Regulation of CD74 expression in response to Human Interferon Gamma and Lipopolysaccharide on Human Trophoblast Derived Cells: Relevance for Human Feto-Maternal Tolerance

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Abstract

During pregnancy, the maternal immune system protects the allogeneic foetus from rejection. At the same time the mother maintains immunity defences against potential pathogens. This study aimed to identify whether immunological receptors associated with antigen presentation and strong inflammatory responses play a role in fetal-maternal tolerance. One such receptor is CD74; a membrane-bound protein involved in HLA class II- mediated antigen presentation and a macrophage migration inhibitory factor (MIF) receptor. CD44 is the signalling component of the MIF-CD74 receptor complex. The expression of CD74, MIF and CD44 was studied in the human trophoblast-derived cell lines JEG-3 and ACH-3P by RT-PCR, flow cytometry, Western blot, immunoprecipitation and fluorescence microscopy. Results obtained showed that untreated JEG-3 and ACH-3P cells did not express CD74 mRNA. By contrast, CD74 mRNA was upregulated in response to IFN-γ or LPS in these cell lineages. Slight upregulation of CD74 was observed following exposure of cells to 500 IU/ml IFN-γ for 12 hr. However, after 5 μg/ml LPS treatment for 4 hr, CD74 was highly upregulated. Results from flow cytometry showed no detectable surface expression of CD74 in the JEG-3 and ACH-3P cell lines even after IFN-γ or LPS treatment. However, IFN-γ and LPS exposure resulted in intracellular expression of CD74 in both cell lines. Western blotting showed the absence of a protein band for CD74 in both cell lines after IFN-γ treatment. However, the 35 kDa isoform of CD74 was detected in JEG-3 and ACH-3P cells treated with LPS. The results of this study indicate that LPS regulates the expression of MIF and CD44 and trophoblast cell proliferation. It was also demonstrated that CD74 positivity significantly increased after incubation with IFN-γ or LPS, and that MIF and CD44 are up-regulated by LPS. No evidence was found for colocalization between CD74 with either CD44 or MIF in JEG-3 and ACH-3P cells using confocal microscopy and coimmunoprecipitation. This indicated that MIF plays an essential role in cell proliferation, whereas both MIF and CD44 play an important role in human pregnancy maintenance. Together, the results suggest that the absence of cell surface expression specific isoforms of CD74 may provide a protective role by minimising inflammatory processes, and thus maximising a healthy pregnancy. In turn, it is reasonable to assume that the overexpression of CD74 in early pregnancy may be related to gestational complications.

Dedication

This work is dedicated to the memory of my father who was always supporting and encouraging me to continue my studies, may Allah rest his soul in peace.

I also dedicate this work to my mother for her love, care and prayers.

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Abbreviations

°C	Celsius
2 DE	Two-dimensional gel electrophoresis
3D	Three dimensional
aa	Amino acids
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cells
APS	Ammonium persulfate
BMP4	Bone morphogenetic protein-4
BSA	Bovine serum albumin
C-terminal	The carboxyl terminal
CAMs	Cell adhesion molecules
CD	Cluster of differentiation
CD44	Cluster of differentiation 44
CD74	Cluster of differentiation 74
cDNA	Complementary deoxyribonucleic acid
CIC	Cancer initiating cells
CIITA	Class II transcriptional transactivator
CLIP	Class-II-accociated invariant chain peptides
CLL	Chronic lymphocytic leukemia
cm	Centimetre
Co-IP	Co-immunoprecipitation
CO_2	Carbon dioxide
CS	Chondroitin sulfate
CTL	Cytotoxic cells
CXCR	Chemokine receptor
DAPI	4', 6'-diamino-2-phenylindole, dihydrochloride
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulphoxids
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphates
DTT	Dithiothreitol
ECM	Extra-cellular matrix
EDTA	Ethylene diamine tetraacetic acid
EMT	Epithelial-mesenchymal transition
EPG	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinases
FACS	Fluorescence-actived cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL	Fluorescence
EVT	Extravillous Trophoblast
FSC	Forward scatter
GAS	Interferon-gamma activated sequence
H. pylori	Helicobacter pylori
HLA	Human leukocyte antigen
hrs	Hours
IDCs	Invasive ductal carcinoma
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
Ii	Invariant chain
JAK	Janus tyrosine kinases
Kb	Kilo base pair
kDa	kilodaltons
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LSCM	Laser scanning confocal microscopy
mAb	Mouse antibody
mAb/MAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinases
MFI	Mean fluorescence intensity

mg	Milligram
MIF	Macrophage migration inhibitory factor
ml	Milliliter
mm	Millimeter
MMPs	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
N-terminal	The amino terminal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural killer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pmol	Picomole
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
PTHrP	Parathyroid hormone-related protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SD	Standard division
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SIRP-α	Signal regulatory protein-alpha
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TAAs	Tumour-associated antigen
TDLUs	Terminal ductal–lobular units
TEBs	Terminal end buds
TEMED	Tetrametylethylenediamine
Tetrazole	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Tm	Annealing temperature
TNBC	Triple-negative breast cancer
TNF-α	Tumour necrosis factor-alpha
TRITC	Tetramethyl rhodamine isothiocyanate
TSAs	Tumour-specific antigens
USF-1	Upstream stimulatory factor-1
α-Tubulin	Alpha- tubulin
β-Actin	Beta-actin
µg	Microgram
µl	Microliter
µm	Micrometer

Chapter 1 Introduction

1.1 Introduction

During mammalian pregnancy, the maternal immune system function in a dual role, protecting both mother and foetus against possible pathogens, and preventing the rejection of the genetically 'foreign' semi-allogeneic fetus and placenta. Therefore, for pregnancy to succeed, the mother and foetus must tolerate each other genetically and immunologically (Fernandez et al., 1999).

Embryonic tissues are allogeneic due to the fact that an embryo is the mating product of histo-incompatible individuals in an outbred population. Embryos express polymorphic major histocompatibility complex (MHC) genes inherited from the father, the detection of such non-self antigens by the maternal immune system would usually lead to allogeneic foetal rejection, but pleural mechanisms of foetal tolerance exist (Koch and Platt, 2003).

It has been suggested that the main immune response triggered by the semi-allogeneic foetus is the adaptive response (Aït-Azzouzene et al., 1998, Tafuri et al., 1995). Therefore, during pregnancy, the maternal immune system is in close contact with cells from the semi-allogeneic foetus. Specific mechanisms must be available to moderate and modulate the maternal immune response, to prevent foetus rejection during pregnancy (Hviid, 2006).

1.2 The placenta

The placenta is a composite structure, composed of tissues derived from the foetus as well as from the maternal uterus that connects the developing foetus to the uterine wall. The chorion is the embryo-derived portion of the placenta, and is formed by extraembryonic mesoderm and two layers of trophoblast. The placenta is the main point of interaction between the foetus and maternal system, termed the fetal-maternal unit. It supplies a very large amount of nutrients to the developing embryo until parturition, such as glucose, the primary energy source for the developing fetus (Jansson and Powell, 2007). It also facilitates transport of metabolic products such as oxygen and carbon dioxide.

The mature human placenta is haemochorial (Figure 1-3), consisting of a mass of cotyledons (villae), making up the fetal-maternal interface (Gill and LOKE (1990), (Pijnenborg et al., 1981). The human placenta is an endocrine organ. It secretes various hormones such as human chorionic gonadotropins (hCG), human placental lactogen (hPL) and progesterone that are vital in maintaining the human pregnancy as well as providing immunological defence to the foetus (Zhang et al., 2013, Petraglia, 1996, Zygmunt et al., 1998, Piccinni et al., 1995, Siiteri and Stites, 1982).

The placenta, and other extraembryonic structures, develops from cells that form an outer layer (trophectoderm) of a blastocyst (Telford et al., 1990, Norwitz et al., 2001, Meban, 1975).

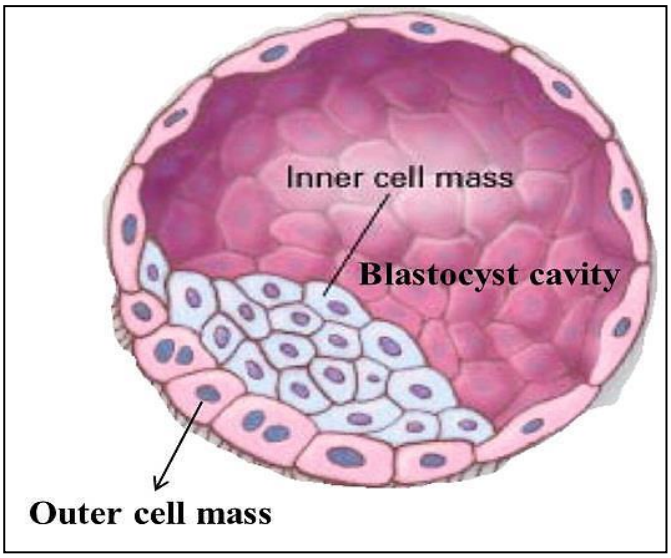

Figure 1-1: The blastocyst stage of development.

The fertilized zygote enters the uterus in 3 to 5 days. In the uterus, the cells continue to divide and develop into a hollow ball of cells called a blastocyst. The blastocyst attaches to the uterine wall (endometrium) of the mother, where it consists of an inner cell mass that develops into a foetus while the outer cell mass develops into placenta (Adapted from (Norwitz et al., 2001)).

Following successful implantation of the blastocyst, an inner and outer layer of trophoblasts is present, the cytotrophoblast (CTB) and syncytiotrophoblast (STB), both of which remain throughout the process of gestation. The STB continues to grow until it completely surrounds the implanted embryo and comes into direct contact with maternal blood (Huppertz and Borges, 2008). Around day 14 after implantation, CTB cells break through the syncytiotrophoblast layer giving rise to extravillous trophoblast (EVT) cells, which are responsible for all of the invasive characteristics of the human placenta. These trophoblast cells play an important role in acting as an interface mediating maternal-to-foetal nutrient transfer (Baergen, 2011, Loke, 1995) (Figure 1-2).

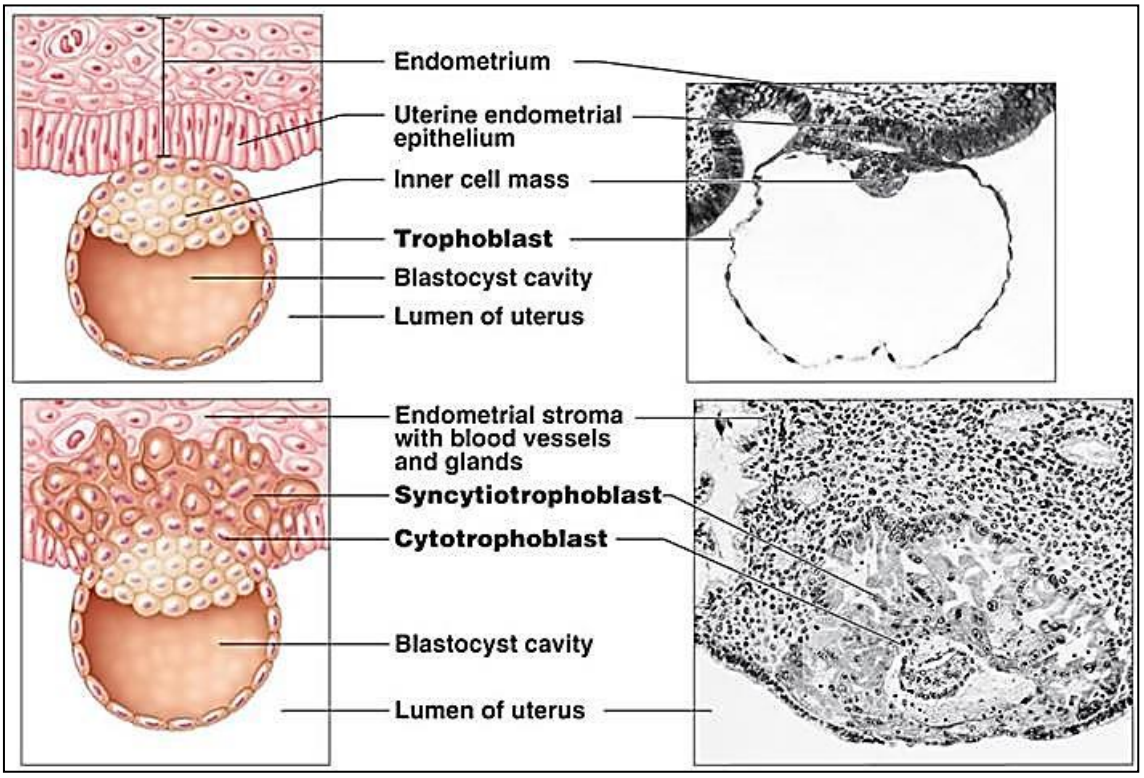

Figure 1-2: Differentiation of human trophoblast.

The process of proliferation of trophoblast in the formation of the placenta (Midlands Feb 8, 2015).

1.3 The fetal-maternal interface

The placenta and fetal membranes are the sites of direct contact between the maternal and fetal tissue, thus forming the human fetal-maternal interface. The fetal-maternal interface is composed of three basic structures: the decidua, the amnion and the chorion. The decidua is the decidualised endometrial lining, which forms the maternal part of the placenta. The amnion is comprised of a translucent membranous tissue that forms (together with the chorion) the amniotic fluid-filled amniotic sac that serves to protect the foetus. The chorion is composed of two layers: an outer reticular adjacent to the amnion

and an inner epithelial layer composed of trophoblast cells (Bryant-Greenwood, 1998, Benirschke and Kaufmann, 1995).

Placental trophoblast cells are specialized cells that play an imperative role in immune tolerance, because they are the only cells of the fetal-placental unit which are in direct contact with the maternal tissue at the fetal-maternal interface (Yie et al., 2006). Trophoblasts are a group of specialized extra-embryonic cells that play an important role in interactions with the decidualised maternal uterus. They are involved in the selective transport of maternal antibodies across the placenta. Moreover, trophoblast cells have the ability to secrete pregnancy factors and hormones that provide local immune suppression (Fernandez et al., 1999).

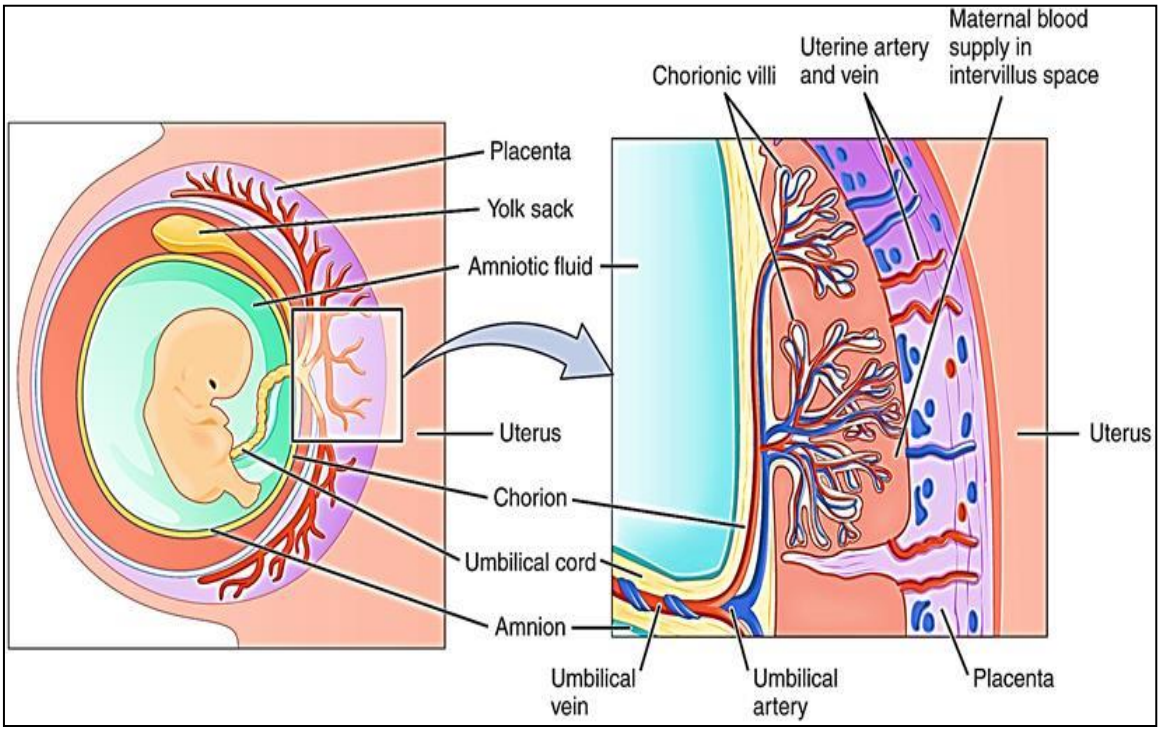

Figure 1-3: The human placenta.

Schematic illustration of the human placenta shows various parts of a fetal-maternal interface. The human placenta forms tiny, that extend into the wall of the uterus. Blood vessels which arise from the embryo pass the placenta through the umbilical cord develop in the villi. The maternal side extends toward the space surrounding the villi (intervillus space) (Midlands Feb 8, 2015).

1.4 Immune cells at human fetal-maternal interface

During normal human pregnancy the decidua is infiltrated by a variety of white blood cells (leukocytes), including natural killer (NK) cells, macrophages and T lymphocytes (Mincheva-Nilsson et al., 1994, Loke and King, 2000). These cells, together with decidua cells and trophoblast cells, secrete large amounts of different cytokines and other regulatory molecules. In addition, natural killer T (NKT) cells are present in the decidua (Tsuda et al., 2001), as well as low numbers of dendritic cells (Gardner and Moffett, 2003), and regulatory T cells (Heikkinen et al., 2004) during the first trimester.

NK cells are phenotypically characterized by the expression of cluster of differentiation (CD) 56 and the lack of expression of CD3. NK cells are divided into two distinct subsets, defined as CD56dim CD16+ CD3− and CD56bright CD16− CD3−. In normal circulating peripheral blood, 90% of NK cells have the 'classical' phenotype CD56dim CD16+ CD3−; the remaining 10% are CD56bright CD16− CD3− (Lanier et al., 1986).

NK cells are the most dominant leukocytes at the human fetal-maternal interface (70% of all leukocytes) in early gestation (Bulmer et al., 1991). However, the proportion of NK cells steadily declines after the first trimester to become nearly absent at term (Moffett-King, 2002).

NK cells may play a number of immunomodulatory roles in human pregnancy. In this respect, these cells are a rich source of many different cytokines that may help to establish a functionally healthy fetal-maternal circulation. These include interleukin (IL)-1, IL-10, tumour necrosis factor α (TNF-α), tissue growth factor-beta 1, leukemia inhibitory factor and interferon (IFN)-γ (Rieger et al., 2002, Lightner et al., 2008). NK cells may produce various angiogenic factors, including vascular endothelial growth factor and placental

growth factor, suggesting that they promote angiogenesis in the human placenta (Hanna et al., 2006). Thus, it seems that NK cells are essential in the establishment of adequate human fetal-maternal circulation.

Macrophages comprise the second largest leukocyte population and the most numerous antigen-presenting cells (APCs) at the fetal-maternal interface and are present throughout pregnancy (Bulmer and Johnson, 1984, Starkey et al., 1988, Williams et al., 2009). Decidual macrophages (DMs) account for about 20-30% of the human decidua at the site of implantation (Vince et al., 1990). It was reported that macrophages are partly suppressive in the placenta as well as in the decidua (Mues et al., 1989, Parhar et al., 1988), suggesting that macrophages play a key role in fighting pathogens during infection.

DMs have a phenotype similar to macrophages present elsewhere in the body. They express the lipopolysaccharide (LPS) receptor CD14, which could potentially be important in an immune response against infection with Gram-negative bacteria. DMs secrete cytokines and growth factors, including IL-10, IL-4 and IL-13 (Roth et al., 1996, Chaouat et al., 1999, Dealtry et al., 1998). These manage local cellular and tissue interactions.

Maternal T lymphocytes comprise 10% of the leukocyte population at the fetal-maternal interface during early human pregnancy (Kurpisz and Fernandez, 1995, Trundley and Moffett, 2004, Shimada et al., 2006). Their number decreases during pregnancy compared to the non-pregnant situation (Maruyama et al., 1992, Haller et al., 1993, Ho et al., 1996).

Decidual T cells express the γδ T cell receptor and are double negative for expression of CD4 and CD8 (Fan et al., 2011, Mincheva-Nilsson et al., 1994). They can produce a variety of type 1 cytokines, including IFN-γ and TNF-α, and type 2 cytokines, including

IL-4, IL-5, IL-9, IL-10 and IL-13 (Chaouat et al., 1990, Lin et al., 1993, Wegmann et al., 1993). It has been suggested that type 1 cytokines are harmful for maintenance of pregnancy compared to type 2 cytokines, which appear to be protective for the foetus (Wegmann et al., 1993, Chaouat et al., 1995). TNF-α stimulates apoptosis, and TNF-α and IFN-γ inhibit the out-growth of trophoblast cells (Yui et al., 1994, Berkowitz et al., 1988, Haimovici et al., 1991). Type 2 cytokines appear to stimulate trophoblast out-growth and invasion (Chaouat et al., 1995, Saito et al., 1996, Das et al., 2002).

1.4.1 Cytokines network during early human pregnancy

Cytokines play a significant role in the immunological mechanisms involved in placental growth and pregnancy maintenance (Bowen et al., 2002, Arcuri et al., 2001). Early pregnancy is characterised by an increase of CD4+ T helper 1 (Th1) lymphocytes/pro-inflammatory cytokines, which are involved in blastocyst implantation, placentation and regulation of trophoblast invasion. The second phase of pregnancy is characterized by an anti-inflammatory state with the predominance of Th2/anti-inflammatory cytokines such as IL-4, IL-5, IL-6 and IL-10. During the third phase of pregnancy Th1/pro-inflammatory cytokines again increase (Wegmann et al., 1993, Lin et al., 1993).

The shift away from Th1/pro-inflammatory cytokine production during pregnancy is beneficial, since Th1/pro-inflammatory cytokines can potentially during pregnancy. For example, human peripheral blood mononuclear cells from females with a history of recurrent spontaneous abortions produced high levels of IFN-γ, but not IL-4 and IL-10. Contrastingly, a Th2/anti-inflammatory response has been found to promote successful fetal outcome (Dinarello, 2000, Opal and Depalo, 2000); Th2/anti-inflammatory cytokines inhibit Th1/pro-inflammatory responses, thereby increasing the probability of fetal survival (Piccinni et al., 2001).

1.4.2 The role of Interferon during pregnancy

IFNs are classified into type I and type II IFNs according to receptor specificity and sequence homology (Pestka et al., 1987, Pestka, 1983, Pestka, 2000). Type I IFNs are comprised of seven classes of molecules that are structurally related: IFN-α, -β, -δ, -ε, -κ, -ω and -τ. These all bind to a common heterodimeric receptor, the type I interferon receptor (IFNAR comprised of IFNAR1 and IFNAR2 chains). IFN-γ is the sole type II IFN.

IFN-γ is a potent a pro-inflammatory cytokine, which is produced by CD4+ T helper 1 Th1 lymphocytes, CD8+ cytotoxic T lymphocytes and NK cells, following their activation by immune and inflammatory stimuli rather than viral infection (Bach et al., 1997, Farrar and Schreiber, 1993). B cells, NKT cells and professional APCs may also secrete IFN-γ (Carnaud et al., 1999, Frucht et al., 2001, Harris et al., 2000). The mature form of human IFN-γ is comprised of a non-glycosylated protein, a polypeptide chain containing 144 amino acid residues, and is encoded by a single gene containing four exons mapping to chromosome 12q14.1 (Ikeda et al., 2002).

The IFN-γ receptor is a cell surface receptor composed of two subunits: an alpha subunit and a beta subunit. The alpha is known as IFN-γ receptor 1, whereas the beta subunit is known as IFN-γ receptor 2 (IFN-γR2) (Soh et al., 1993, Soh et al., 1994, Hemmi et al., 1994). The binding of these receptors causes dimerization and activates the Janus Kinase family members JAK-1 and JAK-2 by tyrosine phosphorylation (Schroder et al., 2004). JAK-1 and JAK-2 subsequently phosphorylate a recognition sequence for signal transducer and activator of transcription (STAT) 1 on IFN-γ (Laurence et al., 2012, Braunstein et al., 2003, Stark et al., 1998). Once STAT1 is phosphorylated by tyrosine, dimerization causes it to translocate to the nucleus and to bind to the interferon gamma activated sequence promoter. This in turn activates the transcription of multiple genes

such as interferon regulatory factor 1 and the intracellular adhesion molecule-1 (Boehm et al., 1997, Stark et al., 1998).

IFN-γ is one of the main cytokines that promotes innate and adaptive immune responses at the fetal-maternal interface (Piccinni et al., 1998, Ito et al., 2000, Guleria and Pollard, 2000). IFN-γ is associated with the decidualization process and can protect against intracellular pathogens (Lloyd and Hawrylowicz, 2009, Bulla et al., 2004, Vilcek, 1982, Young and Hardy, 1990). Jin et al. (2007) reported IFN-γ-dependent resistance against *Toxoplasma* infection in mice in early pregnancy, which resulted in increased immunity levels (JIN et al., 2007). Therefore, it is likely that upholding a normal level of IFN-γ during pregnancy is important for the success of pregnancy.

In early human pregnancy, IFN-γ is secreted in the uterus by uterine natural killer (uNK) cells residing in the endometrium. Trophoblast cells strongly express IFN-γ during early pregnancy, but this declines and by term there is almost no expression (Paulesu et al., 1994). Both IFN-γ receptors are expressed in human placental trophoblast cells throughout human pregnancy with significantly increased levels of IFN-γR2 expression in late pregnancy compared to early pregnancy (Banerjee et al., 2005).

IFN-γ has been reported to induce MHC class II antigen-mediated apoptosis in certain cell types, including ovarian carcinoma and placental cells (Ossina et al., 1997, Xu et al., 1998, Rathbun et al., 2000, Wall et al., 2003, Liu et al., 2002). MHC class II expression has been found to lead to gestational complications such as preeclampsia and allogeneic fetal rejection (Athanassakis-Vassiliadis et al., 1990, Vassiliadis et al., 1994, Morris et al., 2000, Laresgoiti-Servitje et al., 2010). One of the key protective mechanisms in humans is thought to be the lack of MHC class II molecules on trophoblast cells (Murphy and Tomasi, 1998, Sutton et al., 1983, Starkey, 1987).

MHC class II expression can be induced by IFN-γ in cells constitutively expressing MHC class II molecules, such as thymic epithelial cells and APCs. Some cell types, including fibroblasts, epithelial cells and endothelial cells, do not normally express MHC class II antigens, but are induced to do so in response to IFN-γ (Glimcher and Kara, 1992, Kara and Glimcher, 1993).

In contrast, trophoblast cells do not express MHC class II antigens, even after exposure to IFN-γ (Chatterjee-Hasrouni and Lala, 1981, Peyman et al., 1992). The inability of trophoblast cells to express MHC class II proteins is due to the absence of expression of MHC class II transactivator (CIITA), which acts as a master regulator of MHC II expression (Murphy and Tomasi, 1998, Morris et al., 1998, van den Elsen et al., 2000). Thus, silencing of CIITA transcription in trophoblasts is likely to be crucial for the survival of the fetus by protecting it against maternal immune attack (Holtz et al., 2003, Murphy et al., 2004). Under some circumstances, placental trophoblasts of women suffering from chronic inflammation of unknown etiology and recurrent miscarriage display CIITA and MHC class II expression (Labarrere and Faulk, 1990, Athanassakis et al., 1995). Also, CIITA and MHC class II expression may be induced by viral infection or following mechanical injury (Sims et al., 1997).

CD74, also known as human leukocyte antigen (HLA)-DR antigen-associated invariant chain (Ii), is a type II integral membrane protein which is thought to function mainly as an MHC class II chaperone. However, 5% of CD74 is expressed independently of MHC class II at the cell membrane and is believed to be involved in functions including as a survival receptor (Henne et al., 1995). In this respect, CD74 has been reported to be induced by IFN-γ in certain cell types, including dendritic cells and tumour cells (Pessara et al., 1988) and trophoblast cells (Ranella et al., 2005).

Several mechanisms have been suggested to explain the induction of expression of CD74 by IFN-γ. CD74 could be induced directly through intramembrane proteolysis, and then released into the nucleus. This has been suggested for the IL-8 mechanism (Beswick et al., 2005a). Alternatively, CD74 could be induced indirectly via Cathepsin S or by other yet unknown mechanisms (Binsky et al., 2007, Maubach et al., 2007).

1.4.3 Infection and pregnancy

Complications of early pregnancy include issues associated with intrauterine infections and can cause a significant risk to the developing fetus. Pathogens can enter the placenta via three routes: transported in the blood; ascending into the uterus from the lower reproductive tract; descending into the uterus from the peritoneal cavity (Espinoza et al., 2006, Mor, 2008).

Intrauterine infections are associated with up to 40% of cases of pre-term labour (Lamont, 2003). Furthermore, 85% of pre-term deliveries at less than 28 weeks of gestation have evidence of infection (Epstein et al., 2000). Intrauterine infections may occur early in pregnancy, often preceding any observed pregnancy complications (Gonçalves et al., 2002). Miscarriages, which may be due to pre-existing endometritis, are most common during the first trimester of pregnancy. Other complications can occur throughout the pregnancy, such as pre-term labour and preeclampsia (Arechavaleta-Velasco et al., 2002, Romero et al., 2003, Gonçalves et al., 2002).

Bacteria-associated inflammation has been identified as an important cause of pre-term labour and miscarriage (Romero et al., 2010, Kim et al., 2010, Ralph et al., 1999, Hay et al., 1994, Romero et al., 2007). During bacterial infection, toxins are released that activate the production of cytokines, including IL-6 , TNF-α, chemokines (such as IL-8), IL-12, IFN-γ, prostaglandins , proteases and other enzymes (Romero et al., 2007, Filisetti and

Candolfi, 2004, Phillippe et al., 2001). These factors cause a coordinated response that leads to uterine contractions and placental detachment.

The placenta plays a major role in fetal protection, although many of the mechanisms involved are unclear. Placental trophoblasts have been demonstrated to express the toll-like receptors (TLRs) TLR-2 and TLR-4 (Holmlund et al., 2002, Kumazaki et al., 2004, Patni et al., 2009). TLR-2 recognizes bacterial lipoproteins, peptidoglycan and lipoteichoic acid (Yoshimura et al., 1999, Schwandner et al., 1999, Lien et al., 1999), while TLR-4 recognizes Gram-negative bacterial LPS (Poltorak et al., 1998).

LPS, also known as endotoxin, is a major constituent of the outer membrane of Gram-negative bacteria (Poltorak et al., 1998, Qureshi et al., 1999). It is made of both hydrophobic and hydrophilic domains, and is important for bacterial survival, affording protection against hydrolytic degradation by other organisms.

LPS is a large molecule that consists of three component parts: lipid A, core oligosaccharide (OS), and O-specific side chain or O-antigen (Hardy, 2003). O-antigen (LPS O-chain), contained within LPS, is a repetitive glycan polymer. This antigen is linked with the outer core oligosaccharide and is the outermost area of the LPS molecule.

LPS is noted for its diversity. Different bacterial strains can generate different O-antigen LPS structures. For example, *Escherichia coli* strains produce more than 160 different O-antigen structures (Raetz and Whitfield, 2002). Furthermore, LPS exhibits two structural forms, smooth and rough LPS, depending on the presence or absence of the O-antigen. That is, the absence of O-chains makes the LPS rough, whereas smooth LPS is distinguished by the presence of O-chains (Reinhart et al., 2004).

The OS region of LPS links directly to lipid A. Lipid A is a phosphorylated glucosamine disaccharide that links with multiple fatty acids. Its primary function is to attach LPS to

the bacterial membrane (Raetz and Whitfield, 2002). The OS usually contains sugars such as heptose and 3-deoxy-D-manno-octulosonic acid and is divided into the outer and inner cores. The core parts also have non-carbohydrate components, i.e. phosphate and ethanolamine substitutes (Henderson et al., 1996).

In terms of infection, large volumes of data regarding trophoblast responses to bacteria in different aspects of the infections have been investigated. CD74 is an example of a pathogen receptor that recognises the human gastric pathogen *Helicobacter pylori* (Beswick and Reyes, 2009). *H. pylori* binding to CD74 on gastric epithelial cells induces nuclear factor-κB (NF-κB) and subsequent cellular responses, such as the secretion of the pro-inflammatory cytokine IL-8 (Beswick et al., 2005b). In this respect, a previous study reported that CD74 expression was induced by LPS stimulation in the gastric cancer cell line MKN-45 (Zheng et al., 2012). This supports a potential role of CD74 in inflammatory disease.

1.5 Models of placental function

Primary cell lines have been widely used to study placental physiology and function (King et al., 2000, Kovo and Golan, 2008). Three categories of cell line are used: choriocarcinoma cells, including BeWo, JAR and JEG-3 cell lines, spontaneous cell lines, and transfected cells (Sullivan, 2004, Graham et al., 1993).

1.5.1 JEG-3 cell line

The JEG-3 cell line was established in 1971 by Kohler and Brindson (KOHLER and BRIDSON, 1971) and was one of six clonal lines of human choriocarcinoma originating from the same tumour used to establish the BeWo cell line. Choriocarcinomas are highly malignant tumours that arise from trophoblast cells (Berkowitz and Goldstein, 1996).

The morphology of JEG clones has been reported to vary among clones, but all clones, including JEG-3, grow as a monolayer. The JEG-3 cell line has been reported to produce and secrete hCG, hPL and progesterone (Burnside et al., 1985, DEAN et al., 1980, Tuckey, 2005).

The JEG-3 cell line has been widely used for a variety of *in vitro* studies owing to this cell line sharing many of the biological and biochemical characteristics associated with syncytio trophoblast and cytotrophoblast of human placenta (Matsuo and Strauss 3rd, 1994). In addition, JEG-3 is an ideal model for investigating placental functions, including metabolism, transport of substances and molecular mechanisms responsible for cytokine release during pregnancy (Blanchon et al., 2002, Pongcharoen and Supalap, 2009, Hardman et al., 2006, Müller et al., 2004). These studies have established JEG-3 cells as a good *in vitro* model for studying many aspects of the placental physiology and function.

1.5.2 ACH-3P cell line

The novel ACH-3P trophoblast hybrid cell line was established by fusion of primary human first trimester trophoblasts (week 12 of gestation) with a human choriocarcinoma cell line (AC1-1).

The first trimester trophoblasts isolated from human placenta essentially comprised two trophoblast subpopulations: villous cytotrophoblasts and invasive EVT. ACH-3P cells closely resemble primary trophoblast in terms of trophoblast markers (cytokeratin-7, integrins and matrix metalloproteinases), transcriptome and invasion abilities. Resembling AC1-1 cells, they display a uniform polygonal, epithelial-like cytomorphology.

The ACH-3P cell line is composed of a mixed population of HLA-G-negative and HLA-G-positive cells, which can be immuno-separated by HLA-G surface expression. The HLA-G-negative cells represent cytotrophoblast-like cells, while the HLA-G-positive cells represent a population of EVT-like cells.

The ACH-3P cell line is suitable for the study of autocrine and paracrine signalling regulation of various aspects of trophoblast function, such as a novel effect of TNF–α on matrix metalloproteinases (MMP15) (Hiden et al., 2007).

1.6 Major histocompatibility complex molecules

In humans, MHC is located on the short arm of chromosome 6 (p21.3), which spans a number of four million base pairs of DNA and encodes at least 130 genes (Hviid, 2006, Fernandez et al., 1999, Klein, 1986). The human complex genes are expressed in almost all nucleated cells (Kolar et al., 2003). The HLA is generally divided into three groups: class I, II, and III genes and proteins (Figure 1-4). Class I and II genes code for glycoproteins that are involved in antigen presentation (Auffray and Strominger, 1986). HLA-I is classified as classical HLA class I (class Ia; HLA-A, HLA-B, HLA-C) and non-classical HLA class I (class Ib; HLA-E, HLA-G, HLA-F) (Fernandez et al., 1999).

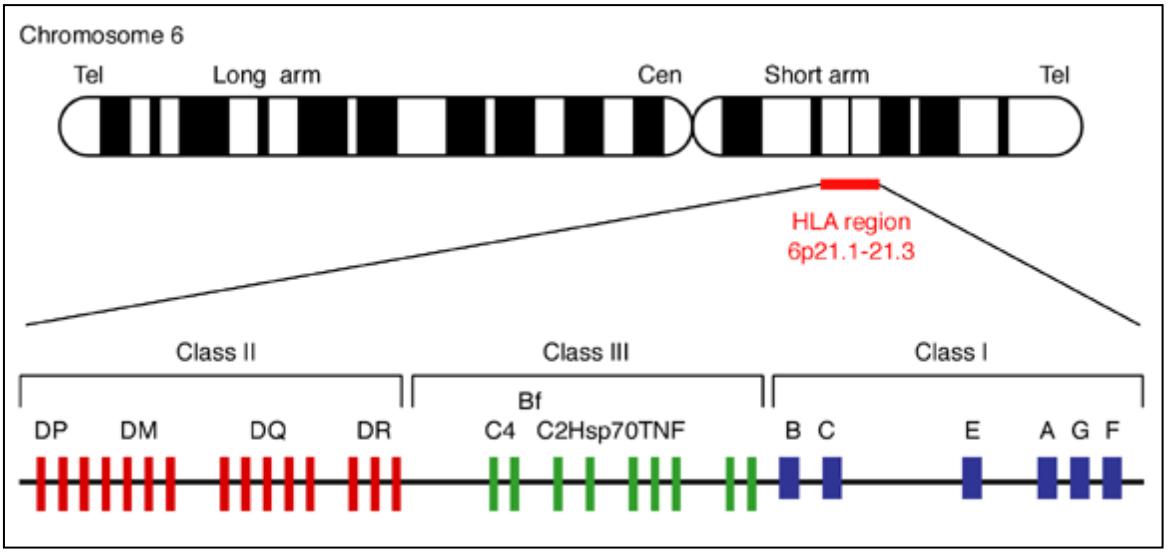

Figure 1-4: The human leukocyte antigen (HLA) complex.

HLA class I, which encodes classical (class Ia) and non-classical (class Ib) HLA proteins is highlighted in blue. Classical HLA antigens are encoded by the HLA-A, -B, and HLA-C genes and are expressed on cell somatic cells. Non-classical HLA antigens are less polymorphic and encoded by HLA-E, -F, and -G. Class III genes, shown in green, are located between class I and II and encode complement system proteins (C2, C4A, C4B, and B1). HLA class II, highlighted in red, encode the HLA-D family of molecules, HLA-DR, HLA-DQ, HLA-DM, and HLA-DP,. HLA class II is expressed only in antigen-presenting cells. Adapted from Yip et al., 2006.

1.6.1 MHC class I molecules

The MHC class I comprises some 20 loci on human chromosome 6 (p21.3), and is most extensive gene system so far identified (Fernandez et al., 1999, Trowsdale et al., 1990). MHC class I molecules appear on the cell surface as heterodimers, which consist of a highly polymorphic heavy (44 kDa) chain, a non-polymorphic light (12 kDa) chain subunit called β2-microglobulin (β2m), and a short (8-11 mer) peptide (Groothuis et al., 2005, Sibilio et al., 2008). The short peptide is a fragment of a larger protein that the cell has cleaved for presentation to the immune system, and it is bound by a cleft in the class I heavy chain (Falk et al., 1991, Bjorkman et al., 1987b).

The class Ia genes, accredited as 'classical', encode HLA-A, -B, -C molecules that are present in almost all nucleated cells. These molecules are involved in the differentiation of non-self from self, presenting peptide antigens to T lymphocytes (Zinkernagel and Doherty, 1974). Trophoblast cells can express HLA-C, but they lack typical MHC class Ia (HLA-A and HLA-B) products (Fernandez et al., 1999, Hutter et al., 1996, Redman et al., 1984). However, HLA-C is expressed at a low level on the cell surface and intracellularly (King et al., 1996); levels of surface HLA-C in normal human first-trimester EVT are 10 fold lower than in normal somatic cells. In addition, the peptide binding groove of HLA-C is less polymorphic and its β2m free heavy chains are more stabilised on the cell surface compared to HLA-A and HLA–B (King et al., 1996).

HLA-C may play an important role during pregnancy in optimal NK cell recognition and allograft protection, along with the non-classical (class Ib) molecules HLA-E, HLA-F and HLA-G. Thus, the presence of HLA-C on the trophoblast prevents killing of trophoblast cells by NK cells by way of the 'missing self' hypothesis (King et al., 1996).

HLA-C has been reported to be associated with pre-eclampsia and recurrent miscarriage. An immunogenetic study indicated that the HLA-C2 group was associated with maternal killer immunoglobulin receptor (KIR) (AA genotype) and increased risks of preeclampsia. Furthermore, the interaction between maternal KIR NK cells and HLA class I molecules on trophoblasts might play a physiological role in placental development (Moffett and Loke, 2004, Hiby et al., 2004, Apps et al., 2008a, Hiby et al., 2008).

HLA class Ib molecules are encoded by HLA-E, -F, and -G genes, which are less polymorphic (oligomorphic) and restricted in terms of cell distribution (Colonna et al., 1997, Geraghty et al., 1987, Koller et al., 1988, Shawar et al., 1994). In 1987, HLA-E was recognised as HLA-6.2 and was mapped to chromosome 6p21.3, between the HLA-A and

HLA-C loci (Koller et al., 1988). The overall structure of HLA-E is similar to that of MHC class Ia molecules, but the gene has limited polymorphism and a lower expression level. Moreover, this gene encodes a class I heavy chain of 41kDa that can associate with β2m (Tripathi et al., 2006). HLA-E expression is found in the majority of cells expressing other HLA class I molecules, including trophoblast cells (Ishitani et al., 2003).

HLA-E is expressed in B cells, activated T lymphocytes and various other cells (Tripathi et al., 2007, Houlihan et al., 1995). Surface expression of HLA-E is regulated by binding of a restricted pool of peptides derived from the leader sequence of other MHC class I molecules including HLA-A, -B, -C and –G (Lee et al., 1998, Braud et al., 1998). For that reason, HLA-E has a low level of cell surface expression (Wainwright et al., 2000). It has low polymorphism and only five alleles, suggesting that it does not play a key role in antigen presentation (Tripathi et al., 2006).

HLA-F has very low allelic polymorphism, and was initially found to be retained intra-cellularly in a number of cell lines (Wainwright et al., 2000, Lepin et al., 2000). It is expressed primarily in trophoblasts (Ishitani et al., 2003). Cell surface expression of HLA-F has also been demonstrated *in vivo* in EVT (Ishitani et al., 2003). HLA-F tetramers have been shown to directly interact with ILT2 (LIR1) and ILT4 (LIR2) receptors (Lepin et al., 2000), therefore indicating their possible role in regulating the activity of immune effector cells.

The genomic structure of HLA-G is very similar to other MHC class I genes, and alternative splicing of the HLA-G gene is known to produce seven messenger RNAs (mRNAs) that encode four membrane bound (HLA-G1, -G2, -G3 and –G4) and three soluble (HLA-G5, -G6, -G7) protein isoforms (Paul et al., 2000, Ishitani and Geraghty, 1992, Vercammen et al., 2008).

The HLA-G molecule consists of a heavy chain, composed of α1, α2 and α3 domains. The heavy chain is non-covalently associated with β2-m. The peptide binding groove is formed by the α1 and α2 helices and peptide is bound with the floor, formed by an antiparallel β sheet derived from the α1 and α2 domains (Clements et al., 2005, Rodgers and Cook, 2005, Bjorkman and Parham, 1990). HLA-G displays remarkably little polymorphism and its expression pattern is highly restricted (Kirszenbaum et al., 1997, LeMaoult et al., 2005). Strong expression of HLA-G is observed in EVT (Bouteiller and Blaschitz, 1999).

Expression of HLA-G has also been found, in human thymic and pancreatic cells, erythroid and endothelial precursor cells, and human cornea, an immune privileged tissue (Le Discorde et al., 2003, Mallet et al., 1999, Menier et al., 2004, Cirulli et al., 2006).

HLA-G expression is up-regulated under certain pathophysiological conditions, and possibly interacts with both the innate and adaptive immune responses (Carosella et al., 2008, Hunt et al., 2007, Favier et al., 2007). HLA-G has been shown to protect fetal cytotrophoblast cells by inhibiting uNK cells through direct interaction with inhibitory receptors on immune cells (Rouas-Freiss et al., 1997, Carosella et al., 1996, Shiroishi et al., 2003, Rajagopalan and Long, 1999, Bainbridge et al., 2001). In addition, HLA-G molecules possibly contribute to prevent the pregnancy complications, such as pre-eclampsia and in unexplained recurrent spontaneous abortions (Hviid, 2006).

1.6.2 MHC class II molecules

The human MHC class II antigen encodes HLA-DM, -DP, -DQ, -DO and –DR genes encoded highly polymorphic membrane-bound glycoproteins that are composed of two polypeptide chains (alpha and beta), which are 230 and 240 amino acids long (Kelly et al., 1991, Karlsson et al., 1991). In addition, each polypeptide chain consists of two

domestic domains; alpha-1 and alpha-2 for the alpha polypeptide, and beta-1 and beta-2 for the beta polypeptide. The peptide-binding region is similarly positioned to that seen in MHC class I molecules, between the alpha-1 and beta-1 domestic domains. It is bound by a beta pleated sheet below and two alpha helices at its sides, and is capable of binding (via non-covalent interactions) a small peptide of about 10 amino acids.

