

Role of hsp-27 in cardiac transplant rejection

**A thesis submitted for the degree of Doctor of Philosophy
and the Diploma of Imperial College**

By

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Abstract

Prevention of allograft rejection following cardiac transplantation is the major obstacle to long-term graft survival. Previous studies have suggested that overexpression of hsp-27 protects against non-transplant atherosclerosis and cardiac allograft vasculopathy in humans and *ex vivo* induced I/R injury in mice. The purpose of this study was to investigate whether overexpression of hsp-27 protects the heart from acute and chronic rejection using mice overexpressing HA-tagged human hsp-27 as donors. Overexpression of HA-tagged hsp-27 was confirmed by western blot and immunocytochemistry. ELISA showed presence of hsp-27 in the serum of transgenic animals. In the acute rejection model, B10.A hearts from transgenic or littermate controls were transplanted into C57BL/6 wild-type recipients. Survival of transgenic allografts was significantly prolonged compared to littermate control allografts. Furthermore, RT-PCR and immunohistochemistry results demonstrated decreased cellular infiltration of CD3+ and CD8+ T-cells and decreased inflammatory cytokines in transgenic allografts compared to controls. A chronic rejection model was established by grafting B10.A donor hearts into CD4+ T-cell depleted CBA recipients. Cardiac allografts harvested at 4, 6 and 8 weeks post-transplant showed reduced intimal thickening and less vessels affected in transgenic allografts compared to littermate controls. This was associated with significant diminution of infiltrating T-cells but augmentation of IL-4 production in transgenic grafts. Flow cytometry analyses showed that hsp-27 may not influence alloantibody production. *Ex vivo* studies suggest that overexpression of hsp-27 significantly decreased the activity of caspase-3, -9 and -1 following ischaemia. In addition, the increase in caspase-3 activity was significantly reduced in transgenic hearts following I/R injury *in vivo*. However, this study failed to demonstrate the immunomodulatory effect of hsp-27 *in vitro*. Our data suggest that hsp-27 protects against acute and chronic rejection. Protective mechanisms include a delay in inflammatory responses and protection against apoptosis of cardiomyocytes.

Declaration

All the work described in this thesis was performed by the author and any contributions from other members are appropriately indicated below or in the text.

The work was undertaken at Harefield Heart Science Centre (HHSC), Imperial College London. The Funding was provided by National Heart & Lung Institute Foundation.

In vitro work including cell culture, Elispot, preparation of monoclonal antibodies and flow cytometry analyses were performed by Ms Ann McCormack. Immunohistochemistry staining of the cardiac grafts in acute rejection and CAV (chapter 4 and chapter 6) were carried by Ms Ann McCormack. Staining for smooth muscle alpha actin and sectioning and staining of paraffin sections for graft vasculopathy were carried out Dr. Padmini Sarathchandra. Echocardiography recordings were performed with the assistance of Dr. Manoraj Navaratnarajah.

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I dedicate the thesis to my Family. Special dedication to my mum and my baby Joshua.

Abbreviations

4HNE: 4-Hydroxy-2-nonenal
AMR: antibody mediated rejection
APC: Antigen presenting cell
BCA: Bicinchoninic acid
BSA: Bovine serum albumin
CAD: caspase activated DNase
CAV: Cardiac allograft vasculopathy
CD: Cluster of differentiation
CD40L: CD40 Ligand
CMV: cytomegalovirus
Ct: Cycle threshold
DAMP: Damage associated molecular patterns
ECL: Enhanced chemiluminescence
ELISA: Enzymed-linked immunosorbent Assay
FCS: Foetal calf serum
H₂O₂: hydrogen peroxide
HHSC: Harefield Heart Science Centre
HLA: Human leukocyte antigen
HOCL: Hyperchloride
HRP: Horseradish peroxidase
Hsp: Heat shock protein
Hsp-27: Heat shock protein 27
I/R: Ischemia reperfusion
ICAM: Intracellular adhesion molecule
IFN: Interferon
Ig: Immunoglobulin
IHC: Immunohistochemistry
IL: Interleukin
ISHLT: International Society for Heart and Lung Transplantation
IVC: Inferior vena cava
KO: Knock-out
LC: Littermate control
LPS: Lipopolysaccharide
mAb: Monoclonal antibody
MAPKAP: Mitogen-activated protein kinase-activated protein

MB: Mannose-binding
MDA: Malodialdehyde
MHC: Major histocompatibility complex
MPC: Magnetic particle concentrator
NK cells: Natural killer cells
 O_2^- : Superoxide
OCT: Optimal cutting temperature
 OH^- :hydroxyl free radicals
PA: pulmonary artery
PAMP: Pathogen associated molecular patterns
PBS: phosphate buffered saline
PBST: phosphate buffered saline Tween-20
PCR: Polymer chain reaction
PKC: Protein kinase C
PRR: Pattern recognition receptor
PVDF: Polyvinylidenedifluoride
ROS: Reactive oxygen species
RPM: Revolutions per minute
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMA: Smooth muscle alpha actin
SMC: Smooth muscle cell
SVC: Superior vena cava
TBS: Tris buffered sulphate
TdT: terminal deoxynucleotidyl transferase
TGF: Transforming growth factor
TLR: Toll like receptor
TNF: Tumour necrosis factor
TRAIL: TNF-related apoptosis inducing ligand
Treg: Regulatory T-cells
VCAM: Vascular cell adhesion molecule
WPB: Weibel Palade Bodies

Publications, Presentations and Prizes

Publications

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Chapter 1: Introduction

1.1 Heart transplantation

Cardiovascular disease and cardiac failure remain the leading causes of death in western countries. Causes of heart failure include acute coronary syndrome, chronic hypertension, (cardiomyopathy) and ischaemic heart diseases including sudden death and atrial fibrillation. Heart transplantation is the gold standard treatment for patients with end-stage heart disease that cannot be treated by conventional techniques.

Tracing the history of heart transplantation gives an insight into the challenges that have been overcome. The first successful human transplant was performed in 1967 by Dr. Christiaan Barnard where the patient died eighteen postoperative days of pseudomonas pneumonia (Cooper, 2001). Over the past 50 years, there have been dramatic changes in cardiovascular sciences, cardiac transplantation and clinical care with a marked decline in morbidity and mortality. As of end 2008, almost 85 thousand heart transplant procedures had been performed world-wide over the past 40 years (Taylor et al., 2009). The 1-year survival rate is approximately 80% and the half life of a heart transplant is around 10 years (Stehlik et al., 2010). With advances in immunosuppression and surgical techniques, the rate of rejection and infection leading to graft failure have greatly declined over the past 20 years (Wong et al., 2005). However the survival rate decreases to 72% at 5 years and the 10-year survival rate is only 50%. The major complications of transplantation are rejection and infection.

1.2 Immune cells involved in transplant rejection

Transplant rejection is the end results of a broad spectrum of immunological insults to the allograft. The immune system is classically divided into innate and adaptive immunity (Figure 1). Innate immunity consists of non specific actions of cells and inflammatory mediators while adaptive immunity consists of antigen specific cell injury. Innate immunity is essential for preventing pathogens from growing freely in the body while adaptive immunity has a long lasting

memory of a specific pathogen. Although previous work has focused on the role of adaptive immunity as the cause of allograft rejection, recent studies show that innate immunity also contributes to graft rejection (Brennan et al., 2010; LaRosa et al., 2007; Millington and Madsen, 2010).

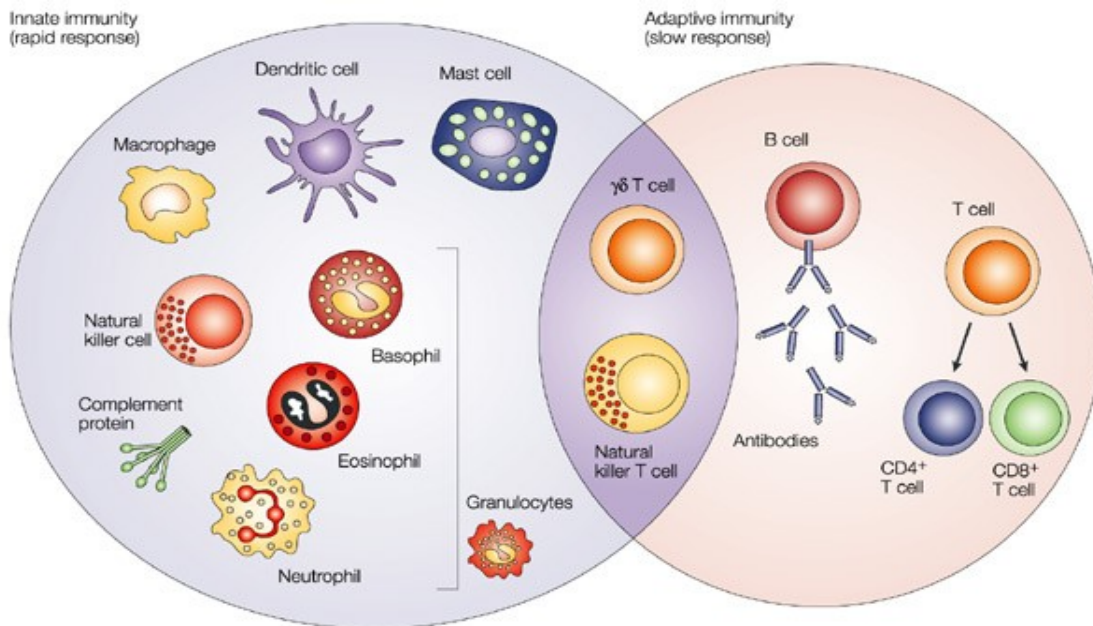


Figure 1: Cells of the innate and adaptive immune systems. Neutrophils are the first cells to arrive to the site of infection, followed by macrophages. Neutrophils, macrophages, natural killer cells and the complement system as well as dendritic cells are the major cells of the innate immune system participating in the immune response against the allograft. Macrophages and especially dendritic cells link the innate and adaptive immune system by stimulating alloreactive T-cells and B-cells (Millington and Madsen, 2010).

1.3 Innate immunity

The innate immune system is the first-line of defence against invading micro-organisms. The cells involved in this non-specific immune system include neutrophils, macrophages, dendritic cells, natural killer (NK) cells, eosinophils, basophils, mast cells as well as the complement system (Akira et al., 2006). The cells of the innate immune system such as neutrophils,

macrophages and dendritic cells possess pathogen recognition receptors (PRR)-which are able to recognize and bind to highly conserved common structures of many bacterial surfaces. These PRRs include the macrophage mannose receptor, the receptor for lipopolysaccharide (LPS) and the scavenger receptors (Takeuchi and Akira, 2010). The toll like receptor (TLR) is a well-defined and most extensively studied PRR that plays an important role in initiation of innate immune responses and shaping the adaptive immune response (Iwasaki and Medzhitov, 2004). Indeed, the innate immune system can discriminate between the surface molecules expressed by the host and those of the pathogen. The latter is defined as pathogen associated molecular patterns (PAMPs) and includes LPS, peptidoglycan and DNA containing unmethylated CpG dinucleotide motifs (Medzhitov and Janeway, 2000). For instance, LPS is recognized by TLR-4 and bacterial DNA containing unmethylated CpG dinucleotide motifs is recognized by TLR-9 (Takeuchi and Akira, 2010).

In addition, the innate immune system can also detect markers of tissue injury, known as damage associated molecular patterns (DAMPs). During transplantation, injury to the tissue and ischaemia reperfusion (I/R) injury leads to generation of DAMPs including reactive oxygen species (ROS), heat shock proteins (HSP), and heparin sulphate (Brennan et al., 2010; Land, 2012).

The recognition of DAMPs/PAMPs by PRRs leads to phagocytosis of the target cell and activation of innate immune responses (Figure 2). This subsequently leads to upregulation of adhesion molecules, co-stimulatory molecules and secretion of cytokines and chemokines (Newton and Dixit, 2012). Cytokines are proteins that are able to modify the behaviour of other cells that have receptors for them, while chemokines are proteins that attract cells with specific chemokines receptors (Janeway et al., 2005). Many different stimuli such as infection and physical damage stimulate phagocytic cells to secrete chemokines. There are two main groups of chemokines: The CC (with two adjacent cysteines near the amino terminus) and the CXC (where the cysteines are separated by a single amino acid).

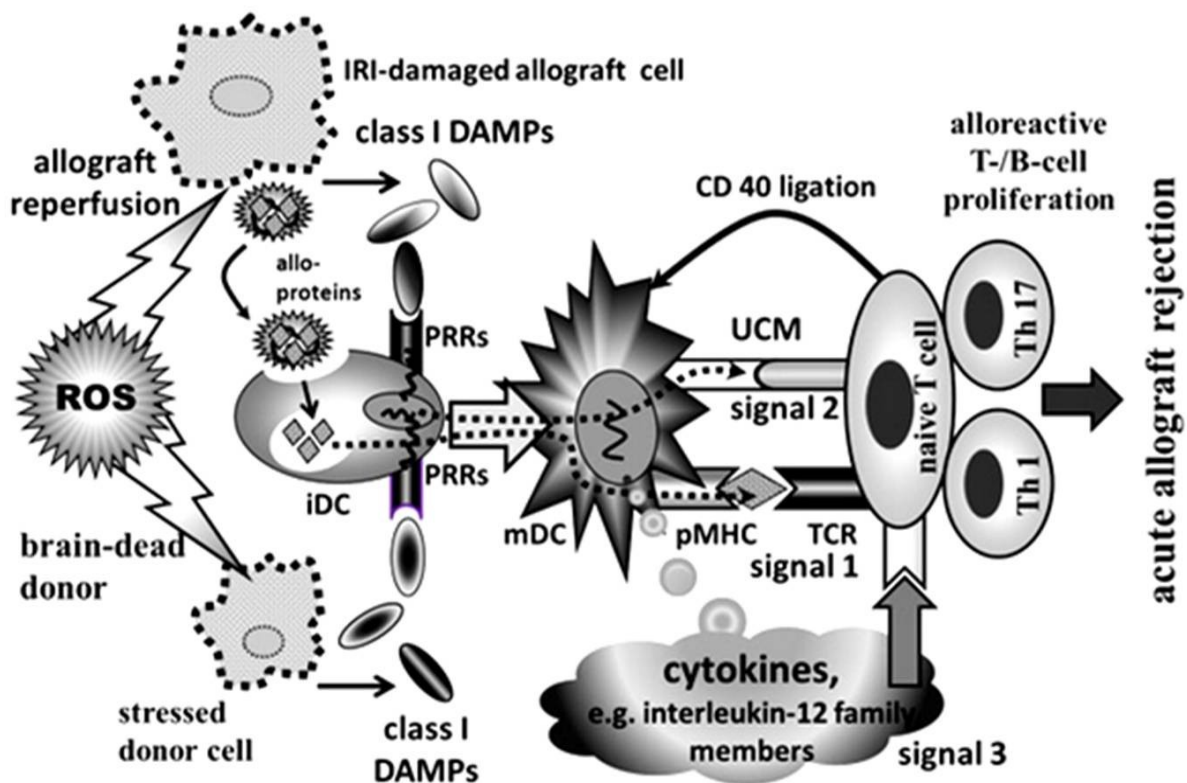


Figure 2: Tissue injury caused during pre and post transplantation leads to induction of DAMPs. Recognition of DAMPs by PRRs subsequently results in maturation of dendritic cells, secretion of cytokines, upregulation of co-stimulatory and adhesion molecules that leads to immune activation and allograft rejection. Diagram taken from (Land, 2012).

The two groups of chemokines are expressed on different cell types and act on distinct receptors: described in Figure 3. For example, CXCL8 acts on neutrophils while CCL2 is a chemoattractant to monocytes.

These chemokines act as chemo-attractants and recruit a large number of cells to the site of infection including leukocytes (neutrophils, monocytes and dendritic cells), lymphocytes and other cells. In addition, the chemokines activates the target cells and help to attack the pathogens that they encounter (Bachmann et al., 2006).

Cell type	CCR expressed	CXCR expressed	Representative surface markers
Immature DC	CCR1, CCR2, CCR5, CCR6, CCR7	CXCR1, CXCR2	CD40 ^{low} , CD80 ^{low} , CD86 ^{low} , MHC class II ^{low}
Licensed DC	CCR7	None	CD40 ^{low/int} , CD80 ^{low/int} , CD86 ^{low/int} , MHC class II ^{int/hi}
Mature DC	CCR7	None	CD40 ^{hi} , CD80 ^{hi} , CD86 ^{hi} , MHC class II ^{hi}
Naive T cell	CCR7	CXCR4	CD62L ^{hi} , CD44 ^{low} , CD69 ^{low}
Activated T cell	CCR2, CCR3, CCR4, CCR5, CCR6, CCR8	CXCR5	CD62L ^{low} , CD44 ^{hi} , CD69 ^{hi}

Figure 3: Expression of chemokines in different cell types. Table taken from (Bachmann et al., 2006) (Hancock et al., 1983)

The main role of the innate immune response is the recruitment of phagocytic cells to the site of infection. The recruitment of leukocytes to the site of infection is a multi-step process that is regulated by cytokines and chemokines described in Figure 4. During inflammation, recognition of DAMPs/PAMPs by PRRs leads to activation of macrophage and secretion of cytokines such as tumour necrosis factor alpha (TNF- α) and leukotriene B4, complement fragment C5a or histamine (secreted by mast cells). Subsequent secretions by macrophage stimulate the endothelium and lead to externalisation of granules called Weibel Palade Bodies (WPB) that contain P-selectin (Janeway and Medzhitov, 2002). In addition, stimulation of endothelial cells leads to upregulation of other selectins such as E-selectin and adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on their cell surface (Newton and Dixit, 2012). P-selectin followed by E-selectin bind to the ligand sulphated sialyl Lewis^x expressed on neutrophils and monocytes. This weak binding and breaking of contact is seen as rolling of leukocytes along the endothelium. This is followed by interaction of lymphocyte-function associated antigen-1 (LFA-1) or CR-3 (leukocytes) with ICAM-1. A conformational change induced by chemokines such as CXCL8, leads to tighter interaction between these molecules and helps the leukocytes to cross the endothelial wall, through interaction with CD31 present on both the leukocytes and the intercellular junctions of endothelial cell wall, a process known as extravasation. Leukocytes then penetrate the

basement membrane (diapedesis) and enter the subendothelial tissue with the help of matrix metalloproteinase enzymes expressed at the cell surface (Liaskou et al., 2012). Neutrophils are the first cells to be recruited to the site of inflammation through the action of chemokine CXCL8. The recruitment of neutrophils is later followed by monocytes that enter the infected tissue under the influence of CCL2 and differentiate into macrophages (Janeway et al., 2005).

The leukocytes recruited at the site of infection have a dual role: they control the destruction of the pathogen and release chemokines and cytokines that act together to recruit more cells to the site of inflammation, which leads to potent activation of inflammation. Following phagocytosis, macrophages and neutrophils produce a variety of toxic products including nitric oxide, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) that destroy the engulfed pathogen (Newton and Dixit, 2012).

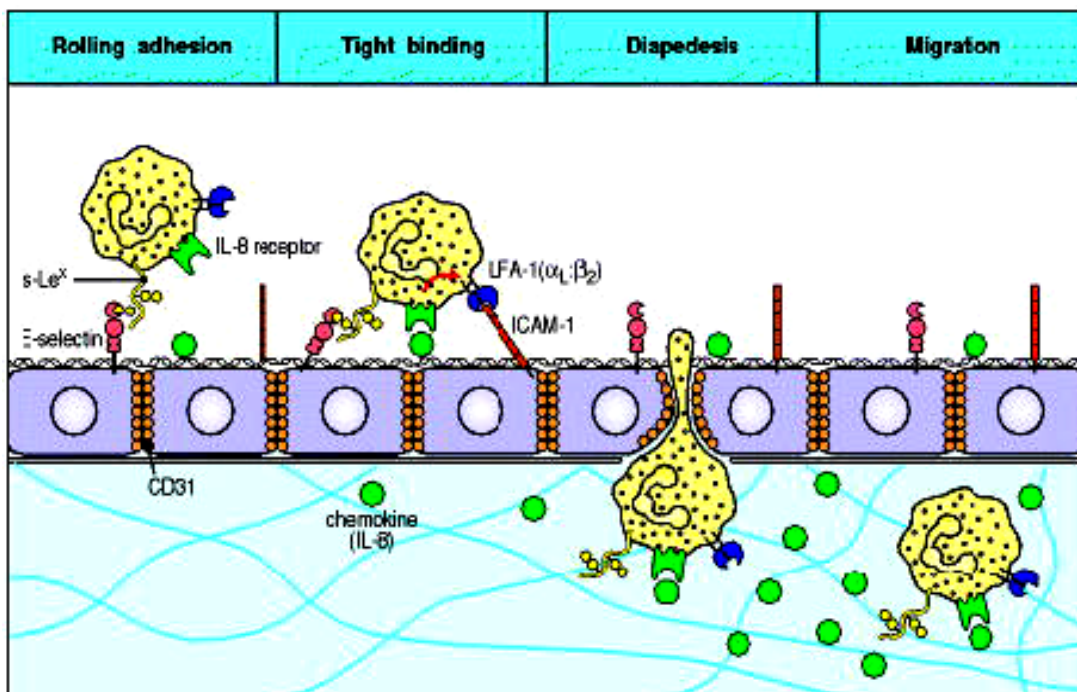


Figure 4: Inflammatory pathway and recruitment of leukocytes to the site of infection. Diagram taken from (Janeway et al., 2005).

The inflammatory infiltrates, once activated, secrete cytokines that define the type of inflammation: pro-inflammatory or anti-inflammatory. The pro-inflammatory cytokines produced by monocytes/macrophages including interleukin-1 (IL-1), IL-6 and TNF- α are involved in

transplant rejection while the anti-inflammatory cytokines such as IL-4 and IL-10 produced by these cells probably play a role in downregulating the immune response and transplant tolerance. Macrophages are inefficient in activating naive T-cells, but following transplantation they accumulate in the graft where they secrete cytokines and effectively present antigen to activated or memory T-cells (Hancock et al., 1983). There is evidence that neutrophils also participate in transplant rejection since preventing infiltration of neutrophils resulted in prolongation of graft survival (Morita et al., 2001).

1.3.1 Complement

The Complement system is a series of stepwise proteolytic events, where circulating enzymes in a precursor form are converted to active proteases. The complement cascade can be activated by three distinct pathways: the classical pathway, the mannose-binding (MB)-lectin pathway and the alternative pathway (Fujita et al., 2004). The classical pathway is activated by binding of the C1q to antibody-pathogen complex or to C-reactive protein bound to the pathogen or by direct binding of C1q to the surface of the pathogen. This leads to conformational changes in C1q, which then binds to other enzymatic components C1r and C1s. This complex cleaves the C4 into C4a and C4b. This exposes the active site of C4b transiently, which can bind covalently to antibody or the surface of endothelial cells via an ester or amide bond. C4b is the ligand for C2, which is cleaved by C1s into C2a and C2b. C2aC4b forms the C3 convertase (Wasowska, 2010). The participation of C4b in the classical convertase pathway is inhibited by the factor I, which cleaves the split product into inactive C4b (iC4b). The iC4b is then further cleaved by factor I into C4c and C4d. The latter is a marker of antibody mediated rejection (Murata and Baldwin, 2009). In fact, following cleavage of C4b, the covalent bond between C4d and the tissue remains intact, thus it can be detected near the site of complement activation. However, no biological function has been identified for this molecule. Interestingly, C4d can also be generated by MB- lectin pathway (Cohen et al., 2012). The MB-lectin pathway is triggered by the interaction of MB-lectin to encapsulated bacteria. Finally, the alternative pathway is activated by binding of spontaneously activated C3 to hydroxyl or amine group on

cell surface of the pathogen (Meri and Jarva, 1998). The activation of all three pathways leads to the same effector function and generates a protease C3 convertase. The latter cleaves C3 to make C3b and C3a. C3b has important effector functions: it binds covalently to the surface of the pathogen and acts as an opsonin to promote up-take and removal by phagocytes. Moreover, C3b together with C3 convertase form C5 convertase, which cleaves C5 to produce C5a and C5b. C3a and C5a are known as anaphylatoxins, *i.e.* these are small peptides and potent mediator of inflammation. They are involved in stimulation and recruitment of phagocytes bearing specific anaphylatoxin receptors to the site of inflammation. They increase vascular permeability; induce the expression of certain adhesion molecules on endothelial cells and acts as a powerful chemoattractant for neutrophils and monocytes to the site of inflammation. Moreover, they activate mast cells and phagocytes to secrete histamine and the cytokine TNF- α (Gasque, 2004).

C5b activates the late event of complement component to form a membrane attack complex (C5b binds to one molecule of C6, C7, C8 and C9 each) that damages the membrane of certain pathogens by forming pores in the membrane.

1.3.2 NK cells

NK cells develop from the bone marrow from the common lymphoid progenitor cells. NK cells have been suggested to have a role in organ transplant rejection (Obara et al., 2005). NK cells are large granular lymphoid-like cells that belong to the innate immune system. Their mechanism of killing is the same as that used by the cytotoxic T-cells from the adaptive immune response. The NK cells express the chemokine receptors CCR2, CCR5, CXCR3 and CXCR5 which facilitate their recruitment to the site of infection (Gregoire et al., 2008; Shi et al., 2011). In fact, they are recruited early to the site of inflammation. The activation of these cells depends on different mechanisms: through interaction of the Fc region of immunoglobulin bound to the pathogen, through recognition of altered molecules on stressed cells or through exposure to cytokines such as interferon (IFN) or IL-12 (Lanier, 2008; Shi et al., 2011). Although they lack antigen specific receptors to recognise pathogens, they seem to have certain mechanisms to

distinguish between self and altered-self. However, the mechanism of how this is accomplished is not yet fully understood. NK cells express two types of receptors on their cell surface: activating receptors and inhibitory receptors (Bryceson et al., 2006). Stimulation of activating receptors lead to killing of target cells while activation of the inhibitory receptors prevents NK cells from killing host cells. The activating receptors include members of C-type lectins while the other set of receptors is composed of immunoglobulin (Ig) like domains that are called Ly49 in mouse and KIR in humans (Shegarfi et al., 2012). The ligands for these cells are not well known; however, the activating receptors recognise changes in cell surface glycoproteins and thus detect virus infected cells. On the contrary, inhibitory receptors have been suggested to be specific to and bind to self-major histocompatibility complex (MHC) class I molecules and thus prevent activation of NK cells (Cheent and Khakoo, 2009). Therefore, absence of self-MHC class I on the allograft could lead to the activation of NK cells and consequently its lysis.

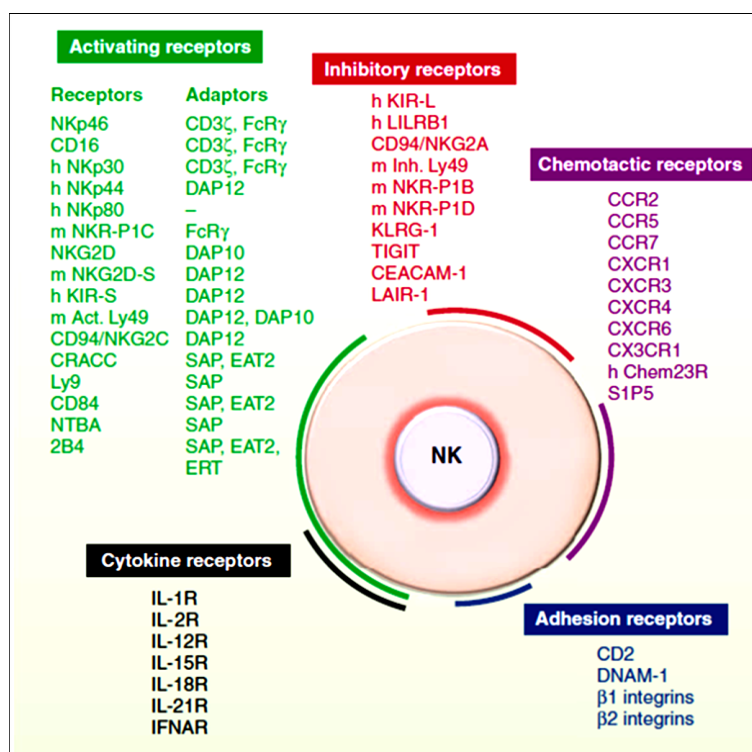


Figure 5: Receptors expressed on NK cells. NK cells possess activating and inhibiting receptors that determine the type of effector reaction of NK cells. Moreover they also express cytokine receptors, chemotactic receptors and adhesion receptors which facilitate the recruitment of NK cells to the site of infection and initiate inflammation. Figure taken from (Vivier et al., 2011).

In summary, the activation of NK cells depends on the overall balance of activating and inhibitory receptors. NK cells exert their effector functions by various means: they produce both pro-inflammatory and anti-inflammatory cytokines: IFN- γ , TNF- α and IL-10 as well as growth factors GM-CSF and G-CSF (Shi and Zhou, 2011). They also exert their function through cytotoxicity: they kill the target cell through induction of apoptosis (Shi and Zhou, 2011). In fact, like CD8+T-cells, NK cells contain cytoplasmic granules perforin (Topham and Hewitt, 2009). In addition, they can also exert the apoptotic activity through TNF-related apoptosis inducing ligand (TRAIL) and FAS-ligand (Shegarfi et al., 2012). In the transplant setting, in the MHC mismatch model, NK cells are activated due to recognition of missing self and this leads to amplification of inflammation and T-cell activation thus promoting graft rejection (Maier et al., 2001; McNerney et al., 2006). Figure 5 shows the full set of receptors present on NK cells.

1.3.3 Dendritic cells: activation of dendritic cells

The antigen independent innate immune response occurs very early after infection/transplantation. However, the innate immune system cannot eliminate the microorganisms or reject the allograft on its own. Activation of dendritic cells is critical for the initiation of adaptive immunity. Figure 6 shows the interaction between innate and adaptive immunity in the context of transplantation. Adaptive immunity starts when a pathogen is ingested by an immature dendritic cell in the infected tissue (Palucka and Banchereau, 1999; Steinman and Hemmi, 2006). At this stage, dendritic cells express low levels of MHC molecules and co-stimulatory molecules. Similarly to the macrophages, dendritic cells also possess PRR that recognise PAMPs/DAMPs, and are recruited to the site of infection, phagocytose the microorganism and subsequently become activated into a highly potent antigen presenting cell (APC) (Munz et al., 2005). Dendritic cells can also take up virus and encapsulated bacteria (other extracellular antigens) by a receptor-independent mechanism called micropinocytosis, where large volumes of surrounding fluids are engulfed (Sallusto et al., 1995). The dendritic cells then migrate carrying the pathogen antigen to the peripheral lymphoid organs where they interact with circulating naive lymphocytes (Altfeld et al., 2011; Banchereau and Steinman,

1998). The activated dendritic cells secrete cytokines (IL-12, IL-15, IL-23, IL-6, TNF, IL-1 β) that influence the innate and adaptive immune responses to determine whether and how the immune systems respond to the presence of the infectious agent (Lanzavecchia and Sallusto, 2001; Liu, 2001). They also secrete chemokines such as CCL18 that specifically attracts naive T-cells.

Within lymph nodes, dendritic cells, macrophages and B-cells present antigen to T-cells. Dendritic cells are the most potent antigen presenting cells (Ni and O'Neill, 1997). As immature cells, in the peripheral tissues they ingest the antigen and migrate to local lymphoid tissue where they mature into APCs that are very efficient at stimulating naive T-cells. However, once matured, they lose the ability of phagocytosis. The maturation of dendritic cells is accompanied by the expression of adhesion molecules including ICAM-1, ICAM-2, LFA-1 and CD58, co-stimulatory molecules such as B7 molecules, increased expression of MHC molecules on their surface which are essential for priming of T-cells (Bodey et al., 2004; Lafferty and Cunningham, 1975; Ni and O'Neill, 1997; Zou and Tam, 2002). Blocking of co-stimulation in a complete MHC mismatch transplant allograft model prolongs survival of the graft (Glysing-Jensen et al., 1997; Larsen et al., 1996; Lin et al., 1993). Moreover, presentation of antigen in the absence of co-stimulation leads to transplant tolerance (Larsen et al., 1996). A diagram of the interaction of innate and adaptive immunity in graft rejection is shown in Figure 6.

Dendritic cells therefore play a major role in directing adaptive immune response by taking the antigen from the graft, processing it and presenting it to naive alloreactive cells present in the secondary lymphoid organs (Larsen et al., 1990).

1.4 Adaptive immune response

The innate immune system cannot always eliminate the microorganisms. The adaptive immune system is made of cells, lymphocytes, which provide a more versatile means of defence. In addition, it initiates a rapid secondary immune response, hence providing increased protection against subsequent re-infection with the same pathogen. In the adaptive immune responses,

antigen is recognized by two distinct sets of highly variable receptor molecules: the immunoglobulins are the antigen receptors on B lymphocytes (B-cells), and the antigen specific receptors of T lymphocytes (T-cells). Lymphocytes arise from common lymphoid progenitor cells. Both B- and T-cells originate from bone marrow. T-cells migrate to the thymus, where they undergo maturation, while B-cells mature in the spleen.

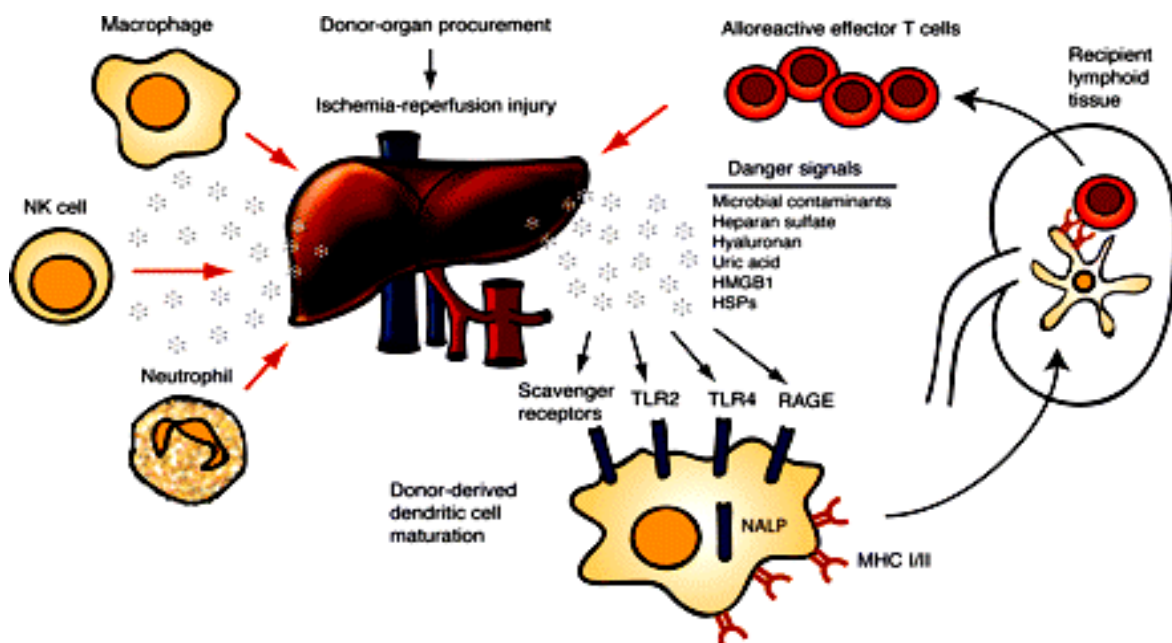


Figure 6: Interaction of innate and adaptive immunity in graft rejection. The cells of the innate immune system are activated by tissue injury and other non-immunological insults thus enhancing immunogenicity and activating the adaptive immune response. (Figure taken from (LaRosa et al., 2007))

1.4.1 T-Lymphocytes

T-cells are a crucial part of the adaptive immune response and they play a major role in cellular immunity. Several different subsets of T-cells are known including CD4⁺ helper or CD8⁺ cytotoxic T-cells, Th17 cells as well as regulatory T-cells (Berner et al, 2000). Each naive lymphocyte in the blood stream bears antigen receptors of a single specificity that was pre-determined by a unique genetic mechanism that operates during lymphocyte development (Janeway et al., 2005). Within the thymus, the developing naive T-cells, whose receptors interact weakly with self antigens (presented by thymic epithelial cells) as a peptide bound to

MHC molecules, are positively selected to survive. However, the cells bearing receptors that bind strongly to self antigens are negatively selected and die without reaching maturity (Saito and Watanabe, 1998; Starr et al., 2003). In this way, the immune system is self- tolerant and prevents autoimmune reactions. Positively selected cells mature and continuously re-circulate between blood stream and peripheral lymphoid tissue until they encounter their specific antigen in the peripheral lymph node, in the form of peptide-MHC complex on the surface of an activated APC (dendritic cell, macrophage, B-cell) (Banchereau and Steinman, 1998).

Upon contact with their specific antigen, naive but mature T-cells become activated to clonally expand and differentiate into armed effector T-cells. Clonal expansion is mediated by IL-2 produced by activated T-cells (Curtsinger et al., 1999).

The effector cells then re-enter the bloodstream and migrate to the site of infection. Activated cells are characterised by secretion and response to IL-2, changes in expression of adhesion molecules and their ability to perform the effector function without the need for co-stimulation. Effector cells have a short life span and most of them undergo apoptosis after the antigen has been eliminated. However, a significant number persists and becomes memory cells. These cells are responsible for rapid and effective responses when they encounter the same antigen a second time.

Naive T-cells are stimulated by activated dendritic cells, which also provide the second signal known as co-stimulation (Frauwirth and Thompson, 2002). However, macrophages and B-cells can also present the antigen to T-cells. The immune system has different strategies to eliminate pathogens from intracellular compartments and the cytosol. The APCs express both class of major histocompatibility complex (MHC) molecules as well as co-stimulatory molecules. The APCs present the antigen to T-cells via MHC class I and MHC class II complex present on their surface (Rescigno et al., 1999). There are different types of T-cells that are distinguished by the co-receptor molecules. The main T-cells subsets involved in adaptive immunity are CD4⁺ T-cells (expressing CD4 co-receptor) and CD8⁺ T-cells (expressing CD8 co-receptor) discussed in more detail below.

1.4.1.1 CD8+ T-cells

MHC class I molecules present peptides from the cytosol (intracellular antigens) to the CD8+ T-cells which then differentiate into cytotoxic T-cells. The function of the CD8+ T-cells is to kill infected target cells, achieved by production of cytokines and membrane associated proteins. CD8+ T-cells produce cytotoxic molecules such as perforin, granzyme, granulysin, Fas ligand and the cytokines TNF- α , TNF- β and IFN- γ (Badovinac et al., 2000). The recognition of antigen on the surface of the target cell stimulates the release of preformed cytotoxic proteins from the granules. The major constituent, perforin, lyse the target cells by creating transmembrane pores (Badovinac et al., 2000; Zhou et al., 2002). Similarly granzyme and granulysin induce apoptosis of target cells. Among the cytokines secreted, TNF- α as well as Fas-ligand can stimulate death by apoptosis. IFN- γ and TNF- α induce increased expression of MHC class I and thus the probability of these cells to be recognized as target cells (Schoenborn and Wilson, 2007). Moreover, TNF- α , TNF- β and IFN- γ activate macrophages into APCs as well as effector cells. The maturation into memory CD8+ T-cells depends on many factors including the type of antigen and it requires the interaction with CD4+ T-cells (Bevan, 2004; Harty and Badovinac, 2008).

1.4.1B CD4+ T-cells

Peptides generated in intracellular vesicles (extracellular pathogens entered through the endocytic pathway) are presented to the CD4+ T-cells via MHC class II molecules. These CD4+ T-cells then differentiate into effector cells that are also known as T helper (Th) cells, due to their role in activating other cells including B-cells and macrophages. CD4+ T-cells can differentiate into four major classes of effector cells termed Th1 cells, Th2 cells, Th17 cells and regulatory T-cells (Treg) (Zhu and Paul, 2008, 2010). The different subset of CD4+ T-cells vary in the cytokines that they produce as well as their effector function (Mosmann and Sad, 1996). The factors that determine the differentiation of CD4+ T-cells into Th1 or Th2 are not yet fully understood; however many factors are thought to be into play such as the cytokines produced by the pathogens, the co-stimulators present on APC that drive the response, as well as the

nature of peptide-MHC complexes (Constant and Bottomly, 1997; Zhu and Paul, 2010). In fact, cytokines present in the environment have a crucial role in the differentiation of CD4⁺ T-cells and in shaping the subsequent adaptive immune response (Farrar et al., 2002).

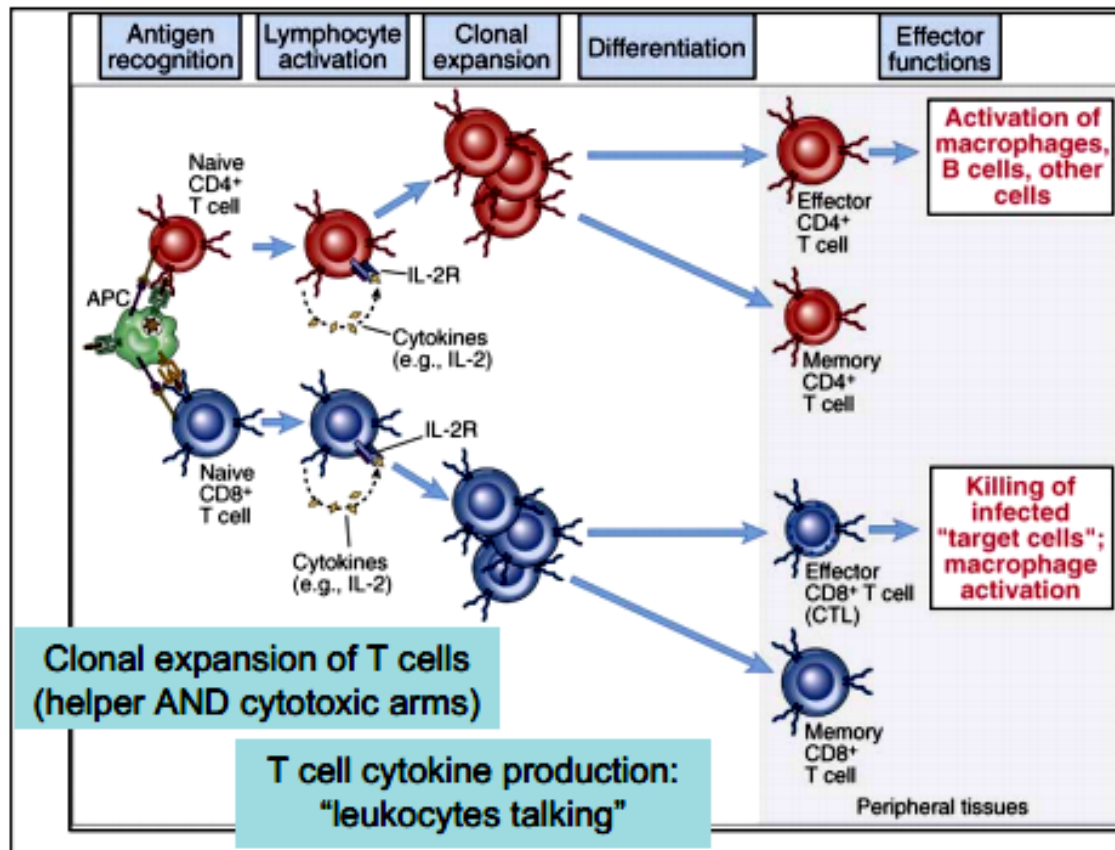


Figure 7: Dendritic cells process and present the antigen to T-cells via the peptide-MHC complex. The recognition of the specific antigen leads to T-cell activation, clonal expansion of cells bearing receptors for that antigen and differentiation to long-lived memory T-cells. Diagram taken from (Abbas et al., 2007).

CD4⁺ T-cells activated in the presence of IL-12 and IFN- γ -expressed by activated macrophages- differentiate into armed Th1 cells, while the presence of IL-4 (especially together with IL-6) promotes differentiation into Th2 cells (Figure 8) (Hsieh et al., 1993; Rogge et al., 1998; Seder et al., 1992). Th1 leads to cell-mediated immunity and production of IgG while Th2 mediate humoral immunity by stimulating B-cells to produce of IgM, IgA and IgE. Figure 7 shows the differentiation of naive T-cells into effector T-cells.

CD4⁺ T-cells perform their effector function by producing cytokines and membrane associated proteins that act by binding to specific receptors on the target cell. Therefore, they act on cells that express MHC class II molecules.

Th1 cells

Th1 cells secrete cytokines including IFN- γ , TNF- α , TNF- β and express CD40 ligand (CD40L) and Fas ligand (Romagnani, 2000). The main effector function of Th1 cells is the activation of macrophages. IFN- γ and TNF- β activate macrophages and inhibit B-cells. IFN- γ together with the CD40 ligand provides activating and sensitising signals respectively, which activate the macrophages into potent effector cells (Schoenborn and Wilson, 2007). The activated macrophages promote killing by producing oxygen radicals, nitric oxide, as well as lysosomal enzymes and proteases. Moreover, the activation is accompanied by increased expression of MHC class II molecules, CD40 and TNF- α receptor on their surface. Th1 produce growth factor IL-3 and GM-CSF that stimulate the generation of new phagocytic cells, while TNF- α , TNF- β and CCL-2 produced are involved in the recruitment and migration of these phagocytic cells to the site of infection (Zenewicz et al., 2009).

Th2 cells

Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13 and express the CD40 ligand on their surface and participate in elimination of extracellular pathogens (Romagnani, 2000). IL-4, IL-5 and IL-13 as well as the CD-40 ligand are involved in activation of B-cells. Notably, IL-4 they trigger isotype switching into IgE in B-cells and promote differentiation of Th2 cells. In addition, IL-10 plays a crucial role in inflammation by inhibiting the activation of macrophages, Th1 proliferation and dendritic cell function (Muraille and Leo, 1998; Zenewicz et al., 2009).

Th17 cells

Th17 is another subset of CD4⁺ T-cells; its development depends on the presence of TGF- β together with IL-21 or IL-6 (Bettelli et al., 2006; Veldhoen et al., 2006; Zhou et al., 2007). Th17 cells promote inflammation; they produce IL-17, and stimulate secretion of numerous

chemokines and cytokines such as IL-6 and IL-8 involved in inflammation and are involved in the recruitment of neutrophils. The expression of IL-21 by Th17 cells may stimulate T-cells, B-cells and NK cells (Schwarzenberger et al., 1998).

Regulatory T-cells

The thymus eliminates autoreactive T-cells, which recognise self-antigens with high affinity by a process known as negative selection. During this process, T-cells that have high affinity for self antigens are deleted. However, existence of autoimmune diseases indicates that the role of thymus in eradicating these autoreactive T-cells is not completely fulfilled (Coutinho et al., 2005). Interestingly, autoreactive cells are also present in healthy controls, but they do not seem to cause the disease. One reason is due to the suppressive effects of Tregs (Lacroix-Desmazes et al., 1998).

The development of Tregs depends on the presence of IL-2 (Nelson, 2004). Tregs participate in the maintenance of immune tolerance by downregulation of immune response. Different types of Treg have been described including Tr1, Tr3 and CD4⁺ CD25⁺ T-cells (Jonuleit and Schmitt, 2003). They have different phenotypes and mechanisms but most suppress immune response by the production of TGF- β and IL-10 (Verbsky, 2007).

T-cells play a central role in the rejection of vascularised allografts. The production of antigen specific T-cells that recognize non-self antigens is the main effector pathway involved in transplant rejection. In experimental model, mice deficient for T-cells or T-cell activation displayed increased graft survival and reduced CAV compared to wild type (Bowles et al., 2000; Glysing-Jensen et al., 1997; Larsen et al., 1996; Lehmann et al., 1997; Youssef et al., 2004). It has been known for many years that acute rejection is a T-cell mediated process since early experiments demonstrated that mice lacking T-cells were unable to reject allografts. This was demonstrated by performing a skin grafts onto nude mice (which lacked T-cells), where the grafts were not rejected (Rosenberg et al., 1987). Graft rejection only occurred when the T-cell population was reconstituted by adoptive transfer of T-cells. The contribution of CD4⁺ and CD8⁺ T-cells in graft rejection was further demonstrated by adoptive transfer experiments of T-

cells into T-cell deficient mice (Bell et al., 1990; Dallman et al., 1982; Whitby et al., 1990). A large body of data support the contribution of Th1 cells as well as the cytokines produced by these cells to graft rejection (Kirkman et al., 1985; Kupiec-Weglinski et al., 1986). Notably, IL-2, IFN- γ and TNF- α were detected in rejecting grafts, while IL-4 –the Th2 cytokine was not (O'Connell et al., 1993).

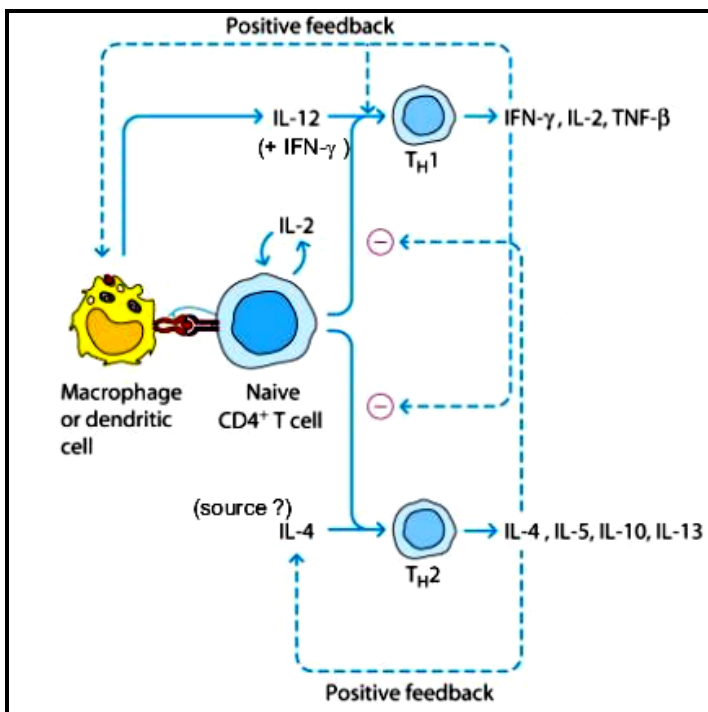


Figure 8: The differentiation of CD4⁺ effector T-cells depend on the cytokines present in the milieu. Predominance in IL-12 and IFN- γ promote the differentiation of CD4⁺T-cells into Th1 helper cells. These cells then secrete the cytokines including IFN- γ , TNF- β and IL-2 that are involved in inflammation. The IFN- γ secreted also positively regulates the macrophages to stimulate production of more Th1 cells. On the other hand, presence of IL-4 encourage the differentiation into Th2 cells that produce IL-4, IL-5, IL-10 and IL-13, which are known as anti-inflammatory cytokines. Diagram taken from immunology lectures by Dr Leonie Taams.

CD4⁺CD25⁺ T-cells play a key role in prevention of alloimmune response and subsequent graft rejection. Pre-treatment with alloantigen and CD4 depletion of the recipients leads to generation of CD4⁺CD25⁺ Treg that are tolerant to donor alloantigens and prevent graft rejection (Kingsley

et al., 2002). There is a growing body of evidence that have shown that Treg are able to prevent acute and chronic rejections (Waldmann et al., 2004; Warnecke et al., 2007; Warnecke et al., 2010). The protection against graft rejection seems to depend on the presence of IL-10 and CTLA-4 expression. (Kingsley et al., 2002; Mellor et al., 2004; Warnecke et al., 2007)

T-cells have been the major focus of transplant research for many years, it is only more recently that it has been realised that B-cells also have a role in transplant rejection,

1.4.2 B-cells

Recently, more studies have been focused on the role of alloantibodies in transplant rejection; the role of B-cells in alloimmune response becomes more evident with the identification of donor specific antibodies and production of *de novo* antibodies in the graft (Dunn et al., 2011; Mahesh et al., 2007; Rose, 2000; Smith et al., 2011; Zhang et al., 2011). B-cells are derived from lymphoid progenitor cells in the bone marrow and mature in the spleen. In the first phase, they rearrange their immunoglobulins in an antigen independent manner. However, this process requires interaction with stromal cells present in the bone marrow (Hardy and Hayakawa, 2001; Lopes-Carvalho and Kearney, 2004). This leads to immature B-cells that carry the antigen receptor in the form of IgM. Similar to T-cell development, immature B-cells that react strongly to self antigens are negatively selected and are removed from the B-cell repertoire. The surviving immature B-cells migrate to the peripheral lymphoid organ where they mature to express IgD in addition to IgM (Ohashi and DeFranco, 2002). At this stage, the naive mature B-cells re-circulate in the peripheral lymphoid tissue and those B-cells that are stimulated by foreign antigen, are activated and are differentiated into antibody-secreting plasma cells or memory cells (Janeway et al., 2005)..

The activation of B-cells requires two signals. The first signal is provided by binding of the antigen to the B-cell antigen receptor. Activated effector T-cells provide the second signal (Klein and Dalla-Favera, 2008). The binding of soluble antigen to the immunoglobulin receptor leads to internalisation of the antigen-receptor complex, degradation of the antigen into fragments, and

presentation of the antigen to CD4⁺ T-cells, in the form of peptide-MHC class II complex. The activated T-cells then provide help for B-cells to proliferate and differentiate into plasma cells that secrete specific antibodies. Activated Th cells express the B-cell stimulatory molecule CD40 on their surface. Interaction of the CD40L (on B-cells) with CD40 expressing activated T-cells causes activation of B-cells (Klein and Dalla-Favera, 2008). However, in certain circumstances, B-cells can also be activated without the help of T-cell, through direct binding of part of the antigen (known as thymus independent antigens) to the surface of B-cells (Lopes-Carvalho and Kearney, 2004).

The cytokines secreted by activated CD4⁺ T-cells serve to direct immunoglobulin isotype switching and hence diversify humoral function. There are 5 classes of antibody: IgA, IgD, IgE, IgG and IgM. All naive B-cells express IgD and IgM, where the early stage of antibody response is directed by the low affinity antibody, IgM. However, IgG and IgA are the predominant isotypes expressed by the plasma cells. The class switching is dependant of CD40L-CD40 interaction. Moreover, different cytokines induce different class switching (Klein and Dalla-Favera, 2008). Thus, IL-4 preferentially induces switching to IgG1 and IgE, while TGF- β induces switching to IgG2b and IgA. On the other hand IFN- γ secreted by Th1 induces switching into IgG2a and IgG3.

Activated B-cells differentiate into either plasma cells or memory cells. Plasma cells secrete high affinity antibody but they have lost the ability to present antigen to T-cells. In contrast, long lived memory B-cells express surface immunoglobulins but do not secrete antibody (Shapiro-Shelef and Calame, 2005).

The antibodies produced can act in three different ways: they can bind to the pathogens and neutralise them, thus preventing their adherence to target cells. Another way is, they coat the pathogens and enhance phagocytosis (through recognition of the Fc region by phagocytic cells): this is referred to as opsonisation. The third possibility is activation of the complement system, which results in opsonisation and lysis of target cells (Janeway et al., 2005; Nakashima et al., 2002).

Antibodies mediate complement activation through the classical pathway. The first step is the binding of antibodies (already attached to the surface of pathogen) to the complement component C1q. The complement independent effector phase is mediated by activation of accessory cells (by binding of Fc region of Ig to Fc receptor) that promote either the phagocytosis of neutralised microorganisms by phagocytic cells or secretion of stored mediators such as histamine, lipid inflammatory mediators and cytokines by non phagocytic cells (Joller et al., 2011). The antibodies (IgG1 and IgG3) bound to the surface of a pathogen can also be recognized by the Fc receptor of NK cells (CD16) and mediate antibody-dependant cell-mediated cytotoxicity (ADCC), which is a similar mechanism of action to cytotoxic T-cells.

Emerging evidences highlight that alloreactive B-cells are critical components of graft rejection (Takemoto et al., 2004). The important role of B-cells in transplant rejection has been demonstrated by the use of anti-CD20 monoclonal antibody (rituximab) to prevent certain transplant rejection (Becker et al., 2006). B-cells not only contribute in the synthesis of alloantibodies but also participate in transplant rejection through other mechanisms such as antigen presentation (Rose, 1998). The exact mechanisms by which B-cells participate in alloimmune response are not fully understood; however, antibodies directed towards the graft endothelium and subsequent endothelial injury has been reported (Ferry et al., 1997; Rose, 2000).

1.5 Allorejection

Allograft transplantation is the transplantation of organs from genetically non-identical members of the same species. Both humoral immunity (antibody-mediated) and cellular immunity play a part in rejection; however the mechanisms underlying cell mediated and antibody mediated rejection (AMR) are not well defined (Schnickel et al., 2004). The term allorecognition refers to discrimination of self versus non-self by T-cells via recognition of genetically encoded polymorphisms displayed on the surface of the cells (Cote et al., 2001). Hence, the primary basis of allograft rejection is the recognition of the MHC antigen present on the graft.

1.5.1 Major and minor histocompatibility:

Genes of the MHC are located on chromosome 6 in humans and on chromosome 17 in mice. They are called Human leukocyte antigen (HLA) system in humans and H-2 in mice. MHC genes are highly polygenic and each gene is highly polymorphic. The cellular immune response depends on the ability of MHC antigens to present alloantigens in the form of a peptide. MHC class I presents the antigen from the cytosol to CD8⁺ T-cells while MHC class II present the peptide originated in the vesicular system to CD4⁺ T-cells.

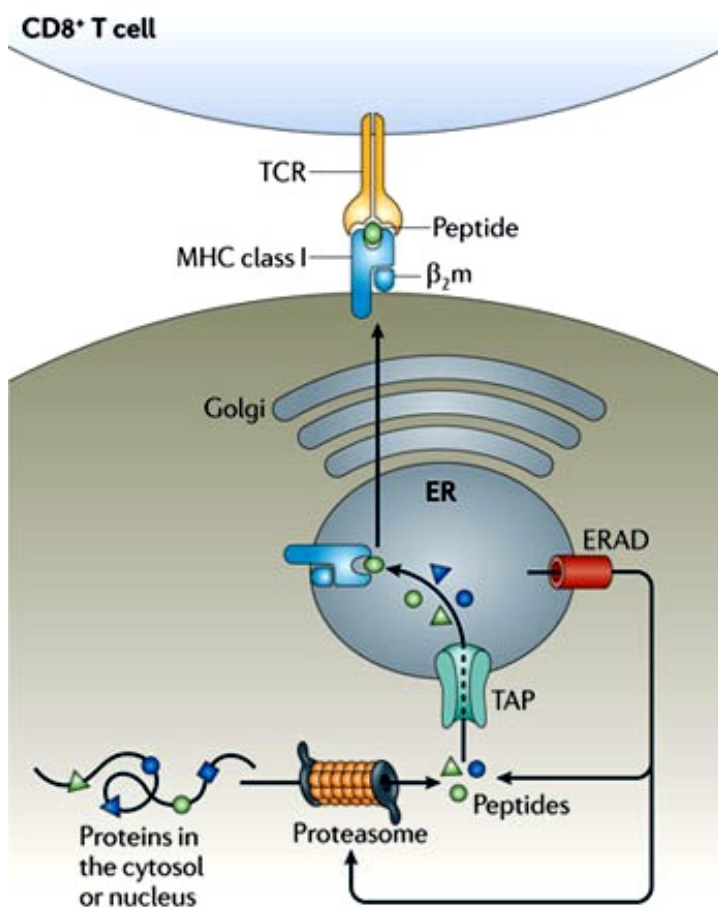


Figure 9: Cytosolic proteins are processed and associate with the MHC class I molecules in the ER. The peptide-MHC complex is then transported to the cell surface via Golgi apparatus, where the antigen is presented to cytotoxic CD8⁺T-cells. Diagram taken from (Neefjes et al., 2011).

The peptides that bind to MHC class I are mostly derived from viruses (Alberts et al., 2002). Thus, intracellular proteins are processed by the proteasome (a large multicatalytic protease)

into short peptides and transported into the endoplasmic reticulum (ER), where they are assembled with MHC class I molecules (Flutter and Gao, 2004). The MHC class I molecules are made of a single α chain non-covalently linked to β_2 microglobulin (Neefjes et al., 2011). The chain first binds to the β_2 microglobulin in the ER, then assembles with the peptide. The assembly of the peptide-MHC class I complex is guided by several chaperone proteins. The peptide-MHC class I complex is then transported through the Golgi apparatus to the cell surface, where it presents the peptide to CD8+T-cells (Figure 9).

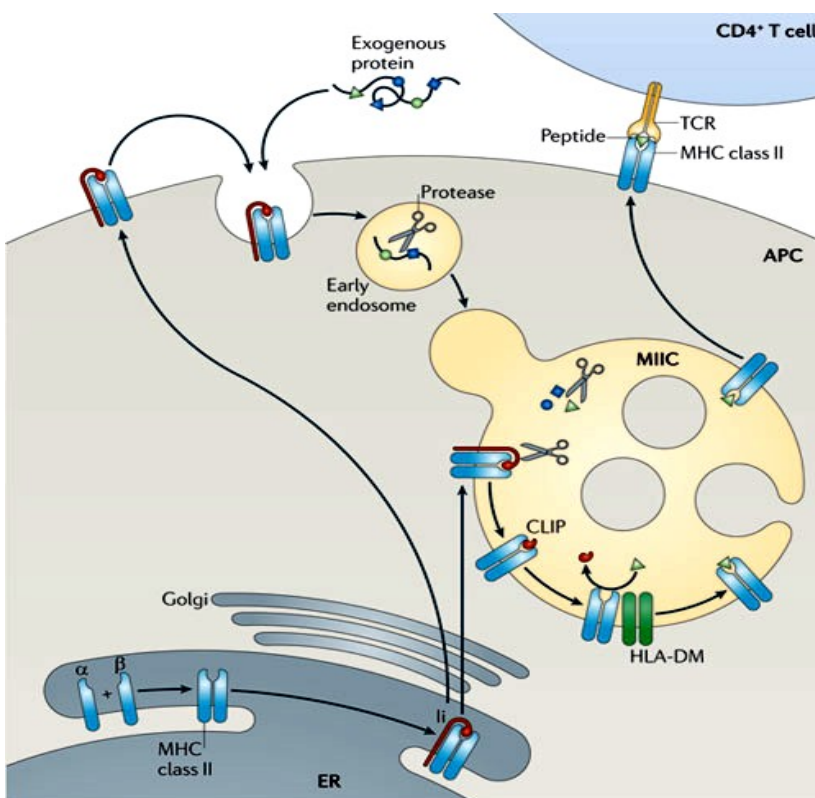


Figure 10: Exogenous proteins are phagocytosed and processed into peptides by the endosome. The α and β chain of the MHC class II are assembled in ER and the otherwise instable molecule bind to the invariant chain. This complex is transported in an acidified vesicle and it fuses with endosome containing the antigenic peptide. The removal of the CLIP (a short peptide of the invariant protein) and the binding of the antigen to the peptide groove is directed by the HLA-DM. The peptide-MHC complex is then transported to the cell surface, where the antigen is presented to CD4+T-cells. Diagram taken from (Neefjes et al., 2011).

