Brunel UNIVERSITY LONDON

# Roles of Surfactant Proteins, SP-A and SP-D, in Pregnancy and Parturition

A thesis

Submitted in partial fulfilment of the requirement for the degree of

**Doctor of Philosophy**

At Brunel University, Biosciences, School of Health Sciences and Social Care, Centre of Infection, Immunity and Disease Mechanisms

By

**Najmunisa Karbani, BSc**

October 2013

*Bismillahirrahmanirrahim– In the name of God most gracious most merciful*

*Blessings of God be upon Muhammad (SAW) and his progeny. O Allah, I ask you, the one who mentions goodness and actualizes it and commands it, remind me of that which the shaytan makes me forget. O Allah! I seek refuge in You from a heart which is not humble; from du'a what is not answered; from a soul which is not satisfied; and from knowledge that is of no benefit. Oh Allah! I entrust you with what I have read and I have studied. Oh Allah! Bring it back to me when I am in need of it. Oh Allah! You do whatever you wish, you are my availer and protector and the best of aid. Ameen.*

# Acknowledgements

My First and foremost appreciation goes to Allah, Lord of the heavens and the Universe, who has created everything within it, the one who possess all knowledge, and the master of the day of judgement. Thank you Allah for guiding me to knowledge on what you have created and helping us gain a deeper understanding to benefit others. Ameen.

I would like to thank…

My ustad, **Dr Kishore** – for always keeping his doors open to us. His help and support throughout has been tremendous from helping me organise ethical approval, establishing collaborations for placentae and arranging the necessary training to carry out my research. I would also like to thank him for being my mentor and making me strong when times were tough and making us laugh whenever we're in his presence.

My second supervisor **Dr Sabrina Tosi** and colleagues **Dr Pathan** and **Dr Tsolaki** for their utmost encouraging support and advice throughout the 4 years of my PhD and sharing their experiences with me.

**Dr Manu Vatish and Professor Andrez Lopez Bernal** – for supplying amniotic fluid, placenta and tissue culture facilities. Placentae were collected by midwives from St. Michael's Hospital, Bristol, Warwick University Hospital and John Radcliffe Hospital, Oxford University, provided to us by my collaborator Professor Andrez Lopez Bernal and Mr. Manu Vatish.

**Uma Singh** – helping me prepare IHC slides

**Dr Robert Phillips** – training in ACD explants and IHC and for the slides which were prepared by the histology team in Bristol University and provided by Robert Phillips.

**Umakanth Garjia** – for plasmid containing SP-A1 gene

**Helen Foster** – for training in QPCR

All my colleagues including **Lina, Asif, Yaghoub, Priyaa, Eswari, Annapurna, Lubna, Munira, Suha, Suhair, Kisten, Abhishek, Sheeba, Punam, Nadia** and all my colleagues from my office **RM123** for their positive professional support professionally and awesome social company.

All My Family including…

My Husband, **Faizal Kasim**, **mum, Zarina Karbani**, and **dad, Nasimul-Haque Karbani** for their support physically, financially and emotionally. Thank you for the delicious meals you cooked to help me save my time, motivating me to stay strong and never giving up. Thank you for all your prayers to keeping my steadfast and focused. Thank you for always motivating me and pushing me to the next level. Thank you for always giving me the best of advice and helping to keep my life balanced. Thank you for being the best family ever. Lastly, but most importantly, thank you for always praying for me, for without Allah's will I couldn't have stayed so strong.

My special little sister, **Hajra Nabila Karbani** – for keeping me sane and being my oxford dictionary when things got too scientific! Always smiling and giving me the best advice no matter what the reason was. Thanks for being the best sister, friend and spine to keeping me strong and always encouraging me to keep going. Thank you for listening to my research even if it made no sense to you. Thank you for sacrificing your studying time for being there for me and remembering me in your prayers.

You all made doing a PhD possible!

Alhumdulillahirabillalameen

# Abstract

Surfactant proteins SP-A and SP-D are important key molecules responsible for pulmonary homeostasis and innate immunity against infectious pathogens. SP-A and SP-D are also found in various parts of the placenta as well as amniotic fluid. The levels of these proteins in the amniotic fluid are good biomarkers of fetal lung maturation. The development of the lungs in fetal growth is important for fetal survival in extrauterine life. In pregnant mice models, a huge increase in SP-A and SP-D levels in the amniotic sac has been reported close to parturition suggesting an important role of these proteins in the hormonal pathway to labour. In this thesis, full length natural and recombinant proteins of human SP-A and SP-D were generated and examined on the maternal-fetal tissues of the placenta (explants of amnion, chorion and decidua) under inflammatory conditions. A range of innate and adaptive immune markers and prostaglandin targets were examined to show that SP-A and SP-D modulate the prostaglandin pathway. Thus, an imbalance in this could potentially lead to disorders such as intrauterine growth retardation and preeclampsia. The cellular basis of immune regulation and prostaglandin pathway was also examined via fractionation of decidual macrophages. Curiously, SP-A and SP-D appears to suppress pro-inflammatory response of decidual macrophages after challenging with LPS. This thesis thus divulges specific and mutually inclusive functions of SP-A and SP-D in the maintenance of pregnancy, protection against intrauterine infection, dampening of inflammation, and premature activation of parturition.

# Table of Contents

# List of Abbreviations

| | |
|---|---|
| **ACD** | Amnion, Chorion, Decidua |
| **Ag** | Antigens |
| **AKR1B1** | Aldo-Keto Reductase Family 1, Member 1 |
| **AKR1C3** | Aldo-Keto Reductase Family 1, Member C3 |
| **AP-1** | Activator Protiein-1 |
| **APC** | Antigen Presenting cells |
| **ARDS** | Acute Respiratory Distress Syndrome |
| **B7-H1** | Human B7 homolog 1 |
| **cAMP** | Cyclic AMP |
| **CAPs** | Contraction-Associated Proteins |
| **CBR1** | Carbonyl Reductase 1 |
| **CL-43** | Collectin of 43 kD |
| **CL-46** | Collectin of 46 kD |
| **CL-K1** | Collectin Kidney-1 |
| **CL-L1** | Collectin Liver-1 |
| **CL-P1** | Collectin Placenta-1 |
| **CRD** | Carbohydrate Recognition Domain |
| **CRH** | Corticotrophin Stimulating Hormones |
| **DC-SIGN** | Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin |
| **DM** | Decidual Macrophages |
| **dNK** | Decidual Natural Killer Cells |
| **DSC** | Dendritic Stem cells |
| **EPTB** | Extreme Preterm Birth |
| **EVT** | Extra-Villous Trophoblasts |
| **FcR** | Fc Receptor |
| **hCG** | Human Chorionic Gonadotrophin |
| **HLA** | Human Leukocyte Antigen |
| **HNF-3** | Hepatocyte Nuclear Factor-3 |
| **hPG** | Human Prostaglandin |
| **hPL** | Human Placental Lactogen |
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |

| | |
|---|---|
| **IPTG** | Isopropyl β-D-1-thiogalactopyranoside |
| **IUGR** | Intrauterine Growth Retardation |
| **LBW** | Low birth weight |
| **LNMP** | Last normal menstrual period |
| **LPS** | Lipopolysaccharide |
| **LPTB** | Late Preterm Birth |
| **MBL** | Mannan Binding Lectin |
| **MLCK** | Myosine Light Chain Kinase |
| **MMPs** | Matrix Metalloproteinases |
| **NK cells** | Natural Killer Cells |
| **ONS** | Office of National Statistics |
| **PAMPs** | Pattern Associated Molecular Patters |
| **$PGD_2$** | Prostaglandin D2 |
| **PGDH** | 15, hydroxyl prostaglandin dehydrogenase |
| **$PGE_2$** | Prostaglandin E2 |
| **PGF2α** | Prostaglandin F2 alpha |
| **$PGH_2$** | Prostaglandin $H_2$ |
| **PGHS** | Prostaglandin $H_2$ synthase |
| **PGHS2/PTGS1** | Prostaglandin H synthase |
| **$PGI_2$** | Prostaglandin I2 |
| **PI** | Phosphatidylinositol |
| **PROM** | Premature Rupture Of Membrane |
| **PRRs** | Pathogen Recognition Receptors |
| **PTD** | Preterm Delivery |
| **PTGIS** | Prostaglandin I Synthase |
| **RDS** | Respiratory Distress Syndrome |
| **SP-A** | Surfactant Protein A |
| **SP-D** | Surfactant Protein D |
| **TGF-β** | Transforming Growth Factor |
| **Th1 / 2** | T helper 1 / 2 |
| **TLRs** | Toll-like Receptors |
| **TNF-α** | Tumour Necrosis Factor |
| **VEGF** | Vascular Endothelial Growth Factor |
| **VPTB** | Very Preterm Birth |
| **WHO** | World Health Organisation |

# List of Figures

## Chapter 1



## Chapter 3

Chapter 4

# List of Tables

# Chapter 1:

# General Introduction

# 1. General Introduction

## *1.1 Pregnancy and Preterm birth*

According to the Office of National Statistics (ONS), in 2005, England and Wales alone had 1 in 13 cases of preterm births, ~8% of all births (Oakley et al., 2009). Preterm complications contribute to ~35% of world-wide (3.1 million) annual neonatal deaths and is the second major cause of death after pneumonia in infants (Liu et al., 2012).

As per the World Health Organization (WHO), the duration of a normal term pregnancy is 37 to 42 weeks of gestation and birth preceding 37 weeks is classified as preterm. The extent of prematurity depends on how soon the infant is born prior to term. Human pregnancy is divided into three trimesters. The first trimester (first 12 weeks of pregnancy) initiates at the time of fertilisation and implantation of the embryo on the endometrial lining from the last normal menstrual period (LNMP). The highest rate of miscarriages occurs within the first trimester. The second trimester starts from 13 weeks to 27 weeks gestation, transitioning to the final, third trimester spanning between 28 to 40 weeks (Figure.1.1).

Preterm births, defined as parturition of the new born before 37 weeks gestation, are the leading cause of prenatal mortality and morbidity. Morbidity includes cerebral palsy, intrauterine growth retardation (IUGR), preeclampsia, hypoxia, impaired learning, visual disorders and lung malfunctions. Lung malfunctions are a result of improper lung maturation with an increased susceptibility to lung infections and risk of chronic diseases during adulthood (Mwaniki et al., 2012).

Preterm birth can be sub-divided according to the timing of birth at different gestational stages. For example, extreme preterm birth (EPTB) is defined as birth between 24 and 28 weeks of gestation. A very preterm birth (VPTB) is between 28 and 32 weeks of gestation, whereas moderate or late preterm birth (LPTB) is between 32 and 37 weeks of gestation. In extreme preterm cases, as recorded in UK and Ireland in 1995 (Costeloe et al., 2000), babies born at 24 weeks have a 50% chance of survival, whereas babies born after 28 weeks have an 81% chance of survival. There is a high correlation between preterm birth and low birth weight, which is associated with increased mortality (within 28 days) and morbidity, ~94% of preterm born babies are of low birth weight (LBW), which is defined as weight less than 5.5 lbs and in extreme cases,

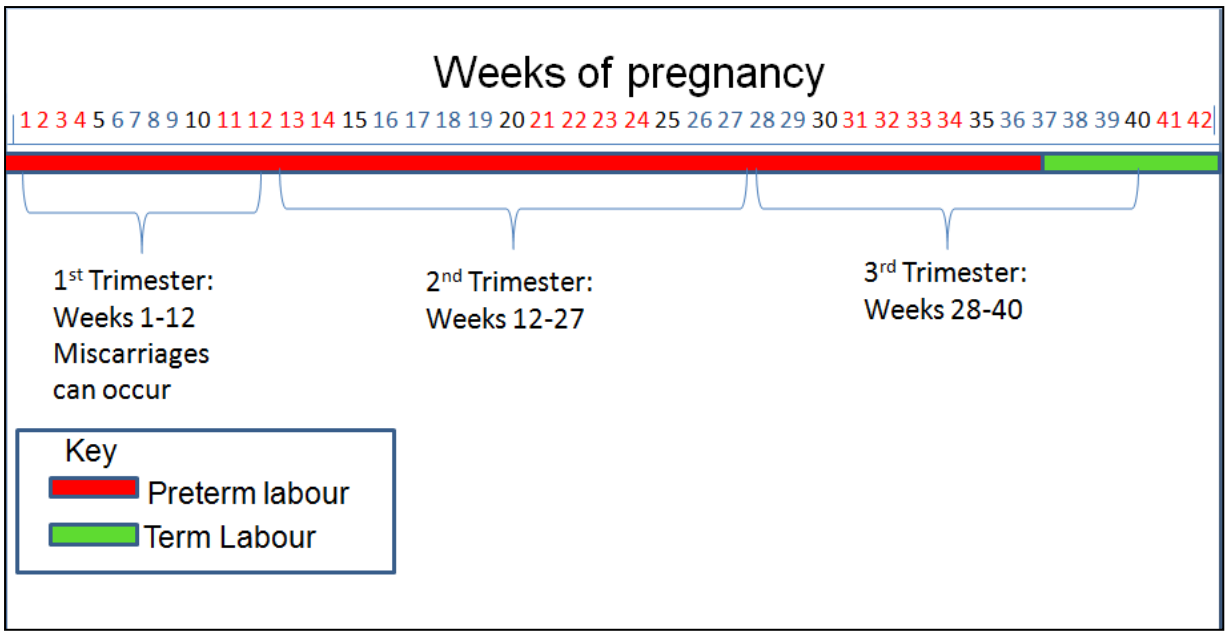



***Figure 1.1 – Three trimesters of gestation – time range of preterm and term birth (this image was created by me for the purpose of this thesis).***

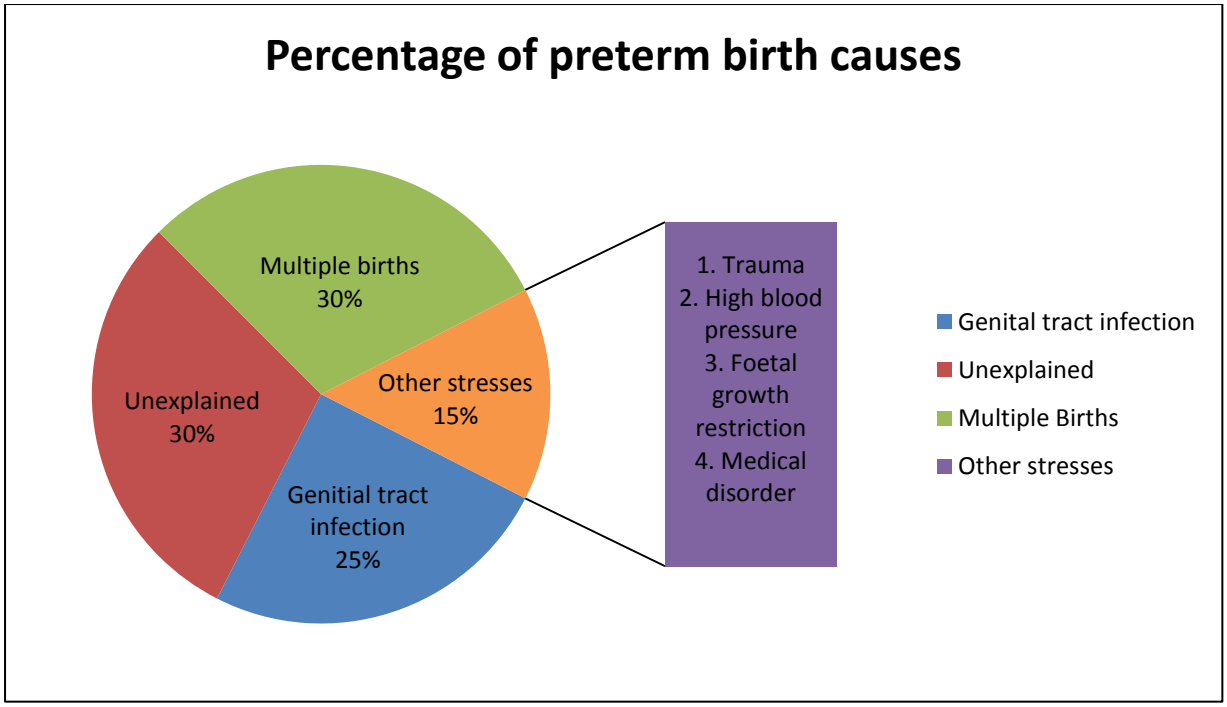



***Figure 1.2 – Factors leading up to preterm births. This image was created by me for the purpose of this thesis adapted from (Haas, 2008).***

less than 2.2 lbs. Women who have given preterm birth earlier have a 2/3rd higher chance of giving preterm births in subsequent pregnancies (Mercer et al., 1999).

Globally, >1 in 10 babies are born premature, which has a huge financial burden on families and the government. A study carried out in 2006 shows, England and Wales alone incur a total cost of ~£3 billion since ~50% of spontaneous preterm births are invariably associated with infection (Lockwood, 2002).

The cause of preterm birth is not fully understood, some cases are spontaneous. However, many cases of preterm births are associated with multiple births, infections or trauma/medical disorders (e.g. high blood pressure and diabetes) during pregnancy. Approximately 30% of spontaneous preterm births are unexplained, and an equal percentage of preterm cases are due to multiple births. Genital tract infections, premature rupture of membrane (PROM), cervical incompetence and uterine abnormalities contribute to 20 to 25% of cases (Figure 1.2). Finally, 15 to 20% of preterm births are due to other stresses including high blood pressure, foetal growth restriction, trauma and other medical disorders (Figure 1.2) (Haas, 2008).

### *1.2 Placental development*

The placenta is a complex organ which houses the foetus/allograft, for 9 months, allowing foetal development to take place. The placenta is a ~500 grams (1lb) organ connecting to the foetus by the umbilical cord (55-60cm). The umbilical cord contains 2 umbilical arteries and 1 umbilical vein (Figure 1.3). The placenta develops on the maternal endometrium from the same blastocyte that forms the embryo, foetus and the maternal placenta. The foetal tissues of the placenta make up the amniotic layer. The amniotic tissues constitute of two types of cells: the epiblast and extraembyonic mesoderm forming around the foetus, otherwise known as the amniotic sac. This sac is filled with amniotic fluid, which bathes the foetus and foetal lungs and increases in volume with foetal growth. This fluid is released at the timing of birth when the amnion membrane ruptures, also referred to as a woman's 'water break'. At 34 weeks of gestation the amniotic fluid is at its greatest, with an approximate volume of 800 ml, however, this decrease closer to term at 40 weeks gestation to 600 ml. The amniotic fluid circulates through the foetal lungs and within the amniotic sac and surrounds the baby carrying the surfactant proteins from the foetal lungs to enter the fluid.

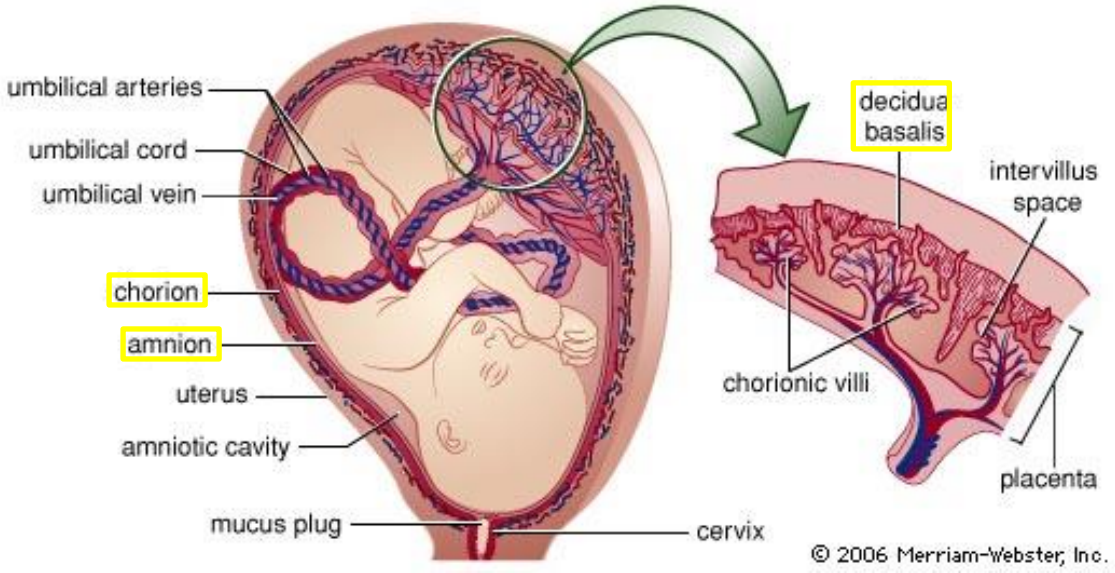



***Figure 1.3 – Different sections of the foetal and maternal membranes including; amnion, chorion and decidua.*** *The umbilical cord joining from the maternal tissue providing nutrients and removing waste can be seen connecting to the foetus. The foetal head is facing the cervix and mechanical stretch triggering the hormonal pathway to initiate contractions occurs here (Sood et al., 2006).*

The maternal tissues of the placenta are constituted of chorionic and decidual tissues (Figure.1.3). The chorion connects the fetal and maternal tissues via chorionic villi. The chorion is adjacent to the amniotic layer and is made up of three main cell types; cytotrophoblasts, syncytiotrophoblasts and extraembryonic somatic mesoderm. The extraembryonic membrane is composed of trophoblasts lined with mesoderm. The chorion separates the amnion from cytotrophoblasts and the chorionic villi penetrates the fetal and maternal tissue to allow vascularisation for the transfer of nutrients from maternal to fetal blood. The outermost layer comprising of maternal tissue is the decidua, surrounding the chorionic layers. This is composed of three main layers; decidua basalis, decidua capsularis and decidua periatelis. The placenta is the organ which separates the mother from the foetus and is the connection between foetal and maternal tissues to allow gaseous and nutrient exchange and the removal of waste via the mother's blood supply. The maternal blood network to the placenta is fully formed within the first trimester and extends as far as the foetal chorionic tissues. The high pressure of the blood allows gaseous exchange to take place. The placenta also forms a barrier against the transmission of microbes to prevent infection. It is important for maternal antibodies to be transported to foetal blood circulation. Hence, syncytiotrophoblast express specific Fc receptors (FcR) and turn away cytotoxic antibodies by expressing complement regulatory proteins (Phillips et al., 2003). In this way, the mother's antibodies penetrate the placenta to provide a memory of the mother's humoral immunity to the foetus.

Besides from serving as an interface for oxygen and nutrient exchange, the placenta is also viewed as an endocrine organ, which secretes hormones such as oestrogen, progesterone, human chorionic gonadotrophin (hCG) and human placental lactogen (hPL). Oestrogen is responsible for mammary gland development for lactation and uterine growth to accommodate the growing foetus. Similarly, progesterone functions in the development of maternal tissues, specifically the endometrium, in order to maintain the lining during pregnancy and prevent myometrial contractility – important for prevention of preterm birth. The hCG synergistically cooperates to maintain oestrogen and progesterone levels secreted by the corpus luteum, thus is important for the maintenance of endometrial lining, preventing it from shedding off. hPL stimulates

mammary gland growth to prepare for lactation and regulates glucose, protein and fat levels to provide the foetus.

### *1.3 Innate immune functions in pregnancy and labour*

Maternal tolerance of the foetus is a major challenge which occurs during all the changes of the uterine environment and is driven by products of the foetal placenta and its associated membranes. Trophoblast cells are involved in protecting the foetus by controlling the expression of HLA molecules preventing an immune response against paternal alloantigens. Restricted expression of HLA molecules also contribute to the tolerance of the foetus. The allograft foetus is protected by foetal trophoblast cells in the placenta and membranes due to its restricted expression of surface antigens that normally stimulate graft rejection, like class Ia antigens on HLA-A and HLA-B and HLA-class II antigens on HLA-D (Hunt and Langat, 2009). Antigen presentation via HLA-G5 or –G6 to encountering uterine (decidual) macrophages stimulates the release of immunosuppressive TGF-β, which is a critical dampener of graft rejection (Figure.1.4).

The pregnant uterus is characterised by innate immune cells which are programmed for an anti-inflammatory response, consistent with tolerating the foetal antigens (Hunt, 1992, Hunt and Robertson, 1996). A high number of natural killer (NK) cells and macrophages and low number of dendritic cells and regulatory T cells ($T_{reg}$) are present in the pregnant uterus (Hunt, 1992) (Figure.1.5). During the first two trimesters of pregnancy, uterine NK cells are most abundant in the decidua (20-40% of decidual stromal cells), which rapidly decline in the third trimester (Moffett-King, 2002, Trundley and Moffett, 2004, Starkey et al., 1988). Uterine NK cells recognise infected and aberrant cells for destruction, however, this does not occur in the human decidua as selective expression of HLA molecules on cytotrophoblast cells suppresses the uterine NK cells (King et al., 2000) (Figure.1.6).

Macrophages are also abundant in the human decidual stromal cells, averaging around 15-20% throughout pregnancy (Bulmer et al., 1988, Hunt, 1990, Vince et al., 1990). Decidual macrophages (DMs) appear to be programmed into a suppressive mode, producing inhibitory cytokines such as IL-10 and TGF-β (McIntire et al., 2004, Heikkinen et al., 2003) and expressing immunosuppressive markers such as Human B7 homolog 1 (B7-H1), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing

Non-integrin (DC-SIGN), ILT3, and factor 13 (Hunt, 1992). Along with IL-10 release from uterine NK cells and macrophages (Heikkinen et al., 2003), TGF-β prevents cellular

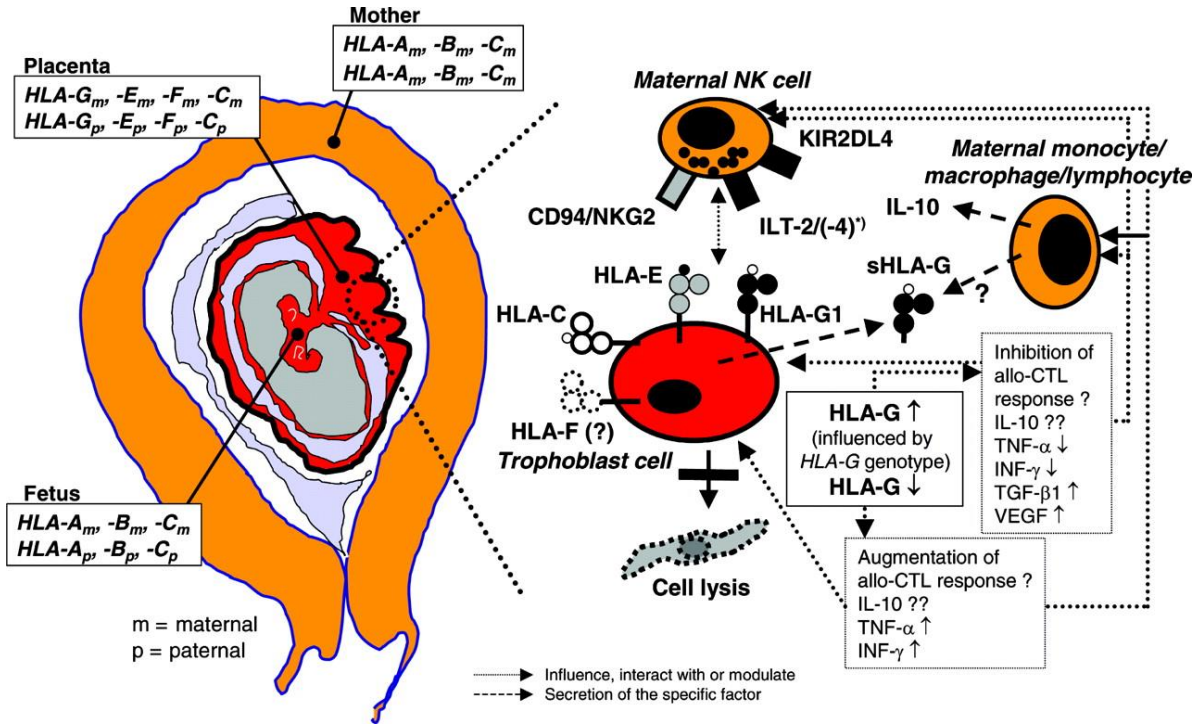



***Figure 1.4 – Foetal tolerance by mother and foetal HLA expression.*** *Human leukocyte antigen expression during pregnancy and expression of HLA-class 1b molecules, especially HLA-G, interacting with NK cell receptors and cytokine expression at the feto-maternal interface. The foetus is semi-allogenic to the mother as it inherits one haplotype of HLA from the mother and one haplotype from the father. Soluble HLA-G and HLA-G expressed on cytotrophoblast escape NK cell mediated lysis by modulating cytokine secretion (Hviid, 2006).*

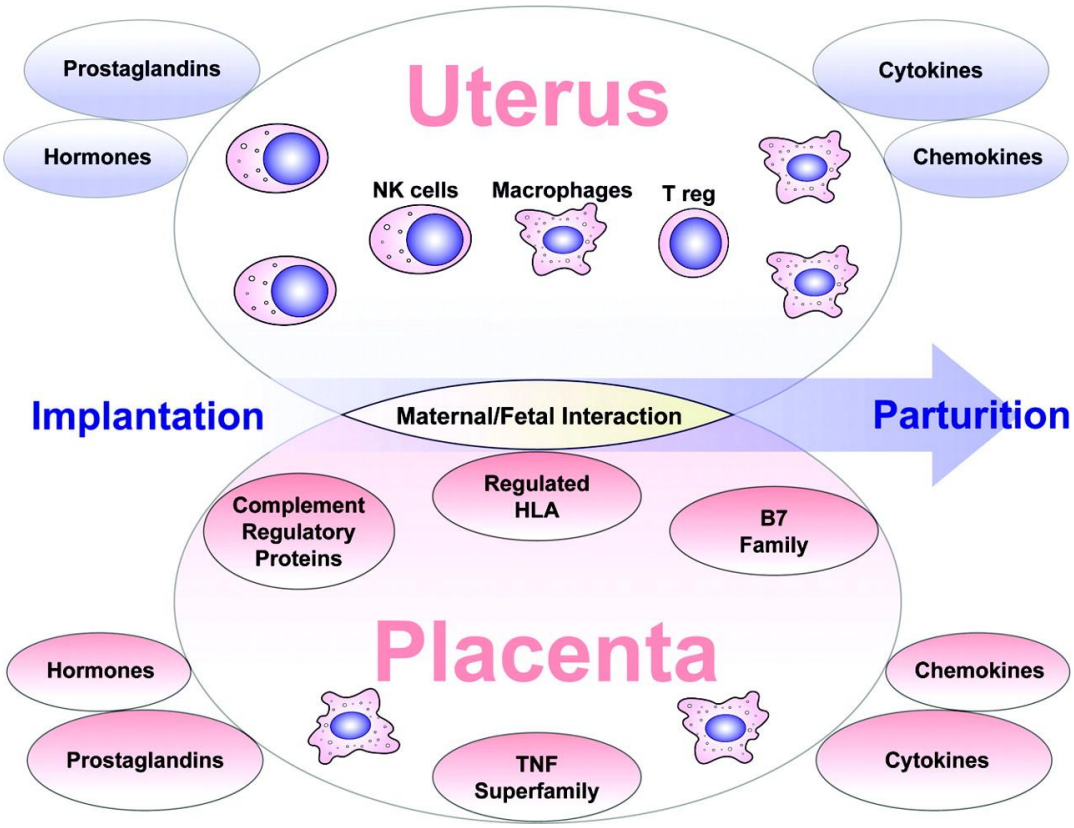



***Figure 1.5 – Hormones and cytokines involved in maintaining pregnancy and inducing parturition.*** *There is a hierarchically defined role for each component at the interface of the foetus-maternal interaction. However, cells and soluble factors in the local vicinity can also affect inflammatory process considerably under conditions of infection and stress (Hviid, 2006).*

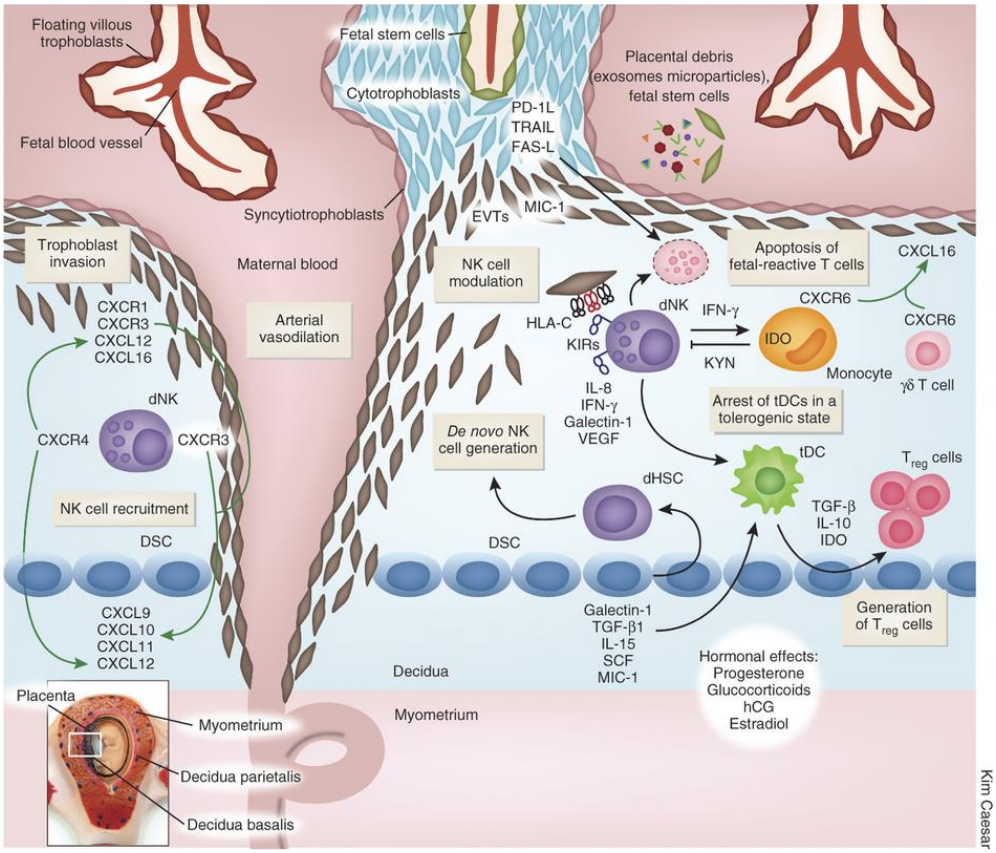



***Figure 1.6 – An anchoring trophoblast connecting from the myometrium to foetal tissues.*** *The anchoring trophoblast extends from the decidua and further differentiates into syncytiotrophoblasts, cytotrophoblast and extra-villous trophoblasts (EVTs) to allow maternal and foetal nutrient and gaseous exchange into the foetal site of the placenta. Chemokines and chemokine receptors surround the EVTs and maternal leukocytes invading the decidua. The EVTs therefore are in direct contact with leukocytes and dendritic stem cells (DSCs). DSCs and/or dendritic NK cells (dNK) produce TGF-β1 to inhibit decidua dendritic cells (dDCs) to provide a tolerogenic state (Arck and Hecher, 2013)*

infiltration. This is aided by prostaglandin $E_2$ and progesterone. Prostaglandins and other hormones are involved in maintaining the status of the non-pregnant uterus due to NK cells, macrophages and $T_{reg}$ cells and their secretory molecules – cytokines and chemokines. During placental development, hormones and prostaglandins are also important for implantation and parturition. During these stages, the placenta is regulated by HLA expression, complement regulatory proteins, B7 family and TNF superfamily. Parturition is designed as an inflammatory process involving chemokines and cytokines (Figure 1.5) (Miyazaki et al., 2003).

DCs are potent antigen presenting cells which only make up 1% of decidual stromal cells (DSC) (Kammerer et al., 2000) and are programmed to exhibit an immunosuppressive phenotype. Mature $CD83^+$ decidual DC secrete less IL-12, a promoter of T-cell activation (Th1), compared to monocyte derived DCs. T lymphocytes are present in the decidua, constituting ~10% of decidual leukocytes (Hunt, 1992) and $T_{reg}$ cells make up 14% of the $CD3^+CD4^+$ T cells in early pregnancy (Heikkinen et al., 2003) whose proliferation is regulated by oestrogen (Polanczyk et al., 2004). $T_{reg}$ cells are responsible for the maintenance of peripheral tolerance via production of the two major immunosuppressive cytokines, IL-10 and TGF-β at the maternal-foetal interface (Itoh et al., 1999, Sakaguchi, 2000, Read and Powrie, 2001) (Figure.1.7).

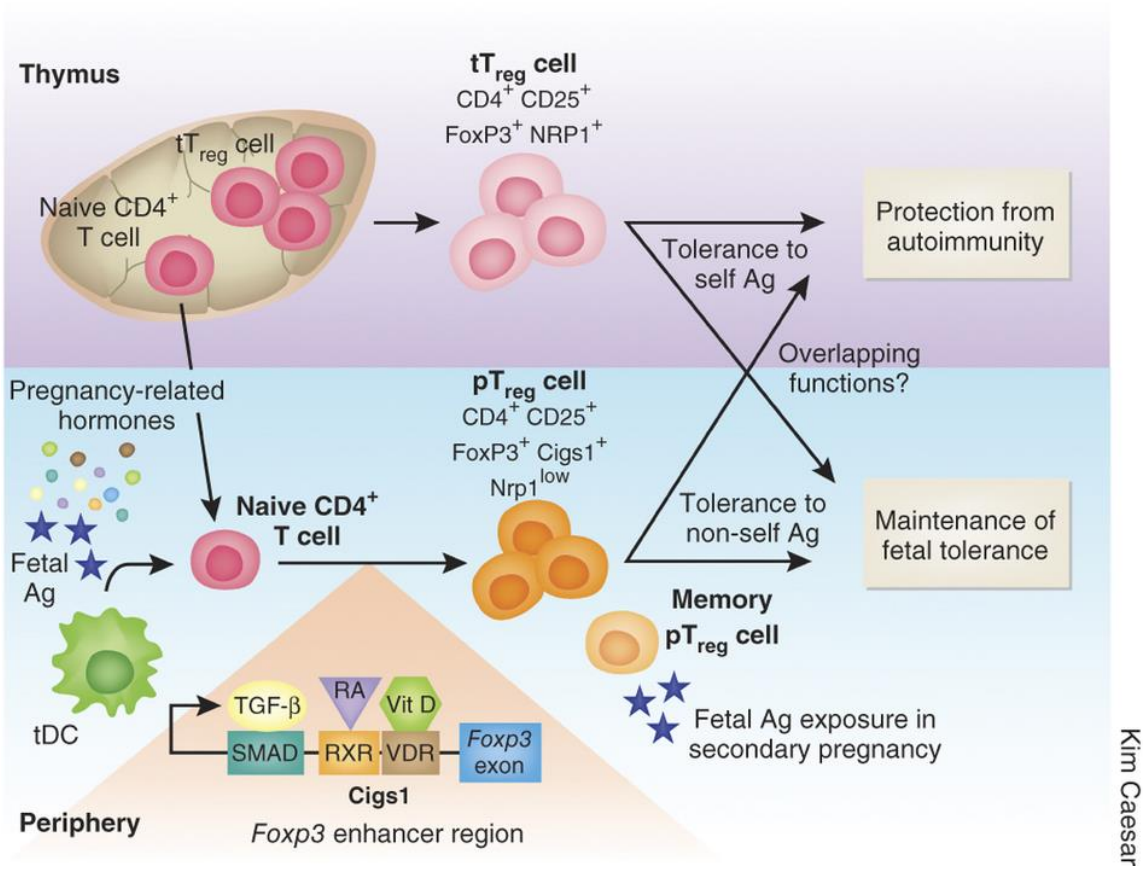



***Figure 1.7 – Functions of $T_{reg}$ cells in maintaining foetal tolerance****. $T_{reg}$ cells stimulated in thymus by naïve CD4+CD44low cells are stimulated by semi-allogeneic foetal antigens. $T_{reg}$ cells released from thymus mediate tolerance to self-antigens (Ag) to protect against autoimmune responses. $T_{reg}$ cells express, CD4, CD25 and FoxP3 and functionally maintain foetal immune tolerance by suppressing decidual inflammation (Arck and Hecher, 2013).*

## *1.4 Physiological processes of labour*

Physiologically, labour can be divided into three main stages including effacement/dilation, expulsion of the foetus and placental stage (Figure.1.8). The first stage, effacement, is the thinning and dilation of the cervix by 3-10 cm in width to allow full passage of the baby. This can last on average 8 hours. The second stage, expulsion, is between the time the cervix has fully dilated and the passage of the baby to extrauterine life. This stage can take minutes to a couple of hours. The third stage is the placental stage, the pushing of the placenta out of the uterus, and is connected to the foetus via umbilical cord. This takes ~10-15minutes (Zhang et al., 2002).

Parturition is characterised by 6 distinct events; cervical ripening, dilation, contractility of myometrium, membrane rupture, placental separation and uterine involution. Biochemical changes occurring near the end of gestation, leads to cervical ripening, membrane rupture and myometrial contractility. The myometrium contractility can be divided into 4 distinct phases: 0) quiescence, 1) activation, 2) stimulation and 3) involution. In phase 0, quiescence occurs from the time of conception to the initiation of parturition where the cervix is soft. Inhibitors such as prostacyclins $PGI_2$ are in action. The inhibitors increase the cAMP levels to transducer signals in order to inhibit $Ca^{2+}$ release which is required for Myosine Light Chain Kinase (MLCK) activity, so no uterine contraction can occur. Therefore, by diminishing the inhibitors, the timing of birth can be controlled and preterm/post term birth prevented. Phase 1 of parturition is the activation of uterine contraction by mechanical stretch. Activation is the beginning of parturition where the cervix undergoes ripening in preparation for labour. Contraction-associated proteins (CAPs) include antagonist receptor, prostaglandins (PGs) and oxytocins (Lye SJ, 1998.). Phase 2 of parturition is the stimulation of the uterus by uterotonins such as prostaglandins, oxytocin and corticotrophin stimulating hormones (CRH) (Lye, 1994). Stimulation involves uterine contractility and cervical dilation up to foetal and placental expulsion. Finally, phase 3 of parturition is the involution after delivery of the foetus and placenta (Gibb and Challis, 2002). Involution is the repair time for the cervix and uterine involution. The cervix is made of collagen, smooth muscle and elastin embedded in connective tissue. The concentration of elastin decreases during pregnancy.

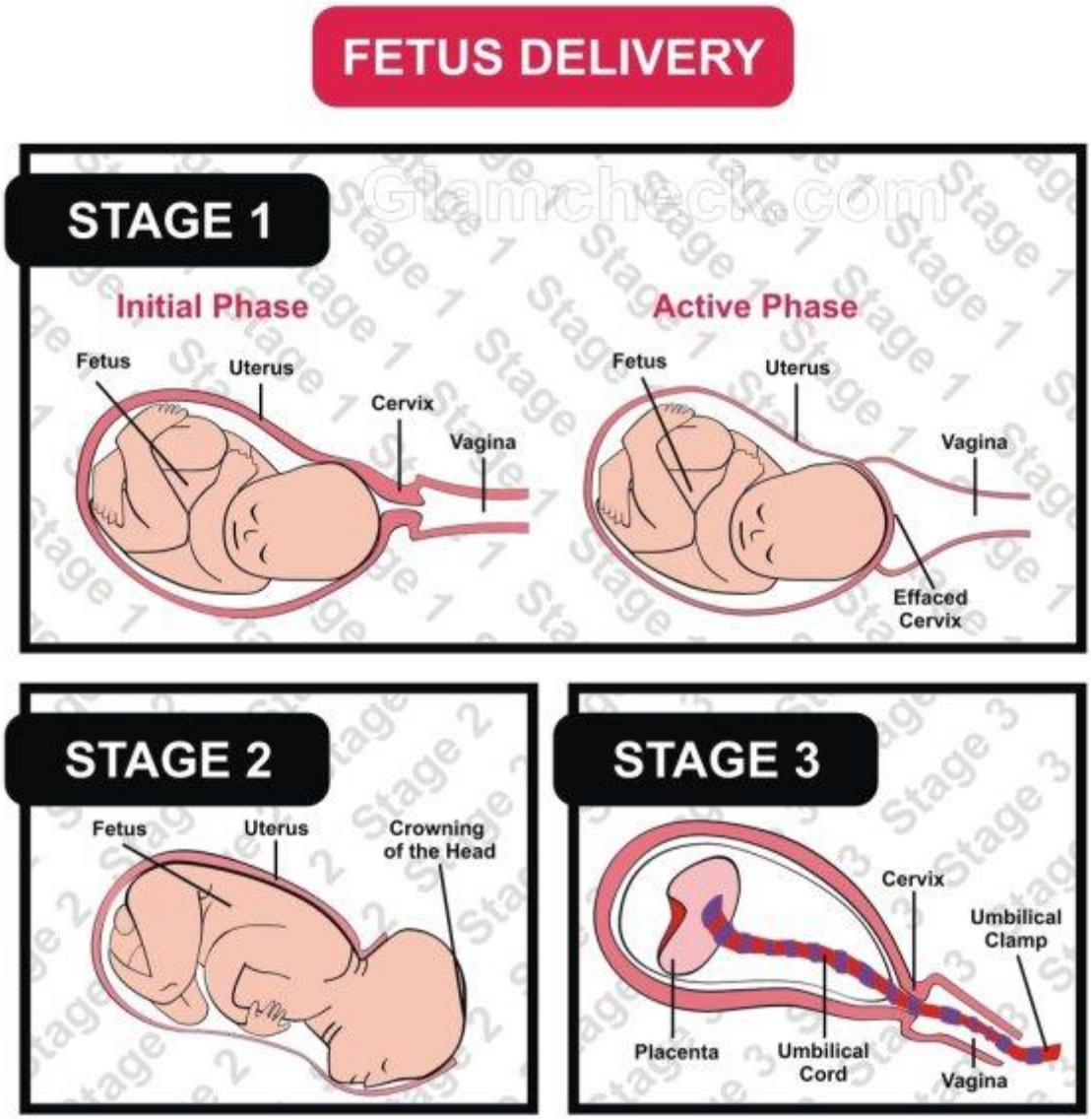



***Figure 1.8 – Physiological stages of labour divided into three main stages.*** *Labour is separated into 3 main stages including dilation (stage 1), expulsion (stage 2) and the placental stage where the placenta is removed (stage 3).*

The collagen is made of fibrils comprising of proteoglycans and glycosaminoglycans and the main cellular components are fibroblasts, which synthesise extracellular matrix and collagen. Cervical ripening and progression of labour is a result of pro-inflammatory response between cells, interleukins and prostaglandins. For instance, SP-D binds to decorin containing glycosaminoglycans (Nadesalingam et al., 2003) and induces the expression of matrix metalloproteinases (MMPs) 1, 2 and 14 (Kolb et al., 2001) to cleave cell receptors and release of apoptotic ligands. This could cause SP-A and SP-D migration to bind and clear the removal of apoptotic cells. Binding to glycosaminoglycans in decorin introduces reduction in levels of collagen for tissue remodelling.

### *1.5 Hormones influencing pregnancy and labour*

Reproductive hormones such as progesterone, hCG, oestrogen, oxytocin, CRH and prostaglandins are some of the important maternal and foetal hormones involved in the labour pathway (Figure.1.9). Progesterone released from the corpus luteum in early gestation enables the maintenance of pregnancy by decreasing prostaglandins and inhibiting oxytocin and oxytocin receptor expression. Cytokines stimulate the synthesis of MMPs, vascular endothelial growth factor (VEGF) and progesterone receptor C isoform, which reduce the response of progesterone during labour (Christiaens et al., 2008). hCG is produced by the syncytiotrophoblasts in the chorion predominantly during pregnancy for its maintenance and regulating the levels of progesterone and oestrogen. Oestrogen, on the other hand, has opposing effects, causing prostaglandin production and promoting expression of oxytocin receptors and stimulating uterine contractility (Challis, 2000). Oxytocin released from the posterior pituitary gland at the end of gestation stimulates further expression of oxytoxin receptors, inducing uterine contractility, and promoting local expression of prostaglandin from foetal membrane and the decidua. Another hormone which reaches at its peak levels at the end of pregnancy is CRH in the placenta. Highly expressed in the placenta, CRH is detected in maternal serum which makes it a great marker for detecting the timing of birth as this further increases prostaglandin production by decidua and placental membranes to drive the labour process (Challis, 2000).

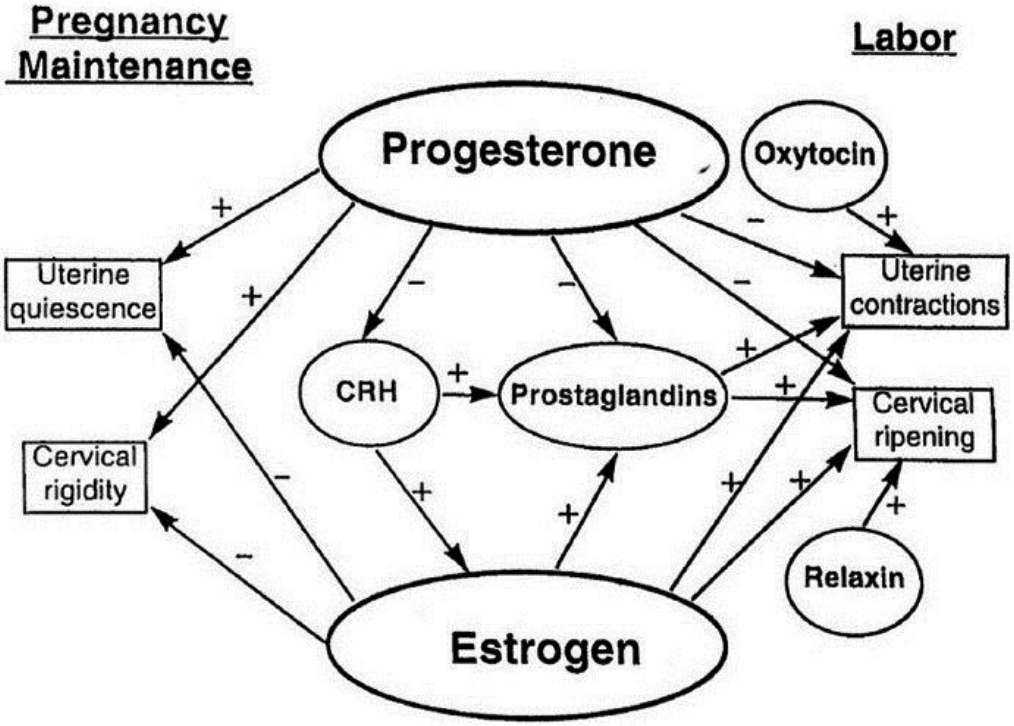



***Figure 1.9 – Effects of hormones in the maintenance of pregnancy and in the initiation of labour****. High levels of progesterone and low levels of oestrogen maintain uterine quiescence and cervical rigidity. Low levels of progesterone and high levels of oestrogen and oxytocin stimulate prostaglandin synthesis and uterine contraction and cervical ripening (Kota et al., 2013).*

### *1.6 Prostaglandin pathway – one of the main hormonal pathways leading upto labour*

It is debated whether the signal for inducing labour is similar in term and preterm labour. In addition, whether the signal is induced by the foetus or mother or if it's a mutual communication from both remains an area of interest in research. It is largely considered the foetus contributes to the timing of birth by secreting components from the lungs into the amniotic fluid which can stimulate foetal membranes and surrounding tissues to initiate the prostaglandin (PG) pathway for the onset of labour (Okita et al., 1983). The importance of PG in promoting parturition was highlighted in a study that used PG endoperoxidase H synthase inhibitor that caused a delay in the onset of labour (Novy et al., 1974).

PGs are a sub-class of eicosanoids derived from a 20-carbon fatty acid which are responsible for promoting contraction and relaxation of smooth muscle tissues (Nelson and Randy, 2005). They are considered as key mediators for the onset of labour (Challis and Vaughan, 1987, Novy and Liggins, 1980, Thorburn and Challis, 1979). They can induce changes in the extracellular matrix metabolism associated with cervical ripening, decidual/membrane activation (Romero et al, 1994) and myometrial contractility (Wiqvist et al., 1983, Calder and Greer, 1991). The three main foetal/maternal membranes of the placenta include amnion, chorion and decidua (ACD). Each of these tissues synthesise different PGs and are expressed in varying levels. The human amnion is the thinnest section, made up of epithelial cells and sub-epithelial mesenchymal cells. These layers are part of the foetal interface. The amnion is made up of four layers including: epithelium, basement membrane, compact layer and fibroblast layer (Figure 1.10). The chorion being the thickest layer of the two comprises of trophoblast cells under the pseudobasement membrane. This is the middle connecting layer between foetal amniotic layer and maternal decidual layer (Figure 1.11). The decidua is a thick vascularised lining between the chorion and myometrium. The decidua consists of a mixture of decidualized stromal cells, bone marrow-derived macrophages, NK cells, decidual cells, lymphocytes, endothelial cells and trophoblasts (Saito et al., 1993, Dudley et al., 1996a).

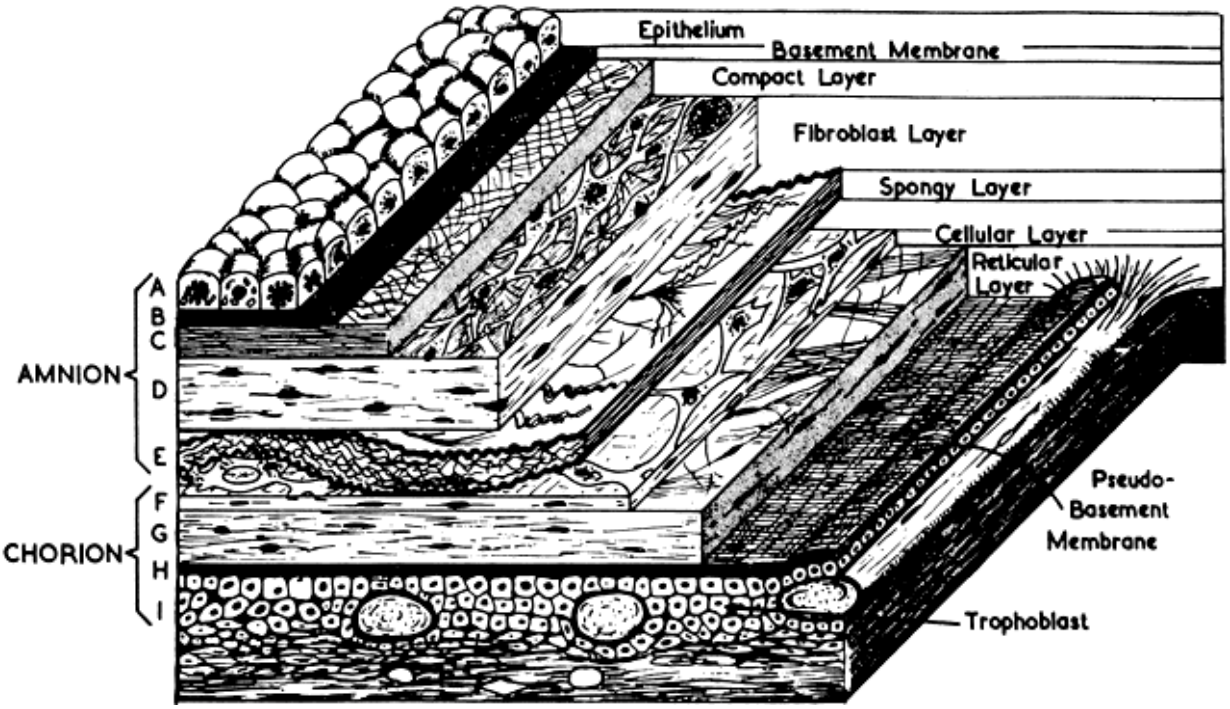



***Figure 1.10 – Layers of amnion and chorion.*** *Different cells distinguish both the amnion and chorion layers surrounding the foetus. Both the amnion and chorion are separated by the spongy layer with the chorion being the thickest layer of the two containing trophoblast cells under the pseudobasement membrane (Bourne, 1960).*

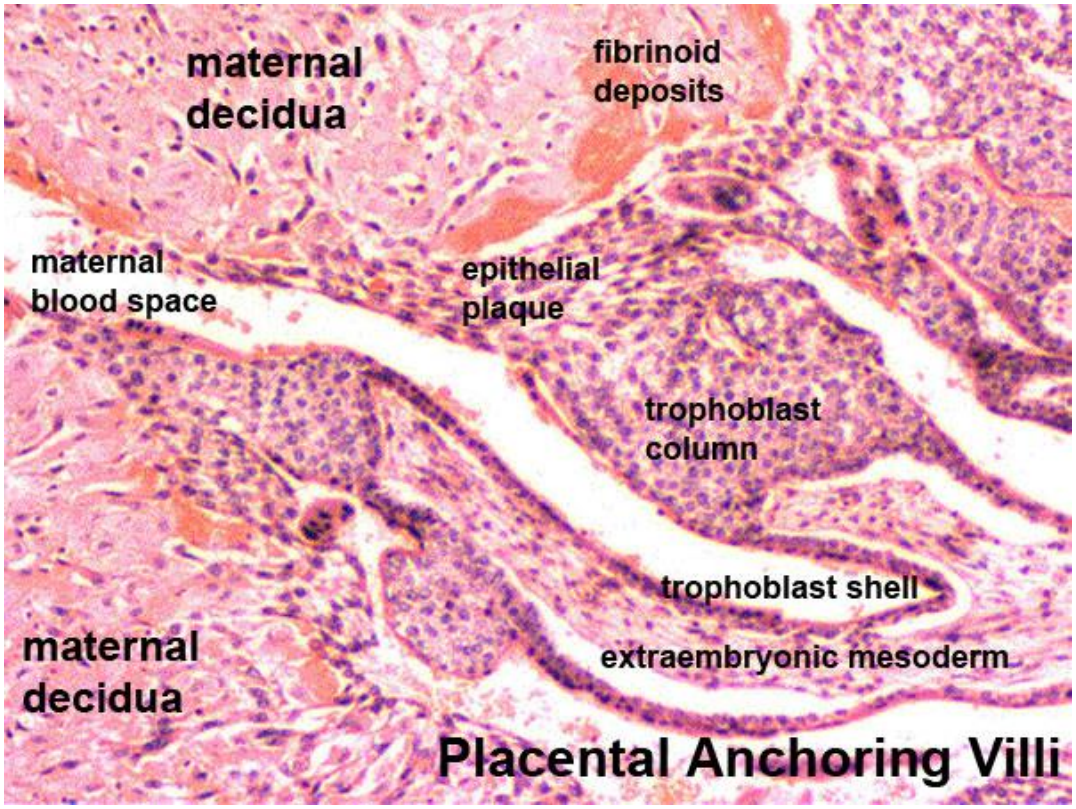



***Figure 1.11 - Histological section of decidua tissue of pregnant placenta.*** *The Section shows the junction between foetal trophoblast (to form syncytiotrophpoblast and cytotrophoblast) and maternal decidua.*

PGs are regulated by their precursor, arachidonic acid, which stimulates and drives the PG metabolism pathway. The PG pathway is dependent on the amount of arachidonic acid available. In response to certain hormones, phospholipase $A_2$ cleaves the phospholipid cell-membrane containing arachidonate to be released. In the smooth ER, arachidonic acid is converted to prostaglandin $H_2$ ($PGH_2$) by COX/prostaglandin $H_2$ synthase (PGHS) (Olsen et al, 2003). Two isoforms of PGHS exist: PGHS1/COX-1 and PGHS2/COX-2 (we refer this to PTGS2 later), which converts arachidonic acid to $PGH_2$, increasing PG levels before parturition (Molnar et al., 1993). Thus, arachidonic acid serves as a substrate for PGHS/COX enzymes, resulting in $PGH_2$ production. It has been proposed that arachidonic acid from the foetus could serve as a source of PG precursor (Lopez Bernal et al., 1988). Although, only 2% of fatty acids in the amniotic fluid are arachidonic acid, the amnion layer has a high turnover of fatty acid, serving as a good source of arachidonic acid from foetal membranes (Liggins et al., 1977). The initiation of the PG pathway is triggered by paracrine lipid mediators such as arachidonic acid, resulting in a sequential oxidation reaction to convert $PGH_2$ to $PGD_2$, $PGE_2$, $PGF_2$ and $PGI_2$ (Figure. 1.12) which are controlled by enzymatic reactions of synthases (Table 1.1). Each PG has its own role for the progression of labour. For example, in human myometrium, $PGD_2$ and $PGI_2$ are involved in the relaxation of muscle tone, whereas $PGE_2$ and $PGF_{2\alpha}$ are involved in myometrial contractility (Senior et al., 1993).

PGs are synthesised in the amnion but their enzymes for catabolic activity is sparse and mainly located in other areas of membrane. PGHS1/COX-1 and PGHS2/COX-2 mRNA are mainly synthesised within the amnion (Hirst et al., 1995) but very little PGDH (15', Hydroxyprostaglandin dehydrogenase). At term, the net basal output of PG produced by amnion mesenchymal layer exceeds the level in amnion epithelial cells (Whittle, 2000). Similarly, very high concentrations of PGHS/COX enzymes are localised in the chorionic layer (Keirse and Turnbull, 1975), suggesting the role of chorion in controlling the transfer of PGs and progression of the PG pathway. Over expression of PGHS2/COX-2and $PGE_2$ levels in the amnion epithelium and mesenchymal fibroblasts has been

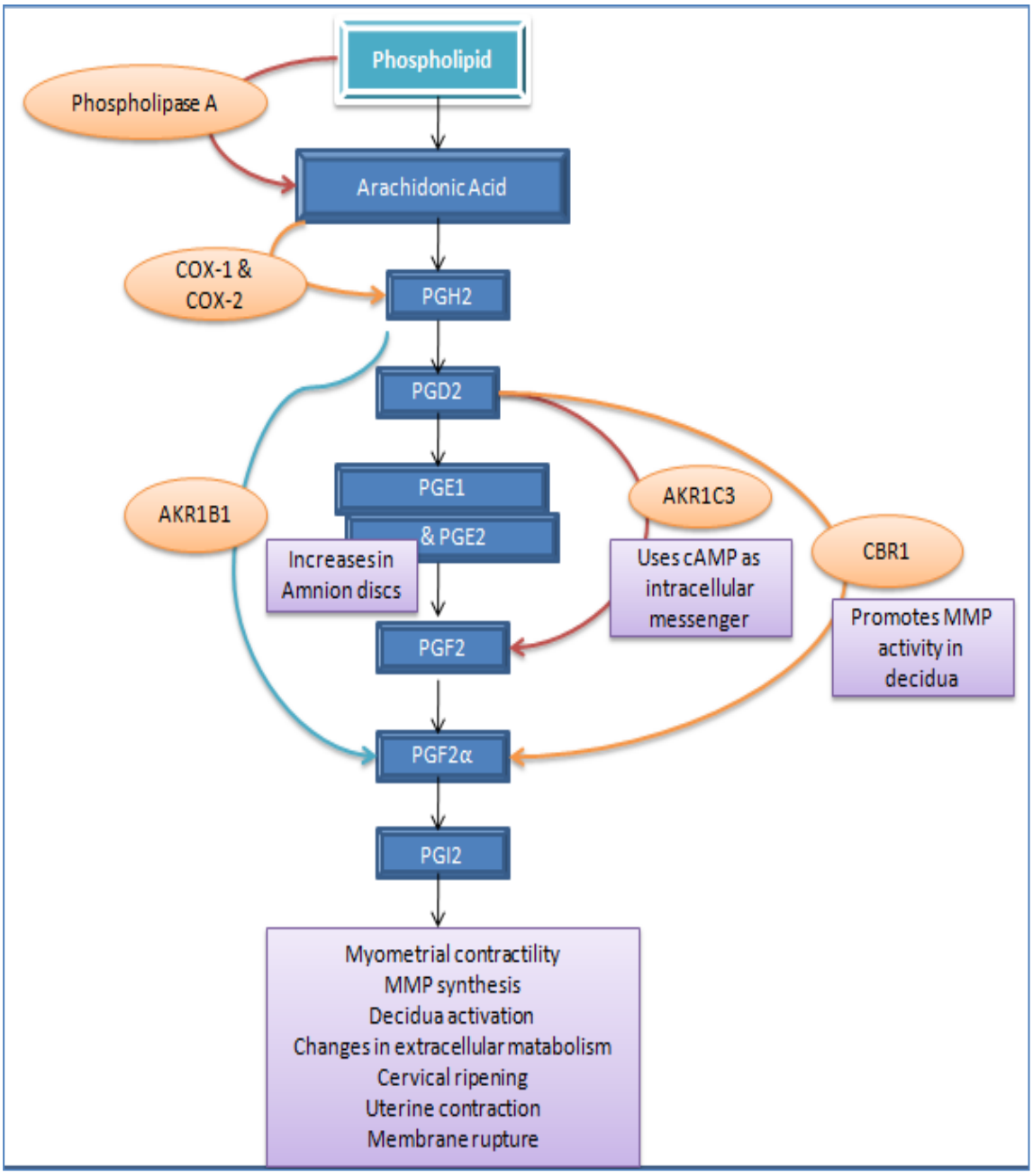



***Figure 1.12 – Enzymes and Hormones of the Prostaglandin pathway in the initiation of parturition.*** *Enzymes (orange circles) and the main hormones involved in the cascade of events leading to myometrial contraction and expulsion of the baby. This image was generated by me for the purpose of this thesis. COX-1/COX-2, prostaglandin G/H synthase 1 / 2; AKR1B1, Aldo-Keto Reductase Family 1, Member B1; AKR1C3, Aldo-keto reductase family 1 member C3; CBR1, Carbonyl reductase 1, PGH2, prostaglandin H2; PGD2, prostaglandin D2; PGE1/2,prostaglandin E1/2; PGF2/2α,prostaglandin F2/2α; PGI2, prostaglandin I2 (Lopez Bernal et al., 1987).*

***Table 1.1 – Prostaglandin pathway and its conversion via different enzymatic activity.***

| SUBSTRATE | ENZYME | PRODUCT |
|---|---|---|
| Membrane phospholipid | Phospholipase (e.g. PLA2G4A) | Arachidonic acid |
| Arachidonic acid | PGHS1/PTGS1, PGHS2/PTGS2 | $PGH_2$ |
| $PGH_2$ | PTGDS, HPGDS | $PGD_2$ |
| $PGH_2$ | PTGES, PTGES2, PTGES3 | $PGE_2$ |
| $PGH_2$ | AKR1B1, AKRIC3 | $PGF_{2\alpha}$ |
| $PGH_2$ | PTGIS | $PGI_2$ |
| $PGD_2$ | AKR1C3 | 9α-11β- $PGF_2$ |
| $PGE_2$ | CBR1 | $PGF_{2\alpha}$ |

*Prostaglandins on the left serve as a substrate for the enzymes in the middle to produce prostaglandin products described on the right of the table. Prostaglandin H2 (*$PGH_2$*), prostaglandin D2 (*$PGD_2$*), prostaglandin E2 (*$PGE_2$*), prostaglandin F2 alpha (PGF2α), prostaglandin I2 (PGI2), 9α-11β-prostaglandin F2 (9α-11β- PGF2), prostaglandin H synthase (PGHS1/PTGS1, PGHS2/PTGS2), Prostaglandin D2 synthase (PTGDS), hematopoietic prostaglandin D2 synthase (HPGDS), prostaglandin E synthase (PTGES, PTGES2, PTGES3), aldo-keto reductase family 1, member 1 (AKR1B1), aldo-keto reductase family 1, member C3 (AKRIC3), prostaglandin I synthase (PTGIS), carbonyl reductase 1 (CBR1).*

associated with cases of preterm birth (Skinner and Challis, 1985, Lopez Bernal et al., 1987, Gibb and Sun, 1996). On the other hand, the decidua has been shown to express low levels of PGHS1/COX-1 and PGHS2/COX-2 and PGDH (Liggins et al., 1977, Hirst et al., 1995), suggesting this tissue may be involved in the end results of the PG pathway.

The levels of PGHS2/COX-2 can increase by 80-fold due to several factors including cytokines (Romero et al., 1991), growth factors: epidermal growth factor (EGF), platelet activating factor (PAF) (Mitchell, 1984), tumour promoters, bacterial endotoxins (Bennett et al., 1987, Lamont, 1999, Soloff et al., 2000), which increase levels of intracellular cAMP (Anteby et al., 1997, Grammatopoulos and Hillhouse, 1999) and glucocorticoids (Potestio et al., 1988, Zakar and Olson, 1989, Gibb and Lavoie, 1990). Stimulation of PGHS2/COX-2 increases PG levels, $PGE_2$ and hydroxyprostaglandin dehydrogenase (PGDH), regulating the control of the PGs metabolism and transfer across the membrane. The high levels of PGs reflect the activity of amnion, which leads to cervix dilation (Keirse, 1990). Due to the short half-life of the PGs, they can only respond locally for cell-cell signalling. However, an increase in PG level enhances the rate of transfer across the decidua and myometrial membrane promoting membrane rupture, cervical ripening and parturition. Nakla et al have shown that the amnion and chorion are the main sites for PG metabolism and that it protects the free transfer of un-metabolised PG across the membrane to the decidua or myometrium (Nakla et al., 1986), which are important in the onset of delivery at term as well as preterm. If this is unregulated by factors including infection or down regulation of PGDH expression, PG transfer cannot be controlled allowing PGs produced from the different tissues to reach the myometrium and induce preterm birth (van Meir et al., 1997).

PG levels are affected by the rates of synthesis and metabolism (Challis et al., 1999). Increased PGDH expression resulted in an increased metabolism in amnion and chorion. However, in 15% of idiopathic preterm labour, patients show no PGDH expression even if trophoblast cells are undamaged and less PG metabolism takes place (Challis et al., 1999). However, concentrations of PGs from other tissue increase the rate to parturition as these PG can bind to agonist PG receptors more effectively. The levels of PGDH have been shown to decrease in the chorion at term and preterm (Van Meir et al., 1996, Sangha et al., 1994). According to Sangha et al, 1994, studies have shown a correlation between low expression of PGDH expression activity in chorion trophoblast (the

absence of infection) and preterm birth. This suggests an important role of PGDH requirement for the biochemical process on the onset of labour. Van Mier et al, (van Meir et al., 1997, Van Meir et al., 1996) have also shown a correlation between infection, and low levels of PGDH due to loss of trophoblast cells in case of preterm delivery. Reduced PGDH expression is not due to trophoblast cell breakdown in chorion, but, due to inability of the decidua to produce PGDH. PGDH decreases the levels of PGs to inactive metabolites so they cannot get transferred across the foetal membranes to the maternal side or vice versa resulting in delayed myometrial contractility (van Meir et al., 1997). The chorion consists of a trophoblast layer containing high concentration of PGDH (15, hydroxyl prostaglandin dehydrogenase), a PG-metabolising enzyme that has been identified to control the production of PGs (Keirse and Turnbull, 1975, Keirse et al., 1976, Keirse, 1978, Keirse et al., 1985, Okazaki et al., 1981a, Okazaki et al., 1981b, van Meir et al., 1997). Several studies have reported that PGDH mRNA concentrations are low in spontaneous labour at term (Lopez Bernal et al., 1987, Skinner and Challis, 1985, Cheung and Challis, 1989, Germain et al., 1994), which would support the progression of labour via the PG pathway. This would allow the PGs produced in the amnion to be metabolised in the chorion inhibiting premature progression of PG synthesis.

PGHS2/COX-2 mRNA expression within the amnion and chorionic layers results in $PGE_2$ production (Slater et al., 1995). The majority of the PGs, mainly $PGE_2$, are produced in the amniotic layer (Gibb and Sun, 1996). An increase in the levels of PGHS2 and $PGE_2$ at term and preterm have been localised within amnion epithelium and mesenchymal layer (Skinner and Challis, 1985) during the onset of labour (Slater et al., 1995).

### *1.7 Innate immunity and pregnancy*

The innate immune system is the first line of defence which modulates the outcome of the adaptive immune response. The cellular components of the innate immunity comprises of neutrophils, monocytes, macrophages, DCs, natural killer (NK) cells, epithelial cells and its secretory materials; cytokines and chemokines, which deal with pathogens in addition to collectively influence the adaptive immune response (Table1.2). Other molecules in the innate immunity which recognise pathogens include specialised pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs),

mannose receptor (MR) and soluble factors including collectins encoded within the germ line. These collectins include SP-A, and -D, mannan binding lectin (MBL), scavenger receptor collectin placenta-1 (CL-P1), collectin liver-1 (CL-L1), collectin kidney-1 (CL-K1), conglutinin, collectin of 43 kD (CL-43), and collectin of 46 kD (CL-46) (Holmskov et al., 2003). The adaptive immune system can be sub-categorised into humoral and cellular immune response. The main players of the adaptive response are B-lymphocytes, antibodies, naive T-lymphocytes, T helper cells 1 (Th1) and 2 (Th2), cytotoxic T-cells, cytokines and chemokines.

***Table 1.2 – Innate and adaptive molecules***

| INNATE IMMUNITY | ADAPTIVE IMMUNITY |
|---|---|
| **Neutrophils** | Dendritic cells |
| **Monocytes** | T lymphocytes |
| **Macrophages** | B lymphocytes |
| **PRRs (e.g. TLRs)** | NK cells |
| **Cytokines** | Immunoglobulins |
| **Chemokines** | |
| **Complement proteins** | |
| **Collectins:** | |
| **Mannose-Bindin Lectin (MBL)** | |
| **Surfactant protein-A (SP-A) and –D (SP-D)** | |
| **C-reactive protein** | |

Although the innate immune system is not as specific as compared to adaptive immune response to produce an immunological memory, it can distinguish between self and non-self as microorganisms contain repeated patterns on their cell surface, called pathogen associated molecular patterns (PAMPs), therefore, pathogens can be recognised by means of receptor (i.e. PRRs) which bind to these common characteristics. There are two types of PRRs, soluble blood plasma proteins such as mannose-binding receptor or collectins such as SP-A and SP-D or membrane bound PRRs expressed on macrophages and phagocytic cells. Phagocytic receptors can be placed in groups including, mannose-receptor, scavenger receptors and toll-like receptors (e.g. TLR-2 and TLR-4). Such phagocytic receptors are able to recognise common features on pathogens, i.e. PAMPs. For example, scavenger receptors recognise low-density lipoproteins and mannose-receptor recognise mannose present on many bacterial and viral cell surfaces. Binding of pathogens (i.e. PAMPs) to TLRs with co-

receptors are capable of activating the adaptive immune response. For example, TLR-4 binds indirectly to LPS of Gram-negative bacteria, through association with CD14 initiating the NF-κB pathway, subsequently producing TNF-α from macrophages. TLR-2 can recognise molecules such as peptidoglycan present on gram-positive bacteria. Cytokines, chemokines and co-stimulatory molecules have the essential role in directing the adaptive immune response (Figure.1.13) (Janeway CA Jr, 2001).

Pathogenic microorganisms which have overcome the first line of defence, invading the epithelial surface of the body, are recognised by the immune cells to mount an innate immune response. Phagocytic neutrophils in the bloodstream recognise these microorganisms through their PRRs. The influx of neutrophils is followed by monocyte invasion which rapidly differentiate in to active macrophages which recognise and ingest the pathogenic microorganisms. Upon phagocytosis, macrophages produce lipid mediators of inflammation such as PGs, leukotrienes, platelet activating factors (PAF), and cytokines and chemokines.

### *1.8 Influence of cytokines in maternal/foetal membranes on the prostaglandin pathway*

Inflammation is an integral aspect of labour. Infiltration of leukocytes producing pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β have been found in increasing levels in foetal and maternal compartments including amniotic fluid, myometrium, decidua, amnion, chorion and maternal serum as pregnancy progresses (Goldenberg et al., 2000). More specifically, pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, have been located in maternal placenta/ trophoblast cells and decidua but only IL-1β and IL-6 in foetal chorion whereas the amnion expresses TNF-α, IL-1β and IL-6 (Menon et al., 1995, Fortunato et al., 1994). Both mRNA and protein levels of IL-6, IL-8 and IL-1β (Keelan et al., 1999, Dudley, 1997) can be detected in membranes and amniotic fluid (Opsjln et al., 1993) perhaps due to granulocyte infiltration in the placentae (Halgunset et al., 1994). These Cytokines are up-regulated during spontaneous term labour and vaginal delivery (Keelan et al., 2003, Marvin et al., 2002) and are known to influence labour (Goldenberg et al., 2000) at term and preterm births even without the presence of infection (Elliott et al., 2001) (Figure.1.14). However, cases of preterm labour with infection (Romero et al., 2006) irrespective of infection (Elliott et al., 2001) have shown

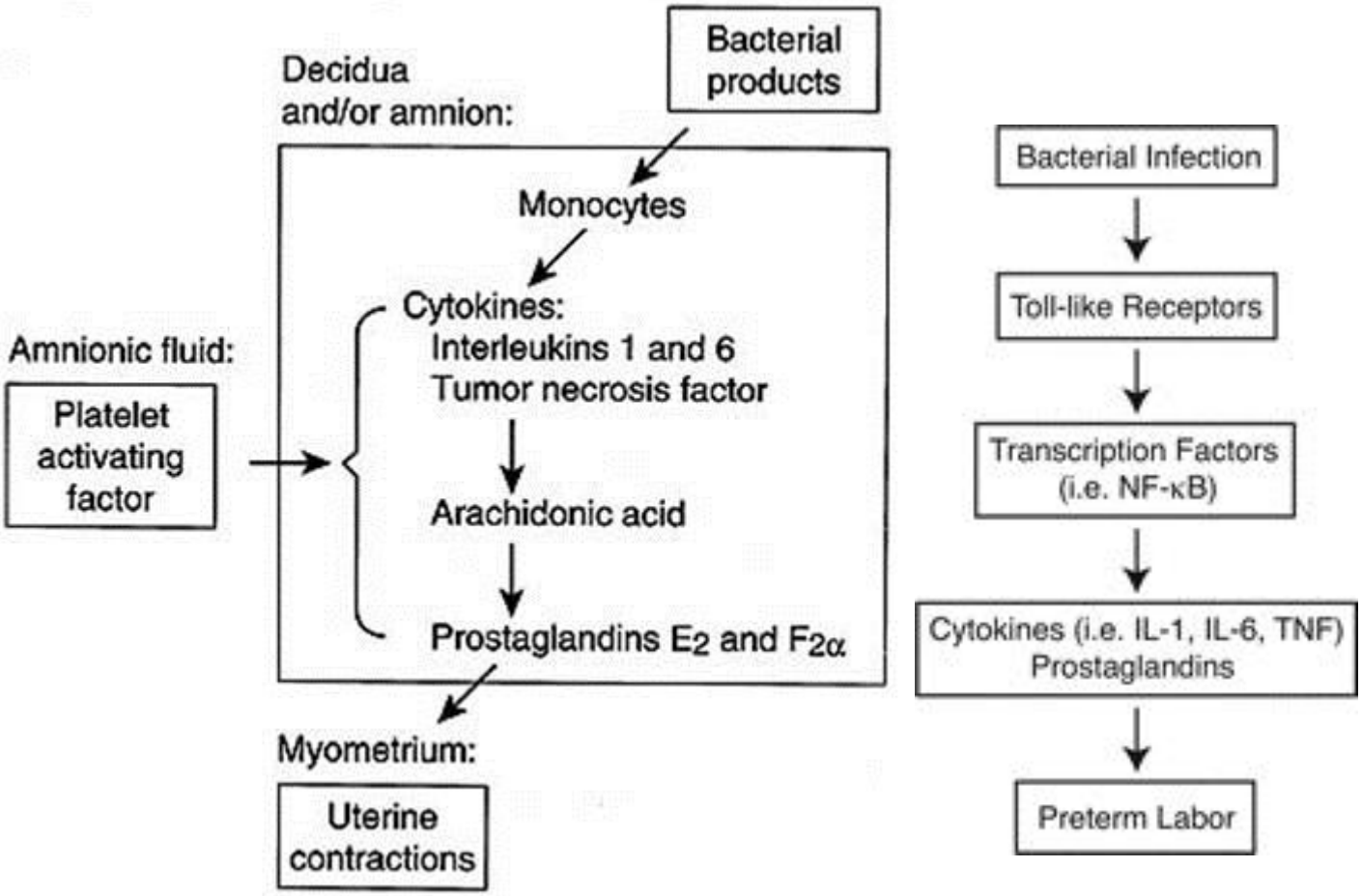



***Figure 1.13 – preterm birth stimulated by bacterial infection.*** *Bacterial invasion from maternal or foetal tissues are recognised by monocytes by PRRs such as toll-like receptors (TLRs), activating NF-κB to transcribe and cytokines. Cytokines such as IL-1, IL-6 and TFN released stimulate arachidonic acid release to promote prostaglandin synthesis, which in turn is responsible for uterine contractility (Ratajczak and Muglia, 2008).*

further elevated levels of IL-1β and IL-8 in the amnion, chorion, decidua and myometrium. Therefore, early initiation and high levels of these cytokines can lead to premature labour.

IL-10 is an anti-inflammatory cytokine which delays the onset of labour as it counterbalances the action of pro-inflammatory cytokines. Fiorentino et al (1991) have shown the strong anti-inflammatory effects of IL-10 in rodents which displayed a delay in the onset of labour and a decrease in the level of pro-inflammatory cytokines, perhaps by desensitising inactivating macrophages (Fiorentino et al., 1991). In contrast, in humans, mRNA and protein levels of IL-10 have been detected in low levels in amnion and chorion at term (Denison et al., 1998, Dudley, 1997, Simpson et al., 1998) but are stable throughout gestation (Roth et al., 1996). The anti-inflammatory environment is overridden by TNF-α, which decreases PGDH expression, promoting the progression of PG pathway, and hence, initiation of labour (Pomini et al., 1999). The release of pro-inflammatory cytokines stimulates uterine activating proteins (UAPs), which promotes PGs synthesis, therefore, cytokines including IL-1β and TNF-α are essential for indirectly influencing the rate of prostaglandin production towards labour (Kniss et al., 1997, Molnar et al., 1993).

IL-6 levels have been shown to increase with labour in gestational tissues (Keelan et al., 1999, Laham et al., 1996) and displays strong immunoreactive staining in endothelial cells of the placenta (Steinborn et al., 1999). There is a close correlation between cytokines present in increased amounts during term labour and the up-regulation of mRNA levels of these cytokines in foetal membranes, suggesting that foetal membranes are one of the main sources of cytokine production in addition to foetal leukocytes and tissues such as the lungs.

IL-1β is expressed from placental macrophages and trophoblasts (Taniguchi et al., 1991). Although IL-1β levels decline during gestation, the levels of IL-6 increase with the onset of labour (Dudley et al., 1996b). IL-6 gene knockout mice show delay of labour when compared to wild type mice, confirming the important role this cytokine plays in the progression of labour (Robertson et al., 2006). All of these cytokines are produced and secreted by macrophages (decidual macrophages: DMs) which can be located in the decidua membranes during late gestation.

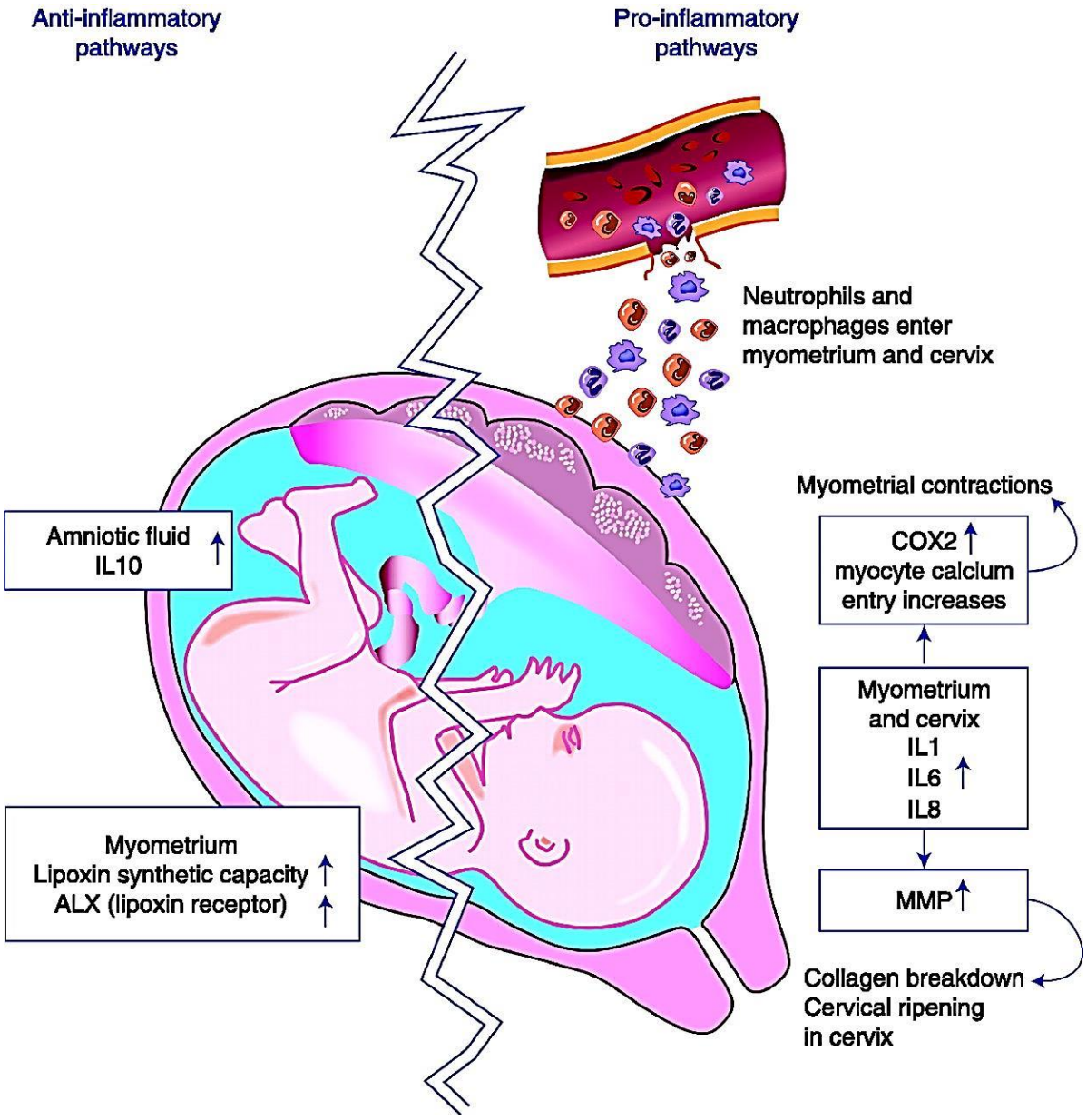



***Figure 1.14 – Pro- and anti-inflammatory molecules in pregnancy and parturition.*** *During pregnancy, amniotic fluid levels of IL-10 increases and in the myometrium levels of lipoxin synthetic capacity and lipoxin receptor expression increases in the maintenance of pregnancy. During labour, the myometrium and cervix expresses high levels of pro-inflammatory cytokines IL-1, IL-6 and IL-8 which initiate COX-2 expression and MMP expression. MMP expression causes collagen breakdown and cervical ripening and COX-2 expression causes myometrial contractions leading to labour (Jabbour, et al. 2009).*

Cytokines have been shown to play a huge role in culminating a successful pregnancy. During labour, macrophages level increases (Osman et al., 2006) which would ultimately result in higher cytokine and PG production, thus, influencing the timing of birth. Different triggers and inhibitors influencing the pro-inflammatory cytokine profile modulate the production of PGs, chemokines, cytokines and collagenases in order to increase myometrial contractility, effacement and dilation and membrane rupture leading to birth (Figure.1.15).

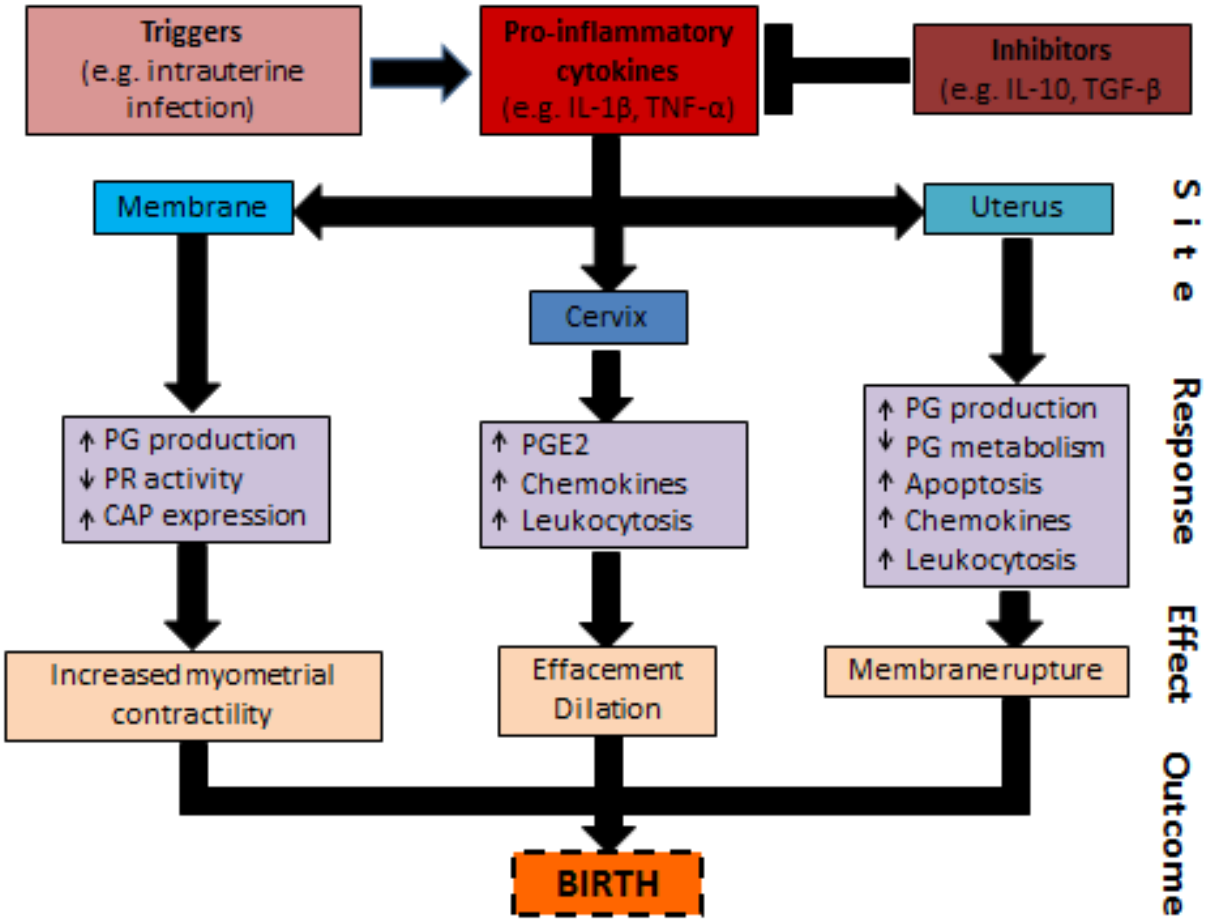



***Figure 1.15 – Cytokines, chemokines, and Prostaglandins in the inflammatory process of parturition.*** *PG, prostaglandin; PR, Progesterone receptor; CAP, contractile associated protein. Produced by me for the purpose of this thesis.*

### *1.9 Complement system and pregnancy*

Scientist, Jules Bordet, discovered a component of the innate immune system which augments the opsonisation and killing of bacteria with and without antibodies named as the complement system (Janeway CA Jr, 2001). The complement system is made of distinct plasma proteins which react with one another in a cascade of events to opsonise pathogens and induce inflammation for the clearance against the infection. The complement system protects against infection by three ways; firstly, it uses complement proteins which opsonise the pathogen which are recognised by phagocytes through their complement binding PRRs. Secondly, complement by-products act as chemoattractants which are involved in phagocytic cell recruitment to the site of infection and activate these phagocytes. Lastly, complement proteins function in forming membrane attack complexes to lyse certain bacteria by creating pores in the membrane. There are three distinct pathways of the complement system which complement proteins can opsonise the pathogen surface for its clearance: the classical, MBL and alternative pathway (Figure1.16). The classical pathway is characterised by the binding of first recognition sub component, C1q, to the pathogen surface in three ways; binding to the surface directly (e.g. lipoteichoic acid on Gram-positive bacteria), or binding to C-reactive protein, as an adapter, that binds to phosphocholine residues on the bacterial polysaccharides or binding to antibody-antigen complex, making a key link between innate and adaptive immunity. MBL pathway is activated recognition of mannose-containing patterns on the bacterial and viral surfaces. The alternative pathway is activated by spontaneous binding of C3 on surface of pathogens. Each of the three pathways leads to a sequence of reactions to generate a protease called a C3 convertase (C4b2b – classical and MBL pathway, C3bBb – alternative pathway). This is pivotal to all three pathways. The C3 convertase enzyme cleaves C3 to C3a and C3b. C3b opsonises the pathogen and binds to complement receptors on phagocytes for recognition of attack by promoting phagocytosis. C3b also serves as a paramount for forming C5 by binding to C3 convertase to form C5 convertase (C4b2b3b – classical and MBL pathway, C3b2Bb – alternative pathway). C5 convertase is responsible for cleaving C5 proteins to produce C5b which inserts into the membrane allowing C6, C7, C8 and C9 proteins to bind to form the membrane attack complex (MAC) for the lysis of cells.

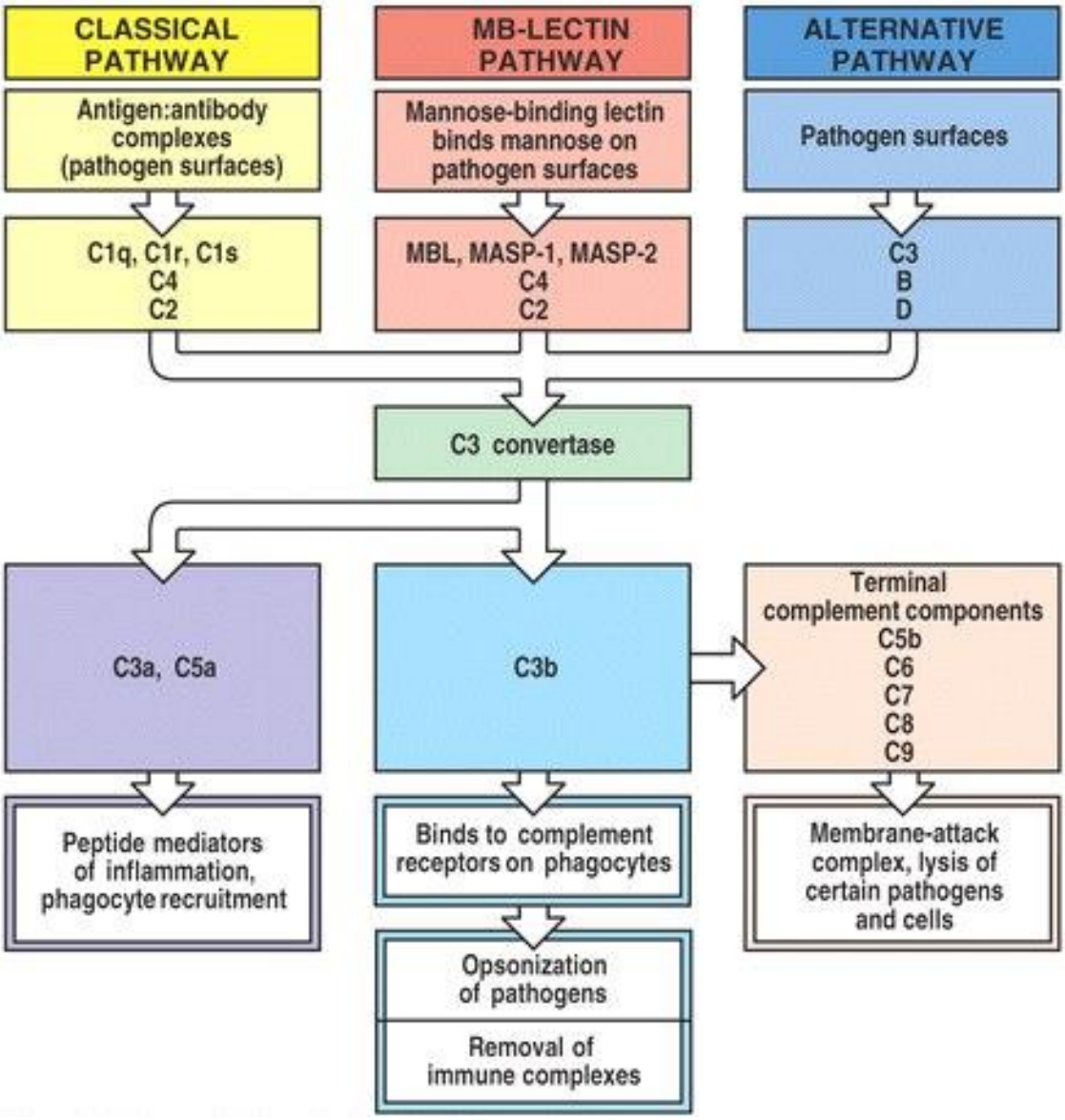



***Figure 1.16 – Complement system is divided into 3 main pathways; classical, MBL, and alternative pathway.*** *Pivotal to all 3 pathways is the C3 convertase. Each pathway leads to phagocytic recruitment, opsonisation, membrane attack complex and lysis of pathogens and cells for clearance (Janeway CA Jr, 2001).*

Product of Factor B, Bb, is a marker of the alternative pathway and does not opsonise the membrane surface, but forms a complex with C3b as part of the C3 convertase of the alternative pathway. Factor H, is a complement-regulatory protein, binding to factor B to displace Bb from the convertase in order to inactivate the complement cascade in initiating the MAC complex. However, factor P (properdin), has a higher affinity to the C3bBb convertase, stabilising the C3 convertase of the alternative pathway by acting as a positive regulator (Janeway CA Jr, 2001).

C1q, MBL and ficolin also contribute to the innate immune response by aiding in phagocytic recruitment, binding to receptors on phagocytes and removal of pathogens by opsonisation to enhance phagocytosis. Phagocytosis triggered by these innate immune cells causes the release of cytokines and chemokines to initiate inflammation. C3a, C4a and C5a, also referred to as 'anaphylatoxins', due to their ability to increase vascular permeability (Schumacher et al., 1991) and stimulate smooth muscle contractility (Gorski et al., 1979), and are products in the complement cascade. Increasing levels of C3a, C4a and C5a anaphylatoxins have been detected in maternal plasma of normal pregnancy, suggesting complement activation of innate immunity during pregnancy. It has also been proposed that complement activation could override the adaptive immune response in order to maintain pregnancy and to protect the host (mother and/or foetus) against pathogens and allergens/autoantigens (Richani et al., 2005). Other studies have shown increase in the levels of C1q, C4 and C3 in pregnancy, but with no complement activation (Adelsberg, 1983).