MHC class II molecules are normally presented only on APCs. Their function is to present antigen peptide fragments to CD4+ T cells responsible for cell-mediated immune responses (Cresswell, 1994).

1.6.3 Antigen presentation by MHC molecules class I and II molecules

T lymphocytes recognize antigen peptide in association with class I and II MHC molecules. MHC class I molecules present antigenic peptides originating in the cytosol to CD8+ T lymphocytes, whereas MHC class II molecules present endocytically acquired peptide to CD4+ T lymphocytes.

In order to access MHC class I molecules, peptides generated by proteasomes are translocated via transport associated with antigen presentation (TAP) into the endoplasmic reticulum (ER) lumen. In the ER, MHC class I heterodimers (heavy 44 kDa chain glycoprotein, and non-covalently-associated β2m soluble light chain) are assembled. In order to stabilize the MHC class I molecule a third peptide component is required which inserts itself deeply into the MHC class I binding groove, which accommodates 8-9 amino acids. In the absence of these peptides, MHC class I molecules are stabilized by ER chaperone proteins, such as calreticulin, ERp57 (known as PDIA3), protein disulphide isomerase (PDI) and the dedicated chaperone tapasin. Tapasin interacts with TAP, thus coupling peptide translocation into the ER with peptide delivery to MHC class I molecules. The chaperones are freed, and assembled peptide-MHC class I

complexes leave the ER via Golgi and travel to the plasma membrane. Peptides and MHC class I molecules that fail to present antigen peptide at the cell surface are returned to the cytosol for degradation (Vyas et al., 2008, Hughes et al., 1997, Koopmann et al., 2000).

The MHC class II transmembrane α- and β- chains are assembled in the ER and associate with the Ii (CD74). The resulting Ii-MHC class II complex is transported to a late endosomal compartment termed the MHC class II compartment. Here, Ii is digested; leaving a residual class II- associated Ii peptide (CLIP; amino acids 91-99) in the peptide-binding groove of the MHC class II heterodimer. In order to facilitate the exchange of the CLIP fragments for specific peptides derived from a protein degraded in the endosomal pathway, MHC class II molecules require HLA DM. After reaching this stage, MHC class II molecules are ready for transporting via the plasma membrane to present their peptides to CD4+ T cells (Denzin et al., 2005, Neefjes et al., 2011) (Figure 1-5).

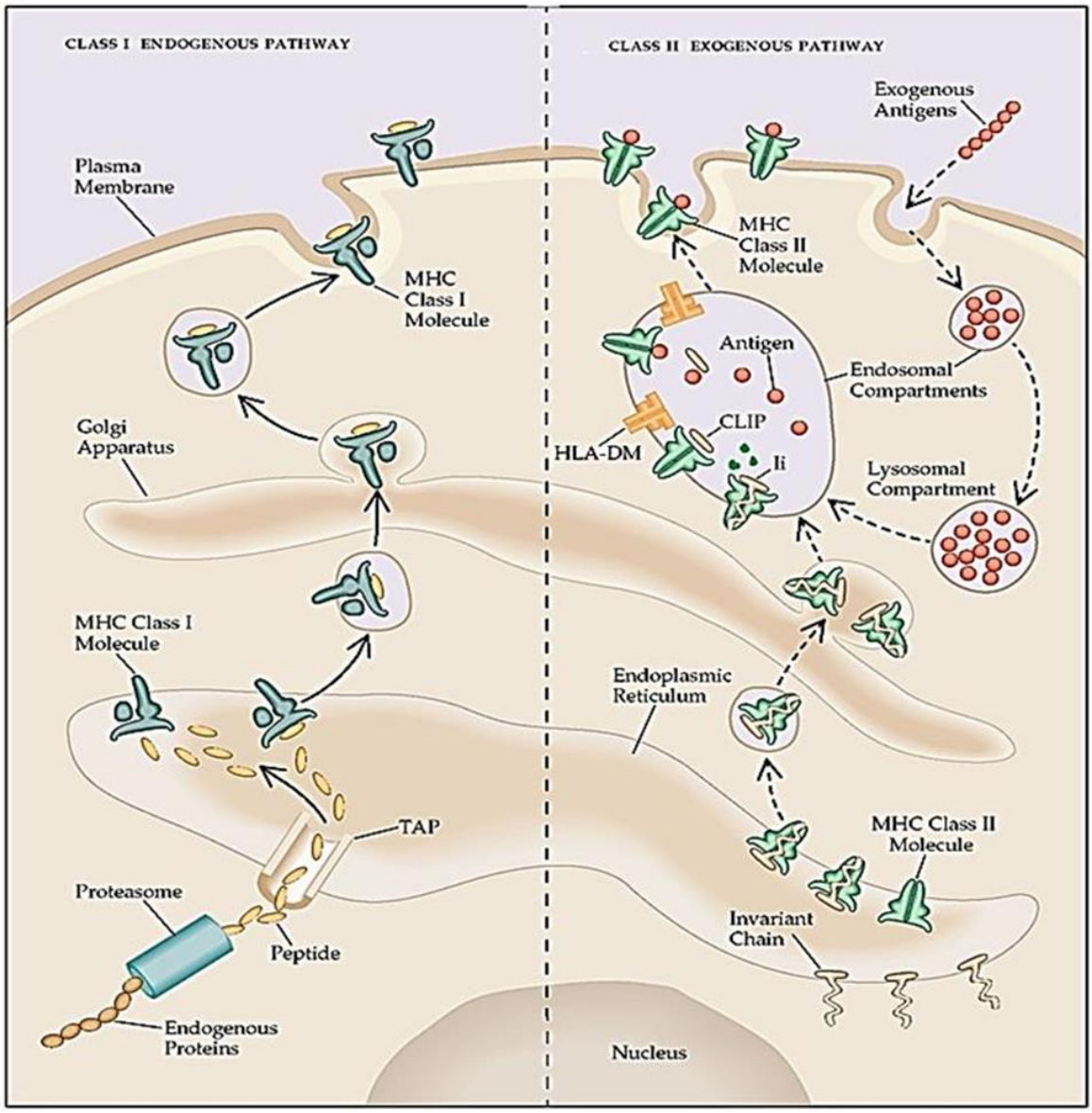

Figure 1-5: MHC class I and II molecule antigen-presenting pathways.

In the MHC class I molecule pathway, endogenous proteins are broken down by proteasomes into smaller peptides. In the endoplasmic reticulum (ER), an antigenic peptide binds to the peptide binding site in an MHC class I molecule. The peptide-MHC complex then migrates through the Golgi apparatus to the cell surface. In the MHC class II molecule pathway, the transmembrane α- and β- chains of the MHC class II molecule bind to the invariant chain (Ii) in the ER. Ii is partially degraded in an endosomal compartment. The portion of Ii that occupies the antigenic peptide binding site on the MHC class II molecule (called CLIP) is removed with the help of HLA-DM, freeing the molecule for binding the processed antigen. Once the antigenic peptide has bound to an MHC class II molecule, the complex migrates to the cell surface (Crankshafts, Feb 28, 2015).

1.7 Invariant chain, CD74

The Ii gene (Figure 1-6), located on chromosome 5 (q32), was first identified in 1979 by Jones and colleagues when the MHC class α chain and Ii were separated on 2D-gels (Jones et al., 1979). However, it was not until 1989 that Ii was found to have a role in antigen presentation through influencing the expression and peptide loading of MHC class II molecules (Sant and Miller, 1994).

In 1995 the Leukocyte Typing Workshop decided that Ii should be designated CD74 (Henne et al., 1995). CD74 is a type II transmembrane glycoprotein which exists in different isoforms, defined by its primary amino acid sequence. There are four CD74 isoforms in human beings, p33, p35, p41 and p43. P33 and p41 are distinguished by alternative splicing of the Ii transcript where the p41 isoform contains an extra exon (exon 6b) (O'Sullivan et al., 1987, Strubin et al., 1986). However, the major and most common isoform in humans is the p33 isoform, which has a short NH2-terminal cytosolic tail of 30 amino acids, intracytoplasmic residues (IC), a 26 amino acid hydrophobic transmembrane region (TM) and 160 amino acid extracytoplasmic domain containing two N-linked carbohydrate chains (EC).

The human-specific p35 isoform contains a longer cytoplasmic tail, due to the use of an alternative translation initiation site, while the p41 and p43 isoforms are the result of alternative splicing (Claesson et al., 1983, Koch et al., 1987, Lipp et al., 1987, Stein et al., 2007, Landsverk et al., 2009, Beswick and Reyes, 2009, Warmerdam et al., 1996).

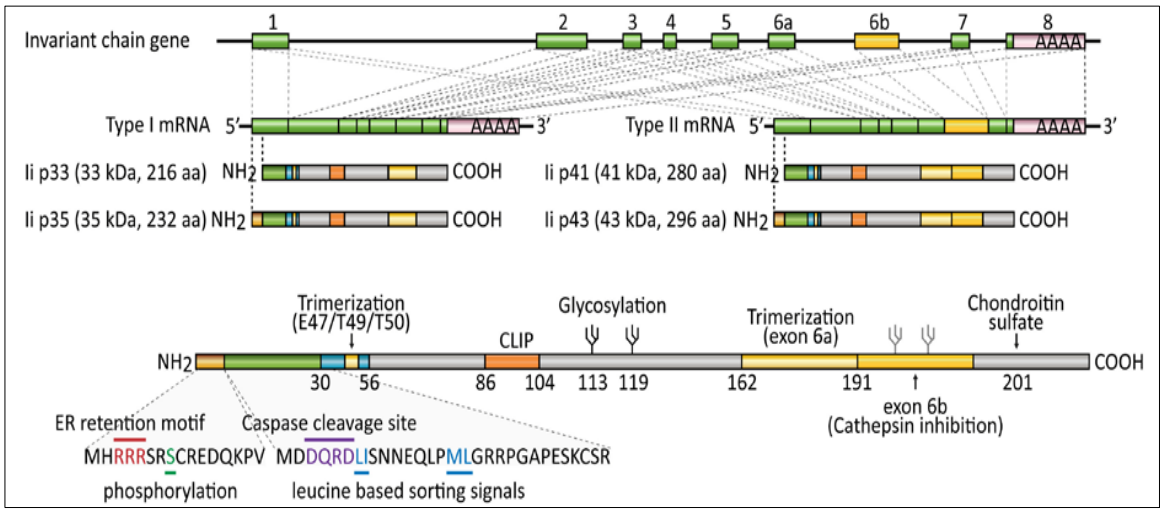

Figure 1-6: Structure of the human invariant chain (Ii) gene.

The corresponding gene of the invariant chain (Ii) consists of nine exons. The p33 and p35 forms of Ii, both encoded by eight exons, differ by 16 N-terminal residues as a result of alternative translation initiation. p41, encoded by nine exons, is generated by alternative splicing of a common pre-mRNA. The exon 6b in p41 and p43 encodes a cysteine rich stretch of 64 amino acids near the C-terminus. All isoforms contain at least two N-linked and two O-linked glycosylations. Adapted from (Strubin et al., 1986, Gregers et al., 2003).

Synthesis of CD74 takes place in the ER in same manner as MHC class II molecules, despite the genes of these molecules being located on different chromosomes (Badve et al., 2002). CD74 assembles into homotrimers immediately after synthesis, forming a nonameric complex (Marks et al., 1990, Roche and Cresswell, 1990, Roche et al., 1991). Once CD74 is synthesized, it associates with DRα and DRβ in the ER. And it then directs the intracellular transport of class II molecules to the trans-Golgi network and subsequently to the endocytosis/lysosomal compartment where CD74 is cleaved and degraded.

The Ii also blocks the peptide binding groove of the secreted MHC molecule (Bakke and Dobberstein, 1990, Roche and Cresswell, 1990). CD74 is then disassociated from MHC class II and charged with antigenic peptides in order to appear on the cell surface and present the peptide to CD4+ T lymphocytes (Moldenhauer et al., 1999, Burton et al., 2004, Stein et al., 2007, Landsverk et al., 2009, Neefjes et al., 2011, Roche et al., 1991). CD74 is a multifunctional protein, but its main function is the association with MHC class

II α and β chains (Borghese and Clanchy, 2011, Maharshak et al., 2010, Stumptner-Cuvelette and Benaroch, 2002b, Pyrz et al., 2010). The role of CD74 in antigen presentation has been divided into three main functions. Firstly, CD74 acts as a chaperone that is responsible for stabilizing nascent HLA-DR αβ-heterodimers through the formation of a trimetric structure. Secondly, by means of various sorting and internalization signals in its N-terminal cytoplasmic tail, CD74 targets HLA-DR molecules to subcellular compartments. Thirdly, CD74 prevents loading of antigenic peptides into the groove of HLA-DR molecules outside endsomes/lysosmes through a stretch called CLIP (Class II-associated Invariant chain Peptide) (amino acids 91-99).

Recent data suggest that there are two distinct pathways by which the Ii-HLA-DR complex reaches the endocytic compartment. In the classical pathway Ii associates with MHC class II in the lumen of the ER and directs the whole assembly to a late endosomal compartment. Subsequently, Ii proteolytically degrades to allow the binding of exogenous peptides; the complex of αβ chains plus the antigenic peptides are then directed to the cell surface (Kvist et al., 1982, Bakke and Dobberstein, 1990, Lotteau et al., 1990, Marić et al., 1994, Blum and Cresswell, 1988). However, the co-localisation of Ii and class II on the cell surface and in early endosomes (Wraight et al., 1990, Koch et al., 1991, Roche et al., 1993, Warmerdam et al., 1996) suggests that HLA-DR is internalised from the cell surface and accumulates in the early endosome. This evidence for an alternative pathway for MHC class II molecules may be of immunological significance (Sercarz et al., 1993, Zhong et al., 1997, Griffin et al., 1997).

CD74 has been shown to have additional functions apparently unrelated to MHC class II transport. Through a pathway involving NF-kB, CD74 has been shown to be involved in B cell maturation and survival (Becker-Herman et al., 2005, Matza et al., 2001, Stein et

al., 2007). Interestingly, NF-kB can be activated by the intracellular domain of CD74, which is liberated from the membrane (Becker-Herman et al., 2005).

Recently, CD74 expression has been confirmed as highly expressed in inflammatory disorders and several types of tumours. For instance, 90% of B-cells cancers and the majority of cell lines derived from these cancers express CD74 in high levels compared to normal tissues (Stein et al., 2007). CD74 is expressed in different cancer cell types, including myeloma cells and prostate cancer cells (Beswick and Reyes, 2009, Verjans et al., 2009).

CD74 can act as a cell surface receptor for macrophage migration inhibitory factor (MIF) in association with CD44 (Leng et al., 2003, Gore et al., 2008), a cytokine that plays versatile roles in both innate and adaptive immunity. Therefore, the signal transduction upon MIF binding to CD74 requires CD44 (Meyer-Siegler et al., 2004, Shi et al., 2006). CD74 also acts as a pathogen receptor, such as for *H. pylori* in gastric epithelial cells (Beswick and Reyes, 2009).

1.8 Macrophage migration inhibitory factor (MIF)

Human MIF is a trimer composed of identical subunits (Figure 1-7), a structure which is unique among cytokines (Sun et al., 1996). The MIF molecule shows a marked topological similarity to three microbial enzymes, CHMI (5-carboxymethyl-2-hydroxymuconate isomerase), 4-OT (4-oxalcrotonate tautomerase) and chorismate mutase (Sun et al., 1996, Suzuki et al., 1996, Subramanya et al., 1996, Sugimoto et al., 1999, Swope and Lolis, 1999). Notably, however, there is no sequence homology among them (Calandra and Roger, 2003, Leng and Bucala, 2006, Baugh and Bucala, 2002).

Unlike any other cytokine or pituitary hormone, the primary structure of MIF consists of 114 amino acids with a molecular weight of 12.5 kDa (Donn and Ray, 2004, Calandra and Roger, 2003). The secondary structure of MIF consists of six β-pleated sheets (β1-β6) and two antiparallel α-helices (α1 and α2) and is closely similar to that of MHC molecules (Bach et al., 2009, Conroy et al., 2010, Bjorkman et al., 1987a).

The human MIF gene is located on chromosome 22 (22q11.1), and is composed of three different short exons of 205, 173 and 183 base pairs and two introns of 189 and 95 base pairs. Within the 5'region, there are several consensus DNA-binding sequences for transcription factors, including activator protein 1, NF-κB, EST GATA, SP1 and cAMP response element binding protein (Weiser et al., 1989, Bernhagen et al., 1994, Wistow et al., 1993, Paralkar and Wistow, 1994).

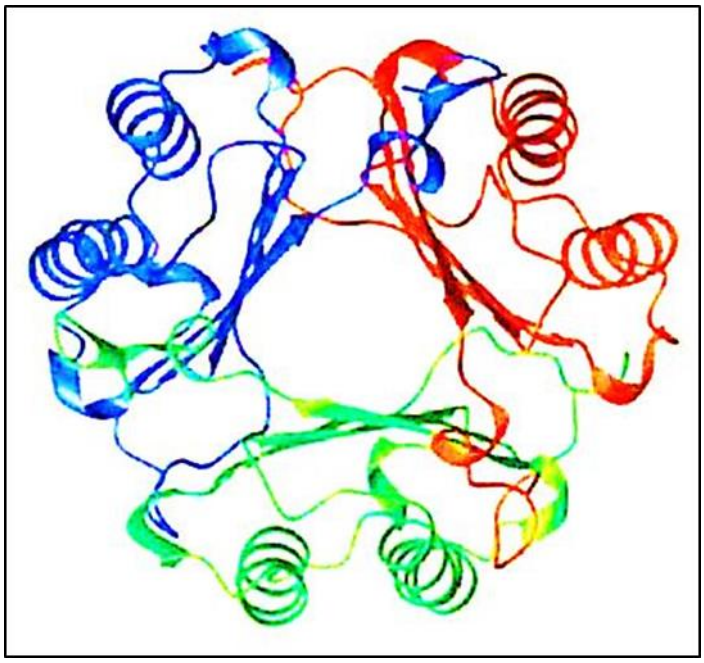

Figure 1-7: Three-dimensional ribbon diagram of human macrophage inhibitory factor.

The homotrimeric subunit structure of MIF. MIF, with a MW of 37.5 kDa comprises three identical monomers. Each monomer consists of two anti-parallel α-helices that pack against six β-pleated sheets. Each colour represents one monomer. Adapted from (Sun et al., 1996, Leng and Bucala, 2006)).

MIF is constitutively expressed and secreted by numerous cell types, and is stored in intracellular pools. Its secretion does not require de novo protein synthesis. Notably, MIF is expressed by cells and tissue in direct contact with the host's natural environment, such as gastrointestinal and genitourinary tracts, the lung and the epithelial lining of the skin. Another feature that makes MIF unique is that it can be highly expressed by some tissues of the endocrine system, specifically by organs that are involved in stress responses (Bach et al., 2008, Calandra et al., 1994, Bacher et al., 1997, Fingerle-Rowson et al., 2003).

MIF plays a pivotal role in both innate and adaptive immunity. It is a multi-functional molecule that has several roles in immune responses (Verjans et al., 2009). It functions as a pro-inflammatory cytokine, an anterior pituitary hormone, and it also possesses enzymatic activity (Calandra and Roger, 2003, Bach et al., 2009, Bernhagen et al., 1998).

MIF is induced by pro-inflammatory agents, such as LPS, IFN-γ and TNF-α (Calandra et al., 1994; Bernhagen et al., 1998; Martiney et al., 2000). Once MIF is released, it has the capacity to induce, rather than be inhibited by glucocorticoid hormones, as do most cytokines (Bacher et al., 1996; Calandra et al., 1995). MIF can also induce the production of TNF-α, nitric oxide, IL-1β, IL-2, IL-6, IL-8 and IL-12 (Jüttner et al., 1998; Calandra and Bucala, 1994; Calandra and Roger, 2003; Calandra et al., 1995).

It has been demonstrated that LPS can induce MIF expression and the up-regulation of Toll-like receptor 4 (TLR4) (Roger et al., 2001a). TLR-4 is the receptor for LPS (Takeda et al., 2003; Poltorak et al., 1998; Hoshino et al., 1999). The expression of TLR-4 is up-regulated by MIF through the ETS-related transcription factor PU.1.

Following binding of LPS to the LPS-binding complex, composed of TLR4, CD14, and lipopolysaccharide-binding protein (LBP), NF-κB is activated for nuclear translocation by a specific signal from the binding complex. Then, NF-κB is translocated into the nucleus to activate genes concerned with inflammatory or immune responses (Medzhitov and Janeway, 2002; Takeda et al., 2003; Abrahams et al., 2005).

Notably, MIF is a negative regulator of p53-mediated growth arrest and apoptosis, creating a link between MIF, inflammation, cell growth and tumorigenesis. Indeed, inhibition of p53 requires serial activation of extracellular signal-regulated kinase (ERK) 1/ ERK2, PLA2, cyclooxygenase 2 (COX2) and PGE2 (Hudson et al., 1999, Mitchell et al., 2002).

Two MIF signalling mechanisms have been identified, entailing an extracellular and an intracellular signalling pathway. The extracellular pathway entails MIF binding to the extracellular domain of CD74, the surface form of Ii, activating the mitogen activated protein kinase (MAPK) pathway and cell proliferation (Leng et al., 2003).

The complex of MIF and CD74 also binds to the adhesion molecule CD44. This binding is required for the phosphorylation of the ERK1/2- MAPK signalling pathway by MIF (Shi et al., 2006, Meyer-Siegler et al., 2004).

The activation of both receptors is also required for MIF-mediated cell proliferation through the initiation of a signalling cascade that causes NF-κB activation via Src kinase (Shi et al., 2006). This results in the phosphorylation of the retinoblastoma gene and subsequently affects cell proliferation (Bach et al., 2009).

In addition, MIF interacts directly with the Jun-activation domain-binding protein-1 (JAB1) in the cytoplasm. The latter is necessary for ERK activation. The interaction between MIF and JAB1 is believed to mediate biological activities such as cell growth, transformation and apoptosis (Kleemann et al., 2000; Lue et al., 2007; Shaulian and Karin, 2002). Additionally, a recent study by Berhagen and colleagues (2007) has revealed that MIF is a noncognate ligand for the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). MIF was observed directly to bind CXCR2 and compete with cognate ligands for CXCR4 and CXCR2. This suggests a new signalling pathway through a functional CXCR2-CD74 complex (Bernhagen et al., 2007).

MIF is expressed in first- and third-trimester villous trophoblasts and EVT and may play a role during implantation, early embryonic development and in the maintenance of pregnancy (Arcuri et al., 1999, Arcuri et al., 2007, de Oliveira Gomes et al., 2011, Ferro et al., 2008). It has been suggested that MIF may serve a physiological role in the creation of a suitable environment for the acceptance of fetal tissues by the maternal uterus (Arcuri et al., 1999).

Previous studies have reported heightened human MIF levels in association with pre-eclampsia and parasitic infections during pregnancy, such as malaria and toxoplasmosis

(Chaisavaneeyakorn et al., 2002;Chaisavaneeyakorn et al., 2005; Chaiyaroj et al., 2004; Todros et al., 2005; Jain et al., 2009). Furthermore, over-production of MIF is associated with the pathogenesis of bacterial sepsis, indicating that MIF could play both protective and pathogenic roles during different infections (Bernhagen et al., 1993). However, recurrent miscarriage in humans is associated with a suppressed level of MIF (Yamada et al., 2003).

1.9 CD44

CD44 is the major receptor for hyaluronic acid (HA), a high molecular weight glycosaminoglycan. Human CD44 is a highly polymorphic transmembrane glycoprotein (Underhill et al., 1987). In humans, it is encoded by a single highly conserved gene, containing at least 20 exons spanning approximately 50-60 kb of DNA, located on the short arm (p13) of chromosome 11 (Goodfellow et al., 1982; Screaton et al., 1992, Ponta et al., 1998). The exons exist as a result of alternative splicing affecting the extracellular domain and are further modified by a range of post-translational modifications (Goodison et al., 1999).

The first five exons (sl-s5), as well as exons 16 to 20 (s6-s10), are almost invariably expressed by a large number of non-epithelial cells. Their product is referred to as the ’standard’ form of CD44; standard isoforms of CD44 are designated CD44s or CD44H. Exons 6 to 15 can be alternatively spliced and are included within the ‘standard’ exons at an insertion site between exons 5 and 16. These encode variant isoforms of CD44, designated CD44v (Screaton et al., 1992, Tölg et al., 1993, Goodison and Tarin, 1998). The variant isoforms differ in peptide units which are included in the extracellular region of the protein.

Theoretically, alternative splicing would result in the generation of more than one thousand CD44 variants (Naor et al., 1997).

The standard form of CD44s consists of 361 amino acids (Screaton et al., 1992). However, its molecular size is considerably increased by posttranslational modifications through N- and O-linked glycosylation and the attachment of glycosaminoglycans (Mackay et al., 1988; Bartolazzi et al., 1996; Dasgupta et al., 1996; Bennett et al., 1995). These posttranslational modifications double the molecular size of CD44s, bringing it from 37-42 kDa to 85-95 kDa (Zhou et al., 1989; Lokeshwar and Bourguignon, 1991). In addition, the chondroitin and heparan sulphate chains increase the size of the human CD44 molecule from 85-95 kDa to 180-200 kDa (Jalkanen et al., 1988).

The human CD44 protein consists of four different domains: the N-terminal, found in the extracellular domain (N-terminal containing two binding sites for HA), a membrane-proximal domain, a transmembrane domain, and a C-terminal cytoplasmic domain (Nottenburg et al., 1989; Peach et al., 1993). Furthermore, exons 1-16 encode the extracellular domain, which can be modified by N- and O-linked glycosylation and contains binding sites for HA (Naor et al., 1997). Exon 18 encodes a short transmembrane domain which is involved in oligomerization and localization (Liu and Sy, 1997; Neame et al., 1995). Exons 19 and 20 encode the cytoplasmic domain (Goodison et al., 1999). Isoforms of CD44 all have the HA-binding domain located at the N-terminal region (Liao et al., 1995; Peach et al., 1993) (Figure 1-8).

The expression of CD44 has been identified on human trophoblast cells. In addition, first and second trimester trophoblast cells express the standard form of CD44 while EVT express an alternatively spliced form of CD44 known as CD44v7-8. The splicing variant of CD44 might play a role in the invasion of human trophoblast into maternal tissue in early human pregnancy (Choi et al., 2006).

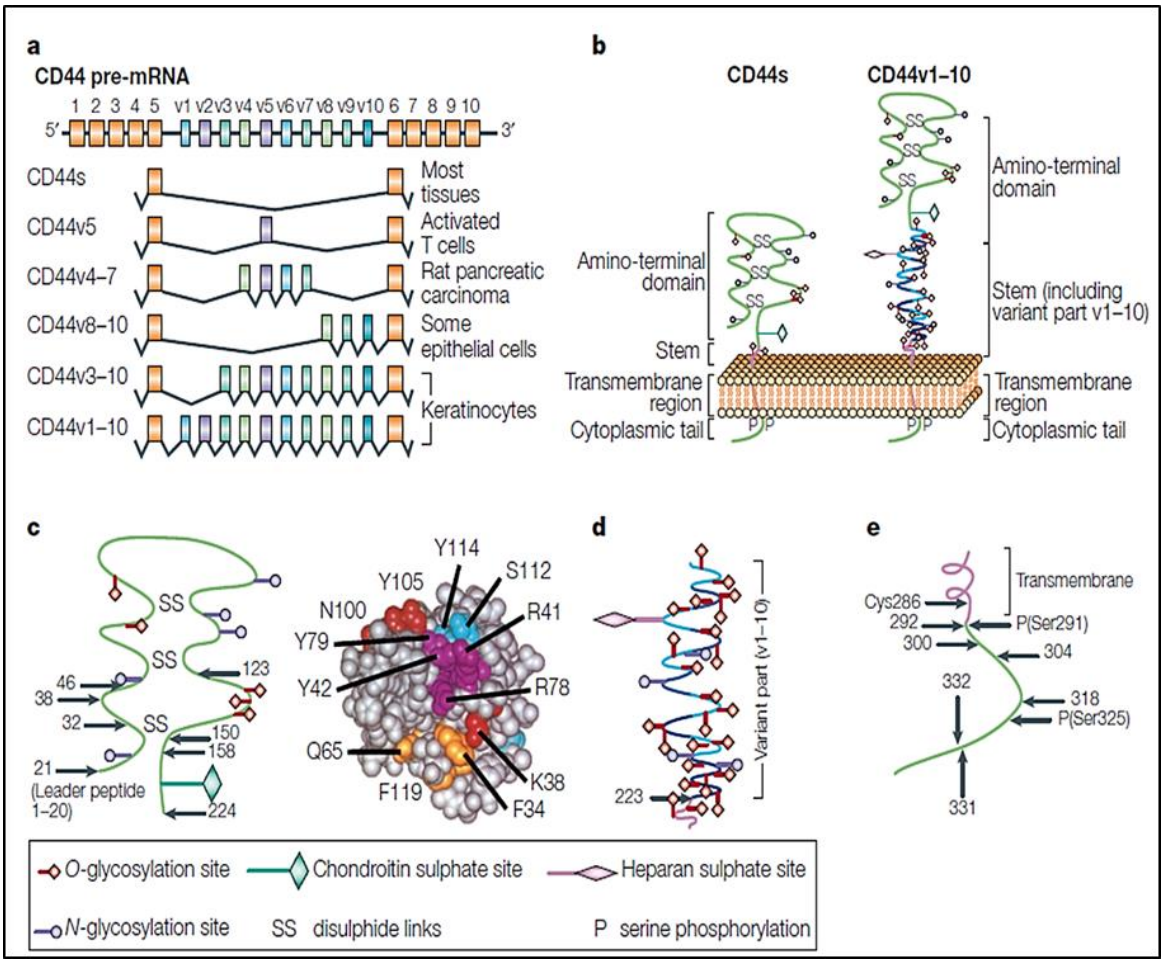

Figure 1-8: The gene and protein structure of human CD44.

A. Exon map of CD44: CD44 pre-mRNA is encoded by 20 exons, 10 of which can be regulated by alternative splicing (variant exons). The smallest CD44 isoform is known as CD44 standard (CD44s). B. the protein structure of CD44s is compared with that of the largest variant isoform CD44v1-10, which shows the sequences encoded by the variant exons. C. The amino-terminal domain of CD44 contains the HA binding sites (amino acids 32-123). D. The structure of CD44s consists of 46 amino acids but can be enlarged by up to 381 variant exon encoded amino acids as shown for CD44v1-10. The heparin sulphate addition site encoded by exon v3 is important for the binding of heparin sulphate dependent growth factors. E. The carboxy terminal cytoplasmic domain supports the binding of proteins with crucial function such as signalling. Adapted from Ponta et al., (2003).

CD44 is expressed in a wide range of cells and tissues, including hematopoietic, endothelial, mesenchymal, central nervous system, lung, liver, epidermis, endothelial, fetal tissue and pancreatic cells (Jalkanen et al., 1986; Cooper et al., 1992; Fox et al., 1994; Mackay et al., 1994). However, the tissue distribution of CD44v is limited, because in this form of CD44 the process of alternative splicing is normally tightly regulated;

CD44v is expressed on a variety of endothelial cells and epithelial lineage in a tissue specific pattern.

CD44 expression within an epithelial tissue can vary between layers and also between cells. For example, Dall and colleagues (1996) have found that the expression of CD44v6 was limited to the stratum basale and the stratum spinosum of normal uterine cervical squamous epithelium (Dall et al., 1996). Korabiowska et al. (1994) reported that normal melanocytes and naevi do not express CD44, although the keratinocytes express CD44v3-10 the expression of CD44 has also been identified in ovarian tissue, placental structures, oocytes, early embryos, spermatozoa and fetal tissues (Goshen et al., 1996; Ohta et al., 1999; Bains et al., 2002; Kaneko et al., 2000; Aplin, 1997).

CD44 is a major hyaluronan receptor (Knudson, 1998), but is also involved in cellular adhesion (cell aggregation, proliferation, angiogenesis and migration), hyaluronate degradation, lymphocyte activation, lymph node homing, angiogenesis and release of cytokines (Jalkanen et al., 1986, Bourguignon et al., 1998; Knudson, 1998, Underhill, 1992, Shimizu et al., 1989; Koopman et al., 1993, Trochon et al., 1996, Webb et al., 1990). CD44 has several additional roles, including cell-cell and cell-matrix interaction, and is also involved in cell motility (Jackson et al., 1992; Knudson and Knudson, 1993; Fraser et al., 1997; Turley, 1992). These biological processes appear to be mediated through receptors for HA which are present on the cell surface (Green et al., 1988; Aruffo et al., 1990). In addition, it has been found that CD44 expression is associated with a number of physiological events such as embryonic development and cell proliferation (Goodison et al., 1999; Callagy et al., 2000). HA and CD44 also play a key role in morphogenesis during embryonic development (Toole, 1991; Campbell et al., 1995). The extracellular matrix of embryonic organs expresses high amounts of HA during early stages of development, which may be required for the migration of mesenchymal cells

and blood vessel development (Toole, 1991). However, during later stages of development the strong expression of HA decreases due to CD44-mediated degradation (Goshen et al., 1996). HA and CD44 play an important role in embryonic development and cell proliferation events during the development of the human placenta. The effect of interaction of HA and its receptor is affiliated to a high proliferation rate, differentiation and invasion, and rapid growth (Benirschke, 1995).

The various physiological events considered above, in which HA and CD44 play an important role, have also been implicated in several inflammatory diseases (Puré and Cuff, 2001). CD44 expression can be up-regulated during inflammation by several inflammatory stimuli, including growth factors and cytokines (Hamada et al., 1998, Bradbury, 2002).

1.10 Aims of this study

Trophoblast cells play a critical role at the fetal-maternal interface by permitting the maternal immune system to accommodate the semi-allogenic fetus whilst maintaining effective immunity.

The overall aim of this thesis was to study the role of CD74, MIF and CD44 expression in the first trimester trophoblast-derived cell lines, JEG-3 and ACH-3P. In order to achieve its objective, the study has been divided into several aspects as follows:

- To analyse and profile the expression of CD74, MIF and CD44 in human trophoblast derived cell lines, JEG-3 and ACH-3P. Validation of the study will be carried out by investigating the expression of CD74, MIF and CD44 in immortalized normal placenta tissue lysate (Abcam, USA) and normal placenta tissue slides.
- Modulation studies: study the effects of recombinant human IFN-γ or LPS on the regulation of CD74 isoform expression in JEG-3 and ACH-3P cells.
- Study of CD74 and its interrelation to MIF and CD44 in JEG-3 and ACH-3P cells. Investigation of the interactive partners of CD74 along with CD44 by target imaging and co immunoprecipitation.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General laboratory chemicals

The majority of chemicals used in this study were from Sigma, Poole, England, UK, particularly, Accutase, Dimethyl Sulphoxid (DMSO), Cellytic M Cell Lysis Reagent, Protease Inhibitor Cocktail, Paraformaldehyde (PFA), Tween-20, Saponin and Triton-X 100. Other chemicals such as Sodium Chloride, Acetone, Methanol and Virkon were from Fisons, Leicester, England, UK; Fetal Calf Serum (FCS) was from Imperials Laboratories, England. Recombinant Human Interferon Gamma (IFN-γ) was from Immuno Tools, Germany. Lipopolysaccaride (LPS) from *E. coli* serotype EH100 was purchased from (Sigma Aldrich, UK). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI were from Fisher Scientific, UK.

2.1.2 Commercial kits

The commercial kits used in this study were:

- Protease Inhibitor Cocktail Set III (Calbiochem, UK)
- Breast (Human) Tissue Lysate - Normal tissue (Abcam, UK)
- Breast Tissue Slide (Normal) (ProSci, USA)
- DNAse treatment kit (Life technologies, UK)
- Placenta (Human) Tissue Lysate - adult normal tissue (abcam, UK)

These kits were used according to the manufacturer’s recommendations.

2.1.3 Cell lines

For the purpose of the present study, we employed two kinds of cell lines. Firstly, we used human placental cell line JEG-3 and ACH-3P as the main cell lines. Secondly, we included other cell lines in the present study as positive controls such as THP1, Raji, CAMA-1, Jurkat, MDA-MB-231, U937 and HeLa. As for the main cell lines, we first used human placental choriocarcinoma cell line JEG-3 provided by Prof. I.L. Sargent, Nuffield Department of Obstetrics and Gynaecology, University of Oxford. This cell line is an epithelial adherent monolayer which is highly proliferative, so it was suitable for use as our trophoblastic model system. This cell line was maintained in DMEM/Hams F-12 which was supplemented with 10% (v/v) FCS, and maintained at 37°C and 5% CO_2. The second type of the cell line employed in this study was ACH-3P which is a new hybrid cell line kindly provided by Dr. G. Desoye, Department of Obstetrics and Gynaecology, Medical University Graz, Austria. This cell line is comprised of two trophoblast subpopulations; villous cytotrophoblasts (VCT) and invasive extravillous trophoblasts. This cell line was maintained in Ham's F-12 with 10% FCS and maintained at 37°C and 5% CO_2.

Secondary cell lines were used in the present study as a positive control since they express a considerable level of CD74, MIF and CD44. The secondary cell lines that were used in this study are as follows: CAMA-1, MDA-MB-231, THP-1, Raji, HeLa and Jurkat. All the types of secondary cell lines were kindly provided by Prof. Nelson Fernandez, (School of Biological Sciences, University of Essex, UK). The cell lines were cultured in RPMI-1640 medium and maintained at 37 °C and 5% CO_2. The cell lines used in this study were obtained from different sources as shown in Table 2-1 below:

Table 2-1: Details of secondary cell lines used in this study.

Cell line	Origin	Cell culture
CAMA-1	Epithelial cells was derived from a human breast carcinoma	RPMI-1640 supplemented with % FCS
MDA-MB-231	Epithelial cells was derived from pleural effusion of breast cancer	D-MEM (high glucose) supplemented with 10 % FCS
THP-1	Human monocyte was derived from patients with Acute Lymphocytic Leukaemia (ALL)	RPMI-1640 supplemented with % FCS
Raji	Human lymphoblastoid cells derived from a Burkitt lymphoma.	RPMI-1640 supplemented with % FCS
Jurkat	Established from the peripheral blood of a 14-year-old boy with Acute Lymphoblastic Leukemia (ALL)	RPMI-1640 supplemented with % FCS
HeLa	Epithelial cells was derived from human cervical carcinoma	D-MEM (high glucose) supplemented with 10 % FCS

2.1.4 Antibodies

The primary and secondary used in this study were obtained from different sources as shown in Table 2-2 below:

Table 2-2: List of the antibodies used for all the applications in the project.

Specificity	Type	Clone	Cat. no.	Company
Mouse anti-human HLA-DR	Primary	LN3	327001	Biolegand
Mouse anti-human CD74	Primary	By2	sc-20062	Santa Cruz
Mouse anti-human CD74	Primary	BU45	Supernatant	Cambridge
Mouse anti-human MIF	Primary	D-2	sc-271631	Santa Cruz
Mouse anti-human MIF	Primary	Polyclonal	ab55445	Abcam
Mouse anti-human CD44	Primary	156-3c11	3570	Cell signalling
Mouse anti-human α Tubulin	Primary	TU-02	sc-8035	Santa Cruz
Mouse IgG2b antibody	Isotype	MPC-11	400301	Biolegand
Rabbit anti-human β-actin	Primary	poly6221	622102	Biolegand
Goat anti-mouse Alexa 488	Secondary	Polyclonal	A-10667	Invitrogen
Goat anti-mouse Alexa 555	Secondary	Polyclonal	A-21422	Invitrogen
Donkey anti-mouse IRDye® 800CW	Secondary	Polyclonal	925-32212	Li-Cor Bioescieces
Goat anti-Rabbit IRDye® 680LT	Secondary	Polyclonal	925-68022	Li-Cor Bioescieces
Mouse IgG2b antibody	Isotype	MPC-11	400301	Biolegand
Goat anti-mouse antibody	Secondary	Poly4053	405305	Biolegand

2.2 Methods

2.2.1 Cell cultures

2.2.1.1 Thawing of cells

Cryogenic vials with 1×10^6 cells/ml in a 9:1(v/v) solution of FCS: dimethyl sulphoxide (DMSO) were removed from liquid nitrogen. Special precautions were taken which included using a protective mask and heavy padded gloves. Cells were thawed rapidly at 37^oC and then transferred using a sterile glass pipette to a 10 ml centrifuge tube containing 5 ml of fresh complete medium to dilute out the DMSO. After centrifugation for 5 minutes at 1000 rpm, the medium was removed, the cell pellet was re-suspended in 5ml of fresh medium, and the number of the cells was counted using a haemocytometer. A final concentration of 1×10^5 cells/ml was transferred to either a flask or petri dish. Cells were cultured at 37^oC in a humidified tissue culture incubator with 5% (v/v) CO_2.

2.2.1.2 Cell cultures of cell lines

The JEG-3 cell line was of immense importance for all the experiments of this study as it served as the control or model with which the new cell line (ACH-3P) could be compared. JEG-3 cells were passaged at 70-80% confluency every 3 to 4 days. The cells were washed with 1X Phosphate buffer saline (PBS) and lifted with 1ml of accutase (Sigma-Aldrich; Dorset, UK) by incubating at 37°C for about 5-10 minutes. The cells were then counted with a hemocytometer and plated out at a density of $1X10^3$ in a new culture flask. ACH-3P, CAMA-1, MDA-MB-231 and HeLa cell lines were expanded in a similar way. The steps followed were exactly as for the JEG-3 cell line. THP1, Jurkat, U937 and Raji are suspension cell lines. The cells were centrifuged at 1000 rpm for 5 minutes, supernatant was aspirated and fresh medium was added to the cell pellet. Then cells were seeded at density of $1X10^5$ in a new culture flask.

2.2.1.3 Cryopreservation of cells

Cells were cryopreserved at 70% confluence. Cells were briefly detached using accutase, counted and checked for viability as mentioned above and then centrifuged. Cells were then re-suspended so that they were about 1.25×10^6 cells/ ml of freezing mixture (90% FCS and 10% Dimethyl sulphide [DMSO]). 1 ml of the cell suspension was then dispensed into each cryo tube (Fisher Scientific, UK) and placed in -80 ^{o}C freezer for 1-2 days before transferring them to liquid nitrogen at -196^{o}C for long-term storage.

2.2.1.4 Growth curve

As both JEG-3 and ACH-3P cell lines grow in culture, there are three distinct phases that can be detected. The first is the 'lag phase', which is usually no further than 1-2 days in length, during which there is a slight increase in cell number. It is assumed that during this period of time, the cells are adjusting to the new media. This is followed by the 'log phase'. During this phase, the cell number increases exponentially. Then, the final phase is the 'confluent phase'. During this phase, the number of cells remains constant. Eventually, the cells die unless they are sub cultured or fresh media is added. Approximately 30,000cells/ml were plated onto 35mm dishes and cell number was determined at approximately 24 hr intervals for 192 hrs. Three dishes were read every day using a haemocytometer and the mean number of the cells in the squares was calculated. The same number multiplied by the amount of media in the dish gave the total number of cells.

2.2.1.5 Cell viability test

Cell viability was tested to identify whether the cells were dead or living, based on the trypan blue exclusion method. Firstly, the cells were washed with PBS. Then accutase treatment was used to split them and the cell suspension was placed in a conical

centrifuge tube. Then a cell suspension of 1:2 dilutions in trypan blue (Sigma, UK) was prepared. Approximately 10µl of diluted cell suspension was loaded into both the haemocytometer chambers, and cells were viewed under the light microscope. The cells were observed as total cells and non-viable (blue) cells. Live cells would have the cell membranes intact and would exclude the trypan blue dye, where dead cells did not.

2.2.2 MTT Cell proliferation assay

2.2.2.1 Principle

This is a colorimetric method for determining the cell viability. The MTT cell proliferation was determined by the Cell Titer 96 Aqueous One Solution Proliferation Assay kit (Promega). This solution contains (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole). Assays were performed by adding a small amount of the solution directly to culture wells and then incubating for 4 hr. the absorbance was measured at 595 nm with a reference wavelength of 620 nm.

2.2.2.2 Experimental protocol

Trophoblast JEG-3 and ACH-3P (1×104 cells/well) cells were cultured in 96 well flat bottom plates at 37°C. After 24 hr incubation to allow cells to adhere, cells were serum starved with low serum (0.1% FBS) culture medium for 4 hrs. Then, cells were exposed to varying concentrations of LPS or no treatment (low serum medium only). Cells were incubated for 12, 24, 48 and/or 72 hrs after treatments. Cell growth and proliferation was assessed by using CellTiter 96 AQueous one solution cell proliferation assyay kit (Promega). Briefly, 20 µl of MTT solution (5 mg/ml in PBS) and 100 µl was added per well and cells were incubated at 37°C with 5% CO2 in a humidified chamber for 4 hr for colour development. The resultant Formosan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured at 595 nm using a micro plate

reader (Versamax). The percentage of cell proliferation was calculated relative to the rate of proliferation in untreated cells.

