

**TISSUE ENGINEERING CELLS FOR ALLOGENEIC
TRANSPLANTATION**

TISSUE ENGINEERING CELLS FOR ALLOGENEIC
TRANSPLANTATION

By

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ABSTRACT

The immune response is a major barrier to the successful transplantation of organs and tissues required in the treatment of many human diseases. Although the field of tissue engineering was created to address the shortage of human organs and tissues, the immune response remains a substantial challenge, impeding the development of allogeneic biological substitutes to repair, replace and regenerate tissues. Specifically, the T cell mediated immune response initiated through the recognition of cell surface Major histocompatibility complex Class I (MHCI) molecules is the primary cause of acute allograft rejection. In nature, viruses have evolved many mechanisms to exploit weaknesses of the T cell response to evade detection. Viral mechanisms to modulate the MHCI molecule can be effectively applied to allogeneic cells in a tissue-engineered construct to evade detection by CD8⁺ Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells of the immune system. We demonstrate the successful application of a retroviral vector to over-express the Kaposi's sarcoma-associated herpesvirus (KSHV) immunomodulatory protein, MIR2, in human monocyte-like leukemia cells to differentially downregulate cell surface MHCI, ICAM-1 and B7-2 molecules. We also developed a novel flow cytometry-based cytotoxicity assay to demonstrate that this differential downregulation of immunoactive molecules has the functional effect of significantly reducing CTL-mediated cytotoxicity, without altering NK-mediated cytotoxicity. We believe that this approach provides a potential solution to circumvent the acute immune rejection of allografts *in vivo*, and can also lead to the development of "universal" donor cells for tissue engineering applications that will not require anti-rejection drugs.

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LIST OF ABBREVIATIONS

AICL	Activation-Induced C-type Lectin
AFS	Amniotic Fluid Stem cells
APCs	Antigen Presenting Cells
CDR	Complementarity-Determining Region
CR	Conserved Region of MIR2
CTLs	Cytotoxic T Lymphocytes
DTH	Delayed Type-Hypersensitivity
ESCs	Embryonic Stem Cells
ER	Endoplasmic Reticulum
EBV	Epstein-Barr Virus
HSV1	Herpes Simplex Virus type 1
HA	Hyaluronic Acid
FasL	Fas Ligand
Ig	Immunoglobulin
ITAMs	Immunoreceptor Tyrosine-based Activation Motifs
ITIMs	Immunoreceptor Tyrosine-based Inhibitory Motifs
ICAM-1	Intercellular Adhesion Molecule-1
IFN- γ	Interferon- γ
IL-2	Interleukin-2
KSHV	Kaposi Sarcoma-Associated Herpes Virus
KIRs	Killer Inhibitory Receptors
LFA-1	Leukocyte Function-associated Antigen-1
LPS	Lipopolysaccharide
LAK	Lymphokine-Activated Killer
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class II
MIC	Major Histocompatibility Complex Class I -related chains
MFI	Mean Fluorescence Intensity
MARCH	Membrane-Associated RING-CH
MAC	Membrane Attack Complex
MSCs	Mesenchymal Stem Cells
mHAgs	Minor Histocompatibility complex Antigens
mtDNA	Mitochondrial DNA
MLR	Mixed Lymphocyte Reaction
MIR2	Modulator of Immune Recognition 2
MAPCs	Multipotent Adult Progenitor Cells
MCMV	Murine Cytomegalovirus
MHV-68	Murine γ -2 Herpesvirus 68
MMF	Mycophenolate Mofetil
NK cell	Natural Killer cell

NFAT	Nuclear Factor of Activated T cells
NT	somatic Nuclear Transfer
PAMPS	Pathogen-Associated Molecular Patterns
PRRs	Pattern Recognition Receptors
PBMCs	Peripheral Blood Mononuclear Cells
PHD	Plant homeodomain
PDGF	Platelet-Derived Growth Factor
PGA	Polyglycolic Acid
PLA	Polylactic Acid
PLGA	Poly(lactic-co-glycolic) Acid
PKPs	Protein Tyrosine Phosphatases
RBCs	Red Blood Cells
RNAi	RNA interference
TCRs	T Cell Receptors
TLRs	Toll-Like Receptors
TGF- β	Transforming Growth Factor β
TM	Transmembrane domain of MIR2
TAP	Transporter associated with Antigen Processing
TNF α	Tumor Necrosis Factor

1. INTRODUCTION

Diabetes, chronic renal failure, ischemic heart disease, end-stage liver disease and many others are all incurable diseases with a known solution: organ transplantation. However, transplanting organs such as pancreas, kidney, heart and liver requires the recipients of these organs to permanently suppress their immune response against the foreign organs with a barrage of anti-rejection drugs. In addition, the extensive list of requirements of matching the donor to the recipient further reduces the number of organs available for transplantation. Together, these factors present a substantial challenge for organ transplantation as a solution for many human diseases. Fortunately, the field of tissue engineering offers many potential solutions to these medical challenges.

Tissue engineering is an interdisciplinary field of study which combines the principles of engineering and life sciences to develop biological substitutes, with the goal of repairing, replacing and regenerating tissues to improve biological function (1). Typically, these biological substitutes include functional cells within a matrix or scaffold to guide tissue development and integration into the body.

Tissue-engineered constructs can be used as a biological substitute for almost any tissue in the body, hence their structure and composition is specifically tailored to the application. For example, a tissue-engineered construct designed to repair damaged cardiac tissue may contain cardiac cells or precursor cells which will differentiate into

cardiac cells. However, this concept of implanting cell-based tissue-engineered constructs raises the same fundamental issue surrounding all allogeneic cell, tissue and organ transplantation: immune rejection. The immune response is arguably the most significant barrier to allogeneic transplantation and this problem affects almost all types of cell-based tissue-engineered constructs. Hence, it is imperative that such tissue-engineered constructs address this important challenge.

In preparing a tissue-engineered construct, the cells required are sourced in advance. This provides the opportunity to use genetic engineering to manipulate these cells such that they evade or suppress the immune response. Viruses have evolved complex genetic mechanisms to evade and suppress detection by the immune system of the host. A tissue-engineered construct with genetically engineered allogeneic cells that also utilizes these viral mechanisms might potentially elicit a similar response, where the construct would not be recognized by the host immune system as ‘foreign,’ thus preventing rejection.

In this project, we examine the feasibility of applying this novel approach to preventing the immune rejection of an allogeneic tissue-engineered construct. In summary, we genetically engineered allogeneic cells to express an immunomodulatory protein called modulator of immune recognition (MIR2) from Kaposi’s sarcoma-associated herpesvirus (KSHV). This protein is known to alter the cell surface expression of immunoactive molecules including Major Histocompatibility Complex Class I (MHCI), intercellular adhesion molecule-1 (ICAM-1) and B7-2, such that the allogeneic cells become less

antigenic and evade the effects of acute immune rejection. Specifically, MIR2 hinders the ability of immune cells (CTLs and NK cells) to detect these genetically engineered allogeneic cells, which is likely to decrease the ability of these immune cells to mediate acute immune rejection. We also tested the feasibility of using a retroviral vector to stably transduce allogeneic cells with MIR2. In order to test the immunoprotective effect of MIR2 expression in allogeneic cells against CTLs and NK cells, we developed a novel *in vitro* cytotoxicity assay in conjunction with flow cytometry to assess immune evasion. Our results show that MIR2 expression in allogeneic cells confers a significant immunoprotective effect against CTLs and NK cells *in vitro*. These results provide a proof-of-principle that this method of employing viral immune evasion strategies can potentially allow cell-based tissue-engineered constructs to delay or even prevent acute immune rejection upon implantation into an allogeneic host. In addition, this strategy immediately provides an alternative method for the creation of ‘universal’ donor cells that can be transplanted into any allogeneic individual.

By extension, our results suggest that this method of employing viral immune evasion strategies would allow tissue-engineered organs to be transplanted into any allogeneic recipient, which could potentially eliminate the need for anti-rejection drugs and improve the prognosis for the recipient.

2. LITERATURE REVIEW

2.1 The immune system

The human immune system is broadly divided into two branches: innate immunity and adaptive immunity. The innate immune system is an evolutionary ancient system of physical and chemical barriers (eg. skin and antimicrobial substances produced at epithelial surfaces), effector cells (phagocytic cells and natural killer (NK) cells), blood-borne molecules (eg. Complement and acute phase proteins) and proteins called cytokines (regulate and coordinate innate immune cells), which together help to protect against pathogens (2). The innate immune system is the first line of defense that has an immediate response (0-4h), with no prior exposure required. In contrast, the adaptive immune system is a more highly evolved defense mechanism with a much greater specificity to target pathogens. This specificity is manifested through cells called lymphocytes and their secreted products. However, this system has a delayed effect and requires prior exposure to the pathogen. In addition, this system has the unique ability to ‘remember’ and respond more vigorously to a repeated exposure to the same pathogen (2).

2.1.1 Innate immune system

The innate immune system has a broad specificity to a variety of molecular structures common to groups of related pathogens. It achieves this by recognizing ubiquitously expressed and evolutionarily conserved molecular structures on pathogens and foreign agents called pathogen-associated molecular patterns (PAMPS). For example, lipopolysaccharide (LPS) is a PAMP found on gram-negative bacterial cell walls. Broadly specific receptors called pattern recognition receptors (PRRs) recognize PAMPS and activate the innate immune system. For instance, innate immune cells such as NK cells, express a type of PRR known as toll-like receptors (TLRs) that activate the innate immune system to carry out their effector functions against pathogens (2).

Among the many cell types involved in innate defense, NK cells are extremely important in their role in clearing (killing) virus-infected cells and secreting IFN- γ . These are large granular lymphocytes and constitute around 5%-20% of mononuclear cells in the blood and spleen. Virus-infected cells are known to modulate their cell surface expression of glycoproteins such as MHCI. MHCI is expressed on all nucleated cells in mammals and NK cells express receptors that recognize MHCI. One of the main functions of NK cells is to recognize cells with missing MHCI, as in the case of virally infected cells, and perform their cytolytic effector function. It is believed that a variety of cell surface ligands may activate NK cells. Although unique NK-activating ligands have not yet been identified, it has been hypothesized that various adhesion molecules such as integrins may be sufficient to activate NK cells (2). However, comparative studies between various NK activation receptors suggest that immunoreceptor tyrosine-based activation motifs

(ITAMs) may be involved in the intracellular transduction of an activating signal. Phosphorylation of the tyrosine residues in the ITAMs leads to the recruitment of protein tyrosine kinases and subsequent activation of downstream pathways, similar to the signal transduction cascade of T cell receptors (TCRs) (2).

In addition, MHCI acts as a ligand and provides a negative signal to NK cells and negatively regulates their cytolytic activity. Previous studies have identified two types of inhibitory receptors for NK cells in humans: Killer inhibitory receptors (KIRs) and CD94/NKG2 receptors. The KIRs are members of the immunoglobulin family and contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains. Ligand binding leads to the clustering and phosphorylation of the receptor in the ITIM domain, which leads to the recruitment of protein tyrosine phosphatases (PKPs). These PKPs remove phosphate groups from various targets, antagonizing the activating kinases recruited by the ITAMs. This blocks the activation of NK cells. Another class of inhibitory receptors on human NK cells consists of heterodimers of CD94 and NKG2. NKG2 is a variable lectin subunit which contains ITIM domains. Similar to the KIRs, the ITIMs function to recruit PKPs and block the activation of NK cells (2).

In humans, many of the Class I alleles of HLA-B, HLA-C and HLA-E loci are recognized by the NK inhibitory receptors (2). However, the various types of MHCI molecules are unequal in ability to negatively regulate NK cells. For example, MHCIB molecules such as HLA-E and HLA-G are more potent in their ability to negatively

regulate human NK cells. Although most human tissues transcribe HLA-E genes, a few tissues such as the placenta express high levels of MHC1b molecules on the cell surface. By definition, a fetus is an allograft since it expresses both maternal and paternal genes. It is believed that the placenta expresses MHC1b molecules to protect the fetus from the mother's immune system (3).

Once activated, NK cells employ mechanisms similar to those used by CTLs to kill target cells, including perforin and granzymes (see section 2.1.2.4). It is interesting to note that NK cells can exhibit enhanced cytotoxic activities upon stimulation with cytokines such as IL-2. Many studies have used high concentrations of IL-2 *in vitro* to differentiate NK cells into lymphokine-activated killer (LAK) cells. In addition to being more cytotoxic, LAK cells also have a broader target range, with the capacity to kill tumor cells and some normal cell types (2). *In vitro*, this enhanced cytotoxic activity makes it easier to quantify the susceptibility of target cells to NK cell-mediated killing.

Activated NK cells also play a role in modulating adaptive immune responses. For example, activated NK cells secrete IFN- γ , which is a potent cytokine that activates macrophages in their phagocytic function and their antigen presentation to T lymphocytes. Thus, NK cells have an important supporting role in mounting a robust adaptive immune response *in vivo*.

2.1.1.1 Complement

The complement system is generally considered to be a component of the innate immune system due to its conserved nature. However, it functions as an important effector system of both innate and adaptive immunity. The complement system consists of a series of heat-labile serum and cell surface proteins, which interact with foreign or pathogenic molecular targets to mediate effector functions of the immune system. Activation of the complement system leads to a sequential activation of precursor enzymes (zymogens), which covalently bind to microbial surfaces, other antigens and antibody-antigen complexes. This process subsequently initiates effector mechanisms involved in the clearance of the opsonized antigen (2).

There are three activation pathways for the complement system, including the classical (C1), lectin (mannose binding lectin) and the alternative (C3) pathways. Briefly, the C1 pathway is activated by certain isotypes of antibodies bound to their antigens, while the lectin pathway is triggered by the binding of plasma-derived mannose-binding lectin to terminal mannose residues present in microbial structures. Similarly, the C3 pathway is initiated by the binding of C3 to microbial surfaces, without requiring antibodies. The lectin and alternative pathways are effector mechanisms of innate immunity, since they rely on PAMPs, while the C1 pathway is an effector mechanism of humoral (adaptive) immunity. Although these three pathways differ in their initiation, they all result in the proteolysis of C3, triggering the enzyme cascade. This cascade results in the formation of a lipid-soluble protein complex called the membrane attack complex (MAC), killing the

target cell via osmotic lysis. In addition, activation of the complement system promotes phagocytosis (by macrophages and neutrophils) of opsonized targets, induces inflammation and also stimulates B cells to produce antibodies (4, 5).

The complement system plays an important role in allograft rejection (6). Acute cellular allograft rejection is primarily initiated through the function of APCs (see section 2.4). Among the professional APCs, macrophages and dendritic cells express receptors, which bind products of complement activation. For example, studies by Navratil *et al.* show that complement proteins opsonize necrotic cells and promote their phagocytosis by APCs (7). Moreover, Sacks and co-workers show that APCs from complement deficient patients are less stimulatory to T cells *in vitro* and *in vivo*, decreasing the response of alloreactive T cells (8-11). Therefore, it is clear that the complement system is involved in the activation of the adaptive immune system, which plays a major role in allograft rejection.

2.1.2 Adaptive immune system

In contrast to the innate immune system, the adaptive system has a delayed onset; however, it can be tuned to be much more effective against the pathogens due to its antigen-specific response. The adaptive immune system specifically eliminates pathogens using two different types of immune responses: humoral immunity and cell-mediated immunity. Humoral immunity is mediated by antibodies, which are secreted by cells

called B lymphocytes. Antibodies are highly specific molecules that recognize and bind target molecules (antigens) in the circulation and other accessible areas in the body. This binding of antibodies to their antigens can neutralize molecules such as bacterial toxins, and also assist other components of the innate and adaptive immune system to eliminate the pathogen. In contrast, cell-mediated immunity is mediated by cells called T lymphocytes and is used to eliminate intracellular pathogens such as viruses. However, both these systems depend on each other to mount an effective immune response. T lymphocytes can be further divided into functionally distinct helper T cells (CD4⁺ T cells) and cytotoxic T cells (CD8⁺ CTLs). Both these cell types specifically recognize protein antigens only when these antigens are presented by other cell surface molecules, which are encoded by genes in the major histocompatibility complex (MHC). CD4⁺ T cells recognize protein antigens presented by MHC Class II (MHCII) molecules, while CD8⁺ T cells recognize protein antigens presented by MHC Class I (MHCI) molecules. Once activated, CD4⁺ cells secrete proteins called cytokines, which stimulate the proliferation and differentiation of T cells and B cells, and recruit other immune cells such as macrophages to the site. On the other hand, MHCI stimulation of CD8⁺ T cells results in the generation of CTLs, whose effector function is to lyse cells presenting the foreign antigen. In order to initiate and develop the adaptive immune response, non-lymphoid cells called accessory cells are employed. These accessory cells include mononuclear phagocytes, dendritic cells and others cell types, which function to display antigens on MHCI and MHCII molecules, subsequently generating an antigen-specific immune response (2).

2.1.2.1 Antigen presentation to naïve T cells

Naïve T cells recognize peptides presented to them on MHC molecules. CD8⁺ T cells recognize MHCI, while CD4⁺ T cells recognize MHCII molecules (12, 13). However, in order to activate T cells, two signals are required: 1) TCR binding to peptide-MHC complex and 2) Costimulation. Both these signals are usually provided via special immune accessory cells called APCs. Activated T cells then begin to perform their effector functions including proliferation, differentiation, upregulation of cell surface immunoactive molecules and cytokine secretion.

2.1.2.1.1 MHCI and MHCII processing and presentation

The MHCI antigen presentation pathway is constitutively active in all nucleated cells and provides a mechanism for cells to display fragments of all types of intracellular proteins present in the cell at any given time (14). This allows CD8⁺ T cells to survey the internal composition of the cell and initiate an immune response if they detect viral or tumor antigens (14). MHCI molecules are composed of two non-covalently linked polypeptide chains: The MHC-encoded heavy chain is 44-47 kD, while the invariant light chain called the β 2-microglobulin is 12 kD. The N-terminals of the heavy chains form the α 1 and α 2 domains and interact together to create the peptide-binding cleft of the MHCI molecule. Peptides of 8 to 11 amino acids can be accommodated into this cleft. However, larger

proteins need to be processed into smaller fragments before they can be loaded onto the MHCI molecule. The $\alpha 1$ and $\alpha 2$ domains contain the polymorphic residues, encoded by the various class I alleles. In humans, heterozygous individuals express a maximum of six different class I molecules on each nucleated cell. The heavy chains of these molecules are encoded by the codominantly expressed alleles of HLA-A, HLA-B and HLA-C genes. Each class I molecule preferentially binds a slightly different subset of peptides, which contributes to the variation in T cell recognition. The $\alpha 3$ portion of the heavy chain forms an Ig domain, with a structure that is conserved in all class I molecules. The Ig domain also serves as a binding site for CD8, which is expressed on CD8⁺ T cells. This $\alpha 3$ domain is anchored onto the cell surface via a stretch of 25 hydrophobic amino acids, which interact with the inner leaflet of the lipid bilayer. Only a small portion of the heavy chain containing the C-terminus extends into the cytoplasm. However, this portion is extremely important for the directed import of the MHCI molecule into the cell. Similar to the structure of the $\alpha 3$ domain, the $\beta 2$ -microglobulin forms an invariant Ig domain which interacts non-covalently with the $\alpha 3$ domain. It is important to note that the heavy chain, the light chain and the bound peptide together assemble to form the heterotrimeric MHCI molecule.

MHCI assembly occurs in the endoplasmic reticulum (ER), where the heavy chain, light chain and the peptide are brought together in a sequence of steps. See Figure 2.1. First, short peptides are generated in the cytoplasm through the action of the proteasome and other cytosolic enzymes (15). These peptides are then transported into the ER lumen by a

specialized heterodimeric protein called the transporter associated with antigen processing (TAP). TAP is known to be upregulated when stimulated by IFN- γ . Newly synthesized heavy chains present in the ER remain sequestered to a chaperone protein called calnexin. Through a series of steps, the heavy chain first associates with the light chain, allowing the calnexin to dissociate. Another transmembrane protein called tapasin forms a physical bridge between the heavy and light chain heterodimer, and the TAP. The peptide is then loaded onto the MHCI via components of the MHCI peptide-loading complex. After successful assembly of the peptide-MHCI complex, it dissociates from the peptide-loading complex and is transported to the cell surface via the constitutively active secretory pathway of the golgi apparatus (14).

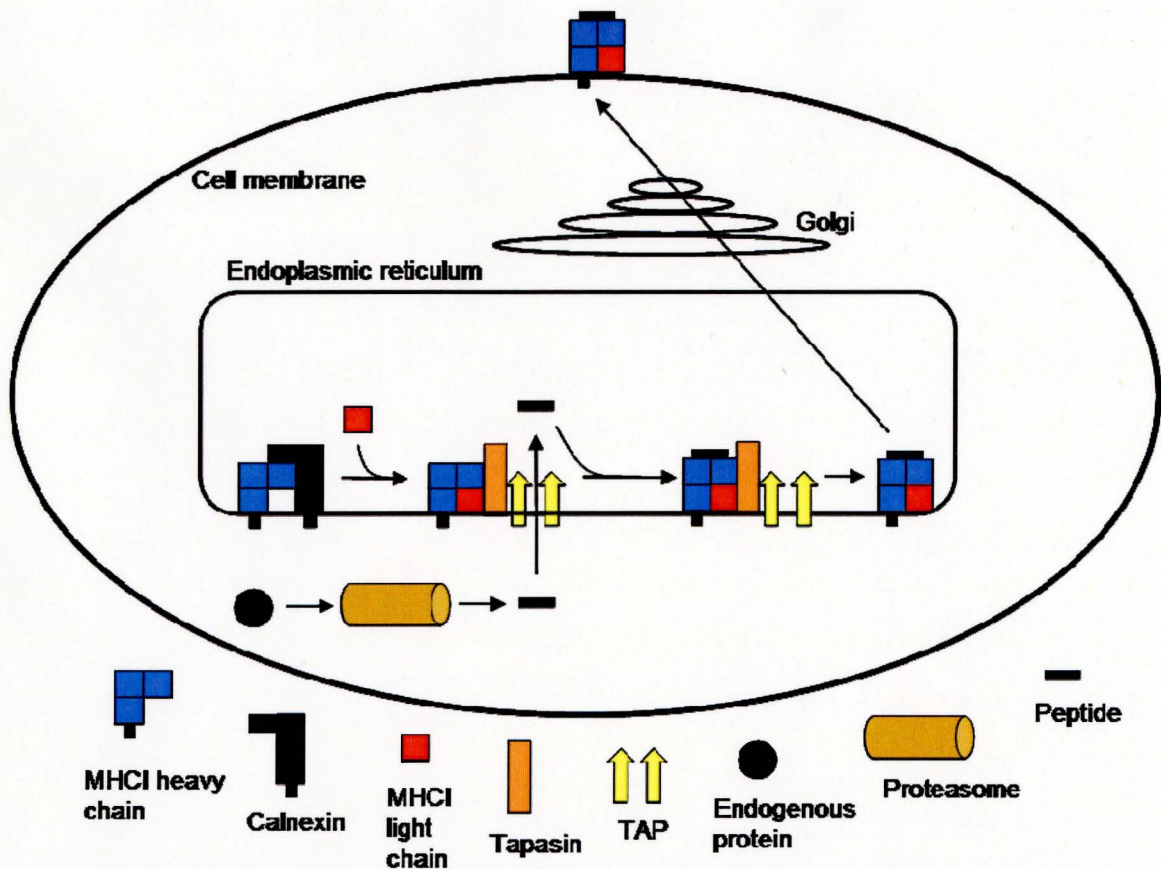


Figure 2.1: *The MHC I peptide presentation pathway.* Shows the steps involved in cytosolic peptide processing (proteasome), peptide transport into the ER (TAP), peptide loading (calnexin, tapasin) and export of the MHC I-peptide to the cell surface through the golgi. Figure adapted from reference (16).

In contrast to the MHC I pathway, the MHC II pathway is constitutively active in APCs such as dendritic cells, macrophages and B cells. MHC II molecules present peptides derived from exogenous proteins which have been internalized by APCs through endocytosis, phagocytosis and other mechanisms. Hence, MHC II molecules allow CD4+ T cells to survey extracellular antigens and regulate adaptive immunity. MHC II

molecules are composed of two polypeptide chains that are non-covalently associated together. Since both the α and the β chain are polymorphic and encoded by the three codominantly expressed MHCII genes, HLA-DR, HLA-DQ, HLA-DP, up to 20 different class II molecules can be expressed by a heterozygous individual. The assembled MHCII heterotrimeric structure is formed when both α and β chains non-covalently associate with the peptide being presented, which varies from 10-30 residues or more. Similar to MHC I assembly, MHCII assembly begins in the ER, where the α and β chains associate to form a heterodimer, which is held together by a type II transmembrane chaperone protein called the invariant chain (17). See Figure 2.2. A retention signal on the invariant chain directs the assembly of MHCII molecules in late endosomal compartments called 'MHCII compartments.' The invariant chain then undergoes a series of proteolytic cleavage events mediated by an acid protease called cathepsin S, which only leaves a short fragment of the invariant chain known as MHCII-associated invariant-chain peptide (CLIP) still occupying the peptide-binding groove (18). In order for the MHCII molecule to present peptides, CLIP must dissociate from the peptide-binding groove. This dissociation of CLIP is mediated by a catalyst-chaperone protein called HLA-DM, which essentially accelerates the peptide exchange process. HLA-DM is a nonpolymorphic molecule which catalyses multiple rounds of peptide exchange, thereby engaging in a process called peptide editing. This process results in the formation of a stable MHCII complex, presenting exogenous or endogenous proteins which have gained access to the endosomal pathway. The fully assembled MHCII complex is then transported to the cell surface for antigen presentation to CD4+ T cells (19).

