

EXPRESSION, REGULATION AND RELEASE OF HUMAN PLACENTAL ANTIGENS:
IMPLICATIONS FOR THE MATERNAL IMMUNE RESPONSE TO THE FETUS

By

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Submitted to the graduate degree program in Anatomy and Cell Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Pregnancy represents a unique immunological arrangement where the maternal immune system must tolerate and support the genetically foreign fetus. The mechanisms by which maternal tolerance to the fetus is achieved and the consequences if this balance is disrupted are not completely understood. In this work, we have shown for the first time that the placenta expresses at least six known human minor histocompatibility antigens (mHAg), including three autosomal antigens, HA-1, BCL2A1 and KIAA0020, and three Y-chromosome-encoded antigens, DDX3Y, RPS4Y1 and KDM5D. In addition, we have shown that expression of at least one of these antigens, HA-1, is decreased in term as compared to first trimester placentas ($P < 0.01$), is regulated by oxygen and is significantly higher in pre-eclamptic placentas as compared to controls ($P = 0.015$). We also examined the protein content of exosomes released from the outermost layer of the placenta (trophoblast-derived exosomes) using mass spectrometry and found that, in addition to several exosome markers, trophoblast-derived exosomes contain the immune molecule HLA-DR, which is not expressed on the surface of the trophoblast. We then treated dendritic cells with trophoblast-derived exosomes either in the presence or absence of the immunostimulatory molecule, LPS. Dendritic cells treated with exosomes alone showed significant increases in IL-8 ($P = 0.05$), VCAM-1 ($P = 0.01$) and CD80 ($P = 0.05$) mRNAs whereas dendritic cells treated with LPS + exosomes showed a significant decrease in CCL7 ($P = 0.03$) and CD40LG ($P = 0.002$) when compared to controls. In conclusion, we have found that the placenta expresses at least six mHAg, that expression of at least one of these mHAg, HA-1, is regulated by oxygen and is increased in placentas from women with pre-eclampsia, and that that exosomes released from placental trophoblast cells contain at least one known fetal antigen, HLA-DR, and can alter dendritic cell phenotype. Taken together, these findings indicate that the placenta expresses and releases antigenic materials into the maternal

blood stream and may affect maternal immune responses to the fetus during pregnancy. These findings may have important implications for the maintenance of a healthy pregnancy, numerous pregnancy complications and many other diseases involving immune responses to foreign and self-antigens.

ACKNOWLEDGMENTS

I would like to thank my family for all of their love and support along this journey. To my husband, Aaron – thank you for always showing me love and kindness, even when I was in my darkest, most frazzled states. To my beautiful boys, Adam and Coen, thank you for every smile and giggle and for being on this journey with me – you mean the world to me. To my parents Bryan and Carolyn Welch – thank you for always encouraging me to follow my dreams and telling me that I can do anything as long as I work hard and love my life. To Noah – I love you and carry your voice with me always. To all of our friends and extended family, thank you for working with us to achieve our dreams – you make a wonderful village.

I would like to thank my lab members, it has been such a wonderful journey and I'm so grateful for your friendship and for all of the things that I have learned from you. I would also like to thank all of our clinical collaborators, especially Dr. Wickstrom, Dr. Maulik, Dr. Heitmann and Dr. Singh as well as all of the patients, nurses and staff members at Saint Luke's Hospital, as well as Dr. Lee and all of the patients, nurses and staff members at the University of Kansas Medical Center.

A big thank you to Dr. Adam Krieg and Lei Qiu for your help with the oxygen experiments; to Rich Hastings and Sonia Soto in the flow core for your help and guidance; Jing Huang and Dr. Sarah Tague in the histology core for your help with histology; Stan Fernald for your help with graphic design; Dr. Lane Christenson for extensive advice and your help with exosome isolation and qRT-PCR questions, Dr. Artigues and Dr. Villar for your help with mass spectrometry and all of my friends, colleagues and mentors at KUMC for your friendship and advice, including: Dr. Susmita Jasti, Dr. Bryce Warren, Michelle McWilliams, Mina Farahbakhsh, Dr. Faezeh Koohestani, Lindsay Reustle, Dr. Jessica Rossol-Allison, Dr. Francesca Duncan and Dr. Julie Christianson.

A huge thank you to all of my committee members: Dr. William Kinsey, Dr. Katherine Roby, Dr. Patrick Fields and Dr. Timothy Fields, and to all of my MD/PhD colleagues and Janice Fletcher, Dr. Brenda Rongish and Dr. Timothy Fields for all of your guidance, support and encouragement.

Finally, thank you, thank you, thank you to my inspirational mentor, Dr. Margaret Petroff. You have taught me so many lessons about science and life, and I feel so fortunate to have your voice and your wisdom to guide me as I move forward – this has been one of the most profound experiences of my life and I cannot thank you enough for your insights, patience and advice.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGMENTS.....	v
TABLE OF CONTENTS	vii
LIST OF TABLES AND FIGURES	xi
CHAPTER 1: REVIEW OF THE LITERATURE.....	1
INTRODUCTION.....	2
OVERVIEW OF REPRODUCTIVE IMMUNOLOGY.....	2
An Historical Perspective on Placenta Structure and Function.....	2
Current Understanding of Placental Structure and Function	4
Maternal Immune Cells.....	5
MINOR HISTOCOMPATIBILITY ANTIGENS	9
Definition of Minor Histocompatibility Antigens	9
The Discovery of Minor Histocompatibility Antigens	16
Minor Histocompatibility Antigens and Transplantation Outcomes	17
MINOR ANTIGENS IN PREGNANCY.....	18
Minor Histocompatibility Antigens are Expressed in the Placenta	22
Clinical implications of Fetal Antigens	23
MICROPARTICLES RELEASED FROM THE PLACENTA MAY INFLUENCE MATERNAL IMMUNE CELLS... 25	
Description of Microparticles	25
Discovery and Classification of Exosomes.....	25

Functions of Exosomes	27
RESEARCH OBJECTIVES	29
CHAPTER 2: EXPRESSION OF FETAL MINOR HISTOCOMPATIBILITY ANTIGENS IN THE HUMAN PLACENTA: A POTENTIAL SOURCE OF MATERNAL IMMUNE EXPOSURE TO FETAL ANTIGENS DURING PREGNANCY	31
ABSTRACT	32
INTRODUCTION.....	33
MATERIALS AND METHODS.....	39
Tissue Procurement	39
Cytotrophoblast Isolation	39
Isolation and Reverse Transcription of RNA and PCR	40
Immunohistochemistry.....	40
RESULTS	41
RNA for mHAg in the placenta and cord blood.....	41
Analysis of mHAg Proteins in the Placenta	44
DISCUSSION	51
CHAPTER 3: TROPHOBLAST EXPRESSION OF THE MINOR HISTOCOMPATIBILITY ANTIGEN HA-1 IS REGULATED BY OXYGEN AND IS INCREASED IN PLACENTAS FROM PREECLAMPTIC WOMEN	54
ABSTRACT	55
INTRODUCTION.....	56
MATERIALS AND METHODS.....	59
Sample Collection	59

Placental Explant Preparation.....	59
Cytotrophoblast Isolation	60
Cobalt Chloride Treatment	60
Oxygen Cultures.....	61
RNA Isolation and RT-PCR.....	61
Immunohistochemistry.....	62
H-score Quantification of HA-1 Expression.....	62
Statistical Analyses.....	63
RESULTS	64
HA-1 is Expressed in Placental Tissues Across Gestation.....	64
Cobalt Chloride Treatment Increases the Expression of HA-1 mRNA in First Trimester Placental Explants and Purified Term Cytotrophoblast Cells	67
Low Oxygen Increases the Expression of HA-1 mRNA in Purified Term Cytotrophoblast Cells	70
HA-1 is Increased in the Syncytiotrophoblast of Preeclamptic Placentas.....	73
DISCUSSION	78
CHAPTER 4: CHARACTERIZATION OF TROPHOBLAST-DERIVED MICROPARTICLES AND THEIR EFFECTS ON THE MATERNAL IMMUNE SYSTEM	81
ABSTRACT	82
INTRODUCTION.....	83
MATERIALS AND METHODS.....	87
Tissue Collection	87
Trophoblast Culture	87
Exosome and Microparticle Collection	88

Nanoparticle Tracking Analysis - NTA	88
Electron Microscopy	89
Mass Spectrometry	89
Dendritic Cell Culture	89
Immunofluorescence	90
RT-PCR.....	90
RESULTS	93
Isolation of Trophoblast-Derived Microparticles and Exosomes	93
Protein Analysis of Exosomes and Microparticles	98
Phagocytosis of Trophoblast-Derived Exosomes and 10K Microparticles by Human Dendritic Cells	103
Effects of Trophoblast-Derived Exosomes on Human Dendritic Cells	106
DISCUSSION	113
CHAPTER 5: GENERAL DISCUSSION	120
PREGNANCY AS AN IMMUNE PARADOX.....	121
EXPRESSION OF MINOR HISTOCOMPATIBILITY ANTIGENS IN THE HUMAN PLACENTA	122
REGULATION OF FETAL MINOR HISTOCOMPATIBILITY ANTIGEN EXPRESSION	122
TROPHOBLAST-DERIVED EXOSOMES AND THE MATERNAL IMMUNE RESPONSE.....	126
CONCLUSIONS.....	128
CHAPTER 6: REFERENCES	130

LIST OF TABLES AND FIGURES

CHAPTER 1: REVIEW OF THE LITERATURE

Table 1.1: Y-Chromosome Encoded Minor Histocompatibility Antigens..... 11

Table 1.2: Autosomally Encoded Minor Histocompatibility Antigens..... 13

CHAPTER 2: EXPRESSION OF FETAL MINOR HISTOCOMPATIBILITY ANTIGENS IN THE HUMAN PLACENTA: A POTENTIAL SOURCE OF MATERNAL IMMUNE EXPOSURE TO FETAL ANTIGENS DURING PREGNANCY

Table 2.1: Human Minor Histocompatibility Antigens Expressed in the Placenta..... 36

Figure 2.1: Minor antigen mRNA expression in placenta, cytotrophoblast cells and fetal cord blood..... 42

Figure 2.2: Minor histocompatibility antigen expression in first and second trimester placentas 45

Figure 2.3: Minor histocompatibility antigen express in term placentas and extraplacental membranes..... 47

CHAPTER 3: TROPHOBLAST EXPRESSION OF THE MINOR HISTOCOMPATIBILITY ANTIGEN HA-1 IS REGULATED BY OXYGEN AND IS INCREASED IN PLACENTAS FROM PREECLAMPTIC WOMEN

Figure 3.1: Expression of HA-1 in the human placenta across gestation..... 65

Figure 3.2: Cobalt chloride increases HA-1 mRNA expression in culture..... 68

Figure 3.3: HA-1 mRNA expression in purified term trophoblasts cultured under low oxygen..... 71

Table 3.1: Clinical characteristics of preeclamptic and matched control placentas..... 74

Figure 3.4: HA-1 expression is increased in preeclamptic placentas as compared to controls... 76

CHAPTER 4: CHARACTERIZATION OF TROPHOBLAST-DERIVED MICROPARTICLES AND THEIR EFFECTS ON THE MATERNAL IMMUNE SYSTEM

Figure 4.1: Comparison of exosomes and 10K microparticles by nanoparticle tracking analysis.....	94
Figure 4.2: Electron microscopy of trophoblast-derived exosomes and microparticles.....	96
Table 4.1: Proteins from trophoblast-derived exosomes and microparticles.....	99
Table 4.2: G-Profiler pathway analysis on trophoblast-derived exosomes.....	101
Figure 4.3: Uptake and trophoblast-derived 10K microparticles and exosomes in human dendritic cells.....	104
Table 4.3: Potentially significant dendritic cell mRNAs identified using PCR array.....	107
Figure 4.4: Treatment of human dendritic cells with trophoblast-derived exosomes significantly increases expression of CD80, IL-8 and VCAM1.....	109
Figure 4.5: Treatment of human dendritic cells with trophoblast-derived exosomes + LPS significantly decreases expression of CCL7 and CD40LG when compared to dendritic cells treated with LPS alone.....	111
Figure 4.6: Comparison of primary trophoblast-derived exosomes to Swan71 and dendritic cell-derived exosomes.....	114

CHAPTER 5: GENERAL DISCUSSION

Figure 5.1: A model of increased HA-1 expression and maternal immune activation in normal vs. preeclamptic pregnancies.....	124
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CHAPTER 1:

REVIEW OF THE LITERATURE

Portions of this chapter have been published in:

Linscheid, C and Petroff MG (2013). “Minor histocompatibility antigens and the maternal response to the fetus during pregnancy.” Am J Reprod Immunol **64**(4):304-14. (Linscheid and Petroff 2013)

INTRODUCTION

The placenta plays a critical role in the success of pregnancy both by facilitating the ever-evolving physiological demands of the developing fetus and by mediating immune interactions between the genetically distinct mother and fetus. Rather than being a passive barrier, the placenta is a dynamic organ with a critical role in maintaining maternal immune tolerance to the fetus and, therefore, a successful pregnancy. In human pregnancy, this tolerance is achieved both actively, via the expression of immuno-modulatory molecules on the surface of the placenta, and passively, through the restricted expression of classical MHC molecules on trophoblast cells (Petroff 2005). Despite these adaptations, the maternal immune system is not naïve to the fetus. Rather, there is a robust and growing body of evidence indicating that, in both mice and humans, the maternal immune system actively responds to fetal antigens (Tarfuri, Alferink et al. 1995, Zhou and Mellor 1998, Aluvihare, Kallikourdis et al. 2004, Piper, McLarnon et al. 2007, Lissauer, Piper et al. 2012).

OVERVIEW OF REPRODUCTIVE IMMUNOLOGY

An Historical Perspective on Placenta Structure and Function

The first scientific description of the placenta occurred c480 BC and has been attributed to the ancient Greek physician, Diogenes of Appolonia (De Witt 1959). Diogenes postulated that the placenta served as a source of nutrition for the developing fetus. This theory was later expanded upon by Aristotle (384-322 BC) and Galen (130-c200 AD), who correctly deduced that because the fetus is surrounded by amniotic membranes during development, fetal nutrition must be derived from the placenta via the umbilical cord, rather than via uterine paps, as was the competing theory of the time (De Witt 1959). More work in this area was conducted by Renaldus Columbus and published in his extensive description of human anatomy, *De re anatomica* (1559), where he correctly stated that, rather than being a source of nourishment, the

amniotic fluid served to cushion and protect the fetus as it grew and developed within the uterus (Power and Schulkin 2012).

The question of the interrelationship between the maternal and fetal circulatory systems was the source of much investigation and speculation for many years. The first proposal that the mother and fetus had separate circulatory systems was by Giulio Cesare Aranzi in his 1564 treatise *De humano foetu* (Longo 2013). This hypothesis was expanded upon in the 18th century by William and John Hunter who, along with Erasmus Darwin, definitively established that the maternal and fetal circulatory systems were separate and that oxygen is one of the major substances transferred from mother to fetus via the placenta (Dunn 2003, Pijnenborg and Vercruyse 2007, Longo and Reynolds 2010, Pijnenborg, Brosens et al. 2010). Modern understanding of the placenta as an organ was greatly enhanced by the development of light microscopy, which precipitated the cellular categorization of placentas of various species by Grosser in the early 20th century (Grosser 1909, Grosser 1927, Enders 1965). Grosser proposed six different placental arrangements based on the number of cell layers separating mother and fetus and the degree of placental invasion into the maternal uterus; epithelia-chorial, syndesmo-chorial, endothelio-chorial, hemo-trichorial, hemo-dichorial and hemo-monochorial, with epithelial-chorial being the least invasive and hemo-monochorial being the most invasive (Grosser 1909, Grosser 1927, Benirschke and Kaufmann 1995). In addition, the 20th century also brought about the extensive description of placental pathologies by Benirschke and Kaufmann (Benirschke and Kaufmann 1995), which has had important implications for numerous pregnancy complications arising from placental dysfunctions. Despite all of these historical advancements, there are still numerous questions surrounding the role of the placenta in maternal and fetal physiology, including the maintenance of fetal tolerance without the threat of overwhelming maternal and fetal infection.

Current Understanding of Placental Structure and Function

The placenta forms the physical interface between the mother and fetus during pregnancy and in addition to its' role as an immune organ, performs many critical biological tasks, including gas and nutrient exchange and numerous endocrine functions, which are central to fetal survival. The placenta originates from the outer cell mass/trophectoderm and differentiates into several distinct trophoblast lineages shortly after implantation, including the invasive extravillous trophoblast and the structural villous trophoblast. The villous trophoblast comprises two cell layers, the cytotrophoblast and the syncytiotrophoblast. The cytotrophoblast is the inner villous trophoblast cell layer that fuses to form the outer, multinucleated syncytiotrophoblast. The syncytiotrophoblast forms the surface of the placenta and is in direct contact with the maternal blood supply (Benirschke, Kaufmann et al. 2006).

There are several different extravillous trophoblast cell (EVT) populations. Some EVTs anchor the placental villi to the uterine wall during pregnancy, whereas others invade into the maternal spiral arteries forming an endovascular trophoblast layer that replaces the maternal endothelium that typically lines the maternal vessels (Tarrade, Lai Kuen et al. 2001). This invasion ensures that the endothelium of the spiral arteries are replaced with the invasive trophoblast cells and that the contractile smooth muscle cells lining the non-pregnant vessels are lost, resulting in a low-pressure, high volume blood delivery system that provides a steady supply of oxygenated blood to the developing fetus with very little vascular reactivity (Burton, Jauniaux et al. 1999, Jauniaux, Watson et al. 2000). Defects in trophoblast spiral artery remodeling and the resulting alterations in placental blood flow have been implicated in several pregnancy complications, including preeclampsia and intrauterine growth restriction. This disruption of placental blood flow ultimately leads to maternal vascular disease, which can result in kidney dysfunction, multi-system organ failure, seizures and ultimately death of both mother

and infant (Stillman and Karumanchi 2007, Gilbert, Nijland et al. 2008, Gilbert, Ryan et al. 2008). Interestingly, preeclampsia also involves increased maternal immune system activation and heightened production of pro-inflammatory cytokines, suggesting a link between placental dysfunction, maternal vascular disease and maternal immune system activation (Redman and Sargent 2003, Redman and Sargent 2004).

Maternal Immune Cells

In addition to the fetally-derived trophoblast, numerous maternal immune cells are involved in maintaining immune tolerance during pregnancy. These cells include uterine NK cells, regulatory CD4⁺ T-cells, maternal antigen presenting cells (macrophages and dendritic cells), and cytotoxic CD8⁺ T-cells. A balance of tolerogenic and cytotoxic maternal immune responses is critical for allowing fetal development while preventing serious infection of both mother and fetus.

Uterine NK cells (uNK) are a unique type of NK cell found in the decidua. Originally defined as a subset of large, granular lymphocytes, NK cells recognize and kill cells that lack the molecules necessary for self-vs.-non-self determination. These molecules are known as Major Histocompatibility Complexes (MHCs) or, in humans, the Human Lymphocyte Antigens (HLAs) (Herberman, Nunn et al. 1975, Kiessling, Klein et al. 1975, Timonen, Ortaldo et al. 1981, Caligiuri 2008). There are several types of human NK cells, and their functions are primarily to lyse target cells and/or to secrete cytokines and chemokines. Uterine NK cells, in contrast to the predominant population of peripheral blood NK cells, are only weakly cytotoxic and do not kill trophoblast cells, despite the fact that trophoblast cells do not express most of the classical (class Ia) HLA molecules (King, Birkby et al. 1989). Instead, extravillous trophoblast cells express HLA-C and the non-classical (class Ib) molecules HLA-E, -F and -G (Yelavarthi, Fishback et al. 1991, King, Burrows et al. 2000, Hunt, Petroff et al. 2005). Interactions between these molecules

and inhibitory NK receptors appear to both limit NK cytotoxicity and promote decidual changes necessary for successful pregnancy (Ponte, Cantoni et al. 1999, Rajagopalan, Fu et al. 2001).

In addition to their lack of cytotoxicity towards trophoblast cells, uterine NK cells are critically important in directing trophoblast invasion and remodeling of the maternal spiral arteries. Research in both mice and humans has provided evidence that uterine NK cells produce chemokines and angiogenic factors, including IL-8 and IP-10, that promote trophoblast invasion and spiral artery remodeling (Hanna, Goldman-Wohl et al. 2006, Zhang, Chen et al. 2011). However, it is important to note that the process of spiral artery remodeling is significantly different in the mouse as compared to the human; for example, the discovery of certain critical factors in the mouse (notably IFN- γ) are of unclear relevance to human biology (Apps, Sharkey et al. 2011).

In addition to uNK cells, one of the most critical immune cell types for a successful pregnancy is a class of T-cells, known as regulatory T-cells (T-regs), which function to promote tolerance of the fetus during pregnancy, and are critical for preventing autoimmune disease (Sakaguchi, Fukuma et al. 1985, Aluvihare, Kallikourdis et al. 2004, Sakaguchi, Sakaguchi et al. 2011). Early studies indicating the possible existence of a fetotolerant class of T-cells showed that in female mice, multiparity imparts tolerance to male skin grafts. This tolerance could be transferred to virgin females by thymocytes and splenocytes, but not by bone marrow cells or sera, indicating that there was a group of T-cells responsible for maintaining tolerance (Smith and Powell 1977, Ruocco, Chaouat et al. 2014). The eventual classification of “regulatory T-cells,” which are phenotypically marked as CD4⁺CD25⁺Foxp3⁺, came with the discovery that their elimination leads to early-onset fulminant systemic autoimmune disease, which subsequently led to insights regarding the role of T-regs in maintaining maternal tolerance to the

fetus (Sakaguchi, Fukuma et al. 1985, Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Sakaguchi, Sakaguchi et al. 2011).

The role of regulatory T-cells in maintaining fetal tolerance was first demonstrated by experiments in mice showing that depletion of CD25⁺ T-cells resulted in allogeneic, but not syngeneic, pregnancy loss, in which fetuses differ genetically from the mother (Aluvihare, Kallikourdis et al. 2004, Darrasse-Jeze, Klatzmann et al. 2006). Further studies revealed that induction of pregnancy-specific regulatory T-cell function may occur as early as copulation (Guerin, Moldenhauer et al. 2011) and studies in humans have shown a correlation between low numbers of uterine regulatory T-cells and unexplained infertility and recurrent pregnancy loss (Jasper, Tremellen et al. 2006, Arruvito, Sanz et al. 2007). Studies in mice have indicated that the expansion of regulatory T-cells is not global, but rather that there is enhanced accumulation of regulatory T-cells with fetal specificity (Rowe, Ertelt et al. 2012).

Recognition of fetal antigens by maternal T-cells has been shown to occur via the indirect antigen presentation pathway, whereby maternal antigen presenting cells process and present fetal antigens to maternal T-cells in the context of MHC molecules (Erlebacher, Vencato et al. 2007, Guerin, Moldenhauer et al. 2011). This is in contrast to the direct recognition pathway, whereby T-cells recognize a foreign MHC/peptide complex directly. The two most widely studied types of maternal antigen presenting cells are macrophages and dendritic cells. Both dendritic cells and macrophages are derived from circulating monocytes (Randolph, Beaulieu et al. 1998) and play important roles in the phagocytosis of foreign and cellular debris and cross-presentation of antigens to immune cells. Macrophages are markedly heterogeneous and may be classified as displaying either an M1 or M2 phenotype depending on the signals received from the surrounding immune environment (Stein, Keshav et al. 1992, Porcheray, Viaud et al. 2005). M1 macrophages are potent pro-inflammatory effector cells, whereas M2 macrophages are

immunomodulatory and are important for preventing excessive inflammation and contributing to homeostatic functions, including angiogenesis (Verreck, de Boer et al. 2004). Decidual macrophages are predominantly M2 and express a high percentage of genes related to immune modulation and tissue remodeling, as compared to macrophages in the periphery (Gustafsson, Mjosberg et al. 2008). Functionally, decidual macrophages have been shown to suppress T-cell activation as compared to peripheral macrophages, likely via production of Prostaglandin E₂, IL-10 and indoleamine deoxygenase (IDO) (Parhar, Yagel et al. 1989, Ishihara, Sullivan et al. 1991, Mizuno, Aoki et al. 1994, Munn, Shafizadeh et al. 1999, Heikkinen, Mottonen et al. 2003, McIntire, Ganacias et al. 2008). Decidual macrophages have also been shown to be intimately involved with extravillous trophoblast remodeling of maternal spiral arteries, a process that is essential for successful human pregnancy (Smith, Dunk et al. 2009).

In comparison to decidual macrophages, which comprise approximately 20-25% of decidual leukocytes (which make up approximately 40% of all decidual cells), dendritic cells are much less abundant in the decidua, amounting to approximately 2% of all decidual leukocytes (Bulmer and Johnson 1984, Lessin, Hunt et al. 1988, Gardner and Moffett 2003). Nonetheless, dendritic cells (DCs) are much more potent stimulators of T-cell responses than macrophages, and, similarly to macrophages, alterations in dendritic cell numbers and phenotype have been implicated in pre-eclampsia (Hart 1997, Banchereau and Steinman 1998, Steinman and Nussenzweig 2002, Darmochwal-Kolarz, Rolinski et al. 2003). Numerous signals in the pregnant decidua, including cytokines produced by uterine NK cells and non-classical MHCs expressed by trophoblast cells, are thought to contribute to the development of a more tolerogenic decidual DC phenotype (Steck, Rieger et al. 2002, Ristich, Liang et al. 2005). In mouse models, defects in DC function and reduced numbers of DCs in the decidua have been implicated in decreased pregnancy success, and the production of IL-10 by DCs has been

hypothesized to be critical for maintaining tolerance, both by promoting T-cell tolerance and by preventing terminal differentiation of decidual DCs (Fiorentino, Zlotnik et al. 1991, Groux, Bigler et al. 1996, Groux, Bigler et al. 1998, Levings, Sangregorio et al. 2001, Piccirillo and Shevach 2001, Gorczynski, Hadidi et al. 2002, Blois, Alba Soto et al. 2004, Blois, Alba Soto et al. 2004, Miranda, Litwin et al. 2006).

In addition to their direct involvement in pregnancy, DCs have been shown to be vitally important in regulating tolerance and rejection of transplanted tissues. Studies in mice have shown that administration of immature dendritic cells prior to allograft transplantation is associated with decreased rejection and improved graft survival (Lu, Li et al. 1997, Lutz, Suri et al. 2000, Roelen, Schuurhuis et al. 2003, Sato, Yamashita et al. 2003, Peche, Trinite et al. 2005, Lan, Wang et al. 2006). Given the historical view of the fetus as the “perfect allograft,” (Medawar 1954) strong evidence of the importance of DCs in transplantation success makes investigation of their role in pregnancy especially interesting and compelling. In transplantation, DCs are involved with both direct (donor-derived DCs) and indirect (recipient-derived DCs) antigen presentation (Larsen, Morris et al. 1990, Auchincloss, Lee et al. 1993, Benichou, Valujskikh et al. 1999). In pregnancy, indirect antigen presentation of fetal antigens has been proposed as the primary mechanism by which the maternal immune system is exposed to fetal antigens, most notably fetal minor histocompatibility antigens (Erlebacher, Vencato et al. 2007).