2.2.3 Cell stimulations

2.2.3.1 Recombinant human interferon-gamma (IFN-γ)

Synthetic IFN-γ is a single, non-glycosylated, polypeptide chain containing 144 amino acids and was purchased from Immuno Tools, Germany in white lyophilized (freeze-dried) powder. IFN-γ was dissolved in 1ml of sterile H_2O (stock 1), 50 µl from the stock was added to 450 µl of sterile H_2O (stock 2) and was stored at -18°C in accordance with the manufacturer's instructions. JEG-3 and ACH-3P cells were serum starved with DMEM/Hams F-12 and Ham's F-12 respectively containing 0.1% FCS, for a period of 4 hrs prior to stimulation. Cells were then stimulated with IFN-γ at a concentration of 500 and 1000U/ml, at the following time points; 12 hr, 24 hr and 48 hr. cells were counted using a haemocytometer, checked for viability and harvested for sample preparation.

2.2.3.2 Lipopolysaccharide (LPS)

LPS is unique for gram-negative bacteria presented in the outer membrane, and is made of both hydrophobic and hydrophilic domains and was purchased from (Sigma Aldrich, UK) in freeze-dried powder. LPS was dissolved in 1ml of sterile 1X PBS (1 mg) by swirling gently until the powder dissolved. Solutions were further diluted to the desired working concentration with additional sterile cell culture media, and were stored at -20°C in accordance with the manufacturer's instructions. JEG-3 and ACH-3P cells were serum starved with DMEM/Hams F-12 and Ham's F-12 respectively containing 0.1% FCS, for a period of 4 hrs prior to stimulation. Cells were then stimulated with LPS at a concentration of 5 and 10 µg/ml, at the following time points; 2 hr, 4 hr, 6 hr and 12 hr.

cells were counted using a haemocytometer, checked for viability and harvested for sample preparation.

2.2.4 Polymerase chain reaction (PCR)

2.2.4.1 Principle

The polymerase chain reaction (PCR) one of the most widely used techniques in molecular biology to amplify a single piece of DNA across several orders of magnitude, by multiplying a particular DNA sequence. PCR involves three steps; denaturation, annealing and extension. This method is useful in the investigation of a large number of diseases using DNA as a target, rather than RNA, due to the higher stability of the DNA molecule as well as the ease with which DNA can be isolated. Qualitative PCR can be used for detecting human, viral and bacterial genes.

2.2.4.2 Primer design

The primers of CD74 and β-actin were designed using primer plus 3 tools. All considerations were taken into account such as annealing temperature (Tm), GC content, the length of primers and the size of the PCR product. The primers were ordered from Fisher Scientific (Invitrogen).

2.2.4.3 RNA extraction

Total RNA from 1x 106 JEG-3, ACH-3Pand THP-1 cells grown in a 75 cm^2 flask was extracted using 1ml of Trisure reagent (Bioline) according to the manufacturer's recommendations (Chomczynski and Mackey, 1995). The reagent was mixed well with a pipette and divided into an equal number of Eppendorf tubes. 200µl of chloroform was added to each tube and mixed well. The tubes were incubated at room temperature for 5 minutes and were then centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant from these was collected and put into new tubes. 500µl of Isopropanol (VWR chemicals,

UK) was added to each tube and mixed well by inverting. The tubes after being incubated at room temperature for 10 minutes were again centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was poured off, and the white pellet retained. The pellet was washed with ice cold 75% ethanol. The supernatant was discarded and the tubes were spun briefly to remove any traces of ethanol, and air – dried for 15-20 minutes.

2.2.4.4 DNase treatment

DNase treatment was carried out for total RNA using a DNase treatment kit. 300 µl of the master mix (3 µl buffer, 1 µl DNase enzyme and 26 µl sterile double distilled H_2O) was added to each tube and the pellet was dissolved into it. The tubes were then placed into a water bath at 37°C for 30 minutes. 5 µl of the inactivation reagent (Fermentas, UK) was then added, and the samples mixed thoroughly. After being incubated at room temperature for few minutes the tubes were then centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant collected consisted of the RNA extracted. 270 µl of sterile double distilled H_2O, 750 µl of ice cold 100% ethanol and 3M Sodium Acetate (Fermentas, UK) were added to the collected supernatant. After an overnight incubation at -20 degrees, the tubes were spun at 13,000 rpm for 20 minutes at 4°C. The supernatant was discarded and pellet washed with 1 ml of ice cold 75% ethanol. The centrifugation step was repeated again at 13,000 rpm for 5 minutes at 4°C. The supernatant was then discarded and the tubes were spun briefly to remove any traces of ethanol. The pellet retained in the tubes was dissolved in 26 µl of sterile double distilled H_2O. The concentration of the RNA was then determined using a NanoDrop. The quality of the RNA was also checked by electrophoresis in a 1% agarose denaturing gel.

2.2.4.5 Complementary DNA conversion

The tubes containing the RNA were vortexed well, then prepared from 2µg of total RNA using 1µl of random hexamers and 1µl of sterile double distilled H_2O (Fermentas, UK). These tubes were then placed into a water bath at 65°C for 10 minutes, and then chilled on ice for about 5 minutes. 8µl of the master mix (4µl of 5x buffer (enzyme buffer), 1µl of sterile double distilled H_2O, 2µl of dNTPs, and 1µl of Reverse Transcriptase) (Fermentas, UK) was added and mixed well by pipetting. The samples were then ready for use in PCR.

2.2.4.6 Reverse transcriptase (RT-PCR)

The CD74 gene was PCR amplified using CD74-specific primers, CD74-F (5' TGACCAGCG CGACCTTATCT 3') and CD74-R (3' GAGCAGGTGCATCACATGGT 5') (Burton et al., 2004). PCR conditions were 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 25 seconds; 5 minutes at 72°C. Control PCR amplification of β-actin using specific primers β-actin-F (5' ATCTGGCACCACACCTTCTACAATGAGCTGCC 3') and β-actin-R (3' CGTCATACTCCTGCTTGCTGATCCACATCTCC 5') was carried out so as to evaluate the RNA in all the samples. PCR conditions were 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; 6 minutes at 72°C. PCR products were then analysed by electrophoresis in a 1% agarose gel stained with ethidium bromide or Safe View (NBS Biologicals, UK). The gel was then analysed using an ultraviolet camera.

2.2.5 Flow cytometry

2.2.5.1 Principle

Flow cytometry is a technique used to analyse and measure various properties of cells. The cells are usually labelled with a fluorescent probe, normally a fluorphore conjugated to an antibody. The cells are suspended in fluid, and the cells carrying fluid pass through a beam of light. This technique uses the principles of light scattering, light excitation, and the emission of fluorochrome molecules to generate specific multi- parameter data from cells. There are three parameters that are measured by photomultiplier tubes. These parameters are forward scatter (FSC), side scatter (SSC) and fluorescence (FL). One advantage of the flow cytometer is its ability to evaluate a large population of cells accurately and quickly with semi quantitative results. This makes the flow cytometry technique an ideal tool for quantitative analysis of certain cellular properties, especially when the cells of interest are a small fraction of other cell types in a cell population. The results are displayed in many ways, including histogram, dot plot, and contour. Results are shown as histograms where mean fluorescence intensity (MFI) is along the horizontal axis (x-axis) versus total cell count on vertical axis (y-axis). As standard practice, an irrelevant or nonspecific isotype antibody was used as a negative control to evaluate the degree of positivity for other surface proteins in terms of the fluorescence intensity.

2.2.5.2 Cell-surface and intracellular staining

For surface staining of specific cell antigens, such as CD74, CD44, MIF or HLA-DR, cultured cells or treated samples. Cell cultures of cell lines were washed twice with 1X PBS and incubated with accutase for 5-10 minutes at 37 ºC. Cell suspensions (1×10^6 cells/ sample) per sample were centrifuged at 300 rpm for 10 minutes. The cells were fixed with a fixation buffer to ensure free access of the antibody to its antigen 4 % PFA (Sigma, UK) followed by a washing step in PBS. Supernatants were discarded and the

cell pellets resuspended in blocking buffer (PBS supplemented with 0.1% (w/v) bovine serum albumin [BSA]). The cells were incubated at 4 ºC for 1 hr with saturating concentrations of the following primary human specific monoclonal antibodies: with an appropriate concentration of By2 (anti-CD74), ab55445 (anti-MIF), 156-3C11 (anti-CD44) and LN3 (anti-HLA-DR). The cells were washed twice with PBS and incubated with secondary antibody fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG at 4 ºC for 1 hr. Cells were used with either only secondary antibody or neither primary nor secondary antibody as negative controls. For intracellular staining, cells were permeabilised with 200 µl permeabilisation buffer (PBS supplemented with 0.1% (v/v) [Triton $_X$-100 (Sigma, UK)]) for 20 minutes to facilitate the entry of antibodies inside the cells. The staining procedure for intracellular staining was the same as for the surface staining. Samples (10,000 cells) were analysed using a BDFACS Aria flow cytometer and a 488 nm beam for detection of the fluorochrome attached to the secondary antibody (PE, FITC). The results were analysed using FlowJo version 8.8.6 (Tree Star Inc., Ashland, OR, USA).

2.2.6 Laser scanning confocal microscopy

2.2.6.1 Principle

Confocal laser scanning microscopy is a valuable technique that has the ability to produce high resolution images of very small objects and produce a three-dimensional image of a specimen. There are many advantages of confocal microscopy over the conventional microscope due to many factors; firstly its ability to control the depth of the field, secondly the reduction of background interference away from the focal plane and thirdly its ability to analyse serial sections from thick specimens.

2.2.6.2 Experimental protocol

Cell monolayer culture and double staining: ACH-3P and JEG-3 cell lines were separately cultured in LabTek 8 well chambers (Thermo Fisher Scientific) at a density of 10×10^3 cell per well, for two days following seeding. For the staining procedure, all the steps were carried out at room temperature. The cells were fixed with 4% PFA (Sigma, UK) for 20 minutes and blocked with 2% (w/v) BSA (Bovine serum albumin) prepared in 1X PBS for 1 hr at room temperature. For intracellular staining, the cells were permeabilized with 0.1% Triton $_X$-100 and then blocked as described above. The incubation steps were also carried out at room temperature. For staining, the cells were incubated with specific monoclonal antibody for 1 hr and washed three times with PBS. For the secondary antibody, anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (Invitrogen, Carlsbad, CA, USA) was used for 1h. For double staining, cells were blocked again for 1h and stained with specific monoclonal antibody for 1 hr. After 3 washes cells were incubated with anti-mouse IgG TRITC conjugated antibody for 1h. Isotype controls were stained with only secondary antibody. Cells were then thoroughly washed and chambers were removed from the slide. Slides were rinsed in a beaker containing 1XPBS and dried. The cells were mounted with anti-fade mounting medium and carefully covered with the cover slip avoiding any air bubbles. Edges of the cover slip were sealed with transparent nail polish and dried in air. Prepared slides were stored at 4^oC in the dark for further analysis. The slides were then examined under a Bio-Rad confocal microscope using an x 60 oil immersion objective (numerical aperture 1.4) and FITC filter for Alexa Fluor® 488 and PE filter for Alexa Fluor® 555. The images obtained were then analysed using the Fiji software and NIS elements.

2.2.7 Preparation of total protein

For Lysate preparation, a pellet of 1×10^6 cells for control, untreated and treated cells were collected and washed twice in ice cold 1X PBS. Following this, 500 µl of cell lysis mixture (Cell Lytic™ MT Reagent and Protease Inhibitor, Sigma, UK) was added. The pellet was resuspended by gentle pipetting and incubated on ice for 20 minutes. Then, the lysate was clarified by centrifugation at 15,000 rpm for 20 minutes. Finally, the protein containing supernatant was moved to a chilled test tube and the lysate was stored at -80°C for long-term use.

2.2.7.1 Bradford assay

This method is used for calculating the protein concentration in the lysate so as to calculate the amount of protein that should be loaded into each well before running the gel. This assay was based on the principal that when the Coomassie Blue Dye is bound to the protein in an acidic medium, a shift in absorbance occurs from 465 nm to 595 nm resulting in a colour change from brown to blue. The amount of complex now present in the solution is a measure for the protein concentration by means of an absorbance reading. Standard concentrations of BSA (Bovine Serum Albumin) 0.015, 0.031, 0.06, 0.125, 0.50, 1, and 2 mg/ml were prepared. A series of dilutions for the sample were prepared in PBS. A volume of 5 µl of the standards and samples respectively were added to the 96 well-plate. Bradford reagent, 250 µl was added to each well and the plate was left for incubation at room temperature for 5 minutes. The Micro-titre plate reader was switched on and set up to an absorbance of 595 nm. The plate was read and results were read three times to minimise experimental error. A graph for concentration vs. absorbance was plotted on Microsoft Excel and the protein concentration was found out using the $y = mx + c$ equation.

2.2.8 One dimensional gel electrophoresis

2.2.8.1 SDS- PAGE

Electrophoresis is currently the most commonly technique used for the isolation and identification of proteins. This technique is based on the unique idea of separating polypeptides based on their molecular weight. The ability of a molecule to move across an electric field is inversely proportional to molecular friction (which depends on the shape and size of the molecule) and directly proportional to the voltage and the charge of the molecule. Total protein complexes can then be separated electrophoretically according to molecular weight in gels containing sodium dodecyl sulphate (SDS), in a semi-solid matrix at a set voltage. In the presence of SDS (sodium dodecyl sulphate), proteins bind the SDS anions, and all become negatively charged and thus separated within a matrix of polyacrylamide gel in an electric field according to their molecular weights. The polyacrylamide gel concentration used determines the effective separation range of SDS-PAGE. For example, low porosity gels 15 % (w/v) total acrylamide concentration are suitable for analysis of small proteins (less than 50 kD), whereas high porosity gels 5% (w/v) total acrylamide concentration should be used for large molecular weight proteins (above 100 kD).

In this study, the protein samples were separated in 12 % resolving gels and 4% stacking gels and prepared in sterile universals, using a BioRad Mini PROTEAN Electrophoresis system. Care was taken during the addition of (TEMED – tetrametylethylenediamine) and (APS- ammonium persulfate) as they enhance the process of polymerization. Thus, these two components were only added after the system was completely set up. Once the resolving and stacking gels were polymerised completely, they were loaded with electrophoresis buffer into to the system. The previously measured protein lysate was used in sample preparation. The samples were diluted in 5X SDS-sample buffer and

heated for 4 minutes at 95^{o}C before loading on 12 % SDS-PAGE. The mixture of 5 µl of sample buffer was added to 20 µl of the lysate loaded into each well. The sample and protein markers thus prepared were loaded into the wells of the gel. The system was connected to a power supply unit and run at 60V until the samples crossed the stacking gel and then increased to 120V until the samples reached the bottom of the resolving gel for approximately 2 hrs. Following separation by SDS-PAGE, the gels were stained with sliver or Coomassie Blue to check the success of the transfer or processed for immunoblotting. Recipes for buffers and solutions are in the (Appendix 1 Buffer Recipes).

2.2.8.2 Coomassie blue staining

The polyacrylamide gel was carefully removed from between the plates and then placed into coomassie blue stain, covering the gel completely, for 2 hrs at RT on a rocking table. Following that, the gel was placed into 100 ml of destain solution and replaced with fresh one until the gel was clear and the stained protein bands were visible. Recipes for buffers and solutions are in the (Appendix 1 Buffer Recipes).

2.2.8.3 Western transfer

Following protein separation by electrophoresis, proteins were moved to polyvinylidene fluoride (PVDF) membrane (Immobilon-FL, Merck Millipore, Merck KGaA, Darmstadt, Germany) by opening the gel system and placing the gels in a transfer buffer. Two cassettes were lined with a sponge and the gels were positioned on a filter paper above the sponge. The methanol pre-soaked PVDF membrane was positioned above the gel while an additional filter paper and sponge were stacked above. A glass pipette was rolled over to eliminate air bubbles, and the two cassettes were positioned into the Bio Rad Mini PROTEAN Electrophoresis system. Then a cooling unit and transfer buffer were added,

and the system was run overnight at 30V. At the end of the transfer period, the system was disconnected from a power supply unit and the gel also stained with coomassie blue stain to check the success of the transfer.

2.2.8.4 Immunoblotting

This technique is used to detect a target protein in a sample through the monoclonal or the polyclonal antibody specific to that protein. The blocking method was the first step to this procedure. A membrane was incubated in a 5% semi skimmed milk solution with PBS-Tween-20 (Sigma-Aldrich) for 1 hr to prevent non-specific protein binding between the membrane and the antibody. The primary antibody was then diluted in PBS-T. Since the primary antibody was specific for the protein, it would not bind to any other protein on the membrane. After incubation, the membrane was washed about 3 times with PBS-T at 10 minutes intervals to eliminate any unbound primary antibody. The membrane was then incubated with a secondary antibody to bind with the primary antibody. The membrane was washed again to remove unbound secondary antibody. Finally, the membrane was scanned with ODYSSEY Infrared Imaging System from LI-COR Biosciences. To estimate the molecular weight of the proteins being studied we used the PageRuler™ Plus Prestained Protein Ladder (#26619) from Fermentas (Thermo Fisher Scientific).

2.2.9 Co-immunoprecipitation

Immunoprecipitaion techniques are very sensitive methods to reveal molecular weights of specific antigens. Immunoprecipitaion is often used to study the interaction of different proteins with each other as part of a complex. As with most other proteins, clock proteins physically interact with one another. Immunoprecipitation is a useful method for isolating proteins of interest from cellular extracts using specific antibodies. Co-immunoprecipitation (Co-IP) is the most straightforward technique to study protein-

protein interactions *in vivo*, if antibodies against the proteins of interest are available. To perform coIP, first an antibody against a target protein was coupled to Sepharose beads through protein A or G, and then the complexes containing the target protein were immunoprecipitated with the antibody-coupled beads by centrifugation. Protein components in the complexes were visualized by western blotting using antibodies specific to the different components.

2.2.9.1 Experimental protocol

During this procedure, cells were lysed with RIPA lysis buffer system (Santa Cruz Biotechnology, USA) and incubated for 20 minutes. This was achieved by mixing the following: 1 ml of RIPA lysis buffer with 10 µl of protease inhibitor cocktail in DMSO, (200mM) PMSF (phenylmethylsulfonyl fluoride) in DMSO (Dimethyl sulfoxide) and (100mM) sodium orthovanadate in water. After incubation, cells were centrifuged at 14,000 rpm for 15 minutes, and the total protein concentration was determined by Bradford assay. 1 mg/ml of each sample was incubated with 4 µg of specific primary antibody for the protein overnight at 4°C. Then, 40 µl of protein A/G PLUS- Agarose (Santa Cruz Biotechnology, USA) was added and kept overnight on a rotator at 4°C. After incubation, the samples were spun down for 30 seconds and the supernatant was discarded. Beads were then washed twice using PBS and the samples were boiled at 100°C after adding 50 µl of SDS-PAGE sample loading buffer containing Dithiothreitol (DTT). Then, 20 µl of each sample was loaded in each well of the gel and they were left for 1 hr and 30 minutes at 120V. After electrophoresis, protein was transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-FL, Merck Millipore, Merck KGaA, Darmstadt, Germany). Membranes were blocked with 5% skimmed milk in PBS-Tween-20 (Sigma) for 1 hr at room temperature and incubated in primary antibody specific for protein co-immunoprecipitation (Co-IP) followed by washing in PBS-T for 30

minutes. The membranes were then incubated with secondary antibody, the IRDye 800CW Donkey anti-Mouse IgG (Li-Cor Bioscience, Licoln, NE, USA) for 1 hr and then by washed in PBS-T for 30 minutes. Signals were detected using the ODYSSEY Infrared Imaging System (Li-Cor Biosciences).

2.2.9.2 Statistical analyses

All results in this research were carried out in at least triplicate experiments. The mean of each set of data was calculated and the standard error (±SD) was also included in order to present the reliability of the mean reading.

Chapter 3 Expression profiling and functional analysis of CD74, MIF and CD44 in human placenta and human trophoblast-derived cell lines

3.1 Introduction

One key mechanism by which human trophoblasts avoid maternal immune rejection is their failure to express highly polymorphic human leucocyte antigen (HLA) class II molecules, and related molecules such as CD74 and selective expression of MHC class I molecules during the first trimester. CD74 was shown to act as a cell surface receptor for macrophage migration inhibitory factor (MIF) in association with CD44 (Leng et al., 2003, Gore et al., 2008), a cytokine that plays versatile roles in both innate and adaptive immunity. Therefore, the signal transduction upon MIF binding to CD74 requires CD44 (Meyer-Siegler et al., 2004, Shi et al., 2006).

Human CD74 (MHC class II-associated invariant chain; Ii), is a non-polymorphic type II integral membrane glycoprotein that is expressed on antigen presenting cells (Burton et al., 2004, Stein et al., 2007, Beswick and Reyes, 2009). CD74 is a multifunctional protein that exists as four glycosylated isoforms, termed p33, p35, p41 and p43. However, CD74's most important function is its association with MHC class IIα and β chains (Borghese and Clanchy, 2011, Maharshak et al., 2010, Stumptner-Cuvelette and Benaroch, 2002). In this role, CD74 acts as a chaperone that is responsible for stabilizing nascent human leukocyte antigen (HLA)-DR αβ-heterodimers by formation of a trimetric structure. It also acts as a signalling factor through the cleavage of its cytoplasmic tail and activation of the NF-κB pathway (Stein et al., 2007, Beswick and Reyes, 2009).

CD74 also function as surface receptors for macrophage migration inhibitory factor (MIF) via the CD44-MIF complex (Duan and Srivastava, 2012, Stein et al., 2007, Shi et al., 2006, Starlets et al., 2006). MIF was one of the first cytokines to be described (George and Vaughan, 1962) and was identified as a T cell-derived factor (David, 1966; Bloom and Bennett, 1966). MIF, as presently understood, is one of the most important cytokines

that regulates the immune response. It is a pleiotropic cytokine and has a pivotal role in innate immunity, adaptive immunity and inflammation (Calandra and Roger, 2003). In addition, MIF plays a role during implantation and early embryonic development (Wada et al., 1997, Arcuri et al., 2001), and is important in the maintenance of human pregnancy (Arcuri et al., 1999, Zeng et al., 1993).

CD44 refers to a multifunctional family of glycoprotein molecules, which are present on the surface of many cell types, including epithelial cells, fibroblasts, lymphocytes and macrophages (Gallatin et al., 1991). CD44 exists in different isoforms, the result of alternative splicing affecting the extracellular domain, as further modified by a range of post-translational modifications (Goodison et al., 1999). CD44 receptors are involved in a number of biological processes, such as cell-cell and cell-matrix interactions (Jackson et al., 1992, Knudson and Knudson, 1993) and cell motility (Fraser et al., 1997, Turley, 1992). These biological processes appear to be mediated through receptors for hyaluronic acid (HA), the principal ligand of CD44, which are present on the cell surface (Green et al., 1988, Aruffo et al., 1990). It has been reported that CD44 is expressed during embryonic development and cell proliferation (Goodison et al., 1999, Callagy et al., 2000. During the development of the human placenta, CD44 plays an important role in cell proliferation events cell differentiation and trophoblastic invasion (Benirschke, 1995). CD44 has been shown to play a key role in the rapid growth of human placenta.

The aim of this study was to investigate the expression profile of CD74, MIF and CD44 antigens in the trophoblast-derived cell lines JEG-3, ACH-3P and in human placenta tissue (whole tissue homogenate lysates; sections of normal placenta specimens). RT-PCR, flow cytometry, microscopy and Western blotting were used to detect the antigens.

3.2 Results

The aim of this study was to investigate the expression of CD74, MIF and CD44 in the trophoblast-derived cells JEG-3 and ACH-3P and human placenta. The findings obtained from the present study may help in the critical analysis of both trophoblasts cell lines which represent a model for placental trophoblast cells in early human pregnancy.

3.2.1 RNA Extraction and Gene Expression

The expression of CD74 gene was examined using RT-PCR to ascertain whether this gene was successfully expressed in the JEG-3 and ACH-3P cell line. RNA of the gene of interest was extracted from JEG-3 and ACH-3P cells and converted into cDNA. The cDNA was then subjected to several PCR cycles. The cDNA was converted back into mRNA, which was separated by electrophoresis through a 0.1% agarose gel. The visualization of the final PCR products was by staining with ethidium bromide and UV irradiation (Figure 3-1).

To check the quality of converted cDNA, messenger RNA (β-actin) was tested by using cDNA converted from isolated RNA obtained from JEG-3 and ACH-3P cells. β-actin bands (800bp) corresponding to the indicated molecular weight on the DNA marker showed that RT-PCR has been carried out successfully (Figure 3-1B). PCR products demonstrated that CD74 mRNA expression was not detectable in JEG-3 and ACH-3P cells, in comparison with THP1 cells as positive control (CD74=384bp).

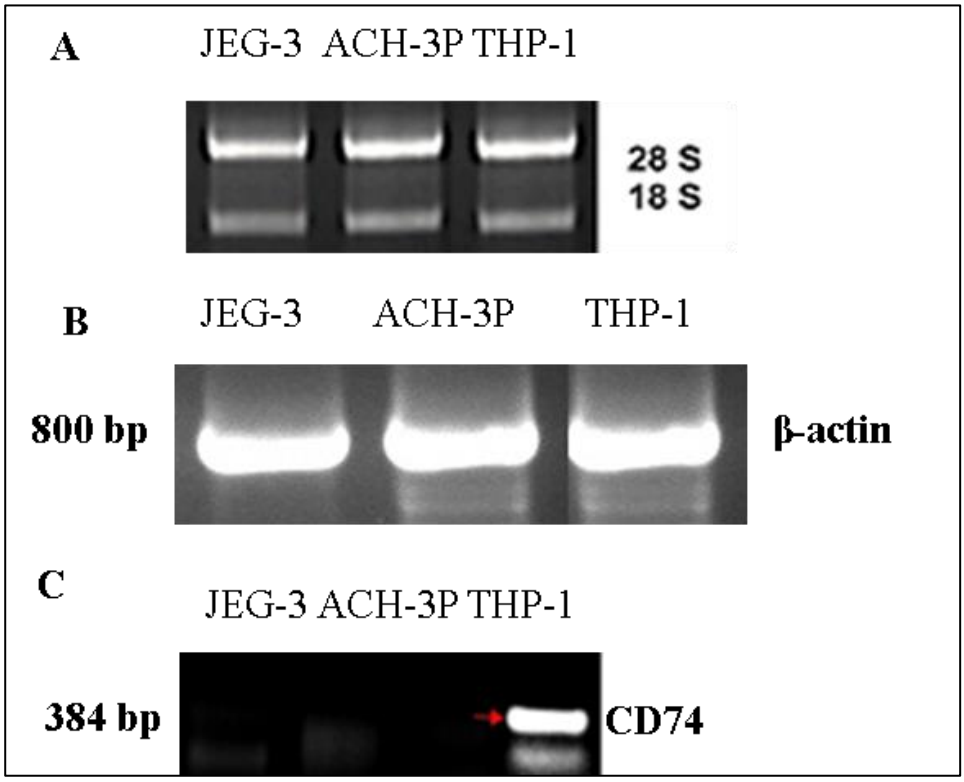

Figure 3-1: Total RNA extracted using TRIzol reagent and CD74 Messenger RNA (mRNA) expression in JEG-3, ACH-3P and THP-1 cells by RT-PCR.

A: Total RNA extraction; RNAs extracted using Trizol reagent. RNA samples isolated from $1X10^6$ cells (JEG-3, ACH-3P and THP-1). Samples were electrophoresed on a 0.1% agarose gel. Visualization was carried out by staining with ethidium bromide and irradiation with UV. The major bands correspond to 28S and 18S rRNA. B: Expression of β-actin mRNA: Messenger RNA (β-actin) was tested with cDNA converted from isolated RNA to check the quality of converted cDNA. The presence of the bands corresponding to the indicated molecular weight on the DNA marker shows that RT-PCR was carried out successfully. The MW marker indicates the molecular size of β-actin (800bp). C: CD74 mRNA: CD74 mRNA expression was not detectable in JEG-3 and ACH-3P cells. The result is representative of at least three replicate experiments. The identity of CD74 mRNA was confirmed by sequencing of the PCR product (CD74=384bp).

3.2.2 Flow Cytometry

The cell surface and intracellular expression of CD74, CD44, MIF and HLA-DR proteins in Raji, HeLa, Jurkat, JEG-3 and ACH-3P cell lines were studied by staining with an appropriate concentration of the primary antibody followed by 1 μl RAM-FITC secondary antibody. Results are shown as histograms where mean fluorescence intensity (MFI) is along the horizontal axis (x-axis) versus total cell count on vertical axis (y-axis).

JEG-3 and ACH-3P cells showed negative expression of CD74 on the cell surface and intracellular membranes compared to the Raji cells, used as a positive control (Figure 3-2).

Surface and intracellular expression of CD44 was investigated in JEG-3 and ACH-3P cells. The expression of CD44 was detected on both the cell surface and intracellular membranes of JEG-3 and ACH-3P cell lines compared to the positive control cell lines (HeLa), which expressed high levels of CD44 (Figure 3-3).

The cell surface and intracellular expression of MIF was analysed for Jurkat, JEG-3 and ACH-3P cells. MIF expression was detected on intracellular membranes, but not on the cell surface, of JEG-3 and ACH-3P cell lines, compared to the positive control cell line (Jurkat), which expressed high levels of MIF (Figure 3-4). MHC class II protein expression is not characteristic of trophoblast cells; therefore, staining of these cells with HLA-DR antibody was carried out to serve as a further positive control (Figure 3-5).

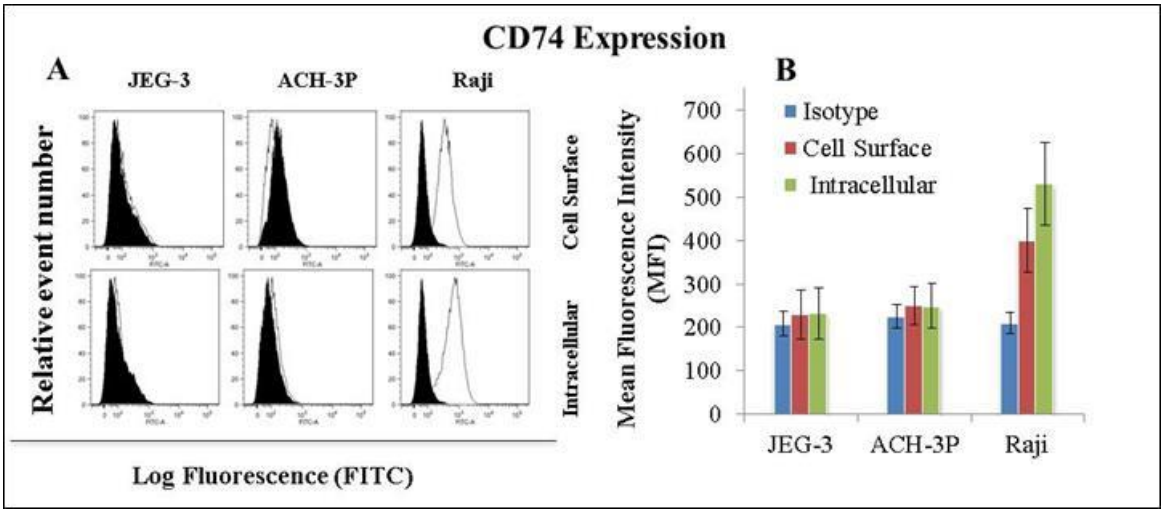

Figure 3-2: CD74 expression in the trophoblast cell lines JEG-3 and ACH-3P.

A: Flow cytometry analysis of the trophoblast cell lines JEG-3 and ACH-3P. Cells were stained with By2 anti-CD74 antibody followed by 1 µl RAM-FITC secondary antibody. The intracellular and cell-surface expression of CD74 is indicated by empty histograms, whereas the black-filled histograms indicate the isotype control for JEG-3 and ACH-3P cell lines. B: Graphical representation of CD74 intracellular and cell surface protein expression in JEG-3 and ACH-3P cells. Mean fluorescence intensity (MFI) values were measured based on geometric means.

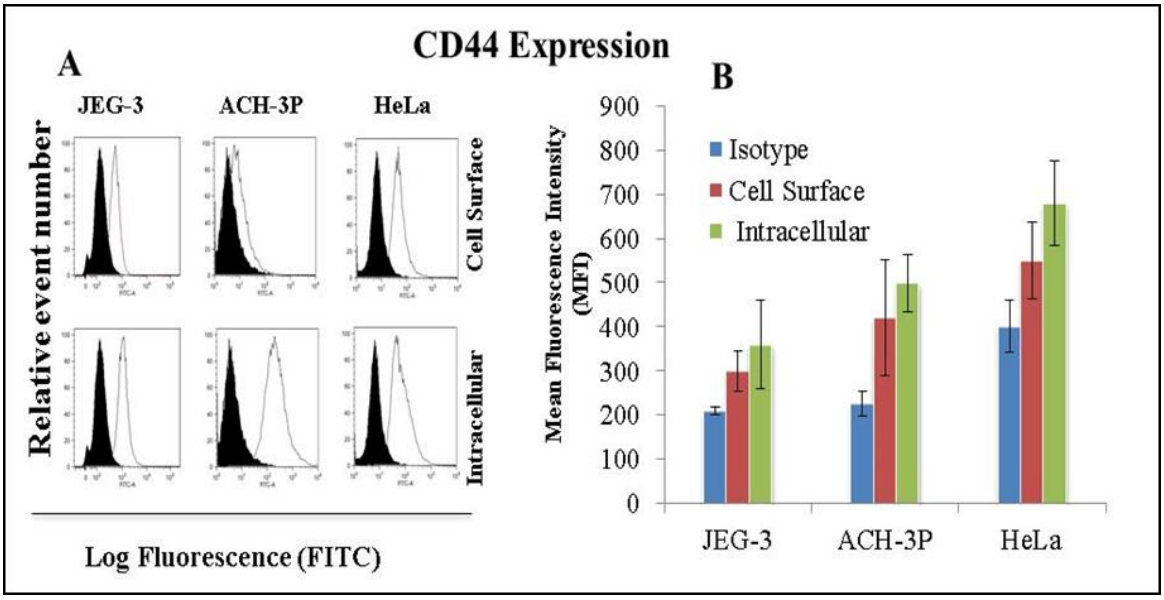

Figure 3-3: CD44 expression in the trophoblast cell lines JEG-3 and ACH-3P.

A: Flow cytometry analysis of the trophoblast cell lines JEG-3 and ACH-3P. Cells were stained with the 156-3c11 mouse mAb anti-CD44 followed by 1 µl RAM-FITC secondary antibody. The intracellular and cell surface expression of CD44 is shown as empty histograms. Black-filled histograms indicate the isotype control for JEG-3 and ACH-3P cell lines. B: Graphical representation of intracellular and cell membrane CD44 expression in JEG-3 and ACH-3P cells. Mean fluorescence intensity (MFI) values were measured based on geometric means.

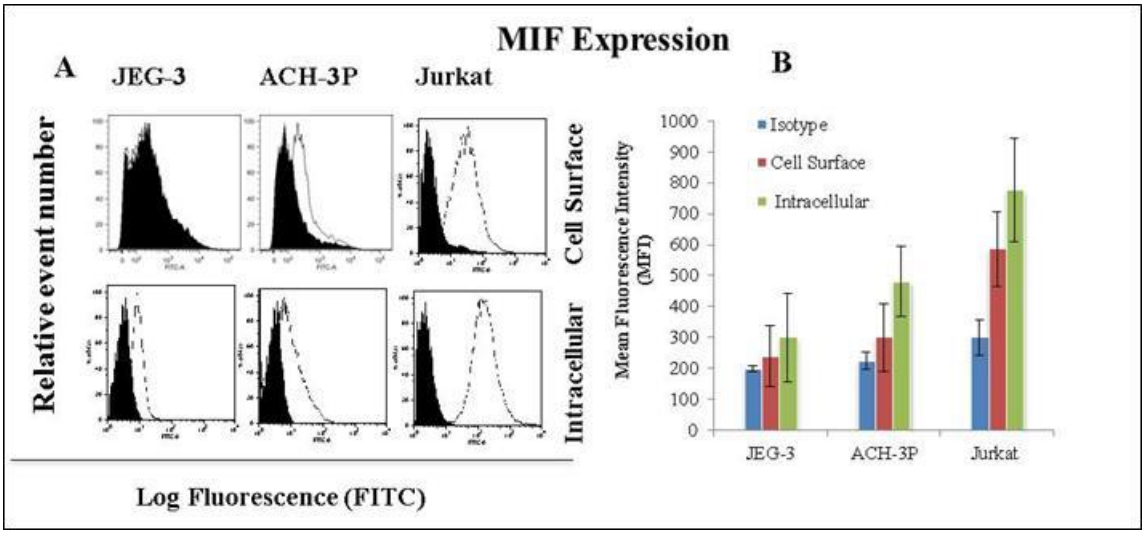

Figure 3-4: MIF expression in the trophoblast cell lines JEG-3 and ACH-3P.

A: Flow cytometry analysis of the trophoblast cell lines JEG-3 and ACH-3P. Cells were stained with Sc-271631 (D-2) anti-MIF followed by 1 µl RAM-FITC secondary antibody. The intracellular and cell surface expression of MIF is shown as empty histograms. Black-filled histograms indicate the isotype control for JEG-3 and ACH-3P cells. B: Graphical representation of intracellular and cell surface MIF expression in JEG-3 and ACH-3P cells. Mean fluorescence intensity (MFI) values were measured based on geometric means.

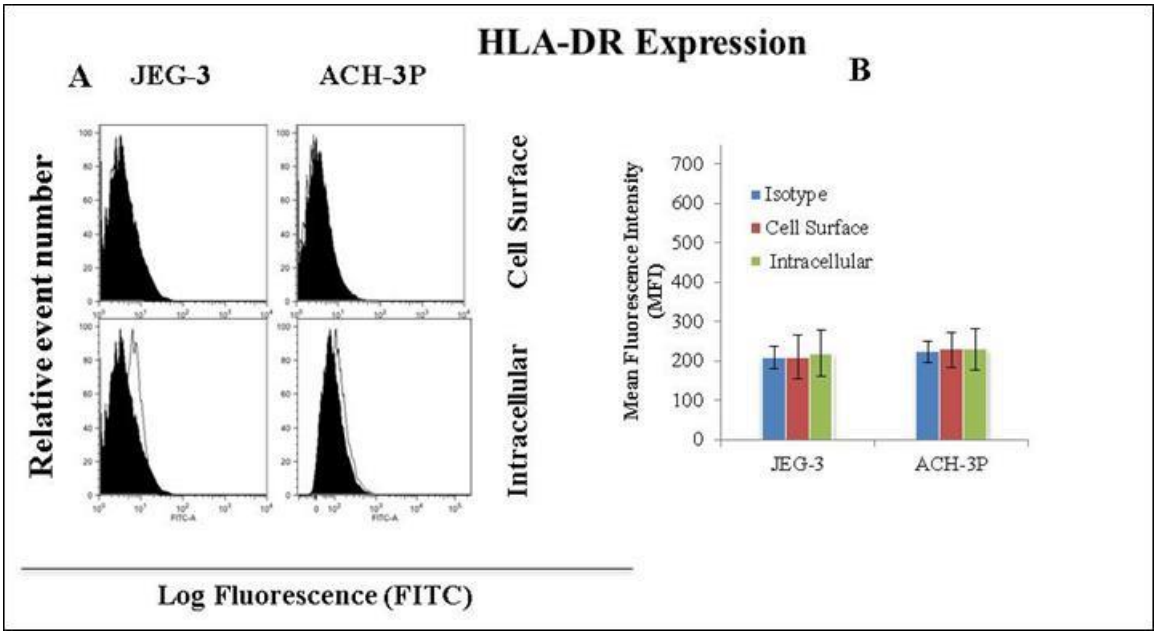

Figure 3-5: HLA-DR expression in the trophoblast cell lines JEG-3 and ACH-3P.

A: Flow cytometry analysis of the trophoblast cell lines JEG-3 and ACH-3P. Cells were stained with L243 anti-HLA-DR followed by 1 µl RAM-FITC secondary antibody. The intracellular and cell surface expression of HLA-DR is shown as empty histograms. Black-filled histograms indicate the isotype control for JEG-3 and ACH-3P cells. B: Graphical representation of intracellular and cell surface HLA-DR expression in JEG-3 and ACH-3P cells. Mean fluorescence intensity (MFI) values were measured based on geometric means.

3.2.3 Immunoblot analysis of CD74, MIF and CD44

To characterise the expression of CD74, MIF and CD44 in HeLa, Raji, Jurkat, JEG-3 and ACH-3P cells, whole cell lysates were analysed by by Western blotting using By2 (anti-CD74), D-2 (anti-MIF), 156-3C11 (anti-CD44) and TU-02 (anti-α- tubulin) loading control. Results showed the molecular weights of CD74, MIF, CD44 and α-Tubulin (loading Control) are 33 kDa, 35 kDa, 41 kDa, 12.5 kDa, 80 kDa and 55 kDa, respectively (Figure 3-6). No signals were detected for CD74 in JEG-3 and ACH-3P human trophoblast-derived cells, indicating the absence of CD74 isoform expression.

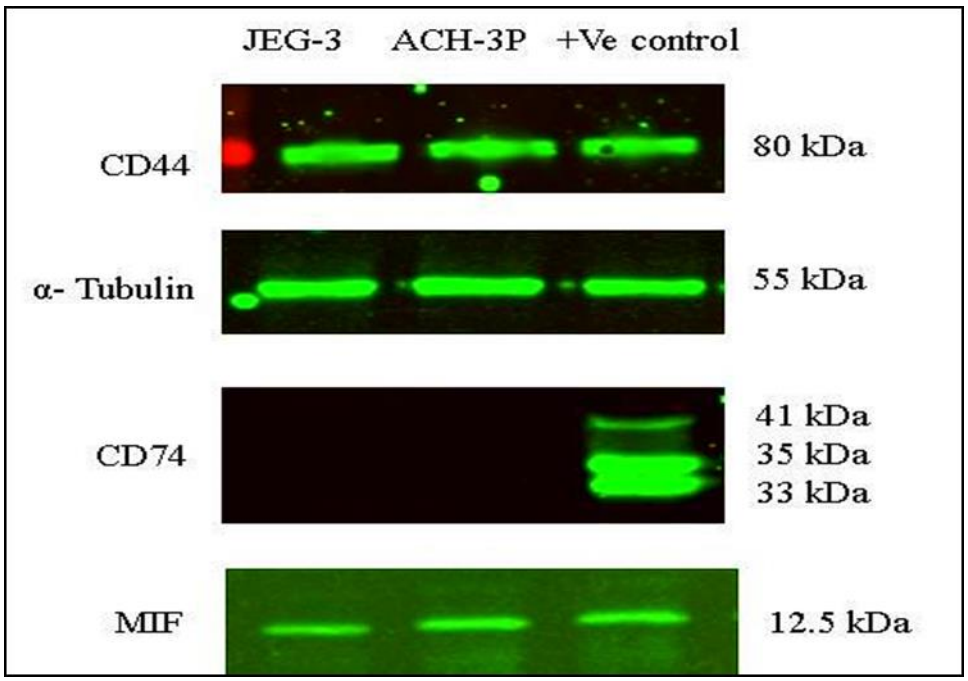

Figure 3-6: Semi-quantitative analysis of the expression of total CD74, CD44 and MIF in trophoblast cell lines JEG-3 and ACH-3P.

Western blot of JEG-3 and ACH-3P cells using By2 (anti-CD74), D-2 (anti-MIF), 156-3c11 (anti-CD44) and TU-02 (anti-alpha-subunit tubulin) monoclonal antibodies (mAb). By2 is specific for CD74 isoforms 31-45 kDa, D-2 is specific for an epitope mapping between amino acids 7-39 at the N-terminus of human MIF, 156-3c11 is a mouse mAb which detects endogenous levels of total CD44 protein that is specific for most CD44 isoforms (80-90 kDa) and TU-02, detects full length α tubulin. A protein band for CD44 was expected to be detected at 80 kDa,which detected in JEG-3 and ACH-3P cells, using the HeLa cell line as a positive control. The absence of the bands corresponding to the expected molecular weight of CD74 confirms that both trophoblast cell lines do not express CD74, compared to the Raji cell line used as a positive control. CD74 was expected to be detected between 31-45 kDa. A protein band for MIF was detected in JEG-3 and ACH-3P cells, which, compared to the Jurkat cell line used as a positive control, was expected to be detected at 12.5 kDa.

3.2.4 Microscopic Studies

Microscopy was utilised to examine the expression of CD74, MIF and CD44 in JEG-3 and ACH-3P cells. Therefore, immunofluorescence staining was carried out (section 2.2.6.2) and microscopic slides were prepared. Figure 3-8 A and B shows that JEG-3 and ACH-3P cells express MIF and CD44, but not CD74, on the cell membrane.

Due to the expression of CD74 in several types of cancer cells, including breast cancer, CAMA-1 cells were used as positive controls (Figure 3-10).