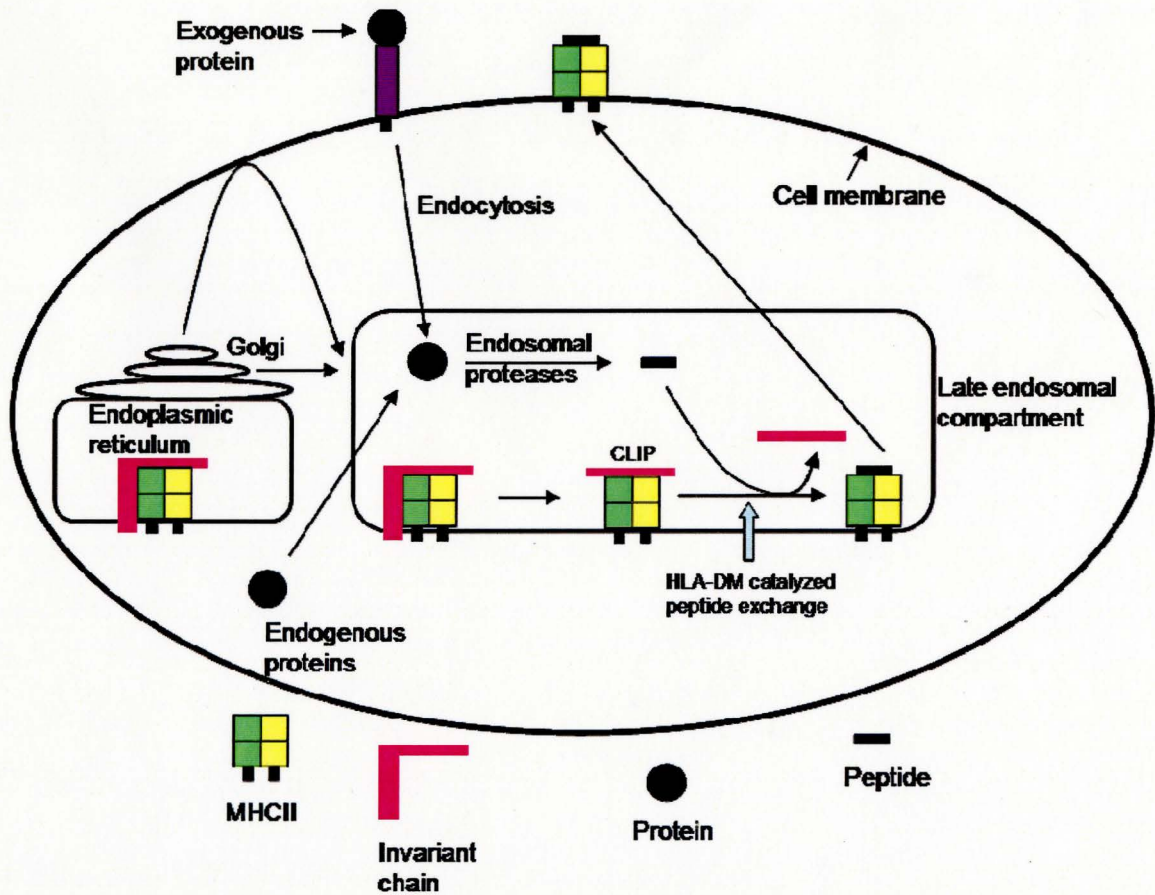


Figure 2.2: *The MHCII peptide presentation pathway.* Shows the steps involved in the formation of the MHCII heterodimer stabilized by the invariant chain in the ER, peptide loading mediated by HLA-DM and export of the MHCII-peptide complex to the cell surface through the endosomal pathway. Figure adapted from reference (16).

2.1.2.1.2 *Costimulation*

Costimulatory molecules are cell surface (or secreted) signaling molecules that serve to provide the ‘second signal’ required to activate naïve T cells. The best-defined costimulatory molecules are two structurally related glycoproteins called B7-1 (CD80) and B7-2 (CD86) molecules expressed on professional APCs including macrophages, B cells and dendritic cells (20). T cells have two specialized receptors, CD28 and CTLA-4, which bind these B7 molecules. CD28 is a homodimer of two polypeptide chains, which is constitutively expressed on greater than 90% of CD4⁺ T cells and over 50% of CD8⁺ T cells. In addition to the first signal provided by the engagement of the MHC to the TCR complex, the binding of B7 to CD28 has many consequences including T cell proliferation and differentiation into effector cells, expression of anti-apoptotic proteins, and the production of growth factors and cytokines such as IL-2. In contrast, CTLA-4 (CD152) is expressed on recently activated CD4⁺ and CD8⁺ T cells and functions to inhibit T cell activation. CTLA-4 is structurally homologous to CD28 molecules and its binding to B7 serves to terminate the T cell response (21). It is interesting to note that in the absence of costimulation, T cells which encounter the first signal either undergo apoptosis or enter a state of unresponsiveness, called anergy, to that particular antigen (2). Currently, many studies are investigating this as a potential mechanism of tolerance induction to foreign antigens. In addition, many viruses have been known to down-regulate costimulatory molecules to control the activation of the adaptive immune system (22).

2.1.2.2 Cross-presentation

As described above, MHCI complexes present peptides from the cytosolic compartment to CD8⁺ T cells, while the MHCII complexes present peptides from the endosomal compartment to CD4⁺ T cells. However, cross-presentation is a process that occurs in APCs like dendritic cells and macrophages, whereby peptides from the endosomal compartment are presented on MHCI molecules, while peptides from the cytosolic compartment are presented on MHCII molecules (23, 24). Studies have shown that TAP-dependent and TAP-independent mechanisms (such as cathepsin S) are both involved in cross-presentation (25). In addition, it is generally believed that cell and particle-associated antigens are more efficient in inducing cross-presentation compared to soluble antigens (14). Recent studies have also shown that cross-presented antigens usually require further processing and may represent a mechanism for the cell to take advantage of the different sets of proteolytic enzymes present in different cell compartments (26, 27). Many studies analyzing the peptides presented on MHC molecules clearly demonstrate that peptides derived from intracellular, extracellular and transmembrane proteins can be presented on both MHCI and MHCII molecules (28). It follows that alloantigens can be presented on both MHCI and MHCII molecules and thus provides another explanation for the robust adaptive immune response to allografts.

2.1.2.3 Molecules involved in the activation of naïve T cells

In order for the adaptive immune system to respond to foreign antigens, naïve T cells must be activated to mount an effective immune response. Naïve T cell activation requires: 1) the engagement of the TCR and the peptide-MHC complex and 2) the interaction of costimulatory and other accessory molecules between T cells and the APCs. The TCR for both MHCI and MHCII molecules is composed of two transmembrane polypeptide chains, α and β , which are covalently linked via disulphide bonds (29). Another type of TCR composed of γ and δ chains are also found on a small subset of T cells, but these TCRs are not MHC restricted and their role in the immune system remains unclear. TCR specificity for the peptide and its MHC restriction is determined by a region of the TCR called the complementarity-determining region (CDR). However, the affinity of TCRs to peptide-MHC complexes is quite low in comparison to antibodies. Hence, other TCR-associated molecules are required for adhesion and signal transduction. For example, all T cells express CD3 and ζ molecules that are noncovalently associated with the TCR α - β heterodimer (forming the TCR complex) and serve to transduce the signal into the cell. Briefly, signal transduction begins with the phosphorylation of tyrosine residues within the ITAMs which occurs in the cytoplasmic tails of CD3 and ζ proteins, leading to T cell activation.

In addition, T cells express other accessory molecules not part of the TCR complex, such as CD8 and CD4. Mature T cells express either CD4 or CD8 molecules, which bind to the non-polymorphic regions of the MHC molecule and serve to transduce the signal delivered through the TCR complex. Hence, CD4 and CD8 molecules are called

coreceptors and may also serve to strengthen the T cell-APC interaction. Moreover, T cells are also known to express CD44, and various types of integrins and selectins to specifically aid the intercellular adhesion of T cells to APCs and other cell types. For example, T cells express heterodimeric cell surface proteins called integrins, which bind specific ligands on other cells and surfaces including APCs, endothelial cells and extracellular matrix proteins to mediate intercellular adhesion. Integrins have cytoplasmic domains anchored to the cytoskeleton, allowing them to also participate in cell migration (30). LFA-1 is an example of an integrin expressed on over 90% of mature T cells, B cells, monocytes and thymocytes. LFA-1 binds specific ligands on other cells, including intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is a membrane glycoprotein expressed on a variety of cells including T and B cells (31). Previous *in vitro* and *in vivo* studies have shown that modulation of the interaction between ICAM-1 and LFA-1 may considerably influence the immune response to allografts (31).

2.1.2.4 Activation of CD8⁺ T cells and effector functions of CTLs

Naive CD8⁺ T cells (pre-CTLs) require activation and subsequent differentiation into CTLs to perform their effector functions. This activation begins with the recognition of peptide-MHCI (signal 1) and costimulation and/or cytokines (signal 2). As described previously, this mode of immune activation primarily samples peptides from the cytosolic compartment, which serves as a mechanism to detect and clear intracellular pathogens. However, this mechanism can also be activated by allogeneic MHC I molecules on

allografts and mediate rejection. In the absence of donor APCs, pre-CTLs receive the first signal from the allogeneic MHCI present on the allograft cells. The second signal can be provided by activated CD4⁺ T cells, which secrete cytokines like IL-2 to stimulate the pre-CTLs to clonally expand and differentiate into CTLs. Although this mechanism of CTL generation does not require APCs directly, the CD4⁺ T cells require APCs to present alloantigens on MHCII molecules for their own activation. Moreover, activated CD4⁺ T cells express CD40L, which binds CD40 on the APCs and activates them to more efficiently stimulate the pre-CTLs directly. This second mechanism through which host APCs directly provide both signals to pre-CTLs is mediated by cross-presentation. This second mechanism is also known as the ‘indirect pathway’ of allograft rejection. See section 2.4. In this mechanism, alloantigens are internalized by APCs via the endocytic pathway and cross-presented onto MHCI molecules. This provides the first signal to pre-CTLs, while B7 costimulatory molecules provide the second signal. Once pre-CTLs proliferate and differentiate into CTLs, they acquire cytolytic functions.

CTL-killing of target cells is highly antigen-specific and contact dependent. CTL-mediated target cell lysis occurs in three steps: First, CTLs must recognize target cells bearing the MHCI-associated peptide via the TCR on CTLs. Signal transduction and TCR complex formation is aided by engagement of the CD8 coreceptor molecule to the MHCI-peptide molecule. Efficient binding of CTLs to the target cell also requires the CD8 coreceptor and accessory molecules such as ICAM-1, which serve to enhance the adhesion of CTLs to the target cells. This results in the activation of CTLs to perform

their cytolytic function. Second, the CTLs deliver cytotoxic granules to the target cells by exocytosis. CTLs induce the fusion of cytoplasmic granules containing proteins called perforin and granzymes to the plasma membrane. Perforin monomers polymerize in the presence of extracellular calcium ions, forming aqueous channels in the plasma membrane of target cells. Osmotic swelling and the influx of calcium ions into the target cell results in cell death. In contrast, granzymes are serine proteases that cleave proteins at specific residues. For example, granzyme B cleaves proteins containing aspartic acid residues. After exocytosis by CTLs, granzymes enter target cells via the perforin-mediated pores in the plasma membrane and begin their proteolytic function, whereby they also activate cellular caspases that induce apoptosis of the target cell (32). This process is highly specific and CTLs only kill cells expressing the same MHC I-associated peptide that trigger the activation and differentiation of pre-CTLs. In addition to the cytoplasmic granules, CTLs also induce target cell death via the Fas ligand (FasL or CD95L). Many cells types express a cell surface protein called Fas (CD95), which binds FasL present on CTLs. This engagement of target cell Fas with FasL present on CTLs results in the activation of target cell caspases, and subsequent induction of apoptosis. Third, the CTLs dissociate after initiating apoptosis of the target cells. This process may involve the decrease in the affinity for accessory molecules like ICAM-1 and occurs shortly after the CTL-mediated induction of apoptosis in target cells (33). In addition to the direct cytolytic functions, CTLs also begin to secrete cytokines such as IFN- γ , lymphotoxin and TNF molecules to induce inflammation and activate phagocytes.

2.2 Tissue engineering

Tissue engineering is a field that combines the principles of biology and engineering as a means to develop biological tissue substitutes and serves as a tool in regenerative medicine. The impetus for the development of this field was the critical gap between the number of human donor organs available for the treatment of end-stage diseases and the growing demand for organs. In essence, the principles and practices of tissue engineering were developed as a means to fill this gap (34). For example, 65,000 people in the US were on the wait list in 2005 for renal transplants and the waiting times exceed 5 years for those without living donors (statistics for Canada are approximately 5% of the US figures). In 2003, over 2,700 patients in the US died while waiting for vital transplants such as the heart, liver and lung. Furthermore, almost 20% of patients on the cadaveric kidney transplant waiting list are patients who are awaiting retransplantation due to a failed first graft (34). In addition, transplanted organs or grafts have a limited life-span in the host due to the immune response against the foreign cells. Immunosuppressive drugs provide a short-term solution (80-95% 1-year graft survival) in cardiac, renal, lung, liver and pancreatic transplantation (35). However, their mode of action is systemic and suppresses the entire body's immune system, therefore increasing the individual's susceptibility to opportunistic infections. The main drawback of long-term immunosuppressive drug therapy is the risk of severe side effects such as cytotoxicity, hypertension and cardiovascular disease (35).

Therefore, there is a real need for a non-immunosuppressive solution to prevent graft rejection. Such a solution could potentially increase the availability of transplantable organs, since it would make available organs that would currently be considered an inappropriate HLA “match.” In addition, a solution that could extend the life of a graft would also reduce the need for retransplantation. Hence, a successful non-immunosuppressive strategy can potentially provide a substantial benefit to the recipient.

However, the goal of creating a complex tissue-engineered organ substitute has not yet been realized. Nevertheless, the vast majority of current tissue engineering approaches have focused on regeneration and restoration of functional tissues such that there is a decreased need for organ transplantation. For example, type 1 and type 2 diabetic patients have a decreased pancreatic cell mass and could potentially benefit from a tissue-engineered islet cell substitute (36). Given that 16 million in the US alone suffer from diabetes mellitus and over 217 million world-wide, it is likely that a successful treatment would have an enormous impact in society (37). Similarly there are many other conditions including Alzheimer’s and Parkinson’s disease (5.5 million US patients), osteoporosis (10 million), spinal cord injuries (0.25 million) and severe burn injuries (0.3 million) that may benefit from tissue-engineered biological substitutes directed at replacement and regeneration of endogenous tissues (38).

2.2.1 Tissue-engineered constructs

A tissue-engineered biological substitute is intended to replace and repair/regenerate damaged tissues and organs. These biological substitutes can be made of scaffolds and/or cells, depending on the intended application.

Scaffolds provide the physical structure for cells to grow and can be made of synthetic and/or natural materials. For example, synthetic scaffolds are usually polymeric and include biomaterials such as polylactic acid (PLA), polyglycolic acid (PGA) and polylactic-glycolic acid (PLGA), while natural scaffolds are made of substances such as alginate, collagen, hyaluronic acid (HA) and chitosan (39). Typically, these materials are non-toxic and/or biodegradable and cell-compatible, and have been shown to enable cell attachment, growth and differentiation. Although natural materials are sometimes more cell-compatible and better promote cell adhesion, their properties (structure, mechanical properties, rate of degradation and porosity) are generally more difficult to control compared to synthetic polymeric scaffolds. A scaffold made of a combination of both synthetic and natural materials could potentially benefit from the desired qualities of both materials. In addition, scaffolds can be three dimensional in structure, such that they provide the proper physical structure for cells to grow into tissues of the desired shape. With time, biodegradable scaffolds degrade and the newly formed tissues gradually integrate with host tissues. For example, scaffolds shaped as tubes can be used to form tubular tissues such as arteries that self-assemble and integrate with host tissues (39).

Tissue-engineered constructs intended for clinical use can employ a wide range of cell types. Hence, one of the major challenges for tissue engineering is exploring different sources of cells. Identifying suitable sources of the desired cell type and generating a sufficient number of cells required for the tissue-engineered construct is the subject of much research and development in the field. There are three genetically distinct classes of cells that can be used in tissue engineering: autologous, allogeneic and xenogeneic. Each class of cells can further be divided into differentiated cells, adult stem cells and embryonic stem cells. Autologous cells are genetically identical to the patient since they are derived from the patient. Hence, an autograft presents no risk of immune rejection, making it highly desirable in tissue engineering. However, generating an autologous construct is likely laborious and costly, providing no opportunity for manufacturing in advance, since it lacks “off-the-shelf” availability for immediate treatment (40). In addition, some differentiated cell types harvested from a single patient might not be sufficient to make the required cell-based tissue-engineered construct, hence prompting the search for other sources of cells. In contrast, allogeneic cells are derived from other human donors, allowing them to be sourced in advance for the development of “off-the-shelf” products that can be used in treating a large number of people. For example, Apligraf (Organogenesis) and Dermagraft (Smith & Nephew) are FDA-approved tissue-engineered skin constructs made of allogeneic cells derived from human foreskins (41, 42). It is interesting to note that these products do not induce an acute immune response although they are not genetically identical to the host. Hence, these products do not require immunosuppressive drugs after implantation. Lastly, xenogeneic cells are cells

derived from a different species. Xenogeneic cells can be considered as a potential source of cells in tissue engineering, however their use remains controversial due to the potential for zoonotic transmission of pathogens to humans and the significant immunological barrier encountered in xenogeneic transplants (40).

Although all three types of cells can be used in creating tissue-engineered constructs, it is currently accepted that allogeneic cells are the most suitable for the treatment of human diseases. Therefore, the remainder of this thesis will discuss the characteristics and utility of allogeneic cells in tissue engineering.

2.2.2 Allogeneic cell sources for tissue engineering

Preliminary studies in the field isolated cells from organs and tissues they wished to regenerate. However, differentiated cells isolated from these sources are difficult to culture *in vitro*. In addition, the shortage of viable human tissues and organs make this an impractical source for differentiated cells. As an alternative to using differentiated cells isolated from human tissues and organs, many groups have proposed the use of stem cells in tissue engineering (43). Stem cells are undifferentiated cells, which can be coaxed to differentiate into multiple cell types. This can be achieved by manipulating the growth environment of stem cells seeded onto a tissue-engineered construct to differentiate and expand cells. This approach also allows for greater control over stem cell characteristics such as self-renewal, differentiation, migration and proliferation of cells before the

construct is transplanted *in vivo*. Moreover, stem cells can also be directed to differentiate into progenitors, which are cells that have committed to a lineage of a certain cell type and can differentiate further into the desired cell type. To date, progenitors and stem cells have been isolated from both adult and embryonic tissues of all types (43). In contrast to differentiated cells, some types of stem cells also seem to suppress the host immune response. In summary, the application of stem cells and progenitors in tissue-engineered constructs provides an opportunity to grow and regenerate complex structures such as tissues and even organs, thereby providing a solution to the shortage of viable organs, tissues and cells required to treat many human diseases. Hence, the proper sourcing of stem cells is an important aspect in the field of tissue engineering.

2.2.2.1 Adult stem cells

Adult stem cells are lineage-restricted stem cells isolated from fetal and postnatal tissues based on their capacity to self-renew and differentiate into several different tissues in culture. However, adult stem cells have a more limited capacity to form different tissues and are termed multipotent, rather than pluripotent nature of embryonic stem cells (ESCs), which can form all tissue types. For example, neural stem cells can differentiate into neurons, astrocytes and oligodendrocytes, while cardiac stem cells can yield cardiomyocytes, smooth muscle cells and endothelial cells (44, 45). Recent studies by Yamada *et al.* have also shown that adult liver stem cells can be stimulated to switch phenotype from hepatic cells to pancreatic endocrine cells. This suggests that adult stem

cells may not be restricted to forming tissues from which they were derived, increasing their potential in tissue-engineering applications (46). Such adult stem cells also have the benefit of being less tumorigenic, making them safer than using ESCs, which can readily form tumors *in vivo*. In addition, adult stem cells can be isolated relatively easily from almost any adult tissue using surface markers, and avoids the ethical dilemma of destroying human embryos to derive hESC lines.

2.2.2.2 Mesenchymal stem cells

Another class of adult stem cells with enormous potential are mesenchymal stem cells (MSCs) derived from the bone marrow. Many studies have explored their multipotent potential and differentiated them into connective tissues such as bone, cartilage, muscle, fat and tendon (47, 48). It is interesting to note that MSCs have demonstrated immune privilege properties in an allogeneic setting. MSCs cultured *in vitro* have also been shown to downregulate immunoactive antigens such as MHCI (49). In addition, a subset of MSCs termed multipotent adult progenitor cells (MAPCs) seem to be almost as multipotent as ESCs, making them highly desirable in tissue-engineering applications (50).

2.2.2.3 Fetal stem cells

Fetal stem cells are another interesting and diverse group of cells derived from fetal tissues like amniotic fluid, chorionic villi, placenta and umbilical cord blood, which possess characteristics that are particularly suited for tissue engineering. First, fetal cells are highly plastic and can be differentiated into cell types representing all the three embryonic germ layers. In addition, De Coppi *et al.* demonstrated that amniotic fluid stem cells (AFS) can be expanded for over 200 doublings without replicative senescence or any indication of telomere shortening (51). A subset of AFS cells called embryonic-like stem cells have also been noted to express embryonic stem cell markers and may also exhibit similar characteristics like pluripotency, which will be useful in generating multiple tissue types (51). In comparison to mature cells, fetal cells are also more responsive to environmental cues, making it easier to direct their differentiation. Second, most fetal cells proliferate faster in culture. This *ex vivo* expansion capability makes them ideal for applications requiring large numbers of cells. For example, MSCs derived from fetal tissues have demonstrated greater plasticity and faster growth rates than bone-marrow derived MSCs. In addition, these cells do not require any special ‘feeder layer’ of supporting cells required by other cultured stem cells, making them easier to culture *in vitro*. Third, fetal MSC’s have embryonic stem cell-like properties, including the lack of MHCII expression. An added advantage of differentiated and undifferentiated cells derived from fetal MSC is that they do not induce the proliferation of alloreactive lymphocytes in a mixed lymphocyte reaction (MLR), even under the presence of IFN- γ . These immunosuppressive properties make fetal MSC particularly suitable for allogeneic transplantation (52). Fourth, fetal cells seem to be more resilient to trauma induced during

in vitro manipulations. For example, fetal cells can survive lower oxygen tensions, which make them more resistant to ischemia-related damage common in transplantation. Fetal cells are also more resilient to damage induced by cryopreservation, making them more versatile and suitable for tissue engineering applications.

2.2.2.4 Embryonic stem cells

Embryonic stem cells are cells that have been traditionally derived from the inner cell mass of a preimplantation blastocyst. These cells have generated enormous interest in the field of tissue engineering due to their unique properties. For example, pluripotent embryonic stem cells have the capacity to self-renew and proliferate indefinitely *in vitro*, while maintaining their potential to differentiate into any cell type in the body (53). This would provide an unlimited source of cultured cells of the desired cell type for tissue engineering applications. However, the process of extracting these cells usually involves destroying the embryo, which poses an enormous ethical dilemma in humans. Nevertheless, many research groups are attempting various other techniques to isolate human embryonic stem cells without destroying human embryos (54).

There are three major challenges associated with the use of human embryonic stem cells in tissue engineering: 1) *In vitro* culture conditions in which hESCs are propagated and differentiated need to be improved in order to minimize the reliance on animal-derived products. 2) Differentiation protocols for hESCs need to be improved such that

homogeneous populations of the desired cell type can be isolated without any contaminating stem cells with tumorigenic potential. 3) The immune response to allogeneic hESCs-derived cells must be overcome.

Many strategies are currently underway to culture and direct the differentiation of stem cells *in vitro*. For example, the development of culture conditions without the need for a supporting feeder layer for stem cell growth and the use of inducers to direct stem cell differentiation (55). Although progress is being made on this front, the immune response to allogeneic cells still remains a major challenge and is the subject of this thesis.

In the absence of immunosuppressive therapy, the immunogenicity of hESC-derived cells would limit their success in allogeneic cell-based tissue engineering applications. The immunogenicity of allogeneic hESC-derived cells is primarily due to the MHC I molecules expressed on these cells, and is the key factor in acute rejection (35). Although many studies have shown that hESCs themselves express low levels of MHC I and may be immune privileged, hESC-derived cells may not share the same properties (56). Drukker *et al.* have shown that hESCs upregulate their expression of MHC I upon differentiation by about 10-fold, sufficient to provoke an acute immune response (53). This group also determined that MHC II and costimulatory antigens are absent in hESCs and are only observed in hESC-derived hematopoietic cells (57). This observation confirmed that allogeneic MHC I are alone sufficient to provoke an immune response in hESC-derived non-hematopoietic cells (56). Moreover, *in vitro* studies by this group also

suggest that hESCs and their derivatives evade NK cell-mediated lysis as they lack NK cell activating ligands (57). Overall, these observations suggest that it will likely be necessary to modulate MHCI molecules expressed on hESC-derived cells or induce active tolerance in the host for these transplanted cells to be successful in tissue engineering applications.

In summary, there are still many challenges that are associated with the use of stem cells and progenitors in tissue engineering constructs developed for human transplantation. Perhaps the most significant challenge associated with the use of cell-based tissue engineering constructs is the immune response of the host. Many studies have shown that the immune response against allogeneic progenitor and stem cells is lesser in comparison to differentiated cells. Although this may be the case, most applications in tissue engineering require the allogeneic progenitor and stem cells to differentiate to form specific cells that serve to replace and regenerate damaged tissues. This poses a problem since many studies provide compelling evidence suggesting that the differentiated cells derived from progenitors and stem cells are immunogenic to an allogeneic host. Hence, using unmodified progenitors and stem cells in tissue engineering constructs still does not provide a solution to the immune rejection problem.

2.3 Mechanisms of rejection

A cell-based tissue-engineered construct can be rejected in multiple ways, depending on the composition of the construct. Factors such as the cell type, biomaterial, presence of biomolecules, degradation products and size contribute to the overall mechanism/pathway of rejection. However, it is likely that some components of the construct are more dominant in determining the specific pathways and the rate at which the construct is rejected. In this review, I will focus on the mechanisms of rejection directed against the foreign allogeneic cells in the construct.

Historically, graft rejection is primarily classified according to the time course of the overall immune rejection rather than the mechanism of rejection. However, it is important to note these classifications since the success or failure of a cell-based tissue-engineered construct may depend on which of these processes dominate in immune rejection. In addition, the specific mechanism of rejection is likely to be a combination of multiple responses of the immune system occurring in tandem. These responses include innate immune response, adaptive immune response, inflammatory response and the wound-healing response. Since immune cells are involved in all of the above host responses, it is instructive to first consider the different types of rejection: hyperacute, acute and chronic.

2.3.1 Hyperacute rejection

Hyperacute rejection primarily occurs in transplanted allogeneic organs and grafts which are complex enough to have a vasculature. This form of rejection is characterized by the

occlusion of blood vessels due to thrombosis (2). Hyperacute rejection begins almost immediately upon anastomosis of the foreign vasculature to the host vasculature and occurs in the time frame of minutes to hours. It has been shown that preexisting antibodies in the host circulation bind the foreign antigens expressed on the graft endothelium and activate the complement system. First, antibody binding and formation of the complement C1q complex activates the endothelial cells in the graft vasculature to secrete von Willebrand factor that mediates the aggregation and adhesion of platelets. This process induces the vesiculation of endothelial cell and platelet membranes, which results in the shedding of lipids and procoagulant factors into the graft vessels. In addition, activated endothelial cells undergo other changes altering their anticoagulant role in the vessel. For example, endothelial lose cell surface expression of heparin sulfate proteoglycans, preventing its interaction with anti-thrombin III, thus creating a procoagulant environment. Second, complement activation physically damages endothelial cells, exposing the basement membrane. The proteins found on the basement membrane further activate platelets and promote coagulation. Together, these processes contribute to the characteristic thrombotic occlusion of graft vasculature, leading to irreversible ischemic damage and death of downstream graft tissues (2).

It is thought that preexisting antibodies (usually IgG) in the host circulation triggers the complex cascade of events leading to hyperacute rejection. In general, preformed antibodies arise due to prior exposure of the host to alloantigens from blood transfusions, previous graft transplantations or multiple pregnancies (2). These antibodies can be

directed against antigens such as ABO, MHC molecules and other antigens on the surface of the graft endothelium. In some cases, the titer of these preformed antibodies can alter the time scale of hyperacute rejection. For example, a lower titer can lead to a slower hyperacute rejection. Allograft transplant recipients today are screened for these preformed antibodies to minimize the chances of hyperacute rejection. In the complete absence of preformed antibodies, an allograft may avoid hyperacute rejection, but it may still be rejected by acute rejection.