MINOR HISTOCOMPATIBILITY ANTIGENS

Definition of Minor Histocompatibility Antigens

Fetal antigens include both the major histocompatibility complex (MHC) and minor histocompatibility antigens (mHAgs) (Tarfuri, Alferink et al. 1995, Goulmy 2006). MHC molecules are responsible for the presentation of foreign peptides to T-cells, which cannot

recognize the peptides directly, and also are mediators of transplant rejection via T-cell recognition of the foreign MHC. Minor histocompatibility antigens are derived from many different types of proteins and can elicit an immune response due to allelic differences between individuals (Goulmy 2006), typically single nucleotide polymorphisms (SNPs), insertions, deletions or presence of the antigen on the Y-chromosome (Tables 1.1&1.2).

In order for mHAGs to be recognized by T-cells, the antigens must be presented to T-cells in the context of specific class I and/or class II MHC molecules (Tables 1.1&1.2). MHC Class II molecules bind to the T cell receptor of CD4⁺ T-cells and are primarily expressed on the surface of antigen presenting cells, most notably dendritic cells, macrophages and B cells. MHC Class I molecules bind to the T-cell receptor of CD8⁺ T-cells and are present on the surface of most nucleated cells where they can present endogenous antigens and facilitate self vs. non-self discrimination by the immune system. In addition, MHC Class I is critical for the indirect antigen presentation pathway, in which antigen presenting cells, typically dendritic cells, phagocytose exogenous material and process it for cross-presentation via the MHC Class I molecule on their cell surface (Bevan 1976, Bevan 1976, Rodriguez, Regnault et al. 1999). This allows recognition of antigens coming from tissues lacking class I MHC molecules, including placental trophoblast cells, by CD8⁺ T-cells, (Petroff 2005).

Table 1.1: Y-Chromosome Encoded Minor Histocompatibility Antigens

Minor Antigen	Gene	HLA	Peptide	Expression (mRNA) ²	Ref.
A1/HY	DFFRY	A*0101	IVDCLTEMY	Ubiquitous	(Pierce, Field et al. 1999)
A2/HY	KDM5D	A*0201	FIDSYICQV	Ubiquitous	(Ofraan, Kim et al. 2010)
B52/HY	RPS4Y1	B*5201	TIRYPDPVI	Ubiquitous	(Ivanov, Aarts et al. 2005)
B60/HY	UTY	B*60	RESEEEESVSL	Ubiquitous	(Vogt, Goulmy et al. 2000)
B7/HY	KDM5D	B*0702	SPSVDKARAEI	Ubiquitous	(Wang, Meadows et al. 1995)
B8/HY	UTY	B*8	LPHNHTDL	Ubiquitous	(Warren, Gavin et al. 2000)
TMSB4Y/A33	TMSB4Y	A*3303	EVLLRPGLHFR	Ubiquitous	(Torikai, Akatsuka et al. 2004)
UTY ₁₃₉₋₁₄₇	UTY	A*2402	YYNAFHWAI	Ubiquitous	(Mortensen, Rasmussen et al. 2012)
DQ5/HY	DDX3Y	DQB1*05	HIEFSDIDMGE	Ubiquitous	(Vogt, van den Muijsenberg et al. 2002)
DRB1/HY	DDX3Y	DRB1*1501	SKGRYIPPHLR	Ubiquitous	(Zorn, Miklos et al. 2004)
DRB3/HY	RPS4Y1	DRB3*0301	VIKVNDTVQI	Ubiquitous	(Spierings, Vermeulen et al. 2003)

Table 1.2: Autosomally Encoded Minor Histocompatibility Antigens

Minor Antigen	Gene	HLA	Peptide ¹	Expression (mRNA) ²	Ref.
ACC-1	BCL2A1	A*2402	DYLQYVLQI, DYLQCVLQI	Restricted	(Bleakley and Riddell 2011), (Kawase, Nannya et al. 2008), (Akatsuka, Nishida et al. 2003)
ACC-2	BCL2A1	B*4403	KEFEDDIINW	Restricted	(Bleakley and Riddell 2011), (Kawase, Nannya et al. 2008), (Akatsuka, Nishida et al. 2003)
ACC-4	Cathepsin H	A*3101	ATLPLL <u>CAR</u>	Restricted	(Torikai, Akatsuka et al. 2006)
ACC-5	Cathepsin H	A*3303	WATLPLL <u>CAR</u>	Restricted	(Torikai, Akatsuka et al. 2006)
ACC-6	HMSD	B*4402, B*4403	MEIFIEVFSHF	Restricted	(Kawase, Akatsuka et al. 2007)
CD19	CD19	A1*05, B1*02, DQ	WEGEP <u>PCLP</u>	Restricted	(Spaapen, Lokhorst et al. 2008)
HA3	Lbc/AKAP13	A*0101	V <u>TE</u> PGTAQY	Ubiquitous	(Spierings, Brickner et al. 2003)
HA1	HMHA1	A*0201, A*0206, B*60, B*40012	VL <u>H</u> DDLLEA, KECVL <u>H</u> DDL	Restricted	(den Haan, Meadows et al. 1998), (Mommaas, Kamp et al. 2002, Torikai, Akatsuka et al. 2007, Bleakley and Riddell 2011)
HA2	MYO1G	A*0201	YIGEVLV <u>SV</u>	Restricted	(de Bueger, Bakker et al. 1992, den Haan, Sherman et al. 1995, Pierce, Field et al. 2001)
HA8	KIAA0020	A*0201	<u>R</u> TLDKVLEV	Ubiquitous	(Brickner, Warren et al. 2001, Bleakley and Riddell 2011)
HB-1	HMHB1	B*4402, B*4403	EEKRGS <u>L</u> HVW, EEKRGS <u>L</u> YVW	Restricted	(Dolstra, Fredrix et al. 1997, Dolstra, Fredrix et al. 1999, Dolstra, de Rijke et al. 2002)
HEATR1	HEATR1	B*0801	ISKERA <u>E</u> AL	Ubiquitous	(Bleakley, Otterud et al. 2010)
HER2_1170	HER2	A*0201	<u>G</u> CC	Restricted	(Wenandy, Kollgaard et al. 2009)
LB-ADIR-1F	ADIR/TOR3A	A*0201	SVAPALAL <u>F</u> PA	Restricted	(van Bergen, Kester et al. 2007)
LB-APOBEC3B-1K	APOBEC3B	B*0702	KPQYHAEMCFL, KPQYHAEMCF, KPQYHAEMC	Restricted	(Van Bergen, Rutten et al. 2010)
LB-ARHGDI-1R	ARHGDI	B*0702	LPRACW <u>R</u> EA, LPRACW <u>R</u> EAR, LPRACW <u>R</u> EART	Restricted	(Van Bergen, Rutten et al. 2010)
LB-BCAT2-	BCAT2	B*0702	QPRRALLFVIL,	Ubiquitous	(Van Bergen, Rutten et al.

¹ Immunogenic amino acid differences are shown in bold and underlined. Peptides generated by alternative splicing are shown in bold. Peptides generated by a frameshift mutation are shown in italics. Peptides generated a translational termination codon are shown in italics and underlined. Peptides generated by a deletion are underlined.

² Tissue expression information was obtained from biogps.org as well as the listed references.

IR			QPRRALLFVI		2010)
LB-EBI3-1I	EBI3	B*0702	RPRARYYIQVA, RPRARYYIQV, RPRARYYIQ, AVRPRARYYI	Restricted	(Van Bergen, Rutten et al. 2010)
LB-ECGF1-1	ECGF1	B*0702	RP <u>H</u> AIRRPLAL	Restricted	(Slager, Honders et al. 2006)
LB-ERAP1-1R	ERAP1	B*0702	HPRQEQIALLA	Ubiquitous	(Van Bergen, Rutten et al. 2010)
LB-GEMIN4-1V	GEMIN4	B*0702	FPALRFVEV	Restricted	(Van Bergen, Rutten et al. 2010)
LB-NUP133-1R	NUP133	B*4001	SEDLIL <u>C</u> RL	Restricted	(Griffioen, Honders et al. 2012)
LB-PDCD11-1F	PDCD11	B*0702	GPDSKTFLLCL, GPDSKTFLL	Ubiquitous	(Van Bergen, Rutten et al. 2010)
LB-PRCP-1D	PRCP	A*0201	FMWDVAEDLK A, FMWDVAEDL	Ubiquitous	(Van Bergen, Rutten et al. 2010)
LB-SON-1R	SON	B*4001	SETK <u>Q</u> RTVL	Restricted	(Griffioen, Honders et al. 2012)
LB-SSR1-1S	SSR1	A*0201	SLAVAQDLT	Restricted	(Van Bergen, Rutten et al. 2010)
LB-SWAP70-1Q	SWAP70	B*4001	MEQLE <u>Q</u> LEL	Restricted	(Griffioen, Honders et al. 2012)
LB-TRIP10-1EPC	TRIP10	B*4001	GE <u>P</u> QDL <u>C</u> TL	Ubiquitous	(Griffioen, Honders et al. 2012)
LB-WNK1-1I	WNK1	A*0201	TLSP <u>E</u> IITV	Ubiquitous	(Van Bergen, Rutten et al. 2010)
LRH-1	P2RX5	B*0702	TPN <u>Q</u> R <u>Q</u> N <u>V</u> C	Restricted	(de Rijke, van Horssen-Zoetbrood et al. 2005, Overes, de Rijke et al. 2008, Norde, Overes et al. 2009)
PANE1 (CTL-7A7)	CENPM	A*0301	<u>R</u> V <u>W</u> D <u>L</u> P <u>G</u> V <u>L</u> K	Restricted	(Brickner, Evans et al. 2006)
SLC1A5	SLC1A5	B*4002	AE <u>A</u> TANGGLAL	Restricted	(Bleakley and Riddell 2011), (Kamei, Nannya et al. 2009)
SP110	SP110	A*0301	SL <u>P</u> R <u>G</u> TSTPK	Restricted	(Warren, Vigneron et al. 2006)
UGT2B17	UGT2B17	A*2902, B*4403	<u>A</u> ELLNIP <u>F</u> LY	Ubiquitous	(Murata, Warren et al. 2003, Terakura, Murata et al. 2007)
UGTB17		A*0206	<u>C</u> VATMIF <u>M</u> I	Ubiquitous	(Kamei, Nannya et al. 2009)
UTA2-1	C12orf35	A*0201	QL <u>L</u> NSVLTL	Restricted	(Oostvogels, Minnema et al. 2012)
LAMA1	LAMA1	DRB1*0301	LLILRAIP <u>K</u> GIRD KGAK	Ubiquitous	(Stumpf, van der Meijden et al. 2009, Stone, Rieck et al. 2012)
LB-LY75-1K	LY75	DRB1*1301	GITYRN <u>K</u> S <u>L</u> M	Restricted	(Stumpf, van der Meijden et al. 2009)
ZNF544	ZNF544	DRB1*0301	KQNSAFIN <u>D</u> EKN GADGK	Ubiquitous	(Stumpf, van der Meijden et al. 2009, Stone, Rieck et al. 2012)

The Discovery of Minor Histocompatibility Antigens

The role of mHAg in eliciting an immune response has been demonstrated using transplantation studies. mHAg were first discovered due to their role in modulating graft rejection and graft-versus-host disease in HLA-matched transplant recipients (Goulmy, Termijtelen et al. 1976, Goulmy, Gratama et al. 1983, Goulmy, Schipper et al. 1996). The first mHAg, a member of the HY family of mHAg, was discovered following the rejection of transplanted HLA-matched bone marrow cells from a male donor by a female recipient (Goulmy, Termijtelen et al. 1976). It was shown that cytotoxic T-lymphocytes (CTLs) isolated from the recipient's blood had the capacity to lyse HLA-matched male cells, suggesting that the immune target was encoded by the Y-chromosome (Goulmy, Termijtelen et al. 1977). Shortly after this discovery, the same group of investigators found that mHAg could contribute to graft-versus-host disease, as donor CTLs can target and lyse recipient cells expressing a Y-chromosome-encoded antigen (Goulmy, Gratama et al. 1983).

Since these original discoveries, fifty unique mHAg derived from forty-three genes have been found (Tables 1.1&1.2). These mHAg arise from SNPs, presence on the Y-chromosome, deletions, insertions, frameshift mutations, nonsense mutations and splice variants. mHAg are encoded on the Y-chromosome (Table 1.1) and on many autosomes (Table 1.2). Expression of some mHAg is restricted to hematopoietic cells or a select group of tissues, whereas other mHAg are expressed ubiquitously. Given the numerous potential HLA combinations as well as the vast number of SNPs present in the population, it is likely that many more mHAg exist that have not yet been identified.

One of the major constraints on research involving mHAg-specific CD8⁺ T-cells is their relative rarity in both peripheral blood and at their target sites. Research using multimeric MHC reagents (MHC multimers) has estimated the prevalence of HY-specific T-cells in peripheral

blood following multiple pregnancies with male babies at 0.0001% to 0.03% of the total CD8+ T-cell population (Piper, McLarnon et al. 2007). MHC multimers are complexes comprised of 2-10 or more linked peptide-MHC ligands that can bind T-cells through the T cell receptor in an antigen-specific manner (Altman, Moss et al. 1996, Batard, Peterson et al. 2006). This allows for identification and quantification of T-cells specific for a particular antigen. However, in order to identify and characterize mHAg-specific T-cells, it is often necessary to expand the *ex vivo* population using cytokines and antigen, thus potentially altering the functionality of these cells both as a consequence of antigen/cytokine exposure and as a result of multimer-binding itself (Lyons, Roszkowski et al. 2006, Laugel, van den Berg et al. 2007, Chattopadhyay, Melenhorst et al. 2008). Therefore, caution should be used when assessing the *in vivo* functional significance of these cells *ex vivo*.

Minor Histocompatibility Antigens and Transplantation Outcomes

Immune responses to mHAgs in the context of transplantation can have both beneficial and detrimental consequences for the patient. mHAgs appear to be the primary mediator of graft-versus-host disease in HLA-matched transplantations (Goulmy, Termijtelen et al. 1976, Goulmy, Termijtelen et al. 1977, Goulmy, Gratama et al. 1983, Goulmy, Schipper et al. 1996), leading to the need for increased immunosuppression as well as other negative health outcomes including dermatitis, kidney failure and even death. However, mHAg specific donor T-cells can also play an important role in mediating graft-versus-leukemia effects or graft-versus-tumor effects (Marijt, Heemskerk et al. 2003, Goulmy 2004), which can significantly prolong the lives of patients who receive transplantations to treat various types of cancers and can help ensure longer periods of disease-free survival.

Recently researchers have proposed that modulation of T-cells specific for mHAgs may provide a unique opportunity for augmenting graft-versus-leukemia and graft-versus-tumor

effects (Kircher, Stevanovic et al. 2002, Warren, Fujii et al. 2010), thus providing a potential avenue for increasing disease-free survival while subsequently reducing the need for immunosuppressive drugs. In this paradigm, donor T-cells specific for a particular mHAg whose expression is restricted to leukemic/tumor cells are isolated from the recipient's blood following the initial transplant and expanded *ex vivo* (Kircher, Stevanovic et al. 2002, Warren, Fujii et al. 2010). These T-cells are then re-infused into the recipient following a disease relapse. A preliminary clinical trial targeting the mHAg HA-1 showed some success in treating disease relapse, although a high percentage of the participants experienced serious side effects (Warren, Fujii et al. 2010).

MINOR ANTIGENS IN PREGNANCY

One important feature of the placenta is the release of large amounts of trophoblast debris into the maternal blood supply during pregnancy. This release has been postulated to result from biological functions of the trophoblast, particularly the continual renewal of the syncytiotrophoblast layer, which occurs via fusion of the underlying cytotrophoblast and release of apoptotic or necrotic syncytiotrophoblast into the periphery (Huppertz, Frank et al. 1998, Huppertz, Kaufmann et al. 2002, Huppertz, Kingdom et al. 2003). As a consequence of this continual syncytiotrophoblast renewal, placental debris containing fetal mHAg could be released into the maternal blood stream, phagocytosed and processed by maternal dendritic cells and presented to maternal CD8⁺ T-cells, thus eliciting a maternal immune response to the fetus (Erlebacher, Vencato et al. 2007).

Histocompatibility Antigen 1 (HA-1) is a widely studied mHAg that has been found on Hofbauer and trophoblast cells in the human placenta as outlined in Chapter 2 (Holland, Linscheid et al. 2012), and immune recognition of HA-1 has been shown to be important for bone marrow transplantation outcomes (Goulmy, Schipper et al. 1996). The antigenic peptide

that arises from HA-1 is the result of a single nucleotide polymorphism (SNP) between the non-immunogenic peptide (KECVLRDDLLEA) and the immunogenic peptide (KECVLHDDLLEA)(den Haan, Meadows et al. 1998). The immunogenic peptide can be presented in the context of at least four different Class I MHCs, including HLA-A*0201 (Table 1.2). As a result of the immunogenic SNP, the binding affinity of the HA-1^H peptide to the HLA-A*0201 peptide binding groove on antigen presenting cells (APCs) is increased (Nicholls, Piper et al. 2009), thus leading to an immunogenic peptide that can be recognized by HLA-A*0201 restricted T-cells.

Recognition of the HA-1 antigen can occur in the pathological situations of graft-versus-host disease and graft rejection, as well as in the physiological situation of pregnancy. In graft-versus-host disease, donor HLA-A*0201-restricted T-cells can recognize the immunogenic peptide in the antigen binding groove, thus eliciting an immune response targeting the recipients' tissues (Goulmy, Schipper et al. 1996, Mutis, Gillespie et al. 1999). Graft rejection occurs when recipient HLA-A*0201-restricted T-cells respond to and target the immunogenic peptide on the graft itself (Krishnan, Higgins et al. 2007). In the case of pregnancy, maternal HLA-A*0201 restricted T-cells can recognize fetal immunogenic HA-1, as evidenced by the presence of HA-1-specific T-cells in maternal blood following pregnancy (Verdijk, Kloosterman et al. 2004). The source of fetal HA-1 could be fetal cells that cross the placenta and enter the maternal blood stream and organs (microchimerism), cells and vesicles released from the placenta, or both. In each of the above situations, the T cells responding to the antigen are both restricted by HLA-A*0201 and are derived from an individual lacking the immunogenic HA-1^H allele.

The probability of this interaction varies depending on the population frequency of both mHAg alleles and the MHC(s) restricting the immunogenic peptide. In the case of HA-1, the population frequency of HA-1^H in a North American Caucasian population is approximately

44% (Tseng, Lin et al. 1998) and the likelihood that an individual will possess one of the four MHCs capable of presenting HA-1^H (A*0201, A*0206, B*60 or B*40012) (den Haan, Meadows et al. 1998, Mommaas, Kamp et al. 2002, Torikai, Akatsuka et al. 2007, Bleakley and Riddell 2011) is at least 48.1% (Gonzalez-Galarza, Christmas et al. 2011). Consequently, the possibility that two individuals will be discordant for HA-1 and that the individual with the non-immunogenic form of HA-1 will have the correct MHC to present the immunogenic peptide is approximately 11.9%:

$$P(HA1^H) \times P(HA1^R) \times P(MHC) = 44\% \times 56\% \times 48.1\% = 11.9\%$$

Thus, histoincompatibility between individuals is far from a rare event: given the large number of mHAGs discovered so far, it is very likely that for a given pregnancy, there will be at least one if not many fetal mHAGs that could be recognized by the mother's immune system.

The role of mHAGs in pregnancy was first considered due to a finding by a number of researchers that parous female donors are more likely to elicit graft-versus-host disease in transplant recipients than non-parous or male donors (Flowers, Pepe et al. 1990, Gratwohl, Hermans et al. 2001, Kollman, Howe et al. 2001, Randolph, Gooley et al. 2004, Takami, Sugimori et al. 2004, Loren, Bunin et al. 2006). These researchers hypothesized that this was due to the formation of mHAG-specific T-cells in the mother during or immediately following pregnancy. This postulate has been confirmed in mice, where researchers have found that CD4+ and CD8+ T-cells can develop in response to the endogenous fetal antigen, HY (James, Chai et al. 2003). Other mouse studies have demonstrated that the presentation of fetal antigens to maternal T-cells can begin as early as copulation and that paternal antigens can be found in the seminal fluid (Robertson 2005, Moldenhauer, Diener et al. 2009, Robertson, Guerin et al. 2009). In women, a number of studies have found T-cells specific for at least three mHAGs, HA-1, HA-

2 and HY, following pregnancy (Verdijk, Kloosterman et al. 2004, Piper, McLarnon et al. 2007, van Halteren, Jankowska-Gan et al. 2009). These T-cells have been found up to twenty-two years following delivery of the baby, suggesting that at least a small cohort of these cells can persist for long periods of time (Verdijk, Kloosterman et al. 2004). It is thought that during normal pregnancy these cells are prevented from attacking the placenta and fetus via numerous tolerogenic mechanisms, thus allowing for a successful pregnancy. A disruption of this tolerance could have significant effects on clinical outcome, as is evidenced by the links between mHAg expression, recognition by the maternal immune system and secondary recurrent miscarriage (Nielsen, Steffensen et al. 2009, Christiansen, Steffensen et al. 2010, Ooi, Russell et al. 2011).

A recent study has confirmed that HY-specific CD8⁺ T-cells are elicited in maternal blood *during* human pregnancy with a male fetus, and not necessarily as a result of parturition (Lissauer, Piper et al. 2012). These cells retained their proliferative capacity as well as their ability to lyse target cells and produce IFN- γ *in vitro*. The authors proposed that this indicates that fetus-specific T-cells are not completely deleted during pregnancy, as previously suggested (Tafari, Alferink et al. 1995, Jiang and Vacchio 1998, Vacchio and Hodes 2003).

As to why fetal antigen-specific T-cells elicited during pregnancy do not normally cause rejection of the fetus, there are at least three non-mutually exclusive possibilities. The first is that the T-cells are incompletely activated during pregnancy, and thus lack effector function *in vivo*. The second hypothesis is that other cell types, most notably regulatory CD4⁺ T-cells, prevent rejection of the fetus by promoting local and systemic tolerance, as indicated by the fact that regulatory T-cells are required for the success of allogeneic pregnancies in mice (Aluvihare, Kallikourdis et al. 2004, Kahn and Baltimore 2010, Shima, Sasaki et al. 2010). The third hypothesis is that the fetal antigen-specific T-cells are unable to traffic to the maternal-fetal interface, and thus cannot mediate direct rejection of the fetus. This hypothesis is supported by

recent work that showed an inability of maternal T-cells to traffic into the decidua during pregnancy as a result of epigenetic modifications leading to the loss of expression of specific chemokine genes by the decidual stromal cells (Nancy, Tagliani et al. 2012). In addition, the highly restricted expression of class I MHC molecules by placental trophoblast (Petroff 2005) most likely renders antigen-specific T-cells wholly or largely unable to target the placenta directly.

Minor Histocompatibility Antigens are Expressed in the Placenta

There are at least two likely sources of fetal mHAGs during pregnancy: the placenta and fetal cells trafficking from the fetus into the maternal blood supply (microchimerism) (Evans, Lambert et al. 1999, Adams, Lambert et al. 2003, Khostrotehrani and Bianchi 2005, Nelson 2008). In Chapter 2, we show that at least six fetal mHAGs are expressed in human placental lysate, fetal cord blood and, most significantly, purified trophoblast cells. These findings provide strong evidence that the human placenta is one likely source of maternal immune exposure to fetal antigens during pregnancy (Holland, Linscheid et al. 2012). The close physical relationship between the syncytiotrophoblast, which covers the outer surface of the placenta, and the maternal blood supply, which surrounds the placenta, provides a likely avenue for maternal immune exposure to fetally-derived mHAGs (Chamley, Chen et al. 2011, Petroff 2011). In addition to this close physical proximity, the syncytiotrophoblast undergoes a continual renewal process wherein the underlying cytotrophoblast fuses to give rise to the multinucleated syncytiotrophoblast and the excess, dead or damaged syncytiotrophoblast debris is released into the maternal blood space. This results in a large volume of fetally-derived placental debris being released into the maternal blood supply during pregnancy.

In addition to the larger, shed debris, there is growing evidence that microvesicles/nanoparticles and exosomes are actively secreted from the surface of the placenta

into the maternal blood space during pregnancy (Taylor, Akyol et al. 2006, Redman and Sargent 2007, Burton and Jones 2009). The total volume of this deported material has been estimated at 1g/day for the term placenta. Most of this placental material is easily cleared by the maternal system during normal pregnancy, but large, multinucleated syncytiotrophoblast debris has been found in the lungs of women who died of eclampsia (Attwood and Park 1961, Ilke 1964, Huppertz, Frank et al. 1998, Huppertz, Kaufmann et al. 2002). Given the robust expression of fetal mHAGs in the syncytiotrophoblast and the large amount of syncytiotrophoblast debris that is released into the maternal system, it seems highly likely that the placenta serves as a source of fetal antigens during pregnancy.

Clinical implications of Fetal Antigens

Of particular interest is the role that the mHAGs expressed in syncytiotrophoblast debris may play in the development or manifestation of numerous pregnancy complications. In Chapter 3, we show that expression of at least one mHAG, HA-1, is upregulated in preeclamptic placentas as compared to normotensive control placentas (Linscheid C and Petroff MG, in revision). We hypothesize that this increase in HA-1 expression may contribute to disruption of the overall immunologic balance by increasing the antigenic load encountered by the maternal immune system in the context of increased proinflammatory cytokine release, a feature of preeclampsia and other pregnancy complications (Kronborg, Gjedsted et al. 2011). Specifically, the increased release of placental debris that is characteristic of preeclampsia (Attwood and Park 1961, Ilke 1964, Huppertz, Frank et al. 1998, Knight, Redman et al. 1998, Huppertz, Kaufmann et al. 2002, Goswami, Tannetta et al. 2006, Chen, Huang et al. 2012), compounded by the upregulation of HA-1 expression in the deported syncytiotrophoblast, combined with an increase in inflammatory cytokines, most notably TNF- α and IL-6 (Kronborg, Gjedsted et al. 2011, Lee, Romero et al. 2012), could alter the phenotype of both the dendritic cells that are presenting

antigen to maternal T-cells as well as the T-cells themselves to promote anti-fetal immune responses, either during the current pregnancy or during subsequent pregnancies.

Dysfunction of the maternal immune system has been implicated in a number of other pregnancy complications, including secondary recurrent miscarriage. Epidemiologic evidence suggests that the recognition of fetal mHAGs may play an important role in secondary recurrent miscarriage, particularly if the preceding live birth was a male baby (Nielsen, Steffensen et al. 2009, Christiansen, Steffensen et al. 2010, Ooi, Russell et al. 2011). Specifically, Christiansen et al. have found that women who experience secondary recurrent miscarriage are more likely than the general population to possess the specific Class II MHC allele that presents Y-chromosome-encoded mHAGs and that secondary recurrent miscarriage is more likely to occur in women who have previously given birth to a male baby (Nielsen, Steffensen et al. 2009, Christiansen, Steffensen et al. 2010). This presents the possibility that the development of secondary recurrent miscarriage is related to the generation of a maternal immune response specific for Y-chromosome-encoded antigens during the preceding, successful pregnancy. One theory regarding this phenomenon is that pregnancy complications late in the first pregnancy may disrupt the tolerogenic environment required for maintaining maternal immune tolerance towards the fetus and that this disruption contributes to the failure of subsequent pregnancies, especially those with a male fetus (Christiansen, Steffensen et al. 2011). It is important to note that disruption of the tolerogenic environment and maternal T-cell recognition of both autosomal and Y-chromosome encoded fetal mHAGs may contribute to many cases of idiopathic infertility as well as numerous pregnancy complications.

