DOES HUMAN LEUKOCYTE ANTIGEN-G (HLA-G) PLAY A ROLE IN IMMUNE MODULATION AND VASCULOPATHY IN HEART TRANSPLANTATION?

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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University of Toronto

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ABSTRACT

HLA-G is a protein normally expressed during pregnancy, protecting the fetus from the maternal immune system. Previous studies have shown an association between HLA-G expression post-transplantation and lower incidences of organ rejection. To further examine this beneficial role, we conducted a prospective study following a cohort of heart transplant recipients for one year and measuring their plasma HLA-G levels at various time points. Preliminary analyses failed to reveal an association between HLA-G and various parameters of rejection and vasculopathy. However, we decided to examine the in vitro effects of HLA-G in a smooth muscle cell (SMC) migration assay and whether HLA-G can be modulated pharmacologically. We made the novel observations that purified HLA-G was capable of inhibiting migration of SMCs, a key event in the development of cardiac allograft vasculopathy. IL-10, an anti-inflammatory cytokine, was capable of upregulating HLA-G in a Jeg-3 cell line. The modulation of HLA-G may represent a strategy to protect against vasculopathy, which is a leading cause of morbidity and mortality in heart transplant recipients.
DEDICATION

To the Creator of all things, I am constantly amazed by the architectural brilliance of the human body, the complexity of which will always be beyond my full comprehension. Studying biology at a molecular level has truly enabled me to appreciate the incredible intricacies of the body. I am grateful for the mysteries that continue to exist, giving our creative minds a chance to solve problems.

To the patients who were part of my clinical study, I am indebted to you for the insight gained on the role of health professionals in the wellness journey. You have taught me invaluable lessons in empathy, courage, and resilience that I will carry forward in my future career.
ACKNOWLEDGEMENTS

The studies depicted in this thesis were performed entirely by me. However, research is definitely a collaborative effort and I would not have been able to carry out all the studies without the contributions of many individuals along the way.

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ABBREVIATIONS

ACR         Acute Cellular Rejection
AMR         Antibody Mediated Rejection
APCs        Antigen-Presenting Cells
BSA         Bovine Serum Albumin
CAV         Cardiac allograft vasculopathy
CMV         Cytomegalovirus
CRP         C-reactive protein
CsA         Cyclosporine
CSB         Cytoskeletal Buffer
CSF         Cerebrospinal fluid
DMSO        Dimethyl sulfoxide
DTT         Dithiothreitol
ECG         Electrocardiogram
ECHO        Echocardiography
ELISA       Enzyme-linked immunosorbent assay
EMB         Endomyocardial biopsy
EVT         Extravillous trophoblast
FBS         Fetal Bovine Serum
FK506       Tacrolimus
HCAEC       Human coronary artery endothelial cells
HCASMC      Human coronary artery smooth muscle cells
HLA         Human Leukocyte Antigen
HLA-G       Human Leukocyte Antigen-G
HRP         Horseradish peroxidase
IgG         Immunoglobulin
IL-10       Interleukin-10
ILT2        Immunoglobulin-like transcript-2 (also known as CD85j/LILRB1)
ILT4        Immunoglobulin-like transcript-4 (also known as CD85d/LILRB2)
ISHLT       International Society of Heart and Lung Transplantation
IVUS        Intravascular ultrasound
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>KIR 2DL4</td>
<td>Killer immunoglobulin-like receptor 2DL4</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane-attack complex</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex (also known as HLA)</td>
</tr>
<tr>
<td>MIT</td>
<td>Mean Intimal Thickness</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate membrane</td>
</tr>
<tr>
<td>PI</td>
<td>Protease Inhibitors</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRA</td>
<td>Panel reactive antibodies</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride membranes</td>
</tr>
<tr>
<td>RAD</td>
<td>Everolimus</td>
</tr>
<tr>
<td>REB</td>
<td>Research Ethics Board</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sHLA-G</td>
<td>Shed HLA-G (HLA-G1) or soluble HLA-G (HLA-G5)</td>
</tr>
<tr>
<td>SMCs</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor β1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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DEFINITIONS

Pre-sensitization: Pre-sensitization of the recipient, is the immunological reactivity to a donor organ that is already present at the time of the transplantation due to processes such as a previous transplantation, blood transfusion or pregnancy.

Vasculitis: heterogeneous group of disorders that are characterized by inflammatory destruction of blood vessels.

Echocardiography (ECHO): Songram of the heart

Electrocardiogram (ECG): Interpretation of the electric activity of the heart over a period of time, as detected by electrodes attached to the outer surface of the skin and recorded by a device external to the body

Magnetic resonance imaging (MRI): Medical imaging technique used in radiology to visualize internal structures of the body in detail.

Cytokines: Secreted proteins that mediate and regulate innate and adaptive immunity; mediators of communication between cells of the immune system

Complement: Serum and cell surface proteins that interact with each other as well as other cells of the immune system in a highly regulated manner to recruit phagocytes to the site of infection, general products that eliminate microbes, opsonize microbes and directly lyse microbes.

Polymorphic: In population genetics, a gene is polymorphic when it has many alleles in a population.

Trophoblasts: Cells forming the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large part of the placenta. They are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg.
Myeloid cells: In hematopoiesis, the term "myeloid cell" is used to describe any blood cell that is not a lymphocyte.

Trogocytosis: Process by which cell membrane fragments are exchanged between lymphocytes (B, T and NK cells) and antigen presenting cells. The molecular reorganization occurring at the interface between the lymphocyte and the antigen-presenting cell during conjugation is also called “immunological synapse”.

Allotransplantation: Transplantation of cells, tissues, or organs, to a recipient from a (genetically non-identical) donor of the same species.

Allograft: Transplanted organ from genetically non-identical donor of same species

Semi-allogenic: Sharing some but not all genes

Choriocarcinoma: Quick-growing form of cancer that occurs in the uterus (womb). The abnormal cells start in the tissue that would normally become the placenta.

Carcinoma in situ: An early form of cancer that is defined by the absence of invasion of tumor cells into the surrounding tissue, usually before penetration through the basement membrane.

Actinic Keratosis: Precancerous condition that affects only the top layer of skin (the epidermis). Ultraviolet rays from the sun damages the skin and if left untreated, 15% of AK can turn into a form of skin cancer called squamous cell carcinoma.

Biological marker: Parameter that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions. For example, sHLA-G has been reported to be a
biomarker for embryo quality in human in-vitro fertilization; the secretion of sHLA-G was shown to be necessary for implantation

**Ectopic expression:** Expression of a gene in an abnormal place in an organism. This can be caused by a disease, or it can be artificially produced as a way to help determine what the function of that gene is.

**Refractory symptom:** A symptom that cannot be adequately controlled despite aggressive efforts to identify a tolerable therapy that does not compromise consciousness.

**Hyperplasia:** Increase in number of cells/proliferation of cells.

**Ischemia-Reperfusion injury:** Tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen. The absence of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

**Intimal hyperplasia:** Term used to describe the universal response of blood vessels to injury, resulting in the thickening of the tunic intima.

**PMSF:** Serine protease inhibitor
1. INTRODUCTION

Human leukocyte antigen-G (HLA-G) was first described in 1986 when Ellis and colleagues found a novel HLA antigen on human extravillous trophoblasts and a choriocarcinoma cell line (1). This protein mapped on chromosome 6 was termed HLA-G and was discovered to be expressed by trophoblast cells of the embryo during pregnancy (2). Today we know that under physiological conditions, HLA-G is primarily expressed by trophoblast cells in the uterus; but it is also upregulated in a wide variety of pathological situations such as cancer, solid organ transplantation, autoimmune diseases and viral infections (3). The immunotolerogenic properties have made HLA-G an attractive candidate to study as a potential therapeutic target in certain disease conditions or to be used as a biological marker or diagnostic tool in various pathologic or non-pathological conditions. In this thesis, I explore the role of HLA-G in heart transplantation through both basic science and clinical studies.

1.1. A Primer on Immunology

The immune system is a network of cells, tissues and organs that defends the body against attacks from foreign pathogens. These are mainly microscopic organisms such as viruses, bacteria and fungi. Proteins found on the cell membrane help the body distinguish between “self” and “non-self” to ensure that the immune system does not attack the body’s own cells (4). Anything that can trigger an immune response is called an antigen. An antigen can be a whole microbe or a component of the microbe (5).
The immune system is divided into two branches, namely the innate immunity and the adaptive immunity. Both systems work in an integrated manner to protect the host organism against potential harm. The innate immune response is native to the body, and is the first line of defense against foreign invaders (5). Its cellular and biochemical defenses produce the same rapid response each time. Cells of the innate system are non-specific and recognize structures common to many pathogens. It does not have a stored memory. Some of the cells of the innate immune system include phagocytic cells (e.g. macrophages, neutrophils, monocytes), certain antigen presenting cells (e.g. dendritic cells) and natural killer (NK) cells, which have direct cytotoxic effects (6). Some components of the humoral or antibody-mediated arm of the innate immune system consist of natural antibodies, complement protein system, cytokines, inflammatory mediators (e.g. C-reactive protein), pentraxins, collectins and defensins (6).

On the other hand, adaptive immunity is ‘acquired’ in that it is slower than innate but increases in magnitude with each successive exposure to foreign bodies (5). This second line of defense provides specific responses to specific antigens and stores a ‘memory’ of the antigen. Adaptive immunity consists of professional antigen presenting cells (e.g. dendritic cells, macrophages, B-cells) and lymphocytes (e.g. T-cells, B-cells) (5). In the next few sections, some components of the immune system pertinent to the investigations in this thesis shall be explored.
1.1.1. Innate Immunity: Complement System

The complement system consists of circulating plasma proteins that interact with each other as well as other cells of the immune system in a highly regulated manner (6). It 'complements' the action of antibodies in destroying foreign pathogens. Complement protein pre-cursors are mainly produced by the liver and are cleaved by proteases to be activated (4). When activated, it functions to recruit phagocytes to the site of infection, produce general products that eliminate microbes, opsonize microbes and directly lyse microbes (4). The main pathway by which the complement system is activated is called the 'classical' pathway.

The classical pathway is activated by the binding of complement component C1 to an antigen-antibody complex. The C1-complex is composed of C1q, C1r and C1s molecules (7). The C1 molecules cleave components C2 and C4 to generate C2b and C4b, which combine to form C3 convertase (7). Once C4b is inactivated, its degradation product becomes C4d, which remains covalently bound to the tissue, enabling it to be a durable marker of complement activation (7,8). C4d is currently a widely used marker for antibody-mediated rejection (9).

C3 convertase cleaves component C3 into C3a and C3b. C3a is released while C3b binds to the original C3 convertase to form C5 convertase, a complex that cleaves the C5 into C5a and C5b (4). C5b recruits components C6, C7, C8 and C9 to form the membrane-attack complex (MAC), which spans the pathogen plasma membrane forming a pore. This membrane pore then can kill or damage the pathogen or cell(4).
1.1.2. Adaptive Immunity: Lymphocytes (T-cells and B-cells)

During lymphocyte development, each cell expresses receptors with antigen specificity. The enormous diversity of lymphocytes is a result of the various possibilities in rearrangement of germline DNA and somatic recombination (5). When a lymphocyte encounters its specific antigen, it is activated and will expand into an effector lymphocyte population.

B cells are lymphocytes whose main function is antibody production (4). Each B cell is programmed to make one specific antibody. When a B cell encounters its specific antigen, it is activated and gives rise to plasma cells that produce and release that antibody (4). The biological function of the antibody is determined by its heavy chain (4). There are five types of heavy chains, resulting in five classes or isotypes of antibodies: IgA, IgD, IgE, IgG and IgM (4).

Like B cells, T cells are also antigen specific, as they have specialized antibody-like receptors on their surface that can recognize antigens on infected or cancerous cells (5). They also have a host of co-receptors that aid in antigen and/or other lymphocyte recognition. There are two main classes of T cells, each with different functions, (a) helper T cells and (b) cytotoxic T cells.

Helper T-cells are identified by their surface co-receptor CD4⁺ (5). They secrete cytokines that direct and regulate other immune cells, such as macrophages or B
cells, to carry out their function (5). Cytotoxic T cells have the co-receptor CD8+ marker on their cell surface (5). They are able to recognize and kill host cells that have been infected with a particular intracellular pathogen, like a virus (5).

1.1.3. Major Histocompatibility Complex

The major histocompatibility complex (MHC) comprises approximately a 3.6 mega base pair cluster of highly polymorphic genes located on chromosome 6 in the mammalian genome. It encodes a set of membrane glycoproteins called the MHC molecules as well as genes coding for other proteins involved in mediating interactions of leukocytes (4,10,11). The MHC was originally discovered in the mouse, but has since been characterized in humans and is referred to as the human leukocyte antigen (HLA) complex (12).

The products of the MHC genes are divided into groups: Class I and Class II MHC molecules function in presenting antigens to T cells (4). A third group, Class III, encodes other immune molecules such as complement proteins and inflammatory cytokines; but is not involved in antigen presentation to T cells (4). Class I is further subdivided into classical (Ia) or non-classical (Ib) HLA molecules (10,13).

All nucleated cells express Class Ia MHC molecules while only professional antigen-presenting cells such as macrophages, B cells and dendritic cells express Class II MHC molecules (11). Three genes encode Class Ia molecules, namely HLA-A,
HLA-B and HLA-C and three pairs of genes encode Class II molecules, namely HLA-DR, HLA-DP and HLA-DQ (4).

MHC Class Ib molecules have been demonstrated to play an important role in cellular immune response (13). They differ from Class Ia molecules in that they exhibit limited polymorphism and have more diverse functional roles (11). Most Class Ib molecules are expressed at much lower levels than Class Ia on the cell surface (14). The genes that encode for Class Ib molecules are found with the MHC loci and are called HLA-E, HLA-F and HLA-G (11). Though the precise roles played by Class Ib molecules are yet to be defined, HLA-G has emerged as an attractive molecule because of its immune tolerance properties in various pathological settings such as organ transplantation. HLA-G shall be further explored in the next section.

MHC molecules play a well-established role in T cell development (11). A given T cell will recognize antigens only when it is bound and presented by a particular MHC molecule. This property is known as MHC restriction or MHC restricted antigen recognition (4). Thus, cell expression of MHC is an essential component of acquired immunity. MHC Class Ia molecules present endogenous antigens, found in the cytoplasm, to CD8+ T cells while MHC Class II molecules utilize an endocytic pathway for presentation of exogenous antigens to CD4+ T cells (11). Most individuals are heterozygous at the level of the MHC loci and display co-dominant expression of MHC genes (4). The specific combination of genes present on one
chromosome is known as the MHC haplotype. The differences at the genetic level enable a wide variety of antigen peptides to be presented to T cells (4).

1.1.4. Antigen-Presenting Cells

Antigen-Presenting Cells (APCs) are cells capable of processing antigens and presenting them on the MHC complex to T cells (4). To initiate acquired immunity, naïve T cells need to be activated by recognizing a specific peptide:MHC complex by APCs (4). APCs fall into two categories: professional or non-professional. Professional APCs express MHC Class II molecules constitutively along with other co-stimulatory signals (5). Professional APCs are dendritic cells, macrophages and B cells while non-professional APCs include fibroblasts, thymic epithelial cells, pancreatic beta cells and vascular endothelial cells (5).

Of the three professional APCs, dendritic cells are the most potent. Immature dendritic cells are exceptionally well-equipped for capturing antigens generated by dying, infected, or allogeneic cells using phagocytosis, macropinocytosis, and receptor-mediated endocytosis (15). Once they have their antigen, the dendritic cells are transported to lymph nodes, where they undergo further maturation and interact with naïve T cells. Once matured, dendritic cells function with high potency to activate helper T cells, cytotoxic T cells and B cells (16).

Macrophages are phagocytic cells that play a major role in both innate and adaptive immune systems (15). They are the mature form of monocytes, which circulate in
the blood and continuously differentiate into macrophages upon migration into tissues (5). Macrophages produce several molecules such as growth factors, chemokines, cytokines, proteolytic enzymes, and adhesion molecules (15). They can be induced to express MHC Class II molecules, which then present exogenous antigens to CD4+ T cells. Additionally, macrophages have the ability to control immune responses by secreting anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor β1 (TGF-β1) (17,18).

1.1.5. Inhibitory Receptors

A successful immune response relies on a balanced equilibrium between activating and inhibitory signals transduced by distinct receptors. Activating receptors, such as B cell receptors, T cells receptors and others recognize foreign pathogens and trigger immune responses (19). Inhibitory receptors recognize endogenous molecules constitutively expressed on normal cells, such as MHC Class I molecules (20), thus preventing unnecessary reactions against ‘self’. Furthermore, when activating signals prevail over inhibitory ones, inhibitory receptors are still required to prevent excessive activation of immune cells and facilitate the extinction of immune responses (19).
1.2. Cardiac Transplantation Immunology

1.2.1. Heart Failure and Heart Transplantation

Heart failure is present “when the heart is unable to pump blood forward at a sufficient rate to meet the metabolic demands of the body (forward failure), or is able to do so only if the cardiac filling pressures are abnormally high (backward failure), or both” (21). Heart failure is usually the final and most severe manifestation of nearly every form of cardiac disease, including coronary atherosclerosis, valvular diseases, hypertension, congenital heart disease and cardiomyopathies (21). The number of patients with heart failure is increasing, not only due an aging population, but also because of medical and technological advancements that allow interventions that prolong survival after damaging cardiac insults (21). The prognosis for heart failure is dismal in the absence of intervention (21). The 5-year mortality rate following diagnosis is between 45-60% (21). Patients with severe symptoms (i.e. New York Heart Association class III or IV) generally have a one-year survival rate of only 40% (21).

Despite the poor prognosis, a heart failure patient’s outlook can be improved with interventions such as medications and devices like cardiac resynchronization therapy (21). However, for patients with end-stage heart failure, heart transplantation is the only therapy once the medical therapy is maximized and the patient still has refractory symptoms (22,23). The scarcity in the number of donors limits the number of transplantations (24). Therefore alternate heart support therapies such as ventricular assist devices are selectively used (21).
The therapeutic success of heart transplantation became possible after the introduction of immunosuppressive drugs such as cyclosporine and later tacrolimus (25,26). Today, with reduced infection and rejection rates, heart transplantation is a highly effective treatment limited only by the number of donors (27). Currently, over 3000 heart transplants are performed globally with a half-life of 10 years and a median survival of 13 years for those surviving to one year (28,29). Although rejection rates continue to decline, the risk of rejection still remains and routine monitoring for rejection is important as clinical manifestations of graft dysfunction often are only detected in advanced stages of rejection (30). Additionally, the effect of immunosuppressive drugs exposes the transplanted patients to higher risks of infections and malignancies (31).