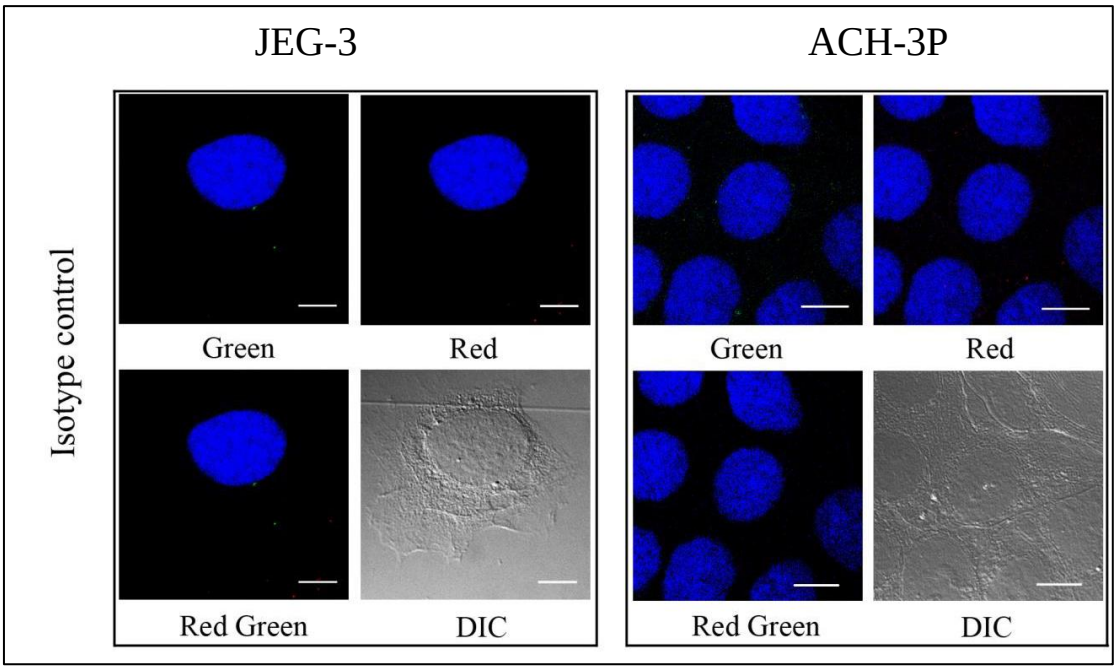

Figure 3-7: Confocal microscopy images of negative control samples of ACH-3P and JEG-3 cells.

Isotype control represents cells stained with Isotype control IgG primary and Alexa Flour 488 and Alexa Fluor 555 secondary antibodies, visualised by using green 488nm and red 555nm wavelength lasers to measure non-specific antibody binding. Blue colour shows DAPI stained cell nuclei, Red Green shows merged image, whereas DIC is differential interference contrast image of corresponding cell. Scale bar 10um.

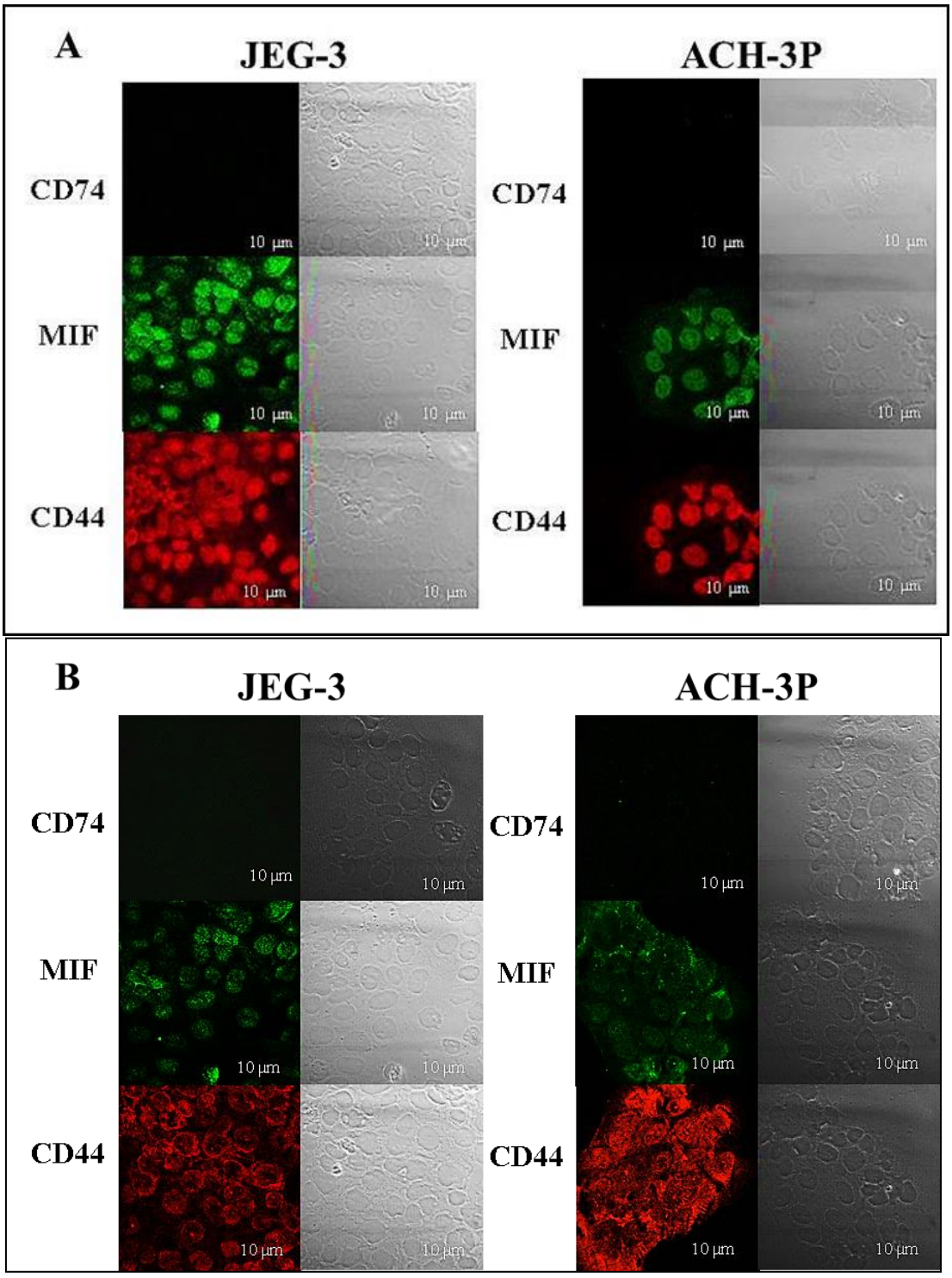

Figure 3-8: Confocal microscopy images of CD74, MIF and CD44 molecule expression on the cell membrane of JEG-3 and ACH-3P cells.

JEG-3 and ACH-3P cells imaged using confocal laser scanning microscopy. A: Cell membrane expression of CD74, MIF and CD44. B: Cell surface expression of CD74, MIF and CD44. The cells were cultured in LabTek 8-well chambers at a density of $6x10^3$ cells per well overnight. Cells were stained with CD74 and MIF primary antibody. Secondary antibodies used are Alexa Fluor 488- and Alexa Fluor 555-labelled goat anti-mouse IgG (green and red, respectively). Scale bar 10 µm.

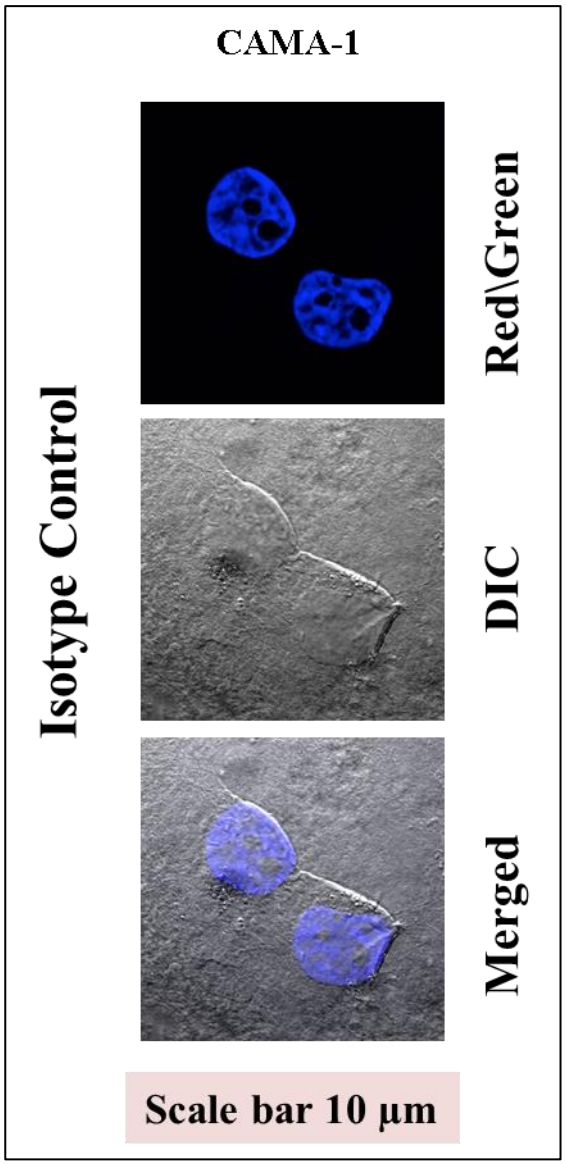

Figure 3-9: Confocal microscopy images of CAMA-1 as isotype- negative controls.

The isotype control comprised cells stained with isotype control IgG primary and Alexa Fluor® 488 and Alexa Fluor® 555 secondary antibodies, visualised by using green 488 nm and red 555 nm wavelength lasers to measure non-specific antibody binding. Blue colour shows DAPI stained cell nuclei, red and green show the merged image, whereas DIC is the differential interference contrast image of corresponding cells. Scale bar 10 µm.

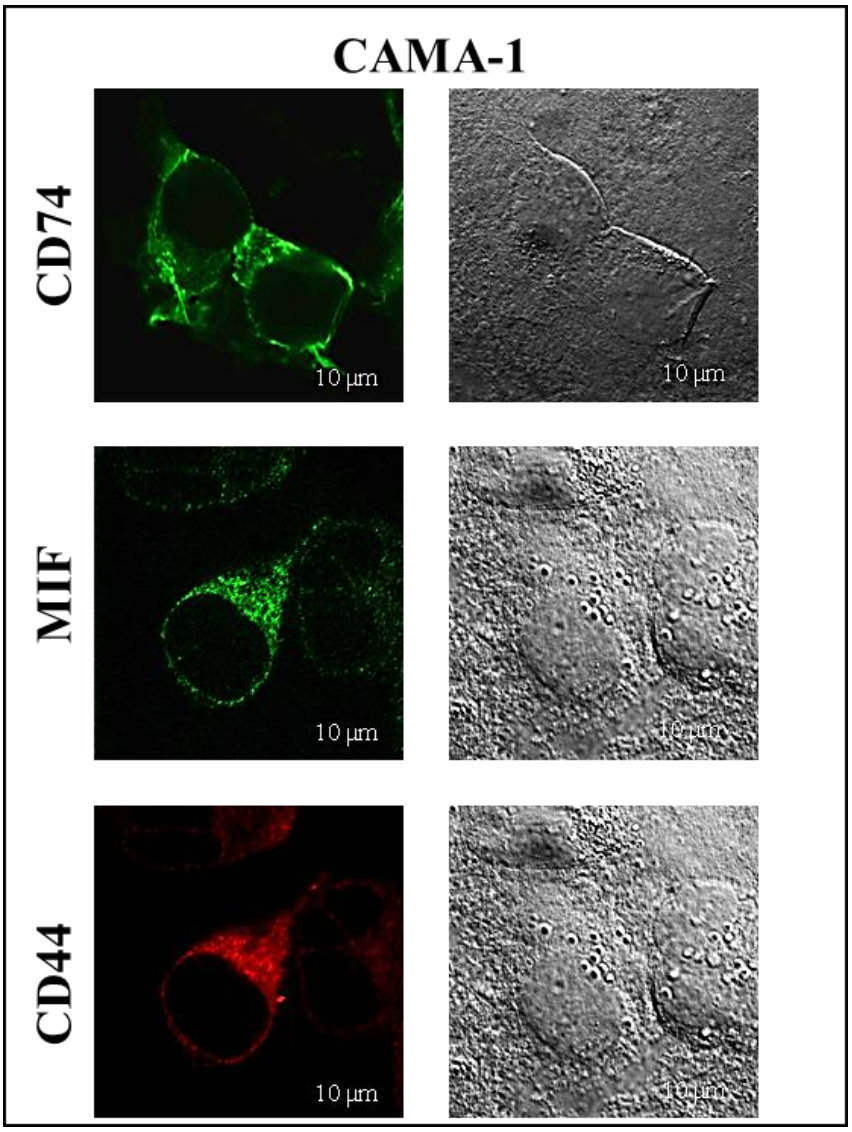

Figure 3-10: Confocal microscopy images of positive control samples of CAMA-1 cells.

Cell membrane expression of CD74, MIF and CD44 in CAMA-1 cells. The cells were cultured in LabTek 8-well chambers at a density of $6x10^3$ cells per well overnight. Cells were stained with CD74 and MIF primary antibody and visualized with Alexa Fluor 488- and Alexa Fluor 555labelled goat anti-mouse IgG (green and red, respectively)., Scale bar 10 µm.

3.2.5 Validation study of human placenta

Validation of normal placenta lysates was used as a positive control to appraise the reliability of β-actin as a loading control respecting its conformity to the full length β-actin (42 kDa).This validation was performed using whole tissue homogenate lysates (40th week of gestation), and normal placenta slides (39th week of gestation). CD74 protein expression in human placenta tissue lysates and human breast tissue lysates were also studied by Western blot analysis using By2 (anti-CD74). Results show that, in Raji cells, the molecular weights of CD74 and β-actin are 33 kDa, 35 kDa, 41 kDa and 42 kDa, respectively No band corresponding to CD74 in both human breast and placenta was detected (Figure 3-11). However, CD74 expression was further validated in a microscopy study of human placenta tissues; CD74 was weakly detected on the cell surface and intracellularly (Figure 3-12).

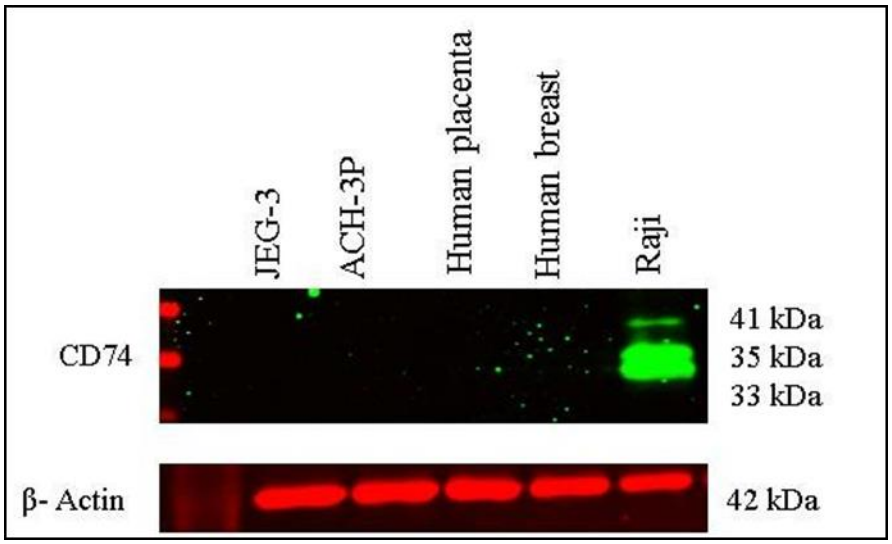

Figure 3-11: Semi-quantitative analysis of the expression of total CD74 in whole human placenta tissue lysate.

Immunoblotting results showing that JEG-3, ACH-3P, human breast tissue and human placenta tissue do not express CD74 in comparison with the Raji cell line, which was used as a positive control. The Raji cell line was expected to express CD74 at a molecular weight between 33-41 kDa. Primary monoclonal antibodies Poly6221 (anti β-actin as loading control), and By2 (anti-CD74) were used. β-actin was detected at a molecular weight of 42 kDa whereas CD74 isoforms were detected at molecular weights 33 and 45 kDa.

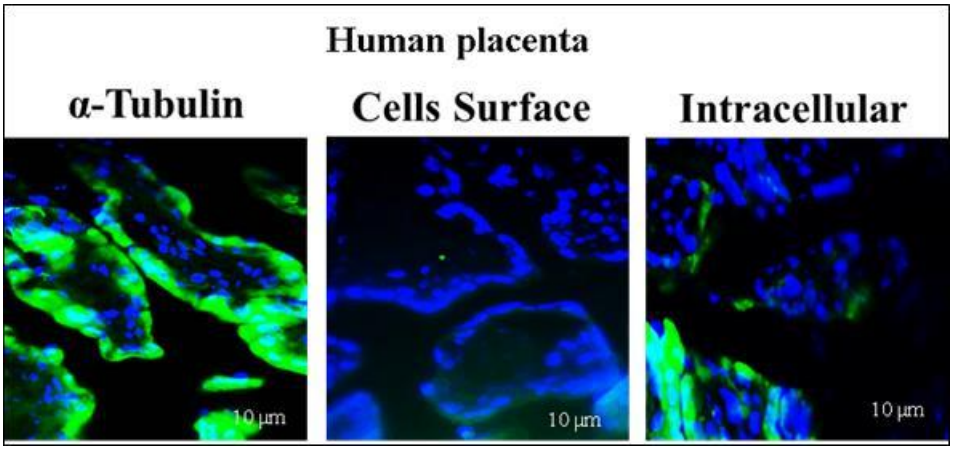

Figure 3-12: Histological immune staining of α-tubulin and CD74 on human placenta tissues.

Detection of α-tubulin and CD74 using ummunostaining. Positive immune staining for α-tubulin using TU-02 was detected. Weak immune staining of CD74 was observed on the cell surface and at intracellular level using a CD74 primary antibody and a labelled secondary antibody (anti-mouse IgG conjugated with FITC Alexa Fluor 488). Scale bar 10 µm.

3.3 Conclusions

The principal aim of this study was to profile and identify the expression of CD74, MIF and CD44 antigens in the trophoblast derived cell lines JEG-3, ACH-3P and human placenta. MIF and CD44 were found to be expressed, and CD74 was not expressed, in human placenta and in JEG-3 and ACH-3P cells. This indicates that JEG-3 and ACH-3P cells proffer good model to better understand trophoblast biology. The following chapter will focus on whether CD74, a membrane-bound protein, is involved in HLA class II-mediated antigen presentation in response to the pro-inflammatory cytokine IFN-γ and lipopolysaccharide (LPS) infection. It will examine, in particular, the regulation of CD74 isoform expression at the fetal-maternal interface.

Chapter 4 CD74 response to lipopolysaccharide (LPS) infection and pro-inflammatory cytokine human interferon-gamma (IFN-γ) in the human trophoblast derived cell lines

4.1 Introduction

During early human pregnancy, the semiallogenic fetus successfully escapes from the maternal immune system. The mechanism by which the fetus avoids rejection is the central question in reproductive immunology posed over fifty years ago by Medawar and Billingham (Medawar, 1953, Billingham, 1964). Specific mechanisms must exist to suppress or modulate the maternal immune system and to protect the developing embryo from rejection. Trophoblast cells must play an imperative role, because they are the only placental cells that interact directly with maternal tissue (Yie et al., 2006). Most notably, trophoblast cells have unique patterns of HLA expression in comparison to other nucleated cells and do not express MHC class II (HLA-DR) antigens. This is one of the key protective mechanisms in the human placenta during the first trimester (Athanassakis-Vassiliadis et al., 1990, Vassiliadis et al., 1994).

It is well known that MHC class II molecules associate with CD74 in antigen presenting cells (APCs) (Ong et al., 1999, Henne et al., 1995). More recently, CD74 expression and function has been examined in cell types other than APCs, such as epithelial cells (Barrera et al., 2005). In addition, some studies suggest that CD74 might be expressed independently of MHC class II, which indicates an additional function for CD74 aside from antigen presentation (Henne et al., 1995, Badve et al., 2002, Momburg et al., 1986).

Various studies have indicated that the expression of CD74 is closely linked to inflammatory/chronic inflammatory conditions, and during Helicobacter pylori infection. H. pylori can use CD74 as a point of attachment to gastric epithelial cells (Beswick et al., 2006, Beswick et al., 2005a, Leng et al., 2003, Beswick and Reyes, 2009, Zheng et al., 2012). Indeed, the expression of CD74 can be up- regulated using several types of

cytokines, including IFN-γ (Ong et al., 1999, Moldenhauer et al., 1999, Murphy and Tomasi, 1998, Pessara et al., 1988, Möller and Moldenhauer, 1999).

The immunology of human pregnancy is heavily influenced by Th1 and Th2 cytokines, present at the fetal-maternal interface. It was confirmed that Th2-type cytokines such as IL-4 and IL-10 are associated with successful pregnancy, while Th1 cytokines, particularly IFN-γ, are incompatible with successful pregnancy (Wegmann et al., 1993, Raghupathy, 1997).

During the first trimester of human pregnancy, placental trophoblast cells express IFN-γ intensely (Paulesu et al., 1994). However, IFN-γ expression appears to be down-regulated from the second to third trimester until there is almost no expression by term. Effecting control of immune reactions against the fetus, such as apoptosis of endothelial cells of the spiral arteries, is the most likely explanation of this change (Banerjee et al., 2005, Jenkins et al., 2000).

In pregnancy the maternal immune system protects the allogeneic foetus from rejection, whilst maintaining immunoprotection against pathogens, allowing the pregnancy to succeed. In early human pregnancy, intrauterine infections have been shown to be strongly associated with certain complications and with pre-term labour in up to 40% of cases (Lamont, 2003). Infection induces a Th1 response during early pregnancy, which may cause abortion by altering the cytokine profile at the fetal-maternal interface (Filisetti and Candolfi, 2004). However, the specific mechanisms regulating this process are unknown.

As a key component of human placenta, trophoblast cells play a major role in recognizing bacterial products through TLRs and induce differential responses at the fetal-maternal interface. The role of TLRs in establishing tolerance to the growing fetus is still not well

known (Koga and Mor, 2008). So, bacterial infection is believed to be associated with the pregnancy complications, such as preterm labour, preeclampsia and septic shock.

A major initiator of septic shock is lipopolysaccharide (LPS), a component of the cell wall envelope of the Gram-negative bacteria. Previous studies have shown that inflammation, as induced by LPS stimulation, can enhance CD74 expression in MKN-45 gastric cancer cell lines (Zheng et al., 2012).

The aim of this study was to investigate the effect of LPS-mediated infection and IFN-γ on the expression of CD74 in JEG-3 and ACH-3P cells at mRNA and protein levels. In particular, the response to LPS and IFN-γ CD74 isoform expression on human trophoblast derived cells, JEG-3 and ACH-3P was investigated. Overall, JEG-3 and ACH-3P cells provided a reliable trophoblastic model system to investigate early human pregnancy and a variety of inflammation and infection, using several detection methods including RT-PCR, flow cytometry, Western blotting and immunoprecipitation (IP).

4.2 Results

4.2.1 Gene Expression

Expression of CD74 on trophoblast cells (JEG-3 and ACH-3P) in response to IFN-γ and LPS was studied at the mRNA level. Both JEG-3 and ACH-3P cells were subjected to various concentrations of IFN-γ and LPS. Results obtained with semi-quantitative RT-PCR, used to quantify the amount of CD74 DNA amplified, are presented in (Figure 4-1). The results indicated significant up-regulation in cells that were treated with IFN-γ and LPS, as compared to untreated cells. In terms of the expression of CD74 on both JEG-3 and ACH-3P cells, slight expression was observed after 12 h stimulation with 1000 IU/ml of IFN-γ and after 5 µg/ml LPS treatment for 4 h.

CD74 mRNA expression was not detectable in both un-stimulated JEG-3 and ACH-3P cell lines using RT-PCR (above). However, a slight concentration- and time-dependent expression of CD74 mRNA after stimulation of both cell lines with IFN-γ and LPS arose. In terms of stimulation with IFN-γ, JEG-3 and ACH-3Pcells showed slight mRNA expression of CD74 after 12 h stimulation with 1000 IU/ml IFN-γ. Interestingly, there was a strong up-regulation of CD74 mRNA expression in both JEG-3 and ACH-3P cells after 24 h stimulation with 500 IU/ml IFN-γ. In contrast, only modest expression of CD74 mRNA occurred after incubating both JEG-3 and ACH-3P cells for 4 h with 5 µg/ml LPS (Figure 4-1).

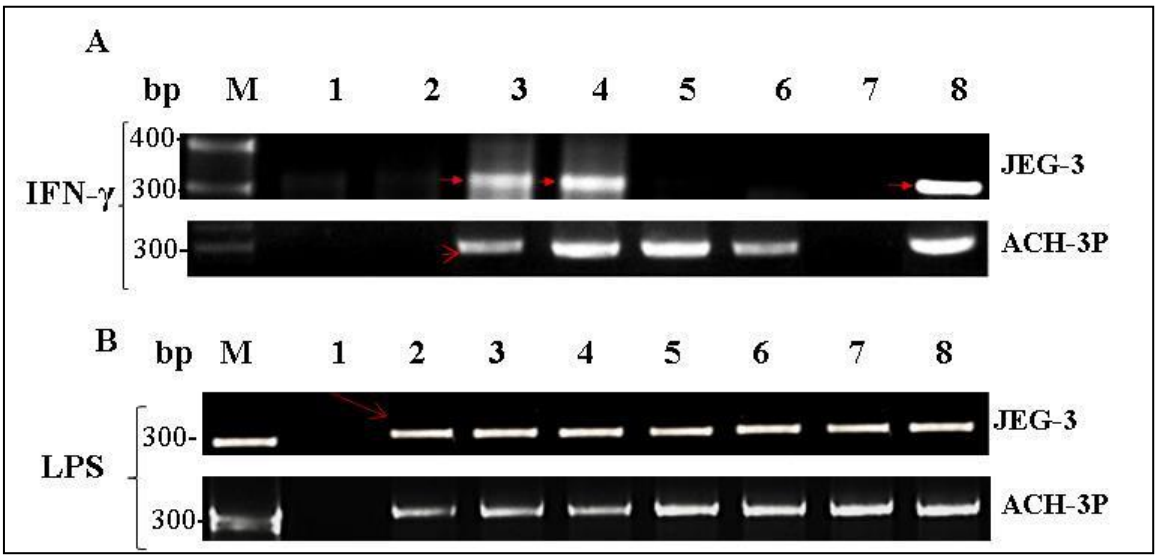

Figure 4-1: CD74 mRNA expression assayed by RT-PCR.

In JEG-3 and ACH-3P cells, in response to pro-inflammatory cytokines, CD74 mRNA was up regulated. Untreated JEG-3 and ACH-3P cells, however, did not express CD74 mRNA. The samples were loaded onto lane numbers as follows: A: 1: un-stimulated JEG-3 or ACH-3P cells; 2: JEG-3 or ACH-3P cells stimulated for 12 h with 500 IU/ml IFN-γ; 3: JEG-3 or ACH-3P cells stimulated for 12 h with 1000 IU/ml IFN-γ; 4: JEG-3 or ACH-3P cells stimulated for 12 h with 500 IU/ml IFN-γ; 5: JEG-3 or ACH-3P cells stimulated for 24 h with 1000 IU/ml IFN-γ; 6: JEG-3 or ACH-3P cells stimulated for 48 h with 500 IU/ml IFN-γ; 7: JEG-3 or ACH-3P cells stimulated for 48 h with 1000 IU/ml IFN-γ; 8: CAMA1 cells as positive control. B: 1: un-stimulated JEG-3 or ACH-3P cells; 2: JEG-3 or ACH-3P cells stimulated for 4 h with 5µg/ml LPS; 3: JEG-3 or ACH-3P cells stimulated for 4 h with 10µg/ml LPS; 4: JEG-3 or ACH-3P cells stimulated for 6 h with 5µg/ml LPS; 5: JEG-3 or ACH-3P cells stimulated for 6 h with 10µg/ml LPS; 6: JEG-3 or ACH-3P cells stimulated for 12 h with 5µg/ml LPS; 7: JEG-3 or ACH-3P cells stimulated for 12 h with 10µg/ml LPS; 8: CAMA1 cells as positive control. Molecular weight marker (80- 1031 bp) is shown on the left (Fermentas, #SM0383). The results are representative of at least three replicate experiments. The identity of CD74 was confirmed by sequencing of the polymerase chain reaction product (CD74=384bp).

4.2.2 Detection of CD74 cell-surface expression by flow cytometry

Cell surface expression of the CD74 antigen was assessed by analysing the cell membrane of JEG-3 and ACH-3P cell lines after IFN-γ and LPS stimulation (Figure 4-2 A and B). The mouse CD74-specific monoclonal antibody By2, which is specific for 31-45 kDa isoforms, was used in an appropriate concentration, followed by 1 µl RAM-FITC secondary antibody. CD74 cell surface expression was not detected in both trophoblast cell lines, even after treatment with IFN-γ (for 12, 24 and 48 h) at a concentration of 500 and 1000U/ml, or with LPS (for 4, 6 and 12 h) at a concentration of 5 and 10µg/ml. Results indicated that there was no induction of cell surface expression of CD74 on JEG-3 cells even after exposure to IFN-γ or LPS (Figure 4-2 A and B).

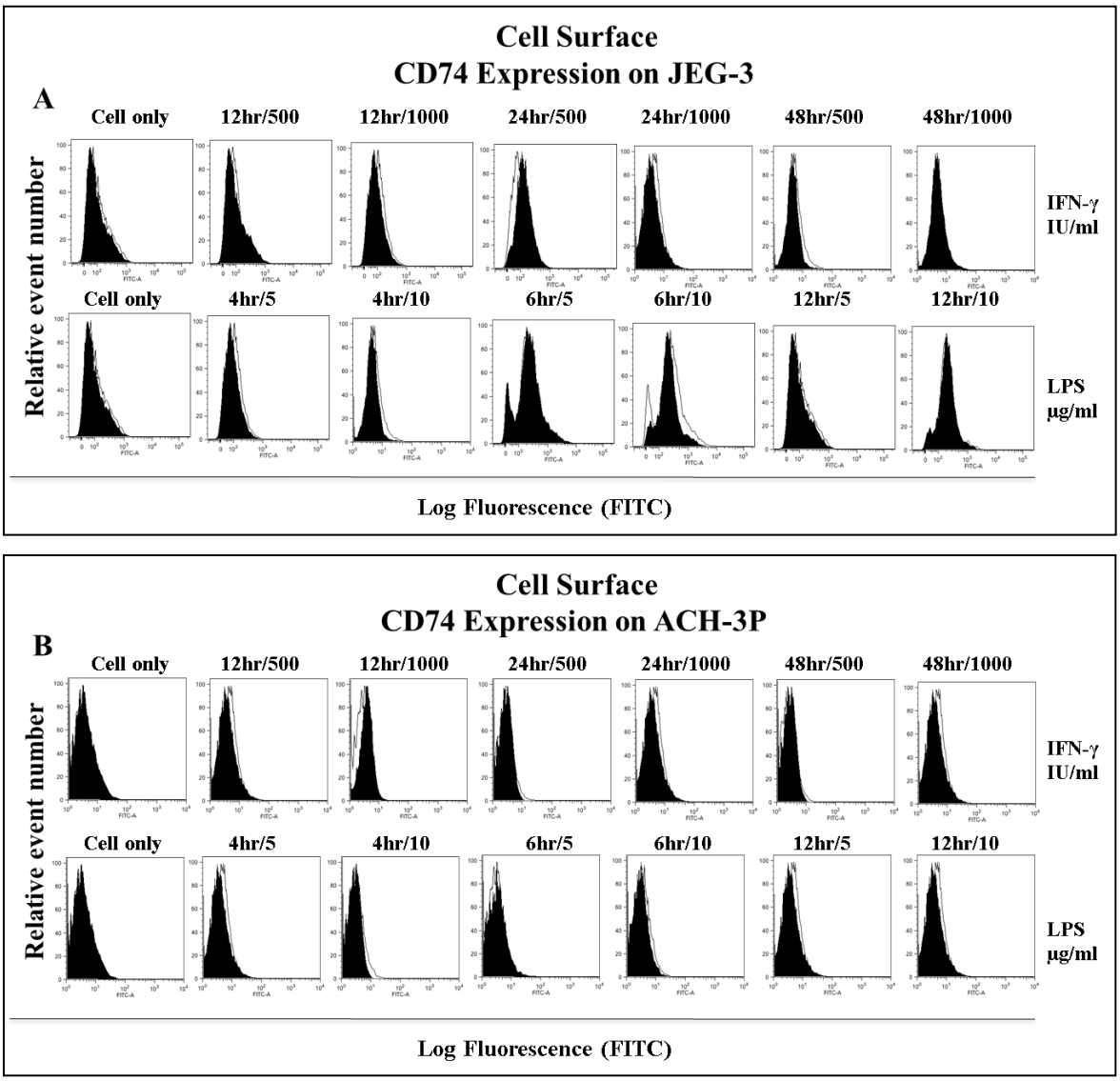

Figure 4-2: Expression of cell surface CD74 by JEG-3 and ACH-3P cells exposed to IFN-γ and LPS.

(A) The black histograms represent cells labelled with FITC-labelled secondary anti-mouse antibody, serving as a negative control. The empty histograms represent the cell surface expression of CD74 under treated and untreated conditions; cells labelled with By2 (anti-CD74) antibody. (B) The black histograms represent the negative control in which the cells were labelled with FITC-labelled secondary anti-mouse antibody. The empty histograms represent the cell surface expression of CD74 in cells labelled with By2 (anti-CD74) antibody, under treated and untreated conditions.

4.2.3 Detection of CD74 intracellular expression by flow cytometry

Intracellular expression of CD74 in the JEG-3 and ACH-3P cell lines in response to IFN-γ and LPS was analysed (Figure 4-3 A and B). Treatment of JEG-3 and ACH-3P cells with IFN-γ or LPS induced intracellular CD74 expression, but no surface expression of CD74 (Figure 4-3 A and B). Significantly, both JEG-3 and ACH-3P cells showed slight upregulation of CD74 after exposure to IFN-γ or LPS, which occurred after 12hr stimulation with 1000 IU/ml of IFN-γ, and with LPS at a concentration of 10µg/ml. CD74 was highly expressed in the JEG-3 and ACH-3P cell lines after 24hr stimulation with 1000 IU/ml of IFN-γ, and a further increase was observed after 48hr stimulation with 500 IU/ml of IFN-γ. In contrast, heightened expression of CD74 in both cell lines was evident after 6hr stimulation with LPS at a concentration of 5 and 10µg/ml, and a further increase was observed after 12hr stimulation with 5µg/ml of LPS. However, a decrease in CD74 expression was seen after 48hr stimulation with 1000 IU/ml of IFN-γ as well as after 12hr stimulation with 10µg/ml of LPS. Graphical representation of CD74 intracellular protein expression on JEG-3 and ACH-3P cells is shown in (Figure 4-4). Because IFN-γ and LPS induced intracellular expression of CD74 in the JEG-3 and ACH-3P cell lines, their effect on HLA-DR expression at the intracellular level was also studied. HLA-DR expression is not characteristic of trophoblast cells (Figure 4-5 A, B).

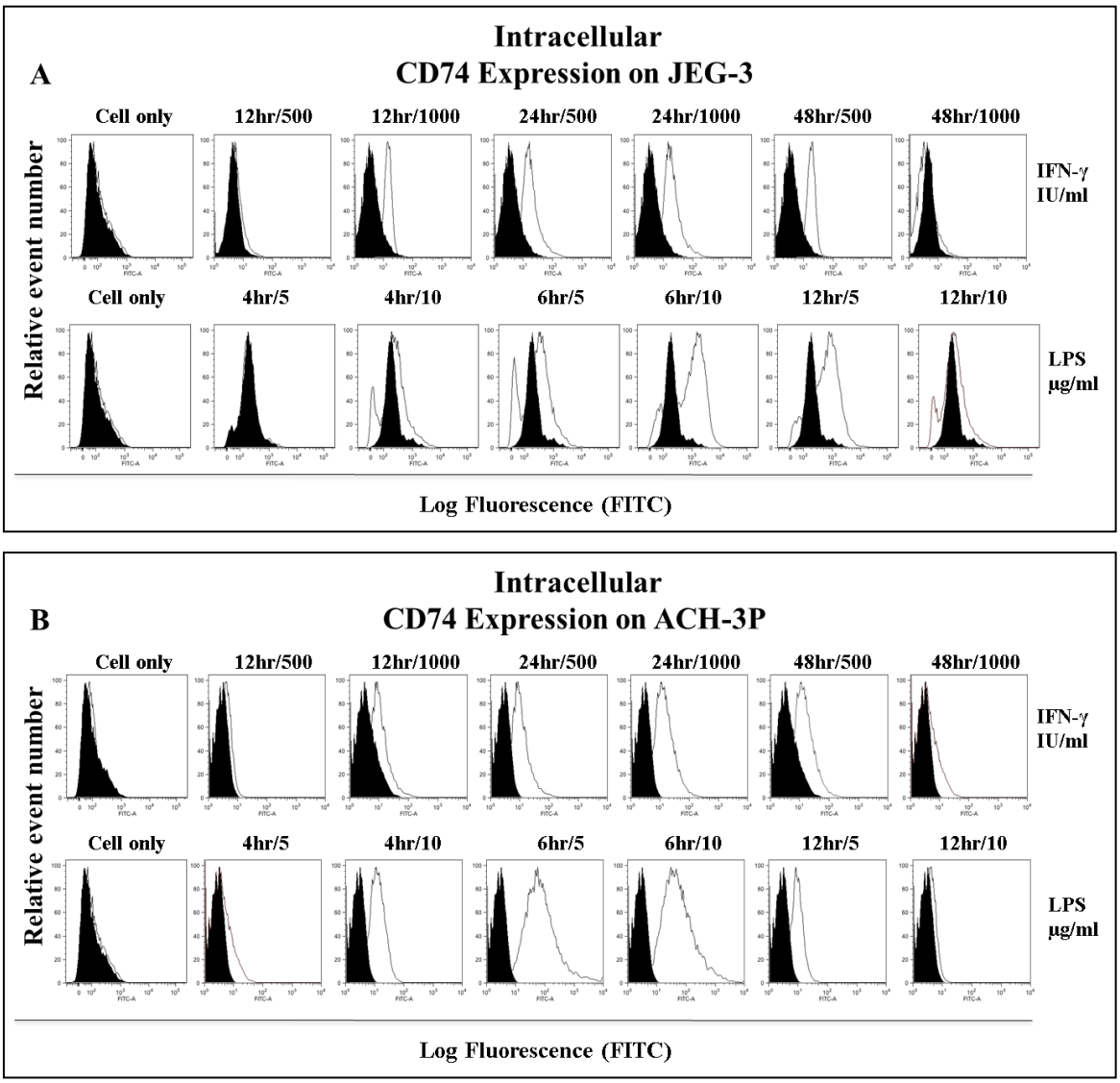

Figure 4-3: Expression of intracellular CD74 in JEG-3 and ACH-3P cells exposed to IFN-γ and LPS.

(A) Black histograms represent cells were labelled with FITC-labelled secondary anti-mouse antibody, serving as negative control. Empty histograms represent the intracellular expression of CD74 under treated and untreated conditions, labeled with By2 (anti-CD74) antibody. (B) Black histograms represent the negative controls, in which cells were labelled with an FITC-labelled secondary anti-mouse antibody. Empty histograms represent intracellular expression of CD74 under treated and untreated conditions in cells labeled with By2 (anti-CD74) antibody.

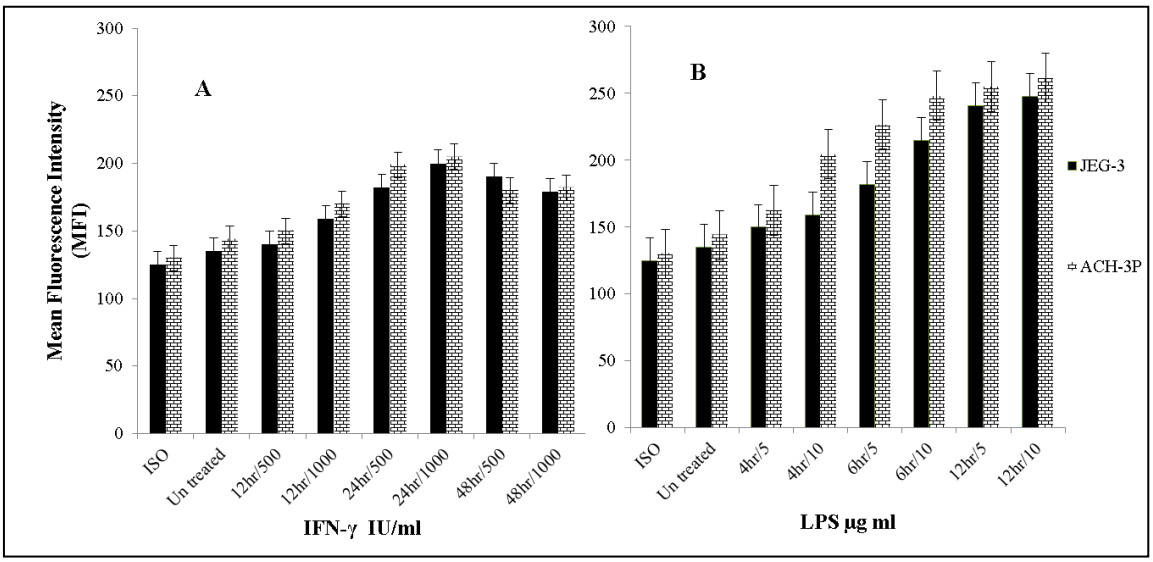

Figure 4-4: Graphical representation of CD74 intracellular protein expression on JEG-3 and ACH-3P cells.

Bar graphs representing the level of CD74 expression in the JEG-3 and ACH-3P cell lines. The level of CD74 expression was significantly higher in both cell lines treated with LPS (B) compared to when treated with IFN-γ (A).

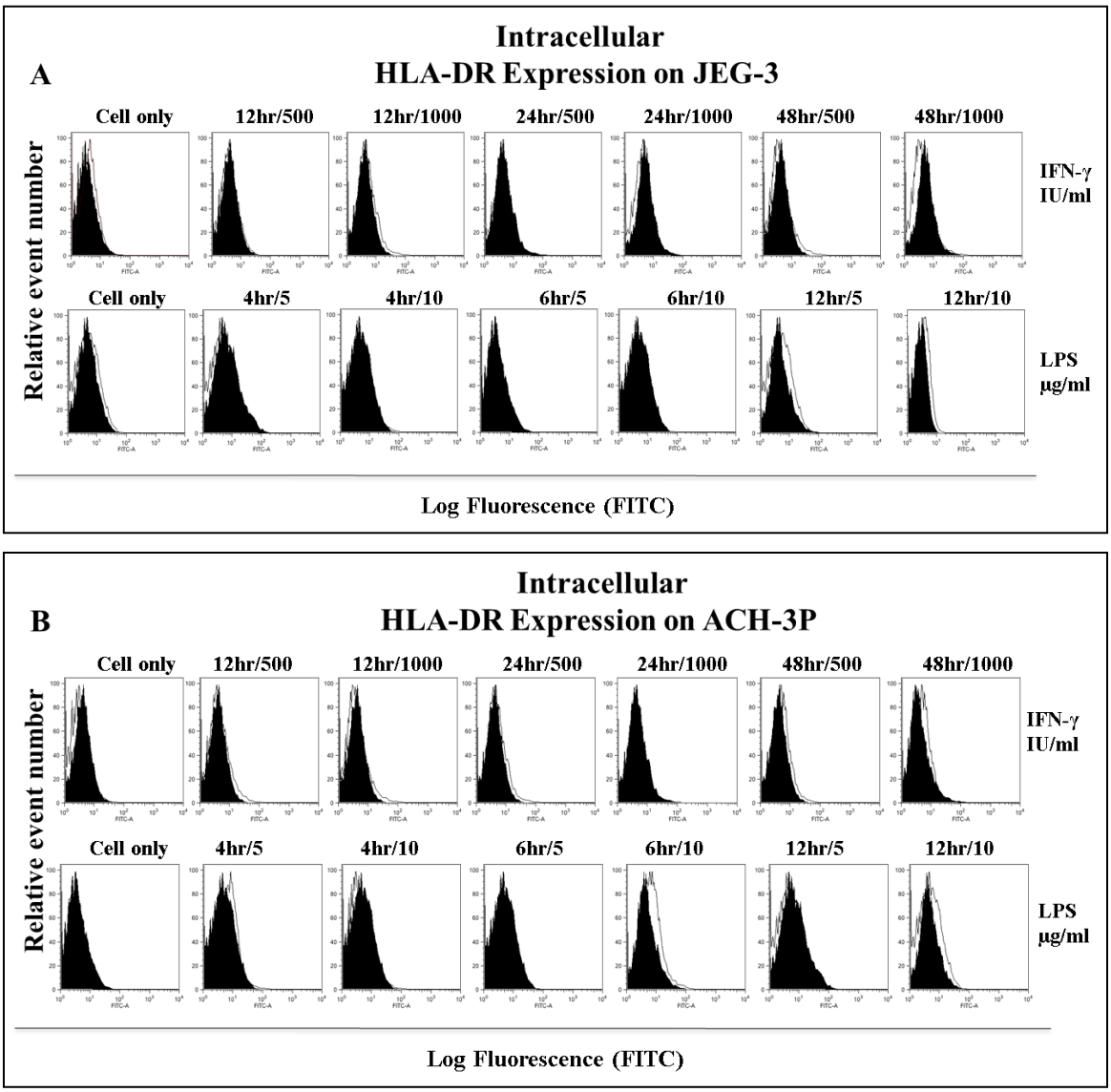

Figure 4-5: Expression of Intracellular HLA-DR by JEG-3 and ACH-3P cells exposed to IFN-γ and LPS.