The intravesicular pathogens however, are taken up into endosomes and are processed into peptides. The MHC class II molecules are formed by heterodimers of α and β chains (Neefjes et al., 2011). The assembly of α and β chains take place in the ER, where an invariant chain is bound to the groove of the molecule. This invariant chain is then cleaved and a short peptide fragment known as CLIP which will be bound to the MHC class II molecule and transported in an acidified endosome (Cresswell, 1994). The fusion of the acidified endosome (containing CLIP-MHC class II complex) with the endosome that contains the processed antigens allows the exchange of the CLIP with the antigenic peptide in the groove of the MHC class II molecule (Bertolino and Rabourdin-Combe, 1996). This assembly is guided by HLA-DM (a class II like molecule). The peptide-MHC class II complex is then transported to the cell surface, where it is presented to CD4+T-cells (Figure 10).

The amino acid (aa) structure of the peptide-binding cleft determines which antigen can bind to it (Matsumura et al., 1992). The majority of peptides that bind to MHC class I are 8-10 aa in length while the peptide presented by MHC class II are formed of between 10-34 aa (Appella et al., 1995).

The normal role of the MHC is to present peptides to T-cells and mediate normal immune responses. The function of the MHC was first discovered due to its involvement in rejection. The allorecognition of the organ is mainly due to disparities between MHC molecules (Janeway et al., 2004). Rejection can also occur due to the recognition of minor histocompatibility antigens (peptide derived from allelically polymorphic host proteins other than MHC molecules). These alloreactive cells recognize alloantigens via distinct, but not mutually exclusive pathways. Two concepts are proposed to explain how the host anti-graft immune response is responsible for rejection of vascularised grafts (Afzali et al., 2007): presentation through the direct pathway and the indirect pathway.

1.5.1.1 Direct Pathway: donor MHC and recipient T-cells

Direct allorecognition (Figure 11) consists of recognition of allogeneic MHC-peptide presented by intact APC of donor origin to host T-cells (Lombardi et al., 1989). Although this concept is in

contradiction with the rule of thymic positive selection for self-MHC restriction, this could be explained by cross-reactivity of T-cells, *i.e.* mimicking of self-MHC molecules by allogeneic MHC molecules. Direct recognition involves presentation of allopeptides by both donor MHC class I to CD8+T-cells and by donor MHC class II to CD4+T-cells. Consequently, this induces rapid and vigorous immune responses that result in acute rejection. This pathway seems to be most active during the first few weeks of transplantation. As donor APCs are destroyed during the priming process, direct alloreactivity is time-limited (Hornick et al., 1998). Thereafter, the T-cells are primed by the indirect pathway.

1.5.1.2 Indirect Pathway: both MHC and T-cells are from recipient cells

With the disappearance of donor APCs from the allograft over time, chronic rejection has been attributed to indirect allorecognition (Weis and von Scheidt, 2000). A number of experimental and clinical studies have demonstrated that patients with chronic rejection are hyporesponsive in the direct pathway but hyperresponsive in the indirect pathway (Hornick et al., 1998; Stanford et al., 2003). In the indirect pathway, host APCs process and present the donor MHC molecules to recipient T-cells (Benichou et al., 1992).

Dendritic cells are potent APC since they have the ability to present antigen to naive CD4+ and CD8+ T-cells. Antigen presentation is done by internalization and processing of alloantigens that are then presented to recipient T-cells in form of peptide bound to MHC (Shoskes and Wood, 1994). This is the same way that T-cells recognise conventional antigens and the allogeneic MHC molecules are recognised in a self-MHC restricted manner. Mismatch at MHC class I alone will result in alloantibody production to mismatched class I antigen and this leads to AMR and possibly chronic rejection (Reed et al., 2006).

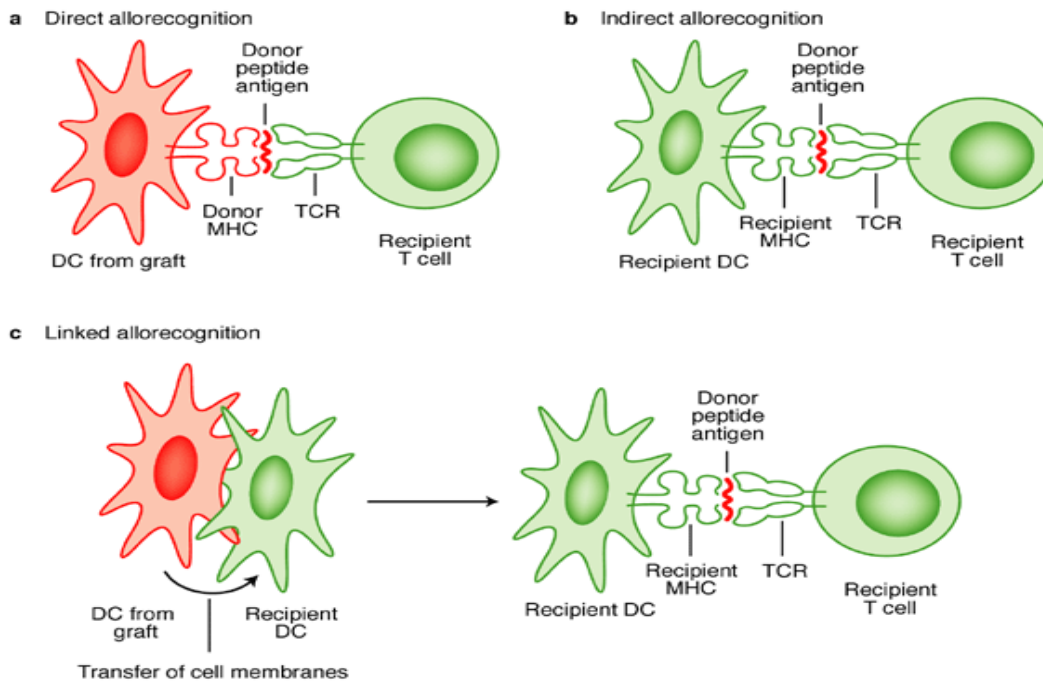


Figure 11: Direct and indirect pathway for presentation of alloantigen and ability of the dendritic cells for cross presentation. In the direct pathway, alloreactive T-cells recognize donor MHC-peptide directly. In contrast, the indirect pathway is characterised by T-cells recognizing processed donor antigen that are presented by host MHC. Diagram taken from (Harshyne et al., 2001).

Dendritic cells are potent APCs because they acquire the ability of cross-priming, *i.e.*, they are able to take up, process and present exogenous antigen with MHC class I to CD8+T-cells (Figure 11). Thus, they can mediate indirect allorecognition through both CD4+ T-cells (primed by MHC class II) and CD8+ T-cells (primed by MHC class I) (Rescigno et al., 1999). Hence, donor dendritic cells are not only responsible for activation of direct pathway but can also be a source of antigen in indirect pathway (Austyn and Larsen, 1990). T-cells recognising the intact donor derived peptides through direct pathway represents 90% of overall alloreactive T-cells specific for donor antigen while less than 5% of overall alloreactive T-cells were involved in the indirect pathway (Benichou et al., 1999; Suchin et al., 2001). This means that acute rejection tends to occur rapidly and early after transplantation whereas indirect recognition takes time to

develop and probably leads to chronic rejection. Therefore the direct pathway and the indirect pathway play a critical role in acute and chronic rejection, respectively.

Although mismatched MHC class molecules are the most important alloantigens for triggering rejection, there are also other factors such as minor histocompatibility molecules, I/R injury, brain death and infection that also contribute to transplant rejection.

1.5.2 Ischaemia reperfusion injury

The pathophysiological processes that contribute to graft rejection involve both alloantigen dependent and alloantigen independent mechanisms. I/R injury is the major factor contributing to alloantigen independent rejection (Gaudin et al., 1994). I/R injury occurring during transplantation has been studied widely (Abu-Amara et al., 2010; Knight et al., 1997; Tanaka et al., 2005b). I/R injury consist of two phases: ischaemia is defined as an inadequate blood flow resulting in reduced availability of glucose, oxygen and delays in clearing metabolic by-products such as lactate and CO₂. This produces irreversible cellular changes, necrosis and apoptosis thus resulting in cellular damage and organ dysfunction. The injury caused by ischaemia is then extended by reperfusion of the organ. In normal conditions, reperfusion leads to re-establishment of blood flow and metabolic function. However, re-oxygenation of the ischaemic tissue may also result in further damage to the organ (Abu-Amara et al., 2010). The end result of reperfusion will depend on the extent of damage caused by ischaemia as well as other variables including tissue type, temperature, perfusion pressures, pharmacologic pre-treatment, and physiologic preconditioning.

Ischaemia begins with the cessation of blood flow which leads to decrease in oxygen levels in the tissue therefore intracellular metabolic changes from aerobic to anaerobic (Carden and Granger, 2000). The consequences of these changes are accumulation of lactate, hydrogen ions and decrease in pH, thus creating acidic conditions and inducing apoptosis (Karwatowska-Prokopczuk et al., 1998). Moreover, ATP reserves are depleted, so the reduced availability of energy to the ionic pumps alters the ion transport system (Thandroyen et al., 1992). Therefore

the transport functions of the membrane are impaired causing an increase in calcium among other ions (Karmazyn et al., 2001). The changes in calcium concentration leads to systolic/diastolic dysfunction, alters the function of mitochondria, activates the enzymes kinases, phosphatases, phospholipases, proteases and calcineurin (Meldrum et al., 1996; Wang et al., 1999). The latter dephosphorylates the pro-apoptotic protein Bad and leads to activation of apoptosis (Springer et al., 2000). Furthermore, the increase in calcium acts as a second messenger to trigger production of inflammatory mediators. The major pro-inflammatory mediators are platelet activating factor (PAF), leukotriene B₄ and thromboxane A₂ (Carroll and Esclamado, 2000). These mediators promote vasoconstriction, activation of platelets and they act as chemoattractants to neutrophils (Chintala et al., 1994).

I/R injury is an inflammatory process wherein the restoration of blood flow facilitates influx of inflammatory cells to the organ. Notably, I/R injury leads to establishment of inflammatory environment, with the production of cytokines and chemokines such as TNF- α , IL-1 β and MCP-1 (monocytes chemoattractant protein)/CCL2 and myeloperoxidase produced by neutrophils (Tanaka et al., 2005a). This leads to recruitment of inflammatory cells. The neutrophils interact with the endothelium and migrate through the vessel wall (Figure 4). The activation of neutrophils is associated with production of oxygen free radicals, secretion of proteolytic enzymes, proteinases and phospholipases (Zimmerman and Granger, 1994). Proteinases and phospholipases are responsible for vascular membrane and endothelial cell damage. In physiological conditions, endothelial cells produce vasodilators such as nitric oxide and other substances that degrade vasoconstrictors. However, during I/R injury, they lose the ability to produce such substances. Moreover, the capillaries are narrowed and become leaky. The induction of inflammation combined with dysfunction of mitochondrial transport chain and activation of vasoconstrictors result in increased blood flow, swelling of endothelial cells and interstitial oedema resulting in narrowed diameter of the capillary (narrow lumen).

The stimulation of neutrophils and macrophages is associated with production of oxygen free radicals and proteolytic enzymes. Macrophages are involved in the initial phase of reperfusion

while neutrophils are activated in the later phase (Tapuria et al., 2008). Previous studies have shown that free radicals such as superoxide anion generated during I/R injury leads to apoptosis of cardiomyocytes (Tanaka et al., 2005b). The damage caused by ischaemia is proportional to the ischaemic time. Increased ischaemic damage is seen in tissues with higher metabolic rates. Studies have attempted to diminish the damage caused by ischaemia by cooling the tissue in order to reduce the metabolic rate. Another way to reduce the damage is through ischaemic preconditioning via induction of a brief period of reversible ischaemia followed by reperfusion in order to reduce the damage caused by longer ischaemic time. Ischaemia preconditioning was first described by Murry et al in 1986 and they showed that preconditioning the myocardium with multiple 5 minutes of ischaemia and reperfusion followed by 40 minutes of sustained ischaemia limited the infarct size considerably compared to the control group that had a single 40 minutes ischaemia (Murry et al., 1986). Furthermore, preconditioning has been showed to confer protection against myocardial injury by reducing the use of ATP, the metabolic rate as well as the rate of anaerobic glycolysis (Murry et al., 1986; Murry et al., 1990). Although the preconditioning method could be used to reduce the damage caused by prolonged ischaemia, this method is rarely used in transplantation (Huang et al., 2009). In contrast, current methods of limiting ischaemic damage during harvesting of hearts for transplantation consist of cooling the hearts and use of preservation and cardioplegic solutions. Moreover, experimental studies have demonstrated that prolonged cold ischaemic time positively correlate with increased level of superoxide anion production and graft damage (Knight et al., 1997; Tanaka et al., 2005b)

I/R of the heart following heart transplantation is associated with increased myocardial injury contributing to increased incidence of myocyte apoptosis. Experimental studies using dogs showed that apoptosis and necrosis were mainly caused by I/R injury rather than ischaemia on its own (Zhao et al., 2000). Prolonged cold ischaemia not only accelerates the progression of acute rejection but also contribute to the development of chronic rejection (Wang et al., 2000; Yilmaz and Hayry, 1993). In fact, vessel injury as well as graft vasculopathy was significantly

more prominent in ischaemic injured allograft compared to non-ischaemic injured allograft (Knight et al., 1997; Wang et al., 2000; Yilmaz and Hayry, 1993). Ischaemia leads to increased expression of MHC II molecules (Waddell et al., 1996). Similarly recovery from I/R is accompanied by increased expression of MHC I and MHC II antigens, which then promote recognition of these cells by immune cells and thus graft rejection (Shackleton et al., 1990).

1.5.3 Oxidative stress

I/R injury leads to formation of free radicals. Free radicals are molecules with unpaired electron on their outer shell. They react violently with stable molecules thus creating more free radicals (Kaul et al., 1993). Free radicals are toxic to all biologic substances, including proteins, polysaccharides, nucleic acids, collagens, fatty acids, and phospholipids. ROS are involved in the regulation of many biological processes. They defend against micro-organisms and can cause direct cell injuries (Buttke and Sandstrom, 1994; Halliwell and Gutteridge, 1990). They play a major role in regulation of cell death through apoptosis and necrosis (Buttke and Sandstrom, 1994). Exposure of cell membranes to oxygen free radicals results in increased permeability and, ultimately, cell death. The main types of oxygen free radicals are O_2^- , H_2O_2 , hydroxyl free radical (OH^-) and hyperchloride ($HOCL$). The xanthine oxidase system and the NADPH oxidase system produce O_2^- , which is functional in activated neutrophils. H_2O_2 is produced by the enzyme superoxide dismutase. OH^- arises from reactions catalyzed by iron molecules.

The free radicals such as superoxide generated following injury cause cell membrane permeability and tissue damage. Moreover, alteration in mitochondrial transport system, and neutrophil activation also produces ROS (Cai and Harrison, 2000). The oxygen derivatives thus produced are highly toxic and damage vascular endothelium and cardiomyocytes. Numerous experimental studies have demonstrated the role of free radicals in inducing myocardial injury as a result of myocardial ischaemia and in particular during reperfusion of the tissue. I/R injury is associated with increased oxidative modifications of mitochondrial proteins leading to formation of free radicals and oxidative damage. 4-Hydroxy-2-nonenal (4-HNE) and malondialdehyde

(MDA) increases following reperfusion of the heart (Kaul et al., 1993; Lucas and Szweda, 1998). One of the main consequences of increased oxygen radicals is the activation of the pro-apoptotic BCL-2 family of proteins which mediate apoptosis. Moreover, oxygen radicals also stimulate various intracellular proteins. Consequences of all these changes leads to apoptosis, necrosis and subsequent organ dysfunction (Singal et al., 1998).

1.5.4 Apoptosis

I/R injury, oxidative stress and subsequent apoptosis have been shown to play an important role in the progression of various diseases including myocardial infarction, hypertension, cardiomyopathy and atherosclerosis (Haunstetter and Izumo, 1998; MacLellan and Schneider, 1997; Moudgil et al., 2001). Apoptosis is a fundamental biochemical process involved in elimination of cells during normal cellular differentiation and development (Kumar and Jugdutt, 2003). It is defined as a highly regulated energy-dependant cell death that does not induce inflammation and the immune response, while necrosis was considered to be an unregulated form of cell death that causes inflammatory responses (Figure 12).

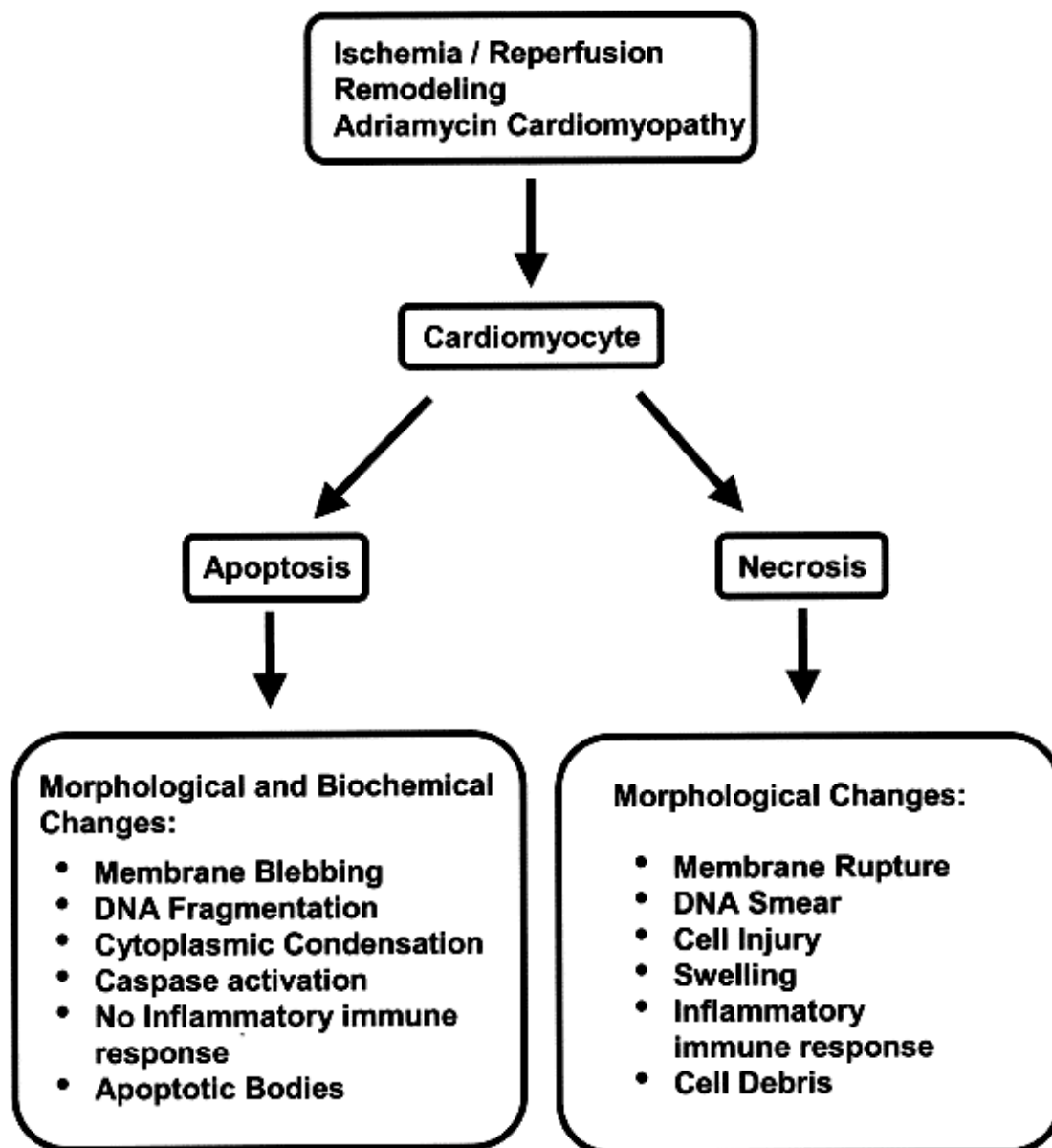


Figure 12: Morphological and biochemical changes induced following apoptosis and necrosis. Diagram taken from (Kumar and Jugdutt, 2003).

Apoptosis was first identified by Kerr et al in 1972 (Kerr et al., 1972). It is involved in tissue homeostasis, aging, pathological processes, and irreversible cell injury (Ellis et al., 1991). Apoptosis is characterised by morphological changes such as chromatin aggregation, cytoplasmic and nuclear condensation, membrane blebbing and cell shrinkage (Wyllie et al., 1980). These changes are due to the activation of a family of cysteine aspartyl proteases known as caspases (Alnemri et al., 1996). Apoptosis is mediated through signal transduction and activation of a cascade of caspases which leads to disintegration of the cell (Logue et al., 2005).

The major signal transduction pathways include intrinsic mitochondrial pathway and the extrinsic Fas and TNF receptor mediated death receptor pathway (Nagata, 1997) as shown in Figure 13.

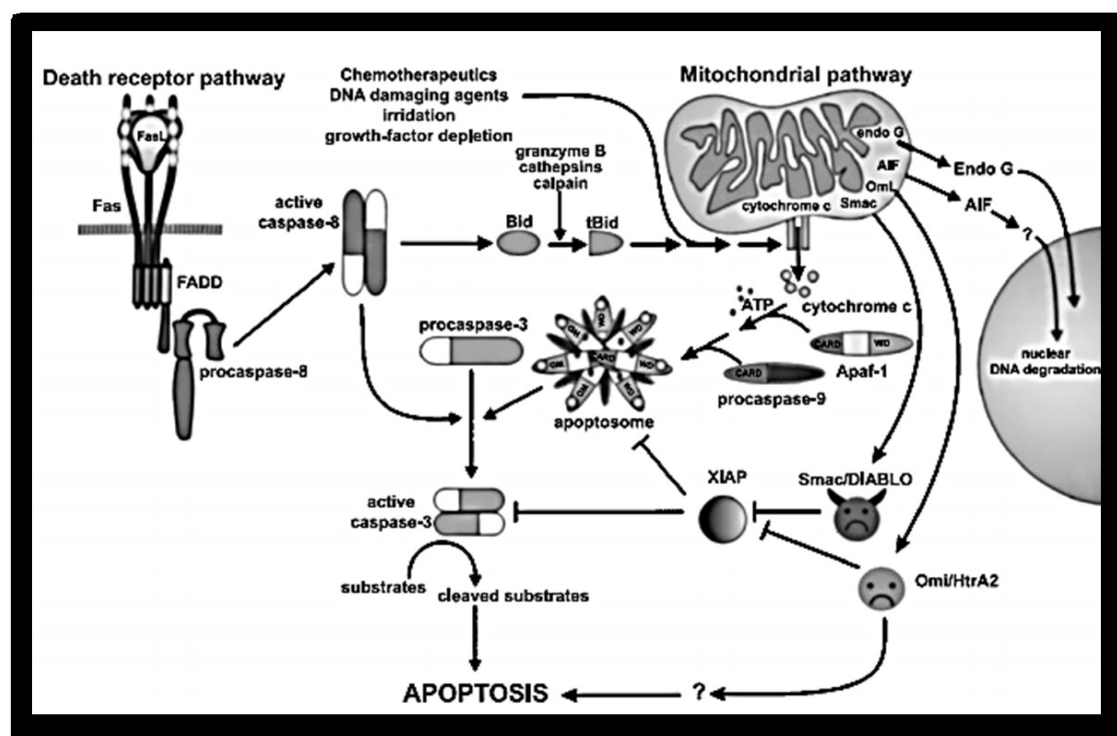


Figure 13: Activation of apoptosis via mitochondrial and death receptor pathway. Diagram taken from (van Loo et al., 2002).

1.5.3.1 Mitochondrial pathway:

The BCL-2 family of proteins include both pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic proteins Bax and Bad are located in the cytosol while the anti-apoptotic protein Bcl-2 is found in the outer mitochondrial membrane, nuclear membrane and ER. Numerous cell damage pathways such as hypoxia, heat shock, cytotoxic drugs, DNA damaging agents and irradiation act on mitochondria and translocate the Bax to the mitochondria and leads to subsequent modifications of mitochondrial structure. In fact, the outer membrane of the mitochondria becomes permeable and releases apoptogenic factors such as cytochrome c, Smac/DIABLO, endonuclease G and apoptosis inducing factor into the cytosol (Narula et al., 2001). Cytochrome c then binds to Apaf-1, dATP, and pro-caspase-9 forming a complex referred to as apoptosome. Apoptosome activates caspase-9, which then acts on the downstream target and proteolytically activates caspase-3 (van Loo et al., 2002). Numerous studies

have targeted the mitochondrial pathway to reduce the damage caused following I/R injury. Transgenic mice overexpressing the anti-apoptotic protein Bcl-2 demonstrate less apoptosis and attenuation of I/R injury (Chen et al., 2001).

1.5.3.2 Death receptor pathway:

In the external pathway, binding of ligand to the FAS or TNF- α receptor present on the cell surface stimulates trimerisation of the receptor and the recruitment of adaptor proteins such as TRADD or FADD. Pro-caspase-8 binds to these adaptor proteins and subsequently leads to cleavage and activation of caspase-8, which in-turn cleave pro-caspase-3 into the active form. This is followed by initiation of the caspase cascade (French and Tschopp, 2003).

Although these two pathways are described separately, both pathways are intimately connected. Caspase-8 activated in the death receptor pathway can cleave Bid, a member of pro-apoptotic family, which then activates the mitochondria to release cytochrome c (Micheau et al., 2002).

Caspase-3 is normally present in the cytosol in the inactive form. Cleavage of pro-caspase-3 leads to its activation. Activated caspase-3 then leads to proteolytic activation of pro-caspase 2, 6, 8 and 10. Activated caspase-3 elicits DNase activation. For example, the enzyme caspase activated DNase (CAD) is normally in its inactive form known as ICAD. Caspase-3 cleaves ICAD into the active form CAD, which then degrades nuclear DNA (Lavrik et al., 2005). The cells that are undergoing apoptosis are ingested by phagocytic cells without induction of co-stimulatory molecules on their surface. Thus, apoptosis is thought not to invoke a pro-inflammatory immune response.

Apoptosis has been described in many diseases including myocardial ischaemia and reperfusion. Death of cardiomyocyte through apoptosis is seen in the early stage of infarcted heart while necrosis is found during later stages (Veinot et al., 1997). Probably dysfunction of the mitochondria and therefore depletion of ATP may promote cell death by necrosis rather than apoptosis (as apoptosis is energy dependant). Taken together, death of cardiomyocytes

mediated through I/R injury and recognition of injured cells by histocompatibility mismatch leads to rejection of the transplanted organ.

1.6 Types of rejection.

Peter Medawar demonstrated that the immune system was responsible for the rejection of transplanted organs. He also suggested the possibility that the immune system could be educated in a way to tolerate the transplanted tissue. He did this by demonstrating that injection of splenocytes from a different strain into a mouse embryo produced a mature mouse which tolerates skin grafts from the splenocyte donor but not from third party strains of mice (Billingham RE and Medawar 1952). These experiments led to the concept of neonatal tolerance and much work has been done on understanding mechanisms of tolerance induction and how it can be induced in humans. Clinically, rejection can be classified into three categories: hyper-acute rejection, acute rejection and chronic rejection.

1.6.1 Hyper-acute rejection

Hyper-acute rejection occurs very quickly, usually within minutes to hours following transplantation. Clinically, this syndrome has been described after heart and kidney transplantation. It is due to the presence of preformed antibodies to HLA antigens of the donor or to blood group antigens. Patients have pre-formed antibodies for three main reasons; pregnancies, multiple surgeries with the use of blood products, or previous organ transplantation. These preformed antibodies bind the antigens present on the surface of the endothelial cells and activate the classical complement pathway (Roos and Daha, 2002). It is well established that human endothelial cells constitutively express MHC class I and MHC class II antigens (Rose et al., 1986). Binding of alloantibodies to endothelial MHC class I or class II antigens results in rapid intra-vascular thrombotic occlusion, haemorrhage and tissue injury. Histologically, deposition of immunoglobulin and complement on the surface of the endothelial cells can be seen. Hyper-acute rejection is characterised by obstruction of small vessels and damage of the endothelial cells and therefore destruction of the graft. In 1969, Patel and

Terasaki published the incidence of hyperacute rejection in renal transplantation and recommended use of the prospective leukocytes cross-match technique to prevent hyperacute rejection (Patel and Terasaki, 1969). Thankfully, due to mandatory use of the prospective cross-match technique, the incidence of hyper-acute rejection after heart or kidney transplantation is now very rare.

1.6.2 Acute rejection

Acute rejection and infection are the two major cause of death in the first year following cardiac transplantation. Acute rejection can occur anytime after transplantation but mainly occurs within the first 6 months. Acute rejection can be sub-classified as AMR or cellular rejection

After heart transplantation, patients undergo endomyocardial procedure at regular intervals in the first three months to detect acute rejection. Acute cellular rejection is histologically defined as infiltration of lymphocytes and macrophages in the myocardium leading to myocardial oedema and myocyte necrosis. It is graded from no rejection to severe rejection according to the International society for Heart and Lung transplantation (ISHLT) ranking grade (Stewart et al., 2005) (Figure 14):

- Grade 0R-for no rejection: absence of mononuclear infiltration such as lymphocyte and macrophages or myocyte damage.
- Grade 1R-for mild rejection: characterised by interstitial and or perivascular infiltrate with up to 1 focus myocyte damage. However the architecture of the cell is not deformed.
- Grade 2R-for moderate rejection: presence of two or more foci of mononuclear cell infiltration with associated myocyte damage
- Grade 3R-for severe rejection: diffuse infiltration of lymphocytes, monocytes as well as polymorphs and plasma cells. In addition, multiple areas of myocyte damage, oedema, haemorrhage and vasculitis can be seen (Stewart S et al, 2005).

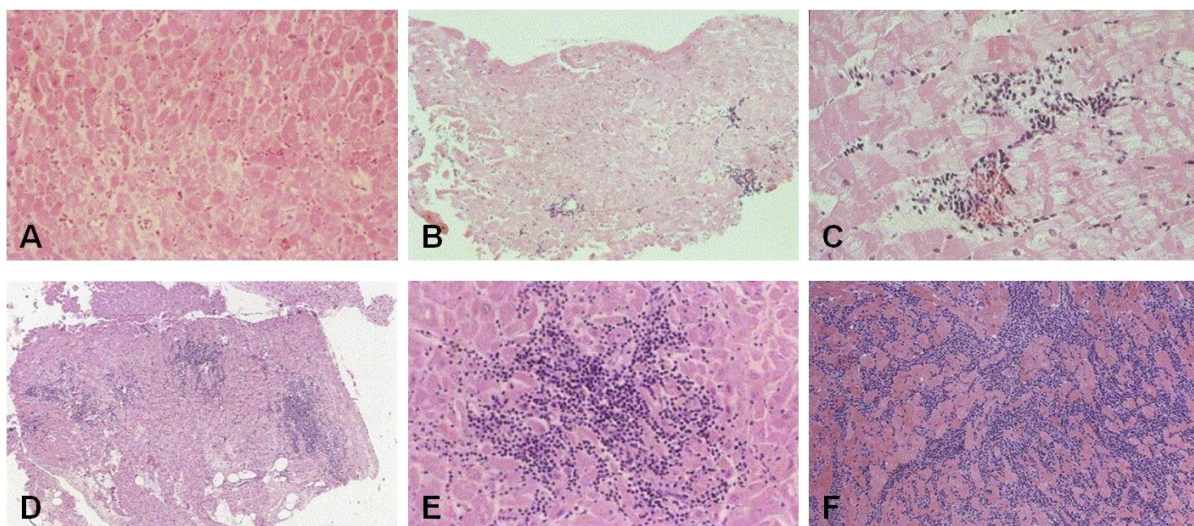


Figure 14: ISHLT standardised grading for acute cellular rejection: in grade 0 R, there is no evidence of cellular infiltration (A), however grade 1 R is characterised by perivascular and interstitial infiltration of mononuclear cells (B and C). Histologic sections D & E show more than one infiltration foci together with myocyte damage which represents grade 2 R. Severe acute cellular rejection is associated with multifocal infiltrate and myocyte damage (F). Sections taken from (Stewart S. et al, 2005).

Acute humoral rejection, also known as AMR is less common than acute cellular rejection and is commonly observed in allosensitised patients including those with previous transplantation, pregnancy or transfusion as well as those who had a ventricular assisted device. AMR is initiated by antibodies that are directed to donor HLA or endothelial cell antigens. It can be detected by the presence of C3d, C4d, and or C1q deposition in the vessels as well as accumulation of macrophages (CD68) in the capillaries of the graft (Rahimi et al., 2004; Stewart et al., 2005). It results in myocardial capillary injury, ventricular dysfunction, endothelial damage and dysfunction (Lindenfeld et al., 2004). Although myocardial damage leads to myocyte necrosis, there may be no evidence of cellular infiltration (Lones MA et al, 1995). The ISHLT has also established a grading system for acute humoral rejection:

AMR 0: no antibody mediated rejection with absence of complement deposition or immunopathologic features of AMR such as capillary injury.

AMR 1: evidence of AMR characterised by presence of intravascular macrophages, interstitial oedema and endothelial cell injury. At this stage, histological studies show positive staining for C4d and CD68 deposition.

Moreover, functional changes have also been reported during immune mediated myocyte damage. Indeed, hemodynamic changes, more specifically systolic dysfunction, occur during acute rejection (Amende I. et al; 1990). In contrast, echocardiographic studies showed that the wall thickness as well as the ventricular mass increased during the episode of rejection, owing to vascular inflammation and interstitial oedema (Barry WH; 1994).

With the advances of immunosuppression and surgical techniques, the rate of acute rejection and infection has declined and chronic rejection has become the major concern of cardiac transplantation.

1.6.3 Chronic rejection

In patients surviving the first year of transplantation, chronic rejection, also known as cardiac allograft vasculopathy (CAV) is the major cause of death. CAV is an accelerated form of atherosclerosis which usually occurs within the first 5 years post-transplant in 30-60% of transplant recipients. The aetiology of chronic rejection is not well understood. Evidence suggests that it is primarily an immunological process (Schmauss and Weis, 2008), which is also enhanced by non-immunological (alloantigen independent) factors (Costanzo-Nordin, 1992; Hosenpud et al., 1992).

Events occurring in the first year of transplantation including HLA mismatches, previous episodes of acute rejection, initial delayed graft function as well as hyperlipidaemia, ischaemia/reperfusion injury, post-transplant infections and an older donor age seem to contribute to the pathogenesis of allograft vasculopathy (Figure 15) (Avery, 2003; Yamani et al., 2004).

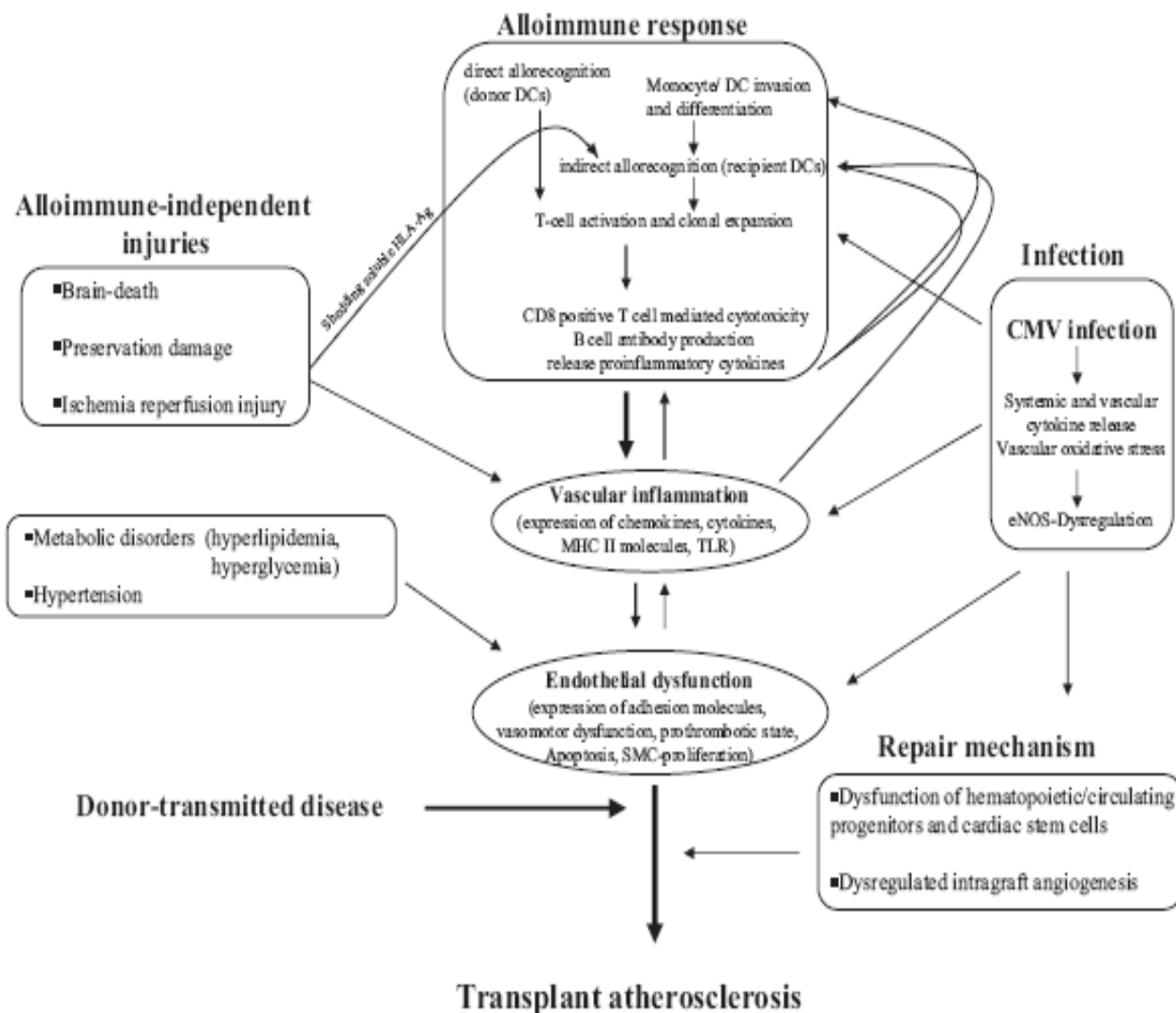


Figure 15: Immunological and non immunological events are responsible for transplant rejection. Both alloantigen dependant and independent factors appear to be important in the development and progression of CAV. Diagram taken from (Schmauss and Weis, 2008).

The hallmarks of chronic rejection of the graft are perivascular inflammation, fibrosis and graft vasculopathy of the major and minor vessels. It is characterised by vascular obliteration, progressive fibrotic changes and intimal thickening of the allograft (Nykanen et al., 2003). These changes occur due to damage to the endothelium, which leads to platelet aggregation, recruitment of leukocytes and migration of smooth muscle cells (SMC) from the media to the intima layer (neointima) of coronary arteries. The SMC then proliferate and result in thickening of blood vessel walls (neointimal formation), hence leading to narrowing of lumen-intimal

hyperplasia (Waller et al., 2003). In fact, Yeung et al showed intimal thickening in 75% of cardiac allograft recipients by the end of the first year after transplantation (Yeung et al., 1995). In experimental models, graft vasculopathy is characterised by an intima consisting of SMC and infiltration of mononuclear cells and macrophages (Figure 16).

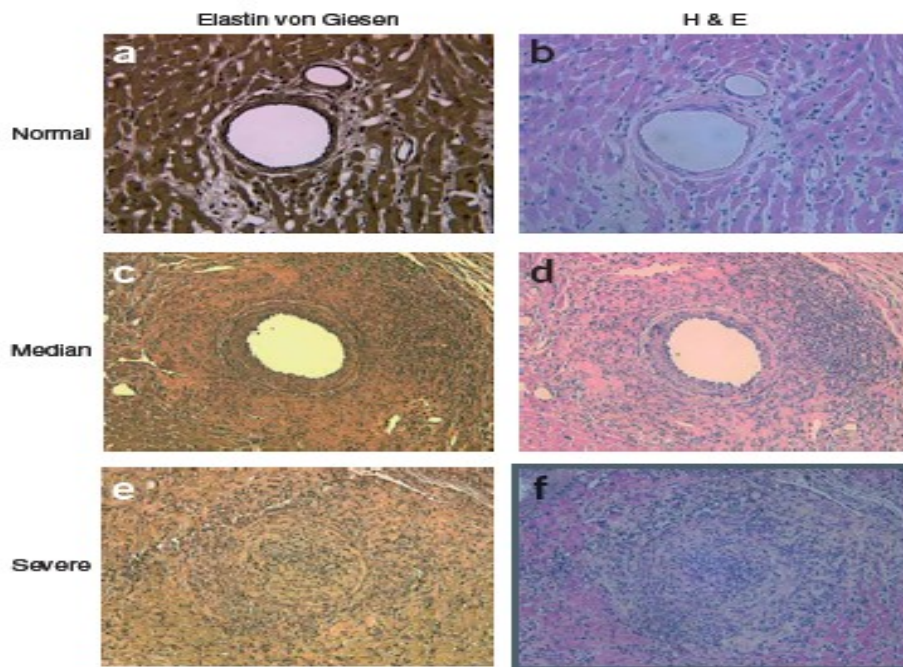


Figure 16: Vessel occlusion seen during CAV. A representative histological section of experimental model of CAV showing marked intimal occlusion and cellular infiltration with increased severity of CAV (a&b: normal, c&d: median CAV, e&f: severe CAV). The elastin staining (a, c and e) shows vessel occlusion and the H & E staining (b, d and f) enable to identify cell infiltration. Section taken from (Hasegawa et al., 2007).

As mentioned CAV is an accelerated form of atherosclerosis and the Ross hypothesis (that explains atherosclerosis) could also be applied to CAV (Ross, 1993). CAV and conventional non-transplant atherosclerosis have similarities and differences in phenotypes and biological mechanisms (Figure 17): in both diseases, intimal smooth muscle cell migration, endothelial cell dysfunction and abnormal apoptosis are seen (Rahmani et al., 2006). However, the progression of the disease in CAV can be very rapid compared with conventional atherosclerosis. Besides,

CAV, as opposed to atherosclerosis, is characterised by diffused, concentric intimal thickening, which results in narrowing and luminal occlusion of the vessels of the graft (Soleimani et al., 2006). This is due to proliferation of SMC and endothelial cells (Valantine, 2003). Endothelial cell damage triggers release of pro-inflammatory cytokines (such as IL-1), chemokines and induces expression of adhesion molecules (Shi et al., 1996). This leads to recruitment and transmigration of immune cells across the endothelial cell barrier into the intima (Wang et al., 1998). Interestingly constrictive remodelling further contributes to the narrowing of vessels (Kobashigawa et al., 2000). Indeed, this could be seen as a sign of inappropriate repair process within the vessel wall (Quaini et al., 2002).

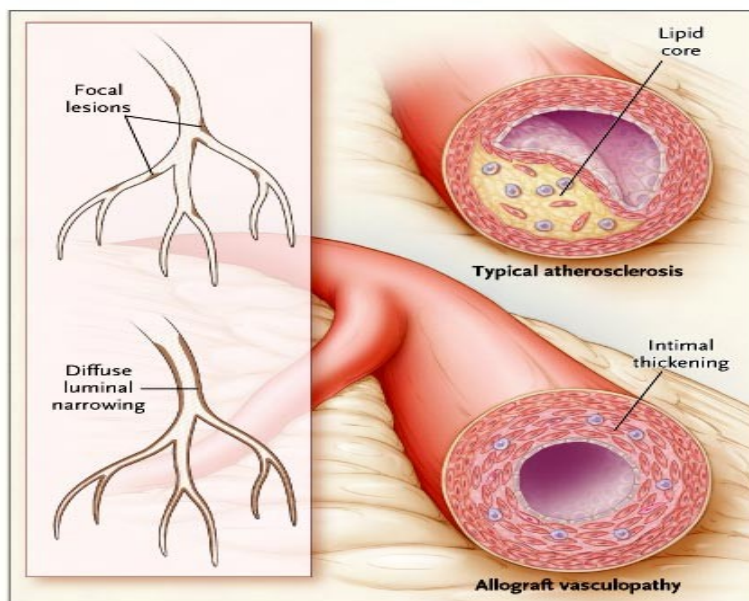


Figure 17: Distinctive luminal narrowing is seen in allograft vasculopathy and atherosclerosis. CAV is characterised by concentric intimal thickening leading to diffuse narrowing of the lumen. On the other hand, luminal narrowing seen in native atherosclerosis is focal and eccentric. Picture taken from (Avery, 2003).

Intimal hyperplasia and neointimal thickening are seen in both CAV and native atherosclerosis (Atkinson et al., 2004). SMC play a critical role in intimal hyperplasia (Kwun and Knechtle, 2009). Nevertheless, the exact origin of the neointimal SMCs is still not known. Previous studies demonstrated conflicting ideas. Experimental studies suggest it results from migration and differentiation of recipient derived myofibroblast or circulating progenitor cells (Han et al., 2001;

Hillebrands et al., 2001; Song et al., 2007). On the other hand, clinical studies suggest SMC remain of donor origin (Atkinson et al 2004) or postulate a role for both donor and recipient derived SMC in the formation of neointimal thickening (Boersema et al., 2009).

Progress in understanding the disease pathogenesis and the natural history of rejection during the past years and advances in diagnosis have paved the way for new therapeutic approaches.

1.7 Possible treatments and drawbacks

Following transplantation, patients routinely undergo intravascular myocardial biopsies for evaluation of graft rejection. Emerging non-invasive techniques including intima-media thickness or peripheral flow-mediated dilatation allow assessment of CAV (Schmauss and Weis, 2008). The treatments for rejection require life-long administration of immunosuppressants such as steroids, anti-proliferative agents, calcineurin inhibitors, anti-lymphocyte preparations and anti-cytokine antibodies (Sulemanjee et al., 2008). Nevertheless, despite significant advances in the treatment of transplant rejection the results are far from perfect. Moreover, immunosuppressants are associated with infections and malignancy. Although re-transplantation could be proposed as a possible treatment for graft failure for a selected few patients, the low success rate of re-transplantation and shortage of donor hearts decreases the likelihood of re-transplantation and makes it more important to find new ways to prevent graft rejection (Srivastava et al., 2000). On the other hand, targeting a single pathway seems to be inefficient, and combinations of immunosuppressive drugs are required to limit transplant damage. For example, combination of rituximab together with cyclosporine seems to be more effective in reducing the severity of CAV (Kelishadi et al., 2010). Modern research is also concentrating on xenotransplantation (implanting animal organs into human) and treatment using mechanical devices.

Heat shock protein 27 (hsp-27) may hold a key role in protection and treatment of cardiac rejection. Using hsp-27 as a combinatorial therapy or on its own might be effective in protecting from cardiac rejection. Therefore, it is pivotal to understand its mechanism of action. Prevention

of initiation of an immune response against the graft is the best way to protect against immune mediated myocyte injury.

1.8 Heat shock protein

1.8.1 General

Heat shock proteins are a family of small proteins found in plant, yeast, bacterial and mammalian cells. They are upregulated in stress conditions. They were originally named hsp because of their ability to increase their expression in response to elevated temperature (Mehta et al., 2005). However, now a wide variety of other stressful stimuli such as hypoxia, heavy metals, osmotic stress, metabolic poisons and I/R have been shown to induce hsps. The heat shock proteins have various roles: some hsps have a role in post-translational folding of proteins, some are involved in removal of protein that are improperly folded while other hsps play a role as chaperone protein and modulate the activity of other hsp (Vos et al., 2008). Actually many of the hsps interact with each other to function efficiently. Hsps are grouped into five different families according to their molecular weight and function: these are hsp-110, hsp-90, hsp-70-72, hsp-55-60 and the small hsps (Concannon et al., 2003). Hsp-27, lens α A and α B crystallin belong to the ubiquitous small hsps (Merck et al., 1993a; Merck et al., 1993b). The small hsps have a molecular weight between 15 and 30kDa, but they are present in the cell as large oligomeric complexes of about 12-40 subunits corresponding to a molecular mass of 800kDa (Behlke et al., 1991). The small hsps are formed with α -crystallin domain flanked by C-terminal and N-terminal, where the N terminal contains a number of phosphorylation sites (Haslbeck et al., 2005). The monomers of hsps interact with each other to form dimers, which is the building block of the large oligomers. Moreover, the small hsps can interact with each other and thus have more functions.

The genes encoding hsps are highly conserved, however small variation occurs in protein size: the hsp-27 is a 27kDa protein in human and 25kDa in rodents (Djamali et al., 2005). The expression of hsp-27 is particularly high in skeletal, cardiac and SMC (Ciocca et al., 1993;

Robinson et al., 2010). Small hsps are characterised by a conserved C-terminal domain and a non-conserved domain in the N-terminal (Figure 18). The primary structure of α B crystalline, in particular, the C-terminal region is homologous to hsp-27 (Merck et al., 1993b). Moreover, hsp-27 also has a WDPF motif that seems to be important in oligomerisation. Human hsp-27 contains 205 amino-acids residues with three phosphorylation sites (on serine 15, serine 78 and serine 82 residues but only serine 15 and serine 86 in rodent hsp-25) that modulate their activities in response to various stimuli including growth factors, differentiating agents, tumour necrosis factor, oxidative stress or heat shock (Landry et al., 1992; Stokoe et al., 1992).

Hsp-27 is present in 4 different isoforms, which are different post-translational modifications of the same proteins. It is present as unphosphorylated, monophosphorylated, biphosphorylated, and triphosphorylated variants of the protein.

Hsp27 (HspB1)



Figure 18: Structure of hsp-27: It is characterised by non conserved domain (also called WDPF domain –the grey box) and a conserved flexible domain in N-terminal (light grey box) and a conserved α -crystallin domain in the C-terminal (black box). At position 137, it contains the amino-acid cysteine, which is responsible for dimer formation. Human hsp-27 contains three phosphorylatable serine residues at position 15, 78 and 82. Diagram taken from (Arrigo et al., 2007).

There are different types of hsp: the inducible and the cognate hsp. The inducible hsps are absent in normal physiological condition and they are upregulated in response to stress (Arrigo et al., 2007). On the other hand, cognate proteins are constitutively synthesised in cells. Hsp-27 belongs to the latter. Hsp-27 is constitutively expressed and its level increases further in response to stress stimuli (Njemini et al., 2006). In normal conditions, hsp-27 is expressed at

low levels and it plays a major role as a molecular chaperone, and facilitating transport, and folding and assembly of polypeptides (Horvitz J, 1992; Hartl FU et al, 1996). Hsp-27 defends against cell injury and cell death by helping proper refolding of non native proteins and removing aberrant protein (Ehrensperger et al., 1997; Haslbeck et al., 2005). In fact, one of the main functions of hsp-27 is to prevent unfolded proteins from entering aggregation pathway in an ATP-independent manner (Ehrensperger et al., 1997; Wyttenbach et al., 2002). In certain condition, it facilitates ubiquitination and degradation of proteins that cannot be refolded by targeting them for proteosomal degradation (Parcellier et al., 2006). Hsp-27 modulates actin polymerisation, thereby stabilising and remodelling actin cytoskeleton during times of stress (Mounier and Arrigo, 2002). In addition, hsp-27 also has an effect on the microfilaments in cell–cell interaction, cell migration, proliferation, and secretion through regulation of actin polymerisation. Actin polymerisation and re-organisation modulates neutrophil chemotaxis and exocytosis in hsp-27 dependant manner (Jog et al., 2007). Under stressful conditions, the expression of small hsps and α - β -crystallin is increased yielding a final concentration of 1% of the total cellular protein (Klemenz et al., 1991; Oesterreich et al., 1990). Overexpression of hsp-27 has been shown to increases thermotolerance (Landry et al., 1989) of cells as well as inhibiting cell proliferation (Knauf et al., 1992).

Post-translational modifications seem to affect the function of hsp-27. These include phosphorylation, S-thiolation and methylglyoxal modification. Modification by methylglyoxal enhances the chaperone like activity and alters the anti-apoptotic activity (Oya-Ito et al., 2006). S-thiolation together with pH and temperature modulate the oligomerisation states as well as chaperon like activity (Eaton et al., 2002; Lelj-Garolla and Mauk, 2006). Similarly, phosphorylation of hsp-27 alters the size of oligomers and interferes with chaperone like activity. Phosphorylation of hsp-27 increases rapidly (within minutes) following stress (Rogalla et al., 1999). Phosphorylation of hsp-27 by stress activated kinases, mainly mitogen-activated protein kinase-activated protein (MAPKAP) leads to disassembly of large hsp-27 oligomeric structures into small multimeric and monomeric units (Abisambra et al., 2010; Larsen et al., 1997; Rogalla

et al., 1999). Cytokines have also been demonstrated to have an effect on phosphorylation of hsp-27 (Hatakeyama et al., 2002). However the exact consequences of phosphorylation are not well defined. In murine models, it has been demonstrated that inhibition of actin polymerisation depends on the degree of hsp-25 phosphorylation and its structural organization (Preville et al., 1998). Contrary to phosphorylated hsp-25 or non-phosphorylated oligomers of hsp-25, non-phosphorylated hsp-25 monomers are able to inhibit actin polymerisation (Benndorf et al., 1994). However other studies demonstrate that phosphorylation is not necessary for polymerisation of actin network and protection against I/R (Hollander et al., 2004). The chaperone like activity depends amongst others on the size of oligomers. Interestingly, studies show that phosphorylation of hsp-27 alters the size of oligomers resulting in small oligomers (Mehlen et al., 1997; Rogalla et al., 1999). Indeed, phosphorylation leads to rapid dissociation of aggregated hsp-27 and thus reduces the size of oligomers from 200-800kDa to 75 to 250kDa in human glial cells and transfected hamster cells (Kato et al., 1994; Lavoie et al., 1995; Rogalla et al., 1999)

Further, hsp-27 has been shown to have an effect on cytoprotection by inhibiting osmotic stress and apoptosis (Arrigo, 2000; Rogalla et al., 1999). The localisation of hsp-27 depends on the differentiation-state of the cell. It is present in the cytosol of weakly differentiated cells, while it is found in the I-bands and Z-discs of differentiated cells (Mymrikov et al., 2011). Interestingly, hsp-27 is co-localised with actin in mature cardiomyocytes underlining its role in actin polymerisation and regulation of cytoskeleton during physiological conditions (Hoch et al., 1996). Heat shock induced nuclear translocation and increased cytoskeleton expression of hsp-27 thus explaining its importance in cell survival of the stress induced cell (Qian et al., 2006).

1.8.2 Apoptosis and hsp-27

Apoptosis is a controlled, energy dependant form of cell death. As mentioned earlier, it comprises the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways. Interestingly, apoptosis induced by neuronal growth factor withdrawal was inhibited in neuronal cells where hsp-27 was upregulated (Dodge et al., 2006). Moreover, it has been reported that it

also protects against ischaemia induced cell death of neurons (Wagstaff et al., 1999). Similarly, in a rat model, it has been demonstrated that hsp-27 protects retinal ganglion cells from ischaemia-reperfusion injury thus prolonging cell survival (Yokoyama et al., 2001). Other studies have also reported the protective effect of hsp-27 on cerebral ischaemia in transgenic mice overexpressing hsp-27 (Latchman, 2005). The effect of hsp-27 in protecting from apoptosis is becoming more evident. Hsp-27 has also been shown to be responsible for protection against cell death in cardiac tissue (Latchman, 2001; Martin et al., 1999; Martin et al., 1997). They showed that pre-treatment of cardiomyocytes with hsp-27 expressing virus conferred protection against apoptosis of cardiac cells exposed to ceramide compared to non-infected cells (Martin et al., 1997). Similarly, overexpression of hsp-27 using adenovirus has also been shown to protect the cardiac cells from ischaemia induced damage (Latchman, 2001).

Interestingly, hsp-27 has been reported to protect from apoptosis by interacting with the apoptotic pathway at different levels: For example, it regulate cell death by acting on signals that trigger the release of cytochrome c, by interacting on cytochrome release itself, and through direct inhibition of caspase activation (Bruey et al., 2000; Paul et al., 2002; Samali et al., 2001). It seems that hsp-27 prevents pore formation, therefore cytochrome c release by regulating the activity of kinases such as PI3K. PI3K acts on AKT and phosphorylate Bax that prevent pore formation (Havasi et al., 2008). However it can also act directly on cytochrome c and thus prevent apoptosome formation (Paul et al., 2002). In addition, hsp-27 seems to regulate the extrinsic pathway by preventing translocation of Bid to the mitochondria (Arya et al., 2007; Paul et al., 2002). Moreover the anti-apoptotic property of hsp-27 could also be due to its regulation of the survival transcription factor nuclear factor κ B (NF- κ B). In fact, hsp-27 leads to degradation of ubiquitinated I κ B α (the inhibitor NF- κ B) through interaction with the proteasome degradation machinery resulting in increased activity of NF- κ B as well as survival (Parcellier et al., 2003b). Concannon et al propose possible mechanisms by which hsp-27 could act on the apoptotic pathway which is displayed in Figure 18 (Concannon et al., 2003). In conclusion, hsp-27 could interact at various levels of the apoptotic pathway and thus promote cell survival.

higher in DOX treated heat shocked cells compared to control cells (Venkatakrishnan et al., 2006). It's is therefore evident that the production of ROS is controlled by presence of phosphorylated hsp-27. This protection against ROS production is accompanied by increased level of glutathione production (Mehlen et al., 1996). Interestingly, increase in glutathione promotes formation of large oligomers (Mehlen et al., 1997).

In fact, the protective activity of hsp-27 may depend on the level of glutathione expression. Glutathione (a major source of cellular thiol) protects cells from oxidative injury and regulates cell death (Yamauchi et al., 1990; Zhong et al., 1993). Moreover, hsp-27 (as well as the lens protein $\alpha\beta$ -crystallin) prevents formation of hydroxyl radicals by reducing intracellular level of iron (Mymrikov et al., 2011).

On the other hand, hsp-27 also protects from actin remodelling following oxidative stress. Oxidative stress caused by H_2O_2 treatment induced phosphorylation of hsp-27 in human umbilical vein endothelial cells and promoted actin re-organization (Huot et al., 1997; Landry and Huot, 1999). Similar results have been obtained in Chinese hamster cells, where the overexpression of hsp-27 prevented actin cytoskeleton from fragmentation and conferred cell survival. However this effect was not seen in cells overexpressed with non phosphorylatable form of hsp-27, thus indicating that phosphorylated hsp-27 protects actin cytoskeleton during oxidative stress and promotes cell survival (Huot et al., 1996).

1.8.4 Hsp-27 and immune reaction

Oxidative stress can cause inflammation and structural damage to various molecules leading to tissue injury. Interestingly, hsp-27 could diminish the damage caused by osmotic stress (Preville et al., 1999). This is probably due to the ability of hsp-27 to regulate the expression of pro and anti-inflammatory genes. Notably, overexpression of hsp-27 conferred protection against TNF- α induced cell death through protection against oxidative stress and apoptosis (Mehlen et al., 1995). In fact, downregulation of hsp-27 results in increased release of pro-inflammatory cytokine, IL-8 (Sur et al., 2008), but on the other hand hsp-27 activates MAPK p38 pathways

and thereby induce the production of the anti-inflammatory cytokine IL-10 (De et al., 2000). Hastie et al showed that hsp-27 plays a protective role in regulating inflammatory response in airway epithelium (Hastie et al., 1997).

1.8.5 Hsp-27 in disease

The gene encoding hsp-27 is situated on chromosome 7q11.23 (Stock et al., 2003). The mutation of this gene has been implicated in various diseases including Charcot-Marie-Tooth disease type 2 and distal hereditary motor neuropathy (Evgrafov et al., 2004). This is primarily due to the altered ability of aggregate formation (Rossor et al., 2012). The neuroprotective effect of hsp-27 has also been shown in Huntington's disease by decreasing the level of ROS in the cells as well as modulating the chaperone activity and regulating apoptosis (Perrin et al., 2007; Wyttenbach et al., 2002). Kainic acid induced neuronal toxicity is an experimental model used to study the temporal lobe epilepsy (Ben-Ari, 1985). Overexpressing hsp-27 has been shown to protect from kainic acid induced neurotoxicity through reduction of seizure severity and cell death (Akbar et al., 2003; Kalwy et al., 2003). On the other hand, increased expression of hsp-27 has been correlated with demented patients in Parkinson's disease. It has been shown that the level of hsp-27 was increased in the astrocytes of patients with Alzheimer disease compared to healthy brains, where hsp-27 was present at lower concentrations (Renkawek et al., 1999).