It was initially postulated that 'foetal-allograft' acceptance is caused by lack of foetal-antigen presentation or immunosuppression between mother and foetus (Billingham et al., 2010), either through lack of foetal antigen presentation to maternal lymphocytes, or through suppression of lymphocytes in recognising foetal antigens. It was also proposed by Wegmann *et al,* maternal immune response would be biased to foetal antigen presentation by selecting a Th2 antibody mediated response (Wegmann et al., 1993). This supported the notion that pregnancy is an immunosuppressive state although mothers are not in a completely disadvantaged state, with a higher susceptibility to contracting infections. Although the foetal allograft is often compared to tissue transplantation, the foetal allograft does not undergo surgical trauma, deliverance of acute and large antigens presented by antigen-presenting cells (APCs) which would lead

to rejection. Therefore, there is a clear difference in the two models. In addition, the placenta is neither inert nor impermeable, and is not generally immunosuppressed. Human pregnancy is its own specialised model. Firstly, the foetus is protected by its own organ, the uterus, protected by a mucosal barrier; the decidua. Secondly, the interaction between foetus and mother is via maternal blood where lipid components, hormones, cytokines and foetal and placental cells, displaying the two main points of foetal and maternal immune interactions are contained within the decidua and blood. The foetus is protected against immune rejection as it lacks MHC class I (HLA-A and HLA-B) or MHC class II expression. HLA-G expressed on extravillous trophoblast inhibits NK cells (Munz et al., 1997) and does not stimulate a cytotoxic T lymphocyte (CTL) (Sargent, 1993) response to placental products such as progesterone (Piccinni et al., 1995), and cytokines such as IL-10 (Wegmann et al., 1993). The general immunosuppressive state to protect the foetus carries a risk of infections either indirectly from the mother or directly to the amniotic fluid through vaginal entry. This is one of the major causes of preterm labour, growth defects, miscarriage, and in worse cases intrauterine death (still birth). Therefore, understanding the over-suppression of the adaptive immune response by innate immunity can help understand the clinical presentation of some infection and preeclampsia (Sacks et al., 1999).

Preeclampsia is strongly associated with foetal growth restriction and is one of the leading causes of preterm birth (Steegers et al., 2010). Although some complement activity is essential for normal placentation and successful pregnancy, previous studies have shown, over activity of complement may contribute to preeclampsia. High levels of C3a produced from all three pathways and Bb levels from the alternative pathway have been documented as markers for preeclampsia compared to normal pregnancies suggesting alternative pathway contributes to the pathogenesis of preeclampsia (Lynch et al., 2011, Lynch et al., 2012).

### *1.10 Surfactant protein SP-A and SP-D in pregnancy and labour*

The main component that lines the alveoli is the pulmonary surfactant secreted by the distal pulmonary epithelia. Pulmonary epithelia is made up of lipids, phospholipids and proteins which forms a thin layer at the air-liquid interface to lower the surface tension (Perez-Gil, 2002) to make breathing more mechanically sustainable (Rooney et al., 1994). Pulmonary surfactants consist of 90% lipids and 10% apoproteins. Hydrophillic

SP-A and SP-D together with hydrophobic collectins SP-B and SP-C make up the 10% of apoproteins. In the alveolar tissue, SP-A comprises 90% of the surfactant proteins (compared to SP-B, SP-C and SP-D) and its levels are ~10 fold greater than SP-D. Surfactant proteins SP-A and SP-D are hydrophilic apoproteins which are involved in the innate immune defence for the pulmonary clearance of infection. In the lungs, SP-A and SP-D are synthesised by alveolar type II cells and Clara cells and are secreted into the lining of the alveolar epithelium from lamellar bodies. SP-A and SP-D are soluble PRRs, and belong to the collectin family which bind with high affinity to infectious PAMPs in a calcium dependent manner. Upon recognition of microbe, the proteins, SP-A and SP-D activate for the clearance of infectious agent via opsonisation, neutralisation, agglutination, and phagocyte activation. The dual binding ability to interact with the pathogen and receptors on host immune cells activates the host innate immune response. These specific proteins modulate an inflammatory and anti-inflammatory response against pathogens and apoptotic cells as well as necrotic cells to promote its clearance.

### *1.11 Location and regulation of SP-A and SP-D gene*

SP-A and SP-D genes are located on chromosome location 10q22.2-23.1 in close proximity to MBL. SP-A is a major surfactant apoprotein consisting of two active genes; SP-A1 and SP-A2 and a pseudogene (White et al., 1985, Korfhagen et al., 1991). SP-A in humans is encoded by two genes unlike rats, rabbits and mice. SP-A1 and SP-A2 are 94% homologous at a nucleotide level and 96% homology in amino acid sequence (Katyal et al., 1992, McCormick et al., 1994), the main difference being, SP-A1 is encoded by 5 exons and SP-A2 encoded by 6 exons (Hoover and Floros, 1998). Approximately 300bp upstream from the DNA sequence for SP-A have been identified for basal promoter activity in lung epithelial cells *in vitro*. Within the 300bp, there is a sequence which is necessary for cAMP induction of promoter activity. SP-D promoter contains activator protiein-1 (AP-1), hepatocyte nuclear factor-3 (HNF-3), GT box, C/EBP elements which are important for SP-D promoter activity (He et al., 2000, He and Crouch, 2002) (Figure.1.17).

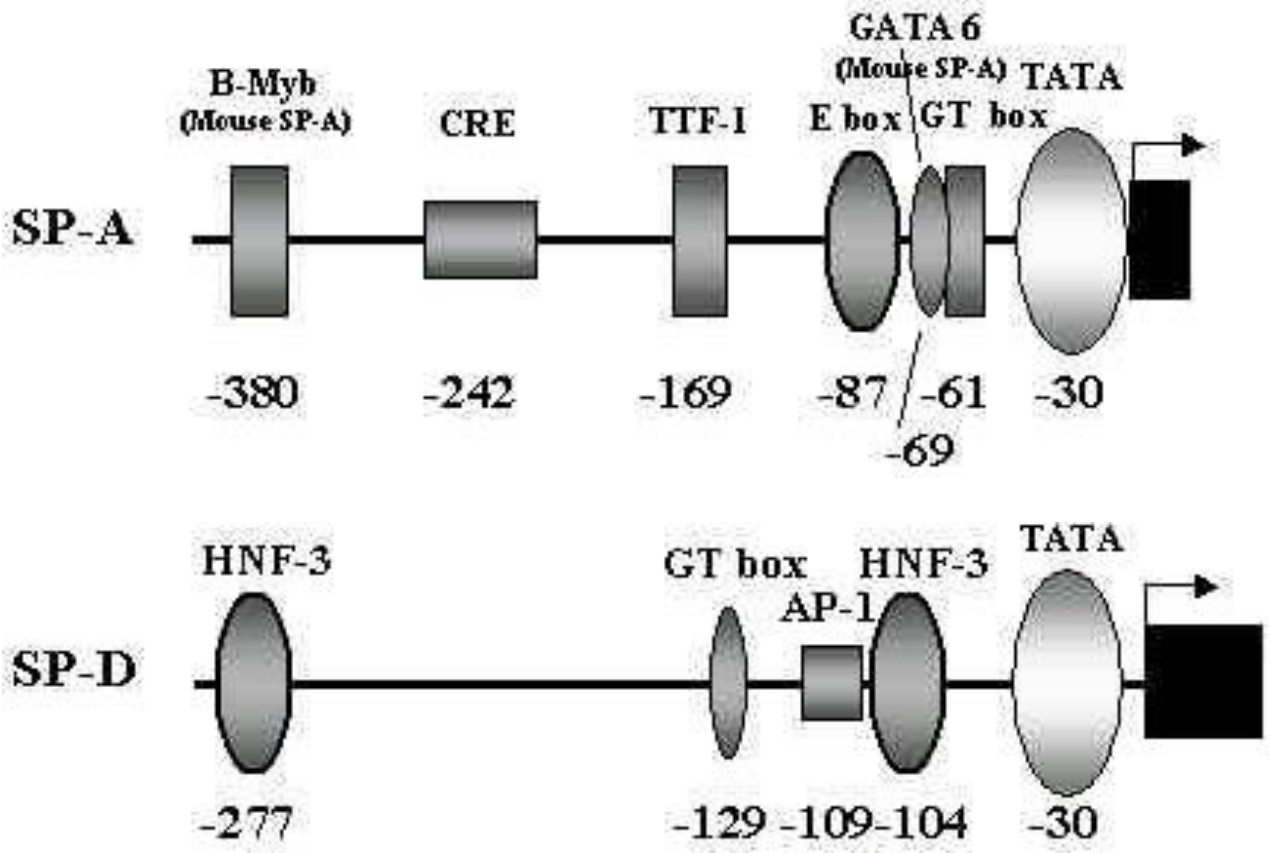



***Figure 1.27 – Schematic diagram of promoter regions of surfactant proteins A and D.*** *Diagram shows minimal promoter regions of mouse surfactant protein A and D genes. The locations (base pairs) are relative to the start of transcription (arrow) (Boggaram, 2003).*

### *1.12 Structural organisation of SP-A and SP-D*

The primary structure of SP-A and SP-D are organized into four regions: (i) a cysteine-containing N-terminus (required for disulphide-dependent oligomerization) that is linked to (ii) a triple-helical collagen region composed of repeating Gly-X-Y triplets (associated with maintaining the molecules shape, dimension, stability and oligomerization), followed by (iii) an α-helical coiled coil neck region (whose main function is protein trimerization), and (iv) a globular structure at the C-terminus comprising a C-type lectin or CRD that mediates calcium-dependent ligand-binding (Kishore et al., 2006) (Figure.1.18). The degree of subunit oligomerization probably affects the recognition of and binding strength to the carbohydrate ligands on the surface of pathogens (Holmskov et al., 2003). The collagen region consists of repeating motifs of Gly-X-Y, where X and Y can be any amino acid (generally proline or hydroxyproline). The relatively large collagen region of SP-D probably offers more freedom to the distal CRD regions to bind target pathogens and cause agglutination.

The structure of SP-A in its mature form consists of 6 trimers ~62 kDa each. In this hexameric form, a single molecule yields to 630 kDa. The hexameric, structure bears close resemblance to C1q with a bouquet-like appearance (Vaandrager and van Golde, 2000); (Kishore et al., 2006). In the presence of calcium, SP-A can bind in order of affinity to: mannose, fucose, glucose, galactose and N-acetylglucosamine (Childs et al., 1992).

SP-D is thought to be encoded by only one gene (Lu et al., 1992). SP-D consists of 4 oligomers and each oligomer comprises of a trimerised polypeptide chains each of 43 kDa and its oligomeric form (after trimerisation) is 130kDa (Figure.1.18). Once oligomerised to its tetrameric cruciform structure (4 of 130 kDa subunits), the protein yields a molecule of 520 kDa, each containing an N-linked oligosaccharide structure at Asn70. Human SP-D is assembled into a 520 kDa tetrameric structure with four of the homotrimeric subunits linked via their N-terminal regions, but trimers, dimers and monomers of the 130 kDa subunit can also be present in SP-D preparations. Up to eight of the 520 kDa tetrameric structures can undergo further oligomerization to give SP-D multimers having a large array, of up to 96 individual chains (12 x 8) (Kishore et al., 2006); (Holmskov et al., 2003).

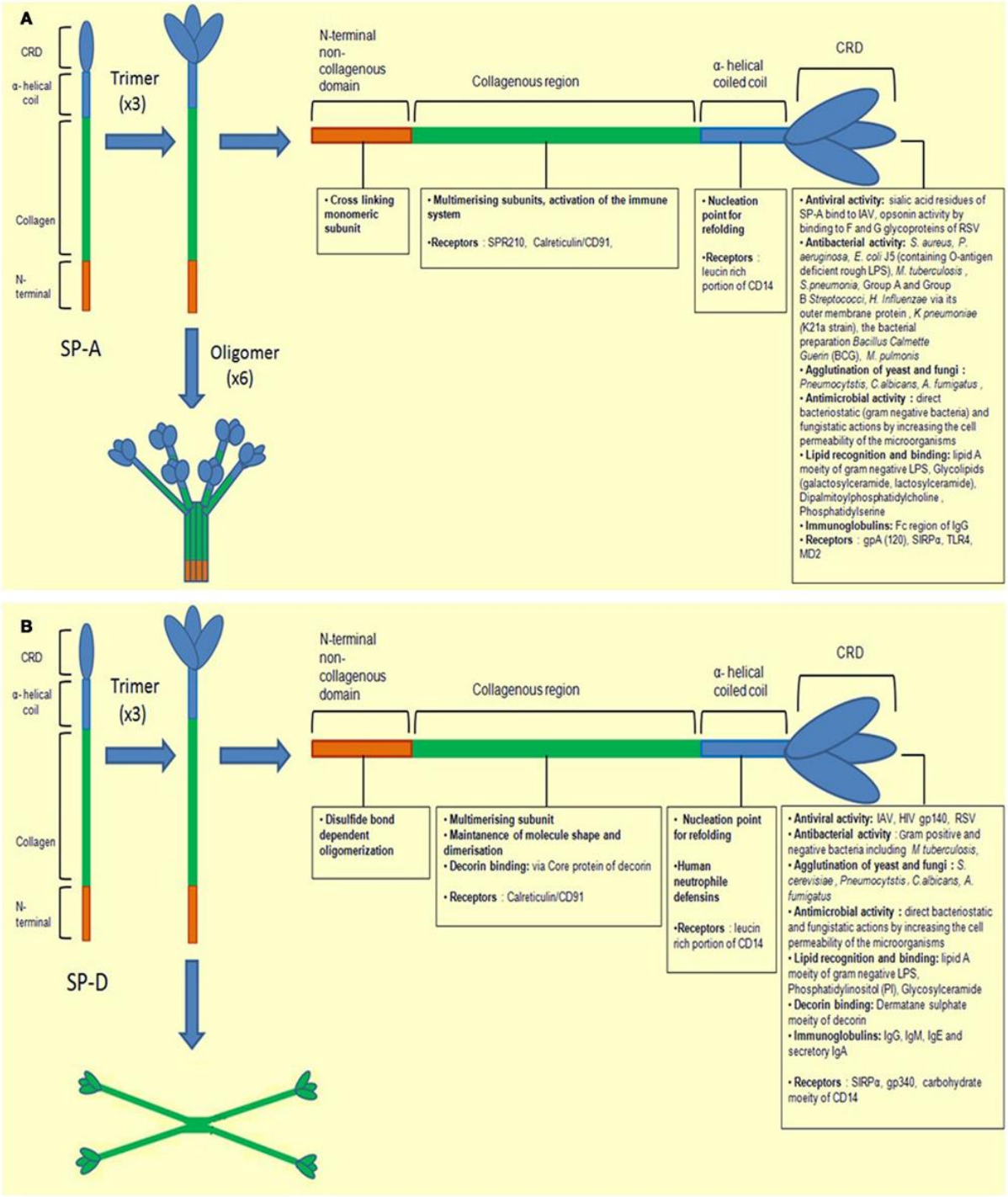



***Figure 1.38 – Sub-components of trimeric SP-A and SP-D and there functions.*** *(a) Trimeric form of SP-A (62 kDa) combining to form hexameric structure of SP-A (630 kDa) (b) Trimeric form of SP-D (43 kDa) combining to form tetrameric structure of SP-D (520 kDa protein). Contains N-terminal and carbohydrate recognition domain (CRD) connected to a collagenous region held by a supercoiled neck region. The key describes each component of the protein structure and its main functions (Nayak et al., 2012).*

### *1.13 Functional aspects of SP-A and SP-D*

SP-A and SP-D recognise specific carbohydrate and lipid structures on microorganisms, i.e. bacteria, viral particles, fungi and protozoa (Schelenz et al., 1995, Reid, 1998, Reading et al., 1998, Lim and Holmskov, 1996) via its C-type lectin/CRD to initiate an effector immune response (table 1.3). The CRD recognises and binds to carbohydrates, pathogens, phospholipids and sometimes receptors on phagocytes in a calcium-dependent manner (Palaniyar et al., 1998) for viral neutralisation, agglutination and opsonisation of bacteria, fungi, apoptotic and necrotic cells for clearance by activating antigen presenting cells such as DCs and phagocytosis by activated macrophages (Kishore et al., 2005). SP-A has a higher affinity to bind to dipamitoylphosphatidylcholine (DPPC), a major constituent of surfactant and is considered to play a major role in surfactant homeostasis and surfactant turnover (Holmskov et al., 2003, Kishore and Reid, 2001). SP-D preferentially binds to phosphatidylinositol (PI) and glucosylceramide sugar moieties and constitutes the minor part of surfactant (Kishore et al., 1996). SP-A and SP-D can recognise specific PAMPs such as rough LPS, mannose and glucose residues on most microbial ligands (Holmskov et al., 2003, Kishore and Reid, 2001). SP-A and SP-D can then engage with macrophages, neutrophils, lymphocytes and dendritic cells (Wright, 2004). They inhibit the IgE-allergen cross-linking and prevent histamine release from basophils and mast cells and modulates the Th cytokine profile and dampen an inflammatory response making them anti-inflammatory.

### *1.14 Biological Functions of SP-A and SP-D in extra-pulmonary tissues*

Human SP-A and SP-D both have pulmonary and extra-pulmonary existence (Nayak et al., 2012). Examples of extra-pulmonary sites include: the placenta, foetal amniotic fluid, umbilical cord, amnion, chorion, and decidua, detected from 26 weeks of gestation (Fisher and Mason, 1995, Madsen et al., 2000b, Madsen et al., 2003, Madsen et al., 2000a, Herias et al., 2007). SP-A and SP-D are secreted from the foetal lungs to the surrounding amniotic fluid and can be detected from the 2$^{nd}$ trimester where the levels increase up to term (Bayer et al., 1973). SP-A and SP-D have been localised by Immunohistochemistry in the foetal tissues (amniotic epithelium and chorionic membrane) and maternal tissues of the uterus (choriodecidual layers) (Miyamura et al., 1994).

***Table 1.3 – Recognition of a range of pathogens by SP-A and SP-D***

| **Viral** | | |
|---|---|---|
| Virus | Role | Reference |
| *Herpes simplex virus* (HSV) | SP-A Opsonisation of HSV via oligosaccharides for macrophages activation | (van Iwaarden et al., 1992) |
| *Influenza A virus* (IAV) | SP-A binding to IAV via sialic acid to stimulate respiratory burst of neutrophils. SP-D binding to hemagglutinin (HA) inhibiting its activity. | (Hartshorn et al., 1996)<br>(Hartshorn et al., 2000) |
| *Respiratory syncytial virus* (RSV) | SP-A binds to the glycoprotein of RSV for opsonisation. | (Hickling et al., 1998) |
| **Bacteria** | | |
| Bacteria | Role | Reference |
| *Staphylococcus aureus* | SP-D binding to peptidoglycan to enhance attachment to macrophages without phagocytosis | (van Iwaarden et al., 1992, van de Wetering et al., 2001) |
| *Escherichia coli* | Containing LPS, SP-A Enhances macrophage phagocytosis | (van Iwaarden et al., 1992) |
| *Streptococcus pneumonia* | Binds, aggregates, and promotes macrophages phagocytosis | (McNeely and Coonrod, 1994) |
| *Haemophilus influenza* | Binds, aggregates, and promotes macrophages phagocytosis | (McNeely and Coonrod, 1994) |
| *Klebsiella pneumonae K21a strain* | Opsonisation and enhances phagocytosis by binding to mannose receptor on macrophages. | (Kabha et al., 1997) |
| *Mycobacterium tuberculosis* | SP-A and SP-D bind via CRD to M.tb to enhance attachment to alveolar macrophages. SP-D however binds to lipoarabinnomannan moiety of Mtb bacilli to reduce uptake of bacteria by macrophages. | (Downing et al., 1995, Ferguson et al., 1999, Ferguson et al., 2002) |
| **Fungal** | | |
| Fungal | Role | Reference |
| *Pneumocystis carinii* | SP-A and SP-D binding to glycoprotein 120 (gpA) and β-glucans | (O'Riordan et al., 1995, Zimmerman et al., 1992) |
| *Candida neoforms* | SP-A and SP-D recognition and binding, SP-D opsonisation enhances phagocytosis of aggregates | (Walenkamp et al., 1999) |
| *Candida albicans* | SP-D inhibits hyphae and fungal growth and inhibits macrophage phagocytosis. SP-A prevents pro-inflammatory cytokine release from macrophages | (Rosseau et al., 1997, Rosseau et al., 1999) |
| *Aspergillus species* | SP-A and SP-D bind and agglutinate *Afu* conidia to enhance phagocytosis by neutrophils and alveolar macrophages, killing of germinating conidia. | (Madan et al., 1997) |

These proteins are considered to play an important role in the development and maturation of foetal lungs for respiratory function in the transition between intra-uterine to extra-uterine environments. SP-A and SP-D may be involved in the maintenance of pregnancy, protecting from infections, possibly in the progression to parturition.

### *1.15 Protective roles of SP-A and SP-D against intra-uterine infections*

SP-A and SP-D are innate immune molecules which can potentially recognise, opsonise and enhance clearance of infection from foetal membranes and amniotic fluid by interacting with the receptors present on amniotic epithelium and decidual macrophages. The clearance of infection is important to prevent threatening complications related to preterm birth such as lung infections in preterm neonates (Awasthi et al., 2001). Infection-associated preterm birth is accompanied by inflammation triggered by bacterial products with increased levels of PGs, cytokines and other inflammatory mediators. Clinically referred to as chorioamnionitis, it is most common at 26-32 weeks gestation when SP-A and SP-D levels are low (Lahra and Jeffery, 2004). Near term, SP-A levels rise sharply (Miyamura et al., 1994). The ability of SP-A to inhibit TNF-α secretion from DMs may prevent a premature activation of pro-inflammatory PG pathways delaying labour (Alcorn and Wright, 2004, Hallman, 2013). In addition, SP-A and SP-D are involved in the removal of necrotic and apoptotic cells therefore, reducing the risk of provoking an inflammatory response.

### *1.16 A role for surfactant in parturition?*

Pulmonary surfactant is secreted from the foetal lungs into the surrounding amniotic fluid, which accumulates in the third trimester. There is a clear association with development of foetal surfactant and survival of newborn, suggesting a possibility that spontaneous labour may be triggered by foetal lung surfactant as they are responsible for lung maturation (Lopez Bernal et al., 1988). Both SP-A and SP-D are synthesised in the 26$^{th}$ week of pregnancy and reaches functional levels at 34 weeks. By 40 weeks gestation, the levels of SP-A in human amniotic fluid rise dramatically, from 3 to 24 μg/ml with a steady rise of SP-D, rising from a 1:1 ratio (SP-A/SP-D at 26-34weeks) to 6:1 (at 38-40 weeks) at the end of gestation of term pregnancies (Miyamura et al., 1994) making it an important factor for foetal lung development before parturition. Therefore,

surfactant replacement therapy is important for babies born premature suffering from respiratory distress syndrome (Gibson, 1997). The presence of SP-A and SP-D in the foetal membranes and the levels of SP-D in the amniotic fluid of mice models giving birth at different gestational periods may differ. The levels of SP-D detected in amniotic fluid ranged from 5ng/ml to 50ng/ml. The levels of SP-A and SP-D could be detected as early as 26 weeks of gestation. The ratio between SP-A and SP-D levels in the amniotic fluid are roughly identical between 26-34 weeks of gestation, curiously, there is a large surge of SP-A between 37-42 weeks of gestation which is the normal time for parturition to deliver the baby at term (Miyamura et al., 1994).

SP-A levels are good indicators of lung maturity which reflects the timing of birth in mice (Condon et al., 2004). Mice SP-A secretion near term activates macrophages in amniotic fluid which promotes SP-A migration from the foetal membranes to maternal side of the uterus, ultimately withdrawing progesterone to initiate the PG pathway to parturition (Condon et al., 2004). As progesterone levels decrease, the NF-κB driven inflammatory response increases which drives the foetus to labour (Condon et al., 2004). SP-A and SP-D from human foetal membranes, amnion and choriodecidua, can directly regulate the human maternal uterine cell function in the process of initiating labour. SP-A can bind to uterine smooth muscle cells and influence its cell signalling pathway to cause uterine contractility (Breuiller-Fouche et al., 2010).

With increased gestational age, SP-D levels in the amniotic fluid and foetal lung tissues increase (Leth-Larsen et al., 2004, Miyamura et al., 1994, Dulkerian et al., 1996). Therefore, SP-D can be used as a good biomarker for lung maturation (Inoue et al., 1994) and prediction of gestational age. Dahl et al, investigated the level of SP-D found in capillary blood in infants and umbilical cord blood of newborns. They found the median level of SP-D detected in umbilical cord blood from 423 infants was 392.1ng/ml. In 130 patients, arterial blood was drawn and showed to have 359.0 ng/ml. The venous umbilical cords from 263 cases were shown to have 420.8 ng/ml. The median SP-D level in the capillary blood of 233 infants determined to be as 779.5 ng/ml of blood, considerably greater than umbilical cord blood. It was evident that low levels of SP-D in umbilical cord made a difference in the timing of birth but there was no correlation between levels of SP-D in capillary blood and timing of birth (Dahl et al., 2005). However, the mode of delivery or the timing of birth did not affect the levels of SP-D. In

fact, with progression, SP-D levels decreased in the umbilical cord and, as reported earlier, levels of SP-D enter into the amniotic fluid from the pulmonary epithelium to the infant (Pfister et al., 2001, Callen et al., 1979, Marino and Rooney, 1981, Berger et al., 1996, Kalache et al., 2002).

### *1.17 Immunomodulatory properties of SP-A and SP-D in pregnancy and parturition*

Garcia-Verdugo et al (2008) have demonstrated the effects of SP-A on human myometrial cells (Garcia-Verdugo et al., 2008). Previous studies show that SP-A binds to LPS and interacts with components of the LPS receptor complex such as TLR4 or CD14 (Sano et al., 2000, Yamada et al., 2006) which modulates macrophages to activate NF-κB resulting in production of proinflammatory cytokines (Guha and Mackman, 2001) which stimulate uterine contractility and parturition (Condon et al., 2004).

LPS is another stimulatory molecule initiating an inflammatory response, particularly activating macrophages through TLR-4, which are present in the cervix, endometrium and uterine tubes (Fazeli et al., 2005). At the time of labour, macrophages can synthesise TNF-α which is upregulated by infection (Chen et al., 1991). Microorganisms such as *Ureaplasma urealyticum,* cytomegalovirus*, Listeria monocytogenes,* parovirus *B19* and *Gardnerella and mycoplasma* can damage foetus or induce preterm birth (Dortbudak et al., 2005), although the link between inflammation and pregnancy loss is evident it is still unclear how to develop strategies for preventing or controlling undesirable outcomes.

SP-A not only activates the transcription factor RELA but also MAPK1/3, protein kinase C zeta (PRKCZ) and $PTGS_2$ which are all modulators of uterine contractility (Garcia-Verdugo et al., 2008). Verdugo et al (2008) also found SP-A binds to LPS receptor complexes such as CD14, TLR4 and LY96, inhibiting LPS-induced NF-kB activity to prevent an inflammatory response (Garcia-Verdugo et al., 2008). SP-A knockout mice show a clear correlation between foetal lung development and the timing of birth. Following injection of SP-A into amniotic fluid, high levels of NF-kB, IL-1β and amniotic fluid macrophages were expressed which induced mice into early labour (Condon et al., 2004). Condon et al also showed that by inhibiting these pro-inflammatory cytokines using inhibitors (e.g. NF-kB peptide inhibitors) the onset of labour can be delayed

(Condon et al., 2004, Haagsman, 2002). Consistent with the previous assessment, injection of high levels of anti-SP-A antibodies delays the progression to labour (Condon et al, 2004), providing strong evidence that SP-A may play a vital role in the parturition pathway in mice.

### *1.18 SP-A and SP-D as biomarkers of lung maturation*

Surfactant levels of SP-A and SP-D can be potentially good biomarkers for foetal lung maturity for its survival in extra-uterine life. Surfactants reduce the surface tension in the lungs to allow sufficient oxygen exchange for function and viability of all organs. Deprivation of oxygen to the brain (cerebral hypoxia) can lead to severe brain damage and intrauterine growth retardation (IUGR). Surfactants play an important role for proper lung function and prevention of acute and chronic pulmonary disorders. Therefore, sufficient levels of SP-A and SP-D are important for foetal survival. During labour, hormone levels increase to accelerate foetal lung maturation. Hence, caesarean sections without labour can lead to lung malfunction such as respiratory distress syndrome (RDS). The ability to assess foetal lung maturations in women with high risk pregnancy emphasises the requirement for surfactant replacement for the infant after birth. Collection of amniotic fluid via trans-abdominal and trans-vaginal amniocentesis is good for clinical diagnostics to assess the risk of RDS. Levels of SP-A rise before birth, and 24hours after delivery the levels reach 1.7 μg/ml in normal healthy babies. 71% of this level suggests RDS and 83% of this level is the minimum value to place in the non-RDS category (Satoh et al., 1992). Levels of SP-A are lower in diabetic mothers, suggesting insufficient lung development in foetus with diabetic mothers. At 30-31 weeks gestation, levels of SP-A in healthy foetus' is ~3μg/ml which rises to 24μg/ml at 40-41 weeks gestation (Snyder et al., 1988). Therefore, the ability to quantify SP-A levels before term is a good potential biomarker for foetal lung maturation and early diagnosis for development of lung malfunctions. SP-A levels from foetal amniotic fluid can have a significant correlation with the maturation of foetal lung. However, other sites which express and have localisation of this protein could also serve as prediction markers for diseases. For example, in foetal cord blood levels of SP-A in infants born preterm, before 32 weeks gestation, have lower levels of SP-A in cases with RDS compared to without. Babies with normal weight born preterm express ~5.8ng/ml SP-A in cases with RDS and three times this value (15.1 ng/ml) without RDS. However, this

comparison could only be made without accounting for heavy weight babies born preterm with RDS, in which these cases had higher levels of SP-A (Cho et al., 2000). This suggests that other factors such as heavy weight babies should be taken into account for diagnostic purposes. In contrast, mice born preterm have similar levels of SP-A and SP-D in amniotic fluid with or without infection (Chaiworapongsa et al., 2008). This shows one of the many difference between mouse and human foetal pregnancy development.

SP-A is expressed in amniotic epithelium and chorionic trophoblasts, with levels appearing higher by 17.4 folds in chorionic membrane in preterm delivery (PTD) with chorioamnionitis than without chorioamnionitis, regardless of labour or no labour. Furthermore, the levels of SP-A appear higher at term compare to preterm infants (Han et al., 2007). SP-A levels have shown to vary with or without labour and in both amniotic fluid as well as cord blood. SP-A levels in cord blood are higher at term with labour (between 36-38wks) with 4.8-50.2 ng/ml compare to term with no labour with 2.7-2 1 ng/ml. Labour shows to increase levels of SP-A at least 2-folds compared to no labour (Cho et al., 1999). In contrast, another study has shown that the levels of SP-A in amniotic fluid are higher at term with no labour, ranging between 2.2-15 μg/ml compare to 1.2-10 μg/ml with labour (Chaiworapongsa et al., 2008). However, levels of SP-A increase as gestation to term parturition progresses. At 36-39 weeks SP-A levels appear 4.8-50.2 ng/ml and this increases by 39-41 weeks to 12.2-44.6 ng/ml. On the other hand, maternal serum shows no significant changes in SP-A levels before and after labour, varying from 7-74 ng/ml before labour and 9.6-73.6 ng/ml after labour (Cho et al., 1999). This suggests that SP-A could potentially signal to parturition by accelerating foetal lung maturation for extrauterine survival and this may be a foetal signal to parturition. Furthermore, maternal SP-A may not be a favourable marker for timing of birth. Another study has shown that overexpression of methionine allele of SP-D gene Met31Thr polymorphism is present in preterm infants compared to term infants and this genetic predisposition was only found in foetal tissue not maternal tissue (Karjalainen et al., 2012). Thus, the timing of birth can be influenced by SP-A and SP-D. Therefore, foetal immune molecules may play an important role in promoting labour.

Levels of SP-A and SP-D have been detected by various methods to predict foetal lung maturity such as ELISA from amniotic fluid (Snyder et al., 1988, Shimizu et al., 1989, Satoh et al., 1992) and maternal serum (Inoue et al., 1994), immunoblotting (Wiehle et

al., 1995), immunohistochemical staining (Khoor et al., 1993, Sati et al., 2010) and stable microbubble test (Kumazawa et al., 2003). The ability to detect the levels of SP-A and SP-D accurately is going to be useful for reducing neonatal morbidity in infants born with RDS.

SP-A and SP-D genes are independently regulated in foetal lung tissue throughout gestation. The mRNA and protein levels can be detected in foetal lungs at the end of the 2nd trimester of pregnancy (after 75% gestation), whereas, SP-B and SP-C can be detected much earlier before differentiation of type II cells. There is some evidence which show SP-D to express in bronchial epithelium and alveolar epithelial type II cells in human foetal lung as early as 12 weeks of gestation which gradually increases during the canalicular and saccular stages, gradually intensifying throughout gestation (Chen et al., 2005). During the second trimester, at 19-20 weeks gestation, SP-A mRNA and protein can be localised in tracheal epithelium in mucosal folds, bronchial epithelium and glands, bronchial cells and pre-type II cells lining the airways of human foetal lungs (Khoor et al., 1993). SP-A, SP-B, SP-C and SP-D can be located in extra-pulmonary sites such as the placenta, in particular; trophoblastic layers of chorionic villous tree, trophoblastic cell columns, stromal cells, hofbauer cells, angiogenic cell cords and vascular hematopoietic cells (Sati et al., 2010). SP-D has been localised in all villous and extra-villous trophoblast subpopulations and in the endometrium at the secretory phase of menstruation. SP-A and SP-D are also present surrounding the placenta such as amniotic fluid, foetal membranes and in foetal serum. This indicates these innate immune molecules serve important function in protection again uterine infections and from the moment of implantation during pregnancy.

### *1.19 Hormonal regulation of SP-A and SP-D biosynthesis*

The two genes, SP-A and SP-D are regulated differentially by cAMP and glucocorticoids (McCormick and Mendelson, 1994). Cyclic AMP analogs such as PGE2 (Odom et al., 1987) increase SP-A mRNA (and SP-A protein) levels (Boggaram and Mendelson, 1988). Glucocorticoid levels are capable of stimulating and inhibiting the expression of SP-A in foetal lung tissues *in vitro* (Boggaram and Mendelson, 1988, Boggaram et al., 1988, Khoor et al., 1994). *In vitro* studies of human foetal lung from mid-trimester (15-18 weeks) have shown that $10^{-9}$ and $10^{-10}$ M glucocorticoid levels stimulate SP-A mRNA levels but any higher level have an inhibitory effect (Boggaram et al., 1989). However,

glucocorticoid receptor antagonist RU487 can reverse these inhibitory effects (Boggaram et al., 1991). Glucocorticoid can also increase SP-D levels *in vitro* (Dulkerian et al., 1996) and rat maternal administration of glucocorticoid stimulates SP-D mRNA (Deterding et al., 1994, Mariencheck and Crouch, 1994) and protein levels (Mariencheck and Crouch, 1994) in foetal lungs. Hormone dexamethasone has also shown to regulate SP-A mRNA levels (McCormick and Mendelson, 1994), which can also be regulated in response to TGF-β. In one study, TGF-β family increases SP-A mRNA in human foetal lung tissues (Ballard et al., 1990), whereas another study appears to show TGF-β to inhibit SP-A mRNA (Beers et al., 1998) and protein (Whitsett et al., 1987) expression in human foetal lung. TNF-α, has been shown to inhibit SP-A mRNA expression via p38 signal transduction pathway (Miakotina and Snyder, 2002). Thus, inhibition via protein kinase C could result in lung tissue damage by inflammation. In preterm labour, levels of IL-1β and SP-A are high except in the incidence of RDS in preterm labour (Watterberg et al., 1996), suggesting IL-1β is also important in regulating surfactant levels and promoting lung maturation.

SP-B and SP-C are independently regulated to SP-A and SP-D, suggesting the levels are regulated by different stimulus. Cyclic AMP and glucocorticoids regulate the levels of SP-A transcriptionally and both at a gene and protein level respectively. Glucocorticoids are clinically used to accelerate foetal lung maturation and can stimulate SP-A and inhibit mRNA stability to regulate its levels. Combination of both cAMP and glucocorticoids synergistically increase SP-A gene transcription (Mendelson and Boggaram, 1990). SP-A is involved in surfactant function and reutilisation by type II cells (Mendelson et al., 1991). Cyclic AMP and glucocorticoids affect level of SP-A gene expression in mid-trimester of human foetal lung in vitro (Mendelson et al., 1991). PGs also play an important role in regulating SP-A levels, as it has been shown, PGE2 and PGF2α, the main stimulators for myometrial contractility, when inhibited by indomethacin inhibits cAMP formation and, thus, SP-A gene expression. However, further stimulation by PGE2 reverses this effect (Mendelson et al., 1991), suggesting that PG indirectly regulates SP-A through cAMP. However, the effects of SP-A and SP-D on PGs have not yet been investigated. Another corticosteroid drug, dexamethasone, has been shown to alter the levels of SP-A by either stimulating or inhibiting its expression in human foetal lungs (Boggaram et al., 1991). At high levels the drug inhibits SP-A

expression and at lower levels inhibits PG expression. This suggests that SP-A is not entirely dependent on cAMP generation by PGs and that other hormones are involved in increasing cAMP levels. Therefore, levels of PGs do not necessarily reflect the level of SP-A production. However, the production of cAMP is important for the development for foetal lung tissue. This has been shown in an earlier study where human foetal lung tissue cultured without serum and hormones increased cAMP levels by 140% within 3-6 days. The addition of isobutylmethylxanthine further doubled cAMP levels and SP-A mRNA and protein levels, which was then inhibited by indomethacin. Indocmethacin also inhibits SP-B expression, phosphatidylcholine and activity of fatty acid synthase, suggesting its possible role to delay labour. However, parallel stimulation of indocmethacin with PGE1 and PGE2 further stimulated cAMP levels, reversing the inhibitory effects in high PG content. As a result, levels of SP-A increase and showing the strong effects of PGs to stimulate the induction to labour (Ballard et al., 1991). This suggests indomethacin near term may not be a strong competitive drug to delay labour due to the stronger effects of PGs. This also suggests PGs, in particular PGE1 and PGE2, are important for accelerating alveolar type II epithelial cell differentiation by increasing cAMP production to accelerate foetal lung maturation near term.

PGs regulate the synthesis of cAMP, PGE stimulates cAMP as a signal for smooth muscle cell contraction of the uterus during labour. $PGE_1$ and $PGE_2$ bind to their hormone receptors which subsequently stimulates cAMP synthesis because the receptor is paired with adenylyl cyclase through its stimulatory G protein (GPC). If an inhibitory G-protein (Gi) is coupled to the receptor, the signal will not be sent across the membrane. Another transporter molecule responsible for regulating the signal across the cell membrane is the human prostaglandin (hPG) transporter (Lu et al., 1996). This transports $PGE_1$, $PGE_2$ and $PGF_{2\alpha}$ with high affinity (Lu et al., 1996, Itoh et al., 2006) with the adenyl cyclase activity. The ability of PGs to bind to transmembrane receptors (G-protein-coupled-receptors) allows their diffusion through tissues.

SP-A regulates surfactant uptake and recycling, and thus its deficiencies results in diseases such as neonatal RDS. Therefore, regulating the levels of SP-A and SP-D before birth is important for proper lung function. Diabetic mothers are hyperinsulinemic and have increased rate of neonatal RDS due to surfactant production deficiency by alveolar type II cells. Studies have shown that insulin inhibits accumulation of SP-A and SP-B, but

not SP-C mRNA levels in human foetal lungs (Sugahara et al., 1994). In this case glucocorticoids are used to accelerate foetal lung maturation. Low levels of cortisol stimulates SP-A by 20% whereas high levels of cortisol decrease SP-A by 50% but increase SP-B and SP-C. Low levels of cortisol, when combined with insulin, also decreases SP-A expression. However, surprisingly, high levels of cortisol when combined with insulin show a stimulatory effect on SP-A mRNA expression (Dekowski and Snyder, 1995). This suggests cortisol in high doses can accelerate foetal lung maturation in diabetic mothers undergoing insulin treatment.

The foetal pituitary gland also regulates hormonal release such as cortisol and PGs. Cortisol is known to stimulate foetal lung maturation and stimulate the conversion of progesterone to oestrogen to promote uterine contractility and cervical ripening. PGs are important for both cervical ripening and myometrial contractility. The foetal membrane produces predominantely $PGE_2$ and the decidua produces mainly $PGE_{2\alpha}$ to promote cervical ripening and myometrial contractility respectively. Labour is associated with increased PG concentrations in both amniotic fluid and maternal membrane as well as systemic circulations. If we know how SP-A and SP-D affects the PG production and influences the timing of birth we could look at the levels of PGs in maternal serum and estimate the risk of mothers to enter preterm birth. Therefore, the levels can be regulated by inhibiting the levels of PG to normal levels.

Cortisol has been shown to stimulate SP-A mRNA expression and SP-A up-regulates PGE2 release in chorionic trophoblasts by induction of COX-2 (also known as, PTGS2) and microsomal PGE synthase (Sun et al., 2006). Recently, it has been shown that SP-A1, SP-A2 and SP-D mRNA expression is predominant in the choriodecidua, and SP-D predominantly in the amnion at the end of pregnancy (Breuiller-Fouche et al., 2010). This suggests the signal for parturition could be initiated from the foetus and SP-A could be a strong contributor to the PG pathway to initiate parturition. The effect of the increasing levels of SP-A and SP-D has been shown to induce stress fibre formation in cultured myometrial cells via Rho-kinase pathway. Thus, it is important to understand the mechanism of myometrial contractility and how the increasing levels of SP-A and SP-D can affect it. SP-A has been shown to exert a paracrine effect on F-actin filament in the organisation of myometrial cells to induce contraction and parturition (Breuiller-Fouche et al., 2010). Again, this study also shows that the level of SP-A and SP-D

affecting the parturition physiology is from the foetal side. This emphasises the foetus plays an important role in parturition and the increasing levels of extra-pulmonary surfactant proteins could be huge contributors to stimulating the pathway to labour and can potentially help predict the timing of birth.

### *1.20 Regulation of pro-inflammatory and anti-inflammatory immune responses by SP-A and SP-D during pregnancy*

Although SP-A and SP-D are well known as the pulmonary innate immune molecules involved in clearing infection, the immunological significance of SP-A and SP-D during pregnancy has not been well understood. The placenta is an interesting, yet complex, organ which expresses both paternal and maternal antigens which can potentially be attacked by the maternal immune system. They escape the maternal immune system by releasing immunosuppressive cytokines such as IL-6 activated IL-6-receptor-mediated signal transduction pathways to produce hCG. IL-1 and TNF-α augments IL-6 production to stimulate hCG production. On the other hand, TGF-β has opposite effects, suppressing IL-6 production and cytokine mediated hCG production (Matsuzaki, 1994, Strayer et al., 1997). In support to this, TGF-β inhibits $Ca^{2+}$ influxes, which is a negative feedback regulator for SP-A (Strayer et al., 1997). This suggests TGF-β regulates and modulates intracellular $Ca^{2+}$ signalling. IL-6 from cord serum has been shown to stimulate SP-A synthesis to promote lung maturation and reduce chances of RDS. IL-6 derives mainly from chorionic trophoblast cells as well as IL-8 and monocyte chemotactic and activating factor (MCAF) which are expressed actively in the 3$^{rd}$ trimester for potential defence against chorioamnionitis during pregnancy (Matsuzaki, 1994). Therefore, these cytokines do not only promote acute-phase protein synthesis but also augment the immune defence against infection. IL-1β has stimulatory effects in increasing SP-A mRNA levels to promote lung maturation as it has been shown in horn foetal lungs (Bry et al., 1997). However, IL-1 activity is high in cases of intraamniotic infection associated with preterm labour although it is a lung stimulatory cytokine to perhaps prepare foetus for extrauterine life. Murine models have shown SP-A to induce the NF-κB pathway, upregulating TNF-α and IL-10 for TLR-4 function in response to pathogens and endogenous host mediators (Guillot et al., 2002). In human foetal immune system, TNF-α augments IL-6 production and IL-6 stimulates SP-A synthesis to

promote lung maturation, whereas, in mice, SP-A shows to regulate TNF-α level to protect the host. Another study by Condon et al, 2004, has also shown in mice foetal lungs, SP-A stimulates IL-1β to activate NF-κB in cultured amniotic fluid macrophages. This leads to the migration of foetal amniotic fluid macrophages to the uterus with advancing gestation.

Parturition has also been associated with prostaglandin release (Keirse, 1978) suggesting a possible relation of surfactant and prostaglandins. Studies show arachidonate as a precursor for prostaglandin synthesis by phopholipase $A_2$ and arachidonate includes 4% of foetal surfactant. However, arachidonic acid is transferred from surfactant arachidonylphophatidylcholine to aminocyte phosphatidylethanolamine and PI by sequential actions from phopholiase A2 and phopholipase C and diacylglycerol lipase (Okita et al., 1982). Moreover, phospholipase C and monoacyl- and diacyl-glycerol lipases directly degrade surfactant released from foetal lungs to contribute to the pool of free arachidonate in amniotic fluid (Bernal and Phizackerley, 2000). Therefore, amniotic epithelium provides a source of arachidonic acid for prostaglandin synthesis during labour.

As previously shown, SP-A up-regulates $PGE_2$ release in chorionic trophoblasts by induction of COX-2 (Sun et al., 2006). However, Lee et al, 2010 cultured human amnion explants incubated with SP-A to see inhibitory cytokine-cytokine receptor interaction, down regulating IL-1β and COX-2, suggesting SP-A maintains pregnancy and promotes an anti-inflammatory environment during pregnancy (Lee et al., 2010). Mice models have shown that SP-A is critical for the onset of labour (Condon et al., 2004) and further investigation supports this view in humans. Labour is characterised by decidual activation with the production of pro-inflammatory mediators. A recent study investigated the effects of SP-A (100μg/ml) in human decidua. The results reveal that SP-A inhibits $PGF_{2\alpha}$ in term decidual stromal cells without affecting other inflammatory mediators and SP-A decreases in decidua with labour (Snegovskikh et al., 2011). This contradicts the reported increasing levels of SP-A as it has been shown in amniotic fluid, amnion and chorionic membrane, therefore the net levels of SP-A in the 3$^{rd}$ trimester has been shown to increase. Perhaps the levels of SP-A used in this study was unrealistically high, and therefore, shows contradictory results. In sheep, LPS instillation in intra-amniotic fluid causes $PGE_2$ expression to increase in foetal lung,

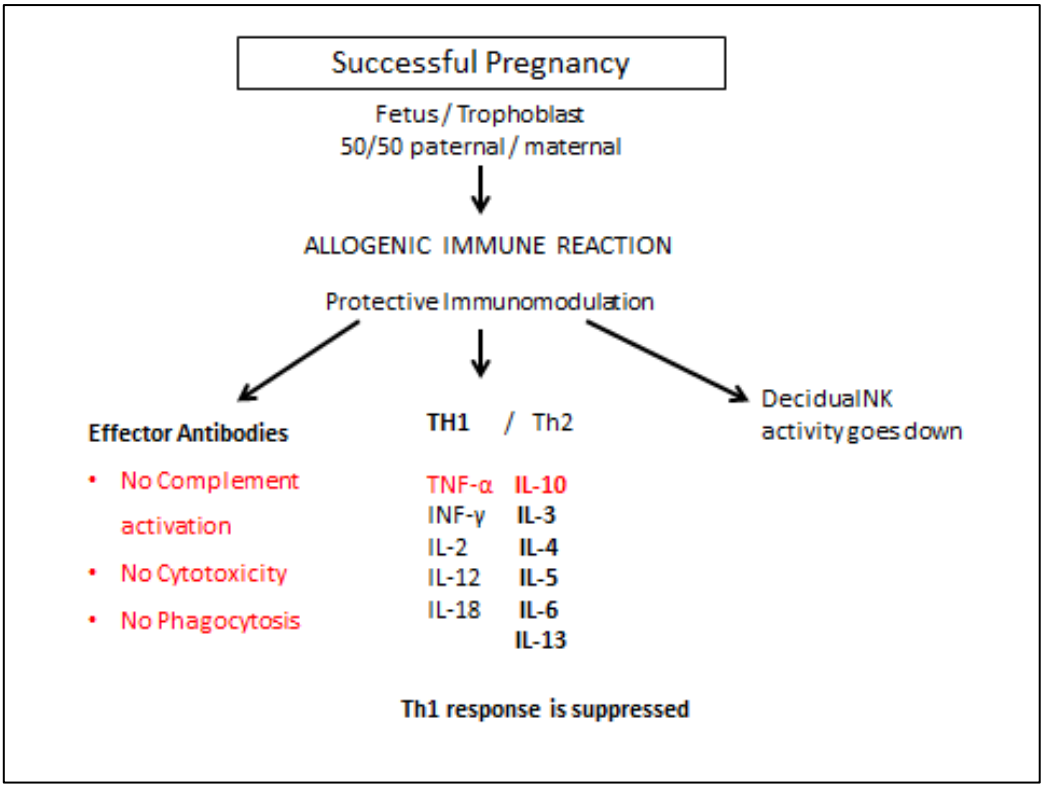
which has been established to promote foetal lung maturation and surfactant production (Westover et al., 2012). SP-D knockout mice, when challenged with LPS, show increased expression of TNF-α, suggesting SP-D promotes an inflammatory response in mice (Salminen et al., 2012). It has been postulated that the maintenance of pregnancy i.e. quiescence is maintained by increasing progesterone receptor transcriptional activity and therefore, spontaneous labour is initiated by biochemical events which negatively influence the progesterone receptor (Mendelson and Condon, 2005). Thus, it could be interesting to see if SP-A could down-regulates the progesterone receptor and therefore serve as a hormone to the initiation of labour.

### *1.21 Lung deficiencies and Preterm birth*

Preterm birth has shown to be the leading cause of infant mortality and morbidity and is associated with chronic respiratory illnesses (Berkowitz, 1993, Kramer et al., 1987, Hillier et al., 1988, Goldenberg et al., 2000). Lung injury and lung immaturity are the major contributors to infant mortality and morbidity (Horbar et al., 2002). The cases of preterm birth are continually on the rise and are associated with diseases. 1/10 preterm born children have been shown to develop a permanent disability such as lung disease and cerebral palsy. 80% of extremely preterm babies (before 26 weeks) develop some form of disability (Marlow et al., 2005). There is great evidence to suggest preterm babies born before 36 weeks in the third trimester have a high incidence of respiratory diseases such as acute respiratory distress syndrome (ARDS) (Wang et al., 2004). This is supported by Rubaltelli et al, who have documented 30.8% of preterm neonates born between 34-36 weeks gestation had respiratory problems (Rubaltelli et al., 1998). The three main respiratory diagnoses associated with preterm labour are: respiratory distress syndrome (RDS), tachypnea, pneumonia and respiratory failure (Hibbard et al., 2010). This highlights the importance of full lung development of the neonate for defence against respiratory pathogens and the maturation of lungs being vital in the full gestational time. There are, however, interventions to aid in lung maturation such as antenatal treatments with corticosteroids to induce lung maturation in infants who are at risk of preterm delivery before 32-34 weeks gestation (Crowley, 2000). However, due to lack of knowledge and understanding of the underlying causes and mechanisms of labour, it is difficult to manage the timing of birth effectively.

## *1.22 Hypothesis/objectives*

The main objectives of this thesis were; to investigate how SP-A and SP-D modulate the PG pathway and the cytokine profile which influences the pregnancy and labour initiating myometrial contractility and membrane rupture. As SP-A and SP-D not only influence the hormonal pathway which leads to birth, but are also important in the clearance of infection. It has been examined how they maintain a balance in playing a protective role during pregnancy and modulate the expression of PGF2α and COX-2, the two potent stimulators of the PG pathway and parturition. The rationale is that SP-A and SP-D serve as a source of arachidonic acid influencing the rate of PG synthesis. The main prostaglandin expression here is $PTGS_2$ which synthesises PGDH – the first prostaglandin protein. The receptor expression being focused is PTGFRN, a negative regulator receptor for $PGF_2$ – the most potent prostaglandin which accelerates the pathway to result in myometrial contractility. IL-1β, which mimics an inflammatory response of labour will be used as a positive control for treating amnion, chorion and decidua to examine the effects of SP-A and SP-D. To examine the protective roles of SP-A and SP-D, the influence of these proteins on the expression of TLRs and cytokines is also the focus of this research (Figure.1.20) This will highlight the balance between protection against infection and maintenance of pregnancy as well as the induction of labour by SP-A and SP-D and any dysfunctions could affect normal pregnancy leading to preterm birth, miscarriages, preeclampsia, IUGR and ARDS.



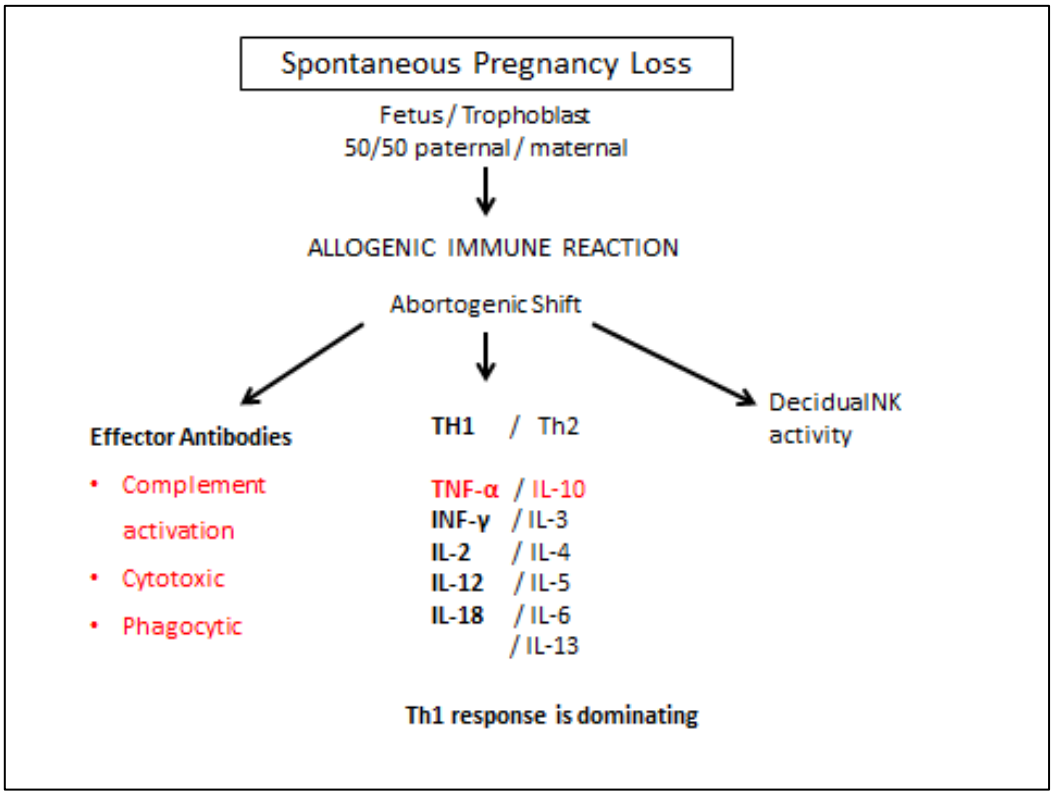




***Figure 1.19 – Representation of immunomodulation of successful pregnancy and pregnancy loss.*** *(Druckmann and Druckmann, 2005). Those highlighted in red were examined in the present study (see chapter 4).*

### *1.23 Synopsis*

In this PhD thesis, the following objectives have been achieved:

**Chapter 3** – Focuses on the production of full length and homotrimeric lectin domains (CRD regions) SP-A and SP-D molecules. In order to purify human native SP-A and SP-D, pooled amniotic fluid from term labour was used via affinity chromatography, ion-exchange and gel filtration. Full length human SP-A1 gene was also cloned and expressed in HEK-293T cells as a transient expression system. The gene fragment containing neck and CRD region of human SP-A was expressed first under bacteriophage T7 promoter system as inclusion bodies, followed by denaturation and re-naturation procedure and affinity purification. A similar version of homotrimeric CRD region of human SP-A was also expressed using gene fusion technology where an *E. coli* maltose-binding protein (MBP) was used as an affinity partner in order to yield soluble form of recombinant protein. The gene fragment containing neck and CRD with 8 Gly-X-Y amino acids from human SP-D gene was expressed under the bacteriophage T7 promoter system as inclusion bodies. This was followed by denaturation and re-naturatation procedure followed by affinity purification using maltose-agarose column. These proteins were characterised for their immunogenicity and primary ligand binding abilities prior to their use using placental tissues. The levels of LPS were also measured in order to obviate potential pro-inflammatory response due to TLR pathway.

**Chapter 4** – presents the effects of SP-A and SP-D and their truncated versions on the hormonal and immunological pathway related to pregnancy and labour. This was achieved by making amnion, chorion and decidua (ACD) explants and treating these with either IL-1β as inducers of pro-inflammatory immune response. This response was also examined in the presence of various versions of SP-A and SP-D molecules in terms of production of pro-inflammatory and anti-inflammatory cytokines, prostaglandin pathway enzymes and innate immune pattern recognition receptors (PRRs) such as toll-like receptors (TLRs).

**Chapter 5** – starts with immunohistochemical (IHC) staining in order to localise SP-A and SP-D (and its cognate receptor calreticulin) in ACD explants, decidual tissue, umbilical cord, placenta and myometrium. Having established the differential existence of SP-A and SP-D in these tissues, this study focuses on localisation and fractionation of

decidual macrophages (DMs) in the decidual tissues. Since DMs are central to protective response against pathogens and inflammations, it was considered imperative to examine the immunomodulatory effect SP-A and SP-D can have on resident DMs under inflammatory conditions.

# Chapter 2:

# General Methods and Materials

# 2. General Methods and Materials

### *2.1 Collection of pooled amniotic fluid from term labour*

Ethical approval was obtained from Brunel University, Bristol Hospital and Warwick University Hospital. All women were consented for obtaining placental, amniotic and BALF samples.

Amniotic fluid is normally collected either following vaginal rupturing of the foetal membranes or during spontaneous rupturing. The collected amniotic fluid is usually equilibration with 5 mM $CaCl_2$ and stored at -80°C until it is required for purification.

1 L of pooled amniotic fluid was completely thawed by incubating at 37°C (~ 5-6 hours) with occasional mixing. The fat and large aggregates/debris from amniotic fluid were sieved through a 3 mm Whatman filter paper three times then through a cell strainer twice for a clearer suspension and to avoid future clogging of maltose-sepharose column. The SP-A rich pellet was separated from the SP-D rich supernatant by centrifugation at 10,000 x g for 40 mins at 4°C.

### *2.2 Preparation of Maltose Sepharose*

Prior to Sepharose 4B activation with divinylsulphone (DVS), Sepharose beads were washed extensively. Firstly, 100 ml of Sepharose 4B was washed with autoclaved deionised water ($dH_2O$) centrifuging at 5,000 rpm for 5 minutes each time. After decanting the water, the Sepharose 4B was suspended in 100 ml of 0.5M sodium carbonate pH9.5 and stirred at room temperature whilst slowly adding 10 ml DVS drop-by-drop. The activated Sepharose was left stirring at room temperature for 1 hour inside a fume hood, then transferred to a Buchner funnel with filter paper and was washed extensively with 3 litres of autoclaved water. The gel was then suspended in 20% maltose solution made in 0.5% sodium carbonate ($Na_2CO_3$) for a further incubation overnight on a magnetic stirrer. Under these conditions the Sepharose couples to the hydroxyl groups of D-maltose. After 24 hours, the gel was washed with 2 litres autoclaved $dH_2O$ through a Buchner funnel lined with filter paper and then washed with 2 litres of 0.5% sodium bicarbonate ($NaHCO_3$). The gel was suspended in 100 ml 0.5% sodium bicarbonate containing 5% 2-merceptoethanol to block excess vinyl groups and mixed for a further 3 hours at room temperature before washing

through a funnel with 2 litres of autoclaved $dH_2O$. The maltose-Sepharose was stored in affinity column buffer containing 0.02% sodium azide at +4°C.

### *2.3 Competent cell and transformation into BL21 (λDE3) and BL21 (λDE3) pLysS*

A single colony of BL21 (λDE3) pLysS was inoculated in 5 ml LB containing 5µl of chloramphenicol (50 mg/ml) and incubated overnight in a 37°C shaker at 2000 rpm. The next day, 1 ml of primary inoculum was transferred into 25 ml LB containing 50 mg/ml chloramphenicol. This was incubated for 2-3 hours in the shaking incubator for cells to grow to log phase indicated by 0.3-0.4 OD against plain LB medium at 600 nm. After correct OD had reached, the sample was centrifuged at 2500 rpm for 10mins to collect BL21 (λDE3) pLysS cells to pellet. The supernatant was discarded the pellet was resuspended in 12.5 ml of 0.1 M $CaCl_2$. The resuspended pellet was incubated on ice for 1 hour to make cells competent. The cells were again centrifuged at low speed centrifugation of 2500 rpm for 5 mins to pellet the cells. After discarding the supernatant, the pellet was resuspended cells in 2 ml 0.1 M $CaCl_2$ that was ready for transformation.

Firstly, 200 µl of competent cells was transferred into a sterile 2 ml eppendorf tube and 100 ng of plasmid was added. This was mixed by flicking the tube to allow DNA to enter the competent cells followed by incubation on ice for 1 hour before applying heat shock at 42°C for 90 secs to allow transfer of DNA material. The nuclear pore was closed by transferring the tube to ice for 5 minutes. 800 µl of LB was added to the cells and placed in a 37°C incubator for 45 mins to allow 1 complete cell cycle replication to take place. 100 µl of competent cells was spread on an LB agar plate containing ampicillin and chloramphenicol

Note: when using BL21 (λDE3) for transforming pMalC-2 containing MBP fused SP-A gene, no antibiotics were used for inoculation of E.coli strain. After transformation, transformed cells were streaked on agar containing ampicillin plates and thereafter, colonies were inoculated in LB media containing ampicillin.

### *2.4 Transformation into OneShot TOP10 competent cells*

Transformation of plasmids into OneShot TOP10 competent cells (Invirtogen, Paisley, UK) were carried out according to manufacturer's recommendation. TOP10 competent cells have a transformation efficiency of 1 x $10^9$ cfu/µg supercoiled DNA and allow

stable replication of high-copy number plasmids. In brief, 2 vials of TOP10 competent cells were thawed on ice and transformation was taken place immediately after thawing. 3 µl of overnight incubated ligation mixture was added directly to one vial of TOP10 competent cells and mixed by flicking the tube gently. The second vial was used as a control so no DNA was added to this. Both vials were incubated on ice for 1 hour then heat shocked for the uptake of plasmid at 42°C for 90 seconds in a water bath. The vials were then placed on ice for 5 minutes to close cellular pores and prepare for replication in S.O.C medium. Aseptically, 250 µl of pre-warmed S.O.C medium was added to each vial and placed in an incubator set at 37°C for 1 hour. 50 µl of the culture was spread on an ampicillin/agar plate and left overnight at 37°C for colonies to form.

## Cloning of recombinant full length SP-A1 in pSecTagC

### *2.5 PCR with High Phusion DNA polymerase*

cDNA of full length SP-A cloned into pSecTagC (primer sequences mentioned on p.91) was used to amplify neck and CRD region to create trimeric SP-A. High-Phusion DNA polymerase (Thermoscientific-F-553 S) has a superior advantage over the tradition *Taq polymerase* as it has an error rate 50-fold lower than *Taq.* High Phusion DNA polymerase has a high thermostability and produces higher yields with lower amount of enzyme and in the presence of RNA inhibitors. This makes it more convenient and reliable for its use making it great to use for high performance PCR and cloning, which is fit for our purpose.

The necessary reagent provided in the kit contains; Phusion DNA Polymerase 2 U/µl, 5X Phusion HF Buffer and dNTP mix (10 mM each). Phusion HF buffer and 1 Unit of DNA was added to <250 ng template DNA per 50 µl reaction. Forward and reverse primers were used at a final concentration of 10 mM. No template DNA was added to the master mix but directly to the tubes and control tube contained water as a substitute to DNA. The final volumes of the components in each tube are listed in table (Table 2.1)

***Table 2.1 – PCR tubes set up to amplify SP-A neck and CRD region using Phusion DNA polymerase.***

| Component | R x n – 50 µl | Master mix 4 x n |
|---|---|---|
| **5 X Phusion HF buffer** | 10 | 40 |
| **10 mM dNTPs** | 1 | 4 |
| **10 mM F.Primer** | 2.5 | 10 |
| **10 mM R.Primer** | 2.5 | 10 |
| **Template DNA** | 1.5 | (6) |
| **Phusion DNA polymerase** | 0.5 | 2 |
| **Nuclease free water** | 32 | 128 |
| **TOTAL** | 50 | 200 |

In the 4 labelled tubes, 48.5 µl of master mix was added and 1.5 µg DNA was added directly to the tubes except the control tube. 1.5 µl of nuclease free waster was added directly to the control tube. The samples were mixed well and loaded into a thermocycler at condition mentioned in the table 2.2. Initially, a denaturation of 2 minutes takes place to separate the double stranded cDNA. This is followed by 30 cycles of denaturation (10 secs @ 98°C), annealing of primers (20 secs @ 59°C) and extension (1 min @ 72°C) to allow complementary bases bind to make a copy of the template strand. At each cycle the amount of DNA doubles. At the end of the reaction there is a final extension to complete the cycle.

***Table 2.2 – Thermocycler conditions for PCR with Phusion DNA polymerase***

| **Thermocycler condition** | | |
|---|---|---|
| **Initial denaturation** | 98°C | 2 mins |
| **30 CYCLES:** | | |
| **Denaturation** | 98°C | 10 secs |
| **Annealing** | 59°C | 20 secs |
| **Extension** | 72°C | 1min |
| **Final extension** | 72°C | 10 mins |
| **Store** | 4°C | |

### *2.6 Agarose-EtBr gel – 0.7%*

0.7% agarose-EtBr gel was prepared by dissolving 0.35 g agarose powder into 50ml 1 X TBE (Tris/Borate/EDTA) buffer and after cooling to hand bearable temperature, 5 µl ethidium bromide was mixed before pouring into the casting gel apparatus. After the gel had solidified (between 30 mins-1 hour), 1 X TBE buffer was poured over the gel and into the tank. The comb was removed and the samples were added. 10 µl of each sample were mixed with 6 X DNA loading dye and loaded into the wells with 5 µl DNA marker (Hyperladder I – Bioline). The DNA was separated at 70 V for 1.5-2 hours and the bands were observed under the transilluminator before capturing an image on the Molecular Image Gel Doc XR+ (Bio-Rad, Hemel Hempstead, UK).

### **2.7 Gel extraction (QIAquick Gel Extraction kit – QIAGEN)**

After fractionating the DNA PCR product on a 0.7% agarose gel, the PCR product was gel extracted using Qiaquick Gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's protocol. In brief, initially the fragment was excised using a sharp blade and added to a 2 ml eppendorf tube. This was weighed and 3 volumes of Buffer QG was added (100 mg ~ 100 µl) and incubated in a water bath at 50°C for 12 mins mixing occasionally to help dissolve the gel. 1 volume of isopropanol was added directly to the dissolved sample and mixed. The suspensions were transferred to a QIAquick spin column provided with a 2 ml collection tube. The sample was centrifuged for 1 min at the highest centrifugation speed to allow DNA to bind and the flow-through was discarded. This was repeated until all content was spun and maximum DNA was collected. As the DNA was to be sent for sequencing the column was washed with 0.5 ml Buffer QG and centrifuged at 13,000 rpm for 1 min. The DNA was washed with 0.75 ml washing buffer, Buffer PE, after incubating for ~5 minutes. Again, the column was spun at 13,000 rpm for 1 minute. The flow-through was discarded and the column was spun again to remove residual wash buffer. In a clean 1.5 ml microcentrifuge tube, the column was added and 50 µl of elution buffer was added and incubated for ~5 minutes before centrifuging at 13,000 rpm for 1 min. The quantity and purity of eluted DNA was assessed via NanoDrop. The plasmid was transformed into TOP10 *E.coli* competent cells and grown for plasmid isolation and digestion.

### *2.8 Plasmid extraction mini kit*

Plasmid isolation (Qiagen, Manchester, UK) was carried out in order to subject each plasmid for restriction digest to check if gene was correctly inserted. Consequently, the plasmids containing the gene of interest were sent for sequencing (Beckman Coultier, UK). Plasmid isolation was carried out according to manufacturer's recommendations. Briefly, 3 colonies of the transformed cells were selected and individually inoculated in 6 ml LB + ampicillin (100 µg/ml) and grown overnight in a shaking incubator at 220 rpm. The next day, 1 ml of the culture was reserved for possible streaking and 5ml of the culture was centrifuged in a swing-bucket centrifuge at 5000 rpm for 7 mins. The pellet was retained and resuspended in 250 µl Buffer P1 (containing RNase) prior to transferring into a clean microcentrifuge tube. 250 µl of Buffer P2 was added and mixed thoroughly to lyse the cells until the solution appeared clear. The reaction was neutralised with 350 µl of Buffer N3 and mixed immediately by thoroughly inverting the tube several times causing the solution to become colourless. This was centrifuged at 13,000 rpm for 10 minutes in a table-top microcentrifuge. The supernatant was passed through a QIAprep spin column by centrifugation at 13,000 rpm for 1 min to allow DNA to bind. The column was washed with 0.5 ml Buffer PB to prevent contamination of DNA by centrifugation prior to further washing of DNA with 0.75 ml Buffer PE containing ethanol and again was centrifuged at 13,000 rpm for 1 minute. The flow-through for each sample were discarded after each step and further centrifuged to remove residual ethanol contaminants from DNA. Finally, to elute the DNA, 50 µl of Buffer EB was added directly to the membrane of the spin column and incubated for ~5mins before centrifugation at 13,000 rpm for 1 min. Each plasmid was subjected to digestion to check if the gene of interest was inserted.

### **2.9** ***Plasmid digestion***

Colonies were selected to verifying insert of gene of interest. Each colony were inoculated in 5 ml LB containing 100 µg/ml ampicillin and grown for 12-17 hours at 37°C. Cultures were harvested at 5,000 x g for 8 minutes before extracting plasmid with mini prep plasmid isolation kit (Qiagen, Manchester, UK) according to manufactures recommendation. For digestion, 10 µl of extracted plasmid from each colony were incubated in a 50 µl reaction containing 2.5 µl EcoRI and XhoI each, 10 µl 10x BSA and 5µl NEB buffer 4 incubating at 37°C for 3 hour. The products were verified on an

agarose-gel and visualised under a UV Transilluminator (Bio-Rad, Hemel Hempstead, UK).

Colony containing and verified for the gene of interest was subjected to large scale plasmid extraction with EZNA FastFilter (VWR, Leicestershire, UK), according to manufactures recommendation. Large scale plasmid extraction was used for transfection in HEK-293T cells.

### 2.10 Plasmid isolation large scale EZNA fastfilter (VWR, Leicestershire, UK)

Large scale plasmid isolation was carried out to prepare large amounts of plasmid pSecTagC and pSecTagC containing full-length human SP-A for expression in mammalian HEK cells. EZNA fastfilter was used according to manufactureer's recommendation. Initially, one colony was inoculated in 100 ml of LB containing Ampicillin and grown overnight in a shaking 37°C incubator. The next day, the culture was pelleted at 3500 rpm for 10 mins. To the pellet, 2.25 ml of Solution A/RNase A was added to resuspend the cells. This was then transferred to centrifugation tube capable of withstanding 12,000 x g and 2.25 ml of Solution II was added and mixed gently by inversion 8-10 times until a flocculent white precipitate was formed. The suspension was again centrifuged at 12, 000 x g for 10 mins at room temperature. To a HiBind DNA Midi column pre-inserted in a 15 ml collection tube (supplied in kit), 1 ml of equilibration buffer was added and incubated for 4-5 mins at room temperature before centrifugation at 3000 x g for 3 minutes in a swing-bucket centrifuge. The supernatant containing DNA was added to the equilibrated column and centrifuged at 3,000 x g for 5 mins, flow-through was discarded and repeated to centrifuge the entire content of supernatant. To the column, 3 ml of Buffer HB was added to allow further binding of DNA to the column and centrifuged at 3,000 x g for 5 mins. The column was washed to remove residues using 3 ml of DNA wash buffer diluted with absolute ethanol to the Midi column and centrifuged as repeated above. The flow-through was discarded and the collection tube was re-used. Washing step was repeated again and then the column was centrifuged to remove excess wash buffer and to remove excess ethanol from the column. The column was transferred to a clean 15 ml centrifuge tube and 0.5-1 ml of elution buffer (10 mM Tris, pH8.5) was added and incubated for 5 minutes before centrifugation at maximum speed for 5 mins to elute DNA.

## Cell culture and Expression of SP-A in mammalian HEK-293T cells

### *2.11 Preparing cell suspension for cell count*

Once cells had grown to 80-90% confluence, the old media was discarded and cells were washed with PBS. The cells were dissociated from flask with 3 ml TripLE Select (Invitrogen, Paisley, UK) and tripsinised as described before. After inactivating the trypsinisation by adding 5 ml supplemented media, cells were used for cell counting.

### *2.12 Cell count*

Cell pellet was mixed evenly with gentle agitation and 1 ml of cells was transferred into a sterile eppendorf tube using Gilson pipette. Using a 100 µl pipette, 100 µl of cells was mixed and transferred into another eppendorf tube containing equal amounts of trypan blue dye. 10µl of dyed cells was loaded on a haemocytometer (both sides of the chamber) and the lines were focused on the haemocytometer with the 10x objective lens. Observing 4 big squares containing 16 small squares, the cells which were not taken up by trypan blue (viable cells) were totalled. Each big square containing 16 small squares indicated number of cells x $10^4$ per ml. By adding the total number of cells in all 4 squares and dividing by 4 gives the average number of cells x $10^4$ per ml. This value is multiplied by 2 to adjust for the 1:2dilution of cells in trypan blue. The calculated sum accounts for the total number of cells x $10^4$ per ml which can be multiplied by the ml the cells are resuspended in. For transfection, 1.5 x $10^6$ cells are added to 2 flasks and 2 x $10^6$ cells were frozen down for future use.

### *2.13 Cryopreservation of Cells*

After cell count, 1 ml containing two-four million cells/ml in DMEM was diluted in 500 µl FBS and 500 µl 10% DMSO and 1 ml was transferred per labelled cryovial. The vials were stored at -80°C overnight and in liquid nitrogen the next day.

## Cloning and expression of trimeric SP-A in pET101/D TOPO

### *2.14 Primer design*

Primers for human SP-A neck and CRD region were designed using the CRD region after the 23gly-Xaa-Yaa repeats. The forward primer is designed to contain four bases – CACC to complement the overhang in the TOPO vector to allow directional cloning to take place by stabilising the PCR product in correct orientation. The forward primer

sequence 5' – CACCATGCATCTAGATGAGGAGCTCCAAGC (amplicon 444 bp) containing the leading pET101/D-TOPO sequence (CACC) which hybridizes to the GTGG overhang on pET101/D-TOPO vector. The CACC sequence is also necessary for directional cloning and a start codon (ATG) to provide optimal spacing for proper translation. The same reverse sequence 3' - GGGCTCGAGTCAGAACTCACAGATGGTCAGTCG containing the XhoI site which was used for cloning both trimeric and full length SP-A in HEK-293 cells. The gene of interest is controlled by the T7 promoter upstream to the pET101 vector. This promoter is regulated by the E.coli strain BL21 (λDE3) pLysS containing expressing the T7 RNA polymerase. This is induced by IPTG.

### *2.15 PCR with High Phusion DNA Polymerase*

PCR was carried out as described earlier with High Phusion DNA polymerase and the products were separated via 0.7% agarose-gel to excise the band containing gene of interest.

### *2.16 Gel extraction*

After fractionating the DNA on a 0.7% agarose gel, the PCR product was gel extracted using Qiaquick Gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's protocol as previously described.

### *2.17 Ligation with pET101/D TOPO vector (Invitrogen)*

The blunt-end PCR purified product was directly cloned into Champion pET101/D-TOPO vector (Invitrogen, Paisley, UK). The kit has 90% efficiency without requiring ligase or restriction enzymes. The pET vector was chosen for its specific features including the T7 *lac* promoter for IPTG inducible expression of the gene of interest (Dubendorff and Studier, 1991; Studier *et al.*, 1990). The vector is designed to contain the lacI gene encoding the lac repressor to reduce basal level of expression from the T7 lac promoter in the vector and from the lacUV5 promoter in the E. coli host chromosome. The vector contains an ampicillin resistant gene for selection of E. coli containing the vector with the gene of interest.

The ligation was set up according to manufaturer's guidance. Briefly, two 1.5 ml eppendorf tubes were set up containing pET101/D TOPO vector, PCR product, salt solution diluted in sterile water. The volumes of each component are listed in table 2.3.

*Table 2.3 – Ligation set for directional cloning of SP-A N/CRD into vector pET101/D TOPO.*

| Reagent | Vector only (µl) | Vector + PCR insert (µl) |
|---|---|---|
| **pET101/D TOPO vector** | 1 | 1 |
| **Salt solution** | 1 | 1 |
| **PCR product** | - | 3 |
| **Sterile water** | 4 | 1 |
| **Final volume** | **6** | **6** |

*Ligation was set-up using pET101/D TOPO. Two reactions were set up, using no PCR product as a control expecting no growth of cells on an ampicillin/agar plate as open vector cannot be transformed and replicated in E. coli. List of reagents and volume were followed as stated in the table. The ligation reaction was carried out at room temperature for 30 minutes before incubating at 4°C overnight.*

### *2.18 Transformation of pET101/D TOPO into OneShot TOP10 competent cells*

The ligated sample of SP-A N/CRD in pET101/D TOPO was transformed into ready-made competent cells (Invitrogen, Paisley, UK) as previously described.

### *2.19 Plasmid isolation for pET101/D TOPO containing N/CRD of human SP-A*

Plasmid isolation (Qiagen, Manchester, UK) was carried out for restriction digest to check if gene was correctly inserted. Consequently, the plasmids containing the gene of interest were sent for sequencing (Beckman Coultier). 6 Plasmid isolations were carried out according to manufacturer's recommendations as previously described.

### *2.20 Restriction digestion of pET101/D TOPO containing SP-A (N/CRD) with SacI and EcoRI*

The plasmids isolated were set up for digestion using restriction enzymes EcoRI and SacI. Six tubes were set up plus one for water control containing no plasmid. A master mix was made to contain 7 µl EcoRI, 7 µl SacI, 7 µl Buffer 4, 7 µl 10 X BSA with no plasmid. Into 7 individual 1.5 ml microcentrifuge tubes, 8 µl of master mix was added and mixed with 2 µl plasmid. For the water control 2 µl nuclease free water was added. The samples were incubated at 37°C for 1.5 hours. After digestion had taken place 6 X loading dye was added and loaded in a 1% agarose-EtBr gel (0.5 g agarose in 50 ml 1 X

TBE buffer). The results were observed under the transilluminator before capturing an image on the ImageStream. The plasmids with the correct inserted gene were transformed in *BL21 (λDE3) pLysS* as previsouly described.

### *2.21 Expression of the recombinant fragments of homotrimeric CRD regions of human SP-A (rfSP-A) and SP-D (rfSP-D)*

Completion of transformation of recombinant fragment SP-A (rfSP-A) colonies of cells were formatted on the ampicillin/chloramphenicol (amp/chl) agar plate. 10 colonies were selected randomly to examine the expression of the gene of interest. 10 single colonies were inoculated individually in 5 ml LB supplement with Amp/chl. before growing in a 37°C shaking incubator overnight. The next day, 500 µl of the primary culture was transferred into 10 x 10 ml LB with amp/chl. and grown in the a 37°C shaking incubator until the cells reach late log phase, indicated by an OD (600 nm) between 0.6-0.8. 1 ml of the secondary culture was transferred in a 2 ml eppendorf tube to represent the un-induced control to observe expression efficiency. The rest of the culture was induced with 0.5 mM IPTG and both un-induced and induced samples were further incubated for 3 hours in a 37°C shaking incubator. After induction was complete, 1 ml of the 'induced' sample was transferred into a 2ml eppendorf tube and along with the 'un-induced' samples, the samples were centrifuged at 13,000 rpm and the pellets were reserved. The supernatant was discarded and the pellet was resuspended in 100 µl of 2 x treatment buffer. The 'un-induced' samples along with 'induced' samples were denatured on a heating block set at 100°C for 10 minutes before briefly centrifuging and loading 10 µl (100 µg/ml) of each samples on a 12% SDS-PAGE. Expected size is between 18-20 kDa. According to the bands visualised on the gel, the colony with the best expression was streaked on an LB amp/chl. plate to grow more colonies. Once the best colony for expression has been established the next step was to upscale it to a large scale expression.

### *2.22 Large scale expression of insoluble proteins rfSP-A and rfSP-D*

A single colony was inoculated individually in 15 ml LB supplement with Amp/chl. before growing in a 37°C shaking incubator overnight. The next day, 12.5 ml of the primary culture was transferred into 250 ml LB with amp/chl. and grown in the a 37°C shaking incubator until the cells reach log phase, indicated by an OD (600 nm) between 0.6-0.8. 1 ml of the secondary culture was transferred in a 2 ml eppendorf tube to

represent the un-induced control to observe expression efficiency. The rest of the culture was induced with 0.5 mM IPTG and both un-induced and induced samples were further incubated for 3 hours in a 37°C shaking incubator. After induction was complete, 1 ml of the 'induced' sample was transferred into a 2ml eppendorf tube and along with the 'un-induced' samples, the samples were centrifuged at 13,000 rpm and the pellets were reserved. The supernatant was discarded and the pellet was resuspended in 100 µl of 2 x treatment buffer. The 'un-induced' samples along with 'induced' samples were denatured on a heating block set at 100°C for 10 minutes before briefly centrifuging and loading 10 µl (100 µg/ml) of each samples on a 12% SDS-PAGE. Expected size is between 18-20 kDa. The 250 ml culture was harvested by centrifugation at 5000 rpm for 15 minutes in a swing-bucket centrifuge and stored at -20°C until required for further processing.

### *2.23 Endotoxin removal from rfSP-A*

The removal of endotoxins from recombinant proteins is important to prevent interference in further studies caused by LPS such as in cell culture. Lipopolysaccharides (LPS) are found on the outer cell membrane of gram-negative bacteria. Thermo Scientific Pierce High capacity endotoxin removal resin in spin columns were used for the removal of LPS. The resins contain porous cellulose beads with covalently attached ε-poly-L-lysine which has a high affinity for endotoxins. The binding capacity is 2 million endotoxin units (EU) per ml, reducing endotoxin levels by 99% and should typically result with a final endotoxin concentration below 5 EU/ml which is of an acceptable range.

The use of the columns was followed as instructed by manufacturers' guidance. Briefly, the column was regenerated incubating the spin column in 5-bed volumes (5ml) 0.2N NaOH in 95% ethanol for 1-2 hours at room temperature. The column was then washed with 5 resin-bed volumes of 2M NaCl followed by a 5 resin-bed volumes of endotoxin free, ultrapure water. The column was equilibrated with 5 resin-bed volumes of sodium phosphate buffer ($Na_2HPO_4$ – 1.42 g in 10ml LPS free water and autoclave, $NaH_2PO_4$ – 1.2 g in 10 ml LPS free water and autoclave → for pH7.4 add 7.74 ml $Na_2HPO_4$ with 2.26 ml $NaH_2PO_4$) at room temperature. The sample was added to the column and incubated overnight at room temperature and eluted the next day at a flow rate of 10-15 ml/h (250 µl/min). The flow-through was collected and the proteins were eluted in 1-2 resin-

bed volume fraction with endotoxin free equilibration buffer in endotoxin free tubes. The flow-through and the fraction were run on a 12% SDS-PAGE. The endotoxin levels were determined using the Limulus Amebocyte Lysate (LAL) assay. The endotoxin column was cleaned by regenerating the column in 5-bed volumes (5ml) 0.2N NaOH in 95% ethanol for 1-2 hours at room temperature followed by 5 resin-bed volumes of 2M NaCl then 5 resin-bed volumes of endotoxin free, ultrapure water. Finally, the column was stored in 20% ethanol at 4°C.

### *2.24 Limulus Amebocyte Lysate (LAL) assay (Lonza, Slough, UK)*

The chromogenic Limulus Amebocyte Lysate (LAL test is a quantitative test for gram-negative bacterial endotoxin. The addition of the LAL reagent causes the protein sample to change colour to yellow. The absorbance is determined spectrophotometrically at 405-410 nm. Since the absorbance is in direct proportion to the endotoxin levels, the concentration can be calculated from a standard curve.

The LAL reagent contains lyophilised lysate from Limulus polyphemus. Endotoxin from the protein sample catalyses a pro-enzyme in the Limulus Amebocyte Lysate to an active enzyme. The activated enzyme then catalyses to split pNA. The release of this substrate is measured spectrophotometrically against a linear gradient of known endotoxin standards.

The standards contain approximate 15-40 EU lyophilised endotoxin. One vial is reconstituted with 1 ml of LAL reagent water at room temperature (to yield a concentration stated on the Certificate of Analysis e.g. 24 EU/ml) and vortexed for 15 minutes to detach the endotoxin from the glass. This can be stored at 4°C for up to 4 weeks. The substrate used is approximated 7 mg of lyophilised substrate which is reconstituted in 6.5 ml of LAL reagent water to yield a concentration of ~2 mM. this reconstituted substrate solution is stable at 4°C for up to 4 weeks if not contaminated with microorganisms. To prepare a set of standards, the reconstituted endotoxin vial was vortexted for 1 minute then was diluted to 1 EU/ml. 0.5ml of this was transferred to another glass vial to and diluted with 0.5 ml LAL reagent water to halve the concentration of EU/ml. 5-10 standards were prepared and vortexted for 1 minute before use. The samples were pipetted from tube to tube at the same rate to allow the same time for reaction. 50 µl of the standard was mixed with 50 µl of LAL reagent,

mixed and incubated for 10 minutes at 37°C. Next, 100 µl of the substrate solution was added and mixed vigorously before incubating at 37°C for 6 minutes. Finally, 100 µl of the Stop reagent was added and mixed immediately before taking a reading at 405 nm. The blank sample contains LAL reagent water instead of the sample and the rest of the steps were identical. After the standard graph was recorded, protein endotoxin levels were determined using the test tube method.

Firstly, 50 µl of the sample was dispensed in an endotoxin free glass reaction tube in a 37°C water bath and at T = 0 minutes, 50 µl of LAL reagent was added and mixed thoroughly without vortex. At T = 10 minutes, 50 µl LAL substrate solution (pre-warmed at 37°C) pipetting same number of times as before when preparing standards. At T = 16 minutes, 100 µl of Stop reagent was added and mixed the same number of times as before. The absorbance of each sample were taken at 405 nm using distilled water to adjust the photometer to zero absorbance.

**Native and reduced SDS-PAGE**

### *2.25 Reduced Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE is a technique widely used in biochemistry to separate protein samples according to their molecular weight. The size of the protein can be estimated using a molecular weight marker. The gel comprises of two parts, a stacking and a resolving gel. The stacking gel is standard regardless of the percentage of the gel. This is polymerized with a separating comb for samples to be loaded into these wells. The resolving gel is made of the same reagents as stacking gel including agarose-bisacrylamide which forms a matrix for protein diffusion separated according to size. Smaller fragments of protein travel further according to their negative charges towards the positive anode. The smaller the protein desired to visualize, the higher the percentage gel is required which forms a network of pores small enough to separate a range of low molecular weight proteins. After the gel has polymerized it is placed in a tank and 1 X running buffer is poured into the tank over the gel and the separating comb is removed. The protein samples are mixed with an equal volume of bromophenol blue loading dye and denatured by heating at 95°C for 6-8 minutes before being briefly centrifuged prior to

loading in the wells. A molecular weight protein marker is also loaded at approximately 3 µl and the gel is run at 120V for 1.5 hours until the bromophenol blue dye front reached near the end of the gel. The negatively charged proteins migrate away from the cathode towards the positively charged anode. The size of the protein will determine the length it travels through the gel matrix. The gel was then stained with staining solution (50% Methanol, 40% acetic acid with 0.1% Coomassie blue). This was left on a rotating rocker for 4hours for proteins to incorporate the dye before the de-staining process. After destaining the gel, only the proteins stained with Coomassie blue were visible as well as the protein marker to estimate the size of the protein. The gel images were taken using the Molecular Imager Gel Doc (Peqlab, Sarisbury Green, UK).

A 12% SDS-PAGE was prepared by making a 12% resolving gel followed by a stacking gel. The resolving gel was made as mentioned in table 2.4:

***Table 2.4 – Composition for preparing 12% resolving SDS-gel***

| **Component (12% Resolving Gel)** | **Volume (µl)** |
|---|---|
| **De-ionised Water (D.W.)** | 330 |
| **30% Acrylamide Mix** | 4000 |
| **1.5M Tris-HCL, pH8.8** | 2500 |
| **10% sodium dodecyl sulfate (SDS)** | 100 |
| **freshly made Ammonium persulfate (APS)** | 100 |
| **TEMED** | 15 |

The resolving gel was left to polymerise at room temperature for 20 minutes. The stacking gel was then prepared and loaded on top with a separating comb fixed in place.

The stacking gel for all SDS-PAGE is made as mentioned in table 2.5:

***Table 2.5 – Composition for preparing 12% resolving SDS-gel***

| Component (Stacking Gel) | Volume (µl) |
|---|---|
| **De-ionised Water (D.W.)** | 3400 |
| **30% Acrylamide Mix** | 830 |
| **1.5M Tris-HCL, pH8.8** | 630 |
| **10% sodium dodecyl sulfate (SDS)** | 50 |
| **freshly made Ammonium persulfate (APS)** | 50 |
| **TEMED** | 15 |

This was left at room temperature to polymerise for 20 minutes before placing it in the chamber and pouring 1 X running buffer and loading wells with denatured protein samples. The gels were all set at 120V for 90 mins.

### *2.26 Native Gels*

There are some exceptions for when preparing and running a native gel. Firstly, no SDS is used in the running buffer and gel preparation. Secondly, no 2-merceptoethanol and SDS is added to the 2 X treatment buffer. Finally proteins are not denatured on a heating block but added directly to the gel.

### *2.27 Western blot*

For western blot preparation a 12%SDS-PAGE was first prepared as described earlier. Either, semi-wet transfer of proteins were carried out or wet-transfer of proteins to membrane. These are described below.

### *2.28 Semi-wet transfer of proteins to membrane*

After the proteins had passed through the gel for separation they were ready to be transferred to PVDF membrane. Initially, the gel, membrane and 4 stacked pieces of 2.5 mm thick Blotting Filter paper were equilibrated in 1 X transfer buffer (80% methanol, 5.8 g Tris-Base, 2.9 g Glycine powder and 0.37 g SDS) for 5 minutes. The membrane is then sandwiched with the gel by forming layers of 2 stacked pieces of 2.5 mm thick blotting filter paper, followed by placing the PVDF membrane on top, then layering the

gel followed by the last 2 stacked pieces of 2.5 mm thick blotting filter papers. This is placed in the semi-dry blotter on the anode plate. Note that between each layer of sandwich the air bubbles must be removed using a blotting roller to ensure efficient transfer of proteins. 100 ml of transfer buffer was poured over the membrane in the semi-dry holder and was covered with the cathode lid. The transfer took place at 14V for 1 hour.

### *2.29 Western blot detection using Chemi-Immunofluorescence (CIF)*

After transfer was complete, the membrane was blocked with PVDF Westernbreeze blocking solution (Invitrogen, Paisley, UK) for 1 hour at room temperature on a rocker. A 1:1000 dilution of primary antibody (e.g. anti-human SP-A, anti SP-D, anti CRT and anti COX-1) was prepared in 0.2% BSA, incubated at room temperature and left on the rocker for 2.5 hrs. The membrane with unbound proteins were washed using Tween 20 detergent (PBS/Tween 20 0.05%) for 5 mins five times. The Luminata Western HRP substrate solution poured over the membrane is a premised solution (GE healthcare Life sciences, Buckinghamshire, UK). The membrane is soaked in the substrate solution for 20 mins. In a dark room the membrane is taken from the substrate solution and blotted dry before placing it on top of the film in a dark cassette holder. After 1 minute of developing, the film is processed in the processor to visualize results.

### *2.30 Wet transfer of proteins*

The gel and western blot material [4 x whatmann paper, 2 x fibropads, nitrocellulose membrane] were equilibrated in transfer buffer for 15 mins. The apparatus was set up in the following order: black side- fibropad- 3 mm whatmann paper-gel-nitrocellulose membrane-3 mm whatmann paper-fibropad-white side in the transfer buffer, removing all air bubbles as this would affect the transfer of proteins. Transfer took place at 320A, for 1.5 hrs whilst stirring and an ice pack was used to ensure even temperature throughout the process.

### *2.31 Western blot detection with 3,3′-Diaminobenzidine (DAB)*

The blot was transferred into blocking solution in 5% milk powder-PBS overnight at 4ºC and the gel was stained as a control to ensure that all proteins had been transferred. The next day, the membrane was incubated in primary antibody (rabbit anti human SP-A & SP-D - (donated by group in Oxford University)) prepared in a 1:1000 dilution in

PBS at room temperature for 1hr. The unbound primary antibody was washed with stringent - PBS Tween-20, 0.05% thrice for 5mins each. The membrane was then transferred in secondary protein A (PA) HRP at 1:1000 dilution in PBS and incubated for 1hr at room temperature (RT). The secondary antibody was discarded and membrane was washed with PBS Tween 20, 0.05% thrice for 5mins each. DAB tablets were dissolved in 15ml $dH_2O$ and the membranes was incubated for 10mins to visualise the bands.

### *2.32 ELISA*

A 96-well microtitre plate was coated with 5µl of cell lysate protein in carbonate buffer and double diluted across the wells. This was carried out in duplicates for each of the protein. The plate was incubated overnight at 4°C for proteins to bind to the microtitre plate. The next day, the buffer and unbound proteins were discarded and unbound sites of wells were blocked with 100 µl 2% BSA blocking buffer. The plate was further incubated at 37°C for 2 hour before washing 3 times with PBS/Tween 0.05%. Primary antibody was diluted by mixing 1 µl rabbit Anti-human SP-A in 5 ml of PBS and 1 µl rabbit anti-human SP-D (donated by group in Oxford University) in 5 ml of PBS (1:5000) in separate tubes. 100 µl of diluted primary antibody was added to the wells. The plate was incubated at room temperature 37°C for 2 hours. Again the wells were washed with PBS/Tween 0.05% thrice before adding secondary antibody. Protein A-HRP was diluted in 1:500 dilution. Again this was mixed in a step-wise manner as described above. 100 µl of dilute secondary antibody was added to all the wells. The plate was incubated for 1hour at 37°C to allow Protein A-HRP to bind to primary antibody. Post incubation, the content was discarded and wells were washed with PBS/Tween 0.05% thrice before developing with OPD. To develop the reaction, OPD substrate was added to 100µl per well and reacted with the HRP conjugated to Protein-A and optical density was determined under a plate reader at 450 nm.

### *2.33 Immunohistochemistry (IHC) using ABC system*

Immunohistochemistry is a method used which applies the detection of antigens from tissue sections. Firstly, the ACD tissue sections were dissected from placenta and embedded in formaline fixed paraffin wax to prevent tissue from decaying or degenerating. The tissue was cut into fine sections of 3-5 microns thick using a

microtome and placed on microscope super frost-free slides. The process of Immunohistochemistry involves heat-mediated antigen retrieval for immune-detection.

The tissue sections were dewaxed using dewax solutions by successive immersions in: histoclear x 2, 100% ethanol, 90% ethanol, 70% ethanol, water for 5mins each. The endogenous peroxidase activity was blocked by immersion in 1% $H_2O_2$ for 15 mins. For antigen retrieval, the slides were placed in a dish filled with heated 0.01M Sodium citrate buffer pH6.0) 0.05% Tween 20 allowing to stand for 5mins thrice. After washing in PBS the buffer was blotted dry and placed at room temperature in a humidifying box to carry out the immunodetection process. 200 µl of 2% normal horse serum in PBS were added to sections directly over the cells and incubated for 20mins before washing in PBS. A 1:1000 dilution of primary antibody (rabbit anti-human SP-A, SP-D and CRT) in 2% BSA/PBS was prepared and 100 µl was added and incubated at room temperature for 20 mins then left in the humidifying chamber for 1 hour followed by an overnight incubation at 4°C. After 24 hour incubation, primary antibody was washed and 1:1000 biotinylated secondary antibody (Horse-anti Rabbit) in PBS was added in 100 µl per slide, followed by further incubation at room temperature for 1 hour. Following the incubation, three successive washes were carried out prior to adding 150 µl of ABC reagent (Vector Lab) for 20 minute incubation at room temperature. After successive washing, the slides were incubated with 200µl of DAB (3,3'-Diaminobenzidine) for 2-10 minutes.

After immunodetection of SP-A, SP-D and CRT, the slides were ready for counterstaining with haematoxylin. The slides were stained in haematoxylin for up to 1 minute and then washed before immersing in 1% sodium carbonate followed by 1% HCl in 70% ETOH (acid/alcohol) for 5 mins each. The tissue sections were destained with acid/alcohol for before running under the tap until slides turn slightly blue. The tissue slides were dehydrated in successive washes of – 70%, 90%, 100% alcohol followed by clearing in histoclear I and II for 5 mins each.

For examining the slides, they were blotted dry before adding adhesive and a coverslip over the tissue. Images were taken using an Axioskop 2 microscope.

### *2.34 Amnion, Chorion and Decidua (ACD) explants culture and stimulation*

All women gave consent for the collection of tissue according to the requirements of Brunel University, School of Health Sciences and Social Care, Research Ethics Committee. Each placenta was obtained from not in labour via caesarean section at 39 weeks. The amnion, chorion and decidua tissues were identified, and a total of 200 mg of tissue was added to 8 x 12-well tissue culturing plates. ACD explants were cultured in Dulbecco's modified eagles medium (DMEM) with 1x Penicillin and streptomycin (Pen/strep.) (i.e 5 ml in 500 ml DMEM) and 10% foetal calf serum (FCS). The cultures were incubated overnight at 37°C for 24 hours in 95% air and 5% $CO_2$ atmosphere. After 24 hours, the medium was changed to DMEM + 0.2% lactoalbumin hydrolysate. Each well contained 3.3 µg/ml of native SP-A, SP-D, rfSP-D Gly-Xaa-Yaa & rfSP-A, with and without IL-1β (200 pMol) in 3ml of media, to examine the direct and indirect effect of these proteins on the hormonal and immunological pathway of pregnancy and labour. The plates were incubated further, for 12 hours at 37°C in 95% air and 5% v/v $CO_2$ atmosphere. 0.1 g of the tissue was subjected to RNA extraction and the rest, 0.1 g of tissue per well was subjected to cell lysate extraction.

### *2.35 Protein extraction and purification from ACD explants*

Protein extraction is commonly carried out to be used in applications such as western blots and Enzyme linked immunoassay (ELISA). RIPA buffer is the main solution used for the extraction which extracts cytoplasmic and membrane proteins. Inhibitors such as protease and phosphatase inhibitors can be added to prevent proteolysis and to prevent phosphorylation of proteins.

Fresh cultured membranes from the wells were taken and placed in bijou tubes adding 0.5ml RIPA lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 0.5% $NP_40$, 0.25% Triton X-100), with an appropriate inhibitor cocktail added immediately prior to use. Samples were kept on ice before and after homogenization to prevent protein degradation and enzyme cleavage. The tissues from each well were individually homogenized with a mechanical rotor-stator homogenizer for 20-25 secs. The lysate was transferred to 1.5 ml microcentrifuge tubes and centrifuge at 10,000 x g for 25 mins at $4^oC$. The supernatant was transferred to clean tubes. The protein extracted can either be stored at $-20^oC$ or the protein content can be measured using the BCA assay which is read on a spectrophometer at 560 nm using PBS/elution buffer as a control. It is

advisable not to keep freeze thawing the protein samples as this will result in low quality protein and can denature the protein.

