THE EFFECT OF CHEMOKINES ON T REGULATORY CELLS FOLLOWING HEART TRANSPLANTATION

A thesis submitted to The University of Manchester for the degree of

MD

in the Faculty of Medical and Human Sciences

2011

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Abstract

The University of Manchester

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Doctor of Medicine (MD)

Title: THE EFFECT OF CHEMOKINES ON T REGULATORY CELLS FOLLOWING HEART TRANSPLANTATION

2011

Heart transplantation (HTx) is now an established therapy for end-stage cardiac failure not responding to medical treatment. Recent decades have seen improved outcome following HTx due to more effective and targeted immunosuppressive therapy. However, acute and chronic rejection remains a major cause of morbidity and mortality. At the same time, immunosuppressive strategies are associated with significant side effects, including development of tumours. Hence, the induction of immunologic tolerance to alloantigen is considered the "holy grail" of transplant research.

T regulatory cells (Tregs) are a subset of T cells that appear to suppress cytotoxic cell and initiate tolerance to foreign tissues. The Tregs suppress cytotoxic cells through specific cytokine pathways and cell-cell contact. In-vivo Treg migration has been a matter of debate in recent years. Treg trafficking is governed by chemokines, which are small secreted proteins, acting via their distinct trans-membrane serpentine receptors.

Experimental work has demonstrated an involvement of distinct chemokine pathways in Tregs migration and localization following cardiac transplantation; however, there is paucity of data in humans. I investigated the effects of chemokines on Tregs in heart transplant recipients through a series of observational studies. My study demonstrated that acute rejection following heart transplantation is associated with a significant elevation of peripheral blood Th1 chemokine levels. I hereby further show that peripheral blood Treg counts in stable heart transplant recipients are not affected by immunosuppression but are significantly lower in patients taking statins. I have demonstrated via *in-vitro* chemotaxis assays a specific pattern of chemotactic response for Tregs and the effector T cells. Using double immunofluorescence staining and immunostaining, I show for the first time that Tregs may migrate to the allograft under the influence of CCL17.

Word count: 32,445

DECLARATION

I hereby declare that no portion of the work referred to in the thesis has been submitted in support of any application for another degree or qualification of this or any other university or other institute of learning

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Acknowledgments

It is an honour for me to thank those who made this thesis possible. First and foremost, I owe my deepest gratitude to my supervisor, Prof Nizar Yonan, whose encouragement, guidance and support enabled me to understand the subject and carry out the necessary experiments. I am extremely grateful to Dr James Fildes, whose dedication to lab and grasp in the subject of immunology provided me with the knowledge base to design the whole study. I would like to thank Dr Peter Wood, whose support and guidance proved invaluable during all this work. I am deeply indebted to several colleagues, who provided helped in various ways during this work. I would like to thank Mrs Mary Krische, for all the guidance and help during long hours of work and discussion in the lab. I would also like to thank Chris Puchalka, Will Critchley, Sami Aljanabi, and Vishal for their sincere support in carrying out the various experiments. I am particularly indebted to the staff at the Transplant centre, including Sister Doreen Simpson, Mrs Anna Baynes, Mrs S Yonan, Mrs S Rhinds, and all the rest in the transplant outpatients and the Jim Quick ward, for their help in the sample collection and their love and support at difficult times.

I am greatly indebted to Dr Peter March, for his advice and support during the immunofluorescence staining and the use of microscopes at the University. I would also take this opportunity to thank Dr Paul Bishop for his kind support during the immuno-histochemistry experiment. At the same time, I am extremely thankful to all the staff in the pathology department, particularly Nigel, for providing the help and support in various ways. I am also grateful to

Mrs Julie Morris, for her unbiased and sincere statistical advices from the start to the finish.

My heart goes to my wife, for her sincere love and devotion gave me strength to stand fast during the late hours of laboratory work. And there has been a person whose affection and prayers made it all possible, my mother. I love you mum.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

DEDICATION

I dedicate this thesis to the loving memory of

my father

Istafa Ullah Khan

and

my brother

Furqan Khan

List of abbreviations

APC: Antigen presenting cell

BCA: B cell chemoattractant

CAV: Cardiac allograft vasculopathy

CD: Cluster of Designation

CNI: Calcineurin inhibitors

CTLA-4: Cytotoxic T-lymphocyte associated antigen 4

DAPI: 4', 6-diamidino-2-phenylindole, dilactate

DNA: Deoxyribonucleic acid

EDTA: Ethylenediamine tetra-acetic acid

e-GFR: Estimated glomerular filtration rate

FFPE: Formalin-fixed, paraffin-embedded

GCSF: Granulocyte colony stimulating factor

GVHD: Graft versus Host Disease

HEV: High endothelial venules

HIER: Heat induced antigen retrieval

IFNγ: Interferon gamma

IgG: Immunoglobulin G

IL: Interleukin

IP-10: Interferon-gamma induced protein-10

LPS: Lipopolysaccharide

MCP-1: Monocyte chemotactic protein-1

Met-RANTES: N-terminal Met, Regulated upon Activation, Normal T cell Expressed and presumably secreted)

MHC: major histocompatibility complex

MIG: Monokine induced by gamma interferon

MIP-1-α: Macrophage inflammatory protein-1-alpha

MIP-1-β: Macrophage inflammatory protein-1-beta

MMF: Mycophenolate mofetil

MPA: Mycophenolic acid

mRNA: messenger ribonucleic acid

NK cells: Natural Killer cells

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PMT: Photomultiplier tube

RPE: R-Phycoerythrin

RPM: rev per minute

SCL: Secondary Lymphoid-tissue chemokine

SLT: Secondary lymphoid tissue

TARC: Thymus and activation regulated chemokine

TBS: Tris buffered saline

TCR: T cell receptor

TGF-β: Transforming growth factor beta

TNF-α: Tumour necrosis factor alpha

Chapter 1 A review of the role of chemokines and Tregs following heart transplantation

1.1 Introduction

Clinical outcome following cardiac transplantation has significantly improved since its first description by Prof. Christian Barnard, who performed this pioneering operation on December the 3rd, 1967 in Cape Town (*1*). Although the first heart transplant recipient survived for 18 days only before succumbing to a chest infection; it sparked enormous interest in the medical community across the globe. This enthusiasm soon faded due to a poor long-term survival owing to inadequate understanding of the early post-operative complications, acute rejection and coronary vasculopathy (*2*). The next two decades saw overcoming of these hurdles, with a better understanding of the immune response across the antigenic barrier, diagnosis for rejection and provision of better immunosuppressive agents (*3*). Current figures demonstrate an excellent long-term survival following heart transplantation, with the median survival in excess of 10 years (*4*). Nevertheless, acute and chronic rejections continue to remain a major cause of morbidity and mortality. In addition, long term use of various immunosuppressive agents risks the development of serious adverse effects, including infections and carcinomas (*4*). Hence, considerable research is focused on obtaining a state of immunological tolerance across the barrier of Major Histocompatibility Complex (MHC). Immunological tolerance is a state of antigen-specific immunological unresponsiveness towards the allograft, thereby avoiding rejection and allowing sustained graft function without the need of immunosuppressive medications.

1.2 Mechanisms of transplant tolerance

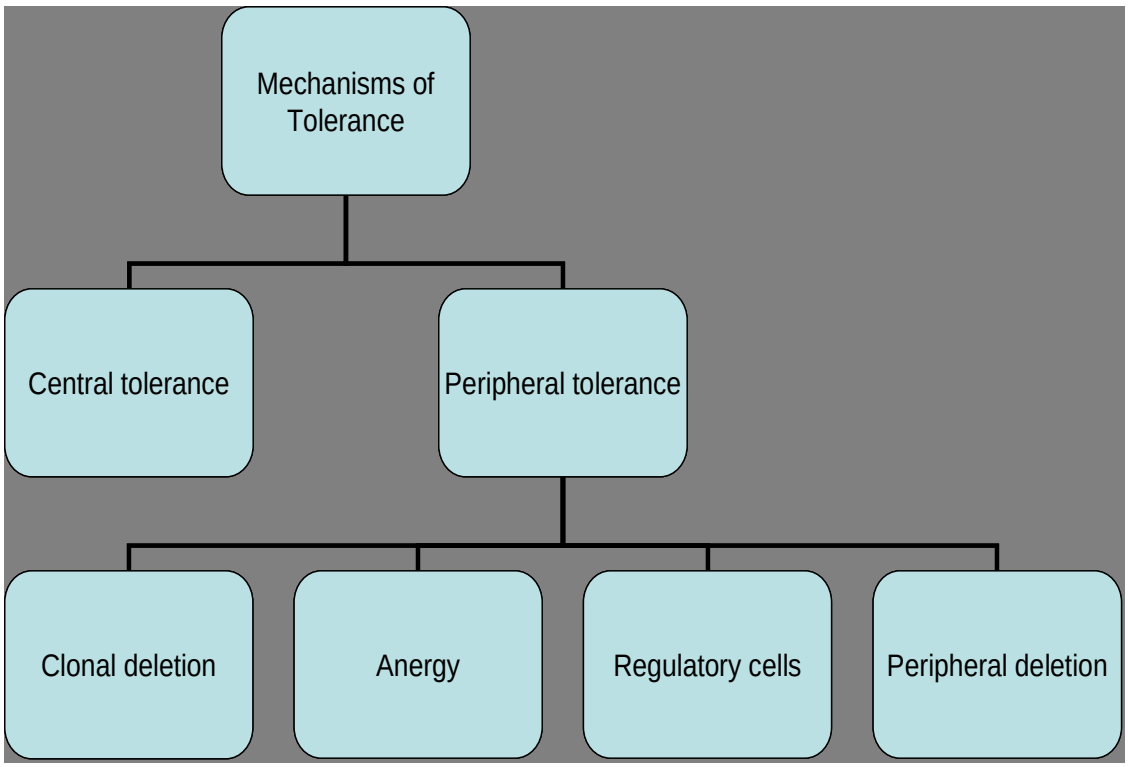
The concept of immunological tolerance emanates from the pioneering work by Sir Peter Medawar during World War II. His work, which started as an attempt to improve the outcome of skin grafting to help the burn victims, formed basis of the immunological mechanisms underpinning future developments in solid organ transplantation (*5*). His elucidation of acquired immunological tolerance constituted part of the overall work leading to the award of Nobel Prize in 1960.

The mechanisms for tolerance induction include central and peripheral tolerance (*6*). **Central tolerance** involves intra-thymic deletion or "negative selection" of T lymphocytes with high avidity for the expressed antigens. In contrast, there are several mechanisms for the induction of **Peripheral tolerance**, including clonal deletion, clonal anergy, exhaustion, ignorance or active suppression by regulatory T cells (*7*).

Clonal deletion is achieved by elimination of all those clones of T lymphocytes that can bind to donor-associated MHC antigens. It can be accomplished by thymic elimination of donor-specific T cells (**central deletion**), as seen in animal models of haematopoietic donor-chimerism (*8*). The donor T cells enter the recipient thymus, where donor-reactive T cell clones are deleted via apoptosis (*9*). Deletion of alloreactive T cells can also be achieved directly by depleting antibodies, immunotoxins, and /or lymphoid irradiation. The emigration of new alloreactive T cells following this non-selective depletion seems to favour, but does not insure tolerance induction (*10*). Therefore, the newly generated alloreactive T cells have to be deleted through ongoing therapy.

Clonal exhaustion (or **activation-induced cell death** (AICD) is a method of T cell apoptosis triggered by repetitive stimulation of the activated T cells with high concentrations of antigen, or exposure to alloantigen in a suboptimal condition (*11, 12*). A classis example is tolerance following MHC-mismatch liver transplantation, due to the presence of a large number of donor antigen presenting cells (*13*).

Figure 1.1 Mechanisms of Tolerance induction. Central tolerance refers to intra-thymic deletion of high-avidity lymphocytes, while the peripheral tolerance can be induced by several mechanisms, including T regulatory cells.



Ignorance is a state of unresponsiveness to alloantigen, developing as a result of interplay between new, alloreactive T cells and immuno-regulatory mechanisms (*14*). This state of unresponsiveness to alloantigen is not a permanent tolerance, as is can be broken by further triggering with alloantigen or exposure to interleukin 2 (IL-2) (*15*).

Peripheral suppression of self- or alloreactive T cells can also be achieved by induction of **anergy**. It is established that T cell activation requires CD28 co-stimulation when the T cell receptor (TCR) binds to peptide: MHC molecule. The expression of co-stimulatory molecules on the antigen presenting cells (APC) is tightly regulated. Therefore, when engagement of TCR (signal 1) occurs without co-stimulation (signal 2) T cell clones cannot proliferate or produce interleukin 2 (IL2), which is a necessary cytokine for T cell proliferation (*16*). Further observations suggest that this anergic state can develop as a result of either intrinsic signalling defects (such as a lack of mitogen-activated protein kinase (MAPK) signalling) or up-regulation of dominant anergic factors (*17*). Various models have been suggested to lead to anergic state, including oral administration of antigens, cross linking of CD3 complexes in vitro, and use of potent calcium ionophore, ionomycin (*18*). Further work provides evidence of reduced activation of LAT (linker of activation of T cells), a transmembrane protein that facilitates various other signalling molecule, as a critical step in the induction of anergy (*19*).

1.3 Tolerance by regulatory cells

The evidence for a peripheral T-cell mediated active immunosuppression was first reported by Gershon *et al* in 1970 (*20*). In 1985, Hall *et al* reported specific suppression of alloreactive cells by CD4+ T helper/inducer cells in a rat model of heart transplantation (*21*). Later, Sakaguchi *et al* showed it for the first time that $CD4^+$ T cells co-expressing CD25 were able to prevent organ specific autoimmune disease (*22*). These "**regulatory T cells**" (Tregs) were mentioned as thymically-derived, as neo-natal thymectomy at day 3 of age led to various organ-specific autoimmune diseases. In turn, adoptive transfer of $CD4^+CD25^+$ T cells from normal mice into thymectomized animals completely prevented manifestations of autoimmune diseases in those animals (*22, 23*). *In vitro* studies suggest that these $CD4^+$ $CD25^+$ cells are anergic i.e. do not proliferate on stimulation with exogenous IL-2 or TCR stimulation alone (*24*) and inhibit the proliferation of other CD4+ and CD8+ effector cells through a cell-cell contact dependent and antigen non-specific mechanism which required T cell receptor signaling (*25, 26*) and is reversible by IL-2 (*27*). They have also been shown to maintain allograft tolerance in vivo via interleukin (IL)-10 (*28*), and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) dependent mechanisms (*29*).
Since then it has been extensively documented that Tregs have a major role in maintaining tolerance in both experimental and clinical transplantation models (*30-34*).

Tregs can be broadly classified into natural and adaptive subsets. Naturally occurring $CD25^+$ T cells comprise 5-10 % of the peripheral blood $CD4^+$ T cells in adult humans and mice (*35-37*). Originally thought to develop only in the thymus by positive selection of naïve T cells (*38*), $CD4^+CD25^+$ Tregs

can also be generated in the periphery from either $CD25^-$ T cells through costimulation with T cell receptor and transforming growth factor beta (*39, 40*), or from highly differentiated memory $CD4^+$ T cells (*41*).

The molecular properties that characterize Tregs remain a matter of debate. Several cell-surface molecules have been identified for this subpopulation of $CD4^+$ T cells (table 1.1).

Table 1.1 Cell markers for Tregs. The markers are listed with their abbreviated and full names, while the third column shows the location of the marker within the cell. The last column shows that some of the cell markers are not specific for Tregs. CD = Cluster of Designation, IL = Interleukin.

Marker	Full / other name	Location	Comments
CD25	IL-2 receptor α chain	Cell membrane	Expressed on activated T cells, high expression on Tregs (*36, 42*)
CTLA4	cytotoxic T-lymphocyte antigen 4 (CD152)	Intracellular Cell membrane	Mainly intracellular, induced after TCR activation (*43, 44*), also expressed on activated $CD25^-$ T cells (*45*) and B cells (*46*).
CD103	$\alpha_E\beta_7$ integrin	Cell membrane	Receptor for E-cadherin (*47*), responsible for mucosal lymphocyte homing (*48*), expressed in naïve (*49*) or effector/memory-like Tregs (*50*), also expressed on $CD25^{-/low}$ cells (*36*).
GITR	Glucocorticoid induced tumour necrosis factor receptor family-related gene, TNFRSF18	Cell membrane	Expressed on both naïve and activated Tregs and activated $CD25^-$ T cells (*49, 51*) , expression level in humans were found uniformly distributed on all CD3+ and CD4+ T cells (*36*).
CD122	B chain of IL2 receptor	Cell membrane	Expressed by naïve $CD4^+CD25^+$Tregs (*52*) as well as $CD8^+$ Treg (*53*), essential for in vivo development and maintenance of Tregs (*54*).

Continued table 1.1

Marker	Full / other names	Location	Comments
CD28		Cell membrane	Naïve and activated Tregs (*55*), co-stimulation via B7 (*56*). Important for anergic state (*57*), expression also seen on $CD3^+$ and $CD4^+$ T cells (*36*).
CD45RB	Protein tyrosine phosphatase (PTP)	Cell membrane	Involved in T cell activation, low on primed effector cells (*58*) and Tregs (*59*). Anti-CD45RB antibody prolongs allograft survival (*60*).
CD62L		Cell membrane	Expressed on 50-60% of naïve Tregs in mouse, not a marker of suppression activity (*61*), also expressed on $CD25^-$ T cell (*24*, *36*).
CD127	IL7Rα	Cell membrane	Low on Tregs, higher expression on most other $CD4^+$ T cells (*62*), inversely correlates with FOXP3 expression (*63*)
FOXP3	Forkhead box P3	Intranuclear	Encodes for forkhead/winged helix transcription factor, important for Treg development and function (*62*, *64*), difficult for functional analysis due to intracellular location (*65*)

Recent evidence suggests that an adoptive transfer of *in-vitro* expanded Tregs can be used for therapeutic purposes in certain autoimmune disorders and to induce transplant tolerance (*33, 66*). In a murine model, the *in-vitro* expanded and adoptively transferred Tregs migrated preferentially to allograft and significantly delayed allograft rejection in the absence of immunosuppression (*67*). Another study on non-human primates documented potent suppressive activity of *in-vitro* expanded Tregs against allospecific xenogeneic stimulation (*68*). Somewhat similar observations have also been made during *in-vitro* experiments on peripherally expanded human Tregs (*69, 70*).

In order to exert optimum homeostatic and inflammatory regulation, Tregs migrate to lymphoid or peripheral tissues including tumours, transplanted organs or other areas of inflammation (*32, 71-73*). A sound knowledge of the mechanisms controlling Treg migration in vivo is therefore crucial for their proper utilization in future cell based therapies. Tissue-specific Treg trafficking is dependent on a complex network of chemotactic signalling from cytokines, chemokines and adhesion molecules.

1.4 Chemokines

Chemokines are a superfamily of 8 to 11 kDa (67-127 amino acids) proteins with discreet roles in leucocyte activation, migration, haematopoiesis and angiogenesis (*74-78*). Because of their specificity, the chemokines direct selective leucocyte recruitment in response to inflammation, thus orchestrating the secretion of inflammatory mediators and tissue damage (*79, 80*). Chemokines are subdivided into four groups (C, CC, CXC, and CX3C) based on the position and separation of the first two amino-terminal cysteine residues of a four-cysteine motif in their primary amino acid sequence (*81*). There are two

nomenclature systems for chemokines, the traditional abbreviations, such as interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1, and a systematic nomenclature that combines structural motifs (CXC, CC, XC, CX3C) with L for ligand and the number of the respective gene.

Chemokines can also be divided into categories reflecting their temporal and spatial expression: the inducible (or inflammatory) and the constitutive (or homeostatic) chemokines (*81-83*). The inducible chemokines are promoted by pro-inflammatory stimuli such as tumour necrosis factor alpha (TNFα), interleukin-1 (IL-1), lipopolysaccharide (LPS) to control the recruitment of effector leukocytes in infection, inflammation, tissue injury, and tumours. Most inducible chemokines demonstrate broad target cell selectivity and act on cells of the innate as well as the adaptive immune system (*84*). The constitutive (or homeostatic) chemokines are produced at non-inflamed sites. They are important for immune surveillance, maintaining homeostatic leukocyte traffic and cell compartmentalization within lymphoid tissues, as well as homing of leukocyte precursors during haematopoiesis (*81*).

1.5 Chemokine receptors

All known chemokines bind to seven-pass, trans-membrane-spanning serpentine, Gi/Go protein-coupled, Bordetella pertussis toxin-sensitive receptors (*75, 85*). Chemokine receptors are designated according to the type of chemokine(s) they bind (CXC, CC, XC, and CX3C), followed by “R” (for receptor) and a number indicating the order of discovery. Binding of chemokines to the specific receptors triggers complex intracellular signalling cascades that rapidly promote the activation of leukocyte integrins and their adhesion to endothelial cells. This process leads to trafficking of immune cells in

response to adequate chemokine signals (*86*). Each chemokine family recruits only specific cell types, while the expression of receptors is further regulated according to the cell subsets, and/or the state of cell activation (*87, 88*). However, there is substantial redundancy in this system, with overlap in the ligand specificities, and some chemokines binding to multiple receptors (*75*). Approximately 50 chemokines and 20 chemokine receptors have been identified in humans (*82*). Some chemokine receptors form homodimers or heterodimers. This post-translational modification is suggested to add flexibility to the overall system, with formation of cell type- or activation-specific receptors (*75, 80*).

1.6 Evidence for role of chemokines in acute rejection following cardiac transplantation

Acute cellular rejection includes an infiltration of mononuclear cells into the allograft. Several studies in animal models have demonstrated an increased expression of CXC-chemokines (CXCL9, CXCL10 and CXCL11), associated with an infiltration of the corresponding receptor (CXCR3) expressing Th1 cells during rejection (*89-91*). Another study showed increased expression of CX3CL1 on rejecting allografts (*92*). Human studies showed variable patterns of the expression of CC and CXC-chemokines and chemokine receptors during acute allograft rejection (*93-96*). The expression levels increase in the later versus earlier rejections, despite no change in the grade of mononuclear infiltrate (*97*). The analysis of sequential human endomyocardial biopsies showed an association of $CD3^+$ T-cell infiltration with the expression of CCR1, CCR3 and CXCR3. However, only CXCR3 and its ligand CXCL10 were up-regulated during acute rejection, suggesting a critical role for this chemokine pathway (*93*). (Table 1.2)

Table 1.2 Evidence for the involvement of chemokines in acute cardiac allograft rejection. All chemokines are mentioned according to the systematic nomenclature. The first column refers to the author and the year of publication. The table also shows whether the study was conducted in humans or other species. The final column shows the relevant conclusions from these studies.

Study (ref)	Chemokine	Receptors	Species	Correlations
Fairchild 1997 (*91*)	CCL2 CCL3, CCL4, CCL5, CXCL10, KC	Not checked	Mouse	Increased gene expression of all chemokines in rejecting allografts; CXCL10 expression increased throughout rejection, while CCL3, CCL4 and CCL5 expression were high during late phase of rejection
Hancock 2000 (*89*)	CXCL9, CXCL10, CXCL11	CXCR3	Mouse	Intragraft mRNA expression of CXC chemokines correlate with rejection and infiltration of CXCR3+ mononuclear cells
Robinson 2000 (*92*)	CX3CL	CX3CR1	Mouse	Increased CX3CL is associated with cellular infiltration and acute rejection
Melter 2001 (*93*)	CCL2, CCL5, CCL11 CXCL9,	CCR1, CCR3, CCR5, CXCR3	Human	CCR1 strongly associated with T cell infiltration ($p<0.001$) but not with rejection CXCR3 strongly associated with

	CXCL10, XCL1, CXCL12			both T cell infiltration and rejection ($p<0.001$) Intragraft CXL10 and CCL5 strongly correlated with acute rejection ($p<0.05$)
Zhao 2002 (*96*)	CXCL9, CXCL10, CXCL11	CXCR3	Human	Intragraft mRNA expression of CXCL9, CXCL10, and CXCR3 correlated with acute rejection ($p<0.05$)
Fahmy 2003 (*94*)	CXCL8, CXCL9, CXCL10, CXCL11, CCL5, CCL2	CXCR3, CCR5	Human	Intragraft mRNA expression of CXCL10, CXCL9, CXCL11, CCL5, CXCR3, and CCR5 correlated with acute rejection ($p<=0.009$)
Karason 2006 (*98*)	CXCL9 CXCL10	Not assessed	Human	Intragraft mRNA expression of CXCL9 correlated with acute rejection ($p<0.05$) Serum levels of chemokines showed no association with rejection

Further studies defined the cellular sources of chemokines during rejection. It is seen that the early expression (within 8 days following transplantation) of CXCL9 and CXCL10 in murine allograft are mediated by host $CD8^+$ T cell, but not by $CD4^+$ T cells or NK cells (*99*). Using human cardiac allograft biopsies, Zhao *et al* showed that CXCL10 and CXCL11 were expressed in vascular smooth muscle cells and CXCL11 in the endothelial cells, while infiltrating macrophages expressed CXCL9 as well as CXCL10 and CXCL11 (*96*). Another study showed that CXCL9 is produced by allograft endothelium, infiltrating macrophages and neutrophils (*100*). The precise pathways regulating differential secretion of these chemokines still remain elusive.

A study using DNA microarray analysis showed that IFN-γ knockout mice rejected the cardiac allografts at the same pace as wild-type mice, with expression of a completely different set of chemokines and receptors genes (*101*). There was an up-regulation of CCL2, CCL3, CXCL12 and CXCR4 despite the absence of IFNγ signalling. The sources of these chemokines were, however, not described.

Morita *et al* investigated the role of sequential chemokine expression on trafficking of T cells into allografts during the progression of acute rejection in an animal model. They showed that the early chemokine cascade, including CCL2, CCL3, CCL4, CXCL1, CXCL10 and CX3CL1 are directed at the recruitment of the cellular component of the inflammatory system (i.e. neutrophils, macrophages, NK cells), which leads to a later upsurge of CCL5, CXCL9, CXCL10, CXCL11 causing recruitment of alloantigen primed T cells into the grafts (*102*).

Based on the observations that chemokines are involved in acute allograft rejection, investigations were carried out to determine if peripheral blood chemokine levels can be used as a marker of rejection. Karason *et al* compared the intra-graft gene expression of CXCL9 and CXCL10 with serum levels at the time of acute rejection in human heart transplant recipients. They concluded that acute rejection leads to a significant up-regulation of CXCL9 mRNA in the graft, while the serum levels of both CXCL9 and CXCL10 remain unaltered (*98*).

1.7. Evidence for role of chemokines in Cardiac allograft vasculopathy:

Cardiac allograft vasculopathy (CAV), a hallmark of chronic rejection, is the leading cause of late death in heart transplant recipients (*103*). CAV results from a combination of complex pathological processes, including insults to the vascular integrity, immune response against the allograft, ischemia-reperfusion injury, viral infections, hypertension, hyperlipidemia, and diabetes mellitus (*103*). Acute rejection episodes are a critical risk factor for the subsequent development of chronic rejection (*104*, *105*). There is evidence that allograft infiltration with activated T lymphocytes and macrophages precedes the development of intimal proliferation, the hallmark of CAV (*106*). *In vivo* data suggests that allogeneic T cells mediate graft endothelial cell dysfunction, followed by vascular smooth muscle cell dysfunction (*107*). Another interesting study suggested that the smooth muscle-like cells forming vascular neointima in the CAV are derived from circulating bone marrow-derived precursors (*108*). Similar to their well documented role in atherosclerosis (*109*), chemokines play a significant part in the development of CAV (table 1.3). Several animal studies have demonstrated that increased intra-graft expression of chemokines like

CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10, along with corresponding receptors (CCR5, CCR2 and CXCR3), is associated with CAV (*110-113*). These results were complemented by chemokine-blocking studies; anti-CCL2 gene therapy in mice attenuated the development of CAV (*114*): blocking CCL5/CCR1 & CCR5 pathway with Met-RANTES reduced infiltration of $CD4^+$, $CD8^+$, and monocytes/macrophages into the allograft, and a subsequent attenuation of intimal thickening (*115*), and use of anti-CXCL9 antibody reduced T cell infiltration and intimal proliferation (*112*). Recent work on human cardiac allograft biopsies showed persistent elevation of CXCL10 and CXCL11 expression (but not CXCL9) in patients who developed significant CAV (*96*). Another study looking at peripheral blood levels of the CCR5 and CXCR3 ligands demonstrated that only CXCL11 levels were elevated in patients with CAV, while levels of CCL3, CCL4, CCL5, CXCL9 and CXCL10 failed to show any correlation (*116*).

Table 1.3 Evidence for a role of chemokines in chronic cardiac allograft vasculopathy. All the chemokines are mentioned in the systematic nomenclature. The first column refers to the author and the year of publication, while the table also shows if the study was conducted in humans or any other species. The final column shows the relevant conclusions.