According to the 2011 International Society for Heart and Lung Transplantation (ISHLT) Registry Report on heart transplantation (32), the leading causes of death post-transplantation are due to acute rejection, cardiac allograft vasculopathy (CAV), malignancy, infection and graft failure. Within the first month post-transplant, graft failure (primary and non-specific) accounted for about 40% of deaths, followed by multi-organ failure (20%) and non-CMV (cytomegalovirus) infection (13%) (32,33). Between the first month and one year post-transplant, non-CMV infection accounted for almost 30% of the deaths, followed by graft failure (18%) and multiple organ failure (17%) (32). Between one to three years post-transplantation, infection is an important cause of death (30%) (32). After five years post-transplantation, CAV and
late graft failure (likely due to CAV) together accounted for 32% of the deaths, followed by malignancies (23%) and non-CMV infections (10%) (33). In the next few sections, we examine the various types of rejection in-depth.

1.2.2. Rejection

Based on the time course, rejection is classified as hyperacute, acute or chronic. Hyperacute rejection develops within minutes to hours after the transplantation. It is caused by pre-sensitization to donor tissue, and is usually mediated by the consequences of alloantibody and complement fixation (7). Pre-sensitization of the recipient, is the immunological reactivity to a donor organ that is already present at the time of the transplantation due to processes such as a previous transplantation, blood transfusion or pregnancy. This can be detected by measuring serum antibodies in the recipient against donor targets (expanded in Section 3.1.5) (7). Acute rejection takes place over days to years following the transplantation and is mediated by antigen-specific T-cell and/or antibodies towards the allograft. Chronic rejection develops over months to years and also involves allo-immunity to the graft by T cells and/or antibodies (7).

Until the introduction of calcineurin inhibitors such as cyclosporine, and, later, tacrolimus (25,26), allograft rejection was a major limitation to successful heart transplantation. As rejection rates declined, heart transplantation was considered increasingly a more therapeutic option for select patients with end-stage heart disease. However, the risk of rejection still remains significant, especially in the early
period following the transplantation, needing routine surveillance for acute cellular and antibody-mediated rejection (27).

1.2.2.1. Acute cellular rejection

Acute cellular rejection (ACR) is histologically defined as a predominantly T cell mediated response by the recipient with infiltration of lymphocytes and macrophages. This inflammatory response may lead to necrosis of myocytes (27). It was the major limitation to a successful transplantation until the advent of effective immunosuppressive drugs (25,26). Nevertheless, ACR still represents the main risk (26) for mortality in the first year post-transplantation, especially in the first three to six months (24). A number of risk factors for ACR have been identified: younger age of recipient, female gender (donor and recipient), higher number of human leukocyte antigens (HLA) mismatches, black recipients, and induction therapy (34,35).

The diagnosis of ACR is made by endomyocardial biopsy (described below) using an internationalized standardized grading scale, which was originally devised by International Society of Heart and Lung Transplantation (ISHLT) in 1990 and subsequently revised in 2004 (36) (Figure 1). Inflammatory changes may however be sporadic throughout the myocardium or may predominantly affect the subendomyocardium (27). This limits the findings of the biopsy to the sampling areas. Therefore, the diagnosis of rejection also relies on clinical presentation and findings on echocardiograms, which may or may not be supported by histology (27).
Typically a cardiac biopsy procedure is performed weekly for the first month post-transplantation, once every two weeks for the second month, monthly until six months and then once every three months until the end of the first post-operative year (27). Beyond the first year, biopsies are likely not of clinically significance due to lower rejection rates (37). Additional biopsies are usually performed about a week after treatment of a documented rejection to confirm its resolution or when there is clinical suspicion of ACR (24). Over 60% of adult heart transplantation recipients experience one or more episodes of ACR within the first six months post-transplantation (ISHLT grade ≥ 1R) (27) . The 12-month incidence of biopsy-proven ACR ISHLT grade ≥ 2R is approximately 25% to 30% (38).

Although the endomyocardial biopsy is an important diagnostic tool, it is invasive, anxiety provoking, uses significant resources and still carries a small risk in the incidence of complications (<2%) (24,39,40). Therefore other non-invasive approaches have been explored to predict ACR. The ideal test would utilize fewer resources and allow early detection of rejection before any myocyte necrosis occurs (27).

Some of the imaging modalities that have been explored for detecting ACR are echocardiography (ECHO) and cardiac magnetic resonance imaging (MRI), while an electrical parameter that has been considered is the intramyocardial electrocardiogram (ECG) (27,30) . ECHO has a strong negative predictive value of 95% but this technique has issues with reproducibility across centres and patient
Cardiac MRI has the potential of detecting early changes that accompany allograft rejection with high sensitivity but further studies are needed for its validation. Additionally, MRI is an expensive modality with limited access and can show non-specific findings. The intramyocardial ECG has a high negative predictive value, which might allow significant reduction in the number of biopsies performed, however, more investigations are needed before widespread adoption. To date, none of these techniques have demonstrated sufficient sensitivity and specificity to replace the biopsy.

A variety of circulating biomarkers have also been studied in allograft rejection. Some of these are markers of myocardial necrosis (e.g. creatinine kinase-MB, cardiac troponin), antibodies to HLA, various cytokines (e.g. interleukin-6, interleukin-8, TNF-α), complement fragments, clotting factors, soluble CD30, endothelin P-selectin and c-reactive protein (CRP). In one study, CRP levels showed a significant difference between rejection (≥ grade 3) and no rejection groups. Unfortunately for all these markers, their accuracy was either suboptimal, specificity was low or preliminary results were not replicable. To be deemed clinically relevant, large-scale replication is necessary with refinement of the most useful timing of measurement.

Though there is limited evidence for imaging techniques and peripheral biomarkers in identifying ACR, the search for surrogate markers continue. To date, the only non-invasive test for detecting cardiac allograft rejection that has reached clinical
use in USA, involves screening for genetic markers. This process helps determine a gene expression profile that may that might represent ACR. A recent study (44) showed that this technique had a high negative predictive value for diagnosing ACR. However, this study only included a majority of low-risk patients who had been over a year post-transplant. Furthermore, it is presently not applicable to monitoring antibody-mediated rejection.

1.2.2.2. Antibody-mediated rejection

Antibody-mediated rejection (AMR) (also known as vascular or humoral rejection) of the cardiac allograft was first recognized by Herskowitz and colleagues (45). Their work showed an association between arterial vasculitis and poor outcomes despite immunosuppressive therapy with cyclosporine. Hammond and colleagues explored this further and were the first to describe immunoglobulin and complement deposition in the microvasculature (46). Since then, AMR has been recognized as a cause of non-cellular rejection (47).

Rejection that is caused by antibodies is mediated by different mechanisms from rejection that is caused by T cells, thereby requiring other approaches to diagnose, treat and prevent the rejection (7). AMR follows a clinical-pathologic continuum starting with the presence of circulating antibodies only (humoral response), followed by a silent phase (circulating antibodies plus C4d deposition) without histologic or clinical alterations, a subclinical stage with circulating antibodies, histologic and
immunopathologic changes, and finally a symptomatic AMR with clinical manifestations (7,24,48).

Antibodies induce rejection acutely through the fixation of complement, resulting in tissue injury and coagulation. The endothelial cells are believed to be the main target of the antibodies. Complement activation recruits macrophages and neutrophils, causing additional endothelial injury. Antibodies and complement also induce gene expression for adhesion molecules by endothelial cells, which is thought to remodel arteries and basement membranes, leading to fixed and irreversible anatomical lesions that permanently compromise graft function (7).

Presently, the blueprint used for cardiac AMR includes capillary endothelial changes, macrophage and neutrophil infiltration, interstitial edema, and vascular deposition of immunoglobulins (IgG) and complement pathway components such as C4d (36).

Although the frequency of ACR has been reduced with the introduction of immunosuppressive drugs, the incidence of AMR remains unchanged. It occurs in about 10% to 20% of post-transplant recipients and is linked to poor outcomes (47) due to the hemodynamically compromised organ, increased development of cardiac allograft vasculopathy and increased mortality (49). Clinically, AMR can present during the first month post-transplantation and is accompanied by a rise in donor-specific antibodies (50). AMR occurring in the first week post-transplantation (acute AMR) is usually an indication that the recipient was pre-sensitized to the donor antigens (50). Asymptomatic patients with untreated biopsy-proven AMR, have a
higher incidence of mortality and greater chance of developing cardiac allograft vasculopathy (51). The diagnostic criteria for acute AMR can be seen in Figure 2 (7).

Some of the risk factors associated with the development of AMR include pregnancy, female gender, elevated panel-reactive antibodies, positive donor-specific crossmatch, cytomegalovirus (CMV), seropositivity, prior implantation of ventricular assist device, and/or re-transplantation (27,36).

Since complement activation is a key event in AMR, the detection of complement split product - C4d - in capillaries, is considered a valid surrogate for AMR (52,53). C4d is bound covalently to the graft tissue and, therefore, serves as a more durable and visible marker of complement activation than other components (53-55). C4d participates in the classic pathway of complement activation.

Other markers can also be considered as part of the diagnosis of AMR. According to the revised formulation for nomenclature in the diagnosis of rejection the immunologic evidence for AMR is considered to be “immunoglobulin (IgG, IgM and/or IgA) plus complement deposition (C3d, C4d and/or C1q) in capillaries by immunofluorescence on frozen sections; and/or CD68 staining of macrophages within capillaries (CD31- or CD34-positive) by immunohistochemistry; and C4d staining of capillaries by paraffin immunohistochemistry.” (36). Figure 3 shows the ISHLT classification of AMR. If there is no histologic or immunopathologic features
of AMR, this is classified as ‘AMR 0’, whereas if there is histologic features associated with AMR along with positive immunofluorescence / immunoperoxidase staining for C4d or CD68, it is classified as ‘AMR 1’.

1.2.2.3. Cardiac Allograft Vasculopathy

Cardiac allograft vasculopathy (CAV) is a leading cause of morbidity and mortality after the first year following heart transplantation (29,56-59). It is a unique form of accelerated coronary artery disease affecting heart transplant recipients (60). CAV is detectable in approximately 7% of recipients within the first year, 30% within five years, and in 50% of survivors within 10 years of transplantation. Early CAV, diagnosed within one year of transplantation, is an independent predictor of mortality at five years (32,60). Risk factors for CAV can be divided into two broad categories: (i) traditional risk factors that are non-immunologic and non-specific to transplantation and (ii) risk factors that are specific to transplantation (60). The former includes hypertension, hyperlipidemia, obesity, tobacco use and diabetes (60). The latter include viral infections, ischemia-reperfusion injury, HLA mismatch, number of rejection episodes, recipient age at the time of transplant, and donor factors such as donor age, comorbidities and body size (29,60).

The arterial wall consists of three layers; from innermost layer of the lumen to the outmost, they are the intima, media and adventitia (21) (Figure 4). The intima acts as a metabolically active barrier between circulating blood and the vessel wall (21). Its inner lining is called the endothelium. The endothelium secretes a variety of
substances such as antithrombotic molecules, vasodilators, vasoconstrictors and chemokines (21). A normal endothelium provides a protective, nonthrombogenic surface with homeostatic vasodilatory and anti-inflammatory properties (21) (Figure 5). The media is the thickest layer, rich in elastic fibres, smooth muscle cells and extracellular matrix (21). The adventitia contains nerves, lymphatics and blood vessels that nourish the vessel wall (21).

The predominant feature of CAV is a concentric intimal hyperplasia and diffuse narrowing along the entire length of the vessel, as opposed to the discrete focal lesions usually seen in native coronary artery disease (60) (Figure 6). This leads to luminal stenosis of the vessels and eventually obstructive lesions. The development of CAV is a multifactorial and complex process initiated by immune and non-immune factors (60). There is sufficient evidence to suggest that endothelial injury and ensuing inflammation is a key inciting event for the development of CAV (60,61). Many factors contribute to endothelial cell injury including organ preservation injury, alloimmune response (cellular and humoral rejection), immunosuppressive regiments, and possibly chronic viral infection (62). Activated endothelial cells secrete various cytokines and growth factors that attract circulating leukocytes such as lymphocytes, macrophages, neutrophils and eosinophils (61). These inflammatory cells infiltrate the tissues by means of adhesion molecules (63). Multiple signals from the activated endothelial cells cause the migration of smooth muscle cells from the media into the intima of the coronary artery. Ultimately, the
accumulation of proliferative smooth muscle cells and extracellular matrix results in thickening of the vessel wall (Figure 5).

Of the several diagnostic methods available, intravascular ultrasound (IVUS) is currently considered to be the gold standard, as it is more sensitive than angiography and it can detect the extent of intimal thickening by imaging vessel wall structure rather than just the luminal diameter (58,64). However, it is an invasive method (65). CAV is classified into mild, moderate or severe according to the ISHLT 2010 nomenclature (59) (Table 1).
1.3. Human Leukocyte Antigen-G

Human Leukocyte Antigen-G (HLA-G) is a MHC Class Ib immune molecule (66) and is primarily expressed in the trophoblast cells of the placenta. It has been reported to play a crucial role in mediating maternal tolerance of the fetal semi-allograft (2,67,68). It is ‘non-classical’ as it differs from MHC Class Ia molecules by its genetic diversity, expression, structure and function (69). Unlike Class Ia, HLA-G exhibits low polymorphism (70,71) and is highly tissue restricted. Besides expression in fetal tissue, HLA-G is constitutively expressed only in the adult thymus (72), cornea (73), pancreatic islets (74), and erythroid and endothelial-cell precursors (75). The expression of HLA-G is upregulated in various pathological conditions such as cancer, organ transplantation, autoimmune diseases and viral infections (3).

1.3.1. HLA-G: Isoforms and structure

While the gene structure of HLA-G is similar to that of other HLA class I molecules, the HLA-G primary mRNA transcript is alternatively spliced, resulting in four membrane bound (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and three soluble (HLA-G5, HLA-G6 and HLA-G7) isoforms (76,77) (Figure 7). HLA-G5, HLA-G6 and HLA-G7 is the soluble counterpart of HLA-G1, HLA-G3 and HLA-G3 respectively. In addition, shed HLA-G can be generated by proteolytic release of the HLA-G membrane-bound forms (3). Alternate splicing stands out because a) it leads to soluble and truncated protein production, and b) it can be regulated as shown by the
fact that depending on the cell type, some isoforms are expressed but others are not (69,78,79).

The ‘complete’ HLA-G molecule, which is the membrane-bound HLA-G1 and its soluble component HLA-G5, are the most investigated isoforms (71). Their structure is identical to other HLA Class I molecules: a heavy chain made of three extracellular globular domains, namely $\alpha_1$, $\alpha_2$, and $\alpha_3$, non-covalently bound to $\beta_2$-microglobulin (13,80,81). However, a unique feature is that the primary transcript contains a stop codon in exon 6, shortly after the coding sequence for the transmembrane domain (82). One extracellular domain is deleted in HLA-G2 ($\alpha_2$), two in HLA-G3 ($\alpha_2$ and $\alpha_3$), and one in HLA-G4 ($\alpha_3$) (83,84). Soluble HLA-G lacks the cytoplasmic and transmembrane segments (76). Their C-terminal is encoded by intron 4 for HLA-G5 and HLA-G6, and by intron 2 for HLA-G7 (76,85,86) (Figure 7) (3,87). HLA-G1 and HLA-G5 can be found as $\beta_2$-microglobulin-free forms (87).

The discovery of peptide presentation by HLA-G1 and HLA-G5 was made when DNA sequence analysis showed that $\alpha_1$ and $\alpha_2$ domains form a peptide pocket made up of nine amino acids (82,88,89). The diversity of peptides presented by HLA-G1 and HLA-G5 is five times less than other classical HLA Class I molecules (88,90) possibly suggesting than HLA-G is not the most ideal candidate for antigen presentation.
1.3.2. HLA-G: Receptors

Unlike classical HLA Class I molecules, HLA-G does not seem to have significant immune stimulatory functions (69). However, HLA-G is known to play an important role in immune tolerance through binding inhibitory receptors found on T cells, B cells, natural killer (NK) cells and other APCs. Three HLA-G inhibitory receptors have been described so far: a) immunoglobulin-like transcript-2 (ILT2), b) immunoglobulin-like transcript-4 (ILT4) and c) inhibitory killer immunoglobulin-like receptor 2DL4 (KIR2DL4) (91-94). According to a revised nomenclature of HLA molecules (69,95), ILT2 is also known as CD85j or LILRB1, ILT4 is also known as CD85d or LILRB2, and KIR2DL4 is also known as CD158d. Studies have also reported that HLA-G binds to CD8+ T cells and cause apoptosis, which is similar to other HLA Class I molecules (96,97).

ILT2 is expressed by B cells, some T cells, some NK cells and all monocytes/dendritic cells (91). ILT4 is myeloid-specific and only expressed by monocytes/dendritic cells (94). The expression of KIR2DL4 is mainly restricted to a CD56-subset of NK cells, which constitute a minority of peripheral NK cells but a majority of uterine NK cells (98-100). ILT2 and ILT4 are clearly inhibitory receptors and bind to both classical and non-classical HLA molecules (91,94). In terms of binding to HLA-G, the difference lies in the structures they recognize: ILT2 recognizes HLA-G associated with β2-microglobulin, whereas ILT4 recognizes β2-microglobulin and the free heavy chains α1, α2, and α3 (101). On the other hand, the KIR2DL4 receptor is known for sending both inhibitory and stimulatory signals.
making its mechanism via HLA-G controversial. HLA-G is the sole ligand for KIR2DL4 (93). Nonetheless, HLA-G is the ligand of highest affinity for ILT2 and ILT4 receptors (102), especially HLA-G multimers (101). Table 2 summarizes the various HLA-G receptors, cellular distribution and HLA-G binding sites (3,87).

HLA-G1 and HLA-G5 can be found as β2-microglobulin-free heavy chains and as homodimers (103,104). Dimerization of HLA-G occurs through the creation of disulphide bonds between 2 unique cysteine residues at position 42 (Cys42-Cys42 bonds) and 147 (Cys147-Cys147) of the HLA-G heavy chains (103,104) and commonly observed using gel electrophoresis (63). Some studies have shown that ILT2 and ILT4 binding sites of HLA-G dimers are more accessible than HLA-G monomers which may explain why HLA-G dimers binds two ILT receptors with a higher affinity and slower dissociation rate than monomers (69,101).

