The Role of HLA Class I Antibody in Endothelial Cell Activation and Allograft Rejection

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Thesis submitted in partial fulfilment of the requirements of the regulations for the degree of Doctor of Philosophy

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September 2013

Abstract

Background: Antibody-mediated rejection is one of the major causes of acute and chronic rejection. This is mediated by endothelium microvascular inflammation and leukocyte migration to the graft. However, the role of donor specific HLA class I antibodies in inducing allograft rejection in the absence of complement is not fully understood. In this project, the mechanisms by which HLA class I antibodies induce endothelial cell-leukocyte interactions were examined.

Methods: Human microvascular endothelial cells (HMEC-1) were stimulated with HLA class I antibody either mouse monoclonal (W6/32) or from sensitized kidney patients. The activation of cell signaling pathways was examined using Western blotting. The expression of adhesion molecules and chemokines were determined using flow cytometry and q-PCR, respectively. Monocyte adhesion and migration was examined using chemotaxis and flow based adhesion assays.

Results: HMEC-1 cells stimulated with W6/32 antibody showed phosphorylation of a transcription factor CREB by a mechanism dependent on the protein kinase A pathway. W6/32 also induced significant expression of the adhesion molecules VCAM-1 and ICAM-1 ($P<0.001$) in a mechanism dependent on the PI3K/Akt pathway. Additionally, stimulated cells showed significant up-regulation in IL-6, CXCL8, CXCL1, CCL5 and CXCL10. The expression of CXCL8 was significantly reduced by knocking down CREB ($p<0.001$). Conditioned media from W6/32-treated cells was able to induce significant THP-1 monocyte migration compared to control ($p<0.001$). Furthermore, monocytes flowing at 0.5 dyne/cm^2 significantly adhered to HMEC-1 cells-treated with $F(ab)_2$-fragments of W6/32 ($p<0.001$). HLA class I alloantibodies from patients induced phosphorylation of CREB and a significant upregulation of VCAM-1, ICAM-1 and CXCL8. Monoclonal human HLA-B58 antibody was also able to induce CREB phosphorylation.

Conclusion: Exposure of microvascular endothelial cells to HLA class I antibodies induce an activation of endothelial cell signaling that are responsible for upregulation of adhesion molecules and chemokines. These mediators enhance the interaction between donor endothelium and recipient leukocytes during cellular rejection. Strategies that block endothelium-leukocyte interaction might reduce the incidence of allograft rejection and improve allograft survival.

Acknowledgements

I would like to thank the funding body for this project, Ministry of Health in Kingdom of Saudi Arabia. I am really grateful for their continuous help and support through my study in the UK.

I would like to express my gratitude to my supervisors at Newcastle University Professor John A. Kirby and Professor Simi Ali for their help, ideas, support and useful comments through the project.

I would like to express my deepest appreciation to my supervisor Dr. Vaughan Carter, NHS Blood and Transplant, Newcastle, for his collaboration in the project, his useful advices, continuous support and time.

I would like also to thank all members who helped me during the project. From Newcastle University, many thanks to Maureen Kirkley, JK laboratory previous technician, Dr. Jeremy Palmer for his help and advice in antibody purification, Liz Shiells for her help and all staff in flow cytometry facility.

Many thanks go to all staff in NHS Blood and Transplant at Newcastle for their assistance during the project.

I would like to thank Dr. A. A. Alhasan and Dr. Eva-Maria for their help and support.

I would like to express a special gratitude to all my family members for their help, support and understanding.

List of Abbreviations

ADCC	Antibody-Dependent Cell Mediated Cytotoxicity
BCA	BiCinchoninic Acid
BSA	Bovine Serum Albumin
CaMK	calcium-moduline kinases
cDNA	complementary DNA
CNX	Calnexin
CREB	cAMP Responsive Element Binding protein
CREM	cAMP-Responsive Element Modulators
DAPI	4, 6-Diamidino-2-Phenylindole
dcell	donor cells
DMSO	DiMethylSulphOxide
DNA	Deoxyribose Nucleic Acid
EDTA	EthyleneDiamineTetraAcetic acid
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular Regulatory Kinase
ELISA	Enzyme Linked ImmunoSorbent Assay
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein IsoThioCyanate
FSC	Forward side scatter
HLA	Human Leukocyte Antigen
HMEC-1	Human Microvascular Endothelial Cells
HRP	Horse Radish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	IntraCellular Adhesion Molecule-1
IFN-γ	Interferon-γ
JAM-A	Junctional Adhesion Molecule-A
LFA-1	Lymphocyte Function Associated Antigen-1
MAPK	Mitogen Activated Protein Kinase
MFI	Median Fluorescence Intensity
MHC	Major Hisocompatibility Complex
MIC	Major Histocompatibility Complex class I related chain A or B
MSK	Mitogen/Stress Kinase

mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear Factor- κB
NK cells	Natural Killer cells
Nrf2	Nf-E2-related factor 2
OPD	O-phenylenediamine
PBMC	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR-SSP	Polymerase Chain Reaction-Sequence Specific Primer
PCR-SSOP	Polymerase Chain Reaction-Sequence Specific Oligonucleotide Probe
PE	PhycoErythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecules
PI	Propidium Iodide
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PSGL1	P-selectin Glycoprotein Ligand-1
PVDF	Polyvinylidene fluoride
q-PCR	Semi-quantitative Polymerase Chain Reaction
rAPC	recipient Antigen Presenting Cells
RNA	Ribose Nucleic Acid
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering RNA
SSC	Side scatter
STS	Staurosporine
TAE	Tris-acetate EDTA electrophoresis buffer
TAP	Transporter Associated with antigen Processing
TNF-α	Tumor Necrosis Factor-α
TPBS	0.1 % tween 20 in PBS
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4

Table of Contents

List of Figures

1. Chapter One-General Introduction

1.1 Historical background

The significance of the human leukocyte antigen (HLA) system in organ transplantation was initially reported in skin allograft transplantation in 1946. In this year, Peter Medawar performed a set of transplantation experiments where skin transplantation was performed between rabbits. He showed that the transplanted skin graft was rejected by the recipient's immune system in around 10 days. However, transplantation of a second graft from the same donor to the same recipient resulted in graft rejection within 5 days. This observation led to the conclusion that the exposure to the skin graft for the first time immunized the recipient against specific antigens expressed on the transplanted graft. This effect was specific to the donor since skin graft from a third party induced rejection similar to the first skin transplantation. In a further experiment, Medawar injected a buffy coat preparation from the donor's blood to the intradermal region of a recipient followed by skin transplantation from the same donor and subsequently observed rejection within 5 days similar to the immunization with the skin graft. This result suggested that the leukocytes have antigens similar to the antigens expressed on skin graft, enhancing graft recognition and rejection (Medawar, 1946a; Medawar, 1946b). Several groups later identified these antigens as transplant antigens. Therefore, the main concept in transplantation has been identified, in which matching for these antigens might improve graft survival (Rapaport et al., 1962). In a parallel manner, it was demonstrated that transplantation of the graft between identical siblings survived longer than that between non-identical which may differ in their HLA expression (Amos *et al.*, 1969). These observations were confirmed by *in vitro* examination where leukocytes from HLA identical sibling failed to react in mixed lymphocyte reaction (Bach and Amos, 1967).

In terms of alloantibody formation, early studies by Peter Gorer showed that skin allograft into mice induced the formation of alloantibody that agglutinated donor red blood cells (Gorer, 1936). However, the contribution of HLA antibodies in inducing skin allograft rejection was excluded when the passive transfer of alloantibodies to mice allo-transplanted with skin graft did not induce rejection (Makela and Mitchison, 1965). This was accompanied by the observation that T-cell deficient mice do not reject an allograft and the transfer of sensitized lymphocytes caused rejection. However, the concept of allograft rejection by humoral response was renewed by

Winn *et al*. in 1973 when a passive transfer of mouse anti-rat sera to rats transplanted with a skin allograft for 2 weeks induced complete graft failure within 2 days (Winn *et al.*, 1973). The failure to induce rejection in the previous model was due to delay in the blood supply to the transplanted skin for the first few days following transplantation; reducing the interaction between the donor endothelium and the antibody (Jeannet *et al.*, 1970). From animal model to humans, it was shown that the presence of circulating donor-specific antibody in sensitized patients induced hyperacute rejection of transplanted kidneys and cross matching of patients serum with donor cells reduced the incidence of the rejection (Kissmeyer-Nielsen *et al.*, 1966). The involvement of *de novo* synthesized antibody in inducing chronic renal rejection was then investigated in cross match negative transplant (Jeannet *et al.*, 1970). Terasaki *et al*. and other groups showed later that circulating HLA antibodies is associated with increased frequency of graft rejection (Terasaki *et al.*, 2007).

1.2 Forms of antibody-mediated rejection

Antibody-mediated rejection can occur in all types of solid organ transplantation including lung, heart, kidney, pancreas and liver. The incidence of organ rejection is accelerated by but not restricted to pre-sensitization. There are four different forms of antibody-mediated rejection which might occur separately or sequentially. They are classified according to histological findings and time frame rather than mechanisms into; hyperacute, acute, chronic and subclinical rejection (Cai and Terasaki, 2005).

1.2.1 Hyperacute antibody-mediated rejection

Before the introduction of cross-matching techniques, hyperacute rejection was the most common and first described form of antibody-mediated rejection post-transplantation. This type of rejection occurs as a result of the presence of preformed circulating donor-specific antibody either against ABO blood group antigens or HLA antigens. Aggressive immune response occurs following antibody binding and complement system activation. As a consequence, histological and morphological damage to the transplanted allograft occurs within a few hours after transplantation. In kidney transplantation, hyperacute rejection is manifested by a rapid swelling of the kidney with hemorrhagic cortical necrosis and vascular thrombosis. Neutrophil and platelet margination along the endothelial cells of the peritubular capillaries and the glomeruli is also seen. With increasing sensitivity of cross-matching techniques and

improvement in blood grouping, the incidence of hyperacute rejection post transplantation is minimal (Puttarajappa *et al.*, 2012).

1.2.2 Acute/accelerated antibody-mediated rejection

Accelerated rejection occurs as a result of the presence of memory cells before transplantation while acute antibody-mediated rejection occurs as a result of the development of antibodies specific to donor antigens following transplantation. These antibodies are mostly formed in pre-sensitized patients who received a preconditioning treatment before transplantation or in patients with a low antibody titer that cannot be detected by screening methods (Sellares *et al.*, 2012). The development of these antibodies induces a microvascular endothelium inflammation associated with leukocyte infiltration resulting in disturbance of organ function. Aggregation of different subsets of leukocytes can be seen in peritubular capillaries of transplanted kidneys associated with fibrinoid necrosis of arteries (Sis *et al.*, 2012), myocardial capillaries in transplanted hearts (Loupy *et al.*, 2011), interacinar capillaries in transplanted pancreas (Drachenberg *et al.*, 2011) and alveolar capillaries in transplanted lungs (Girnita *et al.*, 2006). The common diagnostic criteria in all solid organ transplants include; circulating donor specific antibody, C4d deposition, evidence of histological tissue damage associated with graft dysfunction. Positive staining for C4d may occur in 95% of patients with donor specific HLA class I antibodies as a finger print of complement activation on the endothelium (Cornell *et al.*, 2008). C4d is an inactive fragment of the C4b which forms by C4 breakdown, an essential component of the classical pathway. It has a hidden sulfhydryl group which can form thioester bonds with tissue proteins, forming a long lasting complex which can be detected by immunohistochemistry (Feucht *et al.*, 1991; Nickeleit and Mihatsch, 2003). This type of rejection might occur within months to a year after transplantation (Halloran *et al.*, 2010). It was estimated that around 25% of all acute rejection cases are due to antibodies specific to HLA molecules (Cornell *et al.*, 2008).

1.2.3 Chronic antibody-mediated rejection

This late form of rejection occurs sometimes concurrent with the formation of *de novo* antibodies specific for mismatched donor HLA antigens. Although the term "chronic" is more related to morphological changes rather than time, it has been estimated that circulating HLA antibodies can be followed by chronic rejection within 6 months to 8

years (Lee *et al.*, 2002). Chronic rejection is characterized by serious irreversible morphological and structural changes to transplanted allografts leading to dysfunction and failure (Sellares *et al.*, 2012). In kidney transplantation, chronic rejection is diagnosed by histological evidence of arterial intimal and interstitial fibrosis, duplication of glomerular basement membrane, thickening of peritubular capillaries, loss of endothelial fenestrations with cytoplasmic swelling and C4d deposition with the evidence of circulating donor specific HLA antibodies (Solez *et al.*, 2007). C4d deposition can be used either as a diagnostic marker or as a predictive factor for subsequent chronic-antibody mediated rejection. Deposition of C4d in peritubular capillaries was detected in 61% of biopsies with transplant glomerulopathy of which 88% had donor specific HLA antibodies (Mauiyyedi *et al.*, 2001). In another study, 34% of kidney patients diagnosed with transplant glomerulopathy stained positive for C4d in which the deposition of this factor preceded chronic rejection in around 80% of the studied cases (Regele *et al.*, 2002). However, emerging data show that transplant glomerulopathy can occur without any evidence of C4d deposition in the presence of donor specific HLA antibody. The C4d-negative antibody-mediated rejection phenotype shows also irreversible histological changes such as endothelial swelling and duplication of basement membrane. Therefore, different studies reported the insensitivity of C4d as an antibody-mediated rejection marker (Sis *et al.*, 2007; Einecke *et al.*, 2009).

1.2.4 Subclinical antibody-mediated rejection

The concept of subclinical antibody-mediated rejection has been reported by Haas and Gloor to define a group of patients with histological graft injury accompanied with normal allograft function (Gloor *et al.*, 2006; Haas *et al.*, 2007). This has been observed in renal transplant patients with a positive cross-match who have been transplanted after de-sensitisation process, or patients with low level donor specific antibodies. Histological examination of biopsies shows positive C4d staining and leukocyte infiltration in peritubular capillaries associated with glomerulitis. This type of silent rejection can be a predictive factor and a risk factor for the development of subsequent allograft dysfunction. In a study of 54 kidney transplanted patients with donor specific HLA antibodies, 3 month screening demonstrated that around 31% of patients showed histological evidence of subclinical antibody-mediated rejection with ~49% of these having negative C4d staining. This led to a high rate of allograft

dysfunction associated with the development of transplant glomerulopathy within 1 year after transplantation (Loupy *et al.*, 2009).

1.3 Clinical relevant antibody in solid organ transplantation

Different types of antigens can elicit humoral immune responses following transplantation leading to allograft dysfunction mediated by antibody formation. ABO blood group antigens, HLA, non-HLA antigens and autoantigens can induce the formation of corresponding antibodies following transplantation affecting long term allograft survival.

1.3.1 Blood group antibodies

Shortage in donor organs for transplantation renders the transplantation across the ABO barrier an attractive option in particular for urgent transplantation (Takahashi and Saito, 2013). ABO antigens are carbohydrate antigens expressed on almost all cells including endothelial cells and erythrocytes (Cai and Terasaki, 2005). In ABO-incompatible transplantation, the presence of a high titer of donor specific anti-ABO antibodies at the time of transplantation can cause an immediate hyperacute rejection within hours following reperfusion of the graft. The new immunosuppressive treatments and immunoadsorption permits ABO-incompatible transplantation with a minimum risk of hyperacute rejection. However, the reformation of anti-A or anti-B antibodies following transplantation can induce acute-antibody rejection; inducing allograft dysfunction (Westphal *et al.*, 2013).

1.3.2 HLA antibodies

Donor specific HLA antibodies are a major cause of chronic allograft rejection (Cai *et al.*, 2013). Around 15 to 23% of kidney transplanted patients develop donor specific HLA antibody within 8 months to 4 years following transplantation, with similar frequency in other solid organ transplantation (Kaneku, 2012). The presence of both donor specific HLA class I and HLA class II antibody is associated with poor allograft outcome (Otten *et al.*, 2012). The development of antibodies specific to donor HLA class I antigens can induce acute and chronic allograft rejection in kidney transplantation (Varnavidou-Nicolaidou *et al.*, 2004). Recent study showed that renal transplanted patients who developed HLA class I but not HLA class II donor specific antibodies experienced episodes of rejection within one year following transplantation

(Thiyagarajan *et al.*, 2012). However, other studies showed the negative impact of donor specific HLA class II antibody on allograft function. In kidney transplantation, *de novo* synthesized donor specific HLA class II antibody is associated with a high incidence of acute rejection and a reduction in 5-year allograft survival (Freitas *et al.*, 2013). The development of donor specific HLA class II antibody has also a negative impact on allograft function in lung (Lobo *et al.*, 2013), heart (Ticehurst *et al.*, 2011) and liver transplantation (O'Leary and Klintmalm, 2013).

1.3.3 Polymorphic non-HLA antibodies

Transplantation from a fully HLA matched donor does not guarantee the absence of antibody-mediated rejection episodes following transplantation. Donor specific antibodies against polymorphic non-HLA molecules have been reported to induce allograft rejection. Major Histocompatibility Complex (MHC) class I related chain A or B (MICA-MICB) are polymorphic cell surface glycoproteins expressed on endothelial, epithelial cells, keratinocytes and monocytes but not on peripheral blood lymphocytes. They do not present antigens to T-cells but they bind specific NK receptors. In contrast to HLA class I molecules, the polymorphism in MIC antigens is distributed along the molecules in addition to the polymorphism in the transmembrane region (Zou and Stastny, 2011; Liu *et al.*, 2012). Transplantation across MICA sensitization has been associated with poor allograft outcome as observed in lung transplantation (Lyu *et al.*, 2012). In addition, the development of these antibodies post-transplantation is associated with acute rejection in heart and kidney transplantation (Cox *et al.*, 2011; Zhang *et al.*, 2011a). Antibodies against minor histocompatibility antigens can also induce allograft rejection and reduce graft survival. Minor histocombatibility antigens are polymorphic self-peptides encoded by autosomal gene or Y-chromosome related gene. They are presented by cell surface HLA molecules in HLA allele-specific manner. Examples of minor histocompatibility antigens are HA-1, HA-2, HA-3 and HA-8. The significant of these antigens in solid organ transplantation becomes apparent in a male to female transplantation (Dierselhuis and Goulmy, 2009).

1.3.4 Autoantibodies

The formation of antibodies against self-antigens is implicated in the pathogenesis of chronic allograft rejection. Autoantibodies can form as a result of graft remodeling or

injury following transplantation which might expose cryptic antigenic determinants of self-antigens to T-cells inducing activation. In addition, cytokines profile following transplantation can reduce the activity and number of self-tolerant T-regulatory cells; increasing the autoimmune response (Tiriveedhi *et al.*, 2012a). The development of anti-K-α1 tubulin and collagen V antibodies is associated with the pathogenesis of chronic lung transplant rejection (Goers *et al.*, 2008; Iwata *et al.*, 2008). Patients diagnosed with chronic cardiac vasculopathy can develop autoantibodies to vimentin, myosin and collagen V (Nath *et al.*, 2010). In kidney transplantation, the development of antibody to angiotensin II type 1 receptor and glomerular basement membrane self-proteins such as agrin have been shown to induce chronic nephropathy (Joosten *et al.*, 2005; Dragun *et al.*, 2012).

In addition, alloimmune responses such as the development of donor specific HLA class I antibody might also result in the formation of autoantibodies accelerating chronic allograft rejection (Fukami *et al.*, 2009). Fukami *et al.* (2009) showed that the passive transfer of MHC class I antibody in an animal model of lung transplantation induced chronic rejection manifested by irreversible morphological changes of the grafts and the formation of antibodies to self-antigens collagen V and K-α1 tubulin. Lung transplanted patients diagnosed with bronchiolitis obliterans syndrome have been shown to develop autoantibodies to self-antigens within few months following the development of donor specific HLA antibody (Saini *et al.*, 2011). Autoantibodies can bind their targets inducing cell signaling and activation. Anti-vimentin antibodies induce P-selectin exocytosis from cardiac microvessel endothelial cells, which increases the activation and adhesion of platelets to endothelial cells (Leong *et al.*, 2008). Expression of growth factors, proliferative and inflammatory proteins are also increased; accelerating chronic injury (Tiriveedhi *et al.*, 2012b).

1.4 Mechanism of HLA class I antibody formation

The process of antibody formation is a process that requires the activation of naive B-lymphocytes (primary sensitization) or memory B-lymphocytes (second exposure) to produce antigen-specific antibodies. Naive B-cells, a part of adaptive immune response, mature in the bone marrow and migrate to secondary lymphoid organ, lymph node and spleen. In these organs, antigen presenting cells, dendritic cells, present foreign antigens to CD4+ T-helper cells which induce the activation of naive B-cells in the extrafollicular T-cell area (Stegall *et al.*, 2009). Allorecognition of the

mismatched HLA antigens by T-cells occurs by either a direct or indirect mechanisms. In the direct pathway, T-cells recognize directly the mismatch HLA antigens expressed on the donor passenger antigen presenting cells; CD8+ T-cells recognize HLA class I while CD4+ T-cells recognize HLA class II antigens. In the indirect mechanism, the recipients' T-cells recognize peptides from processed donors' antigens presented by the recipients' antigens-presenting cells (Ali *et al.*, 2013). However, the formation of donor specific HLA class I antibodies results mostly due to indirect recognition of HLA class I-related peptides presented by recipients' HLA class II to CD4+ T-helper cells, which are necessary for B-cells activation and antibody formation. A recent study using an animal model showed that CD4+ T-cells activated by the indirect pathway against MHC class I peptides induce an isotype class switch and produce long-lived plasma cells maintaining alloantibody production (Conlon *et al.*, 2012).

Activated B-cells differentiate into short-lived plasma cells, plasmablasts, producing low affinity antibodies. The formation of a high affinity antigen-specific antibody requires the clonal expansion and differentiation of activated B-cells into high affinity antibody producing plasma cells. This differentiation occurs in a compartmentalized structure called the germinal centers which results from the interaction of activated B-cells with follicular dendritic cells and T-follicular helper cells. In germinal center, B-cells undergo a rapid division, somatic hypermutation and affinity maturation to produce clones of B-cells specific to target antigen. B-cells expressing new immunolglobulin on the cell surface are assessed for antibody affinity by interacting with antigen-specific dendritic cells. Clones producing low binding affinity antibody receives an apoptosis signal while clones producing a high-affinity antibody receive survival signals (Clatworthy *et al.*, 2010).

Surviving B-cell clones can enter an isotype switching process, for example from IgM to IgG, with the aid of helper T-cells. The resultant activated B-cells differentiate into either plasma cells producing high affinity antibody or memory B-cells. A small proportion of plasma cells migrate to the bone marrow where they maintain the serum level of allospecific antibodies (Radbruch *et al.*, 2006). During secondary stimulation, memory B-cells, which account 40-60% of peripheral B-cells, convert to plasma cells producing a high affinity antibody specific to target antigen (Han *et al.*, 2009). Whether the T-helper cells are required for the conversion of memory B-cells to plasma cells is unknown. In murine MHC class I mismatched transplantation,

germinal center formation and humoral responses can develop in the graft itself in addition to secondary lymphoid organs; inducing chronic allograft rejection (Thaunat *et al.*, 2005). Mechanism of antibody formation is summarized in Figure 1.1.

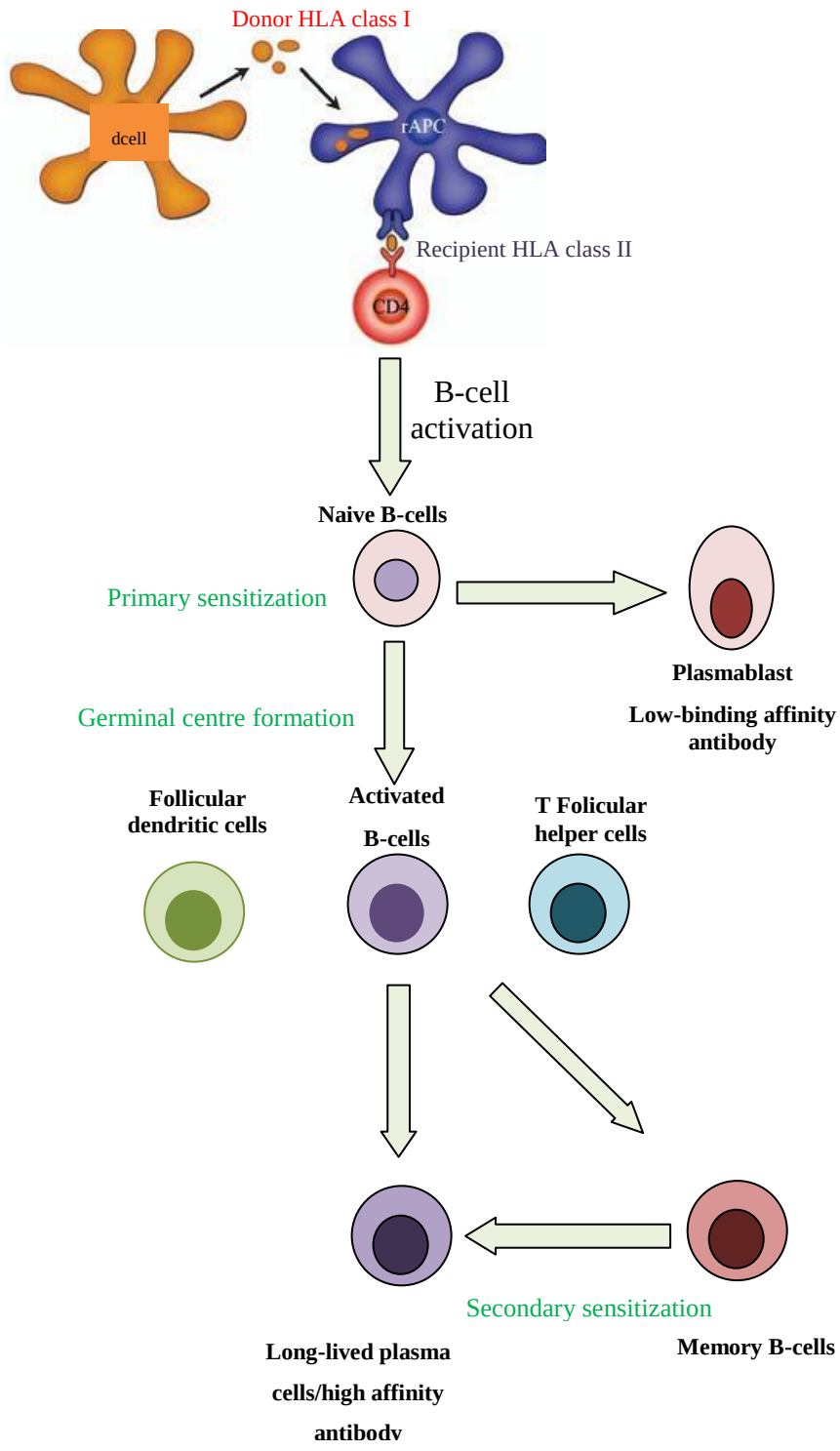


Figure 1.1: Mechanism of sensitization and antibody formation.

Abbreviations: dcell, donor cells. rAPC recipient antigen presenting cell., adapted from (Stegall *et al.*, 2009).

1.5 Mechanisms of antibody action

The main target of antibodies, whether preexisting or synthesized *de novo*, is the endothelial cells of the transplanted graft. Once antibodies interact with corresponding antigens on endothelial cells, they may mediate endothelial damage through either complement dependent or complement independent mechanisms (Colvin and Smith, 2005). This may be related to the ability of specific antibody subclass to fix complement.

1.5.1 Complement-dependent mechanism

Complement fixation is an important pathway by which circulating allospecific antibody induces hyperacute or acute allograft rejection in highly sensitized patients (Tinckam and Chandraker, 2006). In pre-sensitized patients, antibodies can bind directly to the transplanted graft activating the complement system which induces irreversible ischemic damage (Ota *et al.*, 2005). Different IgG subclasses have different potential to activate the complement system, with IgG3 the strongest complement fixing antibody followed by IgG1, IgG2 and IgG4, respectively. The complement system can be activated by either classical, alternative or lectin-dependent pathways; however, the contribution of alternative or lectin pathways to allograft injury is not fully understood. In the classical pathway, the C1s fragment from C1 mediates the cleavage of C4 into two fragments C4a and C4b as shown in Figure 1.2. The inactivation of C4b can occur in the presence of factor I into C4d which covalently binds to tissue as a 'foot-print' for complement activation. Activation of the 'common pathway' occurs through C5 cleavage into C5a and C5b. The latter interacts with other complement components to form the membrane attack complex (C5b-C9) on endothelial cells causing cell lysis. In animal models, C4d deposition has been correlated with the level of circulating alloantibody as well as morphological changes to transplanted allograft (Minami *et al.*, 2006; Qian *et al.*, 2006). Therefore, an agent that impedes complement activation such as anti-C5 monoclonal antibody can improve allograft survival as shown in previous study (Wang *et al.*, 2007a). In addition to formation of the membrane attack complex, complement fragments initiate cascade of events enhancing inflammatory responses and leukocyte migration to the transplanted graft.

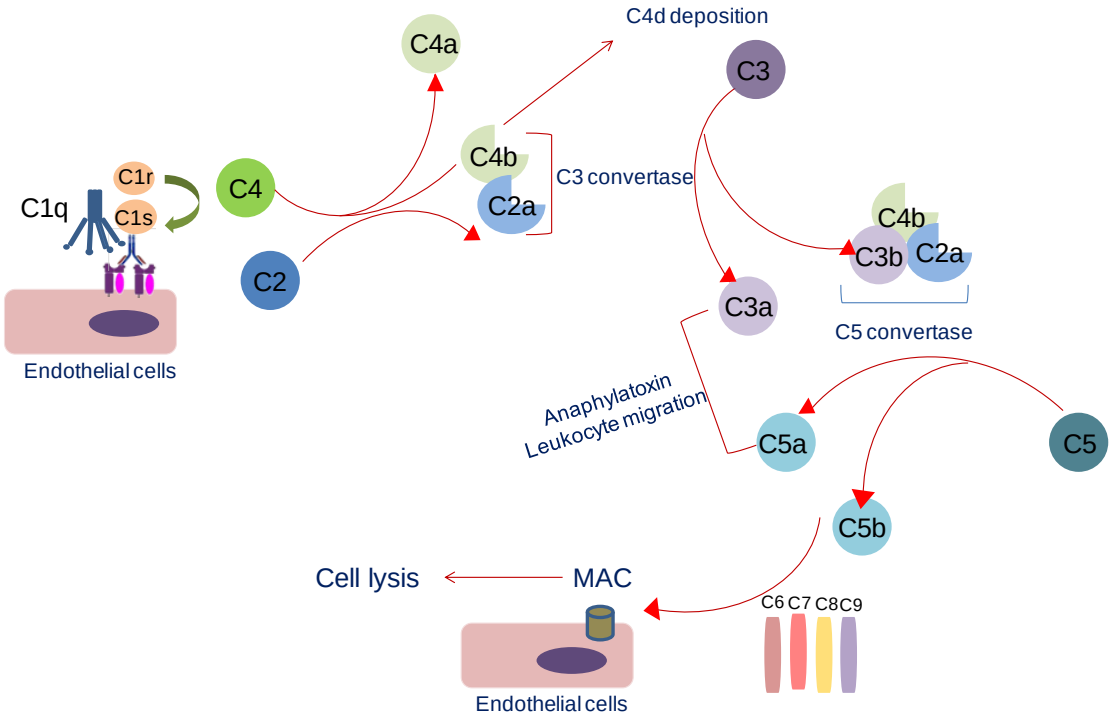


Figure 1.2: Antibody-mediated classical complement activation.

Complement fragments such as C3a/C5a are chemoattractants that increase the expression of different endothelial adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), and enhance the production of various cytokines (IL-6, IL-1α) and chemokines (CXCL8, CCL2). These secretory proteins recruit inflammatory cells (neutrophils) to transplanted grafts and accelerate the rejection process (Colvin and Smith, 2005). In addition, complement fragments bind to corresponding receptors on endothelial cells and can be recognized directly by neutrophils or dendritic cells which express corresponding surface receptors for these fragments (Deitch and Mancini, 1993). Accumulation of soluble-sublytic doses of the C5b-C9 complex may also induce endothelial cell activation and adhesion molecule expression. Inflammatory responses driven by complement components can induce endothelium injury, exposing the basement membrane to the circulation. This effect activates the adhesion of circulating platelets and increase the secretion of von Willebrand factor enhancing thrombosis formation and vascular occlusion (Wehner *et al.*, 2007).

1.5.2 Complement-independent mechanism

Antibody can mediate allograft rejection independent of the complement system by two different mechanisms these are; antibody-dependent cell mediated cytotoxicity (ADCC) or by direct endothelial cell activation. Fc-receptors on natural killer (NK) cells and macrophages are involved in ADCC (Lee *et al.*, 2007). The interaction of Fcγ-receptors on NK cells (FcγRIII, CD16) with the Fc fragment of the immunoglobulin bound to the corresponding antigens on the endothelium results in NK cell activation. This binding enhances the production of various cytokines such as IFN-γ and TNF-α, expression of the T-bet transcription factor, release of cytotoxic granules such as perforin and granzyme B and the activation of death receptors. This results in cell apoptosis and lysis of antibody-coated cells and activation of macrophages' phagocytic process (Cornell *et al.*, 2008). This mechanism plays a role in the development of hyperacute, acute and chronic rejection (Uehara *et al.*, 2005).

In xeno-transplantation, this mechanism has a significant impact in inducing an immediate hyperacute rejection in the presence of non-complement fixing anti-Gal antibody (Yin *et al.*, 2004), while the depletion of NK cells prolongs the graft survival and reduces acute antibody-mediated rejection (Chen *et al.*, 2006a). The contribution of NK cells in the development of chronic-antibody mediated rejection has been

examined in animal models. Passive transfer of MHC class I antibody to NK cell-deficient mice significantly reduced the development of chronic vasculopathy of transplanted hearts either in the presence or absence of complement (Hirohashi *et al.*, 2012). This effect results from the interaction of the Fc-fragment with Fc-receptors on NK cells as confirmed using $F(ab)_2$-fragments. In kidney transplant patients, the expression of NK cells-related transcripts was significantly increased in biopsies diagnosed with chronic antibody-mediated rejection and not on those diagnosed with T-cell mediated rejection (Hidalgo *et al.*, 2010).

1.6 Antibody-mediated graft accommodation

In 1980, a group of scientists showed condition for organ survival following ABO-incompatible kidney transplantation, a transplantation normally associated with hyperacute rejection. They demonstrated that kidneys transplanted from A or B blood group donors to O blood group recipients, after removal of recipients' circulating anti-ABO antibodies by plasmapheresis or immunoprecipitation, survived and functioned normally without any signs of rejection. In addition, when the antibodies levels returned to normal levels or higher, the organ functioned normally without any deterioration. This condition is termed as organ accommodation (Lynch and Platt, 2008). Graft accommodation is different from immunological tolerance, where a recipient's immune system becomes unresponsive to the transplanted graft as is the case of self-antigens (Koch *et al.*, 2004). In xeno-transplantation, depletion of complement along with immunosuppressive treatments induced accommodation of hamster hearts transplanted into rats. The accommodated organs showed expression of anti-apoptotic genes, including Bcl-2, Bcl-xl and A20 and the hemoxygenase enzyme. In addition, the expression of non-complement fixing IgG antibodies and Th2 helper cell-associated cytokines such as IL-4, IL-10 and IL-13 was detected in accommodated organ, while rejected organs showed Th1 helper cell-associated cytokines such as IL-2, IFN-γ and TNF-α along with other classes of IgG antibodies (Bach *et al.*, 1997). An *in vitro* study using endothelial cells showed similar results (Hancock *et al.*, 1998).

Allograft accommodation has been reported in a situation of a sensitization to HLA class I antigens. Antibody concentration plays a fundamental role in inducing this phenomenon. Highly sensitized patients who have undergone preconditioning treatment to remove circulating HLA class I antibodies showed up-regulation of anti-

apoptotic protein, Bcl-xl in the accommodated kidney allografts (Salama *et al.*, 2001). In addition, exposure of HUVEC endothelial cells to sub-saturating concentration of HLA antibodies from those patients induced the expression of Bcl-xl, reduced the expression of adhesion molecules and protected the cells against complement-mediated cell lysis (Salama *et al.*, 2001). By using human aortic endothelial cells, treatment with a sub-saturating concentration of HLA class I antibodies provided protection against cell lysis induced by saturating concentration of antibody in the presence of complement; while exposure to saturating levels of antibodies induced apoptosis through caspase-3-dependent mechanism. In addition, accommodated cells showed an increase in the expression of protective genes such as Bcl-2, Bcl-xl and heme-oxygenase-1 and a phosphorylation of pro-apoptotic gene Bad at serine residues inhibiting its activity. Furthermore, sub-saturating concentration of antibody induced a significant decrease in the expression of adhesion molecules ICAM-1 and VCAM-1. This resulted in a decrease in the adhesion of leukocytes to stimulated endothelial cells under static conditions (Narayanan *et al.*, 2004).

Expression of other antioxidant genes in endothelial cells stimulated with sub-saturating concentration of HLA class I antibody has also been reported. Human aortic endothelial cells stimulated with sub-saturating concentration of HLA class I antibodies expressed ferritin and were protected against complement attack (Iwasaki *et al.*, 2010). In a model of heart transplantation in sensitized recipients, pre-exposure to a low concentration of HLA class I antibodies induced a significant reduction in the expression of adhesion molecules, cytokines and chemokines and significant increase in the expression of the hemoxygenase-1 and anti-apoptotic genes (Fukami *et al.*, 2012).

1.7 Human leukocyte antigen system

1.7.1 Human leukocyte antigen classes

Human leukocyte antigens (HLA) are highly polymorphic cell-surface glycoproteins. They are encoded by a region located on the short arm of chromosome six. The HLA system is a part of the MHC which was identified originally in mice as a gene dense area involved in graft rejection (Klein, 1986). Over 200 genes are located in this region and more than 40 genes encode the human leukocyte antigens. The comparable region for leukocyte antigens in mice is located in chromosome 17 and called H-2 (Klein, 1986). The MHC locus in human, consisting of 3.5 million base pairs, codes for two different classes of HLA antigens (class I and class II) and other immunological proteins. The region codes for HLA class I and class II antigens consists around one third of this region spreading over that sequence. This region also codes for various proteins responsible for various immune responses apart from the HLA system. These proteins are encoded by genes on HLA class III region. These include complement components, some hormones and various intracellular peptides involved in antigen processing. The presence of these genes in the MHC region might suggest a functional association or a shared transcriptional regulation (Klein and Sato, 2000).

1.7.2 Human leukocyte antigen structures

HLA class I and class II are different on a structural and functional basis, the structure of both molecules is shown in Figure 1.3. HLA class I consists of α-polypeptide heavy chain that non-covalently binds to a light chain, β2-microglobulin, to form a 45 kDa molecule. The heavy chain is encoded on HLA region located on chromosome 6 while β2-microglobulin is encoded on chromosome 15. The single membrane bound heavy chain consists of three alpha domains which are divided into the peptide-binding domains α1 and α2 and an immunoglobulin like domain α3. The α3 domain is non-covalently bound to β2-microglobulin. The heavy chain is anchored in the cell membrane by a membrane spanning region (25 amino acids) following the α3 region, followed by a short cytoplasmic tail (30 amino acids). The α1 and α2 domains, through their N-terminal domains, fold together to form α-helical sides and eight anti-parallel β-pleated sheet floors which can bind 8-10 amino acids or peptide protein fragments (Bjorkman *et al.*, 1987). HLA class II genes on chromosome 6 encode for

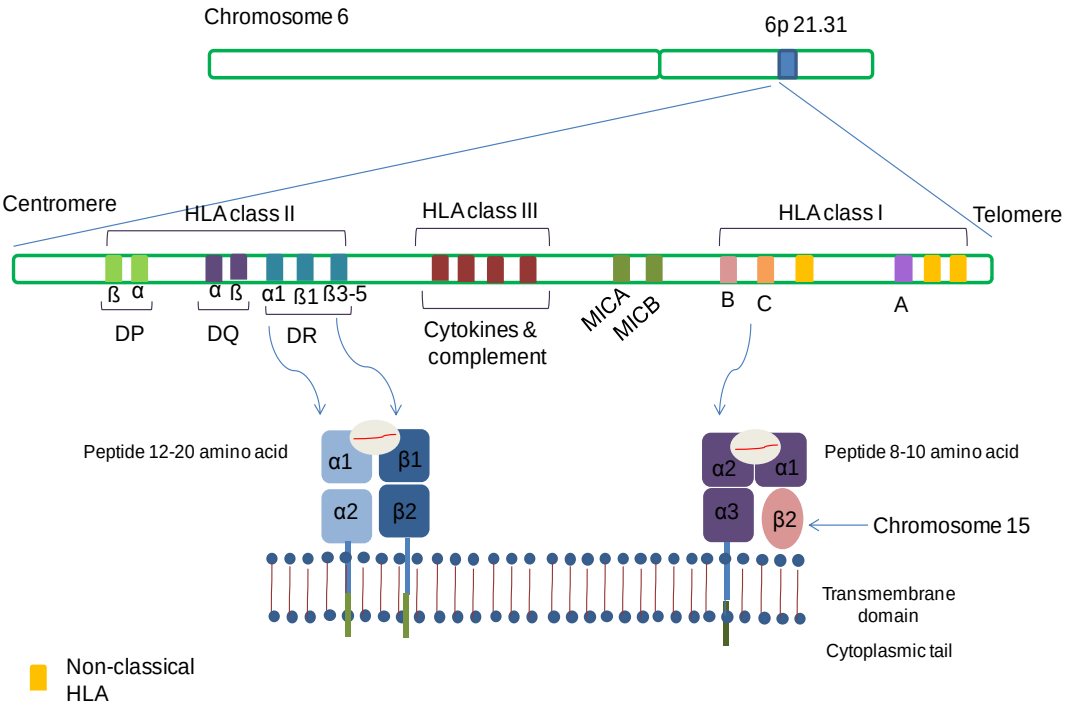


Figure 1.3: Location and structure of human leukocyte antigens.

both α and β polypeptide chains. Each molecule contains four domains: the peptide binding domain (α1 and β1), the immunoglobulin like domain (α2 and β2), the transmembrane region and the cytoplasmic tail. There are several genes that encode classical and non-classical HLA class I molecules. HLA-A, B and C are classical molecules which are involved in antigen presentation, and HLA-D, E, F, G, H, J, K and L are some of the non-classical HLA molecules which are less involved in peptides presentation (Klein and Sato, 2000). Both HLA class I and II occur in a multiple alleles diversity. The polymorphic regions are mainly found in the peptide binding groove resulting from amino acid substitution in α1 and α2 regions for class I and α1 and β1 regions for class II. This substitution and polymorphism occurs as a result of sequence diversity in the second exon for class II and in the second and third exon for class I. In January 2012, around 7130 classical HLA class I and class II alleles were reported in the IMGT/HLA database (report v.3.7.0, http://www.ebi.ac.uk/imgh/hla/index.html). At least three isotypes of HLA class I antigens are expressed by individual cells with two allelic forms for each isotype forming a maximum of six classical HLA class I molecules expressed on the cell surface. HLA class II molecules follow the same pattern of the expression in which six HLA class II antigens are expressed on the cell surface. Therefore, individuals can express 12 HLA antigens, six for HLA class I and six for HLA class II. This highly polymorphic characteristic of HLA system ensures efficient immune responses against various types of antigenic peptide.

1.7.3 HLA class I assembly and antigen precursors

The fully structured HLA class I molecules on cell surfaces are formed by sequential steps involving the formation of heavy and light chain heterodimers followed by peptide loading. The formed class I molecule is then transported to the cell surface to present peptides to circulating T-cells. The loading peptides play an important role in maintaining the stability of HLA class I heterodimer which is rapidly dissociated in the absence of peptides (Townsend *et al.*, 1989; Ljunggren *et al.*, 1990). HLA class I molecules can be loaded with two types of peptides. Normally, endogenous cytosolic proteins are trimmed into small peptides and uploaded into HLA class I molecules. By expressing self-peptides, HLA class I molecules continuously familiarize the circulating cells with intracellular proteins, especially newly synthesized proteins. This occurs during the continuous turn-over process for class I antigens in which various

proteins are loaded each time into newly synthesized HLA class I antigens (Heath *et al.*, 1989; Jardetzky *et al.*, 1991; Corr *et al.*, 1992). In addition, extracellular proteins can be internalized into the cytosol by phagocytosis, processed and presented by HLA class I molecules in a process called cross presentation (Segura and Villadangos, 2011). When cells become infected with virus, class I molecules are loaded with peptides from virus proteins allowing the cells to be identified by cytotoxic T-cells. The circulating T-cells are able to discriminate between HLA class I molecules loaded with self peptides and those loaded with foreign peptides. The educational process of naive T-cells to discriminate between self and non-self peptides occurs in the thymus where the negative and positive selections occurs using self HLA class I molecules displaying various self peptides (Bevan, 1977; Van Kaer *et al.*, 1992).

The process of HLA assembly occurs in different cell compartments and is started by the synthesis of heavy and light chains. Synthesized heavy chains are translocated into the lumen side of the endoplasmic reticulum (ER) and bound to a membrane chaperone called calnexin (CNX). This binding enhances the formation of intrachain disulfide bonds and chain folding. The heavy chain has two intrachain disulfide bonds: one is within the transmembrane IgG-like domain and the other at the peptide binding groove, connecting the α2 region to the β-sheet forming the floor of the groove (Williams, 2006). The folded heavy chain then binds to β2-microglobulin forming a class I heterodimer. The formation of the heterodimer is followed by the replacement of CNX with other protein called calreticulin (CRT). The heterodimer with the CRT is then integrated into peptide loading complex that is containing various proteins for peptide loading. This complex contains transporter associated with antigen processing (TAP), tapasin and ER thiol-oxidoreductase ERp57. All these proteins are involved in the assembly of HLA class I in the ER by a process called complex assembly pathway (Cresswell *et al.*, 2005).

In this process, intracellular proteasome degrades various cytosolic proteins into small peptides suitable for the loading into the groove of the class I molecules. These peptides are transported from the cytosol into the ER lumen in association with TAP heterodimer. This contains two subunits TAP1 and TAP2 which belongs to the transmembrane protein family called ATP-binding cassette family responsible for protein transportation across cellular membranes in an ATP-dependent manner (Scholz and Tampe, 2005). Each of these subunits contains a cytosolic domain and a

transmembrane domain containing multiple membrane spanning regions. Each cytosolic domain of TAP1 and TAP2 is attached to ATP molecule; forming a conformational unit. This unit is hydrolyzed during the translocation process to provide energy required for this process (van Endert *et al.*, 2002). Peptides translocated into the ER might also be trimmed by ER associated aminopeptidase (ERAAPs) into 8-10 peptides that are suitable for HLA class I peptide binding pocket (Serwold *et al.*, 2002).

The peptides and heterodimer are brought into close proximity for loading process. The tapasin protein plays an important role in tethering the TAP complex to the heavy: light heterodimer. The tapasin binds the transmembrane domain of TAP1 and TAP2 stabilizing TAP1/TAP2 heterodimer and maintaining efficient peptides transport (Leonhardt *et al.*, 2005; Procko *et al.*, 2005). The tapasin, through its N-terminal residue, interacts also with the HLA heterodimer through α2 and α3 regions of heavy chain, connecting the heterodimer to the TAP complex (Lehner *et al.*, 1998). CRT and thiol oxido-reductase ERp57 have a role in maintaining an optimal function for the peptide loading complex and in promoting HLA class I folding (Gao *et al.*, 2002; Garbi *et al.*, 2006; Ireland *et al.*, 2008). CRT is a lectin that specifically binds to the carbohydrate side chain of HLA molecules via monoglucosylated N-linked glycans (Williams, 2006). ERp57 forms a conjugate with the tapasin protein to provide protection for the heavy chain-disulfide bond at α2 region from the reductive activity of the ERp57 reductase before peptide loading (Kienast *et al.*, 2007). Following the interaction of class I heterodimer with disulfide linked tapasin and ERp57 the peptide loading is occurred. Fully structured HLA class I structure is then dissociated from the complex and transported to the cell surface through Golgi apparatus in a process require ATP degradation (Knittler *et al.*, 1999). Figure 1.4 shows a diagram for HLA class I assembly.

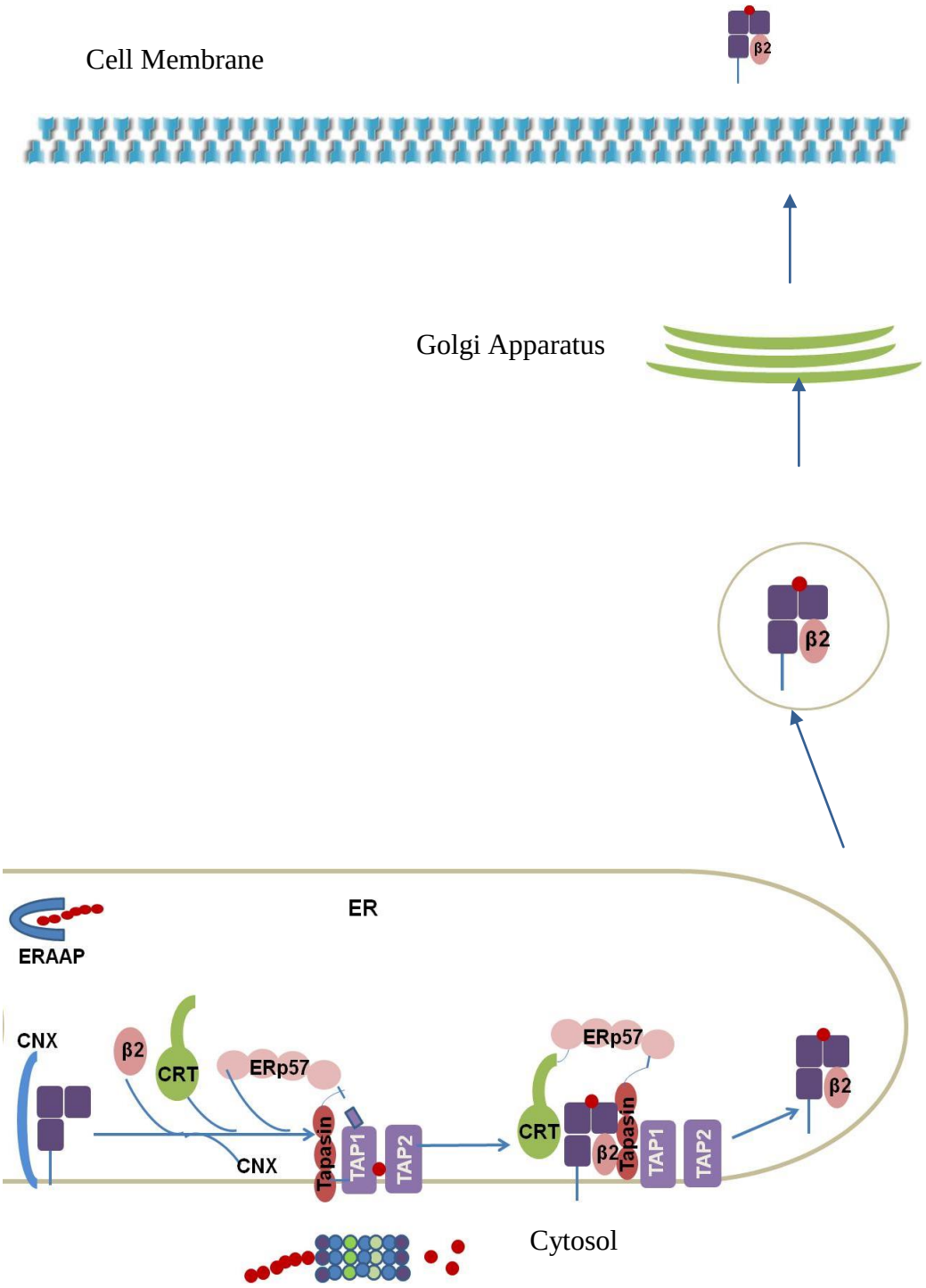


Figure 1.4: HLA class I molecule assembly.

ER, Endoplasmic Reticulum, ERAAP, ER associated aminopeptidase, CNX, calnexin, CRT, calreticulin, B_2m, B_2-microglobulin, TAP, transporter associated with antigen processing, ERp57, ER thiol oxidoreductase, adapted from (Cresswell *et al.*, 2005).

1.8 HLA class I antibody and endothelial cell signaling

1.8.1 Mechanism of HLA class I antibody signaling

The mechanism by which HLA class I molecules transmit intracellular signaling upon cross linking by antibody is still a matter of debate. This is attributed to the fact that HLA class I molecules contain only a short cytoplasmic tail that do not attach to any cytoplasmic signaling motif such as G-protein or intrinsic kinases. Therefore, different studies have suggested that the activation of cell signaling mediated by HLA class I molecules occurs through the interaction with other transmembrane molecules which have a capacity to trigger intracellular signaling pathways (Jindra and Reed, 2007). Early studies showed that HLA class I molecules are able to interact with insulin (Ramalingam *et al.*, 1997) and epidermal growth factor receptors (Schreiber *et al.*, 1984) augmenting their signaling processes. In addition, deletion of the intracytoplasmic domain of HLA class I molecules from Jurkat T-cells did not effect their capacity to transduce signaling after cross linking with antibodies (Gur *et al.*, 1999). However, a recent study showed that cross linking HLA class I antigens with corresponding antibodies induced cell signaling through interaction with cell-surface ß4-integrin expressed by endothelial cells. The interaction with integrins was mediated by the cytoplasmic tail of HLA class I antigens. This result contradicts previous finding on T-cells. In addition, knockdown of this integrin induced inhibition in cell signaling and proliferation in response to cross-linking with HLA class I antibody (Zhang *et al.*, 2011b).

1.8.2 HLA class I antibody and Akt pathway

Akt is a serine/threonine kinase that is responsible for phosphorylation of target proteins at serine and threonine residues. It is also referred to as protein kinase B and was originally identified as an oncogene in a mouse leukemia virus, AKT8 (Bellacosa *et al.*, 1991). Three isoforms of Akt have been identified with specific tissue distributions which are Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Akt1 is predominant in brain, heart and lung while Akt2 is expressed in skeletal muscle and embryonic fat, and Akt3 is mostly expressed in brain, kidney and embryonic heart (Altomare *et al.*, 1995; Brodbeck *et al.*, 1999). The activity of Akt proteins is highly regulated by its phosphorylation at two residues; threonine 308 and serine 473. This phosphorylation is mediated primarily by phosphatidylinositol-3-kinases (PI3Ks)

attached to tyrosine kinase or G-protein coupled receptors. Upon exposure to stimuli, activation of PI3Ks results in the phosphorylation of cell-membrane phospholipid, phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate resulting in the recruitment of 3-phosphoinositide dependent-protein kinase (PDK) and Akt to the cell membrane. PDK translocation to the cell membrane results in the phosphorylation of Akt at serine and threonine residues in stimuli dependent manner. PDK might interact with other cellular proteins to induce the phosphorylation of Akt at serine residue such as integrin-linked kinase (Delcommenne *et al.*, 1998). In endothelial cells, the Akt pathway is downstream of many cytokines, growth factors and various cellular stimuli, regulating the activity of various proteins involved in cell survival, apoptosis, proliferation and angiogenesis (Fujio and Walsh, 1999; Jiang and Liu, 2009; Li *et al.*, 2012).

Exposure of human aortic endothelial cells to mouse HLA class I antibody induces the phosphorylation of Akt at serine residue 473 and threonine residue 308 (Jin *et al.*, 2004; Narayanan *et al.*, 2004). The phosphorylation of Akt in turn induces elevation of the expression of the pro-survival protein Bcl-2, suggesting enhancement of cell survival signals. The pro-survival activity of Bcl-2 is regulated by the phosphorylation of pro-apoptotic gene Bad at serine residues 112 and 136 by Akt, enhancing their dissociation from Bcl-2 proteins and binding to 14-3-3 cytoplasmic protein, resulting in the inhibition of cell apoptosis. Human cardiac allograft biopsies from patients diagnosed with antibody-mediated rejection also showed immunostaining for Bcl-2 expression (Jin *et al.*, 2004). However, the protective effect of Akt might be correlated with antibody concentration, where low concentration of antibody is responsible for inducing cell-survival signaling (Jin *et al.*, 2004). Moreover, pre-exposure of human aortic endothelial cells to sub-saturating concentration of HLA class I antibody provides a protection against the damaging effects induced by the exposure to saturating concentration of HLA class I antibody in a mechanism involving the expression of anti-apoptotic genes Bcl-2, Bcl-xl and hemoxygenase-1 as well as Bad phosphorylation at serine residues 112 and 136. These might occur as a result of the activation of Akt at serine residue 473 (Narayanan *et al.*, 2004).

1.8.3 HLA class I antibody and mammalian target of rapamycin (mTOR) pathway

Mammalian target of rapamycin (mTOR) is a large serine/threonine kinase complex with central function in cell growth and proliferation signaling network. mTOR exists in two different multi protein kinase complexes. mTORC1 consists of mTOR protein kinase, regulatory associated protein (raptor) and G protein Beta subunit Like protein (GBL). The kinase activity of this complex is inhibited by rapamycin, an immunosuppressive drug used as an anti-proliferative agent. The second complex is mTORC2 which consists of mTORC2, sin1, GBL and rictor, and its kinase activity is insensitive to rapamycin treatment (Sarbassov *et al.*, 2005). Activation of these complexes leads to the increase in ribosomal biosynthesis and translation of mRNAs to proteins essential for transition from G1 to S phase, which enhances cell cycle progress and proliferation (Hidalgo and Rowinsky, 2000).

Ligation of HLA class I molecules on endothelial cells by corresponding antibodies enhances mTORC1/C2 complexes assembly, promoting cell proliferation. HLA class I antibody induces mTORC1 phosphorylation at serine residue 2448 which, in turn, phosphorylates p70 S6 kinase (S6K) at threonine residues 389, 421 and 424 leading to the phosphorylation of two downstream regulatory proteins, S6 ribosomal protein (S6RP) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Jindra *et al.*, 2008c). Conversely, mTORC2 mediates the phosphorylation of Akt at serine residue 473 and the expression of Bcl-2 protein (Jindra *et al.*, 2008c). In addition, the activation of mitogen-activated protein kinase such as extracellular regulatory kinase (ERK1/2) has been shown to occur downstream of mTORC2 in response to cross linking of HLA class I molecules with antibodies (Jindra *et al.*, 2008b). ERK is a serine/threanine kinase involved in cell proliferation, differentiation, survival and arrangement of actin cytoskeleton (Chang *et al.*, 2003; Jindra *et al.*, 2008a). The phosphorylation of mTOR, S6K, S6RP, ERK, and Akt has been detected in endothelial cells from cardiac transplanted allografts in mice transfused with donor specific MHC class I antibodies after transplantation (Jindra *et al.*, 2008a).

1.8.4 HLA class I antibody and cytoskeleton proteins

Endothelial cytoskeleton proteins are crucial not only in maintaining cell shape and integrity but also in mediating cell responses to extracellular stimuli. The cytoskeleton of endothelial cells consists of actin microfilaments, microtubules and intermediate

filaments. These structures are essential for maintaining cell-cell and cell-matrix interactions, regulating endothelial permeability and mediating cell migration and proliferation. These proteins are connected to various intracellular proteins creating an intracellular network signaling (Prasain and Stevens, 2009). Actin in resting endothelial cells is present in the cortical fiber rim, which is reorganized to form stress fiber in response to inflammatory agonists, therefore, reducing cell-cell adhesion (Dudek and Garcia, 2001). The attachment of endothelial cells to the extracellular matrix is mediated by the formation of focal adhesion regions where transmembrane integrins bound to extracellular matrix are attached to intracellular actin through various linking proteins. Reorganization of endothelial cytoskeleton and the formation of stress fiber might occur in response to extracellular stimuli such as growth factors and cytokines. Focal adhesion kinase (FAK) is a cytoplasmic non-receptor tyrosine kinase that is responsible for focal adhesion assembly. It is involved in the organization of actin cytoskeleton and regulation of growth factor and integrin signaling, cell survival, proliferation and cell migration (Wu, 2005). This protein induces cell proliferation through phosphorylation and interaction with other signaling molecules such as src family kinase proteins, src and fyn, and paxillin (Schaller, 2001).

Emerging studies show the crucial role of endothelial cytoskeleton-related proteins in cell signaling-mediated by HLA class I antibodies. Ligation of HLA class I antigens with antibodies has been shown to phosphorylate src protein kinase, fyn, paxillin and PI3K/Akt in FAK dependent manner (Jin *et al.*, 2002; Jin *et al.*, 2007). Exposure to HLA class I antibodies induce the formation of actin stress fiber and cell proliferation which is abrogated in the absence of FAK (Jin *et al.*, 2007). In addition, disturbance of the actin cytoskeleton by chemical agents inhibits the ability of HLA class I antibodies to induce FAK phosphorylation, which may suggest the importance of cell cytoskeleton in HLA class I molecule-mediated signaling (Jin *et al.*, 2002). Moreover, ERK1/2 and other proteins belong to Rho GTPase family are essential for the formation of endothelial stress fiber in response to HLA class I antibody (Lepin *et al.*, 2004; Ziegler *et al.*, 2012a). Recently, a number of endothelial cytoskeleton proteins has been identified after treatment with HLA class I antibody, although the exact role of these proteins in the development of allograft rejection is still not clear (Ziegler *et al.*, 2012b).

1.8.5 HLA class I antibody and growth factors

HLA class I antibodies induce cell proliferation by mediating expression of various growth factors and their receptors. Expression of platelet-derived growth factor, epidermal growth factor, insulin-like growth factor 1, and basic fibroblast growth factor (FGF) increases in airway epithelial cells in response to HLA class I antibody, inducing fibroblast proliferation and development of bronchiolitis obliterans syndrome (Jaramillo *et al.*, 2003). A previous study showed that ligation of HLA class I molecules with antibodies increases the expression of fibroblast growth factor receptors in smooth muscle cells (Bian *et al.*, 1998). This effect is enhanced by IFN-γ treatment, which increases the expression of HLA class I molecules (Bian and Reed, 1999). Furthermore, cross linking of HLA class I antigens on endothelial cells stimulates the redistribution of FGF receptors from intracellular stores to the plasma membrane in a concentration dependent manner (Harris *et al.*, 1997). Binding of FGF to its receptors enhances auto-phosphorylation of a tyrosine residue in the cytoplasmic domain of the receptor which in turn may activate mitogen activated protein kinases (MAPK) and nuclear transcription factors involved in cell proliferation. Kerby *et al.* showed an increase in the expression of both FGF and FGF receptor in the vessels of cardiac and renal allografts undergoing chronic rejection (Kerby *et al.*, 1996).

Further study by Bieri et al. (2009) showed that clustering of HLA class I molecules by antibodies induced a production of vascular endothelial growth factor from activated endothelial cells, which might activate vascular endothelial growth factor receptor in an autocrine mechanism. Activated receptors can either enhance cell proliferation or increase membrane permeability through degradation of VE-cadherin, which results in impaired adherence at junctions (Bieri *et al.*, 2009).

1.9 HLA class I antibody induce activation of transcription factors

Exposure of endothelial cells to anti-HLA class I antibody can induce the activation of various transcription factors. The activation of these factors could induce genes responsible for either cell protection or cell damage. Activation of transcription factors during antibody-mediated rejection might lead to modulation in the expression of related genes contributing to modulation of allograft function. Various transcription factors are activated in response to stimulation with HLA class I antibody including; nuclear factor- κB (NF-κB) (Smith *et al.*, 2000), E2F (Nath *et al.*, 1999), Nf-E2-

related factor 2 (Nrf2) (Iwasaki *et al.*, 2010) and cAMP Responsive Element Binding protein CREB (Naemi *et al.*, 2011).

1.9.1 HLA class I antibody and NF-κB

Treatment of human umbilical vein endothelial cells (HUVEC) with monoclonal HLA class I antibody (W6/32) and allospecific class I antibody from sensitized patients induced NF-κB activation which was augmented by cross-linking with rabbit anti-mouse antibodies (Smith *et al.*, 2000). Nuclear factor-κB is a transcription factor that is normally sequestered in the cytosol as an inactive hetero- or homo-dimer. Five different proteins can be combined to form these dimers which are termed, p50, p52, p65 (RelA), Rel B and c-Rel; p50/p65 is the most common dimer. The catalytic activity of NF-κB is inhibited by a family of 7 inhibitory proteins. The predominant inhibitory protein is IKBα which binds the p50/p65 dimer. NF-κB-mediated gene transcription is a sequential process involving the phosphorylation and subsequent separation from inhibitory proteins followed by translocation to the nucleus, where it up-regulates various genes (May and Ghosh, 1998). The contribution of activated NF-κB to the transcription of cytokines, chemokines, growth factors, adhesion molecules, and cytoprotective enzymes, highlights its role in organ transplantation (Tsoulfas and Geller, 2001). NF-κB can be activated early after transplantation in response to oxidative stress during ischemia/reperfusion injury. In addition, pro-inflammatory cytokines such as TNF-α and IL-1, which might be induced after transplantation, can also enhance NF-κB activation (Li and Verma, 2002). However, this activation can induce either organ dysfunction or organ protection.

In ischemia reperfusion injury, NF-κB is elevated after a few hours of reperfusion and accompanied by up-regulation of iNOS activity, suggesting cytoprotective mechanism (Hur *et al.*, 1999). In contrast, genes associated with endothelial cell activation after exposure to allospecific antibodies mostly contain an NF-κB binding site. These genes have a role in leukocyte migration and inflammation during allograft rejection and include E-selectin, ICAM-1, VCAM-1, IL-6, CXCL8 and CCL2 (Hou *et al.*, 1994; Jahnke and Johnson, 1994; Parry and Mackman, 1994). The transcription of VCAM-1 in response to cytokine stimulation was mediated by the binding of NF-κB and interferon regulatory factor-1 (IRF-1) to their consensus sequences on the promoter of the VCAM-1 gene (De Caterina *et al.*, 2001). However, this effect might be cell type and stimulus specific. In bovine aortic endothelial cells, stimulation with TNF-α

produced an up-regulation of VCAM-1 mediated by CREB activation through p38 MAPK (Ono *et al.*, 2006). On the other hand, cell surface expression of endothelial ICAM-1 is mediated by CREB and NF-κB binding in response to TNF-α stimulation (Hadad *et al.*, 2011).

1.9.2 HLA class I antibody and E2F transcription factor

Cross linking HLA class I molecules with antibodies has been associated with the activation of transcription factor, E2F. E2F is a family of transcription factors consisting of nine subclasses. They regulate the expression of various genes involved in cell cycle progression, apoptosis and cell differentiation. E2F-1 is the most prominent member mediating both apoptosis and cell proliferation. The transcriptional activity of this protein is regulated by binding to retinoblastoma (Rb) proteins (Chen *et al.*, 2009). HLA class I antibody-induced E2F activation by inhibiting the activity of Rb protein, rendering the E2F factor active to enhance the expression of proliferative proteins (Nath *et al.*, 1999). However, further studies are needed to explore the effects of this activation.

1.9.3 HLA class I antibody and c-Jun transcription factor

C-Jun is a transcription factor that is a member of activator protein-1 complex. It is present as either a homodimer or heterodimer with other members such as ATF-2 or c-Fos. They regulate the expression of various genes involved in cell proliferation, apoptosis, fibrosis, differentiation and inflammation (Angel and Karin, 1991). The catalytic activity of c-Jun is regulated by the phosphorylation by c-Jun N-terminal kinase. Activation of c-Jun factor has been correlated with the inflammation and fibrosis associated with many human kidney diseases (De Borst *et al.*, 2007). In cellular rejection, the phosphorylation of this factor has been observed in leukocytes infiltrating kidney allografts, and both endothelial cells and infiltrating leukocytes in the case of antibody-mediated rejection (Kobayashi *et al.*, 2010). In addition, the degree of c-Jun phosphorylation in peritubular endothelial cells from chronic antibody-mediated rejection biopsies correlates negatively with allograft function (Kobayashi *et al.*, 2012). Inhibition of c-Jun activation by inhibiting c-Jun N-terminal kinase activity prolonged allograft survival in a model of cardiac transplantation (Tabata *et al.*, 2007).

1.9.4 HLA class I antibody and Nf-E2-related factor 2

Exposure of endothelial cells to HLA class I antibodies is associated with activation of the oxidative stress-dependent transcription factor, Nf-E2-related factor 2 (Nrf2) (Iwasaki *et al.*, 2010). Endothelial cells transfected with this factor are resistant to complement cytotoxicity in the presence of HLA class I antibodies through inducing the expression of heme oxygenase-1 and ferritin H (Iwasaki *et al.*, 2010). Nrf2 is a basic leucine zipper transcription factor that regulates the transcription of oxidative genes containing a consensus region for Nrf2 binding, antioxidant responses element (ARE). The predominant genes which are controlled by this factor are NADPH oxidoreductase, glutathione-s-transferase, glutamyl-cysteine synthase, glutathione peroxidae and HO-1 (Ruiz *et al.*, 2013). Activation of these genes provides a protection against oxidative stress induces lung and renal injury (Cho *et al.*, 2004; Kim and Vaziri, 2010). An *in vivo* study showed that Nrf2-deficient mice exhibited remarkable inflammation in their lungs in response to hypoxic or bleomycin injury (Thimmulappa *et al.*, 2006). In addition, Nrf2 factor might also regulate the expression of various genes that have a role during inflammation and leukocyte migration. Ischemic Nrf2-deficient mice expressed high level of pro-inflammatory proteins including CCL2, TNF-α and cyclo-oxygenase-1 (Ichihara *et al.*, 2010). In a similar manner, up-regulation of endothelial Nrf2 induced a protection against oxidative stress and abolished the ability of TNF-α to induce the expression of CCL2 and VCAM-1, leading to reduction in the adhesion of monocytes to endothelial cells (Chen *et al.*, 2006b). Summary of all currently known signaling induced by HLA class I antibody is shown in Figure 1.5.

1.10 cAMP Responsive Element Binding protein (CREB)

CREB is a nuclear transcription factor that belongs to the basic leucine zipper (bZIP) superfamily. This group contains CREB isoforms and two other factors, cAMP-responsive element modulators (CREM) and activating transcription factors (ATF), which are collectively called the CREB family (Sandoval *et al.*, 2009). Members of this group are both activators and repressors of the transcription process. For example, the binding of some isoforms of CREM proteins to DNA blocks the transcription process and functions as a negative regulator of CREB by competing with the CREB for binding to the specific sequence on DNA. This blockade occurs as a result of the

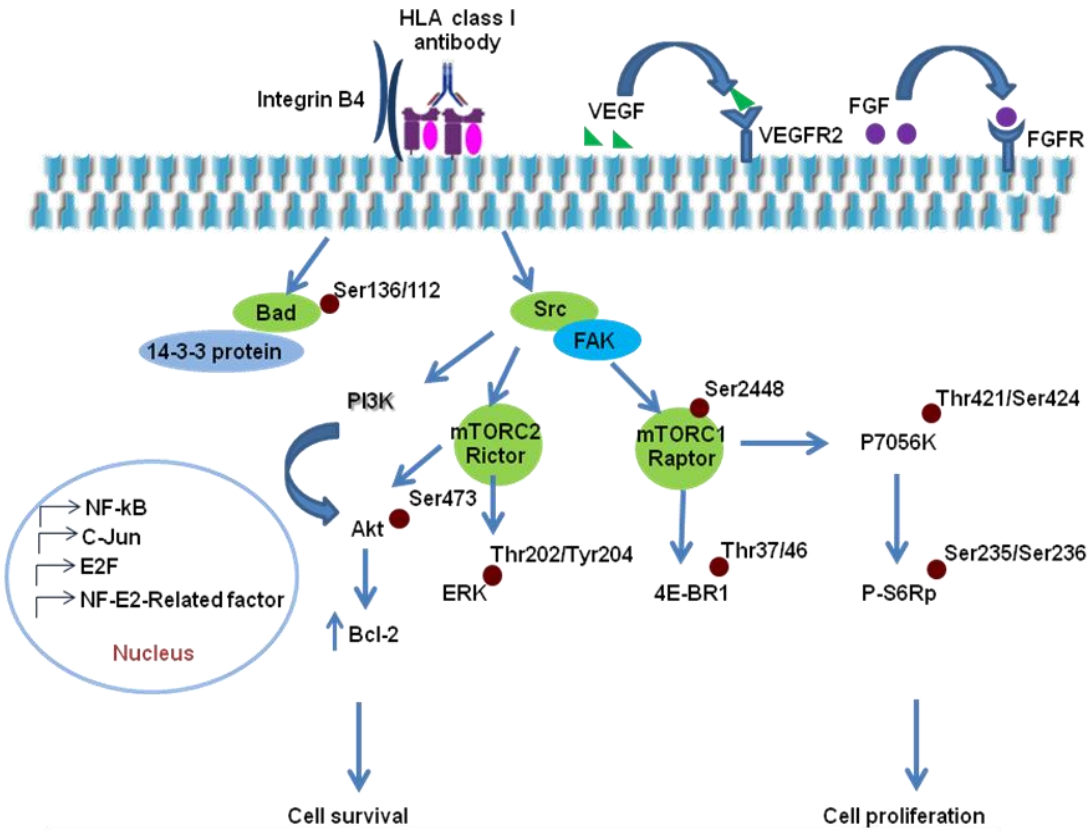


Figure 1.5: Summary of the published data on endothelial cell signaling induced by HLA class I antibody.

missing domains responsible for transcription activation in the repressor CREM (Shaywitz and Greenberg, 1999).

There are more than 100 target genes for CREB binding. They are proteins responsible for a wide range of cell functions such as metabolic regulation, proliferation, apoptosis, differentiation, immune regulation, neuronal activities, hematopoiesis and transcription (Persengiev and Green, 2003). Cell cycle proteins such as cyclins, growth factors, c-fos transcription factor and apoptosis-associated genes such as 14-3-3 protein and Bcl-2 are all regulated by CREB binding (Zhang *et al.*, 2005). Consequently, different studies elucidate a potential role of CREB in the pathogenesis of diverse range of diseases (Chiappara *et al.*, 2007; Mroz *et al.*, 2007).

1.10.1 CREB structure

In humans, mature CREB is 43 kDa protein encoded by gene containing 11 exons. According to the number of expressed exons, there are three isoforms of CREB which are uniformly expressed in all somatic cells. They are CREB-α, CREB-Δ and CREB-β. CREB-α contains an extra 14 amino acid residues at the beginning of the chain called the α-peptide compared to CREB-Δ, the most abundant form of CREB. CREB-β lacks the first 40 amino acid residues which are present on the other two types (Waeber and Habener, 1991). Regulation of gene expression by CREB is facilitated by the presence of different domains in the functional protein. CREB has a basic DNA binding domain, leucine zipper dimerization domain and three transcription domains. DNA binding domain includes mainly sequences of basic amino acid residues, lysine-arginine, in the c-terminal chain. CREB binds to the cis-regulatory element on the promoters of various inducible genes called cAMP responsive element (CRE). This element contains either the 8-9 base pair palindromic consensus sequence, TGACGTCA or, in some genes, a shorter sequence, CGTCA. CREB binds to palindromic CRE with a Kd of 1 nM and to short CRE with a Kd of 5 nM (Johannessen *et al.*, 2004). After its binding, CREB dimerizes to form either a homodimer or heterodimer with other transcription factors, although it is unknown whether this dimerization occurs before or after DNA binding. The dimerization between two transcription factors relies on the leucine zipper domain. This domain is conserved between CREB members and contains repeated residues of leucine (Hoeffler and Habener, 1990).

In the amino terminus, two glutamine rich domains (Q1 and Q2) are present which are separated by the kinase inducible domain (KID). KID, also known as the phosphorylation box, contains different phosphorylation sites that can be a target for multitude of kinases. The glutamine rich domains, next to the binding domain, are necessary for binding the components of the transcription complex such as transcription factor II D (TFIID) and facilitate the accumulation of RNA polymerase. This binding might mediate the transcription activity of CREB, even when in its unphosphorylated state. The most critical and well characterized phosphorylation site is at serine residue 133 (Gonzalez *et al.*, 1991). The phosphorylation of this amino acid by various kinases induces a conformational change in the structure of the transcription domain, which enhances the binding of coactivator protein called CREB-binding protein (CBP). Activation of CREB by growth factors, oxidants and G-protein coupled receptor is mediated by phosphorylation at this residue (Chrivia *et al.*, 1993).

1.10.2 Activation of CREB

Phosphorylation of CREB is mediated by the activation of protein kinase A (PKA) enzyme in cAMP-dependent manner. Stimulus receptors such as neurotransmitter, growth factors and hormone receptors are attached to the cytoplasmic GTP molecules. Upon stimulation, GTP is able to bind and activate adenylyl cyclase which converts ATP to cAMP. Following cAMP production, the two catalytic subunits of PKA dissociate from the two regulatory subunits and translocate to the nucleus. In the nucleus, PKA phosphorylates CREB at serine residue 133 (Gonzalez and Montminy, 1989). Although different studies suggest that phosphorylation of this site does not affect the DNA binding capacity of CREB, this phosphorylation potentially stimulates the transactivation potential of CREB. Substitution of serine 133 residue with alanine produces transcriptionaly inactive CREB upon PKA stimulation (Brindle *et al.*, 1993).

In addition to PKA, CREB can be phosphorylated by multitude of kinases such as calcium-moduline kinases (CaMK), mitogen/stress kinase (MSK), RSK and protein kinase C. The phosphorylation by PKA, MSK, RSK and CaMKIV is associated with an increase in the transcriptional potential of CREB (Sun *et al.*, 1994; Deak *et al.*, 1998). Because some of these kinases are Ca^{2+}-sensitive proteins, CREB might also induce cellular gene transcription in response to a calcium influx. Moreover, some types of adenylyl cyclase are cAMP independent/ Ca^{2+}-dependent which render other stimuli independent on G-coupled protein receptors able to enhance CREB activation.

In neurons, Ca^{2+} influx induced by the binding of neurotransmitters interacts with different cell proteins such as Ca^{2+}-binding protein calmodulin (CaM) inducing the activation of CaMKI, CaMKII and CaMKIV, which all have the capacity to activate CREB (Enslen *et al.*, 1995).

Moreover, Ca^{2+} has been shown to activate various mitogen activated protein kinases such as Ras/ERK and PI3K/Akt pathways which end with CREB phosphorylation (Du and Montminy, 1998). These signaling transduction pathways might be mediated by different MAPK such as RSK1/3 and MSK1/2. Although Akt can activate and phosphorylate CREB, it is still unknown whether Akt directly phosphorylates CREB or works through other intracellular mediators. Furthermore, some of the stress-inducible kinases such as P38-MAPK are activated in response to stress and activate MSK1/2 which in turn phosphorylates CREB.

1.10.3 CREB function and disease

CREB is a key modulator of various cell functions such as proliferation, apoptosis, survival, glucose metabolism, circadian rhythms and synaptic plasticity-associated with memory. All these functions imply CREB as a key effector in the pathogenesis of various diseases including cancers, asthma, diabetes, neuronal disorders and vascular diseases (Mayr and Montminy, 2001).

In immune regulation, CREB regulates the differentiation of various cell lineages such as T and B-cells, allocating CREB as a central regulator of the adaptive immune response. CREB regulates the expression of pro-inflammatory genes such as IL-2, IL-6, TNF-α, cyclooxygenase 2 and macrophage migration inhibitory factor (Brenner *et al.*, 2003; Hughes-Fulford *et al.*, 2005). In macrophages, CREB promotes survival signaling enhancing immune responses against invading harmful agents (Park *et al.*, 2005). Autoimmunity-induced cytokine secretion has been shown to be a causative factor for the destruction of pancreatic islet β-cells through downregulation of CREB activity leading to type-1 diabetes. This reduction in CREB activity has also been reported in both aging and hypertension.

CREB also modulates survival and proliferation processes in response to pro-growth or pro-survival signals. In its role as a proliferative agent, CREB induces the expression of endothelial cell cycle genes such as cyclin D1 and cyclin A in response to growth factors stimulation (Devi *et al.*, 2011). In hematopoietic cancers, over-

expression and phosphorylation of CREB in acute myeloid leukemia patients was associated with a poor prognosis (Pigazzi *et al.*, 2007). This effect occurs as a result of the ability of CREB to enhance proliferation and cell survival upon stimulation with hematopoietic growth factors. CREB has also been shown to enhance the expression of the pro-survival gene, Bcl-2, in response to growth factor stimulation and ischemic conditions (Meller *et al.*, 2005).

Upregulation of CREB during hypoxia and oxidative stress implies that it has cytoprotective effect under these conditions. CREB is also important for the regulation of lipid and glucose metabolism particularly during fasting, by modulating the activity of insulin and glucagon hormones. Through its ability to induce the expression of cytochrome C gene, CREB also regulates the mitochondrial respiration process. In addition, CREB might regulate the transcription of other transcription factors such as C/EBPβ, Erg1 and Nurr1 (Shaywitz and Greenberg, 1999). A recent study highlighted the role of endothelial CREB in maintaining normal endothelial barrier function after exposure to inflammatory mediators. Stimulation of CREB-deficient endothelial cells with growth factor and histamine induced exaggerated endothelial cell permeability (Chava *et al.*, 2012).

1.11 Leukocyte migration in transplantation

Leukocyte migration is a major characteristic that is observed at different stages of organ transplantation. This infiltration occurs early after reperfusion of the transplanted allograft, during the development of acute rejection and has an implication for the pathogenesis of graft vasculopathy, the main feature of chronic rejection. Leukocyte migration into extravascular tissue is a multistep process requiring direct contact between circulating leukocytes and activated or inflamed endothelial cells. This process is characterized by the contribution of distinct types of adhesion molecules expressed on both leukocytes and endothelial cells. The expression of these adhesion molecules might be augmented during inflammatory responses enhancing leukocyte adhesion and migration. In addition, the production of chemoattractant cytokines, chemokines, by transplanted grafts plays a crucial role in directing recipients' leukocytes to the grafts. These chemokines might also mediate leukocyte activation by enhancing the exocytosis of cytotoxic inflammatory mediators, inducing allograft damage (Fairchild, 2005). Traditionally, the main three steps for leukocyte migration were leukocyte rolling, adhesion and transmigration. However,

the discovery of integrins and their ligands as well as chemokines and their receptors has added more steps to this cascade. Chemokine-induced integrin activation, integrin-mediated firm adhesion or arrest and intraluminal crawling are added to define sequentially the adhesion process. In addition, the transmigration process has been expanded to include paracellular migration, intracellular migration and migration through basement membrane (Parish, 2005).

1.12 Mechanism of Leukocyte extravasation

1.12.1 Selectin-mediated rolling

Rolling of circulating leukocytes on endothelial cells require an interaction between cell surface selectins and their carbohydrate ligands expressed on both leukocytes and endothelial cells (Zarbock *et al.*, 2011). Selectins are heavily glycosylated cell surface proteins that bind cell surface oligosaccharide or glycosylated ligands. There are three members that belong to the selectin family; L-selectin, E-selectin and P-selectin. L-selectin is constitutively expressed by most leukocytes. However, E-selectin is up-regulated on endothelial cells in response to stimulation with pro-inflammatory cytokine. P-selectin is stored in intracellular Weibel-Palade bodies and rapidly secreted to the endothelial and platelet cell surface in response to stimulation (Zarbock *et al.*, 2011). P-selectin glycoprotein ligand-1 (PSGL1) is a dominant ligand for all selectins and it is expressed on all leukocytes and certain types of endothelial cells (Zarbock *et al.*, 2009). The binding of selectins with their ligands is essential for leukocyte rolling on endothelial cells under flow conditions and it is supported by the applied shear stress. Under flow, the adherent cells roll on the endothelium according to the selectin transporter phenomena, in which a new bond between selectin and ligand is formed before the old bond is broken (Yago *et al.*, 2007). Ligation of selectins or their ligands on leukocytes triggers activation of various signalling pathways such as phosphoinositide-3 kinase and p38-mitogen activated protein kinases. The activation of these pathways enhances the activation of other adhesion proteins on leukocytes which are integrins. Integrins on leukocytes participate in leukocyte rolling and is also involved in leukocyte firm adhesion mediated by chemokines (Wang *et al.*, 2007b).

1.12.2 Integrin-mediated adhesion

Integrins are cell-surface heterodimers of non-covalently bound α and β chains with a wide range of biological functions in addition to leukocyte trafficking, such as development and cell binding to extracellular matrix. The presence of 18 α chains and 8 β chains creates around 24 intergrin combinations with cell type and ligand specificity (Herter and Zarbock, 2013). Integrins that are involved mostly in leukocyte adhesion include α4ß1 integrin, which is also called very late antigen 4 (VLA-4, CD49d/CD29). VLA-4 binds vascular adhesion molecule-1 (VCAM-1, CD106) on endothelial cells and the ß2 integrin, lymphocyte function associated antigen-1 (LFA-1), that binds intracellular adhesion molecule-1 (ICAM-1, CD54). VLA-4 integrin is expressed by monocytes, T-cells and lymphocytes, while LFA-1 mostly expressed on lymphocytes (Kuwano *et al.*, 2010). Constitutively expressed α4β1 and β2 integrins on circulating leukocytes are in an inactive form with low binding affinity to corresponding ligands. Signalling results from selectin-mediated rolling converts the intergin to intermediate binding affinity to their ligands. However, the high affinity and avidity of integrins to their ligands is achieved by chemokines secreted by activated endothelial cells inducing firm adhesion (Lefort and Ley, 2012).

Chemokines are highly basic low molecular weight proteins. They have fundamental roles beyond inflammation such as organ development, angiogenesis, haematopoiesis and tumor metastasis (Mortier *et al.*, 2012). Up to 50 different chemokines have been identified which include inflammatory and homeostatic chemokines. They are structurally classified in human into four distinct groups based on the numbers of the amino acids (X) between the first two cysteines (C) at the amino terminal. The chemokine families are; C, CC, CXC and CX3C. The CC group contains chemokines CCL1 to 28, CXC group contains chemokines CXCL1 to17, C group contains XCL1 and 2 and only one chemokine in the last group, CX3CL1 or fractalkine (Nomiyama *et al.*, 2010). Chemokines bind to seven trans-membrane G-protein coupled chemokine receptors (GPCR) expressed on effecter cells. There are up to 22 chemokine receptors has been identified (Nomiyama *et al.*, 2010).

Chemokines secreted by endothelial cells during inflammation bind to negatively charged cell surface glycosaminoglycans such as heparan sulfate. This binding is crucial in localizing the chemokines on the endothelial cell surface and preventing the diffusion of chemokines into the blood stream (Wang *et al.*, 2005; Gangavarapu *et al.*, 2012). Binding of chemokines to corresponding receptors induces a rapid leukocyte

arrest within milliseconds, especially under shear stress conditions. Leukocyte arrest results from integrin activation mediated by chemokines; is an inside-out signaling, while integrin binding to corresponding ligands is an outside-in signaling (Kinashi, 2005). Inside-out signaling involves the activation of various intracellular signaling pathways such as phospholipase C and GTPase enzymes (Hyduk *et al.*, 2007). This signaling induces a conformational change in integrins resulting in exposure of the ligand-binding pocket. This also induces the redistribution of integrins increasing integrin clustering and avidity. In addition, the binding of integrins to their ligands, for example the binding of LFA-1 to ICAM-1, initiates an outside-in signaling on integrin-expressing cells. This involves the activation of a cascade of signaling pathways such as focal adhesion kinase, src and Syk families. This signalling strengthens leukocyte adhesion and induces cytoskeleton changes essential for leukocyte arrest and crawling (Abram and Lowell, 2009).

1.12.3 Leukocyte crawling and extravasation

Following firm adhesion, adherent cells 'crawl' on blood vessel endothelial cells mediated by cell surface adhesion molecules mainly ICAM-1 (Schenkel *et al.*, 2004). The crawling step is essential for leukocytes to seek a proper site for extravasation and is dependent on luminal chemokine expression and shear flow. Leukocytes transmigrate to the site of inflammation after crossing three barriers; endothelial cells, basement membrane and pericytes (Cinamon *et al.*, 2004). Adherent leukocytes induce the formation of endothelial projections called transmigratory cups that are rich in ICAM-1 and VCAM-1 molecules. These projections initiate the migration of leukocytes in either paracellular or transcellular mechanisms (Muller, 2011). In paracellular transmigration, adherent leukocytes migrate through the junction of endothelial cells. This process is mediated by contraction of endothelial cells reducing endothelial-endothelial cell contacts. In addition, endothelial junction adhesion molecules such as platelet endothelial cell adhesion molecules (PECAM-1 or CD31), junctional adhesion molecule-A (JAM-A) and CD99 are redistributed and localized to the luminal surfaces (Muller, 2003). This movement creates an adhesive gradient guiding the adhered leukocytes to the site of extravasation. In addition, cross linking of junction molecules induces a signaling pathway resulting in the degradation of VE-cadherin on adjacent cells (Schulte *et al.*, 2011). In transcellular migration, adherent leukocytes cross the endothelial cell by forming a gateway in the cell itself. This type

of migration occurs in more restricted manner in the central nervous system and only in some kind of inflammation (Muller, 2011).

The adherent leukocytes squeeze between the endothelial cells starting the penetration of the basement membrane to the site of inflammation. The basement membrane contains laminin proteins and collagen type IV, which are connected by various molecules such as heparan sulphate and proteoglycan perlecan (Hallmann *et al.*, 2005). Chemokines bound to the luminal heparan sulfate guide the adherent leukocytes to cross the basement membrane. Adhered leukocytes bind to laminins through integrins and secrete protease and heparanase enzymes enhancing basement membrane degradation at particular sites. A summary of this process is shown in Figure 1.6.

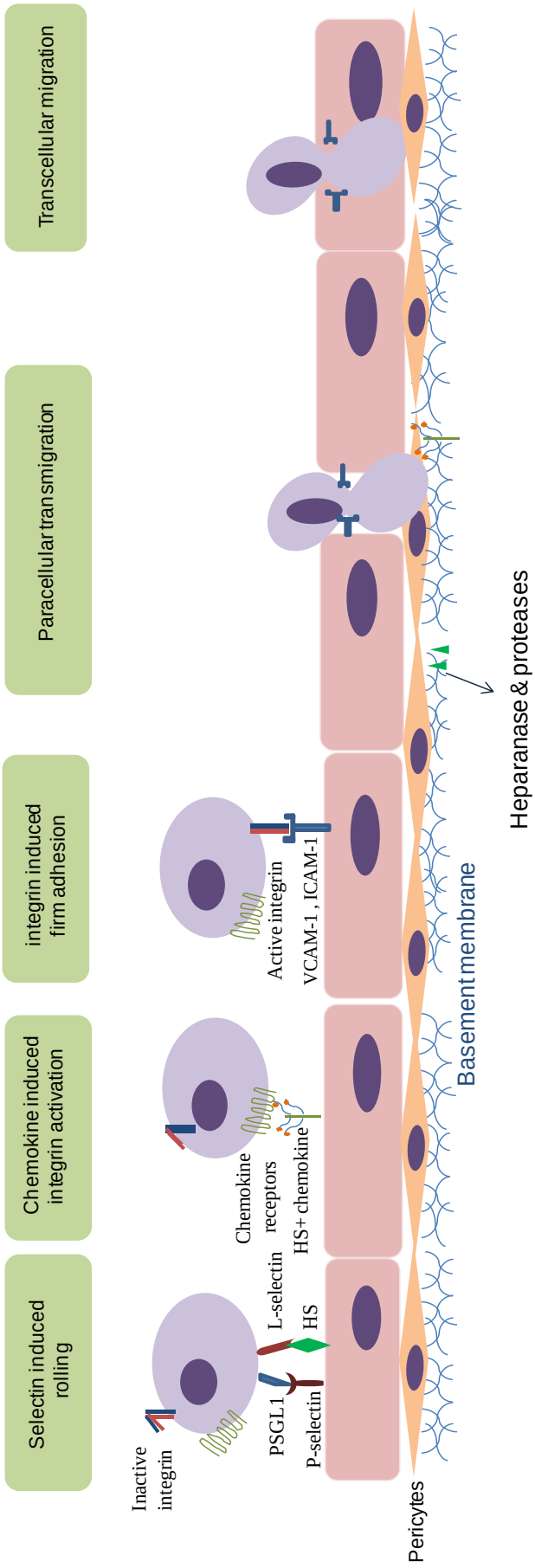


Figure 1.6: Mechanism of leukocyte migration.
Adapted from (Parish, 2005).