### *2.36 RNA extraction and purification*

RNA extraction and purification was carried out using the TRIzol method. TRIzol (Invitrogen, Paisley, UK) is a monophasic solution of phenol and guanidine used for isolation of high quality yield of RNA during the homogenization of sample. The reagent is able to denature proteins and remove RNases which can potentially affect the quality of RNA. Hence, the integrity of RNA remains undisrupted whilst the cellular components are disrupted and dissolved. The addition of chloroform results in a biphasic mixture. Total RNA was recovered by adding 1 ml of TRIzol reagent to each sample of treated membranes and mechanically homogenized for 20-25 seconds. The homogenate was transferred to RNase free 1.5 ml tubes and centrifuged at 12000 rpm for 10 mins at 4°C. The supernatant of each sample were transferred to sterile RNase free tubes and incubated at room temperature for 5 minutes. 200 µl of molecular biology grade chloroform (Sigma, Dorset, UK) was added to each sample and vortexed vigorously for 15 seconds and left to incubate at room temperature for 4 minutes. The chloroform causes TRIzol to separate into a colourless aqueous phase and an organic lower phase containing phenol and chloroform. The tubes were then centrifuged at 12000 rpm for 15 mins at 4°C to separate the upper aqueous phase containing nucleic acids from the phenol, without disrupting the interphase or lower organic phase containing proteins and lipids dissolved by the phenol/chloroform. The aqueous phase of each sample was transferred to sterile 1.5 ml tubes and 3/5V isopropanol (Fisher) was added to each sample and inverted several times. The tubes were then incubated at room temperature for 5minutes, followed by centrifugation at 12000 rpm for 10 minutes at 4°C. The supernatant was transferred to fresh tubes and 440 µl of isopropanol was added inverting several times to precipitate the RNA and incubated at room temperature for 7 minutes. The samples were then centrifuged to form an RNA pellet at 12000 rpm for 10 miutes at 4°C. The supernatant was dispensed without losing RNA pellet and 1 ml ice-cold 75% ethanol was added to precipitate RNA due to its high salt content. The samples were vortexed briefly and centrifuged at 12000 xg for 5 minutes at 4°C. The ethanol was fully dispensed and left to air dry before re-dissolving RNA. RNA was dissolved in 40-50 µl of nuclease-free water and retropipetted several times to fully

dissolve the RNA. Partial dissolving of RNA gives $A_{260/280\ nm}$ ratio of <1.6. The RNA samples were left on ice for 1hour before being stored at -80°C.

### *2.37 Quantifying RNA via NanoDrop*

The absorbance of RNA to determine the quantification of total isolation is read using a spectrophotometer (NanoDrop ND-1000). The $A_{260/280}$ ratio determines the nucleic acid purity. A ratio of ~2.0 is generally accepted as "pure" for RNA samples. A value appreciably lower than 2.0, may indicate the presence of organic contaminants such as phenol. The $A_{260/230}$ ratio also determines the nucleic acid purity, a value lower than 1.8 is usually unacceptable, as this indicates the presence of organic contaminants. This may interfere with gene expression. This lowers the efficiency for applications such as RT-PCR. The absorbance reader was blanked with 1µl of nuclease free water and 1µl of each RNA sample was added singly to measure the $A_{260/280}$ and $A_{260/230}$ ratio.

The RNA after DNase I treatment was quantified using spectrophometer NanoDrop. The machine was blanked with 1µl nuclease-free water and each sample was measured by adding 1 µl of RNA to the NanoDrop. This measures the $A_{260/280}$ and $A_{260/230}$ ratio to estimate RNA quality and presence of any contaminants. The six samples of RNA treated with DNase I were converted to cDNA using High capacity RNA-to-cDNA kit (Applied Biosystems, Paisley, UK) in a 20 µl reaction 2 µg of RNA is converted to single stranded complementary DNA (cDNA).

The samples were centrifuged briefly to bring components together. The tubes placed in a thermo cycler configured to the same settings as described earlier – warmed at 37°C for 60 mins and then heated at 95°C for 5 minutes.

### *2.38 DNase treatment for DNA elimination from RNA samples*

Deoxyribonuclease I (DNase I) is an endonuclease which digests DNA and eliminated RNase activity to prepare RNA for sensitive applications such as RT-PCR. DNase I (Sigma, Dorset, UK). The entire RNA sample (~50 µl) is treated with 5 µl of the 10 reaction buffer and 5 µl of Amplification grade DNase I (1 U/µl) and incubated at room temperature for 15 minutes. The reaction buffer and Amplification Grade DNase I combined, removes contaminating DNA by nucleic digestion at room temperature. The DNase is then inactivated by adding 5µl of the stop solution and heating the sample to

70°C for 10 minutes. The heating process also denatures secondary RNA structures so RNA can be used for reverse transcription. The RNA sample not used for agarose gels or cDNA synthesis can be stored at -80°C for future use.

### *2.39 Agarose- Ethidium Bromide (EtBr) gel electrophoresis to separate and analyze RNA with and without DNase treatment*

The overall RNA quality can be assessed by electrophoresis and also gives a semi-quantitative indication on RNA yield. Seven samples of RNA, five DNase-treated and two not treated have been separated on a 1% agarose-EtBr gel against a DNA sizer III (Peqlab, Sarisbury Green, UK). RNA sample was diluted in 6 X Loading dye (Peqlab, Sarisbury Green, UK) and the DNA sizer III was also diluted down 6 times in $dH_2O$ plus the 6 X loading dye. The voltage was set at 70 V until the fast migrating dye (bromophenol blue) reached 2/3 the length of the gel. The results should indicate 2 distinct bands, 28S and 18S rRNA with a 2:1 ratio of intensity, respectively. Bands of a very low molecular weight indicate complete degradation of RNA. A smeared appearance of rRNA indicates partial degradation, contamination or poly(A) tail near the 0.5 kb to 6 kb. The supercoiled RNA remains higher than the nicked RNA. The native agarose gel separates nucleic acids according to size and separates through the mesh network which decreases in pore size to distinguish large and small sizes of nucleic acids. The larger molecules remain higher up the gel whilst the smaller molecules are able to migrate lower down the gel. The results were visualized under a UV transilluminator and processed under the Alpha Imager.

### *2.40 Purification of RNA with QIAGEN RNeasy Plus mini kit*

In attempt to improve the RNA quality by increasing the $A_{260/230}$ ratio, RNeasy Plus mini kit (Qiagen, Manchester, UK) was used. 50 µl samples of RNA with an equal volume of 70% ethanol was added to 2 V RLT buffer Plusin an RNeasy spin column in a 2 ml collection tube. The samples were centrifuged at 8000xg for 15 seconds. The collection sample was discarded and 700 µl of Buffer RW1 was added to the RNease spin column and centrifuged again at 8000 x g for 15 seconds. The collection tube was fully emptied without allowing the column to contact the flow-through. 500 µl of Buffer RPE containing ethanol was added to the RNease spin column and centrifuged at 8000 x g for 15 seconds. This step was repeated again but centrifuged for a further 2 mins at 8000 x g. This ensures all ethanol was removed and the spin columns were dry

containing the RNA. 30 µl of nuclease free water was added directly over the membrane holding the RNA and was centrifuged at 8000 x g for 1 minute to elute the RNA. RNA samples were quantified and quality was checked using NanoDrop spectrophometer. The spectrophometer was blanked with 1 µl nuclease-free water and an equal volume of sample was added to measure as described in 2.47.

### *2.41 cDNA synthesis*

cDNA synthesis is a process of converting single stranded RNA to complementary DNA using an enzyme, reverse transcriptase. The High capacity RNA-to-cDNA kit (Applies Biosystems) contains a 2 X RT buffer and a 20 X Enzyme mix with RNase inhibitor giving maximum yield to high quality DNA for real-time PCR.

Initially the RNA is transcribed to cDNA which is more stable by an enzyme reverse transcriptase. This contains RNase inhibitors so prevents RNA degradation in the process. In a 20 µl reaction 2 µg of RNA is converted to single stranded complementary DNA (cDNA). Using the values from the NanoDrop analyses 2 µg of RNA was used in a total 20 µl sample to convert to cDNA. All kit components were thawed on ice including RNA samples from -80°C. Table 2.6 indicates the volume of each component used for each RNA sample to transcribe to cDNA:

***Table 2.6 - Samples preparation for cDNA synthesis for 5 samples***

| **Component** | **Component volume/reaction (µl)** | | | | |
|---|---|---|---|---|---|
| | **IL-1β** | **rfSP-A** | **rfSP-D** | **rfSP-A + IL-1β** | **rfSP-D + IL-1β** |
| **2 X RT Buffer** | 10 | 10 | 10 | 10 | 10 |
| **20 X Enzyme Mix** | 1 | 1 | 1 | 1 | 1 |
| **RNA sample** | 1.68 | 2.77 | 1.79 | 3.73 | 1.02 |
| **Nuclease free $H_2O$** | 7.32 | 6.23 | 7.21 | 5.27 | 7.98 |
| **Total per reaction** | **20** | **20** | **20** | **20** | **20** |

The samples were centrifuged briefly to collect components together. The tubes were placed in a thermo cycler. Samples were warmed to 37°C for 60 minutes, then heated to 95°C for 5 mins before cooling down to 4°C.

### *2.40 Real-time PCR*

Real time PCR also known as Q-PCR, amplifies specific gene amplicons over 40 cycles. The specificity of product depends on the primers used. The process requires primers complementary to the forward (sense -5'-3') and reverse (antisense- 3'-5') strands for gene specific amplification. The primers anneal to complementary DNA on the sense and antisense strand and are extended by DNA polymerase. This makes the reaction DNA-specific. The primers are short oligonucleotide sequences and their Tm value indicates the optimum temperature of primer annealing. This is usually 5-6degrees below the Tm of the primers. The result of different temperatures is due to A-T and C-G bonds which have 2 or 3 hydrogen bonds between their complementary bases, respectively. The melting temperature is the temperature required to break the hydrogen bonds between the bases. As primers differ in length and composition, they may have different Tm values.

During each cycle the number of strands double leading to an exponential amplification. This application requires Power SYBR green PCR master mix (A. Biosystems) which is a master mix containing AmpliTaq fast DNA polymerase, SYBR green which incorporates into the minor groves of DNA, dNTP, uracil-DNA glycosylase (UDG) and an internal reference dye. SYBR green is able to amplify specific regions efficiently without formation of primer-dimers and non-specific amplification. The amplicons amplified have SYBR green incorporated into their DNA. The more the amplicon are amplified this results in higher emission of fluorescence. During the dissociation stage the double stranded DNA are hydrolysed releasing the reporter molecule which is converted to a quantitative value (Ct) which can be used to calculate gene expression from the treated tissue. The results are analyzed using the SDS 2.4 (A. Biosystems) and the dissociation curve determined using RQ Manager1.2.1 (A. Biosystems) compatible with the 7500HT fast system (A. Biosystems).

Four master mixes were prepared containing 500 nM primer, SYBR green, and nuclease free water to a total of 10 µl (table 2.7). The 5 cDNA samples used were; IL-1β, rfSP-A,

rfSP-D, rfSP-A + IL-1β & rfSP-D + IL-1β of 100 ng per well. The samples were briefly centrifuged prior to adding 9 µl of master mix. The master mix was added to the allocated wells of a Fast 96-well plate (A.Biosystems) and 1 µl of cDNA were added to the wells directly for a total 10 µl reaction. A negative control was carried out for each target.

***Table 2.7 –Master mix using SYBR green for real-time PCR***

| | **POLR2A** | **PTGS2** | **PTGFRN** | **IL-8** |
|---|---|---|---|---|
| **Component** | **Volume (µl)** | | | |
| **Power SYBR green master mix** | 100 | 100 | 100 | 100 |
| **Forward primer** | 3 | 3 | 3 | 3 |
| **Reverse primer** | 3 | 3 | 3 | 3 |
| **cDNA template** | 0 | 0 | 0 | 0 |
| **Water** | 74 | 74 | 74 | 74 |
| **Total** | **180** | **180** | **180** | **180** |

5µl of sample from the Q-PCR products was mixed with 6 X loading dye and loaded into the wells of a 2% agarose-gel. DNA sizer III (Peqlab, Sarisbury Green, UK) was used as a molecular weight marker to size the PCR product. The gel was run at 80 V until the bromophenol blue line had reached three quaters the length of the gel. The results were visualized using the Alpha Imager under UV light. The products should be size of the amplicon it amplifies in the PCR reaction.

### *2.43 Reverse transcription polymerase chain reaction (RT-PCR) using KAPA-HiFi DNA polymerase*

Reverse transcriptase is a polymerase enzyme and in each chain reaction the product doubles. Initially the RNA was transcribed to cDNA which is more stable. The double stranded molecule is heated to separate the hydrogen bonds and then cooled down allowing the enzyme and oligonucleotides to anneal complementary to the bases of the template. Finally, the temperature is raised to 72°C for the strands to be extended. This cycle is repeated to approximately 40 cycles resulting in $2^{40}(2^{n})$ strands of DNA
(i. e. $1.1 \times 10^{-12}$).

To optimize cDNA concentration for primer specificity to obtain interpretable results, 50ng and 100ng of cDNA were used for each primer using KAPA HiFi DNA polymerase

kit (KAPA HiFi) (table 2.8). A negative control was also carried out to ensure product specificity and a positive control using 18S rRNA forward and reverse primers.

***Table 2.8 – Master mix using KAPA HiFi DNA Polymerase and 500 nM primer concentration***

| Component | End concentration |
|---|---|
| **5 X DNA fidelity buffer** | 1 X |
| **dNTP – 0.3mM** | 0.3mM |
| **Forward primer** | 500 nM |
| **Reverse primer** | 500 nM |
| **KAPA HiFi DNA polymerase** | (1 U/µl) |
| **Water** | quantity sufficient to 25 µl end volume |

The samples were centrifuged briefly to bring the components together. The tubes were placed in a thermo cycler undergoing an initial denaturation followed by 40 cycles of denaturation, annealing of primers and extension to replicate amplicon number of target gene. Initial denaturation was at 95°C for 45 seconds, followed by denaturation at 95°C for 20 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 30 seconds.

Products from RT-PCR to optimize cDNA concentration were visualized on a 1% Agarose-EtBr gel. 10 µl of each sample was mixed with 6 X loading dye and DNA sizer III (Peqlab, Sarisbury Green, UK) was used as a molecular weight marker. The gel ran at 80 V until the bromophenol blue line reached three quarters the length of the gel. For each target, 50 ng cDNA product, 100 ng cDNA product and a negative control were loaded next to each other.

### *2.44 RT-PCR using KAPA-HiFi for primer PTGFRN and cDNA optimization*

PTGFRN required optimization. As 75 nM primer was insufficient for RT-PCR, 250 nM and 500 nM primer concentrations were tested using 3 samples of cDNA from preterm placenta: rfSP-D, rfSP-A and rfSP-A + IL-1β at 100 ng and 200 ng. RT-PCR was carried out using KAPA HiFI DNA polymerase kit (KAPA HiFi). Samples were made up to 25 µl total volume. For each cDNA the tubes contained the following:

*Table 2.9 – Preparation of master mix using KAPA HiFi for primer optimisation*

| **PTGFRN** | Volume (µl) | | | |
|---|---|---|---|---|
| | **250 nM primer + 100 ng cDNA** | **250 nM primer + 200 ng cDNA** | **500 nM primer + 100 ng cDNA** | **500 nM primer + 200 ng cDNA** |
| **5x DNA fidelity buffer** | 5.00 | 5.00 | 5.00 | 5.00 |
| **Water** | 17.00 | 18.00 | 17.00 | 18.00 |
| **dNTP** | 0.75 | 0.75 | 0.75 | 0.75 |
| **Forwards primer** | 0.38 | 0.38 | 0.75 | 0.75 |
| **Reverse primer** | 0.38 | 0.38 | 0.75 | 0.75 |
| **DNA template/ (water for -ve control)** | 1.00 | 2.00 | 1.00 | 2.00 |
| | 0.50 | 0.50 | 0.50 | 0.50 |

The samples were centrifuged briefly to bring components together. The tubes were placed in a thermo cycler and set up the same way as described previously (See 1.2.17). The RT-PCR products were separated on a 1% Agarose-EtBr gel. The results were visualized using the Alphaimager under UV light.

### *2.45 Q-PCR using SYBR green for primer and cDNA optimization*

A Q-PCR was carried out using the optimised conditions of the 12 targets: PTGFRN, PTGS2, IL-8, IL-10, TNF-α, TGF-β, TLR2, TLR4, SP-A, SP-D, C1q and factor H with 18S rRNA as our endogenous control. Each primer was used at a concentration of 500 µM and 100 ng cDNA. The cDNA samples used were from term placenta; rfSP-A, rfSP-D and rfSP-A + IL-1β, rfSP-D + IL-1β, IL-1β and RNA from non-treated cells. Master Mix for each target containing Power SYBR green (Applied Biosystems, Paisley, UK) was added to the fast 96-well plate then cDNA on top.

cDNA samples rfSP-A + IL-1β and primers IL-8 and 18s rRNA were used to optimize cDNA and primer concentrations. The three different cDNA amounts used were: 200 ng, 100ng and 50 ng cDNA and 500 nM primer concentration (table 2.9). The three primer concentrations optimized were 75 nM, 250 nM and 500 nM each with 100 ng cDNA. In

total, for the three primers and 3 different cDNA concentrations, 9 master mixes were made for cDNA optimization.

Each well of fast 96-well plate for cDNA optimization contained the following components:

***Table 2.10 – Components using SYBR green for cDNA optimisation***

| Component | End concentration |
|---|---|
| **Power SYBR green master mix** | 5 µl |
| **Forward primer** | 190 nM |
| **Reverse primer** | 190 nM |
| **cDNA template** | 200 / 100 / 50 ng |
| **water** | quantity sufficient for total 10 µl reaction |

Each well of fast 96-well plate for primer optimization contained the components described in table 2.10:

***Table 2.11 – Components using SYBR green for primer optimisation***

| Component | End concentration |
|---|---|
| **Power SYBR green master mix** | 5 µl |
| **Forward primer** | 75 / 250 / 500 nM |
| **Reverse primer** | 75 / 250 / 500 nM |
| **cDNA template** | 100ng |
| **Water** | quantity sufficient for total 10 µl reaction |

Water was added to the negative control wells as a substitute to cDNA for every target. The plate was briefly centrifuged and the conditions of the template were input into the 7500HT real-time PCR system (table 2.11). The results were shown using SDS 2.4 and the dissociation curve was deduced using the RQ manager 1.2.1. (Applied Biosystems, Paisley, UK).

*Table 2.12 - Settings for thermal cycler*

| **Times and Temperatures** | | |
|---|---|---|
| **Initial Steps** | PCR (each of 40 cycles) | |
| **Power SYBR green DNA polymerase activation** | Melt | Anneal/Extend |
| **HOLD** | CYCLES | |
| **10 mins @95°C** | 15 secs @ 95°C | 1 min @ 57°C |

The final primer and cDNA concentrations used after optimisation were 500nM and 100ng respectively.

### *2.46 Temperature optimization for POLR2A - RT-PCR*

*RT-PCR* was carried out to optimize annealing temperature for primers POLR2A using PeqGOLD Taq polymerase. The gradient of temperature ranged between 49.5°C and 59.2°C using a thermal cycler. The two different primer concentrations used were – 75 nM and 500 nM (table 2.13) to optimize the annealing temperature (table 2.14). Each tube for both primer concentrations contained the following components at their final concentrations in a 10 µl end volume are described in table 2.14.

*Table 2.14 - Optimisation of primer concentration via RT-PCR*

| **Component (end conc.)** | **75 nM primer POLR2A** | **500 nM primer POLR2A** |
|---|---|---|
| **10x reaction buffer (1x)** | 1 | 1 |
| **dNTP mix (800µM)** | 0.8 | 0.8 |
| **Forward primer (75 nM/500 nM)** | 0.15 | 0.38 |
| **Reverse primer (75 nM/500nM)** | 0.15 | 0.38 |
| **Taq DNA polymerase (0.05U/µl)** | 0.1 | 0.1 |
| **cDNA template** | 1 | 1 |
| **Water** | 6.8 | 7.34 |
| **Total** | **10** | **10** |

The samples were centrifuged briefly to collect components together. The temperatures set on the thermal cycler are described in table 2.15.

***Table 2.15 – Thermocyler conditions for temperature gradient PCR***

| **Annealing temperature (°C)** | **75 nM** | **500 nM** |
|---|---|---|
| **49.5** | 1 | 13 |
| **50.1** | 2 | 14 |
| **50.7** | 3 | 15 |
| **51.8** | 4 | 16 |
| **52.9** | 5 | 17 |
| **54** | 6 | 18 |
| **55** | 7 | 19 |
| **56** | 8 | 20 |
| **57.1** | 9 | 21 |
| **58.2** | 10 | 22 |
| **58.8** | 11 | 23 |
| **59.2** | 12 | 24 |

The thermal cycler was configured to a program to denature the double strand DNA at 95°C and allow annealing of primers with a 54.5°C ± 5°C to optimise the best temperature for primer annealing followed by increasing the temperature to 72°C for extension of primers to amplify gene products. This cycle was repeated 40 times where the product doubles in every cycle.

### *2.47 RT-PCR to investigate Taq and dNTP integrity*

As RT-PCR for temperature optimization was unsuccessful, the Taq and dNTPs (Peqlab, Sarisbury Green, UK) efficiency were validated using KAPA HiFi DNA polymerase dNTPs. This RT-PCR was carried out using 18S primers. Duplicate reactions were carried out including negative control which contained water as a substitute of cDNA. 500 nM primer and 100 ng was used in each reaction tube. 10 X Reaction buffer, dNTP mix plus 0.5 U/µl of Taq DNA polymerase were added to each tube of RNA containing

primers. After separating results on gel and visualising the amplicon on gel determined Taq and dNTP integrity.

### *2.48 Statistical analysis for QPCR data*

Results were analysed using the Sequence Detections Software (SDS) V2.4 and RQ Manager. Samples were compared to 18S rRNA, the endogenous control, and calibrated against non-treated samples. The $C_t$ values were calculated at the point where the PCR curve crosses the threshold. For each sample, the geometric mean $C_t$ for each target was subtracted from the geometric mean $C_t$ of the endogenous control to give $\Delta\Delta C_t$ values. The RQ value to give the relative fold change of expression compared to non-treated sample and were calculated by 2 to the power of $-\Delta\Delta C_t$ ($2^{-\Delta\Delta Ct}$). For all subsequent statistical analysis, RQ values were plotted on a logarithmic scale ($\log_{10}$) with the standard error of mean to minimise error. All data were calculated by two-way ANOVA using GraphPad Prism software. Sequences of primers are listed in the table below:

Sequences of primers used in this study are in the table below.

***Table 2.16 – Primer sequences and gene nomenclature***

| Gene Nomenclature /Formally | Primer name | Forward primer sequence | Reverse primer sequence | Amplicon (bp) |
|---|---|---|---|---|
| **18s rRNA** | 18s rRNA | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG | 151 |
| **PTGS2/COX2** | Prostaglandin H synthase 2 | CTCAGACAGCAAAGCCTACC | ATGTGATCTGGATGTCAACAC | 372 |
| **PTGFRN** | Prostaglandin F2-Alpha Receptor Regulatory Protein | CTGTCGTTGGCTCTTTGCCG | CATCATAGTCACTGACGTTGC | 109 |
| **POLR2A** | | GCACCACGTCCAATGACATTG | GTGCGGCTGCTTCCATAAGC | 267 |
| **IL8** | Interleukin 8 | CTGTGTGAAGGTGCAGTTTTG | GTGTTGGCGCAGTGTGGTC | 137 |
| **IL-10** | Interleukin 10 | ACATCAAGGCGCATGTGAAC | TAGAGTCGCCACCCTGATGT | 248 |
| **TNF-α** | Tumour necrosis factor alpha | CACCACTTCGAAACCTGGGA | AGGAAGGCCTAAGGTCCACT | 196 |
| **TGF-β** | Transforming growth factor-beta | CCCAGCATCTGCAAAGCTC | GTCAATGTACAGCTGCCGCA | 190 |
| **TLR2** | Toll-like receptor 2 | GAGACCTATAGTGACTCCCAG | CTGCCCTTGCAGATACCATTG | 150 |
| **TLR4** | Toll-like receptor 4 | CTTCTCAACCAAGAACCTTGG | GTGGCCTTAGGCTCTGATATG | 152 |
| **SP-A** | Surfactant protein A | GTGGGGTGGGATTAGATAAATGC | TACTGAGAGATGTGTGCTTGGTGAG | 196 |
| **SP-D** | Surfactant protein D | TGGTTTCTGAGATGGAGTCGTG | TGGGGCAGTGGATGGAGTGTGC | 184 |
| **C1q** | | ATGGTGACCGAGGACTTGTG | GTCCTTGATGTTTCCTGGGC | 190 |
| **FH** | Factor H | CCCGGGGAAATACAGCCAAA | TCTGGGAGTAGGAGACCAGC | 182 |
| **SP-A** | SP-A/pSecTagC | GGGGGAATTCGAAGTGAAGGACGTTTGTGTTGGAAG | CGACTGACCATCTGTGAGTTCTGACTCGAGCCC | 686 |
| **SP-A** | SP-A/pMalC-2 | GGGGAATTCCATCTAGATGAGGAGCTCCAAGC | GGGGGATCCCTAGAACTCACAGATGGTCAGTCGG | 444 |
| **SP-A** | SP-A/pET-101 | CACCATGCATCTAGATGAGGAGCTCCAAGC | CGACTGACCATCTGTGAGTTCTGACTCGAGCCC | 444 |

# Chapter 3:

# Cloning, expression and purification of full length and truncated forms of human SP-A and SP-D

## Summary

The main objective of this thesis was to examine the effects of pulmonary surfactant proteins SP-A and SP-D on the explants of amnion, chorion and decidua in terms of their ability to modulate immune response under inflammatory conditions and modulation of the prostaglandin pathways. Having established the optimal readouts on the ACD explants, the plan was to narrow it down to decidual macrophages which are the key inflammation modulating immune cells in trimester 2 and 3. Thus, it was imperative to prepare various forms of native and recombinant SP-A and SP-D. Full length native SP-A and SP-D were purified from pooled amniotic fluid using affinity chromatography. Full length cDNA of human SP-A1 was also cloned in a mammalian vector pSecTagC for expression in human embryonic kidney cells (HEK-293T). SP-A was purified from the culture medium. In order to localise the domains of SP-A and SP-D which have immunomodulatory properties, the homotrimeric C-type lectin domains of human SP-A and SP-D were expressed in E. coli. The trimeric fragment of human SP-A designated rfSP-A was expressed as a fusion protein with maltose binding protein. It was also expressed under the T7 promoter as inclusion bodies in E. coli, which were subjected to denaturation-renaturation procedures followed by affinity purification. A similar construct and procedure were applied for generating a recombinant fragment of human SP-D, designated rfSP-D, that is comprised of homotrimeric neck and carbohydrate recognition domain. Each protein preparation were examined for purity and immunological identity followed by LPS content. Wherever possible the contaminating LPS were removed prior to use in bioassay systems.

## *3.1 Introduction*

SP-A and SP-D belong to the collectin family along with mannose-binding protein. The primary structure of SP-A and SP-D are arranged into four subunits: an amino terminal involved in the formation of disulphide bonds, collagen region containing Gly-Xaa-Yaa repeats, an α-helical neck domain to stabilise the carboxyl-terminal carbohydrate recognition domain (CRD) (Holmskov et al., 1994). Three of the polypeptide chains intercalate to form a triple helix with 3 CRD heads. These polypeptides form a cruciform structure in the case for SP-D and a bouquet-like formation for SP-A. SP-A and SP-D recognise pathogens such as house dust mite (Wang et al., 1996) and *Aspergillus fumigatus* (Madan et al., 1997) by its pathogen associated molecular patterns via its CRD regions to aid in macrophage uptake for phagocytosis and respiratory burst (van Iwaarden et al., 1990, van Iwaarden et al., 1991). Thus showing the importance of SP-A in the innate immune system of the lung. SP-A has also shown an importance in formation of tubular myelin and for spreading and adsorption of phospholipids at the air-liquid interface of alveoli making SP-A fundamental in regulating surfactant homeostasis.

### *3.1.1 Purification strategies of native SP-A and SP-D from amniotic fluid*

Amniotic fluid is a clear, yellowish fluid surrounding the baby within the amniotic sac and circulating the foetal lungs with a pH of 7.0 to 7.5. Initially, the water-like fluid enters from the maternal plasma to the amniotic cavity by osmotic and hydrostatic pressure. The amniotic fluid at first consists of electrolytes and water then at approximately 8 weeks gestation, the kidneys develop allowing the passing of foetal urine which enters the amniotic fluid. By around 12-14 weeks gestational age, the liquid builds levels of carbohydrates, proteins, lipids, and phospholipids. Analysis of amniotic fluid has also shown a large quantity of pluripotent stem cells. These amniotic stem cells could be important for therapeutic uses in humans. The volume of amniotic fluid increases positively to foetal growth then reaches a plateau of 800 ml at 28 weeks gestation then reduces to 400 ml at 42 weeks gestation perhaps reducing because of increasing size of foetus (Underwood et al., 2005).

The origin of some proteins found in the amniotic fluid cannot be determined with certainty. There is evidence from studies using electrophorectic techniques (Abbas and Tovey, 1960) and labelled proteins that they originate from the maternal circulation

which are filtered through the foetal membranes. For examples, human placental lactogen (HPL) is found in appreciable amounts in the maternal serum and amniotic fluid, however cannot be demonstrated in cord blood (Tallberg et al., 1965). This alarms the question of whether the protein originates from the mother or foetal circulation.

Amongst the proteins found in the amniotic fluid, exists SP-A and SP-D, which are synthesised in alveolar type II cells in the foetal lungs and are secreted into the surrounding amniotic fluid. Both proteins and lipids are important in the formation and function of foetal lungs for uterine and postnatal life. As these proteins are important in the defence against infection, it was investigated if there was a difference in the level of protein expression in ratio to all proteins in the amniotic fluid in cases with and without intra-amniotic infections. The results suggested that there was no change in amniotic levels of SP-A and SP-D between 18 and 34 weeks gestation in amniotic infection regardless of corticoidsteroid treatment (Chaiworapongsa et al., 2008). Although the findings suggest the proteins level do not change with and without IAI, the levels of SP-A and SP-D do change with gestational age in the third trimester (Miyamura et al., 1994). We decided to purify foetal SP-A and SP-D and investigate if they influence the immune pathway during pregnancy, closest to the native context. We show here the purification of SP-A and SP-D from amniotic fluid (Strong et al., 1998). The amniotic fluid was collected after vaginal rupture at labour and collected by midwives at Bristol University Hospital. The purification protocol involves the separation of SP-A and SP-D from one source using their affinity properties. For SP-D, $MnCl_2$ is used for eluting the protein which is later removed from the protein by dialysis against calcium buffer. SP-A is eluted with EDTA after being bound to maltose on an affinity column. In both situation, the proteins were aggregated with the addition of calcium before separating the pellet rich SP-A from the supernatant rich SP-D at 10,000 x g for 40 minutes and the proteins were recovered by solubilisation with step wise dialysis containing urea starting at 8M concentration. The proteins were analysed for functionality via binding with maltosyl-BSA and determined by ELISA. Each protein were characterised by western blot.

### *3.1.2 Cloning and expression of SP-A and SP-D with mammalian cell system*

#### *3.1.2.1 pSectagC*

In this chapter we have shown cloning of full length human SP-A1 gene into pSecTagC for expression in mammalian HEK-293T cells. pSecTagC vector is a 5.2 kb expression vector designed for stable and transient expression of genes into mammalian host cells (See appendix 8 for map). The proteins are cloned downstream to the Igκ leader sequence with the deletion of the host leader sequence to create a fusion allowing proteins to be secreted into the supernatant.

Post-cloning, the plasmid was transfected into mammalian HEK-293T cells. Transfection is the process of inserting foreign gene into host i.e. mammalian cells, for protein production. This procedure allows the post-translation modification (glycosylation) of SP-A which it cannot do in bacterial *E.coli* cells. Therefore the mammalian cell is an important vehicle for the production and folding of functionally active human proteins. The DNA is a very negatively charged molecule which requires neutralising for transfection to take place. There are many methods of transfection and after optimising with cationic polymer polyethylenimine (PEI), lipofectamine and calcium phosphate co-precipitation; we found the later to be efficient. There are two types of transfection, stable and transient. In stable cell lines, the procedure involves careful integration the foreign DNA into the host's genome to continuously reproduce the gene of interest during each passage, whereas, in transient transfection, there is no integration of foreign gene into the host and the gene is only expressed in one passage of cells. For stable cell lines, a selectable marker is used to select the integrated cells which have taken up the gene of interest and for long term production. Full length human SP-A has been cloned before into Chinese hamster ovarian (CHO) DXB11 cell line (Venkatraman Girija et al., 2010) to create a stable cell line, allowing the efficacy of SP-A production to be greater. We chose transient transfection into HEK-293T cell line using calcium-phosphate co-precipitation to produce a suitable amount of SP-A desired protein for functional and analytical studies. Human embryonic kidney cells often referred to as HEK-293T cells was chosen as they grow fast and can readily take up DNA for transfection and translation. After optimisation experiments were carried out, proteins were produced. HEK-293T cell contains an addition of the SV40 large T-antigen to allow episomal replication of transfected plasmids which contain the SV40-derived

viral sequence, origin of replication, which contributes to T-antigen recognition. The cell line was grown in high-glucose DMEM supplemented with 10% FCS, glutamine, non-essential amino acids and penicillin/streptomycin. The gene was cloned into pSecTagC within EcoRI and XhoI sites after the IgGκ-chain leader sequence. The plasmid was then transfected (50μg) into each culture dish and grown up to 120 hours replenishing the media each day. The media from each day was collected and harvested for protein purification. The protein was dialysed against calcium buffer to aggregate the protein and allow efficient binding to maltose on an affinity column. The proteins were eluted with EDTA and analysed on a denaturing SDS-PAGE and functionality was confirmed in maltosyl-BSA and antibody specific binding.

### *3.1.3 Cloning and expression of homotrimeric fragment of human SP-A and SP-D with without fusion partner*

SP-A and SP-D are important opsonins for interacting with macrophages to initiate an innate immune response. These collectins consist of 3 CRDs bound to a neck domain to create a binding site. We have constructed plasmids containing gene for neck and CRD domains for SP-A and SP-D, SP-D containing the addition of 8 Gly-Xaa,Yaa repeats from the collagen region. This will allow us to elucidate the biological function of these proteins to understand their importance in modulating an immune response in pregnancy and labour. Trimeric SP-A and SP-D were produced in *E. coli* BL21 (λDE3) and BL21 (λDE3) pLysS followed by characterisation via binding ability. Trimeric SP-D was produced as previously described (Singh et al., 2003) using the T7 promoter system and fusion system with MBP (Eda et al., 1997, Kishore et al., 1996). We have adopted both techniques to clone trimeric SP-A.

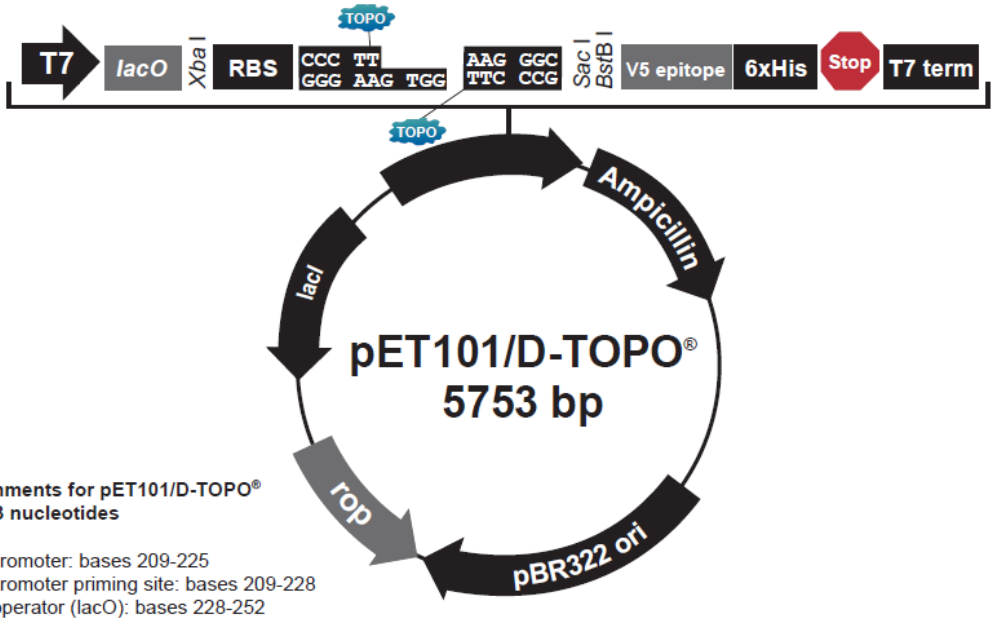
The DNA coding for the trimeric region was pulled out from the same cDNA used for cloning full length SP-A. The primers used are mentioned in chapter 2. Trimeric fragment SP-A to create a fusion protein was inserted into pMalC-2 and that cloned for expression under the T7 promoter system was cloned into BL21 (λDE3) pLysS.

#### *3.1.3.1 pET-101 system*

pET-101 (Invitrogen, Paisley, UK) is a directional cloning vector which contains a topoisomerase I which binds to double stranded DNA and cleaves the phosphodiester

backbone at 5'-CCCTT (Shuman, 1991) (figure 3.2-3.3). The reverse reaction can occur if phosphor-tyrosyl bond between DNA and enzyme can be attacked by 5'hydroxyl of the cleaved strand to release the topoisomerase (Shuman, 1994). In this system, PCR products are cloned with by adding the CACC on the forward primer to allow directional cloning which anneals to the complimentary GTGG overhang of the open vector to stabilise the PCR for annealing (figure 3.3). The Champion pET expression system contains the bacteriophage T7 promoter modified to contain a lac operator. The T7 RNA polymerase recognises this promoter in BL21 (λDE3) to transcribe the gene of interest. This strain contains λDE3 lysogen which contains the lacI gene encoding the lac repressor. T7 RNA polymerase gene is under the control of lacUV5 promoter and small portion of the lacZ gene. The lacI repressing T7 RNA polymerase is overcome with addition of isopropyl β-D-thiogalatosidase (IPTG) allowing the expression of lacUV5 promoter to express T7 RNA polymerase. The disadvantage of the T7 lac promoter system shows that there is always some basal T7 RNA polymerase expression from lacUV5 promoter in λDE3 lysogens even without IPTG (Studier and Moffatt, 1986). This is not usually a problem, but if the gene of interest is toxic, even small expression of the gene of interest may lead to plasmid instability and cell death. The gene of interest (trimeric SP-A) was cloned in to pET-101 and transformed into TOP10 competent cells (Invitrogen). TOP10 cells do not contain T7 RNA polymerase but provides a host for the propagation of recombinant plasmids. The plasmid was transformed into BL21 (λDE3) pLysS for expression but could have been transformed for expression in BL21 STAR, however this requires fusion-tags at the N-terminus and C-terminus.

During cloning the forward primer was designed to contain an initiating ATG codon following the 5'-CACC overhang.




**Comments for pET101/D-TOPO®**
**5753 nucleotides**

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
*lac* operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288, 292-296
TOPO® cloning site (directional): bases 297-310
V5 epitope: bases 333-374
Polyhistidine (6xHis) region: bases 384-401
T7 reverse priming site: bases 455-474
T7 transcription termination region: bases 416-544
*bla* promoter: bases 845-943
Ampicillin (*bla*) resistance gene (ORF): bases 944-1804
pBR322 origin: bases 1949-2622
*ROP* ORF: bases 2990-3181 (complementary strand)
*lacI* ORF: bases 4493-5584 (complementary strand)

***Figure 3.1 Map of pET-101/D-TOPO***

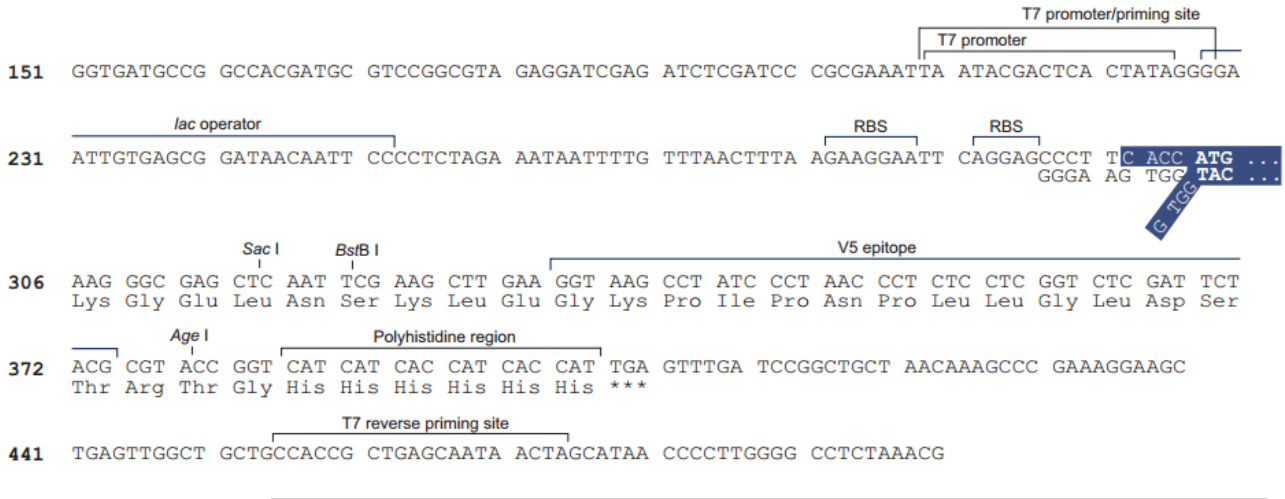



***Figure 3.2 – Sequence and cloning sites of pET101/D-TOPO***

#### *3.1.3.2 pET-3b system*

pET expression vectors derived from pBR322 plasmids which have been engineered to contain T7 bactriophage gene10 to promote high level of transcription and translation for the gene of interest. This is specific to the bacteriophage RNA polymerase so will not be recognised by host RNA polymerase making it very specific. This system is one of the most widely used for cloning and *in vivo* expression system because of its specific bacteriophage T7 RNA polymerase promoter sequence. The T7 gene10 leader sequence can significantly enhance the gene of interest expression more than 40 folds (Olins et al., 1988). The sequence of interest is cloned downstream to the T7 RNA polymerase and T7 gene10 leader sequence. The high level of T7 RNA polymerase activity out-competes transcription by the host RNA polymerase, together with its translation efficiency leads to higher level of expression of target protein to constitute majority of the cellular proteins within a few hours. Expression is achieved with 0.5mM IPTG which allows lacUV5 promoter to express T7 RNA polymerase to transcribe the downstream sequence of the gene of interest. SP-A and SP-D were cloned directly into NdeI/HindIII site in the pET-3b vector downstream to the gene10 initiation codon (figure 3.4). Despite the highly specific activity of T7 RNA polymerase, there are chances of 'leaky expression' which can be toxic to the cell causing cell death. Alternatively, more stringent methods such as the pET-11 vector containing the lac operator can be used for toxic genes.

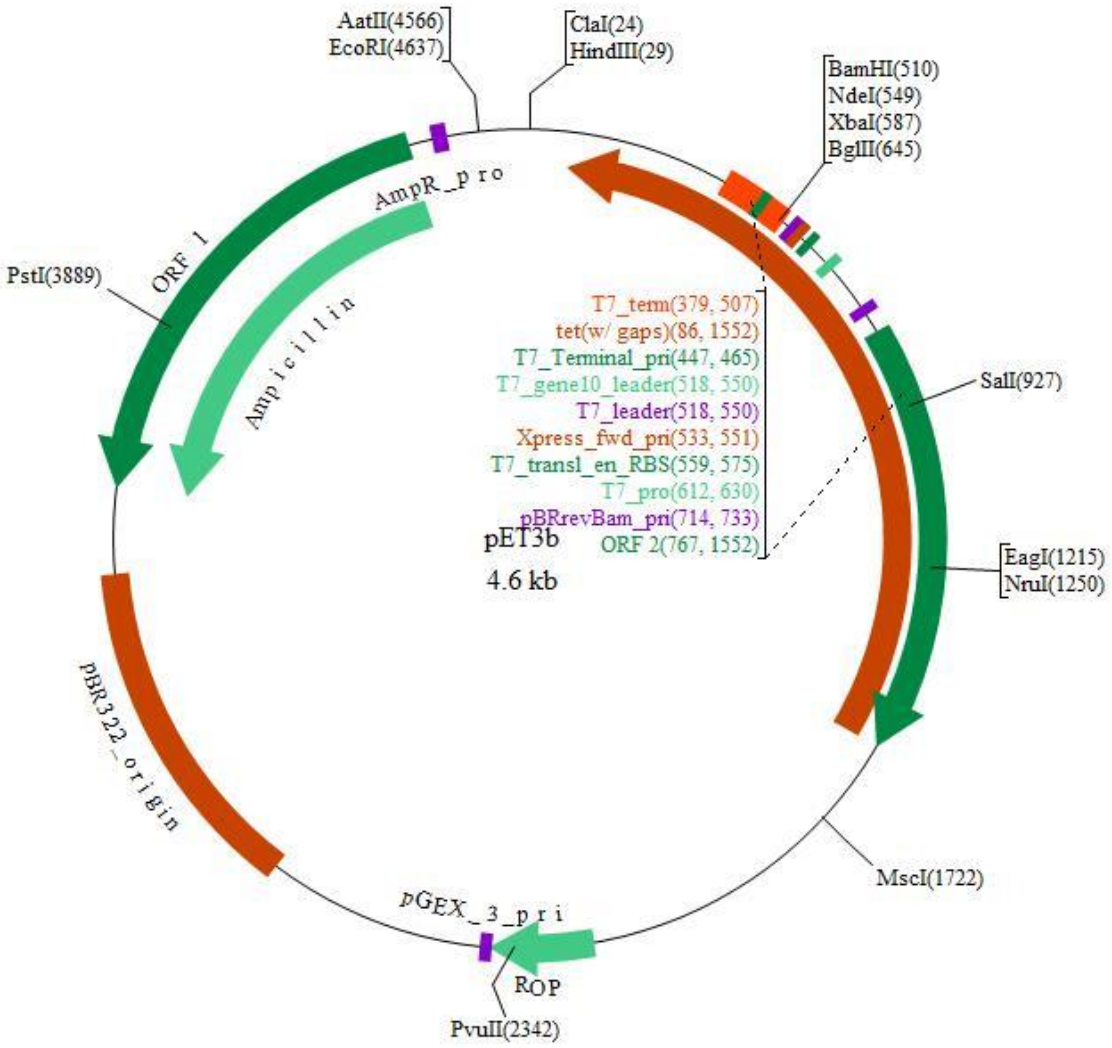
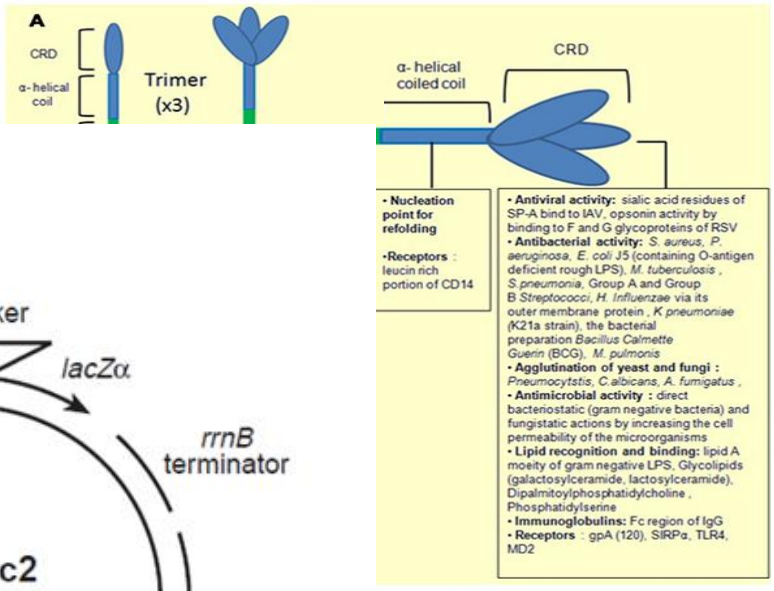


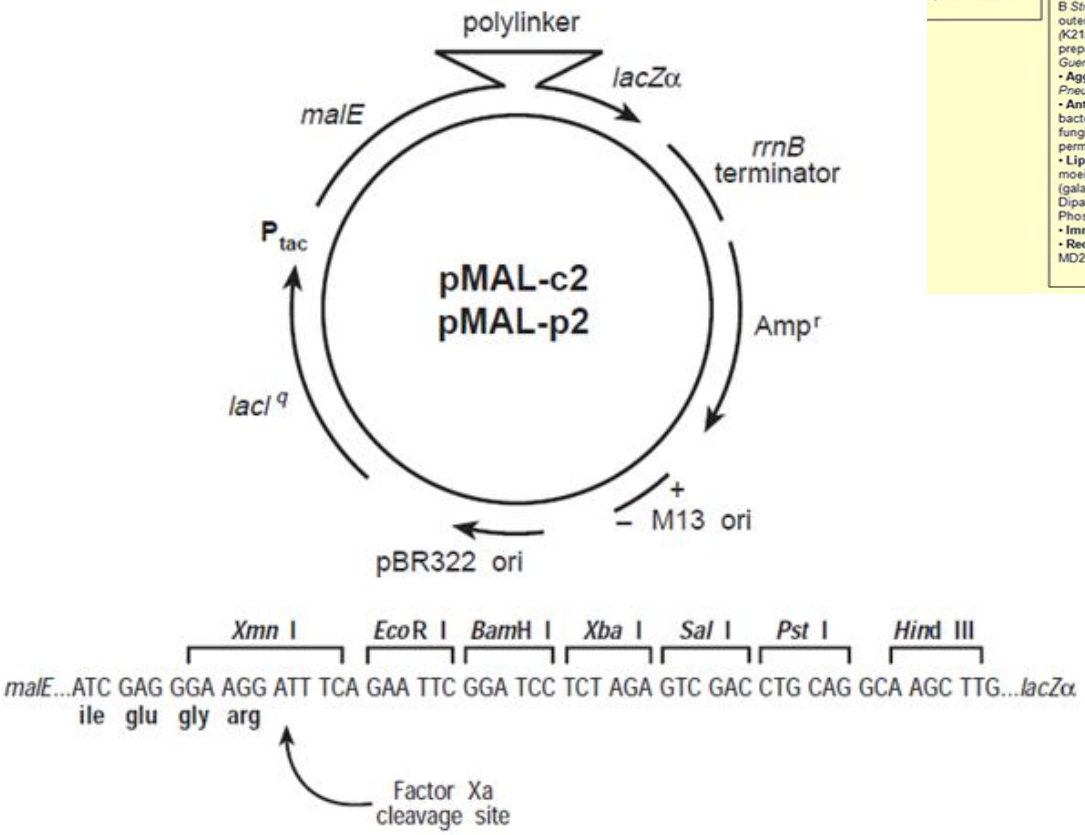

***Figure 3.3 – Map of pET3b and its restriction cloning sites***

#### *3.1.3.3 pMal-c2*

The pMal-c2 vector provides a method for cloning a gene for expressing and purifying a protein. The gene was cloned downstream to the malE gene of E.coli which codes for maltose-binding protein (MBP) to results in a MBP fusion protein (Maina et al., 1988) (figure3.5). This method has many advantages, such as the malE initiation sequence and "tac" promoter to result in high-level of expression of the gene of interest, a laqI gene for lac repressor to keep Ptac low in the absence of IPTG. This system carries a recognition site specific for factor Xa protease, 5' to the polylinker insertion to allow cleaving of MBP from the protein of interest. The one-step purification of the fusion protein makes it easier to purify the soluble fused protein. To produce the clone in pMal-c2, the gene of interest was inserted in frame to the malE gene and has its own stop codon at the 3' end. The pMal-c2 has a deletion of the leader sequence which leads to the cytoplasmic expression of the fusion protein and is more stable than other pMal vectors in its series. Cloning and production of SP-D using this fusion system has been shown before (Eda et al., 1997, Kishore et al., 1996).

SP-A




pMAL™-2 Vectors. pMAL™-c2 (6646 base pairs) has an exact deletion of the *malE* signal sequence. pMAL™-p2 (6721 base pairs) includes the *malE* signal sequence. Arrows indicate the direction of transcription. Unique restriction sites are indicated.

***Figure 3.4 – Map of pMal-c2 and cloning sites***

## 3.2 Materials and methods

### *3.2.1 Purification of native SP-A from Amniotic fluid*

Amniotic fluid is normally collected either following vaginal rupturing of the foetal membranes or during spontaneous rupturing. Method of purification of native SP-A from amniotic fluid has been adapted from Strong et al (Strong et al., 1998). Briefly, 1L of amniotic fluid was equilibrated with 5 mM $CaCl_2$ and the fat and large aggregates/debris were sieved first through a 3 mm Whatman filter paper then through a cell strainer for a clearer suspension. The SP-A rich pellet was separated from the SP-D rich supernatant by centrifugation at 10,000 x g for 40 mins at 4°C. The SP-A rich pellet was extracted with 20 ml of 8 M urea Buffer-I (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5mM $CaCl_2$, 0.02%, $NaN_3$, 8 M Urea) and mixed for 1 hour at 4°C. The solubilised protein was centrifuged at 10,000 x g for 40 mins at 4°C to collect the supernatant containing SP-A. The protein was dialysed against 4 M urea buffer for 2 hours, then 1 M urea buffer for 2 hours followed by dialysis against Buffer-I (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$) overnight. The next day, the dialysate (~20 ml) was centrifuged at 10,000 x g for 20 mins at 4°C and the clear supernatant was used for protein purification by affinity chromatography.

### *3.2.2 Affinity chromatography for purification of SP-A from amniotic fluid*

Maltose-sepharose was washed with 10 bed volumes of autoclaved distilled water, followed by equilibration with 10 bed volumes of Affinity Buffer-I (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$). The matrix was mixed with the supernatant containing SP-A for 30 mins at room temperature to allow binding of native SP-A to maltose-sepharose. Consequently, the sample was passed through an affinity column thrice to allow the bed to settle and the proteins to be collected on the matrix. The column was then washed with 5 bed volumes of Affinity Buffer I containing 1 M NaCl (50 mM Tris-HCl, pH7.4, 1 M NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$). SP-A bound proteins were eluted in 20 x 1 ml with Buffer I containing 10 mM EDTA (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 10 mM EDTA, 0.02% $NaN_3$). Protein purity and integrity were determined by SDS-PAGE.

### *3.2.3 Purification of native SP-D from Amniotic fluid*

The supernatant of amniotic fluid rich is SP-D were calcified to 5mM $CaCl_2$ to increase volume and decrease viscosity, whilst monitoring the pH to 7.5. After stirring for 1hour at 4°C to allow aggregation of the calcium dependent proteins, SP-D, the sample was passed thrice through maltose-agarose equilibrated with 10 bed volumes of Affinity Buffer I (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$). Non-specific bound proteins were washed with 5 bed volumes of 1 M NaCl buffer (50 mM Tris-HCl, pH7.4, 1 M NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$). 10 x 1ml fractions of SP-D were eluted with elution buffer containing manganese chloride (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 10 mM $MnCl_2$, 0.02% $NaN_3$) over ice. Optical density of each fraction is read at 280 nm using Elution Buffer without $MnCl_2$ as a reference.

### *3.2.4 Removal of BSA and manganese chloride and concentration of SP-D protein*

To remove manganese chloride ($MnCl_2$), the fractions with the highest concentrations were pooled and dialysed against 1L elution buffer containing 5mM EDTA overnight at 4°C. To concentrate the protein, the dialysis bag was placed in a beaker containing Polyethylene Glycol MW 6000-10,000 (Sigma, Dorset, UK) for 2 hours until the concentration reached ~500 µg/ml. The proteins were stored in 1ml fractions at -20°C.

### *3.2.5 SDS-PAGE and Western blotting*

SDS-PAGE was performed in a 4% stacking and 12% separating/resolving gel with an unstained protein molecular marker (Peqlab, Sarisbury Green, UK). Protein bands were visualised by Coomassie brilliant blue R-250 (Bio-Rad, Hemel Hempstead, UK). In SDS-PAGE for Western blotting, full-range rainbow molecular weight marker were used (GE healthcare Life sciences, Buckinghamshire, UK).

Immunoblotting was carried out essentially as described previously (Towbin et al., 1979) in a blotting chamber from Bio-Rad (Mini Trans blot) (Bio-Rad, Hemel Hempstead, UK) using nitrocellulose membrane (Millipore, Watford, UK). After transfer (340A for 90 mins), the membrane was blocked overnight at 4°C in 5% v/v non-fat milk-powder in PBS (blocking buffer). After washing in PBS with 0.05% Tween -20 (washing buffer) the membrane was incubated for 1.5 hours at room temperature in 1 µg/ml rabbit anti-human SP-A in blocking buffer. After washing, the membrane was incubated in 1/1000 dilution Protein A-HRP (Invitrogen, Paisley, UK) for 1 hour at room temperature. Subsequently, the blot was developed in DAB (Sigma, Dorset, UK) according to manufactures recommendation.

### *3.2.6 Cloning of full length SP-A in pSecTagC for expression in mammalian HEK-293T cells*

PCR amplification, DNA digestion, separation of fragments by agarose-gel electrophoresis, ligation of DNA fragments and transformation of *E.coli* with plasmid DNA was performed as described by Maniatis et al. (Malke, 1990). cDNA containing genetic code for full length SP-A was used as a template and provided by Umakanth, Leicester University. On the basis of the map for pSecTagC, two PCR primers were designed selecting two restriction sites after the Igκ chain leader sequence which did not cut within the full length sequence of SP-A. SP-A primers were designed excluding its own leader sequence as it will be initiated by the vector leader sequence itself. SP-A gene was amplified with forward oligonucleotide primer (5'-3') – GGGGGAATTCGAAGTGAAGGACGTTTGTGTTGGAAG and reverse primer (5'-3') – CGACTGACCATCTGTGAGTTCTGACTCGAGCCC. These were designed using the CDS region of the cDNA coding for SP-A excluding the leader sequence. Sequence data were from GeneBank/EMBL. Synthetic oligonucleotide primers were prepared by Sigma (Sigma, Dorset, UK).

Template DNA (250 ng) and primers (500 µM each) were incubated in a 50 µl reaction mixture containing 5 x Phusion HF buffer, 10mM deoxytrinucleoside triphosphate (dNTPs), and 2 units/µl High-Phusion DNA polymerase (Thermoscientific, Loughborough, UK), set in a thermocycler at 98°C (10 secs), 59°C (20 secs) and 72°C (1 mins) for 30 cycles. Vector, pSecTagC, was digested with EcoRI/HF and XhoI (NEB, Hitchins, Herts, UK) for 3hrs at 37°C. The products were gel purified with QIAquick gel extraction kit (Qiagen, Manchester, UK) according to manufactures recommendation.

### *3.2.7 Ligation and transformation in TOP10 competent cells*

Prior to transformation of plasmid in TOP10 competent cells (Invitrogen, Paisley, UK), digested vector and insert were ligated with T4 DNA ligase (NEB, Hitchins, Herts, UK). For ligation, 1:3 vector to insert were incubated in a 20 µl reaction containing 1 µl enzyme and 2 µl T4 ligase buffer. This was incubated at room temperature for 20 minutes and overnight at 4°C. The ligated product was transformed in TOP10 competent cells with SOC media, incubating on ice for 1 hour, applying heat chock at 42°C for 2 minutes, ice for 5 minutes and after adding SOC media the culture was incubated at 37°C for 1 hour before plating 50 µl on LB agar/ampicillin plates.

### *3.2.8 Plasmid isolation and digestion*

Colonies were selected for verifying successful cloning of gene of interest. Each colony were inoculated in 5 ml LB containing 100 µg/ml ampicillin and grown for 12-17 hours at 37°C. Cultures were harvested at 5,000 x g for 8 minutes before extracting plasmid with mini prep plasmid isolation kit (Qiagen, Manchester, UK) according to manufactures recommendation. 10 µl of extracted plasmid from each colony were incubated in a 50 µl reaction with 2.5 µl EcoRI and XhoI each, 10 µl 10x BSA and 5 µl NEB buffer 4. After 3 hour digest at 37°C the products were gel fractionated on an agarose gel and visualised under a UV Transilluminator (Bio-Rad, Hemel Hempstead, UK). Colony containing gene of interest was subjected to large scale plasmid extraction with EZNA FastFilter (VWR, Leicestershire, UK) according to manufactures recommendation. Large scale plasmid extraction was used for transfection in HEK-293T cells.

### *3.2.9 HEK 293T transfection with pSecTagC/SP-A*

HEK293T (human embryonic kidney 293T) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated FBS (foetal bovine serum), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C under humidified air containing 5% $CO_2$. After HEK-293T cells were seeded 5 x $10^6$ cells and grown for 12 hours for cells to adhere. Next day, fresh media was added and transfection with calcium phosphate was prepared. 50 µg of DNA was incubated with 2M $CaPO_4$ to make a final volume of 500 µl to form DNA-calcium phosphate complex. This was added to 500 µl 2 x HBS (280 mM NaCl / 1.5 mM $Na_2HPO4$ / 50 mM HEPES, pH 7.05) drop wise and incubated at room temperature for 30 minutes. The complex was added to the cells in fresh media and incubated for 4-5 days. Each day the media was replenished and harvested to purify protein secreted in the supernatant.

### *3.2.10 Protein purification*

Media collected at 24, 48, 72, 96 and 120 hours and was harvested at 5000 x g for 10 minutes and pooled before dialysing against Buffer-I containing 5 mM $CaCl_2$ (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$, pH7.4) for 3 hours before purification by high affinity chromatography through a maltose-agarose matrix. The column was washed with 3 bed volumes of buffer-I followed by 3 bed volumes of buffer-I containing 1 M NaCl to remove non-specific bound proteins. The maltose binding SP-A was eluted

at 1 ml/min with elution buffer containing 5 mM EDTA (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 0.02% $NaN_3$) and collected in 1 ml fractions over ice. These fractions were separated on a 12% SDS-PAGE to observe protein yield, quality and purity.

### *3.2.11* Cloning of recombinant fragment human trimeric-SP-A (rfSP-A) *comprising of neck and CRD regions* in pMal-c2 for expression in E.coli BL21 (λDE3)

Cloning of trimeric fragment SP-A (rfSP-A) was adapted from (Eda et al., 1997, Kishore et al., 1996). PCR amplification, DNA digestion, separation of fragments by agarose-gel electrophoresis, ligation of DNA fragments and transformation of *E.coli* with plasmid DNA was performed as described by Maniatis et al. (Malke, 1990). cDNA containing genetic code for full length SP-A was used as a template and provided by Umakanth, Leicester University. On the basis of the map for pMal-c2, two PCR primers were designed to amplify the CRD region after the 23gly-Xaa-Yaa repeats to clone the neck and CRD of SP-A. Two restriction sites were selected which were within the pMal-c2 vector but not within the N/CRD region of SP-A. This was verified with digest finder from NEB using the sequence data from GeneBank/EMBL. Primers were designed to contain EcoRI site on forward primer and BamHI on the reverse primer. Recombinant fragment of human SP-A gene was amplified with forward oligonucleotde primer (5'-3') – GGGGAATTCCATCTAGATGAGGAGCTCCAAGC and reverse primer (5'-3') – GGGGGATCCCTAGAACTCACAGATGGTCAGTCGG. Synthetic oligonucleotide primers were prepared by Sigma (Sigma, Dorset, UK). The gene of interest was cloned downstream to the *MalE* gene of *E.coli* which encodes the maltose binding protein (MBP) gene to result in a fusion protein for cytoplasmic expression. SP-A neck and CRD cloned into pET-101 was carried out the same way as described for cloning in pMal-c2 except the plasmid used was pET-101 and the forward primer was substituted with – CACCATGCATCTAGATGAGGAGCTCCAAGC. The expression and purification for SP-A cloned into pET-101 was carried out the same way as described for proteins in inclusion bodies.

High-Phusion DNA polymerase (Thermoscientific, Loughborough, UK) was used to amplify the gene of interest. Template DNA (250 ng) and primers (500 µM each) were incubated in a 50 µl reaction mixture containing 5 x Phusion HF buffer, 10mM deoxytrinucleoside triphosphate (dNTPs), and 2 units/µl High-Phusion DNA polymerase (Thermoscientific, Loughborough, UK), set in a thermocycler at 98°C (10

secs), 59°C (20 secs) and 72°C (1 mins) for 30 cycles. Vector, pMal-c2, was digested with EcoRI/HF and BamHI (NEB, Hitchins, Herts, UK) for 3 hours at 37°C. The products were gel purified with QIAquick gel extraction kit (Qiagen, Manchester, UK) according to manufactures recommendation.

### *3.2.12 Ligation and transformation in TOP10 competent cells*

Prior to transformation of plasmid in TOP10 competent cells (Invitrogen, Paisley, UK), digested vector and insert were ligated with T4 DNA ligase (NEB, Hitchins, Herts, UK). For ligation, 1:3 vector to insert were incubated in a 20 µl reaction containing 1 µl enzyme and 2 µl T4 ligase buffer. This was incubated at room temperature for 20 minutes and overnight at 4°C. The ligated product was transformed in TOP10 competent cells with SOC media, incubating on ice for 1 hour, applying heat chock at 42°C for 2 minutes, ice for 5 minutes and after adding SOC media the culture was incubated at 37°C for 1 hour before plating 50 µl on LB agar/ampicillin plates.

### *3.2.13 Isolation of the recombinant construct and characterisation by restriction digestion and DNA sequencing*

Colonies were selected for verifying gene of interest insertion. Each colony was in inoculated in 5 ml LB containing 100 µg/ml Ampicillin and grown for 12-17 hours at 37°C. Cultures were harvested at 5000 x g for 8 minutes before extracting plasmid with mini prep plasmid isolation kit (Qiagen, Manchester, UK) according to manufactures recommendation. 10 µl of extracted plasmid from each colony were incubated in a 50 µl reaction with 2.5 µl EcoRI and BamHI each, 10 µl 10x BSA and 5 µl NEB buffer 4. After 3 hours digest at 37°C the products were gel fractionated on an agarose gel and visualised under a UV Transilluminator (Bio-Rad, Hemel Hempstead, UK). Plasmids were sequenced by BD genomics and varied by BLAST on NCBI. Colony containing gene of interest in pMal-c2 vector was transformed in BL21 (λDE3) and colonies containing gene of interest in pET-3b or pET101 was transformed in BL21 (λDE3) pLysS (Invitrogen, Paisley, UK) for protein production.

### *3.2.14 Expression and purification of soluble proteins – MBP fused SP-A*

Single colony of BL21 (λDE3) with the right construct was subjected to large scale expression in 250 ml for protein production. This was purified by affinity chromatography through a amylose-resin column. Initially, the colony was inoculated in

LB supplemented with 100 µg/ml ampicillin and grown at 37°C shaking for 12-17 hours. 12.5 ml of the primary culture was transferred into 250 ml LB with 100 µg/ml ampicillin and grown at 37°C shaking until the cells reach log phase, indicated by an OD (600 nm) between 0.6-0.8. The culture was induced with 0.5 mM IPTG and further incubated for 3 hours in a 37°C shaking for production of protein. The culture was harvested 13,000 x rpm. The pellet was further processed to extract soluble proteins.

The cell pellet containing protein was separated by breaking aggregates with lysis buffer and sonication. Initially, the pellet was dissolved in 30 ml ice-cold lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 0.25% Tween-20, pH8.0) and stirred for 1h 45 mins at 4°C. PMSF and lysozyme was added to a final concentration of 10 mM and 50 µg/ml respectively to prevent protein degradation and aid the lysis of cells. The suspension was sonicated for 15 cycles at 40 kHz for 30 seconds with 2 minute intervals. All stages of sonication were carried out on ice to prevent proteins denaturing. The sonicated sample was harvested at 13000 rpm for 15 mins to collect protein rich supernatant. The supernatant was diluted 5-fold with column buffer containing tween-20 with an end concentration 100 mM NaCl and applied to the equilibrated amylose resin (GE healthcare Life sciences, Buckinghamshire, UK) column for purification.

### *3.2.15 Affinity chromatography purification of MBP-SP-A*

The amylose column was washed with 3 bed volumes of column buffer containing tween-20 (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.25% Tween-20, 5% glycerol, pH8.0). This was followed by passing the diluted supernatant once and washing with 3 bed volumes of column buffer without tween-20. The column wash then washed with 25 ml factor Xa buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, 5% glycerol, pH8.0) to remove non-specific bound proteins and proteins were eluted with 20 ml elution buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, 10 mM Maltose, 5% glycerol, pH8.0) in 1 ml fractions over ice. The OD was checked at 280 nm to obtain protein yield before cleavage of protein with FactorXa protease.

### *3.2.16 Cleavage of MBP with FactorXa protease*

Proteins were dialysed against column buffer with glycerol but without tween for 3-4 hours. The samples were concentrated to 1 mg/ml with polyethylene glycol. Factor Xa protease (1 U/100 µg of fusion protein) was added to 100 µl same and incubated for 24 hours at R/T until cleavage is complete.

### *3.2.17 Purification of MBP-SP-A with maltose-agarose after cleavage*

Proteins were dialysed against affinity buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$) for 2-3 hours and passed through maltose-sepharose column thrice. The column was washed with 10 ml affinity buffer containing 1 M $NaCl_2$ (50 mM Tris-HCl, pH7.4, 1 M NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$) to remove any impurities and non-specific bound proteins. Proteins were eluted in 0.5 ml fractions with elution buffer containing 5 mM EDTA buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 5 mM EDTA, 0.02% $NaN_3$).

### *3.2.18 Expression and purification of rfSP-A and rfSP-D in T7 system E.coli BL21 (λDE3) pLysS*

Plasmid containing rfSP-A and rfSP-D was provided by Dr Uday Kishore, Brunel University. These plasmids and rfSP-A in pET-101 were transformed in BL21 (λDE3) pLysS. A single colony was selected for large scale expression, inoculated individually in 15 ml LB supplemented with Amp/chl. 100 µg/ml and 50 µg/ml respectively. The inoculum was grown overnight at 37°C shaking. 12.5 ml of the primary culture was transferred into 250 ml LB supplemented with amp/chl. and grown for approximately 3 hours at 37°C until the cells reached log phase, indicated by an OD (600 nm) between 0.6-0.8. 1 ml. The culture was induced with 0.5 mM IPTG and further incubated for 3 hours in a 37°C shaking incubator. Post induction, the culture was harvested at 13000 rpm for 10 min and pellets were stored at -20°C.

### *3.2.19 Lysis and sonication of insoluble proteins in inclusion bodies*

The cell pellet containing protein in inclusion bodies were separated by breaking aggregates with lysis buffer and sonication. Initially, the pellet is dissolved in 25 ml ice-cold lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, pH7.4) with PMSF and lysozyme at an end concentration of 10mM and 50 µg/ml respectively. After lysis for 1 hour at 4°C the suspension of lysed cells was sonicated for 15 cycles at 40 kHz for 30

seconds with 2 minute intervals. All stages of sonication were carried out on ice to prevent proteins denaturing. The sonicated sample was harvested at 13,000 rpm for 15 mins to collect protein rich pellet.

### *3.2.20 Refolding of proteins from inclusion bodies by step-wise dialysis*

The proteins recovered in the pellet were solubilised with 25 ml 8 M urea buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH7.5, 8 M Urea) containing 10 mM 2-merceptoethanol whilst stirring on a magnetic stirrer for 1 hour at 4°C. The solubilised protein was centrifuged at 13,000 rpm for 15 minutes and transferred into a dialysis bag. The proteins were dialysed against dialysis buffer containing 4 M urea and 2-merceptoethanol for 2 hours at 4°C, transferred to dialysis buffer containing 2 M urea with 2-merceptoethanol followed by dialysis buffer containing 1 M urea and fresh 2-merceptoethanol, stirring for 2 hours at 4°C at each step. Finally, the buffer was changed to dialysis buffer with no urea stirring at 4°C overnight. The following day, the proteins were purified by affinity chromatography on a maltose-agarose column.

### *3.2.21 Purification of proteins from inclusion bodies expressed in E.coli via Affinity chromatography*

The next day, the buffer was changed to affinity buffer containing 5 mM $CaCl_2$ to remove EDTA and to calcify the protein in preparation of affinity chromatography. The refolded rfSP-A and rfSP-D-gly-x-y proteins were purified by high affinity chromatography containing a maltose-agarose matrix to allow the proteins to specifically bind. Initially the column was prepared by equilibrating with 5 bed volumes of affinity buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH7.5). Next, the protein was passed through the column thrice before non-specific and weakly bound proteins were washed off the column with affinity buffer containing 1 M NaCl (50 mM Tris-HCl, 1 M NaCl, 5 mM $CaCl_2$, pH7.5). Proteins were eluted with elution buffer containing 5 mM EDTA (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH7.5) in 20 x 1ml fractions over ice. The absorbance readings of each fraction were observed at A280 and the presence and purity of the protein was determined by 12% SDS-PAGE. Endotoxin removal was carried out and LPS levels were determined by LAL assay (Lonza, Slough, UK).

### *3.2.22 Endotoxi removal from rfSP-A with endotoxin removal spin columns (Thermo Scientific)*

The use of the columns was followed as instructed by manufacturers' guidance and as described in chapter 2.

### *3.2.23 Limulus Amebocyte Lysate (LAL) assay (Lonza, Slough, UK)*

The LPS levels in finally purified proteins were measured using the chromogenic Limulus Amebocyte Lysate (LAL) assay as described under chapter 2.

**Characterisation of SP-A and SP-D**

### 3.2.24 Bis-(sulfosuccinimidyl)-suberate ($BS_3$) cross-linking

A 25mM solution of $BS_3$ was prepared by dissolving 2 mg $BS_3$ in 140 µL of DMSO. Using a 20-fold excess approach (20:1 Crosslinker: Protein), $BS_3$ crosslinker solution was mixed with the protein sample so that the final crosslinker concentrations are at 3 different concentrations - 1 mM, 0.1 mM and 0.01mM $BS_3$. This was incubated at room temperature for 45 minutes to 1 hour. As a control, quench and unreacted $BS_3$ mixed in 25 mM to 60 mM Tris-HCl (pH-7.5) was incubated for 10-15 minutes at room temperature. The samples were separated on an SDS-PAGE.

### *3.2.25 Maltosyl-BSA binding with native and recombinant proteins*

96-well ELISA plate was coated with 5µg maltosyl-BSA double diluted 5 times and incubated overnight at 4°C. The next day, the sample was discarded and blocked in 2% BSA-PBS for 2hours at 37°C. Protein at 5µg per well were added in duplicates per dilution of maltosyl-BSA and incubated for 1.5 hours at 37°C. The unbound proteins were discarded and wells were washed thrice with PBS + 0.05% Tween-20. Primary antibody (1:1000 dilution – anti-human SP-A and anti-human SP-D) was added and incubated for 1.5 hours, followed by thrice washes with PBS-Tween 20, 0.05%. Finally secondary antibody, PA-HRP, was added to 1:5000 dilution and incubated for 1.5 hours at 37°C. After the final washing off wells, the binding was detected cholometrically by addition of OPD (Sigma, Dorset, UK) until colour developed. This was detected under an ELISA plate reader (Bio-Rad, Hemel Hempstead, UK) at 450nm.

## 3.3 Results

### *3.3.1 Purification of native and recombinant proteins and binding to maltosyl-BSA*

Native SP-A and SP-D were purified from amniotic fluid and observed under reducing conditions. Both monomeric and dimeric forms of SP-A and SP-D were visible (Figure.3.6, 3.7 & 3.8). These were confirmed by western blot (Figure. 3.7 & 3.10). For characterisation these proteins were coated on an ELISA plate and to ensure specificity of these proteins antibodies against the proteins were used and binding was detected. Results displayed a positive trend of protein dilution binding to antibody. Native SP-A and full length rSP-A bound best to the antibody shown by a stronger signal on the spectrophometer. rfSP-A and rfSP-D showed weaker binding to the antibodies (Figure.3.12). Another method was via Westernblot, which confirmed specificity of the native proteins. Finally, binding of proteins (2.5µg) to maltosyl-BSA (ranging from 5-0.625µg) detected with antibodies specific to the protein, displayed best binding from native SP-A, rSP-A, rfSP-A in order of affinity, however rfSP-D showed stronger binding to maltosyl-BSA at 1.25µg (Figure.3.11).

***Purification of native SP-A from Amniotic fluid***

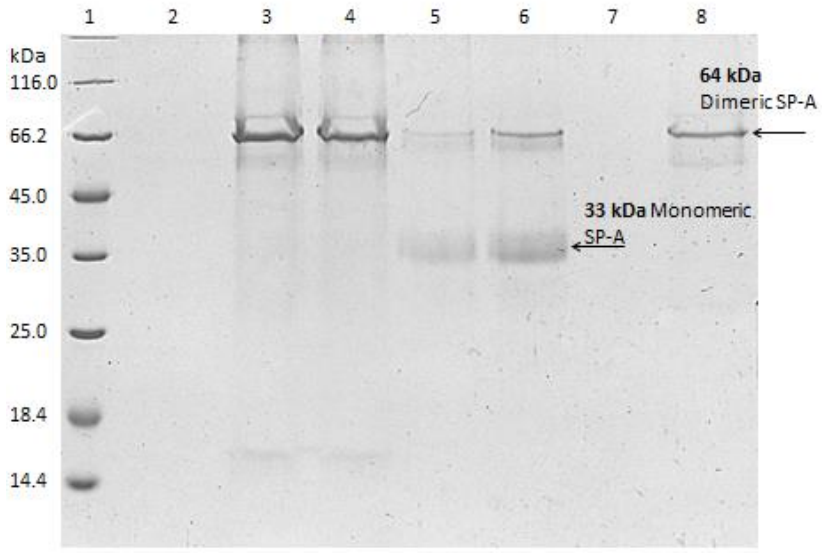



***Figure 3.5 – Purified native SP-A from amniotic fluid.*** *Lane 1 – protein marker, lane 3 – nSP-A F10 batch 2, lane 4 – nSP-A F1 Batch1, lane 5 – nSP-A F5 Batch1, lane 6 – nSP-A F6 Batch 3, lane 6 – nSP-A F.11 Batch 3, lane 8 – nSP-A F.2 Batch 4. Results show bands for dimmers (66 kDa) and monomers (~33 kDa) in Batch 1 (L5 & 6).*

### *Westernblot characterisation of native SP-A from amniotic fluid*

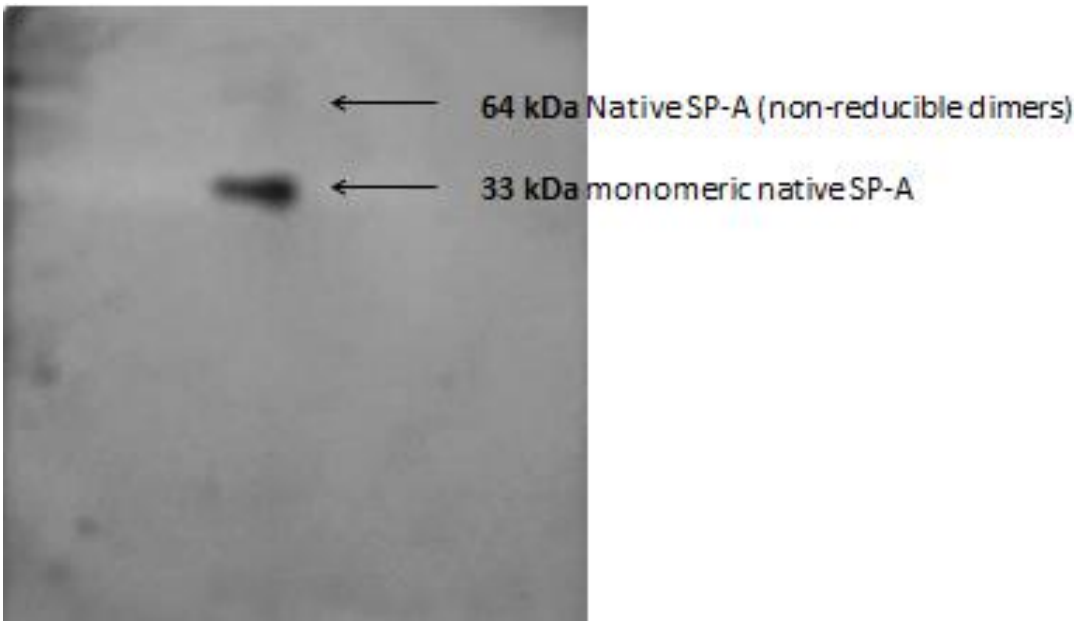



***Figure 3.6 – Western blot to detect native SP-A from amniotic fluid.*** *Initially 1µg and 2 µg proteins were separate on a SDS-PAGE and transferred at 320amps for 90mins. The membrane was blocked with 5% milk-powder/PBS buffer and probed with anti-human SP-A in a 1:5000 dilution. The primary antibody was detected with a HRP conjugated Protein A in a 1:5000 dilution. The bands were visible after DAB reacting with HRP to display protein bands; monomers and dimers of SP-A at 33kDa and 64kDa respectively.*

### *Purification of native SP-D from Amniotic fluid*

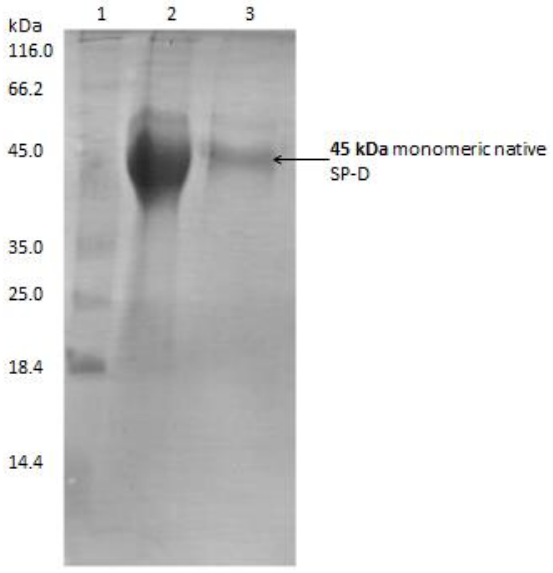



***Figure 3.7 – SDS-PAGE – Purified SP-D from amniotic fluid.*** *Purified SP-D from amniotic fluid purified via affinity chromatography with maltose-agarose separated under reducing conditions on an SDS-PAGE. Protein appears at 46kDa. Lane 1 – protein marker, lane 2 – SP-D fraction 1, lane 3 – SP-D fraction 2.*

***Purification of native SP-D from Amniotic fluid***

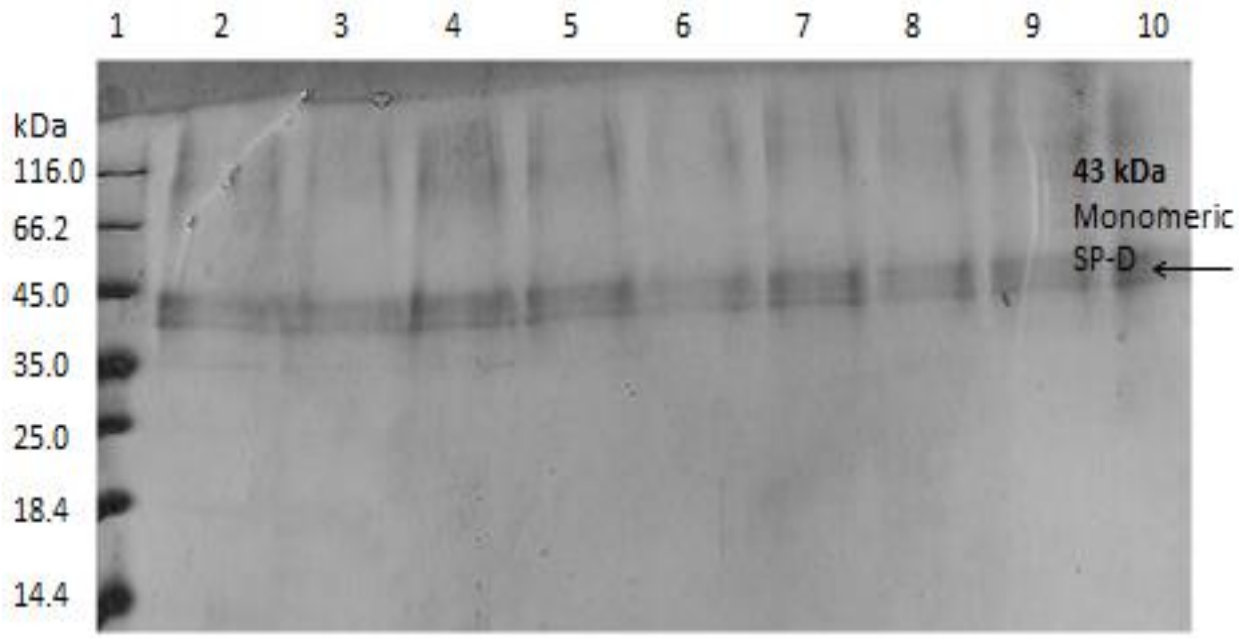



***Figure 3.8 – SDS-PAGE – Purified SP-D from amniotic fluid.*** *Purified SP-D from amniotic fluid purified via affinity chromatography with maltose-agarose separated under reducing conditions on an SDS-PAGE. Protein appears at 46kDa. Lane 1 – protein marker (14.4, 18.4, 25, 35, 45, 66.2, 116kDa), lane 2-10 – fractions 1-9. Protein appears in trimeric form at ~65kDa.separated under reducing conditions, stained with coomassie blue.*

***Westernblot characterisation of native SP-D from amniotic fluid***

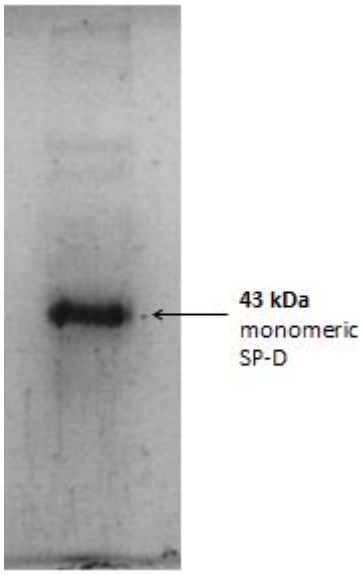



***Figure 3.9 – Western blot detection of nSP-D under reducing conditions.*** *SP-D is composed of a 43 kDa polypeptide chain, with faint bands corresponding to dimers and trimers of the 43kDa chain can also been seen.*

***Characterisation of native and recombinant SP-A and SP-D***

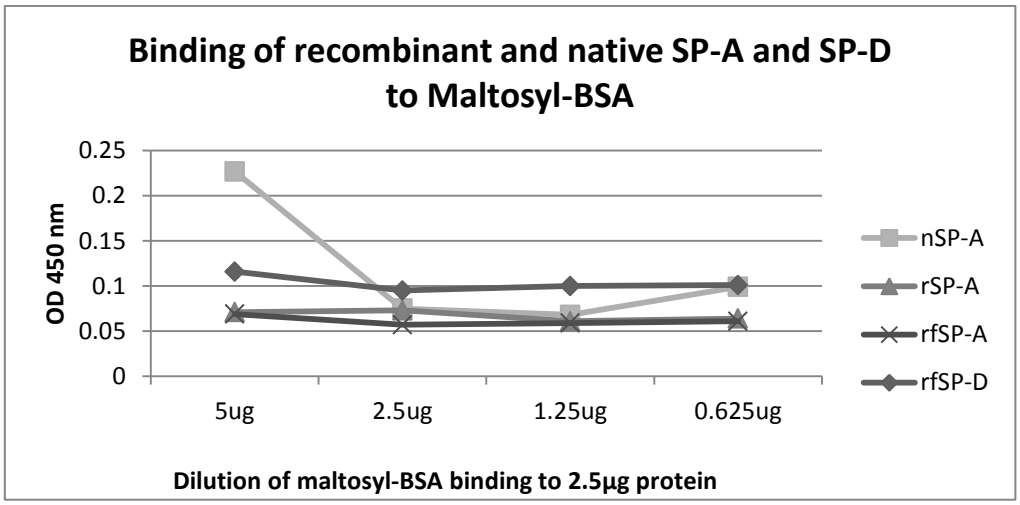



***Figure 3.10 – binding of native and recombinant SP-A and SP-D to maltosyl-BSA.*** *Dilutions from 5 µg of maltosyl-BSA were diluted 4-folds to observe best concentration for binding of protein (2.5 µg). Maltosyl-BSA was coated in a 96-well multititre plate incubated overnight. The next day, the bound proteins were blocked with 5% v/v non-fat milk powder in PBS for 2hours at 37°C. After 3 consecutive washed the proteins were added at a constant concentration of 2.5 µg and incubated for 1.5 hours at 37°C. Following 3 consecutive washed, the secondary antibody at 1 µg/ml was added and incubated for a further 1 hour. The binding of protein-protein interaction was observed with the addition of DAB reacting with the HRP on the secondary antibody protein A and quantified via spectrophometer at 450 nm.*

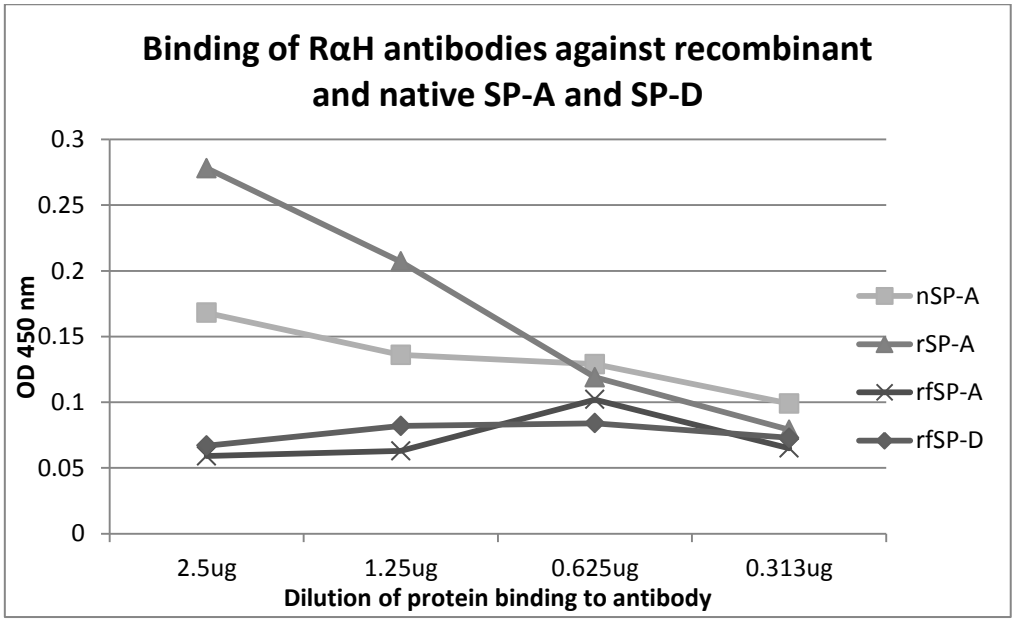



***Figure 3.11 – binding of antibodies against native and recombinant proteins.*** *Proteins were double diluted 4 times and detected with antibodies at 1 µg/ml (1:1000 dilution). Secondary antibody protein-A conjugated with HRP bound to primary antibody which was detected with DAB and results were quantified spectrophotometrically at 450 nm.*

### *3.3.2 Transient expression of recombinant SP-A (rSP-A) in HEK-293T cells with calcium phosphatase*

Full length SP-A (686 bp) was successfully cloned into pSecTagC and expressed in mammalian HEK-293T cells by transient expression. The two restriction sites selected were XhoI and EcoRI which were not present in the human SP-A mRNA. After successful cloning, the insertion of fragment was verified by restriction digests of isolated plasmids, out of all three plasmids, the fragment has dropped out (figure 3.14). The plasmids were sent for sequencing to Beckman Coulter Genomics and there was a 100% match in the sequence (see appendix). The plasmids were transfected by two methods, lipofectamine 2000 (Invitrogen) and calcium phosphate method (figure 3.15-3.16). Both showed efficient uptake therefore, it was chosen to opt for the inexpensive reproducible method using calcium phosphate. This yields 2-3mg per 50µg plasmid in 3 x 80 $cm_3$ culture flask (figure 3.17-3.18).

***Cloning and expression of recombinant full length SP-A in HEK-293T cells***

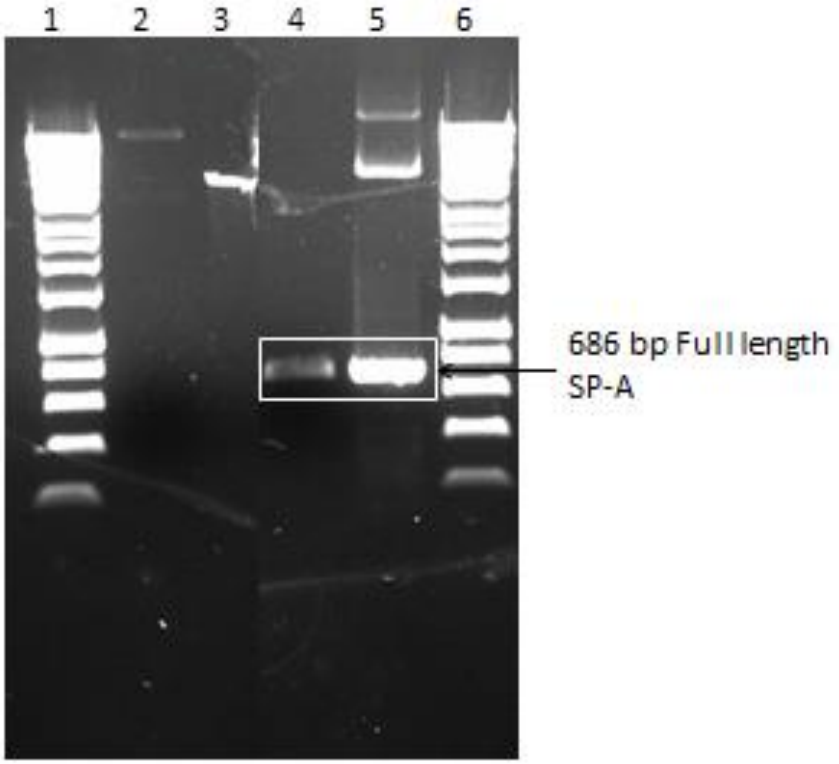



***Figure 3.12 – DNA fractionation of recombinant human full length SP-A on a 0.7% agarose-EtBr gel.*** *0.7% Agarose gel was prepared and 10 µl of each sample including digested and gel purified SP-A1 and pSecTagC were loaded. Lane 1 – DNA hyperladder I, lane 2 – pSecTagC uncut (1:10 dilution), lane 3 – pSecTagC cut with XhoI and EcoRI, lane 4 SP-A1 cut with XhoI and EcoRI, lane 5 – SP-A1 after PCR uncut, lane 6 – DNA hyperladder I.*

***Double digest with EcoRI and XhoI to check for insert***

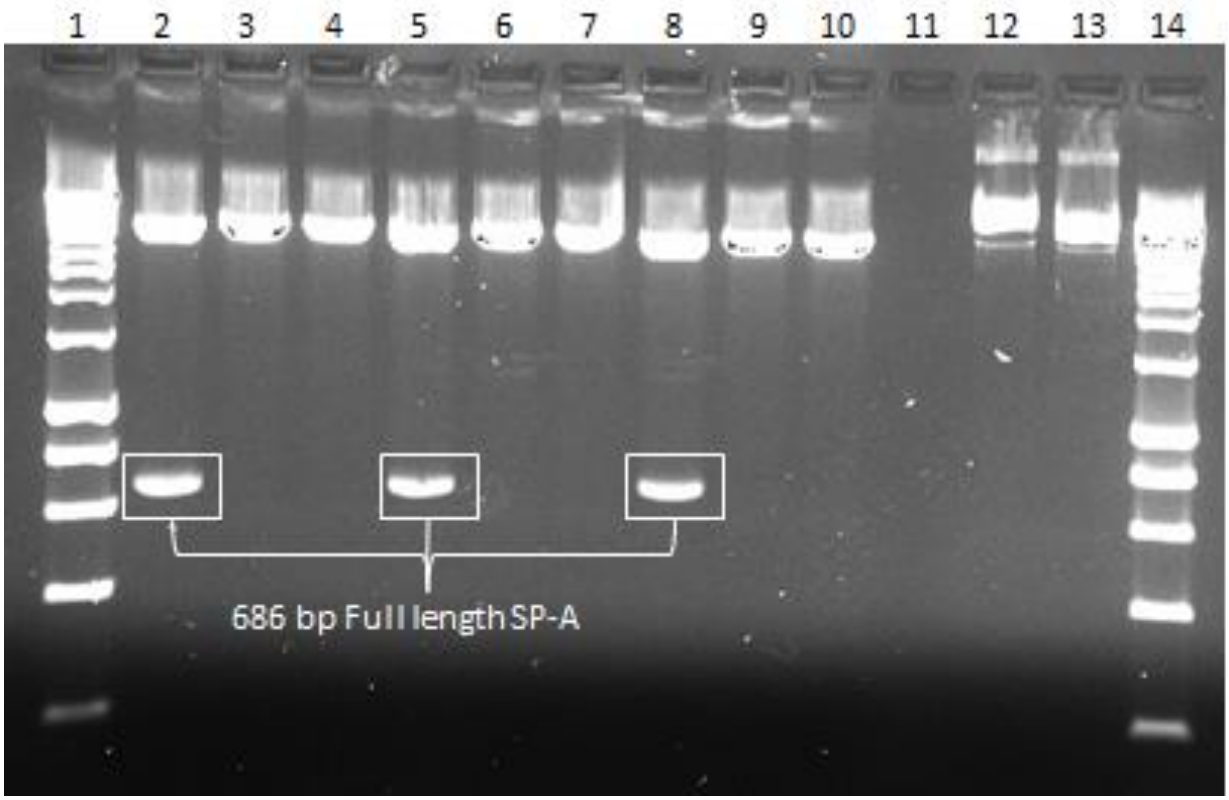



***Figure 3.13 – Restriction digestion of pSecTagC with SP-A gene of interest.*** *Figure 2. 1% Agarose gel - Digested sample were loaded in each well. Three colonies of SP-A1 ligated into pSecTagC were selected for double digestion and each showed a fragment of 686 bp to drop out. Lane 1 – hyperladder I, lane 2 - pSecTagC + SP-A1 E/X col.1, lane 3 - pSecTagC + SP-A1 EcoRI col.1, lane 4 - pSecTagC + SP-A1 XhoI col.1, lane 5 - pSecTagC + SP-A1 E/X col.2, lane 6 - pSecTagC + SP-A1 EcoRI col.2, lane 7 - pSecTagC + SP-A1 XhoI col.2, lane 8 - pSecTagC + SP-A1 E/X col.3, lane 9 - pSecTagC + SP-A1 EcoRI col.3, lane 10 - pSecTagC + SP-A1 XhoI col.3, lane 12 - pSecTagC + SP-A1 uncut, lane 13 - pSecTagC uncut, lane 14 – hyperladder I*

***Purified SP-A expressed in HEK-293T cells***

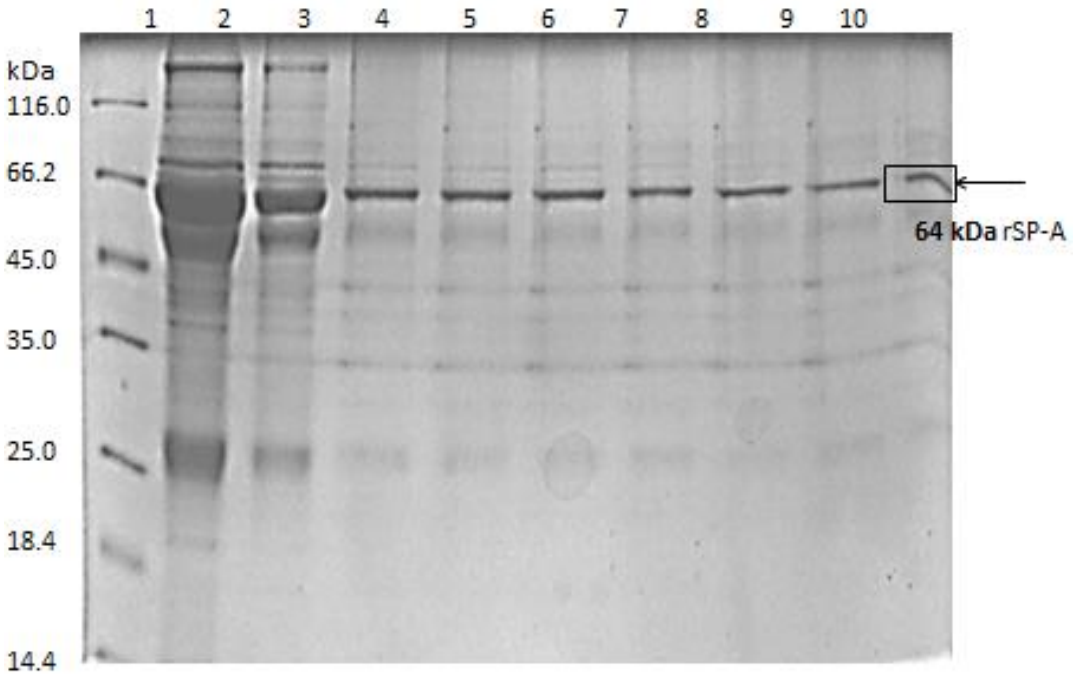



***Figure 3.14 – SDS-PAGE 1/2 – purified SP-A expressed in HEK-293T cells.*** *12% SDS-PAGE – Purified fractions of recombinant full length SP-A after affinity chromatography with maltose-agarose – lane 1 – protein marker, L.2 – flow through, L.3 – 1M NaCl wash, L.4-10 – fractions 1-7*

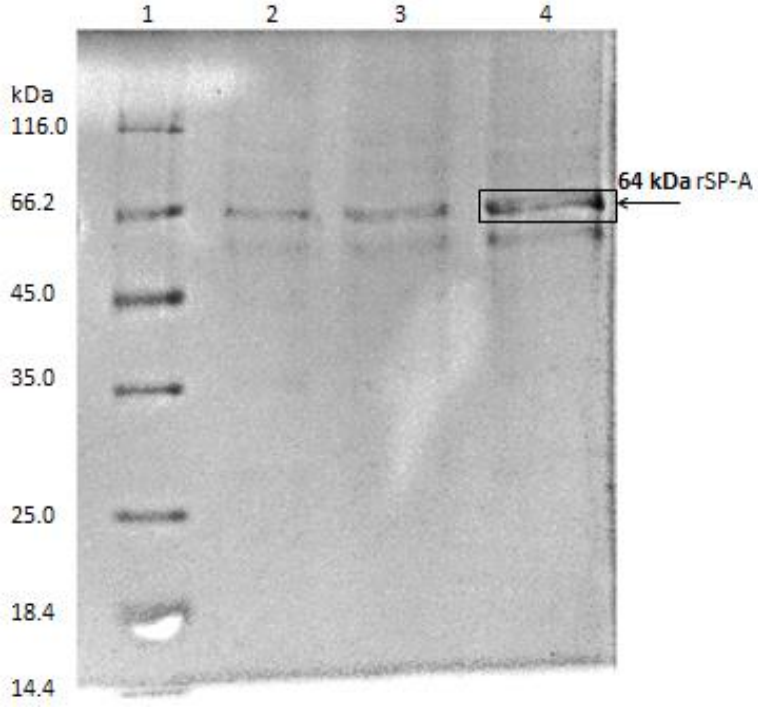



***Figure 3.15 – SDS-PAGE 2/2 – Purified SP-A expressed in HEK-293T cells.*** *12% SDS-PAGE – Purified fractions of recombinant full length SP-A after affinity chromatography with maltose-agarose - lane 1 – protein marker, L.2-4 – Fraction 8-10*

### *3.3.3 Expression of recombinant fragment SP-A (rfSP-A)– neck and CRD region in pET-101*

Neck and CRD fragment of SP-A was cloned into pET-101 at restriction sites EcoRI and SacI. After digestion, fragment of interest had dropped out successfully (figure 3.20); although the fragment drop was very faint the sequence was verified by Beckman Coultiers, showing a 100% match to SP-A gene. The plasmids were transformed into BL21 (λDE3) and pLysS strains of *E.coli.* Cultures transformed into pLysS were unable to grow and those transformed into BL21 (λDE3), OD to reach 0.6-0.8 typically took 4-8 hours which gave slight expression (Figure.3.21-3.23, 3.26). To verify if the protein was present in the induced samples, a western blot was carried out and protein was detected at ~66kDa rather than the expected 18kDa after IPTG induction (Figure.3.24-3.25). The sample was lysed and sonicated to see if protein can be purified, after another western blot analysis; the protein was detected again at ~66kDa and in the supernatant rather than the expected pellet (Figure.3.26-3.27). The proteins are usually found in the inclusion bodies which are collected in the pellet that are later extracted. The protein was detected in the supernatant which was dialysed against affinity buffer and purified via affinity chromatography using a maltose-sepharose column. The purified samples were analysed by immunoblot and no protein was detected in the fractions however was in the flow through (Figure.3.28). This suggests the protein was denatured or was not refolded correctly to be functionally/biologically active, and did not bind to the column. To confirm this was not a one off situation, many pilot and large scale expressions were carried out, each giving the same results.