(A) Black histograms represent cells labelled with FITC-labelled secondary anti-mouse antibody, serving as negative control. Empty histograms represent the intracellular expression of HLA-DR under treated and untreated conditions, labeled with L243 (anti-HLA-DR) antibody. (B) Black histograms represent the negative control, in which cells were labelled with FITC-labelled secondary anti-mouse antibody. The empty histograms represent the intracellular expression of HLA-DR under treated and untreated conditions in cells labeled with L243 (anti-HLA-DR) antibody.

4.2.4 Detection of CD74 protein by immunoblotting

Western blotting was utilised to investigate the expression of CD74 isoforms in the JEG-3 and ACH-3P cell lines. Whole-cell lysates from untreated and IFN-γ or LPS treated cells were fractionated by gel electrophoresis and the presence of CD74 was detected by By2 (anti-CD74), which is specific for 31-45 kDa isoforms. Results showed that the molecular weight of CD74 and α-tubulin (loading control) were 33 kDa, 35 kDa, 41 kDa and 55 kDa, respectively. The absence of protein bands for CD74 on both trophoblast cell lines, stimulated with IFN-γ, JEG-3 and ACH-3P, is shown in (Figure 4-6 A). However, CD74 was observed after LPS treatment as represented in (Figure 4-6 B).

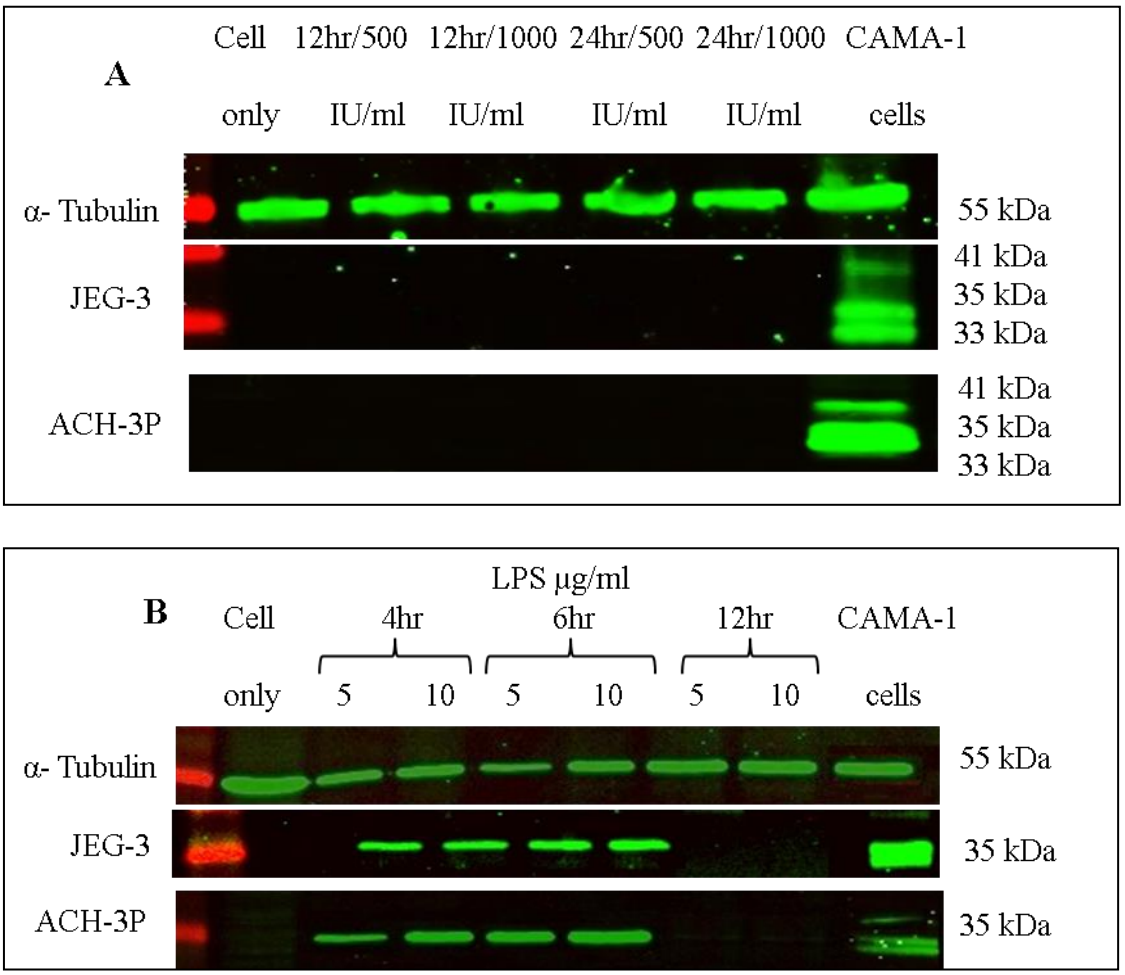

Figure 4-6: Immunoblot analysis to detect CD74 according to its molecular weight after stimulation of CAMA-1, JEG-3 and ACH-3P cells with IFN-γ and LPS.

Western blot analysis of CD74 in JEG-3 and ACH-3P cells untreated or stimulated with IFN-γ (500 IU/ml or 1000 IU/ml) for 12 and 24 hr, and with LPS (5 µg/ml or(10 µg/ml) for 4, 6 and 12 hr . CAMA-1 cells were used as a positive control. The molecular weight marker (Fermentas, #SM0671) is shown on the left. Primary monoclonal antibodies used were TU-02 (anti-alpha-subunit tubulin), which detects full length α-tubulin at a molecular weight of 55 kDa, and By2 (anti-CD74), specific for CD74 isoforms 31-45 kDa. The absence of bands corresponding to the expected molecular weight of CD74 confirmed that JEG-3 and ACH-3P cells do not express CD74, even after stimulation with IFN-γ; However, CD74 specific isoform was detected after LPS treatment (CAMA-1 cells).

4.2.5 Immunoprecipitation

As shown above, the expression of CD74 was detected in both JEG-3 and ACH-3P cells treated with 5 or 10 µg/ml LPS for 4 and 6 hr. Therefore, the expression of CD74 was investigated by immunoprecipitation (IP). To determine if By2 (anti-CD74) antibody was suitable for IP, whole-cell lysate of JEG-3 or ACH-3P cells treated with 5 or 10 µg/ml LPS, for a period of 6 hr, were incubated with By2 antibody or with protein A/G PLUS-agarose as control. This was carried out because the same antibody was used in both IP and Western blot analysis. The blot was then incubated with By2 (anti-CD74) antibody. The resulting bands (Figure 4-7) correspond to the heavy and light chains of CD74 antibody in both JEG-3 and ACH-3P.

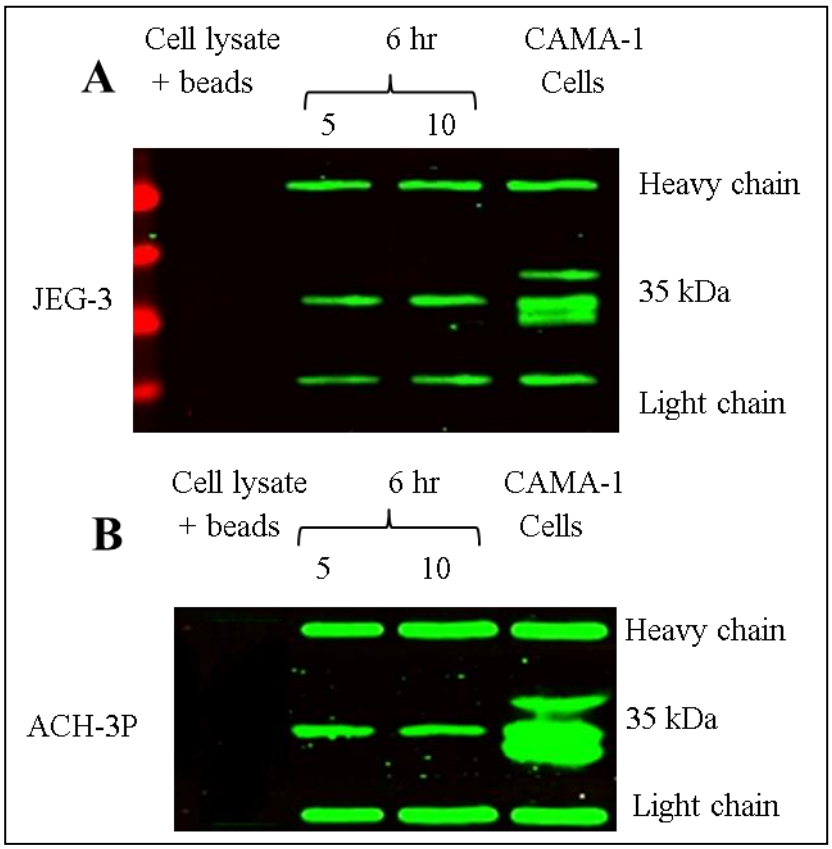

Figure 4-7: Immunoprecipitation analysis of CD74 expression in JEG-3 and ACH3P cells.

Immunoprecipitates were subjected to pull down against CD74. The samples were loaded into lanes as follows: A: JEG-3 cell lysate and beads only; JEG-3 cells were treated with 5 µg/ml LPS for 6 hr; JEG-3 cells treated with 10 µg/ml LPS for 6 hr; CAMA-1 cells, serving as positive control. B: ACH-3P cell lysate and beads only; ACH-3P cells treated with 5 µg/ml LPS for 6 hr; ACH-3P cells treated with 10 µg/ml LPS for 6 hr; CAMA-1 cells, serving as the positive control. Heavy and light chain antibody bands were observed at approximately 55 and 25 kDa respectively.

4.3 Conclusions

This study identified CD74 as functioning independently of class II MHC in JEG-3 and ACH-3P cells. Therefore, the effect of IFN-γ and LPS on CD74 expression in JEG-3 and ACH-3P cells was analysed. It was found that CD74 positivity significantly increased after incubation with IFN-γ or LPS. This suggests that CD74 present in these cells might play a negative role in protecting early human pregnancy. CD74 may also be associated with gestational complications. The findings obtained from this study may help to determine whether CD74 plays a role in fetal-maternal tolerance in early human pregnancy. The following chapter will further focus on MIF, given its role in ensuring that pregnancy succeeds, and CD44, in the response of JEG-3, ACH3P cells to LPS infection.

Chapter 5 Macrophage Migration Inhibitory Factor (MIF) and CD44 expression in lipopolysaccharide (LPS)-stimulated JEG-3 and ACH-3P cells

5.1 Introduction

Maternal immune status is a vital determinant of pregnancy outcome, since the maternal immune system serves to protect the allogeneic foetus from rejection. Concurrently, during pregnancy, immune defences against potential pathogen invasion are maintained. Intrauterine infections have been shown to be strongly associated with certain complications in pregnancy and also are associated with preterm labour in up to 40% of cases (Lamont, 2003). Furthermore, 85% of pre-term deliveries at less than 28 weeks of gestation have evidence of infection (Epstein et al., 2000). Moreover, intrauterine infections may occur early in human pregnancy, preceding any observed pregnancy complications (Gonçalves et al., 2002). Indeed, some complications occur mainly in one part of pregnancy. For example, miscarriage is most common in the first trimester, whereas other complications can occur throughout the pregnancy, such as pre-term labour and preeclampsia (Arechavaleta-Velasco et al., 2002, Romero et al., 2003, Gonçalves et al., 2002). Hence, maternal innate immune responses against pathogens at the fetal-maternal interface may have a significant impact on the success of human pregnancy (Koga et al., 2009).

Bacterial infection has been identified as an important cause of pre-term labour and early miscarriage, particularly during the first trimester of pregnancy, due to several mechanisms related to inflammation (Romero et al., 2010, Kim et al., 2010, Ralph et al., 1999, Hay et al., 1994, Romero et al., 2007). A variety of bacterial products interact with cellular pathogen-associated molecular patterns (PAMPs), including the toll-like receptors (TLRs) (Flo et al., 2002). TLRs play an essential role in the innate immune response by detecting conserved molecular products of bacteria (Medzhitov et al., 1997, Medzhitov, 2001). Trophoblast cells are able to trigger inflammation through the recognition of

bacterial products via TLRs and induce differential responses, which has been proposed to support successful implantation (Abrahams and Mor, 2005).

The bacterial endotoxin lipopolysaccharide (LPS) is the major antigen of the outer membrane in Gram-negative bacteria. It has toxic effects and is a major cause of intrauterine inflammation, which potentially causes preterm parturition (Elovitz and Wang, 2004). LPS binds to toll-like receptor 4 (TLR-4) (Abrahams et al., 2004, Adams et al., 2007, Takeda et al., 2003). Subsequent to the binding of LPS, the innate immune response is activated. Thus, activation of TLR-4 in the placenta triggers nuclear factor kappa B (NF-kB) signalling by releasing several cytokines, including MIF (Martiney et al., 2000, Bennett et al., 1997, Calandra and Roger, 2003), a cytokine expressed at the fetal-maternal interface (Arcuri et al., 2001). Recent observations have also shown that CD44 can serve as an endogenous trigger of TLR signalling and are able to activate an innate immune defence, promoting the production of cytokines by a variety of cell types (Termeer et al., 2002, Jiang et al., 2005).

MIF is one of many cytokines that act during human pregnancy. MIF engages in pro-inflammatory activities and serves multiple other functions in response to infection or the presence of bacterial toxins and pathogen including Gram-positive toxins (TSST-1 and SPEA) and LPS (Bernhagen et al., 1993, Bacher et al., 1997, Das et al., 2013). It plays an important role in the maintenance of human pregnancy by regulating innate and adaptive immunity by affecting the trafficking and behaviour of macrophages and lymphocytes (Viganò et al., 2007). Once secreted, MIF exhibits a broad range of immune and inflammatory activities, including the induction of inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-12, IL-6 and IL-8 (Calandra and Roger, 2003). In human pregnancy, elevated MIF has been detected in many infectious and inflammatory diseases, such as

preeclampsia (Todros et al., 2005). Contrastingly, low levels of MIF have been linked to early gestation in women with recurrent miscarriages (Yamada et al., 2003).

CD44, the principal cell surface receptor for HA, is involved in HA-induced cytokine release by forming a TLR4-CD44 complex (Taylor et al., 2007). CD44 is expressed at the maternal-fetal interface and might play critical roles in maintenance of normal human pregnancy (Wasserman et al., 1983). It has been reported that the regulation of CD44 induction after LPS stimulation may involve a combination of signals delivered by the interaction of LPS with the CD14/TLR complex. This suggests that CD44 may be induced as a result of the signals generated following the association of LPS with lipopolysaccharide binding protein (LBP) in the LPS-LBP-CD14-TLR-4 complex. There is also evidence that the expression of CD44 and CD14 may be induced by LPS and that LPS may modulate the biological effect in inflammation that is mediated by CD44 and CD14 (Gee et al., 2002, Mishra et al., 2005). These observations suggest that CD44 might play a critical role in protecting early human pregnancy against bacterial infection.

This study examined JEG-3 and ACH-3P cell proliferation in response to bacterial LPS. The effect of LPS on MIF and CD44 expression was also investigated.

5.2 Results

5.2.1 Effect of LPS on the proliferation of JEG-3 and ACH-3P cells

Extravillous trophoblast (EVT) cell proliferation and viability is crucial for proper embryo implantation, invasion and a successful pregnancy. EVT cells are first embryo-derived cells, which come into direct contact with maternal immune cells. As a first point of investigation, trophoblast cell viability and proliferation in response to LPS was studied. The effect of varying concentrations of LPS on the proliferation of JEG-3 and ACH-3P cells was examined. Normal cells, without LPS treatment, were used as a control group (Figure 5-1).

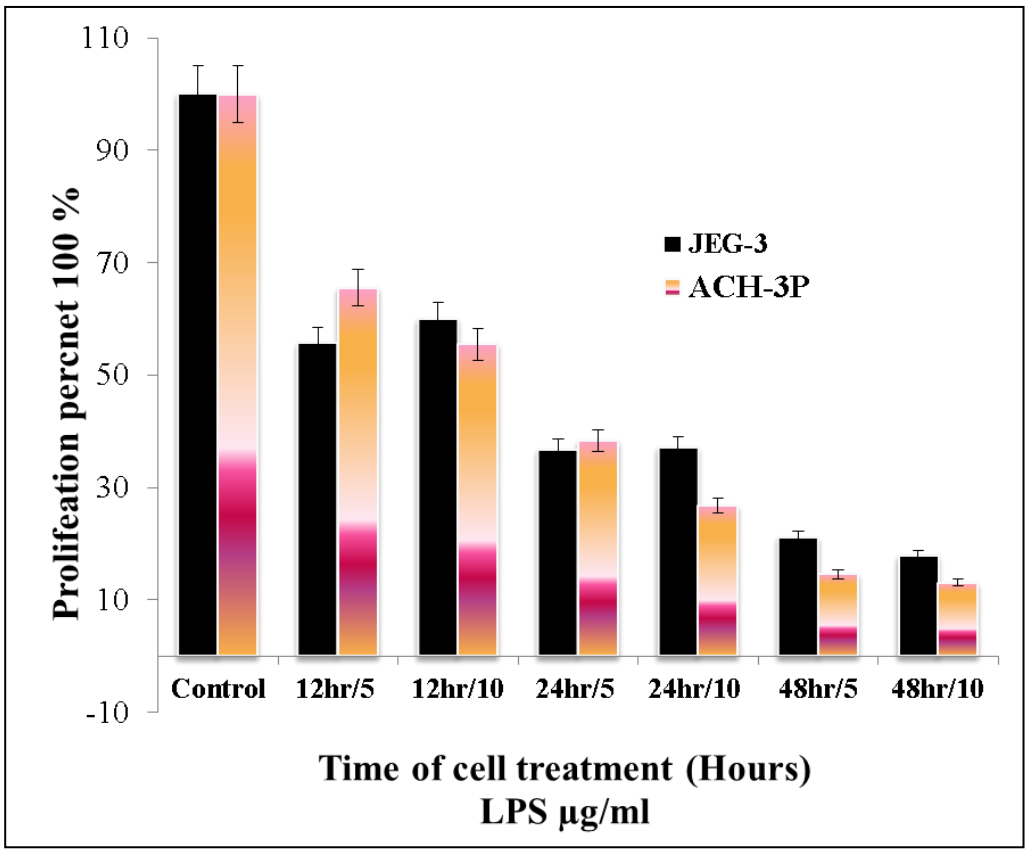

Figure 5-1: JEG-3 and ACH-3P cell proliferation in response to dose and time dependent LPS treatment.

X axis shows time and LPS concentration. Each data point represents a mean of at least three independent experiments with triplicate wells. The percentage of cell proliferation compared to control cells (untreated cells) is depicted. Untreated, positive control sample and the error bars represent the mean of three separate experiments.

5.2.2 Detection of MIF expression by flow cytometry

The effect that LPS may have on cell surface and intracellular expression of MIF in JEG-3 or ACH-3P cells was investigated. To stain for MIF, antibody D-2 (anti-MIF) was used on untreated and LPS-treated JEG-3 and ACH-3P cells, followed by 1 µl mouse anti-mouse IgG conjugated with FITC secondary antibody. The results showed that LPS stimulation appeared to effect both intracellular and surface expression of MIF after 4, 6 and 12hr stimulation of JEG-3 or ACH-3P cells with 5 and 10µg/ml of LPS (Figure 5-2).

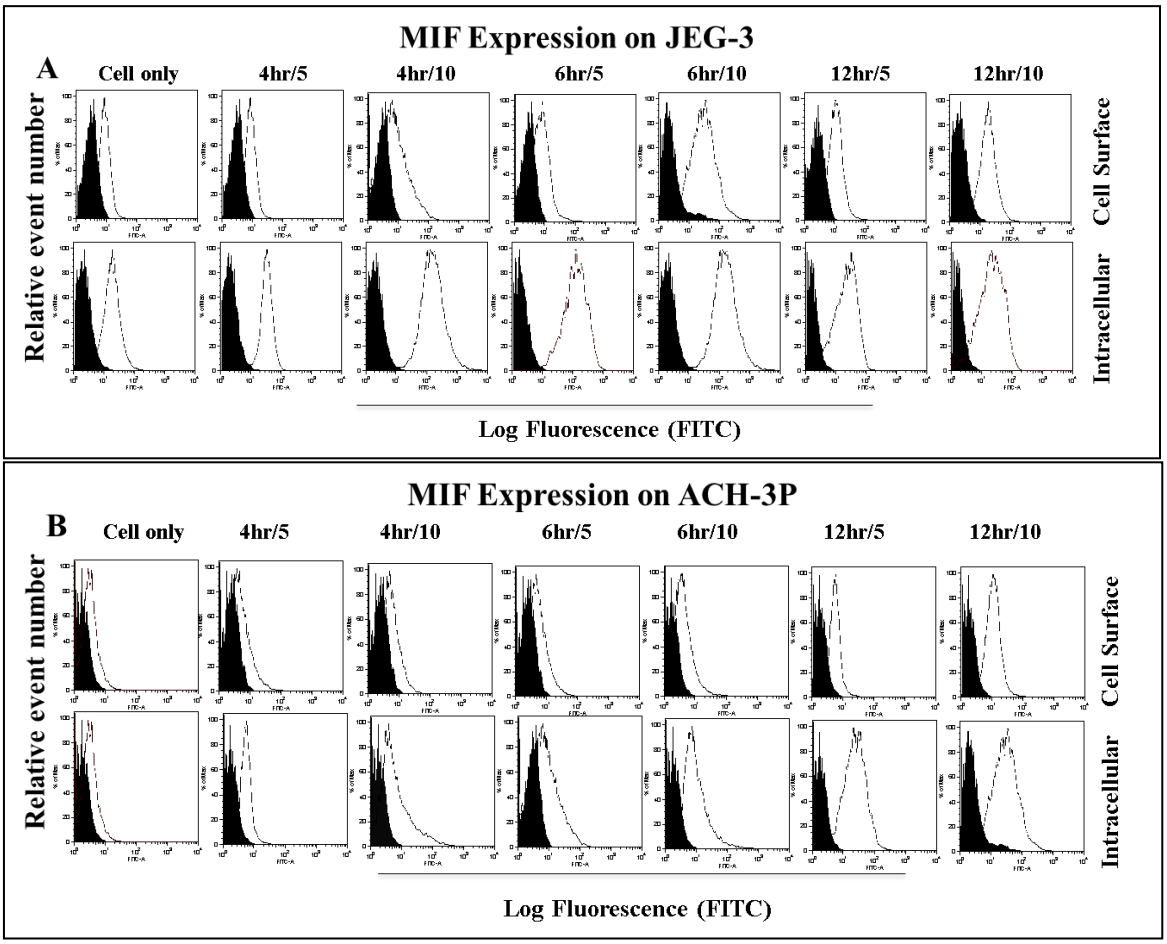

Figure 5-2: Expression of MIF in the trophoblast cell lines JEG-3 and ACH-3P after LPS treatment.

A: Flow cytometric detection of MIF expression in JEG-3 cells. Cells were stained with Sc-271631 (D-2) anti-MIF followed by 1 µl RAM-FITC secondary antibody. The intracellular and cell surface expression of MIF is shown as empty histograms. Black filled histograms represent the isotype control for the JEG-3 cell line. B: Flow cytometry analysis of ACH-3P cells. Cells were stained with Sc-271631 (D-2) anti-MIF followed by 1 µl RAM-FITC secondary antibody. Intracellular and cell surface expression of MIF is shown as empty histograms. Black-filled histograms represent the isotype control for the ACH-3P cell line.

5.2.3 Detection of CD44 expression by flow cytometry

The effect of LPS on CD44 expression in JEG-3 andACH-3P cells was studied by flow cytometry. Untreated and LPS-treated JEG-3 and ACH-3P were stained with 156-3c11 mouse mAb (anti-CD44), and labelled with 1 µl mouse anti- mouse IgG conjugated with FITC secondary antibody. LPS stimulation appeared to influence intracellular and surface expression of CD44 in JEG-3 and ACH-3P cell lines after 4, 6 and 12hr stimulation with 5 and 10µg/ml LPS (Figure 5-3).

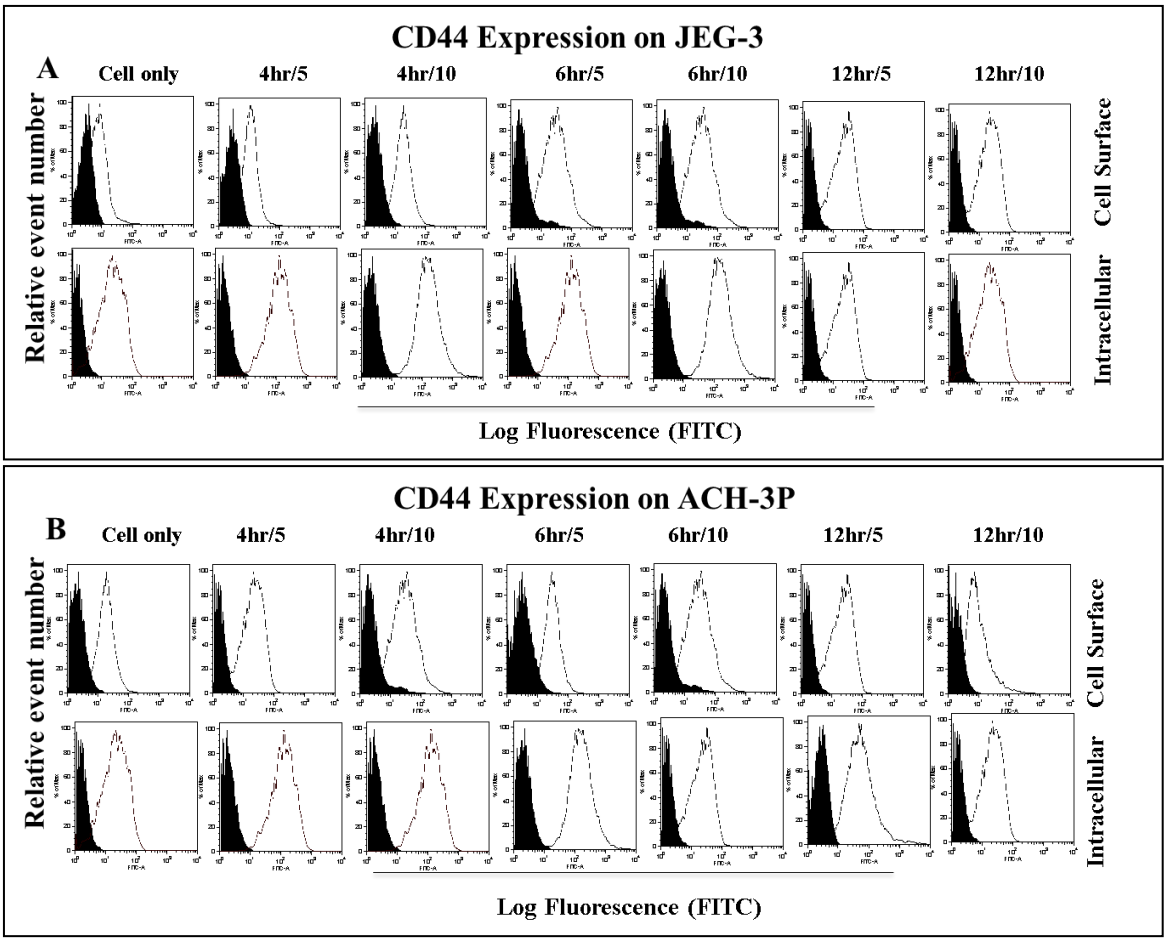

Figure 5-3: Expression of CD44 in JEG-3 and ACH-3P cells exposed to LPS.

A: Flow cytometry analysis of CD44 expression in JEG-3 cells. Cells were stained with 156-3c11 mouse mAb anti-CD44, visualized by reaction with fluorescently-labelled secondary antibody (1 µl RAM-FITC). The intracellular and cell surface expression of CD44 is shown as empty histograms. Black-filled histograms represent the isotype control for the JEG-3 cell line. B: Flow cytometry analysis of CD44 expression in ACH-3P. Cells were stained with the 156-3c11 mouse mAb anti-CD44 and labelled with 1 µl RAM-FITC. The intracellular and cell surface expression of CD44 is shown as empty histograms. Black filled histograms represent the isotype control for the ACH-3P cell line.

5.2.4 Detection of MIF protein by immunoblotting

Western blotting was utilised to investigate the expression of MIF in JEG-3 and ACH-3P cells. Whole cell lysates from untreated and LPS-treated cells were fractionated by gel electrophoresis and the presence of MIF was detected by D-2 (anti-MIF) and TU-02 (anti-α- tubulin) loading control. Western blot analysis demonstrated a time and dose-dependent increase of MIF protein expression in both JEG-3 and ACH-3P cells in response to LPS. The results showed that the molecular weight of MIF and α-tubulin (loading control) were 12.5 kDa and 55 kDa, respectively, compared to the Jurkat cell line which was used as a positive control (Figure 5-4).

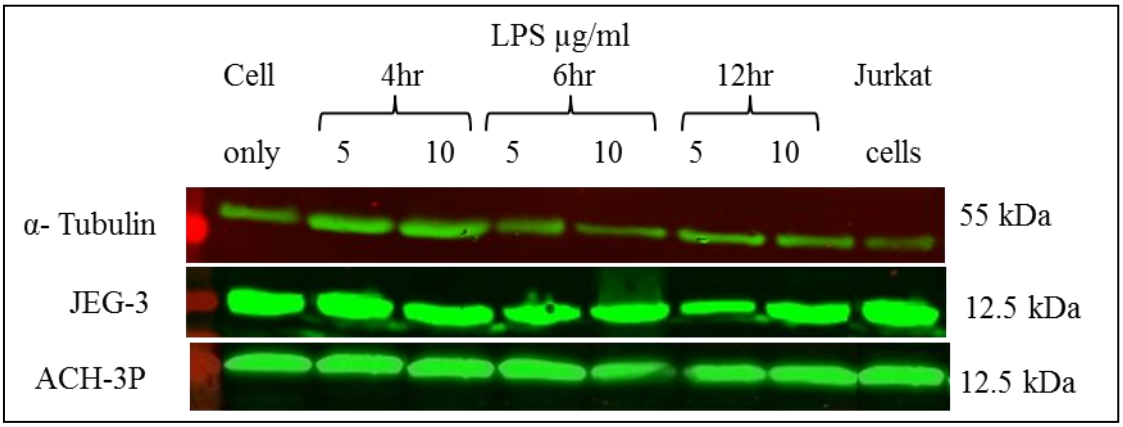

Figure 5-4: MIF expression analysis according to its molecular weight in LPS treated JEG-3 and ACH-3P cells.

Western blot analysis of MIF in JEG-3 and ACH-3P cells treated with LPS (5 µg/ml or 10 µg/ml) for 4, 6 and 12 hr and unstimulated cells. Jurkat cells were used positive controls. The molecular weight marker (Fermentas, #SM0671; 170-10 kDa) is on the left side. TU-02 mAb (anti-alpha-subunit tubulin) detects full length α-tubulin at a molecular weight of 55 kDa. D-2 (anti-MIF) is specific for an epitope mapping between amino acids 7-39 at the N-terminus of human MIF. In JEG-3 and ACH-3P cells, a band for MIF was detected at 12.5 kDa, compared to the positive control (Jurkat cells).

5.2.5 Detection of CD44 protein by immunoblotting

Cell lysates were prepared from untreated and LPS-treated JEG-3 and ACH-3P cells and separated on a 12% SDS-PAGE gel. Alpha-tubulin was detected by immunoblotting. Tubulin bands were observed at the expected molecular weight of 55kDa. Western blot analysis demonstrated a time and dose-dependent increase of CD44 protein expression in both JEG-3 and ACH-3P cells in response to LPS. Results showed that the molecular weight of CD44 was 80 kDa compared to the positive control cell line (HeLa), which expressed high levels of CD44 (Figure 5-5).

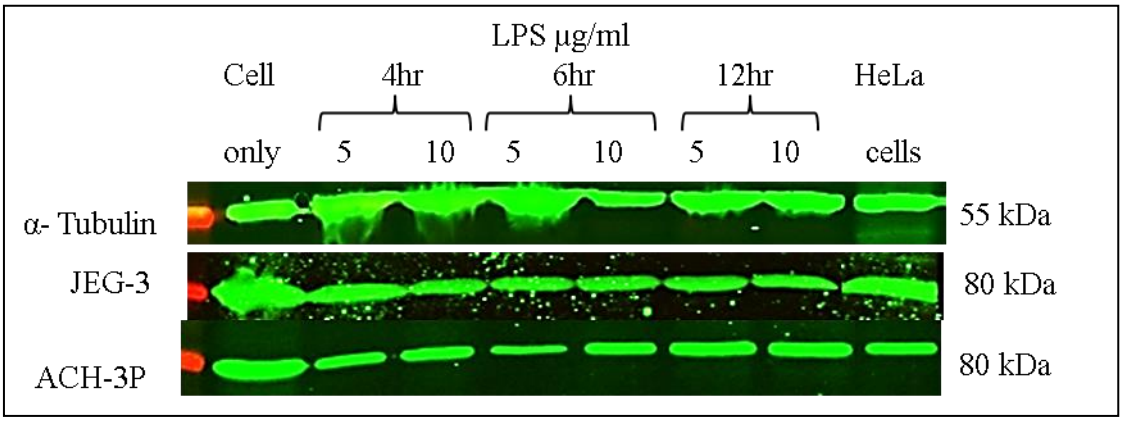

Figure 5-5: CD44 expression analysis according to its molecular weight in LPS treated JEG-3 and ACH-3P cells.

Western blot analysis of CD44 in JEG-3 and ACH-3P cells treated with LPS (5 µg/ml or 10 µg/ml) for 4, 6 and 12 hr, and unstimulated cells. HeLa cells were used as a positive control. The molecular weight marker (Fermentas, #SM0671; 170-10 kDa) is on the left side. TU-02 mAb (anti- alpha- subunit tubulin) which detects full length α-tubulin at a molecular weight of 55 kDa. 156-3c11 (anti-CD44) is a mouse mAb which detects endogenous levels of total CD44 protein, and is specific for most isoforms (80-90 kDa). In JEG-3 and ACH-3P cells a band for CD44, was detected at 80 kDa, compared to the positive control (HeLa) cells.

5.2.6 Histological immune staining of MIF and CD44 in human placenta tissue

Validation of the human placenta experiments was performed using normal placenta tissue slides (39th week of gestation). MIF or CD44 protein expression in human placenta tissue was studied by microscopy using D-2 (anti-MIF) or 156-3c11 (anti-CD44) respectively (Figure 5-6).

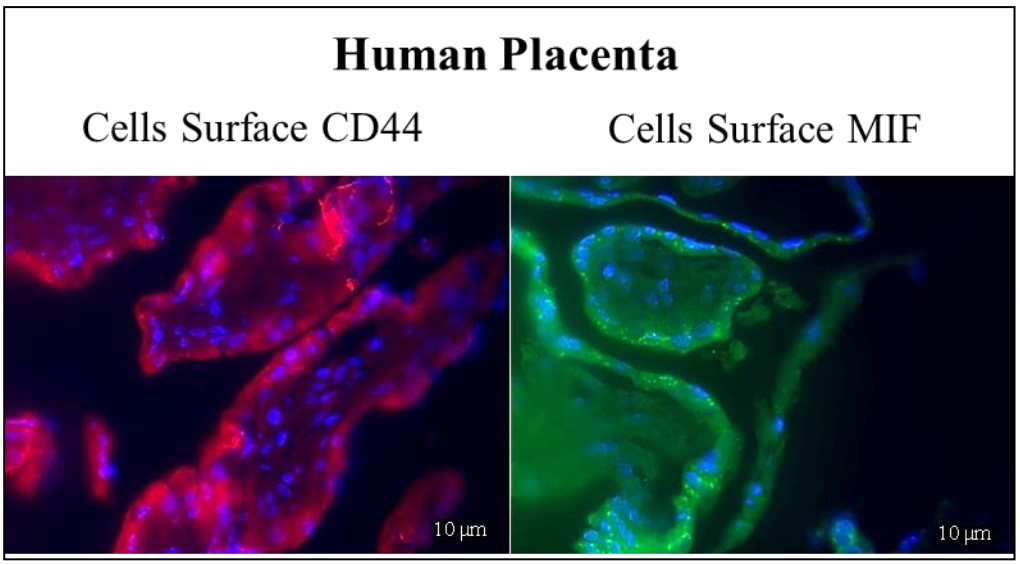

Figure 5-6: Histological immune staining of CD44 and MIF in human placenta tissue.

Positive immune staining of CD44 using 156-3c11 (anti-CD44) was detected. Positive staining of MIF was also detected, using D-2 (anti-MIF) primary antibody. The secondary antibody was anti-mouse IgG conjugated with FITC Alexa Fluor 488 (green) or Alexa Fluor 555 (red), which was visualized at cell surface level. Scale bar 10 µm.

5.3 Conclusions

The present study concludes that MIF and CD44 are upregulated after LPS exposure. Bacterial infection inhibits trophoblast cell proliferation. This indicates that MIF plays an essential role in cell proliferation, whereas both MIF and CD44 play an important role in human pregnancy maintenance at the fetal-maternal interface.

Chapter 6 Evaluation of the relationship between CD74, MIF and CD44 in human trophoblast-derived cell lines

6.1 Introduction

Several studies have shown that MIF can bind to the extracellular domain of CD74 to promote signalling pathways implicated in inflammatory processes, cell proliferation, PGE_2 production, chemokine-mediated signalling and apoptosis (Leng et al., 2003, Shi et al., 2006, Starlets et al., 2006). However, the short cytoplasmic tail of CD74 lacks a signal-transducing intracellular domain, although phosphorylation of the serine residues takes place on the p35 variant of CD74 (Borghese and Clanchy, 2011). It has been demonstrated that the complex of CD74 and MIF binds to CD44, and that this binding is required to activate intracellular signalling pathways (Shi et al., 2006). This binding is required for phosphorylation of the ERK1/2- MAPK kinases signalling pathway by MIF (Shi et al., 2006, Meyer-Siegler et al., 2004). The activation of CD74 and CD44 s also required for MIF-mediated cell proliferation by initiating a signalling cascade activation of NF-κB via Src-tyrosine kinase (Shi et al., 2006). Therefore, CD74 that is modified by the addition of chondroitin sulfate interacts with CD44, a polymorphic transmembrane protein with kinase activating properties, forming a receptor complex (Naujokas et al., 1993, Borghese and Clanchy, 2011). This results in phosphorylation of ERK1/2 and subsequently affects cell proliferation (Bach et al., 2009).

In the present study, it was hypothesized that the lack of human trophoblast-specific isoforms of CD74 at the fetal-maternal interface may protect human pregnancy. The potential colocalization between CD74, CD44 and MIF was investigated by confocal microscopy and the interactive partners of CD74 and CD44 were studied by co-immunoprecipitation of JEG-3 and ACH-3P cells. In order to validate the study, the expression of CD74, MIF and CD44 receptors in immortalized human breast cancer luminal cells (CAMA-1) and human monocyte cells (THP-1) was assessed.

6.2 Results

6.2.1 Interaction of CD74 and CD44

To identify which isoform of CD74 is involved in interaction between CD74 and CD44 in JEG-3 and ACH-3P cells, co-immunoprecipitation was utilized. Cell lysate was extracted from JEG-3 and ACH-3P cells cultured with 5 or 10 µg/ml LPS for 6 hr. The extracted lysate was incubated with anti-CD74 antibody and loaded on a gel. The probe was then incubated with anti-CD44 antibodies (Figure 6-1).

Pull down experiments were carried out to identify CD44 in JEG-3 and ACH-3P cells treated with 5 or 10 µg/ml LPS for 6 hr. The expression of CD44 in both cell lines was investigated by IP. To determine if anti-CD44 antibody (156-3c11) was suitable for IP, whole cell lysate from JEG-3 cells, or ACH-3P cells treated with 5 or 10 µg/ml LPS for a period of 6 hr, was incubated with 156-3c11 antibody or with protein A/G PLUS- agarose as control. This was done because the same antibody was used in both IP and Western blot analysis. Then, the blot was incubated with 156-3c11 antibody (Figure 6-2).

To study the interaction between CD44 and CD74 in JEG-3 and ACH-3P cells, co-immunoprecipitation was utilized. Cell lysate was extracted from JEG-3 and ACH-3P cells cultured with 5 or 10 µg/ml LPS for 6 hr. The extracted lysate was incubated with anti-CD44 antibody and loaded on a gel. The probe was then incubated with anti-CD74 antibodies (Figure 6-3).

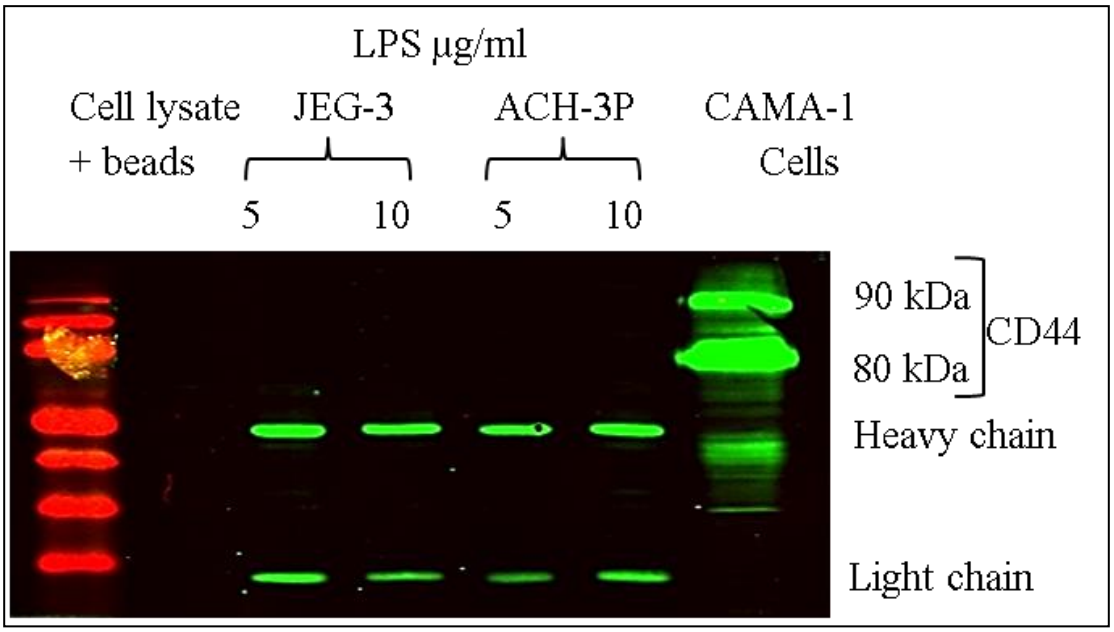

Figure 6-1: Co-immunoprecipitation results indicating no interaction between CD74 and CD44 in JEG-3 and ACH-3P cells.

Co-immunoprecipitation of CD74/CD44 from JEG-3 and ACH-3P cells using antibody (Ab) to CD44 protein. The samples were loaded onto lanes as follows: JEG-3 cell lysate and beads only; JEG-3 cells treated with 5 µg/ml LPS for 6 hr; JEG-3 cells treated with 10 µg/ml LPS for 6 hr; ACH-3P cells treated with 5 µg/ml LPS for 6 hr; ACH-3P cells treated with 10 µg/ml LPS for 6 hr; CAMA-1 cells as the positive control. The blot was probed with mouse anti-CD44 Ab. The Ab heavy and light chain bands are indicated. Treated JEG-3 and ACH-3P cell lysate was incubated with anti-CD74 Ab so that Ab heavy and light chain fragments can be observed at approximately 55 and 25 kDa respectively. The absence of the bands that corresponding to the molecular weight of CD44 confirmed that there was no interaction between CD74 and CD44.

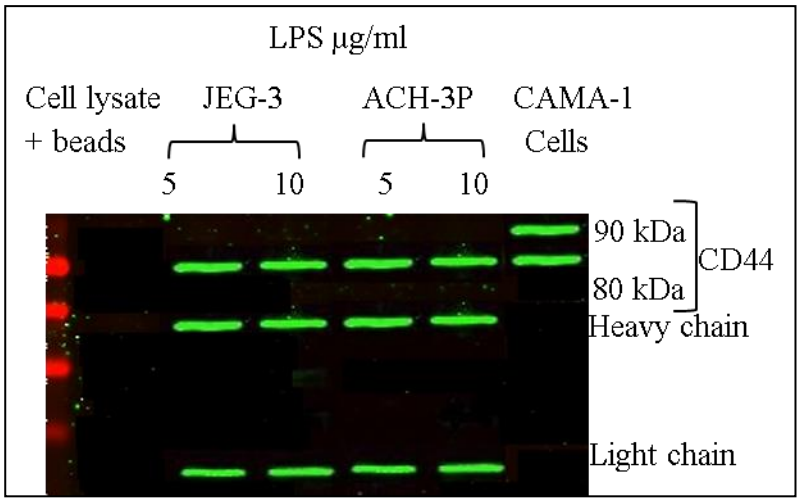

Figure 6-2: Immunoprecipitation analysis, detecting CD44 in JEG-3 and ACH3P cell lines.