Hsp-27 has been primarily investigated due to its involvement in breast cancer. Hsp-27 is overexpressed in various tumours and has been suggested to play a role in the pathogenesis of cancer (Garrido et al., 1998). It has been reported to be upregulated in various tumour tissues including breast, endometrial, ovarian, pancreatic, and colon tumours (Geisler et al., 1999).

Hsp-27 is involved in cell proliferation, differentiation, and drug resistance. Studies of biopsies from breast cancer patients showed that there is an inverse correlation between hsp-27 overexpression and proliferation (Vargas-Roig et al., 1997). Similarly patients with advanced stage of ovarian cancer have higher concentration of hsp-27 compared to patient at initial stage

or patient with benign tumour. The studies also suggest that hsp-27 confers resistance to chemotherapy (Langdon et al., 1995).

1.8.6 Hsp-27 in heart disease

Hsp-27 is abundantly expressed in cardiac and skeletal muscle as well as the brain (Ciocca et al., 1993). In the heart, immunocytochemistry of human tissues has shown hsp-27 to be expressed in cardiomyocytes, SMC as well as endothelial cells (Martin-Ventura et al., 2004; Robinson et al., 2010). In non-transplant atherosclerosis, hsp-27 expression decreases with the progression of the disease. Studies of carotid endarterectomy specimens removed from patients with acute coronary syndrome showed decreased expression of hsp-27 in atherosclerotic plaque compared to adjacent normal appearing vessels (Park et al., 2006). Moreover, they showed that the phosphorylation of hsp-27 was decreased in diseased vessels. Another study, using 2-D electrophoresis, Martin–Ventura compared hsp-27 secretion from atherosclerotic plaques and healthy areas of carotid endarterectomies and found that less hsp-27 was secreted from plaque areas than healthy areas of the same blood vessels (Martin-Ventura et al., 2004). They also performed ELISA for hsp-27 and reported significantly less hsp-27 in plasma of patients with atherosclerosis than healthy individuals. However, another group performing a large prospective study investigated the level of baseline hsp-27 plasma concentration of healthy women who went on to develop myocardial infarction, ischaemic stroke or cardiovascular death and failed to show the link with future cardiovascular event, thus it is not possible to conclude that the secretion of hsp-27 by atherosclerotic plaques reflect the plasma concentration of hsp-27 (Kardys et al., 2008). Hsp-27 has been shown to offer protection against I/R induced by Langendorff perfusion and reduces myocardial infarction in a transgenic mouse model (Efthymiou et al., 2004). It is thus conceivable that upregulation of hsp-27 may protect from ischaemia induced during transplantation and thereby limit the severity of rejection.

1.8.9 Hsp-27 and transplant rejection

In addition, biopsies from cardiac patients undergoing acute rejection were compared to non-rejecting allografts as well as control myocardium from donor heart by western blot. The study showed that hsp-27 was expressed in rejecting heart as well as control heart. However increased level of hsp-27 was seen during acute cardiac allograft rejection. This could suggest that the increased level of hsp-27 during rejection has a protective effect (Schimke et al., 2000). Interestingly, use of 2-dimensional electrophoresis demonstrated that cardiac biopsies from long term cardiac patients showed an increase of 20-fold of the expression of hsp-27 compared to patients who developed graft vascular disease (De Souza et al., 2005). Confirmation that there was less hsp-27 in vessels in biopsies of patients with CAV compared to patients without CAV was shown by immunocytochemistry of cardiac biopsies. Further studies compared by western blot and immunohistochemistry the non-diseased coronary arteries with arteries of patients with dilated cardiomyopathy or ischaemic heart disease and have shown vessels of patients with coronary artery disease have less phosphorylated hsp-27 than healthy coronary vessels (Robinson et al., 2010) thus suggesting that phosphorylation of hsp-27 plays a role in cardioprotection. Taken together, these studies have led us to propose, hsp-27 may confer protection against I/R injury caused during heart transplantation and protect against acute and chronic rejection. Animal models are needed in order to understand the molecular pathogenesis and appreciate the mechanisms involved in disease development and for testing novel therapeutics.

1.9 Hypothesis and Aim

Hsp-27 is a constitutively expressed protein; its expression increases following stress. It is known that hsp-27 protects from apoptosis and ischaemia injury induced *ex vivo*. In addition, a number of clinical studies have suggested that increased expression of hsp-27 protects against non-transplant atherosclerosis and cardiac allograft vasculopathy in cardiac transplant patients (De Souza et al., 2005; Martin-Ventura et al., 2004).

Based on these observations, a role for hsp-27 in cardiac rejection was hypothesised. We hypothesised that increased expression of hsp-27 is beneficial against cardiac rejection.

The main aim of this study was to determine whether overexpression of hsp-27 protects the heart from acute rejection and CAV and if so, to define the mechanisms of its protective effect using transgenic mice overexpressing human hsp-27.

Specific aims of this project were:

- To identify transgenic animals and describe expression of the transgene in various organs including the heart.
- To explore the effect of hsp-27 on acute rejection using an experimental model: by performing heterotopic heart transplantation and assessing whether presence of transgene alters graft survival, influences the infiltration of graft-damaging cells and plays a role in antibody mediated rejection.
- To create an experimental model of CAV: To explore the role of hsp-27 in CAV by performing heterotopic heart transplantation and assessing the severity of blood vessel occlusion, characterisation of the cells infiltrating the grafts and understand the role of hsp-27 in antibody mediated rejection.
- To identify the mechanisms of action of hsp-27 following I/R injury: understand whether over-expression of hsp-27 protects from cell death and if so identify the mechanisms by studying the proteolytic enzymes.

- To investigate whether hsp-27 affects the immune response towards the graft by removing splenocytes from transplanted mice and assessing their ability to recognise donor antigens.

Chapter 2: Materials and Methods

2.1 Animals

B10.A hsp-27 transgenic, their LC, CBA and C57BL/6 mice aged between 8 and 12 weeks were used in this study. Heterozygous hsp-27 transgenic mice were obtained from professor Dominic Wells (Imperial College London, UK). Initially, the transgenic mice were generated using a transgene containing human hsp-27 cDNA with a chicken β -actin promoter and cytomegalovirus enhancer. The transgene was placed together with a hemagglutinin tag (HA tag) in order to track the expression of the transgene. The transgene was then microinjected into pronuclei of fertilized eggs from C57BL/10 x CBA/Ca mice which were transferred to pseudopregnant recipients. These mice were then bred for nine generations with wild-type B10.A, selecting for hsp-27 positive mice at each generations. Hsp-27 transgenic and their LC mice were then bred in the animal facilities at Harefield Heart Science Centre (HHSC) using hsp-27 transgenic female and B10.A wild-type males. B10.A wild-type, C57BL/6 and CBA mice were purchased from Harlan Laboratory. Mice were conventionally housed at HHSC and fed and the experiments were performed under license by the Animals and Scientific Procedures Act (1986).

2.2 Tissue preparation

The animals were anesthetized under 2-2.5% isoflurane inhalation anesthesia (isoflurane-Vet, Merial) and the donor hearts from recipients or the native hearts from non-transplanted animals were procured. The heart was dipped into saline solution and was cut transversely into three parts. The upper part containing the atria was used for histology. The middle and lower part were as snap frozen in liquid nitrogen and was used for obtaining proteins or RNA respectively.

2.3 Polymerase chain reaction

Genotypes of offspring were determined from mice genomic tail DNA using polymerase chain reaction (PCR) assay and PCR reagents from Qiagen (Hot star Taq DNA polymerase, QIAGEN). PCR reaction mix contained 0.5µl of genomic DNA, 5µl of 10×buffer, 10µl of Q solution, 1µl of dNTP (60µl/L, Invitrogen) 1µl of hsp-27 sense primer (5'-TGA-CGT-CAA-TGG-GTG-GAC-TA-3'), 1µl of hsp-27 anti-sense primer (5'-TCA-CCT-CGA-CCC-ATG-GTA-AT-3'), 26.5µl of Millipore distilled water and 0.5µl of Taq DNA polymerase, in a final volume of 50µl. Known hsp-27 transgenic DNA was used as positive control and negative control was without genomic DNA but instead water. Protein kinase C (PKC) was used as a housekeeping gene to check the presence of genomic DNA in the PCR samples. The reaction mixture for PKC contained 0.5µl of genomic DNA, 5µl of 10×buffer, 10µl of Q solution, 4µl of MgCl₂, 1µl of dNTP 1µl of primer 1 (5'-TCT-CCT-GTC-ATC-TCA-CCT-TGC-3'), 1µl of primer 2 (5'-CCA-ATG-CTA-AGG-CAG-CAA-GTC-3'), 1µl of primer 3 (5'-CAT-GGT-AGT-GTT-CAA-TGG-CCT-TC-3'), 21.5µl of Millipore distilled water and 0.5µl of Taq DNA polymerase 33 number of cycles were performed on the samples as follows: denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C at 30 seconds, final extension cycle at 72°C for 2.5 minutes. Reaction products were analyzed in 1-2% agarose gel in which the following bands were expected: LC (-/-): 500bp band for PKC and no band for hsp-27, transgenic (+/-): 500bp band for PKC and 200bp band for hsp-27. A picture of the gel was taken using Transilluminator (Kodak).

2.4 Protein assay

Mouse heart samples were homogenized in liquid nitrogen using pestle and mortar in RIPA lysis buffer (20mM MOPS pH 7.0, 150mM NaCl, 1mM EDTA pH 8.0, 1% NP40, 1% Sodium deoxycholate, 1% SDS) supplemented with protease inhibitors and phosphatase inhibitors. The homogenate was incubated on ice for 20 minutes and centrifuged at 13K revolutions per minute (rpm) for 10 minutes at 4°C. The supernatant was subjected to colorimetric bicinchoninic acid (BCA) protein assay (Pierce) in order to quantify the protein concentration. This method is

based on two reactions: (1) reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium followed by (2) reaction of cuprous cation (Cu^{1+}) with BCA. The resultant purple colour product exhibits a strong linear absorbance at 550nm. The amount of colour produced is proportional to protein concentration present in the protein sample. A standard assay using known concentration of bovine serum albumin (BSA) is performed simultaneously. For the standard curve, 200 $\mu\text{g/ml}$ was prepared from 2mg/ml BSA stock solution diluted in PBS and lysis buffer. The 200 $\mu\text{g/ml}$ was then used to make serial dilutions to give final concentration of 100 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 12.5 $\mu\text{g/ml}$, 6.25 $\mu\text{g/ml}$ and 3.125 $\mu\text{g/ml}$. The blank was 0 $\mu\text{g/ml}$. Some protein samples were highly concentrated, therefore all samples were diluted 1/10, 1/20 and 1/50 in PBS and protein buffer. For the assay, 100 μl of samples or serially diluted standard were added to the 96 well plate. Each standards and samples were added in duplicate to minimise pipetting errors. 100 μl of colour reagent (by diluting reagent B in reagent A by 1/50) was added to each well as instructed by the manufacturer and was incubated for 1h at 37°C. The absorbance of standards and samples were read at 550nm using a μQuant microplate reader and the software v1.41.3 (BIO-TEK Instruments, Inc). A standard curve was generated by plotting the Absorbance at 550nm against the known concentration of standards. A representative standard curve using BSA is shown in Figure 20. The coefficient of determination r^2 was >0.99 indicating a good fit of the data points with the linear regression, hence suggesting the possibility to get highly accurate values from the regression line. The unknown concentrations of the samples are determined by reference to the standard curve.

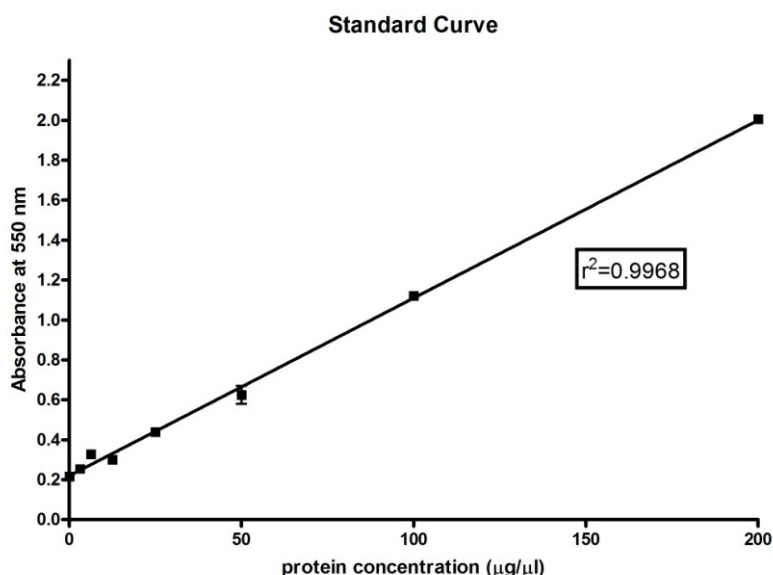


Figure 20: A representative standard curve for protein quantification using the Pierce BCA protein assay. Serial dilutions of known concentration of BSA were made and their absorbance was measured. A standard curve was generated using linear regression and plotting the absorbance at 550nm against the known protein concentration. The concentration of each standard was assayed in duplicate. The coefficient of determination r^2 is indicated.

2.5 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli to separate proteins according to their molecular weight (Laemmli, 1970). SDS is an anionic detergent; it acts by disrupting non-covalent interaction between polypeptide chains. SDS binds to the polypeptide chain in proportion to the molecular weight. This result in a denatured protein where charge to mass ratio is proportional, thus the final separation of the proteins is dependent on the relative molecular mass of polypeptides. SDS-PAGE consists of discontinuous buffer system made of two different layers of acrylamide: stacking gel and resolving gel. The stacking gel, (upper layer) which includes the wells, is used to stack all proteins into a single starting band and the resolving gel enables separation of the proteins according to their molecular weight. The composition of stacking and resolving gel are given in the table below (Table 1).

Table 1: Formulation of Resolving and Stacking gel

Reagents	Resolving gel			Stacking gel
	6%	12%	15%	
MilliQ water	1.75ml	1ml	0.6ml	1.575ml
Lower Tris (pH 8.8)	2.5ml	2.5ml	2.5ml	
Upper Tris (pH 6.8)				625μl
Acrylamide: Bis-acrylamide solution (40%)	0.75ml	1.5ml	1.9ml	225μl
SDS (5%)	100μl	100μl	100μl	80μl
10% APS	50μl	50μl	50μl	40μl
TEMED	5μl	5μl	5μl	4μl

After heating the samples at 100°C for 10 minutes in order to denature the proteins, gel electrophoresis was performed using vertical gel apparatus and 10-15% acrylamide Tris-Glycine gel. The percentage of acrylamide used was dependent on the molecular weight of the target protein. In fact, higher percentage of acrylamide with greater degree of cross-linking was used to detect proteins of smaller sizes. 40μg of total protein was then further solubilised in 1:3 ratio of loading buffer (100mM Tris pH6.8, 4%SDS, 20% glycerol, 0.2% Bromophenol Blue, 40mM DTT and 13ml of water). The bromophenol blue present in the loading buffer facilitate the visualization of protein migration since it migrates through the gel at a similar speed to glycine, the smallest known amino-acid. The 20μl of sample/buffer and 7μl of the rainbow molecular weight markers (Colourburst, Sigma, UK) were run in the electrophoresis running buffer (100ml of 10×Tris-Glycine, 10% SDS and 890ml of distilled water) at a constant voltage of 100volts initially until the dye front reached the resolving gel then at 150volts. The gel run was stopped when bromophenol blue dye in the sample buffer had reached the end of the gel.

2.6 Transfer of proteins

The proteins that have been separated by electrophoresis were transferred from polyacrylamide gel to the Polyvinylidenedifluoride (PVDF) membrane (Amersham Labs, GE Healthcare Ltd). A

standard tank system (wet tank) was used where the membrane and the gel were sandwiched in between filter papers as summarised in figure below (Figure 21).

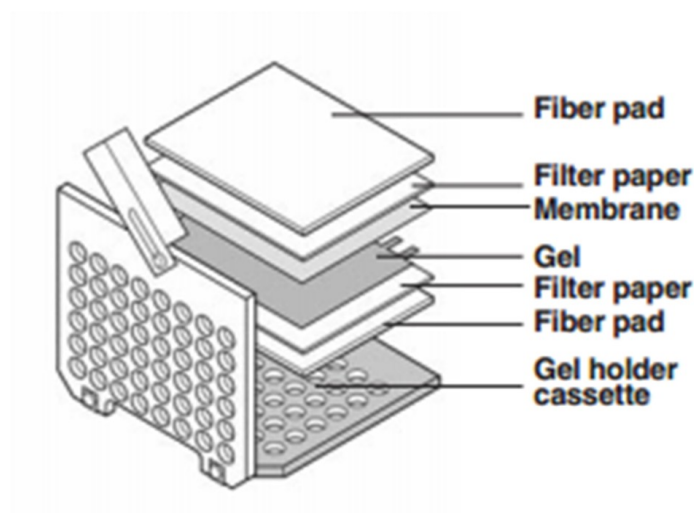


Figure 21: Assembly of trans-blotting system. Figure taken from Bio-Rad Laboratories inc. (<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1703930.pdf>).

After careful assembly and removal of air bubbles the stack was placed between the electrodes in the correct orientation. The tank that was filled with transfer buffer (1 L: Tris base (3 g), Glycine (14.4 g), 20% Methanol) and an ice pack was used as cooling mechanism in order to prevent temperature fluctuations and overheating during transfer. The transfer was carried out at 100V for 2 hours.

2.7 Immunoblotting

The western blotting technique introduced by Towbin et al was used for protein analysis (Towbin et al., 1979). Proteins that were separated in 10-15% acrylamide Tris-Glycine gel were transferred onto nitrocellulose membranes for 1h at 100V. Membranes were then air dried. The efficiency of protein transfer was assessed by staining the membranes with Ponceau to visualize protein bands and to confirm accurate loading between groups. Ponceau binds to the positively charged amino groups of the protein and non-covalently to non-polar regions. The membrane was immersed in Ponceau for 2 minutes at room temperature and washed in 1x Tris-buffered sulphate (TBS) in order to visualize the protein bands that have been transferred. To

destain, the membrane was washed three times in TBS for 5 minutes at room temperature. The membrane was then used for western blot analysis. Non specific bindings were blocked by incubating the membrane for 2h in 5% solution of dried milk powder in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBST). After washing, the membrane was probed for specific proteins by incubating with primary antibody in 5% BSA at room temperature for 1h on a shaker to allow uniform binding of the antibody to the target proteins. This was followed by incubation with secondary antibody directed against the Fc region of primary antibody and conjugated with horseradish peroxidase (HRP) for 1h with constant shaking. Three 5 minutes washes with PBST were performed between each antibody step to remove the non-specific binding. Please see table below for the details of antibodies used in western blotting (Table 2). Enhanced chemiluminescence (ECL) was prepared by mixing ECL reagent A to reagent B in 1:1 ratio (ECL, Amersham hyperfilm *ECL*, GE healthcare Ltd) and was added evenly to the membrane for 1 minute, which was then exposed to autoradiography film for various time periods until optimal bands were detected. To visualize the bands, the film was developed by immersion in developer for 5 minutes, water for 1 minute and fixer for 5 minutes.

Table 2: Summary of antibodies used in Western-blotting

Primary Antibody	Dilution	Host	Company
HA	1/200	Rabbit	Santa Cruz
Hsp-25	1/250	Rabbit	Calbiochem
GAPDH	1/1000	Rabbit	Cell signalling
Phospho-AMP kinase	1/500	Rabbit	Cell Signalling
Hsp-27 phospho-ser82	1/500	Rabbit	Stressgen
Secondary Antibody			
HRP conjugated anti-rabbit	1/1000	Goat	Dako

2.8 Histology

2.8.1 Immunohistochemistry

Immunohistochemistry was used to analyse the presence of HA tagged hsp-27, CD31, CD3, CD4, CD8 and CD11b. Briefly, pieces of tissue were embedded in optimal cutting temperature (OCT, Tissue Tek, Germany) and snap frozen in the liquid nitrogen. They were stored at -80°C until use. Cryostat sections of 7µm thickness were cut using a Leica CM 850 cryostat (Leica Microsystems, UK) and fixed in acetone (100%) for 6 minutes and stored at -20°C until use.

Table 3: Summary of antibodies used for immunohistochemistry

Primary Antibody	Dilution	Host	Company
HA	1/50	Rat	Roche
CD31	1/100	Rat	Serotec
CD11b	1/100	Rat	Serotec
CD3	1/100	Rat	BD pharmingen
CD4	1/100	Rat	HHSC
CD8	1/100	Rat	HHSC
Smooth muscle alpha actin	1/100	Rabbit	Epitomics
Mac 30: rat Ig: isotype control ¹	1/100	Rat	HHSC
Rabbit Ig: isotype control ²	1/50	Rabbit	Dako
Secondary Antibody			
Biotinylated anti-Rat	1/200 or 1/300	Rabbit	Dako
Biotinylated anti-rabbit	1/400	Goat	Dako
1: isotype control for primary antibody raised in rat 2: isotype control for primary antibody raised in rabbit			

Non-specific sites were blocked with 1% BSA /0.1%Tween-20 in PBS for 30 minutes before incubating the sections with the primary antibody (Table 3) for 1h30 at room temperature. After washing in PBS, biotinylated secondary antibodies (see below) in 2% mouse serum was added for 45 minutes followed by avidin-biotin-horseradish peroxidase complex (*Vectastain ABC kit*, Vector laboratories). The sites of peroxidase binding were developed in PBS containing

diaminobenzidine tetrachloride (0.3mg/ml) and hydrogen peroxide (0.01%) (*Sigma fast*, Sigma Aldrich). Slides were counterstained with Mayer's hematoxylin (Sigma). The sections were mounted with DPX mountant (BDH) and then examined under light microscopy using Nikon DMx1200 camera mounted on a Zeiss Axioscope microscope and the NIS elements software V (Nikon Instruments, USA).

2.8.2 Staining paraffin sections

This work was performed by Dr. Padmini Sarathchandra

2.8.2.1 Preparation of paraffin blocks

Myocardial tissues from mice were collected in 10% formal saline. These were dehydrated by successive passage through ascending methanol and xylene series as follows: 70% methanol, 100% methanol twice followed by 100% xylene twice. The tissues were then embedded with paraffin wax overnight in a cassette. The tissues present in the wax block were cut using Leica 2065 microtome (Leica, Germany).

2.8.2.2 Staining for graft vasculopathy

Paraffin sections of mouse myocardium were dewaxed in 100% xylene and 100% methanol. The sections were then stained with Miller's elastin (VWR international, UK) for 2h. Following differentiation in 95% methanol for few minutes in order to remove excess colour, the sections were washed with running tap water, followed by distilled water and stained with Van Gieson stain (VWR international, UK) for 5 minutes. These were then blot dried, rapidly dehydrated in methanol, cleared in xylene and mounted with DPX embedding compound (VWR international, UK).

2.8.2.3 Measurement of graft vasculopathy

Paraffin sections stained with Miller's elastin and Van Gieson were used to determine the percentage of vessel occluded by vasculopathy. Quantitative morphometry was used to outline and measure the neointima (Figure 22 below) and hence calculate amount of neointimal thickening and percentage vessel occlusion.

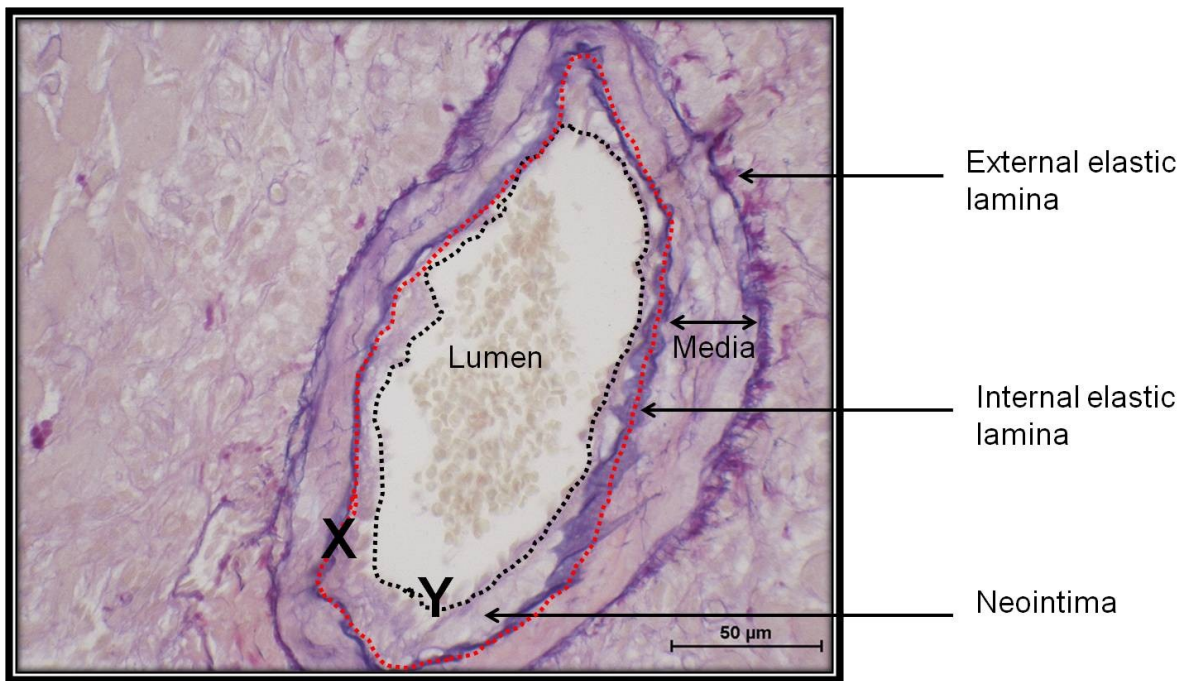


Figure 22: Measurement of graft vasculopathy. The Photomicrograph shows different layers of the blood vessel. The area between the internal elastic lamina (red dotted line-X) and the neointimal thickening (black dotted line-Y) represents the neointimal hyperplasia. The percentage of occlusion was calculated using the formula $((X-Y)*100)/Y$ where X-Y represents the occluded area.

2.8.2.4 Alpha actin staining

In order to assess proliferation and migration of SMC and establishment of graft vasculopathy following heterotopic heart transplantation, the allografts were stained for smooth muscle alpha actin. The paraffin sections were dewaxed and rehydrated by passing through 100% xylene twice, 100% methanol twice. These were then stained with Miller's elastin for 2h, differentiated in 90% ethanol for 30 seconds and washed well with tap water. Antigen retrieval was carried out by microwaving the slides for 10 min in 0.1M citrate buffer (pH=6) and left for further 20 minutes in the same buffer. After water wash, non-specific binding was blocked by incubating the sections with 3% bovine serum albumin BSA (Sigma Aldrich) for 30 minutes followed by rabbit anti-human smooth muscle α -actin monoclonal antibody (Epitomics Inc, CA) at 1:100 in PBS with 3% BSA for 16 hours at 4°C. After washing the sections to remove non-specific binding of the antibody, the sections were then incubated with biotinylated Swine anti-rabbit IgG (DakoCytomation, Denmark) at 1:250 for 1h. The sections were then washed and incubated

with Avidin-Biotin peroxidase complex (Vector Laboratories) for 1h, visualized with DAB substrate supplemented with hydrogen peroxidise and counter stained with Hematoxylin.

2.9 Induction of ischaemia *ex vivo*

During heterotopic heart transplantation in rodents, the donor organ is exposed to 10 minutes of cold ischaemia during procurement of the organ and 40 minutes of warm ischaemia experienced during anastomosis. In order to mimic the effect of ischaemia encountered during conventional heterotopic heart transplantation in mice, hearts were harvested from hsp-27 transgenic and LC mice and subjected to 10 minutes of cold ischaemia (4°C saline) followed by 40 minutes of warm ischaemia (25°C saline). Hearts where no ischaemia was induced were used as controls. Figure 23 represents a schematic diagram of induction of ischaemia *ex vivo*.

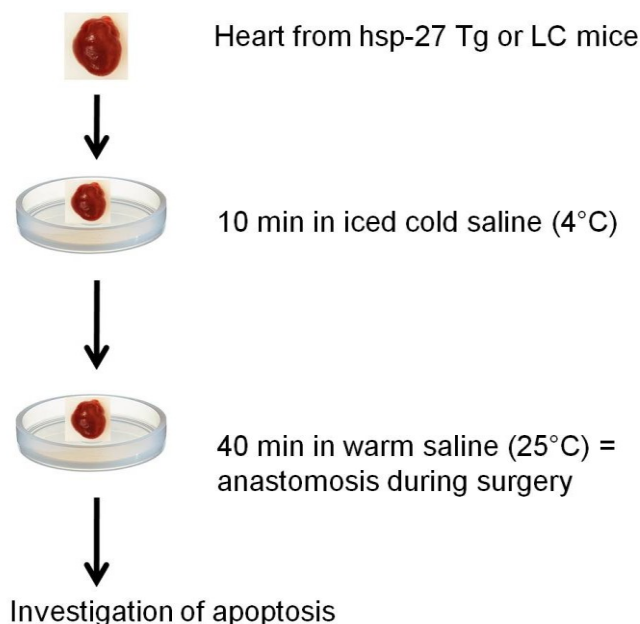


Figure 23: Schematic diagram explaining the induction of ischaemia *ex vivo*. Transgenic and LC hearts were subjected to 10 minutes of cold ischaemia at 4°C and 40 minutes of warm ischaemia at 25°C as encountered in conventional heterotopic heart transplantation in mice.

2.10 TUNEL assay

To identify apoptotic response during ischaemia, apoptotic cells from the hearts of hsp-27 transgenic mice and their LC were detected by TUNEL labelling detection of free 3'-OH groups in fragmented DNA *in situ* (In Situ Cell Death Detection Kit, POD, Roche). The assay was performed as described by Sgnoc R. et al (Sgonc and Wick, 1994). The piece of hearts were frozen in liquid nitrogen and cut into 7µm section using a cryostat. The sections were fixed with 4% paraformaldehyde in PBS, pH7.4 and washed with PBS for 30 minutes. At this stage, the sections could be stored at -20°C after dehydrating them in absolute ethanol for 2 minutes. Non specific binding was blocked with 3% H₂O₂ in methanol at room temperature. After permeabilisation (0.1% Triton X-100 and 0.1% sodium citrate for 2minutes at 2-8°C), the sections were then incubated with 50µl per section of terminal deoxynucleotidyl transferase (TdT), in the presence of nucleotides. Positive controls were obtained by incubating fixed permeabilised cells with DNAase I recombinant, grade I (2000U Tris-HCl, pH 7.5, 10mM MgCl₂, 1mg/ml BSA). Sections incubated with nucleotides, in the absence of TdT, were used as negative controls. The slides were washed three times with PBS. These were then mounted using DAPI mounting media (vectashield, vector) and visualized under fluorescent microscope using an excitation wavelength in the range of 450-500nm. All the Tunel positive cells (brightly fluorescent cells) present in random 50fields/ heart were counted using fluorescent microscope at magnification x40.

2.11 Caspase assay

Heart tissues subjected to ischaemic injury *ex vivo* or I/R injury *in vivo* as well as control non ischaemic heart tissues were homogenized in lysis buffer (125mM HEPES, 12.5mM CHAPS, 12.25mM DTT and protease inhibitor) and protein concentration was determined using Bradford protein assay reagents. 30µl of total protein was incubated at 37°C with 50ul of caspase assay buffer (200mM HEPES, 50mM DTT, 1%CHAPS, 20mM EDTA) and the appropriate caspase substrate. The activity of caspase-3, caspase-1 and caspase-9 were measured using specific

substrates labelled with N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Sigma Aldrich), N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin and N-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin respectively (Tocris) and quantified by spectrofluorimetry (excitation wavelength 360nm and emission wavelength 460nm for caspase-3 and wavelength 360nm and emission wavelength 530nm for caspase-1 and caspase-9).

2.12 Quantitative Real-Time PCR

2.12.1 Sample disruption and homogenisation

Tissues from explanted heart were frozen in liquid nitrogen and stored in -80°C until use. Qiagen mini-kit was used to isolate RNA from heart samples. Immediately before use, 10ul of β -mercaptoethanol was added per 1ml of lysis buffer. β -mercaptoethanol is a reducing agent that disrupts the tertiary and quaternary structure by cleavage of disulphide bonds. The lysis buffer also contains guanidine thiocyanate which disrupts the secondary structure by breaking the hydrogen bonds. These results in inhibition of RNAase activity and promote lysis of the tissue. To prepare RNA from human cardiac tissue, up to 30mg of frozen sample was added to lysis buffer in a 2ml round bottomed microtube. Tissues were disrupted and homogenised in lysis buffer using homogeniser. The tip of the probe was submerged in the lysis reagent and the sample homogenised at maximum speed for up to 1 minute. To assess homogenisation, the lysate was held up to the light and examined for aggregates; if any remained the lysate was homogenised further until it appeared clear. In between samples, the probe was sequentially rinsed in 70% ethanol and DEPC-treated water. Disruption is done to break up the extracellular matrix and open plasma membrane of cells and organelles in order to solubilise all RNA contained in the sample. Homogenisation is performed in order to reduce the viscosity of the disrupted samples by shearing genomic DNA and other high molecular weight cellular components. The disruption/homogenisation step is critical and thawing of the samples (as it will activate the RNAase) prior to this should be avoided to obtain high quality RNA. In order to

achieve complete homogenisation, the lysate was passed through Qiasredder and centrifuged for 1 minute at 13 000rpm at room temperature.

2.12.2 mRNA isolation

RNA was isolated from human tissue samples using RNeasy mini kit. The RNeasy kit is based upon a bench-top centrifuged-sized spin column which contains a silica gel membrane. By altering the salt concentration in the column, RNA can be adsorbed onto or eluted from the membrane. The binding capacity of the membrane stated by the manufacturer is approximately 100µg of RNA. The membrane enriches for longer RNA molecules by excluding RNA less than 200 bases. The lysate (500µl) was then diluted by adding 983µl of water and 17µl of proteinases k in order to digest the proteins. The samples were then incubated for 10 minutes at 55°C, centrifuged at 10K rpm for 3 minutes in order to remove the debris. The supernatant was transferred into a new 2ml microtube and 0.5 volumes of 96-100% ethanol was added to the lysate and mixed by pipetting. In order to adsorb the RNA to the membrane, 700µl of the sample was added to the RNeasy mini spin column placed in 2ml collection tube and centrifuged at 10k rpm for 20 seconds. The flow through was discarded and this step was repeated until all the samples were passed through the column. The column was incubated with 350µl of RW1 wash buffer for 5 minutes and centrifuged at 10k rpm for 20 seconds. To remove the DNA contamination, the mixture of 10µl of DNase I and 70µl of RDD buffer was added carefully on top of the column membrane and incubated for 15 minutes. In order to remove the DNase and any contaminants, the column was washed with RW1 buffer (350µl) followed by washing with ethanol containing RPE buffer (500µl) by centrifuging at 10 000rpm for 20seconds. The column is again washed with 500µl of RPE buffer by centrifuging for 2 minutes at 10K rpm and the flow through is discarded. To avoid any carryover of ethanol, the spin column is placed in a new 2ml microtube and centrifuged at 10K rpm for 1 minute the flow through and the tube was discarded. Finally, to elute the RNA bound to the silica membrane, the column was placed in a new 1.5ml tube and 30µl of water is carefully added on top of the membrane. This was incubated for 15 minutes and centrifuged at 10 000rpm for 1 minute. The column was discarded

and the microtube containing the RNA was placed immediately on ice. Except where specific temperature is given, the protocol is performed at room temperature throughout.

2.12.3 mRNA quantification

The RNA obtained from tissues were quantified using spectrophotometry. At 260nm absorbance of 1unit corresponds to 44µg of RNA per ml. Samples were diluted 1:35 in TE buffer and the absorbance was measured at 260nm using BioPhotometer Plus (Eppendorf, UK). Before measuring the absorbance, the TE buffer on its own was used to zero the spectrophotometer. The concentration of RNA (µg/µl) was calculated as follows: $A_{260} \times \text{dilution factor} \times 44$. The purity of RNA was tested by calculating the ratio of absorbance at A_{260}/A_{280} . Pure RNA sample has a A_{260}/A_{280} ratio of 1.9 - 2.1. The RNA was quantified and stored at -80°C.

2.12.4 cDNA synthesis

The RNA samples were thawed and kept on ice. The cDNA was prepared using the reverse transcription kit (Applied Biosystems, UK). The mastermix of all the reagents were prepared as summarised in table below (Table 4).

The mastermix was prepared and aliquoted on ice. The same quantity of RNA was added to each tube and vortexed. The samples were then centrifuged briefly at 10K rpm at 4°C. cDNA was synthesised in a Techne PHC-3 thermal cycler (Helix technologies Ltd., UK). The cycling conditions used for cDNA synthesis are as follows:

25°C for 10 minutes Allows primer binding

48°C for 30 minutes Extension

95°C for 5 minutes Enzyme denaturation

The cDNA was then diluted to the RNA equivalent of 2ng/µl and stored at -20°C until required.

Table 4: Reagents for cDNA synthesis

Reagent	Final concentration	10µl Rxn	20µl Rxn	30µl Rxn
10x RT buffer	1x	1.0	2.0	3.0
25mM MgCl ₂	5.5mM	2.2	4.4	6.6
dNTPs	500µM each dNTP	2.0	4.0	6.0
Random hexamers	2.5µM	0.5	1.0	1.5
Rnase inhibitor	0.4U/µl	0.2	0.4	0.6
MultiScribe reverse transcriptase	1.25U/µl	0.625	1.25	1.875
RNA-free water	-	3.475	6.95	10.425
RNA	10ng/µl			

2.12.5 Quantitative RT-PCR

Quantitative real-time PCR (RT-PCR) was performed using off the shelf TaqMan® gene expression assays (Applied Biosystems). This technique consists of using fluorogenic probe to monitor the progress of PCR as it occurs (Real-time). The TaqMan probe is an oligonucleotide containing a reporter fluorescent dye (FAM) on the 5' end and a quencher dye (NFQ) on the 3' end. When the probe is intact, the Fluorescence of the reporter dye is transferred to the quencher by a method called fluorescence resonance energy transfer (FRET). In presence of target gene, the probe binds to the target gene and the primer is extended. This results in cleavage of primer by DNA polymerase leads to separation of reporter dye from quencher dye and thus increased signal of the reporter dye. At each cycle, additional reporter dye is cleaved from the probes. Thus the fluorescence intensity is proportional to the amount of amplicon produced. The cycle at which fluorescence of the reporter dye exceeds background threshold fluorescence is called the Cycle threshold (Ct). Ct value is used to calculate the relative gene expression by the 'Comparative Ct method'. Amplification of an endogenous reference gene (18S rRNA) was carried out at the same time as the amplification of target gene in order to calculate the relative gene expression. The 18S was amplified using primers and probe labelled with VIC reporter dye and TAMRA quencher dye. This allows measurement of Ct values for

target gene and control in the same tube and the ΔCt is calculated as the difference between Ct of target gene and Ct of endogenous reference genes. From this, the relative expression of the gene is calculated as: Relative gene expression = $2^{-\Delta [\Delta Ct]}$; where $\Delta [\Delta Ct]$ is the difference between ΔCt of test sample and ΔCt of blank/calibrator sample. Thus, the abundance of the target gene is calculated, normalized to an endogenous reference gene like 18s. Taqman Fast Advanced Master mix (# 4444557), 18s rRNA assay (# 4310893E) and Taqman probes for CD3, CD4, CD8, CD14, CD68, IFN- γ , IL-1 β , TGF- β , TNF- α , IL-4, IL-5 and IL-10 and Th17, Fast Optical 96-well reaction plate (# 4346906) and Optical adhesive film kit Fast Optical 96-well reaction plate (# 4313663) were purchased from Applied Biosystems. The details of the probes used are displayed in Table 5.

The master mix prepared as detailed in Table 6 was aliquoted into 96-well reaction plate. 3 μ l of the 2ng/ μ l cDNA samples were added in duplicates to the wells (4.5 μ l of cDNA was added for genes with lower transcripts expression). The plate was sealed using optical adhesive film and centrifuged for 3 minutes at 1500g. RT-PCR reaction was performed using ABI Prism 7700 Sequence Detection System (Applied Biosystems) and the conditions of the reactions are explained in Table 7. At the end of the RT-PCR cycle, data were collected and the relative gene expression was calculated by the comparative Ct method using MS Excel.

Table 5: Details of RT-PCR probes used in the study. Probes were purchased from Applied Biosystems.

Target gene	Probe code	Catalogue number
CD3	Mm00438095_m1	4331182
CD4	Mm00442754_m1	4331182
CD8	Mm00438116_m1	433112
CD14	Mm00438094_g1	4331182
CD68	Mm03047343_m1	4331182
IL-1 β	Mm01336189_m1	4331182
IL-2	Mm00434256_m1	4331182
IL-4	Mm00445260_m1	4331182
IL-5	Mm00439646_m1	4331182
IL-6	Mm00446190_m1	1038736
IL-10	Mm00439614_m1	4331182
IL-17	Mm00439618_m1	4331182
IFN- γ	Mm00807178_m1	1010220
TGF- β	Mm01178820_m1	4331182
TNF- α	Mm00443258_m1	4331182

Table 6: Real-Time PCR reaction setup

Reagents	Final Concentration	Amount (in μ l) for 20 μ l reaction
2x TaqMan [®] gene expression master mix	1x	10
20x 18s rRNA assay (VIC reporter)	1x	1
20x target gene assay (FAM reporter)	1x	1
cDNA	-	3
DNase/RNase free water	-	5

Table 7: RT-PCR cycling conditions

Stage	Temperature	Time	Reason
Stage 1	50°C	2 mins	UNG incubation
Stage 2	95°C	10 mins	DNA polymerase activation
Stage 3	95°C	15 seconds	Denaturation
Stage 4	60°C	60 seconds	Annealing and extension
Stage 3 and stage 4 were run for 40 cycles			

2.13 Flow cytometry analyses

2.13.1 Confirmation of T-cell depletion

This test was performed to confirm the depletion of T-cells in CBA and C57BL/6 mice that were previously injected with monoclonal CD4 and or CD8 antibodies. Mouse blood was collected in 100 units of heparin and diluted in 1:5 PBS containing 2% foetal calf serum (FCS). The samples were then incubated with directly conjugated antibodies for 15 minutes in the dark at room temperature. The details of antibodies used are listed in Table 8.

400 μ l of 1:10 diluted FACS lysing solution (BD Bioscience, UK) was added to each tube and incubated for 15-20 minutes in the dark at room temperature in order to lyse the red blood cells. Two consecutive washing steps were performed with PBS-2%FCS and centrifuged at 1500rpm for 6 minutes at room temperature and the supernatant was discarded. The cells were then

fixed with 0.5% formaldehyde in PBS (400µl) and the samples were acquired and analyzed using a flow cytometer (FACSaria instrument and FACSDiva software. BD Bioscience, UK).

Table 8: Summary of antibodies used to confirm depletion of T-cells

Antibody	Format	Host	Company	Dilution
IgG 2a	FITC	Rat	Serotec	1.5:50
IgG 2a	PE	Rat	Serotec	1.5:50
CD3	FITC	Rat	Serotec	1:50
CD4	FITC	Rat	Serotec	1:50
CD8	PE	Rat	Serotec	1:50
CD19	PE	Rat	Serotec	1:50

2.13.2 Measurement of alloantibodies

Serum from recipient mice was collected at the time of sacrifice. 100 000 splenocytes were incubated with sera at 1:10, 1:20 and 1:40 dilutions for 30 minutes at 4°C. Samples were washed twice with PBS and incubated with secondary Goat anti-mouse IgG: FITC at 1:30 dilution for 30 minutes at 4°C. Two further washes were performed and the splenocytes were then fixed in 0.5% formaldehyde and analyzed by flow cytometry.

2.14 Isolation of T-cells

Isolation of T-cells and APCs, measurement of alloantibody and Elispot assays were performed by Ann McCormack

This method was used to isolate T-cells by depleting non-T-cells including B-cells, monocytes/macrophages, NK cells, dendritic cells, erythrocytes and granulocytes. Dynal® mouse T-cell negative isolation kit (#114.13D, Invitrogen) was used to isolate the T-cells. Spleens were disrupted and single cell suspension was prepared by passing the suspension through sterile 100 micron mesh. Splenocytes were resuspended in 0.83% ammonium chloride for 3 minutes at room temperature to lyse the red blood cells. This was then washed, counted and re-suspended in RPMI medium supplemented with 10% foetal calf serum (FCS). 15µl of

cell suspension was diluted in 1:1 ratio with acridine orange/ethidium bromide and the number of viable cells was determined using fluorescent microscopy. The cells were suspended in Buffer 1 made of PBS (without Ca^{2+} and Mg^{2+}) with 0.1% BSA and 2mM EDTA, at pH 7.4. In a tube containing 100 μl (1×10^7) leukocytes 20 μl of heat inactivated FCS and 20 μl of antibody mix (containing a mixture of monoclonal antibody against CD45R, CD11b, Ter-119 and CD16/32 in PBS supplemented with 0.02% sodium azide) were added and incubated for 20 minutes on ice. These were then washed in 10mls of Buffer 1 and centrifuged at 300g for 8 minutes at 2-8°C to collect the pellet. The cells were re-suspended in 800 μl of Buffer 1 and 200 μl of pre-washed mouse depletion dynabeads and incubated for 15 minutes at 18-25°C with gentle tilting and rotation, in order for the cells to attach to the beads. 9mls of Buffer 1 was added to tube and the magnet was applied for 2 minutes, which led to isolation of T-cells present in the supernatant. The non-T-cells bound to the beads were discarded. For washing Dynabeads, these were re-suspended in equal volume of Buffer 1 and attached to the magnet in order for the beads to bind to the magnet. After discarding the supernatant, the tube was removed from the magnet and the beads were re-suspended in the same volume of Buffer 1 as the initial volume of Dynabeads.

2.15 Isolation of APC

B10.A and FVB spleens were disrupted and passed through a 100 μm nylon mesh to produce a single cell suspension. Red blood cells were lysed and the remaining lymphocytes incubated with rat anti-mouse CD3 antibody for 20 minutes on ice. After washing with RPMI1640 + 5% FCS the cells were further incubated with sheep anti-rat IgG magnetic beads (Dyna) for 20 minutes at 4°C with gentle rotation. Cells were exposed to a dynal magnetic particle concentrator (MPC) and unbound, T-cell depleted cell suspensions were removed and used as antigen presenting cells in Elispot assays. To prevent any cells within the APC population from potentially producing cytokines, they were treated with mitomycin C before use in the assay. Cells were incubated with mitomycin C (40 $\mu\text{g}/10^7$ cells/ml) in PBS for 20 minutes at 37°C. These were then washed 3x with PBS before being resuspended in RPMI1640 + 10%FCS ready for

use. Wells containing APC alone or with con A were included in the assay to test that there were no cytokine producing cells present in the APC population.

2.16 Elispot

This technique is used for the detection of secreted proteins such as growth factors and cytokines. PVDF 96 well plates were washed with 100µl/well of 70% ethanol followed by washing thrice with 100µl/well of sterile PBS. The wells were coated with monoclonal rat anti mouse capture antibodies to IFN-γ and IL-2 (BD Bioscience, UK) and incubated overnight at 4°C. Next day, the plates were washed with 1%BSA in PBS and blocked with 200µl/well of 1%BSA in PBS for 1h at room temperature. After washing the plates with sterile* PBS, 2×10^5 responder T-cells and 2×10^5 antigen presenting cells were added to the wells in duplicate and incubated for 48h at 37°C in a CO₂ incubator. Positive control wells of T-cells + APC +con A were included. During this incubation, the cytokines secreted by the cells will be captured by the primary antibody. Then, the plates were washed with water in order to lyse the cells followed by washing three times with PBS-0.025%Tween-20 to remove the cells and unbound cytokines. Plates were then incubated with biotinylated monoclonal rat anti-mouse detection antibody to IFN-γ and IL-2 (BD Bioscience, UK) in 1% PBS-BSA for 2h at room temperature. Following this, the plates were incubated with Streptavidin-HRP (Vector Laboratories, UK) for 1-2h and developed with 3-amino-9-ethyl-carbazole (#A-5754, Sigma)/N, N-Dimethylformamide (#D-4551, Sigma) substrate supplemented with hydrogen peroxide. Spots were developed and counted using an Elispot reader and software (AID EliSpot GMBH). Each spot represents a footprint of the original cytokine- producing cells (Czerkinsky et al 1988).

*All steps up to and including 48h incubation were performed aseptically. After that work was done on open bench

2.17 ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to assess plasma levels of hsp-27 in transgenic mice. The hsp-27 ELISA kit was used according to manufacturer's instructions. (#Q1A 119, Calbiochem). Briefly, the serum obtained from transgenic and LC animals were diluted 1/10 in assay buffer. 50µl/well of samples and 50µl/well of serial diluted hsp-27 standards (provided by the manufacturer) and 50µl/well of detection antibody specific for human hsp-27 were added simultaneously to the wells pre-coated with anti-human hsp-27 capture antibody and incubated for 2h at 37°C. Thereafter, 100µl/well HRP conjugate was added to the plates and incubated for 1h at room temperature. Then 100µl/well of TMB substrate was aliquoted to each well, which will react with HRP to produce a blue coloured solution. Following incubation for 15-20 minutes, the enzymatic reaction was stopped by adding 100µl of Stop Solution that resulted in colour change from blue to yellow and the absorbance was measured at 450nm using µQuant microplate reader and the software v1.41.3 (BIO-TEK Instruments, Inc).

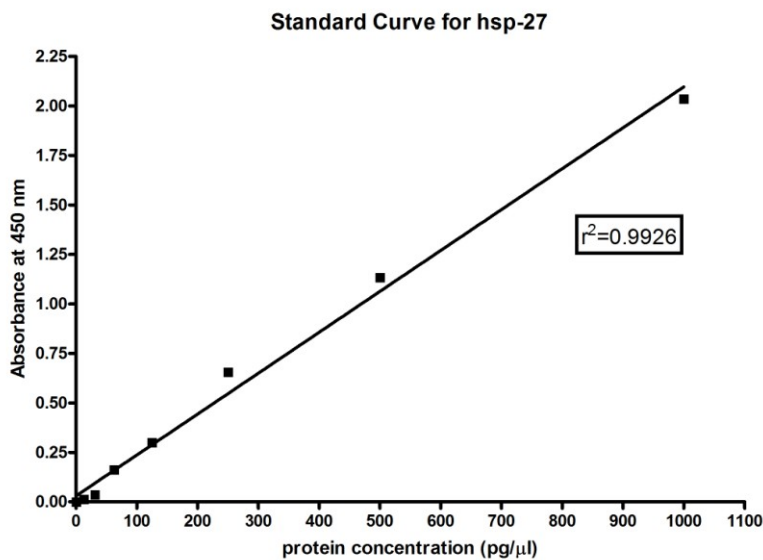


Figure 24: A representative standard curve used for quantification of hsp-27 present in the serum. The standard curve was obtained by plotting the mean absorbance of each standard dilution against the standard concentration of hsp-27 and the best fit curve was obtained by linear regression. The coefficient determinant r^2 is indicated.

A standard curve was plotted of absorbance 450nm to hsp-27 amounts from the standard once the values of the blank were subtracted (Figure 24). Concentration of hsp-27 present in the plasma was calculating by comparing the absorbance of samples with the values obtained from standard curve.

2.18 Heterotopic Heart Transplantation

B10.A ($H-2^a$) hearts were heterotopically transplanted into C57BL/6 ($H-2^b$) or partially T-cell depleted CBA ($H-2^k$) recipients by the technique introduced by Corry et al in 1973 (Corry et al., 1973). Syngeneic controls consisted of either C57BL/6 into C57BL/6 or CBA into CBA. Schematic diagram of heterotopic heart transplant is displayed in Figure 25. Donor and recipient mice were shaved, anaesthetized under 2-2.5% isofluorane inhalation anesthesia (isofluorane-Vet, Merial) and the mice were placed on a heating pad on supine position.

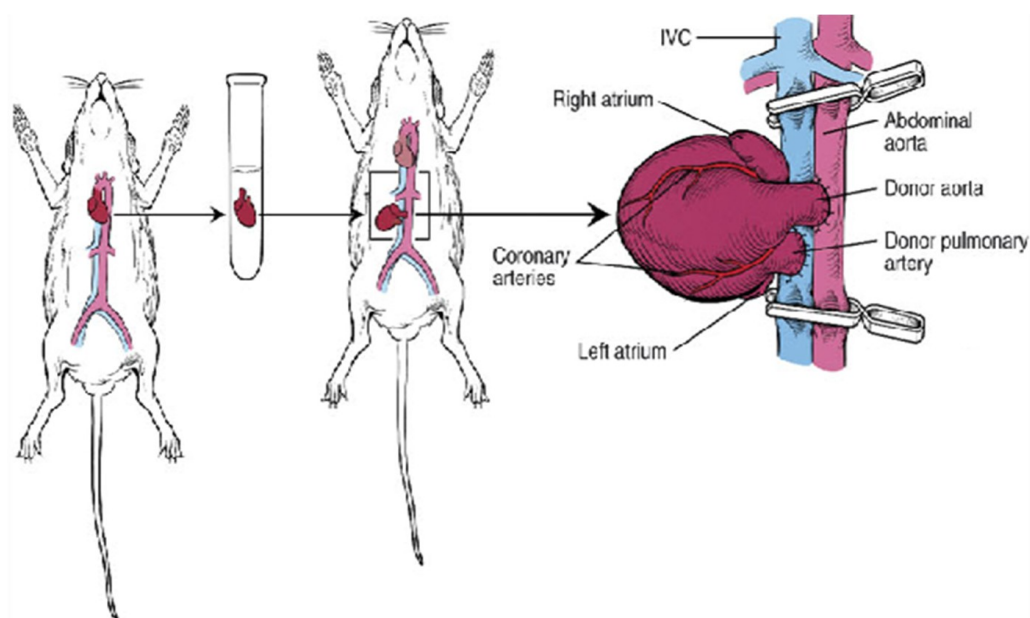


Figure 25: Schematic diagram of heterotopic heart transplantation in murine. The donor aorta and pulmonary artery are anastomosed to recipient aorta and IVC respectively. Figure taken from (Hasegawa et al., 2007).

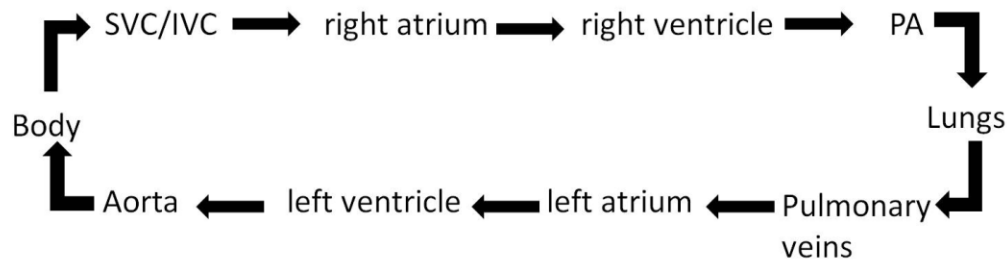
2.18.1 Preparation of the donor

In the donor mice, a long midline abdominal incision was made and donor hearts were perfused with heparinised saline (50U/ml, monoparin, CP Pharmaceuticals Ltd) using a 30G needle into the inferior vena cava (IVC). The animal was allowed to exsanguinate through the divided abdominal aorta. The incision was extended and the donor heart was arrested with cold saline. Superior vena cava (SVC), inferior vena cava (IVC) and azygous vein were ligated using 7-0 silk suture (Mersilk #W817, Ethicon). Aorta and the pulmonary artery (PA) were divided and cut. Finally, all the pulmonary veins were ligated with 7-0 silk and the heart was excised and stored on cold saline at 4°C.

2.18.2 Preparation of the recipient

Anaesthetized recipients were opened through a midline abdominal incision and the abdomen was retracted to the side in order to expose the abdominal aorta and IVC. The microvascular clip was applied proximally and distally to the anastomosing area followed by aortectomy and venectomy. The donor aorta was anastomosed to the recipient infra-renal abdominal aorta and the donor PA was anastomosed to the IVC using 10-0 prolene (#W2790, Ethicon). Removal of the distal clamp followed by proximal clamp allowed reperfusion of the donor heart through coronary arteries (Figure 26). After carefully assessing for any potential bleeding, the intestines were returned into the abdomen and the muscle layer and the skin was closed using 5-0 Vicryl (#W9982, Ethicon) and Mersilk (#W329H, Ethicon) respectively. Recipients were kept in the heating chamber with wet food to recover post-operatively. Ischaemia time, including warm and cold ischaemia, was 45min±10minutes. Function of the allograft was assessed by abdominal palpation daily until the heart rate slows down then by palpating twice a day when the strength of beating declined. Rejection was defined by complete cessation of the transplanted heart and confirmed by direct visualisation.

Blood flow in the native heart



Blood flow in the transplanted heart

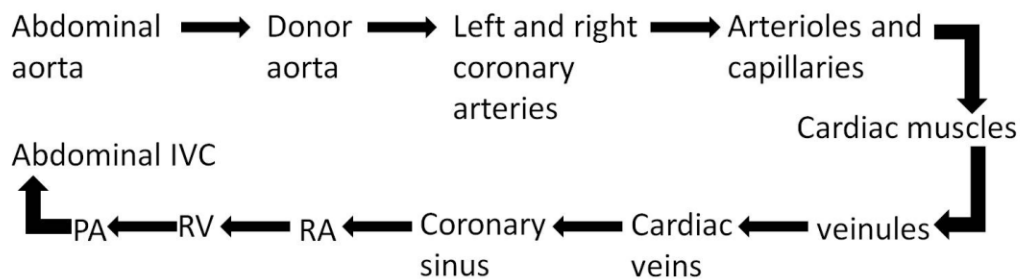


Figure 26: Schematic diagram comparing the blood flow in native heart and in heterotopically transplanted heart. In heterotopic heart transplantation, the donor heart is unloaded and is perfused through coronary arteries.

2.19 Echocardiography

Mice were anesthetized with 1-2% inhaled isoflurane and the chest of the mice was shaved and non-invasive echocardiographic measurements were performed using Ultrasound machine (Siemens). The function of the graft was identified by echocardiography and the hearts were scanned using motion mode (M-mode) and internal diameter at end systole and end-diastole were calculated.

2.20 Statistics

Statistical calculations were performed using GraphPad Prism® V.5 (GraphPad Software, LaJolla). Comparison of only two groups was performed with unpaired t-test. In order to compare three or more groups analysis of variance (One way ANOVA) and Bonferroni post-testing (compare all pairs of columns) was performed while two way ANOVA Bonferroni post-

testing was used to compare two factors. $P < 0.05$ was considered statistically significant with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter 3: Characterisation of animal model

3.1 Introduction

Hsp-27 is a small heat shock protein expressed ubiquitously and highly conserved across species, suggesting it plays a vital role. Hsp-27 plays a major role as a molecular chaperone facilitating transport, folding and assembly of polypeptides. Hsp-27 is a 27kDa protein in human and a 25kDa protein in rodents. Studies in mice have shown that hsp-27 protects hearts and neurones from I/R injury *in vitro*. Our group have previously studied cardiac biopsies of long term patients who have undergone cardiac transplantation and showed that there was a 20-fold increase in expression of hsp-27 in hearts of patients who did not develop cardiac allograft vasculopathy suggesting that hsp-27 could have a protective effect in the development of cardiac allograft vasculopathy. Therefore in order to understand the effect of hsp-27 in transplant rejection, we used transgenic mice overexpressing human hsp-27. The transgenic mice were created in the strain B10.A, using a transgene containing human hsp-27 cDNA under the control of β -actin promoter and cytomegalovirus enhancer. Transgenic mice were identified and characterised. This chapter describes the characteristics of transgenic and litter-mate controls.

3.2 Identification of transgenic mice

B10.A transgenic mice overexpressing human hsp-27 were initially obtained from Professor Dominic Wells, Charring Cross Medical School. Actually a hemagglutinin (HA) tag was placed in contiguous with the transgene in order to trace the expression of the transgene. The transgenic mice were generated by microinjecting the transgene purified linearised cDNA of human hsp-27 into pronuclei of fertilized eggs from C57BL/10 x CBA/Ca mice which were transferred to pseudo-pregnant recipients. These mice were then bred for nine generations with littermate control B10.A, selecting for hsp-27 positive mice at each generation. These mice were then bred at our own facilities using PCR to detect the presence of the transgene in offspring of B10.A littermate control (LC) male and B10A transgenic female.

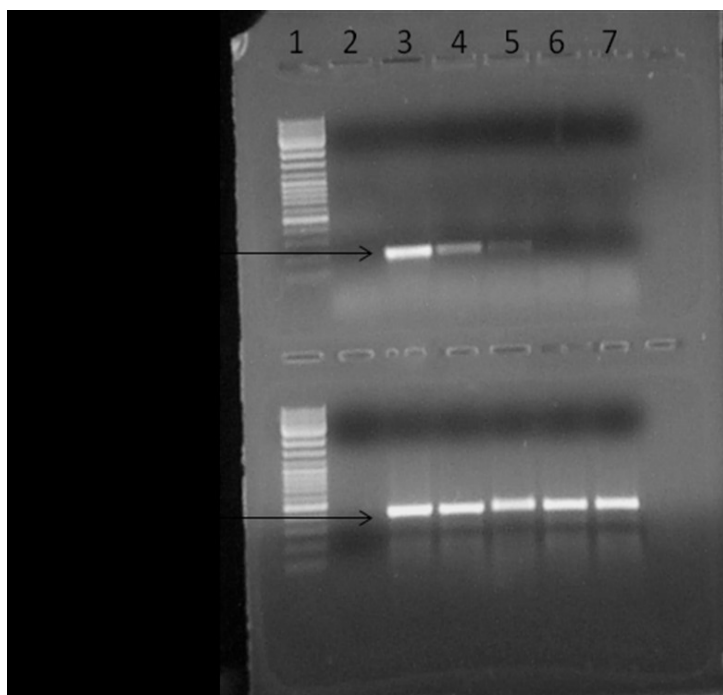


Figure 27: identification of transgenic mice. Panel A shows amplification using hsp-27 primers and PKC, an internal control was run in Panel B. To distinguish hsp-27 transgenic mice, the genotyping PCR reagents (lane 4 to 7) were run alongside amplicon of known transgenic mouse DNA (lane3) and negative control of water only (lane 2). The size of the fragments was determined using 100bp DNA Ladder Plus run in lane 1. Lane 4 and 5 of Panel A shows positive band while lane 6 and 7 shows negative bands.