1.3.3. HLA-G: Function

Figure 8 adapted from Carosella and colleagues summarizes some of the main functions of HLA-G (3,69). HLA-G induces immune tolerance by inhibiting the proliferation of T cells (105,106), the cytotoxic activity of cytolytic T cells (107-109), the lytic functions of natural killer (NK) cells (109-113), the alloproliferative response of CD4⁺ T cells (114,115), and the maturation and function of dendritic cells (71,116,117). Soluble HLA-G5 or shed HLA-G1 (sHLA-G), which is generated by proteasomal cleavage from the cell membrane, have similar functions. The other HLA-G isoforms have been less well studied, and little is known about their function.
except that membrane-bound HLA-G2, HLA-G3, and HLA-G4 can inhibit NK-cell and cytotoxic T cell cytolysis in-vitro. The functions of HLA-G discussed below can be classified into two groups: (i) the direct immuno-inhibitory functions through blocking effector cells, and (ii) the indirect immuno-inhibitory functions through regulatory cell generation via trogocytosis (87).

1.3.3.1. Direct immuno-inhibitory functions of HLA-G

HLA-G is primarily expressed at the maternal-fetal interface by cytotrophoblast cells that do not express HLA class I molecules, other than HLA-C and HLA-G. Rouas-Freiss and colleagues were the first to show that HLA-G protected cytotrophoblasts against NK cell lysis, and HLA-G mediated protection was eliminated when the cytotrophoblasts were treated with an HLA-G specific monoclonal antibody (67). Another experiment by the same group showed in-vitro that the α1 domain, specifically of HLA-G1 and HLA-G2 transfected into a cell line, inhibited the cytotoxic activity of NK cells (118). Thus, HLA-G interacts with the inhibitory receptors at the surface of NK cells leading to their functional inhibition. These studies preliminarily established the tolerogenic function of HLA-G in the context of pregnancy (87).

Other direct interactions of HLA-G1 with ILT2 and ILT4 inhibitory receptors have shown that it inhibits the antigen-specific cytolytic function of T cells (87), the alloproliferative response of CD4+ T cells (114,119), the on-going proliferation of NK and T cells (106,120), and the maturation of dendritic cells (117). Shed HLA-G1, which is HLA-G1 released from the cell surface by proteolytic cleavage, or HLA-G5,
the soluble form of HLA-G1, have generated the same functional outcomes as HLA-G1 except for the maturation of dendritic cells (105,121-124). Studies have demonstrated the capability of HLA-G truncated isoforms (HLA-G2, -G3, -G4) to inhibit NK and cytolytic T cells *in-vitro*; however, the receptors that interact with these isoforms to mediate their function remain unknown (109,125). Recently HLA-G has been shown to inhibit B-cell proliferation, differentiation, and immunoglobulin production for both T-dependent and T-independent antigens (126).

1.3.3.2. **Indirect immuno-inhibitory functions of HLA-G**

Direct HLA-G function is a ‘short-term’ immune inhibition as the inhibitory functions are carried out only as long as HLA-G is bound to its receptor. But, it has now been shown that HLA-G can have ‘long-term’ inhibitory effects as well through the generation of regulatory or suppressor cells through trogocytosis. Regulatory cells are immune effectors that are critically involved in promoting and maintaining immune tolerance because they can inhibit the reactivity of other effectors. Regulatory cells can be of various subsets that include APCs, CD8+ T cells, and CD4+ T cells (87). Trogocytosis is a fast, cell-to-cell contact-dependent uptake of membrane fragments (120). In this process, all the molecules associated with that membrane fragment are transferred as well, even if they are not involved in cell-to-cell crosstalk, thus the transfer is non-specific.

The majority of the work on trogocytosis has been carried out in murine T cells and has shown that CD4+ and CD8+ T cells can acquire MHC Class II and MHC Class I
molecules, respectively, from APCs (127-129). A study by Caumartin showed that HLA-G1 could be acquired via trogocytosis by NK cells from tumor cells or other HLA-G expressing cells (120). Upon acquisition of HLA-G1, activated NK cells stopped proliferating, were no longer cytotoxic, and behaved as suppressor cells (120). Another study by LeMaoult and colleagues investigated whether HLA-G was capable of inducing regulatory T cells. It demonstrated that CD4\(^+\) and CD8\(^+\) T cells that had been stimulated in the presence of HLA-G1 not only lost their capability to respond to antigenic stimulation, but also were able to inhibit allo-proliferative responses through HLA-G1 that they acquired (106) and differentiated into regulatory T cells capable of inhibiting the reactivity of other T cells (119). Such HLA-G-induced regulatory T cells required HLA-G for their generation but not for their function (106,119) These data suggested that the immune inhibitory function of a few HLA-G producing cells could be spread beyond the reach of HLA-G expressing cells, thus enforcing the significance of long-term inhibitory effects of HLA-G.

Since these first experiments, multiple HLA-G-related regulatory cell types have been described, that function through or independent of HLA-G. For example, naturally occurring HLA-G-positive T cells are generated centrally and characterized by their expression of membrane-bound and soluble HLA-G. They are increased at sites of inflammation and may not require HLA-G for generation, but partially function through it (130-132).
Most of the HLA-G functions are described *in-vitro*, mainly because HLA-G is seldom expressed in non-pathological contexts, and its expression is heavily dependent on micro-environmental parameters (87). Yet it has been shown to have an importance in pathological situations like cancer, transplantation and autoimmune diseases. What makes HLA-G unique is the multiplicity of its cellular targets, its capability to inhibit immune responses at every level, and the broad range of its mechanisms of action (87).

### 1.3.4. HLA-G: Role in pregnancy

Once fertilized, a zygote undergoes several cell divisions or cleavages as part of its development. Once it has over 64 cells, it is now called a blastocyst (133). A blastocyst has two layers: an inner cell mass, known as the embryoblast and an outer layer known as the trophoblast (133). The latter combines with the maternal uterine lining to form the placenta (133). Being a derivative of the zygote, the trophoblast is semi-allogenic to the mother. The trophoblast differentiates into the cytotrophoblast and the syncytiotrophoblast (133). The core of the placental villi is surrounded by the cytotrophoblast, which can differentiate into the extravillous trophoblast (EVT). The EVT grows out from the placenta and invades the decidual layer of maternal uterus. In the process, the trophoblast comes in direct contact with the maternal blood to establish the uteroplacental circulation (134,135). This is where the embryo is exposed to the maternal immune system, which identifies the embryo as “non-self” due to paternal antigens. Yet the pregnancy paradox is that the embryo is protected from rejection.
The role of HLA-G was originally discovered and since then extensively studied in the context of pregnancy. The highly polymorphic HLA class I (HLA-A and HLA-B) and class II molecules displayed by somatic cells, which initiate recognition of the allograft and its subsequent rejection, are not displayed by most blastocyst cells (136,137). However, the EVT cells, which invade the uterus, express an unusual combination of HLA class I molecules: small amounts of HLA-C, HLA-E and HLA-F as well as preferential expression of HLA-G (90,137). Figure 9 shows the expression of HLA-G isoforms in trophoblast cell subpopulations. The main leukocytes present at the maternal uterine mucosa are the NK cells and macrophages, not B cells and T cells (136).

Given HLA-G expression patterns, its main physiological relevance is at the fetal-maternal interface, contributing to the tolerance of the fetus by the immune system of the mother (138). So far, no pregnancy in which all of the proteins derived from the HLA-G gene are absent has been reported (139,140). HLA-G has been shown to protect fetal cytotrophoblast cells from maternal NK cells through the interaction with their inhibitory receptors (111,141). Studies have shown that HLA-G expression seemed to be a pre-requisite to embryo implantation and the subsequent pregnancy (142,143). For example, a review by Rizzo and colleagues found that the secretion of soluble HLA-G antigens by early embryos was necessary for a successful implantation and could be a marker of increased pregnancy rate following in-vitro fertilization (143). The importance of HLA-G production by placental trophoblasts is
also evident in pre-eclampsia and in unexplained recurrent spontaneous abortion (138). In these conditions, there is reduced expression of both HLA-G mRNA and protein expression in comparison with control placentas (140,144-146).

1.3.5. HLA-G: Role in cancer

Due to its immunosuppressive properties, HLA-G expression by tumors can be a strategy to escape immune surveillance, especially since HLA-G is advantageous in protecting against both innate and adaptive immune responses. HLA-G expression was first described by Paul and colleagues in melanoma cells, where they found high levels of HLA-G transcription in three melanoma cell lines and differential HLA-G protein expression patterns, which may protect them from NK cell lysis (147). Since its earlier discovery, HLA-G has been studied in the context of cancers. Studies on more than 1000 malignant lesions have confirmed that HLA-G expression has been shown to protect lesions against cytolysis (121,148,149).

Alongside its implication in the escape of tumor cells from host anti-tumor immune responses, HLA-G appears to be detected in the transplant recipient's tissues, mainly those with fewer rejection episodes (150). Since skin carcinomas develop frequently in organ transplant recipients, one study looked at the frequency of various skin cancers in kidney transplant recipients (150). They found that HLA-G was expressed in 35% of skin cell carcinoma specimens, 47% of in situ carcinoma (early form of cancer), 27% of actinic keratosis (pre-cancerous sun damages to the skin) and 14% of basal cell carcinoma (most common type of skin cancer) (150).
Tumors may express various isoforms of HLA-G and regulate their expression. For example, in melanoma, cell surface HLA-G1 expression is lost after tumor cells are cultured, as the expression is switched to intra-cellular HLA-G2 expression (151). From the literature, it is clear that the frequency of HLA-G expression varies noticeably between different types of cancer, between different studies within the same tumor (3,87,152). This might likely be due to the criteria of patient selection and the methodology used to detect the presence of HLA-G in the tumors. HLA-G has been detected in biopsies from cancer patients mostly by immunohistochemistry or PCR. Immunohistochemistry is the preferred method over PCR because it detects the production of HLA-G protein, versus HLA-G mRNA detection by PCR - which does not imply the protein production, due to a post-transcriptional control (87).

Figure 10 adapted from Carosella and colleagues (3) is an excellent summary of studies that examine the percentage of HLA-G positive tumor lesions in various types of cancers (3,152). Increased HLA-G expression has been found with a higher frequency in choriocarcinoma (fast-growing cancer in the uterus), where HLA-G expression was present in 78% of the cases (153) and 93% of intermediate trophoblastic tumors (154). Other gynecological tumors with increased expression of HLA-G are breast (155-158), endometrial (159,160) and ovarian cancers (160,161).
In the digestive system, HLA-G expression was described in esophageal squamous cell carcinoma (162), colorectal cancer (163-165), gastric cancer (166-168) and liver cancer (169,170). Amongst hematological tumors, the presence of HLA-G was noted in cutaneous lymphoma (171), or in acute leukemias (172). In addition, plasma HLA-G levels were increased in B chronic lymphocytic leukemia (173), non-Hodgkin lymphoma (174), Hodgkin lymphoma (175), and multiple myeloma (176). In the respiratory system, HLA-G has been associated with various types of lung cancer (177-179). Finally, in the nervous system, the expression of HLA-G has been associated with neuroblastoma (180). Figure 11 is a summary of HLA-G positive samples in various cancers and the potential utility of HLA-G as a tumor marker in those cancers.

HLA-G expression has not only been detected in lesion biopsies, but also in plasma samples of patients suffering from cancer. Circulating HLA-G is either soluble HLA-G (mostly HLA-G5) or shed HLA-G1 and the source of this protein can be either the tumoral cells or the reactive immune cells. (156,172,181) . The high proportion of HLA-G-expressing tumors contrasts its absence in healthy tissue, suggesting HLA-G upregulation and its association with malignant transformation (87). In spite of a heterogeneous HLA-G expression in tumors, even a low expression may be biologically significant because only a small percentage of HLA-G-positive cells is sufficient to induce tolerance (87). For example, only 10% of HLA-G-positive glioma cells within 90% of HLA-G-negative cells are enough to induce an immune inhibitory effect (121).
1.3.6. HLA-G: Role in inflammatory and autoimmune diseases

The upregulation of HLA-G expression has been shown in several pathological conditions such as inflammatory and autoimmune diseases, which is often the result of an overactive immune system or an immune system unable to discriminate self from non-self (87). The investigated diseases include multiple sclerosis (132,182-184), rheumatoid arthritis (185,186), systemic lupus erythematosus (187,188), myopathic inflammations(189), atopic dermatitis (190,191) and psoriasis (191-193). The expression of HLA-G has been associated with immune suppression, needed to make up for the overactive immune insult in several diseases.

Multiple sclerosis (MS) is an autoimmune disease that was investigated the earliest and in most depth with regards to HLA-G. It is a chronic inflammatory disease that causes demyelination of the brain and spinal cord, with its hallmark being the presence of plaque lesions in the white matter that contain the damaged myelin sheath (194). The active disease is diagnosed by detection of lesions through MRI (87). Patients develop intermittent neurologic symptoms and signs with relapse-free periods that shorten as the disease progresses (87,194).

A study by Fainardi and colleagues examined soluble HLA-G (sHLA-G) in the cerebrospinal fluid (CSF) of 50 MS patients (183). They found that sHLA-G levels were significantly higher in the CSF of patients with MS when compared with 77 controls or patients with other neurological disorders such as meningitis or
Alzheimer’s disease (183). This data suggests that sHLA-G in CSF might be a useful biomarker in the follow-up of the disease activity. It may be easier to obtain sHLA-G from blood serum than the CSF; however, the peripheral blood sHLA-G may not reflect the inflammatory status in the brain. A recent study examined serum sHLA-G and did not find a difference between controls and MS patients (195).

Wiendl and colleagues performed immunohistochemical studies on brain specimens and found that the HLA-G protein was strongly expressed in brain specimens from patients with MS while it was rarely detectable in the non-pathological control specimens (184). ILT2, a receptor for HLA-G, was also found in MS brain specimens, thus emphasizing the relevance of this inhibitory pathway in-vivo (184). The HLA-G immunoreactivity was observed in acute plaques, in chronic active plaques, in perilesional areas as well as in normal appearing white matter. In all these areas, microglial cells, macrophages, and in part endothelial cells were identified as the primary cellular source of expression. Analysis of the CSF of MS patients revealed that monocytes were the main source of HLA-G expression (184). Recently, a novel population of HLA-G positive T-regulatory cells were discovered in the inflamed CSF and in inflammatory demyelinating lesions of MS brain specimens (132). Taken together and using MS as an example, it can be seen that the presence of HLA-G might play an important role in inflammatory and autoimmune diseases.
1.3.7. HLA-G: Role in solid organ transplantation, particularly heart transplantation

Due to its immunosuppressive properties, HLA-G has been recently studied in the context of solid organ transplantation. The presence of HLA-G has been linked to increased graft tolerance and reduced rejection in heart (78,196-201), kidney (202-208), liver (170,209-212), liver/kidney (212-214), and lung transplantation (215). Assessment of HLA-G could potentially be a useful tool to determine tolerance status of the transplanted organ.

In heart transplantation, the presence of myocardial and soluble HLA-G expression has been associated with reduced acute cellular rejection (196,197,200) and antibody-mediated rejection (201). Lila and colleagues examined this link by conducting two single-centre retrospective studies of 51 heart transplant recipients (78,196). They measured the HLA-G expression in myocardial biopsies and serum and correlated this with episodes of rejection (78,196). Acute rejection was assessed by examining endomyocardial biopsies within the first post-operative year. Chronic rejection – examining cardiac allograft vasculopathy (CAV) – post-first operative year was detected using angiography of the coronary arteries (196). The 20% of the patients with HLA-G-positive biopsies had significantly fewer acute rejection episodes than the HLA-G-negative group (196). 86% of patients who never experienced acute cellular rejection were HLA-G-positive (196). For the chronic rejection study, 15 cases of CAV were detected in HLA-G-negative patients, while none of the nine HLA-G-positive patients experienced CAV. These results suggest
that HLA-G expression may play an important role in immune tolerance post-heart transplantation (196). A longitudinal study on the same cohort showed that the HLA-G expression was stable over time (196).

Since the initial investigations, several groups have examined the role of serum HLA-G in heart transplant recipients. While these studies have shown intra- and inter- patient variability, there is a tendency for higher serum HLA-G to be associated with fewer rejection episodes (197,198,216). Two studies by Luque and colleagues showed that patients with higher serum HLA-G levels (>30 ng/mL) pre- and post-transplantation had no recurrent rejection whereas 50% of those with lower serum HLA-G levels (<30 ng/mL), had recurrent severe rejection (197,198). This study also found an increase in serum HLA-G levels 2 hours after the administration of immunosuppressive treatment, showing a potential link for immunosuppressive agents in modulating HLA-G (197,198).

A more recent study by Lila and colleagues correlated graft rejection with soluble HLA-G levels in the serum of heart transplant recipients (216). Patients with high concentrations of soluble HLA-G (>200 ng/mL; n=6) had no episodes of acute or chronic rejection. Amongst patients with HLA-G levels between 90-200 ng/mL (n=12), 16% had low incidence of acute rejection and there was no chronic rejection. Amongst patients with HLA-G levels between 7-60 ng/mL (n=16), there were higher number of acute rejection episodes but no chronic rejection. In patients who had no HLA-G expression (n=40), all of them displayed a high number of acute rejection
episodes and signs of chronic rejection (216). All these patients were retrospectively followed from 1 to 18 year post-transplantation, which showed a higher number of chronic rejection in patients with lower HLA-G (216).

Finally, a study from our research group, retrospectively examined two groups of heart transplant recipients within the first post-operative year: the non-rejection group (n=29), with no moderate or severe rejection episodes (ISHLT Grade < 2R) and the rejecting group (n=38), with at least two moderate or severe rejection episodes (ISHLT Grade ≥ 2R) (200). In the former group, 86% of patients had HLA-G-positive biopsies with 11% in the latter group. Thus, this study showed a significant negative association between myocardial HLA-G expression and acute cellular rejection after cardiac transplantation.

The relationship between HLA-G and transplantation might have therapeutic implications as patients with high HLA-G could have their immunosuppressive therapy reduced, whereas those with a low HLA-G titre would have to be monitored more closely for rejection. Additionally, providing exogenous HLA-G as an immunosuppressive agent could potentially complement other therapies (69).