A recent paper by Rowe et al. (2012) found that in mice there is a substantial expansion of fetal-specific regulatory T-cells (T-regs) during primary and subsequent pregnancies (Rowe, Ertelt et al. 2012). The authors also found that resorption rates were significantly decreased in

second pregnancies, due to a rapid expansion of a memory population of fetal-antigen specific T-regs that were formed during the first pregnancy. The authors propose that this mechanism may help explain why preeclampsia is more common in primiparous women as well as providing some insights as to why preeclampsia risk increases with interpregnancy interval. The role of these fetus-specific T-regs in secondary recurrent miscarriage is unclear, but it seems possible that a failed expansion of fetal-specific T-regs during the first pregnancy or a loss of fetal-specific T-regs between the first and second pregnancies could contribute to the manifestation of secondary recurrent miscarriage.

MICROPARTICLES RELEASED FROM THE PLACENTA MAY INFLUENCE MATERNAL IMMUNE CELLS

Description of Microparticles

A wide variety of vesicles/particles are released from cells, including cellular debris, microvesicles and exosomes. Cellular debris are much larger than microvesicles or exosomes and are generally the result of surface epithelial shedding or cell death (Thery, Boussac et al. 2001). Microvesicles are generally derived from the surface membrane of the cell of origin, whereas exosomes are smaller (50-150nm) and are derived from the late multivesicular endosome.

Discovery and Classification of Exosomes

Exosomes were first discovered independently by two research groups studying reticulocytes using electron microscopy and pulse-chase experiments (Harding, Heuser et al. 1983, Pan, Teng et al. 1985). Exosomes remained minimally studied until researchers found that exosomes secreted from B cells and dendritic cells could have potent immunomodulatory effects (Raposo, Nijman et al. 1996, Zitvogel, Regnault et al. 1998). In addition to reticulocytes,

dendritic cells and B cells, exosomes have been isolated from numerous cell lines, cancer tissues, cultured primary cells and bodily fluids, including T-cells, NK cells, macrophages, cultured trophoblast cells and maternal blood during pregnancy (Chaput, Flament et al. 2006, Iero, Valenti et al. 2008, Cocucci, Racchetti et al. 2009, Hedlund, Stenqvist et al. 2009, Chen, Liu et al. 2010, Atay, Gercel-Taylor et al. 2011, Chaput and Thery 2011, Dragovic, Gardiner et al. 2011, Huotari and Helenius 2011, Clifton, Stark et al. 2012, Filipazzi, Burdek et al. 2012). Currently, exosomes are defined as cup-shaped vesicles (visualized using electron microscopy) that measure 50-150nm in diameter with a density between 1.13-1.19 g/ml. Several different surface markers are currently used to identify exosomes, including various Annexins, Alix, CD9, CD63 and CD81. Given the relatively short period of time that exosomes have been extensively studied, there is still some debate about what parameters qualify a vesicle as an exosome and the methods by which exosomes should be ideally obtained (Chaput and Thery 2011).

Exosomes originate from multivesicular bodies/late endosomes (subsequently referred to as MVBs) and involve the packaging of exosomes for secretion rather than for degradation by the lysosome. Several components of cellular machinery have been identified as important for this process, most notably the ESCRT (endosomal sorting complex required for transport) proteins Alix and Tsg101 and the RAB family of small GTPases (Thery, Boussac et al. 2001, Savina, Fader et al. 2005, Hsu, Morohashi et al. 2010, Ostrowski, Carmo et al. 2010). The ESCRT complex is involved with targeting mono-ubiquitinated proteins to MVBs, where they can either be packaged into exosomes or undergo degradation in the lysosome. However, it is important to note that not all proteins found in exosomes are ubiquitinated, suggesting alternate mechanisms for the inclusion of different cellular materials in exosomes (Katzmann, Stefan et al. 2003, Skinner, O'Neill et al. 2009, Wollert and Hurley 2010). The RAB GTPases are important for trafficking of exosomes to the cell surface, fusion of the multivesicular body with the plasma

membrane and secretion of exosomes into the extracellular environment. Several different RAB proteins have been identified as important for the biogenesis of exosomes, including Rab11, Rab27 and Rab35, but no single protein has been found to be critical for exosome production and secretion (Savina, Fader et al. 2005, Hsu, Morohashi et al. 2010, Ostrowski, Carmo et al. 2010). This provides unique challenges for the study of the functions of exosomes, given that complete elimination of exosomes from a particular biological system – and the possible consequences of such elimination - have not yet been demonstrated. However, numerous studies, primarily in cancer biology, have found potent immune effects for exosomes derived from a variety of sources (Bobrie, Colombo et al. 2011).

Functions of Exosomes

In addition to proteins involved in their formation, exosomes contain a wide variety of proteins, mRNAs and miRNAs, some of which are found in most exosomes studied from a wide variety of biological sources, and others that appear to be more closely related to the cell of origin. For example, syncytiotrophoblast-derived exosomes express a number of highly-conserved exosome markers, including CD9, CD63, CD81 and LAMP-1 as well as a variety of proteins closely associated with the placenta, including B7-H1, B7-H3 and placental alkaline phosphatase (Mincheva-Nilsson and Baranov 2010, Kshirsagar, Alam et al. 2012, Mincheva-Nilsson and Baranov 2014). Notably, expression of HLA-G was only seen in exosomes from placental explants and cytotrophoblast cells and this expression was lost when the exosomes were derived from syncytialized trophoblast cells (Kshirsagar, Alam et al. 2012). This loss of expression is consistent with earlier research indicating the expression of membrane-bound HLA-G on extravillous trophoblast cells, but not the syncytiotrophoblast (Yelavarthi, Fishback et al. 1991, Ishitani, Sageshima et al. 2003, Morales, Pace et al. 2003).

Research regarding the role of exosomes in pregnancy is relatively new, but a number of studies focusing on the immune effects of cancer-derived exosomes provide compelling evidence the exosomes can have potent immunomodulatory effects. Numerous tumor-derived exosomes have been shown to express the pro-apoptotic molecules FasL and TRAIL, contributing to apoptosis of tumor-specific T-cells and tumor evasion of the adaptive immune response (Andreola, Rivoltini et al. 2002, Martinez-Lorenzo, Anel et al. 2004, Abusamra, Zhong et al. 2005, Huber, Fais et al. 2005, Bergmann, Strauss et al. 2009). In addition to promoting apoptosis of cytotoxic CD8⁺ T-cells, cancer-derived exosomes appear to increase the number of regulatory CD4⁺CD25⁺Foxp3⁺ T-cells, potentially contributing to increased tolerance of tumor cells (Szajnik, Czystowska et al. 2010, Wada, Onishi et al. 2010). This increase in regulatory T-cells is mediated, in large part, by TGF- β , which is also vitally important for establishing pregnancy, suggesting potentially overlapping roles for exosomal proteins in both tumor biology and pregnancy (Robertson, Ingman et al. 2002).

Recent studies on pregnancy-associated exosomes have indicated that some pregnancy complications, including pre-term birth and preeclampsia, are associated with alterations in exosome number and function. Specifically, the concentration of exosomes in the plasma of women who delivered prematurely was lower than in women who delivered at term and exosomes from premature delivers were less effective at promoting a tolerogenic T-cell phenotype as compared to those isolated from women who delivered at term, as defined by inhibition of IL-2 production by activated T-cells (Sabapatha, Gercel-Taylor et al. 2006, Taylor, Akyol et al. 2006). In addition, placental exosomes isolated from maternal blood decreased expression of NKG2D receptors on CD8⁺ T-cells, leading to impaired receptor-mediated cytotoxicity (Mincheva-Nilsson, Nagaeva et al. 2006).

The role of placental exosomes in pre-eclampsia is less clear, with some groups reporting increased and other groups reporting decreased exosome/microparticle production in pre-eclamptic pregnancies as compared to controls. These differences are likely related to differences in how exosomes/microparticles were isolated and defined and the markers used to identify the exosomes in maternal plasma (Harlow, Brown et al. 2002, VanWijk, Nieuwland et al. 2002, Bretelle, Sabatier et al. 2003, Gonzalez-Quintero, Jimenez et al. 2003, Holthe, Lyberg et al. 2005, Meziani, Tesse et al. 2006, Lok, Nieuwland et al. 2007). Some researchers have proposed opposite effects for microparticles and exosomes in pre-eclamptic pregnancies, with microparticles associated with increased endothelial dysfunction, hypercoagulability and increased maternal inflammation, and exosomes associated with the opposite effects (Toth, Lok et al. 2007).

However, recent work on the role of exosomes derived from the first trimester trophoblast cell line, Swan71, indicates that placental exosome-associated fibronectin may increase maternal inflammation by upregulating IL-1 β production by macrophages (Atay, Gercel-Taylor et al. 2011, Atay, Gercel-Taylor et al. 2011). There is clearly a lack of research on the similarities and differences between placental exosomes and syncytiotrophoblast microparticles, both with respect to vesicle content and their effects on maternal immune cells.

RESEARCH OBJECTIVES

Pregnancy presents numerous challenges to the maternal immune system, which must simultaneously tolerate the semiallogeneic fetus and protect both the mother and fetus from potentially life-threatening infections. The mechanisms by which this is achieved are varied and include the recognition of fetal antigens by maternal T-cells. There are a number of studies to suggest that this process has important implications for both maternal and fetal health. Developing a better understanding of the cellular interactions that mediate this tolerance may

contribute to both the development of more successful transplantation protocols as well as the prevention and/or treatment of common pregnancy complications.

In this Chapter 2 of this work we investigate the expression of fetal antigens in the human placenta and the possibility that the placenta may act as a source of fetal antigens during pregnancy. Chapter 3 focuses on how expression of one of these antigens, HA-1, is regulated in the human placenta, both across gestation in normal pregnancy and in pre-eclampsia. The final set of experiments in Chapter 4 examines the proteins associated with trophoblast-derived microparticles and exosomes isolated from primary trophoblast cells and the effect of these trophoblast-derived exosomes on dendritic cells. Together, these experiments will provide new and important information about the expression of fetal antigens in the human placenta, how these antigens are expressed and regulated in normal and pre-eclamptic pregnancies and the role of placental exosomes and other shed microparticles in influencing the maternal immune response during pregnancy. As a whole, this work will provide important insights into the role of the immune system in normal pregnancy, pregnancy complications and beyond.

CHAPTER 2:**EXPRESSION OF FETAL MINOR HISTOCOMPATIBILITY ANTIGENS IN THE
HUMAN PLACENTA: A POTENTIAL SOURCE OF MATERNAL IMMUNE
EXPOSURE TO FETAL ANTIGENS DURING PREGNANCY**

Holland OJ*, Linscheid C*, Hodes HC, Nauser TL, Gilliam M, Stone P, Chamley LW, Petroff MG. Am J Pathol. 2012 Jan;180(1):256-66

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ABSTRACT

The purpose of this study was to determine whether human minor histocompatibility antigens are present in the human placenta. To do this, transcript levels of human minor histocompatibility antigens HMHA1, KIAA0020, BCL2A1, KDM5D, DDX3Y and RPS4Y1 in placental lysate, purified trophoblast cells and fetal cord blood were determined using PCR. Protein expression patterns for HMHA1, KIAA0020, BCL2A1 and RPS4Y1 were examined in placental and extraplacental membrane sections across gestation using immunohistochemistry. The autosomal minor histocompatibility antigens, HMHA1, KIAA0020 and BCL2A1 were found in placental lysate, purified trophoblast cells and fetal cord blood for all of the samples analyzed using RT-PCR, whereas the Y-chromosome encoded antigens, KDM5D, DDX3Y and RPS4Y1, were only found in tissues from pregnancies with male infants. Using immunohistochemistry, we discovered protein expression of HMHA1, KIAA0020, BCL2A1 and RPS4Y1 in both placental tissues and extraplacental membranes, with staining patterns that varied according to antigen and gestational age. These results provide new evidence that the placenta can act as a source of fetal antigens during pregnancy and may affect the maternal immune response to the fetus during pregnancy. This phenomenon has important implications for our understanding of transplant immunology as well as numerous pregnancy complications.

INTRODUCTION

Pregnancy is not an immunologically benign event. Rather, it is a series of carefully orchestrated interactions involving the maternal, paternal and fetal systems with the ultimate goal of fetal tolerance leading to the uncomplicated birth of a healthy infant. The numerous interactions required for a healthy pregnancy have long been of interest to researchers. In the 1950s, Medawar proposed the paradox of pregnancy whereby the fetus acts as a semi-allograft, a view that is still discussed today (Medawar 1954). We are interested in the mechanisms by which the maternal system recognizes and tolerates the fetal allograft and the consequences when these mechanisms dysfunction.

Placental and fetal growth and development have been postulated to be associated with chronic exposure of the maternal immune system to fetal antigens. Studies in both mice and women have provided compelling evidence that paternally-inherited antigens are detected by maternal lymphocytes (James, Chai et al. 2003). In mice, maternal lymphocyte exposure to a model paternal antigen can occur as early as insemination. This exposure appears to wane until the placenta matures and the maternal blood supply to the placenta is established, at which point the placenta is inundated with maternal blood. This contact between maternal blood and the placental surface accommodates antigen efflux into the maternal circulation in the form of trophoblast debris, fetal cells and placental exosomes. This efflux persists for the last half of pregnancy in mice and the last two-thirds of pregnancy in women (Erlebacher, Vencato et al. 2007, Moldenhauer, Hayball et al. 2010, Taglauer, Adams Waldorf et al. 2010).

The interface between the maternal blood supply and the fetus consists of the syncytiotrophoblast, which covers the placental villi and is in direct contact with the maternal blood supply. This syncytium is a single, multinucleated cell that is the result of fusion of the underlying cytotrophoblast cells and is of fetal origin (Benirschke, Kaufmann et al. 2006).

During pregnancy, large amounts of syncytial debris are shed into the maternal blood supply, providing a likely avenue for maternal exposure to fetal antigens expressed by the syncytiotrophoblast during pregnancy. This debris may include apoptotic syncytial knots, exosomes, syncytiotrophoblast microparticles and other foreign material that may elicit a tolerogenic response from the maternal immune system (Schmorl 1893, Huppertz, Kingdom et al. 2003, Benirschke, Kaufmann et al. 2006, Lapaire, Holzgreve et al. 2007).

Proteins that have the ability to induce immune responses do so as a result of polymorphisms between two individuals, and are classified as either major or minor histocompatibility antigens (MHC and mHAg, respectively). Antibodies to paternally-inherited MHC are common in multiparous women (Bouma, van Caubergh et al. 1996), and can be detected in mice as well (Tarfuri, Alferink et al. 1995). Experimental systems using mice have shown that T and B cells vigorously respond to naturally inherited MHC and mHAg with evidence that fetal MHC-specific CD8⁺ T cells undergo proliferation, deletion and tolerance during pregnancy (Tarfuri, Alferink et al. 1995). Furthermore, maternal anti-fetal CD4⁺ and CD8⁺ T cells express an array of modulating receptors that may function to ensure safe passage of pregnancy (Taglauer, Yankee et al. 2009, Taglauer, Adams Waldorf et al. 2010). In humans, cohorts of mHAg-reactive maternal CD8⁺ T cells are often expanded as a result of pregnancy, and are reported to possess a “memory” phenotype. These include the Y-chromosome-encoded antigens A2/HY and B7/HY, as well as the autosomally encoded polymorphic antigen HMHA1 (Verdijk, Kloosterman et al. 2004) (James, Chai et al. 2003, Piper, McLarnon et al. 2007). Despite this increasingly well-documented interaction between fetal alloantigens and the maternal immune system, maternal anti-fetal T cells and antibodies are normally harmless, and robust maternal tolerance of these fetal antigens is the norm rather than the exception.

Despite the presumed relationship between maternal immune reactivity to fetal minor histocompatibility antigens, trophoblast shedding, and fetal microchimerism (Gratwohl, Hermans et al. 2001, Christiansen, Steffensen et al. 2010), the identity and sources of fetal antigens have not been systematically investigated. The placenta is a likely source of fetal antigen since murine studies suggest that a model mHAg from trophoblast cells can stimulate maternal lymphocytes (James, Chai et al. 2003) and studies in the human placenta indicate that where the placenta interfaces with maternal blood, there is shedding of large amounts of apoptotic cells and placental debris, both of which could be phagocytosed by maternal antigen presenting cells and presented to maternal T-cells, thus eliciting a maternal immune response (Huppertz, Frank et al. 1998, Adams, Yan et al. 2007, Germain, Sacks et al. 2007).

In this chapter we chose to analyze the expression of six well-characterized minor histocompatibility antigens shown in Table 2.1: HMHA1, KIAA0020 and BCL2A1 (autosomal antigens) and KDM5D, RPS4Y1 and DDX3Y (Y-chromosome-encoded antigens). Of these six antigens, three (HMHA1, KIAA0020 and KDM5D) are known to elicit maternal immune responses during pregnancy (James, Chai et al. 2003, Piper, McLarnon et al. 2007, van Halteren, Jankowska-Gan et al. 2009). Here we show for the first time that minor histocompatibility antigens are expressed in the human placenta during pregnancy and may be shed into the maternal circulation where they could potentially interact with the maternal immune system and contribute to maternal recognition of the fetus.

Table 2.1: Human Minor Histocompatibility Antigens Expressed in the Placenta

Minor Antigen	Gene	Chromosome	HLA-Restriction	Function	Tissue Expression (mRNA)	References
HA-1 ¹	HMHA1	19p13.3	HLA-A*0201, HLA-A*0206, HLA-B*60, HLA-B*40012	Rho GTPase activating protein	Hematopoietic cells	(den Haan, Meadows et al. 1998, Wilke, Pool et al. 1998, Mommaas, Kamp et al. 2002, de Kreuk, Schaefer et al. 2013)
HA-8	KIAA0020	9p24.2	HLA-A*0201	RNA binding protein (putative)	Broad	(Brickner, Warren et al. 2001)
ACC-1, ACC-2	BCL2A1	15q24.3	HLA-A*2402; HLA-B*4403	Negative regulator of apoptosis	Hematopoietic cells	(Akatsuka, Nishida et al. 2003)
A2/HY; B7/HY	KDM5D	Yq11	HLA-A*0201; HLA-B*2705	Protein containing zinc finger domains	Broad; high in hematopoietic cells	(Wang, Meadows et al. 1995)
B52/HY; DRB3/HY	RPS4Y1	Yp11.3	HLA-B*5201; DRB3*0301	RNA binding protein	Broad	(Spierings, Vermeulen et al. 2003, Ivanov, Aarts et al. 2005)
DQ5/HY; DRB1/HY	DDX3Y	Yq11a	DQB1*05; DRB1*1501	RNA helicase, involved in spermatogenesis	Broad; high in testis	(Rosinski, Fujii et al. 2008)

¹Abbreviations: HA, histocompatibility antigen; HMHA1, human minor histocompatibility antigen 1; HLA, human leukocyte antigen; GTP, guanosine triphosphate; BCL2A1, B cell lymphoma 2 related protein A1; KDM5D, lysine demethylase 5D; RPS4Y1, ribosomal protein S4, Y-linked; DDX3Y, DEAD box polypeptide 3, Y-linked.

The role of these antigens is not confined to pregnancy. Recent work in transplant immunology has indicated that male recipients of parous female hematopoietic stem cell transplantations have both higher rates of graft-versus-host disease and graft-versus-leukemia effects, likely as a result of male minor antigen-specific T-cells provided by the female graft (Randolph, Gooley et al. 2004, Takami, Sugimori et al. 2004, Loren, Bunin et al. 2006). A case study involving a matched human histocompatibility leukocyte antigen (Hladunewich, Derby et al.) transplant from a female to a male sibling has confirmed that male minor antigen-specific T cells and antibodies can be generated following male to female transplantation (Zorn, Miklos et al. 2004). Work done by Verdijk et. al. suggests that these minor histocompatibility antigen-specific T cells are not a temporary finding, but may persist up to twenty-two years following pregnancy (Verdijk, Kloosterman et al. 2004). The presence of minor antigen-specific T cells has also been implicated in the development of graft-versus-leukemia effects following cord blood transplantation, which contains a significant number of minor antigen-specific T-cells (Goulmy 2006). These findings suggest that understanding the source of these minor antigens during pregnancy and the mechanism by which they traffic into maternal circulation is of critical importance not only to reproductive immunologists, but to the broader world of transplant biology as well.

MATERIALS AND METHODS

Tissue procurement

This research was conducted in accordance with the Helsinki Declaration and was approved by the University of Kansas Medical Center Institutional Review Board. Term placentas with no associated pathologies of pregnancy were collected following elective Caesarian sections with no associated labor. First and second trimester placentas were collected following elective pregnancy termination in association with the University of Chicago Tissue Collection Core (HD049480) and the Center for Women's Health in Overland Park, KS. For extraction of RNA and protein, villous placenta was frozen in liquid nitrogen and stored at -80° C. For immunohistochemistry, samples of villous placenta with attached basal plate, as well as extraplacental membranes, were obtained and prepared by fixation in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) overnight. Samples were then dehydrated and embedded in paraffin.

Cytotrophoblast isolation

Cytotrophoblast cells were isolated as previously described (Petroff, Chen et al. 2003). Briefly, term placentas were collected following normal, Caesarian deliveries and approximately 40g of villous material was dissected. The tissue was rinsed with 0.9% NaCl saline and subjected to enzymatic digestion using Trypsin and DNase. After washing, the pellet was resuspended in HBSS and layered over a Percoll gradient (Sigma-Aldrich, St. Louis, MO). Cells were further washed and subjected to immuno-magnetic purification, using anti-class I MHC-coated magnetic beads. Cell purity was assessed using anti-cytokeratin 7 by immunohistochemistry or flow, resulting in greater than 90% purity. Cells were then stored at -80°C for RNA isolation.

Isolation and reverse transcription of RNA and PCR

RNA was extracted from whole placenta, purified cytotrophoblast cells and fetal cord blood using TRI Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's directions. The RNA concentration was quantified using spectrophotometry and 1 μ g of RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen, Carlsbad, CA) in a reaction volume of 20 μ l. A parallel reaction that included RNA but no Superscript III Reverse Transcriptase was also included as a control to ensure the absence of genomic DNA in samples. Two microliters of the reverse transcription (RT) reaction were subjected to polymerase chain reaction (PCR) using Taq DNA polymerase (Fermentas, Glen Burnie, MD). Primers for DDX3Y and RPS4Y1 were designed using PrimerBLAST (NCBI). Primers were produced by Integrated DNA Technologies (Coralville, Iowa). Primer sequences were as follows: DDX3Y forward, 5' TGC TGT CGT ATA GCT GTG GG 3'; DDX3Y reverse, 5' TCT CGA GAC CCA AAA CTG CT 3'; RPS4Y1 forward, 5' AGA TTC TCT TCC GTC GCA GA 3'; RPS4Y1 reverse, 5' CAT AGA CCA GGC GGA AAT GT 3'. PCR was carried out for 35 cycles at 63°C. RT-PCR for HMHA1, KIAA0020, BCL2A1 and KDM5D was carried out using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). The assay numbers used were as follows: Hs00299628_m1 for HMHA1, Hs00972109_m1 for KIAA0020, Hs00187845_m1 for BCL2A1 and Hs01104415_m1 for KDM5D. Hs99999903 for beta-actin was used as a control.

Immunohistochemistry

Histological preparations of tissues were cut into 10- μ m sections and were stained as previously described (Holets, Hunt et al. 2006). Antibodies specific for HMHA1, KIAA0020 and RPS4Y1 were purchased from Sigma-Aldrich (St. Louis, MO); BCL2A1 was purchased from Epitomics (Burlingame, CA).

RESULTS

RNA for mHAg in the placenta and cord blood

To determine whether the placenta or fetal blood cells could serve as sources of mHAg, whole placental lysates, purified cytotrophoblast cells and fetal cord blood mRNAs were examined by real time RT-PCR for the presence of four genes known to encode mHAg proteins. Expression of mRNA for HMHA1, KIAA0020, BCL2A1, and KDM5D was found in placental lysates, purified cytotrophoblast cells and fetal cord blood (Figure 2.1A). No expression of the Y-chromosome encoded mHAg KDM5D was found in samples from pregnancies with a female fetus. Because of lack of availability of TaqMan real-time PCR gene expression assay kits that distinguish between the male and female isoforms of the DDX3Y (DDX3X) and RPS4Y1 (RPS4X) genes, we designed primers for conventional RT-PCR that would distinguish these transcripts. Like KDM5D, DDX3Y and RPS4Y1 were identified in whole placenta, cytotrophoblast cells and cord blood from male, but not female, fetuses (Figures 2.1B&C).

Figure 2.1: Minor antigen mRNA expression in placenta, cytotrophoblast cells and fetal cord blood.

Real-time RT-PCR for autosomally-encoded mHAg HMHA1, KIAA0020 and BCL2A1, and the Y chromosome encoded mHAg KDM5D, for placental lysate and purified cell populations. Data are expressed as the mean difference in Ct values between the gene of interest and β -actin for the same samples \pm SEM, calculated as described in the Materials and Methods. Average β -actin values \pm SEM in each sample were: 24.9 ± 0.59 (placenta); 21.4 ± 0.24 (cytotrophoblast); 26.53 ± 1.56 (cord blood mononuclear cells). (B) and (C) Conventional RT-PCR data for placental lysate and purified cells, respectively. G3PDH was used as a control; sex of the infant is indicated by symbols above each lane. Ct, threshold cycle number; CT, cytotrophoblast cells; CB, cord blood mononuclear cells; M, Molecular weight marker.

Analysis of mHAg Proteins in the Placenta

General properties of mHAg immunoreactivities in the placenta

After determining that mRNA for all six minor histocompatibility antigens could be found in the placenta, purified cytotrophoblasts and fetal cord blood, we examined placental tissue sections for minor antigen protein expression using immunohistochemistry. 10 µm sections from first trimester, second trimester and term placental villi and placental membranes were stained for HMHA1, KIAA0020, BCL2A1 and RPS4Y1. All four minor antigens were found in both villous and extravillous trophoblast cells, with substantial variation occurring between samples and across gestation (Figures 2.2 & 2.3). Expression of HMHA1 and RPS4Y1 was exclusively cytoplasmic (Figures 2.2A, B, G, H and 2.3A, B, G, H) whereas both cytoplasmic and nuclear staining was seen for KIAA0020 and BCL2A1 (Figures 2.2 C-F and 2.3 C-F). It is also important to note here that the RPS4Y1 antibody recognized both the male and female versions of the protein, so both male and female placentas stained with the same patterns. We attempted to examine expression of both DDX3Y and KDM5D, but were unable to do so using the currently available antibodies.