1.13 Role of endothelial cells in rejection

Endothelial cells are vessel lining cells with wide range of fundamental physiological functions such as vascular permeability, coagulation and homeostasis. In transplantation, endothelial cells represent the first contact between the recipients' immune system and donor graft beginning at the time of organ reperfusion. Graft endothelial cells play a fundamental role in different aspects of transplant biology either as an initiator of rejection or as participant and a target during rejection. HLA molecules on endothelial cells are able to present foreign antigens to circulating leukocytes, initiating alloimmune responses toward transplanted graft. In addition, inflammatory responses driven by endothelial immunogenicity disturb normal endothelial homeostatic functions and modulate their adhesive properties promoting leukocyte adhesion and migration to the transplanted graft.

1.13.1 Endothelial cells as an initiator of rejection

Endothelial cells *in vivo* express both HLA class I and HLA class II antigens (Page *et al.*, 1992). Therefore, graft endothelial cells also express donor HLA molecules on their cell surfaces. This expression suggests that endothelial cells can initiate T-cell allorecognition, exposing graft antigens to the recipient's immune system. The absence of co-stimulatory molecules on endothelial cells, such as CD80 and CD86, renders the memory T-cells but not naive T-cells the main responder cells (Shiao *et al.*, 2005). This depends on HLA match grade and degree of disparity between the donor and recipients. Endothelial cells can activate memory CD4+ T-cells by a direct allorecognition mechanism as observed using immunodeficient mice transplanted with human skin graft. Activated cells proliferate in response to allogeneic endothelial cells producing inflammatory cytokine such as IFN-γ and IL-2 and causing vessel injury (Shiao, 2007). The production of inflammatory cytokines by activated T-cells is sufficient to induce allograft rejection after renal and cardiac transplantation (Tellides and Pober, 2007). The activation of memory CD4+ T-cells by direct allorecognition is observed exclusively on endothelial cells but not by epithelial cells or fibroblasts stimulated with IFN-γ (Samsonov *et al.*, 2012). Memory CD8+ T-cells can also recognize allogeneic antigens on endothelial cells inducing cell migration to transplanted graft and maturation of CD8+ T-cells into cytolytic cells (Biedermann and Pober, 1998; Walch *et al.*, 2013). It was reported that memory T-cells targeting infectious agents might be able to cross-react with allogeneic HLA antigens expressed

on donor endothelial cells inducing allograft rejection (Adams *et al.*, 2003). In addition to direct recognition, antigenic fragments shed from apoptotic endothelial cells can be processed and presented by host antigen presenting cells inducing activation of both naïve and memory T-cells. T-cells activated by indirect mechanism produced a wider range of inflammatory cytokines including IFN-γ, IL-2, IL-4 and IL-5 (Samsonov *et al.*, 2012). The expression of inflammatory cytokines induces production of other cytokines by endothelial cells which in turn activate different T-cell subsets (Briscoe *et al.*, 1995). Furthermore, another study showed that endothelial cells can capture soluble foreign antigens and present these to antigen presenting cells thus accelerating the graft recognition (Vora *et al.*, 1994).

1.13.2 Endothelial cells as a mediator of rejection

Endothelial cell activation following transplantation results in the disruption of the endothelial homeostatic balance and induces proinflammatory changes augmenting the adhesive properties of the endothelium. Activation of endothelial cells might occur into two phases; a rapid early response (type I) or a slow response (type II). The rapid responses do not require protein synthesis, as endothelial cells secrete stored proteins from weibel-palade bodies to the cell-surface. Production of prostaglandin or NO by endothelial cells and translocation of P-selectin from stores to the cell surface are examples of this response (Pober and Sessa, 2007). Endothelial cells-derived from large vessels stimulated with HLA class I antibodies demonstrated a rapid translocation of P-selectin to the cell surface (Valenzuela *et al.*, 2013a). In type II endothelial activation, new proteins are synthesized in response to inflammation such as in response to inflammatory cytokines TNF-α or IFN-γ. The expression of cell-surface adhesion molecules and chemokines is induced, enhancing endothelial-leukocyte interaction. Endothelial cells are able to synthesize various adhesion molecules, including VCAM-1 and ICAM-1 in a cytokine-dependent manner. Endothelial cells can also produce different chemokines such as CCL2 and CCL5 inducing the adhesion of circulating monocytes which express corresponding receptors CCR1/CCR2 and CCR5, respectively (Grandaliano *et al.*, 1997). In addition, endothelial cells can also express CXCL9, CXCL10 and CXCL11 inducing the adhesion of T-cells expressing CXCR3 (Manes *et al.*, 2006). Exposure of endothelial cells to HLA class I antibodies induces the synthesis of adhesion molecules and different chemokines as will be discussed in chapter five.

Graft vessel thrombosis is one of the major characteristics observed during acute cellular rejection and antibody-mediated rejection (Shimizu and Colvin, 2005). The role of endothelial cells in maintaining blood flow and regulating the coagulation cascade highlights the contribution of endothelial dysfunction in inducing thrombosis. Under resting conditions, endothelial cells prevent platelet adhesion and express tissue factor pathway inhibitors maintaining continuous blood flow. In addition, expression of negatively charged cell-surface heparan sulphate repels negatively charged proteins such as albumin and clotting factors and facilitates the binding of anti-thrombin III, inhibiting thrombin activation (Platt *et al.*, 1990). During inflammation, and especially during type II activation, endothelial cells synthesize and express pro-coagulant proteins such as tissue factor and fibrinogen-like protein 2 and lose the expression of some anti-coagulants such as thrombomodulin (Pober and Sessa, 2007). In addition, shedding of heparan sulphate during inflammation enhances the binding of platelets and clotting factors. Exposure of endothelial cells to donor specific antibodies has been shown to be associated with the shedding of heparan sulfate (Saadi and Platt, 1995).

1.13.3 Endothelial cells as a target during rejection

Circulating leukocytes, antibodies, complement and non-immune agents might all contribute to allograft rejection and graft endothelial dysfunction. Host cytotoxic T-cells directly recognize foreign HLA class I/peptide complexes displayed on graft endothelial cells, inducing apoptosis. This direct recognition is the main mechanism involved in acute cellular rejection (Meehan *et al.*, 1997). Biopsies diagnosed with acute cellular rejection show evidence of the presence of cytolytic T-cells and mRNA related to these cells (Strehlau *et al.*, 1997). The endothelial-cytolytic T-cell contact leads to endothelial lysis mediated by activation of endothelial death receptors or secretion of cytotoxic molecules such as perforin and granzyme B. The perforin permeates the cell membrane facilitating the entry of granzyme B into endothelial cells enhancing activation of the caspase pathway and cell apoptosis.

The infiltration of NK cells to a transplanted allograft post-transplantation also has a role in inducing endothelial injury by ADCC as described earlier in this chapter (Hsieh *et al.*, 2002). Expression of adhesion and chemotactic proteins induces infiltration of monocytes and their maturation to macrophages as observed in acute cellular and antibody mediated rejection (Wyburn *et al.*, 2005). Macrophages also enhance the

development of capillary regression by inducing endothelial apoptosis. The latter may be mediated by the generation of TNF-α and reactive oxygen species such as nitric oxide enhanced also by the presence of IFN-γ (Martinez *et al.*, 2008). Neutrophil recruitment is also associated with both antibody-mediated rejection and T-cell mediated rejection. Activated neutrophils produce reactive oxygen species and degradative enzymes contributing to endothelial cell injury (Morita *et al.*, 2001).

1.14 Aim of the project

In this project, the ability of HLA class I antibody in inducing the activation of allograft endothelial cells was examined. Since all previous studies were performed on large vessel-derived endothelial cells, this study was extended to examine the activation of microvascular endothelial cells. This includes the phosphorylation of various proteins and expression of adhesion molecules and chemokines.This study was performed using two sources of HLA class I antibody. Mouse monoclonal HLA class I antibody (W6/32) and allospecific antibody from patients who had a circulating HLA class I antibody at the time of the rejection of their transplanted kidneys were used. Characterization of endothelial cells was carried out first and then the activation of endothelial cells was assessed. The thesis was divided into chapters as follow:

- Chapter one: General introduction;
- Chapter two: General material and methods;
- Chapter three: Characterization of endothelial cells and purification of mouse monoclonal antibody (W6/32);
- Chapter four: Examination of activation of endothelial cell signaling;
- Chapter five: Expression of endothelial cell surface adhesion molecules and chemokines; and examination of leukocyte migration and adhesion, and
- Chapter six: Endothelial cell activation using allospecific antibodies.

2. Chapter Two- General Material and Methods

2.1 General practice

All laboratory work has been performed in a containment level II laboratory under the University Health and Safety policy. COSHH and BIOCOSH forms have been reviewed and signed before starting work. Procedures of cell culturing were performed in containment level II microbiological safety cabinets. The latter were routinely cleaned with 70% ethanol prior to use and periodically cleaned by "mycoplasma off" spray. All tissue culture equipment was sprayed with 70% ethanol before use. For routine culture, all cell lines were grown in plastic tissue culture flasks (Corning, UK) in two sizes (25 and 75cm^2). All cell types were cultured in specific medium according to ATCC recommendations and were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Adherent cell lines were placed horizontally in the incubator to allow for cell adherence while suspension cell lines were grown in vertical flasks. All culture media were supplemented with 10% Foetal Bovine Serum (FBS), 100 U/ml penicillin, 100μg/ml streptomycin (Sigma) and 4 mM L-glutamine (Sigma). For a specific purpose where specified, cells were incubated in media containing 0.2% FBS and 1% Bovine Serum Albumin (BSA).

2.2 Culture media

2.2.1 MCDB-131 media

MCDB-131 is the media that has been optimized to support the growth of Human Microvascular Endothelial Cells (HMEC-1). This medium (Sigma, M8537-1L) was purchased in a powder form containing L-glutamine. The content of each vial was dissolved in 900 ml of sterile water containing 15.7ml of 7.5% sodium bicarbonate (Sigma, S8761). Complete medium was prepared by adding FBS (10%), 10 ng/ml Epidermal Growth Factor (EGF) (Peprotech), 1μg/ml of hydrocortisone (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin. To obtain sterilized media, the medium was passed through sterile 0.2μm filters using a vacuum pump.

2.2.2 RPMI 1640 media

RPMI 1640 medium (Sigma, 5886) was used to support the growth of suspension cell lines. Cells were normally grown in this medium after the addition of 10% FBS (whole serum or IgG stripped serum), 4 mM L-glutamine, penicillin (100 U/ml) and

streptomycin (100 μg/ml). Medium supplemented with 1% BSA was used in some experiments as specified.

2.2.3 DMEM media

This media (Sigma, D5546) was used to support the growth of the EA.hy926 endothelial cell line. Complete media was obtained after the addition of 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.2.4 DMEM F12 media

DMEM F-12 Ham's media (Sigma, D6421) was used to grow adherent epithelial cell lines. Cells were normally grown in this medium after the addition of the supplements; 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.3 Cell lines and primary cells

2.3.1 Human microvascular endothelial cell line (HMEC-1)

This cell line is a model for microvascular endothelial cells generated from the transfection of human dermal microvascular endothelial cells (HMEC) with a PBR-322-based plasmid containing the coding region for the Simian Virus 40 A gene product, large T antigen, for immortalization purpose (Ades *et al.*, 1992). These cells were cultured in horizontal 75-cm^2 flasks using complete MCDB-131 media in a humidified 5% CO_2 atmosphere at 37^oC. SV40 transformed endothelial cells retained the characteristic cobblestone morphology of the confluent primary endothelial cells and maintained the expression of endothelial cell markers such as CD31 (PECAM-1) molecules. The modulation of the expression of adhesion molecules by inflammatory cytokines renders HMEC-1 cells a good model to study endothelial-leukocyte interaction occurring in micro-blood vessels. They also express a high level of HLA class I antigens and low level of HLA class II molecules. All experiments related to stimulation with HLA class I antibody in this project were performed using this cell line. Confluent monolayer cells were routinely sub-cultured every 3-4 days. Adherent cells were washed twice with sterile phosphate buffered saline (PBS) and detached using trypsin-EDTA and split in the ratio 1:3 or 1:4 depending on cell density.

2.3.2 Human large vessels endothelial cell line (EA.hy926)

EA.hy926 is an immortalized cell line that represents an experimental model of large vessel endothelial cells. These cells were formed by fusion of human umbilical vein endothelial cells (HUVEC) and human lung carcinoma cells (A549) (Edgell *et al.*, 1983). The cells were cultured in 75-cm^2 flask horizontally in complete DMEM media in a humidified 5% CO_2 atmosphere at 37^oC. The cells maintain the major characteristic of primary endothelial cells such as the expression of CD31. Confluent monolayer cells were routinely sub-cultured every 2-3 days. Adherent cells were washed twice with sterile PBS and detached using trypsin-EDTA and split in the ratio 1:4 or 1:5 depending on cell density. Because the cells are a fusion of two cell types, the choice of this cell line for our project was unfavorable since the expression of more than six HLA class I antigens on the cell surface is suspected. These cells were used as a comparison between microvascular and large vessel endothelial cells.

2.3.3 Human kidney epithelial cell lines (HK-2 & HKC-8)

HKC-8 and HK-2 are human renal proximal tubular epithelial cells transfected with adenovirus 12-SV40 (Racusen *et al.*, 1997) and human papilloma virus (HPV 16) E6/E7 genes (Ryan *et al.*, 1994), respectively. These cells were grown in DMEM F-12 media supplemented with 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37^oC. Confluent monolayer cells were routinely subcultured every 2-3 days. Adhered cells were washed twice with sterile PBS and detached using trypsin-EDTA and split in the ratio 1:4 or 1:5 depending on cell density.

2.3.4 Human embryonic kidney 293 cell line (HEK 293 cells)

This cell line produced by transformation of human embryonic kidney cultured cells by adenovirus 5 DNA, which incorporates 4.5 kilobases from its genome into chromosome 19 (Graham *et al.*, 1977; Louis *et al.*, 1997). The cell line is widely used in transfection experiments due to the ease in cell culturing and transfection. In this project, the cell line was used to optimize a transfection protocol and validate different transfection reagents. The cells were grown in complete RPMI 1640 media and sub-cultured into 4 or 5 flasks by washing with PBS and detached by trypsin-EDTA.

2.3.5 Hybridoma cell line (W6/32)

This is a hybridoma cell line produced from a fusion between mouse myeloma B-cells (lymphoblast) and B-cells from spleens of immunized mouse with membrane from human tonsils (Barnstable *et al.*, 1978). These cells produce a mouse IgG2a isotype immunoglobulin in culture media which recognize a monomorphic antigenic determinant common to HLA-A, B and C class I antigens. The antibodies recognize HLA class I molecules expressed on all nucleated cells and platelets (Barnstable *et al.*, 1978). They were grown in 200-cm^2 flask vertically in RPMI-1640 media containing 10% IgG stripped FBS (PAA, A15-706), 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37^{o}C. The cells were left to grow for around two weeks before the conditioned media was collected. The remaining cells were subcultured into 3 or 4 flasks depending on cell density. The collected media was filtered through 0.2μm filter and frozen (-20^{o}C) for subsequent antibody purification.

2.3.6 RAJI B cell line

This is a B human cell line of hematopoietic origin derived from 40 years old Nigerian patient with Burkitt's lymphoma (Pulvertaft, 1964). They were grown in 75-cm^2 flask vertically in RPMI 1640 media containing 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37^{o}C. The cells were split every 4-6 days into 2-3 flasks depending on cell density. Because the cells express a high level of HLA class II antigens, they were used as a positive control in HLA class II molecules staining experiments.

2.3.7 Human monocytic cell line (THP-1)

This is a human monocytic cell line established from the peripheral blood of a 1 year old human male with acute monocytic leukemia (Tsuchiya *et al.*, 1980). THP-1 cells express Fc and C3b receptors and lack the expression of surface and cytoplasmic immunoglobulin. The cells can be differentiated into macrophage-like cells using various chemical agents such as phorbol 12-myristate 13-acetate (PMA). Characterization of the cells showed their expression of CD49d (leukocyte marker) and chemokines receptors; CCR5, CXCR4 and CXCR2. They were grown in 75-cm^2 flask vertically in RPMI 1640 media containing 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37^{o}C.

2.3.8 MOLT-16 T-cell line

This cell line is a human T cell derived from the peripheral blood of a 5-year-old girl with T cell acute lymphoblastic leukemia (Minowada *et al.*, 1984). The cells express CXCR4, CCR1 and CXCR3 chemokine receptors. They were grown in 75-cm^2 flask vertically in RPMI 1640 media containing 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified 5 % CO_2 atmosphere at 37^oC. The cells were split every 4-6 days into 2-3 flasks depending on cell density.

2.3.9 Peripheral blood mononuclear cells (PBMC)

The preparation of PBMC was performed freshly when needed from blood taken from healthy volunteers. Isolation of viable mononuclear cells was performed using a density gradient separation solution, Lympholyte-H (Cedarlane laboratories). Blood (15 ml) was collected in 50 ml conical tube containing heparin at final concentration 25 IU/ml. The blood was diluted with an equal volume of RPM1 1640 media without any additives. Lympholyte reagent was added into diluted blood in ratio 1:2 underneath the blood layer gently avoiding the mixing of the two layers. The tubes were centrifuged at 800 g for 20 minutes with no brake at room temperature. After centrifugation, three layers were formed; a red pellet containing red blood cells and dead cells, top plasma layer and interface layer containing mononuclear cells. The interface layer was collected into fresh tube and washed three times with PBS at 500 g for 5 minutes each. The cells were then counted and re-suspended in 1% BSA RPMI 1640 media at final concentration of 2 million cells per ml and incubated in 25 cm^2 flask horizontally for 1 hour.

2.4 Cell counting

When required, cells were counted using an improved Neubauer chamber haemocytometer. Ten microliter of cells suspended in an appropriate volume of media was inserted onto the chamber covered with a suitable glass slide. Cells within the 25 squares of the chamber were counted. At least two areas were counted and the mean multiplied by 10^4 were considered the number of cells in one milliliter.

2.5 Cell cryopreservation

In order to maintain a stock of the cell lines and to retain cells from an early passage, cryopreserved cells were frozen in liquid nitrogen. Cells in their log growth phase (70-80% confluent) were re-suspended in freezing medium, which consists of 10%

dimethylsulphoxide (DMSO-Sigma) in complete media or FBS. Cells were counted and frozen in different batches of 5-10 million cells per vial in cryovials (Corning, UK). The vials were placed in a Mr. Frosty container (isopropyl alcohol) which maintains a gradual reduction in temperature, 1^0C every minute when stored at -80^0C overnight. On the second day, the cells were transferred into a liquid nitrogen tank for long-term storage. For cell recovery, cells were thawed by rapid incubation in a 37°C water bath. Once thawed, the cells were transferred immediately into 10-15 ml of fresh pre-warmed medium.

2.6 Mycoplasma screening

Mycoplasma infection is one of the major risk factors affecting cells in tissue culture. This is due to the difficulty in realizing the presence of this type of infection which induces biochemical alteration of cell metabolism. This results in morphological and molecular changes at later stage (Macpherson, 1966). During this project, a routine test was performed to ensure the absence of mycoplasma infection in all cell cultures by using a Luminometric assay. This assay was performed on culture supernatant of cells grown for at least 48 hours by using a Mycoalert kit (Lonza, LT37-618). In this assay, the lyophilized reagent and lyophilized substrate were solubilized in 0.6 ml of the assay buffer. A plain assay buffer was used as a negative control while the positive control was prepared by adding 0.3 ml of the assay buffer into lyophilized positive control. The reagents were allowed to stand for 15 minutes at room temperature to equilibrate. Two ml of cell supernatant was centrifuged at 200 g for 5 minutes to pellet any cells. 100 µl of cell supernatant, positive control and assay buffer were added into eppendorf tubes and mixed with 100 µl of reagent. After 5 minutes incubation, integrated readings for one second were recorded (reading A). After that, 100 µl of substrate was mixed into each tube and incubated for 10 minutes before recording (reading B). The ratio between reading B and A was calculated and the results interpreted in accordance with this guide: < 1 Negative, 1-1.2 Borderline, > 1.2 Positive. All cell lines in this project were negative for mycoplasma infection as the all readings were less than 1.

2.7 Flow cytometry

2.7.1 General principle

A flow cytometer is an instrument that has a wide range of applications including: analysing the expression of cell surface or intracellular molecules, estimating the

heterogeneity in a particular cell population, and defining the purity of an isolated cell preparation. These applications are based on the capability of this machine to analyse different physical and chemical characteristics of a single particle including cells, microorganisms and even chromosome preparations. The instrument detects these characteristics for each particle separately while it passes through a laser light source following hydrodynamic focusing on a very small nozzle using pressurized isotonic sheath fluid stream. The cells passing through the laser scatter the light in different directions according to their size and granularity; which can be detected by different detectors. One detector is in front of the light beam captures forward angle light scatter (FSC) and one on the side which captures side scatter (SSC). The FSC represents cell size and the latter represents cell granularity, a larger size and more granular cells produce higher forward and side scatter signals, respectively. In addition, using different fluorescence dyes which can bind specific cell component or intercalate within DNA or RNA can be detected by a specific system on the instrument. Once the bound dye intersects the laser light, it is excited to a higher energy state and emits light at a higher wavelength, called the emission spectra, upon returning to the ground state. The emitted fluorescence light is collected via optics and directed to a series of filters each one responsible for the detection of a narrow range of wavelengths. Each of these detectors, which are called photomultiplyer tubes, can change photon energy into an electrical signal which is recorded by the computer system as fluorescence intensity for each event.

In the immunofluorescence technique, a fluorescence dye is conjugated to an antibody specific for a particular antigen and the fluorescence intensity produced is correlated positively with the amount of that antigen. In this project, three fluorescence dyes were used which are fluorescein isothiocyanate dye (FITC) which has excitation-emission spectra 488-530/30 and propidium iodide and phycoerythrin (PE) which have excitation-emission spectra 488-585/40. As the emission spectra of FITC overlaps the emission spectra of the PE, therefore, a compensation step is performed in experiments where both dyes were used simultaneously. This step involves the subtraction of the contribution of each dye from the other by running each fluorchrome separately first.

2.7.2 Indirect immunofluorescence

In indirect immunofluorescence staining method, a specific antigen is identified by using a fluorescently conjugated secondary antibody that recognizes a primary

antibody bound to that antigen. In this method, adherent cells were washed twice with PBS and detached by using enzymatic free-PBS based cell dissociation buffer at 37^{o}C for 15 minutes. Detached cells were re-suspended in complete media and centrifuged at 500 g for 5 minutes. Cells were counted and 2 x 10^5 cells were used per tube. The cells were washed once with 2% FBS in PBS before staining with primary or isotype-control antibodies. These antibodies were used at concentrations recommended by the manufacturer and incubated at 4^{o}C for 20 minutes. Then, the cells were washed twice with 2% FBS-PBS solution at 500 g for 5 minutes. Secondary antibodies were added and again the cells were incubated at 4^{o}C for 20 minutes. Finally, the cells were washed twice with PBS/FBS solution at 500 g for 5 minutes and re-suspended in 200-400 µl PBS/FBS. Cells were scanned on flow cytometry machine using FACSDiva software acquiring 10,000 events.

2.7.3 Direct immunofluorescence

In this method, one step staining is performed using one antibody specific to the examined antigen rather than using primary and secondary antibodies. In direct staining, an antibody specific to the antigen of interest is conjugated directly to fluorescence dye and the fluorescence intensity of the labeled cells is recorded by flow cytometry. 80 % confluent cells were treated with a particular stimulus either at different concentrations for one time point or at a constant concentration for various time points. After treatment, the cells were detached using cell dissociation buffer and pelleted by centrifugation at 500 g for 5 minutes. 1 X 10^5 cells were incubated with fluorescence-conjugated antibodies or isotype control at 4^{o}C for 20 minutes. Before analysis, cells were washed twice with 2% FBS in PBS and re-suspended in 200-400 µL of this solution and analyzed on flow cytometer machine using linear scale for FCS and SSC.

2.8 Western blotting

2.8.1 General principle

Western blotting or protein immunoblotting is a widely used method to detect a specific protein in a mixture which might be a tissue homogenate or cell lysate. This analytical technique is based on the separation of proteins by gel electrophoresis followed by protein transfer onto a suitable membrane to be accessible and detectable by specific-antibodies. The separation can be performed on the basis of isoelectric points, molecular weight, electric charge or a combination of these factors. In this project, the separation was based on the molecular size by using a reducing agent and a detergent, Sodium Dodecyl Sulphate or SDS. β-mercaptoethanol is a reducing agent that cleaves the disulfide bonds between cysteine residues converting the tertiary structure of the proteins into linear polypeptide chains, while SDS maintains the denatured form of the proteins and coats the proteins with negative charge enabling the separation according to the molecular size. Separated proteins are transferred into a membrane where the specific protein is determined by indirect immunochemistry. Polyvinylidene fluoride or PVDF is the most hydrophobic membrane that is used to immobilize the separated proteins in the transfer process. This membrane is characterized by its capacity to bind various amino acids non-specifically and can tolerate probing with different antibodies. The non-protein bound areas of the membrane are blocked before probing with primary protein specific antibody. After incubation, a secondary antibody conjugated with an enzyme, fluorophore or isotope is added. The chemiluminescence method depends on the label on the secondary antibody. Horseradish peroxidase (HRP) is one of the most common and safe conjugates and was used in this project. The detection of this enzyme, which correlates with the abundance of the examined protein, is determined indirectly by the addition of a peroxide-luminol based reagent. The peroxidase enzyme catalyzes the oxidation of the luminol, resulting in the emission of the light. The emitted light can be captured by a camera imager device or by the exposure to X-ray film.

2.8.2 Preparation of cell lysate

Cells were stimulated as required and lysed immediately using lysis buffer consisting of cell lytic solution (Sigma) supplemented with protease inhibitor tablets (Roche, USA) and kept in aliquots in (-20^0C). The phosphatase inhibitor solution (Thermo scientific, USA) was added in the experiments of the determination of phosphorylated

proteins. Around 40 µl of lysis buffer was added per million cells with continuous mixing. Cell lysates were incubated for 10 minutes on ice with gentle hand shaking every 5 minutes. To ensure complete cell lysis and protein extraction, samples were sonicated using an MSE Soniprep 150 sonication. The sonication was performed twice separated by cooling in ice for one minute to avoid heating causing protein denaturation. The samples were centrifuged at 15000 g for 15 minutes to pellet cells debris and either used immediately or stored at -80^0C.

2.8.3 Determination of protein concentration

The protein concentration of each sample was estimated colorimetrically using a BiCinchoninic Acid (BCA) protein assay kit (Pierce, USA) in accordance with the manufacturer's instructions. This assay is based on the biuret method in which Cu^{1+} ions produced by a reduction of Cu^{2+} ion under an alkaline condition by sample proteins are determined using the BCA reagent. The addition of the latter forms a coloured, water-soluble complex with Cu^{1+} ions that shows a strong absorbance at 562 nm. The extent of the colour on sample is proportional to the amount of the protein presence. The concentration of the unknown protein was evaluated from a bovine serum albumin standard curve which was run in parallel with unknown samples. Ten microlitres of standard samples ranging from 125-2000 µg/ml or from unknowns were mixed with 200 µl of a working solution in a 96 microwell plate. The working solution was prepared prior to use by mixing reagent A (BCA in alkaline buffer) with reagent B (4% cupric sulfate) in a ratio of 50:1. The plate was incubated at 37^0C for 30 minutes before recording the absorbance at 490 nm. A linear regression analysis of the standard curve was calculated and the unknown protein concentration was determined by interpolation. Figure 2.1 represents an example of a standard curve used to determine the protein concentration of unknown samples during this project.

2.8.4 SDS-PAGE electrophoresis

SDS-PAGE is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. SDS-PAGE gels consist of a stacking gel on the top of a resolving gel. The amount of acrylamide in the resolving gel determines the percentage of that gel and its pore size. In this project, 10% gel was used to separate large proteins such as an antibody, and 12% was used for smaller proteins with 60-40 kDa molecular weight. The following

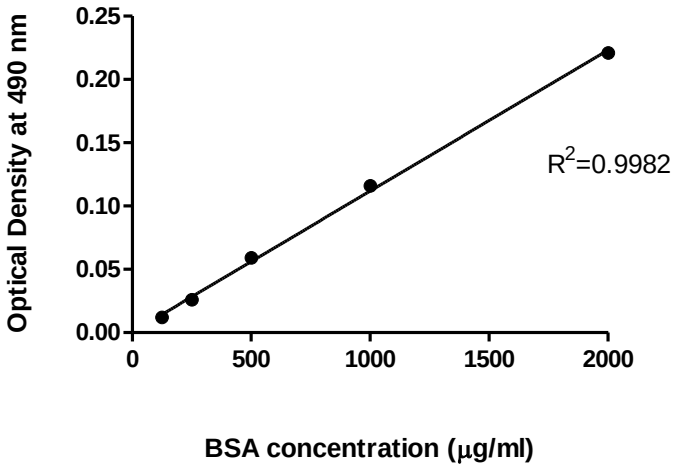


Figure 2.1: Standard curve of protein concentration.

Different known concentrations of BSA were mixed with the BCA reagent provided in BCA assay kit as explained in the methods. After 30 minutes incubation, the absorbance was measured at 490 nm. The linear regression was determined between the concentration and the absorbance. The standard curve was used for the determination of the protein concentration of unknown samples.

constituents, all from Sigma, were mixed to prepare 5 ml resolving gel solution and 3 ml stacking gel solution sufficient to cast one gel and added to glass and alumina plates:

Resolving gel (12%)	**Stacking gel**
2ml of 30% acrylamide solution	0.5ml of 30% acrylamide solution
1.3ml of 1.5M Tris-HCl, pH 8.8	380µl of 1 M Tris-HCl, pH 6.8
1.6ml deionised water	2.1ml deionised water
50µl of 10% (w/v) SDS	30µl 10% (w/v) SDS
50µl of 10% (w/v) ammonium persulphate	50µl 10% (w/v) ammonium persulphate
2µl of N,N,N,N – tetramethylethylenediamine	3µl of N,N,N,N – tetramethylethylenediamine

The resolving gel was left to polymerize for around 30 minutes after the addition of a butanol layer on the top of the gel to prevent air disturbance. After polymerization, the butanol was washed by deionized water and the stacking gel was added on the top. The stacking gel was left to polymerize after insertion of the combs. To prepare cell lysates, samples were mixed with laemmli loading buffer (12% sodium dodecyl sulfate, 60 % (v/v) of 20 % glycerol, 30 % (v/v) β-mercaptoethanol, 0.0012 % bromophenol blue, 0.375 M Tris-base pH 6.8) and boiled for 5 minutes before loading on the gel. The addition of β-mercaptoethanol in the loading buffer was excluded in non-reducing conditions where the intact structure of desired protein is required. The gels were immersed in running buffer consists of 3g/L Tris base, 14.4g/L Glycine and 1g/L SDS at pH 6.8. In parallel, prestained protein marker (Fementas, SM0671) was run on the same gel each run as a guide for the molecular weight of the studied proteins. Gels were run at room temperature at 30 mA for each gel using mini vertical electrophoresis unit (SE260, GE Life Sciences).

2.8.5 Gel staining

In some experiments, staining of separated proteins on gel was required to ensure the presence of purified proteins. For this purpose, Coomassie staining consisting of 10% (v/v) acetic acid, 10% (v/v) Isopropanol and 0.1% (w/v) Coomassie Blue powder was used to visualize the separated protein. After 20 minutes incubation at room temperature on the rocker, the gels were destained in three sequential steps using three different solutions. The stained gels were mixed with destain 1 consisting of 25%

propanol with 10% acetic acid for 10 minutes before the addition of destain 2. After the removal of destain 1, destain 2 consisting of 10% propanol and 10% acetic acid was added to the gel and washed many times until the clear bands were obtained. The gels were stored in destain buffer 3 containing 10% acetic acid until acquisition of the image using Alphaimager software of AlphImager gel documentation system (Alpha innotech, USA).

2.8.6 Wet Protein transferring

After protein separation, the proteins were transferred to PVDF membrane. This membrane was equilibrated in absolute methanol for ten seconds followed by washing with distilled water twice for 5 minutes each. After washing, the membranes were soaked in transfer buffer consisting of 3g Tris base, 14.4g glycine and 100ml methanol made up to 1L in distilled water 10 minutes before performing the transfer sandwich. The gels and transfer electroblotting cassette were soaked in transfer buffer before being assembled. Protein transfer was performed either overnight at 30 V or for 2 hours at 250 mA using a transfer tank (TE22, GE Life Sciences).

2.8.7 Immunoblotting

After transfer, the PVDF membrane was washed once with PBS containing 0.1 % tween 20 (TPBS) for 5 minutes to remove the transfer buffer before blocking. To block the non-specific binding, the membrane was blocked with 3 % BSA for phosphorylated proteins or 5% milk for other proteins in 0.1 % TPBS at room temperature for 1 hour before probing with primary antibody. Primary antibody was diluted in the blocking buffer at a concentration recommended by a manufacture and added to the membrane. This was incubated overnight at $4^{o}C$ with continuous shaking on a rocker. After incubation with primary antibodies, the membrane was washed three times with 0.1 % TPBS for 5 minutes each. A HRP-conjugated secondary antibody was added for 1 hour at room temperature with continuous shaking. After three washes with TPBS, peroxidase activity was detected by using Pierce enhanced chemiluminscent (ECL) substrate (Thermo-Scientific) for 5 minutes. The detection of bound antibodies was dependent on the oxidation of the luminol in the substrate by HRP conjugated to the secondary antibody and consequent emission of light. The resulting bands were visualized by exposure of the membrane to Kodak film (Sigma) using ready to use developer and fixer (Tentenal, Germany). The exposure time was varied according to the expression of the desired protein, starting with 2 minutes

exposure as a guide. As a loading control, membrane was stripped at room temperature for 30 minutes using stripping buffer consisting of; 1.5 % glycine, 0.1 % SDS and 1 % Tween 20 (v/v) followed by washing with TPBS and reprobed with loading control specific antibody by the same procedure.

2.9 Molecular biology

Precautions should be taken when DNA isolation and amplification is performed. DNA isolation and amplification in a standard way occurs in two different areas to avoid contamination of the original samples with an amplified sequence. This principle is applied when performing critical analysis such as tissue typing in transplantation. All reagents used for this procedure should be at a high degree of purity and designed for molecular biology use. These reagents are also RNase free which provide protection during the RNA isolation process. All reagents used for RNA isolation in this project were molecular biology grade available commercially. All areas used for RNA isolation where decontaminated by using RNase removal spray (Sigma). All pipettes were decontaminated by exposure to UV light for 30 minutes prior to use. Filter sterile tips and autoclaved sterile Eppendorfs were used during the whole process.

2.9.1 General principle of RNA isolation

Isolation of RNA allows study of the modulation of a gene expression following exposure to a particular stimulus or its modulation during the development of diseases. In this project, RNA was extracted using guanidinium-thiocyanate-phenol-chloroform. This method was developed by Chomczynski and Sacchi in 1987 (Chomczynski and Sacchi, 1987). In this method, cells were lysed in a mixture of guanidinium isothiocyanate and phenol mixture. The former acts as a denaturing solution that separates rRNA from ribosomes and it denatures cell proteins including RNase enzyme to ensure RNA integrity. The presence of phenol dissolves the proteins and lipids leaving water soluble materials in the aqueous phase. The addition of chloroform followed by centrifugation results in the formation of upper aqueous phase and lower pink phase separated by interphase containing a high amount of DNA. At acidic pH, the isolated RNA retains in the upper phase while lipids and proteins are present in organic phase along with phenol and chloroform. The precipitation of RNA from the aqueous phase is achieved by the addition of propanol. The extraction process is terminated by washing with 70% ethanol and dissolving in RNase free

water. A commercial reagent, Tri-reagent (Sigma), based on this principle was used in this project. This reagent has a high capacity to extract RNA from samples in comparison with other methods such as column based method.

2.9.2 Procedure of RNA Isolation

Tri reagent was used to isolate total RNA from cells according to manufacturer's instructions. The addition of this reagent at a rate of 1 ml per $5x10^6$ cells for 5 minutes at room temperature resulted in a complete dissolution of the cells. When lysing cells in a 12 well plate, 250 µl of Tri reagent was used and the volumes of all further solutions were adjusted accordingly. After incubation, 200 µl of chloroform (Sigma) was added per 1 ml of the reagent and the mixture was mixed vigorously by hand for 15 seconds. The mixture was then allowed to stand at room temperature for 2-3 minutes. After centrifugation at 12000 g for 15 minutes at 4^oC, the upper colourless aqueous layer was isolated into freshly autoclaved Eppendorfs avoiding disturbance of the interfacial layer. Soluble RNA in this layer was precipitated by the addition of 250 µl of isopropanol (Sigma) per 1 ml of Tri reagent. After 10 minutes incubation at room temperature, samples were centrifuged at 12000 g for 10 minutes at 4^oC. The RNA pellet was then washed with 75% (v/v) ethanol at an equal volume of the used Tri reagent and centrifuged at 7500 g for 5 minutes. Isolated RNA was left to dry before it was re-suspended in 10 µl of RNase free water.

2.9.3 Analysis of RNA concentration and purity

A Nanodrop spectrophotometer was used for determination of RNA concentration and purity. The machine measures the absorbance at 230, 260 and 280 nm and calculates these values as a guide to nucleic acid purity and concentration. One microliter of extracted RNA was applied to the Nanodrop after blanking with 1 µl of RNase-free water. The concentration was obtained in ng/µl. RNA absorbs light at a wavelength of 260 nm and the optical density relates proportionally to the amount of RNA in samples. Contaminant proteins and organic chemicals in isolated RNA samples absorb light at 280 and 230 nm, respectively. Therefore, the purity of isolated RNA sample was evaluated by examining 260/280 and 260/230 ratios. The reading around 2 for both of the ratios is considered as a pure sample free from protein and phenol contamination and suitable for cDNA synthesis. Figure 2.2 shows an example of RNA concentration measurement and purity using the Nanodrop.

2.9.4 Analysis of RNA integrity

For analysis of RNA integrity, isolated RNA was run on 1.5 % agarose gels containing ethidium bromide. For a mini gel, 0.75 g of agarose was dissolved in 45ml of distilled water and 5 ml of 10X Tris-acetate EDTA electrophoresis buffer or TAE (48.4 g of Tris base, 11.4 ml glacial acetic acid and 3.7g of EDTA dissolved in 1L). Dissolving the agarose was achieved by gentle boiling in a microwave for 2 minutes with avoidance of extra heating. The solution was left to cool slightly under running tap water followed by the addition of 3 µl of ethidium bromide at final concentration 0.5 µg/ml. The gel was allowed to solidify for around 15 minutes at room temperature. For preparation of RNA sample, 1µg of RNA was suspended in RNase free water and mixed with 3 µl of 5 X loading buffer consisting of 50% Glycerol, 1mM EDTA (pH 8.0) and 0.4% Bromophenol blue in a total volume 15 µl. The sample was run using 1X TAE running buffer at 100 V for around 20 minutes, until the dye front reached the third quarter of the gel. Around 10 µl of 1 kb DNA ladder was run in parallel to check the size of the products. Figure 2.3 shows an example of intact RNA. The intact RNA was confirmed by the presence of 28S and 18S rRNA subunits and the absence of smeared RNA.

2.9.5 cDNA synthesis.

Isolated RNA was reverse transcribed reversibly to complementary DNA (cDNA) using RT first strand kit (Qiagen). This kit contains all the components required for cDNA synthesis, including oligo dT, a random hexamer primer mixture, assay buffer, reverse transcriptase enzyme, dNTP and RNAase free water. Each reaction was performed in a total volume of 20 µl using 5 µg RNA adjusted to the maximum volume 10 µl with RNAase free water. Ten µl of cDNA solution containing 1 µl primer, 4 µl assay buffer, 3µl reverse transcriptase enzyme and 2 µl RNAase free water was mixed with 10 µl of RNA and transcribed in a thermocycler (G-storm) at 42°C for 15 minutes followed by rapid heating at 95°C for 5 minutes. The resultant cDNA was used as a template for q-PCR reactions.

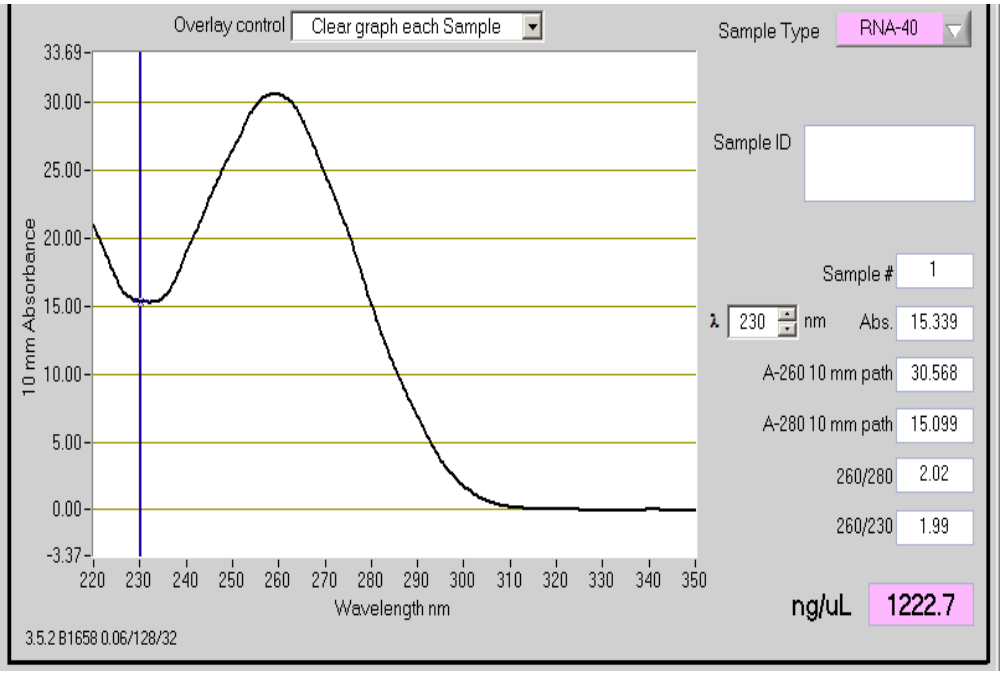


Figure 2.2: Examination of RNA purity and concentration using a Nanodrop instrument.

The system was initialized and blanked with 1 µl of RNase free water followed by 1 µl of RNA sample. The concentration and purity ratios were recorded for each sample.

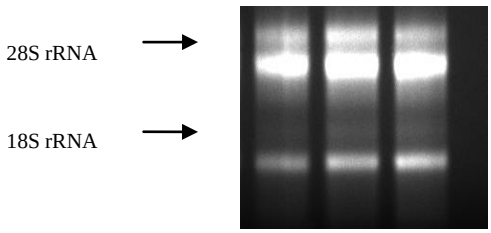


Figure 2.3: Examination of the integrity of isolated RNA.

The integrity of isolated RNA was verified by running on 1% agarose gel from three random samples. The efficiency of the isolation was assessed by the presence of intact rRNA 28S and 18S subunits.

2.10 Real time-polymerase chain reaction

2.10.1 General principle

Quantitative polymerase chain reaction or q-PCR is a precise technique for measurement of changes in gene expression. In this method, a specific sequence of DNA is amplified in a geometric way to produce many copies of the original sequence. The amplified product can be measured either at the end point of the reaction in "traditional" PCR or during the amplification process in the real-time PCR method. The latter has many advantages that overcome the traditional PCR in the context of the processing time and accuracy. In a way that traditional PCR is more qualitative method in determining the presence or absence of a particular gene of interest at the end point of the reaction, q-PCR is able to detect small differences in the level of the expression between examined samples. In contrast to traditional PCR, in q-PCR the amplification and quantification occur simultaneously. An ideal amplification plot has an exponential, linear and plateau phases, in which the amplicon is quantified as the amplification is in progress and reaches plateau when the reaction components are depleted. The PCR product is determined in the exponential phase and crosses the threshold level in a cycle called the threshold cycle. The detection process in this method requires the presence of fluorescent reagents that detect the amount of the amplified product. These reagents are either sequence specific or non-sequence specific. An example of sequence specific reagent is the 5' nuclease probe such as the Taqman assay, while the non-sequence specific reagents are the DNA double stranded intercalating dyes such as SYBR green or Evagreen.

2.10.2 Taqman assay

In this project, Taqman primer-probes (or 5' nuclease assay) was used to determine the modulation in the expression of genes of interest. In this assay, a set of two primers and probe in the presence of exonuclease activity of Taqman DNA polymerase are used. The DNA probe is a non-extendable sequence that is labelled with a fluorescence reporter at the 5' end and quencher at the 3' end. This probe is able to bind complementary sequence on the desired gene in either an exon boundary spanning or non-exon spanning manner. In the intact probe, the presence of the quencher in the close proximity to the reporter enables the former to absorb the fluorescence light emitted by the latter preventing the emission of any light. In the first step of the amplification process, the reaction mixture is heated to 95^0C for 15 seconds which is required for

optimal activation of the DNA *Taq* polymerase enzyme. In the second step, the hybridization of the two oligonucleotide primers and probe to the complementary sequence on the DNA strand occurs at lower temperature of around 40-60^{0}C for one minute; this is called the annealing step. DNA synthesis is initiated as the polymerase extends the primers using the deoxynucleotide triphosphate (dNTPs) forming new complementary strands in the 5' to 3' direction. The elongation process occurs at around 72^{0}C for 1-2 minutes and requires the addition of optimum salt and $MgCl_2$ concentrations which are required for enzyme activity. As the polymerase reaches the probe, the exonuclease activity of the enzyme cleaves the hybridized probe enhancing separation of the reporter from the quenchers. This separation eliminates the quencher role allows the reporter fluorescence to be detected by real-time instrument. This thermal cycle is repeated between 30-40 times before the reaction is ended. During the exponential phase each cycle doubles the amount of the original sequence ending with 2^n copies, where n is the number of cycles.

2.10.3 Primer efficiency

PCR efficiency is defined as the rate at which a PCR product is generated. This is represented as a percentage value with a maximum PCR amplification efficiency almost 100%. If the PCR amplicon is doubled during each cycle, that reflects a 100% efficiency. This value is estimated from a standard curve specific for each primer which is prepared by serial dilutions of cDNA sample. In this curve, a log of input nucleic acids on X-axis is plotted against CT values on the y-axis. The gradient of the semi-log regression is determined to evaluate the amplification efficiency by this equation: efficiency = 10^ (-1/gradient)-1. The gradient value equals to -3.32 represents 100% amplification efficiency. A value that is more negative than this value reflects an amplification process less than 100 % while more positive value reflects sample quality or pipetting problems. In this project three human primer probes with FAM fluorophore and TAMRA as a quencher were used which are glyceraldehyde 3-phosphate dehydrogenase or GAPDH (HS9999905-m1), CXCL8 (HS00174103-m1) and 18S (HS99999901_s1). Figure 2.4 shows the efficiency curves for these primers performed using 10 times serial dilution from cDNA sample.

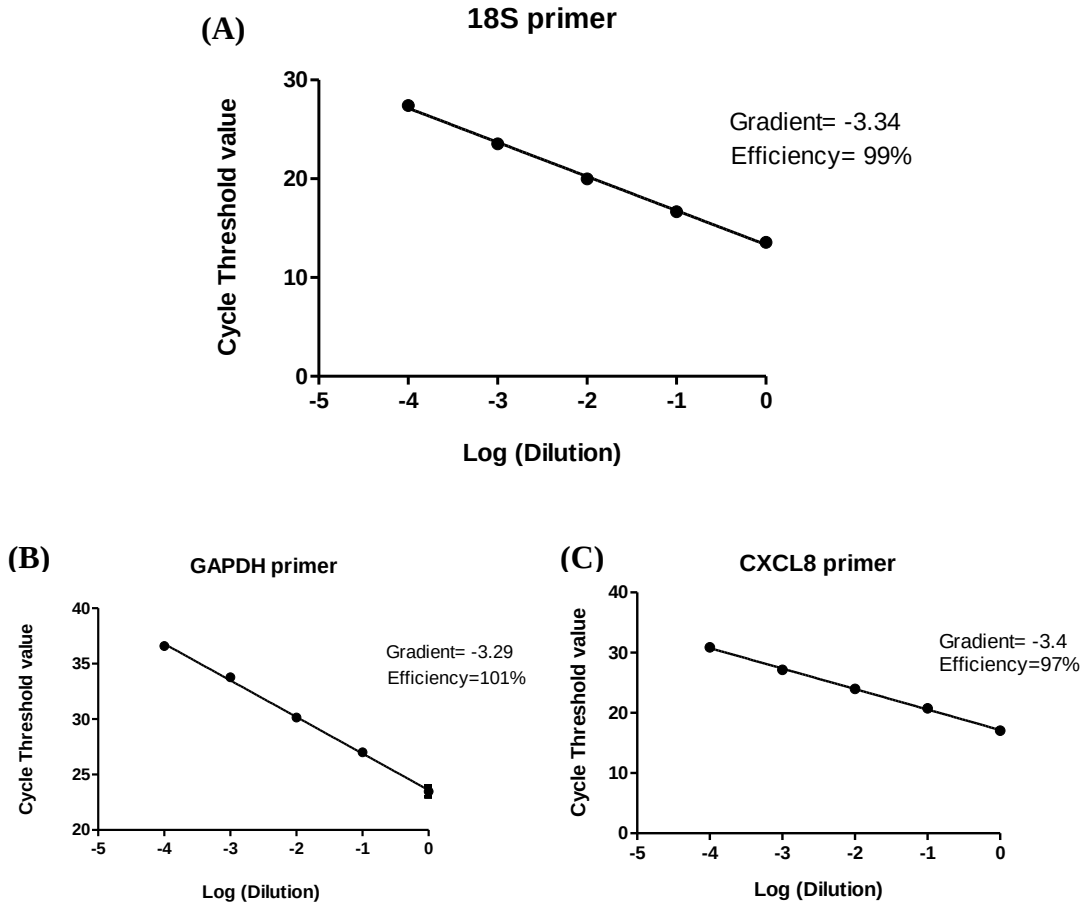


Figure 2.4: The standard curve for tenfold dilution series of cDNA for (A) 18S, (B) GAPDH and (C) CXCL8 transcripts.

The efficiency of GAPDH and 18S primers was confirmed by using ten serial dilutions from cDNA prepared from 5 µg of untreated HMEC-1 cells. While the efficiency of CXCL8 was assessed by using cDNA prepared from 5 µg RNA of 10 ng /ml TNF-α treated cells for 3.5 hours. The reactions were run on an Applied Biosystem instrument (StepOnePlus) for 40 cycles. For gradient and efficiency values, the data were analyzed by linear regression converting x values to a log scale.

2.10.4 Semi quantitative real time-PCR

To determine the change in expression of a particular gene at a molecular level a quantitative PCR reaction was performed using a cDNA template transcribed from the isolated RNA. PCR reaction was prepared in 20 µl total volume by mixing 10 µl of 2X master mix, containing Taq man polymerase enzyme and buffers essential for its activity, 8 µl distilled water molecular grade, 1 µl of cDNA and 1 µl of desired primer in 96 wells plate. The mixture was amplified using Applied Biosystem (Step one plus). The amplification process was carried out for 40 cycles in the following sequential steps: 95^0C for 10 minutes followed by 40 cycles of 95^0C for 15 seconds followed by 1 minute at 60^0C. Each PCR run contains a target gene in parallel with a housekeeping gene. In addition, each run has negative control wells where RNA sample and master mix mixture were checked separately for any contamination.

2.10.5 Data analysis-comparative ΔΔCT value

Quantification of PCR products can be performed by relative expression of target gene compared to control group. The latter requires the measurement of target gene and housekeeping gene (constantly expressed between samples) on both control and treated samples. For the purpose of this project, relative expression of target gene was used in which a comparative ΔΔCT value was calculated and converted to fold change. In this analysis, ΔCT value between target gene and housekeeping gene is calculated for both control and treated samples, ΔCT= CT target gene - CT housekeeping gene. The ΔCT value for the control group is subtracted from the ΔCT value of the treated group, ΔCT treated group - ΔCT control group. This value is then converted to fold change or relative expression calculated as $2^{-(\Delta CT \text{ treated group} - \Delta CT \text{ control group})}$. All PCR data analysis in this project is performed under this principle using REST 2009 software.

2.11 Statistical analysis

All data were plotted using GraphPad prism software version 5. Comparison between different groups were verified using one way analysis of variance (ANOVA) followed by Bonferonni test as a post hoc test considering the significance at $P < 0.05$, or two way ANOVA followed by Bonferroni post-tests. In this study, * refers to $p<0.05$, ** refers to $P<0.01$ and *** $p<0.001$. Comparison between two groups were performed by unpaired Student's t-test at $P<0.05$. All flow cytometry data were analyzed and graphed by WinMDI 2.9 and FACSDiva software using median fluorescence intensity (MFI) value from each sample. Densitometric analysis of western blotting data was performed

using AlphaImager software of AlphaImager gel documentation system and the ratio between analyte and loading control was plotted (Alpha innotech, USA).

3. Chapter Three-Characterization of Cell Lines and Antibody Purification

3.1 Introduction

Macovascular (large vessel) and microvascular (small vessel) endothelial cells show similarity in cell morphology and some of their characteristics. However, endothelial cells from different blood vessels have a distinct gene regulation and protein expression which facilitates their functions. In addition, expression of some types of cell surface antigens and adhesion molecules between the two cell types are different. Expression of HLA class II antigens *in vivo* is detectable on microvascular but not on macrovascular endothelial cells; suggesting their involvement in antigen presentation (Muczynski *et al.*, 2003). Non-stimulated microvascular endothelial cells, in contrast to large vessel endothelial cells, show a constitutive expression of ICAM-1 adhesion molecules which is up-regulated on both cell types by stimulation with different inflammatory cytokines. During inflammation, both of cell types respond differently to some extent to extracellular stimuli. In response to TNF-α stimulation, microvascular endothelial cells express different types of chemokines and cytokines such as IL-β and CXCL5 which is either not expressed or expressed at low level by large vessel endothelial cells (Viemann *et al.*, 2006). The interaction between the endothelial cells and leukocytes, in addition, occurs mostly in post-capillary vessel which is necessary for leukocyte trafficking. Therefore, both of cell types respond differently to the inflammatory responses occurring following solid organ transplantation (Taflin *et al.*, 2011).

Donor specific antibodies can form against HLA class I and class II antigens inducing allograft rejection. Polymorphism of these antigens is mainly in the peptide binding groove which can be identified by different methods to determine the HLA type and antigen specificity. Traditionally, serological typing was used to identify the cell surface expression of HLA antigens. In this method, patient lymphocytes are mixed with sera of known specificity toward particular HLA antigens; sera were collected mostly from multiparous women. After the addition of complement and a vital dye, the antigen-antibody complex is formed activating the complement system and inducing cell death. Dead cells with permeable membrane internalize the vital dye (Acridine Orange or Ethidium bromide) which is visualized under phase contrast microscopy. By excluding and comparing the results between the wells, the HLA type can be identified. However, this method is no longer used in HLA typing (Tinckam, 2009).

The predominantly used HLA typing nowadays is mainly performed using DNA-based methods. These methods are dependent on the amplification of DNA sequence by polymerase chain reaction technique followed by different detection methods. The most commonly used molecular typing methods: are polymerase chain reaction-specific sequence primer (PCR-SSP), sequence specific oligonucleotide probes (PCR-SSOP) and direct DNA sequencing. The highly polymorphic regions of HLA molecules are encoded by second and third exons in the case of HLA class I and second exon in the case of HLA class II. Both PCR-SSP and PCR-SSOP can be performed at low, intermediate or high resolution. Low resolution typing gives similar resolution to the serological typing with identification of allele groups. The intermediate resolution typing provides possibility of group of alleles at more restricted manner. With higher resolution typing, additional information about other exons might be achieved by adding additional primers (SSP) or additional probes (SSOP) to determine the exact HLA type (Nunes *et al.*, 2011).

Luminex assay is the most commonly used in PCR-SSOP method. Isolated DNA is amplified using biotin-conjugated primers specific to either HLA-A, HLA-B or HLA-C locus. Amplified products are then denatured and the denatured strands hybridize with sequence specific oligonucleotides probes attached to microsphere beads panel. Streptavidin-conjugated phycoerythrin is then added and the fluorescence intensity of phycoerythrin in each bead is determined by Luminex flow analyzer. HLA alleles are then assigned using specific software containing sequences listed in IMGT/HLA database. This method is considered slightly expensive compared to other types, since it requires a set of beads and Luminex analyzer (Petersdorf, 2008).

The PCR-SSP is the most widely used PCR-based typing. In this method, a particular HLA allele is amplified by using specific sequence primer in a pre-designed 96 wells tray. The content of the wells is then analyzed by separation into agarose gel. Interpretation of the results is performed by assigning the amplified products in the gel with a corresponding primer in the wells. Among the advantages of this method are: equipment required, test cost and time to perform the assay. The minimum molecular equipment such as thermal cycler and gel electrophoresis are sufficient to perform this test with reasonable cost depending on the cost of the primers. The method is considered the quickest molecular method that can be used for typing a deceased donor. In addition, the typing can be performed with low to high resolution according to the primer mix used (Erlich *et al.*, 2001). Therefore, this method was used in this project.

The majority of previously published studies on HLA class I antibody have used macrovascular endothelial cells (Jin *et al.*, 2004; Jindra *et al.*, 2008c). Therefore, in this project the effect of HLA class I antibody on the activation of microvascular endothelial cells (HMEC-1) was examined. In addition, microvascular endothelial cells are a good model to study leukocyte adhesion and migration. HMEC-1 cells were characterized for the expression of endothelial cell markers, CD31 and CD34 and compared to a model of macrovascular endothelial cells, EA.hy926 cells. The modulation in the expression of HLA class II antigens on both cell types by inflammatory cytokines was also determined. Mouse HLA class I antibody, W6/32, was chosen for this project. W6/32 antibody binds to all HLA class I antigens reacting with the monomorphic determinants present in the α3 domain and β2-microglobulin. The hybridoma cell line (W6/32) was grown and the antibody was purified from the conditioned media and used in this project as will be described in later sections within this chapter.

3.2 Specific Aims

In this chapter the characterization of human endothelial cells was performed. This includes;

- The expression of endothelial cell markers, CD31 and CD34;
- The modulation in the expression of HLA class II molecules by inflammatory cytokines IFN-γ and TNF-α;
- HLA class I and ABO genotyping of HMEC-1 cells, and
- The purification of HLA class I antibody (W6/32).

3.3 Specific materials and methods

3.3.1 Immunofluorescence staining on chamber slides

Immortalized endothelial cells (EA.hy926 and HMEC-1) grown in chamber slides until confluency as described in section 2.3.1 and 2.3.2 were washed twice with PBS after removing the media. Washed cells were fixed with cold methanol at -20^{o}C for 10 minutes. Once the slides were dried, they were covered with 0.1 % triton X-100 /PBS and incubated at 4^{o}C for 10 minutes in a humid atmosphere. After washing twice with PBS for 10 minutes, 5% BSA was added and cells were incubated at 4^{o}C for one hour to block non-specific binding. Primary mouse anti-human CD31 and CD34 antibodies (R&D system) were diluted in BSA at the manufacturer's recommended concentration and cells were incubated at 4^{o}C overnight. After washing three times, secondary antibody, rabbit anti-mouse FITC conjugated antibody (Sigma), was added at an optimum dilution (1:150) followed by incubation in a dark humid atmosphere for two hours at the room temperature. After washing, DAPI was added for five minutes in the dark at a final concentration of 2 μg/ml prepared in PBS. After washing three times, fluorescence mounting media (DAKO) was added and the slides were covered by cover slips and sealed with nail polish solution. Slides were examined by fluorescence using an inverted microscope (Leica LCM microscope).

3.3.2 Treatment of endothelial cell lines with IFN-γ and TNF-α

Interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α) were supplied as lyophilized powder (R&D system). They were reconstituted in PBS in accordance with manufacturer's instructions to make a stock solution of 200 μg/ml and 100 μg/ml, respectively. The stock solution was aliquoted and stored at -20^{o}C. For stimulation, cells were grown in 12-well plates until 50-70 % confluency. Different concentrations of IFN-γ were prepared in complete media just before use, either alone or in combination with TNF-α. Cells were washed once with PBS and then treated with cytokine-containing media. Cells were incubated at 37^{o}C for different time points ranging from 24-72 hours without media change. After cytokine stimulation, cells were detached and analyzed for the expression of HLA class II antigens by flow cytometry.

3.3.3 Direct immunofluorescence staining for HLA class II expression

After treatment, healthy cells were washed once with PBS and detached using enzyme-free cell dissociation buffer (Invitrogen). Cells were counted and 1X10^{5} cells were

washed once with 2% FBS in PBS at 500g for 5 minutes. The cells were stained with mouse anti-human PE conjugated HLA class II antibody (Abcam) specifically for HLA-DR, DQ and DP. After 20 minutes incubation at 4^{o}C, cells were washed twice with 2% FBS in PBS before analysis by flow cytometry as described in section 2.7.3. Unstained cells were used to determine the gates for FSC against SSC and SSC against FL-2 channel. In parallel, cells stained with isotype control antibody (PE-conjugated mouse IgG2a) were used to adjust the background staining. This value was subtracted from the median fluorescence of all stained tubes.

3.3.4 Acid treatment assay

To produce a cell line without expression of HLA class I molecules, HMEC-1 cells were exposed to sodium citrate buffer at pH 3 (Sugawara *et al.*, 1987). This buffer was prepared by mixing 0.123M Na_2HPO_4 containing 1% (w/v) bovine serum albumin with 0.263 M citric acid and adjusting the pH to 3. Adherent cells were treated with this buffer for two minutes before washing with PBS and detachment by enzyme-free cell dissociation buffer. Cells were stained with 12 µg/ml of mouse HLA class I antibody (W6/32) for 20 minutes at 4^{o}C. Following washing with 2% FBS/PBS, incubation with FITC-conjugated rabbit anti-mouse IgG (Sigma) was performed at 4^{o}C for another 20 minutes. After further washing steps, cells were resuspended in 200 µl FBS/PBS and analyzed by flow cytometry. After compensation, propidium iodide was added at a final concentration of 2.5 µg/ml to exclude dead cells. The acid treatment induced not more than 10% of cell death.

3.3.5 Effect of trypsin treatment on cell surface HLA class I expression

To investigate the ability of trypsin to induce proteolytic cleavage and shedding of HLA class I molecules expressed by HMEC-1 cells, adherent cells were detached with either trypsin or non-enzymatic PBS-EDTA cell dissociation buffer and washed in 2% FBS/PBS. Cells were counted and 2X10^{5} cells were stained for the expression of HLA class I molecules by W6/32 antibody followed by FITC conjugated rabbit anti-mouse IgG as previously described. Cells resuspended in 200 µl FBS/PBS were analysed by flow cytometry using FSC/SSC and excitation/emission for FITC dye 488/530.

3.3.6 Genotyping of cell lines for the HLA class I

3.3.6.1 DNA Extraction

A T 75 cm^2 flask of 80-90 % confluent endothelial cells was trypsinized and 8 million cells were re-suspended in 200 µL PBS. These cells were treated with proteinase K and incubated at 50^oC for 15 minutes to dissociate the cell membrane. DNA extraction was performed using Geno-M6 instrument (QIAGEN) located in the National Health Service (Blood and Transplant laboratory (NHSBT), Newcastle). This machine isolates DNA from cell lysates through its binding to the silica surface of magnetic particles in the presence of a chaotropic salt. The bound DNA is then washed and eluted by elution buffer. The DNA concentration was determined by Nanodrop and 60 ng/µl of DNA was used for genotyping.

3.3.6.2 HLA typing using PCR-SSP

Low to medium resolution typing for HLA class I (A,B,C) and HLA class II (DR,DQ) were performed by PCR-SSP using a Bio-Rad HLA SSP kit according to manufacturer's instructions. The kit consists of 96 well plates with each well containing dried primer mix that identifies a group of alleles. Each well also contains an internal positive control primer which amplifies a 1069 bp fragment of human growth hormone to demonstrate successful PCR amplification. The concentration of the control primers is lower than the allele-specific primer pairs as this could render these bands weak in the presence of positive allele-specific product. The HLA class II genotype was also performed in case this was needed for a later analysis, but only the HLA class I genotype was used in this project. PCR reaction was performed by mixing 748 µl of distilled water, 544 µl of PCR cocktail and 8.7 µl of Taq DNA polymerase. 10 µl of the PCR reaction was added to the negative control wells before the addition of the DNA sample. 68 µl of DNA sample was then mixed with the PCR reaction and 10 µl was added to the remaining wells for HLA class I and class II typing. The samples were amplified in thermal cycler using the following protocol:

Initial denaturating	96^oC	2 minutes	
Denaturing	96^oC	10 seconds	10 cycles
Annealing & Extension	65^oC	60 seconds	
Denaturing	96^oC	10 seconds	
Annealing	61^oC	50 seconds	20 cycles
Extension	72^oC	30 seconds	

3.3.6.3 Agarose electrophoresis

Post-PCR, the PCR product in each well was loaded into 2% agarose gels which were prepared by boiling 2 g of agarose in 100 ml of 1X Tris Boric acid EDTA (TBE) until completely dissolved. The solution was cooled to < 60^{o}C, and 4 µl of ethidium bromide was added to 50 ml of the solution. The solution was then poured into sealed gel trays with combs. The gels were left to solidify at the room temperature for 15 minutes. After gel solidification, the gels were placed into the gel tank and covered with TBE buffer. The PCR products from the wells were pipetted into the gel wells. The gels were running at 8 voltage /cm for 15 minutes. A DNA ladder with molecular weight ranging from 50-1000 base pairs was used to check the size of PCR products. The gels were visualized on UV transilluminator and photographed for documentation and interpretation. The results were interpreted using Bio-Rad SSP typing software. In this project, three cell lines were genotyped: HMEC-1 endothelial cells, HK-2 and HKC-8 epithelial cell lines.

3.3.6.4 ABO genotyping of HMEC-1 cell line by PCR-SSP

DNA was extracted from HMEC-1 cell line as described previously in section 3.3.6.1. Since the ABO antigens are carbohydrates, ABO genotyping is performed by determining the presence of glycosyl transferase enzymes located on chromosome 9 responsible for building the sugar blocks. Specific sequences were amplified by traditional PCR in 8 reactions strip containing primers for these enzymes (inno-train, Germany). The amplicon was run on 2% agarose gel as described in the previous section and interpreted manually using the manufacturer's sheet.

3.3.7 Purification of W6/32 antibody

3.3.7.1 Media collection and processing

As a source of HLA class I antibody for this project the W6/32 hybridoma cell line was grown in medium containing IgG stripped serum as described previously in section 2.3.5. The media collected and stored at -20^{o}C was thawed and filtered through a 0.2 µM sterile filter to remove any debris that might block the column during the purification process.

3.3.7.2 **Affinity chromatography**

3.3.7.2.1 General principle

Affinity chromatography is the most commonly used biochemical technique to purify antibody from a complex mixture. This technique is dependent on reversible interaction between protein and its ligand in a highly selective way such as between antigen and antibody. The bound antibody is then eluted or recovered by using other factors such as changes in pH, ionic strength or polarity. The specificity and stability of the system is maintained by the use of bispecific ligands that are supported on chromatography matrices. These ligands can be naturally derived products, bacterial derived receptors, antigens or anti-antibody. The application of bacterial proteins to antibody purification is based on the high affinity between the bacterial protein and antibody. The most frequently used bacterial protein ligands are Staphylococcal protein A and streptococcal protein G, which are extracted from bacterial cell walls of *Staphylococcal aureus* and *Streptococcal group C and G*, respectively. Both proteins are used in the purification of intact antibody and characterized by their capacities to bind different subclasses of antibody.

Protein A contains five domains that can bind different subclasses of immunoglobulin. In the case of IgG, the binding to protein A occurs through the Fc region of the antibody between the CH2 and CH3 domains. A protein A column has high purification efficiency due to its selectivity in binding IgG antibody; in particular, mouse IgG1, IgG2b, IgG3 and very high affinity for IgG2a. In this project, bacterial receptor in the form of a protein A column was used for the purification of W6/32 antibody (mouse IgG2a) from a hybridoma conditioned media as was described in section 2.3.5. Media containing antibody was run through the protein A column at a natural pH (8.2). Elution with acidic buffer at pH 2 was then used for elution of bound antibody. To avoid antibody denaturation and aggregation at low pH, neutralization to a neutral or slightly basic pH was performed.

3.3.7.2.2 Antibody purification using Protein A column

The fast protein liquid chromatography system (FPLC) was used to purify the HLA class I antibody (mouse IgG2a), (Äkta, GE healthcare). Before purification, the protein A column (GE healthcare) was washed with 75% ethanol followed by washing with filtered PBS solution. The media was kept on ice and passed through a pre-equilibrated

protein A column overnight at a rate of 0.5 ml to 1 ml/minute. After running the media, the column was washed with PBS and the bound antibody was eluted using 0.1 M glycine at pH 2.2. The eluates were collected into 1 ml Eppendorf tubes containing 0.5 ml of 1M Tris PH 8.8 to prevent antibody degradation. The protein content of each fraction was detected by an inbuilt detector which estimates the absorbance of each fraction at 280 nm. Figure 3.1 shows the resultant peak that occurs at a high antibody concentration. The protein concentration of the fractions was also assessed using a Nanodrop. The fractions that contain antibodies were combined together and concentrated using a 20 ml centrifuge concentrator tube (Thermo scientific) with cutoff 100 kDa. The protein concentration of the concentrator was determined by BCA assay as it was described in section 2.8.3. Purified antibody was adjusted to 1 mg/ml in sterile PBS, aliquoted into small Eppendorf tubes and stored at -20^0C.

3.3.7.3 **ELISA assay**

The presence of mouse IgG antibody in each fraction was assessed by Enzyme-Linked Immunosorbent Assay (ELISA). Eluted fractions were diluted with carbonate coating buffer (15 mM Na_2CO_3, 35 Mm $NaHCO_3$, pH 9.6), 1/10 and 1/100, and incubated overnight at 4^oC in 96 well plates. The wells were washed three times with 0.1 % tween 20 in PBS (TPBS) and then blocked with 3% BSA for 30 minutes at the room temperature. Anti-mouse HRP antibody (Sigma) was added to the wells at concentration (1:5000). After 1 hour incubation at the room temperature, wells were washed three times with TPBS. The plate was then developed using a solution containing 0.4 mg/ml O-phenylenediamine (OPD) in 50mM citrate at pH 5 in the presence of 0.012% (v/v) H_2O_2. The reaction was stopped after 30 minutes using 2M sulphuric acid and the absorbance was recorded at 490 nm. Blank wells contained all reagents except the W6/32 antibody.

3.3.7.4 **Reducing SDS-PAGE**

The purification of antibody was confirmed by separation on 10% SDS-PAGE gel under reducing condition using β-metcaptoethanol. The addition of this agent dissociates the antibody to its heavy and light chains which appear at 55 and 25 kDa, respectively. Five μg of antibody was mixed with 2 X loading buffer containing β-metcaptoethanol and heated for 10 minutes. The sample as well as the prestained protein marker was run on 10% SDS-PAGE electrophoresis at 30 mA for around 1.5 hour. The gel was stained using coomassie stain and destained as described in section 2.8.5.

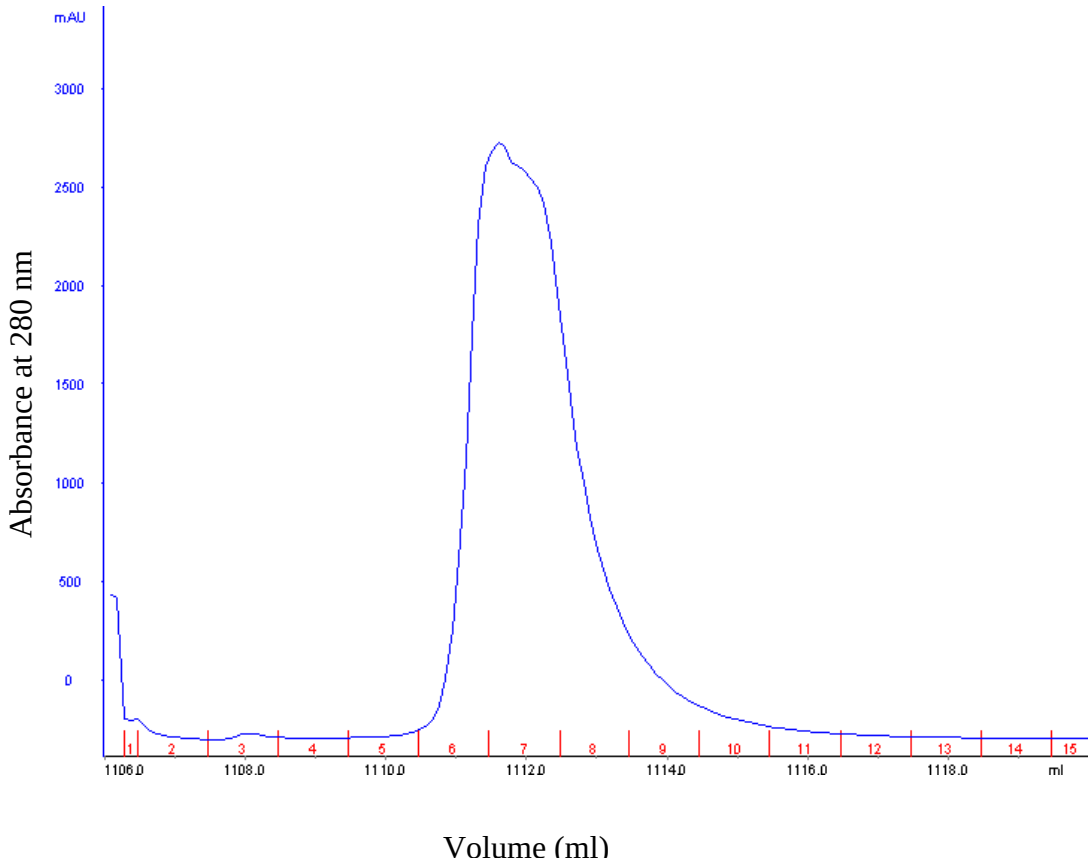


Figure 3.1: Purification of W6/32 antibody using a protein A column.

The media was passed through the protein A column at 0.5 ml/minute overnight. The bound antibody was eluted by 0.1 M glycine (pH 2.2). The eluted protein in each fraction is detected by UV light which gives the absorbance of each fraction at 280 nm.

3.3.7.5 **Indirect immuno-fluorescence staining for HLA class I expression**

To determine the expression of HLA class I on HMEC-1 endothelial cells, the cells were detached using cell dissociation buffer, washed once in 2% FBS/PBS and counted. $2X10^5$ cells were added into each tube and stained with different concentrations of W6/32 antibody ranging from 36 µg/ml to 0.005 µg/ml. After incubation for 20 minutes at 4^0C, cells were washed twice with 2% FBS/PBS. FITC-conjugated rabbit anti-mouse IgG antibody was added at a concentration recommended by the manufacturer and incubated again for 20 minutes at 4^0C. After incubation, cells were washed twice with 2% FBS/PBS and resuspended into 200 µl of FBS/PBS before analysis by flow cytometer.

3.4 Results

3.4.1 Expression of CD31 and CD34 on endothelial cells

Platelet derived endothelial cells adhesion molecule (PECAM-1 or CD31) is a transmembrane glycoprotein with molecular weight 130 kDa. It is a member of the immunoglobulin superfamily molecules. It is expressed on the surface of platelets, neutrophils, monocytes, macrophages and at endothelial intracellular junction. CD31 plays an important role in facilitating the migration of inflammatory cells during inflammation process and is used as an endothelial cell marker. EA.hy926 and HMEC-1 endothelial cells were shown to express CD31 molecules by the indirect immunofluorescence staining as shown in Figure 3.2. To confirm this result, expression of CD31 on EA.hy926 cell line was investigated by flow cytometry at various antibody concentrations as shown in Figure 3.3. CD34 is a transmembrane glycoprotein with molecular weight 110 kDa presents on various cell types including endothelial cells of blood vessels but not lymphatic origin, stem cells, leukemic cells and dendritic cells. The function of CD34 is not clear; however, its location at cell to cell junction suggests a role in leukocyte extravasation to inflammatory sites. In bone marrow, CD34 facilitates the attachment of stem cells to bone marrow. HMEC-1 cell line expressed CD34 at low level as examined by immunofluorescence staining. In contrast, there was no CD34 expression on EA.hy926 cell line as shown in Figure 3.2.

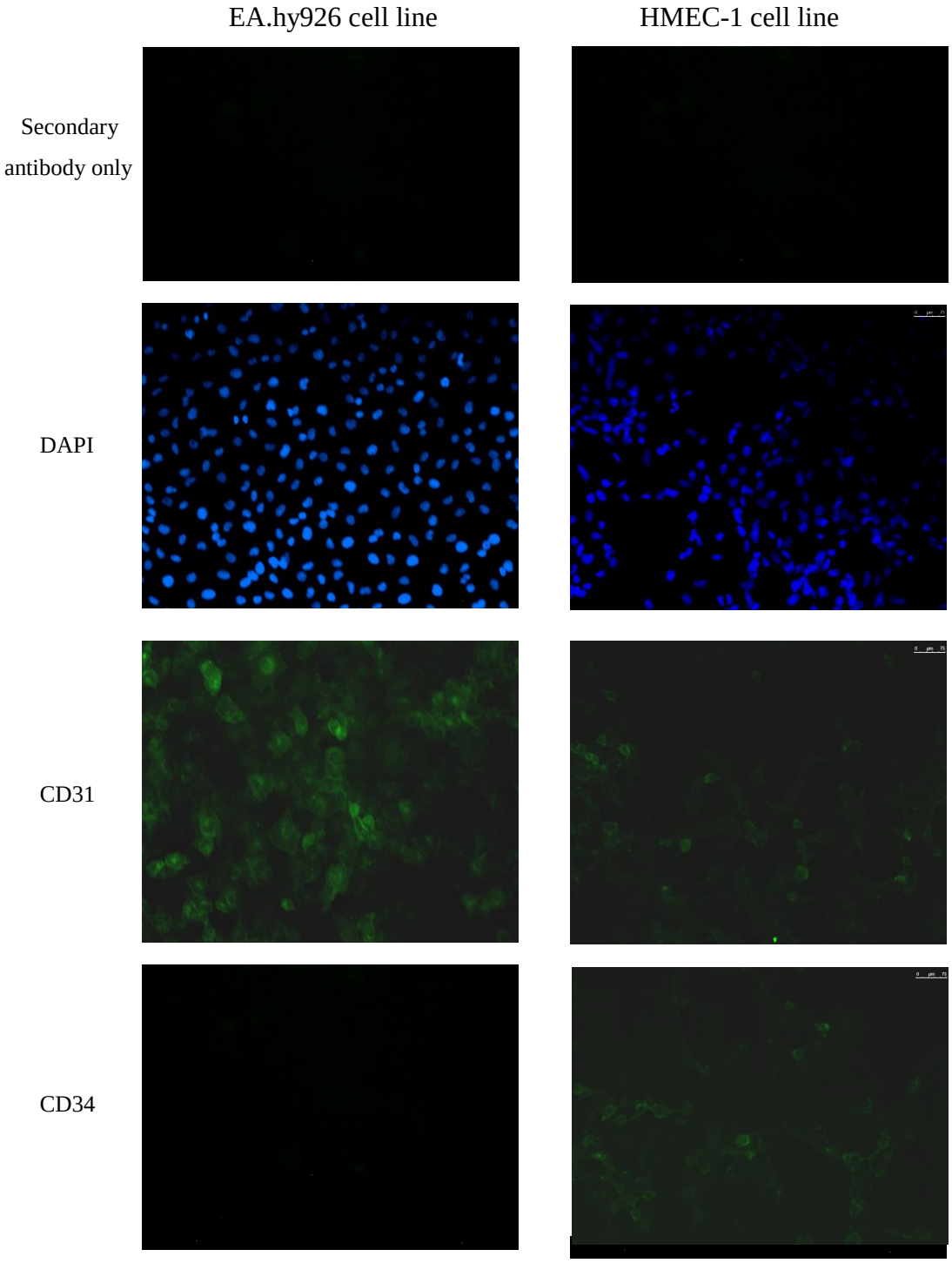


Figure 3.2: Indirect CD31 and CD34 immunofluorescence staining on EA.hy926 and HMEC-1 endothelial cells.

Cells grown on chamber slides were fixed and stained using mouse anti-human antibodies against CD31 and CD34 molecules followed by FITC-conjugated rabbit anti-mouse antibody. Slides were examined by fluorescence microscope (Leica). The data are representative of two independent experiments.

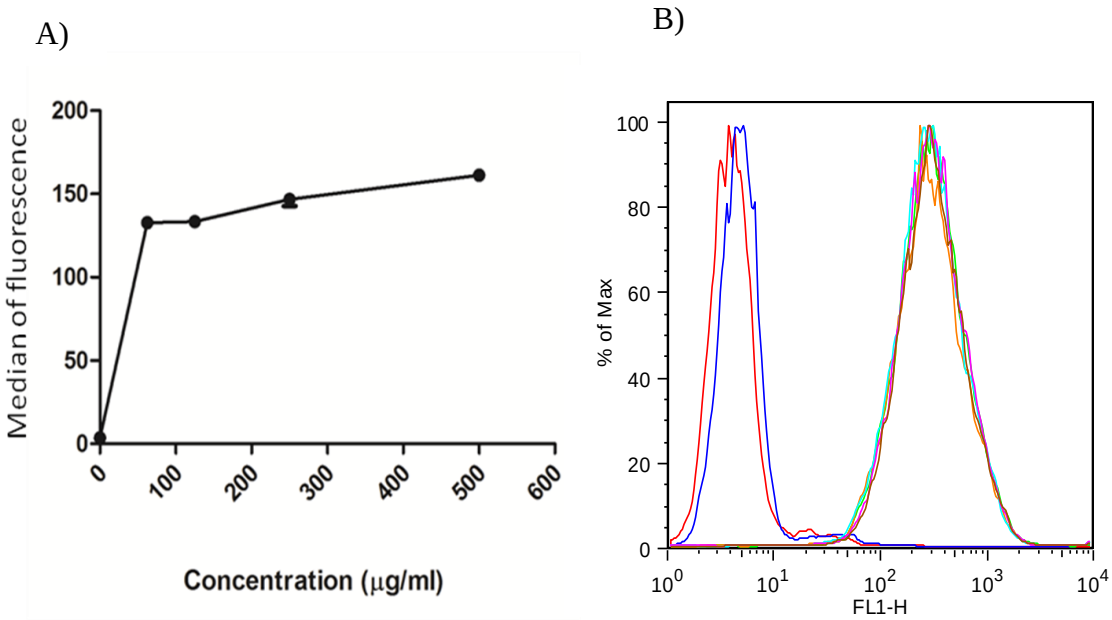


Figure 3.3: Expression of CD31 molecules on EA.hy926 cells examined by flow cytometer.

Panel A shows the expression of CD31 molecules on EA.hy926 cell surface using different concentrations of mouse anti-human CD31 antibody. Panel B shows flow cytometer histogram for CD31 expression showing unstained (red), secondary antibody (blue) and different concentrations of primary CD31 antibody ranging from 100-500 µg/ml (green, purple, yellow). The data are representative of two independent experiments.

3.4.2 Expression of HLA class II antigens on endothelial cells stimulated with IFN-γ

Human endothelial cells *in vivo* express HLA class II antigens at low level and this expression is lost *in vitro* during cell culturing. However, the expression of these molecules can be modulated in a concentration and time dependent manner by different cytokines. Interferon-γ (IFN-γ) is a proinflammatory cytokine produced by a variety of lymphatic lineage cells such as T-cells, B-cells and NK cells. Previous studies have shown that IFN-γ can induce the expression of HLA class II molecules on endothelial cells (McDouall *et al.*, 1997; Muczynski *et al.*, 2003). As shown in Figure 3.4, unstimulated EA.hy926 and HMEC-1 endothelial cells expressed a negligible level of HLA class II molecules which is upregulated significantly following stimulation with IFN-γ. The increase in the expression was dependent on cytokine concentration as shown by using different cytokine concentrations ranging from 0.3 nM to 2.9 nM for 72 hours. Although the expression of HLA class II molecules on both cell types was increased with IFN-γ treatment, HMEC-1 microvascular endothelial cells showed a higher response to lower concentration of IFN-γ compared to EA.hy926 cells.

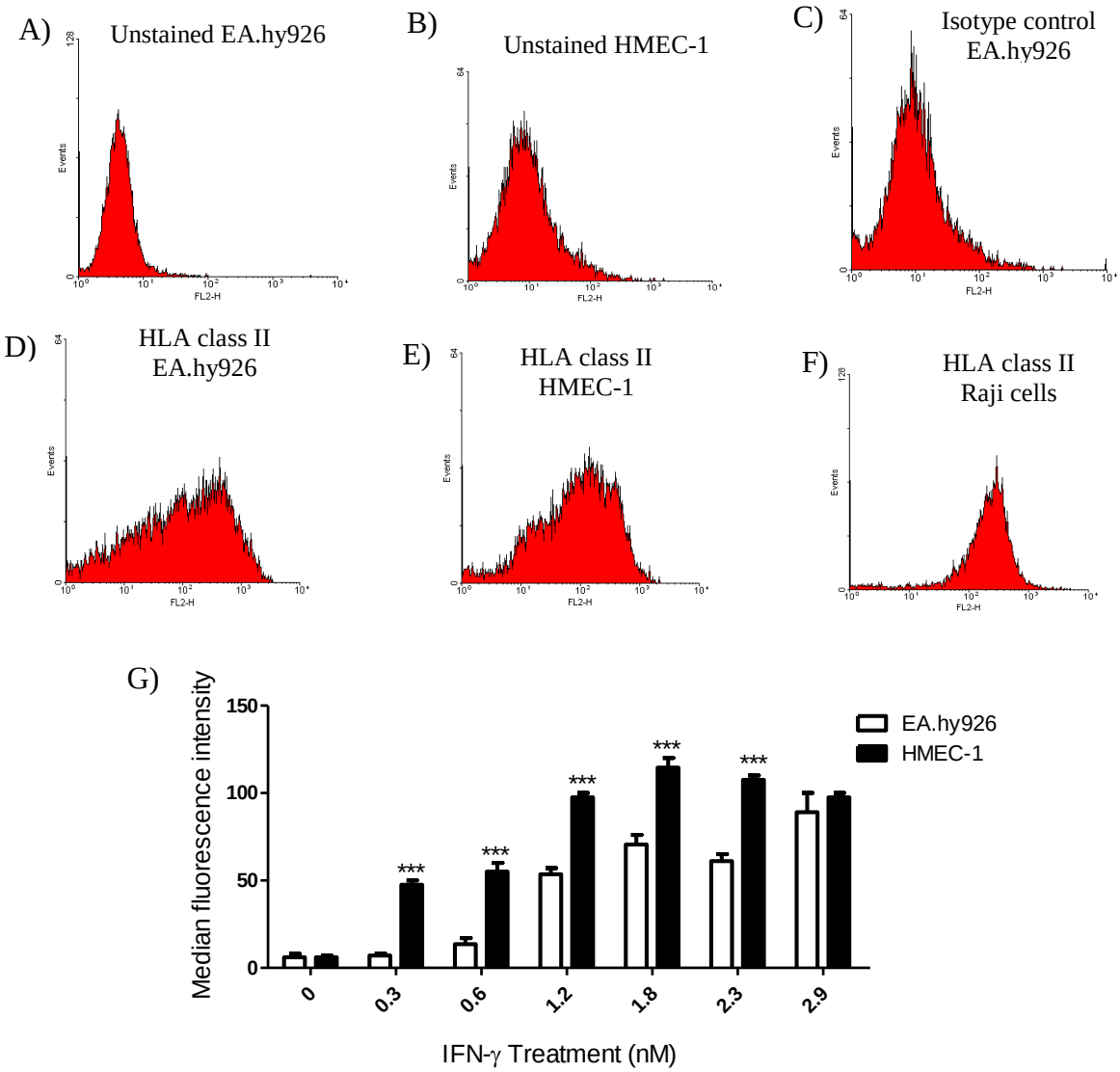


Figure 3.4: Expression of HLA class II antigens on EA.hy926 and HMEC-1 endothelial cells following stimulation with IFN-γ.

Endothelial cells were incubated with different concentrations of IFN-γ for 72 hours before cell detachment. Cells were stained with PE-conjugated mouse anti-human HLA class II antibody and analyzed by flow cytometer. A and B show unstained EA.hy926 and HMEC-1 cells, respectively. C shows isotype control PE-conjugated mouse IgG2a on EA.hy926 cells. D and E show examples of staining with HLA class II antibody on EA.hy926 and HMEC-1 cells, respectively. F shows positive control using Raji B-cells. G Bar chart represents median fluorescence intensity of the two cell types. The data were analyzed by two way ANOVA followed by Bonfferoni test. *** $p<0.001$ compared to EA.hy926 at the same concentration. The data are representative of three independent experiments.

3.4.3 Expression of HLA class II antigens on endothelial cells stimulated with both IFN-γ and TNF-α

Tumor necrosis factor-α (TNF-α) is a non-glycosylated protein with molecular mass of 17 kDa. It is a proinflammatory cytokine secreted by cell types such as mast cells and endothelial cells in response to inflammatory stimulus. The modulation in the expression of HLA class II antigens on endothelial cells in response to a costimulation with IFN-γ and TNF-α was examined on both EA.hy926 and HMEC-1 cells. EA.hy926 cells were treated with varying concentrations of TNF-α ranging from 0.29 nM to 5.7 nM with a constant concentration of IFN-γ (1.5 nM) for 72 hours. It was expected that the addition of these two cytokines collectively to the endothelial cells would produce a synergistic effect on the expression of HLA class II molecules, since both are pro-inflammatory cytokines. However, this treatment significantly abrogated the ability of IFN-γ to induce the expression of HLA class II antigens. In addition, as the concentration of TNF-α increases the inhibitory effect increases accordingly, as shown in Figure 3.5. The addition of TNF-α at 0.29 and 5.7 nM reduced the expression of HLA class II antigens by around 50% and 80%, respectively compared to cells treated with IFN-γ only. However, the increase in expression was still statistically significant compared to the untreated group. Treatment of HMEC-1 cells with the same cytokines combination induced a similar effect as shown in Figure 3.6.

To confirm these findings, HMEC-1 and EA.hy926 cells were treated with the higher concentration of TNF-α (5.7 nM) in combination with different concentrations of IFN-γ, 5, 10 and 25 nM at different time points. As previously observed, treatment of both cell types with different concentrations of IFN-γ did not reverse the inhibitory effects mediated by TNF-α at any time point 24, 48 or 72 hours. As the concentration of IFN-γ increased the inhibition of expression was reduced. In addition, TNF-α treatment alone had no significant effect on the expression of HLA class II molecules on EA.hy926 cells but there was a significant increase on HMEC-1 endothelial cells as shown in Figure 3.7 and Figure 3.8, respectively.