A typical 1-2% gel, stained with ethidium bromide of PCR genotyping reagents is shown in Figure 27. The genomic DNAs from tail extracts amplified by PCR were run in lanes 4 to 7. Panel A shows DNA amplified using human hsp-27 primers from 5 mice and panel B shows amplification of DNA primed with protein kinase C (PKC). PKC is a house keeping gene that was used to confirm the presence of DNA in PCR samples. As shown in panel B of the gel photograph, PKC was positive for all samples (presence of a band at 500bp) with the exception of the negative control that had no band (lane 2). Thus, it confirms the presence of DNA in each sample with the exception of the negative control. Panel A of the gel shows presence or absence of a single strong band at 200bp in lane 4 to7, which were identified using molecular markers run in lane 1. Amplicon of known transgenic mouse DNA was run in lane 3. Comparison of the prominent band present in lane 3 with the bands present in lane 4 or 5

confirms that hsp-27 was present in those extracts. On the other hand, there was no band present in lane 6 and 7. Therefore, human hsp-27 was not expressed in the mice from which these extracts were extracted.

3.3 Widespread expression of HA in Transgenic mice

To explore the tissue distribution of human hsp-27 in transgenic mice, organs were removed from transgenic mice and their littermate controls for analysis by SDS-PAGE and western blot. Figure 28 shows representative data of western blots probed with monoclonal anti-HA and anti-hsp-25 antibody. The protein HA is undetectable in the heart (well 1), lung (well 2), liver (well 3), kidney (well 4) or spleen (well 5) of negative B10.A LC. However, a prominent band in the heart (well 6) and a less prominent band in the lung (well 7) was detected in transgenic mice. The expression of the transgene could not be detected in transgenic liver, kidney or spleen (wells 8, 9 and 10 respectively). On the other hand, widespread expression of the endogenous protein (hsp-25) was seen in heart, lung and spleen of both transgenic and LC mice as well as in LC kidney. But it was not expressed in the kidney of transgenic and in the liver of both transgenic and littermate controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a 37kDa enzyme that is constitutively expressed at high level in every cell (Gilliand G, 1990). In this study, the house keeping gene, GAPDH was used as an internal control to check for equal loading of the protein. As shown in Figure 28, the expression of GAPDH was comparable in various organs examined; suggesting that equal amount of protein was loaded in each well during SDS-PAGE electrophoresis.

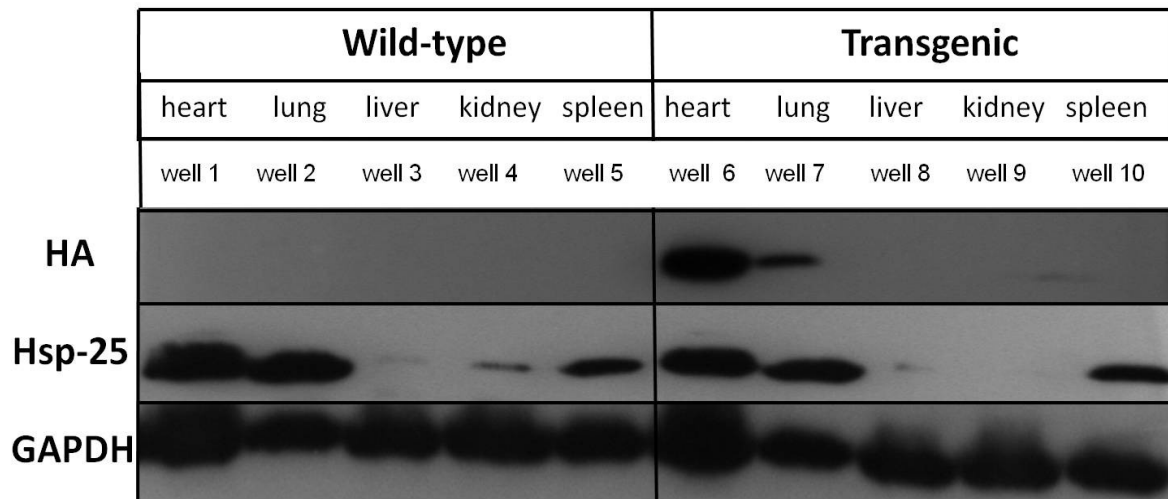


Figure 28: Human hsp-27 is present in transgenic mice while endogenous hsp-25 is present in both transgenic and littermate control. HA: western blot probed with antibody directed against the HA tag. Hsp-25: western blot using antibodies for detection of hsp-25. Proteins present in the heart, lung, liver, kidney and spleen of control littermate (well 1, 2, 3, 4 and 5 respectively) and transgenic (well 6, 7, 8, 9 and 10) were separated by SDS-PAGE, and transferred to nitrocellulose membrane by western-blotting. These were then probed with antibody directed against HA, hsp-25 or the internal control GAPDH followed by an HRP conjugated secondary antibody. Bound antibodies were detected using ECL.

3.4 Overexpression of hsp-27 in cardiomyocytes.

CAV is characterised by endothelial and SMC proliferation and SMC migration. Acute rejection is characterised by destruction of cardiac myocytes. It is therefore essential to understand which cells of the heart express the transgene. The identification of hsp-27 in different cell types is important to understand the function of hsp-27 and appreciate how it might be involved in cardiac rejection. In order to demonstrate the typical distribution of hsp-27 in mouse heart, hearts procured from transgenic and LC mice were subjected to immunohistochemistry as described in materials and methods. Transverse cardiac sections of 7µm thickness were labelled with HA antibody followed by a biotinylated anti-rat Ig, as secondary antibody. Cardiac sections were examined for transgene expression by immunocytochemistry (Figure 29). Photomicrographs of the heart demonstrated expression of HA in cardiomyocytes of hsp-27

transgenic mice, whereas littermate controls did not show expression of the tag (expression of HA in negative littermate mice heart was similar to the negative control which was stained with a non-specific antibody).

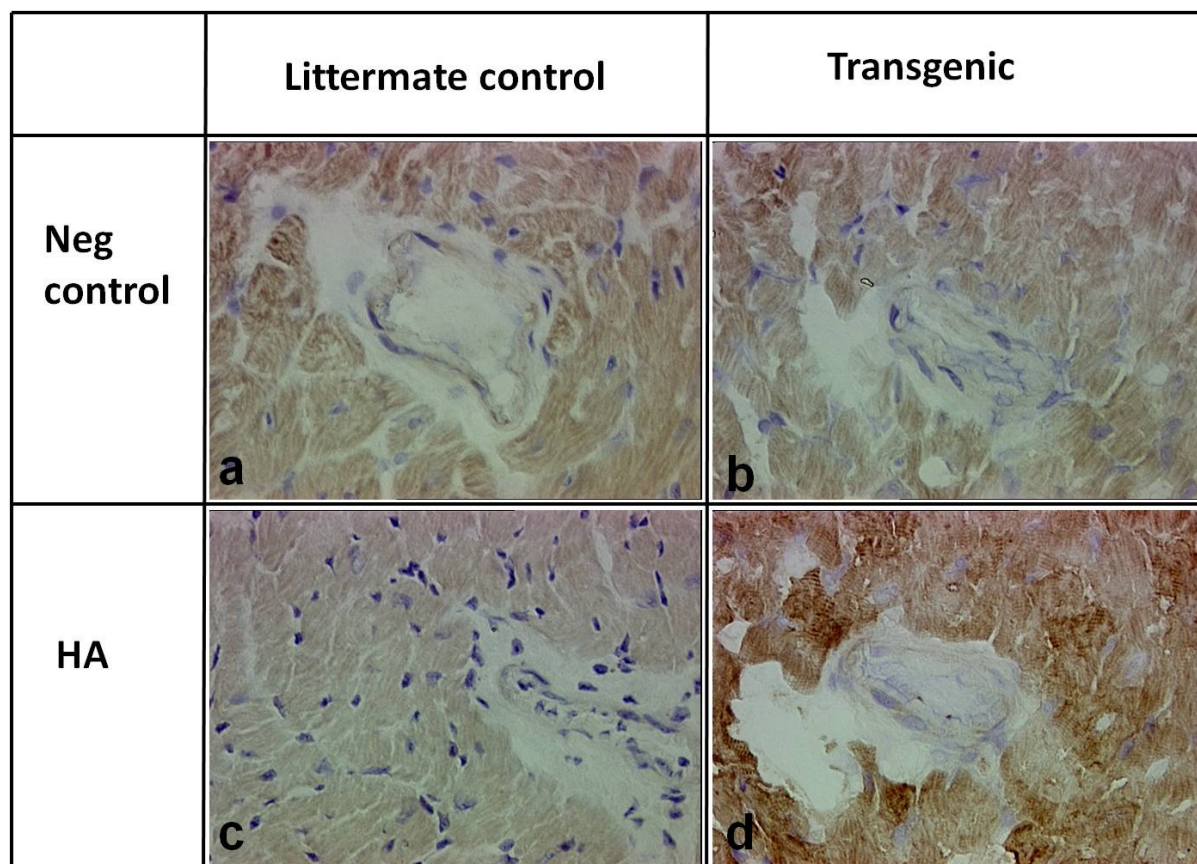


Figure 29: HA was expressed in cardiomyocytes of transgenic mice. Immunoperoxidase staining of cardiac tissue from hsp-27 transgenic and LC mice visualized using light microscope with the magnification x20. HA was overexpressed in cardiomyocytes of transgenic heart compared to LC. LC heart stained with control MAb (A), transgenic heart stained with control MAb (B), LC heart stained with MAb to HA (C) and transgenic heart stained with MAb to HA (D).

3.5 Overexpression of hsp-27 in smooth muscle cells

The aorta is composed of tunica intima (endothelial cells), tunica media (smooth muscle cells) and tunica adventitia (collagen fibres). Immunohistochemistry was carried out on transgenic and LC aorta to investigate whether hsp-27 was present in smooth-muscle cells and endothelial cells using antibody to HA. The histological tissue sections were stained with antibody to CD31

to demonstrate the presence of endothelial cells. Figure 30 shows that HA was expressed in transgenic SMC compared to LC mice. However, the endothelial cells identified by CD31 were not stained positively by HA. Therefore, it was not possible to detect HA tagged hsp-27 in the endothelial cells of transgenic or littermate control mice.

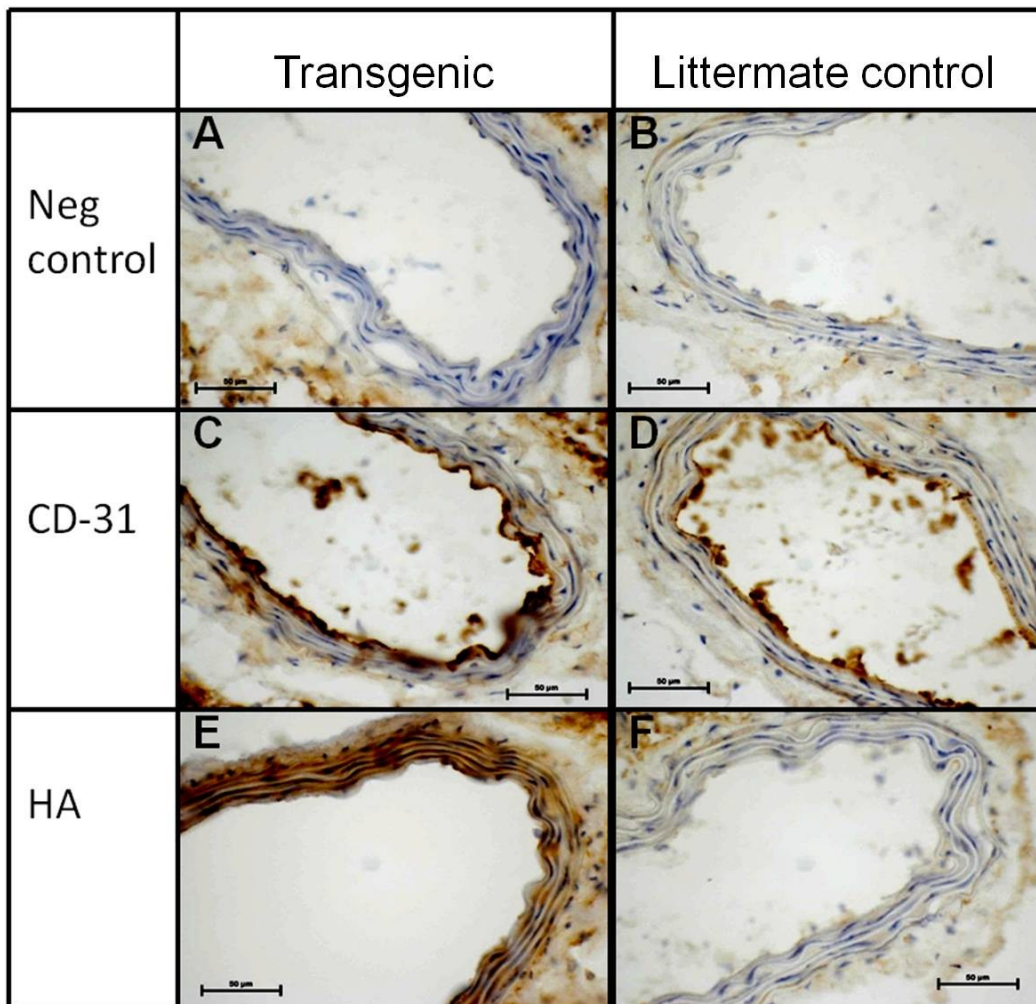


Figure 30: HA is expressed in SMC of transgenic mice. Immunoperoxidase staining of aorta from transgenic and LC mice were visualized under light microscopy at magnification x40. Positive staining of CD31 shows the presence of endothelial cells. HA is expressed in transgenic SMC however no positive staining was detected in LC-SMC or endothelial cells from either LC or transgenic mice. Panels show hearts from transgenic and LC mice stained with control MAb (A, B), transgenic and LC hearts stained with MAb to CD31 (C, D) and transgenic and LC hearts stained with MAb to HA (E, F).

3.6 Secretion of hsp-27

As shown above, hsp-27 is expressed in transgenic cardiomyocytes and SMC. In order to characterise the transgenic mice further, ELISA was used to detect hsp-27 in sera. Analyses of sera collected from transgenic and littermate control mice revealed that transgenic mice secreted hsp-27, as shown by the high level of hsp-27 present in their sera. However no hsp-27 was detected in sera from LC mice (Figure 31).

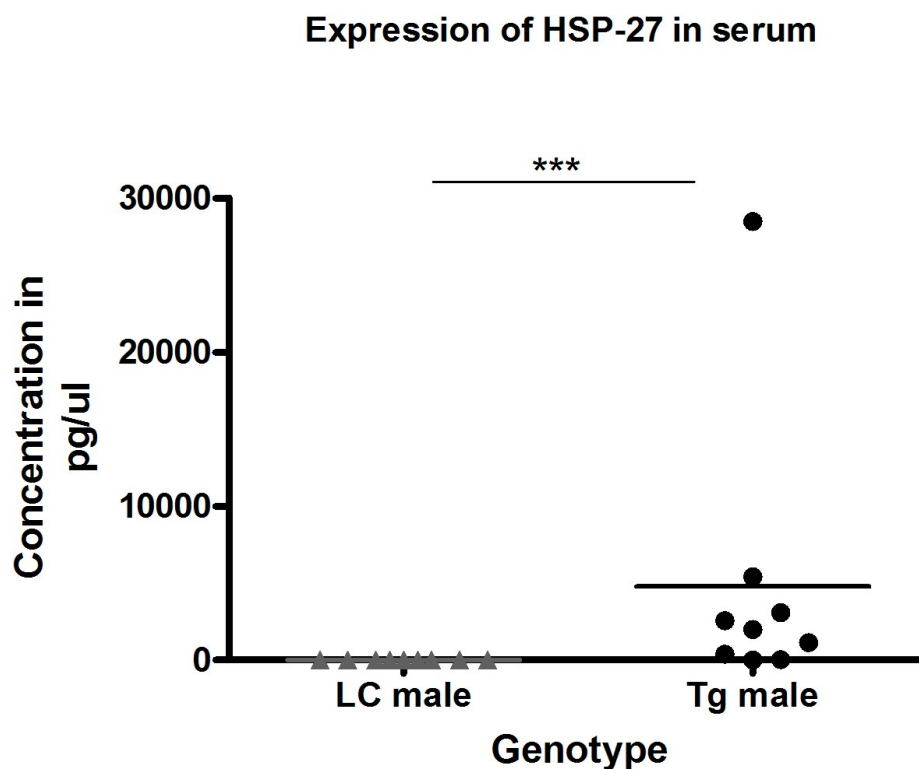


Figure 31: Detection of hsp-27 in the serum. Sera collected from hsp-27 transgenic (n=8) and their littermate controls (n=8) were analyzed by ELISA for the presence of hsp-27. Experiments were performed in duplicate and chromogenic results were read on a spectrophotometer at OD450. The concentration of hsp-27 was determined using the standard curve provided by the manufacturer (Calbiochem). The horizontal bar represents the mean $\pm$ SEM (** $p < 0.001$).

3.7 Discussion

3.7.1 Identification of transgenic and littermate controls

Genetically engineered mouse models have been widely used in biomedical research for studying function of genes *in vivo* and thus understand the mechanisms underlying human diseases. In human studies, expression of hsp-27 has been associated with protection from CAV (De Souza et al., 2005). We therefore utilized a human hsp-27 overexpressing transgenic line to examine the effect of hsp-27 on graft rejection. The purpose of this study was to characterise the transgenic animals. In fact, all studies are dependent on correct genotyping of these animals. Hence, genotyping accurately the transgenic mice is vital for our studies in order distinguish the transgenic animals from their control littermates. Using PCR probed with hsp-27 primers, we were able to identify transgenic (expressing hsp-27) from littermate controls. We showed that approximately 1/2 of the offspring were hsp-27+ as expected according to Mendelian law.

3.7.2 Localisation of hsp-25 and hsp-27 expression

We have shown that HA, *i.e.*, human hsp-27 is overexpressed in heart and lung of transgenic mice compared to littermate control (Figure 28). The study of Akbar et al, using hsp-27 overexpressing transgenic mice is in accord with our results and they showed that hsp-27 was also expressed in kidney, liver and brainstem of transgenic mice (Akbar et al., 2003). By western-blot and immunohistochemistry, they showed widespread expression of hsp-27 in the brain including cerebrum and hippocampus.

It is interesting to note that organ and tissue distribution of transgene varies between the two models. Our mice were produced from the C57BL/10 x CBA mice used by Akbar et al., but they were back crossed for 9 generations with B10.A mice. It is important to underline that the transgenic mice in both studies were created using β -actin promoter and CMV enhancer. This combination of promoter and enhancer has been shown to result in widespread expression of

the transgene (Ikeguchi et al., 2004; Okabe et al., 1997; Sawicki et al., 1998). However, in our study we did not observe expression of the transgene in liver, kidney or spleen of transgenic mice (Figure 28). Since the generation of the founders, the transgenic animal have undergone many back-crossing and breeding cycles. Therefore, it is possible that the expression of transgene could have been lost with the breeding cycle or mutated with time. Indeed, lack of persistent transgene expression may be due to decline in the expression of the promoter (Argyros et al., 2008). On the other hand, DNA methylation of the promoter could also be responsible for the absence or decrease in expression of the transgene (Doerfler, 2005; Palmiter et al., 1982). In addition, the variation in the level of expression of transgene in heart (highly expressed in the heart) and lung could also be explained by DNA methylation (Kearns et al., 2000). Interestingly, previous studies have shown that the host immune system, particularly IFN- γ response against the vector, inhibit the transcription of CMV promoter (Gribaudo et al., 1993; Kropp et al., 2011). Moreover, the strain used in our study (B10.A) is different from the strain used in Akbar et al's paper (2003), suggesting that the differences in strain are likely a contributing factor to the differences observed in these studies.

3.7.3 Expression of hsp-27 in the heart

Although, similar to our study, Akbar et al (2003) showed predominant expression of hsp-27 in the heart, they did not investigate the expression of the transgene in the various cellular compartments of the heart. IHC studies showed that hsp-27 is localized in cardiomyocytes (Figure 29) and SMC (Figure 30) of transgenic heart suggesting a potential role of cardiomyocytes and SMC (through hsp-27) in protecting against both acute rejection and CAV. Interestingly, the concentration of hsp-27 is higher in myocardium and the increased expression of hsp-27 in cardiomyocytes protects against ischaemia injury (Scheler et al., 1999). In fact, study of Jozefowicz-Okonkwo et al validates this concept and they discuss the fact that hsp-27 is not only present in myocardium but could also be produced by endothelial cells and SMC (Jozefowicz-Okonkwo et al., 2009). In this respect, several groups have reported the presence of hsp-27 in human cardiomyocytes, endothelial cells and SMC (De Souza et al., 2005; Lutsch

et al., 1997; Robinson et al., 2010). Immunohistochemistry of biopsies from long term cardiac patients free from cardiac allograft vasculopathy showed expression of hsp-27 in cardiac myocytes, endothelial and SMC (De Souza et al., 2005). Similarly, Robinson et al investigated cardiac biopsies from heart transplant donors and as well as patients with ischaemic heart disease, and they showed by western blot as well as immunohistochemistry cellular expression of hsp-27 and phosphorylated hsp-27 in smooth muscle cells and endothelial cells (Robinson et al., 2010) Our study has also shown expression of transgene in cardiomyocytes and SMC of aorta. Surprisingly, we did not find hsp-27 in endothelial cells. One possible explanation for the fact we have not found hsp-27 in mouse endothelial cells is that the endogenous protein hsp-25 could be expressed by endothelial cells, but the transgene (hsp-27) was not. We have not looked for the expression of hsp-25 in endothelial cells of mice. It would however be interesting to isolate the endothelial cells from transgenic mice and stain with HA tagged hsp-27 to confirm whether hsp-27 is present in endothelial cells.

3.7.4 Secretion of hsp-27

Interestingly, hsp-27 is present in the serum of male transgenic mice. Other groups have also shown presence of hsp-27 in sera of transgenic mice (Rayner et al., 2008) and demonstrated by *in vitro* studies that hsp-27 is secreted by human macrophages following treatment with estrogen (Rayner et al., 2008). In addition, hsp-27 is also produced by monocytes and lymphocytes (Njemini et al., 2006). Considering that the spleen is full of monocytes, macrophages and lymphocytes, it is surprising that we did not detect expression of the transgene in the spleen of hsp-27+ mice. This may be explained by the loss of transgene by the spleen. However, the endogenous protein hsp-25 was present in the spleen of transgenic and littermate control mice.

As mentioned earlier, Rayner et al have shown secretion of hsp-27 is controlled by estrogen, since the secretion only occurred in female mice receiving a high fat diet. This group found negligible levels of hsp-27 in sera of transgenic male receiving a low fat diet. Interestingly, treatment with estrogen antagonist did not completely prevent hsp-27 secretion (Rayner et al.,

2009). In addition, it is important to note that hsp-27 is secreted by healthy coronary arteries and found in the blood of healthy individuals (Martin-Ventura et al., 2004; Park et al., 2006). Taken together, it suggests that there are estrogen-independent mechanisms by which hsp-27 protein is secreted, and these remain to be elucidated.

3.7.5 Summary

In summary the characterisation of the animal model showed that human hsp-27 was overexpressed in lung and heart, in particularly in cardiomyocytes and SMC. Moreover, it was present in the serum of transgenic animals. However the expression of transgene was absent in endothelial cells and spleen. Since cardiomyocytes and SMC are the major cells affected during acute and chronic rejections, this model could be used to understand the role of hsp-27 in transplant rejection.

Chapter 4: Role of hsp-27 in acute rejection

4.1 Introduction

Acute rejection following heart transplantation remains a major cause of first year morbidity and mortality despite the outstanding improvements in pharmacological therapy. Moreover, it affects short and long-term outcome after transplantation. Acute rejection is a complex immune-mediated response usually characterised by molecular, cellular and physiological changes resulting in infiltration of inflammatory cells including lymphocytes and macrophages in the myocardium leading to myocardial oedema and myocyte necrosis (Rocha et al., 2003). Moreover, these infiltrating cells produce cytokines and chemokines that influence the transplant outcome. On the other hand, non-immune factors including infections, pre-existing diseases, donor age and in particular I/R also contribute to allograft rejection (Crudele et al., 2011). In fact, hsp-27 is a heat shock protein that has been shown to be implicated in I/R injury and protect from cell death (Latchman, 2001; Yokoyama et al., 2001). However, the role of hsp-27 in pathogenesis is complex. Higher level of hsp-27 is associated with severe forms of cancer (Geisler et al., 1999) and Parkinson's disease (Renkawek et al., 1999). On the other hand, increased level of hsp-27 protects from temporal lobe epilepsy (Akbar et al., 2003; Ben-Ari, 1985) and Huntington disease (Perrin et al., 2007). Similarly, the level of hsp-27 expression was increased in cardiac transplant patients who were free from cardiac allograft vasculopathy (De Souza et al., 2005). However, so far no studies have explored the effect of hsp-27 in acute rejection. Therefore, the aim of this chapter was to explore the role of hsp-27 in acute rejection. In order to test this, we performed heterotopic heart transplantation using transgenic mice overexpressing human hsp-27 and we assessed time to acute rejection. The effect of over-expressed hsp-27 on cellular infiltration and antibody mediated rejection were also analysed.

4.2 Heart Transplant model

We accomplished heterotopic transplantation to produce a well characterised model of acute rejection. Our surgical success rate was 90% with an ischaemia time of 45min $\pm$ 10minutes

(including cold and warm ischaemia). Surgical success is defined by survival of the animal as well as the presence of heart beat in the abdomen (checked by palpation) for over 2 days post-operatively. This was also confirmed by echocardiography. Figure 32a shows the echocardiogram and Figure 32b the M-mode of the donor heart transplanted into syngeneic recipients. The variation in diastolic and systolic diameter shows that the heart is contracting.

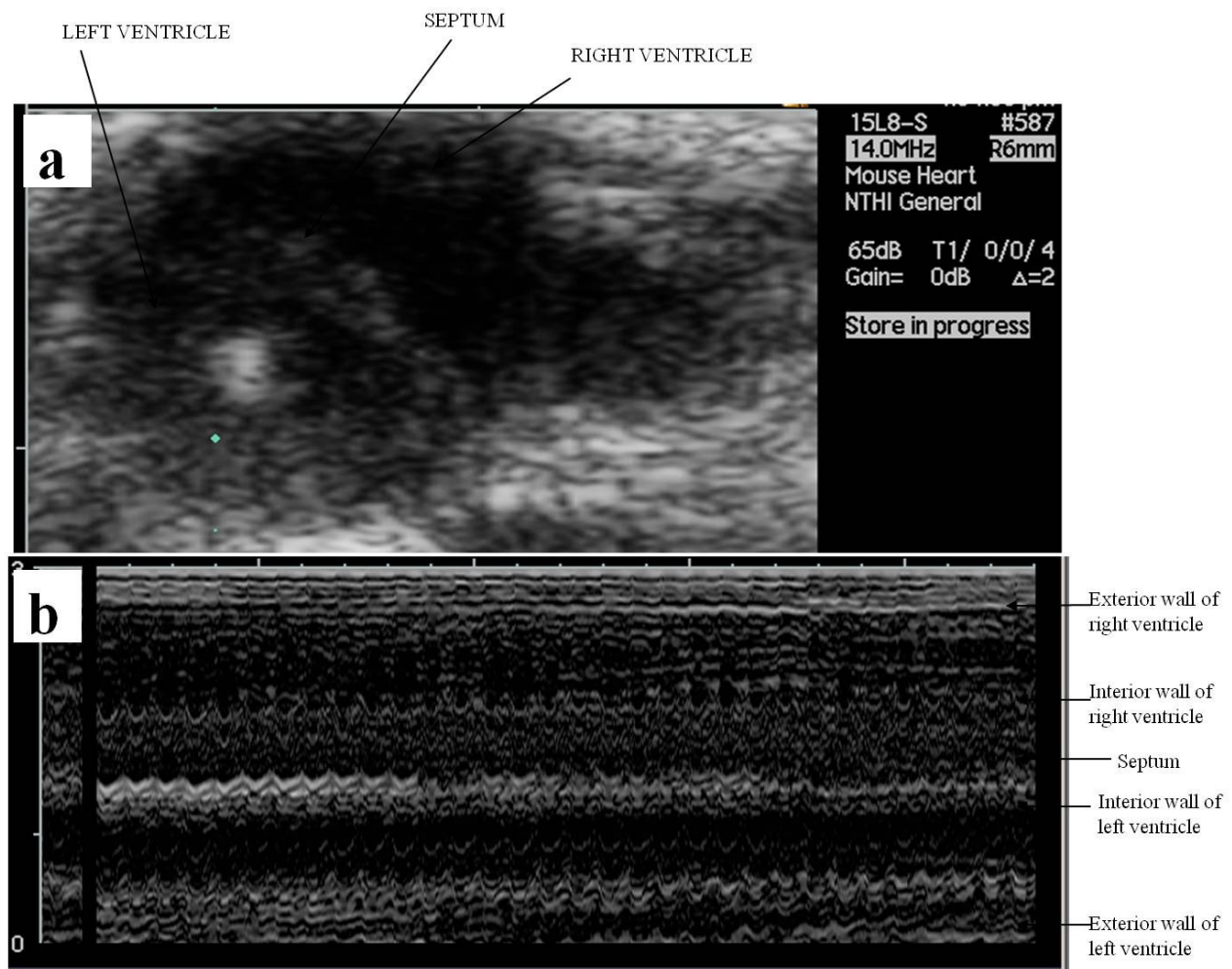


Figure 32: Representative echocardiography and M-mode of transplanted heart. Echocardiography and M-mode of transplanted heart were performed at 7 days post-transplant to assess the contraction of the graft thus the surgical success.

4.3 Survival of the allograft

We have already shown that hsp-27 was overexpressed in cardiomyocytes of transgenic animals (Chapter 3). Acute rejection is characterised by destruction of cardiomyocytes. In order

to determine the effect of hsp-27 on acute rejection, we performed heterotopic heart transplantation using B10.A transgenic or littermate controls as donors and C57BL/6 as recipients. The MHC of B10.A is $H-2^a$ and the MHC of C57BL/6 is $H-2^b$, representing a complete MHC-mismatch. Transplantation of B10.A transgenic or littermate controls into C57BL/6 recipients showed that the survival of transgenic allografts (35 days $\pm$ 10.37) was significantly prolonged compared to littermate control allografts (13.6 days $\pm$ 3.06). Syngeneic C57BL/6 recipients of C57BL/6 donors did not reject their hearts and were sacrificed at 8 weeks post transplantation (Figure 33).

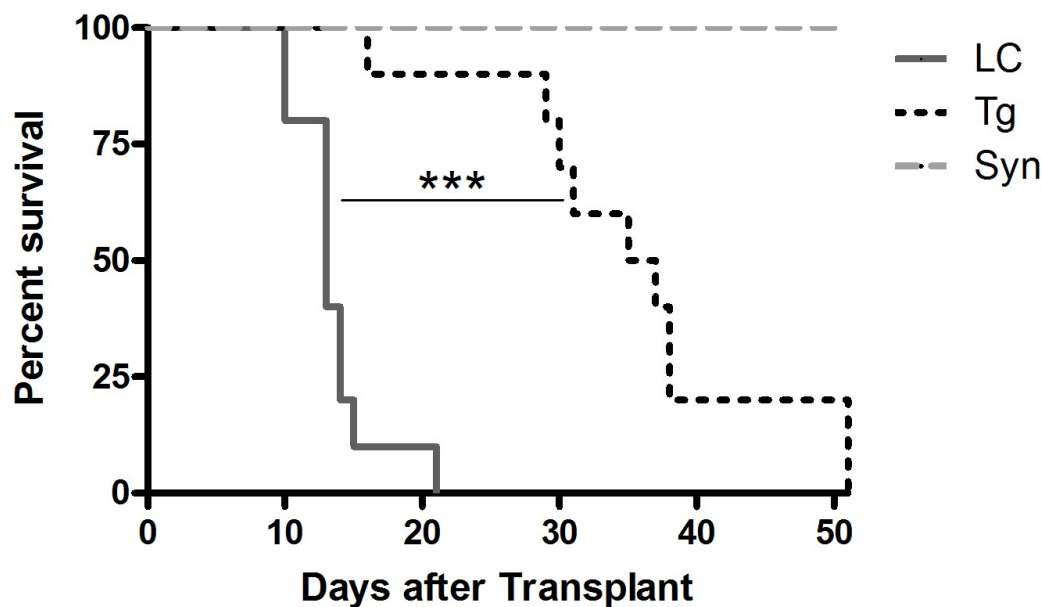


Figure 33: Hearts from hsp-27 transgenic and their littermate controls were transplanted heterotopically into C57BL/6 recipients. LC hearts (n=10) were rejected within 12-20 days while the survival of transgenic allografts (n=10) was significantly prolonged. The grafts from syngeneic donors were not rejected until the end of study (n=6). The survival data was analysed by log-rank test in Kaplan-Meier (***) $p < 0.001$.

4.4 Hsp-27 delays cellular infiltration

To investigate how hsp-27 could delay graft rejection, we studied infiltration of inflammatory cells in allografts by RT-PCR at 2, 5 and 12 days post-transplant (Figure 34).

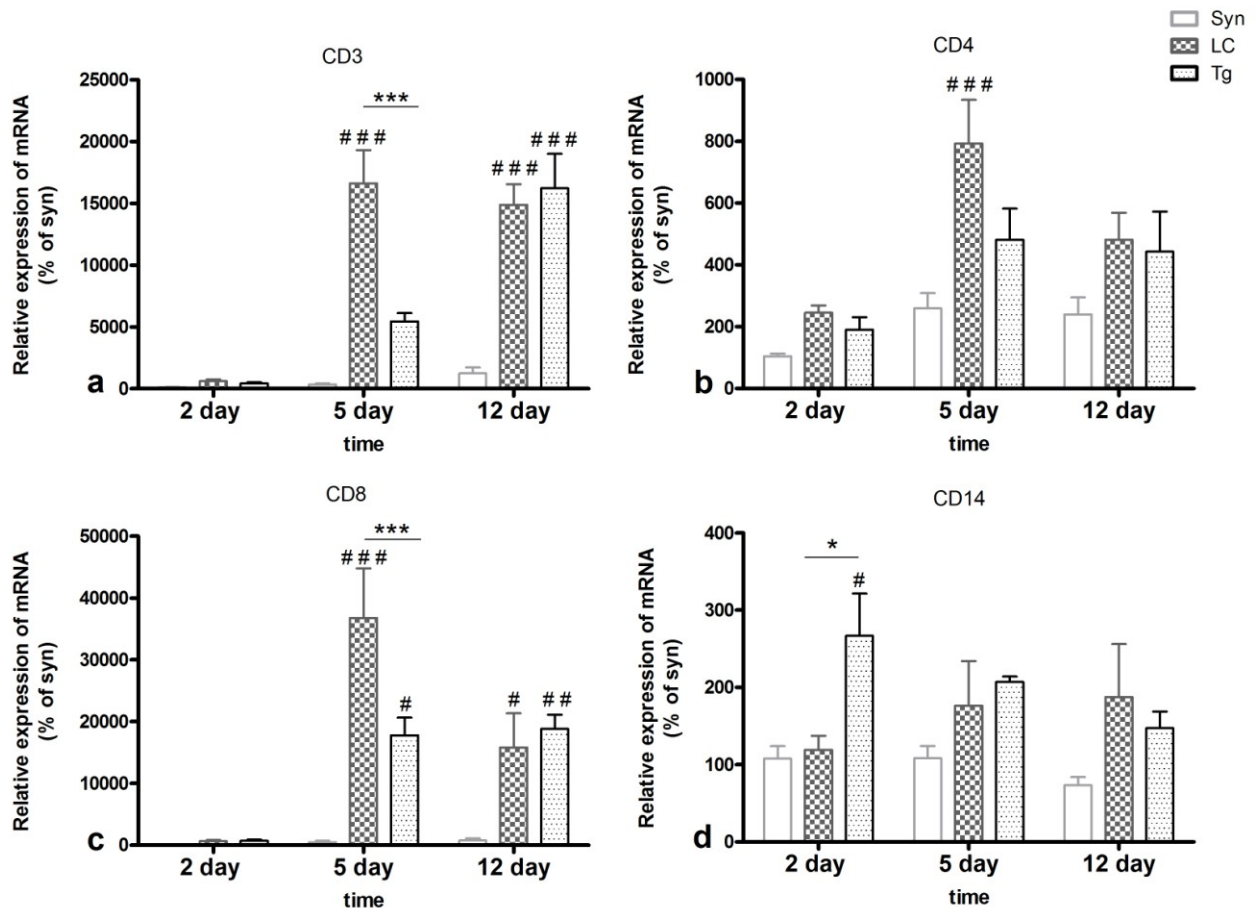


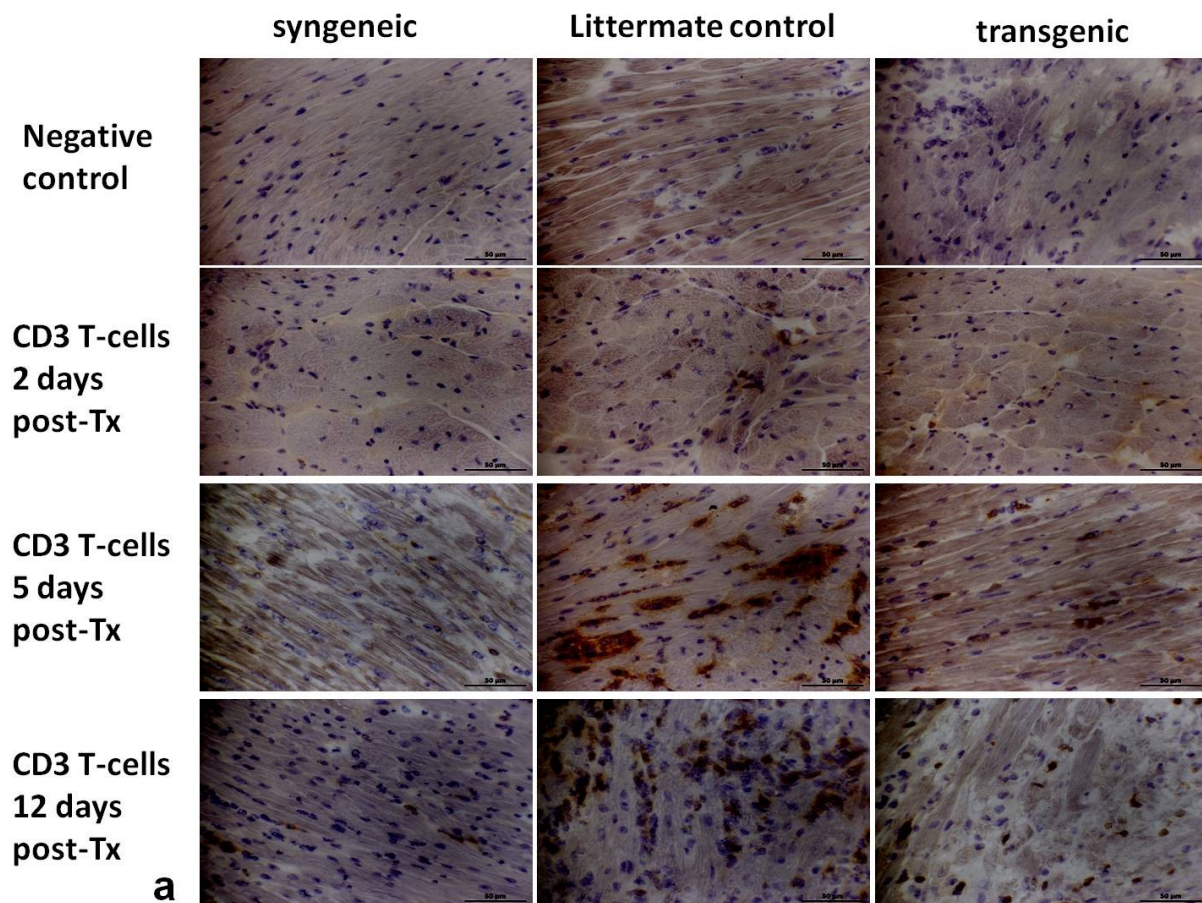
Figure 34: Effect of hsp-27 on infiltrating cells following heart transplantation: Transgenic and littermate control allografts were sacrificed at day 2, 5 and 12 post-transplantation and assessed for infiltration of CD3+ (a), CD4+ (b) and CD8+ (c) T-cells as well CD14+ (d) cells by RT-PCR. The Y-axis shows relative expression of mRNA as a percentage of that present in syngrafts. Transgenic allograft has less infiltrating cells compared to LC allograft. (* or # $p \leq 0.05$; ** or ## $p < 0.01$ and *** or ### $p < 0.001$, * Transgenic versus littermate control, # syngeneic versus littermate control or syngeneic versus transgenic $n=3-6$).

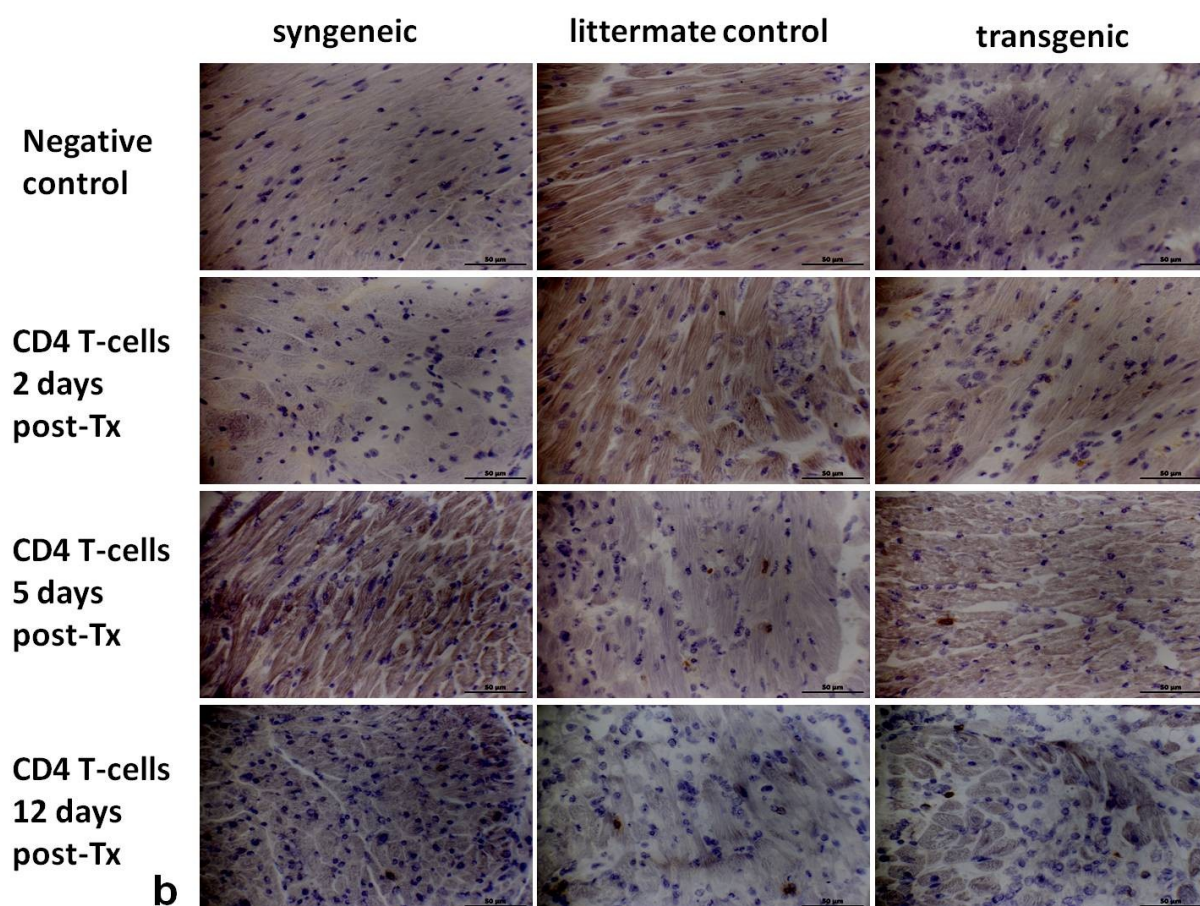
The mRNA expression for CD3, CD4 and CD8 were significantly higher in allografts compared to isografts. Allografts overexpressing hsp-27 showed significantly reduced expression of CD3 and CD8 mRNA at day 5 post-transplant compared to littermate control allografts (Figure 34a and c respectively). However, although not significant, the mRNA expression for CD3+ T-cells increased in transgenic hearts at 12 days post-transplant. On the other hand, hsp-27 did not have an effect on the infiltration of CD4+ T-cells: the expression of CD4+ T-cells was higher

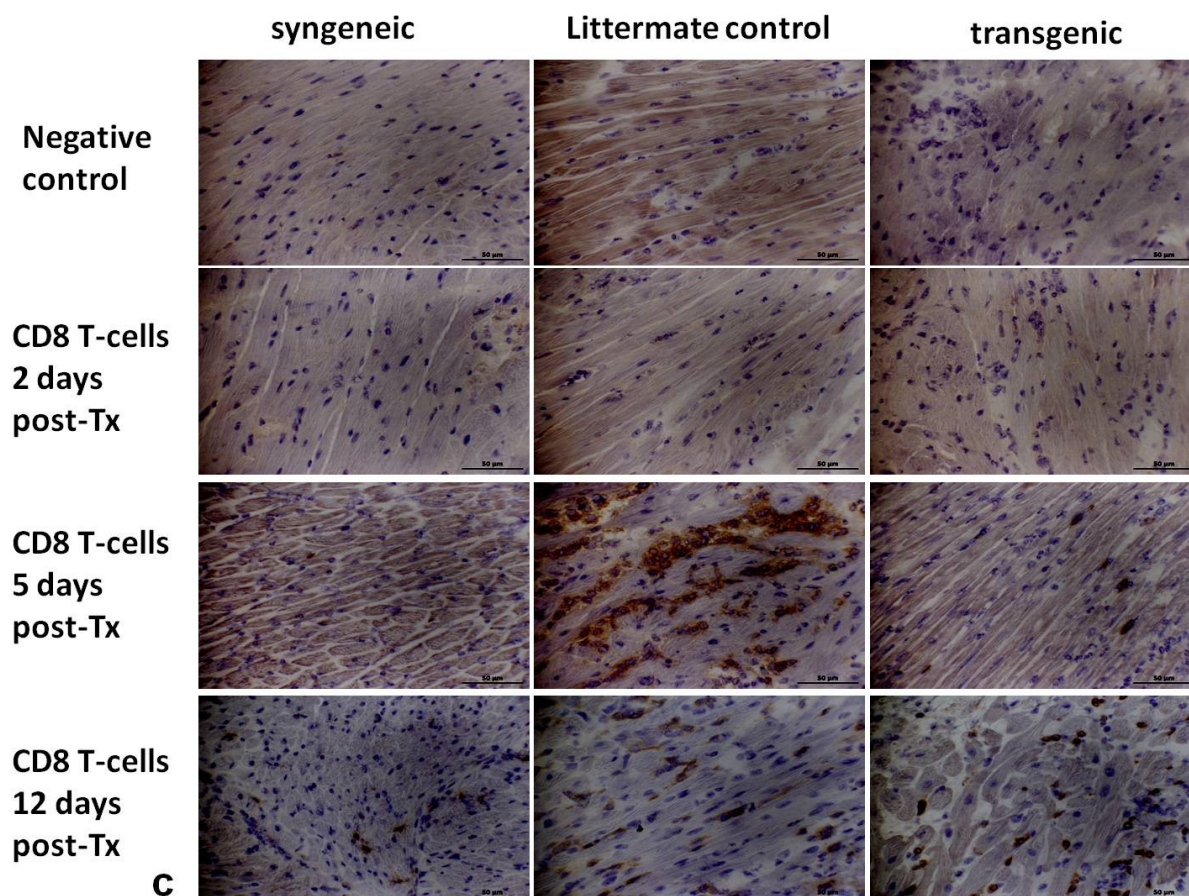
in littermate control allografts at 5days post transplant, but this was not significant (Figure 34b). The expression of mRNA for CD4+ T-cells were very low compared to CD3+ or CD8+ T-cells. It is interesting that the mRNA expression for CD14+ cells were significantly higher in transgenic allografts than in littermate control allografts or isografts at 2 days following transplantation (Figure 7d).

4.5 Expression of infiltrates at protein level.

In order to confirm the results obtained by RT-PCR, we looked for the presence of inflammatory infiltrates at protein level by immunohistochemistry. The sections of grafts collected at days 2, 5 and 12 were examined for the presence of CD3+ (a), CD4+ (b) and CD8+ (c) T-cells as well as monocytes/macrophages (d) population (Figure 35) using immunocytochemistry. Our results showed a progressive time dependent increase in cellular infiltrates. As shown by RT-PCR, immunohistochemistry confirms that there were more CD3+ as well as CD8+ T-cells in littermate controls at day 5 compared to allografts from hsp-27+ mice. There were relatively few infiltrating CD4+ T-cells in either littermate control or transgenic hearts compared to total CD3+ or CD8+ T-cells shown by immunocytochemistry. Of note, similarly to PCR results, CD3+, CD4+ and CD8+ cells were not detected in isografts. Importantly, there was a prominent CD11b+ monocytes/macrophages presence at day 2, day 5 as well as day 12 post-transplant in both transgenic and littermate control groups. Interestingly, infiltration of CD11b+ was also detected in syngrafts, but in much lower number compared to allografts. Immunohistochemistry data for CD3+ T-cells and CD8+ T-cells confirms RT-PCR results. The quantity of mRNA for CD4 gene was slightly higher in LC compared to transgenic, however IHC analysis stained with antibody to CD4 does not show such effect. Moreover, in apparent contrast with RT-PCR results (Figure 34d), IHC analyses (Figure 35d) appears to show that infiltration of monocytes increases progressively and there seemed to be high number of monocytes at day 12 in both LC and transgenic allografts. Unfortunately the interpretation of IHC data is limited due to poor quality of the images and the fact that cells have not been quantified. Interpretation of the immunocytochemistry is subjective.







C

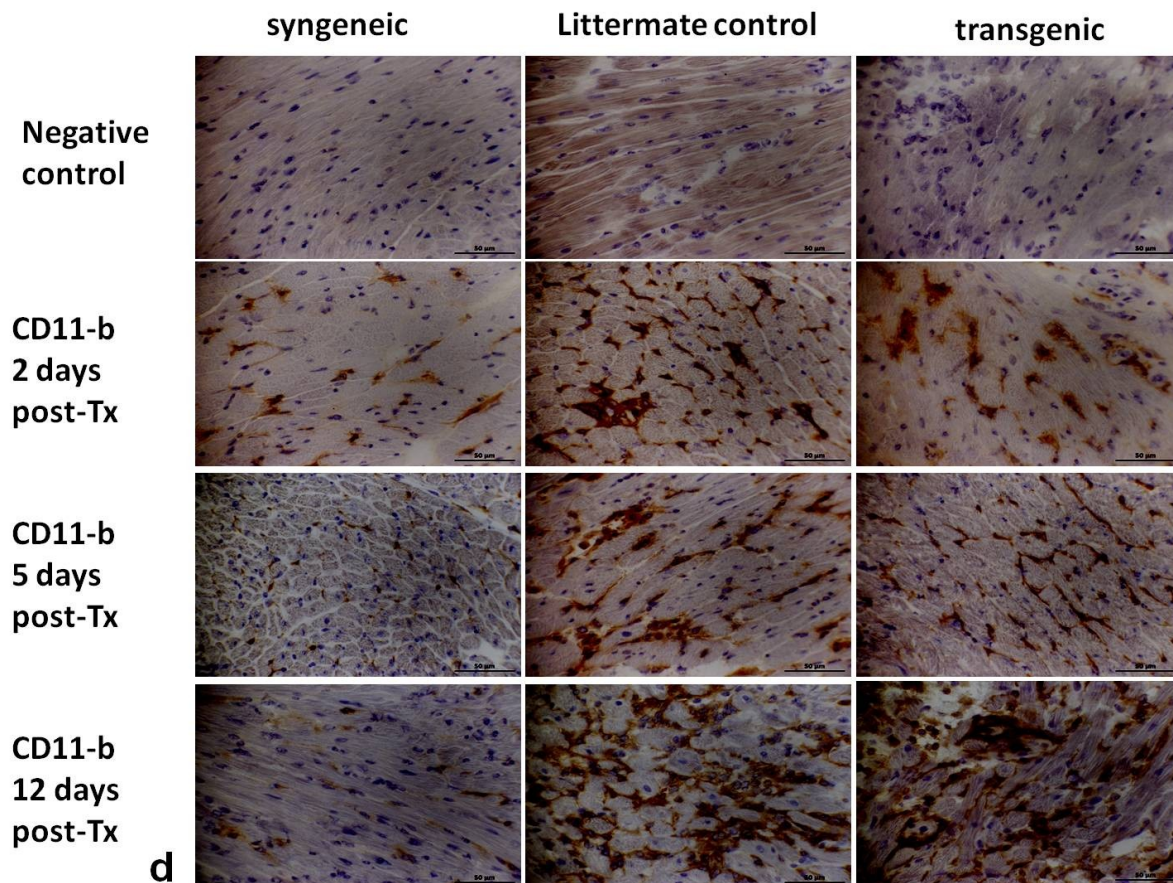


Figure 35: Representative section of cardiac allografts or syngrafts stained for the presence of CD3+, CD4+, CD8+ and CD11b+ cells. Cardiac allografts or syngeneic grafts collected at days 2, 5 and 12 after transplantation were embedded in OCT and cryo-sections of 7µm were obtained. Immunoperoxidase staining was used to identify infiltration of CD3+ (a), CD4+ (b) and CD8+ (c) T-cells as well as CD11-b+ (d) cells as described in Materials and Methods. Photomicrographs are representative data from 4-5 animals (magnification x40).

Immunohistochemistry was also performed to investigate the expression of HA tagged hsp-27 in the graft (Figure 36). As expected, HA was present at day 2, day 5 and day 12 only in transgenic grafts, thus confirming the genotype. Interestingly, HA, expression became more intense with time, but there were fewer cardiomyocytes in the graft. Areas of cardiomyocytes have become replaced by areas of fibrosis.

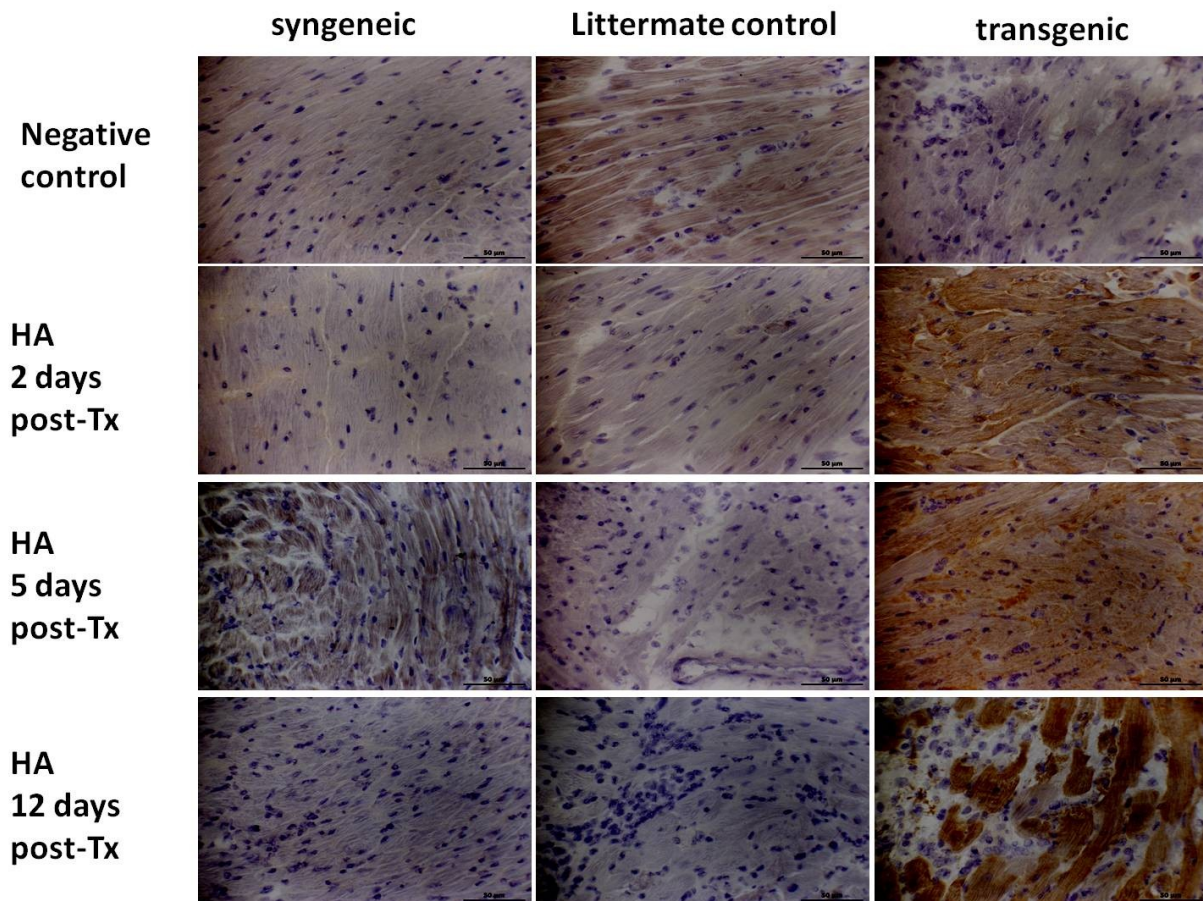


Figure 36: HA staining in B10.A and C57BL/6 cardiac tissue removed from C57BL/6 recipients on days 2, 5 and 12 after transplantation. The allografts and syngrafts were frozen in OCT and 7µm sections were obtained. These were then incubated with primary antibody to HA to visualise the presence of HA tagged hsp-27. It showed expression of HA in grafts obtained from transgenic donors compared to littermate controls or syngeneic donors. Photomicrographs are representative data from 4-5 animals (magnification x40).

4.6 Interaction of hsp-27 at molecular level

To further understand the effect of hsp-27 on graft rejection, we investigated the cytokines secreted by infiltrating cells. All cytokines tested (IFN-γ, IL-2, TNF-α and IL-1β) were significantly higher in allografts compared to isografts. Similar to CD3+ and CD8+ T-cells (Figure 34a and c respectively) the pro-inflammatory cytokine IFN-γ was significantly increased in littermate control allografts at 5 days following transplantation compared to transgenic allografts, but this effect did not persist (Figure 37a). The expression of IFN-γ was higher in

transgenics compared to littermate controls at 12 days post-transplant. In addition, we found that there was a small but significant increase in the expression of IL-2 at 12 days post-transplant in transgenics group compared to littermate controls (Figure 37b). The other pro-inflammatory cytokines TNF- α and IL-1 β did not differ between transgenics and littermate controls at any time point (Figure 37c and d respectively). On the other hand, the expression of IL-17 was below detection level (data not shown).

It is interesting to note that mRNA level of the anti-inflammatory cytokine IL-4 was increased at day 2 (along with CD 14 cells, Figure 34d) in transgenic hearts compared to littermate controls or isografts (Figure 38a). No significant changes were seen regarding the expression IL-5, IL-10 or TGF- β (Figure 38b, c and d respectively). However the expression of TGF- β in transgenic allografts or IL-10 in both littermate control and transgenic allografts were significantly different compared to grafts from syngeneic transplants. On the other hand IL-5 and TGF- β expression were very low compared to IL-4 or IL-10.