1.3.8. HLA-G: modulation

Table 3 summarizes some of the human diseases associated with HLA-G and its clinical applications. Outside of the primary physiological expression during pregnancy, the expression of HLA-G during pathologic conditions such as organ
transplantation, cancer and autoimmune diseases suggest that microenvironmental factors can control the ectopic HLA-G expression in injured cells and tissues either at the transcriptional or post-transcriptional level (3).

It has been identified that IL-10 selectively induced HLA-G transcription in human trophoblast cells and induced cell surface HLA-G expression in peripheral blood monocytes (217). IL-10 is selective in that while it upregulated HLA-G, it downregulated other MHC classical Class I and Class II molecules in monocytes (217). IL-10 is a cytokine with anti-inflammatory and immunosuppressive effects (218). It has a broad range of biological activities. For example, a study by Fainardi and colleagues noted that IL-10 levels in CSF were higher in MS patients without lesional activity on MRI scans versus patients with lesions (183). Furthermore, this study noted a positive correlation between IL-10 and HLA-G levels in the CSF (183).

HLA-G upregulation was also noted with stress induced by heat shock (219). This study was interesting in that it showed an increase in the level of the different HLA-G alternative transcripts without affecting other HLA class I proteins (219). Other factors that affected HLA-G upregulation were nutrient deprivation in cell culture conditions (220), hypoxia (221), and cytokines such as granulocyte-macrophage colony-stimulating factor (222), interferons (223-225), tumor necrosis factor-α (TNF-α) (226), transforming growth factor β (TGF-β) (222), and leukemia inhibitory factor (223).
Some reproductive steroid hormones such as progesterone have been shown to play a role in modulating HLA-G. Progesterone is an essential steroid for the maintenance of pregnancy. Functions attributed to progesterone in pregnancy include the stimulation of growth and differentiation of the endometrium to allow for implantation, inhibition of myometrial contractions, and the induction of immune tolerance to the fetus (227). Yie and colleagues examined the effect of progesterone on HLA-G expression in primary cultures of cytotrophoblasts and Jeg-3 chorioncarcinoma cells, which is an extravillous trophoblast in-vitro cancerous cell line (227). Both cell types displayed increased concentration of HLA-G in a dose-dependent manner after being cultured in the presence of progesterone (227). Another study by the same group suggested that a potential mechanism of immunomodulation by progesterone might be through the regulation of gene expression via a progesterone response element in the HLA-G promoter region (228). Given the association between HLA-G expression and reduced rejection after heart transplantation, our research group examined vascular human coronary artery endothelial, aortic endothelial, and coronary artery smooth muscle cells, none of which express HLA-G at baseline. Following treatment of these cells with progesterone, the three cell types demonstrated an upregulation of cell surface and soluble HLA-G in a dose-dependent manner (229). This important finding confirms that myocardial cells and endothelial cells are capable of expressing HLA-G (230).

The association of reduced rejection and HLA-G expression post-transplantation has led to the suggestion of immunosuppressive therapy as another potential modulator
of HLA-G (197,198,211,231). A study by Luque and colleagues found an increase in serum HLA-G levels 2 hours after the administration of immunosuppressive treatment, which consisted of cyclosporine (CsA) or tacrolimus (FK506), methylprednisone (MP), and mycophenolate mofetil (MMF) (197). In another clinical study, Sheshgiri and colleagues compared soluble HLA-G levels in heart transplant patients receiving two different anti-proliferative agents commonly used post-transplantation: MMF (n=8) and everolimus (RAD) (n=9) (232). Among patients receiving RAD, 78% expressed detectable levels of plasma HLA-G compared with only 25% of patients receiving MMF (232). Given the small sample size, the clinical relevance of this study is still unclear and larger clinical investigations are needed to examine whether RAD is independently associated with increased HLA-G expression (232).
2. INVESTIGATIONS: RATIONALE AND HYPOTHESES

2.1. HLA-G in heart transplant recipients: A prospective study

2.1.1. Rationale

While it has been shown that the presence of HLA-G is correlated to increased graft tolerance and reduced rejection in heart (78,196-201), the studies to date are retrospective. To the best of our knowledge, no prospective investigations have been conducted to determine the role of HLA-G in determining the risk for developing cardiac allograft vasculopathy (CAV). Additionally, to date, no non-invasive test exists to determine if patients have severe endothelial thickening or underlying CAV. The current gold standard for diagnosing CAV is intravascular ultrasound (IVUS) but it is an invasive method (58,64,65). The other standard procedure used to assess rejection is the endomyocardial biopsy, which is also invasive, and is not a risk free procedure (24,39,40). Therefore, it is necessary to explore simpler, non-invasive approaches that can reliably predict rejection.

Given the link between HLA-G expression and reduced rejection scores in transplantation, the serum levels of HLA-G could potentially be a reliable marker to determine tolerance status. This study was, therefore, designed with the purpose of gaining a deeper understanding of the expression of HLA-G and coronary artery disease post-transplantation due to rejection and CAV. For this, we followed a cohort of heart transplant recipients from pre-transplant to post-transplantation for one year.
2.1.2. Hypothesis

We hypothesize that soluble HLA-G expression in the plasma of heart transplant recipients will be negatively correlated with:

a) Acute cellular rejection as evaluated by ISHLT rejection scores based on T-cell infiltration

b) Antibody-mediated rejection as evaluated by ISHLT rejection scores based on the presence of C4d and panel reactive antibodies (PRA)

c) Cardiac allograft vasculopathy as evaluated by intravascular ultrasound

2.2. Effect of HLA-G on Smooth Muscle Migration in-vitro

2.2.1. Rationale

Cell-surface and circulating HLA-G isoforms have been shown to be protective through the inhibition of immune responses directed against the allograft. Previously, a study by Dorling and colleagues found that HLA-G inhibited the migration of natural killer cells across porcine endothelial cells when they were transfected with HLA-G1 (233). Unpublished observation by Bouteiller and colleagues initially found that purified recombinant soluble HLA-G1 down-regulated endothelial cell migration in-vitro (234). Later, the same group confirmed that in-vitro, soluble HLA-G inhibited the migration of human umbilical vein endothelial cells (HUVEC) using a chemotactic migration assay (235). These findings suggest that HLA-G molecules may affect migration of different cell subsets (236). Additionally,
the HLA-G inhibitory receptor ILT4 found on placental vascular smooth muscle cells suggests that HLA-G may have novel functions in these cells (237). Given the possible anti-migratory properties of HLA-G, and its potential biological effect on smooth muscle cells, we aimed to examine the role of HLA-G in human coronary artery smooth muscle cells – which are the main component of the media of arterial blood vessels and play a critical role in the development of cardiac allograft vasculopathy (CAV).

2.2.2. Hypothesis

We hypothesize that HLA-G will inhibit the migration of human coronary artery smooth muscle cells \textit{in-vitro} because of its potential anti-migratory properties, thus contributing to the prevention of CAV.

2.3. Modulation of HLA-G Expression \textit{in-vitro}

2.3.1. Rationale

Previously, it was shown by our research group that \textit{in-vitro} human coronary artery endothelial cells are capable of expressing HLA-G when stimulated with progesterone (229). Another study by our group found that the immunosuppressive agent, everolimus but not MMF, was associated with HLA-G expression in heart transplant recipients (232). Given this correlation, in this experiment, we determined to understand whether everolimus would modulate the expression of HLA-G in
human coronary artery endothelial cells, which is capable of expressing HLA-G though not at baseline, and that of Jeg-3 cells, which are known to express HLA-G even at baseline.

Additionally, we also investigated another HLA-G modulator called IL-10, an anti-inflammatory molecule that has been shown to upregulate HLA-G transcription in trophoblast cells (217). We examined whether IL-10 would modulate HLA-G in Jeg-3 cells. We did not investigate IL-10 in endothelial cells as a previous study in our lab already examined this and found that IL-10 does not impact HLA-G expression in this cell type (229).

2.3.2. Hypothesis

We hypothesize that everolimus will upregulate HLA-G in both human coronary artery endothelial cells and Jeg-3 cells and that IL-10 will upregulate HLA-G in Jeg-3 cells.
3. METHODOLOGY

3.1. Clinical Study

3.1.1. Patient enrollment

This clinical study was approved by the local research ethics board (REB #: 06-0650-A). An informed consent was obtained from all patients on the heart transplant waitlist. 50 consecutive patients that consented and underwent heart transplantation at the Toronto General Hospital between 2007 and 2009 were enrolled in the study. Given that about 25-30 heart transplant surgeries are done at the hospital annually, the number of patients enrolled represent the sample size collected over the course of the Masters degree timeframe (which included two years of patient enrollment and a one-year follow-up).

All patients received induction therapy with anti-thymocyte rabbit globulin. Treatment consisted of a triple therapy: a calcineurin inhibitor (cyclosporine or tacrolimus), an anti-proliferative agent (sirolimus, MMF or everolimus) and steroids (prednisone). Prednisone was discontinued at one year post-transplantation if there were no signs of cellular rejection. Sirolimus was indicated for de novo patients with renal failure after transplantation in place of calcineurin inhibitors. All patients who received everolimus were enrolled in a de novo randomized clinical trial.
3.1.2. Blood collection and processing

To evaluate HLA-G, plasma was collected at pre-transplantation and at 3, 6 and 12 months post-transplantation during routine endomyocardial biopsy procedures. 10 mL of venous blood was collected from each patient in EDTA-coated BD Vacutainer blood collection tubes and centrifuged at 3500 rpm at 4°C for 20 minutes. The plasma was then transferred to microtubes, flash frozen in liquid nitrogen and stored in -80°C for further analysis.

3.1.3. HLA-G measurement: ELISA

Plasma HLA-G levels were determined with an HLA-G–specific enzyme-linked immunosorbent assay (ELISA) kit (Exbio, Praha, Czech Republic). This kit measures soluble HLA-G (sHLA-G), specifically shed HLA-G1 (membrane-bound) and HLA-G5 (soluble) isoforms. The manufacturer’s recommended protocol was followed.

The optical densities were measured at 450 nm and at 620 nm as the reference wavelength (Bio-Tek ELISA reader Instruments, KC4 analysis Software). The sHLA-G concentration was determined according to a standard curve using four-parameter logistic curve fitting. sHLA-G is represented in Units/mL (U/mL). The detection limit was 3 U/mL. From personal communication with the manufacturer, 100 U/mL of HLA-G is approximately 40-50 ng/mL. Samples were analyzed in duplicate.
3.1.4. Acute cellular rejection: biopsy scoring

In the first year post-transplantation, all patients underwent routine endomyocardial biopsies (EMB) during a right heart catheterization. Typically, a cardiac biopsy procedure is performed weekly for the first month post-transplantation, once every two weeks for the second month, monthly until six months and then once every three months until the end of the first post-operative year (27).

All histological specimens were graded according to the 2004 revised International Society of Heart and Lung Transplantation (ISHLT) criteria for acute cellular rejection (ACR) (36) [Figure 1]. ISHLT Grade 0 corresponded to no rejection, while ISHLT Grade 1R, 2R and 3R corresponded to mild, moderate and severe rejection, respectively (36). Mild rejection consists of interstitial and/or perivascular inflammatory cell infiltration with up to one focus of myocyte damage; moderate rejection involves two or more foci of cellular infiltration with associated myocyte damage; finally severe rejection involves diffuse cellular infiltration with multifocal myocyte damage with or without edema, hemorrhage and/or vasculitis (36). One cardiac pathologist at our institution (Dr. J.B.) reviewed all biopsy samples and scored them for rejection, thus eliminating any inter-observer variability.

Patients were divided into two groups based on their rejection profiles. Group A, non-rejectors, has no episodes of moderate or severe rejection (ISHLT < Grade 2R), while Group B, rejectors, has at least one episode of moderate or severe rejection (ISHLT ≥ Grade 2R) within the first year post-transplantation.
3.1.5. Antibody-Mediated Rejection: PRA analysis and C4d staining

The presence of circulating antibodies to anti-human leukocyte antigen (HLA) in the sera of heart transplant recipients, or “humoral sensitization,” has been associated with an increased frequency of acute rejection (238,239,239,240). Pre-sensitization to HLA Class I and/or Class II antibodies predispose heart transplant recipients to higher rates of AMR and worse outcomes (241). New assays have been developed to allow a more accurate and discriminate appraisal of preformed antibodies. As a result, sensitized patients awaiting suitable heart donors can now be better risk-stratified and screened by virtual crossmatch. Panel reactive antibodies (PRA) screening is used to determine the presence of the circulating antibodies (HLA A, B, and DR) in the serum to a random panel of donor lymphocytes (242). The degree of sensitization towards antibodies is evaluated for potential heart transplant recipients (243). In my study, all patients underwent virtual crossmatch prior to transplant.

The PRA results are presented as the percent of panel reactivity (i.e., the number of wells with positive reactivity over the total number of wells tested × 100). For a patient with elevated PRA levels, his/her serum is directly tested against the prospective donor’s lymphocytes, a procedure known as a crossmatch (239). Most commonly, a PRA elevation level of ≥10% is required for the titer to be considered positive and this was used as the criterion in the present study (239).
Since complement activation is a key event in AMR, the detection of complement split product - C4d - in capillaries is considered a valid surrogate for AMR (52,53). Capillary involvement and immunohistochemistry for C4d analysis were performed by pathologists to detect the presence of AMR. C4d staining was classified based on the ISHLT classification, where no histological evidence of Cd4 was considered AMR grade 0 and a positive Cd4 immunostain was considered AMR grade 1 (59) (Figure 3). AMR grade 1 was further classified into three grades, 1 to 3, based on staining intensity as developed by Chantranuwat and colleagues (244).

### 3.1.6. Cardiac allograft vasculopathy: Coronary Angiography and IVUS imaging

Selective coronary angiography and intravascular ultrasound (IVUS) was performed at one month and one year post-transplantation by cardiologists. Coronary angiograms were analyzed and quantified by two independent cardiologists blinded to the HLA-G levels in these patients. The degree of stenosis was expressed as the percent reduction of the internal luminal diameter in relation to the normal reference. Based on these results, patients were classified as having normal or abnormal CAV according to the ISHLT 2010 classification (59). CAV Level 0 was classified as no disease, and CAV Levels 1-3 were classified as increasing severity of disease (Table 1).

IVUS was performed following the completion of the diagnostic coronary angiography. IVUS images were obtained from the left anterior descending artery
using a mechanical 30-MHz system and motorized pullback device. Studies were recorded on 0.5-inch super VHS videotape, and digitized by an image-processing computer for subsequent off-line analysis. The full-motion sequence was examined frame by frame to select the image with the most atherosclerosis for analysis. Maximal intimal thickness (MIT) was measured as the greatest distance from the intimal leading edge to media-adventitia border. The average intimal thickness was also measured for 10 random frames. CAV was defined as a MIT of ≥ 0.5 mm. To measure the progression of coronary disease, the difference in MIT from one month (baseline), to MIT at one year was measured (MIT progression).

3.1.7. Statistics

SPSS for Windows (version 13) was used to perform statistical analysis. To determine the statistical significance of differences between means, we performed unpaired t-tests. All values were presented as mean ± standard error of mean (SEM). To test associations, Pearson’s correlation was used. We considered p < 0.05 to be statistically significant; however, exact p values are provided to enable the reader to determine clinical and statistical significance.

3.2. In-vitro Studies

3.2.1. Materials used

Cells: Human coronary artery endothelial cells (HCAEC), human coronary artery smooth muscle cells (HCASMC), MesoEndo Endothelial Cell Medium and Smooth
Muscle Cell Growth Medium were purchased from Cell Applications Inc., San Diego, CA, USA. The Jeg-3 cells were from American Type Culture Collection, Manassas, VA, USA. Jeg-3 medium, RPMI 1640, FBS and antibiotics (100 U/mL streptomycin and 100 µg/mL penicillin) were obtained from GIBCO, Invitrogen Corp., Carlsbad, CA, USA.

**Antibodies and drugs:** Purified recombinant HLA-G was purchased from Origene, Rockville, MD, USA. Everolimus was purchased from LC Laboratories, Woburn, MA, USA. Recombinant human IL-10 was purchased from R&D Systems Inc., Minneapolis, MN, USA. Monoclonal antibody to HLA-G, 4H84, was purchased from Exbio, Praha, Czech Republic. HRP-conjugated goat anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Finally, β-actin was purchased from Abcam Limited, Cambridgeshire, UK.

**Chemicals/Western blotting:** For protein determination, the Bio-Rad DC protein dye kit from Bio-Rad Laboratories, Hercules, CA, was used. PI cocktail, PMSF, DMSO, BSA, DTT, Na₂HPO₄, HEPES and EDTA were purchased from Sigma Aldrich, St. Louis, MI, USA. NaCl, KH₂PO₄, Tween20, Tris base, 1% Triton-X-100 and SDS were purchased from Fisher Scientific, New Jersey, USA. KCl was obtained from Bioshop Canada Inc., Burlington, Canada. The standard protein ladder used for gel loading was Cruz Marker Molecular Weight Standards from Santa Cruz Biotechnology, Santa Cruz, CA. ECL Plus from GE Healthcare, Little Chalfont, UK, was used for chemiluminescence detection of blots.
**Equipment and software:** The humidified tissue incubator used for cell culture was Model MCO-18AIC, Sanyo Electric Co., Ltd, Moriguchi, Japan. The sonicator used in obtaining cell lysates was Sonic Dismembrator Model 500, Fisher Scientific, New Jersey, USA. The light microscope used for imaging was Nikon TE 300 inverted microscope with a Photometrics CoolSnap HQ camera. For Western blot analysis, Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA) was used. The μQuant Universal Microplate Spectrophotometer and the KC4 software from Bio-Tek Instruments, Vermont, USA were used for protein determination.

**Smooth Muscle Cell Migration Assay:** The cell migration inserts used were BD BioCoat Matrigel Invasion Chamber obtained from BD Biosciences Pharmingen, San Jose, CA. The Amicon Ultra-15 Centrifugal filter unit (ultracel 30kDa filter), which was used as 30 kDa molecular filter, was purchased from Fisher Scientific, New Jersey, USA. The Pierce Detergent Removal Spin Columns was from Thermo Scientific, Rockford, IL, USA. The Diff Quik Staining Kit for smooth muscle cell staining was obtained from Siemens Healthcare Diagnostics, NY, USA.

### 3.2.2. Commercial cell lines: endothelial cells, Jeg-3 cells, smooth muscle cells

Since endothelial cells are strategically located between the circulating blood and the vascular smooth muscle, endothelial cells are crucial in releasing various vaso-
regulatory substances and cytokines that interact with the immune system. Hence we used endothelial cells to study the possible mechanisms of HLA-G on these cells. Commercially available Human Coronary Artery Endothelial Cells (HCAEC), which were isolated from normal human coronary arteries, were used for the experiments (245).

