Adhesion molecule expression by peripheral blood monocytes and regulation of monocyte adhesion to decidual endothelial cells in normal and in type I diabetic pregnancy

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Declaration

The work in this thesis is original research performed by the author in the Perinatal and Renal Research laboratories in the Kolling Institute of Medical Research, University of Sydney at Royal North Shore Hospital, St Leonards, Australia. The Work described in this thesis has not been submitted to any other institution for a higher degree

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Abstract

Background: Pregnant women with Type 1 diabetes mellitus develop vascular lesions similar to atheroma in the placental bed. These lesions are characterised by an accumulation of activated macrophages around spiral arteries, where they can stimulate a vigorous inflammatory reaction and impair vascular integrity and threaten blood flow. Diabetic pregnancies carry a high risk of development and rapid progression of preeclampsia and decreased placental blood flow causing fetal growth restriction.

In the development of the vascular lesions, peripheral blood monocytes are recruited, adhere to endothelium and migrate to subendothelial space where they initiate an inflammatory response. These events are mediated by surface adhesion molecules on both monocytes and endothelial cells. Earlier work from our research group had shown increased *in vitro* adherence of monocytes from diabetic pregnancy to decidual endothelial cells from normal pregnancies,

Experiments described in this thesis explore further the mechanisms subserving this abnormal monocyte adherence to decidual endothelium in diabetic pregnancy. After initial studies comparing adhesion molecule expression by monocytes from normal and diabetic pregnancies, the role of monocyte-derived Mac-1 in monocyte-decidual endothelial cell adherence was further investigated by the use of a blocking antibody.

Methods: Mixed peripheral blood mononuclear cells from three groups of pregnant 3rd trimester subjects, (i) normal, (ii) gestational diabetes, and (iii) type I diabetes, were isolated by density gradient centrifugation through Ficoll-Paque. Monocytes were identified (CD 14+) and their adhesion molecule expression (LFA-1, Mac-1, VLA-4) determined by double staining flow cytometry.

DECs were isolated from decidual biopsies collected from normally pregnant women at elective caesarean section. For subsequent flow cytometry, DECs were identified CD45-, and their ICAM-1 expression was measured by double staining flow cytometry. A highly enriched population of monocytes was then isolated (by density gradient centrifugation through iodixanol) from the blood of normally and type I

diabetic pregnant women. Monocytes underwent incubation under basal and stimulated conditions followed by a further incubation with or without a blocking antibody to Mac-1. Monocytes were then co-cultured with DECs, and their adhesion and expression of Mac-1 were assessed.

Results: Monocytes from type I diabetic pregnancies displayed increased expression of Mac-1 and LFA-1 but not VLA-4. Adhesion molecule expression in gestational diabetes was between normal and type 1 diabetes. There was no significant direct correlation with adequacy of diabetic control as assessed by concomitantly measured blood glucose or HbA1c levels across the three groups in this study. LPS pre-treatment of monocytes from normal pregnancy caused an increase in Mac-1 expression and was associated with an increase in their adherence to normal DECs. ICAM-1 expression by normal DECs was stimulated by the presence of adherent monocytes, but no further increase in this ICAM-1 expression was seen when the monocytes had been pre-stimulated by LPS.

Exposure of monocytes from normal pregnancy to high levels of glucose also caused an increase in Mac-1 expression, associated with an increase in their adherence to normal DECs. The cause-and-effect nature of this Mac-1 expression was shown by monocyte pre-treatment with a Mac-1 monoclonal antibody, which prevented their adhesion to DECs in both basal and stimulated conditions, both in normal and in type I diabetic pregnancy.

Conclusion: The increased adhesion molecule expression of monocytes from type I diabetic pregnancy is paralleled by their increased adhesion to decidual endothelium, and may be causally involved in production of placental bed vascular disease in diabetic pregnancy .Pre-treatment of monocytes with stimuli that increased Mac-1 expression resulted in an increase in their adhesion to decidual endothelium, an effect prevented by specific Mac-1 antibody blockade. The experiments conducted in this study examine only a part of the adhesion interaction of monocytes and decidual endothelial cells. While monocyte Mac-1 expression is clearly of central importance, other factors are involved, some resulting from the initiation of monocyte adherence, such as the observed DEC-derived ICAM-1 which may amplify the adhesion. Other

adhesion molecules could be involved, as could be the release of chemo-attractant peptides.

These results add significantly to our earlier report of increased adhesion of monocytes from pregnant woman with type I diabetes to normal decidual endothelial cells, and provide evidence related to the mechanisms subserving this increased adhesion.

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Dedication

This thesis is dedicated to the memory of "my parents, my mother Rouhaifa Mohsen and Father Mohamed" and their unconditional love that they bestole upon myself.

A tragedy befell our immediate family in my boyhood youth, My mother developed type I diabetes and severe complications. My mother had significant influence in my upbringing. I witnessed her efforts and pains to support financially and morally my brothers Khaled and Omar and all my sisters.

As the youngest child in my family ,the time I did spend with my mother **Rouhaifa** (71 y.o) and father **Mohamed** (83 y.o) was precious and memorable. I have spent whole my life far away from my parents, but my spirit and soul is always around them.

My mother just didn't want us to get by, she wanted the best she could provide for her sons and daughters. So, after developing type I diabetes, she was still deeply involved in providing all of us with all the support by all means.

Morbidity of those close to us, particularly my parents seem to bind our family ties stronger and instill the importance of remembering the past, particularly mother Rouhaifa and Father Mohamed past, and their sacrifices for us.

Within the last four years after my mother had developed an ischemic stroke, and while preparing to start the AMC examination , I was fortunate to be given the opportunity to do a medical research project in diabetes, hoping to achieve more understanding in the development of the vascular disease in diabetes. This research work is only the beginning and soon I will start the medical internship, and after that I will try my best to be involved in diabetic work (clinical and research) and I hope that one day to be able to match between clinical work and research activities in diabetes to help diabetic patients and reduce their suffering.

Oh..Mother... I know you are expecting much more from me, but I swear in God that I tried my best despite the hardships and difficulties, that I have experienced during this candidature... you are always in my mind, particularly when performing the experiments... I love you mother and I promise to make life easier to my kids Rouhaifa and Fouad, to enable them to achieve better results in medical research in diabetes.

Thank You

This work would not be possible without the loving support of so many people, As a man usually too full of words, I find myself overwhelmed in offering them all my thanks in dedicating this book to them.

To my wife, **Nesrene,** who, after 8 years of love and support - 7 years of married life together - is still my strength and purpose in life.

I love you, **Nesrene,** now and forever. You have gone through a very tough time and carried most of the burden so that I didn't have to. How do I say it, except: ***"Thank you, my love!"***

Last step in our long hard battle is coming very soon “AMC Clinical examination” be prepared!

To a loving sister **Hend,** that just never stopped giving of herself in countless ways, both direct and indirect, thank you sister.

To my sister **Fida** and mother in law **Ziena,** thank you for moral support.

To my brother in Law **Abou Rabih,** thank you for support.

To my children, Rouhaifa and Fouad thanks Good for your presence in my life....

Thank you all

Thank You

This work would not be possible without the loving support of so many people, As a man usually too full of words, I find myself overwhelmed in offering them all my thanks in dedicating this book to them.

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Last step in our long hard battle is coming very soon " AMC Clinical examination", and no more drama..

To a loving sister Hend, that just never stopped giving of herself in countless ways, both direct and indirect, thank you sister

To my children, Rouhaifa and Fouad

Abstracts Arising From This Thesis

Abstract presented at RNSH/UTS/USYD Annual Scientific Research Meeting Sydney 2006

Karime B, Xie L, Morris J, McCracken S, Jackson C, Gallery E, 2006

BLOCKING MAC-1 ON HUMAN MONOCYTES OR ICAM-1 ON DECIDUAL ENDOTHELIAL CELLS DECREASED PERIPHERAL BLOOD MONOCYTES ADHESION TO ENDOTHELIAL CELLS IN NORMAL AND TYPE I DIABETIC PREGNANCY

Abstract presented at Perinatal Society of Australia and New Zealand Adelaide 2005

Karime B, Xie L, Morris J, McCracken S, Jackson C, Gallery E, 2005.

DECIDUAL ENDOTHELIAL CELL INTERACTIONS WITH PERIPHERAL BLOOD MONOCYTES IN NORMAL AND TYPE 1 DIABETIC HUMAN PREGNANCY

Abstract presented at RNSH/UTS/USYD Annual Scientific Research Meeting Sydney 2004

Karime Bilal, McCracken Sharon, Gallery Eileen, Morris Jonathan, 2004.

THE ROLE OF THE PERIPHERAL BLOOD MONOCYTE IN ATHEROSIS IN THE PLACENTAL BED VASCULAR DISEASE OF DIABETIC PREGNANCY

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AGE	Advanced glycation end products
ADCC	Antibody dependent cell mediated cytotoxicity
AP	Activation protein-1
Anti	Antibodies
Bp	Blood pressure
BSA	Bovine serum albumin
BSA.PBS	Phosphate buffered saline with added bovine serum
C	Cytosine
CRP	C reactive protein
CCE	Counter current elutriation
Cm	Centimetre
CTL	Cytotoxic T lymphocytes
D	Dilution factor
DC	Dendritic cells
DAB	3, 3 – Diaminobenzidine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylenediaminetetraacetic dihydrate
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
ENOS	Endothelial nitic oxide synthase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
fMLP	N formyl methionyl leucylphenylalanine
G	Grams
GAD	Anti glutamic acid decarboxylase
GDM	Gestational diabetes
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hanks balanced salt solution
HCG	Human chorionic gonadotropin
Hrs	Hours
ICAM	Intercellular adhesion molecules
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
LFA-1	Leucocyte function associated antigen
LPS	Lipopolysacharide
LDL	Low density lipoprotein
LRP	Leucocyte-rich plasma

M	Molarity
MPS	Mononuclear phagocyte system
MHC	Major histocompatibility complex
M-CSF	Macrophage colony stimulating factor
MCP-1	Macrophage chemotactic protein-1
MPO	Myeloperoxidase
MFI	Mean fluorescence intensity
Mg	Milligram
Min	Minutes
Ml	Mililiter
Mmol	Milimole
MMP	Matrix metalloproteinase
Mo	Monocyte
N	Number
NaCL	Sodium chloride
NK	Natural killer
NP	Normal pregnancy
OKM1	Monocyte specific surface antigen
PGE	Prostaglandin E
PMS	Pooled maternal serum
PAMPs	Pathogen-associated molecular patterns
POVPC Phosphorylcholine	Palmytoyl 1-2 (5-oxo-valeroyl)-sn glycerol-3
PDAY	Pathobiological Determinants of Atherosclerosis
	in Youth study
PPARs	Peroxisome proliferator-activated receptors
PBMC	Peripheral blood mononuclear cells
PFA	Paraformaldehyde
PE	Phycoerythin
PBS	Phosphate buffered saline
RNSH	Royal North Shore Hospital
Rpm	Revolutions per minute
SMC	Smooth muscle cells
SSC	Side scatter
TLRs	Toll-like receptors
TIMPs	Tissue inhibitors of metalloproteinases
TNF	Tumor necros factor
TBS	Tris- Borate- EDTA buffer
T1DM	Type I diabetes mellitus
U	International unit
UEA I	Ulex europaeus I .

V/v	Volume per unit volume
VCAM	Vascular cell adhesion molecule
VALT	Vascular associated lymphoid tissue
VLA-4	Very late antigen
W/v	Weight per unit volume

Chapter I

Literature Review

1. Introduction

The studies described in this thesis, conducted in cells obtained from pregnant human subjects, examine the relationship between endothelial cells of the placental bed and the circulating precursors of their surrounding macrophages, peripheral blood monocytes. The overall aim of these studies was to clarify the patho-physiological processes that result in the atheromatous vascular pathology seen in the placental bed in pregnancies complicated by diabetes mellitus.

As background to this work, there follows a review of pertinent literature, including:

(i) A summary of development of the early placenta.

(ii) A review of monocyte function, with particular reference to their adhesion molecule production

(iii) A description of the lesions of atheroma, and of the potential involvement of the immune system, in particular monocytes, in their production.

(iv) An outline of the increase in such vascular pathology seen in diabetes.

(v) Aspects of diabetes and the immune system in pregnancy

1.1 Development of the Placenta

The placenta is the fetus's extension into the mother, and functions as the crossing point between the two. It is a vital and exceptional organ, which allows cells of fetal origin to come into direct contact with maternal blood, to facilitate exchange of oxygen and carbon dioxide, to provide nutrients required for fetal growth and development, and to remove fetal waste products.

The principal cells of the human placenta –the trophoblasts– first appear four to seven days after fertilization as the outer layer of cells of the blastocyst. These early blastocyst trophoblasts differentiate into all other cell types found in the human placenta. Trophoblasts are crucial for successful pregnancy, by mediating such steps as implantation, hormone production, immune protection of the fetus, and regulation of maternal vascular blood flow into the placenta (Moore, 1993).

1.1.1 Early Development

Within a few days of fertilization the embryo develops into a blastocyst (Figure 1.1), a globular structure composed on the outside of trophoblasts and on the inside of an assembly of cells called the inner cell mass (Figure 1.1). The inner cell mass will eventually develop into the fetus. In addition to making human chorionic gonadotrophin (hCG), trophoblasts mediate the implantation process by first attaching to, and then invading into the endometrium. (Kliman, 1994, 1986)

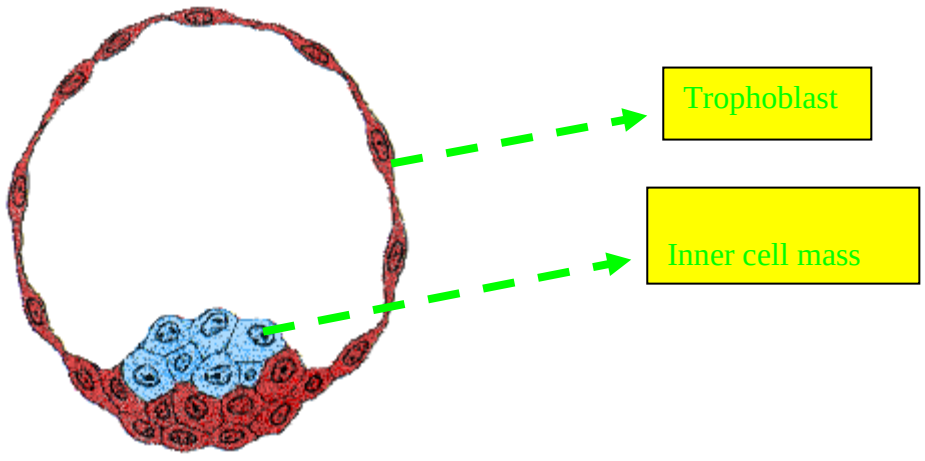

Figure 1.1: Blastocyst

The most difficult task for the floating blastocyst is to become attached to the lining of the uterine cavity (endometrium). The blastocyst is initially held in place by specific long molecules that extend from the endometrium (mucins), followed by a flow of molecules that convey the trophoblasts into closer and closer contact with the endometrium. Once intimate contact is made trophoblasts invade into the endometrium, beginning the process of placentation.

1.1.2 Formation of the early placenta

By nine days after fertilization the blastocyst (embryo) is surrounded by two layers of trophoblasts: the inner mononuclear cytotrophoblasts and the outer multinucleated syncytiotrophoblast layer, both of which remain throughout gestation. During the first 2 weeks of development, the syncytiotrophoblast forms lacunae that encircle the blastocyst. At the same time, they penetrate the endometrial blood vessels, to form inter-trophoblastic maternal blood filled sinuses, which surround the growing trophoblasts.

On day 13 the primitive villous structure appears, and the basic structure of the villous tree is established, which continues to develop throughout gestation (Figure 1.1.2) (Sadler, 1990)

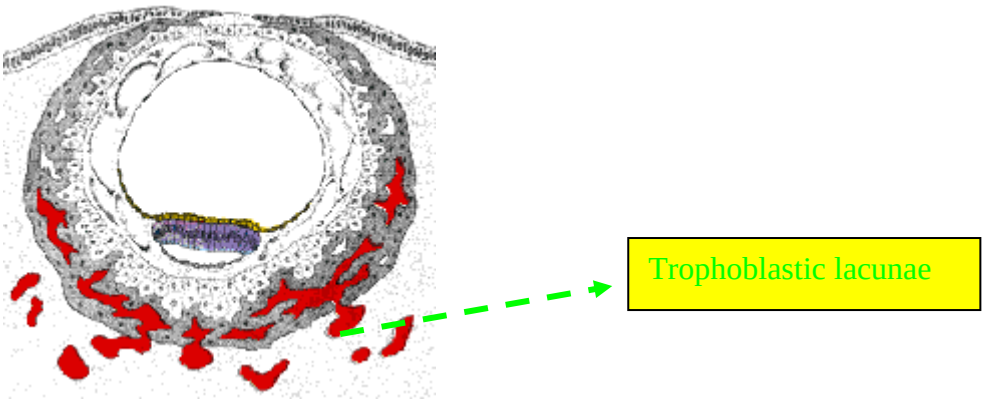

Figure 1.2: Two week implantation site

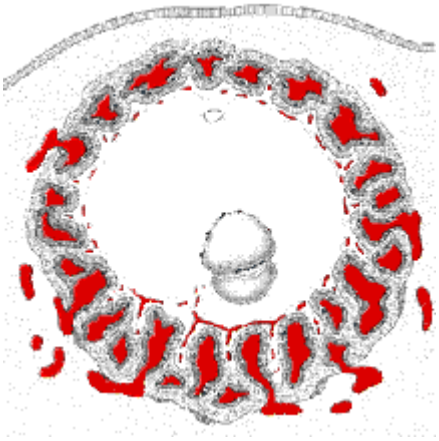

Figure 1.3: Three week implantation site

Within the placenta the syncytiotrophoblasts produce high levels of hCG which shifts cytotrophoblast differentiation towards a non-invasive, hormone secreting, villous-type cell. The nearer the trophoblasts are to the endometrium the less hCG is made, allowing the trophoblasts to differentiate into anchoring type cells which formulate the placental 'glue', the protein trophouteronectin. Trophoblasts that leave the placenta and migrate within the endo- and myometrium are induced to make proteases and protease inhibitors, presumably to facilitate trophoblast regulation of invasion into maternal tissues (Lee, 1986).

The trophoblastic shell continues to grow and proliferate to increase the surface area for maternal-fetal interchange and allow floating villi to bathe directly in maternal blood, and start chorionic villi formation (Loke, 1990). By week 3 embryonic tissue and maternal blood are separated by a layer of cytotrophoblasts and syncytiotrophoblasts. During that time the villous syncytiotrophoblasts secrete vital hormones such as progesterone, estrogen and hCG, hormones critical for maintaining pregnancy (Conley and Mason, 1990)

The chorionic villi next to the maternal blood supply will continue to develop and enlarge into an accumulation of chorionic tissue, which we categorize as the placenta. The chorionic villi farthest away from the maternal blood supply are gradually pushed into the uterine hollow by the growing amnionic sac which surrounds the embryo. These villi eventually degenerate and form the chorionic layer of the external membranes.

At around 12 to 14 weeks of gestation the combined amnion—chorion membrane makes contact with the opposite side of the uterus, where it fuses with the decidualised maternal endometrium and the extra embryonic coelom is usually obliterated by the expanding amnion by 10 to 11 weeks.

Figure 1.4: Terminal chorionic villus.

The fetal circulation branches until it reaches the capillaries of the chorionic villi where exchange of nutrients takes place between the mother and fetus.
Associated with the general expansion of placental construction is the differentiation of three different trophoblast types (Kliman, 1986) (Kurman, 1984). Depending on their eventual function *in vivo*, undifferentiated cytotrophoblasts can expand into the following:

1) Hormonally active villous syncytiotrophoblasts, that secrete the majority of placental hormones, such as hCG, which is essential to early pregnancy since it rescues the corpus luteum from involution, thus maintaining progesterone production by the ovarian granulosa cells.(Conley and Mason, 1990)

2) Extravillous anchoring trophoblastic cell columns. They form the second type of trophoblast–the junctional trophoblast. These cells form the anchoring cell columns that can be seen at the junction of the placenta and endometrium throughout gestation.
3) Invasive intermediate trophoblasts within the villi of the human placenta. There always exists a population of cytotrophoblasts which remain undifferentiated and available for differentiation as necessary.

Biologically, trophoblast-mediated vascular remodelling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. Abnormalities in this invasive process have been correlated with early and mid-trimester pregnancy loss, vascular disease, preeclampsia and eclampsia, and intrauterine growth restriction

1.1.3 Maternal and Fetal cells - genetically and immunologically different.

The body's immune system is unique in its ability to differentiate between the body's own tissues, and unfamiliar invaders, such as micro-organisms (Paul, 1993). This 'self' versus 'non-self' discrimination is best illustrated by the clinical outcomes of solid organ transplantation. Donor grafts that are matched to the recipient's "tissue types" result in successful transplantation, whereas mismatched transplants are rejected. Rejection is mediated by highly polymorphic molecules encoded in a genetic region termed the major histocompatibility complex (MHC) or HLA complex in humans. Transplant rejection is primarily mediated by T lymphocytes that are also responsible for cell-mediated immunity. T cells can recognize molecules encoded within the MHC, such as MHC class I or class II molecules, and can recognize and be stimulated by foreign MHC molecules expressed on the transplanted tissue, resulting in rejection.

Generally, fetal trophoblast cells are in contact with the maternal circulation and should be subject to attack by circulating maternal T cells if paternal MHC molecules are perceived as foreign. One obvious violation of the classic transplantation paradigm is the case of a mother's successful ability to maintain a fetus in the womb. This maternal–fetal "tolerance" is such a notable exception that it has evaded most attempts to explain it. Among the possibilities are that the uterus is an immunologically advantaged site, protected from the immune system, or that maternal immune responses are immunosuppressed during pregnancy (Janeway, 1996, 2002) (Tafuri, 1995).

Studies over the last few years have shown that maternal–fetal tolerance may be due to a specific and direct interaction (or lack thereof) between fetal and maternal cells Rouas and colleagues (Rouas, 1997) provided new evidence involving an MHC class I-like molecule termed HLA-G and natural killer (NK) cells, adding significantly to an emerging literature that implicates these seemingly unrelated molecules and cells.

1.2 The Immune system

1.2.1 Introduction

The human immune system is a complex set of pathways that allow the human body to react to foreign invaders such as infectious agents or antigenically different cells. The human immune system is divided broadly into innate and adaptive arms, described in more detail below.

1.2.2 The innate immune system

The innate arm of the immune system is an ancient, well conserved protective pathway, not targeted to specific pathogens, but primed for immediate broad range defence. It recognizes a range of pathogen-associated molecular patterns present in many different micro-organisms. These include lipopolysaccharide of the gram-negative bacterial cell wall, peptidoglycans, lipotechoic acids from the gram-positive bacterial cell wall, the sugar mannose (common in microbial glycolipids and glycoproteins but rare in humans), bacterial DNA, N-formylmethionine found in

bacterial proteins, double-stranded RNA from viruses, and glucans from fungal cell walls. (Borghnas, 1999, Medzhitov R. 2001).

The innate immune responses involve:

(i) phagocytic cells (neutrophils, monocytes, and macrophages),

(ii) cells that release inflammatory mediators (basophiles, mast cells, and eosinophils), natural killer cells (NK cells);

(iii) molecules such as complement proteins and cytokines.

A detailed consideration of all of these components is outside the scope of the present body of work. Since the studies performed examined aspects of the involvement of monocytes/macrophages in development of placental bed vascular pathology in diabetic pregnancy, there follows a summary of the function and role of cells of this lineage.

1.2.2.1 Phagocytic cells

Monocytes and macrophages are classified as cells of the reticulo-endothelial system (RES). Early in the 19th century, Van Furth anticipated the mononuclear phagocyte system (MPS), and monocytes and macrophages were recognised as basic cell types of this system. Their maturity takes place in the bone marrow and passes through the following steps: stem cell - committed stem cell - monoblast - promonocyte - monocyte (bone marrow) - monocyte (peripheral blood) - macrophage (tissues).
Monocyte differention in the bone marrow proceeds rapidly (1.5 to 3 days). During differentiation, granules are formed in monocyte cytoplasm and these can be divided into at least two types.

Blood monocytes are young cells that possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (Fc$^{\gamma}$R) and iC3b complement. Monocytes go through additional differentiation (at least one day in

duration) to become multifunctional tissue macrophages. Monocytes are generally, therefore, considered to be immature macrophages. However, it can be argued that monocytes represent the circulating macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration. (Aschoff, 1924).

Macrophages can be separated into normal and inflammatory macrophages. Normal macrophages are found in several sites, including connective tissue (histiocytes), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), liver (Kupffer's cells), lung (alveolar macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages), and skin (histiocytes, Langerhans's cell). (Van Furth, 1971)

The macrophage population in a particular tissue is maintained by three mechanisms: arrival of monocytes from the circulating blood, local production and biological turnover. Under normal conditions, the renewal of tissue macrophages occurs through local proliferation of progenitor cells and not via monocyte influx. Tissue macrophages are relatively long-lived cells. Depending on the type of tissue, their viability ranges between 6 and 16 days.

1.2.2.2 Human peripheral blood monocyte subsets

Monocytes account for 3-8% of the circulating leucocyte population, and are the largest of the white cells (up to 20 μm) in diameter. They are highly motile and actively phagocytic. Monocytes are characterised by a large, eccentrically placed nucleus that stains less intensely than that of other leucocytes. Nuclear indentation becomes more pronounced as the cells mature, giving the cell a horseshoe appearance. The cytoplasm contains a variable number of ribosomes and polyribosomes, and monocytes are capable of continuous ribosomal activity. They are members of the human mononuclear phagocyte system, important for non-specific defence against pathogenic organisms and tumour surveillance. Various subtypes are diverse in phenotype and purpose, all generated from $CD34^+$ myeloid progenitors. Upstream in the myeloid lineage there exists a myelo-dendritic progenitor cell from which monocytes, granulocytes and CD14-derived dendritic cells all derive (Santiago, 1999).

In peripheral blood, it is possible to find not only mature monocytes and granulocytes but also precursor cells such as $CD34^+$ progenitor cells (Bender, 1991) (Mattern, 1999) that may experience additional differentiation depending on the local environment. When tissue damage occurs, cytokines and chemokines are produced and transport activation signals for the recruitment of different leukocytes to the inflammatory site (Scapini, 2002). Monocytes receiving such a stimulus enter the inflamed tissue and differentiate into macrophages (Qiao, 1996) (Wintergerst, 1998).

Akiyama distinguished a major population of so-called {regular monocytes} > 80% and a minor subset of {intermediate Mo} < 10% (Akiyama, 1983, 1985).
The regular monocytes are categorized by bigger size, higher expression of the monocyte-specific surface antigen OKM1 and higher accessory capacity in mitogen-induced T cell proliferation.

They exhibit higher peroxidase activity and higher antibody-dependent cell-mediated cytotoxicity (ADCC) than intermediate Mo, whereas the intermediate monocyte is more easily mobilized from extravascular reservoirs. The regular monocytes release a larger amount of prostaglandin E_2 (PGE_2), interleukin-1 (IL-1) and colony-stimulating factor (CSF) than intermediate monocytes, which produce more interferon-α (IFN-α) (Yasaka, 1981).

Weiner identified a population of larger cells with higher myeloperoxidase (MPO) activity, higher superoxide production in response to zymosan, and higher CSF-producing capacity compared to a subset of small cells with high cytotoxicity toward heterologous tumours (Weiner, 1984).

Also, different monocyte subsets with regard to cytokine production have been reported, showing that Mo of high-density fractions from Percoll-gradient preparations release more IL-1 than cells of low-density fractions (Fernandez, 1986).

Definition of monocyte subsets has generally been based on their differences in surface marker expression. Monocyte subsets are phenotypically recognized by their differential expression of FcγR alone or in combination with other surface markers

and functions. Mo expressing FcγR-I and -II, but not those expressing FcγR-III, have been reported to mediate ADCC efficiently (Connor, 1990).

FcγR-I has been found on the majority of monocytes. Similar to the regular Mo described above (Chechimi, 1989), these cells are large cells, represent potent phagocytes (Grage, 1993) and produce higher amounts of pro-inflammatory cytokines such as IL-1, IL-6, tumour necrosis factor α(TNF-α) (Szabo (a), 1990), and also higher amounts of PGE_2 , which in turn is known to suppress APC function and T cell activity (Pryjma, 1992)(Zembala, 1984, 1986). Large monocytes express higher chemotactic activity than small Mo, the FcγR-I Mo has been defined as the main population expressing receptors for C5a and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Ohura, 1987). Separation of monocytes based on size and density results in subsets that share characteristics with subsets defined by FcγR expression but are not the same.

Rather than FcγR-I, FcγR-III is expressed only on a small subset of monocytes, and Ziegler-Heitbrock has described phenotypic and functional differences for those Mo that express or lack FcγR-III (Ziegler-Heitbrock, 1991, 1992). Accordingly, the majority of Mo express the surface marker CD14 and appear as $CD14^+$ low HLA-DR-expressing phagocytic cells with high producing capacity for reactive oxygen radicals and pro-inflammatory cytokines (Ziegler, 1991, 1993).