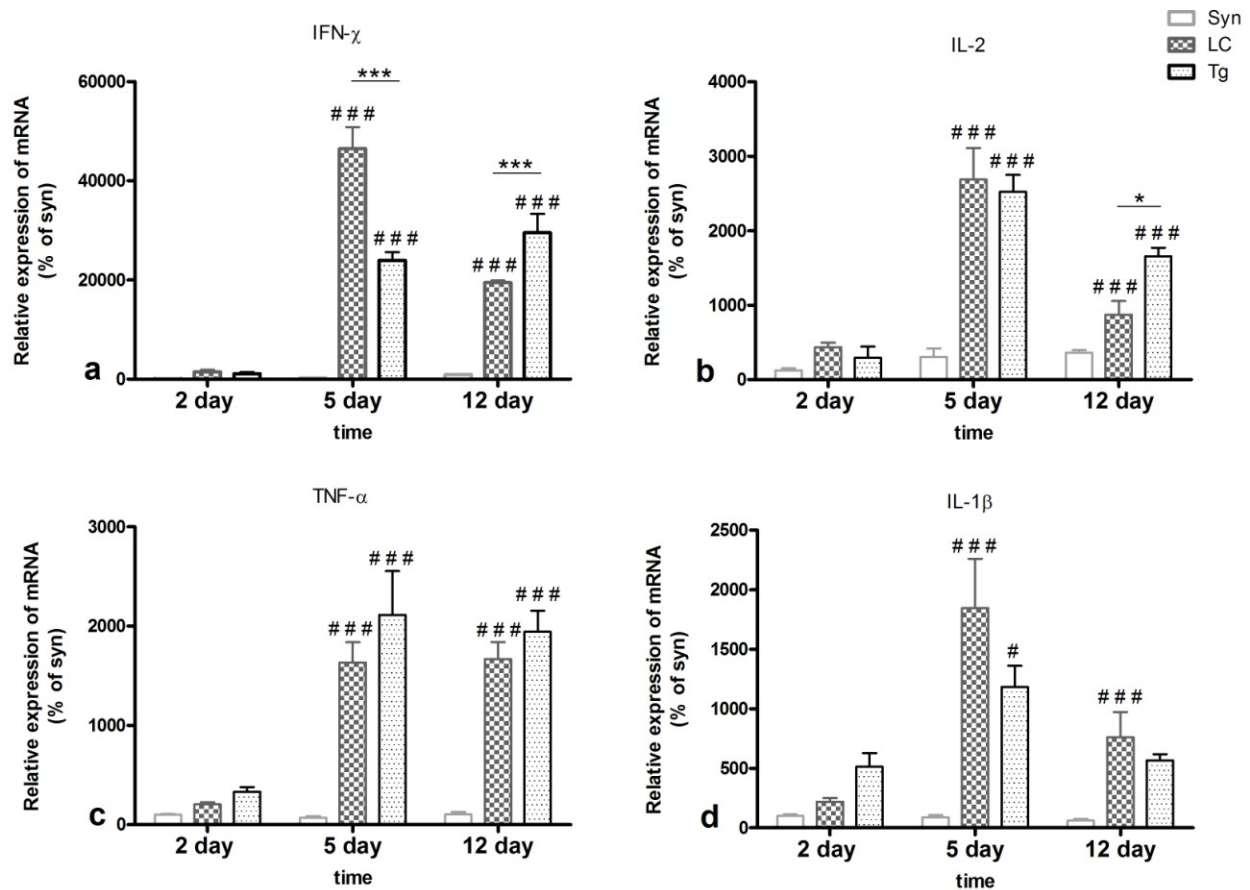


Figure 37: Effect of hsp-27 on infiltrating inflammatory cytokines following heart transplantation: Transgenic and LC allografts were sacrificed at day 2, 5 and 12 post-transplantation and cytokines expression of pro-inflammatory cytokines IFN- γ (a), IL-2 (b), TNF- α (c) and IL-1 β (d) were analysed by RT-PCR. The quantity of mRNA was normalised to the endogenous housekeeping gene 18s rRNA. Two-way Anova followed by Bonferroni post-test was used to calculate the statistical significance. (* or # $p \leq 0.05$; ** or # # $p < 0.01$ and *** or # # # $p < 0.001$, * Transgenic versus littermate control, # syngeneic versus littermate control or syngeneic versus transgenic $n=3-6$).

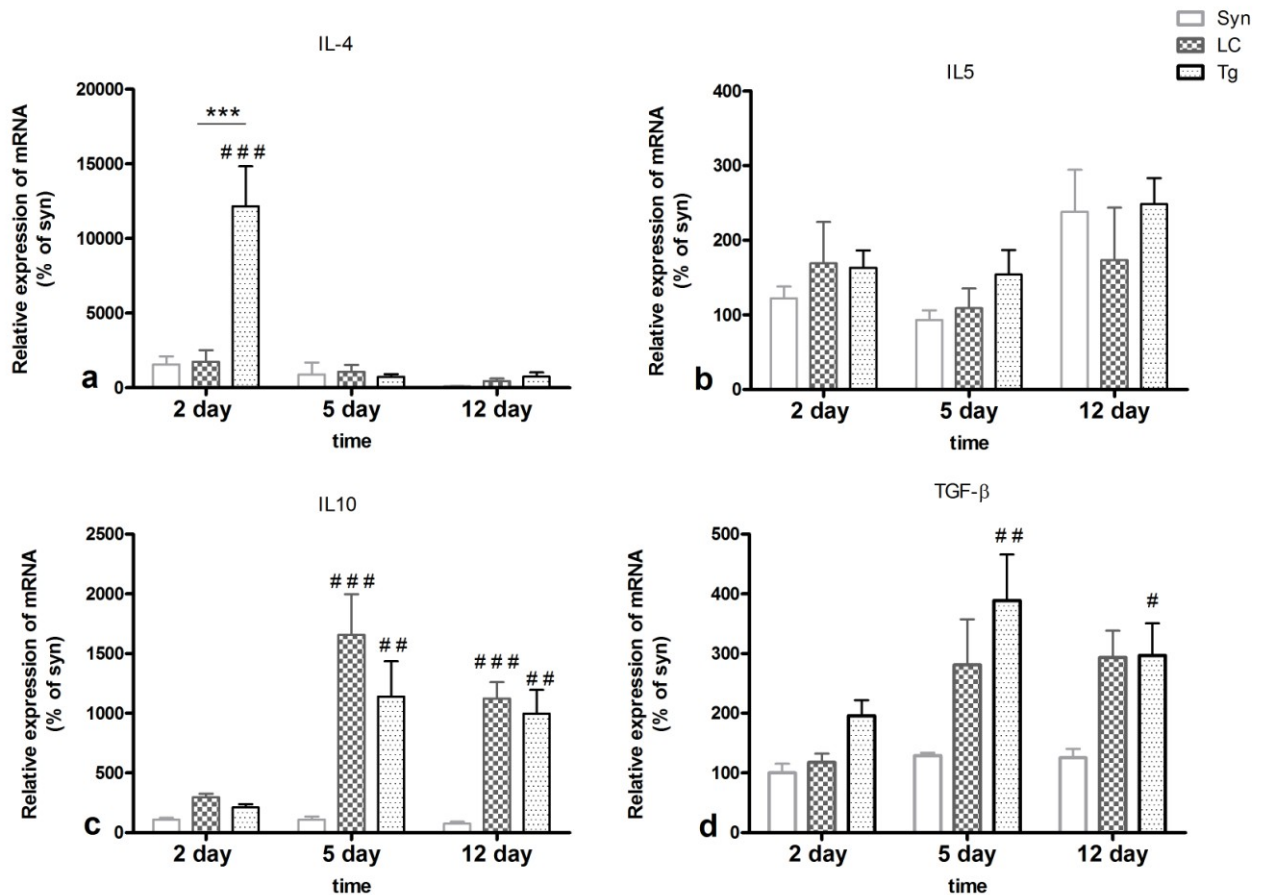


Figure 38: Effect of hsp-27 on infiltrating anti-inflammatory cytokines following heart transplantation: Transgenic and LC allografts were sacrificed at day 2, 5 and 12 following transplantation and cytokines expression of anti-inflammatory cytokines IL-4 (a), IL-5 (b), IL-10 (c) and TGF-β (d) were analysed by RT-PCR. The quantity of mRNA was normalised to the endogenous housekeeping gene 18s rRNA. Two-way Anova followed by Bonferroni post-test was used to calculate the statistical significance. (* or # $p \leq 0.05$; ** or ## $p < 0.01$ and *** or ### $p < 0.001$, * Transgenic versus littermate control, # syngeneic versus littermate control or syngeneic versus transgenic $n=3-6$).

4.7 Effect of hsp-27 on alloantibody

Alloantibody has been associated with poor graft survival and contributes to acute and chronic rejection. Therefore animals were sacrificed at day 2, day 5 or day 12 post-transplant and tested for presence of circulating antibodies in the serum by flow cytometry. No alloantibody response was detected at day 2 and day 5 post-transplant in both recipients of transgenic or LC grafts.

Indeed, the level of alloantibodies present in the serum from these animals were not significantly different from the mice that had a syngraft (Figure 39a and b respectively).

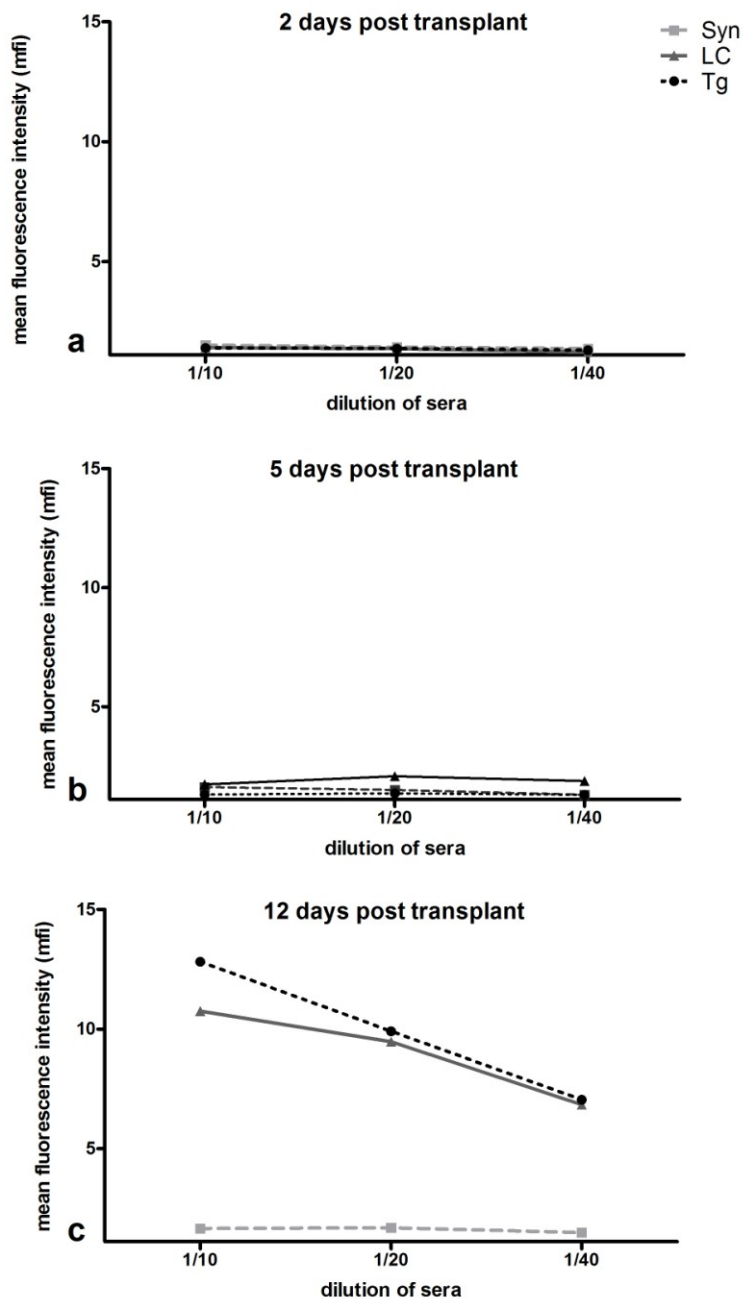


Figure 39: Presence of alloantibodies was measured by flow cytometry. B10.A transgenic or LC or C57BL/6 heart was transplanted into C57BL/6 recipient. Sera from recipients were collected at 2 days (a), 5 days (b) or 12 days (c) post-transplant and incubated with B10.A splenocytes at 1/10, 1/20 and 1/40 dilutions. The intensity of staining was analysed by flow cytometry and is shown as mean fluorescence intensity.

Interestingly, the level of alloantibodies was significantly elevated at 12 days post-transplant, in animal receiving transgenic or LC heart compared to the negative control that had a syngeneic heart (Figure 39c). However, alloantibody response was comparable at all time point between transgenic and LC, suggesting that hsp-27 does not regulate antibody response.

4.8 Discussion

Transplantation between MHC mismatched animals induces an immune response against alloantigens of the donor graft thus leading to graft rejection. The aim of this study was to understand the role of hsp-27 in acute rejection using a transgenic mouse model. We successfully established the model of acute rejection in our research centre. In order to fully elucidate the cardioprotective effect of hsp-27 on transplant rejection, we performed a heterotopic heart transplant using hsp-27 transgenic mice. Although the heterotopically transplanted heart is unloaded and the blood supply is low, the donor heart is still perfused with recipient blood (Korecky and Masika, 1990); hence the myocardium and the vessels are exposed to host immune system. Indeed, in our model the donor heart was perfused and contracting well.

Acute rejection is accompanied by cardiomyocyte destruction. We showed that hsp-27 was overexpressed in cardiomyocytes of transgenic mice. B10.A - C57BL/6 is a complete MHC mismatch model and thus the heart was expected to be rejected rapidly. A previous study using the same strain combination demonstrated that the time to rejection of the allograft was 12-14 days (Wasowska et al., 2001). We also reported that the survival of B10.A littermate control heart transplanted into C57BL/6 was about 14 days. Interestingly, transplantation of transgenic B10.A into C57BL/6 led to prolongation of allograft survival, demonstrating that hsp-27 delays graft rejection. This finding suggests that hsp-27 has a protective role on cardiac rejection.

4.8.1 Hsp-27 protects from cellular rejection

Acute rejection was previously thought to be a T-cell mediated process. Moreover T-cells are the principle components in the histological sections of allograft during acute rejection. Studies

showed that acute transplant rejection is characterised by a Th1 type inflammatory infiltrates (Hunt and Haddad, 2008). Our results are in accord with this. In fact, we showed that T-cells, *i.e.*, CD3+, CD4+ and CD8+ T-cells as well as CD14+ monocytes infiltrated the rejecting hearts since no such infiltration was seen in isografts at any time point. We thus showed that transplantation into complete MHC mismatch (B10A and C57BL/6) leads to infiltration of inflammatory cells. This is supported by another study where a complete mismatch model (BALB/c donors and C3H/He recipients) of cardiac transplant was used and reported that prolongation of graft survival is accompanied by decreased number of infiltrating CD4+, CD8+ and CD11b+ cells and lower expression of IFN- γ mRNA (Kosuge et al., 2006). Interestingly, we reported that there were more CD3+ as well as CD8+ T-cells in littermate controls at 5 days compared to transgenics. Thus suggesting that hsp-27 down-regulates inflammatory infiltration into the graft.

Moreover, CD14+ monocytes were significantly higher at day 2 in transgenic donors compared to LC donors. The literature suggests presence of sub-populations of monocytes with regulatory or pro-inflammatory properties. The monocytes are divided into subsets depending on the chemokine receptors and expression of specific surface markers. The monocytes known as Ly6C^{hi} (in mice) express Ly6C and CD11b together with high levels of chemokine receptor CCR2 but low level of CX3CR1. On the other hand Ly6C^{lo} express low level of Ly6C and CCR2 but high level of CX3CR1 (Shi and Pamer, 2011; Swirski et al., 2009). The subsets of monocytes also differ in their effector function since Ly6C^{hi} are shown to be pro-inflammatory with the production of TNF- α and iNOS while the Ly6C^{lo} has been shown to be involved in inducing tolerance and wound healing (Shi and Pamer, 2011). It is therefore possible that the large numbers of monocytes seen in transgenic grafts (Figure 34) are of an anti-inflammatory nature. Unfortunately, we did not distinguish the two types of monocytes population in our study due to lack of time. However, it would be interesting to FACS sort the monocytes population for Ly6C^{hi} (Ly6C^{hi}CD11b^{hi}) and Ly6C^{lo} (Ly6C^{lo}CD11b^{hi}) as described by Rivollier et al (Rivollier et al., 2012).

4.8.2 Immunomodulatory effects of hsp-27 in transplant rejection

It is well established that IFN- γ plays a significant role in graft rejection (Diamond and Gill, 2000; Koga et al., 1999; Rosenberg et al., 1990). It increases the antigenicity of the graft through induction of MHC expression on epithelial and endothelial cells thereby increasing the efficiency of antigen presentation. In addition, it increases the ability of antigen processing and presentation by APCs, and regulates leukocyte-endothelium interactions during inflammation (Bach et al., 1997; Boehm et al., 1997). In the present study, we reported that IFN- γ was markedly increased in littermate control grafts 5 days post-transplant. IFN- γ is secreted by NK cells and T-cells, mainly CD8⁺ T-cells. Therefore, high numbers of CD8⁺ T-cells seen at 5 days post-transplant may possibly be responsible for the remarkable increase in IFN- γ . In addition, IFN- γ produced by these inflammatory cells may work as a positive feedback loop to further promote differentiation of Th1 cells, cytotoxic activity of CD8⁺ T-cells and macrophages, thus further increasing cell damage. On the other hand, IFN- γ has a pro-apoptotic effect on activated CD4⁺ T-cells (Refaeli et al., 2002), thereby possibly explaining lower number of CD4⁺ T-cells compared to CD8⁺ or CD3⁺ T-cells. It should be noted that, IFN- γ was increased in transgenic grafts compared to littermate control allografts at day 12. This is probably due to the presence of less overall cell population in littermate controls at this time (Figure 34 and Figure 37); therefore less IFN- γ producing cells.

Previous studies showed that the level of TNF- α was upregulated in serum during rejection (Chollet-Martin et al., 1990; McLaughlin et al., 1991). Moreover, antibody treatment with anti-TNF prolonged the survival of the graft in an animal model (Lin et al., 1992). All these results suggest that TNF- α plays a role in graft rejection. However, our results demonstrate that although not significant, there seemed to be more TNF- α in grafts from transgenic donors compared to littermate control donors. In fact, TNF- α has a conflicting role in cardiac injury since pre-treatment of adult cardiomyocytes with TNF- α protects against hypoxic injury (Nakano et al., 1998). On the other hand, it should be noted here that there are two types of TNF receptors: one mediates a pro-inflammatory effect (TNFR1) and the other mediates an anti-inflammatory

(TNFR2) effect (Fujita et al., 2008; Lecour and James, 2011); it is therefore possible that there may be preferential expression of the anti-inflammatory TNFR in transgenic hearts. This would be interesting to investigate.

It has been previously demonstrated that hsp-27 regulates the expression of pro and anti-inflammatory cytokines, thereby controlling inflammation. In fact, some investigators have reported that treatment of monocytes with recombinant hsp-27 resulted in marked increase in IL-10 production and modest increase of TNF- α (De et al., 2000). The immunomodulatory effect of hsp-27 is further strengthened with Rayner et al's study (Rayner et al., 2008). In order to explain the effects of hsp-27 on atherosclerosis in the apoE^{-/-} mice, Rayner et al suggest hsp-27 may downregulate IL-1 β and upregulate IL-10 in transgenic hearts. Although IL-1 β is high in littermate control, we failed to demonstrate regulation of IL-10 or TNF- α by hsp-27, as in our study, there was a slight increase in mRNA level of these cytokines in littermate controls at 5 days compared to transgenic allografts. Interestingly, IL-10 regulates the expression of TNF- α and vice versa (Turner et al., 1997; Wanidworanun and Strober, 1993). Thus it harmonises with the results that transgenic allografts have high TNF- α / low IL-10.

Moreover, we found there was a striking difference in the production of anti-inflammatory cytokine IL-4, since transgenic grafts expressed large amount of IL-4 at early times (day 2) while littermate controls did not (Figure 38), thereby suggesting that hsp-27 favours the expression of anti-inflammatory cytokine IL-4. IL-4 promotes differentiation of Th0 into Th2 cells. In fact, IL-4 activates Th0 by a positive feedback to maintain the cytokine environment where their own differentiation is favoured. In addition, IL-4 inhibits the development of naive T-cells into Th1 effector cells and thus prevents production of pro-inflammatory cytokines by these T-cells (Fiorentino et al., 1989). Therefore the increased level of IL-4 present in transgenic grafts could prevent activation of Th1 effector cells and protect from graft destruction. Our study is in agreement with this since increased IL-4 expression correlates with decreased expression of IFN- γ . However, we failed to show the regulation of other anti-inflammatory cytokines such as IL-5 or TGF- β by hsp-27.

Interestingly IL-2 was increased in transgenic at day 12. It is known that IL-2 can induce apoptotic death of IL-2-R expressing T-cells through regulation of BCL-2 (O'Flaherty et al., 2000) suggesting there may be more downregulation of activated T-cells in transgenic hearts. Unfortunately we did not look for apoptosis in rejecting hearts.

Taken together, the literature and our results suggest compelling evidence for an immunomodulatory function of hsp-27 (see chapter 8). It protects from graft rejection by selectively promoting the anti-inflammatory cytokine IL-4 while downregulating cytokines involved in graft destruction. Further studies are needed to confirm with certainty these effects. It would be interesting to co-culture splenic cells *in vitro* with recombinant hsp-27 and study the secretion of cytokines by ELISA.

4.8.3 Effect of hsp-27 in antibody mediated rejection

Alloantibody has been associated with poor graft survival and it contributes to acute and chronic rejection (Michaels et al., 2003; Racusen et al., 2003; Stewart et al., 2005). In fact, the antibody response to the transplanted organ mainly depends on the disparity of MHC between donor and recipient. Using the strain combination B10.A and C57BL/6, Wasowska et al showed that transplant into Immunoglobulin deficient recipients (deletion of μ heavy chain) leads to prolongation of graft survival (Wasowska et al., 2001). They suggested that, in this strain combination, T-cells are not capable of causing acute rejection on their own (Rahimi et al., 2004; Wasowska et al., 2001). But our results show though alloantibody was detected at 12days post-transplant in recipients of both transgenic and littermate control hearts, the survival of hsp-27+ grafts was prolonged. We showed earlier that cellular infiltration and IFN- γ expression was decreased in transgenic model (Figure 34, Figure 37), thus suggesting that in our model cellular immunity plays a crucial role in contributing to acute rejection.

Rahimi et al showed using similar mouse model to us (B10.A and C57BL/6) that allograft rejection is caused by complement activating and non-complement activating antibodies which lead to endothelial cell injury, thus release of von willebrand factor (VWF) and P-selectin

(Rahimi et al., 2004). Rahimi et al used B-cell deficient C57BL/6 recipients (who could not reject B10A cardiac allografts) and restored severe vascular injury and acute cardiac rejection by injection of monoclonal alloantibody. In our model, alloantibody production was not significantly different between transgenic and LC graft at day 12, but cellular rejection was significantly less at early times (days 2 and 5), and rejection was delayed for transgenic grafts. Our results are not necessarily discrepant with those of Rahimii et al or Wasowska et al; it may be that alloantibody is damaging the grafts in our study, but the changes in the cellular profile are sufficient to delay rejection. In our study, hsp-27 was not expressed in the endothelial cells of transgenic animals. However, it would still be interesting to understand the effect of hsp-27 on antibody mediated rejection by checking the deposition of complement (staining for C4d) on one hand (Reed et al., 2006; Stewart et al., 2005) and release of VWF and P-selectin on the other hand (Morrell et al., 2008).

4.8.4 Summary

In summary, we are the first to show that overexpression of hsp-27 prolongs graft survival following cardiac transplantation. Prolongation of graft survival is accompanied by a decrease in graft infiltrating cells and reduced expression of the inflammatory cytokine IFN- γ . In addition, expression of the immunomodulatory cytokine IL-4 is increased. Taken together, the study suggests that hsp-27 has potent immunomodulatory effects: it delays the Th1 inflammatory response and may promote Th2 response, thus regulating inflammation as well as the adaptive immune response. Moreover, it has now been shown that Damage Associated Molecular Patterns (DAMPs) amplify inflammatory response and activate APC to express co-stimulatory molecules that are necessary for activation of T-cells (Mollen et al., 2006; Schmidt and Tuder, 2010). It is therefore possible that hsp-27 present in cardiomyocytes limits cell injury (see chapter 7) and therefore diminishes the activation of DAMPs. Hence, downregulation of inflammation may protect the transgenic allograft from rapid rejection.

Chapter 5: Development of a chronic rejection model

5.1 Introduction

The aim of this study was to understand the effect of hsp-27 on the development of CAV, also called chronic rejection. As explained in the Introduction (section 1.6.3), after cardiac transplantation chronic rejection is presented as occlusion of the donor coronary arteries with SMC, or CAV. For this, we had originally planned to use an established model of chronic rejection using B10.A as heart donors and B10.BRs as recipients (Russell et al., 1994a). B10.A and B10.BR are congenic lines that are mismatched at MHC class I but matched at MHC class II as well as minor MHC loci. Russell et al have previously demonstrated that transplantation of B10.A donor hearts into B10.BR recipients that have been T-cell-depleted with monoclonal antibodies (mAb) at day-6, -3 and -1 before transplantation leads to development of CAV. Without T-cell depletion, rejection occurs very rapidly and hearts are acutely rejected. The purpose of T-cell depletion is to slow down the immune response and give time for CAV to occur. Therefore, the project was designed to use the MHC class I mismatch model B10.A and B10.BR. Unfortunately B10.BR mice line was discontinued from Harlan, UK and the animals were also unavailable from Charles River, UK. In view of the fact that the gene of interest hsp-27 had been bred into B10A mice; it was necessary to identify a suitable recipient for B10.A that results in chronic rejection.

5.2 Confirmation of depletion of T-cells in C57BL/6

We had previously used C57BL/6 as recipients of B10.A hearts in our acute rejection model. This is a complete MHC mismatch model, where transplantation of LC B10.A donor heart into C57BL/6 resulted in cessation of beating 12-14 days post-transplant (see chapter 4). So the aim was to investigate whether this model could be used to develop chronic rejection. It is well known that T-cells play a major role in acute rejection. Therefore, we postulated that depletion of T-cells may delay the development of acute rejection and thus promote establishment of

CAV. In the first instance, we checked whether depletion of T-cells in C57BL/6 could delay acute graft rejection. A pilot study was conducted to identify the type and dose of antibodies required to deplete CD4⁺ and CD8⁺ T-cells. Our laboratory has previously used the mAb GK1.5 (IgG2b) and 2.43 (IgG2b) to deplete CD4⁺ T-cells and CD8⁺ T-cells respectively in C57BL/6 mice (Mahesh et al., 2010). Thus, C57BL/6 mice (that will be used as recipient in future studies) were administered with 250µg of GK 1.5 and/or 250µg of 2.43 mAb intraperitoneally (i.p.) on days 0, 3 and 6 to deplete CD4⁺ and CD8⁺ T-cells respectively (Russell et al., 1994a). The blood was collected on day 7 (week 1) and weekly thereafter and analysed by flow cytometry to measure numbers of circulating T-cells following depletion as well as their recovery at later time points. Figure 40 displays the frequency of mAb injection and blood collection

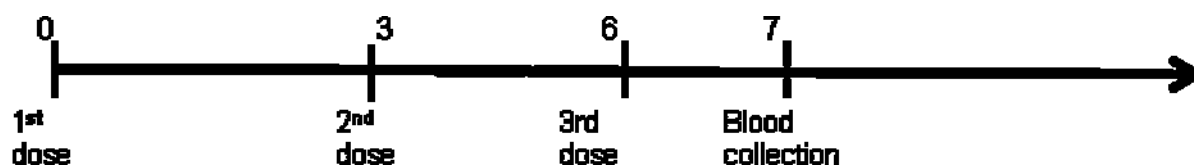


Figure 40: Timeline showing the administration of monoclonal antibodies in order to deplete CD4⁺ and/or CD8⁺ T-cells followed by blood collection in order to assess the depletion of circulating T-cells by flow cytometry.

Injection of mAb to CD4⁺ and CD8⁺ T-cells on days 0, 3 and 6 led to a significant reduction of peripheral circulating CD3⁺ T-cells (which is a common marker for both CD4⁺ and CD8⁺ T-cells) CD4⁺ T-cells as well as CD8⁺ T-cells on week 1 post-injection. CD3⁺ and CD4⁺ T-cells were depleted for two weeks following the initial injection and started to recover progressively thereafter (Figure 41a and Figure 41c respectively). Although CD8⁺ T-cells (Figure 41d) were depleted for 2 weeks post-injection, they did not recover as rapidly as CD3⁺ or CD4⁺ T-cells. In fact, there was a gradual increase in CD8⁺ T-cells and both CD3⁺ as well as CD8⁺ T-cells reached half the level present in blood from non-depleted mice by week 7 post-injection. Interestingly, at 7-weeks post-injection, CD4⁺ T-cells had recovered completely.

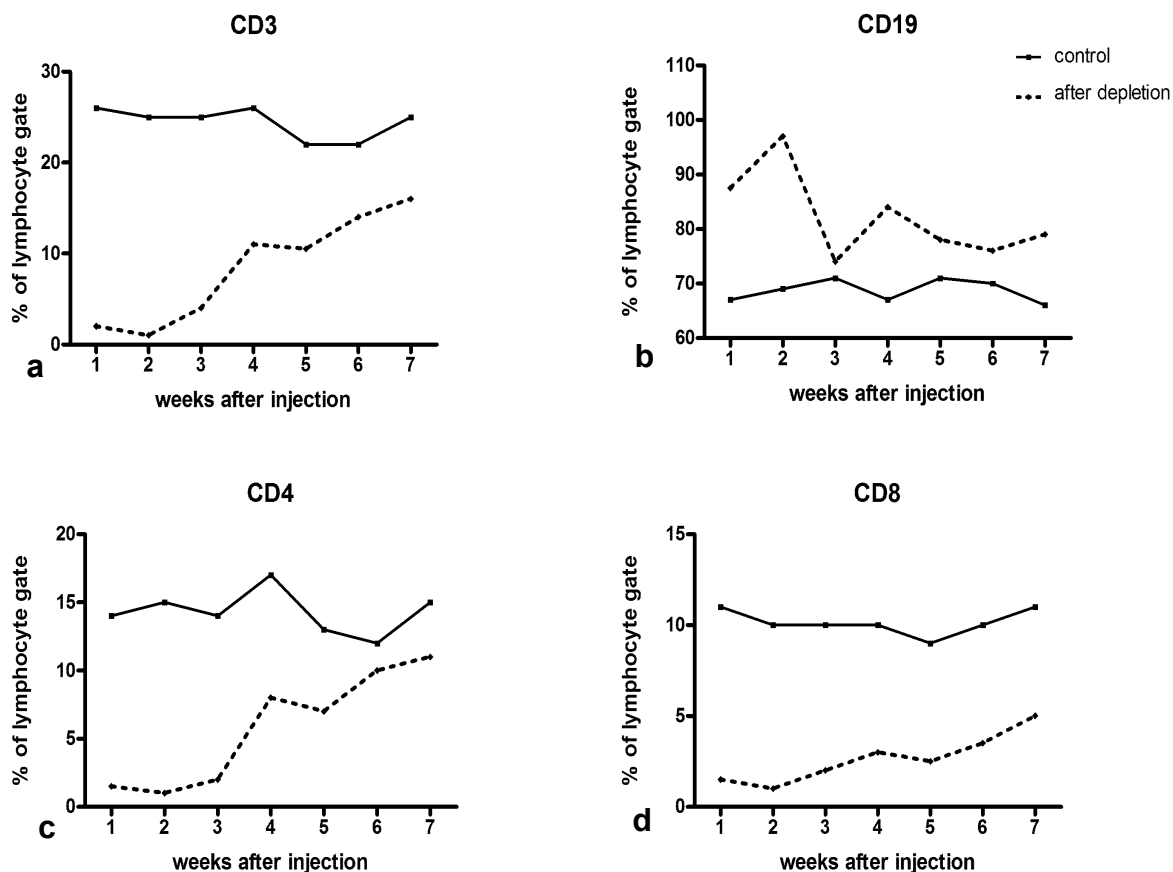


Figure 41: Flow cytometry data showing depletion of peripheral circulating CD4⁺ and CD8⁺ T-cells following administration of 250µg of monoclonal antibodies to murine CD4 (GK1.5, IgG2b) and 250µg of monoclonal antibodies to murine CD8 (2.43, IgG2a) compared to control mice that received saline (n=2-3).

The effect of T-cell depletion on B-cells was tested by measuring the presence of CD19⁺ cells. This is a specific marker for B-cells. As shown in Figure 41b, an apparent increase in the B-cell population was observed in T-cell-depleted mice in the first 2 weeks after depletion, compared to control mice. However, as blood contains various cells including T-cells and B-cells, treatment with mAb to CD4⁺ or CD8⁺ T-cells, will result in enrichment of the B-cell population. As flow cytometry is analysing the percentage of B-cells present compared to total lymphocytes, it is expected that the percentage of the B-cell population will be higher in the antibody treated group compared to control. It is therefore possible to suggest that the depletion was specific to T-cells since the number of B-cells was not reduced compared to control.

5.3 Effect of CD4+ and CD8+ T-cells depletion on allograft rejection in C57BL/6

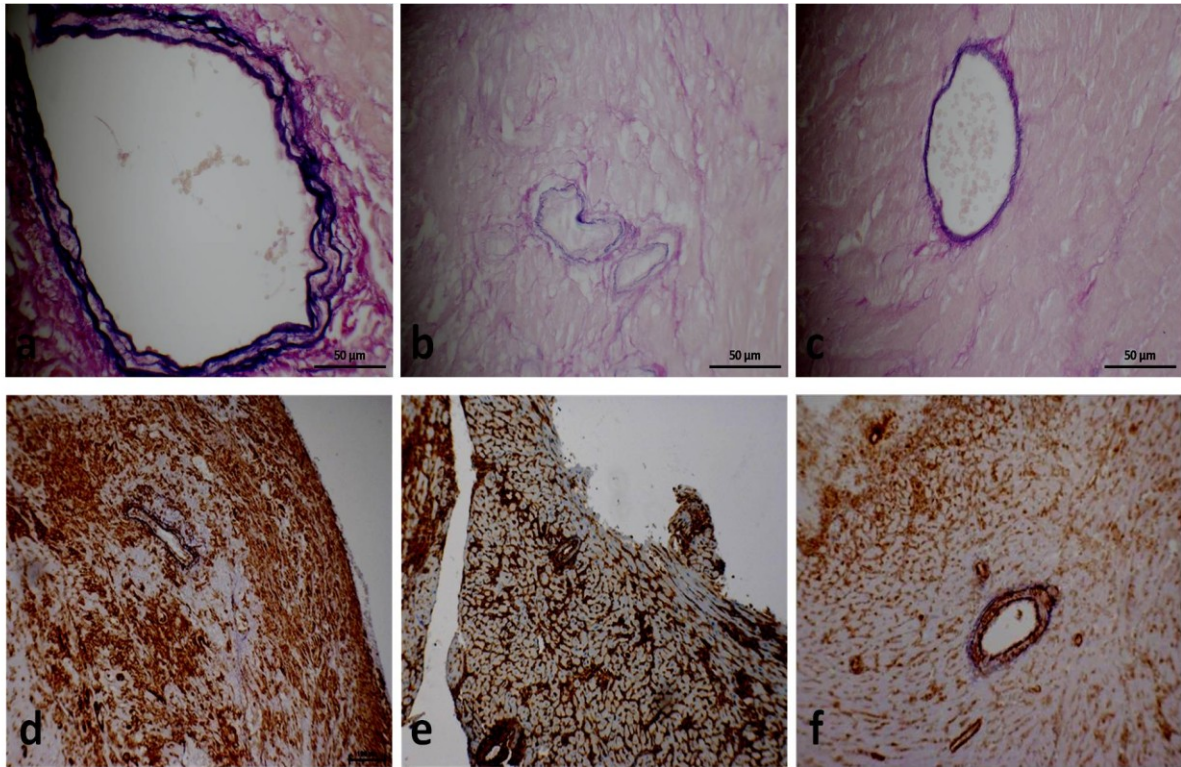


Figure 42: Effect of CD4+ and/or CD8+ T-cells on graft rejection in C57BL/6 mice. Anti-CD4 alone (a and d), anti-CD8 alone (b and e) or anti-CD4 together with anti-CD8 (c and f) mAb were administered to C57BL/6 recipients at 0, -6, -3 and -1 days before transplantation. They received a heart from B10.A at day 0. The animals were sacrificed at either at the time of rejection of the heart (b and e) or at 8 weeks (a, c, d and f) and assessed for graft vasculopathy by staining with elastin and Von Giesen's stain (a-c) or antibody to smooth muscle alpha actin (d to f) (n=3-4).

We showed that the mAb GK1.5 and 2.43 deplete CD4+ and CD8+ T-cells respectively. The aim was then to investigate the effect of T-cell depletion on chronic rejection in C57BL/6 recipients of B10A hearts. The recipient mice were either injected with mAb to CD4+ or CD8+ or both CD4+ and CD8+ T-cells at day -6, -3 and -1 before transplantation. Heterotopic heart transplantation was performed at day 0 using B10.A littermate controls as donors and the hearts were palpated weekly to check possible rejection. Mice that were depleted with antibody to CD4+ alone or antibody to both CD4+ and CD8+ T-cells did not reject the graft up to week 8

post-transplant. These were then sacrificed at week 8 and hearts tested morphologically for graft rejection through examination for vasculopathy and intimal thickening of blood vessels using miller's elastin and Von Giesen's staining (EVG; Figure 42 a-c) or an antibody against smooth muscle α -actin (SMA Figure 42 d-f)) respectively. The grafts obtained from mice that received anti-CD4 alone (n=4) or anti-CD4 and anti-CD8 antibody (n=3) did not show any evidence of occlusion of the graft (Figure 42a & d and Figure 42c & f respectively) at 8 weeks post-transplant as indicated by absence of vasculopathy or actin positive cells in the intima. Hence, there is no sign of CAV present at 8 weeks. Interestingly, depletion of CD8+ T-cells in recipients prior to transplantation (n=3) resulted in cessation of the heart beat, *i.e.* graft rejection at 3 weeks post-transplant. Therefore, the experiments were repeated (n=3) and the heart was palpated every 2 days from week 1 post-transplant. The animals were sacrificed on the 18th day post-transplant and assessed for graft vasculopathy. The EVG staining showed intimal thickening and occlusion of the blood vessels at 18 days post-transplant (Figure 42b). SMA staining confirmed presence of SMC in the intima. Hence, there is evidence of both graft rejection and early CAV at 18 days post-transplant in mice that received anti-CD8 mAb therapy. In summary, B10.A hearts transplanted into CD4+ T-cell-depleted or combined CD4+ and CD8+ T-cell-depleted C57BL/6 recipients survived up to 8 weeks and demonstrated no evidence of CAV. However, rejection occurred rapidly in CD8+ T-cell-depleted model (within 2-3 weeks).

5.4 Identification of an antibody to deplete T-cells in CBA mice

Russell et al showed that transplantation of B10.A into B10.BR that were depleted of CD4+ and CD8+ T-cells prior to transplantation, resulted in development of graft vasculopathy at 8 weeks post-transplant. B10.A and B10.BR are mismatched at MHC class I but matched at MHC class II and minor MHC. Due to unavailability of the B10.BR strain, the CBA strain was used as an alternative. The CBA strain is of haplotype $H-2^k$ and B10.A is of haplotype $H-2^a$ ($H-2K^k$ for class I and $H-2A/E^d$ for class II). Thus, CBA and B10.A are mismatched at MHC class I as well as minor MHC but matched at MHC class II. Therefore, the goal of this study was to investigate the possibility of developing graft vasculopathy using CBA mice as recipients. Our Laboratory has

not previously used the mAb 2.43 to deplete CD8⁺ T-cells in CBA mice. It was therefore necessary to identify a potential antibody that would deplete CD8⁺ T-cells in CBA mice. A literature revision suggested that using mAb 53-6.72 was effective in depleting CD8⁺ T-cells in CBA mice (Majeski et al., 2003; Waki et al., 1992; Yates et al., 2007). So the aim of this study was to examine the experimental conditions –i.e. the antibody type, the concentration and the frequency of injection- in order to achieve optimum depletion of CD4⁺ and CD8⁺ T-cells in CBA mice. In order to identify the concentration of antibody required to deplete T-cells, CBA mice were administered with either 250µg or 500µg of a mixture of GK1.5 and 53-6.72 intraperitoneally at days 0, 3 and 6 to deplete CD4⁺ and CD8⁺ T-cells respectively. Control mice received saline. Blood was collected weekly from the tail vein and the depletion of peripheral circulating T-cells was assessed by flow cytometric analysis. Interestingly both 250µg and 500µg of the cocktail of GK1.5 and 53-6.72 mAb had similar effect on T-cell depletion (Figure 43). CD3⁺ (Figure 43a) as well as CD4⁺ (Figure 43c) T-cells were depleted up to three weeks post-injection and increased progressively thereafter. Analysis of blood from T-cell-depleted mice shows that CD4⁺ T-cells had returned to the normal level by week 7 post-injection. Conversely, CD8⁺ T-cells (Figure 43d) were depleted for up to 5 weeks and recovered very slowly thereafter. Treatment with anti-CD4 or anti-CD8 mAb did not deplete B-cells (Figure 43b). In fact B-cells were significantly higher in both 500µg and 250µg of the antibody depleted groups compared to the control. As discussed in section 6.2.1, the increase in the percentage of B-cells is due to the reduced number of total lymphocytes.

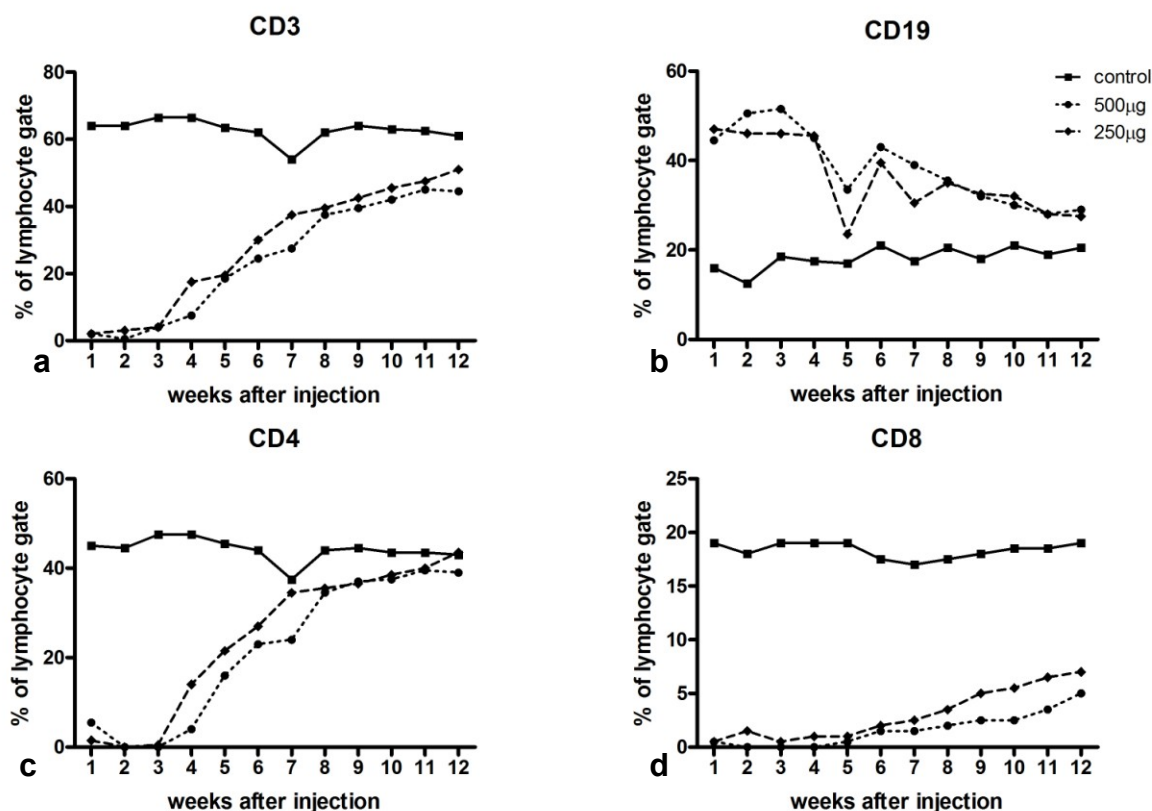


Figure 43: Flow cytometry data showing depletion of peripheral circulating CD4+ and CD8+ T-cells following i.p. administration of either 250µg or 500µg of a mixture of monoclonal antibodies to murine CD4 (GK1.5) and CD8 (53-6.72) compared to control mice that received saline. Mice were treated with depleting antibodies on day 0, day 3 and day 6. Blood was collected from the tail vein on day 7 then weekly to assess T-cell depletion (n=3-4).

Since the administration of 250µg and 500µg of depleting antibodies yielded similar results, 250µg of antibody was used for further studies. Although the mAb Gk1.5 and 53-6.72 were effective in depleting CD4+ and CD8+ T-cells respectively, the T-cells, in particularly CD4+ T-cells recovered rapidly. So in order to prolong the depletion of T-cells, an extra dose (4th dose) of depleting antibody was administered a week following transplantation (which means 2 weeks after the initial injection). Figure 44 shows that administration of an extra 4th dose a week after transplantation resulted in a similar effect as to the mice that received only 3 doses of injection before transplantation. In fact the flow cytometry profiles of peripheral circulating leukocytes followed similar pattern for CD3+, CD19+, CD4+ and CD8+ cells (Figure 44a, b, c and d

respectively), suggesting that administration of an additional dose of antibodies GK1.5 and 53-6.72 does not further prolong the depletion of T-cells. Therefore, the studies were conducted using 250µg of mAb GK1.5 and 53-6.72, administered at day -6, -3 and -1 before transplantation.

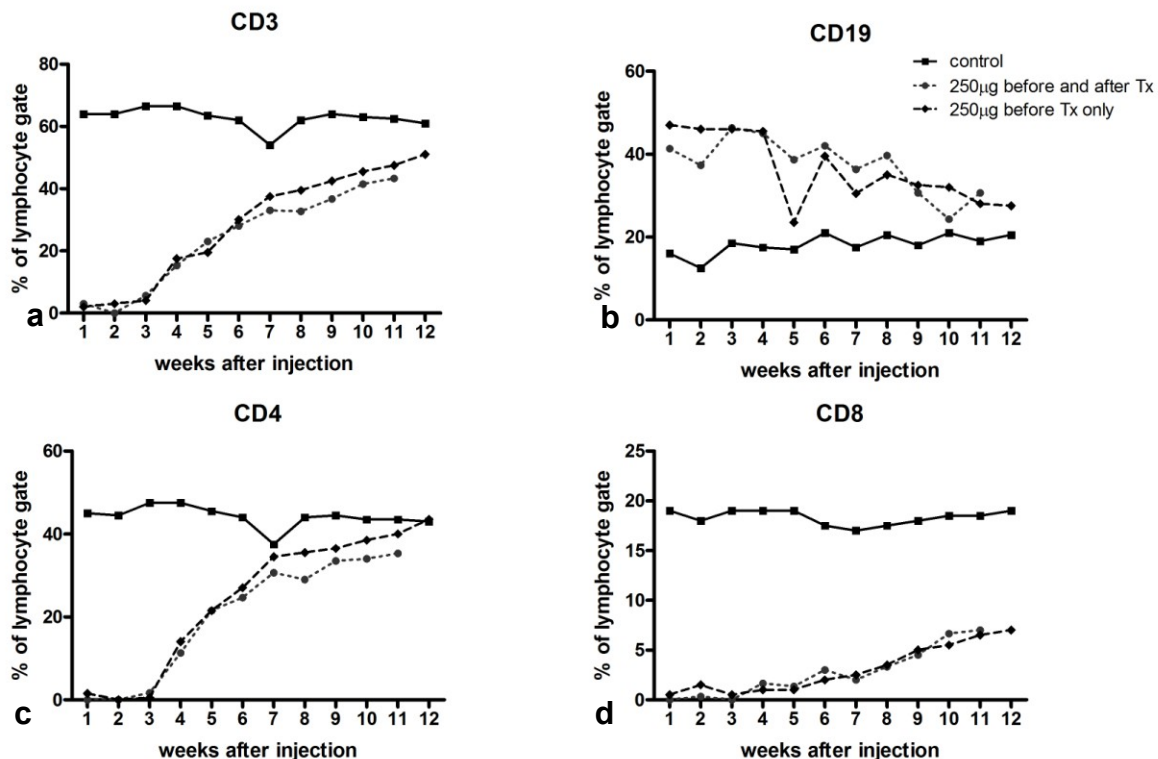


Figure 44: Flow cytometry data showing depletion of peripheral circulating CD4+ and CD8+ T-cells following i.p administration of either 3 doses before transplantation or with an additional dose a week after transplantation of mAb to murine CD4 (GK1.5) and CD8 (53-6.72), compared to control that received saline (n=3).

5.5 Effect of CD4+ and CD8+ T-cell depletion on allograft rejection in CBA

As the antibodies and the experimental conditions have been established, it was possible to use the T-cell-depleted mice to investigate whether this would produce a model of CAV. In this study, B10.A wild-type hearts were transplanted into CBA mice that were depleted of CD4+ T-

cells and/or CD8+ T-cells and assessed for the development of CAV. Recipient mice that were depleted with antibody to CD4+ alone or antibody to both CD4+ and CD8+ T-cells did not reject the graft up to week 8 post-transplant. These were then sacrificed at week 8 and assessed for CAV by staining with EVG stain and smooth muscle α -actin (SMA). Depletion of CD8+ T-cells in recipients prior to transplantation resulted in graft rejection around 3 weeks post-transplant, where the donor heart was beating very slowly. These were then sacrificed at week 3 following transplantation to assess for graft vasculopathy. Histological evaluation showed that the grafts obtained from mice that received antibodies to both CD4+ and CD8+ T-cells displayed no sign of CAV at 8 weeks post-transplant (Figure 45c and f). The vessels of these mice were clean and free from SMC infiltration. On the other hand, donor heart obtained from recipients that were treated with antibody to CD8+ T-cells contained occlusion of most of the blood vessels (Figure 45b and e). In fact, anti-CD8 depletion alone produced rapid rejection of the allograft at 3 weeks post-transplant, characterised by intimal occlusion which consisted of SMC and infiltration of leukocytes. Hence, this suggests development of a mixture of acute rejection and CAV. Similarly, grafts obtained from recipients that were depleted of CD4+ T-cells alone resulted in intimal occlusion (Figure 45a) by the presence of SMA in the intimal region (Figure 45d) 8 weeks post-transplant. As there was no evidence of rejection in recipients that received combined anti-CD4 and anti-CD8 mAb therapy; and as recipients depleted with CD8+ T-cells rejected the heart fairly rapidly, CBA mice that received anti-CD4 mAb treatment were therefore used as the chronic rejection model in our study. Using this model it will be possible to investigate whether hsp-27 has any effect on the development of chronic rejection.

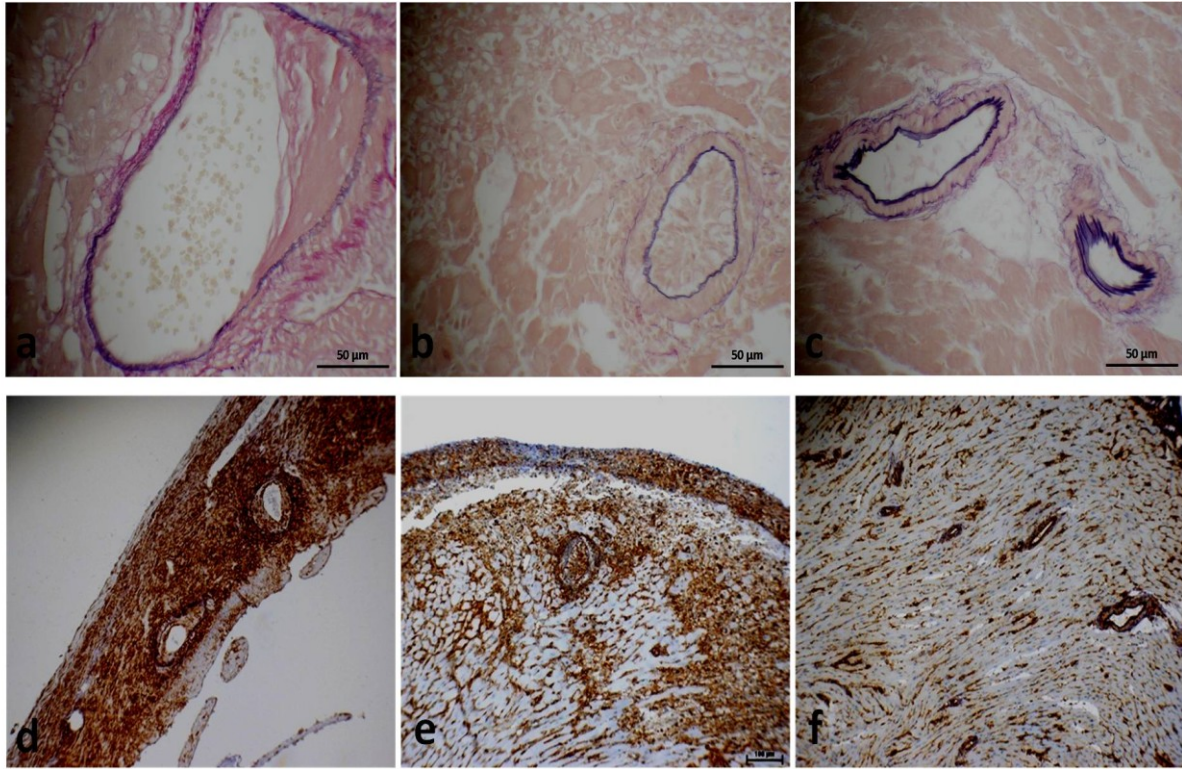


Figure 45: Effect of CD4⁺ and/or CD8⁺ T-cell depletion in development of CAV in CBA mice. CD4⁺ alone (a and d), CD8⁺ alone (b and e) or CD4⁺ together with CD8⁺ T-cells (c and f) of CBA mice were depleted with monoclonal antibody GK 1.5 and 53-6.72 at -6, -3 and -1 before transplantation. Following depletion, B10.A wild-type heart was grafted heterotopically in the abdomen of CBA mice. The animals were sacrificed at 8 weeks (a, c, d and f) or at 3 weeks (b and e) post-transplant, and the graft was assessed for intimal thickening and SMC infiltration by staining with EVG stain or antibody to SMA.

In conclusion the results demonstrate that combined depletion of recipient CD4⁺ and CD8⁺ T-cells before transplantation prolongs graft survival and protects from the development of CAV in both CBA and C57BL/6 recipients. On the other hand, in both of these recipients, removal of CD8⁺ T-cells using mAb in the host, leads to rapid development of CAV and graft rejection. Conversely, anti-CD4 mAb therapy in C57BL/6 recipients delays graft rejection and protects from CAV. However, the same treatment leads to luminal occlusion and CAV when CBA is used as recipients.

	Anti-CD4 mAb	Anti-CD8 mAb	Anti-CD4 and anti-CD8 mAb
C57BL/6	Graft survival at 8 weeks post-transplant. No sign of intimal thickening or vessel occlusion. Absence of CAV	Graft rejected at 18 days post-transplant. Intimal thickening and vessel occlusion by smooth muscle cells. Presence of CAV	Graft survival at 8 weeks post-transplant. No sign of intimal thickening or vessel occlusion. Absence of CAV
CBA	Graft survival at 8 weeks post-transplant. Evidence of intimal thickening and vessel occlusion by smooth muscle cells. Presence of CAV	Graft rejected at 3 weeks post-transplant. Intimal thickening and vessel occlusion by smooth muscle cells. Presence of CAV	Graft survival at 8 weeks post-transplant. No sign of intimal thickening or vessel occlusion. Absence of CAV

Table 9: Table summarizing the effect of T-cell depletion therapy of graft recipients on the development of CAV in a complete MHC disparate (B10.A into C57BL/6) and MHC class I and minor MHC disparate model (B10.A into CBA).

5.6 Discussion

The aim of this section was to identify a suitable model in order to study chronic rejection or CAV. In fact, the aim was to delay acute rejection and promote establishment of chronic rejection using mAb to deplete T-cells. The technique of depleting a T-cell subset was performed by Cobbold et al (Cobbold et al., 1986) so as to understand the role of CD4+ and CD8+ T-cells in allograft rejection. Here, we used this method to understand how we could promote the development of chronic rejection *in vivo*.

5.5.1 Role of T-cells in development of CAV

The results presented here demonstrate the importance of T-cells in transplant rejection. We and others have reported infiltration of T-cells during transplant rejection, accompanied by production of cytokines by these T-cells (Chapter 4). T-cells can be divided into sub-populations depending on the expression of differentiation markers. Both CD4⁺ T-cells and CD8⁺ T-cells have been detected in the rejected heart. This chapter examined the involvement of CD4⁺ and CD8⁺ T-cells in graft rejection following cardiac transplantation.

Transplantation of B10.A hearts into CD4⁺ and CD8⁺ T-cell-depleted recipients resulted in survival of the hearts up to 8 weeks post-transplant in both CBA and C57BL/6 recipients. Moreover, there was no evidence of vasculopathy in these models. It is important to note that B10.A and CBA differ in MHC class I and minor MHC while C57BL/6 and B10.A combination is a complete mismatch model. These results suggest that T-cells are pivotal to graft rejection and graft vasculopathy.

5.5.2 Effect of CD8⁺ T-cells depletion on transplant outcome

Using a different model system, investigators have studied the role of CD8⁺ T-cell subsets in allograft rejection (Bishop et al., 1993; Bolton et al., 1989). Bolton et al showed that adoptive transfer of CD8⁺ T-cells alone in congenitally athymic rats does not cause rejection (Bolton et al., 1989). In our study, both CBA and C57BL/6 recipients that were depleted of CD8⁺ T-cells rejected the B10.A graft rapidly, within 3 weeks. Histological evaluation further confirmed occlusion of blood vessels and presence of infiltrating cells (Figure 42 and Figure 45). The present study thus suggests that CD8⁺ T-cells are not the major effector mechanism of graft rejection in the B10.A-C57BL/6 model, since the animals depleted of CD8⁺ T-cells reject the heart in the same tempo as the non-depleted wild-type recipients (see chapter 5). However, we did not investigate whether rejected hearts from non-depleted recipients developed CAV. The graft was also rejected rapidly in the B10.A into CBA model. In this respect, others have also found that CD8⁺ T-cells do not play an important role in the development of intimal lesions (Shi

et al., 1996) or progression of CAV (Forbes et al., 1994). It is surprising that although there was almost complete depletion of CD8⁺ T-cells (Figure 41 and Figure 43); there was very little influence on graft survival. These results are consistent with the study that used CD8 knock out (KO) recipient, where both skin and heart transplanted in an MHC disparate model were rejected as rapidly as the control (Krieger et al., 1996). This further validates that CD8⁺ T-cells are not critical to transplant rejection.

On the other hand, we have previously shown significant infiltration of CD8⁺ T-cells and production of IFN γ in the acute rejection model (see chapter 5), suggesting this subset of T-cells is involved in graft rejection. However, in the present study, despite presence of CD8⁺ T-cells in the CD4⁺ T-cell-depleted model, grafts were not rejected rapidly, but were still beating at 8 weeks in both the complete MHC mismatch (B10.A and C57BL/6) as well as the MHC class I mismatch and minor mismatch (B10.A and CBA) model. However, grafts obtained from CD4⁺ T-cell-depleted CBA showed CAV. Taken together, these results indicate a possible role for CD8⁺ T-cells in the effector phase of CAV. A possible explanation would be that CD8⁺ T-cells are unable to initiate graft rejection (*i.e.* cessation of heart beat) in complete absence of CD4⁺ T-cells (Bishop et al., 1993; Morton et al., 1993). It is important to note that in the anti-CD4 treated host, there was no detectable CD4⁺ T-cells for the first 3 weeks after transplantation; after this time period they start to recover (Figure 41 and Figure 43); hence, during this time period CD8⁺ T-cells could not become primed. It has been demonstrated that in the absence of CD4⁺ T-cells, *in vitro* primed CD8⁺ T-cells were able to cause rejection (Delfs et al., 2001). Moreover, another study has underlined that unprimed CD8⁺ T-cells, in the absence of CD4⁺ T-cells, required treatment with CD40 activating antibody for the development of intima lesions (Schnickel et al., 2004). Activation of APC is mediated by the interaction of CD40 (expressed on APC) with antigen specific CD4⁺ T-cells. Following activation, the interaction of CD40 L with CD40 expressed on APCs, lead to the activation of CD8⁺ T-cells (Kupiec-Weglinski et al., 2001). In our studies, using CBA as recipients, it is likely that CD8⁺ T-cells remained unprimed until about 4 weeks when CD4⁺ T-cells start to recover. At this point interaction between CD4⁺

T-cells and antigen-primed dendritic cells leads to activated CD4⁺ T-cells and priming of CD8⁺ T-cells. The graft survival in anti-CD8 treated CBA was around 3 weeks, while depletion of CD4⁺ T-cell prolonged survival but leads to CAV and the combination of both CD8⁺ and CD4⁺ depletion delayed rejection and prevented CAV. It is thus clear that both subsets can participate in the alloimmune response but activation of CD8⁺ T-cells may depend on recovered CD4⁺ T-helper cells.

Histological assessments show that, in both complete MHC mismatch (Figure 42 b and e), as well as MHC class I and minor mismatch (Figure 45b and e), depletion of CD8⁺ T-cells resulted in complete occlusion of blood vessels and cardiomyocytes damage. Cardiomyocyte damage is a characteristic feature of acute rejection (cf. acute rejection chapter). Similarly, intimal occlusion is a sign of chronic rejection (cf. chronic rejection chapter). We did not assess for tissue infiltration or necrosis. However, studies have shown infiltration of innate immune cells such as macrophages, NK cells as well as CD4⁺ T-cells in the allograft of anti-CD8 treated recipients (Bishop et al., 1993; Bolton et al., 1989), suggesting these cells might have caused rejection seen in anti-CD8 mAb-treated hosts.

However, the role of CD8⁺ T-cells in graft rejection seems controversial. Our results, as well as other studies show that CD8⁺ T-cells are not essential for rejection of cardiac (Bishop et al., 1993) or kidney allografts (Bolton et al., 1989). On the other hand, these results are in contradiction with other studies demonstrating that CD8⁺ T-cells are sufficient for graft rejection of intestine (Halamay et al., 2002; He et al., 1999). Using a TCR transgenic cardiac transplant model, Halamay et al showed that CD8⁺ T-cells, in the absence of CD4⁺ T-cells (through anti-CD4 mAb therapy), were able to reject the heart acutely. This might be due to an increase number of CD8⁺ T-cells present in the transgenic model.

In summary, in the models used in our study, CD8⁺ T-cells, in the absence of CD4⁺ T-cells seem unable to mediate graft rejection as defined by cessation of the heart beat. However, they do mediate CAV after a period of 8 weeks. CD4⁺ T-cells are able to mediate rapid rejection and

CAV in the absence of CD8⁺ T-cells; this is probably because they are able to recruit many other cells such as B-cells and cells of the innate immune system to the graft.

