The Germinal Centre Reaction and Follicular T Cells in Alloantibody Formation

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A thesis submitted for the degree

Doctor of Philosophy

Michaelmas 2016

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Abstract

Kidney transplantation is the optimal treatment for end-stage renal failure, however graft lifespan remains limited, and a major cause of graft loss is chronic, low-grade antibody-mediated damage. Development of *de novo* donor specific antibodies (DSAs) occurs in up to 30% of patients post transplant and there are currently no biomarkers for predicting which patients may be at risk, in part because antibody production occurs in lymphoid tissue, which cannot easily be sampled in humans. Recently, circulating CD4 T cells have been identified that appear to correlate with active germinal centre reactions in secondary lymphoid tissue. These circulating T follicular helper-like cells (cTfh) increase in association with antibody production, and correlate with antibody levels in autoimmune disease and vaccination. They were therefore of interest in transplantation, as potential biomarkers for *de novo* DSA formation.

This study aimed to recruit a cohort of transplant patients, obtaining paired blood and lymph node (LN) samples to confirm the correlations between circulating and LN cells, and to follow patients for the first two years post transplant, assessing for DSA development and examining the circulating cell profile, and the impact of immunosuppression on these cells.

Results suggested that high cTfh and low circulating T follicular regulatory (cTfr) cells were associated with *de novo* DSA formation, and patients going on to develop *de novo* DSAs had a low cTfr to cTfh ratio at all times post transplant. Interestingly this pattern of low cTfr and high cTfh was also seen in patients treated with alemtuzumab induction and mycophenolate mofetil (MMF) maintenance. These patients were also at highest risk of *de novo* DSA formation. This is a novel observation, which may also shed light on the high rates of antibody-mediated autoimmune disease after alemtuzumab treatment, suggesting a lack of control of germinal centre responses may underlie this phenomenon. Patients treated with azathioprine maintenance had lower numbers of cTfh and were also at the lowest risk of *de novo* DSA formation. This data was supported by *in vitro* work suggesting a greater reduction of antibody formation with azathioprine compared to MMF.

While numbers were small and therefore these results did not reach statistical significance, they are potentially very interesting as they suggest that the current treatment regimen of alemtuzumab and MMF maintenance for patients considered as high immunological risk may not be the best option for preventing *de novo* DSA formation. These observations warrant further investigation to establish which immunosuppressive regimen can maximise transplant outcomes while minimising the risk of *de novo* DSA formation.

Acknowledgements

This body of work could not have been carried out without the support and help of many individuals. I would particularly like to thank the following.

The MRC and Kidney Research UK, for funding my fellowship and providing a platform for presenting my work, helpful discussion and networking in the form of Fellows Day.

My supervisor, Kathryn Wood, for allowing me to come and undertake these studies in the TRIG laboratories, her wealth of transplant knowledge, rigorous and ruthless proofreading skills, and for guiding the direction of the project.

My co-supervisor, Michelle Linterman, for her longstanding support, guidance and expertise in the field of follicular T cells, training in laboratory techniques, reading endless drafts, and for answering any question, no matter how ridiculous, with patience and good humour.

All members of TRIG for help with sample collection and laboratory techniques. Particularly Joanna Hester for all her suggestions on *in vitro* techniques, and Kate Milward for *in vitro* and *in vivo* help and advice, offers of help whenever I looked even slightly lost, as well as constant tea and support. Monica Dolton, who can answer any administrative, organisational or grant related question known to man, and who works so hard to support the lab. With sadness, to the memory of Andrew Bushell, whose sense of humour, hands-on approach and unwavering support for the students of TRIG made all the work much easier.

Professor Sue Fuggle, for guidance on all matters antibody related and kindly allowing me to use the H&I laboratories for sample processing. Also for informal but very much appreciated supervision on donor specific antibodies and transplant matching, including proofreading many drafts; as well as kindness and support whenever it was needed.

To other members of the H&I lab, including Mian Chen and Martin Barnado for their help and training in Luminex techniques, and for help with exporting data, proving to me that learning bioinformatics would speed my data analysis exponentially. Finally, all other members of the H&I lab, without whom this project would never have happened, both for training me in clinical techniques but more importantly, for regularly waking me in the middle of the night and greeting me cheerily no matter the time.

Professor Seph Borrow and Isabela Pedroza-Pacheco for helpful discussions around cTfh and cTfr and their kindness in training me in the co-culture techniques and allowing me to adapt their protocols to my project. I hope to continue collaborations in future.

Helen Ferry, Flow Facility Manager, not only for all her advice on flow cytometry panels, techniques and analysis, but also for a wealth of general laboratory advice and regular pep talks.

Professor Peter Friend, for kindly allowing me to carry out this project in the Oxford Transplant Centre, and supporting the collection of blood and tissue samples. All members of the surgical, anaesthetic and ward teams, who worked hard to facilitate this project.

Sally Ruse, research nurse, for driving merrily around to all the peripheral units to collect samples, and helping with administrative work. To all of the phlebotomists in both the OTC and peripheral units for not balking at the number of tubes I required. Also to the research teams at University Hospital Birmingham, Royal Berkshire Hospital and Southmead Hospital Bristol.

Professor Chris Pugh and Professor Simon Draper, my examiners for my Transfer of Status and Confirmation of Status, who pointed out flaws in my thinking and put me in touch with both Professor Borrow and Dr Clutterbuck, who both provided helpful discussions to guide me in the right direction.

Wilson Wong, for attempting to teach me to transplant a mouse kidney. Although I wasn't able to master the technique sufficiently for this project, the skills I learned made the *in vivo* work I did carry out much easier. David Withers, for advice on immunofluorescence microscopy. Although this data did not make it into this manuscript, the skills I learned were invaluable.

My patients, whose generosity in taking part in research made this project possible, and who gave me the motivation to keep going when things were difficult. It is a privilege to be involved in the care of people who are so keen to improve things for future generations.

My friends and family for their support and understanding of plans abandoned when transplants occurred, and for forcing me to go dancing when it all got too much.

Finally, more than anyone, to Ray, for everything. I couldn't have done any of this without you.

Abbreviations used in this manuscript:

6-MP – 6-mercaptopurine, active metabolite of azathioprine

ABMR – antibody mediated rejection

ADCC – antibody-dependent cell-mediated cytotoxicity

ANOVA – analysis of variance

APC – antigen presenting cell

ATG – anti-thymocyte globulin

ATN – acute tubular necrosis

B + A – basiliximab and azathioprine

B + M – basiliximab and mycophenolate mofetil

BCR – B cell receptor

bnABs – broadly neutralising antibodies (in the context of HIV+ individuals)

BSA – bovine serum albumin

BV – brilliant violet

CDC-XM - Complement-dependent cytotoxicity cross match

CFSE – carboxyfluorescein succinimidyl ester

CLN – contralateral lymph node

CMV – cytomegalovirus

CNI – calcineurin inhibitor

CREG – cross-reactive epitope group (related to HLA matching)

cRF – calculated reaction frequency

CRP – C-reactive protein

CTLA-4 – cytotoxic T lymphocyte antigen 4

DAMPs – damage associated molecular patterns

DBD – donation after brainstem death

DC – dendritic cell

DCD – donation after cardiac death

DGF – delayed graft function

DI – division index

DLN – draining lymph node

DMSO – dimethylsulphoxide

DNA – deoxyribonucleic acid

DSA – donor specific antibody

EDTA – ethylenediaminetetraacetic acid

eGFR – estimated glomerular filtration rate

ELISA – Enzyme-linked immunosorbent assay

ESRF – end stage renal failure

FCS – fetal calf serum

FCXM – flow cytometric cross match

FDC – follicular dendritic cells

GBM – glomerular basement membrane

GC – germinal centre

HIV – human immunodeficiency virus

HLA – human leucocyte antigen

HRP – horseradish protein

ICOS – inducible costimulator

IFTA – interstitial fibrosis and tubular atrophy

LD – living donor

LN – lymph node

MAC – membrane attack complex

MB – memory B

MFI – median fluorescence intensity

MHC – major histocompatibility complex

MMF – mycophenolate mofetil

MP – methylprednisolone

MPA – mycophenolic acid, active metabolite of mycophenolate mofetil

MST – median survival time

mTOR – mammalian target of rapamycin

NaOH – sodium hydroxide

NB – naïve B

NEAA – non-essential amino acids

NK – natural killer

PAMPs – pathogen associated molecular patterns

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PD-1 – programmed death 1

PFA – paraformaldehyde

PI – proliferation index

PRA – panel reactive antibody

PTLD – post transplant lymphoproliferative disorder

REC – research ethics committee

RNA – ribonucleic acid

RRT – renal replacement therapy

SAB – single antigen bead

SD – standard deviation

SEB – staphylococcal enterotoxin B

SHM – somatic hypermutation

SLE – systemic lupus erythematosus

SLO – secondary lymphoid organ

SPA – single-phase assay

SPK – simultaneous pancreas kidney transplant

TB - tuberculosis

TCMR – T cell mediated rejection

TCR – T cell receptor

Tfh – T follicular helper

Tfr – T follicular regulatory

Th – T helper

TLO – tertiary lymphoid organ

TLR – toll like receptors

TMB – tetramethylbenzidine

TMPT – thiopurine-methyltransferase

TNFR – tumour necrosis factor receptor

Treg – regulatory T cell

TRIG – Transplant Research Immunology Group

UTI – urinary tract infection

VPD – violet proliferation dye

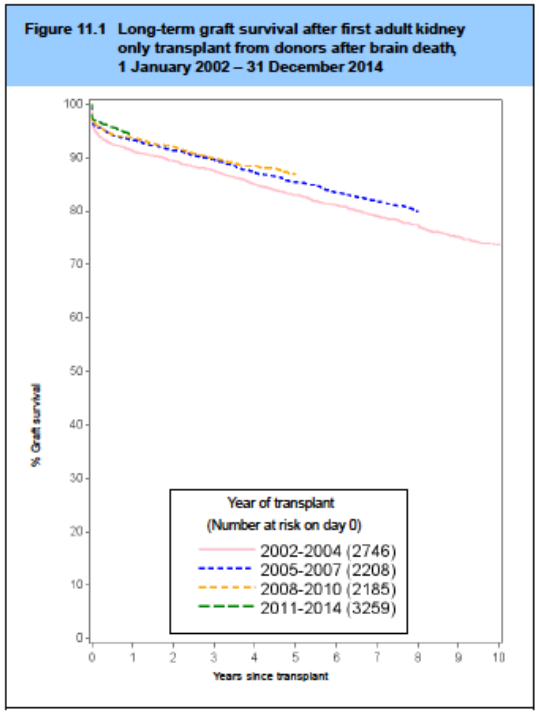
Contents

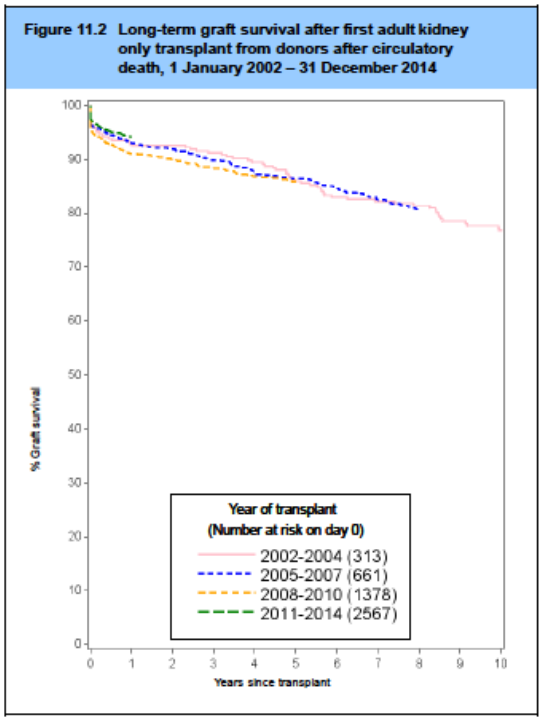
1. The Germinal Centre Reaction and Follicular T Cells in Alloantibody Formation

1.1 Introduction

End stage renal failure (ESRF) requiring renal replacement therapy (RRT) has a prevalence of nearly 60 000[1] in the UK at present. RRT can be provided in several different ways, but over half of those currently living with ESRF have a kidney transplant[1], which is widely recognised as the optimal form of RRT. Although short-term outcomes are excellent, long-term graft attrition has remained relatively unchanged[2-4], with a steady decline and rate of loss after the first year post-transplant (**fig 1.1**)[5]. This is not confined to kidney transplantation; all solid organ transplants show long-term graft attrition that has not improved over the past few decades despite improvements in organ retrieval, organ allocation and immunosuppressive regimens[5-8].

The commonest cause of 'graft loss' is not failure of the graft, but death of the patient from another cause despite a functioning graft[9]. Other common causes of graft failure include complications of surgery or infection, acute rejection, side effects of immunosuppressive agents or recurrent disease[10]. However, in recent years it has been recognised that chronic rejection, associated with and potentially mediated by antibodies[11-13], is a major cause of long term graft loss[14-17]. B cells and alloantibody are becoming increasingly recognised as important targets in transplantation to try to improve long-term outcomes[18-22].





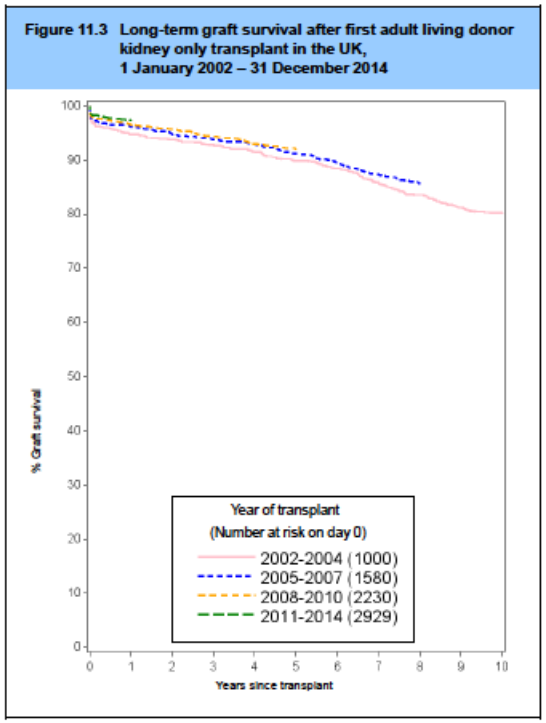


Figure 1.1 Long term graft survival after first kidney transplant. Graphs reproduced from 2016 NHS Blood and Transplant-Organ Donation and Transplantation report 'Survival Rates Following Transplantation'[5]. First panel DBD donors, second panel DCD donors, third panel living donors.

This project was designed to investigate risk factors for and potential biomarkers of alloantibody formation, in particular the use of circulating cells as a biomarker of germinal centre activity, which might allow early intervention prior to antibody formation.

1.2 Generation of an immune response

The immune system is extremely complex, having developed to balance protection of the individual with a response proportional to the potential threat. Protection needs to be both against external threats such as microorganisms, and also internal threats such as malignant transformation of cells. This requires the immune system to be able to respond quickly, but also distinguish self-antigens from non-self or altered-self antigens.

However, recognition of self and non-self is insufficient alone; it is also important for the immune system to distinguish harmful non-self or altered-self, such as invading pathogens, from benign or beneficial non-self, such as intestinal commensal flora. The response, or lack thereof, to a particular antigen must be proportionate to the potential threat posed by that antigen. For example, clearance of an invading pathogen would require initiation of a sufficient response, but also control of that response, to limit damage to host tissues that might occur with unchecked inflammation.

To address these myriad requirements, the immune system is broadly divided into innate and adaptive responses, although there is significant cross talk between the two arms.

1.3 Innate Immune Responses

The innate immune system is capable of providing rapid, broad-spectrum responses to a wide range of insults. It consists of a group of cells and molecules that can respond immediately to potential danger and provide a first line of defence to either clear the potential threat or control it until an antigen-specific response can be mounted by the adaptive immune system[23]. Each component of the innate immune system has a specific function, and these are detailed in the table below (adapted from Francis/Wallin and Wood. Principles of Transplantation Immunology[24,25])

Table 1.1: Components of the innate immune system.

Component	Functions
Complement	• Chemoattraction of immune cells • Opsonisation of pathogens • Lysis of target cells
Dendritic cells	• Cytokine production • Antigen uptake and presentation to lymphocytes
Macrophages	• Phagocytosis • Cytokine production • Secretion of enzymes and inflammatory mediators • Initiation of adaptive immune response
Neutrophils	• Phagocytosis • Cytokine production • Secretion of enzymes and inflammatory mediators
Eosinophils	• Release of cytokines, chemokines and enzymes, particularly damaging to parasites
Basophils	• Phagocytosis • Release of heparin, histamine and cytokines

Component	Functions
Mast cells	• Release of histamine and other inflammatory mediators, particularly relevant in parasitic and allergic disease
NK cells	• Cytotoxicity, particularly to altered self – malignant or virally infected cells.

Innate immune cells recognise molecular patterns of injury through pattern-recognition receptors such as Toll-like receptors (TLRs)[26]. These receptors recognise pathogen associated molecular patterns (PAMPs)[27], which are highly conserved within classes of microbes, but absent from human cells. They can also recognise damage-associated molecular patterns (DAMPs)[28] that are produced as a result of tissue injury and cellular stress, such as heat shock proteins and reactive oxygen species, extracellular RNA and DNA, as well as activated components of the complement cascade. These DAMPs and PAMPs activate innate immune cells through TLR ligation, and lead to release of pro-inflammatory cytokines, chemokines, adhesion molecules and other inflammatory mediators. TLR ligation also activates effector functions such as phagocytosis of necrotic tissue by macrophages and neutrophils[29].

As the innate immune system is designed to respond to broadly expressed DAMPs and PAMPs, rather than to specific antigens associated with particular pathogens, it will respond to both sterile tissue injury[30] and that associated with non-self invasion[31,32]. For example, complement consists of a group of proteins, which form a proteolytic cascade when activated, and generate a range of downstream effects. Three different pathways can activate complement: the classical pathway, the lectin pathway or the alternative pathway. The classical

pathway is activated by the binding of complement component C1q either directly to a pathogen surface, or to C-reactive protein (CRP) bound to pathogen surface, or by antibody complexed to antigen[33,34]. The lectin pathway is activated by binding of mannose-binding lectin or ficolin to carbohydrate residues on the surface of pathogens[35]. The alternative pathway is triggered directly on the surface of pathogens, when complement component C3 is spontaneously activated and binds to pathogens. Whichever pathway is activated, they converge with the conversion of C3 to C3a, an inflammatory mediator and chemoattractant[36], and C3b, the main effector of complement downstream function. C3b can bind to its receptor on the surface of pathogens, aiding phagocytosis in a process called opsonisation[37]. It can also trigger the terminal complement components C5b-9 to form the membrane attack complex and lyse the target cell[38]. These downstream effects, like all mediators of the innate immune system, are not specific to a particular pathogen, and generate the same response to each invader. As such, the components of the innate immune system, for example proteolytic enzymes, may damage host tissues as well as pathogens[39,40].

Despite the speed and efficient mechanisms of the innate immune response, it may be insufficient to clear the pathogen and antigen can accumulate, leading to excessive damage to self. For this reason, components of the innate immune system not only respond by attacking non-self, but also initiate an adaptive immune response[41,42].

Tissue resident dendritic cells (DCs), and monocytes responding to the inflammatory response are able to recognise when non-self is present, and initiate an adaptive immune response[43]. In the case of infection this would be via recognition of PAMPs rather than DAMPs alone. However there is evidence from mouse models that monocytes may be able to recognise non-self antigen in the context of transplantation, although the mechanism of this is not yet understood[44-46]. Monocytes are activated by TLR stimulation, and in the presence of non-self are triggered to differentiate into inflammatory DCs[44]. DCs (whether tissue resident or induced by inflammation) are the most efficient antigen presenting cells (APCs)[47]. When activated by DAMPs and PAMPs, DCs upregulate MHC class II and costimulatory molecules such as CD80/CD86 and migrate to secondary lymphoid organs (SLOs). Here, DCs are able to present antigen to cells of the adaptive immune system and provide the costimulation required for efficient activation of the adaptive immune system[48,49].

1.4 Adaptive Immune Responses

The adaptive immune system generates both effector and memory lymphocytes, capable of clearing a current pathogen, but also generating rapid recall responses should the pathogen invade in future. It consists of B cells and T cells; the latter of which can be divided into CD8 T cells, CD4 T cells, and γδ T cells. The key feature of the adaptive immune system is the precise specificity of its cells; each cell has a unique receptor that is specific for a particular antigen, and only when it encounters that antigen will it become activated and generate a response[23].

The ability of adaptive immune cells to discriminate between self and non-self is achieved because peptides are presented in the context of the major histocompatibility complex (MHC) molecules[50]. There are two classes of MHC molecule responsible for presenting peptides. These molecules are closely related but differ both in structure and tissue distribution[51].

MHC class I consists of a membrane-spanning α chain, bound to a smaller β2 microglobulin chain. The α chain consists of three sections, two of which form a peptide-binding groove, and one of which spans the membrane (**fig 1.2**). The ends of the peptide-binding groove are closed; meaning only short peptide sequences of 8-9 amino acids can be bound[52,53]. MHC class I is constitutively expressed on almost all cells, and is responsible for presenting peptide to CD8 T cells. In most cases this will be self-peptide and is not recognised by CD8 T cells due to self-tolerance mechanisms. In the case of malignant transformation, altered-self will be presented, and in viral infection, viral peptides can be presented, allowing CD8 T cells to recognise and kill the affected cell[54,55].

MHC class II consists of two membrane-spanning domains, an α chain and a β chain, which together form a similar structure to class I. However, unlike class I, the ends of the peptide-binding groove are open, meaning the length of peptide that can be presented is not limited[56]. MHC class II is used by APCs to present peptide to CD4 T cells, and is therefore involved in activation of the immune system when presenting foreign antigen, or tolerance induction when presenting self-antigen[55]. Because of this role, MHC class II is expressed by

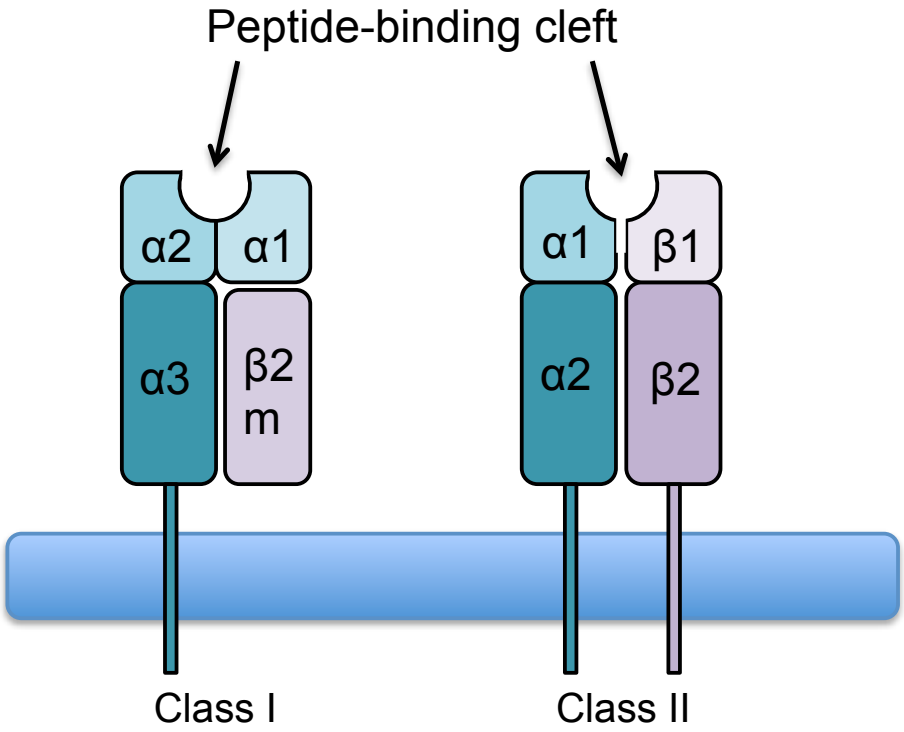


Figure 1.2: MHC molecules. Class I molecules have a closed peptide-binding cleft, the ends of the Class II cleft are open. Adapted from Janeway's Immunobiology, 7th Edition.

specialised antigen presenting cells such as DCs, macrophages, B cells and thymic epithelial cells and not normally expressed on other cell types in their resting state[57].

MHC molecules are highly polymorphic[58] and there is enormous diversity of molecules between individuals. Thus, presentation of a peptide in the context of self-MHC allows the immune system to distinguish self-peptide-self-MHC from foreign-peptide-self-MHC and, in the case of transplantation, peptide-foreign-MHC complexes[57].

In order to recognise the broad range of antigens, in the case of B cells, or peptide-MHC complexes, in the case of T cells, adaptive immune cells must have a diverse range of antigen receptors[23].

Adaptive immune cells are generated in primary lymphoid organs. The bone marrow produces both B and T cell precursors but, while B cell development continues in the bone marrow, T cell development occurs in the thymus. The main purpose of development in primary lymphoid organs is to develop a repertoire of mature antigen receptors that are capable of recognising a wide range of antigens, but to delete those that recognise self[23]. Precursor cells undergo rearrangement of their variable (V), diversity (D) and joining (J) receptor gene segments, yielding different combinations between different cells of the same individual. Many segments of each are present in the genome of the individual, allowing a myriad of combinations that generate diversity of receptors within each individual[59]. The process is similar for both the B cell immunoglobulin

receptor (BCR), and T cell receptor (TCR), both of which have constant regions allowing downstream function, and variable V(D)J regions, allowing antigen specificity[60].

B cell precursors develop their BCR in the bone marrow, undergoing VDJ recombination and interacting with bone marrow stromal cells[61]. Once they have formed a surface immunoglobulin receptor, B cells undergo negative selection, where those binding strongly to self-antigen expressed in the bone marrow are deleted, undergo receptor editing or are rendered anergic, a process known as central tolerance[62-64]. B cells that bind only weakly to self-antigen are allowed to mature, and it is thought that this helps to maintain a broad range of antigen specificity. B cells require help from T cells to become fully activated, and so self-reactive cells can be deleted or undergo receptor editing later on, in a process known as peripheral tolerance[65-67].

T cell precursors migrate from the bone marrow to the thymus, and undergo rearrangement of their TCR in the thymus. The TCR is tested against self-antigen expressed on medullary thymic epithelial cells or thymic DCs in a step-wise fashion[68]. As with B cells, T cells that react strongly to self-antigen can be deleted or rendered anergic. In contrast to B cells, those with only weak binding to self-antigen are not allowed to mature, as T cells can become activated upon antigen recognition without additional help. Thus, any self-reactive T cells exiting the thymus could respond to self-antigen in the periphery and damage self-tissues. Therefore, negative selection of T cells must be more stringent than that of B cells[69,70]. However, weakly self-reactive T cells may also be differentiated

into regulatory T cells (Tregs), in order to control peripheral responses to self-antigen[71,72].

B cells exit the bone marrow as mature naïve B cells; T cells exit the thymus as mature naïve CD4 T cells, CD8 T cells, or γδ T cells. These naïve cells recirculate through the blood, lymph and SLOs, where antigen is constantly presented by DCs and other APCs[55,73]. Cells not encountering their cognate antigen will exit the SLO and re-enter the circulation, however those that recognise their cognate antigen will become activated.

1.4.1 Activation of T cells

Naïve T cells express CCR7, a chemokine receptor that allows them to migrate towards the chemokines CCL19 and CCL21, produced by stromal cells in the T cell zone of SLOs[74]. Moving through the T cell zone along the chemokine gradient, T cells encounter APCs and sample the antigen presented on MHC class II[75,76]. Engagement of the TCR by antigen-MHC provides signal 1 (see **fig 1.3**), but if received in isolation this interaction will lead to deletion or anergy as it suggests self-reactivity[77]. For full activation, T cells require a second signal, provided by the costimulatory molecules that are upregulated on the surface of APCs following ligation of TLRs by DAMPs and PAMPs. Signal 2 (**fig 1.3**) is induced by engagement of molecules on the T cell surface with these co-stimulatory molecules. Without signal 2, T cells cannot be activated[78]. However, these molecules can also suppress an immune response. Activation or inhibition of the T cell depends on the ligand that engages with the T cell surface receptor.

Alongside their TCR (CD3) T cells express surface receptors from two main families: the CD28/B7 family, best characterised by CD28 and CTLA-4 (cytotoxic T lymphocyte antigen-4, CD152), and the tumour necrosis factor receptor (TNFR) superfamily, the best characterised of which is CD40 ligand (CD40L, also known as CD154)[79]. These surface receptors are stimulated by ligands on the surface of antigen presenting cells. For example, CD80 and CD86 bind both CD28 and CTLA-4, whilst CD40 recognises CD40L. However there are many costimulatory molecules that can be both activating and inhibitory, depending on their pairing and the balance of activatory and inhibitory molecules expressed on the cell surface at a particular time in the immune response, see table below (adapted from Principles of Transplantation Immunology, Oxford Textbook of Medicine[24,25]).

Table 1.2: Costimulatory molecules

	T cell molecule	APC molecule
Activating signals	CD28	CD80 (B7-1), CD86 (B7-2)
	CD27	CD70
	CD40L (CD154)	CD40
	ICOS (inducible costimulator)	ICOS ligand (ICOS-L)
	OX40 (CD134)	OX40-L (CD252)
	4-1BB (CD137)	4-1BB-L (CD137L)
Inhibitory signals	CTLA-4 (CD152)	CD80 (B7-1), CD86 (B7-2)
	PD-1 (programmed death-1)	PD-L1 (CD274, B7-H1), PD-L2 (CD273, B7-DC)

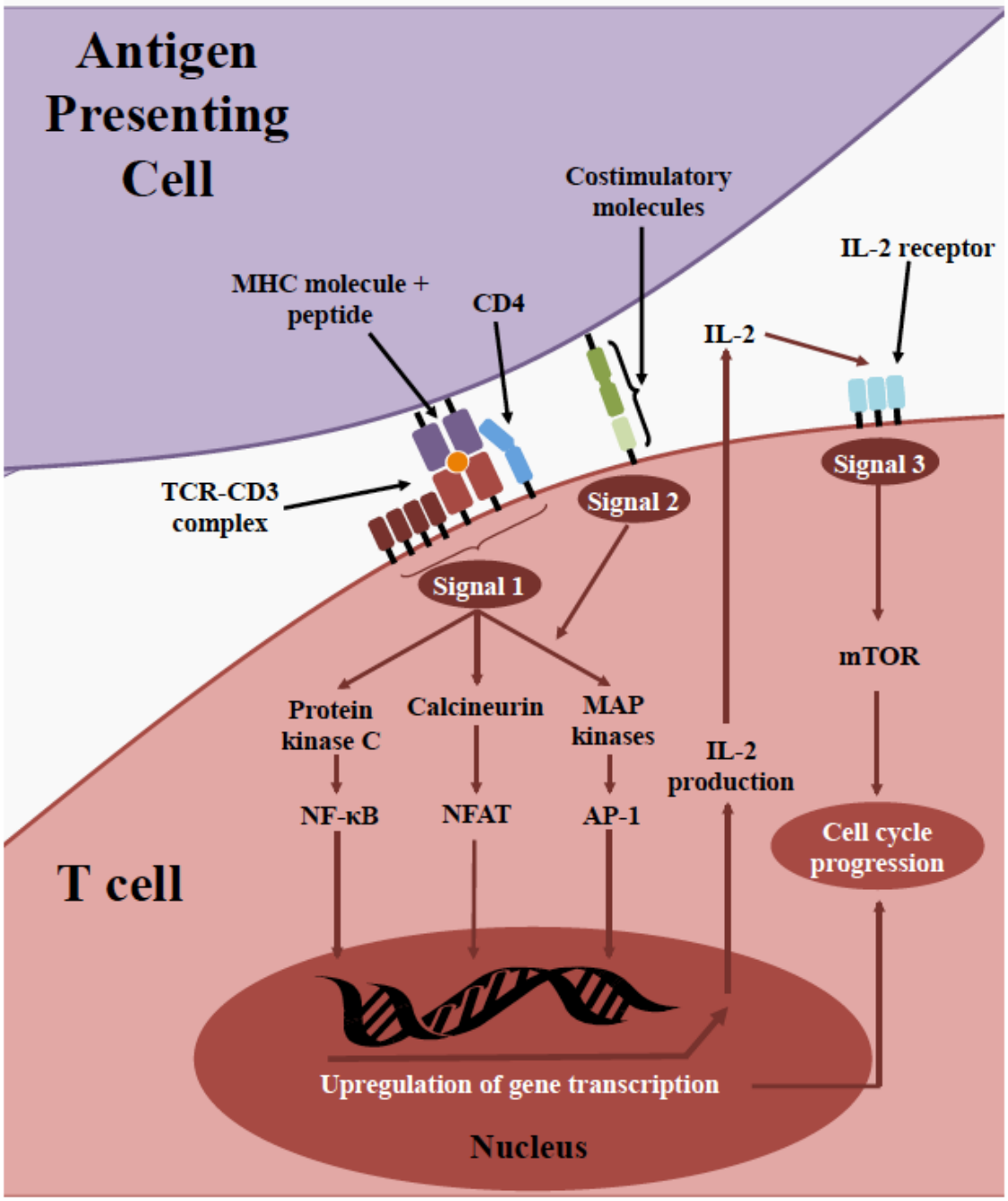


Figure 1.3: Three-signal model of T cell activation. Adapted from Francis and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 5th Edition. In press, Wallin and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 6th Edition.

A third signal comes in the form of cytokines, which can be produced both by innate cells and APCs, but also in an autocrine manner by the T cells (see **fig 1.3**), thus maintaining and polarising the immune response in the presence of persistent antigen. The cytokine signal is important to direct the downstream response of the T cell. For example IL-2 leads to T cell proliferation, whereas IL-4 will induce CD4 T cells to become Th2 cells (see below)[80-82].

The character and magnitude of the T cell response is determined by the strength of interaction between the TCR and peptide-MHC complex, the balance of activatory and inhibitory signals delivered by the costimulatory molecules, and the fate direction provided by cytokine stimulation. These will vary with the degree of inflammation, the stage of the immune response, and any external factors such as immunomodulatory drugs[79].

CD8 T cells are activated broadly in the same way as CD4 T cells, however they require much higher levels of costimulation to become fully activated[83,84]. Mature DCs express high levels of costimulatory molecules and are able to activate CD8 T cells directly. However, inflammatory DCs or other APCs require pre-activation by CD4 T cells to upregulate CD40 and CD80/86 to a sufficient level to fully activate CD8 T cells[85].

Once naïve T cells have been activated, their effector function depends on the type of T cell. CD8 T cells are also known as cytotoxic T cells (Tc), and target either cells infected with intracellular pathogens, such as viruses, or altered cells such as pre-malignant cells. They act mainly by inducing apoptosis of target cells

either through granzymes, or Fas/Fas-ligand interactions, both of which trigger the caspase cascade[23].

CD4 T cells are also known as helper T cells (Th), and are important for the generation of efficient immune responses by secretion of cytokines and expression of costimulatory surface receptors. CD4 T cells activate macrophages and enhance killing of phagocytosed pathogens, but are also important in full CD8 T cell activation and are required for formation of CD8 memory[85]. Additionally, CD4 T cells are required for the production of long-lived antibody responses and B cell memory. This diverse range of functions is produced by polarising Th cells into subsets, with different effector functions. Activated CD4 T cells are polarised by the specific costimulatory signals received alongside TCR engagement, which are differentially induced on APCs according to the type of DAMP or PAMP stimulation encountered. These polarising signals cause upregulation of transcription factors that drive phenotypic changes specific to that subset[85]. Multiple helper subsets have been identified and each have different downstream effector functions, from promotion of inflammatory responses against a particular pathogen, to generation of antibody, or control and suppression of an inflammatory response when the pathogen has been cleared, detailed in the table below:

Table 1.3: T helper subsets (adapted from Janeway's Immunobiology[23])

Subset	Polarising cytokines	Canonical transcription factor	Effector cytokines	Key functions
Th1	IL-12, IFNγ	T-BET	IFNγ, TNF	Activation of macrophages, proinflammatory
Th2	IL-4	GATA3	IL-4, IL-5, IL-13	Parasitic and allergic responses
Th17	IL-6, TGFβ	RORγT	IL-17, IL-21, IL-22	Proinflammatory, recruitment of phagocytes
Tfh	IL-6, IL-21	BCL6	IL-21	B cell help
Treg	IL-2, TGFβ	FOXP3	IL-10, TGFβ	Regulation and resolution of inflammation

Depending on the type of immune response required, multiple helper subsets may be produced.

1.4.2 Activation of B cells

Mature naïve B cells also migrate to SLOs to encounter antigen. These mature B cells express CXCR5, which allows them to migrate towards the chemokine CXCL13, produced by follicular dendritic cells (FDCs) and stromal cells[73,86]. Within the B cell follicle they continuously sample antigen presented by FDCs, DCs or subcapsular sinus macrophages. Unlike T cells, B cells are able to

recognise intact antigen and then take it up, process it and present peptides on their own MHC, as well as upregulate costimulatory molecules[87]. These newly activated B cells downregulate CXCR5 and upregulate CCR7, allowing them to migrate to the edge of the B cell follicle, the T-B border[88]. Similarly, recently activated CD4 T cells upregulate transcription factors such as ASCL2 and BCL6[89], leading them to downregulate CCR7 and upregulate CXCR5, migrating to the border of the B cell follicle and T cell zone[90,91] to become pre-T follicular helper (Tfh) (**fig 1.4**). If two cells recognise cognate antigen, they exchange survival and proliferation signals through CD40-CD40L engagement. B cells begin to proliferate, moving further into the B cell follicle, where they can either form extrafollicular or follicular responses. Extrafollicular responses are rapid and short-lived but produce low-affinity antibody[92]. To form high affinity antibody requires B cells to form into clusters called germinal centres (GCs) and undergo a process called the GC reaction[93].

It has been suggested that pre-Tfh cells can undergo similar fate decisions, exiting the LN as memory-like Tfh cells and entering the circulation, or upregulating CXCR5 further to enter into the B cell follicle and support the GC reaction as Tfh cells[94].

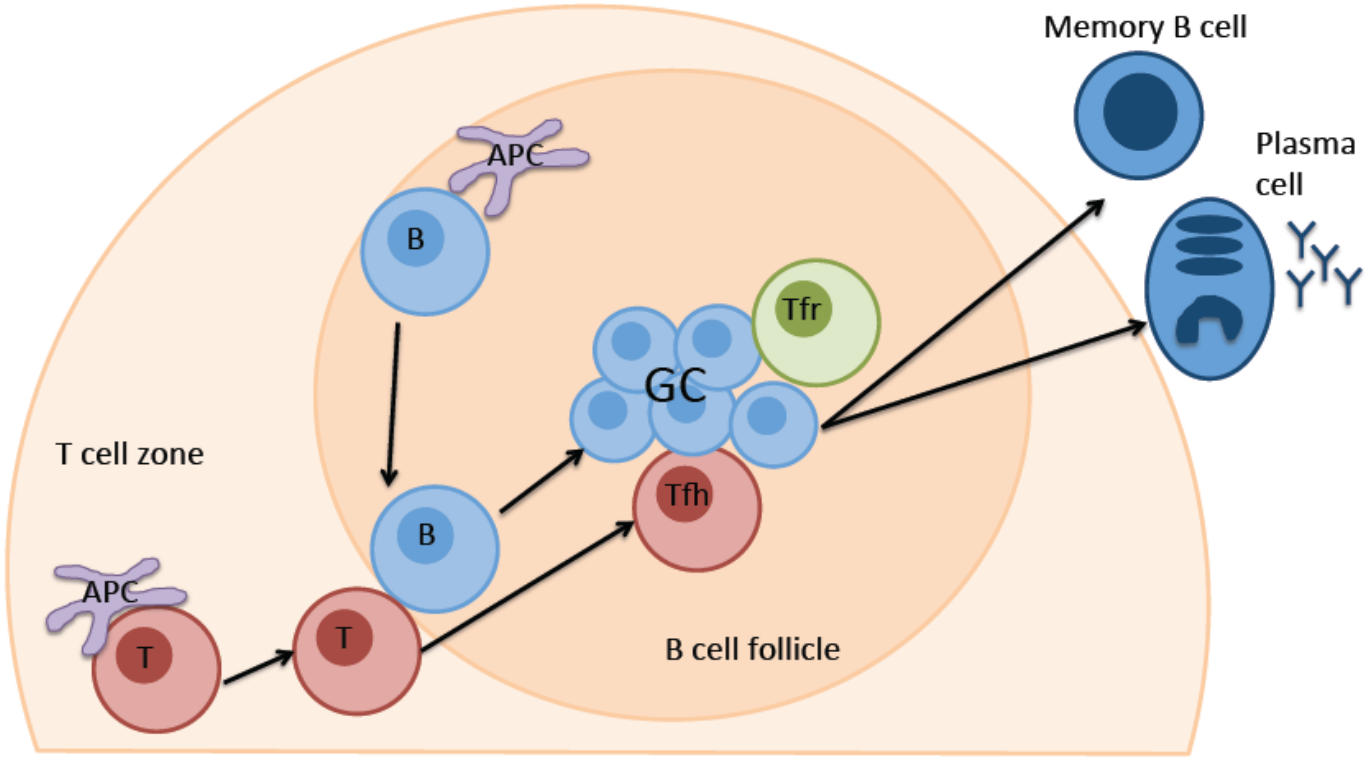


Figure 1.4: Interaction of B cells with T follicular helper and regulatory cells in the germinal centre reaction.
From Wallin & Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 6th Edition.

1.4.3 The germinal centre

Over the lifetime of an individual, the immune system generates a broad spectrum of highly specific, high affinity antibodies that provide protection against the multiple pathogens that are encountered. Over the course of an antibody response, for example to vaccination, the affinity of antibodies for antigen increases, in a process known as affinity maturation[95,96]. In order to increase affinity, proliferating GC B cells undergo mutation of the V region of the BCR at an extremely high rate, far above the rate of normal somatic mutation; hence this process is known as somatic hypermutation[97-99] (SHM). However, random mutation may generate BCRs with both lower and higher affinity for antigen, as well as potentially self-reactive BCRs. In order to ensure only higher affinity B cells go on to produce antibodies, which are soluble forms of the BCR, a selection process takes place within the GC that is dependent on Tfh cells. If the mutation has increased the affinity of the BCR for antigen, the B cell can take up more antigen from FDCs and out-compete lower affinity B cells for T cell help. This is in the form of CD40-CD40L interactions that provide both survival and differentiation signals to the B cells. However lower affinity B cells either do not receive CD40L signalling and undergo apoptosis, or receive signals to induce re-entry into cell cycle and further rounds of SHM[90]. Thus, only the higher affinity cells are able to differentiate into long-lived memory B cells or plasma cells that then migrate to bone marrow or gut niches to form long-lived plasma cells[100].

1.4.4 T cells in the GC response

B cells cannot differentiate and undergo SHM to generate a highly specific, high affinity, long-lived response to protein antigens without T cell help[101,102]. The T cells that provide help to B cells are a specialised subset of CD4 T cells that have formed stable cognate interactions with B cells at the T-B border in the SLO. On exchange of CD40-CD40L, CD28-CD80/86 and ICOS-ICOSL signalling with B cells, as well as cytokine stimulation, pre-Tfh upregulate CXCR5 further and move out of the T cell zone into the B cell follicle[103,104].

Once in the follicle, Tfh cells are key players in the maintenance of the GC response, and selection of GC B cells, with GCs collapsing in the absence of Tfh cells[105]. Tfh cells contain pre-formed CD40L that can be rapidly expressed on the cell surface to provide CD40 signalling to GC B cells during cognate T:B interactions. Tfh cells can also provide help in the form of cytokines. IL-21 is the classical cytokine associated with Tfh cells, maintaining BCL6 expression in GC B cells and stimulating plasmablast development[106]. Tfh cells are also capable of producing other T helper cytokines to alter the antibody profile produced in response to a particular pathogen (see **table 1.3** above). An intracellular pathogen that stimulates a Th1 response will lead to Tfh cells that produce both IL-21 and IFNγ, driving B cells to produce IgG2 class-switched antibodies. Similarly, extracellular pathogens such as parasites will stimulate a Th2 response, and will induce Tfh cells to produce both IL-21 and IL-4 and drive an IgG1 response[107,108].

The GC response produces long-lived antibody-secreting plasma cells and memory B cells; because of this it is the main source of long-lived humoral immunity. The longevity of this response provides potent protection against future re-infection, but can have serious adverse consequences if the GC response is directed against components of self, or, in the context of transplantation, the graft. Indeed, uncontrolled GC responses can lead to spontaneous autoimmunity, as random mutation of BCR genes can lead to self-reactivity. In the presence of excessive Tfh cells these self reactive B cells can receive help to produce autoantibodies[109-111]. Thus, control of this response is essential to minimise adverse outcomes.

Tregs are an important mechanism of immune control. These CD4 T cells can either be thymically derived or peripherally induced and are characterised by expression of the transcription factor FOXP3[71,112,113]. Tregs act through a number of mechanisms, including anti-inflammatory cytokines, such as IL-10 and TGFβ, as well as cell-contact dependent inhibition of T cell activation by inhibitory receptors, such as CTLA-4 (CD152). In addition, Tregs can also express transcription factors traditionally associated with other Th subsets alongside FOXP3 and tailor their suppression to the response generated[114-116]. It is not surprising therefore that Tregs can also co-opt the Tfh pathway, expressing BCL6 alongside FOXP3, and upregulating surface markers associated with Tfh cells, such as CXCR5, ICOS and PD-1[117-119]. Upregulation of CXCR5 allows these cells to enter the B cell follicle, where they limit the size of the GC response, possibly through disruption of CD28-CD80/86 signalling by CTLA-4[120,121]. This inhibitory receptor leads to downregulation of CD80 and CD86 on B

cells and can therefore disrupt the signals between GC B cells and Tfh. However there are likely to be other mechanisms involved in T follicular regulatory (Tfr) cell mediated control of GC responses[122].

The collaboration between DCs, B cells, Tfh cells and Tfr cells leads to a high affinity, highly specific and long-lived antibody response, with the capacity for recall responses that occur much faster than initial responses. GCs normally begin to form around 6 days after initial antigen encounter and increase rapidly over a few days to form a mature GC[123].

If memory T cells are present, this response can form 2-3 days earlier, and can be much larger than a primary response[124]. Peripheral Tfh-like cells have been identified in both humans and mice, and are thought to be generated both before and during GC formation[125,126]. They can be seen in the circulation of both mice and humans early after an immunising event[127-130]. While GC Tfh cells express high levels of CXCR5, PD-1 and the transcription factor BCL6, circulating Tfh-like cells (coined cTfh) do not express BCL6, express lower levels of CXCR5, moderate levels of CCR7, and low or no PD-1[128,131,132]. They have also been shown to be antigen specific and are thought to represent a memory population[133].