Study (ref)	Chemokines	Receptors	Species	Correlations
Pattison, JM 1996 (*111*)	CCL5	Not checked	Human	Expression in mononuclear cells, myofibroblasts, and endothelial cells associated with CAV but not in normal coronary arteries
Yun JJ 2000	CCL2, CCL5, CXCL1, CXCL10, XCL1	Not checked	Mice	Late (day 4 onwards) and persistent intra-graft XCL1, CCL2 and CCL5 expressions were associated with macrophage and T cell infiltration and intimal proliferation
Kao J 2003 (*116*)	CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11	CCR5, CXCR3	Human	Elevated plasma levels of CXCL11 was associated with CAV ($p<0.05$); CXCL11 localized to the endothelial surface of CAV lesions, associated with CXCR3+

				mononuclear cells infiltration
Yun JJ 2002 (*112*)	CXCL9	Not checked	Mice	Increased intragraft CXCL9 levels increased significantly ($p<0.001$) by day 7 and remained elevated, preceding mononuclear cell infiltration and development of CAV. CXCL9 neutralization delays CAV
Horiguchi 2002 (*113*)	Multiple chemokines	Multiple chemokine receptors	Rats	Enhanced intragraft expression of CCR2, CCR5, and CXCR3 genes with corresponding ligands in rats developing CAV ($p<0.167$)
Van Loosdregt J 2006 (*117*)	CXCL9, CXCL10, CXCL11, CCL5, CX3CL1	CXCR3, CCR5, CX3CR1	Human	Intra-coronary expression of CXCL11, CCL5, CX3CL1, CCR5 and CX3CR1 were significantly elevated ($p<0.05$) in allograft with CAV as compared to those without

The inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (commonly known as Statins) are the most common agents to treat hyperlipidemia frequently seen in transplant recipients. It has been well documented that statins can attenuate CAV (*118*). However, separate from a lipid altering mechanism of action, evidence also exists to suggest that statins have important immune-modulating properties. Pravastatin has been shown to inhibit monocytes CCL2 expression (*119*), while Yin *et al* demonstrated that simvastatin administration reduces the intra-graft levels of CCL2, CCL5 and CXCL10 with decreased expression CCR2 and CCR5, and thus inhibited T cell and macrophage infiltration, with attenuation of CAV (*120*). Further evidence suggests that the anti-atherosclerotic potential of statins is independent of the type of statins or cholesterol-lowering properties. A study using pig-model of induced atherosclerosis compared the effects of Atorvastatin and Pravastatin versus placebo. The vascular expression of CCL2 was down regulated by 37% by both statins ($p<0.05$) compared to the placebo. This finding opens new insight into the pleiotropic effects of cholesterol lowering agents in heart transplant recipients. However currently, our knowledge remains limited about the mechanism of any potential immunoregulatory effects of statins within the atherosclerotic lesion.

1.8 Chemokine pathways involved in Treg migration

Several mechanistic studies have attempted to define pathways utilised for T cell trafficking during immune responses. Despite the apparent redundancy in the chemokine system, it appears that discreet chemokine pathways are responsible for migration of specific lymphocyte subsets (*84*). It is generally perceived that Tregs share the same chemokine receptor profiles as effector T

cells (*73*). However, evidence suggests that specific migration of Tregs *in vivo* is a result of differential expression of chemokine receptors, which might be different from the effector T cells. For example, effector T cells express CXC chemokine receptor, while its expression in Tregs is documented to be very low (*37*). Also, various organs can achieve different levels of Treg enrichment, which points to preferential expression of certain receptors in Treg population (*121, 122*). The following discussion emphasizes the importance of various chemokine pathways involved in Treg trafficking following transplantation.

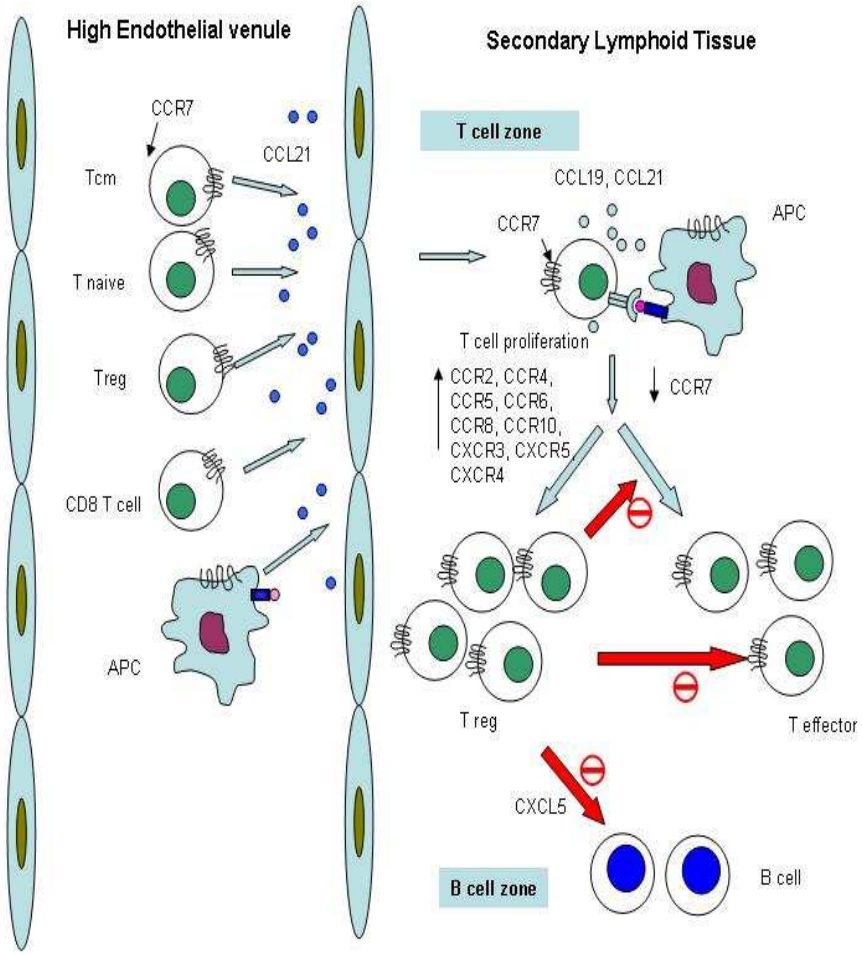
1.9 Lymphoid tissue homing chemokine pathway in transplantation

1.9.1 CCR7 / CCL19, CCL21 pathway

CCL19 (also known as macrophage inflammatory protein-3-beta) and CCL21 (also known as 6Ckine or the chemokine with 6 cysteines), the homeostatic chemokines constitutively expressed in the secondary lymphoid organs, are the only ligands for CCR7 (the CC-chemokine receptor 7) (*123*). The post capillary or high endothelial venules (HEV) of lymph nodes and Peyers patches constitutively express CCL21, while fibroblastic reticular cells within T cell zones of the lymph nodes express CCL21 as well as CCL19 (*124*). This pathway primarily controls movements of CCR7 expressing naïve and central-memory T cells and Tregs through the secondary lymphoid tissue (SLT) in search of specific antigen, as well as trafficking of antigen-presenting dendritic cells from allograft to the SLT to initiate allospecific immune responses (*125*). Following organ transplantation, blood-borne circulation of dendritic cells to the draining lymph nodes is essential for tolerance induction (*126*). Subsequent signalling with CCL19 and CCL21 leads to T cell and DC co-localization in T cell areas, thereby facilitating antigen recognition and proliferation of T cells,

including Tregs that control the effector T cell response (*127*). CCR7 and its ligands are also essential for thymic development and central tolerance (*128*). Mice deficient in CCR7 or its ligands show disturbed lymphoid structure, impairment of central and peripheral tolerance, impaired Treg function, and develop autoimmune disorders (*129*). Menning *et al* demonstrated that more than 80% of murine naïve-like Tregs exhibit high CCR7 expression, while effector-memory like Tregs, albeit positive, show more heterogeneous expression. The also showed that CCR7 deficiency abolishes the lymph-node homing capacity of naïve-like Tregs; however, it favours accumulation of effector/memory-like Tregs in the inflammatory areas, with enhanced suppression of inflammation (*50*). In humans, memory Tregs can be differentiated on the basis of differential expression of CCR7 into central memory ($CCR7^+$) and effector memory ($CCR7^-$) subsets (*130*).

Figure 1.2 T cell and antigen presenting cell (APC) trafficking in the secondary lymphoid tissue. The chemokines CCL19 and CCL21 cause T cell and APC migration from the high endothelial venules towards the T cell zone via the CCR7 receptor. Subsequent activation of the T cells via TCR (T cell receptor) stimulation leads to T cell proliferation and an up-regulation of various CC and CXC chemokine receptors, while causing a down-regulation of the CCR7 receptor. Tregs hence exert an inhibitory effect on the Teffector cells and the B cells (shown as red arrows). (Tcm = Central memory T cell, Tnaive = Naïve T cell, Treg = Regulatory T cell, Teffector = Effector cell)



Further evidence suggests that CCR7-related pathways may represent target of immunotherapy following transplantation. In a study using murine MHC mismatch kidney and cardiac transplantation, CCR7 pathways were blocked using recombinant CCL19-IgG1 (an agonistic chemokine fusion protein, produced to prolong the half life of CCL19 up to 24 hours). Prolonged, high dose stimulation by CCL19-IgG1 resulted in CCR7 downregulation, markedly reduced T cell and DC trafficking to the SLT, reduced allospecific effector T cell proliferation and prolonged allograft survival (from 9 days to 20 weeks). However, this method failed to induce tolerance, since adoptive transfer of splenocytes from long term survivors (after 9 weeks) following CCL19-IgG treatment did not prevent rejection in untreated syngeneic mice (*131*). Another study using NOD.SCID mice showed via transfer that $CD4^+CD25^+CD62L^+$ Tregs, high in CCR7 expression, were able to significantly delay the onset of diabetes when compared to $CD4^+CD25^+CD62L^-$ Treg, which were CCR7 deficient, despite similar *in vitro* regulatory function (*132*). CCR7 expression has been implicated in tumour survival and progression for various cancers (*133, 134*), and blocking CCR7 via monoclonal antibodies and chemotherapy has shown promising results in experimental cancer therapies (*135*).

1.9.2 CXCR5/CXCL13 pathway and Tregs

CXCL13, also known as BCA-1 (B-cell attracting chemokine 1), is a primary B cell chemoattractant (*136*), secreted by germinal centre T helper cells (GC-Th) and follicular dendritic cells (*137*). It plays a major role in the humoral arm of alloresponse or autoimmunity (*138*).

While only a small subset of $CD4^+CD25^+$Tregs naturally express CXCR5 (approximately 10-30%), the expression appears significantly enhanced

following TCR stimulation. Hence, after activation in the T cell zone, Tregs acquire the ability to migrate to the B cell area, where they suppress B cell survival, immunoglobulin synthesis, and activation-induced cytidine deaminase (AID) expression (*139*). Hence, this chemokine pathway is critical in the regulation of adaptive humoral immune response. In a study using an MHC-I mismatched rat transplant model, Tregs activated by the indirect pathway prevented alloantibody mediated rejection (*140*). Further studies are warranted in this area to explore the possibilities of using the CXCR5/CXCL13 pathway for inducing transplant tolerance.

1.10 Peripheral tissue homing chemokine pathways in transplantation

1.10.1 CCR4 / CCL17, CCL22 and CCR8 / CCL1 pathways

Antigen priming in SLT causes an overhaul of chemokine receptors on Tregs, from CCR7 and CXCR5 to effector-memory-like chemokine receptors including CCR2, CCR4, CCR6, CCR8 and CCR9 (*141*). This heterogeneous expression of homing receptors enables Tregs to migrate to different non-lymphoid organs (*142*).

In a study comparing chemotactic profile of human peripheral blood Tregs against their $CD25^-$ counterparts, Tregs showed greater selective expression of CCR8 ($p=0.0001$) and CCR4 ($p=0.03$) than $CD4^+CD25^-$ cells. *In-vitro* chemotaxis assays also demonstrated a significantly greater ($p<0.01$) migration of Tregs compared to $CD25^-$ T cells in response to the CCR4 ligands, CCL17, CCL22, and CCR8 ligand, CCL1, plus a synergistic effect of the suboptimal doses of CCL1 and CCL22 (*37*).

In a murine model of induced allo-tolerance via CD154mAb and donor specific transfusion (DST), tolerance was associated with up-regulation of

CCL22 and CCR4 and infiltration of Tregs in the allograft. $CCR4^{-/-}$ mice rejected the allograft associated with reduced infiltration of Tregs, however, the number and function of peripheral Tregs were normal. The study demonstrated that CCL22/CCR4 axis does not impact on Tregs development, but plays a key role in Tregs migration to the allograft. The authors failed to find any association with CCL17 expression and Tregs allograft infiltration (*143*). However, another study using fully mismatch murine cardiac transplant model with induced tolerance showed that alloantigen-bearing plasmacytoid DC migrated to lymph nodes and affected allospecific Treg development via the CCR4/CCL17 pathway. The same protocol in $CCR4^{-/-}$ mice prevented failure of Treg development, leading to rejection (*144*).

CCR4 and CCR8 are also expressed and used by effector T cells (*145, 146*) and skin homing (but not gut homing) memory T cells, therefore fine spatial and temporal balancing operates in vivo for self tolerance and during an alloresponse (*147*). In the study by Lee *et al* (*143*), CCR8 expression was higher in rejecting allografts, which could be due to infiltration of either the effector T cells or Tregs in response to inflammation. They showed that the CCR8 / CCL1 pathway is not critical for immune tolerance, at least in their model, since CCL1 blockade did not affect tolerance induction. Much still needs to be learnt about the precise in vivo role of these pathways for Tregs trafficking.

1.10.2 CXCR3 / CXCL9, CXCL10, CXCL11 pathways

Th1-associated CXC chemokines, CXCL9 (monokine induced by gamma-Interferon) CXCL10 (Interferon-inducible protein-10) and CXCL11 (Interferon-inducible T-cell alpha chemoattractant) have often been associated with acute allograft rejection (*89, 90*), but it is uncertain if they play a role in

Tregs trafficking. Under homeostatic conditions Tregs express low levels of CXCR3, and show minimal chemotactic response to the three inflammatory CXC chemokines (*37*). The expression probably increases during the stress response of inflammation or rejection following transplantation. Eksteen *et al* demonstrated the presence of $CXCR3^{high}$ Tregs around inflamed bile ducts in chronically inflamed human liver (*72*). A study on kidney allografts in primates demonstrated a significant correlation between rejection and infiltration of the grafts with Tregs (*148*). In a murine model of experimental autoimmune encephalomyelitis, CXCR3 expression was associated with infiltration of Tregs and containment of the tissue damage (*149*).

Hence, it is likely that the CXCR3 axis is involved in Treg homing to the allograft at the time of acute rejection. Hasegawa and colleagues utilized this pathway by developing an *in vitro* expanded, transfected Treg variant expressing high levels of CXCR3. Following adoptive transfer in a GVHD-model of B6D2F1 mice, these Tregs localized to the target organs and suppressed the GVHD in a dose-dependent manner (*150*).

1.10.3 Bone marrow homing chemokine pathway

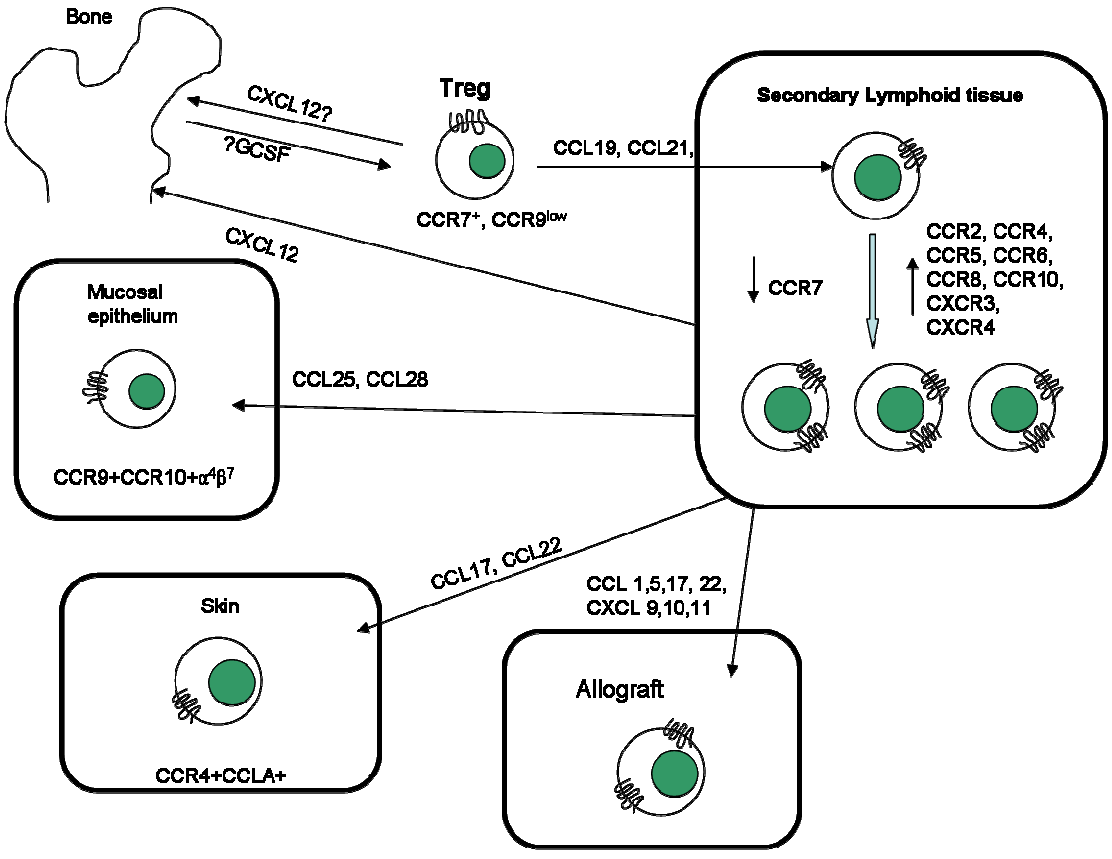
Bone marrow is a part of the lymphocyte recirculation network and particularly enriched in functional $CD4^+CD25^+FOXP3^+$ Tregs (*141, 151*). Zhou *et al* demonstrated that human Tregs preferentially migrate to bone marrow using CXCR4 / CXCL12 axis, express more FOXP3 and were more suppressive than blood-borne Tregs (*121*). It is likely that Treg mobilization explains the amelioration of acute GVHD (graft-versus-host disease) by GCSF (granulocyte colony stimulation factor) treated blood mononuclear cell transplantation, since GCSF mobilizes bone Tregs by decreasing marrow CXCL12 levels (*121, 152*).

A similar mechanism of action may account for another study that showed reduction in the severity of murine experimental allergic encephalomyelitis by GCSF (*153*).

In a fully mismatched rat cardiac allograft model, GSCF injections following transplantation led to enhanced Treg population in the peripheral blood and prolonged allograft survival. By determining ratios of Tregs to the CD4 cell population in bone marrow and spleen, the authors established that Tregs were mobilized from the bone marrow (*154*). However, the prophylactic use of GCSF to prevent rejection in the early postoperative period in a randomized, placebo-controlled, double-blind, multicenter trial following human liver transplantation failed to show any benefits (*155*). This could probably be due to a difference in species, or the simultaneous mobilization of effector T cell populations from the bone marrow.

Hence in order to utilize bone marrow Tregs for the induction of tolerance, there is a need for such agents that can preferentially target CXCR4 on Tregs, without affecting the effector T cells. Recently, some CXCR4 antagonists have been used in non-transplant experimental studies with promising results; TN14003 has shown to block CXCL12-induced migration and invasion of pancreatic tumour cells (*156*), AMD3100 reduced airway inflammation and hyper-reactivity in a mouse model of asthma (*157*), the same compound also suppressed the severity of murine collagen induced arthritis (*158*), CTCE-9908 inhibited migration and division of osteosarcoma cells in vitro and decreased pulmonary metastases in a murine model (*159*). These developments suggest other possible means for mobilizing Tregs from bone marrow for therapeutic applications.

Figure 1.3 Diagram showing chemokine pathways involved in the Treg migration between various organs. Naïve Treg (before activation by antigen stimulation), are $CCR7^{+}CCR9^{low}$ and migrate to the Secondary Lymphoid tissue in response to CCL19 and CCL21. Here, stimulation by an antigen presenting cell leads to an up-regulation of various CC and CXC receptors, and a down-regulation of CCR7. This change of receptor profile enables the Treg to migrate towards other organs, such as the allograft, the skin, mucosal epithelium, and the bone. The various chemokines affecting this migration are shown with arrows.



1.10.4 CCR10 / CCL28 pathway

CCR10 is expressed on T and B cell subsets that constitutively migrate to gastrointestinal and non-intestinal mucosal epithelial sites (such as liver) via CCL28 (also known as MEC or mammary enriched chemokine) (*160*). Liver is considered a tolerogenic organ, where Tregs mediate allograft acceptance across MHC-barriers (*161*). It is likely that Tregs also use the CCL28 / CCR10 pathway for migrating to liver allografts during tolerance induction; however, there is a paucity of supporting data. A study of chemotactic signals for Tregs during chronic hepatic inflammation in humans reported a 25-fold up-regulation of CCL28 in biliary and portal epithelium, associated with the infiltration of functional $CCR10^{+}$ $CXCR3^{+}$ Treg (*72*). Using a rat model of liver transplant rejection (DA-LEW), Pu *et al* showed that adoptive transfer of alloantigen stimulated Tregs prolonged allograft survival (30 vs. 12 days in control group). Surprisingly, a short course of tacrolimus gave an even better outcome (more than 60 days) (*162*). The authors failed to explain the unexpected synergistic effect between tacrolimus and Tregs, since tacrolimus has been reported to impair Treg expansion (*163*). Also, they did not mention migratory pathways responsible for Treg trafficking to the liver allografts.

1.10.5 CCR5 / CCL5, CCL4 pathways:

Expression of the chemokine receptor CCR5 has been associated with pro-inflammatory cellular infiltration in acute and chronic allograft rejection (*102, 164, 165*), tumours (*166*), and inflammatory conditions (*167*). Its ligands include CCL5 (RANTES or regulated upon activation, normal T-cell expressed, and presumably secreted), CCL3 (MIP-1-α or macrophage inflammatory protein-1-alpha) and CCL4 (MIP-1-β or macrophage inflammatory protein-1-

beta). The role of CCR5-related pathways in Treg migration following organ transplantation remains elusive. A study on mice showed that approximately 20% of Tregs in murine SLT constitutively express CCR5 and immune activation leads to enhanced expression of both CCR5 and Foxp3. The same study revealed that CCR5/CCL4 pathway caused preferential accumulation of Tregs in the gravid uterus for tolerance induction (*168*). Further evidence suggests that Tregs up regulate CCR5 expression in certain pathological states for peripheral migration. In chronic inflamed intestine of SAMP/Yit mice, the CCR5 / CCL5 pathway was preferentially used by $Foxp3^+$ Tregs for homing to the inflamed tissues. Interestingly, activated $CD8^+$ T cells were the major source of the secreted CCL5, suggesting a possible role of CCL5 in balancing the effector and regulatory response. It was demonstrated that *in vitro* activation caused significantly more $Foxp3^+$ cells than $Foxp3^-$ cells to express CCR5, and their migration was blocked by TAK-779, a CCR5 antagonist (*169*). In murine pulmonary mycosis, Tregs showed enhanced expression of CCR5 compared to effector T cells and migrated to the fungal lesions, with subsequent dampening of the immune response against the disease (*170*). Another study in murine model of acute GVHD reported that Tregs used CCR5-related pathways for homing to the target organs, and this migration was essential for suppression of the effector response. The investigators did not mention the chemokines involved in this migration (*171*). Taken together, these observations suggest that CCR5-related pathways play a significant role in tolerance induction following organ transplantation.

1.11 Do immunosuppressants modulate chemokine receptors on Tregs?

The effects of various immunosuppressant agents on the proliferation and suppressive capability of Tregs has been well documented (*163*). However, little is known about the effects of these interventions on chemokine receptor profile of Tregs. It is well known that immunosuppressive agents such as cyclosporine alter the chemokine receptor expression and migratory capacity of dendritic cells (*172*). It is likely that Tregs also switch chemokine receptors and homing potentials in response to therapeutic interventions. Knowledge of such effect can provide an opportunity to alter therapy for tolerance induction following transplantation.

Summary:

Treg cells are a heterogeneous group of lymphocytes with potent immunosuppressive capacity. These cells use different chemokine pathways for tissue selective migration, depending on the pathophysiological mechanism. The relevance of various chemokine networks in Tregs migration in the transplant scenario is still elusive. Further knowledge in this area is required to help in designing cell-based immunotherapy, particularly to induce transplant tolerance.

Chapter 2 Materials and methods

2.1 Study Subjects

This part prospective, part retrospective observational, non-randomized, cross-sectional study included adult heart transplant recipients under routine follow up at the University Hospital of South Manchester NHS Foundation Trust (UHSM). The study was approved by the local research ethics committee and group informed consent was obtained from all the participants. All the biological samples were collected and stored in accordance with the Human Tissue Act 2004. The study was carried out in conformation with the spirit and the letter of the declaration of Helsinki, and in accord with the ICH Good Clinical Practice Guidelines.

2.2 Blood samples

A 5 ml peripheral blood sample was collected from research participants in an EDTA (ethylenediamine-tetra-acetic acid) vacutainer, using standard venepuncture technique. The samples were then either used to separate plasma via centrifuge, or used for isolation of peripheral blood mononuclear cells, depending on the experiments.

2.3 Luminex immunoassay

Plasma concentrations of chemokines were measured using multiplex bead-based Luminex® technology (Invitrogen, UK). The Multiplex technology incorporates solid phase immunoassays using spectrally encoded antibody-conjugated beads as the solid support. The assay is performed in a 96-well plate and analyzed with a Luminex® 100™ instrument, capable of simultaneous, precise *in-vitro* quantitative analysis of up to 100 different proteins in a single

well (also called xMAP technology). The use of xMAP technology for bioassays is now well described (fig 4).

In short, the technology used 5.6μm polystyrene beads, called microspheres, internally dyed with red and infrared fluorophores of different intensities and given unique numbers, allowing differentiation of one bead from another (fig 4a). Each bead set is conjugated with capture antibody for a specific bioassay, and added along with samples (including standards, control and test sample) into the wells of the filter bottom microplate. The plate is then incubated for 2 hours, when capture antibodies bind to the specific proteins (analyte) (fig 4b). After washing the beads, protein-specific biotinylated detector antibodies are added and incubated for another hour, when biotinylated antibodies bind to the specific immobilized proteins (fig 4c). After washing excess biotinylated antibodies, streptavidin conjugated to fluorescent protein, R-Phycoerythrin (Streptavidin-RPE) is added and the plate incubated for another 30 minutes. The Streptavidin-RPE binds to the detector antibodies, thus forming a four-member solid phase sandwich, to be analyzed by the Luminex system (fig 4d). By detecting the spectral properties of capture beads and measuring the amount of associated RPE fluorescence, the concentration of one or more proteins is determined.

I used Human Thirty-Plex Antibody bead kit (Invitrogen®, CA) for measuring the peripheral blood chemokine levels. This bead kit comprises analyte specific components for the measurement of several human cytokines and chemokines. For the purpose of this specific experiment, I measured chemokines only. I am aware that the antibody bead kit is mostly used with the serum or the tissue culture medium samples; however, it can be used with

plasma, with satisfactory sensitivity and minimum interassay variation. For the selected chemokines, the assay sensitivity is between 5-15 pg/ml, with an interassay variation between 2.9 to 6.9%.