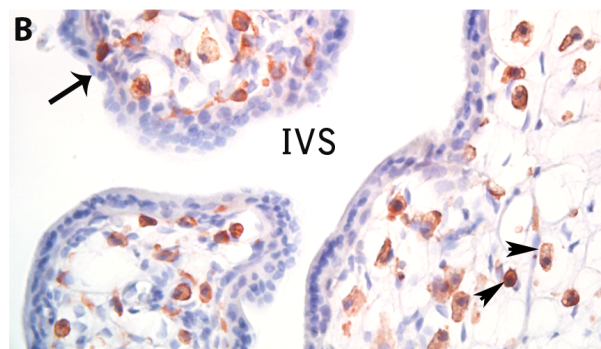
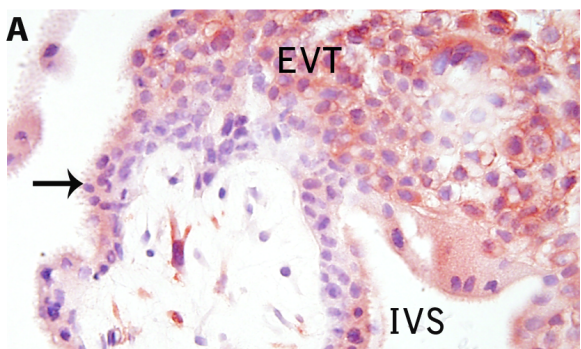
Figure 2.2: Minor histocompatibility antigen expression in first and second trimester placentas.

A, C, E, G, I: first trimester placenta (gestational age 8-12 weeks); B, D, F, H, J: second trimester placenta (gestational age 13-21 weeks). Reddish-brown staining represents specific immunoreactivity of mHAg-specific antibodies with cells; bluish-purple staining represents the hematoxylin counterstain. Arrows indicate syncytiotrophoblast; arrowheads indicate Hofbauer cells. EVT, extravillous trophoblasts; IVS, intervillous space.

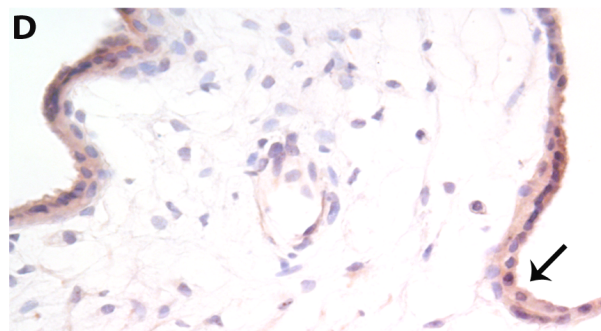
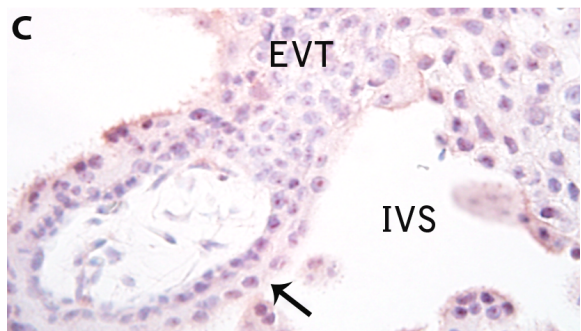
First Trimester

Second Trimester

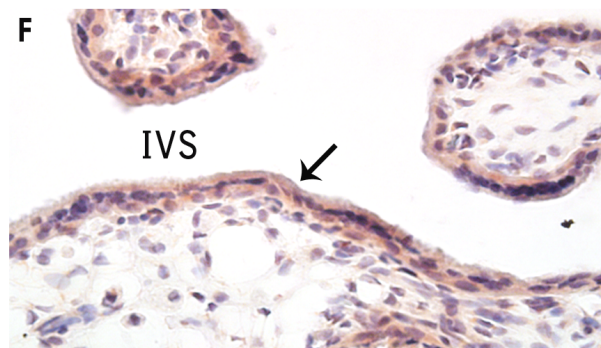
HMHA1



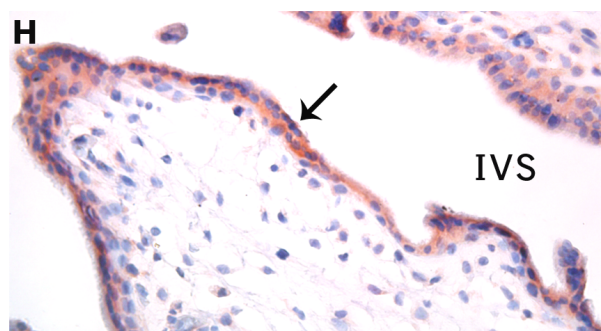
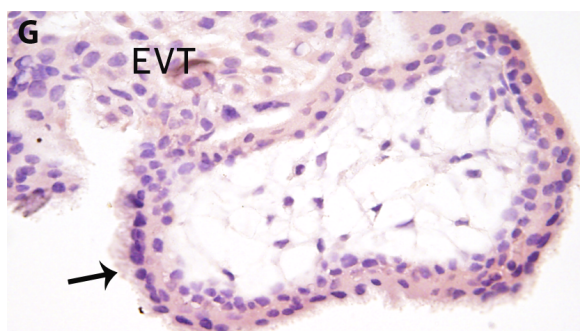
KIAA0020



BCL2A1



RPS4Y1



IgG

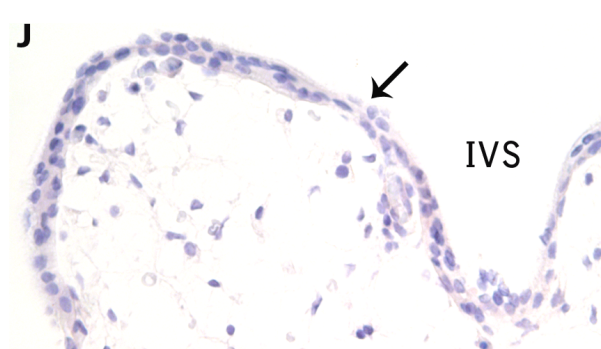
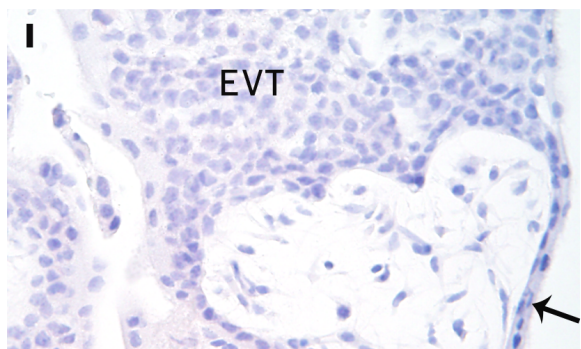


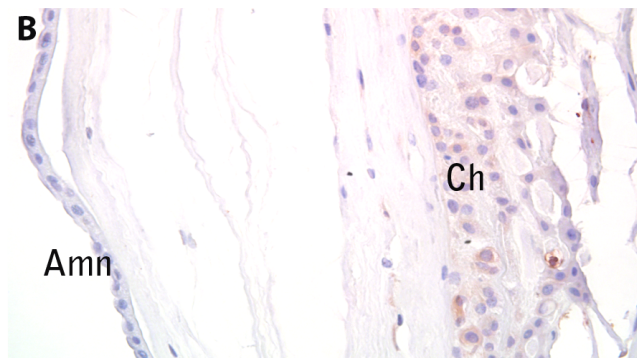
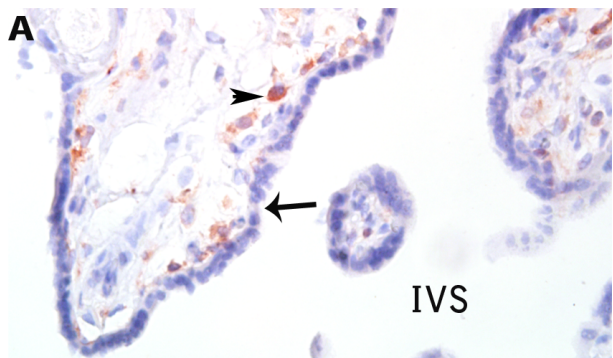
Figure 2.3: Minor histocompatibility antigen expression in term placentas and extraplacental membranes.

A, C, E, G, I: term placenta; B, D, F, H, J: extraplacental membranes. Arrows indicate syncytiotrophoblast; arrowheads indicate Hofbauer cells. Amn, amnion membrane; BP, basal plate; Ch, chorion membrane; IVS, intervillous space; Decid., decidua.

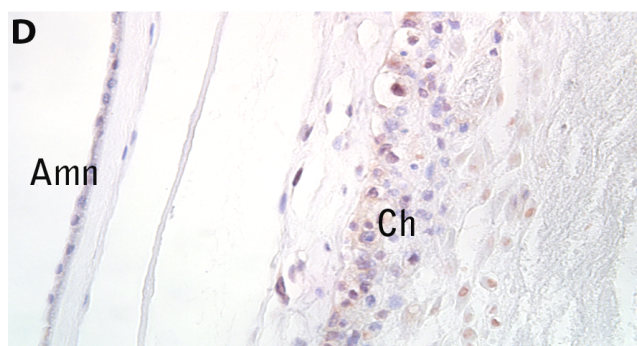
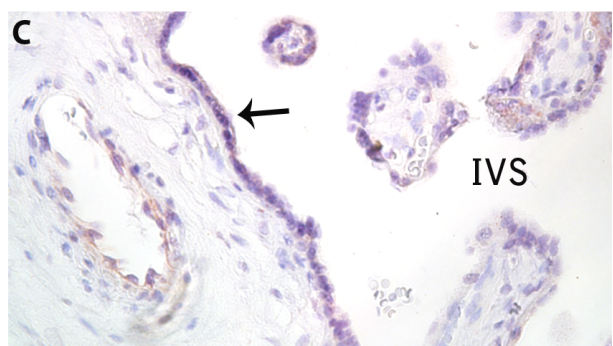
Term

Membrane

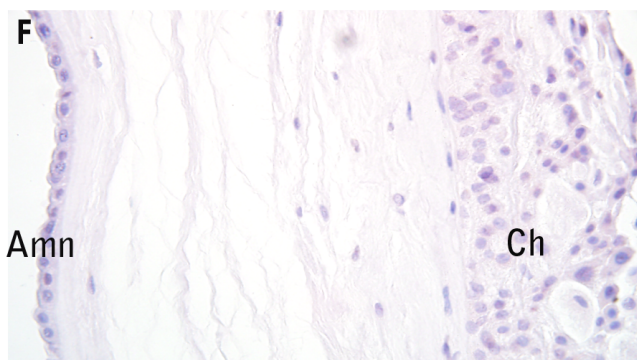
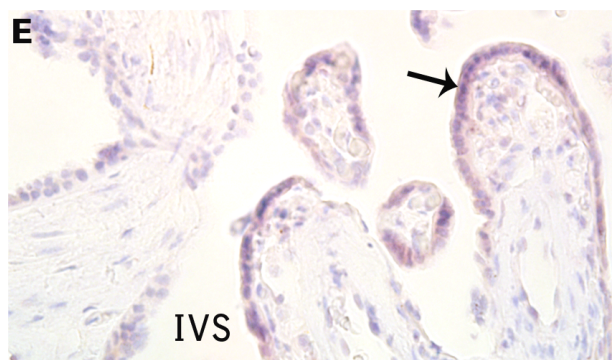
HMHA1



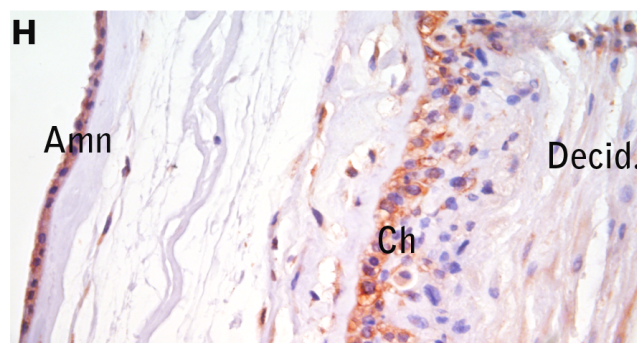
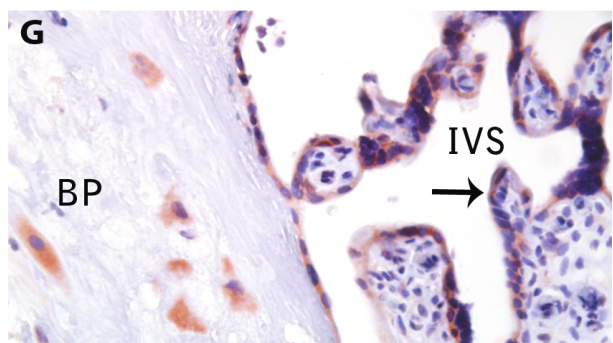
KIAA0020



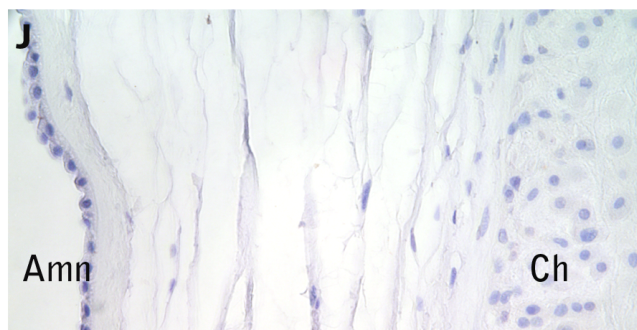
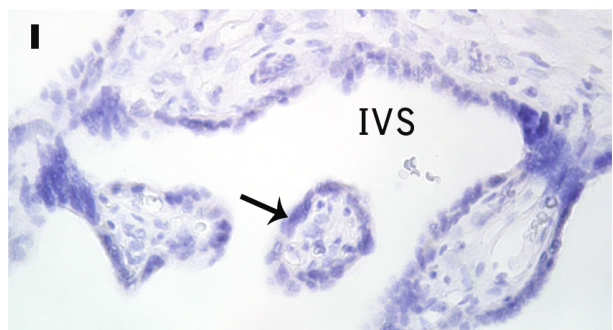
BCL2A1



RPS4Y1



IgG



Villous Trophoblast Cells

The staining of the syncytiotrophoblast was highly variable for all four antigens, with some areas of robust protein expression and other regions with little or no protein expression. Expression of BCL2A1 was generally stronger in the first and second trimester syncytiotrophoblast, as compared to term placentas, and several of the term placentas examined (3/8) had strong BCL2A1 only in areas resembling syncytial knots/aggregates (Figures 2.2E, F and 2.3E).

KIAA0020 and RPS4Y1 were found in the villous trophoblast of all of the samples examined across gestation (Figures 2.2C, D, G, H and 2.3C, G). KIAA0020 was found in the cell cytoplasm throughout gestation and some first trimester sections had nuclear staining as well. Expression of KIAA0020 was often stronger in the syncytiotrophoblast as compared to the cytotrophoblast.

HMHA1 expression was strongest in the first trimester syncytiotrophoblast, with many of the second trimester and all of the term placental sections lacking trophoblast expression of HMHA1 (Figures 2.2A, B and 2.3A).

Extravillous Trophoblast Cells

All four antigens were expressed in extravillous trophoblast cells (EVTs). KIAA0020, HMHA1 and RPS4Y1 were all found in extravillous trophoblast cells throughout gestation (Figures 2.2A, C, G and 2.3B, D, G, H). Expression of BCL2A1 was weaker in the term EVT's when compared to EVT's from first trimester placentas (Figures 2.2E and 2.3F).

Fetal Leukocytes

Fetal leukocytes were generally negative for KIAA0020, BCL2A1 and RPS4Y1 with a few weakly positive cells scattered throughout the samples. HMHA1 stained moderately in term

fetal leukocytes, but was either weak or negative in the fetal leukocytes present in first and second trimester samples.

Hofbauer Cells

Hofbauer cells, also known as placental macrophages, stained strongly for HMHA1 across gestation (Figures 2.2A, B and Figure 2.3A) and were weakly positive for KIAA0020, BCL2A1 and RPS4Y1 in second trimester, but not first or term placentas (Figures 2.2C-H and 2.3C, E, G).

Endothelial Cells

Endothelial cells were positive for KIAA0020, but lacked expression of the other antigens throughout gestation, with the occasional exception of RPS4Y1 (Figures 2.2C,D and Figure 2.3C).

Maternal Leukocytes

KIAA0020, BCL2A1 and RPS4Y1 were generally negative in maternal leukocytes with an occasional weakly positive cell. Maternal leukocytes located both within the peripheral blood and decidual tissues stained moderately for HMHA1 in both first and third trimester tissues (Figures 2.2A, B and 2.3A).

Decidua

BCL2A1 was not expressed in either the basal plate or in decidual tissues associated with extraplacental membranes (Figure 2.3F) whereas the three other minor antigens, HMHA1, RPS4Y1 and KIAA0020, were expressed in decidual tissues (Figure 2.3B, D, H).

DISCUSSION

In this chapter we have shown, for the first time, the expression of minor histocompatibility antigens in the placenta. Using PCR, we demonstrated that mRNA for the autosomal mHAgs HMHA1, KIAA0020 and BCL2A1 and the Y-chromosome encoded mHAgs KDM5D, DDX3Y and RPS4Y1 is found in placental tissues and fetal cord blood. Using immunohistochemistry, we were able to show that four of the six minor histocompatibility antigens studied were expressed by a number of different cell types found in the placenta including syncytiotrophoblast, cytotrophoblast, extravillous trophoblast, leukocytes and macrophages. This study provides support for earlier work indicating that pregnancy can lead to the expansion of minor-antigen specific T-cells (James, Chai et al. 2003, Verdijk, Kloosterman et al. 2004, Mommaas, Stegehuis-Kamp et al. 2005, Piper, McLarnon et al. 2007, Burlingham and Goulmy 2008).

This process has important implications for HLA-identical hematopoietic stem cell transplantation (HSCT). Recent research has shown that multiparous donors are associated with increased rates of graft-versus host disease in both male and female recipients, possibly due to mHAgs immunization during pregnancy (Loren, Bunin et al. 2006). Pregnancy-induced mHAgs immunization can also be beneficial to HSCT recipients in cases where minor antigen mismatch leads to an enhanced graft-versus-leukemia effect (Randolph, Gooley et al. 2004, Takami, Sugimori et al. 2004).

In addition to the importance of minor histocompatibility antigens in transplant biology, recent research has indicated a potential role for Y-chromosome encoded minor histocompatibility antigens in the occurrence of several pregnancy-associated complications

including secondary recurrent miscarriage and villitis of unknown etiology. Secondary recurrent miscarriage (SRM) is defined as three or more consecutive miscarriages following a birth at twenty-four weeks gestation or later. At least two independent studies have found a significant association between the development of SRM and prior birth of a male infant and it has been suggested that this may be due, at least in part, to an aberrant maternal immune response to Y-chromosome encoded antigens (Christiansen, Steffensen et al. 2010, Ooi, Russell et al. 2011). Researchers have also found that maternal carriage of HY-restricting class II alleles is associated with poor pregnancy outcomes in women with a prior live birth of a male infant and a diagnosis of SRM (Nielsen, Steffensen et al. 2009). Our data indicates that maternal exposure to Y-chromosome encoded fetal antigens is likely to occur during pregnancy as a result of both syncytiotrophoblast shedding and fetal microchimerism.

Villitis of unknown etiology (VUE) is a relatively common placental pathology in which maternal immune cells infiltrate placental villi. The etiology and importance of this lesion has been widely debated, with some pathologists proposing a fetal transplant rejection mechanism and others advocating for an as yet unidentified infectious agent (Redline 2007). Two recent studies have shown an increase in the prevalence of VUE in pregnancies resulting from egg donation as compared with other pregnancies conceived using IVF, although it is important to note that in only one of these studies was this finding significant (Styer, Parker et al. 2003, Perni, Predanic et al. 2005). The association of VUE with ovum donation provides support for the theory that VUE represents a maternal immune response to the fetus given that in ovum donation pregnancies the fetus is completely genetically foreign to the mother. Our work in this paper showing that the placenta expresses mHAGs provides a potential mechanism by which the maternal immune system is exposed to fetal antigens. This is of particular importance given the association between VUE and small for gestational age infants (Becroft, Thompson et al. 2005).

More work is needed to elucidate the molecular relationships between fetal mHAg expression, VUE and pregnancy outcomes.

In conclusion, we have demonstrated that fetal mHAgs are expressed in the placenta and fetal cord blood. This finding provides support for the hypothesis that mHAg-specific T-cells are generated during pregnancy as the result of both syncytiotrophoblast shedding from the placenta into the maternal blood supply and fetal microchimerism. This research provides important insight into the mechanisms by which the maternal immune system is induced to tolerate the fetal allograft during pregnancy. Further work is needed to elucidate the interactions between fetal mHAgs and maternal immune cells leading to both tolerance and aberrant maternal immune activation.

CHAPTER 3:**TROPHOBLAST EXPRESSION OF THE MINOR HISTOCOMPATIBILITY ANTIGEN
HA-1 IS REGULATED BY OXYGEN AND IS INCREASED IN PLACENTAS FROM
PREECLAMPTIC WOMEN**

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Traci Nauser and Margaret G. Petroff.

In Revision for Placenta

ABSTRACT

Maternal T-cells reactive towards paternally inherited fetal minor histocompatibility antigens are expanded during pregnancy, and can persist for many years following pregnancy. Placental trophoblast cells express at least four of these antigens, including human minor histocompatibility antigen 1 (HMHA1 or HA-1). In this study, we investigated oxygen as a potential regulator of HA-1 expression, and further, whether HA-1 expression is altered in placentas from preeclamptic patients. Expression and regulation of HA-1 mRNA and protein were examined by qRT-PCR and immunohistochemistry, respectively, using first, second, and third trimester placentas, first trimester placental explant cultures, and purified cytotrophoblast cells. Low oxygen conditions were achieved by varying ambient oxygen, and were mimicked using cobalt chloride. Lastly, HA-1 mRNA and protein expression levels were evaluated in preeclamptic and matched control placentas. HA-1 protein expression was significantly higher in the syncytiotrophoblast of first trimester as compared to second and third trimester placentas ($P<0.01$). HA-1 mRNA was increased in cobalt chloride-treated first trimester placental explants and purified term cytotrophoblast cells ($P=0.04$ and $P<0.01$, respectively) and in purified term cytotrophoblast cells cultured under 2% as compared to 8% and 21% oxygen ($P<0.01$). HA-1 mRNA expression in preeclamptic vs. control placentas was increased 3.3-fold ($P=0.015$). HA-1 protein expression was increased in syncytial aggregates and in the syncytiotrophoblast of preeclamptic vs. control placentas ($P=0.03$). Placenta HA-1 expression is regulated by oxygen and is increased in the syncytiotrophoblast of preeclamptic as compared to control placentas. Increased HA-1 expression, combined with increased preeclamptic syncytiotrophoblast deportation, provides a novel potential mechanism for exposure of the maternal immune system to increased fetal antigenic load during preeclampsia.

INTRODUCTION

Pregnancy is a unique biological process wherein the female is confronted with an antigenically foreign fetus that is permitted and encouraged to flourish, despite a surrounding immunological environment that can recognize it as foreign. Thus, the success of pregnancy depends on maternal immunological acceptance of the fetus. An understanding of the biological mechanisms responsible for this immunological tolerance is of significant interest: not only is the health of both mother and baby of paramount importance, also, the success of the immunological relationship between the two may inform medical success with transplantation, immune-mediated rejection of tumors and avoidance of autoimmune disease.

Preeclampsia is a common and dangerous complication that is specific to pregnancy and affects 5-8% of pregnancies worldwide (Duley 2009). It is a syndrome characterized by new onset maternal hypertension after 20 weeks gestation and proteinuria. If untreated, preeclampsia can lead to maternal seizures, multi-system organ failure and even death of both mother and baby. The only successful treatment of preeclampsia is delivery of the baby and the placenta; making preeclampsia one of the leading causes of iatrogenic prematurity (Pennington, Schlitt et al. 2012). Numerous studies have indicated a central, causative role for the placenta in the manifestation of preeclampsia; for example, preeclampsia can develop in molar pregnancies, in which no fetus is present (Soper, Mutch et al. 2004), and further, delivery of the placenta, not the baby, relieves the maternal symptoms (Redman and Sargent 2003). Although the pathophysiology of preeclampsia is not well understood, alterations in vascular reactivity leading to both increased placental oxidative stress and enhanced maternal immune activation are key components of this syndrome (Tannetta and Sargent 2013).

The placenta plays an important role in modulating the response of the maternal immune system to the baby during pregnancy. While the placenta tightly restricts its expression of the highly polymorphic Human Leukocyte Antigens (Hunt, Andrews et al. 1987), which govern adaptive immune recognition, it does express polymorphic fetal proteins encoded outside the HLA complex, known as minor histocompatibility antigens (Holland, Linscheid et al. 2012), that can be recognized by maternal immune cells. The immunogenic peptides derived from minor histocompatibility antigens (mHAgs) arise due to allelic polymorphisms, presence on the Y-chromosome, or common allelic deletions. Minor antigens are important in the success or failure of hematopoietic stem cell and solid organ transplants (Goulmy, Schipper et al. 1996, Goulmy 2006, Dierselhuis and Goulmy 2009). Exposure of an untolerized individual to these antigens, either as the result of pregnancy or allogeneic transplantation, can lead to the expansion of minor antigen-specific T cells (Verdijk, Kloosterman et al. 2004, Krishnan, Higgins et al. 2007). Further, the presence of minor antigen-specific T cells in allogeneic transplants has been linked to increased rates of graft rejection, graft-versus-host disease, and graft-versus-leukemia effects, indicating that, at least in the context of transplantation, these T cells are functional and pathophysiologically relevant (Goulmy, Termijtelen et al. 1976, Goulmy, Gratama et al. 1983, Goulmy, Schipper et al. 1996, Dierselhuis and Goulmy 2009).

Our work outlined in Chapter 2 has demonstrated that the placenta is a source of fetal antigens during pregnancy, and thus contributes to maternal immunological recognition of the fetus (Holland, Linscheid et al. 2012). T cells with specificity for paternally-inherited fetal antigens expand during pregnancy and can persist for up to 22 years following delivery of the baby (Verdijk, Kloosterman et al. 2004, Piper, McLarnon et al. 2007, Lissauer, Piper et al. 2012). In mice, MHC class I and class II-restricted presentation of paternally inherited fetal minor antigens occurs through the action of maternal antigen presenting cells, and results in expansion

of CD4⁺ and CD8⁺ T cells specific for both natural and model fetal antigens (James, Chai et al. 2003, Darrasse-Jeze, Klatzmann et al. 2006, Moldenhauer, Diener et al. 2009, Robertson, Guerin et al. 2009, Perchellet, Jasti et al. 2013).

HA-1 (also called HMHA1 for *human minor histocompatibility antigen 1*) functions as a Rho GTPase activating protein (de Kreuk, Schaefer et al. 2013), is expressed in the human placenta (Holland, Linscheid et al. 2012) and is important for graft outcomes in HLA-matched hematopoietic stem cell transplantations. A study of 148 HLA-matched bone marrow transplant pairs noted that a mismatch of HA-1 alone was significantly correlated with increased graft-versus-host disease (Goulmy, Schipper et al. 1996). HA-1 is antigenic due to a single nucleotide polymorphism in which the arginine in the peptide sequence KECVLRRDDLLEA is changed to a histidine, giving rise to the immunogenic peptide KECVLHDDLLEA. This single nucleotide polymorphism results in increased binding affinity of the peptide to the appropriate major histocompatibility complex (MHC) for immune recognition, in this case HLA-A*0201, A*0206, B*60 or B*40012 (den Haan, Meadows et al. 1998, Mommaas, Kamp et al. 2002, Torikai, Akatsuka et al. 2007, Nicholls, Piper et al. 2009, Bleakley and Riddell 2011). HA-1-specific CD8⁺ T cells have been identified in human pregnancies where the mother expresses the non-immunogenic form of the peptide and the fetus expresses the immunogenic form (Verdijk, Kloosterman et al. 2004). Given the expansion of specific T cells during pregnancy and the importance of HA-1 in transplantation immunology, we investigated the expression and regulation of HA-1 in the human placenta during pregnancy, when the placenta and fetus together act as an allograft that can be recognized by the maternal immune system.