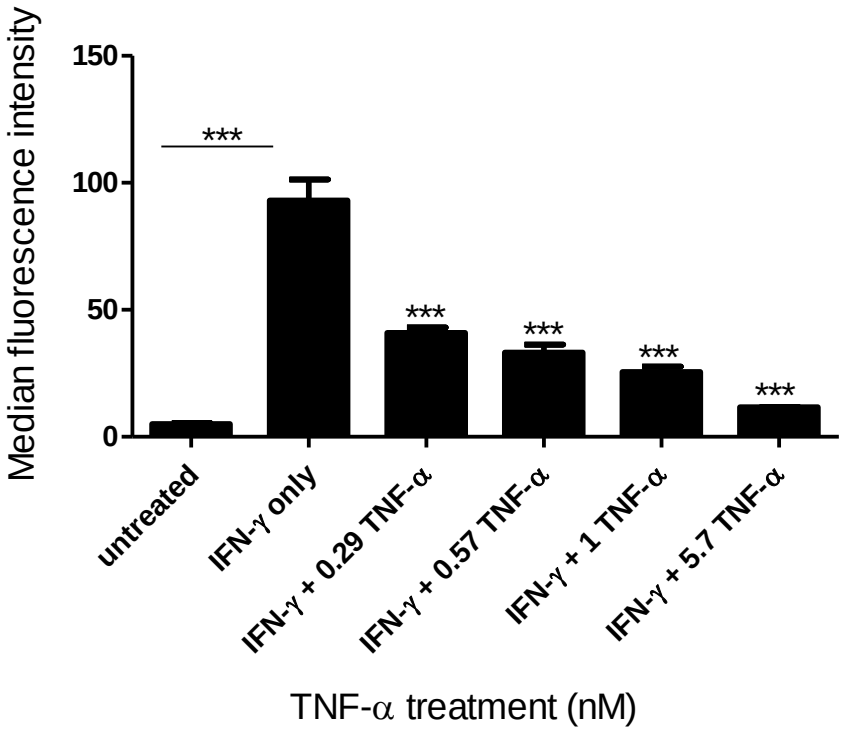


Figure 3.5: Expression of HLA class II molecules on EA.hy926 cells following stimulation with IFN-γ and TNF-α.

Cells were incubated with 1.5 nM IFN-γ and different concentrations of TNF-α ranging from 0.29 to 5.7 nM for 72 hours followed by flow cytometry analysis. Cells treated with combined cytokines were compared to IFN-γ treated group using one-way ANOVA followed by Bonferonni test. The data are representative of two independent experiments. *** $p < 0.001$.

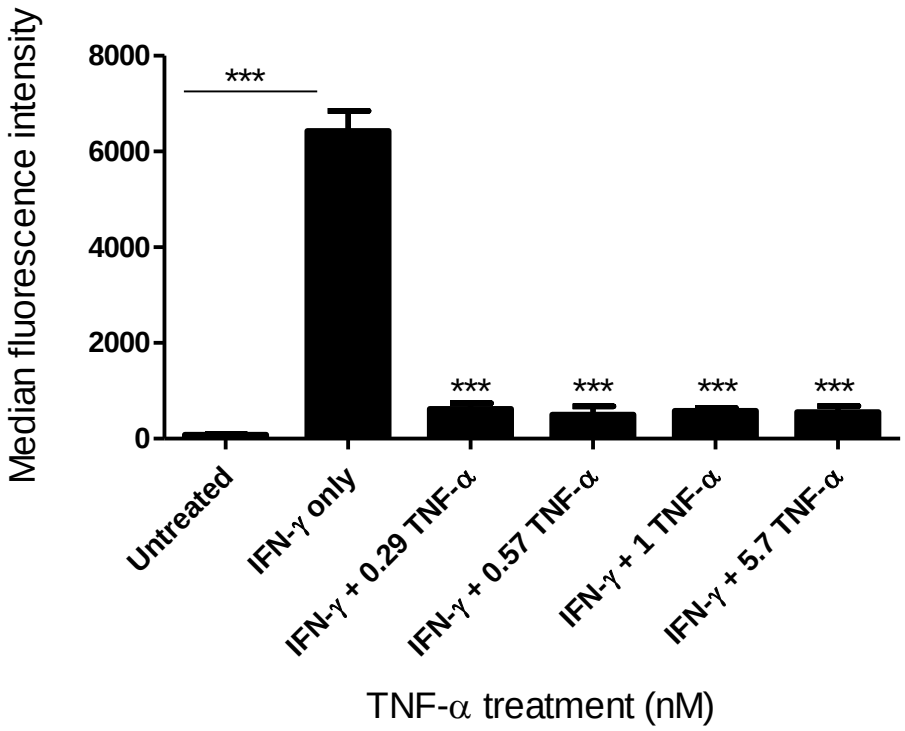


Figure 3.6: Expression of HLA class II molecules on HMEC-1 cells following stimulation with IFN-γ and TNF-α.

Cells were incubated for 72 hours with 1.5 nM IFN-γ combined with TNF-α at different concentrations ranging from 0.29 to 5.7nM followed by analysis using flow cytometer. The analysis between the groups was performed by one-way ANOVA followed by Bonferroni test and compared to IFN-γ treated group. *** $p < 0.001$. The data are representative of two independent experiments.

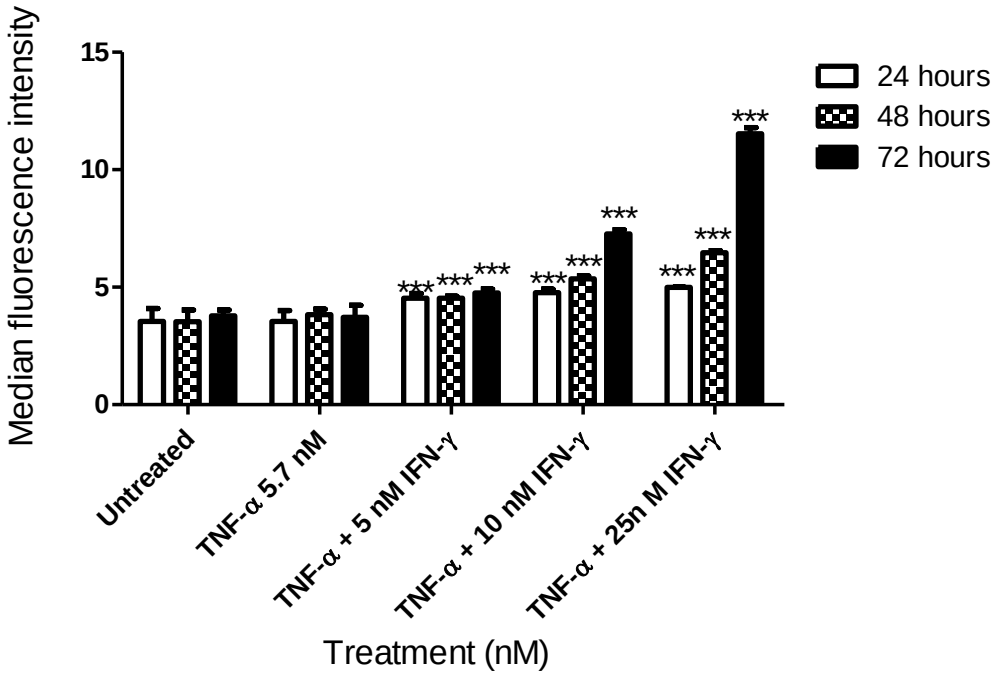


Figure 3.7: Expression of HLA class II molecules on EA.hy926 endothelial cells following stimulation with IFN-γ and TNF-α at different time points.

Cells were incubated for 24, 48 and 72 hours with 5.7 nM TNF-α and different concentrations of IFN-γ (5, 10 and 25 nM) before flow cytometry analysis. Statistical analysis was performed using two-way ANOVA followed by Bonferonni test compared to corresponding untreated group. *** $p<0.001$. The data are representative of three independent experiments.

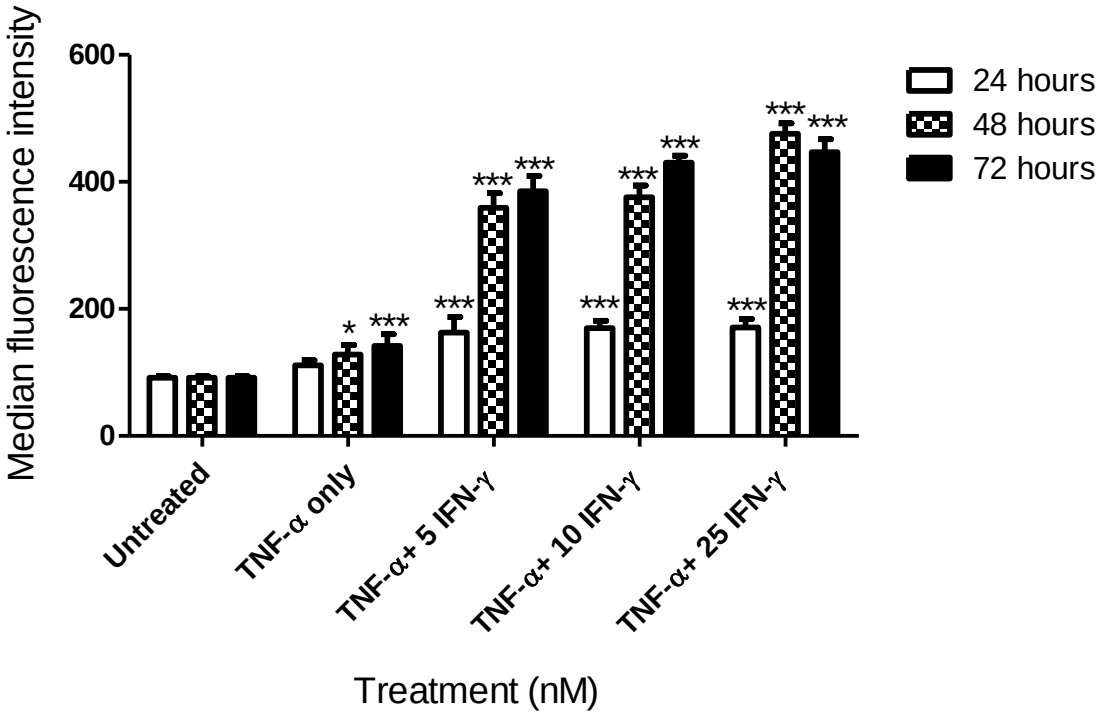


Figure 3.8: Expression of HLA class II molecules on HMEC-1 cell line following stimulation with IFN-γ and TNF-α at different time points.

Cells were incubated for 24, 48 and 72 hours with 5.7 nM TNF-α and different concentrations of IFN-γ (5, 10, 25 nM) before flow cytometry analysis. Statistical analysis was performed using two-way ANOVA followed by Bonferroni test compared to untreated corresponding group. * $p<0.05$ and *** $p<0.001$, respectively. The data are representative of three independent experiments.

3.4.4 Effect of acid treatment on HLA class I expression on endothelial cells

The effect of acid treatment on HLA class I antigen expression was investigated. HMEC-1 endothelial cells were treated with monosodium citrate solution (pH 3) for 2 minutes. Different citrate concentrations were used to determine the lowest concentration that reduced HLA class I expression. Around 80% reduction in the expression of these antigens was achieved with concentrations between 0.06 -0.26 M when compared to untreated group. Treatment with 0.01 and 0.03 M induced a reduction in the antigen expression by 20% and 60%; respectively, compared to untreated groups as shown in Figure 3.9. Treatment with citrate solution at the above concentrations did not induce more than 10% in cell death as shown by staining with PI; however, exposure to citrate concentration higher than 0.26 M induced significant cell death (data not shown). Therefore, 0.06 M was used for further experiments.

The time required to recover the expression of HLA class I antigens post-treatment was examined. HMEC-1 cells were treated with 0.06 M of citrate solution for 2 minutes after which the solution was replaced with fresh media. The cells were detached at different time intervals and stained for the expression of HLA class I antigens using W6/32 antibody. As shown in Figure 3.10, the cells gradually regained the expression of the cell surface HLA class I antigens, this started within 30 minutes after treatment. The complete expression of HLA class I molecules, comparable to untreated cells, was seen after 24 hours. The cell viability was assessed by trypan blue at each time point before analysis by flow cytometry. HMEC-1 microvascular endothelial cells were used for all further experiments.

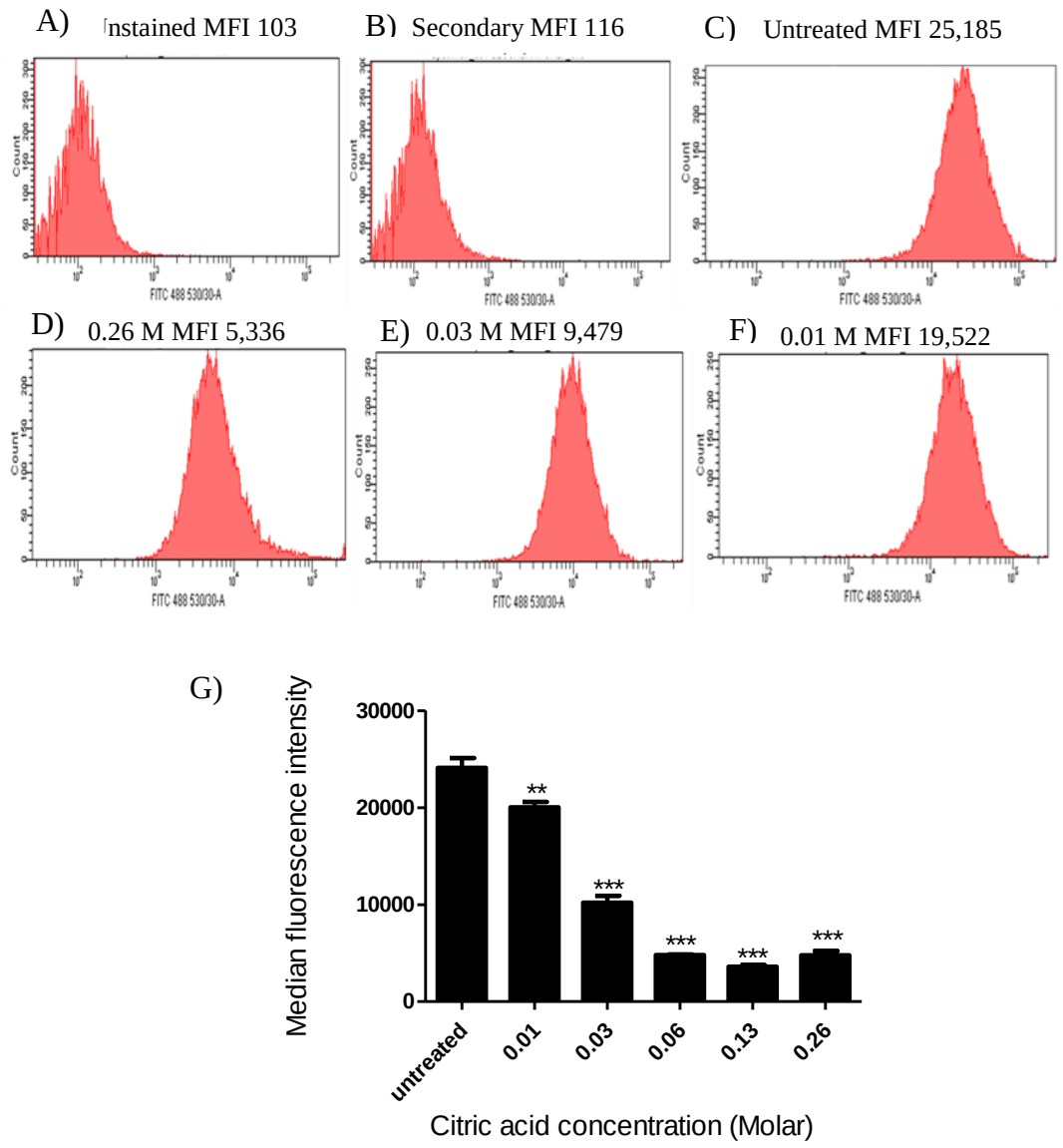


Figure 3.9: Effect of sodium citrate treatment on the expression of HLA class I molecules on HMEC-1 cells.

Adherent HMEC-1 cells were exposed to different concentrations of citric acid solution ranging from 0.26 to 0.01 M for 2 minutes. Cells were detached and stained with 12 µg/ml of W6/32 antibody and analyzed by flow cytometry. Panel A shows unstained, B secondary antibody only, C untreated, D 0.26 M treated, E 0.03 M treated, F 0.01 M treated and G bar chart of the median fluorescence intensity. The data were compared to untreated group by one way ANOVA followed by Bonferroni test. ** $p<0.01$ and *** $p<0.001$. The data are representative of two independent experiments.

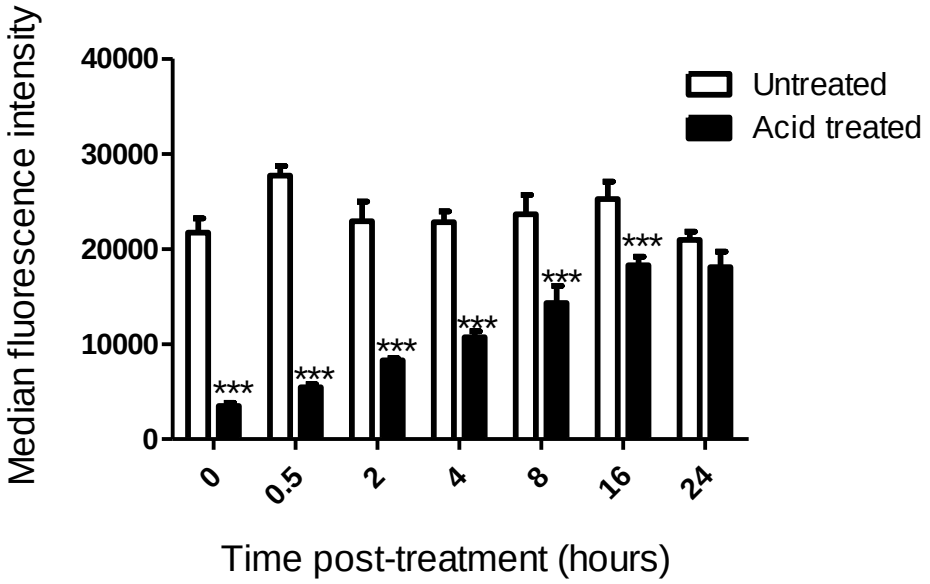


Figure 3.10: Recovery of the expression of HLA class I antigens on HMEC-1 cells following sodium citrate treatment.

Adherent HMEC-1 cells were treated with 0.06 M of sodium citrate (pH 3) for 2 minutes. Cells were detached and stained with 12 μg/ml of W6/32 antibody and analyzed by flow cytometry. The analysis was performed by two-way ANOVA followed by Bonferroni test. *** $p<0.001$. The data are representative of two independent experiments run on duplicate.

3.4.5 Effect of trypsin treatment on the expression of HLA class I antigen on endothelial cells

Trypsin is a serine protease enzyme that cleaves the polypeptide chains at lysine or arginine residues. To assess the effect of trypsin on cell surface HLA class I antigens, adherent HMEC-1 cells were detached with either trypsin or PBS-EDTA based cell dissociation buffer. Following detachment, the cells were stained with W6/32 antibody and examined for the expression of HLA class I antigens by flow cytometry as shown in Figure 3.11. There was no significant difference in the expression of HLA class I antigens when trypsin was used compared to the detachment using cell dissociation buffer. Cells stained with FITC-conjugated anti-mouse secondary antibody were used as a negative control.

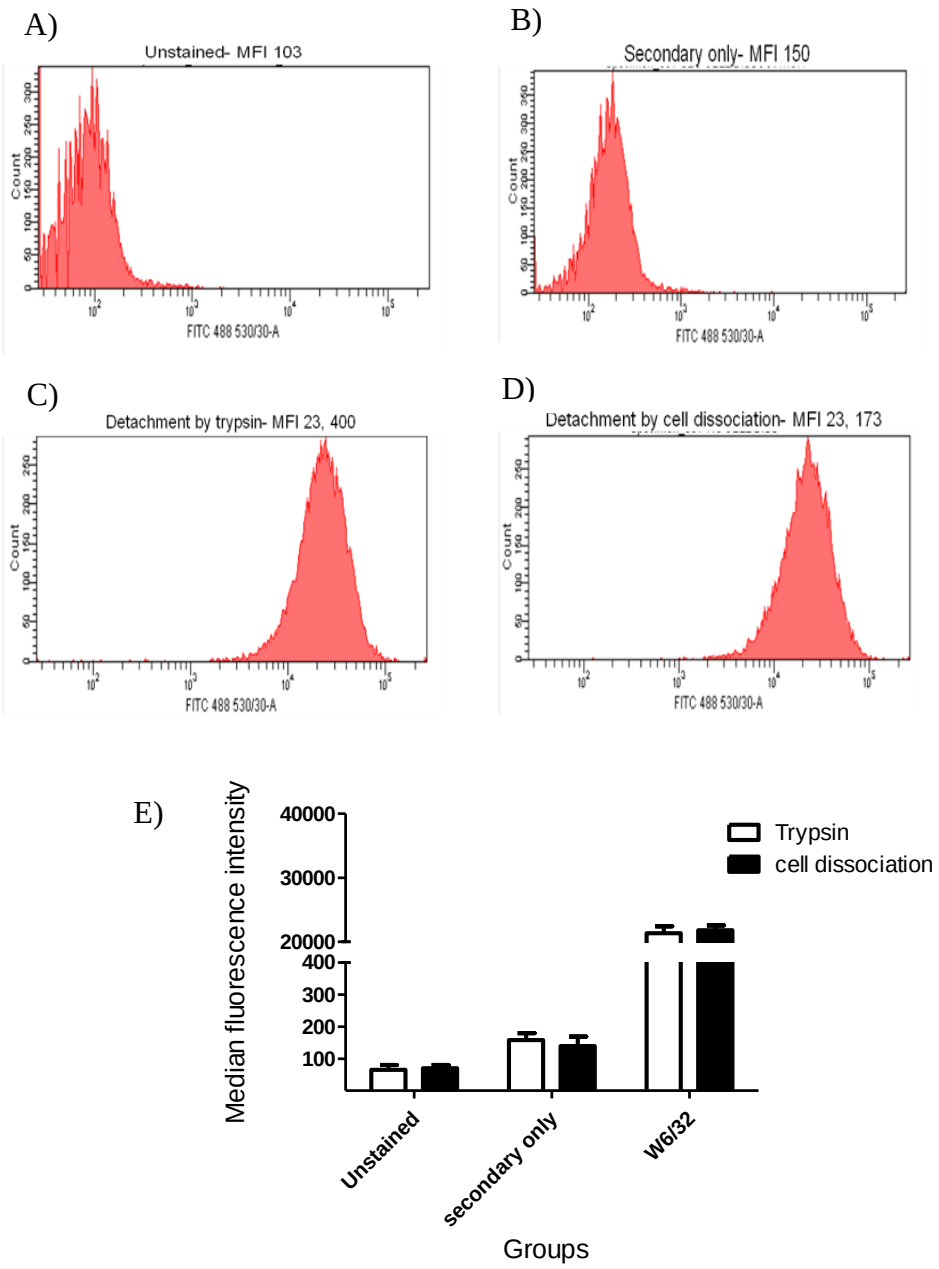


Figure 3.11: Effect of trypsin treatment on the expression of HLA class I molecules.

Adherent HMEC-1 cells were detached using either trypsin or enzyme-free dissociation buffer before cell detachment. Cells were stained using W6/32 antibody at 12 µg/ml followed by rabbit anti-mouse FITC-conjugated secondary antibody. Histograms show A unstained, B secondary antibody only, C staining on trypsin detached cells, D staining on dissociation buffer detached cells and E bar chart of median fluorescence intensity. The data were analysed by two-way ANOVA. The data are representative of two independent experiments.

3.4.6 HLA class I and ABO genotyping on HMEC-1 cells

HMEC-1 cells were genotyped for HLA class I (HLA-A, B and C) and ABO blood group by PCR-SSP method. The identification of the HLA class I type was performed to allow selecting sera from sensitized patients with corresponding antibody specificity. Adherent HMEC-1 cells were detached and the DNA was isolated as described in the methods section. Amplification was performed with primers specific to group of alleles in a tray and the amplicons were run on agarose gel. Figure 3.12 shows the agarose gels for HLA-A, B and C. Each well contains a primer that amplifies the human growth hormone as a positive control (red arrow). The gel was interpreted by using Biotest software to determine the genotype. The HLA class I genotype for HMEC-1 is A*01, A*68, B*35, B*58, C*04, C*06.

To ensure there was no effect of ABO antibodies in the polyclonal sera, The HMEC-1 cells were genotyped for ABO antigens by PCR-SSP. This was performed to eliminate any potential interaction of ABO antibody with the endothelial cells. HMEC-1 DNA was amplified using specific primers for ABO transferase enzymes. The amplicons were run on agarose gel as shown in Figure 3.13. Each well has a primer that amplifies the human growth hormone as a positive control. The amplicon product was run on 1.2% gel and interpreted using Biotest software. The cell line is from an "O" blood group donor.

Cell line	HLA typing	ABO typing
HMEC-1 cell line	A*01, A*68 B*35, B*58 C*04, C*06	'O'

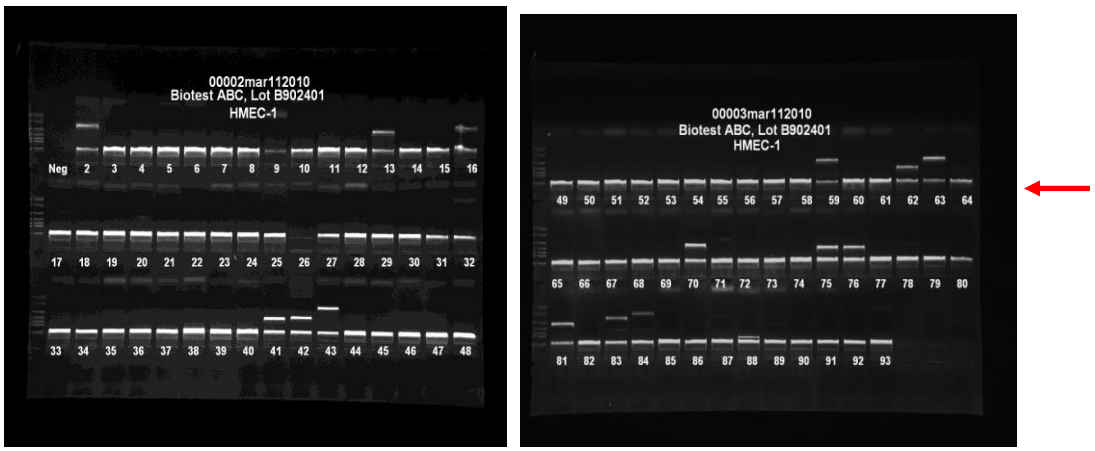


Figure 3.12: Agarose gel of HLA class I genotyping on HMEC-1 cell line using PCR-SSP method.

The amplicon product from each well was run on 1.2% agarose gel and visualized by UV machine. Lanes from 1-25 represents HLA-A genotypes, 26-76 for HLA-B and 77-93 for HLA-C. HMEC-1 class I typing are A*01, A*68, B*35, B*58, C*04, C*06. Interpretation of the data was performed using Biotest SSP typing Software.

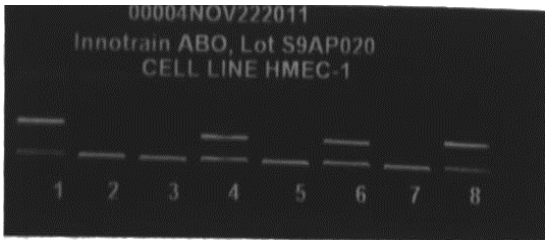


Figure 3.13: Agarose gel of ABO genotype of HMEC-1 cell line using PCR-SSP method.

DNA was extracted and amplified using specific sequence primer for transferase enzymes. The amplicon was run on 1.2% agarose gel. Interpretation of the data was performed using Biotest SSP typing Software.

3.4.7 Purification of W6/32 antibody

The hybridoma cell line (W6/32) which produces a monoclonal mouse HLA class I antibody (IgG2a) was grown in media supplemented with IgG stripped serum as described in section 2.3.5. Conditioned media from W6/32 cells was collected and the antibody was purified using protein A column as described in the methodology part. The concentration of the eluted and concentrated antibody was determined using a BCA protein assay kit. The presence of the antibody was confirmed by SDS gel electrophoresis and ELISA as shown in Figure 3.14 A and B, respectively. The separation on the gel under reducing condition showed clearly two bands for the heavy and light chains at 55 and 25 kDa, respectively. To determine the saturating and subsaturating concentrations of this antibody on HMEC-1 cells, a titration experiment was performed using indirect immunofluorescence staining as shown in Figure 3.14 (C and D). The cells were stained with different concentrations of W6/32 antibody followed by staining with rabbit anti-mouse FITC-conjugated secondary antibody. W6/32 antibody at a concentration was the saturating concentration and concentrations lower than 12 µg/ml were sub-saturating concentrations (0.35-6 µg/ml). Cells stained with secondary antibody only were used as a negative control.

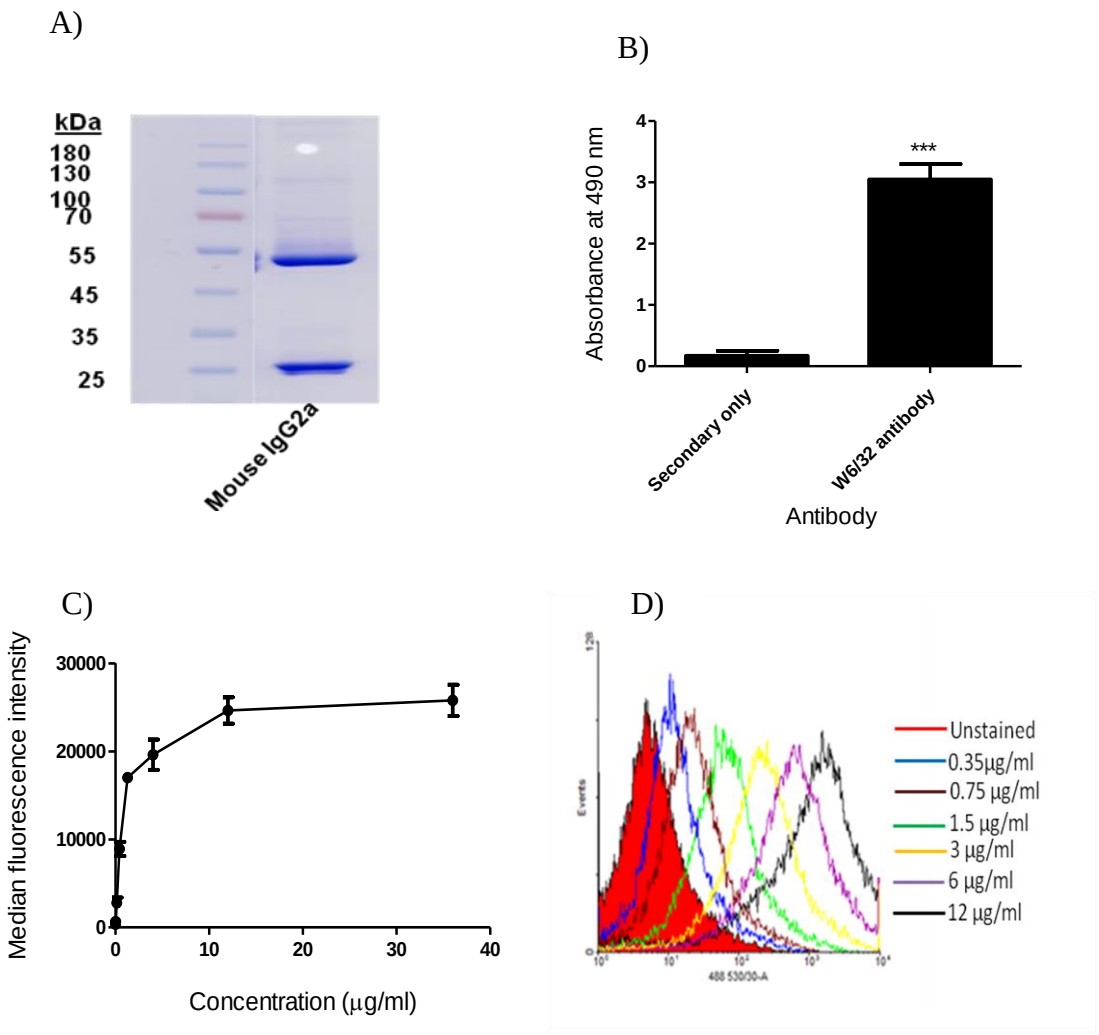


Figure 3.14: Purification of HLA class I antibody (mouse IgG2a) from W6/32 hybridoma cell line.

A shows the reduced antibody on 10% SDS-PAGE which separates into heavy and light chain bands. B shows ELISA of purified concentrated antibody. C shows titration of purified HLA class I antibody on HMEC-1 cells by flow cytometry. D shows flow cytometry histogram of the binding of W6/32 antibody to HMEC-1 cells at different concentrations. The cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody after staining with W6/32 antibody. Cells stained with the secondary antibody only were used as a control. *** $p<0.001$. The data are representative of two independent experiments.

3.5 Discussion

Donor endothelial cells are the first target exposed to recipients' immune system after transplantation. The constitutive expression of polymorphic HLA class I antigens renders these cells the main target for either cellular or donor specific antibody responses. Endothelial cells from both large and small vessels are crucial in maintaining normal allograft function. However, microvascular endothelial cells as an antigen presenting cells might have a more potent role in inducing allograft rejection. Functions such as leukocyte adhesion and migration may predominantly occur in small vessels rather than large vessels where the blood flow is slower facilitating the adhesion and migration processes (Aird, 2007). Therefore, human microvascular endothelial cells (HMEC-1) were used in this project. Characterization of the cell line and comparison to human large vessel endothelial cells (EA.hy926) were carried out.

EA.hy926 and HMEC-1 endothelial cells were characterized for the cell surface expression of CD31 and CD34 antigens. CD31 is a transmembrane glycoprotein with six extracellular immunoglobulin domains and two cytoplasmic immunotyrosine-based inhibitory motifs. This antigen is expressed also on other cell types such as neutrophils, lymphocytes, monocytes and platelets (Ilan *et al.*, 1999). Although it is widely known as a cell-cell adhesion molecule, its role as a cell signaling molecule is emerging. For example, CD31 regulates the activation and detachment of other cells such as T-cells and platelets (Liu and Shi, 2012). In addition, it has an important role in the regulation of leukocyte trafficking to the secondary lymphoid organs during recirculation and inflammation (Ma *et al.*, 2012). Inflammatory cytokines enhance the redistribution of endothelial CD31 and activation of integrins expressed on leukocytes enhancing their transmigration (Romer *et al.*, 1995). In this project, indirect immunofluorescence staining showed that both endothelial cell lines examined express CD31 molecules on their cell surface, confirming the finding from other groups used other techniques such an immmunocytochemistry, flow cytometry and RT-PCR (Ades *et al.*, 1992; Unger *et al.*, 2002; Ali *et al.*, 2008).

CD34 is a transmembrane glycoprotein antigen which is expressed mainly on hematopoietic stem cells (Dedeepiya *et al.*, 2012). Studies have also shown that other cell types such as endothelial and tumor cells might also express this antigen (Fina *et al.*, 1990; Soligo *et al.*, 1991). Although the role of this molecule is not fully understood, different studies have suggested a role in regulating leukocyte adhesion and

infiltration (Nielsen and McNagny, 2008). However, the expression of this antigen on endothelial cells is highly dependent on the origin of these cells, culturing condition and passage number. Some endothelial cells from large veins and arteries such as from placenta and lymphatic endothelial cells do not express this antigen and other cells might lose this expression after a few passage numbers (Fina *et al.*, 1990; Delia *et al.*, 1993). In this project, HMEC-1 cells showed a weak positive staining for CD34 but not on EA.hy926 cell line. The cell surface expression of this antigen on HMEC-1 cells has been previously detected on small subsets of cell population ranging from 10-15% (Nisato *et al.*, 2004; van Beijnum *et al.*, 2008). In addition, the cell surface expression of this antigen on CD34 negative subsets of HMEC-1 cells has been shown to become CD34 positive after re-culturing for 10 days (Siemerink *et al.*, 2012). This result suggests that the expression of this molecule on the cell surface is not constitutive and regulated by different conditions. Primary vascular endothelial cells lose the expression of this molecule after culturing for few passages which can be retained by culturing at high density (Delia *et al.*, 1993). This observation might explain the failure to detect a positive CD34 population on HMEC-1 cell in other studies (Unger *et al.*, 2002). The later study also showed the absence of CD34 antigens on EA.hy926 cells.

Expression of HLA class II antigens on endothelial cells was determined under resting and stimulated conditions. The expression of these molecules is normally restricted to antigen presenting cells due to their role in presenting processed extracellular antigens to T-helper cells, eliciting series of events leading finally to eradication of harmful agents. (Unanue, 1992). Some types of human endothelial cells express HLA class II molecules *in vivo* and lose this expression by culturing. This includes heart and kidney microvascular endothelial cells but not large vessel endothelial cells. However, the expression *in vitro* can be up-regulated by stimulation with pro-inflammatory cytokines such as IFN-γ (McDouall *et al.*, 1997; Muczynski *et al.*, 2003). IFN-γ is an inflammatory cytokine that is secreted by activated T-cells (Th-1) and NK cells in response to viral, bacterial infection or inflammation to enhance the differentiation and activation of other immune cells. A recent study showed that IFN-γ has the potential to induce endothelial to mesenchymal transition implicating a role in fibrogenesis and vascular-related disorders. This is mediated by downregualting the expression of VE-cadherin and upregulating the expression of transformation growth factor-beta2 (TGF-β2) and endothelin-1 (Chrobak *et al.*, 2013). IFN-γ enhances the expression of HLA class II antigens through activation of Janus kinase/signal transducers and activators of

transcription (JAK/STAT) pathway. The binding of IFN-γ to its receptor results in the phosphorylation of JAK1 and JAK2 kinases which subsequently phosphorylate the IFN-γ receptor. The phosphorylation of this receptor induces the recruitment and binding of cytoplasmic STAT1 protein to the IFN-γ receptor. This binding results in the phosphorylation and homodimerization of STAT1. The homodimer translocates to the nucleus where it induces the transcription of interferon regulatory factor-1 (IRF-1). This factor with STAT1 and other constitutive factors bind to the class II transactivator, nuclear transcription factor, promoter inducing its expression (Tsai *et al.*, 2007). In addition, a recent study showed the involvement of another factor called B-associated transcript-3, adjacent to HLA-B locus, in the expression of HLA class II molecules on lymphocytes (Kamper *et al.*, 2012). In this project, the expression of endothelial HLA class II molecules in response to either IFN-γ alone or in combination with TNF-α was examined.

Exposure of endothelial cell lines with different concentrations of IFN-γ for 72 hours induced a significant increase in the expression of HLA class II antigens as shown by direct immunofluorescence staining. Although both cell lines responded to this treatment in a dose dependent manner, HMEC-1 cells exhibited a higher level of expression at lower doses of IFN-γ than EA.hy926 cells. This might be due to the higher sensitivity of microvascular endothelial cells to pro-inflammatory cytokines compared to endothelial cells derived from large vessels (McDouall *et al.*, 1997). The up-regulation in the expression of HLA class II antigens by IFN-γ has been previously reported in many studies using macrovascular and microvascular endothelial cells (Cunningham *et al.*, 1997; McDouall *et al.*, 1997). Through their ability to express HLA class II molecules, endothelial cells can function as an antigen presenting cells to T-cells inducing their recruitment (Shiao *et al.*, 2007; Manes and Pober, 2008). The expression of HLA class II molecules is linked with the expression of class II transactivator which has been detected within hours after IFN-γ treatment in HUVEC endothelial cells (Tsai *et al.*, 2007) and HMEC-1 cells (Fritchley *et al.*, 2000).

The effect of TNF-α stimulation alone or in combination with IFN-γ on endothelial HLA class II antigen expression was also examined. TNF-α is a pro-inflammatory cytokine secreted by activated T-cells, dendritic cells and macrophages in response to inflammatory signals. It can synergise with other cytokines to induce the expression of cell surface molecules or chemokines (Cavalcanti *et al.*, 2012; Griffin *et al.*, 2012). In this project, treatment of HMEC-1 but not EA.hy926 cells with TNF-α alone induced

the expression of cell surface HLA class II antigen. In addition, treatment with a combination of IFN-γ and TNF-α significantly decreased the expression of HLA class II molecules compared to cells treated with IFN-γ alone. The inhibitory effect was dependent on the concentration, as the concentration of TNF-α increased the inhibitory effect increased accordingly. In a recent study by Norder and co-workers, TNF-α was unable to induce the expression of HLA class II molecule on lymphatic endothelial cells (Norder *et al.*, 2012). In addition, the latter study showed no change in the expression of HLA class II in the presence of TNF-α with IFN-γ compared to IFN-γ alone as observed in our study. Previous studies on HUVEC cells showed the inhibitory effect of TNF-α on HLA class II expression induced by IFN-γ (Leeuwenberg *et al.*, 1988; Wedgwood *et al.*, 1988). This inhibitory effect might be attributed to the ability of TNF-α to reduce the transcription of HLA class II genes as shown by a reduction in murine MHC class II β-chain mRNA (Melhus *et al.*, 1991) or through production of nitric oxide (Sicher *et al.*, 1995). In contrast, a synergistic effect between TNF-α and IFN-γ on HLA class II expression has been reported on other human cell types such as tumor cell line (Pfizenmaier *et al.*, 1987) and T-cells (Scheurich *et al.*, 1987). It has previously shown that the effect of TNF-α on IFN-γ treatment is dependent on the cell type and maturation state, as shown by the Watanabe and Jacob (Watanabe and Jacob, 1991). They showed that treatment of immature promyelocytic cells with a TNF-α augmented the ability of IFN-γ to induce HLA class II expression. However, differentiation of these cells inhibited the synergistic effect of TNF-α and IFN-γ. Since endothelial cells might be considered as differentiated cells, they may function in a similar way.

The expression of HLA class I antigens on HMEC-1 cells was confirmed by staining with purified W6/32 antibody. The titration experiment showed that 12 µg/ml was a saturating concentration for HLA class I antigens on HMEC-1 cells and concentrations lower than that were sub-saturating concentrations. Therefore, 12 µg/ml was used for all experiments performed using saturating concentration. HLA class I genotyping was performed by PCR-SSP to determine the HLA specificity of the cell line. The genotype is; A*01, A*68, B*35, B*58, C*04 and C*06 and the cell line from ‘O’ blood group donor. Genotyping also included HLA class II genotypes, and two epithelial cell lines (HKC-8, HK-2) which can be used in other studies (appendices 10.1, 10.2, 10.3). HLA class I antigen expression was not reduced when exposed to trypsin. This suggests that the use of trypsin to detach cells for flow cytometry analysis can be performed without affecting the expression of HLA class I antigen. Acid treatment produced a significant,

but incomplete, cleavage of cell surface HLA class I antigens. In agreement with our finding, treatment of platelets with the sodium citrate solution caused approximately 80-85% of HLA class I cleavage (Zou *et al.*, 2011). Citric acid treatment showed specificity for HLA class I cleavage as treated platelets showed a specific class I cleavage but normal expression of other cell surface antigens (Neumuller *et al.*, 1993). In our study, the expression of HLA class I antigens fully regains after 24 hours of acid treatment.

In this chapter, phenotypic analysis of HMEC-1 endothelial cells was performed and compared to that of EA.hy926 cells. HMEC-1 cells do express CD31, HLA class I and upregulate HLA class II in response to cytokine stimulation. The W6/32 antibody was successfully obtained for this project, with retained binding efficiency to HMEC-1 cells as assessed by flow cytometry.

4. Chapter Four- HLA Class I Antibodies and Endothelial Cell Signalling

4.1 Introduction

Protein phosphorylation is an important post-translational modification and an early response by which cells regulate different cellular processes in response to various extracellular stimuli. This phosphorylation can occur at different amino acid residues such as serine, threonine and tyrosine, changing protein activity to either activation or deactivation, protein localization and effecting downstream protein targets (Hunter, 2007). Different lines of evidence showed that ligation of HLA class I molecules on endothelial cells with antibodies is associated with the phosphorylation of various intracellular proteins by a mechanism independent to the activation of the complement system. These proteins are involved in a variety of signal transduction pathways responsible for regulating various endothelial cell functions such as survival, apoptosis, proliferation and cell migration. Therefore, in this chapter the activation of cell signaling-associated proteins in response to stimulation with HLA class I antibodies was examined using human microvascular endothelial cells.

Phosphorylation of endothelial Akt, ERK1/2, mTOR, cytoskeleton proteins and others has been previously reported in response to stimulation with HLA class I antibody as discussed in details in section 1.8. In addition, activation of endothelial transcription factors such as NF-kB, E2F, c-Jun and Nf-E2-related factor in response to HLA class I antibody has also been observed as explained in details in section 1.9. The activation of transcription factors suggests a modulation in the expression of endothelial proteins upon stimulation. However, all of the cell signaling studies previously performed have used human large vessel endothelial cells. Therefore, activation of microvascular endothelial cells in response to HLA class I antibody was investigated in this study. This includes activation of cell signaling pathways and transcription factors.

HLA class I antibody is associated with the development of chronic allograft rejection, which is characterized by the perivascular fibrosis, neointimal thickness and proliferation of endothelial cells and smooth muscles (Zhang *et al.*, 2011b; Trayssac *et al.*, 2012). Ligation of HLA class I molecules by antibodies *in vitro* also induces epithelial cell proliferation leading to bronchiolitis obliteraus syndrome, the main manifestation of chronic rejection in lung transplantation (Reznik *et al.*, 2000). Cell proliferation results from activation of cell-cycle progression proteins downstream of

the cell signaling transduction pathways. Activation of mTOR, focal adhesion signaling (Jin *et al.*, 2002) and Akt pathways in response to HLA class I antibody showed a correlation with endothelial cell proliferation (Jindra *et al.*, 2008a). In addition, an increase in endothelial cell proliferation might occur as a result of the expression of endothelial growth factors such as vascular endothelial growth factor in response to stimulation with HLA class I antibody (Bieri *et al.*, 2009). In addition to the increase in the expression of growth factors, cells stimulated with these antibodies show an upregulation in the expression of cell surface fibroblast growth factor receptors which increased the sensitivity to fibroblast growth factor (Harris *et al.*, 1997; Bian *et al.*, 1998; Jin *et al.*, 2002).

Apoptosis is a programmed cell death that is crucial for cell development and homeostasis. However, excessive endothelial cell apoptosis in response to extracellular stimuli can cause endothelial dysfunction and vascular injury (Winn and Harlan, 2005). Narayanan *et al.* (2004) reported endothelial cell apoptosis in response to stimulation with saturating concentration of HLA class I antibody in the presence of complement. Apoptotic cell death was inhibited by the pre-exposure to sub-saturating concentration of HLA class I antibody. This effect was mediated by the expression of anti-apoptotic genes upon exposure to sub-saturating concentration providing protection against a high dose of the antibodies (Narayanan *et al.*, 2004). Stimulation of epithelial cells with HLA class I antibody induced cell apoptosis after 48 hours from stimulation (Jaramillo *et al.*, 2003). Using aortic endothelial cells, Yamakuchi and colleagues showed that stimulation with HLA class I antibody alone or in combination with complement C5a did not induce cell death (Yamakuchi *et al.*, 2007). However, the role of HLA class I antibody in inducing microvascular endothelial cell apoptosis independent of complement has not been studied.

In this chapter, the phosphorylation of endothelial cell signaling proteins in response to stimulation with HLA class I antibody was investigated. This was achieved using proteome profiler phospho-array and western blotting. Both methods are the most commonly used methods for detection of phospho-proteins. They share the main principle where a phosphorylated analyte is detected by a specific antibody. The array contains multi-phospho-antibodies attached to the nitrocellulose membrane which provides a quick and convenient method for the detection of a wide range of phosphorylated proteins in response to particular stimulus. The method has a main

advantage of avoiding using highly hazardous materials such as radioisotops. Phosphorylation of Akt, ERK and transcription factor CREB at specific residues in response to stimulation with HLA class I antibody was evaluated. In addition, the effect of the antibodies in inducing endothelial cell proliferation and apoptosis was also assessed. Colorimetric assay for cell proliferation and flow cytometric analysis for cell apoptosis were used.

4.2 Specific Aims

In this chapter, the potential of HLA class I antibody in inducing activation of microvascular endothelial cells was examined. This includes:

- Activation of signal transduction pathways including; p-ERK, p-Akt and p-CREB;
- Examining the ability of W6/32 antibody in inducing cell apoptosis, and
- Assessing the ability of W6/32 antibody to induce cell proliferation.

4.3 Specific materials and methods

4.3.1 Human phosphokinase array on endothelial cells

Analysing the phosphorylation of kinases and their substrates after exposure to the HLA class I antibody was performed using a human phospho-kinase array kit (R&D system). The array contains different capture antibodies specific to wide range of phosphorylated proteins attached to nitrocellulose membranes. HMEC-1 cells were serum starved overnight in 0.2% FBS containing media without EGF or hydrocortisone. The cells were then detached using cell dissociation buffer and resuspended into 200 µl of 0.2% FBS and incubated at 37^0C for 2 hours. The cells were then stimulated for 5 minutes with either saturating concentration of W6/32 antibody (12 µg/ml) or isotype control mouse IgG2a. Stimulation was terminated by the addition of cold PBS followed by rapid centrifugation. The cell lysate was obtained as described in the instruction manual and 500 µg of protein was used per array (membrane A & B). After blocking for one hour with the provided blocking agent, the membranes were incubated with cell lysates on the shaker in cold room (4^0C) overnight. After the washing steps, an antibody cocktail specific for each membrane was added and incubated for 2 hours at room temperature. Streptavidin-HRP was added to the membrane after three washes. The signal produced by HRP activity was detected by using Pierce enhanced chemiluminscent (ECL) substrate and Kodak X-ray films.

4.3.2 Stimulation of endothelial cells for measurement of phosphorylated proteins (Akt, ERK and CREB)

To examine the phosphorylation of cell signalling proteins, HMEC-1 cells were incubated overnight with 0.2% FBS containing media without EGF or hydrocortisone. The cells were then detached using cell dissociation buffer and and resuspended into 200 µl of 0.2% FBS and incubated at 37^0C for 2 hours. The cells were then stimulated with W6/32 antibody at either different time points or different antibody concentrations. The stimulation was terminated by the addition of cold PBS and the tubes were transferred immediately into ice. After centrifugation at 12000g for 3 minutes, the cells were lysed by using lysis buffer containing protease and phosphatase inhibitors as described in section 2.8.2. Protein concentration was determined using the BCA assay as described in section 2.8.3. Between 20 to 40 µg of proteins were separated on 12% SDS-PAGE and transferred into PDVF membrane as described in sections 2.8.4 and 2.8.6, respectively. The membrane was blocked with 3 % BSA in 0.1% TPBS for one

hour before incubation with the primary antibodies. Primary antibodies were diluted in the blocking solution at the recommended concentration by the manufacturer. The primary anti-human antibodies used were: mouse p-ERK1/2 (T202/Y204) (Santa Cruz Biotechnology, USA), rabbit T-ERK (BD Bioscience, USA), rabbit monoclonal p-Akt (Serine residue 473), rabbit total Akt, rabbit monoclonal p-CREB (Serine residue 133) and rabbit total-CREB antibodies (Cell Signaling Technology, USA). The secondary antibodies used were: horseradish peroxidase conjugated goat anti-rabbit IgG (Cell Signaling Technology, USA) and horseradish peroxidase conjugated rabbit anti-mouse IgG (Sigma Aldrich, USA). Membranes were probed with antibodies and developed as described in section 2.8.7.

4.3.3 Stimulation of endothelial cells with forskolin

Forskolin (7-beta-acetoxy-8,13-epoxy-1-alpha, 6-beta, 9-alpha-trihydroxylabd-14-en-11-one Coleonol) is a natural product belonging to the labdane diterpene group and produced by Indian plant called *Coleus forskohlii*. Forskolin is particularly characterized by the presence of a tetrahydopyran-derived heterocyclic ring. It has an antihypertensive and platelet aggregation effects. It is also widely used to increase the level of cAMP through activation of intracellular adenylyl cyclase. The rise in cAMP levels leads to the activation of protein kinase A, enhancing the phosphorylation of cAMP Responsive Elements Binding protein (CREB) at serine residue 133 (Bhat *et al.*, 1983). In this project, forskolin was used as a positive control for endothelial CREB phosphorylation. Forskolin was dissolved in DMSO to prepare a 100 mM stock (Sigma Aldrish, USA). Endothelial cells starved in 0.2% FBS containing media were detached using cell dissociation buffer, re-suspended in 200 µl of 0.2% FBS media and incubated at 37^0C for 2 hours. The cells were stimulated with 10, 50 and 100 µM of forskolin for 5 minutes before the addition of cold PBS. The cells were centrifuged at 12000g for 5 minutes before adding the lysis buffer. The cell lysates were then prepared and analyzed for CREB phosphorylation by western blotting as previously described.

4.3.4 Using specific inhibitors

To examine the contribution of a particular cell protein in the activation of downstream proteins, a specific inhibitor is normally used. In this project, two inhibitors have been used to examine the dependency of CREB phosphorylation at serine residues on the Akt and PKA pathways.

4.3.4.1 **PI3K/Akt inhibitor**

LY294002 is a selective inhibitor for phosphoinositol-3-kinase (PI3K) but not for other kinases such as protein kinase C or MAP kinases (Cell Signalling Technology). The specificity for PI3K is optimal at a concentration of 50 μM as determined in previous study (Vlahos *et al.*, 1994). The inhibitor was dissolved in DMSO at a stock concentration of 50 mM. Endothelial cells were incubated with two different concentrations of the inhibitor (10 and 50 μM) one hour before the stimulation with saturating concentration of W6/32 antibody for 5 minutes. The activity of the inhibitor was verified by probing for p-Akt following stimulation with W6/32 antibody in the presence of the inhibitor.

4.3.4.2 **Protein kinase A inhibitor**

To assess the contribution of protein kinase A in CREB phosphorylation, a protein kinase A (PKA) inhibitor was used. The H-89 inhibitor is a selective and potent inhibitor for PKA, (Cell Signalling Technology). In *in vitro* assay, H-89 has been shown to induce a 50% inhibition of PKA phosphorylation at 50 nM (Chijiwa *et al.*, 1990). The inhibitor was dissolved in DMSO to make a stock solution at a concentration of 20 mM. Optimisation of the concentration of H-89 inhibitor was performed by incubation of HMEC-1 cells with different concentrations of H-89 inhibitor ranging from 0.05 to 50 μM followed by stimulation with 10 μM of forskolin. The cells were incubated with 5 μM of the inhibitor one hour before the stimulation with saturating concentration of W6/32 for 5 minutes. To ensure the activity of the inhibitor, reduction in CREB phosphorylation by forskolin in the presence of H-89 inhibitor was assessed.

4.3.5 Trypan blue staining

Cell viability following serum starvation or following the use of different protein kinase inhibitors was determined by staining with trypan blue before stimulation. Staining with trypan blue is a quick and efficient way to examine cell viability. Dead cells are characterised by the disruption in the cell membrane which facilitates the entrance of the blue stain inside the cells; live cells exclude this stain. Cells with 95% viability were used for all experiments.

4.3.6 Examination of biological functions

4.3.6.1 Cell proliferation assay

To assess the ability of HLA class I antibody to induce endothelial cell proliferation, a CellTiter 96 AQueous One Solution Cell proliferation assay was used (Promega, USA). This assay is a colorimetric method that determines the number of viable cells by using a tetrazolium compound (inner salt) and an electron coupling reagent (phenazine ethosulfate). The tetrazolium compound mixed with phenazine ethosulfate is reduced by NADPH and NADH produced by dehydrogenase enzymes in metabolically active cells to produce a soluble colored formazan product which can be measured at 490 nm. HMEC-1 cells were counted and 20,000 cells were cultured in 96 wells plate. After overnight incubation, cells were treated with a saturating concentration of W6/32 antibody or isotype control and incubated at 37^0C for 24 and 48 hours. The number of viable cells was determined by adding 20 µl of 5 % phenazine ethosulfate in tetrazolium compound to 100 µl of culture media. The plate was incubated for one hour at 37^0C and the absorbance was measured at 490 nm. The efficiency and sensitivity of the colorimetric assay was evaluated by seeding different number of cells in complete media for 24 hours followed by performing the assay as shown in Figure 4.1.

4.3.6.2 Cell apoptosis assay

4.3.6.2.1 General principle

Apoptosis was defined in 1972 as a programmed cell death essential for eliminating unwanted or aged cells from living tissues, or as a part of normal cell turnover (Kerr *et al.*, 1972). This phenomenon is a distinctive mode of cell death that is different from cell necrosis. In the latter, a rapid and non-specific cell death occurs in response to the exposure to high concentration of toxic agents. Each mode of cell death is recognized on the basis of biochemical and morphological changes. The main features of cell apoptosis are cell shrinkage, DNA fragmentation and formation of blebs in the plasma membrane. However, the apoptotic cells preserve cell membrane integrity as well as organelle structures. DNA fragmentation occurs as a result of the activation of calcium and magnesium-dependent endogenous nucleases. These enzymes catalyze the fragmentation of DNA at the sites located between nucleosomes producing mono and oligonucleosome fragments. For the apoptotic cells to be phagocytosed, the translocation of the phosphatidylserine from the inner side of the cell membrane to the outer side occurs as a phagocytic marker for phagocytic cells. In contrast, cell necrosis

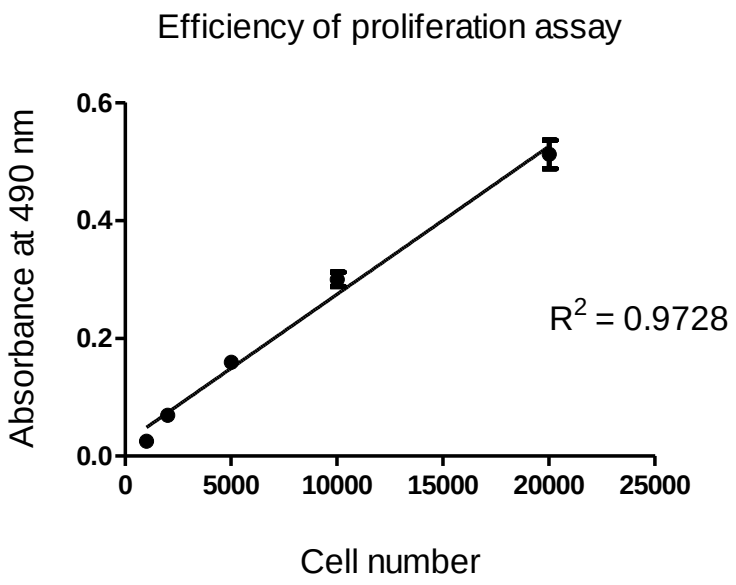


Figure 4.1: Efficiency of proliferation assay using HMEC-1 endothelial cells.

Different number of cells was seeded into 96 wells plate. After 24 hours, viable cells were assessed using colorimetric proliferation assay as described in the methods. The absorbance was measured at 490 nm using ELISA plat reader. A linear regression between cell count and absorbance was evaluated. The correlation coefficient of the line was 0.9728. Absorbance of the reagent alone was used as a background and subtracted from all readings. Each point represents the mean ± standard error of mean. The data are representative of two independent experiments.

is characterized by loss of cell membrane integrity with swelling of cell organelles. On the basis of these differences, there are many assays that can be used to determine cell apoptosis in response to particular stimulus such as colorimetric or radioactive assays, DNA ladders, ELISA and flow cytometry (Elmore, 2007).

The simplest method to detect cell apoptosis is by flow cytometry using DNA intercalating dyes. After cell permeabilization, the dye stains the DNA content of the cells. Staining of healthy cells from normal culture shows cells at three different phases according to DNA contents which are G1, G2 and S phases. The DNA content of cells in the G2 phase is a double of that in the G1 phase, while DNA content of cells in the S phase is in between the G1 and G2 phases. Apoptotic cells are characterized by the presence of DNA content less than that in the G1 phase as a result of DNA fragmentation and leakage from the cells. The differences in DNA content at different stages occur with different fluorescence intensity on flow cytometry after staining with DNA intercalating dye. Different fluorochromes such as Propidium Iodide (PI), DAPI and Acridine Orange can be used to stain the DNA and determine the reduction in DNA content during apoptosis. Therefore, cells stained with these dyes show two different peaks for G1 and G2 phases, where the fluorescence intensity for the G2 peak is double of that in the G1. The cells in the S phase will show a range of fluorescence intensity that lay between G1 and G2 peaks. The apoptotic cells show low DNA staining and appear as a peak with fluorescence intensity lower than that of the G1 population, which is the sub-G1 or sub-diploid peak. Due to cell membrane integrity, different dyes will be excluded from the apoptotic cells but not from necrotic cells which lose cell membrane integrity. Therefore, a detergent such as triton X-100 is used to facilitate the diffusion of dyes into cells and enhance the release of fragmented DNA from the nucleus enabling detection of the reduction in DNA content. In contrast, necrotic cells do not show an immediate reduction in DNA content and the sub-G1 peak is not present. In this project, cells were stained with a mixture of PI and triton X-100 to determine the sub-G1 peak (Riccardi and Nicoletti, 2006).

4.3.6.2.2 Staurosporine treatment

Staurosporine (STS) is a natural product isolated from *Streptomyces staurosporeus* with a wide range of biological activities ranging from antifungal to antihypertensive. The main biological activity of staurosporine is inhibition of protein kinase C through the prevention of ATP binding to the kinase portion. It is used in research as an apoptosis

inducing agent or synchronizing agent. Endothelial cells grown to 80 % confluency in 6 wells plate were treated with STS at a concentration of 2.5 μM prepared by a serial dilution from staurosporine stock (2.1 mM) dissolved in DMSO (Sigma Aldrich).

4.3.6.2.3 Sub-diploid peak assay

Endothelial cells were treated with STS dissolved in complete media at either different concentrations or different time points. After treatment, cells were washed once with PBS and detached using trypsin. Detached cells were washed twice with 2 % FBS in PBS and re-suspended in 1 ml PBS/FBS solution. Cells were permeabilized and stained using 1 % triton X-100 containing PI at a concentration 0.2 μg/ml. This was added to the cells just prior to analysis by flow cytometry. Around 20,000 events were acquired on linear scales for FSC, SSC and PI channel. Cells stained with PI only were used to exclude dead necrotic cells.

4.4 Results

4.4.1 Human phosphokinase array on endothelial cells following stimulation with W6/32 antibody

A human phosphokinase array was chosen as a screening experiment to examine the phosphorylation of endothelial cell signaling proteins in response to stimulation with HLA class I antibody (appendix 10.4). The array contains capture antibodies specific for phosphorylated proteins attached to a nitrocellulose membrane. These proteins include the phosphorylated form of: Akt, mTOR, CREB, MEK1/2, MSK1/2, β-catenin, FAK and STAT6. Endothelial HMEC-1 cells were stimulated with a saturating concentration (12 μg/ml) of W6/32 antibody or isotype control for 5 minutes followed by preparation of cell lysates. As shown in Figure 4.2, HLA class I antibody enhanced the phosphorylation of various kinases including p-Akt, p-CREB, β-catenin, FAK, MEK1/2 and others. Positive spots in both membranes were used to assess the validity of the array and as a loading control between the two samples. The phosphorylation of ERK at tyrosine 202 and threonine 204 residues, Akt at serine residue 473 and phosphorylation of transcription factor CREB at serine residue 133 were chosen for further analysis.

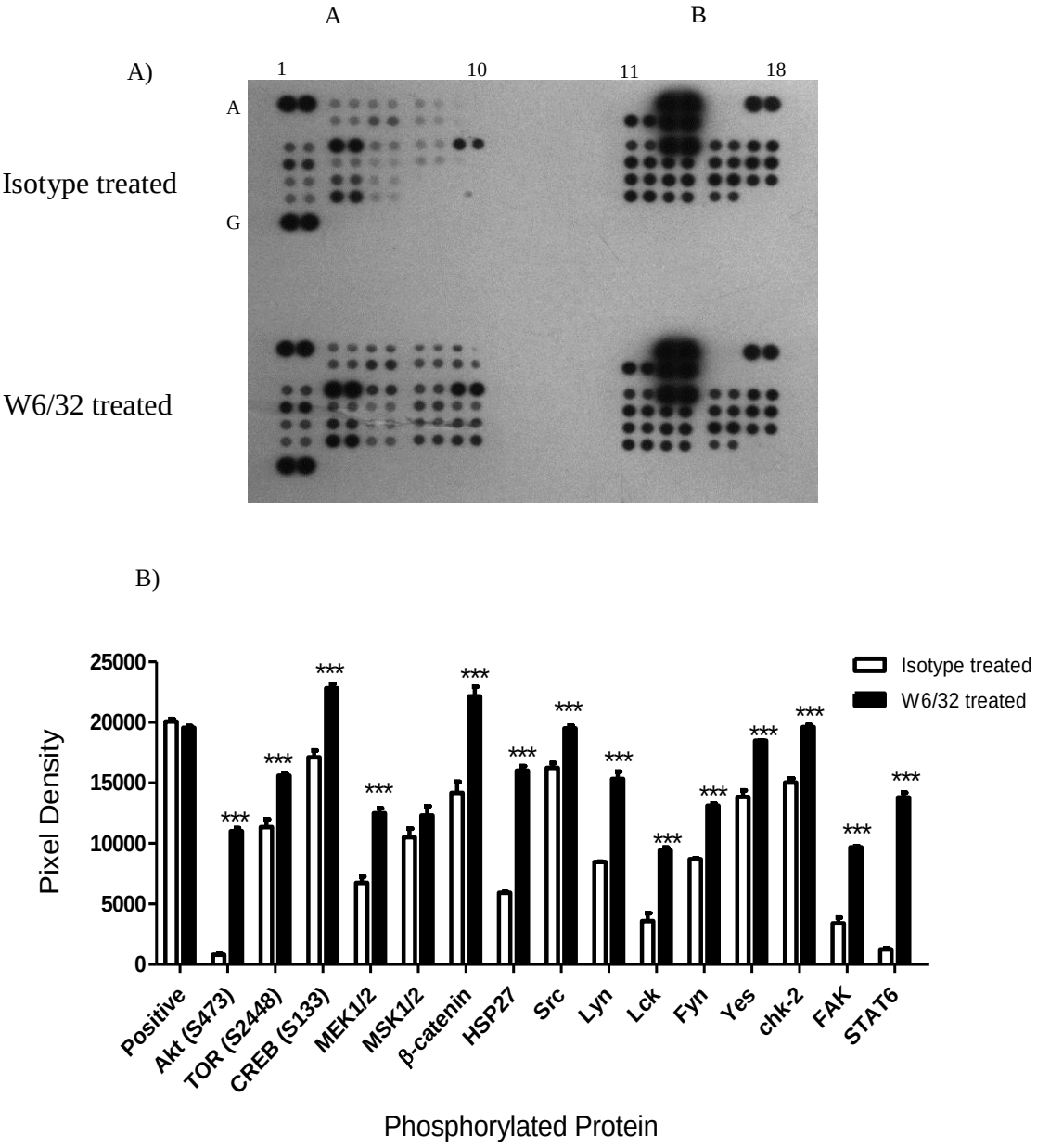


Figure 4.2: Human-phosphokinase array of endothelial cells following stimulation with HLA class I antibody.

HMEC-1 cells were treated with saturating concentration (12 μg/ml) of W6/32 antibody or isotype control for 5 minutes. The cells were lysed and applied to the array. Panel A shows the arrays and panel B shows the densitometric analysis. The density of the dots was measured using gel documentation system and AlphaImager software. Statistical analysis was performed using two way ANOVA followed by Bonfferoni test at $p<0.05$. *** $p<0.001$. The data are representative of two independent experiments.

4.4.2 Endothelial ERK, Akt and CREB phosphorylation following stimulation with W6/32 antibody

To examine further the ability of HLA class I antibody to induce activation of endothelial cell signalling, HMEC-1 cells were treated with HLA class I antibody (W6/32) and then analysed by western blotting for the phosphorylation of selected proteins. Cells were treated with either saturating concentration of W6/32 antibody (12 μg/ml) or isotype control mouse IgG2a for different time points or stimulated with different concentrations of HLA class I antibody for 5 minutes. The lysates were probed for p-Akt at serine residue 473, p-ERK1/2 at tyrosine and threonine residues and p-CREB at serine residue 133.

As a positive control, treatment with H_2O_2 was used for ERK and Akt phosphorylation, and treatment with forskolin was used for CREB phosphorylation. Forskolin is well known as a PKA activator that is used to investigate CREB phosphorylation. Endothelial cells were treated with 100 μM of H_2O_2 for 5 minutes and lysates were probed for p-ERK1/2 and p-Akt antibodies. This concentration was shown to induce significant protein phosphorylation. Cell viability was assessed using trypan blue staining; 90-95% viable cells were observed and used after forskolin or H_2O_2 treatments. For CREB phosphorylation, endothelial cells where stimulated with 10, 50 and 100 μM of forskolin for 5 minutes followed by probing with p-CREB antibody. As shown in Figure 4.3, 10 μM of forskolin was sufficient to induce CREB phosphorylation compared to untreated cells. The antibody used for CREB phosphorylation also detects other protein belonging to the CREB family, p-ATF1 shown as second band on the western blot. P-CREB antibody can bind weakly to pyruvate dehydrogenase which might explain the third band. According to the size of p-CREB, the first band is for p-CREB.

Exposure to a saturating concentration of HLA class I antibody induced ERK1/2 phosphorylation at tyrosine 202 and threonine 204 residues which peaked at 1 minute and declined by 30 minutes as shown in Figure 4.4. The exposure to W6/32 antibody also induced the phosphorylation of Akt and CREB at specified residues as shown in Figure 4.5 (A and B), respectively. The phosphorylation of Akt increased at 5 minutes following stimulation, peaked at 15 minutes and slightly reduced after 30 minutes. The phosphorylation of CREB occurred at early time point within 1 minute, peaked at 5 minutes and declined by 30 minutes following stimulation with HLA class I antibody.

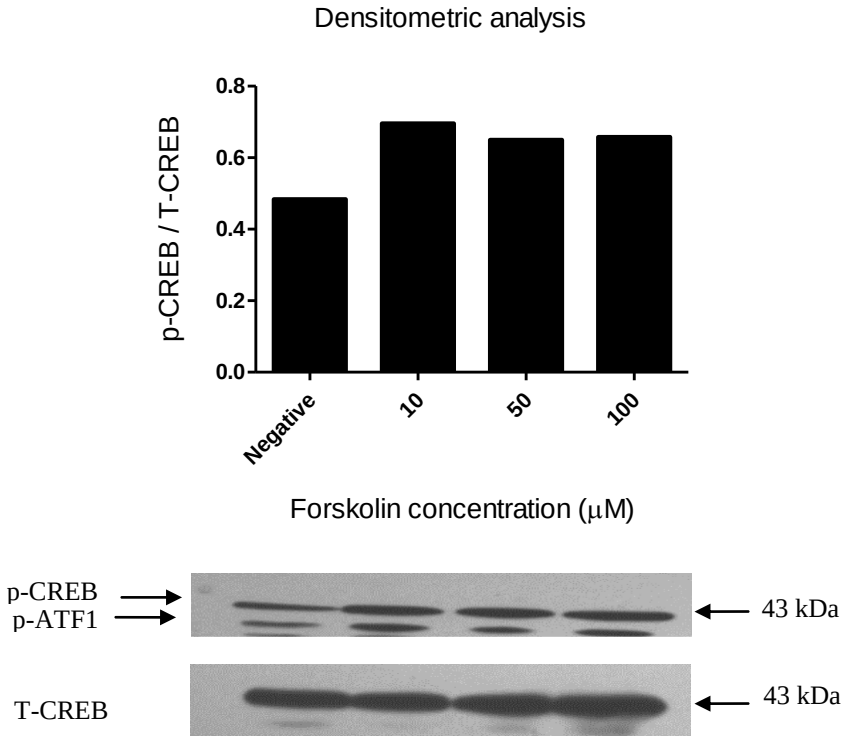


Figure 4.3: Phosphorylation of CREB at serine residue (133) by endothelial cells following stimulation with forskolin.

Serum starved HMEC-1 cells were stimulated with different concentrations of forskolin, 10, 50 and 100 μM for 5 minutes followed by cell lysis and western blotting. The membrane was probed for p-CREB using rabbit anti-human p-CREB antibody. The membrane was stripped and re-probed with mouse T-CREB antibody as a loading control. Densitometric analysis was performed using AlphaImager software. The experiment is representative of two independent experiments.

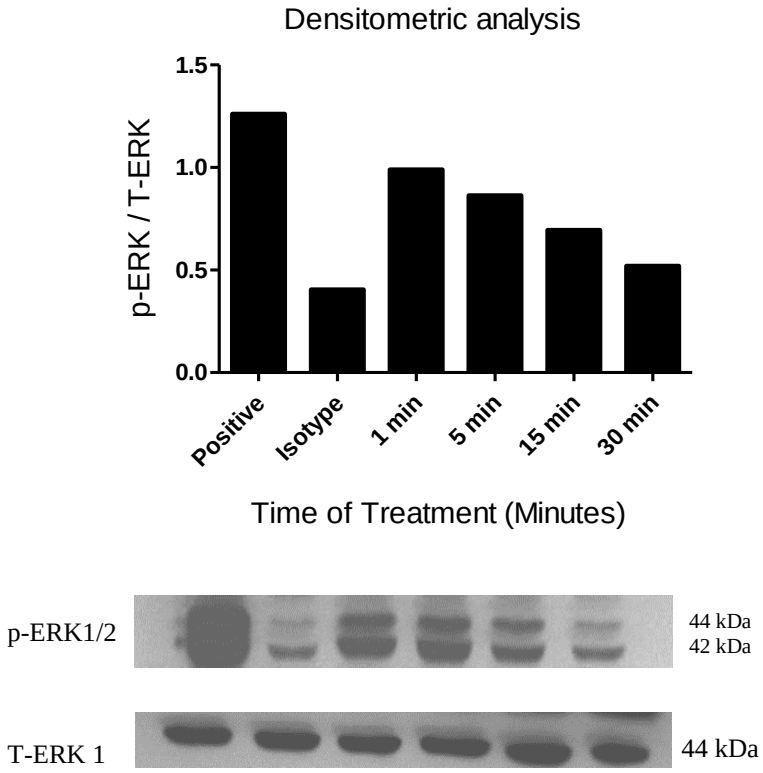


Figure 4.4: Phosphorylation of ERK1/2 at threonine 202 and tyrosine 204 residues by endothelial cells following stimulation with HLA class I antibody.

Serum starved HMEC-1 cells were treated with a saturating concentration (12 µg/ml) of W6/32 antibody or isotype control for various time points before cells were lyzed. Cells treated with 100 µM of H_2O_2 for 5 minutes were used as a positive control. The membrane probed for p-ERK using rabbit anti-human p-ERK antibody. The membrane was then stripped and re-probed for total ERK (T-ERK). The density of the bands was analyzed using AlphaImager software. The data are representative of three independent experiments.

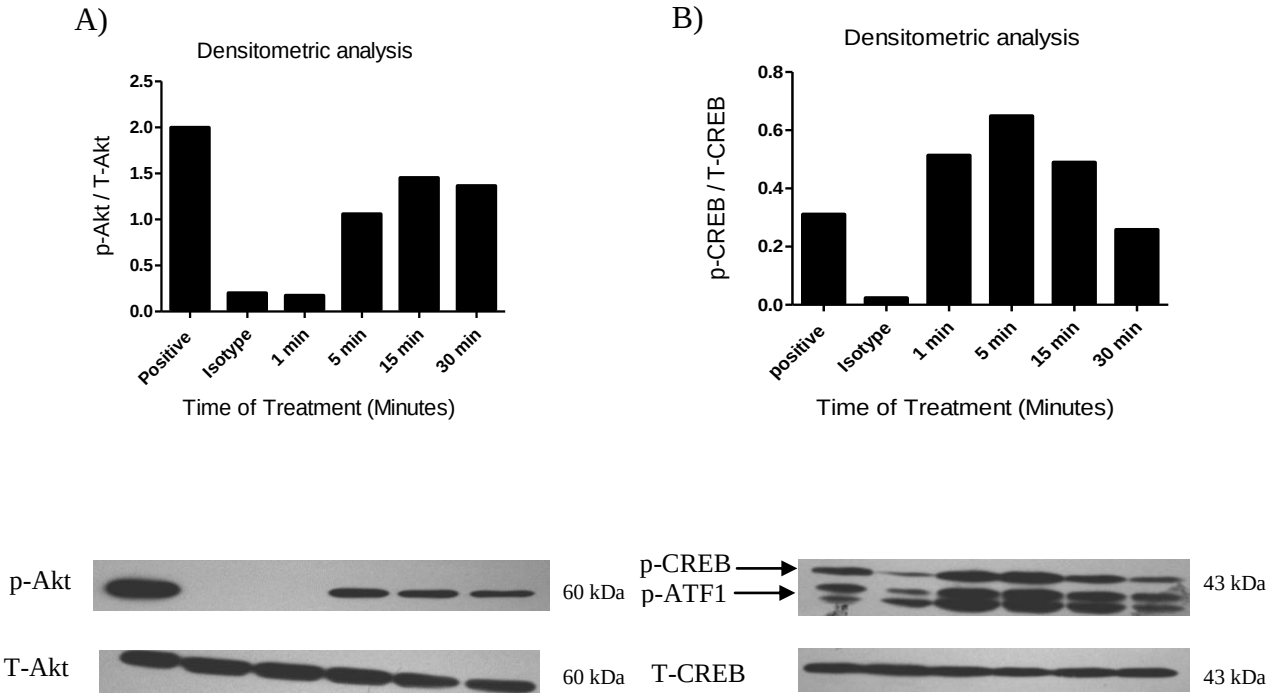


Figure 4.5: Phosphorylation of Akt and CREB by endothelial cells following stimulation with HLA class I antibody.

Serum starved HMEC-1 cells were treated with saturating concentration (12 µg/ml) of W6/32 antibody for various time points before the cells were lyzed. The membranes were probed for p-Akt at serine residue 473 (A) and p-CREB at serine residue 133 (B) using rabbit anti-human p-Akt and p-CREB antibodies, respectively. Cells treated with isotype control mouse IgG2a at the same concentration were used as a negative control. Cells stimulated with 100 µM of H_2O_2 or 10 µM of forskolin were used as a positive control for p-Akt and p-CREB, respectively. The membranes were stripped and re-probed for total Akt (T-Akt) and total CREB (T-CREB). The density of the bands was analyzed by AlphaImager software. The data are representative of three experiments.

4.4.3 Dose response of endothelial Akt and CREB phosphorylation following stimulation with W6/32 antibody

To investigate whether the exposure to sub-saturating concentrations of W6/32 antibody might induce a similar effect on protein phosphorylation, HMEC-1 cells were stimulated with different subsaturating concentrations of HLA class I antibody (W6/32) ranging from 0.35-6 μg/ml and examined by western blotting for Akt and CREB phosphorylation. The subsaturating concentrations were previously determined using flow cytometry as shown earlier in Figure 3.14 (C and D). Endothelial Akt phosphorylation showed a dependency on antibody concentration, the level of phosphorylation declined at concentration from 0.35 to 1.5 μg/ml compared to that between 3-12 μg/ml, as shown in Figure 4.6 (A). In contrast, panel B on the same figure shows the phosphorylation of CREB independent to antibody concentration. The exposure to the lower concentration of W6/32 antibody used is sufficient to induce the phosphorylation of this transcription factor. Corresponding total proteins were used as a loading control for each blot.

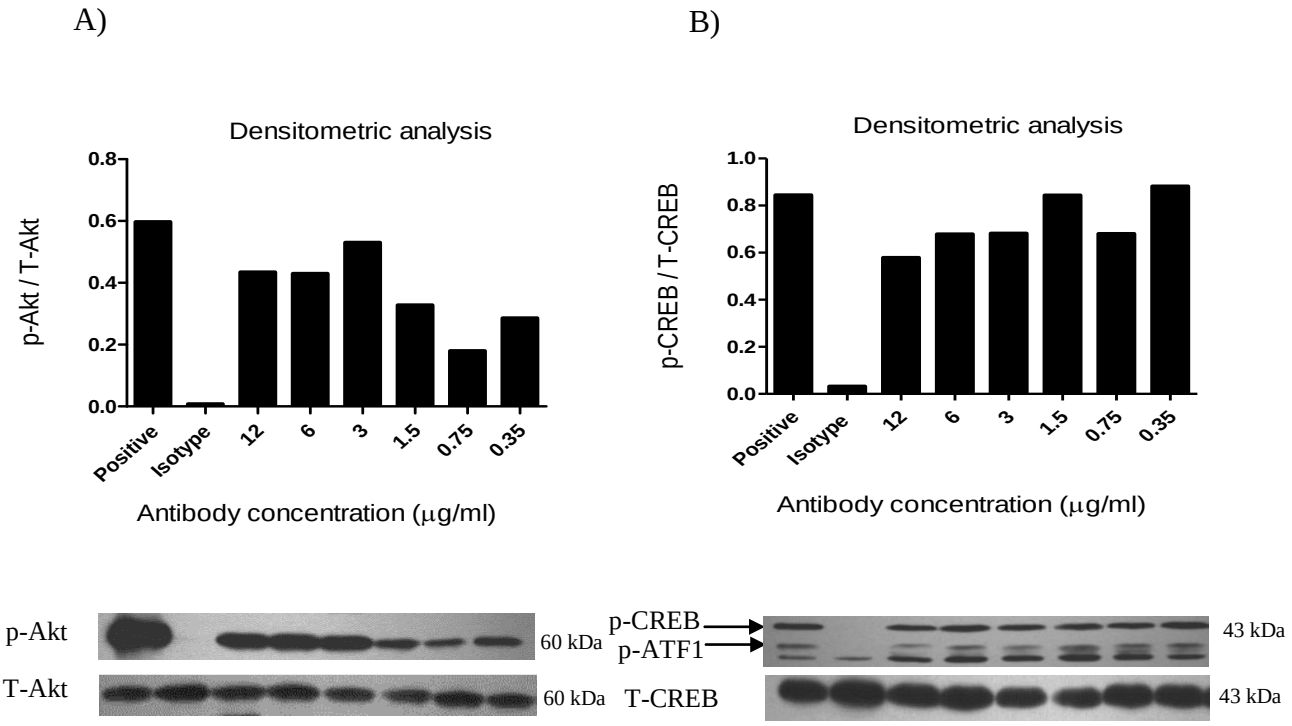


Figure 4.6: Phosphorylation of endothelial Akt and CREB following stimulation with different concentrations of HLA class I antibody.

Serum starved HMEC-1 cells were treated with different concentrations of W6/32 antibody ranging from (0.35-12 μg/ml) for 5 minutes followed by cell lysate preparation. Panel A shows the phosphorylation of Akt at Serine 473 and B shows phosphorylation of CREB at serine 133. Cells treated with isotype control (mouse IgG2a) were used as a negative control. Cells stimulated with 100 μM of H_2O_2 and 10 μM forskolin were used as positive controls for p-Akt and p-CREB, respectively. The membranes were stripped and re-probed for T-Akt and T-CREB. The densitometric analysis was performed using Alphaimager software. The data are representative of three independent experiments.

4.4.4 Effect of the PI3K/Akt pathway on CREB phosphorylation induced by W6/32 antibody

The contribution of Akt to CREB phosphorylation in response to HLA class I antibody was examined using PI3K/Akt inhibitor, LY249002. The inhibitor was used at two concentrations: 10 and 50 μM for 30 minutes before stimulation with a saturating concentration of W6/32 antibody. The concentrations of the inhibitor were recommended by the manufacturer. Cell viability and toxicity related to the inhibitor was assessed by staining with trypan blue before stimulation the cells with W6/32 antibody. The inhibitor did not induce cell toxicity; 90-95% of cell viability was observed after incubation with the inhibitor. The cell lysates were probed for p-Akt and p-CREB simultaneously. Probing for p-Akt was used as a control to ensure the activity of the inhibitor. The phosphorylation of Akt observed in response to W6/32 antibody is reduced significantly in the presence of the LY249002 inhibitor as shown in Figure 4.7 (A). The presence of LY249002 inhibitor before stimulation with the W6/32 antibody did not abrogate CREB phosphorylation at serine residue 133 in both examined concentrations as shown in Figure 4.7 (B). The presence of the inhibitor alone did not show significant Akt or CREB phosphorylation. This result suggests that stimulation with W6/32 antibody activates two distinct pathways and Akt phosphorylation is independent to CREB pathway.

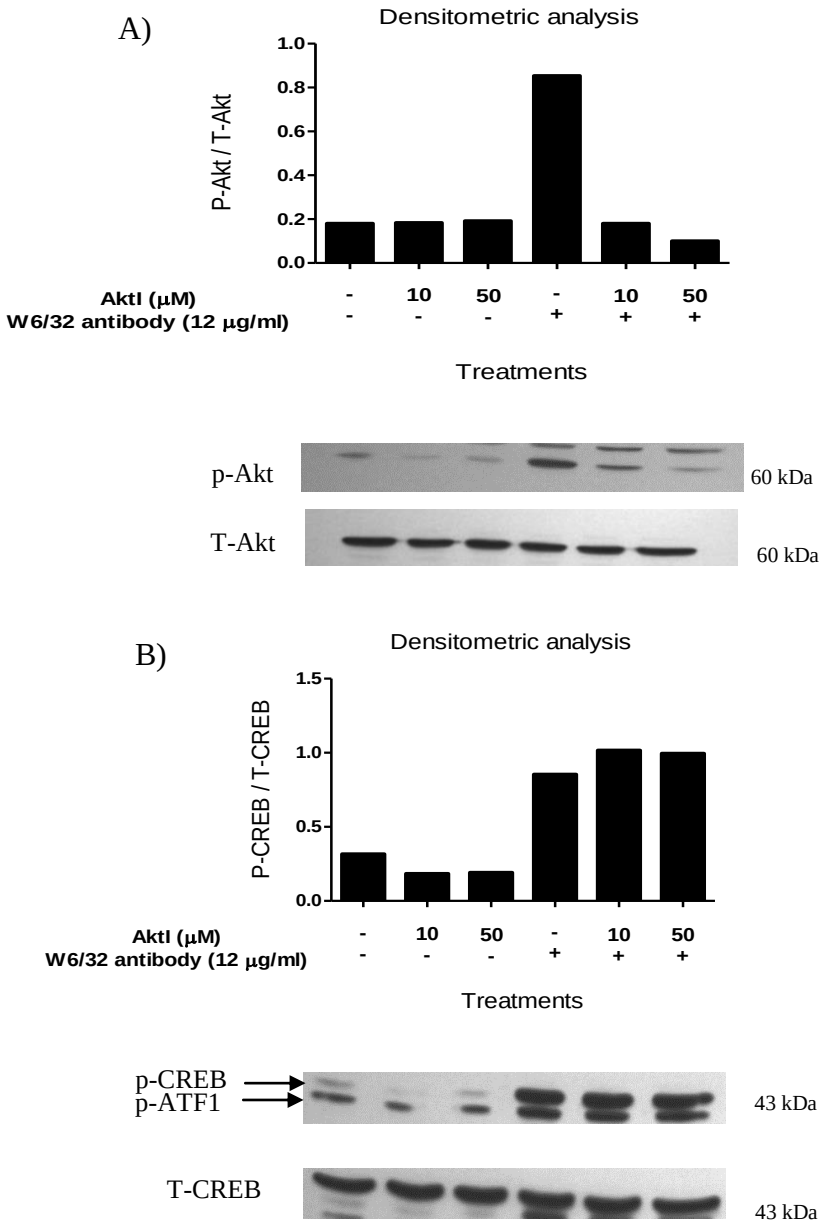


Figure 4.7: Effect of PI3K/Akt pathway on endothelial CREB phosphorylation induced by HLA class I antibody.

HMEC-1 cells were treated with LY249002 inhibitor at 10 and 50 μM for 30 minutes before stimulation with saturating concentration of W6/32 antibody for 5 minutes. Cells were lysed and analysed by western blotting. Untreated cells and cells incubated with the inhibitor alone were used as negative controls. The membranes were probed for p-Akt (A) and p-CREB (B). The membranes were then stripped and re-probed for corresponding total Akt and CREB. The densitometric analysis was performed using Alphaimager software. The data are representative of three independent experiments.

4.4.5 Effect of PKA pathway on endothelial CREB phosphorylation induced by W6/32 antibody

Phosphorylation of the transcription factor CREB following stimulation with W6/32 antibody suggests the involvement of an upstream pathway. It is well known that the PKA pathway is mainly responsible for CREB phosphorylation in response to stimulation with different stimuli. To examine whether CREB phosphorylation is downstream of the PKA pathway, the PKA inhibitor H-89 was used. To optimize the inhibitor concentration, HMEC-1 endothelial cells were incubated with different concentrations of the PKA inhibitor ranging from 50 nM to 50 μM before stimulation with 10 μM of forskolin for 5 minutes. Concentration above 50 μM effected significantly the cell viability as determined by trypan blue staining. As shown in Figure 4.8, both 50 and 5 μM of H-89 inhibited CREB phosphorylation upon exposure to forskolin stimulation compared to cells treated with forskolin only. Therefore, 5 μM was used in further experiments since it is the lower concentration that inhibits significantly CREB phosphorylation in response to stimulation.

For antibody stimulation, HMEC-1 cells were incubated with 5 μM of PKA inhibitor for 30 minutes before stimulation with saturating concentration of W6/32 antibody (12 μg/ml) for 5 minutes. Cells stimulated with the W6/32 antibody following incubation with H-89 inhibitor showed a reduction in the extent of CREB phosphorylation compared to cells treated with W6/32 antibody alone as shown in Figure 4.9.

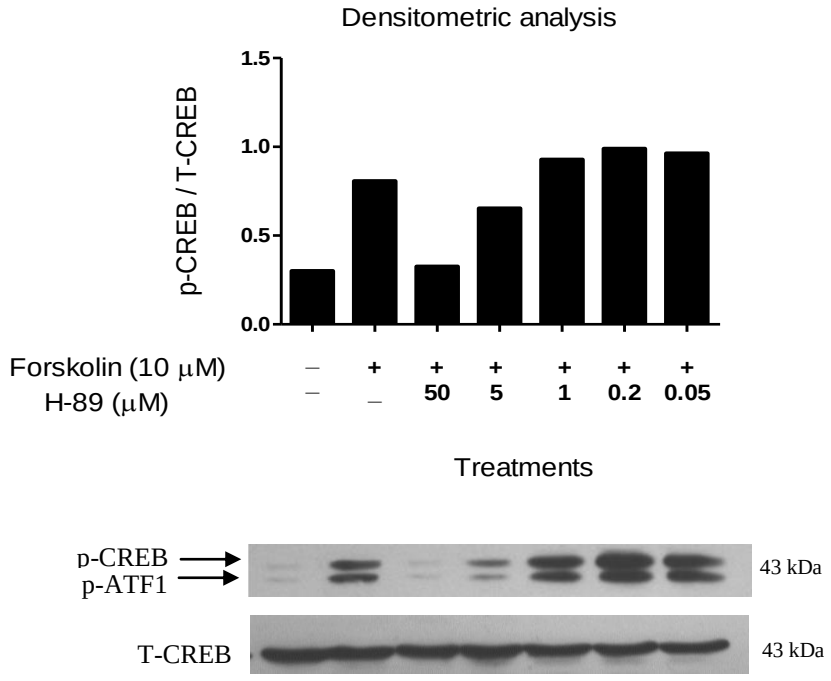


Figure 4.8: Optimization of the concentration of the PKA inhibitor, H-89 on endothelial cells.

HMEC-1 cells were treated with forskolin 10 μM for 5 minutes either in the presence or absence of different concentrations of H-89 inhibitor. The inhibitor was added 30 minutes before stimulation with forskolin and lysates were probed for p-CREB at serine residue 133. The membrane was stripped and re-probed for T-CREB to ensure equal loading. The densitometric analysis was performed using Alphaimager software. The experiment is representative of two independent experiments.