5.5.3 Effect of CD4⁺ T-cells depletion on transplant outcome

We have previously shown that transplantation of a B10.A graft into a non-depleted C57BL/6 results in rapid rejection of the heart within 2 weeks (cf. acute rejection chapter). Unfortunately, we did not assess the survival of the graft in untreated CBA recipients. But precedent studies showed that transplantation of B10 into CBA, in an MHC class I and minor disparate leads to graft loss at around 7 days (van Maurik et al., 2002). Interestingly, pre-treatment of recipients (CBA or C57BL/6) with an anti-CD4 mAb leads to a significant prolongation of the allograft survival for up to 8 weeks; thus, suggesting that CD4⁺ T-cells play a critical role in acute graft rejection. Consistent with this observation, Krieger et al showed that CD4 KO recipients permanently accept heart or skin grafts transplanted across the MHC barrier. In fact, the survivals of these grafts were prolonged for up to 100 days compared to control wild-type recipients that rejected the grafts within 9 days. The same group further confirmed the importance of CD4⁺ T-cells by showing that the adoptive transfer of naive CD4⁺ T-cells in CD4 KO recipients restored rejection; thus, further validating that CD4⁺ T-cells are primordial for the initiation of transplant rejection (Krieger et al., 1996).

Similarly to the fully mismatched model, graft survival was prolonged in MHC class I and minor disparate model following anti-CD4 mAb therapy. However, signs of chronic rejection were observed in this model when assessed at 8 weeks post-transplant. Previous studies have also observed that CD4⁺ T-cell depletion resulted in a delay of graft rejection, but they were not effective in promoting long-term graft survival (Bowles et al., 2000; Orosz et al., 1996). Therefore, antibody treatment with anti-CD4 mAb might prevent acute rejection and lead to cardiac allograft vasculopathy in the B10.A-CBA strain combination.

It maybe that longer depletion of CD4⁺ T-cells would allow survival of the allograft in the absence of CAV. However, in our study, we failed to identify a method to prolong CD4⁺ T-cell

depletion. This could possibly be done by administration of an anti-CD4 mAb for several weeks after the initial depletion (Nagano et al., 1998).

5.5.4 Other mechanisms of rejection in the T-cell-depleted recipient

Antibody treatment may also delay rejection through mechanisms other than T-cell depletion. This notion is supported in our studies since B10.A graft in anti-CD4 treated C57BL/6, as well as CBA recipients were free from acute rejection at 8 weeks after transplantation although many CD4⁺ T-cells had re-emerged by that time (Figure 41 and Figure 43 respectively). Consistent with this observation, He et al showed prolongation of intestinal graft survival despite return of CD8⁺ T-cells in an anti-CD8 antibody treated model (He et al., 1998). They also remarked that the rejected intestinal allograft showed signs of chronic rejection (He et al., 1999). Our results are in agreement with this observation. In fact, graft obtained from anti-CD4 treated CBA shows signs of chronic rejection including intimal hyperplasia and luminal occlusions. This further validates that depletion of CD4⁺ T-cells abrogates acute rejection but not cardiac allograft vasculopathy. Possible existence of mechanisms other than depletion alone is further strengthened by studies that used knock-out models. In fact, mice genetically deficient due to T-cell KO or targeted gene disruption reject the graft in the same tempo as control non-depleted mice (Grusby et al., 1993; He et al., 1998). Conversely, treatment with non-depleting antibody extends graft survival, suggesting that antibody treatment activates alternative mechanisms other than depletion of T-cells alone. Studies using non-depleting antibody demonstrated decreased lymphocyte proliferation *in vitro*, decreased antibody-mediated and cell-mediated immune response, diminished Th1 response or even possible tolerance induction (Binder et al., 1996; Lehmann et al., 1997).

Some studies have discussed the possibility of tolerance induction as one of the mechanisms accountable for prevention of acute rejection in mAb treated hosts (Binder et al., 1996; Lehmann et al., 1997). In our study, depletion of CD4⁺ and CD8⁺ T-cells prevented graft rejection and CAV. Similarly, C57BL/6 recipients treated with anti-CD4 mAb alone are also protected from acute and chronic rejection, suggesting induction of possible tolerance.

However, we did not look for tolerance in our model. Analyses of alloantibodies, T-cell profile and the cytokines present in the graft, could give insight to whether recipients treated with mAb develop tolerance against graft rejection (Binder et al., 1996; Siegling et al., 1994; Stumbles and Mason, 1995).

5.5.5 Role of non T-cells in graft rejection

We have shown the presence of an important number of B-cells in CD8⁺ T-cell-depleted mice (Figure 41 and Figure 43). Interestingly, numerous studies have shown the importance of the humoral response and thus alloantibodies in graft rejection (Blume et al., 2012; Reed et al., 2006). In this respect, several studies have demonstrated presence of antibodies directed against the allograft. Both pre-formed antibodies to MHC antigens (Itescu et al., 1998; Smith et al., 1993; Zhang et al., 2011), as well as de-novo HLA antibodies formed post-transplantation have been shown to contribute to graft rejection (Hodges et al., 2012; Li et al., 2008; Reed et al., 2006; Smith et al., 2011). Moreover, non-HLA antibodies including antibodies against MICA, vimentin and anti-nuclear antibodies also contribute to graft rejection (Jurcevic et al., 2001; Mahesh et al., 2007; Mahesh et al., 2010; Sumitran-Holgersson et al., 2002; Win et al., 2009; Zhang et al., 2011). In our study, recipients were not pre-sensitized; therefore, it is very unlikely that the rapid graft rejection seen in the CD8⁺ T-cell-depleted host was due to pre-formed antibodies. However, we cannot discount the possibility of graft rejection as a consequence of rapid production of de-novo antibodies in anti-CD8 treated recipients. This notion is supported by Morton et al, who showed significant antibody response 3 days after transplantation in anti-CD8 mAb treated group. (Morton et al., 1993). It would have been very interesting to investigate the presence of auto-antibodies in the rejecting heart.

On the other hand, a significant number of B-cells were detected in CD4⁺ T-cell-depleted mice. As discussed earlier, humoral alloreactivity and presence of alloantibodies appear to be critical to graft rejection. It is important to mention that the alloantibody response and antibody class switching are T-cell dependent (Steele et al., 1996). Moreover, in the context of transplantation, studies have shown that CD4⁺ T-cells are required for the production of alloantibodies by

allospecific B-cells (Taylor et al., 2007). Although we did not investigate circulating alloantibodies, previous studies have demonstrated that depletion of CD4⁺ T-cells blocked class switching to IgG and attenuated the humoral response against graft rejection (Binder et al., 1996; Morton et al., 1993; Steele et al., 1996). Thus, this inhibitory effect of anti-CD4 mAb on the humoral response may also have contributed to the prolongation of graft survival experienced in our study.

The possibilities that innate immune cells including monocytes/macrophages and NK cells are involved in graft rejection have been under consideration (Bolton et al., 1989; Habiro et al., 2005). Studies have shown that NK cells can trigger vasculopathy in parental to F1 strain combination of a cardiac transplant model (Uehara et al., 2005a; Uehara et al., 2005b). On the other hand, depletion of macrophages reduced graft vasculopathy by 70% (Kitchens et al., 2007). Further support was obtained from a study using macrophage depletion to prevent acute graft nephropathy (Jose et al., 2003).

Taken together, it is evident that non T-cells also contribute to graft rejection. It is therefore possible that the accelerated graft rejection seen in anti-CD8 mAb treated host was due to immune reaction by CD4⁺ T-cells, as well as B-cells and innate immune cells.

5.5.6 The alloreactivity of T-cells depends on MHC disparity, type of transplant and the model used

Although anti-CD4 mAb delays graft rejection in C57BL/6 and CBA recipients, it is interesting that while the C57BL/6 recipient did not develop CAV, the CD4⁺ T-cell-depleted CBA recipient developed luminal occlusion and CAV. It is notable that B10.A and CBA differ in MHC class I and minor MHC, while C57BL/6 and B10.A are complete mismatch models. This suggests that the development of CAV and the importance of CD4⁺ T-cells may vary depending on the model. Consistent with this hypothesis, previous studies have illustrated that heart grafts were rejected rapidly from fully MHC mismatched strain combinations but were accepted permanently in an MHC class II disparate model (Youssef et al., 2004). Similarly, the survival of kidney transplant

varied depending on the strain used (Zhang et al., 1996). These studies and our work thus underline the importance of MHC restrictions in graft rejection. In contrast, Win et al used an MHC class II mismatch model and showed that passenger CD4⁺ T-cells provide help for autoantibody production, and such autoantibodies lead to CAV (Win et al., 2009). That work hence underlines the importance of the model used.

Besides, allograft rejection varies depending on the organ studied (Zhang et al., 1996). Youssef et al demonstrated that skin grafts were rejected in all strain combinations, while heart grafts were not in minor MHC combinations (Youssef et al., 2004). Similarly, allogenic intestinal graft survival was prolonged in a CD8⁺ T-cell-depleted mouse model, while cardiac transplant was not (He et al., 1999). The possible explanation for conflicting results could be due to the degree of immunogenicity of the organ studied. Therefore, the MHC disparity, the organ studied and the model used are all important factors to consider in order to understand graft immunogenicity (Zhang et al., 1996).

5.5.7 Efficiency of monoclonal antibodies

Results presented here demonstrate that the mAb GK 1.5 depletes CD4⁺ T-cells (Dialynas et al., 1983) in both the CBA strain as well as the C57BL/6 strain. Moreover, the mAb 2.43 depletes CD8⁺ T-cells (Sarmiento et al., 1980) in C57BL/6, while the antibody 53-6.72 depletes CD8⁺ T-cells (Ledbetter and Herzenberg, 1979) in CBA mice. More than 99.5% of the cells have to be depleted to prevent rejection (Cobbold and Waldmann, 1986). The results from our work in fact shows that the antibodies used are effective, since treatment with mAb specific to CD4⁺ or CD8⁺ T-cells resulted in complete depletion of their target cells. As a result, depletion of CD4⁺ alone or CD4⁺ together with CD8⁺ T-cells was able to delay or prevent rejection. Furthermore, we have also confirmed here that these antibodies are specific for T-cell depletion since they did not deplete the B-cell population (Figure 41, Figure 43 and Figure 44).

Both CD4⁺ and CD8⁺ T-cells were recovered after depletion, indicating that depletion was not permanent. As mice were not thymectomized, it is not surprising for the T-cells to recover.

However, following treatment with anti-CD4 and anti-CD8 mAb, T-cells recover with different kinetics. CD8+ T-cell depletion did not get back to control level until week 8 post-injection, while the level of CD4+ T-cells increased from week 3 and reached the control level at week 7 post-injection. These differences could be attributed to the variation in half life of those proteins. In fact, it has been previously reported that following treatment with GK1.5 (anti-CD4) and 2.43 (anti-CD8) mAb, CD8+ T-cells did not re-emerge for several weeks, while CD4+ T-cells returned to 30% of their normal level a few weeks later (Ghobrial et al., 1989).

5.5.8 Summary

In summary, CD4+ and CD8+ T-cells contribute to graft rejection. However, the relative importance of these cell subsets depend on the model used and the organ studied. Depletion of CD4+ T-cells prolongs allograft survival, while anti-CD8 mAb seems to be less effective at preventing rejection. Interestingly, a combination of anti-CD4 and anti-CD8 mAbs inhibits graft rejection and CAV, while CD4+ T-cell depletion alone prevents acute rejection but not chronic rejection. Nevertheless, the development of CAV following T-cell depletion depends on the strain combination used.

Chapter 6: Role of hsp-27 in chronic rejection

6.1 Introduction

Chronic rejection also termed CAV is one of the major causes of cardiac allograft failure and hence death in patients who survive the first year of transplantation (Taylor et al., 2009). Compared to non transplant atherosclerosis, there is a greater inflammatory response characterised by perivascular inflammation, infiltration of inflammatory mediators and fibrosis. Hence, fibro-intimal proliferation of SMC and its subsequent migration leads to graft vasculopathy and occlusion of blood vessels (Julius et al., 2000). Infiltration of monocytes/macrophages, T-cells and fibroblasts are also found within the lesion (Hornick and Rose, 2006; Lu et al., 2011). Similarly to non-transplant atherosclerosis, CAV is also characterised by lipid containing foam cells and cholesterol clefts (McManus et al., 1995). In contrast to non-transplant atherosclerosis, CAV progresses rapidly and consists of diffuse and concentric intimal occlusion affecting both major and minor vessels. The fact that smaller vessels are involved means that treatments such as vessel bypass operations or stenting are not possible (Hornick and Rose, 2006). Hence, re-transplantation is often the only option. The aetiology of vasculopathy is not well understood, however, both alloantigen dependant and independent factors have been shown to contribute to graft rejection (Rahmani et al., 2006). It is accepted that cell mediated immunity contributes vastly to CAV (Jimenez et al., 2001). Recent studies also underline importance of humoral immunity as a contributing factor (Russell et al., 1997; Taylor et al., 2009). Antibody mediated rejection could develop as a result of the presence of preformed antibodies from sensitised patients or due to formation of de novo antibodies post-transplant (Reed et al., 2006). A number of clinical studies have shown that hsp-27 protects from non-transplant atherosclerosis (Martin-Ventura et al., 2004). In addition, previous studies have shown that hsp-27 expression was decreased with the progression of CAV or ischaemic heart disease (De Souza et al., 2005; Robinson et al., 2010). Therefore, we hypothesised that hsp-27 may be protective from chronic rejection following cardiac transplantation. In order to test this hypothesis, we performed heterotopic heart transplantation using hsp-27 transgenic

donors and studied development of allograft vasculopathy over time. The markers for cellular infiltrates as well as production of alloantibodies were measured.

6.2 Development of allograft vasculopathy

We have shown previously by immunohistochemistry analysis that hsp-27 was expressed in the SMC of transgenic mice (see chapter 3). SMC are one of the major cell types found in the intimal occlusion. Hence, the objective of this present study was to investigate whether overexpression of hsp-27 in donor hearts protects from chronic rejection. In order to understand the role of hsp-27 in CAV, heterotopic heart transplant using MHC class I and minor MHC disparate model (B10.A into CBA) was used. In our previous studies, we have demonstrated that treatment with mAb Gk1.5 at -6, -3 and -1 before transplantation depleted circulating CD4⁺ T-cells, which recovered progressively from week 3 post-injection, *i.e.* 2 weeks after transplantation (see chapter 5). In addition, we also showed that transplantation of B10.A (*H-2^a*) into CD4⁺ T-cell-depleted CBA (*H-2^k*) recipient prolonged graft survival and led to CAV (see chapter 5). Hence, in this chapter, donor hearts from hsp-27 transgenic B10.A and their LC were transplanted into CD4⁺ T-cell-depleted CBA. The animals were sacrificed at 4, 6 and 8 weeks post-transplant and the grafts were analysed for intimal thickening as described in materials and methods (section 2.5.2 of materials and methods). Staining with Miller's elastin and Van Gieson shows progressive occlusion of blood vessels over time in both transgenic and LC grafts (Figure 46). At 4 weeks after transplantation, both internal and external elastic lamina could be distinguished with clear lumen suggesting little to no sign of CAV (Figure 46a and b). However, there was a progressive occlusion of the lumen at 6 weeks post-transplant in both LC and transgenic grafts, accompanied by less discernible elastic lamina, especially in LC grafts (Figure 46c and d). Histological sections taken at 8 weeks post-transplant showed that some vessels were fully occluded while others had some occlusion (Figure 46e and f). Notably, a few ghost vessels (indicated by arrow on Figure 46e) that were fully occluded with a very shallow elastic lamina can be identified.

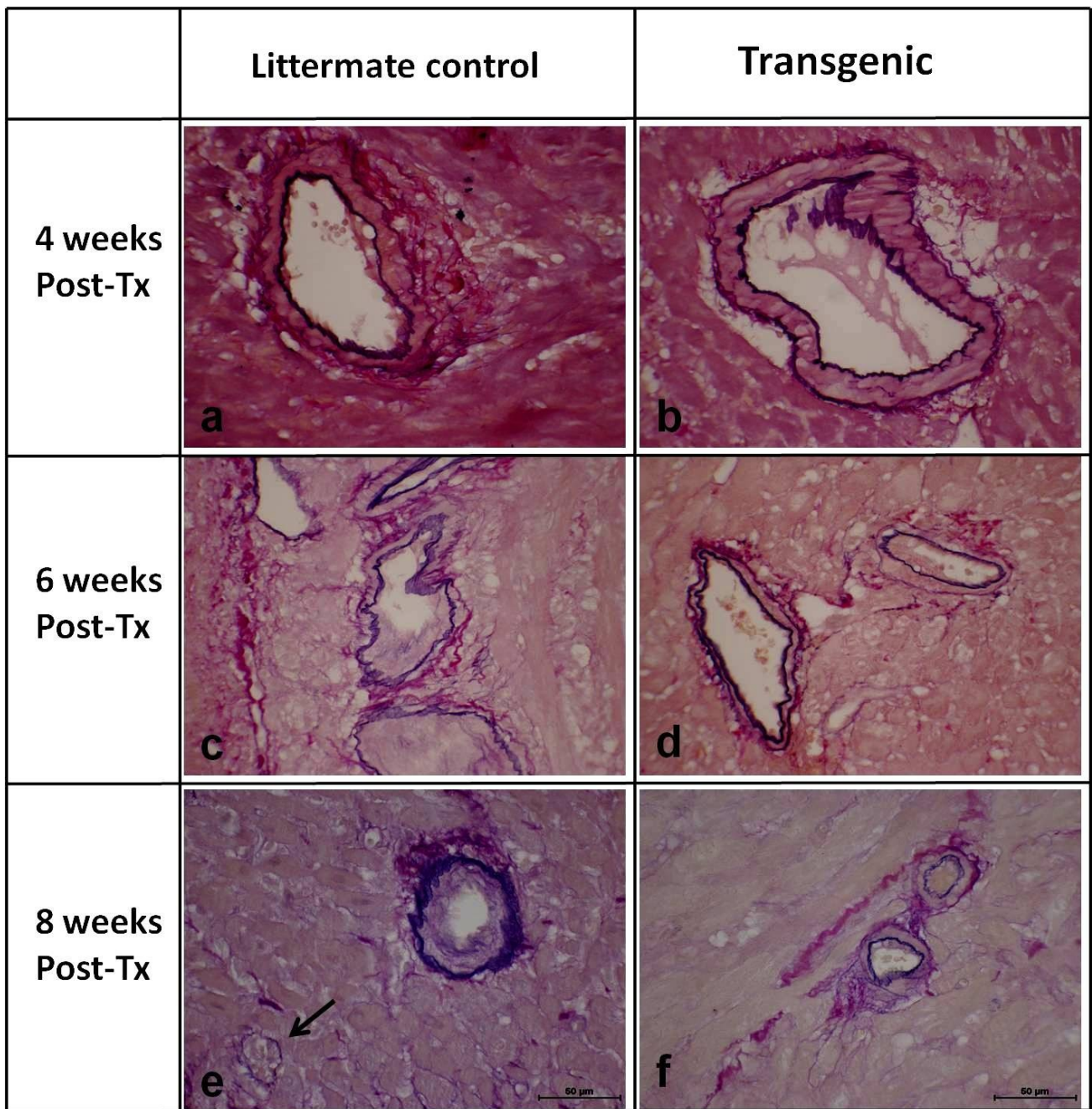


Figure 46: Representative section showing graft vasculopathy and progressive occlusion of blood vessels. Transgenic and LC allografts transplanted into CD4⁺ T-cell-depleted CBA recipients were removed at week 4, week 6 and week 8 post-transplantation and stained by Miller's elastic and Van Gieson staining as described in materials and methods. The arrow indicates the "ghost vessel". (Magnification x40, n=4-5).

The percentage of neointima was defined by measuring the area from the vessel lumen to the internal elastic lamina as shown in Figure 47.

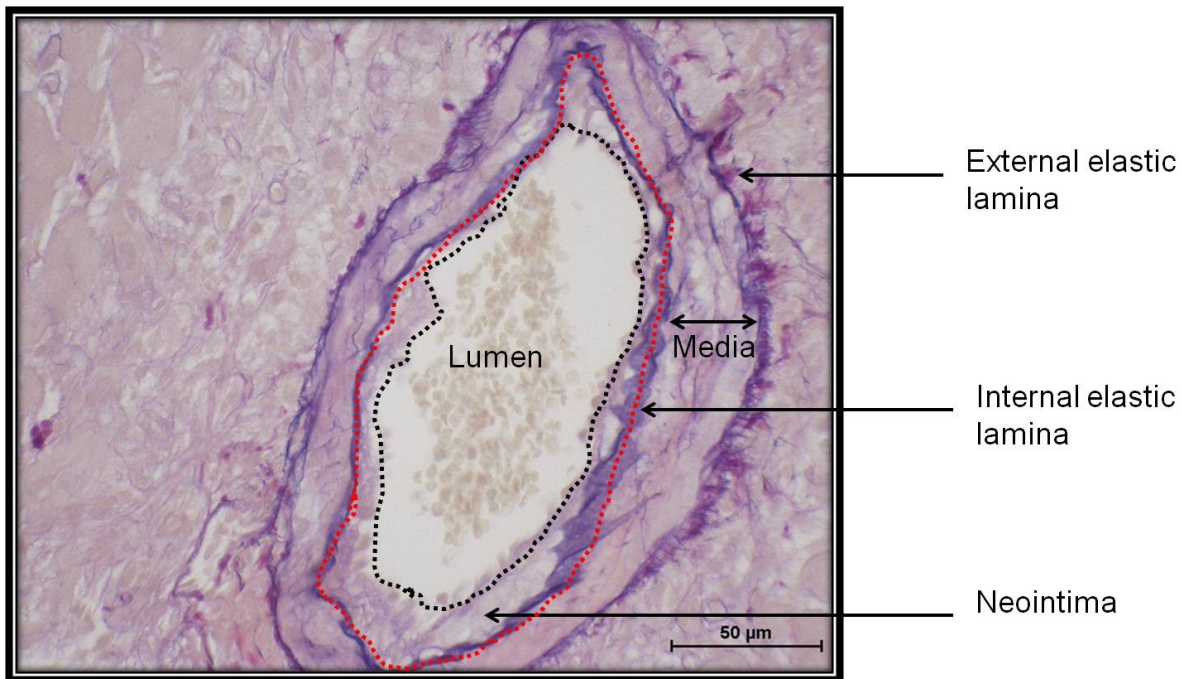


Figure 47: Diagram explaining the measurement of neointimal thickening. The photomicrograph shows different layers of blood vessel. The area between the internal elastic lamina (red dotted line) and the neointimal thickening (black dotted line) represents the neointimal hyperplasia.

Interestingly, the mean percentage of vessel occlusion was significantly lower at week 6 (33 ± 4.77) and week 8 (34.8 ± 3.22) in transgenic allografts compared to LC allografts (50.25 ± 2.84 , $p < 0.05$ and 51.8 ± 3.60 , $p < 0.01$ at week 6 and week 8 respectively). Conversely, there was no significant difference in overall vessel occlusion at week 4 between transgenic and littermate control allografts (Figure 48). To further characterise vascular thickening, the percentage of vessel occlusion was classified according to occlusive severity into 0%-20%, 20%-50% and 50% to 100% occlusion (Figure 49). Although not significant, transgenic grafts consisted of more occluded vessels in 0%-20% and 20%-50% categories compared to LC grafts. However, the latter grafts had significantly higher number of vessels that showed severe intimal thickening (50%-100%) compared to transgenic grafts.

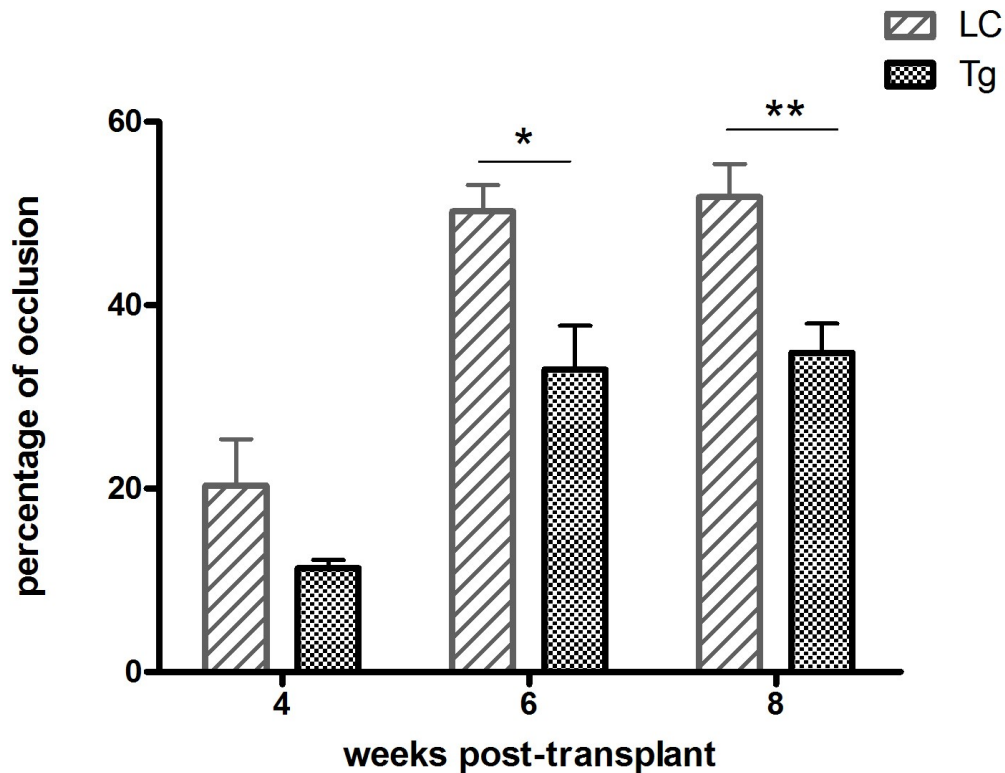


Figure 48: Percentage of vessel occlusion in transgenic and LC B10.A cardiac allografts placed in CD4+ T-cell-depleted CBA recipients. Cardiac allografts were harvested at weeks 4, 6 and 8 post-transplantation. Sections of the heart were stained with Miller's elastin and Van Gieson and the mean percentage luminal occlusion (area from vessel lumen to the internal elastic lamina) of elastin positive vessels were calculated. The Y-axis shows mean $\pm$ SEM (* $p \leq 0.05$; ** $p < 0.01$, $n=4-5$ mice, 49-67 vessels/group).

A striking characteristic of CAV is the presence of SMC in the intima (Billingham, 1994; Libby and Pober, 2001). We therefore performed immunohistochemistry analysis using an antibody against smooth muscle alpha actin. Figure 50 shows the presence of α -actin-positive SMC in the area of lesion. Hence, the luminal narrowing observed in allogeneic grafts was due to accumulation of SMC. Taken together, these results demonstrate that transplantation of B10.A heart into CD4+ T-cell-depleted CBA across MHC class I and minor mismatch leads to CAV, and hsp-27 reduce the severity of CAV.

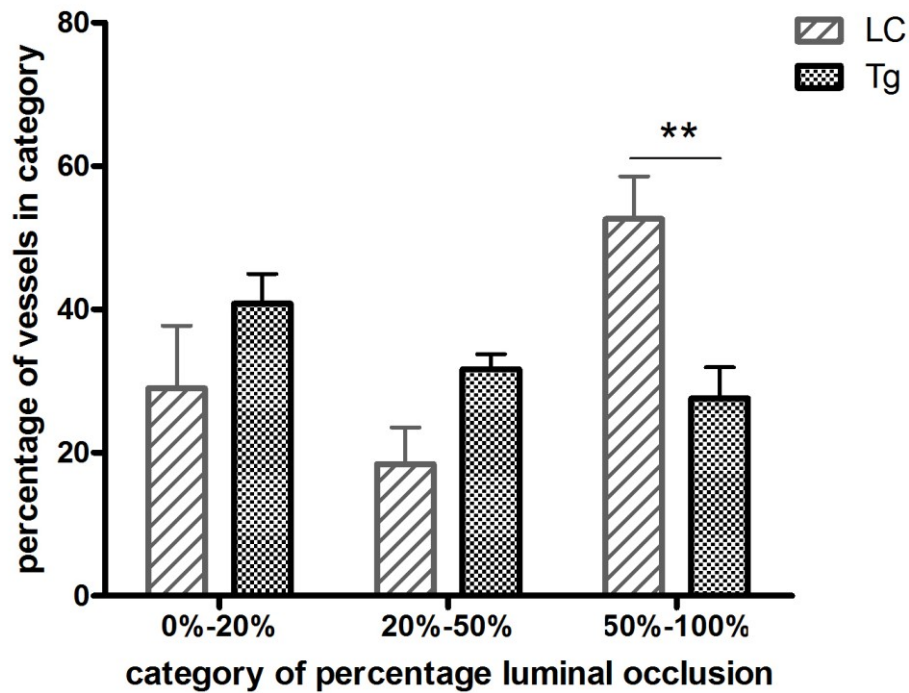


Figure 49: Distribution of vessels by occlusive severity in transgenic and LC 8 weeks after transplantation. The luminal occlusion of each elastin positive vessel was calculated and classified according to the severity of occlusion. Histogram represents mean $\pm$ SEM (** $p < 0.01$, $n=5$).

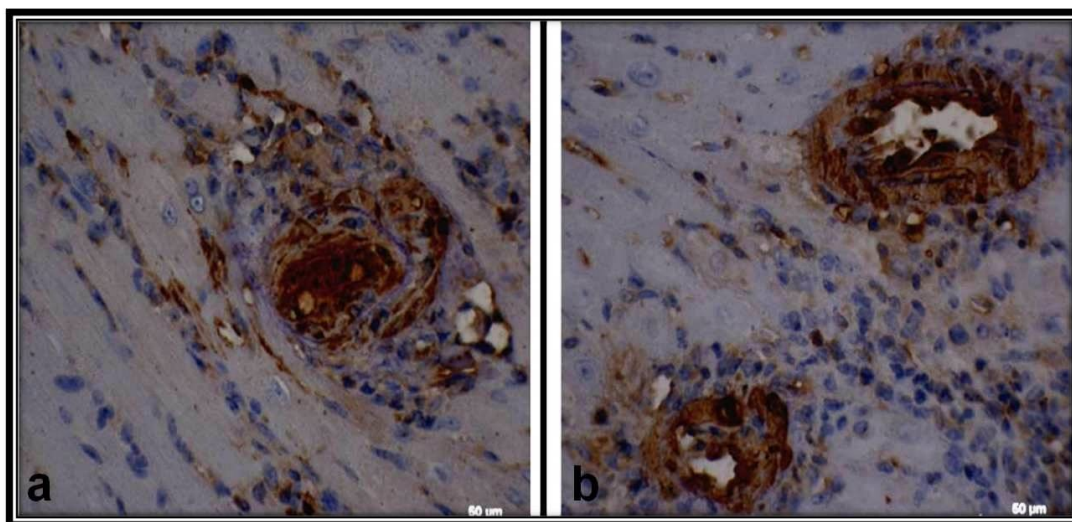


Figure 50: Presence of α -actin positive cells in cardiac allografts transplanted into CD4⁺ T-cell-depleted CBA recipients. LC (a) and transgenic (b) hearts transplanted into CD4⁺ T-cell-depleted CBA hosts were harvested at 8 weeks post-transplantation and stained for the presence of smooth muscle alpha actin. (Magnification $\times 40$, $n=3$).

6.3 Effect of hsp-27 on cell infiltration

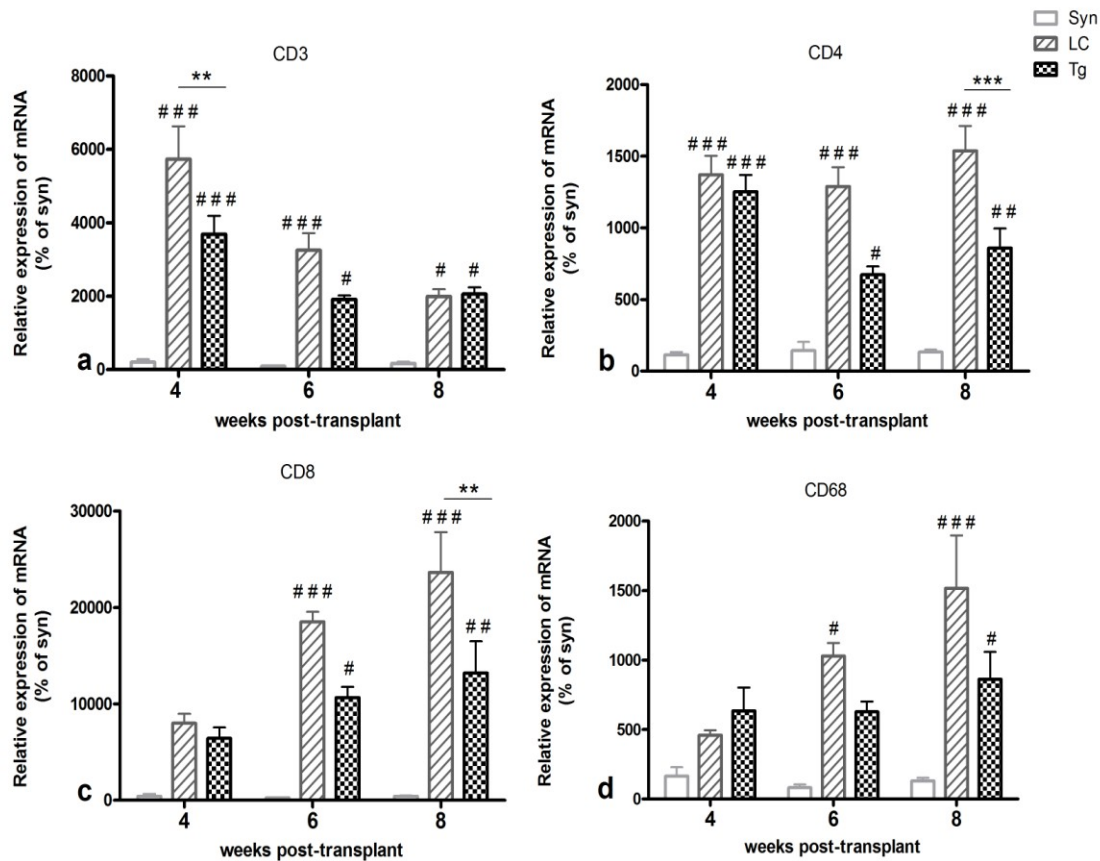


Figure 51: Effect of hsp-27 on infiltrating cells following heart transplantation: Transgenic and LC allografts as well as syngeneic heart grafts were harvested at weeks 4, 6 and 8 post-transplantation and assessed for infiltration of CD3+ (a), CD4+ (b) and CD8+ (c) T-cells as well CD68+ (d) cells by RT-PCR. Histogram shows relative expression of mRNA of each infiltrating cell normalised to that of syngeneic mRNA. (* or # $p \leq 0.05$; ** or ## $p < 0.01$ and *** or ### $p < 0.001$, * Transgenic versus littermate control, # syngeneic versus littermate control or syngeneic versus transgenic $n=3-6$).

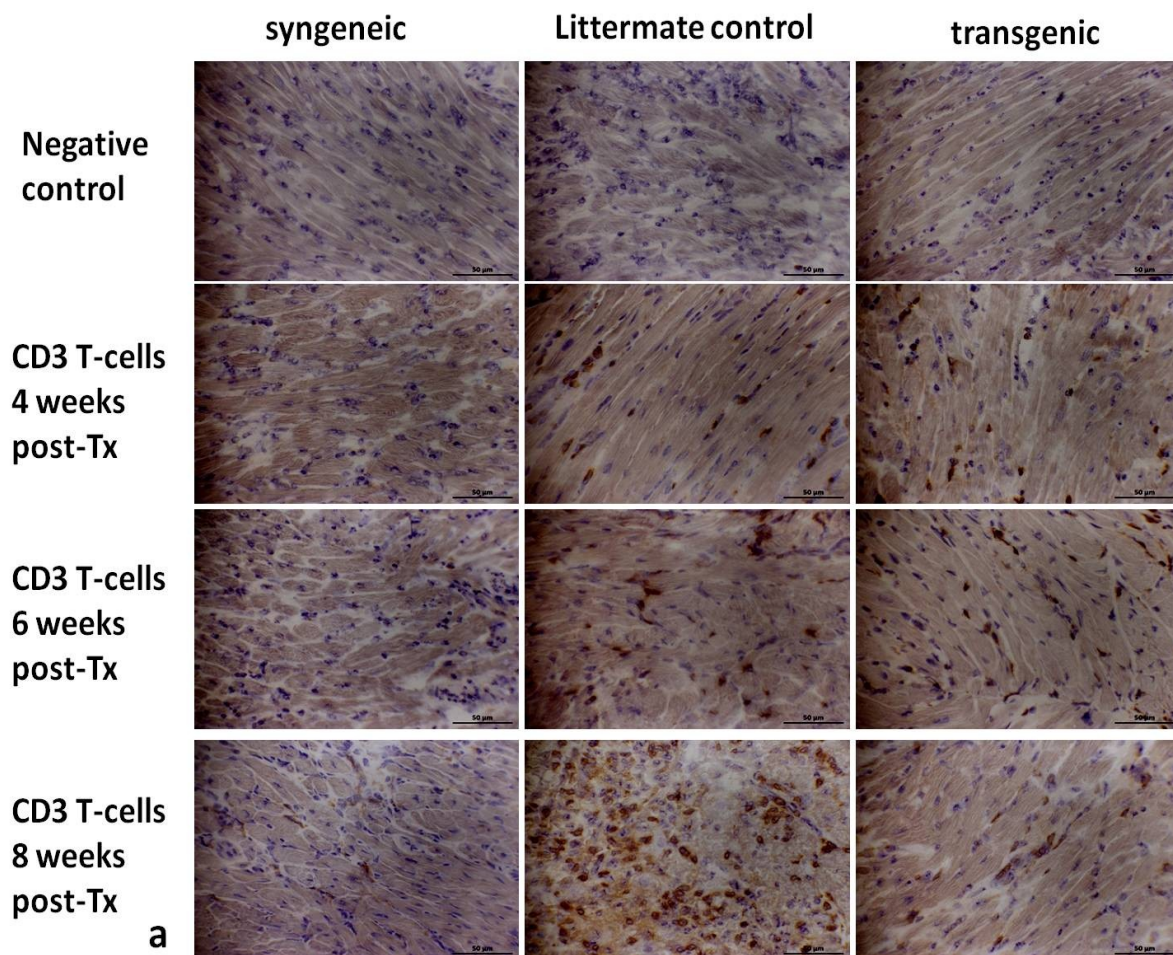
In the light of the fact that hsp-27 attenuated neointimal occlusion, we next investigated the participation of inflammatory infiltrates in the development of CAV and the influence of hsp-27 on infiltration of these cells. Grafts from recipients that received transgenic, LC or syngeneic hearts were harvested at regular time points (4, 6 and 8 weeks post-transplant) and assessed by RT-PCR for the presence of inflammatory cells (Figure 51) and their cytokine productions

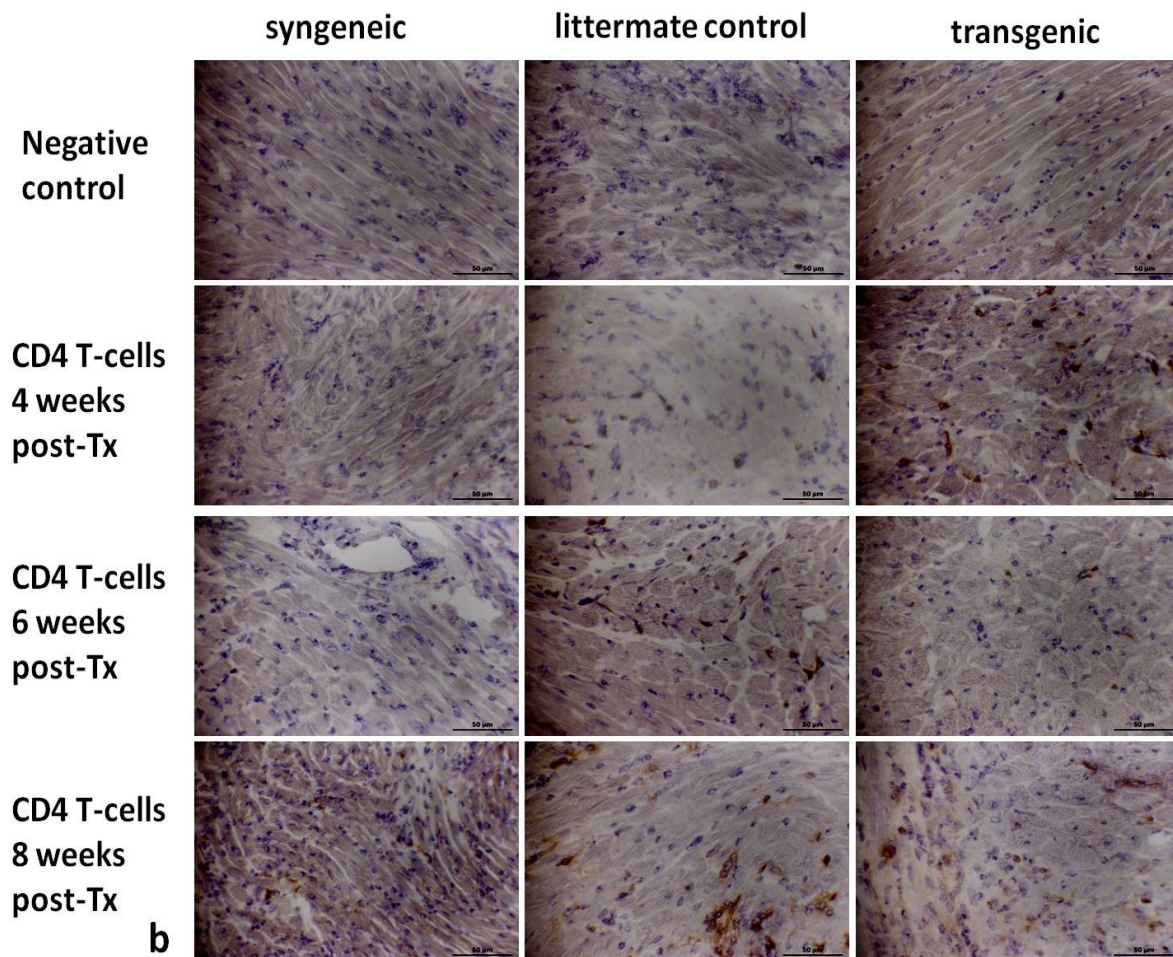
(Figure 54 and Figure 55). Infiltration of CD3+, CD4+, CD8+, and CD68+ cells occurred mainly in the allografts since there were significantly less infiltrating cells in the syngrafts. It is interesting to see that high number of CD3+ T-cells infiltrated the allografts at 4 weeks post-transplant, which then decreased progressively over time. Similarly, the expression of CD4+ T-cells also decreased in transgenic allografts while it increased in allografts from LCs. In contrast, the mRNA expression for CD8+ cells and CD68+ cells increased with the progression of the disease, although the augmentation was relatively small in transgenic allografts. Expression of mRNA for CD3+ T-cells was significantly lower in transgenic grafts compared to littermate control grafts at 4 weeks post-transplantation. Surprisingly both mRNA expression for CD4+ as well as CD8+ T-cells did not differ between both groups at 4 weeks or 6 weeks post-transplant. However, at 8 weeks after transplantation, both expression for CD4+ and CD8+ cells were significantly reduced in transgenic grafts compared to the LC's. Although mRNA transcript for CD68+ cells seemed higher in LC when compared to transgenic at 8 weeks post transplant, it did not reach statistical difference. Moreover, CD68+ cells were considerably higher in LC at 6 weeks post-transplant compared to isografts, but mRNA transcript of such cell did not differ between transgenic and syngeneic grafts. These results suggest that hsp-27 may reduce infiltration of these graft damaging cells.

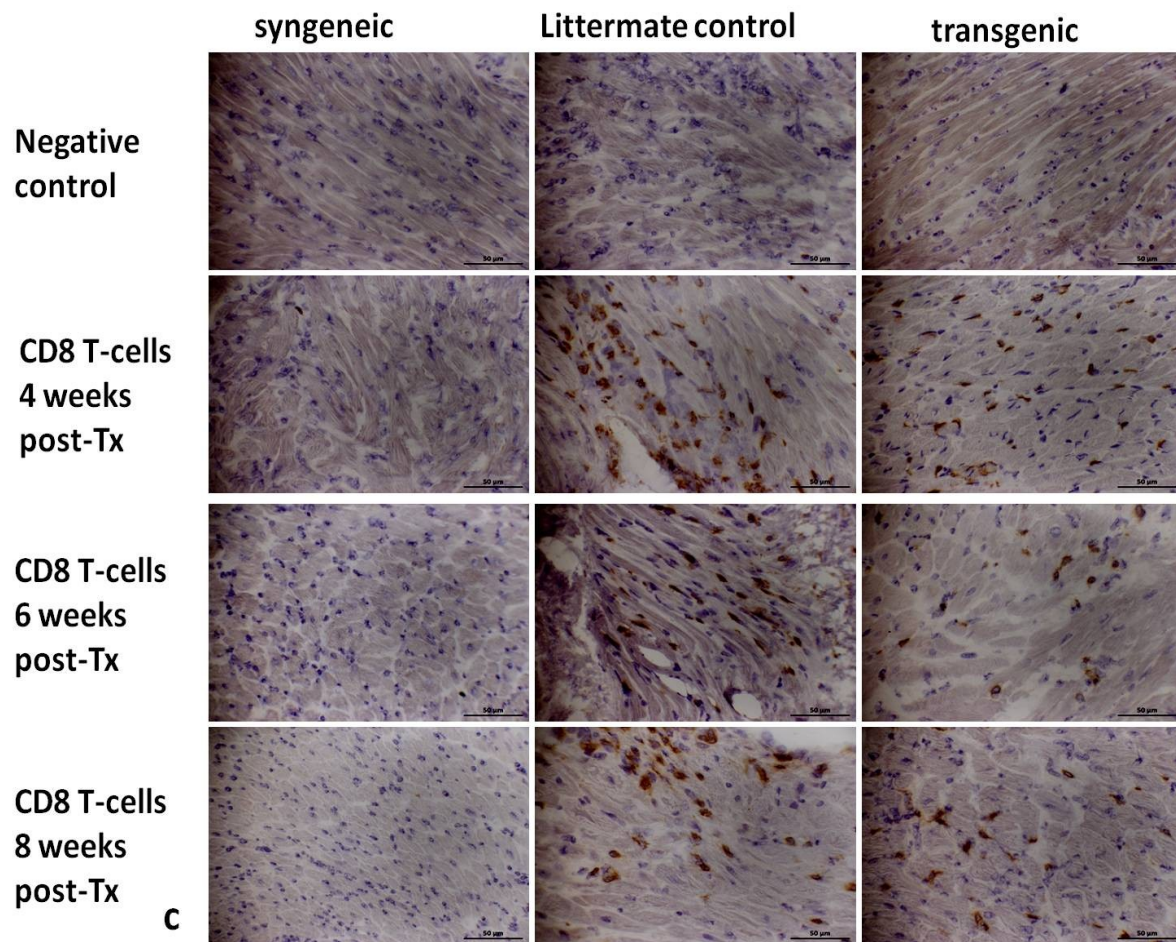
6.4 Expression of infiltrates at protein level.

To gain more insight on cellular constitution intragraft and to confirm the RT-PCR results at protein level, sections of heart grafts harvested at 4 weeks, 6 weeks and 8 weeks post-transplant were subjected to immunological analyses. Immunohistochemistry was performed to identify the presence of CD3+ (a), CD4+ (b), CD8+ (c) and CD11b+ cell population intragrafts (Figure 52). Expression of hsp-27 was associated with reduced number of infiltrating cells. In fact, CD8+ (Figure 52c) and CD11b+ (Figure 52d) cells seemed to be fewer in transgenic grafts compared to LC's. Figure 52a shows that, in contrast to RT-PCR results, CD3+ T-cells seemed to increase from week 4 to week 8; in fact, large numbers of CD3+ cells were manifest in LC at 8 weeks post-transplant, but very few CD3+ T-cells were present in transgenic grafts. The

expression of mRNA for CD8 gene (Figure 51c) also differs with the protein expression for CD8+ T-cells analysed by IHC (Figure 52c). In fact, the progressive increase of CD8 mRNA demonstrated by RT-PCR analyses was not seen in microscopic images. Small numbers of CD4+ T-cells were present in transgenic and LC (Figure 52b). Moreover, CD4+ T-cells seemed to have decreased at 8 weeks post transplant in transgenic group. There seemed to be reduced number of CD11b+ cells in transgenic allografts compared to littermate controls. However, in contrast to RT-PCR, histological analyses did not show progressive increase in monocytic population overtime. On the other hand, histological analyses confirmed the RT-PCR results that CD3+, CD4+ and CD8+ T-cells did not infiltrate the isografts. However, although CD11b+ cells populated the isografts at early time point (4 weeks), the number of these cells declined considerably at 6 week and 8 week post-transplant in isografts.







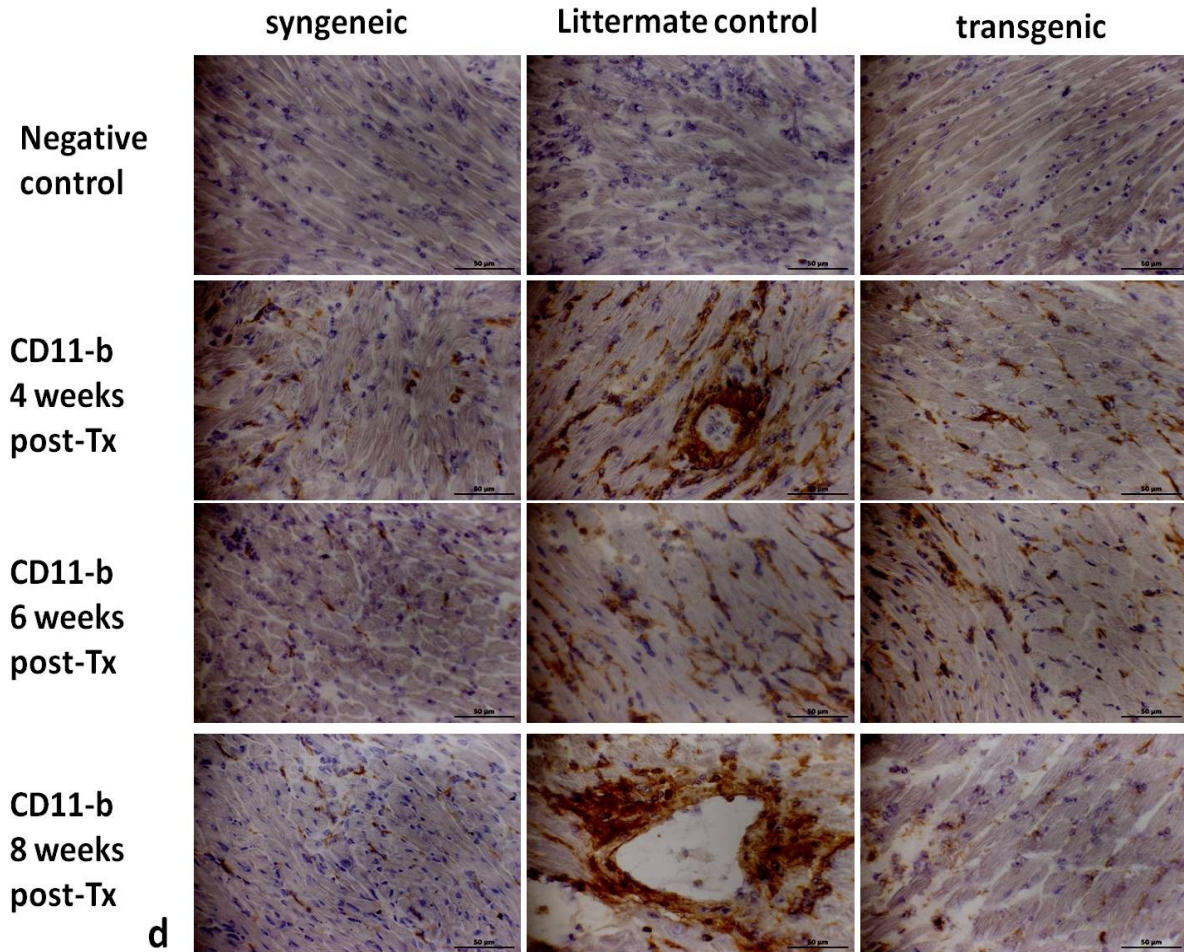


Figure 52: Immunohistochemical analyses of explanted transgenic and LC allografts and control isografts from CD4⁺ T-cell depleted hosts show cellular infiltration. The grafts collected at week 4, 6 and 8 were embedded in OCT and cryo-sections of 7 μ m were obtained. Immunoperoxidase staining was used to identify infiltration of CD3⁺ (a), CD4⁺ (b) and CD8⁺ (c) T-cells as well as CD11-b⁺ (d) monocytes/macrophages cell population. (Magnification x40, n=4-6).

Immunohistochemistry was also performed in the same hearts using antibody to HA in order identify the expression of HA tagged hsp-27. Figure 53 shows that, as expected, only transgenic grafts expressed HA, hence hsp-27. Histological analyses revealed prominent expression of hsp-27 at 4 weeks and this decreased with the progression of CAV. In fact, a weak expression of hsp-27 by few cells was seen at 8 weeks post-transplant.

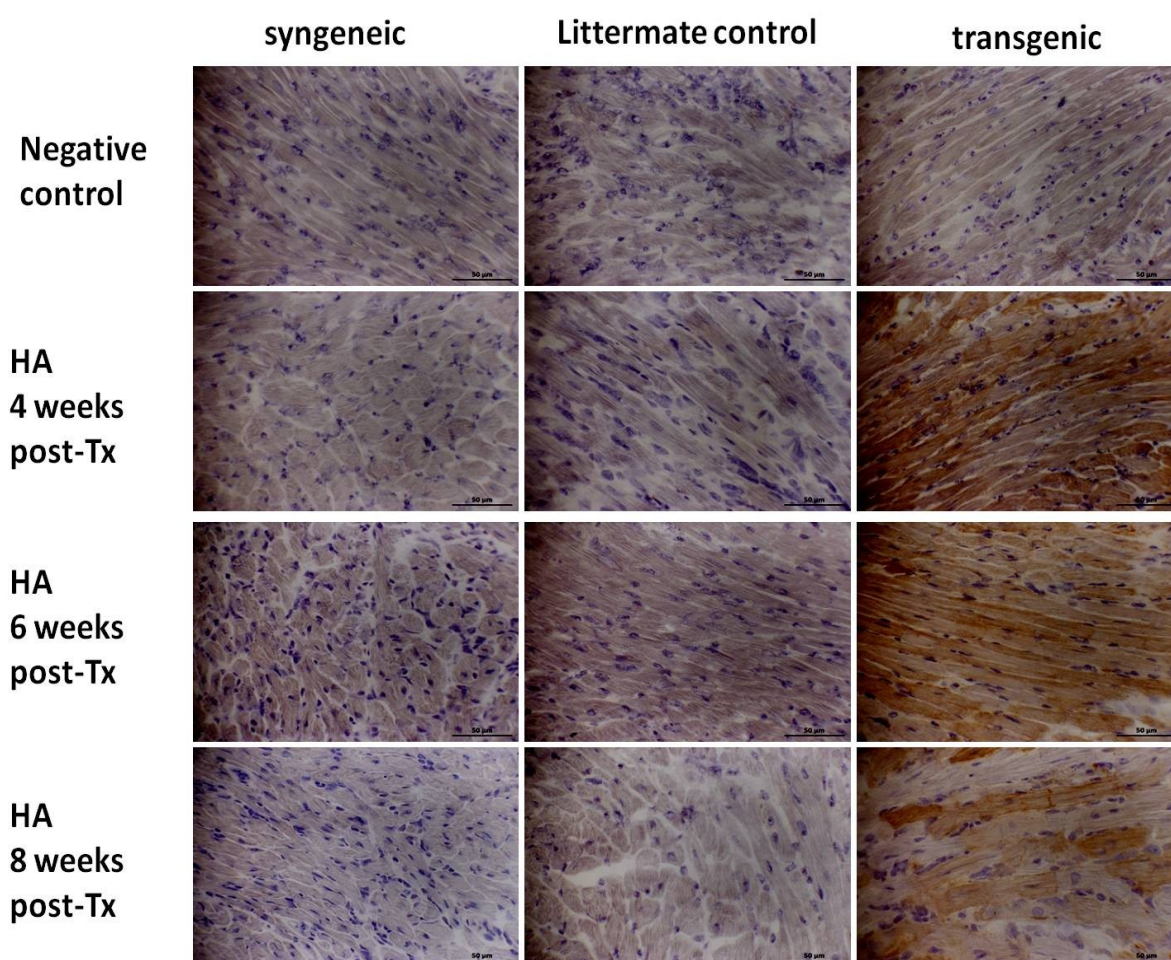


Figure 53: A representative section of transgenic or LC allograft or isograft from B10.A to CBA at 4, 6 and 8 weeks after transplantation. The recipients were pre-treated with anti-CD4 mAb prior to transplantation. Harvested grafts were frozen in OCT and 7µm cryosections were obtained. These were then labelled with primary antibody to HA to visualize the presence of HA tagged hsp-27 at x40 magnification. (n=4-6).

6.5 Effect of hsp-27 on cytokine production

To further delineate the effect of hsp-27 on graft rejection, we investigated whether hsp-27 modulates the cytokine profile present in the allografts (Figure 54 and Figure 55). RT-PCR analyses were performed on transgenic, LC and syngeneic grafts collected at 4, 6 and 8 weeks post-transplantation. RT-PCR results showed that the pro-inflammatory cytokines IFN-γ (Figure 54a) as well as IL-6 (Figure 54b) follow a similar pattern to CD4⁺ cells. In fact, the expression level of IL-6 in LC and IFN-γ in both transgenic and LC allografts were significantly high

compared to syngrafts at 4 weeks post-transplant but the expression level of both of these cytokines decreased and plateaued at 6 and 8 weeks post-transplant.

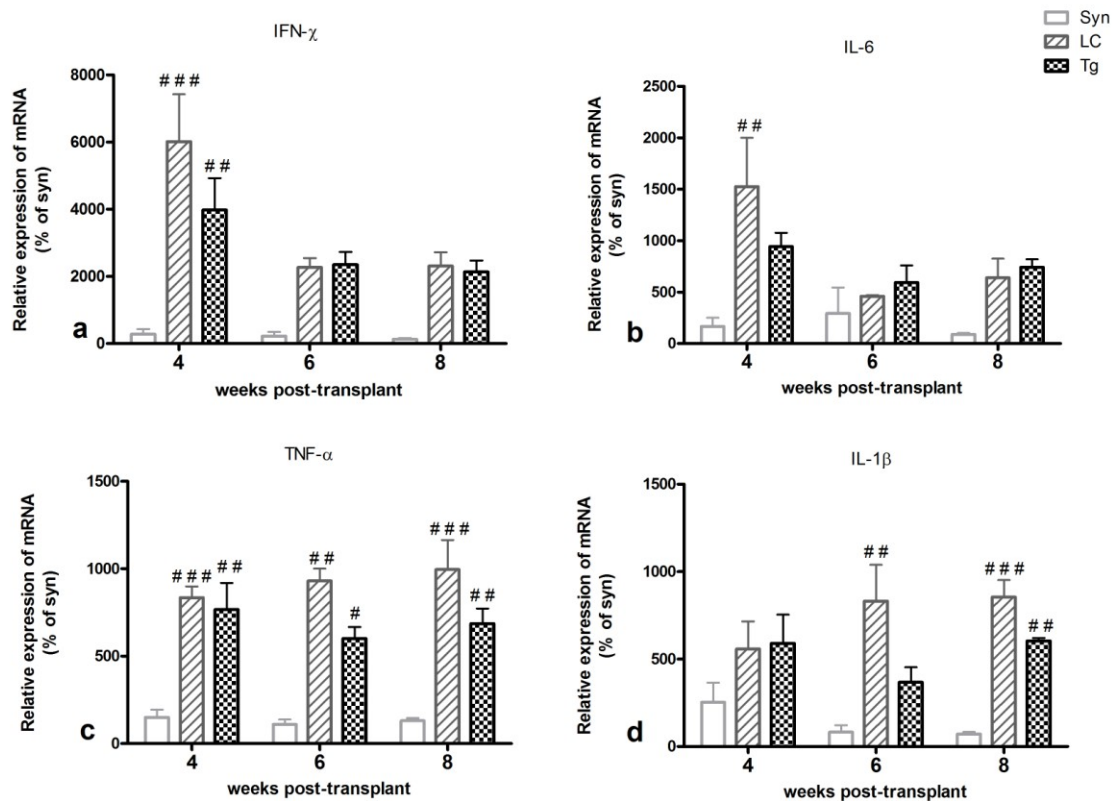


Figure 54: Effect of hsp-27 on the expression of pro-inflammatory cytokines following heart transplantation. Transgenic and LC allografts as well control isografts were harvested at 4, 6 and 8 weeks post-transplantation and the expression of pro-inflammatory cytokines IFN- γ (a), IL-6 (b), TNF- α (c) and IL-1 β (d) were analysed by RT-PCR. Two-way ANOVA followed by Bonferroni corrections were used to calculate statistical significance. Comparison between transgenic and LC did not reach statistical significance for cytokines tested. (# p ≤ 0.05; ## p < 0.01 and ### p < 0.001, # syngeneic versus littermate control or syngeneic versus transgenic n=3-6).