Immunoprecipitates were subjected to pull down of CD44. The samples were loaded onto lanes as follows; JEG-3 cell lysate and beads only; JEG-3 cells treated with 5 µg/ml LPS for 6 hr; JEG-3 cells treated with 10 µg/ml LPS for 6 hr; ACH-3P cells treated with 5 µg/ml LPS for 6 hr; ACH-3P cells treated with 10 µg/ml LPS for 6 hr; CAMA-1 cells as the positive control.

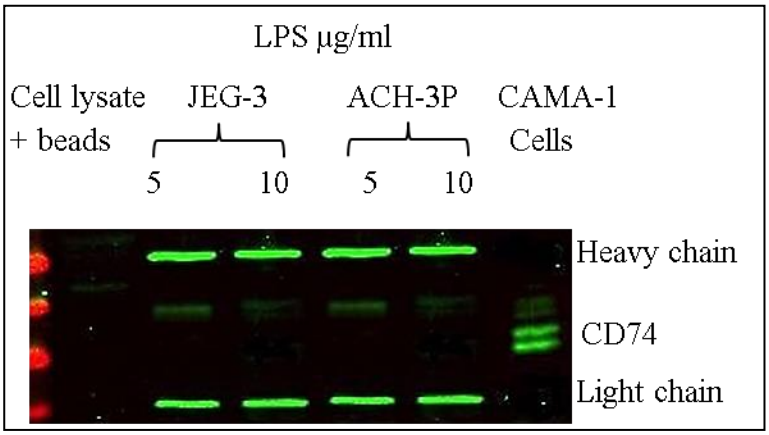

Figure 6-3: Co-immunoprecipitation results for the interaction of CD44 and CD74 in JEG-3 and ACH-3P cell lines.

Co-immunoprecipitation of CD44/CD74 from JEG-3 and ACH-3P human trophoblast cells using antibody to CD44 protein. The samples were loaded onto lane as follows: JEG-3 cell lysate and beads only; JEG-3 cells treated with 5 µg/ml LPS for 6 hr; JEG-3 cells treated with 10 µg/ml LPS for 6 hr; ACH-3P cells treated with 5 µg/ml LPS for 6 hr; ACH-3P cells treated with 10 µg/ml LPS for 6 hr; CAMA-1 cells as the positive control. The blot was probed with mouse anti-CD74 antibody (Ab). The Ab heavy and light chain bands are indicated. Treated JEG-3 and ACH-3P cell lysate was incubated with anti-CD44 Ab so that Ab heavy and light chain fragments can be observed at approximately 55 and 25 kDa respectively. The absence of bands that corresponded to the molecular weight of CD44 confirmed that there was no interaction between CD74 and CD44.

6.2.2 Co-localization of CD74, CD44 and MIF

To investigate whether CD74, CD44 and MIF are co-localized in JEG-3 and ACH-3P cells, both cell lines were immune stained with an appropriate primary antibody, followed by a secondary antibody. Laser scanning confocal microscopy using different wavelengths was used to visualise the intracellular and the cell surface expression of CD74, MIF and CD44 molecules. CD74 was labelled with either Alexa fluor 488 (green) or Alexa fluor 555 (red), MIF was labelled with Alexa fluor 488 (green) and CD44 was labelled with Alexa fluor 555 (red). Merging green and red channels assesses the co-localization and the Pearson's correlation coefficient (PCC) used to analyse the percentage of co-localization. The scale lay between -1 and 1, 1 standing for co-localisation, -1 standing for negative colocalisation and zero standing for no colocalisation (Figure 6-4, Figure 6-5, Figure 6-6 and Figure 6-7).

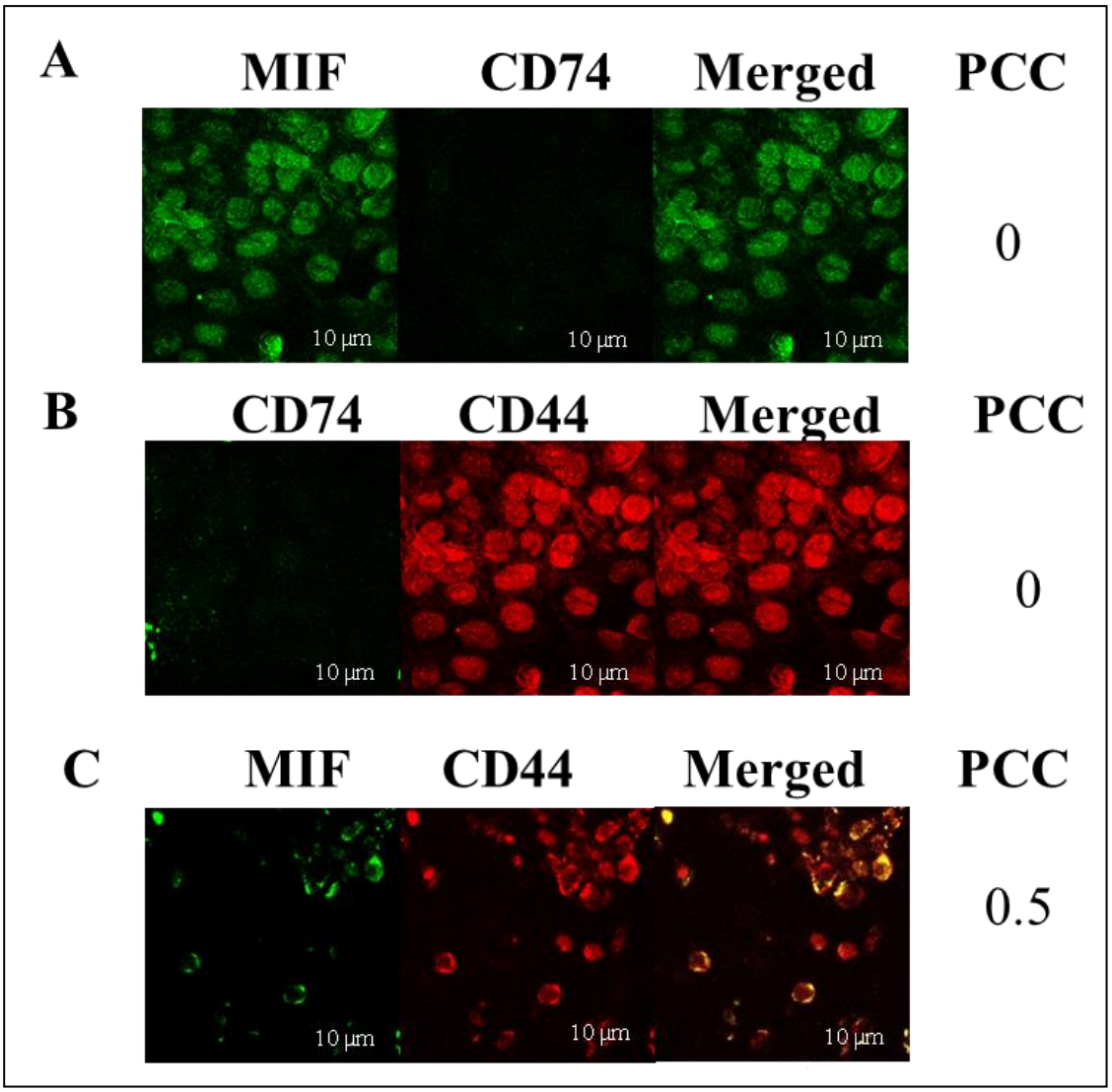

Figure 6-4: Colocalisation of the cell surface CD74, CD44 and MIF proteins in JEG-3 untreated cells.

JEG-3 cells were cultured in LabTek 8-well chambers at a density of 8 x 10^3 cells per well overnight. JEG-3 cells were stained with MIF primary antibody and labelled with FITC Alexa fluor 488 (green). CD74 was stained with either Alexa fluor 488 (green) or TRITC Alexa fluor 555 (red). JEG-3 cells were stained with CD44 primary antibody and labelled with TRITC Alexa fluor 555 (red). Area of co-localisation detected by merging images is shown in yellow. Pearson's correlation coefficient (PCC) was used to analyse the percent of co-localisation and value is shown in the right. Data represents three different experiments. Scale bar 10 µm.

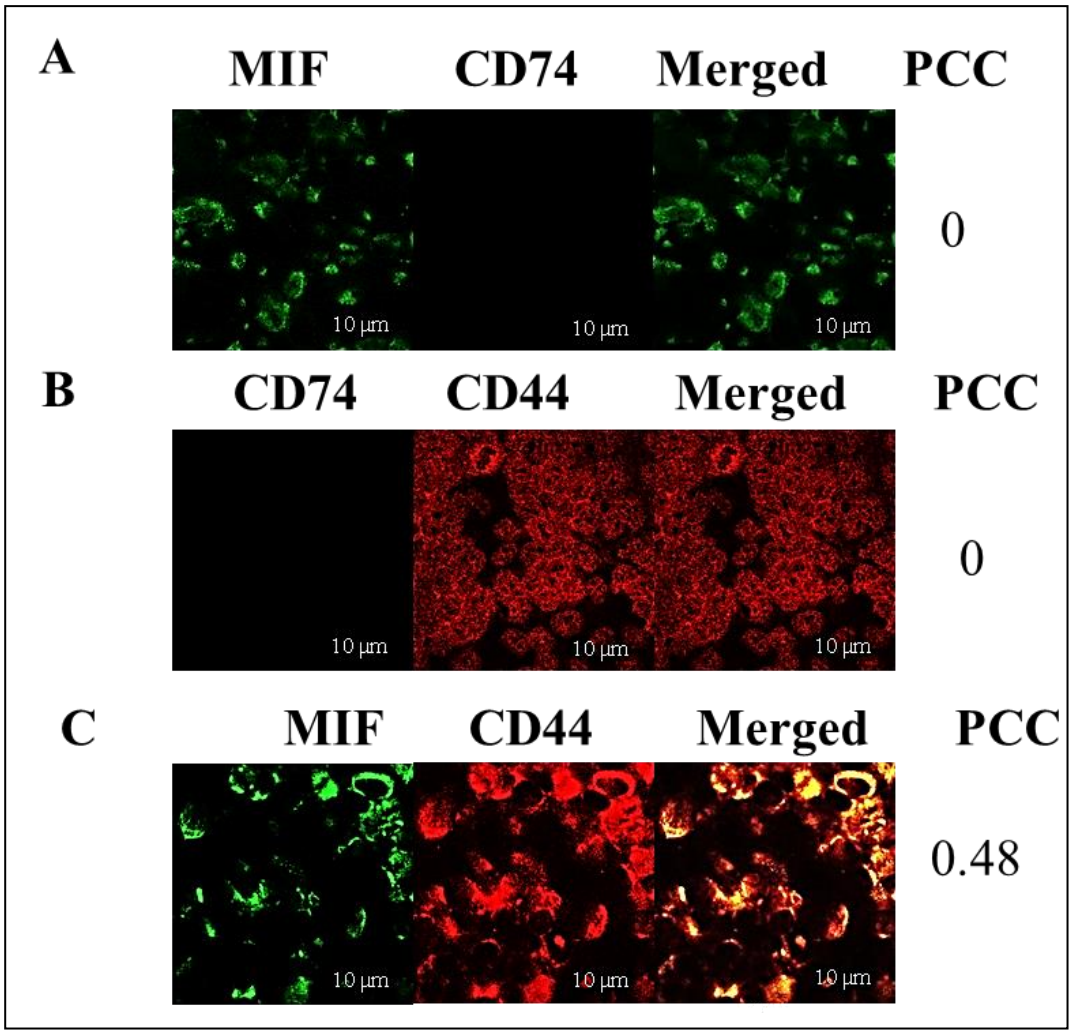

Figure 6-5: Colocalisation of the cell surface CD74, CD44 and MIF proteins in ACH-3P untreated cells.

ACH-3P cells were cultured in LabTek 8-well chambers at a density of 8 x 10^3 cells per well overnight. ACH-3P cells were stained with MIF primary antibody and labelled with FITC Alexa fluor 488 (green). CD74 was stained with either Alexa fluor 488 (green) or TRITC Alexa fluor 555 (red). ACH-3P cells were stained with CD44 primary antibody and labelled with TRITC Alexa fluor 555 (red). The area of co-localization detected by merging images is shown in yellow. Pearson's correlation coefficient (PCC) was used to analyse the percent of co-localization and value is shown in the right. Data represents three different experiments. Scale bar 10 µm.

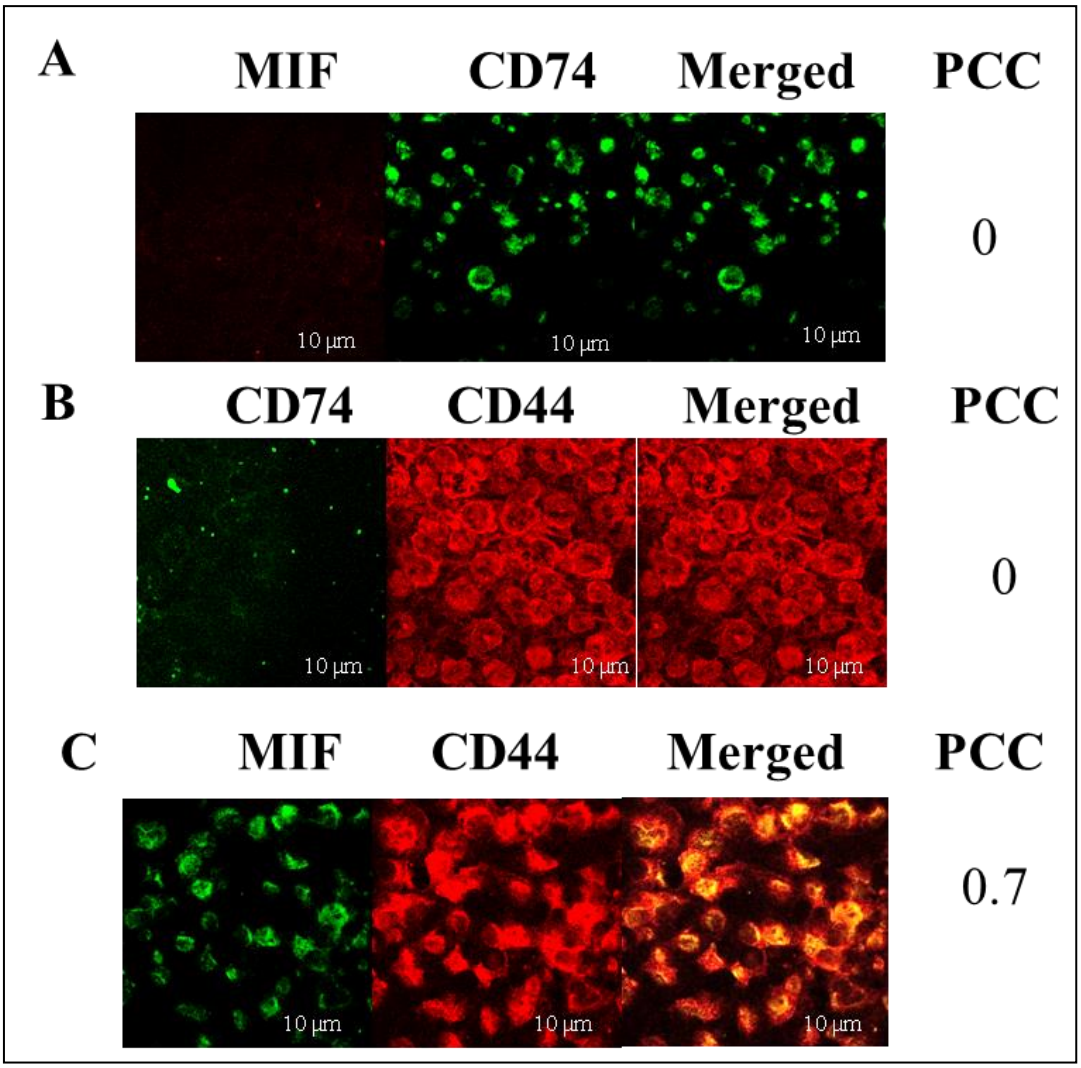

Figure 6-6: Colocalisation of cell surface CD74, CD44 and MIF proteins in JEG-3 cells treated with 10µg/ml LPS for 6 h.

JEG-3 cells were cultured in LabTek 8-well chambers at a density of 8 x 10^3 cells per well. JEG-3 cells were treated with LPS (10µg/ml) for 6 hrs. Treated JEG-3 cells were stained with MIF primary antibody and labelled with FITC Alexa fluor 488 (green). CD74 was stained with either Alexa fluor 488 (green) or TRITC Alexa fluor 555 (red). Treated JEG-3 cells were stained with CD44 primary antibody and labelled with TRITC Alexa fluor 555 (red). The area of co-localisation detected by merging images is shown in yellow. Pearson's correlation coefficient (PCC) was used to analyse the percentage of co-localisation and value is shown in the right. Data represents three different experiments Scale bar 10 µm.

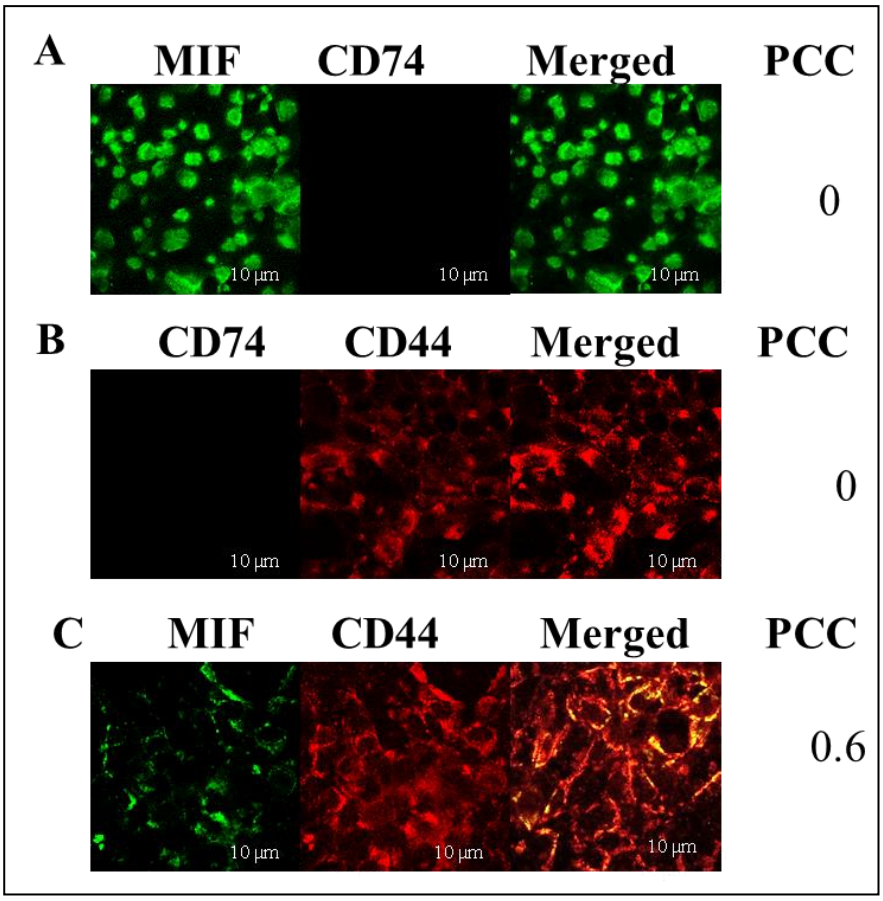

Figure 6-7: Colocalization of cell surface CD74, CD44 and MIF proteins in ACH-3P cells treated with 10µg/ml LPS for 6 h.

ACH-3P cells were cultured in LabTek 8-well chambers at a density of 8 x 10^3 cells per well. ACH-3P cells were treated with LPS (10µg/ml) for 6 hrs. Treated ACH-3P cells were stained with MIF primary antibody and labelled with FITC Alexa fluor 488 (green). CD74 was stained with either Alexa fluor 488 (green) or TRITC Alexa fluor 555 (red). Treated ACH-3P cells were stained with CD44 primary antibody and labelled with TRITC Alexa fluor 555 (red). The area of co-localisation detected by merging images is shown in yellow. Pearson's correlation coefficient (PCC) was used to analyse the percent of co-localisation and the value is shown on the right. Data represents three different experiments. Scale bar 10 µm.

6.2.3 Validation Study

In order to validate the study, the expression of CD74, MIF and CD44 receptors was studied in immortalized human breast cancer luminal cells (CAMA-1) (Figure 6-9) and human monocyte cells (THP-1) (Figure 6-8). Confocal laser scanning microscopy was utilized to study the intracellular and the cell surface expression of CD74, MIF and CD44 in THP-1 and CAMA-1 cells. CD74 was labelled with either Alexa fluor 488 (green) or Alexa fluor 555 (red), MIF was labelled with Alexa fluor 488 and CD44 was labelled with Alexa fluor 555. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (blue) (Figure 6-8 and Figure 6-9). CD74-CD44 interaction in cell lysates, as demonstrated by co-immunoprecipitation, showed that CD74 interacted with CD44 in breast cancer cell lines CAMA-1, MDA-MB-231 and MDA-MB-435 (Figure 6-10).

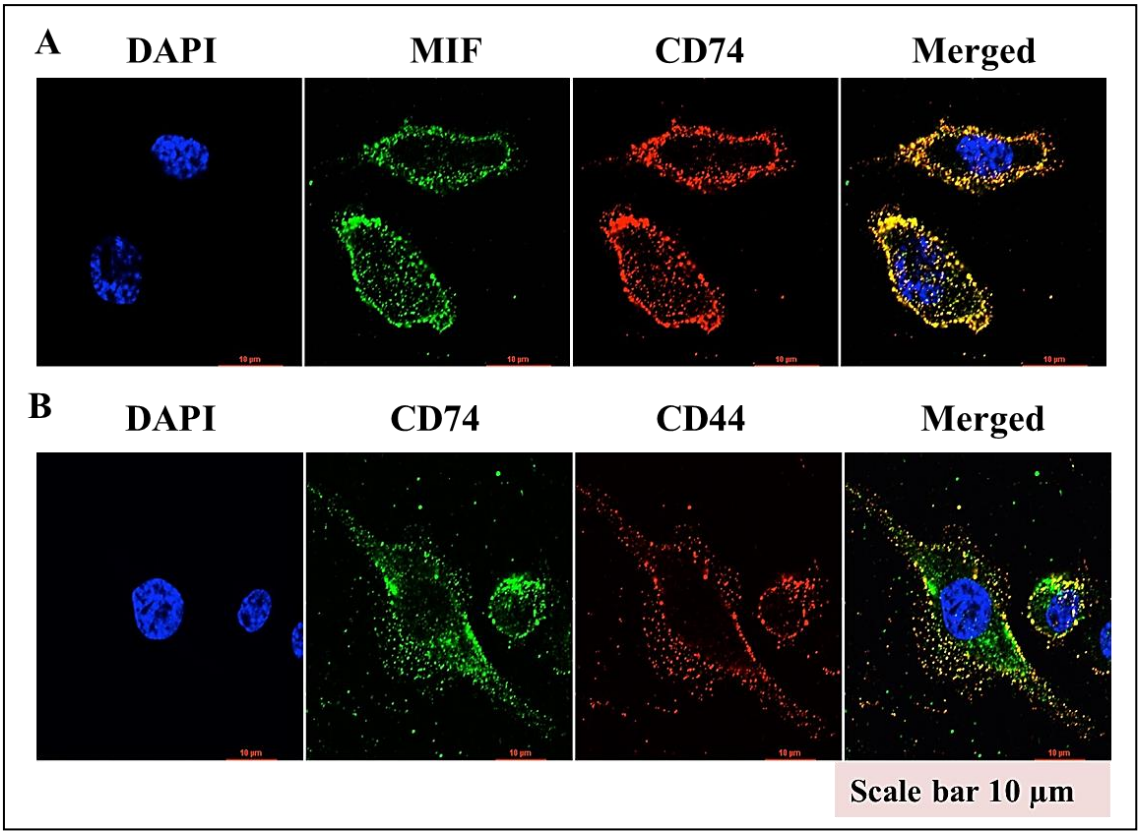

Figure 6-8: Colocalisation of CD74, CD44 and MIF proteins in THP-1 cells.

(A) Cell surface staining of MIF and CD74. (B) Intracellular staining of CD74 and CD44 in THP-1 cell lines, as visualized by confocal laser scanning microscopy. THP-1 cells were stained with CD74 and labelled with Alexa fluor 488 (green) or with Alexa fluor 555 (red). MIF was labelled with Alexa fluor 488 (green) and CD44 was labelled with Alexa fluor 555 (red). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) which is shown in blue. Scale bar 10 µm.

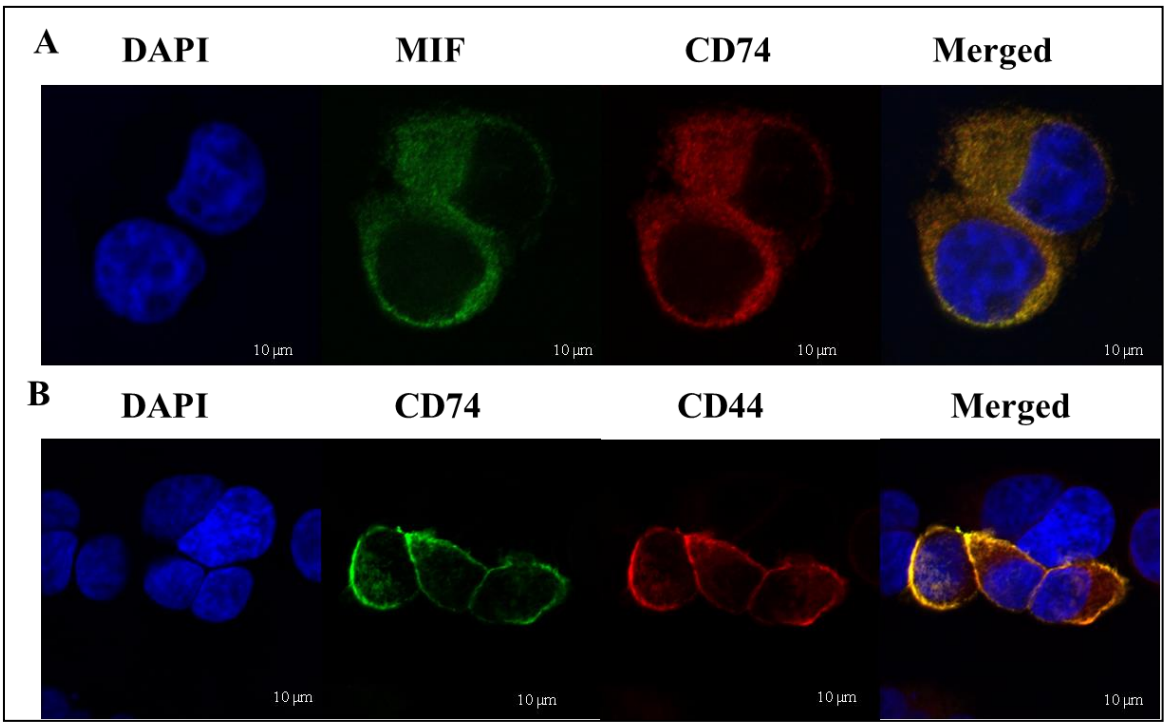

Figure 6-9: Colocalisation of CD74, CD44 and MIF proteins in CAMA-1 cells.

(A) Cell surface staining of MIF and CD74. (B) Intracellular staining of CD74 and CD44 in CAMA-1 cells, as visualized by confocal laser scanning microscopy. CAMA-1 cells were stained with CD74 labelled with Alexa fluor 488 (green) or with Alexa fluor 555 (red). MIF was labelled with Alexa fluor 488 (green) and CD44 was labelled with Alexa fluor 555 (red). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) which is shown in blue. Scale bar 10 µm.

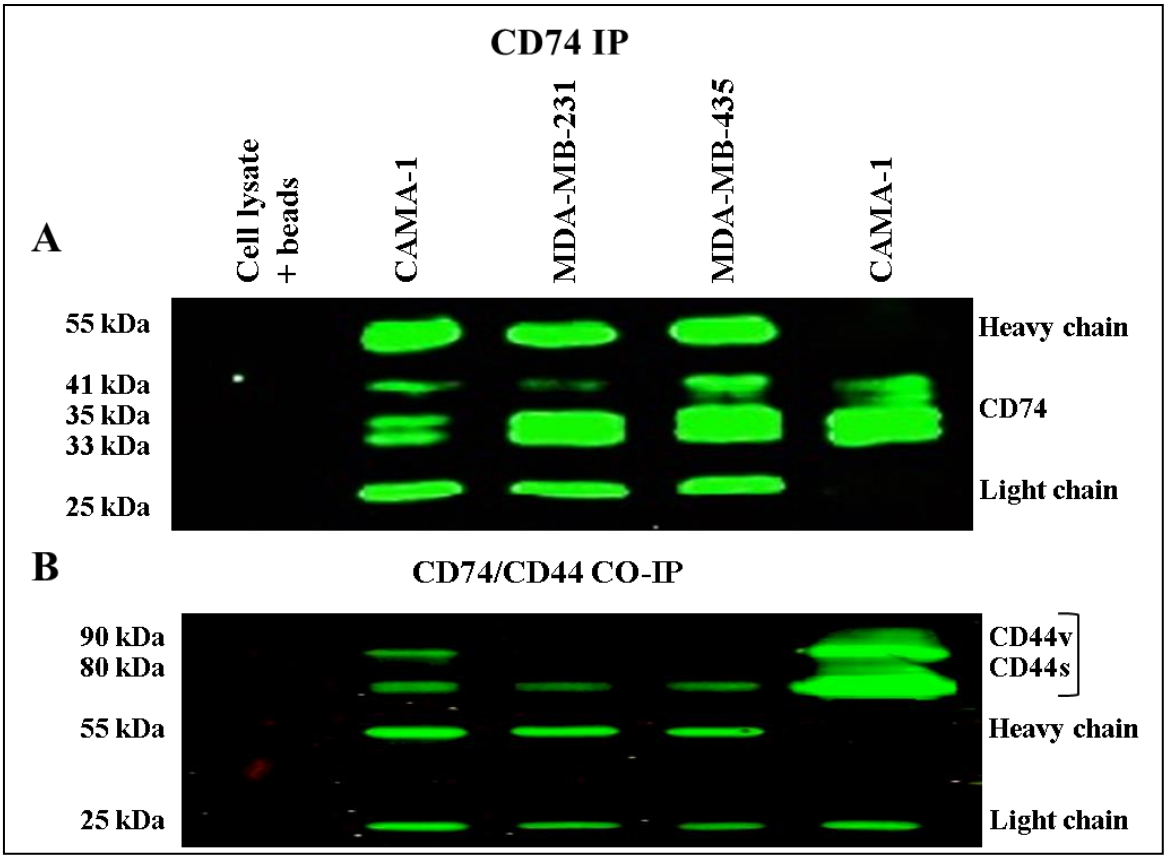

Figure 6-10: Co-immunoprecipitation to study the interaction of CD74 and CD44 in CAMA-1, MDA-MB-231, MDA-MB-435 and THP-1 cells.

(A) Immunoprecipitation was subjected to pull down CD74. The blots were probed with mouse anti-CD74 antibody (Ab). The Ab heavy and light chain bands are indicated so that Ab heavy and light chain fragments can be observed at approximately 55 and 25 kDa, respectively. (B) Co-immunoprecipitation (CO-IP) was applied to study the interaction between CD74/CD44. The CO-IP of CD74 /CD44 confirmed that CD44 interacted with CD74.

6.3 Conclusions

The survival of the human fetus requires diminished immunity at the fetal-maternal interface. This study indicates that the absence of cell surface expression specific isoforms of CD74 may provide a protective role in human pregnancy by minimising inflammatory processes and thus maximising a healthy pregnancy. In turn, it is reasonable to assume that overexpression of CD74 in early human pregnancy may be related to gestational complications.

Chapter 7 Discussion

7.1 General discussion

This study mainly involved the analysis and profiling of CD74, MIF and CD44; namely their expression in human placenta and two human trophoblast-derived cell lines, JEG-3 and ACH-3P. JEG-3 cells are invasive choriocarcinoma cells that serve as a model for *in vitro* studies of the trophoblasts, because they share many biological and biochemical properties with syncytiotrophoblast and cytotrophoblast of human placenta (Matsuo and Strauss, 1994). ACH-3P is a hybrid of primary trophoblasts and choriocarcinoma cells, containing two trophoblast subpopulations, villous cytotrophoblasts (VCT) and invasive extravillous trophoblasts (Hiden et al., 2007). It was established by the fusion of primary human first trimester trophoblasts with the AC1-1 choriocarcinoma cells. the growth curve of JEG-3 and ACH-3P cells (data not shown) indicated that the lag phase was approximately 24 hrs in length. This was followed by a log phase of growth between 24 and 96 hrs duration. The cells required between 96 and 120 hrs to reach confluence. This doubling time for cell culture coincided with that recommended of 48-72 hrs. Real time RT-PCR experiments afforded no detectable expression of CD74 in JEG-3 and ACH-3P cells, compared to THP1 cells that express high levels of CD74 (Burton et al., 2004). This result was confirmed by flow cytometry analysis and Western blot results, by comparison to the Raji cell positive control (Burton et al., 2004).

CD74 is involved in antigen presentation in association with HLA-DR; the molecule present antigenic peptides to regulatory T cells. CD74 is also responsible for stabilizing the HLA-DR molecule (Villadangos, 2001). In the present study, data obtained from flow cytometry showed negative expression of HLA-DR in both JEG-3 and ACH-3P cells, which is in agreement with the findings of previous reports (Murphy and Tomasi, 1998)

(Figure 3-5). Respectively, JEG-3 and JAR cells do not express cell-surface HLA-DR (Ranella et al., 2005; Apps et al., 2008b)

The absence of HLA-DR gene expression in the above cell lines is likely due to epigenetic silencing. Trophoblast cells lack typical MHC class II products (Murphy and Tomasi, 1998, Sutton et al., 1983, Starkey, 1987). This is due to lack of MHC class II transactivator (CIITA) expression, which is essential for the transcriptional regulation of class II (Murphy et al., 2004). This results in the absence of MHC II antigen expression. The lack of MHC class II (HLA-DR) in trophoblast cells, which is evident throughout gestation, supports a mechanism that protects the fetus from maternal immune attack and possible rejection (Athanassakis-Vassiliadis et al., 1990, Vassiliadis et al., 1994).

Trophoblast cells have been reported not to express class II MHC molecules and cannot be induced to do so by IFN-γ or TNF-α (Chatterjee-Hasrouni and Lala, 1981, Hunt et al., 1990a, Hunt et al., 1990b, Lata et al., 1992, Peyman and Hammond, 1992, Peyman et al., 1992).

The expression of MHC class II relies on the expression of CIITA, which is considered to be the "master regulator" of MHC class II gene transcription (Mach et al., 1996, Harton and Ting, 2000). In this respect, it has been confirmed that the CIITA gene is induced by IFN-γ and therefore MHC class II in fibroblasts, epithelial and endothelial cells (Steimle et al., 1994, Chang et al., 1994, Chin et al., 1994). Moreover, the inability of trophoblast cells to express MHC class II genes, even in the presence of IFN-γ, is due to a lack of CIITA gene expression (van den Elsen et al., 2000, Murphy and Tomasi, 1998, Morris et al., 1998). In addition, the CIITA gene contains an IFN-γ responsive promoter (PIV), which in trophoblasts cells is hypermethylated, which leads to the prevention of CIITA expression and consequently inhibits MHC class II expression (Martin et al., 1997).

Moreover, (Murphy and Tomasi, 1998) reported that the lack of CIITA gene expression in trophoblasts treated with IFN-γ is not caused by a defect in the IFN-γ receptor or the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, as the expression of IFN-γ inducible gene encoding the guanylate-binding protein was detected. The absence of the expression of CIITA gene results in the absence of MHC II antigen expression which provides a potential mechanism by which the fetus is protected from the maternal immune system during human pregnancy. (Holtz et al., 2003) revealed that two trophoblast cells lines (JAR and JEG-3) transfected with CIITA expression vectors were able to express MHC class II on the cell surface by activation of class II promoters. JEG-3 cells transfected with either CIITA or MHC class II expression vectors are able to present antigen to antigen-specific T cells, and to stimulate allogeneic T cell responses (van den Elsen et al., 2001, Arvola et al., 1999). Additionally, MHC class II antigens have been found to be expressed in trophoblast cells from the placentas of women suffering from chronic inflammation of unknown etiology and spontaneous recurrent miscarriages (Athanassakis et al., 1995). Therefore, the lack of CIITA expression and the deficiency in MHC class II gene expression is central to the absence of antigen presentation functions in the human trophoblast-derived JEG-3 and JAR cell lines (van den Elsen et al., 2000).

MIF is believed to serve an important role during pregnancy (Wada et al., 1997, Arcuri et al., 2001). MIF protein is expressed in first trimester trophoblast cells, suggesting that MIF might play a role in human implantation and in early embryonic development (Arcuri et al., 1999, Zeng et al., 1993). Calandra and Roger (2003) found that MIF plays a pivotal role in inflammatory and immune diseases including human pregnancy. So, this study investigates the role of LPS on the expression of MIF in human trophoblastic cells.

In the present study, MIF expression was observed in human placenta and in both JEG-3 and ACH-3P cells (Figure 3-4, Figure 3-6 and Figure 3-8). This is in accordance with a number of studies demonstrating that BeWo trophoblast cells release MIF in the first and third trimester (de Oliveira Gomes et al., 2011, Castro et al., 2013). MIF expression was increased in both treated JEG-3 and ACH-3P cells with LPS (Figure 5-2). This study has demonstrated that MIF is a key cytokine in human pregnancy and play a role in both inflammatory response and pathogen defence. The present study demonstrated the presence of CD44s in human placenta and JEG-3 and ACH-3P cells (Figure 3-3 and Figure 3-8). In human trophoblasts CD44 is necessary for embryonic development, and it may play a role in cell proliferation. CD44 is observed in the early human conceptus and in decidual stroma. Hyaluronan-enriched transfer medium significantly increased pregnancy and implantation rates in patients with multiple embryo transfer failures, suggesting that HA is essential for embryo implantation and pregnancy (Zhu et al., 2013).

There was no evidence for the expression of CD74 in human placenta or in breast lysate, relative to Raji control cells (Burton et al., 2004) (Figure 3-11). This result was expected because it has been confirmed that the expression of CD74 is restricted to antigen-presenting cells (Beswick and Reyes, 2009, Stein et al., 2007, Verjans et al., 2009).

Peters et al. (1990) reported a similar absence of expression of MHC class II in lung tissue of human fetuses less than 21 weeks of age, indicating a constitutive expression of MHC II and CD74 in epithelial cells. Furthermore, MacDonald et al. (1988) and Oliver et al. (1988) observed faint and inconsistent expression of MHC class II and CD74 in human fetuses at the tips of the intestinal villi at as early as 18 weeks of gestation (Badve et al., 2002). It has been suggested that prostaglandins, which were used to induce abortions, might have induced this expression (Rognum et al., 1992).

The immunolocalisation studies of CD74 in normal human placental cells (39th week of gestation) demonstrated weak expression of CD74 on the cell surface compared to the intracellularly (Figure 3-12). This might suggest that the presence of CD74 in human placenta tissue serves a critical role in human pregnancy protection by preventing antigen presentation in presence of MHC class II molecules. The absence of CD74 in JEG-3 and ACH-3P cells, which are MHC class II negative, and the presence of MIF and CD44 in these cells provide a suitable environment for the maternal immune system during early human pregnancy. As it has been confirmed that CD74 in association with HLA-DR present the anginas, it is believed that the absence of one these molecules would protect the maternal immune system.

Understanding the regulation of CD74 isoform expression in trophoblast cells is of interest, since trophoblasts lack MHC class II expression, which has been shown to play an important role in protection of the foetus from maternal immune attack (Athanassakis et al., 1995). This *in vitro* study investigated the effect of IFN-γ and LPS on the expression of CD74 in JEG-3 and ACH-3P cells. CD74 expression in a variety of cell types can be induced by several types of cytokines (Pessara et al., 1988, Möller and Moldenhauer, 1999). Maubach et al., (2007) showed that CD74 expression is upregulated when hepatic stellate cells (HSCs) are incubated with IFN-γ (Maubach et al., 2007). The expression of CD74 (mRNA and protein) also increases in vascular smooth muscle cells after IFN-γ treatment (Martín-Ventura et al., 2009). Similarly, (Moldenhauer et al., 1999) showed that the expression of CD74 and HLA-DR was induced when HT-29 cells (colon carcinoma cell lines) were incubated with IFN-γ. This is in agreement with previous findings describing CD74 upregulation by IFN-γ in other cell types (Pessara et al., 1988, Möller and Moldenhauer, 1999).

The expression of CD74 in the human trophoblast cell line JAR was found to increase intracellularly upon treatment with IFN-γ for 6 hr (Ranella et al., 2005). In contrast, HLA-DR was not detected in JAR cells, even after treatment with IFN-γ. Recently, Apps et al., (2008) reported a slight increase of CD74 mRNA in JEG-3 cells, after 24hr stimulation with 500 IU/ml of IFN-γ (Apps et al., 2008b). In the same study they found no evidence of expression of HLA-DR in either JEG-3 or JAR cells, even after stimulation with IFN-γ.

It has been reported that chronic inflammatory conditions and during *H. pylori* infection can increase cell-surface CD74 expression (Beswick et al., 2006). Concomitantly, (Zheng et al., 2012) reported that the CD74 expression increased significantly after exposure to LPS in the gastric cancer epithelial cell line MKN-45. Hence, the dramatic increase in CD74 expression due to Gram-negative infection suggests that LPS can utilize CD74 as a receptor in the same manner of *H. pylori* bacteria (Zheng et al., 2012). LPS an endotoxin that constitutes the major portion of the outermost membrane of Gram-negative bacteria (Poltorak et al., 1998, Qureshi et al., 1999), is a major cause of intrauterine infection.

In the present study, CD74 mRNA expression was not detected in un-stimulated JEG-3 and ACH-3P cells using RT-PCR (Figure 3-1). However, slight dose- and time-dependent expression of CD74 mRNA was observed after JEG-3 and ACH-3P cells were treated with 1000 IU/ml for 12 h IFN-γ or 5 µg/ml LPS treatment for 4 h (Figure 4-1). In the same manner to the results of Apps et al. (2008), who reported only moderate expression of CD74 mRNA in JEG-3 cells after 24hr stimulation with 500 IU/ml IFN-γ. It is interesting to note that higher CD74 levels arose after 24hr stimulation with 500 IU/ml IFN-γ and after 4 hr of 5 µg/ml LPS treatment (Figure 4-1).

The cell surface expression of CD74 molecules in JEG-3 and ACH-3P cells was studied using the mouse CD74-specific By2 mAb. Cell surface CD74 has not previously been observed in JEG-3 and ACH-3P cells, even after exposure to IFN-γ or LPS (Apps et al., 2008). This study has confirmed that JEG-3 and ACH-3P cells do not express CD74, as determined by flow cytometry (Figure 3-2). To investigate whether CD74 was intracellularly permeabilized, JEG-3 and ACH-3P cell lines were stained with By2. IFN-γ or LPS treatment induced intracellular CD74 expression, as compared to absence of CD74 surface expression in both cells lines (Figure 4-2, Figure 4-3). Significantly, both JEG-3 and ACH-3P cell lines showed a clear increase of CD74 expression after 24hr stimulation with 1000 IU/ml IFN-γ and after 6hr stimulation with LPS, at a concentration of 5μg/ml (Figure 4-2, Figure 4-3 and Figure 4-4). In this context, JAR cells have been reported to up regulate the expression of CD74 intracellularly in the presence of IFN-γ at a concentration of 1500 pg/ml for 24hr (Ranella et al., 2005).

In this study, no evidence of HLA-DR intracellular expression was found in the JEG-3 and ACH-3P cell lines (Figure 4-5). This was the case even after stimulation with IFN-γ, the most effective inducer of class II antigens. It was found that JEG-3 and ACH-3P cells failed to express high levels of HLA-DR, in agreement with Apps et al. (2008), who did not detect HLA-DR on JEG-3 cells. Further, Ranella et al. (2005) did not detect HLA-DR cell surface expression in JAR cells (Ranella et al., 2005). Thus, it appears that trophoblasts do not express HLA-DR molecules

In this study CD74 isoforms were found to be absent in the JEG-3 and ACH-3P cell lines (Figure 3-6). This was despite stimulation with IFN-γ (Figure 4-6 A). Contrastingly, the p35 CD74 isoform was detected in both cell lines after treatment with LPS (Figure 4-6 B). Interestingly, this isoform disappeared in both cell lines after LPS treatment for 12hr.

This event was also observed in total protein expression of CD74 when investigated with the IP method (Figure 4-7).

Taken together, the absence of HLA-DR expression and cell surface CD74 expression, and the presence of intracellular expression of CD74 (upon treatment by IFN-γ or LPS) in JEG-3 and ACH-3P cells points to a novel regulatory mechanism of CD74 expression. In the context of early human pregnancy, CD74 appears to function independently of class II MHC molecules. This suggests functional roles for the CD74 molecule other than those established for antigen presentation in the context of MHC class II antigens. Possibly CD74 may act as chaperone in the expression of molecules other than MHC class II antigens during early human pregnancy such as MIF.

JAR and JEG-3 cells express cell surface IFN-γ receptors (IFN-γR) (Peyman and Hammond, 1992, Paulesu et al., 1994, Hampson et al., 1993) IFN-γR binding by IFN-γ leads to activation of the receptor-associated kinases JAK-1 and JAK-2, which phosphorylate and activate Stat1, the intracellular domain of IFN-γR1 (Boehm et al., 1997, Stark et al., 1998, Laurence et al., 2012, Braunstein et al., 2003).