Figure 2.1 Diagrammatic representation of the Multiplex bead-based Luminex® Assay. Fig 2.1(a) shows the first step when the antibody-conjugated beads are added to the well. Fig 2.1(b) shows the analyte capture by the specific antibody when added to the wells with the specific antibody-conjugated beads. Fig 2.1(c) represents the third step when the biotinylated detector antibody binds to the analyte-antibody complex, creating a sandwich. Fig 2.1(d) shows the analyte detection by the fluorescence of the Streptavidine-RPE bound to the detector antibody. (RPE = R Phycoerythrin)

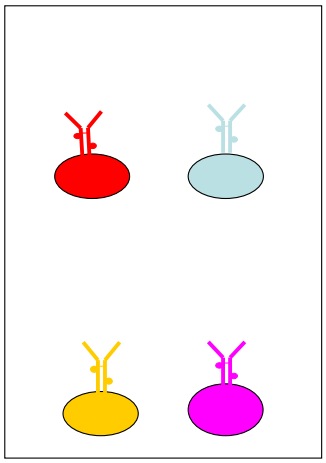


Fig 2.1(a)

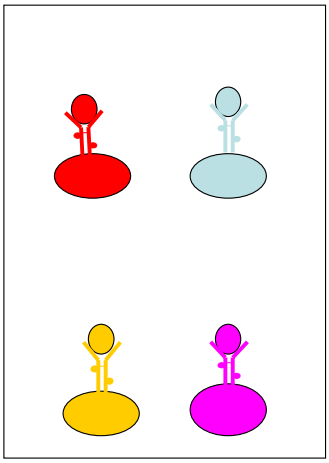


Fig 2.1(b)

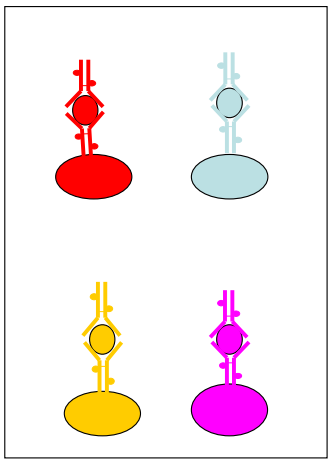


Fig 2.1(c)

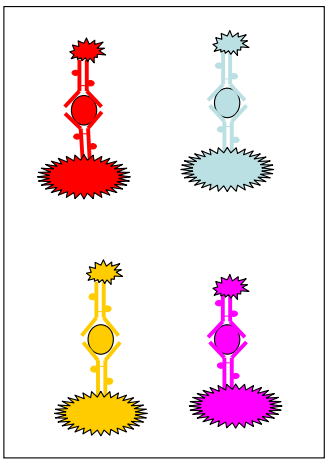


Fig 2.1(d)

2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMC) for flow cytometry

A peripheral blood sample collected in an EDTA vacutainer was used to isolate PBMC by density gradient centrifugation method using Ficoll-Paque as previously described (*173*). The blood is processed within 1-3 hours of collection under aseptic conditions. The blood is diluted 1:1 with room temperature standard RPMI-1640 (Sigma-Aldrich®, MO) in a polypropylene universal container and gently mixed. Eight ml of blood and RPMI mixture is then carefully layered over seven ml of Ficoll-Paque cushion in a polypropylene centrifuge tube, followed by centrifuge at 3000 rpm for 30 min. The PBMC layer is then carefully withdrawn with a pipette, and transferred to another 15 ml centrifuge tube. The cells are then washed in room-temperature phosphate buffered solution (PBS) and centrifuged at 1500 rpm for 15 minutes. The supernatant is aspirated, and the cells resuspended in room-temperature RPMI-1640, to achieve a viable cell density of 1-1.25 x 10^6 cells/ml. The cells are counted using Neuber cell counter.

2.5 Chemokines for Chemotaxis assays

The following recombinant human chemokines were used for *in-vitro* chemotaxis assays; CCL1 (T lymphocyte-secreted protein I-309), CCL2 (Monocyte Chemotactic Protein-1 or MCP-1), CCL5 (Regulated upon Activation, Normal T-cell Expressed, and Secreted or RANTES), CCL17 (Thymus and Activation Regulated Chemokine or TARC), CCL19 (Macrophage Inflammatory protein-3 beta or MIP-3β), CCL21 (6Ckine, Secondary Lymphoid-Tissue Chemokine, or SCL), CXCL9 (Monokine induced by gamma-interferon or MIG), and CXCL10 (Interferon-gamma-induced protein or IP-10)

(purchased from ProSpec-Tany TechnoGene Ltd, Israel). A stock solution of each chemokine is made according to manufacturers' guidelines. It is further diluted according to the required strength for chemotaxis, and aliquots were stored to avoid repeat freeze-thaw cycles. A carrier protein (0.1% bovine serum albumin) was added for long term storage.

2.6 Chemotaxis assay

The use of in-vitro cell migration assays across a barrier membrane with pores of a known size and density has been well documented (*89, 174, 175*). I utilized 24 well insert system purchased from BD Falcon™. It is a cell culture insert assays platform composed of a multiwall insert plate with a microporous PET (Polyethylene Terephthalate) membrane, a 24-well assay plate, or feeder tray and lid. The 24 wells are integrated into a one-piece plate, making it easy to move the insert if necessary. All the inserts are handled under aseptic conditions. 250µl of the cell suspension is added to the insert, while 750µl of chemokine solution in RPMI-1640 is added to the lower well in order to neutralize the effects of hydrodstatic pressure across the membrane according to the manufacturer's guidelines. A negative control is used for each patient, using 750µl of RPMI without chemokine in the lower well. The system is left incubated for 4 hours in 37^{o}C, 5% CO2 and normal humidity. Following incubation, transfer of Treg to the lower chamber was assessed using flow cytometry.

2.7 Flow Cytometry

I used BD™ LSR II flow cytometer for Treg-immunophenotyping. BD LSR II is an air-cooled, multi-laser, bench top flow cytometer with the ability to acquire parameters for a large number of colours. It uses fixed-alignment lasers

that transmit light reflected by mirrors through a flow cell to user-configurable octagon and trigon detector arrays. The octagon is an array of photomultiplier tubes (PMTs) and filters that can detect up to eight signals, while trigon can detect up to three signals. These detectors collect and translate fluorescence signals into electronic signals. Instrument electronics convert these signals into digital data.

The BD LSR II has a fixed-alignment 488-nm blue laser (Coherent Sapphire) with the option of additional fixed-alignment lasers to analyze a stream of fluid containing individual cells. The primary blue laser generates forward scatter (FSC) and side scatter (SSC) signals and four fluorescence signals. The optional red (633-nm laser), violet (405-nm) and UV (355-nm) lasers generate two fluorescence signals each. There are dichroic optical filters, which transmit light of a specific wavelength, while reflecting other wavelengths. Light signals are generated as particles pass through the laser beam in a fluid stream. When these optical signals reach a detector, electrical pulses are created that are then processed by the electronics system.

The flow cytometry requires optimum alignment of the optical and fluidics system to maximize the detection of fluorescence and ensure optimal sensitivity and resolution for forward scatter (FSc) and side scatter (SSc) signals. Hence frequent checks were made to ensure optical alignment was optimised and compensation was corrected for spectral overlap. The use of uniform fluorospheres for optical alignment verification has been well established (*176*). We used BD™ Cytometer Setup and Tracking beads, which consists of equal concentrations of 3-µm bright, 3-µm mid, and 2-µm dim polystyrene beads in PBS with bovine serum albumin (BSA), and sodium azide in a stream-tip

dropper vial. The beads are dyed with a mixture of fluorochromes that are excited by the lasers used in BD digital flow cytometer. Median fluorescence intensity (MFI) and percent robust CV (% rCV) are measure for each bead intensity in all fluorescence detectors. Software algorithms differentiate the fluorescence signals from each bead type based on size and fluorescence intensity in each detector, Linearity, detector efficiency (Qr), optical background (Br), electronic noise and laser delays are all evaluated, PMT voltages are then adjusted to maximize population resolution in each detector, hence providing better resolution of dim populations, fewer compensations artefacts, and reproducible data.

2.8 Treg immunophenotyping

A 100µl of the specimen from a suspension of PBMC or cell suspension from the lower well in the HTS multiwall system was extracted into each analysis tube (5ml polypropylene FACS tube). Treg cells were labelled with the following directly conjugated antibodies: 1. Phycoerythrin-Cy5 (PE-Cy5) conjugated anti-CD4, 2. Phycoerythrin-Cy7 (PE-Cy7) conjugated anti-CD25 (BD Biosciences, UK), for 30 minutes in the dark at 4^{o}C (to avoid denaturing by UV light). Staining with fluorescein isothiocyanate (FITC)-conjugated anti-human FOXP3 (e-Bioscience, UK) was performed with fixation/permeabilization solutions according to the manufacturer's guidelines. In short, following incubation with anti-CD4 and anti-CD25 antibodies, the sample was washed once with 1 ml of PBS and centrifuged at 1500 RPM for 10 min. The cell pellet was resuspended with pulse vortex and 1 ml of freshly prepared Fixation/Permeabilization buffer solution was added to each sample and incubated at 4^{o}C for 45 min in the dark. The sample was then washed once

with 2 ml of 1x permeabilization wash buffer (freshly made from 10x permeabilization buffer) followed by centrifugation at 1500 RPM for 10 min and gentle decanting of the supernatant. The cell pellet was once again suspended with pulse vortex, and stained with FoxP3 FITC antibody at 4^{o}C for 45 min in the dark. The sample was then washed twice with 2 ml of 1x permeabilization buffer, centrifuged at 1500 RPM for 10 min and resuspend in 100µl of PBS before analyzing in the flow cytometer.

2.9 Isotype Controls

A further 100µl of PBMC were stained with combinations of mouse IGg1-PCy5, PCy7, and FITC as isotype control, Isotype controls were included in all experiments, to provide a negative cell reference and also to set up regional quadrants on scatter plot graphs for assessing cell populations (**Figure 2.2 – 2.4).**

Figure 2.2 Dual scatter histogram from an isotype control

This demonstrates an absence of positive fluorescence staining within the gated area (shown by an assigned green colour).

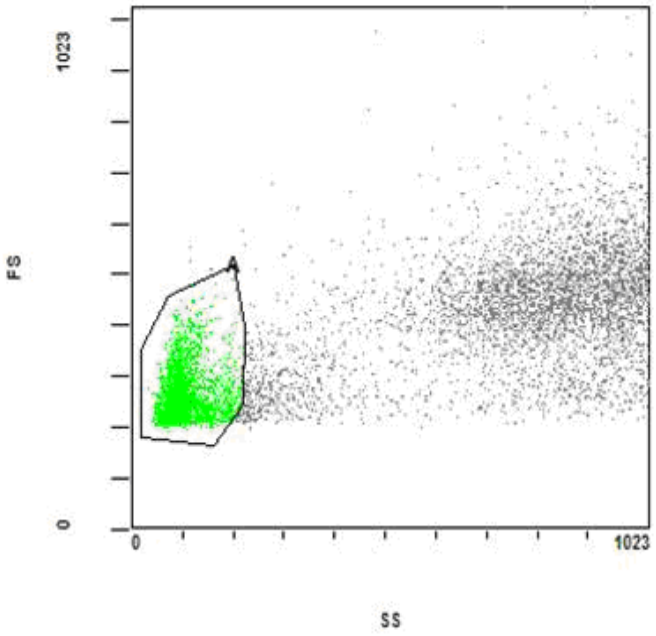


Figure 2.3 Scatter plot from isotype control

This represents a scatter plot constructed from the gated area in figure 2.1. Quadrants have been assigned to determine the 'positively' controlled areas from the isotype control (seen in quadrant F3).

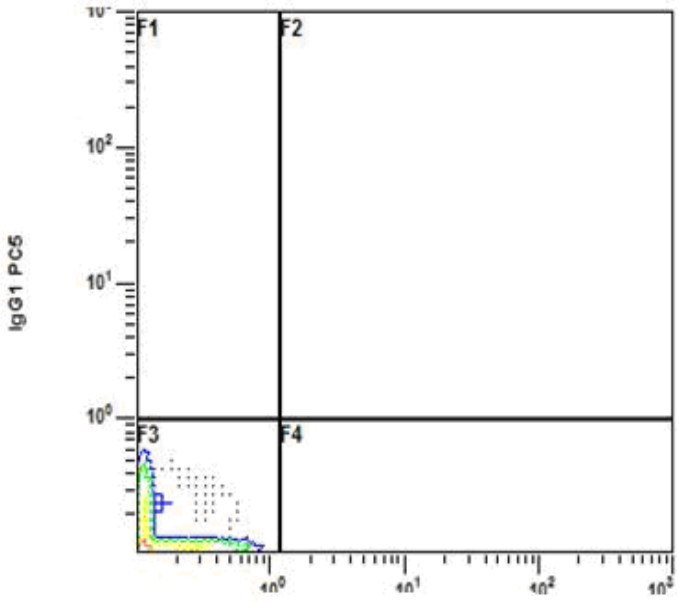
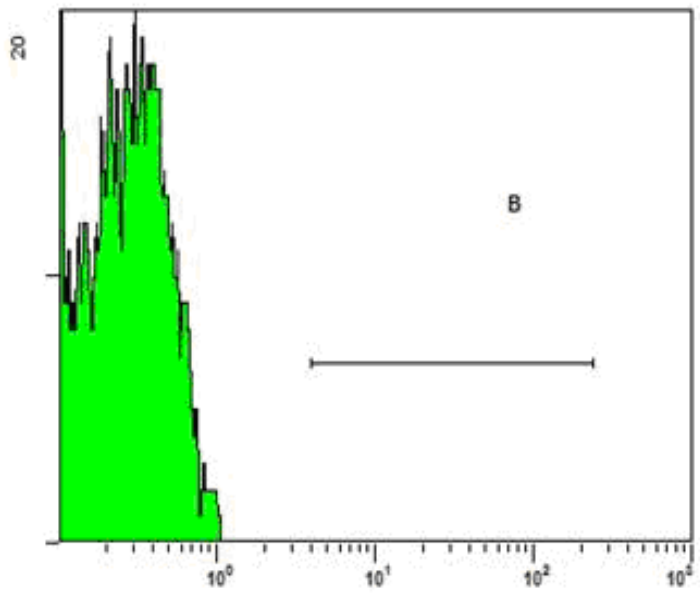


Figure 2.4 Histogram from isotype control. This is a histogram which confirms a control staining area and the absence of positive staining (no activity within area B)

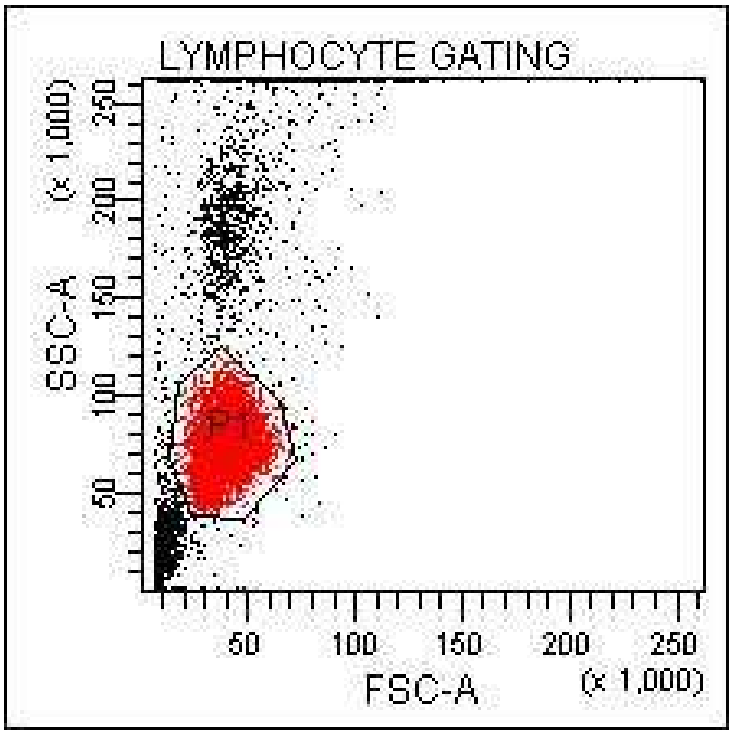


2.10 Gating Strategy

BD FACSDiva™ software was used to create gating strategy protocols. The following protocol was established for analysis of a given sample:

- A forward scatter - side scatter (FSc:SSc) plot was first produced (Figure 2.5). This enabled simple visualisation and differentiation of lymphocytes according to cell size and granularity. A manual gate (labelled Gate P1, red) was then created around the lymphocyte region on the FSc:SSc histogram (Figure 2.5). Further data analysis and collection was then only performed on cells in this region.

Figure 2.5 Scatter plot showing lymphocyte gating strategy. A representative scatter plot showing a manual gate (P1) created around the lymphocyte region. Further analysis was performed on the cells within the lymphocyte gate only.



- In the next step, a forward scatter - side scatter (FSc:SSc) were produced for each colour / antibody, using the cells from the previously gated lymphocytes. Fluorescence outside of the gating regions is considered non-specific (isotype control). To enhance visualisation of cell populations in the FSc:SSc plot or a histogram, each positive antibody gate was assigned a colour (Fig 2.6-2.8).

Figure 2.6 Gating strategies for CD4 (green) on a scatter plot. A representative scatter plot showing the gating strategy for visualizing CD4+ cells (green). The cells outside the gate are the lymphocytes that are CD4- (red).

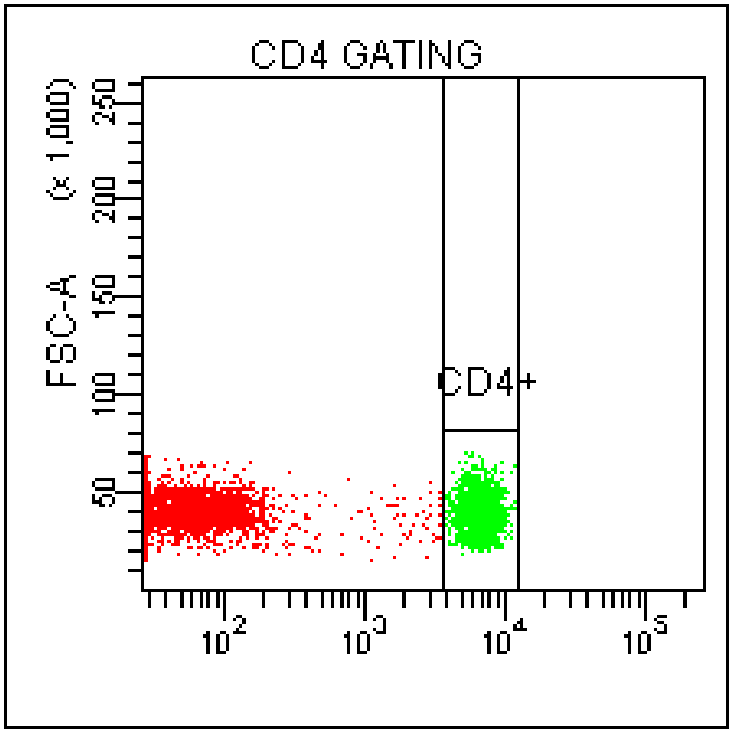


Figure 2.7 Histogram showing CD25 staining. A representative histogram with staining area (P1) in CD25 zone, and an absence of staining outside P1 (control).

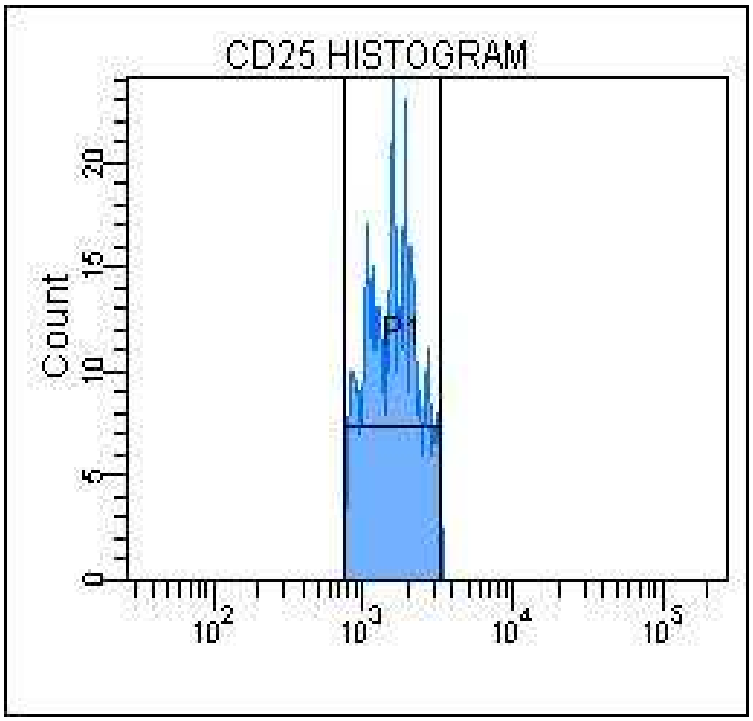
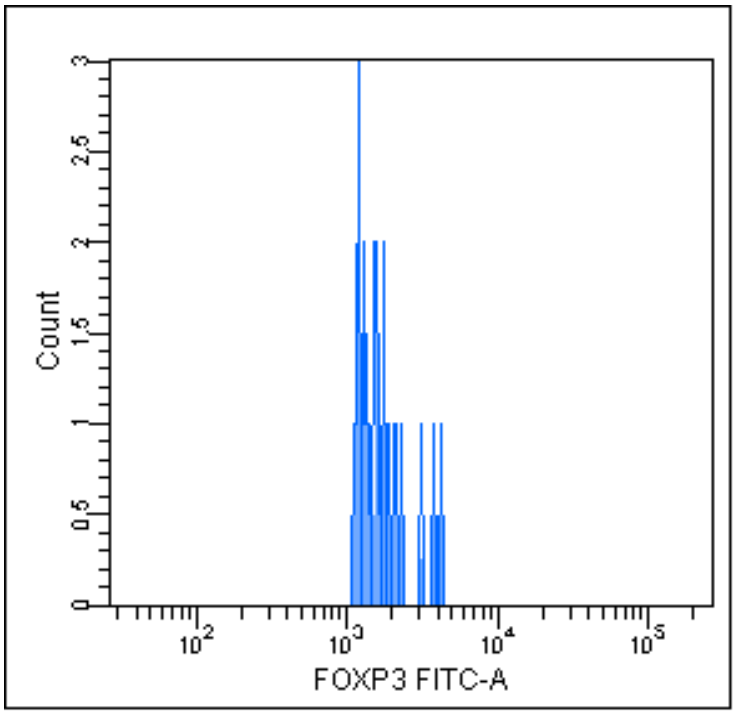
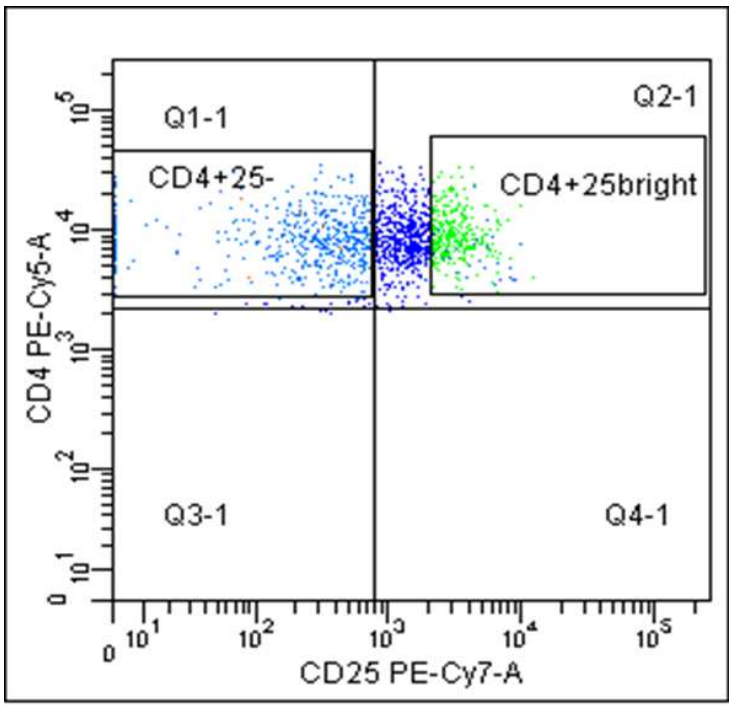


Figure 2.8 Histogram for FOXP3 staining. A representative histogram showing positive staining for FOXP3 within the blue zone, and an absence of staining in the adjacent area (FOXP3-).



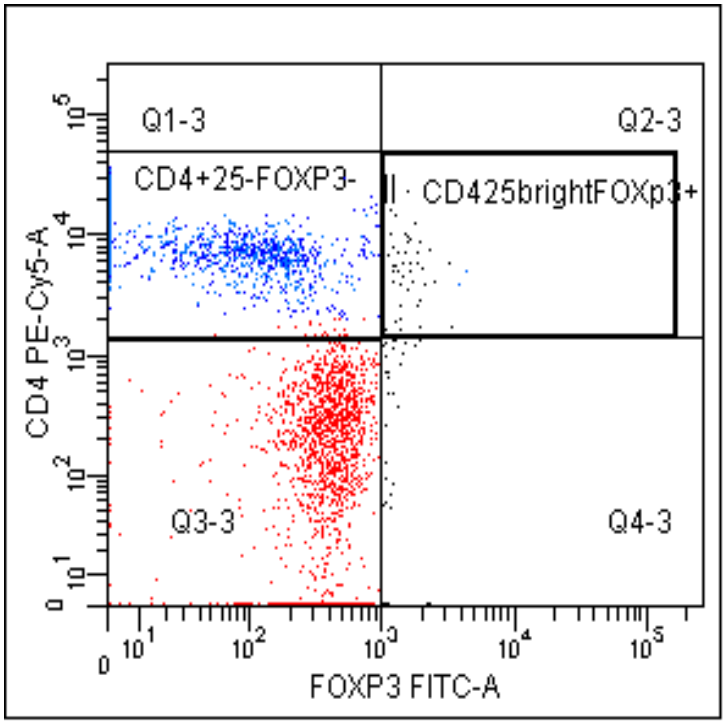
- In the third step, a further gating strategy was defined to identify those cells which are positive for both CD4 and CD25 staining. Hence, another FS:SS plot was created for CD4 and CD25, using cells from the CD4 positive cells only. Another gate was placed to identify $CD4^{+}CD25^{bright}$ cells (shaded green in Fig 2.9)

Figure 2.9 Gating strategies for $CD4^{+}CD25^{bright}$ cells. A representative scatter plot showing dual staining for CD4 and CD25. A further gate was created on the cells staining most brightly for the CD25 ($CD4^{+}25^{bright}$, green cells within the quadrant Q2-1).



- Finally, further scatter plot was created to identify $CD4^{+}CD25^{bright}$ cells staining positive for FOXP3, as shown in figure 2.10.

Figure 2.10 Scatter plot with gating strategy for Treg. A representative scatter plot showing $CD4^{+}CD25^{bright}FOXP3^{+}$ (Tregs), within the quadrant Q2-3. Quadrant Q1-3 shows $CD4^{+}CD25^{-}$ cells which are also $FOXP3^{-}$.



2.11 Immunofluorescence staining

I used immunofluorescent detection technique to co-localize Treg markers with the chemokine in formalin-fixed, paraffin-embedded (FFPE) endomyocardial biopsy tissue from stable and rejecting adult heart transplant recipients. In this method various target antigens are first labelled with primary antibodies, which are then detected by fluorochrome-conjugated secondary antibodies using immunofluorescence scanning microscopy (*177, 178*). The primary antibodies and the secondary, fluorochrome-conjugated secondary antibodies are listed in tables 2.1 and 2.2.

Table 2.1 Primary antibodies used for Immunofluorescence staining in the formalin-fixed, paraffin-embedded endomyocardial biopsy tissue.

Antigen	Isotype / clone, catalogue number	Supplier
CD4	Mouse IgG1 clone BC/1F6, # ab846	Abcam®, Cambridge, UK
FOXP3	Rat IgG2a, κ, clone PCH101, # 14-4776	ebioscience®, CA
CCL17	Goat polyclonal, # AF364	R&D systems, Minneapolis, MN

Table 2.2 Secondary antibodies used for Immunofluorescence staining in the formalin-fixed, paraffin-embedded endomyocardial biopsy tissue.

Antigen	Isotype / clone, catalogue number	Supplier
Alexa Fluor® 488 donkey anti-rat IgG (H+L)	Cat no A-21208	Invitrogen®, UK
Alexa Fluor® 555 donkey anti-mouse IgG (H+L)	Cat no A-31570	Invitrogen®, UK
Alexa Fluor® 647 donkey anti-goat IgG (H+L)	Cat no A-21447	Invitrogen®, UK

DAPI (4',6-diamidino-2-phenylindole dilactate) was used to counter stain nuclei.

The FFPE samples of the endomyocardial biopsies were collected from the archives of the UHSM Pathology department following informed consent. The endomyocardial biopsies are routinely used to diagnose rejection following hear transplantation (*179*). The procedure is usually performed via the right internal jugular vein route or the right common femoral vein route. All tissue samples are routinely fixed in 4% buffered formalin and subsequently embedded in paraffin blocks by conventional techniques. For the diagnosis of rejection, the biopsy samples are stained with haematoxylin and eosin, and then analyzed by experienced histopathologists at the Department of Pathology at UHSM. The biopsies are graded between 0-3R according to the recent International Society of Heart and Lung Transplantation (ISHLT) grading system (*180*).

2.11.1 Tissue slide preparation

From the FFPE tissue blocks, 3-4 μm thick tissue slices were cut using standard microtome. The tissue sections were then floated onto a warm (42°C) water bath from where they are picked up onto X-Tra® adhesive slides (Surgipath®, UK). These slides are made using a special process to produce a permanent positive (+) charged surface which helps to bond tissue sections and cytology preparations without the use of additional adhesives. Once mounted, the slides were left to dry overnight at room temperature, followed by incubation at 60ºC for 30 minutes to help the section adhere to the slide.

2.11.2 Dewaxing and Antigen retrieval

Formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative

staining for immunohistochemical detection of certain proteins. Hence, antigen retrieval is performed in order to facilitate epitope unmasking. Antigen retrieval can be performed by heat-induced (also known as heat-induced epitope retrieval or HIER) or enzymatic methods. HIER can be performed using Citrate buffer (pH 6.0) or Tris/EDTA buffer (pH 9.0), in a pressure cooker, microwave oven, or a water bath. The buffer solution is designed to break the protein cross-links, therefore unmasking the epitopes in FFPE tissue sections. During optimization experiments for individual antibodies, I tested HIER with both buffers in different heating condition, as well as enzymatic retrieval with pepsin.
Prior to antigen retrieval, the tissues were deparaffinised using four 10 minute washes in 100% xylene, agitating for 10 sec every 30 sec. This was followed by rehydration in graded ethanol solutions (100%, 90%, 75%, and 50%) for 3 minutes each, agitating every 20 sec, before rinsing in water.