2.3.2 Acute rejection

Acute rejection can occur in any type of allogeneic transplant including organs, tissues and cells. This form of rejection is characterized by vascular and parenchymal cell damage in organs, and general cell death in transplanted allografts. Acute rejection typically begins after seven days following transplantation. This delay is due to the time it takes for the differentiation of effector T cells and the production of antibodies against the alloantigens. Numerous studies have shown that the MHCI recognition capability of T cells responding to the allograft as the most significant event leading to acute rejection. However, not all T cells are capable of recognizing an allogeneic MHCI complex (2).

As described previously, MHCI molecules, which are present on all nucleated cells, are recognized only by CD8⁺ T cells. Previous studies have shown that as many as 10% of these CD8⁺ T cells respond to allogeneic cells *in vivo* (58). This response is usually

measured by activation of CD8⁺ T cells, which leads to proliferation and differentiation into effector cells known as CD8⁺ CTLs. These CTLs have potent cytolytic activity and specifically kill allogeneic cells (2). This large percentage of cells has the potential to expand and produce a potent immune response against an allograft. In fact, previous adoptive transfer experiments have shown that CTLs alone are sufficient to cause acute graft rejection (2).

In vivo, such CD8⁺ T cell activation occurs in tandem with many other processes such as cytokine production, inflammation, recruitment of other immune cells and a humoral response. Moreover, it has recently been shown that CD4⁺ T cells may also contribute to acute rejection. As described previously, CD4⁺ T cells recognize MHCII molecules, which are generally present on APCs. Once activated, CD4⁺ T cells can secrete cytokines and induce delayed type-hypersensitivity (DTH)-like rejection (2). Some studies have also shown that CD4⁺ T cells alone might be sufficient to induce acute rejection (59).

Antibodies can also mediate acute immune rejection of allografts. The adaptive immune system can generate specific antibodies against cell surface antigens on the allograft. As described above, antibody attachment to its antigen can lead to the activation of the complement system. This leads to necrosis and intravascular acute inflammation in the allograft.

2.3.2.1 Minor histocompatibility antigens

It is important to note that allogeneic cells contain many genes apart from the HLA genes which can differ from the host. Hence, it is expected that the gene-products from these different genes can generate a diverse array of molecules, which can also be presented on MHC molecules. Such molecules are termed minor histocompatibility antigens (mHAgs) and can also lead to immune recognition and subsequent rejection. Any non-MHC gene that encodes a gene product or epitope capable of stimulating CD4⁺ T cells or CD8⁺ T cells after allogeneic transplantation would qualify as a mHAgs (60). However, the major difference between MHC antigens and mHAgs is clinically observed in the time required for the host immune system to reject the allograft. An allograft matched for MHC antigens, with differences only in mHAgs is likely to result in a considerably slower rejection process (60). Hence, mHAgs are not considered as dominant antigens involved in allograft rejection.

2.3.3 Chronic rejection

Chronic rejection primarily occurs in transplanted allogeneic organs and grafts which are complex enough to have a vasculature. This form of rejection is characterized by fibrosis and loss of organ/tissue structure and function over a prolonged period of time following transplantation (2). It has been hypothesized that T cells activated by alloantigens on the graft vessel walls recruit macrophages to the site, where the macrophages begin to secrete smooth muscle growth factors. This leads to a proliferation of smooth muscle cells in the

vascular intima. The resulting intimal thickening occludes the vessels (graft arteriosclerosis) and gradually causes a loss of organ/tissue structures.

The characteristic fibrosis in chronic rejection may represent a wound healing response to the damage initiated by acute rejection. It has been hypothesized that activated macrophages secrete factors such as platelet-derived growth factor (PDGF), which results in fibroblast proliferation and collagen synthesis (2). The accumulation of fibroblasts and collagen is histologically described as fibrosis. In addition, this type of rejection has proven to be difficult to control using immunosuppressive therapies.

2.4 Molecular basis of allogeneic recognition

The immune response to allogeneic grafts involves components of both the innate and adaptive immune system. As described in section 2.3.2, the T cell-response is probably the most important determinant of acute rejection. The T cell response occurs due to the recognition of ‘non-self’ alloantigens present on the allograft. Experiments by George Snell and colleagues identified the MHC complex as the locus of polymorphic genes, which code for the targets of allograft recognition (61). Since MHC I molecules are expressed on all nucleated cells, they can evoke immune responses in virtually all allotransplanted tissues and are responsible for almost all acute rejection. MHC molecules normally function to present peptides to T cells of the adaptive immune

system. The adaptive immune system can be activated by the allograft through at least two separate pathways: direct and indirect. In the direct pathway, host T cells (CD8+ cells and CD4+ cells) respond to allogeneic APCs from the allograft. This occurs when ‘passenger’ leukocytes (APCs) have been transplanted along with the desired allogeneic tissues/organs into the host. See Figure 2.1.

The direct pathway is considered a major mechanism of acute allograft rejection since it begins shortly after transplantation and lasts for a relatively short period of time. One reason for this short-term effect is the depletion of allogeneic APCs over time. This hypothesis is strongly supported by many studies exploring the number of alloreactive T cells. In a classic study by Lechler *et al.*, rat renal allografts were not rejected by the host immune system when the allografts were first ‘parked’ in an intermediate recipient, where it is thought that the donor APCs were depleted (62). Furthermore, these renal allografts were immediately rejected when the host was injected with a small number of donor APCs (63). In another experiment by Lafferty *et al.*, prolonged graft survival was achieved when thyroid allografts were cultured *in vitro* before being transplanted into the host (64). Presumably, the allogeneic APCs from the graft were depleted during the *in vitro* culture, therefore minimizing the direct pathway of allograft recognition. The direct pathway depends critically on the migration of allogeneic APCs (e.g., dendritic cells) from within the grafted tissue to host lymphoid tissue, where they directly present peptide on MHC molecules to naïve T cells and induce their activation (65, 66).

Many studies have established that the strong immune response initiated by the direct pathway is due to the presence of a high frequency of T cells in a host which are cross-reactive to allogeneic MHC-peptide complexes (67). This frequency is estimated to be 100-fold to 1000-fold higher than the frequency of precursor T cells specific for any foreign peptide-self-MHC complex (67, 68). It is generally accepted that this recognition occurs due to the structural similarity of allogeneic MHC molecules to self-MHC molecules. This suggests that although the conventional self-MHC-restricted T cell repertoire dictates specificity in its interaction with MHC molecules, it can encompass a high degree of degeneracy with respect to its interactions with allogeneic MHC molecules. This concept of degeneracy has been proposed to explain the high frequency of alloreactive T cells present in the host, which can generate a robust immune response (28). Therefore, transplanted allografts containing donor APCs pose an immediate risk of acute rejection. It is clear that this problem needs to be addressed before cell-based tissue-engineered constructs containing allogeneic donor tissues can be successfully transplanted across individuals.

In contrast, the indirect pathway is considered a relatively minor mechanism of acute graft rejection, since it requires more time to develop than through the direct pathway. In this mechanism, protein fragments normally shed from allogeneic cells are picked up by host APCs and presented to T cells to induce their activation (65). See Figure 2.1. In general, this process of priming, where host APCs prime host T cells, requires a greater amount of time to manifest itself and lead to immune rejection. Many studies with knock-

out mice have shown that although the indirect pathway is slow to start, it is alone sufficient to cause immune rejection. In a study by Auchincloss *et al.*, it was observed that MHCI knock-out recipient mice could reject allografts from MHCII knock-out donor mice (69). This meant that the recipient mice lacked CD8⁺ T cells capable of recognizing allogeneic MHCI on the donor APCs directly, while the recipient CD4⁺ T cells could only be primed indirectly with allogeneic MHCI and mHAg. This required the allogeneic MHCI molecules and other mHAg to be processed using host APCs and presented on MHCII to host CD4⁺ T cells. This experiment also showed that CD4⁺ T cells are alone sufficient to mediate chronic graft rejection (70). These results lead to the hypothesis that the population of T cells with indirect allorecognition capability are an important driving force of chronic rejection (71, 72). Many clinical studies of patients with established chronic rejection lend support to this hypothesis. For example, several groups have reported an increased frequency of T cells with indirect alloreactivity in patients with chronic rejection of transplanted kidneys, lungs and hearts (72-74).

In addition to the direct and indirect pathways of rejection, the semi-direct pathway has also been proposed as a means to explain recent unexplained observations. For example, Pimenta-Araujo *et al.* recently showed that embryonic thymic epithelium, which naturally lack APCs, are subject to acute rejection in the absence of the indirect pathway (75). This puzzling observation cannot easily be explained by the direct or the indirect pathways of rejection. The semi-direct pathway essentially provides a link between the direct and indirect pathways, where the recipients' dendritic cells (or APCs) acquire intact donor

allogeneic MHC I molecules and present them directly to host CD8+ T cells. Although this semi-direct pathway has not yet been fully supported, it provides an alternative explanation for such observations and is important to consider in the development of allogeneic cell-based tissue-engineered constructs.

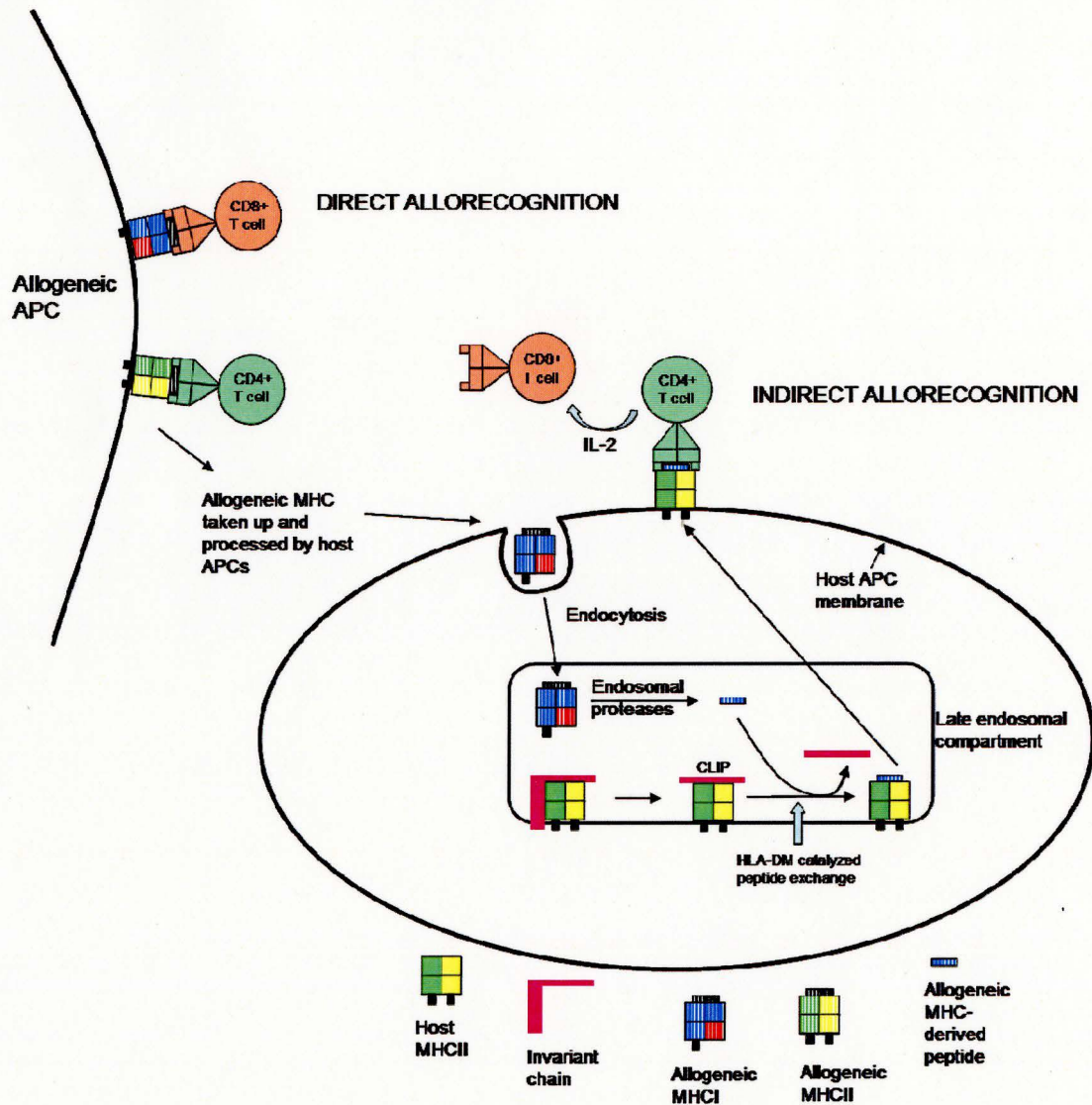


Figure 2.3: Pathways of allorecognition. Shows the direct and indirect pathways of allograft recognition. Figure adapted from reference (76).

2.5 Strategies to prevent and prolong allograft rejection

2.5.1 Immunosuppression

Of the three types of allograft rejection, clinical transplantation experiments have shown that only acute rejection can be prevented and even reversed significantly with immunosuppressive regimes. Hence, the primary focus of current immunosuppressive therapies is to interfere with the generation and function of CD4⁺ T cells and CD8⁺ CTLs, which mediate acute allograft rejection. These T cell responses can be altered in several ways: inhibiting T cell proliferation by blocking cytokine production, killing proliferating T cells, use of antibodies against T cell markers and interfering with T cell recruitment of inflammatory leukocytes (2).

Currently, the primary means of treating allograft rejection is the use of immunosuppressive drugs that inhibit or lyse T cells. A drug that can inhibit important cytokines involved in T cell proliferation would certainly reduce the T cell immune response against the allograft. For example, Cyclosporine is a commonly used immunosuppressive drug which primarily inhibits the transcription of IL-2. Cyclosporine is a fungal-derived cyclic peptide which specifically binds a ubiquitous cellular protein called cyclophilin. This complex then binds and inhibits the phosphorylation activity of calcineurin, which is a calcium/calmodulin-activated protein phosphatase. This inhibition

of calcineurin's phosphorylation (activation) of a transcription factor called nuclear factor of activated T cells (NFAT), results in the lack of transcription of important cytokines such as IL-2. In addition, cyclosporine induces the transcription of an immunosuppressive cytokine known as transforming growth factor (TGF- β). Together, cyclosporine inhibits the IL-2 dependant proliferation and differentiation of T cells, and creates an immunosuppressive environment with TGF- β expression. The clinical introduction of cyclosporine in 1983 has had an immense impact for allograft survival. For example, the five-year survival of cardiac allografts increased almost 25% over a period of 10 years since its introduction. Cyclosporin is sometimes combined with other drugs such as FK506 and rapamycin, which also interfere with IL-2-driven proliferation of T cells, to prolong allograft survival (2).

Another clinical approach to prevent allograft rejection is the use of metabolic toxins that are directed against T cells. These metabolic drugs kill proliferating mature T cells and some are also known to inhibit the maturation of lymphocyte precursors. For example, mycophenolate mofetil (MMF) is an immunosuppressive drug which specifically inhibits proliferation of lymphocytes. In its metabolized form (mycophenolic acid), MMF selectively inhibits the lymphocyte-specific isoform of an enzyme called inosine monophosphate dehydrogenase. Since this enzyme is required for the de novo synthesis of guanine nucleotides, blocking its function prevents lymphocytes from proliferating. In contrast, other drugs such as azathioprine and cyclophosphamide are toxic to lymphocyte precursors, hence preventing their maturation (2).

Antibodies can also be used in conjunction with the innate immune system to modulate the T cell response by targeting cell surface antigens. This approach relies on activating the complement system in the host circulation to selectively eliminate T cells. For example, OKT3 is a mouse monoclonal antibody directed against the human T cell marker CD3. The binding of OKT3 to CD3 on T cells activates the complement system, resulting in the direct lysis of these cells. OKT3 binding to T cells also enhances their selective opsonization by phagocytic cells of the immune system. In addition to these effects, some other antibody-based drugs such as anti-CD25, which binds the α -subunit of the IL-2 receptor, interfere with T cell activation. This further enhances the immunosuppressive effects of the drug. However, injecting mouse-made immunoglobulins (Ig) can evoke an immune response in human hosts. Hence, ‘humanized’ chimeric antibodies are used to reduce their immunogenicity (2).

Allograft rejection is commonly accompanied by inflammation. Hence, anti-inflammatory drugs such as corticosteroids are routinely used to treat allograft rejection. Corticosteroids, and their synthetic analogs, are natural hormones that inhibit the synthesis and secretion of cytokines such as tumor necrosis factor (TNF α) and IL-1 β . This effect reduces the allograft endothelial cell activation and the recruitment of inflammatory cells to the site. In addition, corticosteroids may also interfere with activated macrophage secretion of reactive oxygen species, nitric oxide and

prostaglandins. Other classes of inflammatory drugs use antibodies such as anti-ICAM-1 to block leukocyte adhesion and signaling.

2.5.1.1 Drug toxicity

Drug toxicity is a significant clinical limitation of most immunosuppressants used in allograft transplantation. Most classes of drugs are non-specific and alter the normal functioning of a large part of the immune system. In addition, the non-specific drug toxicity can limit the dose of the drug that can be administered to the patient (2). The systemic effects of these drugs can have profound consequences to the overall health of the host. Hence, it is clear that systemic immunosuppression is not a solution to the immune rejection problem in allogeneic cell-based tissue-engineered construct transplantations as well.

2.5.1.2 Susceptibility to virus-infections and virally-associated malignancies

As explained above, immunosuppressive therapies primarily interfere with the T cell response in the immune system. Since the immune system uses T cells for defense against pathogens such as bacteria and viruses, it is not surprising that immunosuppressive therapies also increase the susceptibility of the host to viral infection and other viral-associated diseases. For example, cytomegalovirus infections are commonly observed in immunosuppressed patients. In addition, allograft recipients are

known to exhibit viral-associated malignancies such as B cell lymphoma, which is a result of uncontrolled Epstein-Barr viral infection and proliferation. Squamous cell carcinomas of the skin, associated with human papillomavirus, are also commonly noted in immunosuppressed allograft recipients. It seems clear that the current immunosuppressive therapies are not ideal in preventing and prolonging allograft survival. New approaches to allograft transplantation are needed to address some of the shortfalls of systemic drug-induced immunosuppression (2).

2.5.2 Immunoisolation

Immunoisolation is based on the premise that a semi-permeable membrane can be used to protect allogeneic cells from the host immune system, without the need for immunosuppression. The semi-permeable membrane would allow adequate exchange of oxygen, nutrients, waste and therapeutic products, allowing the allogeneic cells to proliferate and differentiate within the capsule. However, it is now accepted that the host immune system can still reject allogeneic cells via antigen shedding and the indirect pathway of activating immune responses (77). Encapsulating cells in a membrane does not prevent the indirect pathway of activating the immune system, since protein fragments (8-22 amino acids) shed from allogeneic cells are too small to be stopped by currently used membranes of molecular weight cut-offs of around 100 kDa (77). Once the immune system is activated, either through the direct or the indirect pathway, activated immune cells such as macrophages and granulocytes are recruited to the region. The

activity of these cells induces a general remodeling of the region around the capsules, replacing normal tissue with fibrotic growth, which contributes to chronic nutrient deprivation and necrosis of encapsulated cells (78, 79). Overall, it seems inevitable that immunoisolation alone is not sufficient to prevent allograft rejection and can only be a temporary solution to allogeneic transplantation. Nevertheless, microencapsulation strategies have had limited success in small scale studies, but there are a number of significant challenges that need to be overcome, which makes this approach unlikely to be successful on its own (80).

2.5.3 Induce donor-specific tolerance or suppression

Donor-specific tolerance can be defined as the lack of host immune-mediated allograft injury in the absence of immunosuppression. It follows that this approach will avoid all of the problems associated with immunosuppression. Moreover, tolerance induction may also prevent chronic rejection, which is unaffected by currently used immunosuppressive therapies. Hence, many strategies for tolerance induction are currently being investigated.

2.5.3.1 Hematopoietic chimerism

Induction of hematopoietic chimerism is an immunological state that can induce tolerance specific to the donor. This process involves the transfer of donor hematopoietic cells along with the tissue or organ being transplanted. Persistence of this tolerant state

towards the allograft is maintained through the induction of central tolerance in the thymus. It is believed that APCs from both the donor and recipient cells migrate to the thymus, where they mediate negative selection of alloreactive T cells, thereby inducing central tolerance (81). However, this technique commonly requires the use of irradiation or cytotoxic drugs to ablate mature donor T cells, which have the potential to react with allogeneic host tissues and cause graft-versus-host disease. Thus, this technique is generally reserved for patients already undergoing myeloablative treatments (53).

2.5.3.2 Costimulatory blockade and the induction of regulatory T cells

As described in section 2.1.2.1.2, costimulation is the ‘second signal’ required for the activation of T cell responses. When T cells recognize MHC-peptide complex in the absence of costimulation, a state of ‘anergy’ is induced, where the T cells become incapable of responding to the antigen being presented. Tolerance induction through this method is known as peripheral tolerance, where alloreactive T cells become anergic and unresponsive to the allograft (82). Under such conditions, some T cells that become activated can develop into regulatory T cells (CD4⁺CD25⁺Foxp3⁺), which have the capacity to inhibit the activation and function of similar alloreactive T cells (81). Although many different costimulatory molecules can be targeted, many studies have focused on CD40L and B7 molecules. CD40L is expressed on activated CD4⁺ T cells and binds CD40, which is expressed on APCs, while B7 molecules are expressed on APCs and bind CTLA-4, which is expressed on activated CD4⁺ T cells. Studies by

Elwood *et al.* show that a combination treatment involving the blocking of CD40L with anti-CD40L antibodies, and B7 molecules with CTLA4Ig antibodies during the first week after transplantation can lead to indefinite acceptance of cardiac allografts in mice (83).

2.5.3.3 Generation of isogenic hESCs for use in tissue engineering

An isogenic hESC contains the same set of genes as the host. Hence, the use of isogenic hESC-derived cells is likely the best way to prevent immune rejection. A patient-specific isogenic hESC line can be created by a technique called somatic nuclear transfer (NT), where the nucleus from the patients' somatic cell is transferred to an enucleated oocyte and coaxed to develop to the blastocyst stage (84). An isogenic hESC line can be derived from this stage and subsequently expanded and differentiated for the specific application desired by the patient. Although, the isogenic hESC line contains the same set of genes as the patient, it also contains mitochondrial DNA (mtDNA) inherited from the oocyte used in the NT technique. Apart from the mtDNA mHAg, all other histocompatibility antigens are identical to the patient antigens. It is still not clear whether the mtDNA mHAg are sufficient to cause rejection in humans. However, a recent study by Lanza *et al.* demonstrated that tissues transplanted from cloned animals generated by the NT technique were not rejected in their nuclear donors with mismatched mtDNA (85). This study suggests that mtDNA likely does not contribute significantly to the immune response and that it is reasonable to expect similar results in humans (84).

Similarly, this somatic NT technique could also be applied to hESCs directly, instead of using oocytes. Although this would eliminate the need for human oocytes, this process has proven to be technically challenging. Nevertheless, the concept of hESC nuclear reprogramming via the somatic NT technique is likely feasible, since studies have already shown that the fusion of murine somatic cells with mESCs induces reprogramming such that the hybrid cell retains pluripotency (86). However, the somatic NT technique has a very low efficiency and is ethically very controversial, limiting the wide-spread use of this technique in tissue engineering applications (87).

Isogenic hESCs can also be obtained through a process known as parthenogenesis, where an unfertilized oocyte is activated to proliferate. Such parthenogenesis-derived hESCs would be fully compatible with the oocyte donor and hence will not be rejected. Recently, Cibelli *et al.*, successfully isolated parthenogenic pluripotent stem cells from primate oocytes, suggesting that this technique may also work in humans (88). Parthenogenesis-derived hESCs have the added benefit of being homozygous at most loci, making these cells more useable to a greater proportion of the population than heterozygous hESCs (87, 88). However, this technique relies on the availability of human oocytes, thereby limiting its use in tissue engineering (87).

2.5.3.4 Expression of immunomodulatory ligands

Several groups are currently exploring the expression of immunomodulatory ligands to create an environment that mimics immune privileged sites in the body (87). For example, the cells in the anterior part of the eye express Fas ligand (FasL) that induces apoptosis in Fas (CD95) expressing T cells, and immunosuppressive cytokines such as transforming growth factor β (TGF- β) (89). Many groups have shown that the expression of FasL in organs such as kidney, lung, liver were associated with a reduced immune response against allografts (89). Hence, hESC-derived cells may also benefit from FasL expression. However, such non-specific methods of achieving immunosuppression may be of limited value in tissue-engineered constructs that need to integrate into the body tissues, since FasL expressing cells are also known to induce the destruction of other normal tissues expressing Fas (87).

2.5.4 Reduce immunogenicity of allografts

2.5.4.1 Matching

Matching the donor to the recipient in order to minimize alloantigenic differences is a major strategy to reduce the chances of allograft rejection. The risk of hyperacute rejection can be significantly minimized by checking for preformed alloantibodies and ABO blood group antigen matching between the donor and the recipient. In addition, the donor and the recipient can be ‘tissue-typed’ to determine the identity of the HLA molecules (both MHC I and MHC II), so as to minimize the number of differences and

therefore reduce the chances of acute allograft rejection. Clinical allograft experiments have shown that HLA-A, HLA-B and HLA-DR are the most important loci (of all the MHC Class I and II loci) in determining the outcome of an implanted allograft. This indicates that the MHC molecules generated from these three loci are very important in the immune recognition of allografts and subsequent acute immune rejection. Hence, immunophenotyped hESC banks representing different tissues can be created such that MHC alleles can be matched with a majority of individuals in the population (87).

Although all of these approaches are feasible, most of them involve the creation of a genetically modified or matched hESC-derived cell line to suit the patient. It is expected that this will require a considerable amount of time since it involves differentiating and expanding the cells sufficiently to meet the desired application. Currently, a patient in immediate need of a tissue-engineered allograft is bound to suffer from the consequences of the delay in generating such a construct. Hence, many groups are beginning to explore the creation of a ‘universal’ donor cell-line that can be transplanted into any allogeneic patient. Since the host immune response is one of the greatest problems associated with the use of hESC-derived cells in tissue engineering, many immunomodulatory strategies are currently being investigated to create universal donor cells. It follows that MHC manipulation is likely necessary in the creation of any universal cell line.

2.5.4.2 MHC I modulation

It is clear that the immune rejection problem must be overcome for the successful transplantation of allogeneic tissue-engineered constructs. Many strategies are currently being explored with the ultimate goal of achieving selective suppression of the immune response against the allogeneic tissue-engineered construct, while maintaining its ability to respond to other environmental antigens (90).