Also, regular $CD14^+$subsets differ in their expression of the scavenger receptor and in receptor-mediated binding of low-density lipoprotein (LDL). $CD14^+$cells exhibit higher expression of Sc-receptor class A type I/II and also stronger LDL binding. A recent study also revealed differences in the chemokines receptor expression and chemotactic activity of these subsets. $CD14^+$ cells show expression of CCR2 that is paralleled with higher chemotactic migration responsiveness for monocyte chemotactic protein-1, (Webber, 2000).

Studies on the size and density of Mo in the blood of healthy human donors have identified a major subset of large high-density Mo and minor subsets of less dense cells. The major subset is represented by FcγR-I Mo (Szabo (b), 1990) (Pryjma, 1985, 1986, 1992) (Crage, 1993), which are identical to $CD14^+$ Mo. These cells are classical monocytes with typical phenotype and functions such as high cytokine production

(Schinkel, 1998), high chemotactic (Ohura, 1987) and phagocytic activity (Craige, 1993). In contrast, the different minor subsets of small less dense cells are more heterogeneous than the major typical monocyte population, and their phenotypic and functional characterization reveals common features with macrophages and myeloid or lymphoid dendritic cells.

The phenotypic and functional dissociation between blood Mo subsets, indicates differences in their immunoregulatory roles: the major subset of typical large, high-density FcγR-I Mo represents potent phagocytes that are active in the innate immune response against pathogens and are finally responsible for the down-regulation of an established immune response.

Their high expression of chemotactic ligand receptors indicates high susceptibility to chemotactic agents and chemokines and enables their early recruitment to the site of inflammation where they produce high amounts of proinflammatory cytokines leading to recruitment and activation of other monocytes and other effector cells, and therefore, promotion of an early inflammatory response. Furthermore, the relative resistance of FcγR-I monocytes to T cell killing (Frankenberger, 1996), provides the possibility of prolonged T cell inflammatory responses.

CD 14 was previously reported to be a LPS- binding receptor which is distributed on the cell surface and enhances the LPS signals. But since CD 14 lacks a cytoplasmic domain, it may not be responsible for the signal transduction. Recently, Toll like receptors (TLR), which are type-I transmembrane proteins expressed on immune cells such as macrophages, monocytes and dendritic cells, have been found to be major signalling receptors of the innate immune system. TLR recognise pathogen- specific molecules by their extracellular leucine-rich repeat (LRR) domains, and activate the signalling cascade via cytoplasmic Toll/IL-1 receptor (TIR) domains, leading to the synthesis of inflammatory mediators, like cytokines or nitric oxide. To date more than ten members of the TLR family have been found. Among them, TLR4 is recognised as an LPS receptor.

In conclusion, this variety within the blood monocyte populations may reflect their different states of activation and/or differentiation, enabling them to induce rapid innate and long-lasting adaptive immune responses.

1.2.2.3 Monocyte adhesion molecules

Cell adhesion occurs when a plasma membrane adhesion receptor interacts with a molecule in the extracellular matrix or on the neighbouring cell and when the liganded receptor forms a connection with the cell's own cytoskeleton. The process is controlled by the expression and function of adhesion receptors and by encounter with the corresponding ligands. Since monocyte migration to the perivascular space (where they can initiate and perpetuate inflammatory lesions) must be preceded by adherence of the cells to the endothelium, it is of particular importance to understand the regulation of this adherence, governed by the production of adhesion molecules.

Adhesion molecule classification

The immunoglobulin gene superfamily (Ig): These are type I transmembrane proteins, which may be classified in different subfamilies according to structural and functional homologies. The family comprises more than 70 members. The criterion for inclusion of a protein in this family is the presence of one or more Ig domains. They are expressed generally in leucocytes and endothelium (Ranscht, 1994). Members of the immunoglobulin superfamily bind to other members of the superfamily, to integrins or to a diverse range of additional counter-receptors. (Davies, 1993) (Garrod, 1993).

The Selectins: These are a small family represented by only three members, those expressed on leucocytes (L-selectin), on platelets (P-selectin) and on endothelium (P and E selectin)(Adams, 1994). They bind to carbohydrates and are constructed from three types of protein domains. One of the major functions of selectins is support of leucocytes rolling on endothelium (Lasky, 1995) (Westphal, 1992)

The integrins: The name integrin was coined to signify the presumed role of these proteins in integrating the intracellular cytoskeleton with the extracellular matrix (Ruoslahti, 1990). They are a family of membrane glycoproteins, consisting of two subunits - alpha and beta heterodimers. The β subunits are the main mediators of cell-extracellular matrix adhesion (for example laminin, collagens), and they are also involved in cell-cell adhesion. Sixteen α chains and eight β chains have been characterised.

These associate to form 21 different integrin α / β heterodimers, each showing a defined ligand binding specificity. The integrins were originally classified into three subfamilies: (beta1 integrins, beta 2 and beta 3 integrins) in which a common beta subunit was thought to associate with a number of different alpha subunits, but this classification is not very realistic because until now at least 12 different alpha subunits and 8 beta subunits have been identified.
The ligand binding site of integrins appears to be from both subunits and their cytoplasmic domains form connections with the cytoskeleton endowing integrins with the ability to serve as a link between the cytoskeleton and the extracellular matrix (Westphal, 1992) (Hynes, 1992).

Kishimoto (1989) has shown that three developments underscore the importance of leucocyte integrins as adhesion receptors of the immune system:

- Recognition that these integrins are evolutionarily related to other integrins, such as fibronectin receptor and platelet glycoprotein, which guide cell localization during embryogenesis and wound healing. The leucocyte integrins provide a similar mechanism in the immune system for guiding leucocyte localization during inflammation.

- Identification of intercellular adhesion molecule-1 (ICAM-1),- a ligand for the integrin family member LFA-1 (leucocyte function associated antigen), which is induced during inflammation and can regulate leucocyte migration and localization. This receptor-ligand pair revealed the first known interaction between a member of the integrin family (LFA-1) and a member of the immunoglobulin family (ICAM-1).

- Discovery of immunodeficiency patients genetically deficient in expression of the leucocyte integrins

1.2.2.4 Adhesion regulation and signalling

There is evidence that abnormal cellular immunity, in particular monocyte dysfunction, is central to the development of premature and accelerated vascular disease or atheroma in general, and that this is most marked in diabetic patients. Early in the development of atheromatous lesions, monocytes are preferentially recruited to the arterial intima (Schwartz et al, 1991) (Ross, 1986) (Gerrity, 1981), bind to and migrate across the endothelium. Even in the normal situation, there is a low background level of this monocyte adhesion and transendothelial migration This process ensures maintenance of the macrophage population in the interstitial space, where they may be needed at short notice for phagocytic purposes. In the presence of inflammatory stimuli, the adhesiveness of monocytes is markedly increased as is the recruitment of additional monocytes to augment the resident tissue macrophage population (Issekutz, 1981) (Blusse Van Oud (1983)).

Monocytes express a higher level of adhesion to endothelial cells than do granulocytes or lymphocytes (Beekhiuzen, 1990, 1991) (Wallis, 1985). As they tend to accumulate along the peripheral membrane of individual endothelial cells, it is likely that the surfaces of both cells are more adhesive at the periphery. This has led to speculation about the role of specific adhesion molecules in this process.

The B2 integrins (MAC-1, LFA-1 and VLA-4) are expressed exclusively on leucocytes and particularly on monocytes and so play a vital role in intercellular reaction involving these immune cells ((Khishimoto, 1989) (Arnaout, 1990). Insufficient expression of these adhesion molecules on monocytes is accompanied by impairment of immune surveillance (Arnaout, 1990). The central role of these integrins has been shown in experiments using function perturbing antibodies. Blocking of MAC-1 and LFA-1 by specific monoclonal antibodies results in a fall in extravasation of macrophages to the interstitial space, resulting in impairment of some aspects of local immune function.

Monocytes express a variety of adhesion molecules, each of which plays a vital role in their interactions with endothelial cells. Some, like L-selectin, primarily mediate and regulate rolling of monocytes along the endothelium,), while others have more

direct effects on adhesion. These latter effects, and their regulation, are the subject of my study and a more detailed description of the molecules involved directly in monocyte adhesion follows .

Three members of the B2 integrin subfamily (MAC-1, LFA-1 and VLA-4) have been identified to play a crucial role in the adhesion of monocytes to endothelial cells, .

MAC-1

MAC -1 consists of a 170 Kd α– subunit and a 95 Kd β– subunit and is expressed on monocytes, and to a lesser degree on granulocytes. The expression of β2 integrin MAC-1 is increased significantly after acute hyperglycaemia in both normal and type 2 diabetic subjects (Sampson 2002), and there is a significant increase of MAC-1 on stimulated and unstimulated diabetic monocytes compared with normal (Dosquet 1992). Kunt and colleagues found that monocytes of normolipidemic hyperglycaemic type 1 diabetes express higher level of MAC-1 (Kunt 1999).

LFA-1

LFA-1 consists of a 180-Kd α subunit and a 95-Kd β subunit, is expressed on leucocytes and particularly on monocytes (Krensky, 1983) (Lanier 1985), and has been shown to participate significantly in monocyte interactions with endothelial cells (Velde, 1987) (Dustin, 1988)

LFA-1 plays an important role in the pathogenesis of type I diabetes (Mysliwiec 1999), while in pregnant animals with alloxan-induced diabetes, blocking the B2 integrin component (LFA-1) involved in binding to ICAM-1 on decidual endothelial cells, has been shown to result in significantly reduced monocyte binding (Jeong 1994).

Kretowski found that the expression of monocyte LFA-1 is increased in type I diabetes (Kretowski 1999) and Patrizia found that there is upregulation of monocyte LFA-1 expression in normal human pregnancy (Patrizia 2002)

Koller et al found an upregulation of monocyte LFA-1 expression in inflammatory conditions such as osteoarthritis rheumatoid arthritis

(Koller 1999), and revealed that upregulation of this adhesion molecule is vital for the development of inflammatory processes, explaining the changes of expression and upregulation of LFA-1 on monocytes in diabetic pregnant subjects.

VLA-4

VLA-4 is expressed on monocytes and lymphocytes, and has been shown to contribute to their adhesion to VCAM-1 on endothelial cells (Beekhuizen, 1992) (Osborn, 1989) (Carlos, 1990)

The pathogenesis of adoptive autoimmune diabetes requires an interaction between VLA-4 on monocytes and VCAM-1 on DEC, Baron et al have used antibodies to block VLA-4 on monocytes and one of its ligands and VCAM-1 on DEC, and they were able to delay the onset of diabetes and decrease its incidence in adoptive transfer model. The binding of VLA-4 and VCAM-1 is very important in the development of IDDM (Baron 1993), so what could be the role of VLA-4 in the development of atheroma in T1DM pregnancy? Another scholar found that Blocking VLA-4 on monocyte using monoclonal antibody inhibited the ability of high glucose and Lipoxegenase to induce monocyte adhesion to endothelium (Patrizia 1999).

After rolling along the endothelium and light adherence, monocyte must adhere firmly to the endothelial cell. The regulation of adhesion at the level of receptor-ligand interactions involves modulation of receptor number and type. This occurs by synthesis, secretion from intracellular stores or by increased expression of the integrins. Regulation of integrin functions by affinity modulation through allosteric conformational changes is of major biological significance. These changes are thought to be elicited after cell activation by the interaction of specific cytosolic or cytoskeletal proteins with the cytoplasmic tails of the cell's integrin receptor (Diamond, 1994). The β subunit of integrin plays an important role in this affinity regulation since epitopes for integrin activation antibodies lie on the β chain. Antibodies may affect integrin function by direct stimulation of conformational changes or by an allosteric effect, (ie by binding to the epitope in the β chain induced or attenuated in the ligand-occupied integrin), thereby stabilising the active or the inactive conformation (Mould, 1996).

1.2.3 The adaptive immune system

If an invading pathogen escapes the innate defences, the body can launch a more sophisticated adaptive or specific response against one type of antigen.

Adaptive immunity refers to antigen-specific defence mechanisms that take several days to become protective and are designed to remove a specific antigen. This is the immunity one develops throughout life. There are two major branches of the adaptive immune responses: cell-mediated and humoral (Medzhitov, 1997).

1.2.3.1 Cell-mediated immunity

Cell-mediated immunity does not engage antibodies, but, mediated by T-lymphocytes, involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to an antigen (Welle, 1997).

Its functions include:

- Phagocytosis and killing of intracellular pathogens
- Direct cell killing by cytotoxic T cells
- Direct cell killing by NK and K cells (Palucka, 1999)

These responses are particularly important for destroying intracellular bacteria, eliminating viral infections and destroying tumour cells. While the production of antibody through the humoral immune response can effectively lead to the elimination of a variety of pathogens, micro-organisms that have evolved to invade and multiply within phagocytic cells of the immune response pose a different threat.

The cell-mediated immune response is also involved in rejection of foreign grafts and the elimination of tumours and virus-infected cells. The effector cells involved in these processes are cytotoxic T-lymphocytes (CTLs) and NK-cells.

1.2.3.2 Humoral immunity

Humoral immunity involves the creation of antibodies in response to an antigen and is mediated by B-lymphocytes. The first stage in this pathway is the phagocytosis of foreign matter by macrophages. The macrophages digest the infectious agent and then display some of its components on their surfaces. The helper-T cells recognize this presentation, activate their immune response, and multiply rapidly, inducing the activation phase.

The next phase, the effector phase, involves a communication between helper-T cells and B-cells. Activated helper-T cells use signals to contact B-cells, which then multiply rapidly. B-cell descendants become either plasma cells or B memory cells. The plasma cells manufacture huge quantities of antibodies that will bind to the antigen and prime it for destruction. B memory cells retain a "memory" of the specific antigen that can be used to mobilize the immune system faster if the body encounters the same antigen later in life (Borghans, 1999).

1.2.4 Immune system changes in diabetic patients

Spatz has described an impaired proliferative response of CD4+ T cells to primary antigens in patients with type I diabetes (Spatz, 2003). Also he showed that monocyte-derived dendritic cells from this group of patients expressed elevated levels of CD 86 and reduced amounts of the adhesion molecule CD54 on their cell surface compared to control subjects and patients with type II diabetes (Spatz, 2003), He found that monocytes stimulated with lipopolysaccharide (LPS), produced high levels of the anti inflammatory cytokines interleukin IL-10 and IL-6 (Spatz, 2003).

Studies investigating the behavior of adhesion molecules in diabetic pregnant patients, revealed that levels of E selectin, MAC-1 were significantly higher in type II diabetic pregnant patients compared with clinically normal pregnancy (Pertynska, 2000). Higher expression of VCAM-1 has been described in patients with diabetes compared with the normal population (Rasmussen, 2002), and serum from diabetic patients has been shown to contain components capable of inducing VCAM-1 expression in endothelial cells independent of hyperglycemia. These findings indicate that

augmented induction of endothelial VCAM-1 expression by circulating factors may play a role in the development of atherosclerosis in diabetes ((Rasmussen, 2002).

Gibson found a significant increase in the expression of E selectin and ICAM-1 in nonpregnant diabetic women compared with nonpregnant normal women, while the vascular endothelial cell adhesion molecule VCAM-1 concentration changed significantly in pregnancy in diabetic women only (Gibson, 1997).

1.3 Atherosclerosis

`The definition of atherosclerosis: Clogging, narrowing, and hardening of the body's large arteries and medium-sized blood vessels in response to multiple pathogenic factors. Atherosclerosis is a disease of the arterial wall in which the intima is thickened, resulting in narrowing of the lumen and thus impairment of blood flow.

The arterial wall consists of the following layers:

(i) the innermost (lining the vessel cavity) monolayer of endothelial cells, which is separated from the intima by a basement membrane consisting of connective tissue

(ii) the middle layer, the media, consisting of smooth muscle cells (SMCs), and finally

(iii) an outer connective tissue layer, the adventitia, which embeds the vessel in its surroundings.(Stary, 1994)

Atherosclerotic lesions emerge in the intima as ‘fatty streaks’, cushion-like lipid-rich elevations protruding into the lumen, the earliest stages of the disease that later develops into atherosclerotic plaques

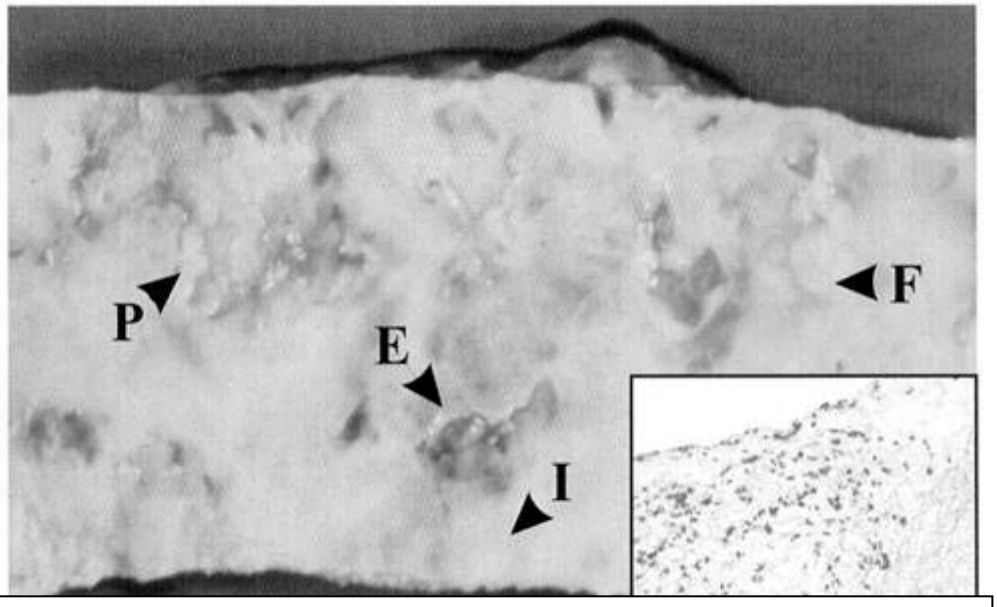

Figure 1.5: Microscopic appearance of the inner surface of a human artery. I, normal artery; F, fatty streak; P, raised plaque; E, exulcerated plaque, The insert in the lower right corner shows immunohistochemical staining of CD3+ T lymphocytes in the shoulder region of an early atherosclerotic lesion. Taken from (Stary, 1995)

Atherosclerosis is an end-stage vascular pathology with a compound pathophysiological structure. Atherosclerosis represents a particular type of thickening and hardening (sclerosis) of the arterial wall that is also characterized by the presence of 'foam cells' (lipid-laden macrophages and smooth muscle cells (SMCs)) that may burst and discharge their filling into the lesional areas.

These severe lesions have a particular propensity to rupture at the edges, the so-called shoulder regions, aggravate thrombus formation, and thus lead to such final complications as myocardial infarction or stroke. Although arteriosclerosis is the wider term for these various forms of the disease, including atherosclerosis, the latter designation is now the generally accepted term (Stary, 1995).

Atherosclerosis is multifactorial with an extensive list of potential causative factors. These include:

(i) high levels of LDL-cholesterol or oxidised LDL. The altered-lipoprotein hypothesis postulates an initiating role of chemically altered lipoproteins, most

notably oxLDL, in forming foam cells and acting as chemoattractants for SMCs and mononuclear cells into the intima (Steinberg 1989, Witztum 2001)

(ii) free radicals (eg caused by cigarette smoking),

(iii) hyperglycemia and advanced glycation end-products (AGE). Accelerated by hyperglycemia, AGE accumulation is believed to contribute to the gradual development of diabetic complications

(iv) infectious agents (Saiku, 1988).

(v) stress.
The increased incidence of atherosclerotic complications seen during the grieving process has been attributed to the development of stress-induced hyperlipidemia, hormone dysfunction, and the increased shear stress of hypertension, which together injures the endothelium and modifies the environment of the endothelial barrier to the passage of blood constituents (Ross 1976, Saiku, 1988).

(vi) genetic vulnerability to endothelial damage (Shi, 2000).

(vii) an immunological origin (Wick 1995, Wick 1997).
This causation is suggested by the results of immunohistological examination of very early atheromatous changes not sufficiently advanced to cause clinical symptoms. It has been shown that the first cell types found in the arterial intima at sites known to be predisposed for later development of atherosclerotic lesions are lymphoid cells, followed by macrophages and SMCs (Xu, 1990).

This last potential mechanism is examined in more detail below.

1.3.1 Atherosclerosis: an immune system disorder?

The association of inflammatory processes (reflected by increased levels of C-reactive protein (CRP) with an increased risk for atherosclerosis is well-recognised

(Ridker 2000, Ridker 2001, Kiechel 2001). Witztum has described defence from atherosclerosis by natural antibodies with a certain idiotype (D15) focussed against oxLDL (Horkko, 1999). Natural antibodies are generated without known antigenic stimulation and belong to the IgM type produced by a special subset of innate B lymphocytes, the B1 cells. These cells are characterized by the expression of restricted germline-encoded B cell receptors conveying specificity for natural or self-antigens. Natural antibodies have been shown to bind oxidized phospholipids, such as palmytoyl 1-2 (5-oxo-valeroyl)-sn glycerol-3 phosphorylcholine (POVPC), and serve to remove oxLDL, thus preventing its uptake by macrophages.

Wiztum suggests that a natural selection process for Bl cells secreting oxidation-specific D15 antibodies has been operative, both for their role in natural immune defence and for housekeeping purposes against oxidation-dependent epitopes under healthy and pathologic conditions.

In early immunohistochemical studies (the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study), there was an opportunity to analyse arterial specimens from more than 3000 arterial fragments collected from young subjects who died from non-cardiovascular causes. The authors found CDIa DC (dendritic cells) in the intima of all subjects. Because DC forms a link between the innate and the adaptive immune systems, it is of interest that Aicher (Aicher, 2003) described a strong DC-activating potential of nicotine. This is reflected by the expression of co-stimulatory molecules (CD86, CD40) and MHC class II and adhesion molecules (LFA-I, CD54), and a sevenfold increase in secretion of the pro-inflammatory Th-1-activating cytokine IL-12. The PDAY study specimens contained substantial numbers of CD19+ B cells, depending on, and growing with severity of lesions.

1.3.1.1 The role of the innate immune system in the development of atherosclerosis

Cells of the innate immune system, in response to immune challenge, produce a set of highly conserved molecules known as peroxisome proliferator-activated receptors (PPARs) . The range of PPARs produced by cells of the innate defence

system is based on a natural selection of germline-encoded genes and includes) and collectins (mannan-binding lectin, surfactant). Probably fewer than 100 different types of receptors exist, each recognising restricted patterns of entire classes of pathogen ligands, called pathogen-associated molecular patterns (PAMPs). PAMPs are present not only on the surface of pathogens, but also in solution, e.g., as bacterial endotoxins (LPS), denatured proteins, bacterial and denatured eukaryotic DNAs, and a variety of other compounds **(**Medzhitov, 2001). Ligand-binding results in internalisation of the ligand-receptor complex, activation of the cell, and the mounting of an inflammatory defensive response via activation of various transcription factors, most notably NF-κB.

Cellular components of the innate immune system are already assembled in the intima of arteries in children at certain branching points, known to be possible sites for later development of atherosclerotic lesions. When the rolling, adhesion, and transendothelial migration of leucocytes is prevented, by lack of expression of either adhesion molecules or chemotactic molecules, atherosclerotic lesions are greatly reduced or do not develop. Therefore, atherosclerosis develops only to a minor extent in hypercholesterolemic mice deficient in VCAM-1(Cybulsky 2001), deficient in P-selectin (Johnson RC. 1997) or deficient in ICAM-I (Collins, 2000). MCP-1 is essential for the recruitment of monocytes into the intima. MCP-1/Apo-E or LDL-R double knockout mice do not develop atherosclerosis (Gu, 1998). Also, crosses of Apo-E^sup -/-^ or LDL-R^sup -/-^ mice with mice deficient in CCR-2 (the receptor for MCP-1) exhibit significantly impaired development of atherosclerotic lesions (Dawson, 1999). Additionally, oxLDL deposited in intima acts as a monocyte chemoattractant, and oxLDL phospholipid constituents stimulate MCP-1 expression by endothelial cells (Cushing 1990).

Arterial intimal macrophages derived from blood-borne monocytes exert both phagocytic functions (via their scavenger receptors) and innate defence functions (via other PPARs). The main representatives of scavenger receptors are CD36, SR-P1, and SR-A. These receptors bind oxLDL and, as they are not saturable, result in the formation of foam cells. Phagocytosis in general, and activation of macrophages via TLRs in particular, lead to the generation of reactive oxygen species - a major microbial killer mechanism - and thus transduction of inflammatory signals. An

additional role for TLRs as target-presenting structures, is to interact with CD14, the non-transmembrane receptor for LPS, involved in the induction of inflammatory reactions (Chen 1999).

Humoral components of the innate immune system include natural antibodies, complement components, acute-phase proteins such as C-reactive protein (CRP), matrix-metalloproteinases (MMPs), and their antagonists (tissue inhibitors of metalloproteinases (TIMPs). Deposits of complement components as a result of activation - via the classical, the alternative, or the mannose-dependent pathways - are known to be present within atherosclerotic lesions and may represent a transitional mechanism between innate and adaptive immunity in atherogenesis (Seifert 1988).

In summary, there is a large body of literature demonstrating a major role for involvement of cellular and humoral components of the innate immune system in the development and progression of atheromatous lesions.

1.3.1.2 The role of the adaptive immune system in the development of atherosclerosis

Complement activation occurs in the arterial intima of rabbits shortly after beginning a cholesterol-rich diet (Seifert 1988, 1989, Vlaicu, 1985). Complement activation via the classical pathway could be the result of the deposition of immune complexes in the arterial intima, or it may reflect the binding of specific antibodies to autologous or exogenous antigens localised in the arterial wall. The former include oxLDL and HSP60, whereas the latter comprise various types of micro-organisms such as EBV, CMV, or bacteria such as Chlamydia. Cholesterol crystals and other lipid components have been shown to activate this pathway *in vitro* (Ishida 2001). That intralesional complement is activated by the classical pathway is supported by its co-distribution with IgG, although the specificity of such locally deposited antibodies has not yet been demonstrated. Infection has an aggravating effect on atherogenesis, which could be due to the endothelial damaging effect of circulating immune complexes (Minick, 1973).

Applying RT-PCR technology, Stemme (1991) showed that T cells in human atherosclerotic plaques are of polyclonal origin, suggesting that they are either recruited to the plaque in an activated stage or activated locally by mechanisms that do not lead to clonal proliferation. Nevertheless, the possibility that these populations contain pathogenic clones with locally relevant antigenic specificity cannot be ruled out. Oligoclonal T cell proliferation has been demonstrated in atherosclerotic lesions of ApoE^sup -/-^ mice (Paulsson, 2000). Knockout mouse models for cholesterol-induced atherosclerosis have contributed considerably to the understanding of atherogenesis. It has to be emphasized, however, that these models do not reflect those situations in common human atherosclerosis that are induced by a variety of risk factors. Murine knockout models, such as LDL-R and ApoE knockout mice, in essence have their counterparts in genetically determined familial hypocholesterolemia in human patients.

In common atherosclerotic lesions, Th1 cells expressing the cytokines IFN-γ and IL-2 prevail over IL-4-, IL-5-, and IL-10-producing Th2 cells (Pinderski 2002, Hubber 2001). This is reflected in significantly elevated serum levels of neopterin, an IFN-γ induced macrophage product (Xu, 1994). IFN-γ has a pro-inflammatory and thus pro-atherogenic effect. This Th 1 cell product locally activates macrophages and inhibits SMC proliferation and collagen synthesis, leading to a higher risk of plaque instability and rupture. The pro-inflammatory effect of IFN-y is augmented by the macrophage-derived cytokines IL-1 and TNF-α, and is reflected in the induction of expression of acute-phase proteins such as IL-6 and CRP in the liver (Teramoto, 2003). The anti-inflammatory cytokine TGF-β produced by macrophages, SMC, and Th3 cells, has a pro-fibrotic effect, and thus promotes plaque stability. Blocking TGF-β signalling by appropriate monoclonal antibodies results in the development of larger and more unstable lesions.

Although there are no definitive data on the route of migration of mononuclear cells to the vascular associated lymphoid tissue (VALT) area, or the specificity of the involved T cells, indirect evidence based on the distribution of certain adhesion molecules on endothelial cells and the composition of extracellular matrix (ECM) molecules provides some clues.

A basic expression of ICAM-1 and P-selectin by endothelial cells in the VALT area of normal carotid arteries has been found. ELAM-1 was not found on endothelial cells in any of the specimens investigated, explaining the lack of neutrophils that are known to adhere preferentially via this adhesion molecule. VCAM-1, expressed by both endothelial cells and macrophages within the VALT, may be instrumental in further recruitment of mononuclear cells and subsequent development of atheromatous lesions. Various stress factors, notably the classical atherosclerosis risk factors, can simultaneously induce expression of HSP60 and upregulation of adhesion molecules ICAM-1, VCAM-1, and ELAM-1 (Amberger, 1997).

The ECM molecules, together with cytokines and growth factors, contribute to the microenvironment of any tissue, including arteries. Cells of the immune system interact with ECM resulting in promotion or inhibition of their migration and function. They support cellular migration, e.g., collagen triple helices acting as conveyer belts for the movement of DC, whereas T cells show a similar pattern of migration depending on the three-dimensional structure of the ECM environment, e.g., via collagen-binding sites of VLA-1 and VLA-2. Ligand binding to these receptors induces T cell stimulation, resulting in the synthesis of cytokines and increased migration.