While most cTfh cells do not express PD-1, a proportion that are $CXCR5^{+}CCR7^{lo}PD\text{-}1^{+}$ are seen at much lower levels and are thought to represent early memory Tfh cells, consistent with an active GC response and Tfh cell differentiation in SLOs[125,128]. These circulating cells have been described in

association with antibody production in vaccination[130], chronic infection[129] and autoimmunity[127,134-140]. These $CXCR5^+CCR7^{lo}PD\text{-}1^+$ Tfh cells are transcriptionally most similar to GC Tfh[129].

As seen in GC Tfh cells, cTfh cells can also express different surface receptors and produce different cytokines consistent with Th1, Th2 and Th17-like skewing. This would be a logical response to a skewed GC Tfh response, meaning that a memory response in future will initiate a pathogen-appropriate antibody response much earlier than a primary response. The surface markers CXCR3 and CCR6 have been used to divide $CXCR5^+$ cTfh cells into $CXCR3^+$ Th1-like cells, $CCR6^+$ Th17-like cells and $CXCR3^-CCR6^-$ Th2 like cells. Morita and colleagues showed that these cells, which they coined Tfh1, Tfh2 and Tfh17, produced different cytokines alongside IL-21 and had different B helper capacity[127]. $CXCR3^+CXCR5^+$ Tfh1 expressed T-bet and IFNγ, and in this study did not secrete IL-21 or induce B cells to produce antibody, although subsequent studies showed that they were efficient helpers of memory B cells, but not naïve B cells[130]. $CXCR3^-CCR6^-CXCR5^+$ Tfh2 expressed GATA3 and secreted IL-4, IL-5 and IL-13 alongside IL-21 and were efficient helpers of both memory and naïve B cells, including production of all antibody classes tested – IgG, IgM, IgA and IgE. $CCR6^+CXCR5^+$ Tfh17 expressed RORγT and produced IL-17A, IL-22 and IL-21. Like Tfh2, Tfh17 were also efficient B cell helpers, and induced production of IgG, IgM and IgA, but not IgE[127].

It has been proposed that cTfh cells represent a peripheral biomarker of GC activity, and therefore could be used to identify an active GC reaction. This has

been demonstrated in influenza vaccination, which normally generates a Th1 response. Following vaccination, subjects showed increased $ICOS^+PD\text{-}1^+CXCR3^+CXCR5^+CD4^+$ Tfh1 cells at 7 days, which correlated both with plasmablast appearance in the circulation and development of strain-specific influenza antibody[130]. There was no alteration in the proportion of $CXCR3^-$ Tfh2 or Tfh17. However, in autoimmune diseases, where Th17 cells have been implicated in pathogenesis[141-143] increased proportions of circulating Tfh17 were identified in patients with active disease, and reduced with successful treatment of the condition[127,138]. $CXCR3^-PD\text{-}1^+CXCR5^+CD4^+$ Tfh cells have also been associated with the production of broadly neutralising antibodies (bnAb) in HIV infected individuals. HIV is a chronic viral infection, where the virus persists and evades the immune system for many years[144]. Antibodies continue to develop throughout the course of infection, but the virus evolves rapidly and few of these antibodies are able to neutralise more than the strain to which they have developed[145]. However, a small number of individuals are able to generate bnAbs, which can clear more than 70% of the currently known strains of HIV. These individuals have significantly higher levels of $CXCR3^-PD\text{-}1^+CXCR5^+CD4^+$ Tfh cells in their circulation than either HIV negative controls or patients who do not develop bnAbs[129].

The alterations in proportions and activation status of cTfh cells have therefore been proposed as biomarkers for GC activity, allowing tracking of tissue responses to antigen through peripheral blood sampling, and reflecting an early time point in the generation of an adaptive antibody response to antigen[146]. Circulating cells resembling Tfr cells have also been identified in humans and

mice[128,147,148]. These cells express CXCR5, PD-1 and ICOS, as well as high levels of CD25, low levels of CD127, and intracellular FOXP3, the canonical transcription factor associated with Tregs. In mice these cells have been shown to have a lower suppressive capacity than lymph node (LN) Tfr cells [128], which themselves have similar suppressive capacity to Tregs[149]. In humans cTfr cells have been identified but characterisation and associations have been limited.

1.5 Response to transplanted tissue

Under normal circumstances, the response to foreign antigen follows the patterns described above, with a primary immunising event and a low number of T cells able to react to a particular antigen, estimated at 1 in 20 000 to 1 in 100 000[150]. However, transplantation is a unique situation for several reasons. First, the process of organ retrieval and implantation is extremely proinflammatory, with ischaemia-reperfusion injury, surgical wounds and donor factors such as infection contributing to the inflammatory milieu[6,151,152]. Second, the precursor frequency of T cells able to react to non-self MHC can be as high as 1 in 10, several orders of magnitude higher than other antigens[153]. Third, and related to this, because of the transfer of donor passenger leucocytes with the transplanted organ, there are multiple pathways of allorecognition. Fourth, there is a high frequency of sensitisation to human leucocyte antigens (HLA) within the population, meaning that memory responses are not uncommon. These factors will be discussed in turn.

Transplantation of tissue can be as an autograft from one part of an individual's body to another, e.g. vascularised flaps for reconstructive surgery; an isograft between genetically identical (syngeneic) individuals, e.g. monozygotic twins; an allograft between genetically different (allogeneic) individuals of the same species; or a xenograft between individuals of different species, e.g. porcine heart valves transplanted into humans. In the context of transplantation for ESRF, the overwhelming majority of transplants are allografts, either from deceased donors or from related or unrelated living donors. Therefore we will focus on allogeneic transplantation and the response to alloantigen.

1.5.1 Initiation of an innate response to the allograft

As described above, the innate immune system represents a pre-formed defence against foreign antigen, capable of an immediate response while the adaptive immune system generates a long-term antigen specific response. In the context of transplantation, there are several processes that can activate the innate immune system. The surgical procedure itself will damage tissues, leading to release of DAMPs, such as heat shock proteins and activation of the complement cascade. In addition to this, donor factors can play a significant role in generating a proinflammatory environment. Depending on the type of donor, the tissue will likely have a degree of ischaemia. There may be damage from the process that led to death, leading to both DAMPs and potentially PAMPs (e.g. if the donor had an infection prior to death) being present in the organ and upregulated after implantation[154-156].

There are two main categories of donor, living donors and deceased donors. Living donors (LD) can donate, for example, one of their two kidneys, or a portion of liver. Operating on someone who is well violates the Hippocratic oath to first do no harm, therefore living donors must be carefully selected and there must be a benefit to the donor to overcome the harm of surgery, for example a parent donating to a child. The advantage of a living donor is that infection, trauma or other damaging processes that can lead to death, are not present. In addition, the time from retrieval of the organ, when the supply of warm, oxygenated blood is stopped and cold tissue ischaemia begins, to reperfusion, when cold ischaemia ceases, is usually shorter. These factors should reduce the DAMPs produced by the tissue, and therefore reduce the initial inflammatory response to the transplanted organ.

Deceased donors can be divided into donation after brainstem death (DBD) and donation after circulatory death (DCD), and make up the majority of donors in the UK, with 1075 live donors and 1364 deceased donors (785 DBD, 579 DCD) in 2015-16[157]. In deceased donors, short-term outcomes are better in DBD than DCD donors, but long-term outcomes are comparable. Outcomes are worse in donors considered to meet ‘expanded criteria’ for donation (ECD) – age over 60 or over 50 with vascular comorbidities such as hypertension. Outcomes are significantly better in LD than deceased donors because of the proinflammatory processes surrounding death.

Death is a complex and active process widely accepted as an irreversible loss of the capacity to breathe, and capacity to remain conscious. This can be

diagnosed on somatic, cardiorespiratory or neurological criteria. Somatic criteria include those that can be identified without the need for medical examination, and would exclude organ donation.

The commonest criteria used to diagnose death are cardiorespiratory, where loss of cardiac activity leads to hypoperfusion of the brain, and hence respiratory centres, with simultaneous loss of consciousness and respiratory effort. DCD donors must have cardio-respiratory support (if in place) withdrawn and must be observed. When cardiorespiratory arrest occurs, a period of observation begins to ensure that spontaneous recovery does not occur. In the UK this is 5 minutes, and any sign of cardiac or respiratory effort necessitates beginning the observation period again. However, DCD transplantation leads to poorer outcomes for donated organs, as cardiorespiratory arrest after withdrawal of life support often involves minutes to hours of hypo-oxygenation and hypo-perfusion of organs, which leads to upregulation of heat shock proteins and other DAMPs, necrosis of cells and production of proinflammatory cytokines.

In the late 1960s, the neurological determination of death was proposed for those circumstances where brain injury is thought to have caused irreversible loss of consciousness and respiratory capacity before terminal apnoea has led to cardiorespiratory arrest. This is particularly relevant in the current era, where cardiorespiratory support can maintain function even in the absence of brainstem input. The internationally accepted neurological criteria for diagnosis of death are:

1. An established aetiology capable of causing structural damage to the brain, which has led to the irreversible loss of the capacity for consciousness combined with the irreversible loss of the capacity to breathe
2. An exclusion of reversible conditions capable of mimicking or confounding the diagnosis of death on neurological criteria
3. A clinical examination of the patient, which demonstrates profound coma, apnoea and absent brainstem reflexes

(Adapted from Gardiner et al, An International Perspective on the Diagnosis of Death, British Journal of Anaesthesia 2012[158]).

If a donor has been diagnosed as dead on neurological criteria, they can become a DBD donor and can then be taken to theatre for organ retrieval while cardio-respiratory support is still in place, meaning that well oxygenated blood is pumped at an adequate pressure to perfuse the organs. This form of donation improves outcomes for organs, however DBD organs will still suffer damage from the catecholamine storm that accompanies brainstem death, which can cause hypotension, cardiovascular instability and fluid and electrolyte imbalances. Careful management of the donor can reduce these[159], but organs from deceased donors of both types are primed to generate an inflammatory response.

LDs avoid any catecholamine storm or warm ischaemia from hypotension, however they are still prone to damage from cold ischaemia. Following retrieval of the organ, it is flushed with cold perfusion fluid and packed in ice to reduce

metabolism until it can be re-perfused with warm, oxygenated blood on implantation. Prolonged cold ischaemia, while not as damaging as warm ischaemia, increases the likelihood of delayed graft function, which is itself associated with acute rejection from an immune response to the transplanted organ[4,6,160].

Ischaemia-reperfusion injury is perhaps the greatest activator of the innate immune system[161]. Ischaemia leads to endothelial activation and an increase in adhesion molecules, as well as upregulation of heat shock proteins. When reperfusion occurs, reactive oxygen species are generated, which, along with endothelial changes, attract innate cells to the graft, activate cells, predominately through DAMP-mediated TLR4 ligation, and activate the complement cascade (see section **1.3** above). Macrophages and other phagocytes are attracted to the graft and begin to ingest necrotic tissue, releasing TNFα, IL-1, IL-6 and other proinflammatory cytokines[162].

Acute inflammation, secondary to tissue damage and invasion of innate immune cells, occurs even in genetically identical transplants by the innate mechanisms outlined above[44]. However, it is clear that the transplant of an allograft generates a much stronger innate response[45] and begins the process of generating an adaptive immune response. Monocytes are thought to be key players in the bridge between innate and adaptive responses[163]. They are one of the earliest cells to invade the graft, and become activated through TLR ligation. Animal models have shown that in the case of genetically identical individuals these monocytes do not differentiate further, and the inflammatory response

subsides[44]. However, in allogeneic transplants the monocytes differentiate into inflammatory DCs, upregulate costimulatory molecules and traffic to SLOs, where they can present antigen with costimulation and activate adaptive immune cells[44]. Depletion of monocytes, or blockade of TLR4 can improve ischaemia-reperfusion injury and allograft outcomes[154,164].

Development of inflammatory DCs that can migrate to SLOs and present alloantigen is one mechanism by which the adaptive immune system can be stimulated to generate a response to the allograft. However in the context of transplantation, there are other mechanisms of allorecognition.

1.5.2 T cell recognition of alloantigen

Transplantation is unique in terms of immunology, as priming of recipient T cells can occur not only by presentation of donor peptide on recipient APCs, but also by donor APCs. Three distinct pathways have been recognised for antigen presentation and allorecognition – direct, semi-direct and indirect (**fig 1.5**)[165].

Direct allorecognition is a unique but short-lived pathway for recipient T cell priming. Transplanted tissue contains donor passenger leucocytes (including donor derived immature DCs), which are activated by the inflammatory milieu surrounding transplantation. Activation leads these cells to differentiate into mature DCs expressing costimulatory molecules, as well as MHC class I and II of donor origin[166]. They migrate to SLOs where they can act as APCs, presenting donor peptides in the context of donor-derived MHC (**fig 1.5**). Recipient CD4 and CD8 T cells recognise the MHC as non-self regardless of the peptide presented

and become activated, initiating a response against the graft[50,167]. Originally, the direct pathway was thought to play the dominant role in transplant responses because T cells recognising alloantigen in the context of donor MHC represent the vast majority of the alloreactive T cell pool in the early response. Furthermore, animal work suggested that depleting donor APCs from the graft prior to transplantation led to long-term graft survival without immunosuppression[168]. However, other studies showed that donor APCs are rapidly killed by recipient NK cells in SLOs, and are unlikely to be able to activate recipient T cells[169,170]. More recent models have suggested that the expanded T cell repertoire post-transplant is stimulated by indirect allorecognition (described below) rather than direct[171,172]. Therefore it is likely that donor cells acting as APCs to recipient T cells plays a much smaller role in alloresponses than originally thought.

However, given the previous evidence supporting the role of donor passenger leucocytes, a new theory has been proposed that, rather than acting as APCs, the impact of donor-derived DCs is through transport of donor peptides from the graft to SLOs. There is evidence to suggest that intact peptide-MHC complexes can be passed between DCs. Thus non-self MHC could prime recipient T cells when it is presented on recipient APCs (**fig 1.5**)[173,174]. There is increasing evidence for this "semi-direct" pathway[175,176], and it has been suggested that it might dominate in early responses to the graft, which do seem to be directed against donor MHC more than other antigens.

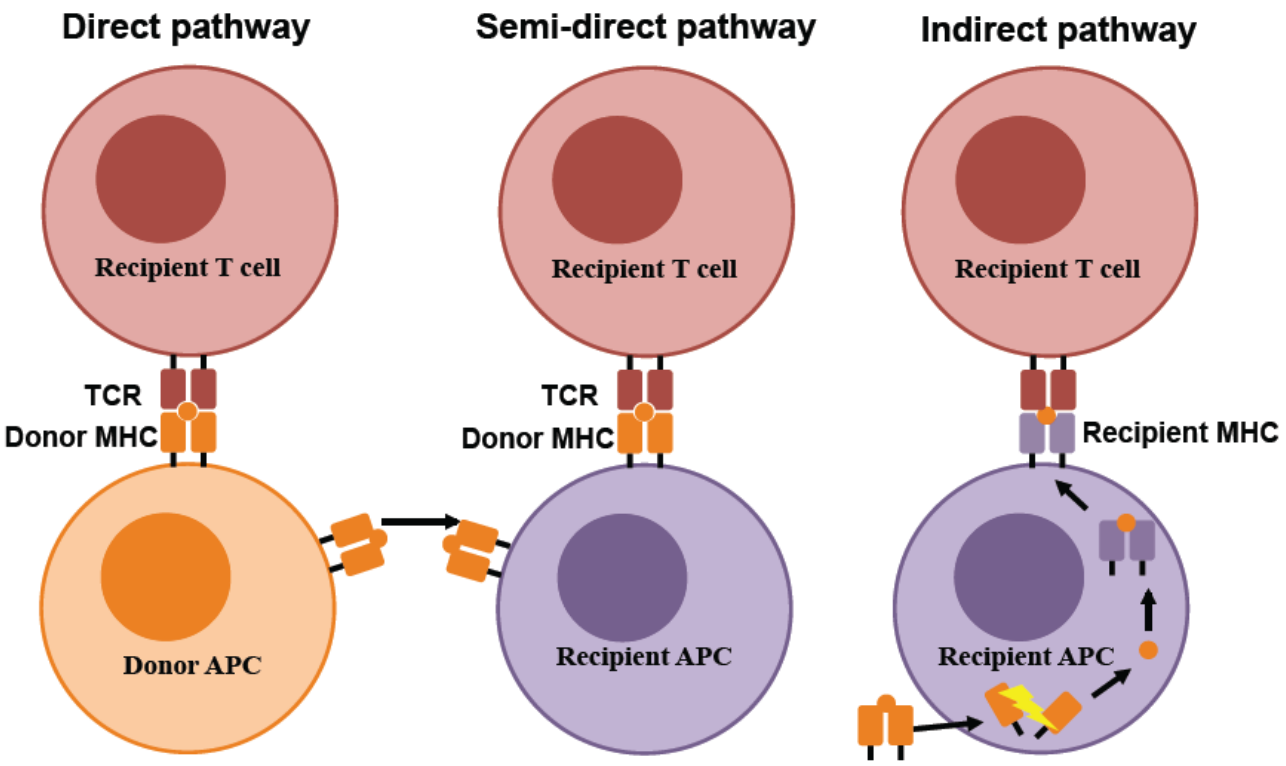


Figure 1.5: Pathways of Allorecognition. Adapted from Francis and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine, 5th Edition. In press, Wallin and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 6th Edition.

These two pathways are unique to transplanted tissue, however the third pathway, indirect allorecognition, dominates in the long term and is the classical pathway of antigen uptake and presentation (see sections **1.4.1** and **1.4.2**). Donor peptides taken up by recipient APCs, whether in the graft from the first wave of invading monocytes differentiating into DCs or in SLOs from donor APCs transporting peptide or through lymphangiogenesis[177], are degraded and presented on recipient MHC. This pathway comes to dominate in the long-term responses to the graft because donor APCs are rapidly destroyed and therefore direct and semi-direct responses can only be generated early post-transplantation[165].

1.5.3 Immunogenicity of MHC molecules and B cell responses to the graft

Whichever pathways of allorecognition occur, the main antigenic peptides presented are the hypervariable regions of the MHC molecules. MHC, which in humans is also known as human leucocyte antigen or HLA, is extremely polymorphic[58]. Each HLA molecule is a heterodimer, as shown in **fig 1.2,** and each polypeptide composed of combinations of amino acid epitopes encoded at multiple genetic loci. The number of serological types of HLA found in the population is wide, with, for example 20 separate HLA-A and 18 separate HLA-DR antigens. However, because of the diversity at the allelic level there are thousands of different proteins for each HLA class I and II molecule[178]. This allows generation of a very diverse repertoire of MHCs within a population, as polymorphisms allow binding of different peptides and thus presentation of a wide range of antigens. It also allows individuals to clearly distinguish self from

non-self by recognition of the HLA molecule itself. The evidence that genetically identical individuals did not reject organs while unrelated donor-recipient pairs had very poor outcomes, combined with detection of donor-specific antibodies[179] and characterisation of HLA molecules[180,181], led to attempts at matching donor and recipient HLA alleles.

The characterisation of HLA molecules was carried out serologically, by testing sera of multiparous women against cells of different individual. This was a stepwise process (detailed in chapter 3) and led to multiple levels of characterisation. Broad antigens, which were the first identified, were subsequently discovered not to be individual molecules, but shared epitopes between multiple molecules. These shared epitopes meant that antibodies directed against the epitope would react with any HLA molecule that shared that epitope, even if a different molecule had triggered the original antibody. This serological hierarchy led to the classification of cross-reactive epitope groups (CREGs) listing the potential cross-reactivity that might be expected between different HLA antigens[182,183].

Unfortunately in the case of transplantation, antibodies that develop against one HLA molecule can potentially sensitise patients to multiple molecules if they develop against a shared epitope. Anti-HLA antibodies are class switched[184-186], and persist in the circulation for many years both pre and post-transplant, suggesting that they have been produced by plasma cells that have come from the GC[100,187]. Circulating HLA-specific B cells with memory markers (CD27, CD28) have also been identified in transplant patients, suggesting a requirement

for GC formation, and hence for T cell help, in the development of a response to the transplanted organ[188]. While class switching and memory formation can both occur in the absence of a GC reaction, there is some evidence from mouse models that alloantibodies are somatically hypermutated and antibody mediated rejection requires the GC reaction[189]. Studies in humans have been more limited because of the difficulty of obtaining secondary lymphoid tissue, but kidneys removed following rejection have shown evidence of somatic hypermutation in intra-graft B cell aggregates[190]. It is therefore likely that the GC reaction is necessary for development of anti-HLA antibodies.

Because of the high precursor frequency of T cells recognising non-self HLA, antibody responses can develop after any exposure to non-self HLA, for example blood product transfusion, pregnancy or transplantation. Sensitisation through blood product transfusion is controversial. In the general population rates of anti-HLA antibody formation following blood product transfusion are generally very low, less than 2%[191,192]. However anti-HLA antibody has been found in up to 33% of previously transfused transplant patients[193], although this is thought to require either multiple transfusion events or very large volumes to have a lasting impact[194,195]. Scornik *et al.* reported that levels of anti-HLA antibodies after transfusion often decreased with time from transfusion and patients rarely had circulating anti-HLA memory B cells detectable, suggesting that extremely high numbers of transfusions may be required to develop lasting allosensitisation[195].

In contrast, women who have been pregnant have an extremely high rate of sensitisation to paternal antigens, whether the pregnancy was carried to term or

not. Antibodies to paternal antigens appear after 28 weeks of pregnancy, between 30-50% of women will become sensitised during their first pregnancy[192,193,196], and this increases with further pregnancies[197].

The highest risk of allosensitisation comes from previous transplantation[198] with 8-10% of recipients developing *de novo* donor-specific anti-HLA antibodies (DSA) within the first year[199,200], and between 15-30% within 10 years[13,201,202]. These antibodies are associated with an increased risk of graft failure[11,13,203,204]. If a failed graft is removed[205-207], or immunosuppression stopped in the light of graft failure[208,209], the rate of antibody formation rises further. The former thought to be due to the inflammatory process surrounding surgery, combined with the release of donor antigens, as well as retention of small quantities of donor antigen, for example vessel patches, which cannot easily be removed. Antibody levels can remain high for years following graft removal, suggesting generation of long lived, bone marrow resident plasma cells. Memory B cells to HLA antigens are also detectable many years after transplantation, whether the graft remains *in situ* or has been removed[210]. This long-lived response is thought to be why second grafts have poorer outcomes if they share mismatched antigens with first grafts, even in the absence of circulating antibodies, and hence such repeat mismatches are generally avoided[211-214].

It is also possible for anti-HLA antibodies to occur in the absence of a defined sensitising event. A large study looking at healthy males with no history of transplantation, blood transfusion or recent infection identified anti-HLA antibodies in over 60% of participants[215]. These were to HLA specificities that are

rare in the general population, and several theories have been proposed for their development. It is possible that heterologous immunity from previous viral infection may cause cross-reactivity to HLA epitopes. Animal models have shown that viral infections can generate populations of alloreactive memory-phenotype T cells, possibly through epitope sharing, or TCR cross reactivity, and the presence of these cells was shown to prevent transplant tolerance induction by protocols that usually resulted in reliable long-term graft acceptance[216,217]. It is possible that a similar mechanism could explain these 'natural' HLA antibodies in humans, as HLA reactive memory T cells have been identified in previously non-sensitised individuals following Epstein-Barr and herpes virus infections[218-220].

Another proposed theory is of homeostatic proliferation following lymphopenia. The size of the T cell pool, and the relative ratios of CD4 T cells to CD8 T cells, as well as of naïve to memory T cells, is tightly controlled. There is considerable evidence from animal models that depletion of the T cell population prior to transplantation speeds rejection and augments memory responses[217,221,222]. Reduction in cell numbers induces proliferation of residual cells even in the absence of cognate antigen. Because of the high precursor frequency of alloreactive T cells, it is highly likely that homeostatic proliferation could generate alloreactive memory T cells. This may explain the development of 'natural' antibodies without sensitisation, but is of particular concern in the case of transplant patients, many of whom receive T-cell depleting therapies either as part of induction therapy or treatment for rejection.

However, it is also possible that these pre-formed antibodies are not of clinical significance, and may be false positives due to the methods of testing, reacting to denatured antigen rather than intact antigen on cells[223-226] (see chapter 3 for more detail of testing methods).

1.5.4 Sensitisation of the UK transplant waiting list

Regardless of the mechanism of sensitisation, it remains a major problem in transplantation. In the UK up to half of the patients on the waiting list have some detectable HLA antibodies. Over a quarter of the patients on the list are considered highly sensitised, with pre-formed HLA antibodies that are calculated to react (calculated reaction frequency, cRF) to over 85% of the UK population, based on known HLA allele frequencies[227]. This is an issue worldwide, as sensitisation reduces the likelihood of a suitable and well-matched transplant donor being identified. Outcomes are worse in sensitised patients[10,228,229] and reducing the development of *de novo* DSAs is a key goal in transplantation.

1.5.5 Rejection of a transplanted organ

With the combination of responses to a graft from the innate immune system, new adaptive responses and memory responses, rejection of a transplanted organ can take several forms. Rejection is broadly categorised by timing from transplant, and the cells involved. These are summarised in the table below, adapted from Wallin & Wood[25].

Table 1.4: Mechanisms of rejection

Timing	Cellular mechanisms	Humoral mechanisms
Hyperacute (minutes – hours)		Preformed, complement fixing anti-HLA or ABO antibodies
Acute (days – weeks)	Allospecific $CD8^+$ T cells – acute T cell mediated rejection	Preformed anti-HLA antibodies – acute antibody mediated rejection
Chronic (weeks – years)	Repeated episodes of acute T cell mediated rejection lead to fibrosis and scarring	Chronic antibody mediated rejection

Hyperacute rejection

Hyperacute rejection occurs within the first 24 hours after transplantation in the context of high titre, pre-formed anti-HLA antibodies, or antibodies directed against ABO blood antigens. It is a classical antibody-mediated process where binding of the antibody leads to complement activation, coagulation cascade activation and widespread endothelial injury, activating platelets and leading to microvascular thrombosis[230]. Thrombosis occurs throughout the graft and leads to rapid infarction, which can occur within minutes of reperfusion. Hyperacute rejection was responsible for many of the early graft failures[231,232] but is now exceedingly rare due to modern matching techniques, immunosuppression and desensitisation regimens.

T cell mediated rejection (TCMR)

Alloreactive T cells that have been activated by APCs in SLOs migrate to the site of inflammation along chemokine gradients. The endothelial activation of the

graft vessels from ischaemia and reperfusion triggers activation of the endothelium of anastomosed recipient vessels, due to complement activation and production of pro-inflammatory cytokines by invading innate cells. Vessels dilate and flow slows to allow activated leucocytes to move from the fast laminar flow in the centre of vessels towards the endothelium. Endothelial cells contain Weibel–Palade bodies with pre-formed P-selectin (CD62P), which they can rapidly upregulate when they become activated[233]. Leucocytes bind to P-selectin, initially forming low affinity interactions that slow the leucocytes further until they begin 'rolling' along the endothelium. Inflammation causes expression of chemokines on endothelial cells, allowing them to bind other adhesion molecules on the leucocytes with higher affinity. These high affinity interactions cause conformational changes in the leucocytes, allowing them to extravasate between endothelial cells and enter the graft along the chemokine gradient[233,23.]

In the graft, activated CD8 T cells target class I HLA on donor cells and form an immunological synapse. These T cells release perforins to form a pore in the cell membrane, through which granzyme B and other cytotoxic mediators can be inserted into the cell to trigger the caspase cascade and apoptosis of the target cell. CD8 T cells can also trigger apoptosis through engagement of Fas ligand (FasL) with Fas on target cells[234].

Although the major responding cells in acute TCMR are CD8 T cells, CD4 T cells also play a role in the acute inflammatory response to a graft[235]. Allospecific Th1 cells release proinflammatory mediators such as IFNγ, TNFα and IL-1. These cytokines promote graft infiltration by both innate and adaptive cells, and

stimulate monocytes and macrophages to produce prostaglandins, nitric oxide and reactive oxygen species. These in turn increase cell permeability and lead to vasodilatation, increasing blood flow to the graft and thus increasing immune cell invasion. This vascular permeability and increased blood flow can lead to oedema and stretching of the graft capsule, which can cause pain in severe acute TCMR[236].

For kidney transplants, the accepted histological criteria for diagnosis of TCMR were described at a meeting of pathologists in Banff, Canada, and are updated regularly to reflect current thinking about the mechanism underlying and the pathology of TCMR (see Appendix 1 for full Banff classifications). The histological criteria for TCMR include interstitial and tubular inflammation, diagnosed with oedema and lymphocyte infiltration. As the inflammation increases and begins to affect the intima of arteries, the grade of TCMR worsens. For example, Banff IA TCMR includes interstitial inflammation with foci of moderate tubulitis; Banff IIB requires severe intimal arteritis with more than 25% of the luminal area involved, alongside interstitial inflammation and tubulitis[16].

The Banff criteria also recognise ‘chronic active TCMR’ as a possible diagnosis, comprising chronic allograft arteriopathy. For diagnosis, biopsies must show evidence of arterial intimal fibrosis with mononuclear cell infiltration and formation of a neo-intima. However, the Banff working groups recognise that these changes may represent the end result of abnormal healing from multiple bouts of acute TCMR, with disordered healing leading to macrophages promoting fibrosis

rather than repair[237-239]. The changes can also come about through antibody mediated damage, and differentiating between chronic damage from TCMR and ABMR is controversial[15].

Antibody mediated rejection (ABMR)

Antibody mediated rejection can be acute or chronic. Antibodies bind to their target antigen via the variable region, or Fab region, but the consequence of binding is generally mediated by the constant region, or Fc region[23]. There are three main mechanisms by which antibodies can protect the individual – neutralisation by binding and blocking, e.g. viral entry; coating the pathogen to promote phagocytosis, or opsonisation; and complement activation via the classical pathway. The main mechanism of acute antibody mediated rejection is, like hyperacute rejection, activation of complement leading to MAC formation and cell lysis. Antibody-dependent cell-mediated cytotoxicity (ADCC), via NK cell and macrophage binding of Fc receptors, provides a second mechanism. The classical pathway of complement activation requires C1q to bind to antigen-antibody complexes and convert from its inactive form to the active form (see section **1.2**). This leads to the conversion of C4 from an inactive to an active form, and its combination with the active forms of C2 to form the C3 convertase, which converts C3 to its active forms. As part of this process, complement breakdown products such as C4d are deposited in tissues, and this formed the cornerstone of diagnosis for acute ABMR in the early Banff criteria.

For acute ABMR three features are required to confirm the presence of graft-damaging antibodies:

1. Evidence of acute tissue injury – microvascular inflammation, arteritis, acute thrombotic microangiopathy or acute tubular injury
2. Evidence of current or recent antibody interaction with endothelium such as linear C4d staining, moderate microvascular inflammation or gene transcripts suggesting endothelial injury
3. Serological evidence of DSAs

However, in recent years, it has been recognised that the presence of antibodies, even without evidence of acute tissue injury or complement breakdown products, is associated with worse graft outcomes[11-13]. A new entity of chronic ABMR has been recognised, which still requires serological evidence of antibodies and evidence of their interaction with the endothelium, but is diagnosed on the basis of chronic tissue injury. This can include transplant glomerulopathy (thickening of the glomerular basement membrane with scarring or sclerosis of the glomerulus), peritubular capillary basement membrane multilayering or arterial intimal fibrosis. All of these features point toward a more chronic, fibrotic process rather than an acute inflammatory process[16].

It is not clear why some alloantibodies cause acute inflammation and rejection, whilst other antibodies, still directed against the graft antigens, can lead to a slow and gradual decline. Hyperacute rejection and early ABMR are frequently associated with high titre antibodies[240], however high titre antibodies can also be associated with stable graft function, so this is clearly not the only factor

associated[241,242]. One possible factor could be the antibody class and Fc region effector function. Different antibody classes have different capacity to generate effector functions, including activation of complement and recruitment of effector cells via Fc receptors [243].

Anti-HLA DSAs are class switched, and generally IgG[244]; IgM and IgA DSA are seen but no convincing evidence of their role in antibody-mediated damage has been shown[245,246]. The majority of rejection episodes are associated with IgG antibodies[246], which themselves can be divided into four subclasses. In the context of infection, these subclasses are induced by different stimuli and cytokines in response to different types of pathogens. In humans, IgG1 and IgG3 are effective against viruses[247], and are the most potent when it comes to complement activation and Fc receptor activation on phagocytes. IgG2 is usually produced in response to encapsulated bacteria and causes moderate Fc activation and only weakly activates complement[243]. The role of IgG4 is not yet clear; it is normally produced in response to large, extracellular parasites[247] as well as food and environmental allergens. Its presence therefore seems to indicate long-term exposure to antigens and it is postulated to have a role in preventing responses to allergens[248,249]. However IgG4 has also been associated with progressive, multi-organ fibrotic diseases such as retroperitoneal fibrosis and sclerosing pancreatitis, as well as organ-specific disorders such as membranous nephropathy[250,251]. IgG4-related systemic disease is characterised by predominately CD4 T cell lymphocytic infiltrates with formation of GC-like structures in affected organs and the presence of $IgG4^+$ plasma cells, surrounded by characteristic storiform (literally ‘cartwheel-like’) fibrosis[250]. As a systemic

disease, it can affect the kidney, leading to a membranous-like pattern of disease and nephrotic range proteinuria[252]. Interestingly, IgG4 has also been associated with idiopathic membranous nephropathy, a separate entity involving expansion of the glomerular basement membrane with deposition of IgG and C3, also leading to nephrotic range proteinuria. It can be secondary to a number of systemic conditions, but the majority of cases are idiopathic. Recent evidence has suggested a role for IgG4, as the recently described anti-PLA2 antibodies[253] that are associated with active disease co-localise with IgG4 staining in renal biopsies in up to 70% of patients with idiopathic membranous nephropathy[254]. The presence of this antibody appears to predict response to immunosuppressive therapy, so a pathogenic role has been postulated[255].

However, IgG4 binds only weakly to Fc**γ**RI, lacks the ability to form immune complexes and is a poor activator of complement. Therefore, in many studies, IgG4 is considered to be 'non-inflammatory', particularly as it binds Fc**γ**RIIb, the only inhibitory Fc receptor[256]. Indeed, IgG4 has been considered to be protective in allergic responses[257] and has been shown to be produced by regulatory B cells[248]. It is not clear if IgG4 is pathogenic and has mechanisms of action yet to be elucidated, or if it is a downstream product of chronic antigen stimulation and an attempt to control inflammation.

In alloantibody research, there has been some interest in the subclass distribution of IgG DSAs, both pre-formed and *de novo* post-transplant. It is clear that IgG1 is the commonest both in terms of *de novo* and pre-formed antibodies, and many groups have shown an association between IgG1 and IgG3 subtypes and acute rejection episodes [258-261]. This would seem logical, given the effector

mechanisms of these subclasses and the histological changes seen with acute ABMR. Interestingly there have been reports of an association between IgG4 and chronic graft deterioration. Lowe *et al.* reported that patients who had chronically failing grafts tended to develop an anti-HLA-DQ antibody, with a subclass skew towards IgG4[262]. In non-HLA antibodies a similar trend has been seen, with pre-formed anti-endothelial cell antibodies skewed towards IgG2 and IgG4 being associated with higher serum creatinine, although in these patients there was also an increased risk of TCMR[263]. More recently, a large cohort study of 125 patients with DSAs showed that IgG3 was strongly associated with acute ABMR, increased microcirculation injury and complement activation (measured by C4d deposition); whereas IgG4 was strongly associated with subacute ABMR with transplant glomerulopathy and interstitial fibrosis with tubular atrophy[264].

Overall it appears that increasing variety of subclasses reflects longer exposure to donor antigen and more sensitising events or proinflammatory stimuli[200,262]. It is conceivable that the shift from IgG3, the first subclass produced, through to IgG4 is associated with a shift from acute ABMR, with an acute inflammatory infiltrate and complement activation, to chronic, low-grade ABMR with interstitial fibrosis. This may be why there is little evidence of acute rejection in the form of C4d staining on many later biopsies. However it is not clear if or how IgG4 can cause damage in these cases, or if it is a consequence of chronic antigen exposure and the fibrosis is driven through other mechanisms.

One question that remains is how and when the shift from IgG3 to IgG4 might occur. In IgG4 related disease, Th2 responses were originally implicated, however, more recent studies have shown that cTfh cells, particularly the Th2-

like subset, may be involved in both generation and maintenance of disease[265,266]. cTfh2 cells were found in higher proportions in the circulation of patients with systemic IgG4 disease and induced B cells to differentiate into IgG4-producing plasma cells *in vitro*. Numbers of $IgG4^+$ plasma cells fell with successful treatment of disease. However, it is yet to be determined if the GC-like structures found in the affected tissue of IgG4 patients contain Tfh2-like cells.

This is an intriguing concept that fits with the theory that cTfh cells may be able to migrate to inflamed tissue and set up tertiary lymphoid organs (TLOs) to induce local antibody production. The development of TLOs has been previously described in areas of chronic inflammation in autoimmune disease, chronic infection, malignancy and allograft rejection[267-269]. In transplantation, TLO formation has been associated with rejection in murine models of heart and skin transplant[270,271]. Infiltrating lymphocytes were found to contain both B cells and follicular-helper like CD4 T cells in rat aortic allografts, and to be associated with antibody production independent of SLOs, suggesting local antibody production[272].

In human transplantation, follicle-like structures have been identified in the biopsies of rejecting grafts by several groups, with $BCL6^+$ Tfh cells co-localising with B cells[273,274], and B cells showing evidence of somatic hypermutation, suggesting local GC formation[275]. Local antibody production has not yet been linked with graft loss in human transplantation, but all biopsies in these studies were taken from rejecting grafts and showed evidence of on-going ABMR and/or TCMR.

1.6 Preventing rejection of transplanted organs

With many potential mechanisms for activating the immune system in transplantation, it is clear that equally detailed methods for preventing activation would be required. Since the early days of transplantation, these have evolved from basic HLA-matching, through the use of single immunosuppressive agents; to the current era of complex HLA and antibody matching, induction and maintenance immunosuppression; and future prospects of improving donor organ condition and tolerance to the graft through cell therapies.

1.6.1 Matching donor with recipient

As described in section **1.5.3** above, and in chapter 3, HLA is extremely polymorphic and matching at an allelic level is neither feasible nor necessary to prevent rejection. Multiple alleles can encode for each HLA molecule, and large studies have shown that outcomes are reasonable if patients are matched at the broad antigen level for HLA-A, B and DR[276-280]. This is the current matching scheme in the UK.

Patients on the waiting list will therefore have their HLA type determined by DNA analysis, and this information is sent to allocation centres (NHSBT-ODT in the UK) to be compared to national databases with matching software to generate a list of acceptable and unacceptable mismatches for each individual.

Patients are also screened for pre-formed anti-HLA antibodies (screening methods covered in detail in Chapter 3). This was originally carried out using a

system known as the complement-dependent cross match, or CDC-XM (**fig 1.6A**). This was a crude measure, only detecting high titre, complement-fixing HLA antibodies and was variable between laboratories depending on the panel of lymphocytes chosen[281]. Cell based assays using flow cytometry improved the sensitivity of antibody detection (flow cross match, FCXM, **fig 1.6B**). This allowed identification of lower titre antibodies binding donor leucocytes, without requiring complement fixation and cell lysis[282,283]. However these assays were still dependent on the choice of lymphocytes for the panel, and therefore could not guarantee that all antibody specificities had been identified.

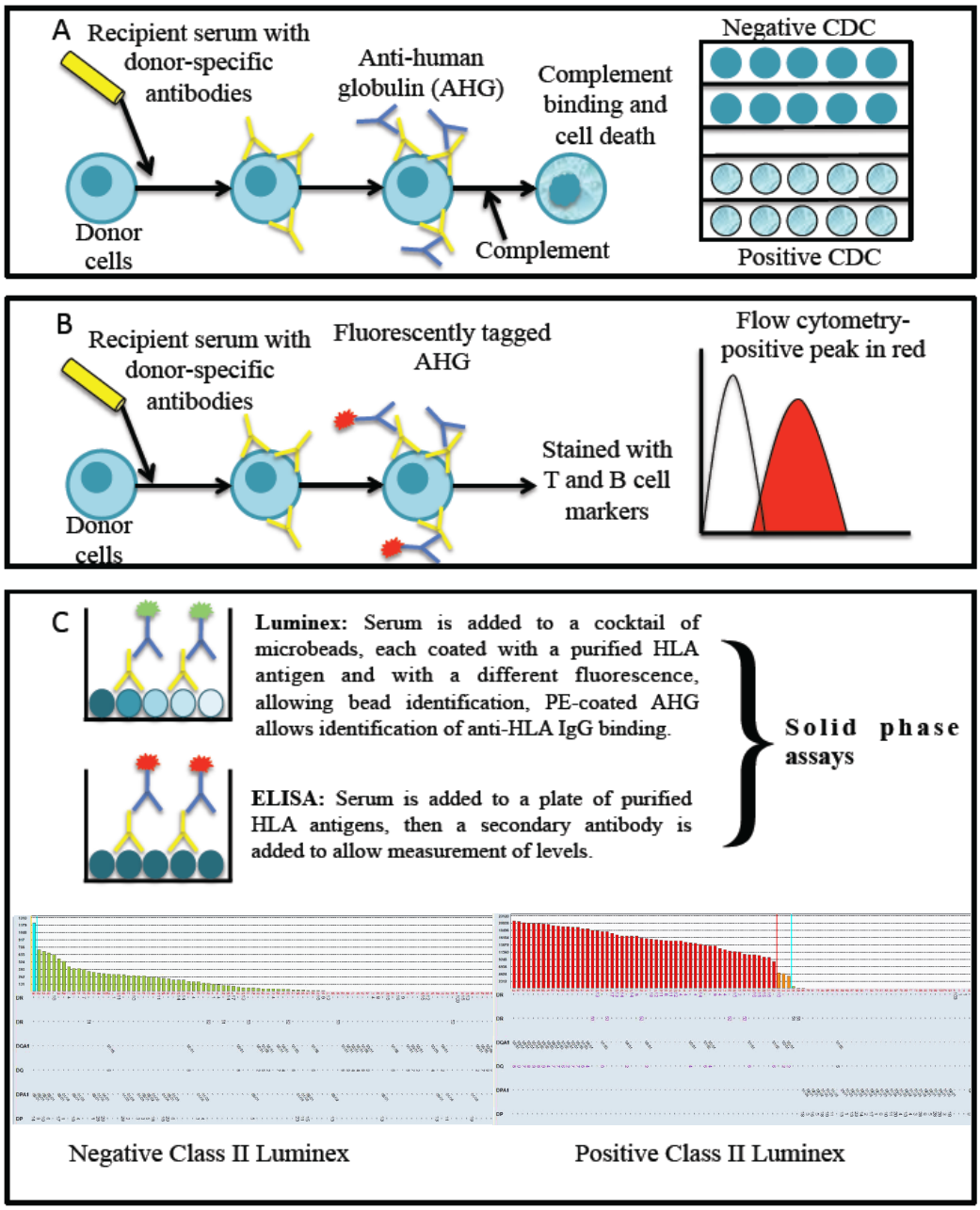


Figure 1.6: Cross matching techniques. A) Complement dependent cytotoxicity cross match, B) Flow cross match, C) Solid phase assays. Adapted from Wallin & Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 6th Edition.

The development of solid phase assays (SPAs) improved screening enormously (**fig 1.6C**). These assays allow detection of all potential antibodies, with the caveats described above in 'natural antibody' sensitisation, that a reaction to denatured HLA may not correlate with a reaction to intact HLA on cells[223-226,284,285].

Pre-transplant screening is now carried out using SPAs, with broad screens initially and then more detailed screens if patients test positive. All positive results are confirmed by repeat testing on a separate occasion, as antibody levels can fluctuate. Repeat testing is also recommended after any sensitising events such as pregnancy, transfusion or transplantation[286,287]. Sensitisation is now reported as cRF, the proportion of the potential donor population that the patient would be calculated to react to.

1.6.2 Pre-transplant crossmatch

Except in liver transplantation, all transplants require a cross match test of recipient against donor to exclude the presence of complement fixing antibodies to donor HLA. In the majority of cases this is done immediately prior to the transplant, with a combination of tests that may include the CDC-XM assay, FCXM assay, and SPAs. The use of these tests has reduced the risk of hyperacute rejection and acute ABMR significantly. Patients can be categorised as high, intermediate or standard risk, depending on which tests are positive and whether positive tests are current or historical (**fig 1.7**)[287].

Table1. Immunological pre-transplant risk assessment based on donor crossmatch and antibody screening results

Donor crossmatch result	Crossmatch method	Current or Historical	Antibody screening results	Interpretation of Immunological Risk
Positive T & B cell	CDC (DTT)	C	IgG HLA class I DSA	High risk* Hyperacute rejection (veto to transplantation)
Positive B cell	CDC (DTT)	C	IgG HLA class II DSA	High risk*
Positive B cell	CDC (DTT)	C	Weak IgG HLA class I DSA	Intermediate risk^
Positive T & B cell	FCXM (CDC neg)	C	IgG HLA class I DSA	Intermediate risk^
Positive B cell	FCXM (CDC neg)	C	IgG HLA class II DSA	Intermediate risk^
Positive T & B cell	CDC (DTT)	H	IgG HLA class I DSA	High risk$
Positive B cell	CDC (DTT)	H	IgG HLA class II DSA	High risk$
Positive B cell	CDC (DTT)	H	Weak IgG HLA class I DSA	Intermediate risk^
Positive T & B cell	FCXM (CDC neg)	H	IgG HLA class I DSA	Intermediate risk^
Positive B cell	FCXM (CDC neg)	H	IgG HLA class II DSA	Intermediate risk^
Positive T & B cell	CDC (neg DTT)	C or H	IgM HLA class I DSA	Standard risk
Positive B cell	CDC (neg DTT)	C or H	IgM HLA class II DSA	Standard risk
Positive T & B cell	CDC (neg DTT)	C or H	IgM non-HLA (often autoreactive)	Standard risk
Positive B cell	CDC (neg DTT)	C or H	IgM non-HLA (often autoreactive)	Standard risk
Negative T & B cell	FCXM	C or H	IgG HLA class I or II DSA (detected by Luminex SAB alone)	Standard risk
Positive T &/or B cell	CDC and/or FCXM	C or H	Negative (Luminex Ab detection and/or SAB)	Standard risk (IgM/IgG non-HLA, often showing in vitro autoreactivity)
Positive T; Negative B cell	CDC and/or FCXM	C or H	Positive (Luminex SAB - not donor-specific) or negative	Standard risk (results suggest antibody is not HLA-specific)
Negative T & B cell	FCXM	C or H	Positive (Luminex SAB) not donor HLA-specific	Standard risk
Negative T & B cell	CDC and/or FCXM	C or H	Negative (Luminex Ab detection and/or SAB)	Standard risk

* High immunological risk: hyperacute rejection is unlikely (reported only in cases with very high titre HLA-DR antibodies) but donor-specific HLA class II antibodies are increasingly recognised as being associated with refractory humoral rejection and poor transplant prognosis.