The human choriocarcinoma cell line Jeg-3 is known to express detectable levels of HLA-G at baseline (2). Hence these cells were used as a positive control for HLA-G and to see if HLA-G can be modulated in these cells. Jeg-3 cells were graciously obtained from Dr. Librach’s research group at Women’s College Hospital.

As reviewed previously, the proliferation and migration of smooth muscle cells are key events in the development of atherosclerosis and CAV. Therefore we studied commercially available Human Coronary Artery Smooth Muscle Cells (HCASMC) which are derived from the tunica intima and tunica media of normal human, fibrous plaque-free coronary arteries (246).

### 3.2.3. Cell culture

Commercially available human coronary artery endothelial cells (HCAEC), human coronary artery smooth muscle cells (HCASMC) and Jeg-3 cells were grown to full confluence in 10 cm cell culture dishes in a sterile, humidified tissue incubator at 37 °C and 5% CO₂ for all experiments. HCAEC, HCASMC and Jeg-3 cells were grown in MesoEndo Endothelial Cell Medium, Smooth Muscle Cell Growth Medium and
RPMI 1640 Medium, respectively. The media were all supplemented with appropriate growth factors as recommended by manufacturers, 10% FBS and antibiotics (100 U/mL streptomycin and 100 µg/mL penicillin).

From the primary culture, cells were passaged once and cryopreserved in solution consisting of culture medium, FBS and dimethyl sulfoxide (DMSO) in a 5:4:1 ratio, respectively, until further use. The cryopreserved cells were subsequently used in experiments between passages 3-5. Cells were split in 1:3 for passaging.

### 3.2.4. Boyden Chamber Migration Assay

To investigate the migration of smooth muscle cells (SMC), we used the Boyden chamber assay model (247,248). The Boyden chamber assay is based on a chamber of two medium-filled compartments separated by a microporous membrane. Cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane towards the lower compartment, in which chemotactic agents are present. After an appropriate incubation time, the membrane between the two compartments is fixed, stained, and the number of cells that have migrated to the lower side of the membrane is determined (247,248) [Figure 13 shows a schematic diagram of Boyden Chamber].

The migration of SMCs was assessed using the Boyden Chamber Assay with a matrigel-coated PET membrane that had 8 µm pores (‘matrigel inserts’). The matrigel-coating mimics basement membrane, which is digested by the SMCs prior
to its migration from the media to the intima in a coronary blood vessel \textit{in-vivo}. SMCs were seeded in the upper chamber, while the lower chamber was filled with control medium to which chemoattractants were added.

The following media compositions were used as controls in the lower chamber:

a) Negative Control (no migration): SMC Basal Medium with 0.5\% Bovine Serum Albumin and no additional growth factors nor FBS (SmBSA)
b) Positive Control (induces migration): Full SMC medium with growth factors and 1\% FBS (Sm-full-1\%FBS).

SMCs were starved for 1 hour in SMC basal medium prior to trypsinizing and doing a cell count. Then 50,000 SMCs were resuspended in 200 \( \mu \)l of SmBSA and added to the upper chamber (that is, the matrix insert). 500 \( \mu \)l of SmBSA ± chemoattractants was added to the wells of a 24-well culture dish (the lower chamber). The SMCs were incubated for 24 hours in a humidified tissue culture incubator at 37°C and 5\% CO\textsubscript{2}. Following the incubation, the upper surface of the matrigel membrane was cleaned to remove any non-migrated cells, while the lower surface was fixed using the Diff-Quik Staining Kit, which stains for nuclei and cytoplasm. The manufacturer’s recommended protocol was followed for staining. Once stained, the membranes were mounted onto microscope slides. The migrated cells were observed at 20x magnification using a light microscope and imaged in four random fields per membrane. The average count of the four fields was utilized for calculations. All migration experiments were done in duplicates. Two imaging
software programs, Image J and GIMP, were utilized to assist with cell counting. To reduce bias, a blinded volunteer counted all images separately.

3.2.4.1. Obtaining sHLA-G from Jeg-3 cells

To observe whether or not HLA-G inhibited SMC migration, Jeg-3 cells were used as a surrogate to obtain soluble and membrane-bound HLA-G because purified HLA-G protein was not commercially available at the time of these experiments. Once Jeg-3 cells were grown to 80% confluence, they were placed in SMC basal medium with growth factors but no FBS for 24 hours. This ‘conditioned’ medium from the Jeg-3 cells now contained shed HLA-G1 and soluble HLA-G5 produced by the cells. The molecular weight of the HLA-G monomer is ~38kDa and that of HLA-G dimer is ~78kDa. The conditioned Jeg-3 medium was concentrated using a 30 kDa molecular protein filter according to the manufacturer’s protocol, which retains all the proteins >30kDa, including HLA-G. sHLA-G ELISA analysis showed that the conditioned medium had ~75 U/mL of sHLA-G, which is comparable to the level of HLA-G in patient blood plasma. The concentrated conditioned medium had ~1000 U/mL of sHLA-G.

3.2.4.2. Membrane-bound HLA-G extraction from Jeg-3 cells

The membrane-bound HLA-G was extracted from the cell membranes of the Jeg-3 cells. The following solutions were prepared:

   a) Phosphate Buffer Saline (PBS) with protease inhibitors (PI) (0.5µl/mL) and phenylmethylsulphonylfluoride (PMSF) (0.5µl/mL).
b) Tris Buffer Saline (TBS) (10 mM Tris and 0.1 M NaCl dissolved in H₂O, pH 7.5) with PI (0.5µl/mL) and PMSF (0.5µl/mL).

c) Cytoskeletal Buffer (CSB) (5 mM EDTA, 30 mM HEPES, 150 mM NaCl and 1% Triton X-100 dissolved in H₂O, pH 7.4, PMSF at 0.5µl/mL).

Unless otherwise noted, all reactions were carried out at 4°C. Once Jeg-3 cells were grown to confluence in 10 cm dishes, cells were washed in PBS twice, and then collected using a cell scraper with 1 mL of ice-cold PBS+PI+PMSF. The cells were then centrifuged at 130,000 rpm for 10 min at 4°C. The supernatant was discarded. Jeg-3 cell pellets were then re-suspended in TBS+PI+PMSF before sonication on ice for 10 seconds. Subsequently, the homogenate was centrifuged at 130,000 rpm for 10 minutes at 4°C, pelleting the cell membranes. The supernatant (cytosolic proteins) was discarded and the membrane pellets were disrupted by re-suspending them in CSB, followed by vortexing thrice with 10 minutes of incubation on ice in between vortexing. After the final vortex, the proteins were centrifuged at 130,000 rpm for 10 min at 4°C. The supernatant, which now contains membrane proteins, was stored in -80°C until further use.

The use of a detergent is necessary to extract membrane-bound proteins; unfortunately, the detergent interferes with downstream experiments and analysis. Therefore, before use in experiments, the detergent was removed from the Jeg-3 membrane protein extract by passing it through a Pierce detergent removal spin column using the manufacturer’s recommendation. The concentration of HLA-G in
membrane-bound protein extracts was >4000 U/mL HLA-G as measured by the sHLA-G ELISA kit.

3.2.4.3. Treatment Groups for Migration Assay

Table 10 summarizes the various treatment groups investigated in this migration assay. In all experiments, 50,000 SMCs were resuspended in 200 µl of SmBSA in the upper chamber.

3.2.4.4. Purified HLA-G protein

The migration studies outlined above were completed prior to the availability of a commercially produced, purified, recombinant HLA-G protein (rh-HLA-G, Origene). Once on the market, a migration study was performed using the purified protein. To test a dose-response, rh-HLA-G at 100, 500 and 1000 ng/mL were added to the lower chamber diluted in Sm-full-1%FBS.

3.2.5. HLA-G modulation in-vitro with everolimus and IL-10

Since the immunosuppressive drug, everolimus (RAD), has been previously associated with the presence of HLA-G in heart transplant recipients (232), we investigated whether it modulates HLA-G in HCAEC and Jeg-3 cells. Additionally, we examined whether the anti-inflammatory cytokine, IL-10, would modulate HLA-G in Jeg-3 cells. IL-10 was not examined in HCAEC, because an earlier study from our laboratory showed that IL-10 did not impact HLA-G levels in this cell type (229).
HCAEC were treated with 10, 50 and 100 ng/mL doses of RAD, while Jeg-3 cells were treated with 100 ng/mL of RAD and 10, 25, 50, 100 and 200 ng/mL of IL-10. Treated cells were placed in a humidified tissue incubator at 37 °C and 5% CO₂ for 24 hours. To determine the expression of HLA-G, whole cell lysates were collected, and the Western immunoblot technique was employed to determine the expression of the HLA-G protein.

### 3.2.6. Whole cell lysate preparation

(See preparation of solutions in Section 4.1.4.2.) Upon completion of the 24-hour treatment, the cells were washed twice with PBS and then collected using a cell scraper with 1 mL of ice-cold PBS+PI+PMSF. Cells were then centrifuged at 130,000 rpm for 10 min at 4°C. The supernatant was discarded. Jeg-3 cell pellets were then resuspended in TBS+PI+PMSF. Subsequently, the solution was sonicated on ice for 10 seconds before vortexing thrice with a 10-minute incubation in ice in between vortexing. Thereafter, the pellet suspension was centrifuged at 130,000 rpm for 10 minutes. The resulting supernatant, containing whole cell lysate, was divided into two aliquots – one for protein concentration determination and the other for Western blot analysis. Proteins were frozen in liquid nitrogen and stored at -80°C until ready for analysis.
3.2.7. Protein Determination

Protein determination was performed using the Bio-Rad DC protein dye kit. Samples were diluted 1:10 and placed into a microtube. Standards were also prepared by serial dilution of bovine serum albumin (10mg/mL) to achieve concentrations of 0.05mg/mL, 0.1mg/mL, 0.25mg/mL, 0.5mg/mL, and 0.77mg/mL. Once prepared, 10µl of samples and standards were transferred into second microtube, where 50µl of Working Reagent A and 400µl of Working Reagent B were added. 200µl of each of these sample solutions was transferred into a 96-well plate and incubated for 15 minutes at room temperature. Following the incubation, the protein concentration was measured spectrophotometrically at an absorbance (Abs) wavelength of 750nm. The KC4 software was used for analysis. Sample absorbance values were corrected by subtracting the absorbance value of blank (empty) wells. The protein concentration was determined by the following equation:

\[
\text{Sample Protein Concentration (mg/mL)} = \left(\frac{(\text{Sample Abs}_{750} - \text{Blank Abs}_{750})}{m}\right) \times \text{dilution factor}
\]

where \(m\) is the slope of the standard curve passing through zero.

3.2.8. Western immunoblotting: HCAEC

20 µg of samples were supplemented with 6X Laemmli loading buffer and denatured for 5 minutes under non-reduced conditions at 95°C. Then the samples were loaded and separated using a 4% stacking gel and 10% tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred to polyvinylidene fluoride (PVDF) membranes. Blocking was performed in 5% milk
solution for 1 hour at room temperature. HLA-G expression was determined with the use of an HLA-G-specific monoclonal antibody, 4H84, at a dilution of 1:1000. 4H84 recognizes the α1 domain, which is found on all isoforms of HLA-G (249). Blots were placed in the primary antibody solution at 4°C for 24 hours. Subsequently, the blots were washed and then incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature at a dilution of 1:5000. After incubation, the blots were washed and then processed using ECL Plus as a substrate for chemiluminescence detection on X-ray films. The films were analyzed with a Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software. β-actin was used as the loading control for all blots. A primary monoclonal β-actin antibody and secondary antibody were used, both at a dilution of 1:15,000, for 1 hour at room temperature. HLA-G expression values was normalized using β-actin as the protein loading control.

3.2.9. Western immunoblotting: Jeg-3 cells

Western blots for Jeg-3 cells were processed similar to that for HCAEC with noted differences. Since HLA-G is capable of forming dimers through the creation of disulphide bonds (103,104), Jeg-3 lysates using dithiothreitol (DTT). 3 µg of samples were supplemented with 6X Laemmli loading buffer and denatured for 5 minutes under DTT-reducing conditions at 95°C. Then the samples were loaded and separated using a 4% stacking gel and 12% tris-glycine SDS-PAGE gel. Once gels were transferred to PVDF membranes and blocked, HLA-G expression was determined with the HLA-G monoclonal antibody, 4H84, at a dilution of 1:30,000 in
4°C for 1 hour. Subsequently, the blots were washed and then incubated with goat anti-mouse HRP-conjugated secondary antibody at a dilution of 1:30,000 for 1 hour at room temperature. Then blots were washed and processed using ECL Plus as a substrate for chemiluminescence detection on X-ray films. The films were analyzed with a Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software. β-actin was used as the loading control for all blots. A primary monoclonal β-actin antibody and secondary antibody were used, both at a dilution of 1:15,000, for 30 minutes at room temperature. HLA-G expression values was normalized using β-actin as the protein loading control.

3.2.10. Statistical Analysis

Statistical analysis was performed using SPSS for Windows (version 13) software. To determine the statistical significance of differences between means, we performed unpaired t-tests. All values are expressed as mean ± standard error of mean (SEM). A one-way ANOVA with Tukey HSD post-hoc was used for comparisons between groups. We considered a p value of < 0.05 to be statistically significant. Exact p values are provided to enable the reader to determine clinical and statistical significance as well.
4. RESULTS

4.1. Clinical Prospective Study

4.1.1. Baseline Characteristics

Of the 50 patients in the study, there were 34 men and 16 women, with a mean age of 49±12 years. The indication for heart transplantation was idiopathic cardiomyopathy (32%), ischemic cardiomyopathy (44%) and other heart diseases (24%). All patients underwent coronary angiography at baseline (within one month post transplantation) and at one-year post transplantation. 35 patients had IVUS imaging done at baseline and at one year post transplantation. Patients were divided into two groups according to the ISHLT CAV classification: Group A had no CAV (ISHLT grade 0) and Group B had evidence of CAV (ISHLT grades 1, 2 and 3). Table 4 shows the baseline characteristics of the patients. Group A consisted of 35 patients (30 males, 5 females) with a mean age of 48±14 years and Group B had 15 patients (12 males, 3 females) with a mean age of 50±9 years at the time of heart transplantation. There were no significant differences in mean age, gender, indication for heart transplantation, or immunosuppressive therapies.

4.1.2. Acute Cellular Rejection: HLA-G Expression and Biopsy Scores

As seen in Figure 12, there was no significant difference between non-rejecting (Group A, n=35) and rejecting (Group B, n=15) patients in sHLA-G levels for pre-transplantation, and post-transplantation at 3, 6 and 12 months. Mean sHLA-G
levels pre-transplantation between Group A and B were 70.45 ± 12.79 and 69.19 ± 8.49 U/mL (p=0.932) respectively; at 3 months post-transplantation, 68.02 ± 7.61 and 74.56 ± 11.14 U/mL (p=0.621), respectively; at 6 months post-transplantation 67.84 ± 4.95 and 59.78 ± 6.54 U/mL (p=0.648), respectively; and at 12 months post-transplantation 59.68 ± 7.11 and 57.57 ± 10.24 U/mL (p=0.885), respectively.

4.1.3. Antibody Mediated Rejection: HLA-G Expression and PRA/C4d

There was no correlation between pre-transplantation PRA levels and HLA-G expression at 3, 6, and 12 months ($r^2=0.00$, p=0.92; $r^2=0.06$, p=0.115; $r^2=0.015$, p=0.45, respectively) (Table 5). Furthermore, there was no correlation between pre-transplantation PRA levels and MIT at 1 month, 1 year and MIT progression from one month to one year ($r^2=0.034$, p=0.34; $r^2=0.004$, p=0.708; $r^2=0.03$, p=0.233, respectively) (Table 5). Similarly, as seen in Table 6, we found no correlation between C4d staining score and HLA-G levels at any time point.

4.1.4. Cardiac Allograft Vasculopathy: HLA-G Expression and IVUS

Table 7a shows the IVUS results at one month, at one year and MIT progression in both patients with and without CAV. Unsurprisingly, MIT and MIT progression was significantly higher in Group B than Group A (At one month, MIT of Group A 0.22±0.08 mm versus Group B 0.57±0.2 mm (p<0.001); at one year, MIT of Group A 0.23±0.08 mm versus Group B 0.67±0.15 mm (p<0.001); MIT progression from one month to one year, Group A 0.014±0.036 mm versus Group B 0.15±0.12 mm...
(p<0.02)). Table 7b shows an IVUS image of a normal blood vessel and Table 9c shows an IVUS image of an abnormal artery with CAV.

We compared sHLA-G levels measured at 3, 6, and 12 months between the two groups (Table 8). Though not significant, there was trend towards lower sHLA-G levels in patients with CAV (Group B mean sHLA-G levels were 65.7±51 U/mL, 60.9±23 U/mL and 69.9±42 U/mL, respectively, at 3,6 and 12 months post-transplantation) than in non-CAV patients at these time points (Group A mean sHLA-G levels were 71.7±39 U/mL, 67.8±28 U/mL and 54.3± 33 U/mL respectively at 3,6 and 12 months post-transplantation).

35 patients underwent IVUS at one month and one year post-transplant to assess the progression of coronary disease. There was no significant correlation between sHLA-G levels and MIT at one year (r²=0.45, p=0.7) and the MIT progression from one month to one year (r²=0.254, p=0.1) (Table 9).

With the sample size of 50, the power calculated was 0.35 for ACR and CAV, meaning that there was a 35% probability of detecting a difference in the two groups. For AMR, the power calculated was 0.05.

4.2. **SMC Migration Assay**

To examine the effect of HLA-G on the migration of SMCs, we used a Boyden chamber migration assay, where we investigated whether the migration of SMCs
towards chemoattractants would be inhibited by HLA-G. Initially, soluble and membrane-bound HLA-G was obtained from Jeg-3 cells, a cell line known to express HLA-G at baseline (2). At a later time, various doses of purified HLA-G were utilized to see if it would inhibit migration.

As seen in Figure 14, the presence of soluble or membrane-bound HLA-G obtained from Jeg-3 cells did not inhibit migration of SMCs in this in-vitro assay. All the treatment groups (Groups 2-5) were significantly higher than Group 1, the negative control group (p <0.0001). Group 2, the positive control, showed significantly less migration than Group 3, the Jeg-3 conditioned medium (p <0.0001) and Group 4, the Jeg-3 >30kDa concentrated conditioned medium (p <0.015). Group 3, the Jeg-3 conditioned medium, had a significantly higher migration than all other treatment groups (Group 2, p <0.0001; Group 4, p <0.021; Group 5, p <0.012).