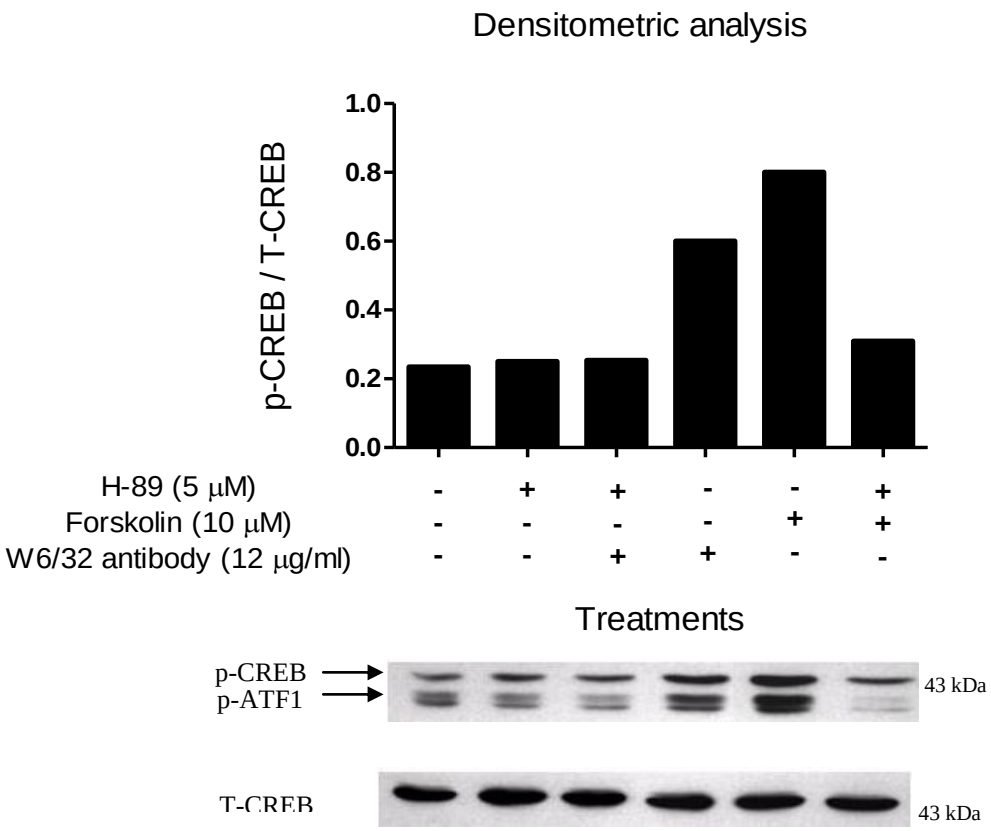


Figure 4.9: Effect of PKA pathway on endothelial CREB phosphorylation induced by HLA class I antibody.

Serum starved HMEC-1 cells were treated with isotype control (mouse IgG2a), 10 μM of forskolin or 5 μM of PKA inhibitor (H-89) 30 minutes before the exposure to either forskolin or W6/32 antibody (12 μg/ml) for 5 minutes. The cell lysates were probed with rabbit anti-human p-CREB antibody at serine residue 133. The membrane was stripped and re-probed for total CREB. The density of the bands was measured by AlphaImager software. This experiment is representative of three independent experiments.

4.4.6 Effect of W6/32 antibody on cell proliferation

The ability of HLA class I antibody to induce endothelial cell proliferation was examined. Optimum cell number of HMEC-1 cells (20,000) were seeded into 96 wells plate and treated with a saturating concentration (12 μg/ml) of either W6/32 or isotype control antibody (mouse IgG2a) for 24 and 48 hours in complete media before performing colorimetric proliferation assay. As shown in Figure 4.10, HLA class I antibody treated cells did not show any significant difference in the rate of cell proliferation compared to isotype treated groups after 24 and 48 hours of stimulation. There was no significant difference between these groups and the untreated group (data not shown).

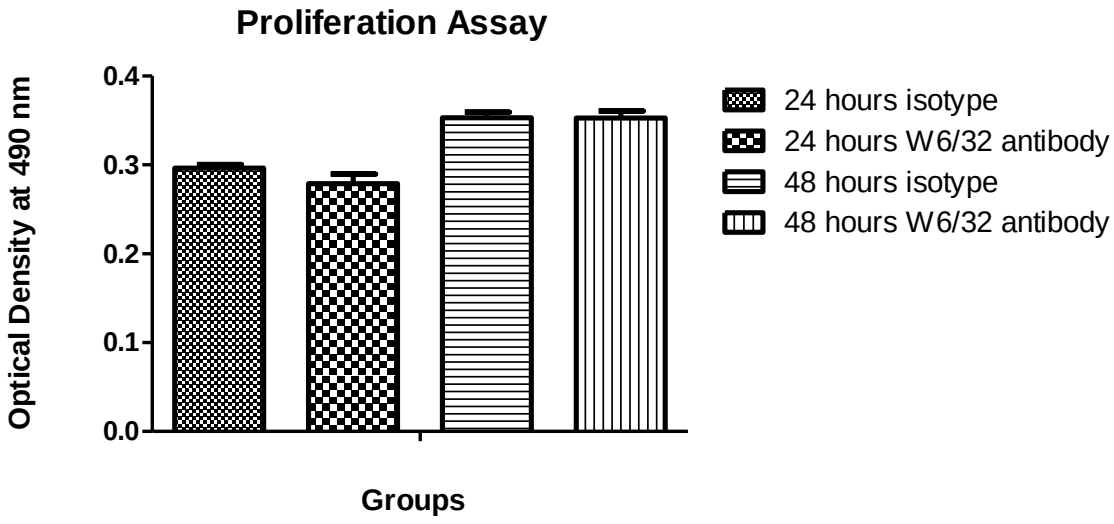


Figure 4.10: Proliferation assay using endothelial cells following stimulation with HLA class I antibody.

20,000 HMEC-1 cells were treated with a saturating concentration of HLA class I antibody (12 μg/ml) or isotype control in complete media for 24 and 48 hours before adding the colorimetric proliferation assay reagents. The plate was incubate at 37^0C for one hour and then measured at 490 nm using ELISA plate reader. Data are representative of three independent experiments.

4.4.7 Optimization of apoptosis of the HMEC-1 cells using staurosporine

Staurosporine (STS) is a potent inhibitor of various protein kinases especially protein kinase C. It is known as an anti-proliferative drug which might cause cell death of various human cell types. To examine the apoptosis of endothelial cells in response to HLA class I antibody, HMEC-1 cells were first examined for cell apoptosis using STS to be used as a positive control. Cells were initially treated with 2.5 μM STS, a concentration optimized by our group for Jurkat T-lymphocyte cell line. Unfortunately, this dose induced an immediate cell necrosis in HMEC-1 cells. Therefore, different concentrations of STS were used to evaluate the optimum concentration which might produce a significant percentage of apoptotic cells. Cells were treated with different concentrations of STS ranging from 25 nM to 600 nM for 24 hours followed by staining with propidium iodide in the presence of permeabilizing agent. As shown in Figure 4.11, a lower concentration (25-200 nM) caused cell cycle arrest at G1 phase and at a higher dose (400-600 nM) cells were arrested at G2 phase. Treatment of HMEC-1 cells with DMSO only did not induce a significant change in cell distribution (data not shown). This data suggests that STS treatment on HMEC-1 cells has a synchronizing rather than an apoptotic effect.

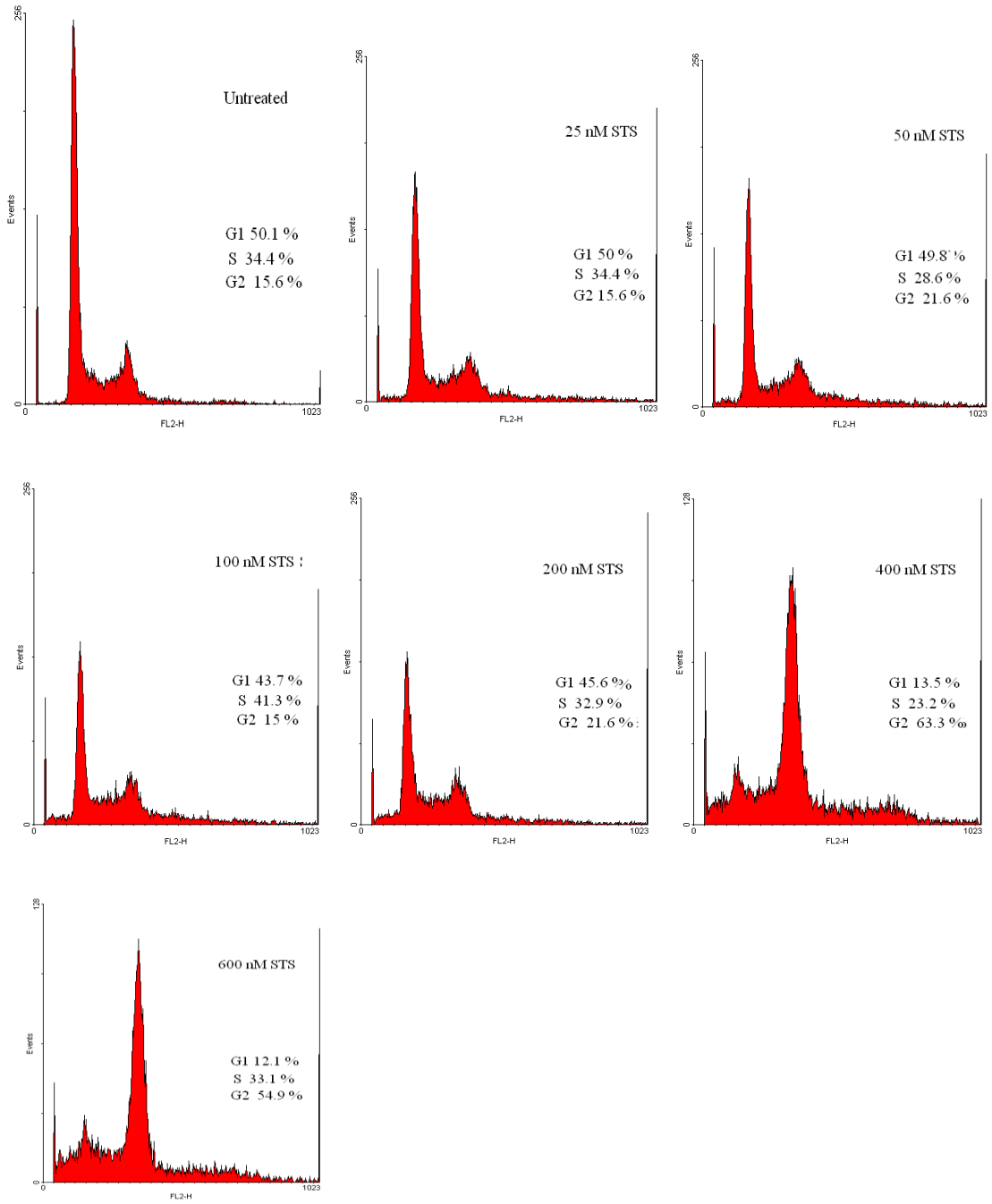


Figure 4.11: Optimization of apoptosis on HMEC-1 endothelial cell line using staurosporine.

Cells were treated with different concentrations of staurosporine for 24 hours before flow cytometry analysis using PI/triton X-100 solution. Data were analyzed by Ventourione software for cell cycle distribution. The data are representative of two independent experiments.

4.4.8 Optimization of apoptosis on EA.hy926 cells using staurosporine

As we were unable to elicit apoptosis on HMEC-1 cells following treatment with STS, another endothelial cell line, EA.hy926, was used to validate STS treatment and used as a comparison to HMEC-1 cells. EA.hy926 cells were treated with 2.5µM of STS for 24 hours followed by flow cytometry analysis. Incubation of confluent cells in a serum free media for 24 hours followed by treatment with STS in complete media induced around 15 % apoptotic cells after 4, 8 and 16 hours and around 42 % after 24 hours as shown in Figure 4.12. To exclude the possibility of non-specific effect of DMSO, cells were treated with 4 % DMSO alone and examined by flow cytometry. DMSO did not show significant difference from untreated group.

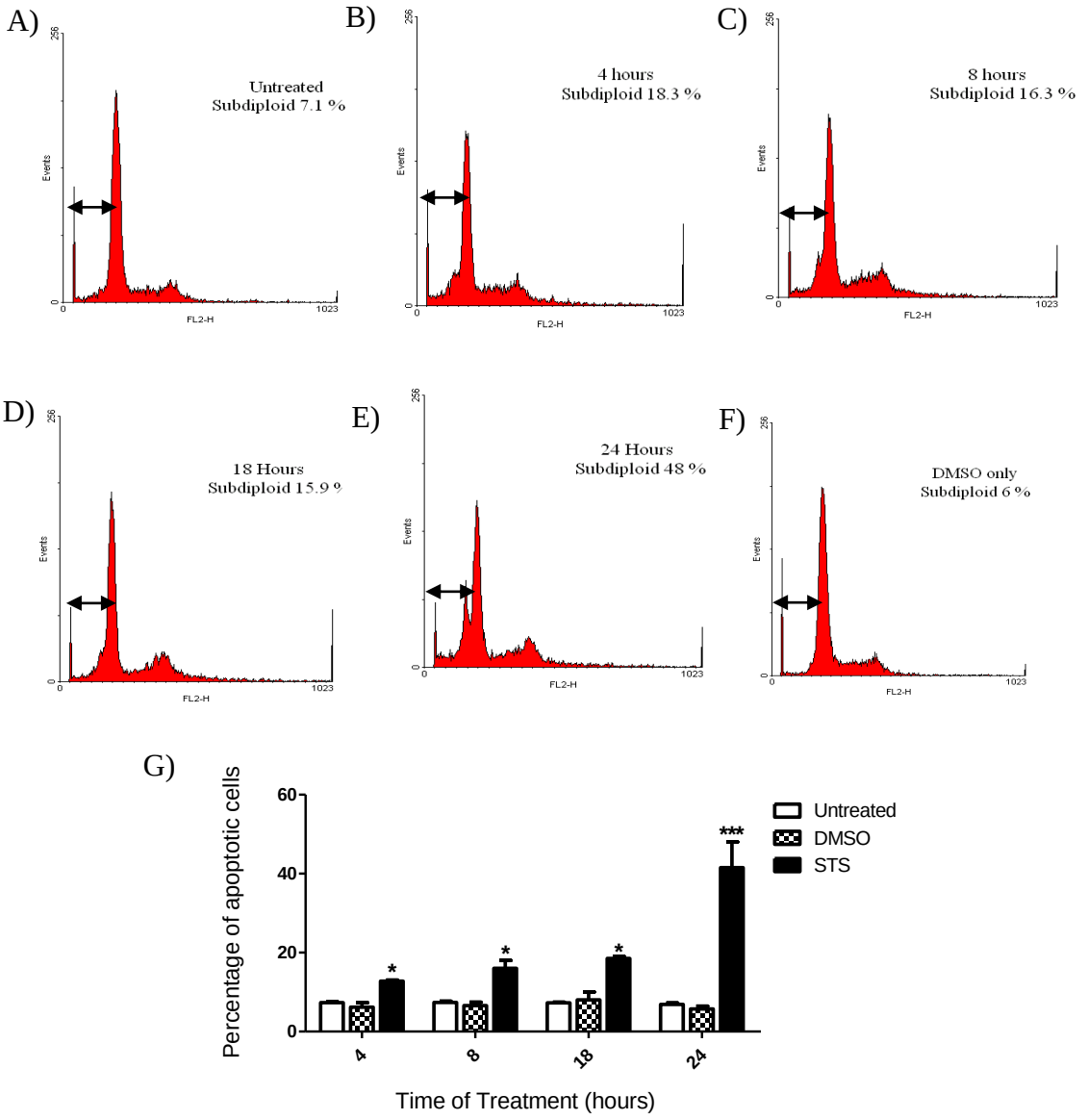


Figure 4.12: Optimization of apoptosis on EA.hy926 cells using staurosporine.

Serum starved cells were incubated with 2.5 μM STS or 4 % DMSO at different time points before analysis by flow cytometry. Detached cells were stained with PI/triton X-100 and analyzed by flow cytometry. Data were analyzed by Ventourione software for cell cycle distribution. Panel A, B, C, D, E and F show untreated (24 hours), 4, 8, 18, 24 hours STS-treated and DMSO-treated (24 hours), respectively. Panel G shows the bar chart of the percentage of apoptotic cells. Data analysis was performed by two way ANOVA followed by Bonferroni test compared to DMSO treated cells at the same incubation time. * $P<0.05$ and *** $p<0.001$. The data are representative of three independent experiments.

4.4.9 Effect of HLA class I antibody on cell cycle distribution of HMEC-1 cell line

From optimization experiments, it seems that HMEC-1 cell line is not a good model to study cell apoptosis. However, examination of the effect of HLA class I antibody on inducing changes in cell cycle of HMEC-1 endothelial cells was assessed. HMEC-1 cells were treated with a saturating concentration of W6/32 antibody (12 µg/ml) for 24 hours followed by analysis using flow cytometry. As shown in Figure 4.13, treated cells showed no significant changes in cell cycle distribution compared to control group in response to HLA class I antibody stimulation after propidium iodide staining. Isotype control treated cells showed similar cell cycle distribution to untreated group (data not shown).

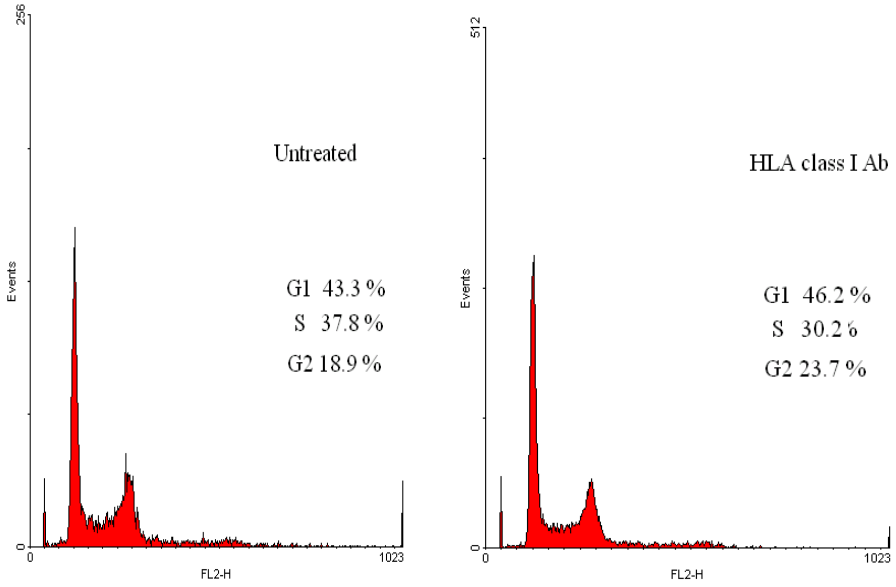


Figure 4.13: Effect of HLA class I antibody on the cell cycle of HMEC-1 cell line.

Cells were treated with saturating concentration of HLA class I antibody for 24 hours before permeabilization with triton X-100 / propidium iodide solution. The cells were analyzed by flow cytometry. Interpretation of the data was performed using Ventourione software. The data are representative of three independent experiments.

4.5 Discussion

Antibody-mediated rejection is one of the major obstacles against successful long-term solid organ transplantation. Donor specific antibody targeting HLA class I antigens induces activation of endothelial cells and microvascular inflammation. This results in allograft acute rejection and the development of transplant vasculopathy, the main manifestation of antibody-mediated chronic rejection (Sis, 2012). In a mechanism independent on the complement system, HLA class I antibody is able to induce activation of large vessel endothelial cells (Jindra *et al.*, 2008c). Cross linking of HLA class I antigens induces a rapid phosphorylation of various endothelial cell signaling mediators; modulating their normal functions. In this chapter, the activation of endothelial cell signaling pathways in response to stimulation with HLA class I antibody was examined.

In this study, treatment of endothelial cells with saturating concentration (12 μg/ml) of HLA class I antibody induced the phosphorylation of different cell signaling proteins such as Akt, FAK, Src, β-catenine, CREB and others assessed using human phospho-kinase array. Although these mediators regulate endothelial cell functions, the role of some of these kinases in inducing endothelial dysfunction during rejection is not fully investigated. Cross linking of HLA class I antigens by saturating concentration of HLA class I antibody enhanced ERK phosphorylation at Thr202/204 residues as determined by western blotting. It is a serine/threonine kinase that mediates the activation of proliferative and cell differentiation genes (Chang *et al.*, 2003). The phosphorylation of ERK has also been observed in primary human aortic endothelial cells treated with the same antibody in a mechanism involving the activation of mTORC2 (Jindra *et al.*, 2008b). In addition, in this study, HLA class I antibody induced Akt phosphorylation at serine residue 473 in a time and dose dependent manner. The expression of β4-integrin on endothelial cells is shown to be responsible for signaling mediated by HLA class I molecules (Zhang *et al.*, 2011b). HMEC-1 endothelial cells express a high level of β4-integrin as reported previously which might explain the cell signaling (Xu *et al.*, 1994).

Akt is a serine/threonine kinase that maintains the balance between cell proliferation and apoptosis depending on cell type and stimulus (Fatrai *et al.*, 2006). The phosphorylation of Akt on aortic endothelial cells by HLA class I antibody has been previously shown as a downstream consequence of mTORC1 activation, following phosphorylation by exposure to HLA class I antibody (Jindra *et al.*, 2008c). In a

previous study, maximum activation of Akt phosphorylation after stimulation with HLA class I antibody has been detected at a lower concentration of antibody (0.01 μg/ml) (Jin *et al.*, 2004). It was assumed that the Akt phosphorylation at lower concentrations of antibody promoted cell survival by up-regulating the expression of anti-apoptotic proteins; enhancing organ accommodation rather than rejection. However, in this project, the extent of Akt phosphorylation was reduced at lower antibody concentration. Whether this contributes to protective mechanism or not is not obvious. However, large vessel endothelial cells and microvascular endothelial cells might response differently to different antibody concentrations.

In this study, the phosphorylation of endothelial transcription factor CREB in response to stimulation with W6/32 antibody was also observed. Using HMEC-1 endothelial cells, CREB phosphorylation at serine residue 133 occurred in a time dependent manner and was less sensitive to antibody concentrations. CREB is a nuclear transcription factor that belongs to the basic leucine zipper (bZIP) superfamily. It regulates a wide range of crucial biological functions such as cell proliferation, survival, inflammation and differentiation via modulating the expression of various genes (Ichiki, 2006). In response to growth factor stimuli, CREB is responsible for enhancing cell survival through up-regulation of anti-apoptotic genes such as Bcl-2 (Bonni *et al.*, 1999; Riccio *et al.*, 1999) and cell proliferation by enhancing the expression of cyclin D1 (Lee *et al.*, 1999; D'Amico *et al.*, 2000; Nagata *et al.*, 2001) and cyclin A (Desdouets *et al.*, 1995).

CREB activation has a role in providing protection during oxidative stress through expression of antioxidant mediators such as manganese superoxide dismutase, thioredoxin and heme oxygenase-1 (Kronke *et al.*, 2003; Chiueh *et al.*, 2005). Various kinases might phosphorylate CREB in cell type and stimuli dependent manners such as ERK, MAPK, Akt and PKA as discussed in details in section 1.10.2. The phosphorylation in our system is independent on Akt signalling and depends on the PKA pathway as shown by using pathway specific inhibitors. Only one previous study has reported the phosphorylation of PKA in aortic endothelial cells stimulated with sub-saturating concentration of HLA class I antibody, but not when stimulated with a saturating concentration (Narayanan *et al.*, 2006). This group also showed that the activation of PKA was associated with a protection of endothelial cells against lysis by complement. The possibility that CREB protects cells from a sub-saturating concentration of antibody still needs to be elucidated.

Proliferation of smooth muscle and endothelial cells is one of the major histological signs of chronic antibody-mediated rejection (Zhang *et al.*, 2012). Therefore, the effect of HLA class I antibody in inducing microvascular endothelial cell proliferation was examined. HMEC-1 cells treated with a saturating concentration of HLA class I antibody did not show any significant difference in the proliferation rate compared to isotype-treated cells after 24 and 48 hours of stimulation. In contrast, human aortic endothelial cells stimulated with 1 µg/ml of HLA class I antibody showed a significant increase in cell proliferation compared to control group after 72 hours stimulation (Jindra *et al.*, 2008c). This was also observed using HUVEC cells treated with W6/32 antibody and allospecific antibody (Smith *et al.*, 2000). This difference might be attributed to the use of microvascular endothelial cell lines or might suggest the inability of microvascular endothelial cells to proliferate in response to HLA class I antibody.

Staurosporine is an alkaloid isolated from bacterium *Streptomyces staurosporeus* which has an indol-2,3-α-carbazol chromophore. It has a wide range of clinical activity ranging from antifungal to antitumor effects (Omura *et al.*, 1977). Its effect is attributed to its ability to inhibit a catalytic domain of protein kinase C enzyme which mediates phosphorylation of various intracellular proteins involved in cell growth and differentiation (Tamaoki and Nakano, 1990). In the research field, STS has been shown to induce apoptosis in embryonic neuronal cells (Wiesner and Dawson, 1996), causing nuclear changes in MOLT-4 cell line (Falcieri *et al.*, 1993) and HL-60 cells (Bertrand *et al.*, 1993), cell differentiation (Okazaki *et al.*, 1988) and inhibition of cell cycle progression (Bruno *et al.*, 1992) and metastasis of tumor cells (Schwartz *et al.*, 1990). In this project, STS was used to assess the ability of EA.hy926 and HMEC-1 cells to undergo apoptotic cell death, provide a positive control in apoptosis experiments using HLA class I antibody.

Treatment of HMEC-1 with doses of STS ranging from 25 to 200 nM caused an arrest of the cell cycle at G1 phase while treatment with higher doses (400-600 µM) arrested the cells at G2 phase. In contrast, treatment of EA.hy926 cells with 2.5 µM STS induced around 41% of cell apoptosis after treatment for 24 hours. Previous results showed that treatment with STS produced both effects in a cell type and cell cycle dependent manner (Bernard *et al.*, 2001). Treatment of fibroblasts with STS showed a similar effect to that observed on HMEC-1 cells. Treatment with lower doses of STS (10 ng/ml) arrested cells at the G1-phase whilst treatment with higher doses of STS induced cell cycle arrest at the G2-phase (Abe *et al.*, 1991). For EA.hy926 cells, apoptotic cell death was

observed previously using STS at different time points and concentrations (Mosnier and Griffin, 2003). This suggests that STS can be used as a cell synchronizing agent in some types of endothelial cells and as an apoptosis inducing agents in the others. Therefore, HLA class I antibody was assessed for its ability to induce a modulation in endothelial cell cycle rather than apoptosis. Exposure to these antibodies at a saturating concentration for 24 hours did not produce any changes in the cell cycle compared to isotype control. This might also confirm the proliferation result, since the increase in the proliferation rate suggests an increase in S and G2-phases which is also not observed.

In conclusion, exposure of human microvascular endothelial cells to HLA class I antibody induces phosphorylation of various intracellular signaling proteins such as ERK, Akt and transcription factor CREB. The phosphorylation of CREB shows sensitivity to PKA but not to the Akt pathway suggesting activation of two independent pathways. Although the stimulation with HLA class I antibody did not induce endothelial cell proliferation or apoptosis, the activation of these pathways suggests a modulation of endothelium function after exposure to HLA class I antibody. Therefore, expression of adhesion molecules and chemokines by stimulated endothelial cells was examined.

5. Chapter Five-HLA class I Antibodies and Endothelial-Leukocyte Interaction

5.1 Introduction

In solid organ transplantation, the presence of circulating donor specific HLA antibodies is associated with a high incidence of acute cellular rejection (Musat *et al.*, 2011). In addition, detection of these antibodies along with cellular rejection in renal transplantation is a marker of poor prognosis of allograft survival compared to grafts with antibody-mediated rejection or cellular rejection alone (Everly *et al.*, 2009). Infiltration of mononuclear cells is not only observed in cellular-mediated rejection but is also one of the main characteristic of microvascular inflammation during antibody-mediated rejection and predicts poor allograft outcome (Tinckam *et al.*, 2005; Molne *et al.*, 2006; Fahim *et al.*, 2007; Papadimitriou *et al.*, 2010). In a mouse model, passive transfer of MHC class I antibodies into mismatched recipients induces monocyte/macrophage infiltration independent of the Fc fragment of the antibody (Jindra *et al.*, 2008a). Infiltrating macrophages can induce endothelial cell proliferation by secreting growth factors and cytokines promoting the development of vasculopathy (Schubert *et al.*, 2008). The mechanism by which endothelial cells induce monocyte adhesion in response to HLA class I antibodies is not fully understood.

Leukocyte migration is a multistep process mediated by the expression of adhesion molecules and chemokines by endothelial cells. The expression of these mediators is increased at different stages following transplantation. During ischemia reperfusion, the expression of cytokines and chemokines such as IL-2, IL-6, CCL2 and CCL5 increased after 30 minutes of allograft reperfusion in an animal model of pancreas transplantation (Lunsford *et al.*, 2013). In cellular rejection, the expression of CCL21 chemokine increased in biopsies from kidney patients and is involved in inducing cell fibrosis (Zhou *et al.*, 2013). Antibody-mediated rejection is also characterized by the expression of different chemokines and adhesion molecules. In a mouse model, passive transfer of MHC class I antibody to mismatched antigens induced the expression of VCAM-1, ICAM-1, IL-6, IL-1β, CXCL8 and IL-12 (Fukami *et al.*, 2012). Chemokine expression is also involved in the development of graft vasculopathy, the main feature of chronic rejection (Ruster *et al.*, 2004). In addition, the detection of soluble adhesion molecules VCAM-1 and ICAM-1 can be used as a marker for early acute rejection and predicts

poor outcome following kidney transplantation (Connolly *et al.*, 2011; Reinhold *et al.*, 2012).

In blood vessels, intravascular flow is necessary for inducing leukocyte migration in a process called chemorheotaxis. In this process, shear stress applied by the blood flow is essential for leukocyte trafficking into inflammatory sites in the presence of immobilized chemokines (Luscinskas *et al.*, 2001). Apical chemokines immobilized on the endothelial cell surface play an essential role in inducing stable leukocyte arrest, while luminal chemokines are essential for inducing leukocyte transmigration (Weber *et al.*, 1999; Cinamon *et al.*, 2001). Shear stress is defined by the magnitude of the force exerted by the blood flow to the specific area on the blood vessels, and is expressed as dyne cm^{-2}. According to the type of blood vessels and their inflammatory status, the blood flow applies shear stress ranging from 1 dyne cm^{-2} in post-capillary venules to more than 10 dyne cm^{-2} in arteries (Fidkowski *et al.*, 2005). Typically, from 1-6 dyne cm^{-2} is a physiological range for leukocyte recruitment in post-capillary and 0.5 dyne cm^{-2} is optimal for *in vitro* leukocyte adhesion (Verdier *et al.*, 2009).

Shear stress applied by blood flow is crucial in maintaining optimum function of cell surface selectins and integrins (Laudanna and Alon, 2006). In addition, lymph node-related chemokines such as CXCL12 induce efficient integrin activation and cell adhesion under shear forces compared to that in a shear-free environment. Endothelial chemokines under flow induce conformational changes to the cell surface integrins, enhancing their binding to corresponding endothelial ligands (Woolf *et al.*, 2007). Furthermore, blood flow enhances a synergistic effect between different chemokines compared to the effect of each chemokine independently in static adhesion assays (Schreiber *et al.*, 2007).

Examination of leukocyte migration in response to particular chemokines is traditionally performed under static conditions. Static chemotaxis assays using transwell chambers, such as Boyden chambers, can be used for a migration assays without applying any shear stress. These assays can be used to assess the ability of the cells to migrate in response to chemokine gradients, without assessing cell adhesion and migration in the presence of physiological shear stress. Although both chemotactic effects and chemokinetics can be analyzed in this assay, diffusion of chemokines after a few hours impairs the chemokine gradients and impedes further cell migration (Toetsch *et al.*, 2009). By using flow based assays, these limitations are overcome; remodeling *in*

vivo leukocyte adhesion that is not present under static condition. Therefore, in this project the role of activated endothelial cells in inducing leukocyte adhesion and migration was examined using both static and flow based assays.

In this chapter, the potential of HLA class I antibody to induce the expression of mediators responsible for leukocyte adhesion and migration was examined. This includes the expression of endothelial cell surface adhesion molecules; VCAM-1, ICAM-1 and E-selectin in addition to the expression of inflammatory cytokines and chemokines. The expression of adhesion molecules was determined using flow cytometry while the expression of inflammatory cytokines was evaluated using human protein cytokine array and q-PCR. Furthermore, the mechanisms responsible for the expression of these molecules were assessed. This was performed by examining the contribution of cell signaling pathways Akt and PKA/CREB in the expression of adhesion molecules and chemokine using pathway-specific inhibitor and CREB-knockdown. The ability of activated microvascular endothelial cells to induce monocyte adhesion and migration was also determined using static chemotaxis and *in vitro* flow-based adhesion assays.

5.2 Specific Aims

The role of HLA class I antibody (W6/32) in inducing the expression of mediators involved in leukocyte adhesion was examined. This involves:

- Evaluating the expression of cell surface adhesion molecules following stimulation with W6/32 antibody;
- Assessing the contribution of the PI3K/Akt pathway in the expression of endothelial adhesion molecules;
- Evaluating the expression of cytokines and chemokines by endothelial cells following the exposure to saturating concentration of W6/32 antibody;
- Examining the pathways responsible for the expression of CXCL8 in response to W6/32 antibody
- Examining the efficiency of W6/32 antibody-treated endothelial cells in inducing human monocytes (THP-1) migration using static chemotaxis assay, and
- Assessing the potential of $F(ab)_2$ fragments in inducing the adhesion of THP-1 cells, under physiological flow condition.

5.3 Specific materials and methods

5.3.1 Stimulation of endothelial cells for adhesion molecules expression

Expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 on endothelial cells was examined following stimulation with tumor necrosis factor (TNF-α). Adherent HMEC-1 cells were grown in 12 well plates until reach 50-60% confluence. The cells were then washed once with PBS and the media was changed to media containing TNF-α at different concentrations. HMEC-1 cells were stimulated with TNF-α at concentrations ranging from (0.1-20 ng/ml) for 16 to 72 hours without media change. Stimulated cells were detached using cell dissociation buffer and $1X10^5$ cells were stained with PE-conjugated mouse anti-human E-selectin, VCAM-1 and ICAM-1 antibodies (eBioscience) and analysed by flow cytometry. Cells stained with isotype control (mouse IgG1) were used as a negative control. For stimulation with W6/32 antibody, adherent cells were incubated with a saturating concentration of W6/32 antibody (12 μg/ml) or isotype control (mouse IgG2a) in complete media at different time points ranging from 8 hours to 72 hours. The cells were then analyzed using flow cytometry.

5.3.2 Stimulation of endothelial cells for measurement of cytokine and chemokine expression

To assess the ability of endothelial cells to produce inflammatory cytokines and chemokines in response to W6/32 antibody cells were stimulated with this antibody and examined for the expression of these mediators at the protein level. A human cytokine array (R&D system) containing 36 different anti-cytokine antibodies attached to a nitrocellulose membrane was used. HMEC-1 cells at 50% confluence were stimulated with saturating concentration of W6/32 antibody (12 μg/ml) or isotype control for 72 hours. Supernatant was collected and analysed against the cytokine array according to the manufacturer's instruction. In brief, membranes were blocked for 1 hour at room temperature with blocking buffer provided in the kit. Conditioned media from W6/32 or isotype treated cells were mixed with biotinylated detection antibody cocktail and incubated for 1 hour at room temperature. The samples were then added to the blocked membranes and incubated overnight at 4^oC with continuous shaking. After incubation, membranes were washed three times followed by incubation with streptavidin-HRP. The membranes were incubated at room temperature for 30 minutes followed by three

washes. Blots were developed using ECL substrates and Kodak films. The density of the dots was measured using AlphaImager software.

5.3.3 Stimulation of endothelial cells for expression of CXCL8

Endothelial cells (HMEC-1) were grown in 12 wells plate until confluence. Cells were stimulated with saturating concentration 12 µg/ml of either isotype control or W6/32 at different time points or with different antibody concentrations ranging from (0.4-12 µg/ml) for 3.5 hours. After stimulation, the cells were washed once with PBS and RNA was isolated as described in section 2.9.2 using 250 µl of Tri reagent per well. The RNA concentration and purity was assessed by Nanodrop as described in section 2.9.3. Two to five µg of RNA was used for cDNA synthesis. The latter was performed using one step cDNA systhesis kit as described in section 2.9.5. The resultant product was used in a q-PCR reaction as described in section 2.10.4 using human CXCL8 and GAPDH specific primer-probes.

5.3.4 Transient transfection (siRNA technology)

5.3.4.1 General principle

The RNA interference is a physiological regulatory pathway which occurs in the cytoplasm of most eukaryotic organisms. In this process, small double stranded RNA mediates the downregulation of different proteins at the post-transcriptional level. The key mediators in this process are small endogenous RNA sequences with around 21-28 nucleotides; they are small interfering RNA (siRNA) and microRNA. From this pathway, the ability to silence a specific gene by using an exogenous siRNA in a transfection process has emerged. In this machinery, a long double stranded RNA, exogenous or endogenous, is cleaved by the Dicer enzyme, a ribonuclease belonging to the RNase III family, generating a short double stranded siRNA of around 22 nucleotides. This sequence is incorporated into the RISC complex (RNA induced silencing complex) producing a single stranded siRNA in a reaction catalyzed by RNA helicase enzyme. The production of single stranded siRNA induces activation of RISC complex which activates the nuclease enzyme, inducing degradation of mRNA that is complimentary to the siRNA as shown in Figure 5.1. Transfection of siRNA induces transient, sequence specific knock down which is applicable for 3-5 cell divisions (Kim, 2003).

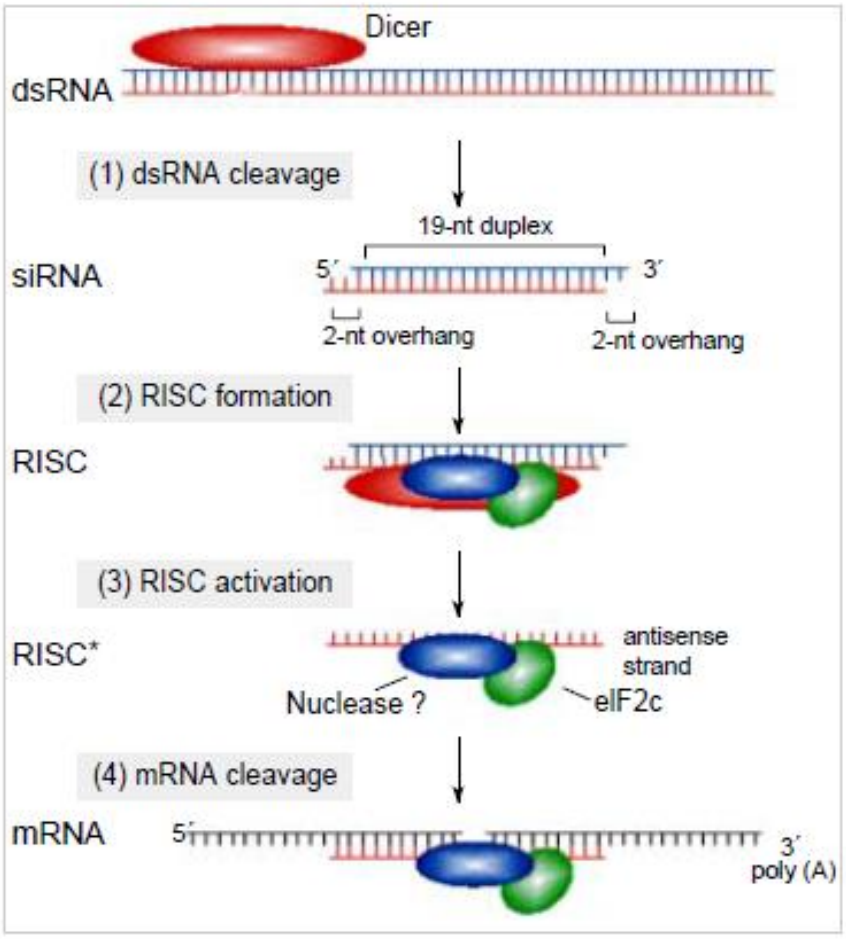


Figure 5.1: Mechanism of siRNA action in degradation of target mRNA.

Source: (Kim, 2003).

The transfection of siRNA into cultured cells can be performed using different transfection reagents and methods. Transfection can be achieved using biological, chemical or physical methods. The chemical agents that can be used involve; cationic polymer, calcium phosphate and cationic lipid based reagents. In this project, siRNA transfection was performed using the chemical method (lipid-based transfection agent) and physical method (electroporation). In the former, a complex is formed between negatively charged siRNA and a cationic lipid attached to liposomes. The latter contains long hydrophobic chains and a positively charged head group which encapsulates the siRNA by lipid bilayer preventing its degradation and facilitating its uptake to the cells. Adherent cells can be transfected by these agents either in their adherent state (traditional transfection) or by reverse transfection. In reverse tranfection method, cells are transfected while they are in suspension and the transfection occurs at the time of cell attachment, minimizing the transfection time and increasing transfection efficiency. In electroporation method, the cells are exposed to electrical pulse forming pores in the cell membrane facilitating the entrance of siRNA. In general, successful transfection depends on many factors and might require optimization. Factors such as cell type, passage number, method used and the half-life of the target protein need to be all considered.

5.3.4.2 **Transfection efficiency using cy3 labelled GAPDH siRNA and lipid based reagents**

Fluorescently labelled siRNA is used as a guide in monitoring siRNA uptake as well as its distribution and localization during the transfection process. Additionally, it can be also used to assess the efficiency of the knockdown of the desired gene. Human silencer cy3 labelled GAPDH siRNA (Applied Biosystems) was supplied in lyophilized form. It was resuspended in RNAase free water to 10 μM concentration. A lipid based transfection reagent, siPORT NeoFX (Applied Biosystems) was used to perform the transfection process using manufacturer's instruction. In brief, 50% confluent HMEC-1 cells were trypsinized and counted. $9X10^4$ cells were added per well in 12 wells plate, re-suspended in 900 μL of HMEC-1 media without antibiotics. For reagent preparation, the complex formation between siRNA and the reagent was performed using HMEC-1 media (MCDB-1 media) without any additives. The siRNA was used at concentrations 30, 50 and 100 nM. For complex formation, 50 μL of media was mixed with 3 μL of labelled GAPDH siRNA to final concentration of 30 nM in Eppendorf tube. In a second

Eppendorf tube, 50 μL of media was mixed with 3 μL of siPORT NeoFX transfection reagent. The contents of the two Eppendorfs were mixed together and left for 10 minutes at room temperature. After incubation, 100 μL of siRNA mixture was added to the cells in the wells with gentle shaking. After 24 hours of incubation, cells then were washed once with PBS and fresh media was added. The cells were examined by fluorescence microscope (Leica, Germany) at excitation 547 nm and emission 563 nm at 24, 48 and 72 hours. Cells transfected with 30 nM of labelled GAPDH siRNA for 24, 48 and 72 hours are shown in Figure 5.2, while transfection with different siRNA concentrations for 24 hours is shown in Figure 5.3.

5.3.4.3 Transfection efficiency using cy3 labelled GAPDH siRNA and electroporation technique

For transfection by electroporation, 0.5 million cells trypsinized from 50% confluent HMEC-1 culture were suspended in PBS. The cells were transfected with labelled siRNA for GAPDH at final concentration of 30 nM. The electroporation process was carried out using a Nucleofector I instrument (Amaxa, Lonza). Suspended cells and siRNA were exposed to electric pulses recommended by the manufacturer for similar cell lines and saved as M-03 program. The cells were resuspended in 1 ml of media without antibiotics and grown in 12 well plates. After 24, 48 and 72 hours, cells were visualised using inverted fluorescence microscope (Leica) at excitation 547 nm and emission 563 nm as shown in Figure 5.4. Electroporation caused excessive cell death. Therefore, transfection using lipid-based reagent was chosen for further experiments.

5.3.4.4 Evaluation of knockdown efficiency using different lipid based reagents and non-fluorescently labelled GAPDH siRNA

Fluorescently labelled GAPDH siRNA showed successful transfection of HMEC-1 cells. However, fluorescently labelled GAPDH siRNA showed a low efficiency in knocking down GAPDH at a protein level as assessed by western blotting (data not shown). Therefore, a non-fluorescently labelled silencer select GAPDH siRNA was chosen. The choice of this sequence was made according to manufacturer's recommendation which is supposed to be effective in knocking down a target sequence at lower siRNA concentrations between 5-20 nM. Silencer select GAPDH siRNA (Applied Biosystems) was supplied in purified and dried form. It was reconstituted in RNAase free water at 5 μM stock solution. To optimize the transfection protocol, the

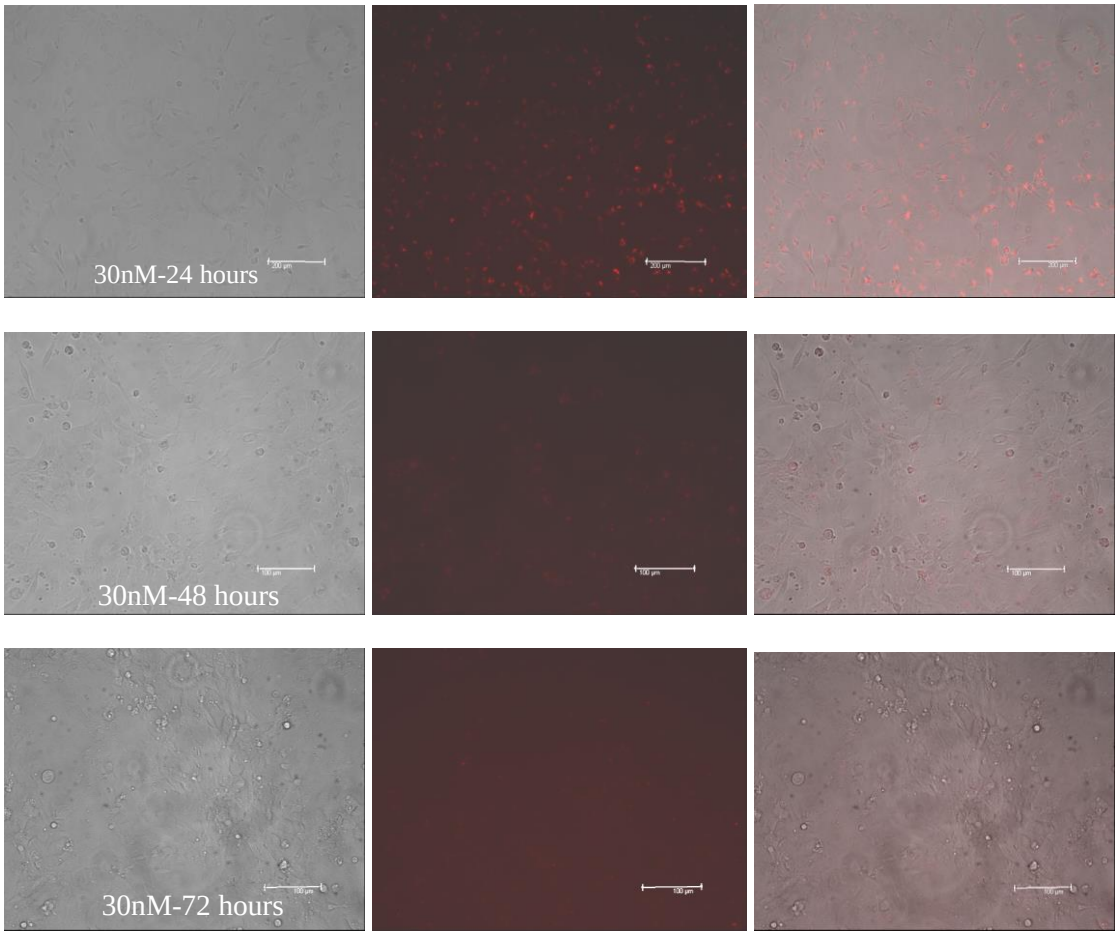


Figure 5.2: Time course of the transfection with cy3 GAPDH siRNA into HMEC-1 cells using lipid based transfection reagent.

HMEC-1 cells were transfected with 30 nM of cy3-GAPDH siRNA using NeoFX siPORT transfection reagent and visualized by fluorescence microscopy (Leica) at excitation 547 nm and emission 563 nm after 24, 48 and 72 hours of transfection.

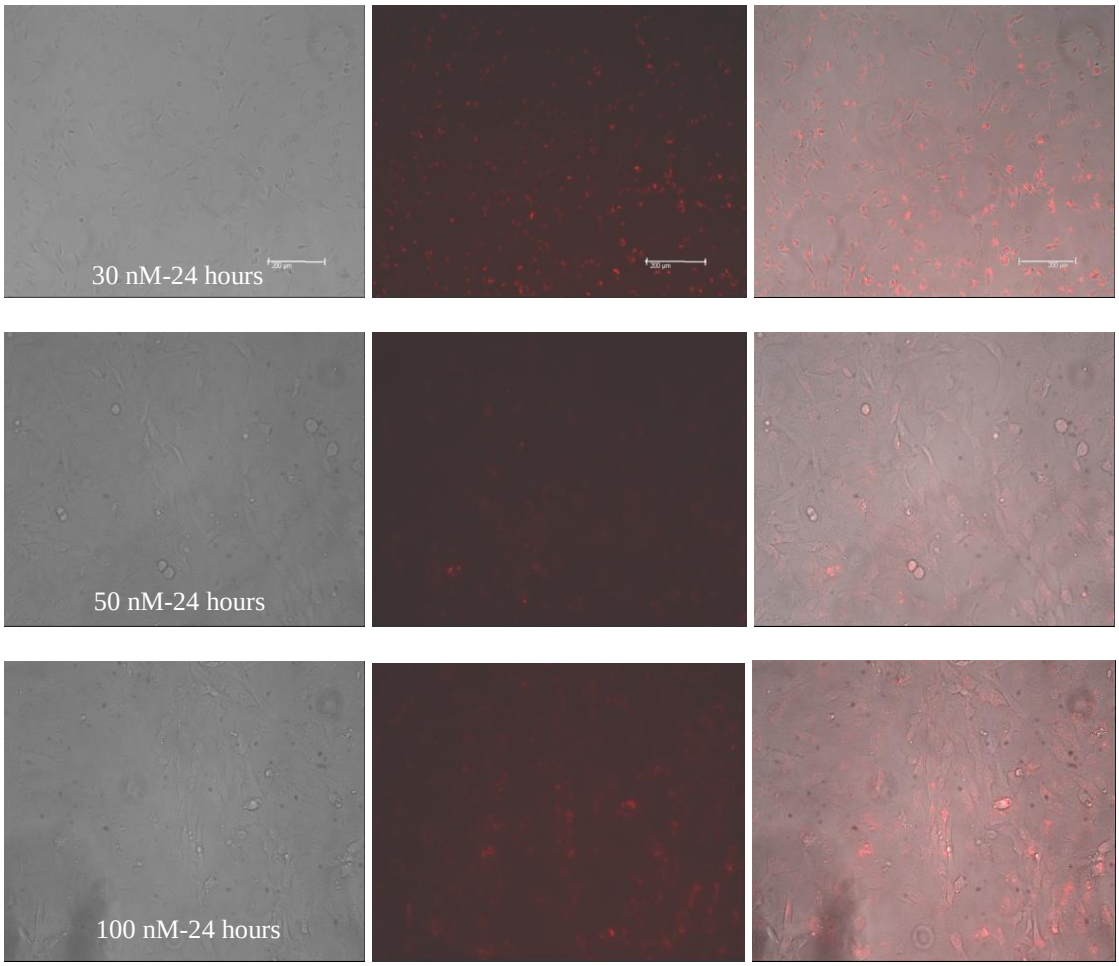


Figure 5.3: Transfection of cy3 GAPDH siRNA at different concentrations into HMEC-1 cells using lipid based transfection reagent.

HMEC-1 cells were transfected with 30, 50 and 100 nM of cy3-GAPDH siRNA using NeoFX siPORT transfection reagent and visualized by fluorescence microscope (Leica) at excitation 547 nm and emission 563 nm after 24 hours of transfection.

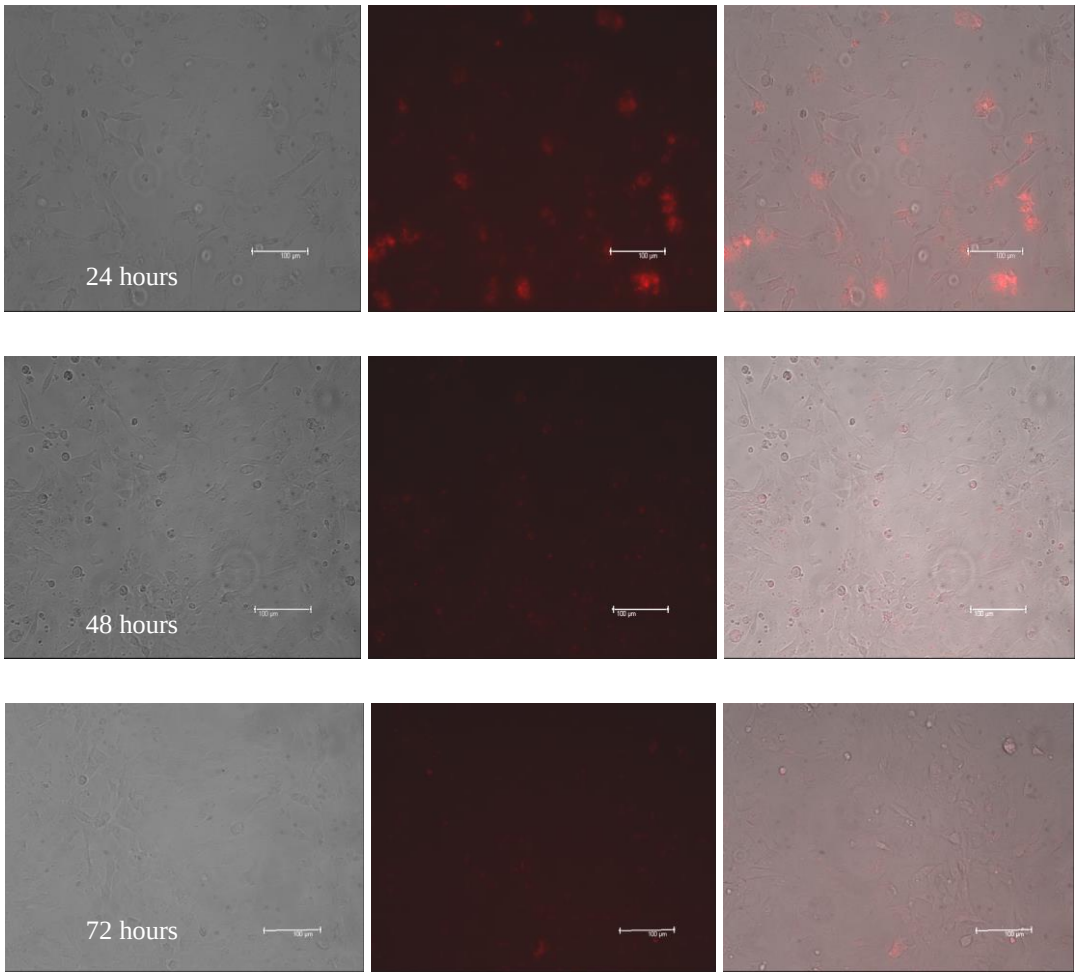


Figure 5.4: Transfection of cy3 GAPDH siRNA into HMEC-1 cells using the electroporation technique.

HMEC-1 cells were resuspended in PBS and transfected with 30nM of cy3-GAPDH siRNA using Nucleofector I instrument with M-03 program. The images were captured by fluorescence microscope after 24, 48 and 72 hours of transfection.

HEK cell line described in section 2.3.4 was used. This cell line is a good model to achieve an efficient transfection and gene silencing as previously shown by our laboratory. Therefore, knocking down of GAPDH in this cell line was assessed in parallel with HMEC-1 cells. Four different lipid based transfection reagents were tested which were supplied by different manufacturers including; siPORT NeoFX transfection reagent (Applied Biosystems), lipofectamine (invitrogen), interferin siRNA transfection reagent (PolyPlus-transfection) and Ribocellin siRNA delivery reagent (BioCellChallenge). All reagents were tested using HEK cells and HMEC-1 cells except the last reagent was tested on HMEC-1 only. The $2X10^5$ of HMEC-1 cells were transfected reversely with 5 nM of GAPDH siRNA in 12 wells plate using four different reagents, following the manufacturer's instructions for each reagent. The cells were incubated at 37^0C for 48 and 72 hours incubation. The knockdown efficiency was determined at mRNA level after 48 hours of transfection and at protein level following 72 hours. Media was changed to fresh transfection media (without antibiotics) after 24 hours of transfection.

5.3.4.5 **Knockdown efficiency at mRNA level**

To determine the efficiency of silencing GAPDH at mRNA level, real time-PCR using a Taqman assay was performed as described in section 2.10.4. After 48 hours of transfection, HEK and HMEC-1 cells were washed once with PBS and RNA was isolated by Tri-reagent as described in section 2.9.2. Cells transfected with Ribocellin siRNA delivery reagent were excluded due to high toxicity and cell death. RNA concentration and purity was assessed by Nanodrop spectrophotometry as described in section 2.9.3. cDNA synthesis was performed using 2-3 μg of RNA as described in section 2.9.5. GAPDH primer-probe was used to analyze GAPDH expression and 18S primer probe was used as a house keeping gene. Because the latter is a non-exon spanning primer, a genomic DNA elimination step was performed before cDNA synthesis using a commercial kit (Promega) following the manufacturer's instructions. No amplification was observed in wells containing RNA samples. Figure 5.5 (A) shows real time PCR data for HEK cell line using different reagents.

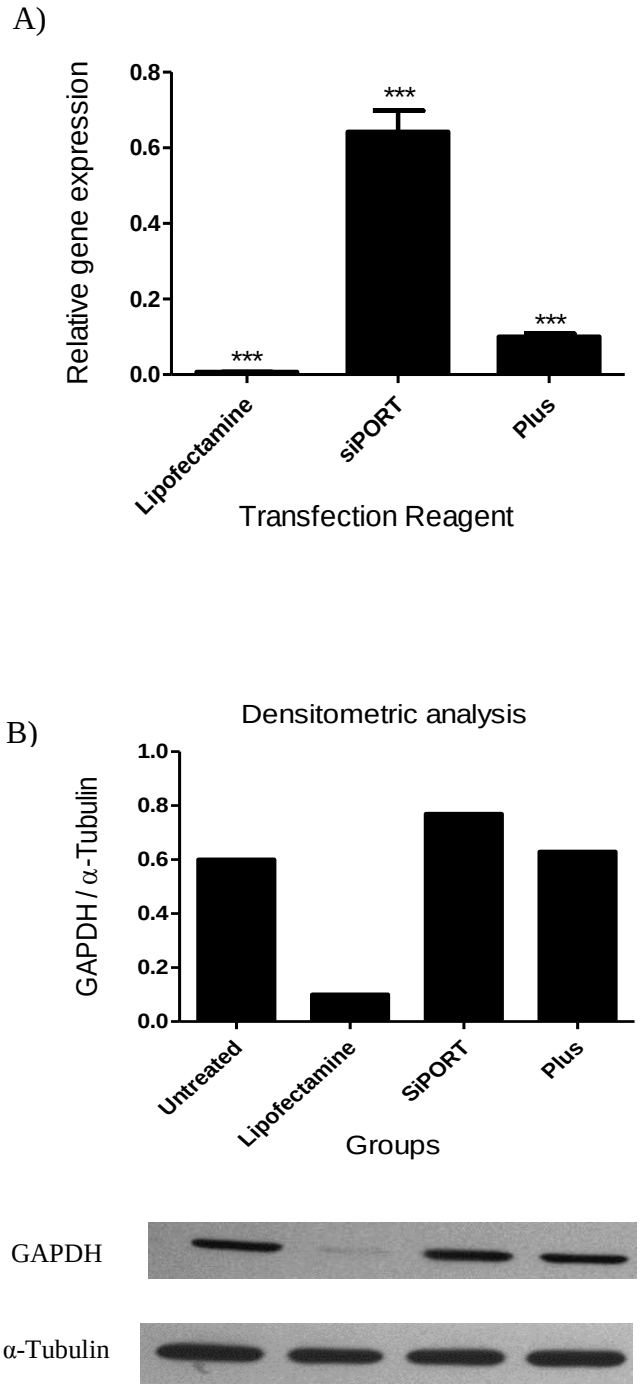


Figure 5.5: Optimization of GAPDH siRNA transfection in HEK cell line.

HEK cells were transfected with 5 nM of silencer select siRNA using different reagents. Changes at mRNA level were determined by real time-PCR as shown in panel A using 18S as a house keeping control. The change at protein level for GAPDH was assessed by western blotting as shown in panel B using tubulin as a loading control. Densitometric analysis was performed using AlphaImager software. The data are representative of three independent experiments.

5.3.4.6 **Knockdown efficiency at protein level**

To examine the efficiency of the knockdown at the protein level after transfection, western blotting was performed as described in section 2.8. Cells were transfected for 72 hours using different reagents on HEK and HMEC-1 cells, and different siRNA concentrations with lipofectamine reagent on HMEC-1 cells to assess the dose effectiveness. Briefly, cells were washed once with PBS and lysed by the addition of cold lysis buffer described in section 2.8.2 (without addition of phosphatase inhibitor) followed by cell scraping. The cells were incubated on ice for 10 minutes followed by sonication, centrifugation and determination of protein concentration as specified in section 2.8.3. Eight µg of proteins were separated on 12% SDS gel and transferred to PVDF membrane as described in section 2.8.4 and 2.8.6, respectively. After blocking for 1 hour with 5% milk, rabbit anti-human GAPDH antibody (Santa Cruz Biotechnology) was used at 1:1000 diluted in 5% milk. HRP-conjugated anti-rabbit secondary antibody was used at 1:4000 and the membrane was developed as described in section 2.8.7. As a loading control, the membrane was washed once with PBS and mouse antibody against tubulin was added (Sigma) followed by HRP-conjugated anti-mouse secondary antibody. Figure 5.5 (B) and Figure 5.6 show western blots for HEK and HMEC-1 cells, respectively.

5.3.4.7 **Transfection of CREB siRNA**

Optimization experiments of transfection showed that lipofectamine reagent produced efficient transfection of HMEC-1 cells with low toxicity; therefore, this was used for further experiments. T-CREB knockdown was performed using CREB siRNA silencer select and assessed by western blotting as shown in Figure 5.7. The CREB siRNA and negative control siRNA was supplied in a purified and dried form (Applied Biosystems). These were reconstituted using RNase free water at 5 µM stock concentration and diluted to 2 µM working solution. 50% confluent HMEC-1 cells were trypsinised and adjusted to $2X10^5$ cells suspended in 900 µl of transfection media. To form a transfection complex containing a final concentration of 5 nM siRNA, 50 µl of HMEC-1 media without any additive containing 2.5 µl of CREB or negative control siRNA (2µM) was mixed with 50µl of the same media containing 2µl of lipofectamine per well in a 12 well plate. Accordingly, higher concentrations were adjusted. The complex was allowed to form by incubation at room temperature for 25 minutes. The complex was added to 900 µl of cell suspension in 12 well plates with gentle mixing.

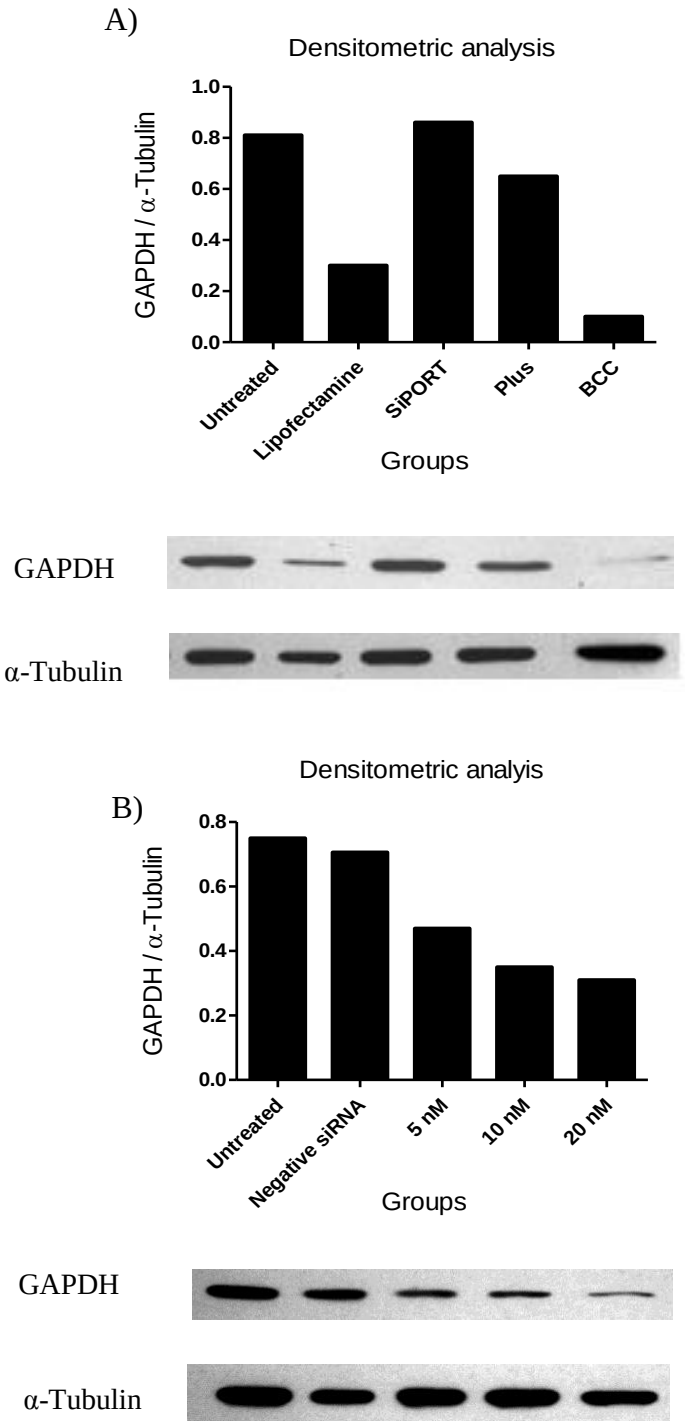


Figure 5.6: Optimization of GAPDH siRNA transfection on HMEC-1 cell line.

The cells were transfected with siRNA for GAPDH and the change at protein level was assessed by western blotting. Panel A shows the transfection with 5 nM GAPDH siRNA using different transfection reagents. Panel B shows knockdown in response to different siRNA concentration using lipofecamine. Tubulin was used as a loading control. The data are representative of two independent experiments.

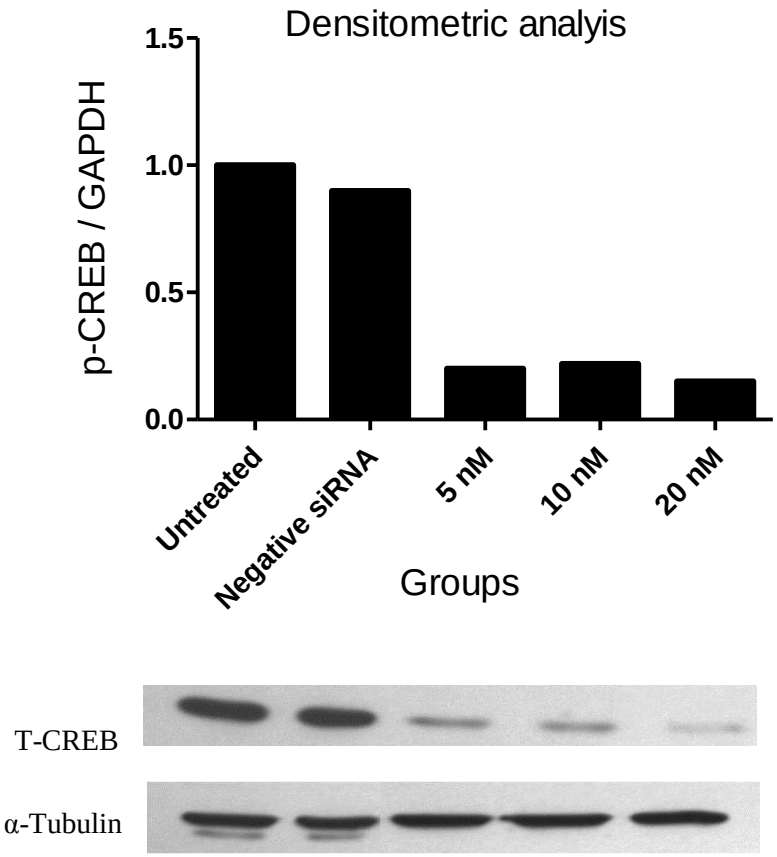


Figure 5.7: Total CREB knockdown in HMEC-1 cells using CREB-specific siRNA and lipofectamine reagent.

Cells were transfected with different concentrations of CREB siRNA (5, 10, 20 nM) or negative control siRNA at concentration of 10 nM for 72 hours before western blot analysis. The membrane was probed with T-CREB, and tubulin was used as a loading control. The data are representative of two independent experiments.

After 72 hours, the cells were lysed and assessed by western blotting as previously described in section 2.8. Mouse anti-human T-CREB and tubulin antibodies were used to validate the knockdown efficiency.

5.3.5 Preparation of $F(ab)_2$ fragment

5.3.5.1 General principle

The antibody consists of two antigen-binding fragments (Fab) and one class specific constant fragment (Fc) which are connected by a hinge region containing disulfide bridge to form a Y shape structure. The disulfide bonds in this region are accessible to different proteolytic enzymes which facilitate the formation of different antibody fragments as shown in Figure 5.8. To study the functional characteristics of each fragment separately, antibody fragmentation can be performed for this purpose. Antibody fragmentation can be achieved using reducing agents or proteases that selectively cleave the immunoglobulin molecule at a certain point into fragments in an agent-dependent manner. Commonly prepared antibody fragments include half-IgG, Fab, $F(ab')_2$ and Fc fragments. Fragments can be produced by reduction of hinge-region disulfides or by digestion with proteolytic enzymes such as papain, pepsin or ficin. In this project, the ability of W6/32 antibody in inducing leukocyte adhesion independent of Fc receptors by preparing $F(ab)_2$ fragments from W6/32 antibody was examined.

Pepsin is a nonspecific endopeptidase enzyme that is widely used for $F(ab)_2$ fragment preparation. Pepsin is active at acidic pH and irreversibly degraded at neutral or alkaline pH. Antibody digestion by this enzyme produces one $F(ab')_2$ fragment with size 110 kDa and various small peptides from the Fc portion. The resulting $F(ab')_2$ fragment consists of two Fab units connected by disulfide bonds. The degraded Fc fragment can be separated from $F(ab')_2$ fragment by dialysis, gel filtration or ion exchange chromatography as shown in Figure 5.9.

5.3.5.2 $F(ab)_2$ fragment preparation

The preparation of $F(ab)_2$ fragment was performed using a $F(ab)_2$ preparation kit (Thermo scientific, USA) according to manufacturer's instruction. The kit consists of immobilized pepsin, a protein A spin column, phosphate buffer saline, IgG elution buffer, $F(ab)_2$ digestion buffer, a desalt spin column and microcentrifuge tubes. The

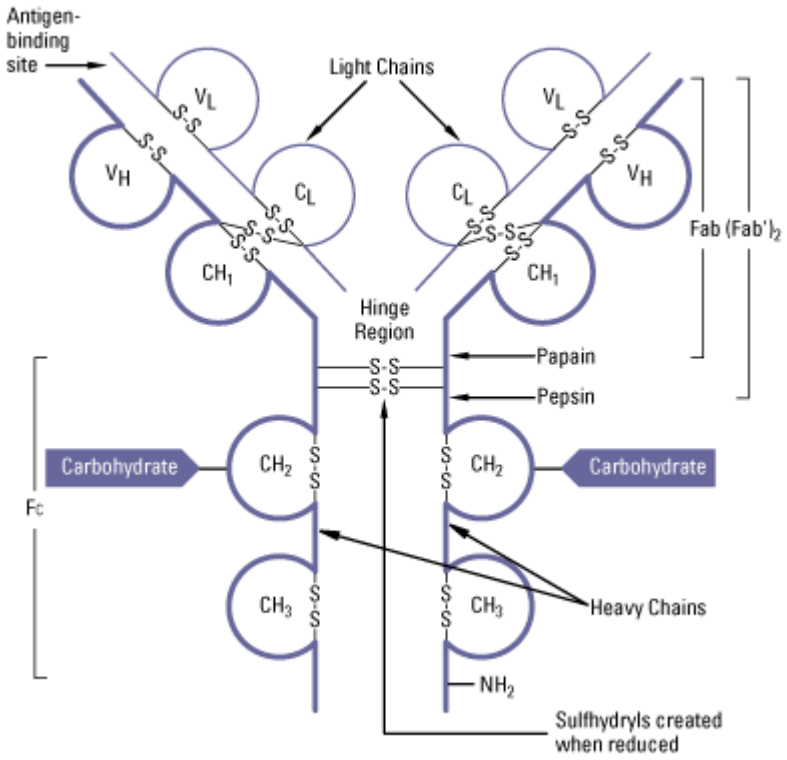


Figure 5.8: Antibody structure and enzymatic cleavage activity.

(thermo scientific, http://www.piercenet.com/browse.cfm?fldID=4E03B016-5056-8A76-4ECA-982DA6CAAC8A).

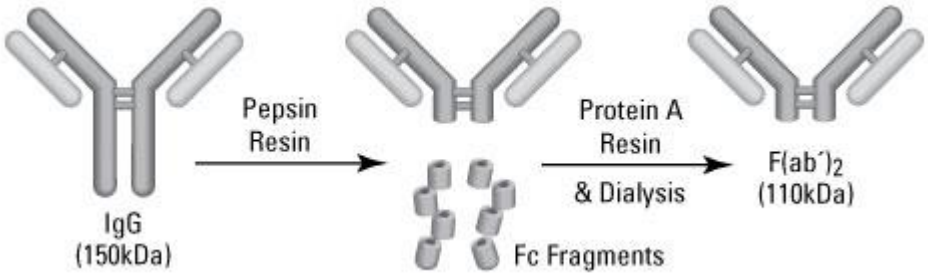


Figure 5.9: Preparation of F(ab)$_2$ fragments by pepsin treatment.

(thermo scientific, http://www.piercenet.com/browse.cfm?fldID=01010504).

immobilized pepsin was equilibrated by washing once with 0.5 ml of digestion buffer in the provided spin column at 5000 g for 1 minute. To resuspend the sample in the digestion buffer, a desalt spin column was used. The latter was centrifuged once at 1500g for 1 minute to remove the storage buffer. Subsequently, 300 µl of digestion buffer was added to the column and this was centrifuged at 1500 g for 1 minute. This step was repeated three times and the buffer was discarded after collection in a separate tube. After placement of the column in a new collection tube, 0.5 ml of 4 mg of W6/32 antibody was applied to the centre of the column and this was centrifuged at 1500 g for 2 minutes. The eluate contains the antibody suspended in the digestion buffer after the exchange step. The antibody was then added to the immobilized pepsin and incubated at 37^{o}C on a shaker for 3 hours. After incubation, the spin column was centrifuged at 5000 g for 1 minutes and the sample was collected into a microcentrifuge tube. To ensure the elution of all digested antibody, the spin column was washed once with PBS. To separate the undigested IgG, the antibody was then passed through protein A column.

The protein A column was placed in a collection tube and centrifuged at 1000 g for 1 minute to remove the storage buffer. To equilibrate the column, 400 µl of PBS was added followed by centrifugation at 1000 g for 1 minute. The digested antibody was applied to the column and mixed on the shaker for 10 minutes. The column was placed into a clean tube and centrifuged at 1000 g for 1 minute. The fraction collected contained $F(ab)_2$ and Fc fragments. The column was washed twice with PBS to ensure the complete recovery of the fragments. To remove the Fc fragment, the digested antibody was passed through spin column with a cut-off of 50 kDa. The recovery of $F(ab)_2$ fragments was confirmed by SDS-PAGE. Five µg of antibody was separated on 10% SDS-PAGE under non reducing condition, without the addition of β-mercaptoethanol followed by staining with comassie stain as described in section 2.8.4 and 2.8.5, respectively. Figure 5.10 shows the comassie staining of the gel. Figure 5.11 shows the binding of $f(ab)_2$ fragments at 12 µg/ml to HMEC-1 cells. This was assessed by flow cytometry using anti-mouse IgG FITC conjugated antibody.

5.3.5.3 **Flow cytometry analysis of $F(ab')_2$ fragment**

To assess the ability of $F(ab')_2$ fragments to bind and activate endothelial cells, HMEC-1 cells were stained and stimulated with $F(ab')_2$ fragments. Adherent cells were detached using cell dissociation buffer and $2X10^5$ cells were incubated with 12 µg/ml of

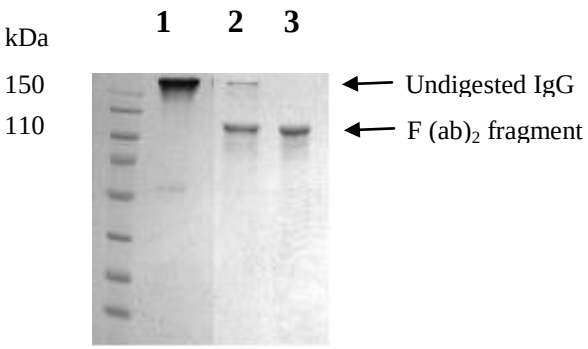


Figure 5.10：The preparation of F(ab)2 fragments from W6/32 antibody.

W6/32 antibody was digested by pepsin treatment and the product was run on 10% SDS-PAGE under non-reducing condition. Lane 1 shows undigested antibody, Lane 2 shows digested antibody before protein A purification and Lane 3 shows purified $F(ab)_2$ fragments.

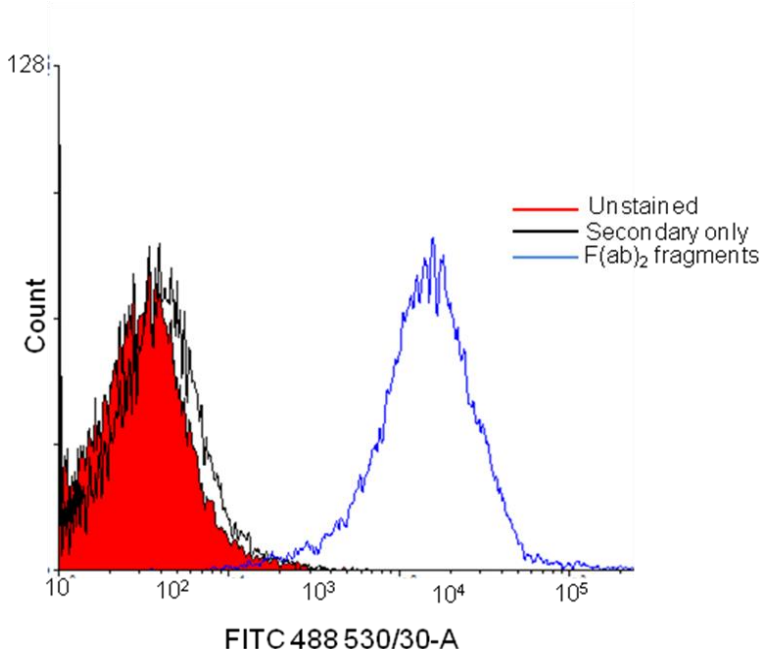


Figure 5.11：Binding of F(ab)2 fragments to HMEC-1 cells assessed by flow cytometry.

HMEC-1 cells were stained with 12 μg/ml of $F(ab)_2$ fragments followed by anti-mouse FITC conjugated antibodies. Cells stained with secondary antibody only were used to assess the background staining.

these fragments for 20 minutes at 4^{o}C. After washing with 2% FBS/PBS, cells were stained with anti-mouse FITC conjugated antibody. The cells were incubated with the secondary antibody for 20 minutes at 4^{o}C. After washing steps, cells were resuspended in 2% FBS/PBS before analysis by flow cytometry. For the activation experiments, adherent cells were incubated with 12 μg/ml of $F(ab')_2$ fragments overnight before analyzing the expression of adhesion molecules. Cells were detached as previously described and $1X10^5$ cells were stained with PE-conjugated mouse anti-human ICAM-1 and VCAM-1 antibody. Stained cells were washed twice with FBS/PBS and analyzed by flow cytometry.

5.3.6 Chemotaxis assay

5.3.6.1 General principle- the Boyden chamber

This assay is used to evaluate the potential of chemotactic agents to induce leukocyte migration, modelling *in vivo* cell migration. The *in vitro* cell migration assay can be performed using a transwell chamber as described by Boyden in 1962 (Boyden, 1962). In this assay, a transwell chamber containing a polycarbonate multipore filter with different pore sizes is used. The size of the pore is dependent on the type of the cells to be investigated but generally it ranges from 3-8 μm for leukocyte migration. The cells to be examined are placed on the upper side of the chamber while the chemoattractant solution is placed in the lower side. During incubation, cells migrate in response to a concentration gradient from the lower chemokine concentration to the higher concentration. Migrant cells are then stained and counted. Figure 5.12 shows a diagram of a transwell chamber.

5.3.6.2 Method for chemotaxis assay

HMEC-1 cells grown in 6 wells plate were stimulated with 12 μg/ml of either W6/32 antibody or isotype control for 72 hours. The media was collected and used to perform chemotaxis assays. Conditioned media was passed through a 100 kDa cutoff concentrator tube to remove any remaining W6/32 antibody. Human THP-1 monocytic cells, expressing the corresponding receptors for the chemokines secreted by HMEC-1 cells, were used in this assay. The ability of THP-1 cells to migrate in response to chemokine was examined by this method using different concentrations of recombinant human (rh) CCL5 (R&D systems) ranging from 1-200 ng/ml as shown in Figure 5.13. 0.5 million THP-1 cells were resuspended in 200 μl of serum free medium containing

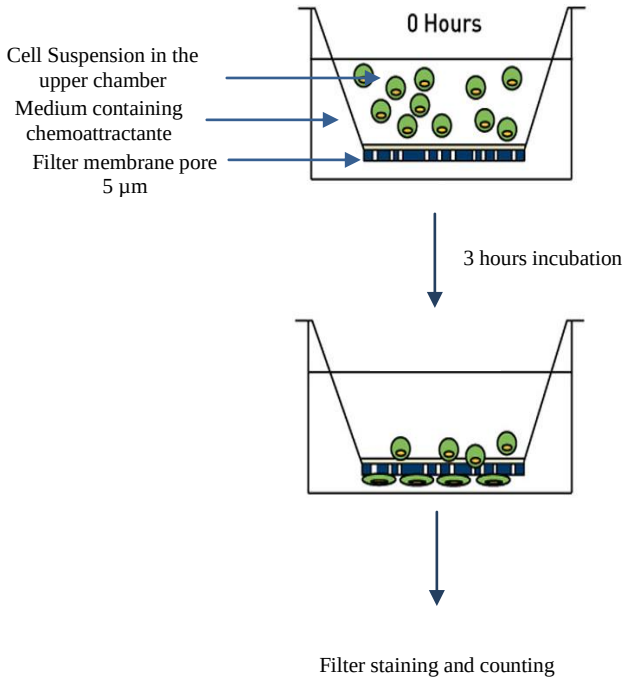


Figure 5.12: Transwell chemotaxis chamber.

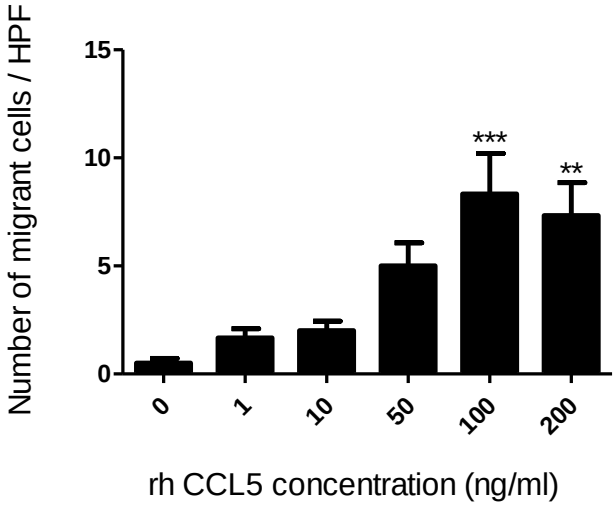


Figure 5.13: Chemotaxis assay using rhCCL5 and human THP-1 monocytic cells.

THP-1 cells were starved for 1 hour in 1% BSA media before placing them in chemotaxis chamber containing different concentrations of rhCCL5 ranging from 1-200 ng/ml for 3 hours at 37^0C. Filters were stained and migrant cells were counted. The data are representative of two independent experiments.

1% BSA for 1 hour. Simultaneously, a 24 wells plate was blocked with 1ml of 1% BSA for 1 hour at room temperature to prevent non-specific binding of the chemokine. 600 µl of conditioned media from both treated groups was placed in the wells and 200 µl of THP-1 cells were placed in the upper chamber with 5 µm pore size. The plate was incubated at 37^0C for 3 hours. The chemotaxis inserts were removed and non-migrant cells were removed from the filter by cotton swab. The filter was fixed in absolute methanol at -20^0C overnight. Filters were then washed with tap water followed by the addition of 1.5 ml of hematoxylin stain. After 10 minutes incubation at room temperature, the stain was removed and filters washed with tap water. The filters were then immersed sequentially in 50%, 75%, 90% and 100% ethanol for 10 minutes each. Filters were then mountened on the slides using DPX mountant (Sigma) and covered with cover slips. Migrant cells were counted by light microscopy. The cells in at least 6 fields from each filter were counted. Migration of cells in the absence of chemokine was used as a negative control.

5.3.7 *In vitro* flow based adhesion assays

5.3.7.1 General principle-Cellix platform

Cellix platform is a novel microfluidic system used to examine cell-cell interactions and the adhesion of leukocytes to recombinant proteins or monolayers of endothelial cells under physiological *in vitro* flow conditions. The system can be used to analyse cell morphology and track different steps of leukocyte migration including rolling and adhesion in a manner that is more physiological than the static chemotaxis assays. Different types of fluids can be used with the cellix plateform such as media and blood. Therefore, the system can be used to examine platelet adhesion, aggregation and thrombi formation by using whole blood samples. The efficiency of a particular drug can be also tested by this system in order to manage drug toxicity and development.

The system consists of bright field and fluorescence microscopy with a motorised stage, microscope cage incubator, fluorescence and temperature controllers and, importantly, an adjustable syringe pump that is all controlled by Venaflux software, as shown in Figure 5.14. The syringe pump accurately maintains the flow rates at a desired fluid ranging from 5 picoliter/minute to 10 microliter/minute producing a wide range of shear stresses, reaching 450 dyne/cm^2, depending on the syringe type. These characteristics facilitate the study of cell-cell interactions in an equivalent manner to that which occurs

in the blood vessels *in vivo*. The vena8 biochips are designed to mimic the blood vessels. Each chip consists of 8 channels which can be coated with different recombinant proteins for adhesion assays. Each channel is 20 mm length and 400 μm in width plus 100 μm in depth, as shown in Figure 5.15 (A). VenaEC biochips are 20 mm in length, 600 μm width and 200 μm depth. For this type of biochip, small square substrate is used to seed and culture endothelial cells after sterilization with UV light. Two microcapillary channels are then formed by placing a chip on the top of the substrate and are sealed via a customised frame as shown in Figure 5.15 (B).

5.3.7.2 **Flow based adhesion assay using recombinant human adhesion molecule**

Recombinant human VCAM-1/Fc supplied as a lyophilised powder (R&D system) was reconstituted in accordance with the manufacturer's instruction at 100 μg/ml stock solution. Ten μl of VCAM-1/Fc was injected into the desired channels of Vena8 chip and used at concentration 10 μg/ml diluted using carbonate buffer consisting of $Na_2CO_3/NaHCO_3$ buffer at pH 9.6. The chip was kept at 4^0C overnight in a humidified sealed box to prevent it from drying. The chip was then blocked with 1% BSA for 30 minutes at room temperature. Chemokines were either immobilized on the chip at 500 ng/ml or added directly at different concentrations to the cells before infusion onto the chip. The effect of immobilised rhCCL5 was examined in inducing THP-1 cells adhesion to VCAM-1/Fc. Furthermore, mixing of rhCCL5 and rhCXCL12 with cells in suspension was also used to examine the adhesion of PBMC to VCAM-1/Fc.

THP-1 cells and PBMC were centrifuged and adjusted to $2X10^6$ cells/ ml in serum free medium containing 1% BSA for 1 hour before the assay. Negative controls included the adhesion of cells to BSA-coated channel and the adhesion of the unstimulated cells to VCAM-1/Fc coated channel in the absence of chemokine. The system was washed twice with filtered water followed by 70% ethanol. The system was then primed with RPMI medium without supplements and used as a flow buffer. The channels were washed once using the system pump by dispensing 40 μl solution at 40 dyne/cm^2 to remove the unbound proteins. The assay was performed by infusing the cells into channels of chip at 10 dynes/cm^2 for 10 seconds followed by 0.5 dynes/cm^2 for 5 minutes where the adhesion was assessed. Images were captured at the last minute and adhered cells were counted in at least 5 fields using DuoCell analysis software.

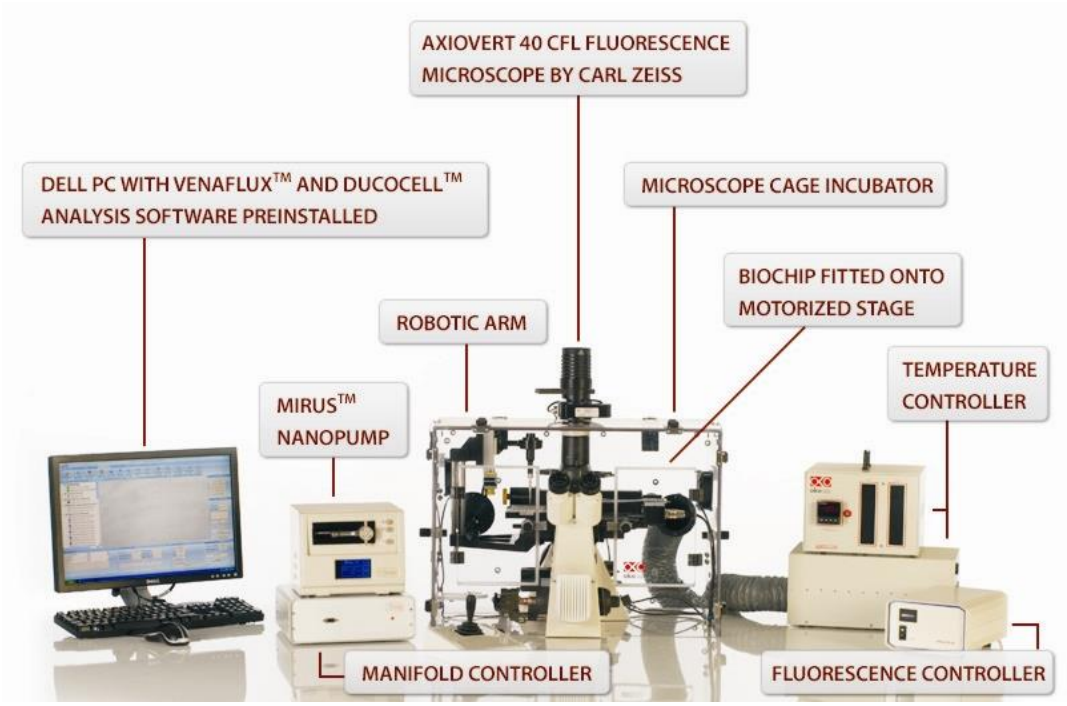


Figure 5.14: Cellix multifluidic plateform.