^ Intermediate immunological risk: transplantation should be avoided if reasonably possible (i.e. short waiting time, easy to avoid unacceptable mismatches) but may be undertaken with appropriate clinical caution; consideration for enhanced immunosuppression, proactive use of clinical intervention strategies and post-transplant antibody monitoring.

$ Risk of anamnestic secondary T and/or B cell response: need to consider high risk immunosuppression strategy, the duration, titre and priming source of antibody and repeat mismatches (pregnancy or regraft). Historical positive crossmatches caused by cross-reactive alloantibodies (avoiding the main specificity and priming stimulus) constitute intermediate immunological risk and are less likely to be associated with refractory T or B cell responses.

Figure 1.7: Risk stratification for pre-transplant crossmatching. Figure from the British Society for Histocompatibility and Immunogenetics, British Transplantation Society 'Guidelines for the detection and characterisation of clinically relevant antibodies in allotransplantation' 2014.

In patients with no antibodies or stable and well-characterised antibodies, it is possible to perform a 'virtual' crossmatch, where the potential donor genotype is compared against the recipient's genotype and antibody profile without the need for a pre-emptive crossmatch[288], which can take several hours and prolong the ischaemic time of the organ. A confirmatory cross match is recommended within 24-48 hours of a virtual match to confirm the lack of donor reactivity[287].

1.7 Immunosuppression

Early transplants were performed without any immunosuppressive agents, and universally failed until the first successful transplant between monozygotic twins in 1954 by Murray, Merrill and Harrison[289,290]. Further transplants between twins were successful while those between less closely related individuals failed within the first few days or months[291]. Immunosuppressive agents were introduced by Roy Calne in the 1960s and revolutionised transplantation[292]. Initially monotherapy with azathioprine was used, and then this was combined with corticosteroids to allow successful transplants between genetically non-identical individuals[293].

Calcineurin inhibitors (CNIs) were discovered in the 1970s[294] and combined with azathioprine and steroids to form the first triple therapy, improving survival in kidney transplantation to over 80% at 1 year, and over 50% at 5 year [295,296]. Modern triple therapy still includes a CNI, but tacrolimus has superseded ciclosporin as a more potent and less nephrotoxic alternative[297,298].

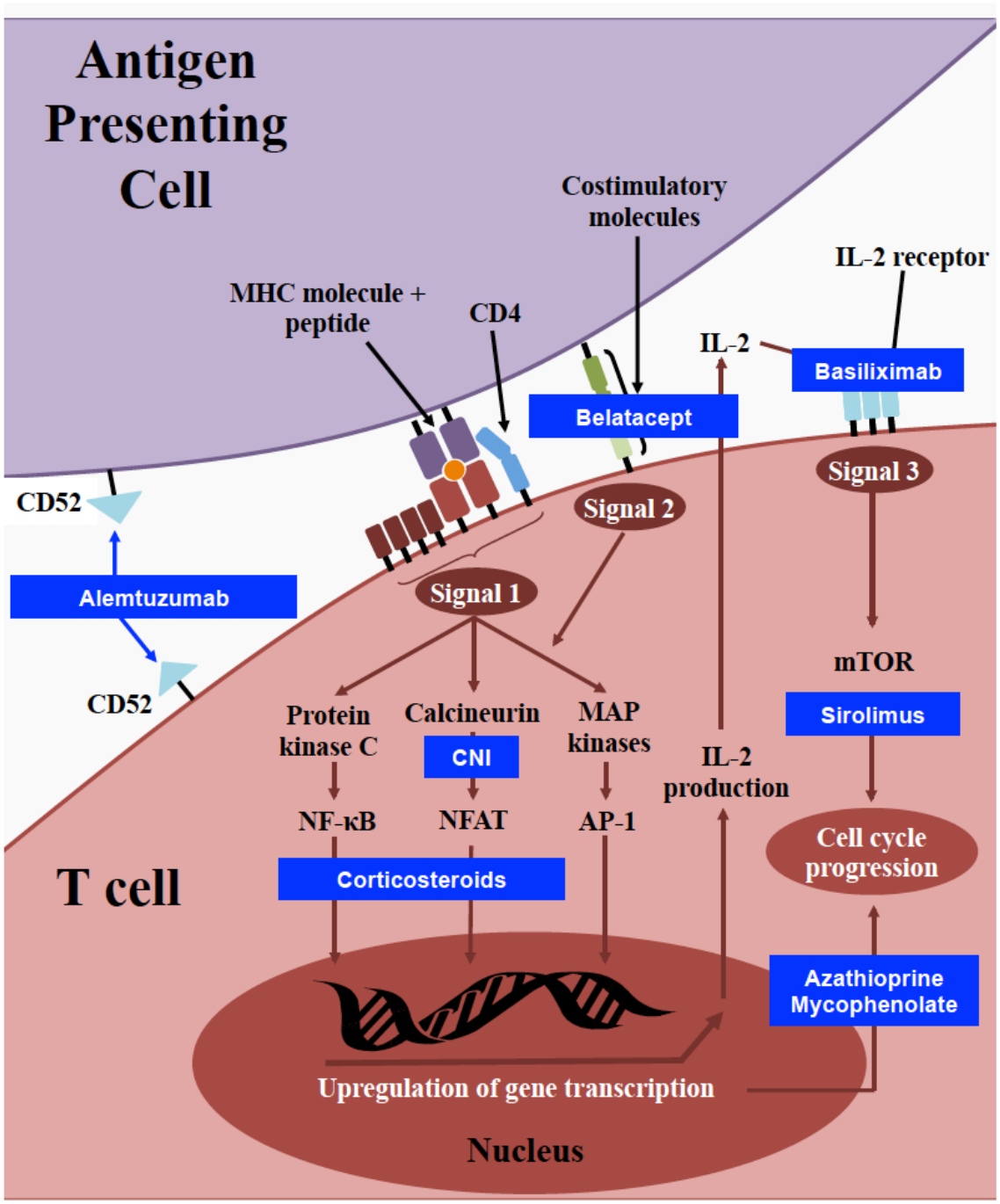


Figure 1.8: Site of action of immunosuppressive agents. Adapted from Francis and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 5th Edition. In press, Wallin and Wood, Principles of Transplantation Immunology, Oxford Textbook of Medicine 6th Edition.

Azathioprine is still in routine use along with a related anti-metabolite mycophenolate mofetil (MMF)[299]. The discovery of immunosuppressive agents is covered in more detail in Chapter 5.

Current treatment for all solid organ transplants consists of induction and maintenance therapy, aimed at targeting multiple aspects of the immune response to the graft (**fig 1.8**)[300,301].

1.7.1 Induction therapy

Induction therapy in most solid organ transplants consists of intravenous corticosteroids to suppress the acute inflammation and reduce innate responses, and either a polyclonal or monoclonal antibody designed to limit adaptive responses to the graft.

Corticosteroids

Developed in the 1950s corticosteroids, a synthetic version of the adrenal glucocorticoids, have complex immunomodulatory effects. Their main mechanism of action is through binding to glucocorticoid receptors, causing translocation to the nucleus, where these receptor complexes alter the transcription of pro-inflammatory mediators such as interleukins, TNFα, leucotrienes and prostaglandins[302]. Glucocorticoids help to suppress the early innate responses to the graft and are used as therapy for rejection episodes as well as induction. Initially used as triple therapy for maintenance, they have fallen

out of favour due to the plethora of side effects associated with steroid therapy including glucose intolerance, weight gain, hyperlipidaemia and osteoporosis[303]. Corticosteroids are given intravenously at the time of transplant and then orally for the first few weeks to months in many patients, but weaned and stopped wherever possible. Steroid-sparing regimens are also used in patients prone to glucose intolerance or with overt diabetes mellitus.

Polyclonal antibodies

Developed early in the history of transplantation, polyclonal antibodies are produced by immunising animals (rabbits or horses) with human lymphocytes. Immunoglobulins directed against human lymphocytes can then be purified for clinical use. Anti-thymocyte globulin (ATG) is the most widely used polyclonal antibody, causing profound lymphodepletion of T cells particularly, with slow reconstitution over many months. However, ATG can cause serious side effects including cytokine release syndrome, a potentially life threatening systemic inflammatory response causing fever, rigors, hypotension and potentially pulmonary oedema[304]. Additionally, the long-lasting lymphopenia associated with ATG leads to an increased risk of infection and malignancy after transplantation, and therefore it is currently recommended as induction therapy only for high-immunological risk patients, and for the treatment of steroid-resistant acute rejection episodes[305]. Given these side effects, alternative induction therapies were designed, and monoclonal antibodies provide more targeted therapy than polyclonal.

Monoclonal antibodies

Two main categories of monoclonal antibody are used for induction therapy, depleting and blocking. The most widely used depleting antibody is alemtuzumab, an anti-CD52 monoclonal antibody that is in routine use in kidney[306-308], pancreas[309] and other solid organ transplants[310,311]. The function of CD52 is not clearly understood, but administration of alemtuzumab causes rapid and profound lymphocyte depletion with slow recovery first of B cells, then CD8 T cells, then CD4 T cells, the latter of which can remain suppressed for years after the initial dose[312,313]. Alemtuzumab is also used for treatment of steroid-resistant rejection in patients who have had alternative induction agents. It can cause similar problems to ATG with cytokine release syndrome, although this is less common and can be controlled with concomitant corticosteroid administration. Importantly, the risk of malignancy and infection appears to be lower with alemtuzumab than ATG[307,314,315].

The most commonly used non-depleting monoclonal antibody is basiliximab, which is a blocking antibody directed against the high affinity IL-2 receptor α-chain CD25. By blocking CD25, it inhibits signal 3 of T cell activation (**fig 1.8**) and hence reduces autocrine and paracrine stimulation of T cell activation and proliferation. Given at the time of transplant, the antibody can only be detected for 1-2 weeks, with its effects on CD25 persisting for 4-6 weeks, and therefore it limits early T cell responses to the graft[316,317]. Studies showed that acute rejection rates were reduced by a third in those receiving basiliximab induction compared to patients receiving no induction therapy other than steroids[7]. It is

now widely used for induction immunosuppression in many solid organ transplants.

1.7.2 Maintenance Therapy

The standard therapy used for most solid organ transplants is triple therapy with a calcineurin inhibitor, an antimetabolite and corticosteroids, although there are moves to reduce or remove steroids from longer term maintenance therapy due to their side effects.

Calcineurin inhibitors

Both ciclosporin and tacrolimus target cytoplasmic immunophilins to form complexes that inhibit the rate-limiting step in TCR signal transduction by blocking the enzyme calcineurin. By blocking calcineurin-dependent signal transduction, the transcription factor NFAT cannot translocate to the nucleus and induce expression of cytokines such as IL-2, IL-4, TNFα and IFNγ, as well as costimulatory molecules such as CD40L[318]. Tacrolimus is more potent than ciclosporin, and is less nephrotoxic, so is gradually replacing use of ciclosporin in modern maintenance regimens[297,298,319]. Both drugs have side effects including hypertension, glucose intolerance and nephrotoxicity, but tacrolimus is associated with a higher risk of new-onset diabetes after transplantation and neurological sequelae, which are usually mild.

Antimetabolites

Azathioprine and MMF, or its active form mycophenolic acid (MPA), are the agents in routine clinical use. Azathioprine is absorbed from the gut with around 80-90% bioavailability. It is a pro-drug, which is broken down slowly and non-enzymatically by reductive removal of a thioether, converting it to the active metabolite, 6-mercaptopurine (6-MP) on the intestinal wall, in the liver and on red blood cells. 6-MP is converted to thioguanine nucleotides and incorporated into the DNA of replicating cells in place of purine nucleotides, blocking cell cycle. Other metabolites also block *de novo* purine synthesis. Many cells have salvage pathways, which can generate purines through guanine conversion, however lymphocytes do not have active salvage pathways and are therefore more dependent on *de novo* purine synthesis than other cell types[320,321]. Azathioprine is broken down by thiopurine-methyltransferase (TPMT) to inactive metabolites, and levels of the TPMT enzyme alter patient responses to the drug[322]. Bone marrow suppression, leading to lymphopenia, thrombocytopenia or anaemia, is the commonest side effect, although metabolites of 6-MP can also cause heptatotoxicity.[301]

MMF also interferes with DNA synthesis via the purine pathways. It is absorbed rapidly with over 90% bioavailability and metabolised to MPA predominately in the liver. MPA is a reversible inhibitor of inosine monophosphate dehydrogenase, which catalyses the conversion of inosine monophosphate to guanosine monophosphate[323]. By blocking the production of purines in this way, cells must rely on the salvage pathway of purine synthesis and hence, as with azathioprine,

lymphocytes are unable to replicate. Because this is the main mechanism of MMF action, it is more lymphocyte-specific than azathioprine, which will block cell division in all cells through incorporation into DNA. MMF can still lead to bone marrow suppression with profound lymphopenia, but is less myelosuppressive than azathioprine. However, it is commonly associated with diarrhoea, which can be severe[301].

1.7.3 Alternative Maintenance Therapies

All immunosuppressive agents have side effects, and treatment is a balance between minimising side effects and preventing rejection. However, the general side effects of immunosuppression – increased risk of malignancy and infection – are easier to balance than the drug-specific side effects. It is not uncommon for patients to find MMF-associated diarrhoea intolerable and be changed to azathioprine maintenance. Equally, some patients are intolerant of CNIs, whether due to nephrotoxicity, diabetes, or neurological symptoms and alternative maintenance therapies have been developed.

mTOR inhibitors

Sirolimus and everolimus bind to FKBP12, the same binding protein that tacrolimus targets, but instead of blocking calcineurin they potently inhibit the mammalian target of rapamycin (mTOR)[324]. mTOR is a serine/threonine kinase involved in cell cycle signalling and blockade of mTOR prevents progression from G1 to the S phase of cell cycling. Like CNIs, mTOR inhibitors block T cell activation and proliferation. However, mTOR inhibitors have many off-target

effects, including blocking fibroblast growth factors and hence inhibiting wound healing[325]. They cannot be used early post-transplant for this reason, but in patients with nephrotoxicity due to CNIs they can improve renal outcomes and have comparable rates of acute rejection to CNIs. mTOR inhibitors have potentially serious side effects of interstitial lung disease and thrombocytopenia, which must be monitored in the first few weeks after a switch[326]. However, they represent a good alternative maintenance therapy for patients intolerant of CNIs or with biopsy-proven CNI toxicity[327].

Belatacept

Belatacept is a biological agent that has come to the forefront in recent years. It is a fusion protein consisting of the extracellular portion of CTLA-4 (CD152) with the Fc portion of IgG. CTLA-4 is an inhibitory molecule that has higher affinity than CD28 (the activatory receptor) for the costimulatory molecules CD80 and CD86. Belatacept is therefore able to block CD28:CD80/86 costimulatory signalling, increasing the activation threshold for T cells (signal 2, **fig 1.8**) and potentially rendering them anergic by providing TCR stimulation in the absence of co-stimulation. It is currently used as maintenance therapy for patients intolerant of both CNIs and mTOR inhibitors. A large prospective study comparing belatacept maintenance with ciclosporin maintenance (BENEFIT-EXT) showed non-inferiority with regards to rejection and superiority with regards to kidney function[328-330]. Comparisons with tacrolimus were also favourable, although there was a slightly increased risk of acute rejection in the first year[331]. However, the greatest benefit seems to come from switching from a CNI to belatacept in the case of CNI-induced nephrotoxicity[332].

1.7.4 Treating rejection of transplants

Despite immunosuppressive regimens, rejection episodes still occur and both TCMR and ABMR are recognised in both acute and chronic forms. Acute TCMR is generally treatable with pulsed intravenous corticosteroids, and episodes of rejection that do not respond are usually treated with a depleting antibody, either ATG or alemtuzumab[303]. Outcomes are usually good with up to 95% of TCMR responding to treatment, although chronic damage can occur with multiple bouts of rejection[333].

ABMR is a much harder entity to treat. Once donor-specific antibodies have developed, they are usually produced by long-lived plasma cells in the bone marrow niche, and are therefore extremely hard to eliminate[334]. Acute ABMR can respond to steroid therapy, depletion of B cells with alemtuzumab or rituximab (an anti-CD20 monoclonal antibody), and reduction of plasma antibody levels with plasmapheresis or immunoabsorption. However, success of these therapies relies on early diagnosis and treatment prior to the establishment of plasma cells. Once established, antibody production is usually long-lived and can be extremely difficult to treat. Therapies directed against plasma cells have been tried as part of desensitisation regimens in patients with multiple HLA antibody specificities, but these are extremely toxic and not always successful[335-337].

Chronic ABMR is harder still to treat and there is no clear consensus on the best option. Some success has been found with rituximab, intravenous

immunoglobulin, plasma exchange, ATG and corticosteroids, however it is not clear how much these treatments were improving co-incident TCMR.[338]

1.8 Summary

Despite the challenges associated with transplantation, outcomes are generally improving, with one-year graft survival rates now between 94-98% in kidney transplants, 87% in pancreas transplants (alone or with kidney), 93-94% in liver transplants, and 75-87% in lung, heart or combined heart/lung transplants[5]. Because of these early improvements, the average lifespan of a kidney transplant is now approaching 15 years, with a 75% graft survival rate at 10 years in kidney alone transplants[339] and 72-78% graft survival rate at 5 years in simultaneous kidney-pancreas (SPK) transplant[340].

However, the waiting list remains long and, despite a recent fall in the waiting list (**fig 1.9**) the number of patients requiring a transplant is still well above the number of transplants performed each year[157]. There is therefore a need to preserve transplants and improve long-term outcomes, and particularly to tackle the significant clinical burden of *de novo* DSA formation and chronic ABMR.

With this in mind, and the recent evidence suggesting a significant role for Tfh cells and the germinal centre response in the production of alloantibody, this project was designed to investigate the role of Tfh cells and Tfr cells in the production of alloantibody, in a prospective cohort of kidney and SPK transplant recipients.

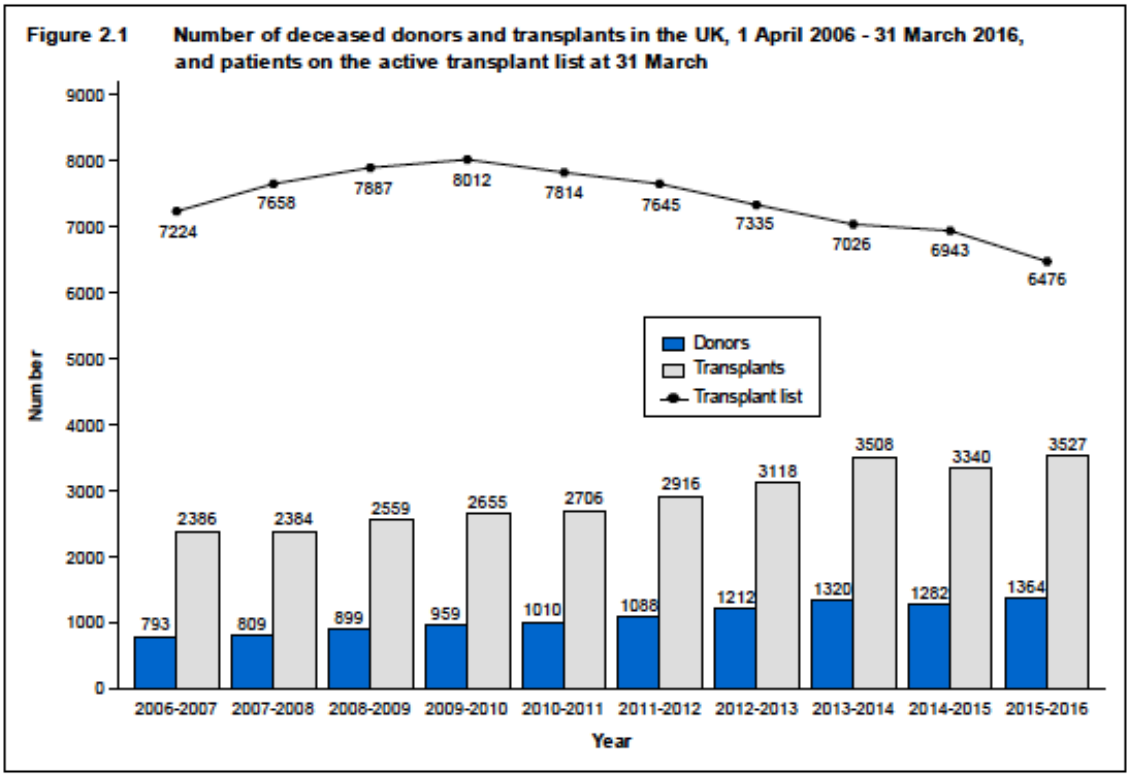


Figure 1.9: Transplant waiting list numbers compared to number of deceased donor transplants. Adapted from NHSBT Organ Donataion and Transplantation Activity Report 2015/16.

1.9 Aims of project

Given the clinically significant problem of cABMR in transplant recipients, alongside the problem of sensitisation against future donors from development of *de novo* anti-HLA antibodies, the aim of this project was to identify potential biomarkers of and risk factors for *de novo* donor-specific antibody formation in transplant recipients. In order to address this, several strategies were planned:

- To recruit a prospective cohort of kidney and SPK transplant patients and follow them for the first two years post-transplant, with regular immunophenotyping and HLA antibody assessment.
- To assess the possibility of using cTfh and cTfr cells as a biomarker of GC activity in transplant patients, by correlating circulating cells with anti-HLA antibody development.
- To investigate the impact of immunosuppressive drugs on the risk of *de novo* anti-HLA antibody formation, and particularly *de novo* DSA formation, in transplant patients.
- To investigate the impact of individual immunosuppressive drugs on antibody production and cTfh and cTfr cells in particular.

2. Methods

Prospective study

1. Ethical approval and recruitment to study
2. Research sample collection
3. Clinical data collection
4. Sample preparation and storage
5. Processing for flow cytometry
6. Flow cytometry panel
7. Flow cytometry data analysis
8. HLA antibody screening with Luminex
9. HLA antibody analysis
10. Data processing
11. Statistical methods

***In Vitro* Experiments**

12. Sample selection
13. Pre-sort enrichment
14. Sorting strategy
15. Co-culture protocol
16. Immunosuppressive drug doses
17. Pre-incubation co-cultures
18. Proliferation dye staining for timed cultures
19. Flow cytometry of retrieved cells
20. ELISA set up and analysis

21. Statistical methods

***In Vivo* Experiments**

22. Transplant technique
23. Immunosuppressive drug dosing
24. Retrieval of samples
25. Flow cytometry analysis
26. Data analysis
27. Statistical methods

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Prospective Study

2.1 Ethical approval and recruitment to study

This study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki, and received ethical approval from the Local Research Ethics Committee, REC reference 14/SC/0091. Written informed consent was obtained from all patients.

This study was designed to recruit enough patients to show a significant difference ($p<0.05$) between patients developing antibodies and those not developing antibodies. From previous publications suggesting a standard deviation of 3.3-6.1% (mean 4.7) in the proportion of cTfh cells[135] a power

calculation was estimated using a 95% confidence interval and confidence level of 95% (therefore z score of 1.96).

$$\text{Sample size} = \frac{\text{z-score}^2 \times SD(1-SD)}{CI^2} = \frac{(1.96 \times 1.96) \times (0.047 \times (1-0.047))}{0.05 \times 0.05}$$

This gave a sample size of 69 patients, with a range of 51-88 patients. On the basis that 10-12% of patients would develop antibodies over the course of the study we estimated that with a 4.7% SD (range 3.3-6.1), 10% antibody development over 2 years, a 95% confidence interval and approximately 150 patients transplanted per year at the Oxford Transplant Centre (OTC), we would require 77 patients to obtain the required difference between groups (range 59-103). These two estimates were combined to give a minimum sample size of 77 with a range up to 103.

Over the three years prior to this study, OTC had transplanted 60-80 SPK and 150-180 kidney recipients per year. Therefore it was considered feasible to recruit around 80-100 patients to the study over the course of 6-8 months.

Patients were recruited over an 8-month period from May to December 2014 from the OTC. At the time of admission for kidney or simultaneous pancreas kidney (SPK) transplantation, patients meeting inclusion criteria were identified by the clinical team. Patients with known pre-formed donor-specific anti-HLA antibodies and those undergoing planned pre-transplant desensitisation with antibody removal were not recruited, but those with a negative pre-transplant

cross-match to donor HLA were approached for study specific consent. All patients received a dose of an antimetabolite (azathioprine or MMF) 2 hours prior to transplantation. Patients receiving kidneys from live donors also received one week of treatment with tacrolimus (aiming for trough levels of 5-15ng/L) prior to transplantation.

Induction therapy was either with the monoclonal antibody basiliximab (anti-CD25) or alemtuzumab (anti-CD52). Basiliximab infusions were commenced 30-120 minutes prior to anaesthesia. Patients receiving alemtuzumab were given 30mg intra-operatively within 3 hours of organ reperfusion. A second dose of basiliximab was given at day 4 post-operation. A second dose of alemtuzumab 30mg was given to certain individuals based on clinician judgement and laboratory lymphocyte count.

Choice of immunosuppressive regimen at the time of study recruitment was dependent on the donor, immunological risk and transplant type.

- SPK patients received alemtuzumab induction with tacrolimus and MMF maintenance therapy, and a steroid-free regimen
- DCD recipients received alemtuzumab induction with tacrolimus and MMF maintenance therapy, with steroid therapy tapered over the first 6-8 weeks, unless the patient had diabetes, in which case a steroid-free regimen was used
- DBD and live donor recipients received basiliximab induction with tacrolimus maintenance therapy, with tapered steroid therapy, unless the patient had diabetes, in which case a steroid-free regimen was used. The

choice of maintenance agent depended on immunological risk:

  - MMF was given to patients with a poorly matched kidney, previous transplant, sensitised or highly sensitised with pre-formed anti-HLA antibodies (not donor specific) or autoimmune cause of ESRF that might recur in the transplant (B+M group)
  - Patients with a well matched kidney who were non-sensitised received azathioprine (B+A group)

- If there was a concern that the patient may be non-compliant, for example young adults (under 25 years) or elderly patients with a history of confusion, these patients received alemtuzumab induction with tacrolimus and azathioprine maintenance (both once daily dosing), regardless of immunological risk

Tacrolimus was given orally at 0.05mg/kg twice a day and then adjusted to trough levels of 5-10ng/ml, aiming for the upper end of this range in the first 3-6 months and then the lower end of the range. If there was a concern over compliance or memory, patients were given a once a day formation of tacrolimus at a dose of 0.15mg/kg.

MMF was given at a dose of 750mg twice a day, reduced down if neutrophil counts fell below 2.

Azathioprine was started at 1.5mg/kg, rounded to the nearest 25mg, for example a 70kg man would receive 100mg once a day. For young adults this was reduced to 1mg/kg.

Steroids, if used, were started at 20mg once a day and weaned over the first 6-8 weeks post-transplant.

2.2 Research sample collection:

After induction of anaesthesia, central venous access was obtained and 50mls of blood was taken. In patients receiving basiliximab the infusion was completed prior to sampling, but all samples were taken prior to methylprednisolone infusion. At subsequent time points, clinical data was collected from clinic visits and 30-45mls of blood was taken at the time of routine clinical sampling.

Lymphoid tissue was removed at the time of transplantation, to allow access to blood vessels as part of the routine operative procedure. Lymph nodes from kidney recipients were from the inguinal region; lymph nodes from SPK recipients were from the para-aortic region.

2.3 Clinical data collection

Clinical data was collected from the electronic patient record and stored securely in line with Good Clinical Practice. Data was collected at the time of transplant, at each follow up visit and at any significant events such as biopsy or graft loss. Data collected at the time of transplant included:

- **Recipient demographics** – age, date of birth, weight, cause of ESRF, current RRT modality (pre-dialysis, peritoneal dialysis, haemodialysis), current mediation and significant history, for example previous transplant,

recent infection. Current creatinine and laboratory lymphocyte count from the time of research sampling were noted.

- **Donor details** – type of donor (LD, DBD, DCD). If deceased, cause of death, any comorbidities or relevant medication, for example antibiotics in the case of peri-mortem infection. Data on warm ischaemic time and cold ischaemic time were not collected, but the research team was informed if the donor was considered to meet 'extended criteria' (age >60 years, comorbidities e.g. hypertension)[341,342]
- **Histocompatibility data** – recipient genotype, donor genotype, HLA mismatch for HLA A-B-DR, any pre-formed anti-HLA antibodies (sensitisation), pre-emptive (or virtual) cross match results
- **Transplant data** – immunological risk decision, induction therapy, maintenance therapy

Data collected at follow up visits included:

- Current creatinine and laboratory lymphocyte count
- Any need for dialysis suggesting DGF or non-functioning transplant
- Any changes to medication – doses or alteration in drug choice
- Any significant events such as biopsy (with results), infection or other complication, or graft failure

Research data collected at the time of transplant included the number of nodes obtained and the cell count and death levels. Research data at all time points included the volume of blood obtained for research samples to ensure this did not exceed the limits stated in the protocol, and PBMC count.

2.4 Sample preparation and storage

Blood samples were collected into 4-5 10ml EDTA tubes (BD biosciences) depending on the time point, and one 5ml gel serum tube (BD biosciences). Within 5 minutes of collection, samples were placed on ice, and processed within 4 hours of sampling. The blood was diluted 50:50 with MACS rinsing buffer (1 x phosphate buffered saline (PBS), 2mM EDTA) and centrifuged over a density gradient (LSM, MP Pharmaceuticals) at 1000g for 20 minutes at room temperature. The PBMC layer was then removed, washed once with rinsing buffer at 1100g, and then washed twice with rinsing buffer at 230g to remove platelets as previously described[343].

Lymph nodes were made into a single cell suspension by passing through a 70nm filter, topped up to 50mls with rinsing buffer and centrifuged once at 1100g at 4^{o}C, causing the fat layer to rise to the top. The fat layer was then removed. The lymph node cells were then washed once with rinsing buffer at 500g.

Each sample was then re-suspended in 5mls of MACS running buffer (1 x PBS, 2mM EDTA and 0.5% of bovine serum albumin) and counted with a haemocytometer.

To minimise variation, samples were frozen in aliquots of 2 x 10^{7} cells and defrosted later for batch processing. All samples were frozen to -80^{o}C at -1^{o}C per minute using a CoolCell cryopreservation container (BioCision) in a 90% fetal

calf serum (FCS), 10% dimethyl sulphoxide (DMSO) mix. When samples had reached -80^{o}C they were transferred to liquid nitrogen for long-term storage.

2.5 Processing for Flow Cytometry

Samples were batch processed in groups of 10. From liquid nitrogen they were transferred to dry ice and then defrosted rapidly in a 37^{o}C waterbath. Samples were then transferred to a 15ml falcon with 0.4µL/ml DNAse, and slowly warmed with RPMI media/2% FCS. Defrosted samples were washed, counted and 3 x 10^{6} cells were added to each polypropylene tube for flow cytometry.

A cocktail of surface antibodies was added, and samples were incubated for 30 minutes at room temperature. They were washed, and streptavidin and live/dead stains were added for a further 10 minutes at room temperature. Samples were then washed again, and fixed using eBioscience Foxp3 staining fixation/permeabilisation buffer for a minimum of 45 minutes at 4^{o}C as per manufacturers protocol. Intracellular stains were then added and samples incubated for 60 minutes at 4^{o}C. Samples were then run on a BD LSR Fortessa cell analyser.

2.6 Flow cytometry panel design

For each sample, separate panels were run to assess B cells, T follicular helper, and regulatory T cells. Antibody panels are shown in **table 2.1**. Each antibody was titrated to find the optimal dilution and panels were constructed to allow

good separation of surface markers while minimising compensation requirements. All samples were incubated with human Fc block (eBioscience) to prevent non-specific binding of antibodies. Antibody cocktails were mixed in brilliant violet stain buffer (BD biosciences) to prevent ‘budding’ of the negative population, as recommended by BD bioscience when combining multiple brilliant violet fluorochromes.

Biotinylated antibodies were combined with brilliant violet (BV) 605 streptavidin (BioLegend). As samples had been frozen, a live-dead marker was included in each panel, with near-infrared fixable live-dead stain kit (Invitrogen) excited by the red laser and emitting at 750nm.

Panel	Violet laser	Blue laser	Red laser
B cells	CD38 BV421 (HIT2, BD), CD19 BV510 (SJ25C1, BD), HLA-DR BV711 (G46-6, BD)	IgD FITC (IA6-2, eBioscience), Bcl6 PE (K112-91, BD), IgM PerCP-Cy5.5 (H1-FB1), BD), CD24, PE-Cy7 (ML5, BioLegend)	CD138 AF627 (MI15, BD), CD27 AF700 (O323, eBioscience), Live/dead near IR.
Tfh	CD45RA BV421 (HI100, BD), CXCR5 biotin (RF8B2, BD), PD-1 BV711 (EH12.1, BD)	CXCR3 AF488 (IC6, BD), CCR7 PE (3D12, eBioscience), ICOS PerCP-Cy5.5 (C398.4A, BioLegend), CCR6 PE-Cy7 (R6H1, eBioscience)	CD57 APC (NK-1, BD), CD4 AF700 (OKT4, eBioscience), Live/dead near IR.
Tregs	IL-7R BV421 (HIL-7R-M21, BD), CXCR5 biotin (as Tfh), PD-1 BV711 (as Tfh)	Foxp3 AF488 (259D, BioLegend), Bcl6 PE (k112-91, BD), ICOS PerCP-Cy5.5 (as Tfh), CD25 PE-Cy7 (M-A251, BioLegend)	CD57 APC (as Tfh) CD4 AF700 (as Tfh), Live/dead near IR.

Table 2.1 – Antibody staining panel Shown as target, fluorochrome (clone, company).

2.7 Fortessa set up and flow data analysis

Samples were run on an LSR Fortessa cell analyser using BD FACSDiva software (BD Biosciences). Experiments were set up and 'duplicated without data' to maintain voltages. Prior to each batch of samples, compensation was run with single-colour stained bead controls and voltages adjusted as required.

Data was analysed using FlowJo LLC software version 10.0.8.

2.8 HLA antibody screening with Luminex

Anti-HLA antibody assessment was undertaken using Luminex screening following the same protocols and using the same machines as used for clinical assessment.

Serum samples were spun at 1500g for 5 minutes, and serum removed and snap frozen to -80^{o}C. For screening samples, serum was defrosted, spun at 13000 rpm in a microcentrifuge to pellet any impurities, and then 10µl incubated for 30 minutes with 2.5µL of LabScreen Mixed beads (OneLambda). Samples were then washed 5 times in wash buffer (OneLambda) and incubated with PE-conjugated goat anti-human IgG for 30 minutes. Samples were washed 5 times to remove residual antibody and re-suspended in PBS. Samples were run on a Liquichip 200 machine (Luminex) with one positive and two negative controls per batch. Samples were analysed with HLA-Fusion software using positive and

negative cut-off levels determined by the control samples, to avoid inter-lot variation.

Samples with indeterminate results or where bead counts were below minimum cut-off were re-run using Adsorb Out (OneLambda), a combination of the same microparticles that are coated with antibody for mixed screens, but with no bound coating. These are combined with blocking solution, and used to reduce background staining caused by non-specific binding of serum to the beads used for the assay. 20μL of serum was incubated with 2.5μL of Adsorb Out beads, incubated for 30 minutes, and then spun at 13000 rpm in a microcentrifuge. 10μL of unbound serum was then removed without disturbing the bead pellet, and used for the assay as previously described.

Panel reactive antibody (PRA) class I or class II screens were performed as described with Mixed Screen beads at 2.5μL of beads to 10μL of serum, except the beads are coated either with HLA class I or HLA class II rather than a combined class I and II bead as in Mixed Screen.

Single antigen beads (SAB) assays were run as described with 2.5μL of beads to 10μL of serum. However all SAB screens were treated with 2.5μL of EDTA to 50μL of serum prior to Adsorb Out treatment, as per clinical protocols. This is designed to avoid the 'prozone' effect where a high titre antibody gives a false negative SPA test due to complement products[344-346].

2.9 HLA antibody analysis

Samples were analysed with HLA Fusion. Each batch was imported into HLA Fusion, and all positive and negative controls reviewed to ensure they fell into recommended limits. Patient samples were checked to ensure that the bead count was above recommended levels, and the positive control bead fluoresced at or above 5000 median fluorescent intensity (MFI) and the negative control bead sat below 150 MFI.

Each sample was then compared to the positive and negative control samples and assessed as positive if MFI was above the positive cut off, negative if below the negative cut off, or indeterminate if between the two MFI levels.

If a sample was assessed as positive on Mixed Screening, the serum was assessed using either PRA or SAB. Samples that looked positive on Mixed Screen but with a similar profile to previous positive screens were assessed using PRA screens. Samples with new positive screens or altered profiles were assessed using SABs.

If samples screened positive on SAB the positive antibody profile was compared with both donor and recipient genotype to establish whether the antibody was donor specific or a non-specific HLA antibody.

2.10 Data Processing

Data from FlowJo were exported to Microsoft Excel using the 'table' function and converted from proportion to absolute count by multiplying the cell count of interest (exported as a proportion of lymphocytes) by the clinical laboratory lymphocyte count taken from the clinic blood sample that was taken alongside the research sample. Luminex data were also exported to Excel.

Data from Excel were then analysed in Graphpad Prism, version 5.0f for Mac OS X, (Software Inc, USA).

2.11 Statistical Methods

Human samples were compared using Mann Whitney testing for two group comparisons or one-way ANOVA for three group comparisons. A p value of <0.05 was considered to be statistically significant.

Where there were fewer than 5 data points at later time points for patients developing *de novo* DSA, if the samples were normally distributed when assessing patients developing HLA, groups were compared using a 2-tailed independent T test with $p<0.05$ as statistically significant.

Data were expressed as mean and standard deviation rather than standard error of the mean due to the low numbers in B+A and B+M sample groups.

In Vitro Experiments

2.12 Sample selection

Cells for *in vitro* co-cultures were obtained from leucocyte cones from platelet donors attending the Oxford Blood Donation Centre at the John Radcliffe hospital. Consent to use of samples was provided to NHS Blood and Transplant.

Leucocyte cones were drained into a 50ml falcon tube and the cone rinsed twice with MACS rinsing buffer. Any cones that had formed blood clots were discarded. The blood was then centrifuged over a density gradient (LSM, MP Pharmaceuticals) at 1000g for 20 minutes at room temperature. The PBMC layer was removed, washed once with rinsing buffer at 1100g, and then washed twice with rinsing buffer at 230g to remove platelets as previously described[343].

The supernatant was discarded and pellet resuspended before adding 10mls of 1 x Pharm Lyse (BD Biosciences) buffer to lyse red cells. This was incubated for 5 minutes at room temperature as per manufacturers protocol and then topped up with PBS to stop the reaction. The sample was spun at 500g for 5 minutes at 4^{o}C and the supernatant discarded.

Cells were resuspended in pure RPMI, counted and frozen in aliquots of 15 x 10^{7} to ensure sufficient cells for each sort were available after defrosting. All samples were frozen to -80^{o}C at -1^{o}C per minute using a CoolCell cryopreservation

container in a 90% FCS, 10% DMSO mix as per PBMC samples, and stored in liquid nitrogen after freezing.

A smaller aliquot of 2 x 10^7 cells from each cone was frozen and then defrosted for analysis to ensure there would be sufficient numbers of each (see chapter 5 for details, **table 5.1**).

2.13 Sorting strategy

Enriched complete media was used for sorting and co-culture:

- 430ml RPMI 1640
- 50mls serum – human AB serum used for pre-Aria processing, FCS used for sorting and co-culture
- 5mls penicillin/streptomycin
- 5mls 100mM sodium pyruvate (Sigma)
- 5mls 100x MEM NEAA (Gibco)
- 5mls 100x Glutamax-I (Gibco)
- 500uL 50mM β mercaptoethanol (Gibco)

The afternoon prior to the planned sort, an aliquot of cells was defrosted and counted. As with clinical samples, from liquid nitrogen cone samples were transferred to dry ice and then defrosted rapidly in a 37^{o}C waterbath. Samples were then transferred to a 50ml falcon with 0.4µL/ml DNAse, and slowly warmed with complete media.

Cells were counted and diluted to 2 x 10^6 cells/ml in complete media with 0.4µl/ml Benzonase (combined RNAse and DNAse, Millipore) and incubated overnight at 37^{o}C.

On the day of sorting, pre-enrichment was carried out for B cells (CD19 Dynabead/Detatchabead kit, Invitrogen) and then CD4 T cells (Miltenyi CD4 negative isolation kit). The incubated samples were counted and then spun down at 500g for 5 minutes at room temperature, and the supernatant discarded.

B cell isolation:

Using the recovered cell count, 1.5µL CD19 Dynabeads per 1 x 10^6 cells were transferred to an eppendorf and washed in MACS running buffer. The supernatant was discarded and the beads resuspended in the original volume of running buffer. The cell pellet was resuspended in running buffer at 25 x 10^6 cells/ml and the washed beads added. Samples were incubated for 30 minutes at 4^{o}C on a lab sample rotator at 6-8 revolutions per minute.

Samples were placed in a magnet for 2 minutes and the supernatant removed to a 50ml falcon tube for later CD4 isolation. The samples were removed from the magnet, running buffer added to wash beads, and replaced in the magnet for 1 minute. The supernatant was transferred to the 50ml tube and the wash procedure repeated for a total of 4 washes with supernatants pooled.

The isolated B cells were then resuspended in complete media with 1% human AB serum at 250µL per 25 x 10^6 cells (from pre-isolation count). Detatchabeads

were added at 15µL per 25 x 10^6 cells and the samples incubated for 45 minutes at room temperature on a lab sample rotator at 6-8 revolutions per minute.

Samples were pipetted vigorously to enhance detachment of cells and then placed in a magnet for 1 minute and the supernatant transferred to a fresh 15ml falcon tube. To obtain residual cells, the beads were washed with 500µL of complete media with 1% serum and pipetted to release cells. The samples were then placed in the magnet for 1 minute and the supernatant added to the tube. This wash procedure was repeated for a total of 5 washes.

The isolated B cells were then topped up to 15mls and centrifuged at 400g for 5 minutes. The supernatant was discarded and the cells resuspended in 5mls of complete media with 10% AB serum, counted and then rested in a 37^{o}C incubator for one hour.

CD4 T cell isolation:

This was carried out as per manufacturers recommended protocol. The pooled supernatants from the first step of B cell isolation were counted and resuspended in 40µL running buffer per 1 x 10^7 cells. 10µL of Biotin-Antibody cocktail was added per 1 x 10^7 cells and the sample incubated for 5 minutes at 4^{o}C. A further 30µL of running buffer was added per 1 x 10^7 cells and then 20µL of Microbead cocktail per 1 x 10^7 cells. The sample was incubated for 10 minutes at 4^{o}C and then separated using an LS Column on a manual MACS separator magnet, collecting the negative fraction of $CD4^+$ cells into a 15ml falcon and discarding the cells bound to the column.

The negative fraction was centrifuged at 500g and resuspended in 5mls complete media with 10% AB serum, counted and then rested in a 37^{o}C incubator for one hour.

When both samples had been isolated and rested, they were stained using the panels below. Samples were spun down and resuspended in 40µL of running buffer, with 60µL of antibody cocktail added. The antibodies were titrated to find the optimal concentration to give good separation of samples at sorting, and all cocktails were made up in BV buffer (BD biosciences).

CD4 staining – DAPI (BioLegend), CD3 BV 605 (OKT3, BioLegend), PD-1 BV 711 (EH12.1, BD) CD4 APC-Cy7 (OKT4, BioLegend), CXCR5 APC (J252D4, BioLegend), CXCR3 PE (1C6/CXCR3, BD Biosciences)
B cell staining – CD3 BV 605 (as CD4 panel), CD19 PE-Dazzle (HIB19 BioLegend), CD27 BV421 (M-T271, BD Biosciences)

Samples were then incubated for 15 minutes at 37^{o}C. After staining 5mls of running buffer was added and samples were centrifuged, supernatants were aspirated completely and stained cells resuspended in 300-500µL of running buffer with 20mM HEPES.

2.14 Sorting strategy

Samples were sorted as described in **Chapter 5, figs 5.2, 5.4 & 5.5.** Using a BD FACSAriaIII T cells were sorted into three populations (Teffector, Tfh1 and Tfh2/27) and B cells into 2 populations (naïve and memory). PD-1 was included in the panel to look at expression prior to co-culture, and not used for sorting cell subsets.

Prior to each sort, single colour bead controls were run to compensate and test sorts were carried out to check alignment and confirm purity of sorted populations. Samples were then bulk sorted at 4^{o}C into sterile polypropylene tubes pre-wetted to prevent cells sticking with 1ml of complete media with 10% FCS.

2.15 Co-culture protocol

Sorted samples were counted to confirm sorter counts, spun down and resuspended in complete media with 10% FCS to give 3 x 10^4 cells per 50µL. Samples were co-cultured in 96-well round-bottomed plates by combining 3 x 10^4 of each cell type to a total of 6 x 10^4 cells per well, plus 50µL of 3µg/ml of staphylococcal enterotoxin B (SEB, Sigma). Total volume per well – 150µL, final concentration of SEB 1µL/ml.

Samples were cultured for 11 days at 37^{o}C in the combinations shown in **table 2.2**. Because early experiments suggested significant evaporation of media, and

high death rates of B cells, at day 6 wells were topped up with 100µL complete media with 10% FCS but no SEB to ensure sufficient nutrients remained to support cell growth.

Naïve B alone (Control)	Memory B alone (Control)	
Naïve B + Teffector	Memory B + Teffector	Teffector alone (Control)
Naïve B + Tfh1	Memory B + Tfh1	Tfh1 alone (Control)
Naïve B + Tfh2/17	Memory B + Tfh2/17	Tfh2/17 alone (Control)

Table 2.2 Control and test culture combinations

After 11 days of culture, plates were spun down and supernatants removed and frozen to be run for IgM and IgG ELISA. Cell pellets were then resuspended and duplicates combined for flow cytometry.

2.16 Immunosuppressive drug doses

In order to assess the impact of drugs on antibody production, drugs were added into the culture medium. *In vivo*, azathioprine is absorbed from the gut with around 80-90% bioavailability. It is a pro-drug, which is broken down slowly and non-enzymatically to the active metabolite, 6-mercaptopurine (6-MP). This takes place on the intestinal wall, in the liver and on red blood cells, and therefore cannot occur *in vitro*. For these co-cultures 6-MP (Sigma, 852678) was used at a dose of 50µM to correlate with human dosing and in keeping with previous *in*

vitro work both published[347] and unpublished from the Transplant Research Immunology Group (TRIG) lab.