1.3.1.3 Endothelial cell dysfunction in causation of atheromatous lesions

Premature and accelerated vascular disease or ‘atheroma’ is an end stage vascular pathology with a complex pathophysiological structure. Amongst other changes, vascular disease in diabetic subjects is associated with abnormal peripheral blood monocyte function, abnormal function of the blood vessel lining cells and a marked infiltration around these blood vessels by fat-filled foamy macrophages (Schwartz, 1991). The endothelium is not just a cell monolayer covering the luminal surface of blood vessels, but a critical “structure” involved in inhibition of platelet adhesion, with many regulatory functions, such as the control of vascular tone, leucocyte rolling and transendothelial migration, and vascular smooth muscle cell growth and migration (Hsueh, 2003) (Issemann, 1990).

An increased frequency of apoptosis has been implicated as a hallmark of endothelial dysfunction (Isner, 1995) (Rossing, 2001) and has been documented in the vascular endothelium of diabetic animals and humans (Mizutani, 1996). Endothelium-derived nitric oxide (NO) plays an important role as an anti-inflammatory factor, because of its ability to reduce the expression of leucocyte adhesion molecules such as intercellular adhesion molecule (ICAM-1) and other inflammatory chemotactic agents like monocyte chemotactic protein (MCP-1), which is involved in migration of monocytes to the intima at the site of plaque formation (Localzo, 2001). A loss of endothelium-derived NO enhances the action of the pro-inflammatory transcription factor NFkB, resulting in increased expression of monocyte adhesion molecules and increased production of chemokines and cytokines. These changes encourage monocyte migration into the intima and the formation of macrophage foam cells, changes characteristic of early atheroma (Creager, 2003) (Dosquet 1992)

Even a small defect in endothelial function can have significant clinical implications (Landmesser, 2004). Beyond the impairment of endothelium-dependent vasomotion, endothelial dysfunction promotes key aspects of the atherosclerotic disease process, ie, vascular inflammation and thrombosis that are relevant at all stages of the disease. Conversely, a systemic inflammatory response that may involve detrimental effects of C-reactive protein on the endothelium augments endothelial dysfunction (Yeh ETH, 2003).

The observation that endothelial dysfunction represents an independent predictor of CV events suggests that assessment of endothelial function integrates variables beyond conventional risk factors, variables that may include the "pathogen burden" (Prassad, 2003), the genetic predisposition, and other not-yet-identified factors causing endothelial activation. Increased vascular production of reactive oxygen species is characteristic of all major conditions predisposing to atherosclerosis (Cai H, 2000), since bioavailability of NO reflects a balance between its production via endothelial nitric oxide synthase (eNOS) and degradation by superoxide oxygen derived free radicals (Costentino, 1997) (McVeigh GE, 1992). The molecular pathways leading to endothelial dysfunction-related events include: activation of protein kinase C and the transcriptional messenger NFkB with subsequent expression

of endothelial cell surface adhesion molecules, as well as increased expression of enzymes that promote oxidative stress (Gonzalez, 2003).

Generally, in diabetic patients, hyperglycemia decreases endothelium derived NO (Tesfamarian, 1991) and activates PKC (Beckman, 2001), whereas excess free fatty acid liberation injures the endothelium via activation of PKC, and exacerbation of dyslipidemia (Dichtl, 1999) (Dresner, 1999) and insulin resistance mediate abnormal endothelial cell function by affecting the synthesis or degradation of NO (King, 1996).

1.4 Diabetes Mellitus

1.4.1 Introduction

Diabetes mellitus represents a group of diseases of heterogenous aetiology, characterised by chronic hyperglycemia and other metabolic abnormalities, related to deficiency of insulin. After long term metabolic instability, particular complications of diabetes may occur such as neuropathy, nephropathy, retinopathy, cardiovascular disease.(Kosaka, 1994). The deficiency of insulin action leads to characteristic abnormalities in the metabolism of carbohydrate, lipid and protein. If the metabolic abnormality is mild, patients may be asymptomatic, while in the presence of overt hyperglycemia, specific symptoms such as thirst, polydipsia, and polyuria and weight loss often occur. In severe cases, ketoacidosis may occur, that leads to disturbance of consciousness, coma and even death without treatment. With long duration of diabetes, small vessel abnormalities will ensue (microvascular disease), that leads to serious complications such as renal failure and blindness. Also diabetes accelerates the development of atherosclerosis, increasing the risks for occlusive artery disease (macrovascular disease) involving the lower limbs, the cerebral and coronary circulations. (Kawasaki, 1996)

1.4.2 Classification of diabetes mellitus

Both aetiological classification and staging of pathophysiology by the degree of deficiency of insulin effect need to be considered:

- Aetiological classification:

    1. Type I diabetes (T1DM), also known as insulin-dependent or juvenile-onset diabetes is characterised by destructive lesions of pancreatic Beta cells usually by an autoimmune mechanism. This type of diabetes accounts for 10 to 15% of all cases of DM. Auto antibodies to islet cell antigens are detected in the majority of patients (70%-90%). Typically it occurs in young patients with acute onset but may occur at any age (Kobayashi, 1994). In the future, T1DM may be further divided into subtypes according to the mode of onset (i.e. acute or slowly progression), HLA antigens, or epitopes of autoantigens ((Imagawa, 2000)

    2. Type II diabetes mellitus is characterised by a variable combination of decreased insulin secretion and decreased insulin sensitivity. The mass of pancreatic Beta cells and their function are preserved to some extent. The majority of patients are obese or have been obese in the past. This type of diabetes classically develops after middle age, but may occur in younger people, particularly if they are obese (Kitagawa, 1994, 1998)

    3. Gestational diabetes mellitus: GDM is a state of glucose intolerance occurring for the first time during pregnancy, where relative insulin resistance is common. This glucose intolerance may affect the infant and mother adversely. Glucose intolerance during pregnancy is often normalised after delivery, but affected subjects have an increased risk of developing diabetes in the future (Kuzuya, 2002) (Knock, 1997)

- Diabetes stage classification
    1. Non insulin requiring
    2. Insulin required for glycemic control
    3. Insulin dependent for survival (Kuzuya,2002)

1.4.3 Diagnosis of diabetes mellitus

The confirmation of chronic hyperglycemia is a prerequisite for diagnosis, and in order to confirm persistent hyperglycemia, at least two plasma glucose measurements on separate days are necessary. Diabetes can be diagnosed following a single plasma glucose measurement with other metabolic abnormalities such as a high HbA1c level. (Kuzuya, 2002). T1DM is confirmed by a history of diabetic ketoacidosis and /or the presence of anti glutamic acid decarboxylase (GAD) antibodies.

Criteria	**Normal range**	**Diabetic range**
Fasting plasma glucose	< 6.1	≥ 7.8
2h postural glucose load	< 8	≥ 11.1

Table 1.1: Criteria for diagnosis of diabetes mellitus
(Report of a WHO consultation. *Diabet Med 1998; 15: 539-553)*

1.4.4 Vascular disease in diabetes

1.4.4.1 Classification of vascular disease

Macrovascular disease, or atherosclerosis, is the basis of more than half of all mortality in developed countries. It is a progressive disease of the large- and medium-sized arteries. The name is derived from the Greek *athero* meaning "gruel" or "paste" and *sclerosis* meaning "hardening." Thus, atherosclerosis is the hardening of the arteries due to the accumulation of this paste (commonly called plaque).

There are three principal sites of macrovascular disease: coronary artery, cerebrovascular, and peripheral vascular.

Microvascular disease describes the thickening of the basement membrane seen in capillaries (LoGerfo and Coffman, 1984), and arterioles (Silhi, 1998) of diabetic patients. Thickening of the basement membrane is thought to be a result of the proliferative response of the vascular endothelium to chronic injury (Silhi, 1998),

brought on by the effects of hyperglycaemia on glucose metabolism, protein synthesis and non-enzymatic glycation of cellular and extracellular components.

The accumulation of sorbitol in the cells due to defective glucose metabolism (Frank, 1994) leads to increased oedema (Watkins et al, 1997), resulting in modified membrane function and permeability. Alterations in basement membrane protein synthesis and advanced glycation end products (AGEs) formed as a result of nonenzymatic glycation lead to functional changes of the microcirculation, causing a reduced ionic charge, allowing albumen across the basement membrane and leading to altered cellular nutrition (Stonebridge, 1996).

1.4.4.2 Incidence and predispositions of vascular disease in diabetes

Macrovascular disease accounts for more than 70% of deaths in people with diabetes, mostly from myocardial infarction, congestive cardiac failure and stroke. Risk factors for the development of macrovascular disease in diabetes include age, race, cigarette smoking, hypertension, raised cholesterol, HbA1c levels, and obesity. There is little support for the suggestion that the macrovascular disease of diabetes is histologically or biochemically different from atherosclerosis in the non-diabetic individual, but there may be some differences in the extent and distribution of disease Diabetic macrovascular disease manifests in the form of atherosclerotic heart disease, peripheral vascular disease, and cerebrovascular disease. The increased risk of macrovascular disease in diabetes mellitus can be attributed to a higher prevalence of predictable risk factors, many of which are part of the insulin resistance syndrome. More "controversial" risk factors, i.e., triglycerides, plasma glucose, and insulin, are uncertain.

In type 2 diabetes, hypertriglyceridemia has long been associated with an increased risk of macrovascular disease (Fontbonne, 1989). Whether hypertriglyceridemia is the connection between increased plasminogen activator inhibitor 1, decreased HDL cholesterol, and/or small dense LDL or is independent of these expected relationships is unclear. Almost all patients with hypertriglyceridemia >200 mg/dl have small dense LDL (Austin, 1990) but only some are at increased risk of macrovascular disease.

Hyperglycemia has been observed as a cause of vascular diseases in animal models and also the 'Diabetes Control and Complications' trial demonstrated that aggressive glycaemic control in type I diabetes reduces significantly the risk of microvascular complications, such as retinopathy and nephropathy. A link between hyperglycaemia and macrovascular disease was shown in the San Antonio Heart Study that revealed a high proportion of cardiovascular related deaths in those with higher fasting blood glucose levels NIDDM (Wei, 1998).

The 'Wisconsin Epidemiologic Study of Diabetic Retinopathy' revealed that an 18% increase in cardiovascular mortality was associated with a 1 % increase in glycated hemoglobin (Klein, 1995). Despres has shown that hyperinsulinaemia and insulin resistance accelerate the development of atherosclerosis (Despres, 1996), and another study of multiple ethnic groups have shown increased carotid intimal and medial thickness (a reliable marker for coronary disease) in subjects with insulin resistance (Howard,1996). The presence of hypertension in diabetic patients increases significantly their risks of both microvascular and macrovascular disease. Several studies have demonstrated a benefit of blood pressure control in reducing these complications (Estacio, 1998), (Prospective Diabetes Study Group,1998)

1.5 Diabetes and the immune system in pregnancy

1.5.1 Diabetes actively affects pregnancy

Pregnant women with type I diabetes have a particularly high level of risk for the development of atheromatous lesions, and the placenta is a specific target organ. The precise mechanisms regulating the initiation and progression of vascular lesions in diabetic pregnant women are unclear. There is evidence that atypical cellular immunity is central to the development of this abnormal atheroma-like lesion within maternal intrauterine blood vessels supplying the placenta in type I diabetic pregnant women over a very short space of time. Saviddu has demonstrated that in pregnant women with Type 1 diabetes, vascular function during the second trimester and late pregnancy is significantly impaired compared with normal healthy pregnant women (Saviddu, 2002), and that this impairment may result from decreased production of

endothelium-derived nitric oxide as a result of reduced nitric oxide synthase activity or decreased availability of L- arginine (Tesfamariam, 1993) (Sobrevia, 1994).

Pregnancies in women with pre-existing T1DM mellitus are complicated by considerably higher than normal rates of severe perinatal complications, and women with poor self-care are at the highest risk. The problems are well demonstrated in a report from Dorte (2004), summarised in Table 1.5.1.1 below. The perinatal mortality rate was 3.1% in T1DM pregnancies compared with 0.75% in the background population (RR 4.1 [95% CI 2.9–5.6]), while the stillbirth rate was 2.1% compared with 0.45 (RR 4.7 [3.2–7.0]). The congenital malformation rate was 5.0% in the study population and 2.8% (1.7 [1.3–2.2]) in the background population. Six of the perinatal deaths (16%) were related to congenital malformations. Only 34% of women performed daily home monitoring of blood glucose at conception, and 58% received preconceptional guidance.

	T1DM	Normal	Relative risks P value
n	1,215	70,089	
Pre-eclampsia*	220 (18.1%)	* 1822 (2.6%)	<0.001
Caesarean section	680 (55.9%)	8,831 (12.6%)	4.4 (4.1–4.8)
Preterm delivery†	507 (41.7%)	4,205 (6.0%)	7.0 (6.3–7.6)
Gestational age at delivery (days)	256 ± 16	280+ 15	<0.0001
Birth weight (g)	3,487 ± 817	3,478+ 816	0.71
Birth weight ≥4,500 g	97 (8.0%)	2,383 (3.4%)	2.3 (1.9–2.9)
Large for gestational age infant‡	761 (62.5%)	—	<0.001
Stillbirth	26 (2.1%)	318 (0.45%)	4.7 (3.2–7.0)
Perinatal mortality	38 (3.1%)	525 (0.75%)	4.1 (2.9–5.6)
Congenital malformations	61 (5.0%)	1,987 (2.8%)	1.7 (1.3–2.2)
Respiratory distress syndrome§	202 (17.1%)	ND	—
Jaundice	215 (18.1%)	ND	—

Table 1.2 : Obstetric complications and fetal characteristics for type 1 diabetic pregnancies and the background population (Dorte, 2004).

Data are mean ± SD or *n* (%). * Blood pressure ≥140/90 mmHg and proteinuria. The frequency in the background population is 2.6% †before 37 completed weeks of gestation; ‡birth weight ≥90th centile. The expected frequency in the background population is 10%, but exact numbers are not available; §use of continuous positive airway pressure for >1 h postpartum;

Pregnancies with serious adverse outcomes (perinatal death and/or congenital malformations) were characterised by higher HbA_{1c} values before and during pregnancy and a lesser degree of maternal self-care and preconceptional guidance. Women who performed daily self-monitoring of blood glucose at any time during pregnancy had lower HbA_{1c} values than women who did not measure their daily profile. Likewise, daily self-monitoring was associated with a reduction in serious adverse outcomes. The caesarean section rate was 55.9% in diabetics and 12.6% in normal women, while the risk of preterm delivery was 41.7% and 6.0%, respectively. These results are consistent across several publications (Casson 1997, Howthorne 1994, Suhonen 2000, Penney 2003, Boulot 2003, Westergaard 1997, Miller, 1981), and others shown in Table 1.3 below.

	Serious adverse outcomes	others	Relative risks P value
n	93	1,125	
Age (years)	28 (25–31)	29 (26–32)	0.49
Preconceptional BMI (kg/m^2)	22.1(19.424.2)	23.0 (21.4–25.3)	0.01
Diabetes duration (years)	12 (6–18)	12 (5–19)	0.75
Daily blood glucose monitoring at conception	18 (22.5%)	363 (34.6%)	0.019
Preconceptional guidance	38 (42.7%)	631 (59.2%)	0.002
HbA_{1c} 0–3 months prior to conception (%)	8.0 (7.3–9.1)	7.6 (6.8–8.5)	0.005
HbA_{1c} during first trimester (%)	7.6 (6.6–8.6)	7.3 (6.6–8.1)	0.037
HbA_{1c} during second trimester (%)	6.9 (6.2–8.0)	6.6 (6.0–7.3)	0.012
HbA_{1c} during third trimester (%)	7.1 (6.5–7.9)	6.7 (6.2–7.4)	<0.001
Retinopathy*	5 (5.8%)	78 (7.3%)	0.58
Nephropathy†	8 (9.0%)	70 (6.4%)	0.34
Hypertension‡	6 (6.7%)	53 (4.8%)	0.45
Pre-eclampsia§	15 (18.1%)	205 (18.1%)	1.0
Large for gestational age infant‖	48 (57.1%)	713 (62.9%)	0.30

Table 1.3: Maternal characteristics in pregnancies with serious adverse outcomes (perinatal death and/or congenital malformations) versus other pregnancies. (Neilsen 2004, Evers 2002, Hanson 1990).

Data are median (interquartile range) or *n* (%). * Proliferative retinopathy; † nephropathy (urine albumin excretion >300 mg/24 h or >200 µg/min); ‡ hypertension (pharmacological treatment) before pregnancy and/or during first trimester; § blood pressure ≥140/90 mmHg and proteinuria; ‖ birth weight ≥90th centile.

1.5.2 Significant changes in the function of the immune system are necessary for successful pregnancy

The 'immunological irony' of pregnancy refers to the observation that fetal development proceeds unimpaired despite the fact that the fetus is an allograft (Bonney, 1997), (Munn, 1998), and an adaptation of the maternal immune system is clearly necessary to prevent rejection of the fetal allograft (Weinberg, 1984), (Vacchio, 1999). Some studies have described normal pregnancy as characterized by an activation of circulating leucocytes as part of a generalized immune response (Faas, 2000), (Sacks1998), and because of the role of adhesion molecules in mediating leucocyte responses to inflammatory stimuli and cell- cell or cell-matrix interactions, it is likely that there are changes in the expression of adhesion molecules present on resting leucocytes or in mobilization of adhesion molecules to the surface of monocytes.

Several investigations have showed contradictory results about changes in the different subsets of maternal peripheral blood leucocytes. In normal human pregnancy for example, Tallon has reported a decrease in CD8 T cells, (Tallon, 1984), whereas other investigators have described an increase (Fiddes, 1986) (Rich,1999). Some authors have found that T lymphocytes from pregnant women have impaired functional activity, and reduced levels of production of inflammatory cytokines (Weinberg, 1984), (Matthiesen, 1995), and reduced levels of production of inflammatory cytokines (Cracker, 2005).

The innate immune system is activated during pregnancy. Monocytes and granulocytes from pregnant women show increased surface expression of activation markers and adhesion molecules (Sacks, 2000). They show increased production of intracellular reactive oxygen species and enhanced phagocytosis compared with the nonpregnant state. Granulocytes from pregnant women have reduced microbial killing activity and chemotaxis as well as a decreased respiratory burst activity (Maallem, 1980) (Bjorksten, 1978). Luppi and his colleagues have found, in normal pregnancy, an increase in the proportion of granulocytes, a decrease in the level of monocytes, but no change in the percentage of distribution of CD3+ T cells and CD 19+ B cells, CD14+ monocytes and CD 56+ natural killer cells in pregnancy, compared with the non pregnant state (Luppi, 2002).

The same group has reported that CD+4 T lymphocytes decreased, while of CD8+ T lymphocytes increased, the percentage of granulocytes (CD15+) that positively stained for CD11a and CD454 was significantly higher in pregnant women than in the nonpregnant control group, also there was a significant increase in the level of monocyte expression of CD 62L, which is important for the initial attachment of circulating monocytes to activated endothelial cells (Von Anderian, 1991). Granulocytes from normal pregnancies showed a significantly higher surface expression of CD11a and CD11b compared with the non pregnant group, whereas monocytes showed higher surface expression of CD11a, CD11b, CD54, CD49d and CD62L in normal pregnancy compared with the non pregnant group (Luppi, 2002).

1.5.3 Interaction of diabetes, the immune system and pregnancy

Pregnancy is a very real immunological and metabolic challenge, and the interplay of pre-existing maternal diabetes, also challenging to the same aspects of physiology, carries a number of potential risks and difficulties in adaptation. In addition to possible effects on general maternal vascular and immune function, abnormal function of cells of the immune system, common in diabetes, may have specific deleterious effects on the development of the vascular bed at the maternal fetal interface. Within the placental bed, despite direct contact between immunologically disparate cells of maternal and feto-placental origin, there is usually no apparent reaction between them. In contrast, women with T1DM who become pregnant have an increased incidence of atherosis, the atheromatous-like lesion seen in their placental bed blood vessels, a lesion associated with reduced feto-placental blood flow resultant fetal compromise. This lesion involves the ingress of maternal monocytes/macrophages, as does atheroma in other vascular beds in the non-pregnant. The mechanisms subserving this placental bed pathology have not yet been investigated. Given the pathophysiological events that precede the development of atheroma in other vascular beds in the non-pregnant, it is likely that it involves increased adhesion of circulating monocytes to the endothelial cells of the decidual microvasculature. The work described in this thesis is therefore directed at further understanding of the regulation of this process.

Summary

In this chapter, I have described some of the relevant background linking immunity, atheroma, diabetes and human pregnancy. I have identified a set of adhesion molecules that are likely to mediate increased monocyte adhesion to the decidual endothelium in type I diabetic pregnant women.

Hypothesis to be tested

The current study investigates the hypothesis that monocytes from type I diabetic pregnancy, compared with normally pregnant women, express higher levels of specific adhesion molecules, and that these molecules play an important role in peripheral blood monocyte adhesion to the endothelial cells of the decidual vasculature.

Specific Aims

- To compare the level of expression of the adhesion molecules LFA1, VLA4 and MAC1 in peripheral blood monocytes from normal pregnant women and pregnant women with Type 1 diabetes mellitus

- Using specific function-perturbing antibodies, to examine aspects of the regulation of monocytes adhesion molecule expression in pregnancy, and the effects of this on monocytes adhesion to decidual endothelium.

Chapter II

General Methodology

2. Introduction

This chapter outlines general procedures for cell isolation and maintenance, and cell culture and co culture techniques used in this project. Specific methods related to individual experiments are described in relevant chapters.

2.1 Subject selection

Subjects involved in the study were women attending Royal North Shore Hospital and North Shore Private Hospital for antenatal care between 2004 and 2006. The project was approved by the Human Research Ethics Committees of both hospitals. All subjects gave written informed consent. All subjects were in the third trimester of pregnancy. Women with known medical disorders other than diabetes or with fetal abnormalities were excluded from study. Women in labour at the time of delivery, or with ruptured membranes prior to caesarean section were excluded from study.

2.1.1 Normal pregnant women

Healthy pregnant women were selected on the basis of a normal medical history and physical and laboratory examination. A woman was considered normal after having, at 26-28 weeks gestation, either negative screening results for gestational diabetes or negative OGTT (oral glucose tolerance test).

2.1.2 Pregnant women with Type I diabetes mellitus (T1DM)

These were women known to have T1DM pre-pregnancy, confirmed by a history of diabetic ketoacidosis and /or the presence of anti glutamic acid decarboxylase (GAD antibodies).

2.1.3 Pregnant women with gestational diabetes

Pregnant women were considered to have gestational diabetes after having a positive screening test for gestational diabetes (50g glucose challenge, 1h blood sugar >7.8mmol/L), performed at 26-28 weeks gestation and a positive 75g oral glucose tolerance test (fasting venous plasma glucose $\geq$ 5.5 mmol/l, and/or $\geq$ 8.0 mmol/l after 2 hours). (AOA criteria)

2.2 Cell isolation

2.2.1 Serum/cell preparation from blood

2.2.1.1 Collection of whole blood for pooled maternal serum (PMS)

Whole blood was collected from normal women in the second trimester of pregnancy (approximately 60 ml in total weekly from 4-6 volunteers) into tubes with a clot activator for serum. This blood was centrifuged immediately (2000g, 15 min), then the serum was transferred to a sterile 50 ml tube and stored at -20^0C for later preparation of culture medium (which contained 40 % pooled maternal serum)

2.2.1.2 Collection of whole blood for peripheral blood monocyte isolation

For isolation of monocytes, 40 ml of venous blood was drawn aseptically via a vacutainer device into tubes containing disodium ethylene diamine tetra-acetic acid (EDTA) (final concentration 5.5mmol/l).

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2.2.2 Monocyte isolation

Density gradient centrifugation has been validated in our laboratory and was chosen as the method for isolating monocytes in my study (Nutt et al 2004). The monocytes in human peripheral blood account for an average of 8% of the leucocyte population. They are larger (15-20 μm) than lymphocytes (6-20 μm) and they have

slightly lower density. These characteristics allow their separation on the basis of flotation from human leucocyte-rich plasma (LRP).

2.2.2.1 Monocyte isolation procedure

Blood was centrifuged at 2000 g for 5 minutes at room temperature. The upper plasma layer was removed and the buffy coat (leukocyte-rich plasma (LRP)) was collected. More than 90% of the leucocytes were recovered in this manner, with a small number of contaminating erythrocytes.

Two density solutions were produced by mixing a 60% solution of iodixanol (OptiPrep (Axis-Shield PoC AS-Denmark)) with a medium comprising RPMI 1640 plus 10% fetal calf serum (endotoxin free).

Density solutions of 1.084 g/ml (1.5ml of the 60% OptiPrep with 4.5 ml of medium) and 1.065 g/ml (3 ml of the 60% OptiPrep with 13.4 ml of medium) were prepared. One volume of LRP was mixed with 0.4 volumes of OptiPrep. Approximately 2 ml of the LRP/ OptiPrep mixture was loaded into each of two 15 ml polypropylene centrifuge tubes, then overlaid successively with 2 ml of the 1.084 g/ml density solution, 4 ml of the 1.065 g/ml, and then a layer of medium (0.5 ml) on top. (While this layer is not required for the separation process, it minimises the banding of cells at a liquid/air interface, and also inhibits cell adhesion to the walls of the tube).

Then the tubes were centrifuged at 2000 g for 15 minutes in a swinging bucket rotor centrifuge without application of the brake during the phase of deceleration. The layer below the upper interface (i.e. the layer which contained the monocytes that had floated into the 1.065 g/ml layer) was collected. The resultant cell suspension was diluted with medium and the cells counted in a Fuchs-Rosenthal counting chamber. The cell suspension was then centrifuged at 1000 g for 5 minutes, and the resultant pellet resuspended in 2% FCS+ $M199^-$ at a concentration of 10^5 cells per ml.

2.2.2.2 Assessment of cell yield

The yield of recovered monocytes was calculated by counting the cells and comparing the numbers with a monocyte count performed on whole blood at the same time by the hospital pathology department.

2.2.2.3 Preparation of cytospins

Cytospins were arranged from the cell suspensions for consecutive immunostaining. Cells from each isolation were adhered to glass microscope slides in a cytocentrifuge (Cytophonel). The sample chambers and slides were assembled, then loaded with 200 µl cell suspension and centrifuged at 1000 revolutions per minutes (rpm) for 5 minutes.

The slides were allowed to dry overnight at room temperature. The cytospins were immunostained the next day or wrapped in aluminium foil and stored at -20^0C for later staining.

2.2.2.4 Immunolabelling of monocytes

Cytospins were fixed in 50% (v/v) acetone/ 50% (vol/vol) methanol for 2 minutes, and then washed twice in wash buffer (0.15 M sodium chloride (TBS) with 0.1 % (wt/vol) bovine serum albumin (BSA) and 0.1 % (v/v) Tween-20) for 5 min. The area surrounding the sample was blot dried and the cytospins encircled with a PAP pen. Samples were covered with wash buffer for 5 minutes then incubated in blocking solution (DAKO) to block non-specific binding; they were then washed again in wash buffer for 5 minutes.

The primary antibody and control antibodies were diluted and applied to samples for one hour at room temperature.

Monocytes were identified with mouse anti human CD68 (clone EBM11, 1: 100 dilution) and lymphocytes with mouse anti human CD3 (1:100) as control. After one hour incubation, samples were washed twice for 5 minutes in wash buffer and a LSAB 2 kit provided the secondary antibody and the peroxidase amplification step: a

biotinylated goat anti mouse secondary antibody (reagent 1) was applied for 10 min preceding incubation with streptavidin conjugate (reagent 2) for an additional 10 min.

The peroxidase substrate 3, 3 - diaminobenzidene (DAB) was applied for approximately 5 minutes to allow visualisation of the immunolabelling as a brown colour. Samples were then washed in water for 5 min, stained for 2 min in Haematoxylin (1:1) in water, and washed again in water for 5 min.
Samples were covered with Glycergel and cover-slipped to be examined later under the microscope. CD68 positive cells were classified as monocytes, CD3 positive cells as lymphocytes.