In the first step, antigen retrieval was performed using pre-warmed buffer in a water bath (warmed to 90ºC). The slides were left in the water bath for 30 minutes, followed by 20 minutes on the bench at room temperature for cooling, and then 10 minutes under running water. However, I found inadequate staining of the various antibodies using this technique.
Subsequently, I used pressure cooker with Citrate buffer or Tris/EDTA buffer for HIER with optimum results for CD4 and FOXP3 antibodies.

In order to make sure that the sections are never dry, all staining procedures were carried out in a humidified chamber. I used a shallow plastic box with a sealed lid and wet tissue paper in the bottom as our incubation chamber. The plastic serological pipettes were cut into lengths and glued at the bottom of the chamber, with the 2 individual pipette tubes of each pair being

placed about 4.0 cm apart. It helped to keep slides off the paper and be laid flat so that the reagents don't drain off.

2.11.3 Blocking Step

In order to minimise background or un-specific staining, I used 5-10% normal horse serum (serum from the host of the secondary antibodies), prepared by diluting normal horse serum in PBS. The slides were removed from water, gently wiped around the section and then 200µl PBS was carefully added to prevent the section from drying out. Dako pen (Dako®, DK) was used to ring the island, in order to provide a barrier to liquids applied to the sections. This was followed by gently pipetting 200µl of 10% blocking serum on to the section before placing the slide in a moist chamber at room chamber for 30 minutes. The slides were then rinsed again in PBS before staining with the primary antibodies.

2.11.4 Immunofluorescence staining for CD4 and FOXP3

To determine optimum staining concentrations for each antibody, different antigen retrieval techniques and dilutions were tested according to the manufacturers' recommendations and published guidelines, along with positive and negative controls. For positive controls, anonymous archival tonsil tissues were obtained from the department of Pathology at UHSM, while the primary antibodies were omitted to create negative controls.

Table 2.3 summarizes the best antigen retrieval method and dilutions for CD4 and FOXP3 antibodies, and their corresponding secondary antibodies.

Table 2.3 Antigen retrieval and staining protocol for CD4 and FOXP3 in the formalin-fixed, paraffin-embedded human endomyocardial biopsy tissue. The method was optimized for mouse antihuman CD4 (clone BC/1F6) and rat anti-human FOXP3 (clone PCh-101). (HIER = Heat Induced Antigen Retrieval, PBS = Phosphate Buffered Saline).

	Mouse anti-human CD4 (BC/1F6)	**Rat anti-human FOXP3 (PCH-101)**
Antigen retrieval	HIER	HIER
Buffer	Citrate buffer (pH 6.0)	Citrate buffer (pH 6.0)
Heating method	Pressure cooker, 4 minutes on full pressure	Pressure cooker, 4 minutes on full pressure
Dilution of the primary antibody	1:50 (2% blocking serum in PBS)	1:50 (2% blocking serum in PBS)
Staining time and temperature	Overnight 4ºC	Overnight 4ºC
Secondary antibody	Alexa Fluor® 555 donkey anti-mouse IgG (H+L)	Alexa Fluor® 488 donkey anti-rat IgG (H+L)
Dilution of the secondary antibody	1:500 (2% blocking serum in PBS)	1:500 (2% blocking serum in PBS)
Staining time and temperature for secondary antibody	1 hr room temperature	1 hr room temperature

During the optimization experiments, I tested simultaneous and sequential methods for staining CD4 and FOXP3, and found significantly better results when using sequential method as follows:

- First blocking step: incubation with 10% normal donkey serum for 1 hour at room temperature
- Incubation with the CD4 antibody diluted in 2% blocking serum in PBS (Phosphate buffered saline) in a humidified chamber for overnight at 4ºC
- Wash in PBS with Tween (0.05%) four times for 5 minutes each
- Incubation with the AF555 antibody for 1 hour at room temperature in moist chamber in dark
- Subsequently all staining steps were carried out in the dark.
- Wash in PBS with Tween (0.05%) four times for 5 minutes each
- Second blocking step: incubation with 10% normal donkey serum for 1 hour at room temperature
- Decant the blocking serum and incubation with the FOXP3 antibody diluted in 2% blocking serum in PBS for overnight at 4ºC
- Wash in PBS with Tween (0.05%) four times for 5 minutes each
- Incubation with the AF488 antibody for 1 hour at room temperature in moist chamber in dark
- Wash in PBS with Tween (0.05%) four times for 5 minutes each

2.11.5 Autofluorescence

During optimization experiments, I encountered significant autofluorescence in the endomyocardial biopsy samples. This natural fluorescence is due to substances like lipofuscin that persists in paraffin sections.

Lipofuscin is composed of cytoplasmic yellow brown pigment granules, the breakdown products of unsaturated fatty acids. Apart from lipids, they also contain various metals including iron, copper and zinc (*181*). Because of their broad excitation and emission spectra, lipofuscin-like autofluorescence causes significant problems during immunofluorescence methods (*182*). Hence I tried different quenching methods, including UV light and Sudan black at various concentrations as previously described (*183*). I found that 10 minutes incubation in 0.5% Sudan black in 70% alcohol reduced the autofluorescence significantly while slightly dampening the intensity of the immunostaining.

Following incubation with 0.5% Sudan Black, the slides were thoroughly washed with PBS before nuclear counterstaining with DAPI (4', 6-diamidino-2-phenylindole, dilactate; 1:10,000 for 2 minutes, Biotium, CA).

The sections were again washed four times in PBS for 5 minutes each, before drying at room temperature. The sections were then mounted with Prolong Gold® anti-fade reagent (Molecular Probes®, OR).

The entire sections were evaluated using epifluorescent microscope (Olympus® BX51, Japan) and images acquired using $CoolSNAP_{HQ}$ Monochrome camera (Roper Scientific®, AZ) with MetaVue™ Imaging System (Molecular Devices®, PA). $CD4^+FOXP3^-$ and $CD4^+FOXP3^+$ cells (Tregs) were counted in 5-10 non-overlapping high power fields (x60).

2.12 Immunofluorescence staining for CCL17, CCR4 and CCR8

I attempted to co-localize Tregs with CCL17, CCR4, and CCR8 using immunofluorescence methods. Despite trying various methods for antigen retrieval and staining protocols as highlighted earlier, I could not obtain optimum staining for either antibody. Hence, I opted to use

immunohistochemistry to stain sequential cuts of the endomyocardial biopsies for CCL17 only.

2.13. Immunohistochemistry

Rabbit Polyclonal anti-human CCL17 antibody was purchased from AbD Serotec® (Oxford, UK). I used ImmPRESS™ peroxidase detection system (Vector® labs, CA) to detect CCL17 staining according to the manufacturer's guidelines. The ImPRESS system is based on attaching polymerized enzymes to the antibodies and provides a high sensitivity with low background staining. The following protocol was used:

- Dewax in xylene three times for 10 minutes each
- Graded alcohol rehydration with 100% alcohol (twice, three minutes each), followed by 3 minutes each in 95% and 75% alcohol
- Endogenous peroxidase activity was blocked by placing slides in 3% solution of freshly made hydrogen peroxide in 70% alcohol for 10 minutes
- Wash in water for 5 minutes
- Antigen retrieval was performed using Citrate Buffer (pH 6.0) in microwave (high power) for 30 minutes.
- Wash in running water for 5 minutes
- The slides were removed from water, gently wiped around the section and then 200µl TBST (Tris Buffered Saline with Tween) was carefully added to prevent the section from drying out. Dako pen (Dako®, DK) was used to ring the island
- Incubation with 2.5% normal horse serum for 30 minutes in a moist chamber at room temperature (blocking step)

- Incubation with the primary antibody (anti-CCL17 antibody) for 30 minutes in a moist chamber at room temperature
- Wash with TBST
- Incubation with ImmPRESS™ peroxidase detection system (Vector® labs, CA)
- Wash twice with TBST
- ImmPACT™ Dab (diaminobenzidine) chromogen (Vector® labs, CA) was freshly prepared by adding 1 drop of the chromogen to 1 ml of the diluent.
- Incubation with ImmPACT™ Dab for 5 minutes at room temperature
- Wash with water for 5 minutes
- Nuclear counter stain with Haematoxylin
- Dehydration by placing the slides in 95% and then 100% alcohol for 2 minutes each
- Placing slides in Xylene for 2 minutes
- mount using DPX resin
- Once again, tonsil tissue was used as the positive control. The primary antibody was excluded for the negative control.

With this method I obtained satisfactory staining for CCL17. The degree of staining was assessed and quantified on a scale of 0-3 by an expert histopathologist at our Pathology department.

2.14 Statistical analysis

All the data was analyzed using SPSS v 15. Quantitative data with normal distribution was described in mean and standard deviation, while non-normal data was described with median and inter-quartile range. Similarly,

continuous variables from two unpaired groups with normal distribution were compared using T-test, otherwise Mann-Whitney U test was used. In paired groups, paired t-test or Wilcoxon test was utilized depending on the distribution of the data. One-way ANOVA was used to compare 3 or more unmatched groups with normal distribution, while Kruksal-Wallis test was used if the data from these groups was non-parametric. Chi-square test was used to compare categorical data.

Chapter 3 Th1 Chemokines are up-regulated in acute rejection following heart transplantation

Abstract

Background

Chemokines are the mediators of immune cell trafficking, and play an important role in defining the alloresponse following solid organ transplantation. Evidence suggests that acute rejection leads to enhanced expression of certain chemokines in the allograft. However, there is paucity of data to show the effects of acute rejection on the peripheral blood chemokine levels.

Methods

This study analyzed the peripheral blood concentration of Th1 and Th2 chemokines including CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CCL11 in 50 adult heart transplant recipients at the time of routine surveillance endomyocardial biopsies. The peripheral blood samples were taken during the biopsy procedure and plasma was stored before batch analysis using bead-based Luminex® technology (Invitrogen®, MA). The biopsy samples were analyzed by experienced histopathologists at UHSM and graded between 0-4 according to the International Society of Heart and Lung Transplantation (ISHLT) grading system (*180*). The chemokine levels were correlated with acute rejection episodes using SPSS v15.

Results

Out of a total of 50 patients, 18 had histological evidence of acute cellular rejection, while the rest showed no rejection. There was no difference between the two groups in terms of demographics, immunosuppression and CMV serostatus. I found significantly higher levels of CCL2 and CCL5 (Th1

chemokines) in the blood samples from patients with acute cellular rejection compared to those with no rejection. There was no difference between the two groups in the levels of CXCL10, CXCL9, CCL3, CCL4 and CCL11.

Conclusion

In this study, I demonstrate for the first time a significant rise in the peripheral blood levels of specific Th1 chemokines during acute rejection in human heart transplant recipients. These findings further highlight the importance of Th1 chemokines in the alloresponse in humans and may lead to novel pathways for prevention and treatment of acute rejection.

Background

Despite recent advances in immunosuppressive strategies and improvement in survival, acute rejection remains a significant cause of morbidity and mortality following heart transplantation (*184*). In addition, acute rejection imposes a direct and cumulative effect on the development of chronic rejection, also known as cardiac allograft vasculopathy, hence affecting the long term survival (*185*). Acute cellular rejection involves infiltration of mononuclear cells into the interstitium including T lymphocytes, monocytes and macrophages (*186*). Recent advances in the understanding of the molecular mechanisms governing this cellular infiltration suggest an involvement of both Th1 or Th2 cytokines, including Interferon-gamma (IFN γ), Tumour necrosis factor-alpha (TNF α), or Interleukin 4 (IL4) (*187-189*).

Chemokines are small chemotactic proteins responsible for directing various immune cells to the target environment. With over 50 chemokines and more than 20 corresponding receptors, the system is immensely complex yet highly organized. Some of the chemokines are particularly inclined to recruit Th1 effector cells, while others mediate Th2 inflammatory responses (*190*). An association between intragraft chemokine proteins or gene expression and acute allograft rejection has been documented in several models of renal, skin and other solid organ transplants, including the heart (*186, 191-193*). Further studies have dissected out associations between expression of Th1 chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, with the cellular infiltrate during acute rejection episodes following heart transplantation (*91, 93, 94*). On the other hand, links between Th2 chemokines and acute rejection have been less well defined. One study mentioned that peripheral blood levels of a Th2

chemokine CCL11 is associated with eosinophilia prior to acute rejection in human heart transplant recipients (*194*). Another recent study using rat model of acute cardiac allograft rejection showed significant increase in the intragraft CCL11 expression during rejection (*195*).

Most of the aforementioned studies have looked at the chemokine proteins or gene expression within the allograft; however, there is very little information on the relevance of peripheral blood levels of such chemokines during acute rejection. Hence I prospectively investigated an association between the peripheral blood levels of Th1 and Th2 chemokines and moderate to severe acute cellular rejection in adult heart transplant recipients.

Methods

Patient demographics and sample collection

50 adult heart transplant recipients undergoing routine surveillance endomyocardial biopsies were recruited. The exclusion criteria included patients with acute infections, severe hepatic dysfunction, and failure to obtain an informed consent. The timing of the biopsy was determined according to the clinical need, and it was performed via right internal jugular route under fluoroscopy guidance. Just prior to the biopsy a sample of the peripheral blood was obtained in an EDTA (ethylene diamine tetra-acetic acid) vacutainer. Plasma was separated from the blood by centrifuge at 1500 RPM (rev per minute) for 10 minutes. Approximately 500 microliters aliquot of the separated plasma was immediately stored in polypropylene tube at -80º Celsius for batch analysis. The biopsy samples were fixed in 4% formalin and transferred to the pathology department for routine histological grading according to the ISHLT grading system (*180*). The patients were thus divided into two groups: Group 1

(with acute rejection) and Group 2 (showing no rejection). Clinical data was collected from patient notes for risk stratification. The use of immunosuppressive agents (Prednisolone, Cyclosporine, Tacrolimus, Mycophenolate Mofetil and Azathioprine), and haematological white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets) were also collected.

The study was designed to investigate the differences in the peripheral blood chemokine levels between stable, non-rejecting patients and those with at least grade 2R (moderate rejection). Due to the very limited number of patients with this degree of rejection, it was not possible to obtain sufficient number of consecutive samples during the limited time of the study. Hence, patient's consent was obtained for using some plasma aliquots stored over a period of nearly 3 years (between 2005 and 2007) for the purpose of the immunological marker study in the Transplant department at my hospital. With this method, I could obtain a group of 18 heart transplant recipients with grade 2R rejection, to compare against a randomly selected group of 32 heart transplant recipients with no rejection. Hence, the study was part prospective, part retrospective, observational study. Due to the same reason of limited number of samples with grade 2R rejection, it was not possible to sufficiently power the study for observing minor effect size for the individual chemokines. The study was approved by the local regional ethics committee and performed according to the declaration of Helsinki. All participants provided written informed consent. All tissue samples were stored according to the Human Tissue act.

Luminex Immunoassay

I used Luminex® (Invitrogen®, MA) to measure the levels of Th1 and Th2 chemokines including CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CCL11 in the plasma separated form the peripheral blood samples. The Luminex® implies multiplex solid phase immunoassays using spectrally encoded, antibody conjugated beads (figure 2.1, chapter 2). This technology is capable of simultaneous measurement of several proteins in a single well and is now well described (*196, 197*). The method is capable of measuring the chemokine levels with a high sensitivity and low interassay variability, as mentioned in chapter 2.

Statistical analysis

All the data was analyzed using SPSS. The two groups were compared in terms of demographics, clinical data and serum chemokine levels. Students t test or Mann-Whitney U test were used for comparing the continuous data depending on the normality of distribution of the variables, while Chi-square test was used for comparing the categorical variables. A p-value of less than 0.05 was considered statistically significant.

Results

Group 1 (with evidence of grade 2R rejection on the endomyocardial biopsy) comprised of 18 patients, while Group 2 included 32 patients with no evidence of rejection on the endomyocardial biopsy. Clinical and laboratory data was available for all the patients. I found no difference between the two groups in terms of the demographic data. The two groups were similar in terms of immunosuppression and CMV serostatus as well. Table 3.1 summarizes some of

the demographic variables while table 3.2 shows the peripheral blood counts for the two groups.

Table 3.1 Demographic data for rejecting versus non-rejecting patients.

Age is described in mean (standard deviation), while the duration since transplant is shown in median (25^{th}, 75^{th} percentiles) due to non-parametric distribution. (CNI, Calcineurin inhibitors)

		Group 1 (n=18)	Group 2 (n=32)	P value
Age (years)		49.83 (13.35)*	50.63(13.01)*	0.55
Duration since transplant (days)		126.0 (27.5, 185.25)	174.5 (59.0, 558.0)	0.08
Sex:	Male	77.8%	75.0%	0.82
	Female	22.2%	25.0%	
Pre-transplant diagnosis	ICM	50%	53.12%	0.83
	DCM	44.4%	37.5%	
	Others	5.6	9.37%	
CMV serostatus	Positive	5.5%	21.8%	0.13
	Negative	94.5%	78.2%	
CNI	Cyclo	88.8%	87.5%	0.87
	FK	11.1%	12.5%	

Table 3.2 Comparison of the peripheral blood counts between the two study groups.

***** denotes data in mean (±standard deviation), § data in median (25th, 75th percentiles)

	Group 1 (n=18)	Group 2 (n=32)	P value
WBC ($x10^9$/l)	6.36 (1.84)*	7.14 (3.2)*	0.38
Neutrophils ($x10^9$/l)	4.64 (1.59)*	5.64 (2.92)*	0.39
Lymphocytes ($x10^9$/l)	0.74 (0.5, 0.9) §	0.62 (0.5, 0.91)	0.69§
Monocytes ($x10^9$/l)	0.37 (0.09)*	0.6 (0.38)*	0.14
Eosinophils ($x10^9$/l)	0.1 (0.07, 0.1)§	0.05 (0.02, 0.1) §	0.19
Basophils ($x10^9$/l)	0.04 (0.03)*	0.02 (0.02)*	0.14
Platelets ($x10^9$/l)	251.53 (94.93)	230.85 (92.62)	0.51

When comparing the chemokine levels between the two groups, I found that patients with acute rejection had significantly higher peripheral blood levels of CCL2 and CCL5 (Fig 3.1, 3.2). However, there was no significant difference in the levels of CCL3, CCL4, CXCL9, CXCL10 and CCL11 between the two groups.

Fig 3.1 Box plot showing mean concentration of CCL2 in the peripheral blood samples in acute (n=18) versus non-rejecting (n=32) heart transplant patients.

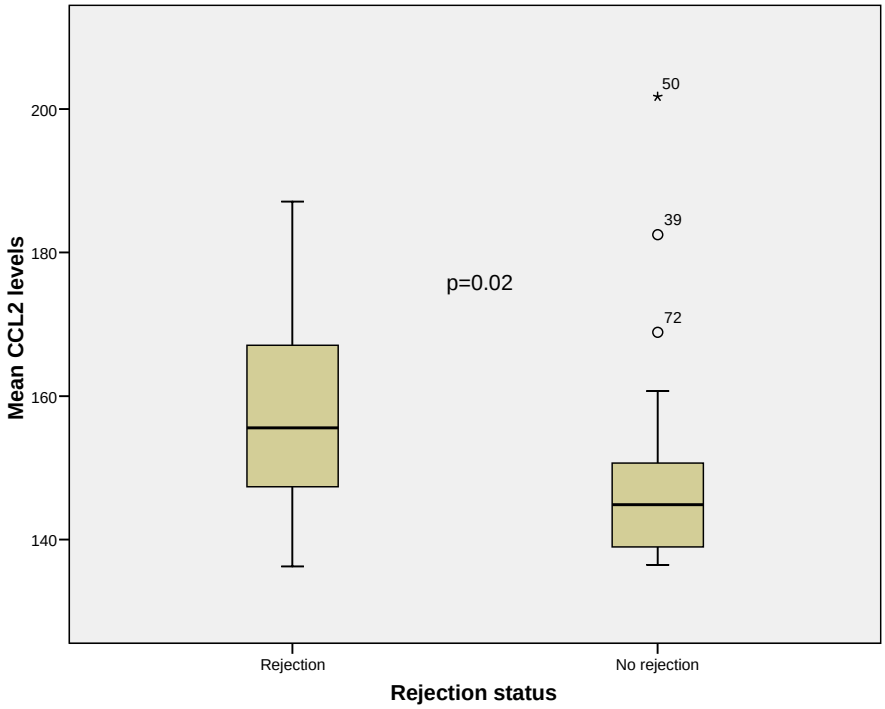
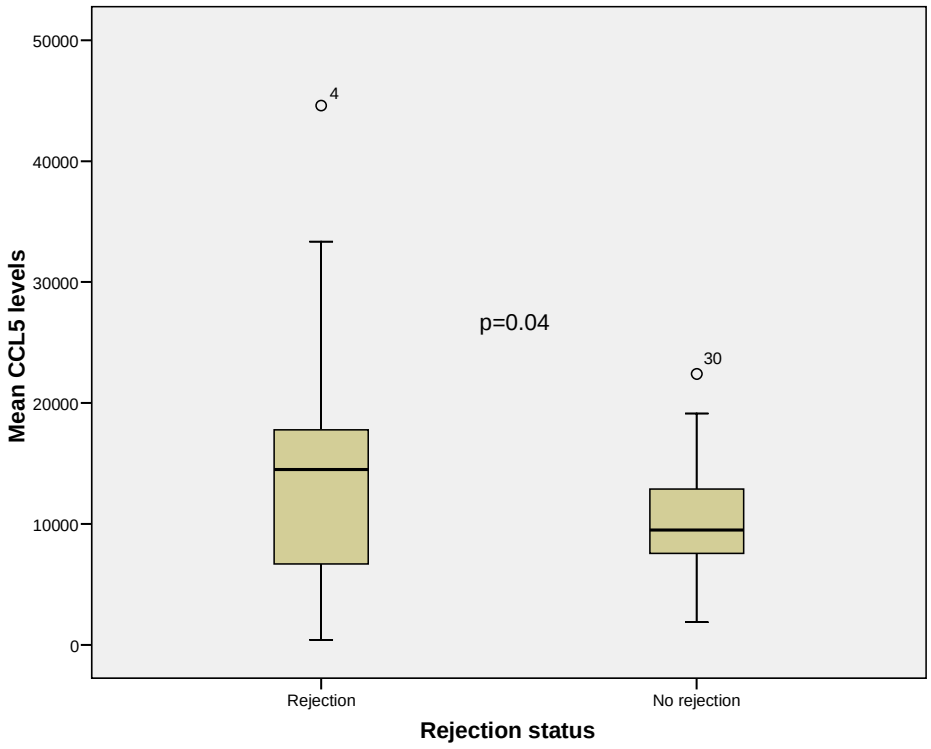


Fig 3.2 Box plots showing mean peripheral blood concentration of CCL5 in acute (n=18) versus non-rejecting (n=32) heart transplant patients.



Discussion

Chemokines are important mediators of leucocyte trafficking, and undoubtedly play a key role in acute rejection following solid organ transplantation. Despite the up-regulation of various chemokines and their receptors in the allograft during rejection, there had yet been no evidence to suggest any associations with allograft rejection and the peripheral blood concentrations of chemokines. Herein I demonstrate for the first time an association between peripheral blood chemokine levels and acute cellular rejection following heart transplantation.

As previous evidence suggests that acute rejection following solid organ transplantation may involve Th1 or Th2 response (*198*), I evaluated a range of Th1 and Th2 chemokines in the peripheral blood of rejecting and non-rejecting patients. Interestingly, only CCL2 and CCL5 concentrations were significantly elevated during the acute rejection episode, while I found no associations with the levels of CXCL9, CXCL10, CCL3, CCL4 and CCL11. This finding is interesting because previous authors have reported that intragraft expressions of CCL2 (a ligand for CCR2), and CCL5 (ligands for CCR1 and CCR5) have been associated with cellular infiltration that lead to acute rejection following heart transplantation (*91*).

The findings of my study further complement a previous study by Karason et al, who found that the peripheral blood levels of CXCL9 and CXCL10 did not correlate with acute rejection, despite a significant up-regulation of corresponding genes in the rejecting myocardium (*98*). Similar to Karason et al, my study did not demonstrate an association between peripheral blood CXCL9 and CXCL10 levels and acute rejection. The purpose of Karason

study was to define non-invasive biomarkers of rejection in heart transplant recipients, hence they selected CXC chemokines that are well described to up-regulate during acute rejection (*93, 97*). However, they did not evaluate relationship between CCL2 and CCL5 and acute rejection. Hence, my novel findings may help in determining valuable non-invasive adjuncts for the detection of acute rejection in solid organ transplantation.

Apart from transplantation, various studies have documented serum chemokine levels as indicative of a systemic response to the pathological state, including amyotrophic lateral sclerosis (*199*) rheumatoid arthritis (*200*) hepatic inflammation (*201*) and psoriasis (*202*). While none of the included patients in my study had significant systemic inflammation as shown by the normal peripheral blood white cells and neutrophil counts, one can argue that more stringent inclusion criteria and measuring high-sensitivity C-reactive protein (CRP) might have helped in excluding any confounding factors. I acknowledge that this remains one of the limitations of this study.

The findings of this study may still have implications towards prevention of acute rejection or induction of allograft tolerance. Experimental models have suggested that allograft survival can be prolonged by blocking specific chemokine pathways. For example, Horuk *et al* demonstrated that treating a rat model of heart transplant rejection with BX471 (an orally active CCR1 antagonist) resulted in significant prolongation of allograft survival ($p=0.004$), with further synergistic effects when used with a sub-therapeutic dose of cyclosporin ($p=0.0009$) (*203*). The study suggested that BX471 inhibits the adhesion of activated mononuclear cells to inflamed epithelium. Another study used TAK779 (a CCR5 and CXCR3 antagonist) on murine model of cardiac and

islet allograft rejection. They also found a significant dampening of the local immune response, reduced infiltration of CD4, CD8 and CD11c cells into the allograft, and significant prolongation of the allograft survival (*204*). Hence, cellular infiltration of the allograft can be altered by interfering with the systemic effects of CCL2 and CCL5, thus helping to prevent allograft rejection.

One limitation of my study was the small underpowered sample size due the reasons explained above. In addition, I also acknowledge the fact that this study was conducted on plasma instead of serum samples. However, I believe that this small observational study still provides an important finding relevant to the heart transplant population.

To conclude, I herein demonstrate that acute rejection in human heart transplant recipients lead to a significant rise in the peripheral blood levels of Th1 chemokines CCL2 and CCL5, without any significant change in the levels of CCL3, CCL4, CXCL9, CXCL10 and CCL11. Further studies may demonstrate if these findings can be used for non-invasive determination of acute rejection, or to prolong allograft survival via blocking specific chemokine pathways.

Chapter 4 The effects of immunomodulatory drugs on peripheral blood Treg levels in adult heart transplant recipients

Abstract

Background

Establishing an immunologic tolerance is the holy grail of transplantation. The $CD4^{+}CD25^{bright}FOXP3^{+}$ T cells (Tregs) are documented to play a pivotal role in the allograft tolerance. At present various immunosuppressive medications in different combinations are used to prevent rejection. These are, however, associated with debilitating side effects, including hypercholesterolemia. As a result, statins are frequently used following heart transplantation. Previous studies have shown conflicting evidences regarding the effects of these medications on the peripheral blood levels of Tregs.

Methods

90 stable adult heart transplant recipients were prospectively recruited for this observational study. All patients received standard immunosuppression according to the unit protocol, including cyclosporin or tacrolimus (CNI), Mycophenolic acid (MPA) or azathioprine (Aza), and Prednisolone (Pred). Statin use was determined according to clinical necessity and tolerability. The peripheral blood samples were collected in EDTA vacutainer during routine follow up and Tregs were phenotyped by cell surface expression of CD4 and CD25, and intracellular FOXP3 expression.

Results

Following risk stratification, I found no difference in the levels of Tregs between patients taking cyclosporin (n=78) or tacrolimus (n=12). Patients were then divided into three groups according to the use of CNI+MPA±Prednisolone

(Group1), CNI+Azathioprine±Prednisolone (Group 2), or CNI+Prednisolone (Group 3). Once again I found no difference in Treg levels or FOXP3 expression between the three groups. However, patients who were treated with a statin (n=75) had significantly reduced numbers of circulating $CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs compared to patients not treated with a statin (n=15, p=0.01). The percentage of Tregs in the T cell compartment was also significantly lower compared to the non statin group (p=0.02).

Conclusion

In this observational study, I found no effects of immunosuppressive medications on the numbers of circulating Tregs in patients following heart transplantation. However, statin use was associated with significantly reduced peripheral blood Treg counts. This may significantly affect recipient immune responses to graft tissue, as Tregs promote specific T cell unresponsiveness to alloantigen, via modulation of allospecific CD4 T cell responses.