MMF is also a pro-drug, which is absorbed with >90% bioavailability, and then rapidly hydrolysed to MPA in the intestine, liver and blood. This leads to the same issues as with azathioprine, and hence MPA (Sigma, M3536) was used in these co-cultures at 50µg/ml to correlate with human dosing and in keeping with previous *in vitro* work[348], both published and unpublished from the TRIG lab.

Tacrolimus (Sigma F4679) was used at 8ng/ml, aiming for the mid-range of recommended dosing for patients, which is 5-15ng/ml[315,349] and in keeping with previous *in vitro* work from the TRIG lab[350].

Drugs were dissolved in an appropriate diluent (see **table 2.3**) and stored at -20^{o}C. They were added to culture medium along with SEB at 3 times the required concentration, so 50µL of medium per well would give the final required concentration in the well volume of 150µL.

Drug	**Diluent**	**Storage concentration**	**Desired concentration**	**Vol/ml media for 3 x desired concentration**
Tac	DMSO	2.5µg/ml	8ng/ml	9.6µL
MPA	DMSO	50mg/ml	50µg/ml	3µL
6-MP	NaOH	50mg/ml*	50µM*	26µL

Table 2.3: Drug diluent and storage concentrations *50mg/ml is 294µM 6-MP based on molecular weight of 170.2, therefore 50µM requires a concentration of 8.5mg/ml.

The combinations of test and control wells listed in **table 2.2** were run untreated, with vehicle (DMSO or NaOH) and test drug for each co-culture. When media was topped up at 6 days, drug or vehicle was added to the media for top up. SEB was not added to any media used for top-ups.

2.17 Pre-incubation co-cultures

Pre-incubation co-cultures were set up as standard drug co-cultures but rather than 6-MP being added to the medium in 3 x required concentration, a 1 x concentration was added to complete media with 10% FCS. T cell samples were counted after sorting, each separated into three equal aliquots, centrifuged and supernatant discarded. Each aliquot was resuspended in media either without drug, with NaOH vehicle control, or with 6-MP. They were incubated for one hour at 37^{o}C to ensure uptake of 6-MP, centrifuged, supernatant discarded, and then washed with fresh media, resuspended and recounted. The standard co-culture was then set up and left for 11 days with a top up at 6 days of complete media with 10% FCS but no drug.

2.18 Proliferation dye staining for timed cultures

To assess proliferation[351], cells were stained with proliferation dyes prior to culture. B cells were stained with carboxyfluorescein succinimidyl ester (CFSE, ThermoScientific) and T cells with violet proliferation dye (VPD, BD Bioscience).

VPD staining:

Post-sort T cells were centrifuged at 500g for 5 minutes, supernatant discarded, and then washed in PBS, centrifuged and supernatant discarded. T cells were resuspended at 3 x 10^7 cells/ml in PBS and 1uL of 1mM VPD added per ml of PBS, giving a final concentration of 1μM. Cells were mixed well and incubated in the dark for 10 minutes at 37^{o}C and then washed twice in 10mls PBS with 5% FCS. Supernatants were discarded and cells resuspended in complete media with 10% FCS and recounted.

CFSE staining:

B cells were washed in PBS and supernatant was discarded as per VPD protocol and resuspended in 900μL RPMI. CFSE was diluted down to 50μM in pure RPMI and 100μL added to the cell suspension and mixed well. Samples were incubated in the dark for 10 minutes at 37^{o}C and then washed twice in 10mls RPMI with 2% FCS. Supernatants were discarded and cells resuspended in complete media with 10% FCS and recounted.

Samples were analysed at days 1, 3 and 5. To allow enumeration of recovered cell numbers, 1 x 10^4 counting beads were included with each sample. Division peaks were gated as shown in **fig 5.23** and the proportion of cells converted to a cell count using the bead count and the following formula:

$$\text{Cell count} = \frac{\text{Recovered cell count} \times 10\,000}{\text{Recovered bead count}}$$

The division index is calculated in FlowJo as the mean number of cell divisions per progenitor cells. This takes into account undivided cells, and gives a good estimate of proliferation across the whole population, but does not estimate how frequently any individual cell has divided. It is calculated as the sum of cell divisions divided by precursor cells using the formula:

$$\sum_{1}^{i} i\ x\ \frac{N_i}{2^i}$$

Where i is the generation number and N is the number of cells in that generation.

The proliferation index (PI) is also calculated in FlowJo, and is the mean number of divisions amongst cells that have divided, so it does not include the undivided peak. This is a more sensitive measure of how many divisions an individual cell has undergone, and is calculated using the formula:

$$\sum_{0}^{i} \frac{N_i}{N^i/2^i}$$

When there is no proliferation and all cells are in the undivided peak, the formula will be $\frac{N_0}{N^0/2^0}$ = 1 Giving the lower limit of the PI as one.

2.19 Flow cytometry of retrieved cells

Two separate panels were run on retrieved cells, one to elucidate the phenotypes of recovered cells from standard and drug-treated co-cultures, and the second to assess for apoptosis alongside the proliferation dyes. All antibodies were titrated to find optimal concentrations for staining.

A. Standard and drug-treated co-cultures

Duplicate wells were combined and transferred from the 96 well plate to facs tubes. Samples were incubated with human Fc block, then a cocktail of surface antibodies was added and samples incubated for 30 minutes at room temperature. The live/dead marker was added in for the last 10 minutes only. Samples were then washed, fixed in 2% paraformaldehyde (PFA) and run immediately on a BD LSR Fortessa.

Violet laser: CD27 BV421, CD38 BV605, PD-1 BV711 (all BD Bioscience)

Blue laser: CXCR3 AF488 (BD Bioscience), CD19 PE-Dazzle (BioLegend), CD20 PE-Cy7 (eBioscience)

Red laser: CXCR5 APC (BioLegend), CD4 AF700 (eBioscience), Live/dead near IR (Invitrogen)

B: Panel for assessing apoptosis and proliferation

Alongside VPD (excited 405nm violet laser, emits 450/50 channel) and CFSE (excited 488nm blue laser, emits 530/30 channel) samples were stained with CD3 BV605 (BioLegend, as above), CD19 APC-efluor780 (HIB19, eBioscience), 7AAD viability stain (eBioscience, excited 488nm blue laser, emits 610/20 channel) and annexin V APC (eBioscience).

7-aminoactinomycin D (7AAD) is a fluorescent marker that binds to DNA, therefore when cells are in late apoptosis and lose cell membrane integrity, 7AAD can enter the cells and bind to DNA. Annexin V is one of a family of phospholipid binding proteins and specifically binds phosphatidylserine. Under

normal circumstances phosphatidylserine is located on the inner layer of the cell membrane, however when apoptosis is triggered it is translocated to the outer layer of the cell membrane as a signal to phagocytes that the cell is undergoing apoptosis. Annexin V binds to phosphatidylserine and hence stains both early apoptotic cells that have a cell membrane intact enough to exclude 7AAD, as well as late apoptotic cells that will stain for both annexin V and 7AAD. Details of gating are shown in **chapter 5, fig 5.25.**

To stain proliferation dye-labelled cells the antibody cocktail was prepared in annexin-V binding buffer (eBioscience). Cells were harvested from 96 well plates into facs tubes, centrifuged and then resuspended in 100µL of annexin V binding buffer. The antibody cocktail was added, the sample mixed and incubated for 30 minutes at room temperature in the dark. 1ml of annexin V binding buffer was added per tube, the samples centrifuged at 500g for 5 minutes, and supernatants discarded. Samples were resuspended in 200µL of annexin B binding buffer and run on the Fortessa within one hour of staining.

2.20 ELISA set up and analysis

ELISAs were run using the eBioscience Ready-SET-Go! ELISA kits for human total IgG (catalogue 88-50550) and human IgM (catalogue 88-50620), using polystyrene high-binding 96 well plates and according to manufacturers recommended protocol.

Briefly, plates were coated with 100µL per well of diluted capture antibody (purified anti-human IgM or IgG monoclonal antibody diluted in PBS), sealed and incubated overnight at 4^{o}C. Plates were then washed twice with 400µL/well of wash buffer (1 x PBS with 0.05% Tween-20) allowing 1 minute soaking time per wash.

Plates were blocked using 250µL/well of blocking buffer (2 x assay buffer A from kit – PBS with 1% Tween-20 and 10% BSA), sealed and incubated overnight at 4^{o}C. Plates were then washed twice with 400µL/well of wash buffer allowing 1 minute soaking time per wash.

For each plate, two columns of standards were run according to manufacturers recommendations, from 100ng/ml for IgG (1000ng/ml for IgM) with 6 serial dilutions performed and two blank wells at the bottom of the first two columns of the ELISA plate. A similar serial dilution strategy was used for test samples with each test sample being run in serial dilutions from 1 in 4 (saturating dilution) down in 2-fold serial dilutions to 1 in 512. Control samples were run at 1 in 2 only. All dilutions were performed in 1 x assay buffer A as per manufacturers recommendation.

Samples were sealed and incubated for 2 hours at room temperature, and then washed 4 times with 400µL/well of wash buffer allowing 1 minute soaking time per wash. 100µL/well of diluted detection antibody (HRP-conjugated anti-human IgG or IgM as appropriate, diluted in PBS), plates were sealed and incubated for 1 hour at room temperature.

Plates were washed 4 times with 400µL/well of wash buffer allowing 1 minute soaking time per wash and then 100µL/well of substrate solution (tetramethylbenzidine, TMB) added. Plates were sealed and incubated for 15 minutes and then 100µL of stop solution (2N H_2SO_4) added to each well.

Plates were read immediately at 450nm on a plate reader, and emission data transferred to an excel spreadsheet. For each sample a standard curve was set using the dilution factor and emission data. Data points on the linear portion of the curve were selected for each duplicate and the emission data for these points compared to the standard curve for that plate using the linear regression function in Prism. Interpolated mean concentrations for replicates were calculated from the linear regression of the known standard concentrations with the emission of the blank wells subtracted to remove background fluorescence.

A representative example of the standard curve generated for samples and optimal dilution selection is shown in **fig 2.1**. Two to three optimal dilutions were chosen for each sample. If there was no optimal dilution, for example in naïve B + Tfh1 co-cultures where no IgG was produced, the top dilutions of 1 in 4 and 1 in 8 were selected.

The interpolated mean concentration was then multiplied by the dilution factor to give a final concentration for each sample.

2.21 Statistical methods

Individual samples were compared using Mann Whitney testing for two group comparisons. A p value of <0.05 was considered to be statistically significant.

Flow data with more than two groups was compared using one-way ANOVA comparing treated samples to the untreated control, with Bonferroni multiple-test correction applied. A p value of <0.05 was considered to be statistically significant. ELISA data was compared using 2-way ANOVA comparing T cell subset in co-culture to treatment condition.

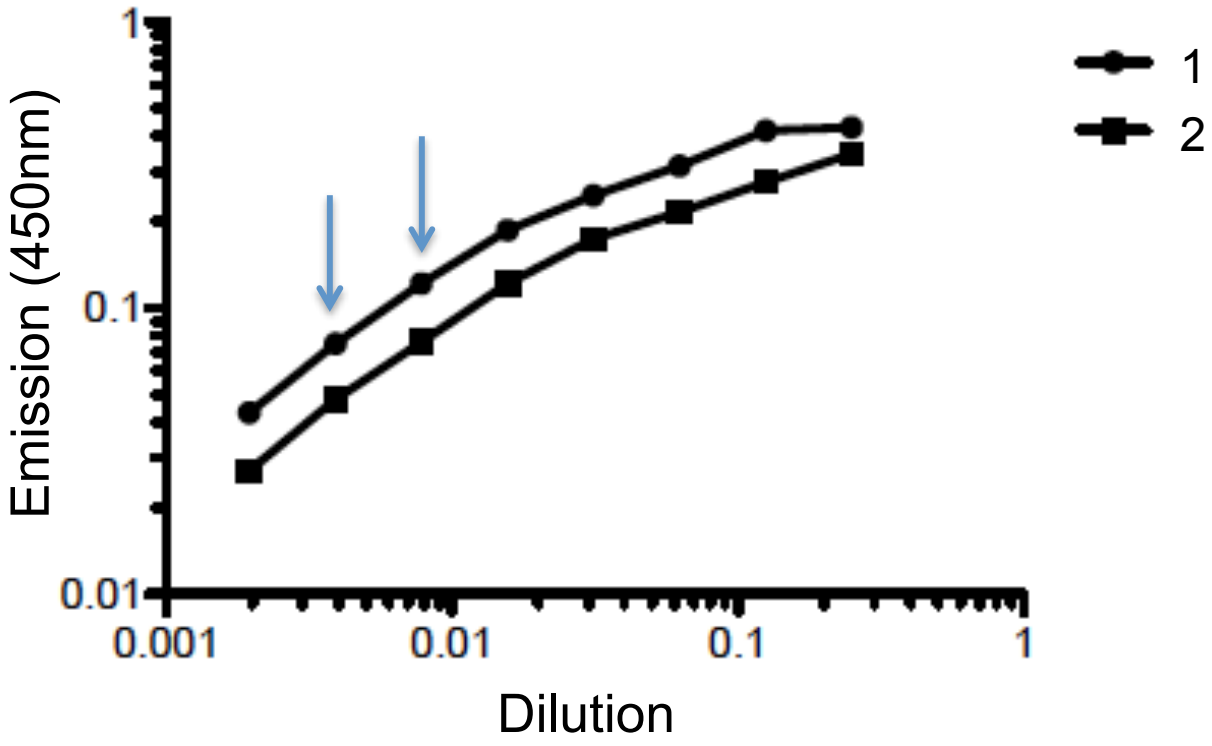


Figure 2.1 Standard curve for sample dilutions. Emission data was plotted against serial dilutions, with duplicate 1 and duplicate 2 plotted in separate curves. In order to generate the curves, all samples started at 1 in 4 dilution, plotted as 0.25 and were serially diluted to 1 in 512, plotted as 0.00195. Optimal dilutions were selected from the linear portion of the curve, here 1/256 and 1/128 (blue arrows).

Dilutions run:	X-axis value plotted
1 in 4	0.25
1 in 8	0.125
1 in 16	0.0625
1 in 32	0.03125
1 in 64	0.015625
1 in 128	0.0078125
1 in 256	0.00390625
1 in 512	0.001953125

In Vivo Experiments

2.22 Transplant technique

CBA/Ca (CBA, H-2k) and C57BL/6 (B6, H-2b) mice were bred in the John Radcliffe BMS unit and housed in specific pathogen free (SPF) conditions. This study was conducted under Home Office project license 40/3580.

Full thickness B6 tail skin grafts were applied to the left flank of CBA animals and held in place with tissue glue and dressings. Animals were monitored daily post op and sacrificed if there was any concern over welfare or sign of illness. Dressings were removed at day 7 and grafts monitored for rejection 3 times per week.

2.23 Immunosuppressive drug dosing

Protocols were established for azathioprine, MMF and tacrolimus, although I was only able to use MMF in this project. All protocols were based on a comparison of human doses and murine absorption.

Dosing of MMF in adult humans is not based on body weight but is fixed and adjusted based on neutrophil count. Area-under-curve and trough level measurements can be undertaken, but in practice these are complex and do not necessarily correlate with outcomes[352]. Paediatric patients receive doses based on body surface area at 600mg/m^2 but this is very difficult to calculate in mice.

Previous animal work in our lab had shown that MMF given by oral gavage at doses of 1mg/kg increased median survival time (MST) in B6 to CBA heterotopic heart transplants. Several dosing regimens were tested:

- MMF 1mg/kg/day on days 0 to 6 led to MST of 87.5 days
- MMF 1mg/kg on days 0, 1, 3 and 5 led to MST of 80.5 days
- MMF 1mg/kg on days 0, 1 and 5 led to MST of 73.5 days
- MMF 1mg/kg/day on days 0 to 3 led to MST of 24.5 days

Without immunosuppression, the MST of heart transplants in these experiments was 8 days (A. Whatcott, unpublished data).

These doses correlated with previous published work[353] although this was in bone marrow transplantation. Work with MMF in murine skin experiments was limited, and either used derivatives of MPA in transplantation[354] or IP dosing for skin hypersensitivity[355].

Given the detailed data from our lab and in bone marrow transplantation, I chose a dose of 1mg/kg on days 0, 1, 3 and 5 to be given by oral gavage. MMF oral suspension (CellCept 1g/5ml powder for reconstitution, Roche Pharmaceuticals) was diluted down to a concentration of 0.2mg/ml in order to give a maximum volume of 100-200μL per mouse.

- 20g mouse requires 0.02mg - 100μL of 0.2mg/ml solution
- 25g mouse requires 0.025mg - 125μL of 0.2mg/ml solution
- 30g mouse requires 0.03mg - 150μL of 0.2mg/ml solution

On the basis that a mouse stomach can hold approximately 400μL but may contain some food prior to dosing, and therefore a maximum of 200μL should be

given by oral gavage. Diluted MMF was frozen in small aliquots and defrosted and warmed to room temperature prior to dosing.

2.24 Retrieval of samples

Groups were sacrificed at fixed time points by exsanguination via IVC puncture under terminal anaesthesia, and then removal of draining (left axillary) and contralateral (right axillary) LNs. Blood was left to clot for 45 minutes at room temperature and then spun in a microcentrifuge for 5 minutes. The serum was removed without disturbing the clot, and then spun again for 5 minutes to remove any residual red cells. The serum was transferred to a fresh eppendorf and frozen at -20.

2.25 Flow cytometry analysis

Tissue samples were passed through 70nm filters to form a single cell suspension, counted and then 2-3 x 10^6 cells added to each facs tube. To optimise staining for CXCR5 this was added to each sample first, and incubated for 40 minutes at room temperature, before the rest of the antibody cocktail was added and the sample incubated for a further 20 minutes. Samples were then washed, and fixed using eBioscience Foxp3 staining fixation/permeabilisation buffer for a minimum of 45 minutes at 4^{o}C. Intracellular stains were then added and samples incubated for 60 minutes at 4^{o}C. Samples were then run on a BD FACS Canto II analyser for untreated experiments and on a BD LSR Fortessa

cell analyser for MMF treated experiments. Antibodies were titrated for optimum concentrations, and re-titrated when transferred between analysers.

Antibody cocktail:

Violet laser: Foxp3 BV421 (clone MF-14, BioLegend)

Blue laser: Ki67 FITC (SolA15, eBioscience), Bcl6 PE (K112-91, BD), CD4 PerCP-Cy5.5 (GK1.5, BioLegend), PD-1 PE-Cy7 (29F.1A12, BioLegend)

Red laser: CXCR5 APC (2G8, BD), B220 APC-eFluor780 (RA3-6B2, eBioscience)

2.26 Data analysis and statistical methods

Flow data was analysed in FlowJo. Numeric data was then analysed in Prism. Survival data was plotted and analysed using Kaplan Meier estimates. All other samples were analysed using unpaired T test to compare DLN with CLN or MMF treated with untreated.

3. Recruitment and Clinical Outcomes

3.1 Introduction

End-stage renal failure (ESRF) leads to major morbidity and increased mortality compared to the general population. However it can be optimally treated by kidney transplantation[1], and can be combined with a pancreas transplant in patients with ESRF secondary to type 1 diabetes[356]. Outcomes in solid organ transplant are steadily improving with a 75% graft survival rate at 10 years in kidney alone transplants[339] and 72-78% graft survival rate at 5 years in simultaneous kidney-pancreas (SPK) transplant[340]. However, most of the improvements in outcome have come from increases in early graft survival and the rate of long-term decline remains fairly stable[2-4]. The commonest cause of transplant failure is death of the patient with a functioning graft, usually from malignancy or infection[1]. Second to death with a functioning graft, the failure of transplanted kidneys is often slow and non-specific, associated with interstitial fibrosis and tubular atrophy (IFTA) on biopsy, which can be due to a number of different processes. In recent years, antibodies reacting against donor HLA molecules have been identified as being associated with, and possibly responsible for many cases of chronic graft decline. These donor specific antibodies (DSA) can significantly reduce graft lifespan even in the absence of histological markers of acute antibody mediated damage such as complement deposition or inflammatory cell infiltration into the graft [11-13].

3.2 Development of HLA matching for transplantation

DSAs were first detected in 1969 when Patel and Terasaki developed a cell-based assay, using donor lymphocytes incubated with recipient serum[179]. This complement-dependent cytotoxicity cross match (CDC-XM) showed that patients with pre-formed, high titre antibodies that caused cell lysis were more likely to lose their grafts to immediate rejection. This, alongside the characterisation of HLA molecules, formed the basis of early attempts at donor-recipient matching to improve outcomes[180,181]. Initially only cell-based assays were available; the CDC-XM was developed to pre-screen potential recipients by combining a panel of 25-60 donor cells with recipient serum. The donors for the panel were chosen to attempt to reflect the HLA distribution of potential organ donors, and recipient serum was classed according to the percentage of the panel there was reactivity to, hence the panel reactive antibody (PRA) assay. This was a crude measure, and only detected high titre HLA antibodies that caused complement mediated cell lysis. This test was variable between laboratories depending on the panel of lymphocytes chosen[281]. With the development of flow cytometry, more detailed cell-based assays were developed[282], allowing identification of lower titre antibodies that would bind to donor leucocytes, even if they were insufficient to cause cell lysis[283]. Donor leucocytes are incubated with recipient serum and then stained with fluorescently labelled anti-human-IgG and IgM antibodies to detect antibody binding (see Chapter 1, **fig 1.6**).

However, the genetics of HLA are extremely complex, there are multiple genetic loci encoding each molecule, making the HLA antigens extremely polymorphic throughout the population. While each HLA molecule has a unique combination of amino acid epitopes, many epitopes are shared with other HLA molecules.

HLA antigens were originally identified serologically using the cell-based assays above, and based on the reactivity of serum from multiparous women to cells of different individuals. There was unparalleled international collaboration between histocompatibility laboratories, the antigens were named serologically based on the order of discovery and the nomenclature became more detailed and complex as more specificities were unearthed[202,357].

As more sera were analysed, it became clear that some sera reacted not against specific HLA antigens but against epitopes that were shared between multiple antigens, for example HLA-Bw4 is not a separate HLA antigen, but an epitope shared between around 20 different HLA-A and HLA-B antigens[357,358]. This allowed HLA molecules to be linked according to these cross-reactive epitope groups (CREGs), as it became clear that antibodies to these public, shared CREG epitopes would react to all members of that CREG. However antibodies can also be directed against epitopes that are unique to a particular molecule, rather than to the shared epitopes between molecules. As an example, HLA-A9 was originally identified, but more detailed testing revealed that this was an epitope shared between HLA-A23 and HLA-A24. Some sera contained antibodies directed against HLA-A23 alone, some against HLA-A24 alone, and some against epitopes shared by both molecules, giving the broad specificity that

was originally identified as HLA-A9. A donor specific antibody is therefore one that will react with the donor HLA, whether it is specifically directed against the donor HLA or at shared CREG epitopes.

Various methods of HLA matching donors and recipients were tested, but evidence showed that the majority of the benefit came from matching patients at HLA-A, HLA-B, and HLA-DR[276-280], with little additional benefit from matching for other loci in kidney transplantation. There is some evidence that matching at other HLA loci may reduce rejection rates[359-362] and HLA-C and HLA-DQ types are routinely reported because a patient may have unacceptable HLA-C and DQ mismatches and this information is therefore required for kidney allocation, but matching is still based on HLA-A, B and DR as the dominant alleles.

When HLA alleles were genetically sequenced, the number of alleles was found to be far in excess of the serological HLA antigens, as most antigens are encoded by a group of alleles. All patients are now HLA typed using DNA sequencing technology but HLA matching is carried out at a serological level[287].

Although cell based assays are still in use for immediate pre-transplant cross matching, routine antibody detection and specification when listing for transplant is carried out using solid phase assays (SPA) e.g. Luminex. Patients are initially screened with a mixed panel of beads, where two or more bead populations are present (**fig 3.1A**), each population coated with either purified HLA class I (A, B and C) or purified HLA class II (DR, DQ and DP)

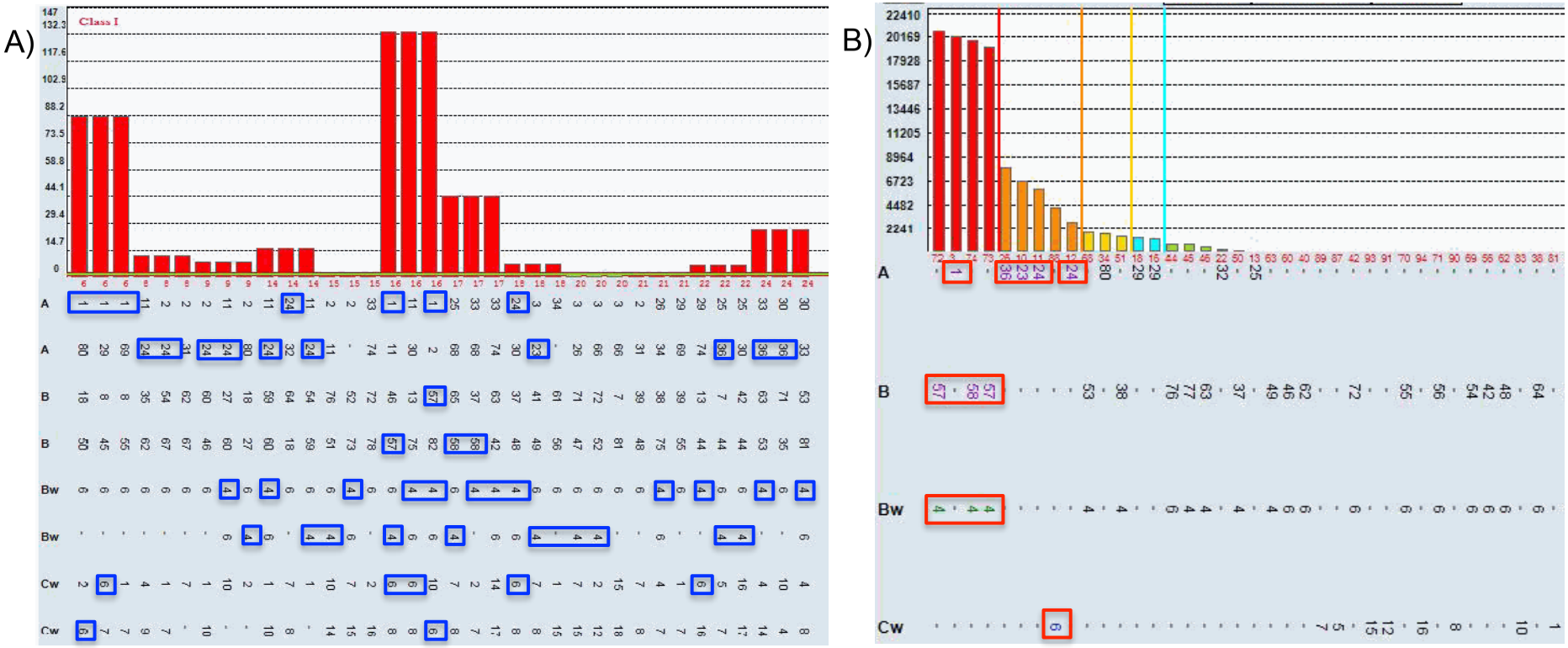


Figure 3.1: Mixed screen compared to single antigen bead. A) Mixed screen showing broad class I positivity but with multiple potential antigens. B) Class I single antigen bead for the same patient showing the positive antibodies highlighted in red boxes, the same antigens are highlighted in blue boxes on the mixed screen, showing the diversity of the mixed screen beads compared to single antigen beads.

antigens. This allows detection of HLA antibody without allowing determination of specificity beyond class I or class II. Although each bead will have several antigens, and the whole HLA spectrum should be represented in each lot, LabScreen Mixed Beads show significant variability between lots[363] with different patterns of HLA on different beads and therefore potentially different signal strengths with the same amount of antibody. For this reason, if HLA antibody is detected, specificity determination requires more detailed assays[286,287].

PRA bead assays are available; where each bead population contains defined HLA class I or HLA class II proteins from an individual donor, meaning it is possible to further define specificities. However the most sensitive and specific SPA is single antigen bead (SAB) testing (**fig 3.1B**), where each bead population is coated with a single recombinant HLA class I or class II protein, allowing clear definition of the antibody, with the caveat that binding with the isolated antigen on the bead may not correlate with binding *in vivo.* This has been postulated to be due to the nature of production of SABs, where denaturing of the antigen may expose epitopes that are not present, or not visible on the HLA molecule when it is on the cell surface[284,285].

At the time of transplant, all potential recipients will undergo a cross match against donor cells. In non-sensitised individuals with no HLA antibodies this can be carried out as a 'virtual' crossmatch, where the patients HLA type is compared with the potential donor HLA type and the predicted reactivity is used to determine the likelihood of a positive crossmatch. There is evidence to suggest that a negative virtual crossmatch is a suitable substitute for a pre-emptive

crossmatch in selected individuals[6,288], although it should be followed up with a laboratory cross match within 24-48 hours. However in patients with known HLA antibodies and/or recent sensitising events, a pre-emptive crossmatch is carried out. This involves cell-based assays such as flow cytometric cross match and may involve testing with SPAs, although this is not carried out in all laboratories. The role of the pre-emptive cross-match is to detect new antibodies directed against donor antigens and new HLA specificities against non-donor antigens that have appeared since the most recent pre-transplant sample[287].

Post-transplant, there is no consensus on the indications for routine screening for anti-HLA antibodies in the absence of graft dysfunction. Kidney alone transplant recipients who have not undergone desensitisation for pre-formed antibodies are not routinely screened, in part because there is also no consensus on how to manage DSAs in the absence of rejection. Screening would be carried out according to clinician preference or in the case of graft dysfunction to determine the likelihood of ABMR in conjunction with histology[16]. There is debate as to whether screening should be carried out routinely in SPK recipients[364], as they seem to be at higher risk of *de novo* DSA development. However, with the literature suggesting up to a third of patients developing *de novo* DSA across the lifetime of a solid-organ graft[13,202,365], reducing both the graft lifespan and the likelihood of receiving a subsequent transplant in future, there is a need for a reliable method of predicting the development of *de novo* DSA and identifying potential areas for intervention.

3.3 Aims

To recruit a prospective cohort of kidney and SPK transplant patients and follow them for the first 2 years post-transplant, monitoring for graft function and development of both donor-specific and non-specific HLA antibodies.

The hypothesis was that 10-15% of these patients would develop *de novo* antibodies over the first 2 years post-transplant, based on the international experience[13,199,202,365].

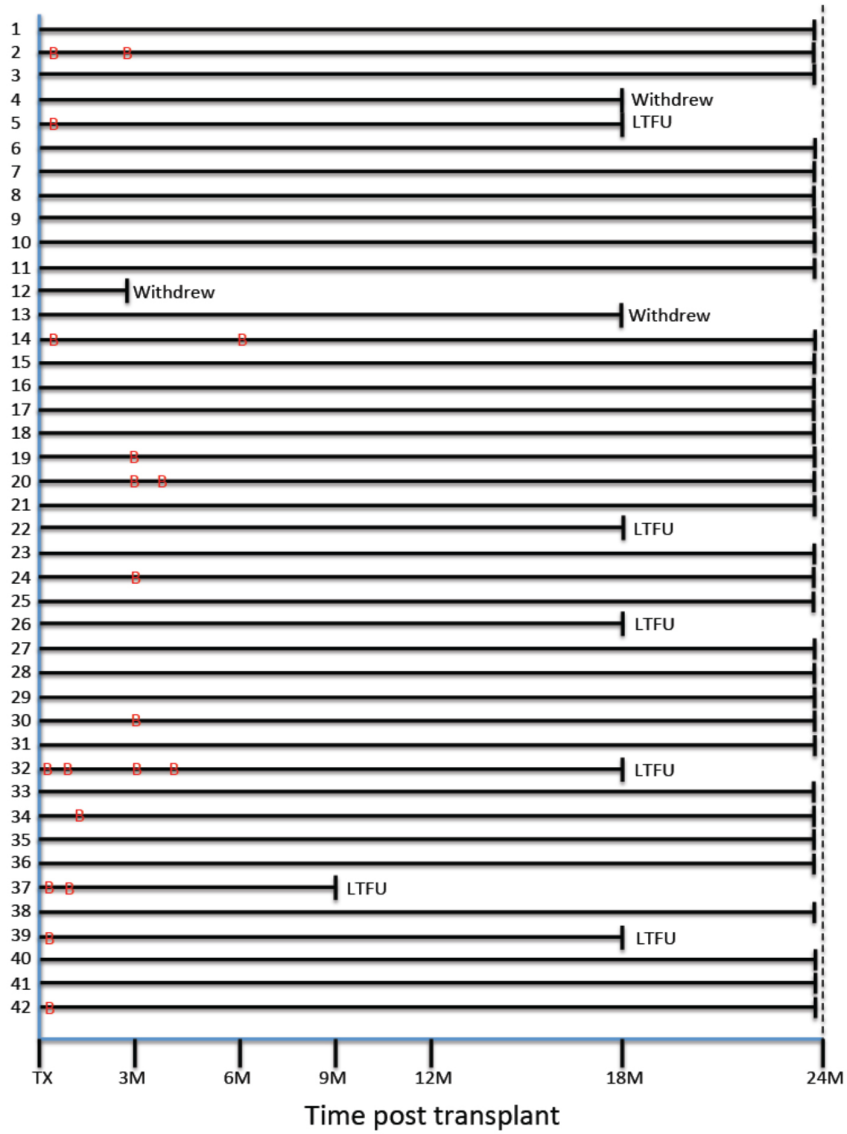
3.4 Results

3.4.1 Recruitment and patient outcomes

After ethical approval (REC 14/SC/0091) 61 patients were recruited to the study between May 2014 and December 2014. Clinical characteristics of the patients at recruitment are shown in **table 3.1**. Patients were followed up until December 2016. During the course of the study a number of patients withdrew or were lost to follow up, as detailed in **fig 3.2**. In total 31 of 42 kidney patients (74%) and 10 of 19 SPK patients (53%) were followed up to 2 years.

	Kidney	Kidney Pancreas
Number recruited	42	19
Sex M/F	31/11	9/10
Median age (range)	54 (26-74)	47 (30-59)
Donor type		
Living	16	n/a
DBD	11	16
DCD	15	3
Drug treatment regimen		
Alemtuzumab	19	19
Basiliximab + MMF	13	n/a
Basiliximab + Aza	10	n/a
Cause of ESRF		
Autoimmune	8	19
ADPKD	7	
Hypertensive/vascular	14	
Anatomical (e.g. reflux)	1	
Other/unknown	12	

Table 3.1 Table of patient characteristics at recruitment. 61 patients were recruited in total. DBD = donor after brainstem death, DCD = donor after cardiac death, MMF = mycophenolate mofetil, Aza = azathioprine, ESRF = end-stage renal failure, ADPKD – Autosomal dominant polycystic kidney disease. Causes defined as autoimmune included type 1 diabetes, ANCA-associated vasculitis, primary membranous nephropathy, IgA nephropathy. 'Other' causes included medullary cystic kidney disease, TB kidney, primary focal segmental glomerulosclerosis (FSGS) and tubulointerstitial nephritis.



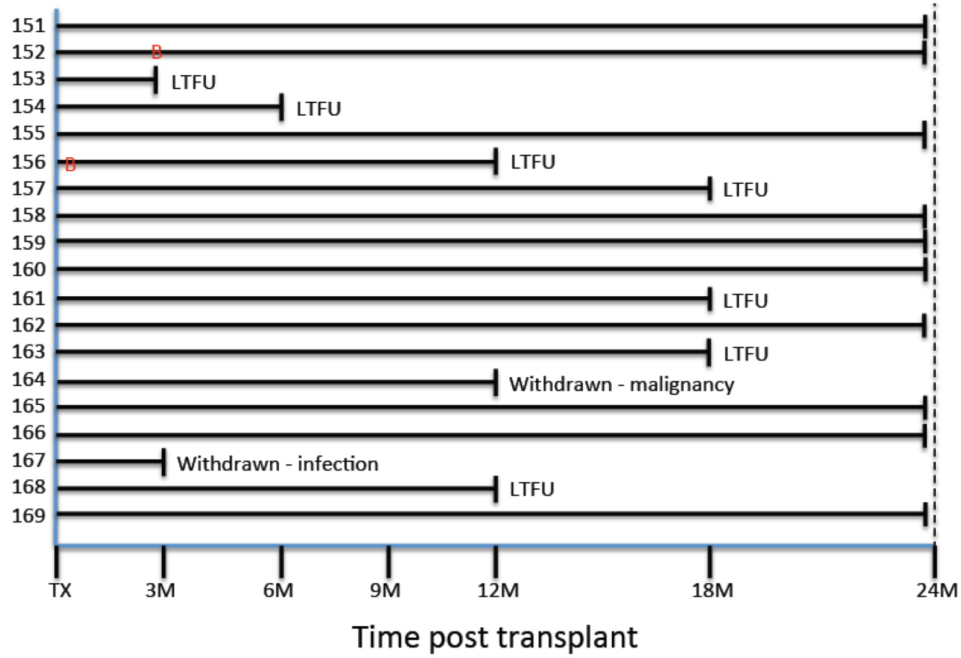


Figure 3.2
Follow up of patient samples. Kidney transplant patients (1-42) and simultaneous kidney pancreas (SPK) transplant patients (151-169) are shown. B = transplant biopsy. LTFU = lost to follow up.

Of the kidney-alone transplant patients, three withdrew consent to further sampling, one at 3 months and the others at 18 months. Six were lost to follow up, one at 9 months, the other five after the 18 month time point. All patients lost to follow up had been referred from peripheral centres and returned to local follow up. One patient died 6 months after completion of the study.

Of the SPK patients, two were withdrawn for clinical reasons; one developed a disseminated tuberculosis (TB) early post-transplant and was withdrawn when therapy was commenced. The second developed a post-transplant lymphoproliferative disorder (PTLD) requiring chemotherapy. Seven patients were lost to follow up, all returning to peripheral referral centres where research samples could no longer be obtained.

3.4.2 Graft outcomes

Over the course of the study 21 biopsies were performed in 14 patients, **table 3.2** shows details of biopsy findings and treatments. In total there were seven episodes of biopsy proven acute TCMR in 6 patients, therefore 9.8% of patients having at least one episode of acute rejection, in keeping with both UK and international averages[315,366,367]. No patients receiving alemtuzumab induction (treatment group 1) had an episode of TCMR, 3 of 6 received basiliximab induction with MMF maintenance (treatment group 2), and the remaining 3 basiliximab induction with azathioprine maintenance (treatment group 3). This is consistent with previously published work suggesting a lower rate of acute rejection with alemtuzumab induction[315,368].

Pt	Rx	Time of biopsy	Results	Treatment
2	3	10 days	ATN	None required
		2 months	ATN with chronic damage	
5	2	7 days	Borderline TCMR	Pulsed MP
14	2	5 days	TCMR Banff 2A	Alemtuzumab and MP
		5 months	TCMR, CMV and possible ABMR (glomerulitis)	Pulsed MP
19	3	4 months	CNI toxicity	Tacrolimus level reduced
20	3	3 months	Borderline TCMR	Pulsed MP, azathioprine switched to MMF
		4 months	No rejection	
24	1	3 months	Nephrocalcinosis + 40% IFTA	None required
30	1	3 months	CNI toxicity	Tacrolimus changed to sirolimus, MMF stopped
32	3	7 days	Tubulitis due to UTI	
		3 weeks	TCMR Banff 1B	Alemtuzumab, azathioprine switched to MMF
		3 months	BK virus + 50% IFTA	None required
		4 months	Tubulitis, BK resolved	None required
34	1	1 month	CNI toxicity	Tacrolimus level reduced
37	3	4 days	TCMR Banff 1B	Pulsed MP
		2 months	ATN, CNI toxicity	Tacrolimus level reduced
39	2	4 days	ATN	None required
42	2	7 days	TCMR Banff 1B	Alemtuzumab and MP
152	1	3 months	CNI toxicity	Tacrolimus level reduced
156	1	7 days	Acute CNI toxicity	Tacrolimus level reduced

Table 3.2: **Results of biopsies.** Pt = patient. Rx = treatment group – 1 = alemtuzumab, 2 = basiliximab + MMF, 3 = basiliximab + azathioprine. ATN = acute tubular necrosis. TCMR = T cell mediated rejection.
MP = methylprednisolone. CMV = cytomegalovirus. ABMR = antibody mediated rejection. CNI = calcineurin inhibitor. IFTA = interstitial fibrosis and tubular atrophy. UTI = urinary tract infection.

Five of the six patients responded to either pulsed methylprednisolone (MP) alone or MP with the lymphocyte depleting agent alemtuzumab (see **table 3.2**). Two patients who had received basiliximab induction with azathioprine were switched to MMF maintenance therapy and analysed as group 2 patients. All three patients receiving alemtuzumab for treatment of rejection were analysed as group 1 rather than their original treatment groups, because this lymphocyte depleting agent was given within the first few days after transplantation and prior to the first post-transplant study sample, therefore phenotyping would reflect the reconstitution of lymphocytes. One additional patient (G009) received alemtuzumab therapy for presumed rejection without biopsy, therefore final numbers were:

- Group 1, alemtuzumab treated (19 SPK at induction, 19 kidney at induction, 4 kidney for rejection): n= 42 (40 MMF maintenance, 2 azathioprine maintenance as per 'young adult' policy)
- Group 2, basiliximab induction, MMF maintenance (B+M): n=10
- Group 3, basiliximab induction, azathioprine maintenance (B+A): n=9.

One patient had an early steroid unresponsive TCMR, which was treated with alemtuzumab, but whose creatinine did not fully recover. Repeat biopsy at 5 months showed persistent TCMR and possible ABMR with glomerulitis, but with co-existing cytomegalovirus (CMV) infection. With coincident infection suggesting over-immunosuppression, and rejection suggestion under-immunosuppression, treatments options were limited and were ultimately unsuccessful. The patient lost the transplant to on-going rejection at 7 months. Following this,

immunosuppression was reduced to allow recovery from infection, but continued at low level to try and prevent *de novo* DSA development.

The patient who was withdrawn due to PTLD returned to dialysis shortly after completing chemotherapy, as the kidney graft failed due to rejection.

3.4.3 Kidney function

23 of 61 patients required haemodialysis within the first week post-transplant, giving a delayed graft function (DGF) rate of 37.7%. As expected[369] the majority of these were in those receiving DCD kidneys with 13 of the 18 DCD recipients requiring dialysis in the first 7 days (72%) compared to 7 of 27 DBD recipients (26%) and 3 of 16 live donor recipients (19%). The rate of dialysis requirements in live donor recipients is high compared to the national average, but all were biopsied within the first 7 days, and two had TCMR, one an acute tubulitis suggestive of urinary sepsis.

In general, creatinine fell in all patients over the course of the study, and, with the exception of the two who lost their grafts, remained steady across the 2 years (**fig 3.3**). Separating patients by drug group suggested more stability in the basiliximab and azathioprine treated group compared to either alemtuzumab or basiliximab with MMF treatment.

3.4.4 Positivity on anti-HLA antibody detection assays

Patients were screened regularly during the study for anti-HLA antibodies using the Luminex platform used for clinical tissue histocompatibility

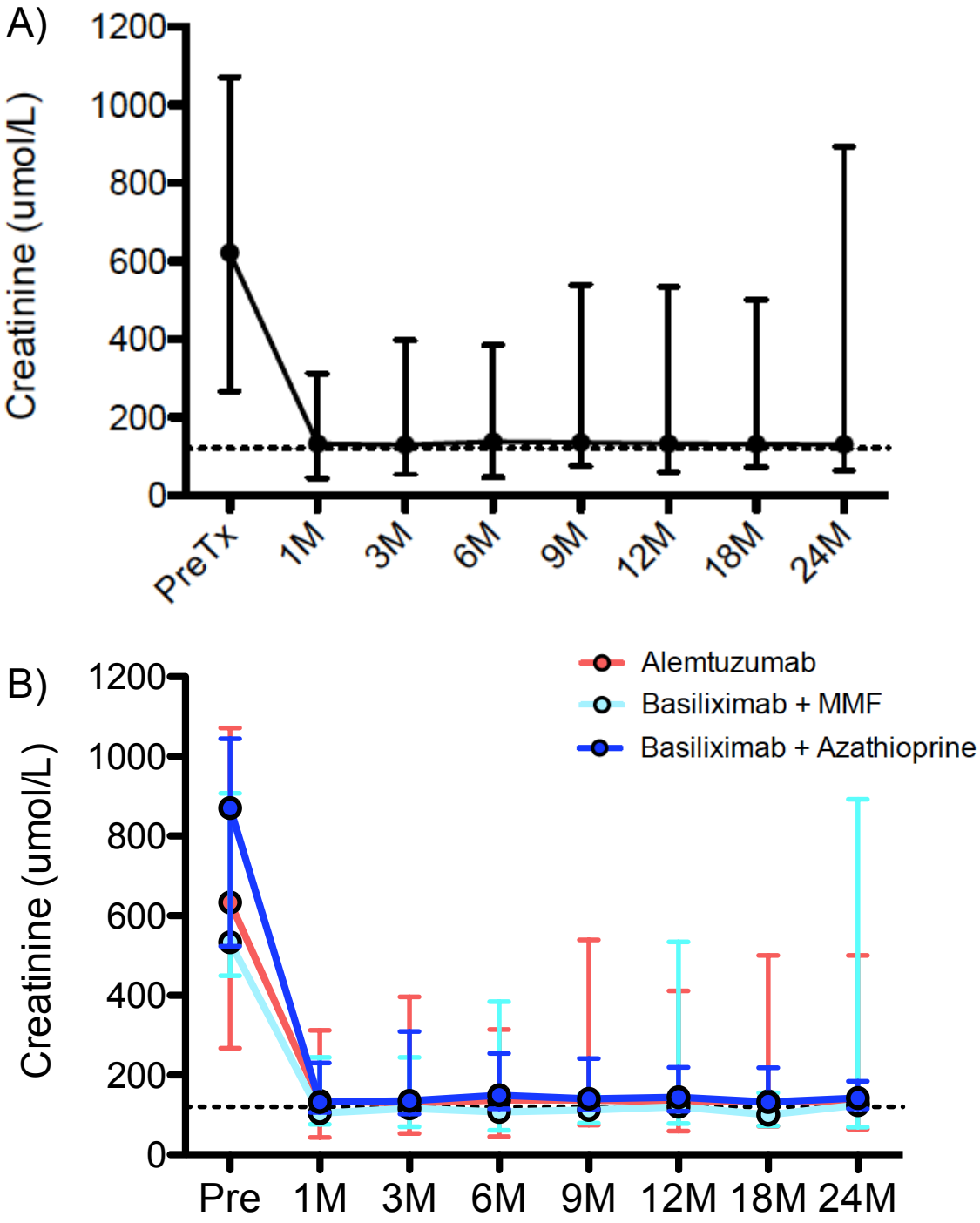


Figure 3.3: Renal function. Renal graft function in A) All patients across the two years, shown as median and range. B) Patients separated by treatment regimen showing median creatinine and range.

assessment. Antibody detection was undertaken using LabScreen Mixed beads (OneLambda), which contain HLA class I and class II coated beads. Results were analysed using HLA Fusion software to determine which were positive, and therefore required further testing, and which were negative.