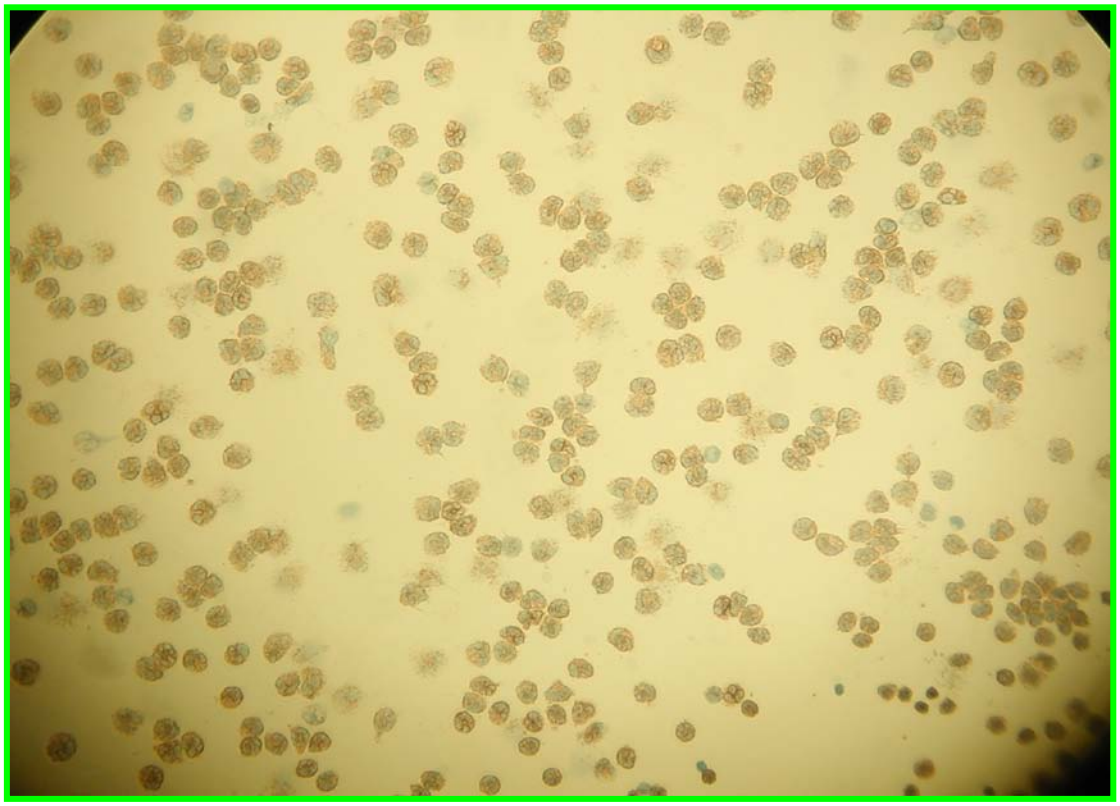

Figure 2.1**:** Monocytes isolated from peripheral blood by density gradient centrifugation. Population of >95% purity (Brown colour shows the immunostained CD68 positive cells, and the small number of lymphocytes are stained blue)

2.2.2.5 Assessment of cell purity

The purity of the monocyte population isolated from each sample was determined in the immunostained cytospins, using the following formula:

Percentage purity = (number of CD68 cells/ total cell count) x 100

2.2.3 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (a mixture of lymphocytes and monocytes) (PBMCs) were isolated from whole blood using the density gradient medium Ficoll Paque Plus.

2.2.3.1 PBMC isolation from whole blood using Ficoll-Paque

Anticoagulated blood (24 ml, 12 ml per tube) was diluted 1:1 in Hanks balanced salt solution (HBSS), with added 2.214 mM sodium bicarbonate, 0.499mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), 100 U/ml benzyl penicillin and 100 μg/ml Streptomycin under strictly aseptic, endotoxin-free conditions, then gently layered over the Ficoll Paque in polypropylene 50 ml Falcon tubes, at a ratio 3 ml of Ficoll Paque to 4 ml of diluted blood.

These layered samples were then centrifuged with fast acceleration and slow deceleration at 1550 g for 40 min at 20^0 C in a Beckman Coulter Allegra 6R centrifuge (Beckman, USA). The plasma layer was discarded using a plastic transfer pipette. The buffy coat was collected from each tube with a sterile plastic transfer pipette into 50 ml sterile Falcon tubes and combined into one tube. Cells were washed twice in PBS and centrifuged at 1550 g for 10 minutes at 20^0C. The resultant cell pellet was resuspended in a small volume of PBS, counted in a Neubauer haemocytometer.

For counting, cells were diluted 1/20 with Turks White Cell count (1% Toluidine blue, 1% glacial acetic acid), that stains nuclei.

The number of PBMCs in each of the four large squares of the counting chamber was counted and the average cell number was determined.

The following formula was used to determine the number of cells per ml of suspension:

Cells /ml= number of cells counted x 20x10000

Flowcytometry:

PBMCs (10^5 /ml) were resuspended in PBS/0.1%BSA, washed twice, then placed in a 1.5 ml eppendorf tube. PBMCs were pelleted by centrifugation at 14000 rpm for 20 seconds at 20^0C in an Eppendorf centrifuge 5417 R. (Hamburg, Germany). Cells were resuspended in PBS/0.1/BSA (final volume 50uL), the appropriate conjugated antibody was added (5 µl/ 1ml), and the cells were incubated with the antibody for 40 minutes at 4^0C.

After 40 minutes the PBMCs were washed in 1 ml PBS/0.1/BSA and then centrifuged at 14000 rpm for 20 seconds, fixed in 250 µl 1% paraformaldehyde (PFA) in PBS/0.1/BSA and stored at 4^0C until proceeding with flow cytometry

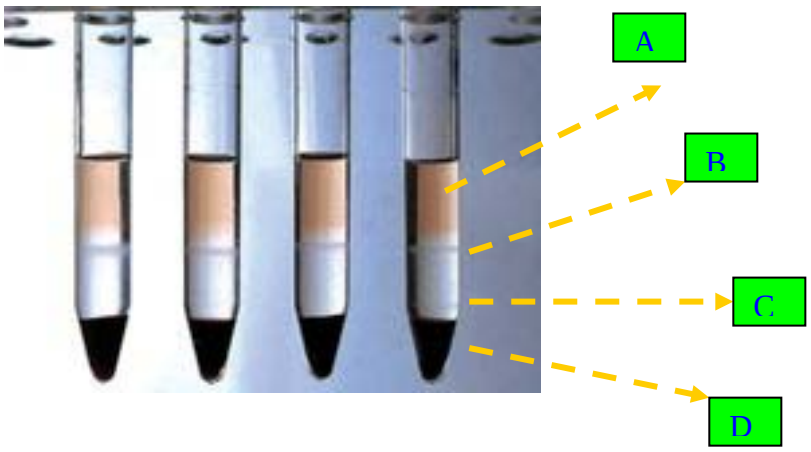

Figure 2.2**:** Ficoll Paque plus density gradient medium

A-Plasma layer, **B**-Buffy coat layer containing the PBMCs, **C**-Ficoll Paque layer separating Buffy coat from erythrocytes, and **D**- erythrocyte layer

2.3 Decidual endothelial cells (DEC)

Endothelial cells were isolated from decidual biopsies by standard methods in our research group (Gallery et al 1991). This method utilises the ability of endothelial cells to bind selectively to the lectin *Ulex europaeus* I (UEA I).

Decidual biopsies were collected from the interior surface of the uterus after delivery of the placenta by the obstetrician at elective caesarean section at Royal North Shore Hospital. The biopsy was immediately placed into a sterile plastic jar containing cold calcium- and magnesium-free Hanks' balanced salt solution (HBSS) and kept at 4^{o}C until processing.

2.3.1 DEC isolation procedure

Decidual biopsies were transferred into a 100 mm diameter Petri dish containing 10 mL of HBSS and sliced into 2-3 mm cubes using a scalpel blade. The tissue was then incubated at 37^{o}C for 6-8 minutes in 0.12% trypsin and 0.8% pronase in 10 mL of HBSS (corrected to pH 7.4 with sodium hydroxide), with gentle agitation every 2 minutes. The resultant fragments were washed in 10 mL of HBSS with gentle agitation to dilute the enzymes.

The HBSS was poured off and after four further washes the tissue was transferred to a sterile Petri dish containing 10 mL of 5% FCS in HBSS. Pieces of tissue were then transferred independently to a sterile 100 mm diameter Petri dish containing 10 mL of HBSS. Using the flat edge of a scalpel blade, capillary segments were squeezed from the enzyme-digested edges of the tissue fragment by downward pressure. The resultant mixed cell population included endothelial cells, fibroblasts, and tissue fragments. The suspension was collected into a 15 mL tube and centrifuged at 1000 rpm for 5 minutes.

The pellet was resuspended in 80 μL of 5% FCS in HBSS and transferred to a 5 mL tube. UEA 1-coated polystyrene-coated magnetic beads (Dynabeads, coated anti-HLA

class I antibody, Dynal, Oslo, Norway) (20 μL) were added and the suspension incubated on a rotator for 10 minutes at 4°C. After incubation, 1 mL of 5% FCS in HBSS was added to the tube, mixed gently to resuspend cells and beads, and the suspension transferred to a 14 mL round-bottom polypropylene tube. This was repeated with another 1 mL of 5% FCS in HBSS to ensure that all contents were transferred. The tube was then attached to a magnetic particle concentrator for 1 minute.

Endothelial cells that had bound to the UEA I-coated Dynabeads were collected along the wall of the tube, and contaminating cells were tipped off into a second 15 mL centrifuge tube. This rinse procedure was repeated four times. Cells bound to the UEA I-coated beads were washed twice with 5 mL of 5% FCS in HBSS and transferred to a 15 mL centrifuge tube. Both tubes (one containing the purified endothelial cells and the other containing the non-endothelial mixed cell population that had been separated from the bead-containing cells) were centrifuged at 1000 g for 5 minutes. Each cell pellet was resuspended in 100 μL of decidual endothelial cell growth medium containing 40% pooled human pregnancy serum.

The cell suspensions were seeded into the centre of 35 mm diameter culture dishes that had been pre-coated for 5 minutes with 100 μL of gelatin 0.2%, and these dishes were placed into a 100 mm diameter Petri dish. Cells were incubated for at least 5 hours or overnight in a humidified incubator at 37°C in a 95% air, 5% carbon dioxide atmosphere to allow cell attachment to the gelatin-coated plastic. The cells were then washed in HBSS to remove non-adherent cells, and fresh growth medium was added (2 mL/dish). Cells were grown at 37°C with replacement of growth medium every second day. The Dynabeads were slowly released from the endothelial cells with continued cell reproduction and removed from the culture dish with refeeding and subsequent passaging.

Endothelial cells were identified under a phase contrast microscope. They appeared as large polygonal cells with centrally located nuclei, forming a confluent homogeneous monolayer with a cobblestone appearance. In addition to their morphology, endothelial cells were positively identified by immunocytochemical staining for the

presence of Von Willebrand factor (vWF) antigen. Cells were purified by positive selection with lectin ulex europeus 1 (UEA1; Sigma, St. Louis, MO), giving a population of endothelial cells of >95% purity (determined visually). However, to achieve this, vigilant daily monitoring under a phase contrast microscope was required to identify and remove any contaminating cells, which were eliminated by mechanical weeding using a Pasteur pipette attached to a vacuum pump. This procedure was performed with the 35 mm diameter culture dish placed on the stage of a phase contrast microscope in the laminar flow hood. Contaminating cells were located under the microscope and aspirated with sharp, precise movements, taking care not to remove the surrounding endothelial cells nor allow the culture dish to dry out during the procedure.

2.3.1.1 Cell passaging

Decidual endothelial cells were passaged when they reached confluence, usually within 7-10 days of initial isolation. Passaging was performed using a solution of 0.01% (w/v) trypsin and 1 mM EDTA in HBSS (pH 7.4). The growth medium was removed from the culture dish and the cells washed with 3 mL of HBSS. 0.4 mL of HBSS was added plus an equal volume of the trypsin/EDTA solution, and the dish was incubated at 37^{o}C for up to 10 minutes. Every 3 minutes the dish was gently agitated and examined under a phase contrast microscope. Once most of the cells had lifted from the dish, 1.5 mL of growth medium was added to terminate trypsin activity. The cells were resuspended and transferred into a fresh 35 mm culture dish precoated with gelatin. The cells were incubated for at least 5 hours or overnight at 37^{o}C, then were rinsed with HBSS and re-fed with 2 mL of growth medium.

Once the 35 mm culture dish was confluent, the passaging practice was repeated and the cells were transferred into a gelatin-coated 80 cm^2 flask with an additional 8 mL of growth medium. The medium was replaced every second day until confluence (an average of four weeks after the initial decidua isolation).

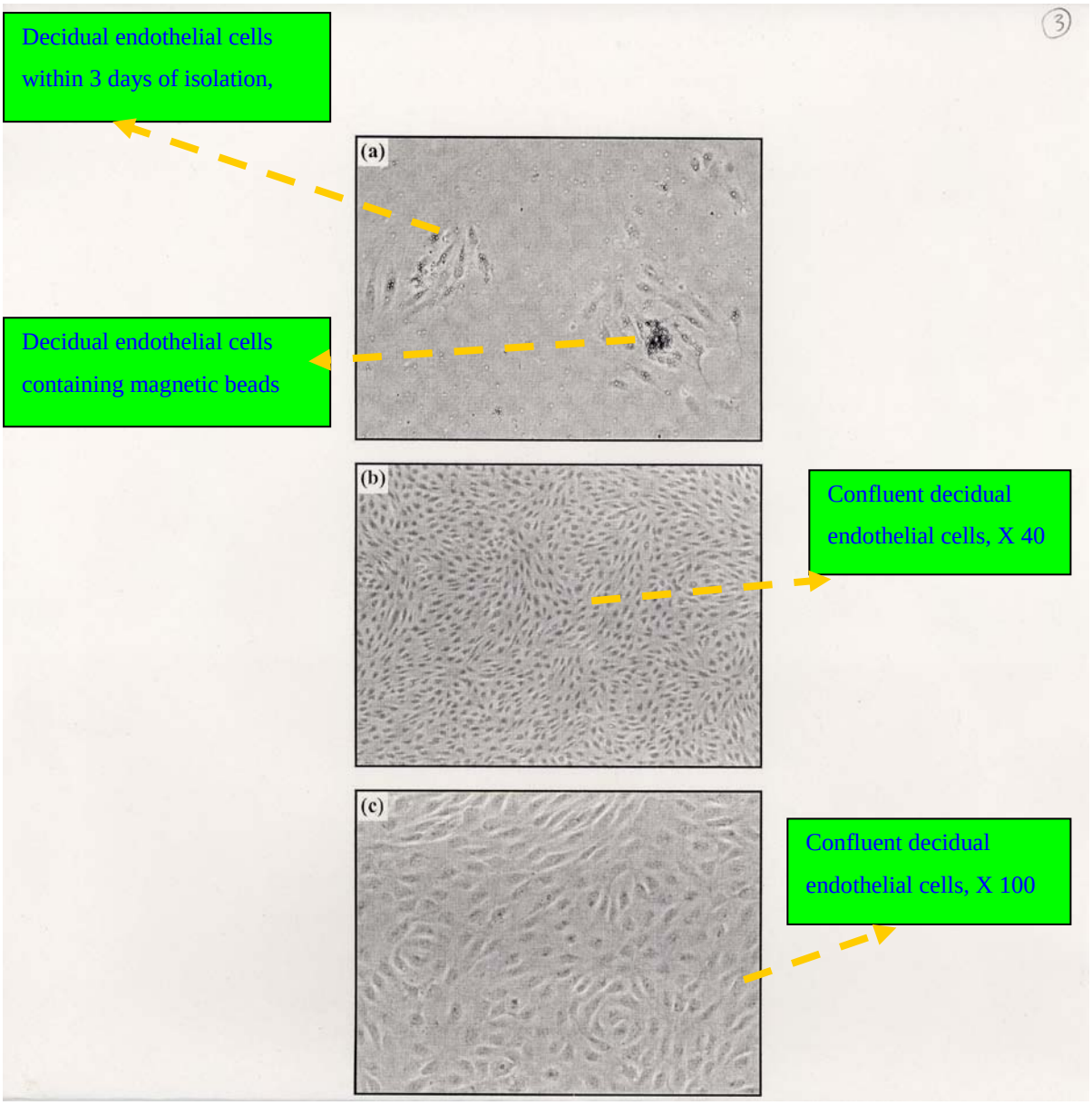

Fig 2.3: Isolated Decidual endothelial cells from normal pregnant women

2.3.1.2 Cell freezing and thawing

Confluent cells at passage two were washed in 10 mL of HBSS, and then lifted from the flask using 2 mL of HBSS plus 2 mL of trypsin/EDTA solution. The suspended cells were centrifuged at 1000 g for 5 minutes and the cell pellet resuspended in a cryogenic solution comprised of 100 μL dimethyl sulfoxide (DMSO) and 900 μL of growth medium or pooled maternal serum ($M199^+$ + ECG + 40% PMS). The cell suspension was immediately transferred into a 1.8 mL cryotube, placed in a pre-cooled cryofreezing container and stored at -70^oC overnight. The following day the cells were transferred to a liquid nitrogen tank. Cells were stored in liquid nitrogen until required for experiments.

To recover cells from liquid nitrogen, the cryogenic tube was thawed quickly in a 37^oC water bath and the contents immediately transferred to a 15 mL centrifuge tube containing 10 mL of growth medium. The tube was centrifuged at 1000 g for 5 minutes, the cell pellet resuspended in 8 mL of growth medium, and transferred to a gelatin-coated 80 cm^2 flask. Decidual endothelial cells, examined by phase contrast microscopy, appeared as large polygonal cells with centrally located nuclei, forming a confluent homogeneous monolayer with a characteristic cobblestone appearance.

2.3.1.3 Pooling of decidual endothelial cells, and preparation of aliquots for co-culture experiments

Cells from ten decidual endothelial cell isolations were thawed and grown to confluence in 75 mm flasks, which had been pre-coated with gelatin. When the decidual endothelial cells were confluent, each flask was trypsinised, and the cells were divided between two coated flasks. All flasks were trypsinised when confluent, the decidual endothelial cells were pooled and 20 aliquots made and stored, as previously described, in liquid nitrogen.

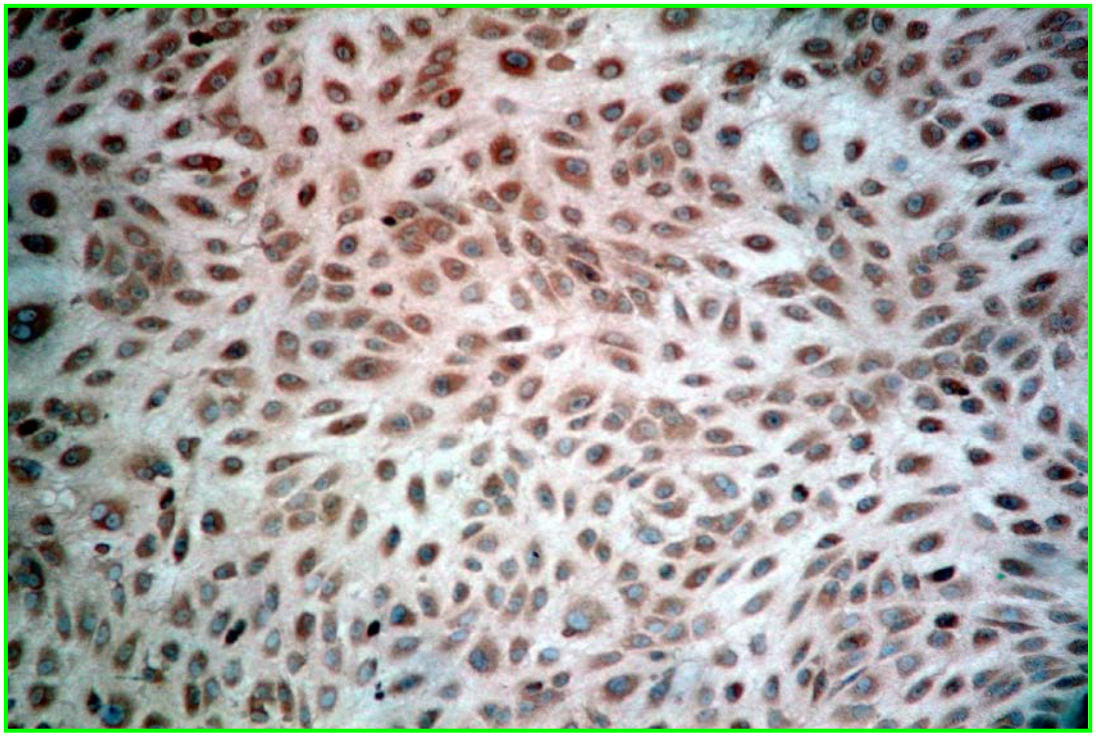

Figure 2.4 This Figure shows a population of decidual endothelial cells positively selected with Ulex Europaeus-1-coated magnetic beads, pooled from 20 normal subjects at passage 2, prior to preparation and freezing of aliquots

2.3.1.4 Preparation of UEA 1-coated dynabeads

UEA I was covalently conjugated to Dynabeads M-450 (containing a ferrous core). The Dynabeads (4 x 10^8 beads/mL) were first washed in borate buffer (0.1 M, pH 8.5) for 5 minutes in a 5 mL tube, and then collected using a magnetic particle concentrator. The supernatant was discarded and the wash repeated. Equal volumes of UEA 1 (0.2 mg/mL in 0.1 M borate buffer, pH 8.5) and Dynabeads suspension (4 x 10^8 beads/mL) were mixed on a rotator at 4^{o}C for 24 hours. The beads were collected using a magnetic particle concentrator and the supernatant discarded. The beads were washed twice with 0.1% (w/v) bovine serum albumin (BSA) in 0.1 x calcium- and magnesium-free phosphate buffered saline (PBS), as above, and then mixed overnight on a rotator at 4^{o}C in the same buffer. The beads were collected as before and resuspended in 0.1% (w/v) BSA/HBSS to a final concentration of 4 x 10^8 beads/mL and stored at 4^{o}C.

2.4 Cell Culture

2.4.1 Preparation of decidual endothelial cell monolayer substrate:

An aliquot of pooled decidual endothelial cells was recovered from liquid nitrogen, the cryogenic tube was thawed quickly in a 37^{o}C water bath and the contents immediately transferred to a 15 mL centrifuge tube containing 10 mL of growth medium (40% pooled maternal serum +60% M199^{+} + 700µl endothelial cell growth supplement (ECGS)). The tube was centrifuged at 1000 g for 5 minutes and the cell pellet was resuspended in 10 mL of growth medium, transferred to a gelatin-coated 80 cm^{2} flask, then the flask transferred into the incubator at 37^{o}C in 5% CO2.

The following morning (day 2), the endothelial cells were washed and re-fed with growth medium. At this stage, glass coverslips were put in 6-well or 12-well plates (according to specific experiments), were coated with collagen IV, (125 or 250 µl/well), and the wells left to dry overnight in the laminar flow hood.

On the third day, 0.5 mL (12-well plates) or 1 mL (6-well plates) 70% ETOH (v/v ETOH and sterile water) was added into each collagen-containing well, mixed well, aspirated, and the well rinsed with 1ml sterile water which was then aspirated. The decidual endothelial cells in the flask were washed in 9 mL of HBSS and 2mL of HBSS plus 2 mL of trypsin/EDTA solution was added for 5 minutes. When the cells detached, they were put into a 15 ml tube, any cells remaining in the flask were washed out with another 2 ml of HBSS. The trypsin was inactivated by addition of 100 µl FCS, the endothelial cell suspension was centrifuged at 1000 g for 5 min, the supernatant discarded, and cells resuspended with growth medium and added to the collagen-coated wells in the multi-well plates at a final concentration of 2.5x 10^{5} cells/ml (12 well plates) or 5x 10^{5} cells/ml (6 well plates)

On the third day monocytes were isolated as described above and pre-incubated in the different conditions (as described below), prior to adhesion co-culture experiments in the multi-well plates.

2.4.2 Pre- incubation of monocytes

Isolated monocytes were suspended in culture medium 2% FCS + $M199^-$ (this medium was suitable for monocyte culture because it maintains good cell viability with minimum activation) at a concentration of 10^5 monocytes / tube in a total volume of 1 ml.

Cells were pre-incubated in Immuno Minisorp tubes (these are designed to inhibit cell adherence) at 37^0C in 5 % CO_2 for 15 minutes and then for 1 hour in the following conditions:

(i) Basal,
(ii) With 100μg/ml of bacterial lipopolysaccharide (LPS),
(iii) With 25mM Glucose
(iv) With 5μl/ml anti Mac-1 blocking antibodies and
(v) With isotype Mouse IgG.

2.4.3 Adhesion assay

Confluent endothelial cell monolayers in multi-well plates were washed with 2% FCS+M199$^-$, and monocytes seeded onto this monolayer in 2% FCS+M199$^-$. After one hour at 37^0 C in 5 % CO_2, non adherent cells were removed by gentle washing.

After that the cells were treated in one of two ways:

(i) 12 well plates

Wells with coverslips (containing monocytes adherent to endothelial cells) were fixed with 4% paraformaldehyde and stored at 4^0C until later immunostaining for monocyte cell counts

(ii) 6-well plates and some 12-well plates

Cells were removed from the coverslips by trypsinisation with 1mM EDTA for 5-7 minutes, then 0.1 % BSA in 50 ml PBS, was added to each well to inactivate the trypsin. The resultant mixed cell population (containing monocytes and decidual endothelial cells) was transferred into a corresponding 15 ml tube.

To ensure complete cell harvesting, 0.5 ml PBS/0.1% BSA was added to each well, rinsed well and transferred into the same 15 ml tube. The mixed cell populations were centrifuged for 5 min at 1000 rpm and transferred to Eppendorf tubes, where they were washed twice more with 1 ml of PBS/0.1% BSA prior to flow cytometry.

2.5 Flow cytometry

For analysis of adhesion and adhesion molecules, and after the relevant experiments cell populations of (a) monocytes alone, (b) PBMCs alone (c) endothelial cells alone and (d) the 2 cell populations together (monocytes and endothelial cells) in co culture were processed for flow cytometry.

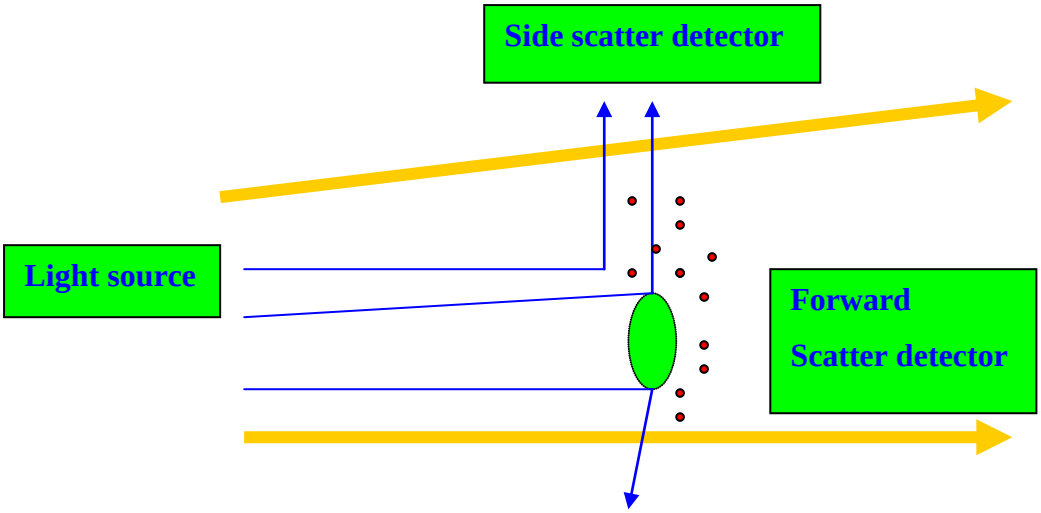

Fig 2.4 Forward scatter is sensitive to surface properties of particles and is equivalent to particle size, whereas side scatter is more sensitive to inclusions within cells, and is able to distinguish granulated from non-granulated cells

2.5.1 Antibody labelling for Flow cytometry

2.5.1.1 Identification of monocytes and decidual endothelial cells.

Adhesion of monocytes was quantified by immunostaining. Monocytes were identified as CD14 or CD 45 positive cells depending on the experiments, decidual endothelial cells were identified as CD45 negative cells.

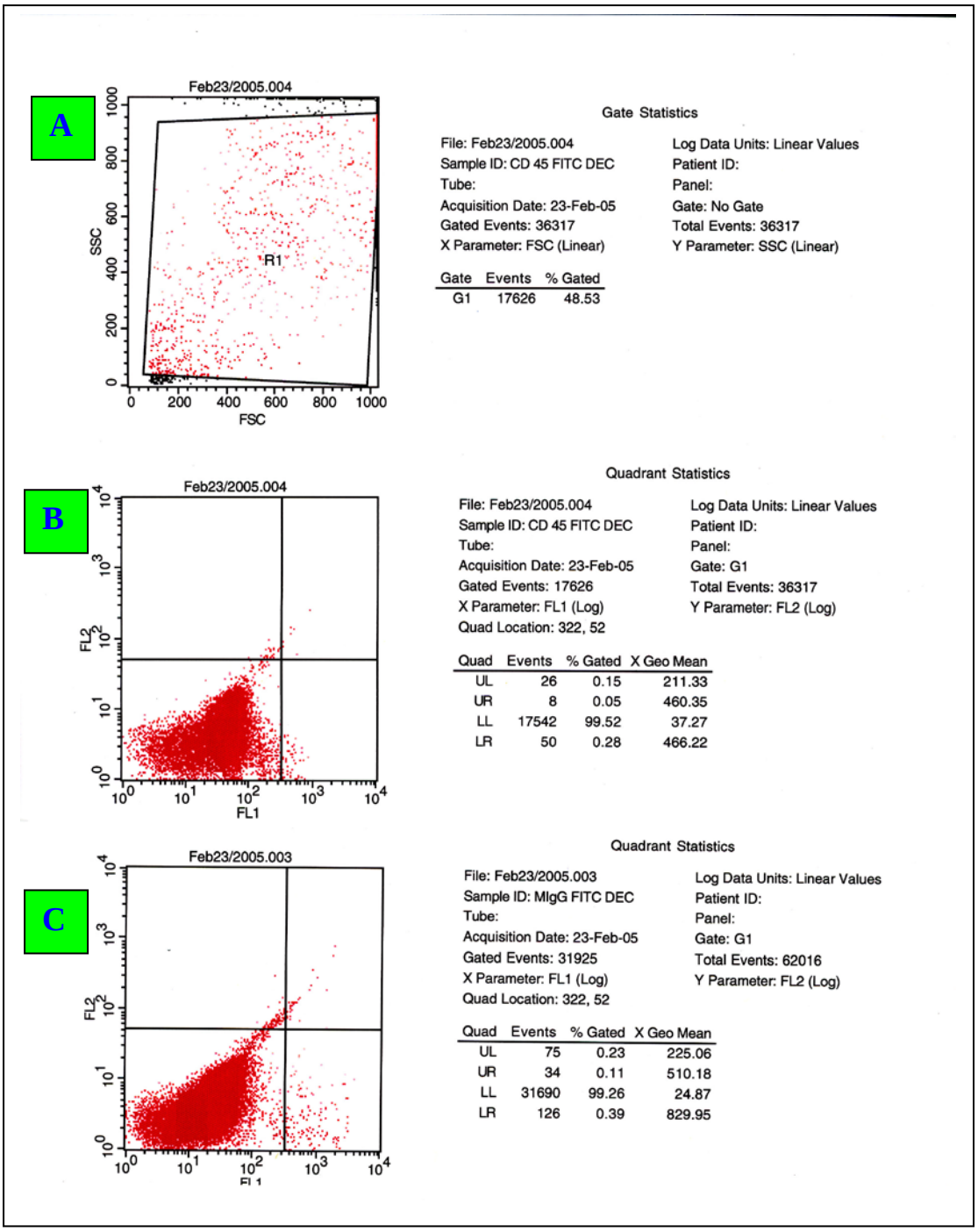

Fig 2.5 Decidual endothelial cells isolated from normal pregnant women.