Background

The alloresponse following transplantation comprises an effector arm to reject the transplanted organ, and a regulatory arm that checks the effector response and induces and maintains homeostasis. The $CD4^{+}CD25^{bright}FOXP3^{+}$ T cells (Tregs) are widely recognized as the most important part of the regulatory armamentarium, maintaining specific unresponsiveness and operational tolerance to donor antigen (*30, 33*). These cells comprise 5-10% of the peripheral blood CD4+ compartment in humans (*35*). Studies have demonstrated that the peripheral blood counts of Tregs increase following solid organ transplantation, and that these levels vary according to the state of immunological stability (*205*).

Another factor that may have an effect on Tregs is the use of various non-specific immunosuppressive agents such as Cyclosporin, Tacrolimus, Mycophenolic acid derivatives, Azathioprine and Prednisolone, which are still widely used to prevent or treat rejection (*206*). Cyclosporin and Tacrolimus (together labelled as Calcineurin inhibitors or CNI) bind to the intracellular immunophilins (calcineurin), blocking the T cell receptor (TCR)-dependent activation of the calcineurin, thus inhibiting nuclear translocation of the Nuclear factor of activated T cells (NF-AT) and suppressing T cell activation and cytokine gene transcription, including Interleukin 2 (IL2) (*207*). Mycophenolic acid (MPA) pro-drugs include Mycophenolate mofetil (MMF or CellCept®) and Myfortic®, the slow release preparation. MPA are powerful inhibitors of Inosine Monophosphate Dehydrogenase (IMDH), a rate-limiting enzyme in de-novo synthesis of guanosine nucleotides, particularly in the activated T and B cells (*208, 209*). Azathioprine acts as a non-specific anti-proliferative drug by the

formation of intracellular thiopurine ribonucleotides (*210*). There is conflicting evidence in the literature regarding the effects of immunosuppression on Tregs. Some studies suggest a negative effect of these immunosuppressive agents, particularly the CNI on the Treg population (*211*). However, in-homogeneity to define Tregs phenotype leads to considerable confusion and further studies are required.

Hyperlipidaemia is another significant problem in the post heart transplant population and occurs for several reasons which include inappropriate diet, reduced physical activity and adverse effects of immunosuppressive therapy, especially from Cyclosporine and steroids (*212*). Hyperlipidaemia has also been associated with early onset vasculopathy and rejection (*213*).

As a result, between 60 to 80 percent of cardiac transplant recipients receive cholesterol lowering agents (*214*). The most frequently used agents are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, commonly referred to as statins. In addition to lipid lowering effects, statins have been reported to modulate immune cells which are directly involved in graft rejection, such as macrophages, T cells, and natural killer (NK) cells (*215*). However, the effects of statins on Tregs are yet to be determined.

On these grounds I prospectively explored the effects of routine immunosuppression and statin administration on the numbers of peripheral blood Tregs following heart transplantation.

Methods

90 stable adult heart transplant recipients were prospectively recruited into this observational, non-randomized study. The exclusion criteria included acute rejection, acute infections, severe hepatic dysfunction, and failure to give

informed consent. All patients received standard immunosuppression according to our unit protocol comprising cyclsopsorin or tacrolimus, MPA or Aza, and Prednisolone. Treatment and the doses of statins were determined by clinical necessity as governed by the medical team. Clinical data was collected from the patient notes. Demographic data (*age, gender, time post transplantation, pre transplant diagnosis*) were collected for risk stratification. The use of immunosuppressive agents and haematological white blood cell counts (*neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets*) were collected.

Flow Cytometry

Sample Collection: Whole blood was collected into EDTA vacutainers (BD) via venepuncture from patients attending for the routine follow up.

Sample Preparation: Peripheral blood mononuclear cells (PBMC) were isolated from whole blood sample by density gradient centrifugation method using Ficoll-Paque as previously described (*173*). 100µl of PBMC were stained with anti-human CD4 phycoerythrin-Cy5 (PE-Cy5) and anti CD25 phycoerythrin-Cy7 (PE-Cy7) for 30 minutes in the dark (to avoid denaturing by ultra violet light). Staining with fluorescein isothiocyanate (FITC)-conjugated anti-human FOXP3 (e-Bioscience, UK) was performed with the fixation and permeabilization solutions according to the manufacturer's guidelines. The combinations of mouse IgG1 PE-Cy5, PE-Cy7, and FITC were used as isotype controls. Flow cytometric analysis was performed using a BD™ LSR II flow cytometer with linear forward scatter (FSc), linear side scatter (SSc), and log fluorescence 1 (FL1), 2 (FL2) and 3 (FL3) detection. A standard FS/SS lymphocyte gating strategy was used (fig 2.5), along with software generated

bright and dim gating strategy. Data was collected for either a maximum of 300 seconds or ten thousand events for all antibody combinations.

Laboratory Data Analysis: Additional gating strategies (including CD25 bright subsets (fig 2.6-2.10) and data analysis were performed using BD FACSDiva™ software EXPO32 ADC Analysis software (Beckman Coulter). Tregs were grouped as $CD4^{+}CD25^{bright}FOXP3^{+}$. Fluorescence values from total number of cells were calculated into Microsoft Excel. Treg counts and FOXP3 mean expression levels were compared to the various demographic data and immunosuppressive data at the time of sampling.

Results

The cohort included 73 males (81.1%) and 17 females (18.9%), at a mean age of 52.8 (± 13.7) years, and mean duration 8.7 (± 6.0) years following transplantation. There were no correlations of age and duration since transplant with the counts of $CD4^{+}$ cells, Tregs, or $CD4^{+}CD25^{-}$ effector cells, or the Treg FOXp3 expression. The cell counts or the FOXP3 expression were also not correlated with any of the demographic variables or the immunosuppressive medications (tables 4.1-4.6)

Table 4.1 The peripheral blood $CD4^+$, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the sex (n=90) (* denotes data in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile) due to non-parametric distribution).

Cell types	Male (81.1%)	Female (18.9%)	P value
$CD4^+$	1128.49 (809.69)*	1549.35 (1223.31)*	0.24
Tregs	3.0 (1.0, 14.0)	7.0 (2.5, 23.0)	0.15
Tregs % of $CD4^+CD25^{bright}$	17.61 (15.55)*	18.39 (13.69)*	0.85
Tregs % of CD4	0.6 (0.1, 1.35)	0.6 (0.35, 1.6)	0.45
Treg FOXp3 expression	1494.0 (1252.5, 1919.5)	1326 (1081.0, 1757.0)	0.15
$CD4^+CD25^-$	661 (315.5, 980.0)	960 (293.5, 1503.0)	0.28
$CD4^+CD25^-$ % of CD4	61.44 (18.8)*	60.53 (15.53)*	0.85

Table 4.2 The peripheral blood CD4+, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the presence or absence of Diabetes (n=90) (* denotes data in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile).

Cell types	Diabetes (15.6 %)	No diabetes (84.4%)	P value
$CD4^+$	1468.35 (1169.9)*	1160.02 (853.8)*	0.24
Tregs	5.5 (2.0, 15.75)	3 (1.0, 16.5)	0.27
Tregs % of $CD4^+CD25^{bright}$	17.62 (13.3)*	17.79 (15.55)*	0.96
Tregs % of CD4	0.6 (0.35, 1.85)	0.5 (0.1, 1.37)	0.38
Treg FOXp3 expression	1423.5 (1194.0, 1672.7)	1487.5 (1214.0, 1923.0)	0.35
$CD4^+CD25^-$	818.5 (302.25, 1018.25)	678 (317.0, 1122.0)	0.81
$CD4^+CD25^-$ % of CD4	57.65 (16.66)*	61.93 (18.44)*	0.42

Table 4.3 The peripheral blood CD4+, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the presence or absence of hypertension (n=90) (* denotes data expressed in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile).

Cell types	Hypertension (87.8%)	No hypertension (12.2%)	P value
$CD4^+$	1092 (483.0, 1641.0)	1297 (644, 2188)	0.33
Tregs	3.0 (1.0, 12.0)	18.0 (5.0, 22.0)	0.054
Tregs % of $CD4^+CD25^{bright}$	17.68 (15.34)*	18.32 (14.37)*	0.89
Tregs % of CD4	0.5 (0.1, 1.3)	0.8 (0.6, 2.1)	0.15
Treg FOXp3 expression	1479.0 (1212.0, 1923.0)	1448.0 (1109.5, 1672.5)	0.35
$CD4^+CD25^-$	686.0 (311.0, 1022.0)	680.0 (320.0, 1475.0)	0.64
$CD4^+CD25^-$ % of CD4	62.08 (18.49)*	55.4 (14.94)*	0.25

Table 4.4 The peripheral blood CD4+, $CD4^+CD25^-$, Treg, and FOXP3 expression according to renal impairment (estimated GFR greater or less than 60) (n=90) (* denotes data expressed in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile).

Cell types	eGFR <60 (83.3%)	eGFR>60 (16.7%)	P value
$CD4^+$	1248.78 (924.86)*	1004.0 (825.63)*	0.34
Tregs	3.0 (1.0, 17.0)	4.0 (2.0, 12.0)	0.97
Tregs % of $CD4^+CD25^{bright}$	16.99 (14.66)*	21.51 (17.36)*	0.29
Tregs % of CD4	0.89 (1.07)*	0.96 (0.80)*	0.81
Treg FOXp3 expression	1460.0 (1189.7, 1762.7)	1811.5 (1379.5, 2120.0)	0.06
$CD4^+CD25^-$	680.0 (320.0, 1050.0)	861.0 (56.0, 1212.0)	0.74
$CD4^+CD25^-$ % of CD4	60.69 (18.22)*	64.16 (18.15)*	0.50

Table 4.5 The peripheral blood CD4+, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the Pre-transplant diagnosis (n=90) (* denotes data expressed in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile).

Cell type	DCM (n=49)	ICM (n=33)	VHD (n=3)	Others (n=5)	p value
$CD4^+$	1018 (497, 1662)	1148 (559, 1680)	1109 (23, 1567)	1627 (494, 2534)	0.83
Tregs	4 (1, 21)	3 (1, 8.5)	2 (0, 24)	20 (3, 26)	0.26
Tregs % of $CD4^+CD25^{bright}$	16.7 (5.9, 30.5)	12.5 (2.5, 27.3)	7.1 (0, 7.4)	20 (2.7, 28.8)	0.27
Tregs % of CD4	0.6 (0.1, 1.7)	0.4 (0.1, 1.1)	0.2 (0, 1.5)	1.4 (0.4, 1.8)	0.16
Treg FOXp3 expression	1475 (1263.5, 1773)	1464 (118, 2063)	3157 (1801, 4514)	1448 (1119, 3188)	0.38
$CD4^+CD25^-$	661 (298, 1033.5)	771 (311, 1132)	519 (1, 780)	676 (260, 1371.5)	0.75
$CD4^+CD25^-$ % of CD4	62.1 (50.3, 74.6)	69.5 (56.5, 73.8)	33.1 (4.3, 70.3)	45.1 (33.7, 64.1)	0.08

Table 4.6 The peripheral blood CD4+, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the type of CNI (n=90) (* denotes data expressed in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile) due to non-parametric distribution).

Cell type	Cyclosporin (n=78)	Tacrolimus (n=12)	p value
$CD4^+$	1127 (528, 1676)	959.5 (629.7, 1651.7)	0.84
Tregs	4 (1, 15)	3 (1, 22)	0.84
Tregs % of $CD4^+CD25^{bright}$	17.5 (15.4)*	19 (13.6)*	0.76
Tregs % of CD4	0.5 (0.1, 1.3)	0.9 (0.2, 1.7)	0.37
Treg FOXp3 expression	1464 (1216, 1886.5)	1632 (106, 1923)	0.93
$CD4^+CD25^-$	683 (302, 1062)	636 (364, 1149)	0.96
$CD4^+CD25^-$ % of CD4	60.7 (19.0)*	64.9 (10.7)*	0.45

Patients were further divided into three groups according to the immunosuppressive regimen: CNI+MPA±Prednisolone (Group1, n=55), CNI+Azathioprine±Prednisolone (Group 2, n=15), or CNI+Prednisolone (Group 3, n=20). Following risk stratification, the three groups showed no difference in terms of CD4, Treg or CD4+CD25- effector cell counts, or Treg FOXP3 expression (table 4.7).

Table 4.7 Distribution of the peripheral blood $CD4^+$, $CD4^+CD25^-$, Treg, and FOXP3 expression according to the immunosuppression protocol: Group 1= CNI+MPA±Pred, Group 2 = CNI+Aza±Pred, Group 3 = CNI+Pred. (* denotes data in mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile).

Cell type	Group 1 (n=55)	Group 2 (n=15)	Group 3 (n=20)	p value
$CD4^+$	971 (555, 1609)	1297 (1018, 1849)	954 (271, 1706))	0.27
Tregs	4 (2, 15)	6 (2, 24)	3 (0.2, 16)	0.70
Tregs % of $CD4^+CD25^{bright}$	16.6 (15.1)*	19.3 (14.3)*	19.8 (16.3)*	0.66
Tregs % of CD4	0.9 (1.1)*	0.9 (0.8)*	0.8 (0.8)*	0.91
Treg FOXp3 expression	1510 (1195.2, 1838.7)	1414 (1205, 1989.5)	1464 (1287, 1923)	0.96
$CD4^+CD25^-$	655 (311, 943)	906 (549, 1323)	1650 (148.5, 1213.5)	0.20
$CD4^+CD25^-$ % of CD4	60.5 (15.9)*	66.6 (18.1)*	59.1 (23.5)*	0.43

The effects of statins on Tregs

As a sub-group analysis, I compared the patients taking statins (ST, n=75) versus patients not on statins (NOST, n=15). The patients in ST group had been on statins for more than 6 weeks prior to inclusion in the study. NOST patients had never been treated with any cholesterol lowering agents. Clinical and laboratory data was available for all study participants. There were no differences in clinical or demographic characteristics between the two treatment groups as shown in table 4.8. Both groups were at a similar time post transplantation (ST group were a mean of 3049 ± 2139 days since transplantation and NOST patients were a mean of 3994.3 ± 2490.4 days since transplantation (p=0.1). In terms of the types of statins, 57.3% of ST patients were taking Pravastatin, 26.7% were taking Atorvastatin, 10.7% were on Simvastatin, and the rest on Fluvastatin or Rosuvastatin. All patients received prednisolone (pred), ranging from 5 to 15 mg per day, with no difference in pred dose between the 2 study groups (6.5 mg/ day vs. 6.0 mg/day for ST and NOST respectively p=0.6). When comparing treatment with routine immunosuppressive agents, 85.3% (ST) vs. 93.3% (NOST) patients received Cyclosporine (CsA), and 14.7% (ST) vs. 6.7% (NOST) received Tacrolimus (Tac). When comparing secondary immunosuppressive agents, I found that 16% (ST) vs. 20% (NOST) patients received Azathioprine (Aza), and 62.7% (ST) vs. 53.3% (NOST) received Mycophenolate Mofetil (MMF). However, there were no differences between CsA/Tac administration and numbers of $CD4^+$ (p=0.845), $CD4^+25^{bright}$ (p=0.840), Treg (p=0.844), Treg mean FOXP3 expression (0.935), Treg percentage of CD4 (p=0.378) or $CD4^+CD25^-$ (p=0.96) lymphocytes, or MMF/Aza administration and numbers of $CD4^+$ (p=0.124),

$CD4^{+}25^{bright}$ (p=0.875), Treg (p=0.499), Treg mean FOXP3 expression (0.833), Treg percentage of CD4 (p=0.954) or $CD4^{+}CD25^{-}$ (p=0.068) lymphocytes. When comparing T cell phenotypes between ST and NOST, I found that the patients in NOST group had significantly higher numbers of circulating Tregs compared to ST patients (Fig 4.1). Interestingly, the number of circulating $CD4^{+}$ cells (even $CD4^{+}CD25^{bright}$) without FOXP3 did not differ between the two groups. I also looked at the relative proportion of Tregs (Tregs as the percentage of $CD4^{+}CD25^{bright}$ cells) between ST and NOST, and again found a significantly lower proportion of Tregs in the ST group (p=0.047) (Fig 4.2)

Table 4.8 Demographics according to the use of Statins

(* denotes data in mean (standard deviation), eGFR = estimated glomerular filtration rate)

	Statin group (n=75)	No statin group (n=15)	P value
Age (years)	53.2 (13.8)*	51.1 (13.6)*	0.58
Male	81.3%	80.0%	0.68
Pre-Transplant diagnosis	DCM (52.0%) ICM (38.7%), Others (9.3%)	DCM (66.7%) ICM (26.7%), Others (6.7%)	0.46
Duration since Transplant (days)	3049 (2139.7)*	3994.3 (2490.4)*	0.14
Hypertension	86.7%	93.3%	0.68
Diabetes Mellitus	17.3%	6.7%	0.45
eGFR	45.3 (17.7)*	43.4 (19.2)*	0.71
Serum Cholesterol	5.0 (0.9) *	5.2 (1.3) *	0.70

Table 4.9 Immunosuppressive medications in patients with and without statins. (* denotes data in mean ± standard deviation, § shows data in median (25^{th}, 75^{th} percentile) due to non-parametric distribution).

	Statin group (n=75)	No statin group (n=15)	P value
Cyclosporin (as % of CNI)	85.3%	93.3%	0.68
Mycophenolic acid	62.7%	53.3%	0.79
Azathioprine	16%	20%	0.7
Cyclosporin level	81 (53, 114)§	101 (60, 149) §	0.28
Prednisolone dose (mg)	6.5 (4.4)*	5.9 (5.2) *	0.68

Table 4.10 Peripheral blood counts in patients with and without statins.

All data is expressed in mean (standard deviation).

	Statin group (n=75)	No statin group (n=15)	P value
WBC ($x10^9$/l)	7.19 (2.05)	6.62 (2.78)	0.37
Neutrophil ($x10^9$/l)	5.15 (2.02)	4.43 (2.68)	0.28
Lymphocytes ($x10^9$/l)	1.26 (0.67)	1.33 (0.52)	0.74
Monocytes ($x10^9$/l)	0.65 (0.19)	0.69 (0.25)	0.80
Eosinophils ($x10^9$/l)	0.1 (0.09)	0.13 (0.08)	0.54
Basophils ($x10^9$/l)	0.02 (0.01)	0.02 (0.01)	0.92
Platelets ($x10^9$/l)	242.96 (57.8)	238.29 (102.9)	0.48

Fig 4.1 Box plot comparing peripheral blood Treg counts in patients with or without statins.

Group 0 denotes patients not on statins (n=15), while the group 1 denotes patients on statins (n=75).

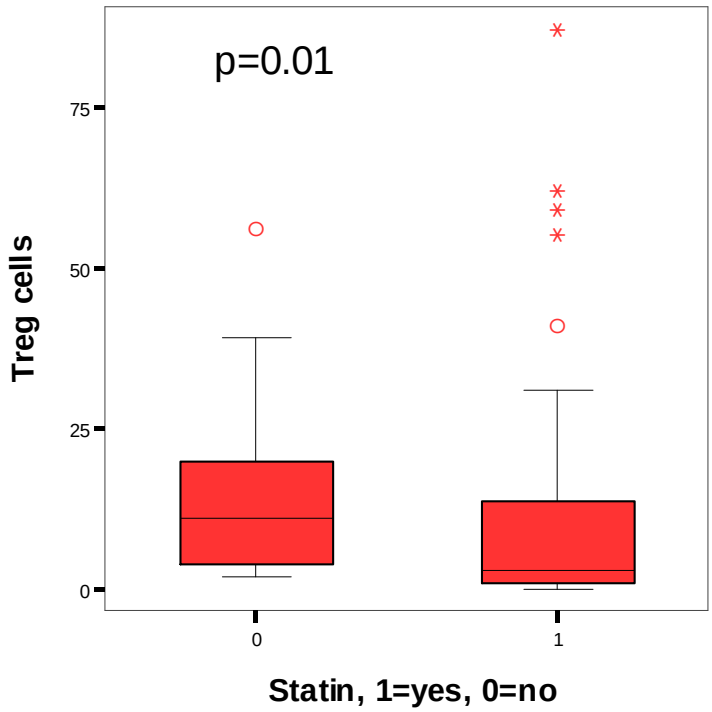
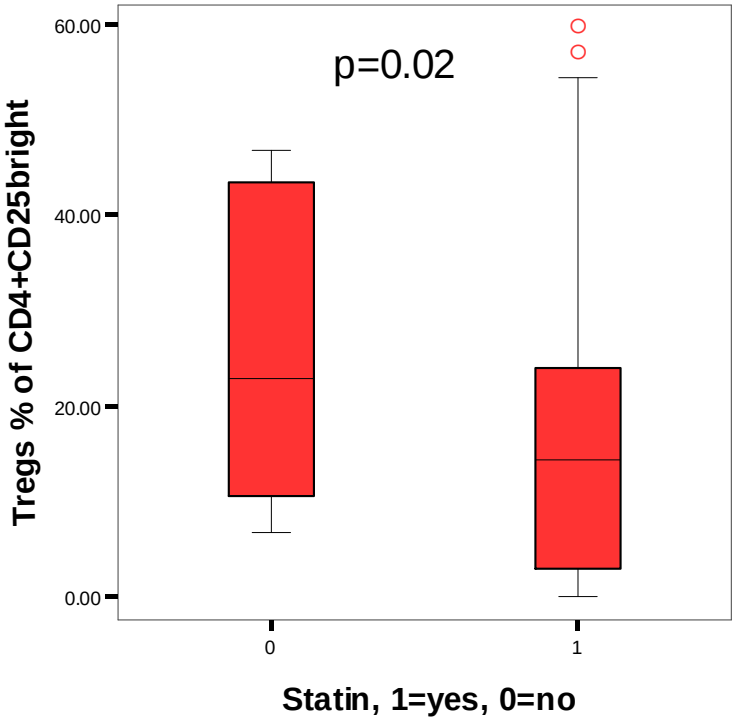


Fig 4.2 Box plot showing peripheral blood Tregs as percentage of the $CD4^{+}CD25^{bright}$ lymphocytes in patients with or without statins.

Group 0 denotes patients not on statins (n=15), while the group 1 denotes patients on statins (n=75).



Discussion

This is the first study in the literature describing an immunomodulatory effect of statins on Tregs following heart transplantation. In terms of immunosuppressive strategies, my results are consistent with the previously published work in lung transplant cohort, showing no association between the individual or combinations of immunosuppressive medications with the peripheral blood Tregs (*205*). I also demonstrate a lack of association between immunosuppression and FOXP3 expression in Tregs, which is considered as the marker of immunoregulatory potential.

The implications of my results to the solid organ transplant recipient are widespread. Tregs have been reported to induce and maintain immunologic tolerance to alloantigen. (*6*). Tregs also disrupt the allospecific T cells that play a pivotal role in the cellular responses leading to graft rejection. This occurs via the deletion of clonally expanded allospecific T cells, or the induction of ignorance and/or anergy (*7*). The removal of Tregs in murine models results in the activation of self reactive T cells causing autoimmune diseases such as gastritis with pernicious anaemia, Hashimotos thyroiditis, insulin dependent diabetes mellitus, systemic lupus erythematosis and rheumatoid arthritis (*22*).

It has been extensively documented that IL2 confers optimal suppressive function to the Tregs, partly via intracellular kinase dependent pathways (*216*). Hence non-specific suppression of IL2 through CNI is expected to render diminished induction and function of Tregs, alongside inhibition of the effector T cell population. Indeed, Baan *et al* showed that CNI inhibit *in-vitro* induction of FOXP3 in a mixed lymphocyte reaction (*211*). Subsequent *in-vivo* studies showed the inhibitory effects of cyclosporin administration on peripheral blood

Treg population in a transplant cohort (*217, 218*). However, the effects of tacrolimus on Tregs are still controversial. One study looking at ischemia/reperfusion (I/R) model showed that the treatment with tacrolimus prevented I/R injury along with an augmentation of the Treg population (*219*). A somewhat similar observation was made by another study where *in-vitro* proliferation of $CD4^+$ cells was observed. Tacrolimus inhibited TCR-stimulated cell division in the conventional $CD4^+$ cells, but Tregs showed enhanced cell division in the presence of Tacrolimus (*220*). Since all the patients in my study were taking CNI as primary immunosuppression, I could not elicit a difference with non-CNI group. However, I found no difference in Treg counts or FOXP3 expression in patients taking CsA or Tacrolimus.

Corticosteroids (CS) (such as Prednisolone, methyl Prednisolone) have long been used in transplant patients due to potent immunosuppressive and anti-inflammatory properties. CS exert their immunosuppressive effects via several direct and indirect pathways, leading to the modulation of adhesion molecules, suppression of cytokine synthesis, and T cell activation (*221*). There is evidence to suggest that part of the CS mechanism of action may involve an augmentation of the Treg population. This was shown in a study where administration of CS to asthmatic patients resulted in a significant increase in the expression of FOXP3 mRNA (*222*). In another study using murine model of autoimmune encephalomyelitis, short term treatment with Dexamethasone and IL2 significantly enhanced the proportion of Tregs in peripheral lymphoid tissue and prevented the disease (*223*). However, there is a paucity of data to suggest CS effects on Treg in human transplant patients. In the present study, I did not find any correlations of Tregs with the Prednisolone dose. However, the study was

not sufficiently powered to measure the effect, and further studies with larger population are required.

In order to optimise immunosuppression and reduce the side effects, use of multi-drug immunosuppressive regimen targeting different pathways is a common strategy following clinical transplantation. The MPA compounds (MMF® or Myfortic®) target de novo synthesis of guanosine nucleotides by inhibiting IMDH (*209*). Since the lymphocytes are more dependent on this pathway than other cells such as neutrophils, the cytostatic effects are more specifically pronounced in lymphocytes, particularly the activated lymphocytes. In addition, MPA drugs induce apoptosis of activated lymphocytes, suppress the expression of certain adhesion molecules and prevent tissue damage by production of NO (*208*). Hence, MPA drugs have largely replaced Azathioprine that induces non-specific DNA and nucleotide synthesis resulting in significant side effects (*224*). In addition, the use of MPA has now been acknowledged as a CNI-sparing strategy with potentially less side effects, as shown in a recent large randomized control trial (*225*). Taking it further, there is evidence to suggest that MPA drugs may actually be helpful in promoting Treg population compared to CNI-based regimen. This was shown by Demirkiran *et al* in their study on liver transplant recipients, where conversion from CNI to MMF resulted in an enrichment of the peripheral blood Tregs (*226*). However, my present work did not show any correlations between either the use or the dose of MPA and the peripheral blood counts of Tregs. One possible reason may be the effect of the concomitant CNI drugs. Also, the Demirkiran study was conducted in liver transplant patients with possible cohort differences compared to my study. In this context, I want to emphasize that small sample size was one of the main

limitations of my study. Hence it could not be statistically powered to detect an absolute difference between the Treg counts for each combination of immunosuppressive medications.

An interesting finding in my study was the effect of statins on Treg population. In a non-transplant setting, statins have largely been used as the cholesterol lowering agents with an ability to halt the progression or even induce regression of atherosclerotic plaques (*227-229*). The mechanism of atherosclerotic regression is considered to occur via the reduction of low density lipoprotein levels (LDL-C) and the increase of high density lipoproteins (HDL-C) (*230*). However, separate from a lipid altering mechanism of action, evidence suggests that statins have other important anti-inflammatory properties. The so called 'pleiotropic' effects of statins were highlighted in the conclusion of two large international trials (*231, 232*). These trials demonstrated beneficial effects of statin use after an acute coronary syndrome, which included a significant reduction of further plaque rupture events. One of the hypothesized explanations for this was an anti-inflammatory effect on the vulnerable plaque, supported by a decline in the inflammatory markers such as the C-reactive protein (CRP) in the statin treated group (*233*). My study did not include a measurement of the CRP and was underpowered to check the effects of statins on anti-inflammatory markers.

It should be appreciated that there are subtle differences between the atherosclerotic processes in a transplant setting, compared to native coronary disease. This is evidenced by concentric rather than eccentric lesions, reduced lipid content and higher numbers of inflammatory cells in the transplant atherosclerotic lesion (*234, 235*). Whether or not statins have a different

magnitude of effect within each setting remains to be answered. My study would argue against any beneficial effect of statins on Tregs action in transplant atherosclerosis. Indeed, the results actually demonstrate a potentially detrimental effect on plaque burden, when the conclusions of a recent study by Warnecke and colleagues are taken into account (*236*). They demonstrated that Tregs were capable of reducing intimal occlusion in transplant atherosclerosis. They proposed this route of regression occurred via the regulation of effector $CD4^+$ $CD25^-$ T cells by $CD4^+CD25^+$ T cells. Taking together, we can say that currently our knowledge remains limited about the mechanism of any potential immunoregulatory effects of statins within the atherosclerotic lesion.

Although some of the patients in the Statin group were also taking Ezetimibe (a cholesterol lowering agent that acts by reducing cholesterol absorption in the intestine), this data was not included as part of the study. Hence, I was unable to detect any changes in Tregs due to Ezetimibe. None of the NOST group patient was taking Ezetimibe.

Despite my findings however, statins remain beneficial to transplant patients and are proven to both prolong survival and reduce the development of coronary graft vasculopathy (*237*). This would suggest therefore that despite a negative effect on Tregs, there are other compensatory properties of statins existing that outweigh my findings, resulting in a net benefit to the patient. Further investigation into the immunomodulatory effects of statins would be beneficial to enhance our knowledge in this area and potential ability to treat vasculopathy more effectively.