22 patients were negative pre-transplant and remained so throughout the study (36%), a representative example is shown in **fig 3.4A.** 10 patients (16%) had pre-transplant HLA antibodies that were not donor specific, in many cases these were directed against previous donors, or in women with history of pregnancy, against their partner's HLA. These patients were assessed with either PRA beads, or single antigen beads and the antibody profile was found to be unchanged. A representative example is shown in **fig 3.4Bi & 3.4Bii.** 4 patients (7%) had pre-transplant HLA antibodies that were not donor specific, and became negative across the course of the study, all were male and all antibodies were directed against class I antigens. A representative example is shown in **fig 3.4C.** These may be examples of 'natural' antibody formation as described in section **1.5.4** of chapter 1, or may be responses to blood transfusions, which all patients had received prior to transplantation as part of management of their ESRF and are known to cause HLA antibody formation in patients with ESRF[193,195]. Sensitisation to blood product transfusion usually does not produce long lasting antibodies, levels decrease with time and studies have shown that memory B cells directed against HLA are rarely detectable in the blood of patients where transfusion is the only sensitising event[195].

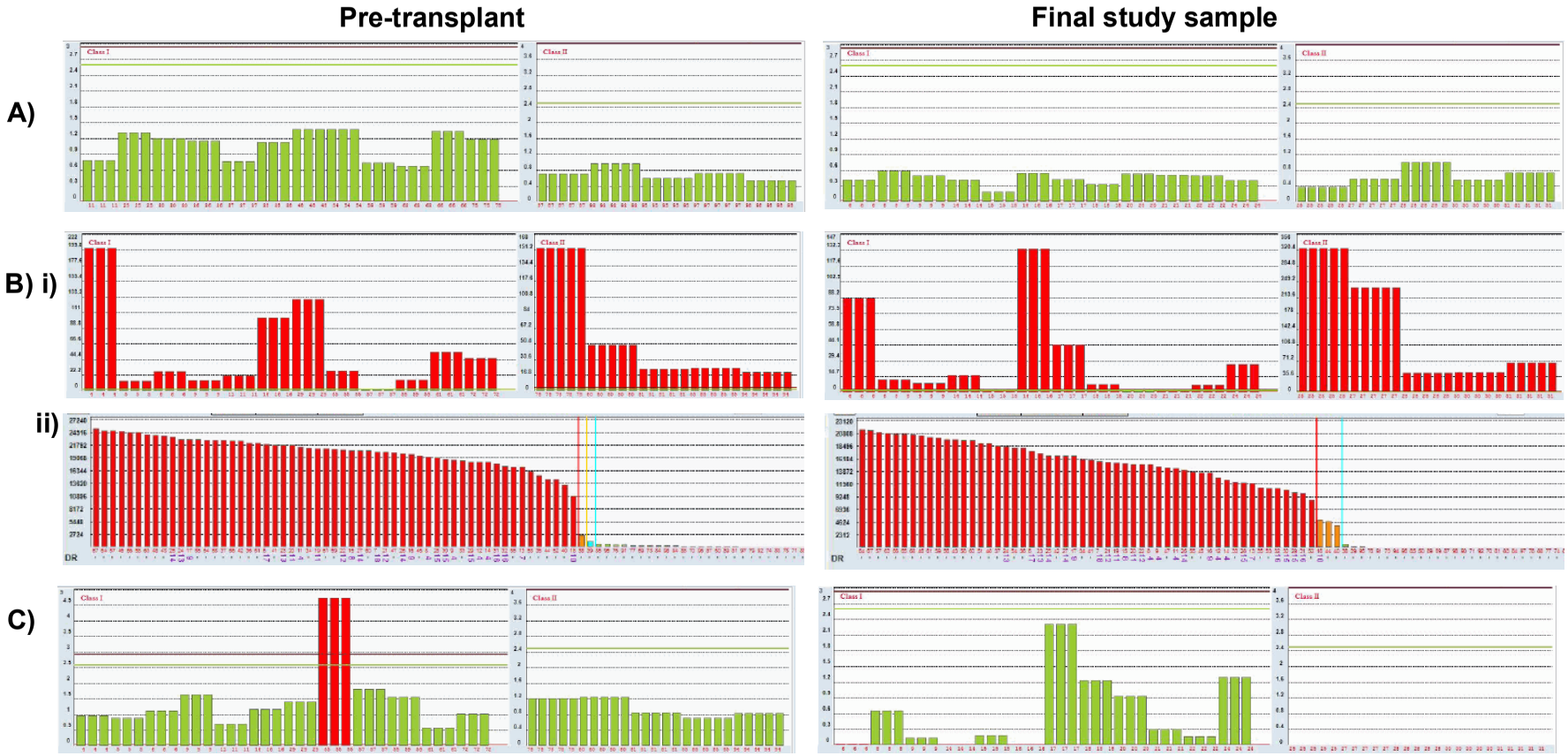


Figure 3.4: Example mixed screens for patients without DSAs. A) Representative patient with negative screens pre-transplant and remained negative throughout the study. B)i) representative patient with multiple pre-existing anti-HLA class I and II antibodies, including to a previous donor, with a calculated reaction frequency (cRF) of 99%. In the final study sample, the positive screen was followed up with single antigen bead (SAB) testing, class II shown in (ii), which revealed an unchanged profile compared to pre-transplant SAB. C) Representative patient who positive for HLA-B37 pre-transplant, but lost antibody positivity over the course of the study. The horizontal positive cut-off line is shown in red, negative cut off in yellow. Negative samples are shown by vertical green bars and positive by vertical red bars.

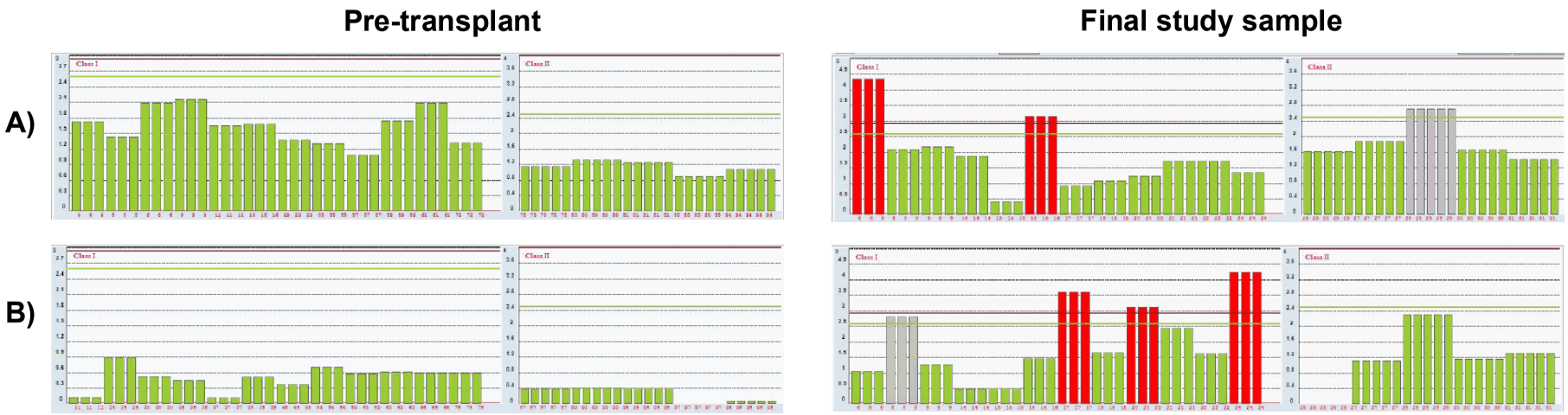


Figure 3.5: Example mixed screens. All serum samples were screened using mixed HLA class I and II beads initially, examples of pre-transplant (left) and post-transplant (right) screens are shown for two representative patients who developed new HLA antibodies. The horizontal positive cut-off line is shown in red, negative cut off in green. Negative samples are shown by vertical green bars, intermediate by vertical grey bars, and positive by vertical red bars.

Across the course of the study, 27 patients (44%) developed new positivity on mixed screens or an altered profile. In the majority of cases their screens had been negative pre-transplant (representative examples seen in **fig 3.5 A, B**) and became positive during the course of the study. In other cases, patients had pre-existing HLA antibodies that were not donor specific, but over the course of the study the profile of their Mixed Screens changed. Because of the significant lot variation in LabScreen Mixed Beads[363] (see above) more detailed assays were required to determine whether antibody specificity had changed or if this reflected this variability.

3.4.5 Detailed assessment of HLA antibody screens

Prior to transplantation, patients who are positive on mixed screen or those who were considered sensitised should be further assessed using either PRA or SAB with Luminex testing[287]. In Oxford, all patients are assessed and will be screened 3 monthly as per British Transplantation Society/British Society for Histocompatibility and Immunogenetics (BTS/BSHI) guidelines[287] using more detailed tests to confirm the absence of antibodies that either could not be detected or were indeterminate on mixed screens.

Post-transplant, mixed screens that were newly positive or suggested an altered profile compared to previous positive screens were tested with more detailed screening (27/61 patients). In 5 patients, PRA or SAB screening was negative or showed a stable profile with no *de novo* HLA antibodies.

22 patients who were further evaluated developed *de novo* HLA antibodies. In the majority of cases (16/22) these were not directed against donor HLA. More details are shown in **table 3.3**, where a specific antibody has been detected, this is detailed, if patients developed an increase in multiple class I or class II antibodies this has been noted. Timing of *de novo* antibody formation followed a bimodal distribution, with 14/22 developing a positive or altered mixed screen within the first 6 months, most at either the 1 or 3 month time points. There was a second peak of detection at 12-24 months, although this may reflect the less frequent sampling beyond the first year missing antibodies developing at earlier time points. Many of the *de novo* HLA antibodies that were not donor specific occurred early post-transplant, which may reflect inflammatory events such as infection or sensitising events such as blood product transfusion, particularly in the SPK patients. No HLA antibodies were specifically associated with biopsy proven rejection.

Pt	Pre Tx	Post Tx changes						De novo HLA	De novo DSA
		3M	6M	9M	12M	18M	24M		
3	Neg						X	B13	
14	Pos						X	Multiple DP, DQ	DQ6
15	Neg	X		X				A43, DP1	B8
16	Neg				X			DP1, DP5	
17	Neg						X		Cw9
19	Neg	X						B76, B82, Cw15, DR4, DR13	
23	Neg		X					B13	
25	Neg						X	DP11	
32	Neg	X						B57	
34	Neg	X						Multiple I + II	
36	Neg	X						DR4, DR16	
39	Neg		X					DP11	A2
42	Pos	X						A10, A28, B75	DQ4, DQ6
152	Neg		X					DQ6	
154	Neg				X			B75, Cw18	
157	Pos	X						Multiple I + II	
158	Neg						X	DP11, DP14	
161	Neg				X			DR18, DP6	B58
164	Pos				X			B8, DR16, DP28	
165	Neg	X						Multiple class I, DR8	
168	Pos	X						A29, A33, A66, A68, B76	
169	Pos	X						B7, B27, B48, B81, B82	

Table 3.3 Outcomes of HLA antibody screening. Pre transplant antibodies are shown with the first alteration in profile indicated by X. If the profile changed again a second X is shown.

3.4.6 De novo DSA developed in 6 patients

In 6 patients, anti-HLA antibodies were reactive to donor antigens. 4 patients developed these within the first 6 months, one at 12 months and one at 24 months. Details of each patient are shown in **figs 3.6 – 3.17**

G014

This patient had had a previous kidney transplant in 2002, which failed 2 months prior to the current transplant in 2014, the patient having been re-listed pre-emptively. The patient had developed anti-HLA antibodies to the first donor, seen in **fig 3.6A** highlighted in blue. The second, living donor (LD) was well matched at class I but with mismatches at class II HLA DR and DQ. HLA-DP was not typed as the recipient had no anti-DP antibodies detected pre-transplant. Mixed screens were positive for both class I (not DSA) and class II (previously identified DSA to DR53) prior to the transplant. Class I positivity fell below the positive cut-off level within the first year of the transplant (**fig 3.6B**) but class II remained positive throughout. Induction therapy was with basiliximab, and maintenance therapy with tacrolimus and MMF. Tacrolimus was started one week prior to transplant as per local policy for living donor transplants. As seen in **table 3.2** this patient had a difficult post-operative course requiring multiple biopsies. The creatinine failed to fall despite the living donor, and a biopsy at 5 days (first blue arrow in **fig 3.6C**) showed a moderate acute TCMR with arteritis, Banff 2A *(please see Appendix 1 for Banff classification of renal transplant histological changes).* The creatinine did not fall with pulsed methylprednisolone (MP) and so alemtuzumab treatment was given. Renal function improved (**fig 3.6C**) to three months but then began to rise and a repeat biopsy (second blue arrow) showed

on-going acute TCMR with glomerulitis suggestive of ABMR, and coincident infection with CMV. The patient received pulsed MP but renal function continued to declined, with graft failure at 7 months post-transplant and a return to dialysis (purple arrow in **fig 3.6C**). Immunosuppression was slowly weaned with MMF being stopped on return to dialysis, and tacrolimus weaned and stopped 15 months after the transplant.

SAB assessment of the patient serum is shown in **fig 3.7**. Pre-transplant SAB showed the known HLA-DR53 to the previous donor, but no specificities directed against the current donor (**fig 3.7A**). Mixed screens of study samples continued to show positivity, but detailed assessment showed only the pre-existing HLA-DR53 until the 24 month sample, which was taken after immunosuppression was ceased. **Fig 3.7B** shows the 24 month class II SAB with the emergence of multiple class II antibodies to DQ6 (DSA) and other DQ and DP antigens (not donor-specific).

A)

	A		**B**		**Bw**		**C**		**DR**		**DRB3 45**		**DQ**	
Recipient	1	32	8	44	4	6	5	7	17	-	52	-	2	-
Donor 1 (failed)	1	**3**	8	**53**	4	6	**4**	7	**7**	17	52	**53**	2	-
Current donor	1	32	44	-	4	-	5	-	**15**	17	**51**		2	**6**

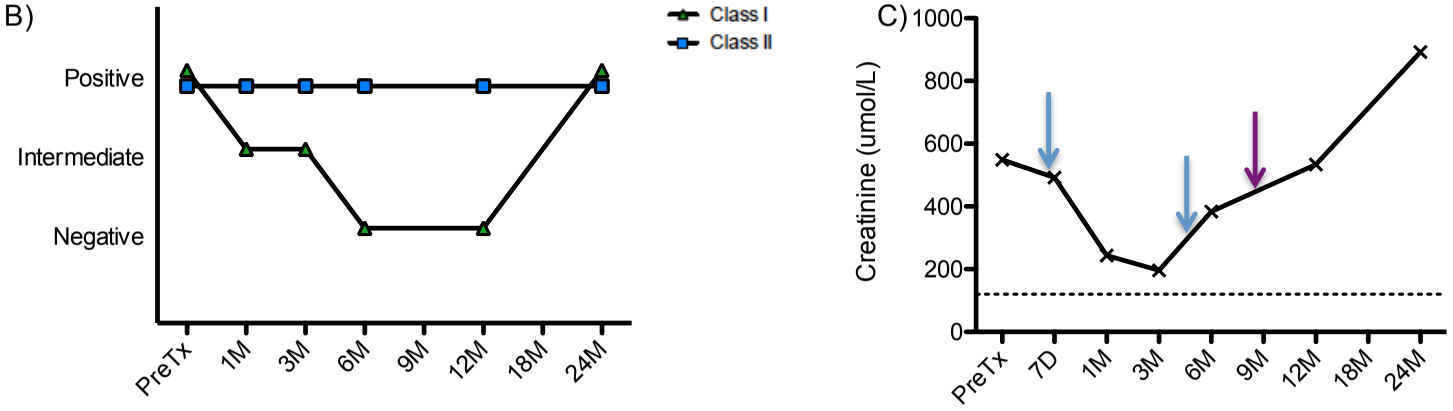


Figure 3.6: G014 Clinical Outcomes. A) Genotype of recipient compared to first donor (transplant failed) and current donor. Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-), de novo DSA highlighted in red, previous DSA shown in blue. B) Mixed screen results across the study, showing HLA class II positivity at all time points, but fall in HLA class I antibodies until 12 months when immunosuppression was withdrawn. C) creatinine over the course of the study, showing biopsy proven rejection episodes - blue arrows, and return to dialysis - purple arrow.

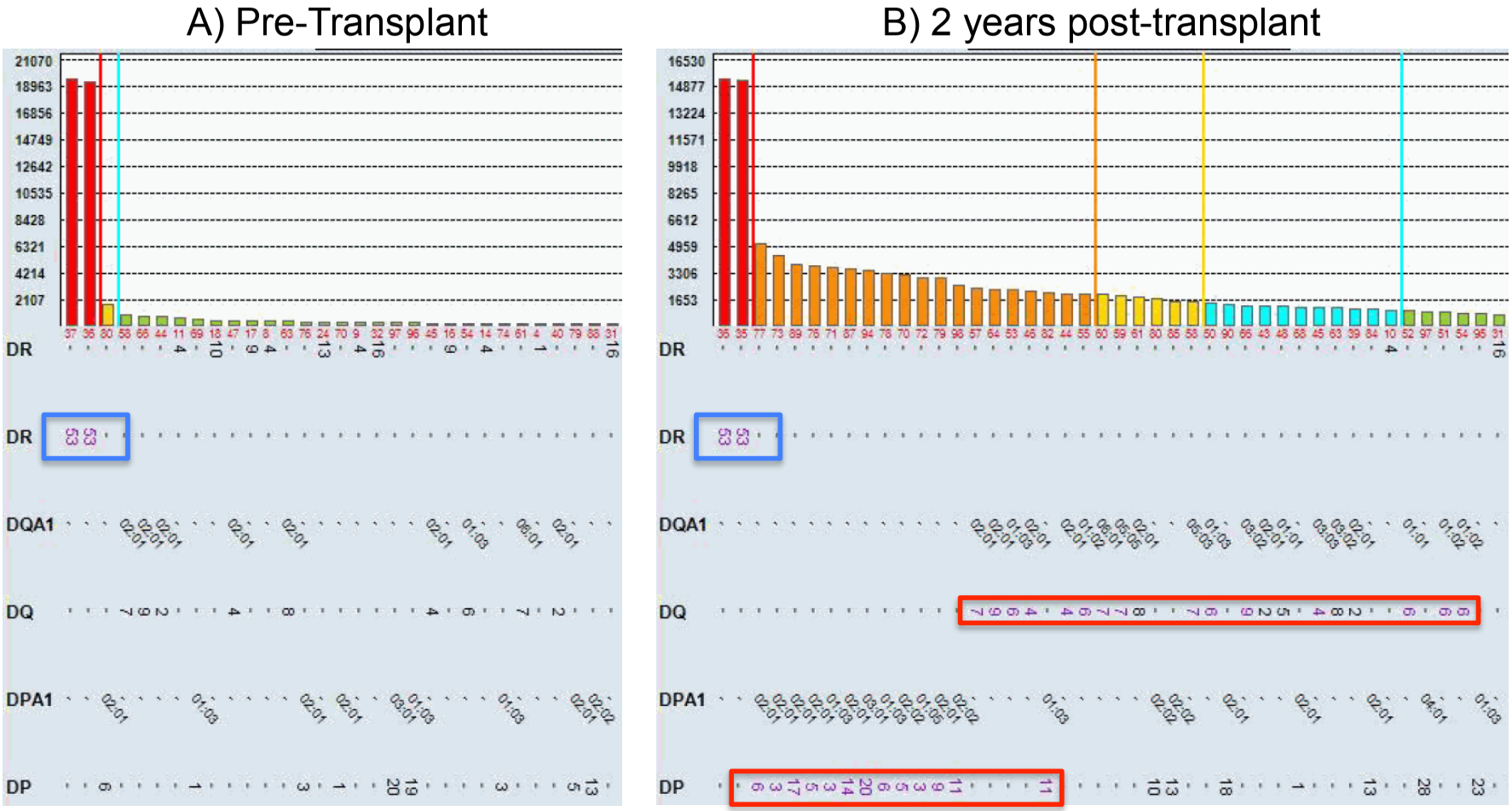


Figure 3.7: Single antigen bead profile G014. A) Single antigen bead test sample from pre-transplant showing pre-existing anti-HLA-DR53 antibodies against first donor. B) Single antigen bead test sample from 2 years post-transplant showing de novo DSA against multiple HLA-DQ and HLA-DP epitopes

G015

This patient received a LD kidney transplant with immediate graft function. As seen in **fig 3.11A** the donor was matched at one of two loci for each antigen. Mixed screens pre-transplant showed negative class I and II screens (**fig 3.11B**), which was supported by negative PRA screening. Induction therapy was with alemtuzumab, and maintenance therapy with tacrolimus and azathioprine. Tacrolimus was started one week prior to transplant as per local policy for living donor transplants. Normal policy would be to combine alemtuzumab with MMF, but in younger individuals where there is a concern over non-concordance with medication, the once-a-day dosing of azathioprine is preferred. The patient had excellent renal function (**fig 3.8C**), and only a minor rise in creatinine at 1 month, which rapidly fell, and then remained stable over the course of the study.

Mixed screens became positive at three months post-transplant for class I, and intermediate for class II. Although both fell at 6 months, class I then became persistently positive on mixed screens (**fig 3.8B**). As pre-transplant mixed screens were negative, the patient was screened with class I PRA bead screen pre-transplant (**fig 3.9A**) on two separate occasions. As both PRA screens were negative this patient was not screened with SAB pre-transplant. When mixed screens became positive at 3 months post-transplant, SAB testing showed emergence of *de novo* HLA-A43 (not donor specific) and HLA-B8 (donor specific) antibodies. At 24 months (**fig 3.9B**) these persisted, but there was still no evidence of graft dysfunction (**fig 3.8C**).

A)

	A		B		Bw		C		DR		DRB345		DQ	
Recipient	2	30	44	51	4	-	2	5	4	11	52	53	7	-
Donor	**1**	2	**8**	51	4	**6**	2	**7**	11	**17**	52	-	**2**	7

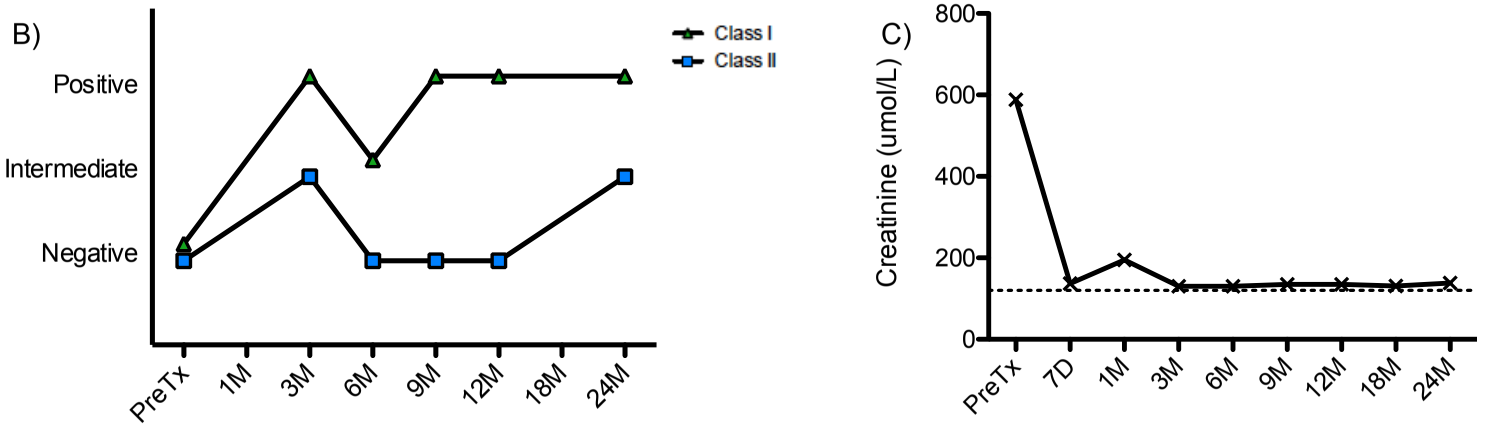


Figure 3.8: G015 Clinical Outcomes. A) Genotype of recipient compared to donor. Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-), de novo DSA highlighted in red. B) Mixed screen showing positive class I HLA from 3 months, persisting across the 2 years, and occasionally positive class II. C) Creatinine over the course of the study, this patient was not biopsied.

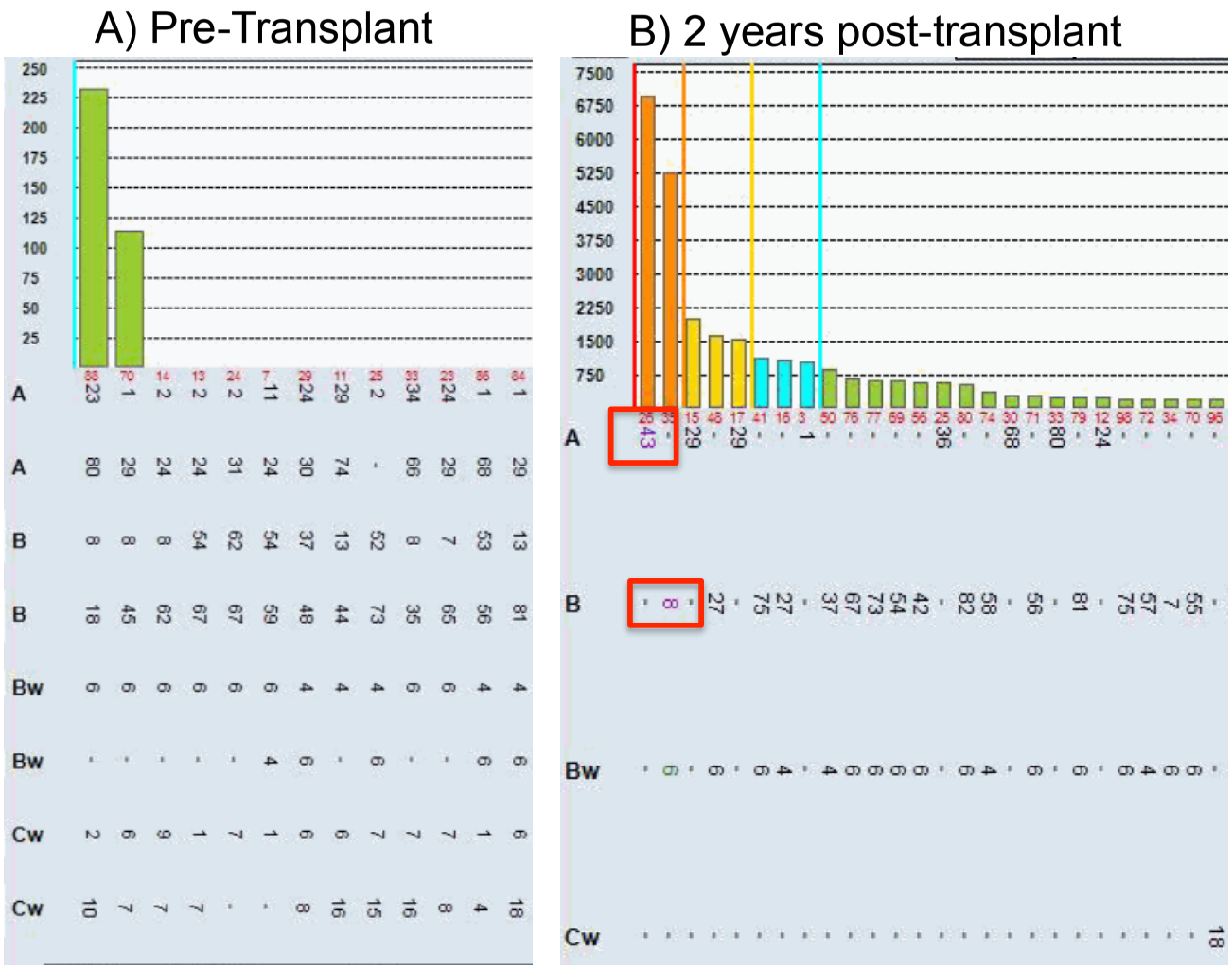


Figure 3.9: Single antigen bead profile G015.
A) Class I panel-reactive antibody (PRA) bead screen pre-transplant negative screen. This patient was not screened with single antigen beads (SAB) as both mixed screen and PRA were negative.
B) Single antigen bead screen at 2 years showing de novo antibodies against HLA-A43 (not donor-specific) and HLA-B8 (donor-specific). These specificities were identified at 3 months and persisted over the 2 years of the study.

G017

This patient received a DCD kidney that was poorly matched across all HLA loci (**fig 3.10A**). The patient was non-sensitised and had a negative crossmatch pre-transplant, but was 74 years old at the time of transplant. In the UK, advanced age is not a contraindication to transplantation[8] but clinicians may accept a less well matched kidney in an older individual to balance the lower risk of graft decline against the higher risk of remaining on dialysis[356]. Pre-transplant mixed screen was negative (**fig 3.10B**) as was PRA screening pre-transplant (**fig 3.11A**). As this was a DCD transplant, alemtuzumab induction with tacrolimus and MMF maintenance was used. The patient had DGF (**fig 3.10C**) but was not biopsied as DGF is common in patients receiving a DCD kidney[369]. The creatinine began to fall at day 10 and remained steady across the study, although renal function remained within the chronic kidney disease range.

At 2 years, having previously been negative, mixed screening became positive for class I antibodies (**fig 3.10B**). As pre-transplant PRA (**fig 3.11A**) had been negative, this mixed screen warranted further investigation. SAB testing showed a de-novo antibody to Cw9 (**fig 3.11B**). Cw9, along with Cw10, is in the Cw3 broad antigen group and therefore this antibody to Cw9 would be considered to be a donor specific antibody because of the shared epitope with Cw10.

A)

	A		B		Bw		C		DR		DRB345		DQ	
Recipient	1	23	8	18	-	6	5	7	1	17	52	-	2	5
Donor	**11**	**25**	**35**	**60**	-	6	**4**	**10**	**7**	**8**	**53**	-	2	**4**

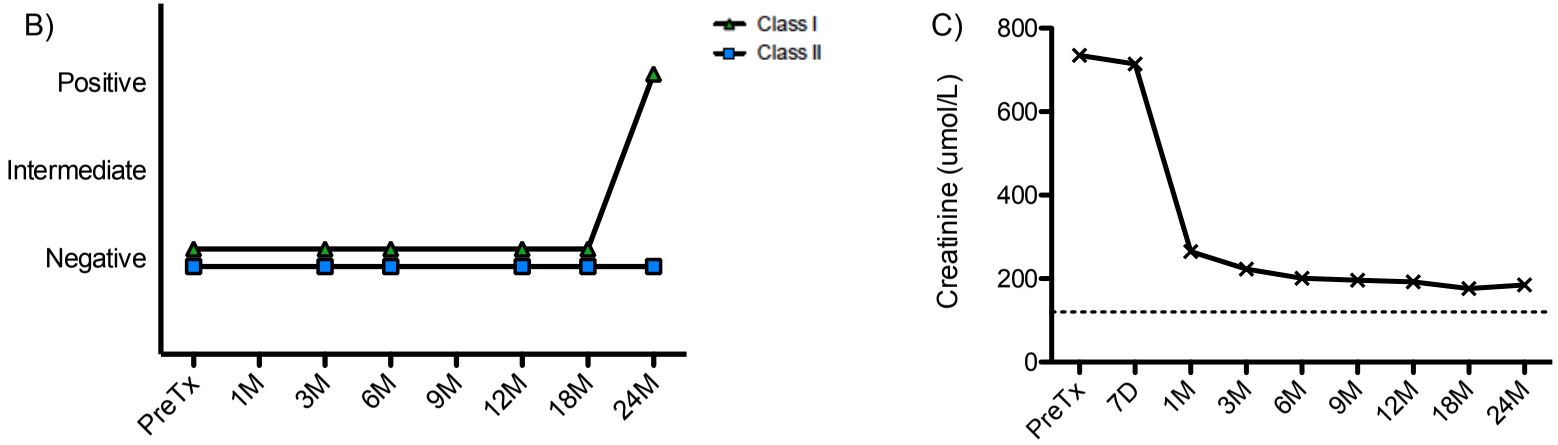


Figure 3.10: G017 Clinical Outcomes. A) Genotype of recipient compared to donor showing an almost complete HLA mismatch. Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-), de novo DSA highlighted in red. B) Mixed screen showing negative screens at all time points until 2 years, when class II has become positive. C) Creatinine over the course of the study, this patient was not biopsied.

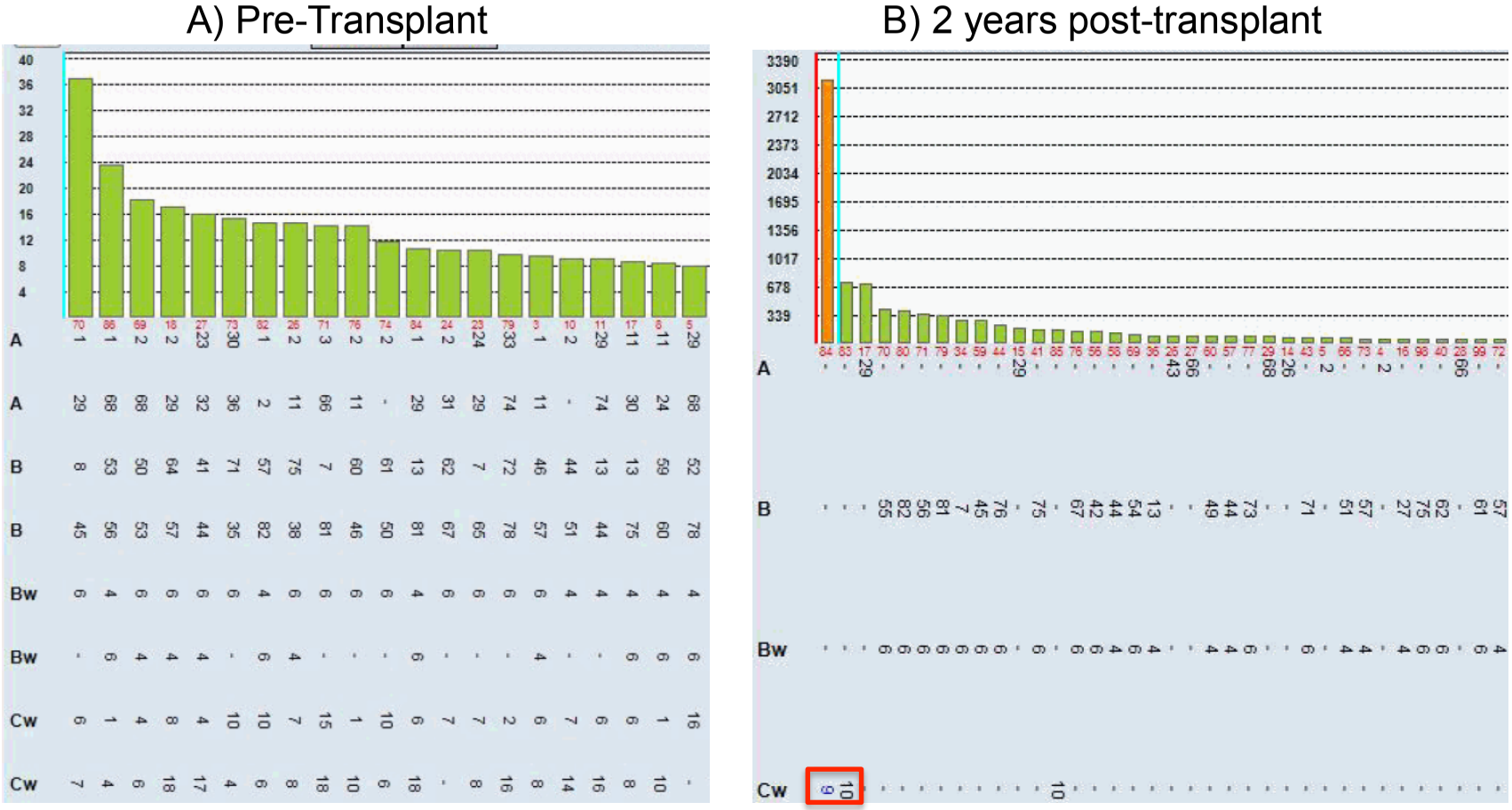


Figure 3.11: Single antigen bead profile G017. A) Class I panel-reactive antibody (PRA) bead screen pre-transplant negative screen. This patient was not screened with single antigen beads (SAB) as both mixed screen and PRA were negative. B) Class I single antigen bead test sample from 2 years post-transplant showing de novo antibody to HLA-Cw9.

G039

This complex patient had previously received two transplants. ESRF was due to type 1 diabetes, and the patient had received a SPK transplant, the pancreas failing 1 month after transplantation. A pancreas alone transplant was performed 8 years prior to study entry and was functioning well at the time of the third transplant, however the kidney from the first transplant failed a few months prior to the third transplant, for which the patient had been pre-emptively listed. The third donor was a living donor, and relatively poorly matched (**fig 3.12A**), in particular sharing some mismatched class I antigens with the first donor – HLA-A2, B44, Bw4; and others with the second donor (highlighted in green in **fig 3.12A**). Because of the previous transplants, the patient had previously received alemtuzumab induction, and therefore induction therapy for this transplant was with basiliximab with maintenance therapy of tacrolimus and MMF, continued from pre-transplant therapy for the functioning pancreas graft. Pre-transplant mixed screen was negative (**fig 3.12B**) but became positive within the first three months post-transplant. The patient had delayed graft function, which is unusual in a living donor recipient and therefore a biopsy was performed (**fig 3.12C,** blue arrow) which showed only acute tubular necrosis and no signs of rejection. Following this, creatinine fell and remained stable across the course of the study.

Because of the complex history, and shared antigens between previous donors and the current donor, regular SAB screening was undertaken. Pre-transplant screening showed a low-level HLA-A2 antibody, however the level fell below the positive cut-off on multiple separate screens and was therefore classed as negative, a representative example is shown in **fig 3.13A**. Post-transplant

screening showed *de novo* HLA-DP antibodies that were not donor specific, as well as an increase in MFI of the antibody to HLA-A2, one of the antigens shared between the first and third donor (**fig 3.13B**). The patient had had previous pre-transplant positive screens for HLA-A2 but had been negative on multiple screens prior to the third transplant and had a negative pre-transplant crossmatch; therefore this was considered an acceptable immunological risk[287]. Since this study was undertaken, the use of paired exchange schemes across the UK has increased, and, if this transplant had been carried out in 2016, it is likely that this scheme would have been used to gain a better HLA match without compromising the benefit gained from a living donor kidney.

A)

	A		B		Bw		C		DR		DRB345		DQ	
Recipient	1	3	60	62		6	9	10	1	4	53	-	5	8
Donor 1 (SPK)	2	3	44	57	4	-	9	10	11	12	52	-	5	6
Donor 2 pancreas	1	29	8	44	4	6	7	16	7	17	52	53	2	-
Donor 3 kidney	2	29	44	-	4	-	5	16	4	7	53	-	2	7

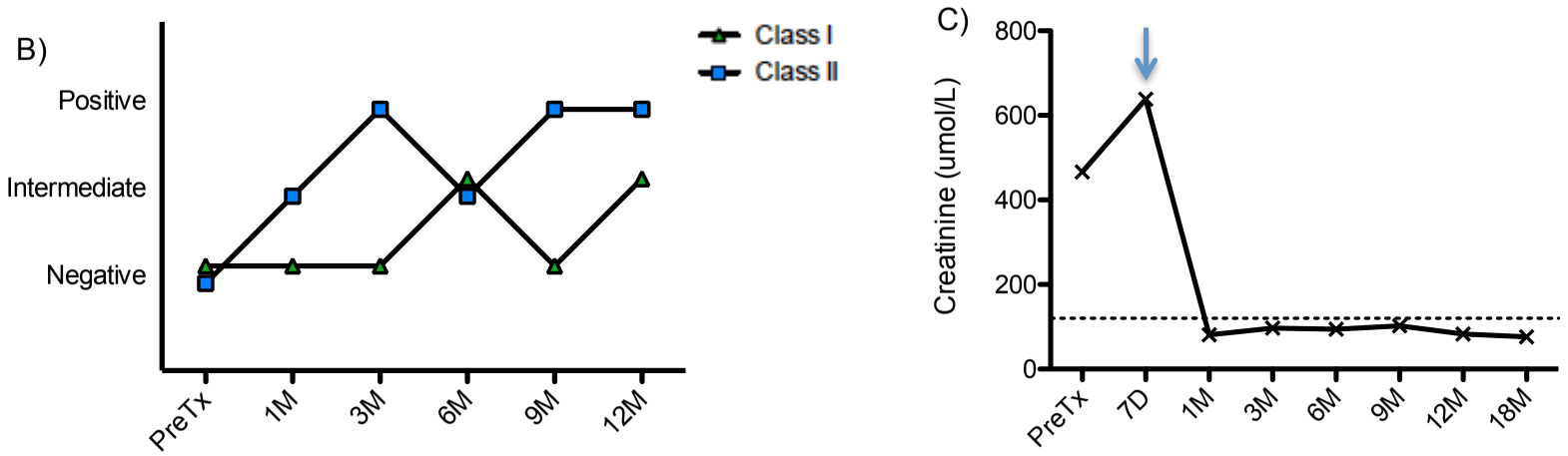


Figure 3.12: G039 Clinical Outcomes. A) Genotype of recipient compared to previous donors and current donor. Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-). Those shared between donors are in green. Antigen to which DSA formed is shown in red, shared with donor 1 (blue) B) Mixed HLA screen results showing negative screen pre transplant, but rapid development of class I positivity by 3 months, and persistent positive screen at 12 months. C) Creatinine over the course of the study. A biopsy was performed at day 7 (blue arrow).

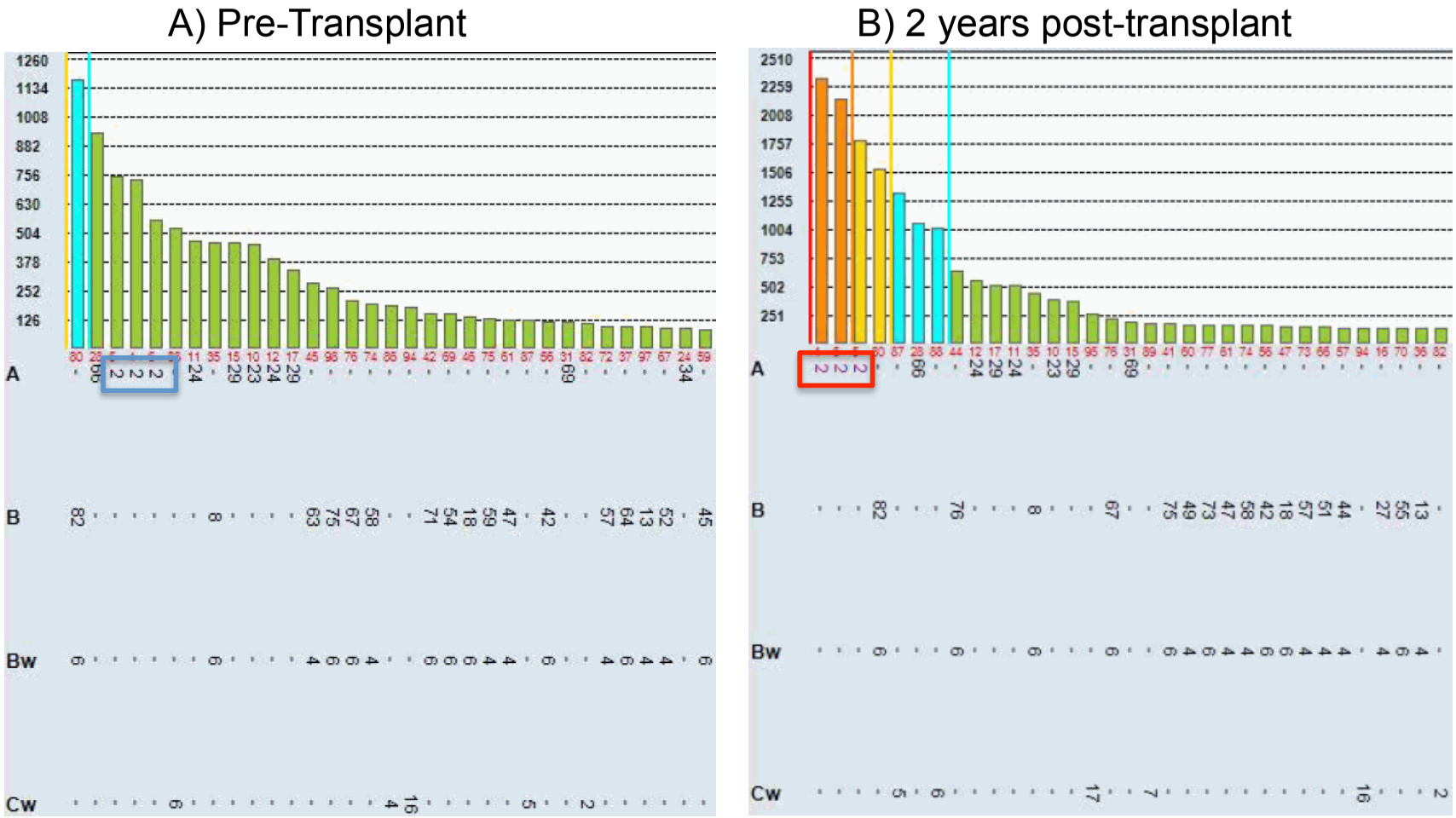


Figure 3.13: Single antigen bead profile G039. A) Class I single antigen bead results showing low MFI A2 antibody, but falling below positive cut off at the time of transplant, B) Class I single antigen bead showing positive HLA-A2 DSA at 12 months.

G042

This patient received a LD kidney that was relatively poorly matched (**fig 3.14A**). On mixed screening the recipient was positive for both class I and II HLA antibodies both pre-transplant and across the study (**fig 3.14B**) but class I SAB screens showed longstanding HLA-A34 (not donor specific). Class II SAB pre-transplant showed a low level HLA-DP DSA (**fig 3.15A**), which was considered weak enough to represent an acceptable immunological risk. This patient had no previous transplants, and was male, therefore sensitisation is likely to have come about through blood product transfusion while on dialysis.