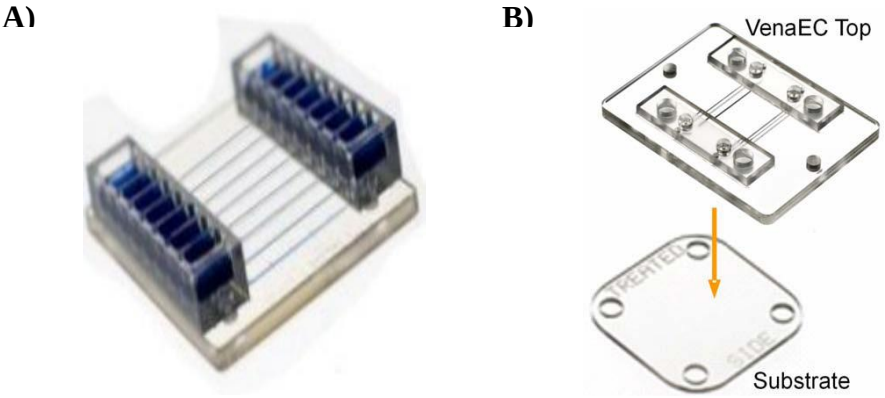


Figure 5.15: Cellix biochips.

Panel A shows Vena8 biochip which consists of 8 channels that can be coated with recombinant proteins. Panel B shows VenaEC biochip in which endothelial cells are grown on the substrate until confluency followed by chip assembly.

5.3.7.3 **Flow based adhesion assays using a monolayer of endothelial cells**

Cellix substrates of VenaEC chips were kept in 6 wells plate and sterilised for one hour in UV light before use. Substrates were precoated with 50 μg/ml of fibronectin (Sigma) for 30 minutes at room temperature. HMEC-1 endothelial cells were then sub-cultured on VenaEC substrate and incubated until confluency at 37^0C. The endothelial cells were then either treated overnight with 10 ng/ml of TNF-α, 12 μg/ml of W6/32 antibody or $F(ab)_2$ fragments purified from W6/32 antibody. Where specified, rhCCL5 and rhCXCL12 chemokine at concentration 100 ng/ml or conditioned media from W6/32 or isotype control were immobilised on endothelial cells for 20 minutes. The system and the cells were prepared for the assay as described in the previous section. Monolayer endothelial cells were washed once by dispensing 40 μl of the media at 10 dynes/cm^2. THP-1 and MOLT-16 cells were infused on the endothelial layer at 0.5 dyne/cm^2 for 5 minutes. Adhered cells seen in at least 5 fields were counted and plotted.

5.4 Results

5.4.1 Effect of TNF-α stimulation on the expression of adhesion molecules on HMEC-1 cells

Adhesion molecules are cell surface proteins that mediate the adhesion of circulating leukocytes enhancing their migration to inflammatory sites. Under normal condition endothelial cells express a limited number of these molecules, which are up-regulated upon exposure to inflammatory cytokines. To address the ability of HMEC-1 cells to express adhesion molecules, cells were stimulated with TNF-α and examined for the up-regulation of VCAM-1, ICAM-1 and E-selectin by flow cytometry. Cells initially were stimulated with (20 ng/ml) of TNF-α for various time points. Figure 5.16 shows that under resting condition HMEC-1 cells showed negligible expression of VCAM-1 molecules which was up-regulated 20 fold in response to stimulation with 20 ng/ml of TNF-α for 16 hours. VCAM-1 expression remained significantly high up to 72 hours after stimulation. The expression of these molecules was dependent on the concentration of TNF-α. Figure 5.17 shows that treatment of endothelial cells with TNF-α for 24 hours at concentrations 0.1, 0.5, 1, 5, 10 and 20 ng/ml increased VCAM-1 expression by 1, 3, 6, 8, 10 and 15 fold, respectively.

In contrast, resting HMEC-1 cells expressed a detectable basal level of ICAM-1 antigen which was increased 40 fold in response to stimulation with 20 ng/ml of TNF-α for 16 hours as shown in Figure 5.18. The expression of this adhesion molecule also showed dependency on the TNF-α concentration with a 10 to 50 fold increase in response to the treatment with TNF-α ranging from 0.1 to 20 ng/ml, respectively, as shown in Figure 5.19.

Figure 5.20 shows that resting HMEC-1 cells expressed E-selectin at a very low level but this was significantly up-regulated after stimulation with 20 ng/ml of TNF-α for 16 and 24 hours compared to untreated group. The expression of this antigen was significantly increased after 48 and 72 hours of stimulation compared to 24 hours. All antibodies used were mouse anti-human IgG1 PE-conjugated, therefore, isotype control mouse IgG1 antibody was used in every experiment.

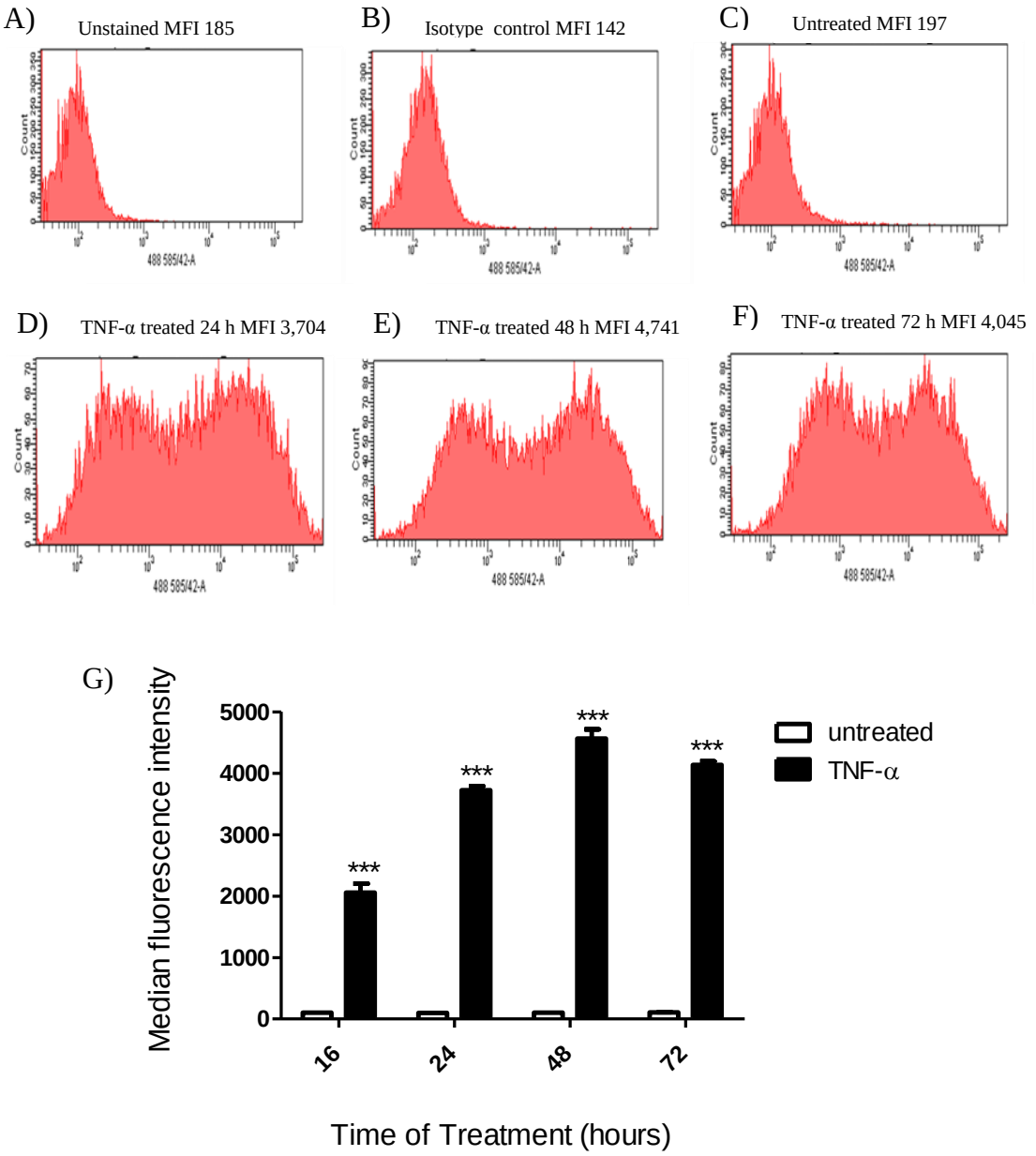


Figure 5.16: Expression of VCAM-1 molecules by endothelial cells following stimulation with TNF-α at different time points.

HMEC-1 cells treated with 20 ng/ml of TNF-α at different time points were stained with PE-conjugated mouse anti-human VCAM-1 antibody and were analysed by flow cytometry. A, B, C, D, E and F represent unstained, isotype control, untreated, 24, 48 and 72 hours treated groups, respectively. Median fluorescence intensity was plotted in bar chart, G. The data was analysed by two-way ANOVA followed by Bonferroni test. *** $p<0.001$. The data are representative of three independent experiments.

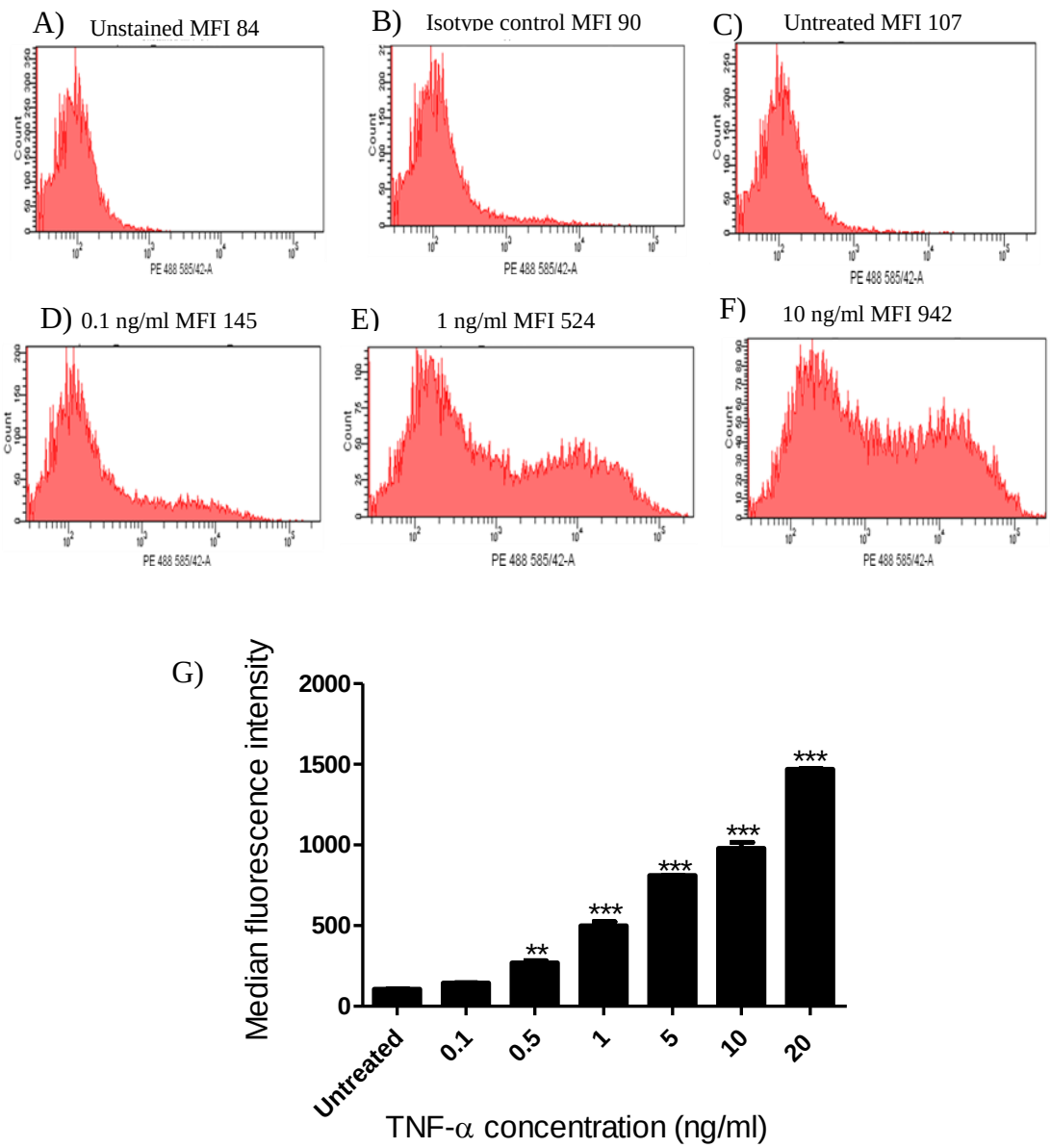


Figure 5.17: Effect of different concentrations of TNF-α on VCAM-1 expression by endothelial cells.

HMEC-1 cells treated with different concentrations of TNF-α for 24 hours were stained with PE-conjugated mouse anti-human VCAM-1 antibody before flow cytometric analysis. A, B, C, D, E and F represent unstained, isotype control, untreated, 0.1 ng/ml, 1 ng/ml and 10 ng/ml treated groups, respectively. Panel G is a bar chart of median fluorescence intensities. The data was analyzed by one-way ANOVA followed by Bonferroni test. ** $p<0.01$ and *** $p< 0.001$. The data are representative of three independent experiments.

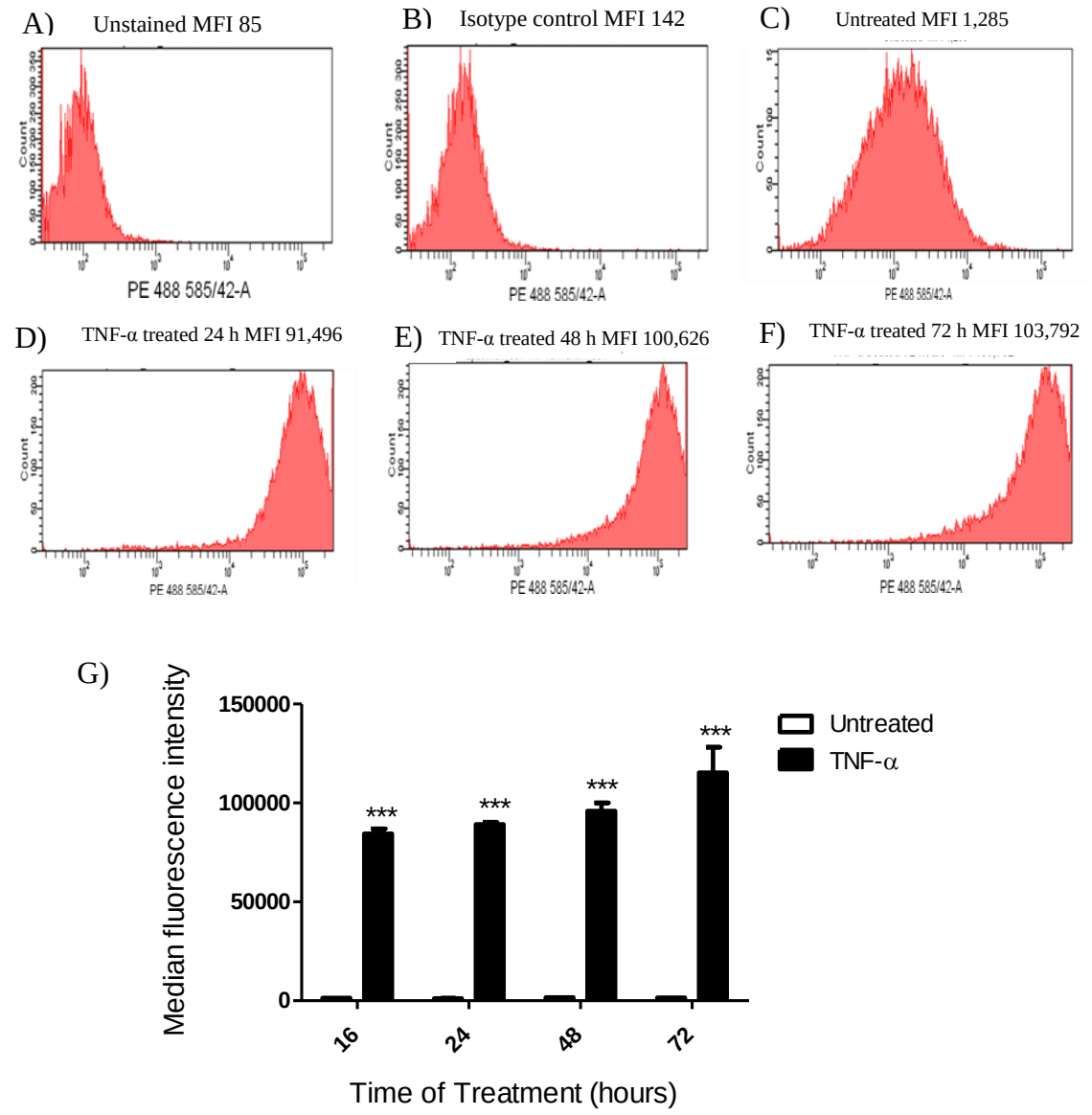


Figure 5.18: Expression of ICAM-1 by endothelial cells following stimulation with TNF-α at different time points.

HMEC-1 cells treated with 20 ng/ml of TNF-α at different time points were stained with PE-conjugated mouse anti-human ICAM-1 antibody and analyzed by flow cytometry. A, B, C, D, E and F represent unstained, isotype control, untreated, 24, 48 and 72 hours treated groups, respectively. Panel G shows a bar graph of the median fluorescence intensity of each group. The data were analyzed by two way ANOVA followed by Bonferroni test. *** $p<0.001$. The data are representative of three independent experiments.

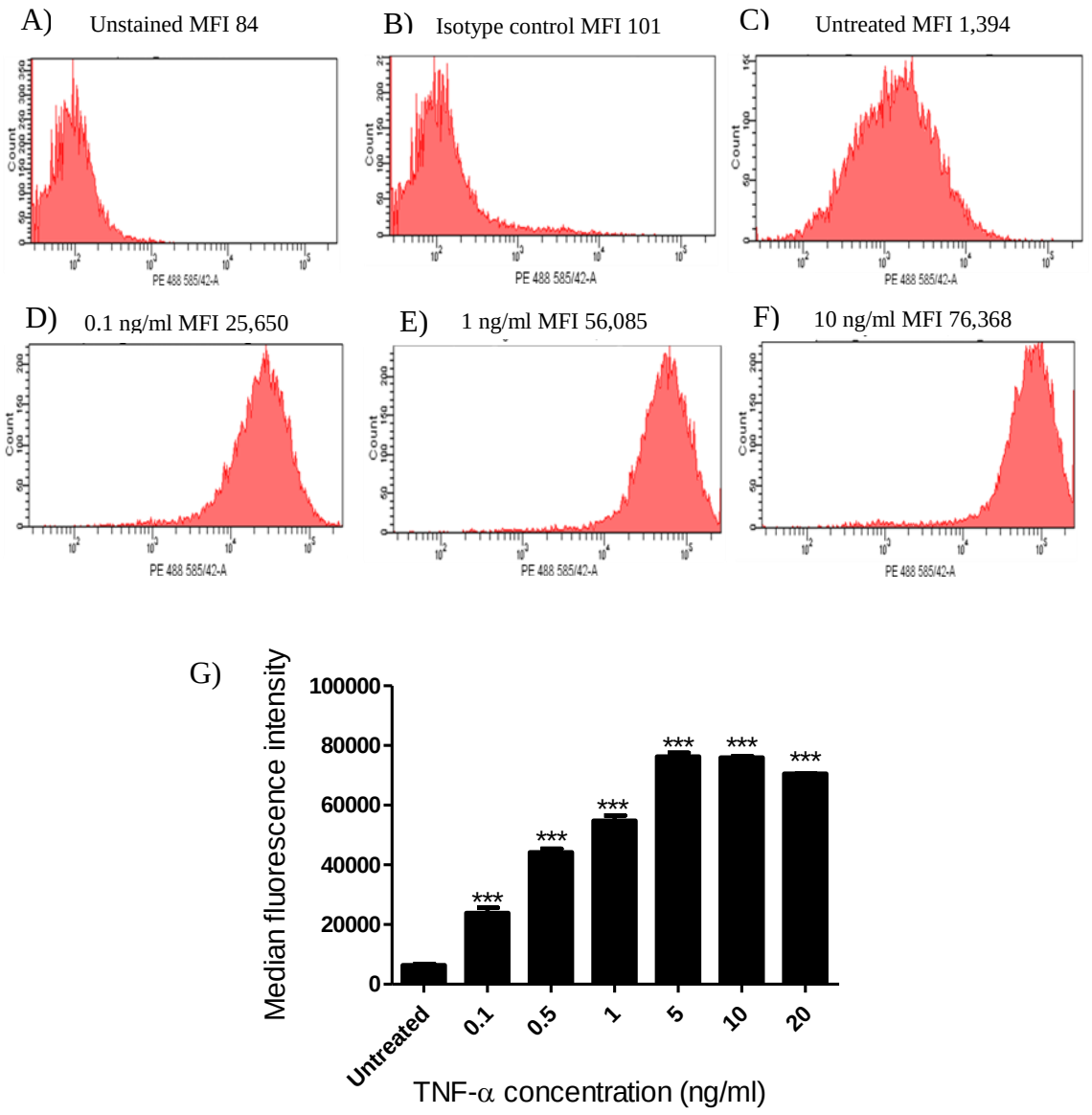


Figure 5.19: Effect of different concentrations of TNF-α on ICAM-1 expression by endothelial cells.

HMEC-1 cells treated with different concentrations of TNF-α for 24 hours were stained with PE-conjugated ICAM-1 antibody and analyzed by flow cytometry. A, B, C, D, E and F represent unstained, isotype control, untreated, 0.1 ng/ml, 1 ng/ml and 10 ng/ml treated groups, respectively. The bar chart represents median fluorescence intensity, G. The data were analyzed by one way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representative of three independent experiments.

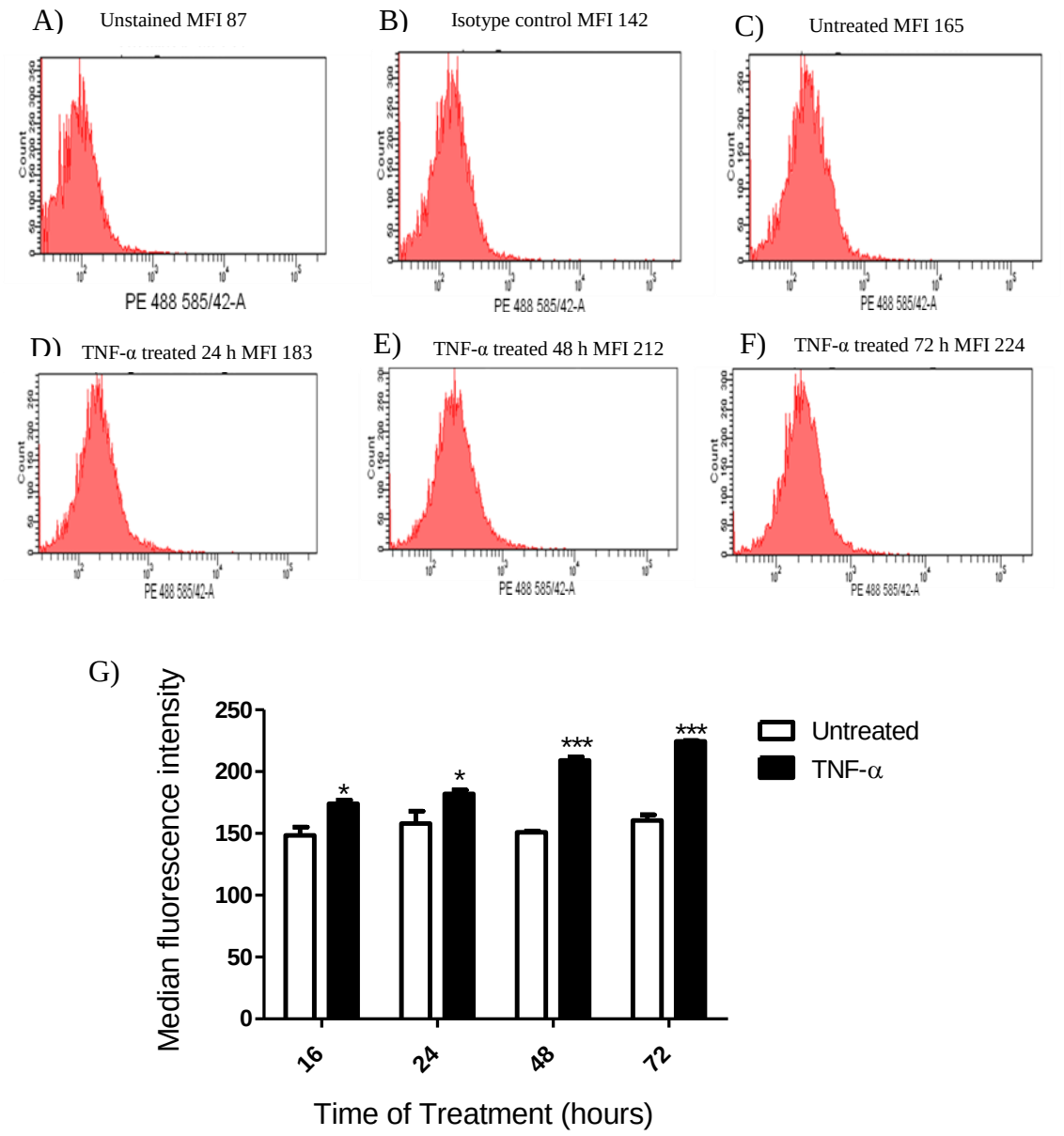


Figure 5.20: Expression of E-selectin on endothelial cells by TNF-α at different time points.

HMEC-1 cells treated with 20 ng/ml of TNF-α at different time points were stained with PE conjugated antibody against E-selectin molecules and analyzed by flow cytometry. A, B, C, D, E, and F represent unstained, isotype control, untreated, 24, 48 and 72 hours treated group. Bar chart of the median fluorescence intensity is displayed on panel G. The data were analyzed by two way ANOVA followed by Bonferroni test. * $p<0.05$ and *** $p<0.001$. The data are representative of three independent experiments.

5.4.2 Effect of W6/32 antibody on the expression of adhesion molecules on endothelial cells

To investigate the ability of HLA class I antibody to induce the expression of adhesion molecules, HMEC-1 endothelial cells were stimulated with a saturating concentration of W6/32 antibody or isotype control for various time points and examined for the expression of VCAM-1, ICAM-1 and E-selectin by flow cytometry. Stimulation with 12µg/ml of W6/32 antibody induced a significant expression of VCAM-1 and ICAM-1 on HMEC-1 cells in a time dependent manner as shown in Figure 5.21 and Figure 5.22, respectively. The expression of VCAM-1 peaked at 12 hours by 3 fold, while the expression of ICAM-1 peaked at 8-12 hours by 30 fold compared to isotype-treated group. Treatment with HLA class I antibody for 24-72 hours gradually decreased the expression of both antigens but remained statistically significant compared to untreated or isotype-treated groups. Figure 5.23 shows that the un-stimulated cells express E-selectin at very low level and the stimulation of HMEC-1 cells with HLA class I antibody had a negligible effect on the expression of this antigen.

It was previously reported that large vessel endothelial cells treated with sub-saturating concentrations of HLA class I antibody had a reduction in the expression of adhesion molecules in response to stimulation with inflammatory cytokines (Narayanan *et al.*, 2006). In this study, the effect of pre-exposure of the microvascular endothelial cells to different concentrations of HLA class I antibody on up-regulation of adhesion molecules induced by TNF-α stimulation was examined. HMEC-1 cells were treated with saturating and sub-saturating concentrations of W6/32 antibody or isotype control for 48 hours followed by stimulation with 10 ng/ml of TNF-α for 24 hours. The cells were then analysed for the expression of VCAM-1 and ICAM-1 by flow cytometry. Figure 5.24 and Figure 5.25 show that the pre-exposure to W6/32 antibody for 48 hours did not abrogate the ability of TNF-α to induce VCAM-1 or ICAM-1 expression, respectively.

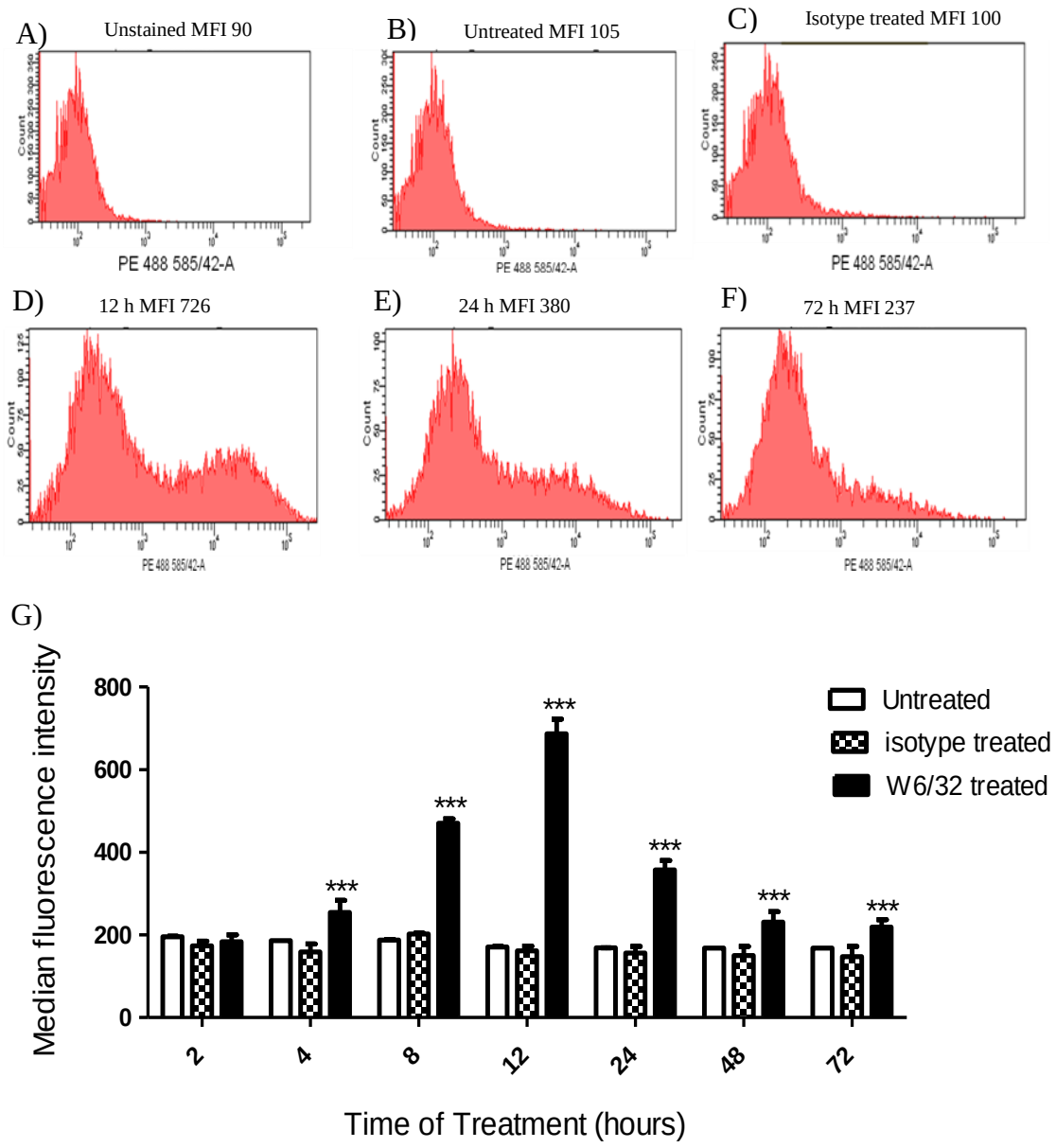


Figure 5.21: Expression of VCAM-1 on endothelial cells following stimulation with HLA class I antibody.

HMEC-1 cells were left untreated or treated with saturating concentration 12µg/ml of W6/32 antibody or isotype control at various time points followed by flow cytometric analysis. A, B, C, D, E and F show the unstained, untreated, isotype treated group, 12, 24 and 72 hours of W6/32 treated groups. Bar chart of median fluorescence intensity is represented in G. The data were analyzed by two-way ANOVA followed by Bonferroni test compared to isotype-treated group. *** $p<0.001$. The data are representative of three independent experiments.

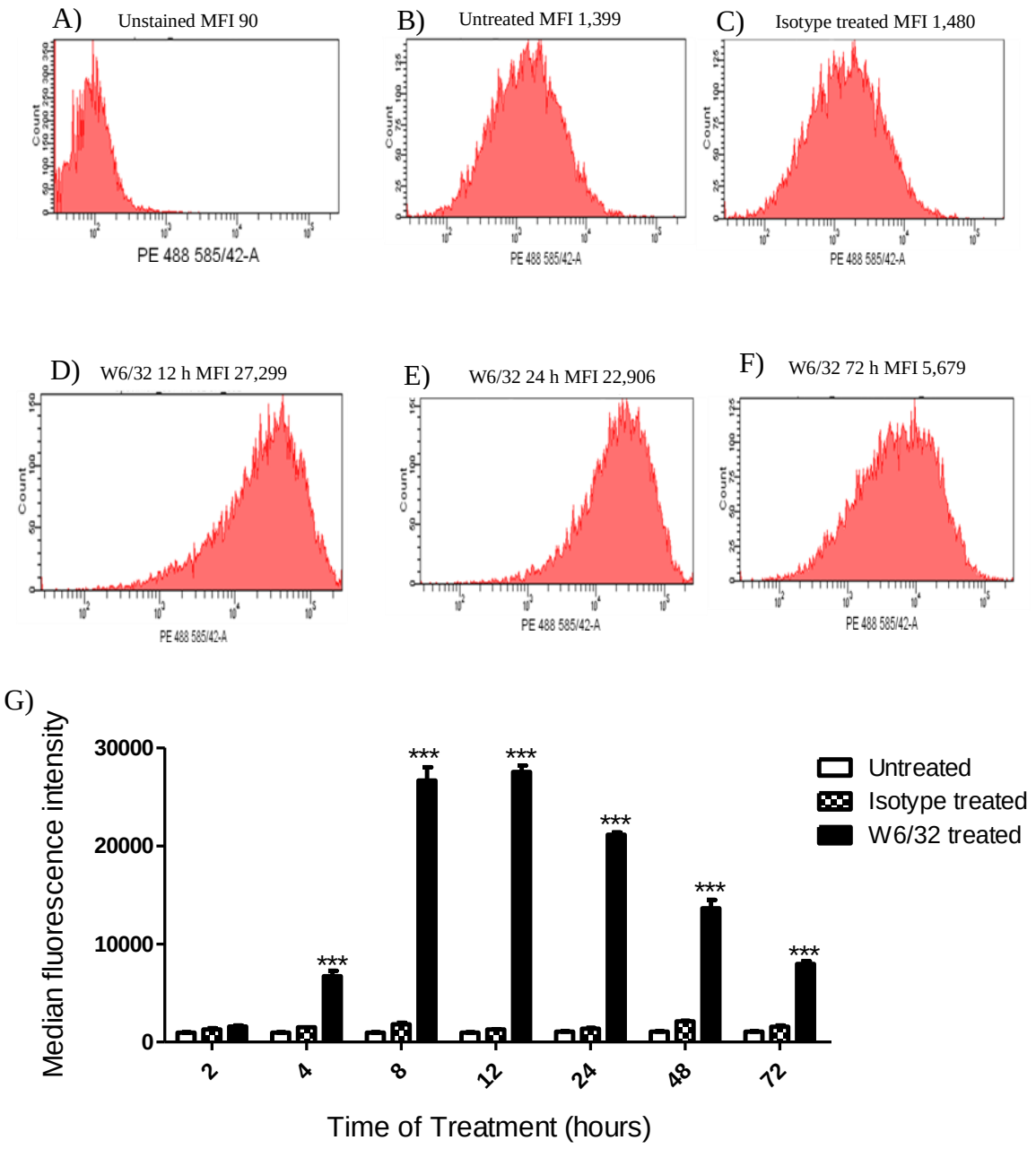


Figure 5.22: Expression of ICAM-1 on endothelial cells following stimulation with HLA class I antibody.

HMEC-1 cells were left untreated or treated with saturating concentration 12 µg/ml of W6/32 antibody or isotype control for various time points before analysed using flow cytometry. A, B, C, D, E and F represent unstained, untreated, isotype treated, 12, 24 and 72 hours treated groups. Median fluorescence intensity is shown in bar chart, G. The data were analyzed by two-way ANOVA followed by Bonferroni test compared to isotype treated group. *** $p<0.001$. The data are representative of three independent experiments.

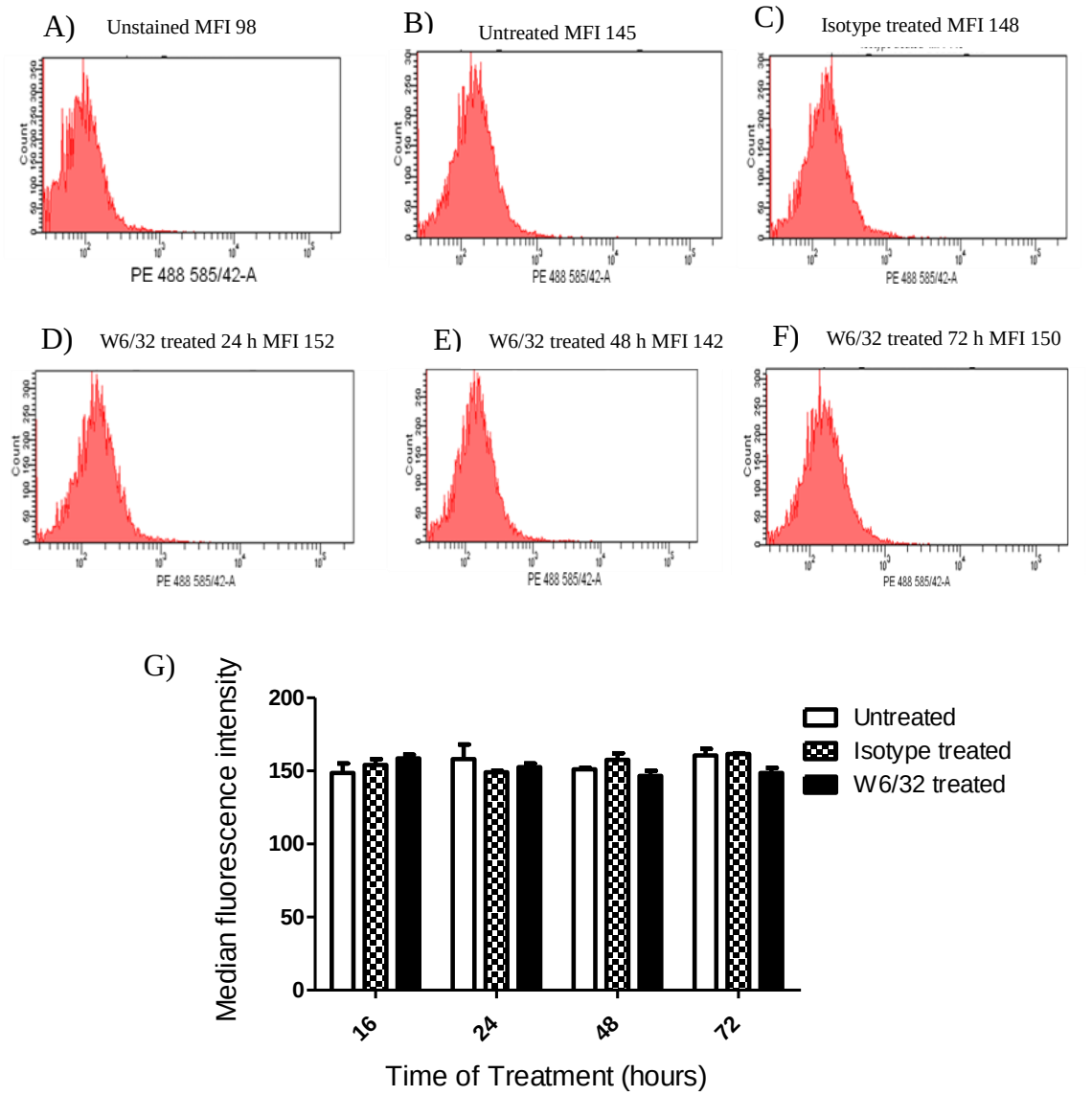


Figure 5.23: Expression of E-selectin by endothelial cells following stimulation with HLA class I antibody.

HMEC-1 cells were left untreated or treated with saturating concentration (12µg/ml) of W6/32 antibody or isotype control at various time points followed by staining with mouse anti-human E-selectin antibody. The cells were analysed by flow cytometry. A, B, C, D, E and F represent unstained, untreated, isotype treated, 12, 24 and 72 hours treated groups. Bar chart, G, represents median fluorescence intensity. Statistical analysis was performed using two-way ANOVA followed by Bonferroni test compared to isotype control. The data are representative of three independent experiments.

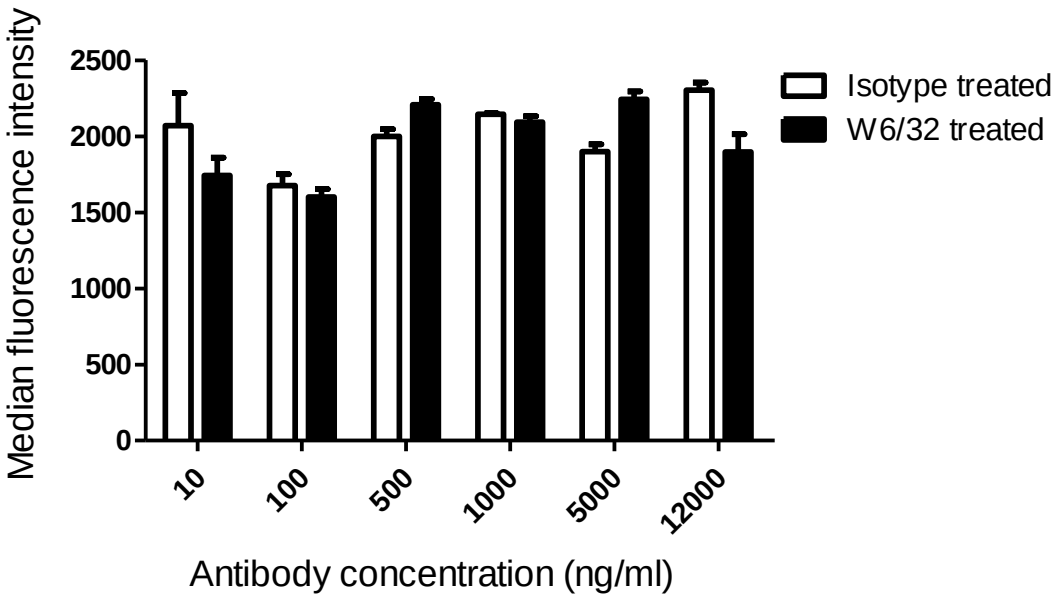


Figure 5.24: Effect of the pre-exposure to HLA class I antibody on TNF-α-induced VCAM-1 expression by endothelial cells.

HMEC-1 cells were treated with different concentrations of W6/32 antibody for 48 hours followed by treatment with TNF-α (10 ng/ml) for 24 hours. Cells were washed and stained with PE-conjugated mouse anti-human VCAM-1 antibody. Statistical analysis was performed by two-way ANOVA followed by Bonferroni test. The data are representative of two independent experiments.

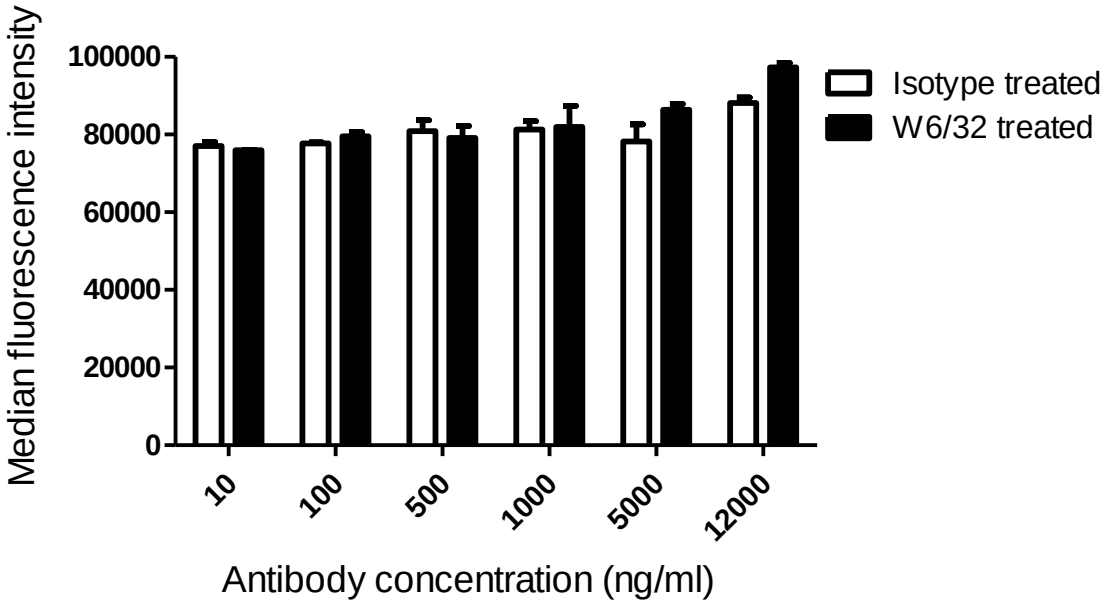


Figure 5.25: Effect of the pre-exposure to HLA class I antibody on TNF-α-induced ICAM-1 expression by endothelial cells.

HMEC-1 cells were treated with different concentrations of W6/32 antibodies for 48 hours followed by treatment with TNF-α (10 ng/ml) for 24 hours. Cells were washed and stained with PE-conjugated mouse anti-human ICAM-1 antibody. Statistical analysis was performed by two-way ANOVA followed by Bonferroni test. The data are representative of two independent experiments.

5.4.3 Effect of the PI3K/Akt pathway on the expression of adhesion molecules induced by W6/32 antibody

To examine the effect of the PI3K/Akt pathway on the increase in the expression of endothelial cell surface adhesion molecules in response to HLA class I antibody, PI3K/Akt pathway inhibition was carried out. The examination of this pathway in particular stems from the involvement of the Akt in the activation of a wide range of other pathways involved in regulation of the expression of different proteins. The Akt pathway inhibitor LY294002 was used at concentration 50 μM to inhibit this pathway 30 minutes before stimulation with a saturating concentration of W6/32 antibody for 8 hours. The activity of this inhibitor was validated as described in section 4.4.4. Before analysis of treated cells for the expression of VCAM-1 and ICAM-1 antigens the cell viability was assessed using trypan blue staining. The inhibition of PI3K/Akt pathway significantly abrogated the expression of both VCAM-1 and ICAM-1 ($P<0.01$) as shown in Figure 5.26 and Figure 5.27, respectively. The contribution of the PKA pathway in regulation of the expression of these antigens was also assessed using H-89 inhibitor. The inhibition of this pathway did not induce a significant reduction in the expression of either of the antigens (data not shown).

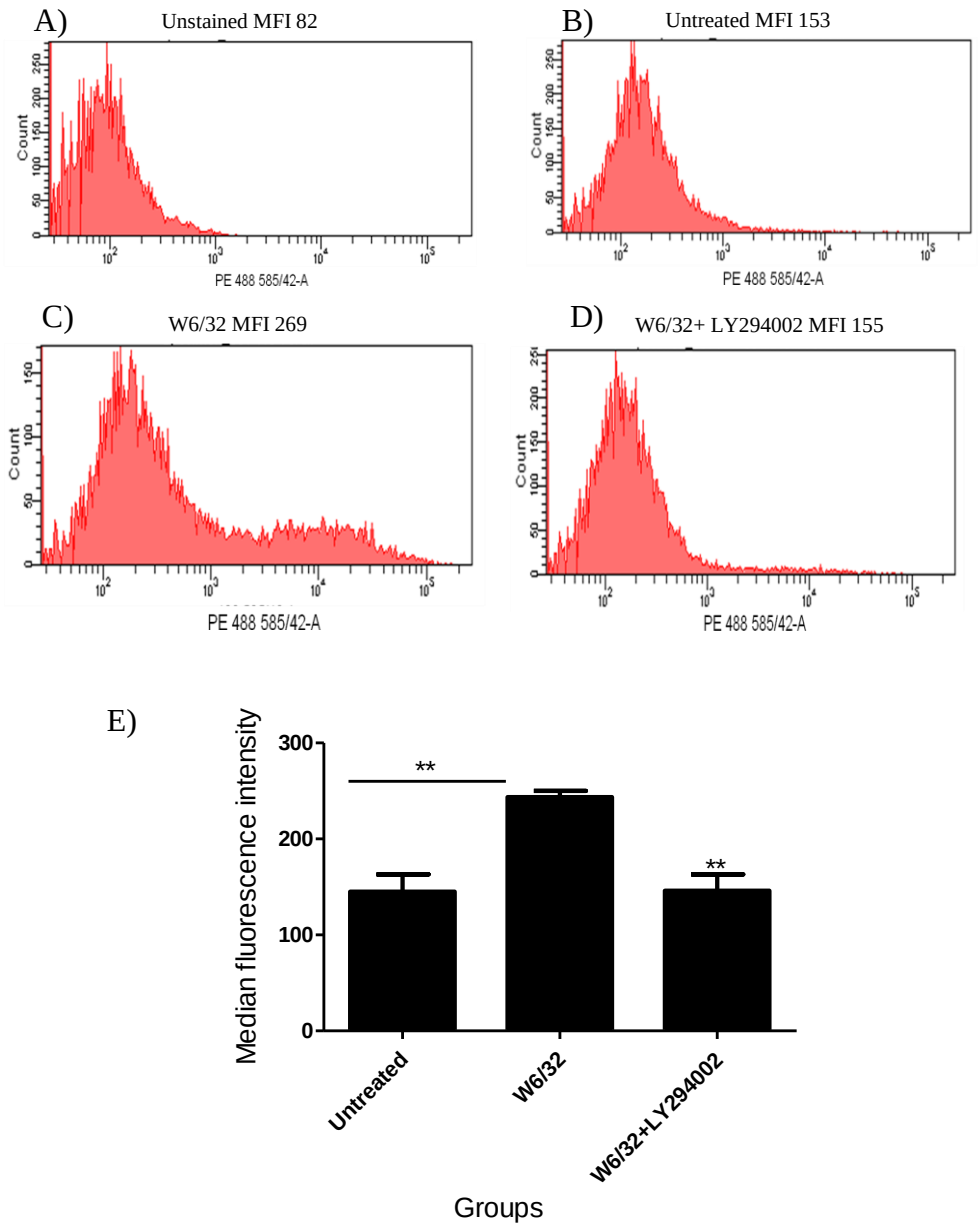


Figure 5.26: Effect of the PI3K/Akt pathway on VCAM-1 expression induced by HLA class I antibody.

Adherent HMEC-1 cells were incubated with 50 µM of LY294002 inhibitor 30 minutes before stimulation with saturating concentration of W6/32 antibody (12 µg/ml) for 8 hours. Cells were analysed by flow cytometry after staining with PE-conjugated mouse anti-human VCAM-1 antibody. A, B, C, D and E represent unstained, untreated, W6/32, W6/32+LY294002 treated cells and a bar chart of median fluorescence intensity. The data were analyzed by one-way-ANOVA followed by Bonferroni test. ** $p<0.01$ compared to W6/32 treated group. The data are representative of three independent experiments.

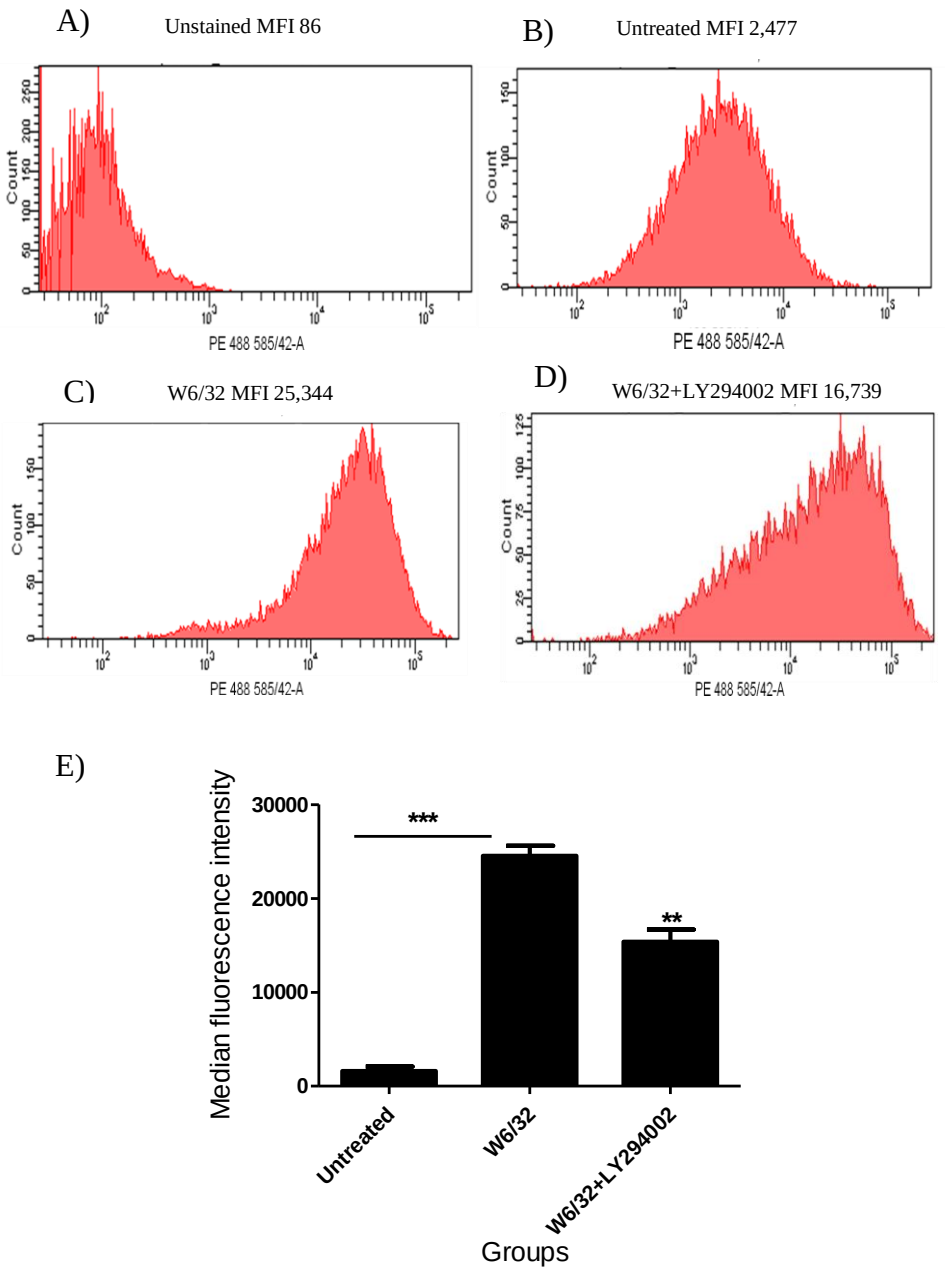


Figure 5.27: Effect of the PI3K/Akt pathway on endothelial ICAM-1 expression induced by HLA class I antibody.

Adherent HMEC-1 cells were incubated with 50 µM of LY294002 inhibitor 30 minutes before stimulation with saturating concentration of W6/32 antibody (12 µg/ml) for 8 hours. Cells were analyzed by flow cytometry after staining with PE-conjugated anti-human ICAM-1 antibody. A, B, C, D and E represent unstained, untreated, W6/32, W6/32+LY294002 treated cells and median fluorescence intensity bar chart. The data were analyzed by one-way ANOVA followed by Bonferroni test. ** $p<0.01$ and *** $p<0.001$. The data are representative of three independent experiments.

5.4.4 Effect of W6/32 antibody on the expression of endothelial cytokines and chemokines

Leukocyte recruitment and inflammatory responses observed during antibody-mediated rejection suggest a possible role for the expression of inflammatory cytokines and chemokines produced by endothelial cells. HMEC-1 endothelial cells were examined for their ability to up-regulate the expression of various chemokines and cytokines in response to stimulation with HLA class I antibody. The cells were stimulated with 12 μg/ml of isotype control or a saturating concentration of W6/32 antibody for 24 and 72 hours. Conditioned media was collected and applied to a human protein cytokine array. The array contains antibodies to specific chemokines and cytokines attached to a nitrocellulose membrane (appendix 10.5). Figure 5.28 shows that W6/32 treated cells significantly up-regulated various cytokines and chemokines compared to the isotype control-treated group after 72 hours. The treated group showed significant upregulation of CXCL8, IL-6, CXCL10, CXCL1 and CCL5. The positive spots on the membranes were used as an internal control. The HMEC-1 cells did not show any expression of IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-13, IL-16, IL-17, IL-23, IL-27 and IL-32α, which were included in the array, in either treated or control groups. However, treatment with W6/32 antibody significantly induced the secretion of C5a complement fragment by endothelial cells compared to isotype control ($P<0.001$). The presence of soluble ICAM-1 (sICAM) and colony stimulating factor-2 (CSF-2) were also significantly increased in HLA class I treated group when compared to isotype treated cells. Twenty four hours stimulation produced a similar profile as 72 hours stimulation, however, the spot intensity was lower at 24 hours (data not shown).

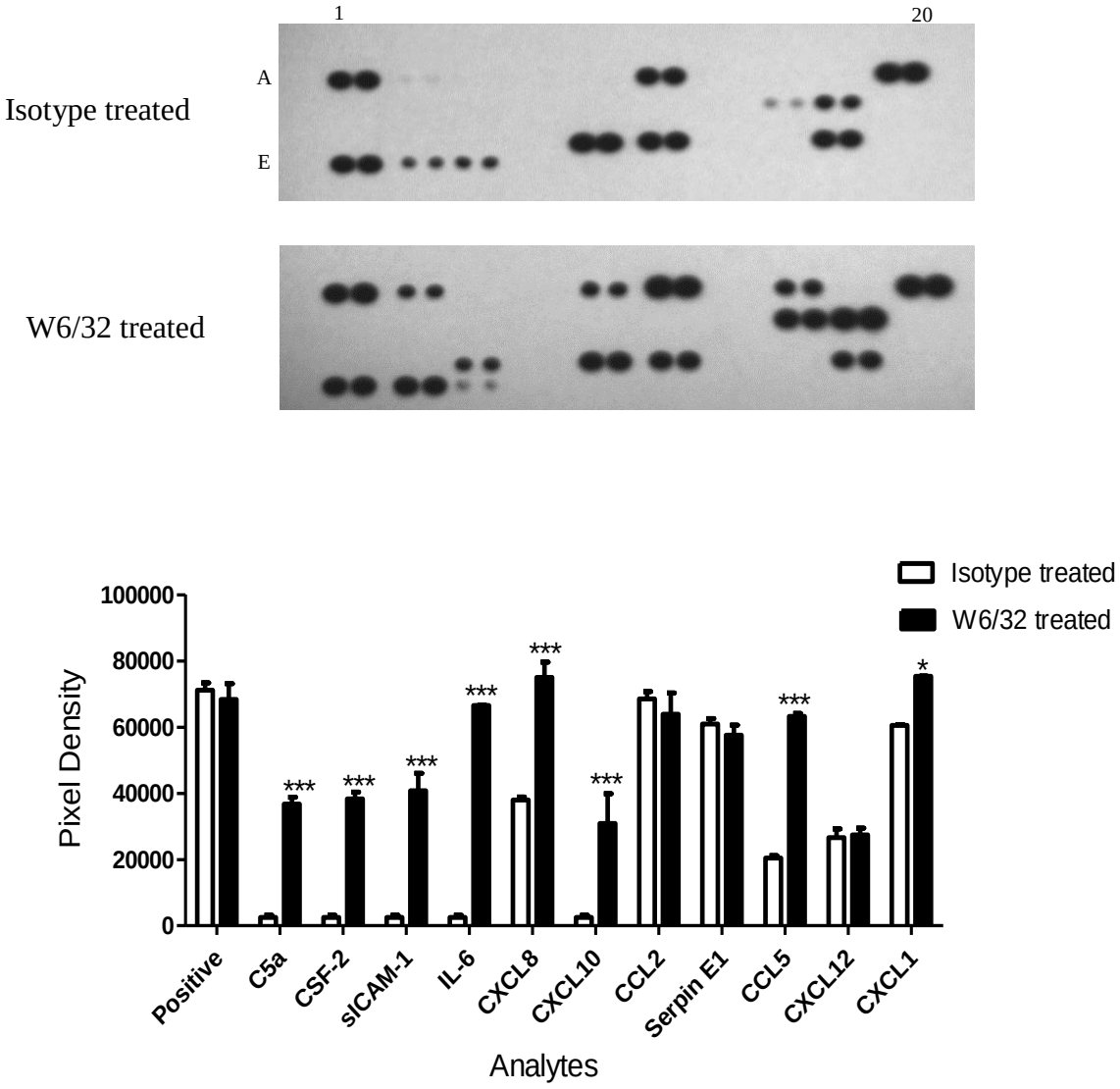


Figure 5.28: Expression of cytokines/chemokines by HMEC-1 endothelial cells following treatment with HLA class I antibody.

Cells were treated with saturating concentration of W6/32 antibody or isotype control (12 μg/ml) for 72 hours. Supernatants were collected and examined for the protein expression of various cytokines and chemokines using human cytokine array. The density of the spots was determined by AlphaImager software. The data were analysed by two-way ANOVA followed by Bonferroni test. * $p<0.01$ and *** $p<0.001$. The data are representative of two independent experiments.

5.4.5 Dose and time response of W6/32 antibody on endothelial CXCL8 expression.

To allow quantitative analysis, the expression of endothelial chemokine was assessed at the molecular level. HMEC-1 cells were treated with saturating concentration of W6/32 antibody or isotype control (12μg/ml) at different time points (3.5, 16 and 24 hours) and examined for mRNA CXCL8 expression by real time-PCR using the Taqman assay. Before performing PCR, isolated RNA was assessed for integrity as described in section 2.9.4. Figure 5.29 shows that exposure of endothelial cells to W6/32 antibody induced a 50 fold increase in the expression of CXCL8 within 3.5 hours compared to isotype treated group, and normalized to GAPDH expression ($p<0.001$). The gene expression of CXCL8 increased more than 60 fold at 16 hours, and then decreased by around 20 % after 24 hours of stimulation. The dose response of treatment was also examined by treating HMEC-1 cells with sub-saturating concentrations of W6/32 antibody. The expression of this chemokine showed a high dependency on antibody concentration as illustrated in Figure 5.30. No reactions were observed in negative wells containing master mix or RNA samples.

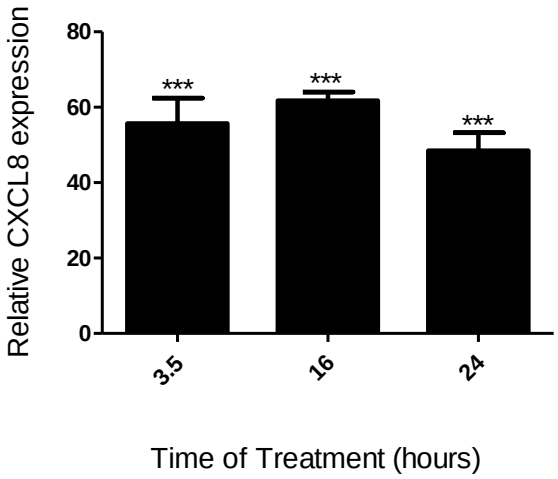


Figure 5.29: Time course of mRNA CXCL8 expression by HLA class I antibody.

HMEC-1 cells were treated with isotype control or saturating concentration of W6/32 antibody (12 μg/ml) at various time points before RNA isolation. cDNA was synthesised and amplified to assess the expression of CXCL8. GAPDH was used as a housekeeping gene. Data analysis was performed by REST 2009 software. *** $p<0.001$. The data are representative of three independent experiments.

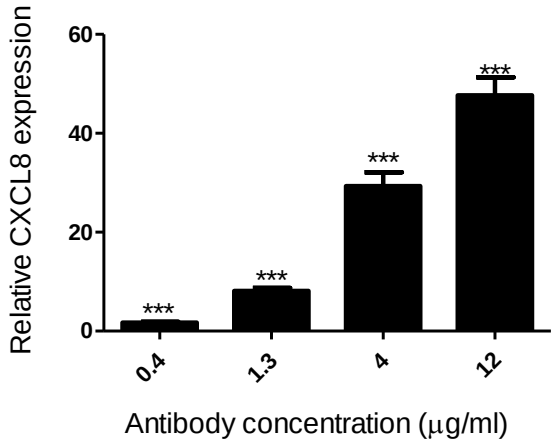


Figure 5.30: Effect of the concentration of HLA class I antibody on endothelial CXCL8 expression.

HMEC-1 cells were treated with different concentrations of W6/32 antibody ranging from 0.4 μg/ml to 12 μg/ml for 3.5 hours. After RNA extraction, cDNA was synthesised and amplified to assess the expression of CXCL8. GAPDH was used as housekeeping. *** $p<0.001$. The data were analyzed using REST 2009 software. The data are representative of two independent experiments.

5.4.6 Effect of PI3K/Akt and PKA/CREB pathways on CXCL8 expression induced by HLA class I antibody

The signalling pathways which might have a role in the expression of CXCL8 in response to HLA class I antibody were investigated. The inhibition of PI3K/Akt and PKA pathways was performed using LY294002 and H-89 inhibitors, respectively. As shown in Figure 5.31 (A), treatment of HMEC-1 cells with 50 µM of LY294002 or 5 µM of H-89 for 30 minutes followed by stimulation with W6/32 antibody (12 µg/ml) for 3.5 hours resulted in 52% and 35% reduction, respectively, in the expression of CXCL8 when compared to the control group. The role of PKA pathway was further explored by CREB knockdown with siRNA. The knockdown efficiency was examined at protein level by western blotting 72 hours after transfection as shown previously in Figure 5.7. CREB siRNA transfected cells or a negative siRNA control for 72 hours were stimulated with 12 µg/ml of W6/32 antibody for 3.5 hours. As shown in Figure 5.31 (B), silencing of CREB using 5 nM and 10 nM of CREB specific siRNA induced 40% and 50% reduction, respectively, in the molecular expression of CXCL8 as determined by real time-PCR data. Transfection of endothelial cells with negative siRNA produced no significant effect on CXCL8 expression compared to stimulated untransfected cells (data not shown).

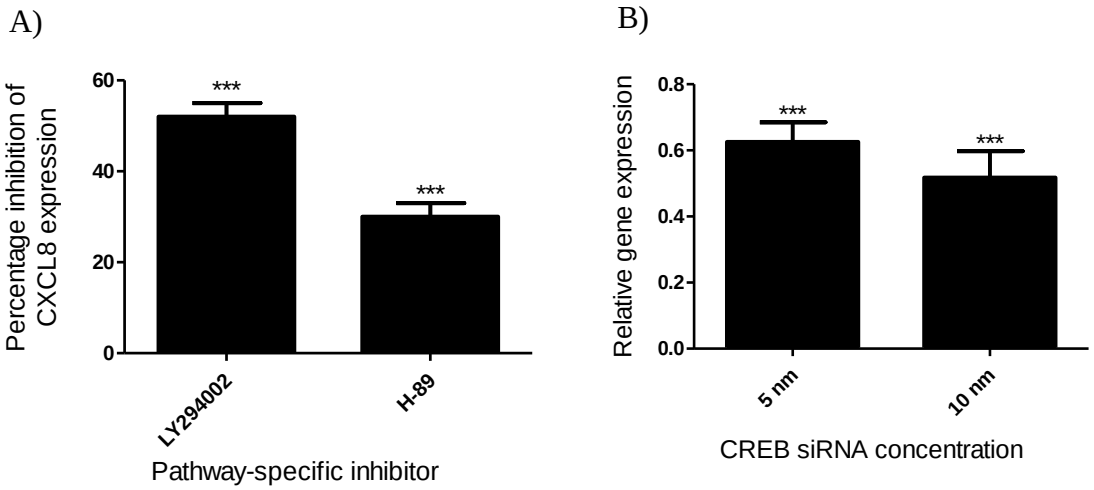


Figure 5.31: Effect of PI3K/Akt and PKA pathways on the expression of CXCL8 in response to stimulation with HLA class I antibody.

Panel A, HMEC-1 cells were treated with 50 µM of LY294002 or 5 µM of H-89 30 minutes before stimulation with 12µg/ml of W6/32 antibody for 3.5 hours. RNA was isolated and assessed for the expression of CXCL8 by real time-PCR. Panel B, HMEC-1 cells were transfected with 5 nM and 10 nM of either CREB siRNA or negative control siRNA for 72 hours. Transfected cells were stimulated with 12 µg/ml of W6/32 antibody for 3.5 hours followed by RNA isolation. After cDNA synthesis, expression of CXCL8 was determined by qRT-PCR. The data was analyzed by REST 2009 software using GAPDH as a reference gene. *** $p<0.001$. The data are representative of three independent experiments.

5.4.7 Effect of W6/32 conditioned media on monocyte migration

To examine the potential of HLA class I antibody-treated endothelial cells to induce leukocyte migration, a chemotaxis assay was performed using THP-1 human monocytes. THP-1 cells were chosen for this assay due to the known expression of CCR5 and CXCR2 chemokine receptors corresponding to the chemokines CCL5 and CXCL8, respectively, secreted by endothelial cells in response to HLA class I antibody. THP-1 cells migration was assessed in response to conditioned media from endothelial cells treated for 72 hours with either isotype control or saturating concentration of W6/32 antibody. Remaining antibody in the media was removed by passing through a 100 kDa cutoff concentrator tube. THP-1 cells showed a significant cell migration in response to the conditioned media from HLA class I treated cells compared to isotype control ($P<0.001$) after 3 hours of incubation. The presence of migrant cells was assessed by staining the filters from both groups with hematoxilin staining. Figure 5.32 shows the mean number of migrant cells per high power field.

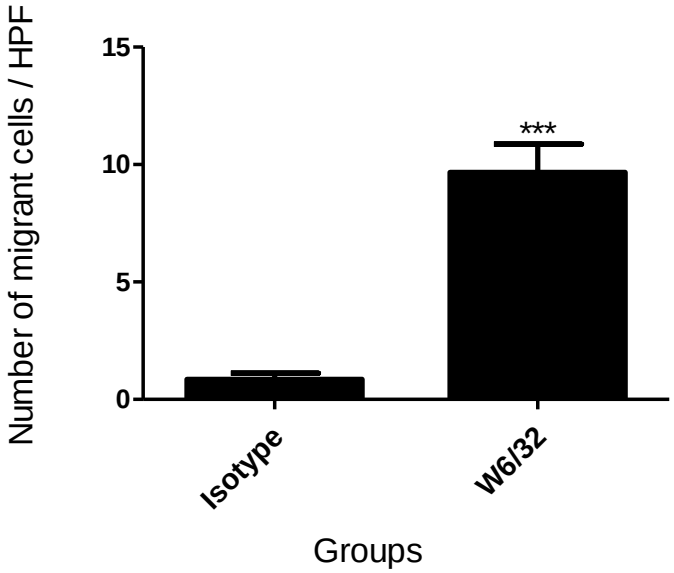


Figure 5.32: Chemotaxis assay using THP-1 human monocyte cells.

THP-1 cells were examined for their ability to migrate in response to supernatant of HMEC-1 cells treated with either 12 µg/ml of either isotype control or W6/32 antibody for 72 hours. Cells were incubated for 3 hours at 37^{o}C, fixed and stained. Migrant cells were counted by light microscope. The data represent the number of migrant cells per high power field. The data are representative of three experiments run on duplicate. The data were analysed by un-paired Student's t-test. *** $P < 0.001$.

5.4.8 *In vitro* flow based adhesion assay using VCAM-1/Fc and THP-1 cells

To optimise an *in-vitro* flow based adhesion assay using the Cellix platform, recombinant human (rh) VCAM-1/Fc was assessed for its ability to bind the ligand VLA-4 on THP-1 cells and PBMC in the presence or absence of chemokine. The optimisation involves evaluating immobilised and solubilised chemokines in inducing leukocyte adhesion under physiological flow condition. Vena8 chips were coated with VCAM-1/Fc (10 μg/ml) alone or in combination with chemokine rhCCL5 at concentration 500 ng/ml. THP-1 cells were infused at a flow of 0.5 dyne/cm^2 for 5 minutes. At the last minute of flow, images from different fields were captured and the number of adherent cells was counted. As shown in Figure 5.33, the presence of immobilized rhCCL5 with VCAM-1/Fc induced THP-1 cells adhesion compared to chips VCAM-1/Fc only. THP-1 cells also showed a significant binding to chip coated with VCAM-1/Fc alone compared to BSA-coated chip. But this binding is augmented in the presence of chemokine as observed that rhCCL5 significantly increased the adhesion of the cells by around 30%.

By using monocyte-reduced PBMC preparations, the flow adhesion assay was performed using different concentrations of rhCCL5 (5-100 ng/ml) or rhCXCL12 ranging from 5 to 60 ng/ml which was mixed with the cells just before starting the flow on VCAM-1/Fc coated chip. As shown in Figure 5.34 (A), soluble rhCCL5 at concentration higher than 10 ng/ml induced a significant adhesion of PBMC to VCAM-1/Fc above the background level in concentration dependent manner. Maximum adhesion of these cells was observed at concentration of 50 ng/ml and using higher concentration reduced the capability of these cells to adhere. Soluble rhCXCL12 also induced the adhesion of PBMC in a concentration dependent manner as shown in Figure 5.34 (B). Cells started to adhere at 5 ng/ml of rhCXCL12 and adhesion became statistically significant at 20 ng/ml. The adhesion reached a plateau at a concentration 40 ng/ml with no extra enhancement of cell adherence at concentration 60 ng/ml.

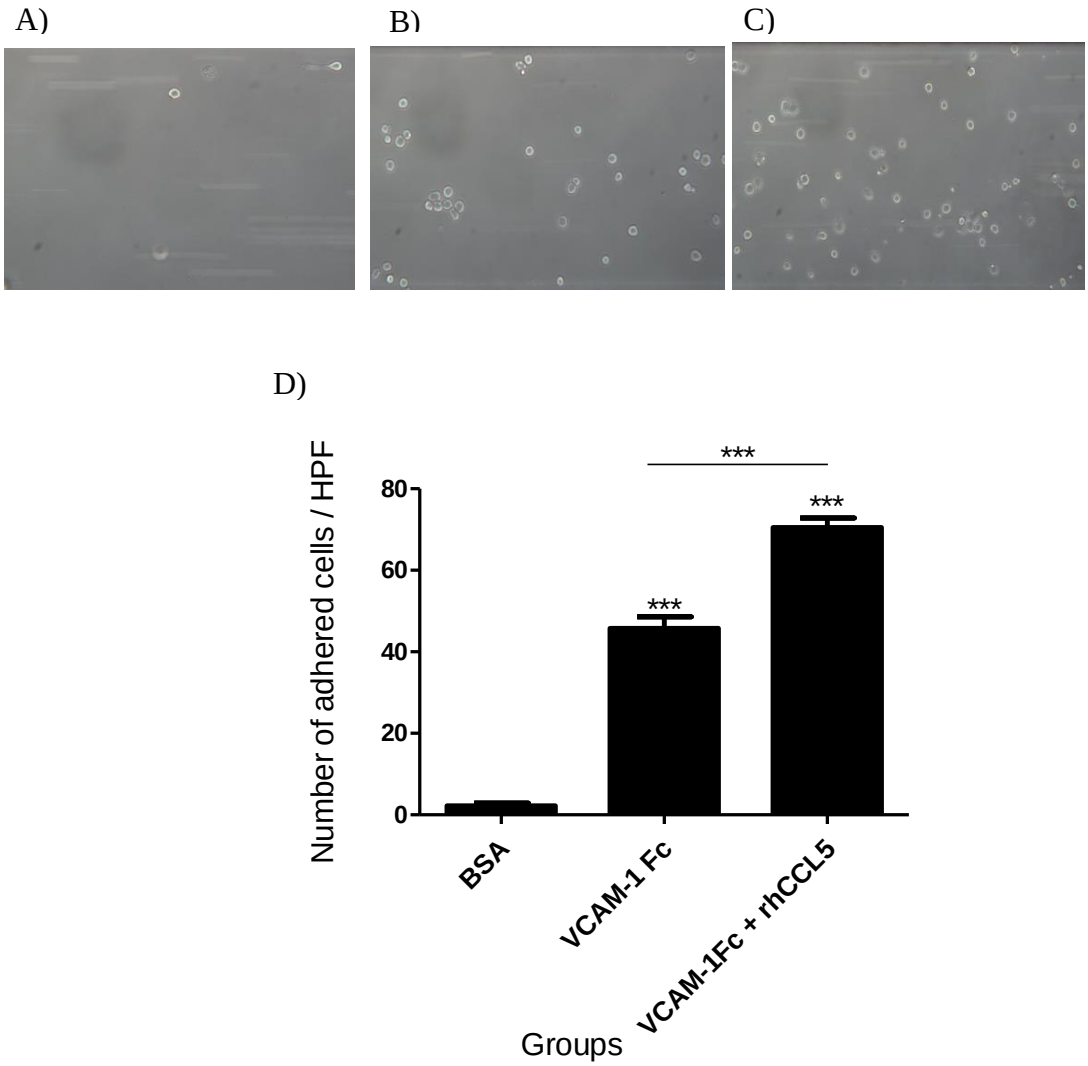


Figure 5.33: *In vitro* flow based adhesion assay using recombinant VCAM-1/Fc and THP-1 human monocytic cell line.

The Vena8 cellix chip was coated with 10 µg/ml of VCAM-1/Fc before flow of THP-1 cells was performed at 0.5 dyne/cm^2 for 5 minutes. This assay was applied on chips coated with VCAM-1/Fc alone or in combination with 500 ng/ml of immobilized rhCCL5. (A) shows the cells adhered to BSA coated channel as a negative control, (B) shows the cells adhered to VCAM-1/Fc coated channel, (C) shows the cells adhered to channel coated with VCAM-1/Fc and rhCCL5 and (D) bar chart showing the mean of six fields from each group, means were calculated and plotted. Comaprison between groups was performed by one-way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representitive of three indepenent experiments.

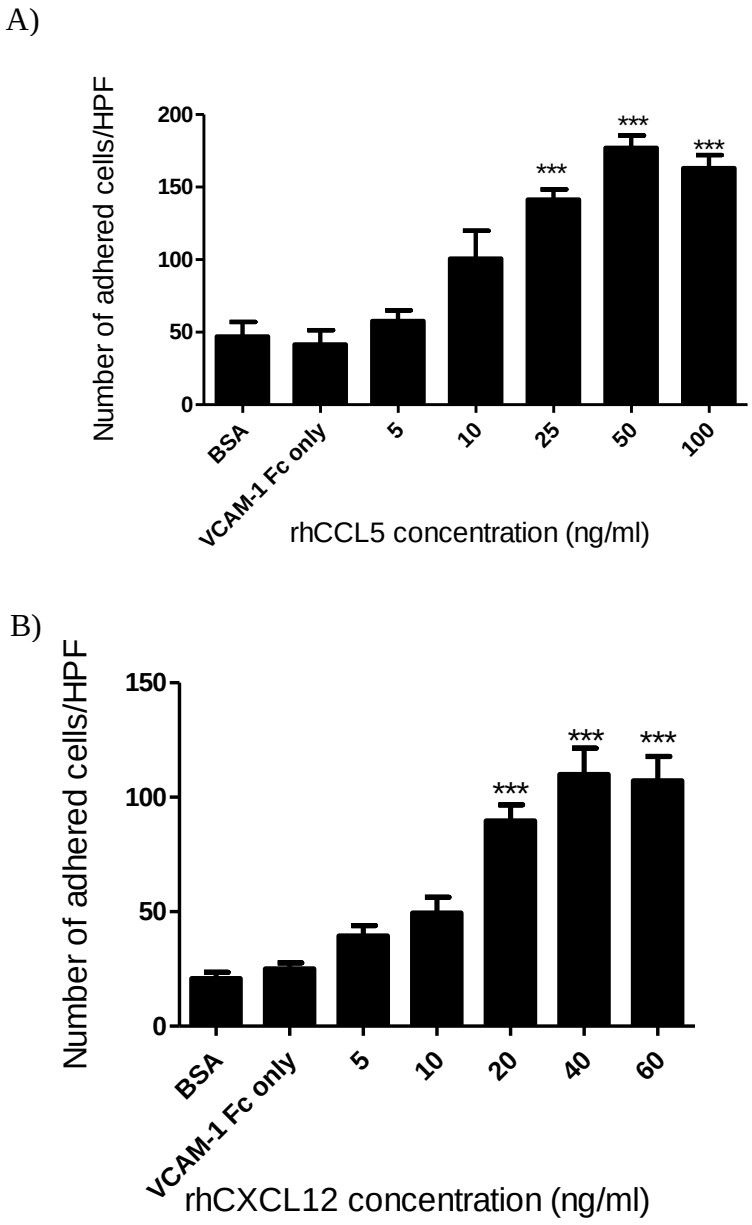


Figure 5.34: *In vitro* flow based adhesion assay using recombinant VCAM-1/Fc and PBMC preparation.

Vena8 channels were coated with 10 µg/ml of VCAM-1 Fc before infusion of freshly isolated PBMC at 0.5 dyne/cm^2 for 5 minutes. Flow assay was performed using unstimulated PBMC or after stimulation with different concentrations of rhCCL5 (A) or rhCXCL12 (B) immediately before starting the flow. Images were captured at the last minute and six fields were counted. Comparison between groups was performed by one way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representitive of two indepenent experiments.

5.4.9 *In-vitro* flow based adhesion assay on TNF-α activated HMEC-1 using THP-1 cells and chemokines

Adhesion of leucocytes to activated endothelial cells under flow condition was optimized using THP-1 monocytic cell line and MOLT-16 T-cell line. In these experiments, HMEC-1 endothelial cells were grown on specific chip until confluent followed by stimulation with TNF-α at concentration 10 ng/ml overnight to induce the expression of adhesion molecules and chemokines. After stimulation, cells were infused at 0.5 dyne/cm^2 for 5 minutes in the absence or presence of immobilized chemokine at 500 ng/ml. As shown in Figure 5.35, stimulation of endothelial cells with TNF-α induced significant adhesion of monocytes at the examined shear stress compared to untreated control. Immobilization of rhCCL5 on activated HMEC-1 cells augmented remarkably the adhesion of THP-1 cells by 50% compared to that in the absence of rhCCL5. Immobilization of rhCCL5 on untreated cells has a negligible effect on leukocyte adhesion (data not shown).

Figure 5.36 shows an *in vitro* flow adhesion assay using rhCXCL12 and MOLT-16 cells on TNF-α stimulated endothelial cells. Activated HMEC-1 cells enhanced significantly the adhesion of T-cells compared to untreated endothelial cells, while the presence of rhCXCL12 augmented significantly cell adhesion by around 45% compared to that in the absence of chemokine. Immobilization of rhCXCL12 on untreated endothelial cells resulted in a non-significant cell adhesion (data not shown).

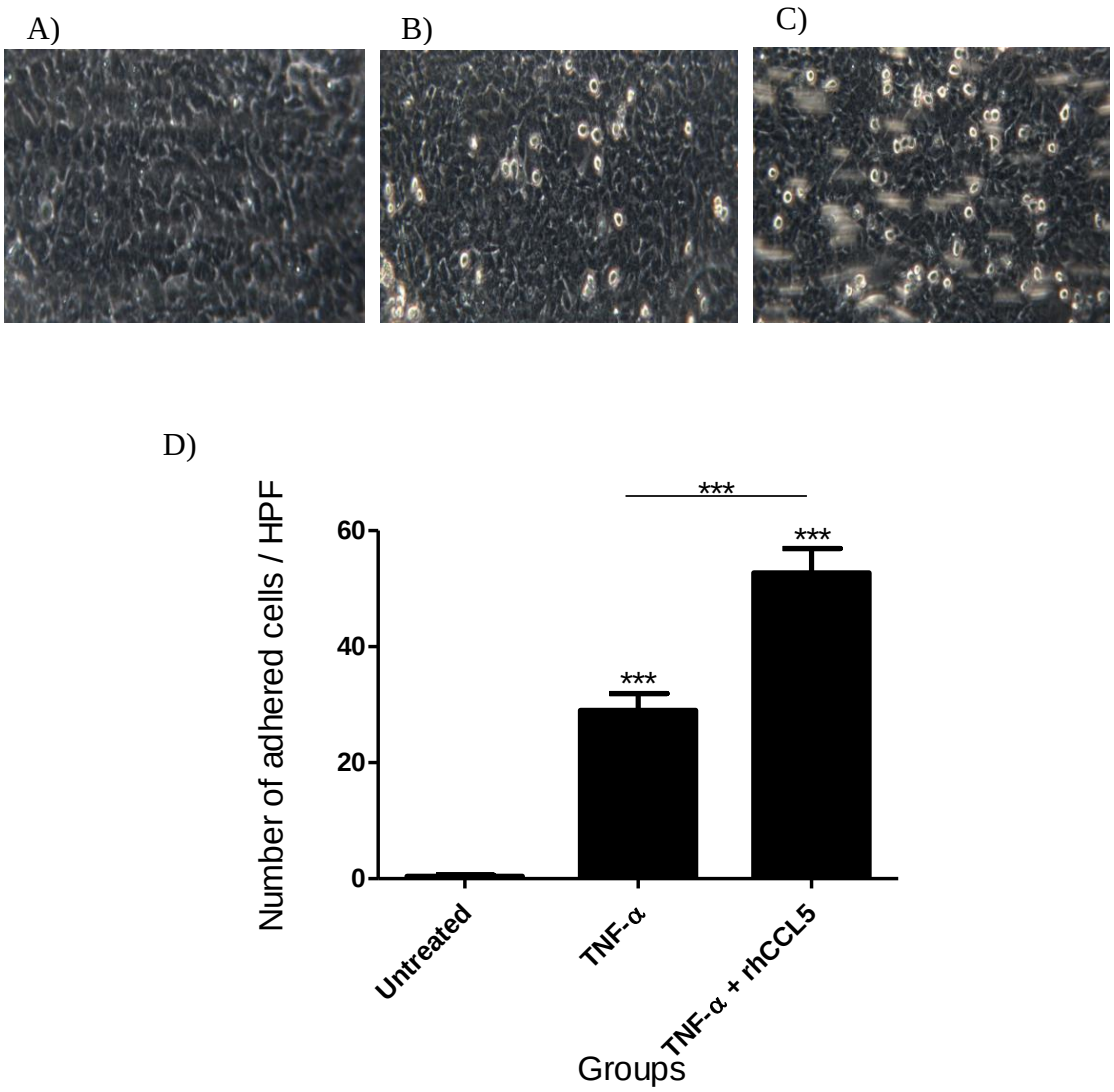


Figure 5.35: *In vitro* flow based adhesion assay on TNF-α stimulated HMEC-1 cells using rhCCL5 and THP-1 cells.

Endothelial cells grown on the endothelial chip (VenaEC) were stimulated with 10 ng/ml of TNF-α overnight. Flow of THP-1 cells were infused at 0.5 dyne/cm^2 for 5 minutes on either (A) untreated or (B) TNF-α stimulated cells or (C) TNF-α stimulated cells where 500 ng/ml of rhCCL5 was immobilized. The mean values of at least six fields of each group were calculated and plotted as shown in (D). The data were analyzed by one way ANOVA followed by Benforroni test at $p<0.05$. *** $p<0.001$. The data are representitive of three independent experiments.

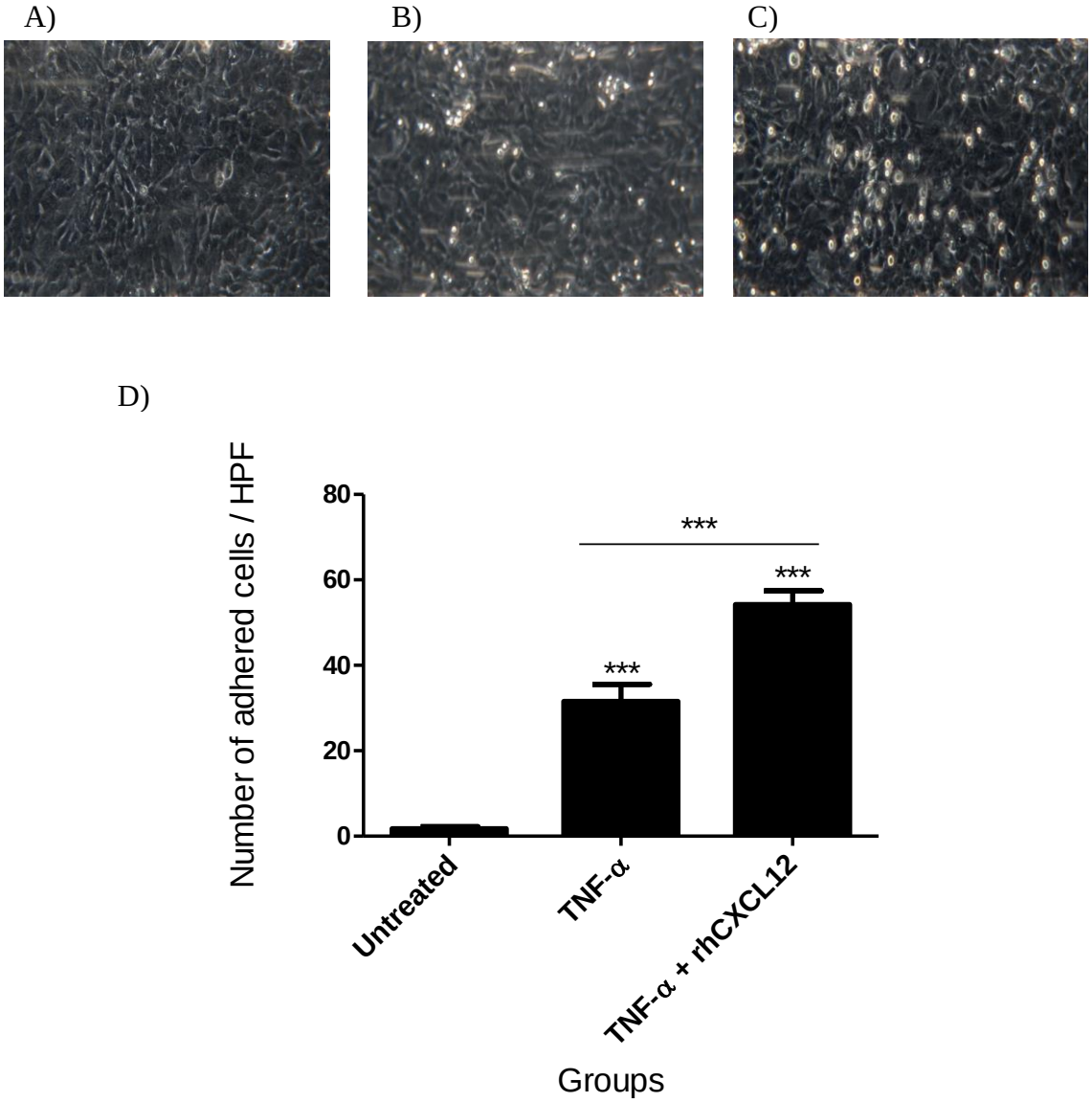


Figure 5.36: *In vitro* flow based adhesion assay on TNF-α stimulated HMEC-1 cells using rhCXCL12 and MOLT-16 cells.