One promising approach is to reduce the immunogenicity of the allograft, such that immunoactive molecules are selectively removed from the cell surface of the allogeneic cells. This ‘donor antigen modification’ at the protein level provides greater control over the mechanism through which the allograft evades the host immune system. This strategy involves identifying the dominant antigens recognized by the host immune system and masking, removing or down-regulating them. Moreover, this approach does not alter the normal functioning of the host immune system.

Early studies by Spits and co-workers on CTLs and their ability to kill target cells showed that MHCI molecules were involved in both adhesion and activation of CTLs (91). They used antibodies against MHCI, which effectively blocked CTLs from lysing target cells. MHCI molecules are highly polymorphic and essentially serve to distinguish between ‘self’ and ‘foreign.’ In essence, the Spits study demonstrated that these MHCI molecules were indeed one of the dominant antigens recognized by the immune system. Hence, many researchers have focused on MHCI modulation as a strategy to alter the immune response against allografts.

2.5.4.2.1 Approaches for modulating MHC1 expression

Antibodies have commonly been used to mask donor MHC1 antigens from a recipient's immune system. For example, Osorio and colleagues used F(ab') fragments against allogeneic MHC1 molecules on donor islet cells in a diabetic mouse model. Although this procedure prolonged allograft survival, it was insufficient to prevent immune rejection (92, 93).

Enzyme ablation of the MHC1 molecules is another approach to reduce the antigenicity of allografts. This technique uses specific enzymes such as papain to cleave conserved hydrophilic regions of the MHC1 molecules, without cleaving other cell surface molecules. This results in the reduction of cell surface MHC1 molecules, comparable to antibody masking (39).

An interesting strategy at the genetic level is to use RNA interference (RNAi) technology to down-regulate the expression of cell surface MHC1. RNAi works by inhibiting translation of MHC1 molecules through the selective degradation of mRNA transcripts of the MHC1 α chain. Although Ramanathan and coworkers showed that this RNAi technology can be used to reduce the possibility of rejection, this technology has not yet been established in the field of allograft transplantation (94).

Gene ablation is another genetic method to remove MHC I molecules from the cell surface. While RNAi works at the translational level, gene ablation works at the DNA level, with a permanent and complete deletion of the target gene. However, the biggest challenge with this technology is in the creation of ‘knock-outs,’ which is not feasible in most human transplant cases requiring a fully differentiated organ. Nevertheless, Markmann and coworkers applied this technology to create β 2-microglobulin-deficient mice and transplanted their pancreatic islet cells into allogeneic normal mice. They found that greater than 80% of allografts survived indefinitely (more than 100 days), and functioned normally (95). In contrast, other studies of tissues transplanted from MHC I and MHC II knockout mice into an allogeneic host still resulted in rejection through other T-cell independent mechanisms (involving B cells) (96). It is not yet clear whether MHC I knockout in hESCs will result in immune rejection in an allogeneic host (87).

2.6 Virology

Viruses are obligate intracellular parasites, which rely on cellular machinery to replicate and propagate themselves (2). Viruses have coexisted with their hosts for millions of years, during which they have learned to manipulate host defense mechanisms to propagate themselves. With such a great diversity of viruses found in nature, it is generally accepted that almost all living species can be a potential host for viruses. However, the hosts have also evolved mechanisms such as the innate and adaptive

immune system to control viral infections (97). In order for viruses to successfully propagate in human hosts, they must modulate the immune system such that they evade immune detection.

2.6.1 Viral strategies for MHC I modulation

Many human viruses modulate the MHC I presentation pathway, since this is the hosts' primary mechanism to detect and eliminate intracellular pathogens. As described in section 2.1.2.1, APCs employ MHC I molecules to prime antiviral CTL responses and these CTLs use MHC I molecules to recognize and destroy virally infected cells. Therefore, viral downregulation of the MHC I-peptide presentation pathway interferes with the adaptive immune system's ability to generate virus-specific CTLs. In addition, viral downregulation of MHC I in infected tissue cells also interferes with the ability of CTLs to recognize and kill these cells.

Peptide presentation by MHC I molecules on the cell surface involves multiple steps that occur within the cell, including transcription of HLA genes, translation, proteasome-mediated peptide processing, transport into the ER, MHC I-peptide complex assembly and transport to the cell surface. See Figure 2.2. Viruses are capable of interfering with virtually all of these steps in order to ensure their replication and persistence within the cell. However, there is a great diversity among viruses in their specific approach to modulating MHC I molecules. See Figure 2.4.

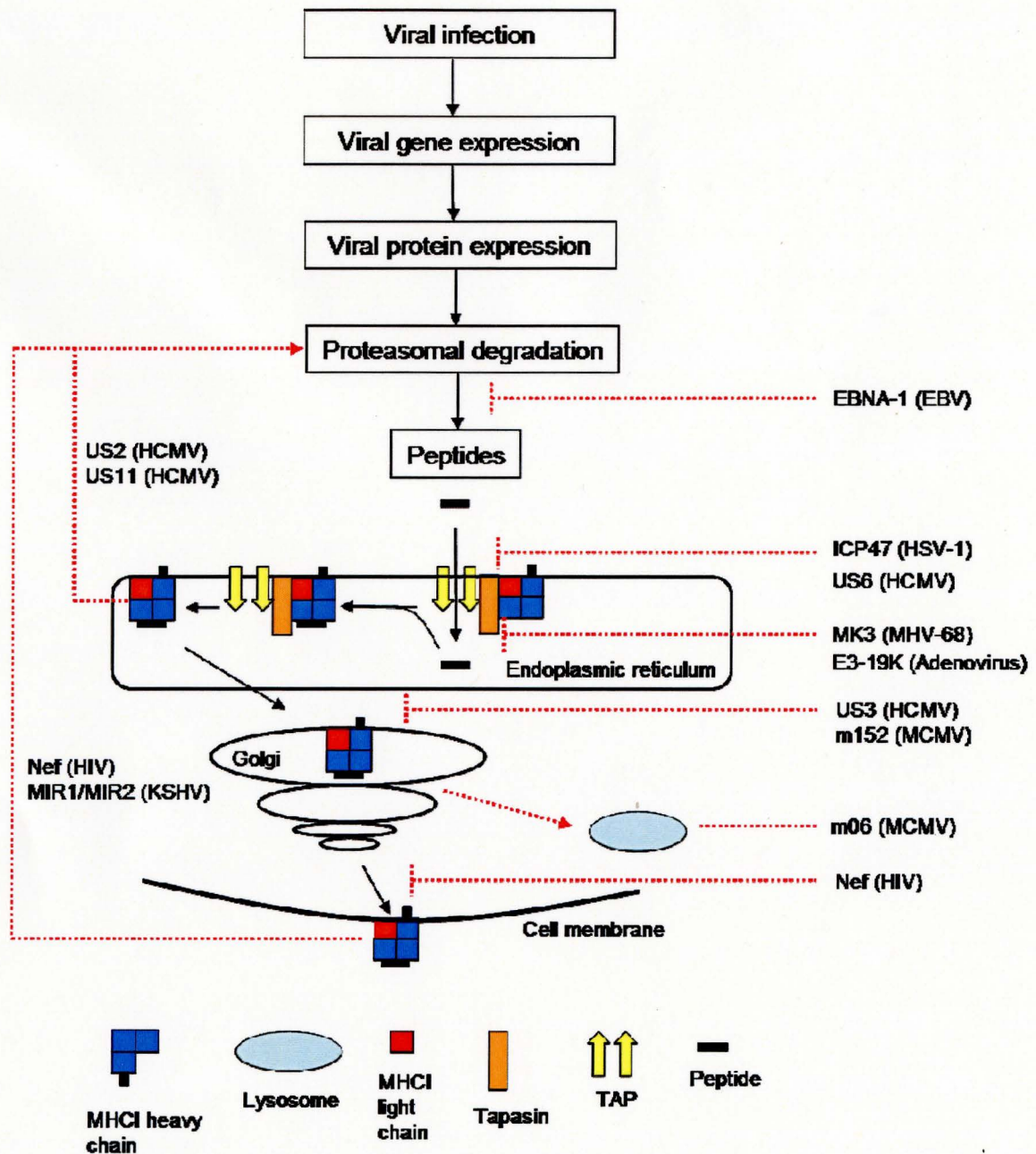


Figure 2.4: *Viral interference of MHC I-peptide processing and presentation.* EBV, Epstein-Barr virus; HSV1, Herpes simplex virus type 1; MCMV, murine cytomegalovirus; KSHV, Kaposi sarcoma herpesvirus; MHV-68, murine γ -2 herpesvirus 68. Figure adapted from reference (98).

The first step to the formation of MHC I molecules is the transcription of HLA genes. Many viruses interfere with the transcription of HLA genes to stop production of MHC I molecules. For example, HIV-1 encodes a protein called Tat, which functions to repress several gene promoters involved in MHC I and β -2 microglobulin transcription (98-100). The second important step in the construction of the MHC I complex is the proteasome-mediated processing of cytosolic proteins. Hence, viruses like EBV express proteins directed at inhibiting the proteasome. EBV encodes an essential viral nuclear protein called EBNA-1, which inhibits its own degradation by the 26S proteasome, thereby reducing EBNA-1 fragments from being presented on MHC I (101). The third step in MHC I synthesis is the transport of proteasome-processed cytosolic peptides into the ER. Viruses influence this pathway by inhibiting TAP, the heterodimeric complex of TAP-1 and TAP-2 involved in the peptide loading complex (PLC), which transports peptides from the cytosol into the ER. For example, HSV-1 expresses a protein called ICP47 that binds the cytosolic portion of TAP and blocks its function (102). In contrast, some viruses interfere with MHC I presentation by targeting the MHC I α chain for proteasome-mediated degradation while being a part of the PLC, thus blocking the formation of mature MHC I-peptide molecules. For example, HCMV encodes a protein called US2 that binds MHC I molecules during their glycosylation and promotes retrograde transport and subsequent degradation in the cytoplasm (103). Finally, some viruses downregulate mature MHC I-peptide molecules after they have been expressed on the cell surface. These viruses downregulate MHC I molecules by enhancing their endocytosis using the

ubiquitin-mediated protein degradation system. For example, Kaposi's sarcoma-associated herpesvirus (KSHV) expresses modulator of immune recognition 1 (MIR1) and MIR2 proteins that function at the cell membrane to downregulate MHC I molecules.

Although a global downregulation of MHC I molecules (HLA-A, B, C, E) may prevent CTLs from recognizing virus-infected cells, this strategy is also likely to increase the susceptibility of these cells to NK cell-mediated cytotoxicity (98). As described in section 2.1.1, HLA-C and HLA-E are ligands that bind the inhibitory NK receptor CD94/NKG2A. This prevents NK cells from lysing target cells. A decrease in the expression of these inhibitory ligands can therefore increase NK cell killing of target cells. In light of this problem, many viruses have evolved strategies to differentially downregulate MHC I molecules such that they evade both CTLs and NK cells. For example, KSHV expresses MIR2, a multifunctional protein that downregulates HLA-A and HLA-B strongly, while only weakly affecting HLA-C and HLA-E (22, 104). This strategy reduces CTL recognition of virally infected cells, while maintaining the negative-regulatory signal recognized by NK cells.

In addition, MIR2 also has the ability to strongly downregulate ICAM-1 (CD54), a molecule involved in forming immunological synapses, and B7-2 (CD86) costimulatory molecules involved in the activation of T cells (105). The functional consequence of this downregulation of ICAM-1 and CD86 is to reduce T cell responses, which is manifested through the decrease in CD4⁺ T cell activation, cytokine release and costimulatory

signals required for CTL generation. Moreover, ICAM-1 downregulation also interferes with the ability of NK cells to bind target cells, hence it inhibits NK cell-mediated killing.

It is becoming increasingly clear that MIR2 may also have a more direct role in manipulating NK cell activation. NK cell activation is regulated by a number of its cell surface activating receptors, including NKG2D and NKp80. NKG2D recognizes several ubiquitously expressed target cell surface ligands including MHC I-related chains (MIC) A and B. A recent study by Thomas *et al.*, showed that MIR2 downregulates MICA and MICB via its E3 ligase activity (106). Similarly, MIR2 was also found to downregulate activation-induced C-type lectin (AICL), which is a ligand found on the surface of myeloid cells that specifically binds NKp80. Like NKG2D, NKp80 is also a C-type lectin-like homodimeric receptor found on NK cells that activates NK cell-mediated cytotoxic functions (106, 107).

Furthermore, MIR2 is known to downregulate CD1d molecules in a manner similar to ICAM-1 and CD86 (108). CD1d proteins belong to the class of MHC Ib molecules and function to present self glycolipids to T cells. CD1d proteins are present only on a limited number of cell types including APCs such as B cells and dendritic cells. The functional consequence of MIR2 downregulation of CD1d proteins is the reduction of CD1d-restricted T cell activation (108). Since CD1d-restricted T cells are known to regulate NK cell activity, this strategy of CD1d modulation may also be involved in modulating the innate immune system (108, 109).

Many recent studies suggest that MIR2 manifests its actions primarily by ubiquitinating the lysine residues on the cytoplasmic chains of target cell surface molecules. This ubiquitination leads to the endocytosis and subsequent degradation of the molecule in the proteasome or lysosome. The pathway of this downregulation occurs after the target molecule has been expressed on the cell surface. Experiments on MHCI downregulation suggest that target proteins are synthesized and assembled normally in the ER and also reach the cell surface normally. However, these target proteins are preferentially endocytosed due to the action of MIR2 at the cell surface (110, 111). It is interesting to note that the majority of MIR2 expressed in a cell are localized in the ER, where they perform no known function. It is estimated that <5% of the intracellular pool of MIR2s reach the plasma membrane, where they function to ubiquitinate target proteins (112). There is some evidence that over-expression of MIR2 may exaggerate the downregulation of target cell surface molecules (113). For example, MIR2 over-expression induces a 20-30 fold reduction in the cell surface expression of MHCI (111).

In terms of structure, MIR2 is a 29 kDa protein (computed MW) composed of a NH₂-terminal zinc finger of the plant homeodomain (PHD) family, two transmembrane (TM.) domains, and a conserved region (CR) at the C-terminus (112). See Figure 2.5. Studies by Sanchez *et al.*, have shown that the TM domains play an important role in determining the specificity of the target protein. Once the target protein has been selected, the PHD and CR domains function to recruit cellular machinery, which regulate the internalization

of target molecules. Specifically, the PHD domain belongs to the membrane-associated RING-CH-containing (MARCH) family of E3 ligases (114). These E3 ubiquitin ligases are enzymes that add ubiquitin to lysine residues within a protein, which targets the protein for proteasome or lysosome-dependent degradation, as part of the cellular recycling machinery for receptors and misfolded proteins (115). The specific function of the CR domain still remains elusive. However, it is known that deletion of the PHD or CR domains inactivates the function of MIR2 (112).

It is interesting to note that MIR2 may have a mammalian origin. Large DNA viruses like herpesviruses are known to encode viral homologues of mammalian proteins. Since KSHV is designated as a herpesvirus (HHV-8), it is possible that it may have obtained MIR2 from a mammalian homologue. It was recently shown that c-MIR/MARCH VIII is indeed a mammalian homologue of MIR2 (116). Although c-MIR only has 18% amino acid identity to MIR2, they share key components in their secondary structures, including the PHD domain that catalyzes the E3 ubiquitin-mediated degradation of target proteins (117). However, c-MIR seems to have a more limited functionality in comparison to MIR2. Currently, c-MIR is only known to downregulate B7-2, but it may also downregulate MHCII molecules in B cells (118, 119). Although the physiological role of c-MIR is not yet known, it is possible that it is also involved in modulating T cell responses. This is supported by the observation that c-MIR is expressed abundantly in the thymus, which is an organ where T cell maturation occurs (120).

It is clear that MIR2 is multifunctional protein that functions to simultaneously modulate both the adaptive and the innate immune system. An application of MIR2 in cell-based tissue-engineered constructs may provide another strategy to modulate the immune response and increase the chances of allogeneic transplant success.

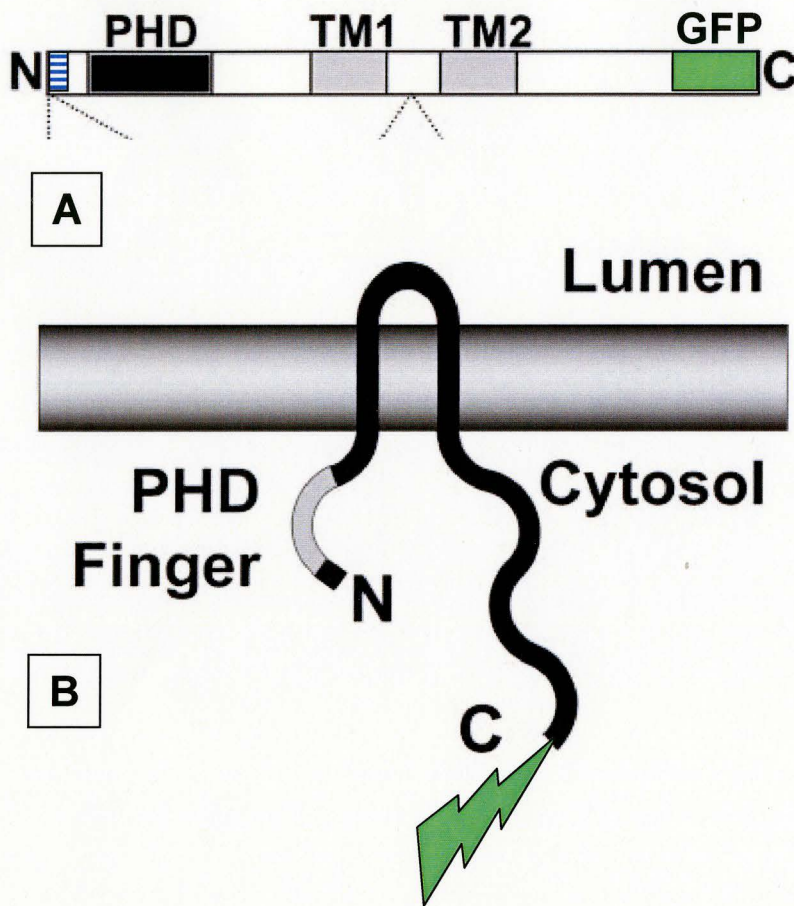


Figure 2.5: *Structural organization of MIR2-GFP*. A) shows the functional organization of the MIR2-GFP protein (56 kDa), with a FLAG sequence (blue striped box) at the N-terminus and a GFP sequence (solid green box) at the C-terminus. B) represents the distribution of the MIR2 domains with respect to the cell membrane. Figure adapted from reference (112).

2.7 Retroviral gene delivery

Retroviruses are small (80 nm-120 nm) enveloped RNA viruses that can be used to deliver genes up to 10 kb in length. Retroviruses have three major features that make them suitable as vectors: First, their life cycle includes an integration step, where the viral RNA is transcribed into dsDNA and is stably incorporated into the transduced cell chromosome (121). This implies that transgene expression is long-term compared to other non-integrating methods of gene delivery. Second, a retroviral cell-type specific promoter can be designed to transcribe high levels of the transgene (121). Third, the retroviral genome can accommodate changes and therefore it can be easily reconfigured to produce virions tailored structurally and genetically to suit the desired application (121). However, retroviral integration can also lead to deleterious effects on the cell, including transformation into cancer cells. Although these ‘insertional effects’ are possible, many current systems retroviral systems are being developed with a suicide gene, which is activated in the event of such a deleterious transformation. Hence, a retroviral gene delivery vector combines high transduction efficiency with stable (long-term) gene expression in a platform that provides a convenient and reliable method of delivering various genes to many different cell types. These characteristics make retrovirus ideal for use in cell-based tissue-engineered constructs.

3. RATIONALE

Immune rejection of allogeneic cells is a major limitation in the field of tissue engineering, where cells and biomaterials typically form the implanted construct. Specifically, acute rejection mediated primarily through the action of CTLs adversely affects the success of allogeneic transplantations. As described earlier, systemic immunosuppressive drugs have many undesirable side effects, limiting their use. Hence, there is a need for selectively suppressing the alloimmune response, while maintaining normal immune system function. It has previously been proposed that donor antigen modification is a viable means of selectively modulating the immune response against allogeneic cells, and potentially provides an alternative to systemic immunosuppression. Although there are many ways to modify donor antigens, we propose that using viral immunomodulatory proteins to differentially downregulate donor cell surface antigens will successfully mimic viral immune evasion of the immune response. Furthermore, we propose an *ex vivo* strategy using a retroviral vector to deliver the viral immunomodulatory gene into allogeneic donor cells to mediate antigen modification. We believe that this strategy might also provide an alternative means for generating ‘universal’ donor cells, capable of surviving in any allogeneic host. Therefore, we tested the feasibility of this approach in successfully evading the alloimmune response *in vitro*.

3.1 Hypothesis

Viral mechanisms to modulate immunoactive cell surface molecules, such as MHC Class I, ICAM-1 and CD86, can be selectively applied to allogeneic cells in tissue engineering to simultaneously minimize cytotoxicity by CD8⁺ Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells of the immune system.

3.2 Objectives

- 1) Construct the MIR2-GFP retroviral plasmid and produce replication-defective MMLV retrovirus.
- 2) Show that a human model cell line can be stably transduced with a retroviral vector to express the immunomodulatory MIR2-GFP chimeric protein.
- 3) Show that MIR2-GFP expression differentially downregulates cell surface proteins such that, HLA-ABC, ICAM-1 and CD86 are downregulated, while the expression of HLA-E remains unaltered.
- 4) Develop a novel flow cytometry-based assay to measure CTL and NK-specific cytotoxicity against allogeneic cells.
- 5) Show that MIR2-GFP expression results in immune evasion of allogeneic cells as measured by reduced cytotoxicity by CTLs and NK cells.

4. MATERIALS AND METHODS

4.1 Cell lines and cell culture

U937

This cell line is non-adherent (cells in suspension) and relatively large compared to NK cells and CTLs, making them easy to distinguish (gating) and ideal for use with flow cytometry. In addition, these cells do not need to be treated with trypsin for use with flow cytometry, which can potentially cleave cell surface proteins. U937 cells are also known to express high levels of MHC Class I, ICAM-1 and costimulatory molecules such as B7-2 (CD86). The viral protein used in this project, MIR2, was expected to downregulate these molecules, hence this cell line allowed us to quantify this effect. This cell line was generously provided by Dr. Karen Mossman's lab.

U937 cells were cultured in monocyte culture medium (see section 4.1.3) in 0.2 µm vented tissue culture flasks at 37° C (humidified, 5% CO₂). U937-MIR2-GFP and U937-LacZ cells were cultured in G418 selection medium. These suspension cells were subcultured every 3-4 days.

293T

293T is a human kidney-derived cell line. This immortalized, adherent cell line has been stably transduced with the temperature sensitive SV40 T-antigen gene and is also known

to be highly transfectable. This cell line was generously provided by Dr. Karen Mossman's lab.

293T cells were cultured in 100 mm tissue culture dishes at 37° C (humidified, 5% CO₂) and subcultured every 4 days using '293T/U2OS cell culture medium.' See section 4.1.3. This cell line was used as the packaging cell line, which was cotransfected with three plasmids (see section 4.3.1) to generate retroviral vector (pseudotyped-MMLV) carrying the MIR2-GFP or the LacZ gene.

U2OS

U2OS is a human osteosarcoma cell line. This immortalized, adherent cell line has an epithelial-like morphology. This cell line was generously provided by Dr. Karen Mossman's lab.

U2OS cells were cultured in 100 mm tissue culture dishes at 37° C (humidified, 5% CO₂) and subcultured every 4 days using '293T/U2OS cell culture medium.' See section 4.1.3. This cell line is known to be transducible by the pseudo-typed retroviral vector used in this study. Hence, it was used as a control cell line to compare the efficiency of transduction to the U937 test cell line.

HCEC

HCEC is a human corneal epithelial cell line. This immortalized, adherent cell line was used in this study as a viral transduction control to compare the expression of MIR2 with non-adherent U937 cells. This cell line was generously provided by Dr. Heather Sheardown's lab.

HCECs were cultured in 100 mm tissue culture dishes at 37° C (humidified, 5% CO₂) and subcultured every 4 days using 'HCEC cell culture medium.' See section 4.1.3.

4.1.1 Freezing cells

All cell lines were frozen with 25% FBS (PAA Labs Inc, Cat No. A15-701), 7.5% DMSO (Sigma, Cat No. D2650) in RMPI 1640 (Invitrogen, Cat No. 11875-093) media using cryovials placed in a Mr. Freeze cell-freezing container at -80° C.

4.1.2 Thawing cells

Cells were thawed rapidly in a 37° C water bath and transferred into 10 ml pre-warmed culture medium in a tissue culture. The culture medium was replaced after 24 h of incubation at 37° C to remove the DMSO present.

4.1.3 Media preparation

Several types of media were prepared according to the cell type. In each case, media components were first mixed together and then filter sterilized with a 0.22 μm filter to remove particulate debris and other biological contaminants.

293T/U2OS cell culture medium

DMEM (Invitrogen, Cat No. 11965-092) supplemented with 10% (v/v) heat-inactivated FBS (PAA Labs Inc, Cat No. A15-701), 1% Penn/Strep (Invitrogen, Cat No. 15140-122), 0.1% Gentamycin (Invitrogen, Cat No. 15710-064), 0.1% Fungizone (Invitrogen, Cat No. 15290-018).

Monocyte cell culture medium

RPMI 1640 (Invitrogen, Cat No. 11875-093), 10% heat-inactivated FBS (PAA Labs Inc, Cat No. A15-701), 1% Penn/Strep, 0.1% Gentamycin, 0.1% Fungizone.

HCEC cell culture medium

Keratinocyte-Serum Free Medium (KSFM) (Gibco 17005-042), 0.05 mg/ml bovine pituitary extract (BPE) (Gibco 17005-042), 5 ng/ml epidermal growth factor (EGF) (Gibco 17005-042), 1% Penn/Strep, 0.1% Gentamycin, 0.1% Fungizone.

Lymphocyte media

Monocyte culture medium, with 50 μ M 2-mercaptoethanol (Invitrogen, Cat No. 21985-023), 1 mM sodium pyruvate (Invitrogen, Cat No. 11360-070) and 0.1 mM non-essential amino acids (Invitrogen, Cat No. 11140-050).

G418 selection media

Monocyte culture medium, with G418 Sulfate (1 mg/ml during initial selection or 250 μ g/ml for maintenance; Invitrogen, Cat No. 11811-023).

4.2 Molecular biology

The molecular biology component of this project involved the generation and amplification of plasmids used in making the VSV-G pseudotyped retroviral vectors. In order to generate retrovirus carrying the MIR2-GFP or the LacZ gene, several plasmids had to be acquired, amplified and/or modified. Stratagene's Viraport™ retroviral gene expression system was employed in making the retrovirus. An outline of the procedure used is described in section 4.3.1.

4.2.1 Primer design

In order to sequence the MIR2-GFP gene from the pBMN-MIR2-GFP vector, primers were designed and checked for stability and the absence of secondary structures using a software package from NET PREMIER Biosoft International, available at: www.PremierBiosoft.com.