***DNA fractionation of recombinant human SP-A (N/CRD) on a 0.7% agarose-EtBr gel***

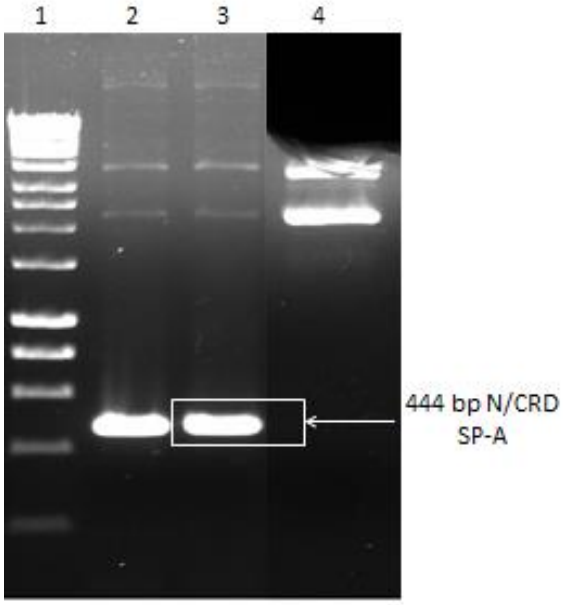



***Figure 3.16 – agarose gel – PCR amplification of human gene, neck and CRD region of SP-A. DNA separation of SP-A (N/CRD) after PCR reaction.*** *PCR products were separated through a 0.7% agarose-EtBr gel using the cDNA of full length SP-A cloned in pSecTagC as a control. This shows a 444bp product against the DNA marker to suggest amplification of the gene of interest. Lane 1: DNA marker (Hyperladder I – Bioline 14 bands from 200 bp - 10,037 bp), Lane 2-3: PCR reaction 1, 2, Lane 4: cDNA template containing full length SP-A (686 bp) in pSecTagC (5.2 kbp).*

Trimeric fragments of SP-A were amplified and separated on an agarose gel (figure. 3.19). The fragment and digested plasmid were purified and ligated before transfection in Top10 competent cells. *S*ix colonies were selected for plasmid extraction and digested with EcoRI and SacI. Five out of six plasmids of rfSP-A cloned in pET-101 appeared to have the gene of interest drop out after restriction digestion. Subsequently, 4 plasmids were selected for sequencing at Beckman Coultier. Sequencing required a single reverse primer, T7 rev., as the fragment is very small (444 bp). This checks each base for any errors or mismatches which is compared to the human genome to identify a close match. Plasmids 2 and 5 gave the best match for cloning SP-A successfully. This was used for transformation in BL21 (λDE3) pLysS.

***Restriction digestion of pET101/D TOPO containing SP-A (N/CRD) with SacI and ECORI***

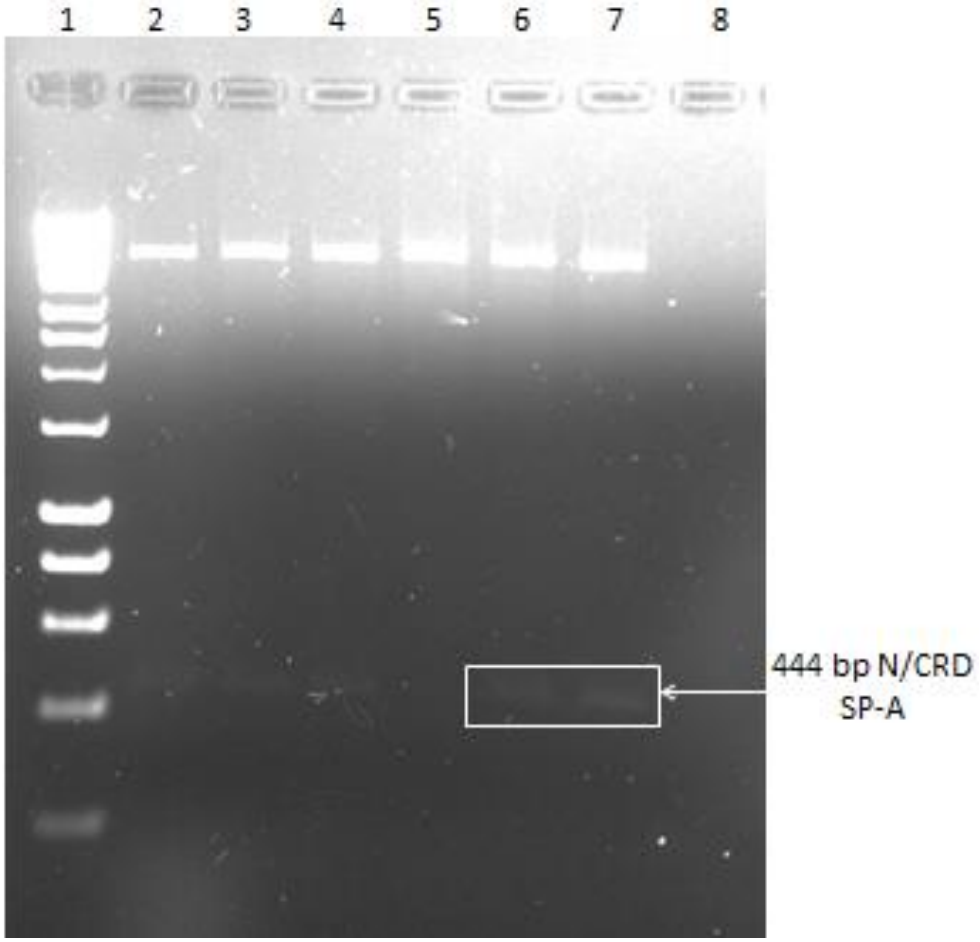



***Figure 3.17 – Restriction digestion of pET101-D/TOPO with EcoRI/SaCI.*** *1% Agarose gel – 6 plasmid restriction digestion with EcoRI and SacI. Recombinant trimeric SP-A (444 bp) in pET101 after restriction digestion with EcoRI and SacI*

***Pilot scale expression of recombinant fragment SP-A (N/CRD)***

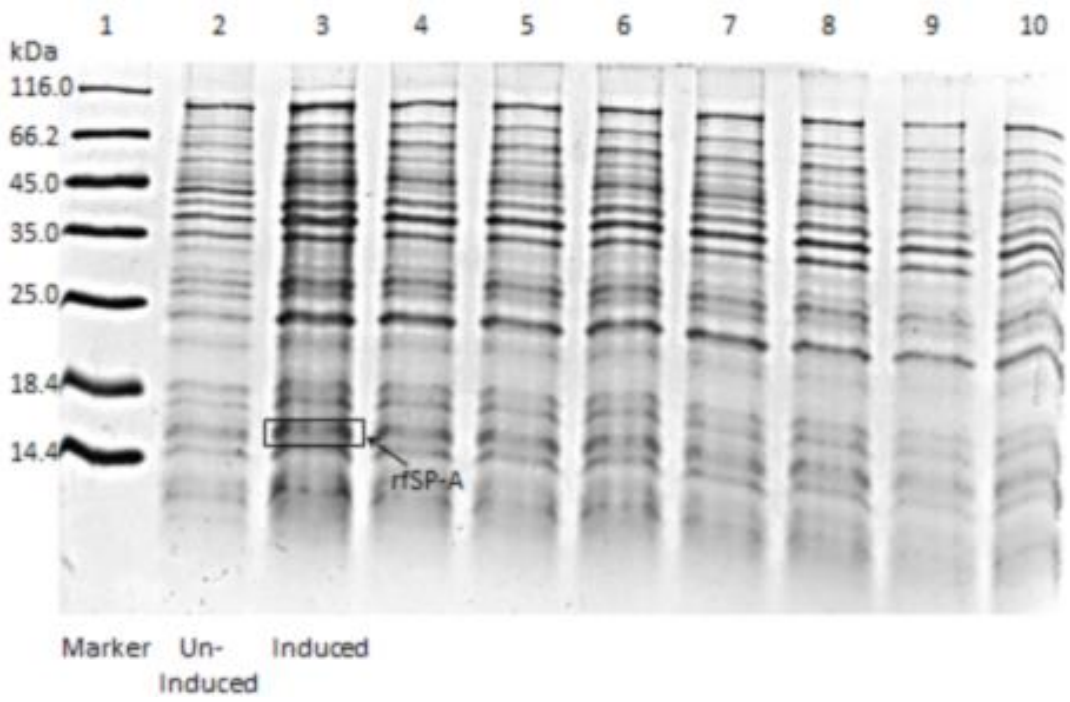



***Figure 3.18 – SDS-PAGE – Pilot scale expression of recombinant fragment SP-A in pET-101.*** *12% SDS-PAGE – Pilot scale expression of 4 colonies from 2 different plasmids. Lane 1 PM, lane 2 – Un-induced (UI), lane 3-6 colonies 1-4 from plasmid 2, lane 7-10 colonies 1-4 from plasmid 5.*

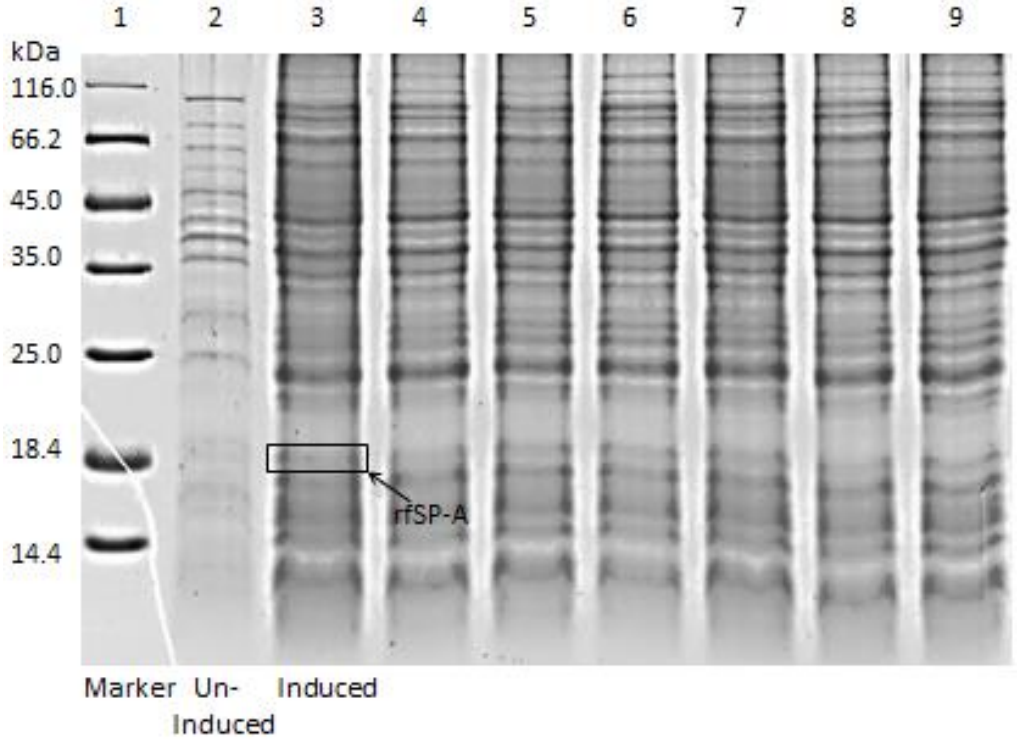



***Figure 3.19 – SDS-PAGE – Pilot scale expression of recombinant fragment SP-A in pET-101.*** *12% SDS-PAGE – Pilot scale expression of recombinant human SP-A trimeric. 3 colonies from 2 different plasmids subjected to pilot scale expression. Lane 1 PM, lane 2 – Un-induced (UI) at 0hrs, lane 3 – UI at 3hrs, lane 4-6 colonies 1-3 from plasmid 2, lane 7-9 colonies 1-3 from plasmid 5.*

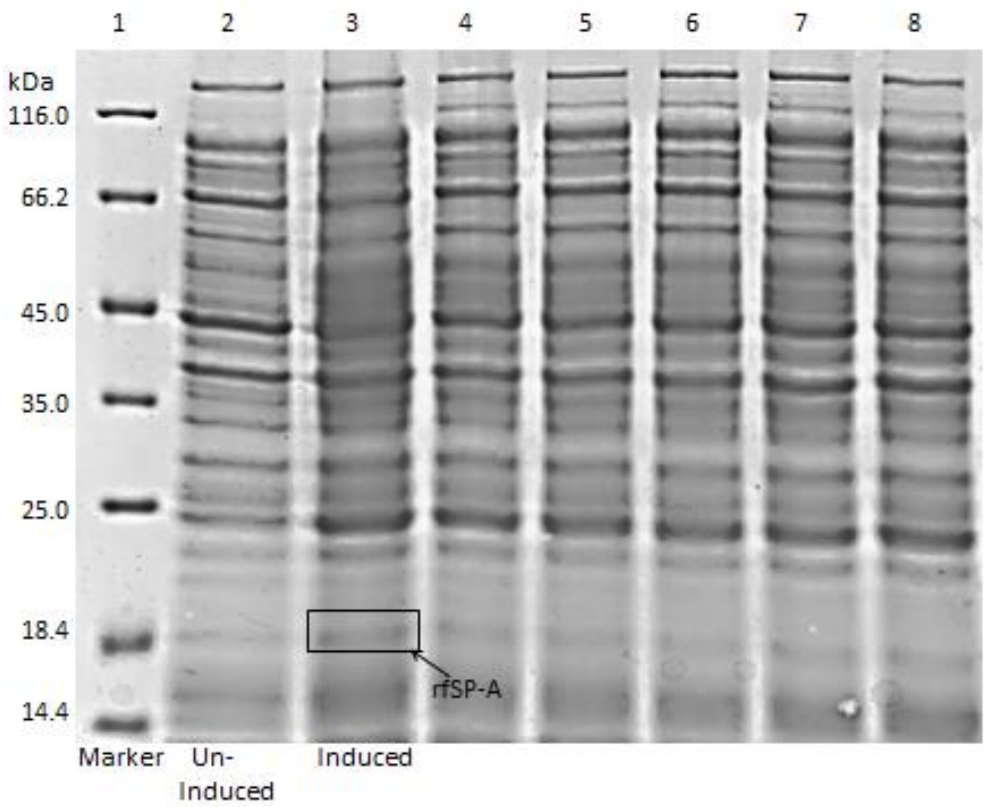



***Figure 3.20 – SDS-PAGE – Pilot scale expression of recombinant fragment SP-A in pET-101.*** *12% SDS-PAGE – pilot scale expression of recombinant trimeric SP-A (pET101/D TOPO). Lane 1 – Protein marker, lane 2 – Un-induced at 0 hrs, lane 3 – Un-induced at 3 hrs, lane 4-8 induced col.1-5.*

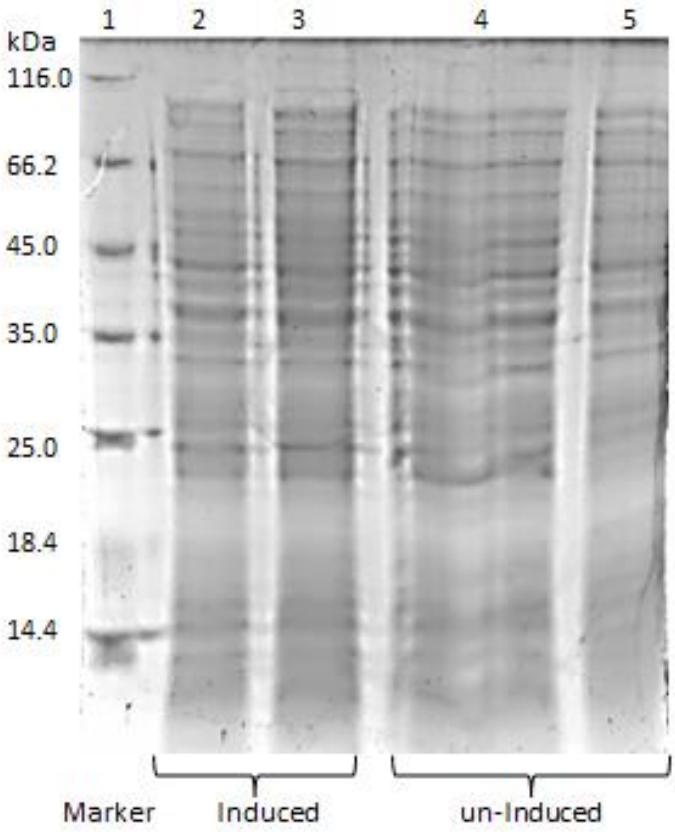



***Figure 3.21 – SDS-PAGE – for western blot to detect recombinant fragment SP-A in pET-101 after pilot scale expression.*** *12% SDS-PAGE of 2 colonies selected from pilot scale expression to detect protein expression via western blot. Lane 1 – PM, lane 2 – Induced col. 1, lane 3 – induced col.2, lane 4 – UI col.1, lane 5 – UI col.2*

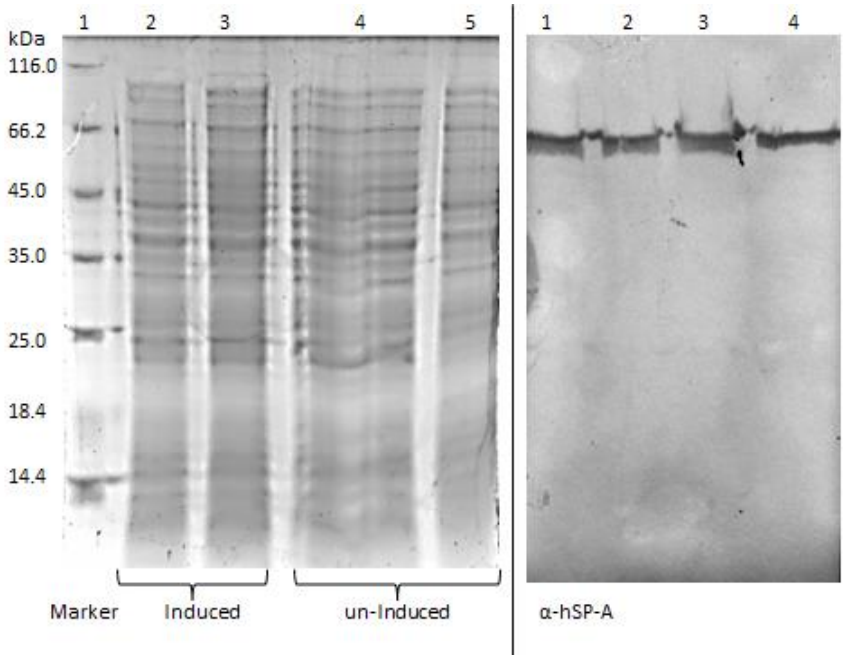



***Figure 3.22 – Western blot to detect recombinant fragment SP-A in pET-101 after pilot scale expression.*** *Western blot of 2 colonies selected from pilot scale expression to detect protein expression via western blot – lane 1 – Induced col. 1, lane 2 – induced col.2, lane 3 – UI col.1, lane 4 – UI col.2*

***Large scale expression of rfSP-A (pET101), lysis and sonication***

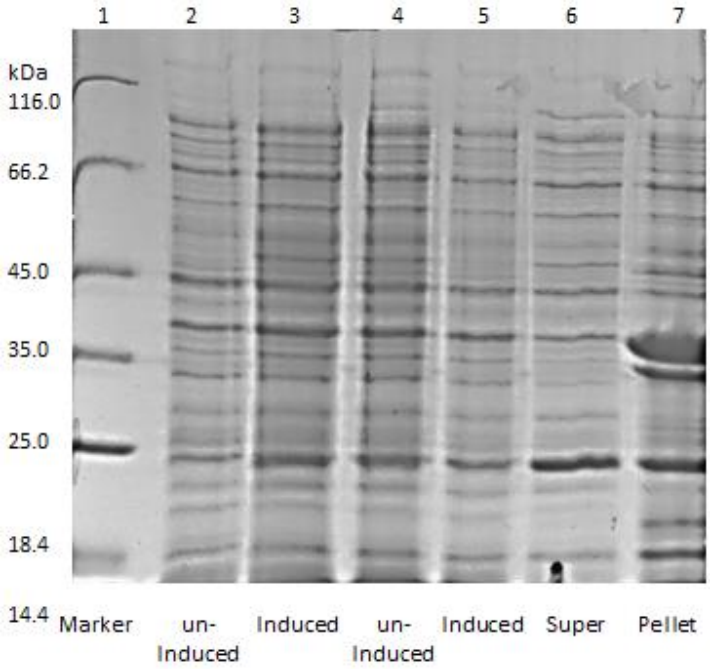



***Figure 3.23 – SDS-PAGE – large scale expression of recombinant fragment SP-A in pET-101 and after lysis and sonication of proteins from inclusion bodies.*** *12% SDS-PAGE – Large scale expression of rfSP-A and lysis/sonication of protein from inclusion bodies. Lane 1 –PM, lane 2 – UI bacth 1, lane 3 – Induced batch 1, lane 4 – UI batch 2, lane 5 – Induced batch 2, lane 6 – supernatant after lysis and sonication, lane 7 pellet after lysis and sonication.*

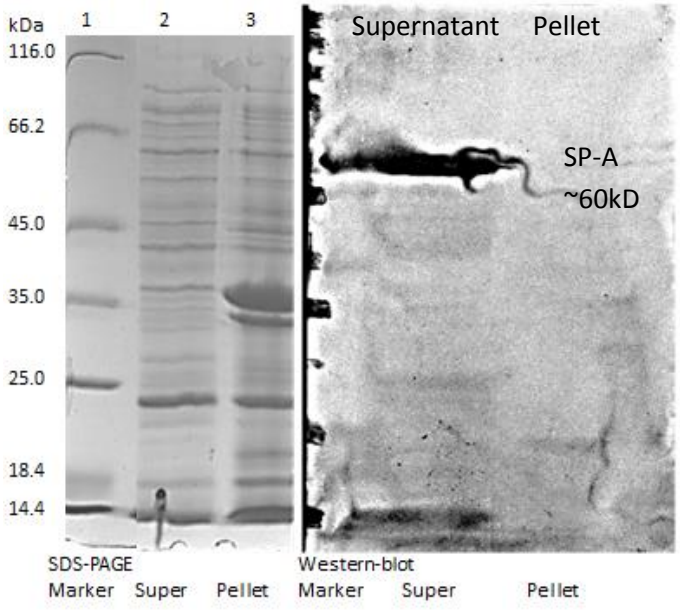



***Figure 3.24 – Western blot – detection of recombinant fragment SP-A in pET-101 after lysis and sonication of proteins from inclusion bodies.*** *Westernblot after lysis and sonication of rfSP-A (pET101). After transfer, membrane was blocked in 5% non-fat milk powder/PBS overnight at +4°C. the next day the membrane was incubated in primary antibody; rabbit anti human SP-A (1:1000) for 1.5hrs at room temperature, after washing 3times in PBS tween 20, 0.05%, secondary antibody; Protein A (1:1000) was added and incubated for 1hour. After washing, DAB tablets dissolved in water were used to detect for SP-A. Protein was detected in supernatant.*

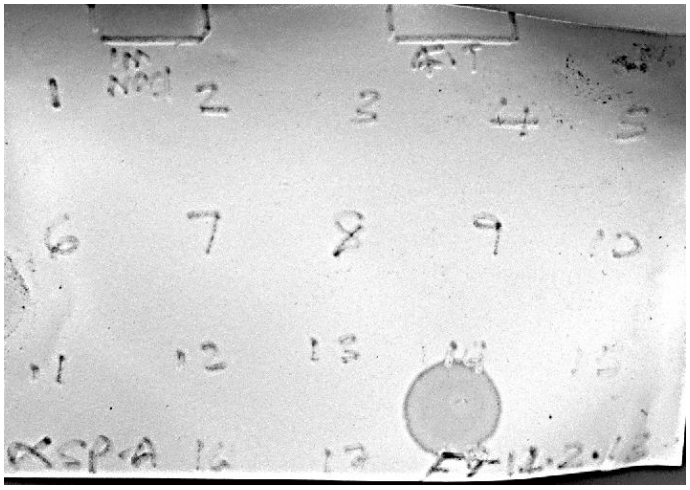



***Figure 3.25 – dot blot – detection of purified recombinant fragment SP-A in pET-101.*** Dot blot of pure rfSP-A protein. No protein was detected in the fraction except the flow through, suggesting protein was degraded and was unable to bind to the column or there is non-specific binding by protein A to other contaminants in the flow through.

### 3.3.4 Cloning and Expression of human trimeric SP-A in pMal-c2 - *Expression of soluble recombinant fragment SP-A (rfSP-A) in pMal-c2 – MBP fusion system*

Neck and CRD of SP-A was cloned into pMal-c2 to create a fusion protein with MBP as this system provided a higher yield and makes the proteins soluble for the ease of purification. After amplifying the gene of interest (Figure. 3.29) and cloning into pMal-c2 at site EcoRI and BamHI, the transformed cloned plasmids were subjected to restriction digest to verify presence of gene of interest. The fragment dropped at 444 bp (Figure.3.30) and expressed the fused MBP-SP-A protein at 60 kDa (Figure.3.31-3.33). Proteins were purified with amylose resin and eluted with 5mM EDTA. Fused proteins expressed at 60 kDa and MBP alone expressed at ~43 kDa (Figure.3.35) as seen in the eluted samples. To separate the fused proteins, factor Xa protease was added at 1µg per µg of protein in a 50 µl reaction at room temperature for at least 6 hours to separate MBP from SP-A (Figure.3.36). The cleaved protein was washed through amylose resin allowing MBP to bind and SP-A to be collected in the flow through (Figure.3.37).

***Digestion of pMal-c2 and PCR gel extracted product***

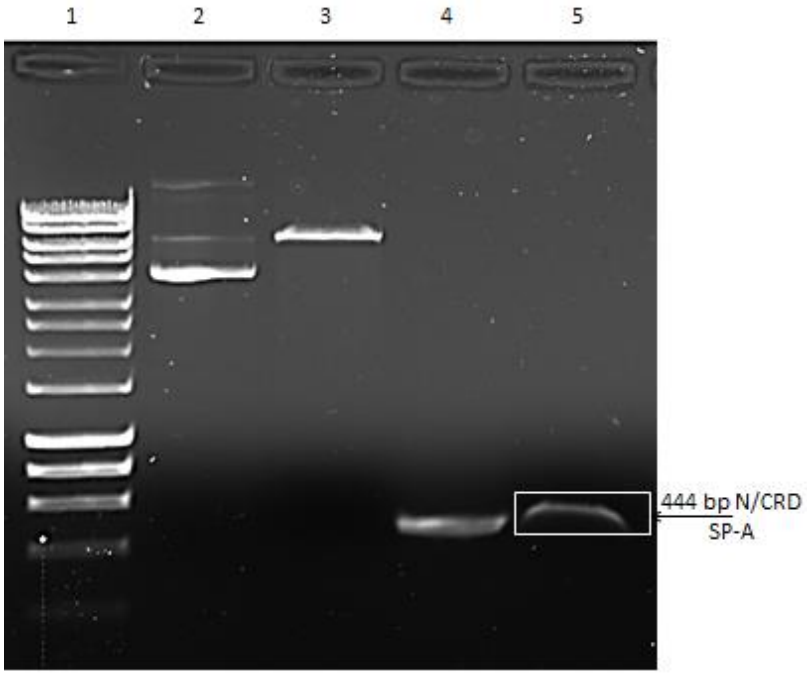



***Figure 3.26 – cut and uncut vector pMal-c2 and digested and undigested SP-A gene fragment.*** *0.7% agarose gel –Lane 1 hyperladder I, Lane 2 – pMal-c2 uncut (10ul), lane 3 – pMal-c2 cut E/B – 30ul, lane 4 –SP-A tri after PCR – 10ul, lane 5 – SP-A tri after PCR and gel extracted and digested E/B – 30ul*

### *Agarose gel after digestion of 3 plasmids*

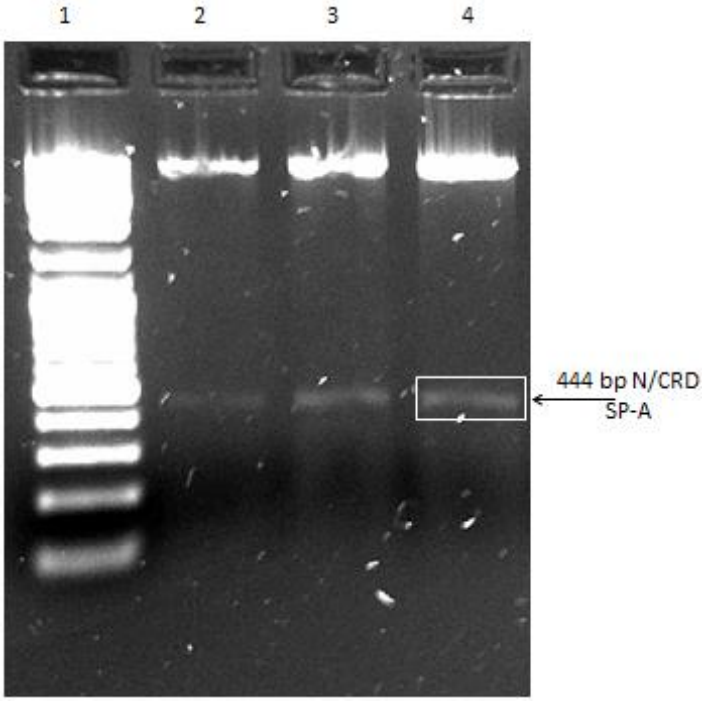



***Figure 3.27 – Digested MBP-SP-A from pMal-c2 with EcoRI and SaCI to check for gene of interest.*** *1% agarose gel – SP-A is 444bp which can be seen against the DNA marker (Peq DNA ladder 100 - 10,000 bp) has been digested with EcoRI and SacI.*

### *Pilot scale expression of MBP-SP-A after transforming in TOP10 competent cells*

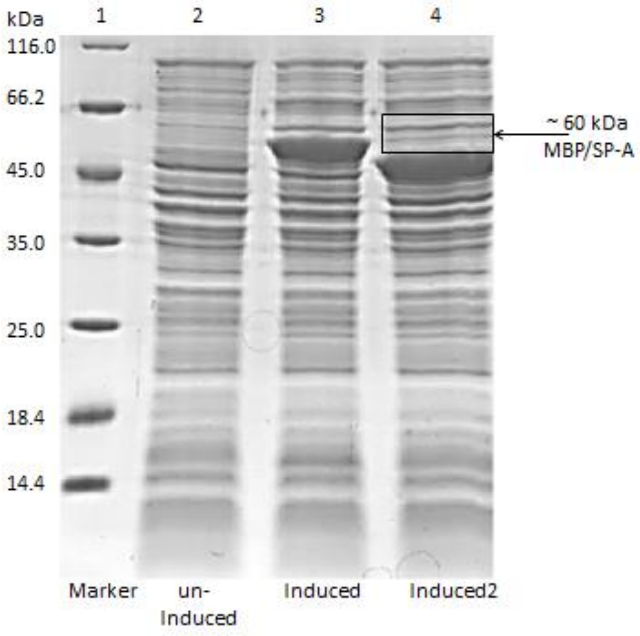



***Figure 3.28 – SDS-PAGE – Pilot scale expression of MBP fused SP-A after transformation in TOP10 E.coli.*** *12% SDS-PAGE – pilot scale expression of MBP-SP-A in TOP10. Lane 1 – PM, lane 2 UI, Lane 3-4 – col.1-2 induced with 0.5mM IPTG. Estimated size at 60kDa.*

***Pilot scale expression of recombinant fragment SP-A (pMal-c2)***

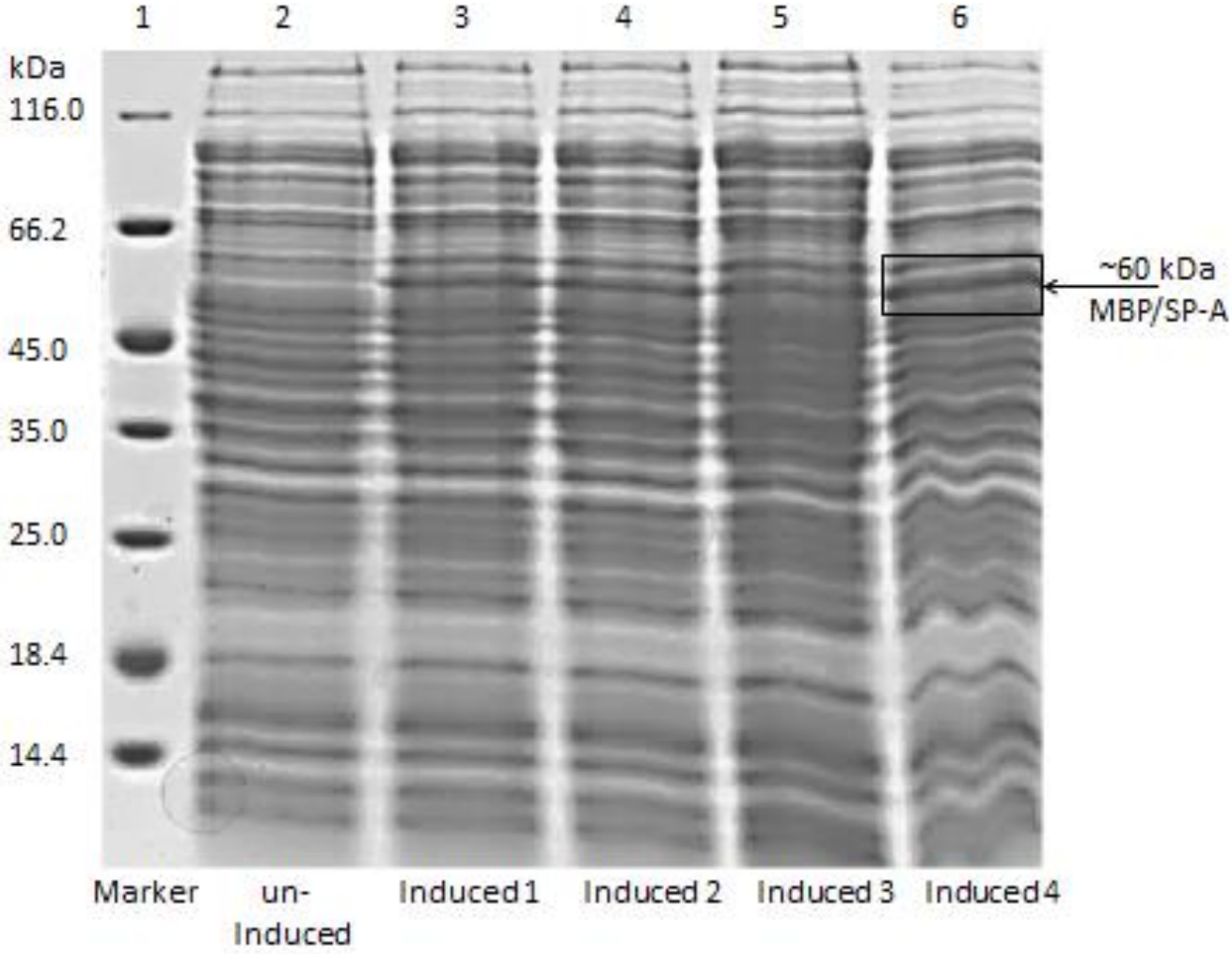



***Figure 3.29 – SDS-PAGE – Pilot scale expression of MBP fused SP-A after transformation in BL21 (λDE3).*** *12% SDS-PAGE – pilot scale expression of MBP fused SP-A. lane 1 – protein marker, lane 2 – PSE Un-Induced col.1 – plasmid 1, lane 3 – PSE Induced col.1 – plasmid 1 , lane 4 – P SE Induced col.2 – plasmid 1, lane 5 – PSE Induced col.1 – plasmid 2, lane 6 – PSE Induced col.2 – plasmid 2.*

***Large scale expression and purification of fusion proteins – SP-A-tri-MBP***

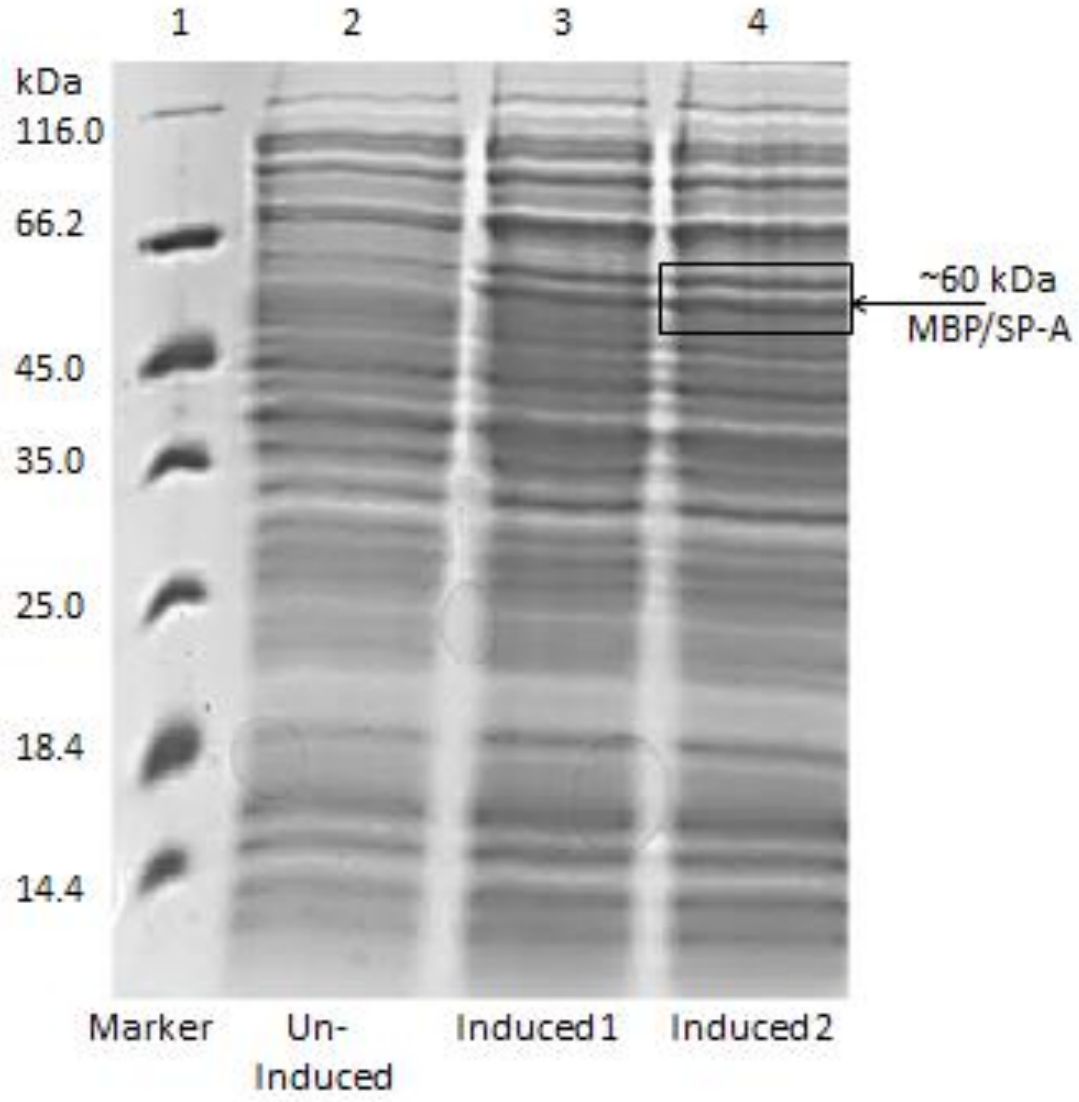



***Figure 3.30 – SDS-PAGE – large scale expression of MBP fused SP-A.*** *12% SDS-PAGE – large scale expression of MBP-SP-A. lane 1 – PM, lane 2 –un-induced MBP-SP-A, lane 3-4 – induced batch 1 and 2 large scale expression.*

**Affinity chromatography purification of MBP-SP-A**

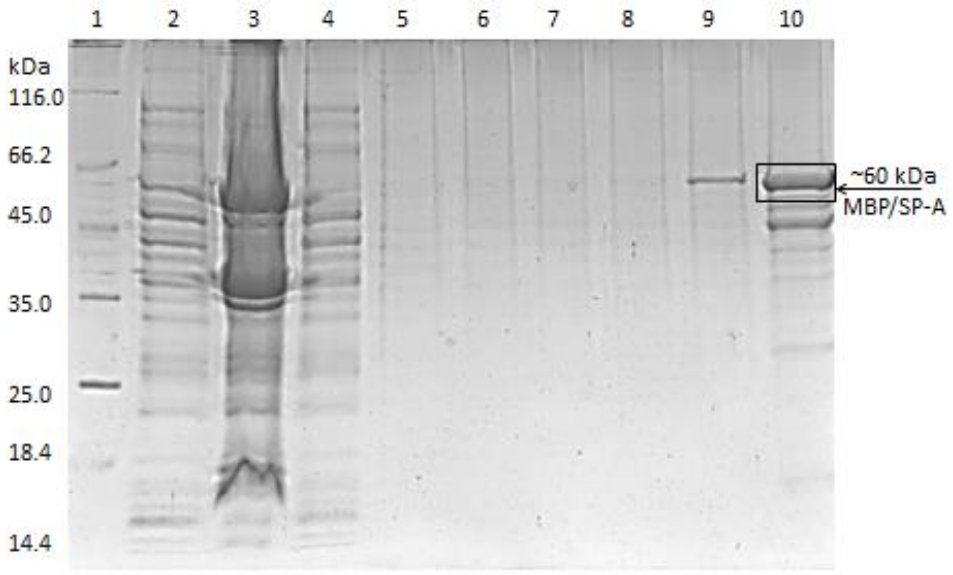



***Figure 3.31 – SDS-PAGE – Lysis, sonication and purification of MBP fused SP-A.*** *12% SDS-PAGE. Lane 1 PM, Lane 2 – diluted supernatant with column buffer, lane 3 – pellet after lysis and sonication, lane 4 – flow through, lane 5 – 1M NaCl wash, lane 6-10 fractions 1-5.*

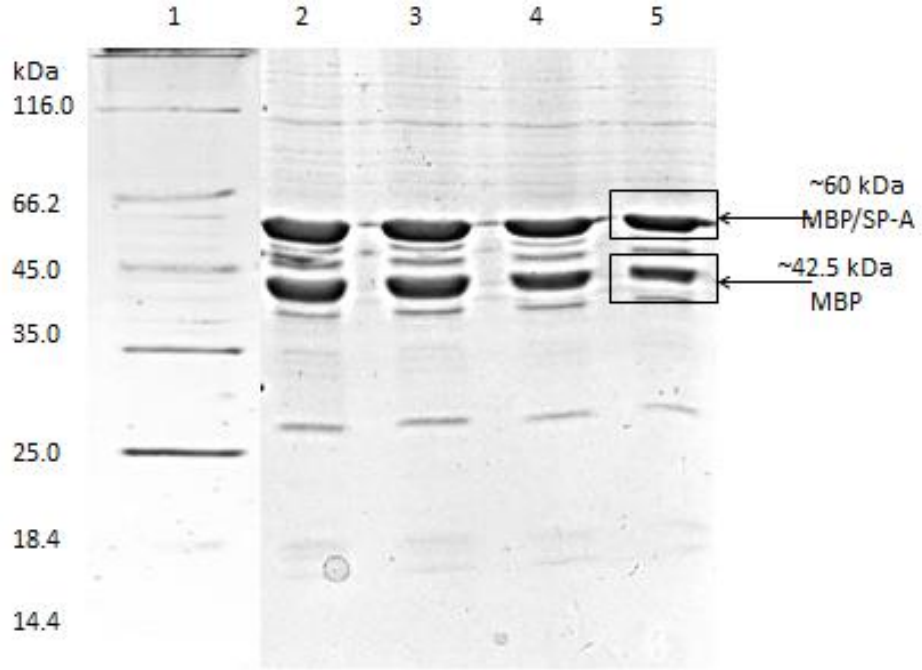



***Figure 3.32 – SDS-PAGE – Purified fractions of MBP fused SP-A through maltose-agarose column. 12% SDS-PAGE of purified fractions of MBP-SP-A.*** *Lane 1 PM, Lane 2-5 – fractions 6-9.*

## Cleavage of MBP with FactorXa

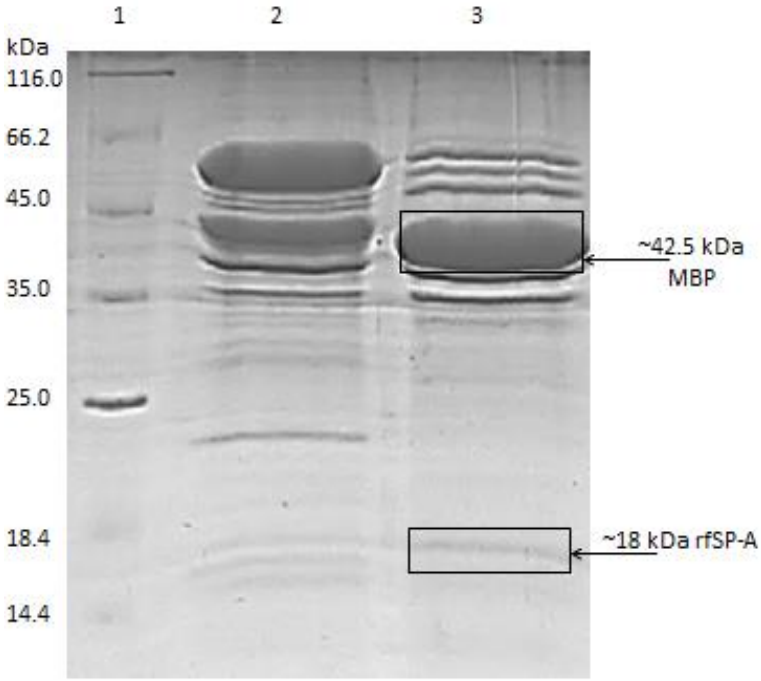



***Figure 3.33 – SDS-PAGE – MBP fused SP-A cut with Factor Xa protease, before and after cleavage.*** *12% SDS-PAGE – MBP-SP-A after cleavage with Factor Xa protease. MBP is seen at 42.5kDa and SP-A is approximately 16-17kDa. Lane 1 – PM, lane 2 – Uncut MBP-SP-A tri, lane 3 – Cut MBP-SP-A tri.*

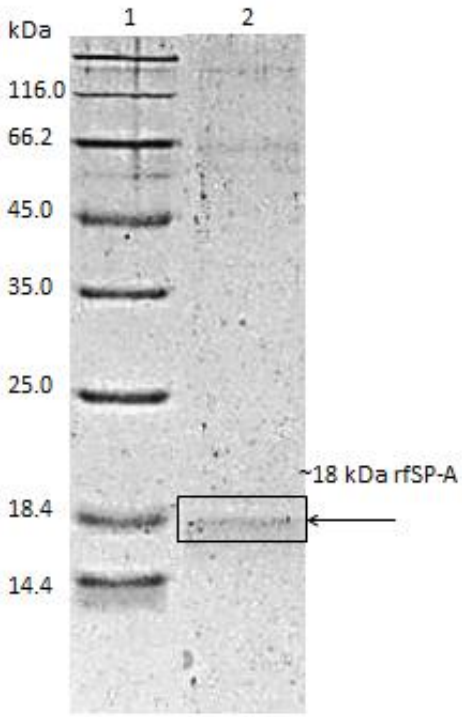



***Figure 3.34 – Purified rfSP-A through maltose-agarose after cleavage from MBP with Factor Xa.*** *12% SDS-PAGE of SP-A separated from MBP after cleavage with Faxtor Xa and purification via maltose-agarose.*

### *3.3.5 Expression of insoluble recombinant fragment SP-D (rfSP-D) in BL21 (λDE3) pLysS*

Neck and CRD region with 8 Gly-Xaa-Yaa repeats from the collagen region were expressed in pET-3b at NdeI/HindIII restriction sites under the T7 promoter system. The ampicillin resistant plasmid was transformed into BL21 (λDE3) pLysS and induced at log phase for expression of gene of interest (Figure.3.38). The protein expressed well in 2 out the 3 colonies which was subjected to extraction from inclusion bodies. The insoluble proteins in inclusion bodies were lysed and sonicated to extract the over-expressed proteins from inclusion bodies and were collected in the pellet after another round of centrifugation (Figure.3.39). The pellet of proteins was solubilised with urea at 8M concentration and via step-wise dialysis the proteins were re-folded and purified via affinity chromatography using maltose-agarose matrix (Figure.3.40-3.41). Approximately 5 mg per 1L of culture was retrieved.

***Expression of rfSP-D (pUK-D1)***

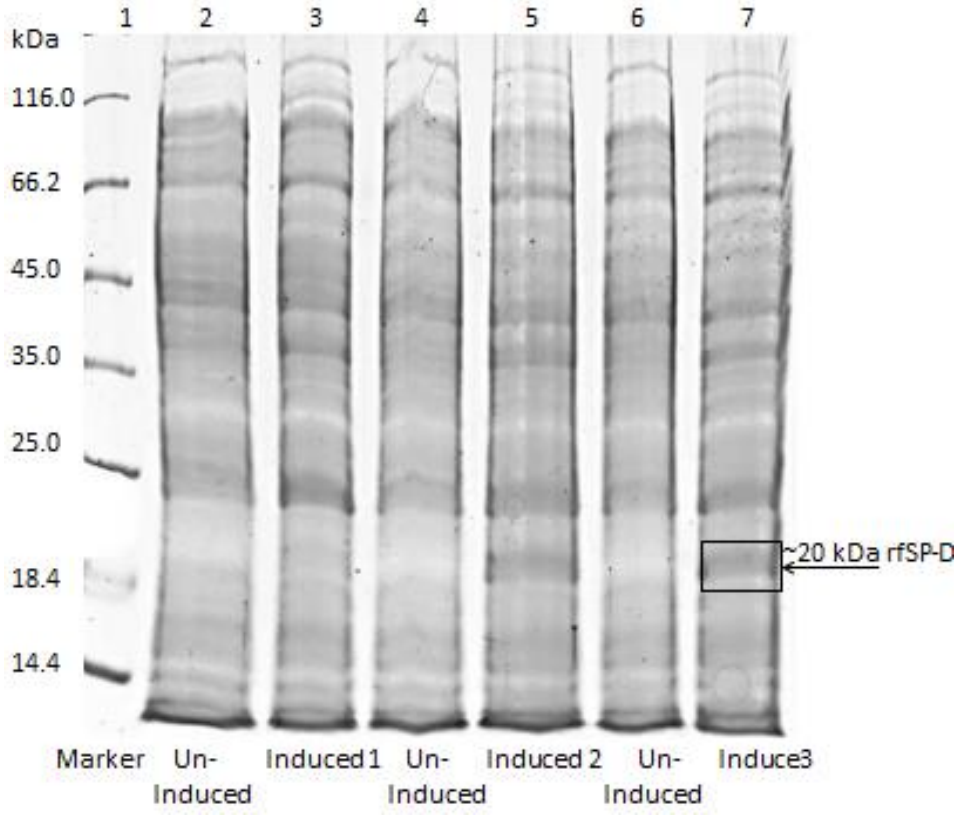



***Figure 3.35 – large scale expression of rfSP-D.*** *12% SDS-PAGE – 3 colonies selected of rfSP-D expressed in 1L batches. Protein expressed at ~20kDa. Lane 1 – protein marker, lane 2 – un-induced, lane 3 – Induced, lane 4 – un-induced, lane 5 – Induced, lane 6 – un-induced, lane 7 – Induced.*

### *Lysis and sonication of rfSP-D*

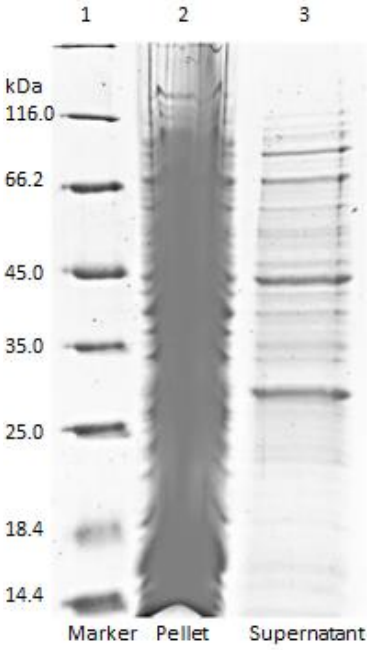



***Figure 3.36 – Lysis and sonication of rfSP-D (pUK-D1).*** *12% SDS-PAGE – proteins in inclusion bodies were lysed and sonication. After centrifugation, insoluble proteins were visible in the pellet at ~20kDa. Lane 1 – protein marker, lane 2 – pellet after lysis and sonication, lane 3 – supernatant after lysis and sonication.*

### *Purification of rfSP-D*

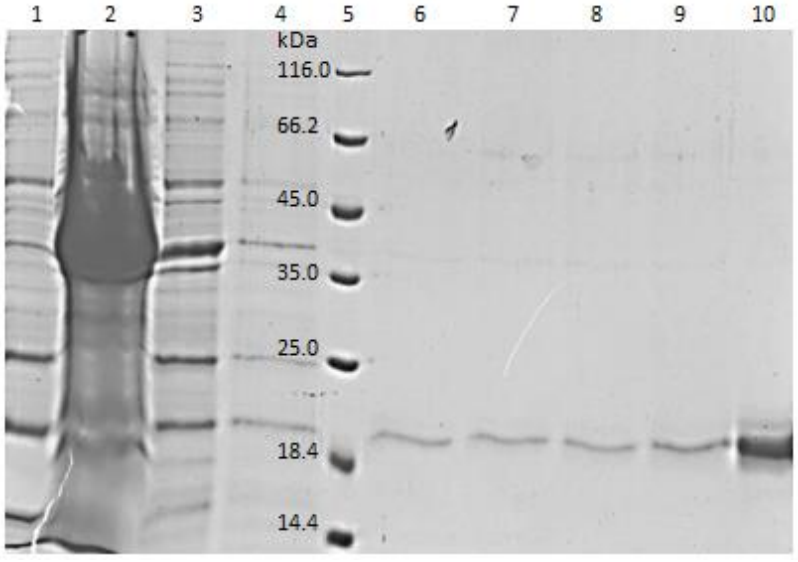



***Figure 3.37 – purification of rfSP-D (pUK-D1) via affinity chromatography.*** *12% SDS-PAGE – rfSP-D (pUK-D1) was purified through maltose-agarose matrix by affinity chromatography eluted with 5mM EDTA. Purified proteins are seen at 20kDA. Peak fraction is fraction 5. Lane 1 – supernatant before purification, lane 2 – pellet before purification and after dialysis, lane 3- flow through, lane 4 – flow through after 1M NaCl wash, lane 5 – protein marker, lane 6-10 – fraction 1-5.*

***Purified rfSP-D***

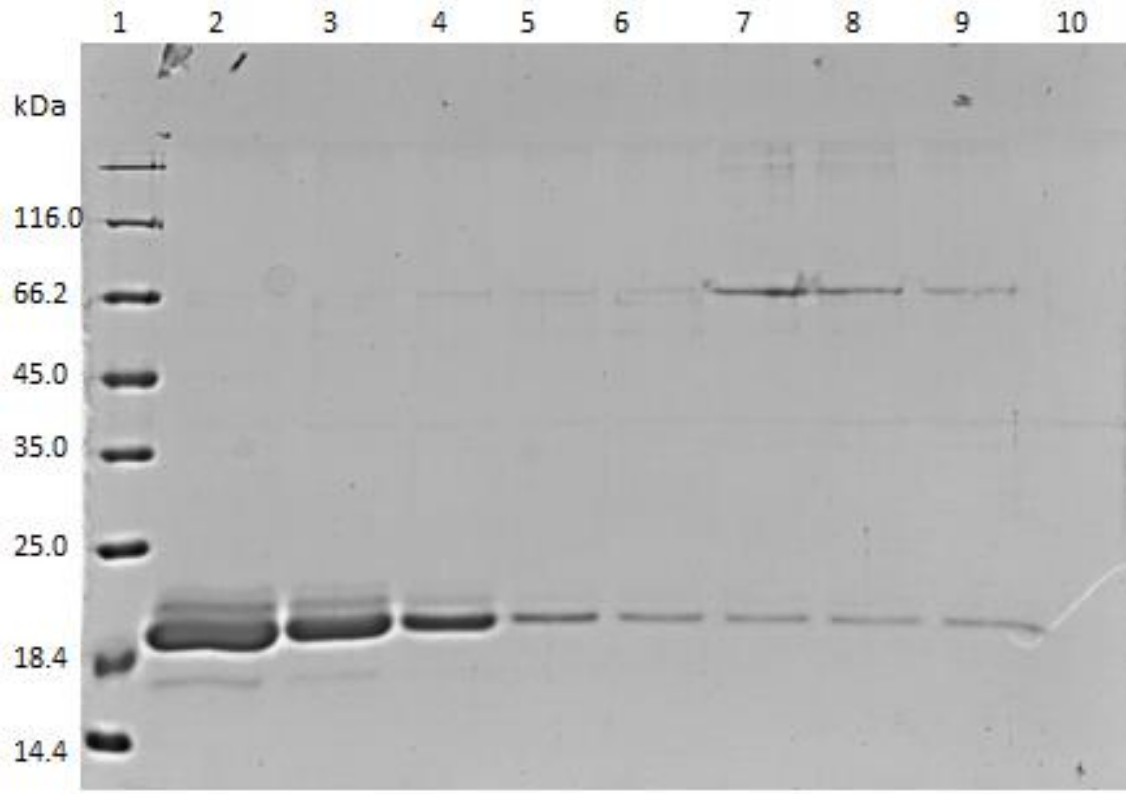



***Figure 3.38 – purification of rfSP-D (pUK-D1) via affinity chromatography.*** *12% SDS-PAGE – rfSP-D (pUK-D1) was purified through maltose-agarose matrix by affinity chromatography eluted with 5mM EDTA. Purified proteins are seen at 20kDA. Peak fractions include fraction 6-8. Lane 1 – protein marker, lane 2-10 – fractions 6-13*

### *3.3.6 Expression of insoluble recombinant fragment SP- A (rfSP-A) in BL21 (λDE3) pLysS*

Neck and CRD region of human SP-A was expressed in pET-3b at NdeI/HindIII restriction sites influenced under the T7 promoter system. The ampicillin resistant plasmid was transformed into BL21 (λDE3) pLysS and induced at log phase for expression of gene of interest (Figure.3.42). The protein expressed well in both colonies, expressed at ~17 kDa which were subjected to extraction from inclusion bodies (Figure.3.42). The insoluble proteins in inclusion bodies were lysed and sonicated to extract the over-expressed proteins from inclusion bodies and were collected in the pellet after another round of centrifugation (Figure.3.43). The pellet of proteins was solubilised with urea at 8M concentration and via step-wise dialysis the proteins were re-folded and purified via affinity chromatography using maltose-agarose matrix (Figure.3.44). Approximately 1 mg per 1L of culture was retrieved. The proteins were characterised by trimerization assays which displayed monomers, dimers and trimers of recombinant fragment proteins SP-A and SP-D (figure 3.45-3.46).

***Expression and purification of rfSP-A (pET-3b)***

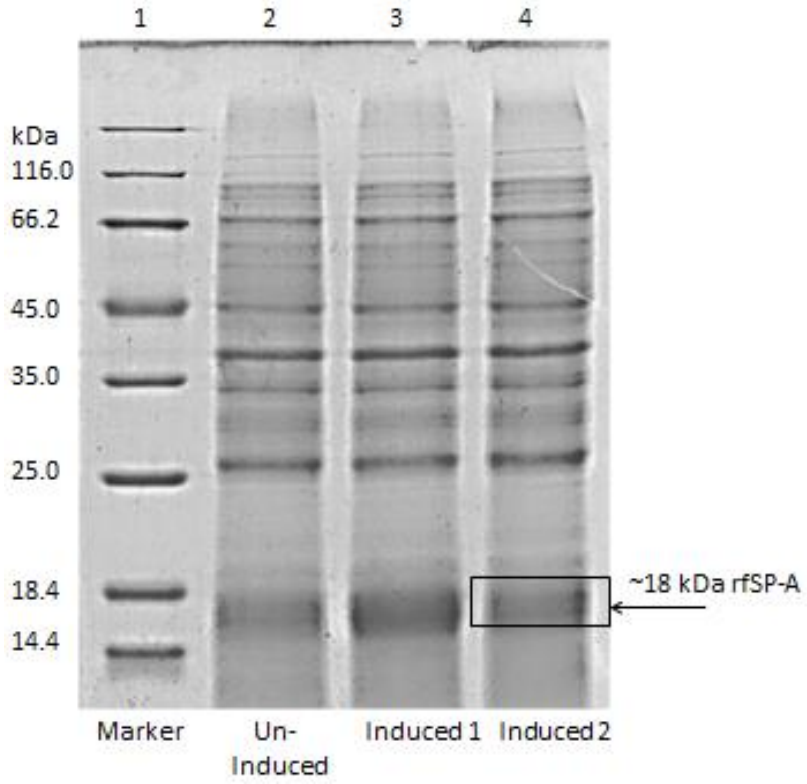



***Figure 3.39 – large scale expression of rfSP-A (pUK-A1 in pET-3b).*** *12% SDS-PAGE – 2 colonies selected of rfSP-D expressed in 1L batches. Protein expressed at ~17kDa. Lane 1 – protein marker, lane 2 – un-induced, lane 3 – Induced, lane 4 – induced.*

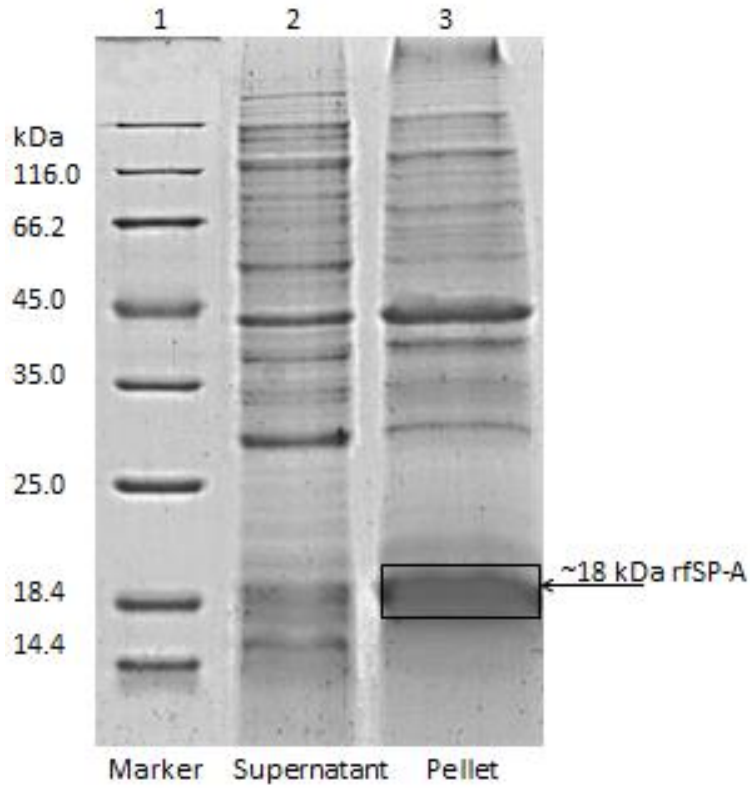



***Figure 3.40 – lysis and sonication of rfSP-A (pUK-A1).*** *12% SDS-PAGE – Protein expressed at ~17kDa. Lane 1 – protein marker, lane 2 – supernatant after lysis and sonication, lane 3 – pellet after lysis and sonication.*

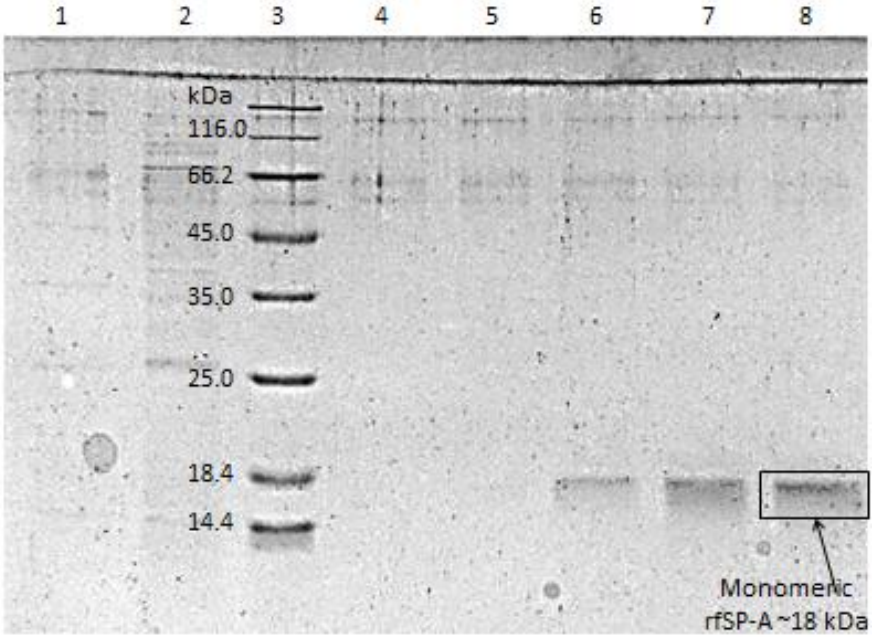



***Figure 3.41 – purification of rfSP-A (pUK-A1) via affinity chromatography.*** *12% SDS-PAGE – rfSP-A (pUK-A1) was purified through maltose-agarose matrix by affinity chromatography eluted with 5mM EDTA purified proteins are seen at ~17kDA. Peak fractions are at 3-5. Lane 1 – flow through, lane 2 – flow through after 1M NaCl wash, lane 3 – protein marker, lane 4-8 – fractions 1-5.*

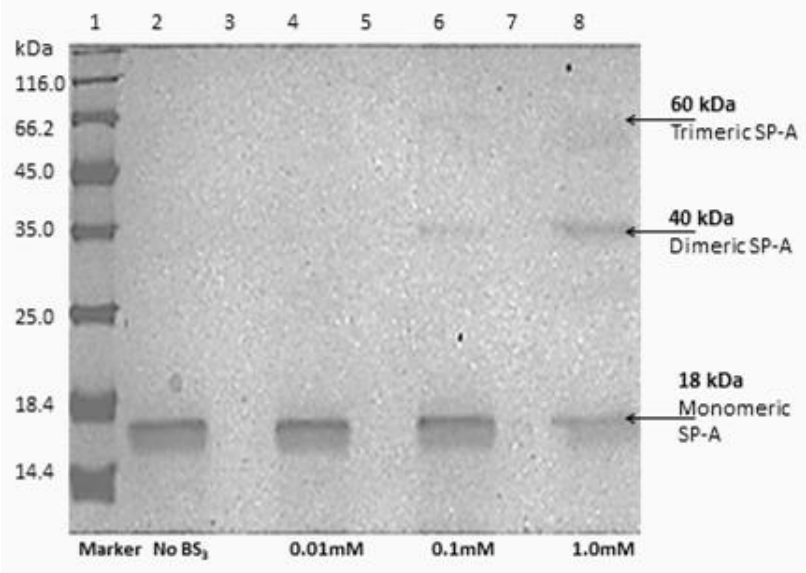



***Figure 3.42 – Trimerization of rfSP-A using chemical cross-linker BS3 and SDS –PAGE analysis – SDS-PAGE 15 % (w/v) gel under reducing conditions after BS$^3$ cross linking and stained with coomassie blue.*** *5µl of 0.01mM, 0.1mM and 1.0mM concentration of BS$^3$ was incubated with 45 µl of the dialysate for 1 min, 2 mins and 4 mins at room temperature. The cross-linking reaction was separated by electrophoresis on a 15 % (w/v) SDS-PAGE gel under reducing conditions after adding equal volume of treatment buffer at each time point and stored at room temperature. After all reactions were complete and treatment buffer was added, the samples were boiled together at 95$^0$C for 10 minutes. Upon reaction with cross liking agent at various incubation time higher oligomers such as monomer (18 KDa), dimer (~40 KDa), and trimer (~60KDa) were seen. Lane 1 – protein marker, Lane 2 - No BS3 + protein, Lane 4 - 0.01mM + protein, Lane 6 – 0.1mM + protein, Lane 8 – 1.0mM BS3 + protein incubated for 4mins.*

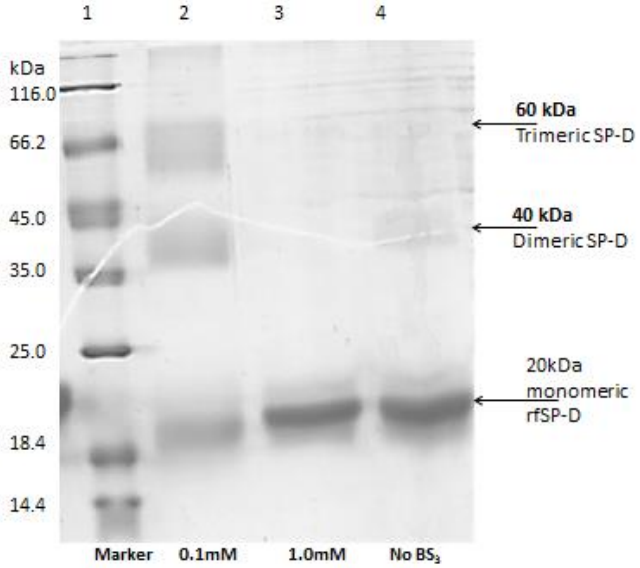



***Figure 3.43 – SDS–PAGE (12% w/v) bis (sulfosuccinimidyl) suberate (BS3) cross-linking of recombinant fragment of human SP-D containing trimeric neck + CRD region (rfSP-D).*** *Treatment of rfSP-D with two different concentrations of BS3: 0.1mM (lane 2), 0.01mM (lane 3) and 1mM (lane3) analysed against protein containing no BS3.*

### *3.4 Discussion*

SP-A and SP-D are synthesised within the alveolar type II cells in the foetal lungs and are secreted into the surrounding amniotic fluid making it a good source for purification of SP-A and SP-D. SP-A and SP-D are also found in large amounts in the bronchoalveolar lavage (BALF) of alveolar proteinosis patients (APP). APP patients have shown to have either GM-CSF deficiencies or anti-GM-CSF neutralising antibodies. In this disease there is a dysregulated secretion of a number of pulmonary proteins including SP-A and SP-D. Although the occurrence of this disease and availability in the clinic are quite rare, lung washings from APP patients provide an extra-ordinary good source of extracting SP-A and SP-D as compared to amniotic fluid. Most of the SP-A remains bound to the phospholipid pool and thus goes to the pellet following centrifugation, thus the supernatant becomes a good source for purifying SP-D while the pellet requires butane extraction or denaturation/renaturation procedures using chaotropic agents such as urea and guanidine hydrochloride. While SP-D can be recovered from the supernatant using affinity column chromatography, the refolded material in solution out of phospholipid pellet offers a generous amount of SP-A.

Because of the low levels of SP-A and SP-D in amniotic fluid and rarity of APP patients visiting the anaesthesia award it was considered empirical to express full length SP-A in mammalian cells. Thus the use of HEK cells was designed to maximise the post-translational features of SP-A. After cloning the entire open reading frame of human SP-A1 in pSecTagC, the transfection and transient expression system was established. This protein present in the supernatant and after purification appeared to retain the biological properties of SP-A. Although the yield of the protein was on the lower scale for HEK expression system this offers opportunities to examine mutational analysis of the collagen, neck and CRD regions of human SP-A.

We decided to opt for mammalian expression system to express and purify human SP-A1. Mammalian system was chosen for expressing the full length protein because good quality and biologically active SP-A would require post-translational modification of collagen regions for refolding of the triple helical structure which cannot be achieved in baculovirus or *E. coli.* We chose to opt for mammalian HEK cells against CHO cells as HEK cells are the closest to human environment for protein production.

In order to localise the domains of human SP-A and SP-D which are most likely to interact with the cells of amnion, chorion and decidua, we considered it important to express homotrimeric fragments of neck and CRD regions of human SP-A and SP-D in *E. coli*. To this effect bacteriophage T7 promoter system was used to express both proteins in BL21 (λDE3) pLysS. Although this expression system yielded into inclusion bodies cytoplasmically, denaturation and renaturation procedure using a gradient of urea helped to recover a good amount of the trimers of SP-A and SP-D C-type lectins. When the construct containing neck and CRD regions of SP-A and SP-D were transformed in to DE3 no expression was observed (data not shown), it is likely, that the 2 human protein fragments were being toxic to E. coli cells, thus, we used pLysS strain which offers a tight regulation on T7 promoter by virtue of producing T7 lysozyme cloned on a pACYC plasmids (low copy number plasmid). T7 lysozyme thus acts as an inhibitor of T7 RNA polymerase. Both proteins went to insoluble inclusion bodies following cell lysis, sonication and high speed centrifugation. Proteins expressed using pLysS system did not require any lysozyme to lyse the bacterial cells. After following a dialysis gradient starting from 6M urea, 4M, 2M, 1M and no urea, the soluble proteins were recovered in the supernatant. Only 10% of soluble fraction bound to the maltose-affinity column suggesting that although refolding protein produced soluble trimmers only a small proportion was intact in terms of three-dimensional structures, thus it bound to affinity column. The final yield of 2-5mg/L was considered sufficient for further biochemical characterisations and biological assays. Since these proteins were meant to be examined on ACD explants and decidual macrophages it was important that most LPS content was removed. This was achieved as validated by measurement of endotoxin level by amoebocyte lysate method. As the literature suggests, a small amount of LPS contamination can skew the balance of macrophage response towards an excess of TNF-α production.

Since the α-helical coiled-coiled neck region is considered to be the nucleation centre for the trimerization of C-type lectin domains of SP-A and SP-D, we also confirmed that trimerising properties of affinity purified homotrimeric rfSP-A and rfSP-D. It was possible to see a gradient of monomer, dimer and trimer in a dose and time dependent manner when $BS^3$ was used as a chemical cross-linker. This also established the homogeneity of the finally purified recombinant fragments. The investigation also

passed through a phase when neck and CRD region of human SP-A were cloned using pET-3b and pET-101, however for a some strange reason the one cloned in pET-101 didn't express very well and the rfSP-A in pET-3b expressed very poorly. To expedite the process of further experimentation, an interim construct expressing neck and CRD region of neck and CRD region of SP-A was cloned downstream to E. coli maltose-binding protein (MBP). The fusion protein expressed under the Ptac promoter comprised of MBP, factor Xa cleavage site and the SP-A. The fusion protein was soluble intracellularly and non-toxic to the host cell. It was possible to separate the rfSP-A away from MBP through factor Xa cleavage. This construct may be quite useful as an affinity matrix where a possible receptor on the cell surface can be passed through this column. The fusion protein can also be used to target CRD ligands of SP-A using anti-MBP antibodies in situations where anti-SP-A antibodies interfere with SP-A ligand interactions.

Thus, this chapter describes the procedures involved in purifying human SP-A and SP-D and expressing full length and truncated recombinant proteins. These products were used in subsequent studies.

# Chapter 4:

# Immunomodulatory effects of SP-A and SP-D on amnion, chorion and decidua explants derived from normal term placentae

## Summary

The placenta hosts an allogeneic fetus allowing a genetically different individual to coexist in the mother. The ambiguous nature of how the mother can sustain an allograft is not clearly understood yet it is tolerated for approximately 9 months. As the initiation of labour is not clearly understood, the mechanism to control the timing of birth is not well established. During fetal maturation, the lungs, secrete fetal surfactant proteins SP-A and SP-D into the amniotic fluid and may signal for fetal maturation. Near term, the levels of SP-A and SP-D have been shown to increase, with SP-A levels surging just before labour. During labour, the levels decrease dramatically, suggesting the levels of SP-A and SP-D could travel to maternal tissues influencing the inflammatory pathway to labour. We propose that SP-A and SP-D could modulate the immune pathway in controlling the inflammatory response through interaction with decidual macrophages and suppressing the cytokine profile and prostaglandin production.

In this chapter, cultured amnion, chorion and decidua (ACD) explants from term placentae were exposed to IL-1β with and without SP-A and SP-D. Targets chosen for QPCR are PTGS2, PTGFRN, TLR2, TLR4, IL-8, IL-10, TNF-α, TGF-β, C1q, Factor H (FH), SP-A and SP-D which are linked in the immune pathway to analyse if SP-A and SP-D modulate a pro inflammatory or anti-inflammatory response. For protein analysis, ELISAs were carried out to examine the changes in C1q, FH, SP-A, SP-D and PTGF2a levels to see if SP-A or SP-D treated explants affect the pro- and anti-inflammatory pathways.

## 4.1 Introduction

### *4.1.1 Immunomodulations of pregnancy and labour*

Each year, there are approximately 15 million babies worldwide born preterm which is the leading cause of neonatal mortality and morbidity in developing countries (Blencowe et al., 2012). Preterm birth, defined as birth before 37 weeks gestation is caused by infection in 20-30% of cases, premature rupture of membranes (PROM) in 25-30% of cases and 40-45% of cases are idiopathic (Romero et al., 1988, Green et al., 2005, Goldenberg et al., 2008). Mice models have been used to identify a cellular and molecular pattern in preterm and term labour (Hirsch and Wang, 2005, Condon et al., 2004). Although infection and spontaneous birth lead to the same end result of contraction and birth, the initiating initial events are distinct. The initiation of labour from an immunological and endocrinological perspective is multifactorial. However, both term and preterm births are associated with inflammatory markers such as IL-1β, TNF-α, IL-8 and IL-6. We have used IL-1β as a stimulant of the inflammatory pathway and examined the levels of TNF-α at a transcriptional levels and both TNF-α and IL-8 at a protein level in maternal and fetal tissues (Cox et al., 1997). This occurs during macrophage and neutrophil infiltration (Thomson et al., 1999, Osman et al., 2003) within fetal membranes, cervix, decidua and myometrium (Osman et al., 2003). The inflammatory onset triggers activation of pro-inflammatory transcription factors such as NF-κB which increases expression of contraction associated proteins (CAP) genes. Examples of CAP genes include cyclooxygenase 2 (COX2), which is critical for contraction (Mendelson, 2009). This study examines whether SP-A and SP-D affect the expression of this gene and expression of the potent prostaglandin protein, PGF2α, a contractile protein.

### *4.1.2 SP-A and SP-D in pregnancy and labour*

The fetus appears to provide a critical signal for parturition through sequential inductions of SP-A from the fetal lung and amniotic fluid (Montalbano et al., 2013). Mice studies have recently shown that SP-A and SP-D induce a pro-inflammatory effect on activating amniotic fluid macrophages through interaction of TLR2. This increases CAP genes expression and accelerated timing of labour. On the other hand, mice deficient in SP-A and SP-D have delayed parturition, especially in their second and third pregnancies. Mice deficient in TLR2 expression had significantly lower levels of SP-A

and SP-D expression. This suggests that SP-A and SP-D interaction with their receptors promote expression of pro-inflammatory cytokines to contribute to the pathway of labour (Montalbano et al., 2013). Although a huge proportion of preterm births are due to infection, studies have shown, that in cases of intrauterine infection in women amniotic fluid did not affect the levels of SP-A and SP-D expression (Chaiworapongsa et al., 2008). A recent study has shown the expression of SP-A and SP-D in amnion and choriodecidua at a protein level and transcriptional level, where higher levels of SP-A were localised in choriodecidua and SP-D was predominant in the amnion. SP-A was only expressed in the supernatant of human fetal membranes; amnion and choriodecidua (Breuiller-Fouche et al., 2010). SP-A has been shown to induce F-actin filament organisation in myometrial cells to induce stress fiber formation in the motion of contractility. This would indicate that SP-A from fetal amniotic fluid and tissues regulate the formation of myometrial stress fibers to induce contractility (Breuiller-Fouche et al., 2010). SP-A and SP-D have also been localised in the first trimester of pregnancy, and thus, are likely to be important in the protection of the fetus against infection at the early stages of pregnancy. SP-A and SP-D were localised in the trophoblastic layers of the chorionic tree, particularly in trophoblastic cell columns, stromal cells, Hofbauyer cells, angiogenic cell cords, and vascular endothelium (Sati et al., 2010). SP-A appears more apparent in syncytiotrophoblasts and trophoblasts, and moderate levels in hofbauyer cells, vascular cords and angiogenic cell cords. SP-D was apparent in low levels in angiogenic cell cords, moderate in Hofbauyer cells but was more apparent in the vascular endothelium (Sati et al., 2010). The importance of SP-A and SP-D in the protection against environmental pathogens after birth has shown of great significance in mice models. SP-A deficiency was related to high mortality compared to wild-type and SP-D deficient mice and the mortality was more associated to gastrointestinal pathologies rather than lung pathologies, and oral administration of SP-A to pups improved their survival in their bacterial-laden environment (George et al., 2008). This suggests that SP-A is more important than SP-D in the protection of the new-born against infection and levels of SP-A are important for reducing mortality. C/EBPα (transcription factor protein) has been shown to be significant for fetal lung maturation especially in the third trimester. Deficiency in the gene has shown to affect normal synthesis of SP-A and SP-D and is related to causing RDS, a common cause of morbidity and mortality in preterm infants (Martis et al., 2006). This suggests that the

gene is essential for perinatal lung maturation and surfactant homeostasis in order to allow mechanical breathing possible. Preterm infants with IUGR have revealed an increase in developing acute and chronic pulmonary disorders. Mice models have revealed that hypoxic conditions lead to preterm birth and IUGR, which is significantly altered in the development of the surfactant system (Gortner et al., 2005). SP-A and SP-D has been shown to be regulated by glucocorticoids and hydrocortisone in fetal rat experiments (Deterding et al., 1994). Human and rabbit lung cultures also show that cAMP and glucocorticoids are important for SP-A synthesis. cAMP regulates transcription of SP-A, whereas, glucocorticoids stimulate SP-A transcription and enhances mRNA stability (Mendelson and Boggaram, 1990). Levels of SP-A and SP-D appear to express mostly in the third trimester. Levels of SP-A in amniotic fluid have been shown to increase sharply from 32 weeks towards term and decrease sharply during labour whereas SP-D levels increase moderately from 32 weeks and decrease during labour (Miyamura et al., 1994). This could be due to the amniotic rupture of membrane that would release the amniotic fluid containing SP-A and SP-D.

### *4.1.3 Complement activation in pregnancy and labour*

The complement system is crucial in the innate and adaptive immune defence but, excessive activity can lead to disorders (Oksjoki et al., 2007). Animal studies suggest activation of the complement system and deposit of C3b and release of anaphylatoxins (C3a, C5a), promotes leukocyte infiltration, generating the lytic membrane attack complex (MAC). Over complement deposits leads to destruction of fetal-placental tissue ultimately resulting in fetal loss (Munn et al., 1998, Mellor et al., 2001, Xu et al., 2000, Holers et al., 2002, Caucheteux et al., 2003). However, regulatory proteins for the complement system limit complement activation (Sjoberg et al., 2009). There are evidence of recent reports of early activation of the complement system is associated with the pathogenesis to preeclampsia. Factor Bb were significantly higher in maternal serum from 20 weeks gestation in preeclamptic pregnancies (Lynch et al., 2008). This suggests the activation of the alternative pathway. However, factor H levels have been reported in high levels (Derzsy et al., 2010), suggesting alternative pathway regulation even in the presence of apoptotic cells, suggesting, Factor H:C3 ratios would be significant in the regulation of the alternative path in pregnancies to prevent the

activation of the complement cascade and cell lysis which ultimately would lead to inflammation and early onset of labour. Factor H is a complement regulator protein of the alternative pathway by promoting proteolytic degradation of C3b to prevent lysis of cells (Kemper and Hourcade, 2008). The alternative pathway takes place on cell surfaces of pathogenic microorganisms including yeast, bacteria and bacteria and can also be triggered through IgG and IgA antibody-antigen complexes (Carroll and Sim, 2011). This pathway is triggered through a conformational change of C3 though cleavage of a single site by serine protease C3 convertase (Carroll and Sim, 2011). Cleavage of C3 leads to generation of to C3a and C3b. Factor B anchors the C3b on cell surfaces in the presence of Mg2+ cleaving to form C3bB. This allows the recognition and binding of Factor D, a serine protease to bind and cleave factor B into Ba and Bb fragments. The Bb fragment bound to C3b forming C3bBb, generates C3 convertase of the alternative pathway. Under physiological conditions C3bBb is not very stable but is stabilized by binding of another complement protein, properdin, to form, C3bBbP, this allows this C3 convertase to generate many C3 molecules. The C3 convertase, C3bBb binding to adjacent C3b molecules leads to the formation of C5 convertase which cleaves C5 molecules into C5a and C5b. Generation of C5b initiates the formation of the MAC complex to causes cell lysis. Factor H, a regulator protein of the alternative pathway, inhibits C3 convertase through binding with C3b and accelerating the half-life of the C3 convertase to inactivate the alternative pathway (Sim et al., 1993), therefore, functions as a downregulator of the alternative pathway. Although there are evidence of recent reports of early activation of the compliment system is associated with the pathogenesis to preeclampsia, the complement system has not yet completely been elucidated.

It has recently been reviewed that SP-A and SP-D do not initiate the pathway to labour, however may contribute to the maintenance of pregnancy (Yadav et al., 2011). Our study investigates the possible roles of SP-A and SP-D in modulating the immune and prostaglandin pathway to delay labour and perhaps help us understand the immunoregulatory roles of SP-A and SP-D in pregnancy and labour to help prevent preterm birth in human pregnancies.

We propose SP-A and SP-D could modulate the cytokine profile and prostaglandin production using ACD explants of term placentae and via QPCR, PTGS2 and PTGFRN

were examined. PTGS2, otherwise known as COX2, is an initiator of the PG pathway that converts arachidonic acid to PGH2. PGFRN, is a negative regulator of the PG pathway. Additional targets including TLR2 and TLR4, C1q and FH are also examined. These are associated with directing immune milieu to an inflammatory response through modulation of macrophages or complement system, How SP-A and SP-D can affect the levels of cytokine expression was examined by examining IL-8, IL-10, TNF-α and TGF-β.

## 4.3 Methods and materials

### *4.3.1 Amnion, Chorion and Decidua (ACD) explants culture and stimulation*

#### *4.3.1.1 Collection of tissue*

This study was approved by the Research Ethics Committee, School of Health Sciences and Social Care, Brunel University, London. Placentae were collected by midwives from St. Michael's Hospital, Bristol and Warwick University Hospital and John Radcliffe Hospital, Oxford University. Each placenta was obtained from women at elective caesarean term-not-in-labour (TNIL) at 39 weeks (N=4). The women were of mixed parity and delivered live births with no sign of infection. Tissues were dissected immediately after delivery, washed in sterile saline for preparation of cell culture.

#### *4.3.1.2 Amnion, Chorion and Decidua (ACD) cell culture*

The amnion, chorion and decidua tissues from TNIL were washed in saline solution and a total of 200 mg of tissue was added to a 12-well tissue culturing plate per patient (Table 4.1). ACD explants were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 10% fetal calf serum (FCS). The cultures were incubated overnight at 37°C for 24 hours in 95% air and 5% $CO_2$ atmosphere. The media was then removed and replaced with DMEM containing 0.2% lactoalbumin hydrolysate. Each well of 200 mg tissue was treated with 10 µg of native SP-A, SP-D, rfSP-D-Gly-Xaa-Yaa & rfSP-A, with and without IL-1β (200 pMol) and incubated for 12 hours at 37°C in 95% air and 5% v/v $CO_2$ atmosphere. 0.1 g of the tissue was subjected to RNA extraction and 0.1 g of tissue per well was subjected to cell lysate extraction procedures.

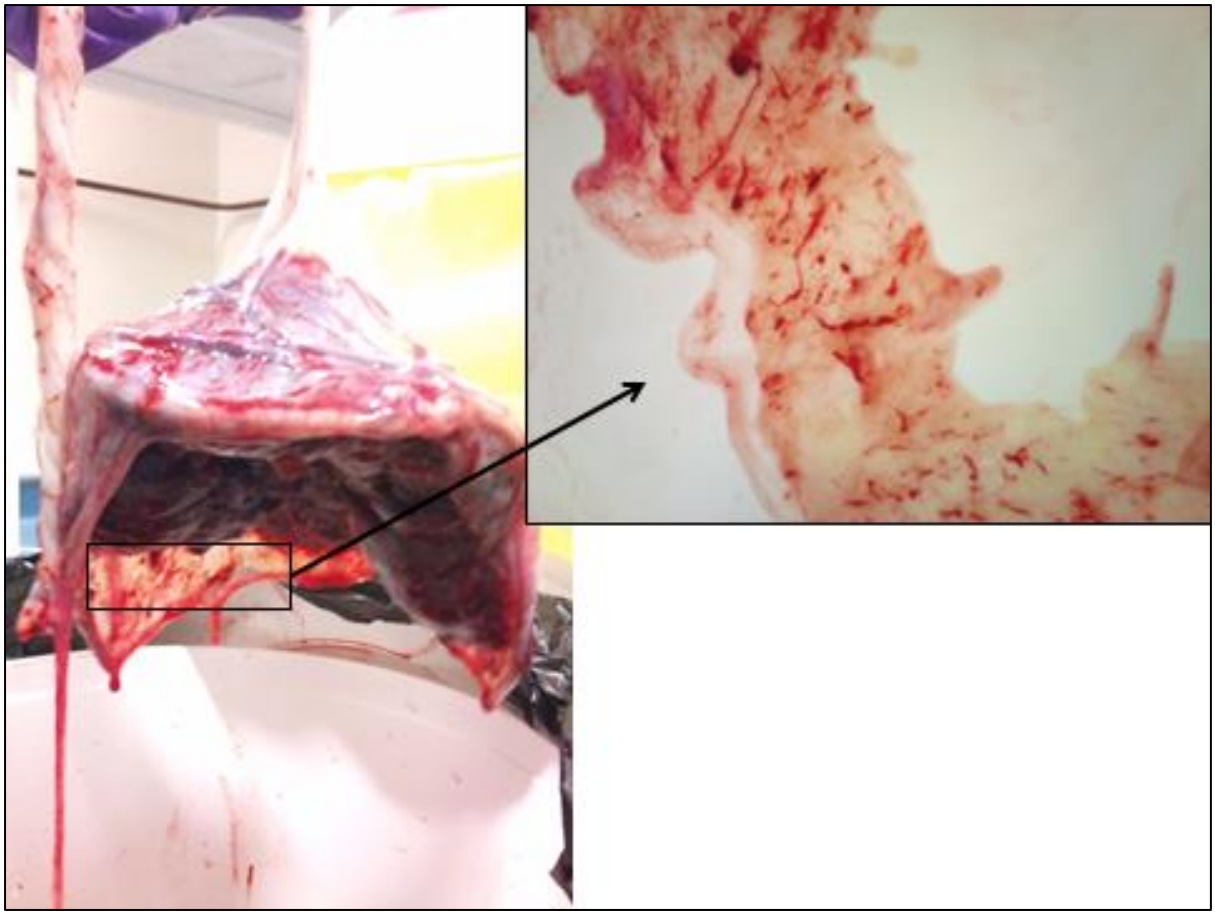


***Figure 4.1 – Image of placenta and ACD section from placenta.***

***Table 4.1 – Plate layout for experimental design***

| | | | |
|---|---|---|---|
| 10 µg rfSP-A | 10 µg rfSP-A + IL-1β | 200 pMol IL-1β | No Treatment (NT) |
| 10 µg rfSP-D + IL-1β | 10 µg rfSP-D + IL-1β | | |
| | | | |

### *4.3.2 RNA extraction*

RNA was extracted as described in chapter 2 and the concentration of RNA was determined by a spectrophometer (NanoDrop ND-1000).

### *4.3.3 DNase treatment and cDNA synthesis*

Prior to cDNA synthesis, deoxyribonuclease I (DNase I) treatment to digest DNA and eliminate RNase activity was carried out to prepare RNA for RT-PCR. DNase I (Sigma, Dorset, UK) treatment was carried out according to the manufacturer's recommendation. Briefly, the RNA sample (~50 µl) was treated with 5 µl of the 10 x reaction buffer and 5 µl of Amplification grade DNase I (1 U/µl) and incubated at room temperature for 15 minutes. The reaction buffer and Amplification Grade DNase I combined, removes contaminating DNA by nucleic digestion at room temperature. The DNase was then inactivated by adding 5 µl of the stop solution and heating the sample to 70°C for 10 minutes. The overall RNA quality was assessed by electrophoresis and also gave a semi-quantitative indication on RNA yield. Samples of RNA were separated on a 1% agarose-EtBr gel against a DNA sizer III (Peqlab, Sarisbury Green, UK) at 70V until the fast migrating dye (bromophenol blue) reached 2/3 the length of the gel. The integrity of RNA was indicated by 2 distinct bands, 28S and 18S rRNA with a 2:1 ratio of intensity, respectively. The results were visualized under a UV transilluminator (Bio-Rad, Hemel Hempstead, UK).

RNA samples were quantified and quality was checked using NanoDrop spectrophometer. 2µg of total RNA were used as a template for cDNA synthesis primed by random primers using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Paisley, UK). Briefly, in a 20 µl reaction, 2 µg of RNA was incubated with 2 X RT Buffer, and 20 X enzyme mix. This was placed in a thermocycler at 37°C (60 minutes) and 95°C (5 mins) and stored at 4°C.

### *4.3.4 Real-time PCR and statistical analysis*

In a 10 µl reaction, 100 ng of cDNA and 500 µM forward and reverse primers were mixed with powerSYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK). These were set in a qPCR thermocycler for 40 cycles of 95°C (15 mins) 57°C (1 min) followed by a dissociation curve using a 7900HT Real-Time PCR system. Results were analysed using the Sequence Detections Software (SDS) V2.4 and RQ Manager. Samples were compared to 18S rRNA, the endogenous control, and calibrated against non-treated

samples. The $C_t$ values were calculated at the point where the PCR curve crosses the threshold. For each sample, the geometric mean $C_t$ for each target was subtracted from the geometric mean $C_t$ of the endogenous control to give $\Delta\Delta C_t$ values. The RQ value to give the relative fold change of expression, were calculated by 2 to the power of $-\Delta\Delta C_t$ ($2^{-\Delta\Delta Ct}$). For all subsequent statistical analysis, RQ values were plotted on a logarithmic scale ($log_{10}$) with the standard error of mean to minimise error. Sequences of primers used in this study are in the table below.