Endothelial cells were grown on the endothelial chip (VenaEC) and stimulated with 10 ng/ml of TNF-α overnight. MOLT-16 cells were infused at 0.5 dyne/cm^2 for 5 minutes on either (A) untreated or (B) TNF-α stimulated cells or (C) TNF-α stimulated cells where 500 ng/ml of rhCXCL12 was immobilized. The mean values of at least six fields of each group were calculated and plotted as shown in (D). The data were analyzed using one way ANOVA followed by bonferonni test at $p<0.05$. *** $p<0.001$. The data are representitive of three independent experiments.

5.4.10 *In vitro* flow based adhesion assay using whole molecule of W6/32 antibody

The presence of infiltrating leukocytes in antibody-mediated rejection highlights the necessity for the examination of the potential of HLA class I antibody in inducing leukocyte adhesion to endothelial cells under physiological conditions, the crucial step in leukocyte migration. To achieve this, HMEC-1 endothelial cells were grown until confluency on VenaEC chip coated with fibronectin as described in the section 5.3.7.3. The cells were treated with 12 µg/ml of either isotype control or W6/32 antibody overnight. THP-1 cells were then infused at 0.5 dyne/cm^2 for 5 minutes and the adhered cells were counted at the last minute of the flow. As shown in Figure 5.37, cells pre-treated with HLA class I antibody (B) induced significant monocyte adhesion compared to isotype control (A). The mean of cells adhered in 10 high power fields were calculated and plotted as shown in panel (C) of the same figure.

To assess the ability of chemokines secreted by endothelial cells to induce leukocyte adhesion, conditioned media from W6/32 treated endothelial cells were used for flow based adhesion assay. HMEC-1 cells grown in VenaEC chip were treated with TNF-α at concentration 10 ng/ml overnight. After stimulation, conditioned media from both isotype and W6/32 treated cells were immobilised on stimulated endothelial cells. Conditioned media were passed through 100 kDa cutoff filtration tube to remove the remaining antibody from the conditioned media, eliminating interaction of the Fc fragments with the Fc receptors on THP-1 cells. The cells were infused on stimulated endothelial cells at 0.5 dyne/cm^2 for 5 minutes and the adhered cells were then counted. As shown in Figure 5.38, human monocytes significantly adhered to endothelial cells where conditioned media from W6/32-treated cells (B) was used compared to isotype conditioned media (A), $p<0.001$. Since inflammatory cytokines treatment induce the secretion of chemokines and upregulation of adhesion molecules, THP-1 cells also significantly adhered to cells treated with TNF-α only. The mean of cells adhered at each group was plotted in panel (C).

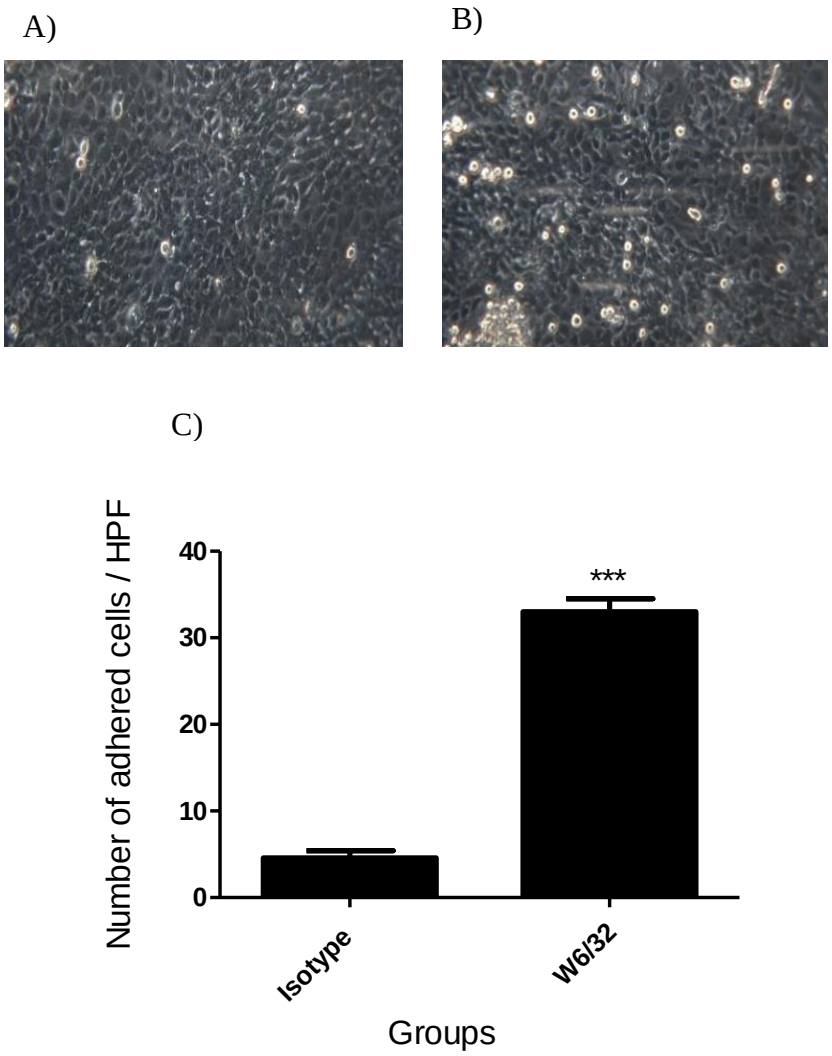


Figure 5.37: *In vitro* flow based adhesion assay on HMEC-1 cells stimulated with HLA class I antibody.

Endothelial cells grown on endothelial chip (VenaEC) were stimulated with 12 μg/ml of either W6/32 or isotype antibody overnight. Infusion of THP-1 cells was performed at 0.5 dyne/cm^2 for 5 minutes on either (A) isotype treated cells or (B) W6/32 stimulated cells. The mean values of 10 fields of each group were graphed, (C). Unpaired Student's t-test was performed at $p<0.05$. *** $p<0.001$. The data are representitive of three independent experiments.

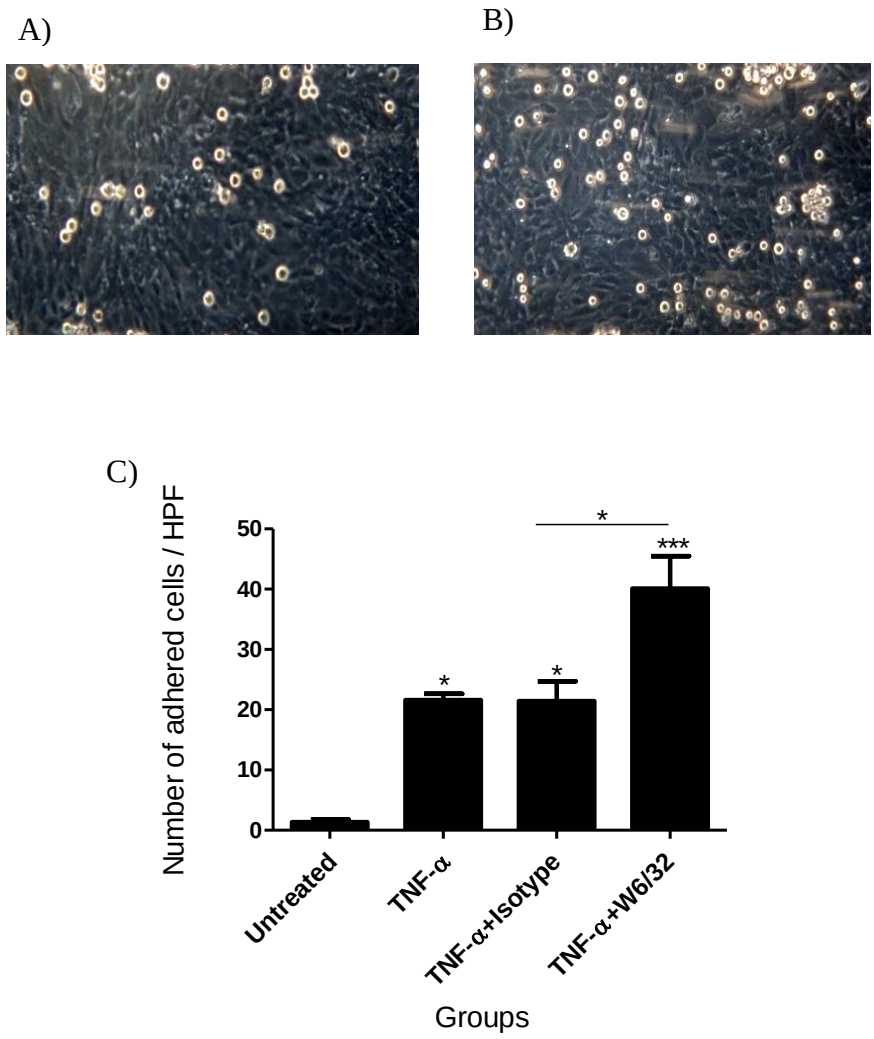


Figure 5.38: *In vitro* flow based adhesion assay on TNF-α stimulated endothelial cells using conditioned media from W6/32 or isotype treated groups.

HMEC-1 cells grown on endothelial chip (VenaEC) were stimulated with 10 ng/ml of TNF-α overnight. Conditioned media from cells treated with either isotype or W6/32 antibody for 72 hours were immobilised on the activated endothelial cells. THP-1 cells were infused at 0.5 dyne/cm^2 for 5 minutes on TNF-α stimulated cells where conditioned media from either (A) isotype or (B) W6/32 treated cells was immobilised. The mean value of six fields of each group were plotted as shown in (D). Two way ANOVA followed by Bonferroni test was perfomed. * $p < 0.05$ and *** $p<0.001$. The data are representitive of three independent experiments.

5.4.11 Expression of VCAM-1 and ICAM-1 by endothelial cells following stimulation with F(ab)$_2$ fragment of W6/32 antibody

To eliminate the interaction through Fc receptors, F(ab)$_2$ fragments from W6/32 antibody were prepared and used for this assay. Cells were treated with these fragments at concentration 12 μg/ml overnight followed by examining the expression of VCAM-1 and ICAM-1 antigens. The expression of these molecules was determined by flow cytometry using PE-conjugated mouse anti-human antigen specific antibodies. The exposure to these fragments significantly induced the expression of these antigens in comparison to untreated group. The expression of VCAM-1 was induced by around 4 fold and the expression of ICAM-1 was induced by approximately 40 fold compared to untreated group. Figure 5.39 shows the flow cytometry histograms for VCAM-1 and ICAM-1 staining for both untreated and treated groups. The median fluorescence intensity for each group is represented in bar chart, F.

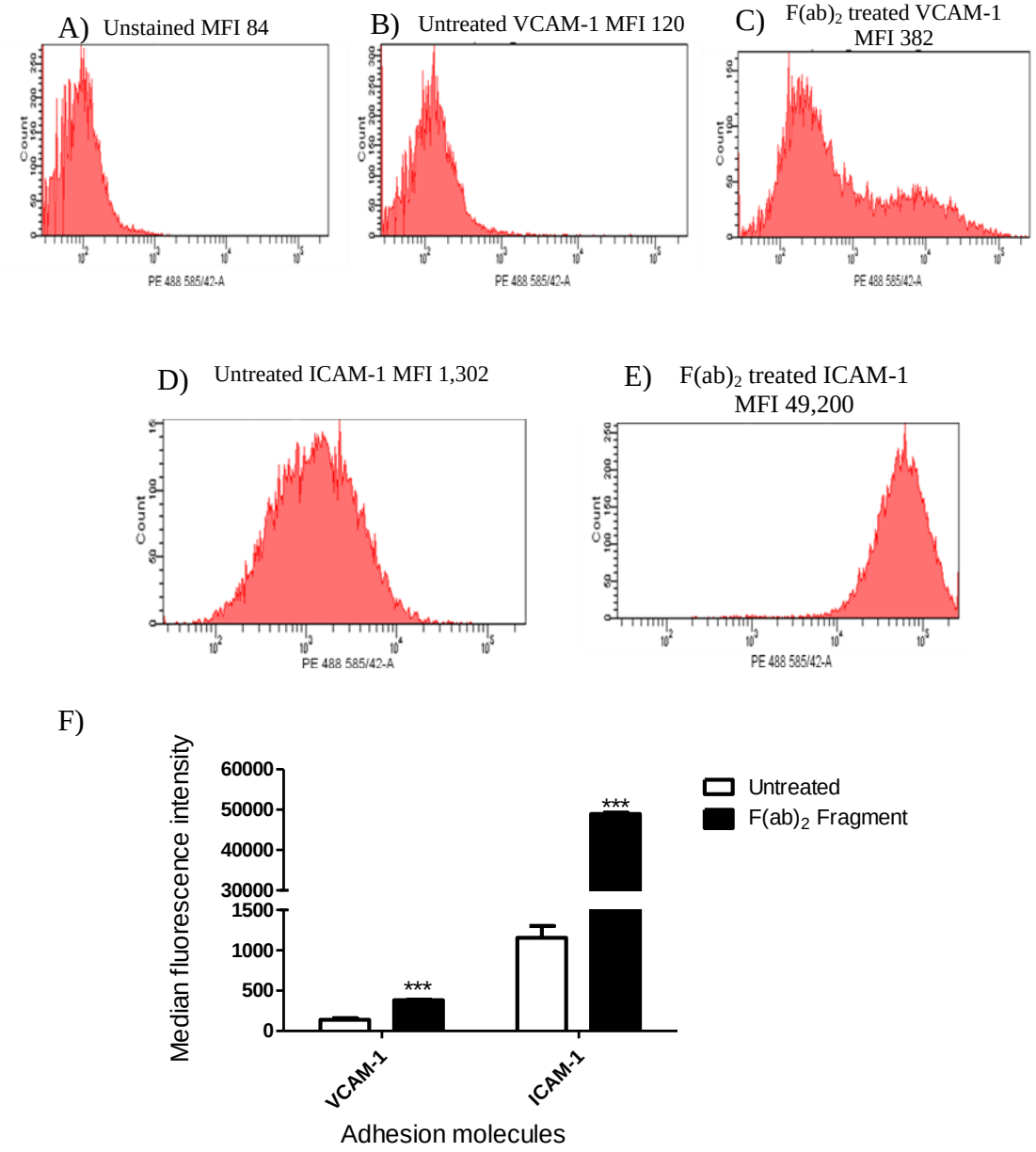


Figure 5.39: Expression of endothelial VCAM-1 and ICAM-1 after stimulation with F(ab)$_2$ fragments of W6/32 antibody.

HMEC-1 cells were incubated with these fragments overnight before staining with PE-conjugated mouse anti-human VCAM-1 and ICAM-1 antibodies. Panel A shows unstained, panel B VCAM-1 staining of untreated cells, panel C VCAM-1 staining of treated cells, panel D ICAM-1 staining of untreated cells, panel E ICAM-1 staining of treated cells and panel F shows the median fluorescence intensity of the expression of both molecules. The analysis between untreated and treated groups was performed by unpaired Student's t-test. *** $p<0.001$. The data are representative of two independent experiments.

5.4.12 *In vitro* flow based adhesion assay using $F(ab)_2$ fragment of W6/32 antibody

The potential of HLA class I antibody to induce monocyte adhesion was examined using $F(ab)_2$ fragments produced from W6/32 antibody by pepsin treatment. HMEC-1 endothelial cells were grown on fibronectine coated Vena EC chip and stimulated with 12 μg/ml of $F(ab)_2$ fragments overnight. THP-1 cells were infused over the monolayer endothelial cells at 0.5 dyne/cm^2 for 5 minutes and the images were captured at the last minute. $F(ab)_2$ fragment treated endothelial cells showed a significant monocyte adhesion compared to untreated endothelial cells ($p<0.001$). As shown in Figure 5.40, $F(ab)_2$ fragment treated endothelial cells (B) induced 6 fold increase in the number of adhered cells compared to untreated group (A).

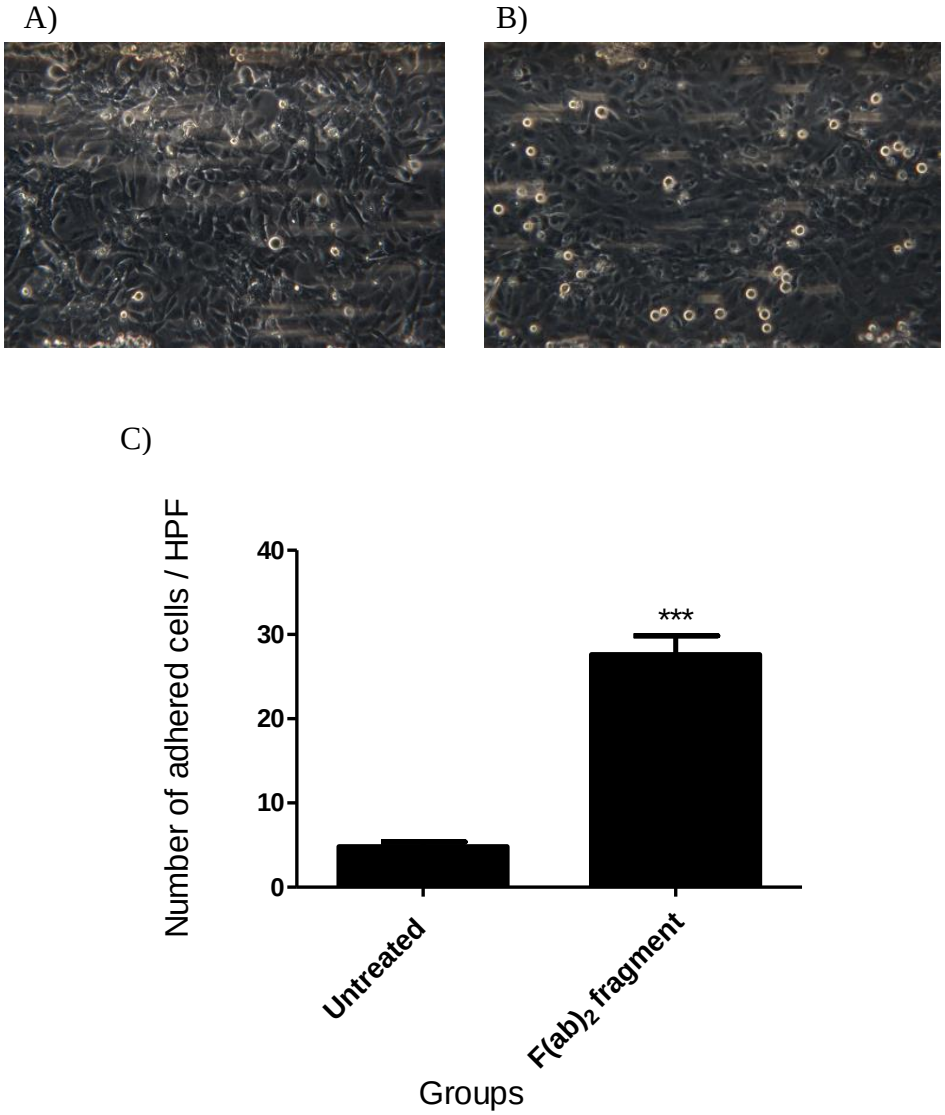


Figure 5.40: *In Vitro* flow based adhesion assay using F(ab)$_2$ fragments of W6/32 antibody.

Cells grown on cellix chip until confluent were treated with 12 μg/ml of F(ab)$_2$ fragments overnight. THP-1 monocyte cells were infused through (A) untreated and (B) treated endothelial cells at 0.5 dyne/cm^2 for 5 minutes. Six fields from each group were counted and the means were plotted (C). Statistical analysis was performed using unpaired Student's t-test. *** $p<0.001$. The data are representative of three independent experiments.

5.5 Discussion

Leukocyte infiltration to the transplanted grafts is one of the main characteristics observed during antibody-mediated rejection. The histological evidence of cellular rejection is increased in patients who develop donor-specific HLA antibody (Caro-Oleas *et al.*, 2012; Yousem and Zeevi, 2012). The link between antibody formation and cell-mediated rejection is not fully understood. However, the transmigration of recipients' leukocytes into grafts suggests the contribution of endothelial adhesion molecules and chemokines in this process. In this chapter, the possible interaction between endothelial cells and leukocytes in the presence of donor-specific HLA class I antibody was examined. In particular, the expression of adhesion molecules, VCAM-1, ICAM-1 and E-selectin, and chemokines was assessed. In addition, the potential of HLA class I antibody to induce monocyte adhesion and migration was determined.

In response to inflammatory cytokines, microvascular endothelial cells up-regulate the expression of cell-surface adhesion molecules (Xu *et al.*, 1994). HMEC-1 endothelial cells were assessed for the expression of these molecules in response to stimulation with TNF-α at different time points and cytokine concentrations. In resting conditions, HMEC-1 cells express a negligible level of VCAM-1 and E-selectin but express a detectable level of ICAM-1. In response to TNF-α stimulation, HMEC-1 cells up-regulate in a time and concentration-dependent manner the expression of VCAM-1 and ICAM-1 antigens and, to a lesser extent, the expression of E-selectin. Characterization of the cell line in early studies showed a similar profile in the expression of these molecules in resting and stimulated states (Xu *et al.*, 1994). The ability to express cell surface adhesion molecules in response to inflammatory cytokines renders these cells a good model to study leukocyte adhesion.

In response to antibody stimulation, treatment of HMEC-1 cells with a saturating concentration of W6/32 antibody (12 µg/ml) induced a significant up-regulation of adhesion molecules VCAM-1 and ICAM-1 in a time dependent manner. The expression of both antigens decreased after 72 hours compared to 12 hours treatment but remained significantly elevated compared to an isotype control-treated group. Resting cells upregulated E-selectin at low level in response to TNF-α, but there was no modulation in the expression of this adhesion molecule following stimulation with W6/32 antibody compared to control. The up-regulation of VCAM-1 and ICAM-1 molecules has been previously reported for a model of large vessel endothelial cells stimulated with W6/32

antibody (Narayanan *et al.*, 2006). The expression of these adhesion molecules has also been observed in *in vivo* model. Following single HLA mismatched heart transplantation in mice, the expression of endothelial adhesion molecules has been increased in the transplanted grafts measured at a molecular level (Fukami *et al.*, 2012). The expression of these molecules suggests the involvement of HLA class I antibody in inducing allograft rejection rather than accommodation. In organ accommodation, it was previously reported that pre-exposure of endothelial cells to sub-saturating concentration of W6/32 antibody reduced the expression of adhesion molecules in response to TNF-α stimulation (Narayanan *et al.*, 2006). In this project, the increase in the expression of adhesion molecules by TNF-α was also examined following the pre-treatment with sub-saturating concentrations of W6/32 antibody. The treatment of HMEC-1 cells with saturating and sub-saturating concentrations of W6/32 antibody for 48 hours followed by 10 ng/ml of TNF-α for 24 hours did not show a decrease in the expression VCAM-1 and ICAM-1 antigens. This might suggest that pre-treatment with sub-saturating concentrations of W6/32 antibody has no effect in providing protection, or longer incubation time to activate the protective pathways in endothelial cell line might be required.

Adhesion molecules have a crucial role in exposing endothelial cells to circulating leukocytes and enhancing their adhesion. VCAM-1 mediates the adhesion of lymphocytes, monocytes and eosinophils, which express very-late antigen-4 (VLA-4, α4 β1 integrin) while ICAM-1 binds to LFA-1 molecules expressed on lymphocytes and neutrophils (Bevilacqua, 1993; Lay *et al.*, 2007). This binding is one of the main steps in leukocyte adhesion to activated endothelium. In order to understand the mechanism responsible for the expression of these adhesion molecules, inhibitors for both PI3K/Akt and PKA pathways were used. The inhibition of PI3K/Akt pathway (LY294002) but not PKA pathway (H-89) induced a significant reduction in the expression of both VCAM-1 and ICAM-1 compared to cells treated with W6/32 antibody alone. The role of PI3K/Akt pathway in the expression of adhesion molecules in response to inflammatory cytokine (TNF-α) was observed using human intestinal endothelial cells (Binion *et al.*, 2009). This expression was mediated by the activation of transcription factor, NF-kβ.

Exposure of endothelial cells to a saturating concentration of W6/32 antibody for 24 and 72 hours in this study induced the secretion of IL-6 and various chemokines into culture media. CXCL8, CXCL10, CXCL1 and CCL5 were significantly induced by endothelial

cells treated with W6/32 antibody compared to cells treated with isotype-control. The expression of CXCL8 was further assessed at the mRNA level. HMEC-1 cells treated with HLA class I antibody showed a significant expression of CXCL8 in a time and concentration dependent-manner compared to isotype-treated cells. The expression of inflammatory cytokines by microvascular endothelial cells showed a similarity to W6/32-stimulated large vessel endothelial cells. The expression of CXCL8, IL-6, TNF-α and IL-1β was previously observed using human iliac artery endothelial cells treated with W6/32 antibody (Reyes-Vargas *et al.*, 2009). Furthermore, a recent study showed that stimulation of human microvascular glomerular endothelial cells with sera containing HLA class I antibodies induced the expression of CCL5 chemokine (Mannam *et al.*, 2013). In our study, endothelial cells stimulated with W6/32 antibody showed an increase in the expression of complement fragment C5a and soluble ICAM-1 molecule. The expression of complement fragment C5a becomes in agreement with previous study showed the ability of glomerular and microvascular endothelial cells to produce complement components C4 in response to the stimulation with HLA class I antibody (Hamer *et al.*, 2012). The expression of complement fragments might augment the cytotoxicity induced by HLA class I antibody. Fragments produced from complement activation such as C5a are anaphylatoxin which are able to activate both endothelial cells and circulating leukocytes, enhancing leukocyte recruitment to the inflamed sites (Monsinjon *et al.*, 2003). Moreover, stimulation of endothelial cells with a combination of W6/32 antibody and C5a induced a significant exocytosis of endothelial von Willebrand Factor compared to the treatment with antibody alone (Yamakuchi *et al.*, 2007). In our project, soluble ICAM-1 was also observed following stimulation with W6/32 antibody compared to control. The serum level of this molecule has been shown to be associated with the increase in the rate of mortality following renal transplantation (Connolly *et al.*, 2011).

The mechanism responsible for the expression of CXCL8 in response to stimulation with HLA class I antibody was further examined by inhibiting the PI3K/Akt and PKA pathways using a pathway-specific inhibitor. Inhibition of the PI3K pathway (LY294002) led to a 52% reduction in the expression of CXCL8, while the inhibition of PKA pathway (H-89) resulted in a 35% reduction. Therefore, synergism between the two pathways in inducing CXCL8 expression is possible. Since the transcription factor CREB is a down-stream of PKA pathway, the contribution of this factor in the expression of CXCL8 was examined using siRNA. Transfection with CREB-siRNA

induced an 80% reduction in CREB expression compared to negative control siRNA as shown at the protein level by western blotting. Stimulation of CREB-siRNA transfected cells with W6/32 antibody produced a significant 50% reduction in the expression of CXCL8 compared to control. This result suggests that the transcriptional activity of CREB is essential for the expression of endothelial CXCL8 in response to HLA class I antibody. A previous study showed that the expression of CXCL8 in response to stimulation with cytokine was dependent on a synergistic effect between CREB and NF-kB transcription factors (Sun *et al.*, 2008). Therefore, the participation of NF-kB downstream of PI3K/Akt pathway cannot be excluded.

Leukocyte infiltration into the transplanted graft is observed in biopsies diagnosed with antibody-mediated rejection and it is used as a diagnostic marker of antibody-mediated rejection in the presence of circulating donor specific antibody (Higgins *et al.*, 2010). In addition, depletion of macrophages or blocking monocyte-related chemokines before transplantation reduces the incidence of intimal thickening and improves allografts survival (Belperio *et al.*, 2001; Kitchens *et al.*, 2007). Therefore, the chemotactic potential of conditioned media from either W6/32 or isotype-treated endothelial cells was examined using static chemotaxis assays. THP-1 human monocytic cells significantly migrated in response to antibody-free media from endothelial cells treated with saturating concentration of W6/32 antibody for 72 hours compared to media from isotype control-treated cells. Characterization of THP-1 cells showed the expression of chemokine receptors CCR5 and CXCR2 that bind CCL5 and CXCL8 respectively, which might explain the cell response and migration. A recent study showed the ability of HLA class I antibody to induce monocyte adhesion using aortic endothelial cells and in *in vivo* model (Valenzuela *et al.*, 2013a).

The ability of HLA class I antibody to induce leukocyte adhesion was assessed using a flow-based adhesion assay. This assay models *in vivo* leukocyte adhesion and overcomes the limitations of static chemotaxis assays. Micro-biochips resembling blood microvessels were coated with human recombinant adhesion molecule VCAM-1/Fc. To optimize our system, immobilized and soluble chemokines were assessed for their efficiency in inducing significant leukocyte adhesion. THP-1 monocytic cells and MOLT-16 T-cells were examined for their ability to bind VCAM-1/Fc. MOLT-16 cells express chemokine receptor CXCR4 which binds CXCL12 chemokine as shown earlier by our group (data not shown). Both cell types express VLA-4 and LFA-1 integrins

which bind VCAM-1 and ICAM-1 molecules, respectively (McGilvray *et al.*, 1997; Sandig *et al.*, 1997).

THP-1 monocytic cells significantly adhered to VCAM-1/Fc in the presence of immobilized rhCCL5. In a similar manner, MOLT-16 T-cells adhere significantly to the same adhesion molecule in the presence of immobilized rhCXCL12. Immobilization of chemokines into channels models the presentation of chemokine by endothelium where the chemokines are protected from the blood flow. In agreement with this study, immobilization of CXCL8 chemokine on VCAM-1 adhesion molecule induced significant adhesion of B-cells expressing VLA-4 (Campbell *et al.*, 1996). THP-1 cells also significantly bound to VCAM-1/Fc in the absence of chemokines. This might be attributed to the presence of active low binding affinity of cell-surface integrins which can bind to the VCAM-1/Fc (Chan *et al.*, 2000). However, this binding was significantly augmented in the presence of chemokine.

In addition, peripheral blood mononuclear cells isolated from healthy volunteers were assessed for the adhesion to biochips coated with VCAM-1/Fc. To examine the role of chemokine concentration on leukocyte adhesion, soluble chemokines rhCCL5 or rhCXCL12 were used. Cells mixed with each chemokine showed significant adhesion to VCAM-1/Fc in a concentration-dependent manner. Although different studies have addressed the necessity of localized chemokine in inducing leukocyte arrest (Grabovsky *et al.*, 2000), soluble chemokines can induce leukocyte adhesion *in vitro* (Gerszten *et al.*, 1999; DiVietro *et al.*, 2001). This might be attributed to the adsorption of chemokines under flow to the coated chips, causing localization of chemokines which is necessary for efficient integrin activation. However, localized chemokine might be more relevant to *in vivo* leukocyte adhesion where the chemokine is protected from dilution by the blood flow (Weber *et al.*, 1999).

Endothelial cells show a constitutive expression of some kinds of chemokines which are upregulated upon exposure to inflammatory cytokines. Apical chemokine along with endothelial adhesion molecules results in the increase of leukocyte adhesion. Therefore, TNF-α stimulated HMEC-1 cells were assessed for the adhesion of human monocytic cells infused at 0.5 dyne/cm^2 for 5 minutes. Monocytic cells significantly adhered to the TNF-α-activated cells compared to untreated cells. However, the adhesion of these cells was augmented by the presence of immobilized chemokine rhCCL5. In a parallel manner, the adhesion of MOLT-16 cells to activated endothelial cells was enhanced in

the presence of immobilized rhCXCL12 compared to that in the absence of chemokine. This result shows that inflamed endothelial cells can induce the adhesion of leukocytes under flow condition which is augmented in the presence of immobilized chemokines. In agreement with this study, immobilization of chemokines on human umbilical vein endothelial cells induces lymphocyte adhesion under shear forces (Shamri *et al.*, 2002; Lee *et al.*, 2009).

HLA class I antibody was examined for its potential to induce monocyte adhesion to microvascular endothelial cells. Treatment with whole molecule of W6/32 antibody induced a significant monocyte adhesion to endothelial cells compared to isotype control. This is attributed to the ability of mouse IgG2a antibody to bind human Fc-receptors as observed in previous study (Valenzuela *et al.*, 2013b). Therefore, $F(ab)_2$ fragments from W6/32 antibody were used to examine monocyte adhesion independent to Fc-receptors. HMEC-1 cells treated with these fragments overnight induced a significant binding of monocytes under flow compared to control group. This is attributed to the expression of adhesion molecules and chemokines by treated endothelial cells as shown earlier using flow cytometry and cytokine array, respectively. In addition, conditioned media from endothelial cells treated with HLA class I antibody also produced a significant increase in the adhesion of monocytes to stimulated endothelial cells compared to the conditioned media from the isotype-treated group. The presence of excess chemokines in antibody-treated conditioned-media might explain monocyte adhesion under flow conditions.

In conclusion, endothelial cells treated with saturating concentration of HLA class I antibody showed an up-regulation of the expression of cell surface adhesion molecules, chemokines and cytokines. These mediators induced the interaction between donor endothelium and recipient leukocytes, enhancing their migration to the transplanted grafts.

6. Chapter Six-Allospecific Antibodies and Endothelial Cell Activation

6.1 Introduction

Antibody-mediated rejection accounts for around 20-30% of all acute rejection episodes following kidney transplantation, reducing the one year allograft survival by 15-20% (Lucas *et al.*, 2011). Donor specific HLA antibodies are one of the major causes of antibody-mediated rejection (Lachmann *et al.*, 2009) and are a risk factor for the development of chronic rejection (Hill *et al.*, 2011). The contribution of donor-specific HLA antibody to allograft rejection occurs also in other types of solid organ transplantation such as heart (Chih *et al.*, 2012), lung (Hachem, 2012), pancreas (Torrealba *et al.*, 2008) and liver transplantation (Abu-Elmagd *et al.*, 2012). In addition to complement fixing antibody, complement-independent mechanism can also play a role in allograft rejection. In mice transplantation models, donor specific antibody to MHC class I molecules induces subsequent chronic transplant arteriopathy in a mechanism independent to complement activation (Hirohashi *et al.*, 2010). The poor prognosis of antibody-mediated rejection is attributed to the lack of responsiveness to most anti-rejection treatments which mainly target cellular rejection pathways (Jirasiritham *et al.*, 2010).

De novo synthesized donor specific antibodies or preformed antibodies have both been shown to have a deleterious effect on allograft survival (Li *et al.*, 2008; Hidalgo *et al.*, 2009). Transplantation across HLA class I or class II antibodies might occur due to prior use of low sensitivity detection techniques for HLA antibodies or after pre-conditioning treatment in highly sensitized patients (Gloor *et al.*, 2004). However, *de novo* HLA antibodies provide a more potent contribution to late allograft dysfunction (Gaston *et al.*, 2010; Halloran *et al.*, 2010). In the absence of pre-transplant antibody, the chance to develop donor specific HLA antibodies might reach 15% within the first 4-7 years post-transplantation, as reported recently in kidney transplantation (Wiebe *et al.*, 2012). In addition to antibody-mediated rejection, the presence of HLA antibodies also has an impact on the development of T-cell mediated rejection embedding a long term allograft survival (Caro-Oleas *et al.*, 2012). Accordingly, 10 year allograft survival reduced from 96% in patients without donor specific antibody to 56% in patients with *de novo* donor specific HLA antibodies (Caro-Oleas *et al.*, 2012).

Donor-specific HLA class I antibodies can be of any antibody isotype; IgG, IgA or IgM as determined earlier on the serum of kidney recipients after 1 month post-transplantation (Groth *et al.*, 1996). A recent study showed that the IgA antibody isotype is mostly formed against HLA class I antigens rather than HLA class II antigens, while the IgG isotype can form against both HLA class I or class II antigens (Arnold *et al.*, 2013). HLA antibodies can be of any IgG subclasses or a mixture of different subclasses. Recently, IgG1 was identified as the most common HLA IgG subclasses followed by IgG2>IgG3>IgG4 in sensitized kidney patients. The mixture of antibodies may contain strong complement fixing antibodies; IgG1 and IgG3 or a mixture of strong and weak complement fixing antibodies; IgG1 or IgG3I with gG2 or IgG4 (Honger *et al.*, 2011).

In this project, activation of microvascular endothelial cells was determined first using mouse monoclonal antibody (W6/32) against HLA class I antigens. The binding between HLA class I antigens and W6/32 antibody occurs through monomorphic conformational epitopes shared by all HLA class I antigens. Amino acid residues present on β2-microglobulin and α3 domain are crucial for W6/32 antibody binding (Ladasky *et al.*, 1999). In contrast, allospecific antibodies are formed against polymorphic determinants of HLA class I antigens where the majority of polymorphism occurs in the α1 and α2 domains. Therefore, examining the role of these antibodies in inducing endothelial cell activation is fundamental. In this chapter, allospecific antibodies from multiparous females and sensitized kidney transplant patients who had specific HLA class I antibodies were used. Selection of the samples was carried out according to the specificity of HLA class I antigens expressed on HMEC-1 cells. As confirmation, monoclonal human HLA specific antibodies were also obtained to assess the activation of microvascular endothelial cells.

6.2 Specific Aims

In this chapter, allospecific HLA class I antibodies from both multiparous females and sensitized kidney patients who had a circulating HLA class I antibodies at the time of the rejection were used to assess changes in;

- The phosphorylation of endothelial cell signaling proteins Akt, ERK and CREB;
- The expression of cell surface adhesion molecules VCAM-1 and ICAM-1, and
- The expression of mRNA CXCL8.

6.3 Specific materials and methods

6.3.1 Sources of allospecific antibodies

HLA allospecific antibodies were obtained from previously characterized serum samples collected from multiparous females who had developed HLA class I antibodies during pregnancy. Samples were previously used as reagents in serological typing in NHSBT (Ethical approval reference: 11/NE/0091). In addition, serum samples from sensitized kidney patients who had circulating HLA class I antibodies at the time of graft rejection were also used. Serum samples were selected according to the presence of HLA antibodies specific to HMEC-1 endothelial cell line; determined using Luminex single antigen bead IgG assay. Antibodies were purified from 400-700 µl of sera. Samples from non-sensitized volunteers were processed in the same way and used as a negative control. In addition, serum sample from patients with HLA class I antibodies against antigens not present on HMEC-1 were also used as a negative control.

6.3.2 Antibody purification

For IgG antibody purification, between 400-700 µl of serum was diluted with PBS to a total volume of 1 ml. The antibody was injected into equilibrated protein G column (GE healthcare) which has a capacity to bind all subclasses of human IgG at high affinity. The column was washed with PBS to remove the unbound material and the elution step was performed using 0.1 M glycine (pH 2). The concentration of antibodies was determined using the BCA protein assay as described previously in section 2.8.3. The purified antibodies were adjusted to 4.5 mg/ml with sterile PBS.

6.3.3 Gel electrophoresis

To assess the presence and integrity of the purified antibodies, they were separated on 10% SDS gel under reducing condition to visualize the heavy and light chains. Eight µg of protein was loaded into each lane after heating with β-mercaptoethanol for 10 minutes. The gel was run at 30 mA for 1 hour and 30 minutes followed by staining with coomassie blue stain for 20 minutes at room temperature and destained as described previously in section 2.8.5. Figure 6.1 and Figure 6.2 show the coomassie blue staining of the gels for antibodies purified from multiparous females and sensitized kidney samples, respectively.

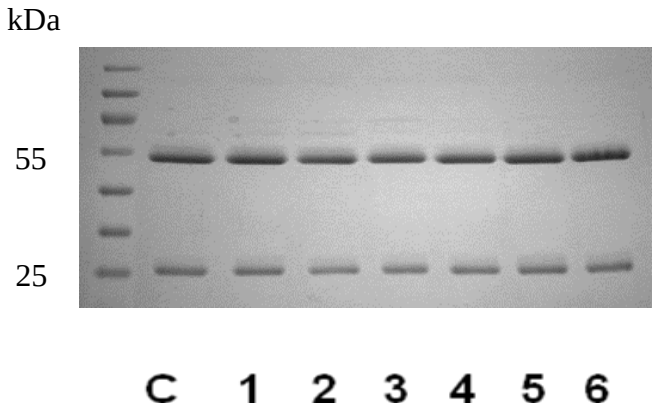


Figure 6.1: Gel electrophoresis of purified IgG from multiparous female samples.

Serum samples from multiparous females were passed through protein G column. 8 µg/ml of purified antibodies were run under reduced condition on 10 % SDS-PAGE gel. Gel was stained with comassie blue stain to visualize the heavy and light chains at 55 and 25 kDa, respectively. First band (C) is for negative control sample from non-sensitized volunteer and 1, 2, 3, 4, 5 and 6 for serum 1, serum 2, serum 3, serum 4, serum 5 and serum 6, respectively.

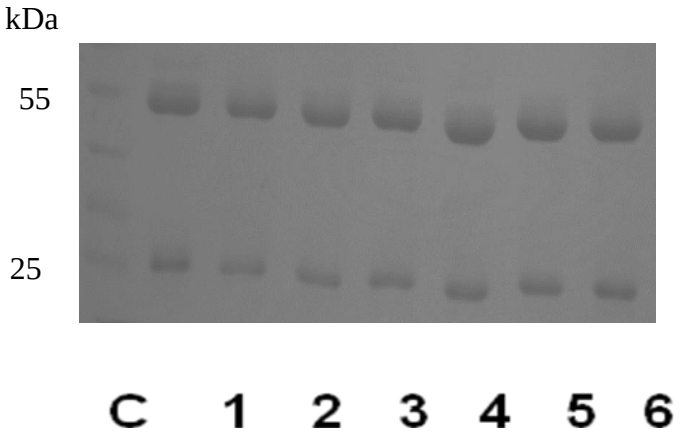


Figure 6.2: Gel electrophoresis of IgG antibodies purified from sensitized kidney patients.

Total IgG antibodies were purified from sensitized patient samples using protein G column. 8 µg of the purified IgG were separated under reduced condition on 10 % SDS-PAGE gel. Gel was stained with comassie blue to visualize the heavy and light chains. Band C is for the control sample containing HLA class I antibodies for antigens do not express on HMEC-1 cells and 1, 2, 3, 4, 5 and 6 for Patient 1, Patient 2, Patient 3, Patient 4, Patient 5 and Patient 6, respectively.

6.3.4 Indirect immunofluorescence

The ability of allospecific antibodies purified from human sera or monoclonal HLA class I antibody to bind HMEC-1 cells was examined by flow cytometry as described in section 2.7.2. In brief, $2X10^5$ HMEC-1 cells were stained with different concentrations of antibodies and incubated for 20 minutes at 4^0C. Following washing steps with 2% FBS/PBS, cells were incubated with anti-human IgG FITC conjugated antibody for 20 minutes at 4^0C followed by two washes with FBS/PBS solution. Cells were resuspended in 200 µl of FBS/PBS and analyzed by flow cytometry. Due to the limited amount of antibodies available, 0.18 mg/ml of purified IgG antibodies were used for all further experiments. Figure 6.3 shows the indirect immunofluorescence staining of the purified IgG from both multiparous and patient samples. Cells stained with secondary antibody only were used as a negative control.

6.3.5 Antibody screening using Luminex cytometer

6.3.5.1 General principle

Luminex flow cytometry is a solid phase assay which can be used to determine the presence of specific HLA antibodies. It is a more sensitive and specific technique than complement-dependent cytotoxicity and ELISA assays. The main advantages of this assay are specificity and sensitivity for HLA antibodies, while the other assays show positive reactions to other types of antibodies such as autoantibody and non-HLA antibodies. Another advantage of using Luminex assay is the ability to detect both complement and non-complement fixing antibodies, whilst complement-dependent cytotoxicity assays detect complement fixing antibody only. Luminex-antibody screening is performed by incubating patient serum with HLA antigen-coated beads followed by staining with fluorescence-conjugated secondary antibody followed by analysis using the Luminex platform. The beads are groups of polystyrene microspheres that have HLA molecules on their surfaces and each bead having a unique fluorochrome intensity that is specific for a bead group. There are three types of assays differentiated according to the attached HLA molecules. The first type contains beads that are attached to a large number of HLA class I and class II antigens which are used as a screening for the presence or absence of HLA antibodies. In the second type of assays, each bead contains two sets of antigens from either HLA class I or class II prepared from platelets (class I) or cell lines (class I or class II) in a manner similar to that present in human nucleated cells. The third type of assay contains recombinant single HLA

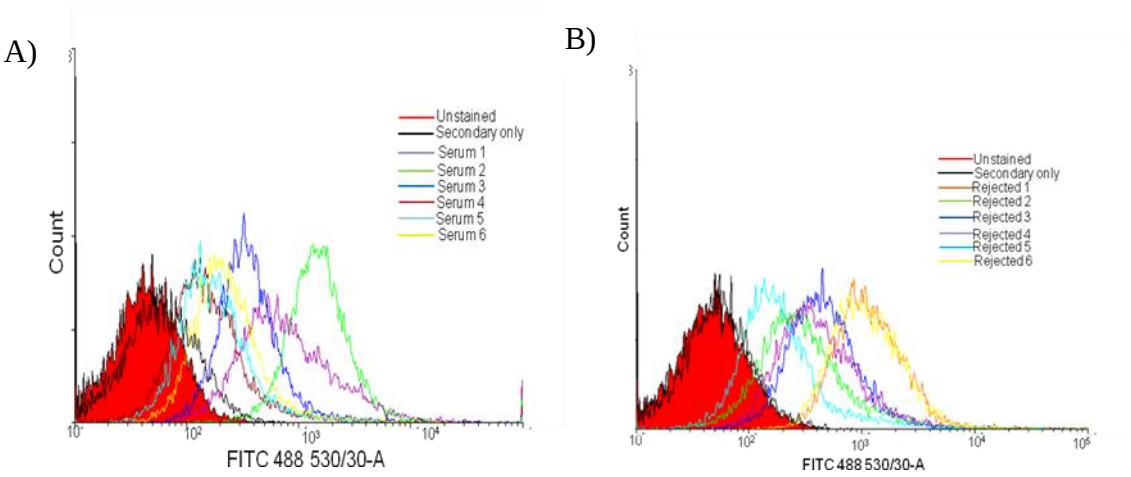


Figure 6.3: Indirect immunofluorescence staining of allospecific antibodies on HMEC-1 endothelial cells.

The cells were stained with allospecific purified IgG antibodies at concentration 0.18 mg/ml followed by anti-human IgG FITC conjugated antibody. Stained cells were run on a flow cytometer. Cells stained with secondary antibody only were used to show background staining. Panel A shows the staining of the purified antibodies from multiparous women samples and panel B shows the staining of purified antibodies from patients' sera. The data are representative of two independent experiments.

molecules attached to beads in form of two separate sets for class I or class II. This assay is called a single antigen bead and is used for the determination of HLA antibodies in sera with high panel reactive antibodies.

In all assays, the patient serum is incubated with beads at room temperature for around 30 minutes to allow antibody binding. Following washing steps, a secondary anti-human IgG PE-conjugated antibody is added which bind to any attached HLA antibody. Washed beads are resuspended in PBS and run on the Luminex platform. When the cells pass through the detector, the fluorochrome in the beads is excited by one laser on the instrument while the other laser excites the PE attached to the secondary antibody. The signals from these molecules collectively determine the antibody specificity in serum sample. The presence of HLA class I antibodies specific to HMEC-1 cells in the purified IgG antibodies was confirmed by single bead antigen Luminex assay.

6.3.5.1 **Antibody screening using Luminex assay**

Screening for HLA class I antibodies was performed using single antigen bead kit (One Lambda). The kit contains all the reagents required for the assay. Plate filters were washed once with diluted buffer followed by mixing 20µl of buffer, 8 µl of purified antibodies and 2 µl of beads. The plate was incubated at 22^0C for 30 minutes in dark on a rotating shaker. Following five washes, 40 µl of PE-conjugated antibody is added. The plate was incubated again at 22^0C for 30 minutes in a dark on a rotating shaker. After five washes, 80 µl of sheath fluid was added to each well and then run through Luminex LX200 cytometer. The data were analyzed using HLA Fusion software with cut-off at 500. Table 6.1 shows the purified samples with their specificities and median fluorescence intensity by both flow cytometry and Luminex assay. Figure 6.4 shows an example of the output sheet of the screening of purified IgG by single antigen bead Luminex assay.

6.3.6 Stimulation of endothelial cells using allospecific antibodies

For endothelial cell signalling, cells were stimulated with allospecific antibodies as described in section 4.3.2. In brief, adherent HMEC-1 cells were serum starved overnight in media containing 0.2% FBS. The cells were dissociated and resuspended in

Table 6.1: Allospecific human IgG purified from HLA sensitized individuals.

Sample	Antibodies	Luminex MFI	* Flow cytometry MFI
Serum 1 sensitized female	A1, A68	13129-4883	752
	B35, B58	2459-709	
	Cw4, Cw6	610-793	
Serum 2 sensitized female	A68	14632	3358
	B58	11343	
	Cw4	1281	
Serum 3 sensitized female	A1, A68	3146-7410	1342
	B35, B58	11785-3161	
	Cw6	1272	
Serum 4 sensitized female	B58	4369	925
	Cw6	1694	
Serum 5 sensitized female	A68	791	863
	Cw4, Cw6	8957-5732	
Serum 6 sensitized female	Cw4, Cw6	8952-14771	959
Patient 1 Kidney patients	B35, B58	2971-1833	4462
	Cw4, Cw6	11477-17766	
Patient 2 Kidney patients	A1, A68	7795-5083	728
	Cw4, Cw6	1126-620	
Patient 3 Kidney patients	B35, B58	17741-12712	1256
Patient 4 Kidney patients	A1, A68	11619-2589	844
	B35, B58	11399-7849	
	Cw4, Cw6	672-728	
Patient 5 Kidney patients	A1, A68	7210-4845	2854
Patient 6 Kidney patients	B35-, B58	910-970	3410
	Cw4, Cw6	9208-15649	
Negative control	A66	813	68
Non-specific HLA class I antibody	B7, B27	3569, 6728	80

* MFI, Median Fluorescence Intensity. Each sample represents the absolute value after the subtraction the MFI of secondary antibody stained cells which are equal to 166 in the multiparous females' sera and 201 in patient samples.

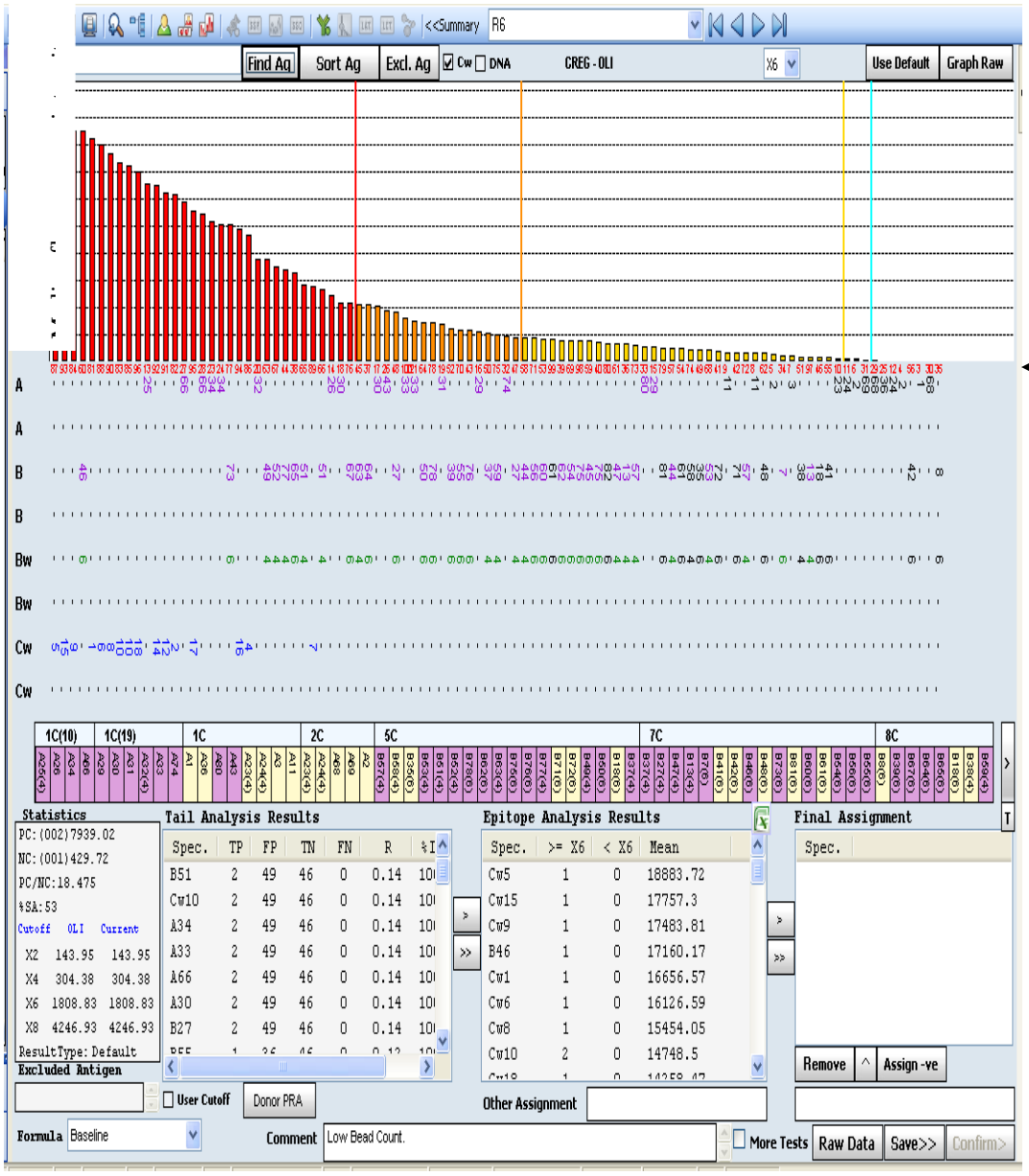


Figure 6.4: Output of a single antigen bead assay for HLA class I antibody determination.

Purified antibodies were screened by single bead antigen assay using labscreen kit. The samples were run through Luminex LX200 and analyzed using HLA Fusion software.

0.2% FBS containing media and incubated at 37^0C for 2 hours. Cells were then stimulated with 0.18 mg/ml of human IgG for 5 minutes. The stimulation was terminated by adding cold PBS and centrifuged at a cold centrifuge at 10,000 g for 3 minutes. Cells were then lysed in a lysis buffer containing phosphatase inhibitor as described previously in section 2.8.2. Cell lysates were used for the determination of p-ERK, p-Akt and p-CREB as described in section 4.3.2.

To determine the expression of adhesion molecules by endothelial cells, adherent cells were stimulated overnight in a 12 well plate with purified antibodies at concentration 0.18 mg/ml. Following incubation, adherent cells were detached using enzyme-free PBS-based cell dissociation buffer. $1X10^5$ cells were stained with mouse anti-human PE-conjugated antibody for VCAM-1 and ICAM-1 for 20 minutes at 4^0C. Following washing, the expression of adhesion molecules was determined by flow cytometry.

6.3.7 Assessment of the specificity of isolated IgG antibodies to endothelial HLA class I antigens.

Polyclonal human sera were selected according to the presence of HLA specific antibodies confirmed by Luminex assay. As these samples are polyclonal, experiments were designed to confirm that the cell signalling was due to the HLA class I antibodies. Acid treatment of HMEC-1 cells as described in section 3.3.4 and knocking down of HLA class I antigens were used to confirm the specificity of the signalling.

6.3.7.1 Acid treatment of HMEC-1 cells

Endothelial cells were treated with citrate solution (pH 3) as described in section 3.3.4. After treatment, around $2X10^5$ cells were stained with allospecific antibodies at final concentration of 0.18 mg/ml for 20 minutes at 4^0C. After two washes with 2% FBS/PBS, cells were stained with anti-human IgG FITC conjugated antibody for another 20 minutes at 4^0C. After washing, cells were resuspended in 200 µl FBS/PBS and analyzed by flow cytometry. Cell viability was assessed by PI staining. Treatment did not induce more than 10% cell death.

6.3.7.2 **Knockdown efficiency of HLA class I antigens (siRNA) using W6/32 antibody**

To knockdown the expression of HLA class I antigens, three different pre-designed siRNA were used with following specificity: HLA-A locus heavy chain siRNA, HLA-B locus heavy chain siRNA and HLA-C locus heavy chain siRNA (Applied Biosystems). These were supplied as purified and dried forms which were reconstituted using RNase free water at stock solution 5 μM and working solution at 2 μM. The transfection process was performed as described in section 5.3.4.7. To knockdown all three HLA class I antigens, three different siRNA were mixed together at final concentration 5, 10 or 20 nM for each siRNA. Where specified, B locus siRNA alone was used to assess the efficiency of HLA-B antigen knockdown. The transfection efficiency was determined by examining the expression HLA class I antigens at different time points and siRNA concentrations. Non-transfected cells, negative control siRNA and locus-specific siRNA HLA class I transfected cells were stained with 12 μg/ml mouse W6/32 antibody followed by FITC conjugated rabbit anti-mouse secondary antibody as described in section 3.3.7.5. The cells were detached and analyzed by flow cytometry.

6.3.7.3 **Knockdown efficiency using human monoclonal HLA class I antibody**

To assess the knockdown efficiency for each locus, monoclonal HLA specific antibodies were used. Human monoclonal antibody against specific HLA class I antigen expressed on HMEC-1 cells was kindly provided by Dr. Arned mulder, Leiden University Medisch Centrum, Department Immunohaematology and Blood Transfusion. Four different IgG1 antibodies (250 μg of each constituted in sterile PBS) at different concentrations were obtained with the following specificities; A2/B17, A2/A28, A1/A9 and B51/B35 which bind B58, A68, A1 and B35 on HMEC-1 cells, respectively. These antibodies were obtained by heterohybridoma followed by antibody purification through protein A column (Mulder *et al.,* 2010). The concentration of each antibody was adjusted to a working concentration of 1mg/ml. The binding of these antibodies to HMEC-1 endothelial cells were examined by flow cytometry as described in section 6.3.4. Cells were stained with these antibodies followed by staining with FITC-conjugated anti-human secondary antibody and flow cytometry analysis. Figure 6.5 shows the staining of HMEC-1 cells with 40 μg/ml of human monoclonal HLA class I specific antibody. Due to limited amount of these antibodies, this concentration was chosen for all subsequent experiments.

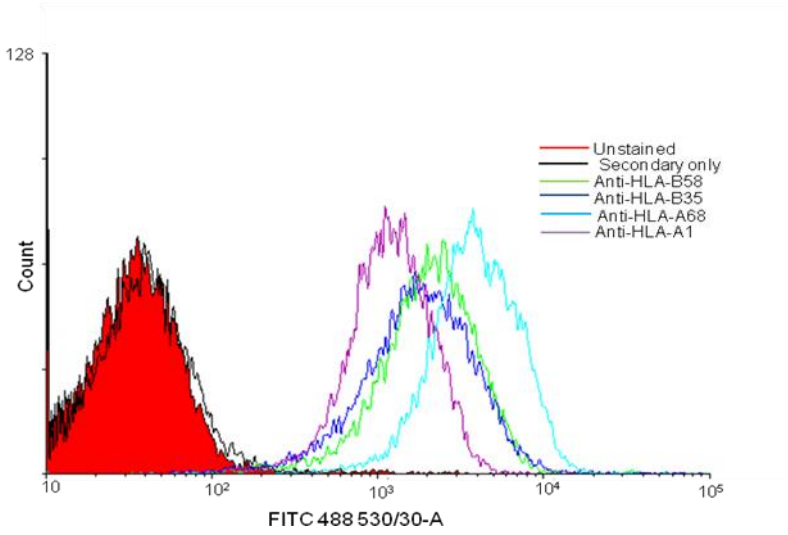


Figure 6.5: Indirect immunofluorescence staining of HMEC-1 cells with human monoclonal HLA class I antibody.

HMEC-1 cells were stained with 40 µg/ml of each antibody followed by FITC conjugated anti-human IgG secondary antibody. Cells stained with secondary antibody only were used to determine background staining. Cells were analyzed by flow cytometry and interpreted by WinMDI 2.9 software.

6.3.8 p-CREB Cell based ELISA

Phosphorylation of CREB at serine residue 133 was performed using cell based ELISA kit (R&D system). The kit contains all reagents necessary for the determination of human p-CREB including; rabbit p-CREB, goat T-CREB, HRP-conjugated donkey anti-rabbit, alkaline phosphatase-conjugated donkey anti-goat antibodies, fixing solution, blocking buffer and substrates. The assay was performed on HMEC-1 cells transfected with HLA-B siRNA in response to HLA-B58 antibody stimulation. HMEC-1 cells were grown in 96 well plates until confluency. Nontransfected and transfected HMEC-1 cells were serum starved in media containing 0.2% FBS for 2 hours. Cells were left un-stimulated or were stimulated for 5 minutes with 40 µg/ml of human monoclonal antibody or different concentrations of forskolin ranging from 1-50 µM as a positive control. Media was removed and 100 µl of fixing solution was added immediately. The following steps were performed following manufacturers' instruction. The plate was read on fluorescence plate reader (Biotek, UK) at excitation 360 nm and emission 450 nm for T-CREB and at excitation 540 nm and emission 600 nm for p-CREB. The ratio between the p-CREB and T-CREB was calculated and plotted. Figure 6.6 shows p-CREB cell-based ELISA on HMEC-1 cells stimulated with forskolin.

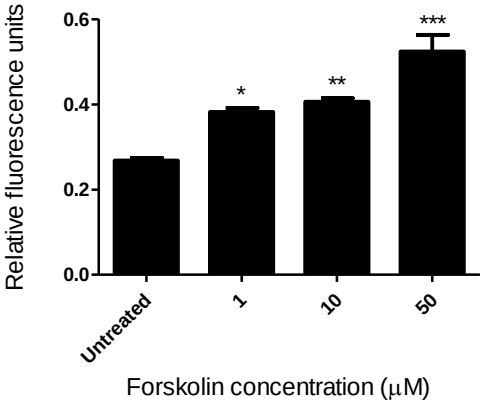


Figure 6.6: p-CREB cell based ELISA on HMEC-1 endothelial cells stimulated with forskolin.

Adherent HMEC-1 cells were stimulated with different concentrations of forskolin for 5 minutes followed by cell fixation. Cells were incubated with antibodies specific to p-CREB and T-CREB followed by conjugated secondary antibodies. Fluorescence intensity was measured using fluorescence plate reader at excitation 360 and 540nm and emission 450 and 600nm for T-CREB and p-CREB, respectively. The data was analyzed by one-way ANOVA followed by Bonferroni test (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$). The data are representative of two independent experiments.

6.4 Results

6.4.1 Effect of allospecific antibodies on endothelial cell signalling

6.4.1.1 Effect of allospecific antibodies from multiparous females on endothelial cell signalling

The effect of allospecific HLA class I antibodies purified from sensitized individuals on activation of endothelial signalling was determined. Six samples from multiparous females containing HLA class I antibodies specific to HMEC-1 cells were used. Samples were screened for the presence of HLA antibodies using single antigen bead Luminex assay both before and after purification. Purified IgG antibodies were adjusted to 4.5 mg/ml and used at concentration 0.18 mg/ml for endothelial cell activation. HMEC-1 cells were stimulated with these antibodies for 5 minutes and assessed for the phosphorylation of ERK, Akt and CREB proteins by western blotting. Stimulation of endothelial cells with allospecific antibodies for 5 minutes induced the phosphorylation of these proteins at specific residues. Figure 6.7 shows the phosphorylation of ERK1/2 at tyrosine 202 and threonine 204 residues in response to these antibodies when compared to negative controls. Untreated cells, cells treated with purified IgG from HLA negative sample were used as negative controls. Figure 6.8 shows the Akt phosphorylation at serine residue (473) on stimulated HMEC-1 endothelial cells compared to negative control. The phosphorylation of the transcription factor CREB at serine residue 133 and ATF-1 in response to these antibodies is shown in Figure 6.9.

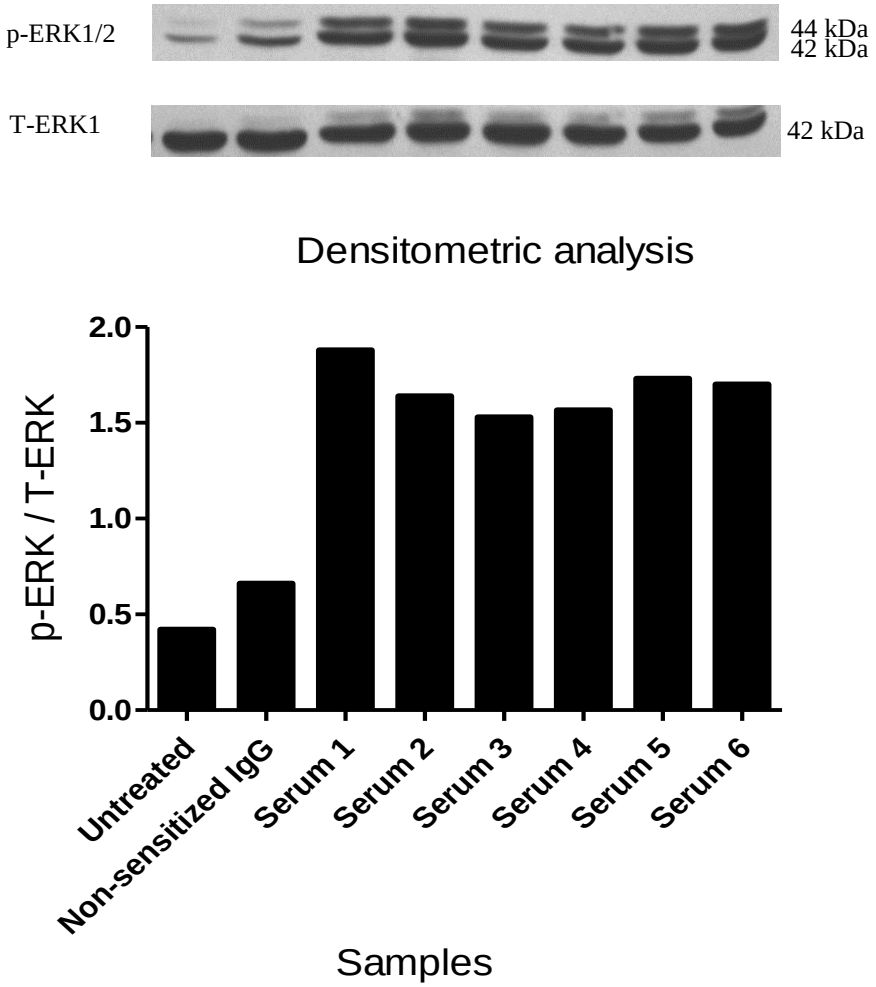


Figure 6.7: Effect of HLA class I allospecific antibodies purified from multiparous female samples on endothelial ERK phosphorylation.

HMEC-1 cells were stimulated for 5 minutes with purified 0.18 mg/ml IgG antibodies from samples followed by cell lysis. Cell lysates were probed with p-ERK1/2 antibody. The membrane was stripped and probed for T-ERK using T-ERK antibody. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

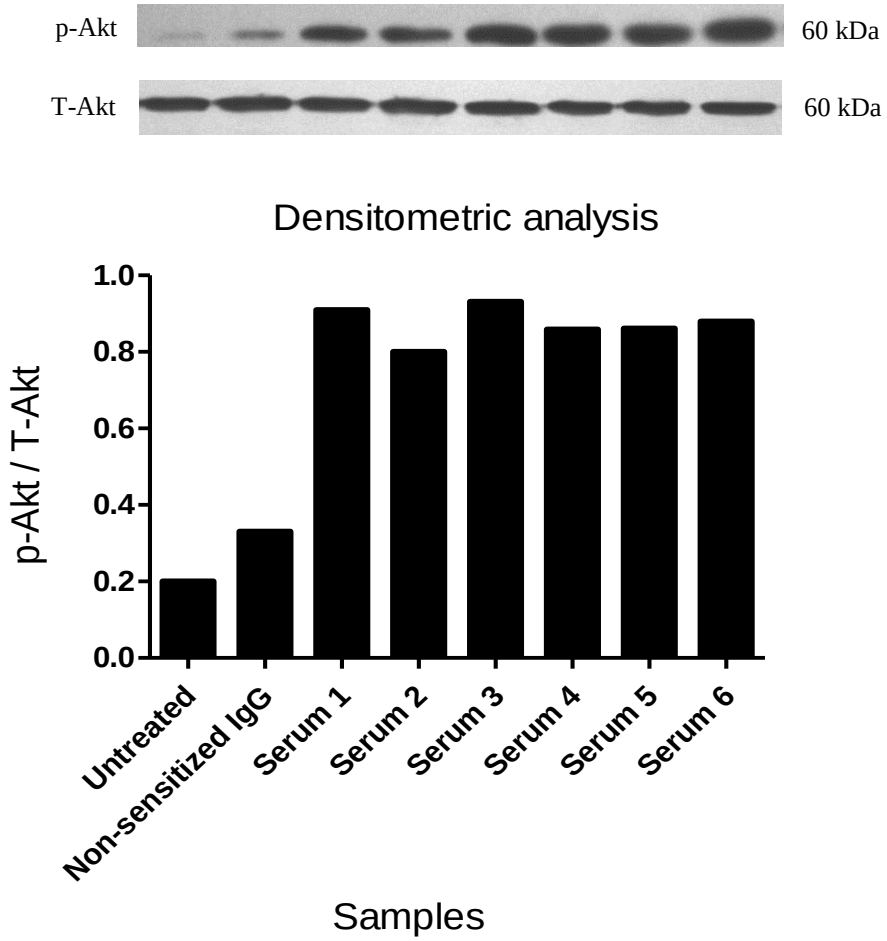


Figure 6.8: Effect of HLA class I allospecific antibodies purified from multiparous female samples on endothelial Akt phosphorylation.

HMEC-1 cells were stimulated for 5 minutes with 0.18 mg/ml of IgG antibodies purified from samples before probing with p-Akt antibody (serine residue 473). The membrane was stripped and probed for T-Akt using rabbit T-Akt antibody. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

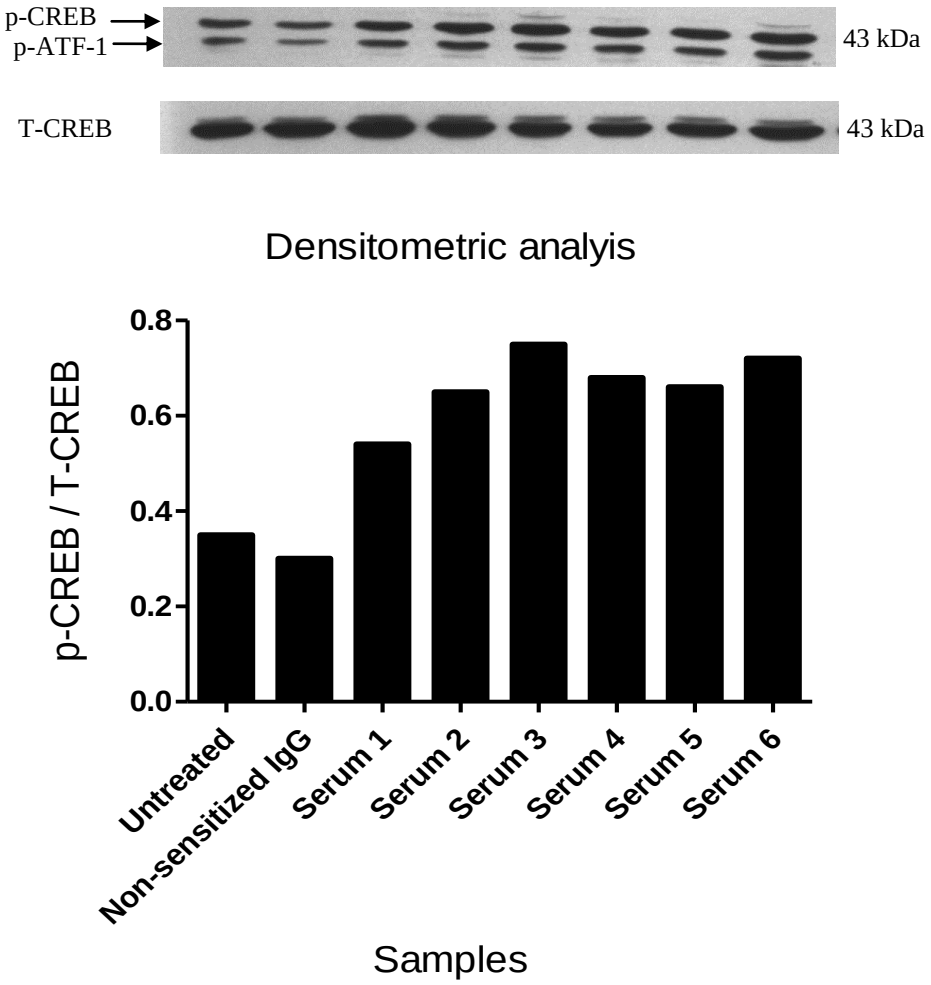


Figure 6.9: Effect of HLA class I allospecific antibodies purified from multiparous female samples on endothelial CREB phosphorylation.

HMEC-1 cells were stimulated with 0.18 mg/ml IgG antibodies purified from samples for 5 minutes before probing with rabbit p-CREB antibody (serine residue 133). The membrane was stripped and probed for T-CREB. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

6.4.1.2 **Effect of allospecific antibodies from sensitized patients on endothelial cell signalling**

The activation of endothelial cell signalling in response to HLA class I allospecific antibodies from sensitized kidney patients was performed. Six patient samples were selected and total IgG antibodies was purified. These patients developed high level of HLA class I antibodies, detectable at the time of allograft rejection. The concentration of each preparation was adjusted to 4.5 mg/ml and used at concentration of 0.18 mg/ml. HMEC-1 endothelial cells were stimulated with these antibodies and examined for the phosphorylation of ERK, Akt and CREB. Treatment of endothelial cells with these antibodies induced a significant increase in the level of the phosphorylated forms of all proteins examined. Figure 6.10 shows the phosphorylation of ERK at tyrosine 202 and threonine 204 residues following 5 minutes of stimulation compared to the control groups; untreated cells, cells treated with IgG from non-sensitised volunteer and non-specific HLA class I IgG antibodies. In addition, the phosphorylation of Akt at serine residue 473 is shown in Figure 6.11. Stimulation of endothelial cells with control IgG did not induce significant Akt phosphorylation. Figure 6.12 illustrates the phosphorylation of endothelial transcription factor CREB at serine residue 133 and ATF-1 in response to antibodies from kidney patients compared to controls.

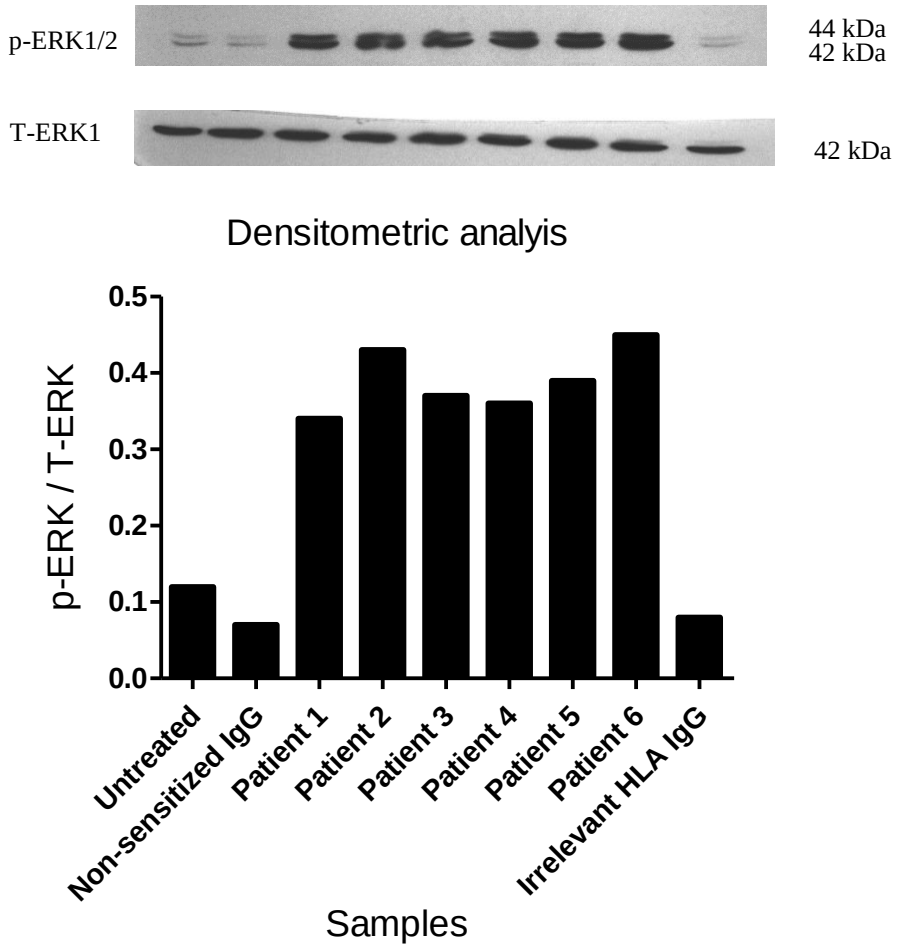


Figure 6.10: Effect of allospecific HLA class I antibodies purified from sensitized kidney patients on endothelial ERK phosphorylation.

HMEC-1 cells were stimulated with 0.18mg/ml of IgG antibodies purified from sensitized kidney patients for 5 minutes before probing with rabbit p-ERK antibody. The membrane was stripped and probed for T-ERK antibody. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

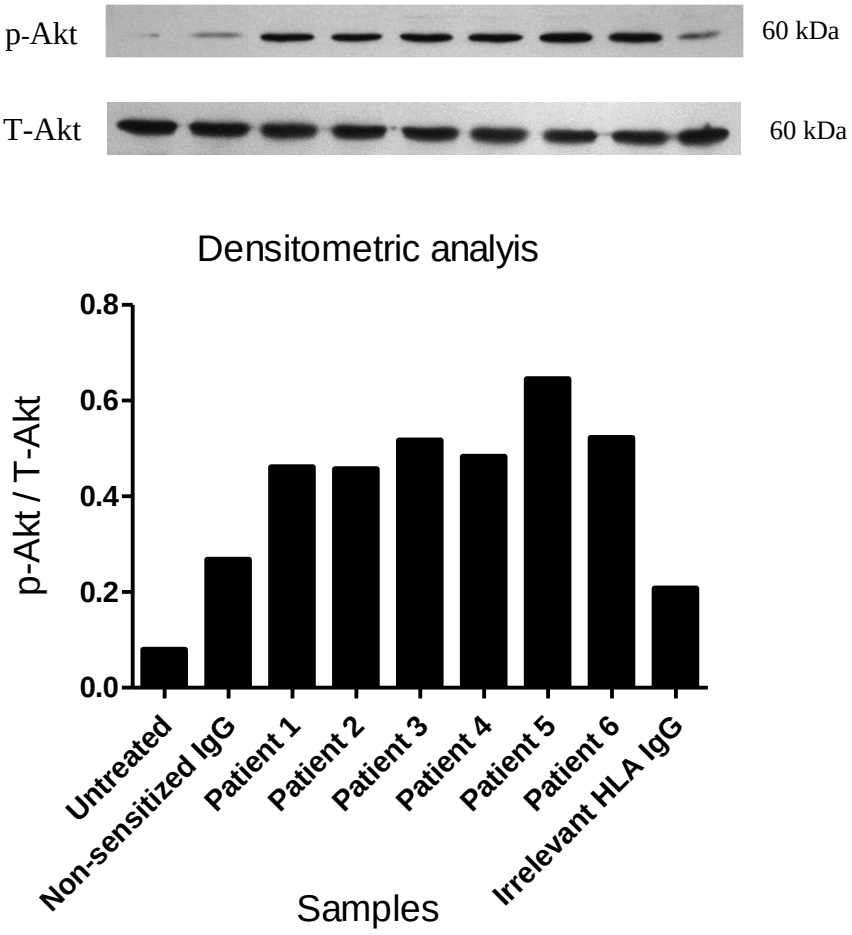


Figure 6.11: Effect of allospecific HLA class I antibodies from kidney patients on Akt phosphorylation.

HMEC-1 cells were stimulated with 0.18 mg/ml of IgG antibodies purified from kidney patients for 5 minutes before probing with rabbit p-Akt antibody. The membrane was stripped and probed with rabbit T-Akt antibody. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

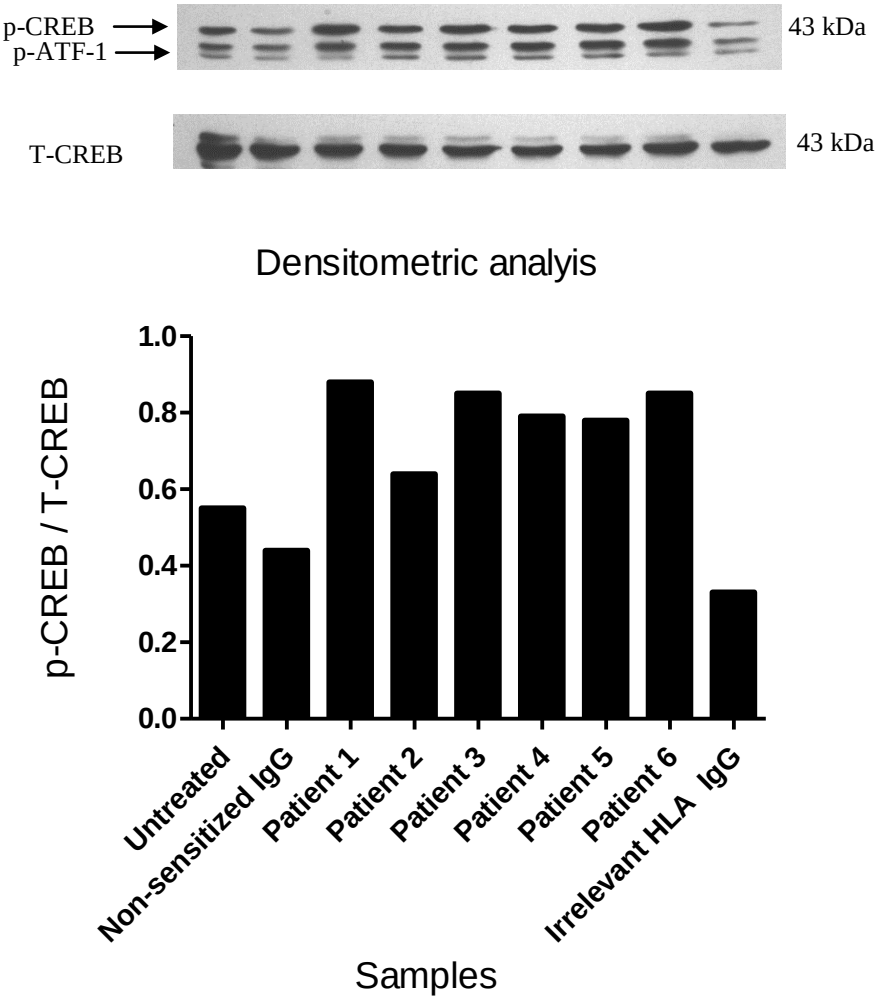


Figure 6.12: Effect of allospecific HLA class I antibodies from sensitized kidney patients on CREB phosphorylation.

HMEC-1 cells were stimulated with 0.18mg/ml of IgG antibodies purified from sensitized kidney patients for 5 minutes before probing with rabbit p-CREB antibody. The membrane was stripped and probed for T-CREB. The densitometric analysis was performed using Alphaimager software. The data are representative of two independent experiments.

6.4.2 Effect of allospecific antibodies on adhesion molecules expression

6.4.2.1 Effect of antibodies from Multiparous females on adhesion molecules expression

To explore the effect of allospecific HLA class I antibodies on inducing the adhesion properties of endothelial cells, the modulation of the expression of VCAM-1 and ICAM-1 antigens in response to these antibodies was examined. IgG antibodies from six different multiparous female samples was examined for the activation of these cell surface antigens on HMEC-1 cells. Endothelial cells were stimulated overnight with antibodies at a concentration of 0.18 mg/ml. After stimulation, cells were stained with PE-conjugated anti-human VCAM-1 and ICAM-1 antibodies and analysed by flow cytometry. Untreated cells and cells stimulated with IgG antibodies from non-sensitised volunteer were used as negative control groups. As shown in Figure 6.13, expression of VCAM-1 molecules on HMEC-1 cells was significantly induced after stimulation with allospecific antibodies compared to both control groups. The upregulation in the expression of VCAM-1 was variable between samples and ranged between 2 to 3 fold. Similarly, the expression of endothelial ICAM-1 was significantly induced in response to these antibodies as shown in Figure 6.14, ($p<0.001$). Treatment induced around 10 to 30 fold increase in the expression of ICAM-1 compared to both control groups.

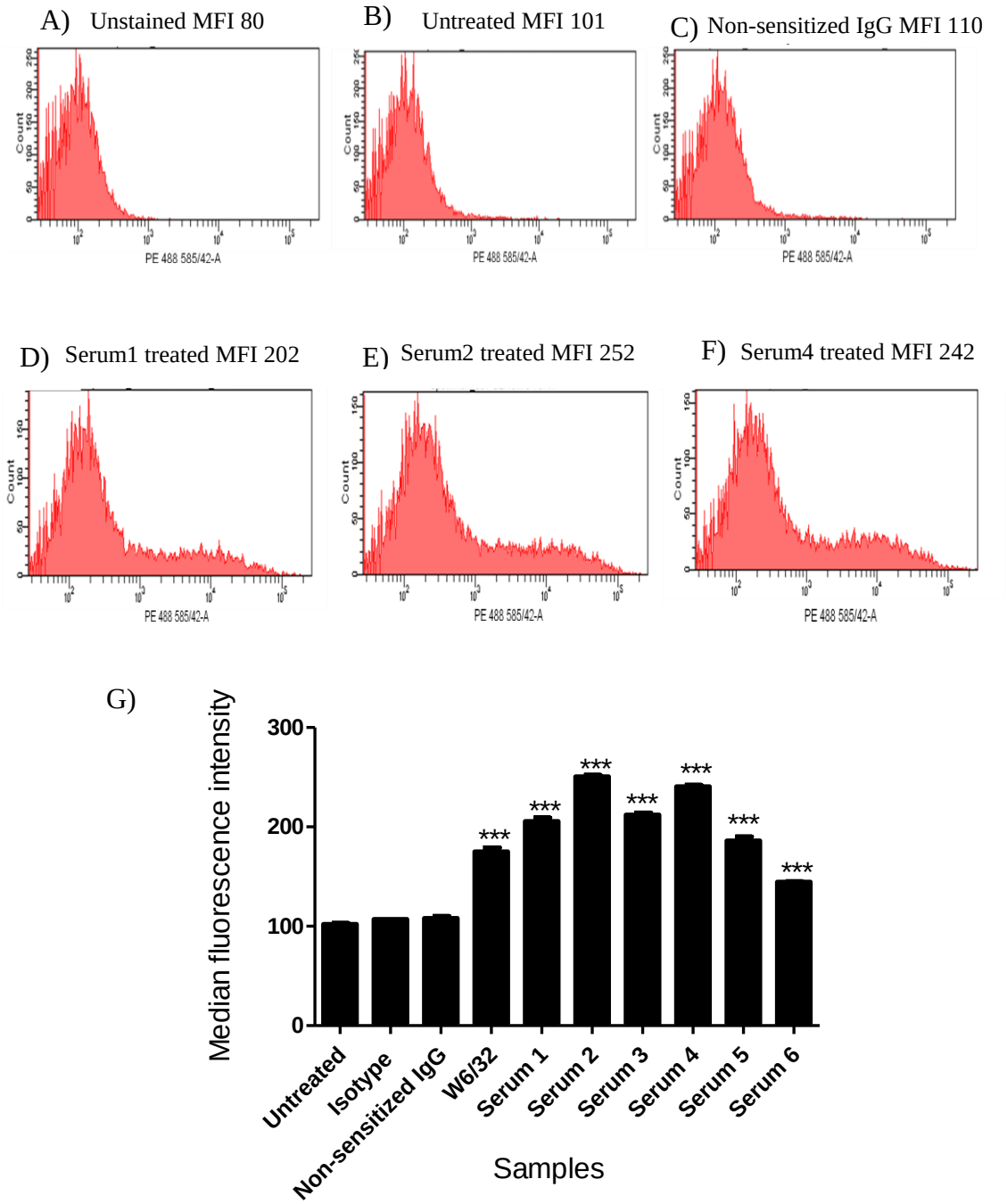


Figure 6.13: Effect of allospecific HLA class I antibodies purified from multiparous female samples on VCAM-1 expression.

HMEC-1 cells were stimulated overnight with 0.18 mg/ml of IgG antibodies purified from multiparous samples before the analysis of the expression of VCAM-1 molecules by flow cytometry. Histograms A, B, C, D, E and F represent unstained, untreated, normal IgG, serum 1, serum 2 and serum 4 treated, respectively. Analysis was performed by one-way ANOVA followed by Bonferroni test. *** $p<0.001$. The data are representative of two independent experiments.

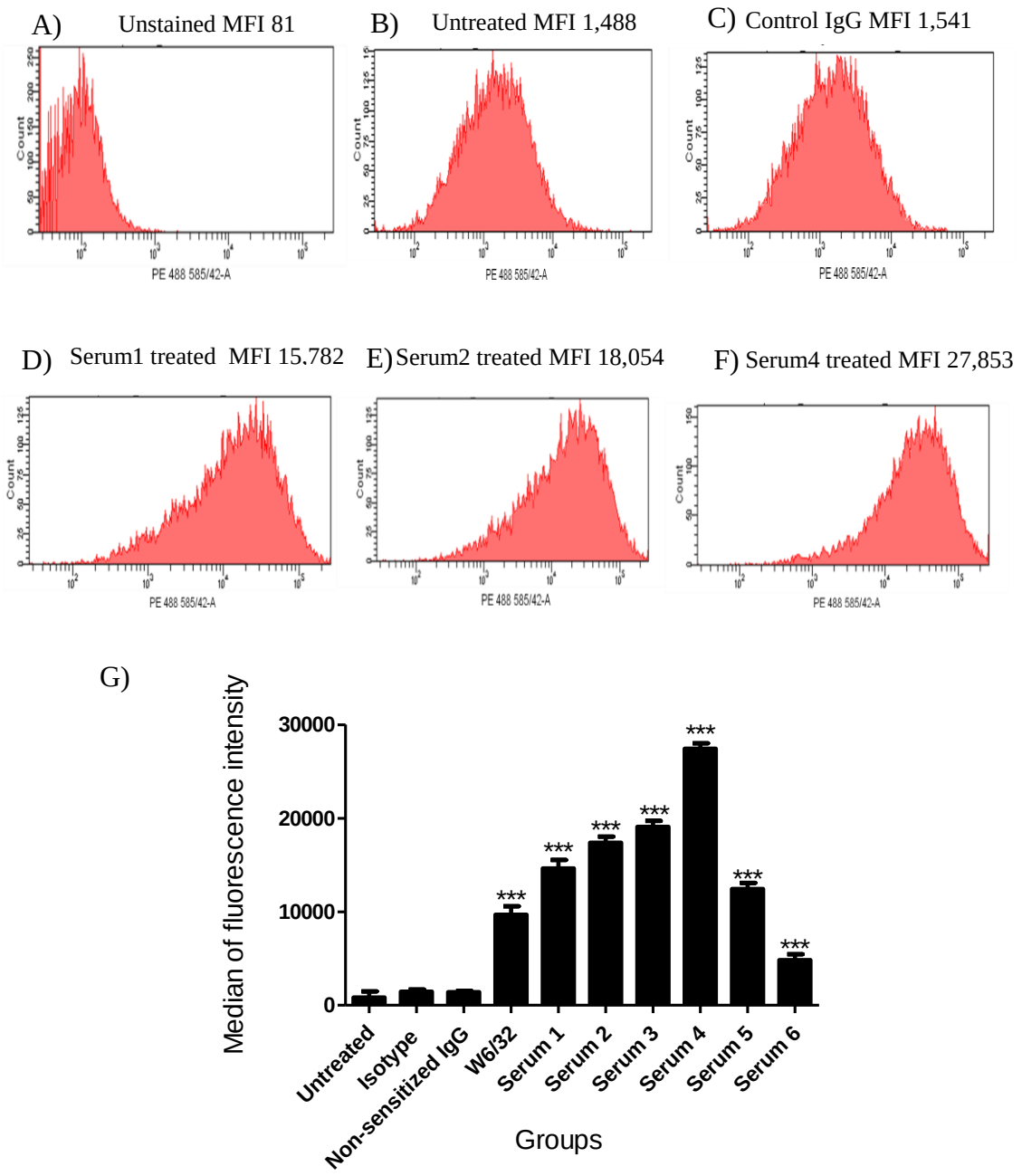


Figure 6.14: Effect of allospecific HLA class I antibodies purified from multiparous female samples on ICAM-1 expression.