A Cell population based on forward scatter (FSC) and side scatter (SSC), this figure shows a representative flow cytometric analysis of one population decidual endothelial cells. Based on FSC or cell size, a gate R1 which represents decidual endothelial cells, was set on the FSC vs. SSC plot to allow analysis of cells

B 99% of Decidual endothelial cells are seen in lower left quadrant, and it was identified as CD45 negative cells **C** Negative control

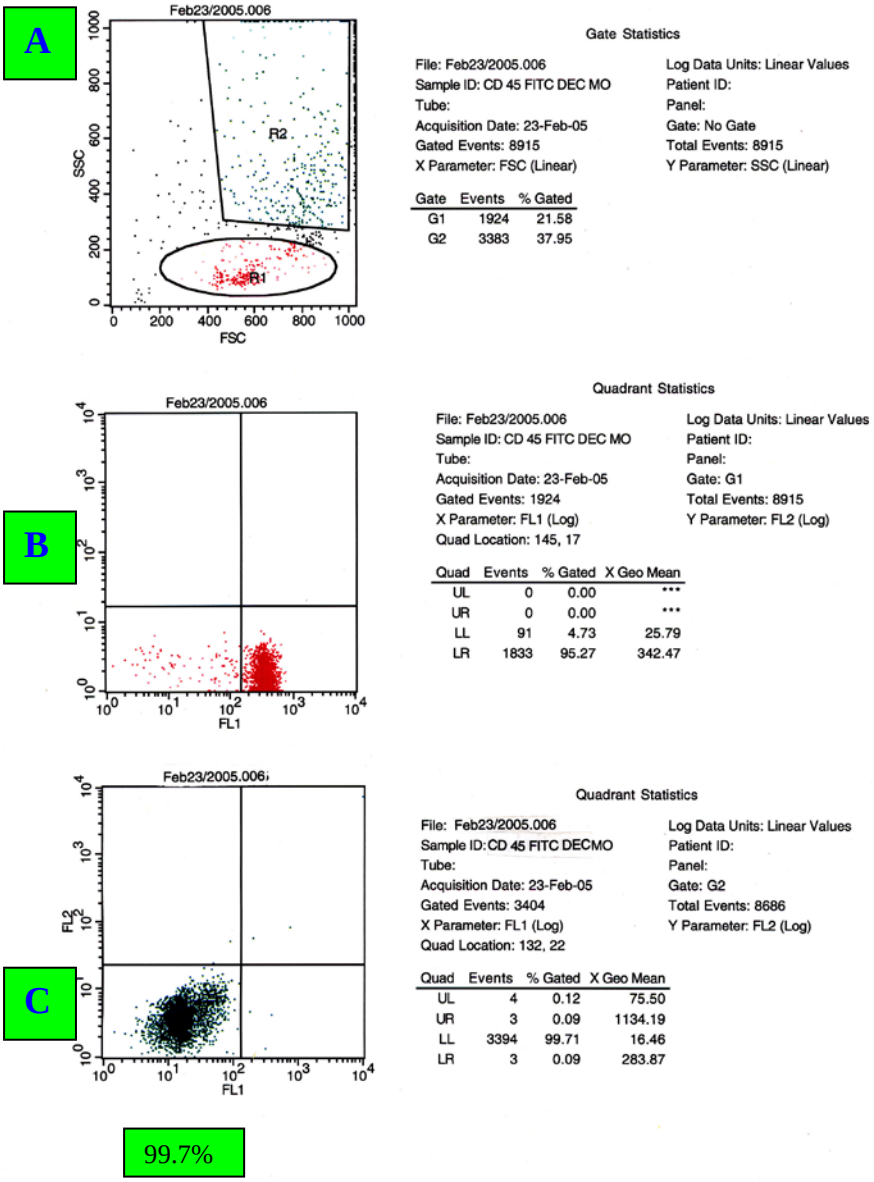

Fig 2.6 **A** Cell subpopulations based on forward scatter (FSC) and side scatter (SSC), This figure shows a representative flow cytometric analysis of two populations - monocytes and decidual endothelial cells. Based on FSC or cell size, a gate R1 which represents monocytes, and R2 which represents DEC, was set on the FSC vs. SSC plot to allow analysis of the two cell types. **B** Monocytes are seen in the LR quadrant as CD 45 Positive cells, while **C** 99.7% of Decidual endothelial cells are identified in LL quadrant as CD 45 negative cells.

2.5.1.2 Identification of adhesion molecules on monocytes and decidual endothelial cells

To examine expression of adhesion molecules by monocytes, double labelling with CD14/PE and adhesion molecule/FITC was used. The adhesion molecules examined were CD11b (Mac-1), CD11a (LFA-1) and CD49 d (VLA-4).
The expression of the decidual endothelial cell adhesion molecule ICAM-1 was examined in co culture with monocytes in normal and stimulated conditions, by double staining with CD45PE.

Cells (monocytes alone, decidual endothelial cells alone, or the 2 cells from co-cultured wells) were resuspended in 50 µl (final volume) of PBS/0.1% BSA, the conjugated antibody of interest was added (5 µl/ ml), and the cells were incubated with the antibody for 40 minutes at 4^0C.
After 40 minutes the mixed cells were washed in 1 ml PBS/0.1% BSA and centrifuged in an Eppendorf centrifuge (14000 g , 20 seconds) and fixed in 250 µl 1% PFA in PBS/0.1%BSA and refrigerated at 4^0C until proceeding with flow cytometry.

The cells were analysed on a Becton Dickinson flow cytometer (BD FACSVantage SE) with an argon laser at 488nM for analysis of PE and FITC. Ten thousand events were collected from a mixed (monocyte-decidual endothelial cell) population or from a pure monocyte population (depending on the experiments). Specific cells were identified from forward and side scatter dot plots.

Data collected from the flow cytometer were stored electronically for later analysis on Cell Quest software.

2.5.1.3 Flow cytometric analysis of monocyte adhesion molecules

For analysis of monocyte adhesion molecules and level of monocyte adherence to decidual endothelial cells, isolated PBMCs and mixed cells including monocytes and decidual endothelial cells were processed for flow cytometry

acquisition using 2.5x 10^5 cells per test or 5x 10^5 cells per test depending on the experiment.

Monoclonal antibodies Mac-1, LFA-1 and VLA-4 conjugated to fluorescein isothiocyanate (FITC) were used in mixed PBMC cell populations to detect monocyte adhesion molecules, using double staining with a monoclonal antibody to CD14 conjugated to Phycoerythin (PE) to identify the peripheral blood monocytes.

PBMCs were aliquoted into 1.5 ml Eppendorf tubes and pelleted by centrifugation at 14000 g for 15 seconds at 20^0C.

The cell pellet was resuspended in 50 µl of PBS/0.1%/BSA. 5 µl of anti Mac-1 FITC, anti LFA-1 FITC, anti CD14 PE and anti VLA-4 FITC were incubated with the cells. Mouse IgG FITC (5 µl) and mouse IgG PE (5 µl) were used as negative controls.

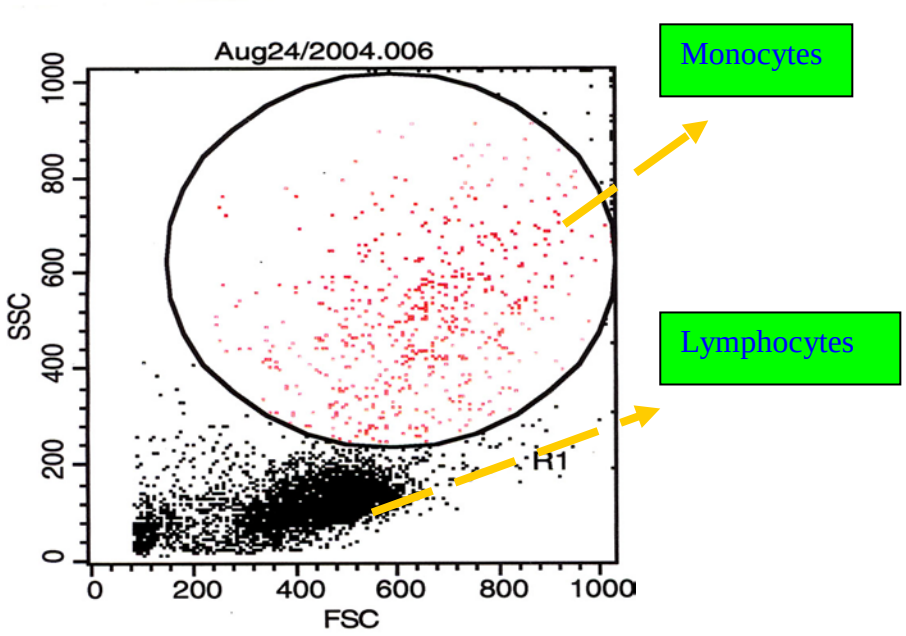

Fig 2.7 Cell subpopulations based on forward scatter (FSC) and side scatter (SSC). It shows a representative flow cytometric analysis of PBMCs. Based on FSC or cell size, a gate R1 was set on the FSC vs. SSC plot to allow analysis of monocytes only.

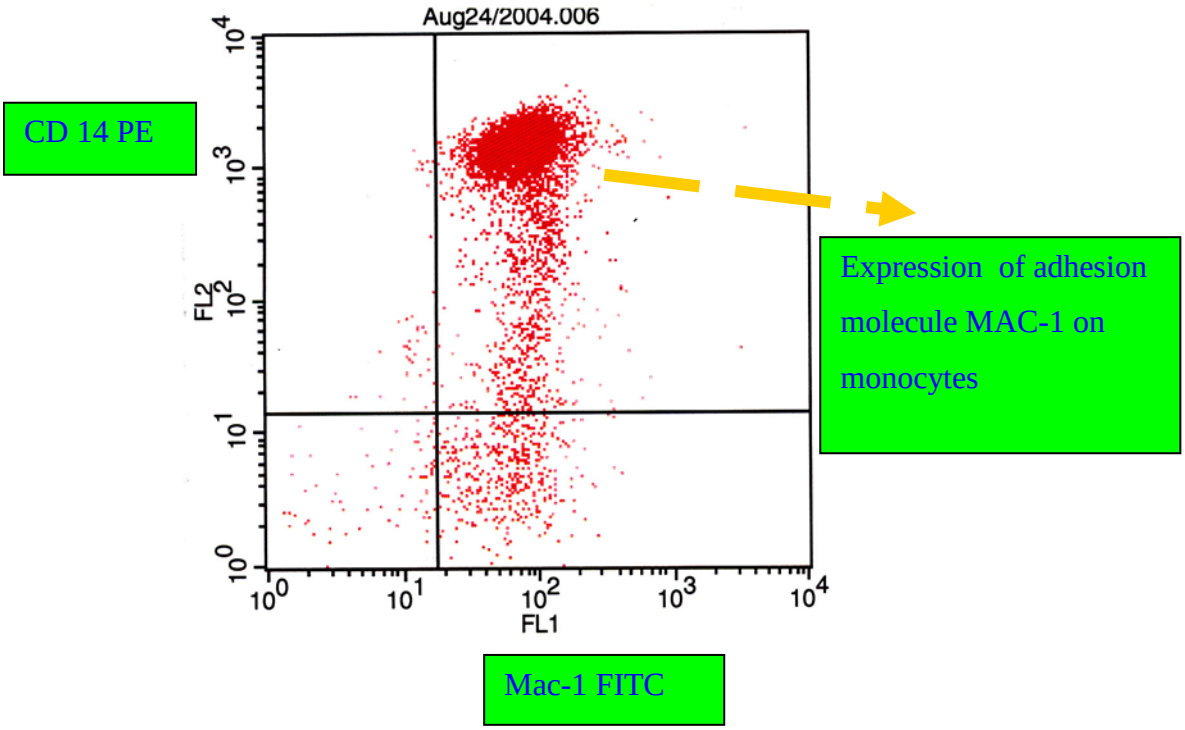

Fig 2.8 Quadrant markers divide two-parameter plots into four sections to distinguish populations that are considered double negative, single positive, or double positive. The lower left quadrant displays events that are negative for both parameters. The upper left quadrant contains events that are positive for the Y axis (CD14PE), but negative for the X axis (Mac-1 FITC). The lower right quadrant contains events that are positive for the X axis (Mac-1 FITC), but negative for the Y axis (CD14 PE). The upper right quadrant contains events that are positive for both parameters (CD14PE/Mac-1 FITC).

2.5.1.4 Flow cytometric identification of decidual endothelial cells

For identification of decidual endothelial cells after co-culture experiments with monocytes, staining with a monoclonal antibody to CD45 (conjugated to Phycoerythin (PE) and a monoclonal antibody to ICAM-1 (conjugated to fluorescein isothiocyanate) was used.

The antibodies were incubated with the cells for 40 minutes at room temperature in the dark, and then cells were washed twice in 1 ml of PBS/0.1%/ BSA, pelleted by centrifugation at 14000 rpm for 15 seconds at 20^0C (Eppendorf Centrifuge 5417R), and fixed with 1 % paraformaldehyde.

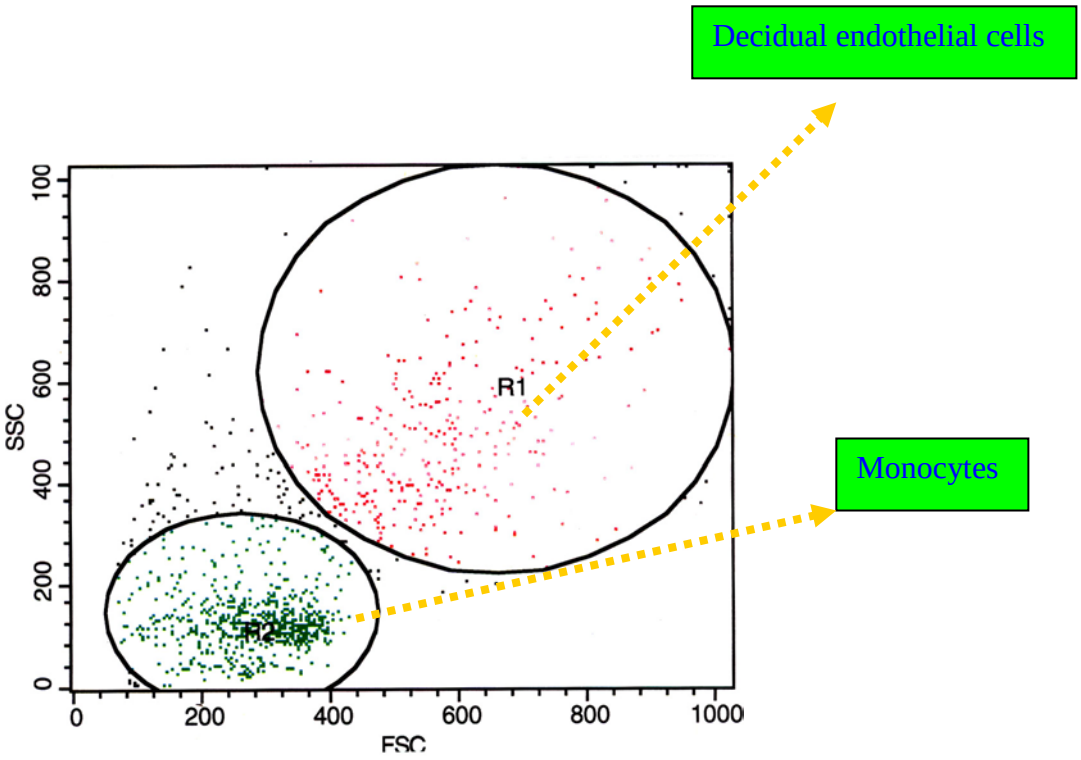

Fig 2.9 Cell subpopulations based on Forward scatter (FSC) and Side scatter (SSC). Figure 2.11 shows a representative flow cytometric analysis of mixed cell population of monocytes, endothelial cells. Based on FSC or cell size, a gate R1 was set on the FSC vs. SSC plot to identify the decidual endothelial cells, R2 to identify the monocytes.

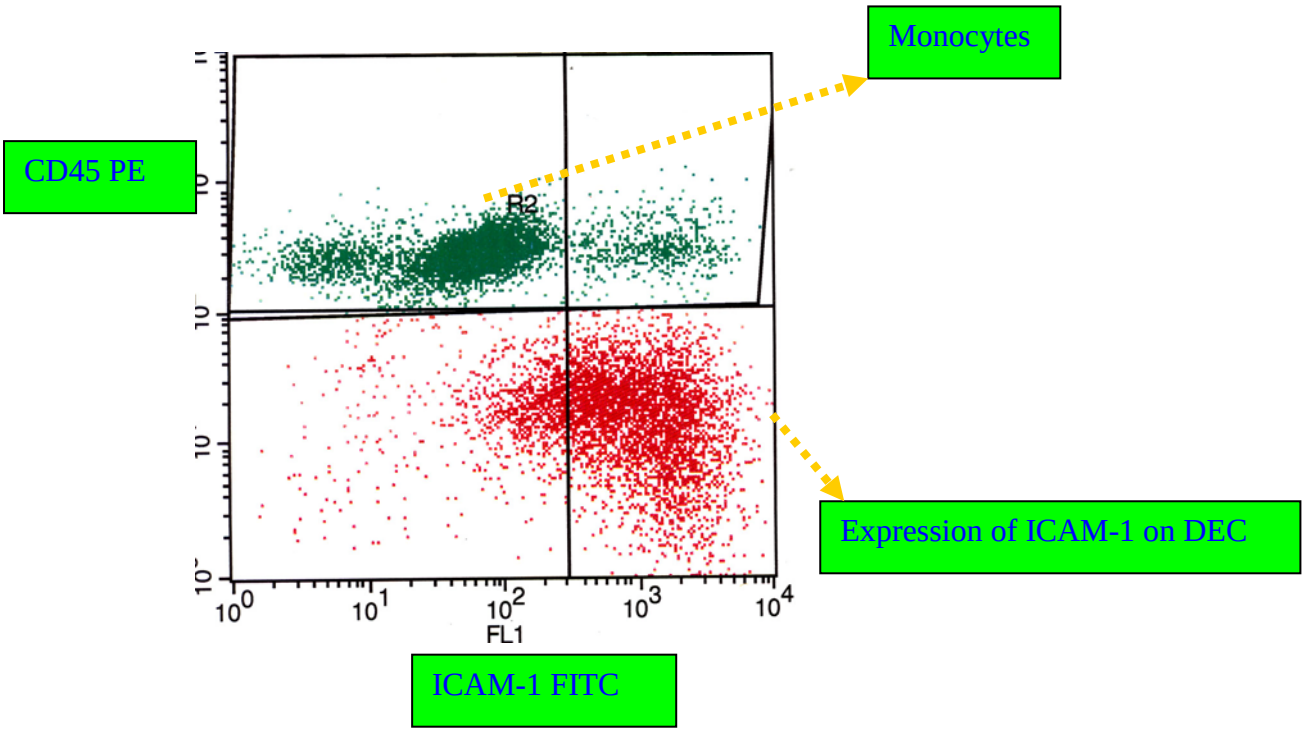

Fig 2.10 Dot plots, shown in Figure 2.12 provide a two-parameter display of data in co culture. Quadrant markers divide two-parameter plots into four sections to distinguish populations that are considered double negative, single positive, or double positive. The lower left quadrant displays events that are negative for both parameters. The upper left quadrant contains events that are positive for the Y axis (CD45PE), but negative for the X axis (ICAM-1 FITC). The lower right quadrant contains events that are positive for the X axis (ICAM-1 FITC), but negative for the Y axis (CD45 PE). The upper right quadrant contains events that are positive for both parameters (CD45PE/ICAM-1 FITC).

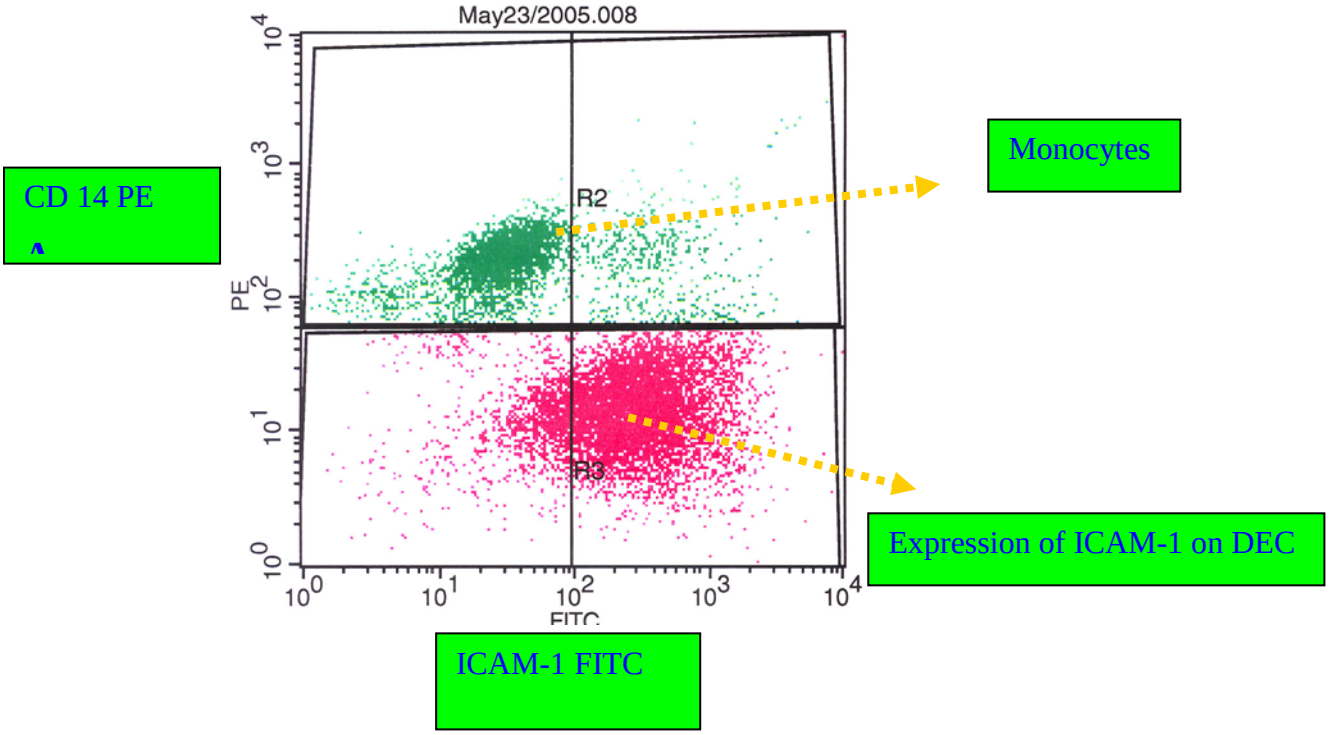

Fig 2.11 Dot plots provide a two parameter display of data.
Adhered monocytes were identified as CD14 PE positive cells.

2.6 Statistical analysis

Where relevant, group data are shown as mean and standard deviation, after initial examination to determine the normality of data distribution. Examination of the effects of stimulation within groups was performed by paired Student t test, while differences between groups (normal and diabetic) were analysed by the Kruskal-Wallis test for non parametric variables. Differences were considered statistically significant at $P < 0.05$.

Chapter III

Adhesion molecule expression by peripheral blood monocytes and regulation of monocyte adhesion to decidual endothelial cells in normal and in type I diabetic pregnancy

3. Introduction

As described in Chapter 1, in pregnant women with T1DM, a vascular abnormality, atherosis, similar to atheroma in the non-pregnant, develops over a relatively short space of time within the maternal blood vessels supplying the placenta. There is a growing body of evidence that monocytes are preferentially recruited to the arterial intima early in the development of atheromatous lesions (Schwartz et al, 1991).

Monocyte adherence to endothelial cells is the first step required for migration of monocytes from blood into tissue. Exposure to a variety of cytokines can result in activation of adhesion molecules on monocytes and endothelium that cause firm adhesion to the endothelium (Beekhuizen, 1993). Adhesion molecules on monocytes that have been implicated include very late antigen (VLA-4), leucocyte function antigen (LFA-1) and complement receptor MAC-1 (Beekhuizen et al., 1990), whereas endothelial cell adhesion molecules likely to be involved include intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), P selectin (CD62) and E-selectin (ELAM-1) (Pober JS et al., 1990; Benkhuizen H, et al., 1991).

The definitive mechanisms regulating the initiation and progression of vascular lesions in diabetic pregnancy have yet to be elucidated. Earlier work in our group had shown increased *in vitro* adhesion of monocytes from T1DM pregnancy to decidual endothelial monolayers (Galettis et al., 2004). The experiments reported in this chapter were performed to examine in more detail the regulation of this process. In particular are described experiments examining the expression of relevant adhesion molecules by monocytes from normally pregnant women and those with diabetes mellitus, and factors that influence both the adhesion of monocytes to decidual endothelial cells isolated from the pregnant human uterus and their expression of relevant adhesion molecules.

3.1 Aims

The specific aims of the experiments reported in this chapter were:

i) To identify the monocyte adhesion molecules associated with increased peripheral blood monocyte adhesion to decidual endothelial cells in T1DM pregnant women.

ii) To define mechanisms involved in regulation of monocyte adhesion to decidual endothelial cells in normal pregnancy and in T1DM pregnancy

A series of preliminary experiments was performed to select the most appropriate antibodies for monocyte identification.
Then dose response curves were performed to optimise antibody-antigen interactions for relevant monocyte adhesion molecules (LFA-1, MAC-1 and VLA-4).

3.2 Identification of monocytes

Peripheral blood monocytes were isolated by gradient density centrifugation, as described in chapter 2, then the cells were analysed for level of expression of CD14 and CD68 to determine which was of greater utility for recognition of the majority monocytes

A double-staining method was used for surface markers CD14 (conjugated and unconjugated) and CD68 (conjugated and unconjugated) (Figure 3.2.1)
There was no specific staining when using the unconjugated CD68 compared with the negative control, whereas the mean fluorescence intensity of conjugated CD14 was significant, compared with the negative control.

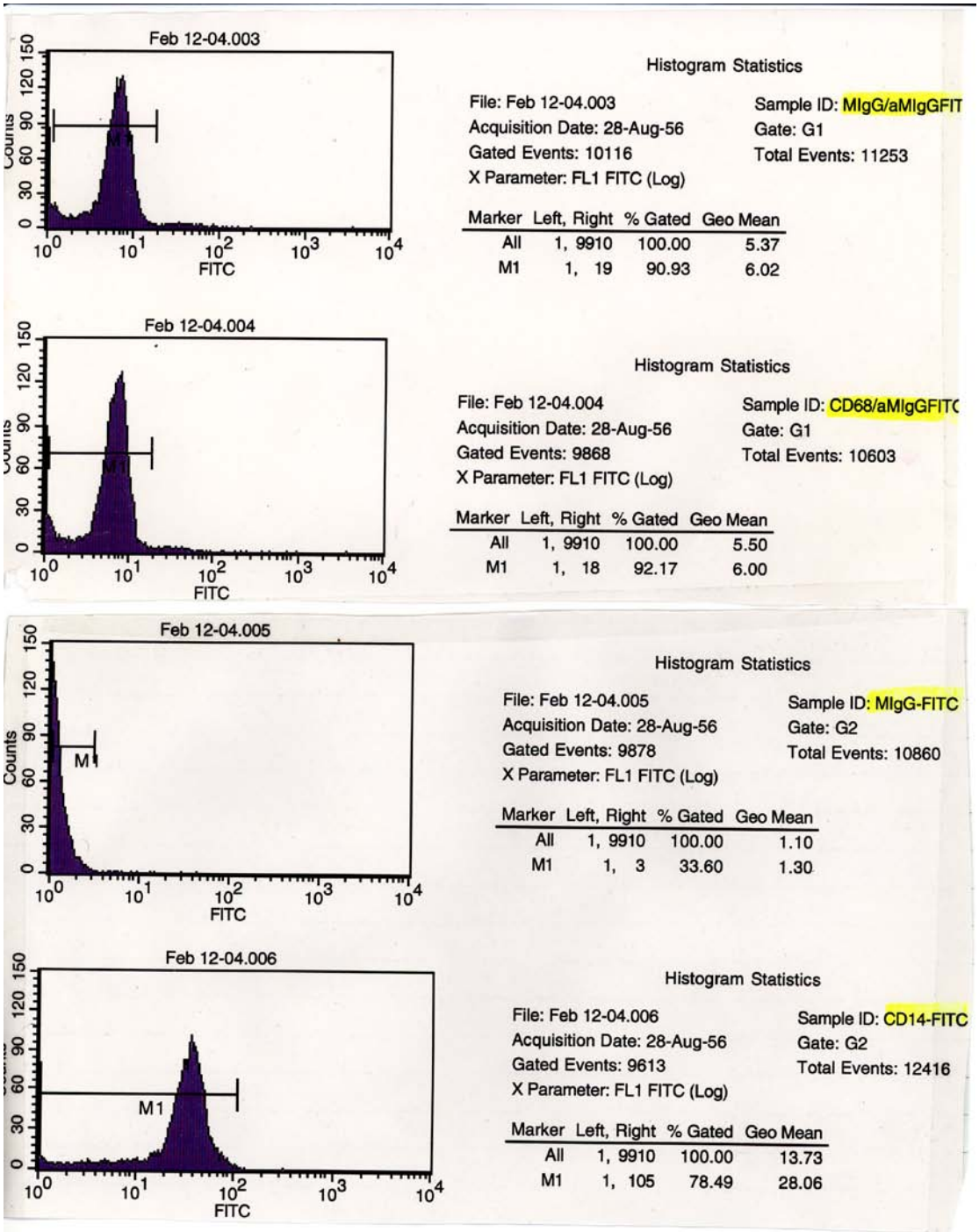

Figure 3.1 a representative histogram charting the number of cells counted and the fluorescence intensity (MFI). The MFI of the CD14 FITC was significant compared with the unconjugated CD68.