Chapter 5 Chemotactic profile of T regulatory cells in a heart transplant cohort

Abstract

Background

Tregs ($CD4^{+}CD25^{bright}FOXP3^{+}$ T cells) are widely recognized as key elements in the transplant related tolerance. Following antigen stimulation, naïve Tregs change their phenotype, including a switch of their chemokine receptors. This critical step leads to the Treg migration to specific peripheral organs including secondary lymphoid tissue (SLT) or the allograft, etc. Various studies suggest specific chemotactic pathways guiding Tregs to inflammatory sites (*71*). However, there is lack of such data in humans following heart transplantation.

Methods

After thorough literature search, following chemokines were selected to detect chemotactic properties of peripheral blood Tregs in stable adult heart transplant recipients: CCL1, CCL2, CCL5, CCL17, CCL19, CCL21, CXCL9, and CXCL10. In-vitro chemotactic assays were performed for Tregs in 24 well insert systems (BD Falcon™) using 10 and 100 ng/ml solutions for each chemokine and compared against a negative control. Following the incubation, transfer of Treg to the lower chamber was assessed using flow cytometry. Direct comparison of migrated cell numbers and the "chemotactic index" (defined as the cells migrated in response to chemokine solution divided by the migrated cell count in negative control) was carried out. Demographic data and data on immunosuppressive medications were collected from the patient notes for risk stratification. All data was analyzed using SPSS v 15.

Results

A total of 128 patient samples were used to carry out chemotactic assays using chemokines CCL5, CCL17, CCL19, CCL21, CXCL9, CXCL10 (n=18 each), and CCL1, CCL2 (n=10 each). I found that CCL17 and CCL5 caused dose-dependent Treg specific migration ($p<0.05$). None of the other chemokines showed any specific Treg migration, while $CD4^+$ lymphocytes without regulatory phenotype i.e. $CD4^+CD25^-$ effector cells showed significant specific migration in response to CCL19 ($p=0.04$).

Conclusion

This study demonstrates for the first time a specific pattern of chemotaxis for Tregs in heart transplant patients. These results provide another avenue of research to determine therapeutic manipulations to guide circulating Tregs into the allograft.

Background

Despite an improved overall survival, most of the heart transplant patients are administered a combination of two-three immunosuppressive medications to prevent acute and chronic rejection (*184*). These drugs have several undesirable side effects, including hypertension, renal failure, metabolic diseases, and tumours, which result in significant morbidity and mortality (*238*). Hence, induction of immunologic tolerance is highly desirable following transplantation. Tolerance denotes an ideal state of antigen-specific immunological unresponsiveness towards the allograft, thereby avoiding rejection and allowing sustained graft function without the need of immunosuppressive medications. A significant body of evidence suggests that immune regulation governed by T regulatory cells (Tregs) plays a key role for peripheral tolerance in both experimental and clinical transplantation models (*30, 33, 34*). The naturally occurring Tregs, first described by Sakaguchi *et al* (*22*) as $CD4^+CD25^+$ T cells, comprise 5-10 % of the peripheral blood $CD4^+$ T cells in healthy adult humans and mice (*35, 36*). These cells have been reported to inhibit the proliferation of other $CD4^+$ and $CD8^+$ effector cells. Due to similarities with effector T cells, functional characterization of Tregs has remained a challenge. However, it is now largely accepted that $CD4^+CD25^{bright}$ cells expressing FOXP3 (a transcription factor) represent the Tregs (*65*).

Immune regulation by Tregs is complex, involving non-specific cell-cell contact mechanisms as well as secretion of IL-10 and TGF-β (*25, 29*). Hence, appropriate co-localization of Tregs with the effector cells is essential for Tregs to exert their regulatory function and control allo-reactivity (*239*). Indeed, like the effector T cells, Tregs require complex intra- and inter-compartmental

migration, from thymus to secondary lymphoid organs, and then to the peripheral tissues before recirculation (*73, 240*). This system is intricately controlled by a cohort of different chemokines and adhesion molecules. We postulated that transplantation may incur a difference in the dynamics of chemotaxis between $CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs and $CD4^{+}CD25^{-}$effector cells.

With this background, I prospectively investigated the chemotactic properties of peripheral blood Tregs and the effector T cells in human heart transplant recipients.

Methods

Subjects and sample collection

This was a prospective, observational, non-randomized study, conducted on stable (non-rejecting) adult heart transplant recipients attending the transplant outpatients department at the University Hospital of South Manchester. The exclusion criteria included evidence of acute rejection, acute infections, severe hepatic dysfunction, and inability to get an informed consent. Following informed consent, peripheral blood samples were collected from 71 participants. All the samples were collected in 5 ml EDTA (ethylenediamine-tetra-acetic acid) vacutainers, using standard venepuncture technique. According to our unit protocol, all patients were receiving combination of immunosuppression comprising cyclsopsorin or tacrolimus, mycophenolic acid derivatives (MPA) or Azathioprine (Aza), and Prednisolone (Pred). Clinical and demographic data were collected from patient notes for risk stratification.

Isolation of peripheral blood mononuclear cells

The blood samples were processed within 1-3 hours of collection and used to isolate peripheral blood mononuclear cells (PBMC) by density gradient centrifugation method using Ficoll-Paque as previously described (*173*). The cells were then suspended in RPMI-1640 medium (Sigma-Aldrich®), achieving up to 1-1.25 x 10^6 cells/ml.

Chemokines and antibodies

CCL1, CCL2, CCL5, CCL17, CCL19, CCL21, CXCL9, and CXCL10 were purchased from ProSpec-Tany TechnoGene Ltd, Israel. Antibodies for surface molecules including anti-CD4 Phycoerythrin-Cy5 (PE-Cy5) and anti-CD25 Phycoerythrin-Cy7 (PE-Cy7) were purchased from BD Biosciences, UK. Fluorescein isothiocyanate (FITC) conjugated anti-FOXP3 antibody was purchased from e-Bioscience, UK.

Chemotaxis assays

All the chemokines were diluted according to the required strength for chemotaxis, and aliquots were stored at -20ºC to avoid repeat freeze-thaw cycles. A carrier protein (0.1% bovine serum albumin) was added for long term storage. In-vitro cell migration assays were performed on PBMC using 8μm pore, 24 well insert system purchased from BD Falcon™. A 250μl of the cell suspension was added to the insert, while 750μl of chemokine solution in RPMI-1640 was added to the lower well. A negative control was used for each patient, using 750μl of RPMI-1640 without chemokine in the lower well. The system was incubated for 4 hours at 37^{o}C, 5% CO2 and normal humidity. Following incubation, transfer of Treg to the lower chamber was assessed using flow cytometry. Specific migration was calculated by direct measurement of Treg

numbers in the chemokine versus control solution. Chemotactic index for each chemokine was calculated as a ratio of the Treg counts migrated in response to the chemokine solution and those in the control medium. Similar calculations were carried out for $CD4^{+}CD25^{-}$ effector cells.

Treg immunophenotyping

The cell suspension from the lower well of the HTS multi-well system was extracted into a 5 ml polypropylene tube, washed with PBS (phosphate buffered saline) at room temperature, and then centrifuged at 1500 rpm for 10 minutes. The cell pellet thus created was stained with anti-CD4 PE-Cy5 and anti-CD25 PE-Cy7 for 30 minutes in the dark at $4^{o}C$ (to avoid denaturing by UV light), followed by intracellular staining with FITC conjugated anti-human FOXP3 using fixation/ permeabilization solutions according to the manufacturers guidelines. Isotype controls using mouse antibodies were included to provide a negative cell reference.

Flow cytometry

I used BD™ LSR II flow cytometer with linear forward scatter (FSc), linear side scatter (SSc), and log fluorescence 1 (FL1), 2 (FL2) and 3 (FL3) detection. A standard FS/SS lymphocyte gating strategy was used along with bright and dim gating strategy using FACSDiva™ software. Data was collected for either a maximum of 300 seconds or ten thousand events for all antibody combinations and was analyzed using EXPO32 ADC Analysis software (Beckman Coulter).

Statistical analysis

For statistical comparisons and clinical correlations, data analysis was performed using SPSS v15. Intergroup comparisons were made using t-test or

Mann Whitney U test, depending on the normality of distribution. Comparisons between multiple chemokine groups were made using one-way ANOVA. Dose response was evaluated using generalized linear model. A p value of < 0.05 was considered statistically significant.

Results

A total of 128 chemotaxis assays were carried out with PBMC from 71 adult heart transplant patients. Tregs ($CD4^{+}CD25^{bright}FOXP3^{+}$) comprised a small fraction of the $CD4^{+}$ population (median 0.5, range 0-6.7), while the $CD4^{+}CD25^{-}$ effector cells comprised 66.4 % (±19.2) of the CD4 cells. Rest of the $CD4^{+}$ cells belonged to the $CD4^{+}25^{dim}$ group which were not assessed. Due to the small numbers of peripheral Tregs it was not possible to perform simultaneous chemotaxis for all the selected chemokines on each patient. However, inter-group comparisons of the clinical and demographic data were performed for risk stratification. There was a significant difference between the groups in terms of age (p=0.029), with CCL2 group being the youngest at a mean age of 47.5 (±15.4). However, I found no other differences between the groups in terms of demographics or immunosuppression (table 5.1-5.3).

Table 5.1 Demographics for the various groups of chemokines as shown in the first column. Age and duration since transplant are expressed in mean (standard deviation). eGFR = estimated glomerular filtration rate, vMDRD (4 variable Modification of Diet in Renal Disease formula).

Group	Age	Sex (% male)	Duration since Tx (days)	Diabetes (%)	Hypertension (%)	eGFR (vMDRD, ml/min)
CCL1 (n=10)	60.1 (8.25)	100	2372.1 (1951.05)	30.0	100	49.7 (16.41)
CCL2 (n=10)	47.5 (15.47)	70	3270.2 (2636.18)	30.0	80.0	41.6 (22.87)
CCL5 (n=18)	60.44 (7.22)	94.4	3222.06 (2102.35)	50.0	94.4	38.06 (12.63)
CCL17 (n=18)	50.94 (15.79)	83.3	3709.94 (2228.83)	22.2	88.9	45.06 (18.15)
CCL19 (n=18)	51.61 (12.47)	72.2	2732.5 (2220.04)	22.2	94.4	44.11 (17.38)
CCL21 (n=18)	57.44 (9.25)	94.4	2763.94 (1724.06)	22.2	100	45.61 (13.8)
CXCL19 (n=18)	56.5 (8.54)	83.3	3933.56 (2472.57)	33.3	83.3	35.83 (16.61)
CXCL10 (n=18)	55.0 (9.43)	83.3	3994.78 (2523.81)	33.3	83.3	35.83 (18.04)
P value	0.029	0.28	0.36	0.64	0.41	0.23

Table 5.2 Immunosuppressive and immunomodulatory drugs used for the various chemokine groups. (Pred dose in mean (standard deviation) (Cyclo=cyclosporin, FK=tacrolimus, Aza=azathioprine, MMF=mycophenolate mofetil, Pred= prednisolone, ACEi = Angiotensin converting enzyme inhibitors)

Chemokine Group	Cyclo/FK (% on Cyclo)	Aza/MMF (% on MMF)	Pred dose (mg/day)	Beta blockers (%)	ACEi (%)	Statin (%)
CCL1	100	70.0	7.75 (2.18)	10	80	100
CCL2	70.0	50.0	5.50 (4.83)	0	60	70
CCL5	94.1	72.2	7.20 (2.77)	23.5	64.7	88.2
CCL17	94.4	50.0	5.97 (4.21)	27.8	61.1	83.3
CCL19	83.3	66.7	5.55 (4.33)	11.1	61.1	83.3
CCL21	83.3	72.2	7.63 (3.87)	33.3	72.2	88.9
CXCL19	94.4	61.1	5.69 (4.68)	33.3	72.2	77.8
CXCL10	94.4	61.1	4.72 (4.84)	44.4	72.2	77.8
P value	0.279	0.94	0.34	0.12	0.94	0.68

Table 5.3 Peripheral blood cell counts for the different groups of chemokines measured at the time of sampling for the chemotaxis assays. All cell counts in $x10^9$/L. *****mean ± standard deviation, rest of the data is expressed in median (25^{th}, 75^{th} percentile) (WBC: white blood cells, Neutro: neutrophils, Lympho: lymphocytes, Mono: monocyte, Eosino: eosinophils.)

Group	WBC	Neutro	Lympho	Mono	Eosino	Basophil	Platelet
CCL1	*7.72 (2.50)	*5.91 (2.32)	0.9 (0.29, 3.32)	0.64 (0.34, 0.89)	0.05 (0.01, 0.3)	0.05 (0.01, 0.3)	232.17 (27.23)
CCL2	*6.81 (3.23)	*4.91 (2.65)	1.11 (0.2, 2.04)	0.66 (0.09, 1.06)	0.1 (0.01, 0.21)	0.02 (0.01, 0.03)	302.7 (86.68)
CCL5	*7.6 (1.92)	*5.63 (1.83)	0.94 (0.34, 3.0)	0.64 (0.3, 1.08)	0.08 (0.0, 0.33)	0.01 (0.0, 0.08)	247.99 (59.81)
CCL17	*7.43 (1.73)	*5.14 (1.51)	1.43 (0.57, 3.32)	0.6 (0.45, 1.26)	0.08 (0.03, 0.27)	0.02 (0.01, 0.05)	253.16 (70.54)
CCL19	*6.45 (1.57)	*4.37 (1.51)	1.1 (0.75, 3.34)	0.64 (0.28, 1.0)	0.06 (0, 0.17)	0.01 (0.0, 0.04)	265.66 (54.50)
CCL21	*7.91 (2.12)	*5.74 (1.79)	1.1 (0.71, 3.32)	0.66 (0.37, 1.27)	0.08 (0.02, 0.3)	0.02 (0.01, 0.05)	253.50 (75.91)

Continued Table 5.3

Group	WBC	Neutro	Lympho	Mono	Eosino	Basophil	Platelet
CXCL19	6.81 (1.56)	4.72 (1.40)	1.13 (0.57, 3.0)	0.61 (0.28, 1.08)	0.09 (0.0, 0.33)	0.02 (0.0, 0.08)	244.61 (45.46)
CXCL10	6.36 (1.58)	4.46 (1.54)	1.09 (0.55, 1.90)	0.58 (0.28, 1.0)	0.11 (0.0, 0.27)	0.02 (0.0, 0.06)	240.50 (49.05)
P value	0.16	0.10	0.24	0.86	0.70	0.56	0.24

Tregs show specific migration in response to CCL17 and CCL5

Amongst the homeostatic chemokines, peripheral blood Tregs showed specific migration to CCL17, with a significant dose-response curve ($p=0.03$) (Fig 5.1 and 5.2). In terms of the inflammatory chemokines, only CCL5 caused specific Treg chemotaxis ($p=0.04$), as shown in Fig 5.3 and 5.4. I did not find any statistically significant chemotaxis of Tregs in response to CCL1, CCL2, CCL19, CCL21, CXCL9, or CXCL10 (Fig 5.5 and 5.6)

Fig 5.1 Box plot showing *in-vitro* Treg migration in response to the two different strengths of CCL17 (10 and 100 ng/ml) compared to a negative control (no chemokine) (on the x-axis). The results represent a mean of 18 assays for each of the chemokine solutions. The y-axis represents the Treg counts.

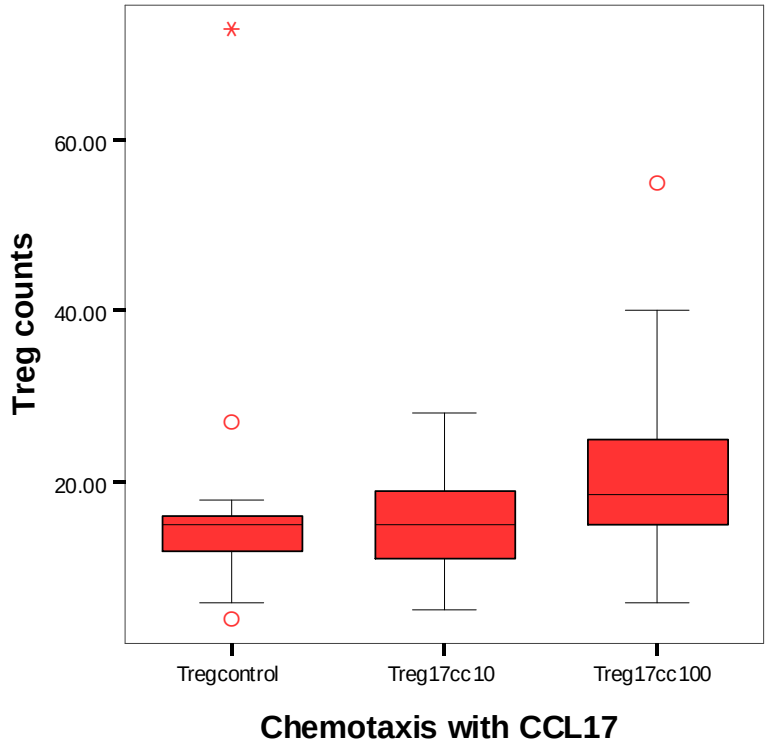


Fig 5.2 Graph showing dose-dependent *in-vitro* migration of Tregs in response to CCL17. The x-axis shows the two different concentrations of CCL17 (10 ng/ml and 100 ng/ml), and the control (no chemokine). The y-axis shows the logarithmic means of Tregs for each set of chemokine strength (mean of 18 assays for each group).

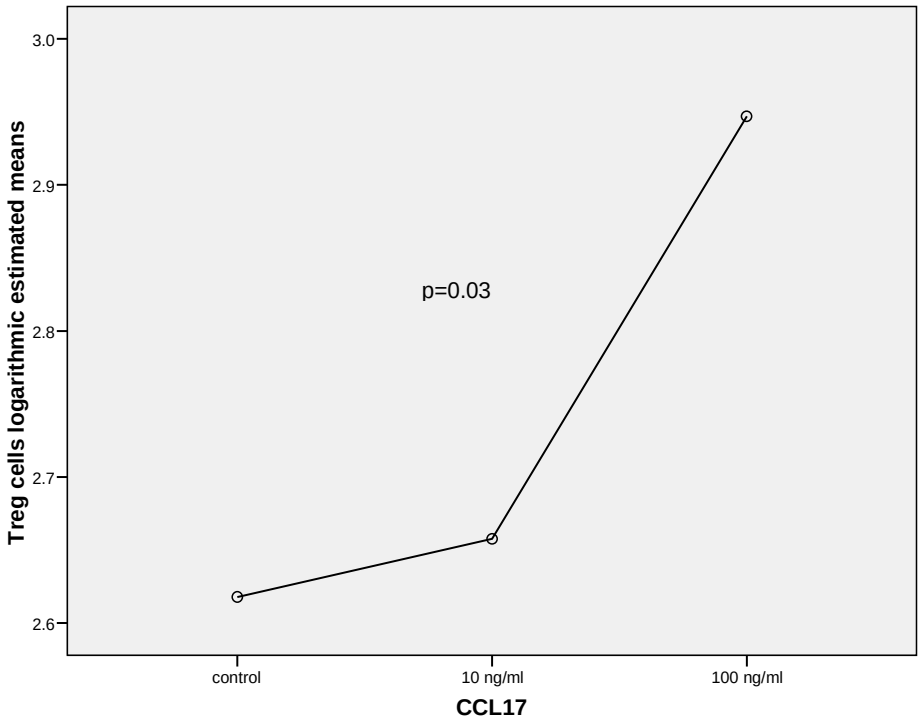


Fig 5.3 Box plot showing *in-vitro* Treg migration in response to the two different strengths of CCL5 (10 and 100 ng/ml) compared to a negative control (no chemokine) (on the x-axis). The results represent a mean of 18 assays for each of the chemokine solutions. The y-axis represents the Treg counts.

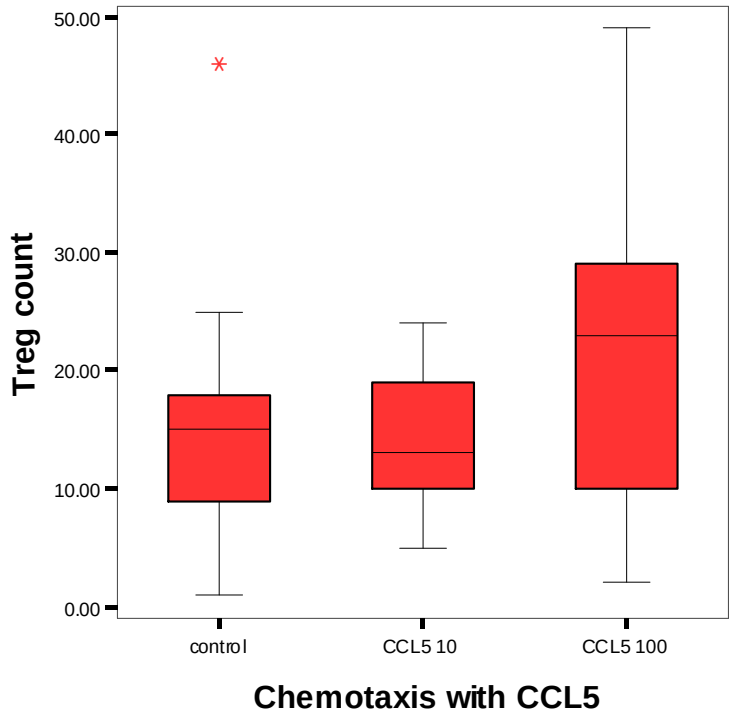


Fig 5.4 Graph for the result of *in-vitro* chemotaxis assay showing dose-dependent migration of Tregs in response to CCL5. The x-axis represents the two different concentrations of CCL5 (10 ng/ml and 100 ng/ml) compared against the control (no chemokine), while the y-axis shows the logarithmic means of Treg counts (mean of 18 assays for each group).

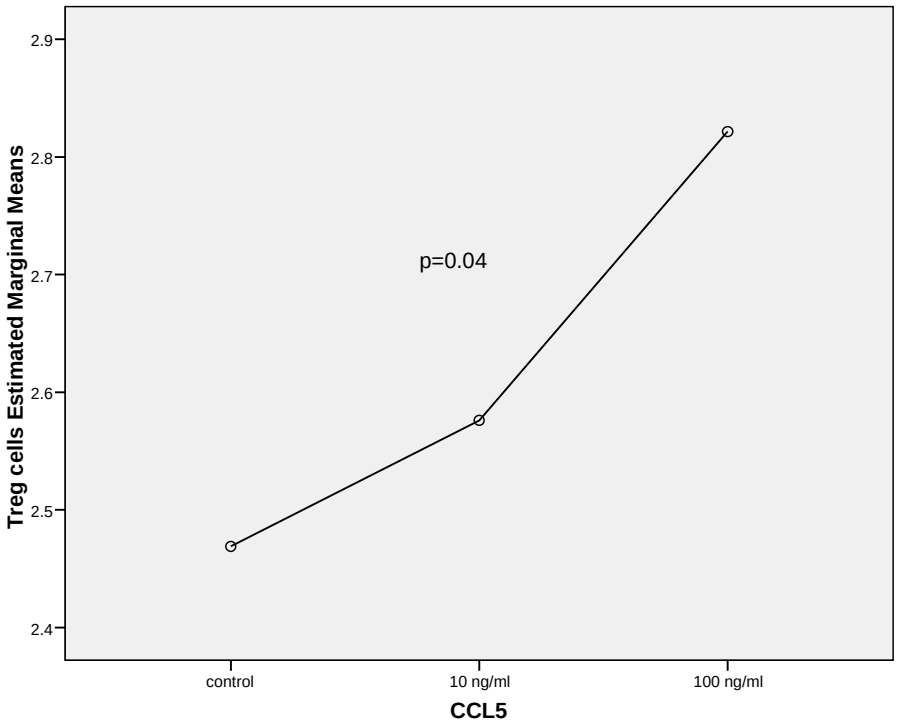


Fig 5.5 Box plots showing *in-vitro* Treg migration in response to CCL1 (5.5a), CCL2 (5.5b), CCL19 (5.5c) and CCL21 (5.5d). The x-axis shows the two different concentrations of the chemokines (10 ng/ml and 100 ng/ml), and the control (no chemokine). The y-axis represents the Treg counts. The results represent a mean of 10 assays for CCL1 and CCL2, and mean of 18 assays for CCL19 and CCL21 solutions.

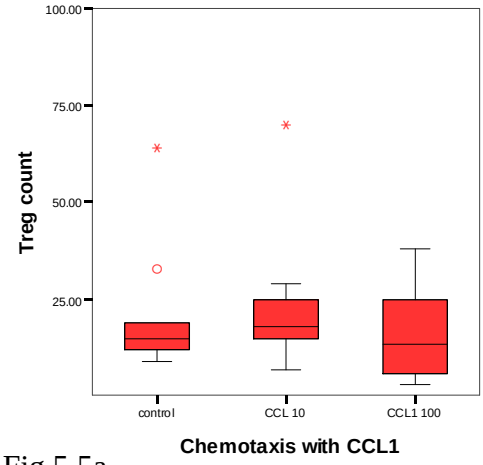


Fig 5.5a

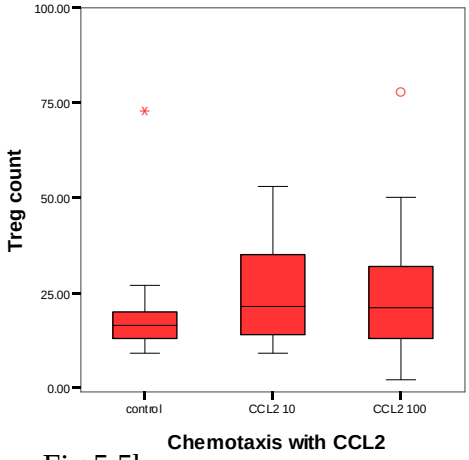


Fig 5.5b

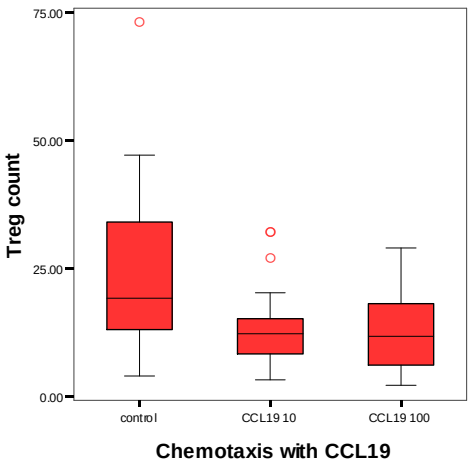


Fig 5.5c

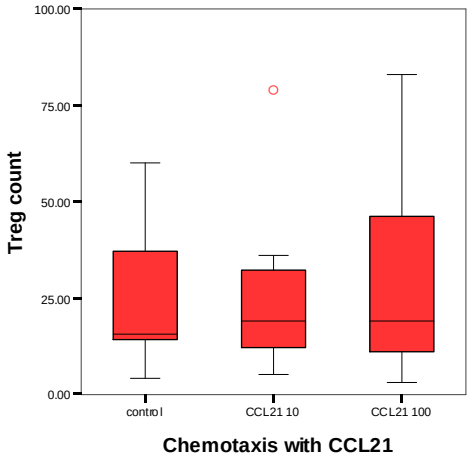


Fig 5.5d

Fig 5.6 Box plots showing *in-vitro* Treg migration in response to CXCL9 (5.6a) and CXCL10 (5.6 b). The x-axis shows the two different concentrations of the chemokines (10 ng/ml and 100 ng/ml), and the control (no chemokine). The y-axis represents the Treg counts. The results represent a mean of 18 assays for each chemokine solutions.

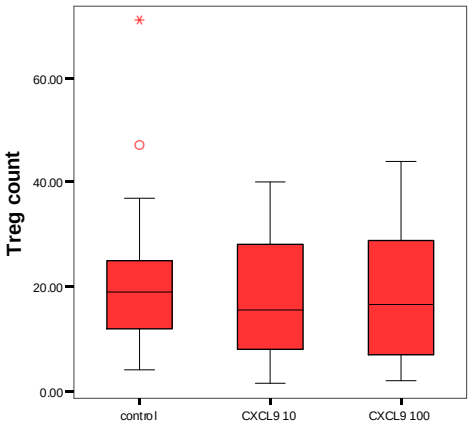


Fig 5.6a **Chemotaxis with CXCL9**

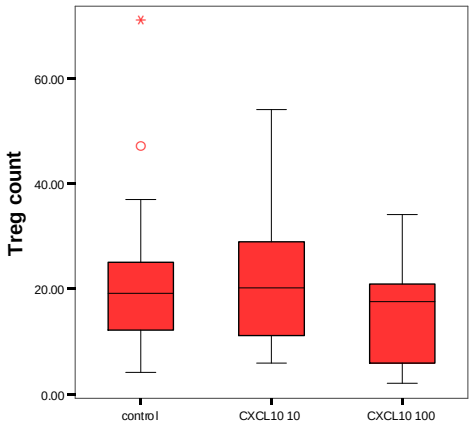


Fig 5.6b **Chemotaxis with CXCL10**

Tregs possess different chemotaxis properties compared to effector cells

I then looked at the chemotactic profile of $CD4^{+}CD25^{-}$ effector cells. These cells did not show the same pattern of chemotaxis as Tregs; instead the effector cells were found to be migrating more specifically in response to CCL19. There was no significant chemotaxis of these cells with any of the other chemokines. (Fig 5.7-5.9)

Fig 5.7 Graph showing dose-dependent *in-vitro* chemotaxis of $CD4^{+}CD25^{-}$ lymphocytes in response to CCL19. The x-axis represents the two different concentrations of CCL19 (10 ng/ml and 100 ng/ml) compared against the control (no chemokine), while the y-axis shows the $CD4^{+}CD25^{-}$ counts (mean of 18 assays for each group).