Forward Primer (MIR2-up): 5'-GGCTGCCGACCCCGGGGGTGG-3' with binding site 2260-2280 in the pBMN-MIR2-GFP vector. $T_m = 70^\circ \text{C}$

Reverse Primer (MIR2-down): 5'-GCCAGGTTTCCGGGCCCTCAC-3' with binding site 3873-3893 in the pBMN-MIR2-GFP vector. $T_m = 64^\circ \text{C}$

The following formula was used to calculate the melting temperature (T_m) of the primers:

$$T_m = 4*(G+C) + 2*(A+T)$$

4.2.2 Reagents

Luria-Bertani (LB) medium

Prepared according to Molecular Cloning protocol given in Appendix 2, section A2.2. LB agar using Bacto Agar for plates was prepared according to section A2.5. All reagents were acquired from Dr. Carlos Filipe's lab.

4.3 Retroviral vectors and transduction

4.3.1 Replication-defective viral vector production

The MMLV vector, pseudotyped with the VSV-G envelope protein, was generated with a modified version of Stratagene's pVPack Vectors™ protocol. Briefly, a packaging cell line, 293T cells, was acquired from Dr. Karen Mossman's lab and cotransfected with pVPack-GP, pVPack-VSV-G and pFB-MIR2-GFP-Neo. After 48 h of incubation, the supernatant containing the virus was collected and used to transduce the U937 model cell line.

Day 1: 293T packaging cell line preparation

Seeded cells at about 5×10^6 cells per 100 mm tissue culture dish in growth medium (see '293T/U2OS cell culture medium' in section 4.1.3) and incubated at 37° C for 24 h.

Day 2: Cotransfecting packaging cell line

Cotransfection was done using a modified ExGen500 protocol (Fermentas, Cat No. R0511). In a 1.5 ml microcentrifuge tube, 18 µg (6 µg of each of the three vectors) of DNA were mixed in a total volume of 400 µl, containing 150 mM NaCl. The solution was vortexed and centrifuged (minicentrifuge) at 6000 rpm for 30 seconds, after which

59.23 μl (6 equivalents of DNA, see ExGen500 protocol) of ExGen500 reagent was added and vortexed immediately for 10 seconds. After a 10 minute incubation period at RT, the ExGen500/DNA mixture was evenly dispersed onto the 293T cells (approx 80% confluent) containing 4 ml media. Even dispersal of the ExGen500/DNA precipitate was ensured by rocking the dish a few times. The cells were then incubated at 37° C for 48 h.

Day 4: Harvesting the viral supernatant

The virus-containing supernatant was collected and filtered (0.22 μm syringe filter) to remove cell debris and contaminants. The viral supernatant was then snap-frozen in cryovials using liquid nitrogen. A similar procedure with the alternate transgene vector pFB-LacZ-neo was used to generate control virus carrying the β -galactosidase gene.

4.3.2 Transducing U937 cells

U937 cells were plated two different concentrations ($0.5 \times 10^6/250 \mu\text{l}$ and $0.25 \times 10^6/250 \mu\text{l}$) in a 24-well tissue culture plate. Viral supernatant stock was thawed rapidly using a 37° C water bath and DEAE-dextran (Sigma, Cat No. D9885; 0.22 μm filter sterilized) was added to a final concentration of 10 $\mu\text{g/ml}$. DEAE-dextran was added to increase transduction efficiency by neutralizing the surface charge of the virus. 250 μl of the viral supernatant stock with DEAE-dextran was added to each of the wells and mixed. After incubating the cells at 37° C for 3 h, 1.5 ml of U937 media was added to each well and

further incubated for 16-18 h. The cells were then cultured in 0.5 mg/ml G418 antibiotic media to select and amplify transduced cells. After a week of incubation, the selection media was replaced with fresh media containing 1 mg/ml G418. After three weeks of selection, a limiting dilution procedure was setup to select for clones. See section 4.3.3.

4.3.3 Isolation of MIR2-GFP clones by limiting dilution

The heterologous U937-MIR2-GFP culture was diluted to 60 cells/ml using G418-supplemented (1 mg/ml) media in a sterile tray. Performed a 2x serial dilution (transfer 100 μ l using a multi-channel pipetter) from 6 cells/well to 0.2 cells/well (6, 3, 1.5, 0.8, 0.4, 0.2 cells/well) in a 96-well round-bottom culture plate. All 48 wells (8 wells for each cell concentration) had 100 μ l of G418 antibiotic media. Incubated cells at 37° C (humidified, 5% CO₂) for about 35 days, replacing G418-media every 10 days. After 35 days, 7 wells were identified (cell-pellet visible) among the 0.4-cell and 0.2-cell wells and expanded in separate tissue culture flasks with G418 media. MIR2-GFP expression level was assessed for each clone using flow cytometry (BC FC500) and the highest expressing clone (labeled '0.4A/clone 7') was cultured for use in all subsequent experiments. The remaining MIR2-GFP clones were frozen and stored at -80° C.

4.3.4 Microscopy

All transmission light images were collected using an Axiovert 200 (Carl Zeiss MicroImaging, Inc., Germany) inverted fluorescence microscope. Images were acquired using AxioVision 3.1 (Carl Zeiss Vision, USA) software and processed with Adobe Photoshop. Fluorescence images were collected using the aforementioned setup as well as with a Leica DM IRE2 inverted fluorescence microscope system (LEICA) in conjunction with Openlab imaging software (IMPROVISION).

4.3.5 β -Galactosidase assay

This assay was used to determine the transduction efficiency of the retrovirus by quantifying the number of cells that were transduced compared to a control cell line (U2OS, osteosarcoma). Cells transduced by the retrovirus encoding the LacZ gene should express the β -galactosidase enzyme, which catalyzes the hydrolysis of β -galactosides. In transduced cells, β -galactosidase cleaves X-Gal, a β -galactoside analog, to produce a blue stain. The transduction efficiency is determined by counting the stained (blue) and unstained cells using light microscopy and calculating the percentage of stained cells in the total population. Approximately 1×10^6 U937 cells were fixed in 1 ml formalin for 5 minutes at RT. Cells were then washed with PBS and resuspended in 0.5 ml staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 10% DMSO, buffer stabilizer, PBS) containing X-Gal (1 mg/ml). After incubating the cells at 37° C for 12 h, the cells were washed with PBS and resuspended in PBS. All reagents were acquired from Dr. Karen Mossman's lab.

4.4 Flow cytometry

Flow cytometry is a fluorescence-based method to analyze a heterogeneous population of particles/cells. A Beckman Coulter FC 500 system (Beckman Coulter Canada Inc., Mississauga, ON, Canada) was used to analyze the cells with the CXP software provided.

4.4.1 Counting cells

Cells were counted using a hemocytometer after labelling with Trypan blue (Invitrogen, Cat No. 15250-061) in a 1:1 or 1:10 dilution (Trypan blue volume: volume of solution containing cells), depending on the experiment. After a 5 minutes incubation step at RT, 10 μ l of the trypan-labelled cells were pipetted into the hemocytometer. A Wilovert 30 (Hund Wetzlar, Germany) light microscope was used for enumerating cells.

Trypan blue staining was used to calculate viability from the cell count. Dead/dying cells with a compromised cell membrane stain positive (blue) for trypan blue. However, living cells have an intact cell membrane, hence remain translucent.

Hemocytometer cell count

Total number of cells in a given volume = (number of cells in 4 squares/4) x (10000) x (dilution factor) x (total volume in milliliters)

4.4.2 Antibody labelling of cells for flow cytometry

An antibody staining technique was developed to minimize non-specific binding to cell surface receptors. This staining technique was tested for all classes of antibodies used in this project and found to be just as reliable as using isotype controls. Thus, isotype controls were not used for most experiments in this project.

Approximately 5×10^5 cells to be stained were pelleted in a 1.5 ml microcentrifuge tube. The supernatant was aspirated and the cells resuspended in 70 μ l of heat-inactivated horse serum (Sigma, Cat No. H0146) to block Fc receptors on the cell surface. After incubation for 10 minutes at RT, an appropriate amount of antibody (as determined by titration) was added, such that the final volume was 100 μ l. The cell pellet was resuspended and incubated on ice for 30 minutes. After incubation, the cells were washed by adding 1 ml 0.5% BSA in PBS (Ca^{2+} and Mg^{2+} free) (Invitrogen, Cat No. 14190-144) and centrifuged (minicentrifuge) for 1 min at 4000 rpm. The supernatant was aspirated and the wash procedure repeated once more. The cells were finally resuspended in 500 μ l of PBS (Ca^{2+} and Mg^{2+} free) and transferred into flow tubes. When staining lymphocytes, flow tubes with a 40 μ m cell strainer were used to remove any cell clumps.

When measuring antibody stained cells using the flow cytometer, the live cell population was identified and gated to further minimize a false positive signal arising from non-specific binding of fluorescent antibodies to dead cells.

4.4.2.1 *Antibodies and fluorescent dyes*

Anti-HLA-ABC-PE	Beckman Coulter, Cat No. IM0107
Anti-HLA-E (MEM-08)	Abcam, Cat No. ab11821
Anti-mouse IgG1-APC (2° antibody for HLA-E)	BD, Cat No. 550874
Anti-CD8-PC7	Beckman Coulter, Cat No. 6807102
Anti-CD11a-PE	Beckman Coulter, Cat No. IM1433U
Anti-CD54-PE	Beckman Coulter, Cat No. IM1239
Anti-CD56-PE	Beckman Coulter, Cat No. IM2073U
7-AAD Viability dye	Beckman Coulter, Cat No. IM3422
Calcein-AM	Invitrogen, Cat No. C1430

4.5 Immune Assays

4.5.1 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by exposing RBCs to hypotonic (relative to the RBCs) ammonium-chloride (ACK) solution (See section 4.5.1.1). This procedure preferentially lyses the RBCs, leaving mostly undamaged PBMCs. It is important to note that there is a possibility of leukocyte activation using this procedure. Please refer to section 8 for further discussion. This study was approved by the Research Ethics Board at McMaster University and blood collection was done by a licensed phlebotomist.

According to the experimental needs, 20-60 ml of whole blood was collected from human male or female donors with informed consent. Briefly, 10 ml of whole blood (with acid-citrate-dextrose (ACD) anticoagulant) was collected in each 45 ml centrifuge tube. 30 ml of ACK lysis solution was added and the solution mixed by inverting 2-3 times. The solution was incubated at RT for 5 minutes and then centrifuged (BC) at 1500 rpm for 5 minutes. A pasteur glass pipette was then used to remove the supernatant, leaving about 500 μ l of solution over the cell pellet. This was done to minimize the risk of aspirating the desired cells. A second lysis step was performed by adding another 10 ml of ACK lysis solution and resuspending the cell pellet by pipetting gently. After incubating for another 5 minutes at RT, 30 ml of PBS (1x) was added and the solution centrifuged at

1500 rpm for 5 minutes. The supernatant was aspirated as described above. 3.5 ml of RPMI 1640 was added and the cells were resuspended by pipetting gently. Cell were counted using Trypan blue (10 μ l solution + 90 μ l Trypan blue + 900 μ l PBS) staining for 5 minutes at RT and enumerated using a hemocytometer.

4.5.1.1 Ammonium-Chloride (ACK) red blood cell (RBC) lysis solution preparation

8.29 g	NH ₄ Cl (0.15 M)
1.00 g	KHCO ₃ (10.0 mM)
37.2 mg	Na ₂ EDTA (0.1 mM)

Added 800 ml distilled H₂O to the reagents listed above and adjusted pH to 7.2-7.4 with 1 N HCl. After raising the final volume to 1 L, the solution was filter sterilized through a 0.22 μ m filter and stored at RT. Procedure adapted from Dr. Jonathan Bramson's lab protocols.

4.5.2 Positively selecting NK cells from fresh PBMCs

PBMCs were used as a source of NK cells for the NK assay. Briefly, magnetic antibody-coated beads (StemCell Technologies) were used to positively select CD56⁺ cells from PBMCs. In this experiment, CD56⁺ cells are referred to as NK cells, since the vast

majority of CD56⁺ cells are indeed NK cells. Please refer to section 5.3.2.2 for further discussion.

Adjusted PBMCs to 100×10^6 cells/ml in the recommended buffer solution (2% FBS and 1 mM EDTA in PBS, (Ca²⁺ and Mg²⁺ free). Followed protocol as described in the Human CD56 Selection Kit (EasySep 18055, StemCell Technologies), however, performed only two rounds of magnetic selection to improve cell recovery. After the last separation step, NK cells were resuspended in 4 ml lymphocyte media. A portion of the cells were stained with fluorescent anti-CD56 antibody (BC) to quantify the proportion of CD56⁺ cells in the positively selected cell population. See Figure 5.18.

4.5.3 Activating NK cells

NK cells that encounter cellular targets *in vivo* are exposed to certain soluble factors, which activate them to perform their cytolytic function. NK cells isolated from fresh PBMCs *in vitro* are not exposed to such soluble factors in the NK assay. Hence, isolated NK cells require *in vitro* activation prior to using them in the NK assay.

In order to activate the NK cells, 10 µg/ml Poly I:C (Sigma, Cat No. P1038) and 50 U/ml rh IL-2 (Invitrogen, Cat No. PHC0027) was added and the cells were incubated at 37° C (humidified, 5% CO₂) in a 6-well tissue culture plate for 48 h.

4.5.4 Negatively selecting CD8⁺ T cells from fresh PBMCs

PBMCs were used as a source of CD8⁺ T cells for generating CTLs, which were used in the CTL assay. This negative selection procedure was used to minimize activation of CD8⁺ T cells prior to their directed differentiation into CTLs. In this thesis, all CD8⁺ cells isolated through negative selection are referred to as ‘CD8⁺ T cells,’ since this population comprises the vast majority of CD8⁺ cells in PBMCs.

Adjusted PBMCs to 50×10^6 cells/ml in the recommended buffer solution (2% FBS and 1mM EDTA in PBS, Ca²⁺ and Mg²⁺ free). Followed protocol as described in the Human CD8⁺ T Cell Enrichment Kit (EasySep 19053, StemCell Technologies), however, performed a second round of magnetic selection on the enriched CD8⁺ T cells to improve cell recovery. The negatively selected cell population was then resuspended in 5ml lymphocyte media (supplemented RPMI 1640). A portion of the cells were stained with fluorescent anti-CD8 antibody to quantify the proportion of CD8⁺ T cells in the negatively selected cell population. See section 5.3.2.1.1.

4.5.5 CTL generation

Only a small proportion of naïve CD8⁺ T cells respond to allogeneic cells. In order to expand this ‘responder’ population, negatively isolated CD8⁺ T cells were stimulated with allogeneic ‘stimulator’ cells (U937-LacZ) *in vitro*. It was determined through test

experiments that 2:1 was the optimal Responder (CD8⁺ cells): Stimulator (U937-LacZ) cell ratio.

Prepared a sufficient number of exponentially growing U937-LacZ cells in RPMI media. In order to use these U937-LacZ stimulator cells, they were first treated with mitomycin C to prevent their cell division, so that they would proliferate and overwhelm the responder cells during the stimulation period. Pilot experiments (data not shown) showed that Mitomycin C treatment was more suitable than γ -radiation as a way to suppress the proliferative capacity of the stimulator cells, hence it was added to a final concentration of 25 $\mu\text{g/ml}$ and the solution mixed gently with a pipet. After incubating the solution at 37° C for 20 minutes, 12 ml of fresh RPMI was added and the resulting solution centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated with a pasteur glass pipette and the wash step repeated two more times in excess 1x PBS (Ca²⁺ and Mg²⁺ free). The mitomycin C treated cells were then resuspended in 3 ml lymphocyte media. These cells were enumerated using a hemocytometer. The responder and stimulator cells were then cocultured at the appropriate ratio (4x10⁶ responder: 2x10⁶ stimulator cells) in a total volume of 10 ml lymphocyte media supplemented with 10 U/ml rh IL-2 in a 75 cm² tissue-culture flask positioned (upright and angled) to maximize the contact between the cells. This coculture was incubated at 37° C (humidified, 5% CO₂) for 96 h to allow time for the responder population (CD8⁺ T cells) to proliferate and differentiate into CTLs. A portion of these cells were stained with fluorescent anti-CD8 antibody to

quantify the proportion of CTLs (which also express CD8) in the coculture population.

See section 5.3.2.1.2.

4.5.6 CTL Assay

The cocultured cells were first enumerated with the hemocytometer and stained with fluorescent anti-CD8 antibody (BC) to quantify the proportion of CTLs, which also express CD8 molecules. Appropriate Effector (CTLs): Target (U937-MIR2-GFP or U937-LacZ) ratios were determined (2:1 and 10:1) and the number of cells required was calculated. The target cell number was held constant at 50,000 cells, while the effector cell number was varied according to predetermined ratios. In setting up the CTL assay, a sufficient number of target cells (as determined by the ratio) were centrifuged at 1000 rpm for 5 minutes and resuspended in media and labelled with 100 nM Calcein for 15-20 minutes at RT. The cells were centrifuged and the supernatant aspirated. This wash step was repeated two more times with PBS (Ca^{2+} and Mg^{2+} free) and the cells were finally resuspended in lymphocyte media at $0.5 \times 10^6/\text{ml}$. The effector cells and target cells were adjusted such that a 100 μl of each would achieve the predetermined ratios during coculture, which was performed in 96-well round bottom tissue culture plates. Control wells had 100 μl of the target cells and 100 μl of media, so that the total volume in each well was 200 μl . Each E:T ratio had three repeats for each of the target cell lines. After incubating the coculture for 4 h at 37° C (humidified, 5% CO_2), 20 μl of 7-AAD was

added to each well. This was followed by another incubation of 20 minutes at RT (protected from light). The contents of each well were added to 400 μ l of PBS (Ca^{2+} and Mg^{2+} free) in separate flow cytometry tubes for analysis.

4.5.7 NK Assay

Activated NK cells were first enumerated with the hemocytometer and stained with fluorescent anti-CD56 antibody (BC) to quantify the proportion of NK cells. Appropriate Effector (NK cells): Target (U937-MIR2-GFP or U937-LacZ) ratios were determined (0.1:1, 0.25:1 and 1:1) and the number of cells required was calculated. The NK assay was setup in a manner similar to the CTL assay. See section 4.5.6.

5. RESULTS AND DISCUSSION

The overall objective of this project was to show that viral immune evasion strategies can be successfully applied to human tissues such that they would be less prone to NK and CTL-mediated acute rejection in the context of allogeneic transplantation.

In summary, we first showed that a model human cell line can be stably transduced with a viral immunomodulatory protein, MIR2-GFP, using a retroviral vector. Second, we showed that this immunomodulatory protein differentially downregulates cell surface proteins such that, HLA-ABC, ICAM-1 and CD86 are downregulated, while the expression of HLA-E remains unaltered. Third, we showed that this differential downregulation has the functional effect of significantly reducing CTL-mediated cytotoxicity, without altering the NK-mediated cytotoxicity.

5.1 Retroviral transduction of human cells

5.1.1 Construction of MIR2-GFP and LacZ retroviral plasmids

In order to make the viral vector carrying the MIR2 gene, Stratagene's Viraport™ retroviral gene expression system was employed. This system contained three plasmids (pVPack-GP, pVPack-VSV-G and pFB-Neo), designed to make the MMLV vector

carrying the MIR2 gene on the pFB-Neo plasmid. An alternate transgene vector (pFB-LacZ-neo, carrying the β -galactosidase gene) was used as a control. All plasmids were a generous gift from Dr. Karen Mossman's lab. The MIR2 gene (pBMN-MIR2-GFP) was a gift from Dr. Laurent Coscoy (122). See Figure 5.1.

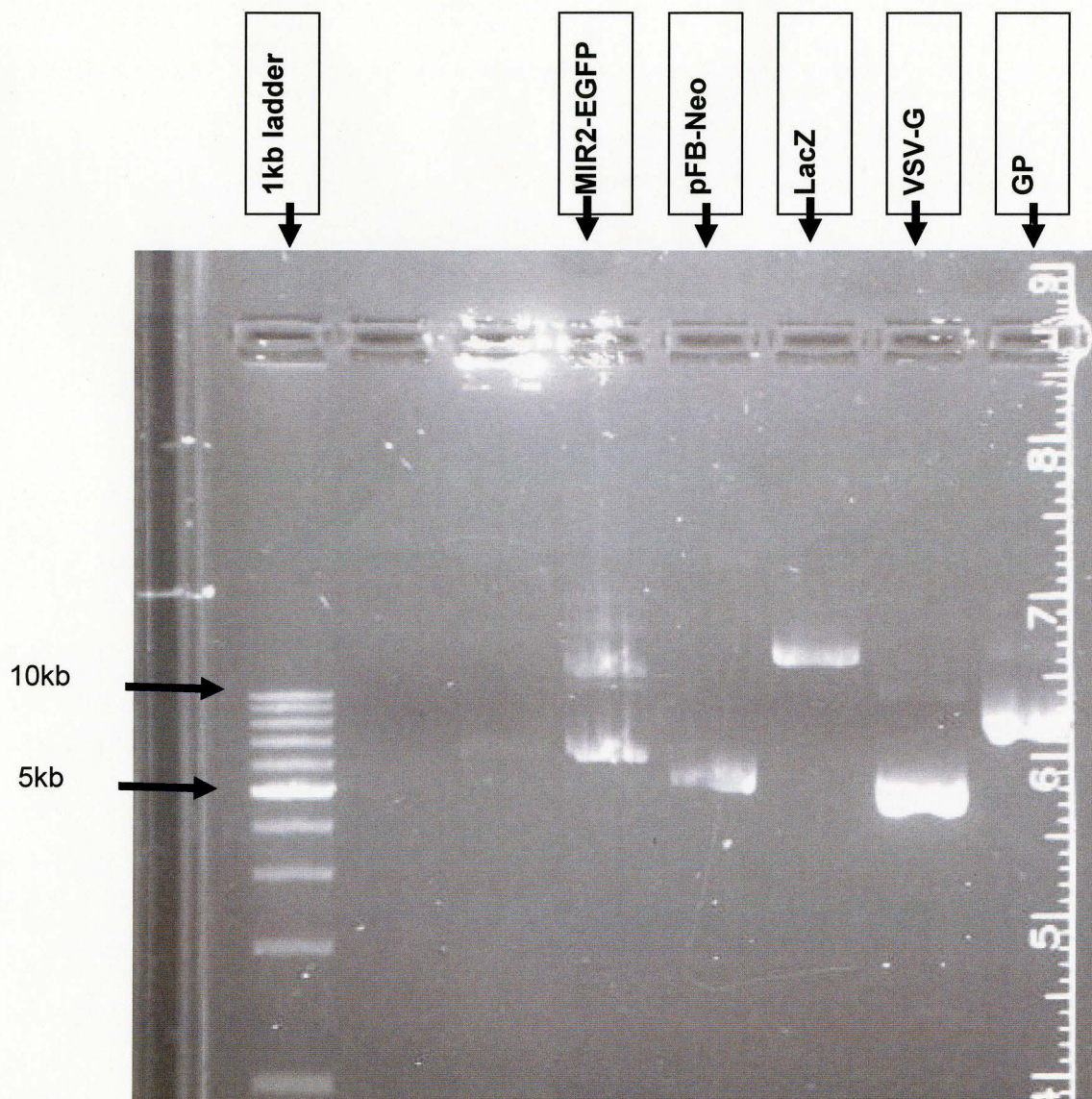


Figure 5.1: *Electrophoresis gel confirming sizes of retroviral vectors.* This (0.8%) agarose gel confirms the approximate sizes of circular supercoiled plasmids (generally appear smaller in size) pBMN-MIR2-GFP (7.9 kb), pFB-Neo (6.6 kb), LacZ (9.6 kb), VSV-G (6.6 kb), GP (10.7 kb).

5.1.1.1 Amplification and transformation of competent Stbl-4 *E. coli* bacteria

Stbl-4 strain (generous gift from Dr. Karen Mossman's lab) of *E. coli* were amplified in LB agar and treated with CaCl_2 to make them chemically competent, according to Molecular Cloning protocol 25 (section 1.116) (123). Each plasmid was separately introduced into Stbl-4's using a heat-shock transformation procedure (see Molecular Cloning, section 1.116, protocol 25) (123). Transformed bacteria were plated on ampicillin-treated agar plates and colonies were selected for amplification in LB media. A Qiagen HiSpeed Maxi kit (Qiagen, Cat No. 12662) was then used to isolate the plasmid from the bacterial cultures. All plasmids were run on a diagnostic 0.8% agarose gel to verify their approximate size, and their concentrations were quantified using a DNA spectrophotometer. Primers were designed to amplify the MIR2-GFP sequence for verification. Sequencing the pBMN-MIR2-GFP vector confirmed that the XhoI restriction site was corrupted; hence the HindIII site was used for subsequent excision of the MIR2-GFP sequence. See Figure 5.3. All molecular biology reagents were acquired from Dr. Carlos Filipe's lab.