The patient received basiliximab induction with tacrolimus and MMF maintenance therapy. After initially falling, creatinine levels rose on days 6 and 7 and a biopsy was performed (**fig 3.14C**), which showed Banff 1B TCMR but no evidence of ABMR *(see appendix 1)*. SAB screens showed persisting class I and *de novo* HLA class II antibodies that were not donor specific. The TCMR did not respond to pulsed MP and therefore the patient was given alemtuzumab, which led to a fall in creatinine (**fig 3.14C**).

SAB screening was carried out for both clinical indications and on study samples early post-transplant. Pre-transplant SAB screens (**fig 3.15A**) showed only a low level antibody to HLA-DP14. This antibody showed low median fluorescence intensity (MFI) only just above the negative cut-off, but was directed against an epitope shared across many DP alleles, therefore was relevant for matching purposes. By three months post-transplant, this antibody had fallen below the negative cut-off MFI on SAB (**fig 3.15B**) and there was no evidence of *de novo*

antibody formation. Previously detected class I HLA-A34 antibodies persisted but were not directed against donor antigens. At 18 months research SAB screens showed development of *de novo* DSAs against HLA-DQ4 and DQ6 (**fig 3.15C**) despite a stable creatinine.

A)

	A		B		Bw		C		DR		DRB345		DQ	
Recipient	2	-	60	58	4	6	7	10	4	8	53N	-	7	8
Donor	**1**	2	**44**	**62**	4	6	**1**	**5**	8	**15**	**51**	-	**4**	**6**

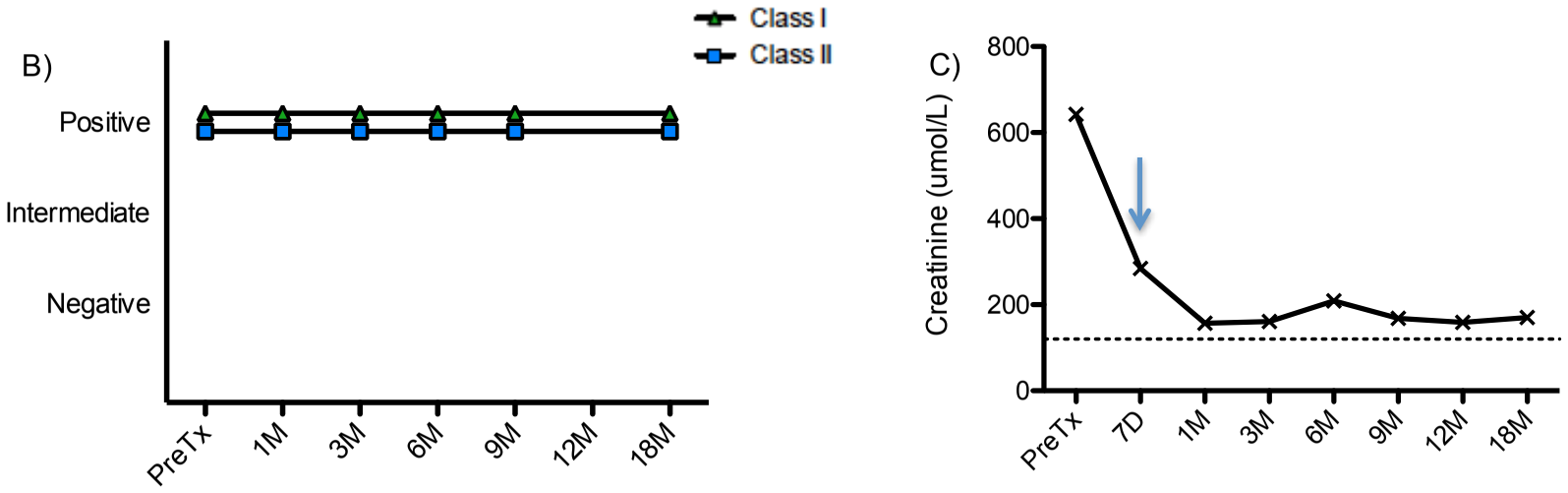


Figure 3.14: G042 Clinical Outcomes. A) Genotype of recipient compared to donor Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-), de novo DSAs highlighted in red. B) Mixed screens positive for class I and II HLA antibodies throughout study. C) Creatinine over the course of the study, this patient was biopsied at day 7 (blue arrow).

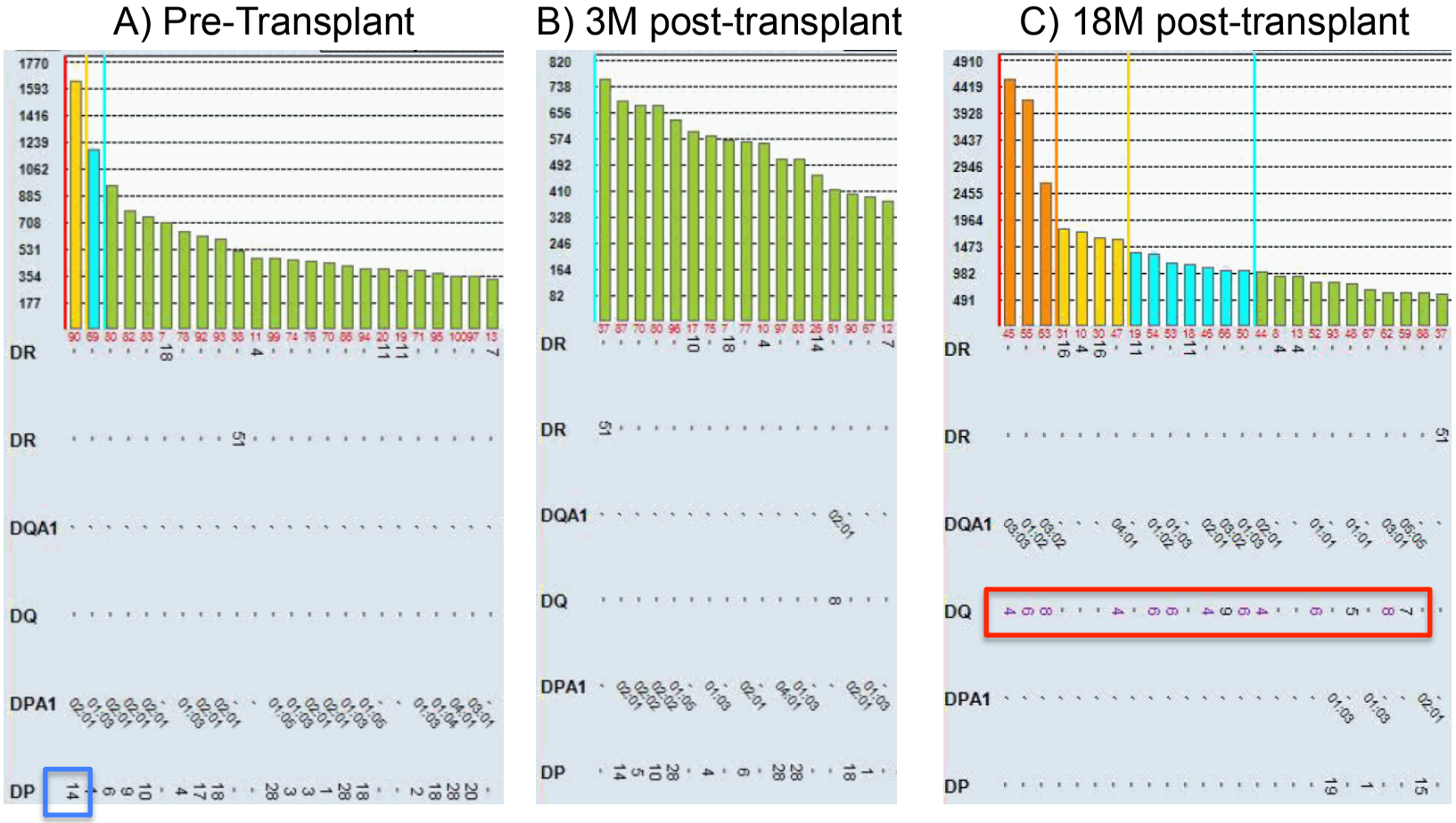


Figure 3.15: Single antigen bead profile G042. A) Class II single antigen bead (SAB) results showing low-level HLA-DP14 DSA at the time of transplant. B) Class II SAB at 3 months showing reduction in median fluorescence intensity of HLA-DP14 antibody below negative cut-off. C) Class II SAB at 18 months post transplant showing de novo DSA against HLA-DQ4 and HLA-DQ6.

G161

This patient received a SPK transplant from a relatively poorly matched donor (**fig 3.16A**) with alemtuzumab induction and tacrolimus with MMF maintenance therapy as standard. Kidney pancreas patients are often more poorly matched at class II than single organ recipients (particularly if the recipient holds the type 1 diabetes risk allele HLA-DR4) to try and prevent recurrence of type 1 diabetes in the graft, which occurs in 5-6% of recipients[370,371]. Pre-transplant antibody screens were negative (**fig 3.16B**). The patient had excellent primary graft function and stable creatinine over the first year post-transplant, with only a slight rise towards 18 months (**fig 3.16C**), not significant enough to warrant a biopsy.

Pre-transplant antibody assessment had been carried out using PRA screens, which were negative on multiple occasions (**fig 3.17A**). However, mixed screens began to show positivity for both class I and class II antibodies at 12 months post-transplant (**fig 3.16B**). SAB testing at 12 and 18 months showed *de novo* DSAs against HLA-B58 (**fig 3.17B, 3.17C**), and *de novo* class II antibodies that were not donor specific (data not shown).

A)

	A		B		Bw		C		DR		DRB345		DQ	
Recipient	1	11	35	64	-	6	4	8	1	17	52	-	2	5
Donor (SPK)	11	**33**	**7**	**58**	**4**	6	**7**	**10**	**11**	17	52	-	2	**7**

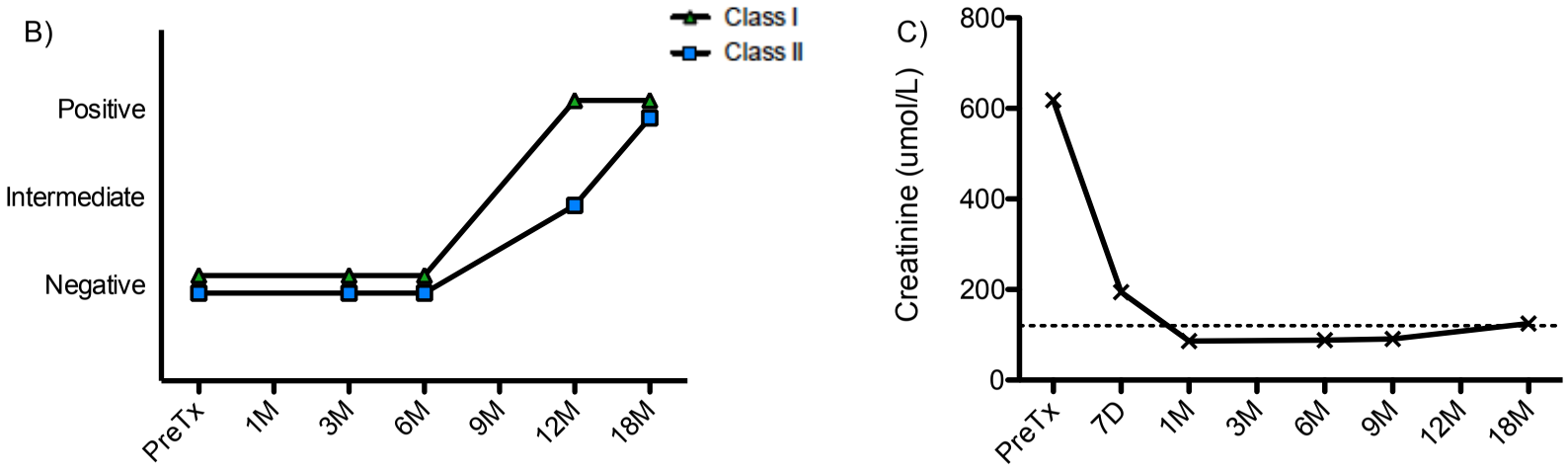


Figure 3.16: G161 Clinical Outcomes. A) Genotype of recipient compared to kidney-pancreas donor. Mismatched antigens shown in bold, underlined. Homozygous antigens shown by dash (-), de novo DSA highlighted in red. B) Mixed screens negative for class I and II until 12 months C) Creatinine over the course of the study, this patient was not biopsied.

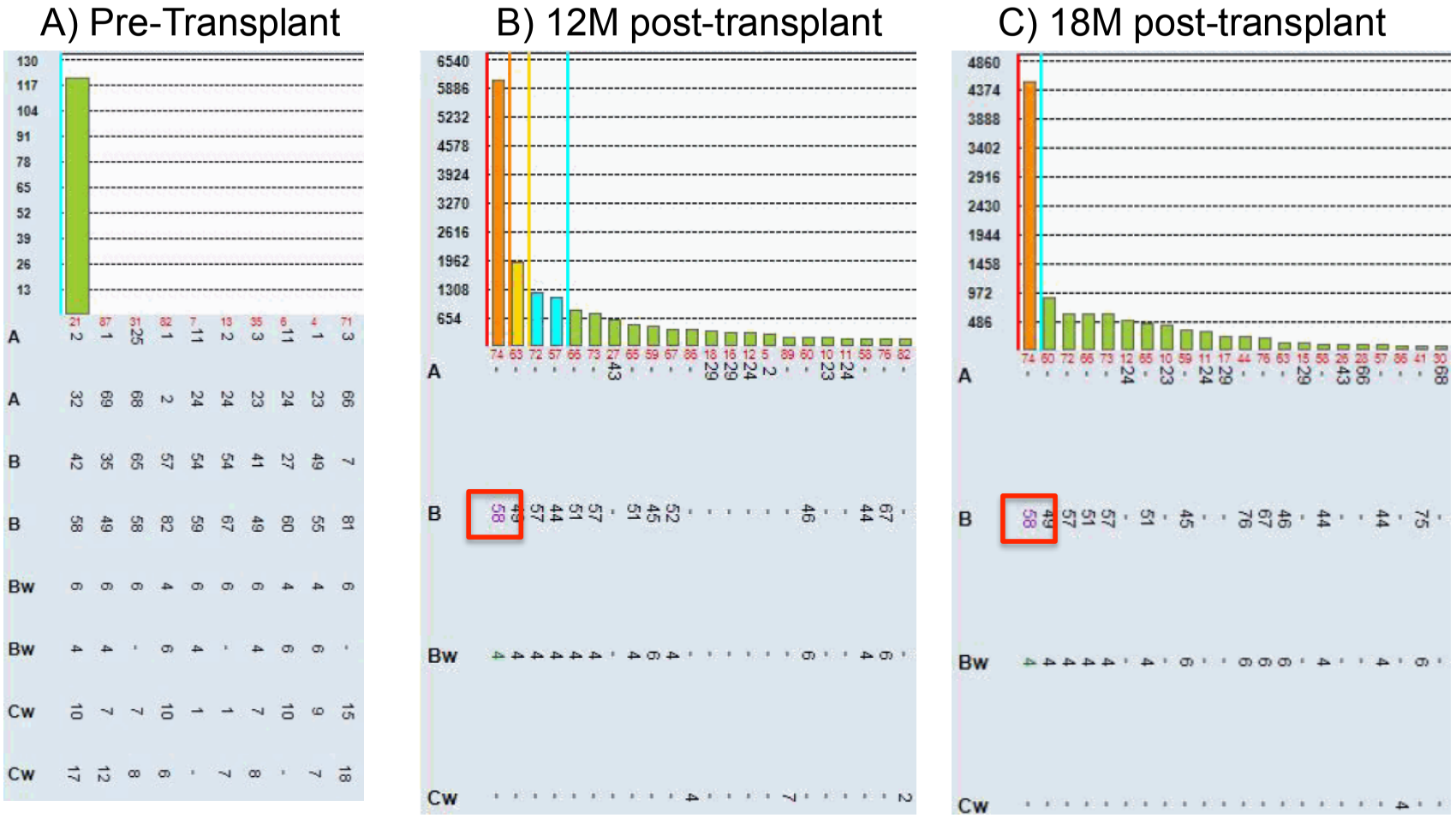


Figure 3.17: Single antigen bead profile G161. A) Class I panel-reactive antibody (PRA) bead screen pre-transplant negative screen. This patient was not screened with single antigen beads (SAB) as both mixed screen and PRA were negative. B) Class I SAB at 12 months showing de novo DSA to HLA-B58. C) SAB at 18 months showing persistent DSA.

3.4.7 Patients developing de novo DSA showed no common factors

Of the 6 patients developing *de novo* DSA there were no common factors. The donors were varied (4 live donor, 2 deceased donor). In terms of accepted risk factors, 2 patients had early rejection episodes, 2 had delayed graft function, and 2 functioned well early on with no rejections. While one patient with rejection (G014) had much higher antibody levels on SAB than the other 5 patients, this came from a longstanding pre-transplant antibody, and the MFI of the *de novo* antibodies was under 5000 (**see fig 3.7**), in keeping with the MFI range of the other 5 patients. However, this must be interpreted with caution as MFI is only semi-quantitative and is not directly comparable between patients, as it represents only the amount of antibody bound to a particular bead, not the titre of the antibody.

In one patient there was a concern over compliance but tacrolimus levels remained stable, the other 5 filled all prescriptions and had steady drug levels. Patients were not well matched for HLA, but in only one patient, G039, was there a concern over historical DSAs. There were therefore no definitive indicators that each of these patients would develop DSAs.

3.5 Conclusions

22 of 61 patients (36%) developed *de novo* HLA antibodies across the course of this study. In the majority this was a general increase in sensitisation to HLA antigens without being donor directed, however in 6 of the patients these antibodies were directed against the donor HLA. Only one of these patients lost

their graft, and this was due to on going TCMR with no DSA detected at this time. DSA developed after reduction of immunosuppression when the patient returned to dialysis. In the 5 remaining patients, creatinine was stable despite DSA development and there was therefore no clinical indication to biopsy and look for evidence of acute or chronic ABMR, and hence no clinical indication to screen for HLA antibodies.

There is evidence that the poorer the HLA match, the worse the longer term graft outcome, which may be due to a number of factors including an increased risk of acute TCMR as well as the development of *de novo* DSA. In this cohort, there was an increased risk of anti-HLA antibody formation with increasing HLA mismatch (**fig 3.18**), but many patients with poorly matched grafts did not generate *de novo* antibodies. Given the relative importance of HLA-DR matching[372] we have grouped patients as:

	Well matched	**Moderately matched**		**Poorly matched**	
HLA- A and/or B	1-2 mismatch (MM)	3-4 MM	1-2MM	3-4MM	Any
HLA-DR	0 MM	0MM	1MM	1MM	2MM

With these criteria no well-matched patients (n= 9) developed *de novo* HLA antibodies. 8 of 25 moderately matched patients developed *de novo* HLA antibodies of which 6 were non-specific (24%) and 2 donor specific (8%). Of poorly matched patients, 14 of 27 developed *de novo* HLA antibodies, of which 10 were non-specific (37%) and 4 donor specific (15%).

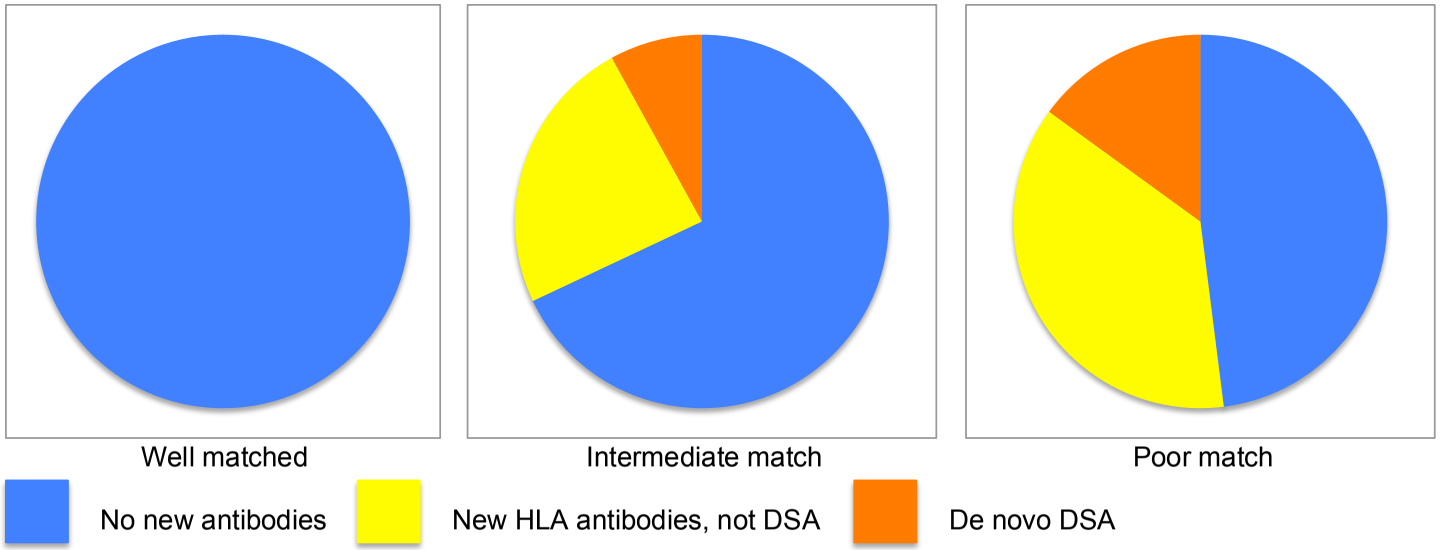


Figure 3.18: Outcomes of HLA matching. Risks of new non-donor specific HLA antibody formation, DSA formation or stable HLA antibody levels by original HLA match with donor. Well matched – 0 HLA DR mismatches (MM), 1-2 MM at A and/or B. Moderately matched – 0 MM DR and 3-4 MM at A and B, OR 1 MM DR and 1-2 at A and/or B. Poorly matched – 1 MM DR and 3-4 MM at A and B, or 2 MM DR

While it is clear that increasing mismatch increases the likelihood of antibody development, and well-matched kidneys are less likely to develop antibodies, there are clearly more complex factors at play and neither the degree of mismatch at HLA A, B and DR antigens, nor graft function can be used alone as a predictive factor for HLA antibody development.

This highlights the need for alternative biomarkers that would indicate the possibility of HLA antibody formation and allow potential intervention much earlier than a rise in creatinine, which may occur late in the process when a majority of the kidney has become damaged[373-375]. This led us to look for alternative markers of antibody formation.

4. Immunophenotyping of Transplant Recipients and Correlation with Clinical Outcomes

4.1 Introduction

In the current era of transplantation, short-term outcomes are excellent. With modern HLA-matching techniques, immunosuppressive regimens and improvements in surgical technique, graft survival within the first year has improved to 94-98% for kidney recipients, 94% for liver recipients, 87% for SPK and heart/lung recipients, 84% for heart alone and 75-81% for lung alone recipients[5]. However, long-term transplant outcomes remain fairly consistent with a gradual decline in graft function that is now thought to be associated with, or possibly caused by anti-HLA antibodies. Previously this was thought to be due to chronic allograft nephropathy (also existing in other solid organ transplants, e.g. bronchiolitis obliterans, cardiac graft vasculopathy) or the effects of CNIs[3,5] but there is evidence that antibodies play more of a role than originally considered.

Many studies have been undertaken, predominately in kidney and liver transplant recipients, to look for biomarkers of operational tolerance, where patients have stable graft function in the absence of immunosuppression[376-381]. Yet more studies have been done looking for biomarkers of acute rejection[382-385] and of chronic rejection[386,387] and patients with stable graft function in whom withdrawal or minimisation of immunosuppression might be feasible[382,388,389]. However, no studies have yielded definitive biomarkers that might be used to predict chronic antibody mediated rejection, the diagnosis of which is still

debated[15,16,390]. Rather than predicting chronic ABMR, a biomarker to predict which patients are at risk of antibody formation may be of use. *De novo* DSA formation occurs in 20-30% of transplant patients and both increases the risk of graft loss and makes future transplantation more difficult and less likely to succeed[13,202,391].

DSAs are long-lived and class switched[244,260,262,264], suggesting a requirement for T cell help for their production[392]. HLA-specific memory B cells have been identified in the circulation of sensitised transplant patients[188,393,394], and HLA-specific plasma cells in the bone marrow[187], suggesting a requirement for the GC reaction in their generation (see chapter 1, section **1.4.3, 1.4.4**). This is supported by animal work suggesting alloantibody production and graft rejection are T cell dependent[395-398].

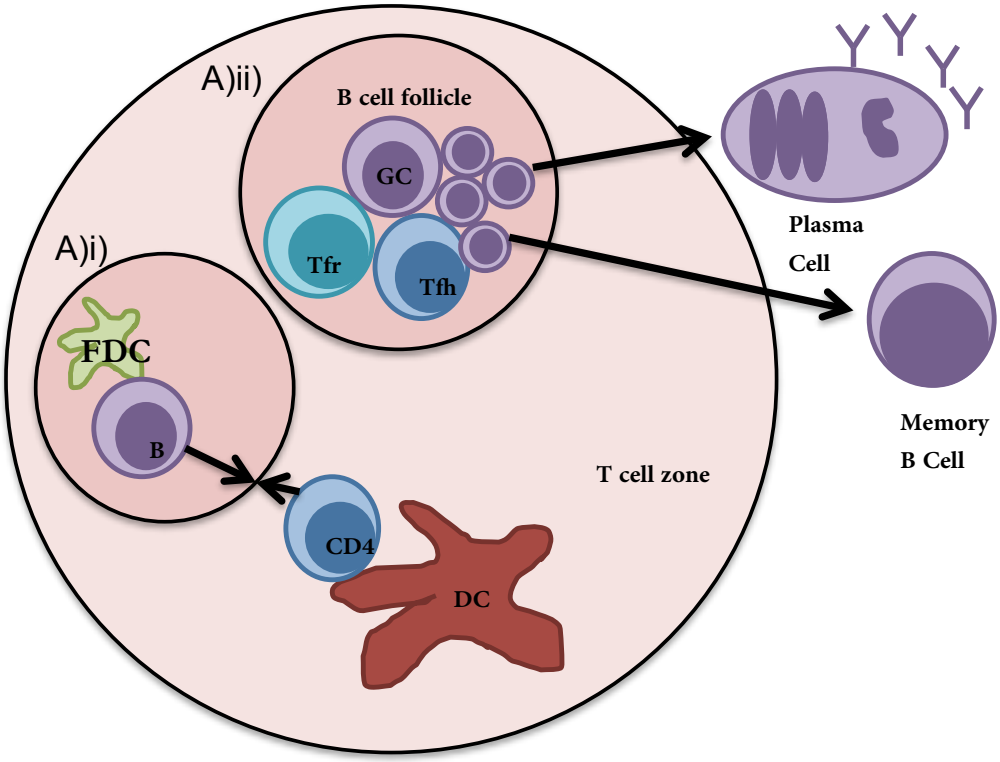
4.2 Circulating cells as a biomarker of GC responses

Initiation of an immune response against the graft is a physiologically normal process, albeit a clinically undesirable one. Cells of the immune system mount a response against the non-self antigens (the graft) presented in the context of an inflammatory stimulus (surgery). Immunosuppressive drugs, targeting various aspects of antigen presentation and cell activation, are used to attempt to control this process. Drug regimens are excellent at controlling early, predominately CD8 T cell responses to the graft, and HLA matching and antibody-screening schemes ensure that acute antibody-mediated rejection is now extremely rare[399].

The role of antibodies in chronic graft dysfunction has not been fully elucidated, but it is becoming clear that antibodies can be associated with the development of IFTA[12,13,16,400-402]. It is certainly plausible that this is a causal relationship. IFTA is a scarring process that can occur secondary to many processes, but is often seen in failing grafts without significant histological features of rejection such as inflammatory cell infiltrate or complement deposition[11,13]. Interest has grown in the role of antibodies in IFTA as there is work showing that those patients who develop *de novo* DSA after transplantation have a much shorter graft lifespan than those with graft dysfunction but no antibodies[13,199,200,403,404].

Work done in the fields of autoimmunity, vaccination and chronic infection has well characterised the immunological processes involved in both undesirable (a reaction to self) and desirable antibody production (vaccination responses or clearance of e.g. chronic viral infection). The focus of this work has converged on the role of the many different cell types that interact within the GC of SLOs – GC B cells, T follicular helper (Tfh) cells and T follicular regulatory (Tfr) cells (**fig 4.1A**). By acting in concert, these cells support the development of memory B cells, and long-lived plasma cells that home to the bone marrow and secrete high affinity antibodies.

Figure 4.1: T and B cell interactions forming the Germinal Centre. A)i) Antigen is presented to naïve B and T cells by dendritic cells (DCs) within their respective areas of secondary lymphoid tissue. A)ii) Following establishment of a GC interactions between T follicular helper cells (Tfh) and GC B cells allow the formation of memory B cells and plasma cells. The size of this response is controlled by follicular regulatory T cells (Tfr). B) Surface markers and transcription factors (red) expressed by follicular T cells.



B)

Marker	CD4	CXCR5	ICOS	PD-1	IL-21	BCL6	FOXP3
Tfh	++	+++	++	++	+	++	-
Tfr	++	++	+++	++	-	+	+

Circulating counterparts of Tfh and Tfr cells have been described in mice and in humans; they share the same surface markers as GC cells (**fig 4.1B**) and are thought to be memory cells, capable of rapid initiation of antibody responses on re-encounter with antigen[127-130]. Circulating Tfh cells have been further subdivided into subsets expressing different cytokines and having different B helper capacity. Morita *et al*. described three distinct populations of circulating Tfh cells based on expression of chemokine receptors CXCR3 and CCR6[127]. $CXCR3^{+}CXCR5^{+}CD45RA^{-}CD4^{+}$ cells expressed IFNγ, classically a Th1 cytokine, as well as IL-21, the classical Tfh cytokine, and were dubbed cTfh1. $CCR6^{+}CXCR5^{+}CD45RA^{-}CD4^{+}$ cells expressed IL-17 in addition to IL-21 and were coined cTfh17 cells, and those cells expressing neither CCR6 nor CXCR3 expressed Th2 cytokines such as IL-4 and are referred to as cTfh2 cells. These cells have different capacity for helping B cells produce antibody; cTfh2 and cTfh17 are capable of helping both naïve and memory B cells to produce antibody *in vitro*; and cTfh1 cells were able to provide help to memory B cells, but not naïve cells[127-130]. Furthermore, the helper capacity of cTfh1 cells was restricted to those expressing high levels of ICOS and PD-1[130].

cTfh cells have been identified in multiple disease states, and are increased in frequency in the peripheral blood after vaccination. The expression of the chemokine receptors CXCR3 and CCR6, and the activation markers ICOS and PD-1 on cTfh cells, have been used to stratify patient subgroups to gain insight into which cell types are linked with particular disease states. Initial work done in human autoimmune disease identified a subgroup of systemic lupus erythematosus (SLE) patients with active disease and high levels of

autoantibodies, who also had high levels of $CXCR5^+PD\text{-}1^+CD4^+$ cells in the circulation. These cells correlated with autoantibody levels and end organ damage[134]. Other studies in autoimmune disease followed, showing a similar phenotype in myasthenia gravis[135,136], rheumatoid arthritis[405,406], and autoimmune thyroid disease[140]. In juvenile dermatomyositis it was specifically the CCR6 expressing cTfh17 cells that were found to be high in association with active disease and to reduce in proportion with effective treatment[127]. The same association was seen in Sjögren's syndrome[138] and SLE[137]. It has therefore been suggested that an imbalance in the proportions of cTfh cell subsets within the circulation can be used as a biomarker of an active germinal centre response to a particular antigen, with a lower proportion of Tfh1 and a higher proportion of Tfh2 and Tfh17 cells promoting autoantibody production by increased provision of T cell help to B cells[146].

The interest in the potential for cTfh as a biomarker of GC activity is not confined to autoimmune disease. For example, in influenza vaccination, Bentebibel *et al.,* showed an increase in $CXCR3^+CXCR5^+$ Tfh cells 7 days after vaccination, and showed these cells also upregulated expression of ICOS and expressed high levels of PD-1. These cells correlated with anti-influenza antibody production and plasmablast production, and were capable of helping memory B cells produce antibodies *in vitro*[130].

These varied findings of increased cTfh1 in vaccination, but increased cTfh2 and cTfh17 in autoimmune disease are thought to reflect the skewing of the original antigen. It has been proposed that cTfh represent a peripheral biomarker of GC

activity, and therefore could be used to identify an active GC reaction to an immunising antigen. For example, influenza normally generates a Th1 response to infection, and the role of Th17 responses in autoimmunity is well documented[141-143].

This is also in keeping with evidence seen in chronic viral infection, particularly HIV, where the virus persists and evades the immune system for many years[144]. Antibodies continue to develop throughout the course of infection, but the virus evolves rapidly and few of these antibodies are able to neutralise more than the strain to which they have developed[145]. However a small number of individuals are able to generate broadly neutralising antibodies (bnAbs), which can clear more than 70% of the currently known strains of HIV. These individuals have significantly higher levels of $CXCR3^{-}PD\text{-}1^{+}$ $CXCR5^{+}$ cTfh cells in their circulation than either HIV negative controls or patients who do not develop bnAbs[129], indicative of an ongoing Tfh/GC response in the lymph nodes. These cells were also transcriptionally similar to GC Tfh, and are purported to be a circulating memory population of Tfh cells[128,129].

Given the clear links between cTfh cells and active GC reactions in LN, their role in transplantation was of interest to many. However, it was not clear how or if they would be of relevance in DSA production. While the graft is a persistent non-self antigen, much like a chronic viral infection, immunosuppressive agents are predominately T cell directed, and therefore may suppress both cTfh and LN Tfh. Animal studies have suggested that LN Tfh cells are required for DSA formation[396] and therefore it is likely they play a similar role in human

transplantation, although studies have been limited, in part because of the difficulty of obtaining SLO samples. Using cTfh as a biomarker of Tfh activity in SLOs, and potentially in tertiary lymphoid follicles within the graft itself, would therefore be of great interest. Some work has been done looking at cTfh in transplantation; one study suggested increased numbers of $CXCR5^{+}CD4^{+}$ cells after transplantation in patients with pre-formed DSA compared to those without, but no differences in those developing *de novo* DSA[273]. More recently increased numbers of $CXCR5^{+}CD4^{+}$ cells with low PD-1 expression have been described in a small cohort of patients with chronic rejection, compared to those with stable renal function. However the group was heterogeneous with varied immunosuppressive regimens[407].

4.3 Aims

Given the limited human work in transplantation, this study aimed to:

1. Collect a cohort of patients with paired blood and LN samples pre-transplant and compare these to assess whether cTfh and cTfr were appropriate correlates of LN Tfh and Tfr for post-transplant follow up
2. Using biomarkers identified above, as well as in other published work, to follow cTfh, cTfr and B cells in the blood over the first two years post transplant, assessing the impact of immunosuppressive agents
3. To correlate cell numbers and proportions with the development of *de novo* DSAs to assess their potential as biomarkers, hypothesizing that cTfh would be higher in those developing *de novo* DSAs

4.4 Results and discussion

Aim 1 – Comparison of LN and circulating cells and the impact of pre-transplant tacrolimus to allow identification of potential biomarkers

4.4.1 Pre-transplant immunosuppression does not impact on B cell subsets

Patients were recruited to the study at the time of transplant; those who had received a transplant from a living-donor (n=16) had been treated with the CNI tacrolimus for one week before surgery, aiming for trough levels of 5-10ng/ml. Blood samples were taken after induction of anaesthesia, immediately prior to knife-to-skin and before the initial dose of methylprednisolone. LN samples were taken prior to organ implantation, shortly after knife-to-skin. Samples were compared with patients receiving a deceased donor kidney or SPK transplant (n=45) who had not received any CNI, to see if this short duration of immunosuppressive therapy had any impact on B cell subsets.

B cells were divided into $CD38^{hi}BCL6^{+}$ GC B cells, $CD38^{hi}CD24^{-}$ plasmablasts, $CD38^{hi}CD24^{hi}$ transitional cells, and naïve and memory subsets based on expression of CD27 and IgD (gating strategy shown in **fig 4.2**). Comparison of LN B cells showed no significant difference in $CD38^{hi}BCL6^{+}$ GC B cells (**fig 4.3**), $CD38^{hi}CD24^{-}$ plasmablasts or $CD38^{hi}CD24^{hi}$ transitional cells (**fig 4.4**). There were also no significant differences in $CD27^{+}IgD^{-}$ class-switched memory cells, $CD27^{+}IgD^{+}$ unswitched memory cells, $CD27^{-}IgD^{+}$ naïve cells or $CD27^{-}IgD^{-}$ cells (**fig 4.5**).

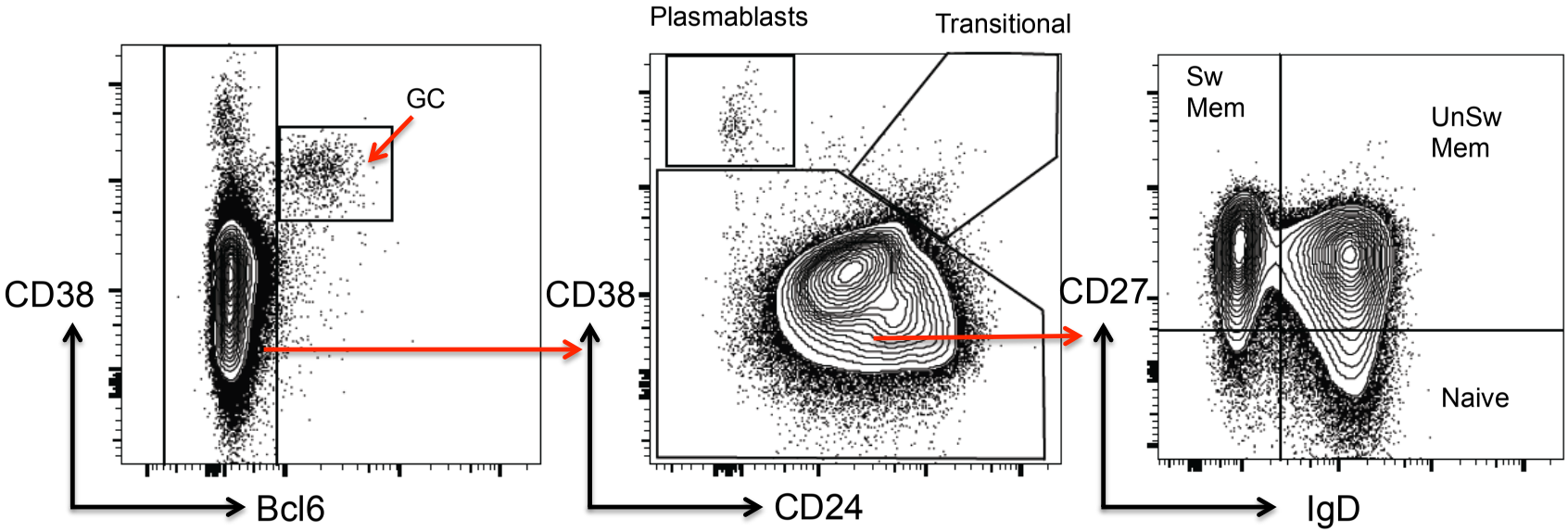


Figure 4.2: Gating strategy for lymph node B cells. $CD38^{hi}Bcl6^{+}$ germinal centre (GC) cells were gated first, then $CD38^{hi}CD24^{-}$ plasmablasts and $CD38^{hi}CD24^{hi}$ transitional cells. Finally cells were split into $CD27^{+}IgD^{-}$ switched memory cells, $CD27^{+}IgD^{+}$ unswitched memory cells, $CD27^{-}IgD^{+}$ naïve cells and $CD27^{-}IgD^{-}$ double negative cells.

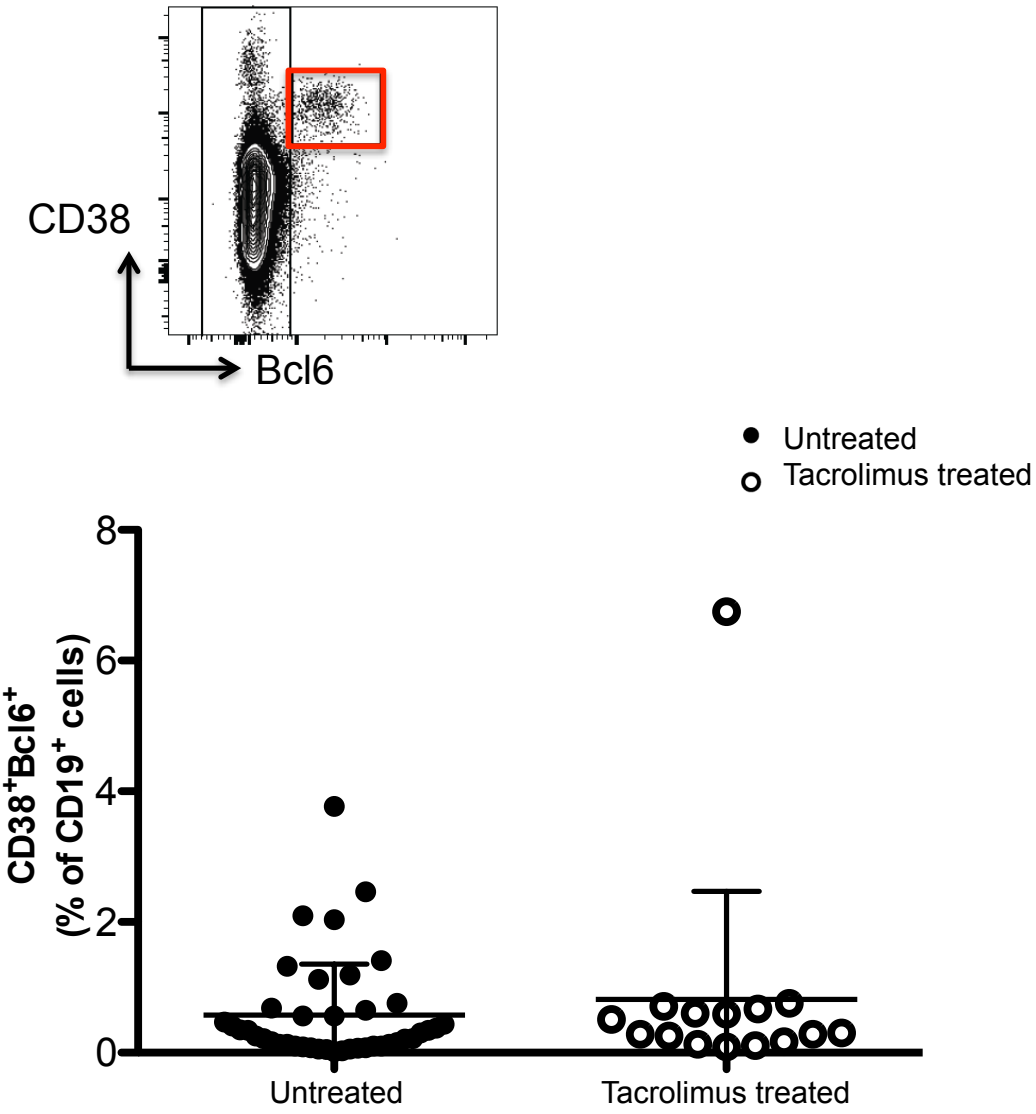


Figure 4.3: LN GC B cell proportions. $CD38^{hi}Bcl6^{+}$ germinal centre B cells in deceased donor recipients, receiving no immunosuppression (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean with SD, each symbol represents one patient. Mann Whitney testing not significant.

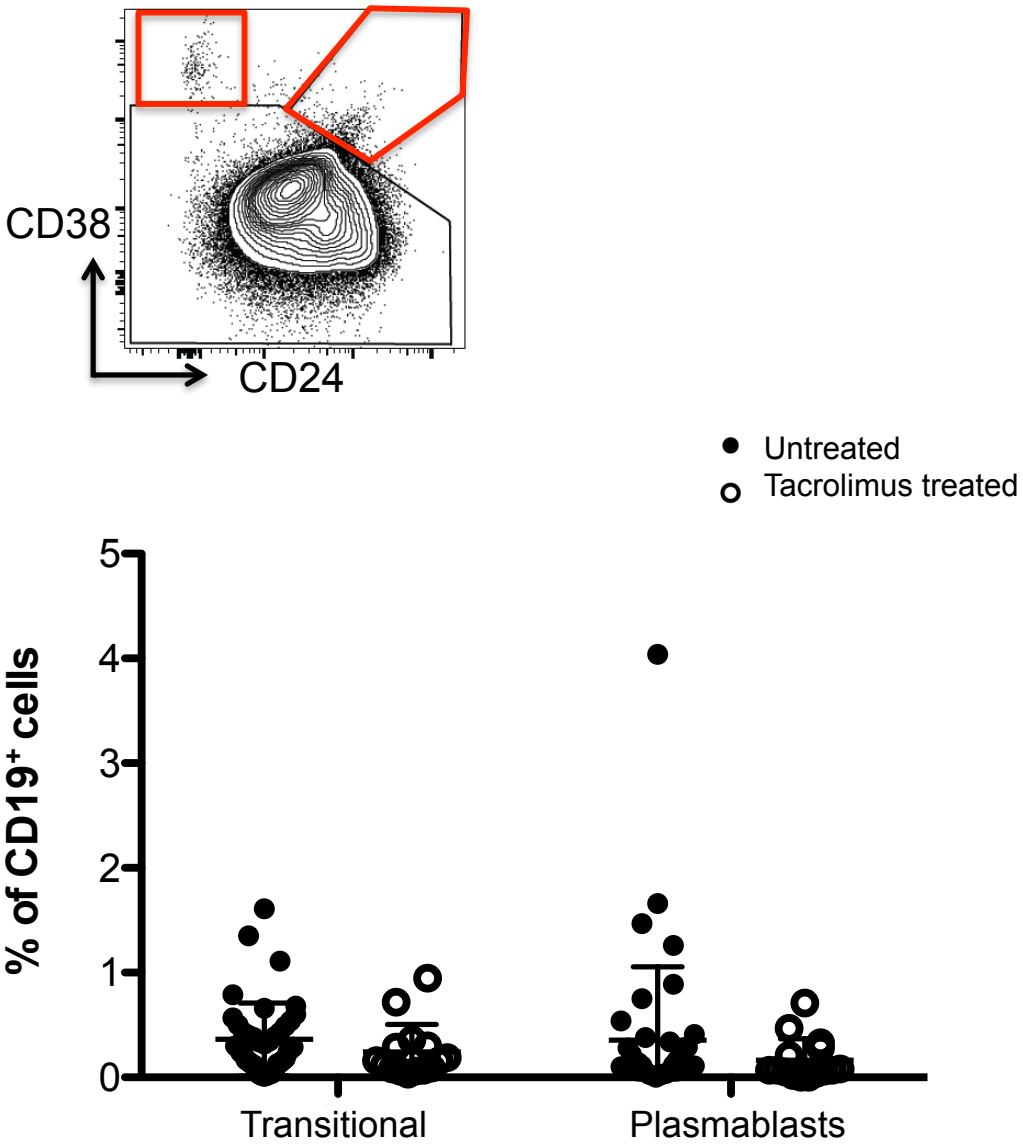


Figure 4.4: LN transitional cells and plasmablasts. $CD38^{hi}CD24^{-}$ plasmablasts and $CD38^{hi}CD24^{hi}$ transitional cells in deceased donor recipients, receiving no immunosuppression (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean with SD, each symbol represents one patient. Mann Whitney test not significant.