The experiment was repeated using conjugated CD14, unconjugated CD68 and unconjugated CD 14 (the reason for using unconjugated CD14 was to test the secondary antibody, which is Goat anti mouse FITC, to clarify whether the lack of

staining of the unconjugated CD68 was due to antibody malfunction or because of an error in the secondary antibody).

The mean fluorescence intensity for unconjugated CD14 was significantly higher than the negative control, and equal to that of the conjugated CD14, whereas the unconjugated CD68 failed to show a specific and significant binding compared with the background staining of negative control (Figure 3.2)

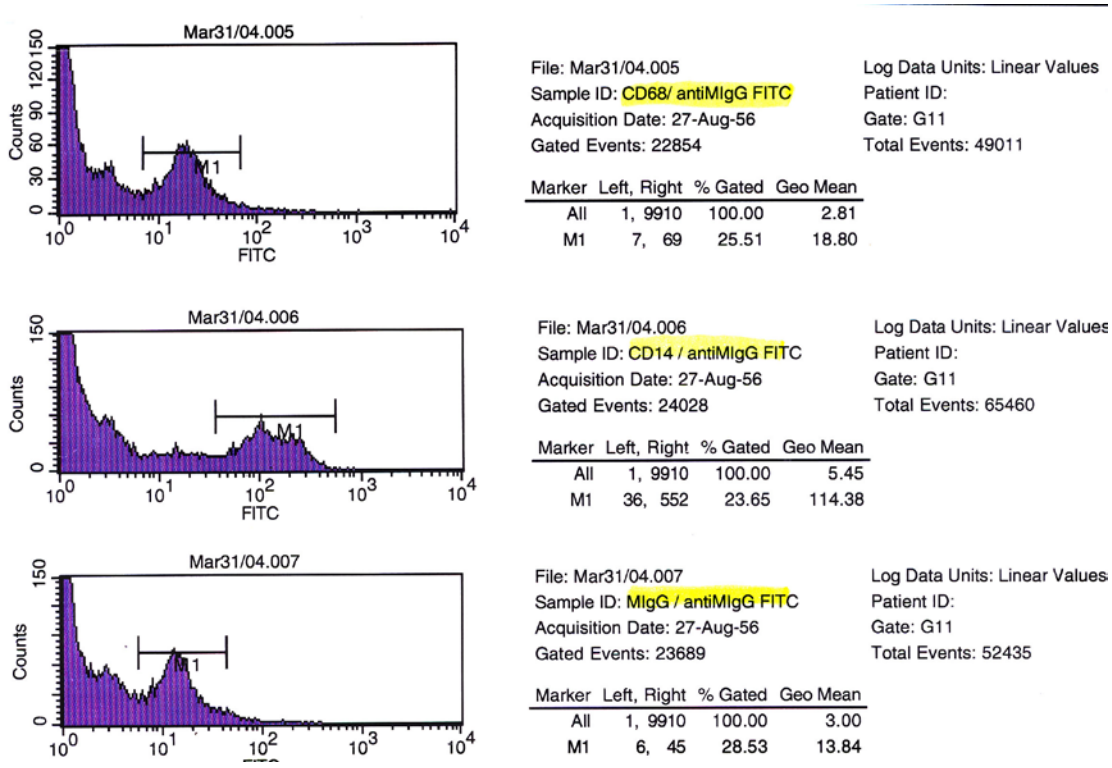

Figure 3.2 a representative histogram charting the number of cells counted and the fluorescence intensity (MFI). The MFI of the unconjugated CD14 was significant, whereas there was a lack of staining with the unconjugated CD68

Because CD68 FITC is directed against an intracytoplasmic antigen, permeabilisation of the cells was necessary before incubation.

Both conjugated CD14 and CD68 were able to recognise the majority of the monocyte population, however using flowcytometry, conjugated CD14 was positive in a greater proportion of the monocyte population (figure 3.2)

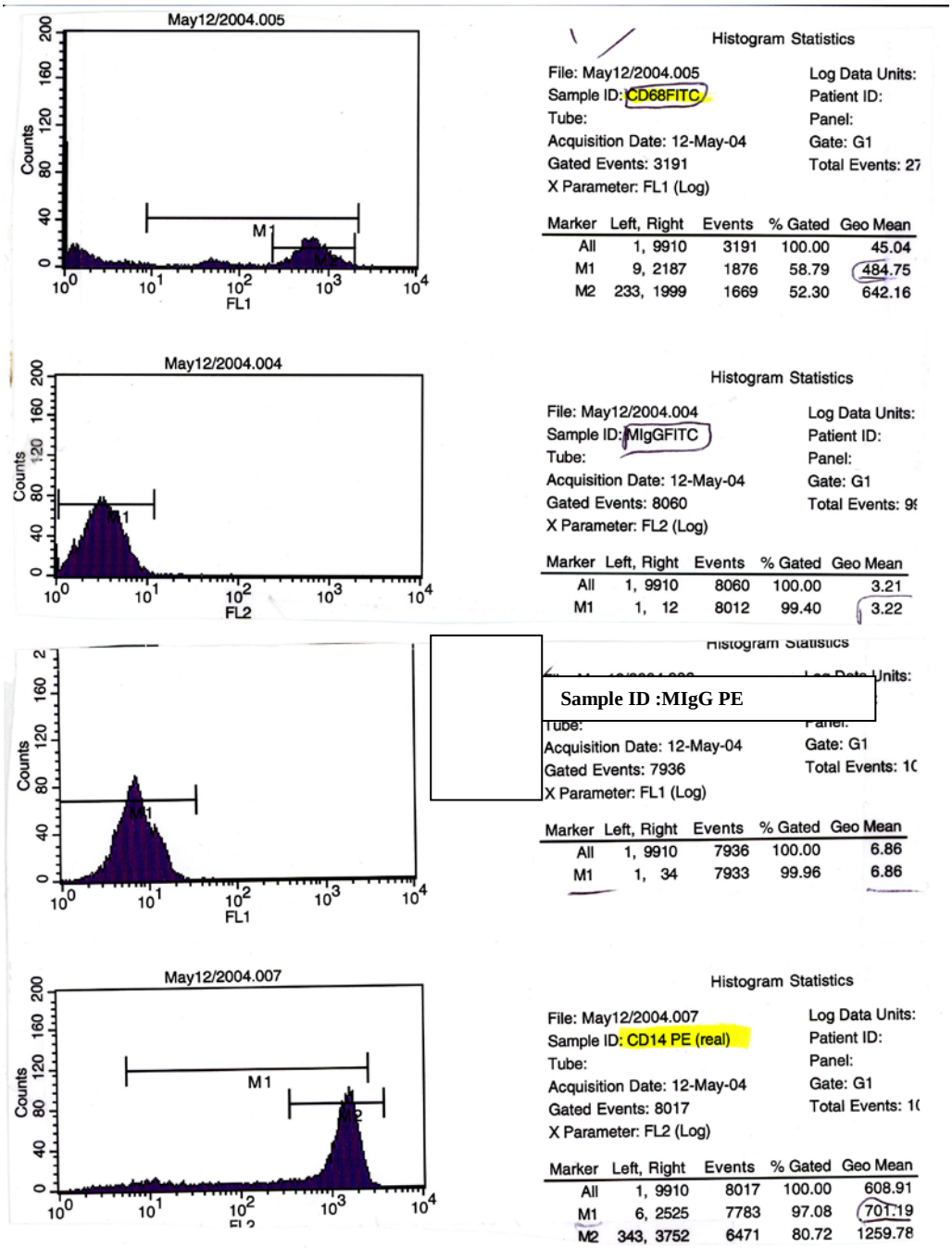

Figure 3.3 a representative histogram charting the number of cells counted and the fluorescence intensity (MFI). The MFI of the CD14 FITC was significant compared with the unconjugated CD68.CD14 PE labelling was used for subsequent adhesion molecule experiments.

3.3 Adhesion molecule dose-response curves

Antibody-antigen dose response curves for adhesion molecules LFA-1, VLA-4 and Mac-1 were done to determine the optimal antibody concentration for subsequent experiments . This is an essential optimisation step, because too high a concentration of antibodies causes increased levels of non specific background of staining, and too low a concentration results in poor display of antigen expression.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as described in chapter 2. The cells were immunolabelled by using the double staining method with conjugated CD 14 to identify the monocytes and the adhesion molecule antibody to identify the specific adhesion molecule under examination, then the samples were analysed by flow cytometry. Results are shown in figures (3.1-3.2 and 3.3)

MFI of cells increased in dose dependent manner for each adhesion molecule examined (LFA-1, VLA-4 and MAC-1)

3.3.1. LFA-1 dose response curve

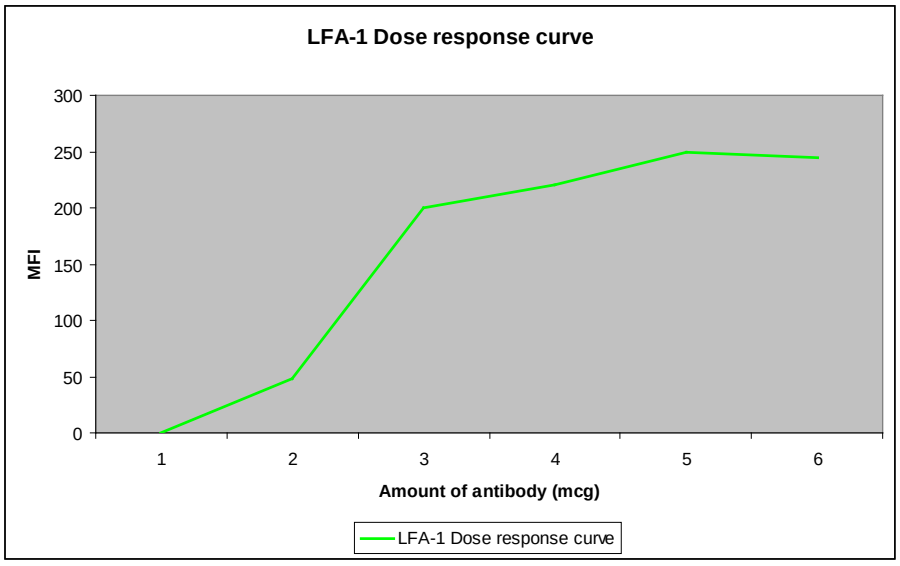

Figure 3.4 a dose-response curve for LFA-1: maximum MFI of LFA-1 was at a final antibody concentration of 5 mcg/ ml ($5x10^5$ cells)

3.3.2 Mac-1 dose response curve

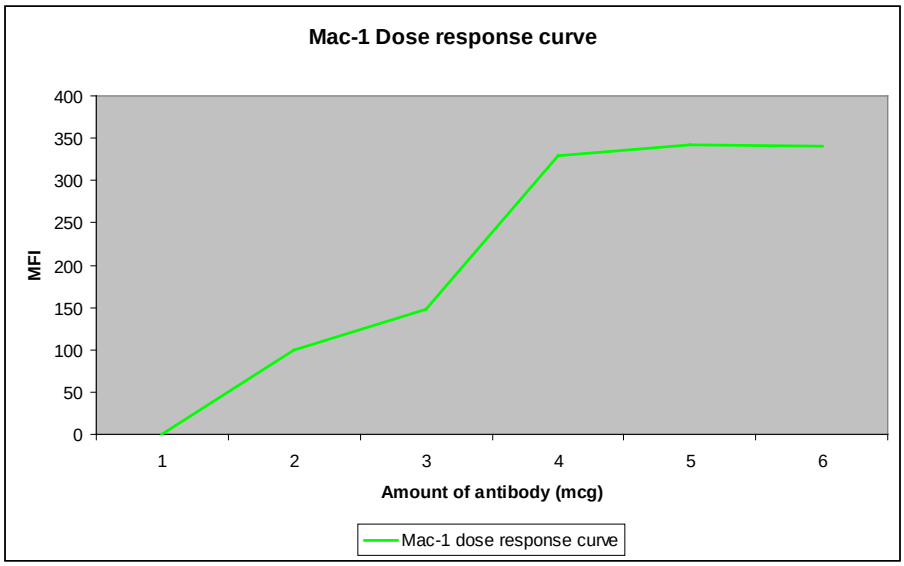

Figure 3.5 a dose-response curve for MAC-1: maximum MFI of Mac-1 was at a final antibody concentration of 5 mcg/ml ($5x10^5$ cells)

3.3.3 VLA-4 dose response curve

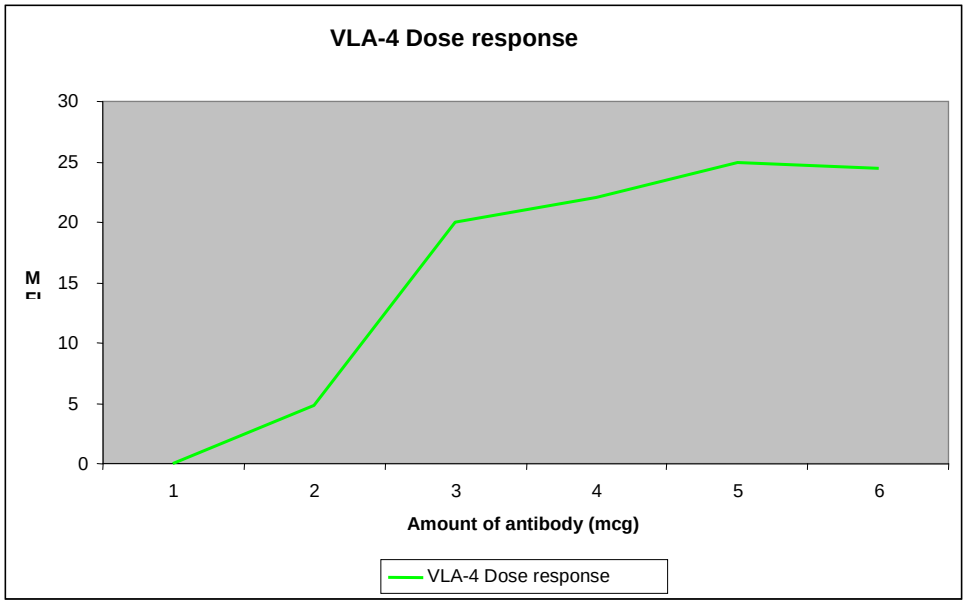

Figure 3.6 a dose-response curve for VLA-4: maximum MFI of VLA-4 was at a final antibody concentration of 5 mcg/ml ($5x10^5$ cells)

3.4 Comparison of adhesion molecule expression by monocytes from normal pregnant women and those with diabetes mellitus.

3.4.1 Subjects

As outlined in Chapter 2, three groups of subjects (n=14 in each group), aged between 23 and 41 years, were studied in the third trimester of pregnancy,

(i) normal (N)

(ii) type I diabetes (T1DM) and

(iii) gestational diabetes (GDM)

3.4.2 Methods

A mixed population of peripheral blood mononuclear cells and monocytes were isolated from peripheral blood as described in Chapter 2 (sections 2.2.2.1 and 2.2.3.1)

To examine expression of adhesion molecules by monocytes, double labelling with CD14/PE and adhesion molecule/FITC was used. The adhesion molecules examined were MAC-1, LFA-1 and VLA-4. Cells were analysed on a Becton Dickinson flow cytometer (BD FACSVantage SE) with an argon laser at 488nM for analysis of PE and FITC. Ten thousand events were collected from a pure monocyte population. The monocytes were identified from forward and side scatter dot plots. Data collected from the flow cytometer were stored electronically for later analysis on Cell Quest software.

3.4.3 Results

3.4.3.1 Level of expression of adhesion molecule LFA-1

Peripheral blood monocytes from T1DM pregnant women expressed higher levels of adhesion molecule LFA-1 than monocytes from both normal pregnant women and those with gestational diabetes (P<0.01) (Figure 3.4.3.1.1). There was no difference in the level of expression of LFA-1 in normal pregnancy compared with gestational diabetes.

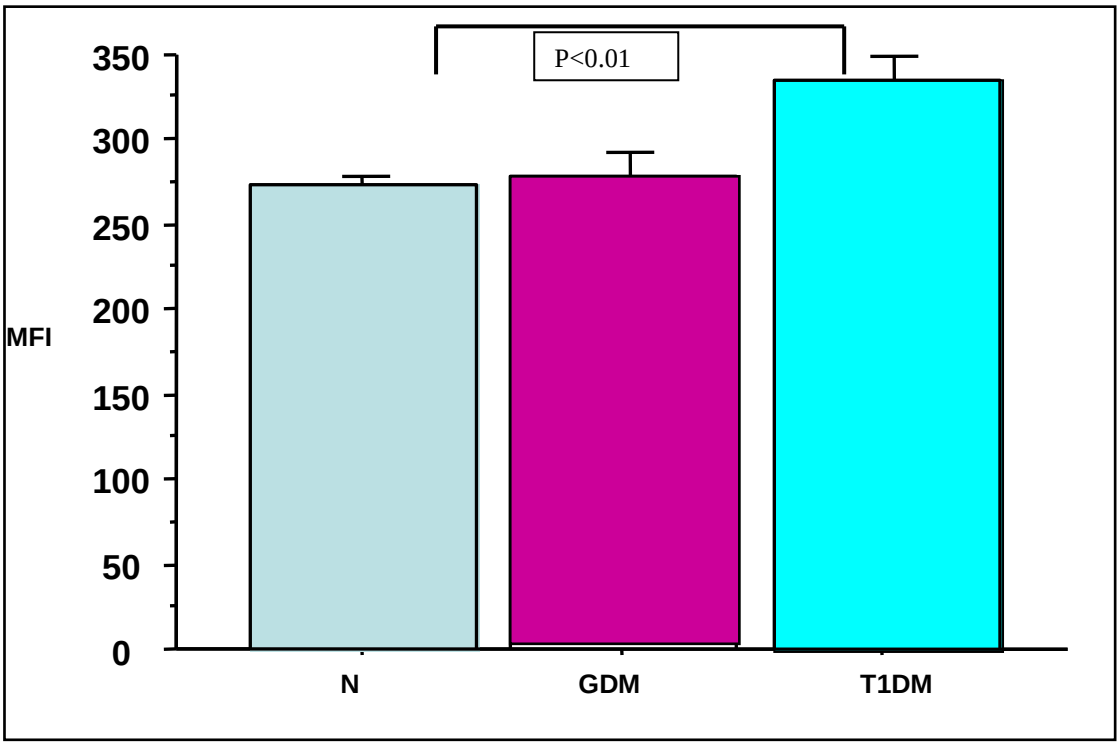

Figure 3.7: Bar chart representing levels of expression of adhesion molecule LFA-1 from Gestational diabetic, Type I diabetic and normal pregnancy x= P<0.01

3.4.3.2 Expression of adhesion molecule MAC-1

Peripheral blood monocytes from T1DM pregnant women expressed higher levels of adhesion molecule MAC-1 compared with monocytes from normal pregnancy and GDM (P<0.01) (Figure 3.4.3.2.1) whereas there was no significant difference in the level of expression of MAC-1 between normal pregnancy and GDM.

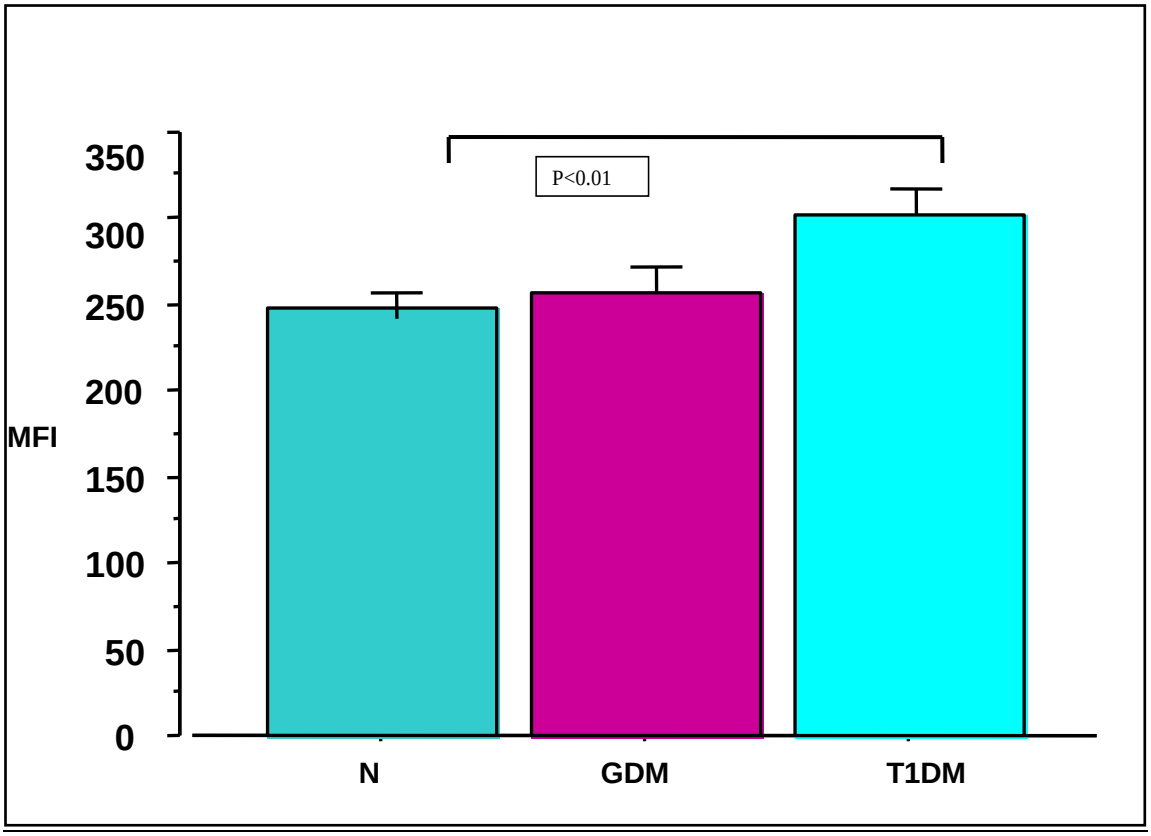

Figure 3.8 Bar chart representing levels of expression of adhesion molecule Mac-1 from Gestational diabetic, Type I diabetic and normal pregnancy x=P<0.01

3.4.3.3 Expression of adhesion molecule VLA-4

There was no significant difference in the expression of monocyte adhesion molecule VLA-4 from T1DM pregnant women compared with normal pregnant women or those with GDM (Figure 3.4.3.3.1)

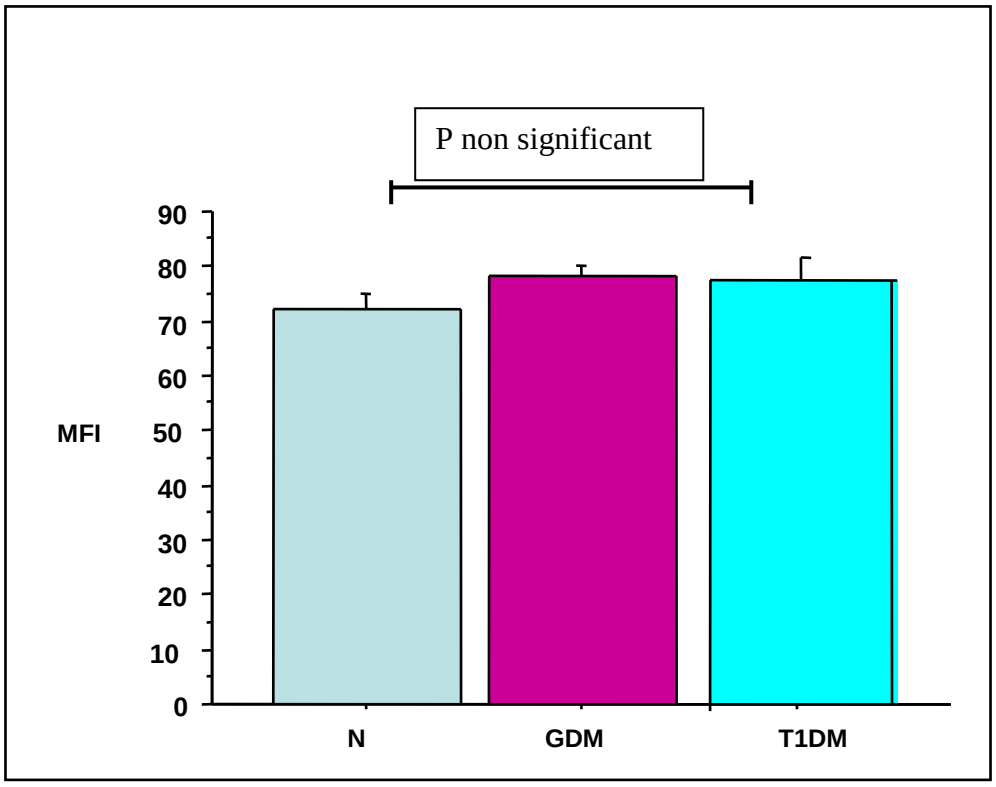

Figure 3.9 Bar chart representing levels of expression of adhesion molecule VLA-4 from Gestational diabetic, Type I diabetic and normal pregnancy. There were no significant differences amongst groups

3.5 Mechanisms involved in upregulation of monocyte adhesion molecules and adhesion to decidual endothelial cells in normal pregnancy and in type I diabetic pregnancy

3.5.1 Introduction

The adhesive properties of blood monocytes are important under normal steady-state (i.e. non- inflammatory) conditions as well as in response to inflammation. Under non- inflammatory conditions the adhesion properties of blood monocytes are not stimulated and the efflux of monocytes from the blood stream to the tissues ensures maintenance of the macrophage population in a given tissue compartment. In response to inflammation, adhesion and sequestration of circulating monocytes is increased and the recruitment of large numbers of blood monocytes to the tissues satisfies the increased demand for tissue macrophages. (Issekutz, 1981) (Blusse, 1983). Under steady-state conditions integrin molecules on the monocyte cell surface are in a relatively inactive state and upon stimulation , these molecules convert to an active state with high affinity for their ligands (Hynes, 1992) (Dobrina, 1991). After initial experiment comparing normal and diabetic monocytes , mechanisms of regulation of adhesion molecules was further explored using cells of normal and diabetic pregnant women.

3.5.2 LPS dose- and time- response curve

Activation of monocytes with mediators of inflammation such as bacterial lipopolysaccharide (LPS), results in rapid degranulation of intracellular stores and increased surface expression of various adhesion molecules (Bainton, 1987) (Miller, 1987). We therefore studied the effects of LPS on monocytes from normal and diabetic pregnant women

In preliminary experiments LPS dose- and time- response curves were determined. Monocytes isolated from peripheral blood were incubated with 10ng/ml, 100 ng/ml and 1000 ng/ml of LPS for 20 min, 60 min and 120 min, and then were double stained with (CD14) PE and (MAC-1) FITC.

The results are summarised in Figures 3.10 and 3.11 Upregulation of MAC-1 expression was maximal at 30 min and with LPS concentration at 100 ng/ml. The time and dose response experiments were repeated three times.

This LPS stimulation experiment was undertaken on monocytes from normal pregnancies only, as other projects in our group examined the response to stimulation of monocytes from diabetic subjects. The current set of experiments used a proinflammatory mediator (LPS) to change the status of normal monocytes towards that of diabetic monocytes in term of inflammation.