The concentration of HLA-G as measured by ELISA was as follows: Jeg-3 supernatant had ~75 U/mL, Jeg-3 >30kDa concentrated supernatant had ~1000 U/mL and the Jeg-3 membrane extract had >4000 U/mL HLAG. From Figure 14, it is interesting to note that as the concentration of HLA-G increased in the lower chamber, the number of migrated cells significantly decreased (Group 4 < Group 3). Figure 15 shows images of migrated SMCs for the different treatment groups.

When the purified HLA-G protein became available, the SMC migration assay was investigated once again. As seen in Figure 16, HLA-G at 1000 ng/mL (256 ± 2
migrated SMCs) caused significantly reduced migration than the positive control group (346 ± 20 migrated SMCs) (p <0.03). The HLA-G doses of 100 and 500 ng/mL did not significantly reduce the migration of SMCs from the positive control. Figure 17 shows representative images of migrated cells under control versus HLA-G 1000 ng/mL conditions.

4.3. HLA-G modulation in-vitro in Jeg-3 cells and HCAEC

Previously, it has been shown that HLA-G can be modulated by a number of factors such as progesterone and IL-10 (217,229). A small clinical study by our research group, an association was found between the presence of HLA-G in plasma of heart transplant recipients and the immunosuppressive agent, everolimus (RAD) (232). Given the potential immunotolerogenic role that HLA-G may have in protecting a transplanted organ from rejection, we examined how HLA-G can be modulated in two cell types: human coronary artery endothelial cells (HCAEC) and Jeg-3 cells using Western blots. HCAEC does not express HLA-G at baseline, whereas Jeg-3 cells do.

As seen in Figure 18a, IL-10 at 50 ng/mL upregulated HLA-G significantly in Jeg-3 cells from control (p<0.003; mean HLA-G level of control cells was 100±7 % versus mean HLA-G level of IL-10 treated cells was 149±12 %). Everolimus did not upregulate HLA-G from baseline (p = 0.98; mean HLA-G level of control cells was 100±7 % versus mean HLA-G level of everolimus-treated cells was 97±7 %). Figure
18b shows a representative Western blot image of control versus IL-10 50ng/mL Jeg-3 HLA-G protein expression with the β-actin loading control.

Since IL-10 was shown to upregulate HLA-G in Jeg-3 cells, we further examined this result to see if there was a dose-dependent response. Jeg-3 cells were exposed to 10, 25, 50, 100 and 200 ng/mL of IL-10 for 24 hrs. Then the cell lysates were collected and HLA-G protein expression was determined through Western blots. As seen in Figure 19a, HLA-G expression was once again significantly upregulated by the 50 ng/mL dose of IL-10 but not with any other doses (p=0.002; mean HLA-G level of control cells was 99 ± 5 % versus mean HLA-G levels of IL-10 50 ng/mL treated cells was 150±10%). Figure 19b shows a representative Western blot image of HLA-G expression in Jeg-3 cells after exposure to a range of IL-10 doses.

Finally, as seen in Figure 20, HLA-G was not at all expressed by HCAEC before or after treatment with three doses of everolimus (10, 50, 100 ng/mL).
5. FIGURES AND TABLES
Acute cellular rejection. This figure by Patel and colleagues shows the International Society for Heart and Lung Transplantation (ISHLT) standardized cardiac biopsy grading score for acute cellular rejection (27).

<table>
<thead>
<tr>
<th>2004</th>
<th>No rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0 R\textsuperscript{a}</td>
<td>No rejection</td>
</tr>
<tr>
<td>Grade 1 R, mild</td>
<td>Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage</td>
</tr>
<tr>
<td>Grade 2 R, moderate</td>
<td>Two or more foci of infiltrate with associated myocyte damage</td>
</tr>
<tr>
<td>Grade 3 R, severe</td>
<td>Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Where “R” denotes revised grade to avoid confusion with 1990 scheme.

<table>
<thead>
<tr>
<th>1990</th>
<th>No rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>No rejection</td>
</tr>
<tr>
<td>Grade 1, mild</td>
<td>Focal perivascular and/or interstitial infiltrate without myocyte damage</td>
</tr>
<tr>
<td>A—Focal</td>
<td>Diffuse infiltrate without myocyte damage</td>
</tr>
<tr>
<td>B—Diffuse</td>
<td>One focus of infiltrate with associated myocyte damage</td>
</tr>
<tr>
<td>Grade 2 moderate (focal)</td>
<td>Multifocal infiltrate with myocyte damage</td>
</tr>
<tr>
<td>Grade 3, moderate</td>
<td>Diffuse infiltrate with myocyte damage</td>
</tr>
<tr>
<td>A—Focal</td>
<td>Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage ± vasculitis</td>
</tr>
<tr>
<td>B—Diffuse</td>
<td>Diffuse infiltrate with myocyte damage</td>
</tr>
<tr>
<td>Grade 4, severe</td>
<td>Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage ± vasculitis</td>
</tr>
</tbody>
</table>
FIGURE 2

Antibody-mediated rejection. This figure by Colvin and colleagues show the diagnostic criteria for acute antibody-mediated rejection (7).

Box 1 | **Diagnostic criteria for acute antibody-mediated rejection**

- Clinical evidence of acute graft dysfunction
- Histological evidence of acute tissue injury:
  - that is, neutrophils, macrophages or thrombi in capillaries, fibrinoid necrosis, or acute tubular injury
- Immunopathological evidence for the action of antibodies:
  - that is, complement component 4d (C4d) deposited in peritubular capillaries, or antibodies or C3 in arteries
- Serological evidence of HLA-specific antibodies or other donor-specific antibodies at the time of biopsy
**FIGURE 3**

Antibody-mediated rejection. This figure by Patel and colleagues outline the International Society for Heart and Lung Transplantation (ISHLT) recommended grading score for antibody-mediated rejection (27).

<table>
<thead>
<tr>
<th>2004</th>
<th>1990</th>
</tr>
</thead>
</table>
| **AMR 0** | Negative for acute antibody-mediated rejection  
No histologic or immunopathologic features of AMR |
| **AMR 1** | Positive for AMR  
Histologic features of AMR  
Positive immunofluorescence  
or immunoperoxidase staining  
for AMR (positive CD68, C4d) |
| | Humoral rejection (positive immunofluorescence,  
vasculitis or severe edema in absence of cellular infiltrate)  
recorded as additional required information |
FIGURE 4

Structure of arterial wall. The three layers of an arterial wall consists of the innermost layer, the tunic intima, the middle layer, tunica media and finally the outermost layer, tunic adventitia.
**FIGURE 5**

Endothelial and smooth muscle cell activation by inflammation. This figure adapted from Lily shows that normal endothelial and smooth muscle cells maintain the integrity and elasticity of the normal arterial wall while limiting immune cell infiltration. Inflammatory activation of these vascular cells corrupts their normal functions and favors proatherogenic mechanisms that drive plaque development (21).

<table>
<thead>
<tr>
<th>NORMAL</th>
<th>ACTIVATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENDOTHELIAL CELLS</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>• Impermeable to large molecules</td>
<td>• ↑ permeability</td>
</tr>
<tr>
<td>• Anti-inflammatory</td>
<td>• ↑ inflammatory cytokines</td>
</tr>
<tr>
<td>• Resist leukocyte adhesion</td>
<td>• ↑ leukocyte adhesion molecules</td>
</tr>
<tr>
<td>• Promote vasodilation</td>
<td>• ↓ vasodilatory molecules</td>
</tr>
<tr>
<td>• Resist thrombosis</td>
<td>• ↓ antithrombotic molecules</td>
</tr>
<tr>
<td>SMOOTH MUSCLE CELLS</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>• Normal contractile function</td>
<td>• ↑ inflammatory cytokines</td>
</tr>
<tr>
<td>• Maintain extracellular matrix</td>
<td>• ↑ extracellular matrix synthesis</td>
</tr>
<tr>
<td>• Contained in medial layer</td>
<td>• ↑ migration and proliferation</td>
</tr>
</tbody>
</table>
FIGURE 6

Cardiac allograft vasculopathy (CAV). (a) This image shows the difference between a typical atherosclerosis and CAV; (b) This image shows a cross-sectional stain of an artery with CAV. The defining feature of CAV is the concentric narrowing of the lumen due to smooth muscle cell migration into the intima and its proliferation. (Images obtained from Dr. Mitesh Badiwala).
FIGURE 7

Figure adapted from González and colleagues showing the structural isoforms of the HLA-G. To date, seven HLA-G isoforms have been identified, four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three soluble (HLA-G5, -G6 and -G7) isoforms. The transmembrane domain common to HLA-G1 to -G4 enables membrane anchoring. The HLA-G5 to -G7 structures are the soluble counterparts of HLA-G1 to -G3, respectively. HLA-G1 and -G5 possess the full mRNA transcript. This figure shows the various exons that code for the different isoforms of HLA-G. Exon 1 codifies the leader peptide (L), exon 2, 3 and 4 codify the α1, α2 and α3 domains, respectively; exon 5 codifies the transmembrane domain (Tm); exon 6 codifies the cytoplasmic domain (Cyt). Red lines indicate stop codons (87).
FIGURE 8

HLA-G functions. This table adapted from Carosella and colleagues indicates the diverse functions of HLA-G expression on natural killer (NK) cells, CD8\(^+\) T cells, CD4\(^+\) T cells and antigen-presenting cells (APCs). The receptors, which mediate these functions, are also shown (69).

<table>
<thead>
<tr>
<th>Effector cell, HLA-G function</th>
<th>Receptors involved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NK cells</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of cytotoxic function</td>
<td>ILT2, KIR2DL4</td>
</tr>
<tr>
<td>Indirect inhibition of cytotoxic function through stabilization of cell-surface HLA-E</td>
<td>CD94/NKG2A (interacts w/HLA-E)</td>
</tr>
<tr>
<td>Inhibition of proliferation</td>
<td>ILT2</td>
</tr>
<tr>
<td>Up-regulation of inhibitory receptors</td>
<td>CD8</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
</tr>
<tr>
<td>Increased proliferation and IFN-γ production</td>
<td>KIR2DL4</td>
</tr>
<tr>
<td>Increased secretion of pro-angiogenic factors</td>
<td>Internalized KIR2DL4</td>
</tr>
<tr>
<td>Inhibition of transendothelial migration</td>
<td>ILT2</td>
</tr>
<tr>
<td><strong>CD8(^+) T cells</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of cytotoxic function</td>
<td></td>
</tr>
<tr>
<td>Inhibition of proliferation</td>
<td>ILT2</td>
</tr>
<tr>
<td>Generation of CD8(^+) regulatory T cells</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>CD8</td>
</tr>
<tr>
<td><strong>CD4(^+) T cells</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of alloreactivity</td>
<td>ILT2, ILT4(^\sim)</td>
</tr>
<tr>
<td>Inhibition of proliferation</td>
<td>ILT2</td>
</tr>
<tr>
<td>Up-regulation of inhibitory receptors</td>
<td>ILT2</td>
</tr>
<tr>
<td>Generation of regulatory T cells, which include CD4(^+) T cells</td>
<td></td>
</tr>
<tr>
<td><strong>APC</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of DC maturation, antigen presentation, trafficking, and induction of regulatory T cells</td>
<td>ILT4</td>
</tr>
<tr>
<td>Up-regulation of inhibitory receptors</td>
<td></td>
</tr>
<tr>
<td>PBMC: secretion of Th2 cytokines</td>
<td>CD160</td>
</tr>
<tr>
<td>Endothelial cells: apoptosis</td>
<td></td>
</tr>
</tbody>
</table>

— indicates not available.
FIGURE 9

Figure adapted González and colleagues showing the expression of HLA-G isoforms in trophoblast cells subpopulations. There is an increasing gradient of HLA-G expression in more invasive cells of the embryo in the uterus. There are different structures and isoforms expressed, with HLA-G5 being the most abundant isoform expressed (87).
FIGURE 10

Figure adapted from Carosella and colleagues summarizing HLA-G expression in tumor lesions. For each type of tumor, the total number of patients and the corresponding percentage of HLA-G-positive patients were determined by taking into account all of the studies published to date in the literature from multiple independent laboratories. These data show that the vast majority of human tumors can express HLA-G to varying extents, and that this might reflect a potential mechanism by which the tumors escape immunosurveillance. HLA-G expression was determined by immunohistochemistry and/or Western blot analysis of tumor lesions (3).
FIGURE 11

Table adapted from González and colleagues showing HLA-G expression in non-hematological tumor biopsies. The potential utility of HLA-G as a tumor marker in various tumors is indicated and the number of HLA-G-positive patients evaluated in these studies (87).

<table>
<thead>
<tr>
<th>Tumor</th>
<th>HLA-G positive/Total</th>
<th>Potential utility as a tumor marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>16/39</td>
<td>Pronostic of lower disease-free survival</td>
</tr>
<tr>
<td></td>
<td>155/235</td>
<td>Diagnostic of advanced disease</td>
</tr>
<tr>
<td></td>
<td>41 / 58</td>
<td>Pronostic of lower survival rate</td>
</tr>
<tr>
<td></td>
<td>209/501</td>
<td>Associated with disease stage</td>
</tr>
<tr>
<td>Cervical</td>
<td>9/40</td>
<td>Diagnostic</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>28/36</td>
<td>Differential diagnosis</td>
</tr>
<tr>
<td></td>
<td>11/11</td>
<td>Differential diagnosis</td>
</tr>
<tr>
<td>Colorectal</td>
<td>130/201</td>
<td>Independent prognostic factor</td>
</tr>
<tr>
<td>Endometrial</td>
<td>24/44</td>
<td>Associated with disease stage</td>
</tr>
<tr>
<td>Esophageal SCC</td>
<td>110/121</td>
<td>Independent prognostic factor</td>
</tr>
<tr>
<td>Gastric</td>
<td>89/179</td>
<td>Prognostic of tumor progression</td>
</tr>
<tr>
<td></td>
<td>113/160</td>
<td>Prognostic of shorter survival time</td>
</tr>
<tr>
<td></td>
<td>52/115</td>
<td>Prognostic of longer survival time</td>
</tr>
<tr>
<td>Liver</td>
<td>110/219</td>
<td>Diagnostic of advanced disease</td>
</tr>
<tr>
<td></td>
<td>99/174</td>
<td>Unfavourable prognostic factor</td>
</tr>
<tr>
<td>Lung</td>
<td>9/34</td>
<td>Associated with disease stage</td>
</tr>
<tr>
<td></td>
<td>79/106</td>
<td>Independent prognostic factor or shorter survival</td>
</tr>
<tr>
<td>NSCLC</td>
<td>42/101</td>
<td>Associated with disease stage</td>
</tr>
<tr>
<td>Melanoma</td>
<td>22/79</td>
<td>Diagnostic</td>
</tr>
<tr>
<td>Ovarian</td>
<td>40/41</td>
<td>Diagnostic of advanced disease</td>
</tr>
<tr>
<td></td>
<td>10/20 Serous 2/2 Clear</td>
<td>Associated with disease stage</td>
</tr>
<tr>
<td></td>
<td>49/148 Effusions</td>
<td>Prognostic of better overall survival</td>
</tr>
<tr>
<td>Renal</td>
<td>47/95 clear cell</td>
<td>Diagnostic</td>
</tr>
<tr>
<td></td>
<td>2/4 chromophobe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/6 collecting duct</td>
<td></td>
</tr>
</tbody>
</table>

*NSCLC indicates non-small-cell lung carcinoma and SCC, squamous cell carcinoma.*
FIGURE 12

HLA-G and acute cellular rejection in rejecting (Group A) versus non-rejecting (Group B) patients. (a) This table shows the differences in mean sHLA-G levels ± SEM between the two groups and number of patients in each group for each time point. (b) As seen in this graphical representation, there was no significant difference between rejecting and non-rejecting patients in their mean sHLA-G levels. Mth = month; tx = transplant.

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Group A sHLA-G ± SEM (U/mL)</th>
<th>Group B sHLA-G ± SEM (U/mL)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Tx</td>
<td>70.45 ± 12.79</td>
<td>69.19 ± 8.49</td>
<td>0.932</td>
</tr>
<tr>
<td>3 mth Post-tx</td>
<td>68.02 ± 7.61</td>
<td>74.56 ± 11.14</td>
<td>0.621</td>
</tr>
<tr>
<td>6 mth Post-tx</td>
<td>67.84 ± 4.95</td>
<td>59.78 ± 6.54</td>
<td>0.648</td>
</tr>
<tr>
<td>12 mth Post-tx</td>
<td>59.68 ± 7.11</td>
<td>57.57 ± 10.24</td>
<td>0.885</td>
</tr>
</tbody>
</table>

(a)

(b)
FIGURE 13

Schematic diagram that describes Boyden chamber assay. Diagram adapted from article by Soncin and colleagues (250).
FIGURE 14

Smooth muscle cell migration assay with HLA-G source being Jeg-3 cells. Treatment groups is as shown in Figure 23. The concentration of sHLA-G as measured by ELISA is as follows: Jeg-3 supernatant (Group 3) had ~75 U/mL HLA-G, Jeg-3 concentrated supernatant medium (Group 4) had ~1000 U/mL and the Jeg-3 membrane extract (Group 5) has >4000 U/mL HLAG. Statistical test used: ANOVA with Tukey post-hoc.

# of migrated smooth muscle cells (± SEM) in response to soluble and membrane bound HLA-G obtained from Jeg-3 cells

All the groups were significantly higher than Group 1, the negative control group (p < .0001)
** Group 2 was significantly lower than Group 3 (p < .0001) and Group 4 (p < 0.015)
* Group 3 was significantly higher than Group 2 (p < .0001), Group 4 (p < 0.021) and Group 5 (p < 0.012)
FIGURE 15

Images of migrated cells in smooth muscle cell migration assay, 20x mag.
(a) Group 1: Negative control (SmBSA; no migration)
(b) Group 2: Positive control (Sm-full-1%FBS; migration)
(c) Group 3: Jeg-3 conditioned supernatant
(d) Group 4: >30KDa Jeg-3 conditioned supernatant
(e) Group 5: Jeg-3 membrane extract
FIGURE 16

Purified HLA-G in smooth muscle cell migration assay shows HLA-G at 1000 ng/mL reduced migration relative to the positive control (which was Sm-full-1%FBS) and the other doses of HLA-G (100, 500 ng/mL) used.
FIGURE 17

SMC Migration Assay with Purified HLA-G. Images taken under a light microscope at 20x magnification showing migrated cells in (a) control: SmBSA + Growth Factors + 1% FBS) versus (b) purified recombinant HLA-G at 1000 ng/mL.