***Table 4.2 – Primer sequences for RT-PCR***

| Gene Nomenclature/Formally | Primer name | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|
| **18s rRNA** | 18s rRNA | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG |
| **PTGS2/COX2** | Prostaglandin H synthase 2 | CTCAGACAGCAAAGCCTACC | ATGTGATCTGGATGTCAACAC |
| **PTGFRN** | Prostaglandin F2-Alpha Receptor Regulatory Protein | CTGTCGTTGGCTCTTTGCCG | CATCATAGTCACTGACGTTGC |
| **POLR2A** | | GCACCACGTCCAATGACATTG | GTGCGGCTGCTTCCATAAGC |
| **IL8** | Interleukin 8 | CTGTGTGAAGGTGCAGTTTTG | GTGTTGGCGCAGTGTGGTC |
| **IL-10** | Interleukin 10 | ACATCAAGGCGCATGTGAAC | TAGAGTCGCCACCCTGATGT |
| **TNF-α** | Tumour necrosis factor alpha | CACCACTTCGAAACCTGGGA | AGGAAGGCCTAAGGTCCACT |
| **TGF-β** | Transforming growth factor- beta | CCCAGCATCTGCAAAGCTC | GTCAATGTACAGCTGCCGCA |
| **TLR2** | Toll-like receptor 2 | GAGACCTATAGTGACTCCCAG | CTGCCCTTGCAGATACCATTG |
| **TLR4** | Toll-like receptor 4 | CTTCTCAACCAAGAACCTTGG | GTGGCCTTAGGCTCTGATATG |
| **SP-A** | Surfactant protein A | GTGGGGTGGGATTAGATAAATGC | TACTGAGAGATGTGTGCTTGGTGAG |
| **SP-D** | Surfactant protein D | TGGTTTCTGAGATGGAGTCGTG | TGGGGCAGTGGATGGAGTGTGC |
| **C1q** | | ATGGTGACCGAGGACTTGTG | GTCCTTGATGTTTCCTGGGC |
| **FH** | Factor H | CCCGGGGAAATACAGCCAAA | TCTGGGAGTAGGAGACCAGC |

All primers were stored at a stock concentration of 5µM in 10mM Tris pH7.5 at -20°C. The final concentration of 500nM was used in the qPCR reactions.

### *4.3.5 Protein Extraction*

Proteins were extracted as described in chapter 2 for further protein analysis

### *4.3.6 Immunoblotting and ELISA*

ELISA plates were coated in duplicates with 5, 2.5, 1.25, 0.625, 0.3125 µl proteins per microtitre well and incubated overnight at 4°C. The next day, the unbound proteins were discarded and un-coated wells were blocked with 5% non-fat w/v milk powder/PBS for 2 hour at 37°C. Following 3 consecutive washes with PBS containing 0.05% Tween-20, the wells were incubated with primary antibody of 1/500 dilution (rabbit α-human SP-A, rabbit α-human SP-D, rabbit α-human C1q, PTGF2-α) and 1/5000 (rabbit α-human Factor h) for 2 hours at 37°C and washed prior to adding secondary antibody, 1/1000 Protein-A conjugated to horse radish peroxidase (HRP) and incubated at 37°C for 1 hour. After three consecutive washes, the wells were developed with OPD (Sigma, Dorset, UK) according to manufactures recommendation and optical density was checked at 450nm. The treated cell lysates were compared to non-treated cell lysate results to understand the effects of SP-A and SP-D with and without IL-1β on the expression of innate immune and prostaglandin proteins.

## 4.4 Results

### *4.4.1 RNA extraction, characterisation and validation*

As seen from figure 4.2, samples treated with DNase Amplification grade (Sigma, Dorset, UK) displays 2 clear bands depicting RNA integrity. Samples not treated with DNase contain DNA which has remained in the wells. The two bands seen are 18S and 28S which are the small and large subunits of RNA (rRNA) in eukaryotes. The DNase treatment of RNA was successful as it is visible in samples not DNase treated due to the large size of DNA and small pore sizes of the agarose gel.

A high yield of RNA was achieved using the TRIzol method. The RNA extracted was from 200 ng of tissue with end results of 50 µl concentrated RNA. The 260/280 reading represents the purity of the RNA, in the case of these samples, they are pure and free from amino acids.

Similarly, RNA from term placenta treated in the same way as preterm were also extracted and purified using the TriZol (Invitrogen, Paisley, UK) method. The samples were; non-treated (control sample), IL-1β, SP-A, SP-D, SP-A + IL-1β, SP-D + IL-1β and each were DNase treated before quantifying RNA concentration, see table below.

Each of the term samples of RNA were converted to cDNA using High capacity RNA-to-cDNA kit (Applied Biosystems, Paisley, UK). The values of the 260/230 ratio were below 2.0, however, after converting 2µg of RNA to cDNA, the samples 260/230 ratio values were between the optimum 2.0-2.2. This shows contamination level to be diluted giving suitable 260/230 ratios.

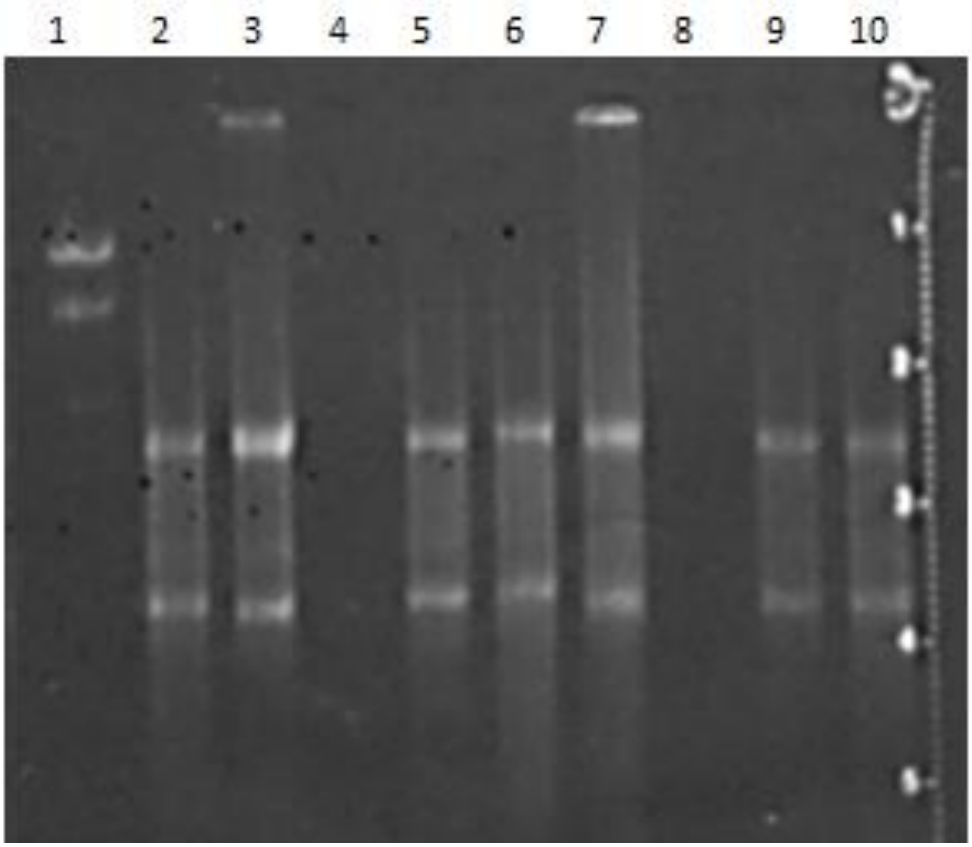



***Figure 4.2 – RNA samples after DNase treatment on agarose gel.*** *1% Agarose gel of RNA samples. 5 samples are DNAase treated and two are not. DNA remains in wells. The DNA marker used is DNA Sizer III containing 13 fragments with molecular weight (largest first): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125 base pairs. Lane 1 – DNA sizer III (Peqlab), lane 2 – IL-1β (DNase treated), lane 3 – IL-1β (not DNase treated), lane 5 – 10µg SP-A (DNase treated), lane 6 – 10 µg SP-D (DNase treated), lane 7 – 10 µg SP-A + IL-1β (not DNase treated), lane 9 – 10 µg SP-D + IL-1β (DNase treated), lane 10 – non-treated sample (DNase treated).*

An RT-PCR experiment was carried out to optimise cDNA concentration for each primer set. The two amounts of cDNA used were 25 ng and 100 ng. We had chosen to use 25 ng to follow a published protocol, however, due to unsuccessful results an RT-PCR was carried out. The results displayed higher amplification of amplicon with a higher concentration of cDNA. Unfortunately 18S product and PTGFRN product was mixed hence the bands in lanes 15 and 18 are identical (figure 4.3). The product for PTGS2 was very faint when 25 ng or 100 ng of cDNA suggesting primer optimisation may be required. Alternatively, PTGS2 primer may be necessary for redesigning as the amplicon size is very large and would be difficult to break the G-C rich bonds for QPCR analysis.

A Q-PCR experiment was carried out after optimising the best primer concentration and the targets were identified from each treated sample as a qualitative method. In figure 4.4, the endogenous control 18S rRNA showed equal amount of expression in ACD explants regardless of treatment or no-treatment.

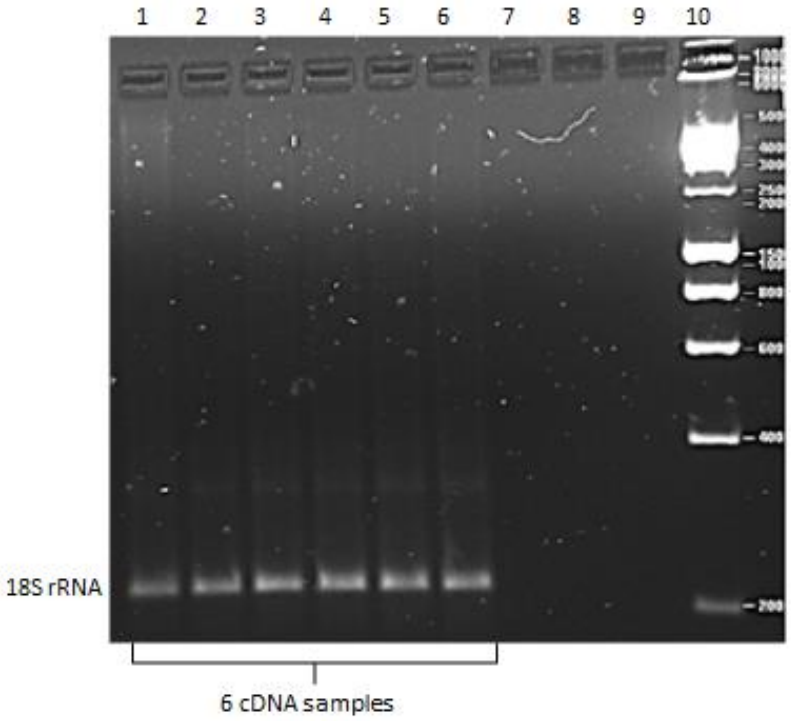



***Figure 4.3 – 2% Agarose-EtBr Gel to separate 18S rRNA products from 6 cDNA samples***

The results show expression of the endogenous control 18S rRNA, in treated and non-treated samples (figure 4.4). Negative controls (NTC) were also separated on the gel to show experimental quality that no expression took place without cDNA therefore validating minimal contamination error. The same validation process for PTGS2, PTGFRN, IL-8, TLR-2 and TLR-4 were carried out to collate qualitative results for target gene expression. In figure 4.5, PTGS2 shows a clear 372bp amplicon size and 109bp for PTGFRN amplicon size (figure 4.5-4.6).

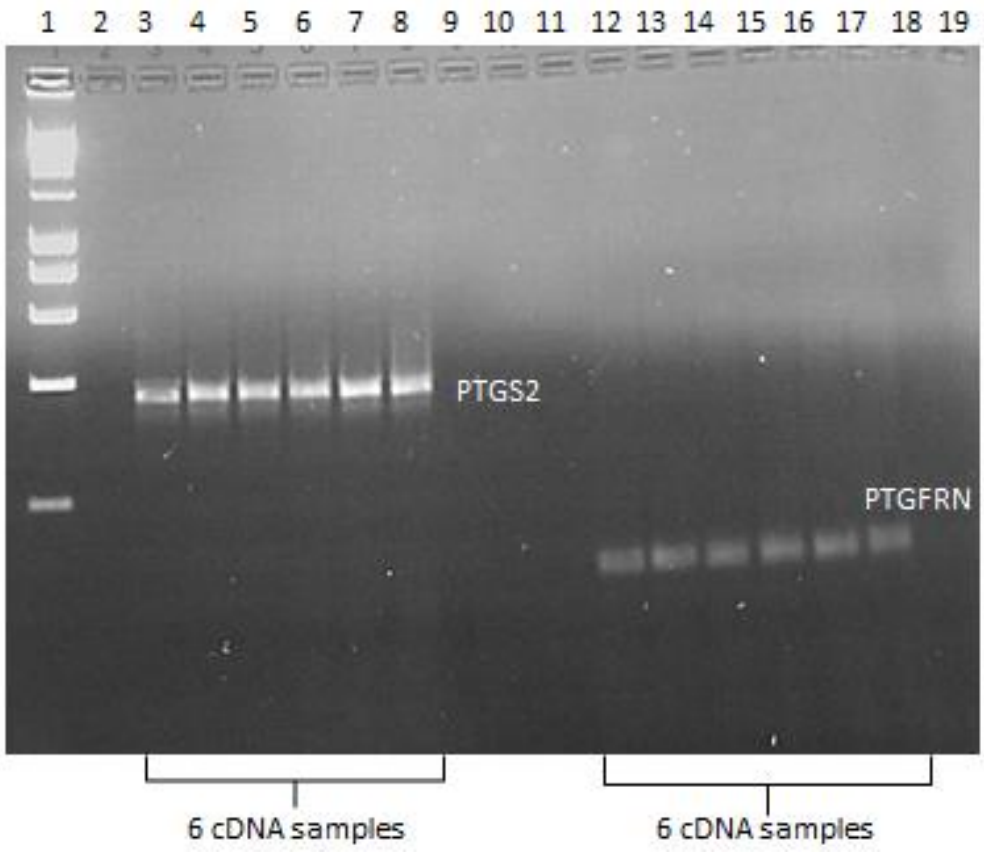



***Figure 4.4 – 2% Agarose-EtBr gel was carried out to visualise QPCR products for PTGS2 and PTGFRN.*** *For PTGS2 there was no amplification of the amplicon as seen in the gel. PTGFRN and POLR2A gave positive results. 18S was an additional endogenous control used as it is consistent in placental tissue and is expressed in high amounts. Lane 1 – Hyperladder I (Peqlab), lane 3 – non-treated sample, lane 4 – IL-1β, lane 5 – rfSP-A, lane 6 – rfSP-D, lane 7 – rfSP-A + IL-1β, lane 8 – SP-D + IL-1β, lane 9 – NTC, lane 12 – non-treated sample, lane 13 – IL-1β, lane 14 – rfSP-A, lane 15 – rfSP-D, lane 16 – rfSP-A + IL-1β, lane 17 – SP-D + IL-1β, lane 18 – NTC*

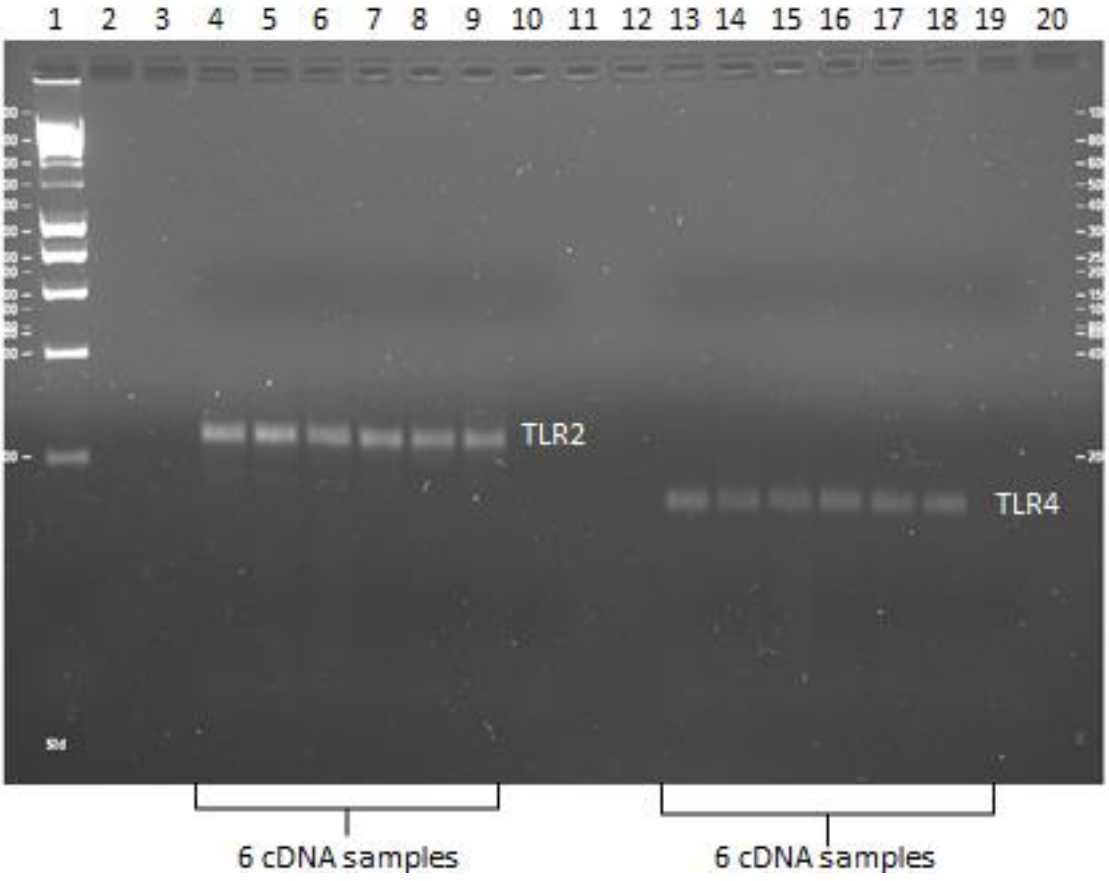
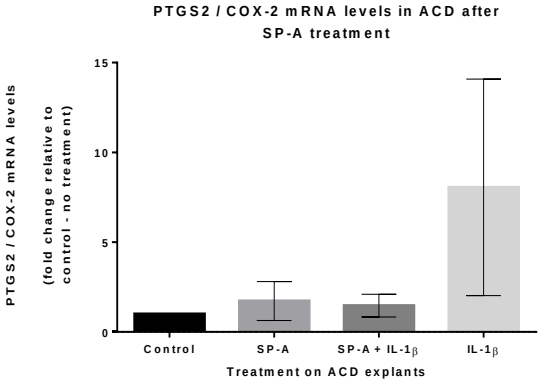



***Figure 4.5 – 1% Agarose-EtBr gel to separate QPCR products of TLR2 and TLR4 expressed in all treated and non-treated samples separated.***

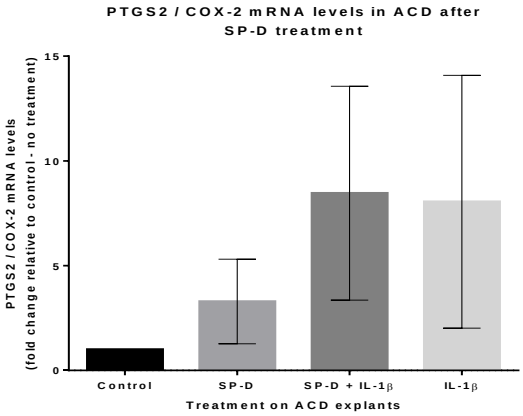
### *4.4.2 Effect of rfSP-A and rfSP-D on mRNA levels in ACD explants*

As seen in figure 4.6, rfSP-A increases PTGS2 levels by 10-fold without IL-1β and more than 100-fold with IL-1β compared to 'no treatment'. IL-8, PTGFRN levels are slightly suppressed in the presence of IL-1β although their expressions do not vary as much compared to non-treated samples. IL-10 levels are relatively the same with and without IL-1β although there is an up-regulation of IL-10 compared to non-treated samples. rfSP-A causes a large increase in TNF-α expression by almost 10-folds. This is supressed in the presence of IL-1β. There is a slight up-regulation of TGF-β by rfSP-A, but this is downregulated in the presence of IL-1β. rfSP-A down regulates TLR-2, which is further enhanced when combined with stimulation of IL-1β. There is an up-regulation of TLR-4 in ACD explants, which is very slightly dampened by IL-1β. However, the change is not considerable. rfSP-A shows to suppress its own gene expression, which is reversed when stimulated with rfSP-A + IL-1β. There is a slight up-regulation of SP-D in ACD when stimulated with rfSP-A, which does not show to be effected by IL-1β. There is a slight suppression of C1q in ACD when stimulated with rfSP-A with and without IL-1β however the level of expression doesn't vary so much compared to non-treated samples. There is a huge up-regulation of Factor H in ACD explants compared to non-treated samples, varying by at least 5-fold (log-10) regardless of IL-1β treatment. As expected, IL-1β, has a more stimulatory effect on the genes in the absence of SP-A and SP-D. Each experiment was carried out in triplicates three times.

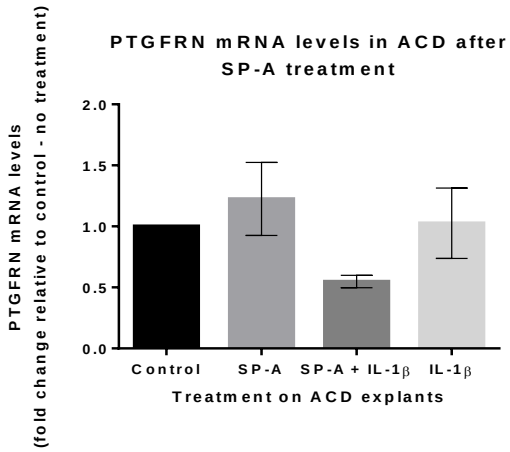
***Qualitative gene expression of prostaglandins, cytokines and innate immune proteins after surfactant protein stimulation***



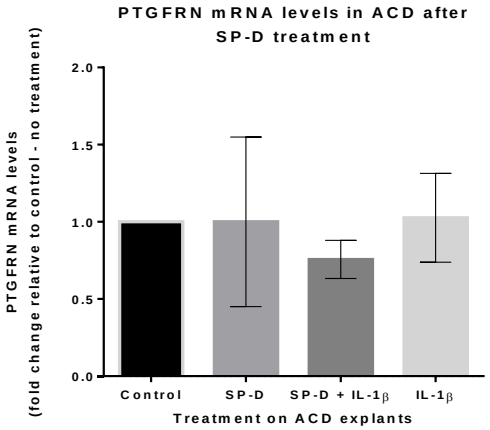

(a)



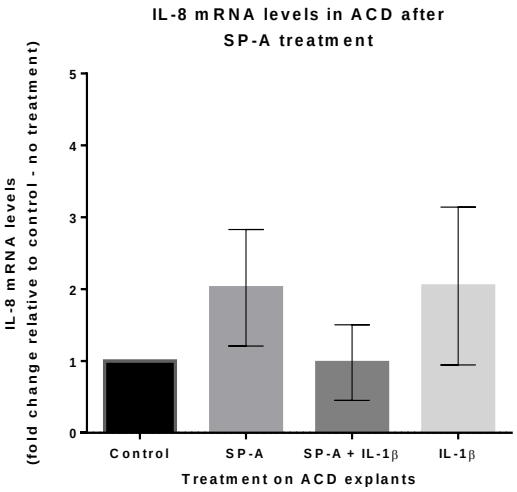

(b)



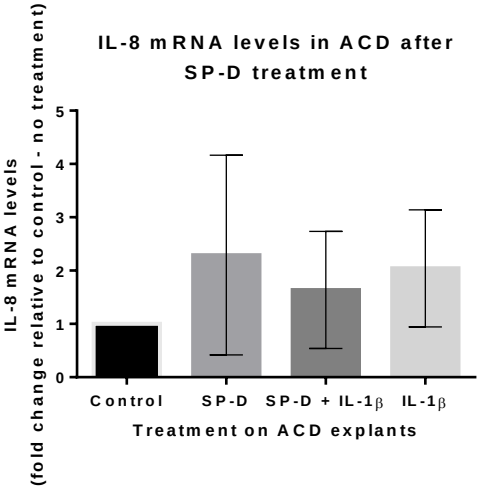

(c)



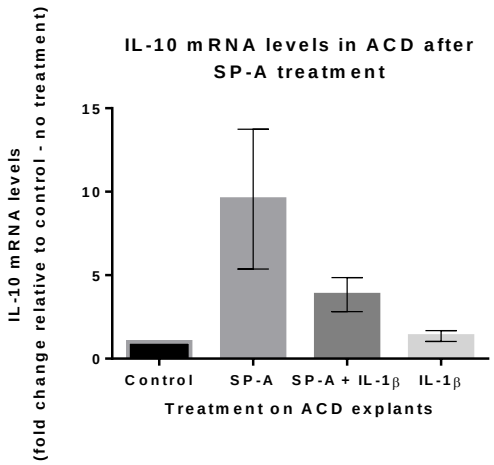

(d)



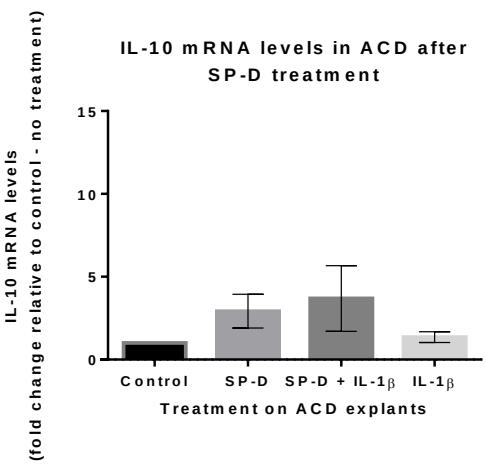

(e)



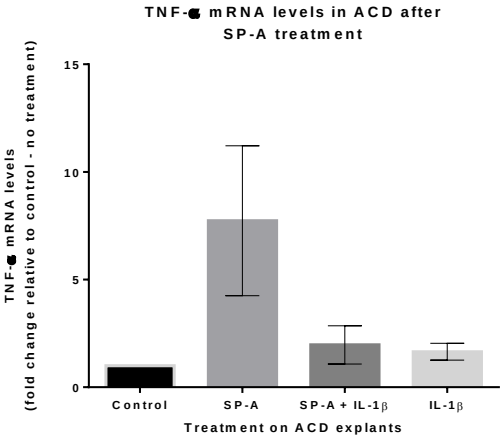

(f)


IL-10 mRNA levels in ACD after SP-A treatment
IL-10 mRNA levels (fold change relative to control - no treatment)
15
10
5
0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

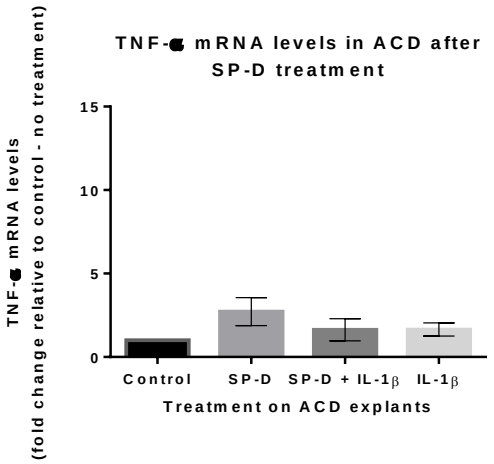
(g)


IL-10 mRNA levels in ACD after SP-D treatment
IL-10 mRNA levels (fold change relative to control - no treatment)
15
10
5
0
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

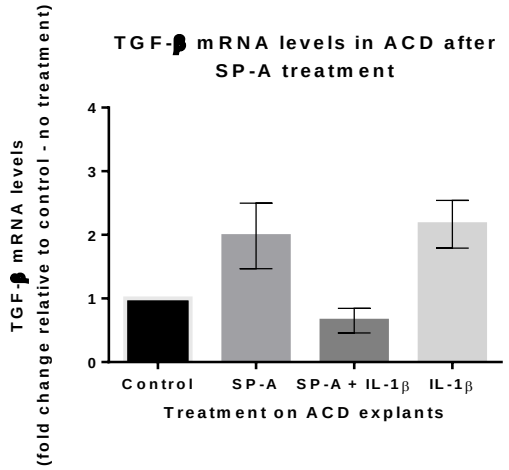
(h)


TNF-α mRNA levels in ACD after SP-A treatment
TNF-α mRNA levels (fold change relative to control - no treatment)
15
10
5
0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

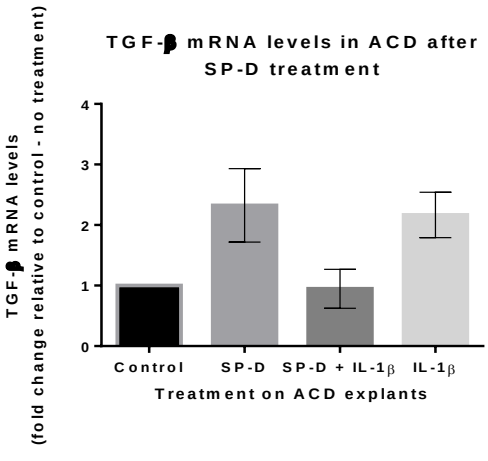
(i)


TNF-α mRNA levels in ACD after SP-D treatment
TNF-α mRNA levels (fold change relative to control - no treatment)
15
10
5
0
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

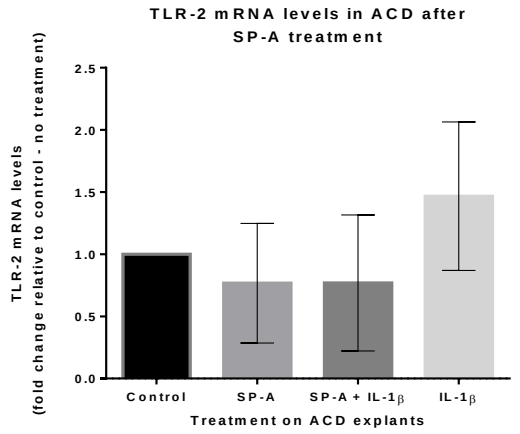
(j)


TGF-β mRNA levels in ACD after SP-A treatment
TGF-β mRNA levels (fold change relative to control - no treatment)
4
3
2
1
0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

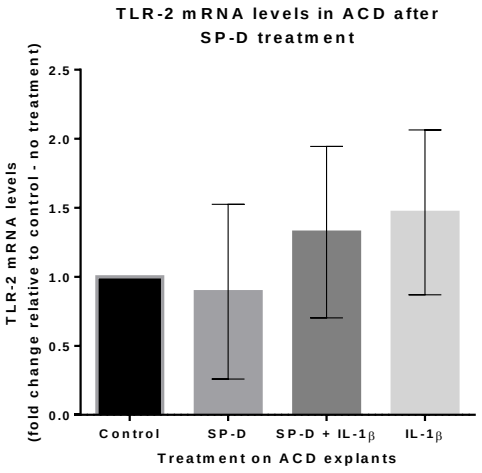
(k)


TGF-β mRNA levels in ACD after SP-D treatment
TGF-β mRNA levels (fold change relative to control - no treatment)
4
3
2
1
0
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

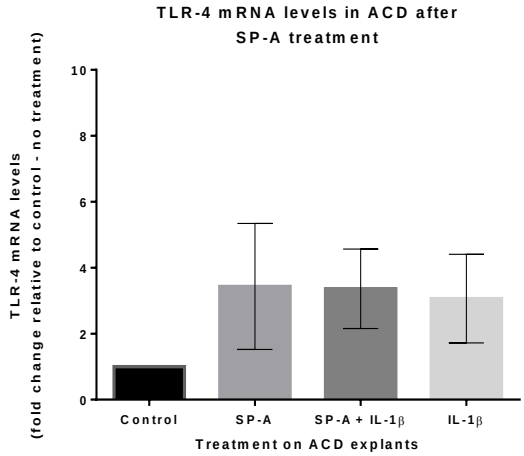
(l)


TLR-2 mRNA levels in ACD after SP-A treatment
TLR-2 mRNA levels (fold change relative to control - no treatment)
2.5
2.0
1.5
1.0
0.5
0.0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

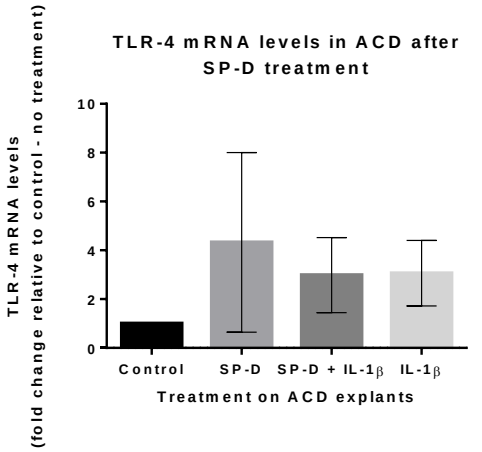
(m)


TLR-2 mRNA levels in ACD after SP-D treatment
TLR-2 mRNA levels (fold change relative to control - no treatment)
2.5
2.0
1.5
1.0
0.5
0.0
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

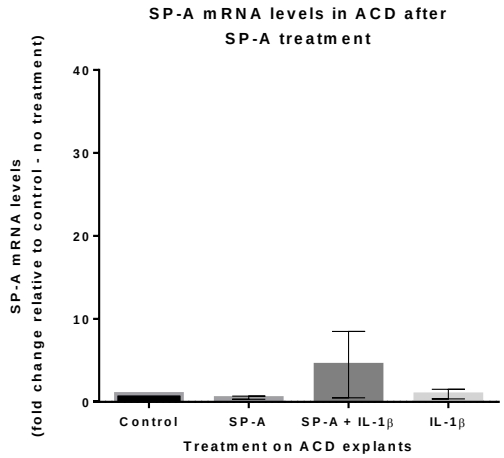
(n)


TLR-4 mRNA levels in ACD after SP-A treatment
TLR-4 mRNA levels (fold change relative to control - no treatment)
10
8
6
4
2
0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

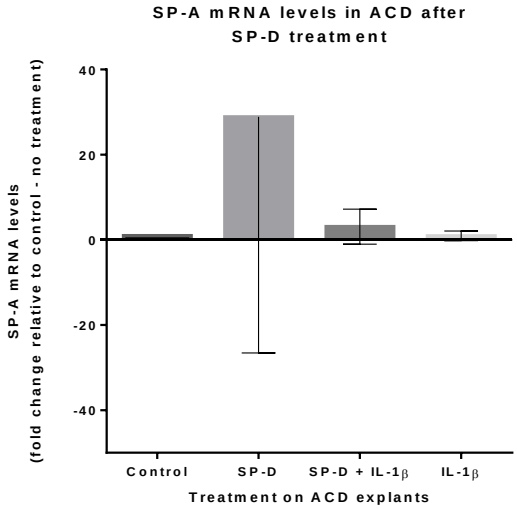
(o)


TLR-4 mRNA levels in ACD after SP-D treatment
TLR-4 mRNA levels (fold change relative to control - no treatment)
10
8
6
4
2
0
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

(p)


SP-A mRNA levels in ACD after SP-A treatment
SP-A mRNA levels (fold change relative to control - no treatment)
40
30
20
10
0
Control
SP-A
SP-A + IL-1β
IL-1β
Treatment on ACD explants

(q)


SP-A mRNA levels in ACD after SP-D treatment
SP-A mRNA levels (fold change relative to control - no treatment)
40
20
0
-20
-40
Control
SP-D
SP-D + IL-1β
IL-1β
Treatment on ACD explants

(r)

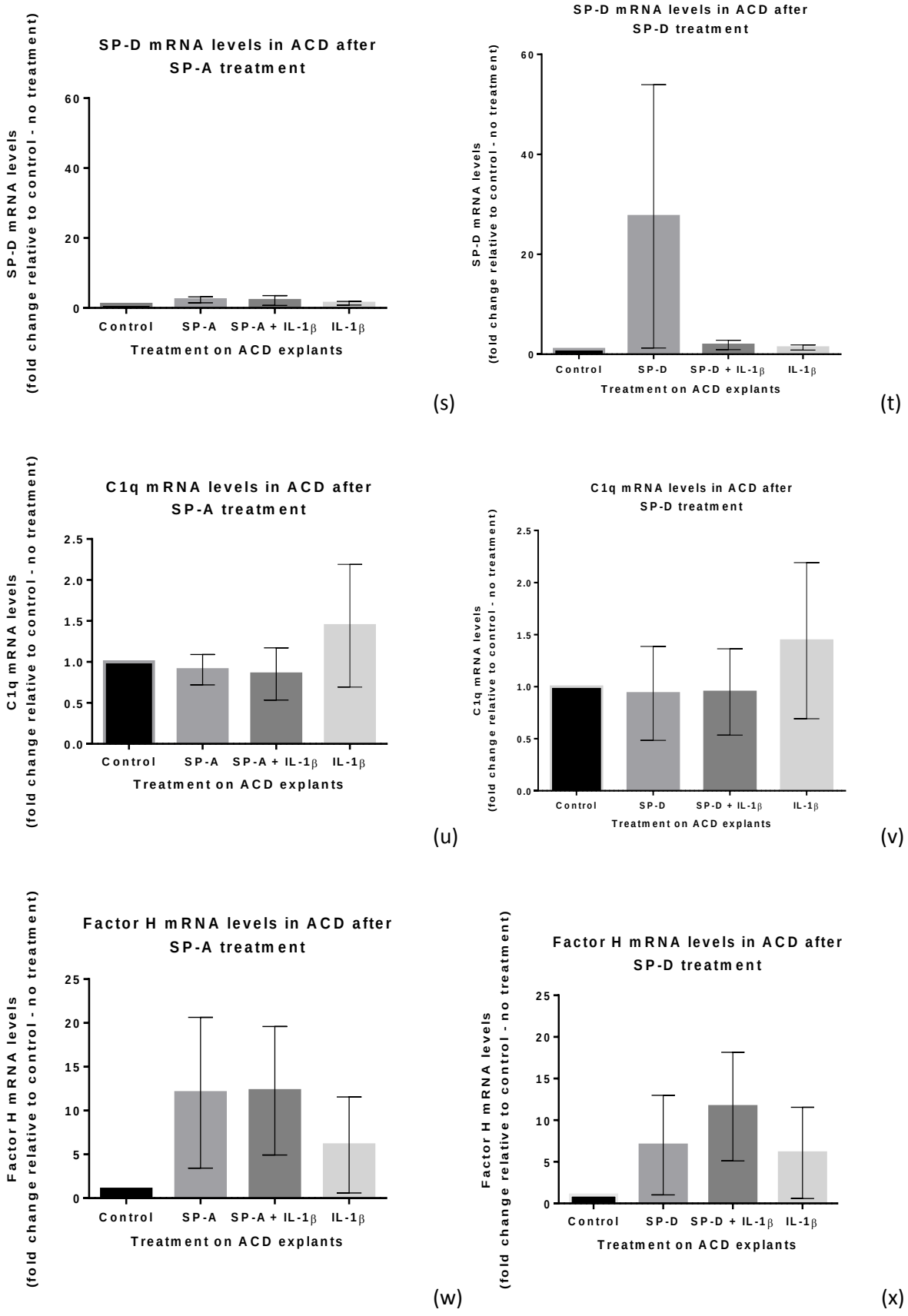
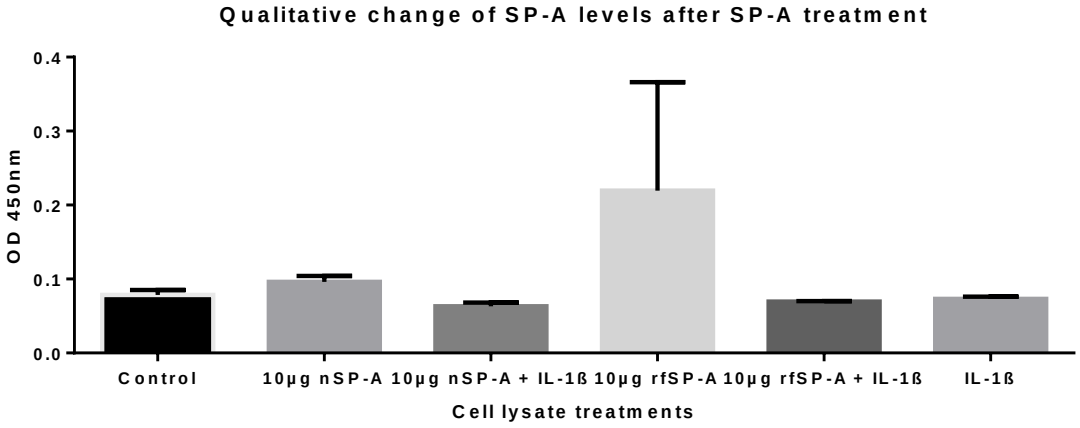


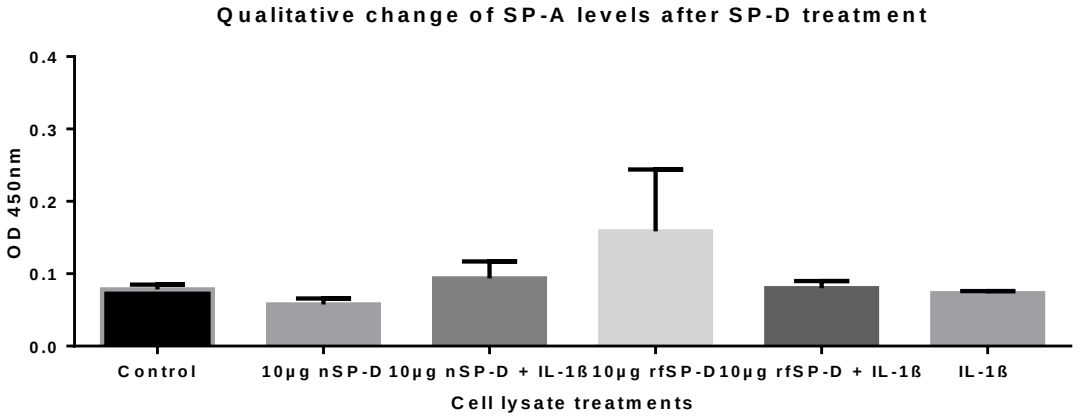

**Figure 4.6 –** ***Qualitative gene expression of prostaglandins, cytokines and innate immune proteins after surfactant protein stimulation.*** Fold change in mRNA levels relative to non-treated control sample (mean ± SEM). ACD explants were treated with 10µg of either SP-A or

SP-D with or without 200pMol IL-1β n=4. RNA was extracted using standard Trizol method and mRNA levels were quantified by quantitative-PCR using SYBR green. Each experiment was carried out in triplicates and measured by two-way ANOVA as shown from a-x. a) Change in PTGS2 mRNA levels after SP-A treatment of ACD explants, b) Change in PTGS2 mRNA levels after SP-D treatment of ACD explants, c) Change in PTGFRN mRNA levels after SP-A treatment of ACD explants, d) Change in PTGFRN mRNA levels after SP-D treatment of ACD explants, e) Change in IL-8 mRNA levels after SP-A treatment of ACD explants, f) Change in IL-8 mRNA levels after SP-D treatment of ACD explants, g) Change in IL-10 mRNA levels after SP-A treatment of ACD explants, h) Change in IL-10 mRNA levels after SP-D treatment of ACD explants, i) Change in TNF-α mRNA levels after SP-A treatment of ACD explants, j) Change in TNF-α mRNA levels after SP-D treatment of ACD explants, k) Change in TGF-β mRNA levels after SP-A treatment of ACD explants, l) Change in TGF-β mRNA levels after SP-D treatment of ACD explants, m) Change in TLR-2 mRNA levels after SP-A treatment of ACD explants, n) Change in TLR-2 mRNA levels after SP-D treatment of ACD explants, o) Change in TLR-4 mRNA levels after SP-A treatment of ACD explants, p) Change in TLR-4 mRNA levels after SP-D treatment of ACD explants, q) Change in SP-A mRNA levels after SP-A treatment of ACD explants, r) Change in SP-A mRNA levels after SP-D treatment of ACD explants, s) Change in SP-D mRNA levels after SP-A treatment of ACD explants, t) Change in SP-D mRNA levels after SP-D treatment of ACD explants, u) Change in C1q mRNA levels after SP-A treatment of ACD explants, v) Change in C1q mRNA levels after SP-D treatment of ACD explants, w) Change in Facto H mRNA levels after SP-A treatment of ACD explants, x) Change in Factor H mRNA levels after SP-D treatment of ACD explants. Data shows no significance between tests.

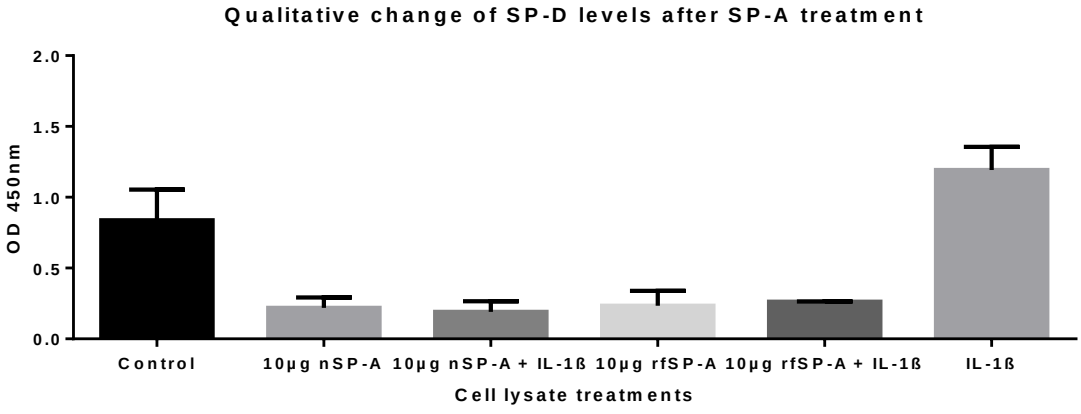
### *4.4.3 Effect of SP-A and SP-D on SP-A, SP-D, PGF2α, C1q and Factor H protein levels in ACD treated placentae*



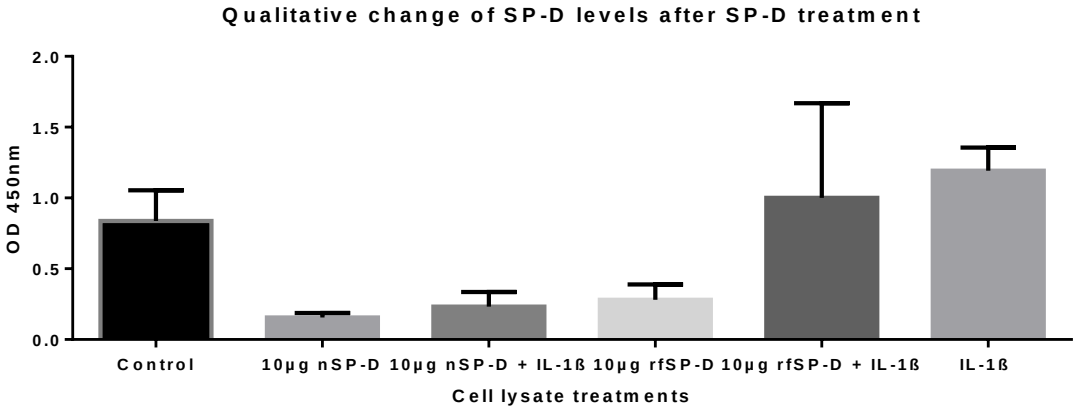

(a)



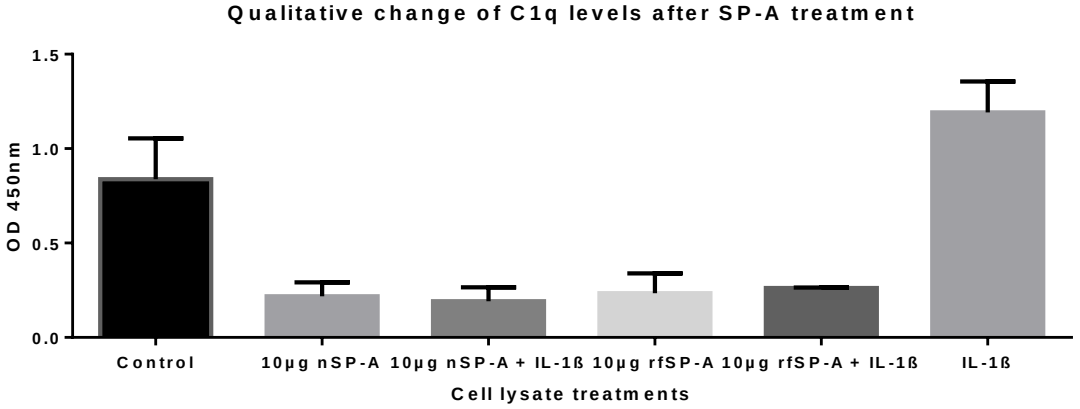

(b)



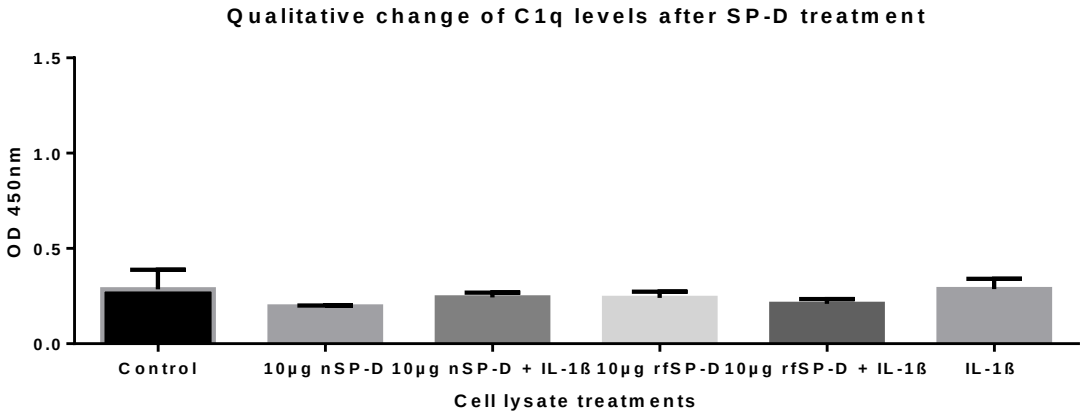

(c)


Qualitative change of SP-D levels after SP-D treatment
OD 450nm
2.0
1.5
1.0
0.5
0.0
Control
10µg nSP-D
10µg nSP-D + IL-1ß
10µg rfSP-D
10µg rfSP-D + IL-1ß
IL-1ß
Cell lysate treatments

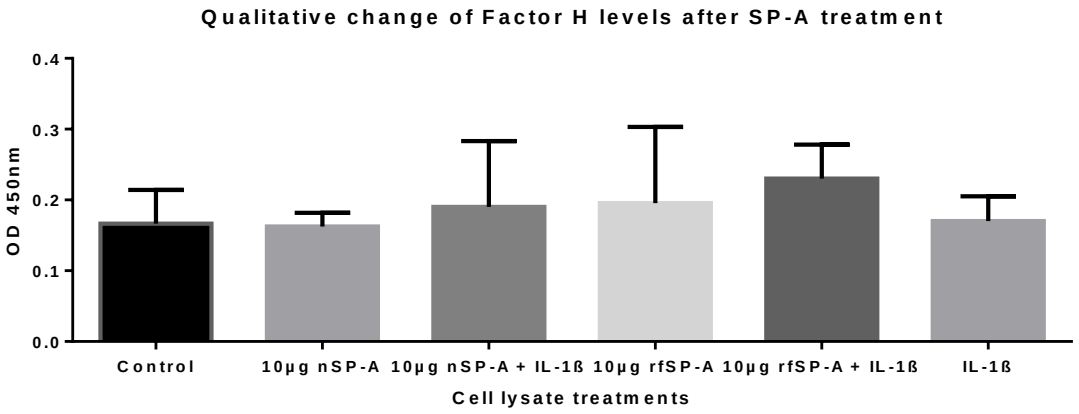
(d)


Qualitative change of C1q levels after SP-A treatment
OD 450nm
1.5
1.0
0.5
0.0
Control
10µg nSP-A
10µg nSP-A + IL-1ß
10µg rfSP-A
10µg rfSP-A + IL-1ß
IL-1ß
Cell lysate treatments

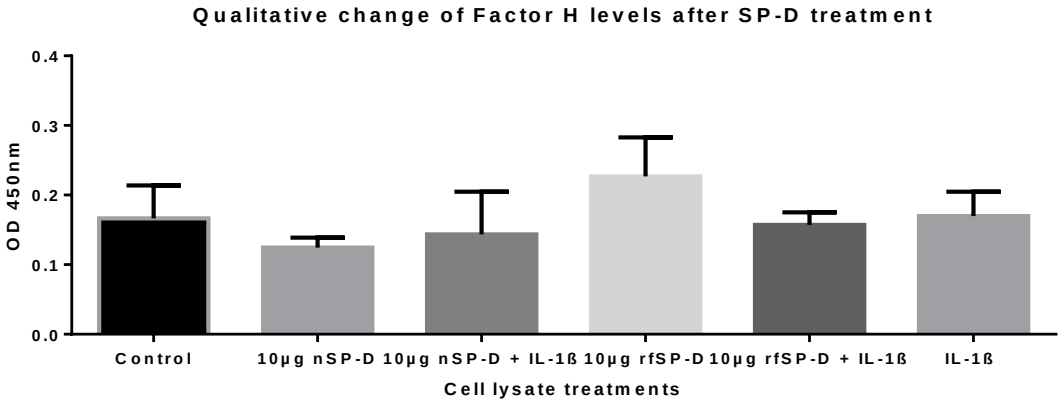
(e)


Qualitative change of C1q levels after SP-D treatment
OD 450nm
1.5
1.0
0.5
0.0
Control
10µg nSP-D
10µg nSP-D + IL-1ß
10µg rfSP-D
10µg rfSP-D + IL-1ß
IL-1ß
Cell lysate treatments

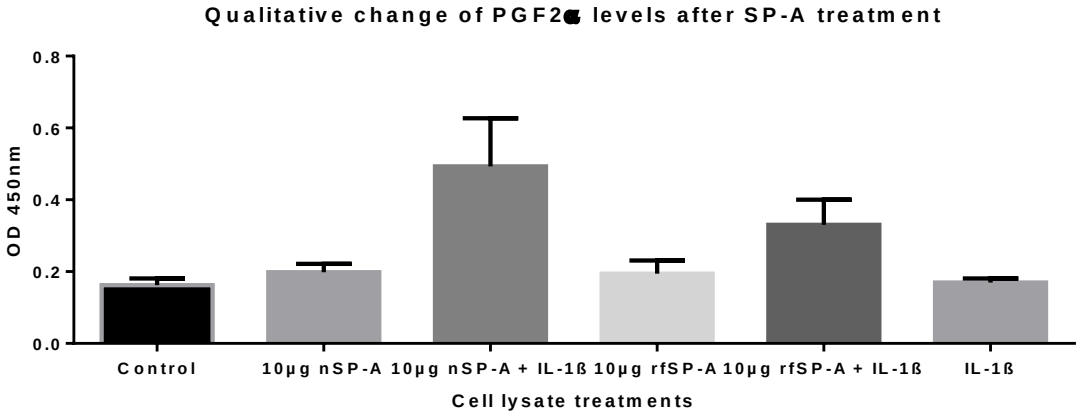
(f)


Qualitative change of Factor H levels after SP-A treatment
OD 450nm
0.4
0.3
0.2
0.1
0.0
Control
10µg nSP-A
10µg nSP-A + IL-1ß
10µg rfSP-A
10µg rfSP-A + IL-1ß
IL-1ß
Cell lysate treatments

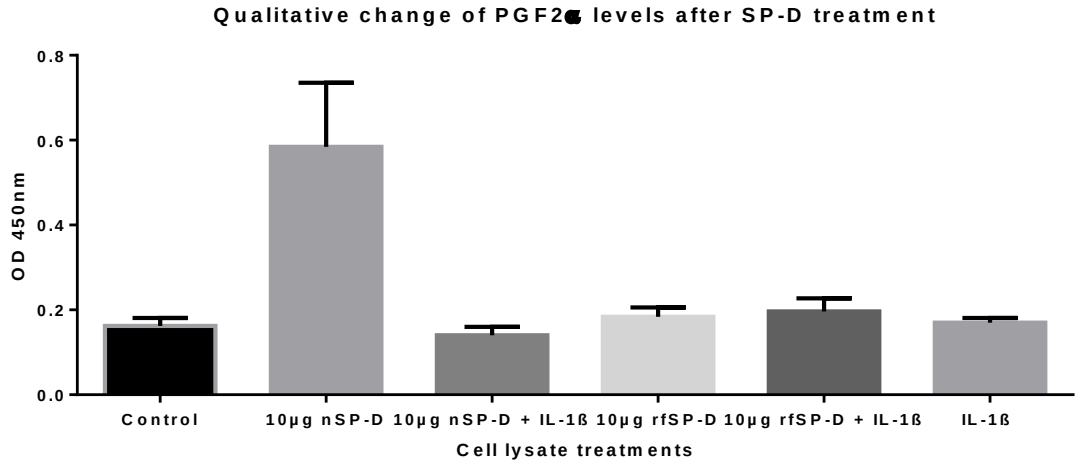
(g)


Qualitative change of Factor H levels after SP-D treatment
OD 450nm
0.4
0.3
0.2
0.1
0.0
Control
10µg nSP-D
10µg nSP-D + IL-1ß
10µg rfSP-D
10µg rfSP-D + IL-1ß
IL-1ß
Cell lysate treatments

(h)


Qualitative change of PGF2 levels after SP-A treatment
OD 450nm
0.8
0.6
0.4
0.2
0.0
Control
10µg nSP-A
10µg nSP-A + IL-1ß
10µg rfSP-A
10µg rfSP-A + IL-1ß
IL-1ß
Cell lysate treatments

(i)




(j)

**Figure 4.7 – Qualitative change in protein levels relative to non-treated control sample** (mean ± SEM). ACD explants were treated with 10µg of either SP-A or SP-D with or without 200pMol IL-1β n=4. Cell lysates were extracted by standard Trizol method and analysed by ELISA using polyclonal antibodies either specific to SP-A or SP-D. Each experiment was carried out in triplicates and measured by two-way ANOVA as shown from a-x. a) Qualitative changes in SP-A protein levels after SP-A treatment of ACD explants with and without inflammatory IL-1β stimulation, b) Qualitative changes in SP-A protein levels after SP-D treatment of ACD explants with and without inflammatory IL-1β stimulation, c) Qualitative changes in SP-D protein levels after SP-A treatment of ACD explants with and without inflammatory IL-1β stimulation, d) Qualitative changes in SP-D protein levels after SP-D treatment of ACD explants with and without inflammatory IL-1β stimulation, e) Qualitative changes in C1q protein levels after SP-A treatment of ACD explants with and without inflammatory IL-1β stimulation, f) Qualitative changes in C1q protein levels after SP-D treatment of ACD explants with and without inflammatory IL-1β stimulation, g) Qualitative changes in Factor H protein levels after SP-A treatment of ACD explants with and without inflammatory IL-1β stimulation, h) Qualitative changes in Factor H protein levels after SP-D treatment of ACD explants with and without inflammatory IL-1β stimulation, i) Qualitative changes in PGF2α protein levels after SP-A treatment of ACD explants with and without inflammatory IL-1β stimulation, j) Qualitative changes in PGF2α protein levels after SP-D treatment of ACD explants with and without inflammatory IL-1β stimulation. Data shows no significance between tests.

## Statistics

Results are the average of four placentae from term caesarean at 39 weeks. QPCR and ELISA experiments were carried out thrice in triplicates and error bars are ± SEM determined by two-way ANOVA. Significance was accepted for $P < 0.05$. Results show statistical significance of $P > 0.05$, therefore treated as not-significant.

## 4.5 Discussion

SP-A and SP-D are pulmonary surfactant proteins also known as collectins due to their collagen and lectin domains. They reside in pulmonary tissues such as alveolar type II tissues and Clara cells and extra-pulmonary tissues such as placentae. These hydrophilic, pathogen recognition receptors can recognise glycoproteins on pathogens binding in a calcium dependent manner to promote phagocytic clearance, thus, providing a good link between innate and adaptive immune response. This makes SP-A and SP-D important molecules for the clearance of infection, one of the contributing factors of preterm birth (i.e. chorioamnionitis). Preterm birth accounts for the highest cause of infant mortality and be a cause of many complications including cerebral palsy, preeclampsia, intrauterine growth retardation (IUGR), hypoxia, vision/learning impairment and lung infection.

Parturition is characterised as an inflammatory response, characterised by vascular dilation, cell recruitment and muscle contraction. During the second stage of pregnancy, levels of decidual macrophages are the most dominant immune cells in the maternal tissue. Thus, we have investigated the roles of SP-A and SP-D in modulating the innate immune response and the main hormonal pathway of labour (the prostaglandin pathway) and in chapter 5 we have examined further how SP-A and SP-D modulate decidual macrophages in an inflammatory situation.

Studies have shown levels of SP-A and SP-D to increase at a 6:1 ratio in human amniotic fluid between 34-42 weeks (Miyamura et al., 1994). Complimenting this investigation, another study has shown, administration of SP-A to mice activates macrophage NF-κB to stimulate prostaglandin production resulting in induction of birth within 6-12 hours (Condon et al., 2004). This lead to explore if there is a link between SP-A and SP-D and the biochemical pathway, and how they could contribute to modulating the inflammatory pathway to initiation of labour in humans using term placentae.

As the initiation of labour is not well understood and the mechanisms to control the timing of birth are not well established, it is well documented SP-A and SP-D are a signal for lung maturation and levels increase just before labour. As lung maturation is important for foetal survival after birth, we propose they play a key purpose in modulating the inflammatory pathway to labour. Hence we have looked at how stimulation of SP-A and SP-D under inflammatory condition affect the prostaglandin

(PG) and cytokine profile on fetal/maternal tissues. Although the QPCR revealed no level of significance, the results suggest very interesting trends worth mentioning.

rfSP-D had a positive up-regulatory effect on PTGS2, increasing the gene expression by 5-fold – this is doubled in the presence of IL-1β. Thus, under inflammatory conditions the level of PTGS2 increases, however, under no inflammatory stimuli the levels PTGS2 expressions are low. Comparing this to protein level of study, native SP-A appears to suppress PGF2α levels suggesting, SP-A in its full form suppresses the progression of the prostaglandin pathway hence dampening the inflammatory response. rfSP-D has shown to down-regulate PTGFRN gene regardless of IL-1β.

Interestingly, in their trimeric form, SP-A and SP-D suppress the levels of IL-8 again supporting the notion that they increases the pro-inflammatory threshold by suppressing an inflammatory response via IL-8. IL-8 is a neutrophil recruitment factor, thus suppressing neutrophil activation and MMP-8 secretion preventing cervical ripening.

In support of the suppression of inflammatory profile, SP-A shows to increase levels of IL-10 regardless of inflammatory environment and both SP-A and SP-D show to increase levels of IL-10 under non-inflammatory situation. Thus suggests SP-A and SP-D modulates macrophages towards an anti-inflammatory response and under inflammatory conditions, SP-A further stimulates IL-10 levels by 5-fold. Similarly, SP-A protects the foetus from an inflammatory attack by suppressing TNF-α levels by 5-fold under inflammatory conditions. This suggests SP-A to play a vital role in the suppression of inflammation, increase in the anti-inflammatory threshold and provide protection in its trimeric form. SP-A protects the foetus by increasing levels of TGF-β under non-inflammatory conditions i.e during pregnancy, to promote vascular growth as low levels of TGF-β have shown to be associated with preeclampsia. An increase in TGF-β may suppress TNF-α expression by macrophages and indirectly suppressing PG production as TNF-α promotes PGF2α expression, again SP-A suggests to protect human foetus from attack.

Our studies to date have shown SP-A to modulate macrophages via TLR-2 ligand suppression, especially by SP-A under inflammatory conditions by 5-fold. SP-A suppress TLR-2 expression (confirmed by previous studies in mice -Agarwal et al, 2013)

suggesting SP-A down regulates TLR-2 pathway induced inflammatory response. TLR-4 on the other hand, appears to be less affected by SP-A and SP-D suggesting they protect the foetus against infection from gram negative bacteria. Furthermore, IL-10 increases TLR-4 levels, therefore SP-A and SP-D could increase TLR-4 indirectly.

Factor H expression increased by 8 fold, compared to normal, although there was a low level of factor H gene present, compared to normal, the little increase shows a big difference, hence a higher fold change compared to negligible amounts of factor H in non-treated samples. Factor H appeared to increase slightly when stimulated with nSP-A + IL-1β, nSP-D, nSP-D + IL-1β, rfSP-A + IL-1β and rfSP-D.

SP-D levels were mostly suppressed with SP-A treatments (full length and recombinant fragments) with and without IL-1β. However, the suppression is less with SP-D treatment (with and without IL-1β), but sharply increases with rfSP-D with IL-1β treatment. On the other hand, SP-A levels were consistently low regardless of treatment, especially with SP-A and SP-D treatment, suggesting a negative feedback mechanism to regulate surfactant levels.

Treatment of fetal maternal tissue with nSP-A + IL-1β, protein levels of C1q were detected the highest, followed by factor H, then SP-D and lastly SP-A. C1q levels increases most with rfSP-A + IL-1β and increased slightly with nSP-A + IL-1β suggesting there is a strong pro-inflammatory effect controlled by C1q.

Progress in the prevention and management of preterm labour continues to be frustrated by our lack of understanding of the mechanism of parturition in women. Surfactant proteins SP-A and SP-D are biologically active proteins secreted by the fetal lung into the amniotic fluid, which appear to have important roles in the onset of labour by controlling the activation of the prostaglandin cascade in the fetal membranes and decidua. Thus, we used natural and recombinant forms of human SP-A and SP-D and measure their effect on prostaglandin and cytokine release by ACD explants. These experiments provided insight into paracrine events in the maternal/fetal interface that may be involved in the onset of labour at term, and highlight possible pathophysiological alterations in preterm labour. Although to identify possible alterations in spontaneous preterm labour was not the main thrust of the current study,

the specific aims of the study was designed to establish the role of SP-A and SP-D in the regulations of cytokine and prostaglandin release by ACD in relation to parturition.

Birth defects and preterm birth contribute significantly to perinatal health and are the leading causes of infant mortality. The association between birth defects, preterm birth and low birth weight has been well documented, but the explanation for this association remains unknown (Dolan et al., 2007). Effective measures to reduce birth defects and preterm birth are desperately needed, but this requires continued research to understand the pathophysiological mechanisms and the shared risks factors of these adverse neonatal conditions. Moreover, the relative risks of cerebral palsy, mental retardation and psychiatric disorders in preterm survivors increase exponentially with decreasing gestational age at birth (Moster et al., 2008). Alterations in the timing of birth per se are the leading, but potentially preventable, cause of neonatal mortality and morbidity. Thus, there is an urgent need to investigate the mechanism of spontaneous preterm labour, which may or may not result from the same endocrine and intracellular pathways as the physiological onset of labour at term.

The onset of labour is the result of cascade of intrauterine biochemical events that culminates in cervical softening and dilatation and increased uterine contractility. Lopez Bernal et al, have proposed that fetal surfactant (a mixture of lipids and apoproteins) is a signal for parturition because it provides a link between fetal lung maturation which is essential for extrauterine life, and the onset of labour through stimulation of prostaglandin production. It has been shown that amniotic fluid surfactant, which reflects fetal lung maturation, is an important intrauterine source of arachidonic acid, and increases the rate of prostaglandin synthesis in the fetal membranes (Bernal and Phizackerley, 2000). This effect is due to the release of fatty acids, including arachidonate, from the lipids of fetal surfactant by the sequential action of phospholipase C and diglyceride lipase activities in amniotic fluid and by the transfer of arachidonate from surfactant phosphatidylcholine to phosphatidylethanolamine and phosphatidylinositol in amnion cells (Bernal and Phizackerley, 2000). These lipid pathways provide a link between the process of maturation of the fetus in preparation for birth and the labour-initiating prostaglandin activation.

Moreover, in addition to the effect of its lipid components, fetal surfactant is likely to be involved in the process of parturition through the effects of the surfactant associated

proteins SP-A and SP-D (Kishore et al., 2005). SP-A and SP-D concentrations in amniotic fluid rise sharply from about 26 weeks gestation to term and the proteins are found in the fetal membranes and decidua (Miyamura et al., 1994). The evidence in support of a likely role of SP-A and SP-D in pregnancy and parturition are as follows: (i) SP-A and SP-D perform immunomodulatory functions in the lungs via macrophage activation, control of inflammation, recognition and clearance of microbes, apoptotic and necrotic cells. (ii) SP-A interacts with toll-like receptors TLR-2 and TLR-4 on macrophages and increases the expression of interleukin IL-1β and NF-κB (Crouch and Wright, 2001). (iii) In mice, intra-amniotic injection of SP-A provokes premature delivery by a process which involves NF-κB activation with pro-inflammatory outcomes (Condon et al., 2004). (iv) SP-A has direct stimulatory effects on human myometrial cells through activation of NF-κB and mitogen-activated protein (MAP) kinase pathways (Garcia-Verdugo et al., 2008). (v) SP-D has been shown to modulate expression of matrix metalloproteases for tissue remodelling and macrophage homeostasis. (vi) SP-D binds DNA from bacteria and on the surface of dying cells, enhancing the clearance of nucleic acids and limiting inflammatory reactions at sites of infection or apoptosis. (viii) SP-D is localised in the endometrium of the secretory phase and in placental villi and may have a role in the prevention of intrauterine infections at the time of implantation and during pregnancy (Leth-Larsen et al., 2004). This is important because infection-associated preterm labour is associated with the worst perinatal outcomes.

A possible role of surfactant proteins SP-A and SP-D in modulating the induction of proteins involved in the prostaglandin pathway being reported here is a novel and very important aspect of the study. Prostaglandin synthesis in fetal membranes and decidua is a key event in parturition and is driven by the availability of arachidonic acid which is converted to $PGH_2$ by the cyclooxygenases (COX-1 and COX-2) (Olson, 2003). The production of PGs of the D, E and F series depends on specific terminal PG synthases. Moreover, detailed temporal in vivo studies in animal models and women suggest that a critical step for the onset of labour is a relative increase in intrauterine PGF release, with lower or unchanged production of PGE2, PGD2 and prostacyclin. This suggests that changes in the availability of PGF synthase may be a key step for parturition. The human prostaglandin transporter (hPGT) regulates the transfer of PGs across the cell membrane and has an important role in promoting the decidualization of human endometrium (Kang et al., 2006). Prostaglandin dehydrogenase (PGDH) is the major PG

catabolic enzyme in choriodecidua and may be a target for regulatory control of PG availability at parturition. We used real-time PCR probes for these key genes and suitable antibodies to measure their expression at the protein level. The purpose of this study was to determine the direct effect of highly purified natural and recombinant forms of surfactant proteins SP-A and SP-D on the activation of the prostaglandin cascade by pro-inflammatory stimuli IL-1β that is a good representation of response induced by bacterial products (LPS) and pro-inflammatory mediators at the interface of the decidua-fetal membranes. There is a strong association between infection (chorioamnionitis) and preterm labour, and it is likely that inflammatory mediators are involved in the preparation for parturition by promoting cervical ripening and increased myometrial contractility.

In summary, IL-1β dampens the effect of IL-8, TNF-α, TGF-β, TLR-2, TLR-4, SP-A1 and SP-D expression when stimulated by rfSP-D. However, SP-A appears to upregulate COX-2/PTGS2 levels of expression suggesting its involvement in setting up PG pathway. This is coupled with up-regulation of IL-10 by SP-A which may raise the threshold of antigen sensitisation and promote a regulatory, anti-inflammatory environment. Thus, in addition to participating in labour, SP-A is likely to create an immunologically safe environment for the foetus. SP-D appears to have a homeostatic role during pregnancy. This is explained by a massive surge in SP-A levels prior to labour and birth while the level of SP-D does not get affected.

# Chapter 5:

# Localisation, identification and immunological characterisation of decidual macrophage in term placenta

## Summary

As evident from the previous chapter, SP-A and SP-D being resident within placentae can have remarkable and distinct effects on the pro- and anti- inflammatory response which may have directly or indirectly influenced the PG pathway. Thus, here we sought to examine the localisation of SP-A and SP-D in various constituents of placentae including, amnion, chorion and decidua membranes, placenta, umbilical cord and myometrium. Since calreticulin (CRT)-CD91 complex is considered to be a bonafide candidate receptor for the collagen region of SP-A and SP-D, we also examined the concomitant localisation of human calreticulin. Having established the presence of these proteins and receptors in placental tissues/membranes, we wanted to localise the cellular component responsible for immune activity and prostaglandin production. The obvious target cell was DM which is one of the most abundant cells in 2$^{nd}$ trimester of pregnancy. Thus, DMs were sequestered from decidual tissue in cell suspension and analysed for CD14 positivity. Their response to LPS challenge via pattern recognition receptors like TLR were also examined using production TNF-α as a read-out, here we show, that a recombinant fragment of SP-D composed of homotrimeric neck and lectin domain, can suppress TNF-α production by LPS induced DM in a dose and time-dependent manner. These read-outs are consistent with our observations when IL-1β activated ACD explants over expressed TNF- α, which in turn, were suppressed by recombinant proteins. Thus, modulation of DMs by SP-A and SP-D is likely to play an important role in suppressing or keeping homeostasis under conditions of inflammation.

## 5.1 Introduction

### *5.1.1 Decidual macrophages in pregnancy and labour*

Decidual macrophages (DMs) constitute an important component of the innate immune system in the third trimester. Responsible for intrauterine defence mechanism, DMs protect the fetus against infection which could lead to preterm labour. The decidua is part of the maternal endometrial tissue during pregnancy which is shed during parturition. The decidua hosts a number of immune molecules such as macrophages, natural killer cells (NK), granulocytes and B and T lymphocytes (Bulmer et al., 1988, Vince et al., 1990). At term, 47% of cells are of bone-marrow origin, 18% of which constitute of macrophages (Vince et al., 1990), 20-30% are local leukocyte population located at the implantation site during the third trimester (Vince et al., 1990, Heikkinen et al., 2003) and NK cells which are constant throughout pregnancy (Mor and Abrahams, 2003). However, full understanding of the roles played by each of these cells is very important. DMs play a big role in the recognition and clearance of infection (table 5.1), where infection is the cause of more than a third of preterm births between 23 and 36 weeks gestation (Lettieri et al., 1993). Bacterial products such as LPS entering the amniotic fluid stimulates phospholipase A2 production from DMs, which leads to the production of prostaglandin E2 and F2α (potent stimulants of labour) (Casey et al., 1989). In response to an infection, DMs can produce inflammatory cytokines such as IL-1β, TNF-α and IL-6 (figure 5.1). The early onset activity of DMs can therefore lead to preterm birth (McGregor et al., 1988). Studies of the role of DMs in intrauterine immunity have been examined through their interaction with yeast zymosan and pathogenic bacteria resulting in phagocytosis, release of superoxide in respiratory burst and production of pro-inflammatory cytokines such as TNF-α (Singh et al., 2005). This suggests that the major immune cell constituent of decidua, DMs, are responsible for the clearance of infection and production of TNF-α which selectively stimulates production of prostaglandin F2α by human DMs (Norwitz et al., 1991). Thus, failure of DMs in the clearance of infection could lead to preterm birth. Previous studies have also shown, isolated macrophages from amniotic fluid from pregnant mice secrete IL-1β and activated the NF-κB pathway in response to SP-A which could activate uterine activation and labour (Condon et al., 2004) and as it is known the levels of SP-A rise sharply towards term (Miyamura et al., 1994), therefore, we sought to examine the effects of SP-A on human DMs.

***Table 5.1 – Ligands and receptors on macrophages and its immunomodulatory functions to emphasise the importance in pregnancy and labour***

| Ligand/receptor on DMs | Function | Reference |
|---|---|---|
| **B7-H1** | Co-stimulatory molecule binds to corresponding receptor PD-1 to negatively regulate T-cell function by suppressing IFN-γ production. Present in early pregnancy not at term. Stimulated by IFN-γ Possible role in regulating local IFN-γ during pregnancy. | (Sayama et al., 2013) |
| **Secretion of TGF-β** | Prevents lytic activity after decidual NK cell killing extravillous cytotrophoblast (CTB) and macrophage phagocytose to clear the cellular debris for successful pregnancy. | (Co et al., 2013) |
| **FasL ligand** | Increases with corticotrophin-releasing hormone (CRH) in vitro. Expressed on decidual macrophages. CRH levels higher in preeclamptic patients to induce apoptosis of extravillous trophoblasts (EVT) disturbing placentation | (Petsas et al., 2012) |
| **Class A scavenger receptor** | Recognition receptor for *Clostridium sordelli* for phagocytosis | (Thelen et al., 2010) |
| **PGF2-α** | Secreted by DMs stimulated by TNF-α in human term decidua. | (Norwitz et al., 1992a) |
| **Prostaglandin D2 (PGD2)** | Major source of PGD2 production by bone-marrow defined macrophages in decidua. HLA-DR had the highest PGD2 production rate and not influenced by labour. | (Norwitz et al., 1992b) |
| **TNF-α** | Secreted by decidual macrophages (and chorionic and decidual villous tissues) at term. | (Vince et al., 1992) |
| **HLA-DR** | Expressed in DMs and are of maternal origin, those expressed on macrophages in amnion and chorionic villi are of fetal origin | (Sutton et al., 1983) |

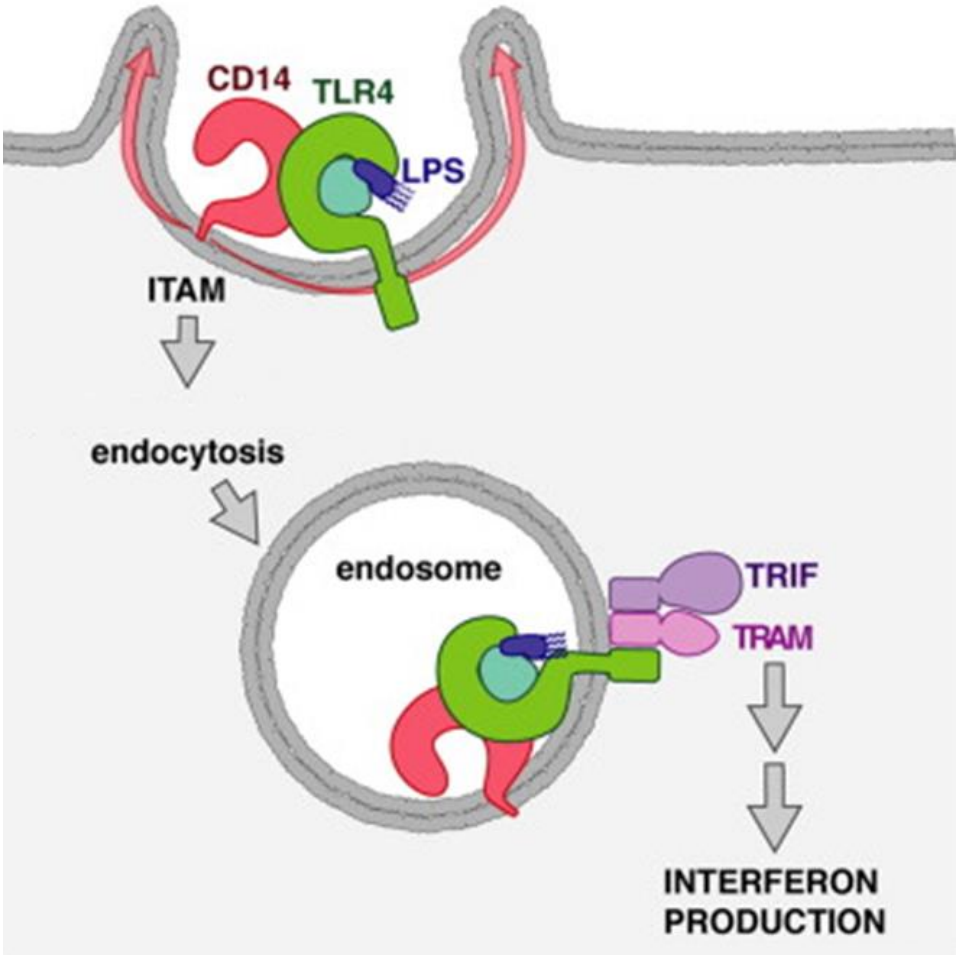



***Figure 5.1 – function of receptor CD14 expressed on DMs for LPS recognition to promote TLR-4 induced endocytosis and interferon expression.*** *Inflammatory mediators such as LPS stimulates up-regulation of CD14 receptors on macrophages to secrete inflammatory cytokines to induce an inflammatory response (Zanoni et al., 2011).*

### *5.1.2 Immune modulations of decidual macrophages during pregnancy and labour*

There is a fine balance which needs to be met for pregnancy progression and preventing pregnancy complications and miscarriages. The semi-allogenic fetus carries half its genes from the father; therefore, the uterine environment is in an immunocompetitive state to achieve fetal tolerance at the maternal/fetal interface. The maternal fetal interface is the maternal tissue interacting with fetal trophoblasts in the decidua (Lewis et al., 1993). The decidua, mainly the decidua basalis is infiltrated with large number of leukocytes, including macrophages, dendritic cells and natural killer cells (Loke and King, 2000). The cells interact with fetal trophoblast cells throughout pregnancy. These molecules play pivotal roles in pregnancy and the inflammatory pathway of labour. Macrophages make up 20-30% of decidual cells from the time of implantation and throughout pregnancy (Vince et al., 1990). Macrophages are responsible for vascular growth and placental development. Amongst these functions consists of effector functions, including removal of infection causing pathogens and tumour cells, thus, producing an inflammatory or anti-inflammatory milieu depending on its stimulus. Macrophages are present in one of two states, represented as M1 or M2. Activation of macrophages to a pro-inflammatory, M1 phenotype occurs through Th1 cytokines such as IFN-γ, TNF-α or bacterial LPS (Gustafsson et al., 2008). Inflammatory suppression can be achieved through anti-inflammatory cytokines including IL-4, IL-10 and IL-13 stimulating monocyte/macrophages to skew to an alternative activated, M2 phenotype (Gordon and Taylor, 2005). Macrophages have also shown to be altered by its ligand binding, HLA-G antigens. Trophoblast cells express non-classical MHC class I, HLA-G antigens, which are ligands for leukocyte immunoglobulin-like receptors (LILR), present on all leukocytes as a membrane bound receptor, although soluble isoforms of HLA-G do exist. Although other HLA-I molecules can also bind to these receptors, HLA-G is the only HLA-I antigen which has a higher affinity for these receptors for antigen presentation to macrophages, DCs and NK cells for fetal tolerance (Apps et al., 2007). The HLA-G gene family was first identified by Geraghty et al in 1987 (Geraghty et al., 1987). Each molecule is composed of a heavy chain composed of 3 main domains; the α1, α2, α3, a transmembrane region and a cytoplasmic tail. The α3 chain is non-covalently bound to a β2-microglobulin which is a non-classical MHC encoded light chain (Hviid, 2006). Soluble HLA-G have been located in peripheral maternal blood, on placental trophoblasts, including villous cytotrophoblasts and syncytiotrophoblast, cord

blood and amniotic fluid (Le Bouteiller and Blaschitz, 1999). HLA-G is not highly polymorphic suggesting it has not gone under evolution pressure. Therefore, maternal and paternal HLA-G genes must be similar or identical. the HLA-G genes present antigens to leukocytes with higher affinity to other HLA molecules, to suppress the other ligands in presenting non-self-antigens, allowing the fetus to escape attack from maternal immune system (Hunt and Langat, 2009). HLA-G protects the fetus by inhibiting IFN-γ production by NK cells (Carosella et al., 2008), presenting antigens through APC to $T_{reg}$ cells in order to differentiate naïve T cells into $CD4^+$ and $CD8^+$ suppressor T cells (Carosella et al., 2008) and inducing apoptosis of activated $CD8^+$ T cells. HLA-G is involved in direct suppression of maternal immune cells such as $CD4^+$ T cells from stimulating B cells production of allo-antibodies (inhibiting complement activation) against fetal antigens by inhibiting proliferation and inducing T cell anergy (LeMaoult et al., 2004), thus, contributing to fetal tolerance. As mentioned earlier, macrophages are responsible for producing pro- and anti-inflammatory cytokines to protect the fetus. HLA-G molecules has shown to directly influence these levels by suppressing pro-inflammatory Th1 cytokines including IL-2, TNF-α and IFN-γ and stimulates pro-inflammatory Th2 cytokines including IL-4, IL-10 and IL-13 (Le Bouteiller et al., 2003). The influences of hormones, cytokines, chemokines and pattern recognition receptors (PRRs) regulates the phenotype and response of macrophages in the decidua (Stout and Suttles, 2004) to a M2 phenotype for protection in the host and for tissue repair (Varin and Gordon, 2009).

### *5.1.3 NK cells at the decidua during pregnancy*

The decidua is also infiltrated with a high number of NK cells amongst other active leukocytes. Maternal decidual NK cells (dNK cells) express polymorphic killer Ig-like receptor (KIR) genes which recognise HLA-C expressed on trophoblast cells (figure 5.3), stimulating GM-CSF which aids in trophoblast migration for the development of placentation (Xiong et al., 2013). HLA-G can also down regulate decidual NK cell activity through direct interaction with inhibitory receptors on decidual NK cells (Hviid, 2006) (figure 5.3). Co-culture experiments of cells with HLA-G positive cells resulted in increased production of IL-10 from DCs (Apps et al., 2007) and macrophages (McIntire et al., 2004). HLA-G can also bind to macrophage cells through inhibitory receptors

Human inhibitory receptors Ig-like transcript 2 (ILT2) and 4 (ILT4) down-regulating the migration of dendritic cells to prevent antigen presentation to naïve T cells (Liang et al., 2008) preventing T cell mediated cell lysis (Hviid, 2006, Rouas-Freiss et al., 1997). Activation of NK cells results in the release of pro-inflammatory cytokine IFN-γ, which activates macrophages causing them to come into close contact with trophoblast cells expressing HLA-G, and these can interact with ILT2 and ILT4 receptors on macrophages providing inhibitory signals for apoptosis and cell lysis and modulate the production of cytokines for Th2 cytokine bias, which would result in inflammatory suppression, macrophage recruitment and T and B lymphocyte tolerance, suggesting the importance of HLA-G to be important in protection of fetal tissues against maternal immune attack.

Placentation is important for fetal survival and inadequate invasion is associated with IUGR, preeclampsia and miscarriages (Brosens et al., 1977, Khong et al., 1986, Norwitz, 2006) (figure 5.2). Decidual NK cells have been shown to play an important role in human trophoblast invasion for successful pregnancies (Ashkar et al., 2003, Ashkar and Croy, 2001, Croy et al., 2003, Croy et al., 2002, Parham, 2004).

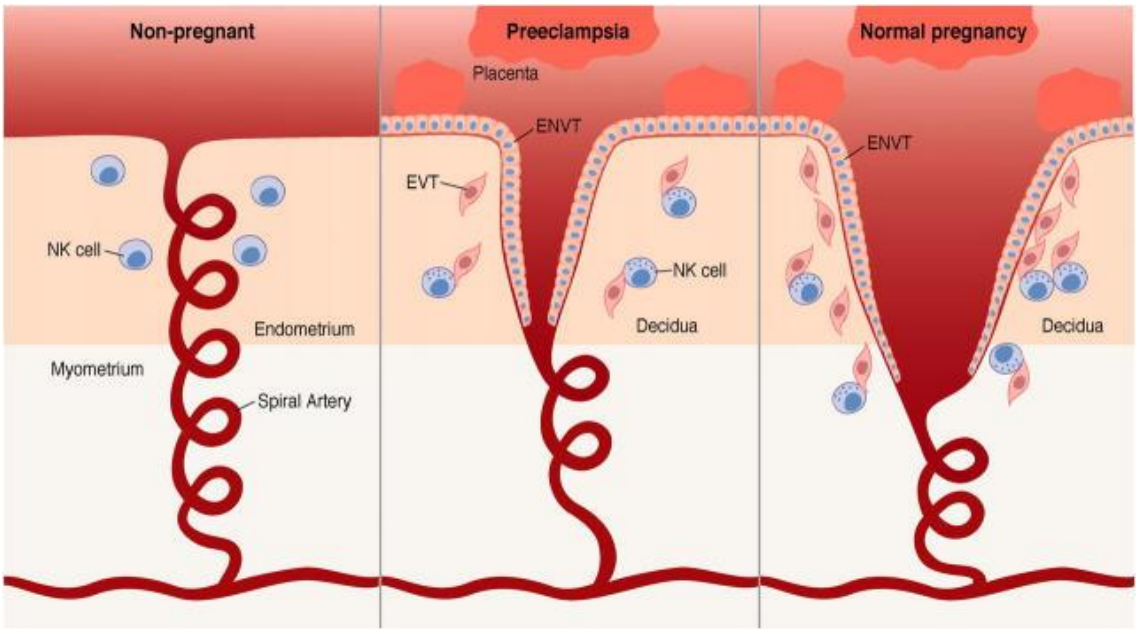



***Figure 5.2 – trophoblast invasion of normal and pre-eclamptic situations.*** *The spiral arteries are remodelled by trophoblasts and NK cells. The left image is of a non-pregnant endometrium in the secretory phase of the menstrual cycle just before menstruation. The right panel shows a normal pregnant endometrium with spiral arteries penetrating up to the endometrium. The middle panel shows a preeclamptic situation of the spiral arteries do no remodel to the depths of the myometrium and the large vessels become invaded with endovascular trophoblast cells (ENVT). Image taken from (Moffett-King, 2002).*

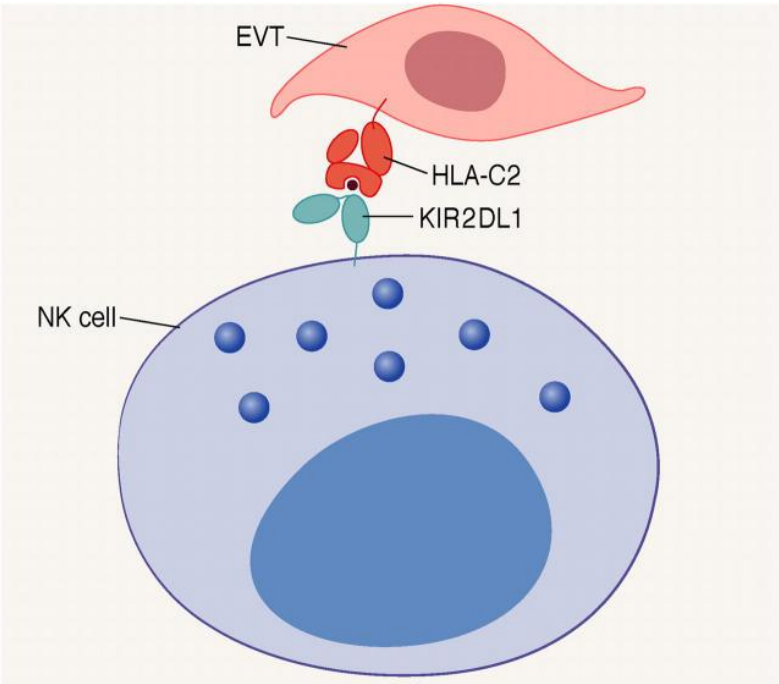



***Figure 5.3 – NK cells inhibiting HLA-C2 molecules recognised by EVT cells.*** *extravillous trophoblasts cells (EVT) expressing HLA-C2 (MHC-Class I receptor) interacting with HLA-C2 which is recognised by its inhibitor on NK cells, the KIR2DL1 receptor. This inhibition favours preeclampsia. Image taken from (Moffett-King, 2002).*

Decidual NK cells are key molecules expressing receptors and interacting with fetal cells and secreting cytokines, chemokines and growth factors. Decidual NK cells secrete IL-8 to regulate trophoblast invasion and interferon-inducing protein (IP)-10 have direct contact with fetal trophoblast cells to allow cell invasion for vascular growth (Lockwood et al., 2013). They also play an important role during pregnancy for angiogenesis for fetal development and survival (Li et al., 2001).

### *5.1.4 Leukocyte activation in pregnancy complications*

Leukocyte activity and regulation are fundamental for vascularisation to provide blood to the fetus, trophoblast invasion and maintenance of immunosuppression, as a defect would lead to pregnancy complications such as preeclampsia, IUGR and miscarriages. It has been reported in preeclamptic women and IUGR cases have a low level of macrophages secreting more pro-inflammatory cytokines including IL-1β, TNF-α and IFN-γ which can induce apoptosis in extravillous trophoblasts, restricting trophoblast invasion (Reister et al., 2001, Abrahams et al., 2004). Therefore in pregnancy loss, there

may be a higher populations of inflammatory cytokine-producing macrophages (Varin and Gordon, 2009).

### *5.1.5 DCs in decidua during pregnancy*

In humans, DCs have shown to migrate to the decidua basalis from as early as 9 weeks gestation (Schuster et al., 2009) (figure 5.5). As the antigens presented on trophoblasts are of fetal origin, antigen presenting cells mediating presentation of alloantigens is exclusively to maternal origin. hDC are found in low levels in decidua and unmarked high levels infiltrated in decidua basalis associated with preeclampsia in humans (Huang et al., 2008) and decidual T-cells clusters surround decidual DC regardless of placental pathologies (Kammerer et al., 2000) (figure 5.6). This suggests that constant antigen presentation to T-cell is common and with higher level of antigen presentation leads to increased chances of fetal rejection. Although the decidua is proposed to have a immunosuppressive nature, they do show to possess many immunocompetent markers such as $CD45^{+}$, $CD40^{+,}$ HLA-$DR^{++}$ and CD83 on decidual DC during early pregnancy (Kammerer et al., 2000). This has shown to be of particular importance for fetal skin development and skin immune maturation to exhibit a protective role against infections after birth (Schuster et al., 2009).

### *5.1.6 SP-A and SP-D as a modulator of macrophage functions*

SP-A and SP-D have shown to bind to receptors SIRPα, a signal-inhibitory regulatory protein α, controlling alveolar macrophage activity, suppressing macrophage phagocytosis and inflammation (Janssen et al., 2008, Gardai et al., 2003). During LPS stimulation, SIRPα can be post-transcriptionally modulated which results in reduction of SIRPα and increases macrophage inflammatory response (Zhu et al., 2013) (figure 5.4). Further studies have shown the ability of MMP-9 to cleave SP-D to inactivate its innate immune functions. As MMP-9 is over-expressed in several diseases such as asthma and cystic fibrosis, these patients can be more susceptible to pulmonary infection possibly due to MMP-9 contribution. However, cleaved SP-D and un-cleaved SP-D have shown to still bind to LPS (Bratcher et al., 2012) suggesting the CRD region was still intact, therefore, SP-D can communicate to macrophages through CRD region, but its effect will be much less. SP-D has also shown more efficient clearance of late

apoptotic cells in lungs through opsonisation by SP-D and clearance through alveolar macrophages without eliciting an inflammatory response. This suggests SP-D can clear late apoptotic cells whilst dampening the inflammatory effects of LPS (Litvack et al., 2010). It has recently been shown that despite increase in inflammatory cells, SP-D dampens LPS inflammatory effects through GM-CSF-dependent pathway during indirect lung injury in mice (King and Kingma, 2011). A bacterial respiratory pathogen, *Legionella pneumophilia* (*L. pneumophilia*) can replicate within macrophages to cause opportunistic infections. However it has been recently shown in mice models that SP-A and SP-D can bind to LPS on *L. pneumophilia* and blocks the growth of the pathogen providing protective measures (Sawada et al., 2010). SP-D functions in suppressing macrophage activity after LPS stimulation from bacteria is not located only in the lungs but also has been shown to suppress nitric oxide (NO) formation in the whole organism depending on the phenotype of macrophages (Atochina-Vasserman et al., 2009). It is important for SP-D to be in its correct oligomeric form, to fulfil its activity as previous studies have shown, coiled-neck and lectin domain of SP-D binding to LPS to dampen an inflammatory response to macrophages through TLR4/MD-2 complex. However, it is not as strong as native SP-D in inhibiting macrophage activation through LPS (Yamazoe et al., 2008). A study opposing the protective effects of SP-A in mice levels suggests LPS decreases TLR2 and 4, SP-A and SP-D expression leading to preterm birth. Moreover, there is evidence of SP-A expression in maternal uterus to suggest that this could have pro-inflammatory effects whereas the fetal surfactants from lung provide protective anti-inflammatory effects yet clearing infection (Salminen et al., 2008). SP-D has been shown to downregulate LPS through inhibition of CD14/TLR4 receptors (Liu et al., 2005, Senft et al., 2005). SP-D has been shown to regulate CD14 through MMP-12 and MMP-9, in SP-D deficient mice, MMP-9 and MMP-12 cleave CD14 into a soluble form and showed that with LPS stimulation, there was less TNF-α production (Senft et al., 2005). In this chapter, preparation and fractionation of DMs are being reported. Their response to LPS challenge in the presence or absence of SP-A and SP-D is also being reported.

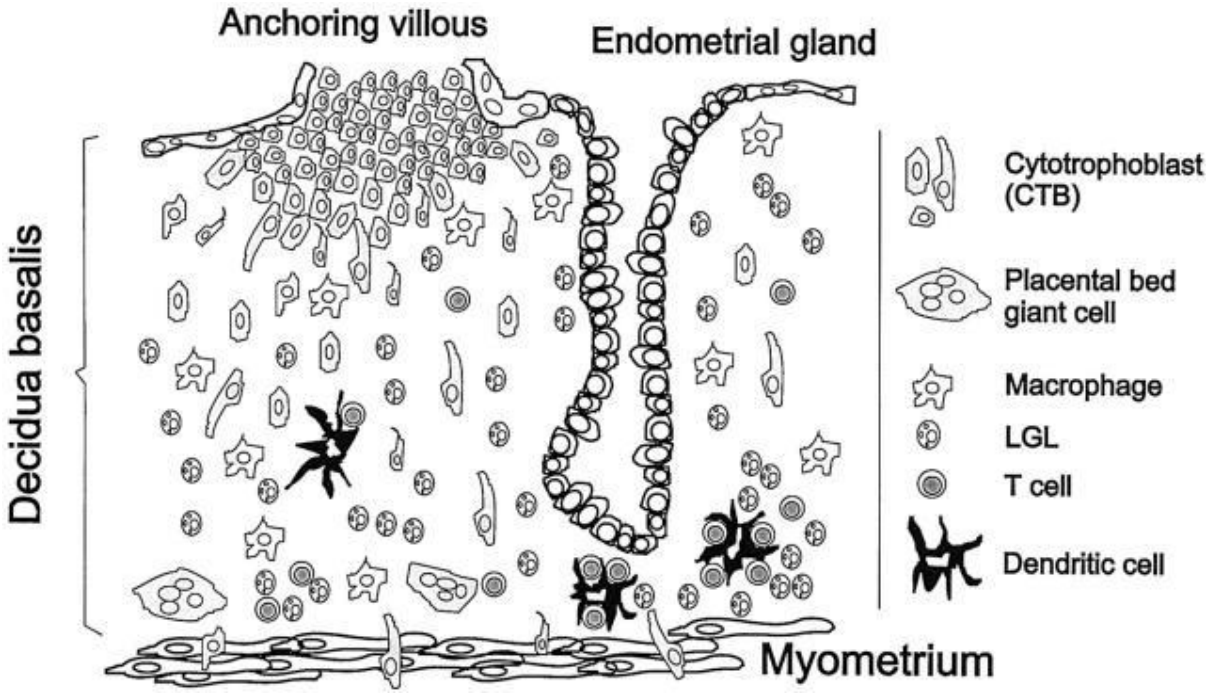



***Figure 5.4 – schematic diagram of anchoring villous with invading macrophages, T cells and dendritic cells.*** *Majority of the dendritic cells are located at the basal layer of the decidual basalis interacting with some T cells and decidual macrophages are scattered throughout the decidual layer. Image taken from (Tagliani and Erlebacher, 2011).*

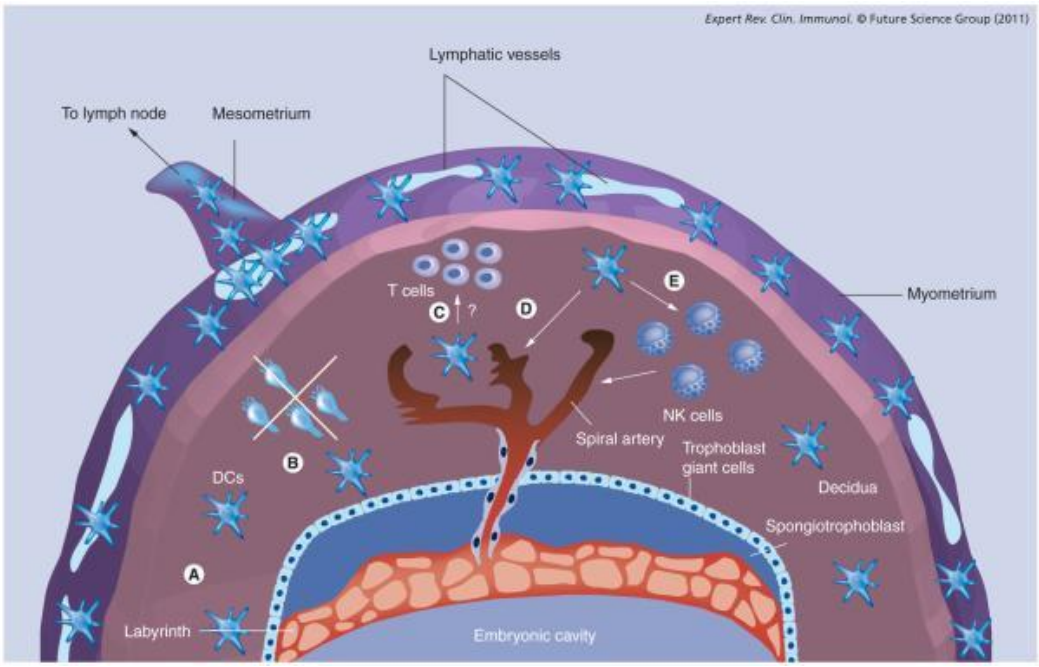



***Figure 5.5 – a schematic representation of the pregnant mouse uterus and the decidual cells, NK cells and T cells interaction at the maternal-fetal interface.*** *There are NK cells and DC cells interaction with T cells for trophoblast remodelling in the decidua. There is less DC in decidua for less exposure of alloantigens to T cells. The decidual cells share the same features as the human uterus. Taken from (Tagliani and Erlebacher, 2011).*

## 5.2 Methods and Materials

### *5.2.1 Preparation of histological slides for antigen retrieval*

The histology slides containing fresh ACD membranes, placenta, umbilical cord and myometrium were embedded in wax and sliced with a microtome to place on microscope slides for antigen detection by the histology team in Bristol University. Antigen detection was carried out using ABC system (Vector Labs, Peterborough, UK). The decidual cells and tissue slides were prepared as described by Singh et al (2005). The process of Immunohistochemistry involves heat-mediated antigen retrieval for immune-detection. Initially, the tissue sections were de-waxed by successive immersions in: histoclear twice, 100% ethanol, 90% ethanol, 70% ethanol followed by water for 5mins each. The endogenous peroxidase activity was blocked by immersion in 1% $H_2O_2$ for 15mins. For antigen retrieval, the slides were placed warm 0.01M Sodium citrate buffer (pH6.0) containing 0.05% Tween-20. After 15 minute incubation, slides were washed in PBS then blotted dry and placed at room temperature in a humidifying box to carry out the immunodetection process.