5.1.1.2 Making the transgene vector

The pFB-Neo vector (generous gift from Dr. Karen Mossman's lab), derived from the Moloney murine leukemia virus (MMLV), encoding a bacterial origin of replication, ampicillin-resistance gene (pBR322), a packaging signal (ψ^+), a multiple cloning site and

5', 3' long terminal repeat sequences was amplified in an overnight liquid culture and the plasmid isolated using the QIAprep Spin Miniprep kit (Qiagen, Cat No. 27104). This was restriction digested with BamHI and HindIII to generate sticky ends and to drop out the unwanted DNA fragment. This unwanted DNA fragment was separated from the vector backbone using gel electrophoresis. The vector backbone was then gel extracted and prepared for ligation. No phosphatase treatment was necessary since the sticky ends were digested using different restriction enzymes and hence were not complementary. Next, the pBMN-MIR2-GFP vector (generous gift from Dr. Laurent Coscoy), encoding the MIR2-GFP insert was also digested with BamHI and HindIII to generate sticky ends and separated from the backbone using gel electrophoresis. See Figure 5.2, 5.3. The MIR2-GFP insert was then gel extracted and prepared for ligation with the vector backbone. See Figure 5.3. Ligations were performed using Formula 1 to calculate the appropriate volumes of the insert and the vector to be mixed per ligation reaction. The 3 insert: 1 vector molar ratio is known to maximize the chances of a single insert being incorporated into a single vector backbone. See Molecular Cloning III (123). After 16 h incubation at RT, the ligation mix was transformed into Stbl-4's and plated overnight. Three colonies were picked and amplified in LB media overnight. A restriction digest (R/D) was performed to screen these colonies and verify the MIR2-GFP insert. See Figure 5.4. The resulting pFB-MIR2-GFP-Neo plasmid construct was used for subsequent viral production. All molecular biology reagents were acquired from Dr. Carlos Filipe's lab.

$$\frac{(X \mu\text{l vector}) * (3) * (\text{vector concentration}) * (\text{insert size})}{(\text{Insert concentration}) * (\text{vector size})} = Y \mu\text{l insert}$$

Formula 1: Ligation formula with ratio of 3 insert: 1 vector

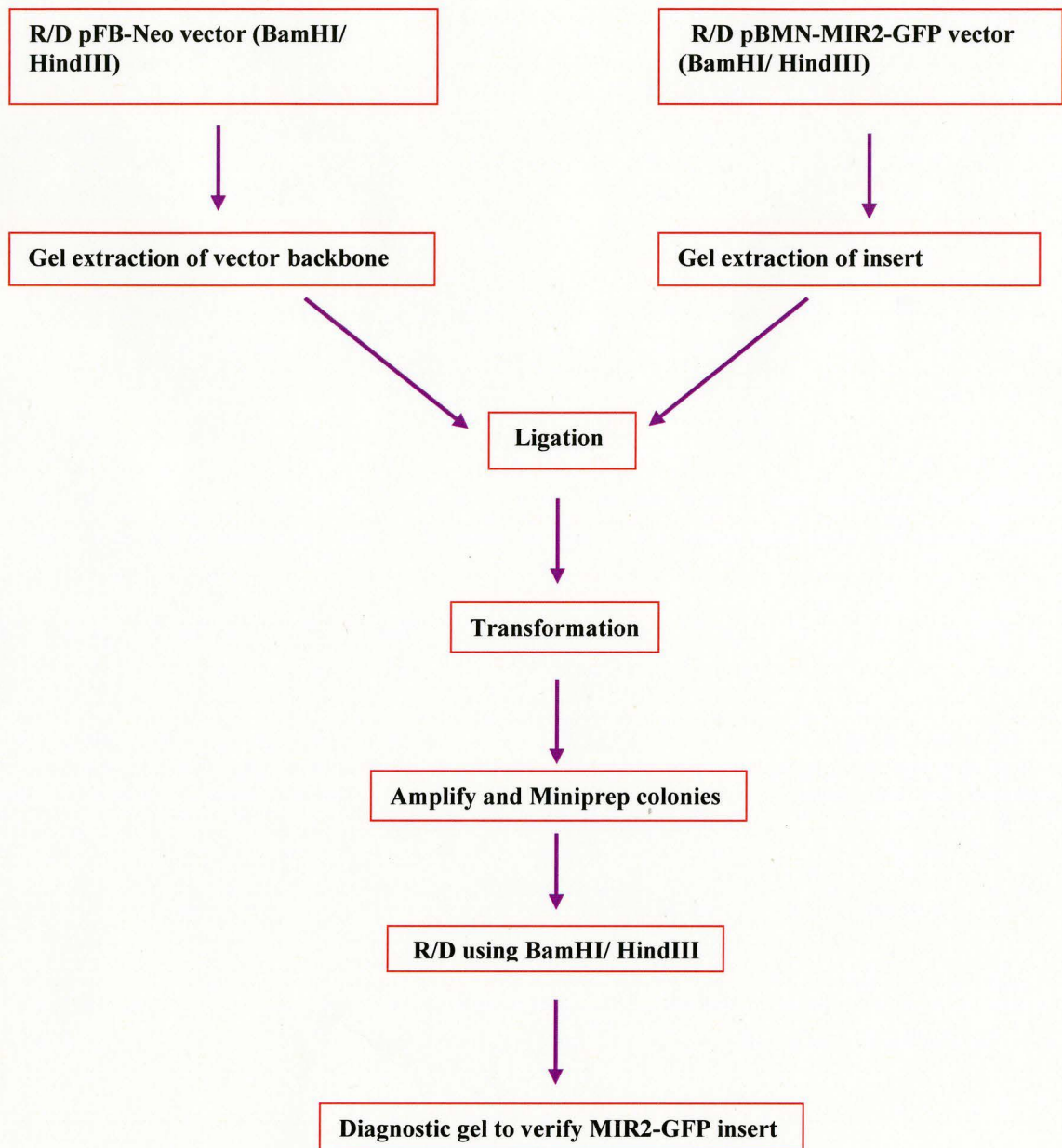


Figure 5.2: *Molecular biology scheme used to construct retroviral vector.* This scheme shows the insertion of MIR2-GFP sequence into the pFB-Neo backbone plasmid.

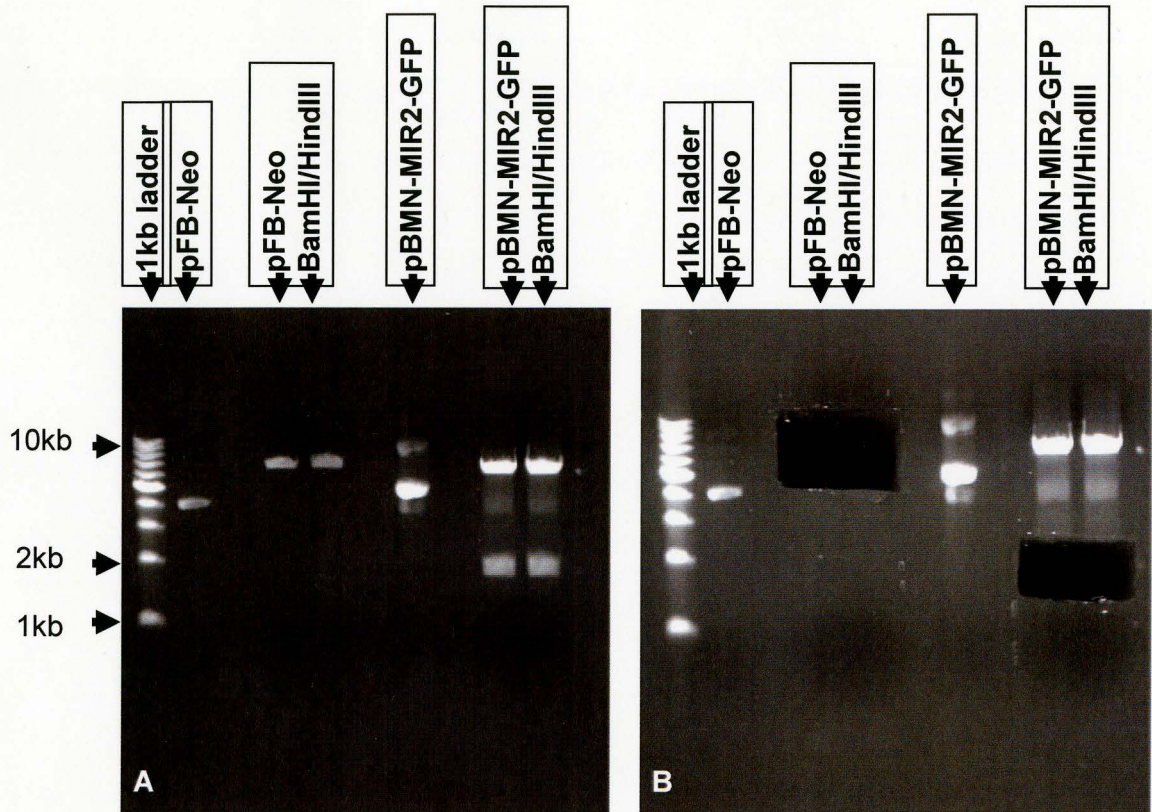


Figure 5.3: Separation of the pFB-Neo backbone and the MIR2-GFP insert. A) pFB-Neo backbone vector and the pBMN insert-containing vector were digested with BamHI and HindIII to separate and then gel extract (B) the pFB-Neo vector backbone (6.6 kb) and the MIR2-GFP insert (1.5 kb).

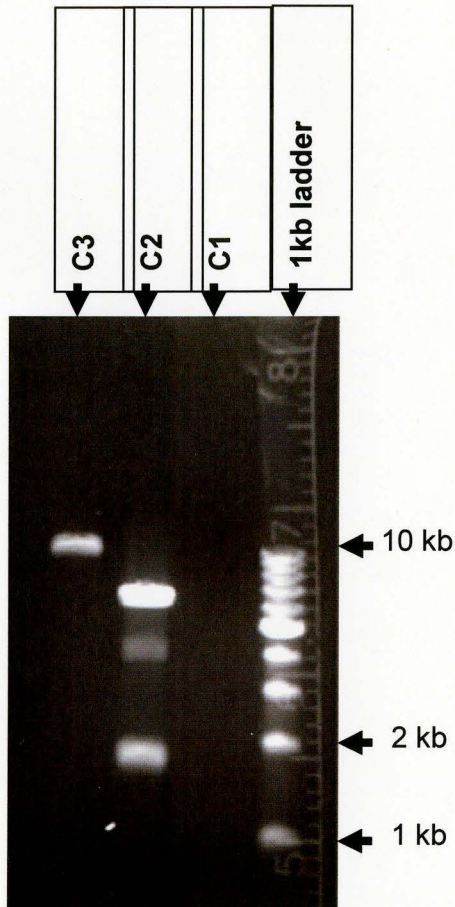


Figure 5.4: *Screening for the pFB-MIR2-GFP-Neo retroviral vector.* Screening of plasmids (R/D with BamHI and HindIII) isolated from three bacterial colonies that had been transformed with the ligation mix. The desired plasmid (C2) contains the MIR2-GFP insert (1.5 kb) with the pFB-Neo vector backbone (6.6 kb).

5.1.2 Retroviral transduction of MIR2-GFP

MMLV is a naturally occurring retrovirus with the capacity to infect certain types of mammalian cells with varying efficiencies. The transduction efficiency depends on the composition of the viral coat and its affinity to the target cell surface. The MMLV used in this study was pseudotyped, such that it expressed the VSV-G coat, which is known to have a broad specificity to almost any mitotic cell. In order to study the transduction efficiency of this VSV-G pseudotyped virus in U937 cells, a test transduction was setup and compared to osteosarcoma (U2OS) cells, which are known to be transduced with this virus. MMLV carrying the LacZ gene was generated and both U937 cells and U2OS cells transduced at the same titer. Figure 5.5 shows blue staining of β -galactosidase expressing cells, indicating that U937 cells were indeed transducible by VSV-G pseudotyped MMLV. However, U937 cells were transduced at a lower efficiency compared to U2OS cells. This difference may be explained by the nature of the cell type (adherent or suspension cells), cell surface composition, cell-division rate and cell-size.

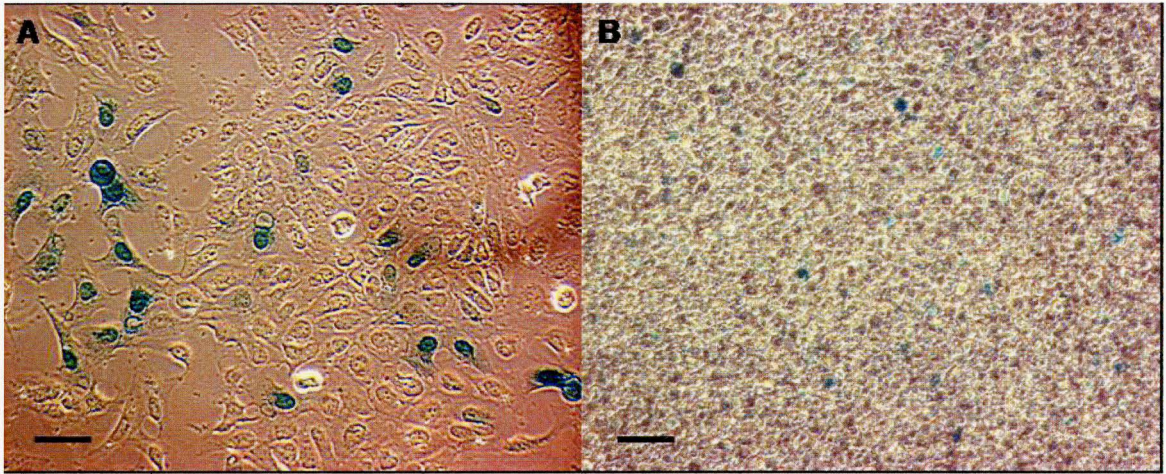


Figure 5.5: *Relative transduction efficiency of U2OS and U937 cell lines using MMLV.* Images show β -galactosidase staining (blue cells) of U2OS cells (A) and U937 cells (B). U937 cells are clearly transducible by retrovirus (MMLV), but much less in comparison to U2OS cells. Scale bar is 20 μm .

After establishing the transducibility of U937 cells, MMLV carrying the MIR2-GFP gene was generated and used in subsequent transductions. Figure 5.6 shows a strong GFP signal, indicating the likely expression of the chimeric MIR2-GFP protein. This result indicates that the VSV-G pseudotyped MMLV is suitable to deliver genes to U937 cells.

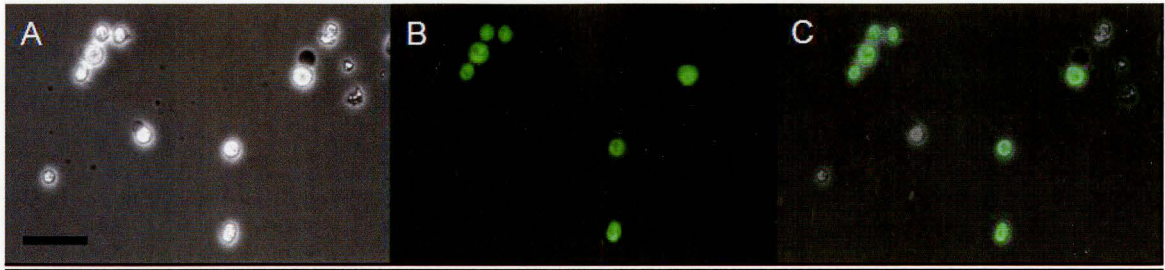


Figure 5.6: *MIR2-GFP* expression in U937 cells after MMLV transduction. U937 cells express MIR2-GFP (green) after transduction with retrovirus. Images collected in transmission (A) and fluorescence (B) channels. Image C overlays the two channels. Scale bar is 20 μm .

As a proof-of-principle, this retrovirus was used to transduce HCECs, with a result comparable to U937 cell transduction. See Figure 5.7. Taken together, these results suggest that the VSV-G pseudotyped MMLV can indeed be used as a gene delivery vehicle to transduce multiple human cell lines and express the MIR2-GFP chimeric viral gene. The versatility of this VSV-G pseudotyped virus makes it ideal for applications in tissue transplantation, where a number of cell types may need to be genetically modified simultaneously.

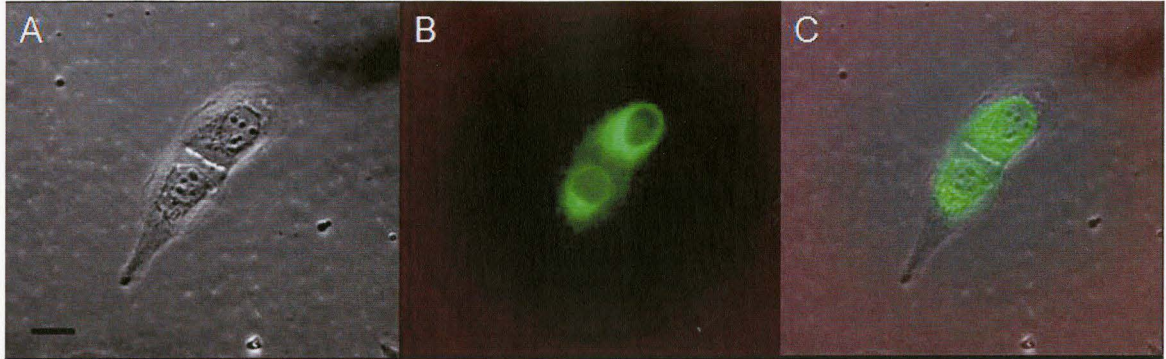


Figure 5.7: *MIR2-GFP* expression in HCECs after MMLV transduction. HCEC cells express MIR2-GFP (green) after transduction with retrovirus. Images collected in transmission (A) and fluorescence (B) channels. Image C overlays the two channels. Scale bar is 20 μm .

5.1.3 Viability of MIR2-GFP transduced U937 cells

Transduction and over-expression of a transgene such as MIR2-GFP may potentially alter normal cell functioning. Hence, it was important to check the viability of cells. As shown in Figure 5.8, cell viability was measured using a dead cell stain, 7-AAD, via flow cytometry and found to be about 6.5%. This value was confirmed using the FS/SS plot, in which live and dead cells appear in distinct regions. A similar measurement showed that this value for cell viability was not significantly different from untransduced cells, which is about 5% (data not shown).

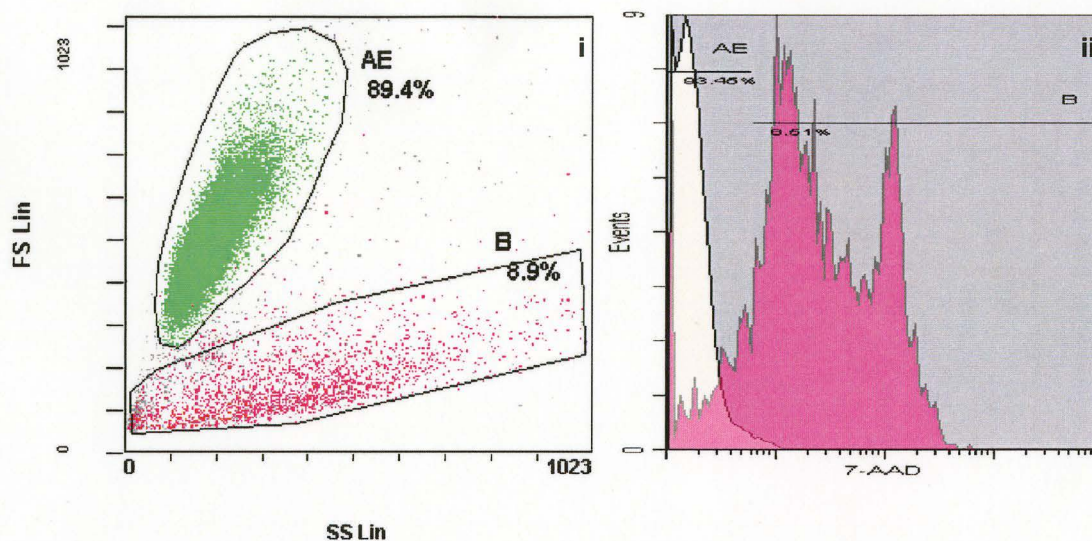


Figure 5.8: *Viability of MIR2-GFP transduced cells.* FS/SS plot of live (box AE, green) and unhealthy/dead cells (box B) in a population of approximately 100,000 MIR2-GFP transduced U937 cells run through a flow cytometer. Image (ii) shows the proportion of all cells, which stain positive for 7-AAD, a dead cell stain. This population of dead cells (6.51%) is coloured purple in the FS/SS plot (i).

5.2 Characterization of MIR2-GFP transduced cells

5.2.1 Differential downregulation of cell surface proteins

The transduction of MIR2-GFP into cells is expected to alter the cell surface expression of various molecules important to immune detection. Hence, untransduced U937 cells, U937-LacZ cells and U937-MIR2-GFP cells were compared to determine the specific

effect of MIR2 on the cell surface expression of HLA-ABC, CD54, CD86, HLA-E, HLA-DR, CD11a and CD14. In Figure 5.9, first panel, the detection of one strong GFP peak in only U937-MIR2-GFP cells confirmed the identity of the cell line and that this was a clonal population of cells. In the second panel, HLA-ABC is moderately down-regulated in U937-MIR2-GFP cells compared to controls. It is important to note that the actual level of MHC Class Ia (MHCIa) downregulation is likely to be greater than the amount being quantified using the anti-HLA-ABC antibody. As explained in the literature review (section 2), MIR2 is known to strongly downregulate HLA-A and HLA-B, and only weakly affects HLA-C. However, the anti-HLA-ABC antibody binds all HLA-A, HLA-B and HLA-C gene products, masking the potential strong downregulation of HLA-A and HLA-B as expected. This antibody only provides a measure of the overall decrease in the cell surface expression levels of HLA-ABC. In the third panel, CD54 is strongly downregulated in U937-MIR2-GFP cells compared to controls. In the fourth panel, CD86 is also strongly downregulated in U937-MIR2-GFP cells compared to untransduced U937 cells. However, there is also an unexpected downregulation of CD86 in U937-LacZ cells. β -galactosidase, the gene product of LacZ, is not known to have any effect on the expression of CD86, hence this result may have been observed anomalously or due to the positional effect of transduction. The LacZ gene may have integrated into a region of the genome that perhaps resulted in a decrease in the cell surface expression of CD86. This observation can be confirmed by measuring the cell surface CD86 level in other U937-LacZ clones. A normal cell surface CD86 expression in these cells,

comparable to CD86 expression in untransduced U937 cells, would support the positional effect theory.

In Figure 5.10, panel one, the cell surface level of HLA-E is virtually undetectable in all three cell lines. This may have occurred due to several reasons: First, it is known that the cell surface expression level of HLA-E is generally low in U937 cells. This would make it difficult to detect. Second, the specific subclass (MEM-08) of antibody used to detect HLA-E may not have been suitable (low binding affinity). A different subclass of anti-HLA-E antibody, perhaps MEM-06, may have been better suited to detect HLA-E on U937 cells. Panel two shows that the low/negligible cell surface level of HLA-DR remains unaffected by the expression of MIR2. This result suggests that MHCII is unlikely to contribute to any observed effects resulting from MIR2 expression. Panel three shows that the moderate cell surface expression level of CD11a remains consistent across the cell lines. CD11a is the α -L chain of leukocyte function-associated antigen-1 (LFA-1), which is a heterodimeric integrin expressed on all leukocytes. U937 are histiocytic mononuclear cells, hence they also express LFA-1. The presence of CD11a on U937 cells, confirms its identity. The identity of U937 cells was also confirmed in the fourth panel, which showed that negligible/undetectable levels of CD14 were expressed on the cell surface. CD14 is a glycoprotein present on the cell surface of macrophages and granulocytes, but is not known to be expressed on the cell surface of U937 cells. The presence of CD11a and the absence of CD14 on the cell surface of U937 cells was a

practical means of confirming cell identity, since a readily available marker for U937 cells was not available.

Overall, this characterization of cell surface expression showed that MIR2 differentially downregulates molecules such that CD54 levels are decreased strongly, HLA-ABC levels are decreased moderately-strongly and CD86 levels are decreased moderately. As described in the literature review, all these molecules are important in allogeneic tissue rejection. Thus, it is expected that this alteration in the cell surface expression levels of immunoactive molecules is likely to have a functional consequence in the process of cellular acute rejection.

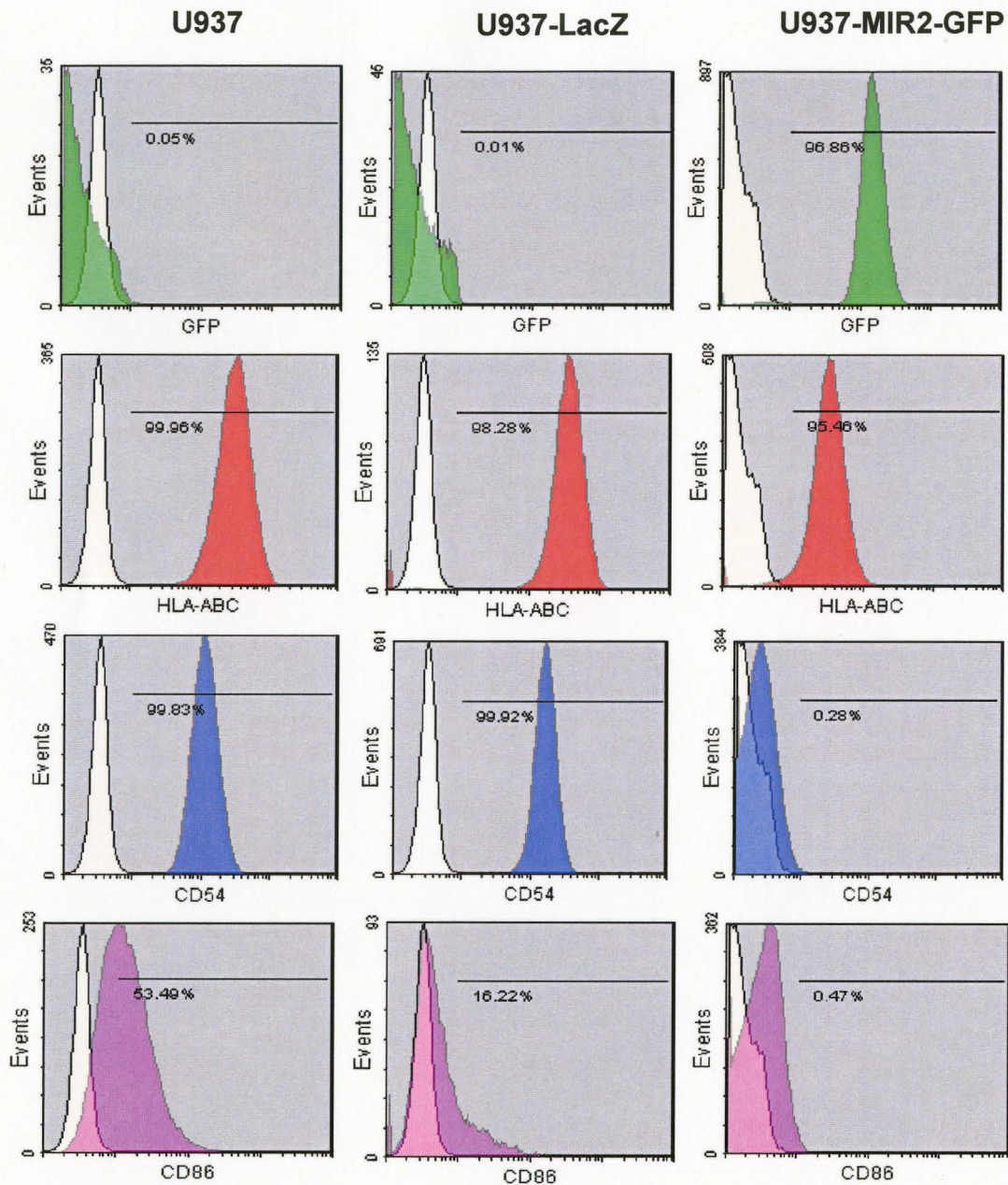


Figure 5.9: Cell surface characterization of U937, U937-LacZ and U937-MIR2-GFP cells. This plot compares the relative amount of GFP, HLA-ABC, CD54 and CD86 detected in each of the cell lines. GFP was quantified by its fluorescence, while fluorescently labelled antibodies were used to quantify the other markers. (n = 1)

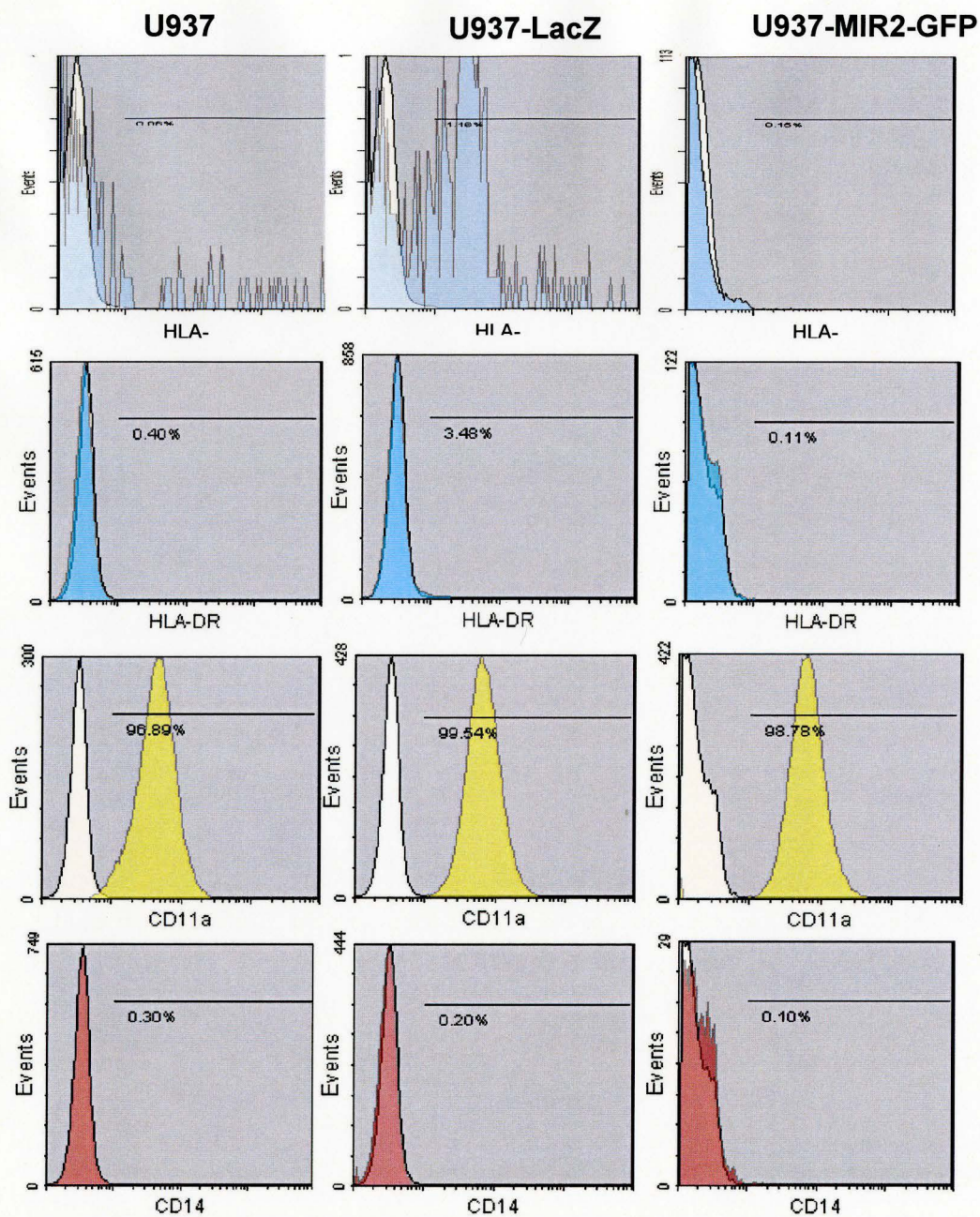


Figure 5.10: Cell surface characterization of U937, U937-LacZ and U937-MIR2-GFP cells. This plot compares the relative amount of HLA-E, HLA-DR, CD11a and CD14 detected in each of the cell lines using fluorescently labelled antibodies. (n = 1)

5.2.2 MIR2-GFP dose-response effect

In the previous section, it was established that GFP, presumably MIR2-GFP, was being expressed in transduced U937 cells. However, it is conceivable that GFP expression was not associated with the expression of MIR2. For example, this can occur in cases where there is a gene rearrangement in the viral expression plasmid sequence. Thus, it was important to correlate the GFP signal strength with the level of MIR2 expression.