MATERIALS AND METHODS

Sample Collection

Samples were collected in accordance with the Helsinki Declaration using protocols approved by the Institutional Review Boards of the University of Kansas Medical Center and Saint Luke's Hospital. First trimester placentas were obtained from women undergoing elective pregnancy terminations at the Center for Women's Health in Overland Park, Kansas. Normal term placentas, defined as placentas from pregnancies with no noted complications other than fetal malpresentation (including intrauterine growth restriction and diabetes), were obtained following non-laboring Caesarian deliveries at the University of Kansas Medical Center and Saint Luke's Hospital. Fixed and frozen tissues from preeclamptic placentas, together with gestational age- and mode of delivery-matched control placentas were obtained from the Research Centre for Women's and Infants' Health Biobank at Mount Sinai Hospital, Toronto, Canada. Control placentas were selected primarily based on the mode of delivery and gestational age and could not be from pregnancies complicated by pre-eclampsia, gestational hypertension or intrauterine growth restriction. Clinical data for these samples are provided in Table 3.1. The only significant difference between the control and preeclampsia groups was that infants in the preeclampsia group weight significantly less than those in the control group ($p < 0.01$). This is not unexpected given the high concordance of preeclampsia and intrauterine growth restriction.

Placental Explant Preparation

First trimester ($n=6$) villi were dissected and explants of approximately 500mg were cultured in 12-well plates fitted with Netwell inserts (Corning, Tewksbury, MA). Explants were cultured in DMEM-F12 media (Corning Cellgro, Herndon, VA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5ng/ml epidermal growth factor (PeproTech,

Rocky Hill, NJ), 5 μ g/ml insulin (Sigma-Aldrich, St. Louis, MO), 10 μ g/ml transferrin (Sigma-Aldrich), 100 μ g/ml L-glutamine (Corning Cellgro), 20nM sodium selenite (Sigma-Aldrich), 400 IU hCG (Sigma-Aldrich), 100 μ g/ml streptomycin (Corning Cellgro) and 100 IU penicillin (Corning Cellgro).

Cytotrophoblast Isolation

Cytotrophoblast cells were isolated as previously described (Petroff, Chen et al. 2003, Petroff, Phillips et al. 2006). Briefly, term placentas were collected following normal, non-laboring, Caesarian deliveries and approximately 40g of villous material was dissected. The tissue was rinsed with 0.9% NaCl and subjected to enzymatic digestion using Trypsin (Gibco, Grand Island, NY) and DNase (Sigma-Aldrich). After washing, the cell pellet was resuspended in Hank's balanced salt solution (HBSS) with 25mM HEPES buffer (Cellgro, Manassas, VA) and layered over a Percoll gradient (Sigma-Aldrich). Cells were further washed and subjected to negative immuno-magnetic selection, using an anti-class I (W6/32) MHC antibody and magnetic beads. Cell purity was assessed by flow cytometry using an anti-cytokeratin 7 antibody (Millipore, Billerica, MA). Only cell preparations resulting in greater than 90% purity were used.

Cobalt Chloride Treatment

First trimester placental explants (n=6) and purified term cytotrophoblast cells (n=5) were plated and cultured overnight at 37°C with 5% carbon dioxide, 95% normal air. Following overnight culture, the media was removed and replaced with control media (no cobalt chloride) or media containing 100 μ M cobalt chloride. Samples were cultured for 24 hours and then collected. Purified cytotrophoblast cells were lysed in TRI Reagent (Sigma-Aldrich) and

processed for RNA isolation as described below; placental explants were frozen in liquid nitrogen prior to mRNA analysis.

Oxygen Cultures

Purified cytotrophoblast cells (n=6) were plated and cultured overnight at 37°C with 5% carbon dioxide, 95% normal air. Culture media were pre-equilibrated for at least twelve hours in the oxygen chambers. After overnight plating, cytotrophoblast cells were washed with pre-equilibrated medium to remove any cellular debris. Cells were then cultured under 2% or 8% oxygen or ambient air (21% oxygen) for 24 hours using an InVivo 300 (Ruskin/Baker, Sanford, ME) or an H35 (Hypoxigen, Frederick, MD) hypoxia chamber or in a standard incubator for the ambient air condition. After 24 hours, cells were collected in TRI Reagent and processed as described below.

RNA Isolation and RT-PCR

RNA was extracted from purified cytotrophoblast cells, first trimester placental explants and frozen placental tissues using TRI Reagent according to the manufacturer's directions. The RNA concentration was quantified using spectrophotometry and 0.5µg of RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen, Carlsbad, CA) in a reaction volume of 20 µl. A parallel reaction that included RNA but no reverse transcriptase was included as a control to ensure the absence of genomic DNA in the samples. 2 µl of the reverse transcription (RT) reaction was subjected to polymerase chain reaction (PCR) using Taq DNA polymerase (Fermentas, Glen Burnie, MD). RT-PCR for HA-1 was carried out using a TaqMan Gene Expression Assay (HA-1 assay number Hs00299628_m1, Applied Biosystems, Foster City, CA). Assay number Hs99999903 for beta-

actin was used as a control. Average fold change for each sample was calculated using ddCt values and normalized to the beta-actin control.

Immunohistochemistry

Paraformaldehyde-fixed (4%), paraffin embedded placentas cut to a thickness of 5µm were collected from preeclamptic (n=8) and gestational age- and mode of delivery-matched control women (n=8) at the Research Center for Women's and Infants' Health Biobank at Mount Sinai Hospital, Toronto, Canada. Slides were deparaffinized in Histo-clear (Fisher Scientific, Pittsburgh, PA), rehydrated and stained as previously described (Holets 2006). Briefly, tissues were subjected to heat antigen retrieval using Reveal buffer (BioCare Medical, Walnut Creek, CA). Nonspecific antibody binding was blocked in 10% goat serum (Sigma-Aldrich). A rabbit polyclonal primary antibody targeting human HA-1 (Sigma-Aldrich, 12 µg/ml) or a rabbit IgG control antibody (Vector Labs, Burlingame, CA, 12ug/ml) was added to tissue sections, which were then incubated overnight at 4°C. Following addition of biotinylated goat anti-rabbit secondary antibodies (Vector Labs, 10 µg/ml) and depletion of endogenous peroxidases, binding of primary antibody was detected using streptavidin-peroxidase and aminoethyl carbazole (Invitrogen, Carlsbad, CA).

H-score Quantification of HA-1 Expression

Expression of HA-1 in first trimester, second trimester, term uncomplicated and preeclamptic placental tissues was calculated using the H-score method (Goulding, Pinder et al. 1995). Briefly, 10-20 400X images were taken of each placenta using a Nikon 80i microscope. The expression of HA-1 in each placental villus was scored by a blinded reviewer as 3 (strong immunoreactivity), 2 (moderate), 1 (weak), or 0 (no immunoreactivity). At least 20 villi were quantified for each placenta. The H-score was quantified using the following formula: $H\text{-score} = 3*(\%strong) + 2*(\%moderate) + 1*(\%weak)$.

Statistical Analyses

All statistical analyses were conducted using the SigmaStat software (Systat, Chicago, IL). The differences between HA-1 mRNA expression in cobalt chloride-treated and untreated first trimester placental explants and purified term cytotrophoblast cells were calculated using two-tailed, paired T-tests. The differences in HA-1 mRNA expression across gestation and in purified term cytotrophoblast cells cultured at 2%, 8% and 21% oxygen were calculated using one-way ANOVAs on Ranks with Student-Newman-Keuls post-tests. The differences between HA-1 mRNA expression and H-scores in preeclamptic and normal placental tissues were calculated using a two-tailed, paired T-test. All results with a P-value of ≤ 0.05 were considered significant different.

RESULTS

HA-1 is Expressed in Placental Tissues Across Gestation

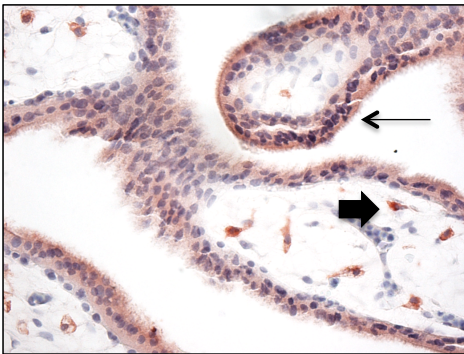
As illustrated in Chapter 2, HA-1 and other mHAGs are expressed in placental lysate, purified cytotrophoblast cells and fetal cord blood (Holland, Linscheid et al. 2012). Nonquantitative analysis suggested that HA-1 is strongly expressed in the syncytiotrophoblast of first trimester, but is reduced in second and third trimester placentas. To further explore the possibility of differential regulation of HA-1 in the placenta across gestation, we used semiquantitative analysis to examine expression of HA-1 protein in the syncytiotrophoblast of first (n=5), second (n=4) and third (n=5) trimester placentas (Figure 3.1). The overall cellular distribution of HA-1 agreed with our previous findings: Hofbauer cells from all stages of gestation exhibited strong staining for HA-1, while immunoreactivity in trophoblast populations was most evident in the first trimester. Furthermore, H-score analysis revealed that HA-1 protein was more than 4- and nearly 40-fold higher in the syncytiotrophoblast of the first trimester placentas as compared to the second ($P=0.001$) and third ($P<0.001$) trimester placentas, respectively. In contrast, second trimester syncytiotrophoblast HA-1 expression did not differ from that observed at term ($P>0.05$). Although there was a small subset of second trimester and term placentas with clear expression of HA-1 in the syncytiotrophoblast, most second trimester and term placentas showed little or no HA-1 expression in the syncytiotrophoblast, suggesting that in many cases HA-1 expression in the syncytiotrophoblast may be confined to the first trimester.

Figure 3.1: Expression of HA-1 in the human placenta across gestation.

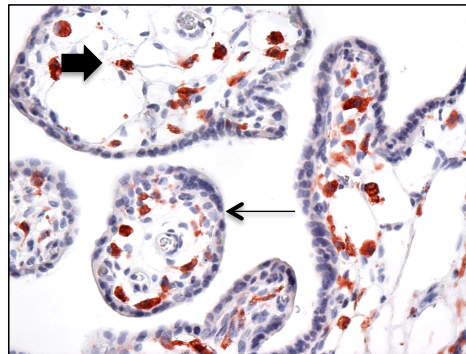
HA-1 expression in the syncytiotrophoblast (small arrows) and Hoffbauer cells (large arrows) of first trimester (n=5), second trimester (n=4) and term placental sections (n=5) was examined.

Expression of HA-1 protein in the syncytiotrophoblast was quantified by a blinded reviewer using the H-score method. Expression of HA-1 in the syncytiotrophoblast was significantly higher in first trimester placentas as compared to second trimester ($P < 0.01$) and third trimester placentas ($P < 0.01$). There was no significant difference in HA-1 protein expression between the second and third trimester placentas ($P = 0.277$). * = $P < 0.01$, ns=no significance

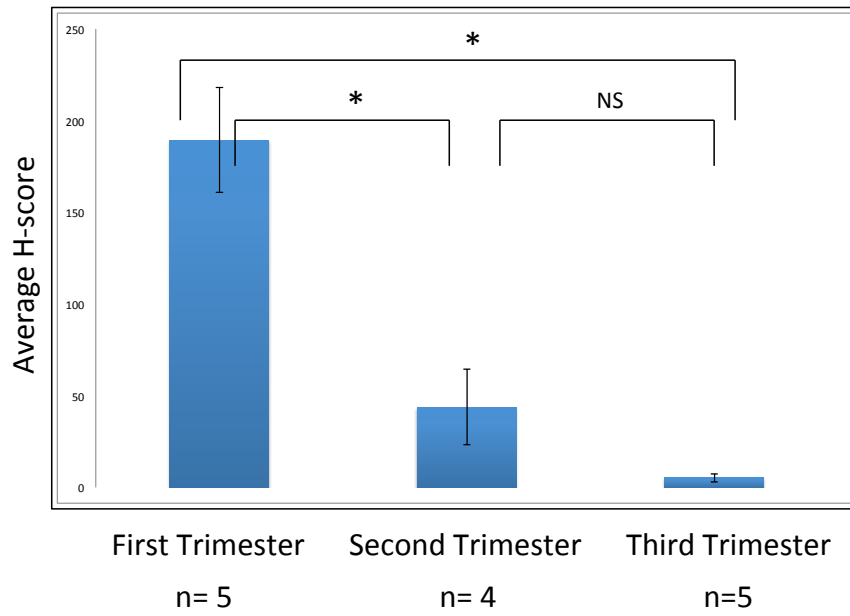
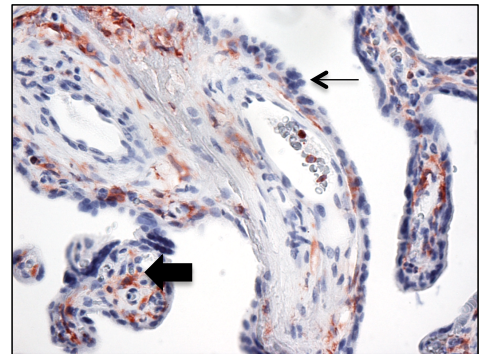
First Trimester



Second Trimester



Third Trimester

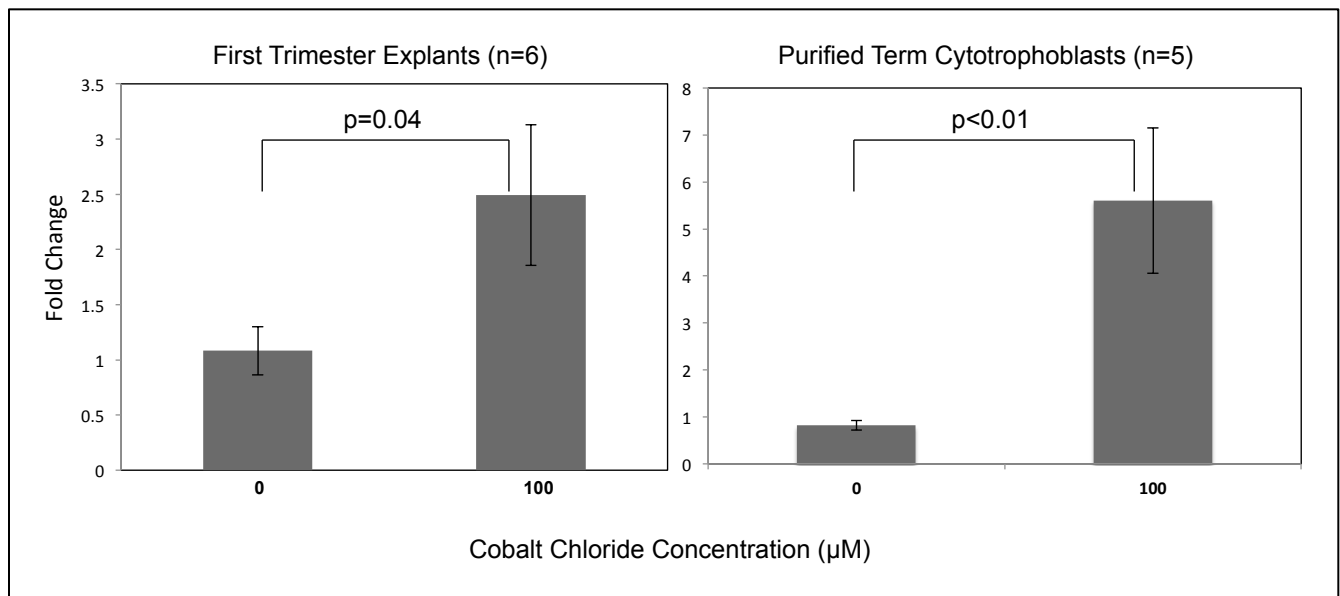


Cobalt Chloride Treatment Increases the Expression of HA-1 mRNA in First Trimester Placental Explants and Purified Term Cytotrophoblast Cells

Oxygen tension in the placenta increases dramatically from the first to the second trimester of pregnancy as a result of trophoblast remodeling of the maternal spiral arteries, suggesting that oxygen could be, at least in part, responsible for the observed alterations in HA-1 expression across gestation (Jauniaux, Watson et al. 2000). Therefore, to examine potential regulation of HA-1 expression by oxygen, we treated first trimester placental explants and purified term cytotrophoblast cells with 100 μ M of the hypoxia mimetic, cobalt chloride (CoCl₂). Cells or explants were treated in the presence or absence of 100 μ M CoCl₂ for twenty-four hours, and HA-1 mRNA was quantified by real time RT-PCR (Figure 3.2). There was a significant increase in HA-1 mRNA expression in the first trimester placental explants (n=6, p=0.04) and in the purified term cytotrophoblast cells (n=5, p<0.01) treated with cobalt chloride as compared to the untreated controls.

Figure 3.2: Cobalt chloride increases HA-1 mRNA expression in culture.

First trimester explants (n=6) and purified term trophoblast cells (n=5) were cultured in the presence of cobalt chloride, a hypoxia mimetic. HA-1 mRNA expression, determined using qRT-PCR, was normalized to beta-actin and fold change relative to the untreated controls was calculated using the ddCt method. HA-1 mRNA expression was significantly elevated in the first trimester placental explants and purified term cytotrophoblast cells treated with 100 μ M cobalt chloride as compared to the untreated controls (P=0.04 and P<0.01, respectively).

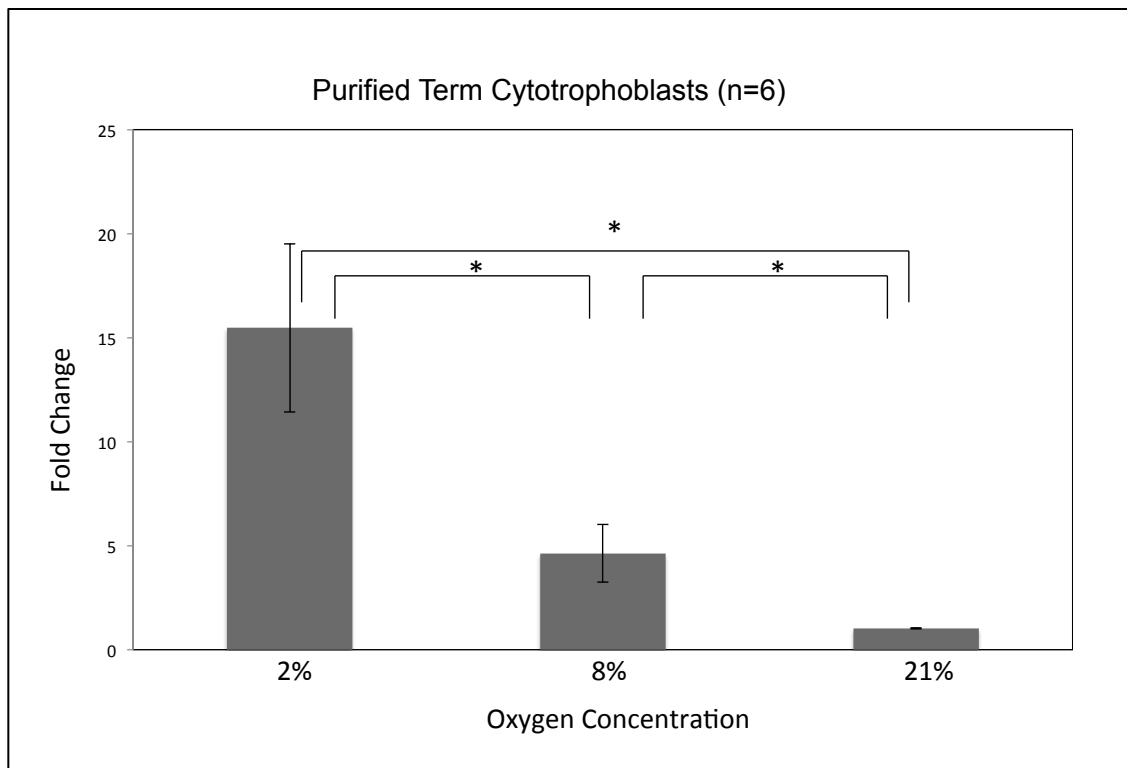


Low Oxygen Increases the Expression of HA-1 mRNA in Purified Term Cytotrophoblast Cells

To directly examine a potential role of oxygen in regulating HA-1 mRNA expression, purified term cytotrophoblast cells (n=6) were cultured at 2% (hypoxia for the term placenta), 8% (normoxia for the term placenta) and 21% (ambient) oxygen for twenty-four hours. The expression of HA-1 mRNA was then determined using qRT-PCR. HA-1 mRNA expression was significantly different between all three groups ($p < 0.01$), with the highest expression seen in the cells cultured at 2% oxygen (Figure 3.3).

Figure 3.3: HA-1 mRNA expression in purified term trophoblasts cultured under low oxygen.

Purified term trophoblast cells from normal, C-section deliveries (n=6) were cultured in 2%, 8% or 21% oxygen for twenty-four hours. HA-1 mRNA expression, determined using qRT-PCR, was normalized to beta-actin and fold change relative to the 21% oxygen condition was calculated using the ddCt method. HA-1 mRNA expression was significantly higher in the 8% and 2% oxygen conditions as compared to 21% oxygen ($P<0.01$). HA-1 mRNA expression was also significantly higher in the 2% oxygen condition as compared to the 8% oxygen condition ($P<0.01$). *= $P<0.01$



HA-1 is Increased in the Syncytiotrophoblast of Preeclamptic Placentas

Previously published observations of increased expression of HIF-1 α in preeclamptic placentas (Caniggia and Winter 2002), the disrupted placental blood flow characteristic of this disease (Steel, Pearce et al. 1990, North, Ferrier et al. 1994), and the observed upregulation of HA-1 mRNA by low oxygen, led us to postulate that HA-1 expression may be dysregulated in placentas from preeclamptic patients. Preeclamptic and control placentas were matched for gestational age and mode of delivery (Table 3.1). Using qRT-PCR, we found that HA-1 mRNA expression (n=9 matched pairs) was significantly increased in placentas from preeclamptic pregnancies as compared to control placentas (Figure 3.4, p=0.015). We also used immunohistochemistry to determine the expression of HA-1 protein in the syncytiotrophoblast of preeclamptic and control placentas (n=8 matched pairs; pair 4 was excluded due to poor tissue fixation). HA-1 protein expression, as scored by a blinded reviewer using the H-score method, was significantly higher in the syncytiotrophoblast of preeclamptic placentas when compared to the matched control placentas (Figure 3.4, p=0.03). Interestingly, although the expression of HA-1 in the syncytiotrophoblast of the control placentas was significantly decreased as compared to the preeclamptic placentas, there was still a high level of HA-1 expression in the Hofbauer cells (placental macrophages) of both the preeclamptic and control placentas.

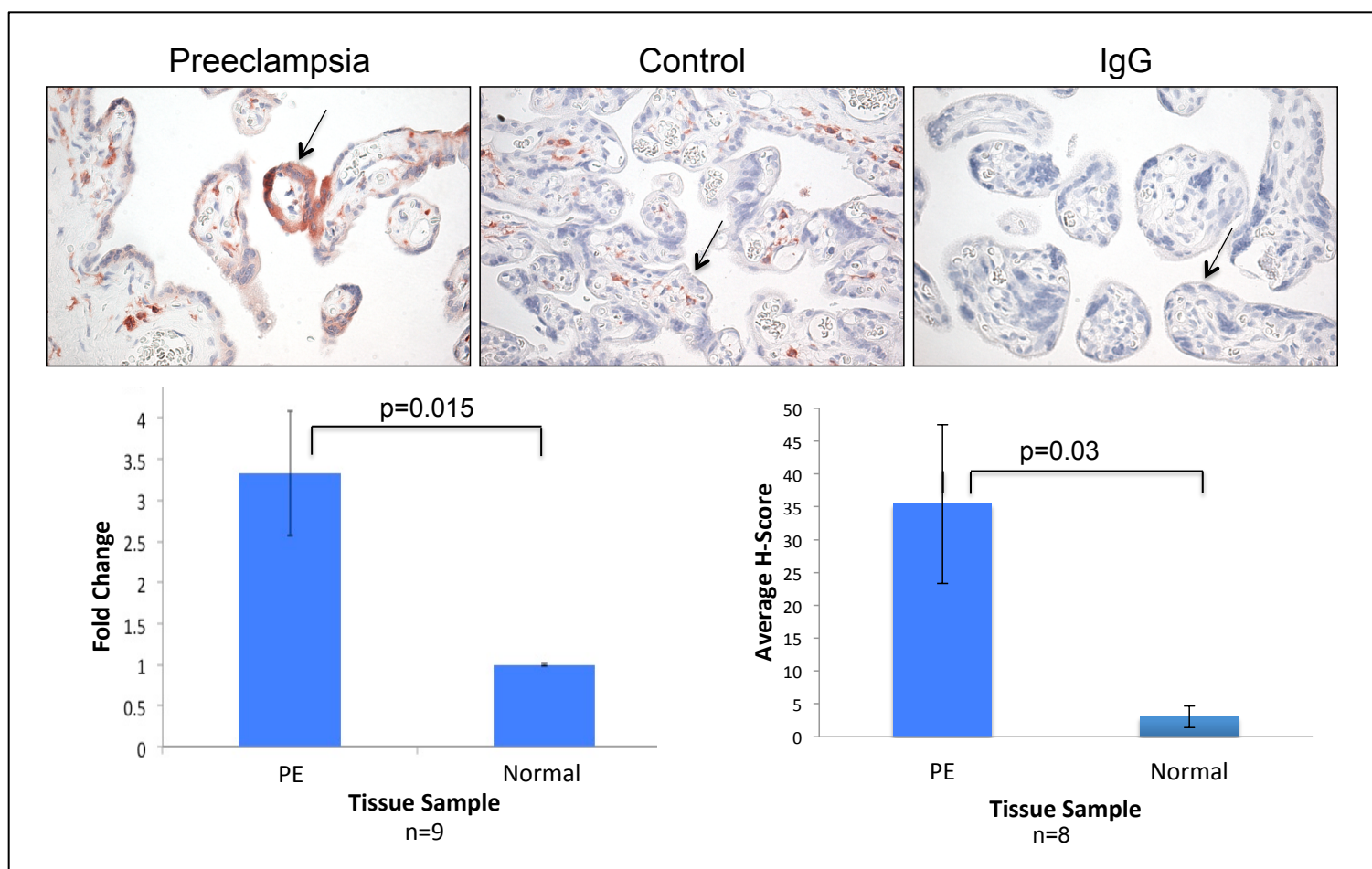
Table 3.1: Clinical Characteristics of Preeclamptic and Matched Control Placentas

Sample	Clinical Diagnosis	GA (weeks/days)	Ethnicity	Age	Parity (G/P)	Antenatal glucocorticoids? (Y/N)	Maternal cigarette use? (Y/N)	BMI	Mode of Delivery	Fetal Birth Weight	Fetal Sex (M/F)
1 - PE	PE	34/6	East Indian	37	3/1	N	N	19.8	Vaginal	2140g	F
1 - C	PTL	34/5	Caucasian	31	1/0	N	N	25.9	Vaginal	2710g	M
2 - PE	PE	37/2	Caucasian	37	3/0	N	N	19.4	Vaginal	2710g	M
2 - C	Labor	37/0	Asian	30	1/0	N	N	21.5	Vaginal	2870g	M
3 - PE	PE/HELLP	36/5	Caucasian	31	1/0	N/A	N	24.3	C-section	2640g	M
3 - C	Placenta praevia	36/3	Black	35	2/1	N	N	26.7	C-section	2665g	M
4 - PE	PE/HELLP/IUGR	32/4	Caucasian	39	2/0	Y	N	33.9	C-section	1220g	F
4 - C	PTL	32/4	Caucasian	33	1/0	Y	N	21.1	C-section	2080g	M
5 - PE	PE	36/2	Asian	38	1/0	N	N	21.6	Vaginal	2265g	M
5 - C	PTL	35/6	Asian	32	1/0	Y	N	19.8	Vaginal	2920g	F
6 - PE	PE/IUGR	34/1	Black	24	1/0	N	N	23.6	C-section + labor	1550g	F
6 - C	PTL	34/1	Caucasian	27	4/1	Y	N	26.1	C-section + labor	2360g	M
7 - PE	PE	36/0	Caucasian	37	1/0	N	N	26.4	C-section + labor	2280g	M
7 - C	PTL	35/4	Other	36	1/0	N	N	21.1	C-section + labor	3030g	F
8 - PE	PE/IUGR	31/5	Black	38	4/1	Y	N	26.2	Vaginal	890g	F
8 - C	PTL	32/1	Caucasian	29	2/1	Y	N	17.9	Vaginal	1810g	M
9 - PE	PE/IUGR	34/4	Native Canadian	37	4/0	Y	N	25.1	C-section + labor	1673g	F
9 - C	PTL	34/6	Caucasian	36	2/1	N	N	35.1	C-section + labor	2180g	M

Table 3.1: GA = Gestational Age, G/P = Gravidity/Parity, PE = Preeclampsia, C = Control, PTL = Pre-term Labor, HELLP = Hemolysis, Elevated Liver enzymes, Low Platelets, IUGR = Intrauterine Growth Restriction, N/A = Not Available, BMI = Body Mas Index, M = Male, F = Female

Figure 3.4: HA-1 expression is increased in preeclamptic placentas as compared to controls.