In human trophoblast cells, Stat1 and IRF-1, the regulatory factors of the CIITA gene, are unable to assemble at promoter IV (PIV). Furthermore, Stat1 and IRF-1 are present in trophoblasts and can activate a reporter gene in an expression assay. The reason that Stat1 and IRF-1 could not be assembled was determined to be the result of methylation of PIV (Morris et al., 2000).

Because CIITA expression is completely silenced in human trophoblast cells (above), it acts as a switch for the up-regulation of class II MHC-related genes by IFN-γ (Boss, 1997) and activated (phosphorylated) Stat1 is present only very transiently. The regulatory factors are unlikely factors that might result in up-regulating CD74 expression in human trophoblasts. Human trophoblast cells have been reported to be one of the only normal cell types that repress IFN-γ signalling (Murphy et al., 2009). Taken together,

these observations suggest that inhibition of the JAK/STAT signalling pathway in response to IFN-γ in human trophoblast cells contributes to successful early human pregnancy, by preventing responses such as apoptosis which could lead to gestational complications.

CD74 is classified as a cytokine receptor, and was recently shown to act as a cell surface receptor for MIF (Gore et al., 2008, Leng et al., 2003). This suggests that its expression might be induced directly after cytokine treatment (Beswick and Reyes, 2009). For example, it has been found that MIF induces the expression of CD74 in B cell chronic lymphocytic leukemia via activation of NF-kB, resulting in regulation of IL-8 (Binsky et al., 2007). Therefore, in the same manner as MIF, IFN-γ might directly induce CD74 expression in human trophoblast cells, as recently shown for IL-8 (Binsky et al., 2007) .

The expression of CD74 upon IFN-γ treatment might be increased indirectly via cathepsin S up-regulation (Maubach et al., 2007). In this respect, there are many different proteases involved in the processing of CD74 during antigen presentation. The most effective proteases involved in the last step of this process are cathepsins S and L. These enzymes release CLIP from leupeptin induced polypeptide, which participates, depending on the cell type, in the latter steps of degradation of CD74 (Nakagawa et al., 1998, Riese et al., 1998, Beers et al., 2005, Driessen et al., 1999). Maubach *et al*. (2007) showed that the increase in cathepsin S expression results in heightened CD74 expression when HSCs are incubated with IFN-γ for 30 hr (Maubach et al., 2007). However, Schönefuß et al (2010) showed that the expression of MHC class II and CD74 was down regulated after inhibition of cathepsin S with CATS-inhibitor in the human T-cell line HuT-78 and the human keratinocyte cell line HaCaT, even after IFN-γ treatment (Schönefuß et al., 2010). This indicates that the expression of CD74 might be upregulated through cathepsin S via IFN-γ treatment.

It has been reported that CD74 expression in gastric epithelial cells increases significantly under inflammatory conditions (Beswick et al., 2005b). Therefore, the dramatic increase in CD74 expression during infection suggests that *H. pylori* can use CD74 as a bacterial receptor (Zheng et al., 2012). Beswick and colleagues (2005) investigated the interaction of *H. pylori* urease with CD74, which is highly expressed by gastric epithelial cells. They suggested an important role for CD74 in gastric epithelial cell interaction with *H. pylori* that leads to activation of NF-kB and ERK1/2 as well as the predication of IL-8 (Beswick et al., 2005a, Beswick et al., 2005b). It was also shown that *H. pylori* can bind directly to CD74 in the absence of class II MHC in gastric epithelial cells. The binding of *H. pylori* to CD74 was confirmed by a series of independent approaches, notably immunoprecipitation, which revealed that *H. pylori* predominantly bind to the 33 kDa isoform of CD74. Also, blocking of CD74 with antibodies significantly reduced the binding of *H. pylori* to gastric epithelial cells. In addition, an increase in CD74 expression by gastric epithelial cells is accompanied by an increase in IL-8 production in response to *H. pylori*. This response declines when CD74 is blocked (Beswick and Reyes, 2009, Beswick et al., 2005a).

Other studies have shown that CD74 expression increases in the gastric cancer epithelial cell line MKN-45 under LPS stimulation; the highest expression was observed when cells were incubated with 1000 ng/ml LPS. It has been suggested that the expression of CD74 is increased via MIF induction (Zheng et al., 2012). Interestingly, it has been found that diabetic patients overexpress MIF and CD74. Similarly, high glucose also upregulated MIF and CD74 expression in cultured HK2 human proximal tubule cells (Sanchez-Niño et al., 2009). This suggests that the interaction between CD74 and LPS might take place in the polysaccharide core of LPS. In this respect, *H. pylori* is thought to bind directly to CD74 on the surface of cells via urease, a common bacterial protein involved in the

catalysis of urea to CO_2 and NH_3 (Beswick and Reyes, 2009, Borghese and Clanchy, 2011). Therefore, it is believed that LPS binds to CD74 via CO_2 and NH_2 chains.

In the present study, no evidence was found for the expression of CD74 in un-stimulated JEG-3 and ACH-3P cells. It was also shown that LPS induces only intracellular expression of CD74 in JEG-3 and ACH-3P cells. Further, Western blot analysis detected the 35 kDa isoform of CD74 (Figure 4-6 and Figure 4-7). In contrast to CAMA-1 cells, JEG-3 and ACH-3P cells stimulated with LPS expressed only the 35 kDa isoform of CD74. Accordingly, the P33 and P35 kDa isoforms of CD74 are known to regulate antigen presentation (Beswick and Reyes, 2009), which could explain the lack of the P35 kDa isoforms in JEG-3 and ACH-3P cells and its presence in CAMA-1 cells (Zheng et al., 2012).

In parallel to the activity of antigen presentation, CD74 has emerged as a MIF receptor in breast tumour cells (Zheng et al., 2012, Beswick and Reyes, 2009) and as a pathogen receptor, as is *H. pylori*, in gastric epithelial cells (Beswick and Reyes, 2009, Leng et al., 2003). It is likely that in trophoblasts the 43 kDa CD74 isoform could be involved in both mechanisms. Interestingly, the 35 kDa isoform appeared in JEG-3 and ACH-3P cells after LPS exposure, and disappeared after 12 hr LPS treatment. The interpretation of the presence of CD74 after LPS treatment is believed that the 35 kDa isoform triggers the cells involvement in antigen presentation as to be recognised as antigen. However, it is suggested that JEG-3 and ACH-3P cells are highly immunogenic, since they are highly stable even after exposure to IFN-γ and LPS (van Nieuwenhoven et al., 2003).

The intracellular expression of CD74 and the absence of its cell surface expression in JEG-3 and ACH-3P cells indicate a novel mechanism for CD74 expression in trophoblast cells. The absence of CD74 on the cell surface causes low MHC class II expression levels and floppy MHC class II molecules (Bikoff et al., 1993). In this context, the complete lack of MHC class II (HLA-DR) in human trophoblast cells might be the reason for

CD74 being expressed intracellulary. In addition, The CD74 intracellular domain is only 46 amino acids long and it lacks homology with tyrosine or serine/threonine kinases, or with the interaction domains for nonreceptor kinases or nucleotide binding proteins (Shi et al., 2006). So, it is believed that the presence of CD74 for short period especially after LPS treatment due to the resistance for the infection.

The purpose of this study was to determine the role of IFN-γ in the regulation of CD74 isoform expression in human trophoblast cells, and in fetal-maternal tolerance. The expression of CD74 in the absence of class II MHC suggests alternative functions for CD74 aside from antigen presentation. It is proposed that over expression of CD74 in early pregnancy can cause gestational complications. The present data suggest that a bacterial pathogens or a bacterial by-product such as LPS, has a stronger up-regulatory effect than IFN-γ. This is in contrast to the expression of most immunological receptors, which are strongly up-regulated by IFN-γ. It is hypothesised that the lack of cell surface expression of CD74 may provide a protective role in pregnancy by minimising inflammatory processes and thus maximising a healthy pregnancy. In turn, it is reasonable to assume that overexpression of CD74 in early human pregnancy may correlate with gestational complications.

The effect of different concentrations of LPS on proliferation of JEG-3 and ACH-3P cells was investigated. A significant decrease in cell growth was observed in both cell lines during LPS infection. Additionally, LPS significantly diminished cell proliferation in both cell lines within 48 hrs of LPS infection (Figure 5-1). LPS-induced lowering of proliferation in the JEG-3 and ACH-3P cell lines. This indicates that trophoblast cells respond rapidly to bacterial toxins. A number of studies have reported that trophoblast cell death is increased in pregnancies complicated by preeclampsia and intrauterine

growth restriction, leading to inadequate trophoblast invasion (Allaire et al., 2000, Crocker et al., 2003, Kakinuma et al., 1997, Smith et al., 1997).

This study confirmed that, following LPS treatment, the expression of CD44 was up-regulated in the JEG-3 and ACH-3P lines, as seen using flow cytometry and Western blotting (Figure 5-3 and Figure 5-5). Results showed that in both cell lines, the expression of CD44 was increased when the cells were exposed to LPS. Similarly, Siegelman and colleagues (1998) found that stimulation with LPS resulted in up-regulated surface expression of CD44 as a result of up-regulation of HA in primary endothelial cells. Additionally, Zbysek Sladek and Dusan Rysanek (2009) suggested that high expression of surface CD44 is related to the involvement of surface CD44 as a competent phagocytic receptor on macrophages in the processes leading to restitution of injured tissues (Sladek and Rysanek, 2009).

Van Der Windt et al. (2010) showed that CD44 is a key controller in various stages of the host response to Gram-negative bacteria, such as in pneumonia (van der Windt et al., 2010). CD44 has been also reported to positively regulate the induction of negative regulators of TLR signalling (Liang et al., 2007). Moreover, CD44 is known to bind and to internalize HA fragments (Harada and Takahashi, 2007, Culty et al., 1992). It has become clear that HA fragments induce NF-kB (Noble et al., 1996), and thereby pro inflammatory gene expression through TLR-2 and TLR-4 signalling (Voelcker et al., 2008, Scheibner et al., 2006).

TLR-4, the first toll-like receptor to be identified (Medzhitov et al., 1997), is the specific receptor for LPS (Poltorak et al., 1998). Several studies have reported that the expression and activation of TLR-4 in human trophoblast cells induces the expression and release of several pro- and anti-inflammatory cytokines (Koga et al., 2009).

MIF, a potent proinflammatory cytokine, plays an important role in the maintenance of pregnancy (Arcuri et al., 1999). The results obtained using the JEG-3 and ACH-3P cell lines are in accordance with up-regulation of MIF under stress conditions. Indeed, MIF was shown to up-regulate and to be up-regulated by pro-inflammatory stimuli (Calandra and Roger, 2003, Roger et al., 2001). This result is also supported by the findings of Chaisavaneeyakorn et al. (2005), who confirmed that LPS up-regulates MIF expression in BeWo cells (Chaisavaneeyakorn et al., 2005). De Oliveira Gomes et al., (2011) have shown that MIF secretion was increased in Jar trophoblast cells infected with *T. gondii* (de Oliveira Gomes et al., 2011).

In terms of human pregnancy protection, these results might suggest a role for MIF in the response to intrauterine infection. On the other hand, it has been reported that there is a relationship between MIF and recurrent miscarriage (RM). Yamada et al., (2003) measured MIF in the blood of women with uncomplicated pregnancy and in those with recurrent abortion. Decreased serum MIF concentrations were observed in women experiencing recurrent miscarriage with normal chromosome karyotype compared to controls. Thus MIF might be related to the aetiology of miscarriage. Moreover, another hypothetical explanation for the decreased serum MIF concentration is that low levels of MIF production may possibly be associated with impairment of trophoblast proliferation, embryo development, and angiogenesis of the placenta (Yamada et al., 2003). This observation suggests that MIF may play a role in human pregnancy failure. However, a study on animal models showed that female MIF gene-knockout mice were fertile and their new-borns developed normally in size (Bozza et al., 1999). This insinuates that MIF is not essential for reproduction.

This study suggests that MIF plays an essential role in cell proliferation as well as contributing to the maintenance of normal human pregnancy. Traditionally, the major

focus of MIF research has been on its role as a pro-inflammatory mediator and in the process of labor (Simón et al., 1998). Recent studies have shown that MIF functions are not limited to immune system. The identification of CD74/CD44 as a cell-surface receptor that mediates the binding of extracellular MIF was not discovered until very recently (Leng et al., 2003, Shi et al., 2006). To date, co-expression of CD74 and MIF has not been studied in the JEG-3 and ACH-3P cell lines. Whether CD74 and CD44 are associated, and the role of regulation of CD74 expression in fetal-maternal tolerance, warrant investigation.

The main aim of this study was to validate the hypothesis that the lack of human trophoblast-specific isoforms of CD74 at the fetal-maternal interface may have a protective role in pregnancy. This is the first report considering whether a specific isoform of CD74 is involved in the interaction of CD74 and CD44 in the JEG-3 and ACH-3P cell lines. The association of CD74 and CD44 was studied in the monocyte cell line THP-1 and the mammary cancer cell line CAMA-1. Potential interaction and co-localisation of these molecules was investigated using co-immunoprecipitation and bioimaging.

In the present study, intracellular CD74 expression increased while MIF and CD44 were expression on the cell surface increased on JEG-3 and ACH-3P cells after exposure to LPS (Figure 6-4, Figure 6-5, Figure 6-6 and Figure 6-7). This result is in agreement with earlier findings from this study (chapter 3) showing no evidence of CD74 expression in un-stimulated JEG-3 and ACH-3P cells (Figure 3-8). The validation results revealed that CD74 is expressed on the cell surface of THP-1 cells and of CAMA-1 cells (Figure 6-8 and Figure 6-9). This result confirmed that the expression of CD74 was restricted in antigen presenting cells or under inflammatory conditions (Stein et al., 2007).

Several studies have reported that CD74 and CD44 are involved in signalling with MIF. Therefore, CD74, CD44 and MIF expression were examined by imaging in order to study their co-localisation. The results showed no evidence for co-localisation of CD74 with either CD44 or MIF in JEG-3 and ACH-3P cells treated with LPS (Figure 6-4, Figure 6-5, Figure 6-6 and Figure 6-7). CD74 is a cell receptor for MIF and able to bind MIF when bound to surface-expressed CD44 (Leng et al., 2003). Recent data suggests that the interaction between CD74, MIF takes place in monocyte cells and CD44 is required to mediate MIF signalling pathway (Leng and Bucala, 2006). However, the interaction between CD74 and CD44 has been reported to occur on the cell surface. Shi *et al.* (2006) confirmed that mammalian COS-7 cells did not bind MIF, unless engineered to express CD74 (Leng et al., 2003). Moreover, it has also been confirmed that CD74 alone is sufficient to mediate MIF binding to cells (Shi et al., 2006). Consequently, CD74 alone is insufficient to signal with MIF in the absence of CD44. It has also been confirmed that although phosphorylation of serine takes place in the p35 variant of Ii, the short cytoplasmic sequence of CD74 does not appear to signal directly, and depends on CD44 (Borghese and Clanchy, 2011). Shi *et al*. (2006) confirmed that COS-7 cells are not able to be involved in signalling when engineered to express CD74 and a truncated CD44 lacking its cytoplasmic signalling domain (Shi et al., 2006).

JEG-3 and ACH-3P cells were found not to express CD74 on the cell surface or at the intracellular level. They do, however, express MIF and CD44. Co-IP failed to demonstrate interaction between CD74 and CD44 in JEG-3 and ACH-3P cells treated with LPS. As a consequence, results obtained from Co-IP confirmed that CD44 interacted with only p41, the largest CD74 isoform (Figure 6-1, Figure 6-2 and Figure 6-3). Correspondingly, a study carried out by Zheng et al. (2012) confirmed that CD74 (p41) interacted with MIF and TLR-4 in the gastric cancer cell line MKN-45 (Zheng et al.,

2012). However, a recent study by Barbosa et al. (2014) found that BeWo cells (which are a model for the villous trophoblast) expressed only the 43 kDa isoform of CD74 (Barbosa et al., 2014).

This finding is in contrast to the present results, which showed that both cell lines JEG-3 and ACH-3P express only the 35 kDa isoform of CD74 upon treatment with LPS. Thus, it is believed that the 35 kDa CD74 isoform might trigger the cells to become involved in antigen presentation. However, it is suggested that both cell lines (JEG-3 and ACH-3P) are highly immunogenic since they are highly stable even after being exposed to IFN-γ and LPS (van Nieuwenhoven et al., 2003).

It can be concluded that the lack of specific isoforms of CD74 in trophoblast cells serves to ensure survival and development of the foetus during pregnancy. This is via tolerance mechanisms which can break down in clinical conditions, such as unexplained recurrent spontaneous abortion or recurrent miscarriage.

7.2 Concluding Remarks

This study investigated the expression of CD74, MIF and CD44 in the human trophoblast derived cells lines JEG-3 and ACH-3P and human placenta. This study also aimed to obtain knowledge on the effect of IFN-γ and LPS, particularly in regulating CD74 isoform expression. The role of JEG-3 and ACH-3P cell proliferation in response to LPS was additionally investigated to assess the effect of MIF and CD44 expression during infection at maternal-placental interface. The final aim of this research was to study the interaction of CD74 and CD44 in JEG-3 and ACH-3P cells.

MIF and CD44 expression was detected in both JEG-3 and ACH-3P cell lines, but CD74 could not be detected. RT-PCR also revealed no evidence for the expression of the CD74 in both cell lines, compared to THP1 cells which were used as a positive control. This was further confirmed by flow cytometry analysis and Western blot results. The complete lack of CD74 in JEG-3 and ACH-3P cells presumably reflects the absence of MHC class II expression in trophoblast.

This study investigated whether the immunological receptor CD74 is associated with antigen presentation and if it plays a role in fetal-maternal tolerance and intrauterine infection. CD74 isoform expression in JEG-3 and ACH-3P was also studied. It has been previously shown that there is no evidence for the expression of CD74 in un-stimulated JEG-3 and ACH-3P cells. However, moderate expression of CD74 mRNA was noted after JEG-3 and ACH-3P cells were incubated with IFN-γ or LPS, in a dose and time-dependent manner.

Whether CD74 expression is up-regulated intracellularly, or at the cell surface level, was investigated by incubating JEG-3 and ACH-3P cells with IFN-γ or LPS. JEG-3 and ACH-

3P cells showed intracellular CD74 expression but no surface expression, even with IFN-γ or LPS treatment, as determined by flow cytometry.

JEG-3 and ACH-3P cells stimulated with IFN-γ, JEG-3 and ACH-3P did not express CD74. However, the p35 CD74 isoform was detected in JEG-3 and ACH-3P cells treated with LPS. Using the IP technique, the same result was obtained.

Taken together, the absence of HLA-DR and CD74 expression in JEG-3 and ACH-3P cells, upon treatment with IFN-γ or LPS, point to a novel regulatory mechanism of CD74 expression at the fetal-maternal interface. In the context of early human pregnancy, CD74 appears to possess functions independent of class II MHC molecules. This suggests that CD74 might play a negative role in early human pregnancy. It may be related to gestational complications.

The role of trophoblasts in the maintenance of utero-placental tolerance during infection was explored. Intrauterine infections have been associated with pre-term labour in up to 40% of cases (Lamont, 2003). Furthermore, 85% of pre-term deliveries are at less than 28 weeks of gestation have evidence of infection (Epstein et al., 2000). Intrauterine infections may occur early in human pregnancy, preceding any observed pregnancy complications (Gonçalves et al., 2002). To investigate the effect of bacteria on various cellular biological functions, LPS was used for *in vitro* studies utilizing the JEG-3 and ACH-3P cell lines. This study investigated trophoblast cell proliferation and also the effect of LPS on MIF and CD44 expression on the surface and inside the cells LPS was found to inhibit trophoblast cell proliferation. In the same manner, MIF and CD44 were up-regulated during LPS exposure. Taking into consideration the above information, it can be suggested that MIF plays an essential role in cell proliferation, whereas both MIF and CD44 play an important role in pregnancy maintenance.

The lack of trophoblast-specific isoforms of CD74 at the fetal-maternal interface may protect pregnancy. Data from interaction and co-localisation studies confirmed that there

was no evidence for co-localisation or interaction of CD74 with either CD44 or MIF in either treated or untreated cell lines (JEG-3 and ACH-3P cells).

It is concluded that the absence of cell surface expression of isoforms of CD74 might invoke pregnancy protection by minimising inflammatory processes. In turn, it is reasonable to assume that the overexpression of CD74 during early pregnancy may be related to gestational complications.

7.3 Limitations of this study

To extend knowledge of the role of CD74 during early human pregnancy, clinical samples of first trimester placental (abortion) tissue should be considered.

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Appendices

Appendix 1 Buffer Recipes

1X PBS (1000 ml)

Reagent	Amount	Final Concentration
NaCl	8.0gm	138 mM
KCl	0.25gm	2.6 mM
KH_2PO_4	0.25gm	1.5 mM
Na_2HPO_4	0.25gm	6.3 mM
Distilled Water	1000 ml	-

(PH 7.3; Store at 4°C).

4% Paraformaldehyde (100 ml)

Reagent	Amount
PFA	4.0 g
PBS	100 ml

(PH 7.3; Store at 4°C).

Blocking buffer (1000 ml)

Reagent	Amount	Final concentration
FCS	10 ml	1 %
PBS	1000 ml	-

(PH 7.3; Store at 4°C).

Coomasie Blue stain (1000 ml)

Reagent	Amount	Final concentration
Coomasie Blue R-250	1 g	0.1 %
Methanol	450 ml	45 %
Glacial acetic acid	100 ml	10 %
Distilled Water	450 ml	

Appendix 1

Coomasie Blue destain (1000 ml)

Reagent	Amount	Final concentration
Methanol	100 ml	10 %
Glacial acetic acid	100 ml	10 %
Distilled Water	800 ml	-

5X electrophoresis running buffer (1000 ml)

Reagent	Amount	Final concentration
Tris	15.1 g	25 mM
Glycine	72 g	250 mM
10% SDS	5 g	0.1 %

1X electrophoresis Buffer (1000 ml)

Reagent	Amount
5X electrophoresis running buffer	200 ml
Distilled Water	800 ml

Ammonium Persulfate (10%) (1 ml)

Reagent	Amount
Ammonium Persulfate	0.1 g
Distilled Water	1 ml

Transfer buffer (1000 ml)

Reagent	Amount	Final concentration
Tris	3.03 g	25 mM
Glycine	14.4 g	192 mM
Methanol	200 ml	20 %
Distilled Water	800 ml	

(PH 8.3; Store at 4°C).

Appendix 1

Wash buffer (PBS-T) (1000 ml)

Reagent	Amount	Final concentration
PBS	999 ml	-
Tween 20	1 ml	0.1 %

Blocking buffer (100 ml)

Reagent	Amount	Final concentration
Non- fat dry milk	5 g	5 %
PBS-T	100 ml	-

Sodium dodecyl sulfate SDS (10 %)(100 ml)

Reagent	Amount
Sodium dodecyl sulfate	10 g
Distilled Water	100 ml

1.5 M Tris-HCl, pH 8.8 (100 ml)

Reagent	Amount
Tris base	10 g
Distilled Water	100 ml Adjust to pH 8.8 with HCl

(PH 8.8; Store at 4°C).

0.5 M Tris-HCl, pH 6.8 (100 ml)

Reagent	Amount
Tris base	6.6 g
Distilled Water	100 ml Adjust to pH 6.8 with HCl

(PH 6.8; Store at 4°C).

Appendix 1

Resolving and stacking gel

Reagent	Resolving gel (ml)	Stacking gel (ml)
Distilled Water	4.9	6.1
1.5 M Tris-HCL	3.8	-
0.5 M Tris-HCL	-	2.5
Bis acrylamide	6	1.33
10% SDS	0.15	0.1
APS	0.15	0.05
TEMED	0.006	0.01

Appendix 2 Chemicals

1. Accutase Sigma-Aldrich Dorset, UK
2. Acrylamide 30% BIO-RAD, UK
3. Agarose Invitrogen, UK
4. Brilliant Blue G-Colloidal Concentrate Sigma-Aldrich, UK
5. Bromophenol blue sodium salt Sigma-Aldrich, UK
6. Calcium chloride Merck, Darmstadt
7. CHAPS AppliChem, Darmstadt Chloroform Merck, Darmstadt
8. Ethanol Fisher Scientific, UK
9. Ethidiumbromide Roth, Karlsruhe
10. Giemsa stain Sigma-Aldrich, UK
11. Glutathione Amersham, Freiburg Glycerol Merck, Darmstadt
12. Glycine Sigma-Aldrich, Steinheim
13. Guanidine hydrochloride Sigma-Aldrich, Steinheim
14. Imidazole Fluka, Steinheim
15. Isopropylthio-β-D-galactoside Serva, Heidelberg
16. Leupeptin Sigma-Aldrich, Steinheim
17. Lipopolysaccharide Sigma-Aldrich, Steinheim
18. Magnesium chloride Merck, Darmstadt
19. Magnesium sulfate Sigma-Aldrich, Steinheim
20. Methanol Fisher Scientific, UK
21. N,N,N',N’-Tetramethylethylenediamin Roth, Karlsruhe
22. Non-fat dry milk Bio-Rad, UK
23. Paraformaldehyde Sigma, Germany
24. Phenylmethylsulfonyl fluoride Sigma-Aldrich, Steinheim
25. Ponceau S Roth, Karlsruhe
26. Potassium chloride Fisher Scientific, UK
27. Sodium acetate Roth, Karlsruhe
28. Sodium azide Merck, Darmstadt
29. Sodium carbonate Roth, Karlsruhe
30. Sodium chloride Sigma-Aldrich, Steinheim
31. Sodium citrate Merck, Darmstadt
32. Sodium dodecyl sulfate Merck, Darmstadt

33. Sodium periodate Sigma-Aldrich, Steinheim

34. Sodium thiosulfate Roth, Karlsruhe

35. Sodium thiosulfate Roth, Karlsruhe

36. Tris (hydroxymethyl) aminomethane Roth, Karlsruhe

37. Triton $_{X}$-100 Sigma-Aldrich, UK

38. Trypan blue Gibco, USA

39. Tween-20 Roth, Karlsruhe

40. Urea Merck, Darmstadt

41. β-Mercaptoethanol AppliChem, Darmstadt

Appendix 3 Training sessions attended during the period of PhD programme

1. Advance project management
2. Assertive communications boards
3. Building an academic profile
4. Communicating your research
5. Communication skills for sciences postgraduates
6. Completing your PhD
7. Confocal microscopy training (Nikon and Bio-Rad Training)
8. Correct referencing and avoiding plagiarism
9. Diversity and quality training course
10. Effective use of voice
11. EndNote training
12. Engaging your audience
13. Flow cytometry training
14. Focus group session
15. General safety in the department and regulations in biology and risk assessment
16. Getting published in science
17. Graduate laboratory assistant
18. How to be an effective researcher
19. Intellectual property in bioscience research
20. Poster presentation
21. Presentations skills
22. Surviving the viva
23. The seven secretes of highly successful research students

Appendix 4 Conferences attended during the period of PhD programme

Two abstracts have been submitted to European Society for Immunodeficiencies (ESID) conference, which was held in Prague, Czech Republic from the 29th October to the 1st November 2014.

1. **The tittle**: "Lack of Trophoblast-specific isoforms of CD74 may protect human pregnancy"

   The authors: Waleed Al Abdulmenaim, Hussain Alssadh and Nelson Fernández

The abstract: During pregnancy the maternal immune system protects the allogeneic foetus from rejection. At the same time the mother maintains immunity defences intact against potential pathogens. We wish to know whether immunological receptors associated with antigen presentation and hence strong inflammatory responses play a role at the feto-maternal tolerance. One such receptor is CD74, a membrane-bound protein involved in HLA Class mediated antigen presentation and as Macrophage Migration Inhibitory Factor (MIF) receptor. Consequently, we wish to investigate the expression of CD74 at mRNA and protein levels in trophoblast derived cells, JEG-3 and ACH-3P and human placenta. We also wish to obtain knowledge on the effect of IFN-γ and LPS-mediated infection, in particular in the regulation of CD74 isoform expression.

University of Essex

Lack of Trophoblast-specific isoforms of CD74 may protect human pregnancy

Waleed Al Abdulmenaim , Hussain Al Ssadh and Nelson Fernández
School of Biological Sciences, University of Essex, Colchester, UK

Introduction

During pregnancy the maternal immune system protects the allogeneic foetus from rejection. At the same time the mother maintains immunity defences intact against potential pathogens. We wish to know whether immunological receptors associated with antigen presentation and hence strong inflammatory responses play a role at the feto-maternal tolerance. One such receptor is CD74, a membrane-bound protein involved in HLA Class mediated antigen presentation and as Macrophage Migration Inhibitory Factor (MIF) receptor. Consequently, we wish to investigate the expression of CD74 at mRNA and protein levels in trophoblast derived cells, Jeg-3 and ACH-3P and human placenta. We also wish to obtain knowledge on the effect of IFN-γ and LPS-mediated infection, in particular in the regulation of CD74 isoform expression [1,2,4 & 5].

Methods

RT-PCR: mRNA expression of CD74 was studied by RT-PCR. It was extracted from 1x 10^6 Jeg-3 and ACH-3P cell lines.

Flow cytometry: Cells were washed, fixed and blocked. Then, they were incubated with anti-CD74 and followed by 3 washed steps with 1X PBS. Next, they were incubated with secondary antibody (FITC) conjugated goat anti-mouse IgG.

Western Blot: Cells were lysed with CelLytic reagent. After electrophoresis, protein was transferred to PVDF membrane. Membranes were blocked with 5% skimmed milk in PBS-Tween-20, and then incubated in anti-CD74. Next they were incubated with anti-Mouse IgG.

Microscopy: Cells or tissues were fixed, permeabilized and then blocked with 2% (BSA/PBS). Then, they were incubated with anti-CD74 for 1 hour followed by anti-mouse IgG, conjugated with Alexa Fluor®.

Results

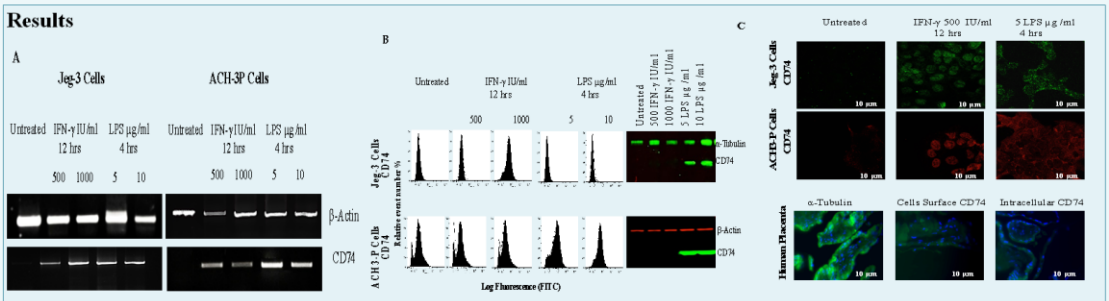

Figure A: CD74 mRNA expression was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). Both untreated trophoblast cell lines (Jeg-3 and ACH-3P) do not express CD74 mRNA. However, in response to infection and pro-inflammatory cytokine on both cell lines, CD74 mRNA was upregulated. After exposure to IFN-γ for 12 hr 500 IU/ml, the result showed slight upregulation of CD74 . However, after LPS treatment for 4 hr 5 µg/ml, CD74 was highly upregulated. **Figure B**: Flow cytometry analysis of surface expression of CD74. Empty histograms shows surface expression of CD74 on Jeg-3 and ACH-3P cell line, whereas black histograms shows the negative control. It can be clearly seen that both cell lines do not express CD74 on the cell surface .Whole cell lysates extracted from Jeg-3 and ACH-3P cell line were subjected to immunoblotting with α Tubulin, β Actin and CD74. **Figure C:** Microscopic images of Jeg-3, ACH-3P cells and Human placenta. In the images CD74 is stained in green or red as indicated with CD74 primary antibody, and Secondary antibody anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555.

Conclusions

- Jeg-3 and ACH-3P lack expression of CD74 isoforms at the cell surface in response to LPS infection and pro-inflammatory cytokine IFN-γ. However, CD74 mRNA is upregulated in response to interferon-γ and LPS. LPS infection has a stronger up regulatory effect than IFN-γ. This is at variance with expression of most immunological receptors, which are strongly upregulated by IFN-γ.
- We hypothesise that the lack of cell surface expression of CD74 may provide a protective role in pregnancy by minimising inflammatory processes and thus maximising a healthy pregnancy. In turn, it is reasonable to assume that overexpression of CD74 in early human pregnancy may be related to gestational complications.

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2. **The tittle**: "Analysis of the expression and interrelationship between the CD74 and CD44 receptors expressed on human breast cancer derived cells"

The authors: Hussain Alssadh, Waleed Al Abdulmenaim and Nelson Fernández

The abstract: CD74 is a transmembrane protein that functions as a chaperone of MHC class II and is also thought to be involved in signalling via MIF and CD44. CD44 is a transmembrane glycoprotein that acts as the receptor for hyaluronan and it is considered as a member of cell adhesion molecules. The relationship between these two proteins is not well understood; one hypothesis postulates that the expression of CD74 and CD44 is associated with inflammatory disorders including cancer. We have examined the expression of CD74 and CD44 by flow cytometry and western blot in three breast tumour cell lines, CAMA-1, MDA-MB-231 and MDA-MB-435. The cell-surface membrane expression of CD44 is higher than CD74 in all examined cell lines. On the other hand, the level of expression of CD74 in MDA-MB-435 cell lines was higher than in CAMA-1 and MDA-MB-231. To evaluate the physical association of these two proteins, co-localization experiments using bioimaging were carried out. It was observed that CD74 and CD44 are highly co-localized suggesting a possible mode of function in facilitating signalling.

Analysis of the expression and interrelationship between the CD74 and CD44 receptors expressed on human breast cancer derived cells

University of Essex

Hussain Alssadh, Waleed Al Abdulmenaim and Nelson Fernández
School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester

10th Biennial Meeting of the EUROPEAN SOCIETY FOR IMMUNODEFICIENCIES
Prague, Czech Republic
29 October - 1 November, 2014

Background

The expression of CD74 and CD44 has been identified in several types of cancer and is believed to have a major role in immunotolerance .Their selective expression is assumed to activate certain mechanisms/ signal transduction pathways that suppress or modulate the immune system and protect the developing cancerous cells.

CD74 is a transmembrane protein that functions as a chaperone of MHC class II and is also thought to be involved in signaling via MIF and CD44. **CD44** is a transmembrane glycoprotein that acts as the receptor for hyaluronan and it is considered as a member of cell adhesion molecules. A recent study has shown that CD74 and CD44 are over expressed on various cancer cells, i.e. prostate cancer cells, B lymphomas, or gastric carcinomas. So, it is believed that CD74 and CD44 might be considered as tumour marker.

Aims

To characterize the surface and total protein expression of CD74 and CD44 on human breast cancer cell lines (CAMA-1 and MDA-MB-231). Study of the interactive partners of CD74 and CD44 by target imaging. A hypothesis in search of a function of these receptors.

Methods

- Surface expression of CD74 and CD44 was studied by flow cytometer and the result was analysed, gated and compensated by flowJo 8.8.6 software.
- Total protein was extracted and subjected to western blot for CD74 and CD44. The blot were finally scanned using the ODYSSEY Infrared Imaging System from LI-COR Biosciences.
- Colocalization study of CD74 and CD44 was studied by bio image using confocal microscopy. Data was analysed using NIS elements and Fiji software.

Results

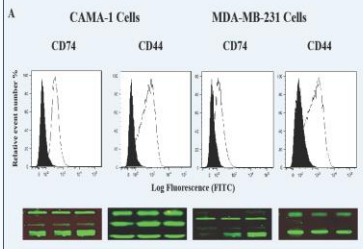

Figure A: Flow Cytometry analysis showing CD74 and CD44 expression (empty histogram), negative control (black filled histogram) as MFI at the cell surface of CAMA-1 and MDA-MB-231 cell lines. Total protein was extracted and subjected to Western Blot for CD74, CD44 and α-Tubulin (control). CAMA-1 and MDA-MB-231 express three different isoform of CD74 whereas CAMA-1 only express two isoform of CD44.

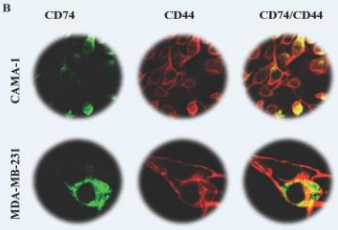

Figure B: Double immunostaining of CD74 and CD44 in CAMA-1 and MDA-MB-231 cell lines by confocal laser scanning microscopy. CD74 was stained with Alexa Fluor® 488 whereas CD44 was stained with Alexa Fluor® 555.Yellow/ orange florescence reveals the co localisation of CD74 and CD44.

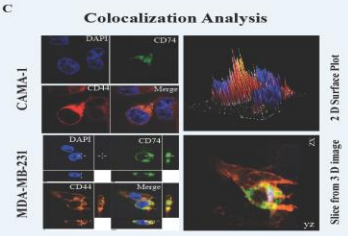

Figure C: Colocalization percent of CD74 and CD44 was analysed using Pearson's correlation coefficient. 2 D surface plot was acquired that shows the intensity merged channels (green and red) using Fiji software. 3 D images were acquired in stack, with z-direction step size 0.14 µm. Single-plane of z-stack is shown in three direction as xy, yz and zx. Scale bar 10 µm.

Conclusions

- CD74 and CD44 are expressed on the breast cancer cells CAMA-1 and MDA-MB-231.
- Both cell lines express three different isoforms of CD74 (p31, p35 and p41 kDa).
- CD74 and CD44 are colocalized at the cell membrane. This suggests that these receptors might be responsible for tumor processes functionality.
- Our finding suggests that CD74 and CD44 might be promising target in tumor therapy.

Future Work

- The colocalisation between CD74 and CD44 will be studied by the technique of CO-IP.
- The expression of MIF will be studied on both cell lines; the interrelationship between MIF and both CD74 and CD44 will be more investigated.
- The isoforms of CD74 and CD44 will be further analysed using 2-D gel techniques and proteomics in order to search for a measurable biomarker in association with cancer.

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Publications

A manuscript has been in progress for publications as described below:

1. Title: Interactions between CD74 and CD44 receptors expressed by human breast cancer cells
   Authors: Al Ssadh Hussain, Alabdulmenaim Waleed and Nelson Fernández
   Journal: Journal of Immunology

Manuscript in the process of writing entitled

2. Lack of Trophoblast-specific isoforms of CD74 may protect human pregnancy

Author(s): Waleed Al Abdulmonaim, Hussain Al Ssadh and Nelson Fernàndez.

Journal: Journal of Placenta

Interactions between CD74 and CD44 receptors expressed by human breast cancer cells

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Abstract

Increasingly the interactions of pairs of membrane-bound receptors acting in synergy have been linked to tumor progression; in this study we show that, indeed, CD74 and CD44 act in synergy and thus could promote breast cancer progression. The transmembrane receptor, which is also known as Cluster of Differentiation (CD) 74 functions as a chaperone of MHC class II biosynthesis, it is also involved in signaling via (macrophage migration inhibitory factor) MIF and the adhesion receptor CD44. The latter is also a transmembrane receptor, which is a member of a large family of cell adhesion molecules that is responsible for mediating communication and adhesion between adjacent cells, and between cells and the extracellular matrix (ECM). We have employed flow cytometry and western blot to examine the breast tumor cell lines, CAMA-1, MDA-MB-231 and MDA-MB-435 for CD74 and CD44. We observed that CD44 was higher than CD74 in all examined cancer cells. On the other hand, the level of expression of CD74 in MDA-MB-435 cell lines was higher than in CAMA-1 and MDA-MB-231. We conclude that CD74 and CD44 showed significant quantitative variations and they associate in cytoplasmic compartments, suggesting a mechanistic and functional mode of action. Co-localization experiments using bioimaging indicate that CD74 and CD44 are physically associated suggesting a possible mode of function in facilitating signaling and hence tumor progression.

Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide and the most common cause of malignancy-associated death (Götte and Yip, 2006, Trapé and Gonzalez-Angulo, 2012, Richard et al., 2014). Breast cancer is found in breast sites and it is more common in females than males (Richard et al., 2014). Each year, 458,000 women die from breast cancer globally, making it the most common cause of female cancer death in both the developed and developing world (Eccles et al., 2013). In the UK, the age-standardised incidence of breast cancer in women has increased by 6% over the last decade, between 1999 to 2001 and 2008 to 2010 (Ferlay et al., 2010). Statistical surveys have estimated that around 550,000-570.000 people are diagnosed with breast cancer in the United Kingdom (Maddams et al., 2009). The number of women who are diagnosed with breast cancer is expected to increased three times by 2040 (Maddams et al., 2012). Recently, breast cancer research indicates that the annual cost of breast cancer healthcare increased over a third compared to the last 10 years (Eccles et al., 2013).

The expression of CD74 and CD44 has been identified in several types of cancer and is believed to play a role in immunotolerance (Becker-Herman et al., 2005). Recently, Greenwood et al. (Greenwood et al., 2012) have determined that high expression of Stat1, Max1 and CD74 is associated with triple-negative breast cancer. It has also been demonstrated that adhesion of tumour cells on the reconstituted membrane matrix Matrigel increases after INF-γ-induced over-expression of CD74 (Greenwood et al., 2012). Jiang et al (Jiang et al., 1999) have shown that WNT5A, a lipid-modified glycoprotein, inhibits metastasis and alters splicing of CD44 in breast cancer cells. Therefore, it is believed that selective expression of CD74 and CD44 activates certain mechanisms and signal transduction pathways that suppress or modulate the immune system and protect the development of cancerous cells and metastasis (Beswick and Reyes, 2009).

CD74, also known as invariant chain (Ii) or macrophage migration inhibitory factor (MIF) receptor, is classified as a type II transmembrane glycoprotein that is expressed on antigen-presenting cells (Burton et al., 2004, Stein et al., 2007, Beswick and Reyes, 2009). However, recent studies have demonstrated that CD74 is also expressed in numerous types of cancer cell, including prostate, bladder and breast cancer cells (Pyrz et al., 2010, Meyer-Siegler et al., 2006). Additionally, CD74 is thought to be involved in signalling via MIF, suggesting it has a crucial role in tumor progression (Meyer-Siegler et al., 2004, Meyer-Siegler et al.,

2006). However, because the short cytoplasmic sequence of CD74 does not appear to signal directly, MIF-induced extracellular signal-regulated kinase (ERK) signalling appears to be reliant upon CD44 (Shi et al., 2006, Meyer-Siegler et al., 2006). Thus CD74 is modified by the addition of chondroitin sulfate, permitting interaction with CD44 to form a receptor complex of MIF-CD74-CD44 (Naujokas et al., 1993, Borghese and Clanchy, 2011).

CD44 is an integral membrane glycoprotein expressed in many cell types that serves as the principal receptor for hyaluronan, a glycosaminoglycan constituent of extracellular matrix (Fillmore and Kuperwasser, 2007). Through alternative splicing, cells produce a family of CD44 including (CD44s and CD44v) protein isoforms that are involved in multiple distinct cellular functions, including proliferation, adhesion, and migration. The expression of CD44 has been associated to particular cancer types such as prostate tumours, head and neck squamous tumours, nervous system tumours, respiratory track tumours, melanomas and breast cancer (Assimakopoulos et al., 2002, Sneath and Mangham, 1998, Iczkowski, 2011, Eberth et al., 2010). CD44 expression in human breast cancer has been correlated with both favourable and unfavourable clinical outcomes (Götte and Yip, 2006, Louderbough and Schroeder, 2011).

The present study aimed to characterize the cell-surface and total protein expression of CD74 and CD44 on human breast cancer cell lines (CAMA-1, MDA-MB-231 and MDA-MB-435). I also studied the interactive partners of CD74 and CD44 by target imaging and coimmunoprecipitation. We investigated the expression of CD74 and CD44 in immortalized normal breast luminal cells (226LDM) to validate the study.

Materials and Methods

Cell lines and cell culture

Three human mammary gland cell lines, CAMA-1, MDA-MB-231 and MDA-MB-435, all derived from malignant pleural effusion, were used. The CAMA-1 and MDA-MB-435 cell lines were maintained in RPMI 1640 medium (LONZA-Belgium), supplemented with 10% of (v/v) fetal calf serum (FCS; Imperials Laboratories). Although MDA-MB-435 has been debated to be melanoma cells due to its gene profile expression, it is yet widely used as breast cancer cells (Chambers, 2009). The MDA-MB-231 cell line was maintained in D-MEM (high glucose), supplemented with 10 % FCS. 226LDM immortalized normal breast luminal cells (kindly provided by Elena Klenova, School of Biological Sciences, University of Essex), were used as positive control cells. Raji cells (human negroid Burkitt's lymphoma) and HeLa cells (human cervical cancer), expressing high levels of CD74 and CD44, respectively, served as additional positive controls. The 226LDM cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12, supplemented with 10% FCS, 5 mg/ml gentamicine, 5 ug/m insulin, l ng/ml hydrocortine, 20 ng/ml epidermal growth factor and 20 ng/ml cholera toxin. Raji and HeLa cells were cultured in RPMI -1640 (LONZA-Belgium) containing 10% FCS and cultured in a humidified atmosphere of 5% CO_2 and 37°C. HeLa cell line was generous gift from Professor Glyn Stanway, School of Biological Sciences, University of Essex .All media used for this study were purchased from PAA Laboratories GmbH (Pasching, Austria).