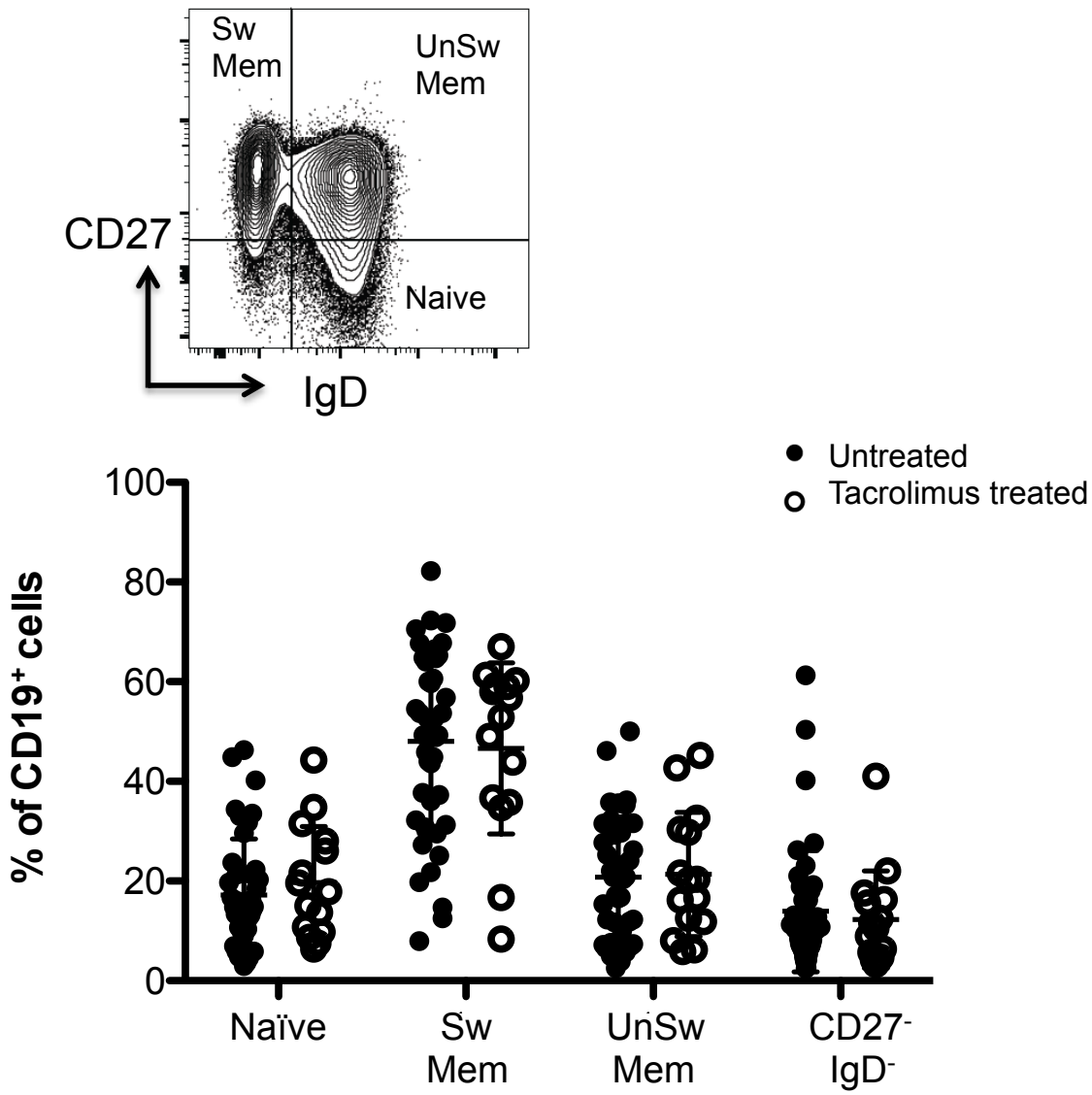


Figure 4.5: LN naïve and memory subsets. $CD27^{+}IgD^{-}$ class-switched memory cells, $CD27^{+}IgD^{+}$ unswitched memory cells, $CD27^{-}IgD^{+}$ naïve cells and $CD27^{-}IgD^{-}$ cells in deceased donor recipients, receiving no immunosuppression (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean with SD, each symbol represents one patient. Mann Whitney test not significant for any subset.

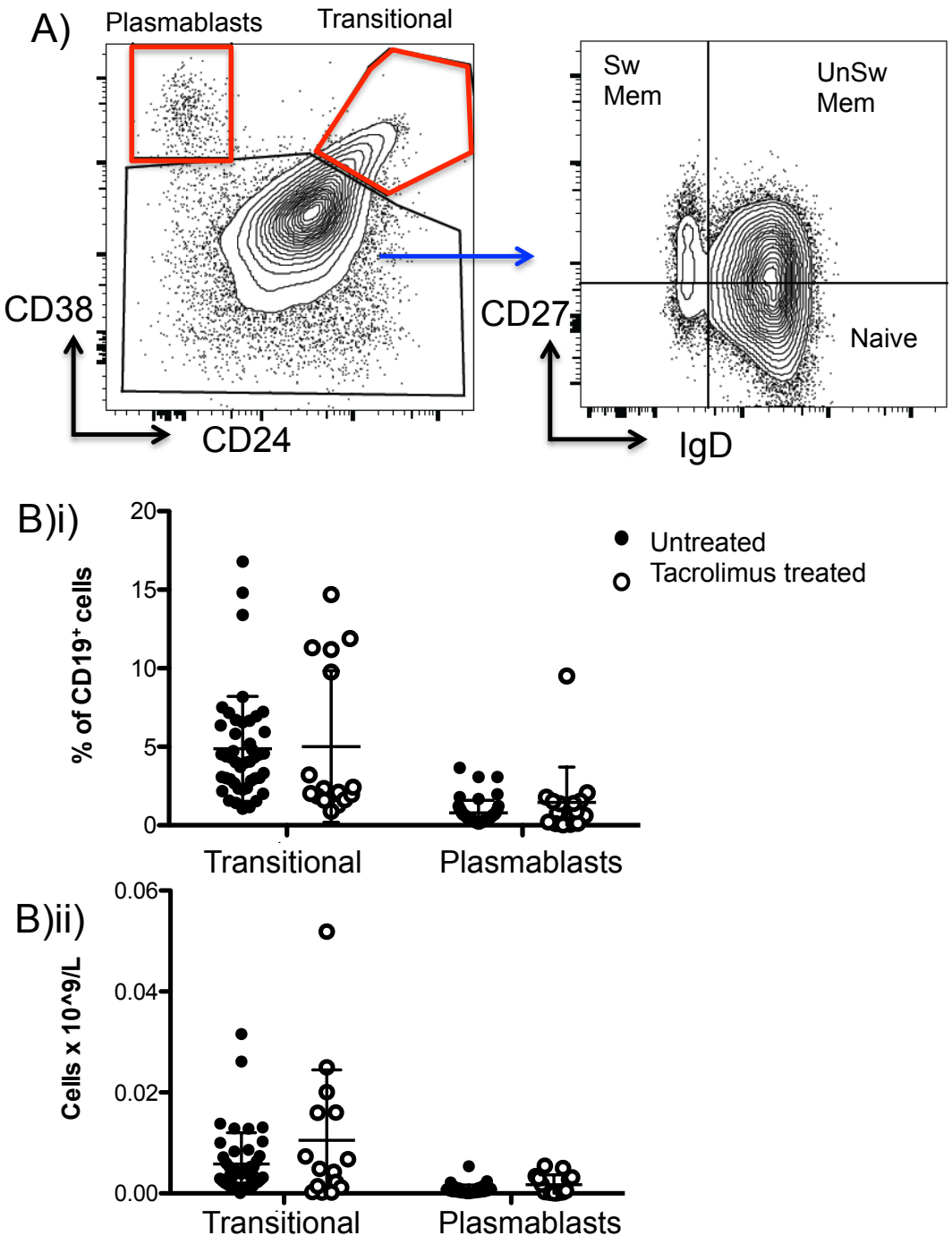


Figure 4.6: Peripheral blood B cells. A) Gating strategy for peripheral blood B cells. B) $CD38^{hi}CD24^{-}$ plasmablasts and $CD38^{hi}CD24^{hi}$ transitional cells shown as i) proportion of B cells and ii) absolute cell count comparing deceased donor recipients, receiving no immunosuppression (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean with SD, each symbol represents one patient. There were no significant differences on Mann Whitney testing.

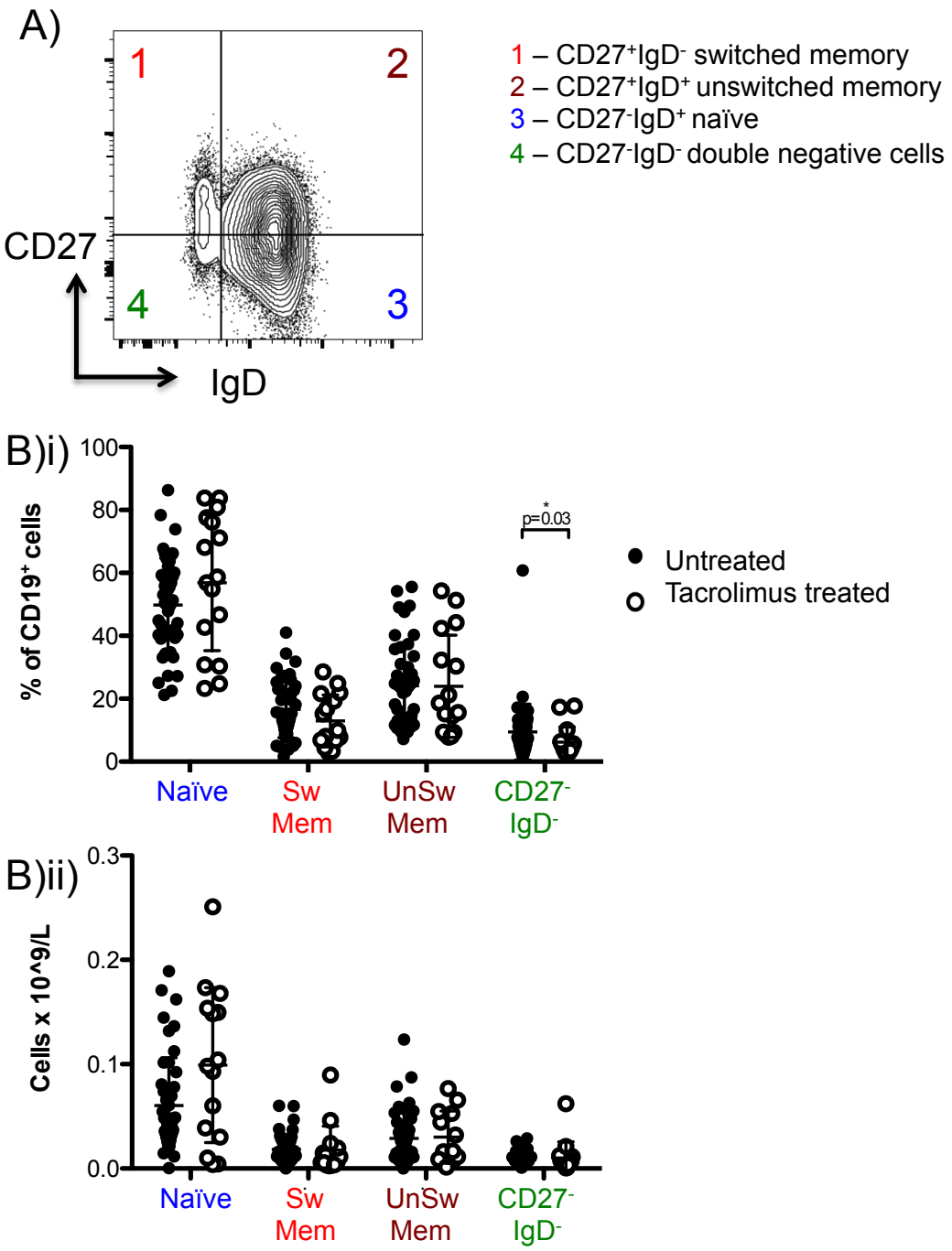


Figure 4.7: Peripheral blood memory cells. A) Representative flow plot with subsets analysed shown. B) $CD27^+IgD^-$ class-switched memory cells, $CD27^+IgD^+$ unswitched memory cells, $CD27^-IgD^+$ naïve cells and $CD27^-IgD^-$ cells shown as i) proportion of B cells and ii) absolute cell count comparing deceased donor recipients, receiving no immunosuppression (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean with SD, each symbol represents one patient. Significant differences on Mann Whitney testing are shown, all others were non significant.

Peripheral blood samples were analysed similarly, with the exception of GC B cells, which were not assessed as they are not present in the blood (**fig 4.6A**). There was no significant difference in $CD38^{hi}CD24^{-}$ plasmablasts or $CD38^{hi}CD24^{hi}$ transitional cells either in proportion of $CD19^{+}$ cells (**fig 4.6B)i**) or absolute cell count (**fig 4.6B)ii**). Peripheral blood naïve and memory B cells were identified using CD27 and IgD expression as in LN samples (**fig 4.7AB**). There was a slight reduction in the proportion of $CD19^{+}$ cells falling into the $CD27^{-}IgD^{-}$ gate (p=0.03, **fig 4.7B)i**) in tacrolimus treated patients but no difference in absolute cell count (**fig 4.7B)ii**) and no differences in $CD27^{+}IgD^{-}$ class-switched memory cells, $CD27^{+}IgD^{+}$ unswitched memory cells or $CD27^{-}IgD^{+}$ naïve cells.

Overall these data show that there were no significant differences in absolute B cell numbers in the circulation or the proportion of LN B cells between patients treated with tacrolimus for one week (treated patients) and those with no pre-transplant tacrolimus treatment (untreated patients).

4.4.2 Pre-transplant immunosuppression does not impact on Tregs or Tfr

Because some patients received basiliximab, an anti-CD25 monoclonal antibody, as induction therapy, we made the decision to identify regulatory T cells (Tregs) using $IL\text{-}7R^{lo}FOXP3^{+}$ (**fig 4.8A)i**) instead of the more commonly used $CD25^{hi}FOXP3^{+}$ phenotype. Previous work has shown that the IL-7R (CD127) identifies Treg cells with equal, if not greater accuracy than expression of CD25, which is more subjective and upregulated on activation of non-Treg cells[350,408]. Tfr were identified as $CXCR5^{+}IL\text{-}7R^{lo}FOXP3^{+}CD4^{+}$ cells (**fig 4.8A)ii**).

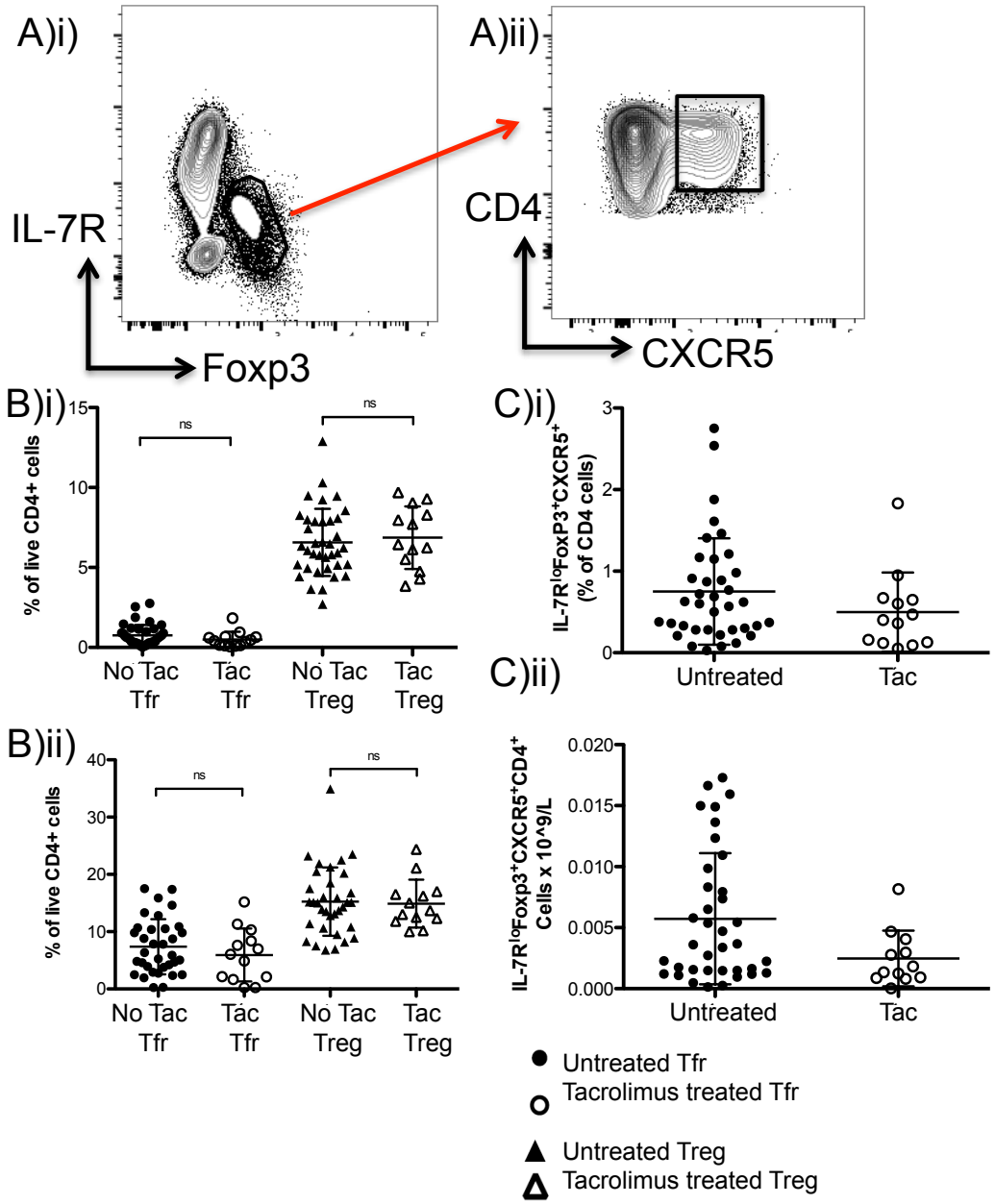


Figure 4.8: T reg gating and Tfr gating. A)i) Regulatory T cells were gated as IL-7R^{lo}Foxp3^{+} then ii) T follicular regulatory cells were gated as CXCR5^{+} Tregs. B) There were no significant differences between Tregs or Tfr in untreated deceased donor recipients (No Tac) and live donor recipients (tac) in i) blood or ii) lymph node (LN) pre transplant. C) There was a trend towards lower Tfr both in i) peripheral blood and ii) LN but this did not reach statistical significance (Mann Whitney testing). Shown as mean with SD, each symbol represents one patient, n=45 untreated, n=16 tacrolimus treated.

Before organ implantation, there were no significant differences in the proportion of Tregs or Tfr cells in the peripheral blood (**fig 4.8B)i**) or LN (**fig 4.8B)ii**) in treated patients compared to untreated patients. There was a trend towards lower Tfr in the peripheral blood of treated patients both in proportion of $CD4^+$ cells and absolute cell count but this did not reach statistical significance (**fig 4.8C**).

4.4.3 Pre-transplant immunosuppression reduces LN $CXCR5^+PD\text{-}1^+$ Tfh

Activated Tfh cells were identified in the LN as $CXCR5^+PD\text{-}1^+CD4^+$ cells (**fig 4.9A**) and showed a significant reduction (p=0.048) in tacrolimus treated compared to untreated patients (**fig 4.9B**) on Mann Whitney testing. Based on previous work, $CXCR5^+CD4^+$ T cells were divided into Tfh1, Tfh2 and Tfh17 subsets as described above (**fig 4.10A**). There were no differences in the proportion of any subset of Tfh (**fig 4.10B**).

4.4.4 Pre-transplant immunosuppression significantly reduces cTfh

Peripheral blood activated $CXCR5^+PD\text{-}1^+$ Tfh were significantly reduced in both proportion (p=0.036) and absolute cell count (p=0.0065) on Mann Whitney testing in treated patients compared to untreated (**fig 4.11**). Given the reduction in both LN and cTfh, this demonstrates that cTfh cells can be considered a biomarker of LN Tfh cells. This is concordant with previously published work[125,129,130,132,409], and is important for the clinical follow-up of these patients, as only blood samples are available after transplantation. There was also a global reduction in all Tfh subsets, both in proportion and absolute cell count (**fig 4.12**) in treated compared to untreated patients.

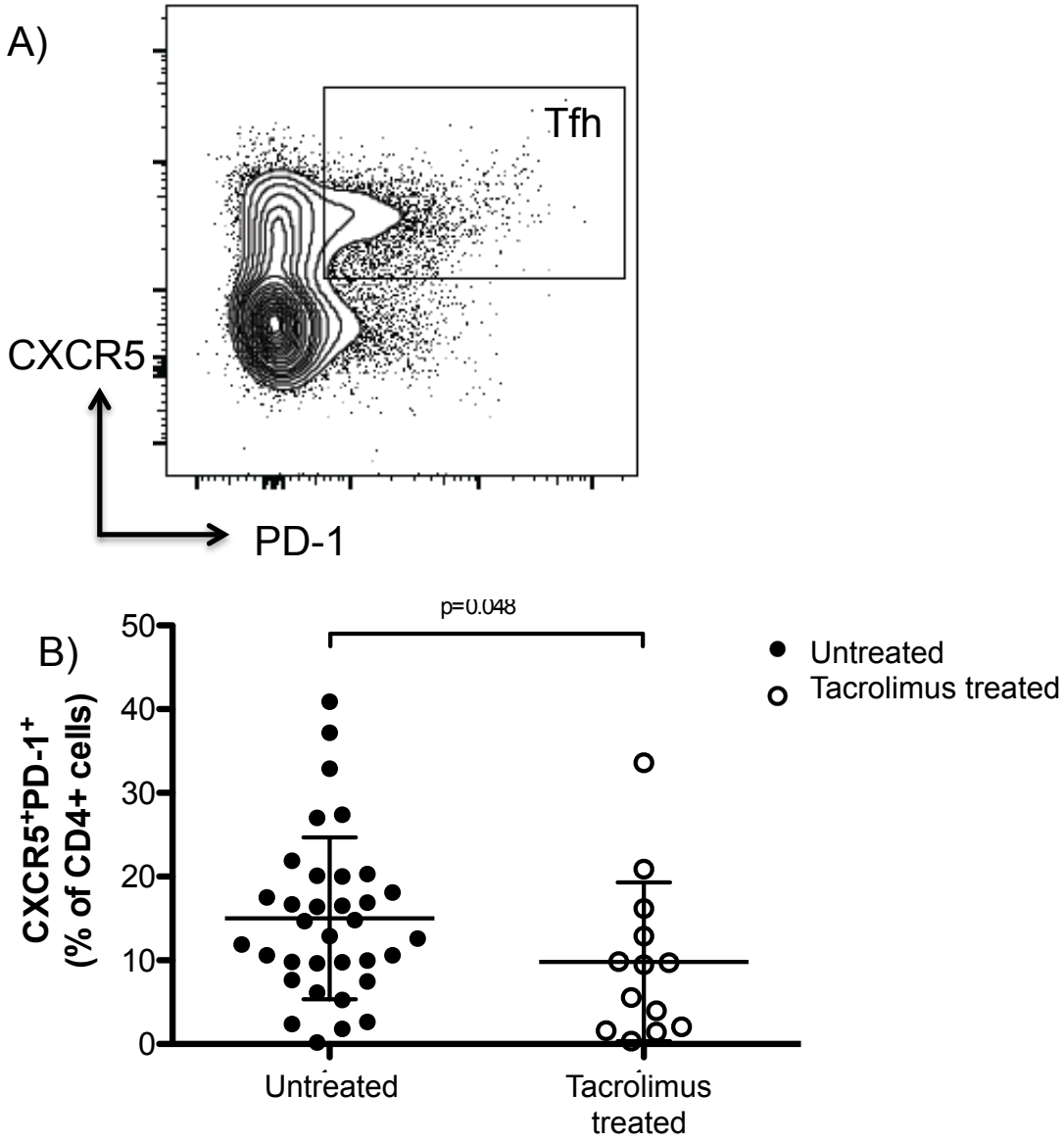


Figure 4.9: LN activated Tfh. Samples were gated on lymphocytes then live $CD4^+$ cells, Tfh were identified as $CXCR5^+PD\text{-}1^+$. B) LN Tfh showed a significant reduction in live donor recipients who had received a week of tacrolimus (tacrolimus treated, n=16) compared to untreated deceased donor recipients (untreated, n=45). Shown as mean and SD, each symbol represents one patient. Samples compared with Mann Whitney test.

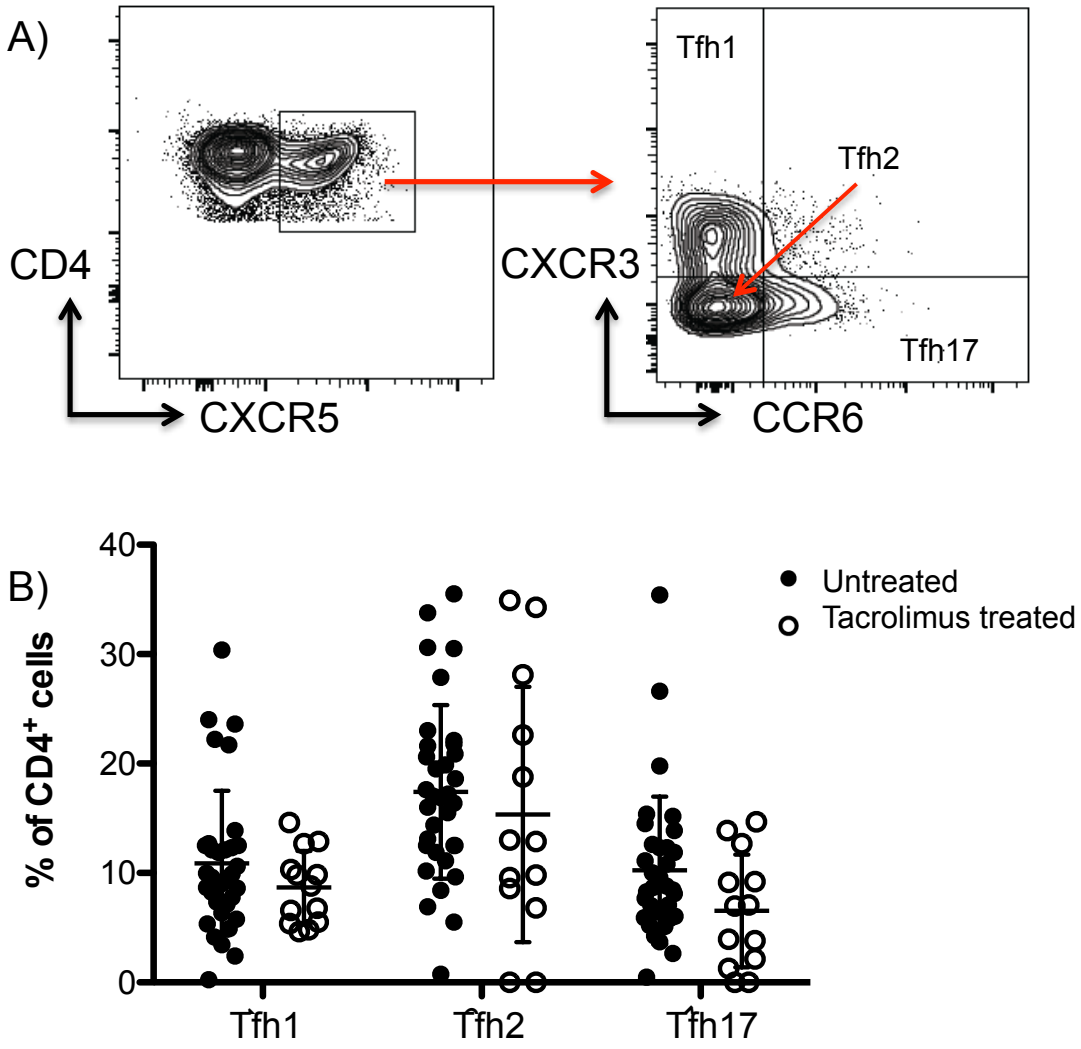


Figure 4.10: LN Tfh subsets. A) Samples were gated on lymphocytes then live $CD4^+$ cells, Tfh were identified as $CD4^+CXCR5^+$ then subdivided into $CXCR3^+CCR6^-$ Tfh1, $CXCR3^-CCR6^-$ Tfh2 and $CXCR3^-CCR6^+$ Tfh17. B) $CXCR3^+CCR6^-$ Tfh1, $CXCR3^-CCR6^-$ Tfh2 and $CXCR3^-CCR6^+$ Tfh17 subsets in live donor recipients (untreated, n=45) and live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16). Shown as mean and SD, each symbol represents one patient. There were no significant differences on Mann Whitney testing.

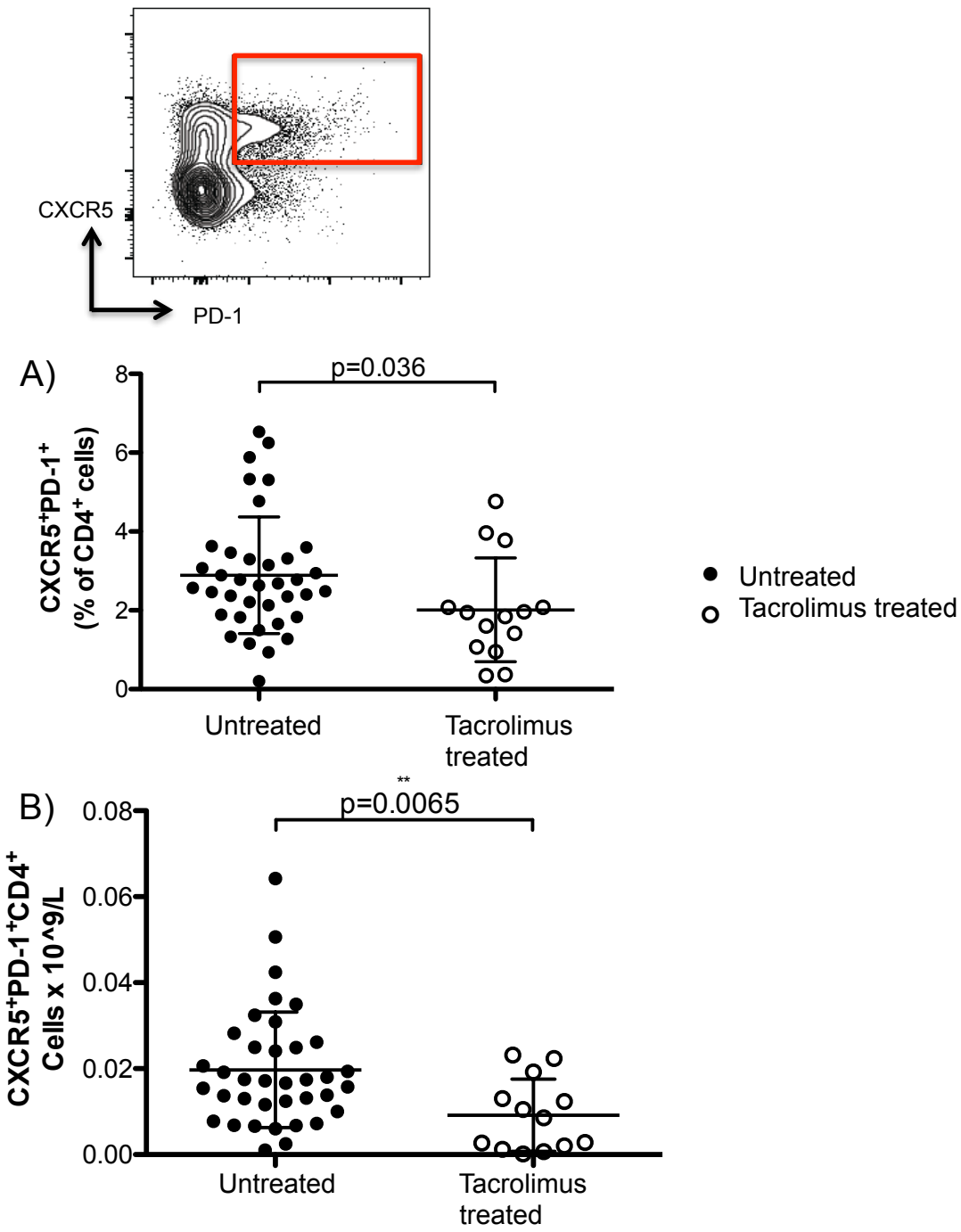


Figure 4.11: Peripheral blood Tfh. As in lymph nodes, Tfh were identified as $CXCR5^+PD\text{-}1^+CD4^+$. Peripheral blood Tfh showed a significant reduction in live donor recipients receiving a week of tacrolimus (tacrolimus treated, n=16) compared to untreated deceased donor recipients (untreated, n=45) in both (A) proportion of $CD4^+$cells and (B) absolute cell count. Shown as mean and SD, each symbol represents one patient. Samples compared with Mann Whitney test.

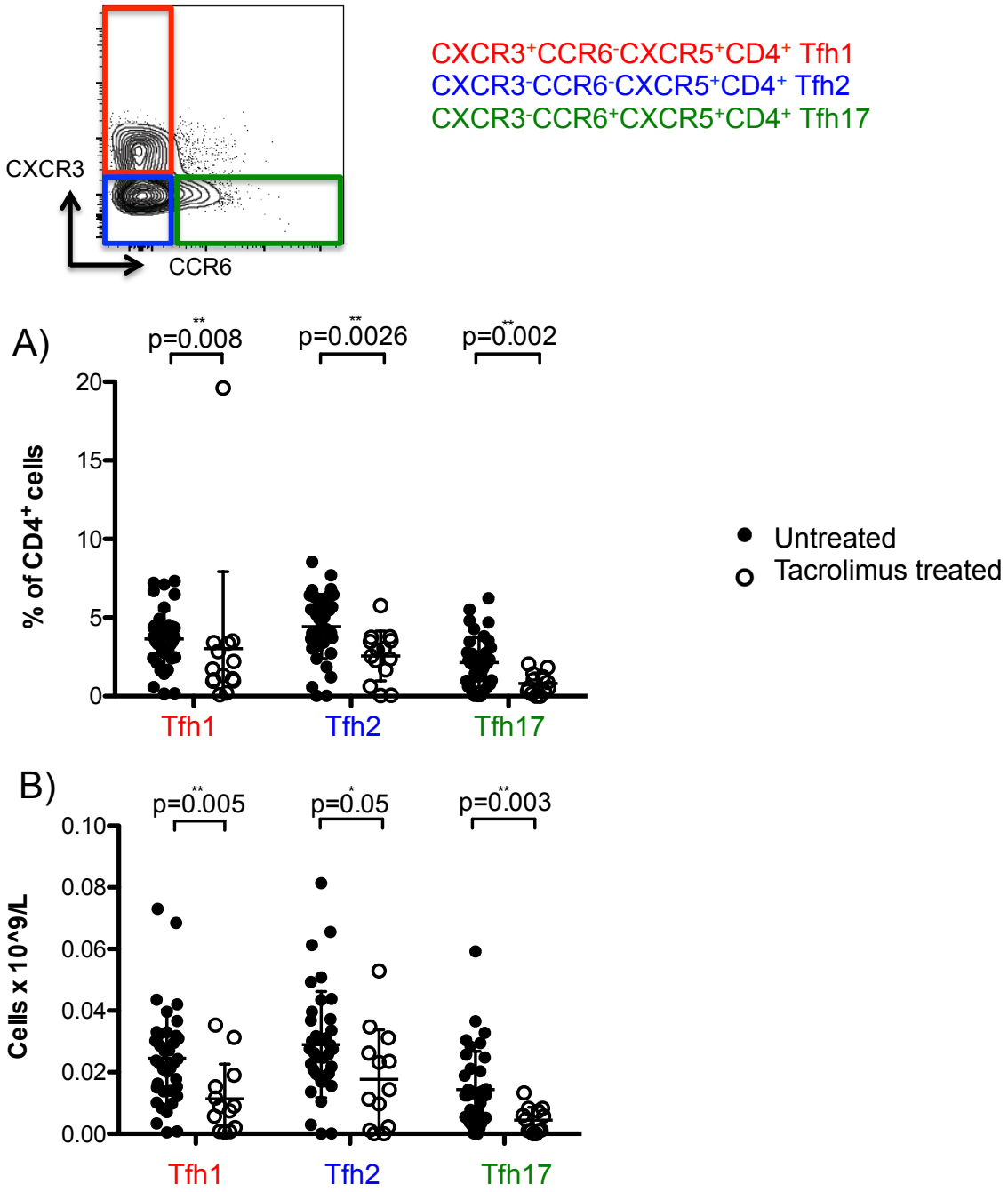


Figure 4.12: Peripheral Tfh subsets. $CXCR3^+CCR6^-CD4^+CXCR5^+$ Tfh1, $CXCR3^-CCR6^-CD4^+CXCR5^+$ Tfh2 and $CXCR3^-CCR6^+CD4^+CXCR5^+$ Tfh17 in live donor recipients who had received a week of tacrolimus (tacrolimus treated, n=16) compared to untreated deceased donor recipients (untreated, n=45) as (A) proportion of $CD4^+$ cells and (B) absolute cell count. Shown as mean and SD, each symbol represents one patient. Significant differences on Mann Whitney testing are shown.

Summary – Aim 1:

With these initial results suggesting that alterations in cTfh with tacrolimus treatment reflected alterations in LN Tfh, and that these cells were therefore appropriate biomarkers to be followed up we planned longitudinal follow up of this cohort of patients. While the differences seen initially were related to tacrolimus treatment, post transplant all patients would be treated with tacrolimus. However maintenance antimetabolite differed between azathioprine and MMF, and there are well-described alterations in circulating cells with alemtuzumab treatment[312,313,410-412], which would not be present in patients receiving induction with basiliximab. With this in mind the cohort was split into three treatment groups:

- **Group 1** – any patient receiving alemtuzumab either as induction therapy or for rejection within the first month post transplant, prior to the 28 day blood sample (n=42). For details see chapter 3, **table 3.2**.
- **Group 2** – Patients with basiliximab induction and MMF maintenance therapy (n=10)
- **Group 3** – patients with basiliximab induction and azathioprine maintenance therapy (n=9)

Therefore, to address **aim 2** the cohort was followed for two years post transplant, looking at B cell numbers, Tregs, cTfr and subsets of cTfh to assess the impact of both induction and maintenance immunosuppression on these cells.

4.4.5 Azathioprine has a greater impact on B cells compared to MMF

Basiliximab and azathioprine (B+A) treated patients had a low proportion of B cells throughout the course of the study (**fig 4.13A**), and absolute cell counts fell below pre-transplant levels and remained stably low to the 2 year time point (**fig 4.13B**). The reduction of B cell numbers in patients on azathioprine has been previously reported[413]. Basiliximab and MMF (B+M) treated patients had a fall in B cell levels after transplant but while proportions remained stable across the course of the study (**fig 4.13A**) the absolute count of B cells rose to above pre-transplant levels (**fig 4.13B**). Alemtuzumab treated patients showed an initial fall in B cell levels in keeping with lymphodepletion and then a rise above pre-transplant levels, similar to the pattern seen in B+M treated patients and in keeping with previous studies[412].

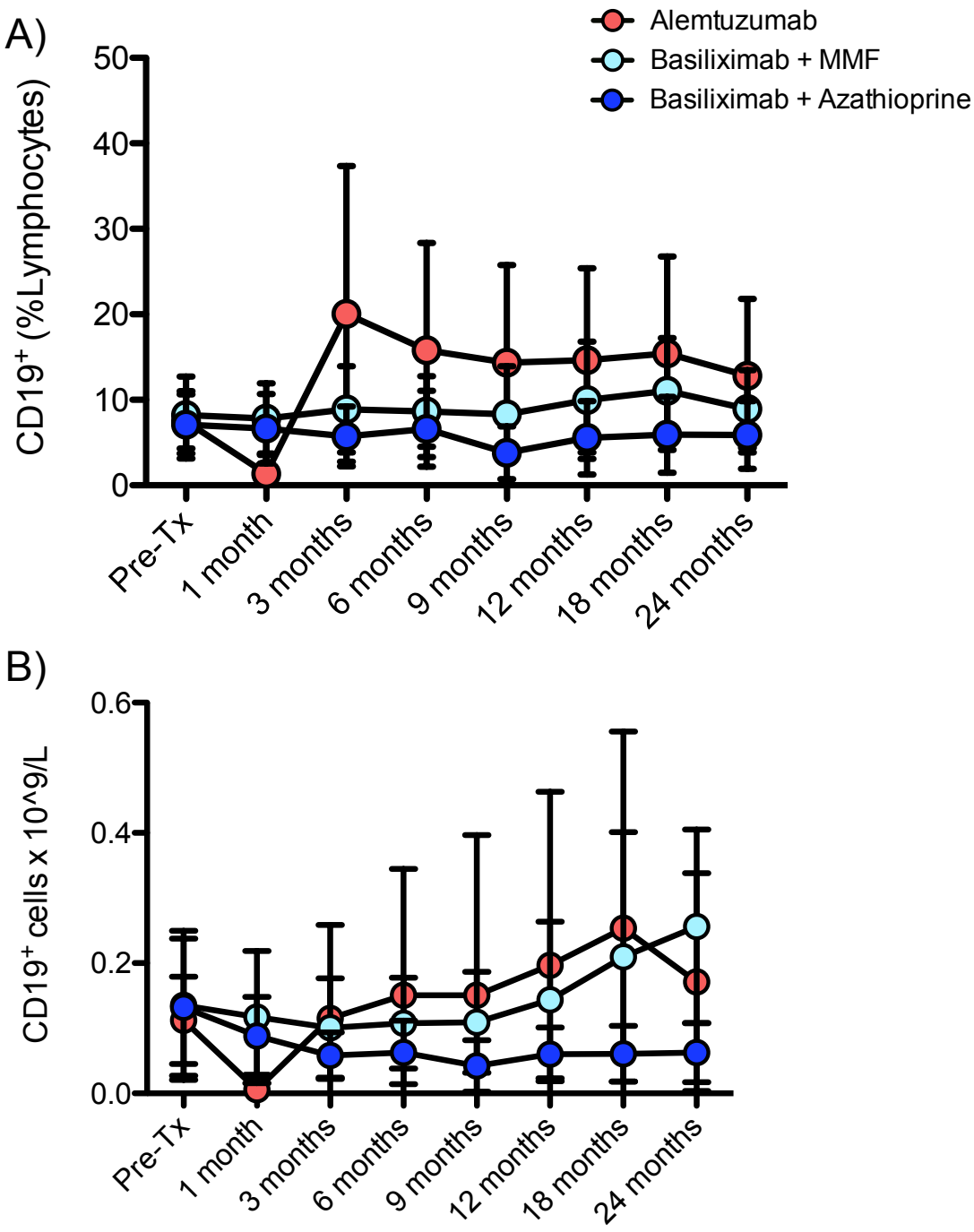


Figure 4.13: B cell proportions and count. $CD19^+$ cells pre and post transplant as a) a proportion of lymphocytes and b) absolute cell count calculated from laboratory lymphocyte count. Graphs split by treatment group showing mean and SD. Alemtuzumab n=42, B+M n=10, B+A n=9.

4.4.6 Lymphocyte reconstitution post alemtuzumab leads to a spike in transitional cells, plasmablasts and CD27$^-$IgD$^-$ B cells

Following global lymphocyte depletion with alemtuzumab, B cell reconstitution begins at 6-12 weeks[414]. There was a spike in the proportion of CD38$^+$CD24$^+$CD19$^+$ transitional B cells at 1 month, which peaked at 3 months after transplantation with a significant difference between treatment groups ($p<0.00001$ at 3 months) and then began to decline ($p=0.0017$ at 6 months, $p=0.015$ at 9 months, **fig 4.14A**) and while absolute counts were extremely low at 1 month, the recovering cell population reflected this increase in transitional cells at 3 months ($p=0.029$) but differences were not significant by 6 months (**fig 4.14B**). This is consistent with previous reports of post-alemtuzumab reconstitution[412]. There was a slight increase in the proportion of transitional B cells early post-transplant in B+M treated patients, but absolute numbers remained steady, in contrast B+A treated patients showed an initial fall and then low proportion and absolute count across the course of the study (**fig 4.14**).

CD38^{++}CD24$^-$CD19$^+$ plasmablasts also showed a slight increase in proportion at 1 month in alemtuzumab treated patients, but otherwise were consistently low across the course of the study (**fig 4.15A**) with a steady increase in absolute cell count in all treatment groups, consistent with the improving lymphocyte count following induction immunosuppression and early, higher dose maintenance therapy (**fig 4.15B**).