Whole placental lysate and placental sections were obtained from preeclamptic and gestational-age and mode of delivery matched controls. HA-1 mRNA expression was determined using qRT-PCR normalized to beta-actin. HA-1 mRNA expression (fold change) was significantly higher in the preeclamptic placentas as compared to the control placentas (n=9, P=0.015). HA-1 protein expression the syncytiotrophoblast layer (arrows) of preeclamptic and control placentas was determined by a blinded reviewer using the H-score method. HA-1 protein expression in the syncytiotrophoblast was significantly elevated in the preeclamptic placentas as compared to the controls (n=8, P=0.03). PE = Preeclampsia.



DISCUSSION

This chapter is the first work to investigate the mechanisms that regulate the expression of the minor histocompatibility antigen HA-1. We found that expression of HA-1 is significantly higher in the syncytiotrophoblast of first as compared to second and third trimester placentas. We also found that treatment with cobalt chloride, culture in hypoxic conditions, and preeclampsia all increase trophoblast expression of HA-1. One of the major changes that occurs during the transition from the first to the second trimester of pregnancy is a dramatic increase in placental oxygen delivery as a result of maternal spiral artery remodeling by invasive trophoblast cells and the loss of cytotrophoblast cell aggregates which block arterial blood flow to the placenta during early pregnancy (Burton, Jauniaux et al. 1999, Jauniaux, Watson et al. 2000). This process may help explain why HA-1 expression in the syncytiotrophoblast decreases as normal gestation progresses but appears to be maintained in preeclampsia, where the remodeling of the spiral arteries is defective.

The increased expression of HA-1 in purified cytotrophoblast cells following treatment with CoCl_2 and culture in a low oxygen environment suggests that HA-1 expression may be regulated, at least in part, by oxygen *in vivo*. This regulation likely occurs via the oxygen-sensitive transcription factor, hypoxia inducible factor (HIF) 1α . Under normoxic conditions, HIF- 1α is hydroxylated by prolyl hydroxylase, which directs its ubiquitination and degradation, thus preventing its action, while under hypoxic or stress conditions, or *in vitro* in the presence of cobalt ions, the prolyl hydroxylase is impaired and HIF- 1α function is maintained (Maxwell, Wiesener et al. 1999, Lee, Keith et al. 2001, Semenza 2004). In support of the idea that HIF- 1α regulates HA-1 expression in trophoblast cells, there are at least 3 sequences (5'-GCGTG-3') in the upstream promoter region of the HA-1 gene that could serve as HIF- 1α binding sites (800, 219 and 100 base pairs upstream of the HA-1 transcription start site). HIF- 1α expression is also

increased in preeclamptic as compared to normal placentas (Caniggia and Winter 2002). HIF-1 α expression in the preeclamptic placenta may be due to changes in oxygen tension, but HIF-1 α can also be upregulated by other processes that occur in preeclampsia, including oxidative stress (Wang, Leng et al. 2011) and heightened proinflammatory cytokines (Thornton, Lane et al. 2000). One notable limitation of this study as a model of pre-eclampsia is the culture of purified cytotrophoblast cells at static oxygen conditions, rather than cycling conditions of hypoxia and normoxia, which would more closely mimic pre-eclampsia in vitro (Burton 2009). We chose to examine HA-1 expression at static oxygen conditions in order to better clarify the precise role of oxygen in directly regulating HA-1 expression and remain interested in future studies examining the role of varying oxygen conditions in regulating HA-1 expression.

HA-1-specific CD8⁺ T cells have been found in the peripheral blood following human pregnancy (Verdijk, Kloosterman et al. 2004), indicating that exposure of the maternal immune system to fetal HA-1 occurs during normal pregnancies. Several factors may alter maternal exposure to HA-1, and possibly other minor histocompatibility antigens, in preeclampsia. Initially, the alteration in placental blood flow at the maternal-fetal interface in preeclamptic women leads to areas of ischemia/reperfusion (Hung, Skepper et al. 2001), which results in the production of reactive oxygen species (ROS) (Hung, Skepper et al. 2002, Jauniaux, Poston et al. 2006, Burton 2009, Burton, Woods et al. 2009) and an increased deportation of syncytiotrophoblast fragments (Knight, Redman et al. 1998, Huppertz, Kaufmann et al. 2002, Huppertz, Kingdom et al. 2003, Goswami, Tannetta et al. 2006, Reddy, Zhong et al. 2008). The increased release of syncytiotrophoblast microparticles into the peripheral blood, together with increased HA-1 expression could mean that the maternal immune system is exposed to higher concentrations of HA-1 in preeclampsia than in normal pregnancies.

Second, the production of acute phase reactants and pro-inflammatory cytokines, specifically TNF- α and IL-6 (Kronborg, Gjedsted et al. 2011), is characteristic of preeclampsia. This increase in pro-inflammatory cytokines together with increased expression of fetal antigen could combine to elicit a heightened antigen-specific maternal immune response in pregnancies complicated by preeclampsia. Although heightened fetal antigen-specific immune responses in preeclampsia have not been definitively shown to date, some authors have suggested the possibility that disruptions in maternal-fetal tolerance (Redman and Sargent 2003, Redman and Sargent 2004, Martinez-Varea, Pellicer et al. 2014), a shift from Th2-mediated immunity to Th1-mediated immunity (Sakai, Tsuda et al. 2002, Szarka, Rigo et al. 2010), and a reduction in quantity or function of regulatory T cells (Hsu, Santner-Nanan et al. 2012) may play important roles in the pathophysiology of preeclampsia.

In conclusion, we have shown that low oxygen levels as well as preeclampsia increase HA-1 expression by trophoblast cells of the human placenta. The increased expression of HA-1 in the syncytiotrophoblast of preeclamptic placentas combined with increased syncytiotrophoblast deportation and elevated levels of proinflammatory cytokines may have significant effects on maternal immune activation during preeclamptic as compared to normal pregnancies. The effects of this enhanced immune activation on current and subsequent pregnancies is unknown and may provide important insights into the manifestation of preeclampsia, other pregnancy complications and the success or failure of HA-1-mismatched transplantations. Further, given the function HA-1 as a Rho GTPase activating protein, and the potential for HA-1 expression to influence actin cytoskeletal remodeling (de Kreuk, Schaefer et al. 2013), the regulation of HA-1 expression is likely to be important for understanding not only the maternal immune response to the fetus during uncomplicated and preeclamptic pregnancies, but other biological processes as well.

CHAPTER 4:

**CHARACTERIZATION OF TROPHOBLAST-DERIVED MICROPARTICLES AND
THEIR EFFECTS ON THE MATERNAL IMMUNE SYSTEM**

ABSTRACT

Pregnancy is a unique, naturally-occurring immunological event wherein two genetically distinct individuals co-exist in one body. The placenta forms the interface between these two individuals and is an important mediator of maternal-fetal immune interactions. It has long been known that the placenta releases large amounts of syncytiotrophoblast-derived material into the maternal circulation. Recently, it has been discovered that this material includes actively secreted exosomes, which have been implicated in a number of disease states, most notably cancer. In this chapter, we analyzed the protein content of primary human trophoblast-derived exosomes and microparticles and examined the effects of trophoblast-derived exosomes on human dendritic cells, both in the presence and absence of lipopolysaccharide (LPS). Using mass spectrometry, we found that trophoblast-derived exosomes contain numerous exosome markers, including Alix and Syntenin, as well as proteins related to intracellular transport and localization, cellular stress responses, coagulation and hemostasis, and inflammatory responses. Surprisingly, trophoblast exosomes expressed HLA-DR, which is not expressed on the surface of trophoblast cells. In addition, we found that primary trophoblast-derived exosomes and microparticles can be taken up by human dendritic cells and that treatment of dendritic cells with trophoblast-derived exosomes resulted in increased mRNA levels of CD80 ($P=0.05$), an activation marker, as well as IL-8 ($P=0.05$) and VCAM-1 ($P=0.01$), both of which are involved in angiogenesis, when compared to vehicle-treated controls. In addition, dendritic cells that were treated with LPS + exosomes showed significantly decreased mRNA expression of CD40LG ($P=0.002$) and CCL7 ($P=0.03$), which are important for dendritic cell activation and migration, when compared to dendritic cells treated with LPS alone. These findings provide important insights into the mechanisms by which trophoblast exosomes may alter maternal immune function during pregnancy.

INTRODUCTION

Pregnancy provides a unique opportunity to study how two genetically distinct individuals can co-exist without either immune system mounting a severe and detrimental immune response to the other. The mechanisms by which the maternal immune system recognizes but does not reject the developing fetus are not yet completely understood. Further investigation of how rejection and tolerance are mediated in this naturally occurring system will help us better understand how successful pregnancies are maintained and the role of the immune system in many biological processes, including solid organ and hematopoietic transplantations. In this chapter we will examine how cellular materials released from the placenta during pregnancy can influence the maternal immune response to the fetus.

The placenta is a highly proliferative organ and large amounts of cellular materials are released from the surface of the placenta into the maternal blood supply during pregnancy. These materials include large, multinucleated syncytial knots, apoptotic debris and cell fragments, microvesicles and exosomes (Jones and Fox 1980, Huppertz, Frank et al. 1998, Huppertz, Kaufmann et al. 2002, Sargent, Germain et al. 2003). This release of placental materials into the maternal bloodstream is one route by which the maternal immune system may be exposed to fetal antigens during pregnancy (Chamley, Chen et al. 2011, Holland, Linscheid et al. 2012). In this chapter we will use mass spectrometry to examine the contents of shed exosomes and larger microparticles as well as investigate the effects of trophoblast-derived exosomes on dendritic cells (DCs), a subset of immune cells responsible for the presentation of both foreign and innate antigens to CD8⁺ T-cells.

Dendritic cells comprise 2-8% of the maternal immune cells in the decidua during pregnancy (Bartmann, Segerer et al. 2014) and play an important role in mediating the

relationship between the innate and adaptive immune systems. In part, dendritic cells are responsible for the phagocytosis, processing and presentation of antigens to T-cells. This is important for immune recognition and response to foreign antigens (including fetal antigens), as well as self antigens. Despite their relatively low proportions in the decidua, dendritic cells are one of the primary cell types involved in indirect antigen presentation to T-cells, a process that is critical for maternal immune recognition of fetal antigens (Erlebacher, Vencato et al. 2007), making it highly likely that they are involved in maternal immune responses during pregnancy. Resting dendritic cells that lack the co-stimulatory molecules necessary for CD8⁺ T-cell activation can lead to tolerance rather than CD8⁺ T-cell-mediated cytotoxicity (Probst, McCoy et al. 2005). Despite a well-established role in CD8⁺ T-cell activation, relatively little is known about peripheral dendritic cells during pregnancy. Recent work in mice suggests that the decidual environment may block dendritic cell migration, even in the presence of a strong immuno-stimulatory molecule such as LPS, suggesting that intrinsic characteristics of the pregnant uterus may prevent migration of decidual dendritic cells to peripheral lymph nodes where antigen cross-presentation to maternal T-cells could occur (Collins, Tay et al. 2009). However, this does not address the possibility that fetal antigens released into the peripheral blood may encounter maternal dendritic cells, leading to cross-presentation of fetal antigens to maternal CD8⁺ T-cells. In addition, myeloid dendritic cells isolated from pregnant women prior to 37-weeks gestational age showed lower levels of the activation markers, CD80, CD86 and HLA-DR, suggesting that basal activation of myeloid dendritic cells is decreased during pregnancy (Bachy, Williams et al. 2008). Dendritic cells from the blood of pregnant women also demonstrated increased production of the anti-inflammatory cytokine, IL-10, when stimulated with either TNF- α or IL-1 β , as compared to non-pregnant dendritic cells (Bachy, Williams et al. 2008). The phenotype of dendritic cells during pregnancy is particularly important given their

role in cross-presenting antigens to T-cells. We are interested in how materials from the placenta may affect dendritic cell phenotype and function during human pregnancy.

Exosomes and larger microparticles provide one potential source for maternal immune exposure to fetal antigens. Exosomes are small, cup-shaped nanoparticles (50-150nm) derived from late multi-vesicular endosomes that were first discovered in the context of their release from tumor cells and are actively secreted rather than passively shed (Taylor and Doellgast 1979, Trams, Lauter et al. 1981, Chaput and Thery 2011). In addition to tumor cells, exosomes are released by many different cell types including reticulocytes, dendritic cells and placental trophoblasts (Pan, Teng et al. 1985, Knight, Redman et al. 1998, Zitvogel, Regnault et al. 1998, Mincheva-Nilsson and Baranov 2010). Increased release of microparticles from the placenta has been implicated in preeclampsia, a common pregnancy complication characterized by elevated blood pressure and proteinuria and a leading cause of premature delivery (Knight, Redman et al. 1998, Goswami, Tannetta et al. 2006, Reddy, Zhong et al. 2008, Burton and Jones 2009). Recent work suggests that placental exosomes may play a role in maternal immune modulation (Taylor, Akyol et al. 2006, Hedlund, Stenqvist et al. 2009). Specifically, circulating exosomes from women who delivered prematurely expressed significantly lower levels of the Class II MHC, HLA-DR, and were less effective at preventing activation of T-cells when compared to exosomes from women who delivered at term, suggesting that during normal pregnancy, exosomes may play an important role in modulating maternal immune responses (Taylor, Akyol et al. 2006). Expression of NKG2D ligands on exosomes from first-trimester placental explants has also been shown to decrease activation of CD8⁺ T-cells, providing further evidence for an immunosuppressive role of placental exosomes (Hedlund, Stenqvist et al. 2009). Interestingly, recent work using a trophoblast cell line, Swan71, has indicated that trophoblast-derived exosomes are capable of recruiting monocytes and stimulating the production of the pro-

inflammatory cytokine, IL-1 β , via exosome-associated fibronectin (Atay, Gercel-Taylor et al. 2011, Atay, Gercel-Taylor et al. 2011). This work suggests that placental exosomes may have both tolerogenic and pro-inflammatory effects on maternal immune cells. It is not clear if these differences are primarily related to the source of the exosomes (peripheral blood vs. placental explant vs. cell line) or the target cell type (T-cells vs. monocytes).

In order to address some of these questions, we will examine the effects of primary trophoblasts on dendritic cells directly, in an attempt to create in vitro model for the role of trophoblast-derived exosomes in modulating maternal immune responses to fetal antigens. Notably, our lab has found that the cancer antigen, 5T4 (also known as trophoblast glycoprotein), is expressed at high levels in placental exosomes, suggesting that exosomes may act as a source of fetal antigen during pregnancy (M. Petroff, unpublished). This work is the first investigation of the effects of placental exosomes and microparticles on dendritic cell phenotype and will provide important information about how antigenic materials released from the placenta can modulate the maternal immune response to the fetus during pregnancy.

MATERIALS AND METHODS

Tissue Collection

All tissues were collected in accordance with protocols approved by the University of Kansas Medical Center Institutional Review Board. Trophoblast cells were isolated from term placentas obtained from non-laboring Caesarian sections as previously described (Petroff, Chen et al. 2003, Petroff, Phillips et al. 2006). Briefly, approximately 40g of villous tissue was dissected and rinsed in sterile saline (0.9% NaCl). The tissue was subjected to enzymatic digestion with Trypsin (Gibco, Grand Island, NY) and DNase (Sigma-Aldrich, St. Louis, MO). The cell digest was then resuspended in Hank's balanced salt solution with 25mM HEPES buffer (Cellgro, Manassas, VA) and layered over a Percoll (Sigma-Aldrich) gradient (5%-70% Percoll in HBSS + 25mM HEPES). The cell fraction containing trophoblasts (approximately 40-50% Percoll, densities 1.052-1.065 g/ml) was carefully aspirated and washed repeatedly with IMDM media (Cellgro) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 1X L-glutamine (Cellgro) and 1X antibiotic/antimycotic (Cellgro). Cell purity was assessed by flow cytometry using an antibody against cytokeratin 7 (Millipore, Billerica, MA). Only preparations with >90% purity were used in experiments.

Trophoblast Culture

Purified cytotrophoblast cells were plated at a density of 20×10^6 cells per 60mm culture dish overnight in exosome-free cytotrophoblast culture media at 37°C with 5% carbon dioxide, 95% normal air. Cells were then washed in pre-equilibrated exosome-free media to remove any cellular debris and cultured at 8% oxygen in cytotrophoblast culture media containing 5 ng/ml human epidermal growth factor (hEGF, Peprotech, Rocky Hill, NJ) for 3 days using an H35

hypoxia chamber (Hypoxygen, Frederick, MD). Following culture, exosomes and larger microparticles were collected as described below.

Exosome and Microparticle Collection

Following trophoblast culture, exosomes and larger microparticles (referred to as 10K microparticles) were isolated and previously described (Kshirsagar, Alam et al. 2012, Cronqvist, Salje et al. 2014). Briefly, cell culture supernatants were subjected to repeated centrifugations at 300xg, 2000xg and 10,000xg (10, 20 and 30 minutes, respectively) at 4°C. The supernatant and pellet obtained following the 10,000xg centrifugation were separated and the pellet was resuspended in 1ml of 1X PBS (Cellgro) and stored at -80°C for analysis as the 10K microparticle fraction. The remaining supernatant was centrifuged at 150,000xg for 70 minutes at 4°C to isolate the exosomal fraction. The exosome pellet was then washed twice in 1X PBS (100,000xg for 70 minutes at 4°C) and then resuspended in 50-100µl 1X PBS and stored at -80°C for exosome experiments. Exosome protein concentrations were determined with the DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's directions.

Nanoparticle Tracking Analysis - NTA

All exosome and microparticle preparations were analyzed using nanoparticle tracking analysis using a NanoSight LM14, software version 2.3 Build 0011 (NanoSight Ltd. Amesbury, UK). Samples were diluted in sterile 1XPBS and the absence of particles in the PBS was confirmed prior to each recording. Samples were vortexed extensively prior to loading and each sample was recorded 5 times for 60 seconds using the default recording settings at ambient temperature.

Electron Microscopy

Exosomes and 10K microparticles were fixed in 1% glutaraldehyde and embedded on formvar-carbon grids. Exosomes and microparticles were imaged by the University of Kansas Electron Microscopy Core using a JEOL JEM-1400 Transmission Electron Microscope. Grid squares examined for morphology and purity were chosen at random to avoid selection bias.

Mass Spectrometry

Exosome and 10K microparticles were washed in 1ml of 1X PBS and centrifuged at 100,000xg for 70 minutes at 4°C. The pellets were resuspended in sample buffer containing 1M Tris-HCl (Sigma-Aldrich), 20% SDS (Bio-Rad), 20% glycerol (Acros, Waltham, MA), 5% beta-mercaptoethanol (Sigma) and 0.48g 8M Urea (Expedeon, San Diego, CA). Samples were run on a 12% SDS-PAGE gel (Bio-Rad) for 1 hour at 120V. Each lane was cut into 12 equal bands and each band was subjected to trypsin digestion using 12.5 µg/mL trypsin in 25mM NH₄HCO₃. Individual peptides were identified using HPLC MS/MS with multidimensional protein identification technology (MUD PIT) and only proteins with two more identified peptides were included in our analyses.

Dendritic Cell Culture

Dendritic cells were derived in vitro from peripheral mononuclear cells (PBMCs) isolated from a healthy male donor as previously described (Kanof, Smith et al. 1996). Approximately 100ml of peripheral blood was collected in heparinized tubes and diluted in an equal volume of 1X PBS. Whole blood was centrifuged for 15 minutes at 200xg. The pellet was diluted in an equal volume of 1X PBS, layered over a cushion of Histopaque 1077 (Sigma-Aldrich) and centrifuged for 30 minutes at 900xg (20°C, no brake). The mononuclear fraction was carefully aspirated and washed several times in RPMI-1640 (Cellgro) containing 10% FBS. The isolated PBMCs were differentiated into dendritic cells as previously described (O'Neill and Bhardwaj

2005). Briefly, PBMCs were cultured for 2 hours in RPMI-10 and washed to remove non-adherent cells. Adherent monocytes were cultured in RPMI-10 for 5 days with 200 IU/ μ l interleukin-4 (IL-4, R&D Systems, Minneapolis, MN) and 100 IU/ μ l granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems). Cytokines were added on day 3 and immature dendritic cells were harvested on day 5, counted and replated (1.5×10^6 cells per condition) overnight. The dendritic cells were then treated for 18 hours with vehicle (PBS), LPS alone (1 μ g/ml, Sigma-Aldrich), LPS (1 μ g/ml) + exosomes (20 μ g) or exosomes (20 μ g) alone. Treated dendritic cells were then collected for analysis of purity by flow cytometry or collected in TriReagent (Ambion, Austin, TX) for RT-PCR analysis. Dendritic cell purity was >80% as assessed using an antibody against HLA-DR (Biolegend, San Diego, CA).

Immunofluorescence

To determine if human dendritic cells were able to phagocytose placental exosomes and 10K microparticles, the exosomes and 10K microparticles were centrifuged at 10,000 \times g for 30 minutes at 4°C (for the 10K microparticles) or at 150,000 \times g for 70 minutes at 4°C (for the exosomes) and were labeled with the green fluorescent dye PKH67 (Sigma), according to the manufacturer's directions, and co-cultured overnight with PKH26-labeled dendritic cells, such that only those dendritic cells able to phagocytose PKH26 would exhibit red fluorescence. The dendritic cells treated or not treated with labeled exosomes were imaged using a Nikon TE-2000U confocal microscope to determine if the exosomes and 10K microparticles would co-localize with the phagocytosis dye, PKH26, indicating possible phagocytosis of the exosomes and 10K microparticles.

RT-PCR

RNA was extracted from treated dendritic cells using an RNeasy Mini Kit (Qiagen, Germantown, MD) for PCR Array experiments or TRI Reagent (Sigma) for RT-PCR

confirmation experiments. All extractions were carried out according to the manufacturer's directions and RNA concentrations were determined using the NanoChip on a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). For PCR Array experiments, cDNA was synthesized from 100ng total RNA using the RT First Strand Kit (Qiagen) and RT-PCR was carried out using the RT Profiler PCR Array for human dendritic and antigen presenting cells (Qiagen, cat# 330231 PAHS-406ZA). All samples were run in triplicate and average fold-change for each sample was calculated using the ddCt method, using beta-2-microglobulin for normalization. Genomic DNA contamination and reverse transcription controls were used for each plate to ensure validity. Eleven potential mRNA targets were identified from the array and selected for RT-PCR confirmation. For RT-PCR confirmation, we repeated the experiment three more times using different exosome preparations and freshly isolated dendritic cells from the same male donor. Dendritic cell RNA was isolated as described above and 0.5µg of RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen) in a reaction volume of 20 µl. A parallel reaction that included RNA but no reverse transcriptase was included as a control to ensure the absence of genomic DNA in the samples. 2 µl of the reverse transcription (RT) reaction was subjected to polymerase chain reaction (PCR) using Taq DNA polymerase (Fermentas, Glen Burnie, MD). RT-PCR for eleven target genes and the housekeeping gene, beta-2-microglobulin, was carried out using the following TagMan Gene Expression Assays (Applied Biosystems, Foster City, CA). The mRNAs assayed were: CD40 ligand (assay number: Hs00163934_m1), CD40 (assay number: Hs01002913_g1), CCL7 (assay number: Hs00171147_m1), CD1B (assay number: Hs00957537_m1), CD80 (assay number: Hs00175478_m1), CLEC4C (assay number: Hs01092462_m1), FLT3 (assay number: Hs00174690_m1), FLT3 ligand (assay number: Hs00957747_m1), IL-8 (Hs00174103_m1), RAC1 (assay number: Hs01902432_s1), and

VCAM1 (assay number: Hs01003372_m1). All samples were run in triplicate and average fold change for each sample was calculated using ddCt values and normalized to the beta-2-microglobulin control (assay number: Hs00984230_m1).

RESULTS

Isolation of Trophoblast-Derived Microparticles and Exosomes

Exosomes and microparticles were isolated from primary trophoblast cells as described above. We used both electron microscopy and Nanoparticle Tracking Analysis to confirm that the isolated fractions contained exosomes and microparticles. The exosomal fraction was relatively homogenous and consisted of rounded and/or cup-shaped structures, consistent with previous reports of placental exosomes (Dragovic, Gardiner et al. 2011) (Figures 4.1A&C, 4.2A). In addition, NTA analysis of the exosomes indicated that the average size was 131nm and that the sample preparations were relatively homogeneous. The microvesicle fraction, in contrast, was heterogeneous when viewed by electron microscopy, with particles ranging in size from 30nm to 1 μ m and had an average size of 176nm when analyzed using NTA (Figures 4.1B&D and 4.2B).

Figure 4.1: Comparison of exosomes and 10K microparticles by nanoparticle tracking analysis.

Exosomes and 10K microparticles were isolated for purified term trophoblast cells cultured at 8% oxygen with 5ng/ml hEGF for 3 days. A. Representative NTA profile for 5 replicates from a single exosome experiment. Y-axis measures the number of particles (10^8 /ml). B. NTA profile for 5 replicates from a single 10K experiment. C. Representative NTA image for primary trophoblast-derived exosomes. D. Representative NTA image for primary trophoblast-derived 10K microparticles.