(a) Control (SmBSA+growth factors + 1% FBS) (count average: 346 ± 20 cells)

(b) Migration after HLA-G 1000 ng/mL (count average: 256 ± 2 cells)
FIGURE 18

HLA-G protein expression quantified through Western blot analysis after Jeg-3 cells, which is known to express HLA-G at baseline, was exposed to 50ng/mL of IL-10 and 100 ng/mL of everolimus (RAD).
FIGURE 19

HLA-G protein expression quantified through Western blot analysis after Jeg-3 cells were exposed to varying doses of IL-10 from 0 to 200 ng/mL. Only significant result was between control (no IL-10 treatment) and IL-10 at 50ng/mL. (99 ± 5 % control mean HLA-G vs. 150±10% mean HLA-G for IL-10 50ng/mL; p=0.002)
FIGURE 20

HLA-G protein expression quantified through Western blot analysis after human coronary artery endothelial cells (HCAEC) were exposed to varying doses of everolimus (RAD).

Lane 3: Positive Control Jeg 3 cell (20ug)
Lane 5-6: Negative Control (HCAEC)
Lane 7-8: HCAEC treated with DMSO Control
Lane 9-10: HCAEC treated with RAD 10 ng/mL
Lane 11-12: HCAEC treated with RAD 50 ng/mL
Lane 13-15: HCAEC treated with RAD 100 ng/mL

[Image of Western blot with HLA-G 38 kDa and β-actin 42 kDa markers, Jeg-3 cell lysate and positive control notes]
Table adapted from Mehra et al. showing the ISHLT recommended nomenclature and classification for cardi allograft vasculopathy (CAV) (59).

<table>
<thead>
<tr>
<th>ISHLT CAV</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV₀ (not significant)</td>
<td>No detectable angiographic lesion</td>
</tr>
<tr>
<td>CAV₁ (mild)</td>
<td>Angiographic left main (LM) &lt;50%, or primary vessel with maximum lesion of &lt;70%, or any branch stenosis &lt;70% (including diffuse narrowing) without allograft dysfunction</td>
</tr>
<tr>
<td>CAV₂ (moderate)</td>
<td>Angiographic LM &lt;50%; a single primary vessel ≥70%, or isolated branch stenosis ≥70% in branches of 2 systems, without allograft dysfunction</td>
</tr>
<tr>
<td>CAV₃ (severe)</td>
<td>Angiographic LM ≥50%, or two or more primary vessels ≥70% stenosis, or isolated branch stenosis ≥70% in all 3 systems; or ISHLT CAV₁ or CAV₂ with allograft dysfunction (defined as LVEF ≤45% usually in the presence of regional wall motion abnormalities) or evidence of significant restrictive physiology (which is common but not specific)</td>
</tr>
</tbody>
</table>

Definitions:
- A “Primary Vessel” denotes the proximal and Middle 33% of the left anterior descending artery, the left circumflex, the ramus and the dominant or co-dominant right coronary artery with the posterior descending and posterolateral branches.
- A “Secondary Branch Vessel” includes the distal 33% of the primary vessels or any segment within a large septal perforator, diagonals and obtuse marginal branches or any portion of a non-dominant right coronary artery.
- Restrictive cardiac allograft physiology is defined as symptomatic heart failure with echocardiographic E to A velocity ratio >2 (>1.5 in children), shortened isovolumetric relaxation time (<60 msec), shortened deceleration time (<150 msec), or restrictive hemodynamic values (Right Atrial Pressure >12mmHg, Pulmonary Capillary Wedge Pressure >25 mmHg, Cardiac Index <2 l/min/m²).
**TABLE 2**

Receptors for HLA-G. The cellular distribution and HLA-G binding site for the three main HLA-G receptors, KIR2DL4, ILT-2 and ILT-4, are summarized below in this table adapted from Carosella and colleagues (3).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cellular distribution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HLA-G binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DL4 (CD158d)</td>
<td>NK, T</td>
<td>α1 domain</td>
</tr>
<tr>
<td>ILT-2 (CD85j)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NK, T, B, DC, monocytes, and Mf</td>
<td>α3/β2m domain</td>
</tr>
<tr>
<td>ILT-4 (CD85d)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DC, monocytes, and Mf</td>
<td>α3</td>
</tr>
<tr>
<td>CD8</td>
<td>T, NK</td>
<td>α3</td>
</tr>
</tbody>
</table>

Abbreviations: B, B cells; DC, dendritic cells; Mf, macrophages; NK cells, natural killer cells; T, T cells.

<sup>a</sup> Although ILT-2 is expressed by B cells, interaction with HLA-G was not described in this context.

<sup>b</sup> HLA-G dimers bind to these receptors with a higher affinity and slower dissociation rates than monomers. Therefore, the inhibitory function of HLA-G bound to ILTs is mostly due to dimers.
TABLE 3

Figure adapted Carosella and colleagues summarizing the human diseases associated with HLA-G. The physiologic and pathologic situations under which HLA-G is expressed, biological function of HLA-G in that setting and the clinical applications is listed below (3).

<table>
<thead>
<tr>
<th>Physio-pathology</th>
<th>Expression and biological functions</th>
<th>Clinical applications</th>
</tr>
</thead>
</table>
| Pregnancy        | • Protection of fetus from uterine NK cell cytolysis  
|                  | • Embryo implantation and placental development  
|                  | • Reduced expression associated with fetal loss | • Marker of efficient implantation after IVF |
| Transplantation  | • Neo-expression in grafted organs and increased plasma levels various types of solid-organ transplantations  
|                  | • Positive associateion with better graft function and survival  
|                  | • Negative association with HLA alloantibody titers in patients | • Marker of clinical follow-up to select patients expected to better accept their allograft and to benefit from reduced immunosuppressive treatment |
|                  | • Positive correlation with high plasma levels of IL-10, sCD4, and sCD8 and verrepression of peripheral blood CD3+CD4\textsuperscript{low} and CD3+CD8\textsuperscript{low} T cells  
|                  | • Induction of allograft acceptance through inhibition of T cell alloreponses and induction of CD3+CD4\textsuperscript{low} and CD3+CD8\textsuperscript{low} suppressor T cells and tolerogenic dendritic cells | |
| Inflammatory      | • Neo-expression in digestive tract diseases (coeliac disease, ulcerative colitis)  
| and autoimmune    | • Neo-expression in cutaneous diseases (psoriasis, atopic dermatitis)  
| disorders         | • Reduced plasma levels and lower expression by monocytes in MS patients | • Differential diagnostic tool between ulcerative colitis and Crohn’s disease  
|                  | | • Marker of clinical follow-up negatively associated with disease activity in MS  
| | | • Marker of IFN treatment efficacy in MS |
| Cancer            | • Neo-expression in malignant lesions and increased plasma levels in cancer patients associated with bad clinical evolution | • Marker of clinical follow-up positively associated with aggressiveness and poor prognosis in ovarian cancers, B-CLL, and in gastric and colorectal tumors |
| Infectious        | • Expression after infections with CMV, HIV, and neurotropic viruses  
| diseases          | • Increased plasma levels in patients with septic shock | • Predictive marker of survival in patients with septic shock |

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TABLE 4

Baseline characteristics of patients with and without cardiac allograft vasculopathy (CAV). Patients were divided into two groups based on ISHLT classification of CAV as seen in Figure 7. There were no significant differences in the sex, age, heart failure etiology and immunosuppressive therapy between the two groups of patients.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Group A, No CAV (n=35)</th>
<th>Group B, CAV (n=15)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>30/5</td>
<td>12/3</td>
<td>0.89</td>
</tr>
<tr>
<td>Mean Age</td>
<td>48±14</td>
<td>50±9</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Reason For Transplantation</strong></td>
<td></td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>Idiopathic Cardiomyopathy</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Ischemic Cardiomyopathy</td>
<td>14</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Immunosuppressive Treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>24</td>
<td>9</td>
<td>0.46</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>9</td>
<td>5</td>
<td>0.62</td>
</tr>
<tr>
<td>Mycophenolate Mofetil</td>
<td>18</td>
<td>10</td>
<td>0.97</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>5</td>
<td>5</td>
<td>0.13</td>
</tr>
<tr>
<td>Everolimus</td>
<td>9</td>
<td>3</td>
<td>0.62</td>
</tr>
</tbody>
</table>
TABLE 5

Correlation between PRA levels pre-transplantation, and sHLA-G levels at 3, 6 and 12 months; MIT at 1 month and 1 year; and MIT progression. PRApretx = PRA levels pre-transplantation; MIT = mean intimal thickness; MIT progression = difference between MIT at 1 month and 1 year; r = Pearson’s Correlation.

<table>
<thead>
<tr>
<th>PRAPreTx</th>
<th>HLA-G 3 mth</th>
<th>HLA-G 6 mth</th>
<th>HLA-G 12 mth</th>
<th>MIT 1 mth</th>
<th>MIT 1 year</th>
<th>MIT progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>-0.016</td>
<td>-0.244</td>
<td>-0.123</td>
<td>-0.185</td>
<td>-0.067</td>
<td>0.174</td>
</tr>
<tr>
<td>r²</td>
<td>0.000</td>
<td>0.060</td>
<td>0.015</td>
<td>0.034</td>
<td>0.004</td>
<td>0.030</td>
</tr>
<tr>
<td>p-value</td>
<td>0.920</td>
<td>0.115</td>
<td>0.454</td>
<td>0.337</td>
<td>0.708</td>
<td>0.233</td>
</tr>
<tr>
<td>n</td>
<td>44</td>
<td>43</td>
<td>39</td>
<td>29</td>
<td>34</td>
<td>26</td>
</tr>
</tbody>
</table>
**TABLE 6**

Correlation between sHLA-G levels post-transplantation at 3, 6, 12 months, and C4d staining at 3, 6 and 12 months. $r =$ Pearson Correlation

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>$r$</th>
<th>$r^2$</th>
<th>p-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4d 3 mth</td>
<td>HLA-G 3 mth</td>
<td>-0.08</td>
<td>0.0064</td>
<td>0.60</td>
<td>42</td>
</tr>
<tr>
<td>C4d 6 mth</td>
<td>HLA-G 6 mth</td>
<td>0.21</td>
<td>0.0441</td>
<td>0.23</td>
<td>34</td>
</tr>
<tr>
<td>C4d 12 mth</td>
<td>HLA-G 12 mth</td>
<td>-0.05</td>
<td>0.0025</td>
<td>0.81</td>
<td>30</td>
</tr>
</tbody>
</table>
TABLE 7

Difference in mean MIT levels between rejecting (Group A) and non-rejecting (Group B) patients as measured by intravascular ultrasound. An MIT of ≥ 0.5 mm was selected as the criteria for defining CAV. MIT = maximum intimal thickness.

<table>
<thead>
<tr>
<th></th>
<th>Group A, No CAV (n=35)</th>
<th>Group B, CAV (n=15)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIT 1 month</td>
<td>0.22 ± 0.08 mm</td>
<td>0.57 ± 0.2 mm</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MIT 1 year</td>
<td>0.23 ± 0.08 mm</td>
<td>0.67 ± 0.15 mm</td>
<td>0.0001</td>
</tr>
<tr>
<td>MIT progression</td>
<td>0.014 ± 0.036 mm</td>
<td>0.15 ± 0.12 mm</td>
<td>&lt;0.022</td>
</tr>
</tbody>
</table>

(a)

(b) Normal vessel, no CAV  
(c) Abnormal vessel with CAV (MIT ≥ 0.5 mm)
TABLE 8

Difference in mean sHLA-G levels between patients with and without CAV. CAV = cardiac allograft vasculopathy.

<table>
<thead>
<tr>
<th>Time post-transplant</th>
<th>Group A, No CAV (n=35)</th>
<th>Group B, CAV (n=15)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-G 3 mth</td>
<td>71.7±39 U/mL</td>
<td>65.7±51 U/mL</td>
<td>0.67</td>
</tr>
<tr>
<td>HLA-G 6 mth</td>
<td>67.8±28 U/mL</td>
<td>60.9±23 U/mL</td>
<td>0.41</td>
</tr>
<tr>
<td>HLA-G 12 mth</td>
<td>69.9±42 U/mL</td>
<td>58.3±33 U/mL</td>
<td>0.35</td>
</tr>
</tbody>
</table>
TABLE 9
Correlation between sHLA-G levels measured at 12 months, with MIT at one year, and MIT progression from 1 month to 1 year. MIT = mean intimal thickness; mth = month; r = Pearson’s Correlation.

<table>
<thead>
<tr>
<th></th>
<th>MIT at 1 year</th>
<th>MIT progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>r²</td>
</tr>
<tr>
<td>HLA-G 12 mth</td>
<td>0.07</td>
<td>0.0049</td>
</tr>
</tbody>
</table>

HLA-G 12 mth (x-axis) and MIT at 1 year (y-axis) Pearson Correlation

HLA-G 12 mth (x-axis) and MIT progression (y-axis) Pearson Correlation
**TABLE 10**

Treatment Groups for smooth muscle cell migration assay.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Lower chamber (total volume = 500 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control for migration: SmBSA (500 µl)</td>
</tr>
<tr>
<td>2</td>
<td>Positive control for migration: Sm-full-1%FBS (500 µl)</td>
</tr>
<tr>
<td>3</td>
<td>sHLAG: Jeg-3 conditioned medium (500 µl)</td>
</tr>
<tr>
<td>4</td>
<td>sHLA-G: Jeg-3 conditioned &gt;30 kDa concentrated medium (20 µl supernatant + 480 µl Sm-full-1%FBS = 500 µl)</td>
</tr>
<tr>
<td>5</td>
<td>Membrane-bound HLA-G: 480 µl of Sm-full-1%FBS + 20 µl (at 1 µg/µl) Jeg-3 membrane extracted proteins [Detergent-free] = 500 µl</td>
</tr>
</tbody>
</table>
6. DISCUSSION

6.1. Clinical Findings

In transplantation, tolerance induction is a major challenge and depends on the modulation of the pathways of acute and chronic rejection (199). Previous retrospective studies showed that the presence of HLA-G was associated with less cellular rejection in heart transplant recipients (78,196,200,201). One previous study suggested a possible association between HLA-G and graft vasculopathy (196). However, this study was retrospective and only a small number of patients underwent coronary angiography to evaluate vasculopathy. In order to understand the role of HLA-G further, we prospectively followed heart transplant recipients to investigate the association between sHLA-G at different time points with acute cellular rejection (ACR), antibody mediated rejection (AMR) and cardiac allograft vasculopathy (CAV) post-transplantation.

As seen in Figure 12, there was no significant difference in sHLA-G levels between rejecting and non-rejecting patients at various times points based on ACR. Of interest, it can be noted that the sHLA-G levels were highest in the pre-transplant patient population. A similar trend was discovered in a study by Almasood and colleagues, who reported that HLA-G is upregulated in the heart failure population (251). A possible reason for this may be that the body upregulates HLA-G in association with underlying pathological processes, but there may be insufficient production of HLA-G receptors for the HLAG itself to be active.
As seen in Table 7, we found that IVUS results at one month, at one year and MIT progression was significantly higher in the rejecting group (Group B) than in the non-rejecting group (Group A). This was anticipated given that those with higher grades of rejection were more likely to have vasculopathy.

Our preliminary analyses failed to reveal an association between HLA-G and various parameters of rejection and vasculopathy. However, there are a number of contributing factors that may have failed to bring results to significance. Our sample size was limited (n=50) due to the small number of heart transplants that happen at our centre every year (around 25-30 heart transplants occur at the Toronto General Hospital annually). This resulted in the study being heavily underpowered. With our sample size of 50, the power calculated was 0.35 for ACR and CAV, meaning that there was only a 35% probability of detecting a difference in the two groups. To obtain a power of 0.8, we would need a sample size of at least 153 subjects in each group. For AMR, the power calculated was even lower, at 0.05. In our study, only 15/50 patients (30%) had CAV at the one-year post-transplantation time point. 6/50 patients (12%) had PRA levels over 10%, which was the cut-off used for the presence of circulating antibodies and only 8/50 (16%) patients had detectable levels of C4d deposition, the surrogate indicators for AMR. Finally, even with a liberal definition for acute rejection – where, if the patient had even one episode of moderate/severe rejection within the first year post-transplantation, they were classified in the ‘rejecting’ group – only 15/50 (30%) patients experienced acute rejection.
cellular rejection. In fact, only 4 of the 15 patients (8% of the total study population) with acute cellular rejection had more than one moderate/severe rejection episode.

Secondly, the improvements in medical management of heart transplant recipients meant that rejection was treated more aggressively. Generally, mild cellular rejection (ISHLT Grade 1R) does not require an increase in dosage of immunosuppressive therapy as the vast majority of episodes of rejection spontaneously resolve themselves. However, higher grades are treated immediately with supplemental immunosuppression. Therefore, we did not find many episodes of moderate-high cellular rejections in our patients during the one-year follow-up.

Thirdly, although HLA-G expression might be relevant to rejection, it may not be the most reliable indicator for vasculopathy, nor does it influence the levels of circulating anti-HLA antibodies or C4d deposition.

Although CAV can be detected as early as one-year post-transplantation, its development and progression increases dramatically after the first year. Using coronary angiography, CAV is detectable in 7.1% of recipients within the first year, 31.5% within five years, and in 52.7% of survivors within 10 years of transplantation (22,60). Therefore, a longer follow up of 3 to 5 years is definitely necessary to understand the relationship between HLA-G and CAV, if any. According to a study by Kapadia and colleagues, using IVUS, the prevalence of CAV lesions was 27% for one-vessel imaging at one year post-transplantation (252). In our study, 15 out of 50 patients (30%) had CAV at the one-year post-transplantation time point. This
higher number, relative to angiographic detection, might be due to the more sensitive method of detecting CAV using IVUS. The follow up of patients up to 3 or 5 years post-transplantation using IVUS instead of angiography might capture the CAV earlier.

Though not significant, there was a trend towards a negative correlation between sHLA-G levels and MIT progression from one month to one year, suggesting that a higher sHLA-G level might be associated with decreased progression of intimal thickening (Table 9). This would need to be further explored with a larger sample size.