### *5.2.2 Immunohistochemistry (IHC) using ABC system (Vector Labs, Peterborough, UK)*

ACD membranes, placenta, umbilical cord and myometrium were stained for SP-A, SP-D and calreticulin (CRT), a bonafide receptor in complex with CD91 for SP-A and SP-D. This was carried out with the ABC system (VectorLabs, Peterborough, UK). Initially, 200µl of 2% normal horse serum in PBS was added to sections directly over the cells and incubated for 20mins, followed by washing in PBS. 100µl 1:1000 dilution of primary antibody (rabbit anti-human SP-A, SP-D and CRT) in 2% BSA/PBS was added to the cells and incubated at room temperature for 20 minutes, 1 hour in a humidifying chamber and overnight at 4°C. After 24 hour incubation, primary antibody was washed and slides were incubated in 100µl 1:1000 biotinylated secondary antibody (Horse-anti Rabbit) in PBS at room temperature for 1hour. Finally after 3 successive washes, slides were incubated in 150µl of ABC reagent (Vector Labs, Peterborough, UK) for 20minute at room temperature followed by another round of washing and developed by Incubating cell sections with 200µl of DAB (3,3'-Diaminobenzidine) for 2-10minutes.

After immunodetection of targets e.g. SP-A, SP-D and CRT, the slides were counterstained with haematoxylin. Briefly, the slides were immersed in haematoxylin for 1minute, washed, immersed in 1% sodium carbonate followed by immersion in 1% HCl in 70% ETOH (acid/alcohol) for 5mins at each step. The tissue sections were destained with acid/alcohol before running under the tap water until slides turn slightly blue. The tissue slides were dehydrated in successive washes of – 70%, 90%, 100% alcohol followed by clearing in histoclear I and II for 5mins each. For examining the slides, they were blotted dry before adding adhesive and a coverslip over the tissue. Images were taken using an Axioskop 2 microscope.

### *5.2.3 Human Decidual Tissue collection*

Term placentae were collected after spontaneous or caesarean delivery with informed consent and processed within 45 min of delivery. All women delivered healthy singleton infants.

### *5.2.4 Alkaline phosphatase–anti-alkaline phosphatase (APAAP) staining of decidual sections*

Rolls were made from amnion choriodecidua, as previously described (Sutton et al., 1986). Frozen sections were cut at 7 mm thick and fixed in acetone and blocked in 40 µl rabbit serum for 5 minutes. After washing thrice in PBS, slides were incubated with 50 µl monoclonal mouse anti-humanCD14 monoclonal antibody (Serotech, Oxford, UK) for 30 minutes. Slides were washed in washing buffer (50 mM Tris-HCl, 0.15M NaCl, pH7.6) for 2 minutes followed by incubation in rabbit anti-mouse serum (DAKO, Cambridge, UK) for 30 minutes and incubation in anti-mouse APAAP complex (DAKO, Cambridge, UK) for 30 minutes. Slides were stained in a solution containing 160 mM Napthol (Sigma, Dorset, UK) diluted in 200 µl Dimethylformamide (Sigma, Dorset, UK), 9.8 ml 0.1M Tris-buffer (pH 8.2), 1 mM Levamisole (Sigma, Dorset, UK), 1 mM Phe-Gly-Gly and 10 mg Fast Red (Sigma, Dorset, UK), for 15 minutes at room temperature. Slides were then counterstained with haematoxylin/eosin for 15 seconds. The same procedure was carried out with other targets of macrophage receptors. Please refer to table 5.2.

### *5.2.5 Decidual cell suspension from term placenta*

The decidual cell suspension was prepared as described by Vince et al (Vince et al., 1990). For each placenta, 2 to 9 g of decidual tissue was obtained, decidual cells was

scraped from fetal membranes and washed in Dulbecco's PBS to remove red blood cells. Decidual tissue was suspended in RPMI-1640 medium (10ml/g of tissue) supplemented with L-Glutamine (Life technologies LTD, Paisley, UK), 100 U/ml benzylpenicillin and 100 µg/ml streptomycin and incubated with Dispase II (5 mg/ml; from Bacillus polymyza; Boehringer Ingelheim Limited, Bracknell, UK) for 30 minutes at 37 °C. The digested tissue was washed in PBS, centrifuged at 600 x g, 5 minutes, and the pellet was resuspended in RPMI-1640 containing 0.5 mg/ml collagenase type IV (Sigma, Dorset, UK), 2 mg/ml hyaluronidase type I-S (Sigma, Dorset, UK), 50 µg/ml DNAse type IV and 10% v/v heat-inactivated fetal calf serum (FCS; Sigma, Dorset, UK) incubating at 37 °C for 1 hour. This was followed by centrifugation at 650 x g for 10 minutes and cells were syringed through a 40 µM filter and allowed to recover overnight at 4 °C in RPMI-1640 containing 10% v/v heat-inactivated FCS. To obtain live cells, the cell pellet was re-suspended in 25% Percoll, underlayered by 50% Percoll and centrifuged at 650 x g for 30 min. The live cells were in the interface and were washed in PBS and examined using trypan blue to count in a haemocytometer. The cells were washed and re-suspended in PBS containing 0.1% w/v bovine serum albumin (BSA) to adjust the concentration to 1 x $10^6$ cells per 50 µl.

### *5.2.6 CD14 staining with cytospin*

The slides and filters were inserted into the appropriate slots in the cytospin to allow cells to reach the slides. The cells were diluted in Tris buffered saline containing 0.1% w/v BSA to adjust 0.025 x $10^4$ cells per 100 µl and added to the slides quickly. The samples were cytospun at 150 x g for 6 minutes. After examining the slides to ensure the cells have annealed properly the slides were left to dry in a desiccation chamber overnight. The cells were stained for CD14 as described for FACS.

### *5.2.7 CD14-Antibody labelling for flow cytometry*

50 µl of Decidual cells were suspended in PBS containing 20 mM glucose and 5% v/v normal human serum (PGN) and were incubated with mouse anti-human CD14-phcoerythrin (RPME) conjugate (Serotec, Kidlington, UK) for 30 minutes at 4 °C. Cells were washed in PBS containing 20 mM glucose and 5% BSA (PGB) and resuspended in 300 µl PBS containing FCS and analysed by flow cytometry. To identify non-viable cells,

50 μg/ml propidium iodide was added to one tube. Mouse IgG isotype-RPE (Beckman Coulter, High Wycombe, UK) was used as a non-specific control.

### *5.2.8 Intracellular cytokine staining for CD14 and TNF-α*

Decidual cells were suspended in 1 ml RPMI-1640 supplemented with 10% v/v FCS and 100 μM monensin (Sigma, Dorset, UK) stimulated with 0.5 μg/ml LPS and were incubated in a non-adherent 24-well tissue culture plate at 37 °C in a 5% v/v $CO_2$ incubating chamber. The cells were then washed and incubated in anti-CD14-RPE for 30 minutes over ice then fixed using PBS containing 0.25% v/v paraformaldehyde and 2% w/v glucose for 10 minutes at 4°C. The cells were then permeabilised using PBS containing 0.1% saponin (Sigma, Dorset, UK) and 0.1% w/v BSA, incubating for 20 minutes at 4°C followed by incubation inn mouse anti-human TNF-α-FITC (Serotec) for 30 minutes over ice. Cells were washed again and then analysed by two-colour FACS. Decidual cells stimulated with 0.5 μg/ml LPS without monensin were used as a control. Each experiment was carried out 3 times and 2-3 placenta were required for one experiments to collect sufficient decidual cells.

## 5.3 Results

### *5.3.1 Localisation of SP-A and SP-D in fetal/maternal tissues from normal term patients*

IHC results of the same amnion, chorion and decidua (ACD) sections were stained with haematoxylin and location of SP-A and SP-D were detected with antibodies conjugated with biotin. The brownish detection is due to the biotin-avidin interaction. Figure 5.7 and 5.8 show ACD and placental sections respectively stained for SP-A, SP-D and CRT (Vandivier et al, 2002). The amnion sections show SP-A and SP-D to be present at equal levels. The chorion displays less SP-A and SP-D, however, the decidua again shows the greatest staining but more for SP-D than SP-A.

In addition, to localise SP-A, SP-D and CRT in ACD membranes, we looked at SP-D and CRT location in myometrium (figure.5.10), which is the maternal membrane. Results display a strong indication of SP-D localisation, however, the CRT expression appears fairly faint, suggesting that the maternal membrane may control the effects of SP-D on local tissue only in the suppression of inflammatory molecules to support the maintenance of pregnancy and avoid premature myometrial contractility. The umbilical cord displays similar results (figure.5.9). There is a strong indication of SP-A and SP-D staining with lower level of CRT expression, suggesting the proteins provoke an anti-inflammatory mileu protecting the fetus against blood borne pathogens entering via arterial canal.

### *5.3.2 Immunological properties of human decidual macrophages*

Decidual tissues and identification of decidual macrophages using the APAAP methods revealed CD14 macrophages within the decidual layer identification of decidual macrophages in decidual layer and amniotic membrane.

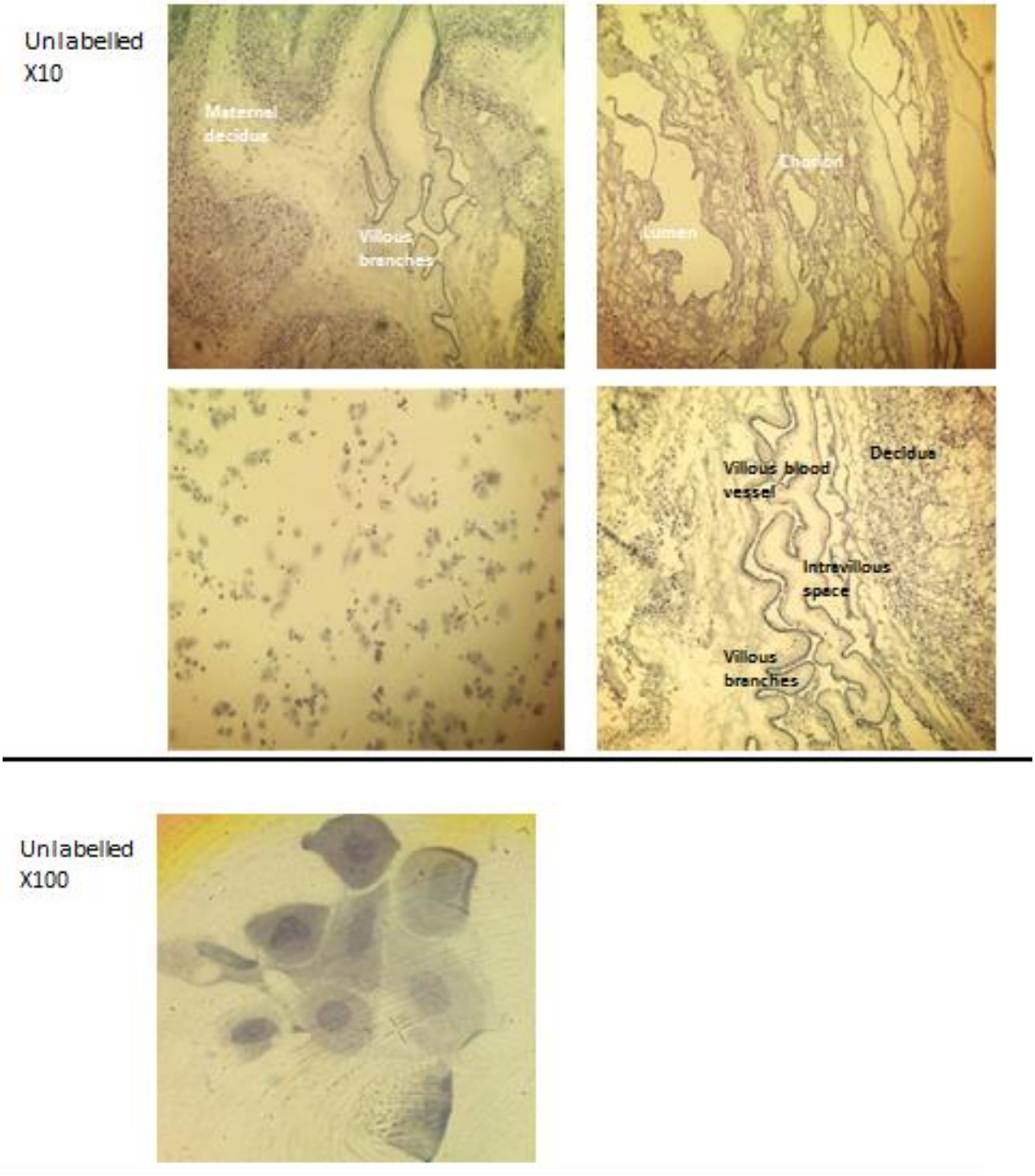



***Figure 5.6 – Unlabelled, negative control of placenta sections (x10 mag.) and decidual macrophages (x100.).***

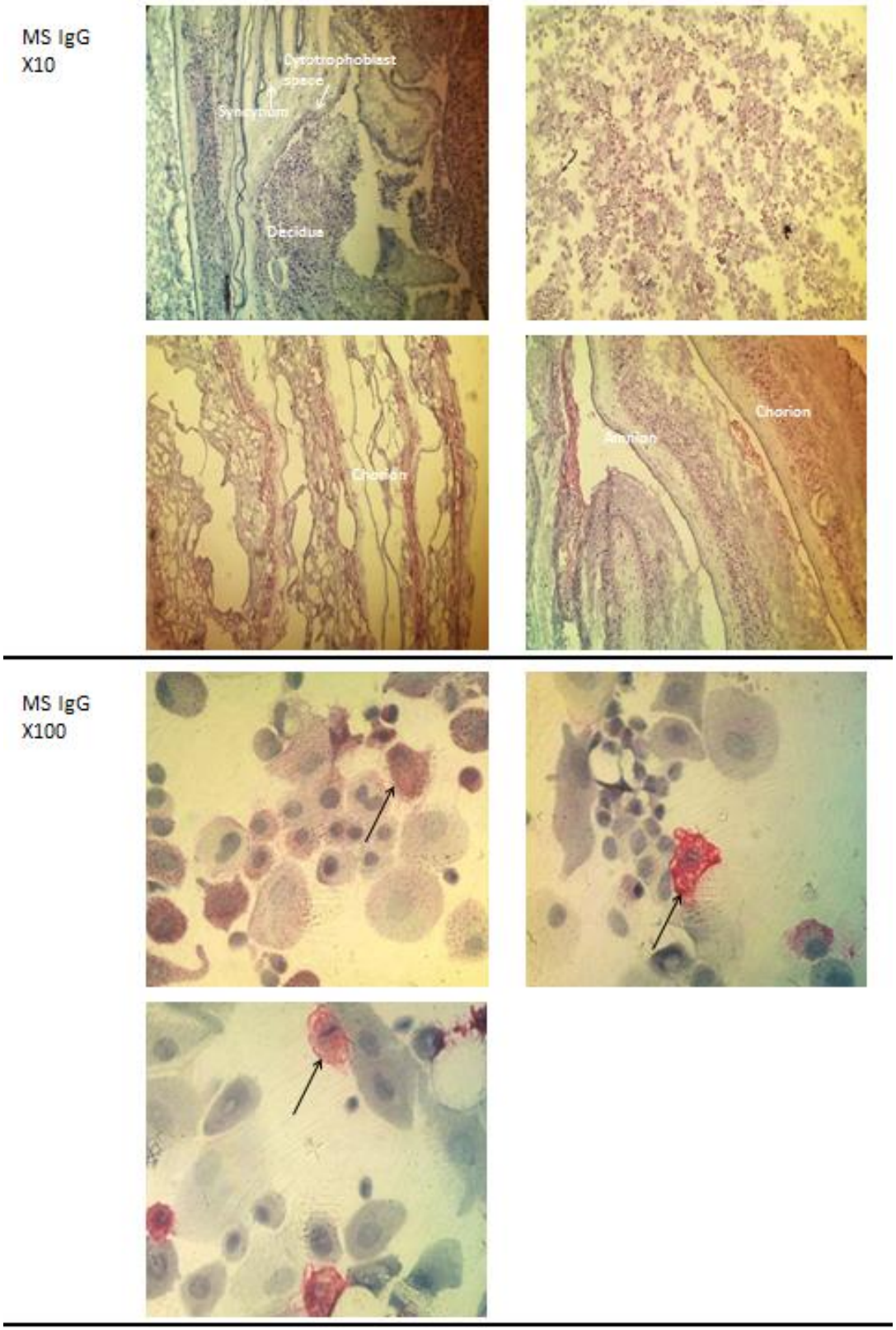
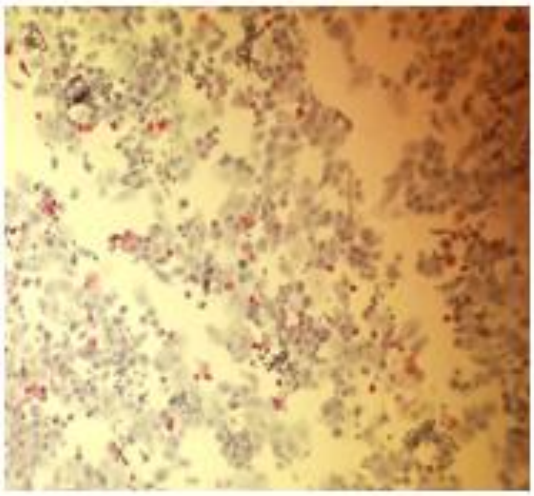



***Figure 5.7 – Mouse IgG labelled, positive control of placenta sections (x10.) and decidual macrophages (x100.).*** *A significant number of macrophages (stained red using an anti-CD14-conjugate), can be seen in the decidua (De), and to a lesser extent, amniotic (Am) and trophoblast (Tr) layers of the fetal membranes.*

CD14
X10



CD14
X100

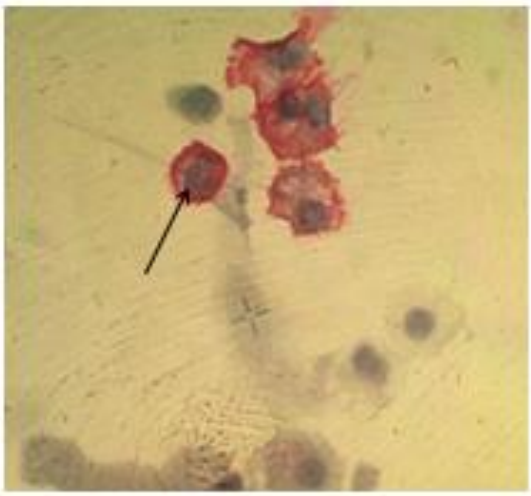


***Figure 5.8 – CD14 labelled placental sections (x10.) and decidual macrophages (x100.).***
*CD14 – surface antigen present on monocytes/macrophages and neutrophils, binds to bacterial lipopolysaccharides and delivers it to MD-2/TLR-4 complex to initiate an innate immune response for the removal of infection. CD14 lacks a cytoplasmic signaling domain and requires coupling to elicit a biological response. Binding to LPS and interactin with TLR4 to result in the production of cytokines and chemokines (da Silva Correia and Ulevitch, 2002) allowing phaocytosis of bacteria (Schiff et al., 1997), lipid transportation (Yu et al., 1997) and clearance of apoptotic cells (Gregory, 2000). Binding of soluble CD14, results in the down-regulation of monocyte/macrophage activation (Schutt, 1999, Bazil and Strominger, 1991).*

### *5.3.3 Effects of SP-A and SP-D on decidual macrophage TNF-α expression in the presence of LPS reduces TNF-a production compared to SP-A alone*

A number of antibody markers were used to show resident and perhaps infiltrated immune cells, in the ACD, and most predominating in the decidua (figure 5.7-5.22). There was a lack of SP-A staining in the decidua samples whereas SP-D was found to be in good amounts at the transcript level (figure 5.23). A number of resident DMs were found in the decidual tissue. These DMs were fractionated using CD14 positive cell and sorted suspension (figure 5.25a) following stimulation with LPS, DMs produced TNF-α, as expected, that peaked at 6 and 48 hours post-challenge. The double positive DMs (CD14 and TNF-α using anti-human CD14 and anti-human TNF-α antibodies) were visualised under microscopy.

We have shown local SP-A and SP-D production in term placentae (figure.5.23). After treating DMs with LPS there was clear up-regulation of TNF-α production from $CD14^+$ cells (figure.5.24). As seen in figure.5.25, SP-A and SP-D appears to suppress TNF-α levels with LPS, but SP-A up regulates TNF-α at lower amounts 5ug and SP-D down regulates TNF-α with higher amounts. Thus, SP-A could induce inflammation at higher levels but in the presence of infection, they suppress the activation of macrophages. Comparing lower and higher levels of SP-A with LPS shows that lower levels of SP-A provides dampening effects of TNF-α and at higher levels of SP-A, there is higher macrophage interaction that stimulates the levels of TNF-α production inducing inflammation. This is on the contrary to SP-D, which show no difference regardless of its level whereas in another situation, SP-D at a lower level has suppressive response to TNF-α and hardly any suppressive effects at higher levels of SP-D.

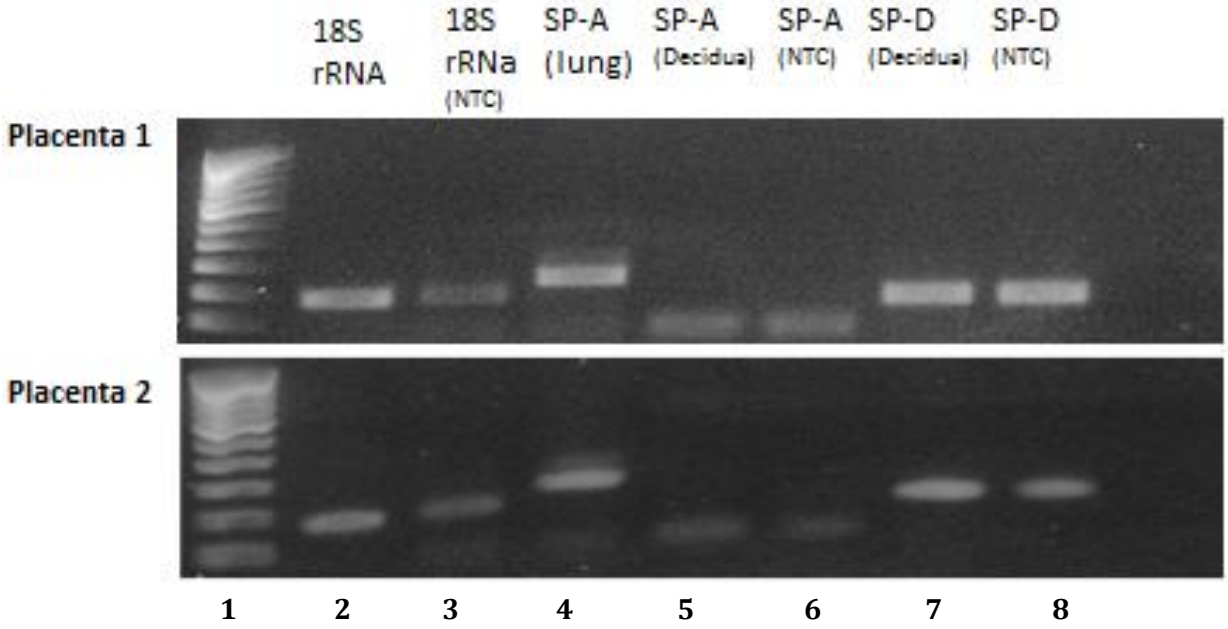



***Figure 5.9 - Real time RT-PCR amplified products separated by agarose gel electrophoresis.*** *Gene expression of SP-A & SP-D in the decidua at term in two placentae (1) and (2). One µg total RNA in decidua was used for RT-PCR. Lane 1: Ladder (100bp); Lane 2: 18s; Lane 3: 18s NTC; Lane 4: SP-A positive control (lung); Lane 5: SP-A decidua; Lane 6: SP-A NTC; Lane 7: SP-D positive control (lung); Lane 8: SP-D decidua; Lane 9: SP-D NTC.*

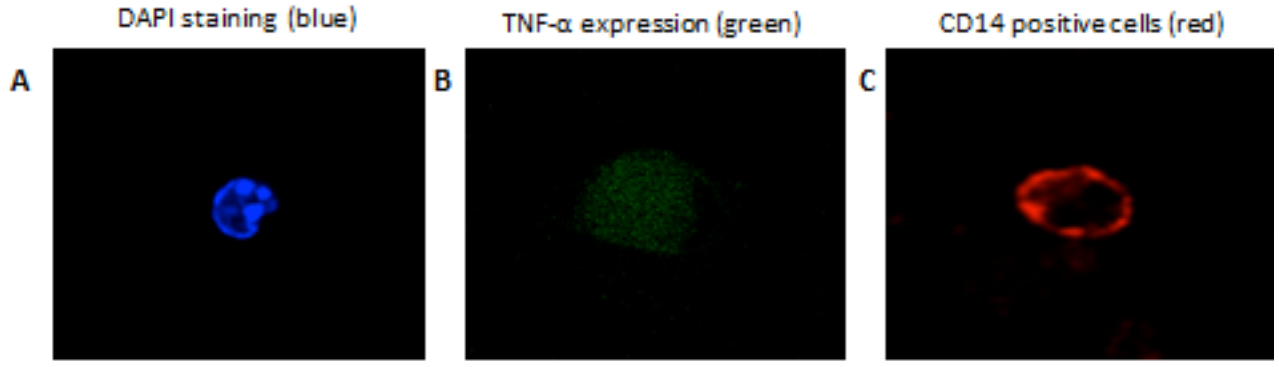



***Figure 5.10 – Confocal images on a Zeiss 510 meta microscope performed by counterstaining the decidual cells with DAPI.*** *a) CD14 positive cells (red); b) TNF-α expression (green) c) DAPI staining (blue).*

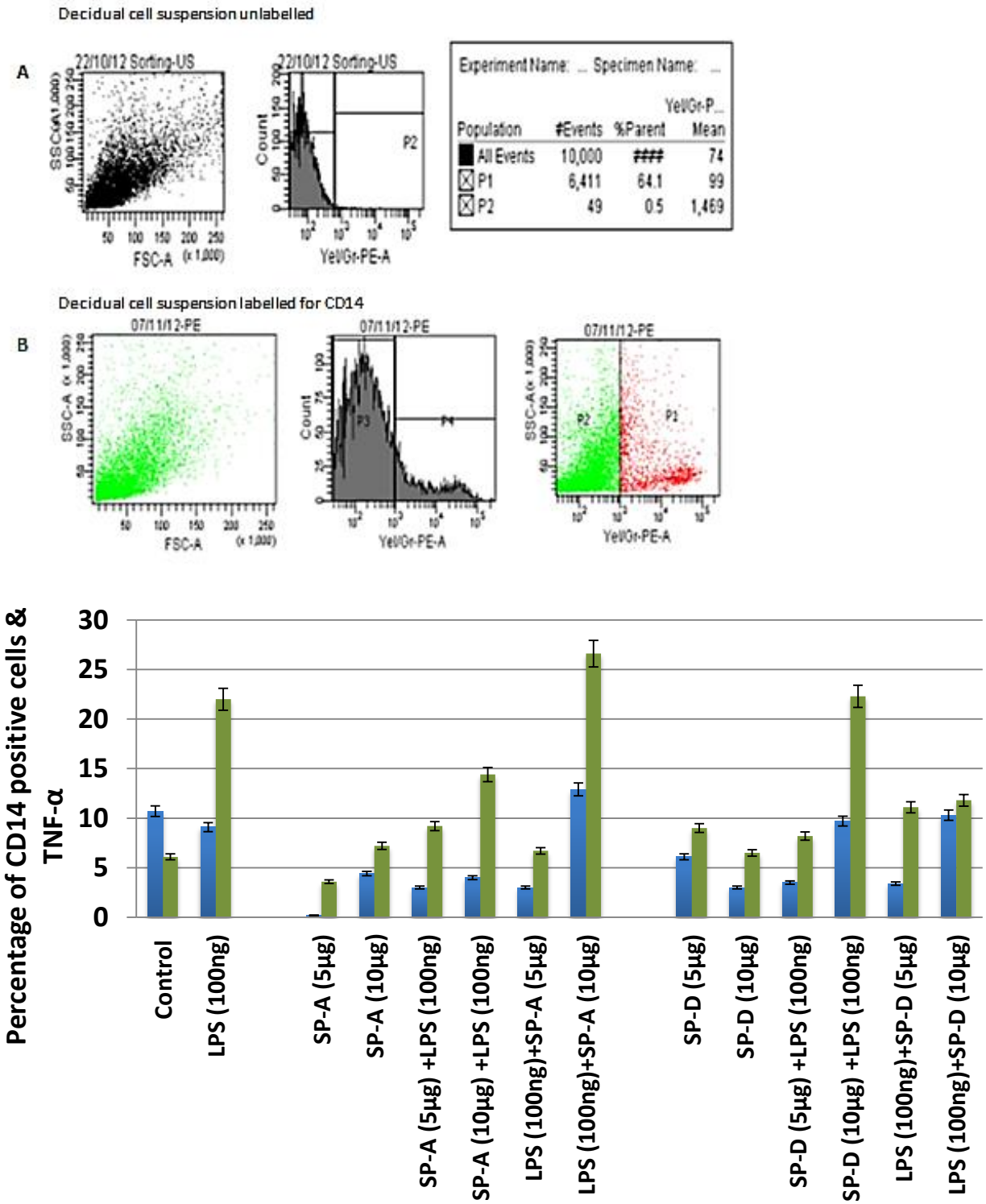



***Figure 5.11 – Effects of SP-A & SP-D on the production of TNF-α, pro-inflammatory cytokine by intracellular cytokine assay.*** *Decidual cell were isolated, and then stimulated with rfSP-A or rfSP-D (5 and 10µg) or LPS (100ng, derived E. coli strain 011:B4) for 1 hour. Percentage of CD14 positive cells and TNF-α cytokine was measured by two color flow cytometry. TNF-α was highly up regulated (2 fold) in the presence of LPS. Low dose of rfSP-A (5µg) &high dose of rfSP-D (10µg) more significantly down regulates (more than 3 fold) TNF-α production by macrophages. Similar down regulatory effect was also seen when decidual cells were pretreated with LPS followed by low-dose rfSP-A /rfSP-D (5µg) blue – TNF-α levels and green – CD14⁺ cells.*

## 5.4 Discussion

We have fractionated $CD14^+$ DMs in decidual cell suspension and examined the effects of LPS with and without SP-A and SP-D. The intracellular staining allows us to compare secretion of TNF-α in CD14 positive decidual cells. The stimulation with recombinant trimeric SP-D (rfSP-D) shows suppression of this inflammatory response to link to our previous data that shows that SP-D is an anti-inflammatory molecule important in the maintenance of pregnancy. This is consistent with our QPCR data showing that SP-A and SP-D (more SP-D) suppresses TNF-α to prevent an inflammatory response by modulating DMs. After showing the impact DMs have on stimulating PGF2α levels with and without SP-D suggests that DMs play an important role in modulating the inflammatory pathway in the third trimester. Therefore, DMs which play an important role in pregnancy and labour can be influenced by SP-A and SP-D. LPS can bind to TLR-4/CD14 complex to stimulate more CD14 expression thus increasing TNF-α levels to induce inflammation.

The mechanism of labour in women remains elusive and as a consequence the prevention and management of preterm labour is poor, resulting in potentially severe and lasting complications in the new born. Premature activation and disruption of decidual cells due to infection, haemorrhage or other stimuli may provoke preterm labour. The decidua provides a place of immune tolerance and also a line of defence to protect the fetus and the placenta from infections. Thus, we characterised resident macrophage populations in human decidua referred to as decidual macrophages (DMs) and their response to LPS, a non-self PAMP, and how that can be modulated in the presence of SP-A and SP-D, especially the production of pro-inflammatory cytokines such as TNF-α. We wished to investigate whether at parturition, DMs change to a classical phenotype characterised by the production pro-inflammatory cytokines (IL-1; TNF-α), which can impact upon the release of prostaglandin F which provokes uterine contractility through stimulatory FP receptors. We aimed at increasing our understanding of the local biochemical equilibrium that allows the maintenance of pregnancy, and will highlight pathways that increase the release of prostaglandin F by DMs leading to preterm birth.

According to literature, DMs are of immunosuppressive nature, perhaps SP-A and SP-D contribute to this suppression, as we have shown in our findings. In addition, during an infection which causes preterm birth or miscarriages (post-stimulation with LPS), SP-A and SP-D appear to suppress macrophages, however, they appear to secrete more pro-inflammatory cytokines without them, suggesting that DMs during normal pregnancy are of M1 phenotype and during an infection, macrophages switch to M2 phenotype causing a pro-inflammatory response as seen before (Guenther et al., 2012). As mentioned in earlier studies, the response from macrophages after SP-D stimulation depends on macrophage phenotype (Atochina-Vasserman et al., 2009).

It has recently been reported that SP-A suppresses inflammation via TLR-2 interaction. SP-A, an endogenous ligand for TLR-2 suppresses IL-1β and TNF-α in pregnant mice, preventing preterm birth and inflammation (Agrawal et al., 2013). We have also shown that SP-A down regulates TLR-2 in human ACD explants, which is further suppressed when treated with IL-1β, whereas, SP-D does not have a prominent effect. However, we have also shown that SP-A and SP-D up regulate TLR-4, as SP-A and SP-D can bind to TLR-4, inhibiting LPS-induced phosphorylation of p65 producing IL-1β, preventing inflammation (Rakhesh et al., 2012). This thus inhibits an LPS-induced inflammatory response. This mechanism may be relevant in IUGR, preeclampsia and miscarriages.

DMs make a large contribution to prostaglandin and cytokine release during labour due to their high expression of LPS and chemokine receptors (Norwitz et al., 1991, Bulmer and Johnson, 1984, Gordon and Taylor, 2005). Given the proximity of decidual tissue to myometrial smooth muscle cells, it is likely that release of prostaglandins and other inflammatory mediators in this area will have a direct effect on myometrial function.

DMs secrete TNF-α and are particularly sensitive in an autocrine fashion to TNF-α as a stimulus for PGF production (Norwitz et al., 1992a, Singh et al., 2005). Thus, modulation of LPS-induced TNF-a production by SP-A and SP-D can also alter the output of prostaglandin $F_{2\alpha}$ (PGF2α) by DMs in response to TNF-α in the presence and in the absence of surfactant proteins, something that needs to be examined.

DMs exhibits 'classically activated' (pro-inflammatory) or 'alternatively activated' (anti-inflammatory) phenotypes as a balance in immune reactions (Gordon and Taylor, 2005, Goerdt et al., 1999, Gordon, 2003). A population of alternatively activated DMs provides important immunosuppressive functions in early human pregnancy through the secretion of IL-10 and TGF-β (Lidstrom et al., 2003). It is thought that progesterone provides the stimulus for the alternatively activated status and for the inhibition of the pro-inflammatory phenotype of DMs (Kudo et al., 2004), either directly or through cross-stimulation of glucocorticoid receptors (Cupurdija et al., 2004). By contrast, classical macrophages, characterised by the production of pro-inflammatory cytokines (IL-1, TNF-α), in response to interferon (IFN-γ) or bacterial LPS, appear to be less prominent in normal first trimester decidua. Uterine natural killer (NK) cells are a dominant source of IFN-γ and DMs express IFN-γ receptors. The induction of classically activated macrophages at term may be associated with the onset of labour (Cupurdija et al., 2004, Heikkinen et al., 2003). In term decidua, nearly 50% of cells are of bone marrow origin, comprising up to 20% macrophages (Vince et al., 1990). Here, we have shown that DMs prepared from women delivered at term produce TNF-α when challenged with LPS. Moreover TNF-α and IL-1β have strong autocrine and paracrine effects on DMs, stimulating the release of PGF2α (Norwitz et al., 1992a).

In summary, we have localised DMs in term placenta and developed a method to generate decidual cell suspension that can be subjected to confocal microscopic and FACS analysis. Through intracellular cytokine analysis, LPS was shown to trigger TNF-α production by $CD14^+$ DMs. The cytokine expression was considerably reduced by co-incubation with recombinant SP-A and SP-D, suggesting that CRDs are capable of modulating interaction of LPS (PAMPs) with DMs surface PRRs that in turn can down regulate pro-inflammatory response, most likely involving NF-κB transcription factor.

# Chapter 6:

# General conclusions and future perspectives

## 6. General conclusion and future perspective

The immunological basis of foetus-mother interaction is still being investigated in terms of maintenance of pregnancy and parturition. Although a number of studies have examined a range of immune cells presented in various parts of the placenta, the importance of soluble factors which are capable of molecular pattern recognition and innate immune effector functions are beginning to be investigated. The interaction and importance of SP-A and SP-D in pregnancy and parturition become pertinent since there is a maternal as well as fetal component involved in terms of origin and biosynthesis of these two innate immune molecules. The presence of SP-A and SP-D in amniotic fluid, predominantly originating from fetal lungs and local production of SP-A and SP-D in maternal placental tissues appear to suggest that their functions may have dichotomous relevance during pregnancy. The constitutive level of SP-A and SP-D during pregnancy and massive up-regulation of SP-A biosynthesis prior to parturition appeared to suggest a link between these proteins and prostaglandin pathways involved during birth. There is plenty of evidence from murine work which have been extrapolated on human placental tissues in the current study.

Having established the above mentioned, experiments and evidence, it will be interesting to pursue a range of ideas in future that will validate the hypothesis arising from this thesis.

1. As highlighted in the Introduction, a number of miscarriages and preterm births are induced by intrauterine infections that itself can generate a robust pro-inflammatory response. Almost 30% of preterm births accounts for amniochorionitis. The current study has used IL-1β as a pro-inflammatory stimulus. Thus, the effect of a range of LPS from bacteria that have been shown to cause intrauterine infections during pregnancy need to be examined using ACD explants model system. We have attempted to mimic this context in the case of fractionated decidual macrophages; however, the interplay of various cells intact within the layers of amnion, chorion and decidua when exposed to bacteria or LPS will be very interesting.
2. The effect of the first recognition sub-component of the classical complement pathway, C1q, on ACD explants with and without pro-inflammatory stimuli will again be quite important to understand. The classical pathway activation and its

possible regulation by Factor H and factor H like molecules is likely to play an important role in the maintenance of pregnancy under inflammatory conditions due to infection, injury or stress.

3. Having established the ACD explant model to understand the relationship between surfactant protein SP-A and SP-D and immune cells, it will be worthwhile to examine how ACD explants compare between normal and IUGR, preeclampsia and miscarriages. These studies should also be extended to fractionated decidual macrophages.
4. Stimuli for the parturition could come from fetus and may affect the maternal tissue. We have looked at the levels of SP-A and SP-D expression in ACD gene and protein levels. Examining their as well as PG levels in the myometrium will be worthwhile. Whether PG levels is a rich source for PGF2α synthesis as it houses high level of DMs to stimulate myometrial contractility and timing of birth are obvious questions to answer. Clearly, differences between normal placentae and those coming from abnormal cases such as preterm, IUGR and miscarriages will help understand the importance of the roles of DMs and how influential they are in the presence and SP-A and SP-D.
5. IL-6, a pro- and anti- inflammatory cytokine, stimulates prostaglandin synthesis, PGE2 – a potent stimulator for myometrial contractility. IL-6 is responsible for lung maturation and SP-A synthesis. Thus, in chorioamnionitis cases, levels of IL-6 are high, and low in cases of RDS as lung have matured. This suggests SP-A could have an indirect effect on PG synthesis.
6. This study used IL-1β to stimulate PG synthesis and mimic inflammatory pathway to labour. It is known that IL-1β releases arachidonic acid. If IL-1β affects SP-A and SP-D biosynthesis, one can correlate if the levels of arachidonic acid released by IL-1β are proportional to SP-A synthesis.
7. Understanding the events in early parturition may lead to more effective therapeutic strategies. Leukocyte infiltration event occurs early in parturition and is 4-fold higher in term labour than non-infection induced preterm birth which is 2.5-folds higher, however neutrophils which were unchanged in term labour, increased from 5- to 53-folds higher in preterm with infection (Hamilton et al., 2012). Macrophages make up 20-30% of leukocytes and therefore could play a fundamental role in regulating parturition.

8. TNF-α has previously been shown to enhance both prostaglandin $E_2$ (PGE2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) (Rauk and Chiao, 2000). Therefore, if SP-A and SP-D can suppress TNF-α secretion levels in DMs, this could have an indirect effect on PGE and PGF synthesis - most potent PGs for inducing labour.

# 7. List of References


**ABBAS, T. M. & TOVEY, J. E. 1960. Proteins of the liquor amnii.** ***Br Med J,*** **1, 476-9.**

**ABRAHAMS, V. M., KIM, Y. M., STRASZEWSKI, S. L., ROMERO, R. & MOR, G. 2004. Macrophages and apoptotic cell clearance during pregnancy.** ***Am J Reprod Immunol,*** **51, 275-82.**

**ADELSBERG, B. R. 1983. The complement system in pregnancy.** ***Am J Reprod Immunol,*** **4, 38-44.**

**AGRAWAL, V., SMART, K., JILLING, T. & HIRSCH, E. 2013. Surfactant protein (SP)-A suppresses preterm delivery and inflammation via TLR2.** ***PLoS One,*** **8, e63990.**

**ALCORN, J. F. & WRIGHT, J. R. 2004. Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway.** ***Am J Physiol Lung Cell Mol Physiol,*** **286, L129-36.**

**ANTEBY, E. Y., JOHNSON, R. D., HUANG, X., NELSON, D. M. & SADOVSKY, Y. 1997. Transcriptional regulation of prostaglandin-H synthase-2 gene in human trophoblasts.** ***J Clin Endocrinol Metab,*** **82, 2289-93.**

**APPLEDORN, D. M., MCBRIDE, A., SEREGIN, S., SCOTT, J. M., SCHULDT, N., KIANG, A., GODBEHERE, S. & AMALFITANO, A. 2008. Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors.** ***Gene Ther,*** **15, 1606-17.**

**APPS, R., GARDNER, L., SHARKEY, A. M., HOLMES, N. & MOFFETT, A. 2007. A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1.** ***Eur J Immunol,*** **37, 1924-37.**

**ARCK, P. C. & HECHER, K. 2013. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health.** ***Nat Med,*** **19, 548-56.**

**ASHKAR, A. A., BLACK, G. P., WEI, Q., HE, H., LIANG, L., HEAD, J. R. & CROY, B. A. 2003. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy.** ***J Immunol,*** **171, 2937-44.**

**ASHKAR, A. A. & CROY, B. A. 2001. Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy.** ***Semin Immunol,*** **13, 235-41.**

**ATOCHINA-VASSERMAN, E. N., ABRAMOVA, E. V., TOMER, Y., SCOTT, P., NAZAROV, V. A., KRUGLOV, S. V., BEERS, M. F., GOW, A. J. & MALYSHEV, I. Y. 2009. SP-D-dependent regulation of NO metabolism in lipopolysaccharide-stimulated peritoneal macrophages.** ***Bull Exp Biol Med,*** **147, 415-20.**

**AWASTHI, S., COALSON, J. J., YODER, B. A., CROUCH, E. & KING, R. J. 2001. Deficiencies in lung surfactant proteins A and D are associated with lung infection in very premature neonatal baboons.** ***Am J Respir Crit Care Med,*** **163, 389-97.**

**BALLARD, P. L., LILEY, H. G., GONZALES, L. W., ODOM, M. W., AMMANN, A. J., BENSON, B., WHITE, R. T. & WILLIAMS, M. C. 1990. Interferon-gamma and synthesis of surfactant components by cultured human fetal lung.** ***Am J Respir Cell Mol Biol,*** **2, 137-43.**

**BAYER, H., BONNAR, J., PHIZACKERLEY, P. J., MOORE, R. A. & WYLIE, F. 1973. Amniotic fluid phospholipids in normal and abnormal pregnancy.** ***J Obstet Gynaecol Br Commonw,*** **80, 333-7.**

**BAZIL, V. & STROMINGER, J. L. 1991. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes.** ***J Immunol,*** **147, 1567-74.**

**BEERS, M. F., SOLARIN, K. O., GUTTENTAG, S. H., ROSENBLOOM, J., KORMILLI, A., GONZALES, L. W. & BALLARD, P. L. 1998. TGF-beta1 inhibits surfactant component expression and epithelial cell maturation in cultured human fetal lung.** ***Am J Physiol,*** **275, L950-60.**

**BENNETT, P. R., ELDER, M. G. & MYATT, L. 1987. The effects of lipoxygenase metabolites of arachidonic acid on human myometrial contractility.** ***Prostaglandins,*** **33, 837-44.**

**BERGER, P. J., SMOLICH, J. J., RAMSDEN, C. A. & WALKER, A. M. 1996. Effect of lung liquid volume on respiratory performance after caesarean delivery in the lamb.** ***J Physiol,*** **492 ( Pt 3), 905-12.**

**BERKOWITZ, R. L. 1993. Should every pregnant woman undergo ultrasonography?** ***N Engl J Med,*** **329, 874-5.**

**BERNAL, A. L. & PHIZACKERLEY, P. J. 2000. Fetal surfactant as a source of arachidonate in human amniotic fluid.** ***Prostaglandins Other Lipid Mediat,*** **60, 59-70.**

**BILLINGHAM, R. E., BRENT, L. & MEDAWAR, P. B. 2010. 'Actively acquired tolerance' of foreign cells. 1953.** ***J Immunol,*** **184, 5-8.**

**BLENCOWE, H., COUSENS, S., OESTERGAARD, M. Z., CHOU, D., MOLLER, A. B., NARWAL, R., ADLER, A., VERA GARCIA, C., ROHDE, S., SAY, L. & LAWN, J. E. 2012. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.** ***Lancet,*** **379, 2162-72.**

**BOGGARAM, V. 2003. Regulation of lung surfactant protein gene expression.** ***Front Biosci,*** **8, d751-64.**

**BOGGARAM, V. & MENDELSON, C. R. 1988. Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung.** ***J Biol Chem,*** **263, 19060-5.**

**BOGGARAM, V., QING, K. & MENDELSON, C. R. 1988. The major apoprotein of rabbit pulmonary surfactant. Elucidation of primary sequence and cyclic AMP and developmental regulation.** ***J Biol Chem,*** **263, 2939-47.**

**BOGGARAM, V., SMITH, M. E. & MENDELSON, C. R. 1989. Regulation of expression of the gene encoding the major surfactant protein (SP-A) in human fetal lung in vitro. Disparate effects of glucocorticoids on transcription and on mRNA stability.** ***J Biol Chem,*** **264, 11421-7.**

**BOGGARAM, V., SMITH, M. E. & MENDELSON, C. R. 1991. Posttranscriptional regulation of surfactant protein-A messenger RNA in human fetal lung in vitro by glucocorticoids.** ***Mol Endocrinol,*** **5, 414-23.**

**BOHLSON, S. S., FRASER, D. A. & TENNER, A. J. 2007. Complement proteins C1q and MBL are pattern recognition molecules that signal immediate and long-term protective immune functions.** ***Mol Immunol,*** **44, 33-43.**

**BORRON, P., MCCORMACK, F. X., ELHALWAGI, B. M., CHRONEOS, Z. C., LEWIS, J. F., ZHU, S., WRIGHT, J. R., SHEPHERD, V. L., POSSMAYER, F., INCHLEY, K. & FRAHER, L. J. 1998. Surfactant protein A inhibits T cell proliferation via its collagen-like tail and a 210-kDa receptor.** ***Am J Physiol,*** **275, L679-86.**

**BOURNE, G. L. 1960. The microscopic anatomy of the human amnion and chorion.** ***Am J Obstet Gynecol,*** **79, 1070-3.**

**BRATCHER, P. E., WEATHINGTON, N. M., NICK, H. J., JACKSON, P. L., SNELGROVE, R. J. & GAGGAR, A. 2012. MMP-9 cleaves SP-D and abrogates its innate immune functions in vitro.** ***PLoS One,*** **7, e41881.**

**BREUILLER-FOUCHE, M., DUBOIS, O., SEDIKI, M., GARCIA-VERDUGO, I., PALANIYAR, N., TANFIN, Z., CHISSEY, A., CABROL, D., CHARPIGNY, G. & MEHATS, C. 2010. Secreted surfactant protein A from fetal membranes induces stress fibers in cultured human myometrial cells.** ***Am J Physiol Endocrinol Metab,*** **298, E1188-97.**

**BROSENS, I., DIXON, H. G. & ROBERTSON, W. B. 1977. Fetal growth retardation and the arteries of the placental bed.** ***Br J Obstet Gynaecol,*** **84, 656-63.**

**BROWN, N. L., ALVI, S. A., ELDER, M. G., BENNETT, P. R. & SULLIVAN, M. H. 1998. Interleukin-1beta and bacterial endotoxin change the metabolism of prostaglandins E2 and F2alpha in intact term fetal membranes.** ***Placenta,*** **19, 625-30.**

**BRY, K. & HALLMAN, M. 1992. Transforming growth factor-beta opposes the stimulatory effects of interleukin-1 and tumor necrosis factor on amnion cell prostaglandin E2 production: implication for preterm labor.** ***Am J Obstet Gynecol,*** **167, 222-6.**

**BULMER, J. N. & JOHNSON, P. M. 1984. Macrophage populations in the human placenta and amniochorion.** ***Clin Exp Immunol,*** **57, 393-403.**

**BULMER, J. N., MORRISON, L. & SMITH, J. C. 1988a. Expression of class II MHC gene products by macrophages in human uteroplacental tissue.** ***Immunology,*** **63, 707-14.**

**BULMER, J. N., PACE, D. & RITSON, A. 1988b. Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function.** ***Reprod Nutr Dev,*** **28, 1599-613.**

**CALDER, A. A. & GREER, I. A. 1991. Pharmacological modulation of cervical compliance in the first and second trimesters of pregnancy.** ***Semin Perinatol,*** **15, 162-72.**

**CALLEN, P., GOLDSWORTHY, S., GRAVES, L., HARVEY, D., MELLOWS, H. & PARKINSON, C. 1979. Mode of delivery and the lecithin/sphingomyelin ratio.** ***Br J Obstet Gynaecol,*** **86, 965-8.**

**CAROSELLA, E. D., MOREAU, P., LEMAOULT, J. & ROUAS-FREISS, N. 2008. HLA-G: from biology to clinical benefits.** ***Trends Immunol,*** **29, 125-32.**

**CARROLL, M. V. & SIM, R. B. 2011. Complement in health and disease.** ***Adv Drug Deliv Rev,*** **63, 965-75.**

**CASEY, M. L., COX, S. M., BEUTLER, B., MILEWICH, L. & MACDONALD, P. C. 1989. Cachectin/tumor necrosis factor-alpha formation in human decidua. Potential role of cytokines in infection-induced preterm labor.** ***J Clin Invest,*** **83, 430-6.**

**CAUCHETEUX, S. M., KANELLOPOULOS-LANGEVIN, C. & OJCIUS, D. M. 2003. At the innate frontiers between mother and fetus: linking abortion with complement activation.** ***Immunity,*** **18, 169-72.**

**CHABY, R., GARCIA-VERDUGO, I., ESPINASSOUS, Q. & AUGUSTO, L. A. 2005. Interactions between LPS and lung surfactant proteins.** ***J Endotoxin Res,*** **11, 181-5.**

**CHAIWORAPONGSA, T., HONG, J. S., HULL, W. M., ROMERO, R. & WHITSETT, J. A. 2008. Amniotic fluid concentration of surfactant proteins in intra-amniotic infection.** ***J Matern Fetal Neonatal Med,*** **21, 663-70.**

**CHALLIS, J. R., PATEL, F. A. & POMINI, F. 1999. Prostaglandin dehydrogenase and the initiation of labor.** ***J Perinat Med,*** **27, 26-34.**

**CHALLIS, J. R. & VAUGHAN, M. 1987. Steroid synthetic and prostaglandin metabolizing activity is present in different cell populations from human fetal membranes and decidua.** ***Am J Obstet Gynecol,*** **157, 1474-81.**

**CHALLIS, J. R. G. 2000. Mechanism of parturition and preterm labor.** ***Obstet Gynecol Surv,*** **55, 650-60.**

**CHEN, H. L., YANG, Y. P., HU, X. L., YELAVARTHI, K. K., FISHBACK, J. L. & HUNT, J. S. 1991. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation.** ***Am J Pathol,*** **139, 327-35.**

**CHEUNG, P. Y. & CHALLIS, J. R. 1989. Prostaglandin E2 metabolism in the human fetal membranes.** ***Am J Obstet Gynecol,*** **161, 1580-5.**

**CHILDS, R. A., WRIGHT, J. R., ROSS, G. F., YUEN, C. T., LAWSON, A. M., CHAI, W., DRICKAMER, K. & FEIZI, T. 1992. Specificity of lung surfactant protein SP-A for both the carbohydrate and the lipid moieties of certain neutral glycolipids.** ***J Biol Chem,*** **267, 9972-9.**

**CHRISTIAENS, I., ZARAGOZA, D. B., GUILBERT, L., ROBERTSON, S. A., MITCHELL, B. F. & OLSON, D. M. 2008. Inflammatory processes in preterm and term parturition.** ***J Reprod Immunol,*** **79, 50-7.**

**CHRONEOS, Z. C., ABDOLRASULNIA, R., WHITSETT, J. A., RICE, W. R. & SHEPHERD, V. L. 1996. Purification of a cell-surface receptor for surfactant protein A.** ***J Biol Chem,*** **271, 16375-83.**

**CO, E. C., GORMLEY, M., KAPIDZIC, M., ROSEN, D. B., SCOTT, M. A., STOLP, H. A., MCMASTER, M., LANIER, L. L., BARCENA, A. & FISHER, S. J. 2013. Maternal decidual macrophages inhibit NK cell killing of invasive cytotrophoblasts during human pregnancy.** ***Biol Reprod,*** **88, 155.**

**CONDON, J. C., JEYASURIA, P., FAUST, J. M. & MENDELSON, C. R. 2004. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition.** ***Proc Natl Acad Sci U S A,*** **101, 4978-83.**

**COOK, J. L., ZARAGOZA, D. B., SUNG, D. H. & OLSON, D. M. 2000. Expression of myometrial activation and stimulation genes in a mouse model of preterm labor: myometrial activation, stimulation, and preterm labor.** ***Endocrinology,*** **141, 1718-28.**

**COSTELOE, K., HENNESSY, E., GIBSON, A. T., MARLOW, N. & WILKINSON, A. R. 2000. The EPICure study: outcomes to discharge from hospital for infants born at the threshold of viability.** ***Pediatrics,*** **106, 659-71.**

**COX, S. M., CASEY, M. L. & MACDONALD, P. C. 1997. Accumulation of interleukin-1beta and interleukin-6 in amniotic fluid: a sequela of labour at term and preterm.** ***Hum Reprod Update,*** **3, 517-27.**

**CRESWELL, W. H., JR. & JANEWAY, T. M. 1974. A comprehensive health education program for Illinois schools.** ***J Sch Health,*** **44, 336-9.**

**CROUCH, E. & WRIGHT, J. R. 2001. Surfactant proteins a and d and pulmonary host defense.** ***Annu Rev Physiol,*** **63, 521-54.**

**CROWLEY, P. 2000. Prophylactic corticosteroids for preterm birth.** ***Cochrane Database Syst Rev*****, CD000065.**

**CROY, B. A., CHANTAKRU, S., ESADEG, S., ASHKAR, A. A. & WEI, Q. 2002. Decidual natural killer cells: key regulators of placental development (a review).** ***J Reprod Immunol,*** **57, 151-68.**

**CROY, B. A., HE, H., ESADEG, S., WEI, Q., MCCARTNEY, D., ZHANG, J., BORZYCHOWSKI, A., ASHKAR, A. A., BLACK, G. P., EVANS, S. S., CHANTAKRU, S., VAN DEN HEUVEL, M., PAFFARO, V. A., JR. & YAMADA, A. T. 2003. Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling.** ***Reproduction,*** **126, 149-60.**

**CUPURDIJA, K., AZZOLA, D., HAINZ, U., GRATCHEV, A., HEITGER, A., TAKIKAWA, O., GOERDT, S., WINTERSTEIGER, R., DOHR, G. & SEDLMAYR, P. 2004. Macrophages of human first trimester decidua express markers associated to alternative activation.** ***Am J Reprod Immunol,*** **51, 117-22.**

**DA SILVA CORREIA, J. & ULEVITCH, R. J. 2002. MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor.** ***J Biol Chem,*** **277, 1845-54.**

**DAHL, M., JUVONEN, P. O., HOLMSKOV, U. & HUSBY, S. 2005. Surfactant protein D in newborn infants: factors influencing surfactant protein D levels in umbilical cord blood and capillary blood.** ***Pediatr Res,*** **58, 908-12.**

**DENISON, F. C., CALDER, A. A. & KELLY, R. W. 1999. The action of prostaglandin E2 on the human cervix: stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor.** ***Am J Obstet Gynecol,*** **180, 614-20.**

**DENISON, F. C., KELLY, R. W., CALDER, A. A. & RILEY, S. C. 1998. Cytokine secretion by human fetal membranes, decidua and placenta at term.** ***Hum Reprod,*** **13, 3560-5.**

**DERZSY, Z., PROHASZKA, Z., RIGO, J., JR., FUST, G. & MOLVAREC, A. 2010. Activation of the complement system in normal pregnancy and preeclampsia.** ***Mol Immunol,*** **47, 1500-6.**

**DETERDING, R. R., SHIMIZU, H., FISHER, J. H. & SHANNON, J. M. 1994. Regulation of surfactant protein D expression by glucocorticoids in vitro and in vivo.** ***Am J Respir Cell Mol Biol,*** **10, 30-7.**

**DOLAN, S. M., GROSS, S. J., MERKATZ, I. R., FABER, V., SULLIVAN, L. M., MALONE, F. D., PORTER, T. F., NYBERG, D. A., COMSTOCK, C. H., HANKINS, G. D., EDDLEMAN, K., DUGOFF, L., CRAIGO, S. D., TIMOR-TRITSCH, I., CARR, S. R., WOLFE, H. M., BIANCHI, D. W. & D'ALTON, M. E. 2007. The contribution of birth defects to preterm birth and low birth weight.** ***Obstet Gynecol,*** **110, 318-24.**

**DORTBUDAK, O., EBERHARDT, R., ULM, M. & PERSSON, G. R. 2005. Periodontitis, a marker of risk in pregnancy for preterm birth.** ***J Clin Periodontol,*** **32, 45-52.**

**DOWNING, J. F., PASULA, R., WRIGHT, J. R., TWIGG, H. L., 3RD & MARTIN, W. J., 2ND 1995. Surfactant protein a promotes attachment of Mycobacterium tuberculosis to alveolar macrophages during infection with human immunodeficiency virus.** ***Proc Natl Acad Sci U S A,*** **92, 4848-52.**

**DRUCKMANN, R. & DRUCKMANN, M. A. 2005. Progesterone and the immunology of pregnancy.** ***J Steroid Biochem Mol Biol,*** **97, 389-96.**

**DUDLEY, D. J. 1997. Pre-term labor: an intra-uterine inflammatory response syndrome?** ***J Reprod Immunol,*** **36, 93-109.**

**DUDLEY, D. J., BRANCH, D. W., EDWIN, S. S. & MITCHELL, M. D. 1996a. Induction of preterm birth in mice by RU486.** ***Biol Reprod,*** **55, 992-5.**

**DUDLEY, D. J., COLLMER, D., MITCHELL, M. D. & TRAUTMAN, M. S. 1996b. Inflammatory cytokine mRNA in human gestational tissues: implications for term and preterm labor.** ***J Soc Gynecol Investig,*** **3, 328-35.**

**DULKERIAN, S. J., GONZALES, L. W., NING, Y. & BALLARD, P. L. 1996. Regulation of surfactant protein D in human fetal lung.** ***Am J Respir Cell Mol Biol,*** **15, 781-6.**

**EDA, S., SUZUKI, Y., KAWAI, T., OHTANI, K., KASE, T., FUJINAGA, Y., SAKAMOTO, T., KURIMURA, T. & WAKAMIYA, N. 1997. Structure of a truncated human surfactant protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus.** ***Biochem J,*** **323 ( Pt 2), 393-9.**

**ELLIOTT, C. L., LOUDON, J. A., BROWN, N., SLATER, D. M., BENNETT, P. R. & SULLIVAN, M. H. 2001. IL-1beta and IL-8 in human fetal membranes: changes with gestational age, labor, and culture conditions.** ***Am J Reprod Immunol,*** **46, 260-7.**

**FAZELI, A., BRUCE, C. & ANUMBA, D. O. 2005. Characterization of Toll-like receptors in the female reproductive tract in humans.** ***Hum Reprod,*** **20, 1372-8.**

**FERGUSON, J. S., VOELKER, D. R., MCCORMACK, F. X. & SCHLESINGER, L. S. 1999. Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages.** ***J Immunol,*** **163, 312-21.**

**FERGUSON, J. S., VOELKER, D. R., UFNAR, J. A., DAWSON, A. J. & SCHLESINGER, L. S. 2002. Surfactant protein D inhibition of human macrophage uptake of Mycobacterium tuberculosis is independent of bacterial agglutination.** ***J Immunol,*** **168, 1309-14.**

**FERRY, B. L., STARKEY, P. M., SARGENT, I. L., WATT, G. M., JACKSON, M. & REDMAN, C. W. 1990. Cell populations in the human early pregnancy decidua: natural killer activity and response to interleukin-2 of CD56-positive large granular lymphocytes.** ***Immunology,*** **70, 446-52.**

**FIORENTINO, D. F., ZLOTNIK, A., VIEIRA, P., MOSMANN, T. R., HOWARD, M., MOORE, K. W. & O'GARRA, A. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells.** ***J Immunol,*** **146, 3444-51.**

**FISHER, J. H. & MASON, R. 1995. Expression of pulmonary surfactant protein D in rat gastric mucosa.** ***Am J Respir Cell Mol Biol,*** **12, 13-8.**

**FORTUNATO, S. J., MENON, R. & SWAN, K. F. 1994. Expression of TNF-alpha and TNFR p55 in cultured amniochorion.** ***Am J Reprod Immunol,*** **32, 188-93.**

**FRAGA, T. R., BARBOSA, A. S. & ISAAC, L. 2011. Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system.** ***Scand J Immunol,*** **73, 408-19.**

**FUENTES, A., SPAZIANI, E. P. & O'BRIEN, W. F. 1996. The expression of cyclooxygenase-2 (COX-2) in amnion and decidua following spontaneous labor.** ***Prostaglandins,*** **52, 261-7.**

**GARCIA-VERDUGO, I., LEIBER, D., ROBIN, P., BILLON-DENIS, E., CHABY, R. & TANFIN, Z. 2007. Direct interaction of surfactant protein A with myometrial binding sites: signaling and modulation by bacterial lipopolysaccharide.** ***Biol Reprod,*** **76, 681-91.**

**GARCIA-VERDUGO, I., TANFIN, Z., DALLOT, E., LEROY, M. J. & BREUILLER-FOUCHE, M. 2008. Surfactant protein A signaling pathways in human uterine smooth muscle cells.** ***Biol Reprod,*** **79, 348-55.**

**GARDAI, S. J., XIAO, Y. Q., DICKINSON, M., NICK, J. A., VOELKER, D. R., GREENE, K. E. & HENSON, P. M. 2003. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation.** ***Cell,*** **115, 13-23.**

**GEORGE, C. L., GOSS, K. L., MEYERHOLZ, D. K., LAMB, F. S. & SNYDER, J. M. 2008. Surfactant-associated protein A provides critical immunoprotection in neonatal mice.** ***Infect Immun,*** **76, 380-90.**

**GERAGHTY, D. E., KOLLER, B. H. & ORR, H. T. 1987. A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment.** ***Proc Natl Acad Sci U S A,*** **84, 9145-9.**

**GERMAIN, M., KROHN, M. A., HILLIER, S. L. & ESCHENBACH, D. A. 1994. Genital flora in pregnancy and its association with intrauterine growth retardation.** ***J Clin Microbiol,*** **32, 2162-8.**

**GIBB, W. & CHALLIS, J. R. 2002. Mechanisms of term and preterm birth.** ***J Obstet Gynaecol Can,*** **24, 874-83.**

**GIBB, W. & LAVOIE, J. C. 1990. Effects of glucocorticoids on prostaglandin formation by human amnion.** ***Can J Physiol Pharmacol,*** **68, 671-6.**

**GIBB, W. & SUN, M. 1996. Localization of prostaglandin H synthase type 2 protein and mRNA in term human fetal membranes and decidua.** ***J Endocrinol,*** **150, 497-503.**

**GIBSON, A. T. 1997. Surfactant and the neonatal lung.** ***Br J Hosp Med,*** **58, 381-4, 397.**

**GOERDT, S., POLITZ, O., SCHLEDZEWSKI, K., BIRK, R., GRATCHEV, A., GUILLOT, P., HAKIY, N., KLEMKE, C. D., DIPPEL, E., KODELJA, V. & ORFANOS, C. E. 1999. Alternative versus classical activation of macrophages.** ***Pathobiology,*** **67, 222-6.**

**GOLDENBERG, R. L., CULHANE, J. F., IAMS, J. D. & ROMERO, R. 2008. Epidemiology and causes of preterm birth.** ***Lancet,*** **371, 75-84.**

**GOLDENBERG, R. L., HAUTH, J. C. & ANDREWS, W. W. 2000. Intrauterine infection and preterm delivery.** ***N Engl J Med,*** **342, 1500-7.**

**GORDON, S. 2003. Alternative activation of macrophages.** ***Nat Rev Immunol,*** **3, 23-35.**

**GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity.** ***Nat Rev Immunol,*** **5, 953-64.**

**GORSKI, J. P., HUGLI, T. E. & MULLER-EBERHARD, H. J. 1979. C4a: the third anaphylatoxin of the human complement system.** ***Proc Natl Acad Sci U S A,*** **76, 5299-302.**

**GORTNER, L., HILGENDORFF, A., BAHNER, T., EBSEN, M., REISS, I. & RUDLOFF, S. 2005. Hypoxia-induced intrauterine growth retardation: effects on pulmonary development and surfactant protein transcription.** ***Biol Neonate,*** **88, 129-35.**

**GRAMMATOPOULOS, D. K. & HILLHOUSE, E. W. 1999. Basal and interleukin-1beta-stimulated prostaglandin production from cultured human myometrial cells: differential regulation by corticotropin-releasing hormone.** ***J Clin Endocrinol Metab,*** **84, 2204-11.**

**GREEN, N. S., DAMUS, K., SIMPSON, J. L., IAMS, J., REECE, E. A., HOBEL, C. J., MERKATZ, I. R., GREENE, M. F. & SCHWARZ, R. H. 2005. Research agenda for preterm birth: recommendations from the March of Dimes.** ***Am J Obstet Gynecol,*** **193, 626-35.**

**GREGORY, C. D. 2000. CD14-dependent clearance of apoptotic cells: relevance to the immune system.** ***Curr Opin Immunol,*** **12, 27-34.**

**GUENTHER, S., VREKOUSSIS, T., HEUBLEIN, S., BAYER, B., ANZ, D., KNABL, J., NAVROZOGLOU, I., DIAN, D., FRIESE, K., MAKRIGIANNAKIS, A. & JESCHKE, U. 2012. Decidual Macrophages Are Significantly Increased in Spontaneous Miscarriages and Over-Express FasL: A Potential Role for Macrophages in Trophoblast Apoptosis.** ***Int J Mol Sci,*** **13, 9069-80.**

**GUHA, M. & MACKMAN, N. 2001. LPS induction of gene expression in human monocytes.** ***Cell Signal,*** **13, 85-94.**

**GUSTAFSSON, C., MJOSBERG, J., MATUSSEK, A., GEFFERS, R., MATTHIESEN, L., BERG, G., SHARMA, S., BUER, J. & ERNERUDH, J. 2008. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype.** ***PLoS One,*** **3, e2078.**

**GYOMOREY, S., LYE, S. J., GIBB, W. & CHALLIS, J. R. 2000. Fetal-to-maternal progression of prostaglandin H(2) synthase-2 expression in ovine intrauterine tissues during the course of labor.** ***Biol Reprod,*** **62, 797-805.**

**HAAGSMAN, H. P. 2002. Structural and functional aspects of the collectin SP-A.** ***Immunobiology,*** **205, 476-89.**

**HAAS, D. M. 2008. Preterm delivery and cytokine gene polymorphisms.** ***J Reprod Med,*** **53, 70; author reply 70-1.**

**HALGUNSET, J., JOHNSEN, H., KJOLLESDAL, A. M., QVIGSTAD, E., ESPEVIK, T. & AUSTGULEN, R. 1994. Cytokine levels in amniotic fluid and inflammatory changes in the placenta from normal deliveries at term.** ***Eur J Obstet Gynecol Reprod Biol,*** **56, 153-60.**

**HALLMAN, M. 2013. The surfactant system protects both fetus and newborn.** ***Neonatology,*** **103, 320-6.**

**HAMILTON, S., OOMOMIAN, Y., STEPHEN, G., SHYNLOVA, O., TOWER, C. L., GARROD, A., LYE, S. J. & JONES, R. L. 2012. Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor.** ***Biol Reprod,*** **86, 39.**

**HARPER, P. S. 1983. Genetic counselling and prenatal diagnosis.** ***Br Med Bull,*** **39, 302-9.**

**HARTSHORN, K. L., REID, K. B., WHITE, M. R., JENSENIUS, J. C., MORRIS, S. M., TAUBER, A. I. & CROUCH, E. 1996. Neutrophil deactivation by influenza A viruses: mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies.** ***Blood,*** **87, 3450-61.**

**HARTSHORN, K. L., WHITE, M. R., VOELKER, D. R., COBURN, J., ZANER, K. & CROUCH, E. C. 2000. Mechanism of binding of surfactant protein D to influenza A viruses: importance of binding to haemagglutinin to antiviral activity.** ***Biochem J,*** **351 Pt 2, 449-58.**

**HE, Y. & CROUCH, E. 2002. Surfactant protein D gene regulation. Interactions among the conserved CCAAT/enhancer-binding protein elements.** ***J Biol Chem,*** **277, 19530-7.**

**HE, Y., CROUCH, E. C., RUST, K., SPAITE, E. & BRODY, S. L. 2000. Proximal promoter of the surfactant protein D gene: regulatory roles of AP-1, forkhead box, and GT box binding proteins.** ***J Biol Chem,*** **275, 31051-60.**

**HEIKKINEN, J., MOTTONEN, M., KOMI, J., ALANEN, A. & LASSILA, O. 2003. Phenotypic characterization of human decidual macrophages.** ***Clin Exp Immunol,*** **131, 498-505.**

**HERIAS, M. V., HOGENKAMP, A., VAN ASTEN, A. J., TERSTEEG, M. H., VAN EIJK, M. & HAAGSMAN, H. P. 2007. Expression sites of the collectin SP-D suggest its importance in first line host defence: power of combining in situ hybridisation, RT-PCR and immunohistochemistry.** ***Mol Immunol,*** **44, 3324-32.**

**HERTELENDY, F., ROMERO, R., MOLNAR, M., TODD, H. & BALDASSARE, J. J. 1993. Cytokine-initiated signal transduction in human myometrial cells.** ***Am J Reprod Immunol,*** **30, 49-57.**

**HIBBARD, J. U., WILKINS, I., SUN, L., GREGORY, K., HABERMAN, S., HOFFMAN, M., KOMINIAREK, M. A., REDDY, U., BAILIT, J., BRANCH, D. W., BURKMAN, R., GONZALEZ QUINTERO, V. H., HATJIS, C. G., LANDY, H., RAMIREZ, M., VANVELDHUISEN, P., TROENDLE, J. & ZHANG, J. 2010. Respiratory morbidity in late preterm births.** ***JAMA,*** **304, 419-25.**

**HICKLING, T. P., MALHOTRA, R. & SIM, R. B. 1998. Human lung surfactant protein A exists in several different oligomeric states: oligomer size distribution varies between patient groups.** ***Mol Med,*** **4, 266-75.**

**HILLIER, S. L., MARTIUS, J., KROHN, M., KIVIAT, N., HOLMES, K. K. & ESCHENBACH, D. A. 1988. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity.** ***N Engl J Med,*** **319, 972-8.**

**HIRSCH, E. & WANG, H. 2005. The molecular pathophysiology of bacterially induced preterm labor: insights from the murine model.** ***J Soc Gynecol Investig,*** **12, 145-55.**

**HIRST, J. J., TEIXEIRA, F. J., ZAKAR, T. & OLSON, D. M. 1995. Prostaglandin H synthase-2 expression increases in human gestational tissues with spontaneous labour onset.** ***Reprod Fertil Dev,*** **7, 633-7.**

**HOLERS, V. M., GIRARDI, G., MO, L., GUTHRIDGE, J. M., MOLINA, H., PIERANGELI, S. S., ESPINOLA, R., XIAOWEI, L. E., MAO, D., VIALPANDO, C. G. & SALMON, J. E. 2002. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss.** ***J Exp Med,*** **195, 211-20.**

**HOLMSKOV, U., MALHOTRA, R., SIM, R. B. & JENSENIUS, J. C. 1994. Collectins: collagenous C-type lectins of the innate immune defense system.** ***Immunol Today,*** **15, 67-74.**

**HOLMSKOV, U., THIEL, S. & JENSENIUS, J. C. 2003. Collections and ficolins: humoral lectins of the innate immune defense.** ***Annu Rev Immunol,*** **21, 547-78.**

**HOOVER, R. R. & FLOROS, J. 1998. Organization of the human SP-A and SP-D loci at 10q22-q23. Physical and radiation hybrid mapping reveal gene order and orientation.** ***Am J Respir Cell Mol Biol,*** **18, 353-62.**

**HORBAR, J. D., BADGER, G. J., CARPENTER, J. H., FANAROFF, A. A., KILPATRICK, S., LACORTE, M., PHIBBS, R. & SOLL, R. F. 2002. Trends in mortality and morbidity for very low birth weight infants, 1991-1999.** ***Pediatrics,*** **110, 143-51.**

**HOUSER, B. L. 2012. Decidual macrophages and their roles at the maternal-fetal interface.** ***Yale J Biol Med,*** **85, 105-18.**

**HOUSER, B. L., TILBURGS, T., HILL, J., NICOTRA, M. L. & STROMINGER, J. L. 2011. Two unique human decidual macrophage populations.** ***J Immunol,*** **186, 2633-42.**

**HUANG, S. J., CHEN, C. P., SCHATZ, F., RAHMAN, M., ABRAHAMS, V. M. & LOCKWOOD, C. J. 2008. Pre-eclampsia is associated with dendritic cell recruitment into the uterine decidua.** ***J Pathol,*** **214, 328-36.**

**HUNT, J. S. 1990. Current topic: the role of macrophages in the uterine response to pregnancy.** ***Placenta,*** **11, 467-75.**

**HUNT, J. S. 1992. Immunobiology of pregnancy.** ***Curr Opin Immunol,*** **4, 591-6.**

**HUNT, J. S. & LANGAT, D. L. 2009. HLA-G: a human pregnancy-related immunomodulator.** ***Curr Opin Pharmacol,*** **9, 462-9.**

**HUNT, J. S., PETROFF, M. G., MCINTIRE, R. H. & OBER, C. 2005. HLA-G and immune tolerance in pregnancy.** ***FASEB J,*** **19, 681-93.**

**HUNT, J. S. & ROBERTSON, S. A. 1996. Uterine macrophages and environmental programming for pregnancy success.** ***J Reprod Immunol,*** **32, 1-25.**

**HVIID, T. V. 2006. HLA-G in human reproduction: aspects of genetics, function and pregnancy complications.** ***Hum Reprod Update,*** **12, 209-32.**

**INOUE, T., MATSUURA, E., NAGATA, A., OGASAWARA, Y., HATTORI, A., KUROKI, Y., FUJIMOTO, S. & AKINO, T. 1994. Enzyme-linked immunosorbent assay for human pulmonary surfactant protein D.** ***J Immunol Methods,*** **173, 157-64.**

**ITOH, H., NASU, K., NISHIDA, M., MATSUMOTO, H., YUGE, A. & NARAHARA, H. 2006. Human oviductal stromal fibroblasts, but not oviductal epithelial cells, express Toll-like receptor 4: the site-specific mucosal immunity of the human fallopian tube against bacterial infection.** ***Am J Reprod Immunol,*** **56, 91-101.**

**ITOH, M., TAKAHASHI, T., SAKAGUCHI, N., KUNIYASU, Y., SHIMIZU, J., OTSUKA, F. & SAKAGUCHI, S. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance.** ***J Immunol,*** **162, 5317-26.**

**JABBOUR, H. N., SALES, K. J., CATALANO, R. D. & NORMAN, J. E. 2009. Inflammatory pathways in female reproductive health and disease.** ***Reproduction,*** **138, 903-19.**

**JANEWAY CA JR, T. P., WALPORT M, ET AL. 2001.** ***Immunobiology: The Immune System in Health and Disease.*****, New York: Garland Science. .**

**JANEWAY, T. & MOSEBERG, W. H. 1977. Tuberculous paraplegia with lateral vertebral dislocation. A case report.** ***J Bone Joint Surg Am,*** **59, 554-6.**

**JANEWAY, T. C. 1917. Address in Medicine. Slight variations from normal structure and function, and their clinical significance.** ***Can Med Assoc J,*** **7, 589-608.**

**JANEWAY, T. C., OPIE, E. L., WELLS, H. G., PEARCE, R. M. & ROUS, P. 1917. Report of the Subcommittee on Pathology.** ***Science,*** **45, 233.**

**JANEWAY, T. C. & PARK, E. A. 1912. The Question of Epinephrin in the Circulation and Its Relation to Blood Pressure.** ***J Exp Med,*** **16, 541-57.**

**JANSSEN, W. J., MCPHILLIPS, K. A., DICKINSON, M. G., LINDERMAN, D. J., MORIMOTO, K., XIAO, Y. Q., OLDHAM, K. M., VANDIVIER, R. W., HENSON, P. M. & GARDAI, S. J. 2008a. Surfactant proteins A and D suppress alveolar macrophage phagocytosis via interaction with SIRP alpha.** ***Am J Respir Crit Care Med,*** **178, 158-67.**

**JANSSEN, W. J., MCPHILLIPS, K. A., DICKINSON, M. G., LINDERMAN, D. J., MORIMOTO, K., XIAO, Y. Q., OLDHAM, K. M., VANDIVIER, R. W., HENSON, P. M. & GARDAI, S. J. 2008b. Surfactant Proteins A and D Suppress Alveolar Macrophage Phagocytosis via Interaction with SIRPα.** ***American Journal of Respiratory and Critical Care Medicine,*** **178, 158-167.**

**KABHA, K., SCHMEGNER, J., KEISARI, Y., PAROLIS, H., SCHLEPPER-SCHAEFFER, J. & OFEK, I. 1997. SP-A enhances phagocytosis of Klebsiella by interaction with capsular polysaccharides and alveolar macrophages.** ***Am J Physiol,*** **272, L344-52.**

**KALACHE, K. D., CHAOUI, R., MARKS, B., WAUER, R. & BOLLMANN, R. 2002. Does fetal tracheal fluid flow during fetal breathing movements change before the onset of labour?** ***BJOG,*** **109, 514-9.**

**KAMMERER, U., SCHOPPET, M., MCLELLAN, A. D., KAPP, M., HUPPERTZ, H. I., KAMPGEN, E. & DIETL, J. 2000. Human decidua contains potent immunostimulatory CD83(+) dendritic cells.** ***Am J Pathol,*** **157, 159-69.**

**KANG, J., CHAPDELAINE, P., LABERGE, P. Y. & FORTIER, M. A. 2006. Functional characterization of prostaglandin transporter and terminal prostaglandin synthases during decidualization of human endometrial stromal cells.** ***Hum Reprod,*** **21, 592-9.**

**KARJALAINEN, M. K., HUUSKO, J. M., TUOHIMAA, A., LUUKKONEN, A., HAATAJA, R. & HALLMAN, M. 2012. A study of collectin genes in spontaneous preterm birth reveals an association with a common surfactant protein D gene polymorphism.** ***Pediatr Res,*** **71, 93-9.**

**KATYAL, S. L., SINGH, G. & LOCKER, J. 1992. Characterization of a second human pulmonary surfactant-associated protein SP-A gene.** ***Am J Respir Cell Mol Biol,*** **6, 446-52.**

**KEELAN, J. A., BLUMENSTEIN, M., HELLIWELL, R. J., SATO, T. A., MARVIN, K. W. & MITCHELL, M. D. 2003. Cytokines, prostaglandins and parturition--a review.** ***Placenta,*** **24 Suppl A, S33-46.**

**KEELAN, J. A., MARVIN, K. W., SATO, T. A., COLEMAN, M., MCCOWAN, L. M. & MITCHELL, M. D. 1999. Cytokine abundance in placental tissues: evidence of inflammatory activation in gestational membranes with term and preterm parturition.** ***Am J Obstet Gynecol,*** **181, 1530-6.**

**KEIRSE, M. J. 1978. Biosynthesis and metabolism of prostaglandins in the pregnant human uterus.** ***Adv Prostaglandin Thromboxane Res,*** **4, 87-102.**

**KEIRSE, M. J. 1990. Progestogen administration in pregnancy may prevent preterm delivery.** ***Br J Obstet Gynaecol,*** **97, 149-54.**

**KEIRSE, M. J., ERWICH, J. J. & KLOK, G. 1985. Increase in placental 15-hydroxy-prostaglandin dehydrogenase in the first half of human pregnancy.** ***Prostaglandins,*** **30, 131-40.**

**KEIRSE, M. J., HICKS, B. R. & TURNBULL, A. C. 1976. Prostaglandin dehydrogenase in the placenta before and after the onset of labour.** ***Br J Obstet Gynaecol,*** **83, 152-5.**

**KEIRSE, M. J. & TURNBULL, A. C. 1975. Metabolism of prostaglandins within the pregnant uterus.** ***Br J Obstet Gynaecol,*** **82, 887-93.**

**KELLY, R. W. 2002. Inflammatory mediators and cervical ripening.** ***J Reprod Immunol,*** **57, 217-24.**

**KEMPER, C. & HOURCADE, D. E. 2008. Properdin: New roles in pattern recognition and target clearance.** ***Mol Immunol,*** **45, 4048-56.**

**KHONG, T. Y., DE WOLF, F., ROBERTSON, W. B. & BROSENS, I. 1986. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants.** ***Br J Obstet Gynaecol,*** **93, 1049-59.**

**KHOOR, A., STAHLMAN, M. T., GRAY, M. E. & WHITSETT, J. A. 1994. Temporal-spatial distribution of SP-B and SP-C proteins and mRNAs in developing respiratory epithelium of human lung.** ***J Histochem Cytochem,*** **42, 1187-99.**

**KING, A., ALLAN, D. S., BOWEN, M., POWIS, S. J., JOSEPH, S., VERMA, S., HIBY, S. E., MCMICHAEL, A. J., LOKE, Y. W. & BRAUD, V. M. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells.** ***Eur J Immunol,*** **30, 1623-31.**

**KING, B. A. & KINGMA, P. S. 2011. Surfactant protein D deficiency increases lung injury during endotoxemia.** ***Am J Respir Cell Mol Biol,*** **44, 709-15.**

**KISHOR, U., MADAN, T., SARMA, P. U., SINGH, M., URBAN, B. C. & REID, K. B. 2002. Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by Aspergillus fumigatus.** ***Immunobiology,*** **205, 610-8.**

**KISHORE, U., BERNAL, A. L., KAMRAN, M. F., SAXENA, S., SINGH, M., SARMA, P. U., MADAN, T. & CHAKRABORTY, T. 2005. Surfactant proteins SP-A and SP-D in human health and disease.** ***Arch Immunol Ther Exp (Warsz),*** **53, 399-417.**

**KISHORE, U., GREENHOUGH, T. J., WATERS, P., SHRIVE, A. K., GHAI, R., KAMRAN, M. F., BERNAL, A. L., REID, K. B., MADAN, T. & CHAKRABORTY, T. 2006. Surfactant proteins SP-A and SP-D: structure, function and receptors.** ***Mol Immunol,*** **43, 1293-315.**

**KISHORE, U., WANG, J. Y., HOPPE, H. J. & REID, K. B. 1996. The alpha-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids.** ***Biochem J,*** **318 ( Pt 2), 505-11.**

**KNISS, D. A., ZIMMERMAN, P. D., GARVER, C. L. & FERTEL, R. H. 1997. Interleukin-1 receptor antagonist blocks interleukin-1-induced expression of cyclooxygenase-2 in endometrium.** ***Am J Obstet Gynecol,*** **177, 559-67.**

**KOBAYASHI, H. 2012. The entry of fetal and amniotic fluid components into the uterine vessel circulation leads to sterile inflammatory processes during parturition.** ***Front Immunol,*** **3, 321.**

**KORFHAGEN, T. R., GLASSER, S. W., BRUNO, M. D., MCMAHAN, M. J. & WHITSETT, J. A. 1991. A portion of the human surfactant protein A (SP-A) gene locus consists of a pseudogene.** ***Am J Respir Cell Mol Biol,*** **4, 463-9.**

**KOTA, S. K., GAYATRI, K., JAMMULA, S., KRISHNA, S. V., MEHER, L. K. & MODI, K. D. 2013. Endocrinology of parturition.** ***Indian J Endocrinol Metab,*** **17, 50-9.**

**KRAMER, R. M., CHECANI, G. C. & DEYKIN, D. 1987. Stimulation of Ca2+-activated human platelet phospholipase A2 by diacylglycerol.** ***Biochem J,*** **248, 779-83.**

**KUDO, Y., HARA, T., KATSUKI, T., TOYOFUKU, A., KATSURA, Y., TAKIKAWA, O., FUJII, T. & OHAMA, K. 2004. Mechanisms regulating the expression of indoleamine 2,3-dioxygenase during decidualization of human endometrium.** ***Hum Reprod,*** **19, 1222-30.**

**LAHAM, N., BRENNECKE, S. P., BENDTZEN, K. & RICE, G. E. 1996. Differential release of interleukin-6 from human gestational tissues in association with labour and in vitro endotoxin treatment.** ***J Endocrinol,*** **149, 431-9.**

**LAHRA, M. M. & JEFFERY, H. E. 2004. A fetal response to chorioamnionitis is associated with early survival after preterm birth.** ***Am J Obstet Gynecol,*** **190, 147-51.**

**LAMONT, R. F. 1999. The prevention of preterm birth with the use of antibiotics.** ***Eur J Pediatr,*** **158 Suppl 1, S2-4.**

**LE BOUTEILLER, P. & BLASCHITZ, A. 1999. The functionality of HLA-G is emerging.** ***Immunol Rev,*** **167, 233-44.**

**LE BOUTEILLER, P., LEGRAND-ABRAVANEL, F. & SOLIER, C. 2003. Soluble HLA-G1 at the materno-foetal interface--a review.** ***Placenta,*** **24 Suppl A, S10-5.**

**LEMAOULT, J., KRAWICE-RADANNE, I., DAUSSET, J. & CAROSELLA, E. D. 2004. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells.** ***Proc Natl Acad Sci U S A,*** **101, 7064-9.**

**LETH-LARSEN, R., FLORIDON, C., NIELSEN, O. & HOLMSKOV, U. 2004. Surfactant protein D in the female genital tract.** ***Mol Hum Reprod,*** **10, 149-54.**

**LETTIERI, L., VINTZILEOS, A. M., RODIS, J. F., ALBINI, S. M. & SALAFIA, C. M. 1993. Does "idiopathic" preterm labor resulting in preterm birth exist?** ***Am J Obstet Gynecol,*** **168, 1480-5.**

**LEWIS, M. P., MORLESE, J. F., SULLIVAN, M. H. & ELDER, M. G. 1993. Evidence for decidua-trophoblast interactions in early human pregnancy.** ***Hum Reprod,*** **8, 965-8.**

**LI, X. F., CHARNOCK-JONES, D. S., ZHANG, E., HIBY, S., MALIK, S., DAY, K., LICENCE, D., BOWEN, J. M., GARDNER, L., KING, A., LOKE, Y. W. & SMITH, S. K. 2001. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells.** ***J Clin Endocrinol Metab,*** **86, 1823-34.**

**LIANG, S., RISTICH, V., ARASE, H., DAUSSET, J., CAROSELLA, E. D. & HORUZSKO, A. 2008. Modulation of dendritic cell differentiation by HLA-G and ILT4 requires the IL-6--STAT3 signaling pathway.** ***Proc Natl Acad Sci U S A,*** **105, 8357-62.**

**LIDSTROM, C., MATTHIESEN, L., BERG, G., SHARMA, S., ERNERUDH, J. & EKERFELT, C. 2003. Cytokine secretion patterns of NK cells and macrophages in early human pregnancy decidua and blood: implications for suppressor macrophages in decidua.** ***Am J Reprod Immunol,*** **50, 444-52.**

**LIGGINS, G. C., FORSTER, C. S., GRIEVES, S. A. & SCHWARTZ, A. L. 1977. Control of parturition in man.** ***Biol Reprod,*** **16, 39-56.**

**LIM, B. L. & HOLMSKOV, U. 1996. Expression of the carbohydrate recognition domain of bovine conglutinin and demonstration of its binding to iC3b and yeast mannan.** ***Biochem Biophys Res Commun,*** **218, 260-6.**

**LITVACK, M. L., DJIADEU, P., RENGANATHAN, S. D., SY, S., POST, M. & PALANIYAR, N. 2010. Natural IgM and innate immune collectin SP-D bind to late apoptotic cells and enhance their clearance by alveolar macrophages in vivo.** ***Mol Immunol,*** **48, 37-47.**

**LIU, C. F., CHEN, Y. L., CHANG, W. T., SHIEH, C. C., YU, C. K., REID, K. B. & WANG, J. Y. 2005. Mite allergen induces nitric oxide production in alveolar macrophage cell lines via CD14/toll-like receptor 4, and is inhibited by surfactant protein D.** ***Clin Exp Allergy,*** **35, 1615-24.**

**LIU, L., JOHNSON, H. L., COUSENS, S., PERIN, J., SCOTT, S., LAWN, J. E., RUDAN, I., CAMPBELL, H., CIBULSKIS, R., LI, M., MATHERS, C. & BLACK, R. E. 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000.** ***Lancet,*** **379, 2151-61.**

**LOCKWOOD, C. J., HUANG, S. J., CHEN, C. P., HUANG, Y., XU, J., FARAMARZI, S., KAYISLI, O., KAYISLI, U., KOOPMAN, L., SMEDTS, D., BUCHWALDER, L. F. & SCHATZ, F. 2013. Decidual cell regulation of natural killer cell-recruiting chemokines: implications for the pathogenesis and prediction of preeclampsia.** ***Am J Pathol,*** **183, 841-56.**

**LOKE, Y. W. & KING, A. 2000. Decidual natural-killer-cell interaction with trophoblast: cytolysis or cytokine production?** ***Biochem Soc Trans,*** **28, 196-8.**

**LOPEZ BERNAL, A., HANSELL, D. J., CANETE SOLER, R., KEELING, J. W. & TURNBULL, A. C. 1987. Prostaglandins, chorioamnionitis and preterm labour.** ***Br J Obstet Gynaecol,*** **94, 1156-8.**

**LOPEZ BERNAL, A., NEWMAN, G. E., PHIZACKERLEY, P. J. & TURNBULL, A. C. 1988. Surfactant stimulates prostaglandin E production in human amnion. *Br J Obstet Gynaecol,* 95, 1013-7.**

**LU, J., WILLIS, A. C. & REID, K. B. 1992. Purification, characterization and cDNA cloning of human lung surfactant protein D. *Biochem J,* 284 ( Pt 3), 795-802.**

**LU, R., KANAI, N., BAO, Y. & SCHUSTER, V. L. 1996. Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA(hPGT). *J Clin Invest,* 98, 1142-9.**

**LUNDIN-SCHILLER, S. & MITCHELL, M. D. 1990. The role of prostaglandins in human parturition. *Prostaglandins Leukot Essent Fatty Acids,* 39, 1-10.**

**LUNDIN-SCHILLER, S. & MITCHELL, M. D. 1991. Prostaglandin production by human chorion laeve cells in response to inflammatory mediators. *Placenta,* 12, 353-63.**

**LYNCH, A. M., ECKEL, R. H., MURPHY, J. R., GIBBS, R. S., WEST, N. A., GICLAS, P. C., SALMON, J. E. & HOLERS, V. M. 2012. Prepregnancy obesity and complement system activation in early pregnancy and the subsequent development of preeclampsia. *Am J Obstet Gynecol,* 206, 428 e1-8.**

**LYNCH, A. M., GIBBS, R. S., MURPHY, J. R., GICLAS, P. C., SALMON, J. E. & HOLERS, V. M. 2011. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. *Obstet Gynecol,* 117, 75-83.**

**LYNCH, A. M., MURPHY, J. R., BYERS, T., GIBBS, R. S., NEVILLE, M. C., GICLAS, P. C., SALMON, J. E. & HOLERS, V. M. 2008. Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol,* 198, 385 e1-9.**

**MADAN, T., EGGLETON, P., KISHORE, U., STRONG, P., AGGRAWAL, S. S., SARMA, P. U. & REID, K. B. 1997. Binding of pulmonary surfactant proteins A and D to Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. *Infect Immun,* 65, 3171-9.**

**MADSEN, J., KLIEM, A., TORNOE, I., SKJODT, K., KOCH, C. & HOLMSKOV, U. 2000. Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J Immunol,* 164, 5866-70.**

**MADSEN, J., TORNOE, I., NIELSEN, O., KOCH, C., STEINHILBER, W. & HOLMSKOV, U. 2003. Expression and localization of lung surfactant protein A in human tissues. *Am J Respir Cell Mol Biol,* 29, 591-7.**

**MAINA, C. V., RIGGS, P. D., GRANDEA, A. G., 3RD, SLATKO, B. E., MORAN, L. S., TAGLIAMONTE, J. A., MCREYNOLDS, L. A. & GUAN, C. D. 1988. An Escherichia coli vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. *Gene,* 74, 365-73.**

**MALKE, H. 1990. J. SAMBROCK, E. F. FRITSCH and T. MANIATIS, Molecular Cloning, A Laboratory Manual (Second Edition), Volumes 1, 2 and 3. 1625 S., zahlreiche Abb. und Tab. Cold Spring Harbor 1989. Cold Spring Harbor Laboratory Press. $ 115.00. ISBN: 0-87969-309-6. *Journal of Basic Microbiology,* 30, 623-623.**

**MARIENCHECK, W. & CROUCH, E. 1994. Modulation of surfactant protein D expression by glucocorticoids in fetal rat lung. *Am J Respir Cell Mol Biol,* 10, 419-29.**

**MARINO, P. A. & ROONEY, S. A. 1981. The effect of labor on surfactant secretion in newborn rabbit lung slices. *Biochim Biophys Acta,* 664, 389-96.**

**MARLOW, N., WOLKE, D., BRACEWELL, M. A. & SAMARA, M. 2005. Neurologic and developmental disability at six years of age after extremely preterm birth. *N Engl J Med,* 352, 9-19.**

**MARTIS, P. C., WHITSETT, J. A., XU, Y., PERL, A. K., WAN, H. & IKEGAMI, M. 2006. C/EBPalpha is required for lung maturation at birth. *Development,* 133, 1155-64.**

**MARVIN, K. W., KEELAN, J. A., EYKHOLT, R. L., SATO, T. A. & MITCHELL, M. D. 2002. Use of cDNA arrays to generate differential expression profiles for inflammatory genes in human gestational membranes delivered at term and preterm. *Mol Hum Reprod,* 8, 399-408.**

**MATSUMOTO, T., SAGAWA, N., YOSHIDA, M., MORI, T., TANAKA, I., MUKOYAMA, M., KOTANI, M. & NAKAO, K. 1997. The prostaglandin E2 and F2 alpha receptor genes are expressed in human myometrium and are down-regulated during pregnancy.** ***Biochem Biophys Res Commun,*** **238, 838-41.**

**MCCORMICK, S. M., BOGGARAM, V. & MENDELSON, C. R. 1994. Characterization of mRNA transcripts and organization of human SP-A1 and SP-A2 genes.** ***Am J Physiol,*** **266, L354-66.**

**MCCORMICK, S. M. & MENDELSON, C. R. 1994. Human SP-A1 and SP-A2 genes are differentially regulated during development and by cAMP and glucocorticoids.** ***Am J Physiol,*** **266, L367-74.**

**MCGREGOR, J. A., FRENCH, J. I., LAWELLIN, D. & TODD, J. K. 1988. Preterm birth and infection: pathogenic possibilities.** ***Am J Reprod Immunol Microbiol,*** **16, 123-32.**

**MCINTIRE, R. H., MORALES, P. J., PETROFF, M. G., COLONNA, M. & HUNT, J. S. 2004. Recombinant HLA-G5 and -G6 drive U937 myelomonocytic cell production of TGF-beta1.** ***J Leukoc Biol,*** **76, 1220-8.**