Figures 5.11 and 5.12 show that there is a general negative relationship between the expression level of MIR2-GFP and the cell surface HLA-ABC level. It is expected that as the number of molecules of MIR2-GFP being expressed within a cell increase (higher mean fluorescence intensity (MFI) of GFP measured), the greater the downregulation of HLA-ABC. Thus, as the MIR2-GFP expression (higher MFI) increases, lower levels (lower MFI) of HLA-ABC should be detected on the cell surface. As shown in Figure 5.12, this effect was indeed observed. Figure 5.12 also suggests that there is a non-linear relationship between the expression level of MIR2-GFP and HLA-ABC. This effect may have occurred due to the various cellular processes occurring in living cells. Firstly, there may be cellular processes that are activated above a certain threshold level of MIR2-GFP expression. The dissociation/ enzymatic cleavage of MIR2 from the GFP or the subsequent sequestering/ selective degradation of MIR2 could reduce the amount of MIR2 present in the cytoplasm. Second, transduced cells may alter the transcription levels of HLA genes upon the downregulation action of MIR2. This effect can also make

the MIR2 function less effectively. Third, MIR2 is a ubiquitinase capable of ubiquitinating any lysine residue present in the cell. Hence, a fraction of the MIR2 is actively ubiquitinating proteins other than the intended MHCIIa targets. Thus, this fraction is unavailable to downregulate MHCIIa. This may explain the linear decrease in the HLA-ABC MFI, which follows a log increase in the MIR2-GFP MFI. In all the cases explained above, the GFP molecule is likely unaffected and thus its MFI appears to increase faster than the decline in the HLA-ABC MFI.

Moreover, it is interesting to note that even low expression of MIR2-GFP results in a large downregulation of HLA-ABC. This provides further support to this approach of expressing viral proteins as a tissue engineering strategy to evade the immune system.

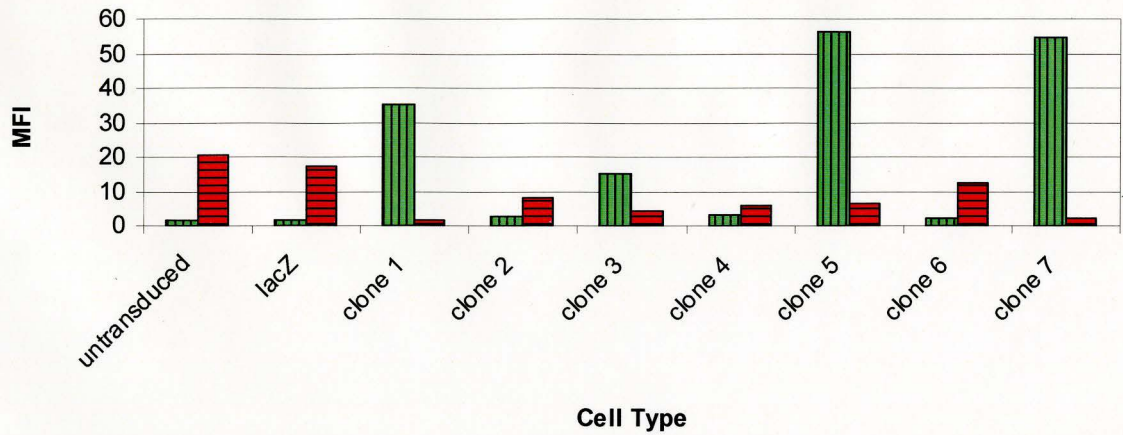


Figure 5.11: *MIR2-GFP* dose-response effect on *HLA-ABC* expression. A plot comparing the relative MFI of *MIR2-GFP* (green) and *HLA-ABC* (red) as measured using flow cytometry in untransduced U937 cells, U937-LacZ cells and seven U937-*MIR2-GFP* clones. (n = 1)

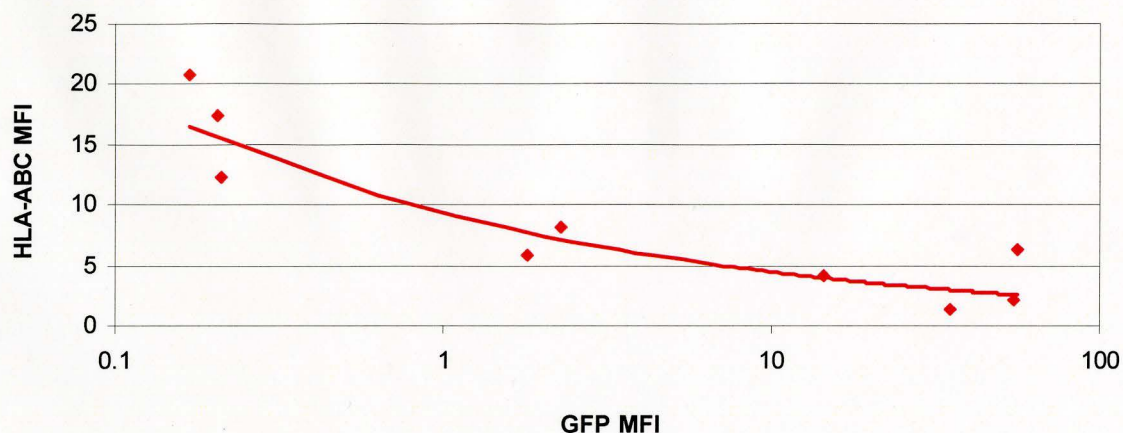


Figure 5.12: *Trend showing MIR2-GFP dose-response effect on HLA-ABC expression.*

Shows a generally decreasing trend in the cell surface HLA-ABC level with increasing levels of MIR2-GFP expression as detected using flow cytometry in each of the transduced U937 clones. (n = 1)

5.2.3 Stability of MIR2-GFP expression

In tissue engineering cells for allogeneic transplantation, it is important that the introduced viral gene be expressed for a sufficient length of time in order for it to be useful as a general strategy. Hence, the long-term stability of retroviral gene transfer is an important characteristic to be assessed. Figure 5.13 shows that, after 100 days, although the GFP signal decreases to about 40% of its original value on day 0, the relative HLA-ABC level does not increase noticeably. Based on this result, it would be expected that the degree of immune evasion of tissue-engineered allogeneic cells should remain

relatively constant over at least a 100 day period. It is interesting to note that many research groups have previously used lentiviral gene delivery vectors for genetically modifying stem cells. However, a recent study by Barrette *et al.*, showed that the efficiency of lentiviral gene delivery might be comparable to MMLV-based retroviral vectors for some types of stem cells (124). Hence, we believe that MMLV-based retroviral gene delivery vectors can be used in tissue engineering for long-term gene expression in various cell types, including some types of stem cells.

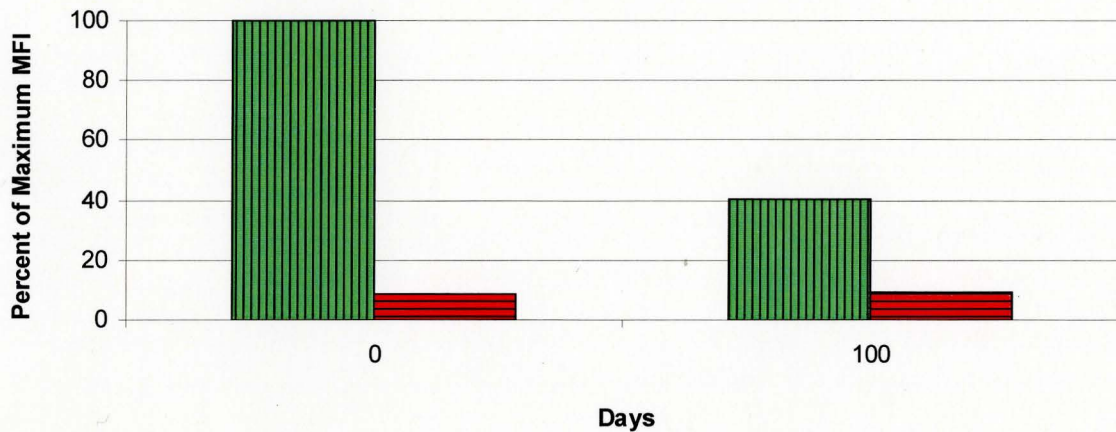


Figure 5.13: Effect of *MIR2-GFP* on *HLA-ABC* expression in transduced cells over 100 days. The green bars (vertical stripes) show the percent of maximum MFI of the *MIR2-GFP* signal relative to the MFI on day 0. The red bars (horizontal stripes) show the percent of maximum MFI of the *HLA-ABC* signal relative to the MFI signal of *HLA-ABC* for U937-LacZ cells on the corresponding days. These measurements were acquired from a clonal population of U937-*MIR2-GFP* cells in active culture for 100 days. Data presented from 100,000 events collected. (n = 1)

5.3 Immune evasion of MIR2-GFP transduced cells

5.3.1 A novel flow cytometry-based cytotoxicity assay

We developed a novel flow cytometry assay to measure specific cytotoxicity in cocultures. Unlike the traditional Chromium-release plate-reader CTL and NK cytotoxicity assays, our assay takes advantage of the single cell sensitivity and high throughput capacity of flow cytometry. It can not only provide a measure of viability, but can also provide an assessment of the composition and health of a heterogeneous mixture of cells. Moreover, it does not require the use of radioactive substances for quantification of viability.

Briefly, target cells are first labelled with Calcein, a live cell-specific dye. These cells are then cocultured with effector cells at various ratios. Effector cells use several means including granzymes (cytolytic granules) and Fas ligand mediated apoptosis to induce target cell death. Dead cells are either completely lysed into multiple fragments, or remain as one entity with a compromised cell membrane. Thus, the physical size of dead cells and/or degree of Calcein staining distinctly varies from healthy, live cells. These distinguishing characteristics were used to calculate specific target cell cytotoxicity.

Flow cytometry data analysis for the CTL and NK assays was based on loss of Calcein fluorescence from target cells. Briefly, live target cells were gated based on their Calcein

staining intensity and their relative size (FS). This population of healthy-bright Calcein stained target cells was first determined in the control sample, which contained the same number of cells (50,000) as the test samples, but without any effector cells. After coculturing effector and target cells together, the population of healthy-bright Calcein stained target cells was determined again. The difference between the two values (control and test sample) was the percent specific lysis. See Formula 2.

$$\% \text{ Specific lysis} = \frac{(Y_{\text{control}}) - (X_{\text{test}})}{(Z_{\text{coculture}})}$$

Formula 2: Calculating % Specific lysis in CTL and NK assays

Where:

X = % Calcein-bright cells after coculture test

Y = % Calcein-bright cells in control

Z = theoretical % of Calcein-bright cells of all cells in coculture

5.3.1.1 CountBright™ beads for enumerating cells using flow cytometry

As described in the section 5.3.1, a flow cytometer analyses and counts fluorescent/non-fluorescent particles in a suspension; however, it cannot determine the concentration of particles/cells since the volume of solution used for analysis is not recorded. CountBright™ beads (Molecular Probes, MP 36950) are small (7 µm) fluorescent microspheres that are added to a single-cell suspension in order to determine the absolute cell count. These beads are brightly fluorescent, with a wide absorption and emission spectrum. This property allows them to be gated unambiguously in a fluorescence emission channel to determine the bead count. Since the concentration of beads and the ratio of the bead solution to cell solution are known, the concentration of cells can be calculated.

In our flow cytometry based cytotoxicity assays, it was important to determine whether Calcein labeled target cells were being lysed and fragmented by effector cells as opposed to staying intact with a compromised cell membrane. In order to determine the fraction of cells being lysed and fragmented by effector cells, we calculated the absolute number of Calcein-labelled target cells in control samples (Either U937-LacZ or U937-MIR2 only) and compared them with test samples (coculture of effector and target cells). This absolute cell count was measured using the CountBright™ beads. The cell concentration was calculated according to the Molecular Probes formula provided:

Calculation of cell concentration:

$$(A/B) \times (C/D) = \text{concentration of sample as cells}/\mu\text{L}$$

Formula 3: Calculating cell concentration using CountBright™ beads

Where:

A = number of cell events

B = number of bead events

C = 51000 beads/50 μL

D = volume of sample (μL)

To determine the accuracy of this method of counting cells, a known number of Calcein-labelled cells (counted using a hemocytometer) were mixed with CountBright™ beads and run through the flow cytometer.

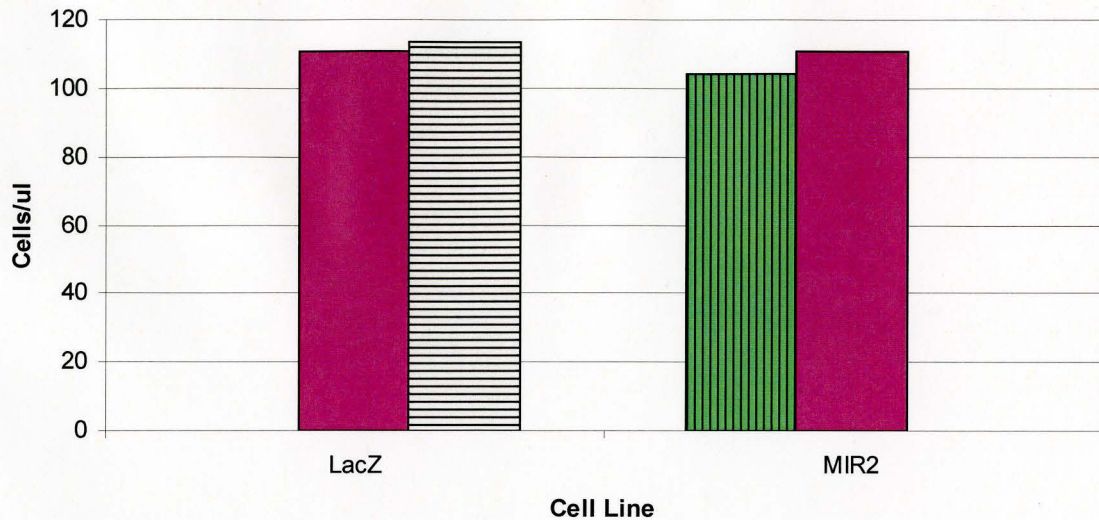


Figure 5.14: *CountBright™ Calcein cell count*. Shows the expected cell concentration (solid, purple bars) determined by the hemocytometer, and actual cell concentration calculated using *CountBright™* beads (flow cytometry) for U937-LacZ (horizontal stripes, white bars) and U937-MIR2-GFP (vertical stripes, green bars) cells. Flow cytometry data presented from at least 30000 events collected. (n = 1)

The *CountBright™* absolute cell counts were not significantly ($P < 0.05$) different from cell counts determined by a hemocytometer (average of four readings). See Figure 5.14. This test supported the validity of using the *CountBright™* method in estimating cell concentrations in the CTL assay.

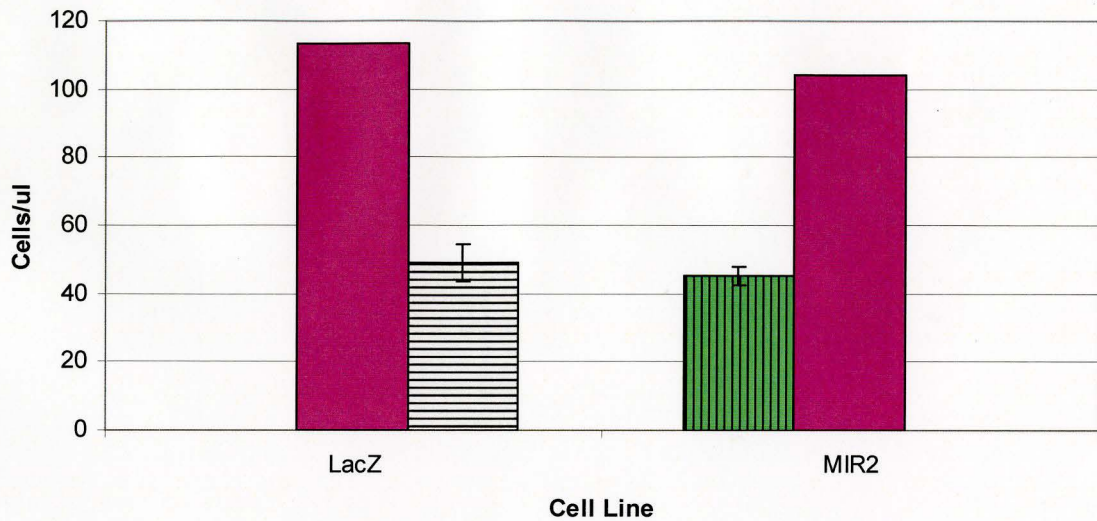


Figure 5.15: *CTL assay CountBright™ Calcein cell count*. Shows the expected Calcein labeled target cell concentration (solid, purple bars) determined by the hemocytometer and actual Calcein cell concentration calculated using CountBright™ beads (flow cytometry) for U937-LacZ (horizontal stripes, white bars) and U937-MIR2-GFP (vertical stripes, green bars) cells. (Error bars indicate standard deviation; $n = 3$)

However, the result shown in Figure 5.15 suggests that there was a significant ($P < 0.05$) loss of Calcein-labelled target cells in the CTL assay. This indicated that the CTLs were lysing a large proportion (approximately 50%) of the target cells. This lysis effect had to be taken into account when calculating CTL-specific cell death of target cells. Therefore, specific-cell death was calculated by taking the difference between the number of Calcein-labelled target cells in test samples (with effector cells) as compared to controls (without effector cells). We refer to this calculation as the ‘*target cell Calcein-loss method*’ for estimating specific-cell death. See Formula 2.

The target cell Calcein-loss method was used as opposed to other methods of calculating specific lysis since it likely provides the best approximation to the real value. To our knowledge, no other commonly used method for determining cytotoxicity analyzes such a large number of cells (>30000) at the single cell level. In addition to accounting for the number of lysed target cells, this method also excluded (since healthy cells can be gated in the FS/SS plot) fluorescent cell fragments, which are generated when a cell is lysed. These fragments are counted by the flow cytometer and can overestimate the number of target cells present in the sample. Hence, specific killing of target cells by effector cells was calculated using the *target cell Calcein loss method* in both the CTL and the NK assays.

5.3.2 Immune cell isolation

CD8⁺ T cells were negatively isolated and differentiated into CTLs, which were used as effector cells in the CTL assay. CD56⁺ NK cells were positively isolated and activated *in vitro*, after which, they were used as effector cells in the NK assay. Both these isolation methods were based on specific antibody recognition of characteristic surface markers.

5.3.2.1 CTL generation

5.3.2.1.1 CD8⁺ T cell isolation

CD8⁺ T cells were negatively isolated using the StemCell™ CD8 isolation kit. Negative selection was used in order to minimize the chance of activating CD8⁺ T cells, which can result in unwanted proliferation and generation of CTLs not specific to target cells. Figure 5.16 shows that negative selection isolated CD8⁺ T cells with a relatively low efficiency, increasing its concentration from 2.4% to 30.6%. Although multiple rounds of isolation can potentially increase purity of the desired cell type, it also decreases the overall efficiency of cell isolation. Hence, the procedure in section 4.5.4 was considered optimal for use in these experiments. Moreover, subsequent cell death of unwanted cells and proliferation of CD8⁺ T cells into CTLs in the *in vitro* culture would increase the relative concentration of CD8⁺ T cells. It is important to note that using a single characteristic surface marker (CD8) for cell isolation will also isolate double positive immune cells such as CD8⁺, CD4⁺ T cells. However, these subpopulations of cells comprise a very small percentage (<0.1%) of PBMCs, and would not likely have a significant impact on the outcome of the results. For simplicity, CD8⁺ cells isolated are referred to as ‘CD8⁺ T cells’ in this thesis.

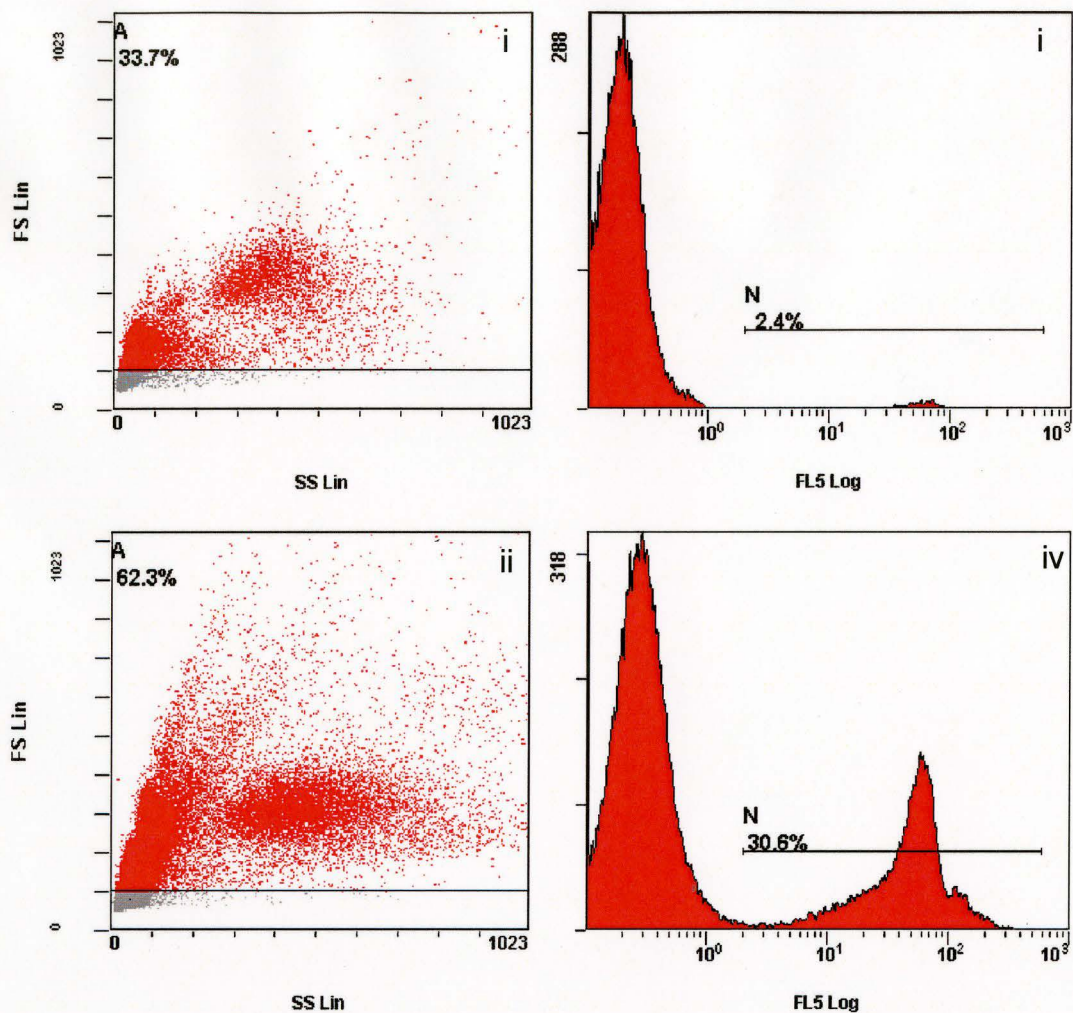


Figure 5.16: *Negative selection of CD8⁺ T cells.* Plot (i) shows the FS/SS distribution of PBMCs and histogram (ii) shows the percentage of PBMCs that stain positive (2.4%) for CD8 on the cell surface. Plot (iii) shows the FS/SS distribution of cells after negative selection using the StemCell CD8 isolation kit, while histogram (iv) shows the percentage of negatively selected cells which express CD8 (30.6%) on the cell surface. Data presented from 100,000 events collected.