Other factors, including immunosuppressive therapy and co-morbidities such as hyperlipidimia, hypertension and diabetes, may influence the development of CAV and antibody mediated rejection at a rate far greater than the potential inhibitory effects of HLA-G, thereby masking effects of HLA-G, if any was present. In future studies, donor characteristics also need to be examined to see its impact on CAV.

HLA-G polymorphisms may also contribute to the development of CAV. Previously, our group showed that a 14 base pair deletion polymorphism in the 3’ UTR of the HLA-G gene was associated with a lower risk for acute rejection in heart transplant recipients (253). However, it is unknown if this or other polymorphism may have an impact on the development of antibodies or CAV post transplantation (254).
Finally, the accuracy of the quantification of circulating sHLA-G in heart transplant recipients may be jeopardized by the different isoforms of HLA-G that can be released and the methods employed for their detection. Currently, there is only one commercially available HLA-G ELISA assay, which measures two isoforms, the shed HLA-G1 and the soluble HLA-G5. Additionally, this assay uses anti-human β2-microglobulin as the detection antibody, which means it does not detect β2-microglobulin free HLA-G1/HLA-G5 isoforms, nor other truncated isoforms (87). Thus, the level of sHLA-G detected in the transplant patient could be underestimated. Further studies with more sensitive measures are needed in order to determine what effects, if any, the other isoforms may have on the progression of CAV.

The expression of HLA-G expression in transplant patients could be a valuable therapeutic strategy to limit allograft rejection and vasculopathy. However, in this study and contrary to previous publications, we did not observe an association between sHLA-G expression on ACR, AMR and CAV, suggesting that HLA-G might not be a reliable prognostic indicator for these morbidities within the first year following the transplantation. Larger studies with longer follow-up time points are needed to fully comprehend the role and the pathways of action and the role HLA-G may play in heart transplantation.
6.2. **Effect of HLA-G on migration of smooth muscle cells**

Even though our preliminary clinical data failed to show a significant association between HLA-G and various parameters of rejection and vasculopathy, the study was underpowered possibly masking the importance of HLA-G. Nevertheless, given the immunotolerogenic properties, we pursued to understand whether HLA-G had any impact on the mechanisms of vasculopathy *in-vitro*. Our study using a migration assay found that purified HLA-G was capable of inhibiting migration of SMCs.

SMCs play a key role in the development of pathological processes such as atherosclerosis and CAV. These processes develop secondary to endothelial injury due to traditional risk factors such as smoking, diabetes mellitus, hypertension, and hyperlipidemia, and non-traditional risk factors associated with transplantation such as ischemia-reperfusion injury, HLA mismatch, the number of rejection episodes, recipient age at the time of transplant, and donor factors such as donor age, comorbidities and body size (29,60,255). Once this injury occurs, endothelial cells, platelets, and inflammatory cells release mediators, such as growth factors and cytokines that induce many effects such as phenotypic changes to SMCs, their proliferation and migration from the media to intima of the coronary blood vessel (58,255). This results in intimal thickening and narrowing of the vessel lumen. CAV is the leading cause of morbidity and mortality long-term following heart transplantation (58,59). Therefore, understanding the mechanisms that underlie the formation of CAV is necessary to prevent this long-term illness.
Previous studies have shown that the presence of cell-surface and circulating HLA-G isoforms have been correlated with reduced rejection after various forms of solid-organ transplantation. In addition to its various properties and inhibitory effects on the immune system, studies have found that HLA-G1 has the ability to inhibit migration of natural killer (NK) cells across porcine endothelial cells (233) and the migration of human umbilical vein endothelial cells (235). The HLA-G inhibitory receptor, ILT4, was found on placental vascular smooth muscle cells, suggesting that HLA-G may have novel functions in these cells (237). Given the potential anti-migratory properties of HLA-G, and its possible biological effect on smooth muscle cells, we investigated a biological model of migration assay to examine whether the presence of HLA-G would inhibit the migration of human coronary artery SMCs.

After several trials, we found that SMC basal media plus growth factors plus 1% FBS proved to be an effective positive control in inducing migration of SMCs (Figure 14). Initially, HLA-G-positive Jeg-3 cell lines were used as a surrogate source of soluble and membrane-bound HLA-G. Later, purified HLA-G became commercially available. Our first results, seen in Figure 14, showed that soluble and membrane-bound HLA-G obtained from Jeg-3 cells did not inhibit migration of SMCs. However, it was interesting to note a trend in reduced migration when the concentration of HLA-G was increased in the lower chamber: Group 5 had >4000 U/mL sHLA-G versus Group 4 had ~1000 U/mL sHLA-G versus Group 3, which had ~75 U/mL of HLA-G.
When purified HLA-G was added to the lower chamber, HLA-G at 1000 ng/mL significantly reduced the migration of SMCs (Figure 15).

A potential mechanism by which SMCs inhibit migration might be through the downstream mechanisms of the inhibitory receptors on SMCs. At least one study found the HLA-G inhibitory receptor, ILT4, on vascular SMCs of the placenta (237). A further study to follow up our finding is to examine the receptor profile of coronary artery SMCs, specifically to see if the HLA-G inhibitory receptors, KIR2DL4, ITL2 and ILT4, can be found on the membrane of these cells.

This study had several limitations. Prior to the availability of purified HLA-G, the use of Jeg-3 conditioned medium meant that this medium not only had HLA-G but other proteins produced and released by the Jeg-3 cells. Jeg-3 cells are obtained from a cancerous cell line, and these cells do release several substances with proliferative properties. Therefore, we cannot say with certainty that the sHLA-G in the supernatant was responsible for the reduction in migration. The use of a 30kDa filter mitigated some of these effects, as most of the growth factors are <30kDa and would have been filtered out and FBS was not added to the conditioned medium that would be used in later experiments.

Going forward, SMC migration assays with purified HLA-G protein doses higher than 1000 ng/mL need to be examined as well to see of there is a dose-response with
increased HLA-G and reduced migration. Secondly, an HLA-G blocking antibody or an HLA-G siRNA need to be deployed to block the effects of HLA-G or its production would reverse the results obtained with migration. Finally, other potential HLA-G modulators such as everolimus, progesterone and IL-10 (217,229,256) can be added to the lower chamber to see if there is a combined or synergistic effect of these pharmacological agents and HLA-G in inhibiting migration.

### 6.3. HLA-G modulation in-vitro in HCAEC and Jeg-3 cells

#### 6.3.1. HLA-G in Jeg-3 cells is modulated by IL-10

Previously, it has been shown that HLA-G can be modulated by a number of factors such as progesterone and IL-10 (217,229). A small clinical study by our research group, an association was found between the presence of HLA-G in plasma of heart transplant recipients and the immunosuppressive agent, everolimus (RAD) (232). Given the association of HLA-G and reduced rejection post-transplantation in many types of solid organ transplantations, we sought to understand mechanisms by which HLA-G can be modulated through in-vitro experiments in endothelial cells and Jeg-3 cells. Western blots were performed to capture HLA-G expression using 4H84 HLA-G monoclonal antibody.

Jeg-3 cells were exposed to everolimus and varying doses of IL-10. As seen from Figure 18 and Figure 19, everolimus did not affect HLA-G expression, however only one dose of IL-10, 50 ng/mL, upregulated HLA-G. This was an interesting phenomenon. A possible explanation could be that increased HLA-G expression by
Jeg-3 cells could block more HLA-G from being expressed or result in a negative feedback mechanism, resulting in no further HLA-G expression. Another postulation is that higher doses of IL-10 may not be well tolerated by Jeg-3 cells.

IL-10 is a potent and pleiotropic anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines and also has a suppressive effect on pro-inflammatory cytokine like TNF-alpha, IFN-gamma and IL-12. It is also directly involved in the modulation of other aspects of inflammation, particularly cytokine responses and inflammatory cell infiltration (257).

### 6.3.2. HLA-G is not modulated by HCAEC by Everolimus

As noted in Figure 20, HLA-G was not expressed in HCAEC, neither at baseline nor following exposure to Everolimus. This could suggest that the immunosuppressive effects of Everolimus do not act through HCAEC. However, there may be other factors present that might have affected the ability of the HCAEC to express HLA-G. In cell culture, primary endothelial cells often undergo phenotypic changes as they are passaged. It is possible that the HCAEC did not upregulate HLA-G in response to Everolimus, as they may have lost their ability to express HLA-G due to aging. The cells used in the studies were between passage (P) 3 and P5. To confirm whether the age of the cells had an impact in the observed results, HCAEC at an earlier stage, P1 or P2, should be examined.
Everolimus is an inhibitor of the mammalian target of rapamycin complex (mTOR) pathway, whose downstream signals causes longer survival of certain cells like tumors (258). Everolimus has been shown to reduce cell proliferation, glycolysis, and angiogenesis in solid tumours in vivo (258).

Everolimus has been used as an immunosuppressive agent for heart transplant recipients in several clinical trials (259). For example, Howard and colleagues showed in a randomized, double-blind, clinical trial that everolimus reduced the severity and incidence of cardiac-allograft vasculopathy compared to another purine inhibitor immunosuppressive agent, azathioprine (260). Given this association between everolimus and reduced vasculopathy, and because vasculopathy is a complex clinical issue with multiple mediators, there may be other effector cells by which everolimus carries out its immunosuppressive functions. To note, a key event in vasculopathy is the proliferation and migration of smooth muscle cells. Further investigations need to be done to examine the effect of everolimus on the proliferation and migration in this cell type.

### 6.4. Future Directions

To examine the potential of HLA-G in heart transplant recipients and to fully comprehend whether it plays a role in the development of acute cellular rejection, antibody-mediated rejection and vasculopathy, we strongly believe that the patients must be followed long-term. Patient plasma HLA-G levels at three and five year post-transplantation must be examined in the context of their rejection. Additionally,
all isoforms of HLA-G must be detected to determine if specific isoforms play a role in rejection.

We showed in our in-vitro studies that purified HLA-G was capable of reducing migration of smooth muscle cells. Going forward, an HLA-G blocking antibody or an HLA-G siRNA can be used to block the effects of HLA-G or its production respectively to see if this would reverse the results obtained with migration. To understand the possible mechanism of HLA-G in the development of vasculopathy, it is necessary to examine which HLA-G receptors are present on the cell surface of smooth muscle cells in the coronary arteries. Additionally, since everolimus has been shown to reduce the incidence and severity of vasculopathy, the impact of everolimus on smooth muscle cell migration could be examined in the in-vitro assay. Finally, it is important to examine the potential of everolimus on inducing HLA-G expression in smooth muscle cells, as this might be a possible mechanism by which everolimus carries out its immunosuppressive function and help in reduced vasculopathy.

7. CONCLUSION

HLA-G is a protein that might have therapeutic value given its expression under the physiological condition pregnancy and other pathological conditions such as organ transplantation, cancer and autoimmune diseases. In this study, our novel findings were that HLA-G can be modulated by the anti-inflammatory cytokine, IL-10 at the
dose of 50 ng/mL in Jeg-3 cell line, and that smooth muscle cell (SMC) migration was inhibited by purified HLA-G \textit{in-vitro}. This is significant as SMC migration is a key mediator in the development of cardiac allograft vasculopathy and is a leading cause of long-term morbidity and mortality in heart transplant recipients. Therefore the inhibition of migration might prove to be an effective therapeutic strategy. Additionally the ability to modulate the expression of HLA-G might be a strategy to protect the transplanted heart, given the immuno-tolerogenic properties of HLA-G. We also examined prospectively whether there is any association between HLA-G levels and parameters of rejection and vasculopathy in heart transplant patients. The preliminary analysis revealed no association; however, the study was underpowered and a longer follow-up of the patients is necessary in order to fully understand the clinical presence of HLA-G and its potential as a biomarker to identify patients who experience rejection.
8. APPENDIX

The following documents have been included as accompanying documents to the clinical study depicted in this thesis:

8.1 Research Ethics Board (REB) Approval Form
8.2 Patient Consent Form

8.1. Research Ethics Board (REB) Approval Form

University Health Network
Research Ethics Board
8th Floor South, Room 6-23
700 University Ave
Toronto, Ontario, M5G 1Z5
Phone: (416)946-4438

Notification of REB Continued Approval

Date: October 2nd, 2009
To: Dr. Diego Dolgado
Rm 1201, 11th Floor, New Clinical Services Building, TGH
Re: 06-0650-AE
Role of the Human Leukocyte Antigen-G Pre and Post-Cardiac Transplantation

REB Review Type: Expedited
REB Initial Approval Date: October 2nd, 2006
REB Annual Approval Date: October 2nd, 2009
REB Expiry Date: October 2nd, 2010

Consent Form(s) Currently Approved for Use:
Consent Form - Main Version date: February 23rd, 2009

The above-named study has received continued approval from the University Health Network Research Ethics Board until the expiry date noted above. If the study is expected to continue beyond the expiry date, you are responsible for ensuring the study receives re-approval. The REB must also be notified of the completion or termination of this study and a final report provided.

If, during the course of the research, there are any serious adverse events, confidentiality concerns, changes in the approved project, or any new information that must be considered with respect to the project, these should be brought to the immediate attention of the REB. In the event of a privacy breach, you are responsible for reporting the breach to the UHN REB and the UHN Corporate Privacy Office [in accordance with Ontario health privacy legislation Personal Health Information Protection Act, 2004]. Additionally, the UHN REB requires reports of inappropriate/ unauthorized use of the information. As the Principal Investigator, you are responsible for the ethical conduct of this study.

The UHN Research Ethics Board operates in compliance with the Tri-Council Policy Statement, ICH/GCP Guidelines, the Ontario Personal Health Information Protection Act (2004), and Part C, Division 5 of the Food and Drug Regulations of Health Canada.

Sincerely,
Lorraine Balaajay
Research Ethics Coordinator

For: Ronald Heslograve, Ph.D.
Chair, University Health Network Research Ethics Board

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8.2. Patient Consent Form

Consent Form for Participation in a Research Study

Title: Human Leukocyte Antigen-G Expression in Heart Transplant Recipients

Investigators: Dr. Diego Delgado (Tel: 416-340-4800 ext. 3940)

Dr. Vivek Rao
Dr. Heather Ross
Ms. Jemy Joseph

You are being asked to participate because you are a patient on the heart transplant waiting list. In order to be enrolled in this study you must understand the purposes, benefits, and risks of participation. You may decide not to participate in this study, which will not affect your health care in any way. Prior to deciding, it is important that you fully understand this consent form. If any word or idea is unclear, please ask Dr. Diego Delgado for clarification. Once you have read this form, you should be able to decide if you wish to participate.

Purpose: The reason for this study is to analyze the presence of a protein called human leukocyte antigen-G (HLA-G) in your blood before and after heart transplantation. The purpose of this study is to investigate the relationship between the pre-transplant levels of HLA-G in blood and rejection post-transplant.

In a single study with limited number of patients, HLA-G was present in approximately 30% of transplanted patients. These patients tend to have significantly fewer problems associated with their graft than patients who do not express HLA-G. Therefore, we are interested in finding out whether patients express HLA-G prior to transplantation, and whether these patients will experience reduced cardiac rejection after transplantation. We also hope to determine if the presence of the protein HLA-G decreased other antibodies in the blood that may affect your heart after the transplant.

Procedures: You will be asked to donate blood for this study. Blood samples will be taken once before your transplant and at one week, one, three and six months after the transplant. You should fast overnight, and no smoking is recommended two hours before the extraction. Thirty milliliters of blood (approximately 2 tablespoons) from a vein of your arm will be drawn.

There will be no changes in your clinical care during this study, except for the one additional blood sample. The molecule HLA-G will be analyzed from your blood. Also, other antibodies in your blood will be investigated at one, three and six months after the transplant.

After transplantation, heart biopsy samples will be regularly obtained from you to monitor the health of the graft by searching for signs of rejection. One of the samples will be used to assess for HLA-G expression at one week and one, three and six months after transplant.
Your previous medical record will be reviewed to compare your treatment history to the results of the blood and biopsy tests. The investigators will have access to your medical records to collect information that is needed for the study for a period of six months from the time of your transplant.

**Risks:** Minor complications may occur from the removal of blood, including minor bruising. Infections may occur but are extremely unusual.

**Benefits:** You will not receive any direct benefit from participating in this study. Future heart transplant recipients might benefit, since your participation will help us understand HLA-G expression and how it potentially protects against graft rejection. This may contribute to the development of new therapeutic strategies for future patients in the same situation as yourself.

**Confidentiality:** Your confidentiality will be respected. Only a study number will be used to identify your biopsy and blood samples. The results of these blood tests will not be placed in your health record. Your blood sample will only be used for the research purposes outlined in this consent, and will be destroyed after the study ends.

Only Dr. Delgado and his research staff will access your records to collect information that is needed for the study, and this information will be kept in a locked secure area. Records containing patients’ information would be available to the study team or the University Health Network Research Ethics Board. The Research Ethics Board may access the study records and the participants’ personal health information to ensure that the information collected is correct and to make sure that the study was done according to the applicable laws and guidelines. All the information collected during the study, including personal health information will be kept confidential and will not be released to anyone outside the hospital, and in the event of premature withdrawal from the study, the information about participants that was collected before that time point will still be used, but no further information will be collected. The biopsy samples for HLA-G determination will be destroyed once the study ends, and will not be used for any other purpose.

**Participation:** Your participation in this study is entirely voluntary. You may withdraw from this study at any time if you wish without any impact on your medical care.

**Compensation:** If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this study. In no way does signing this consent form waive your legal rights nor does it relieve the investigators or involved institutions from their legal and professional responsibilities.

**Questions:** If you have any questions after reading this consent form please contact Dr. Diego Delgado at 416-340-4800 ext. 3940. If you have any questions about your rights as a research participant, please call Dr. R. Heslegrave, Chair of the University Health Network
Research Ethics Board at (416) 340-4557. This person is not involved with the research project in any way and calling him will not affect your participation in the study.

Consent: I have had the opportunity to discuss this study and my questions have been answered to my satisfaction. I consent to take part in the study with the understanding that I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form.

I voluntarily consent to participate in this study.

_________________________________  ________________________________  ________________
Study Participant’s Name (Please Print)  Study Participant’s Signature  Date

_________________________________  ________________________________  ________________
Name of Person Obtaining Signature  Date
Consent (Please Print)

The investigator confirms that the nature and purpose of the study has been explained to the study participant. All the questions he/she had have been answered.

_________________________________  ________________________________  ________________
Name of Investigator  Signature  Date
Obtaining Consent
9. REFERENCES

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