**MCNEELY, T. B. & COONROD, J. D. 1994. Aggregation and opsonization of type A but not type B Hemophilus influenzae by surfactant protein A.** ***Am J Respir Cell Mol Biol,*** **11, 114-22.**

**MELLOR, A. L., SIVAKUMAR, J., CHANDLER, P., SMITH, K., MOLINA, H., MAO, D. & MUNN, D. H. 2001. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy.** ***Nat Immunol,*** **2, 64-8.**

**MENDELSON, C. R. 2009. Minireview: fetal-maternal hormonal signaling in pregnancy and labor.** ***Mol Endocrinol,*** **23, 947-54.**

**MENDELSON, C. R. & BOGGARAM, V. 1990. Hormonal and developmental regulation of pulmonary surfactant synthesis in fetal lung.** ***Baillieres Clin Endocrinol Metab,*** **4, 351-78.**

**MENON, R., SWAN, K. F., LYDEN, T. W., ROTE, N. S. & FORTUNATO, S. J. 1995. Expression of inflammatory cytokines (interleukin-1 beta and interleukin-6) in amniochorionic membranes.** ***Am J Obstet Gynecol,*** **172, 493-500.**

**MERCER, B. M., GOLDENBERG, R. L., MOAWAD, A. H., MEIS, P. J., IAMS, J. D., DAS, A. F., CARITIS, S. N., MIODOVNIK, M., MENARD, M. K., THURNAU, G. R., DOMBROWSKI, M. P., ROBERTS, J. M. & MCNELLIS, D. 1999. The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network.** ***Am J Obstet Gynecol,*** **181, 1216-21.**

**MIAKOTINA, O. L. & SNYDER, J. M. 2002. TNF-alpha inhibits SP-A gene expression in lung epithelial cells via p38 MAPK.** ***Am J Physiol Lung Cell Mol Physiol,*** **283, L418-27.**

**MITCHELL, M. D. 1984. The mechanism(s) of human parturition.** ***J Dev Physiol,*** **6, 107-18.**

**MITCHELL, M. D., DUDLEY, D. J., EDWIN, S. S. & SCHILLER, S. L. 1991. Interleukin-6 stimulates prostaglandin production by human amnion and decidual cells.** ***Eur J Pharmacol,*** **192, 189-91.**

**MITCHELL, M. D., EDWIN, S. & ROMERO, R. J. 1990. Prostaglandin biosynthesis by human decidual cells: effects of inflammatory mediators.** ***Prostaglandins Leukot Essent Fatty Acids,*** **41, 35-8.**

**MITCHELL, M. D., GOODWIN, V., MESNAGE, S. & KEELAN, J. A. 2000. Cytokine-induced coordinate expression of enzymes of prostaglandin biosynthesis and metabolism: 15-hydroxyprostaglandin dehydrogenase.** ***Prostaglandins Leukot Essent Fatty Acids,*** **62, 1-5.**

**MIYAMURA, K., MALHOTRA, R., HOPPE, H. J., REID, K. B., PHIZACKERLEY, P. J., MACPHERSON, P. & LOPEZ BERNAL, A. 1994. Surfactant proteins A (SP-A) and D (SP-D): levels in human amniotic fluid and localization in the fetal membranes.** ***Biochim Biophys Acta,*** **1210, 303-7.**

**MIYAZAKI, S., TSUDA, H., SAKAI, M., HORI, S., SASAKI, Y., FUTATANI, T., MIYAWAKI, T. & SAITO, S. 2003. Predominance of Th2-promoting dendritic cells in early human pregnancy decidua.** ***J Leukoc Biol,*** **74, 514-22.**

**MOFFETT-KING, A. 2002. Natural killer cells and pregnancy.** ***Nat Rev Immunol,*** **2, 656-63.**

**MOLL, F. C., SCHWAB, L., HALL, T. C., KIRK, M., ZANDT, C. V. & JANEWAY, C. A. 1950. Experimental hypersensitivity: a study of the relationship of antibody production, serum complement activity and the development of pathologic lesions in response to single, large injections of heterologous plasma protein.** ***Am J Dis Child,*** **79, 1130-1.**

**MOLNAR, M., ROMERO, R. & HERTELENDY, F. 1993. Interleukin-1 and tumor necrosis factor stimulate arachidonic acid release and phospholipid metabolism in human myometrial cells.** ***Am J Obstet Gynecol,*** **169, 825-9.**

**MONTALBANO, A. P., HAWGOOD, S. & MENDELSON, C. R. 2013. Mice deficient in surfactant protein A (SP-A) and SP-D or in TLR2 manifest delayed parturition and decreased expression of inflammatory and contractile genes.** ***Endocrinology,*** **154, 483-98.**

**MOR, G. & ABRAHAMS, V. M. 2003. Potential role of macrophages as immunoregulators of pregnancy.** ***Reprod Biol Endocrinol,*** **1, 119.**

**MOSTER, D., LIE, R. T. & MARKESTAD, T. 2008. Long-term medical and social consequences of preterm birth.** ***N Engl J Med,*** **359, 262-73.**

**MUNN, D. H., ZHOU, M., ATTWOOD, J. T., BONDAREV, I., CONWAY, S. J., MARSHALL, B., BROWN, C. & MELLOR, A. L. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism.** ***Science,*** **281, 1191-3.**

**MUNZ, C., HOLMES, N., KING, A., LOKE, Y. W., COLONNA, M., SCHILD, H. & RAMMENSEE, H. G. 1997. Human histocompatibility leukocyte antigen (HLA)-G molecules inhibit NKAT3 expressing natural killer cells.** ***J Exp Med,*** **185, 385-91.**

**MWANIKI, M. K., ATIENO, M., LAWN, J. E. & NEWTON, C. R. 2012. Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review.** ***Lancet,*** **379, 445-52.**

**MYATT, L. & LYE, S. J. 2004. Expression, localization and function of prostaglandin receptors in myometrium.** ***Prostaglandins Leukot Essent Fatty Acids,*** **70, 137-48.**

**NAKLA, S., SKINNER, K., MITCHELL, B. F. & CHALLIS, J. R. 1986. Changes in prostaglandin transfer across human fetal membranes obtained after spontaneous labor.** ***Am J Obstet Gynecol,*** **155, 1337-41.**

**NAYAK, A., DODAGATTA-MARRI, E., TSOLAKI, A. G. & KISHORE, U. 2012. An Insight into the Diverse Roles of Surfactant Proteins, SP-A and SP-D in Innate and Adaptive Immunity.** ***Front Immunol,*** **3, 131.**

**NORWITZ, E. R. 2006. Defective implantation and placentation: laying the blueprint for pregnancy complications.** ***Reprod Biomed Online,*** **13, 591-9.**

**NORWITZ, E. R., LOPEZ BERNAL, A. & STARKEY, P. M. 1992a. Tumor necrosis factor-alpha selectively stimulates prostaglandin F2 alpha production by macrophages in human term decidua.** ***Am J Obstet Gynecol,*** **167, 815-20.**

**NORWITZ, E. R., STARKEY, P. M. & LOPEZ BERNAL, A. 1992b. Prostaglandin D2 production by term human decidua: cellular origins defined using flow cytometry.** ***Obstet Gynecol,*** **80, 440-5.**

**NORWITZ, E. R., STARKEY, P. M., LOPEZ BERNAL, A. & TURNBULL, A. C. 1991. Identification by flow cytometry of the prostaglandin-producing cell populations of term human decidua.** ***J Endocrinol,*** **131, 327-34.**

**NOVY, M. J. & LIGGINS, G. C. 1980. Role of prostaglandins, prostacyclin, and thromboxanes in the physiologic control of the uterus and in parturition.** ***Semin Perinatol,*** **4, 45-66.**

**NOVY, M. J., THOMAS, C. L. & LEES, M. H. 1974. Uterine contractility and regional blood flow responses to oxytocin and prostaglandin E2 in pregnant rhesus monkeys.** ***Trans Pac Coast Obstet Gynecol Soc,*** **42, 8-21.**

**O'RIORDAN, D. M., STANDING, J. E., KWON, K. Y., CHANG, D., CROUCH, E. C. & LIMPER, A. H. 1995. Surfactant protein D interacts with Pneumocystis carinii and mediates organism adherence to alveolar macrophages.** ***J Clin Invest,*** **95, 2699-710.**

**OAKLEY, L., MACONOCHIE, N., DOYLE, P., DATTANI, N. & MOSER, K. 2009. Multivariate analysis of infant death in England and Wales in 2005-06, with focus on socio-economic status and deprivation.** ***Health Stat Q*****, 22-39.**

**ODOM, M. J., SNYDER, J. M., BOGGARAM, V. & MENDELSON, C. R. 1988. Glucocorticoid regulation of the major surfactant associated protein (SP-A) and its messenger ribonucleic acid and of morphological development of human fetal lung in vitro.** ***Endocrinology,*** **123, 1712-20.**

**ODOM, M. J., SNYDER, J. M. & MENDELSON, C. R. 1987. Adenosine 3',5'-monophosphate analogs and beta-adrenergic agonists induce the synthesis of the major surfactant apoprotein in human fetal lung in vitro.** ***Endocrinology,*** **121, 1155-63.**

**OKAZAKI, T., CASEY, M. L., OKITA, J. R., MACDONALD, P. C. & JOHNSTON, J. M. 1981a. Initiation of human parturition. XII. Biosynthesis and metabolism of prostaglandins in human fetal membranes and uterine decidua.** ***Am J Obstet Gynecol,*** **139, 373-81.**

**OKAZAKI, T., SAGAWA, N., BLEASDALE, J. E., OKITA, J. R., MACDONALD, P. C. & JOHNSTON, J. M. 1981b. Initiation of human parturition: XIII. Phospholipase C, phospholipase A2, and diacylglycerol lipase activities in fetal membranes and decidua vera tissues from early and late gestation.** ***Biol Reprod,*** **25, 103-9.**

**OKITA, J. R., MACDONALD, P. C. & JOHNSTON, J. M. 1982. Mobilization of arachidonic acid from specific glycerophospholipids of human fetal membranes during early labor.** ***J Biol Chem,*** **257, 14029-34.**

**OKITA, J. R., SAGAWA, N., CASEY, M. L. & SNYDER, J. M. 1983. A comparison of human amnion tissue and amnion cells in primary culture by morphological and biochemical criteria.** ***In Vitro,*** **19, 117-26.**

**OKSJOKI, R., KOVANEN, P. T., MERI, S. & PENTIKAINEN, M. O. 2007. Function and regulation of the complement system in cardiovascular diseases.** ***Front Biosci,*** **12, 4696-708.**

**OLINS, P. O., DEVINE, C. S., RANGWALA, S. H. & KAVKA, K. S. 1988. The T7 phage gene 10 leader RNA, a ribosome-binding site that dramatically enhances the expression of foreign genes in Escherichia coli.** ***Gene,*** **73, 227-35.**

**OLSON, D. M. 2003. The role of prostaglandins in the initiation of parturition.** ***Best Pract Res Clin Obstet Gynaecol,*** **17, 717-30.**

**OLSON, D. M., MIJOVIC, J. E. & SADOWSKY, D. W. 1995. Control of human parturition.** ***Semin Perinatol,*** **19, 52-63.**

**OLSON, D. M., SMIEJA, Z., ZAKAR, T., MACLEOD, E. A., WALTON, J. & MILNE, K. 1991. Regulation of prostaglandin synthesis in the human amnion.** ***Reprod Fertil Dev,*** **3, 413-9.**

**OPSJLN, S. L., WATHEN, N. C., TINGULSTAD, S., WIEDSWANG, G., SUNDAN, A., WAAGE, A. & AUSTGULEN, R. 1993. Tumor necrosis factor, interleukin-1, and interleukin-6 in normal human pregnancy.** ***Am J Obstet Gynecol,*** **169, 397-404.**

**OPSJON, S. L., AUSTGULEN, R. & WAAGE, A. 1995. Interleukin-1, interleukin-6 and tumor necrosis factor at delivery in preeclamptic disorders.** ***Acta Obstet Gynecol Scand,*** **74, 19-26.**

**OSMAN, I., CRAWFORD, M., JORDAN, F., YOUNG, A., NORMAN, J. & THOMSON, A. 2004. Expression and localization of cell adhesion molecules in human fetal membranes during parturition.** ***J Reprod Immunol,*** **63, 11-21.**

**OSMAN, I., YOUNG, A., JORDAN, F., GREER, I. A. & NORMAN, J. E. 2006. Leukocyte density and proinflammatory mediator expression in regional human fetal membranes and decidua before and during labor at term.** ***J Soc Gynecol Investig,*** **13, 97-103.**

**OSMAN, I., YOUNG, A., LEDINGHAM, M. A., THOMSON, A. J., JORDAN, F., GREER, I. A. & NORMAN, J. E. 2003. Leukocyte density and pro-inflammatory cytokine expression**

**in human fetal membranes, decidua, cervix and myometrium before and during labour at term.** ***Mol Hum Reprod,*** **9, 41-5.**

**OSMERS, R. G., ADELMANN-GRILL, B. C., RATH, W., STUHLSATZ, H. W., TSCHESCHE, H. & KUHN, W. 1995. Biochemical events in cervical ripening dilatation during pregnancy and parturition.** ***J Obstet Gynaecol (Tokyo 1995),*** **21, 185-94.**

**PALANIYAR, N., RIDSDALE, R. A., HOLTERMAN, C. E., INCHLEY, K., POSSMAYER, F. & HARAUZ, G. 1998. Structural changes of surfactant protein A induced by cations reorient the protein on lipid bilayers.** ***J Struct Biol,*** **122, 297-310.**

**PARHAM, P. 2004. NK cells and trophoblasts: partners in pregnancy.** ***J Exp Med,*** **200, 951-5.**

**PEEBLES, D. M. & MYATT, L. 2006.** ***Inflammation and Pregnancy*****, Taylor & Francis.**

**PEREZ-GIL, J. 2002. Molecular interactions in pulmonary surfactant films.** ***Biol Neonate,*** **81 Suppl 1, 6-15.**

**PERI, K. G., QUINIOU, C., HOU, X., ABRAN, D., VARMA, D. R., LUBELL, W. D. & CHEMTOB, S. 2002. THG113: a novel selective FP antagonist that delays preterm labor.** ***Semin Perinatol,*** **26, 389-97.**

**PETSAS, G., JESCHKE, U., RICHTER, D. U., MINAS, V., HAMMER, A., KALANTARIDOU, S., TOTH, B., TSATSANIS, C., FRIESE, K. & MAKRIGIANNAKIS, A. 2012. Aberrant expression of corticotropin-releasing hormone in pre-eclampsia induces expression of FasL in maternal macrophages and extravillous trophoblast apoptosis.** ***Mol Hum Reprod,*** **18, 535-45.**

**PFISTER, R. E., RAMSDEN, C. A., NEIL, H. L., KYRIAKIDES, M. A. & BERGER, P. J. 2001. Volume and secretion rate of lung liquid in the final days of gestation and labour in the fetal sheep.** ***J Physiol,*** **535, 889-99.**

**PHILLIPS, T. A., NI, J. & HUNT, J. S. 2003. Cell-specific expression of B lymphocyte (APRIL, BLyS)- and Th2 (CD30L/CD153)-promoting tumor necrosis factor superfamily ligands in human placentas.** ***J Leukoc Biol,*** **74, 81-7.**

**PICCINNI, M. P., GIUDIZI, M. G., BIAGIOTTI, R., BELONI, L., GIANNARINI, L., SAMPOGNARO, S., PARRONCHI, P., MANETTI, R., ANNUNZIATO, F., LIVI, C. & ET AL. 1995. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones.** ***J Immunol,*** **155, 128-33.**

**POLANCZYK, M. J., CARSON, B. D., SUBRAMANIAN, S., AFENTOULIS, M., VANDENBARK, A. A., ZIEGLER, S. F. & OFFNER, H. 2004. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment.** ***J Immunol,*** **173, 2227-30.**

**POLLARD, J. K. & MITCHELL, M. D. 1996a. Effects of gestational age on prostaglandin production and its regulation in human myometrial cells.** ***J Matern Fetal Med,*** **5, 93-8.**

**POLLARD, J. K. & MITCHELL, M. D. 1996b. Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women.** ***Am J Obstet Gynecol,*** **174, 682-6.**

**POMINI, F., CARUSO, A. & CHALLIS, J. R. 1999. Interleukin-10 modifies the effects of interleukin-1beta and tumor necrosis factor-alpha on the activity and expression of prostaglandin H synthase-2 and the NAD+-dependent 15-hydroxyprostaglandin dehydrogenase in cultured term human villous trophoblast and chorion trophoblast cells.** ***J Clin Endocrinol Metab,*** **84, 4645-51.**

**POTESTIO, F. A., ZAKAR, T. & OLSON, D. M. 1988. Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor-mediated mechanism.** ***J Clin Endocrinol Metab,*** **67, 1205-10.**

**QASEEM, A. S., SONAR, S., MAHAJAN, L., MADAN, T., SORENSEN, G. L., SHAMJI, M. H. & KISHORE, U. 2013. Linking surfactant protein SP-D and IL-13: implications in asthma and allergy.** ***Mol Immunol,*** **54, 98-107.**

**RAKHESH, M., CATE, M., VIJAY, R., SHRIKANT, A. & SHANJANA, A. 2012. A TLR4-interacting peptide inhibits lipopolysaccharide-stimulated inflammatory responses,**

**migration and invasion of colon cancer SW480 cells.** ***Oncoimmunology,*** **1, 1495-1506.**

**RAMWELL, P. W. & SHAW, J. E. 1970. Biological significance of the prostaglandins.** ***Recent Prog Horm Res,*** **26, 139-87.**

**RAUK, P. N. & CHIAO, J. P. 2000. Interleukin-1 stimulates human uterine prostaglandin production through induction of cyclooxygenase-2 expression.** ***Am J Reprod Immunol,*** **43, 152-9.**

**READ, S. & POWRIE, F. 2001. CD4(+) regulatory T cells.** ***Curr Opin Immunol,*** **13, 644-9.**

**READING, P. C., HOLMSKOV, U. & ANDERS, E. M. 1998. Antiviral activity of bovine collectins against rotaviruses.** ***J Gen Virol,*** **79 ( Pt 9), 2255-63.**

**REID, K. B. 1998. Functional roles of the lung surfactant proteins SP-A and SP-D in innate immunity.** ***Immunobiology,*** **199, 200-7.**

**REISTER, F., FRANK, H. G., KINGDOM, J. C., HEYL, W., KAUFMANN, P., RATH, W. & HUPPERTZ, B. 2001. Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women.** ***Lab Invest,*** **81, 1143-52.**

**REPNIK, U., TILBURGS, T., ROELEN, D. L., VAN DER MAST, B. J., KANHAI, H. H., SCHERJON, S. & CLAAS, F. H. 2008. Comparison of macrophage phenotype between decidua basalis and decidua parietalis by flow cytometry.** ***Placenta,*** **29, 405-12.**

**RICHANI, K., SOTO, E., ROMERO, R., ESPINOZA, J., CHAIWORAPONGSA, T., NIEN, J. K., EDWIN, S., KIM, Y. M., HONG, J. S. & MAZOR, M. 2005. Normal pregnancy is characterized by systemic activation of the complement system.** ***J Matern Fetal Neonatal Med,*** **17, 239-45.**

**ROBERTSON, S. A., SKINNER, R. J. & CARE, A. S. 2006. Essential role for IL-10 in resistance to lipopolysaccharide-induced preterm labor in mice.** ***J Immunol,*** **177, 4888-96.**

**ROMERO, P., MUNOZ, M., RIVAS, I., SIRI, M. T. & CORDOVA, M. 1991. [Etiology of vulvovaginitis in childhood].** ***Rev Chil Pediatr,*** **62, 321-2.**

**ROMERO, R., DURUM, S., DINARELLO, C. A., OYARZUN, E., HOBBINS, J. C. & MITCHELL, M. D. 1989a. Interleukin-1 stimulates prostaglandin biosynthesis by human amnion.** ***Prostaglandins,*** **37, 13-22.**

**ROMERO, R., ESPINOZA, J., KUSANOVIC, J. P., GOTSCH, F., HASSAN, S., EREZ, O., CHAIWORAPONGSA, T. & MAZOR, M. 2006. The preterm parturition syndrome.** ***BJOG,*** **113 Suppl 3, 17-42.**

**ROMERO, R., MANOGUE, K. R., MITCHELL, M. D., WU, Y. K., OYARZUN, E., HOBBINS, J. C. & CERAMI, A. 1989b. Infection and labor. IV. Cachectin-tumor necrosis factor in the amniotic fluid of women with intraamniotic infection and preterm labor.** ***Am J Obstet Gynecol,*** **161, 336-41.**

**ROMERO, R., MAZOR, M., WU, Y. K., SIRTORI, M., OYARZUN, E., MITCHELL, M. D. & HOBBINS, J. C. 1988. Infection in the pathogenesis of preterm labor.** ***Semin Perinatol,*** **12, 262-79.**

**ROONEY, S. A., YOUNG, S. L. & MENDELSON, C. R. 1994. Molecular and cellular processing of lung surfactant.** ***FASEB J,*** **8, 957-67.**

**ROSSEAU, S., GUENTHER, A., SEEGER, W. & LOHMEYER, J. 1997. Phagocytosis of viable Candida albicans by alveolar macrophages: lack of opsonin function of surfactant protein A.** ***J Infect Dis,*** **175, 421-8.**

**ROSSEAU, S., HAMMERL, P., MAUS, U., GUNTHER, A., SEEGER, W., GRIMMINGER, F. & LOHMEYER, J. 1999. Surfactant protein A down-regulates proinflammatory cytokine production evoked by Candida albicans in human alveolar macrophages and monocytes.** ***J Immunol,*** **163, 4495-502.**

**ROTH, I., CORRY, D. B., LOCKSLEY, R. M., ABRAMS, J. S., LITTON, M. J. & FISHER, S. J. 1996. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10.** ***J Exp Med,*** **184, 539-48.**

**ROUAS-FREISS, N., GONCALVES, R. M., MENIER, C., DAUSSET, J. & CAROSELLA, E. D. 1997. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis.** ***Proc Natl Acad Sci U S A,*** **94, 11520-5.**

**RUBALTELLI, F. F., DANI, C., REALI, M. F., BERTINI, G., WIECHMANN, L., TANGUCCI, M. & SPAGNOLO, A. 1998. Acute neonatal respiratory distress in Italy: a one-year prospective study. Italian Group of Neonatal Pneumology.** ***Acta Paediatr,*** **87, 1261-8.**

**RUS, H., CUDRICI, C. & NICULESCU, F. 2005. The role of the complement system in innate immunity.** ***Immunol Res,*** **33, 103-12.**

**SACKS, G., SARGENT, I. & REDMAN, C. 1999. An innate view of human pregnancy.** ***Immunol Today,*** **20, 114-8.**

**SAITO, S., NISHIKAWA, K., MORII, T., ENOMOTO, M., NARITA, N., MOTOYOSHI, K. & ICHIJO, M. 1993. Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua.** ***Int Immunol,*** **5, 559-63.**

**SAKAGUCHI, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance.** ***Cell,*** **101, 455-8.**

**SALMINEN, A., PAANANEN, R., VUOLTEENAHO, R., METSOLA, J., OJANIEMI, M., AUTIO-HARMAINEN, H. & HALLMAN, M. 2008. Maternal endotoxin-induced preterm birth in mice: fetal responses in toll-like receptors, collectins, and cytokines.** ***Pediatr Res,*** **63, 280-6.**

**SANGHA, R. K., WALTON, J. C., ENSOR, C. M., TAI, H. H. & CHALLIS, J. R. 1994. Immunohistochemical localization, messenger ribonucleic acid abundance, and activity of 15-hydroxyprostaglandin dehydrogenase in placenta and fetal membranes during term and preterm labor.** ***J Clin Endocrinol Metab,*** **78, 982-9.**

**SANO, H., CHIBA, H., IWAKI, D., SOHMA, H., VOELKER, D. R. & KUROKI, Y. 2000. Surfactant proteins A and D bind CD14 by different mechanisms.** ***J Biol Chem,*** **275, 22442-51.**

**SARGENT, I. L. 1993. Maternal and fetal immune responses during pregnancy.** ***Exp Clin Immunogenet,*** **10, 85-102.**

**SATI, L., SEVAL-CELIK, Y. & DEMIR, R. 2010. Lung surfactant proteins in the early human placenta.** ***Histochem Cell Biol,*** **133, 85-93.**

**SAWADA, K., ARIKI, S., KOJIMA, T., SAITO, A., YAMAZOE, M., NISHITANI, C., SHIMIZU, T., TAKAHASHI, M., MITSUZAWA, H., YOKOTA, S., SAWADA, N., FUJII, N., TAKAHASHI, H. & KUROKI, Y. 2010. Pulmonary collectins protect macrophages against pore-forming activity of Legionella pneumophila and suppress its intracellular growth.** ***J Biol Chem,*** **285, 8434-43.**

**SAYAMA, S., NAGAMATSU, T., SCHUST, D. J., ITAOKA, N., ICHIKAWA, M., KAWANA, K., YAMASHITA, T., KOZUMA, S. & FUJII, T. 2013. Human decidual macrophages suppress IFN-gamma production by T cells through costimulatory B7-H1:PD-1 signaling in early pregnancy.** ***J Reprod Immunol*****.**

**SCHELENZ, S., MALHOTRA, R., SIM, R. B., HOLMSKOV, U. & BANCROFT, G. J. 1995. Binding of host collectins to the pathogenic yeast Cryptococcus neoformans: human surfactant protein D acts as an agglutinin for acapsular yeast cells.** ***Infect Immun,*** **63, 3360-6.**

**SCHIFF, D. E., KLINE, L., SOLDAU, K., LEE, J. D., PUGIN, J., TOBIAS, P. S. & ULEVITCH, R. J. 1997. Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism.** ***J Leukoc Biol,*** **62, 786-94.**

**SCHONBECK, U., MACH, F. & LIBBY, P. 1998. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing.** ***J Immunol,*** **161, 3340-6.**

**SCHUMACHER, W. A., FANTONE, J. C., KUNKEL, S. E., WEBB, R. C. & LUCCHESI, B. R. 1991. The anaphylatoxins C3a and C5a are vasodilators in the canine coronary vasculature in vitro and in vivo.** ***Agents Actions,*** **34, 345-9.**

**SCHUSTER, C., VACULIK, C., FIALA, C., MEINDL, S., BRANDT, O., IMHOF, M., STINGL, G., EPPEL, W. & ELBE-BURGER, A. 2009. HLA-DR+ leukocytes acquire CD1 antigens in**

**embryonic and fetal human skin and contain functional antigen-presenting cells.** ***J Exp Med,*** **206, 169-81.**

**SCHUTT, C. 1999. CD14.** ***Int J Biochem Cell Biol,*** **31, 545-9.**

**SENFT, A. P., KORFHAGEN, T. R., WHITSETT, J. A., SHAPIRO, S. D. & LEVINE, A. M. 2005. Surfactant protein-D regulates soluble CD14 through matrix metalloproteinase-12.** ***J Immunol,*** **174, 4953-9.**

**SENNSTROM, M. B., EKMAN, G., WESTERGREN-THORSSON, G., MALMSTROM, A., BYSTROM, B., ENDRESEN, U., MLAMBO, N., NORMAN, M., STABI, B. & BRAUNER, A. 2000. Human cervical ripening, an inflammatory process mediated by cytokines.** ***Mol Hum Reprod,*** **6, 375-81.**

**SENNSTROM, M. K., BRAUNER, A., LU, Y., GRANSTROM, L. M., MALMSTROM, A. L. & EKMAN, G. E. 1997. Interleukin-8 is a mediator of the final cervical ripening in humans.** ***Eur J Obstet Gynecol Reprod Biol,*** **74, 89-92.**

**SHUMAN, S. 1991. Recombination mediated by vaccinia virus DNA topoisomerase I in Escherichia coli is sequence specific.** ***Proc Natl Acad Sci U S A,*** **88, 10104-8.**

**SHUMAN, S. 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase.** ***J Biol Chem,*** **269, 32678-84.**

**SILVEYRA, P. & FLOROS, J. 2012. Genetic variant associations of human SP-A and SP-D with acute and chronic lung injury.** ***Front Biosci (Landmark Ed),*** **17, 407-29.**

**SIM, R. B., DAY, A. J., MOFFATT, B. E. & FONTAINE, M. 1993. Complement factor I and cofactors in control of complement system convertase enzymes.** ***Methods Enzymol,*** **223, 13-35.**

**SIMPSON, K. L., KEELAN, J. A. & MITCHELL, M. D. 1998. Labor-associated changes in interleukin-10 production and its regulation by immunomodulators in human choriodecidua.** ***J Clin Endocrinol Metab,*** **83, 4332-7.**

**SINGH, M., MADAN, T., WATERS, P., PARIDA, S. K., SARMA, P. U. & KISHORE, U. 2003. Protective effects of a recombinant fragment of human surfactant protein D in a murine model of pulmonary hypersensitivity induced by dust mite allergens.** ***Immunol Lett,*** **86, 299-307.**

**SINGH, U., NICHOLSON, G., URBAN, B. C., SARGENT, I. L., KISHORE, U. & BERNAL, A. L. 2005. Immunological properties of human decidual macrophages--a possible role in intrauterine immunity.** ***Reproduction,*** **129, 631-7.**

**SJOBERG, A. P., TROUW, L. A. & BLOM, A. M. 2009. Complement activation and inhibition: a delicate balance.** ***Trends Immunol,*** **30, 83-90.**

**SKINNER, K. A. & CHALLIS, J. R. 1985. Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor.** ***Am J Obstet Gynecol,*** **151, 519-23.**

**SLATER, P. E., BEN-ZVI, T., FOGEL, A., EHRENFELD, M. & EVER-HADANI, S. 1995. Absence of an association between rubella vaccination and arthritis in underimmune postpartum women.** ***Vaccine,*** **13, 1529-32.**

**SOLOFF, M. S., JENG, Y. J., COPLAND, J. A., STRAKOVA, Z. & HOARE, S. 2000. Signal pathways mediating oxytocin stimulation of prostaglandin synthesis in select target cells.** ***Exp Physiol,*** **85 Spec No, 51S-58S.**

**SOOD, R., ZEHNDER, J. L., DRUZIN, M. L. & BROWN, P. O. 2006. Gene expression patterns in human placenta.** ***Proc Natl Acad Sci U S A,*** **103, 5478-83.**

**STARKEY, P. M., SARGENT, I. L. & REDMAN, C. W. 1988. Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry.** ***Immunology,*** **65, 129-34.**

**STEEGERS, E. A., VON DADELSZEN, P., DUVEKOT, J. J. & PIJNENBORG, R. 2010. Pre-eclampsia.** ***Lancet,*** **376, 631-44.**

**STEINBORN, A., NIEDERHUT, A., SOLBACH, C., HILDENBRAND, R., SOHN, C. & KAUFMANN, M. 1999. Cytokine release from placental endothelial cells, a process associated with preterm labour in the absence of intrauterine infection.** ***Cytokine,*** **11, 66-73.**

**STOUT, R. D. & SUTTLES, J. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments.** ***J Leukoc Biol,*** **76, 509-13.**

**STRONG, P., KISHORE, U., MORGAN, C., LOPEZ BERNAL, A., SINGH, M. & REID, K. B. 1998. A novel method of purifying lung surfactant proteins A and D from the lung lavage of alveolar proteinosis patients and from pooled amniotic fluid.** ***J Immunol Methods,*** **220, 139-49.**

**STUDIER, F. W. & MOFFATT, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.** ***J Mol Biol,*** **189, 113-30.**

**SUGAHARA, K., IYAMA, K., SANO, K. & MORIOKA, T. 1994. Differential expressions of surfactant protein SP-A, SP-B, and SP-C mRNAs in rats with streptozotocin-induced diabetes demonstrated by in situ hybridization.** ***Am J Respir Cell Mol Biol,*** **11, 397-404.**

**SUN, K., BROCKMAN, D., CAMPOS, B., PITZER, B. & MYATT, L. 2006. Induction of surfactant protein A expression by cortisol facilitates prostaglandin synthesis in human chorionic trophoblasts.** ***J Clin Endocrinol Metab,*** **91, 4988-94.**

**SUNYER, J. O., BOSHRA, H., LORENZO, G., PARRA, D., FREEDMAN, B. & BOSCH, N. 2003. Evolution of complement as an effector system in innate and adaptive immunity.** ***Immunol Res,*** **27, 549-64.**

**SUTTON, L., GADD, M., MASON, D. Y. & REDMAN, C. W. 1986. Cells bearing class II MHC antigens in the human placenta and amniochorion.** ***Immunology,*** **58, 23-9.**

**SUTTON, L., MASON, D. Y. & REDMAN, C. W. 1983. HLA-DR positive cells in the human placenta.** ***Immunology,*** **49, 103-12.**

**TAGLIANI, E. & ERLEBACHER, A. 2011. Dendritic cell function at the maternal-fetal interface.** ***Expert Rev Clin Immunol,*** **7, 593-602.**

**TALLBERG, T., RUOSLAHTI, E. & EHNHOLM, C. 1965. Immunological studies in human placental proteins and the purification of human placental lactogen.** ***Ann Med Exp Biol Fenn,*** **43, 67-71.**

**TANIGUCHI, T., MATSUZAKI, N., KAMEDA, T., SHIMOYA, K., JO, T., SAJI, F. & TANIZAWA, O. 1991. The enhanced production of placental interleukin-1 during labor and intrauterine infection.** ***Am J Obstet Gynecol,*** **165, 131-7.**

**THELEN, T., HAO, Y., MEDEIROS, A. I., CURTIS, J. L., SEREZANI, C. H., KOBZIK, L., HARRIS, L. H. & ARONOFF, D. M. 2010. The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages.** ***J Immunol,*** **185, 4328-35.**

**THOMSON, A. J., TELFER, J. F., YOUNG, A., CAMPBELL, S., STEWART, C. J., CAMERON, I. T., GREER, I. A. & NORMAN, J. E. 1999. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process.** ***Hum Reprod,*** **14, 229-36.**

**THORBURN, G. D. & CHALLIS, J. R. 1979. Endocrine control of parturition.** ***Physiol Rev,*** **59, 863-918.**

**TODD, H. M., DUNDOO, V. L., GERBER, W. R., CWIAK, C. A., BALDASSARE, J. J. & HERTELENDY, F. 1996. Effect of cytokines on prostaglandin E2 and prostacyclin production in primary cultures of human myometrial cells.** ***J Matern Fetal Med,*** **5, 161-7.**

**TOWBIN, H., STAEHELIN, T. & GORDON, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** ***Proc Natl Acad Sci U S A,*** **76, 4350-4.**

**TRUNDLEY, A. & MOFFETT, A. 2004. Human uterine leukocytes and pregnancy.** ***Tissue Antigens,*** **63, 1-12.**

**UNDERWOOD, M. A., GILBERT, W. M. & SHERMAN, M. P. 2005. Amniotic Fluid: Not Just Fetal Urine Anymore.** ***J Perinatol,*** **25, 341-348.**

**VAANDRAGER, A. B. & VAN GOLDE, L. M. 2000. Lung surfactant proteins A and D in innate immune defense.** ***Biol Neonate,*** **77 Suppl 1, 9-13.**

**VAN DE WETERING, J. K., VAN EIJK, M., VAN GOLDE, L. M., HARTUNG, T., VAN STRIJP, J. A. & BATENBURG, J. J. 2001. Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of gram-positive bacteria.** ***J Infect Dis,*** **184, 1143-51.**

**VAN IWAARDEN, F., WELMERS, B., VERHOEF, J., HAAGSMAN, H. P. & VAN GOLDE, L. M. 1990. Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages.** ***Am J Respir Cell Mol Biol,*** **2, 91-8.**

**VAN IWAARDEN, J. F., VAN STRIJP, J. A., EBSKAMP, M. J., WELMERS, A. C., VERHOEF, J. & VAN GOLDE, L. M. 1991. Surfactant protein A is opsonin in phagocytosis of herpes simplex virus type 1 by rat alveolar macrophages.** ***Am J Physiol,*** **261, L204-9.**

**VAN IWAARDEN, J. F., VAN STRIJP, J. A., VISSER, H., HAAGSMAN, H. P., VERHOEF, J. & VAN GOLDE, L. M. 1992. Binding of surfactant protein A (SP-A) to herpes simplex virus type 1-infected cells is mediated by the carbohydrate moiety of SP-A.** ***J Biol Chem,*** **267, 25039-43.**

**VAN MEIR, C. A., MATTHEWS, S. G., KEIRSE, M. J., RAMIREZ, M. M., BOCKING, A. & CHALLIS, J. R. 1997a. 15-hydroxyprostaglandin dehydrogenase: implications in preterm labor with and without ascending infection.** ***J Clin Endocrinol Metab,*** **82, 969-76.**

**VAN MEIR, C. A., RAMIREZ, M. M., MATTHEWS, S. G., CALDER, A. A., KEIRSE, M. J. & CHALLIS, J. R. 1997b. Chorionic prostaglandin catabolism is decreased in the lower uterine segment with term labour.** ***Placenta,*** **18, 109-14.**

**VAN MEIR, C. A., SANGHA, R. K., WALTON, J. C., MATTHEWS, S. G., KEIRSE, M. J. & CHALLIS, J. R. 1996. Immunoreactive 15-hydroxyprostaglandin dehydrogenase (PGDH) is reduced in fetal membranes from patients at preterm delivery in the presence of infection.** ***Placenta,*** **17, 291-7.**

**VAN ROZENDAAL, B. A., VAN GOLDE, L. M. & HAAGSMAN, H. P. 2001. Localization and functions of SP-A and SP-D at mucosal surfaces.** ***Pediatr Pathol Mol Med,*** **20, 319-39.**

**VARIN, A. & GORDON, S. 2009. Alternative activation of macrophages: immune function and cellular biology.** ***Immunobiology,*** **214, 630-41.**

**VENKATRAMAN GIRIJA, U., FURZE, C., TOTH, J., SCHWAEBLE, W. J., MITCHELL, D. A., KEEBLE, A. H. & WALLIS, R. 2010. Engineering novel complement activity into a pulmonary surfactant protein.** ***J Biol Chem,*** **285, 10546-52.**

**VEZZA, R., ROKACH, J. & FITZGERALD, G. A. 2001. Prostaglandin F(2alpha) receptor-dependent regulation of prostaglandin transport.** ***Mol Pharmacol,*** **59, 1506-13.**

**VINCE, G., SHORTER, S., STARKEY, P., HUMPHREYS, J., CLOVER, L., WILKINS, T., SARGENT, I. & REDMAN, C. 1992. Localization of tumour necrosis factor production in cells at the materno/fetal interface in human pregnancy.** ***Clin Exp Immunol,*** **88, 174-80.**

**VINCE, G. S., STARKEY, P. M., JACKSON, M. C., SARGENT, I. L. & REDMAN, C. W. 1990. Flow cytometric characterisation of cell populations in human pregnancy decidua and isolation of decidual macrophages.** ***J Immunol Methods,*** **132, 181-9.**

**VOORHOUT, W. F., VEENENDAAL, T., KUROKI, Y., OGASAWARA, Y., VAN GOLDE, L. M. & GEUZE, H. J. 1992. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung.** ***J Histochem Cytochem,*** **40, 1589-97.**

**WALENKAMP, A. M., VERHEUL, A. F., SCHARRINGA, J. & HOEPELMAN, I. M. 1999. Pulmonary surfactant protein A binds to Cryptococcus neoformans without promoting phagocytosis.** ***Eur J Clin Invest,*** **29, 83-92.**

**WANG, J. Y., KISHORE, U., LIM, B. L., STRONG, P. & REID, K. B. 1996. Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens.** ***Clin Exp Immunol,*** **106, 367-73.**

**WANG, M. L., DORER, D. J., FLEMING, M. P. & CATLIN, E. A. 2004. Clinical outcomes of near-term infants.** ***Pediatrics,*** **114, 372-6.**

**WATTERBERG, K. L., DEMERS, L. M., SCOTT, S. M. & MURPHY, S. 1996. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops.** ***Pediatrics,*** **97, 210-5.**

**WEDGWOOD, R. J. & JANEWAY, C. A. 1953. Serum complement in children with collagen diseases.** ***Pediatrics,*** **11, 569-81.**

**WEGMANN, T. G., LIN, H., GUILBERT, L. & MOSMANN, T. R. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?** ***Immunol Today,*** **14, 353-6.**

**WHITE, R. T., DAMM, D., MILLER, J., SPRATT, K., SCHILLING, J., HAWGOOD, S., BENSON, B. & CORDELL, B. 1985. Isolation and characterization of the human pulmonary surfactant apoprotein gene.** ***Nature,*** **317, 361-3.**

**WHITSETT, J. A., WEAVER, T. E., LIEBERMAN, M. A., CLARK, J. C. & DAUGHERTY, C. 1987. Differential effects of epidermal growth factor and transforming growth factor-beta on synthesis of Mr = 35,000 surfactant-associated protein in fetal lung.** ***J Biol Chem,*** **262, 7908-13.**

**WHITTLE, B. J. 2000. COX-1 and COX-2 products in the gut: therapeutic impact of COX-2 inhibitors.** ***Gut,*** **47, 320-5.**

**WINKLER, M., FISCHER, D. C., HLUBEK, M., VAN DE LEUR, E., HAUBECK, H. D. & RATH, W. 1998a. Interleukin-1beta and interleukin-8 concentrations in the lower uterine segment during parturition at term.** ***Obstet Gynecol,*** **91, 945-9.**

**WINKLER, M., OBERPICHLER, A., TSCHESCHE, H., RUCK, P., FISCHER, D. C. & RATH, W. 1999. Collagenolysis in the lower uterine segment during parturition at term: correlations with stage of cervical dilatation and duration of labor.** ***Am J Obstet Gynecol,*** **181, 153-8.**

**WINKLER, M., RATH, W., FISCHER, D. C., VAN DE LEUR, E. & HAUBECK, H. 2000. Regulation of interleukin-8 synthesis in human lower uterine segment fibroblasts by cytokines and growth factors.** ***Obstet Gynecol,*** **95, 584-8.**

**WINKLER, M., RUCK, P., HORNY, H. P., WEHRMANN, M., KEMP, B., KAISERLING, E. & RATH, W. 1998b. Expression of cell adhesion molecules by endothelium in the human lower uterine segment during parturition at term.** ***Am J Obstet Gynecol,*** **178, 557-61.**

**WIQVIST, N., LINDBLOM, B., WIKLAND, M. & WILHELMSSON, L. 1983. Prostaglandins and uterine contractility.** ***Acta Obstet Gynecol Scand Suppl,*** **113, 23-9.**

**WRIGHT, J. R. 2004. Host defense functions of pulmonary surfactant.** ***Biol Neonate,*** **85, 326-32.**

**WRIGHT, J. R. 2005. Immunoregulatory functions of surfactant proteins.** ***Nat Rev Immunol,*** **5, 58-68.**

**XIONG, S., SHARKEY, A. M., KENNEDY, P. R., GARDNER, L., FARRELL, L. E., CHAZARA, O., BAUER, J., HIBY, S. E., COLUCCI, F. & MOFFETT, A. 2013. Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation.** ***J Clin Invest,*** **123, 4264-4272.**

**XU, C., MAO, D., HOLERS, V. M., PALANCA, B., CHENG, A. M. & MOLINA, H. 2000. A critical role for murine complement regulator crry in fetomaternal tolerance.** ***Science,*** **287, 498-501.**

**XU, H., SHI, X. P., TANG, W. P. & LI, L. X. 2002. [Expression and clinical significance of matrix metalloproteinase-9 and vascular endothelial growth factor in oral squamous cell carcinomas].** ***Ai Zheng,*** **21, 983-8.**

**YADAV, A. K., MADAN, T. & BERNAL, A. L. 2011. Surfactant proteins A and D in pregnancy and parturition.** ***Front Biosci (Elite Ed),*** **3, 291-300.**

**YAMADA, C., SANO, H., SHIMIZU, T., MITSUZAWA, H., NISHITANI, C., HIMI, T. & KUROKI, Y. 2006. Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response. Importance of supratrimeric oligomerization.** ***J Biol Chem,*** **281, 21771-80.**

**YAMAZOE, M., NISHITANI, C., TAKAHASHI, M., KATOH, T., ARIKI, S., SHIMIZU, T., MITSUZAWA, H., SAWADA, K., VOELKER, D. R., TAKAHASHI, H. & KUROKI, Y. 2008.**

**Pulmonary surfactant protein D inhibits lipopolysaccharide (LPS)-induced inflammatory cell responses by altering LPS binding to its receptors.** ***J Biol Chem,*** **283, 35878-88.**

**YOSHIDA, M., SAGAWA, N., ITOH, H., YURA, S., TAKEMURA, M., WADA, Y., SATO, T., ITO, A. & FUJII, S. 2002. Prostaglandin F(2alpha), cytokines and cyclic mechanical stretch augment matrix metalloproteinase-1 secretion from cultured human uterine cervical fibroblast cells.** ***Mol Hum Reprod,*** **8, 681-7.**

**YU, B., HAILMAN, E. & WRIGHT, S. D. 1997. Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids.** ***J Clin Invest,*** **99, 315-24.**

**ZAKAR, T. & OLSON, D. M. 1989. Dexamethasone stimulates arachidonic acid conversion to prostaglandin E2 in human amnion cells.** ***J Dev Physiol,*** **12, 269-72.**

**ZANONI, I., OSTUNI, R., MAREK, LORRI R., BARRESI, S., BARBALAT, R., BARTON, GREGORY M., GRANUCCI, F. & KAGAN, JONATHAN C. 2011. CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4.** ***Cell,*** **147, 868-880.**

**ZHANG, J., TROENDLE, J. F. & YANCEY, M. K. 2002. Reassessing the labor curve in nulliparous women.** ***Am J Obstet Gynecol,*** **187, 824-8.**

**ZHU, D., PAN, C., LI, L., BIAN, Z., LV, Z., SHI, L., ZHANG, J., LI, D., GU, H., ZHANG, C. Y., LIU, Y. & ZEN, K. 2013. MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein alpha.** ***J Allergy Clin Immunol,*** **132, 426-36 e8.**

**ZIMMERMAN, P. E., VOELKER, D. R., MCCORMACK, F. X., PAULSRUD, J. R. & MARTIN, W. J., 2ND 1992. 120-kD surface glycoprotein of Pneumocystis carinii is a ligand for surfactant protein A.** ***J Clin Invest,*** **89, 143-9.**

# 8. Appendix

## *8.1 Sequencing of full length recombinant SP-A in pSecTagC*

Plasmids of cloned full length SP-A in pSecTagC were sent to Beckman Coultier for sequencing. Results revealed an exact match for all three colonies and were all in frame of pSecTagC.

### *SP-A1 in pSecTagC colony 1*

>NKA22021_03_T7P_G23

NNNNGNNNNCNNGANNNGANCACTCCTGCNATNGGTACTGCTGCTCTNGGTTCAGGTTCCACTGGTGACGCGG
CCCAGCCGGCCAGGCGCGCGCGCCGTACGTACGAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGG
AATTCGAAGTGAAGGACGTTTGTGTTGGAAGCCCTGGTATCCCCGGCACTCCTGGATCCCACGGCCTGCCAGGCA
GGGACGGGAGAGATGGTCTCAAAGGAGACCCTGGCCCTCCAGGCCCCATGGGTCCACCTGGAGAAATGCCATGTC
CTCCTGGAAATGATGGGCTGCCTGGAGCCCCTGGTATCCCTGGAGAGTGTGGAGAGAAGGGGGAGCCTGGCGAGA
GGGGCCCTCCAGGGCTTCCAGCTCATCTAGATGAGGAGCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCT
GCAGACAAGGGGAGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCA
GTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGA
GGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAG
CCCTGGAGACTTCCGCTACTCAGACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCG
GGGAAAAGAGCAGTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGA
CCATCTGTGAGTTCTGACTCGAGTCTAGAGGGCCCGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCG
TCGACCATCATCATCATCATTGAGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATC
TGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAG
GAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGNNTNGGGTGGGGCAGGACAGCAAGGGGG
AGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTNGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGC
TGGGGCTCTAGGGGGTATCCCCNNNNGNCCTGGNACGGCGCATTAANNNNGNNGGGGNNGGTGGTTACCGCCA
CCNTGNCCNNNNNNNTTGC

***SP-A1 in pSecTagC colony 2***

***Sequence from Beckman***

>NKA22021_02_T7P_E23

NNGGTGCNNNNGANNNGANNACTCCTGCTATGGGTACTGCTGCTCTNGGTTCAGGTTCCACTGGTGACGCGGCC
CAGCCGGCCAGGCGCGCGCGCCGTACGTACGAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAA
TTCGAAGTGAAGGACGTTTGTGTTGGAAGCCCTGGTATCCCCGGCACTCCTGGATCCCACGGCCTGCCAGGCAGG
GACGGGAGAGATGGTCTCAAAGGAGACCCTGGCCCTCCAGGCCCCATGGGTCCACCTGGAGAAATGCCATGTCCT
CCTGGAAATGATGGGCTGCCTGGAGCCCCTGGTATCCCTGGAGAGTGTGGAGAGAAGGGGGAGCCTGGCGAGAGG
GGCCCTCCAGGGCTTCCAGCTCATCTAGATGAGGAGCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCTGC
AGACAAGGGGAGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGT
CCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGG
AAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCC
CTGGAGACTTCCGCTACTCAGACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGG
GAAAAGAGCAGTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACC
ATCTGTGAGTTCTGACTCGAGTCTAGAGGGCCCGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTC
GACCATCATCATCATCATTGAGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTG
TTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGA
AATTGCATCGCATTGTCTGAGTAGGTGCATTCTATTCTGGGGGNNNNGGGTGGGGCAGGACAGCAAGGGGGAGG
ATTGGGAAGACAATANCAGGCATGCTGGGGATGCGGTNGGCTCTATGGCTTCTGAGNCGNNAAGAANCAGCTNG
GGNNCTAGGGGNATCCCNNNNNNCCTGGNACNGCCATTAACCNNGGCGGGNTGGGGGNTTANCNNNNNNTGA
CCNTTACTTNNNNNGGNCCTAAGNNNCNNNCNTTTCGNTTTTCCCNNCCT

***SP-A1 in pSecTagC colony 3***

***Sequence from Beckman***

>NKA22021_03_T7P_G23

NNNNGNNNNCNNGANNNGANCACTCCTGCNATNGGTACTGCTGCTCTNGGTTCAGGTTCCACTGGT
GACGCGGCCCAGCCGGCCAGGCGCGCGCCGTACGTACGAAGCTTGGTACCGAGCTCGGATCCACTAG
TCCAGTGTGGTGGAATTCGAAGTGAAGGACGTTTGTGTTGGAAGCCCTGGTATCCCCGGCACTCCTGG
ATCCCACGGCCTGCCAGGCAGGGACGGGAGAGATGGTCTCAAAGGAGACCCTGGCCCTCCAGGCCCCAT
GGGTCCACCTGGAGAAATGCCATGTCCTCCTGGAAATGATGGGCTGCCTGGAGCCCCTGGTATCCCTG
GAGAGTGTGGAGAGAAGGGGGAGCCTGGCGAGAGGGGCCCTCCAGGGCTTCCAGCTCATCTAGATGAG
GAGCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCA
GGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCA
TTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCC
ATTGCAAGCTTCGTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGG
AGACTTCCGCTACTCAGACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTC
GGGGAAAAGAGCAGTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTC
CCGACTGACCATCTGTGAGTTCTGACTCGAGTCTAGAGGGCCCGAACAAAAACTCATCTCAGAAGAGG
ATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTAAACCCGCTGATCAGCCTCGACT
GTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCC
ACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTAT
TCTGGGGGNNTNGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGG
GGATGCGGTNGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCNNN
NGNCCTGGNACGGCGCATTAANNNNGNNGGGGNNGGTGGTTACCGCCACCNTGNCCNNNNNNNTTG
C

EcoRI – GAATTC

XhoI - CTCGAG

### *8.2 Cloning and expression of trimeric SP-A in pET101/D TOPO*

### *Primer-BLAST*

The primers were checked to ensure they are correct and specific for this gene. To check if primers are specific for the desired gene, the primer sequence was checked using primer-basic local alignment search tool (Primer-BLAST) software provided by NCBI was used. This suggested a 100% match for the human SP-A gene (see fig below)

**Primer pair 1**

| | Sequence (5'->3') | Length | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|
| **Forward primer** | CATCTAGATGAGGAGCTCCAAGC | 23 | 60.31 | 52.17 | 10.00 | 4.00 |
| **Reverse primer** | TCAGAACTCACAGATGGTCAGTCG | 24 | 62.11 | 50.00 | 4.00 | 2.00 |

Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

>NM_001098668.2 Homo sapiens surfactant protein A2 (SFTPA2), mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        401  ......................   423

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        844  ........................  821
```

>NM_001164647.1 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 4, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        371  ......................  393

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        814  ........................  791
```

>NM_005411.4 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 1, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        442  ......................  464

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        885  ........................  862
```

>NM_001164646.1 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 6, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        295  ......................  317

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        738  ........................  715
```

>NM_001164645.1 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 5, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        287  ......................  309

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        730  ........................  707
```

>NM_001164644.1 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 3, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        401  ......................  423

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        844  ........................  821
```

>NM_001093770.2 Homo sapiens surfactant protein A1 (SFTPA1), transcript variant 2, mRNA

```
product length = 444
Forward primer  1    CATCTAGATGAGGAGCTCCAAGC  23
Template        431  ......................  453

Reverse primer  1    TCAGAACTCACAGATGGTCAGTCG  24
Template        874  ........................  851
```

***Figure 8.1 – Primer-BLAST using tool from NCBI.*** *Forward and reverse sequences for SP-A trimeric region checked for specificity to clone this region specifically. All parameters are in acceptable range and the no base mismatches were identified.*

## *8.3 – Sequencing of SP-A (N/CRD)cloned in pET101/D TOPO*

**Homo sapiens surfactant protein A2 (SFTPA2), mRNA**

NCBI Reference Sequence: NM_001098668.2

>gi|257743448|ref|NM_001098668.2| Homo sapiens surfactant protein A2 (SFTPA2), mRNA

AACTTGGAGGCAGAGACCCAAGCAGCTGGAGGCTCTGTGTGTGGGTCGCTGATTTCTTGGAGCCTGAAAA
GAAGGAGCAGCGACTGGACCCAGAGCCATGTGGCTGTGCCCTCTGGCCCTCACCCTCATCTTGATGGCAG
CCTCTGGTGCTGCGTGCGAAGTGAAGGACGTTTGTGTTGGAAGCCCTGGTATCCCCGGCACTCCTGGATC
CCACGGCCTGCCAGGCAGGGACGGGAGAGATGGTGTCAAAGGAGACCCTGGCCCTCCAGGCCCCATGGGT
CCGCCTGGAGAAACACCATGTCCTCCTGGGAATAATGGGCTGCCTGGAGCCCCTGGTGTCCCTGGAGAGC
GTGGAGAGAAGGGGGAGGCTGGCGAGAGAGGCCCTCCAGGGCTTCCAGCTCATCTAGATGAGGAGCTCCA
AGCCACACTCCACGACTTCAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCAGGGCTCCATA
ATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCATTCAGGAGGCAT
GTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGT
GAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCA
GATGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCTGCAGGTCGGGGAAAAGAGCAGTGTG
TGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACCATCTGTGAGTT
CTGAGAGGCATTTAGGCCATGGGACAGGGAGGATCCTGTCTGGCCTTCAGTTTCCATCCCCAGGATCCAC
TTGGTCTGTGAGATGCTAGAACTCCCTTTCAACAGAATTCACTTGTGGCTATTAGAGCTGGAGGCACCCT
TAGCCACTTCATTCCCCTGATGGGCCCTGACTCTTCCCCATAATCACTGACCAGCCTTGACACTCCCCTT
GCAAACCATCCCAGCACTGCACCCCAGGCAGCCACTCCTAGCCTTGGCCTTTGGCATGAGATGGAGGCCT
CCTTATTCCCCATCTGGTCCAGTTCCTTCACTTACAGATGGCAGCAGTGAGGCCTTGGGGTAGAAGGATC
CTCCAAAGTCACACAGAGTGCCTGCCTCCTGGTCCCCTCAGCTCTGCCTCTGCAGCCCACTGCCTGCCCA
GTGCCATCAGGATGAGCAGTACCGGCCAAGCATAATGACAGAGAGAGGCAGATTTCAGGGAAGCCCTGAC
TGTGTGGAGCTAAGGACACAGTGGAGATTCTCTGGCACTCTGAGGTCTCTGTGGCAGGCCTGGTCAGGCT
CTCCAGGTGGTCAGAGGGCCCAGTGGTGCCCCAGCACGGTGGTGCCCAAGCCAACCCTGTGACTGACATG
TACGATTCACTCCTTTGAGTCTTTGGATGCCAACTCAGCCCCCTGACCTGGAGGCAGCCGGCCAAGGCCT
CTAGGGAAGAGCCCCCCACTGCAGACATGACCCGAGTAACTTTCTGCTGATGAACAAATCTGCACCCCAC
TTCAGACCTCGGTGGGCATTCACACCACCCCCCATGCCACCGGCTCCACTTTCCCCTTTTATTAATACAT
TCACCCAGATAATCATTAAAATTAACATGTGCCAGGTCTTAGGATGTGTCTTGGGGTGGGCACAGTACCC
GGTGACTCTTGGGGATATTTATTTATTTTCCCTGAGCCTATATCTTCATCTGTGAAATGGGGATAAAAAT
ACTTGTTGCTGTCACAATTATTACCATCTCTCCAGCTAGCAAAATTACTACCAGAGCCGTTACTACACAC
AAAGGCTATTGACCGAGCACATACCATGTGCCACACACCTTGACAAAATCTTTTAATACAGTTTATTATG
TACTATTCAATCTTTACACAATGTCACGGGACCAGTATTGTTTACCCAATTTTTTATAAGGACACTGAAG
CTTAGAGGAGTGAAATGTTTTGAGTGTTATTTCAGAGAGCAAATGGCAAAGACTGGATCCAAACCCATCT
TCCTGGACCTGAAGTTCATGCTCCCAGCCACCCCACCCCTGAGCTGAATAAAGATGATTTAAGCATAATA

AATCGTTAGTGTGTTCACATGAGTTTCCATA

## 8.4 – CDS sequence for SP-A

atg tggctgtgcc ctctggccct

121 caccctcatc ttgatggcag cctctggtgc tgcgtgcgaa gtgaaggacg tttgtgttgg

181 aagccctggt atccccggca ctcctggatc ccacggcctg ccaggcaggg acgggagaga

241 tggtgtcaaa ggagaccctg gccctccagg ccccatgggt ccgcctggag aaacaccatg

301 tcctcctggg aataatgggc tgcctggagc ccctggtgtc cctggagagc gtggagagaa

361 gggggaggct ggcgagagag gccctccagg gcttccagct catctagatg aggagctcca

421 agccacactc cacgacttca gacatcaaat cctgcagaca aggggagccc tcagtctgca

481 gggctccata atgacagtag gagagaaggt cttctccagc aatgggcagt ccatcacttt

541 tgatgccatt caggaggcat gtgccagagc aggcggccgc attgctgtcc caaggaatcc

601 agaggaaaat gaggccattg caagcttcgt gaagaagtac aacacatatg cctatgtagg

661 cctgactgag ggtcccagcc ctggagactt ccgctactca gatgggaccc ctgtaaacta

721 caccaactgg taccgagggg agcctgcagg tcggggaaaa gagcagtgtg tggagatgta

781 cacagatggg cagtggaatg acaggaactg cctgtactcc cgactgacca tctgtgagtt

841 ctga

## 8.5 – Original N/CRD sequence for human SP-A gene

CATCTAGATGAGGAGCTCCA
AGCCACACTCCACGACTTCAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCAGGGCTCCATA
ATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCATTCAGGAGGCAT
GTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGT
GAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCA
GATGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCTGCAGGTCGGGGAAAAGAGCAGTGTG
TGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACCATCTGTGAGTT
CTGA

## 8.6 – Sequence 1 – SP-A N/CRD col. 1

GNNNNNTCCTNTAGAATATTTTGTTTACTTTAAGAAGGAATTCAGGAGCCCTTGGGCTCGAGTCAGAACT
CACAGATGGTCAGTCGGGAGTACAGGCAGTTCCTGTCATTCCACTGCCCATCTGTGTACATCTCCACACACT
GCTCTTTTCCCCGACCTGCGGGCTCCCCTCGGTACCAGTTGGTGTAGTTTACAGGGGTCCCGTCTGAGTAGC
GGAAGTCTCCAGGGCTGGGACCCTCAGTCAGGCCTACATAGGCATATGTGTTGTACTTCTTCACGAAGCTTG
CAATGGCCTCATTTTCCTCTGGATTCCTTGGGACAGCAATGCGGCCGCCTGCTCTGGCACATGCCTCCTGAA
TGGCATCAAAAGTGATGGACTGCCCATTGCTGGAGAAGACCTTCTCTCCTACTGTCATTATGGAGCCCTGCA
GACTGAGGGCTCCCCTTGTCTGCAGGATTTGATGTCTAAAGTCGTGGAGTGTGGCTTGGAGCTCCTCATCTA
GATGCATGGTGAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCG

ATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTGATCCGGCTGCTAACAAAGCCCGAAAGGAA
GCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
GGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAG
CCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACACGG
TGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAAA
CATGAGAATTAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA
TAATGGGTTTCTTAGACGTCAGGGNGGCACTTTTTCGGGGGAAATGNNGCGCGGNAACCCCCTANTTGGTT
TANTTTTCCTAAATACATTNCNAATATGGTATCCNNNTCATGNAAACAATAANCCNTGGANNAATGGCTT
CCAATNAANATTGGAANAAANGGANNAANNNTGANNNATTCCAACANTTTTCCGNNGGTCNNCCNNTT
NNNTCCCCNTTTTTTTGGGGGNATTTTTNGCCTTNCCNNGGNTTTTTGGCNNNNCCCCAAAAANNCCCTT
NG

The sequence was used in NCBI database BLAST to evaluate accuracy of gene cloned. The results show the sequence matches up with SP-A

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Max ident | Accession |
|---|---|---|---|---|---|---|
| Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0 | 701 | 1623 | 34% | 0.0 | 100% | NW_004078068.1 |
| Homo sapiens chromosome 10 genomic scaffold, alternate assembly HuRef SCAF_1103279188106 | 701 | 1629 | 34% | 0.0 | 100% | NW_001837987.2 |
| Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly | 701 | 1635 | 34% | 0.0 | 100% | NT_030059.13 |

Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0

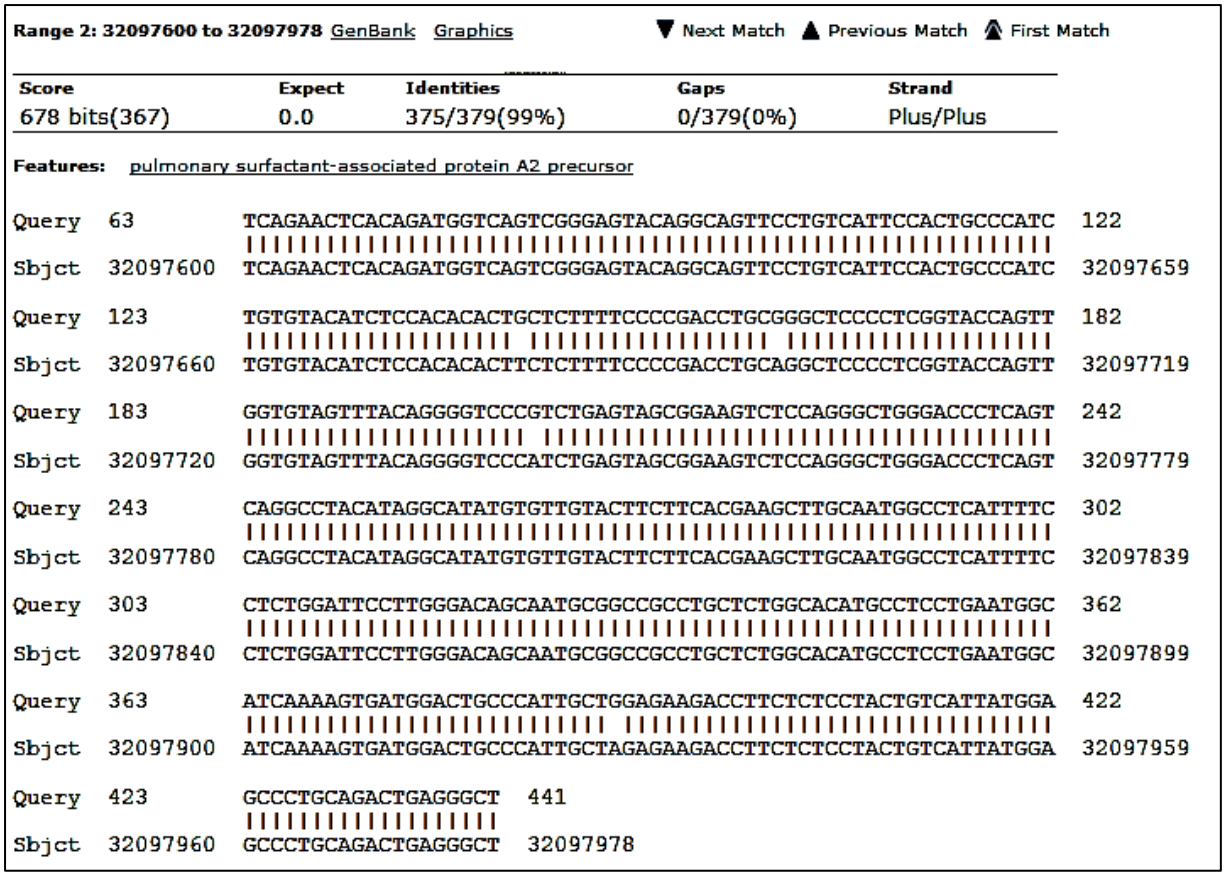
**Sequence ID:** ref|NW_004078068.1| **Length:** 79490249 **Number of Matches:** 4

**Range 1: 32154069 to 32154447** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 701 bits(379) | 0.0 | 379/379(100%) | 0/379(0%) | Plus/Minus |

**Features:** pulmonary surfactant-associated protein A1 isoform 2 prec...
pulmonary surfactant-associated protein A1 isoform 3 prec...

```
Query  63        TCAGAACTCACAGATGGTCAGTCGGGAGTACAGGCAGTTCCTGTCATTCCACTGCCCATC  122
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154447  TCAGAACTCACAGATGGTCAGTCGGGAGTACAGGCAGTTCCTGTCATTCCACTGCCCATC  32154388

Query  123       TGTGTACATCTCCACACACTGCTCTTTTCCCCGACCTGCGGGCTCCCCTCGGTACCAGTT  182
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154387  TGTGTACATCTCCACACACTGCTCTTTTCCCCGACCTGCGGGCTCCCCTCGGTACCAGTT  32154328

Query  183       GGTGTAGTTTACAGGGGTCCCGTCTGAGTAGCGGAAGTCTCCAGGGCTGGGACCCTCAGT  242
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154327  GGTGTAGTTTACAGGGGTCCCGTCTGAGTAGCGGAAGTCTCCAGGGCTGGGACCCTCAGT  32154268

Query  243       CAGGCCTACATAGGCATATGTGTTGTACTTCTTCACGAAGCTTGCAATGGCCTCATTTTC  302
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154267  CAGGCCTACATAGGCATATGTGTTGTACTTCTTCACGAAGCTTGCAATGGCCTCATTTTC  32154208

Query  303       CTCTGGATTCCTTGGGACAGCAATGCGGCCGCCTGCTCTGGCACATGCCTCCTGAATGGC  362
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154207  CTCTGGATTCCTTGGGACAGCAATGCGGCCGCCTGCTCTGGCACATGCCTCCTGAATGGC  32154148

Query  363       ATCAAAAGTGATGGACTGCCCATTGCTGGAGAAGACCTTCTCTCCTACTGTCATTATGGA  422
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154147  ATCAAAAGTGATGGACTGCCCATTGCTGGAGAAGACCTTCTCTCCTACTGTCATTATGGA  32154088

Query  423       GCCCTGCAGACTGAGGGCT  441
                 |||||||||||||||||||
Sbjct  32154087  GCCCTGCAGACTGAGGGCT  32154069
```



**Range 2: 32097600 to 32097978** GenBank Graphics ▼ Next Match ▲ Previous Match ▲ First Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 678 bits(367) | 0.0 | 375/379(99%) | 0/379(0%) | Plus/Plus |

**Features:** pulmonary surfactant-associated protein A2 precursor

```
Query  63        TCAGAACTCACAGATGGTCAGTCGGGAGTACAGGCAGTTCCTGTCATTCCACTGCCCATC  122
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097600  TCAGAACTCACAGATGGTCAGTCGGGAGTACAGGCAGTTCCTGTCATTCCACTGCCCATC  32097659

Query  123       TGTGTACATCTCCACACACTGCTCTTTTCCCCGACCTGCGGGCTCCCCTCGGTACCAGTT  182
                  |||||||||||||||||||| |||||||||||||||||| ||||||||||||||||||||
Sbjct  32097660  TGTGTACATCTCCACACACTTCTCTTTTCCCCGACCTGCAGGCTCCCCTCGGTACCAGTT  32097719

Query  183       GGTGTAGTTTACAGGGGTCCCGTCTGAGTAGCGGAAGTCTCCAGGGCTGGGACCCTCAGT  242
                  |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  32097720  GGTGTAGTTTACAGGGGTCCCATCTGAGTAGCGGAAGTCTCCAGGGCTGGGACCCTCAGT  32097779

Query  243       CAGGCCTACATAGGCATATGTGTTGTACTTCTTCACGAAGCTTGCAATGGCCTCATTTTC  302
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097780  CAGGCCTACATAGGCATATGTGTTGTACTTCTTCACGAAGCTTGCAATGGCCTCATTTTC  32097839

Query  303       CTCTGGATTCCTTGGGACAGCAATGCGGCCGCCTGCTCTGGCACATGCCTCCTGAATGGC  362
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097840  CTCTGGATTCCTTGGGACAGCAATGCGGCCGCCTGCTCTGGCACATGCCTCCTGAATGGC  32097899

Query  363       ATCAAAAGTGATGGACTGCCCATTGCTGGAGAAGACCTTCTCTCCTACTGTCATTATGGA  422
                  |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct  32097900  ATCAAAAGTGATGGACTGCCCATTGCTAGAGAAGACCTTCTCTCCTACTGTCATTATGGA  32097959

Query  423       GCCCTGCAGACTGAGGGCT  441
                  |||||||||||||||||||
Sbjct  32097960  GCCCTGCAGACTGAGGGCT  32097978
```

*Sequence 2 – N/CRD for SP-A col.2*

GNTTCCNTCNNAATATTTTGTTTACTTTAAGAAGGAATTCAGGAGCCCTTCACCATGCATCTAGATGAGGA
GCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCAGGGCTC
CATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCATTCAGGAGG
CATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTC
GTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCA
GACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCAGTGTGT
GGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACCATCTGTGAGTTCT
GACTCGAGCCCAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCG
ATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTGATCCGGCTGCTAACAAAGCCCGAAAGGAA
GCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
GGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAG
CCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACACGG
TGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAAA
CATGAGAATTAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA
TAATGNTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
TAAATACATTCCAANTATGTATCCNGCTCATGAAANCAATAACCCCTGANTNAATGCTTCAATNAATATT

NGAAAAANNGAANGAGTATNNAGTATTCAACANTTTCCNNGGCCGCCNNTAANTCCNNTTTTTTGCGGN
ANTTTTNNCCTTCCTGGTTTTTGNNNNNNCCNNAANNCNNNGGNNGAAANGTAAAANNNNNNNTNNA
AANNNNNNTTTGGGGNGNN

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Max ident | Accession |
|---|---|---|---|---|---|---|
| Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0 | 701 | 1623 | 34% | 0.0 | 100% | NW_004078068.1 |
| Homo sapiens chromosome 10 genomic scaffold, alternate assembly HuRef SCAF_110327918810 | 701 | 1629 | 34% | 0.0 | 100% | NW_001837987.2 |
| Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly | 701 | 1635 | 34% | 0.0 | 100% | NT_030059.13 |

## Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0

**Sequence ID:** ref|NW_004078068.1| **Length:** 79490249 **Number of Matches:** 4

**Range 1: 32154069 to 32154447** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 701 bits(379) | 0.0 | 379/379(100%) | 0/379(0%) | Plus/Plus |

**Features:** pulmonary surfactant-associated protein A1 isoform 2 prec...
pulmonary surfactant-associated protein A1 isoform 3 prec...

```
Query  123       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  182
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154069  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  32154128

Query  183       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  242
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154129  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32154188

Query  243       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  302
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154189  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32154248

Query  303       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  362
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154249  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  32154308

Query  363       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  422
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154309  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  32154368

Query  423       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  482
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154369  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32154428

Query  483       GACCATCTGTGAGTTCTGA  501
                  ||||||||||||||||||
Sbjct  32154429  GACCATCTGTGAGTTCTGA  32154447
```

**Range 2: 32097600 to 32097978** GenBank Graphics ▼ Next Match ▲ Previous Match ⍋ First Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 678 bits(367) | 0.0 | 375/379(99%) | 0/379(0%) | Plus/Minus |

**Features:** pulmonary surfactant-associated protein A2 precursor

```
Query  123       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  182
                  ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  32097978  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCTAGCAATGG  32097919

Query  183       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  242
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097918  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32097859

Query  243       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  302
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097858  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32097799

Query  303       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  362
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  32097798  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGATGG  32097739

Query  363       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  422
                  |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |
Sbjct  32097738  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCTGCAGGTCGGGGAAAAGAGAA  32097679

Query  423       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  482
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097678  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32097619

Query  483       GACCATCTGTGAGTTCTGA  501
                  ||||||||||||||||||
Sbjct  32097618  GACCATCTGTGAGTTCTGA  32097600
```

## Sequence for colony 3 – N/CRD of SP-A

AANTNCCTCNNAATATTTTGTTTACTTTAAGAAGGAATTCAGGAGCCCTTCACCATGCTTCTAGATGAGGA
GCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCAGGGCTC
CATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCATTCAGGAGG
CATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTC
GTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCA
GACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCAGTGTGT
GGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACCATCTGTGAGTTCT
GACTCGAGCCCAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCG
ATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTGATCCGGCTGCTAACAAAGCCCGAAAGGAA
GCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
GGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAG
CCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACACGG
TGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAAA
CATGAGAATTAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA
TAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
TNAATACATTCAAATATGTATCCGCTCATGAANCAATAACCNTGATNAATGCTTCCATAATATTNNAAAA

GGAAGANNANGAGTATTCAACATTTCCGGGNCGNCCTTATTCCCTTTTTTGCGGNATTTTNCCTTCCTGN
NTTTGCTCANCCNNNAACNCTTNGGGAAAGNNAAAGNNNCTGAAAANNNNNTTGGGGGNNNNNNNNN
GGGTTAATNNNAACTGGNNNNNCNAAGCGGNAAAANCTCTTNNAANNNTTNCNCCCNNNAAAANGNTT
TCCAANANNNNAANCNTTTAANNNNNNNNTNNNNGNNNGGNNNNNNNCCC

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| | Description | Max score | Total score | Query cover | E value | Max ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0 | 701 | 1616 | 31% | 0.0 | 100% | NW_004078068.1 |
| ☐ | Homo sapiens chromosome 10 genomic scaffold, alternate assembly HuRef SCAF_1103279188106 | 701 | 1622 | 31% | 0.0 | 100% | NW_001837987.2 |
| ☐ | Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly | 701 | 1627 | 31% | 0.0 | 100% | NT_030059.13 |

Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0

**Sequence ID:** ref|NW_004078068.1| **Length:** 79490249 **Number of Matches:** 4

**Range 1: 32154069 to 32154447** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 701 bits(379) | 0.0 | 379/379(100%) | 0/379(0%) | Plus/Plus |

**Features:** pulmonary surfactant-associated protein A1 isoform 2 prec...
pulmonary surfactant-associated protein A1 isoform 3 prec...

```
Query  123       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  182
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154069  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  32154128

Query  183       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  242
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154129  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32154188

Query  243       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  302
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154189  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32154248

Query  303       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  362
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154249  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  32154308

Query  363       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  422
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154309  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  32154368

Query  423       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  482
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154369  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32154428

Query  483       GACCATCTGTGAGTTCTGA  501
                  ||||||||||||||||||
Sbjct  32154429  GACCATCTGTGAGTTCTGA  32154447
```

**Range 2: 32097600 to 32097978** GenBank Graphics ▼ Next Match ▲ Previous Match ⩓ First Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 678 bits(367) | 0.0 | 375/379(99%) | 0/379(0%) | Plus/Minus |

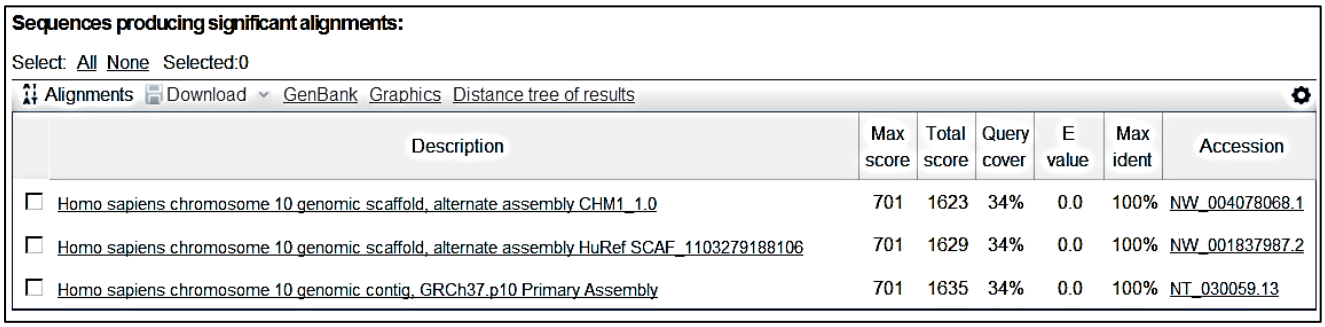
**Features:** pulmonary surfactant-associated protein A2 precursor

```
Query  123       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  182
                  ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  32097978  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCTAGCAATGG  32097919

Query  183       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  242
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097918  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32097859

Query  243       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  302
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097858  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32097799

Query  303       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  362
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  32097798  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGATGG  32097739

Query  363       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  422
                  |||||||||||||||||||||||||||||||||||||||| |||||||||||||||| |
Sbjct  32097738  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCTGCAGGTCGGGGAAAAGAGAA  32097679

Query  423       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  482
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097678  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32097619

Query  483       GACCATCTGTGAGTTCTGA  501
                  ||||||||||||||||||
Sbjct  32097618  GACCATCTGTGAGTTCTGA  32097600
```

## Sequence for colony 5 for N/CRD SP-A

NNGNNTTCCTNNNAATATTTTGTTTACTTTAAGAAGGAATTCAGGAGCCCTTCACCATGCATCTAGATGA
GGAGCTCCAAGCCACACTCCACGACTTTAGACATCAAATCCTGCAGACAAGGGGAGCCCTCAGTCTGCAGGG
CTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGGGCAGTCCATCACTTTTGATGCCATTCAGG
AGGCATGTGCCAGAGCAGGCGGCCGCATTGCTGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGC
TTCGTGAAGAAGTACAACACATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTAC
TCAGACGGGACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCAGTG
TGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACTGACCATCTGTGAGT
TCTGACTCGAGCCCAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTC
TCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTGATCCGGCTGCTAACAAAGCCCGAAAG
GAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTG
AGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACC
AAGCCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACA
CGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTC
AAACATGAGAATTAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGA
TAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGNTTATTT

TTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAANCCTGATAAATGCTTCAATAATATTGNAA
AAGGGANNNGTATGAGTATTCAACATTTCCGTNTNNCCCTTATTCCCTTTTTTGNNGNNNTTTTGCCTTC
CTNNNTTTTNCTNNNCCANAAACGCTGGNNNNANNNAAANNTNNTGAAAANNANTTGGGTGNNCNNA
NNNGGTTAATTNNAANNGG

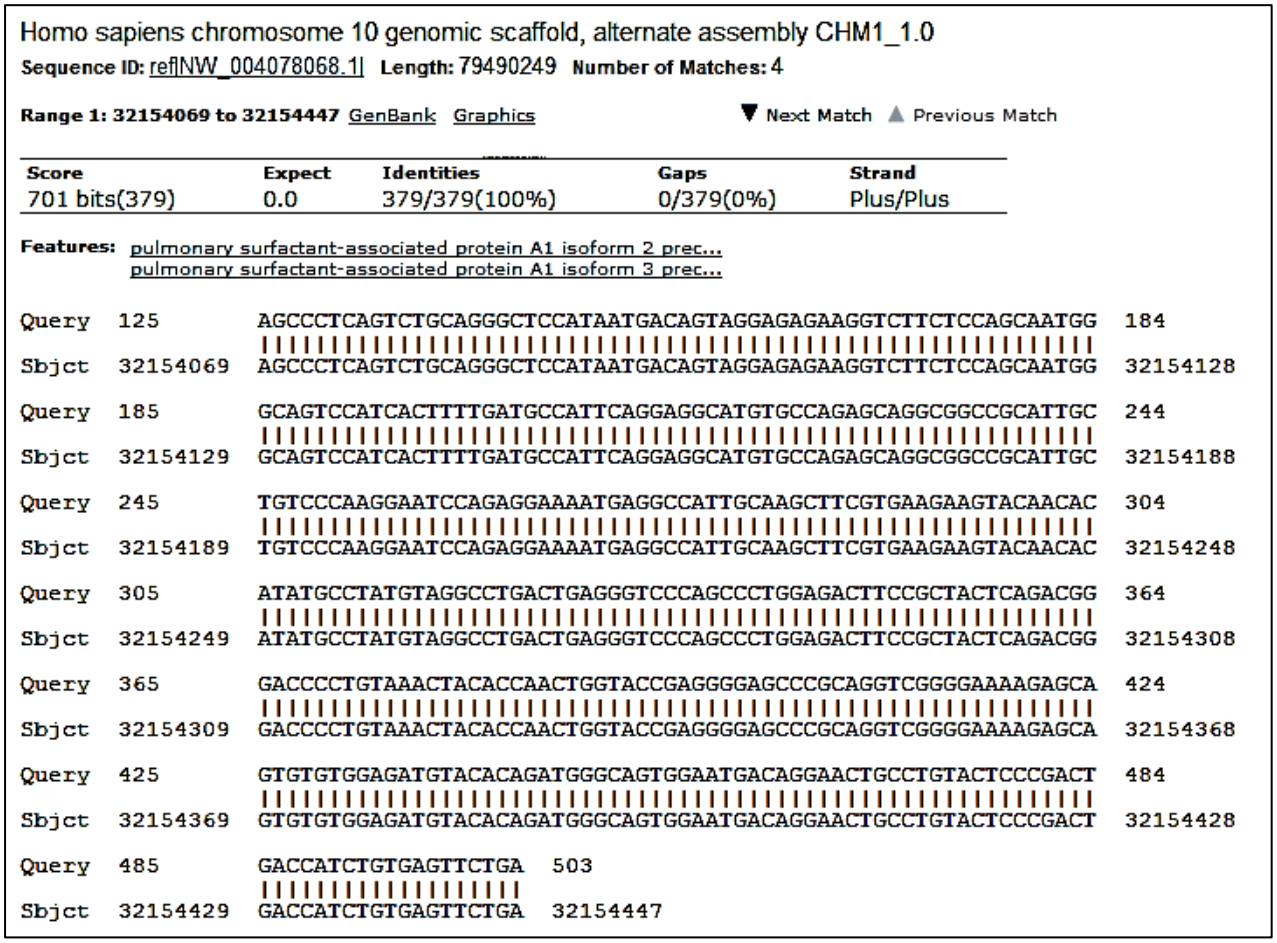


**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| | Description | Max score | Total score | Query cover | E value | Max ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0 | 701 | 1623 | 34% | 0.0 | 100% | NW_004078068.1 |
| ☐ | Homo sapiens chromosome 10 genomic scaffold, alternate assembly HuRef SCAF_1103279188106 | 701 | 1629 | 34% | 0.0 | 100% | NW_001837987.2 |
| ☐ | Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly | 701 | 1635 | 34% | 0.0 | 100% | NT_030059.13 |



Homo sapiens chromosome 10 genomic scaffold, alternate assembly CHM1_1.0

**Sequence ID:** ref|NW_004078068.1| **Length:** 79490249 **Number of Matches:** 4

**Range 1: 32154069 to 32154447** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 701 bits(379) | 0.0 | 379/379(100%) | 0/379(0%) | Plus/Plus |

**Features:** pulmonary surfactant-associated protein A1 isoform 2 prec...
pulmonary surfactant-associated protein A1 isoform 3 prec...

```
Query  125       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  184
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154069  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  32154128

Query  185       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  244
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154129  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32154188

Query  245       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  304
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154189  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32154248

Query  305       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  364
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154249  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  32154308

Query  365       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  424
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154309  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  32154368

Query  425       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  484
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32154369  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32154428

Query  485       GACCATCTGTGAGTTCTGA  503
                  ||||||||||||||||||
Sbjct  32154429  GACCATCTGTGAGTTCTGA  32154447
```

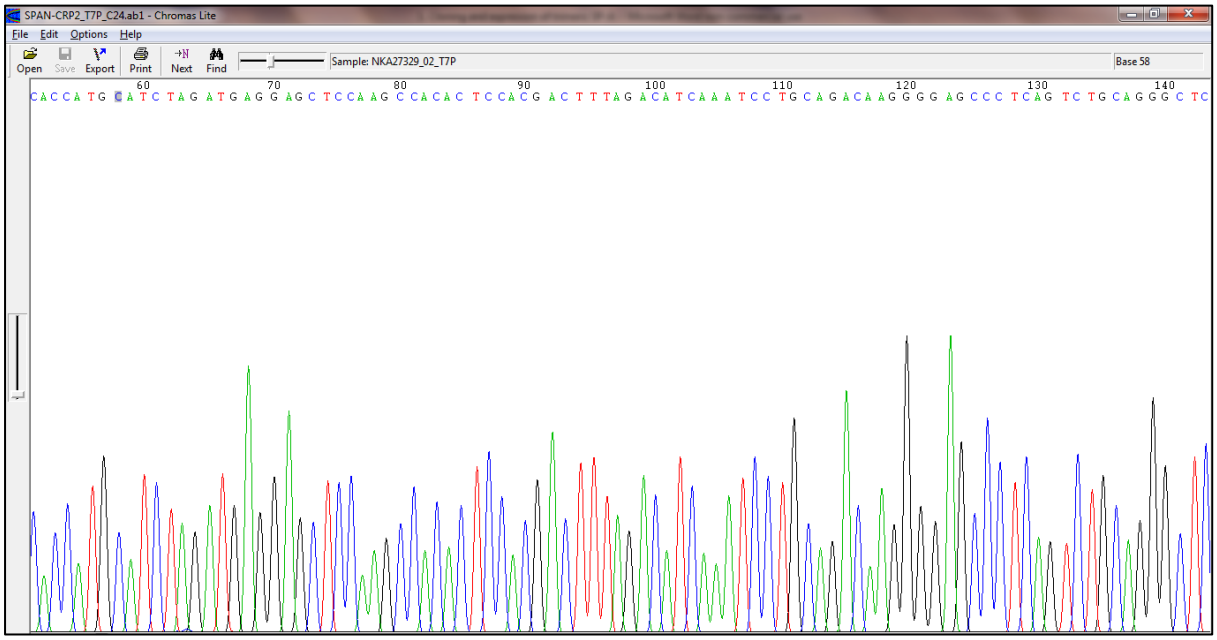
**Range 2: 32097600 to 32097978** GenBank Graphics ▼ Next Match ▲ Previous Match ⍋ First Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 678 bits(367) | 0.0 | 375/379(99%) | 0/379(0%) | Plus/Minus |

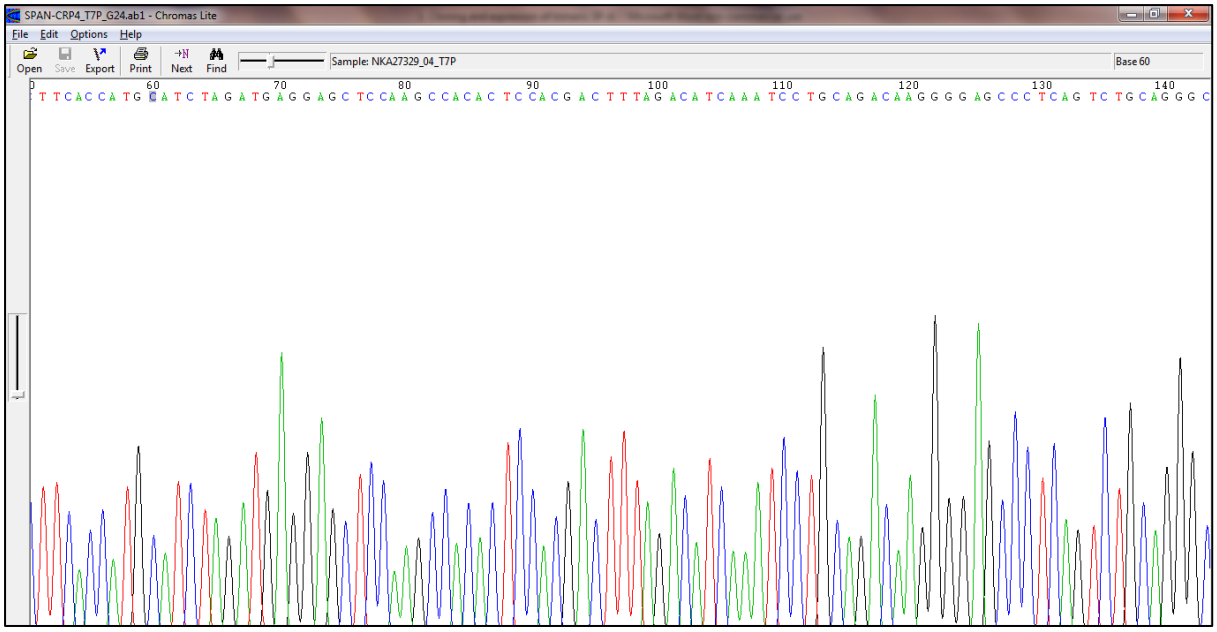
**Features:** pulmonary surfactant-associated protein A2 precursor

```
Query  125       AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCCAGCAATGG  184
                  ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  32097978  AGCCCTCAGTCTGCAGGGCTCCATAATGACAGTAGGAGAGAAGGTCTTCTCTAGCAATGG  32097919

Query  185       GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  244
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097918  GCAGTCCATCACTTTTGATGCCATTCAGGAGGCATGTGCCAGAGCAGGCGGCCGCATTGC  32097859

Query  245       TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  304
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097858  TGTCCCAAGGAATCCAGAGGAAAATGAGGCCATTGCAAGCTTCGTGAAGAAGTACAACAC  32097799

Query  305       ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGACGG  364
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  32097798  ATATGCCTATGTAGGCCTGACTGAGGGTCCCAGCCCTGGAGACTTCCGCTACTCAGATGG  32097739

Query  365       GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCCGCAGGTCGGGGAAAAGAGCA  424
                  ||||||||||||||||||||||||||||||||||||||||| |||||||||||||||| |
Sbjct  32097738  GACCCCTGTAAACTACACCAACTGGTACCGAGGGGAGCCTGCAGGTCGGGGAAAAGAGAA  32097679

Query  425       GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  484
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32097678  GTGTGTGGAGATGTACACAGATGGGCAGTGGAATGACAGGAACTGCCTGTACTCCCGACT  32097619

Query  485       GACCATCTGTGAGTTCTGA  503
                  ||||||||||||||||||
Sbjct  32097618  GACCATCTGTGAGTTCTGA  32097600
```

Once the sequences had been received, they were examined by ChromasLite. For colony 2 the ChromasLite sequencing image is shown below



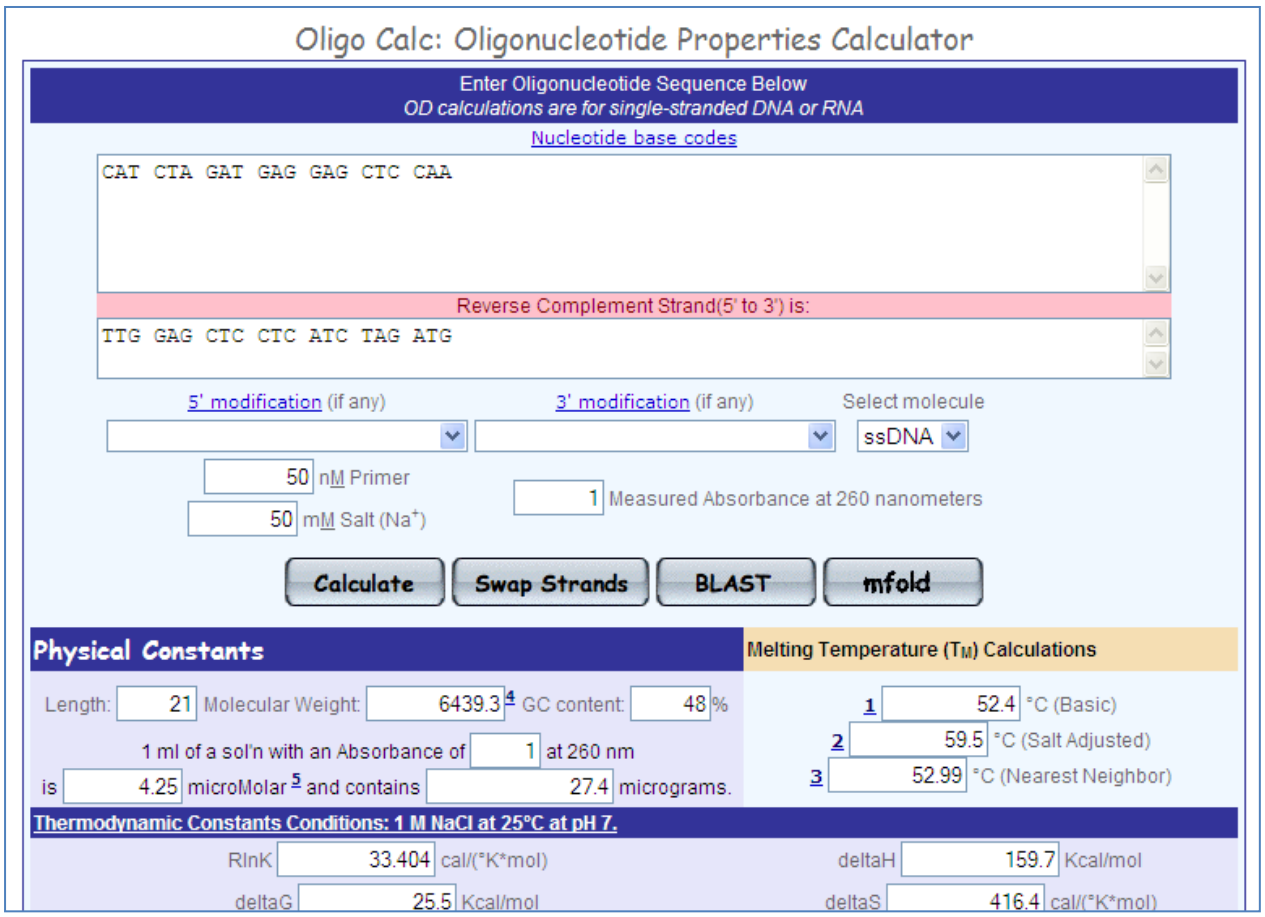

For colony 5 the ChromasLite sequencing image is shown below


SPAN-CRP4_T7P_G24.ab1 - Chromas Lite
File Edit Options Help
Open Save Export Print Next Find
Sample: NKA27329_04_T7P
Base 60
60 70 80 90 100 110 120 130 140

## 8.7 – Primer design for trimeric SP-A to clone in pMalC-2

Forward strand 5' – SP-A1

CAT CTA GAT GAG GAG CTC CAA (tm 57oC)




***Enzyme*** – forward – EcoRI – GAATTC

GGGGAATTC CAT CTA GAT GAG GAG CTC CAA

**Reverse Primer**

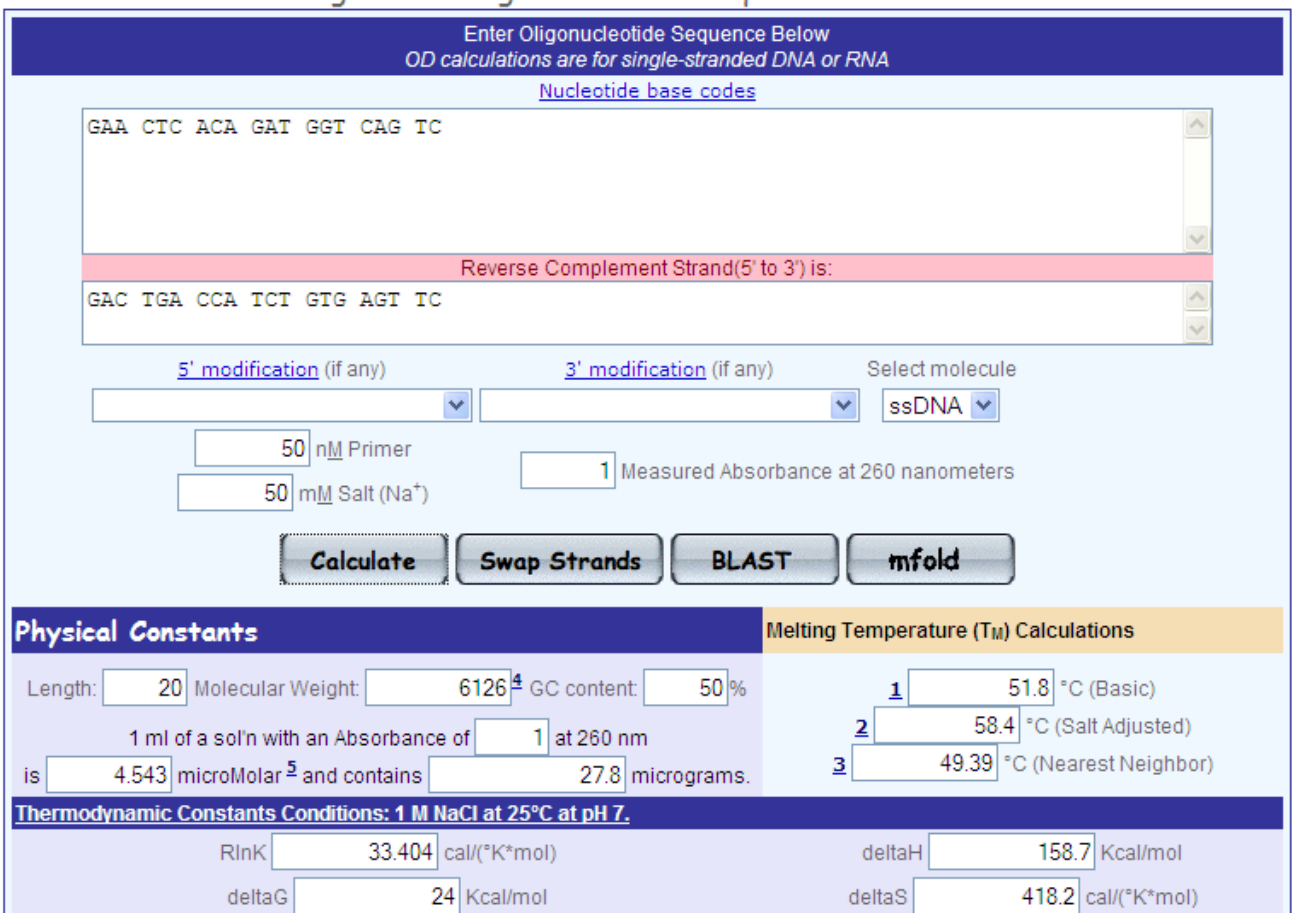
***Enzyme*** – REVERSE – BamHI – GGATC

CCGACTGACCATCTGTGAGTTC (tm 57oC)

Reverse strand 3' (reverse and complement) – SP-A1

GAACTCACAGATGGTCAGTC

# Oligo Calc: Oligonucleotide Properties Calculator



*8.8 – enzymes that don't cut within trimeric region of SP-A*

# Enzymes that don't cut

Help Comments

**SP-A tri**

Number of cuts 0

| # | Enzyme | Specificity |
|---|---|---|
| 1 | AatII | G▴ACGT▾C |
| 2 | AccI | GT▾MK▴AC |
| 3 | AclI | AA▾CG▴TT |
| 4 | AcuI | CTGAAG(N)$_{14}$▴NN▾ |
| 5 | AfeI | AGC▾▴GCT |
| 6 | AflII | C▾TTAA▴G |
| 7 | AflIII | A▾CRYG▴T |
| 8 | AgeI | A▾CCGG▴T |
| 9 | AleI | CACNN▾▴NNGTG |
| 10 | AlwI | GGATCNNNN▾N▴ |
| 11 | ApaI | G▴GGCC▾C |
| 12 | ApaLI | G▾TGCA▴C |
| 13 | ApeKI | G▾CWG▴C |
| 14 | ApoI | R▾AATT▴Y |
| 15 | AscI | GG▾CGCG▴CC |
| 16 | AseI | AT▾TA▴AT |
| 17 | AsiSI | GCG▴AT▾CGC |
| 18 | AvaI | C▾YCGR▴G |
| 19 | AvrII | C▾CTAG▴G |
| 20 | BaeGI | G▴KGCM▾C |
| 21 | BaeI | ▴(N)$_5$▾(N)$_{10}$ACNNNNGTAYC(N)$_7$▴(N)$_5$▾ |
| 22 | BamHI | G▾GATC▴C |

| | | |
|---|---|---|
| 23 | BbvCI | CC▼TCA▲GC |
| 24 | BbvI | GCAGC(N)$_8$▼NNNN▲ |
| 25 | BceAI | ACGGC(N)$_{12}$▼NN▲ |
| 26 | BcgI | ▲NN▼(N)$_{10}$CGA(N)$_6$TGC(N)$_{10}$▲NN▼ |
| 27 | BciVI | GTATCC(N)$_5$▲N▼ |
| 28 | BclI | T▼GATC▲A |
| 29 | BfuCI | ▼GATC▲ |
| 30 | BglI | GCCN▲NNN▼NGGC |
| 31 | BglII | A▼GATC▲T |
| 32 | BlpI | GC▼TNA▲GC |
| 33 | BmgBI | CAC▲GTC |
| 34 | BmrI | ACTGGGNNNN▲N▼ |
| 35 | BmtI | G▲CTAG▼C |
| 36 | Bpu10I | CC▼TNA▲GC |
| 37 | BpuEI | CTTGAG(N)$_{14}$▲NN▼ |
| 38 | BsaAI | YAC▲GTR |
| 39 | BsaBI | GATNN▲NNATC |
| 40 | BsaHI | GR▼CG▲YC |
| 41 | BsaI | GGTCTCN▼NNNN▲ |
| 42 | BsaWI | W▼CCGG▲W |
| 43 | BsgI | GTGCAG(N)$_{14}$▲NN▼ |
| 44 | BsiWI | C▼GTAC▲G |
| 45 | BsmBI | CGTCTCN▼NNNN▲ |
| 46 | BsmI | GAATG▲CN▼ |
| 47 | BsoBI | C▼YCGR▲G |
| 48 | BspDI | AT▼CG▲AT |
| 49 | BspEI | T▼CCGG▲A |
| 50 | BspHI | T▼CATG▲A |

| | | |
|---|---|---|
| 51 | BspQI | GCTCTTCN▼NNN▲ |
| 52 | BsrBI | CCG▲CTC |
| 53 | BsrFI | R▼CCGG▲Y |
| 54 | BssHII | G▼CGCG▲C |
| 55 | BssSI | C▼ACGA▲G |
| 56 | BstAPI | GCAN▲NNN▼NTGC |
| 57 | BstBI | TT▼CG▲AA |
| 58 | BstEII | G▼GTNAC▲C |
| 59 | BstUI | CG▲CG |
| 60 | BstXI | CCAN▲NNNN▼NTGG |
| 61 | BstYI | R▼GATC▲Y |
| 62 | BstZ17I | GTA▲TAC |
| 63 | Bsu36I | CC▼TNA▲GG |
| 64 | BtgI | C▼CRYG▲G |
| 65 | BtgZI | GCGATG$(N)_{10}$▼NNNN▲ |
| 66 | BtsCI | GGATG▲NN▼ |
| 67 | ClaI | AT▼CG▲AT |
| 68 | CspCI | ▲NN▼$(N)_{11}$CAA$(N)_5$GTGG$(N)_{10}$▲NN▼ |
| 69 | DpnI | GA▲TC |
| 70 | DpnII | ▼GATC▲ |
| 71 | DraI | TTT▲AAA |
| 72 | DraIII | CAC▲NNN▼GTG |
| 73 | DrdI | GACNN▲NN▼NNGTC |
| 74 | EarI | CTCTTCN▼NNN▲ |
| 75 | EciI | GGCGGA$(N)_9$▲NN▼ |
| 76 | EcoP15I | CAGCAG$(N)_{25}$▼NN▲ |
| 77 | EcoRI | G▼AATT▲C |
| 78 | EcoRV | GAT▲ATC |

| | | |
|---|---|---|
| 79 | FokI | GGATG(N)$_9$▼NNNN▲ |
| 80 | FseI | GG▲CCGG▼CC |
| 81 | FspI | TGC▼▲GCA |
| 82 | HaeII | R▲GCGC▼Y |
| 83 | HgaI | GACGC(N)$_5$▼(N)$_5$▲ |
| 84 | HhaI | G▲CG▼C |
| 85 | HinP1I | G▼CG▲C |
| 86 | HincII | GTY▼▲RAC |
| 87 | HpaI | GTT▼▲AAC |
| 88 | HpaII | C▼CG▲G |
| 89 | HphI | GGTGA(N)$_7$▲N▼ |
| 90 | Hpy99I | ▲CGWCG▼ |
| 91 | HpyCH4IV | A▼CG▲T |
| 92 | KasI | G▼GCGC▲C |
| 93 | MboI | ▼GATC▲ |
| 94 | MfeI | C▼AATT▲G |
| 95 | MluCI | ▼AATT▲ |
| 96 | MluI | A▼CGCG▲T |
| 97 | MlyI | GAGTC(N)$_5$▼▲ |
| 98 | MmeI | TCCRAC(N)$_{18}$▲NN▼ |
| 99 | MscI | TGG▼▲CCA |
| 100 | MseI | T▼TA▲A |
| 101 | MspA1I | CMG▼▲CKG |
| 102 | MspI | C▼CG▲G |
| 103 | MwoI | GCNN▲NNN▼NNGC |
| 104 | NaeI | GCC▼▲GGC |
| 105 | NarI | GG▼CG▲CC |
| 106 | NciI | CC▼S▲GG |

| | | |
|---|---|---|
| 107 | NcoI | C▼CATG▲G |
| 108 | NgoMIV | G▼CCGG▲C |
| 109 | NheI | G▼CTAG▲C |
| 110 | NmeAIII | GCCGAG(N)$_{19}$▲NN▼ |
| 111 | NruI | TCG▲CGA |
| 112 | NsiI | A▲TGCA▼T |
| 113 | PacI | TTA▲AT▼TAA |
| 114 | PaeR7I | C▼TCGA▲G |
| 115 | PciI | A▼CATG▲T |
| 116 | PflFI | GACN▼N▲NGTC |
| 117 | PflMI | CCAN▲NNN▼NTGG |
| 118 | PleI | GAGTCNNNN▼N▲ |
| 119 | PluTI | G▲GCGC▼C |
| 120 | PmeI | GTTT▲AAAC |
| 121 | PmlI | CAC▲GTG |
| 122 | PshAI | GACNN▲NNGTC |
| 123 | PsiI | TTA▲TAA |
| 124 | PspOMI | G▼GGCC▲C |
| 125 | PspXI | VC▼TCGA▲GB |
| 126 | PvuI | CG▲AT▼CG |
| 127 | PvuII | CAG▲CTG |
| 128 | RsrII | CG▼GWC▲CG |
| 129 | SacII | CC▲GC▼GG |
| 130 | SalI | G▼TCGA▲C |
| 131 | SapI | GCTCTTCN▼NNN▲ |
| 132 | Sau3AI | ▼GATC▲ |
| 133 | SbfI | CC▲TGCA▼GG |
| 134 | ScaI | AGT▲ACT |

| | | |
|---|---|---|
| 135 | SexAI | A▼CCWGG▲T |
| 136 | SfiI | GGCCN▲NNN▼NGGCC |
| 137 | SfoI | GGC▼▲GCC |
| 138 | SgrAI | CR▼CCGG▲YG |
| 139 | SmaI | CCC▼▲GGG |
| 140 | SmlI | C▼TYRA▲G |
| 141 | SnaBI | TAC▼▲GTA |
| 142 | SpeI | A▼CTAG▲T |
| 143 | SphI | G▲CATG▼C |
| 144 | SspI | AAT▼▲ATT |
| 145 | SwaI | ATTT▼▲AAAT |
| 146 | TaqI | T▼CG▲A |
| 147 | TseI | G▼CWG▲C |
| 148 | Tsp45I | ▼GTSAC▲ |
| 149 | TspMI | C▼CCGG▲G |
| 150 | Tth111I | GACN▼N▲NGTC |
| 151 | XcmI | CCANNNN▲N▼NNNNTGG |
| 152 | XhoI | C▼TCGA▲G |
| 153 | XmaI | C▼CCGG▲G |
| 154 | ZraI | GAC▼▲GTC |

# Chapter 21

# Purification of Native Surfactant Protein SP-A from Pooled Amniotic Fluid and Bronchoalveolar Lavage

**Najmunisa Karbani, Eswari Dodagatta-Marri, Asif S. Qaseem, Priyaa Madhukaran, Patrick Waters, Anthony G. Tsolaki, Taruna Madan, and Uday Kishore**

## Abstract

Surfactant protein SP-A is a hydrophilic glycoprotein, similar to SP-D, which plays an important role in pulmonary surfactant homeostasis and innate immunity. SP-A is actively expressed in the alveolar type II cells and Clara cells. Their basic structure consists of triple-helical collagen region and a C-terminal carbohydrate recognition domain (CRD). By binding to the infectious microbes, SP-A (like SP-D) are involved in pathogen opsonization and agglutination and subsequent clearance of the microorganism, via recruitment of phagocytic cells via receptors for the collagen region. SP-A has also been localized at extrapulmonary sites such as salivary epithelium, amniotic fluid, prostate glands, and semen. The presence of SP-A in fetal and maternal tissue and amniotic fluid suggests it is involved in pregnancy and labor. Native SP-A can be purified from amniotic fluid and bronchiolar lavage fluid (BALF) via affinity chromatography. In addition, we also report here a procedure to express and purify a recombinant form of trimeric CRD in *Escherichia coli*. The availability of highly pure native SP-A and CRD region can be central to studies that examine the diverse roles that SP-A play in surfactant homeostasis, pulmonary infection and inflammation and pregnancy.