HMEC-1 cells were stimulated overnight with IgG antibodies purified from samples before analysing the expression of ICAM-1 molecules using flow cytometry. Histograms A, B, C, D, E and F represent unstained, untreated, normal IgG, serum 1, serum 2 and serum 4 treated, respectively. Data analysis was performed using one-way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representative of two independent experiments.

6.4.2.2 **Effect of allospecific antibodies from sensitized patients on the expression of endothelial adhesion molecules**

Allospecific HLA class I antibodies purified from kidney patients who formed antibodies to HLA antigens to previous graft were also used to examine the expression of endothelial adhesion molecules. Expression of both VCAM-1 and ICAM-1 were determined using flow cytometry after stimulation with 0.18 mg/ml of antibodies. Both normal IgG antibodies as well as irrelevant HLA class I antibodies were used as control groups. Stimulation with antibodies from sensitised patients significantly induced the expression of both antigens compared to cells treated with irrelevant antibodies. As shown in Figure 6.15, these antibodies significantly induced the expression of VCAM-1 molecules in comparison to control groups. The stimulation induced around a 2 fold increase in the cell surface expression of VCAM-1 when compared to IgG antibodies from non-sensitized volunteer. Figure 6.16 shows the upregulation in the expression of ICAM-1 antigens in response to allospecific HLA class I antibodies from kidney patients. The expression was increased by around 10 to 30 fold when compared with serum containing HLA class I antibodies which were not specific for the HLA class I antigens on HMEC-1 cells, $p<0.001$.

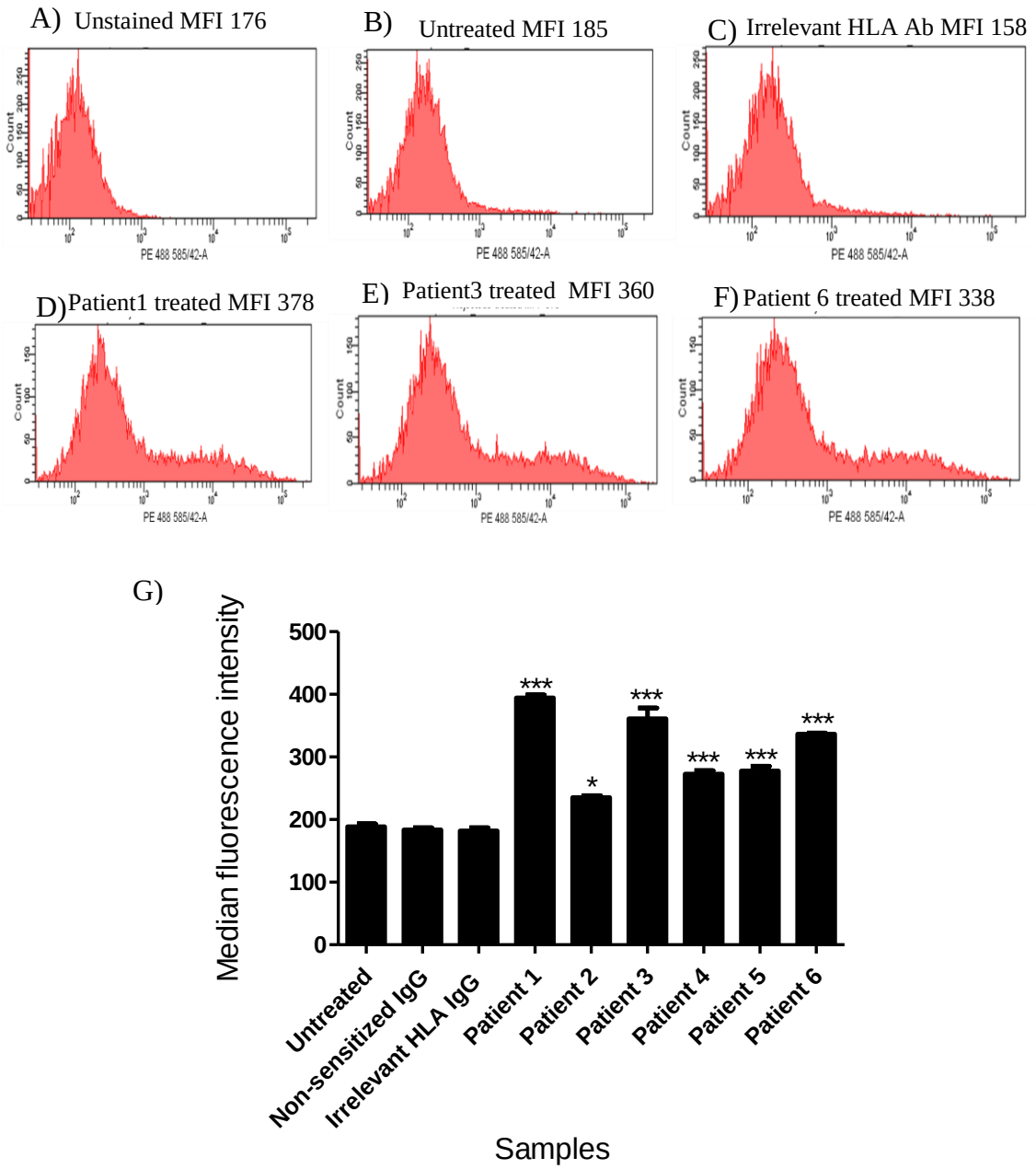


Figure 6.15: Effect of allospecific HLA class I antibodies purified from sensitized kidney patients on VCAM-1 expression.

HMEC-1 cells were stimulated overnight with 0.18 mg/ml of IgG antibodies purified from sensitized kidney patients before analysing the expression of VCAM-1 molecules using flow cytometry. Histograms A, B, C, D, E and F represent: unstained, untreated, irrelevant IgG, patient 1, patient 3 and patient 6, respectively. The data analysis was performed using one-way ANOVA followed by Bonferroni test at $p<0.05$. * & *** $P<0.05$ and $p<0.001$, respectively. The data are representative of two independent experiments.

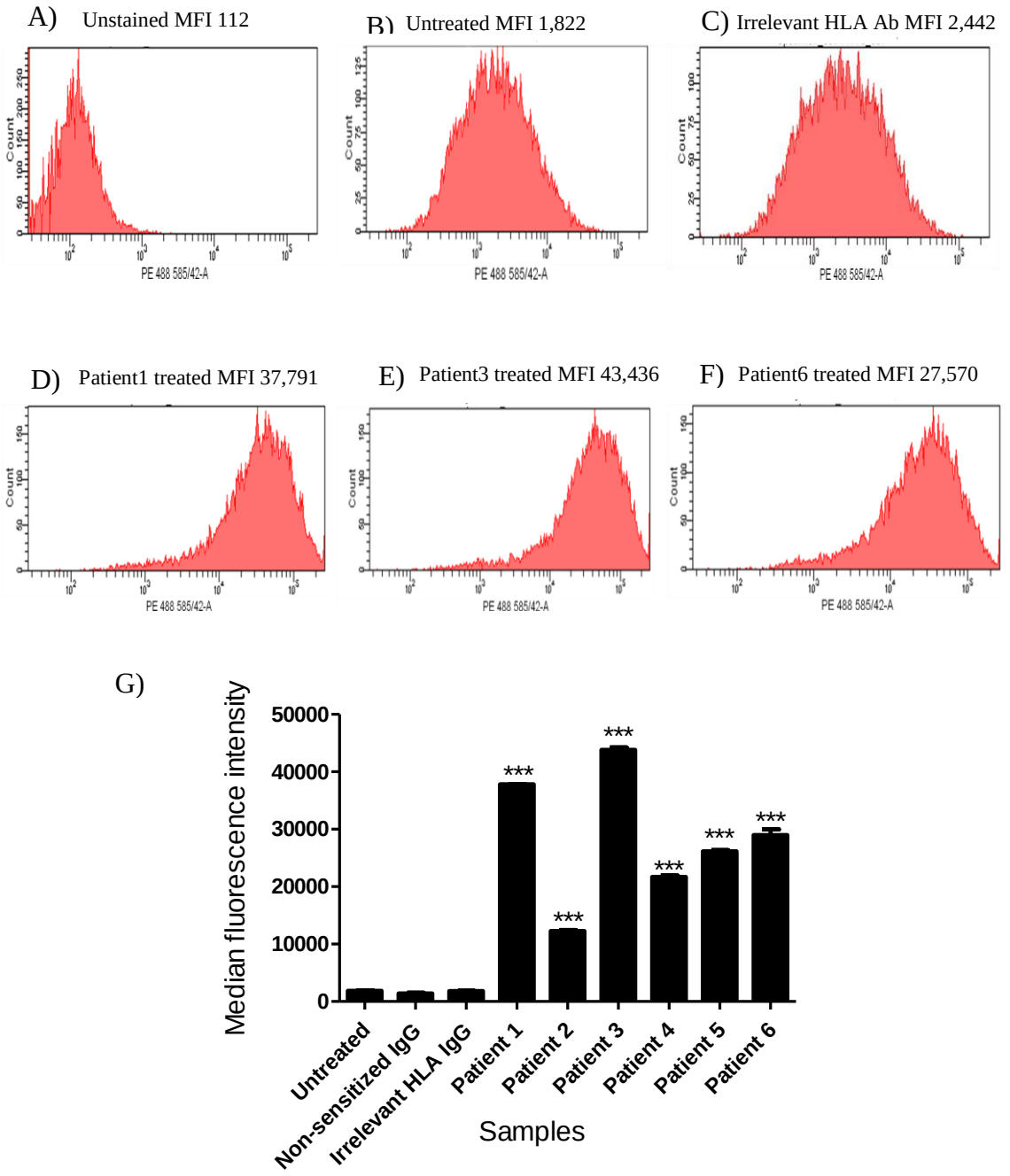


Figure 6.16: Effect of allospecific HLA class I antibodies purified from sensitized kidney patients on ICAM-1 expression.

HMEC-1 cells were stimulated overnight with 0.18 mg/ml of IgG antibodies purified from sensitized kidney patients before analysing the expression of ICAM-1 molecules using flow cytometry. Histograms A, B, C, D, E and F represent unstained, untreated, irrelevant HLA class I antibodies, patient 1, patient 3 and patient 6, respectively. The data were analysed by one-way ANOVA followed by Bonferroni test. *** $p<0.001$. The data are representative of two independent experiments.

6.4.3 Effect of allospecific HLA class I antibodies on CXCL8 expression

Purified allospecific IgG antibodies were examined for their ability to induce the expression of chemotactic the chemokine, CXCL8, at the molecular level. Both groups of samples were used for this experiment. Purified antibodies from multiparous female samples and from sensitized patients were used at concentration 0.18 mg/ml. Endothelial cells (HMEC-1) were stimulated with these antibodies overnight before RNA was extracted. The expression of CXCL8 was then determined by q-PCR. As shown in Figure 6.17, stimulation with IgG from multiparous female significantly induced the expression of endothelial CXCL8. The increase ranged between 3 to 14 fold when compared to the negative controls. IgG from non-sensitised volunteer was used as a negative control and they did not show significant difference compared to untreated group. The fold change for each sample was calculated in comparison to the normal IgG sample using GAPDH as a housekeeping gene. Figure 6.18 shows the expression of CXCL8 at molecular level following stimulation with purified IgG allospecific antibodies from kidney patients. The expression was significantly induced by 3 to 300 fold change when compared to non-specific HLA class I IgG allo-antibodies. Stimulation with HLA class I antibodies not specific for antigens on HMEC-1 cells showed a non significant change in the expression of mRNA CXCL8 when compared to the untreated group. GAPDH was used as a housekeeping gene.

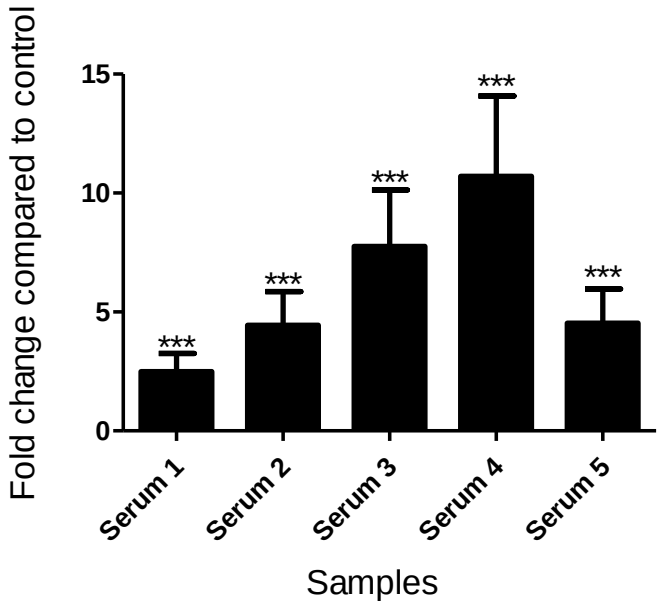


Figure 6.17: Effect of allospecific HLA class I antibodies purified from multiparous female samples on CXCL8 expression.

HMEC-1 cells were stimulated overnight with 0.18 mg/ml of IgG antibodies before analysing the expression of mRNA CXCL8 by q-PCR. The fold change was calculated in comparison to cells treated with IgG antibodies from non-sensitised sample using GAPDH as the normalizing gene. The data analysis was performed using REST 2009 software. *** $p<0.001$. The data are representative of two independent experiments.

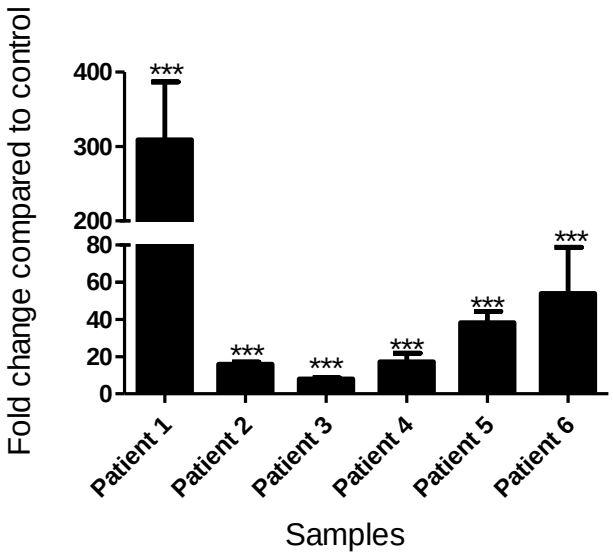


Figure 6.18: Effect of allospecific HLA class I antibodies from kidney patients on CXCL8 expression.

HMEC-1 cells were stimulated overnight with 0.18 mg/ml of IgG antibodies purified from kidney patients before analysing the expression of CXCL8 by q-PCR. The fold change was calculated in comparison to HLA class I antibodies that not specific to HMEC-1 cells and normalized to GAPDH. The data analysis was performed using REST 2009 software. *** $p<0.001$. The data are representative of two independent experiments.

6.4.4 Examination of the specificity of the purified IgG antibodies by acid treatment

Human sera were chosen according to the specificity of the HLA class I antibodies, determined by the Luminex assay. However, confirmatory experiments were performed to ensure that activation was due to HLA class I antibodies. Endothelial cells were treated with citrate buffer (pH 3) for 2 minutes followed by staining with purified allospecific IgG. Stained cells were analysed by flow cytometry. As shown in section 3.4.4, this treatment induced 70-80% reduction in the expression of HLA class I molecules as observed by staining with W6/32 antibody. Purified IgG antibodies were tested for their ability to bind sodium citrate-treated cells. Staining of treated cells with purified IgG from multiparous female samples showed different degrees of binding. Sample 2 showed ~30-40% reduction in the binding to treated endothelial cells. Other samples showed a reduction in the binding to treated cells although this was not statistically significant. The median fluorescence intensity of all six multiparous female samples before and after acid treatment is shown as a bar chart in Figure 6.19. Histograms for two samples are displayed using secondary anti-human antibody as a background staining. Purified IgG from sensitized kidney patients were examined by the same method. As shown in Figure 6.20, purified IgG from sensitized patients showed ~40-60% reduction in antibodies binding to acid treated endothelial cells compared to untreated cells, although sample 4 did not show significant reduction in binding. Median fluorescence intensity for each sample on HMEC-1 endothelial cells before and after acid treatment was plotted in bar chart. Histograms from two patient samples are shown as an example. Cell viability was assessed by staining with PI. There was no significant difference in cell death between acid treated and untreated cells.

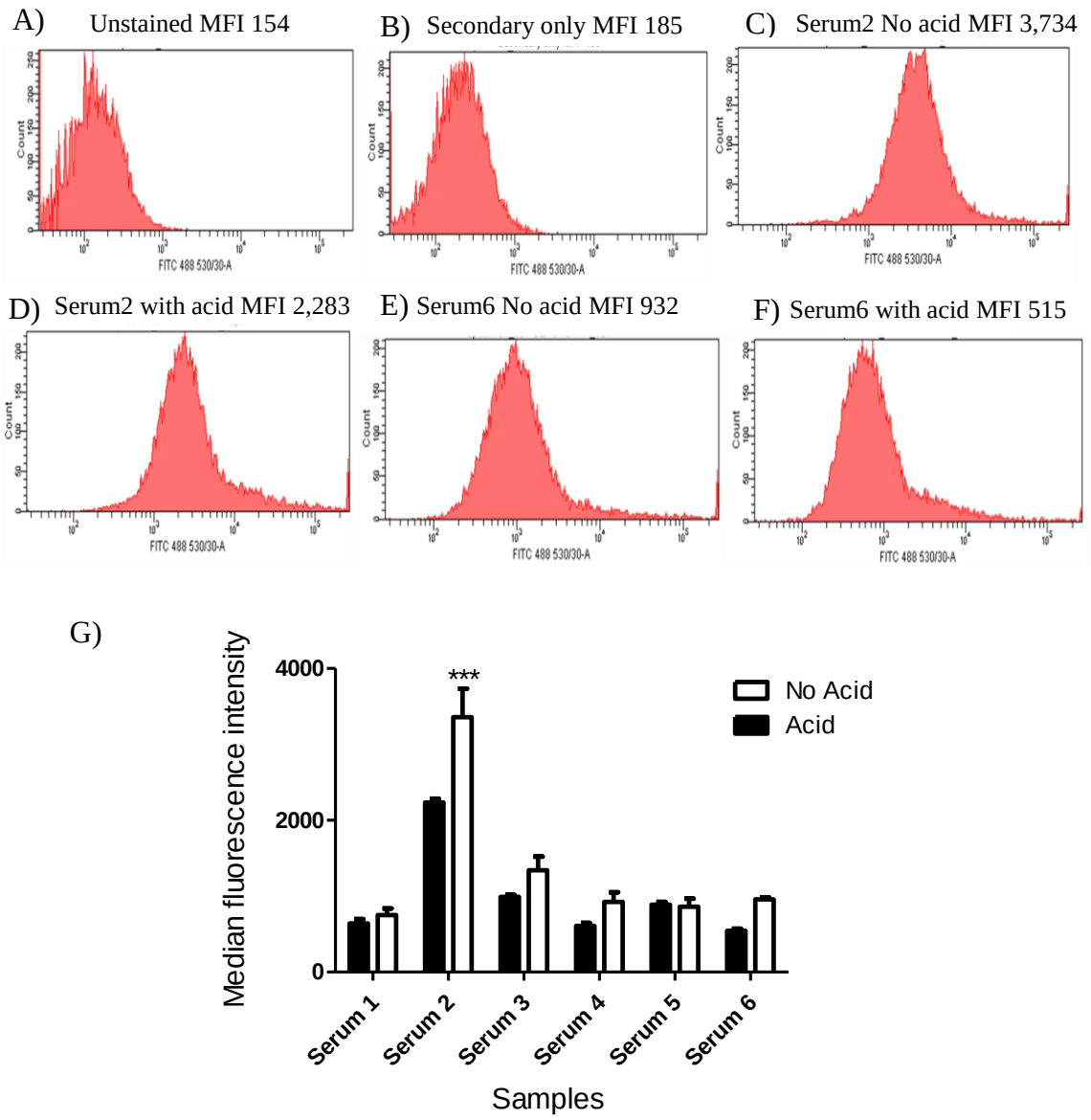


Figure 6.19: Examination of the binding of purified human IgG from multiparous female to acid treated endothelial cells.

HMEC-1 cells treated with citrate buffer (pH 3) for 2 minutes were stained with purified human IgG isolated from multiparous female and compared to untreated endothelial cells. A, B, C, D, E and F show unstained, secondary antibody only, serum 2 with no acid treatment, serum 2 after acid treatment, serum 6 with no acid treatment and serum 6 after acid treatment, respectively. G represents bar chart of median fluorescence intensity. The data analysis was performed using one-way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representative of three independent experiments.

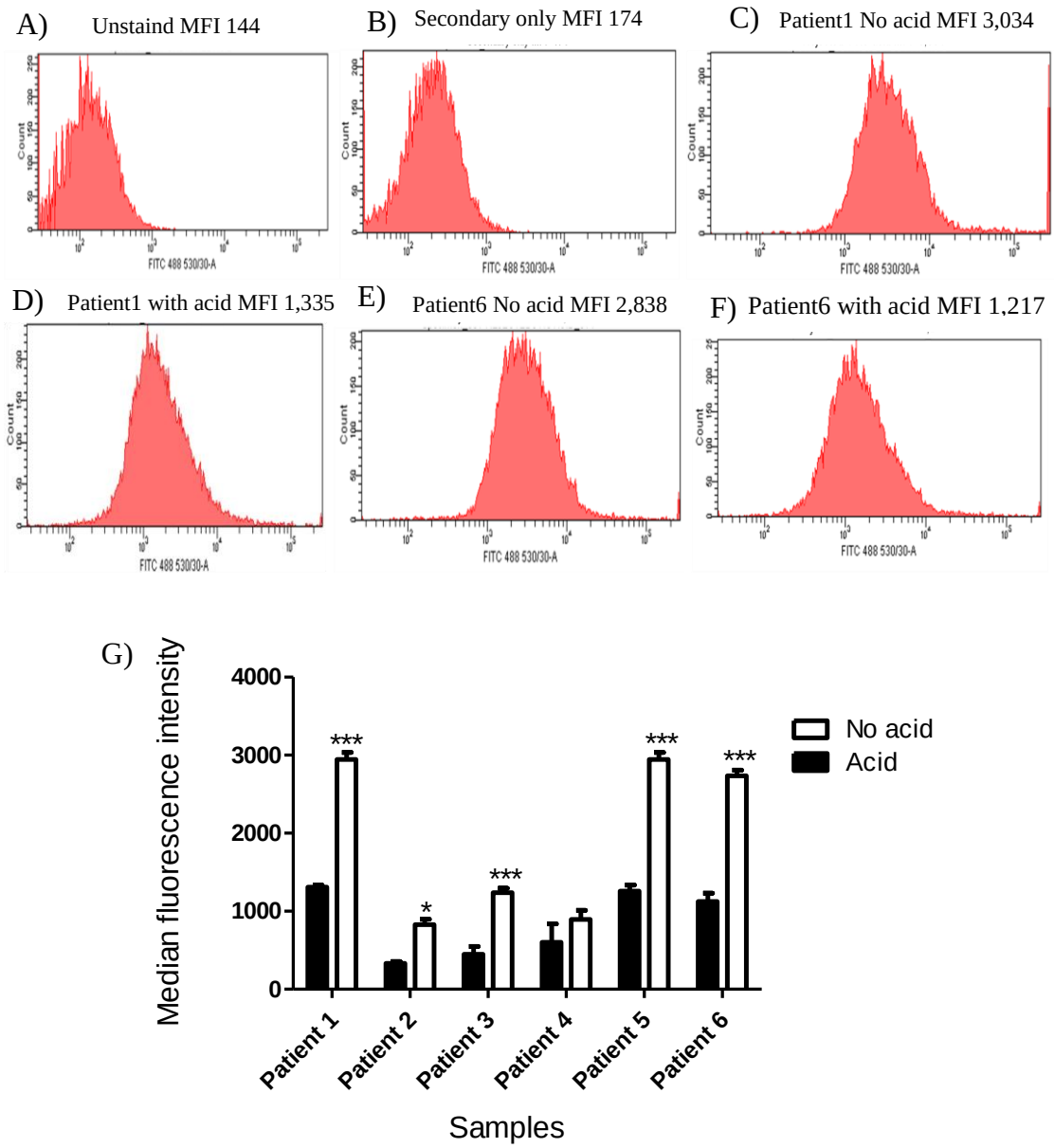


Figure 6.20: Examination of the binding of purified human IgG from sensitized patients to acid treated endothelial cells.

HMEC-1 cells exposed to citrate buffer (pH 3) for 2 minutes were stained with purified human IgG isolated from sensitised kidney patients and compared with untreated endothelial cells. A, B, C, D, E and F shows unstained, secondary only, patient 1 with no acid treatment, patient 1 after acid treatment, patient 6 with no acid treatment and patient 6 after acid treatment, respectively. The data analysis was performed by one-way ANOVA followed by Bonferroni test at $p<0.05$. *** $p<0.001$. The data are representative of three independent experiments.

6.4.5 Knock down of HLA class I antigens on HMEC-1 cells

Knocking down the expression of HLA class I antigens was performed to analyze the effects of locus specific HLA antibody in inducing endothelial cell activation. The transfection efficiency was initially assessed using monoclonal W6/32 antibody.The silencing process was performed using three sequences of siRNA specific for HLA-A, HLA-B and HLA-C heavy chains. HMEC-1 cells were transfected transiently with 5 nM of each siRNA or negative control siRNA at different time points ranging from 24 to 96 hours, followed by staining with W6/32 antibody. As shown in Figure 6.21, transfection with these siRNA significantly reduced the expression of cell surface HLA class I antigens compared to both non-transfected and negative siRNA transfected groups. The expression was reduced by 60 % after 24 hours and by 68, 63 and 50% after 48, 72 and 96 hours, respectively, with $p<0.001$. Transfection for 48 hours induced a greater reduction in the expression of cell surface HLA class I antigens, although complete knock down in the expression of these molecules could not be achieved. Therefore, assays using higher siRNA concentrations were performed to obtain efficient silencing. Both 10 nM and 20 nM siRNA were transfected into HMEC-1 cells for 48 hours followed by staining with W6/32 antibody. Using higher siRNA concentrations resulted in only 5 and 7% extra reduction at 10 and 20 nM, respectively, compared to 5 nM siRNA (data not shown). Therefore, 5 nM for 48 hours was used for following experiments. Staining of transfected cells with W6/32 anibody would not provide an information about which locus is successfuly knocked down. Because the knock down of HLA class I molecules produced similar results to acid treatment, the binding of purified IgG to transfected cells was excluded.

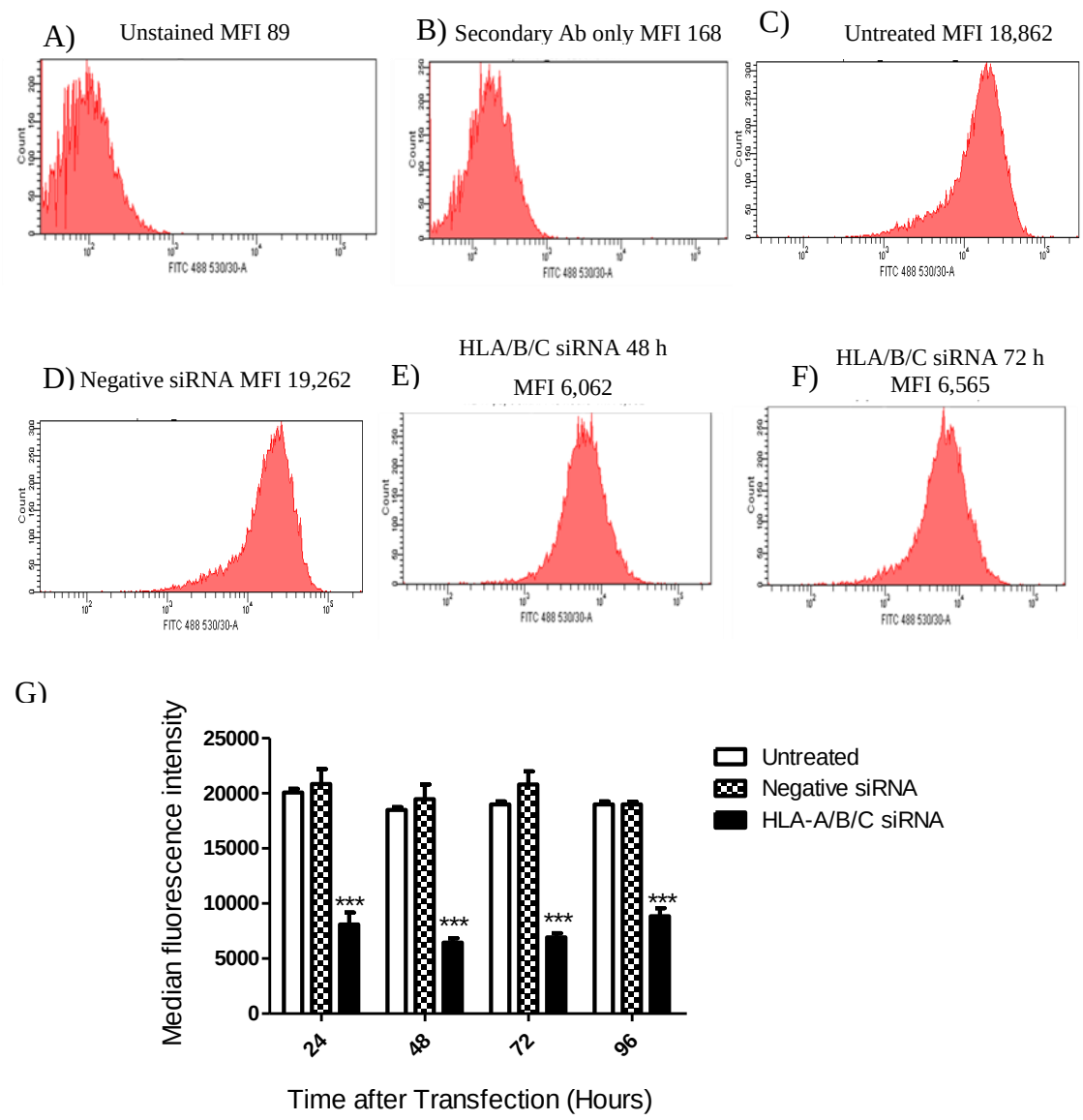


Figure 6.21: Knockdown of HLA class I antigens on endothelial cells using siRNA technique.

HMEC-1 cells were transiently transfected with a mixture of siRNA specific for HLA-A, B and HLA-C or negative siRNA at different time points at concentration of 5 nM. Cells were detached and stained with 12 µg/ml of W6/32 antibody followed by anti-mouse FITC conjugated antibody and analyzed by flow cytometry. Panel A shows unstained, B secondary antibody only, C untreated, D example of negative siRNA, (E) HLA siRNA after 48 hours, (F) HLA siRNA after 72 hours and (G) median fluorescence intensity. The data was analyzed using Two-way ANOVA followed by Bonferroni test. *** $p<0.001$ compared to negative siRNA. The data are representative of three independent experiments.

6.4.6 Determination of knockdown efficiency using human monoclonal HLA class I antibody

Human monoclonal antibodies specific for paticular HLA antigens were obtained from Dr. Arend mulder to evaluate the transfection efficiency of HLA class I antigens. HMEC-1 endothelial cells were transfected with a mixture of 5 nM of HLA siRNAs for class I antigens for 48 hours followed by staining with human monoclonal antibody separately at concentration 40 μg/ml. Figure 6.22 shows the staining with human HLA-A1 and HLA-A68 antibodies on untreated, negative siRNA and HLA siRNA transfected cells. The transfection induced a negligable silencing of HLA-A1 molecules but induced ~50% reduction in the expression of HLA-A68 antigens. This reduction was statisticaly significant compared to negative siRNA stained with the same antibody at the same concentration ($p<0.001$). The transfection of negative control siRNA did not induce a significant change in the expression of specific HLA antigens when compared to the untreated group. Figure 6.23 shows staining of transfected HMEC-1 cells with HLA-B35 and HLA-B58 antibodies. The transfection significantly reduced ($p<0.01$) the expression of HLA-B35 and HLA-B58 antigens by around 90% and 80%, respectively, compared to negative siRNA control. The latter did not induce a significant change in the expression of these antigens when compared to untreated group. Because the antibody specific to HLA-C locus was not available; therefore, assessing the knocking down of HLA-C antigens could not be determined. Therefore, the binding of purified antibodies to transfected cells in attempt to determine the specificity was not investigated.

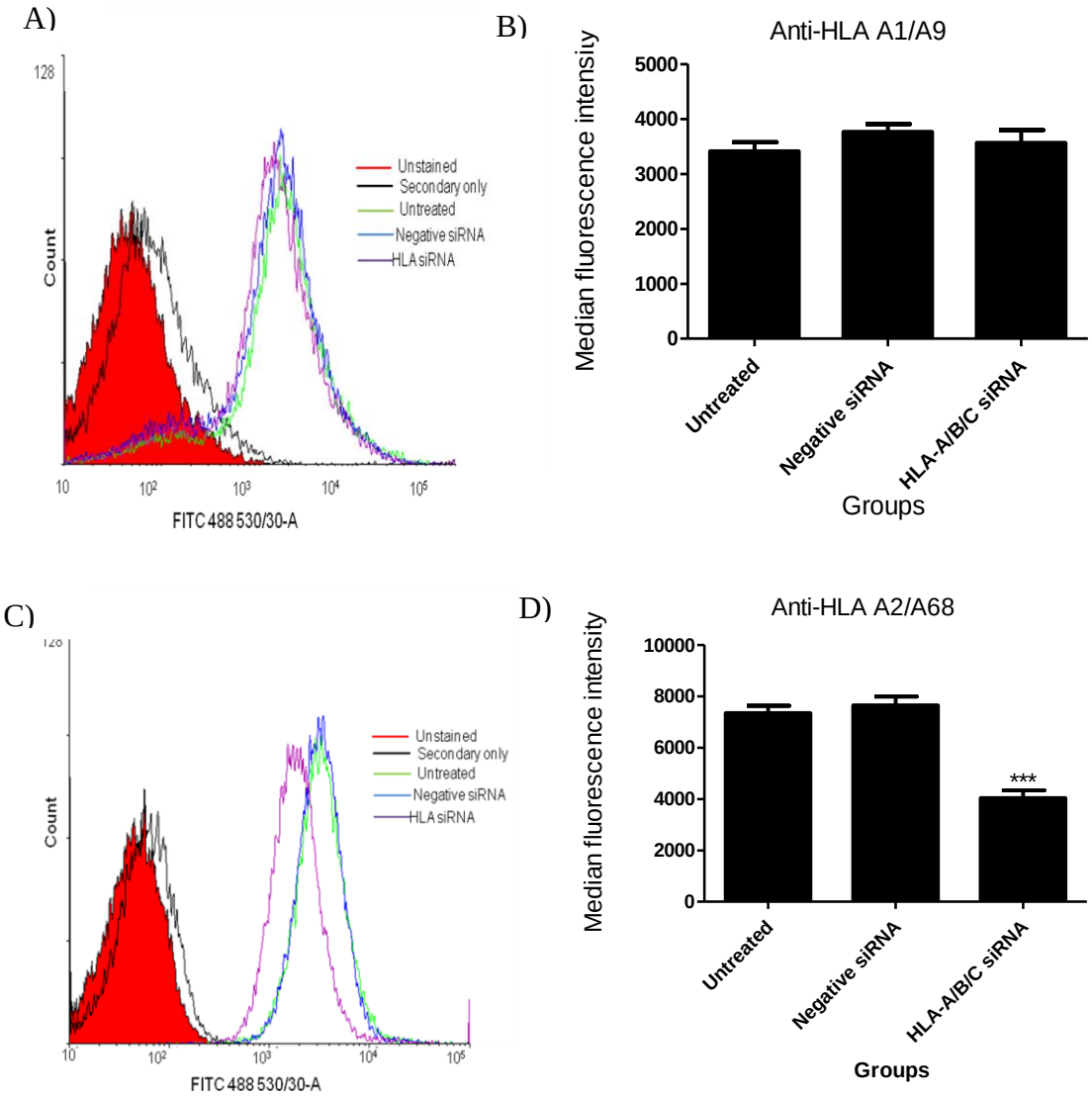


Figure 6.22: HLA class I transfected endothelial cells stained with human monoclonal antibody specific to HLA-A antigens.

HMEC-1 cells were transfected with a mixture of siRNA specific to HLA-A, B and C for 48 hours at 5 nM each followed by staining with HLA class I antibody specific to HLA-A1 and HLA-A68 at concentration 40 µg/ml. Following staining with anti-human IgG FITC conjugated antibody, cells were run on flow cytometer. Panel A shows histograms for HLA-A1/A9 staining and panel B shows the median fluorescence intensity. Panel C shows histograms for HLA-A2/A68 staining and panel D shows the median fluorescence intensity. The data were analyzed by WinMDI software. *** $p<0.001$ by one way ANOVA followed by Bonferroni test. The data are representative of three independent experiments.

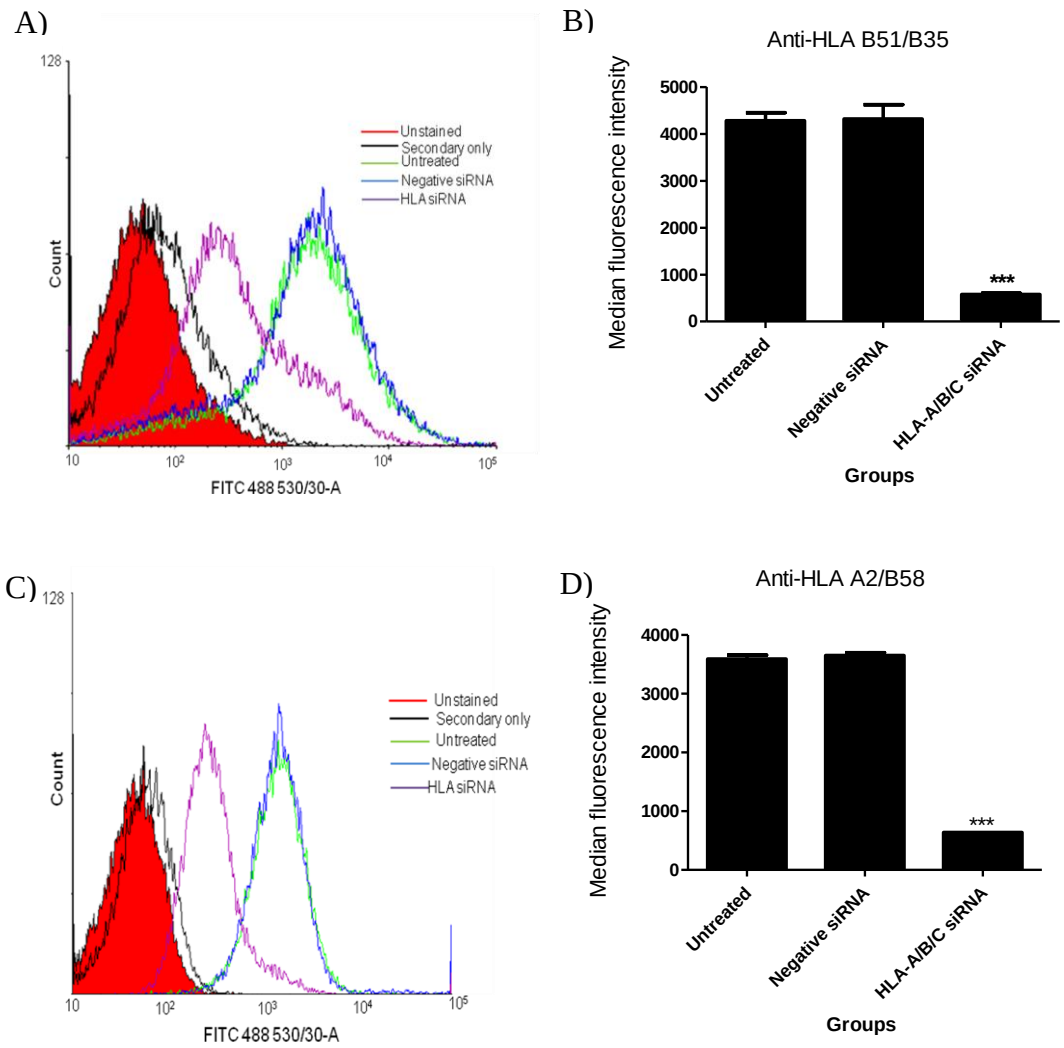


Figure 6.23: HLA class I transfected endothelial cells stained with human monoclonal antibody specific to HLA-B antigens.

HMEC-1 cells were transfected with a mixture of siRNA specific for HLA-A, B and C for 48 hours at 5 nM each followed by staining with HLA antibody specific to HLA-B58 and HLA-B35 at concentration 40 µg/ml. Following staining with anti-human IgG FITC conjugated antibody, cells were analyzed by flow cytometry. Panel A shows histograms for HLA-B35 staining and panel B shows the median fluorescence intensity. Panel C shows HLA-B58 staining and panel D shows the median fluorescence intensity. *** $p<0.001$ by one way ANOVA followed by Bonferroni test. The data are representative of three independent experiments.

6.4.7 Phosphorylation of CREB by monoclonal allospecific antibody

Human monoclonal antibodies were used to determine whether antibody to specific HLA locus induced endothelial activation. P-CREB was assessed following stimulation with monoclonal HLA-B58 antibody which was chosen for this experiment due to the efficient knockdown of HLA-B locus. HMEC-1 cells were transfected with either negative siRNA or HLA-B siRNA at 5nM for 48 hours followed by stimulation with 40 µg/ml of HLA-B58 antibody for 5 minutes. Cells were detached and analyzed by flow cytometry to examine knockdown efficiency. Transfection induced ~85% reduction in the expression of this antigen compared to control siRNA transfected cells (data not shown). Figure 6.24 shows that stimulation of non-transfected or negative siRNA cells with HLA-B58 antibody significantly induced the phosphorylation of CREB at serine residue 133. However, knocking down HLA-B locus significantly abrogated this phosphorylation to baseline level. Cells stimulated with 50 µM forskolin were used as a positive control.

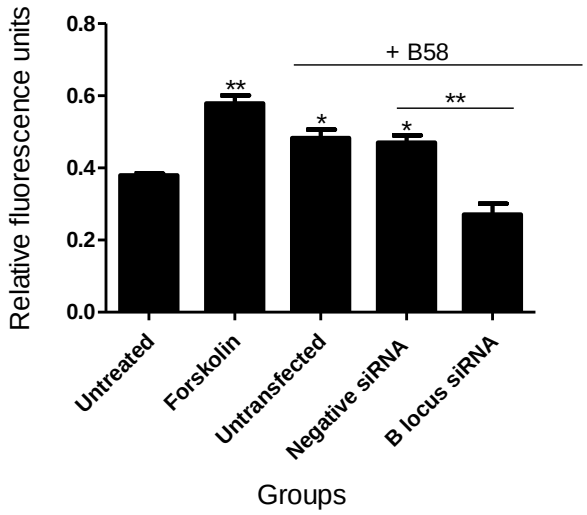


Figure 6.24: p-CREB cell-based ELISA of HMEC-1 cells stimulated with HLA-B58 antibody.

Endothelial cells were transfected with either 5 nM of HLA-B locus siRNA or negative control siRNA followed by stimulation with 40 µg/ml of HLA-B58 antibody. Cells were fixed and the fluorescence intensity was measured using a fluorescence plate reader at excitation 360 and 540 nm and emission 450 and 600 nm for T-CREB and p-CREB, respectively. The data were analyzed by one-way ANOVA compared to untreated followed by Bonferroni test at $p<0.05$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. The data are representative of three independent experiments.

6.5 Discussion

The development of donor specific HLA class I antibodies following transplantation causes the deterioration of allograft function. This is due to subsequent acute and chronic allograft rejection. In this chapter the effect of allospecific HLA class I antibodies in inducing the activation of donor endothelial cells was studied. This was compared to the effects produced by mouse monoclonal HLA class I antibody. Allospecific antibodies were chosen according to their specificity to HLA class I molecules expressed on HMEC-1 endothelial cells. Total IgG antibodies were purified from sera of two groups of samples; sera from multiparous female who develop HLA class I antibodies from multiple pregnancies, and sera from sensitized kidney patients with antibodies formed against HLA class I antigens. The ability of purified antibodies to induce the phosphorylation of endothelial cell signaling proteins and the expression of adhesion molecules and CXCL8 chemokine was examined.

The presence and specificity of these antibodies was confirmed by single antigen bead Luminex assay both before and after antibody purification. Samples showed reactivity against different HLA class I antigens expressed on HMEC-1 cells. Purified IgG antibodies were also examined for their ability to bind HMEC-1 endothelial cells by flow cytometry and compared to the binding of negative controls. Allospecific antibodies showed a strong binding to endothelial cells when compared to the binding of IgG from HLA negative control or IgG from patient who had HLA class I antibodies not specific to antigens express on HMEC-1 cells. Antibodies at 0.18 mg/ml showed a significant binding to HMEC-1 endothelial cells and were used in all experiments.

Exposure of endothelial cells to allospecific antibodies from both multiparous females and patient samples produced a significant phosphorylation of ERK, Akt and CREB compared to negative controls. This activation is the same as was shown with the phosphorylation produced by W6/32 antibody. The extent of phosphorylation produced was comparably similar with all samples. This might be attributed to the polyclonal nature of these samples with high antibody concentration. If maximum phosphorylation was induced by samples with lower HLA antibody content, using a higher concentration might not increase the phosphorylation further. The phosphorylation of intracellular proteins by treatment with allospecific antibodies was previously described using a model of large vessel endothelial cells (Narayanan *et al.*, 2004). In addition, activation

of endothelial transcription factor NF-kB was reported in response to stimulation with sera from patients containing HLA class I specifc antibodies (Smith *et al.*, 2000).

In this project, the potential of allospecific HLA class I antibodies to induce the expression of cell surface adhesion molecules VCAM-1 and ICAM-1 was also examined. Endothelial cells were stimulated with 0.18 mg/ml of allospecific antibodies overnight before examining the expression of adhesion molecules by flow cytometry. Exposure to these antibodies induced a significant expression of both VCAM-1 and ICAM-1 compared to negative controls. Different samples induced different levels of adhesion molecule expression which might be attributed to different factors. Antibodies specific for particular HLA class I locus (A, B or C) might have different potential in inducing endothelial cell activation. In addition, different subclasses of IgG might be present in each sample with different potential to activate cell signaling. In a heart transplantation model, a passive transfer of IgG2b but not IgG1 antibodies against mismatch MHC class I antigens has been shown to induce acute rejection and endothelium activation characterized by the release of von-willebrand factor and P-selectin and platelet aggregation (Wasowska *et al.*, 2001). The expression of ICAM-1 and VCAM-1 adhesion molecules by artery endothelial cells has been previously observed in an *in vitro* study using human polyclonal HLA class I antibodies (Narayanan *et al.*, 2004). In addition, a recent study showed that stimulation of human microvascular glomerular endothelial cells with sera containing HLA class I antibodies induced the expression of both VCAM-1 and ICAM-1 molecules (Mannam *et al.*, 2013). The upregulation in the expression of endothelial adhesion molecules suggests an increase in the adhesion of recipient leukocytes to donor endothelium.

The expression of endothelial CXCL8 chemokine in response to W6/32 antibody was confirmed using allospecific antibodies. Endothelial cells stimulated with antibodies from multiparous females and from sensitized patients showed a significant increase in the expression of this chemokine compared to negative controls, as examined using q-PCR. This suggests that endothelial cells could produce chemotactic factors that can induce leukocyte recruitment. In a previous *in vitro* study, stimulation of human artery endothelial cells with HLA class I antibody showed a significant expression of IL-6, IL-1β, CXCL8 and TNF-α (Reyes-Vargas *et al.*, 2009). In a mice model of lung transplantation, passive transfer of donor specific MHC class I antibody induced the

expression of different cytokines and chemokines such as IL-10, IL-17, CCL9, CXCL1 and CXCL12 (Fukami *et al.*, 2009).

Although serum samples were selected according to their HLA antibody specificity, additional experiment was performed to confirm that endothelial cell activation was due to HLA class I antibodies. The binding of purified antibodies from both sources to citrate-washed endothelial cells were assessed. HMEC-1 cells were treated with citrate buffer pH 3 for 2 minutes and assessed for their ability to bind purified allospecific IgG. Some samples showed a significant reduction in binding to treated cells compared to untreated cells. The acid treatment did not result in complete HLA class I antigen reduction; with 20-30% remaining on the cell surface. Therefore, not all samples showed a significant reduction in binding to acid-treated cells.

In this study, the activation of endothelial cells in response to stimulation with locus specific HLA antibody was assessed. This was examined on normal non-transfected cells and on HLA-B knockdown cells. Silencing the expression of cell surface HLA class I antigens by HMEC-1 cells was achievable using the siRNA technique. The transient transfection was performed using a mixture of siRNA against HLA-A, B and C loci. This was performed to examine the binding of purified allospecifc IgG to HLA class I-knocked down cells. The knockdown efficiency was examined using flow cytometry after staining with W6/32 antibody. Although not complete, HMEC-1 endothelial cells showed a significant but partial silencing for the expression of cell surface HLA class I antigens. Staining of transfected cells with W6/32 antibody showed around 60% reduction in the expression of HLA class I antigens after 48 hours of transfection. Since W6/32 antibody binds all classes of HLA class I antigens, human monospecific antibodies were obtained and used to examine the specific knockdown efficiency. Staining of HMEC-1 transfected cells with monospecific antibody against HLA-A1, HLA-A68, HLA-B35 and HLA-B58 showed a significant knockdown in HLA-B antigens but not all HLA-A antigens. Monospecific antibody targeting HLA-C antigens was not available. Because all of the polyconal samples have mixtures of antibodies against HLA-A, HLA-B and C antigens, binding of purified IgG to silenced cells was not investigated. Silencing of HLA class I antigens have been previously achieved in aortic endothelial cells using siRNA specific to HLA-A or B heavy chains or β2-microglobulin (Zhang *et al.*, 2011b).

The HLA-B locus knockdown model was used to assess the phosphorylation of CREB at serine residue 133 using monospecific HLA-B58 antibody. Stimulation of endothelial cells with 40 µg/ml of HLA-B58 antibody induced a significant CREB phosphorylation compared to untreated cells examined by p-CREB cell based ELISA. Stimulation of HLA-B locus siRNA transfected cells with the same concentration of HLA-B58 antibody showed a reduction in the extent of CREB phosphorylation compared to negative control. The knockdown induced 85% reduction in the cell surface expression of HLA-B58 as confirmed using flow cytometry staining. This reduction in the CREB phosphorylation suggests that the antibody against HLA-B antigen alone can induce endothelial activation. In a previous study, antibody specific to HLA-A2 antigen has been shown to induce the activation of aortic endothelial cells manifested by the phosphorylation of intracellular proteins and expression of adhesion molecules; suggesting the ability of antibody specific to HLA-A antigens to signal (Bian and Reed, 1999; Narayanan *et al.*, 2004).

In conclusion, in this chapter we showed that the development of donor specific antibody against polymorphic determinants of HLA class I antigens induces microvascular endothelium activation. This is manifested by the upregulation of adhesion molecules and expression of chemokines in a manner inducing endothelium-leukocyte interaction. In addition, HLA-B specific antibody is able to induce activation of endothelial cells as observed by phosphorylation of transcription factor CREB.

7. Chapter Seven-Discussion and Concluding Marks

Circulating donor specific HLA antibody post-transplantation is one of the major factors that causes late antibody-mediated rejection. It is associated with subsequent acute and chronic rejection of kidney (Sellares *et al.*, 2012), lung (Hachem, 2012), heart (Chih *et al.*, 2012), pancreas (Torrealba *et al.*, 2008) and liver allografts (Abu-Elmagd *et al.*, 2012). Microvascular endothelial inflammation and infiltration of mononuclear leukocytes through the capillaries are features associated with antibody-mediated rejection (Drachenberg and Papadimitriou, 2013). In addition to complement fixing antibody, non-complement fixing antibodies, IgG2 and IgG4, have been shown to have a crucial role in inducing allograft rejection (Heinemann *et al.*, 2006). Non-complement fixing antibodies might be responsible for the phenotype of C4d-negative antibody mediated rejection. This project was designed to examine the mechanisms by which antibodies targeting donor HLA class I antigens can induce endothelium inflammation and allograft rejection in a complement independent mechanism. This was achieved by focusing on modulation of the expression of adhesion molecules and chemokines by endothelial cells. In addition, the consequence of this expression on endothelial-leukocyte interaction was examined using a powerful flow-based adhesion assay that recapitulates the *in vivo* situation.

Human microvascular endothelial cells (HMEC-1) were used in this study. These immortalized endothelial cells resemble primary endothelial cells in their cobblestone structure. They express the CD31 endothelial marker and high levels of HLA class I antigens. The HLA genotype of the cell line is A*01, A*68, B*35, B*58, C*04, C*06 tested by PCR-SSP. The cell line is from a blood group ‘O’ donor, which eliminates the possibility of the interaction with ABO antibodies. Monoclonal mouse HLA class I antibody was purified from W6/32-hybridoma cell line using affinity chromatography and used in this project. The binding to HMEC-1 cells was confirmed by flow cytometry and 12 µg/ml was the saturating antibody concentration. Allospecific HLA class I antibodies from multiparous females and kidney transplant patients were used to assess their potential to induce endothelial activation. Samples were chosen according to their specificities assessed by Luminex single antigen bead assay. Activation of endothelial cells was determined using both mouse monoclonal antibody and allospecific antibodies.

Phosphorylation of intracellular proteins provides evidence of cell activation in response to an extracellular stimulus (Hunter, 2007). In this project, treatment of HMEC-1 cells with HLA class I antibody induced phosphorylation of a wide range of endothelial cell signaling proteins involved in cytoskeleton rearrangement, inflammatory responses and cell proliferation assessed by human phosphokinase array. The phosphorylation was further assessed by western blotting for Akt at serine residue 473, ERK at tyrosine 202 and threonine 204 residues and transcription factor CREB at serine residue 133 at different time points and antibody concentrations. Endothelial cells showed different degrees of phosphorylation in response to saturating and sub-saturating concentrations of the antibody. The phosphorylation of Akt was sensitive to antibody concentration; however, CREB phosphorylation still occured at the lower concentration of antibody used (0.35µg/ml). As far as we know, this is the first demonstration of the phosphorylation of CREB transcription factor by endothelial cells in response to HLA class I antibodies. The phosphorylation of cell signaling proteins such as ERK, Akt, FAK and others in response to stimulation with HLA class I antibodies has previously reported using large vessel endothelial cells (Jin *et al.*, 2004; Jin *et al.*, 2007; Jindra *et al.*, 2008b). The phosphorylation of nuclear transcription factor CREB suggests a modulation in the endothelial transcriptional profile upon antibody ligation. Using pathway-specific inhibitors, CREB phosphorylation showed sensitivity to PKA pathway but not to the PI3K/Akt pathway. The phosphorylation of CREB by PKA is consistant with the increase in the level of cAMP previously observed on endothelial cells stimulated with HLA class I antibodies (Narayanan *et al.*, 2006).

Endothelial cells also respond to extracellular stimuli by activating the transcriptional machinery to synthesise new proteins. In this project, microvascular endothelial cell showed this type of activation in response to HLA class I antibodies. Treatment of endothelial cells with saturating concentration of HLA class I antibody induced expression of the cell surface adhesion molecules VCAM-1 and ICAM-1. The expression of these antigens peaked after 8-12 hours and declined after 72 hours. Activation of the endothelial signaling pathway was responsible for the expression of these antigens in response to antibody stimulation. PI3K/Akt pathway mediates the significant expression of VCAM-1 and ICAM-1 as examined by inhibiting this pathway. These results are in agreement with previous reports showed the expression of adhesion molecules on large vessel endothelial cells *in vitro* (Narayanan *et al.*, 2006)

and in a MHC class I mismatched mouse transplantation model *in vivo* (Fukami *et al.*, 2012).

Leukocyte migration is a process that is highly dependent on the presence of endothelial adhesion molecules along with chemokines at the site of inflammation. Therefore, the expression of endothelial adhesion molecules and chemokines in response to HLA class I antibody was assessed. HMEC-1 endothelial cells treated with saturating concentration of HLA class I antibody produced the inflammatory cytokine IL-6 and chemokines CCL5, CXCL8, and CXCL1 determined at the protein level. The upregulation of endothelial CXCL8 was time-dependent and sensitive to antibody concentration assessed at mRNA level. The expression of chemokines has been previously observed *in vitro* using aortic endothelial cells stimulated with W6/32 antibody (Reyes-Vargas *et al.*, 2009) and in an *in vivo* MHC mismatched transplant model (Fukami *et al.*, 2012). The expression of CXCL8 was dependent on both the PKA/CREB and PI3K/Akt pathways. The relative expression of CXCL8 mRNA was reduced by 52% and 35% in the presence of specific-inhibitor to PI3K and PKA pathways, respectively. In addition, CXCL8 mRNA was reduced by 50% in knockdown-CREB treated cells suggesting the involvement of CREB transcriptional activity in the expression of CXCL8. CREB-dependent CXCL8 expression was previously observed in epithelial cells in response to cytokine stimulation (IL-β) (Sun *et al.*, 2008). The increase in the expression of chemotactic proteins in response to donor specific antibody suggests modulation of the leukocyte-endothelial interaction.

Extravasation of donor leukocytes to the transplanted grafts occurs not only in cellular rejection but also in antibody-mediated rejection. Histological examination shows that monocyte infiltration occurs in kidney patients with circulating donor specific antibodies (Fahim *et al.*, 2007; Papadimitriou *et al.*, 2010). Therefore, the potential of endothelial cells treated with HLA class I antibody in inducing monocyte adhesion and migration was examined. Conditioned media from endothelial cells treated with HLA class I antibodies induced significant migration of human monocytes examined using static chemotaxis assays. In addition, reflecting *in vivo* adhesion, human monocytes flowing at 0.5 dyne/cm^2 significantly adhered to endothelial cells treated with $F(ab)_2$ fragments of W6/32-antibody. Furthermore, the secretion of chemokines by W6/32–treated endothelial cells significantly augmented the adhesion of monocytes under inflammatory condition as observed using TNF-α-treated endothelial cells. The

adhesion was also independent to the interaction with Fc-receptors of THP-1 cells. Yamakuchi *et al.* observed monocyte adhesion to endothelial cells treated with whole molecule of W6/32 antibody (Yamakuchi *et al.*, 2007). However, the contribution of Fc-receptors in the latter study could not be excluded. These results collectively consolidate the potential of HLA class I antibody in inducing leukocyte adhesion and migration. Furthermore, the presence of the antibody in the inflammatory environment augments the migration of recipient leukocytes. This is the first demonstration of monocyte adhesion under conditions remodeling *in vivo* blood flow.

The results obtained from this project were confirmed using HLA class I IgG allospecific antibodies purified from multiparous females and transplanted kidney patients. After purification, the specificity of the samples was determined using a Luminex single antigen bead assay and measurement of binding to HMEC-1 cells using flow cytometry. Treatment of HMEC-1 cells with these antibodies induced endothelial cell activation evidenced by phosphorylation of endothelial cell signaling, expression of adhesion molecules, and CXCL8 compared to controls. Phosphorylation of ERK, Akt and CREB is induced in response to HLA class I allospecific antibodies compared to HLA class I antibodies not specific to HMEC-1 antigens. Furthermore, expression of the endothelial cell surface molecules VCAM-1 and ICAM-1 was significantly induced by allospecific HLA class I antibodies when compared to control groups. Our results agree with other *in vitro* and *in vivo* results (Fukami *et al.*, 2012). In *in vitro* study, stimulation of human microvascular glomerular endothelial cells with sera containing HLA class I antibody induced the expression of these adhesion molecules (Mannam *et al.*, 2013). In addition, the expression of CXCL8 mRNA was also significantly induced in response to these antibodies. Different samples produced distinct degree of expression which is probably dependent on the IgG subclass of the antibodies or their concentrations.

The potential of locus specific HLA class I antibodies in inducing endothelial cell activation was examined. Human monoclonal HLA-B58 specific antibody was used to examine the phosphorylation of CREB at serine residue 133 using quantitative cell-based ELISA. Stimulation with HLA-B58 antibody induced significant phosphorylation of CREB at specified residues. To confirm this result, the phosphorylation was examined on HLA-B siRNA transfected cells and compared to non-transfected cells. Silencing the expression of HLA class I antigens was performed using siRNA specific

to the heavy chain of each locus. Because the knockdown of HLA-B antigens was achieved at a high efficiency, HLA-B58 antibody was used for this assay. The extent of CREB phosphorylation in response to HLA-B58 antibody was reduced in trasnfected cells compared to the control group. Activation of endothelial cells in response to HLA-A antibody has been previously reported (Narayanan *et al.*, 2004). This result suggests that antibody against single HLA class I mismatch can induce endothelial cell activation.

To sum up, graft microvascular endothelial cells are activated in response to the circulating donor specific HLA class I antibody. This involves the activation of various intracellular signaling pathways which contribute to the expression of endothelial adhesion molecules and chemokines. The upregulation in the expression of inflammatory mediators exposes donor endothelium to circulating leukocytes inducing their adhesion and migration. Additionally, recipient leukocytes can bind Fc fragment of the antibody inducing their recruitment to the transplanted graft. Antibody specifc to HLA-B antigen are able to induce endothelial cell activation in a manner comparable to monoclonal HLA class I antibody. Strategies that block endothelial-leukocyte interaction might provide a level of protection for the transplanted graft following transplantation and prolong allograft survival. Summary of this study is shown in figure 7.1.

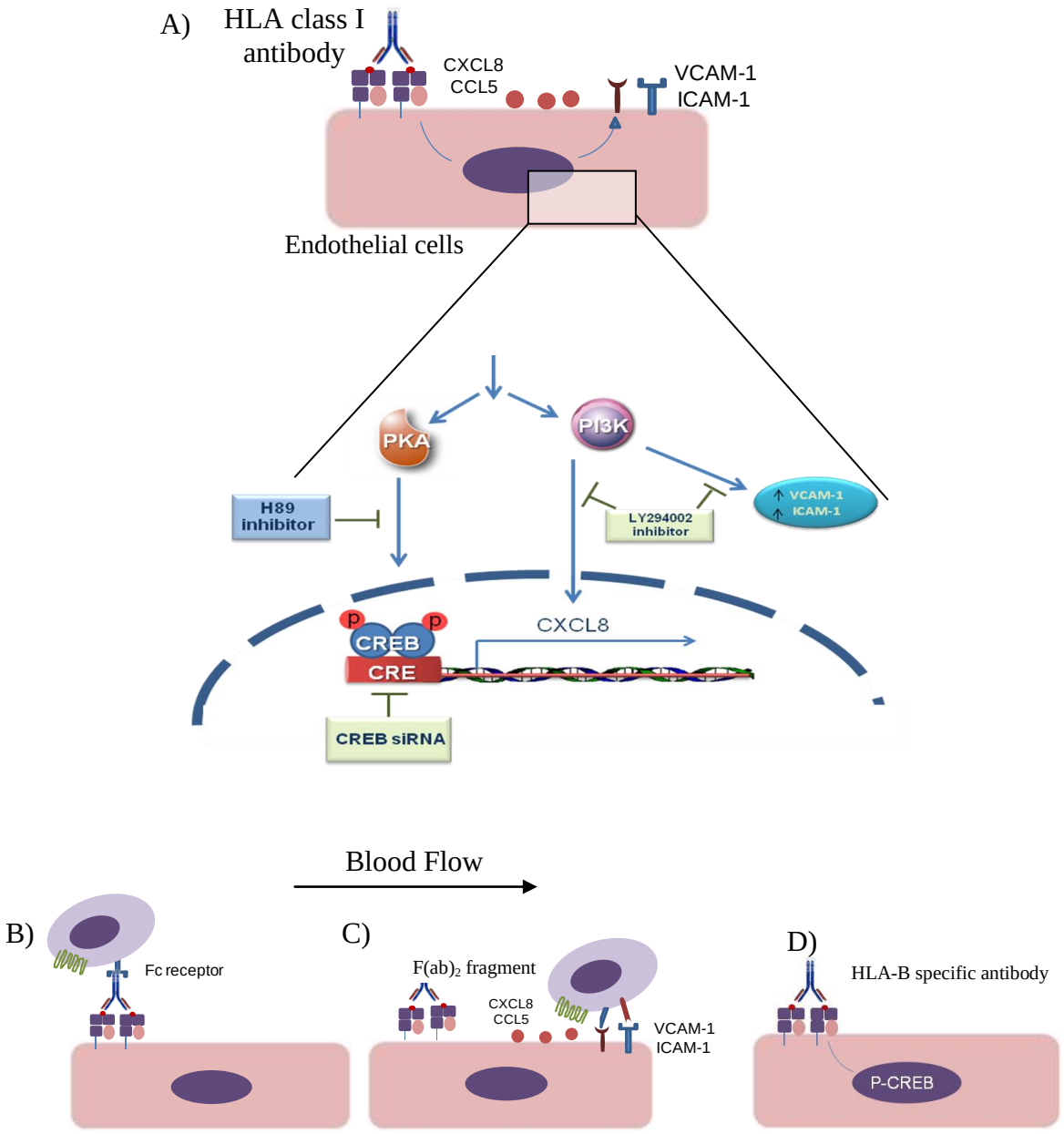


Figure 7.1: Mechanisms of HLA class I antibody in inducing microvascular endothelial-leukocyte interaction.

Panel A shows that the cross linking of HLA class I antigens with intact HLA class I antibody induces activation of endothelial cell signaling and expression of adhesion molecules and chemokines. Panel B shows the binding of circulating leukocytes to the Fc-region of the HLA class I antibody. Panel C shows the activation of endothelial cells and leukocyte adhesion in response to stimulation with $F(ab)_2$ fragments from W6/32 antibody. Panel D shows endothelial CREB activation in response to stimulation with antibody specific to HLA-B antigen.

8. Future directions

In this project, inflammation of human microvascular endothelial cells in response to mouse HLA class I antibody (W6/32, IgG2a) was examined. The project could be extended to examine the effect of different IgG subclasses and different isotypes. Different antibodies might have different mechanisms of inducing endothelial cell activation depending on their ability to bind Fc-receptors or activating complement system. In addition, examination of the potential of isotypes developed at the primary sensitisation, such as IgM, in inducing endothelium activation might address the role of antibody developed in non-sensitised patients in inducing allograft rejection. The pentameric IgM and dimeric IgA might not bind directly to the postcapillary endothelium but still might have a role in inducing large vessel endothelial cells or epithelial activation. However, HLA class I specific IgM and IgA antibodies are not easily available.

In this study, we showed that the expression of chemokine CXCL8 is dependent on transcription factor CREB. However, activation of CREB and ATF-1 could be studied in depth. There are different genes such as proliferative and protective genes which are regulated by these transcription factors and might play a role in the pathogenesis of allograft rejection. Different techniques such as chromatin immunoprecipitation can be used to identify a set of target genes that are regulated by a particular transcription factor (Kirmizis and Farnham, 2004). Identifying genes that are regulated by these transcription factors would address whether targeting these factors will provide any protection to the grafts and prolong their survival.

This project could be taken from bench to bed side if blocking the interaction between endothelium and leukocytes was examined. Due to time limitation, this could not be examined. However, different strategies can be used to block this interaction. Antibodies that targeted adhesion molecules such as P-selectin and ICAM-1 showed a beneficial effect in reducing monocyte adhesion following treatment with HLA class I antibody (Valenzuela *et al.*, 2013a). However, different leukocytes bind different adhesion molecules and blocking each molecule might need extensive efforts. Therefore, blocking chemokine binding to endothelial cells might be more effective in preventing leukocyte extravasation. In our group, negatively charge molecules such as heparin have been shown to block chemokine binding to endothelium by inhibiting binding to heparan sulphate in a competitive manner (Harvey *et al.*, 2007). These agents

might fulfil the requirement of inhibiting leukocytes adhesion and migrations in a broader way.

Future studies can be performed to cover different aspects in the mechanism of HLA class I antibody inducing allograft rejection:

1- Endothelial cells stimulated with HLA class I antibody express complement fragments as reported in this study (C5a) and by other groups (C4) (Hamer *et al.*, 2012). Complement fragments are anaphylatoxin agents that induce leukocyte migration to the graft and augment the inflammatory response. Previously, treatment of endothelial cells with HLA class I antibody combined with C5a increased the exocytosis of von Willebrand Factor compared to cells treated with antibody alone (Yamakuchi *et al.*, 2007). Profiling the endothelial cells for the expression of complement-related proteins in response to HLA I class antibodies could be performed. This might highlight an indirect mechanism of injury mediated by these fragments particularly in non-complement fixing antibody and may identify a useful therapeutic target.

2- The effect of circulating HLA class I antibody on other cell types rather than endothelial cells can also be examined. Previous studies showed that HLA class I antibody can induce the production of growth factors by epithelial and smooth muscle cells inducing cell proliferation (Jaramillo *et al.*, 2003; Reed, 2003). In addition, HLA class I antibody are able to activate platelets in a manner that induce their adhesion to monocytes through P-selectin activation (Takahashi *et al.*, 2012). Different cells can be activated in response to HLA class I antibody, in addition, mediators secreted by specific cell type can induce activation of other cells such as endothelium. Examining the activation of different graft-related cells in response to HLA class I antibody might identify a set of proteins or cell types that are more potent in inducing cell injury. In addition, coculturing of different cell types together in the presence of HLA class I antibody might remodel cell-cell interactions *in vivo*.

3- Chronic antibody-mediated rejection is characterised by irreversible arterial fibrosis (Harris *et al.*, 1997). In lung transplantation, the expression of fibroblast growth factor and transformation growth factor-β are increased upon passive transfer of MHC class I antibody (Takenaka *et al.*, 2012). The expression of these factors is responsible for the activation of fibrogenic pathway. One the other hand, transformation growth factor-β is responsible for endothelial-to-mesenchymal transition enhancing graft fibrosis

(Zeisberg *et al.*, 2007). Therefore, examining the role of HLA class I antibody in inducing allograft fibrosis could be examined. Graft fibrosis can occur directly by HLA class I antibody or indirectly by mediator cells activated by the antibody. Investigating the possibility and the mechanism of this process might aid in understanding the mechanism of allograft injury and discovery of novel therapeutic targets.

4- Finding diagnostic biomarkers for antibody-mediated rejection might identify organs at a risk of rejection before allograft failure. Emerging studies have shown that small non-coding RNA (microRNA) play a role in the pathogenesis of different diseases and can be used as a diagnostic marker. They act on a target mRNA at a post-transcriptional stage effecting the translation process, therefore, the gene expression (Shan *et al.*, 2011). Different types of microRNA were identified in biopsies with rejection compared to functional graft in kidney transplantation (Anglicheau *et al.*, 2009). In addition, a recent study in kidney transplantation showed that biopsies from patients diagnosed with antibody-mediated rejection had a distinct microRNA profile that was different from biopsies from those diagnosed with cellular rejection (Wilflingseder *et al.*, 2013). microRNA can also be detected in the plasma in acute cellular rejection and tissue injury (Hu *et al.*, 2013). Therefore, examining the modulation in microRNA in response to HLA class I antibody using different cell types might identify a set of microRNA that plays a role in the pathogenesis of the rejection.

9. References

Abe, K., Yoshida, M., Usui, T., Horinouchi, S. and Beppu, T. (1991) 'Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases', *Exp Cell Res*, 192(1), pp. 122-7.

Abram, C.L. and Lowell, C.A. (2009) 'The ins and outs of leukocyte integrin signaling', *Annu Rev Immunol*, 27, pp. 339-62.

Abu-Elmagd, K.M., Wu, G., Costa, G., Lunz, J., Martin, L., Koritsky, D.A., Murase, N., Irish, W. and Zeevi, A. (2012) 'Preformed and De Novo Donor Specific Antibodies in Visceral Transplantation: Long-Term Outcome with Special Reference to the Liver', *Am J Transplant*.

Adams, A.B., Williams, M.A., Jones, T.R., Shirasugi, N., Durham, M.M., Kaech, S.M., Wherry, E.J., Onami, T., Lanier, J.G., Kokko, K.E., Pearson, T.C., Ahmed, R. and Larsen, C.P. (2003) 'Heterologous immunity provides a potent barrier to transplantation tolerance', *J Clin Invest*, 111(12), pp. 1887-95.

Ades, E.W., Candal, F.J., Swerlick, R.A., George, V.G., Summers, S., Bosse, D.C. and Lawley, T.J. (1992) 'HMEC-1: establishment of an immortalized human microvascular endothelial cell line', *J Invest Dermatol*, 99(6), pp. 683-90.

Aird, W.C. (2007) 'Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms', *Circ Res*, 100(2), pp. 158-73.

Ali, J.M., Bolton, E.M., Bradley, J.A. and Pettigrew, G.J. (2013) 'Allorecognition Pathways in Transplant Rejection and Tolerance', *Transplantation*.

Ali, S., Jenkins, Y., Kirkley, M., Dagkalis, A., Manivannan, A., Crane, I.J. and Kirby, J.A. (2008) 'Leukocyte extravasation: an immunoregulatory role for alpha-L-fucosidase?', *J Immunol*, 181(4), pp. 2407-13.

Altomare, D.A., Guo, K., Cheng, J.Q., Sonoda, G., Walsh, K. and Testa, J.R. (1995) 'Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene', *Oncogene*, 11(6), pp. 1055-60.

Amos, D.B., Seigler, H.F., Southworth, J.G. and Ward, F.E. (1969) 'Skin graft rejection between subjects genotyped for HL-A', *Transplant Proc*, 1(1), pp. 342-6.

Angel, P. and Karin, M. (1991) 'The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation', *Biochim Biophys Acta*, 1072(2-3), pp. 129-57.

Anglicheau, D., Sharma, V.K., Ding, R., Hummel, A., Snopkowski, C., Dadhania, D., Seshan, S.V. and Suthanthiran, M. (2009) 'MicroRNA expression profiles predictive of human renal allograft status', *Proc Natl Acad Sci U S A*, 106(13), pp. 5330-5.

Arnold, M.L., Heinemann, F.M., Horn, P., Ziemann, M., Lachmann, N., Muhlbacher, A., Dick, A., Ender, A., Thammanichanond, D., Fischer, G.F., Schaub, S., Hallensleben, M., Mytilineos, J., Hitzler, W.E., Seidl, C., Doxiadis, II and Spriewald, B.M. (2013) '16(th) IHIW: anti-HLA alloantibodies of the of IgA isotype in re-transplant candidates', *Int J Immunogenet*, 40(1), pp. 17-20.

Bach, F.H. and Amos, D.B. (1967) 'Hu-1: Major histocompatibility locus in man', *Science*, 156(3781), pp. 1506-8.

Bach, F.H., Ferran, C., Hechenleitner, P., Mark, W., Koyamada, N., Miyatake, T., Winkler, H., Badrichani, A., Candinas, D. and Hancock, W.W. (1997) 'Accommodation

of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment', *Nat Med*, 3(2), pp. 196-204.

Barnstable, C.J., Bodmer, W.F., Brown, G., Galfre, G., Milstein, C., Williams, A.F. and Ziegler, A. (1978) 'Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis', *Cell*, 14(1), pp. 9-20.

Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) 'A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region', *Science*, 254(5029), pp. 274-7.

Belperio, J.A., Keane, M.P., Burdick, M.D., Lynch, J.P., 3rd, Xue, Y.Y., Berlin, A., Ross, D.J., Kunkel, S.L., Charo, I.F. and Strieter, R.M. (2001) 'Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome', *J Clin Invest*, 108(4), pp. 547-56.

Bernard, B., Fest, T., Pretet, J.L. and Mougin, C. (2001) 'Staurosporine-induced apoptosis of HPV positive and negative human cervical cancer cells from different points in the cell cycle', *Cell Death Differ*, 8(3), pp. 234-44.

Bertrand, R., Solary, E., Jenkins, J. and Pommier, Y. (1993) 'Apoptosis and its modulation in human promyelocytic HL-60 cells treated with DNA topoisomerase I and II inhibitors', *Exp Cell Res*, 207(2), pp. 388-97.

Bevan, M.J. (1977) 'Killer cells reactive to altered-self antigens can also be alloreactive', *Proc Natl Acad Sci U S A*, 74(5), pp. 2094-8.

Bevilacqua, M.P. (1993) 'Endothelial-leukocyte adhesion molecules', *Annu Rev Immunol*, 11, pp. 767-804.

Bhat, S.V., Dohadwalla, A.N., Bajwa, B.S., Dadkar, N.K., Dornauer, H. and de Souza, N.J. (1983) 'The antihypertensive and positive inotropic diterpene forskolin: effects of structural modifications on its activity', *J Med Chem*, 26(4), pp. 486-92.

Bian, H., Harris, P.E. and Reed, E.F. (1998) 'Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation', *Int Immunol*, 10(9), pp. 1315-23.

Bian, H. and Reed, E.F. (1999) 'Alloantibody-mediated class I signal transduction in endothelial cells and smooth muscle cells: enhancement by IFN-gamma and TNF-alpha', *J Immunol*, 163(2), pp. 1010-8.

Biedermann, B.C. and Pober, J.S. (1998) 'Human endothelial cells induce and regulate cytolytic T cell differentiation', *J Immunol*, 161(9), pp. 4679-87.

Bieri, M., Oroszlan, M., Farkas, A., Ligeti, N., Bieri, J. and Mohacsi, P. (2009) 'Anti-HLA I antibodies induce VEGF production by endothelial cells, which increases proliferation and paracellular permeability', *Int J Biochem Cell Biol*, 41(12), pp. 2422-30.

Binion, D.G., Heidemann, J., Li, M.S., Nelson, V.M., Otterson, M.F. and Rafiee, P. (2009) 'Vascular cell adhesion molecule-1 expression in human intestinal microvascular endothelial cells is regulated by PI 3-kinase/Akt/MAPK/NF-kappaB: inhibitory role of curcumin', *Am J Physiol Gastrointest Liver Physiol*, 297(2), pp. G259-68.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. (1987) 'Structure of the human class I histocompatibility antigen, HLA-A2', *Nature*, 329(6139), pp. 506-12.

Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A. and Greenberg, M.E. (1999) 'Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms', *Science*, 286(5443), pp. 1358-62.

Boyden, S. (1962) 'The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes', *J Exp Med*, 115, pp. 453-66.

Brenner, S., Prosch, S., Schenke-Layland, K., Riese, U., Gausmann, U. and Platzer, C. (2003) 'cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation', *J Biol Chem*, 278(8), pp. 5597-604.

Brindle, P., Linke, S. and Montminy, M. (1993) 'Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressors', *Nature*, 364(6440), pp. 821-4.

Briscoe, D.M., DesRoches, L.E., Kiely, J.M., Lederer, J.A. and Lichtman, A.H. (1995) 'Antigen-dependent activation of T helper cell subsets by endothelium', *Transplantation*, 59(11), pp. 1638-41.

Brodbeck, D., Cron, P. and Hemmings, B.A. (1999) 'A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain', *J Biol Chem*, 274(14), pp. 9133-6.

Bruno, S., Ardelt, B., Skierski, J.S., Traganos, F. and Darzynkiewicz, Z. (1992) 'Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes', *Cancer Res*, 52(2), pp. 470-3.

Cai, J., Qing, X., Tan, J. and Terasaki, P.I. (2013) 'Humoral theory of transplantation: some hot topics', *Br Med Bull*, 105, pp. 139-55.

Cai, J. and Terasaki, P.I. (2005) 'Humoral theory of transplantation: mechanism, prevention, and treatment', *Hum Immunol*, 66(4), pp. 334-42.

Campbell, J.J., Qin, S., Bacon, K.B., Mackay, C.R. and Butcher, E.C. (1996) 'Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells', *J Cell Biol*, 134(1), pp. 255-66.

Caro-Oleas, J.L., Gonzalez-Escribano, M.F., Gentil-Govantes, M.A., Acevedo, M.J., Gonzalez-Roncero, F.M., Blanco, G.B. and Nunez-Roldan, A. (2012) 'Clinical Relevance of Anti-HLA Donor-Specific Antibodies Detected by Luminex Assay in the Development of Rejection After Renal Transplantation', *Transplantation*, 94(4), pp. 338-44.

Cavalcanti, Y.V., Brelaz, M.C., Neves, J.K., Ferraz, J.C. and Pereira, V.R. (2012) 'Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis', *Pulm Med*, 2012, p. 745483.

Chan, J.R., Hyduk, S.J. and Cybulsky, M.I. (2000) 'Alpha 4 beta 1 integrin/VCAM-1 interaction activates alpha L beta 2 integrin-mediated adhesion to ICAM-1 in human T cells', *J Immunol*, 164(2), pp. 746-53.

Chang, F., Steelman, L.S., Lee, J.T., Shelton, J.G., Navolanic, P.M., Blalock, W.L., Franklin, R.A. and McCubrey, J.A. (2003) 'Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention', *Leukemia*, 17(7), pp. 1263-93.

Chava, K.R., Tauseef, M., Sharma, T. and Mehta, D. (2012) 'Cyclic AMP response element-binding protein prevents endothelial permeability increase through transcriptional controlling p190RhoGAP expression', *Blood*, 119(1), pp. 308-19.

Chen, D., Weber, M., Lechler, R. and Dorling, A. (2006a) 'NK-cell-dependent acute xenograft rejection in the mouse heart-to-rat model', *Xenotransplantation*, 13(5), pp. 408-14.

Chen, H.Z., Tsai, S.Y. and Leone, G. (2009) 'Emerging roles of E2Fs in cancer: an exit from cell cycle control', *Nat Rev Cancer*, 9(11), pp. 785-97.

Chen, X.L., Dodd, G., Thomas, S., Zhang, X., Wasserman, M.A., Rovin, B.H. and Kunsch, C. (2006b) 'Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression', *Am J Physiol Heart Circ Physiol*, 290(5), pp. H1862-70.

Chiappara, G., Chanez, P., Bruno, A., Pace, E., Pompeo, F., Bousquet, J., Bonsignore, G. and Gjomarkaj, M. (2007) 'Variable p-CREB expression depicts different asthma phenotypes', *Allergy*, 62(7), pp. 787-94.

Chih, S., Chruscinski, A., Ross, H.J., Tinckam, K., Butany, J. and Rao, V. (2012) 'Antibody-mediated rejection: an evolving entity in heart transplantation', *J Transplant*, 2012, p. 210210.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) 'Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells', *J Biol Chem*, 265(9), pp. 5267-72.

Chiueh, C.C., Andoh, T. and Chock, P.B. (2005) 'Induction of thioredoxin and mitochondrial survival proteins mediates preconditioning-induced cardioprotection and neuroprotection', *Ann N Y Acad Sci*, 1042, pp. 403-18.

Cho, H.Y., Reddy, S.P., Yamamoto, M. and Kleeberger, S.R. (2004) 'The transcription factor NRF2 protects against pulmonary fibrosis', *FASEB J*, 18(11), pp. 1258-60.

Chrivia, J.C., Kwok, R.P., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) 'Phosphorylated CREB binds specifically to the nuclear protein CBP', *Nature*, 365(6449), pp. 855-9.

Chrobak, I., Lenna, S., Stawski, L. and Trojanowska, M. (2013) 'Interferon-gamma promotes vascular remodeling in human microvascular endothelial cells by upregulating endothelin (ET)-1 and transforming growth factor (TGF) beta2', *J Cell Physiol*, 228(8), pp. 1774-83.

Cinamon, G., Shinder, V. and Alon, R. (2001) 'Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines', *Nat Immunol*, 2(6), pp. 515-22.

Cinamon, G., Shinder, V., Shamri, R. and Alon, R. (2004) 'Chemoattractant signals and beta 2 integrin occupancy at apical endothelial contacts combine with shear stress

signals to promote transendothelial neutrophil migration', *J Immunol*, 173(12), pp. 7282-91.

Clatworthy, M.R., Espeli, M., Torpey, N. and Smith, K.G. (2010) 'The generation and maintenance of serum alloantibody', *Curr Opin Immunol*, 22(5), pp. 669-81.

Colvin, R.B. and Smith, R.N. (2005) 'Antibody-mediated organ-allograft rejection', *Nat Rev Immunol*, 5(10), pp. 807-17.

Conlon, T.M., Saeb-Parsy, K., Cole, J.L., Motallebzadeh, R., Qureshi, M.S., Rehakova, S., Negus, M.C., Callaghan, C.J., Bolton, E.M., Bradley, J.A. and Pettigrew, G.J. (2012) 'Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells', *J Immunol*, 188(6), pp. 2643-52.

Connolly, G.M., Cunningham, R., McNamee, P.T., Young, I.S. and Maxwell, A.P. (2011) 'Elevated soluble cellular adhesion molecules are associated with increased mortality in a prospective cohort of renal transplant recipients', *BMC Nephrol*, 12, p. 23.

Cornell, L.D., Smith, R.N. and Colvin, R.B. (2008) 'Kidney transplantation: mechanisms of rejection and acceptance', *Annu Rev Pathol*, 3, pp. 189-220.

Corr, M., Boyd, L.F., Frankel, S.R., Kozlowski, S., Padlan, E.A. and Margulies, D.H. (1992) 'Endogenous peptides of a soluble major histocompatibility complex class I molecule, H-2Lds: sequence motif, quantitative binding, and molecular modeling of the complex', *J Exp Med*, 176(6), pp. 1681-92.

Cox, S.T., Stephens, H.A., Fernando, R., Karasu, A., Harber, M., Howie, A.J., Powis, S., Zou, Y., Stastny, P., Madrigal, J.A. and Little, A.M. (2011) 'Major histocompatibility complex class I-related chain A allele mismatching, antibodies, and rejection in renal transplantation', *Hum Immunol*, 72(10), pp. 827-34.

Cresswell, P., Ackerman, A.L., Giodini, A., Peaper, D.R. and Wearsch, P.A. (2005) 'Mechanisms of MHC class I-restricted antigen processing and cross-presentation', *Immunol Rev*, 207, pp. 145-57.

Cunningham, A.C., Zhang, J.G., Moy, J.V., Ali, S. and Kirby, J.A. (1997) 'A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells', *Immunology*, 91(3), pp. 458-63.

D'Amico, M., Hulit, J., Amanatullah, D.F., Zafonte, B.T., Albanese, C., Bouzahzah, B., Fu, M., Augenlicht, L.H., Donehower, L.A., Takemaru, K., Moon, R.T., Davis, R., Lisanti, M.P., Shtutman, M., Zhurinsky, J., Ben-Ze'ev, A., Troussard, A.A., Dedhar, S. and Pestell, R.G. (2000) 'The integrin-linked kinase regulates the cyclin D1 gene through glycogen synthase kinase 3beta and cAMP-responsive element-binding protein-dependent pathways', *J Biol Chem*, 275(42), pp. 32649-57.

De Borst, M.H., Prakash, J., Melenhorst, W.B., van den Heuvel, M.C., Kok, R.J., Navis, G. and van Goor, H. (2007) 'Glomerular and tubular induction of the transcription factor c-Jun in human renal disease', *J Pathol*, 213(2), pp. 219-28.

De Caterina, R., Bourcier, T., Laufs, U., La Fata, V., Lazzerini, G., Neish, A.S., Libby, P. and Liao, J.K. (2001) 'Induction of endothelial-leukocyte interaction by interferon-gamma requires coactivation of nuclear factor-kappaB', *Arterioscler Thromb Vasc Biol*, 21(2), pp. 227-32.

Deak, M., Clifton, A.D., Lucocq, L.M. and Alessi, D.R. (1998) 'Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB', *EMBO J*, 17(15), pp. 4426-41.

Dedeepiya, V.D., Rao, Y.Y., Jayakrishnan, G.A., Parthiban, J.K., Baskar, S., Manjunath, S.R., Senthilkumar, R. and Abraham, S.J. (2012) 'Index of CD34+ Cells and Mononuclear Cells in the Bone Marrow of Spinal Cord Injury Patients of Different Age Groups: A Comparative Analysis', *Bone Marrow Res*, 2012, p. 787414.

Deitch, E.A. and Mancini, M.C. (1993) 'Complement receptors in shock and transplantation', *Arch Surg*, 128(11), pp. 1222-6.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998) 'Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase', *Proc Natl Acad Sci U S A*, 95(19), pp. 11211-6.

Delia, D., Lampugnani, M.G., Resnati, M., Dejana, E., Aiello, A., Fontanella, E., Soligo, D., Pierotti, M.A. and Greaves, M.F. (1993) 'CD34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells in vitro', *Blood*, 81(4), pp. 1001-8.

Desdouets, C., Sobczak-Thepot, J., Murphy, M. and Brechot, C. (1995) 'Cyclin A: function and expression during cell proliferation', *Prog Cell Cycle Res*, 1, pp. 115-23.

Devi, T.S., Singh, L.P., Hosoya, K. and Terasaki, T. (2011) 'GSK-3beta/CREB axis mediates IGF-1-induced ECM/adhesion molecule expression, cell cycle progression and monolayer permeability in retinal capillary endothelial cells: Implications for diabetic retinopathy', *Biochim Biophys Acta*, 1812(9), pp. 1080-8.

Dierselhuis, M. and Goulmy, E. (2009) 'The relevance of minor histocompatibility antigens in solid organ transplantation', *Curr Opin Organ Transplant*, 14(4), pp. 419-25.

DiVietro, J.A., Smith, M.J., Smith, B.R., Petruzzelli, L., Larson, R.S. and Lawrence, M.B. (2001) 'Immobilized IL-8 triggers progressive activation of neutrophils rolling in vitro on P-selectin and intercellular adhesion molecule-1', *J Immunol*, 167(7), pp. 4017-25.

Drachenberg, C.B. and Papadimitriou, J.C. (2013) 'Endothelial injury in renal antibody-mediated allograft rejection: a schematic view based on pathogenesis', *Transplantation*, 95(9), pp. 1073-83.

Drachenberg, C.B., Torrealba, J.R., Nankivell, B.J., Rangel, E.B., Bajema, I.M., Kim, D.U., Arend, L., Bracamonte, E.R., Bromberg, J.S., Bruijn, J.A., Cantarovich, D., Chapman, J.R., Farris, A.B., Gaber, L., Goldberg, J.C., Haririan, A., Honsova, E., Iskandar, S.S., Klassen, D.K., Kraus, E., Lower, F., Odorico, J., Olson, J.L., Mittalhenkle, A., Munivenkatappa, R., Paraskevas, S., Papadimitriou, J.C., Randhawa, P., Reinholt, F.P., Renaudin, K., Revelo, P., Ruiz, P., Samaniego, M.D., Shapiro, R., Stratta, R.J., Sutherland, D.E., Troxell, M.L., Voska, L., Seshan, S.V., Racusen, L.C. and Bartlett, S.T. (2011) 'Guidelines for the diagnosis of antibody-mediated rejection in pancreas allografts-updated Banff grading schema', *Am J Transplant*, 11(9), pp. 1792-802.

Dragun, D., Catar, R., Kusch, A., Heidecke, H. and Philippe, A. (2012) 'Non-HLA-antibodies targeting Angiotensin type 1 receptor and antibody mediated rejection', *Hum Immunol*, 73(12), pp. 1282-6.