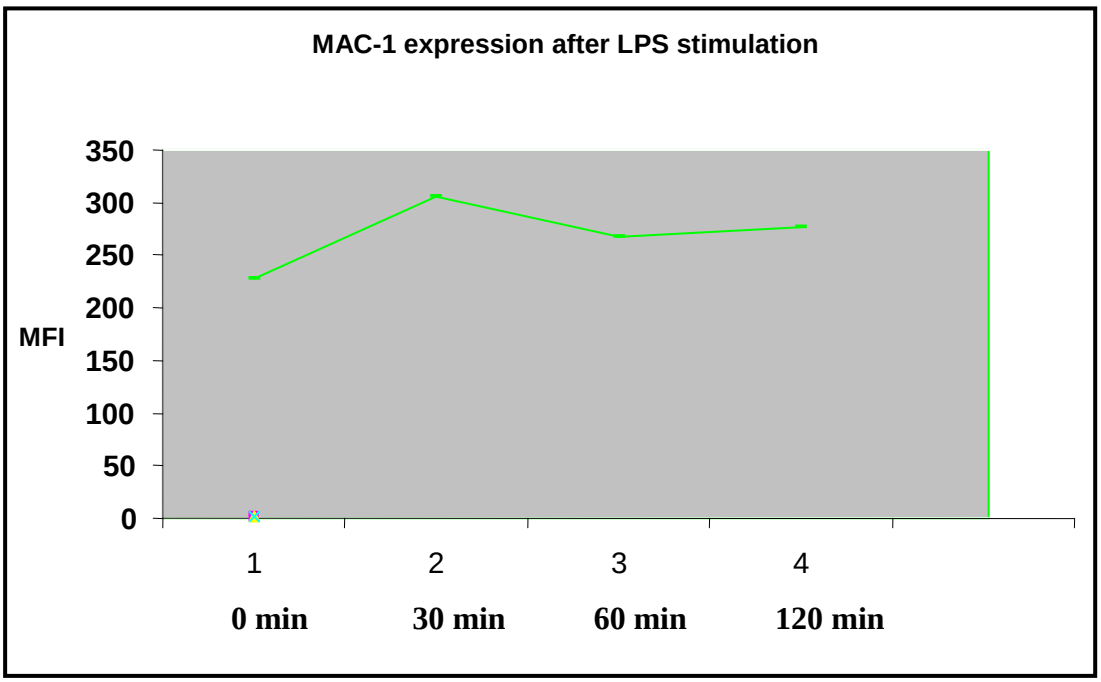

Figure 3.10 Time response curve of adhesion molecule expression in response to 100ng LPS stimulation. Upregulation of MAC-1 expression was maximal at 30 min.

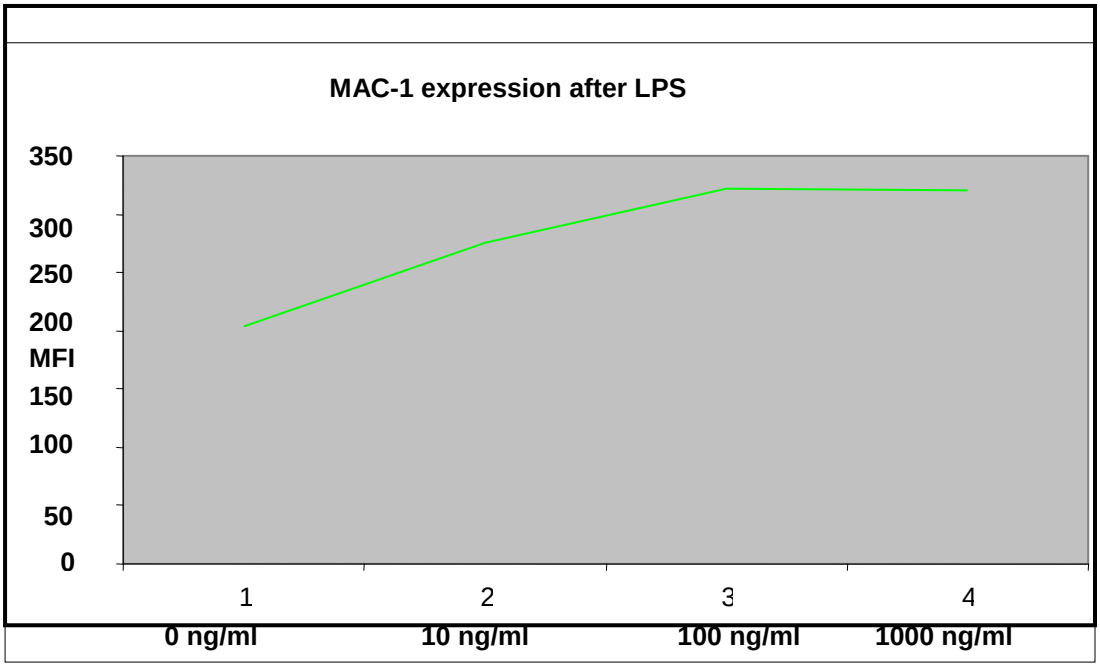

Figure 3.11 Dose response curve of adhesion molecule expression in response to LPS stimulation. Upregulation of MAC-1 expression was maximal with LPS concentration at 100 ng/ml.

In subsequent experiments, monocytes were isolated by density gradient centrifugation as described earlier in chapter 2 (section 2.2.2.1) pre-incubated in Immuno Minisorp tubes with 100 ng/ml of bacterial lipopolysaccharide (LPS), at 37^0C in 5 % CO_2 for 30 minutes. The effect of stimulation by LPS was examined in normal pregnant women (N=14), in the third trimester of pregnancy, aged between 23 and 41 years. Experiments examined MAC-1 expression, known to be significant for diabetic subjects.

3.5.3.1 LPS stimulation of isolated monocytes

Monocytes were suspended in culture medium 2% FCS + $M199^-$ at a concentration of 10^5 cells / tube in a total volume of 1 ml. Cells were pre-incubated in Immuno Minisorp at 37^0C in 5 % CO_2 for 30 minutes with 100μg/ml of bacterial lipopolysaccharide (LPS), then fixed, stained with CD14/PE and MAC-1/FITC, and analysed by flow cytometry.

3.5.3.2 Results

Figure 3.5.3.2.1 is a graphic representation of flow cytometry analysis There was a significant increase of MAC-1 expression in monocytes stimulated by LPS, compared with the unstimulated group.

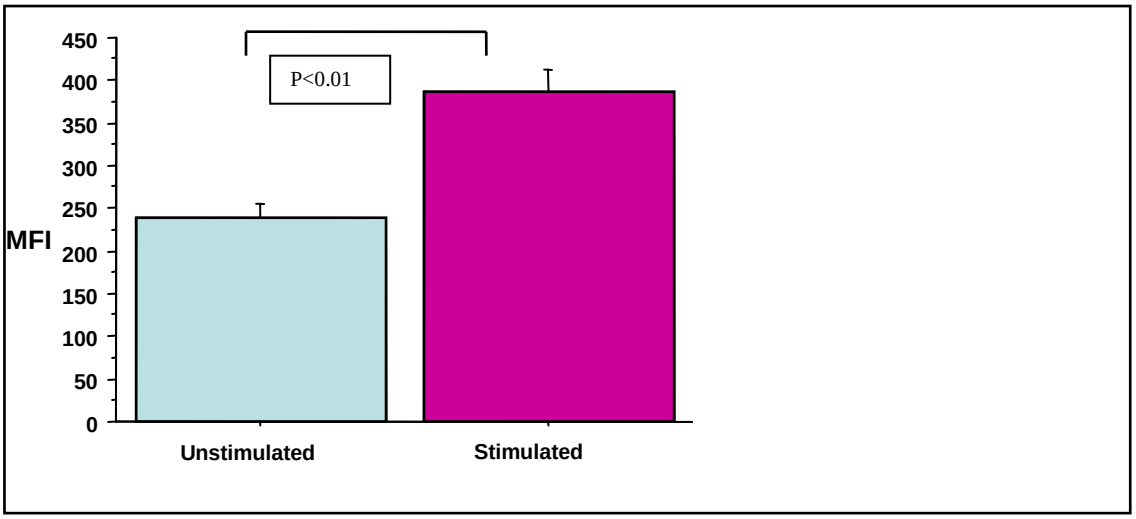

Figure 3.12 MAC-1 expression on $CD14^+$ monocytes from normal pregnant women. Pre-stimulation with LPS caused an up-regulation of MAC-1 P< 0.01

3.5.4 Adhesion assay: regulation of adherence of monocytes from normal pregnant women to decidual endothelial cell

This series of experiments examined the effect of pre-treatment of monocytes with LPS on their adhesion to decidual endothelial cells

Cell isolation and for preparation is described in detail in Chapter 2.

3.5.4.1 Assessment of monocyte-decidual endothelial cell adhesion

Confluent endothelial cell monolayers in 6 well plates were washed with 2% FCS+$M199^-$, and monocytes in 2% FCS+$M199^-$ (control and LPS-stimulated) were seeded onto this monolayer. After one hour incubation at 37^0 C in 5 % CO_2, non adherent monocytes were removed by gentle washing. Cells were then removed from the 6 well plates with trypsin+EDTA (5-7 min), then 0.1 % BSA in 50 ml PBS was added to each well to inactivate the trypsin.

The resultant mixed cell population (containing monocytes and decidual endothelial cells) was transferred into a corresponding 15 ml tube. The mixed cell populations were centrifuged for 5 min at 1000 rpm and transferred to Eppendorf tubes, where they were washed twice more with 1 ml of PBS/0.1% BSA.

Cells were resuspended in 50 µl (final volume) of PBS/0.1% BSA, for flow cytometry. Aliquots were double stained with (CD45 PE + ICAM-1 FITC) for identification of monocytes (CD45 PE positive) and for identification of endothelial cells (ICAM-1 FITC positive).
(See figure Fig 2.6, 2.7 and 2.12 in Chapter 2).

Antibody was added (5 mcg/ ml), and the incubation conducted at 4^0C. After 40 minutes the mixed cells were washed in 1 ml PBS/0.1% BSA , centrifuged in an Eppendorf centrifuge (14000 g , 20 seconds), fixed in 250 µl 1% PFA in PBS/0.1%BSA and refrigerated at 4^0C until proceeding with flow cytometry. The cells were analysed on a Becton Dickinson flow cytometer (BD FACSVantage SE) with an argon laser at 488nM for analysis of PE and FITC. Ten thousand events were collected from a mixed (monocyte-decidual endothelial cell) population or from a pure monocyte population (depending on the experiments). Data collected from the flow cytometer were stored electronically for later analysis on Cell Quest software.

3.5.4.2 Results

3.5.4.2.1 Adhesion

As shown in Figure 3.5.4.2.1 LPS pre-treatment of monocytes resulted in significant stimulation of adhesion to the decidual endothelial cell monolayer (30$\pm$% vs 45$\pm$%, $p< 0.01$)

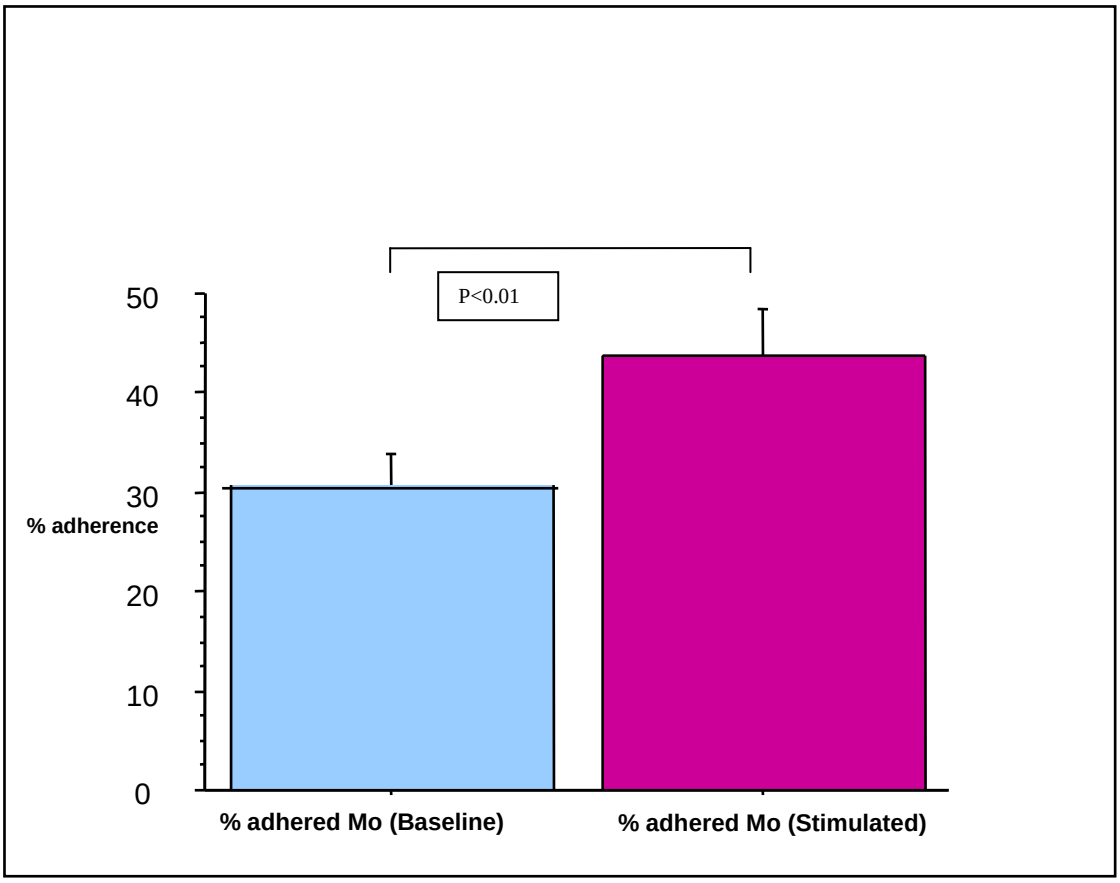

Figure 3.13 Pre-stimulation of monocytes from normal pregnant women with LPS causes an up-regulation of the number of adhered monocytes to decidual endothelial cell

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Monocyte adhesion, to the decidual endothelial cell monolayer was not associated with any further increase in MAC-1 expression (not shown)

3.5.4.2.2 Effect of co-culture with monocytes on ICAM-1 expression by decidual endothelial cells

ICAM-1 a single-chain glycoprotein, expressed on decidual endothelial cells, has MAC-1 as a major ligand. An interaction between these molecule is a potential mechanism for regulation of adherence of monocytes to vascular endothelium, and the co-culture experiments conducted afforded an opportunity to examine the effect of the presence of monocytes on the expression of ICAM-1 by decidual endothelial cells.(Rothlein, 1986) (Marlin, 1987) (Markgoba,1988).

The expression of the decidual endothelial cell adhesion molecule ICAM-1 was examined in co culture with monocytes in normal and stimulated conditions, by double staining (CD45PE + ICAM-1 FITC)

3.5.4.3.3 ICAM-1 Results

The MFI of ICAM-1 on normal pooled decidual endothelial cells (NDEC) in co-culture with non activated monocytes was significantly higher than the levels of ICAM-1 expression of the DEC alone. Interestingly, although LPS stimulated both MAC-1 expression in monocytes, and their adhesion to DEC, such prior stimulation of the monocytes had no effect on DEC ICAM-1 expression.

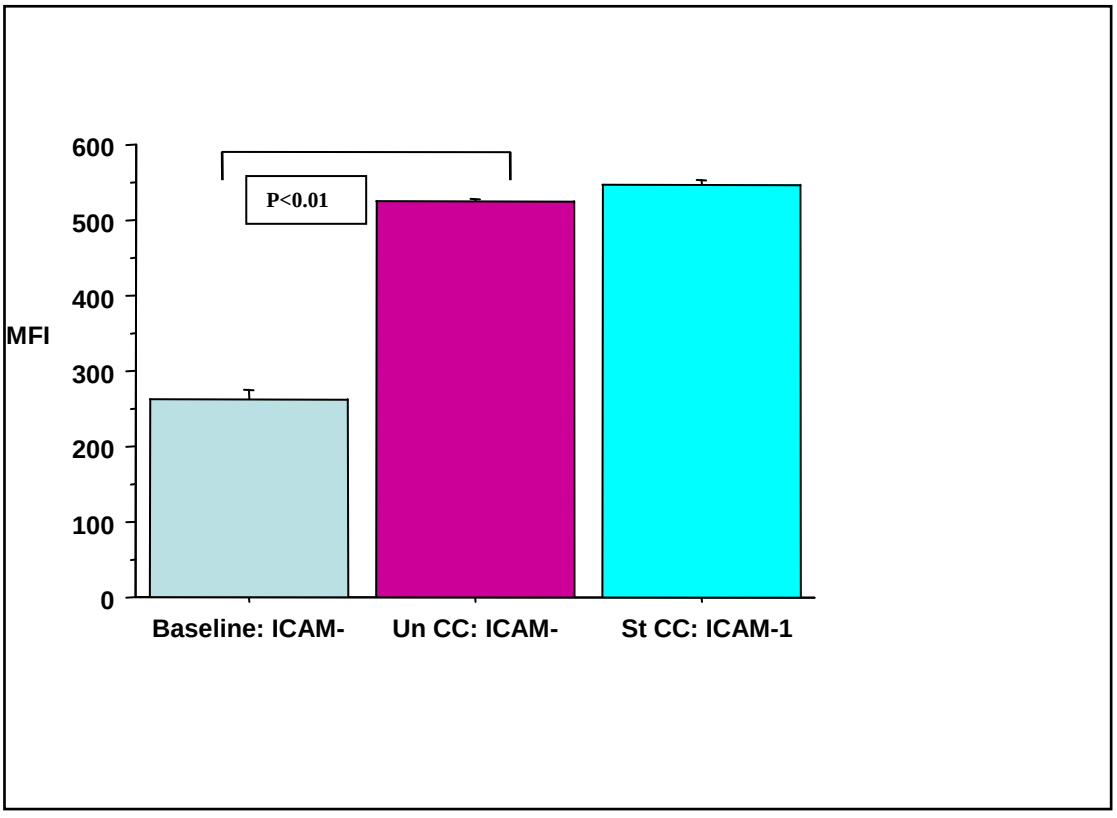


Figure 3.14 There was an increase in expression of ICAM-1 from normal DEC in co culture (CC) with non activated monocytes compared with their baseline ICAM-1 expression, but there was no significant further increase with the pre-stimulated monocytes

3.6 Effect of blocking MAC-1 expression on monocytes on their adhesion to decidual endothelial cells.

3.6.1 Introduction

Earlier work from our group (Galettis et al 2004) had shown increased adherence of monocytes from pregnant women with type I diabetes to normal decidual endothelial cells. Following demonstration that MAC-1 is up-regulated in monocytes from type I diabetic pregnant women, that these monocytes adhere more to DEC and that activation of MAC-1 on monocytes from normal subjects stimulates their adherence to DEC, the effect of blocking MAC-1 expression on monocytes was investigated. These experiments were conducted using monocytes from both normal pregnant women and those with T1DM. The effect of this antibody blockade was examined in control and LPS-stimulated monocytes. The effect of high glucose concentrations was also examined, as this is relevant to the study of adhesion molecule expression in T1DM. (Baumgartner- Parzer, 1995).

3.6.2 Blocking MAC-1 on monocytes in normal subjects

3.6.2.1 Subjects

Subjects involved in this part of the study were normally pregnant women in the third trimester of pregnancy, as described in Chapter 2 (n=12)

3.6.2.2 Materials and Methods

The preparation of normal pooled decidual endothelial cells was as described in chapter 2.

2.6.2.2.1 Pre- incubation of monocytes

Isolated monocytes were suspended in culture medium 2% FCS + $M199^-$ at a concentration of 10^5 monocytes / tube in a total volume of 1 ml. Cells were pre-incubated in Immuno Minisorp tubes 37^0C in 5 % CO_2 in the following conditions:

(i) Control
(ii)With 5µl/ml anti Mac-1 blocking antibodies for 15 minutes
(iii) With 100µg/ml of bacterial lipopolysaccharide (LPS) for 30 min and then with 5µl/ml anti MAC-1 blocking antibodies for 15minutes
(iv) With 25mM Glucose for one hour 5µl/ml anti MAC-1 blocking antibodies for 15 minutes**.**

3.6.2.2.2 Assessment of monocyte-decidual endothelial cell adhesion

Confluent endothelial cell monolayers in 12 well plates were manipulated as described above.

Assessment of monocyte adherence

(A) Immunostaining: Wells with coverslips (containing monocytes adherent to endothelial cells) were fixed with 4% paraformaldehyde and stored at 4^0C until later

immunostaining with CD68 for monocyte cell counts to confirm visually the flowcytometry data .

(B) Flow cytometry: Cells (containing monocytes and decidual endothelial cells) were removed from the well and separated by trypsin/EDTA for 5-7 minutes, then 0.1 % BSA in 50 ml PBS was added to each well to inactivate the trypsin. The resultant mixed cell population was transferred into a corresponding 15 ml tube.
To ensure complete cell harvesting, 0.5 ml PBS/0.1% BSA was then added to each well, rinsed well and transferred into the same 15 ml tube. Wells were visually inspected after this step to ensure there were no residual cells. The mixed cell populations were centrifuged for 5 min at 1000 rpm and transferred to Eppendorf tubes, where they were washed twice more with 1 ml of PBS/0.1% BSA prior to flow cytometry.

Cells were resuspended in 50 µl (final volume) of PBS/0.1% BSA, Monocytes were identified as CD14+ positive cells and decidual endothelial cells as CD45- negative cells. After 40 minutes the mixed cells were washed in 1 ml PBS/0.1% BSA and centrifuged in an Eppendorf centrifuge (14000 rpm , 20 seconds) and fixed in 250 µl 1% PFA in PBS/0.1%BSA and refrigerated at 4^0C until proceeding with flow cytometry as described earlier.

3.6.3 Blocking MAC-1 in baseline in normal subjects results

As shown in (figure 3.15), antibody blockade of MAC-1 resulted in significant inhibition of monocyte adherence to decidual endothelial cells from normal pregnancy.

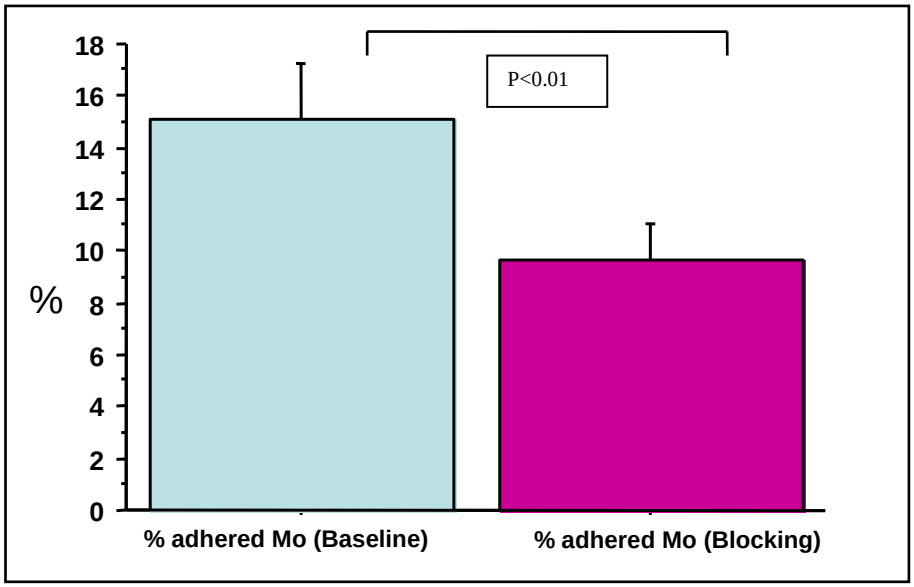


Figure 3.15 There was a decrease in monocyte adherence to DEC in co culture after blocking MAC-1 P<0.01

3.6.4 Blocking MAC-1 after LPS stimulation in normal subjects

MAC-1 antibody blockade virtually abrogated the stimulation of monocyte adhesion induced by LPS, as shown in Figure 3.16

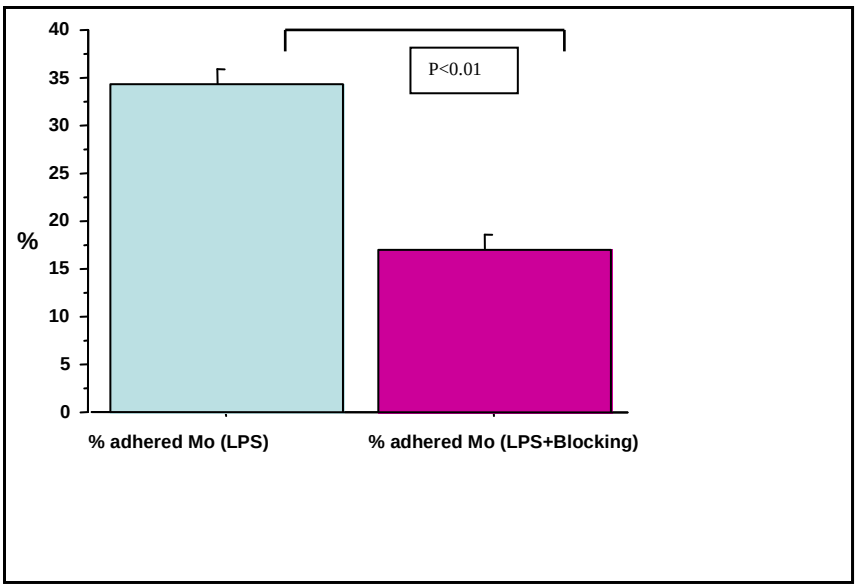


Figure 3.16: There was a decrease in monocyte adherence to DEC in co culture after stimulation with LPS and blocking the MAC-1 (P<0.01)

3.6.5 Blocking MAC-1 after Glucose stimulation in normal subjects

As can be seen in Figure 3.17, pre-incubation of monocytes with high glucose levels (25 mmol/l) resulted in a significant stimulation of their subsequent adherence to the decidual endothelial cell monolayer. Blockade of MAC-1 inhibited this adherence.

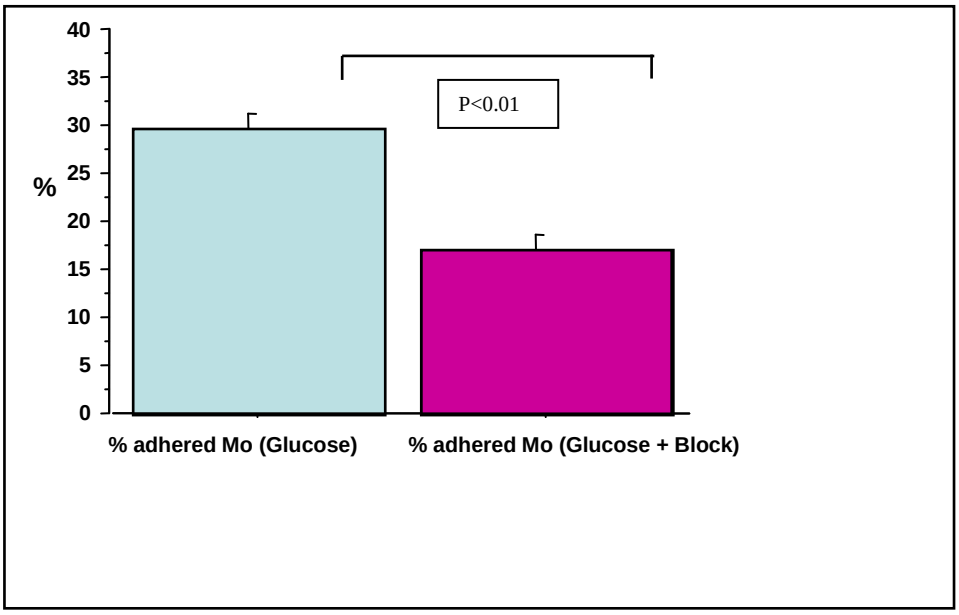


Figure 3.17 There was a decrease in monocytes adherence to DEC in co culture after stimulation with Glucose and blocking the MAC-1 (P<0.01)

3.6.6 Immunostaining of blocking MAC-1 in normal subjects results

The wells with coverslips (containing monocytes adherent to endothelial cells) were fixed with 4% paraformaldehyde and stored at 4^0C until later immunostaining for monocyte cell counts. Monocytes were Immunolabelled and identified with mouse anti human CD68 (clone EBM11, 1: 100 dilution)

As shown in Figure 3.6.3 immunostaining analysis confirmed the flow results., Blockade of MAC-1 on monocytes, resulted in significant inhibition of their subsequent adherence to decidual endothelial cells.

Throughout this body of work, flow cytometry was used to identify adhesion molecule expression, while monocyte-decidual endothelial cell adhesion was assessed by immunocytochemistry.