Interestingly, levels of TNF- α as well as IL-1 β were also stable throughout the study period, while there was a slight increase in LC grafts (Figure 54c and d respectively). Although it was not statistically significant TNF- α and IL-1 β seemed to be considerably high in LC compared to transgenic at 6 weeks and 8 weeks post-transplant. On the other hand, expression of IL-2 was very low and not measurable in all samples tested (data not shown). Overall, the study demonstrated that the inflammatory cytokines IFN- γ , IL-6, TNF- α and IL1- β contribute to the

development of CAV since their levels were significantly different in allografts compared to syngrafts. However, the expression of these inflammatory cytokines appeared to be reduced in transgenic grafts compared to LC grafts.

Having observed that hsp-27 may reduce the expression of inflammatory cytokines, we then investigated whether overexpression of hsp-27 affects the expression of anti-inflammatory cytokines. To achieve this, transgenic, LC and syngeneic grafts were harvested at 4, 6 and 8 weeks post-transplant and looked at the expression of anti-inflammatory cytokines IL-4, IL-5, IL-10 and TGF- β by RT-PCR (Figure 55). It is interesting to note that IL-4 was significantly and considerably higher in transgenic allografts compared to LC at 4 weeks post-transplant (Figure 55a). The expression level of IL-4 increased slowly in LC grafts over a period of weeks and reached similar levels to those seen in transgenic grafts at 6 and 8 weeks. There was a trend for increased mRNA levels of the anti-inflammatory cytokines IL-5 and IL-10 in transgenic grafts compared to LC at 4 weeks following transplantation (Figure 55b and c respectively). However, IL-10 mRNA levels were comparable between transgenic and LC grafts at 6 and 8 weeks post-transplant. Conversely, although the expression of TGF- β was low at 4 weeks and 6 weeks post-transplant in both transgenic and LC grafts, the expression was upregulated in LC at 8 weeks post-transplant (Figure 55d). In fact, though not statistically significant, TGF- β was considerably higher in LC compared to transgenic allografts at 8 week. Taken together, these results suggest that hsp-27 may possibly delay the development of CAV by stimulating IL-4 production. There is also the possibility that hsp-27 may enhance the production of other anti-inflammatory cytokines.

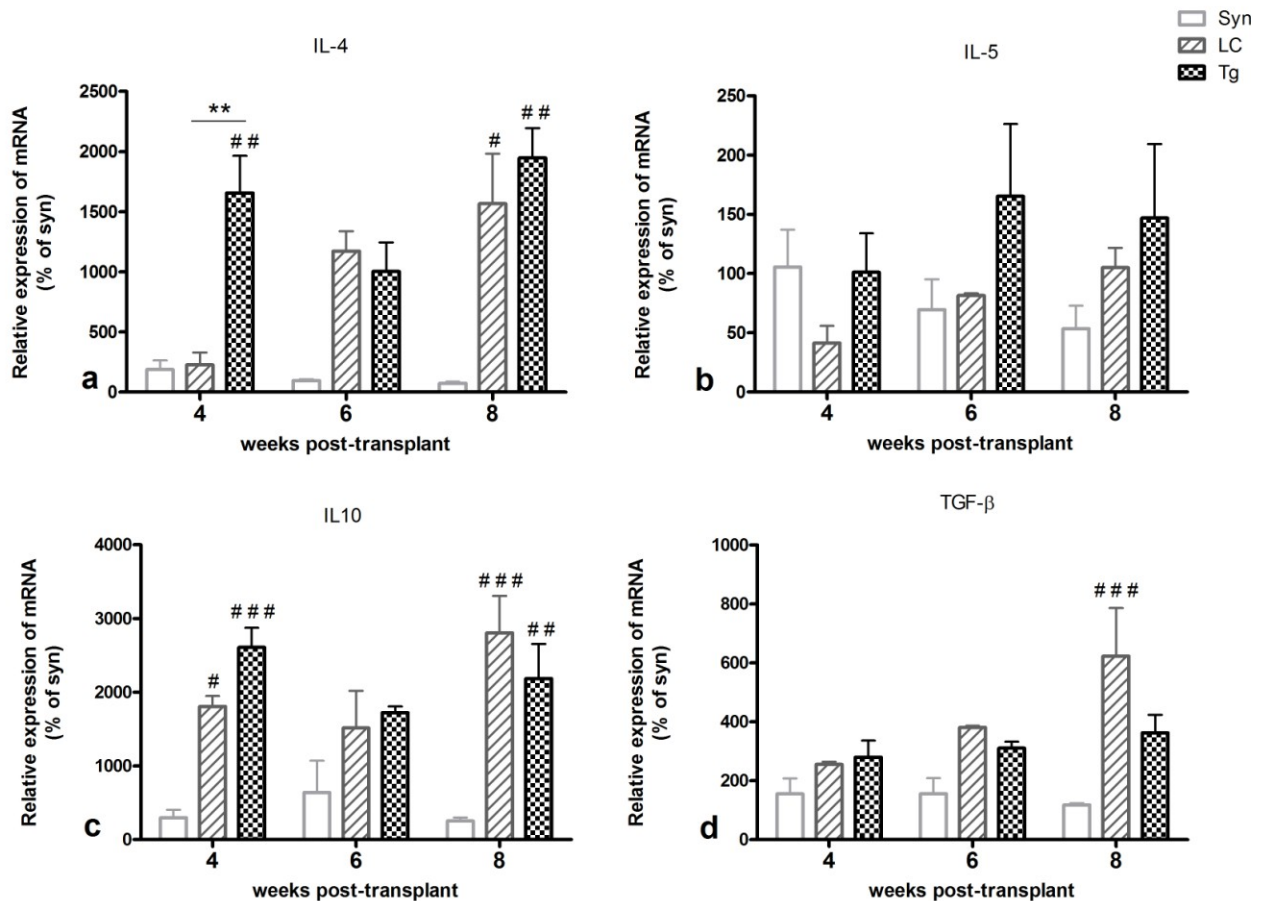


Figure 55: Effect of hsp-27 on the expression of anti-inflammatory cytokines following heart transplantation. Transgenic and LC allografts as well control isografts were collected at 4, 6 and 8 weeks post-transplantation and the expression of anti-inflammatory cytokines IL-4 (a), IL-5 (b), IL-10 (c) and TGF-β (d) were analysed by RT-PCR. Two-way ANOVA followed by Bonferroni corrections were used to calculate statistical significance. (* or # $p \leq 0.05$; * or ## $p < 0.01$ and *** or ### $p < 0.001$, * transgenic versus LC; # syngeneic versus littermate control or syngeneic versus transgenic $n=3-6$).

6.6 Effect of hsp-27 on alloantibody production.

Antibody mediated rejection is associated with worse survival outcome than cell mediated rejection and contribute significantly to graft vasculopathy. Previous works have demonstrated that alloantibodies contributed to the development of CAV (Russell et al., 1997; Russell et al., 1994b; Smith et al., 2005; Soleimani et al., 2006). Sera from CD4⁺ T-cell-depleted recipient mice that have received transgenic or LC heart or isograft were collected at 8 weeks post-

transplantation and analysed by flow cytometry for presence of alloreactive antibodies against the donor (Figure 56). The level of alloantibodies present in the serum of recipient that received LC and transgenic graft were comparable, suggesting that hsp-27 may not influence alloantibody production. However, our results suggest there is production of alloantibody from the recipients that received allogeneic grafts, but the level of circulating antibodies did not statistically differ between allogenic and syngeneic grafts at 8 weeks. It may be possible that antibody mediated response was elicited at early time points, and the alloantibodies are adsorbed by the graft.

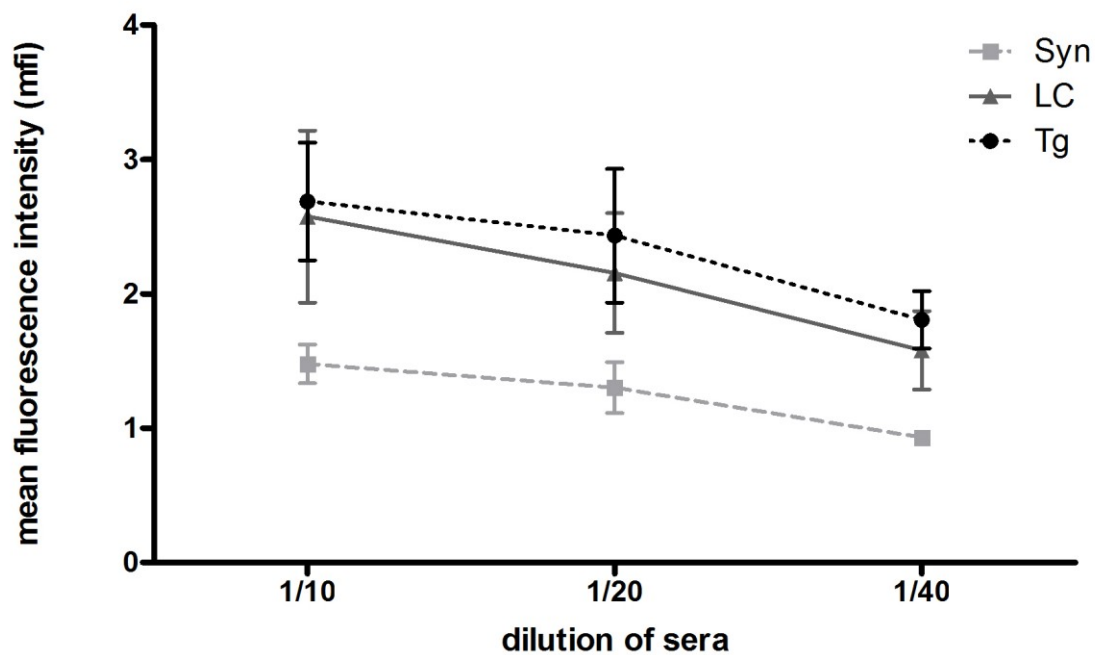


Figure 56: Alloantibody titre after heterotopic cardiac transplantation in CD4⁺ T-cells depleted CBA recipients. Serum collected from recipients that received transgenic, LC or syngeneic hearts at the time of sacrifice, at 8 weeks post-transplant, were incubated with B10.A splenocytes at 1/10, 1/20 and 1/40 dilutions and the presence of alloantibody against the donor was tested by flow cytometry. Alloantibody titre was expressed as mean fluorescence intensity. Two-way Anova with Bonferroni corrections were performed and found no statistical difference between groups tested. (n=4-5).

6.7 Discussion

6.7.1 Confirmation of CAV development

The study had addressed the role of hsp-27 in development of CAV. In this study, hsp-27 transgenic and their LC were transplanted into CBA recipients that were previously depleted with anti-CD4 mAb. Treatment with anti-CD4 therapy in this MHC class I and minor mismatched model has been shown to delay the graft rejection and allow the graft to develop CAV (see chapter 5). CAV is associated with intimal thickening and luminal occlusion, where SMC play a major role. Interestingly, hsp-27 is constitutively expressed on cardiomyocyte and smooth muscle of transgenic animals used in this study (see chapter 3). Human studies have also reported its expression on endothelial cells (De Souza et al., 2005; Jozefowicz-Okonkwo et al., 2009; Robinson et al., 2010). Previous studies have shown that, the expression hsp-27 was 20 fold higher in long term cardiac patients who did not reject the heart compared to patients who developed CAV (De Souza et al., 2005). Moreover, hsp-27 was also reported to be involved in ischaemic heart disease, where patients with coronary artery disease had decreased level of hsp-27 compared to healthy coronary vessels (Robinson et al., 2010). These studies implied that hsp-27 may protect from ischaemic heart disease and CAV. In line with these studies, here we report that overexpression of hsp-27 reduced the severity of CAV (Figure 48 and Figure 49).

Smooth muscle cell proliferation and migration are central to the pathogenesis of CAV (Poon et al., 1996). In recent years, attempts have been made to inhibit such proliferation in order to prevent graft rejection using proliferation signal inhibitors including rapamycin (also known as sirolimus) and everolimus (Poon et al., 1996; Raichlin and Kushwaha, 2008). Interestingly, a previous study using various form of hsp-27 (hyperphosphorylated, hypophosphorylated and wild-type hsp-27) and *in vitro* techniques demonstrated that hsp-27 inhibits proliferation of a human microvascular endothelial cell line and human telomerase reverse transcriptase subunit SMC (Trott et al., 2009). Another experimental *in vitro* study showed that overexpression of mutant hsp-27 where serine was substituted with alanine (which results in hypophosphorylation)

inhibited smooth muscle cell migration (Hedges et al., 1999). We have shown that hsp-27 is overexpressed in smooth muscle cell of transgenic animals used in this study. Hence, it is possible that the decline in vascular thickening seen in transgenic allografts may be due to inhibition of smooth muscle cell proliferation and migration.

At this stage, it is important to underline that the origin of cells leading to intimal hyperplasia has been much debated. Hillebrands et al showed using an experimental aortic transposition transplant model that neointimal SMC as well as endothelial cells were of recipient origin (Hillebrands et al., 2001). They then further confirmed this with whole heart transplants. In fact, they performed allogeneic cardiac transplantation using female as donor and male as recipient and identified by single cell PCR analysis using probe specific for Y-chromosome DNA sequence, that SMC in the neointimal lesion are of recipient origin (Hillebrands et al., 2000). Based on these observations, this group postulated that the mechanisms that control the normal healing process (of vessel wall following alloimmune mediated injury) is impaired and thus the healing process continue even after vascular restoration (Hillebrands et al., 2000), thereby leading to graft vasculopathy (Hillebrands et al., 2000; Hillebrands et al., 2001). In contrast, studies in humans argue that SMC found in atherosclerotic lesion area are of donor origin (Atkinson et al., 2004). These authors postulated that a pre-existing intimal lesion contributes to the development of CAV. Following vascular injury, migration of donor derived SMC from media to intima and its proliferation during vascular remodelling is responsible for vascular injury. In our study, we did not investigate on the origin of SMC. If they were of donor origin, it is possible that hsp-27 prevents migration and proliferation of SMC as discussed previously. In contrast, in the event that population of neointimal SMC are of recipient origin, it is not known how hsp-27 could prevent atherosclerotic lesion. However, it was shown that vascular healing due to severe medial damage resulted in recruitment of recipient cells in the intima (Campbell et al., 2001; Han et al., 2001). It is thus conceivable that hsp-27 may protect from medial destruction caused by apoptosis (anti-apoptotic effect of hsp-27) and hence prevents replacement of SMC by

recipient cells (Hirsch et al., 1998). Unfortunately, the interpretation of this chapter is limited due to restricted knowledge of the origin of SMC in the hyperplasia.

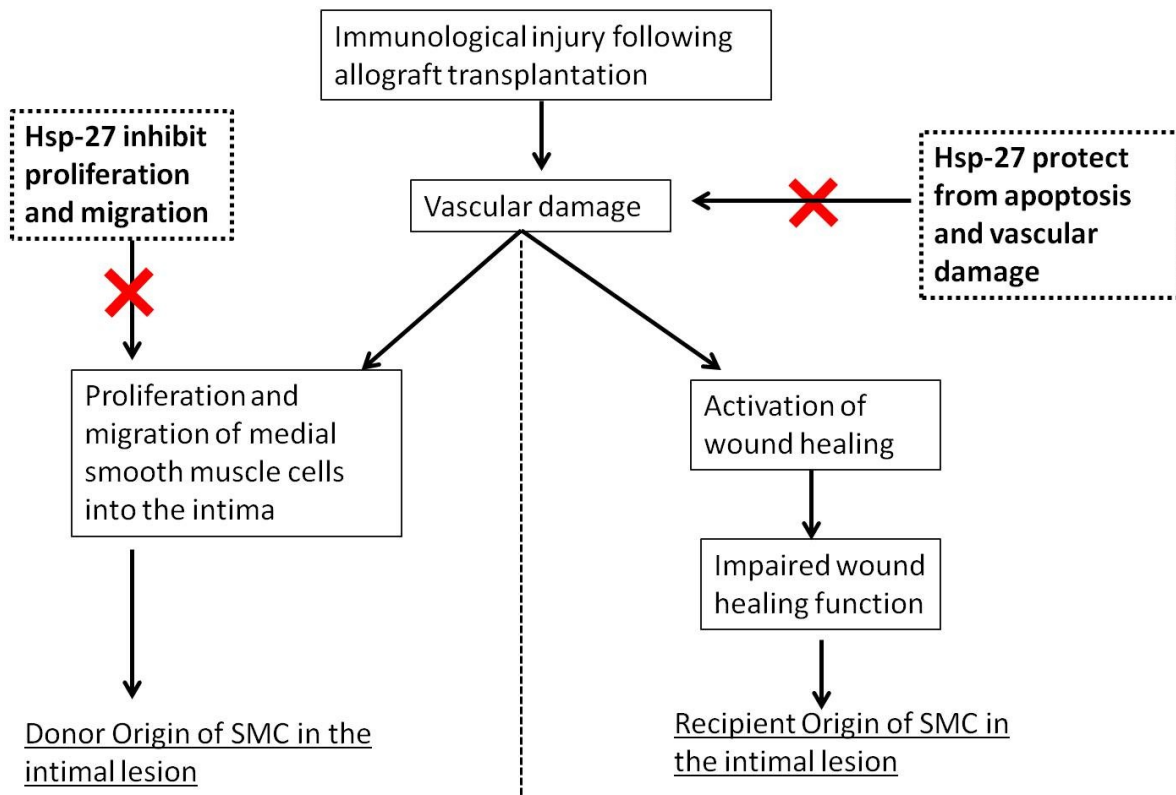


Figure 57: A representative diagram explaining possible origin of neointimal SMC and mechanisms of how hsp-27 may prevent neointimal vasculopathy.

On the other hand, CAV is characterised by interstitial fibrosis and accumulation of extra cellular matrix in the fibrotic area. Although the exact cause and origin of graft fibrosis is not known, endothelial to mesenchymal transition has been proposed as one of the possible contributing mechanism in this effect (Zeisberg et al., 2007). However, in oncology studies, hsp-27 has been shown to promote epithelial mesenchymal transition (EMT) (Mizutani et al., 2010; Wei et al., 2011). Therefore, it would be interesting to investigate whether fibrosis develops in transgenic cardiac grafts. In relation to this, we showed that TGF- β -a cytokine that drives development of fibrosis in the graft- is upregulated in LC grafts but not in transgenic allografts or syngrafts at 8 weeks post-transplant (Figure 55d). It is thus conceivable that hsp-27 inhibits fibrotic lesion through downregulation of TGF- β . However, the role of TGF- β is complex. It has been shown that TGF- β modulates cellular immune responses by promoting Th2 cells (anti-inflammatory) on

one hand but differentiation of Th17 cells (pro-inflammatory) on the other hand (Hatton, 2011; Ludviksson et al., 2000; Qin et al., 2009). We did not investigate Th17 cytokines in this study.

6.7.2 Role of hsp-27 in cellular immunity and CAV

Understanding the mechanisms that leads to CAV is crucial in order to prevent chronic rejection. Here we investigated the participation of cellular immunity in graft vasculopathy. Previous studies have demonstrated the central role of CD4⁺ and CD8⁺ T-cells in the immune response towards CAV (Nagano et al., 1998; Obata et al., 2005; Rosenberg et al., 1987; Uehara et al., 2006). T-cell depleted recipients showed reduced CAV (Nejat et al., 2008; Skaro et al., 2005; Szeto et al., 2002; Uehara et al., 2006). Shi et al used various experimental mutant mice model as recipients for carotid artery transplantation to show that B-cells, CD4⁺ T-cells and macrophages participate in the genesis of CAV, while CD8⁺ T-cells and NK cells were not involved in the development of CAV (Shi et al., 1996). In addition, T-cells mediate endothelial and vascular SMC dysfunction through IFN- γ secretion and dysregulation of the expression of nitric oxide syntase (Koh et al., 2004). Our study further consolidates this, since we provide evidence that CD3⁺, more specifically CD4⁺ as well as CD8⁺ T-cells participate in CAV as demonstrated by high levels of these cells in allografts and low level of such infiltrates in isografts (Figure 51). In addition, we also reported the infiltration of allografts by monocytic population (Figure 51d). These results are in line with other studies that demonstrated presence of macrophages as well as CD3⁺ T-cells in the intima, media and adventitia of arteries and veins of explanted hearts from patient undergoing re-transplantation (Lu et al., 2011). Similarly, production of cytokines involved in inflammation occurs in allogeneic but not syngeneic transplants, implying that their expression is dependent on alloantigen stimulation. In addition, RT-PCR results of cytokine expression showed that most of inflammatory cytokines were increased in allogeneic grafts compared to isografts (Figure 54). This further strengthens the observation of an alloimmune mediated response towards the graft and confirms the observation that Th1 responses towards the graft are predominantly found during chronic rejection (Obata et al., 2005).

Interestingly, cellular infiltrations -CD3+, CD4+ and CD8+ T-cells- were significantly reduced in transgenic allografts, suggesting that hsp-27 regulates cellular immunity (Figure 51 and Figure 52). Moreover, the cytokines secreted by these cells are also regulated. Particularly, pro-inflammatory cytokines had a tendency to be higher in LC grafts compared to mRNA transcripts present in transgenic grafts. Our findings showed increased level of IFN- γ in both transgenic and LC allografts compared to isografts. However, it was insignificantly lower in transgenic grafts than in LC grafts. IFN- γ is one of the central pro-inflammatory cytokine and its role in the development of graft atherosclerosis seems critical (Nagano et al., 1998; Nagano et al., 1997). In relation to this, MHC class II disparate cardiac transplant into recipients with IFN- γ gene deficiency (as a result of targeted gene disruption or treatment with neutralising antibody) developed no intimal thickening (Nagano et al., 1997). This was associated with decreased expression of MHC class II as well as adhesion molecules VCAM-1 and ICAM-1. Interestingly, IFN- γ induces expression of MHC class I and MHC class II (Takei et al., 2000) and promote cellular immunity by activating T-cells, macrophages and NK cells (Mosser, 2003; Schroder et al., 2004; Takei et al., 2000). In this regard, Tellides et al used donor human arterial grafts in SCID mice and demonstrated that IFN- γ was able to induce transplant associated atherosclerosis in the absence of leukocytes through sustained expression of MHC class I and MHC class II and growth factor induced cell division of vascular SMC (Tellides et al., 2000).

On the other hand, our results showed that the expression of anti-inflammatory cytokines were increased in transgenic compared to LC grafts. In particular, IL-4 was significantly and considerably increased in transgenic grafts, at early times after transplantation. However, previous study using CD4+ T-cell depleted-IL-4 KO mice as recipient showed that the graft survival was only slightly but not significantly prolonged in IL-4 KO mice compared to control (Mottram et al., 1998a). Moreover, these long-surviving hearts displayed signs of CAV. However, it is possible that the IL-4 KO mice may have developed alternative mechanisms that lead to transplant rejection and CAV. In fact, another study by Mottram et al using IL-4 KO mice showed increased TNF- α , IL-12 and allograft inflammatory factor-1 (AIF-1) intragraft of IL-4 KO

recipients compared to wild-type recipients, hence highlighting the IL-4 KO displayed elevated inflammatory activity by macrophages (Mottram et al., 1998b). In addition, we showed in the acute rejection model (see chapter 4), that prolongation of graft survival was associated with increased infiltration of monocytes, but these monocytes are probably of anti-inflammatory nature since the transgenic grafts had decreased IFN- γ and increased IL-4 expression ((Nahrendorf et al., 2007; Swirski et al., 2009)). It is well known that IL-4 is a -potent anti-inflammatory cytokine that hinders Th1 differentiation, hence cellular immunity. In light of this, studies of cardiac transplant patients showed that IL-4 producing helper T-cells were reduced in patients who suffered from episode of rejection compared to those who had no signs of rejection, hence suggesting that IL-4 is associated with protection from rejection (Bijlsma et al., 2002). However, the interpretability of this chapter is limited and detailed investigation of the monocytic population and their involvement in CAV is required to confirm whether increased IL-4 is beneficial against development of vasculopathy.

In addition, TGF- β was increased in LC grafts at 8 weeks post-transplant. However, the role of TGF- β is controversial. It is required for graft acceptance in the early days following transplantation (Bickerstaff et al., 2001). But others have examined myocardial biopsies and reported that cardiac allograft rejection is correlated with increased expression of TGF- β as well as IL-6 (Zhao et al., 1993). Interestingly, we also reported increase in mRNA transcripts for IL-6 and TGF- β in LC graft compared to isograft at 4 weeks and 8 weeks respectively. In consistent with our studies, allografts undergoing chronic rejection displayed hypertrophy of cardiomyocytes and increased expression of IL-6. However, treatment with neutralizing anti-IL-6 mAb prevented cardiac hypertrophy and fibrosis (Diaz et al., 2009). Moreover, IL-6 has also other role in chronic rejection including expression of adhesion molecules, SMC growth and endothelial cell dysfunction (Ikeda et al., 1993; Seino et al., 1994; Weis et al., 1999) . Surprisingly, the levels of IL-6 and TGF- β cytokines from transgenic allografts were comparable to isograft, suggesting that hsp-27 may modulate cytokine production.

The role of IL-10 in chronic rejection is complex and has been contested (Ikeda et al., 1993; Raisanen-Sokolowski et al., 1998; Raisanen-Sokolowski et al., 1997). Although IL-10 is known as a potent anti-inflammatory cytokine, it is associated with pathogenesis of chronic rejection (Qian et al., 1996). However, observations from studies using different dose of IL-10 either pre-operatively, peri-operatively or post-operatively seem to resolve this debate (Li et al., 1999; Lowry et al., 1995). Li et al showed that administration of recombinant mouse IL-10 pre-operatively prolonged graft survival and was associated with reduction of CD8⁺ T-cells in the graft. However, post-operative administration of such cytokine exacerbated rejection. Hence it was suggested that the role of IL-10 is dependent on timing and dosage (Li et al., 1999). Our study also supports this theory, as transgenic grafts that display less severe vasculopathy had higher expression of IL-10 at 4 weeks but the level of such cytokine was increased and was more important in LCs at 8 weeks post-transplant where important CD8⁺ T-cells infiltrates as well as severe intimal occlusion were seen. Nevertheless, it was postulated that IL-10 is required for neointimal accumulation of SMC. Transplantation into IL-10^{-/-} recipients had accelerated vascular occlusion, with leukocyte infiltration but very few SMC were present (Raisanen-Sokolowski et al., 1998). This might explain the increase in IL-10 seen at later stage in our study or the exacerbation of chronic rejection in IL-10 treated recipients in other studies (Li et al., 1999).

Taken together, the protective role of hsp-27 is likely due to two effects:

1. Affect on SMCs either by inhibiting early destruction of donor SMC and hence migration of SMC precursors from the recipient or inhibition of migration of donor intimal cells from the media to the intima
2. Affect on infiltration of recipient leukocytes and cytokine production.

6.7.3 Role of hsp-27 in humoral immunity and CAV

Numerous clinical studies have underlined the importance of humoral response in CAV (Ferry et al., 1997; McKenna et al., 2000; Reinsmoen et al., 2004). In fact, these studies linked the

presence of alloantibodies and complement deposition with poor prognosis of the graft. Experimental studies are more mixed, with some demonstrating a need for alloantibody and CAV, others not. Notably, Russell et al showed that transplantation of allograft B10.BR into C-17 SCID recipients developed none or minimal vascular lesion. However adoptive transfer of donor specific anti-serum into host that received B10.BR graft developed severe intimal thickening (Russell et al., 1994b). Similarly, B10.BR graft into B10.A recipient showed anti-donor antibody production and was associated with significant intimal lesion compared to the reverse combination (B10.A into B10.BR) where no antibody response was detected. In another study, this group confirmed that presence of neointimal SMC correlates with presence of alloantibodies (Russell et al., 1997; Russell et al., 1994b). Soleimani et al also reaffirmed this observation. This group demonstrated that transplantation into B-cell knockout mice led to intimal hyperplasia where there was significant infiltration of macrophages. Hence, they showed that an alloantibody response was not essential for the formation of intimal hyperplasia. However, such response was necessary for the proliferation and migration of SMC (Soleimani et al., 2006). In our study, the mechanisms leading to neointimal lesion remains unclear. In fact, we demonstrated progressive luminal occlusion by smooth muscle alpha actin positive cells in both transgenic and LC allografts, but we failed to detect significant increase in antibody production in allogeneic hosts. Nevertheless, we cannot confirm with certainty that no alloantibody was present at earlier time point in our study. In light of this, Soleimani et al showed that following transplantation of B10.A in a non-immunosuppressed mice model, the alloantibody production increased progressively from 1 week post-transplant, reaching the maximum at 3 weeks post-transplant. Hence, in our model, alloantibody produced at earlier time point could have been responsible for intimal hyperplasia by SMC. The other possible reason for lack of alloantibody in the serum tested could be due to CD4⁺ T-cell depletion. In fact, the model used in our study is a CD4⁺ T-cell-depleted model, wherein CD4⁺ T-cells are absent at the time of transplantation and subsequently re-emerge from 3 week post-transplant. B-cell activation and antibody class switching requires help from T helper cells, but such cells are absent in our study at the time of transplantation. This may explain lack of antibody production in our model. Hence, though

alloantibody response is not essential for development of intimal lesion by SMC, we cannot completely exclude the possibility that alloantibody may contribute to formation of such lesion.

6.7.4 Alloantigen independent factor

Surprisingly, the quantity of mRNA for CD8⁺ T-cells was exceptionally high, and superior to CD3⁺ cells. As CD8⁺ T-cells is a subset of CD3⁺ cells, it was not expected to exceed the quantity of CD3⁺ T-cells. Interestingly, recent study has shown that an important number of CD8⁺ cells exhibit cell surface phenotype such as CD16⁺ or CD56⁺ that are characteristics of NK cells (Campbell et al., 2008). It is therefore possible that CD8⁺ cells identified by RT-PCR consist of mixture of CD8⁺ T-cells and NK cells. The contribution of NK cells in acute rejection and CAV has been reviewed (Villard, 2011). Role of NK cells in transplant rejection is becoming more evident (Kroemer et al., 2008; Pratschke et al., 2009). It was shown that CD28^{-/-} (therefore absence of co-stimulation between CD28 on T-cell and B.7 on activated APC) C57BL/6 recipients rejected the cardiac allografts from BALB/c rapidly, but depletion of NK receptor bearing cells in such model prolonged graft survival. Moreover, semi-identical grafts were not rejected in CD28^{-/-} recipients. Hence, this group demonstrated that in absence of co-stimulation, NK cells that are activated due to absence of self MHC can provide help for T-cell (Maier et al., 2001). In contrast, other studies have shown that blockade of costimulatory pathways prolonged allograft survival (Lin et al., 1997; Turka et al., 1992). Taken together, the importance of NK cells in allograft rejection varies according to MHC mismatch combination. In addition, the role of NK cells in transplant outcome is dependent on the balance of activating and inhibitory receptors that they express (Lanier, 2005). Although we did not investigate the presence of NK cells, high amount of CD8⁺ mRNA transcripts observed in allografts indicates the presence of other cells than CD8⁺ T-cells solely that may potentially express CD8 marker. It would be therefore interesting to initially identify the CD8⁺ T-cell by co-labelling with CD3⁺ and CD8⁺ cell surface marker. Moreover, the hypothesis that NK cells infiltrates the graft and participate in the rejection could also be verified using CD16⁺ or CD56⁺ marker. If this was found to be the case, it would suggest that not only antigen dependent but also antigen-

independent immune responses participate in graft rejection. However, many studies have shown that antigen-independent immune responses alone are insufficient to cause graft rejection (Shi et al., 1996). Our findings also support this observation since syngeneic mice experience I/R injury (one of the antigen independent factors) but do not develop CAV. In this regard, it was previously shown that non-immunogenic factors are not sufficient to mediate rejection on their own but aggravate CAV in an allograft model (Knight et al., 1997).

6.7.5 Summary

In summary, transplantation of B10.A donor heart into anti-CD4 treated host leads to CAV characterised by infiltration of immune cells and SMC leading to luminal narrowing and occlusion. Hsp-27 seems to confer protection from chronic rejection by reducing the severity of CAV. Hsp-27 appears to work through various mechanisms: one explanation is inhibition of migration and proliferation of SMC hence protection against vessel occlusion. The other one is protection against graft damage through immunomodulation of cellular response by promoting Th2 response while down regulating Th1 response. In addition, previous episodes of acute rejection contribute to the development of CAV (Brunner-La Rocca et al., 1998; Schutz et al., 1990). We previously showed that hsp-27 delays graft rejection (see chapter 4) and may subsequently reduce the severity of CAV. However, it was not possible to elucidate whether hsp-27 have any effect on alloantibody or auto antibody responses. Further studies needs to be done in order confirm its effect on humoral immunity.

Chapter 7: Role of hsp-27 in ischaemic injury

7.1 Introduction

An unavoidable consequence of organ transplantation is ischaemia and I/R injury. Several types of injury are experienced by the transplanted organ. The earliest is probably caused by catecholamines released during brain death of the donor (Marasco et al., 2013; McKeown et al., 2012). This is followed by injury caused by organ procurement, cold storage during transport, warm ischaemia immediately after implantation and finally reperfusion caused by reperfusion of the organ with warm blood. Ischaemia is the transient interruption of blood supply to tissue and reperfusion is the re-establishment of blood supply to it. This leads to a catastrophic cascade of events which results in organ damage. This is characterised by increased production of ROS, necrosis, apoptosis and vascular injury (Abu-Amara et al., 2010; Hansen, 1995). Cardiac ischaemia leads to rapid deterioration of cardiac function, which has been characterised by dysfunction of coronary microvasculature, infiltration of inflammatory cells and cardiomyocyte damage (Liem et al., 2007; Logue et al., 2005). Among the alloantigen independent injury leading to graft rejection such I/R injury, donor age, brain death; I/R injury is one of the major factors that causes graft damage and subsequent development of CAV (Gaudin et al., 1994). Numerous studies have attempted to limit the damage caused by I/R (during organ retrieval and transplantation) by possibly preventing oxidative stress, generation of ROS, myocardial apoptosis and infiltration of inflammatory cells. Hsp-27 is a chaperone protein that is upregulated following stressful stimuli and modulates actin stabilisation and proper folding of denatured proteins (Marasco et al., 2013; Parcellier et al., 2003a). Moreover, it is known as an anti-apoptotic protein that is involved in protection from cell death (Concannon et al., 2003). Hsp-27 possesses 3 phosphorylatable sites at serine 15, 78 and 82 and the phosphorylation depends on MAPKAP Kinase (Guay et al., 1997). The function of hsp-27 is influenced by the ability to form oligomers and its phosphorylation status (Kostenko and Moens, 2009; Martin et al., 1999). Previous studies have shown that hsp-27 is involved in the protection of cell death (Efthymiou et al., 2004; Hollander et al., 2004; Huot et al., 1996; Martin et al., 1997). However,

the role of hsp-27 on I/R caused during cardiac transplantation is not well understood. The aim of this study was to investigate the protective effect of hsp-27 on I/R encountered during cardiac transplantation.

7.2 Confirmation of ischaemic model: phospho-AMP kinase

It has been previously shown that recombinant adenovirus overexpression of hsp-27 confers protection of adult rat cardiomyocytes against ischaemia injury by decreasing creatine kinase or LDH release (Martin et al., 1997). To confirm these findings and to evaluate whether overexpression of hsp-27 was able to protect mouse heart from ischaemia/ reperfusion injury caused during transplantation, an *ex vivo* model of ischaemia was designed. Hearts from transgenic and LC mice were subjected to ischaemia as encountered in conventional heterotopic heart transplantation in the mouse model. Hearts were subjected to 10 minutes of cold ischaemia -in ice cold saline (at 4°C) followed by 40 minutes of warm ischaemia in warm saline (at 37°C). In control hearts, no ischaemia was induced. The hearts were then frozen in liquid nitrogen and stored at -80°C until use.

To confirm the model of ischaemia, tissues from ischaemic and non-ischaemic hearts were analyzed by SDS-PAGE and western-blot. Figure 58 shows Western blots probed with antibody to phospho-AMP-kinase (Figure 58a) and its graphical representative data (Figure 58b). The 5'-AMP-activated protein kinase (AMPK) is a serine–threonine kinase and plays a key role in regulation of energy homeostasis. AMP-kinase is activated by an increased AMP/ATP ratio due to stressful stimuli such as heat shock, osmotic stress, oxidative stress, hypoxia and ischaemia which leads to phosphorylation of threonine 172 residue by up-stream AMPK-kinase (Russell et al., 2004). Normally, the unbound AMP is present at a very low concentration in the heart, however, the level increases rapidly following ischaemia or stressful stimuli.

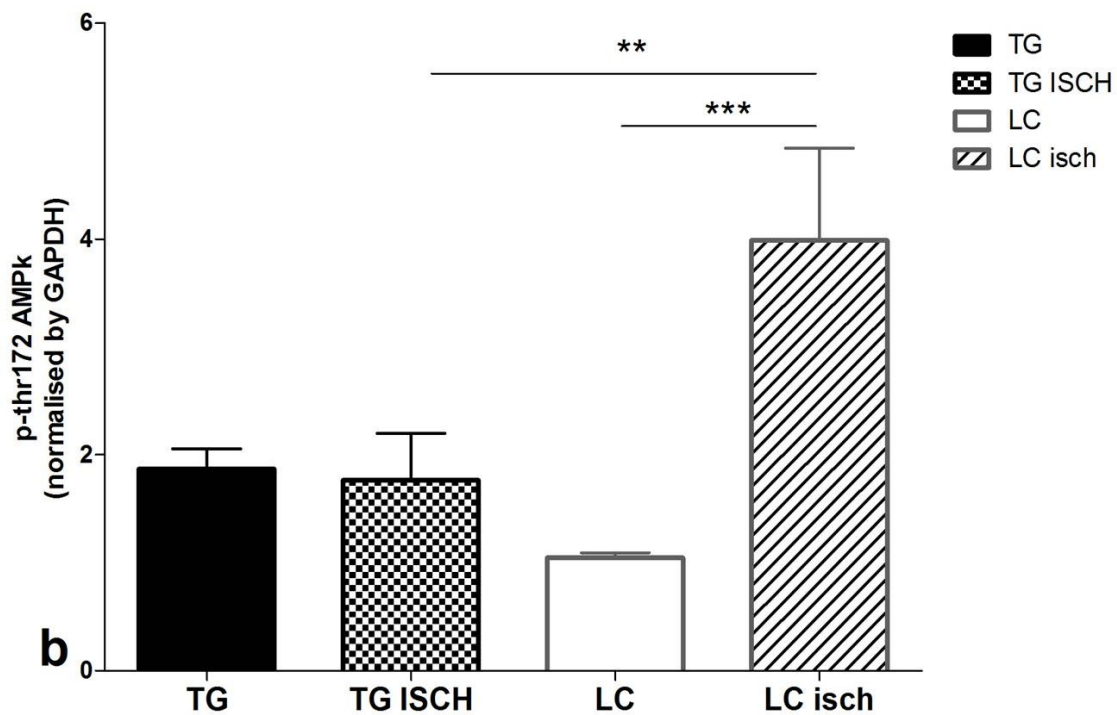
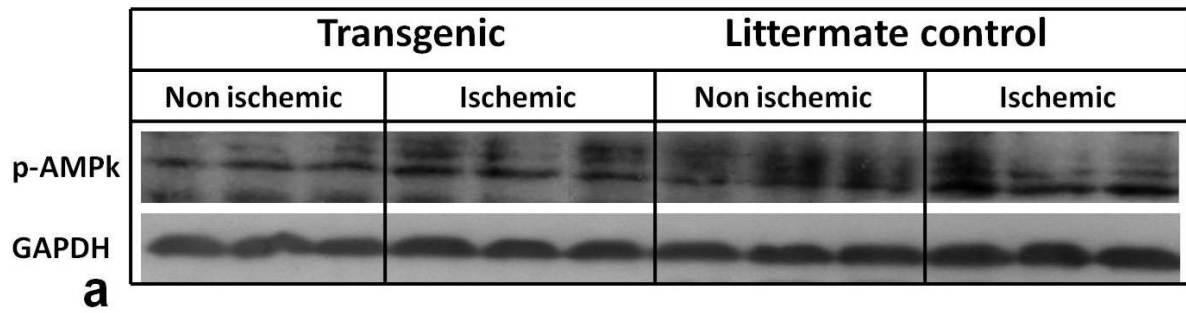


Figure 58: Phosphorylation of AMPk following ischaemia injury ex vivo. Hearts from transgenic and LC mice were incubated for 10 minutes in 4°C iced-cold saline (cold ischaemia) and 40 minutes at 37°C (warm saline). The phosphorylation of AMPk was examined by western-blot and quantified. Histograms represent the phosphorylation of phosphor-threonine172 AMPk normalized to the internal control GAPDH (mean± std). One way Anova was used to calculate the statistical significance. (* $p \leq 0.05$; ** $p < 0.01$; $n=3$ per group).

As shown in Figure 58, the amount of phosphorylated AMP-kinase did not differ between LC and transgenic mice under non-ischaemic conditions. AMP-kinase is activated following

environmental stress. Indeed phosphorylation of AMP-kinase is increased considerably following ischaemia in LC mice. However, significantly less AMP-kinase were phosphorylated in transgenic ischaemic group. This result validates the model of ischaemia used in this study and further shows that hsp-27 might be protecting from ischaemia injury.

7.3 Hsp-27 and cell death

In order to understand the effect of hsp-27 on cell death following ischaemia, cryo-sections of ischaemic and non-ischaemic heart from transgenic and LC mice were subjected to TUNel assay as described in material and methods.

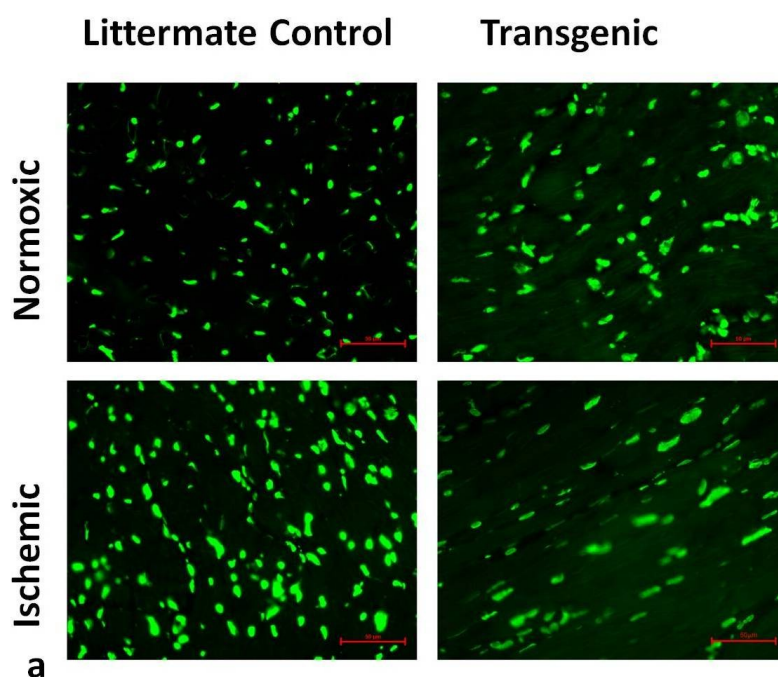


Figure 59a: Effect of hsp-27 on ischaemia induced cell death. Photomicrographs of cryosections of hearts from transgenic (n=3) and wild-type (n=3) mice which had been subjected to 10 minutes of cold ischaemia and 40 minutes of warm ischaemia ex vivo. The extend of apoptosis was assessed by the TUNel assay as described in Materials and Methods, brightly fluorescent cells are apoptotic cells.

Figure 59 shows the effect of ischaemia on transgenic and LC hearts. Presence of apoptotic cells were detected by TUNEL assay. Apoptotic cells were present in both normal and

ischaemia induced hearts (Figure 59). The number of apoptotic cells was significantly increased in both LC hearts (14 to 34 apoptotic cells/ field) and transgenic hearts cells (13 to 19 apoptotic cells/field) following ischaemia *ex vivo*. However, apoptosis due to ischaemia was significantly diminished in transgenic heart compared to LC heart, with a striking two fold decrease from 153% to 250% respectively. These results suggest that hsp-27 protects cardiac myocytes from apoptosis following ischaemia.

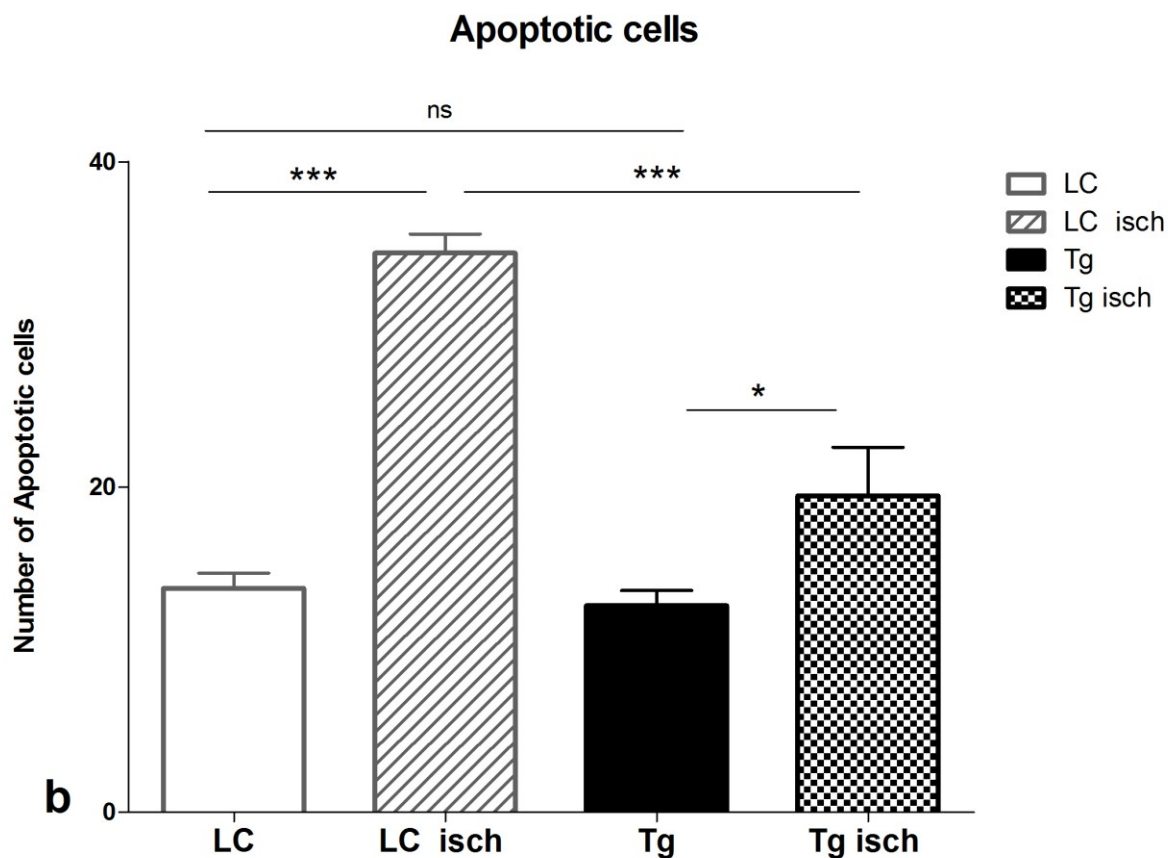


Figure 59b: Effect of hsp-27 on ischaemia induced cell death. Hearts from LC and transgenic mice were subjected to ischaemia and cryosections treated to reveal presence of TUNEL positive cells. Cryosections were examined by fluorescent microscopy and numbers of TUNEL positive cells were counted (on the y-axis) using x40 magnification. Data is representative of mean of 50 fields/heart (** $p < 0.01$ and *** $p < 0.001$, $n = 3$ per group).

7.4 Effect of hsp-27 on caspase-3 activity

Hsp-27 has been shown to protect from cell death following ischaemia encountered *ex vivo*. To gain some insight into the mechanisms contributing to cell death, we analysed the activity of caspase-3 (Figure 60). Caspase-3 plays a crucial role in initiating the process of apoptosis. Cleavage of caspase-3 leads to its activation which in turn is responsible for proteolytic cleavage of many key cellular proteins. This study looked at the effect of hsp-27 on caspase-3 activity.

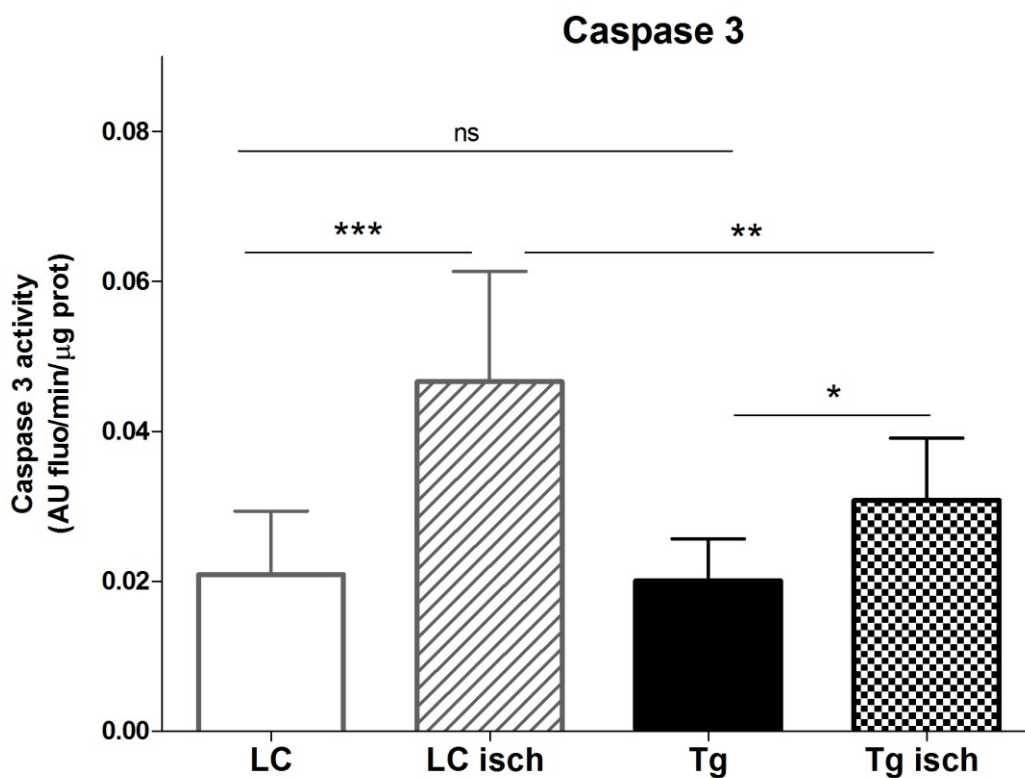


Figure 60: Effect of hsp-27 on caspase-3 activity following ischaemia injury *ex vivo*. LC and transgenic hearts were subjected to ischaemia *ex vivo* and the activity of caspase-3 was determined using the substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin. The data shows the activity of caspase-3 measured as OD460/ug/h. One way Anova with Bonferroni correction, data are the mean $\pm$ SEM (** $p < 0.01$ and *** $p < 0.001$, $n = 6$ per group).

Figure 60 shows similar activity of caspase-3 in transgenic and LC normoxic hearts. Ischaemia led to significantly increased activity of caspase-3 both in LC and transgenic (223% increase

and 153% increase respectively). However, the activity of caspase-3 was significantly higher in LC ischaemic heart compared to transgenic ischaemic heart, suggesting that hsp-27 protects from ischaemia by downregulating the activation of caspase-3.

7.5 Effect of hsp-27 on caspase-9 activity

Apoptosis is triggered through two main signalling pathways: the intrinsic and extrinsic pathway (Crow et al., 2004; Czerski and Nunez, 2004; Scoltock and Cidlowski, 2004). Both of these pathways leads to activation of caspase-3: the intrinsic pathway activates caspase-9 while the extrinsic pathway leads to activation of caspase-8. To understand whether hsp-27 acts via the intrinsic or the extrinsic pathway, the activity of caspase-9 was studied. The activity of capsase-9 was comparable in non-ischaemic, hearts from LC and transgenic mice (Figure 61).

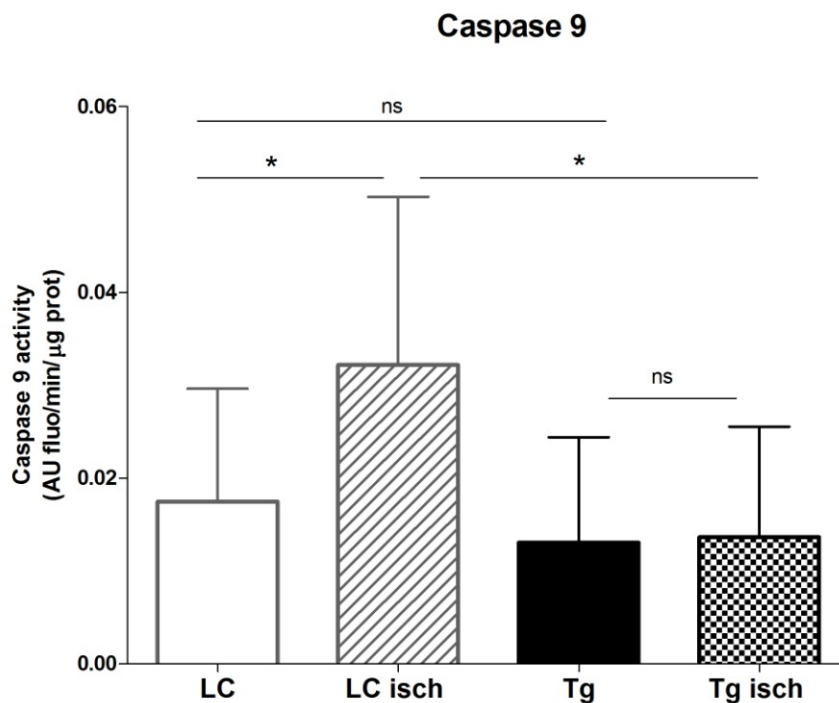


Figure 61: Effect of hsp-27 on caspase-9 activity following ischaemia injury ex vivo. LC (4) and transgenic (4) hearts were subjected to ischaemia ex vivo and the activity of caspase-9 was determined using the substrate N-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin. The data shows the activity of caspase-9 determined as OD460/ug/h. One way Anova with Bonferroni correction, data are the mean $\pm$ SEM (* $p < 0.05$ and ** $p < 0.01$, $n=4$ per group).

Ischaemia induced about 184% increase in caspase-9 activity in LC group. Interestingly, no such increase of caspase-9 activity was seen in transgenic group following ischaemia (Figure 61). In this model it appears that, ischaemia leads to activation of the intrinsic pathway, via the activation of caspase-9. Hsp-27 abolished the activity of caspase-9 and likely led to the decrease in apoptotic cells observed in the earlier experiments.

7.6 Effect of hsp-27 on caspase-1 activity

Caspase-1 is a mediator of an inflammatory pathway which, once activated processes the cytokine precursor pro-IL-1 β and pro-IL-18 into their mature forms. These cytokines play a key role in recruitment and activation of inflammatory cells. Moreover, caspase-1 also plays a role in ischaemia induced cell death (Zhang W. et al, 2003).

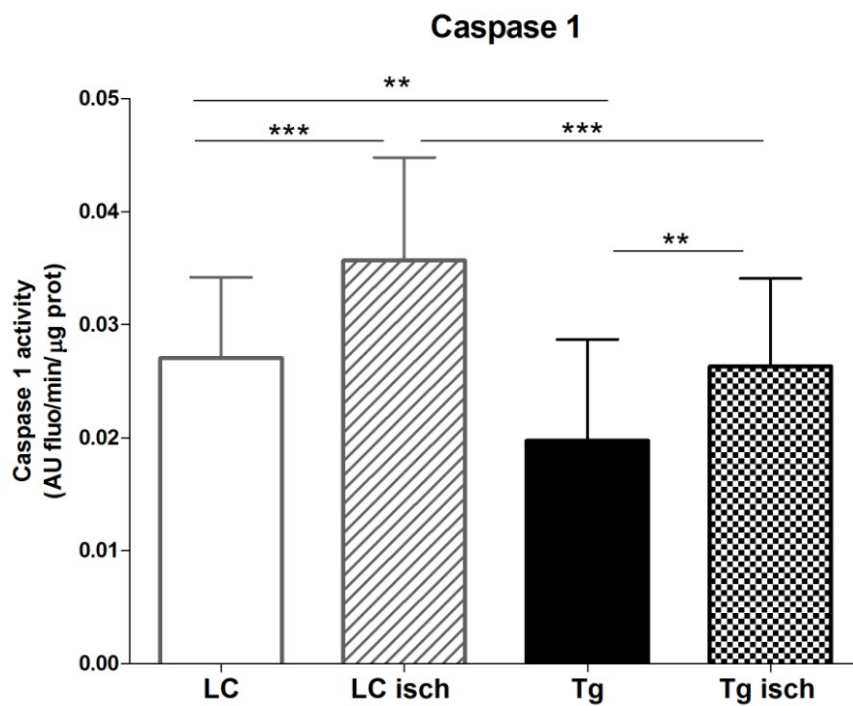


Figure 62: Effect of hsp-27 on caspase-1 activity following ischaemia injury ex vivo. LC and transgenic hearts were subjected to ischaemia ex vivo and the activity of caspase-1 was determined using the substrate N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin. The data shows the activity of caspase-1 measured as OD460/ug/h. One way Anova with Bonferroni correction, data are mean $\pm$ SEM (** $p < 0.01$, $n = 4$ per group).

To investigate whether hsp-27 participates in regulation of cell death through caspase-1, the activity of caspase-1 was examined. As shown in Figure 62, the activity of caspase-1 was increased following ischaemia in LC and transgenic hearts. The percentage of increase was comparable in LC (132% increase) and transgenic (133% increase) ischaemic hearts. Interestingly, the activity of caspase-1 was significantly lower in transgenic non-ischaemic heart (approximately 27% decrease) compared to LC normoxic heart. Taken together, this study suggests that hsp-27 does not regulate the activity of caspase-1 but most probably the expression of caspase-1.

7.7 Role of hsp-27 in ischaemia-reperfusion injury

During transplantation, the donor organ undergoes a phase of ischaemia and a phase of reperfusion. To understand the effect of hsp-27 in ischaemia-reperfusion *in vivo*, hearts from LC and transgenic B10.A mice were transplanted into the abdomen LC B10.A. The animals were sacrificed at either 4h or 24h post-transplant and their hearts were stored at -80°C until use. The activity of caspase-3 of LC and transgenic donor hearts was analyzed following ischaemia-reperfusion injury (Figure 63). The baseline caspase-3 activity was comparable in LC and transgenic normoxic group. The data of Figure 63 shows that there is a small increase in the activity of caspase-3 in LC I/R group at 4h. However, this was not significantly different from LC normoxic or from transgenic ischaemia/reperfusion at 4h. The analyses show a remarkable increase in the activity of caspase-3 following 24h ischaemia/reperfusion injury in LC group. However, there was no significant increase in caspase-3 activity in hearts from transgenic mice following 24 hrs of I/R injury. Taken together, these results suggest that extent of ischaemia/reperfusion damage is higher at 24h post-transplant compared to 4h post transplant. Moreover, hsp-27 protects from I/R injury by downregulating the activity of caspase-3.

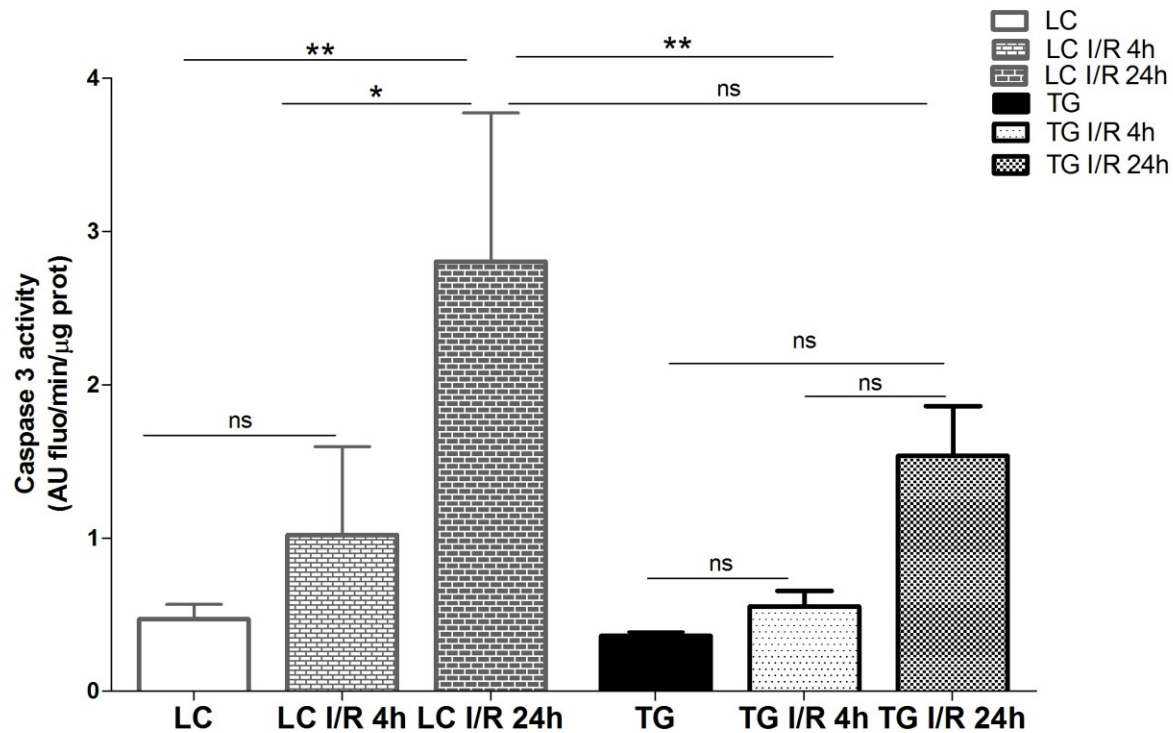


Figure 63: Effect of hsp-27 on caspase-3 activity following I/R injury. LC and transgenic hearts were subjected to I/R injury in vivo and the activity of caspase-3 was determined using the substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin. The data show the activity of caspase-3 at 4h and 24h of reperfusion post-transplant determined as OD460/ug/h. One way Anova with Bonferroni correction, data are mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, $n=3$ per group).