5.3.2.1.2 Stimulating CD8+ T cells to generate CTLs

Negatively selected CD8+ T cells were stimulated by U937 cells (Mitomycin C treated) to induce proliferation and differentiation into CTLs. Without such stimulation, naïve CD8+ T cells are known to apoptose under extended *in vitro* culture conditions. Figure 5.17 shows that 28.6% of the Responder: Stimulator (R:S = 2:1) coculture was counted as live CD8+ T cells. If there was no proliferation or apoptosis of CD8+ T cells after *in vitro* coculture of CD8+ T cells and allogeneic cells for 96 h, then a maximum of 66% CD8+ T cells would be expected from the flow data. In addition, it is known that only about 10% of CD8+ T cells proliferate upon encountering allogeneic cells. Taken together, only 6.6% of the coculture would be expected to comprise the CD8+ T cells. Hence, the measured 28.6% CD8+ T cell population in the coculture was likely an indication of proliferation and differentiation of CD8+ T cells into CTLs. Furthermore, the presence of CTLs could be verified using an antibody specific to a surface marker such as C1.7, which is specific for CTLs.

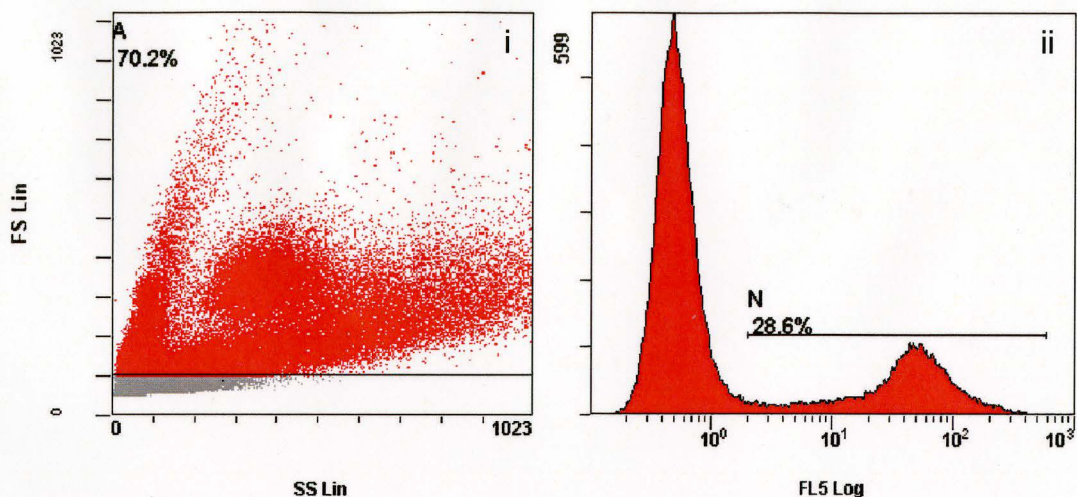


Figure 5.17: CTL fraction after stimulation of CD8⁺ T cell-enriched PBMCs with U937-LacZ cells. Plot (i) shows the FS/SS distribution of the CTL containing cell mixture and histogram (ii) shows the percentage of the cell mixture which stains positive (28.6%) for CD8 on the cell surface, corresponding to the presence of CTLs. Data presented from 100,000 events collected.

5.3.2.2 NK cell isolation

NK cells were positively isolated using the StemCell isolation kit. Positive selection was employed since NK cells comprise only about 5-15% of PBMCs, which required high isolation efficiency. Negatively isolated cell mixtures are known to be less efficient and contain a greater fraction of unwanted cell types, hence making negative selection impractical for use in this experiment. Although there was a chance of potentially activating NK cells using the positive selection technique, it was unlikely to affect the outcome of the NK assay, since all NK cells were activated using poly I:C reagent for 48

h before use. Figure 5.18 shows that positive isolation was highly efficient, as it increased CD56+ cell purity from 1.3% to 90.0%. The vast majority of these cells are likely to be NK cells. Nevertheless, it is important to note that using a single characteristic surface marker (CD56) for cell isolation is also likely to isolate double positive immune cells such as NKT cells (CD56+, CD3+ cells). However, these subpopulations of cells comprise a very small percentage of PBMCs (typically 1%), and would not likely have a significant impact on the outcome of the results.

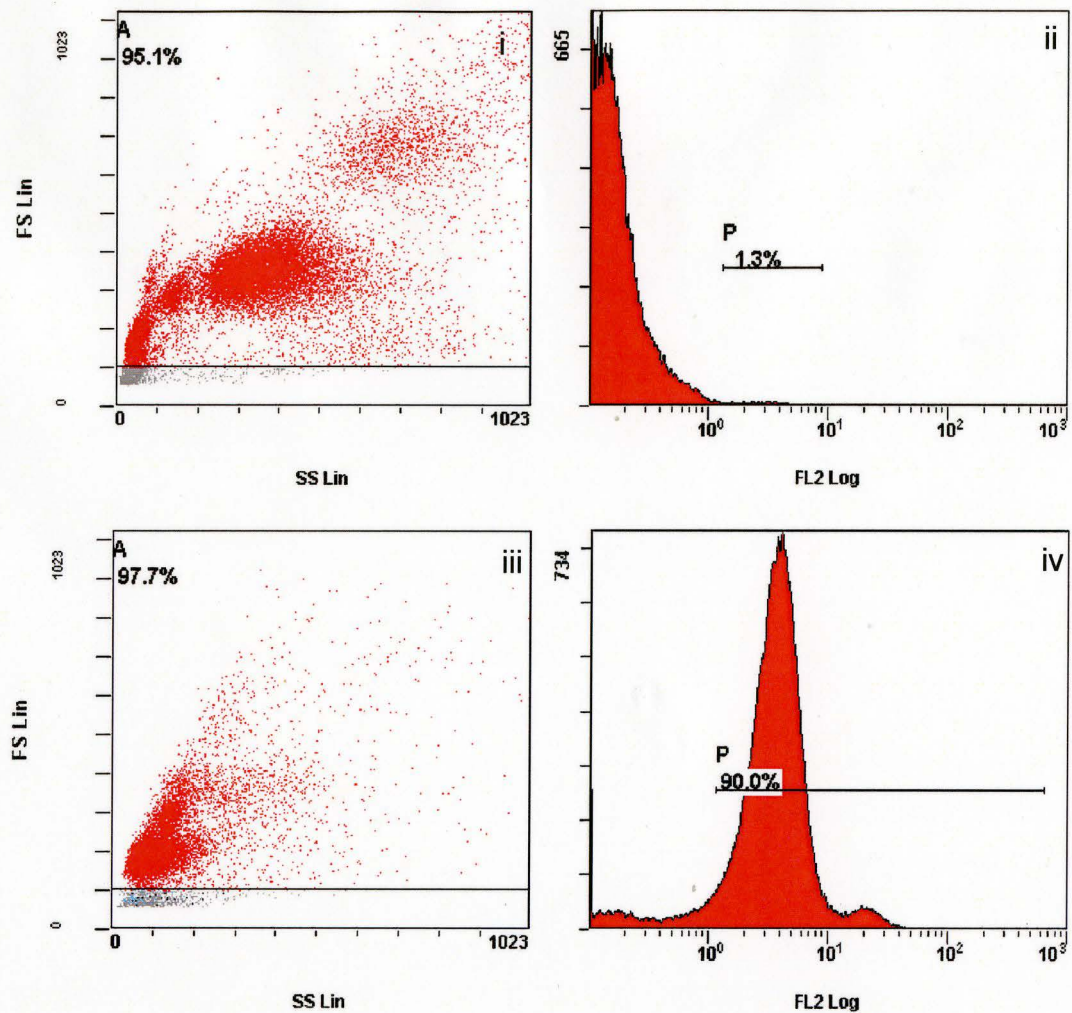


Figure 5.18: *Positive selection of NK cells.* Plot (i) shows the FS/SS distribution of PBMCs and histogram (ii) shows the percentage of PBMCs that stain positive (1.3%) for CD56 on the cell surface. Plot (iii) shows the FS/SS distribution of cells after positive selection using the StemCell NK isolation kit, while histogram (iv) shows the percentage of positively selected cells which express CD56 (90.0%) on the cell surface. Data presented from 100,000 events collected.

5.3.3 CTL Assay

The CTL assay shows a statistically significant decrease in the specific lysis of target cells transduced with MIR2 at two different ratios. See Figure 5.19. Specifically, at the 2:1 E:T ratio, there is 50% reduction in the specific lysis of MIR2 cells. The protection conferred by MIR2 at the 10:1 E:T ratio is less than that measured at the 2:1 E:T ratio. This suggests that there is likely to be an upper limit for the protective effects of MIR2, which can be abrogated by significantly increasing the E:T ratio. However, E:T ratios above 10:1 are relatively high and may not be encountered by transplanted allogeneic cells *in vivo*. Overall, this assay shows that viral immunomodulatory gene expression can indeed significantly decrease the cytolytic effects of CTLs and perhaps extends the life of allogeneic cells.

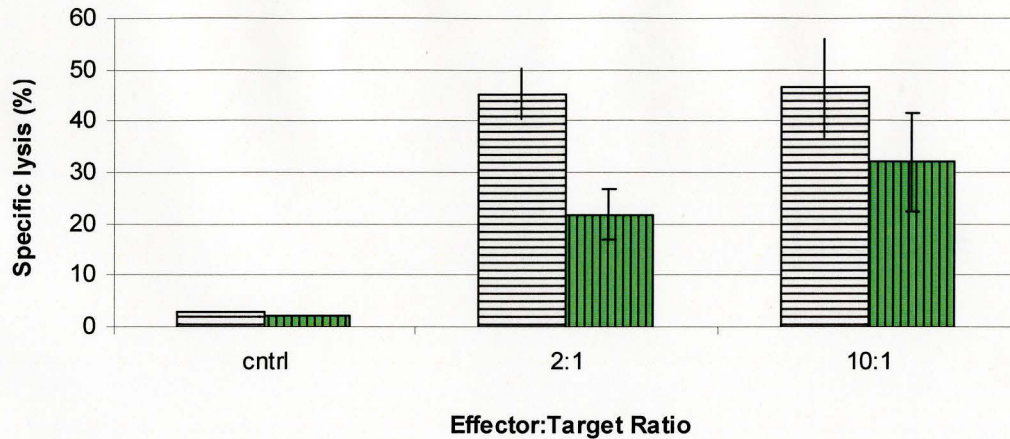


Figure 5.19: *CTL Assay*. Shows percent specific lysis of Calcein-labelled target cells (U937-LacZ [horizontal stripes, white bars] or U937-MIR2-GFP [vertical stripes, green bars] cells) in control samples and test samples with varying Effector:Target ratios. (Statistics: $p \leq 0.05$ between cell lines for both Effector:Target ratios; Error bars indicate mean square error; $n = 3$)

5.3.4 NK Assay

The NK assay showed no statistically significant difference in the specific lysis of target cells transduced with MIR2 when compared to target cells transduced with LacZ at various E:T ratios. See Figure 5.20. As explained in the literature review, NK cell cytolytic activity is inversely proportional to the amount of MHC I present on the cell surface. As the MHC I levels decrease on the cell surface, a greater percentage of the target cells should be specifically killed by NK cells. In this experiment, U937 cells

expressing MIR2 are differentially downregulating MHCI, CD54 and CD86, as shown in Figure 5.9. If there was no immunoprotective effect of this downregulation of cell surface molecules, an increase in the specific lysis of U937-MIR2-GFP cells would be expected. In this NK assay, we did not measure any increase in the specific lysis of U937-MIR2-GFP cells, when compared to U937-LacZ cells. This result suggests that MIR2 expression confers a significant protective effect against NK specific cell lysis. A number of factors may have contributed to this observed protective effect. First, the HLA-E expression on the cell surface might have been sufficient to negatively regulate NK cells and prevent lysis of MIR2 expressing cells. Although, our specific MEM-08 subclass of antibody did not detect the presence of HLA-E, it is possible that a different subclass of HLA-E antibody might have been able to detect cell surface expression of HLA-E. Second, cell surface HLA-C, which is weakly affected by MIR2 expression, might have been sufficient to negatively regulate NK cell specific lysis of target cells. Third, the strong downregulation of CD54 might have significantly lowered the capacity of NK cells to adhere to MIR2 expressing cells, resulting in decreased specific cell lysis. Fourth, MIR2-mediated downregulation of other U937 cell surface molecules such as MICA, MICB and AICL might have reduced the activation signals necessary for NK cell activation. Fifth, a combination of the above factors may have resulted in the observed protective effect of MIR2 expression against NK cell specific lysis of target cells.

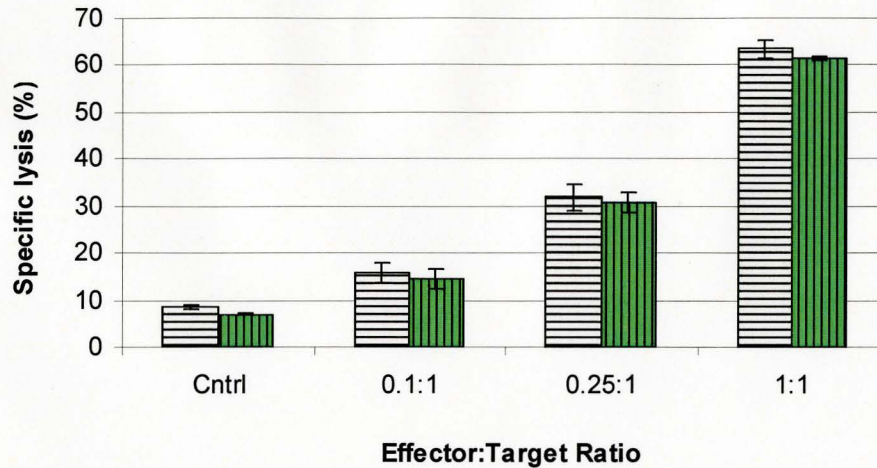


Figure 5.20: *NK Assay*. Shows percent specific lysis of Calcein-labelled target cells (U937-LacZ [horizontal stripes, white bars] or U937-MIR2-GFP [vertical stripes, green bars] cells) in control samples and test samples with varying Effector:Target ratios. (Statistics: No significant difference between cell lines ($P < 0.05$); Error bars indicate standard deviation; $n = 3$)

6. SIGNIFICANCE

In tissue engineering, the immune response to allogeneic cells is a major barrier impeding successful transplantation of tissue-engineered constructs. We propose an alternative method to engineer immune acceptance, such that allogeneic cells are selectively modified to use viral strategies to evade the alloimmune response, while retaining normal immune function. This strategy has an immense potential in tissue engineering since it provides an alternative to using systemic immunosuppressive drugs, allowing a broader spectrum of diseases to be treated and potentially increasing the success of allogeneic transplants.

To our knowledge, this is the first study showing that the employment of multifunctional viral immunomodulatory proteins to differentially downregulate cell surface molecules in allogeneic cells can result in CTL and NK cell evasion *in vitro*. In addition, we provide evidence and propose that a retroviral vector can be used to efficiently modify cells *ex vivo* for cell-based tissue engineering applications.

Another research group recently suggested that viral stealth mechanisms can be exploited for stem cell transplantation. Lee *et. al.* transfected human cytomegalovirus (hCMV) US genes into human neuronal stem cells (hNSCs) and downregulated cell surface MHC I molecules by 20-50% (125). Another study by the same group transfected hCMV US genes into a human breast luminal epithelial cell line, which is known to have stem cell

properties, and achieved a 40-60% downregulation of cell surface MHCII molecules(126). As discussed in the literature review, downregulation of cell surface MHCII molecules is likely to evade the acute CTL response against allogeneic cells. However, their approach only attempted to globally downregulate MHCII molecules. As explained previously, cell surface MHCII molecules, especially HLA-C and HLA-E, also serve to negatively regulate NK cells. Hence, a global downregulation of all types of MHCII molecules is likely to make the cells more susceptible to NK-mediated cytotoxicity. The authors of these studies argue that the small amount of MHCII still expressed on US gene transfected cells is likely to be sufficient to evade NK-mediated cytotoxicity. However, this explanation assumes that all MHCII molecules are equivalent in their potential to negatively regulate NK cells. It is well established that HLA-C and HLA-E are potent negative regulators of NK cells. A downregulation of these MHCII molecules is likely to disproportionately influence NK-mediated cytotoxicity. In contrast, our strategy to express MIR2 results in a differential downregulation of MHCII molecules, strongly reducing HLA-A and HLA-B expression, while having a negligible effect on HLA-C and HLA-E. As described in section 2.5.4.1, clinical experiments have shown that HLA-A and HLA-B are the most important loci (of all the MHCII genes) in determining the outcome of an implanted allograft. Hence, this strategy is more likely to evade both CTLs and NK cells simultaneously.

In addition, neither of the studies discussed above test the functional effect of this global downregulation of MHCII molecules. Our CTL and NK assays provide evidence *in vitro*

that MIR2 expression in allogeneic cells significantly reduces CTL-mediated cytotoxicity and completely evades NK cell cytotoxicity. Moreover, this dual (CTL and NK cell) evasion may significantly improve transplant acceptance, since the proinflammatory cytokines produced in the local area after transplant surgery are known to upregulate MHCI expression and recruit immune cells including CD8+ T cells and NK cells to the region (125). In this case, MIR2 expression may serve to reduce the induction of alloimmune responses, which may increase allograft success.

Furthermore, both of these studies employed electroporation as a means to deliver genes to cells. However, electroporation and other transfection techniques in general are relatively inefficient. Moreover, there is a high degree of variability in gene expression and gene expression is mostly transient. In contrast, we used a retroviral vector to deliver MIR2 into cells. It is well established that retroviral vectors are more efficient, transducing a greater proportion of cells in comparison to transfection, and with a lower variability in gene expression strength. Retroviral gene delivery also results in long-term gene expression, providing a significant advantage for tissue engineering applications which frequently employ stem cells (and progenitors) that differentiate into other cell types over an extended period of time. Moreover our *ex vivo* strategy to modify cells using a replication-defective retroviral vector does not introduce potentially immunogenic viral particles *in vivo*, making this approach relatively safe.

Although using a retroviral vector may be relatively safe, the expression of viral proteins *in vivo* can potentially generate a potent immune response in itself. In our approach, donor antigen modification using MIR2, a viral protein, has a number of other implications with respect to immune evasion. Although MIR2 over-expression can potentially generate an adaptive immune response, it is unlikely that this response will significantly impact cells expressing MIR2. First, MIR2 downregulates MHCI, the primary mechanism of cells to present intracellular peptides to CD8+ T cells. Second, MIR2 downregulates B7-2, a costimulatory molecule present on APCs. As described in the literature review, an MIR2-expressing donor APC lacking costimulatory molecules may induce anergy in alloreactive T cells, thereby tolerizing the allograft. Third, most of the MIR2 expressed in the cell is localized to the ER membrane, with only a small fraction transported to the cell surface. This makes it harder for antibodies generated against MIR2 to bind to its target, which protects the cell from the effects of complement activation and the action of phagocytic cells. Fourth, MIR2 is homologous to the human protein c-MIR, which is likely to reduce its immunogenicity.

Our method of modifying donor cell surface molecules may also have other benefits with respect to the alloimmune response. MIR2's differential downregulation of MHCI molecules implies that there is a significant reduction of some classes of MHCI molecules; however, it does not remove all MHCI molecules from being expressed. The almost unabated expression of HLA-C and HLA-E not only serve to inhibit NK cell-mediated cytotoxicity, they may also serve to induce active host tolerance to the allograft.

In fact, many studies have previously shown that some expression of the immunoantigen is beneficial to maintaining active tolerance. A study by Faustman and Coe showed that incomplete masking of MHCI molecules using antibodies can potentially lead to tolerance in murine hosts (127). This study indicates that donor MHC density is important in inducing tolerance. Hence, our differential downregulation approach to MHCI modulation may effectively create a greater window of opportunity for the induction of tolerance.

Donor antigen modification using viral mechanisms of immune evasion may also pave the way to generating ‘universal’ donor cells. An immediate advantage of universal donor cells in tissue engineering is that it enables the development of “off-the-shelf” technology, which can function immediately in any individual. This technology will provide an alternative to isogenic stem cell technology, which is often laborious and requires time to create, since it involves the differentiation and expansion of isogenic stem cells for applications in tissue engineering.

In addition, this method to modify donor antigens would greatly increase the flexibility of producing tissue-engineered constructs. For example, living cells used in making tissue-engineered constructs can be harvested from any donor and can then be transplanted into any recipient. It follows that this technology will enhance the development of semi-synthetic organs and therefore increases the availability of transplantable organs to better match the demand. This would also significantly decrease the cost and the delays

incurred with current medical procedures involving donor organs. Furthermore, this form of tissue engineering would accelerate the development of biomaterials that better integrate into the recipient and provide new mechanisms for drug delivery.

We predict that this strategy will provide a simple *ex vivo* method to effectively minimize the antigenicity of allogeneic tissues being transplanted in a clinical setting and may indeed be able to extend the life of an allogeneic graft. The viral vector approach also provides us with the flexibility of testing many other viral proteins that may potentially minimize the immune response against the allogeneic tissue.

7. CONCLUSIONS

We characterized the effect of expressing a viral immunomodulatory protein (MIR2) in a model human cell line, with respect to the cell surface levels of immunoactive molecules and also assessed the ability of these MIR2-expressing cells to evade allogeneic CTLs and NK cells *in vitro*. Flow cytometry analysis of MIR2-expressing cells showed that MHC I molecules are strongly downregulated. In addition, other cell surface proteins involved in immune cell adhesion and signaling, such as ICAM-1 and B7-2 respectively, were also downregulated. Furthermore, we developed a novel flow cytometry-based assay to measure CTL and NK-mediated cytotoxicity against MIR2-expressing cells. The CTL assay demonstrated that MIR2-expressing cells are significantly less susceptible to allogeneic CTL-mediated cytotoxicity, while the NK assay showed that these MIR2-expressing cells also completely evade NK cell-mediated cytotoxicity *in vitro*. Hence, we provide evidence that this strategy of employing viral immunomodulatory mechanisms can simultaneously result in the evasion of acute rejection mediated by CTLs and NK cells. In addition, we show that this strategy can be easily implemented in a tissue-engineered construct using an *ex vivo* retroviral gene delivery approach, which circumvents the need for *in vivo* tissue targeting.

Donor antigen modification using viral immunomodulatory strategies to evade the immune response against allogeneic cells represents a new paradigm in the field of tissue engineering. We believe that this simple approach to selective immunosuppression has an

enormous potential in tissue engineering and deserves further investigation. Based upon our results, we expect that viral strategies of immune evasion can be successfully employed to extend the life of a cell-based tissue-engineered construct in an allogeneic host. We hope that this research can lead to the development of “universal” cells for transplantation that will not require anti-rejection drugs.

8. RECOMMENDATIONS

A number of additional studies can be performed to fully investigate the efficacy of using viral evasion mechanisms in cell-based tissue-engineered constructs to modulate the alloimmune response.

In vitro, it would be interesting to investigate additional cell lines derived from various tissues to determine the general range of MIR2's effectiveness in conferring immunoprotection against allogeneic CTLs and NK cells. Transduced cell lines could also be evaluated for MIR2 expression strength using anti-MIR2 antibodies (or anti-FLAG antibodies or anti-GFP antibodies), stability of expression over time, insertional mutagenesis and for the multiplicity of infection (MOI), which determines the average number of insertions per cell. This data would provide information to evaluate the effectiveness of the retroviral vector in gene delivery. It would also be interesting to assess the effect of MIR2 expression on various cell surface ligands such as MHCI, B7-2, ICAM-1, MICA, MICB and AICL. In addition, it would be particularly instructive to delineate the effect of MIR2 on all relevant MHCI alleles (HLA-A, HLA-B, HLA-C, HLA-E) separately, using monoclonal antibodies. This would help clarify the extent to which MIR2 influences the cell surface expression of each of the different HLA genes. Furthermore, ubiquitously expressed 'house-keeping' genes such as GAPDH and β -actin should also be quantified using transcription and translation assays. Normal expression of such 'house-keeping' genes would indicate normal functioning of the cell expressing

MIR2. It may also be interesting to genetically modify MIR2 to improve its function. For example, it is known that only a small proportion of the total MIR2 expressed is transported from the ER membrane to the cell membrane, where it ubiquitinates the cytoplasmic tails of cell surface molecules. If MIR2 is targeted to the cell membrane, a greater proportion of the expressed MIR2 would exert its effects on cell surface molecules. This modification might enhance the downregulation capabilities of MIR2. As an alternative to genetically modifying MIR2, it might also be worth considering the use of other viral immunomodulatory proteins. For example, human papillomaviruses express a protein called E5, which downregulates HLA-A and HLA-B, but not HLA-C and HLA-E, similar to MIR2's effects (128). Perhaps, a chimeric protein composed of the important domains of multiple viral immunomodulatory proteins may provide the ideal multifunctional protein with optimal immune evasion capabilities.

However, it is essential to optimize the isolation and proper stimulation of CD8+ T cells and NK cells. Ideally, these cells should be purified through multiple rounds of negative selection to minimize their chance of activation before proper stimulation *in vitro*. If PBMCs are to be used as the source of CD8+ T cells and NK cells, then a Ficoll-Hypaque column is preferred over ACK lysis of whole blood. After isolation and proper stimulation of CD8+ T cells and NK cells, it is important to use another means to isolate CTLs and activated NK cells, such that stimulator cells, dead cells and debris are excluded. A CTL marker and activated NK cell marker can be used in conjunction with flow cytometry to determine the purity of the isolated cells.

In order to further assess the immunoprotective effects of MIR2 expression, other functional *in vitro* tests can also be considered. First, it would be instructive to setup proper control cells lacking MHCI, since this molecule is the most important contributor to acute cellular rejection. It may be possible to use anti-HLA antibodies to mask MHC molecules, so that the control cells are the same as the test cells. This would provide a more accurate measure of the degree of immune evasion of MIR2 expressing cells. Second, *in vitro* CTL and NK assays can be performed on MIR2-transduced cells that have been exposed to proinflammatory cytokines such as IFN- γ , IL-1 β and TNF- α . This experiment would more closely simulate the conditions a cell-based tissue-engineered construct is likely to be exposed to soon after transplantation. Since these cytokines are known to modulate MHCI expression, it would be instructive to measure the immunoprotective effect of MIR2-expressing cells under these conditions. Third, it would be interesting to assess the CD8+ T cell response to MIR2-transduced allogeneic APCs. Since MIR2 downregulates B7-2 costimulatory molecules, this test would indicate the stimulation potential of MIR2-transduced APCs, which are known to be the primary mediators in the 'direct' pathway of allograft rejection. A lack of CD8+ T cell stimulation by these APCs may also induce active tolerance through anergy.

In vivo, it would certainly be interesting to transplant a MIR2-transduced stem, progenitor or terminally differentiated cell-seeded tissue-engineered construct into an allogeneic host to assess the efficacy of using viral evasion mechanisms to modulate the alloimmune

response. Finally, it may also be important to assess the potential risks of using viral immunomodulatory proteins such as the tumorigenic potential of MIR2-transduced cells, disease transmission from intracellular pathogens and the host immune response to MIR2 protein expression.

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