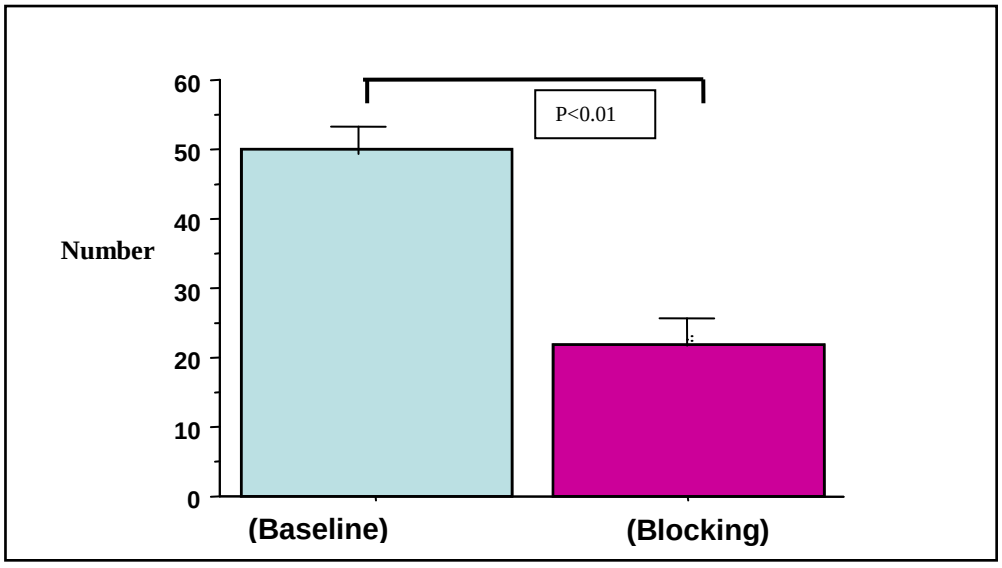
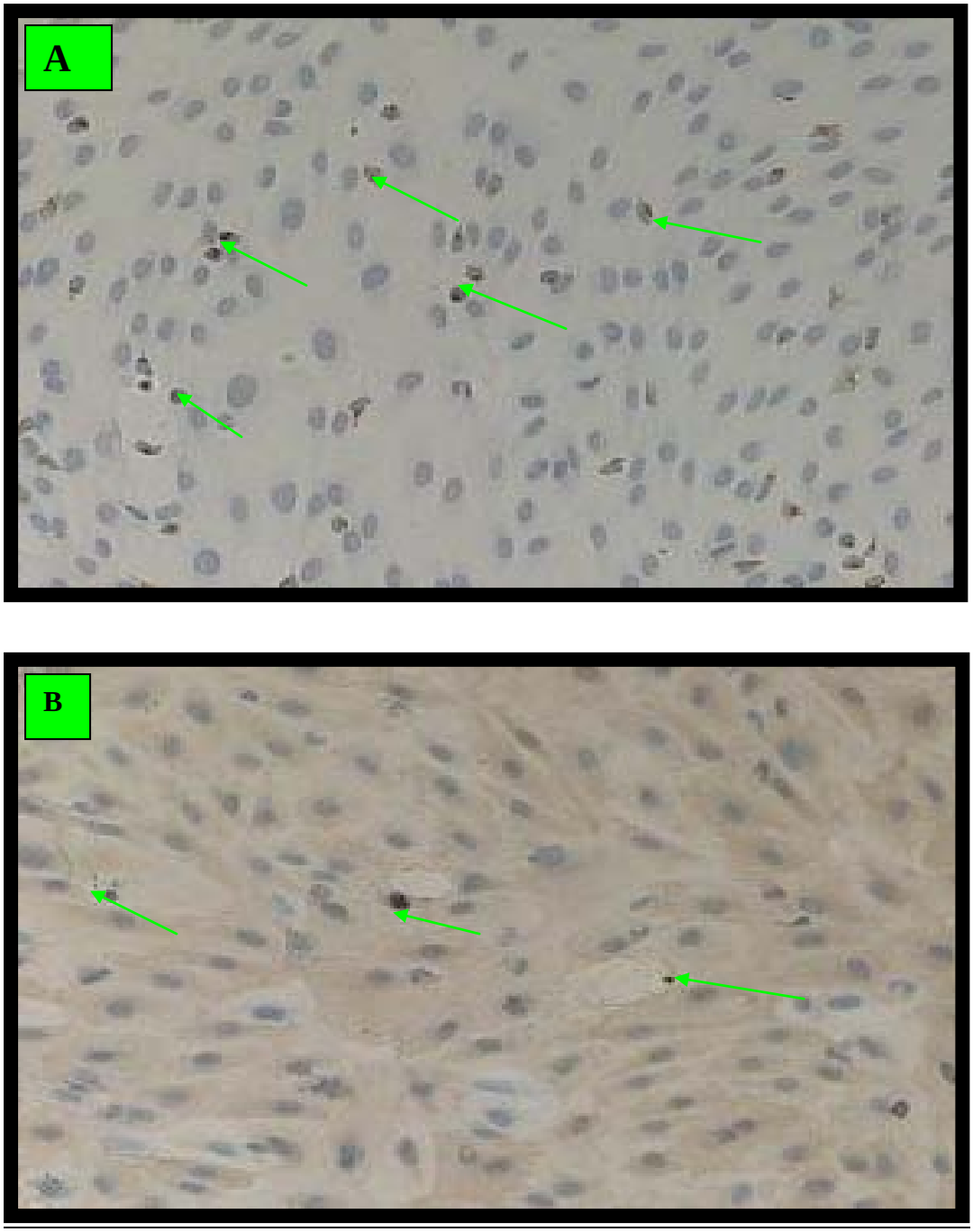


Figure 3.18: There was a decrease of the number of adhered monocytes to DEC from normal subjects in co culture after blocking with anti MAC-1 P<0.01



Picture 3.1 Immunostaining of co culture experiment in normal subjects. Brown colour shows the Immunostained CD68 positive cells , expressing the adhered monocytes from normal pregnancy, A- baseline, B- blocking.

3.6.7 Blocking MAC-1 on monocytes in T1DM pregnant subjects

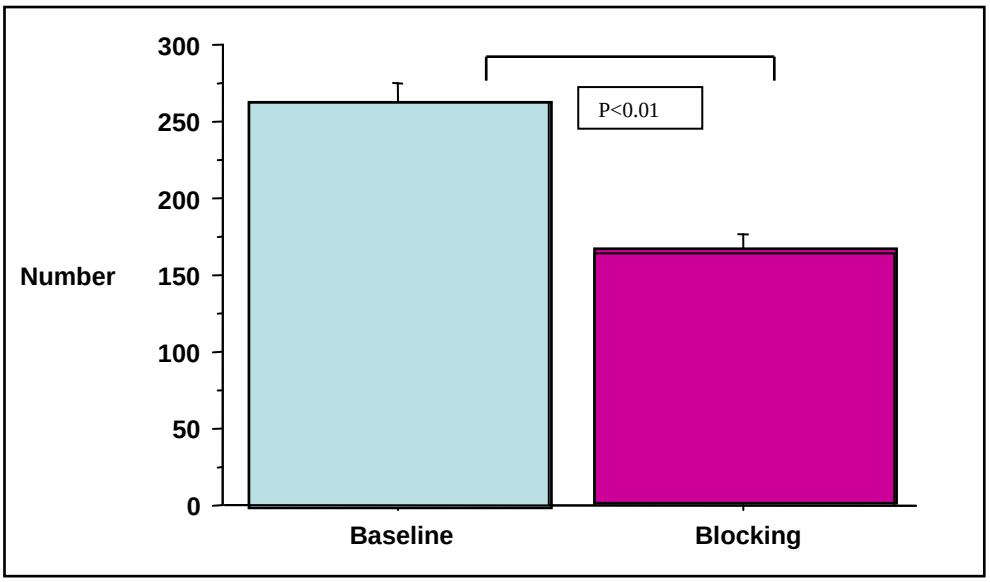
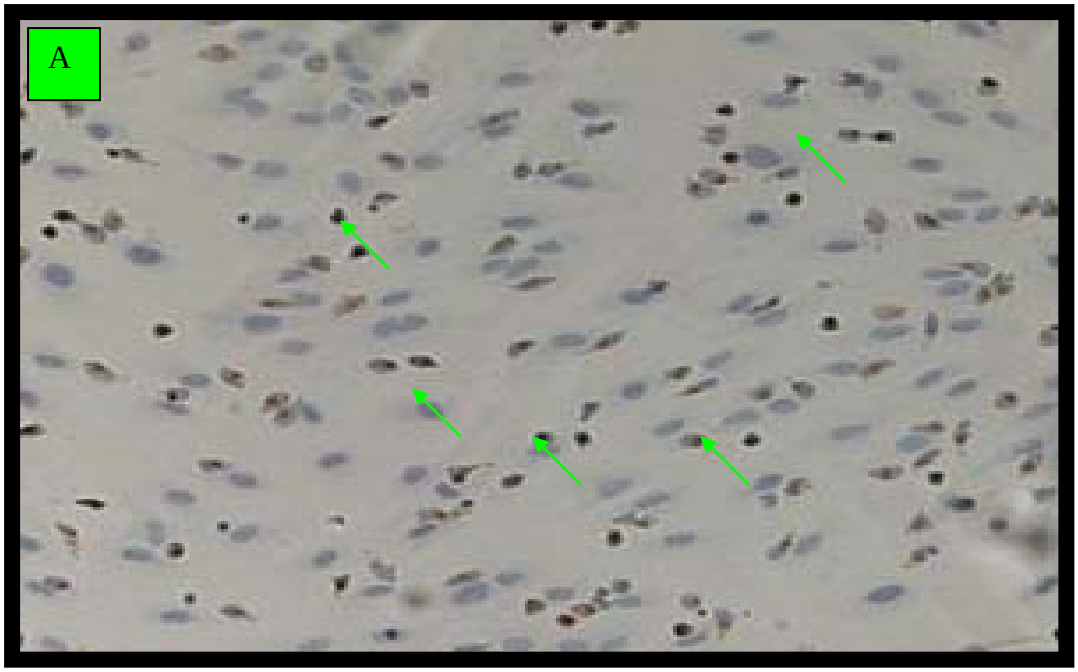
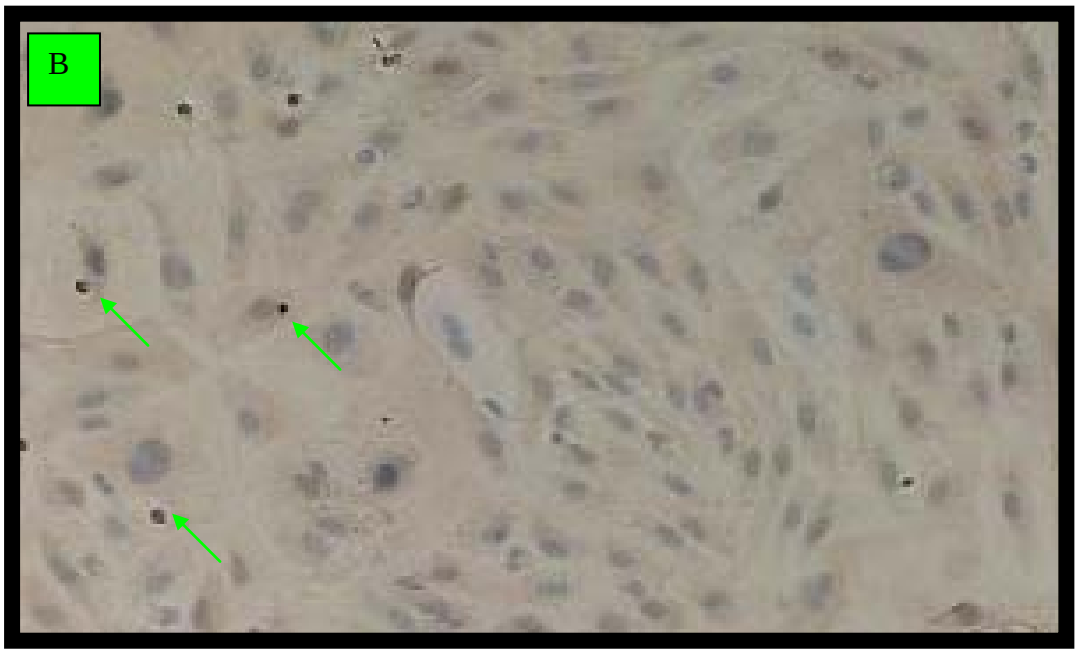


Figure 3.19: There was a decrease of the number of adhered monocytes from type I diabetic pregnancy to DEC in co culture after blocking with MAC-1 P<0.01





Picture 3.2 Immunostaining of co culture experiment in T1DM subjects Brown colour shows the Immunostained CD68 positive cells , expressing the adhered monocytes from type I diabetic pregnancy, A- baseline and B- after blocking

Chapter IV

Discussion

4.1 Introduction

The immune system is complex and the changes necessary to accommodate successful pregnancy are incompletely understood. Published evidence of both immunosuppression and immune activation supports the concept of a unique alteration in the balance between innate and adaptive immune systems. The results presented in this thesis further expand the knowledge of pregnancy-associated changes in the activation and function of monocytes, central players in the innate immune system, and the potentially deleterious abnormalities that are seen in type 1 diabetes mellitus (T1DM).

Using a co culture model, mechanisms that regulate the adhesion between monocytes and endothelial cells have been investigated. Better understanding of these regulatory mechanisms may eventually assist therapeutic approaches to the prevention or treatment of diabetic vascular complications, particularly in pregnancy.

4.2 Non-immune functions of immune cells

Cells of the immune system have multiple functions, and diseases not classically thought of as immune in origin may have an immune component as evidenced by abnormal function of cells of the immune systems. Atherosclerosis is one such macrovascular disease. Atherosclerosis is common in patients with long term diabetes mellitus (Bilato and Grow, 1996; Vlassara, 1996; King and 1996). Hyperglycemia and advanced glycation end products (AGE) (Brownlee, 1995) are the two best recognised important factors in the development of macrovascular disease .

The atheroma of diabetes mellitus is associated with abnormal local tissue macrophage function, and there is a growing body of evidence that this cell's precursor in the circulation, the blood monocyte, also shows abnormal function.

A number of reports have attested to activation of the innate immune system in normal human pregnancy (Fear on, 1996**,** Luppi, 2002, Sacks 1999,) and indicate a significant role for monocytes. In normal pregnancy, activation of the innate immune system is necessary to maintain maternal health in the presence of relative suppression of the adaptive immune system. Such a fine balance is essential, and potential problems are encountered in mothers with underlying vascular pathology or immune disorders that reduce her capacity to maintain a tight regulation of adaptation to pregnancy. Diabetes is accompanied by significant vascular pathology and pathophysiology. Although the majority of women with underlying diabetes have successful pregnancies, they are at increased risk of systemic micro- and macrovascular complications. If this vascular pathology manifests within the specialised uteroplacental circulation, there is an increased chance of reduced placental blood flow, putting the fetus at particular risk, both of growth restriction and of death.

The recognition that an atheroma-like pathology, with prominent monocyte-macrophage infiltration, occurs within an accelerated time frame in the maternal blood vessels of the placental bed raised the possibility that maladaptation of the peripheral blood monocyte, already activated to accommodate pregnancy, is causally involved in production and progression of these vascular lesions. This led to the

studies conducted in this thesis, to investigate factors involved in monocyte adherence to endothelial cells of the placental bed, a necessary preliminary step in their migration through the decidual endothelium into the perivascular space.

4.3 Hyperglycaemia as a cause of increased monocyte-endothelial cell adhesion

Our group had previously described increased adhesion to decidual endothelium of monocytes from pregnant women with type 1 diabetes (Galettis et al, 2004) . Such an increase could be the initial step in the development of placental bed vascular disease. The changes may nevertheless have been related to duration and adequacy of glycemic control, as HbA_{1c} levels were higher in the type 1 diabetic group. Using this novel model, the current body of work has investigated mechanisms by which this increased adhesiveness to placental bed endothelium occurs in diabetic pregnancy.

The findings of this study demonstrate pre-existing activation of the peripheral blood monocyte in T1DM in pregnancy. It is clear that a marked increase in expression of integrins LFA1 and MAC-1 accompanies their elevated levels of adhesion to endothelial cells from the deciduum. Monocytes from both normal and diabetic pregnancies were responsive to stimulation by LPS and by high ambient levels of glucose, but cells from pregnancies with T1DM had more florid responses to all the stimuli examined, most likely due the chronic inflammatory status of diabetic monocytes and the chronic presence of inflammatory cytokines.

One possible reason for these abnormalities is the presence of hyperglycaemia. Hyperglycaemia increases intracellular diacyglycerol synthesis via glycolysis and

activates protein kinase C (Xia et al., 1994). This second messenger activation leads to enhancement of glycolysis, thought to enhance the expression of monocyte integrins (Kim et al., 1994), a suggestion supported by the upregulation of monocyte expression of integrin MAC-1 found in T1DM pregnancies, and by their increased level of adherence to DEC. However, the results indicated that the increased levels were not due solely to high glucose levels, as values for women with gestational diabetes (who had comparable levels of blood glucose) were not as high as those with T1DM. Neither were they correlated simply with HbA1c levels at the time of blood collection, although it is likely that long term exposure to elevated levels of AGEs are at least in part responsible for the stimulation.

Hyperglycaemia is undoubtedly of significance in increased monocyte-endothelial cell adhesion, at least some of which effect is due to stimulation of endothelial expression of adhesion molecules. Consistently, hyperglycemia has been shown to induce monocyte adhesion molecule expression on the endothelium, and increased accumulation of white blood cells on the endothelium has been observed in models of streptozotocin- or alloxan-induced diabetes and hyperglycemia (Booth, 2002). Our research group has recently shown that exposing decidual endothelial cells to high glucose levels in vitro caused an increase in adhesion molecule expression and monocyte adhesion, indicating that hyperglycemia is sufficient for initiation of macrophage-rich lesions (Renard, 2004, Xie et al submitted for publication). In addition, monocytes isolated from normolipidemic hyperglycemic patients with T1DM have an increased binding ability to endothelial cells in vitro (Kunt, 1999).

4.4 Advanced glycation end-products (AGEs) as a cause of increased monocyte-endothelial cell adhesion

Another possible cause of increased monocyte-endothelial cell adhesion is the presence of high levels of advanced glycation end-products (AGEs) in T1DM subjects. Chronic hyperglycaemia causes no enzymatic glycation of circulating cells and proteins, resulting in production of advanced glycation end products (AGEs) (Kunt, 1999, Xia, 1994), with reduced nitric oxide synthase activity or decreased availability of L arginine, the substrate for nitric oxide (Tesfamariam, 1991, 1993), (Sobrevia, 1994). This, in combination with free radicals such as superoxide anions, which directly inactivate NO, are responsible for reducing the bioavailability of nitric oxide, the main factor responsible for vasodilation of endothelium and vascular smooth muscle, by elevating tissue levels of cyclic guanosine monophosphate (Ignaro, 1987) .

Nitric oxide (NO) inhibits key early processes of atheroma formation beginning with the signalling pathways which promote endothelial cell-monocyte interactions. The ability of monocytes to bind to endothelial cells in vitro is inhibited within minutes by exogenous NO in a dose dependent manner, suggesting that NO is a potent modulator of monocyte-vessel wall interactions (Tsao, 1995).

Because of the rapid time course, this effect must be due to inhibition of signalling pathways involved in adhesion. More chronic exposure to NO suppresses gene expression of adhesion molecules and chemokines involved in monocyte adhesion and infiltration (Tsao and al 1997), By contrast, inhibition of NO synthesis increases the expression of endothelial proteins required for monocyte adhesion Tsao, Wang, Buitrago, and Cooke 1996)

4.5 Specific adhesion molecules involved in monocyte-endothelial cell adhesion.

Monocyte adhesion to vascular endothelium is mediated by integrin receptors with a shared ß2 subunit and alpha subunit. (Jang, 1994), (Carlos, 1994). These include LFA-1 and Mac-1 on monocytes and their endothelial ligand intracellular adhesion molecule-1 (ICAM-1). ICAM-1 has low levels of constitutive expression on vascular endothelium (Dustin 1988, 1989), and is upregulated upon stimulation by chemoattractant inflammatory proteins or when in contact with other cells types, particularly monocytes.

VLA-4 is a ß1 integrin ligand that promotes monocyte adherence to endothelial VCAM-1 and to connective tissue components, particularly the connecting segment part of fibronectin (Wayner, 1989). Our finding that VLA-4 is not over-expressed in monocytes from T1DM pregnant women and therefore does not appear to play a major role in glucose mediated binding is consistent with that of Kim (1994) in non-pregnant diabetic subjects. The results are also consistent with those of (Xiao- zhou (1998), who found that LFA-1 and Mac-1 alone can mediate monocyte adherence to endothelial cells and migration even when VLA-4 is blocked with monoclonal antibody.

Mac-1 cell surface expression can increase rapidly as a result of translocation of stored receptor (Edwards, 1998), (Fontana, 2001). The highly significant increase observed in these experiments in the membrane expression of monocyte Mac-1 within 30 minutes of stimulation with different stimuli suggests increased translocation of stored Mac-1 to the cell surface rather than increased Mac-1 mRNA and protein

synthesis. Such a mechanism could regulate the monocyte's ability to adhere to endothelial cells and migrate to the extravascular space (Miller, 1987). The regulation of integrin mediated adhesion appears to be a purely quantitative change in expression of these adhesion molecules on the surface rather than a change in their affinity for the respective ligands Stacker (1991).

The significance of Mac-1 in regulating monocyte-DEC adherence was demonstrated clearly in this study in experiments with a specific monoclonal blocking antibody to Mac-1. The low level of adherence of monocytes to DEC seen in the absence of stimulation was reduced in the presence of antibody blockade, while the elevated levels induced by LPS pre-treatment were virtually abolished.

4.6 Comparison of results in the present study with previous reports

Few researchers have addressed the specific question of monocyte adhesion to endothelial cells of the placental bed in human pregnancy. In non-pregnant subjects, Sampson (Sampson, 2002) found an equivalent glucose-induced increase in Mac-1 expression in both diabetic and control groups, and hypothesised that this pattern of response was due to an "all or none" Mac-1 translocation in response to any glycaemic excursion irrespective of basal plasma glucose. The findings of the current study in pregnant subjects in contrast, show a different degree of responsiveness in normal and T1DM pregnant subjects. While the findings reported here are consistent, in direction if not in degree, with most previous reports dealing with non-pregnant subjects, some authors have found markedly different results.

Martin et al (1991) found that the percentage of adhesion molecule LFA-1-expressing monocytes was slightly lower than normal in T1DM, with a further decrease after

activation with either lipopolysaccharide (LPS) or interferon gamma. These findings are not in agreement with the majority of authors, who have stressed the role of these same inflammatory mediators, cytokines and activators, in induction of adhesion molecules and in their mobilisation from intracellular granules (Springer et al, 1984, Lanier, 1985, Bainton, 1987). LPS in particular is recognised as a ligand for integrins such as Mac-1. There is general recognition that the endothelial dysfunction of T1DM causes production of cytokines that activate adhesion molecules and their ligands on endothelial cells and monocytes.

Hoogerbrugge et al. (1996) found that monocytes from non-pregnant T1DM subjects, did not differ from those of healthy volunteers concerning their adhesion to endothelial cells in vitro, whereas the present study found that monocytes from pregnant women with T1DM expressed higher level of adhesion molecules Mac-1 and LFA-1 than cells from normal pregnant subjects, and that this was associated with increased adhesion to endothelial cells isolated from the placental vascular bed directly involved in atherosis.

4.7 Significance of the findings in this study

Monocytes from normal pregnant women were exquisitely sensitive to even short-term stimulation by inflammatory and metabolic stimuli. Monocytes from pregnant women with T1DM have elevated levels of expression of adhesion molecules. We have previously shown that they have increased adhesiveness, and an augmented response to standard stimuli. This interaction between $ß_2$ integrins and endothelial cell ICAMs is a critical step in the transition from cell rolling to firm arrest, and the findings of this study indicate prior priming of a nature conducive to

development and progression of atherosis of the placental bed blood vessels. Investigation of agents that can block Mac-1/ICAM-1 interactions may be of value in prevention or minimisation of this pathology.

4.8 Potential future work examining monocyte-endothelial cell adhesion.

A number of factors not studied in this body of work undoubtedly play a part in the adhesion of monocytes to endothelial cells. For example, chemoattractants such as MCP-1 belonging to the chemokine superfamily are produced and secreted by monocytes in response to activation or monocyte binding to endothelium. It is also likely that endothelial cell adhesion molecules are involved in increased monocyte-DEC adhesion in diabetic pregnancy. ICAM-1 is a ligand for both Mac-1 and LFA-1. LFA-1 binds to domain 1 and Mac-1 binds to domain 3 (Staunton, 1989), (Marlin, 1987), (Diamond 1991) (Xie, 1995). Certainly, an upregulation of DEC expression of ICAM-1 (the principal ligand for Mac-1) was seen in response to adherence of monocytes. Pre-treatment of monocytes by LPS resulted in upregulation of both Mac-1 expression and monocyte adhesion, but not in further stimulation of DEC expression of ICAM-1, so other factors must be in play. ICAM-2, known to be a ligand for LFA-1, with controversial findings for Mac-1 (De Fougerolles, 1991) (Xie, 1995), is also a molecule of interest. These are potential candidates for future work, but were not examined in the current study.

Appendix

Appendix 1: List of Reagents

The following list contains the source of general chemicals, cell culture and biological reagents, plastic ware and equipment.

Cell Culture and Biological Reagents and General Chemicals

Reagents/Chemical	Source
Antibodies	
Monoclonal mouse anti-human CD68 (Clone EBM11)	DAKO, Glostrup, Denmark
Monoclonal goat anti-mouse IgG	DAKO, Glostrup, Denmark
Monoclonal mouse anti human CD45,	DAKO, Glostrup, Denmark
Leukocyte Common Antigen	DAKO, Glostrup, Denmark
Monoclonal mouse anti-human CD14	DAKO, Glostrup, Denmark

General Reagents/ Chemicals

3'3' Diaminebenzadine (DAB)	Sigma, St Louis, USA
Ammonia Persulphate (APS)	Sigma, St Louis, USA
Ammonium Chloride	Sigma, St Louis, USA
Benzyl penicillin	Sigma, St Louis, USA
Biotin (NHS-L6)	Pierce, Illinois, USA
Bovine Serum Albumin	CSL, Parkville, Australia
Dimethyl sulfoxide (DMSO)	Sigma, St Louis, USA
Ethanol	Scharlau Chemie, Barcelona, Spain
Fetal Bovine Serum (FBS), heat	Thermo Trace, Nobel Park, Australia
Fetal Calf Albumin (FCA)	Invitrogen, Mount Waverley, Australia
Fetal Calf Serum (FCS)	Sigma, St Louis, USA

Ficoll (type 400)	Pfizer, New York, USA
Ficoll-Paque plus	Pfizer, New York, USA
Formaldehyde	Sigma, St Louis, USA
Glycergel	DAKO, Glostrup, Denmark
Haematoxylin, Harris	Polysciences, Warrington, USA
HBSS, without calcium and magnesium	Sigma, St Louis, USA
Hydrogen peroxide	Sigma, St Louis, USA
PBS, tablets	ICN Biochemical's, Cleveland, USA
Sodium acetate (trihdrate)	Sigma, St Louis, USA
Sodium bicarbonate	BDH Ltd, Poole, England
Sodium carbonate (anhydrous)	Sigma, St Louis, USA
Sodium chloride	Sigma, St Louis, USA
Sodium EDTA- Disodium	Sigma, St Louis, USA
Streptomycin	Sigma, St Louis, USA

Hardware and Equipment

Equipment	
3 ml Vacuette tube EDTA	Greiner, Frichenhausen, Germany
3 ml Vacuette tube with clot activator	Greiner, Frichenhausen Germany
50 ml sterile tubes	BD Biosciences, Bedford, USA
50 ml syringe	Terumo Medical, Elkton, USA
Beckman Centrifuge J-21B 0-6000 rpm	Beckman Instruments, Fullerton, USA
Beckman elutriation chamber incorporated into a JE-6 rotor	Beckman Instruments, Fullerton, USA
Beckman J2- HS centrifuge	Beckman Instruments, Fullerton, USA
Class II Biological Safety Cabinet	Clyde-Apac Woodville, North Australia
Clements GS 100	Clements, North Ryde, Australia
Coverslip Glass Square 22x22 mm	Menzel-Glazer, Germany
Cryotube 1.8ml	Nunc, Roskilde, Denmark
Cytospin centrifuge	Thermo Shandon Pitts burg, USA
Haemocytometer-Improved Neubauer	Sigma, St Louis, USA
Incubator, 37 ºC, water jacketed, 5% CO_2, model 3336	Forma Scientific Inc., Marietta, USA
Lamina flow hood	Gelman Science, Australia
Micropipettes (1-10, 5-40)	Lab Systems, Helsinki, Finland
Microwave oven, 650W	NEC, Australia
MilliQ water purification system	Millipore, Molshein, France

Minisorp tubes	Nunc, Roskilde, Denmark
Multichannel pipettes	Lab System, Helsinki, Finland
Needles 19 G	Terumo Medical Corporation, Elkton, USA
Needles 21 G miniset vein infusion set	Baxter Healthcare Toongabbie, Australia
Nikon CoolPix 990 digital camera	Nikon, Tokyo, Japan
Nikon Inverted Stage Microscope	Nikon, Tokyo, Japan
PAP pen	DAKO, Glostrup, Denmark
Pasteur	Chase Instrument Corp., Glen Falls, USA
PC Automate program	Flow Sciences, Inc., Wilmington, USA
PCR tubes	Scientific Specialties Inc., Lodi, CA
pH meter	Schott Gerate, Holfeim, Germany
Pipette Aid	Drummond Scientific, Broomall, USA
Syringes 5mL, 10mL, 50mL	Terumo Medical Corporation, Elkton, USA
Transfer pipette disposable, Falcon	BD Biosciences, Bedford, USA
Tubes 15mL, 50mL, V- base, Falcon	BD Biosciences, Bedford, USA
Tubes Centrifuge 1.5ml Microfuge tube	Eppendorf, Hamberg, Germany

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