**Key words** Surfactant protein-A, SP-A, Purification, Amniotic fluid, BALF, Recombinant, Inflammation, Affinity chromatography

## 1 Introduction

The respiratory system gets exposed to inhaled toxins, infectious microbes, and allergens. Surfactant proteins are important for lowering surface tension to allow inhalation and expiration without difficulty and to mediate the innate immune response against pathogen causing diseases for the clearance of inhaled microorganisms to maintain lung sterility [1]. There are four surfactant proteins characterized so far including; SP-A, SP-B, SP-C, and SP-D. Surfactant protein A (SP-A) are phospholipid-binding proteins

Mihaela Gadjeva (ed.), *The Complement System: Methods and Protocols*, Methods in Molecular Biology, vol. 1100,
DOI 10.1007/978-1-62703-724-2_21, 

synthesized by alveolar type II epithelial cells and stored as intracellular inclusion organelles called “lamellar bodies”. During deep inhalation, the surfactants are secreted covering the alveolar space minimizing surface tension at the air-liquid interface to prevent lungs from collapsing. They are collagen-containing, calcium dependent C-type lectins belonging to the collectin family [2, 3]. SP-A is the most abundant protein amongst the other surfactant proteins, making up 90 % of the proteins out of SP-A, SP-B, SP-C, and SP-D. SP-A is expressed by two alleles; SP-A1 and SP-A2 which are localized on the long arm of chromosome 10 at position 10q21-24 forming a cluster with SP-D and MBL [4]. In comparison to SP-D, SP-A is tenfolds higher in level of expression and plays a major role in surfactant turnover and homeostasis [5, 6]. The primary structure of SP-A consists of an amino-terminus, triple-helical collagen-like domain of tripepetide Gly-X-Y repeats, a lectin connector neck region which stabilizes the trimers and a C-terminus C-type lectin carbohydrate recognition domain (CRD). SP-A forms a hexameric shape consisting of 18 polypeptide (6 trimerized) chains with seven amino acids at the N-terminal, 23 Gly-x-y repeats at the collagen region and 148 amino acids at the globular C-terminal [7, 8]. The overall structure of SP-A resembles that of C1q with bouquet-like conformation. The hexameric, hydrophobic protein of 630 KDa is made up of $6 \times 105$ kDa subunits. These subunits are made up of three polypeptide chains of 35 KDa each [9]. The three polypeptides are bound together by the disulfide bonds at the N-terminal region.

SP-A plays a major role in the pulmonary innate immunity. In the trimeric form, the CRD can recognize and bind to carbohydrates moieties and pattern associated molecular patterns (PAMPs) on the surfaces of viruses, bacteria, yeast, and fungi in a $Ca^{2+}$-dependent manner [10, 11]. They can also recognize and opsonize pathogenic microorganisms via CRD region for chemotaxis by interacting to receptors on immune molecules such as macrophages and neutrophils [12]. SP-A is known to exert both inflammatory as well as anti-inflammatory effects depending on the receptor it binds to. The interaction of SP-A via calcium-dependent CRD to signal inhibitory regulatory protein α (SIRPα) results in an anti-inflammatory effect and the binding of SP-A collagen domain to CD91/calreticulin activates the pro-inflammatory pathway [13].

SP-A proteins are lipid and carbohydrate recognition molecules which in most cases bind to glucose and mannose residues part of most microbial ligands. SP-A can recognize both gram-positive bacteria such as *Staphylococcus aureus* [12] and *Mycobacterium tuberculosis* [14] and gram-negative bacteria. In the case of *M. tuberculosis,* SP-A enhances the interaction of *M. tuberculosis* to alveolar macrophages [14, 15] suggesting its antimicrobial activity for its clearance. SP-A is relevant for yeast and fungi agglutination and phagocytosis. In cases of *Candida albicans* SP-A suppresses

pro-inflammatory cytokine and chemokine production, i.e., TNF-α, IL-1β, MIP-α, and MCP-1 by alveolar macrophages and monocytes [16, 17].

By interacting with phagocytes, SP-A not only modulates the release of several cytokines, but also primes innate immune response against pathogens. In the situation of a viral infection caused by influenza A virus (IAV), the virus can enter the host lungs via its trimeric hemagglutinin (HA) molecule binding to sialic acid residues on the surface of epithelial cells of the lungs. SP-A benefits the host in two main ways: Firstly, the sialic acid present on the CRD of SP-A allows IAV to bind to promote agglutination inhibiting the infectivity [18]. Secondly, the binding of SP-A to IAV enhances virus to stimulate the respiratory burst of neutrophils [19].

SP-A is considered one of the primordial agents of innate immune defense. It can protect against allergenic challenges via allergen scavenging, inhibition of allergen-IgE cross-linking and histamine release, suppression of mast cells, basophils, B and T-cell proliferation, modulation of dendritic cells and macrophages, and Th cell polarization [20]. Subsequently, SP-A has been shown to bind to house dust mite extract *Dermatophagoides pteronyssinus; Derp* and 3-week culture filtrate (3wcf) as well as glycoprotein allergens; gp55 and gp45 of *Aspergillus fumigatus* in a carbohydrate specific and calcium dependent manner, blocking histamine release [21, 22]. This shows the significance of SP-A protection against in pulmonary hypersensitivity by resisting allergenic challenges and dampening hypersensitivity reactions in the lungs.

Using RT-PCR, SP-A is shown not to only synthesize in type II alveolar cells and Clara cells in the lungs, but extra-pulmonary sites of expression have also been shown. These include serum, amniotic fluid [23], sputum [24], thymus, prostate and mesentery [25], and also synoviocytes, lacrimal and salivary epithelia [26] as well as the human colon [27]. In pregnancy, the expression of SP-A is induced as early as 20 weeks gestation during fetal lung development. In the third trimester, there is a sudden surge of SP-A at 26 weeks rising six times more than SP-D between 26 and 40 weeks gestation [28], this could be linked to a possible signal to labor by preparing fetal lung maturation for survival in extra-uterine environment and pathogen clearance to prevent intra-amniotic infection.

In this chapter we describe protocols illustrating the purification of native SP-A from amniotic fluid and bronchoalveolar lavage fluid (BALF) adapted from Strong et al. [29], via Affinity Chromatography. We also describe the expression of a recombinant fragment of human SP-A (rhSP-A) in *Escherichia coli* and its purification via affinity chromatography. The rhSP-A contains the homotrimeric neck and CRD region inserted into the pET-101 vector. The bacterial cloning vector, pET-101 contains a T7 promoter site and an ampicillin resistant gene. pET-101 is then

transfected into the competent plasmid, BL21 (λDE3) pLysS. This plasmid has a T7 lysozyme inserted to suppress the basal levels of T7 promoter by naturally inhibiting T7 RNA polymerase. The recombinant protein is localized in inclusion bodies due to over-expression. Following denaturation–renaturation cycles with urea gradient, the rhSP-A is finally purified using a maltose–sepharose matrix in affinity chromatography.

## 2 Materials

### 2.1 Purification of Native SP-A from Amniotic Fluid

1. 8 M Urea buffer I: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$, 8 M urea.
2. Buffer I: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$.

### 2.2 Affinity Chromatography for Native SP-A Purification

1. Affinity buffer I: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$.
2. Affinity buffer I with 1 M NaCl: 50 mM Tris–HCl, pH 7.4, 1 M NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$.
3. SP-A elution buffer with EDTA: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.02 % $NaN_3$.

### 2.3 Purification of SP-A from BALF

1. Affinity buffer: 10 mM $CaCl_2$, 20 mM Tris–HCl, pH 7.5.
2. SP-A elution buffer: 10 mM EDTA, 20 mM Tris–HCl, pH 7.5.

### 2.4 BL21 (λDE3) pLysS: Competent Cell of Transformation and Expression

1. 1 L LB media: 10 g Tryptone, 10 g yeast, 5 g NaCl.
2. 100 mg/ml Ampicillin: 1 g Ampicillin in 10 ml of autoclaved $dH_2O$—filter-sterilized by passing through 0.2 μm filter.
3. 50 mg/ml Chloramphenicol: 0.5 g Chloramphenicol in 10 ml ethanol (100 %).
4. 1 M IPTG—2.83 g in 10 ml of autoclaved $dH_2O$—filter-sterilized by passing through 0.2 μm filter.

### 2.5 Cell Lysis and Inclusion Bodies Preparation in Order to Extract Recombinant SP-A Fragment Protein Containing Homotrimeric Neck and CRD Domain (rhSP-A)

1. 50 mg/ml Lysozyme: 0.5 g in 10 ml of autoclaved $dH_2O$ use.
2. 100 mM Phenylmethylsulfonyl fluoride (PMSF) stock: 0.174 g PMSF dissolved in 10 ml isopropanol.
3. 50 ml/L Pellet Lysis buffer: 50 mM Tris–HCl, 200 mM NaCl, 5 mM EDTA, 0.1 %Triton-X, 1 mM PMSF, 0.50 mg/ml lysozyme (add PMSF and lysozyme (1:100 dilution of stock) fresh into lysis buffer just before use).

### 2.6 Denaturation of Inclusion Bodies and Renaturation of rhSP-A

1. 50 ml Resolubilization buffer with 6 M urea: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 70 µl 2-mercaptoethanol (β-ME), 0.02 % $NaN_3$, 6 M urea.
2. 1 L 4 M-Urea buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.02 % $NaN_3$, 4 M urea.
3. 1 L 2 M-Urea buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.02 % $NaN_3$, 2 M urea
4. 1 L 1 M-Urea buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.02 % $NaN_3$, 1 M urea.
5. 1 L Final dialysis buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.02 % $NaN_3$.
6. 1 L Calcium buffer/Affinity buffer: 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$.

### 2.7 Purification of rhSP-A via Affinity Chromatography Using Maltose–Sepharose Purification Column

1. 1 L Affinity buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$.
2. 500 ml High salt buffer: 1 M NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$, 0.02 % $NaN_3$.
3. SP-A elution buffer containing 5 mM EDTA: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.02 % $NaN_3$.

### 2.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

1. SDS-gel: 30 % Bis-acrylamide, 1.5 M Tris–HCl, pH 8.8 (resolving gel), 1.0 M Tris–HCl, pH 6.8 (stacking gel), 10 % dodecylsulfate (SDS), 10 % ammonium persulfate (APS), TEMED.
2. 1× Running buffer (1 L): 3 g Tris base, 14.4 g glycine, 10 ml SDS (10 %), 990 ml $dH_2O$.
3. 2× SDS sample buffer (10 ml): 1.25 ml 0.5 M Tris–HCl, pH 6.8, 2 ml 50 % glycerol, 2 ml 10 % SDS, 0.5 ml β-ME, 0.5 % Bromophenol blue, 4 ml $dH_2O$.
4. Coomassie blue stain (1 L): 100 ml Glacial acetic acid, 400 ml methanol, 1 g Coomassie Blue R250, and 500 ml $dH_2O$.
5. De-stain solution (1 L): 100 ml Glacial acetic acid, 400 ml methanol, and 500 ml $dH_2O$.

### 2.9 Western Blot Buffers

1× Transfer buffer (1 L): 2.9 g Glycine, 5.8 g Tris base, 0.37 g SDS, 200 ml methanol, 800 ml $dH_2O$.

## 3 Methods

### 3.1 Purification of SP-A from Human Amniotic Fluid

Amniotic fluid is normally collected following vaginal rupturing of the fetal membranes or during spontaneous rupturing. The collected amniotic fluid is usually stored at –80 °C after equilibration with 5 mM $CaCl_2$ until it is required for purification.

1. Thaw 1 L of pooled amniotic fluid completely by incubating at 37 °C (usually it takes 5–6 h) with occasional mixing. *See* **Note 1**.
2. Pass amniotic fluid through 3 mm Whatman filter paper three times for a clearer suspension and to avoid future clogging of maltose-sepharose column.
3. Pass amniotic fluid through a cell strainer to remove fat and other large aggregates/debris.
4. Centrifuge at 13,800 × *g* for 40 min at 4 °C to separate SP-A rich pellet from the supernatant that is rich in SP-D.
5. Add 20 ml of 8 M urea buffer I to the pellet for further SP-A extraction. The urea helps to extract and unfold the protein which is then folded back to correct conformation by urea gradient dialysis.
6. Stir for 1 h at 4 °C using a magnetic stirrer to allow solubilization of SP-A.
7. Centrifuge the suspension at 13,800 × *g* in a microcentrifuge for 20 min at 4 °C and collect the supernatant.
8. Transfer the supernatant into a dialysis membrane and dialyze against 1 L of Buffer I containing no urea inside a measuring cylinder to remove urea from SP-A. Leave cylinder on a magnetic stirrer overnight at 4 °C.
9. Next day, collect dialysate (~20 ml) in a centrifugation tube and spin at 10,000 × *g* for 20 min at 4 °C and discard the pellet. Solubilized and correctly folded SP-A should be in the supernatant.
10. Proceed to affinity chromatography.

### 3.2 Affinity Chromatography

1. Wash 5 ml bed volume maltose-sepharose with 50 ml autoclaved distilled water (the preparation of maltose-sepharose is described in Chapter 22), followed by equilibration with four bed volumes of Affinity Buffer I.
2. Add the equilibrated maltose-sepharose into the supernatant containing SP-A and allow it to mix on a magnetic stirrer for 30 min at room temperature. This allows binding of soluble SP-A to maltose-sepharose.
3. Pass this supernatant containing maltose-sepharose bound to SP-A through the column three times in order to allow the bed to settle.
4. Wash the column after collecting all the flow through with five bed volumes of Affinity Buffer I containing 1 M NaCl
5. Elute 20 × 1 ml fractions of SP-A with Buffer I containing 10 mM EDTA. Collect all 1 ml fractions over ice (*see* **Note 2**).
6. 25 µl of each fraction is separated on a 12 % SDS-PAGE (*see* Table 1) to assess protein and its purity (Fig. 1).

**Table 1**
**Components and volume for preparing 12 and 15 % SDS-PAGE**

| Resolving gel | 12 % | 15 % | Stacking gel | |
|---|---|---|---|---|
| **Components** | **Volume (ml)** | | **Components** | **Volume (ml)** |
| $dH_2O$ | 3.3 | 2.3 | $dH_2O$ | 3.4 |
| 30 % Bis-acrylamide mix | 4 | 5 | 30 % Bis-acrylamide mix | 0.83 |
| 1.5 M Tris–HCl, pH 8.8 | 2.5 | 2.5 | 1.0 M Tris–HCl, pH 6.8 | 0.63 |
| 10 % SDS | 0.1 | 0.1 | 10 % SDS | 0.05 |
| 10 % APS | 0.1 | 0.1 | 10 % APS | 0.05 |
| TEMED | 0.015 | 0.015 | TEMED | 0.014 |

Prepare the stacking and resolving gel separately and add resolving gel first to the casting plates. After this has polymerized, add TEMED to the stacking gel and mix. Add this on top of the resolving gel inserting a comb to create wells after polymerization. The gel is then ready to be placed in a holder and into the tank containing 1× running buffer for wells to be filled with samples and protein marker. Set voltage to 100 V for 90 min for proteins to be separated according to size and charge

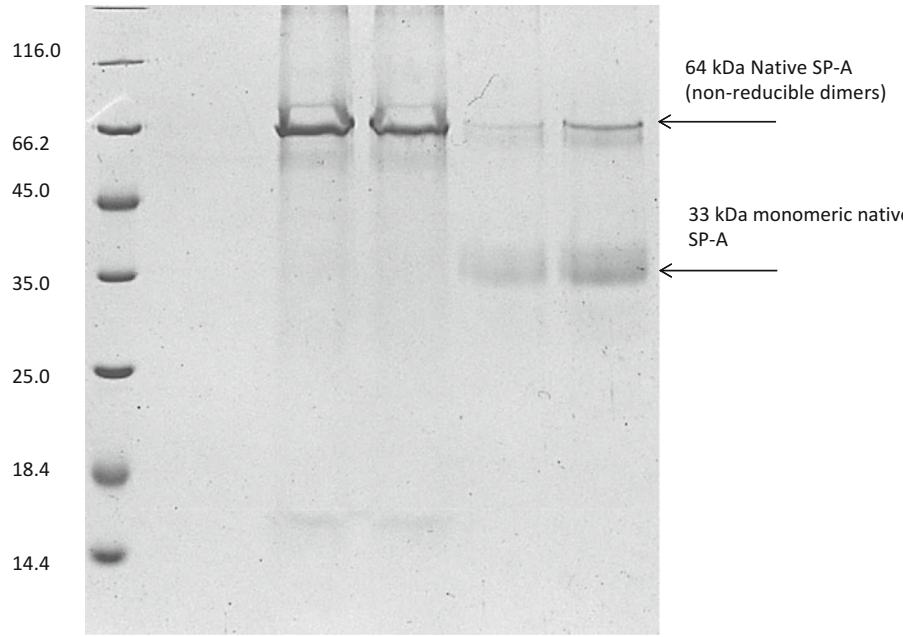



**Fig. 1** SDS-PAGE (10 % w/v) analysis of purified native SP-A under reducing conditions purified from BAL fluid. Majority of SP-A is the monomeric polypeptide chain of SP-A (33 kDa) and the lighter band corresponds to the non-reducible dimer proportion of SP-A. There are slight traces of the SP-A in its oligomeric form. This is confirmed by western blotting

### 3.3 SDS-PAGE: 12 % and 15 %

To prepare a 12 % or 15 % SDS-PAGE, follow the instructions below:

1. In two separate 50 ml beakers label one "resolving gel" and the other "stacking gel".
   For a standard size gel, 10 ml of resolving gel and 5 ml of stacking gel is sufficient.
2. Assemble the clean glass plates onto the glass holder and stand and check for no leakages.
3. Prepare resolving gel for either 12 % or 15 % (Table 1). *See* **Note 3**.
4. Once the TEMED has been added to the resolving gel the polymerization reaction will have begun, mix well then add the resolving gel to the plates (*see* **Note 4**).
5. After 20 min the gel will have polymerized. Add 10 % APS and TEMED to the stacking gel and pipette directly on top of the resolving gel, inserting the comb to form wells as the gel polymerizes.
6. Mix the protein samples with equal amounts of 2 × SDS sample buffer and heat to 95 °C for 10 min for protein denaturation.
7. The sample is then cooled to room temperature and centrifuged at 13,800 × *g* for 1 min before loading one sample per well.
8. Protein standard marker is added to the first well to determine the size of protein.
9. Add 1× running buffer to the tank as instructed by manufacturer.
10. Set the power pack connected to the gel apparatus to 100 V for 90 min to allow the proteins to be separated according to size, with the smaller molecules separating through the gel matrix faster with the larger molecules trailing behind.
11. The gel is then placed in staining solution overnight to be taken up by the proteins and destained to remove background staining for the protein bands to become visible.

### 3.4 Purification of SP-A from BALF

1. Equilibrate maltose-sepharose column with 100 ml Affinity buffer. Discard flow through.
2. Pass the BALF through the column four times to ensure efficient binding.
3. Wash the sepharose column with 10 ml of Affinity buffer I with 1 M NaCl. Discard flow through. This removes unbound non-specific proteins.
4. Elute the proteins with 20 ml SP-A eluting buffer containing EDTA in 1 ml fractions over ice (Fig. 2).

### 3.5 Competent Cell Preparation of BL21 (λDE3) pLysS for Recombinant SP-A Production

1. Inoculate a single colony of BL21 (λDE3) pLysS in 5 ml LB containing 5 μl of chloramphenicol (50 mg/ml).
2. Incubate overnight (~16 h) in a 37 °C shaker at 700 × *g*. Primary inoculum is now ready.

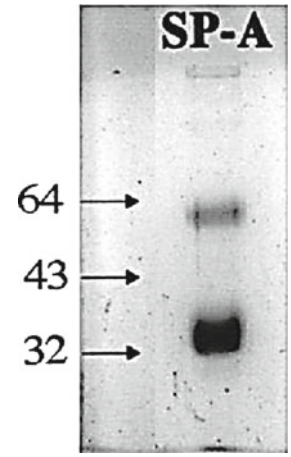



**Fig. 2** Western blot to detect native SP-A from amniotic fluid. Initially 1 and 2 μg proteins were separate on a SDS-PAGE and transferred at 320 amp for 90 min. The membrane was blocked with 5 % milk-powder/PBS buffer and probed with anti-human SP-A in a 1:5,000 dilution. The primary antibody was detected with a HRP conjugated Protein A in a 1:5,000 dilution. The bands were visible after DAB reacting with HRP to display protein bands; monomers and dimers of SP-A at 33 kDa and 64 kDa, respectively

3. Next day, transfer 1 ml of primary inoculum into 25 ml LB containing chloramphenicol.
4. Incubate for 2–3 h in the shaking incubator for cells to grow to log phase indicated by 0.3 against plain LB medium at $OD_{600\ nm}$.
5. Centrifuge the sample at 1,800 × *g* for 10 min to collect BL21 (λDE3) pLysS cells to pellet.
6. Discard supernatant in disinfectant and resuspend pellet in 12.5 ml 0.1 M $CaCl_2$.
7. Incubate pellet on ice for 1 h to make cells competent.
8. Centrifuge cells again at low speed centrifugation 1,800 × *g* for 5 min to pellet the cells.
9. Discard supernatant and resuspend cells in 2 ml 0.1 M $CaCl_2$.
10. The cells are now ready for transformation.

### *3.6 Transformation of Recombinant SP-A-Trimeric (pUK-A1) in BL21 (λDE3) pLysS*

1. Take 200 μl of competent cells and add to a 15 ml Falcon tube.
2. Add 2 μl of plasmid directly to the competent cells and incubate on ice for 1 h.
3. Heat shock the cells to take up the plasmid at 42 °C for 90 s.
4. Transfer the culture to ice for 5 min.

5. Add 800 µl of LB to the sample to make 1 ml total volume, and incubate at 37 °C for approximately 45 min to allow one full cell cycle to take place to increase transformed cells.
6. Add 100 µl of transformed cultured to an LB agar + ampicillin/chloramphenicol plate and incubate overnight at 37 °C (*see* **Note 5**).

### 3.7 Pilot-Scale Expression

1. Inoculate four separate colonies in 5 ml LB containing 5 µl of ampicillin and chloramphenicol to grow overnight in shaking incubator at 37 °C.
2. Next day, transfer 500 µl of primary culture into 10 ml LB with 10 µl ampicillin and chloramphenicol and incubate in the 37 °C shaking incubator to grow to log phase, indicated by a reading of 0.6–0.8 at 600 nm against plain LB medium.
3. When optimum OD has been reached, take 1 ml of un-induced sample and add 5 µl of 1 M IPTG to make a final concentration of 0.5 µM to the 10 ml culture.
4. Incubate both induced and un-induced in a 37 °C shaking incubator for a further 3 h to allow induction to take place and to over-express protein of interest.
5. After 3 h, take 1 ml of un-induced and induced samples and centrifuge at 13,800 × *g* for 15 min to pellet cells.
6. Discard supernatant.
7. Add 100 µl of 2× treatment buffer and denature proteins to separate 10 µl of each sample on a 15 % SDS-PAGE (*see* Table 1) (*see* **Note 6**).
8. After observing gel, the colony giving best expression can be streaked on a LB agar plate containing ampicillin and chloramphenicol using the primary inoculum.
9. This plate of colonies can be used to make multiple large-scale batches before reseeding the plate of colonies the following month.
10. Proceed to large-scale expression.

### 3.8 Large-Scale Expression

1. Inoculate one colony from SP-A-trimeric culture plate into 25 ml LB medium with 25 µl ampicillin and chloramphenicol.
2. Incubate in a 37 °C shaking incubator for 16 h.
3. Transfer 25 ml of the primary culture into 1 L LB medium with 1,000 µl ampicillin and chloramphenicol.
4. Incubate this secondary culture at 37 °C shaker for approximately 3 h until the OD reaches between 0.6 and 0.8.
5. Pipette 1 ml sample from culture for negative control ("un-induced sample").

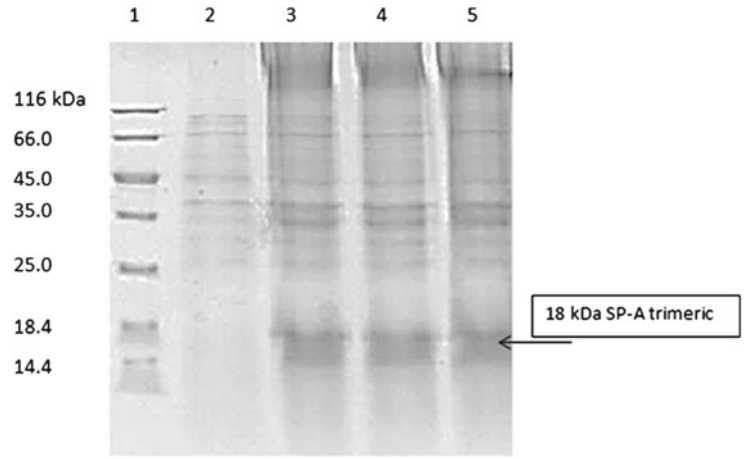



**Fig. 3** SDS-PAGE after large-scale expression of SP-A-trimeric. Expression of recombinant SP-A showing un-induced (*lane 2*) and induced (*lane 3–5*) of 3 × 1 L batches. *Lane 1*: Protein marker contains molecular weight ranging from lowest to highest: 14.4, 18.4, 25.0, 35.0, 45.0, 66.2, 116.0, *Lane 2*: un-induced, *Lane 3–5*: induced batches 1–3. 10 μl of sample including 2× treatment buffer was loaded to each well

6. To the rest of the sample, "induce" add 500 μl 0.5 mM IPTG from 1 M stock and re-incubated in the 37 °C shaker for 3 h with "un-induced" sample.
7. After 3 h, take 1 ml from "induced" sample and centrifuge along with 1 ml "un-induced" sample at high speed 13,800 × *g* for 15 min.
8. Discard supernatant and add 100 μl of 2× treatment buffer to the pellet. Denature at 95 °C and separate on a 15 % SDS-PAGE (Table 1) to determine protein expression (Fig. 3).
9. Centrifuge the entire 1 L "induced" culture at 13,800 × *g* at 4°C, for 15 min to collect pellet containing proteins in inclusion bodies.
10. The pellet can be stored at −20 °C or used straight away for lysing and sonication.

### 3.9 Lysing Inclusion Bodies to Extract Protein

1. If pellet was stored at −20 °C, it should be thawed on ice.
2. Resuspend the pellet in 50 ml lysis buffer and stir at 4 °C.
3. Whilst stirring, add PMSF to 1 mM and 0.5 mg/ml lysozyme to the lysis buffer. Allow to stir for ~2 h (until there are no visible lumps).
4. Keep suspension on ice and sonicate at 4 kHz for 30 s with 2 min interval between each cycle to allow sonicator to cool down, repeating for 15 cycles.

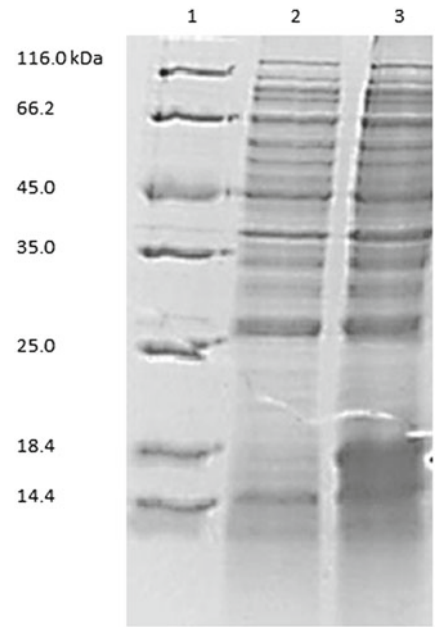



**Fig. 4** 15 % SDS-PAGE after lysis, sonication. Pellets containing SP-A in inclusion bodies are lysed and sonicated. After centrifugation of sample, protein is present in pellet before solubilsation. *Lane 1*: Protein marker contains molecular weight ranging from lowest to highest: 14.4, 18.4, 25.0, 35.0, 45.0, 66.2, 116.0, *Lane 2*: supernatant after centrifugation of lysed and sonicated sample, *Lane 3*: pellet after centrifugation of lysed and sonicated sample. 10 μl of sample including 2× treatment buffer was loaded to each well

5. Centrifuge sample to collect the protein rich pellet out of inclusion bodies at 13,800 × *g* for 15 min at 4 °C.
6. Collect 50 μl sample of supernatant and pellet to separate on a 12 % SDS-PAGE (Table 1) to confirm protein is visible in pellet (Fig. 4) (*see* **Note 7**).

### *3.10 Denaturing and Refolding Recombinant Proteins*

1. To the pellet, add 50 ml resolubilization buffer containing 6 M urea.
2. Incubated pellet at 4 °C whilst on a magnetic flea containing resolubilization buffer for exactly 1 h.
3. Centrifuge sample at 13,800 × *g*, at 4 °C, for 15 min to separate the debris rich pellet and supernatant containing protein. Take a sample of the supernatant to separate on an SDS-PAGE to ensure protein is in the supernatant and has been extracted out of pellet.

### *3.11 Stepwise Dialysis to Refold Protein and Remove Urea Traces*

1. Transfer the supernatant into a dialysis bag and add to a 1 L measuring cylinder containing 1 L 4 M-urea buffer and fresh β-ME.
2. Dialyze the sample for 2 h on a magnetic stirrer at 4 °C.

3. Transfer the dialysis bag to another 1 L cylinder containing 2 M-urea buffer with fresh β-ME.
4. Repeat **step 2**.
5. Transfer the dialysis bag to another 1 L cylinder containing 1 M-urea buffer with fresh β-ME and repeat **step 2**.
6. Transfer the dialysis bag to another 1 L cylinder containing 0 M-urea final dialysis buffer.
7. Dialyze sample overnight at 4 °C whilst stirring.
8. The next day, clarify dialysate by centrifugation at 13,800 × *g*, at 4 °C, for 15 min.
9. Transfer the supernatant into a new dialysis bag and dialyze against 1 L of calcium buffer for 3 h to completely remove traces of urea.
10. Clarify dialysate by centrifugation at 13,800 × *g*, at 4 °C, for 15 min.
11. The sample is now ready for affinity chromatography.

### 3.12 Recombinant SP-A Purification by Affinity Chromatography

1. Wash maltose-sepharose column thoroughly with 100 ml autoclaved $dH_2O$.
2. Equilibrate the maltose-sepharose column with 75 ml affinity buffer (with 5 mM $CaCl_2$).
3. Pass the supernatant through the maltose-sepharose column four times.
4. Wash column with 20 ml high-salt buffer (1 M NaCl) to remove any debris and unbound proteins.
5. Elute proteins with 20 ml elution buffer in 1 ml fractions over ice.
6. Estimate protein concentration at $OD_{280}$, using elution buffer as a reference.
7. Separate proteins on a 12 % SDS-PAGE (Table 1) to determine protein estimation and purity (Fig. 5).

### 3.13 Western Blot

1. Initially, prepare a 12 % SDS-PAGE (Table 1) and separate desired protein of interest to be probed.
2. After the dye front has reached the end of the gel, remove gel and equilibrate in 1× transfer buffer along with fibro pads, nitrocellulose membrane and filter paper for 10 min.
3. Assemble the sandwich in the following order: fibro pads, 2× filter paper, gel, membrane, 2× filter paper and fibro pads (*see* **Note 8**).
4. After closing the cassette, place inside the holder and into the tank containing 1× transfer buffer with the black side of cassette facing the black side of holder.

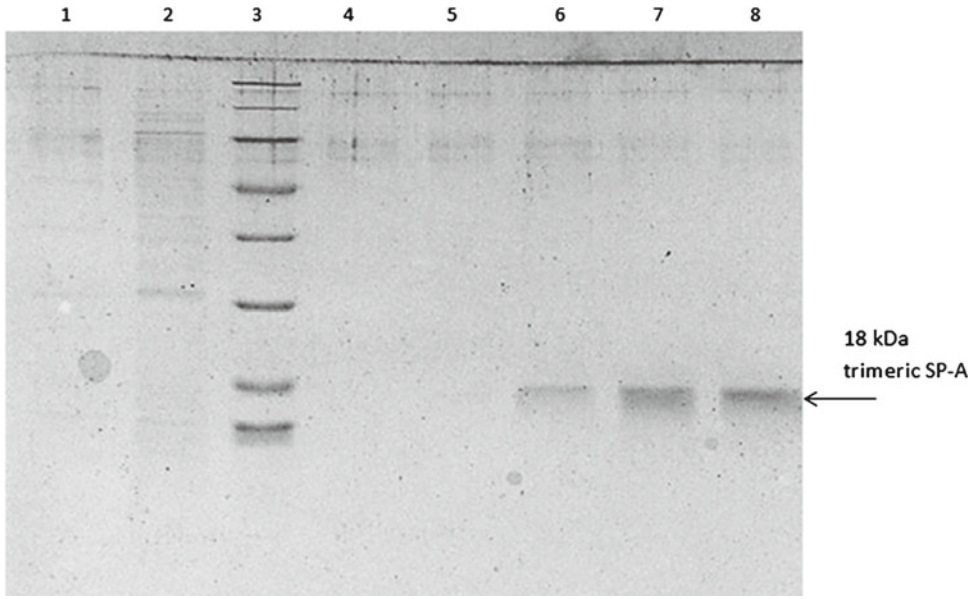



**Fig. 5** 12 % SDS-PAGE of purified SP-A samples after affinity chromatography. The eluted samples were separated on a protein gel to determine size and purity. As controls the flow through after passing sample and flow through after washing column with affinity buffer was loaded to check for protein loss and contaminant removal. *Lane 1*: flow through from sample containing recombinant trimeric SP-A, *Lane 2*: flow through after washing column with affinity buffer containing 1 M NaCl, *Lane 3*: Protein marker contains molecular weight ranging from lowest to highest: 14.4 kDa, 18.4 kDa, 25.0 kDa, 35.0 kDa, 45.0 kDa, 66.2 kDa, 116.0 kDa, *Lane 4–8*: eluted samples 1–5 containing pure protein. 20 μl of sample including 2× treatment buffer was loaded to each well

5. For transfer to take place, set power pack to 340 amp for 90 min for proteins to be transferred to the nitrocellulose membrane.
6. Block the membrane with 5 % nonfat milk powder/PBS buffer overnight at room temperature to prevent nonspecific binding.
7. The next day, decant the blocking buffer and wash membrane with 20 ml 1× PBS for 10 min.
8. Add 10 ml of 1:500 dilution of primary antibody (rabbit anti-human SP-A) (1 mg/ml) to the membrane and incubate for 2 h at room temperature on a rocker.
9. Decant the antibody and wash the membrane thrice with PBS containing 0.05 % Tween-20, for 10 min each removing old wash buffer each time.
10. Add 10 ml of 1:1,000 dilution of secondary antibody (protein A-HRP) (1 mg/ml) over the membrane and leave for a 1 h incubating at room temperature on a rocker.

11. Again, decant the antibody and wash the membrane with PBS Tween 20, 0.05 % thrice for 10 min each.
12. Whilst the final wash is taking place, prepare two tablets of DAB (sigma) dissolved in $dH_2O$ as recommended by manufacturer.
13. After removing wash buffer, pour DAB over the membrane and incubate for 2–10 min until sufficient color develops for bands to be visible.

## 4 Notes

1. Proteins can be cleaved by proteases if left for too long at room temperature.
2. If the concentration is low then the samples can be pooled and concentrated using polyethylene glycol.
3. Keep at least 1 cm distance from the wells to the resolving gel to allow proteins to stack evenly before being separated through the resolving gel.
4. Do not add TEMED until just before adding to the glass plates as this will start the polymerization reaction.
5. Add the rest of the culture (i.e., 900 μl) to 5 ml LB and grow overnight to streak next day. This is done as a backup, in case the number of transformed colonies is very low.
6. Un-induced sample is used as a control to detect SP-A. SP-A should be expressed in induced sample only, which is indicated as a thick band most highly expressed at ~18 kDa.
7. Supernatant can be stored at −20 °C in case there was still some protein that needed further extraction.
8. Ensure that there are no air bubbles in between the gel and membrane as this will affect the transfer of proteins.

### References


1. Crouch E, Wright JR (2001) Surfactant proteins A and D and pulmonary host defense. Annu Rev Physiol 63:521–554
2. Lu J et al (2002) Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochim Biophys Acta 1572(2–3):387–400
3. Holmskov U, Thiel S, Jensenius JC (2003) Collections and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol 21:547–578
4. Sorensen GL, Husby S, Holmskov U (2007) Surfactant protein A and surfactant protein D variation in pulmonary disease. Immunobiology 212(4–5):381–416
5. Ogasawara Y et al (1994) Chimeras of surfactant proteins A and D identify the carbohydrate recognition domains as essential for phospholipid interaction. J Biol Chem 269(47):29785–29792
6. Haagsman HP, van Golde LM (1991) Synthesis and assembly of lung surfactant. Annu Rev Physiol 53:441–464
7. Tenner AJ et al (1989) Human pulmonary surfactant protein (SP-A), a protein structurally homologous to C1q, can enhance FcR- and CR1-mediated phagocytosis. J Biol Chem 264 (23):13923–13928
8. Wright JR (2005) Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 5(1): 58–68

9. Voss T et al (1988) Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36. Structural homology with the complement factor C1q. J Mol Biol 201(1):219–227
10. King RJ et al (1986) Interaction between the 35 kDa apolipoprotein of pulmonary surfactant and saturated phosphatidylcholines. Effects of temperature. Biochim Biophys Acta 879(1):1–13
11. Hawgood S et al (1987) Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28–36 in surfactant lipid adsorption. Proc Natl Acad Sci U S A 84(1):66–70
12. Geertsma MF et al (1994) Binding of surfactant protein A to C1q receptors mediates phagocytosis of *Staphylococcus aureus* by monocytes. Am J Physiol 267(5 Pt 1):L578–L584
13. Gardai SJ et al (2003) By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115(1):13–23
14. Gaynor CD et al (1995) Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. J Immunol 155 (11):5343–5351
15. Pasula R et al (1997) Surfactant protein A (SP-A) mediates attachment of *Mycobacterium tuberculosis* to murine alveolar macrophages. Am J Respir Cell Mol Biol 17(2):209–217
16. Rosseau S et al (1997) Phagocytosis of viable *Candida albicans* by alveolar macrophages: lack of opsonin function of surfactant protein A. J Infect Dis 175(2):421–428
17. Rosseau S et al (1999) Surfactant protein A down-regulates proinflammatory cytokine production evoked by *Candida albicans* in human alveolar macrophages and monocytes. J Immunol 163(8):4495–4502
18. Benne CA et al (1995) Interactions of surfactant protein A with influenza A viruses: binding and neutralization. J Infect Dis 171(2): 335–341
19. Hartshorn KL et al (1997) Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins. Am J Physiol 273(6 Pt 1):L1156–L1166
20. Kishor U et al (2002) Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by *Aspergillus fumigatus*. Immunobiology 205 (4–5):610–618
21. Wang JY et al (1996) Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. Clin Exp Immunol 106(2):367–373
22. Wang JY et al (1998) Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. Am J Respir Crit Care Med 158(2):510–518
23. Miyamura K et al (1994) Surfactant proteins A (SP-A) and D (SP-D): levels in human amniotic fluid and localization in the fetal membranes. Biochim Biophys Acta 1210(3): 303–307
24. Masuda T et al (1991) Surfactant apoprotein-A concentration in sputum for diagnosis of pulmonary alveolar proteinosis. Lancet 337 (8741): 580–582
25. Chailley-Heu B et al (1997) Expression of hydrophilic surfactant proteins by mesentery cells in rat and man. Biochem J 328(Pt 1): 251–256
26. Dobbie JW (1996) Surfactant protein A and lamellar bodies: a homologous secretory function of peritoneum, synovium, and lung. Perit Dial Int 16(6):574–581
27. Eliakim R et al (1997) Isolation and characterization of surfactant-like particles in rat and human colon. Am J Physiol 272(3 Pt 1): G425–G434
28. Khoor A et al (1993) Developmental expression of SP-A and SP-A mRNA in the proximal and distal respiratory epithelium in the human fetus and newborn. J Histochem Cytochem 41(9):1311–1319
29. Strong P et al (1998) A novel method of purifying lung surfactant proteins A and D from the lung lavage of alveolar proteinosis patients and from pooled amniotic fluid. J Immunol Methods 220(1–2):139–149

# Chapter 22

# Purification of Surfactant Protein D (SP-D) from Pooled Amniotic Fluid and Bronchoalveolar Lavage

**Eswari Dodagatta-Marri, Asif S. Qaseem, Najmunisa Karbani, Anthony G. Tsolaki, Patrick Waters, Taruna Madan, and Uday Kishore**

## Abstract

Surfactant protein SP-D is a multimeric collagenous lectin, called *collectin*. SP-D is a multifunctional, pattern recognition innate immune molecule, which binds in a calcium dependent manner to an array of carbohydrates and lipids, thus offering resistance to invading pathogens, allergen challenge, and pulmonary inflammation. SP-D is predominantly found in the endoplasmic reticulum of type 2 pneumocytes and in the secretory granules of Clara or non-ciliated bronchiolar cells. The highest expression of SP-D is observed in the distal airways and alveoli. There is also an extra pulmonary existence of SP-D. The common sources of native full-length human SP-D are bronchoalveolar lavage (BAL) washings from normal or preferably patients suffering from alveolar proteinosis who overproduce SP-D in the lungs. Amniotic fluid collected at the term during parturition is another reasonable source. Here, we describe a simple and rapid method of purifying native SP-D away from SP-A which is also present in the same source. We also describe procedures of expressing and purifying a recombinant fragment of human SP-D (rhSP-D) comprising trimeric neck and carbohydrate recognition domains that has been shown to have therapeutic effects in murine models of allergy and infection.

**Key words** Surfactant protein-D, SP-D, Purification, Amniotic fluid, Recombinant SP-D, Inflammation, Affinity chromatography

## 1 Introduction

The respiratory surface in the lungs inevitably interacts with a variety of inhaled toxins, microbes and other particulate matter. Host defense mechanisms within the lungs must facilitate clearance of inhaled pathogens and particles, while minimizing an inflammatory response that could damage the thin delicate gaseous-exchange lining of the alveoli and distal airways. Pulmonary surfactant is a complex of lipids and proteins that are physiologically essential. There are of four types of surfactant proteins; SP-A [1], SP-B [2], SP-C [3], and SP-D, which constitute 5.3, 0.7, 0.4, and 0.6 % of

Mihaela Gadjeva (ed.), *The Complement System: Methods and Protocols*, Methods in Molecular Biology, vol. 1100,
DOI 10.1007/978-1-62703-724-2_22, 

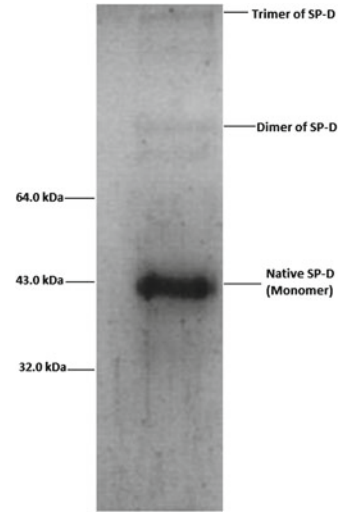



**Fig. 1** SDS–PAGE (10 % w/v) analysis of purified SP-D under reducing conditions. SP-D is composed of a 43 kDa polypeptide chain, with *faint bands* corresponding to dimers and trimers of the 43 kDa chain can also been seen

total surfactant, respectively [4–7]. The gene encoding the SP-D protein is localized at the genomic region of 10q22.2–23.1 near the MBL gene [8] of chromosome 10 in humans. The SP-D polypeptide chain is 355 residues long, with N-terminal 25 non-collagen residues, 177 collagenous residues and 153 residues of the C-terminal lectin domain [9] (Fig. 1). The lectin domain is the globular head region of the protein which binds to pathogen-associated molecular patterns (PAMPs), whilst the collagenous region initiates phagocytosis [10].

Both SP-A and SP-D bind to carbohydrate structures on the surfaces of pathogens, such as viruses [11], bacteria [12], yeast [13], fungi [14], pollen, apoptotic and necrotic cells [15–18]. SP-D also directly binds to type II cell apical membranes [19] and alveolar macrophages [20]. Most of SP-D is found in the aqueous bronchoalveolar lavage fluid (BALF), which binds to phosphotidylinositol (PI) and glucosylceramide, which are sugar moieties present in surfactants [21]. As a potent opsonin, SP-D interacts with a range of pathogens and engineers clearance mechanism including aggregation/agglutination, growth inhibition, enhancement of phagocytosis by phagocytic cells, and production of superoxidative burst and nitric oxide [22].

SP-D has also been implicated in protecting lungs against allergenic challenge [23]. SP-D can bind allergens derived from house dust mite [24] and *Aspergillus fumigatus* [25] inhibit specific IgE binding to allergens and dampen histamine release by sensitized basophils [14]. The interaction with allergens is mediated via CRD region. Thus, the rhSP-D composed of trimeric

neck and CRD region only, produced in *Escherichia coli*, has been shown to be protective in murine models of lung allergy [26–28].

A significant level of SP-D has been detected immunohistochemically in human trachea, brain, testis, salivary gland, heart, prostate, kidneys, small intestine, pancreas, and placenta. Lower levels of expression have also been detected in spleen, adrenal gland, uterus, and mammary glands [29–31]. SP-A, on the other hand, is found to be at low levels in the human and rat small and large intestines [32], mesentery, and colon [33–35]. SP-A is found in human prostate, thymus, amniotic fluid and epithelium, and salivary glands in very low quantities [34]. SP-A has also been detected in the entire human lacrimal apparatus including lacrimal gland, nasolacrimal ducts, conjunctiva, cornea, and human tear fluid [35].

Here, we describe a step-by-step method to purify human SP-D from pooled amniotic fluid and BAL fluid. The BAL washings from pulmonary alveolar proteinosis patients are excellent source of native SP-D [36]. The fact that SP-D binds to maltose reasonably well, via its CRD region, has been exploited for purification that involves use of maltose agarose or sepharose as an affinity matrix.

## 2 Materials

### 2.1 Protein Purification Buffers

1. Low salt affinity buffer: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$.
2. High salt affinity buffer: 50 mM Tris–HCl, pH 7.4, 1 M NaCl, 10 mM $CaCl_2$.
3. Elution buffer: 50 mM Tris–HCl, pH 7.4, 50 mM $MnCl_2$ and 0.02 % $NaN_3$.
4. EDTA buffer: for $MnCl_2$ removal 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA and 0.02 % $NaN_3$.
5. Buffer I + 8 M urea: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.02 % $NaN_3$ and 8 M urea.
6. Buffer I: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ 0.02 % $NaN_3$.

### 2.2 Ion Exchange Chromatography

1. High salt affinity buffer: 50 mM Tris–HCl, pH 7.4, 1 M NaCl, 5 mM $CaCl_2$,0.02 % $NaN_3$.
2. Low salt affinity buffer: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ 0.02 % $NaN_3$.
3. SP-D eluting buffer with maltose: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 25 mM maltose, 0.02 % $NaN_3$.

### 2.3 SDS-PAGE

1. SDS-gel: 30 % Bis-acrylamide, 1.5 M Tris–HCl, pH 8.8 (resolving gel), 1.0 M Tris–HCl, pH 6.8 (stacking gel), 10 % SDS, 10 % APS, TEMED.
2. 1× Running buffer (1 l): 3 g Tris base, 14.4 g glycine, 10 ml SDS (10 %), 990 ml $dH_2O$.
3. 2× SDS sample buffer (10 ml): 1.25 ml 0.5 M Tris–HCl, pH 6.8, 2 ml 50 % glycerol, 2 ml 10 % SDS, 0.5 ml β-mercaptoethanol, 0.5 % Bromophenol blue, 4 ml $dH_2O$.
4. Coomassie blue stain (1 L): 100 ml Glacial acetic acid, 400 ml methanol, 1 g Coomassie blue R250, and 500 ml $dH_2O$.
5. De-stain solution (1 L): 100 ml Glacial acetic acid, 400 ml methanol, and 500 ml $dH_2O$.

### 2.4 Western Blot Buffers

1. 1× Transfer buffer: 2.9 g Glycine, 5.8 g Tris base, 0.37 g SDS, 200 ml methanol, 800 ml $dH_2O$.

### 2.5 Maltose–Sepharose

1. Divinylsulfone (DVS).
2. Sepharose 4B.
3. 0.5 M Sodium carbonate.
4. 0.5 M Sodium bicarbonate.
5. 20 % D-maltose in 0.5 M sodium carbonate.

### 2.6 Bis(Sulfosuccinimidyl)Suberate Cross-linking

1. HEPES buffer [10 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA].
2. DMSO (Dimethyl sulfoxide).

## 3 Methods

### 3.1 Purification of Surfactant protein D

#### 3.1.1 Separation of SP-D from Amniotic Fluid

1. Amniotic fluid can be collected following vaginal rupturing of the fetal membranes which is carried out to induce or facilitate the progress of labor or during spontaneous rupturing from pregnant women at term following appropriate ethical approval. This is usually stored at –80 °C until required for purification.
2. Thaw amniotic fluid from –80 °C at 37 °C for 5–6 h or overnight at 4 °C. Make sure (whilst at 37 °C) that it is regularly mixed to keep temperature consistent to avoid overheating of proteins.
3. Pool all amniotic fluid to allow purification of protein in large amounts.
4. Centrifuge the pooled amniotic fluid in a table top bench centrifuge at 10,000 × *g* for 40 min at 4 °C to separate SP-A, mainly in the pellet, from the supernatant containing SP-D.
5. The amniotic fluid is then passed through a cell strainer to remove fat and other large aggregates. This will remove phospholipids and large unwanted material.

6. To further separate other cellular material, the amniotic fluid is passed through muslin cloth three times for a clearer suspension and to avoid future clogging of maltose–sepharose column during affinity chromatography.
7. The amniotic fluid is calcified to 5 mM $CaCl_2$ to increase the volume and decrease viscosity ($CaCl_2$ is added drop-by-drop whilst monitoring pH to 7.5).
8. Stir at 4 °C for 1 h. This allows aggregation of the calcium dependent proteins SP-A and SP-D.
9. Proceed to affinity chromatography.

*3.1.2 Affinity Chromatography*

1. Maltose–sepharose (see the preparation of affinity matrix below) is washed with 100 ml autoclaved distilled water and then equilibrated with four bed volumes of low salt affinity buffer I (*see* Subheading 2.1, **item 1**).
2. The maltose–sepharose bed is then transferred to the amniotic fluid supernatant obtained from the previous step to allow SP-D to bind to the maltose–sepharose for efficient purification. This is left to stir at 4 °C for a further 1 h.
3. Pass the amniotic fluid containing the maltose–sepharose through the column and allow the maltose–sepharose to settle, collect the flow through and pass through the column thrice in total.
4. Remove nonspecifically bound proteins with four bed volumes of affinity buffer containing 1 M NaCl (see Subheading 2.1, **item 2**).
5. 10 × 1 ml fractions of SP-D are eluted with elution buffer I over ice (see Note 1).
6. Optical density of each fraction is read at 280 nm using elution buffer without $MnCl_2$ as a reference (*see* **Notes 2** and **3**) (Fig. 2).

*3.1.3 Removal of BSA and Manganese Chloride and Concentration of SP-D Protein*

7. 100 µl of each fraction is used for TCA precipitation and this is separated on a 15 % SDS–PAGE to visualize protein concentration and purity.
8. The fractions with the highest concentrations are pooled and dialysis bag is immersed in 1 L EDTA buffer to remove $MnCl_2$ (*see* Subheading 2.1, **item 4**).
9. The dialysis bag is left in a cylinder on a magnetic stirrer overnight at 4 °C (*see* **Note 4**).
10. The dialysis bag is then placed in a beaker containing polyethylene glycol MW 6,000–10,000 (Sigma) for 2 h until the volume of the dialysate is reduced by half.
11. The content from the dialysis bag can then be aliquoted into 1 ml fractions for storage at –20 °C. 2× 100 µl fractions will be required for protein estimation using BCA assay (see below).

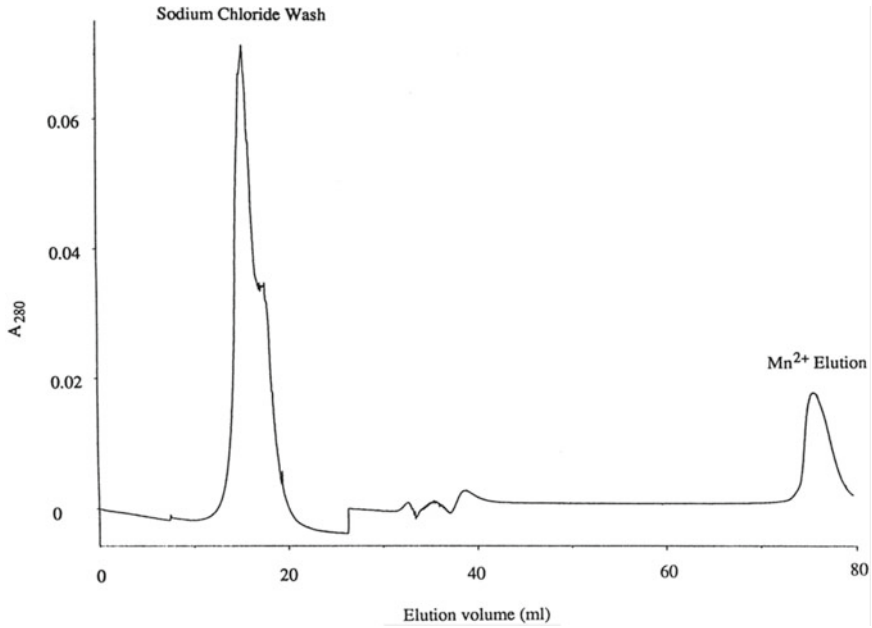



**Fig. 2** NaCl wash and elution of SP-D with $MnCl_2$ from the maltosyl–agarose column. Several proteins, including HSA and histone degradation products bind nonspecifically to maltosyl–agarose and are readily eluted with high salt wash in the presence of calcium while SP-D remains bound. SP-D is specifically eluted by replacing $CaCl_2$ with $MnCl_2$ when changing to elution buffer containing 50 mM $MnCl_2$ after 25 ml of the sodium chloride high salt wash, as outlined in Subheading 2

*3.1.4 Gel Filtration Chromatography*

To further clean the purified SP-D away from minor contaminants SP-A and DNA etc., gel filtration chromatography can be performed.

12. Prepare Superose 6 preparative column; add concentrated SP-D preparation onto the column.
13. Equilibrate with name of the buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA).
14. SP-D elutes as a single peak with molecular weight of >1,000 kDa and SP-A elutes as broad peak with molecular weight of 670 kDa.
15. Other contaminants that co-purify with the proteins on the affinity column elute late, close to the inclusion volume of the column.
16. The fractions with good protein concentrations can be pooled and concentrated adopting the method described above with polyethylene glycol.

### *3.2 Preparation of Competent Cells*

1. Inoculate a single colony of *E. coli* BL21 (λDE3) pLysS (Invitrogen) in 10 ml of Luria broth (LB) media with 10 μl of chloramphenicol (stock: 50 mg/ml dissolved in ethanol) overnight at 37 °C shaker.
2. Following morning, remove 500 μl from BL21 (λDE3) pLysS culture and inoculate into a 50 ml Falcon tube containing 25 ml of LB medium with 25 μl of chloramphenicol.

3. Incubate at 37 °C on a shaker (200 rpm) and measure optical density ($A_{600}$) every hour until it reaches a value between 0.3 and 0.4 (early log Phase).
4. Centrifuge the culture at 2,000 × *g* for 5 min.
5. After centrifugation, discard the supernatant, and add 12.5 ml of sterile 0.1 M $CaCl_2$ to the cell pellet.
6. Resuspend the cells and place them in the temporary state of competence at ice for 1 h.
7. After 1 h, centrifuge the cells for 5 min at 2,000 × *g*.
8. Discard the supernatant, and resuspend the pellet in 2 ml of 0.1 M $CaCl_2$ and store in ice, the cells are now ready to be transformed.

### 3.3 Transformation of Cells

1. Remove 200 μl of cells from the competent cells prepared earlier, and place inside a 10 ml Falcon tube containing 1–2 μl of (pUK-D1) construct.
2. Place the mixture on ice for 1 h.
3. Give a heat shock to the mixture at 42 °C for 90 s.
4. Following heat shock, place the cells on ice for a further 5 min, and then resuspend in 800 μl of LB.
5. Place the mixture inside a 37 °C incubator for approximately 45 min.
6. After incubation, streak the cells on an LB agar plate containing (Amp/Chl) and leave inside a 37 °C incubator to grow overnight.

#### 3.3.1 Protein Expression

1. Grow a single colony of transformed (pUK-D1) cells in 25 ml LB along with 100 μg/ml ampicillin + 50 μg/ml chloramphenicol overnight in a shaker at 37 °C.
2. On the following morning, inoculate the overnight primary culture of 25 ml bacterial culture into 1 L LB along with 100 μg/ml ampicillin + 50 μg/ml chloramphenicol and grow inside a 37 °C shaker, until the absorbance $A_{600}$ reaches 0.6–0.8.
3. After the cells reach log phase ($A_{600}$ at 0.6–0.8) induce with 0.4 mM IPTG for 3 h and harvest the cells by centrifugation at 9,000 × *g* for 15 min.
4. Following centrifugation, discard the supernatant and store the pellet at −20 °C for further processing (Fig. 3).

#### 3.3.2 Cell Lysis

1. Resuspend the cell pellet obtained after IPTG induction in ice cold lysis buffer containing (50 mM Tris–HCl, 200 mM NaCl, 5 mM EDTA, 0.1 % v/v Triton X-100, 0.1 mM PMSF, pH 7.5, 50 μg/ml lysozyme) and vortex for 1 h in cold room at 4 °C and then sonicate the sample (ten cycles at 30 s each).

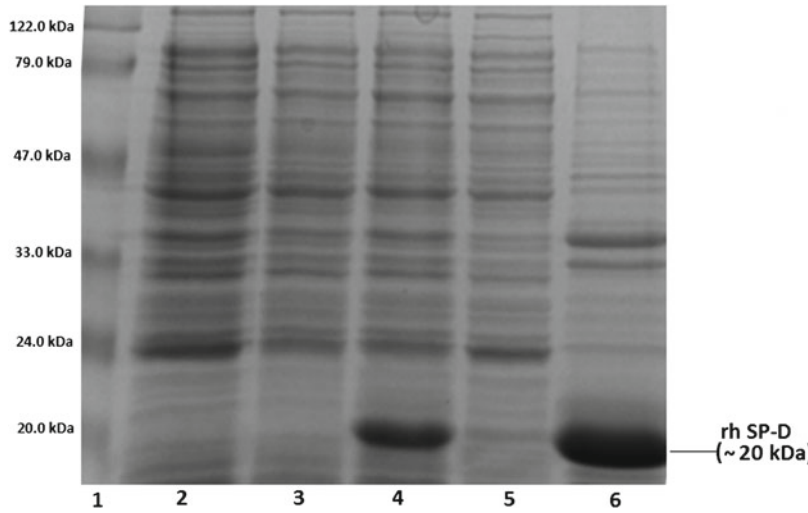



**Fig. 3** SDS–PAGE (12 % w/v) analyses of rhSP-D at various stages of expression and purification. A recombinant fragment of human SP-D containing homotrimeric neck and CRD regions (rhSP-D) was expressed as inclusion bodies using *Escherichia coli* BL21 (λDE3) pLysS as hosts under T7 promoter. 3 h after induction with 0.4 mM IPTG, the rhSP-D accumulated as an over-expressed protein of 20 kDa (*lane 4*) compared with uninduced cells (*lane 2*) and induced cells at zero hr (*lane 3*). Bacterial cells were sonicated and the inclusion bodies (*lane 6*) containing insoluble rhSP-D were recovered in the pellet. The supernatant of the sonicate (*lane 5*) did not contain rhSP-D

*3.3.3 Dialysis*

1. Harvest the sonicate by centrifugation at 9,000 × *g* for 15 min and solubilize the recovered rh SP-D in the inclusion bodies in 100 ml buffer I (50 mM Tris–HCl pH 7.5, 100 mM NaCl) containing 10 mM 2-mercaptoethanol and 8 M Urea.
2. Carry out a step wise dialysis of the resolubilized material against buffer I containing 4 M urea, 2 M urea, 1 M urea and no urea, each for 2 h.
3. Centrifuge the dialysate at 9,000 × *g* for 15 min to clarify, and then dialyze with calcium buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05 % sodium azide) for 3 h to completely remove urea from the dialysate (Fig. 4, *lane 1*).

*3.3.4 Protein Purification by Affininty Chromotography*

1. The rhSP-D is purified by affinity chromatography using maltose–sepharose column.
2. Rinse the maltose–sepharose column with distilled water prior to use. After thorough rinsing of the column with $dH_2O$, pass 50 ml of affinity column buffer containing (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, sodium azide-0.05 %) through the column.
3. After rinsing the column pass the protein sample through the column once.
4. Wash the column with affinity column buffer.

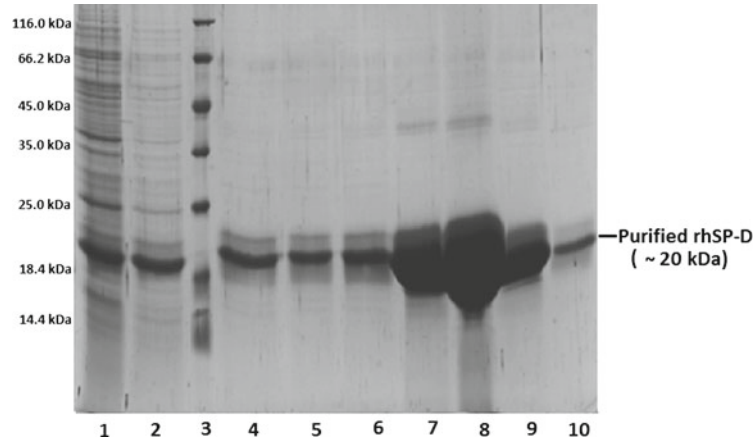



**Fig. 4** SDS–PAGE (12 % w/v) analyses of rhSP-D of purification. Insoluble rh SP-D were refolded via a denaturation and renaturation procedure and the soluble fraction was further purified using Q-sepharose ion-exchange chromatography. The refolded protein (*lane 1*) was passed through maltose-agarose. A significant proportion of the refolded rh SP-D did not bind to the column (*lane 2*). *Lane 3* is standard protein marker. The peak fractions containing rhSP-D were subsequently affinity purified on a maltose–agarose column (*lanes 4–10*)

5. Elute the bound protein by using elution buffer containing 5 mM EDTA (25 ml) (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.05 % sodium azide) (Fig. 4). The affinity eluted protein ran as a trimer on a Superose-12 gel filteration column (Fig. 5).

### *3.4 Endotoxin Removal from Recombinant SP-D*

1. 5 ml of Polymyxin B agarose gel (Sigma) is packed in a 20 ml column.
2. Wash with 50 ml of 1 % sodium deoxycholate and then further rinse with 50 ml of autoclaved distilled water to completely remove 1 % sodium deoxycholate.
3. After rinsing the column, the protein is loaded onto the column, and left to incubate at room temperature for half an hour for the protein to bind completely.
4. After the incubation period the protein is eluted and collected in 1.0-ml fractions.
5. The LPS free rhSP-D protein is then checked for purity by running it on 15 % SDS gel to ensure the removal of LPS.
6. The endotoxin level was examined by QCL-1000 Limulus amebocyte lysate system (BioWhittaker, Walkersville, MD, USA) and was found to be ~4 pg $\mu g^{-1}$ of rhSP-D.
7. The protein is further quantified by measuring the absorbance at 280 nm of the eluted and the concentration of the sample was determined via Spectrophotometry.

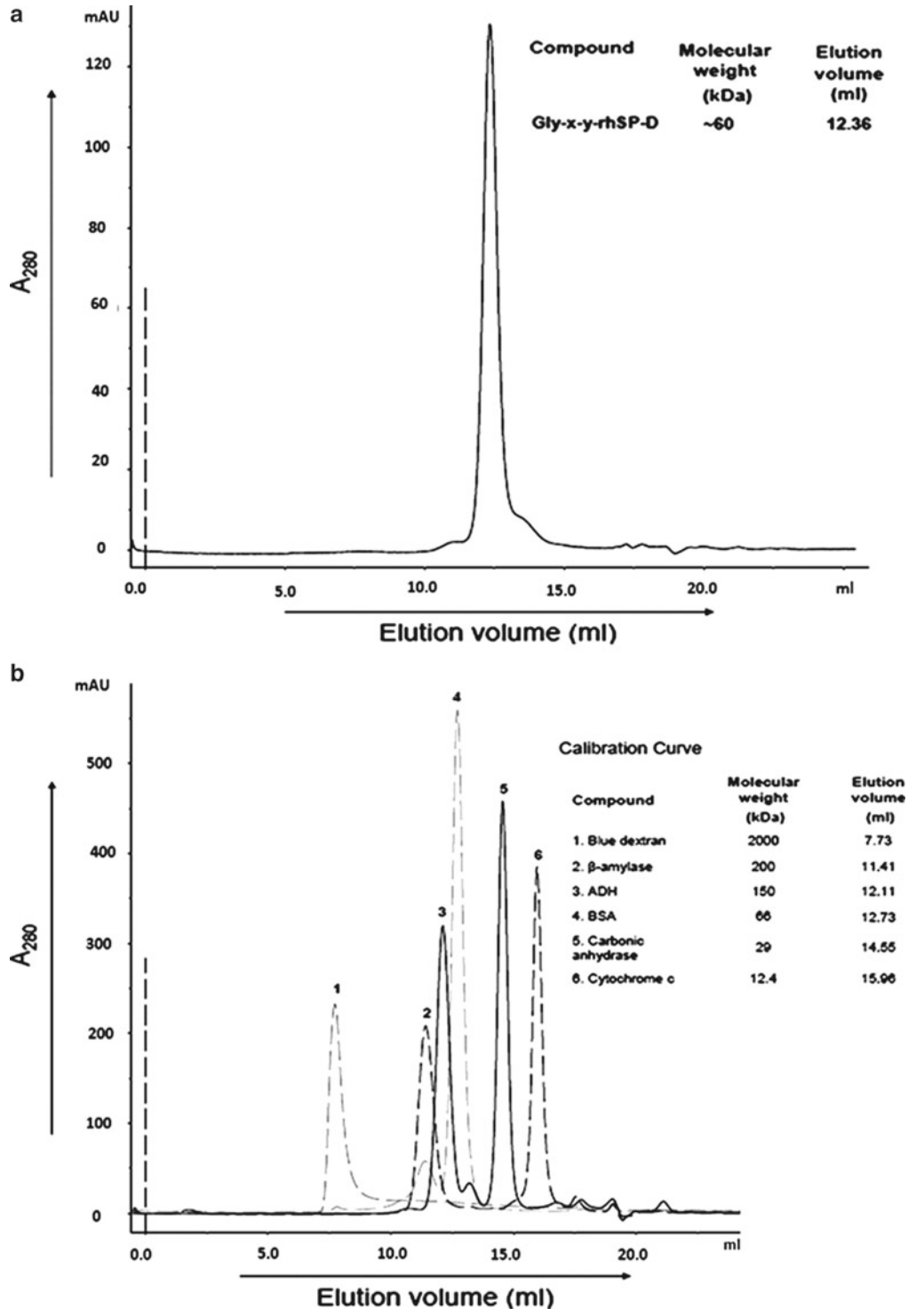



**Fig. 5** (**a**) Elution profile of rhSP-D from an FPLC Superose 12 column (HR 10/30). The column was eluted with Tris-buffered saline–EDTA buffer at a flow rate of 0.3 ml/min. (**b**) Calibration of the column was carried out using the following marker proteins: blue dextran (2,000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa)

### 3.5 Preparation of Maltose–Sepharose Column

Preparation of D-maltose-sepharose 4B by divinylsulfone activation method: (immobilizing hydroxyl-containing compounds) Sepharose is first activated with the bifunctional reagent divinyl sulfone (DVS) (Sigma) and then coupled under basic conditions to the hydroxyls of D-mannose. Reductive amination, epoxy and DVS activation provide three excellent alternatives for the obtaining an optimal affinity support.

Immobilization: (DVS to activate sepharose) (*see* **Note 5**).

1. Activate sepharose 4B (100 ml settled gel with DVS); DVS is a bifunctional cross-linking reagent, used to activate agarose and other hydroxyl matrices (*see* **Note 6**).
2. DVS introduces reactive vinyl groups into the matrix that will couple to amines, alcohols, sulfhydryls, and phenols (see Note 7).
3. DVS activated gels are more reactive than epoxy-activated gels, and therefore coupling proceeds rapidly and completely (*see* **Note 8**).

#### 3.5.1 Activation

1. Wash sepharose 4B (100 ml) settled gel with 1 L water in a sintered glass funnel, suction dry to a wet cake and transfer to a 500 ml beaker.
2. Suspend the gel in 100 ml 0.5 M sodium carbonate and stir the suspension.
3. Slowly add, drop wise, DVS (10 ml) with constant stirring over a 15 min period. After addition is completed, stir gel suspension for 1 h at room temperature.
4. Extensively wash the activated gel with water until the filtrate is no longer acidic (*see* **Note 9**).

#### 3.5.2 Immobilization

1. Wash the DVS-activated sepharose 4B with 1 L water, suction dry to a moist cake and add to a D-maltose solution (20 % D-maltose in 0.5 M sodium carbonate).
2. Stir the reaction mixture at room temperature for 24 h.
3. Filter and wash the gel successively with 2 L each of water and 0.5 M sodium bicarbonate containing 5 ml β-mercaptoethanol.
4. Mix at room temperature for 2 h to block the excess vinyl groups.
5. Finally wash the gel with 2 L of water.
6. D-maltose-sepharose 4B can be stored in 0.02 % sodium azide at 4 °C.

### 3.6 Bis(Sulfosuccinimidyl)Suberate ($BS_3$) Cross-linking

1. Prepare a 25 mM solution of BS3, by dissolving 2 mg BS3 in 140 µl of DMSO.
2. Using a 20-fold excess approach (20:1 cross-linker–protein), add BS3 cross-linker solution to the protein sample so that the final cross-linker concentration is between 0.5 and 5 mM.

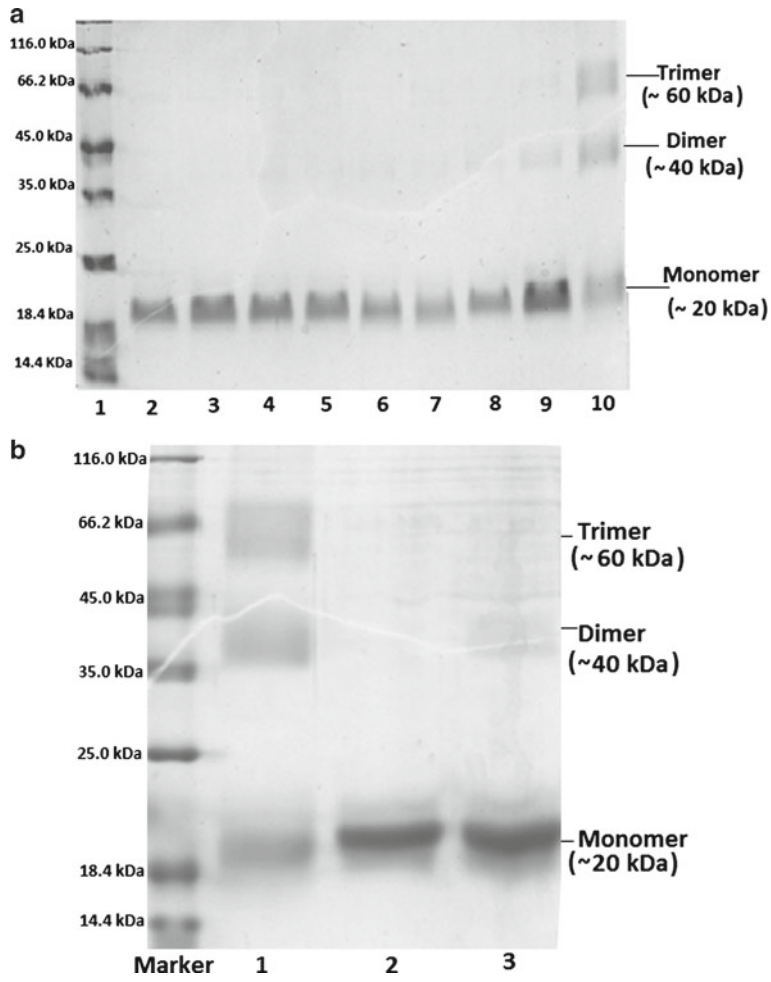



**Fig. 6** (**a**) SDS–PAGE (12 % w/v) bis (sulfosuccinimidyl) suberate (BS3) cross-linking of recombinant fragment of human SP-D containing trimeric neck containing CRD region (rhSP-D). Treatment of rhSP-D with 0.01 mM BS3 at different time intervals of 0, 1, 2, 4, 6, 8, 16, 32 min, respectively (*lanes 3–9*) and the *lane 10* showing 1 mM BS3. (**b**) SDS–PAGE (12 % w/v) bis (sulfosuccinimidyl) suberate (BS3) cross-linking of recombinant fragment of human SP-D containing trimeric neck containing CRD region (rhSP-D). Treatment of rhSP-D with three different concentrations of BS3: 0.1 mM (*lane 1*), 0.01 mM (*lane 2*), and 1 mM (*lane 3*)

3. Incubated rhSP-D with different concentrations (1 mM, 0.1 mM, and 0.01 mM BS3).
4. Allow the sample to react at room temperature for 45 min to 1 h. Allow slightly longer if reaction must be done on ice (this reaction rate is only slightly slower at low temperatures).
5. Quench and unreacted BS3 with 25 to 60 mM Tris–HCl (pH 7.5) and allow reacting for 10–15 min at room temperature.
6. Run the SDS-PAGE for the detection of cross-linking (Figs. 6 and 7).

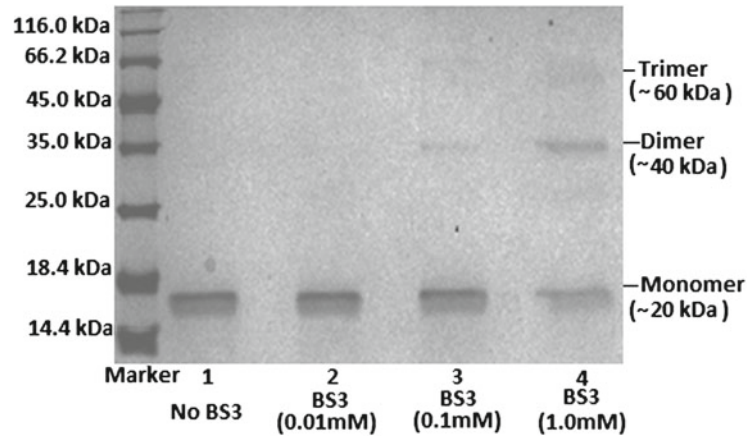



**Fig. 7** SDS–PAGE (12 % w/v) bis (sulfosuccinimidyl) suberate (BS3) cross-linking of recombinant fragment of human SP-D containing trimeric neck containing CRD region (rhSP-D). Treatment of rhSP-D with three different concentrations of BS3: 0.01 mM (*lane 3*), 0.1 mM (*lane 4*), and 1 mM (*lane 5*), and untreated rhSP-D (*lane 2*)

### 3.7 TCA Assay

1. Take 200 µl of protein and mix with equal volumes of 20 % TCA (trichloroacetic acid) in a 2 ml Eppendorf tube, keep on ice for 20 min. if concentrations are low then keep 5 min on ice and 15 min at –20 °C.
2. Centrifuge for 15 min at 20,000 × g at 4 °C, decant the supernatant and add 200 µl of 70 % ethanol, flick and mix content, centrifuge for 15 min at 20,000 × g at 4 °C and decant supernatant.
3. Allow excess ethanol to air dry for 5–10 min. Do not allow excessive drying as this makes the pellet difficult to dissolve.
4. Add 30 µl of 2× protein treatment buffer and pipette up and down for equal mixing.
5. Denature protein on heating block for 10 min at 95 °C.
6. Add each sample to a well of SDS-PAGE.

### 3.8 Bicinchoninic Acid Protein Assay

BCA assay is carried out according to manufacturer's instructions. See below for protocol using BCA (Thermo Scientific).

1. To carry out protein estimation, it is important to perform a standard curve by preparing protein standards using the BSA of 2 mg/ml provided in the kit.
2. Nine vials are labeled A-I and BSA is diluted to range from 20 µg to 2 mg/ml according to Tables 1 and 2 using PBS as a diluent.
3. Prepare working reagent by mixing reagent A and reagent B at a 1:50 dilution. 2 ml of working reagent is required per sample including for the protein standards which is repeated twice.

**Table 1**
**Dilution of protein standards to perform standard curve for protein estimation**

| Dilution scheme for standard test tube protocol and microplate procedure (working range = 20–2,000 μg/ml) | | | |
|---|---|---|---|
| **Vial** | **Volume of diluent (μl)** | **Volume and source of BSA (μl)** | **Final BSA concentration (μg/ml)** |
| A | 0 | 300 of stock | 2,000 |
| B | 125 | 375 of stock | 1,500 |
| C | 325 | 325 of stock | 1,000 |
| D | 175 | 175 of vial B dilution | 750 |
| E | 325 | 325 of vial C dilution | 500 |
| F | 325 | 325 of vial E dilution | 250 |
| G | 325 | 325 of vial F dilution | 125 |
| H | 400 | 100 of vial G dilution | 25 |
| I | 400 | 0 | 0 = Blank |

**Table 2**
**Components and volumes for preparing 12 and 15 % SDS-PAGE**

| **Resolving gel** | **12 %** | **15 %** | **Stacking gel** | |
|---|---|---|---|---|
| **Components** | **Volume (ml)** | | **Components** | **Volume (ml)** |
| $dH_2O$ | 3.3 | 2.3 | $dH_2O$ | 3.4 |
| 30 % Bis-acrylamide mix | 4 | 5 | 30 % Bis-acrylamide mix | 0.83 |
| 1.5 M Tris–HCl, pH 8.8 | 2.5 | 2.5 | 1.0 M Tris–HCl, pH 6.8 | 0.63 |
| 10 % SDS | 0.1 | 0.1 | 10 % SDS | 0.05 |
| 10 % APS | 0.1 | 0.1 | 10 % APS | 0.05 |
| TEMED | 0.015 | 0.015 | TEMED | 0.014 |

The following formula can be used to calculate the total working reagent required (no. of standards + no. of unknown samples) × (2 replicates) × (2 ml volume of working reagent per sample).

4. Label 2× test tubes for each sample containing 100 μl of sample + 2 ml working reagent.
5. This is incubated at 37 °C for 30 min. The reagent will turn from purple to green, depending on the protein concentration. The higher the concentration the greener the solution.

6. The test tubes are then cooled down to room temperature, however measuring the absorbance within the first 10 min.
7. Using a spectrophotometer at 562 nm, each sample optical density is detected and the average of the duplicate sample is taken for a more accurate measurement of protein. The working reagent is used as a reference.
8. A standard curve can be made using the standard protein samples and the optical density detected for the unknown samples can be used to detect the approximate protein content in the sample.

### 3.9 SDS-PAGE: 12 % and 15 %

*Protocol*:

To prepare a 12 % or 15 % SDS-PAGE, follow the instructions below:

1. In two separate 50 ml beakers label one "resolving gel" and the other "stacking gel".
2. For a standard size gel, 10 ml of resolving gel and 5 ml of stacking gel is sufficient.
3. Assemble the clean glass plates onto the glass holder and stand and check for no leakages.
4. Prepare the following for either 12 % or 15 % resolving gel then the standard stacking gel. Do not add TEMED until required just before adding to the glass plates.
5. Once the TEMED has been added the polymerization reaction will have begun, therefore add the resolving gel immediately.
6. After 20 min the gel will have polymerized and then 10 % APS and TEMED can be added to the stacking gel and loaded on top of the resolving gel with the comb inserted for the gel to polymerize and form wells.
7. The gel with the glass plates can be assembled into the gel holder with the anode and cathode conductors and a buffer dam can be added to the opposite side to allow current to flow.
8. The protein samples for separation are mixed with equal amounts of 2× SDS sample buffer and heated to 95 °C for 10 min for denaturation.
9. The sample is then cooled to room temperature and microfuged at 12,000 × *g* for 1 min before loading one sample per well.
10. Protein standard marker is added to the first well to determine unknown protein sample size.
11. 1× Running buffer is added to the tank as instructed by manufacturer.
12. Set the power pack connected to the gel apparatus to 100 V for 90 min to allow the proteins to be separated according to

size, with the smaller molecules passing through the gel matrix quickly with the larger molecules trailing behind.

13. The gel is then placed in staining solution overnight and destained the next day for the protein bands to be visible.

## 4 Notes

1. Add $MnCl_2$ fresh on the day you need to use it as $MnCl_2$ oxidizes.
2. Due to color changes over time of $MnCl_2$ giving improper protein estimations.
3. Both supernatant and flow through and buffers should always be on ice.
4. Note if the color is still dark; allow dialysis to continue for up to 48 h.
5. Since DVS is highly toxic, all operations involved in activation and coupling should be done in the fume hood.
6. DVS activated gels are very useful for immobilizing sugars through hydroxyl groups.
7. Immobilized ligands prepared by the DVS method are unstable at alkaline pH (not stable above pH-8.0-amine linked gels and above pH-9.0- or 10.0-hydroxyl-linked gels).
8. Primary stability of this procedure is caused by the liability of the ether bond formed between DVS and the matrix at the time of activation.
9. Gel can be used to couple to ligands or stored for further use. For storage, extensively wash the activated gel with acetone and keep as suspension in acetone at 4 °C.

### References

1. King RJ, Klass DJ, Gikas EG, Clements JA (1973) An uncommon phosphatidylcholine specific for surface-active material in canine lung. Am J Physiol 224(4):788–795
2. Glasser SW, Korfhagen TR, Weaver T, Pilot-Matias T, Fox JL, Whitsett JA (1987) cDNA and deduced amino acid sequence of human pulmonary surfactant-associated proteolipid SPL(Phe). Proc Natl Acad Sci U S A 84(12):4007–4011
3. Warr RG, Hawgood S, Buckley DI, Crisp TM, Schilling J, Benson BJ et al (1987) Low molecular weight human pulmonary surfactant protein (SP5): isolation, characterization, and cDNA and amino acid sequences. Proc Natl Acad Sci U S A 84(22):7915–7919
4. Weaver TE, Whitsett JA (1991) Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J 273(Pt 2):249–264
5. Kishore U, Reid KB (2001) Structures and functions of mammalian collectins. Results Probl Cell Differ 33:225–248
6. Weaver TE (1998) Synthesis, processing and secretion of surfactant proteins B and C. Biochim Biophys Acta 1408(2–3):173–179
7. Pérez-Gil J, Keough KM (1998) Interfacial properties of surfactant proteins. Biochim Biophys Acta 1408(2–3):203–217
8. Crouch E, Rust K, Veile R, Donis-Keller H, Grosso L (1993) Genomic organization of

human surfactant protein D (SP-D). SP-D is encoded on chromosome 10q22.2-23.1. J Biol Chem 268(4):2976–2983
9. Lu J, Willis AC, Reid KB (1992) Purification, characterization and cDNA cloning of human lung surfactant protein D. Biochem J 284(Pt 3):795–802
10. Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE et al (2003) By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115(1):13–23
11. Hartshorn KL, Crouch EC, White MR, Eggleton P, Tauber AI, Chang D et al (1994) Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. J Clin Invest 94(1):311–319
12. Kuan SF, Rust K, Crouch E (1992) Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. J Clin Invest 90(1):97–106
13. Schelenz S, Malhotra R, Sim RB, Holmskov U, Bancroft GJ (1995) Binding of host collectins to the pathogenic yeast *Cryptococcus neoformans*: human surfactant protein D acts as an agglutinin for acapsular yeast cells. Infect Immun 63(9):3360–3366
14. Madan T, Kishore U, Shah A, Eggleton P, Strong P, Wang JY et al (1997) Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. Clin Exp Immunol 110(2):241–249
15. Kishore U, Strong P, Perdikoulis MV, Reid KB (2001) A recombinant homotrimer, composed of the alpha helical neck region of human surfactant protein D and C1q B chain globular domain, is an inhibitor of the classical complement pathway. J Immunol 166(1):559–565
16. Wright JR (2005) Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 5(1):58–68
17. Kishore U, Greenhough TJ, Waters P, Shrive AK, Ghai R, Kamran MF et al (2006) Surfactant proteins SP-A and SP-D: structure, function and receptors. Mol Immunol 43(9):1293–1315
18. Mahajan L, Madan T, Kamal N, Singh VK, Sim RB, Telang SD et al (2008) Recombinant surfactant protein-D selectively increases apoptosis in eosinophils of allergic asthmatics and enhances uptake of apoptotic eosinophils by macrophages. Int Immunol 20(8):993–1007
19. Herbein JF, Savov J, Wright JR (2000) Binding and uptake of surfactant protein D by freshly isolated rat alveolar type II cells. Am J Physiol Lung Cell Mol Physiol 278(4):L830–L839
20. Miyamura K, Leigh LE, Lu J, Hopkin J, López Bernal A, Reid KB (1994) Surfactant protein D binding to alveolar macrophages. Biochem J 300(Pt 1):237–242
21. Ogasawara Y, Kuroki Y, Akino T (1992) Pulmonary surfactant protein D specifically binds to phosphatidylinositol. J Biol Chem 267(29):21244–21249
22. Barnes P, Liew F (1995) Nitric oxide and asthmatic inflammation. Immunol Today 16(3): 128–130
23. Erpenbeck VJ, Schmidt R, Günther A, Krug N, Hohlfeld JM (2006) Surfactant protein levels in bronchoalveolar lavage after segmental allergen challenge in patients with asthma. Allergy 61(5):598–604
24. Wang JY, Kishore U, Lim BL, Strong P, Reid KB (1996) Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. Clin Exp Immunol 106:367–373
25. Madan T, Reid KB, Singh M, Sarma PU, Kishore U (2005) Susceptibility of mice genetically deficient in the surfactant protein (SP)-A or SP-D gene to pulmonary hypersensitivity induced by antigens and allergens of *Aspergillus fumigatus*. J Immunol 174(11): 6943–6954
26. Madan T, Kishore U, Singh M, Strong P, Hussain EM, Reid KB et al (2001) Protective role of lung surfactant protein D in a murine model of invasive pulmonary aspergillosis. Infect Immun 69(4):2728–2731
27. Singh M, Madan T, Waters P, Parida SK, Sarma PU, Kishore U (2003) Protective effects of a recombinant fragment of human surfactant protein D in a murine model of pulmonary hypersensitivity induced by dust mite allergens. Immunol Lett 86(3):299–307
28. Fisher JH, Mason R (1995) Expression of pulmonary surfactant protein D in rat gastric mucosa. Am J Respir Cell Mol Biol 12(1):13–18
29. Madsen J, Kliem A, Tornoe I, Skjodt K, Koch C, Holmskov U (2000) Localization of lung surfactant protein D on mucosal surfaces in human tissues. J Immunol 164(11):5866–5870
30. Herías MV, Hogenkamp A, van Asten AJ, Tersteeg MH, van Eijk M, Haagsman HP (2007) Expression sites of the collectin SP-D suggest its importance in first line host defence: power of combining in situ hybridisation. RT-PCR and immunohistochemistry. Mol Immunol 44(13):3324–3332
31. Lin Z, deMello D, Phelps D, Koltun W, Page M, Floros J (2001) Both human SP-A1 and

Sp-A2 genes are expressed in small and large intestine. Pediatr Pathol Mol Med 5:367–386
32. Chailley-Heu B, Rubio S, Rougier J, Ducroc R, Barlier-Mur A, Ronco P et al (1997) Expression of hydrophilic surfactant proteins by mesentery cells in rat and man. Biochem J 328:251–256
33. Rubio S, Lacaze-Masmonteil T, Chailley-Heu B, Kahn A, Bourbon J, Ducroc R (1995) Pulmonary surfactant protein A (SP-A) is expressed by epithelial cells of small and large intestine. J Biol Chem 270:12162–12169
34. Madsen J, Tornoe I, Nielsen O, Koch C, Steinhilber W, Holmskov U (2003) Expression and localization of lung surfactant protein A in human tissues. Am J Respir Cell Mol Biol 29:591–597
35. Brauer L, Kindler C, Jäger K, Sel S, Nölle B, Pleyer U et al (2007) Detection of surfactant proteins A and D in human tear fluid and the human lacrimal system. Invest Ophthalmol Vis Sci 48:3945–3953
36. Honda Y, Kuroki Y, Matsuura E, Nagae H, Takahashi H, Akino T et al (1995) Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. Am J Respir Crit Care Med 152(6 Pt 1):1860–1866