Du, K. and Montminy, M. (1998) 'CREB is a regulatory target for the protein kinase Akt/PKB', *J Biol Chem*, 273(49), pp. 32377-9.

Dudek, S.M. and Garcia, J.G. (2001) 'Cytoskeletal regulation of pulmonary vascular permeability', *J Appl Physiol*, 91(4), pp. 1487-500.

Edgell, C.J., McDonald, C.C. and Graham, J.B. (1983) 'Permanent cell line expressing human factor VIII-related antigen established by hybridization', *Proc Natl Acad Sci U S A*, 80(12), pp. 3734-7.

Einecke, G., Sis, B., Reeve, J., Mengel, M., Campbell, P.M., Hidalgo, L.G., Kaplan, B. and Halloran, P.F. (2009) 'Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure', *Am J Transplant*, 9(11), pp. 2520-31.

Elmore, S. (2007) 'Apoptosis: a review of programmed cell death', *Toxicol Pathol*, 35(4), pp. 495-516.

Enslen, H., Tokumitsu, H. and Soderling, T.R. (1995) 'Phosphorylation of CREB by CaM-kinase IV activated by CaM-kinase IV kinase', *Biochem Biophys Res Commun*, 207(3), pp. 1038-43.

Erlich, H.A., Opelz, G. and Hansen, J. (2001) 'HLA DNA typing and transplantation', *Immunity*, 14(4), pp. 347-56.

Everly, M.J., Everly, J.J., Arend, L.J., Brailey, P., Susskind, B., Govil, A., Rike, A., Roy-Chaudhury, P., Mogilishetty, G., Alloway, R.R., Tevar, A. and Woodle, E.S. (2009) 'Reducing de novo donor-specific antibody levels during acute rejection diminishes renal allograft loss', *Am J Transplant*, 9(5), pp. 1063-71.

Fahim, T., Bohmig, G.A., Exner, M., Huttary, N., Kerschner, H., Kandutsch, S., Kerjaschki, D., Brambock, A., Nagy-Bojarszky, K. and Regele, H. (2007) 'The cellular lesion of humoral rejection: predominant recruitment of monocytes to peritubular and glomerular capillaries', *Am J Transplant*, 7(2), pp. 385-93.

Fairchild, R.L. (2005) 'Raising the direction signposts that guide T cell trafficking into allografts', *Transplantation*, 79(6), pp. 646-7.

Falcieri, E., Martelli, A.M., Bareggi, R., Cataldi, A. and Cocco, L. (1993) 'The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in MOLT-4 cells without concomitant DNA fragmentation', *Biochem Biophys Res Commun*, 193(1), pp. 19-25.

Fatrai, S., Elghazi, L., Balcazar, N., Cras-Meneur, C., Krits, I., Kiyokawa, H. and Bernal-Mizrachi, E. (2006) 'Akt induces beta-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity', *Diabetes*, 55(2), pp. 318-25.

Feucht, H.E., Felber, E., Gokel, M.J., Hillebrand, G., Nattermann, U., Brockmeyer, C., Held, E., Riethmuller, G., Land, W. and Albert, E. (1991) 'Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection', *Clin Exp Immunol*, 86(3), pp. 464-70.

Fidkowski, C., Kaazempur-Mofrad, M.R., Borenstein, J., Vacanti, J.P., Langer, R. and Wang, Y. (2005) 'Endothelialized microvasculature based on a biodegradable elastomer', *Tissue Eng*, 11(1-2), pp. 302-9.

Fina, L., Molgaard, H.V., Robertson, D., Bradley, N.J., Monaghan, P., Delia, D., Sutherland, D.R., Baker, M.A. and Greaves, M.F. (1990) 'Expression of the CD34 gene in vascular endothelial cells', *Blood*, 75(12), pp. 2417-26.

Freitas, M.C., Rebellato, L.M., Ozawa, M., Nguyen, A., Sasaki, N., Everly, M., Briley, K.P., Haisch, C.E., Bolin, P., Parker, K., Kendrick, W.T., Kendrick, S.A., Harland, R.C. and Terasaki, P.I. (2013) 'The Role of Immunoglobulin-G Subclasses and C1q in De

Novo HLA-DQ Donor-Specific Antibody Kidney Transplantation Outcomes', *Transplantation*, 95(9), pp. 1113-1119.

Fritchley, S.J., Kirby, J.A. and Ali, S. (2000) 'The antagonism of interferon-gamma (IFN-gamma) by heparin: examination of the blockade of class II MHC antigen and heat shock protein-70 expression', *Clin Exp Immunol*, 120(2), pp. 247-52.

Fujio, Y. and Walsh, K. (1999) 'Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner', *J Biol Chem*, 274(23), pp. 16349-54.

Fukami, N., Ramachandran, S., Narayanan, K., Liu, W., Nath, D.S., Jendrisak, M., Chapman, W. and Mohanakumar, T. (2012) 'Mechanism of accommodation in a sensitized human leukocyte antigen transgenic murine cardiac transplant model', *Transplantation*, 93(4), pp. 364-72.

Fukami, N., Ramachandran, S., Saini, D., Walter, M., Chapman, W., Patterson, G.A. and Mohanakumar, T. (2009) 'Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection', *J Immunol*, 182(1), pp. 309-18.

Gangavarapu, P., Rajagopalan, L., Kolli, D., Guerrero-Plata, A., Garofalo, R.P. and Rajarathnam, K. (2012) 'The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment', *J Leukoc Biol*, 91(2), pp. 259-65.

Gao, B., Adhikari, R., Howarth, M., Nakamura, K., Gold, M.C., Hill, A.B., Knee, R., Michalak, M. and Elliott, T. (2002) 'Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin', *Immunity*, 16(1), pp. 99-109.

Garbi, N., Tanaka, S., Momburg, F. and Hammerling, G.J. (2006) 'Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57', *Nat Immunol*, 7(1), pp. 93-102.

Gaston, R.S., Cecka, J.M., Kasiske, B.L., Fieberg, A.M., Leduc, R., Cosio, F.C., Gourishankar, S., Grande, J., Halloran, P., Hunsicker, L., Mannon, R., Rush, D. and Matas, A.J. (2010) 'Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure', *Transplantation*, 90(1), pp. 68-74.

Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y.C., Yoshida, M., Ding, H.A., Gimbrone, M.A., Jr., Luster, A.D., Luscinskas, F.W. and Rosenzweig, A. (1999) 'MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions', *Nature*, 398(6729), pp. 718-23.

Girnita, A.L., McCurry, K.R., Yousem, S.A., Pilewski, J. and Zeevi, A. (2006) 'Antibody-mediated rejection in lung transplantation: case reports', *Clin Transpl*, pp. 508-10.

Gloor, J.M., Cosio, F.G., Rea, D.J., Wadei, H.M., Winters, J.L., Moore, S.B., DeGoey, S.R., Lager, D.J., Grande, J.P. and Stegall, M.D. (2006) 'Histologic findings one year after positive crossmatch or ABO blood group incompatible living donor kidney transplantation', *Am J Transplant*, 6(8), pp. 1841-7.

Gloor, J.M., DeGoey, S., Ploeger, N., Gebel, H., Bray, R., Moore, S.B., Dean, P.G. and Stegall, M.D. (2004) 'Persistence of low levels of alloantibody after desensitization in crossmatch-positive living-donor kidney transplantation', *Transplantation*, 78(2), pp. 221-7.

Goers, T.A., Ramachandran, S., Aloush, A., Trulock, E., Patterson, G.A. and Mohanakumar, T. (2008) 'De novo production of K-alpha1 tubulin-specific antibodies: role in chronic lung allograft rejection', *J Immunol*, 180(7), pp. 4487-94.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H. and Montminy, M.R. (1991) 'Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB', *Mol Cell Biol*, 11(3), pp. 1306-12.

Gonzalez, G.A. and Montminy, M.R. (1989) 'Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133', *Cell*, 59(4), pp. 675-80.

Grabovsky, V., Feigelson, S., Chen, C., Bleijs, D.A., Peled, A., Cinamon, G., Baleux, F., Arenzana-Seisdedos, F., Lapidot, T., van Kooyk, Y., Lobb, R.R. and Alon, R. (2000) 'Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions', *J Exp Med*, 192(4), pp. 495-506.

Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) 'Characteristics of a human cell line transformed by DNA from human adenovirus type 5', *J Gen Virol*, 36(1), pp. 59-74.

Grandaliano, G., Gesualdo, L., Ranieri, E., Monno, R., Stallone, G. and Schena, F.P. (1997) 'Monocyte chemotactic peptide-1 expression and monocyte infiltration in acute renal transplant rejection', *Transplantation*, 63(3), pp. 414-20.

Griffin, G.K., Newton, G., Tarrio, M.L., Bu, D.X., Maganto-Garcia, E., Azcutia, V., Alcaide, P., Grabie, N., Luscinskas, F.W., Croce, K.J. and Lichtman, A.H. (2012) 'IL-17 and TNF-alpha sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation', *J Immunol*, 188(12), pp. 6287-99.

Groth, J., Schonemann, C., Kaden, J. and May, G. (1996) 'Dynamics of donor-reactive IgG, IgA and IgM antibodies against T and B lymphocytes early after clinical kidney transplantation using flow cytometry', *Transpl Immunol*, 4(3), pp. 215-9.

Gur, H., Geppert, T.D., Wacholtz, M.C. and Lipsky, P.E. (1999) 'The cytoplasmic and the transmembrane domains are not sufficient for class I MHC signal transduction', *Cell Immunol*, 191(2), pp. 105-16.

Haas, M., Montgomery, R.A., Segev, D.L., Rahman, M.H., Racusen, L.C., Bagnasco, S.M., Simpkins, C.E., Warren, D.S., Lepley, D., Zachary, A.A. and Kraus, E.S. (2007) 'Subclinical acute antibody-mediated rejection in positive crossmatch renal allografts', *Am J Transplant*, 7(3), pp. 576-85.

Hachem, R. (2012) 'Antibody-Mediated Lung Transplant Rejection', *Curr Respir Care Rep*, 1(3), pp. 157-161.

Hadad, N., Tuval, L., Elgazar-Carmom, V. and Levy, R. (2011) 'Endothelial ICAM-1 protein induction is regulated by cytosolic phospholipase A2alpha via both NF-kappaB and CREB transcription factors', *J Immunol*, 186(3), pp. 1816-27.

Hallmann, R., Horn, N., Selg, M., Wendler, O., Pausch, F. and Sorokin, L.M. (2005) 'Expression and function of laminins in the embryonic and mature vasculature', *Physiol Rev*, 85(3), pp. 979-1000.

Halloran, P.F., de Freitas, D.G., Einecke, G., Famulski, K.S., Hidalgo, L.G., Menge, L.M., Reeve, J., Sellares, J. and Sis, B. (2010) 'An integrated view of molecular changes, histopathology and outcomes in kidney transplants', *Am J Transplant*, 10(10), pp. 2223-30.

Hamer, R., Molostvov, G., Lowe, D., Satchell, S., Mathieson, P., Ilyas, R., Mitchell, D.A., Lam, F.T., Kashi, H., Tan, L.C., Imray, C., Fletcher, S., Briggs, D., Krishnan, N., Higgins, R. and Zehnder, D. (2012) 'Human leukocyte antigen-specific antibodies and gamma-interferon stimulate human microvascular and glomerular endothelial cells to produce complement factor C4', *Transplantation*, 93(9), pp. 867-73.

Han, M., Rogers, J.A., Lavingia, B. and Stastny, P. (2009) 'Peripheral blood B cells producing donor-specific HLA antibodies in vitro', *Hum Immunol*, 70(1), pp. 29-34.

Hancock, W.W., Buelow, R., Sayegh, M.H. and Turka, L.A. (1998) 'Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes', *Nat Med*, 4(12), pp. 1392-6.

Harris, P.E., Bian, H. and Reed, E.F. (1997) 'Induction of high affinity fibroblast growth factor receptor expression and proliferation in human endothelial cells by anti-HLA antibodies: a possible mechanism for transplant atherosclerosis', *J Immunol*, 159(11), pp. 5697-704.

Harvey, J.R., Mellor, P., Eldaly, H., Lennard, T.W., Kirby, J.A. and Ali, S. (2007) 'Inhibition of CXCR4-mediated breast cancer metastasis: a potential role for heparinoids?', *Clin Cancer Res*, 13(5), pp. 1562-70.

Heath, W.R., Vitiello, A. and Sherman, L.A. (1989) 'Mapping of epitopes recognized by alloreactive cytotoxic T lymphocytes using inhibition by MHC peptides', *J Immunol*, 143(5), pp. 1441-6.

Heinemann, F.M., Roth, I., Rebmann, V., Arnold, M.L., Spriewald, B.M. and Grosse-Wilde, H. (2006) 'Characterization of anti-HLA antibodies eluted from explanted renal allografts', *Clin Transpl*, pp. 371-8.

Herter, J. and Zarbock, A. (2013) 'Integrin Regulation during Leukocyte Recruitment', *J Immunol*, 190(9), pp. 4451-7.

Hidalgo, L.G., Campbell, P.M., Sis, B., Einecke, G., Mengel, M., Chang, J., Sellares, J., Reeve, J. and Halloran, P.F. (2009) 'De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure', *Am J Transplant*, 9(11), pp. 2532-41.

Hidalgo, L.G., Sis, B., Sellares, J., Campbell, P.M., Mengel, M., Einecke, G., Chang, J. and Halloran, P.F. (2010) 'NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection', *Am J Transplant*, 10(8), pp. 1812-22.

Hidalgo, M. and Rowinsky, E.K. (2000) 'The rapamycin-sensitive signal transduction pathway as a target for cancer therapy', *Oncogene*, 19(56), pp. 6680-6.

Higgins, R., Zehnder, D., Chen, K., Lowe, D., McKinnell, J., Lam, F.T., Kashi, H., Tan, L.C., Imray, C., Fletcher, S., Krishnan, N., Hamer, R. and Briggs, D. (2010) 'The histological development of acute antibody-mediated rejection in HLA antibody-incompatible renal transplantation', *Nephrol Dial Transplant*, 25(4), pp. 1306-12.

Hill, G.S., Nochy, D., Bruneval, P., Duong van Huyen, J.P., Glotz, D., Suberbielle, C., Zuber, J., Anglicheau, D., Empana, J.P., Legendre, C. and Loupy, A. (2011) 'Donor-specific antibodies accelerate arteriosclerosis after kidney transplantation', *J Am Soc Nephrol*, 22(5), pp. 975-83.

Hirohashi, T., Chase, C.M., Della Pelle, P., Sebastian, D., Alessandrini, A., Madsen, J.C., Russell, P.S. and Colvin, R.B. (2012) 'A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody', *Am J Transplant*, 12(2), pp. 313-21.

Hirohashi, T., Uehara, S., Chase, C.M., DellaPelle, P., Madsen, J.C., Russell, P.S. and Colvin, R.B. (2010) 'Complement independent antibody-mediated endarteritis and transplant arteriopathy in mice', *Am J Transplant*, 10(3), pp. 510-7.

Hoeffler, J.P. and Habener, J.F. (1990) 'Characterization of a cyclic AMP regulatory element DNA-binding protein', *Trends Endocrinol Metab*, 1(3), pp. 155-8.

Honger, G., Hopfer, H., Arnold, M.L., Spriewald, B.M., Schaub, S. and Amico, P. (2011) 'Pretransplant IgG subclasses of donor-specific human leukocyte antigen antibodies and development of antibody-mediated rejection', *Transplantation*, 92(1), pp. 41-7.

Hou, J., Baichwal, V. and Cao, Z. (1994) 'Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1', *Proc Natl Acad Sci U S A*, 91(24), pp. 11641-5.

Hsieh, C.L., Obara, H., Ogura, Y., Martinez, O.M. and Krams, S.M. (2002) 'NK cells and transplantation', *Transpl Immunol*, 9(2-4), pp. 111-4.

Hu, J., Wang, Z., Tan, C.J., Liao, B.Y., Zhang, X., Xu, M., Dai, Z., Qiu, S.J., Huang, X.W., Sun, J., Sun, Q.M., He, Y.F., Song, K., Pan, Q., Wu, Y., Fan, J. and Zhou, J. (2013) 'Plasma microRNA, a potential biomarker for acute rejection after liver transplantation', *Transplantation*, 95(8), pp. 991-9.

Hughes-Fulford, M., Sugano, E., Schopper, T., Li, C.F., Boonyaratanakornkit, J.B. and Cogoli, A. (2005) 'Early immune response and regulation of IL-2 receptor subunits', *Cell Signal*, 17(9), pp. 1111-24.

Hunter, T. (2007) 'The age of crosstalk: phosphorylation, ubiquitination, and beyond', *Mol Cell*, 28(5), pp. 730-8.

Hur, G.M., Ryu, Y.S., Yun, H.Y., Jeon, B.H., Kim, Y.M., Seok, J.H. and Lee, J.H. (1999) 'Hepatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF-kappaB', *Biochem Biophys Res Commun*, 261(3), pp. 917-22.

Hyduk, S.J., Chan, J.R., Duffy, S.T., Chen, M., Peterson, M.D., Waddell, T.K., Digby, G.C., Szaszi, K., Kapus, A. and Cybulsky, M.I. (2007) 'Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants', *Blood*, 109(1), pp. 176-84.

Ichihara, S., Yamada, Y., Liu, F., Murohara, T., Itoh, K., Yamamoto, M. and Ichihara, G. (2010) 'Ablation of the transcription factor Nrf2 promotes ischemia-induced neovascularization by enhancing the inflammatory response', *Arterioscler Thromb Vasc Biol*, 30(8), pp. 1553-61.

Ichiki, T. (2006) 'Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both?', *Arterioscler Thromb Vasc Biol*, 26(3), pp. 449-55.

Ilan, N., Mahooti, S., Rimm, D.L. and Madri, J.A. (1999) 'PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated beta-catenin', *J Cell Sci*, 112 Pt 18, pp. 3005-14.

Ireland, B.S., Brockmeier, U., Howe, C.M., Elliott, T. and Williams, D.B. (2008) 'Lectin-deficient calreticulin retains full functionality as a chaperone for class I histocompatibility molecules', *Mol Biol Cell*, 19(6), pp. 2413-23.

Iwasaki, K., Miwa, Y., Haneda, M., Uchida, K., Nakao, A. and Kobayashi, T. (2010) 'Significance of HLA class I antibody-induced antioxidant gene expression for endothelial cell protection against complement attack', *Biochem Biophys Res Commun*, 391(2), pp. 1210-5.

Iwata, T., Philipovskiy, A., Fisher, A.J., Presson, R.G., Jr., Chiyo, M., Lee, J., Mickler, E., Smith, G.N., Petrache, I., Brand, D.B., Burlingham, W.J., Gopalakrishnan, B., Greenspan, D.S., Christie, J.D. and Wilkes, D.S. (2008) 'Anti-type V collagen humoral immunity in lung transplant primary graft dysfunction', *J Immunol*, 181(8), pp. 5738-47.

Jahnke, A. and Johnson, J.P. (1994) 'Synergistic activation of intercellular adhesion molecule 1 (ICAM-1) by TNF-alpha and IFN-gamma is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor Stat1 alpha (p91) that can be activated by both IFN-gamma and IFN-alpha', *FEBS Lett*, 354(2), pp. 220-6.

Jaramillo, A., Smith, C.R., Maruyama, T., Zhang, L., Patterson, G.A. and Mohanakumar, T. (2003) 'Anti-HLA class I antibody binding to airway epithelial cells induces production of fibrogenic growth factors and apoptotic cell death: a possible mechanism for bronchiolitis obliterans syndrome', *Hum Immunol*, 64(5), pp. 521-9.

Jardetzky, T.S., Lane, W.S., Robinson, R.A., Madden, D.R. and Wiley, D.C. (1991) 'Identification of self peptides bound to purified HLA-B27', *Nature*, 353(6342), pp. 326-9.

Jeannet, M., Pinn, V.W., Flax, M.H., Winn, H.J. and Russell, P.S. (1970) 'Humoral antibodies in renal allotransplantation in man', *N Engl J Med*, 282(3), pp. 111-7.

Jiang, B.H. and Liu, L.Z. (2009) 'PI3K/PTEN signaling in angiogenesis and tumorigenesis', *Adv Cancer Res*, 102, pp. 19-65.

Jin, Y.-P., Fishbein, M.C., Said, J.W., Jindra, P.T., Rajalingam, R., Rozengurt, E. and Reed, E.F. (2004) 'Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells', *Human Immunology*, 65(4), pp. 291-302.

Jin, Y.P., Korin, Y., Zhang, X., Jindra, P.T., Rozengurt, E. and Reed, E.F. (2007) 'RNA interference elucidates the role of focal adhesion kinase in HLA class I-mediated focal adhesion complex formation and proliferation in human endothelial cells', *J Immunol*, 178(12), pp. 7911-22.

Jin, Y.P., Singh, R.P., Du, Z.Y., Rajasekaran, A.K., Rozengurt, E. and Reed, E.F. (2002) 'Ligation of HLA class I molecules on endothelial cells induces phosphorylation of Src, paxillin, and focal adhesion kinase in an actin-dependent manner', *J Immunol*, 168(11), pp. 5415-23.

Jindra, P.T., Hsueh, A., Hong, L., Gjertson, D., Shen, X.D., Gao, F., Dang, J., Mischel, P.S., Baldwin, W.M., 3rd, Fishbein, M.C., Kupiec-Weglinski, J.W. and Reed, E.F. (2008a) 'Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts', *J Immunol*, 180(4), pp. 2214-24.

Jindra, P.T., Jin, Y.-P., Jacamo, R., Rozengurt, E. and Reed, E.F. (2008b) 'MHC class I and integrin ligation induce ERK activation via an mTORC2-dependent pathway', *Biochemical and Biophysical Research Communications*, 369(2), pp. 781-787.

Jindra, P.T., Jin, Y.P., Rozengurt, E. and Reed, E.F. (2008c) 'HLA class I antibody-mediated endothelial cell proliferation via the mTOR pathway', *J Immunol*, 180(4), pp. 2357-66.

Jindra, P.T. and Reed, E.F. (2007) 'Signal transduction through major histocompatibility complex molecules', *Current Opinion in Organ Transplantation*, 12(4), pp. 426-431 10.1097/MOT.0b013e32823ecff2.

Jirasiritham, S., Khunprakant, R., Techawathanawanna, N. and Mavichak, V. (2010) 'Treatment of simultaneous acute antibody-mediated rejection and acute cellular rejection with alemtuzumab in kidney transplantation: a case report', *Transplant Proc*, 42(3), pp. 987-9.

Johannessen, M., Delghandi, M.P. and Moens, U. (2004) 'What turns CREB on?', *Cell Signal*, 16(11), pp. 1211-27.

Joosten, S.A., Sijpkens, Y.W., van Ham, V., Trouw, L.A., van der Vlag, J., van den Heuvel, B., van Kooten, C. and Paul, L.C. (2005) 'Antibody response against the glomerular basement membrane protein agrin in patients with transplant glomerulopathy', *Am J Transplant*, 5(2), pp. 383-93.

Kamper, N., Franken, S., Temme, S., Koch, S., Bieber, T. and Koch, N. (2012) 'gamma-Interferon-regulated chaperone governs human lymphocyte antigen class II expression', *FASEB J*, 26(1), pp. 104-16.

Kaneku, H. (2012) '2012 annual literature review of donor-specific HLA antibodies after organ transplantation', *Clin Transpl*, pp. 207-17.

Kerby, J.D., Verran, D.J., Luo, K.L., Ding, Q., Tagouri, Y., Herrera, G.A., Diethelm, A.G. and Thompson, J.A. (1996) 'Immunolocalization of FGF-1 and receptors in glomerular lesions associated with chronic human renal allograft rejection', *Transplantation*, 62(2), pp. 190-200.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) 'Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics', *Br J Cancer*, 26(4), pp. 239-57.

Kienast, A., Preuss, M., Winkler, M. and Dick, T.P. (2007) 'Redox regulation of peptide receptivity of major histocompatibility complex class I molecules by ERp57 and tapasin', *Nat Immunol*, 8(8), pp. 864-72.

Kim, H.J. and Vaziri, N.D. (2010) 'Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure', *Am J Physiol Renal Physiol*, 298(3), pp. F662-71.

Kim, V.N. (2003) 'RNA interference in functional genomics and medicine', *J Korean Med Sci*, 18(3), pp. 309-18.

Kinashi, T. (2005) 'Intracellular signalling controlling integrin activation in lymphocytes', *Nat Rev Immunol*, 5(7), pp. 546-59.

Kirmizis, A. and Farnham, P.J. (2004) 'Genomic approaches that aid in the identification of transcription factor target genes', *Exp Biol Med (Maywood)*, 229(8), pp. 705-21.

Kissmeyer-Nielsen, F., Olsen, S., Petersen, V.P. and Fjeldborg, O. (1966) 'Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells', *Lancet*, 2(7465), pp. 662-5.

Kitchens, W.H., Chase, C.M., Uehara, S., Cornell, L.D., Colvin, R.B., Russell, P.S. and Madsen, J.C. (2007) 'Macrophage depletion suppresses cardiac allograft vasculopathy in mice', *Am J Transplant*, 7(12), pp. 2675-82.

Klein, J. and Sato, A. (2000) 'The HLA system. First of two parts', *N Engl J Med*, 343(10), pp. 702-9.

Klein, J.F.F. (1986) 'The evolution of class I MHC genes.', *Immunol. Today*, 7, pp. 41-44.

Knittler, M.R., Alberts, P., Deverson, E.V. and Howard, J.C. (1999) 'Nucleotide binding by TAP mediates association with peptide and release of assembled MHC class I molecules', *Curr Biol*, 9(18), pp. 999-1008.

Kobayashi, A., Takahashi, T., Horita, S., Yamamoto, I., Yamamoto, H., Tanabe, K., Yamaguchi, Y. and Hosoya, T. (2012) 'Clinicopathological impacts of activated transcription factor c-Jun in peritubular capillary endothelial cells in chronic antibodymediated rejection after kidney transplantation', *Clin Nephrol*, 77(1), pp. 32-9.

Kobayashi, A., Takahashi, T., Horita, S., Yamamoto, I., Yamamoto, H., Teraoka, S., Tanabe, K., Hosoya, T. and Yamaguchi, Y. (2010) 'Activation of the transcription factor c-Jun in acute cellular and antibody-mediated rejection after kidney transplantation', *Hum Pathol*, 41(12), pp. 1682-93.

Koch, C.A., Khalpey, Z.I. and Platt, J.L. (2004) 'Accommodation: preventing injury in transplantation and disease', *J Immunol*, 172(9), pp. 5143-8.

Kronke, G., Bochkov, V.N., Huber, J., Gruber, F., Bluml, S., Furnkranz, A., Kadl, A., Binder, B.R. and Leitinger, N. (2003) 'Oxidized phospholipids induce expression of human heme oxygenase-1 involving activation of cAMP-responsive element-binding protein', *J Biol Chem*, 278(51), pp. 51006-14.

Kuwano, Y., Spelten, O., Zhang, H., Ley, K. and Zarbock, A. (2010) 'Rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils', *Blood*, 116(4), pp. 617-24.

Lachmann, N., Terasaki, P.I., Budde, K., Liefeldt, L., Kahl, A., Reinke, P., Pratschke, J., Rudolph, B., Schmidt, D., Salama, A. and Schonemann, C. (2009) 'Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts', *Transplantation*, 87(10), pp. 1505-13.

Ladasky, J.J., Shum, B.P., Canavez, F., Seuanez, H.N. and Parham, P. (1999) 'Residue 3 of beta2-microglobulin affects binding of class I MHC molecules by the W6/32 antibody', *Immunogenetics*, 49(4), pp. 312-20.

Laudanna, C. and Alon, R. (2006) 'Right on the spot. Chemokine triggering of integrin-mediated arrest of rolling leukocytes', *Thromb Haemost*, 95(1), pp. 5-11.

Lay, A.J., Donahue, D., Tsai, M.J. and Castellino, F.J. (2007) 'Acute inflammation is exacerbated in mice genetically predisposed to a severe protein C deficiency', *Blood*, 109(5), pp. 1984-91.

Lee, C.Y., Lotfi-Emran, S., Erdinc, M., Murata, K., Velidedeoglu, E., Fox-Talbot, K., Liu, J., Garyu, J., Baldwin, W.M., 3rd and Wasowska, B.A. (2007) 'The involvement of FcR mechanisms in antibody-mediated rejection', *Transplantation*, 84(10), pp. 1324-34.

Lee, J.Y., Buzney, C.D., Poznansky, M.C. and Sackstein, R. (2009) 'Dynamic alterations in chemokine gradients induce transendothelial shuttling of human T cells under physiologic shear conditions', *J Leukoc Biol*, 86(6), pp. 1285-94.

Lee, P.C., Terasaki, P.I., Takemoto, S.K., Lee, P.H., Hung, C.J., Chen, Y.L., Tsai, A. and Lei, H.Y. (2002) 'All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies', *Transplantation*, 74(8), pp. 1192-4.

Lee, R.J., Albanese, C., Stenger, R.J., Watanabe, G., Inghirami, G., Haines, G.K., 3rd, Webster, M., Muller, W.J., Brugge, J.S., Davis, R.J. and Pestell, R.G. (1999) 'pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells', *J Biol Chem*, 274(11), pp. 7341-50.

Leeuwenberg, J.F., Van Damme, J., Meager, T., Jeunhomme, T.M. and Buurman, W.A. (1988) 'Effects of tumor necrosis factor on the interferon-gamma-induced major histocompatibility complex class II antigen expression by human endothelial cells', *Eur J Immunol*, 18(9), pp. 1469-72.

Lefort, C.T. and Ley, K. (2012) 'Neutrophil arrest by LFA-1 activation', *Front Immunol*, 3, p. 157.

Lehner, P.J., Surman, M.J. and Cresswell, P. (1998) 'Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220', *Immunity*, 8(2), pp. 221-31.

Leong, H.S., Mahesh, B.M., Day, J.R., Smith, J.D., McCormack, A.D., Ghimire, G., Podor, T.J. and Rose, M.L. (2008) 'Vimentin autoantibodies induce platelet activation and formation of platelet-leukocyte conjugates via platelet-activating factor', *J Leukoc Biol*, 83(2), pp. 263-71.

Leonhardt, R.M., Keusekotten, K., Bekpen, C. and Knittler, M.R. (2005) 'Critical role for the tapasin-docking site of TAP2 in the functional integrity of the MHC class I-peptide-loading complex', *J Immunol*, 175(8), pp. 5104-14.

Lepin, E.J., Jin, Y.P., Barwe, S.P., Rozengurt, E. and Reed, E.F. (2004) 'HLA class I signal transduction is dependent on Rho GTPase and ROK', *Biochem Biophys Res Commun*, 323(1), pp. 213-7.

Li, Q. and Verma, I.M. (2002) 'NF-kappaB regulation in the immune system', *Nat Rev Immunol*, 2(10), pp. 725-34.

Li, W., Wang, H., Kuang, C.Y., Zhu, J.K., Yu, Y., Qin, Z.X., Liu, J. and Huang, L. (2012) 'An essential role for the Id1/PI3K/Akt/NFkB/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells in vitro', *Mol Cell Biochem*, 363(1-2), pp. 135-45.

Li, X., Ishida, H., Yamaguchi, Y. and Tanabe, K. (2008) 'Poor graft outcome in recipients with de novo donor-specific anti-HLA antibodies after living related kidney transplantation', *Transpl Int*, 21(12), pp. 1145-52.

Liu, L. and Shi, G.P. (2012) 'CD31: beyond a marker for endothelial cells', *Cardiovasc Res*, 94(1), pp. 3-5.

Liu, X., Li, L., Pan, F. and Tian, W. (2012) 'MICB polymorphism in a southern Chinese Han population: the identification of two new MICB alleles, MICB*005:06 and MICB*026', *Hum Immunol*, 73(8), pp. 818-23.

Ljunggren, H.G., Stam, N.J., Ohlen, C., Neefjes, J.J., Hoglund, P., Heemels, M.T., Bastin, J., Schumacher, T.N., Townsend, A., Karre, K. and et al. (1990) 'Empty MHC class I molecules come out in the cold', *Nature*, 346(6283), pp. 476-80.

Lobo, L.J., Aris, R.M., Schmitz, J. and Neuringer, I.P. (2013) 'Donor-specific antibodies are associated with antibody-mediated rejection, acute cellular rejection, bronchiolitis obliterans syndrome, and cystic fibrosis after lung transplantation', *J Heart Lung Transplant*, 32(1), pp. 70-7.

Louis, N., Evelegh, C. and Graham, F.L. (1997) 'Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line', *Virology*, 233(2), pp. 423-9.

Loupy, A., Cazes, A., Guillemain, R., Amrein, C., Hedjoudje, A., Tible, M., Pezzella, V., Fabiani, J.N., Suberbielle, C., Nochy, D., Hill, G.S., Empana, J.P., Jouven, X., Bruneval, P. and Duong Van Huyen, J.P. (2011) 'Very late heart transplant rejection is associated with microvascular injury, complement deposition and progression to cardiac allograft vasculopathy', *Am J Transplant*, 11(7), pp. 1478-87.

Loupy, A., Suberbielle-Boissel, C., Hill, G.S., Lefaucheur, C., Anglicheau, D., Zuber, J., Martinez, F., Thervet, E., Mejean, A., Charron, D., Duong van Huyen, J.P., Bruneval, P., Legendre, C. and Nochy, D. (2009) 'Outcome of subclinical antibody-mediated rejection in kidney transplant recipients with preformed donor-specific antibodies', *Am J Transplant*, 9(11), pp. 2561-70.

Lucas, J.G., Co, J.P., Nwaogwugwu, U.T., Dosani, I. and Sureshkumar, K.K. (2011) 'Antibody-mediated rejection in kidney transplantation: an update', *Expert Opin Pharmacother*, 12(4), pp. 579-92.

Lunsford, K.E., Baird, B.J., Sempowski, G.D., Cardona, D.M., Li, Z., Weinhold, K.J., Sudan, D.L. and Brennan, T.V. (2013) 'Upregulation of IL-1beta, IL-6, and CCL-2 by a Novel Mouse Model of Pancreatic Ischemia-Reperfusion Injury', *Transplantation*, 95(8), pp. 1000-1007.

Luscinskas, F.W., Lim, Y.C. and Lichtman, A.H. (2001) 'Wall shear stress: the missing step for T cell transmigration?', *Nat Immunol*, 2(6), pp. 478-80.

Lynch, R.J. and Platt, J.L. (2008) 'Accommodation in organ transplantation', *Curr Opin Organ Transplant*, 13(2), pp. 165-70.

Lyu, D.M., Grazia, T.J., Benson, A.B., Cagle, L.R., Freed, B.M. and Zamora, M.R. (2012) 'Pre-transplant presence of antibodies to MICA and HLA class I or II are associated with an earlier onset of bronchiolitis obliterans syndrome in lung transplant recipients', *Clin Transpl*, pp. 237-46.

Ma, L., Cheung, K.C., Kishore, M., Nourshargh, S., Mauro, C. and Marelli-Berg, F.M. (2012) 'CD31 exhibits multiple roles in regulating T lymphocyte trafficking in vivo', *J Immunol*, 189(8), pp. 4104-11.

Macpherson, I. (1966) 'Mycoplasma in tissue culture', *J. Cell Sci.*, I, pp. 145-168.

Makela, O. and Mitchison, N.A. (1965) 'The role of cell number and source in adoptive immunity', *Immunology*, 8(6), pp. 539-48.

Manes, T.D. and Pober, J.S. (2008) 'Antigen presentation by human microvascular endothelial cells triggers ICAM-1-dependent transendothelial protrusion by, and fractalkine-dependent transendothelial migration of, effector memory CD4+ T cells', *J Immunol*, 180(12), pp. 8386-92.

Manes, T.D., Pober, J.S. and Kluger, M.S. (2006) 'Endothelial cell-T lymphocyte interactions: IP[corrected]-10 stimulates rapid transendothelial migration of human

effort but not central memory CD4+ T cells. Requirements for shear stress and adhesion molecules', *Transplantation*, 82(1 Suppl), pp. S9-14.

Mannam, V.K., Lewis, R.E. and Cruse, J.M. (2013) 'The fate of renal allografts hinges on responses of the microvascular endothelium', *Exp Mol Pathol*, 94(2), pp. 398-411.

Martinez, F.O., Sica, A., Mantovani, A. and Locati, M. (2008) 'Macrophage activation and polarization', *Front Biosci*, 13, pp. 453-61.

Mauiyyedi, S., Pelle, P.D., Saidman, S., Collins, A.B., Pascual, M., Tolkoff-Rubin, N.E., Williams, W.W., Cosimi, A.A., Schneeberger, E.E. and Colvin, R.B. (2001) 'Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries', *J Am Soc Nephrol*, 12(3), pp. 574-82.

May, M.J. and Ghosh, S. (1998) 'Signal transduction through NF-kappa B', *Immunol Today*, 19(2), pp. 80-8.

Mayr, B. and Montminy, M. (2001) 'Transcriptional regulation by the phosphorylation-dependent factor CREB', *Nat Rev Mol Cell Biol*, 2(8), pp. 599-609.

McDouall, R.M., Batten, P., McCormack, A., Yacoub, M.H. and Rose, M.L. (1997) 'MHC class II expression on human heart microvascular endothelial cells: exquisite sensitivity to interferon-gamma and natural killer cells', *Transplantation*, 64(8), pp. 1175-80.

McGilvray, I.D., Lu, Z., Bitar, R., Dackiw, A.P., Davreux, C.J. and Rotstein, O.D. (1997) 'VLA-4 integrin cross-linking on human monocytic THP-1 cells induces tissue factor expression by a mechanism involving mitogen-activated protein kinase', *J Biol Chem*, 272(15), pp. 10287-94.

Medawar, P.B. (1946a) 'Immunity to homologous grafted skin; the relationship between the antigens of blood and skin', *Br J Exp Pathol*, 27, pp. 15-24.

Medawar, P.B. (1946b) 'Immunity to homologous grafted skin; the suppression of cell division in grafts transplanted to immunized animals', *Br J Exp Pathol*, 27, pp. 9-14.

Meehan, S.M., McCluskey, R.T., Pascual, M., Preffer, F.I., Anderson, P., Schlossman, S.F. and Colvin, R.B. (1997) 'Cytotoxicity and apoptosis in human renal allografts: identification, distribution, and quantitation of cells with a cytotoxic granule protein GMP-17 (TIA-1) and cells with fragmented nuclear DNA', *Lab Invest*, 76(5), pp. 639-49.

Melhus, O., Koerner, T.J. and Adams, D.O. (1991) 'Effects of TNF alpha on the expression of class II MHC molecules in macrophages induced by IFN gamma: evidence for suppression at the level of transcription', *J Leukoc Biol*, 49(1), pp. 21-8.

Meller, R., Minami, M., Cameron, J.A., Impey, S., Chen, D., Lan, J.Q., Henshall, D.C. and Simon, R.P. (2005) 'CREB-mediated Bcl-2 protein expression after ischemic preconditioning', *J Cereb Blood Flow Metab*, 25(2), pp. 234-46.

Minami, K., Murata, K., Lee, C.Y., Fox-Talbot, K., Wasowska, B.A., Pescovitz, M.D. and Baldwin, W.M., 3rd (2006) 'C4d deposition and clearance in cardiac transplants correlates with alloantibody levels and rejection in rats', *Am J Transplant*, 6(5 Pt 1), pp. 923-32.

Minowada, J., Mathe, G., Barcos, M., Ginsbourg, M., Preisler, H., Canon, C. and Reizenstein, P. (1984) 'Cytological and immunological study of 139 patients with acute leukemia', *Med Oncol Tumor Pharmacother*, 1(1), pp. 3-7.

Molne, J., Nyberg, G., Blohme, I., Rydberg, L., Breimer, M.E. and Svalander, C.T. (2006) 'Glomerular C3c deposition and intravascular macrophage accumulation in early humoral renal allograft rejection signifies a poor short-term outcome', *APMIS*, 114(10), pp. 700-11.

Monsinjon, T., Gasque, P., Chan, P., Ischenko, A., Brady, J.J. and Fontaine, M.C. (2003) 'Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells', *FASEB J*, 17(9), pp. 1003-14.

Morita, K., Miura, M., Paolone, D.R., Engeman, T.M., Kapoor, A., Remick, D.G. and Fairchild, R.L. (2001) 'Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection', *J Immunol*, 167(5), pp. 2979-84.

Mortier, A., Van Damme, J. and Proost, P. (2012) 'Overview of the mechanisms regulating chemokine activity and availability', *Immunol Lett*, 145(1-2), pp. 2-9.

Mosnier, L.O. and Griffin, J.H. (2003) 'Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor', *Biochem J*, 373(Pt 1), pp. 65-70.

Mroz, R.M., Holownia, A., Chyczewska, E., Drost, E.M., Braszko, J.J., Noparlik, J., Donaldson, K. and Macnee, W. (2007) 'Cytoplasm-nuclear trafficking of CREB and CREB phosphorylation at Ser133 during therapy of chronic obstructive pulmonary disease', *J Physiol Pharmacol*, 58 Suppl 5(Pt 2), pp. 437-44.

Muczynski, K.A., Ekle, D.M., Coder, D.M. and Anderson, S.K. (2003) 'Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression', *J Am Soc Nephrol*, 14(5), pp. 1336-48.

Mulder, A., Kardol, M.J., Arn, J.S., Eijsink, C., Franke, M.E., Schreuder, G.M., Haasnoot, G.W., Doxiadis, II, Sachs, D.H., Smith, D.M. and Claas, F.H. (2010) 'Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation', *Mol Immunol*, 47(4), pp. 809-15.

Muller, W.A. (2003) 'Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response', *Trends Immunol*, 24(6), pp. 327-34.

Muller, W.A. (2011) 'Mechanisms of leukocyte transendothelial migration', *Annu Rev Pathol*, 6, pp. 323-44.

Musat, A.I., Agni, R.M., Wai, P.Y., Pirsch, J.D., Lorentzen, D.F., Powell, A., Leverson, G.E., Bellingham, J.M., Fernandez, L.A., Foley, D.P., Mezrich, J.D., D'Alessandro, A.M. and Lucey, M.R. (2011) 'The significance of donor-specific HLA antibodies in rejection and ductopenia development in ABO compatible liver transplantation', *Am J Transplant*, 11(3), pp. 500-10.

Naemi, F.M., Ali, S. and Kirby, J.A. (2011) 'Antibody-mediated allograft rejection: the emerging role of endothelial cell signalling and transcription factors', *Transpl Immunol*, 25(2-3), pp. 96-103.

Nagata, D., Suzuki, E., Nishimatsu, H., Satonaka, H., Goto, A., Omata, M. and Hirata, Y. (2001) 'Transcriptional activation of the cyclin D1 gene is mediated by multiple cis-elements, including SP1 sites and a cAMP-responsive element in vascular endothelial cells', *J Biol Chem*, 276(1), pp. 662-9.

Narayanan, K., Jaramillo, A., Phelan, D.L. and Mohanakumar, T. (2004) 'Pre-exposure to sub-saturating concentrations of HLA class I antibodies confers resistance to endothelial cells against antibody complement-mediated lysis by regulating Bad through the phosphatidylinositol 3-kinase/Akt pathway', *Eur J Immunol*, 34(8), pp. 2303-12.

Narayanan, K., Jendrisak, M.D., Phelan, D.L. and Mohanakumar, T. (2006) 'HLA class I antibody mediated accommodation of endothelial cells via the activation of PI3K/cAMP dependent PKA pathway', *Transpl Immunol*, 15(3), pp. 187-97.

Nath, D.S., Ilias Basha, H., Tiriveedhi, V., Alur, C., Phelan, D., Ewald, G.A., Moazami, N. and Mohanakumar, T. (2010) 'Characterization of immune responses to cardiac self-antigens myosin and vimentin in human cardiac allograft recipients with antibody-mediated rejection and cardiac allograft vasculopathy', *J Heart Lung Transplant*, 29(11), pp. 1277-85.

Nath, N., Bian, H., Reed, E.F. and Chellappan, S.P. (1999) 'HLA class I-mediated induction of cell proliferation involves cyclin E-mediated inactivation of Rb function and induction of E2F activity', *J Immunol*, 162(9), pp. 5351-8.

Neumuller, J., Tohidast-Akrad, M., Fischer, M. and Mayr, W.R. (1993) 'Influence of chloroquine or acid treatment of human platelets on the antigenicity of HLA and the 'thrombocyte-specific' glycoproteins Ia/IIa, IIb, and IIb/IIIa', *Vox Sang*, 65(3), pp. 223-31.

Nickeleit, V. and Mihatsch, M.J. (2003) 'Kidney transplants, antibodies and rejection: is C4d a magic marker?', *Nephrol Dial Transplant*, 18(11), pp. 2232-9.

Nielsen, J.S. and McNagny, K.M. (2008) 'Novel functions of the CD34 family', *J Cell Sci*, 121(Pt 22), pp. 3683-92.

Nisato, R.E., Harrison, J.A., Buser, R., Orci, L., Rinsch, C., Montesano, R., Dupraz, P. and Pepper, M.S. (2004) 'Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span', *Am J Pathol*, 165(1), pp. 11-24.

Nomiyama, H., Osada, N. and Yoshie, O. (2010) 'The evolution of mammalian chemokine genes', *Cytokine Growth Factor Rev*, 21(4), pp. 253-62.

Norder, M., Gutierrez, M.G., Zicari, S., Cervi, E., Caruso, A. and Guzman, C.A. (2012) 'Lymph node-derived lymphatic endothelial cells express functional costimulatory molecules and impair dendritic cell-induced allogenic T-cell proliferation', *FASEB J*, 26(7), pp. 2835-46.

Nunes, E., Heslop, H., Fernandez-Vina, M., Taves, C., Wagenknecht, D.R., Eisenbrey, A.B., Fischer, G., Poulton, K., Wacker, K., Hurley, C.K., Noreen, H. and Sacchi, N. (2011) 'Definitions of histocompatibility typing terms: Harmonization of Histocompatibility Typing Terms Working Group', *Hum Immunol*, 72(12), pp. 1214-6.

O'Leary, J.G. and Klintmalm, G.B. (2013) 'Impact of donor-specific antibodies on results of liver transplantation', *Curr Opin Organ Transplant*, 18(3), pp. 279-84.

Okazaki, T., Kato, Y., Mochizuki, T., Tashima, M., Sawada, H. and Uchino, H. (1988) 'Staurosporine, a novel protein kinase inhibitor, enhances HL-60-cell differentiation induced by various compounds', *Exp Hematol*, 16(1), pp. 42-8.

Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaya, J., Tsuchya, H., Takahashi, Y. and Masuma, R. (1977) 'A new alkaloid AM-2282 OF Streptomyces origin. Taxonomy,

fermentation, isolation and preliminary characterization', *J Antibiot (Tokyo)*, 30(4), pp. 275-82.

Ono, H., Ichiki, T., Ohtsubo, H., Fukuyama, K., Imayama, I., Iino, N., Masuda, S., Hashiguchi, Y., Takeshita, A. and Sunagawa, K. (2006) 'CAMP-response element-binding protein mediates tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in endothelial cells', *Hypertens Res*, 29(1), pp. 39-47.

Ota, H., Fox-Talbot, K., Hu, W., Qian, Z., Sanfilippo, F., Hruban, R.H. and Baldwin, W.M., 3rd (2005) 'Terminal complement components mediate release of von Willebrand factor and adhesion of platelets in arteries of allografts', *Transplantation*, 79(3), pp. 276-81.

Otten, H.G., Verhaar, M.C., Borst, H.P., Hene, R.J. and van Zuilen, A.D. (2012) 'Pretransplant donor-specific HLA class-I and -II antibodies are associated with an increased risk for kidney graft failure', *Am J Transplant*, 12(6), pp. 1618-23.

Page, C., Rose, M., Yacoub, M. and Pigott, R. (1992) 'Antigenic heterogeneity of vascular endothelium', *Am J Pathol*, 141(3), pp. 673-83.

Papadimitriou, J.C., Drachenberg, C.B., Munivenkatappa, R., Ramos, E., Nogueira, J., Sailey, C., Klassen, D.K. and Haririan, A. (2010) 'Glomerular inflammation in renal allografts biopsies after the first year: cell types and relationship with antibody-mediated rejection and graft outcome', *Transplantation*, 90(12), pp. 1478-85.

Parish, C.R. (2005) 'Heparan sulfate and inflammation', *Nat Immunol*, 6(9), pp. 861-2.

Park, J.M., Greten, F.R., Wong, A., Westrick, R.J., Arthur, J.S., Otsu, K., Hoffmann, A., Montminy, M. and Karin, M. (2005) 'Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis--CREB and NF-kappaB as key regulators', *Immunity*, 23(3), pp. 319-29.

Parry, G.C. and Mackman, N. (1994) 'A set of inducible genes expressed by activated human monocytic and endothelial cells contain kappa B-like sites that specifically bind c-Rel-p65 heterodimers', *J Biol Chem*, 269(33), pp. 20823-5.

Persengiev, S.P. and Green, M.R. (2003) 'The role of ATF/CREB family members in cell growth, survival and apoptosis', *Apoptosis*, 8(3), pp. 225-8.

Petersdorf, E.W. (2008) 'Optimal HLA matching in hematopoietic cell transplantation', *Curr Opin Immunol*, 20(5), pp. 588-93.

Pfizenmaier, K., Scheurich, P., Schluter, C. and Kronke, M. (1987) 'Tumor necrosis factor enhances HLA-A,B,C and HLA-DR gene expression in human tumor cells', *J Immunol*, 138(3), pp. 975-80.

Pigazzi, M., Ricotti, E., Germano, G., Faggian, D., Arico, M. and Basso, G. (2007) 'cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression', *Haematologica*, 92(10), pp. 1435-7.

Platt, J.L., Vercellotti, G.M., Lindman, B.J., Oegema, T.R., Jr., Bach, F.H. and Dalmasso, A.P. (1990) 'Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection', *J Exp Med*, 171(4), pp. 1363-8.

Pober, J.S. and Sessa, W.C. (2007) 'Evolving functions of endothelial cells in inflammation', *Nat Rev Immunol*, 7(10), pp. 803-15.

Prasain, N. and Stevens, T. (2009) 'The actin cytoskeleton in endothelial cell phenotypes', *Microvasc Res*, 77(1), pp. 53-63.

Procko, E., Raghuraman, G., Wiley, D.C., Raghavan, M. and Gaudet, R. (2005) 'Identification of domain boundaries within the N-termini of TAP1 and TAP2 and their importance in tapasin binding and tapasin-mediated increase in peptide loading of MHC class I', *Immunol Cell Biol*, 83(5), pp. 475-82.

Pulvertaft, J.V. (1964) 'Cytology of Burkitt's Tumour (African Lymphoma)', *Lancet*, 1(7327), pp. 238-40.

Puttarajappa, C., Shapiro, R. and Tan, H.P. (2012) 'Antibody-mediated rejection in kidney transplantation: a review', *J Transplant*, 2012, p. 193724.

Qian, Z., Lee, C.Y., Murata, K., Liu, J., Fox-Talbot, K., Wasowska, B.A. and Baldwin, W.M., 3rd (2006) 'Antibody and complement mediated injury in transplants following sensitization by allogeneic blood transfusion', *Transplantation*, 82(7), pp. 857-64.

Racusen, L.C., Monteil, C., Sgrignoli, A., Lucskay, M., Marouillat, S., Rhim, J.G. and Morin, J.P. (1997) 'Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines', *J Lab Clin Med*, 129(3), pp. 318-29.

Radbruch, A., Muehlinghaus, G., Luger, E.O., Inamine, A., Smith, K.G., Dorner, T. and Hiepe, F. (2006) 'Competence and competition: the challenge of becoming a long-lived plasma cell', *Nat Rev Immunol*, 6(10), pp. 741-50.

Ramalingam, T.S., Chakrabarti, A. and Edidin, M. (1997) 'Interaction of class I human leukocyte antigen (HLA-I) molecules with insulin receptors and its effect on the insulin-signaling cascade', *Mol Biol Cell*, 8(12), pp. 2463-74.

Reed, E.F. (2003) 'Signal transduction via MHC class I molecules in endothelial and smooth muscle cells', *Crit Rev Immunol*, 23(1-2), pp. 109-28.

Regele, H., Bohmig, G.A., Habicht, A., Gollowitzer, D., Schillinger, M., Rockenschaub, S., Watschinger, B., Kerjaschki, D. and Exner, M. (2002) 'Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection', *J Am Soc Nephrol*, 13(9), pp. 2371-80.

Reinhold, S.W., Straub, R.H., Kruger, B., Kaess, B., Bergler, T., Weingart, C., Banas, M.C., Kramer, B.K. and Banas, B. (2012) 'Elevated urinary sVCAM-1, IL6, sIL6R and TNFR1 concentrations indicate acute kidney transplant rejection in the first 2 weeks after transplantation', *Cytokine*, 57(3), pp. 379-88.

Reyes-Vargas, E., Pavlov, I.Y., Martins, T.B., Schwartz, J.J., Hill, H.R. and Delgado, J.C. (2009) 'Binding of anti-HLA class I antibody to endothelial cells produce an inflammatory cytokine secretory pattern', *J Clin Lab Anal*, 23(3), pp. 157-60.

Reznik, S.I., Jaramillo, A., Zhang, L., Patterson, G.A., Cooper, J.D. and Mohanakumar, T. (2000) 'Anti-HLA antibody binding to hla class I molecules induces proliferation of airway epithelial cells: a potential mechanism for bronchiolitis obliterans syndrome', *J Thorac Cardiovasc Surg*, 119(1), pp. 39-45.

Riccardi, C. and Nicoletti, I. (2006) 'Analysis of apoptosis by propidium iodide staining and flow cytometry', *Nat Protoc*, 1(3), pp. 1458-61.

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A. and Ginty, D.D. (1999) 'Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons', *Science*, 286(5448), pp. 2358-61.

Romer, L.H., McLean, N.V., Yan, H.C., Daise, M., Sun, J. and DeLisser, H.M. (1995) 'IFN-gamma and TNF-alpha induce redistribution of PECAM-1 (CD31) on human endothelial cells', *J Immunol*, 154(12), pp. 6582-92.

Ruiz, S., Pergola, P.E., Zager, R.A. and Vaziri, N.D. (2013) 'Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease', *Kidney Int*, 83(6), pp. 1029-41.

Ruster, M., Sperschneider, H., Funfstuck, R., Stein, G. and Grone, H.J. (2004) 'Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts', *Clin Nephrol*, 61(1), pp. 30-9.

Ryan, M.J., Johnson, G., Kirk, J., Fuerstenberg, S.M., Zager, R.A. and Torok-Storb, B. (1994) 'HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney', *Kidney Int*, 45(1), pp. 48-57.

Saadi, S. and Platt, J.L. (1995) 'Transient perturbation of endothelial integrity induced by natural antibodies and complement', *J Exp Med*, 181(1), pp. 21-31.

Saini, D., Weber, J., Ramachandran, S., Phelan, D., Tiriveedhi, V., Liu, M., Steward, N., Aloush, A., Hachem, R., Trulock, E., Meyers, B., Patterson, G.A. and Mohanakumar, T. (2011) 'Alloimmunity-induced autoimmunity as a potential mechanism in the pathogenesis of chronic rejection of human lung allografts', *J Heart Lung Transplant*, 30(6), pp. 624-31.

Salama, A.D., Delikouras, A., Pusey, C.D., Cook, H.T., Bhangal, G., Lechler, R.I. and Dorling, A. (2001) 'Transplant accommodation in highly sensitized patients: a potential role for Bcl-xL and alloantibody', *Am J Transplant*, 1(3), pp. 260-9.

Samsonov, D., Geehan, C., Woda, C.B. and Briscoe, D.M. (2012) 'Differential activation of human T cells to allogeneic endothelial cells, epithelial cells and fibroblasts in vitro', *Transplant Res*, 1(1), p. 4.

Sandig, M., Negrou, E. and Rogers, K.A. (1997) 'Changes in the distribution of LFA-1, catenins, and F-actin during transendothelial migration of monocytes in culture', *J Cell Sci*, 110 (Pt 22), pp. 2807-18.

Sandoval, S., Pigazzi, M. and Sakamoto, K.M. (2009) 'CREB: A Key Regulator of Normal and Neoplastic Hematopoiesis', *Adv Hematol*, 2009, p. 634292.

Sarbassov, D.D., Ali, S.M. and Sabatini, D.M. (2005) 'Growing roles for the mTOR pathway', *Curr Opin Cell Biol*, 17(6), pp. 596-603.

Schaller, M.D. (2001) 'Paxillin: a focal adhesion-associated adaptor protein', *Oncogene*, 20(44), pp. 6459-72.

Schenkel, A.R., Mamdouh, Z. and Muller, W.A. (2004) 'Locomotion of monocytes on endothelium is a critical step during extravasation', *Nat Immunol*, 5(4), pp. 393-400.

Scheurich, P., Thoma, B., Ucer, U. and Pfizenmaier, K. (1987) 'Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on human T cells and TNF-alpha-mediated enhancement of T cell responses', *J Immunol*, 138(6), pp. 1786-90.

Scholz, C. and Tampe, R. (2005) 'The intracellular antigen transport machinery TAP in adaptive immunity and virus escape mechanisms', *J Bioenerg Biomembr*, 37(6), pp. 509-15.

Schreiber, A.B., Schlessinger, J. and Edidin, M. (1984) 'Interaction between major histocompatibility complex antigens and epidermal growth factor receptors on human cells', *J Cell Biol*, 98(2), pp. 725-31.

Schreiber, T.H., Shinder, V., Cain, D.W., Alon, R. and Sackstein, R. (2007) 'Shear flow-dependent integration of apical and subendothelial chemokines in T-cell transmigration: implications for locomotion and the multistep paradigm', *Blood*, 109(4), pp. 1381-6.

Schubert, S.Y., Benarroch, A., Ostvang, J. and Edelman, E.R. (2008) 'Regulation of endothelial cell proliferation by primary monocytes', *Arterioscler Thromb Vasc Biol*, 28(1), pp. 97-104.

Schulte, D., Kuppers, V., Dartsch, N., Broermann, A., Li, H., Zarbock, A., Kamenyeva, O., Kiefer, F., Khandoga, A., Massberg, S. and Vestweber, D. (2011) 'Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability', *EMBO J*, 30(20), pp. 4157-70.

Schwartz, G.K., Redwood, S.M., Ohnuma, T., Holland, J.F., Droller, M.J. and Liu, B.C. (1990) 'Inhibition of invasion of invasive human bladder carcinoma cells by protein kinase C inhibitor staurosporine', *J Natl Cancer Inst*, 82(22), pp. 1753-6.

Segura, E. and Villadangos, J.A. (2011) 'A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells', *Traffic*, 12(12), pp. 1677-85.

Sellares, J., de Freitas, D.G., Mengel, M., Reeve, J., Einecke, G., Sis, B., Hidalgo, L.G., Famulski, K., Matas, A. and Halloran, P.F. (2012) 'Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence', *Am J Transplant*, 12(2), pp. 388-99.

Serwold, T., Gonzalez, F., Kim, J., Jacob, R. and Shastri, N. (2002) 'ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum', *Nature*, 419(6906), pp. 480-3.

Shamri, R., Grabovsky, V., Feigelson, S.W., Dwir, O., Van Kooyk, Y. and Alon, R. (2002) 'Chemokine stimulation of lymphocyte alpha 4 integrin avidity but not of leukocyte function-associated antigen-1 avidity to endothelial ligands under shear flow requires cholesterol membrane rafts', *J Biol Chem*, 277(42), pp. 40027-35.

Shan, J., Feng, L., Luo, L., Wu, W., Li, C., Li, S. and Li, Y. (2011) 'MicroRNAs: potential biomarker in organ transplantation', *Transpl Immunol*, 24(4), pp. 210-5.

Shaywitz, A.J. and Greenberg, M.E. (1999) 'CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals', *Annu Rev Biochem*, 68, pp. 821-61.

Shiao, S.L., Kirkiles-Smith, N.C., Shepherd, B.R., McNiff, J.M., Carr, E.J. and Pober, J.S. (2007) 'Human effector memory CD4+ T cells directly recognize allogeneic endothelial cells in vitro and in vivo', *J Immunol*, 179(7), pp. 4397-404.

Shiao, S.L., McNiff, J.M. and Pober, J.S. (2005) 'Memory T cells and their costimulators in human allograft injury', *J Immunol*, 175(8), pp. 4886-96.

Shimizu, A. and Colvin, R.B. (2005) 'Pathological features of antibody-mediated rejection', *Curr Drug Targets Cardiovasc Haematol Disord*, 5(3), pp. 199-214.

Sicher, S.C., Chung, G.W., Vazquez, M.A. and Lu, C.Y. (1995) 'Augmentation or inhibition of IFN-gamma-induced MHC class II expression by lipopolysaccharides. The

roles of TNF-alpha and nitric oxide, and the importance of the sequence of signaling', *J Immunol*, 155(12), pp. 5826-34.

Siemerink, M.J., Klaassen, I., Vogels, I.M., Griffioen, A.W., Van Noorden, C.J. and Schlingemann, R.O. (2012) 'CD34 marks angiogenic tip cells in human vascular endothelial cell cultures', *Angiogenesis*, 15(1), pp. 151-63.

Sis, B. (2012) 'Endothelial molecules decipher the mechanisms and functional pathways in antibody-mediated rejection', *Hum Immunol*, 73(12), pp. 1218-25.

Sis, B., Campbell, P.M., Mueller, T., Hunter, C., Cockfield, S.M., Cruz, J., Meng, C., Wishart, D., Solez, K. and Halloran, P.F. (2007) 'Transplant glomerulopathy, late antibody-mediated rejection and the ABCD tetrad in kidney allograft biopsies for cause', *Am J Transplant*, 7(7), pp. 1743-52.

Sis, B., Jhangri, G.S., Riopel, J., Chang, J., de Freitas, D.G., Hidalgo, L., Mengel, M., Matas, A. and Halloran, P.F. (2012) 'A new diagnostic algorithm for antibody-mediated microcirculation inflammation in kidney transplants', *Am J Transplant*, 12(5), pp. 1168-79.

Smith, J.D., Lawson, C., Yacoub, M.H. and Rose, M.L. (2000) 'Activation of NF-kappa B in human endothelial cells induced by monoclonal and allospecific HLA antibodies', *Int Immunol*, 12(4), pp. 563-71.

Solez, K., Colvin, R.B., Racusen, L.C., Sis, B., Halloran, P.F., Birk, P.E., Campbell, P.M., Cascalho, M., Collins, A.B., Demetris, A.J., Drachenberg, C.B., Gibson, I.W., Grimm, P.C., Haas, M., Lerut, E., Liapis, H., Mannon, R.B., Marcus, P.B., Mengel, M., Mihatsch, M.J., Nankivell, B.J., Nickeleit, V., Papadimitriou, J.C., Platt, J.L., Randhawa, P., Roberts, I., Salinas-Madriga, L., Salomon, D.R., Seron, D., Sheaff, M. and Weening, J.J. (2007) 'Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN')', *Am J Transplant*, 7(3), pp. 518-26.

Soligo, D., Delia, D., Oriani, A., Cattoretti, G., Orazi, A., Bertolli, V., Quirici, N. and Deliliers, G.L. (1991) 'Identification of CD34+ cells in normal and pathological bone marrow biopsies by QBEND10 monoclonal antibody', *Leukemia*, 5(12), pp. 1026-30.

Stegall, M.D., Dean, P.G. and Gloor, J. (2009) 'Mechanisms of alloantibody production in sensitized renal allograft recipients', *Am J Transplant*, 9(5), pp. 998-1005.

Strehlau, J., Pavlakis, M., Lipman, M., Shapiro, M., Vasconcellos, L., Harmon, W. and Strom, T.B. (1997) 'Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation', *Proc Natl Acad Sci U S A*, 94(2), pp. 695-700.

Sugawara, S., Abo, T. and Kumagai, K. (1987) 'A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3', *J Immunol Methods*, 100(1-2), pp. 83-90.

Sun, H., Chung, W.C., Ryu, S.H., Ju, Z., Tran, H.T., Kim, E., Kurie, J.M. and Koo, J.S. (2008) 'Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis', *Cancer Prev Res (Phila)*, 1(5), pp. 316-28.

Sun, P., Enslen, H., Myung, P.S. and Maurer, R.A. (1994) 'Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity', *Genes Dev*, 8(21), pp. 2527-39.

Tabata, A., Morikawa, M., Miyajima, M., Bennett, B.L., Satoh, Y., Huang, J., Tamura, Y., Sato, N. and Abe, T. (2007) 'Suppression of alloreactivity and allograft rejection by SP600125, a small molecule inhibitor of c-Jun N-terminal kinase', *Transplantation*, 83(10), pp. 1358-64.

Taflin, C., Charron, D., Glotz, D. and Mooney, N. (2011) 'Immunological function of the endothelial cell within the setting of organ transplantation', *Immunol Lett*, 139(1-2), pp. 1-6.

Takahashi, D., Fujihara, M., Azuma, H., Wakamoto, S., Sato, S., Kato, T. and Ikeda, H. (2012) 'Adhesive interaction between peripheral blood mononuclear cells and activated platelets in the presence of anti-human leukocyte antigen Class I alloantibody causes production of IL-1beta and IL-8', *Vox Sang*, 102(3), pp. 250-7.

Takahashi, K. and Saito, K. (2013) 'ABO-incompatible kidney transplantation', *Transplant Rev (Orlando)*, 27(1), pp. 1-8.

Takenaka, M., Subramanian, V., Tiriveedhi, V., Phelan, D., Hachem, R., Trulock, E., Gelman, A.E., Patterson, G.A., Hoshinaga, K. and Mohanakumar, T. (2012) 'Complement activation is not required for obliterative airway disease induced by antibodies to major histocompatibility complex class I: Implications for chronic lung rejection', *J Heart Lung Transplant*, 31(11), pp. 1214-22.

Tamaoki, T. and Nakano, H. (1990) 'Potent and specific inhibitors of protein kinase C of microbial origin', *Biotechnology (N Y)*, 8(8), pp. 732-5.

Tellides, G. and Pober, J.S. (2007) 'Interferon-gamma axis in graft arteriosclerosis', *Circ Res*, 100(5), pp. 622-32.

Terasaki, P.I., Ozawa, M. and Castro, R. (2007) 'Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival', *Am J Transplant*, 7(2), pp. 408-15.

Thaunat, O., Field, A.C., Dai, J., Louedec, L., Patey, N., Bloch, M.F., Mandet, C., Belair, M.F., Bruneval, P., Meilhac, O., Bellon, B., Joly, E., Michel, J.B. and Nicoletti, A. (2005) 'Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response', *Proc Natl Acad Sci U S A*, 102(41), pp. 14723-8.

Thimmulappa, R.K., Lee, H., Rangasamy, T., Reddy, S.P., Yamamoto, M., Kensler, T.W. and Biswal, S. (2006) 'Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis', *J Clin Invest*, 116(4), pp. 984-95.

Thiyagarajan, U.M., Bagul, A., Frost, J., Horsburgh, T. and Nicholson, M.L. (2012) 'Role of human leukocyte antigen, donor-specific antibodies, and their impact in renal transplantation', *Transplant Proc*, 44(5), pp. 1231-5.

Ticehurst, E.H., Molina, M.R., Frank, R., Kearns, J., Lal, P., Goldberg, L.R., Tsai, D., Wald, J. and Kamoun, M. (2011) 'Antibody-mediated rejection in heart transplant patients: long-term follow up of patients with high levels of donor-directed anti-DQ antibodies', *Clin Transpl*, pp. 409-14.

Tinckam, K. (2009) 'Histocompatibility methods', *Transplant Rev (Orlando)*, 23(2), pp. 80-93.

Tinckam, K.J. and Chandraker, A. (2006) 'Mechanisms and role of HLA and non-HLA alloantibodies', *Clin J Am Soc Nephrol*, 1(3), pp. 404-14.

Tinckam, K.J., Djurdjev, O. and Magil, A.B. (2005) 'Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status', *Kidney Int*, 68(4), pp. 1866-74.

Tiriveedhi, V., Angaswamy, N., Brand, D., Weber, J., Gelman, A.G., Hachem, R., Trulock, E.P., Meyers, B., Patterson, G. and Mohanakumar, T. (2012a) 'A shift in the collagen V antigenic epitope leads to T helper phenotype switch and immune response to self-antigen leading to chronic lung allograft rejection', *Clin Exp Immunol*, 167(1), pp. 158-68.

Tiriveedhi, V., Sarma, N. and Mohanakumar, T. (2012b) 'An important role for autoimmunity in the immunopathogenesis of chronic allograft rejection', *Int J Immunogenet*, 39(5), pp. 373-80.

Toetsch, S., Olwell, P., Prina-Mello, A. and Volkov, Y. (2009) 'The evolution of chemotaxis assays from static models to physiologically relevant platforms', *Integr Biol (Camb)*, 1(2), pp. 170-81.

Torrealba, J.R., Samaniego, M., Pascual, J., Becker, Y., Pirsch, J., Sollinger, H. and Odorico, J. (2008) 'C4d-positive interacinar capillaries correlates with donor-specific antibody-mediated rejection in pancreas allografts', *Transplantation*, 86(12), pp. 1849-56.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.G., Foster, L. and Karre, K. (1989) 'Association of class I major histocompatibility heavy and light chains induced by viral peptides', *Nature*, 340(6233), pp. 443-8.

Trayssac, M., Negre-Salvayre, A. and Thomsen, M. (2012) 'Mechanisms of human smooth muscle cell proliferation and transplant vasculopathy induced by HLA class I antibodies: In vitro and in vivo studies', *Hum Immunol*, 73(12), pp. 1253-60.

Tsai, Y.C., Hsieh, H.J., Liao, F., Ni, C.W., Chao, Y.J., Hsieh, C.Y. and Wang, D.L. (2007) 'Laminar flow attenuates interferon-induced inflammatory responses in endothelial cells', *Cardiovasc Res*, 74(3), pp. 497-505.

Tsoulfas, G. and Geller, D.A. (2001) 'NF-kappaB in transplantation: friend or foe?', *Transpl Infect Dis*, 3(4), pp. 212-9.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *Int J Cancer*, 26(2), pp. 171-6.

Uehara, S., Chase, C.M., Kitchens, W.H., Rose, H.S., Colvin, R.B., Russell, P.S. and Madsen, J.C. (2005) 'NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts', *J Immunol*, 175(5), pp. 3424-30.

Unanue, E.R. (1992) 'Cellular studies on antigen presentation by class II MHC molecules', *Curr Opin Immunol*, 4(1), pp. 63-9.

Unger, R.E., Krump-Konvalinkova, V., Peters, K. and Kirkpatrick, C.J. (2002) 'In vitro expression of the endothelial phenotype: comparative study of primary isolated cells and cell lines, including the novel cell line HPMEC-ST1.6R', *Microvasc Res*, 64(3), pp. 384-97.

Valenzuela, N.M., Hong, L., Shen, X.D., Gao, F., Young, S.H., Rozengurt, E., Kupiec-Weglinski, J.W., Fishbein, M.C. and Reed, E.F. (2013a) 'Blockade of p-selectin is sufficient to reduce MHC I antibody-elicited monocyte recruitment in vitro and in vivo', *Am J Transplant*, 13(2), pp. 299-311.

Valenzuela, N.M., Mulder, A. and Reed, E.F. (2013b) 'HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging FcgammaRs', *J Immunol*, 190(12), pp. 6635-50.

van Beijnum, J.R., van der Linden, E. and Griffioen, A.W. (2008) 'Angiogenic profiling and comparison of immortalized endothelial cells for functional genomics', *Exp Cell Res*, 314(2), pp. 264-72.

van Endert, P.M., Saveanu, L., Hewitt, E.W. and Lehner, P. (2002) 'Powering the peptide pump: TAP crosstalk with energetic nucleotides', *Trends Biochem Sci*, 27(9), pp. 454-61.

Van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L. and Tonegawa, S. (1992) 'TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells', *Cell*, 71(7), pp. 1205-14.

Varnavidou-Nicolaidou, A., Doxiadis, II, Iniotaki-Theodoraki, A., Patargias, T., Stavropoulos-Giokas, C. and Kyriakides, G.K. (2004) 'HLA class I donor-specific triplet antibodies detected after renal transplantation', *Transplant Proc*, 36(6), pp. 1732-4.

Verdier, C., Couzon, C., Duperray, A. and Singh, P. (2009) 'Modeling cell interactions under flow', *J Math Biol*, 58(1-2), pp. 235-59.

Viemann, D., Goebeler, M., Schmid, S., Nordhues, U., Klimmek, K., Sorg, C. and Roth, J. (2006) 'TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells', *J Leukoc Biol*, 80(1), pp. 174-85.

Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) 'A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)', *J Biol Chem*, 269(7), pp. 5241-8.

Vora, M., Yssel, H., de Vries, J.E. and Karasek, M.A. (1994) 'Antigen presentation by human dermal microvascular endothelial cells. Immunoregulatory effect of IFN-gamma and IL-10', *J Immunol*, 152(12), pp. 5734-41.

Waeber, G. and Habener, J.F. (1991) 'Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB', *Mol Endocrinol*, 5(10), pp. 1431-8.

Walch, J.M., Zeng, Q., Li, Q., Oberbarnscheidt, M.H., Hoffman, R.A., Williams, A.L., Rothstein, D.M., Shlomchik, W.D., Kim, J.V., Camirand, G. and Lakkis, F.G. (2013) 'Cognate antigen directs CD8+ T cell migration to vascularized transplants', *J Clin Invest*, 123(6), pp. 2663-71.

Wang, H., Arp, J., Liu, W., Faas, S.J., Jiang, J., Gies, D.R., Ramcharran, S., Garcia, B., Zhong, R. and Rother, R.P. (2007a) 'Inhibition of terminal complement components in presensitized transplant recipients prevents antibody-mediated rejection leading to long-term graft survival and accommodation', *J Immunol*, 179(7), pp. 4451-63.

Wang, H.B., Wang, J.T., Zhang, L., Geng, Z.H., Xu, W.L., Xu, T., Huo, Y., Zhu, X., Plow, E.F., Chen, M. and Geng, J.G. (2007b) 'P-selectin primes leukocyte integrin activation during inflammation', *Nat Immunol*, 8(8), pp. 882-92.

Wang, L., Fuster, M., Sriramarao, P. and Esko, J.D. (2005) 'Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses', *Nat Immunol*, 6(9), pp. 902-10.

Wasowska, B.A., Qian, Z., Cangello, D.L., Behrens, E., Van Tran, K., Layton, J., Sanfilippo, F. and Baldwin, W.M., 3rd (2001) 'Passive transfer of alloantibodies restores acute cardiac rejection in IgKO mice', *Transplantation*, 71(6), pp. 727-36.

Watanabe, Y. and Jacob, C.O. (1991) 'Regulation of MHC class II antigen expression. Opposing effects of tumor necrosis factor-alpha on IFN-gamma-induced HLA-DR and Ia expression depends on the maturation and differentiation stage of the cell', *J Immunol*, 146(3), pp. 899-905.

Weber, K.S., von Hundelshausen, P., Clark-Lewis, I., Weber, P.C. and Weber, C. (1999) 'Differential immobilization and hierarchical involvement of chemokines in monocyte arrest and transmigration on inflamed endothelium in shear flow', *Eur J Immunol*, 29(2), pp. 700-12.

Wedgwood, J.F., Hatam, L. and Bonagura, V.R. (1988) 'Effect of interferon-gamma and tumor necrosis factor on the expression of class I and class II major histocompatibility molecules by cultured human umbilical vein endothelial cells', *Cell Immunol*, 111(1), pp. 1-9.

Wehner, J., Morrell, C.N., Reynolds, T., Rodriguez, E.R. and Baldwin, W.M., 3rd (2007) 'Antibody and complement in transplant vasculopathy', *Circ Res*, 100(2), pp. 191-203.

Westphal, S., Hansson, S., Stelin, G., Holgersson, J., Mjornstedt, L. and Friman, S. (2013) 'Successful Treatment of Severe ABO Antibody-Mediated Rejection Using Bortezomib: A Case Report', *Transplant Proc*, 45(3), pp. 1213-5.

Wiebe, C., Gibson, I.W., Blydt-Hansen, T.D., Karpinski, M., Ho, J., Storsley, L.J., Goldberg, A., Birk, P.E., Rush, D.N. and Nickerson, P.W. (2012) 'Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant', *Am J Transplant*, 12(5), pp. 1157-67.

Wiesner, D.A. and Dawson, G. (1996) 'Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway', *J Neurochem*, 66(4), pp. 1418-25.

Wilflingseder, J., Regele, H., Perco, P., Kainz, A., Soleiman, A., Muhlbacher, F., Mayer, B. and Oberbauer, R. (2013) 'miRNA profiling discriminates types of rejection and injury in human renal allografts', *Transplantation*, 95(6), pp. 835-41.

Williams, D.B. (2006) 'Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum', *J Cell Sci*, 119(Pt 4), pp. 615-23.

Winn, H.J., Baldamus, C.A., Jooste, S.V. and Russell, P.S. (1973) 'Acute destruction by humoral antibody of rat skin grafted to mice', *J Exp Med*, 137(4), pp. 893-910.

Winn, R.K. and Harlan, J.M. (2005) 'The role of endothelial cell apoptosis in inflammatory and immune diseases', *J Thromb Haemost*, 3(8), pp. 1815-24.

Woolf, E., Grigorova, I., Sagiv, A., Grabovsky, V., Feigelson, S.W., Shulman, Z., Hartmann, T., Sixt, M., Cyster, J.G. and Alon, R. (2007) 'Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces', *Nat Immunol*, 8(10), pp. 1076-85.

Wu, M.H. (2005) 'Endothelial focal adhesions and barrier function', *J Physiol*, 569(Pt 2), pp. 359-66.

Wyburn, K.R., Jose, M.D., Wu, H., Atkins, R.C. and Chadban, S.J. (2005) 'The role of macrophages in allograft rejection', *Transplantation*, 80(12), pp. 1641-7.

Xu, Y., Swerlick, R.A., Sepp, N., Bosse, D., Ades, E.W. and Lawley, T.J. (1994) 'Characterization of expression and modulation of cell adhesion molecules on an immortalized human dermal microvascular endothelial cell line (HMEC-1)', *J Invest Dermatol*, 102(6), pp. 833-7.

Yago, T., Zarnitsyna, V.I., Klopocki, A.G., McEver, R.P. and Zhu, C. (2007) 'Transport governs flow-enhanced cell tethering through L-selectin at threshold shear', *Biophys J*, 92(1), pp. 330-42.

Yamakuchi, M., Kirkiles-Smith, N.C., Ferlito, M., Cameron, S.J., Bao, C., Fox-Talbot, K., Wasowska, B.A., Baldwin, W.M., 3rd, Pober, J.S. and Lowenstein, C.J. (2007) 'Antibody to human leukocyte antigen triggers endothelial exocytosis', *Proc Natl Acad Sci U S A*, 104(4), pp. 1301-6.

Yin, D., Zeng, H., Ma, L., Shen, J., Xu, H., Byrne, G.W. and Chong, A.S. (2004) 'Cutting Edge: NK cells mediate IgG1-dependent hyperacute rejection of xenografts', *J Immunol*, 172(12), pp. 7235-8.

Yousem, S.A. and Zeevi, A. (2012) 'The histopathology of lung allograft dysfunction associated with the development of donor-specific HLA alloantibodies', *Am J Surg Pathol*, 36(7), pp. 987-92.

Zarbock, A., Ley, K., McEver, R.P. and Hidalgo, A. (2011) 'Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow', *Blood*, 118(26), pp. 6743-51.

Zarbock, A., Muller, H., Kuwano, Y. and Ley, K. (2009) 'PSGL-1-dependent myeloid leukocyte activation', *J Leukoc Biol*, 86(5), pp. 1119-24.

Zeisberg, E.M., Tarnavski, O., Zeisberg, M., Dorfman, A.L., McMullen, J.R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W.T., Roberts, A.B., Neilson, E.G., Sayegh, M.H., Izumo, S. and Kalluri, R. (2007) 'Endothelial-to-mesenchymal transition contributes to cardiac fibrosis', *Nat Med*, 13(8), pp. 952-61.

Zhang, Q., Cecka, J.M., Gjertson, D.W., Ge, P., Rose, M.L., Patel, J.K., Ardehali, A., Kobashigawa, J.A., Fishbein, M.C. and Reed, E.F. (2011a) 'HLA and MICA: targets of antibody-mediated rejection in heart transplantation', *Transplantation*, 91(10), pp. 1153-8.

Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J.R., Emerson, B., Hogenesch, J.B., Unterman, T., Young, R.A. and Montminy, M. (2005) 'Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues', *Proc Natl Acad Sci U S A*, 102(12), pp. 4459-64.

Zhang, X., Rozengurt, E. and Reed, E.F. (2011b) 'HLA class I molecules partner with integrin beta4 to stimulate endothelial cell proliferation and migration', *Sci Signal*, 3(149), p. ra85.

Zhang, X., Valenzuela, N.M. and Reed, E.F. (2012) 'HLA class I antibody-mediated endothelial and smooth muscle cell activation', *Curr Opin Organ Transplant*, 17(4), pp. 446-51.

Zhou, H.L., Wang, Y.T., Gao, T., Wang, W.G. and Wang, Y.S. (2013) 'Distribution and expression of fibroblast-specific protein chemokine CCL21 and chemokine receptor CCR7 in renal allografts', *Transplant Proc*, 45(2), pp. 538-45.

Ziegler, M.E., Jin, Y.P., Young, S.H., Rozengurt, E. and Reed, E.F. (2012a) 'HLA class I-mediated stress fiber formation requires ERK1/2 activation in the absence of an increase in intracellular Ca2+ in human aortic endothelial cells', *Am J Physiol Cell Physiol*, 303(8), pp. C872-82.

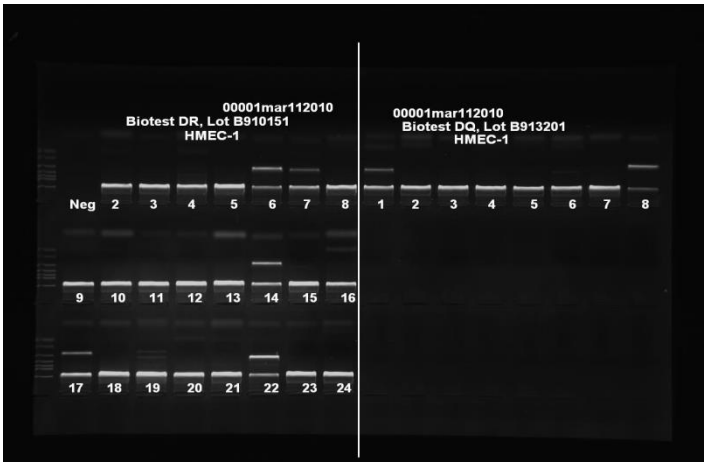
Ziegler, M.E., Souda, P., Jin, Y.P., Whitelegge, J.P. and Reed, E.F. (2012b) 'Characterization of the endothelial cell cytoskeleton following HLA class I ligation', *PLoS One*, 7(1), p. e29472.

Zou, Y. and Stastny, P. (2011) 'Antibodies against major histocompatibility complex class I-related chain A in transplant recipients', *Chin Med J (Engl)*, 124(5), pp. 764-70.

Zou, Y., X., L., S., L., W., L., Y., L., X., T., F., L., Q., Y., G., L., Z., L., J., L. and D., L. (2011) 'Acid treatment of platelets for removal of class I human leukocyte antigen (HLA) antigens', *Scientific Research and Essays*, 6(21), pp. 4539-4544.

10. Appendix

10.1 HLA class II (DR,DQ) typing for HMEC-1 cells



HLA class II genotyping of HMEC-1 endothelial cells using PCR-SSP method. The HLA class II genotype are DRB1*03, DRB1*12, DQ1*04, DQB1*05.

10.2 HLA class I and II typing for HKC-8 cell line

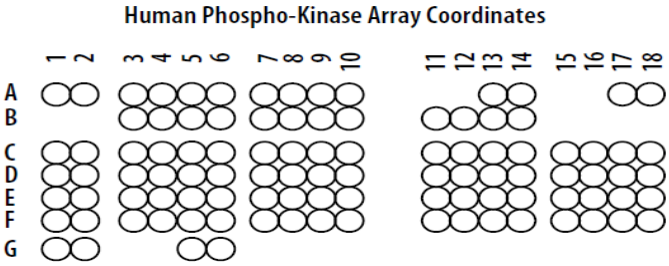
Typing of HKC-8 cell line using PCR-SSP method. HKC-8 typing is A*24, A*29, B*37, B*44, C*06, C*16, DRB1*10, DRB1*15, DQB1*05, DQB1*06. The first two gels for HLA-A, B and C. Lanes from 1-25 represents HLA-A types, 26-76 for HLA-B and 77-93 for HLA-C. The third gel left hand side for DR and right side for DQ. Interpretation of data was performed using Biotest SSP typing Software.

10.3 HLA class I and II typing for HK-2 cell line

Typing of HK-2 cell line by PCR-SSP method. HKC-8 typing is A*02, A*30, B*13, B*44, C*02, C*06, DRB1*01, DRB1*16, DQB1*05, DQB1*05. The first two gels for HLA-A, B and C. Lanes from 1-25 represents HLA-A types, 26-76 for HLA-B and 77-93 for HLA-C. The third gel left hand side for DR and right side for DQ. Lanes from 1-25 represents HLA-A types, 26-76 for HLA-B and 77-93 for HLA-C. Interpretation of data was performed using Biotest SSP typing Software.

Cell line	HLA typing
HKC-8 cell line	A*24, A*29, B*37, B*44, C*06, C*16, *DRB1*10, DRB1*15, DQB1*05, DQB1*06*
HK-2 cell line	A*02, A*30, B*13, B*44, C*02, C*06, *DRB1*01, DRB1*16, DQB1*05, DQB1*05*

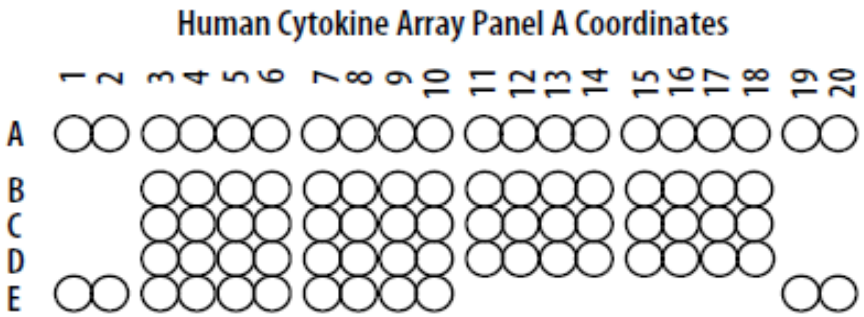
10.4 Human phospho-kinase array



Membrane/Coordinate	Target/Control	Phosphorylation Site
A-A1, A2	Reference Spot	——
A-A3, A4	p38α	T180/Y182
A-A5, A6	ERK1/2	T202/Y204, T185/Y187
A-A7, A8	JNK pan	T183/Y185, T221/Y223
A-A9, A10	GSK-3α/β	S21/S9
B-A13, A14	p53	S392
B-A17, A18	Reference Spot	——
A-B3, B4	MEK1/2	S218/S222, S222/S226
A-B5, B6	MSK1/2	S376/S360
A-B7, B8	AMPKα1	T174
A-B9, B10	Akt	S473
B-B11, B12	Akt	T308
B-B13, B14	p53	S46
A-C1, C2	TOR	S2448
A-C3, C4	CREB	S133
A-C5, C6	HSP27	S78/S82
A-C7, C8	AMPKα2	T172
A-C9, C10	β-Catenin	——
B-C11, C12	p70 S6 Kinase	T389
B-C13, C14	p53	S15
B-C15, C16	p27	T198
B-C17, C18	Paxillin	Y118
A-D1, D2	Src	Y419
A-D3, D4	Lyn	Y397
A-D5, D6	Lck	Y394
A-D7, D8	STAT2	Y689
A-D9, D10	STAT5a	Y694
B-D11, D12	p70 S6 Kinase	T421/S424
B-D13, D14	RSK1/2/3	S380/S386/S377
B-D15, D16	p27	T157
B-D17, D18	PLCγ-1	Y783
A-E1, E2	Fyn	Y420
A-E3, E4	Yes	Y426
A-E5, E6	Fgr	Y412
A-E7, E8	STAT3	Y705
A-E9, E10	STAT5b	Y699
B-E11, E12	p70 S6 Kinase	T229
B-E13, E14	RSK1/2	S221/S227
B-E15, E16	c-Jun	S63
B-E17, E18	Pyk2	Y402
A-F1, F2	Hck	Y411
A-F3, F4	Chk-2	T68
A-F5, F6	FAK	Y397
A-F7, F8	STAT6	Y641
A-F9, F10	STAT5a/b	Y694/Y699
B-F11, F12	STAT1	Y701
B-F13, F14	STAT4	Y693
B-F15, F16	eNOS	S1177
B-F17, F18	PBS (Negative Control)	——
A-G1, G2	Reference Spot	——
A-G5, G6	PBS (Negative Control)	——

*A change in phosphorylation for this protein was detected in multiple cell sources.

10.5 Human cytokine array



Coordinate	Target/Control	Alternate Nomenclature
A1, A2	Reference Spot	—
A3, A4	C5/C5a	Complement Component 5/5a
A5, A6	CD40 Ligand	CD154
A7, A8	G-CSF	CSFβ, CSF-3
A9, A10	GM-CSF	CSFα, CSF-2
A11, A12	GROα	CXCL1
A13, A14	I-309	CCL1
A15, A16	sICAM-1	CD54
A17, A18	IFN-γ	Type II IFN
A19, A20	Reference Spot	—
B3, B4	IL-1α	IL-1F1
B5, B6	IL-1β	IL-1F2
B7, B8	IL-1ra	IL-1F3
B9, B10	IL-2	—
B11, B12	IL-4	—
B13, B14	IL-5	—
B15, B16	IL-6	—
B17, B18	IL-8	CXCL8
C3, C4	IL-10	—
C5, C6	IL-12 p70	—
C7, C8	IL-13	—
C9, C10	IL-16	LCF
C11, C12	IL-17	—
C13, C14	IL-17E	—
C15, C16	IL-23	—
C17, C18	IL-27	—
D3, D4	IL-32α	—
D5, D6	IP-10	CXCL10
D7, D8	I-TAC	CXCL11
D9, D10	MCP-1	CCL2
D11, D12	MIF	GIF, DER6
D13, D14	MIP-1α	CCL3
D15, D16	MIP-1β	CCL4
D17, D18	Serpin E1	PAI-1
E1, E2	Reference Spot	—
E3, E4	RANTES	CCL5
E5, E6	SDF-1	CXCL12
E7, E8	TNF-α	TNFSF1A
E9, E10	sTREM-1	—
E19, E20	Negative Control	—

Publication arising from this study

Published papers

1- Naemi FM, Carter V, Kirby JA, Ali S (2013) Anti-Donor HLA Class I Antibodies: Pathways to Endothelial Cell Activation and Cell-Mediated Allograft Rejection. Transplantation, 96: 258-266.

2- Naemi FM, Ali S, Kirby JA (2011) Antibody-mediated allograft rejection: the emerging role of endothelial cell signaling and transcription factors. Transplant immunology, Sep; 25 (2-3): 96-103.

Conferences

1- 15th International Congress of Immunology (2013), Milan/Italy, Poster presentation.

2- American Transplant Congress, 2012, Boston/USA, American Journal of Transplantation, volume 12, issue supplement s3, pages 27-542 (abstract 79). Oral presentation.

3- British Society of Histocompatibility and Immunogenetics Conference, Tissue Antigen, volume 79, issue 6, pages 399-597 (abstract O 31), Oral presentation.

4- British Transplantation Society Congress, 2012, Glasgow: Abstract 2. Poster Presentation

http://www.bts.org.uk/Documents/Congress%20Archive/Abstract%20Book%202012.pdf

5- BSI 2010, Immunology Journal, Volume 131, issue supplement s1, pages 1-204. Poster session NO. 185. Poster Presentation

Newcastle University activity

The role of HLA class I antibody in allograft rejection, Immunology North East Annual Symposium, June 2010 (Oral presentation).