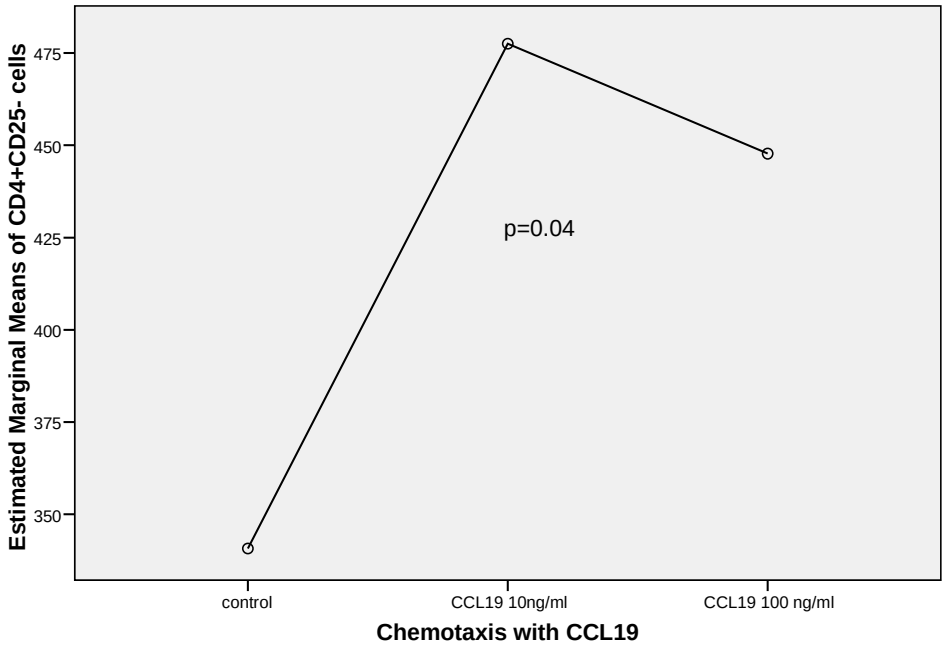


Fig 5.8: Box plots showing *in-vitro* chemotaxis of $CD4^{+}CD25^{-}$ lymphocytes in response to CCL1 (5.8a), CCL2 (5.8b), CCL5 (5.8c) and CCL17 (5.8d). The x-axis shows the two different concentrations of the chemokines (10 ng/ml and 100 ng/ml), and the control (no chemokine). The y-axis represents the $CD4^{+}CD25^{-}$ counts. The results represent a mean of 10 assays for CCL1 and CCL2, and mean of 18 assays for CCL19 and CCL21 solutions.

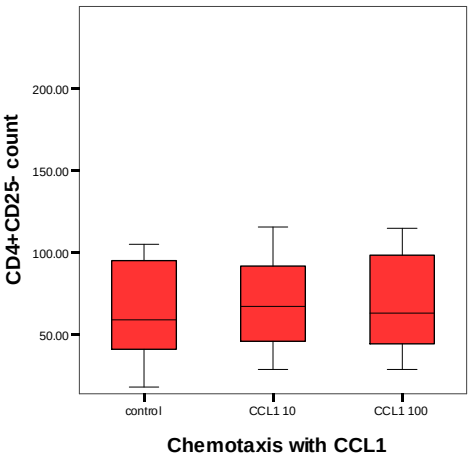


Fig 5.8a

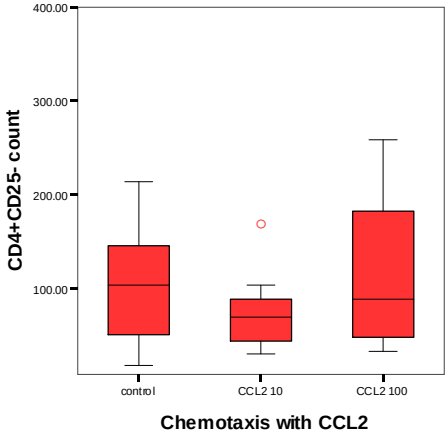


Fig 5.8b

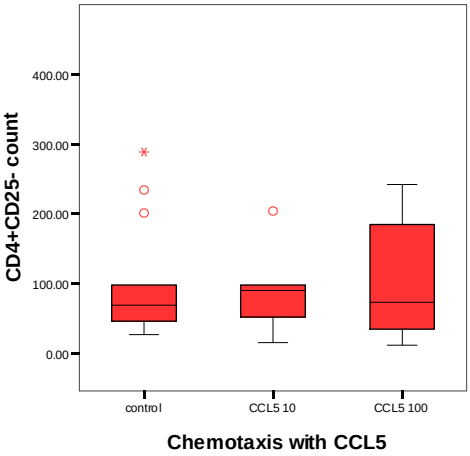


Fig 5.8c

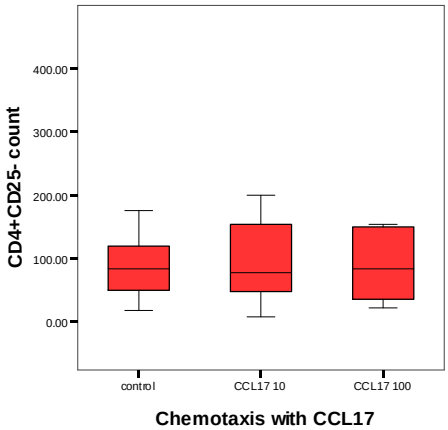


Fig 5.8d

Fig 5.9: Box plots showing *in-vitro* chemotaxis of $CD4^{+}CD25^{-}$ lymphocytes in response to CCL19 (5.9a), CCL21 (5.9b), CXCL9 (5.9c) and CXCL10 (5.9d). The x-axis shows the two different concentrations of the chemokines (10 ng/ml and 100 ng/ml), and the control (no chemokine). The y-axis represents the $CD4^{+}CD25^{-}$ counts. The results represent a mean of 18 assays for each solution.

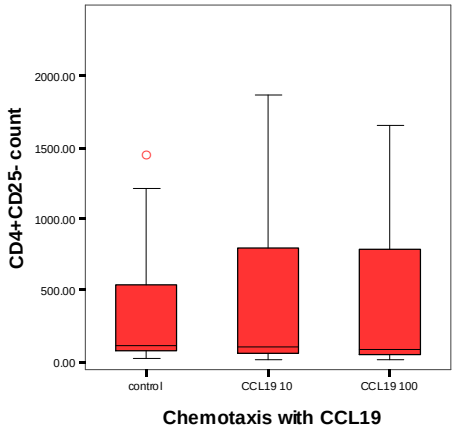


Fig 5.9a

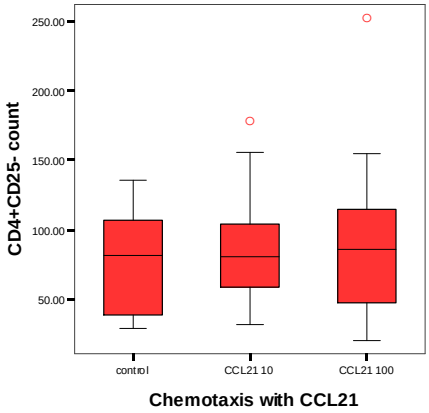


Fig 5.9b

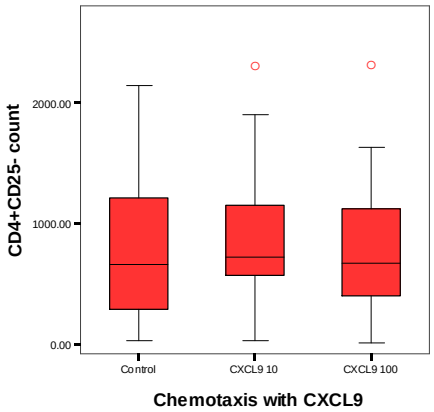


Fig 5.9c

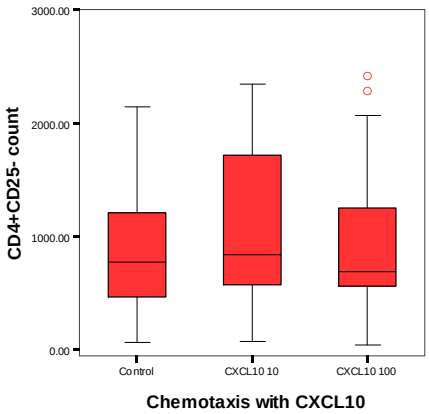


Fig 5.9d

Chemotactic index of Treg versus effector cells

Having seen a difference in the overall cell migration in response to different chemokines, I compared the difference in chemotactic response of Tregs versus the effector cells under same dose of chemokines. CCL19 showed significant specificity for $CD4^{+}CD25^{-}$ effector cells, while there was no statistically significant difference in the chemotactic indices for Tregs or the effector cells for other chemokines (Fig 5.10, 5.11)

Fig 5.10 Chemotactic indices for Tregs and $CD4^+CD25^-$ lymphocytes in response to CCL1 (5.10a), CCL2 (5.10b), CCL5 (5.10c) and CCL17 (5.10d). The blue lines represent the Tregs, while the green lines represent the $CD4^+CD25^-$ lymphocytes. The results represent mean of 10 experiments for CCL1 and CCL2, and mean of 18 experiments for CCL5 and CCL17.

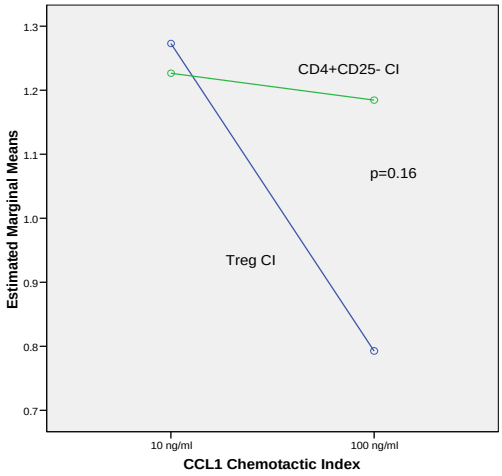


Fig 5.10a

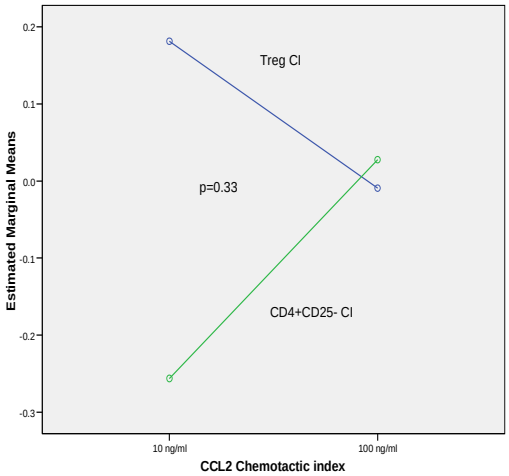


Fig 5.10b

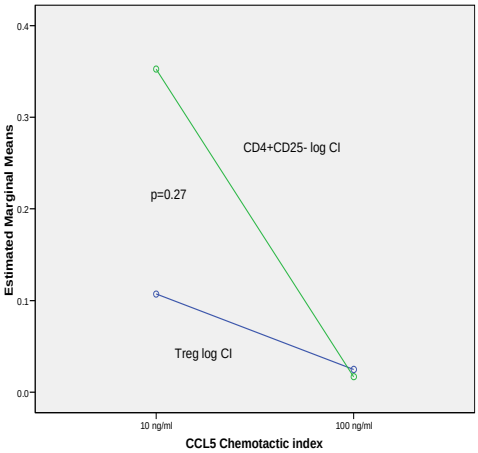


Fig 5.10c

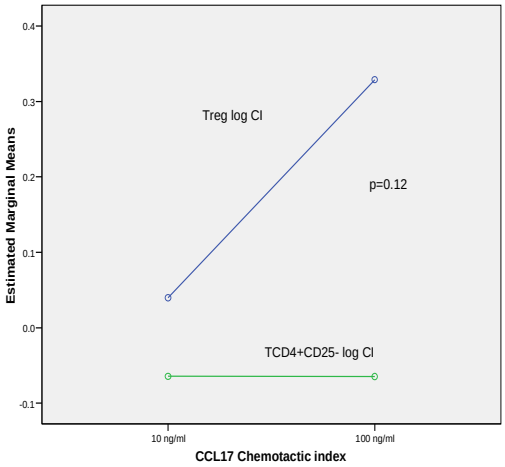


Fig 5.10d

Fig 5.11 Chemotactic indices for Tregs and $CD4^{+}CD25^{-}$ lymphocytes in response to CCL19 (5.11a), CCL21 (5.11b), CXCL9 (5.11c) and CXCL10 (5.11d). The blue lines represent the Tregs, while the green lines represent the $CD4^{+}CD25^{-}$ lymphocytes. The results represent mean of 18 experiments for each assay.

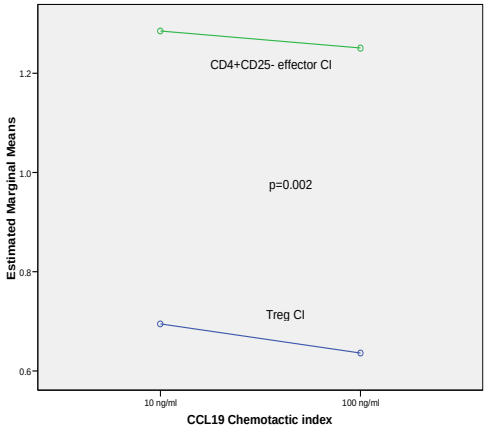


Fig 5.11a

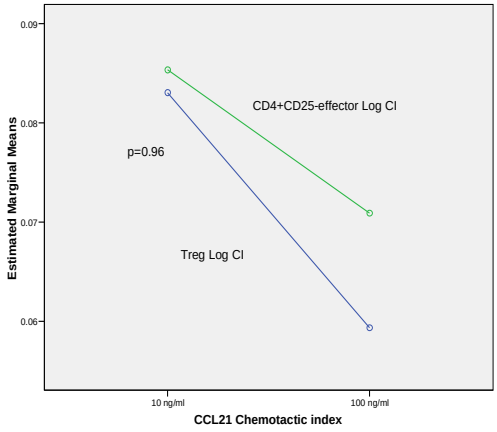


Fig 5.11b

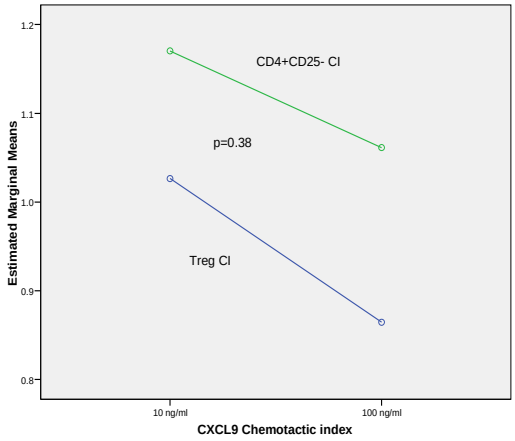


Fig 5.11c

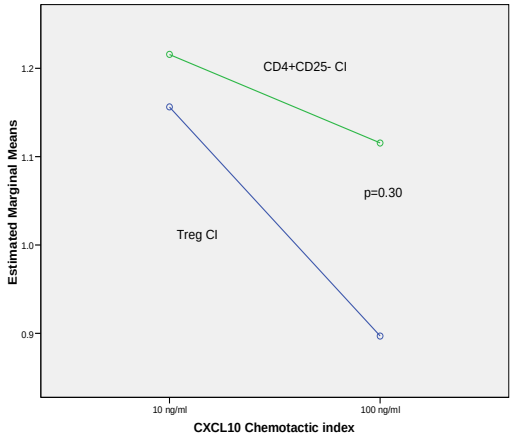


Fig 5.11d

Discussion

This study demonstrates for the first time that the peripheral blood $FOXP3^+$ Tregs in heart transplant recipients possesses specific chemotactic response profile different from the conventional effector lymphocytes. It provides an important insight of the potential differences in Tregs and effector cell mobilization within the body, with consequences for allogeneic immune response.

Like the effector T cells, Tregs require effective in-vivo mobilization between thymus, lymphoid tissue and the allograft in order to execute optimum regulatory function. However, Tregs possess slightly different chemokine receptor profile even at the precursor stage in thymus; CXCR4 is expressed by more $FoxP3^+$ cells than $FoxP3^-$ cell, while the reverse is true for CCR9 expression (*141*). Following transplantation, Tregs are stimulated via TCR activation, through either direct or indirect allo-recognition pathways. This antigen priming happens in the SLT and leads to an overhaul of chemokine receptors on Tregs; from CCR7 to effector-memory-like chemokine receptors including CCR2, CCR4, CCR6, CCR8 and CCR9 (*141*). Iellem *et al* showed that peripheral blood Tregs in healthy individuals exhibit greater selective expression of CCR8 ($p=0.0001$) and CCR4 ($p=0.03$) than $CD4^+CD25^-$ cells. Their *in-vitro* chemotaxis assays demonstrated a significantly greater ($p <0.01$) migration of Tregs compared to $CD25^-$ T cells in response to CCL17 and CCL22 (CCR4 ligands) and, CCL1 (CCR8 ligand), plus a synergistic effect of the suboptimal doses of CCL1 and CCL22 (*37*). My findings were different from this study, since I found significant *in-vitro* chemotaxis in response to CCL17 (CCR4 ligand), but not to CCL1.

CCL17 is secreted by several inflammatory cells, including antigen presenting dendritic cells, and monocytes (*241*). This chemokine probably plays an important role in recruiting Tregs to antigen presenting cells and the area of inflammation. The importance of CCL17 / CCR4 axis is particularly highlighted in several studies on cancer patients. Ishida *et al* showed that Hodgkin's Lymphoma cells produce CCL17 to attract Tregs via CCR4, so as to evade host immune defence by suppressing anti-tumour reactive lymphocytes (*242*). The clinical relevance was provided in the study by Curiel *et al*, who showed that specific migration of $CCR4^{+}$ Tregs to the tumour tissue provides immune privilege and is associated with poor survival (*71*). Another study on patients with neoplastic meningitis demonstrates similar "tumour-protective" specific recruitment of Tregs (*243*).

In transplant setting, there is evidence to suggest that the specific Treg recruitment via CCR4 and its ligands is essential for tolerance. In a murine model of induced allo-tolerance via CD154mAb and donor specific transfusion (DST), tolerance was associated with an infiltration of Tregs in the allograft. The same study also showed that CCR4-/- mice rejected the allograft associated with reduced infiltration of Tregs, however, the number and function of peripheral Tregs were normal. Hence, this study suggested that CCR4 ligands do not impact on Tregs development, but play a key role in Tregs migration to the allograft (*143*). In another fully mismatch murine cardiac transplant model with induced tolerance, Ochando *et al* showed that alloantigen-bearing plasmacytoid DC migrate to lymph nodes and affected allospecific Treg development via the CCR4 / CCL17 pathway. The same protocol in $CCR4^{-/-}$ mice prevented failure of Tregs development, leading to rejection (*144*).

CCL5 is the ligand for the chemokine receptor CCR5 that has been associated with pro-inflammatory cellular infiltration in acute and chronic allograft rejection (*102, 164*). A study on mice showed that approximately 20% of Tregs in murine SLT constitutively express CCR5 and immune activation leads to enhanced expression of both CCR5 and Foxp3. The same study revealed that CCR5 related pathway caused preferential accumulation of Tregs in the gravid uterus for tolerance induction (*168*). Kang *et al* demonstrated in a murine model of chronic inflamed intestine that CCR5 / CCL5 pathway was preferentially used by $Foxp3^+$ Tregs for homing to the inflamed tissues. Interestingly, activated $CD8^+$ T cells were the major source of the secreted CCL5, suggesting a possible role of CCL5 in balancing the effector and regulatory response. It was demonstrated that *in vitro* activation caused significantly more $Foxp3^+$ cells than $Foxp3^-$ cells to express CCR5, and their migration was blocked by TAK-779, a CCR5 antagonist (*169*). Another study in murine model of acute graft-versus-host disease reported that Tregs used CCR5-related pathways for homing to the target organs, and this migration was essential for suppression of the effector response. The investigators did not mention the chemokines involved in this migration (*171*). My results show that in clinically stable heart transplant recipients, CCL5 can cause significant in-vitro migration of $FOP3^+$ Tregs. This suggests that CCL5 / CCR5 axis may play an important role in allograft immune homeostasis.

CXCR3 ligands CXCL9 and CXCL10 are often associated with acute allograft rejection, but it is uncertain if they play a role in Treg trafficking (*89, 90*). Under homeostatic conditions Tregs express low levels of CXCR3, and show minimal chemotactic response to the three inflammatory CXC chemokines

(*37*). My study demonstrates a similar pattern, with a non-significant migration of Tregs in response to CXCL9 and CXCL10 under stable conditions. The expression of CXCR3 probably increases during the stress response of inflammation or rejection following transplantation. An evidence was provided by Eksteen *et al* demonstrated the presence of $CXCR3^{high}$ Tregs around inflamed bile ducts in chronically inflamed human liver (*72*). In another study involving murine model of experimental autoimmune encephalomyelitis, CXCR3 expression was associated with infiltration of Tregs and containment of the tissue damage (*149*).

Recognition of the specific chemotactic profile of effector cells and Tregs may help in developing novel strategies for tolerance induction. For example, I found that in contrast to Tregs, the effector T cells showed specific migration in response to CCL19. This is a homeostatic chemokine, secreted at the high endothelial venules and fibroblastic reticular cells within T cell zones of the SLT and is responsible for T cell homing to the SLT (*124*). It is therefore important for the induction of alloresponse, and one can speculate that blocking this chemokine may have a role in preventing rejection without affecting Tregs. In fact, a study using the experimental models of kidney and heart transplantation showed by blocking CCR7 (CCL19 ligand) that allospecific effector T cell proliferation was reduced and the allograft survival was significantly prolonged from 9 days to 20 weeks (*131*). Such work paves way for exciting new avenues for future studies, which may involve either blocking or alteration of chemokine expression via immunomodulatory drugs and genetic modifications.

In conclusion, my limited observational study shows a difference in the dynamics of chemotaxis between the Tregs and effector cells in stable heart transplant recipients. Further work may demonstrate relevance during states of acute and chronic rejection.

Chapter 6 Role of CCL17 in Treg migration following heart transplantation

Abstract

Background

FOXP3$^+$ T regulatory cells (Tregs) possess suppressive properties against alloreactive effector cells and are considered pivotal in tolerance induction following transplantation. Tregs migrate to the allograft under influence of certain chemotactic cytokines. My previous data on *in-vitro* chemotaxis suggests that peripheral blood Tregs specifically migrate in response to the chemokine CCL17. However, it is uncertain if CCL17 has a role for in-vivo migration of Tregs to the allograft.

Methods

In this observational study, double immunofluorescence labelling was performed to identify CD4$^+$FOXP3$^+$ Tregs in 12 endomyocardial biopsies from 4 adult heart transplant recipients. The rejection grade was identified by expert histopathologists according to the International Society of Heart and Lung Transplantation (ISHLT) criteria. Further immunostaining was performed on sequential cuts from the same samples to detect CCL17 expression that was correlated with the Treg and effector cell infiltration. Demographic and immunosuppression data was collected from patient records. Data was analyzed using SPSS v15.

Results

Acute rejection was present in 4 out of 12 biopsies (33.3%). CD4$^+$ FOXP3$^-$ effector cells were present in both rejecting and non-rejecting samples; however, CD4$^+$FOXP3$^+$Tregs were identified in the rejecting samples only.

Although there was no statistically significant rise in CCL17 expression during the rejecting state, the CCL17 expression pattern was significantly correlated with both $CD4^+$ $FOXP3^-$ effector ($p=0.02$) and $CD4^+FOXP3^+$ Treg counts ($p<0.01$) in the rejecting samples.

Conclusion

This study demonstrated that CCL17 is associated with $CD4^+FOXP3^-$ cells and Treg infiltration during acute rejection following heart transplantation. Tregs increase in number in the allograft during acute rejection, and that CCL17 secretion may be responsible for Treg migration to the allograft. This finding may have important implications, since Tregs play pivotal roles in the alloresponse and their selective migration can be utilized as a tool for tolerance induction following transplantation.

Background

Transplantation leads to an activation of the allospecific immune response causing infiltration of mononuclear cells into the allograft, which is the hallmark of acute rejection (*186*). At the same time, however, there occurs an activation of a special subset of T cells, called T regulatory cells (Tregs) that are capable of suppressing the allo-specific immune response leading to a state of immune privilege known as peripheral tolerance (*30, 34*). Initially described as important for maintaining tolerance against self antigens (*22*), the role of Tregs in transplant-related tolerance is now widely acknowledged (*33, 244, 245*). Tregs have been identified as a small subset (approximately 5-10%) of the peripheral blood CD4+ T cells, expressing high levels of cell surface CD25 and the transcription factor FOXP3 (*35, 65, 246*). It appears that the level of FOXP3 expression is directly related to the regulatory potential of Tregs (*247*). Its deletion or mutations lead to a variety of autoimmune diseases in both animals and humans (*248*), while retroviral transfer to naïve T cells renders them the regulatory profile (*249*).

Naturally occurring Tregs are generated in thymus. Subsequently they migrate to the secondary lymphoid tissue (SLT) where they get activated via TCR stimulation (*250*). Further migration of allo-specific Tregs to the site of antigenic challenge is the key step towards suppression of effector response (*251*). This is due to the fact that Tregs function via local secretion of cytokines or cell-cell contact dependent mechanism (*25, 239*).

Chemokines are small chemotactic proteins secreted by various cells to facilitate leucocyte migration towards specific sites of interest (*81*). The chemokines bind to seven-pass, trans-membrane-spanning serpentine, Gi/Go protein-

coupled, Bordetella pertussis toxin-sensitive receptors. Approximately 50 chemokines and 20 chemokine receptors have been identified in humans and mice (*82*). Evidence suggests that different subsets of lymphocytes including Tregs show unique chemotactic response profiles during homeostasis, inflammation and following transplantation (*73, 252, 253*). In this context, CCL17 (also known as Thymus and Activation Regulated Chemokine or TARC) appear to cause Treg specific migration in healthy individuals and certain cancers (*37, 242, 254*). There is evidence to suggest that Tregs specifically migrate to the allograft in human heart transplant recipients (*255*). However, the specific chemokine signal responsible for this Treg migration to the allograft is yet to be established.

My previous experiment on *in-vitro* chemotaxis in stable heart transplant recipients shows that CCL17 causes specific migration of peripheral blood Tregs. Hence, I postulated that CCL17 may be responsible for *in-vivo* migration of Tregs following heart transplantation.

Methods

This was a retrospective, observational, non-randomized study conducted on the adult human heart transplant recipients. The study was designed to correlate the pattern of CCL17 expression with Treg infiltration in three serial endomyocardial biopsies from each patient, including a biopsy with no rejection (Biopsy 1), followed by a biopsy showing at least moderate rejection (Biopsy 2), followed by another biopsy with no rejection (Biopsy 3). To fulfil this criterion, I could only identify 4 heart transplant recipients who had biopsies from April 2008 till March 2009, and showed at least moderate degree of rejection in one biopsy. Hence, the study could not be sufficiently powered to detect minor effect size. The 12 formalin-fixed, paraffin embedded endomyocardial biopsy

specimens were then obtained following informed consent. The biopsies were performed by the clinical team responsible for the patients according to the local guidelines and were graded for rejection according to the revised ISHLT criteria (*180*). As mentioned earlier, 4 samples had evidence of acute cellular rejection on routine H&E staining, while 8 samples had no rejection. Clinical and demographic data including the use of immunosuppressive agents (Prednisolone (Pred), Cyclosporine (Cyclo), Tacrolimus (FK), Mycophenolate Mofetil (MMF) and Azathioprine (Aza) administration) and haematological white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets) were collected from patient records. The study was approved by the local ethics committee.