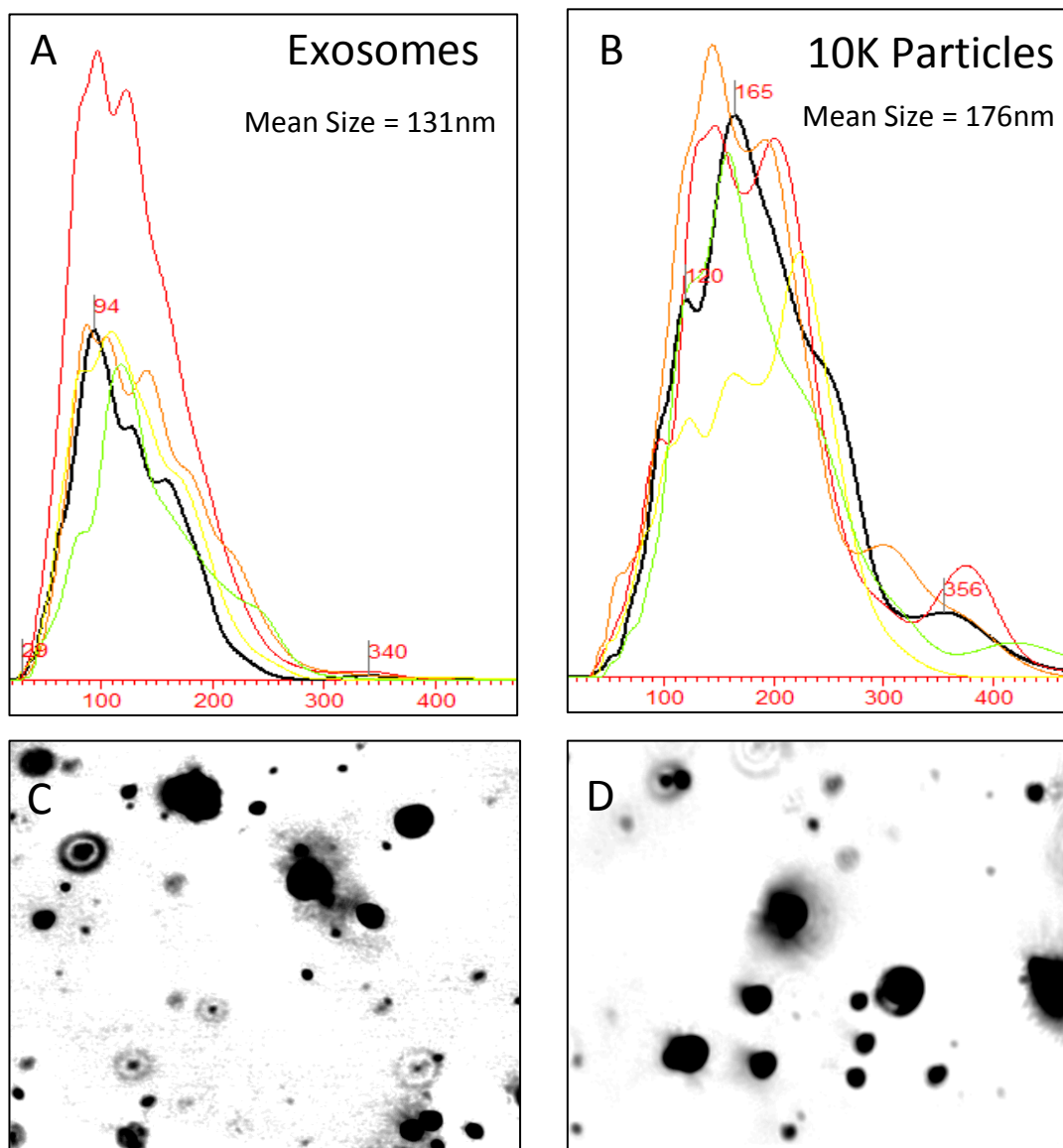
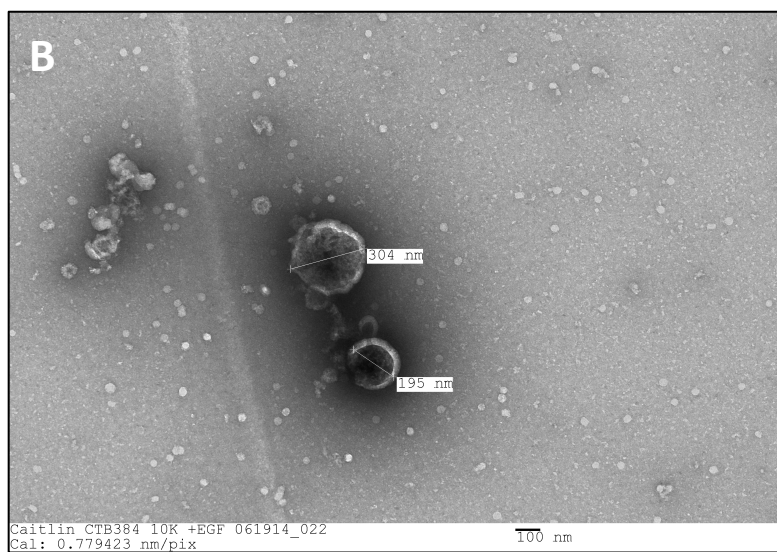
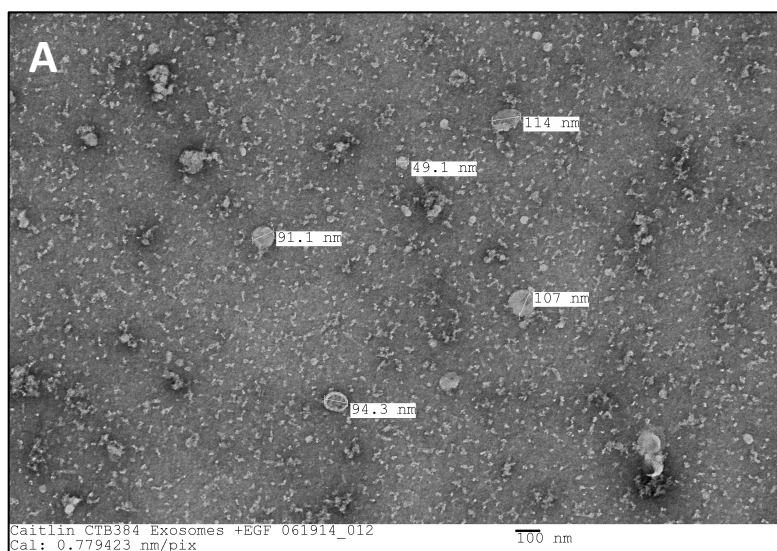


Figure 4.2: Electron microscopy of trophoblast-derived exosomes and microparticles.

Exosomes and 10K microparticles were isolated from purified term trophoblast cells by ultracentrifugation. Exosome and microparticle pellets were fixed in 1% gluteraldehyde and imaged using a JEOL JEM-1400 Transmission Electron Microscope. A. Representative electron microscopy image of trophoblast-derived exosomes. Exosome sizes range from 49.1nm to 114 nm. B. Representative electron microscopy image of trophoblast-derived 10K microparticles. Microparticle sizes range from 195nm to 304nm.



Protein Analysis of Exosomes and Microparticles

Exosomes have been shown to have a wide variety of contents, including microRNAs, mRNAs and proteins. To determine the protein content of trophoblast-derived exosomes and microparticles, 35 µg of protein from matched exosome and 10K samples was loaded onto a 12% SDS PAGE gel and each band was separately excised, subjected to trypsin digestion and analyzed using HPLC MS/MS with multidimensional protein identification technology (MUD PIT). Proteins were only considered positive identifications if two or more separate peptides were identified using HPLC MS/MS. 8 proteins were identified in both the exosome and 10K fractions, including several cytoskeletal proteins. Interestingly, only 2 of the identified proteins were unique to the 10K fraction, whereas there were 32 proteins that were found in the exosome fraction but not in the 10K fraction (Table 4.1). The proteins unique to the exosome fraction included several proteins that have been identified as exosome markers, including Hsc70, GAPDH, Annexin A2, Alpha-enolase, Syntenin, Annexin A5 and Alix (Mathivanan and Simpson 2009, Mathivanan, Fahner et al. 2012, Simpson, Kalra et al. 2012).

All of the identified proteins were analyzed using the open-source pathway analysis program, G-Profiler (Reimand, Arak et al. 2011). Four primary functional pathways were identified using this approach: localization and transport, stress response, coagulation and hemostasis and inflammatory response (Table 4.2). In addition, three cytoskeletal keratins were identified in our exosomes (KRTs 1, 2, and 9), all of which had been previously identified in exosomes from other cell types (Mathivanan and Simpson 2009, Mathivanan, Fahner et al. 2012, Simpson, Kalra et al. 2012).

Table 4.1: Proteins from Trophoblast-derived Exosomes and Microparticles

Exosomes Only	10K Microparticles Only	Both Exosomes and 10K Microparticles
Actin-Fimbrin Complex	Keratin 16	Alpha-2 Macroglobulin
Albumin	Keratin 17	Apolipoprotein B
Alix		Fibronectin 1
Alpha tubulin		Inter-alpha inhibitor H2
Alpha-2-HS-glycoprotein		Keratin 1
Alpha-enolase		Keratin 2
Alpha-fetoprotein		Keratin 9
Annexin A1		Vitamin D-binding protein X1
Annexin A11		
Annexin A2		
Annexin A3		
Annexin A5		
Annexin A6		
Apolipoprotein E		
Complement C3		
DaxxH3.3		
Dipeptidyl-peptidase 4		
Facilitated glucose transporter member 1		
GAPDH		
Gelsolin		
Hemoglobin		
Hemoglobin Bart's		
Histone H2A type 2-C		
HLA-DR		
Hsc70		
Inter-alpha inhibitor H3		
Lumican		
Protein 14-3-3		
Serpin peptidase inhibitor, clade F		
Syndecan binding protein (syntenin)		
Thrombin		
Thrombospondin-1		

Table 4.2: G:Profiler Pathway Analysis on Trophoblast-derived Exosomes

Exosome Markers	Localization and Transport	Stress Response	Coagulation and Hemostasis	Inflammatory Response
Alix	Actin-Fimbrin Complex	Actin-Fimbrin Complex	Actin-Fimbrin Complex	Alpha-2-HS-glycoprotein
Alpha-enolase	Albumin	Albumin	Albumin	Annexin A1
Annexin A2	Alix	Alpha-2-HS-glycoprotein	Alpha feto-protein	Complement C3
Annexin A5	Alpha feto-protein	Annexin A1	Alpha-2-HS-glycoprotein	HLA-DR
GAPDH	Alpha tubulin	Annexin A2	Alpha-enolase	Serpin peptidase inhibitor, clade F
Hsc70	Alpha-2-HS-glycoprotein	Annexin A3	Annexin A1	Thrombospondin
Syndecan binding protein (syntenin)	Annexin A1	Annexin A5	Annexin A2	
	Annexin A2	Complement C3	Annexin A3	
	Annexin A3	DaxxH3.3	Annexin A5	
	Annexin A6	Dipeptidyl-peptidase 4	Annexin A6	
	Annexin A11	Facilitated glucose transporter 1	Complement C3	
	Complement C3	GAPDH	DaxxH3.3	
	Dipeptidyl-peptidase 4	Gelsolin	Dipeptidyl-peptidase 4	
	Facilitated glucose transporter 1	Hemoglobin	Facilitated glucose transporter 1	
	Gelsolin	Hsc70	GAPDH	
	Hemoglobin	Serpin peptidase inhibitor, clade F	Gelsolin	
	Hsc70	Syndecan binding protein (syntenin)	Hemoglobin	
	Protein 14-3-3	Thrombin	Hsc70	
	Syndecan binding protein (syntenin)	Thrombospondin-1	Inter-alpha inhibitor H3	
	Thrombin		Lumican	
	Thrombospondin-1		Protein 14-3-3	
			Serpin peptidase inhibitor, clade F	
			Syndecan binding protein (syntenin)	
			Thrombin	
			Thrombospondin-1	

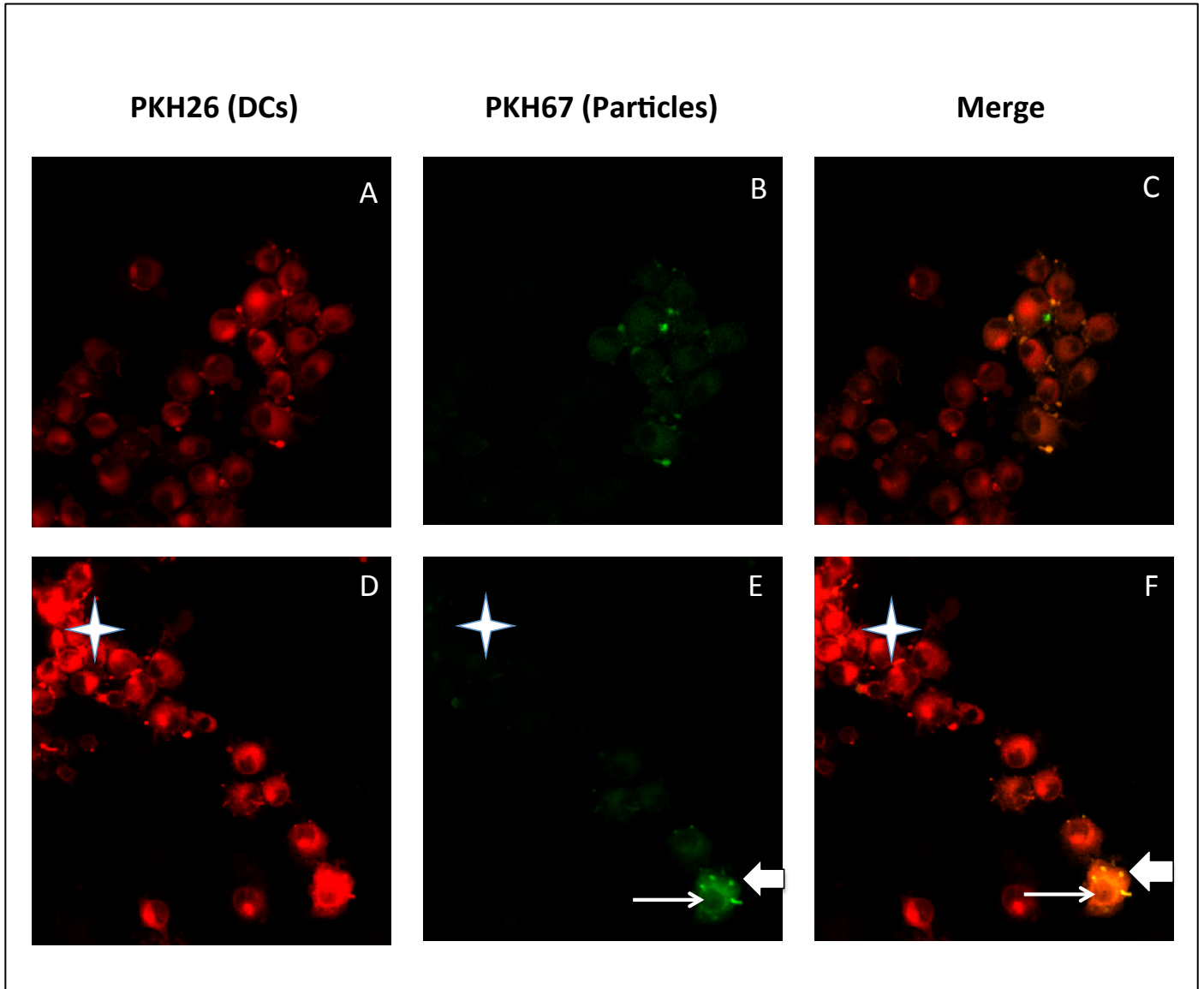
Phagocytosis of Trophoblast-Derived Exosomes and 10K Microparticles by Human Dendritic Cells

To begin determining the potential functional impact of trophoblast-derived exosomes on human dendritic cells, we investigated whether monocyte-derived dendritic cells are capable of phagocytosing trophoblast-derived exosomes and/or microparticles. To do this, exosomes and microparticles were stained with a green fluorescent dye, PKH67, and co-cultured individually with dendritic cells that were labeled with PKH26, a red fluorescent dye that does not bind to the cell surface, but rather only remains visible when it is phagocytosed, making it a good marker of dendritic cells with phagocytic capabilities (Atay, Gercel-Taylor et al. 2011).

Many of the dendritic cells did not show co-localization of the phagocytic dye and the exosomes, suggesting that most of the dendritic cells were functional but did not phagocytose the exosomes (Figure 4.3). However, there were a substantial number of dendritic cells that displayed co-localization of the phagocytic dye and the exosomes (Figure 4.3E,F). The patchy distribution of the green staining (exosomes) and co-localization with the red phagocytic dye is to be expected if the exosomes are enclosed within phagocytic vesicles, rather than being adhered to the outside of the cells, and is similar to a previous report in a paper outlining phagocytosis of trophoblast-derived exosomes by macrophages (Atay, Gercel-Taylor et al. 2011). Co-localization of the green 10K microparticles and the red phagocytic dye was less convincing, with most, if not all, of the dendritic cells displaying only red staining (Figure 4.3B,C).

Figure 4.3: Uptake of Trophoblast-derived 10K Microparticles and Exosomes by Human Dendritic Cells.

Human dendritic cells were co-cultured with PKH67-stained, human trophoblast-derived 10K microparticles (A-C) and exosomes (D-F). The dendritic cells were treated with a red dye, PKH26, to confirm their ability to phagocytose. Co-staining of green and red (yellow) indicates dendritic cells containing either microparticles or exosomes. The star indicates a group of dendritic cells that are stained only with the red, phagocytic dye, indicating DCs capable of phagocytosis that have not phagocytosed labeled exosomes or microparticles. The small arrow shows an area of a dendritic cell containing neither red nor green dye, whereas the large arrow shows the patchy distribution of green exosomes co-localized with the red phagocytic dye, suggesting that the exosomes are likely contained within phagocytic vesicles.



Effects of Trophoblast-Derived Exosomes on Human Dendritic Cells

Human dendritic cells were isolated as described above and treated for 18 hours with vehicle (PBS), LPS alone (1 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$) + exosomes (20 μg) or exosomes (20 μg) alone. We used LPS to stimulate the dendritic cells because we wanted to examine the effects of trophoblast-derived exosomes in both non-inflammatory and pro-inflammatory conditions. This is particularly important given the possibility that trophoblast-derived exosomes may have different effects on dendritic cells depending on whether or not the dendritic cells are being activated by another stimulus. The effects of the four treatment conditions were first examined using a commercially-available PCR array for human dendritic and antigen-presenting cells. Using the RT² PCR Data Analysis Software from Qiagen, we identified 11 mRNAs that appeared to differ between experimental and control groups ($p < 0.1$) (Table 4.3).

In order to confirm these effects, we repeated the experiment 3 more times using additional dendritic cells and exosome preparations. We analyzed the candidate mRNAs identified during the first set of experiments using qRT-PCR. In order to completely account for the total biological variability between samples, we combined the data from both experiments, and found that treatment of dendritic cells with trophoblast-derived exosomes increased expression of CD80, IL-8 and VCMA1 mRNAs when compared to vehicle-treated controls ($p = 0.05$, 0.05 and 0.01 , respectively, Figure 4.4). In addition, treatment of dendritic cells with LPS + exosomes significantly decreased expression of CD40LG and CCL7 when compared to samples treated with LPS alone ($p = 0.002$ and 0.03 , respectively, Figure 4.5). Notably, CD80 was originally predicted to differ between the LPS and LPS + exosome-treated conditions, but was only significantly different between the vehicle and exosome-treated conditions.

Table 4.3: Potentially Significant Dendritic Cell mRNAs Identified Using PCR Array

Vehicle-Alone vs. Exosomes	LPS Alone vs. LPS + Exosomes
RAC1	CD1B
IL-8	CD40LG
VCAM1	CD40
	CD80
	CLEC4C
	FLT3
	FLT3LG
	CCL7

Figure 4.4: Treatment of Human Dendritic Cells with Trophoblast-Derived Exosomes Significantly Increases Expression of CD80, IL-8 and VCAM1.

Dendritic cells (1.5×10^6 cells/condition, n=6) were treated with vehicle (PBS) or trophoblast-derived exosomes suspended in PBS (20 μ g exosomes) for 18 hours. Expression of CD80, IL-8 and VCAM1 mRNAs was significantly increased (p=0.05, 0.05 and 0.01, respectively) in the dendritic cells treated with exosomes as compared to vehicle-treated controls.

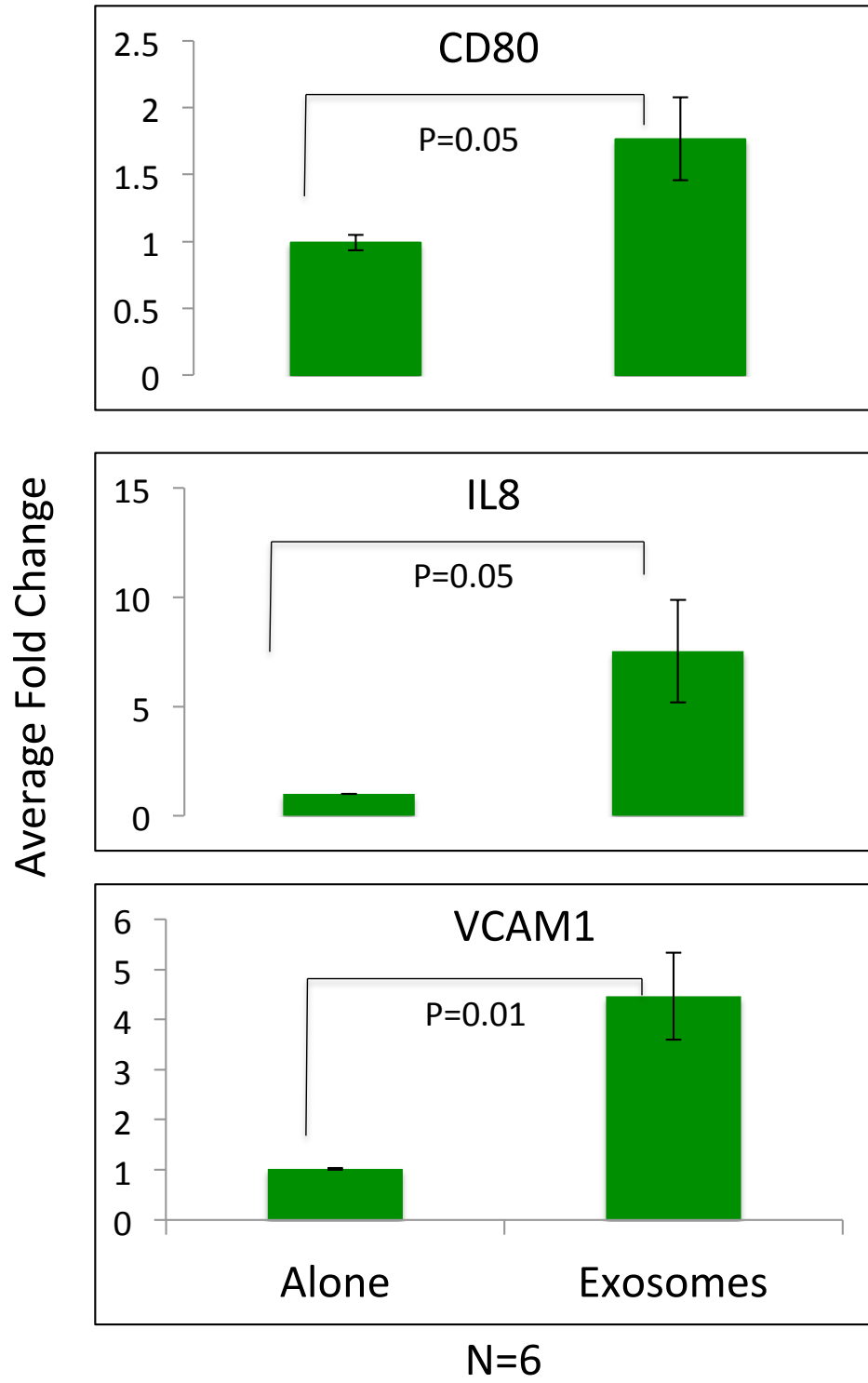
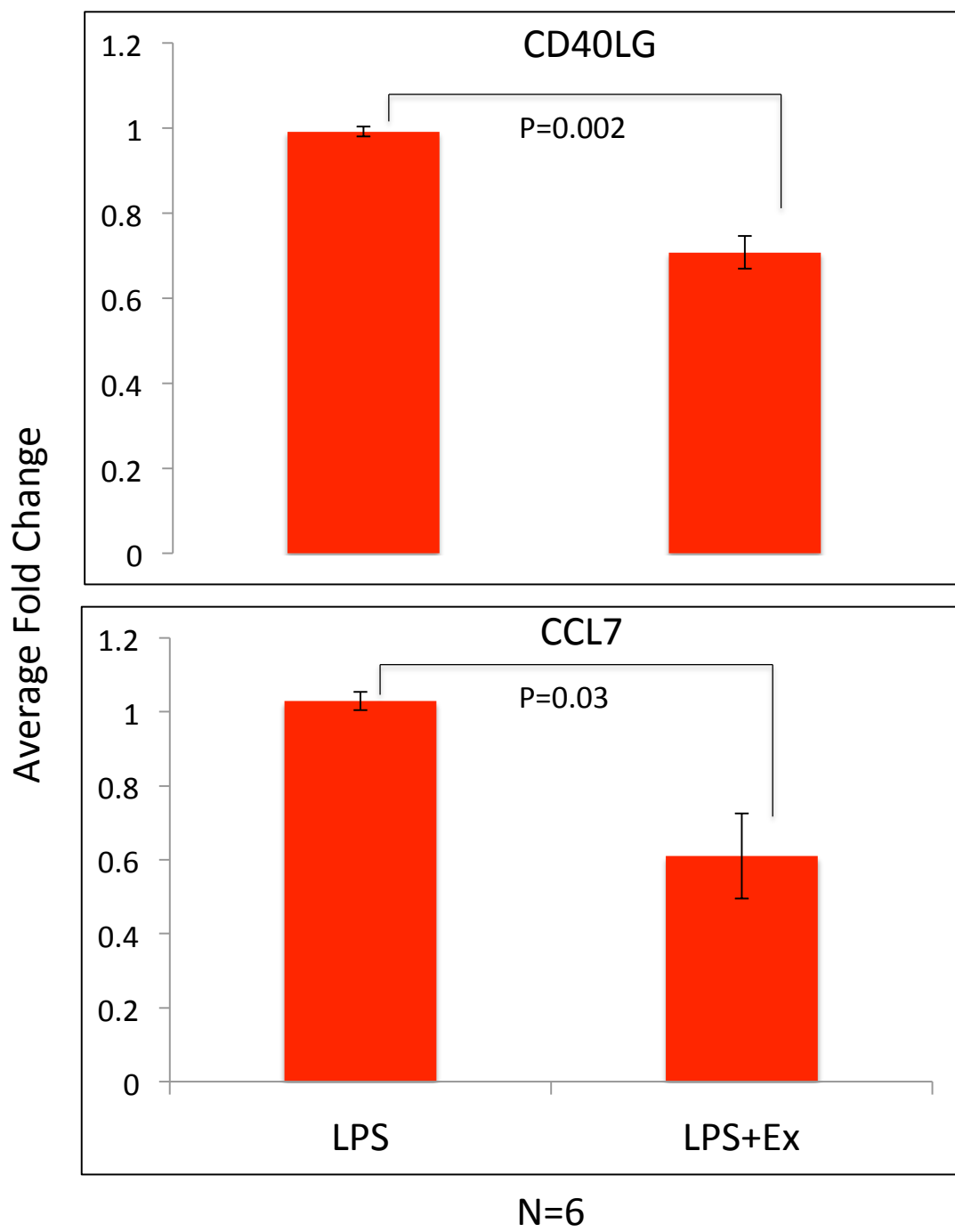


Figure 4.5: Treatment of Human Dendritic Cells with Trophoblast-Derived Exosomes + LPS Significantly Decreases Expression of CCL7 and CD40LG When Compared to Dendritic Cells Treated with LPS Alone.