7.8 Phosphorylation of hsp-27

Hsp-27 is regulated by post-translational modification including phosphorylation. Previous studies suggest that the phosphorylated hsp-27 confer protection against atherosclerosis and CAV (De Souza et al., 2005; Park et al., 2006). We therefore aimed to investigate whether the phosphorylation of hsp-27 was altered during ischaemia. For this, transgenic and LC hearts were subjected to 10 minutes of cold ischaemia and 40 minutes of warm ischaemia. Phosphorylation of hsp-27 was detected by western-blot using antibody to hsp-27 serine 82 and serine 78 as described in materials and methods.

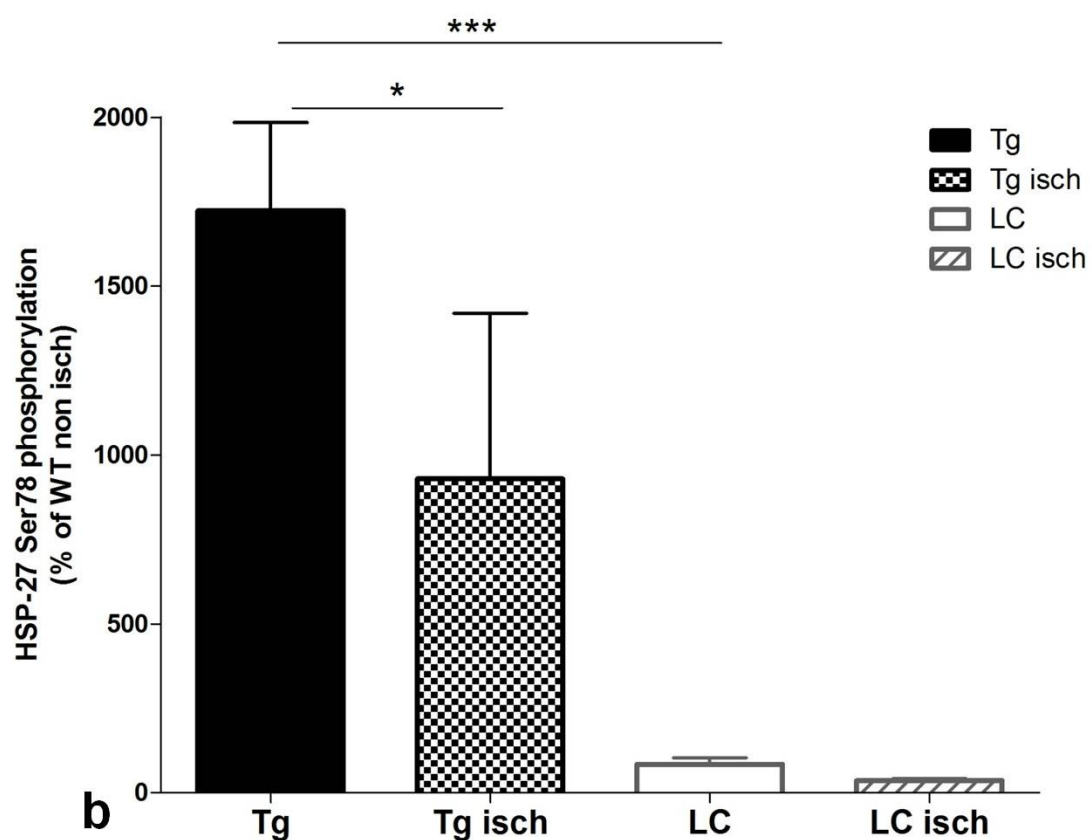
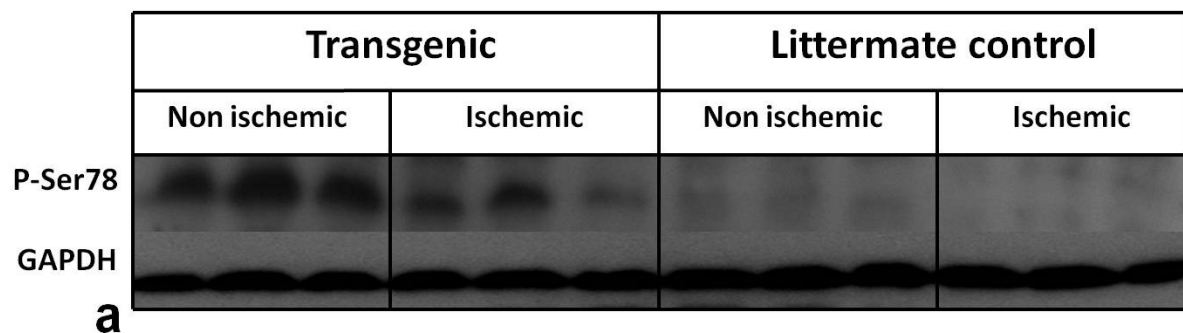


Figure 64: Phosphorylation of hsp-27 at serine78 in transgenic and LC at normoxic and ischaemic condition. Western blot and its graphical representation following treatment of transgenic and LC hearts to 10 minutes cold and 40 minutes warm ischaemia. Respective non-ischaemic hearts were used as controls. Histograms represent the phosphorylation of hsp-27 normalized non-ischaemic LC. Data are the mean $\pm$ SEM (* $p \leq 0.05$; ** $p < 0.01$; $n=3$ per group).

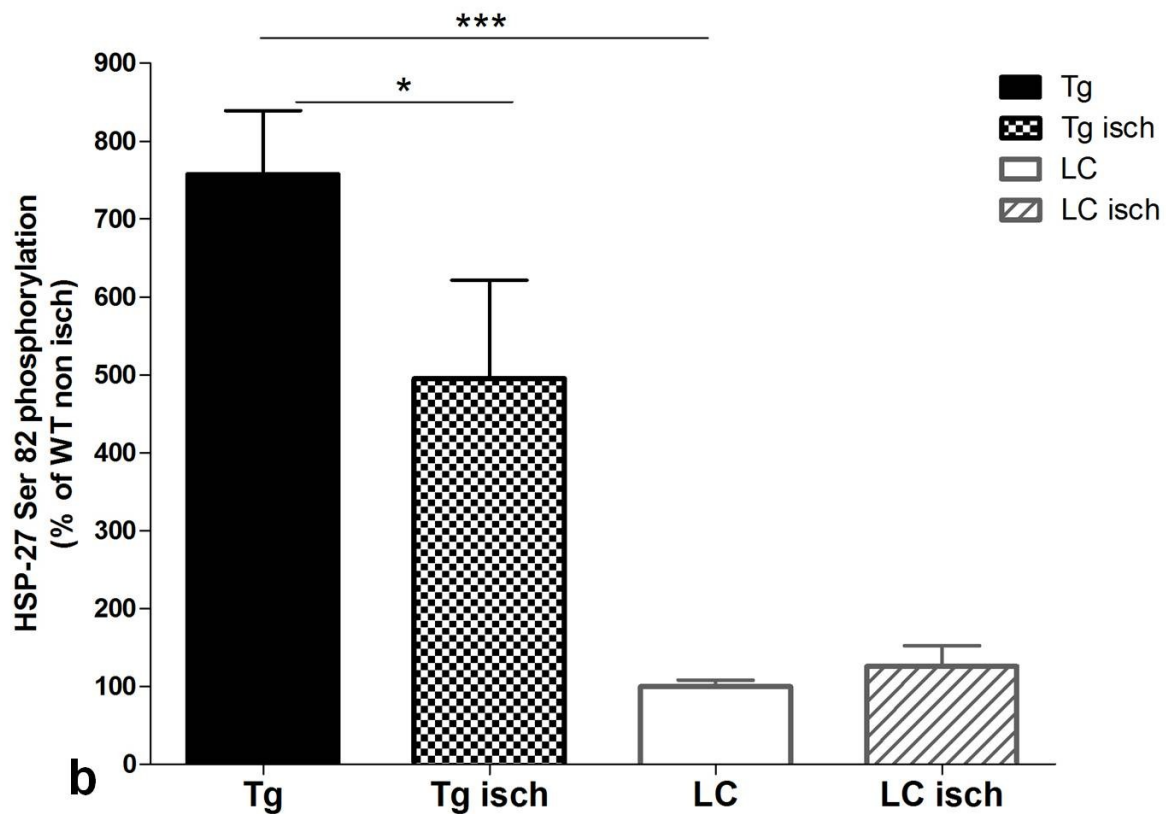
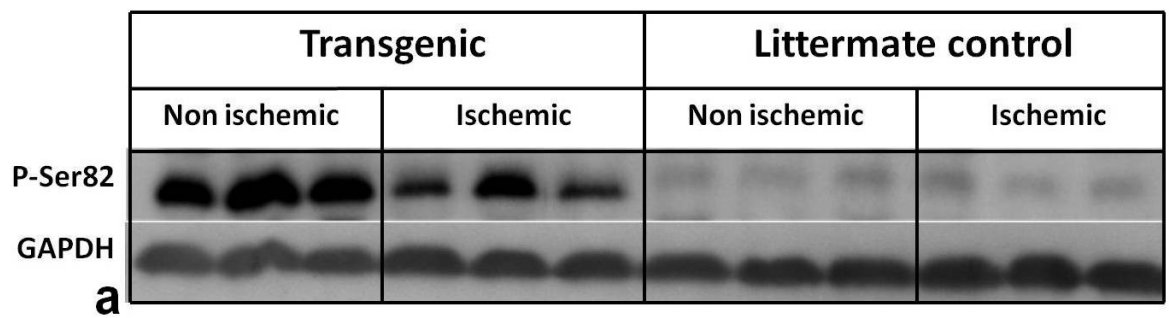


Figure 65: Phosphorylation of hsp-27 at serine82 in transgenic and LC at normoxic and ischaemic condition. Western blot and its graphical representation following treatment of transgenic and LC hearts to 10 minutes cold and 40 minutes warm ischaemia. Respective non-ischaemic hearts were used as controls. Histograms represent the phosphorylation of serine 82 of hsp-27 normalized non-ischaemic LC. Data are the mean $\pm$ SEM (* $p \leq 0.05$; ** $p < 0.01$; $n=3-4$ per group).

At normoxic condition, considerably high level of phosphorylation of hsp-27 at serine 78 was observed in transgenic mice overexpressing hsp-27 (Figure 64). Following ischaemia,

phosphorylated hsp-27 at serine 78 was significantly reduced in transgenic heart compared to non-ischaemic control. Similarly, hsp-27 was also highly phosphorylated at serine 82 in transgenic mice but it decreased when these hearts were subjected to ischaemia (Figure 65). As expected virtually no phosphorylation of hsp-27 at serine 78 or serine 82 was observed in LC groups, since these animals do not express the transgene. Phosphorylation at serine 15 was not detected

7.9 Discussion

7.9.1 Establishment of ischaemic model

Although studies were performed to understand the protective role of hsp-27 in I/R injury, to our knowledge, this is the first study using transgenic mice overexpressing hsp-27 to investigate preventive effect of hsp-27 on myocardial apoptosis and I/R injury following cardiac transplantation. This chapter examined the effect of hsp-27 overexpression in response to ischaemia and I/R injury. Western-blot analysis of p-AMPk confirms that the method used does induce ischaemia *ex vivo*. Subjection of hearts to ischaemia *ex vivo* mimics ischaemia as encountered during heterotopic heart transplant in mice. The TUNEL assay demonstrated less apoptosis following ischaemic injury in transgenic hearts compared to littermate control hearts. However, Zhao et al reported that cardiomyocyte death occurs mainly during I/R injury but not during ischaemia alone induced by LAD ligation. The discrepancy with our results, where there are TUNEL positive cells after ischaemia alone, could be due to the different methods of inducing ischaemia as well as the animal model (dog) used for their study (Zhao et al., 2000).

7.9.2 Ischaemia Reperfusion injury *in vitro* or *ex vivo*

Efthymiou et al have also shown that hearts from mice overexpressing hsp-27 displayed reduced infarct size and were protected from myocardial infarction using the Langendorff perfusion technique (Efthymiou et al., 2004). Another group also using the Langendorff method demonstrated that hearts from hsp-27 transgenic mice preserved contractile function and were

protected from I/R injury (Hollander et al., 2004). Our findings confirm this, and we showed that overexpression of hsp-27 protects from apoptosis of cardiac cells (Figure 59). Apoptosis involves a series of signals and cell activation. Caspase-3 is the terminal executor of apoptosis. Activation of caspase-3 during hypoxic injury has been shown to play a role in cardiomyocyte apoptosis (de Moissac et al., 2000). Consistent with this, we showed increased caspase-3 activity (Figure 60) and myocardial apoptosis (Figure 59) in LC heart subjected to ischaemic injury. However, we demonstrated that hsp-27 confers protection from apoptosis by reducing caspase-3 activity following ischaemia. This suggests that hsp-27 regulates the apoptotic pathway through modulation of caspase-3 activity. The apoptotic process could be activated by two major signal transduction pathways: intrinsic mitochondrial pathway and the extrinsic death receptor pathway (Crow et al., 2004; Czerski and Nunez, 2004). We studied the effect of hsp-27 on the mitochondrial pathway: caspase-9 activity was abolished in transgenic mice overexpressing hsp-27, suggesting that hsp-27 regulates the apoptotic pathway via modulation of the intrinsic apoptotic pathway. In fact, a study using U937 human leukaemic cells also suggests this is the case. The leukaemic cells transfected with hsp-27 cDNA showed that hsp-27 interacts with cytochrome c and thus prevents interaction with caspase-9 and Apaf-1, thereby preventing formation of the apoptosome (Bruey et al., 2000). The cytochrome/Apaf-1 pathway is activated by the mitochondrial protein Smac. During apoptosis the latter is released into cytosol and interacts with inhibitors of apoptosis protein (IAP), hence promoting apoptosis (Du et al., 2000). Interestingly, hsp-27 prevents the release of Smac from mitochondria thus adding another level of protection from apoptosis (Chauhan et al., 2003). In this study, we did not investigate the extrinsic pathway directly. But using antibody to detect caspase-8 by western-blot, one could determine whether hsp-27 also modulates the extrinsic pathway. Interestingly, hsp-27 has been shown to regulate the death receptor Fas pathway, independently from caspase (Charette and Landry, 2000). Their study demonstrates that hsp-27 regulates the extrinsic pathway by interacting with Daxx (binds to FAS and mediates the downstream action) and thus preventing its translocation from the nucleus to the cytosol.

Moreover, this protective function depends on the phosphorylation of hsp-27 (Charette and Landry, 2000; Charette et al., 2000).

7.9.3 Ischaemia reperfusion injury *in vivo*

We further showed that the protective effect of hsp-27 seen *ex vivo* is also replicated *in vivo*. In our study ischaemia followed by 24h of reperfusion significantly decreased caspase-3 activity in hsp-27 transgenic donor hearts (Figure 63). In contrast, Chen et al failed to see such protection *in vivo* in a renal I/R injury model (Chen et al., 2009a). They showed that overexpression of hsp-27 resulted in increased I/R injury and tubular necrosis after 3h as well as 24h after reperfusion. The reason for this discrepancy is not clear but may be due to differences in the organs studied namely kidney and heart. In support of this, overexpression of hsp-27 is associated with protection against liver injury following I/R injury *in vivo* (Chen et al., 2009b). Moreover, they reported that following 24h of reperfusion, there was less apoptosis and cleaved caspase-3 as observed in our study. Although, we did not see any protection following 4h of reperfusion, the transgenic donor hearts displayed significant decrease in caspase-3 activity following 24h of reperfusion. Surprisingly, ischaemia followed by 4h of reperfusion did not lead to significant modification of caspase-3 activity in both LC and transgenic grafts (Figure 63). It is however interesting to note that, other groups have shown that caspase-3, -8 and -9 activities as well as the production of TNF- α were significantly increased at 4h of reperfusion compared to native hearts (Tanaka et al., 2005b). In these studies, the 4h of reperfusion was preceded by prolonged cold ischaemia, whereas in our study the cold ischaemic time was only 10 minutes. However, even with 30 minutes of total ischaemic time (cold and warm ischaemia) Tanaka et al still see an increase in caspase-3 activity (Tanaka et al 2005). The reasons for their results being different to ours are not known. However, we confirm significant increase in caspase-3 activity in ischaemia followed by 24h of reperfusion in the LC heart, suggesting longer reperfusion time leads to greater caspase mediated cell death. We conclude therefore that hsp-27 protects from I/R injury.

7.9.4 Should hsp-27 be phosphorylated?

The function of hsp-27 is regulated by its phosphorylation state (Rogalla et al., 1999). Hollander et al showed that mice transgenic for both wild-type hsp-27 as well as mutant hsp-27 (mutant for serine 15, 78 and 82, hence not able to be phosphorylated) were able to protect against cellular damage induced by I/R injury (Hollander et al., 2004). Moreover, the non-phosphorylated form of hsp-27 could further reduce oxidative damage initiated by I/R injury. Another study also confirmed this observation (Martin et al., 1999). Other studies suggest that the protective function does depend on the phosphorylation status of hsp-27. Rabbit myocardium subjected to varying ischaemic and reperfusion time showed phosphorylation of hsp-27 following ischaemia and I/R injury (White et al., 2006). Similarly, analyses of myocardial tissue following heat shock of rats demonstrated that hsp-27 was phosphorylated (Arnaud et al., 2004). We showed in our report that the phosphorylation of hsp-27 at serine 78 and serine 82 decreased following ischaemia. Previous study by De Souza et al showed that the phosphorylated form of hsp-27 decreased with the progression of the disease and underlined that the phosphorylated form was associated with protection from CAV (De Souza et al., 2005). Phosphorylated hsp-27 also decreased in atherosclerotic plaque of patients with acute coronary syndrome (Park et al., 2006). These studies stated that the phosphorylated form has been consumed -therefore phosphorylation is essential for protection against ischaemia. Phosphorylation of hsp-27 blocks extrinsic apoptosis cascade by binding to Daxx, however the non-phosphorylated hsp-27 was not able to interact with Daxx (Charette and Landry, 2000; Charette et al., 2000). It is important to note that, in our study, though there was a decrease in phosphorylated hsp-27, the number of phosphorylated hsp-27 was still elevated in ischaemic heart, suggesting that both phosphorylated and non-phosphorylated hsp-27 are involved in the anti-apoptotic function of hsp-27. As explained by Parcellier et al, it is therefore possible that the modulation of the mitochondrial pathway is independent of phosphorylation while the protection through death receptor pathway requires phosphorylation (Parcellier et al., 2003a). However, it is difficult to conclude on the phosphorylative status of hsp-27. The decrease in the

phosphorylated form of hsp-27 could be a consequence of ischemic injury or decrease in overall cell number through apoptosis. It is important to note that in clinical setting, the donor heart is perfused with cold preservative solution such as cardioplegia, then explanted and stored at 4°C to slow hypothermic metabolic activity, minimise ischemic injury, thus cell death. However, saline preservation used in our experiment does not offer such protection. In order to determine whether the post-translational modification through phosphorylation of hsp-27 was due to ischemic injury, we could isolate the cardiomyocytes, and compare the changes in phosphorylative state before and after ischemia. It would also be interesting to study the changes in hsp-25 following ischemic injury.

7.9.5 Hsp-27 and oxidative stress

An unwanted consequence of I/R injury is generation of high levels of ROS. These play a pivotal role in oxidative damage resulting in cell death by apoptosis or necrosis. Studies have attempted to prevent the oxidative damage caused during I/R using scavenger proteins or detoxify ROS (Powis et al., 1995). Tanaka et al used a scavenger protein (copper/Zinc superoxide dismutase) against oxidative stress and showed that there was less apoptosis, reduced TNF- α , MCP-1/CCL2 production and decreased neutrophils infiltration at 4h of reperfusion in transgenic donors compared to control (Tanaka et al., 2004). We demonstrated that hsp-27 could function as inhibitor of apoptosis. However, our attempts to investigate the regulation of hsp-27 on oxidative stress using antibodies to MDA and 4HNE by western blot were not successful (data not shown). Interestingly, it was previously shown that hsp-27 could also protect from oxidative stress by lowering the level of ROS (Huot et al., 1996; Wyttenbach et al., 2002). Cardiac cells overexpressing hsp-27 generated significantly less ROS compared to control cells following oxidative stress induced by H₂O₂ treatment (Liu et al., 2007). Rogalla et al also demonstrated that hsp-27 protects from oxidative stress (Rogalla et al., 1999). Moreover, they highlighted that phosphorylation of hsp-27 prevents the protective effect of hsp-27 against apoptotic damage. Interestingly, we showed that phosphorylation of hsp-27 decreases following ischaemia; hence the resultant non-phosphorylated hsp-27 may participate in the protection

from oxidative stress (Figure 64 and Figure 65). Hsp-27 modulates redox activity and maintains redox homeostasis thus preventing from ROS (Arrigo et al., 2005a; Concannon et al., 2003). The ability to maintain the redox stability by hsp-27 depends on the regulation of glucose 6 phosphate dehydrogenase glutathione reductase that maintains glutathione in a reduced form (Arrigo et al., 2005b).

7.9.6 Hsp-27 and inflammation

I/R injury leads to production of cytokines and facilitates the formation of an inflammatory environment. Studies have shown production of cytokines TNF- α , IL-1 β , IL-6 as well as expression of adhesion molecules increases following I/R injury (Sawa et al., 1998; Yamauchi-Takahara et al., 1995). Moreover, this accelerates the infiltration of inflammatory cells, mainly neutrophils and monocytes to the inflammatory site. In fact, suppression of I/R has been effective in preventing cell infiltration, hence limiting the danger caused by I/R. Tanaka et al used a scavenger protein (copper/Zinc superoxide dismutase) against oxidative stress and showed that there was less apoptosis, reduced TNF- α , MCP-1/CCL2 production and decreased neutrophils infiltration at 4h of reperfusion (Tanaka et al., 2004). We have not investigated whether hsp-27 overexpression in the graft diminishes the inflammation caused by I/R injury. It would be interesting to study the cell infiltration and cytokine production following I/R injury in transgenic mice overexpressing hsp-27.

Recent studies report the involvement of caspase-1 in ischaemia injury and heart failure (Merkle et al., 2007; Syed et al., 2005). In our study, the increase in caspase-1 activity following ischaemia was comparable between littermate control and transgenic mice (Figure 62). However the baseline caspase-1 activity was reduced in transgenic compared to wild-type in normoxic condition. This suggests that hsp-27 might not regulate the activity but the expression of caspase. Caspase-1 is involved in the maturation of pro-IL-1 β and pro-IL-18 into mature forms (Bryant and Fitzgerald, 2009; Latz, 2010; Siegmund, 2002). Both of these cytokines are important mediators of inflammatory response and are involved in inflammation (Latz, 2010; Siegmund, 2002). Moreover IL-1 β stimulation induced production of IL-6 (Yamauchi-Takahara et

al., 1995). IL-6 is also implicated in inflammation and enhances recruitment of leukocytes (Youker et al., 1992). Therefore decreased level of caspase-1 in transgenic heart may be beneficial by limiting inflammation.

On the other hand, hsp-27 was shown to prevent neutrophils apoptosis through interaction with AKT (Rane et al., 2003). These authors further demonstrated that phosphorylation of hsp-27 leads to dissociation of hsp-27 from AKT, hence promoting neutrophils apoptosis. It is thus conceivable that phosphorylation of hsp-27 may confer protection against inflammation.

7.9.7 Summary

In summary, overexpression of hsp-27 protects from ischaemia and I/R injury through regulation of caspase activity, notably by decreasing caspase-3 activity and inhibiting the activity of caspase-9. Hsp-27 is therefore potent in preventing cellular damage and apoptosis caused by I/R injury. Moreover hsp-27 seems to prevent oxidative damage caused by I/R injury. The protective function of hsp-27 was suggested to be regulated by its phosphorylation. The phosphorylation decreases following ischaemic insult; however it's still highly phosphorylated making it difficult to come to a conclusion regarding the necessity for phosphorylation and hsp-27 protection against ischaemia in our model. It maybe that both phosphorylated hsp-27 and unphosphorylated hsp-27 are required for the protective function of hsp-27.

Chapter 8: Hsp-27 and immune regulation

8.1 Introduction

Hsp-27 is involved in a range of functions including protein folding, actin polymerisation and cytoprotection from a variety of stimuli. Such protection was thought to be due to the ability of hsp-27 to modulate actin cytoskeleton and inhibit apoptosis. High levels of hsp-27 were found in heart biopsies from long-term heart transplant patients without CAV as well as in the coronary vessels of patients free from ischaemic heart disease (De Souza et al., 2005; Robinson et al., 2010). In addition, we have now shown that acute rejection was delayed in cardiac allografts overexpressing human hsp-27 (see chapter 4). Similarly, overexpression of hsp-27 in the donor heart also minimised intimal thickening and reduced the severity of CAV (see chapter 6). Detailed analyses demonstrated hsp-27 to be influential on cytokine secretion, since our study underlined that LC allografts had increased numbers of inflammatory cells as well as inflammatory cytokines compared to transgenic allografts. The aim of this study was to elucidate whether hsp-27 could be exerting a direct effect on immune cells.

8.2 Role of hsp-27 in IL-2 production by T-responder cells

It was previously shown that the presence of hsp-27 intragraft modulated inflammatory infiltrates and cytokines expression; principally reflected by IFN- γ reduction and IL-4 upregulation (see chapters 4 and 6). However, it was not clear how overexpression of hsp-27 in donor heart could possibly influence host immune response. During allograft rejection, intact MHC antigens of donor origin are displayed on donor APCs and presented to recipient T-cells. With time, donor APCs are eliminated from the graft and rejection occurs due to interaction of host T-cells with host MHC/APC that present the alloMHC in the form of peptide (Auchincloss and Sultan, 1996). One possible explanation for a potential immunoregulatory role of hsp-27 is that the latter has a direct immunomodulatory effect on APC; hence donor APC in hsp-27 overexpressing hearts may not be as efficient at stimulating recipient T-cells as APC in hearts from LCs. In order to test this hypothesis, we isolated the T-cells from spleens of recipients that received transgenic,

LC or syngeneic hearts and looked for evidence that they had been primed against B10.A antigens. This was achieved by looking at their ability to produce IL-2 and IFN- γ in response to B10.A and third party antigens *in vitro*. In the first set of experiments the IL-2 response was examined (Figure 66). T-cells were isolated at 5 days (Figure 66a) or 12 days post-transplant (Figure 66b) and these responder T-cells were then cultured in presence of transgenic, LC or third party mouse (FVB) APCs. The haplotype of FVB is $H-2^q$ while B10.A is $H-2^k$ and $H-2A/E^d$. Elispot assays were performed in order to detect the secretion of IL-2 and IFN- γ as described in materials and methods. Lack of IL-2 production by the negative control, where splenocytes were cultured in absence of APC confirmed that T-cells were unable to produce cytokines in presence of media alone and they required stimulation from APCs to be stimulated and produce cytokines. Surprisingly, all splenic T-cells isolated from animals that received syngrafts or allografts were stimulated to produce IL-2. Of note, syngraft is transplantation between genetically identical individual (mice in our case). T-cells obtained from recipients of syngrafts should not be proliferating in response to APC which are genetically identical to themselves. It was also surprising to see that third party control FVB APC added to the culture also induced production of IL-2 by recipient T-cells. It is unlikely that the positive response to FVB-cells *in vitro* represents priming *in vitro*. In the Elispot assay, cells are cultured with stimulator cells for a period of only 48hrs, this is not long enough to invoke a primary IL-2 response, therefore all positive responses in the Elispot assay must be from cells primed *in vivo*. There was no significant difference between the magnitudes of IL-2 responses between recipient T-cells from recipients of syngeneic or allogeneic hearts. In addition, the source of APC -whether it's from the transgenic animal or LC animal- did not alter the results obtained.

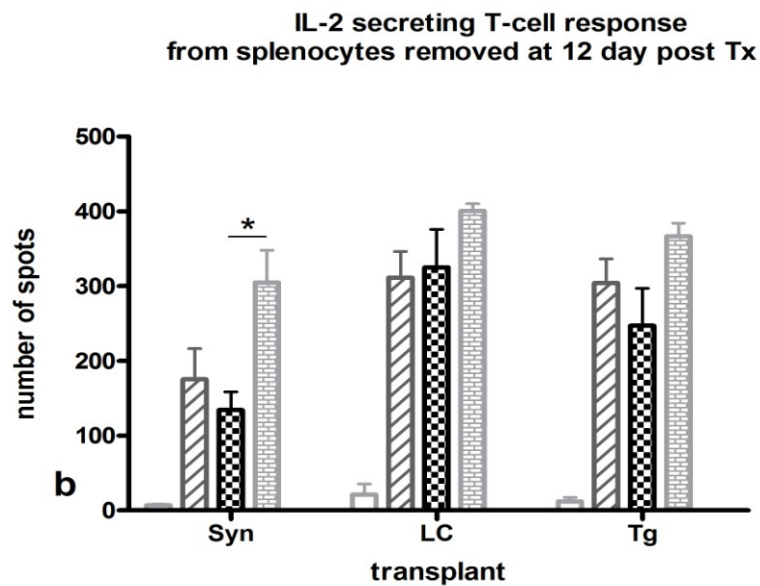
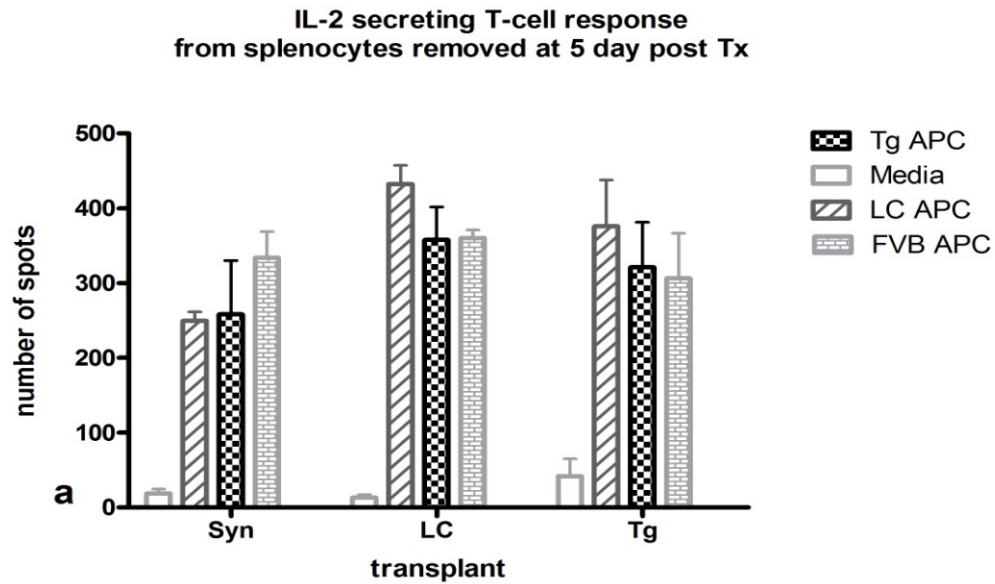


Figure 66: Frequency of IL-2 producing T-cells in response to allogeneic or third party control APC stimuli. Spleen cells harvested from C57BL/6 recipients that received transgenic, LC or syngeneic graft at 5 days or 12 days post-transplant were mixed with APC isolated from transgenic, LC or third party control and tested by Elispot for IL-2 production. Responder cells cultured with medium alone was used as negative control. The frequency of IL-2 producing cells is expressed as number of spots per million of splenocytes. Each spot represents one single cell secreting the IL-2 cytokine. (n=3-6, * p< 0.05).

This experiment was repeated with splenic T-cells harvested from recipients at 12 days post-transplant. The results obtained for T-cell assays conducted with 12 days-post-transplant samples were comparable to the response obtained at 5 days post-transplant. In fact, a T-cell response was induced regardless of the source of responder cells – (whether it was from allogeneic or syngeneic transplant) or the type of stimuli (whether it was by allospecific or third party control APC). Unfortunately, because the negative controls (syngeneic grafts and responses to third party FVB-cells) produced aberrant results, these experiments are not informative.

8.3 Role of hsp-27 in IFN- γ production by responder T-cells

We then sought to determine whether the frequency of IFN- γ producing T-cells was influenced by hsp-27. For this, we isolated T-cells from spleens of hosts that received transgenic, LC or syngeneic grafts at 5 days (Figure 67a) or 12 days (Figure 67b) post-transplant and evaluated the frequency of IFN- γ producing cells following stimulation by LC, transgenic or FVB APCs. APCs from FVB were used as third party controls. As expected, the Elispot analyses show that in absence of stimulation by APC (media alone); no IFN- γ was detected. Similarly, no T-cell activity was observed in splenocytes obtained from mice transplanted with isografts in response to transgenic APC or LC APC, demonstrating, as expected, that IFN- γ producing T-cells are not primed in syngeneic transplantation. On day 5 post-transplant (Figure 67a), spleen cells from mice transplanted with allogeneic heart –either LC or transgenic- showed T-cell activity against target cells expressing B10.A donor alloantigens, but such activity was significantly reduced when cultured with third party control, thus underlining that IFN- γ is produced by primed alloreactive T-cells and these T-cells are specific for B10.A antigens. Interestingly, when compared with third party control stimulation, splenocytes stimulated by LC APC produced consistently more IFN- γ than that stimulated by transgenic APC; however these differences did not reach statistical significance.

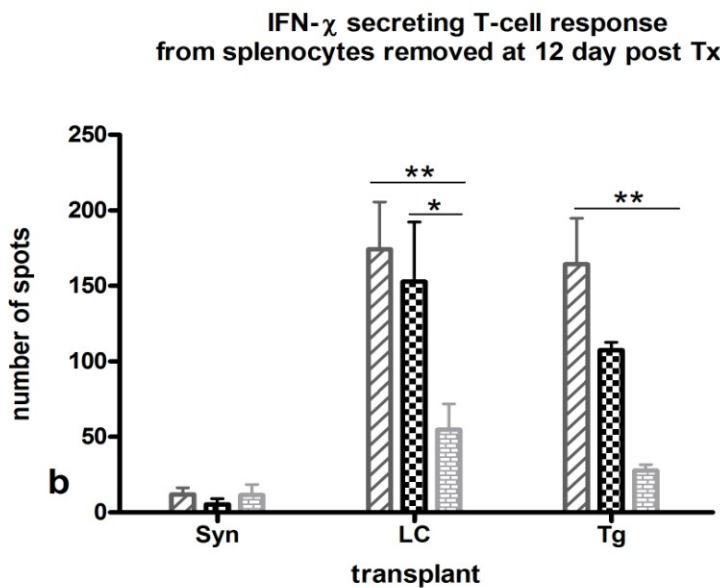
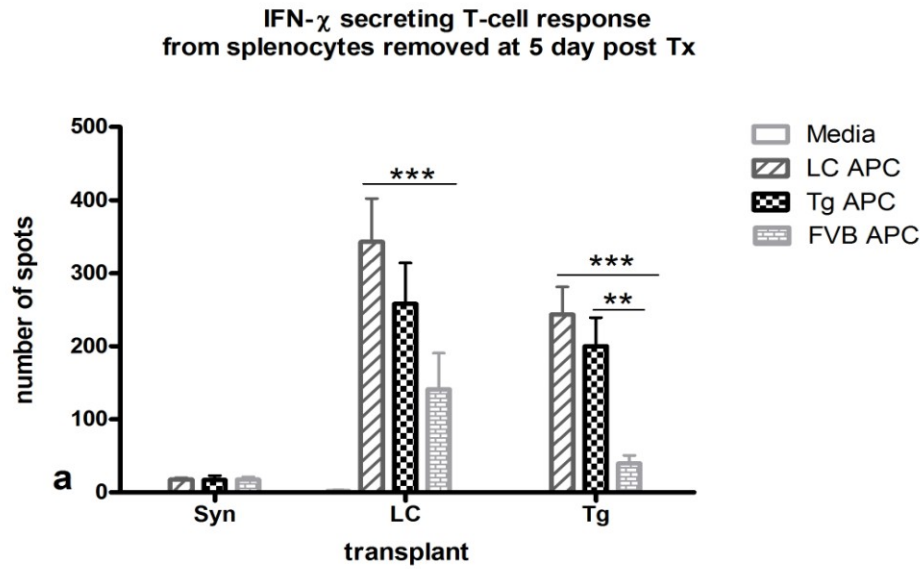


Figure 67: Frequency of IFN- γ producing T-cells in response to allogeneic or third party control APC stimuli. Splenic T-cells obtained from C57BL/6 recipients that received transgenic, LC or syngeneic graft at 5 days (a) or 12 days (b) post-transplant were mixed with APC isolated from transgenic, LC or third party control and tested by Elispot for IFN- γ production. Responder cells cultured with medium alone served as negative control. The frequency of IFN- γ producing cells is expressed as number of spots per million of splenocytes. Each spot represents an IFN- γ producing cell. (n=3-8, * p< 0.05 and ** p<0.01).

In contrast, regardless of the nature of stimulation; mice exposed to transgenic graft were associated with a trend (but were not statistically significant) in decreased IFN- γ producing cells compared to mice exposed to LC graft, suggesting less frequent IFN- γ positive cells in presence of hsp-27. IFN- γ Elispot results of 12 days post-transplant (Figure 67b) were comparable to the response observed at 5 days post-transplant, although there was an overall decrease in the frequency of IFN- γ producing cells. Yet again, it is interesting to note that mice exposed to transgenic graft had much less IFN- γ production when stimulated with transgenic APC compared to LC APC, but again the differences did not reach statistical significance. Taken together, these results suggest that hsp-27 may dampen the frequency of IFN- γ producing cells and thus modulate the immune response.

8.4 Discussion

In this study, we used Elispot assay to analyse the T-cells response towards donor alloantigens and to investigate whether hsp-27 influenced such responses. We looked at the IFN- γ response because others have shown that this is a sign of primed T-cells. Indeed, other studies have shown that when naive T-cells (identified by CD45RA⁺/CD45RO⁻) and memory T-cells (identified by CD45RO⁺/CD45RA⁻) were re-stimulated with the same alloreactive APCs, only memory T-cells were able to produce IFN- γ (Gebauer et al., 2002). Our findings confirm that IFN- γ producing T-cells are allospecific since we observed a decreased response to third party stimulation compared to B10.A allogeneic APC. The IL-2 results have to be discounted.

8.4.1 Hsp-27 and antigen presentation

In this study, we investigated the memory response of *in vivo* primed T-cells in response to B10.A allogeneic antigen. Upon re-stimulation by LC or transgenic APC, IFN- γ was produced by LC primed memory T-cells. Interestingly, this response was slightly but not significantly lower in splenic T-cells obtained from hosts that received transgenic grafts in response to both LC APC and transgenic APC, suggesting that hsp-27 may interfere with T-cell priming. In fact, a recent study confirmed that silencing of hsp-27/hsp-25 leads to generation of T-cell memory response

(Nagaraja et al., 2012). This group showed that primed splenic CD8⁺ T-cells extracted from tumour free mice as a results of 4T1-hsp-25shRNA injection (leading to long-term silencing of hsp-25) were able to lyse the target cells but not the third party control. In addition, adoptive transfer of these CD8⁺ T-cells in tumour bearing mice (that were previously injected with 4T1-controlshRNA cells) resulted in tumour regression. However, these effects were not seen in CD8⁺ T-cells extracted from 4T1-controlshRNA injected cells, thus suggesting that silencing of hsp-25 enhance the memory response.

Since the responder cells used in our assay are T-cells isolated from the recipients and the APCs were of donor origin, the Elispot results represents cytokines production by T-cells activated via the direct allorecognition pathway. Our experiment was focused on alloreactivity that occurs at early time points following transplantation where acute rejection takes place. It is well established that direct allorecognition is the dominant pathway at early times post-transplantation (Lechler and Batchelor, 1982a, b). In our study, stimulation of primed allogeneic T-cells with donor APCs resulted in IFN- γ secretion. However, in the same group, this response was only marginally reduced when challenged with transgenic APCs compared to the respective LC APCs, thus implying that hsp-27 has little effect on alloimmune response that occurs during direct recognition. It is important to note that, in this study we used T-cells that have been already primed. Hence it is possible that hsp-27 is not able to regulate the responder cells that are already activated. It is however probable that hsp-27 may interfere with the activation of naive T-cells. But we did not investigate the effect of hsp-27 on T-cell activation. We could test this hypothesis by culturing naive T-cells with recombinant hsp-27 and a T-cell activator such as anti-CD3 or Concanavalin *in vitro* and analyse T-cell response by Elispot. Activation of naive T-cells requires presentation of antigen displayed by MHC complex present on the surface of APC, as well as co-stimulatory signal that is also provided by the APC (Abbas et al., 2007). Proteins that are presented by MHC class I are degraded into peptide by a protease complex called proteasome. Interestingly, hsp-27 has been shown to repress proteasome activity, thus hindering the presentation of antigen by MHC class I to CD8⁺ T-cells (Nagaraja et al., 2012).

Moreover, these authors demonstrated that silencing hsp-25/hsp-27 improved antigen presentation and tumour killing. It has been demonstrated that peripheral circulating human monocytes cultured with recombinant human hsp-27 resulted in decreased expression of MHC class II antigen (HLA-DR) as well as the co-stimulatory molecule CD86 (Banerjee et al., 2011). These studies raise the possibility that APC in hsp-27 transgenic donor hearts express less cell-surface MHC class I, MHC class II and CD86, hence resulting in less effectual antigen presentation. Ineffective antigen presentation has also been shown to lead to anergy of T-cells. In fact, a recent study has demonstrated that T-cells cultured with macrophages pre-exposed to hsp-27 were unresponsive and did not proliferate upon stimulation (Banerjee et al., 2011). Moreover, hsp-27 inhibits the differentiation of monocytes into immature dendritic cells and this inhibition could be due to increased production of IL-10 (Laudanski et al., 2005). Taken together, it may be possible that hsp-27 protects the graft from rejection through hsp-27 mediated dampening of antigen presentation.

8.4.2 Hsp-27 and cytokine production

Cytokine production by immune cells and regulation of such production also influence the immune response. In this chapter, we investigated production of IFN- γ and IL-2 by primed allospecific T-cells. These are characteristic cytokines of Th1 inflammatory cells. We did not investigate the presence of cytokines produced by Th2 cells with anti-inflammatory properties such as IL-4, IL-10 or IL-13. Hence, in this present study, it was not possible to conclude with certainty that hsp-27 may modulate cytokine production directed towards the allograft and further studies are required to confirm this. In order to assess whether hsp-27 regulates the immune response to the allograft, we could stimulate T-cells in presence or absence of hsp-27 and study the production of Th1 and Th2 cytokines profiles by ELISA. However, De et al showed that treatment of human monocytes with recombinant hsp-27 resulted in increased production of IL-10 (De et al., 2000). Thus, regulation of cytokines production could be a possible route through which hsp-27 prevents graft rejection. Interestingly, we showed that

overexpression of hsp-27 in donor heart was associated with diminished IFN- γ expression while elevated IL-4 expression (see chapters 4 and 6).

8.4.3 Summary

In Summary, hsp-27 may modulate the immune response through multiple mechanisms including dampening antigen presentation and regulation of cytokine production. Furthermore, regulation of other immune pathways including humoral immunity or innate immunity could also be possible. Further studies are required to fully elucidate the role of hsp-27 in modulating the immune response to allografts.

Chapter 9: Final Discussion, Limitations and Further Work.

9.1 Introduction

Cardiac transplantation is one of the gold-standard treatments for patient with terminal cardiac disease. The main drawback of transplantation is rejection of the graft and the requirement of life-long immunosuppression. Our laboratory previously observed with proteomic analyses of biopsies from long term cardiac transplant patients that hsp-27 was considerably more highly expressed in patients without CAV compared to those who developed CAV (De Souza et al., 2005). This key clinical finding was the stem of our hypothesis. We hypothesised that hsp-27 must be protecting the graft from developing CAV. The aim of this study was to elucidate the role of hsp-27 in cardiac transplantation using transgenic mice overexpressing human hsp-27. This is the first study to have looked at the effects of overexpressing hsp-27 in cardiac transplantation.

9.2 Overview of key findings

Cardiomyocytes are the principal cells damaged in acute rejection while SMC and endothelial cells are involved in CAV. We demonstrated that the transgene is overexpressed in cardiomyocytes and SMC; hence the transgenic model could be used for further studies.

We established a chronic rejection model by transplanting B10.A into CD4⁺ T-cell depleted CBA.

Our work revealed that overexpression of hsp-27 in donor hearts delayed acute rejection. Analyses of the graft indicated that the increased expression of hsp-27 led to a significant diminution of infiltration of the inflammatory cells. In addition, overexpression of hsp-27 resulted not only in considerable decrease of inflammatory cytokines but also upregulation of protective cytokines. These findings demonstrated that hsp-27 is cardioprotective during acute rejection and implied that hsp-27 might modulate the immunological responses of the host.

Similarly, using a murine model of CAV we demonstrated that increased expression of hsp-27 attenuated intimal thickening by reducing the severity and the number of vessels affected. Our findings further revealed that the protection from CAV was associated with reduced cellular infiltration and graft damaging cytokines. However, it enhanced the secretion of the regulatory cytokine-IL-4.

I/R injury is the main alloantigen independent factor that is involved in both acute and chronic rejection. Our findings demonstrated that hsp-27 inhibited the activity of caspase-3 and abolished the activity of caspase-9. Moreover it seemed to regulate the expression of caspase-1. Hence, it offered a significant protection against cell death caused by I/R injury which is encountered during cardiac transplantation.

Hsp-27 seemed to have the ability to induce immune deviation, whereby transgenic grafts showed an elevated expression of intragraft IL-4 and diminished expression of intragraft IFN- γ . We attempted to verify whether hsp-27 modulated the memory response of T-cells. Although there was a trend for decreased secretion of IFN- γ by splenic T-cells exposed *in vivo* to hsp-27+ cardiac allografts, this did not statistically differ to the response of T-cells exposed to LC hearts. This implied that we could neither demonstrate the immune deviation by hsp-27 *in vitro* nor conclude about the immunomodulatory effect of hsp-27 in the context of our study.

9.3 Possible mechanisms of protective effect of hsp-27 on graft rejection

9.3.1 Hsp-27 prevents oxidative stress

I/R injury causes oxidative stress that further damages the graft. Studies have shown that hsp-27 is involved in maintenance of redox homeostasis and reduction of the generation of ROS following oxidative stress. In addition, others and we have demonstrated that, hsp-27 resulted in less apoptotic cells in the graft. It is thus conceivable that hsp-27 may delay graft rejection by limiting oxidative stress and apoptosis.

Limitations and Further studies: Some individuals make antibodies to autoantigens exposed on apoptotic cells (Casciola-Rosen et al., 1994; Dieker and Muller, 2009; Rosen and Casciola-Rosen, 1999) – such as vimentin antibodies or anti-nuclear antibodies. It would have been interesting to look at production of autoantibodies in our murine recipients and see if hsp-27 overexpression in donor hearts limited production of antinuclear or anti-vimentin antibodies.

9.3.2 Hsp-27 limits inflammation

Hsp-27 may attenuate inflammation. The maturation of cytokines IL-1 β and IL-8 -involved in inflammation- are regulated by caspase-1 (Bryant and Fitzgerald, 2009; Siegmund, 2002). Increased expression of hsp-27 reduced the activity of caspase-1 at normoxic and ischaemic condition, hence suggesting that hsp-27 may regulate the expression of this caspase. It is thus possible that the decreased activity of caspase-1 resulted in reduced production of pro-inflammatory cytokines IL-1 β and IL-8, thereby reducing the inflammatory response directed towards the graft.

Limitations and further studies: one caveat to this study is that we did not investigate the expression of IL-1 β or IL-8 following I/R injury. Although the expression of IL-1 β seemed higher in LC grafts in both acute rejection and CAV, this was insignificant. Further studies need to explore whether hsp-27 affects the activation of IL-1 β and IL-8 following I/R injury.

9.3.3 Hsp-27 dampens antigen presentation

Intracellular proteins are processed by proteasomes and presented to CD8⁺ T-cells by MHC class I, while exogenous antigens are presented to CD4⁺ T-cells by MHC class II. Furthermore, the activation of T-cells requires presentation of antigen by MHC/APC complex on the surface of APC and simultaneous co-stimulation provided APC. Others have shown that hsp-27 represses proteasome activity thus leading to ineffective presentation of antigen by MHC class I (Nagaraja et al., 2012). In addition, others have shown that hsp-27 reduces the expression of MHC class II and the co-stimulatory molecule CD86 on the surface of monocytes *in vitro* (Banerjee et al., 2011). Furthermore, other studies also revealed that T-cells stimulated by macrophages (a

potent APC) pre-exposed to hsp-27 became unresponsive (cannot initiate an immune response) and resulted in inhibition of their proliferation as well as diminished cytokine secretion. We could thus argue that the enhancement of graft survival was due to interference with antigen presentation and subsequent T-cell anergy.

Limitations and future studies: Investigate MHC class II expression in hsp-27 transgenic mice – look at circulating monocytes as well as APC in the spleen and hearts. We could also have taken APC from transgenic mice spleen and tested their ability to induce a primary mixed lymphocyte response with allogeneic lymphocytes *in vitro*.

9.3.4 Hsp-27 and immune deviation

Attempts have been made to prevent rejection by promoting tolerance. Peripheral tolerance can be maintained by mechanisms such as anergy (unresponsive T-cells), immune deviation (from Th1 to Th2) or immune regulation (by regulatory T-cells). Hsp-27 seems also to possess the ability of strengthening peripheral tolerance. We discussed in the previous section that others have suggested that hsp-27 induces anergy. Our results demonstrated that grafts overexpressing hsp-27 displayed enhanced IL-4 secretion. IL-4 is a regulatory cytokine since it modulates the secretion of other cytokines and it also regulates the Th1 response. IL-4, produced by Th2 cells, activates Th0 (non-differentiated T-cells) by a positive feedback where it promote their own differentiation. In addition, it prevents Th1 differentiation and thus prevents production of pro-inflammatory cytokines. We have shown that increased expression of hsp-27 resulted in decreased secretion of IFN- γ . In addition, De et al showed that hsp-27 considerably enhanced the production of IL-10 but only moderately enhanced the production of TNF- α by monocytes (De et al., 2000). Thus, we could postulate that hsp-27 may enhance graft survival due to immune deviation from Th1 to Th2.

Further studies could investigate the ratio of Th1 to Th2 cytokines produced by spleen cells from transgenic and LC in primary responses to allogeneic APC.

9.3.5 Hsp-27 and SMC migration

CAV, which is the major long-term complication following cardiac transplantation consists of luminal occlusion of donor cardiac vessels by SMC. Two concepts have been proposed to explain the origin of SMC within the intima: the first is that SMC of donor origin migrate to the lumen from the media and proliferate leading to neointimal hyperplasia (Atkinson et al., 2004). The second theory was that cells of recipient origin cause luminal occlusion as a consequence of vascular remodelling; there is evidence that medial cells are destroyed early on after transplantation (in non-immunosuppressed experimental models) and it has been suggested that stem cells from the recipient are mobilised for repair and they invade the intima where they proliferate (Hillebrands et al., 2003; Shimizu et al., 2001). Hsp-27 could prevent neointimal hyperplasia in both instances. The increased expression of hsp-27 present in donor SMC may prevent their migration and proliferation, thus reducing neointimal population by SMC of donor origin. On the other hand, overexpression of hsp-27 in SMC in the media may protect from medial damage, thereby reducing the need for vascular remodelling hence preventing neointimal hyperplasia by recipient origin. It would be very informative to elucidate the origin of the intimal SMC in our model. To identify the origin of SMC, one could transplant heart from GFP positive female (donor) into male recipient and identify the origin of cells populating the neointima

9.4 Other limitations and further studies

9.4.1 Antibody mediated rejection and hsp-27

One of the main disadvantages is our understanding of the contribution of AMR towards graft rejection. We showed the presence of alloantibody directed towards the donor in acute rejection model. In CAV model, we could not firmly affirm the presence of alloantibody in host serum. However, other studies have shown that AMR seems to be playing a critical role in the development of neointimal lesion. It is very likely that in our study, the treatment with anti-CD4 mAb has prevented production of alloantibody. Therefore, in our CAV model, we need to

investigate the presence of antibody at earlier time point. In addition, the significance of hsp-27 on antibody production and antibody mediated rejection needs to be explored. Although, hsp-27 did not alter the alloantibody production in the acute rejection model, it may act on the production of de novo antibody. This might add to the mechanistic link of hsp-27 on graft rejection. In order to understand the involvement of antibodies in acute and chronic rejection, we could look for the deposition of the complement component C4d in both acutely rejecting grafts as well as hearts showing CAV.

9.4.2 Identification of CD14+ cells

One of the most interesting result of our study was the observation that allografts from mice overexpressing hsp-27 displayed considerably increased infiltration of CD14+ cells in the acute rejection model. We failed to characterise further these monocytic populations. We discussed in chapter 4 that there were two types of monocytic population distinguished by their surface marker Ly6C^{hi} and Ly6C^{lo} which have inflammatory and regulatory properties respectively. In addition, studies have shown that overexpression of hsp-27 increases the life span of monocytes, which otherwise undergo spontaneous apoptosis (Voss et al., 2007). It would therefore be interesting to identify the type of monocytes that were enhanced by flow cytometry by gating CD11b^{hi}/Ly6C^{hi} and CD11b^{hi}/Ly6C^{lo} (Rivollier et al., 2012; Swirski et al., 2009).

9.4.3 Immunomodulation by hsp-27

Our studies suggest potential immunomodulatory properties of hsp-27. However, we failed to demonstrate such function *in vitro* using memory T-cells. It is however possible that hsp-27 may modulate naive T-cell response by preventing the development of naive T-cells into Th1cells and promote differentiation into Th2 cells, which we failed to investigate in this study. It is well known that Th0 cells stimulated in presence of IFN- γ and IL-12 differentiate into Th1 cells while IL-4 promotes the differentiation of Th0 cells into Th2 cells (Le Gros et al., 1990; Seder et al., 1993; Swain et al., 1990; Zhu and Paul, 2010). We showed that transgenic heart overexpressing hsp-27 produced less IFN- γ and high level of IL-4. It is therefore possible that

hsp-27 promotes differentiation Th2. In addition, hsp-27 may also regulate the immune response by ineffective antigen presentation. As described in section 9.3.4 above, we could perform further studies to investigate whether APC overexpressing hsp-27 alters naïve T-cell activation and causes deviation from a Th1 response to a Th2 response.

A major consideration in interpretation of our results is that it remains elusive whether the protection against graft rejection was due to direct action of hsp-27 on immune cells or whether it was secondary to decreased cell damage thus resulting in an attenuated immune response.

Therefore further studies are required to verify whether hsp-27 modulate alloimmune response directly by either acting on cytokine environment, hence on naïve T-cells or on APCs.

9.4.4 Phosphorylation of hsp-27

Hsp-27 has three phosphorylatable sites at serine 15, 78 and 82. The function of hsp-27 depends on its phosphorylation status and subsequent oligomer size. Others have shown that phosphorylation was required for hsp-27 to protect against mitochondrial apoptosis and oxidative stress. Our results suggest it to be involved in graft survival. Unfortunately the level of understanding of the phosphorylative effect of hsp-27 in graft rejection is minimal in our study. Further studies of cardiac transplantation using transgenic donors overexpressing either non-phosphorylatable hsp-27 or phosphomimicking hsp-27 would be able to give more insight on the phosphorylative function of hsp-27 on graft rejection.

9.4.5 Anti-hsp-27 antibodies may prevent the function of hsp-27

Our study demonstrated that overexpression of hsp-27 protects from graft rejection. Similarly, expression of hsp-27 is high in cardiac patients free from CAV or IHD. These findings imply that hsp-27 protects from graft rejection. Interestingly, the expression of hsp-27 was reduced in CAV or IHD patients. In addition, we showed that hsp-27 decreased with the progression of disease in mice rejecting the graft. It is therefore intriguing why does the expression of hsp-27 decrease in diseased subjects. One explanation would be that anti-hsp-27 autoantibodies are made against the hsp-27. Anti-hsp-27 autoantibodies have been found in serum of patients with

coronary artery disease as well as other diseases (Pourghadamyari et al., 2011; Wood et al., 2010). This could target the hsp-27 expressing cells. Although the exact mechanism is not known, we postulate that increased expression of hsp-27 may stimulate the production of anti-hsp-27 autoantibodies. This may suggest a reason for the lower expression of hsp-27 seen in CAV patients or in the rejecting hearts of our animal models. Further studies needs to investigate the presence of anti-hsp-27 autoantibody in the serum of host rejecting the graft.

In addition, another possibility is that human hsp-27 is a foreign antigen in the mouse heart of the model used in our study. It is thus possible that following damage to the myocardium, it will be recognised as heterologous by the mouse immune system, hence production of antibodies against human hsp-27. Further studies are required to check for the presence of antibodies against human hsp-27.

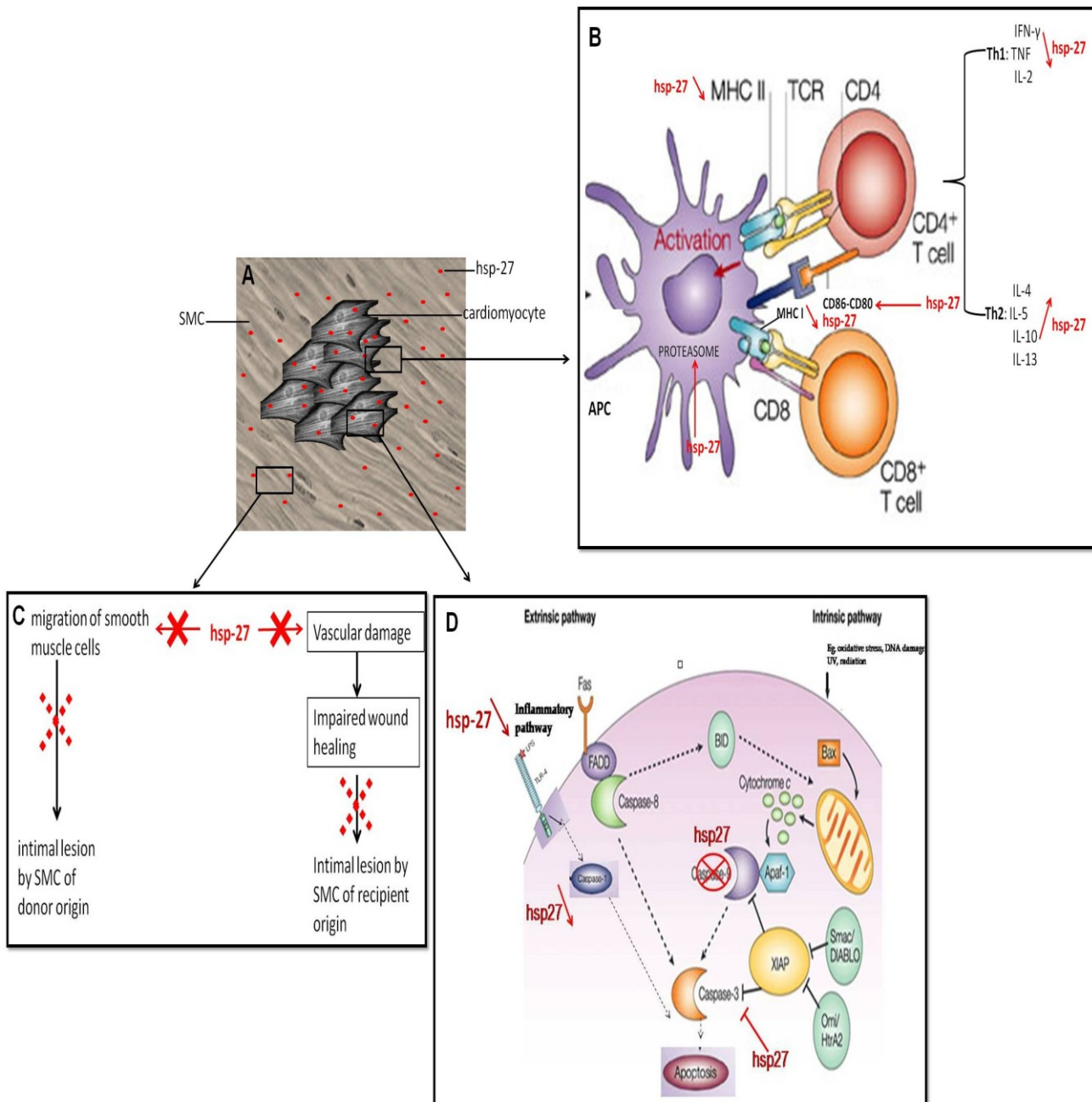


Figure 68: Schematic diagram of role of hsp-27 in graft survival. Adapted from (Bevan, 2004; Tschopp et al., 2003), <http://www.abcam.com>, <http://www.gwc.maricopa.edu>, <http://www.ebi.ac.uk>).

Legend for Figure 68

Schema to illustrate different mechanisms whereby hsp-27 protects the graft from rejection.

A-shows that hsp-27 is predominantly expressed in cardiac myocytes and SMC.

B-shows how hsp-27, if present in APC could be suppressing the activation of CD4+ and CD8+ T-cells by APC.

C-shows how hsp-27 could be inhibiting intimal proliferation by SMC, depending on whether SMC originate from the donor or the recipient.

D-shows how hsp-27 prevents apoptosis of donor cardiac myocytes.

9.5 Conclusion

The present study shows that hsp-27 delays acute rejection and lessens intimal lesion of CAV. Other studies conducted using hsp-27 shows that hsp-27 has pleiotropic effects on many cellular systems hence could prevent graft damage by regulating oxidative stress, inflammation, antigen presentation, immune deviation and SMC migration. These concepts are illustrated in Figure 68; however, further studies need to be designed to investigate whether these effects of hsp-27 are beneficial for graft survival.

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