Immunofluorescence labelling for Tregs

All the samples were serially sectioned to 4μm thickness using the standard microtome. The sections were deparaffinised and rehydrated in graded alcohol before heat-induced antigen retrieval in a pressure cooker using Citrate buffer (pH 6.0) as previously described. Following incubation with 10% normal donkey serum to block non-specific staining, the sections were labelled with monoclonal mouse anti-human CD4 (1:50 dilution, clone BC/1F6, Abcam®, UK) overnight at 4ºC followed by Alexa Fluor® 555 donkey anti-mouse IgG (H+L) (Invitrogen®, UK) for 1 hour. This was followed by second staining sequence, which again started with 10% donkey serum for 1 hour, then rat anti-human FOXP3 (1:50 dilution, clone PCH101, eBioscience, UK) overnight at 4ºC followed by Alexa Fluor® 488 donkey anti-rat IgG (H+L) (Invitrogen®, UK) for 1 hour. Due to high background auto fluorescence, sections were incubated with 0.5% Sudan black for 10 minutes before nuclear counterstaining

with DAPI (4', 6-diamidino-2-phenylindole dilactate; 1:10,000, Biotium, CA). The sections were then mounted with Prolong Gold® anti-fade reagent (Molecular Probes®, OR). The entire sections were evaluated using epifluorescent microscope (Olympus® BX51, Japan) and images acquired using $CoolSNAP_{HQ}$ Monochrome camera (Roper Scientific®, AZ) with MetaVue™ Imaging System (Molecular Devices®, PA) (Fig 6.1). $CD4^+FOXP3^-$ and $CD4^+FOXP3^+$ cells (Tregs) were counted in at least 5 non-overlapping high power fields (x60). Tonsil tissue was used as the positive control, while primary antibodies were omitted for a negative control.

Immunohistochemistry for CCL17

Sequential 4μm cuts from the 12 FFPE endomyocardial samples selected earlier were stained with polyclonal rabbit anti-human CCL17 (1:800, AHP1919, AbD Serotec®, Oxford, UK). I used ImmPRESS™ peroxidase detection system (Vector® labs, CA) to detect CCL17 in the samples. In short, after deparaffinization in xylene and rehydration in graded alcohol, the sections were subjected to heat-induced antigen retrieval using citrate buffer (pH 6.0). The endogenous peroxidise activity was quenched by incubation in 0.3% hydrogen peroxide solution in alcohol. Following blockade with 2.5% normal horse serum the sections were stained with the anti-CCL17 antibody for 30 minutes each. Further 30 minutes incubation was performed with ImPRESS™ reagent before application of ImmPACT™ DAB (diaminobenzidine) (Vector® labs, CA) for 5 minutes. Nuclear counter stain was performed with Haematoxylin and mounted using DPX resin (Fig 6.2). Once again, tonsil tissue was used as the positive control. The primary antibody was excluded for the

negative control. The degree of staining was assessed and quantified on a scale of 0-3 by two expert histopathologists at our Pathology department.

Statistical analysis

The data was tabulated using excel and analyzed with SPSS v15. Paired analysis was performed to compare the demographic, clinical and staining data for the three biopsy time points. Data with normal distribution was assessed with paired sample t test or the one way ANOVA, while non-parametric data were assessed by Mann-Whitney U test or the Friedman one-way repeated measures analysis of variance by ranks. Analysis of correlations between $CD4^{+}FOXP3^{-}$ cells, $CD4^{+}FOXP3^{+}$ cells, and CCL17 expression were performed by Spearman's rank correlation test. A two-sided p value <0.05 conferred statistical significance.

Results

All patients were males, with a mean age of 40.4 (±14.4) years. The median duration of the three serial biopsies since transplant were 217.5 (68.2, 633.0), 313.0 (118.5, 993.5), 349.5 (140.0, 1085.5) days respectively. There was no significant difference between rejecting versus non-rejecting states in terms of immunosuppression, immunomodulatory drugs (including statins), or renal dysfunction (table 6.1). At the time of the first and second biopsies, all patients were taking Cyclo only, however 2 patients were switched to FK by the time of their third biopsy. In terms of MMF and Aza, only one patient was on Aza when he had his first biopsy, while the others were taking MMF. Subsequently, all patients were receiving MMF. The median prednisolone doses were 10.0 mg (10.0, 21.25), 8.7 mg (7.5, 21.25), and 11.0 mg (8.12, 21.75) respectively at the time of the three serial biopsies (p=0.36). Table 6.1 summarizes the differences

in estimated glomerular filtration rate (eGFR) and peripheral blood counts at the times of serial biopsies.

Table 6.1 Renal function and peripheral blood cell counts at serial biopsy time points.

All data in mean (SD). Biopsy1, 2 and 3 correspond to the three serial endomyocaridal biopsies performed in four subjects. (eGFR = estimated glomerular filtration rate, WBC = White blood cells)

	Biopsy #1	Biopsy #2	Biopsy #3	P value
eGFR (ml/min)	67.0 (24.89)	52.0 (24.75)	47.25 (26.6)	0.21
WBC ($x10^9$/l)	10.1 (2.39)	8.37 (1.93)	11.07 (4.18)	0.52
Neutrophils ($x10^9$/l)	8.18 (1.92)	7.28 (1.66)	8.34 (3.19)	0.26
Lymphocytes ($x10^9$/l)	1.14 (0.8)	1.05 (0.8)	1.79 (1.1)	0.18
Monocytes ($x10^9$/l)	0.67 (0.37)	0.5 (0.18)	0.8 (0.23)	0.54
Eosinophils ($x10^9$/l)	0.08 (0.1)	0.12 (0.1)	0.09 (0.3)	0.89
Basophils ($x10^9$/l)	0.015 (0.01)	0.03 (0.03)	0.025 (0.02)	0.51
Platelets ($x10^9$/l)	265.5 (79.38)	235.75 (71.92)	243.75 (108.11)	0.26

Fig 6.1 Double immunofluorescence labelling of Tregs with CD4 and FOXP3 antibodies in a representative endomyocardial biopsy.

Blue staining denotes nuclear counter-stain with DAPI (4', 6-diamidino-2-phenylindole, dilactate). CD4 stain was seen with red (Cy3) filter staining the membrane (red arrows), while the white arrow points to intranuclear FOXP3 staining (green with FITC filter).

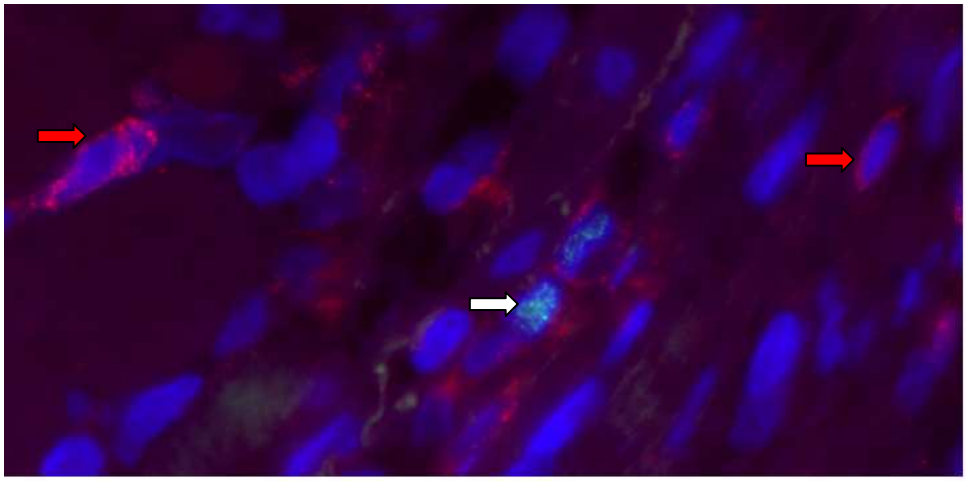
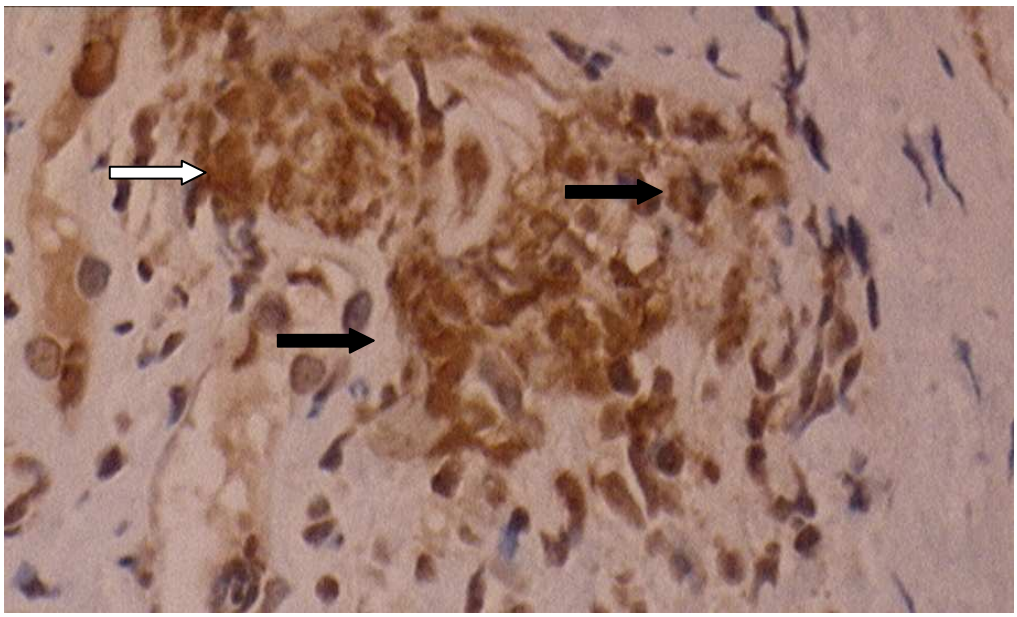


Fig 6.2 Immunoperoxidase staining of a representative section of the formalin-fixed, paraffin embedded endomyocaridal biopsy showing CCL17 staining within the lymphocytic infiltrate.

White arrow points to CCL17 staining, while black arrow points to a lymphocyte. Myocardial nuclei are stained blue with haematoxylin (x100 magnification).



Tregs versus CD4$^+$FOXP3$^-$ effector cells in the endomyocardial biopsy

Out of a total of twelve biopsy samples, CD4$^+$FOXP3$^-$ cell infiltration was identified in 2 rejecting (median count 6.0 (0, 27.75) and 2 non-rejecting (median count 2 (0, 24.25) samples. One patient had CD4$^+$FOXP3$^-$ cell infiltration in both non-rejecting (Biopsy 1) and rejecting (Biopsy 2) samples. The other two samples belonged to one rejecting and one non-rejecting patient. Interestingly, no CD4$^+$ cells were observed in the third series of biopsies (Biopsy 3).

Despite the presence of CD4$^+$FOXP3$^-$ cells in the non-rejecting samples, FOXP3$^+$ Tregs were identified in two rejecting samples only, comprising 6.45% (0, 21.97%) of the CD4$^+$ cell counts. I found no significant association between CD4$^+$FOXP3$^-$ counts or Tregs and the demographic data.

Changes in CCL17 expression during acute rejection

In the next step, the sequential staining pattern of CCL17 was compared during the acute rejection and non-rejecting states. While there was an enhanced staining of CCL17 in samples with acute rejection, I did not find a statistically significant difference compared to the non-rejecting samples (Fig 6.3).

Association of CD4$^+$ FOXP3$^-$ cells and Tregs with CCL17

In the samples with acute rejection (Biopsy 2), both CD4$^+$FOXP3$^-$ and Treg counts were significantly associated with the degree of CCL17 staining ($p=0.02$ for CD4$^+$FOXP3$^-$ and $p<0.01$ for Tregs) (Fig 6.4, and 6.5).

Due to the small numbers of Tregs, I wanted to check if Treg: CD4$^+$ ratio was associated with CCL17 expression. However, I found no significant association between Treg: CD4$^+$ ratio and CCL17 ($p=0.22$). There was also no

association between $CD4^+$ counts and CCL17 expression in the non-rejecting samples.

Fig 6.3 Graph showing mean CCL17 expression in serial endomyocardial biopsies in non-rejecting, rejecting, and then non-rejecting states. Each point refers to a mean of 4 samples.

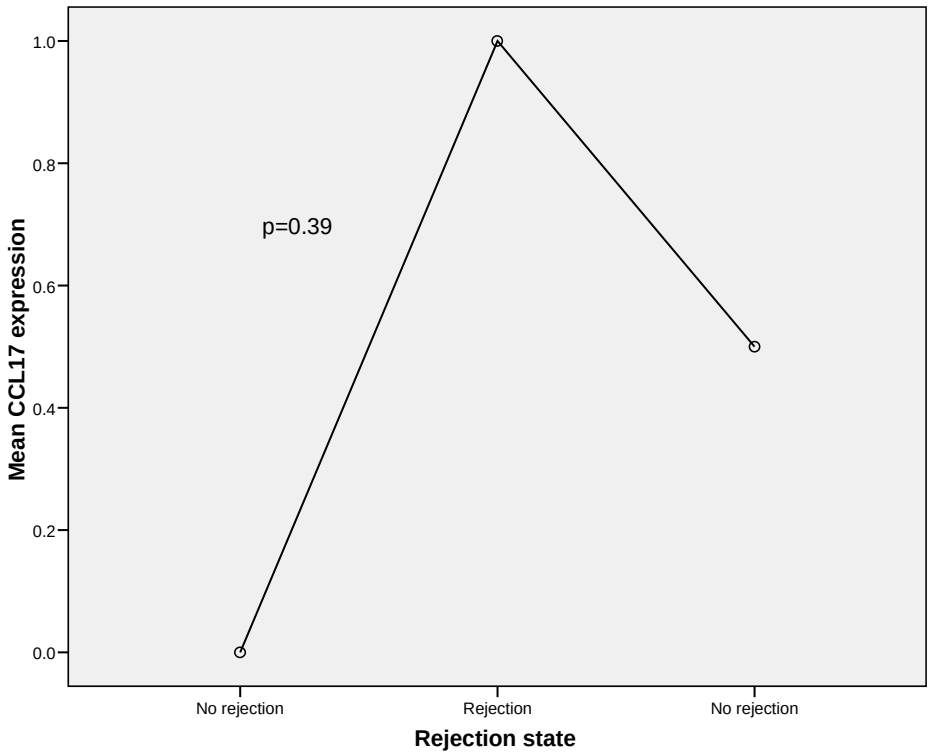


Fig 6.4 Graph showing $CD4^{+}FOXP3^{-}$ lymphocyte counts (y axis) versus CCL17 expression (x axis) in the rejecting endomyocardial biopsy tissues (n=4). P value denotes the two-tailed significance value from Spearman's correlation (r=Spearman's correlation coefficient).

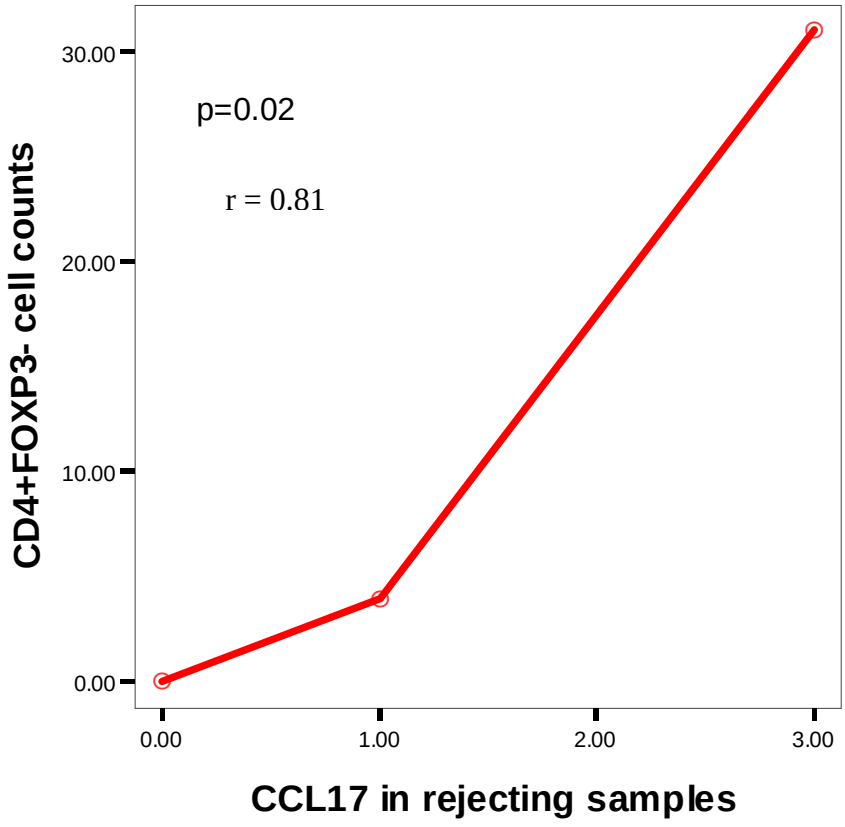
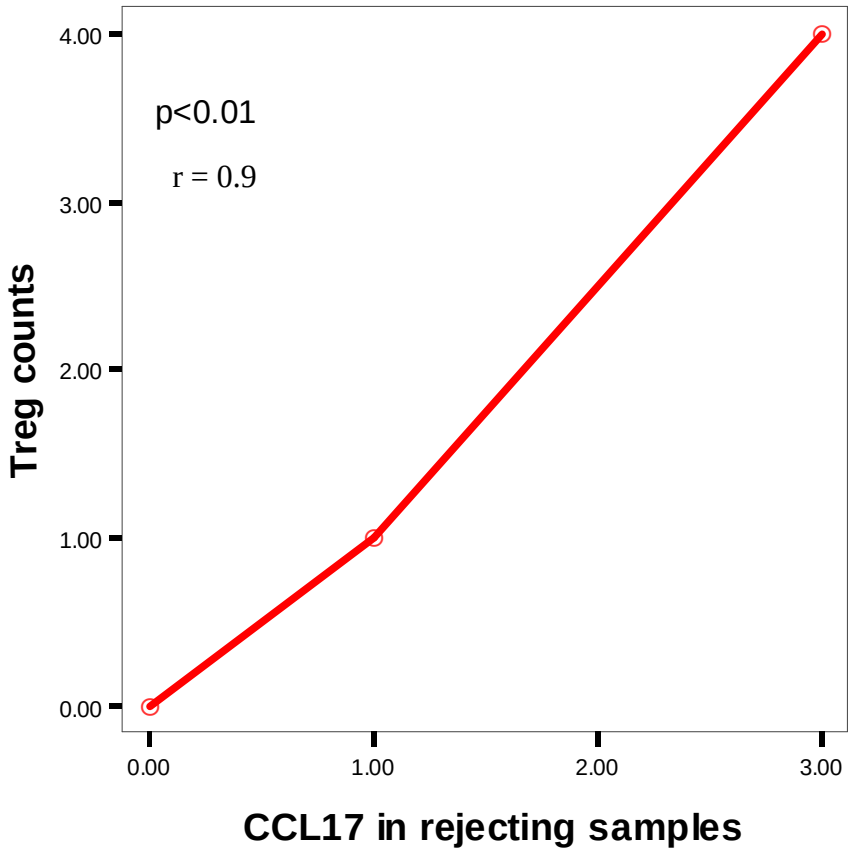


Fig 6.5 Graph showing Treg counts (y axis) versus CCL17 expression (x axis) in the rejecting endomyocardial biopsy tissues (n=4). P value denotes the two-tailed significance value from Spearman's correlation (r= Spearman's correlation coefficient). .



Discussion

To my knowledge, this is the first study to assess the correlation of intragraft CCL17 with *in-vivo* allograft migration of $FOXP3^+$ Tregs following clinical heart transplantation. Herein I demonstrated a significant association between CCL17 staining and infiltration of $CD4^+FOXP3^-$ and Treg lymphocytes in the allograft during acute rejection.

Within the past couple of decades Tregs have increasingly been realized to have an important role in peripheral tolerance against self and allo-antigen (*6, 22, 33*). Consistent with these findings, experimental studies show that therapeutic use of ex-vivo expanded Tregs prevents allograft rejection and perhaps induces state of tolerance (*66*). In humans, heart transplantation has seen improved outcome in recent years owing to provision of better immunosuppression, which unfortunately leads to several other side effects (*184*). At the same time, the incidence of chronic rejection remains high (*256*). Hence, induction of peripheral tolerance is highly desirable, and Tregs can play a major role.

It has been shown that Tregs migration from thymus to the SLT and then to the site of antigen challenge is vital to their function (*73*). This compartmentalization is largely governed by the chemokines, which are small secreted cytokines, acting via their specific transmembrane receptors. It has been shown that Tregs possess specific chemotactic potential that is different from the effector T cells. Iellem *et al* reported in healthy individuals that Tregs show specific expression of the chemokine receptors CCR4 and respond to its ligands CCL17 and CCL22 in the supernatant of maturing dendritic cell culture (*37*). However, antigen presentation and activation of TCR in all T cells leads to up-

regulation of CCR4 and CCR8, with the ability to respond to corresponding chemokines CCL17 and CCL1 (*145*). Hence, secretion of these chemokines by various inflammatory cells such as activated dendritic cells within the allograft may lead to a competitive migration of both the effector cells and Tregs. This may explain my results of a significant association between CCL17 and $CD4^+$ lymphocytic infiltration in the rejecting allograft.

My limited data shows the presence of Tregs in the rejecting endomyocardial biopsy tissue only, while CD4+ cells were present in non-rejecting samples as well. Despite the small number of cases in my series, the finding is consistent with a previous study by Dijke *et al* showing an association between acute rejection and FOXP3 mRNA expression within the endomyocardial biopsies from heart transplant recipients (*257*). Similar work in renal (*258*) and liver allograft recipients (*259*) also showed an increase in $FOXP3^+$ Tregs in the allograft during acute cellular rejection. Taken together, these findings suggest that Tregs form part of the cellular infiltrate during acute rejection, probably in an attempt to suppress the effector response. However, the study by Veronese *et al* with a large cohort of 80 human renal transplant recipients demonstrated no beneficial effect in survival with Treg infiltrate during acute cellular rejection (*258*). Hence, the significance of Treg infiltration and its association with the outcome following acute rejection, chronic rejection or long term survival in transplant recipients is yet to be established.

Despite this uncertainty, an understanding of and attempts to manipulate the physiological differences between effector and regulatory T cells present exciting new opportunities for tolerance induction following transplantation. In this regards, knowing exact chemokine pathways that lead to Treg migration to

the allograft bears significant importance. This is an established fact that Tregs sow specific migration towards the allograft. Schmidt-Lucke *et al* recently documented via trans-cardiac gradient specific uptake of Tregs in the cardiac allografts (*255*). They were the first to demonstrate the actual presence of $FOXP3^+$ Tregs in the human cardiac allografts using double immunofluorescence labelling. However, they did not investigate the chemotactic pathway responsible for Treg migration, and also did not compare the results between rejecting versus non-rejecting biopsies. My data is unique in the sense that we have provided a serial, objective assessment of $CD4^+FOXP3^-$ counts, Treg counts, and CCL17 expression in a cohort of heart transplant recipients in both rejecting and non-rejecting states.

Studies on murine knock-out models has paved way to targeting specific cytokines and chemokines in various inflammatory, neoplastic and transplant condition (*131, 260, 261*). Most of the transplant experiments involved blocking one or two chemokine pathways, hence leading to reduced effector cell infiltrate and prolonging allograft survival. However, it has not yet been possible to augment a specific chemokine expression that could lead to isolated effects on desirable cell population within the body. This is due to the fact that chemokine system is quite redundant, with several overlapping pathways operating together. The aim of my study was to determine if CCL17 could provide unique chemotactic response to the Tregs in isolation. However, I found that CCL17 expression is also correlated with the infiltration of $CD4^+FOXP3^-$cells. In addition, the ratio of Treg: $CD4+FOXP3^-$ cells did not show any correlations with CCL17. A corroborative example comes from the study on murine models of colonic carcinoma where intra-tumoral injections of mutant adenoviral

vectors encoding for CCL17 caused significant effector T cells and macrophage infiltration, leading to tumour regression (*262*). Hence, any immune therapy using CCL17 to guide Tregs into the allograft may invariably lead to effector cell infiltration, and provoke a worse outcome.

One of the major limitations of my study was that it was hugely underpowered to detect substantial changes in chemokines or cellular infiltrations. The obvious reasons were ability to obtain the required serial samples to run the histological tests in this novel study. Another limiting factor was that some sections from the FFPE samples might have missed the level where lymphocytic infiltration was present. This might have had an effect on staining both the $CD4^{+}FOXP3^{-}$ cells and the Tregs, and is the likely explanation of the inability to identify the cells in two rejecting samples.

In conclusion, my limited observational study shows that FOXP3+ Tregs infiltrate the myocardium during acute rejection in human heart transplant recipients. Treg and $CD4^{+}FOP3^{-}$ infiltration during acute rejection is significantly associated with CCL17 expression in the allograft. Further studies will determine long term effects of CCL17 expression on allograft vasculopathy and long term survival.

Chapter 7 Conclusion

This is the first study to explore the chemotactic response of Tregs in human heart transplant recipients. The results of this study show that acute rejection following heart transplantation leads to an up-regulation of peripheral blood Th1 chemokine levels. I have further shown in stable heart transplant recipients that peripheral blood $CD4^{+}CD25^{bright}FOXP3^{+}$ lymphocytes show specific and dose dependent migration in response to Th2 chemokine CCL17 and Th1 chemokine CCL5, in contrast to the effector T cells which migrate in response to CCL19. Through double immunofluorescence labelling and immunohistochemistry I have also shown that in-vivo migration of Tregs to the allograft is associated with CCL17 secretion.

Tregs, first described by Sakaguchi *et al* as naturally occurring $CD4^{+}CD25^{+}$ cells capable of maintaining self tolerance, are now widely recognized as the prime tolerance mediating cells following transplantation (*33, 34, 263, 264*). In fact, experimental studies have documented a role for Treg immunotherapy in transplant models with some success (*245, 265*). Any such attempt in humans needs extra caution and thorough understanding of the physiological alterations in Tregs under varying conditions.

Mindful of the fact that Tregs mechanism of action involves close cell-cell contact, Treg migration in vivo has been scrutinized by several authors (*73, 240*). Naïve like Tregs circulate to the antigen presenting sites under the influence of CCL19 and CCL21, where they co-localize with antigen presenting cells (APC) (*50, 124*). This interaction not only activates Tregs via t cell receptor (TCR) stimulation, but also leads to up-regulation of CCR4 and CCR8,

the receptors for CCL17 and CCL22 secreted by the APC (*145*). Also, the same chemokines are secreted by various inflammatory cells within the site of antigen challenge, leading to the peripheral migration of Tregs (*266*). My data shows CCL17 staining within the myocardium during both stable and rejecting states, with significant association between CCL17 and Treg infiltration during acute rejection. However, the chemotactic effect of CCL17 is not exclusive to Tregs, since $CD4^{+}FOXP3^{-}$ cells also showed strong correlation with CCL17 expression during acute rejection. This suggests that CCL17 secretion forms part of the alloresponse during acute rejection, leading to migration of both the effector and regulatory cells within the allograft.

I have further demonstrated that acute rejection in heart transplant recipients leads to a significant rise in the peripheral blood levels of specific Th1 chemokines, including CCL2, and CCL5. Intragraft expressions of these chemokines have been associated with acute rejection; however, association with peripheral blood levels was never established before (*91, 98*). Using in-vitro chemotaxis study we further demonstrated that CCL5 causes specific migration of Tregs but not of the $CD4^{+}CD25^{-}$ effector cells. Taken together, it suggests that CCL5, which is an inflammatory Th1 chemokine secreted by various inflammatory cells including APC, may have an important role in Treg migration during acute rejection. Further studies may investigate the balance between CCL17 and CCL5 stimuli during stable and acute rejection following transplantation.

Transplant poses a different environment not only due to the alloantigen presentation, but also from various immunosuppressive medications. My results demonstrate that Tregs in stable heart transplant recipients are unaffected by

various immunosuppressive drugs. However, a surprising result in my study was that the use of statins was associated with significantly reduced Treg counts. Statins are commonly used in transplant recipients to counter hyperlipidaemia in an effort to prevent allograft vasculopathy and prolong survival (*214*, *237*). Statins are pleiotropic molecules, with the ability to affect, either directly or indirectly, several immune cells and cytokines (*215*). Since both statins and Tregs reduce the burden of atherosclerosis following heart transplantation (*236*), the findings of my study need further exploration. It is uncertain whether statins lead to a decreased population of Tregs associated with other compensatory mechanisms or they cause Treg migration to the allograft.

This study further highlighted the problems of conducting such investigations in humans. In particular, co-localizing Treg cellular markers in the precious biopsy tissues posed a significant challenge due to various reasons, including high autofluorescence from FFPE samples and non-specific staining from experimental or polyclonal antibodies. Somewhat similar observations have been made by previous authors (*255*). Hence, further dissection of other chemokine pathways within the allograft was not possible, which formed a limitation of my study.

In summary, I have demonstrated for the first time an association between the presence of Tregs within rejecting myocardium and the chemokine CCL17. The absence of Tregs from the non-rejecting hearts suggests Treg infiltration as part of the alloresponse. Prospective longitudinal studies will have to determine the fate and function of these cells in the development of CAV and long term survival. Therapeutic manipulations to augment selective Treg

migration may provide exciting opportunities to reduce the incidence of acute or chronic rejection and perhaps, induce tolerance.

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