Dendritic cells (1.5×10^6 cells/condition, n=6) were treated with LPS (1 μ g/ml) or LPS (1 μ g/ml) + trophoblast-derived exosomes (20 μ g) for 18 hours. Expression of CCL7 and CD40LG mRNAs was significantly decreased in the dendritic cells treated with LPS + exosomes as compared to dendritic cells treated with only LPS (p=0.002 and 0.03, respectively).

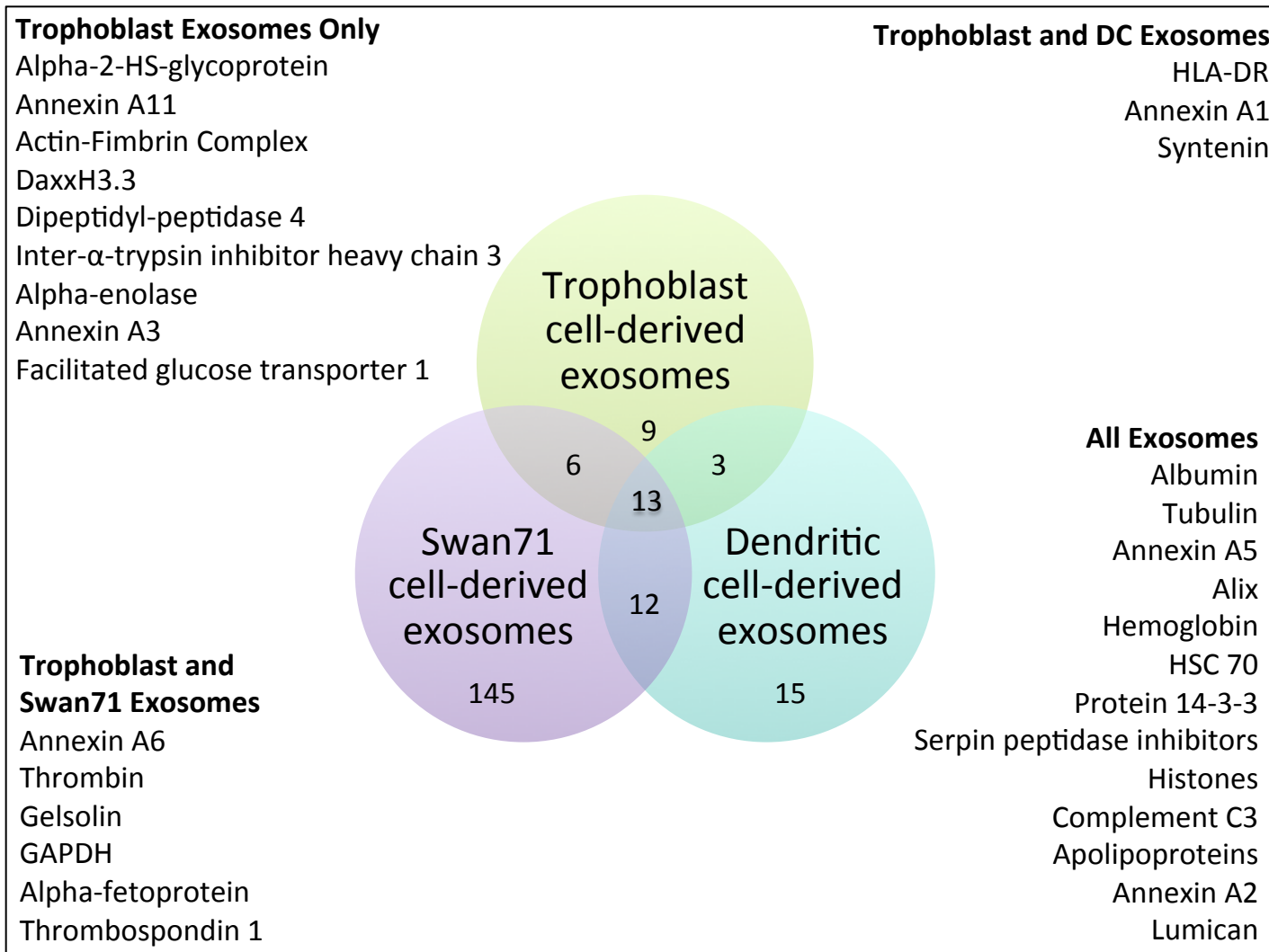


DISCUSSION

In this chapter we have shown that human dendritic cells can take up trophoblast-derived exosomes and microparticles and that trophoblast-derived exosomes contain a unique set of proteins and can affect dendritic cell phenotype. In order to determine how the protein contents of primary trophoblast-derived exosomes compare to other published descriptions of exosome contents, we compared the proteins found in our preparations of primary trophoblast-derived exosomes to published lists of proteins found in exosomes from Swan71 cells (Atay, Gercel-Taylor et al. 2011), a commonly used extravillous trophoblast cell line, and human dendritic cells (Thery, Boussac et al. 2001) (Fig. 4.6). In addition to their size and morphology (Figure 4.1 & 4.2), the trophoblast-derived exosomes contained a number of proteins identified as exosome markers, including ALIX, numerous Annexins, Hsc70 and Syntenin. Interactions between Syntenin and ALIX have recently been shown to be important for exosome biogenesis (Baietti, Zhang et al. 2012) and various Annexins and Hsc70 have been widely used as exosome markers in a variety of cell types and model systems (Hegmans, Bard et al. 2004, Mathivanan and Simpson 2009, Mathivanan, Fahner et al. 2012, Simpson, Kalra et al. 2012).

Figure 4.6: Comparison of Primary Trophoblast-Derived Exosomes to Swan71 and Dendritic Cell-derived Exosomes.

The protein contents of primary trophoblast-derived exosomes were compared to published lists of proteins found in exosomes from a commonly used extravillous trophoblast cell line, Swan-71, and primary human dendritic cells. Of the 32 proteins found in primary trophoblast-derived exosomes, 13 were found in all three exosome types, 3 were found in both primary trophoblast and dendritic cell-derived exosomes, 6 were found in both primary trophoblast and Swan71-derived exosomes and 9 were unique to the trophoblast-derived exosomes.



One of the most surprising findings was that the primary trophoblast-derived exosomes contained the Class II MHC, HLA-DR. It has long been known that trophoblast cells do not express Class II MHCs on the surface, presumably to avoid rejection of the placenta by the maternal immune system (Chatterjee-Hasrouni, Montgomery et al. 1983, Hunt and Hsi 1990, Murphy, Choi et al. 2004). However, there has been at least one report of intracellular, but not extracellular, HLA-DR in human trophoblast cells (Ranella, Vassiliadis et al. 2005) and researchers studying placental exosomes found in peripheral maternal blood have also reported presence of HLA-DR (Taylor, Akyol et al. 2006). Taken together, these results suggest that trophoblast-derived exosomes may contain HLA-DR, which could have important implications for maternal immune recognition of the fetus during pregnancy.

Intriguingly, other researchers have proposed a link between maternal and paternal HLA-DR and HLA-DQ disparity and a decrease in rheumatoid arthritis symptomology during pregnancy. This suggests a connection between maternal immune recognition of paternally - inherited fetal MHC Class II molecules and decreased maternal rheumatoid arthritis symptomology during pregnancy (Nelson, Hughes et al. 1993). However, how the maternal immune system could be exposed to paternally-inherited antigens during pregnancy has never been clearly defined. Our findings here suggest that trophoblast-derived exosomes could be a source of maternal immune exposure to paternal HLA-DR during pregnancy.

In addition, research by de Luca Brunori et al. has indicated that preeclampsia is significantly more common in pregnancies where there is a high level of similarity between maternal and paternal HLA-DR molecules (de Luca Brunori, Battini et al. 2000, de Luca Brunori, Battini et al. 2003). In these studies, the authors hypothesized that maternal immune

exposure to paternal HLA-DR likely occurred during sexual intercourse (de Luca Brunori, Battini et al. 2000, de Luca Brunori, Battini et al. 2003). Our findings that HLA-DR is expressed by purified term trophoblast-derived exosomes provides another possible explanation, that exposure to paternal HLA-DR occurs throughout the pregnancy and not just following exposure to paternal semen.

At least two other proteins found in the exosomes have been associated with pre-eclampsia. Alterations in expression levels of alpha-2-HS-glycoprotein (also known as Fetuin A) in trophoblast cells and in maternal serum have been associated with pre-eclampsia (Molvarec, Kalabay et al. 2009, Gomez, Anton et al. 2012). In addition, decreased levels of the anti-angiogenic factor, thrombospondin-1, in maternal circulation has been associated with HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome, a severe form of pre-eclampsia (Stenczer, Molvarec et al. 2012). In both cases, the connections between materials contained in exosomes from normal trophoblasts, the altered release of trophoblast debris and exosomes from the placenta in pre-eclampsia and the effects of the contents of these exosomes on maternal cells is unclear. However, we hope that by documenting the inclusion of these proteins in trophoblast-derived exosomes, we can come one step closer to understanding the intricate balance of released trophoblast-derived microparticles and the maternal immune system that is important for successful pregnancy outcomes (Chua, Wilkins et al. 1991, Knight, Redman et al. 1998, Goswami, Tannetta et al. 2006, Redman and Sargent 2007, Redman and Sargent 2008).

Interestingly, treatment of human dendritic cells with trophoblast exosomes lead to increased expression of both IL-8 and VCAM-1, both of which are closely associated with angiogenesis (Koch, Polverini et al. 1992, Fukushi, Ono et al. 2000, Simonini, Moscucci et al. 2000, Hou, Ryu et al. 2014). Recent work on the effects of exosomes derived from chronic

myelogenous leukemia cells on endothelial cells also showed increased expression of IL-8 and VCAM-1 (Taverna, Flugy et al. 2012). Studies in mice have indicated that uterine dendritic cells (uDCs) are required for successful pregnancy and that depletion of uDCs leads to embryo resorption. MRI studies in these mice indicated defective decidual vascular expansion, likely as a result of decreased release of molecules involved in angiogenesis from the uDCs (Plaks, Birnberg et al. 2008). Together, these data suggest that trophoblast-derived exosomes may be interacting with uDCs to increase the release of pro-angiogenic molecules important for a successful pregnancy.

Treatment of dendritic cells with trophoblast-derived exosomes also resulted in increased mRNA expression of the dendritic cell activation marker, CD80. Increased dendritic cell activation is associated with increased pro-inflammatory cytokine production, another component of pre-eclampsia (Benyo, Smarason et al. 2001, Hung, Charnock-Jones et al. 2004, Redman and Sargent 2004, Redman and Sargent 2005, Sharma, Norris et al. 2010, Szarka, Rigo et al. 2010). This may help explain the relationship between the increased release of placental microvesicles in pre-eclamptic as compared to control pregnancies and increases in pro-inflammatory cytokine production (Knight, Redman et al. 1998, Tannetta, Dragovic et al. 2013). Taken together, these findings may help elucidate a recently proposed hypothetical link between trophoblast-derived exosomes, spiral artery remodeling and preeclampsia pathogenesis (Salomon, Yee et al. 2014).

Interestingly, two of the proteins found in exosomes (Complement C3 and Gelsolin) have been implicated in modulating the maternal immune response to foreign pathogens. Expression of Complement C3 by first trimester trophoblast cells occurs in response to high levels of IFN-gamma, suggesting a potential role in trophoblast response to exogenous pathogens (Bulla, Bossi et al. 2009). Perhaps more intriguing is the possibility that the role of Gelsolin in binding LPS in

the amniotic fluid and decreasing LPS-mediated maternal immune system activation (Sezen, Bongiovanni et al. 2009), could also occur via exosome-associated Gelsolin, either directly at the maternal-fetal interface or in maternal peripheral blood during pregnancy.

This could explain, at least in part, the decreased dendritic cell mRNA expression of both CD40 LG and CCL7 in dendritic cells treated with LPS + trophoblast exosomes as compared to dendritic cells + LPS alone. Both CD40LG and CCL7 are important in dendritic cell activation and migration (de la Rosa, Longo et al. 2003, Gunn 2003) and by blocking LPS-induced up regulation of CCL7 and CD40LG, trophoblast-derived exosomes may help prevent dendritic cell activation and migration to lymph nodes, thus decreasing fetal antigen cross-presentation to maternal T-cells in the context of increased inflammation.

Taken together, these data provide compelling evidence that trophoblast-derived exosomes may have multiple effects on the maternal immune system, and may be involved in both the maintenance of normal pregnancy and the development of pregnancy complications, such as pre-eclampsia. More work is needed to clarify the causal relationships between trophoblast-derived exosomes and maternal immune responses.

CHAPTER 5:

GENERAL DISCUSSION

PREGNANCY AS AN IMMUNE PARADOX

In the early 1950s, Sir Peter Medawar proposed for the first time the idea that pregnancy posed a unique “immune paradox,” an idea that has provided the foundation for the field of reproductive immunology. Medawar put forth three potential explanations for why the genetically foreign fetus was not rejected by the maternal immune system: the fetus and the mother are physically separated; the fetus does not express the antigens necessary to elicit an immune response; and/or that the mother’s immune system does not respond to fetal antigens (Medawar 1954, Billington 2003). We now know that none of these theories completely explain the complex immunologic relationships between the mother and fetus during pregnancy. This dissertation seeks to clarify some of the relationships between the maternal and fetal immune systems; specifically, how fetal antigens are released into the maternal blood during both normal and preeclamptic pregnancies and how the maternal immune system responds to these antigens.

Numerous pregnancy complications, including preeclampsia, have been linked to placental dysfunction and increased activation of the maternal immune system (Benyo, Smarason et al. 2001, Goldman-Wohl and Yagel 2002, Redman and Sargent 2004, Germain, Sacks et al. 2007, Gilbert, Nijland et al. 2008, Gilbert, Ryan et al. 2008, Kronborg, Gjedsted et al. 2011, Lee, Romero et al. 2012). Preeclampsia affects 5-8% of pregnancies worldwide (Duley 2009) and, if untreated, can lead to maternal kidney dysfunction, multi-system organ failure, and even death of both the mother and infant. Currently, the only successful treatment for preeclampsia is delivery of the placenta, making preeclampsia one of the leading causes of iatrogenic prematurity (Pennington, Schlitt et al. 2012). However, the precise role of the placenta in both the manifestation and continued pathogenesis of preeclampsia has yet to be definitively determined.

EXPRESSION OF MINOR HISTOCOMPATIBILITY ANTIGENS IN THE HUMAN PLACENTA

In this work, we have shown that that the placenta expresses at least six known fetal minor histocompatibility antigens (mHAgs) and that both shed trophoblast debris and fetal leukocytes may act as sources of fetal antigen exposure for the maternal immune system during pregnancy (Holland, Linscheid et al. 2012). This discovery provides new insights into the sources of fetal antigens during pregnancy and is a significant contribution to the increasing body of literature showing that maternal T-cells specific for fetal antigens are expanded during pregnancy and can persist for many years following parturition (James, Chai et al. 2003, Piper, McLarnon et al. 2007, Moldenhauer, Hayball et al. 2010).

This finding has important implications for both pregnancy and transplantation immunology, where mHAgs have been shown to be important for graft-versus-tumor effects as well as graft-versus-host disease (Randolph, Gooley et al. 2004, Takami, Sugimori et al. 2004, Loren, Bunin et al. 2006). By better understanding the mechanisms by which mHAgs are regulated in both grafted tissues and in the placenta, as well as the different immune molecules governing activation vs. tolerance to mHAgs, we can gain important insights into the mechanisms by which tumors, transplants and fetuses are either rejected or tolerated by the immune system. Chapter 2 provides important insights into the localization of mHAgs in the placenta and the importance of the placenta in modulating maternal immune responses to the fetus during pregnancy.

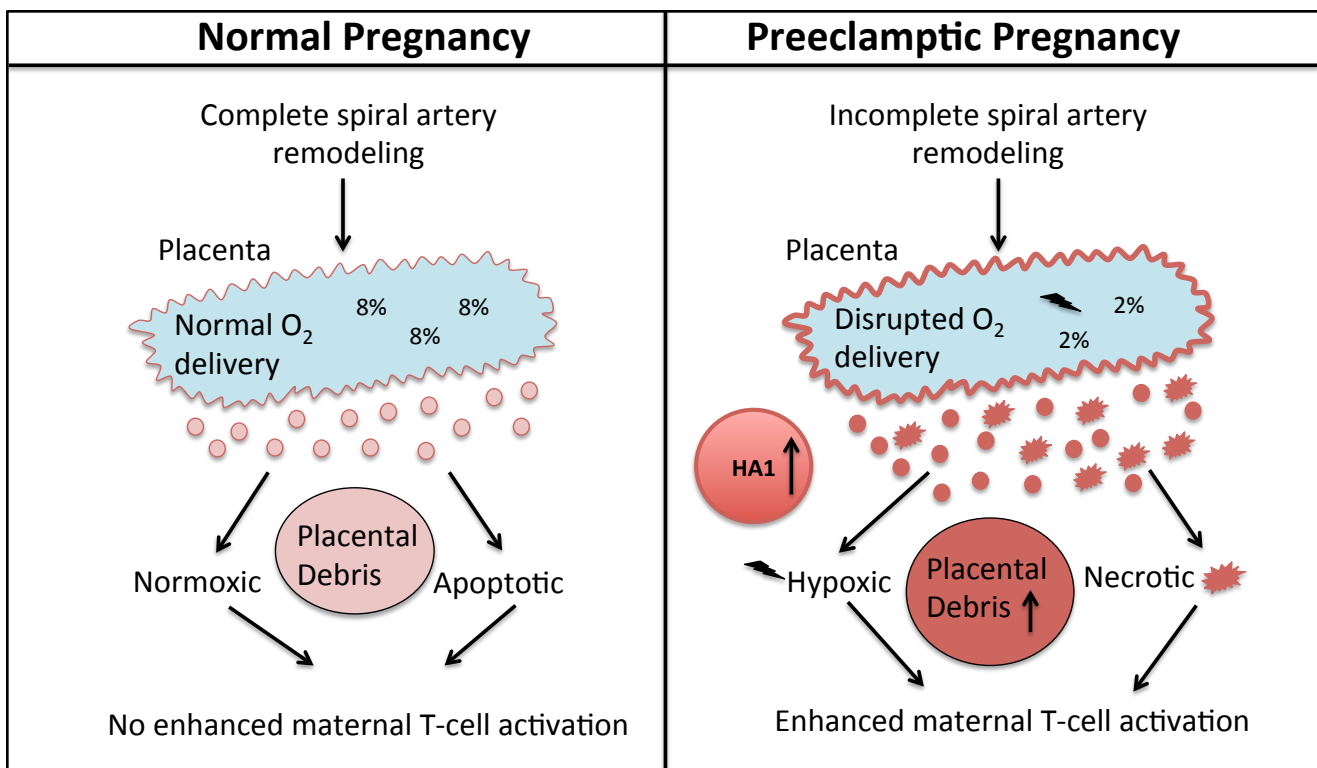
REGULATION OF FETAL MINOR HISTOCOMPATIBILITY ANTIGEN EXPRESSION

In addition to showing that fetal mHAgs are expressed in the placenta, this work also demonstrates that expression of least one mHAg, HA-1, decreases with increasing gestational

age (Figure 3.1), is regulated by oxygen (Figures 3.2 and 3.3) and is increased in the placentas of women with preeclampsia as compared to control placentas (Figure 3.4). We have hypothesized that increased expression of HA-1, and potentially other mHAGs, in concordance with the increased release of syncytiotrophoblast debris and production of proinflammatory cytokines (Redman and Sargent 2001, Redman and Sargent 2003, Redman and Sargent 2004, Redman and Sargent 2005), both characteristic of preeclampsia, could shift the maternal immune system from a more tolerogenic to a more activated phenotype (Figure 5.1).

Figure 5.1: A Model of Increased HA-1 Expression and Maternal Immune Activation in Normal vs. Preeclamptic Pregnancies.

Preeclampsia is characterized by impaired remodeling of the maternal spiral arteries, leading to altered blood flow to the placenta and areas of ischemia/reperfusion. This altered oxygen delivery leads to increased expression of the minor histocompatibility antigen, HA-1, as well as increased release of necrotic trophoblast debris into the maternal blood supply. Together, these factors may contribute to the enhanced maternal immune system activation seen in preeclampsia as compared to normal pregnancies.



In addition to a potential role in the altered maternal immune responses that are characteristic of preeclampsia, the altered expression of HA-1 in hypoxic tissues may provide important information for transplant rejection, where the graft may express higher levels of HA-1 and possibly other antigens as a result of decreased blood flow, which often occurs in grafted tissues following transplantation (Rosenberger, Mandriota et al. 2002). In Chapter 3, we showed that increased expression of HA-1 in response to low oxygen is likely mediated by the transcription factor, Hypoxia Inducible Factor-1 α (HIF-1 α), which is increased in both preeclamptic placentas (Caniggia and Winter 2002) and in transplanted tissues, where it is also correlated with an increased risk of rejection (Rosenberger, Pratschke et al. 2007). Taken together, these findings suggest a possible role for altered blood flow, increased antigen expression and altered immune responses in both preeclamptic pregnancies and in the rejection of transplanted tissues.

TROPHOBLAST-DERIVED EXOSOMES AND THE MATERNAL IMMUNE RESPONSE

In addition to the expression and regulation of fetal mHAGs in the placenta, we also found evidence that one of the classical MHC Class II molecules, HLA-DR, that is not expressed on the surface of the placenta, presumably to avoid maternal immune detection, may have another route of maternal immune exposure via release of exosomes from trophoblast cells (Chapter 4). Major Histocompatibility Complexes (MHC or HLAs, in humans) are the structures on the surface of cells that are responsible for self versus non-self discrimination and the presentation of antigens, whereas minor histocompatibility antigens (mHAGs) are numerous proteins with a variety of different functions that can be presented by specific MHCs and can elicit immune responses due to differences between individuals, typically single nucleotide polymorphisms (SNPs), allelic insertions or deletions, or presence on the Y-chromosome

(Goulmy 2006). For example, certain mHAgS, including the Y-chromosome encoded antigens DDX3Y and RPS4Y1, can be presented by specific HLA-DRs (Spierings, Vermeulen et al. 2003, Zorn, Miklos et al. 2004).

Our finding that HLA-DR is expressed in exosomes derived from purified trophoblast cells (Table 4.1, Figure 4.6) was surprising, given the historical assertion that the surface of the placenta does not express the classical MHC molecules (Chatterjee-Hasrouni, Montgomery et al. 1983, Hunt and Hsi 1990). However, this discovery is in agreement with work by other researchers indicating that HLA-DR is expressed inside trophoblast cells, but not on the surface, and that placental exosomes found in peripheral maternal blood during pregnancies also contain HLA-DR (Ranella, Vassiliadis et al. 2005, Taylor, Akyol et al. 2006). Taken together, our findings that mHAgS are expressed in the placenta (Chapter 2), that at least one mHAg, HA-1, is regulated by oxygen and is increased in preeclampsia (Chapter 3) and that exosomes from trophoblast cells contain HLA-DR (Chapter 4), it is clear that the fetus is neither entirely physically separate from the mother, nor is the fetoplacental unit entirely lacking in potentially antigenic materials, two of the original hypotheses put for by Medawar (Medawar 1954, Billington 2003). This leaves us with examination of Medawar's third hypothesis; that the maternal immune system is unable to respond to fetal/placental antigens.

Work by other researchers on exosomes, small cup-shaped nanovesicles containing microRNAs, mRNAs and proteins, suggests that they can have potent immunomodulatory effects (Raposo, Nijman et al. 1996, Bodey, Bodey et al. 1997, Skokos, Le Panse et al. 2001, Clayton, Mitchell et al. 2008). In order to determine the effects of trophoblast-derived exosomes in the context of maternal immune recognition of fetal antigens, we decided to investigate the response of dendritic cells, which are critically important for cross-presentation of antigens to T-cells, to exosomes from cultured primary trophoblast cells. Using qRT-PCR, we found that treatment of

human dendritic cells with trophoblast exosomes lead to upregulation of CD-80, IL-8 and VCAM-1 mRNAs (Figure 4.4). CD-80 is a marker of dendritic cell activation, whereas IL-8 and VCAM-1 are both associated with angiogenesis (Koch, Polverini et al. 1992, Fukushi, Ono et al. 2000, Simonini, Moscucci et al. 2000, Hou, Ryu et al. 2014). This is particularly interesting given work by other researchers indicating that defective spiral artery remodeling, a process that is similar to angiogenesis, is implicated in the development of preeclampsia (Lockwood, Huang et al. 2011, Plaisier 2011).

When dendritic cells were treated with trophoblast-derived exosomes in the presence of the pro-inflammatory molecule, LPS, exosomes lead to decreased mRNA expression of CD40LG and CCL7 (Figure 4.5), both of which are involved in dendritic cell activation and migration (de la Rosa, Longo et al. 2003, Gunn 2003). Notably, the dendritic cell mRNAs that were altered by the presence of trophoblast-derived exosomes were different in the untreated cells as compared to those treated with LPS, suggesting that there may be multiple factors in the exosomes that influence dendritic cell phenotype depending on the surrounding immune environment.

CONCLUSIONS

These data clearly indicate that trophoblast cells produce exosomes that can influence maternal immune cell phenotype, indicating that the maternal immune system does, in fact, respond to materials released from the placenta during pregnancy, some of which express fetal antigens. This is a significant contribution to the wider field of placental immunology, where many other researchers have previously shown that the maternal immune system responds to the fetus and placenta in a variety of different ways (Tafari, Alferink et al. 1995, Jiang and Vacchio 1998, Petroff, Chen et al. 2003, Aluvihare, Kallikourdis et al. 2004, Hiby, Walker et al. 2004, Erlebacher 2010, Hiby, Apps et al. 2010) and that the immune relationship during pregnancy is a

complex and dynamic interplay of both fetal and maternal signals, rather than a lack of interaction, as was originally proposed by Medawar in his transformative treatise that gave rise to the field of placental immunology (Medawar 1954). Given the rapid expansion of this field of study, our findings that the placenta expresses fetal mHAgS (Chapter 2), that at least one of these antigens is regulated by oxygen and upregulated in preeclampsia (Chapter 3), and that trophoblast-derived exosomes contain a number of immune molecules, including HLA-DR, and can alter maternal dendritic cell phenotype (Chapter 4), are significant immunological advances and may provide the foundation for future investigations in the field of placental immunology and, potentially, beyond.

CHAPTER 6:

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