Reagents

The monoclonal primary antibodies mouse anti-human CD74 (clone: By2) and mouse anti-human α-tubulin (clone: TU-02) were purchased from Santa Cruz Biotechnology, USA. Mouse antibody CD44 (clone: 156-3c11) was purchased from Cell Signaling Technology, USA. Rabbit anti-human β-actin (clone: poly 6221) was purchased from (BioLegend, UK). The secondary antibody used for flow cytometry was a goat anti-Mouse antibody conjugated with the fluorophore FITC (clone: poly4053) and was purchased from Bio-legend, UK. The secondary antibody used for Western blotting was either goat anti-mouse (IRDye 800CW) or goat anti-rabbit (IRDye 680 LT), purchased from LI-COR Biosciences. Finally, Alex 488 (green) and Alex 555 (red) antibodies were purchased from Life Technologies, UK.

Flow cytometry analysis

Cell lines were lifted with accutase (Sigma) and $1x10^6$ cells were used per sample. Monoclonal antibodies By2 (anti-CD74) and 156-3c11 (anti-CD44) were employed in indirect immunofluorescence staining. Cells were preincubated with saturating concentrations of primary antibody, followed by washing and labeling with FITC-conjugated goat anti-mouse IgG (Bio-legend). For cell-surface staining, cells were fixed with 4% formaldehyde solution, and washed with 1X PBS. The cells were then blocked with blocking buffer (PBS/0.1 %BSA) followed by washing steps in PBS. Primary and secondary antibodies were diluted with 0.1% BSA in PBS. Samples were analysed on BD FACSAria and analyzed by FlowJo 8.8.6.

Western blotting and immunedetection

Cells were lysed with CelLytic reagent (Sigma) and total protein concentration was determined by Bradford assay. The Total cell lysate was separated on a 12% SDS-PAGE gel. A total of 40 µg protein was loaded per well, and, after electrophoresis, protein was transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-FL, Merck Millipore, Merck KGaA, Darmstadt, Germany). Membranes were blocked with 5% skimmed milk in PBS-Tween-20 (Sigma) for 1 hr at room temperature and incubated in anti-CD74 (clone: By2) at a concentration of 1:200, and anti- CD44 (clone: 156-3C11) at a concentration of 1:1000. As a control, an alpha subunit-specific tubulin mouse monoclonal antibody was used to probe the cell extracts at a concentration of 1:200, followed by washing in PBS-T for 30 min. The membranes were then incubated with IRDye 800CW Donkey anti-Mouse IgG (Li-Cor Bioscience, Licoln, NE, USA) at a concentration of 1:1000 for 1 hr followed by washing in PBS-T for 30 min. Signals were detected using the ODYSSEY Infrared Imaging System (Li-Cor Biosciences). Fermentas PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used in order to estimate the molecular weight of the respective protein bands.

Immunofluorescence staining

CAMA-1, MDA-MB-231 and MDA-MB-435 cell lines were cultured in LabTek 8-well chambers (Thermo Fisher Scientific) at a density of 6×10^3 cells per well for two days and following that they were seeded. The cells were fixed with 4% paraformaldehyde for 20 min on ice. For immunofluorescence staining all procedures were carried out at ambient

temperature. Cells were permeabilized and then blocked with 2% (w/v) bovine serum albumin (BSA) prepared in 1x phosphate-buffered saline (PBS) for 1 h at room temperature. For single staining of each antigen, cells were incubated with anti-CD74 (clone: By2) at a concentration of 1:500, and anti- CD44 (clone: 156-3C11) at a concentration of 1:400, for 1 h followed by three washes with PBS. Secondary antibody, anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (Invitrogen, Carlsbad, CA, USA), was used at a dilution of 0.25 µg/100ml for 1 h. For double staining, cells were blocked again with 2% BSA and the staining process was repeated for each desired pair. 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) counter stain (Vector Laboratories, Burlingame, CA, USA) was used at a 1:250 dilution. Cells were thoroughly washed with PBS, the chambers removed, and the slide was mounted with anti-fade mounting medium (Vector Shield) covered with a cover slip (Chance proper LTD, West Midlands, England) and sealed with Marabu fixogum rubber cement (Marabuwerke GmbH & Co. KG, Tamm, Germany).

Confocal microscopy

For image acquisition, a Nikon A1Si confocal microscope (Nikon Instruments Inc.) was used with a plan-apochromatic VC1.4 N.A. 60x magnifying the oil-immersion objective. NIS-Elements Advanced Research Software (version 4.13.01, build 916; Nikon Instruments Inc.) was used for image acquisition. Images were acquired in three channels, using one-way sequential line scans. DAPI was excited at 398.7 nm with laser power 1.6 arbitrary units, and its emission was collected at 450 nm with a PMT gain of 86. Alexa Fluor 488 was excited at 488 nm with laser power 5.8, its emission was collected at 525 nm with a PMT gain of 117. Alexa Fluor 555 was excited at 560.5 nm with laser power 3.7, and was collected at 595 nm with a PMT gain of 98.The scan speed was ¼ frames/s (galvano scanner). The pinhole size was 35.76 µm, approximating 1.2 times the Airy disk size of the 1.4-NA objective at 525 nm. Scanner zoom was centered on the optical axis and set to a lateral magnification of 60 nm/pixel. Axial step size was 105 nm, with 80-100 image planes per z-stack.

Image processing

NIS-Elements software (version 3.21.03, build 705; Nikon Instruments Inc.) was used for image processing. CD74 (green) and CD44 (red) channels were segmented using regional maxima detection tools followed by manual threshold. The generated binary areas were visually inspected; the overlap of the green and red channels which were generated by the overlay tool, resulted in a new layer (yellow) that represents the intersection of CD74 and

CD44. Finally, automated volume measurement was carried out for CD74, CD44 and their intersection was measured by a volume measurements tool.

Immunoprecipitation and coimmunoprecipitation

Cells were lysed with the RIPA Lysis Buffer System (Santa Cruz Biotechnology, USA) and total protein concentration was determined by Bradford assay.1 mg/ml of each sample was incubated overnight with 4 µg of anti-CD74 (clone: By2), at 4°C. Following that 40 µl of protein A/G PLUS- Agarose (Santa Cruz Biotechnology, USA) was added. To promote immunoglobulin binding, this solution was kept overnight on a rotator at 4°C. The samples were spun down for 30 s and the supernatant was discarded. Beads were then washed twice using PBS and the samples were boiled at 100°C after adding 50 µl of SDS-PAGE sample loading buffer containing dithiothreitol. Then 20 µl of each sample was loaded in each well of the gel that was left for 1 hr and 30 min at 120 V. After electrophoresis, protein was transferred to a PVDF membrane. Membranes were blocked with 5% skimmed milk in PBS-Tween-20 (Sigma) for 1 h at room temperature and incubated in either anti-CD74 (clone: By2) at a concentration of 1:200, or anti-CD44 (clone: 156-3C11) at a concentration of 1:1000, and flowed by washing in PBS-T for 30 min. The membranes were then incubated with secondary antibody (IRDye 800CW Donkey anti-Mouse IgG ;Li-Cor Bioscience, Licoln, NE, USA) at a concentration of 1:1000 for 1 h, followed by washing in PBS-T for 30 min. Signals were detected using the ODYSSEY Infrared Imaging System (Li-Cor Biosciences).

Results

Identification and quantification of CD74 and CD44

The cell surface expression of CD74 and CD44 was analyzed for CAMA-1, MDA-MB-231, and MDA-MB-435 and Raji cell lines. Non-permeabilized cells were stained with an appropriate concentration of By2 (anti-CD74) and 156-3C11 (anti-CD44) antibodies followed by 1 µl RAM-FITC secondary antibody. Cells only, without staining, and isotype cells, stained with only secondary antibody, were used as a negative control. CD74 and CD44 were detected on the cell-surface membrane of CAMA-1, MDA-MB-231 and MDA-MB-435 cells (Fig 1).

Immunostaining of CD74 and CD44

Laser scanning confocal microscopy using different wavelengths was used to visualize intracellular expression of CD74 and CD44 molecules. The CAMA-1, MDA-MB-231 and MDA-MB-435 cell lines all showed expression of CD74 and D44 on the intracellular membrane (Fig 2).

Immunoblot analysis of CD74 and CD44 proteins

CD74 and CD44 protein expression in CAMA-1, MDA-MB-231, MDA-MB-435, Raji and HeLa cells was studied by Western blot analysis using By2 (anti-CD74), 156-3C11 (anti-CD44) and Poly6221 (anti β-Actin). By2 (anti-CD74) is specific for CD74 isoforms 31-45 kDa and 156-3C11 (anti-CD44) is a mouse mAb which detects endogenous levels of total CD44 protein that is specific for most isoforms 80-90 kDa.

Results in figure 3 show that CD74, CD44 and anti β-actin have molecular weights of 33-41 kDa, 80-90 kDa and 45 kDa respectively. β-actin was used as a loading control. Most of the cell lines expressed three different isoforms of CD74 (Fig. 3*A*). However CAMA-1 cells expressed two different isoforms of CD44 (Fig. 3*A*). To evaluate the differences in protein loading during the experiment, the percentage of expression was calculated after the intensity of each band was adjusted according to its respective β-actin band intensity using the Image Studio Lite software (LI-COR Biosciences) (Fig. 3*B*).

Validation study of tumour antigens

In order to validate our study, we investigated the expression of CD74 and CD44 receptors in immortalized normal breast luminal cells (226LDM). Cell-surface and intracellular expression of both CD74 and CD44 was assessed by flow cytometry (Fig 4.*A*). Total protein of CD74 and CD44 was detected by Western blotting and α-Tubulin was used as a loading control (Fig 4.*B*). Confocal laser-scanning microscopy was utilized to study the intracellular staining of CD74 and CD44 in 226LDM cells (Fig 4.*C*). The results show that 226LDM cells do not seem to express CD74 however they do express CD44. Additionally, The expression of CD44 is very weak in the cell surface but it is higher intracellularly.

Colocalization analysis of CD74 and CD44

To investigate whether CD74 and CD44 are co-localized in CAMA-1, MDA-MB-231 and MDA-MB-435 cells, all cell lines were immunostained with an appropriate primary antibody followed by a secondary antibody. CD74 was labeled with FITC Alexa Fluor 488 (green) and CD44 was labeled with Alexa Flour 555 (red) (Fig 5 A.B.C). All breast cancer cells show expression of CD74 and CD44. Colocalization was assessed by merging green and red channels and the Pearson's correlation coefficient was used to analyse the degree of colocalization. The scale lay between -1 and 1, where 1 stands for colocalization, -1 stands for negative colocalization and 0 stands for no colocalization Fig 5D shows the comparison of colocalization in all cell lines versus the negative control. The colocalization of DAPI against FITC was used as a negative control. For more accuracy, 3D images were acquired in stack, with z-direction and segmented by NIS elements to calculate the exact degree of colocalized molecules of CD74 and CD44 against the total volume in each of the images in (Fig 6.A.B.C). The results colocalization were obtained by correlation coefficient and total segmented volume showed that CAMA-1 cells seem to have the highest degree of colocalized CD74 and CD44 molecules followed by MDA-MB-435 and then MDA-MB-231.

Interaction of CD74 and CD44

CD74-CD44 interaction in cell lysates as demonstrated by coimmunoprecipitation showed that there is an interaction between CD74 and CD44 in breast cancer cells (CAMA-1, MDA-MB-231 and MDA-MB-435) (Fig 7.*B*). It was further revealed that

CD74 interacts with both CD44s and CD44v isoforms, in CAMA-1 cells. MDA-MB-231 and MDA-435 cells express only CD44s isoform, which also interact with CD74. To test whether CD44s and CD44v interact with all isoforms of CD74 we performed the Co-IP of CD44-CD74. The results obtained showed that CD44s and CD44v bind only to p41 of CD74 (Fig 7.*C*).

Discussion

Characterization and identification of tumour antigens that are highly immunogenic in human tumours are considered important in tumour immunology (Parmiani et al., 2007). Recent data suggest that CD74 and CD44 can play a significant role in the pathogenesis of various solid tumours (Meyer-Siegler et al., 2004, Meyer-Siegler et al., 2006, Gore et al., 2008). For example, CD74 can mediate MIF binding, and MIF-induced ERK1 and ERK2 kinase phosphorylation requires the co-expression of CD44 (Shi et al., 2006). Greenwood et al. (Greenwood et al., 2012) found that Stat1, Max1 and CD74 are expressed at high levels in triple-negative breast tumours . It has also been shown that CD44 expression is associated with a high rate of cell division and proliferation status (Sneath and Mangham, 1998).

Our data have confirmed that cell-surface CD74 and CD44 molecules are detectable by flow cytometry on CAMA-1, MDA-MB-231 and MDA-MB-435 cells. We also confirmed intracellular expression of CD74 and CD44 by confocal microscopy in all breast cancer cell lines. Our immunoblot results revealed that CAMA-1, MDA, MB-231 and MDA-MB-435 cells express total protein of CD74 and CD44. In addition, our data, obtained from Western blotting and immuneprecipitation, reveal that the CAMA-1, MDA-MB-231 and MDA-MB-435 cell lines express three different isoforms of CD74 (Fig. 4). These findings differ from that made by Verjans et al. [27] who found that MDA-MB-231 and MDA-MB-468 cells express only one isoform of CD74 (Verjans et al., 2009). Similarly, Metodieva et al. (Metodieva et al., 2013) found that MDA-MB-435 express one isoform of CD74.

It is generally accepted that CD74 facilitates export of MHC II/CD74 complexes from the endoplasmic reticulum, while blocking premature loading of peptides on to the MHC II molecules (Neefjes et al., 2011, Genève et al., 2012, Datta et al., 2000, Bergmann, 2012). In humans, 80% of the CD74 protein pool is composed of the p33 isoform, and p35 is considered the most enigmatic isoform [32]. Genève et al. (Genève et al., 2012) have suggested that p33 and p35, together, facilitate antigen presentation, and this process does not require the co-expression of any other CD74 isoform (Beswick and Reyes, 2009, Genève et al., 2012). However, it has been recently suggested that high levels of CD74 expression might prevent tumour antigen presentation by blocking the MHC class II peptide-binding cleft, thus preventing binding of antigenic peptides for

presentation to T cells, rendering tumors less immunogenic (Beswick and Reyes, 2009, Zheng et al., 2012). Moreover, several studies have shown that the level of CD74 expression is proportionality associated with tumour grade (Zheng et al., 2012, Jiang et al., 1999, Chao et al., 2012). Chao et al. (Chao et al., 2012) have shown that upregulation of CD74 expression influences tumor growth and dissemination.

It was previously shown that MIF binds to the CD74 extracellular domain on macrophages and B cells to facilitate signaling (Leng et al., 2003, Gore et al., 2008). However, the interaction of MIF and CD74 requires CD44, due to lack of direct signal in the cell surface domain of CD74 (Starlets et al., 2006). When CD74 forms a complex with CD44, CD74 is modified by the addition of chondroitin sulfate, which is essential for the MIF-induced signaling cascade (Stumptner-Cuvelette and Benaroch, 2002a, Maharshak et al., 2010, Gil-Yarom et al., 2014).

CD44, which is a multifunctional, class I transmembrane glycoprotein that generally acts as a specific receptor for hyaluronic acid, promotes migration in normal cells. It is widely expressed in almost all cells (Jaggupilli and Elkord, 2012), and in its standard or variant form CD44 expression is found in most cancer cells (Sieuwerts et al., 2009). CD44 has been the subject of extensive research for more than three decades because of its role in breast cancer. It has been demonstrated that CD44's role in breast cancer is twofold (Louderbough and Schroeder, 2011). It was shown that CD44 activates and inhibits oncogenic signalling by both promoting and inhibiting tumour progression in response to extracellular cues (Marhaba and Zöller, 2004, Louderbough and Schroeder, 2011). CD44 has also been linked to cancer due to its ability to increase the proliferation and invasion of tumour cells (Götte and Yip, 2006).

We have shown, by flow cytomtery, Western blotting and imaging, that CD44 is expressed in the CAMA-1, MDA-MB-231 and MDA-MB-345 cell lines. Equivalently, Sieuwerts et al. (Sieuwerts et al., 2009) observed that MDA-MB-435, ZR 75 and MDA-MB-231 cells express high levels of CD44. Western blot results have shown that CAMA-1 cells express two different CD44 isoforms: CD44s and CD44v. Jung et al. (Jung et al., 2009) showed that some breast cancer cell lines, such as MDA-MB-468 and SUM149, express several isoforms of CD44s and CD44v. It is possible that CD44 expression in breast cancer is associated with highly aggressive breast tumour subtypes or highly invasive breast cancer cells (Montgomery et al., 2012).

To validate our study we have used immortalized normal breast luminal cells (226LDM) as a model. We have investigated the expression of CD74 and CD44 by flow cytometry, Western blotting and bioimaging. Our findings confirmed that 226LDM cells do not express CD74 on the cell surface and intracellularly, while them do express CD44. This result was expected because it has been confirmed that the expression of CD74 can only be found antigen-presenting cells, including B cells, monocytes, macrophages, dendritic cells, and Langerhans cells (Gold et al., 2011). The presence of CD44 in normal breast cells is normal, since CD44 has a wide range of functions in normal tissues.

In view of the fact that CD74 and CD44 are involved in signalling with MIF (Borghese and Clanchy, 2011), several groups have studied the association of CD74 and CD44 in cancers . Meyer-Siegler et al. (Meyer-Siegler et al., 2004) have shown that CD44 binds to the p35 isoform of CD74 in bladder cancer cells, although this isoform has been suggested to be involved in antigen presentation (Genève et al., 2012). Meyer-Siegler et al. (Meyer-Siegler et al., 2004) did not specify, however, whether bladder cancer cells express different CD74 isoforms. Yet, they found that CD44v interacts with CD74 and MIF, creating a complex in prostate cancer cells (DU-145). However, human benign prostate hyperplasia epithelial cells (BPH-1) and LNCaP prostate cancer cells do not express CD74 on the cell surface. For this reason, both cells do not interact with CD44; however, LNCaP has been demonstrated to interact with MIF (Meyer-Siegler et al., 2006).

Our study has confirmed that CD74 and CD44 are expressed on the cell surface and intracellularly in the breast cancer cell lines CAMA-1, MDA-MB231 and MDA-MB435. Confocal microscopy allowed for the determination that CD74 and CD44 are highly colocalized in cytoplasmic domain in all breast cancer cells. Pearson's correlation coefficient was used to analyse the colocalization of CD74 and CD44 (Bolte and Cordelieres, 2006). The segmentation of the images also showed the percentage of colocalized CD74 and CD44 molecules. The result from Co-IP confirmed that CD74 interacts with CD44s and CD44v. However, CD44s and CD44v interact only with p41, the most abundant isoform of CD74. Shi et al. (Shi et al., 2006) have shown that mammalian COS-7 cells do not bind MIF unless engineered to express CD74 (Leng et al., 2003). It has been confirmed that CD74 alone is sufficient to mediate MIF binding to cells (Shi et al., 2006) however, CD74 alone is insufficient to signal with MIF in the absence of CD44. It has also been demonstrated that although phosphorylation of serine

takes place in the p35 variant of Ii, the short cytoplasmic sequence of CD74 does not appear to signal directly and depends on CD44 (Borghese and Clanchy, 2011). Shi et al. (Shi et al., 2006) showed that COS-7 cells cannot instigate signalling when engineered to express CD74 and a truncated CD44 lacking its cytoplasmic signalling domain. Gore et al. (Gore et al., 2008) have also shown that CD74 binds to CD44 to initiate a signaling pathway involving with MIF in monocytes and macrophages. Furthermore, CD44-deficient cells do not have any activity that might involve in signaling pathway (Gore et al., 2008).

In summary, the present study has confirmed that all above used cell lines express CD74 and CD44. In addition, CAMA-1, MDA-MB-231 and MDA-MB-435 cells have significant amounts of cell surface CD74, an event that was not observed immortalized normal breast luminal cells 226LDM. Colocalization analysis has demonstrated that CD74 and CD44 are colocalized at cytoplasmic membrane suggestion a crucial role in breast cancer cells. In specific, coimmunoprecipitation result has confirmed that CD44 interact only with p41, the most abundant isoform of CD74. The emerging role of CD74 and CD44 in breast tumorigenesis may have selective, therapeutic modalities for women with breast cancer.

Acknowledgment

We would like to thank a Professor Elena Klenova Research Group Coordinator (Molecular and Cellular Bioscience) Director of Essex Biomedical Sciences Institute for providing us with breast normal cells 226LDM. We would like also a Professor Glyn Stanway the Chair Biological Hazards Committee for providing us HeLa cell line

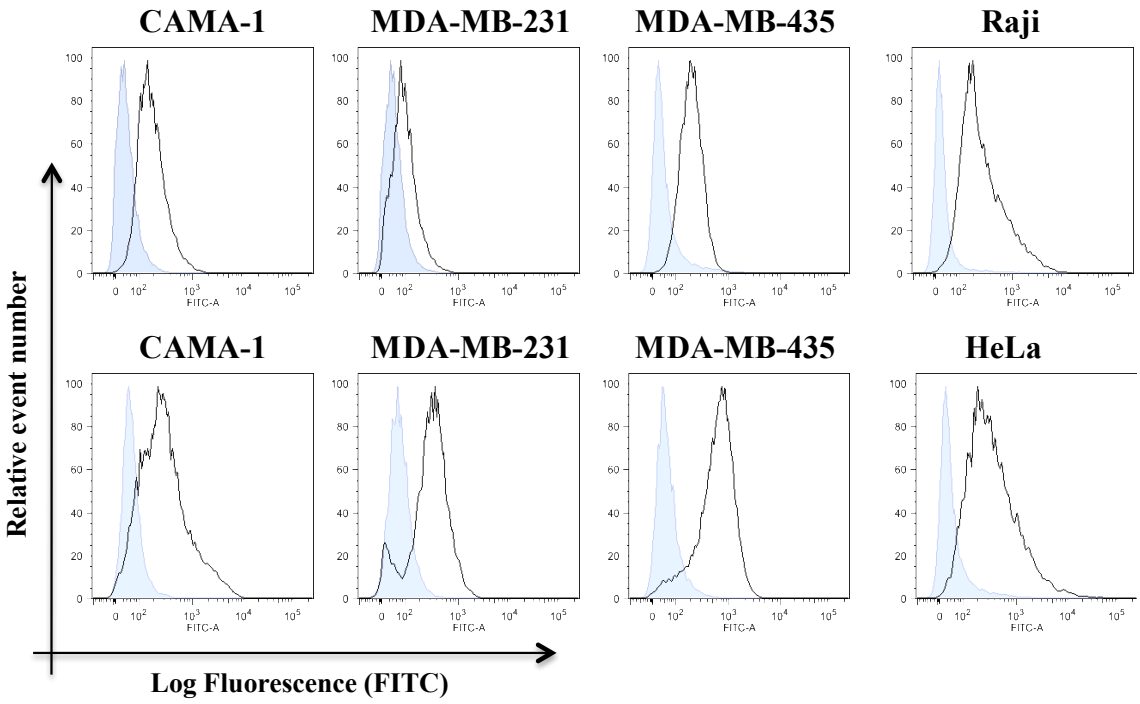

FIGURE 1: Cell surface expression of CD74 and CD44 on CAMA-1, MDA-MB-231, MDA-MB-435, Raji and HeLa cells. All cells were cultured in the appropriate media and were acquired by flow cytometry using By2 (anti-CD74) and 156-3C11 (anti-CD44). (A and B) Empty histograms represent the aforementioned cell lines labeled with anti-CD74 and anti-CD44 antibody. Blue filled histograms show the isotype as negative controls. Cells were labelled with an FITC-labelled secondary anti-mouse antibody. Expression levels were analyzed by flow cytometry (Aria cell sorter) and FlowJo 8.8.6 software was used to analyze the data. Mean fluorescence intensity (MFI) values were measured based on geometric means. Mouse IgG was used as a negative control.

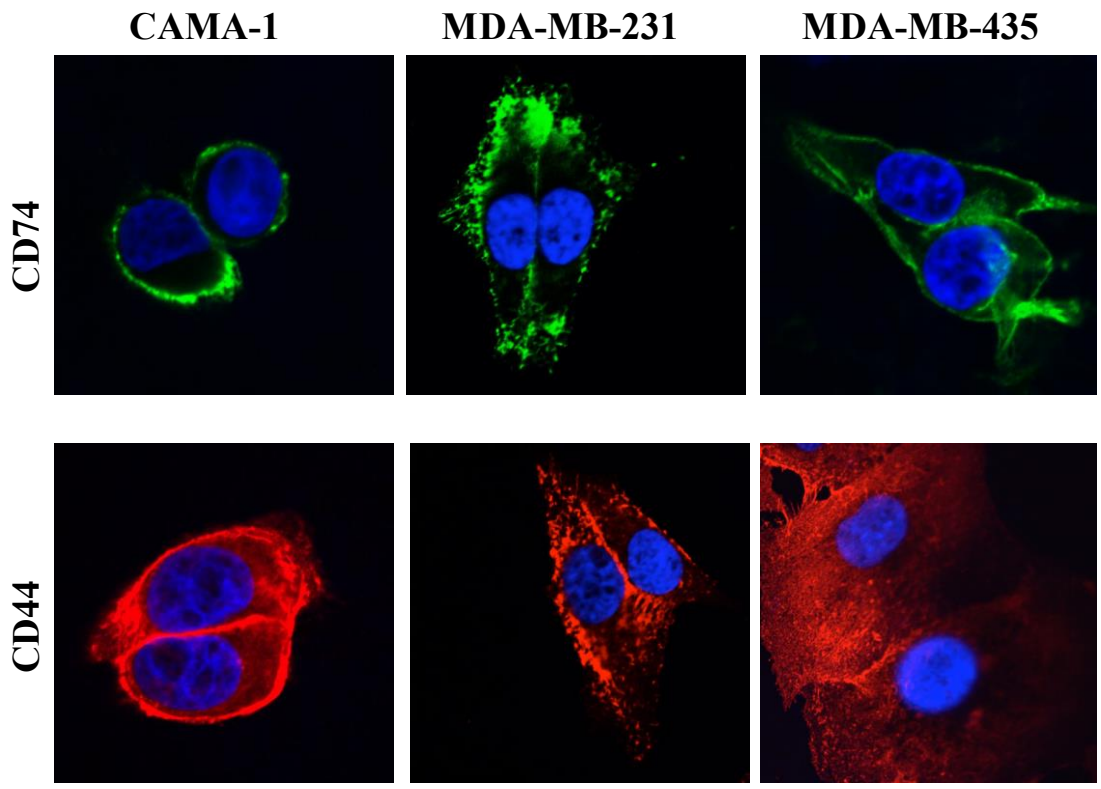

FIGURE 2: Confocal laser scanning microscopy images of intracellular staining of CD74 and CD44 in CAMA-1, MDA-MB-231 and MDA-MB-435 cells. The cells were cultured in LabTek 8-well chambers at a density of 6 x 10^3 cells per well overnight. CD74 was labelled with Alexa Fluor 488 (green) and CD44 with Alexa Fluor 555 (red). 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear staining (blue). Fluorochromes were acquired separately to evaluate the expression of CD74 and CD44 using the image-analysis software platform Fiji. Scale bar 10 µm.

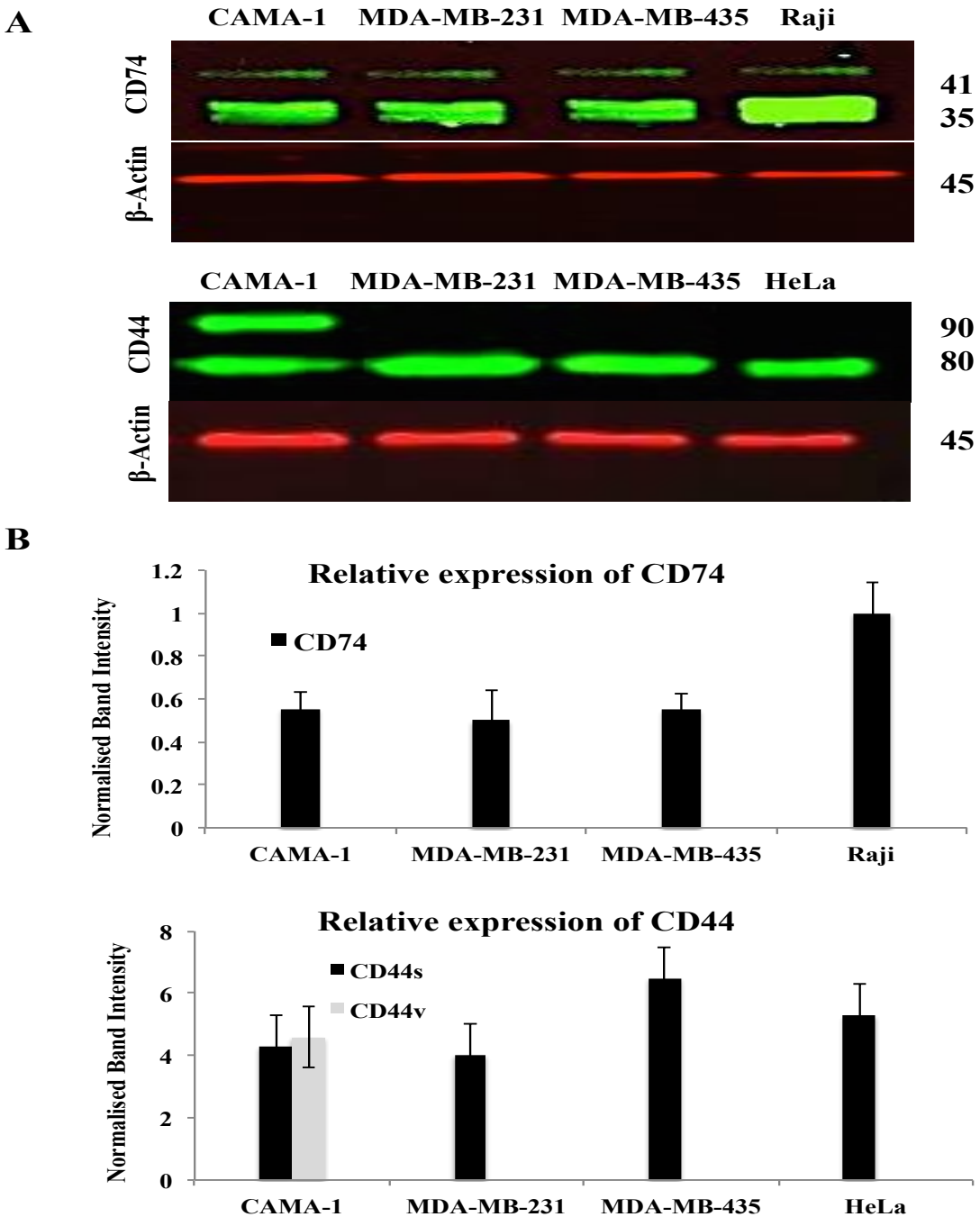

FIGURE 3: Western blotting was utilized to detect CD74, CD44 and β-actin expression in CAMA-1, MDA-MB-231, MDA-MB-435 and Raji cell lines. (A) Primary monoclonal antibodies Poly6221 (anti β-actin as loading control), By2 (anti-CD74) and 156-3C11 (anti-CD44) were used. β-actin is detected at a molecular weight of 50 kDa; CD74 isoforms were detected at molecular weights 33 and 45 kDa, whereas CD44 isoforms were detected at molecular weights 80 and 90 kDa. (B) CD74 and CD44 levels are normalized against β-actin. To account for the difference in protein loading during the experiment, the percentage of expression was calculated after the intensity of each band was adjusted according to its respective β-actin band intensity using the Image Studio Lite software (LI-COR Biosciences).

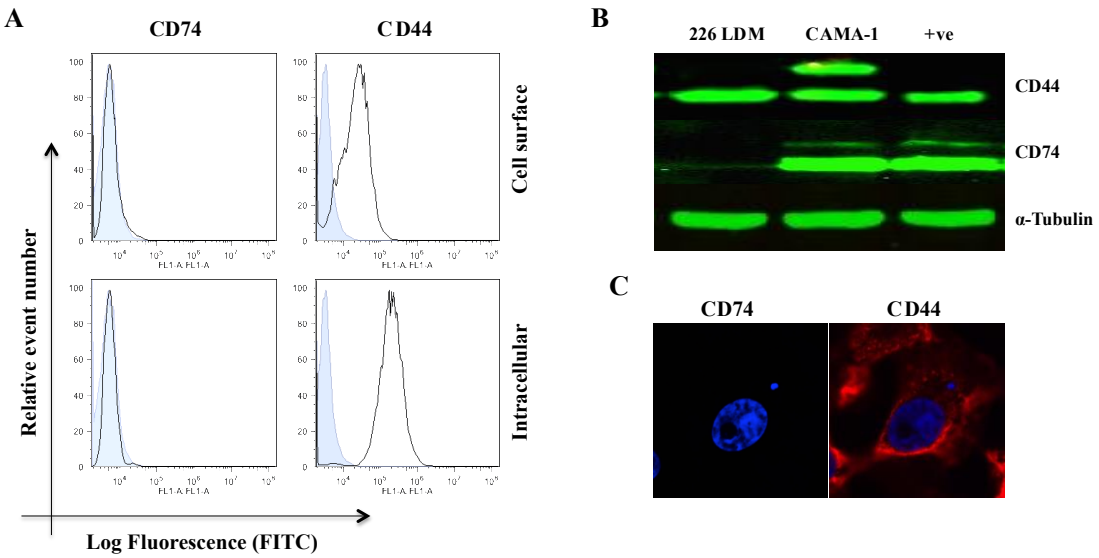

FIGURE 4: The expression of CD74 and CD44 receptors in immortalized normal breast luminal cells (226LDM). (A) Cell-surface and intracellular expression of CD74 and CD44 was acquired by flow cytometry using By2 (anti-CD74) and 156-3C11 (anti-CD44). Grey histograms represent the 226 LDM cells stained with indicated antibody. White histograms show the isotype as negative controls. (B) Total protein of CD74 and CD44 was detected by Western blotting and α-tubulin was used as a loading control. (C) Intracellular staining of CD74 and CD44 in 226LDM cells by confocal laser scanning microscopy. Scale bar 10 µm.

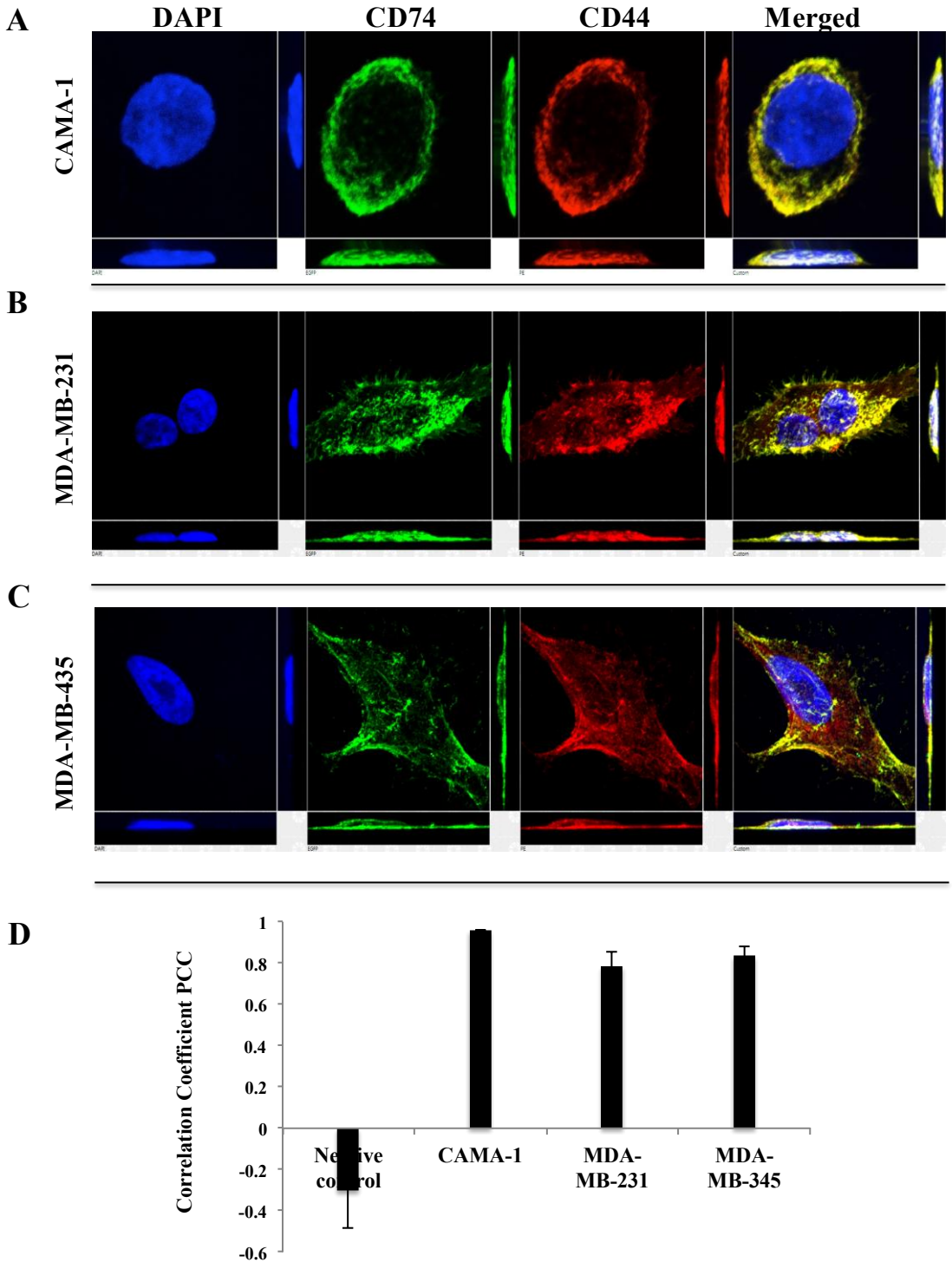

FIGURE 5: Colocalization of CD74 and CD44 at intracellular level on (A) CAMA-1, (B) MDA-MB-231 and (C) MDA-MB-435 cells, determined by confocal microscopy analysis. Yellow/orange fluorescence reveals the potential colocalization of two antigens. 3 D images were acquired in stack, with z-direction step size 0.14 µm using NIS element. Single-plane of z-stack is shown in three directions as xy, yz and zx. (D) Graphical representation of colocalization analysis based on PCC on each cells.

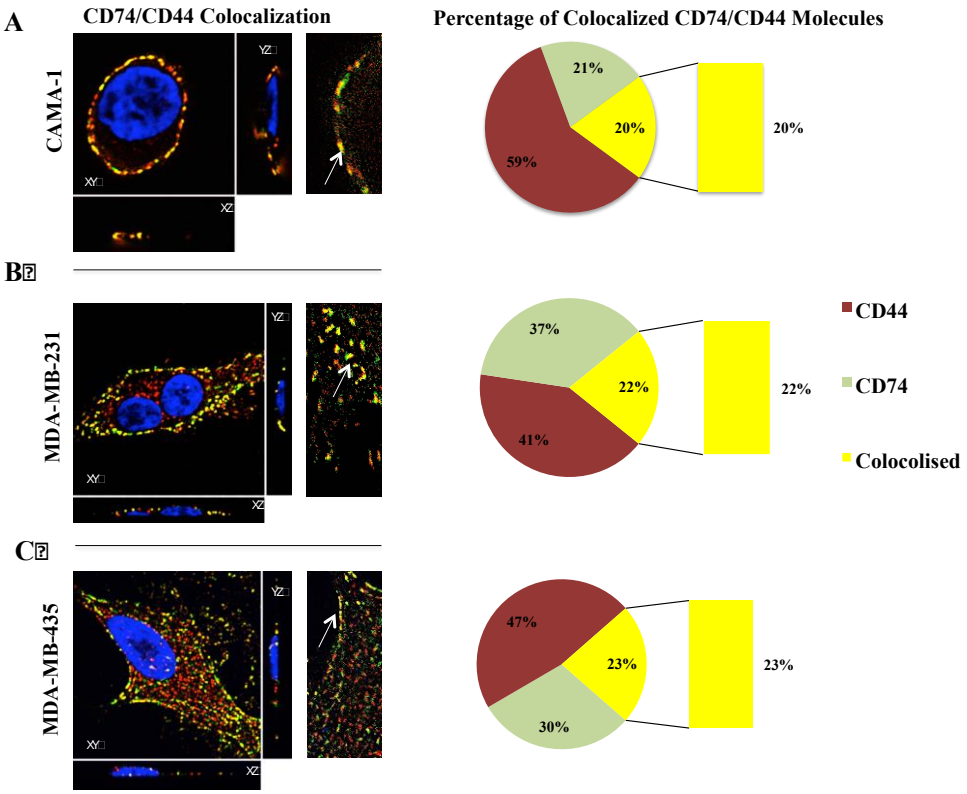

FIGURE 6: The colocalisation of CD74 with CD44 was quantified on segmented 3D images. Maximum intensity projections of CAMA-1, MDA-MB-231 and MDA-MB-435 cells are represented in A, B and C. CD74 was labelled with Alexa Fluor 488 (green) and CD44 with Alexa Fluor 555 (red). Nuclei are visualized with blue 4', 6-diamidino-2-phenylindole (DAPI) staining. Segmentation results from both channels merged with the DAPI. The percentages of the total volumes of CD74, CD44 and the colocalisation from each image are represented by pie charts. The result shows that CAMA-1 cells have the highest correlation between CD74 and CD44 followed by MDA-MB435 and MDA-MB231.

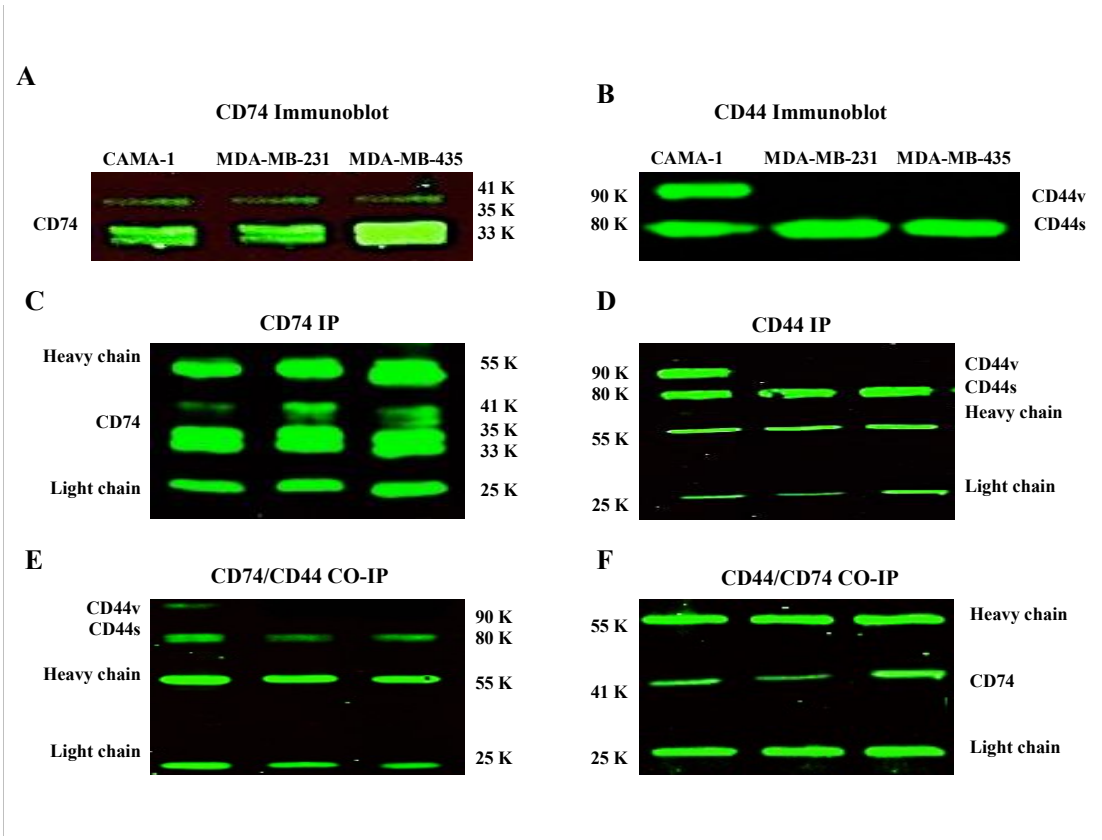

FIGURE 7: Coimmunoprecipitation to study the interaction of CD74 and CD44 in CAMA-1, MDA-MB-231 and MDA-MB-435 cell lines. (A and B) Immunoblot of CD74 and CD44 from breast cancer cell lysate. The blots were probed with either mouse anti-CD74 or anti-CD44 antibodies. (C and D) IP was subjected to pull down either CD74 or CD44.The antibody (Ab) heavy and light chain bands are indicated so that Ab heavy and light chain fragments can be observed at approximately 55 and 25 kDa respectively. (E and F) Co-IP was applied to study the interaction of CD74/CD44 and CD44/CD74. The Co-IP of CD74 /CD44 confirmed that all CD44 isoforms, including CD44s and CD44, interact with CD74. However, Co-IP of CD44/CD74 confirmed that only p41 of CD74 interacts with CD44.

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