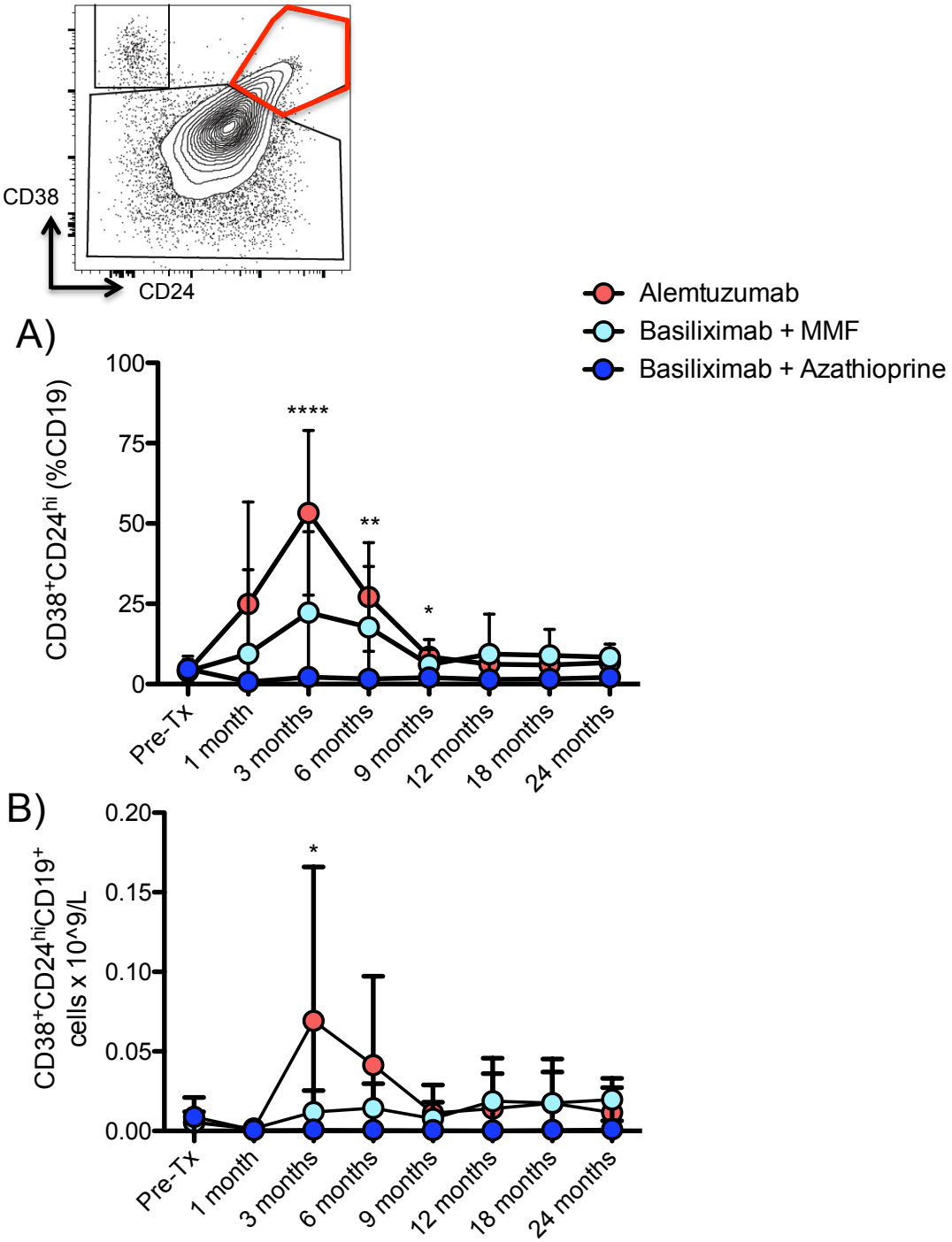


Figure 4.14: Peripheral blood transitional cells. A) $CD38^{++}CD24^{+}CD19^{+}$ transitional B cells as proportion of B cells, alemtuzumab treated patients had significantly higher transitional cells at 3 months ($p<0.00001$), 6 months ($p=0.0017$) and 9 months ($p=0.015$). B) absolute cell count of $CD38^{+}$ $^{+}CD24^{+}CD19^{+}$ transitional B cells, showing significant difference in alemtuzumab treated patients at 3 months ($p=0.029$). Shown as mean and SD for each treatment group. Analysed with 1 way ANOVA. Alemtuzumab n=42, B +M n=10, B+A n=9.

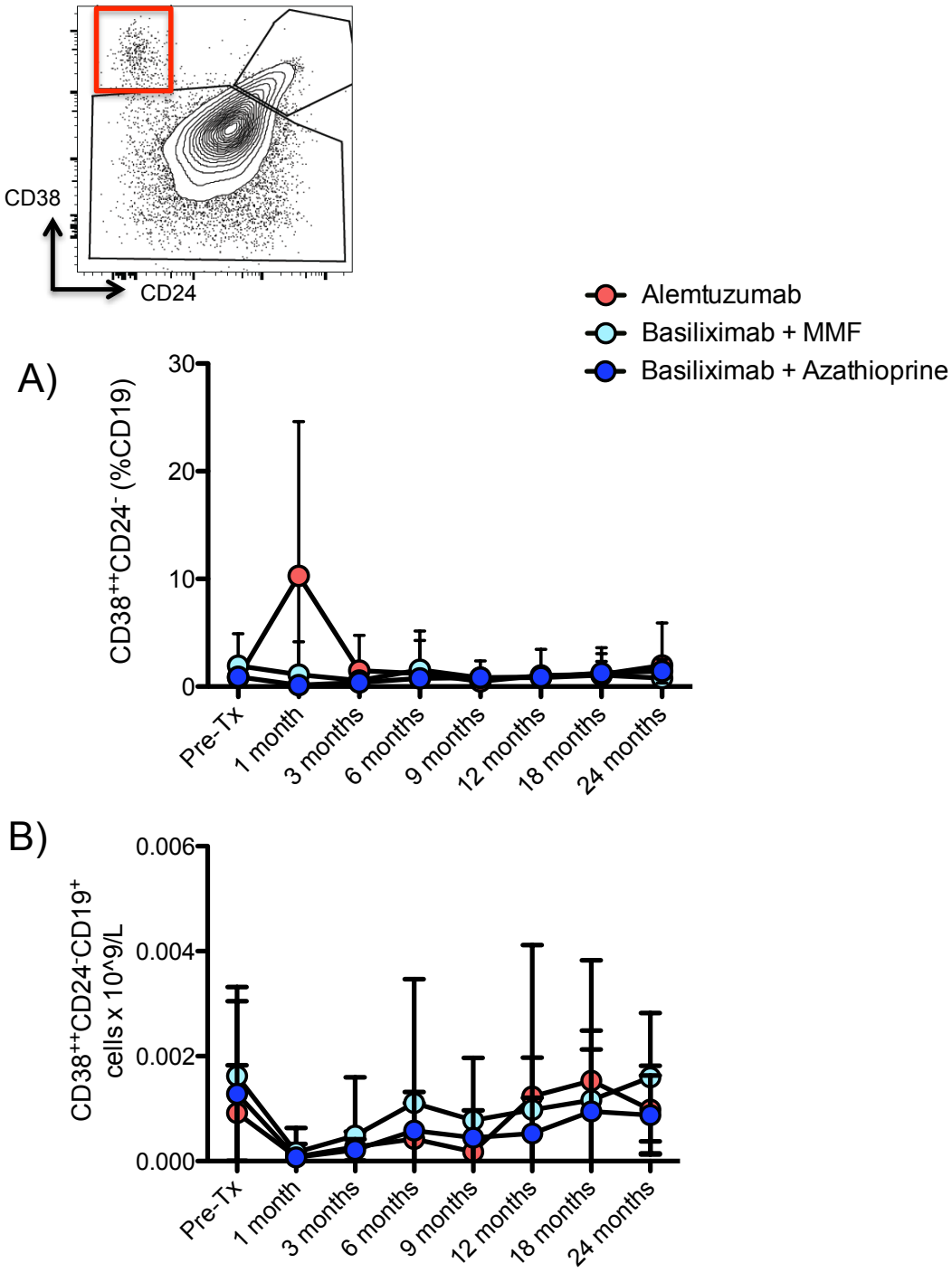


Figure 4.15: Peripheral blood plasmablasts. $CD38^{++}CD24^{-}CD19^{+}$ plasmablasts as A) proportion of B cells, and B) absolute cell count shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

Naïve and memory subsets were analysed as pre-transplant. A similar pattern to that seen for plasmablasts was observed in $CD27^{-}IgD^{-}CD19^{+}$ cells, with a spike in cell proportion at 1 month in alemtuzumab treated patients but steady proportions at all other time points in the other treatment groups (**fig 4.16A**). These cells slowly increased in absolute number following transplantation in all treatment groups, reaching pre-transplant levels in B+A treated patients, but increasing compared to pre-transplant in alemtuzumab and B+M treated patients (**fig 4.16B**). These cells are thought to be part of the memory B cell compartment despite lacking CD27 expression, the classical marker of B cell memory[415,416].

4.4.7 Switched memory B cells are relatively resistant to azathioprine

In contrast to the changes seen in $CD27^{-}IgD^{-}CD19^{+}$ cells, $CD27^{+}IgD^{-}CD19^{+}$ class switched memory cells remained remarkably steady as a proportion of B cells across the course of the study (**fig 4.17A**) although fell slightly in alemtuzumab and B+M treated patients. Absolute counts fell in all patients following transplantation (**fig 4.17B**) but then stabilised across the study, remaining at low levels in all groups. This finding has potential implications for immunity in transplant patients as a reduction in circulating switched memory cells may lead to reduced protective immunity, increasing the risk of infectious diseases.

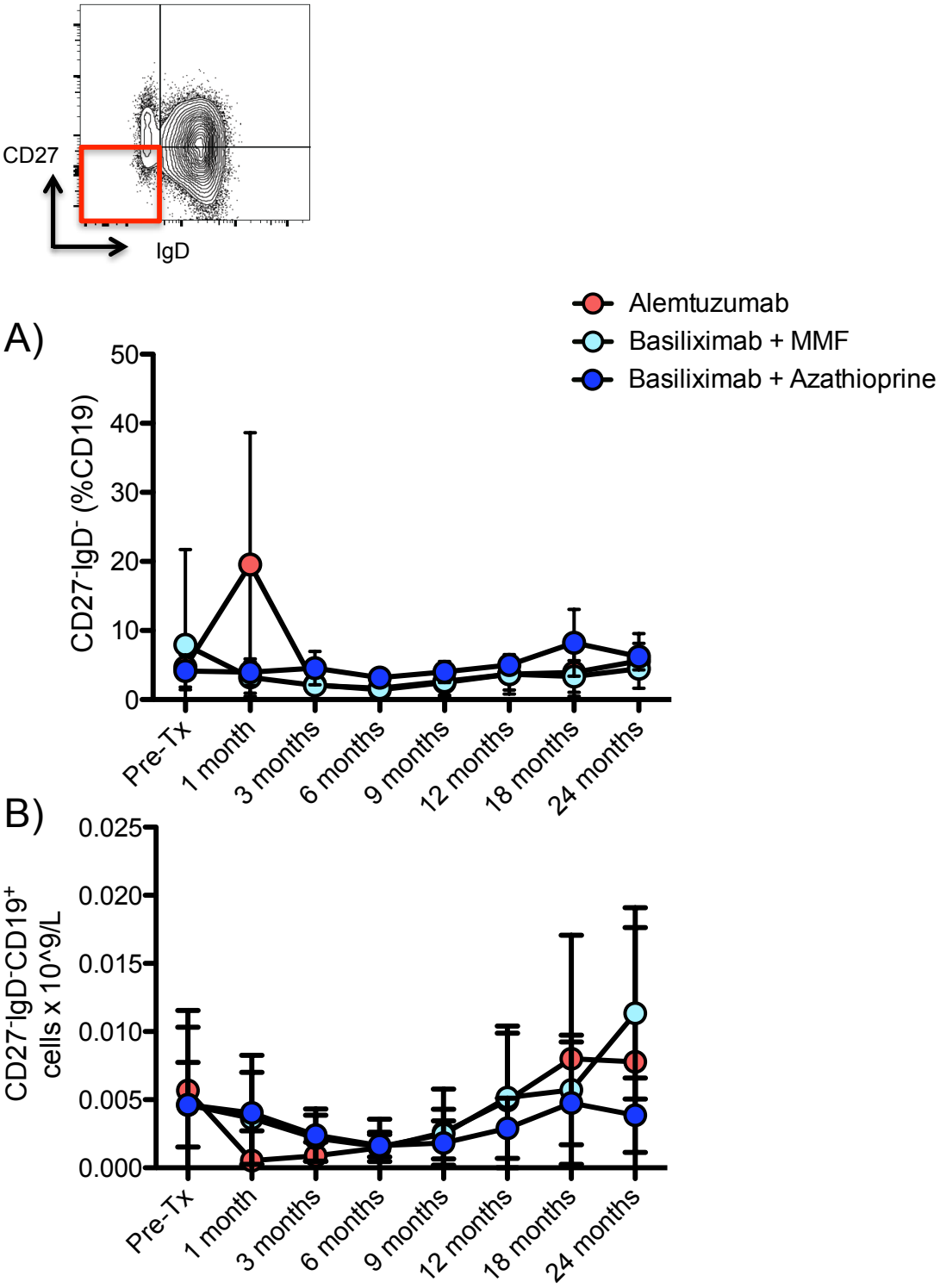


Figure 4.16: Peripheral blood CD27$^-$IgD$^-$ cells. CD27$^-$IgD$^-$CD19$^+$ cells as A) proportion of B cells, and B) absolute cell count shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

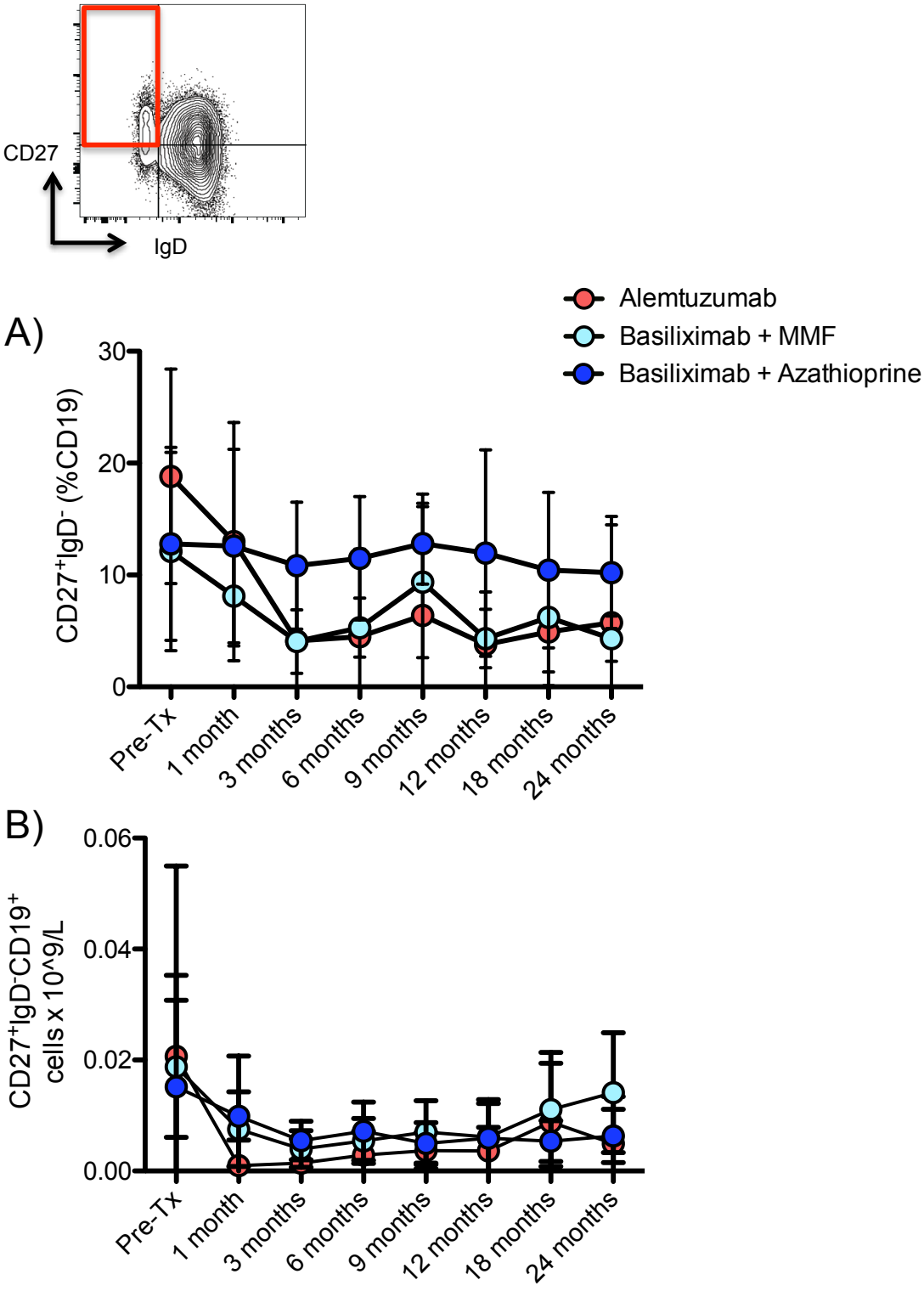


Figure 4.17: Peripheral blood class-switched memory B cells. $CD27^{+}IgD^{-}CD19^{+}$ class switched memory B cells as A) proportion of B cells, and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

4.4.8 Unswitched memory B cells predominated in basiliximab induction

$CD27^+$ memory B cells can be split into those that have downregulated IgD and are considered class-switched classical memory cells (above) and those expressing IgD, which are considered to be non-class switched cells, and generally express IgM alongside IgD. $CD27^+IgD^+CD19^+$ unswitched memory cells appeared to be most susceptible to the effect of alemtuzumab, falling sharply both in proportion (**fig 4.18A**) and absolute cell count (**fig 4.18B**) immediately post-transplant, but recovering between 3 and 6 months post-transplant. In comparison, both B+M and B+A treated patients maintained steady proportions of unswitched memory cells until one year when proportions fell, likely reflecting recovery of other B cell subsets. The absolute count of $CD27^+IgD^+CD19^+$ unswitched memory cells varied considerably in alemtuzumab and B+M treated patients but declined steadily across 2 years in the B+A treated cohort.

4.4.9 Naïve B cells predominated in the long term

In all treatment groups $CD27^-IgD^+$ naïve B cells formed a high proportion of $CD19^+$ lymphocytes post-transplant and rose over the first two years to comprise over 50% of B cells in all treatment groups (**fig 4.19A**). In alemtuzumab treated and B+M treated patients the absolute count of naïve B cells rose to above pre-transplant levels, but this remained consistently low in B+A treated patients (**fig 4.19B**) at similar levels to pre-transplant.

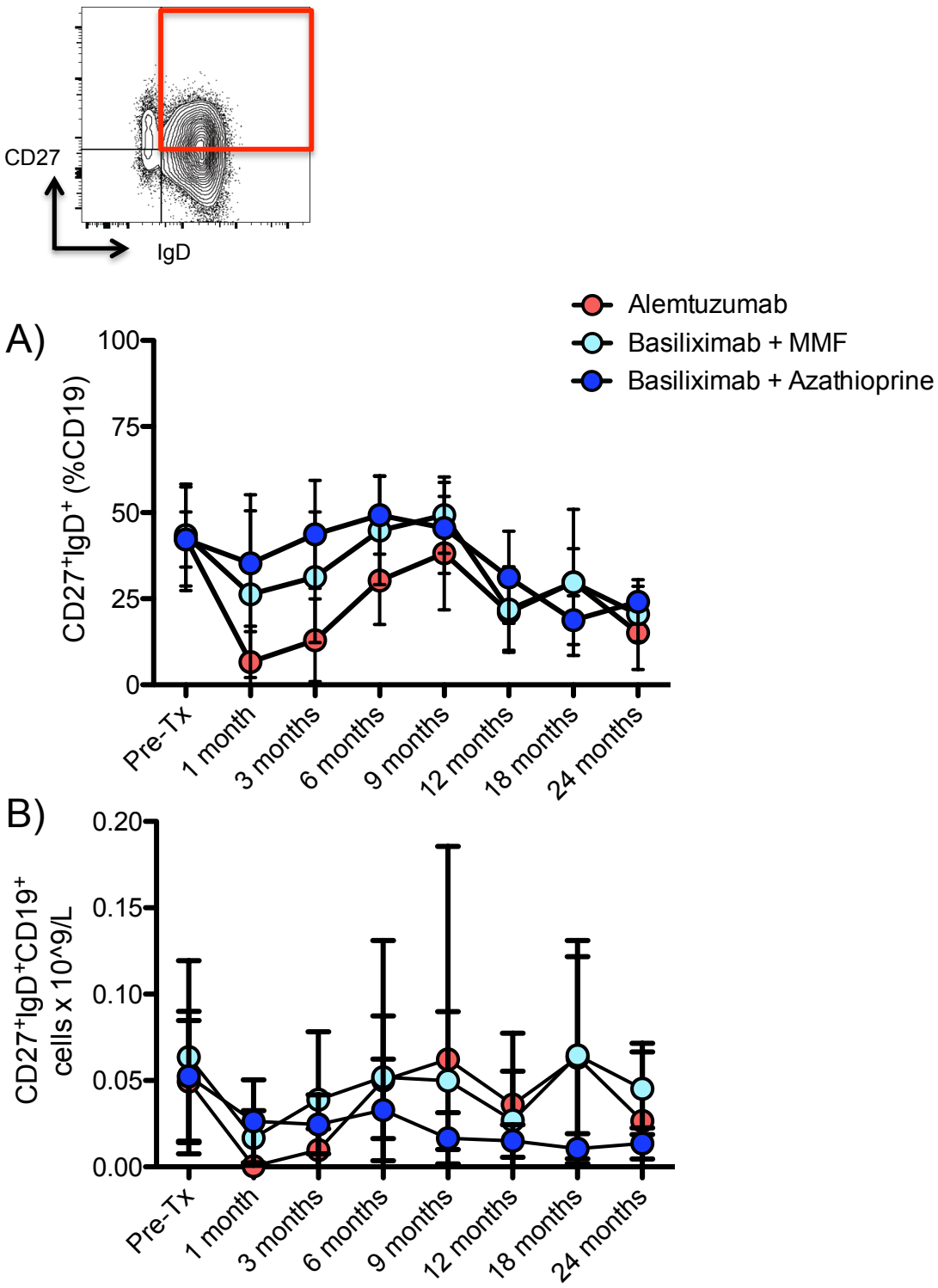


Figure 4.18: Peripheral blood unswitched memory B cells. $CD27^{+}IgD^{+}CD19^{+}$ unswitched memory B cells as A) proportion of B cells, and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

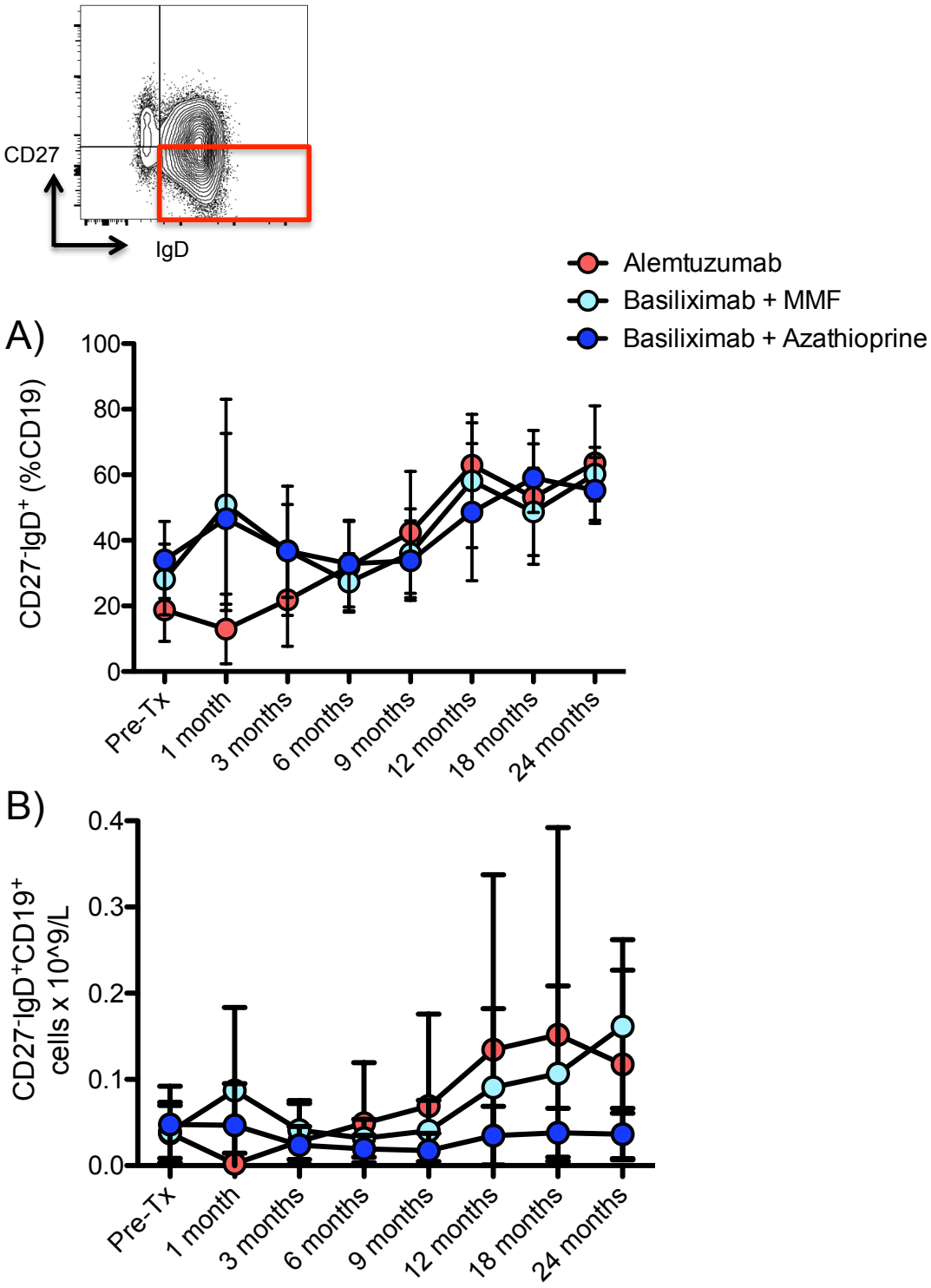


Figure 4.19: Peripheral blood CD27$^-$IgD$^+$ naïve B cells. CD27$^-$IgD$^+$CD19$^+$ naïve cells as A) proportion of B cells, and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

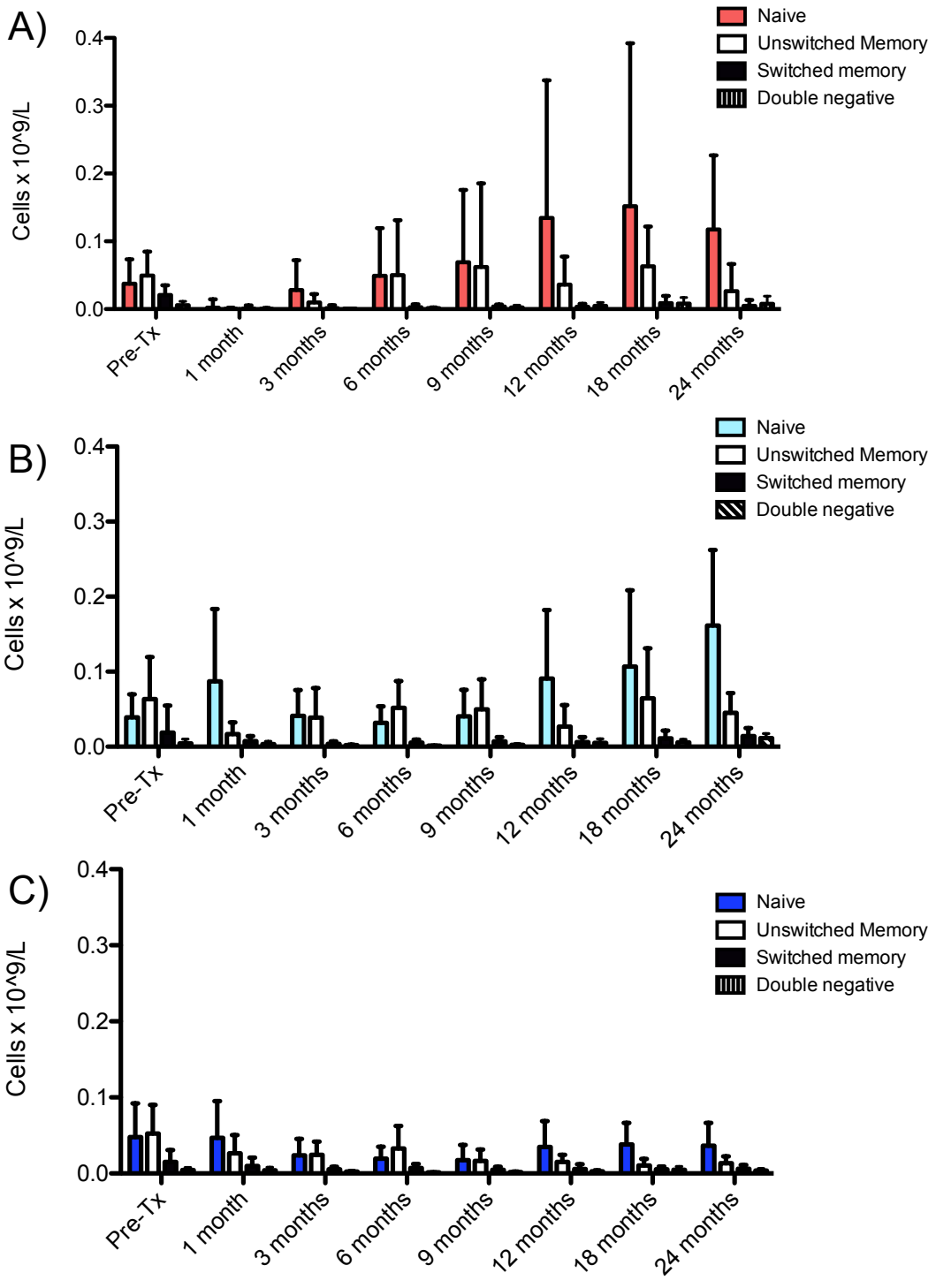


Figure 4.20: B cell proportions by drug group. Naïve, unswitched memory, switched memory and double negative cells split by A) Alemtuzumab treated patients, B) Basiliximab with maintenance MMF, C) Basiliximab with maintenance azathioprine. Each bar represents mean with standard deviation. Alemtuzumab n=42, B+M n=10, B+A n=9.

This predominance of naïve cells with low switched memory cells in alemtuzumab and B+M treated groups, and flattening of all B cell subsets in B+A treated patients is seen in the longitudinal graphs of **fig 4.20**, showing absolute cell count for each subset per treatment group. The effects of azathioprine are particularly clear when compared to other treatment groups, with low numbers of B cells in all subsets at all time points, rather than recovering post-transplant as seen in B+M and alemtuzumab treated cohorts.

4.4.10 Persistent CD4 depletion with alemtuzumab treatment

In keeping with previous work in autoimmunity[417] and transplantation[418], CD4 depletion with alemtuzumab was profound. Both proportion (**fig4.21A**) and absolute cell counts (**fig4.21B**) remained low across the 2 years of the study. In contrast, proportions and numbers remained relatively steady in both B+A and B+M treated patients. The increased proportion of $CD4^+$ cells in B+A treated compared to B+M patients likely reflects the reduced proportion of $CD19^+$ cells, as absolute cell counts were similar in both groups.

4.4.11 Treg cells transiently increase in proportion with alemtuzumab

Treg cells were gated as pre-transplant, as IL-$7R^{lo}FOXP3^+CD4^+$ cells (**fig 4.22A**) and then subdivided into $CXCR5^+$ Tfr and $CXCR5^-$ Tregs. An increase in the proportion of $FOXP3^+$ cells following lymphocyte depletion with alemtuzumab has been previously described[417,419] and was also seen in this patient cohort, with a steady proportion in B+A and B+M treated patients (**fig4.22Bi**). In keeping with the profound lymphocyte depletion, absolute

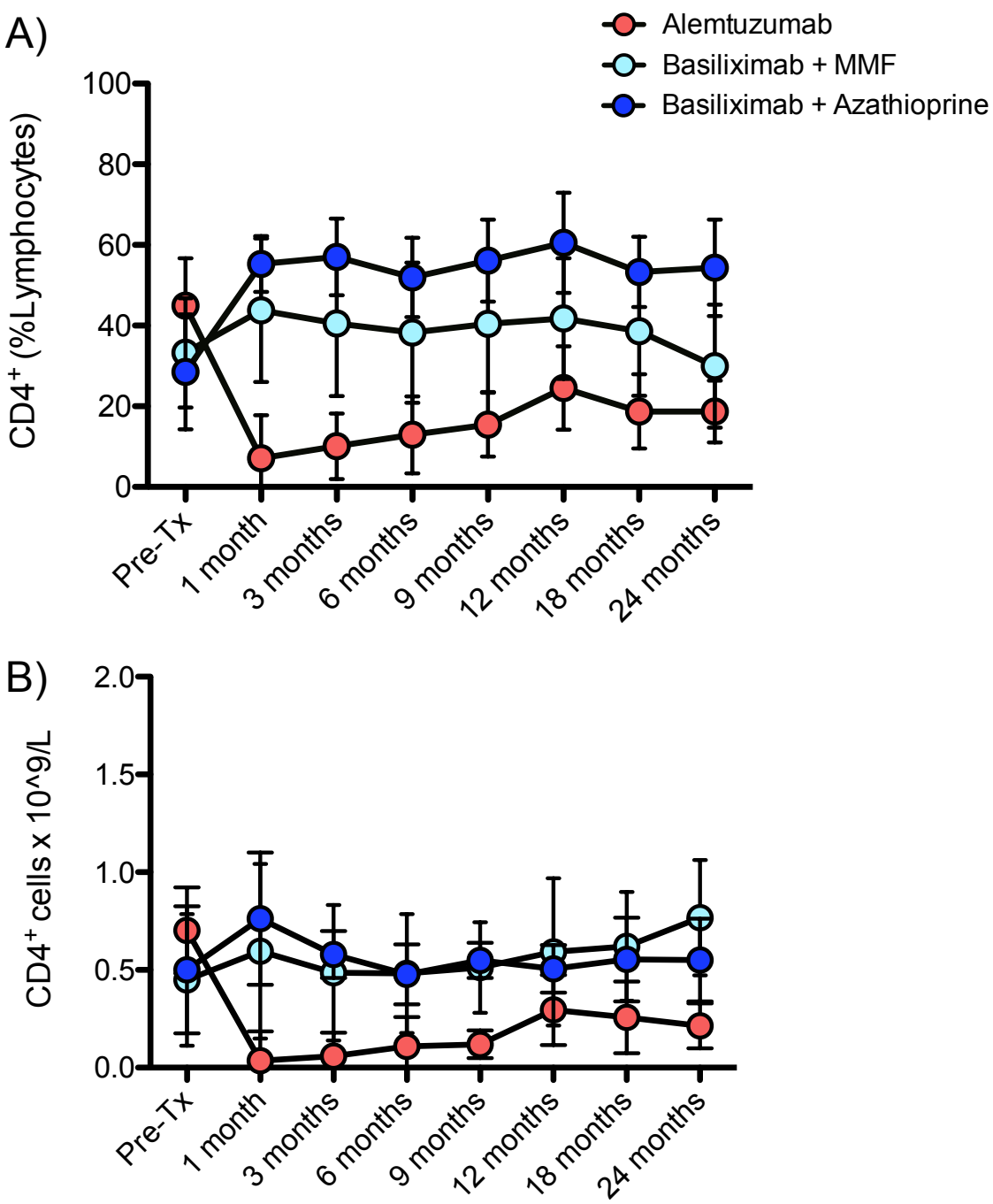


Figure 4.21: CD4 T cell proportions and count. $CD4^+$ cells pre and post transplant as a) a proportion of lymphocytes and b) absolute cell count calculated from laboratory lymphocyte count. Graphs split by treatment group showing mean and SD. Alemtuzumab n=42, B+M n=10, B+A n=9.

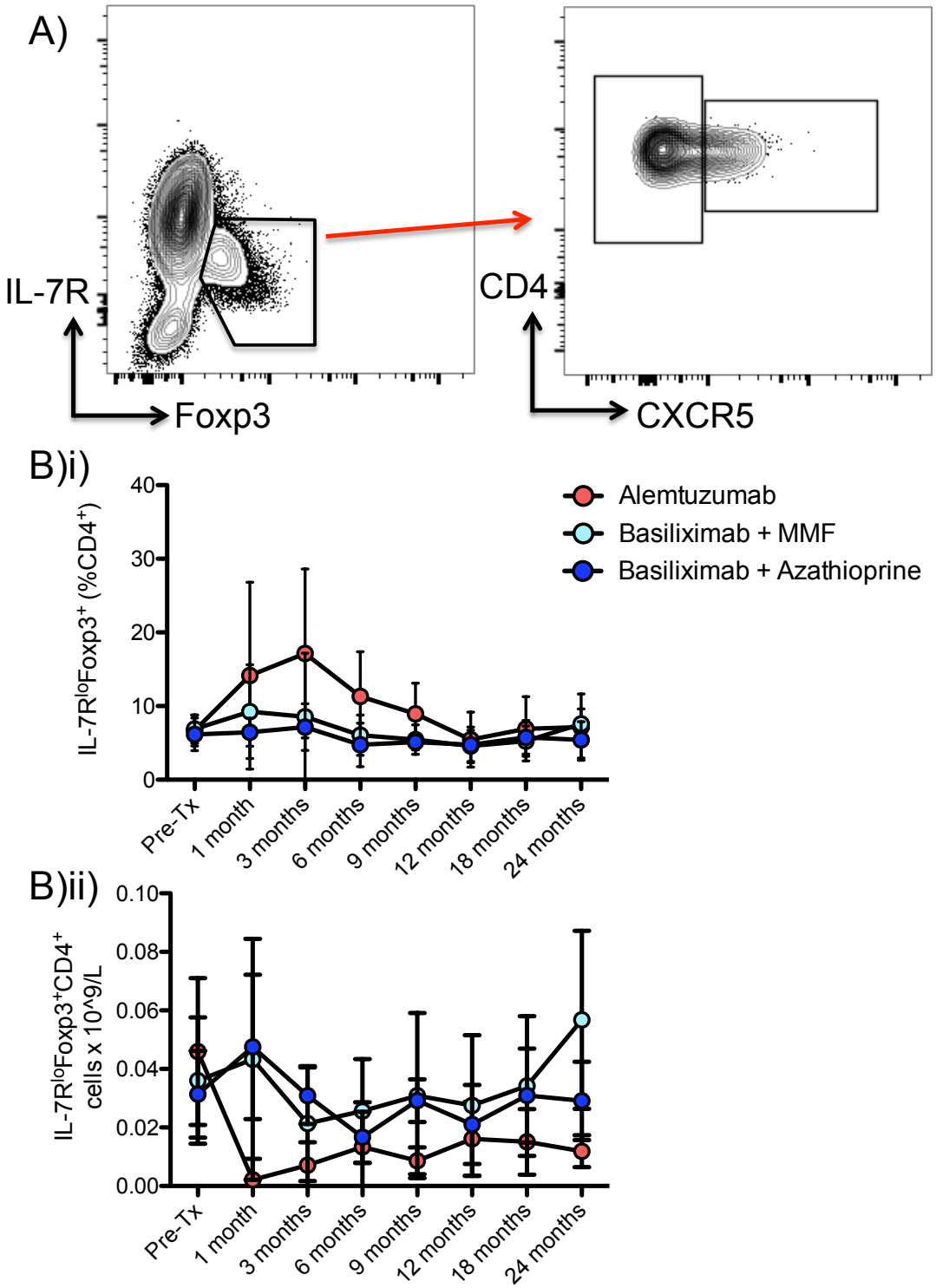


Figure 4.22 Regulatory T cells. A) Gating strategy for Tregs and Tfr, samples were gated on lymphocytes, live $CD4^+$ then IL-$7R^{lo}Foxp3^+$ for all Tregs, then divided into CXCR5$^-$IL-$7R^{lo}Foxp3^+CD4^+$ and CXCR5$^-$IL-$7R^{lo}Foxp3^+CD4^+$ Tfr. B) IL-$7R^{lo}Foxp3^+CD4^+$ Tregs as i) proportion of $CD4^+$ T cells and ii) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

counts of IL-7R^{lo}FOXP3^{+} Treg cells were lower in alemtuzumab treated patients (**fig4.22Bii**).

These cells were further subdivided into CXCR5^{-}IL-7R^{lo}FOXP3^{+} Treg cells, which followed similar alterations in proportion and absolute cell count as seen in all IL-7R^{lo}FOXP3^{+} CD4 T cells (**fig 4.23**). There was a significant increase in proportion of CXCR5^{-}IL-7R^{lo}FOXP3^{+} Treg cells (**fig 4.23A**) in alemtuzumab treated patients at 1 month (p=0.016), 3 months (p=0.008), 6 months (p=0.003) and 9 months (p=0.004), but normalised at later time points. There were no significant differences in the groups on one-way ANOVA from 12-24 months.

There was considerable variation in the absolute counts of CXCR5^{-}IL-7R^{lo}FOXP3^{+} Treg cells, with significant differences at several time points (**fig 4.23B**) on one-way ANOVA, but the standard deviation within groups is large, and it is not clear how meaningful these differences are given the diversity of the patient groups.

4.4.12 CXCR5^{+}IL-7R^{lo}FOXP3^{+} Tfr cells fall after alemtuzumab treatment

Interestingly, despite the increase in proportion of all FOXP3^{+} cells the opposite was seen in CXCR5^{+}IL-7R^{lo}FOXP3^{+} Tfr, where both proportion and absolute count in alemtuzumab treated patients fell below baseline and below that seen in other treatment groups (**fig4.24**). Levels remained low at all time points after transplantation, below pre-transplant levels. In contrast, in both B+M and B+A treated patients, the proportion of CXCR5^{+}IL-7R^{lo}FOXP3^{+} Tfr rose steadily to above pre-transplant levels. There were significant differences between the

groups on one-way ANOVA at 3 months (p=0.0008), 9 months (p=0.0004), 12 months (p=0.0024), 18 and 24 months (both p<0.0001) (**fig4.24A**).

Similarly for absolute cell counts (**fig4.24B**) there were significant differences between the groups on one-way ANOVA at all time points except 6 months (p=0.054). Pre-transplant results must be interpreted with caution as B+M and B+A patients had received induction therapy with basiliximab prior to sampling, whereas alemtuzumab patients had not received any induction therapy. At later time points, alemtuzumab treated patients had consistently lower levels of cTfr cells than B+M or B+A treated patients, although there was considerable variability. As seen in $CXCR5^-$ Tregs, this likely reflects the diversity of the patient groups.

Previous work looking at post-alemtuzumab reconstitution in autoimmune disease[417,419] has focussed on all $FOXP3^+CD4^+$ cells and not separated out Tfr from Tregs. In this study it was notable that $CXCR5^-$Tregs rose in proportion while $CXCR5^+$Tfr fell. This was particularly relevant after 12 months, when $CXCR5^+$Tfr were still statistically lower in alemtuzumab treated patients, but $CXCR5^-$Tregs had normalised in proportion. This interesting observation may go some way towards explaining the high rate of antibody-mediated autoimmune disease seen after alemtuzumab treatment[420,421]. However, results must be interpreted with caution as the absolute cell counts for cTfr were low at all time points in all groups.

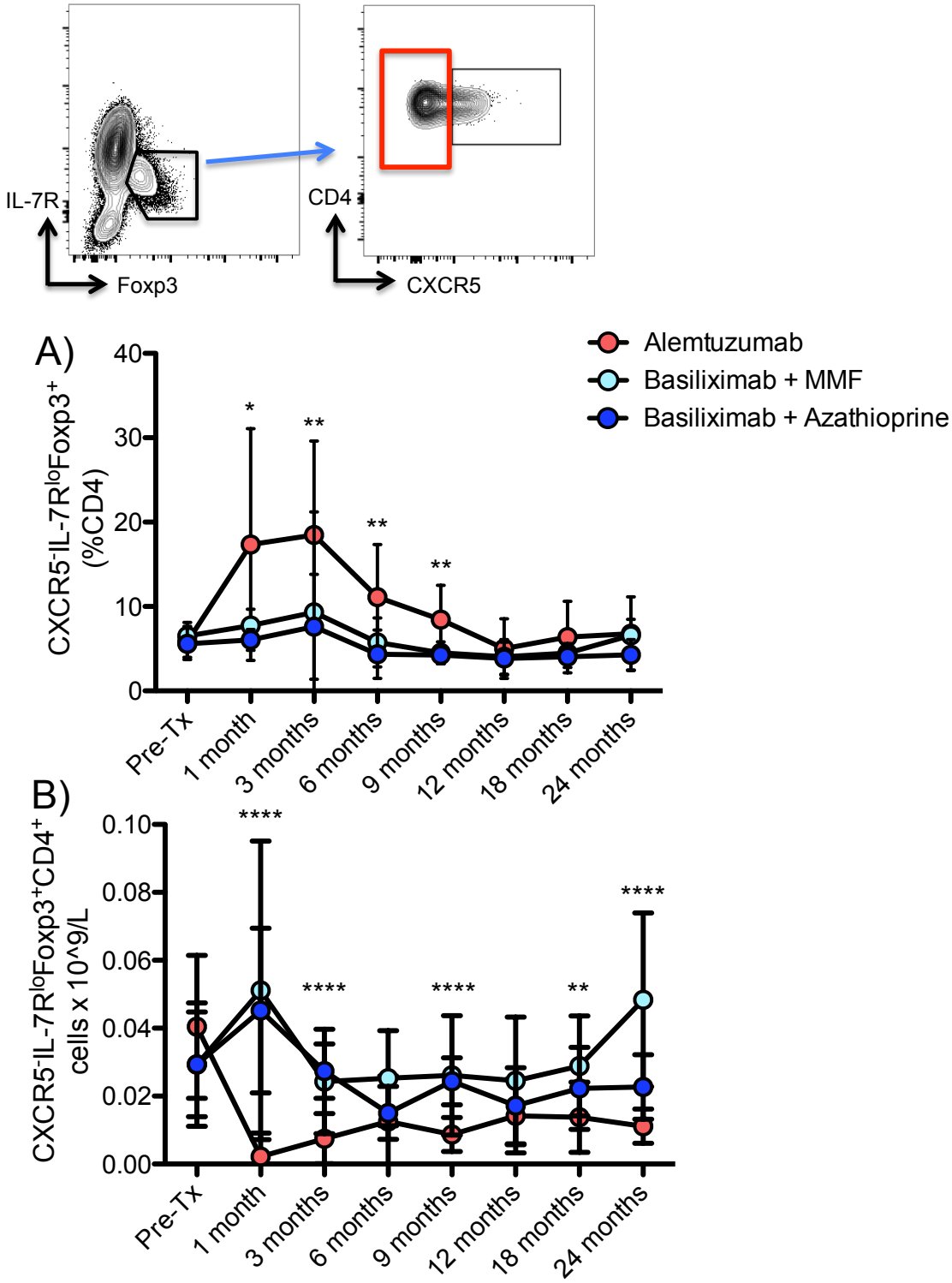


Figure 4.23 CXCR5$^-$ Regulatory T cells. CXCR5$^-$IL-7R^{lo}Foxp3$^+$CD4$^+$ regulatory T cells as A) proportion of CD4$^+$ T cells and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 on 1-way ANOVA.

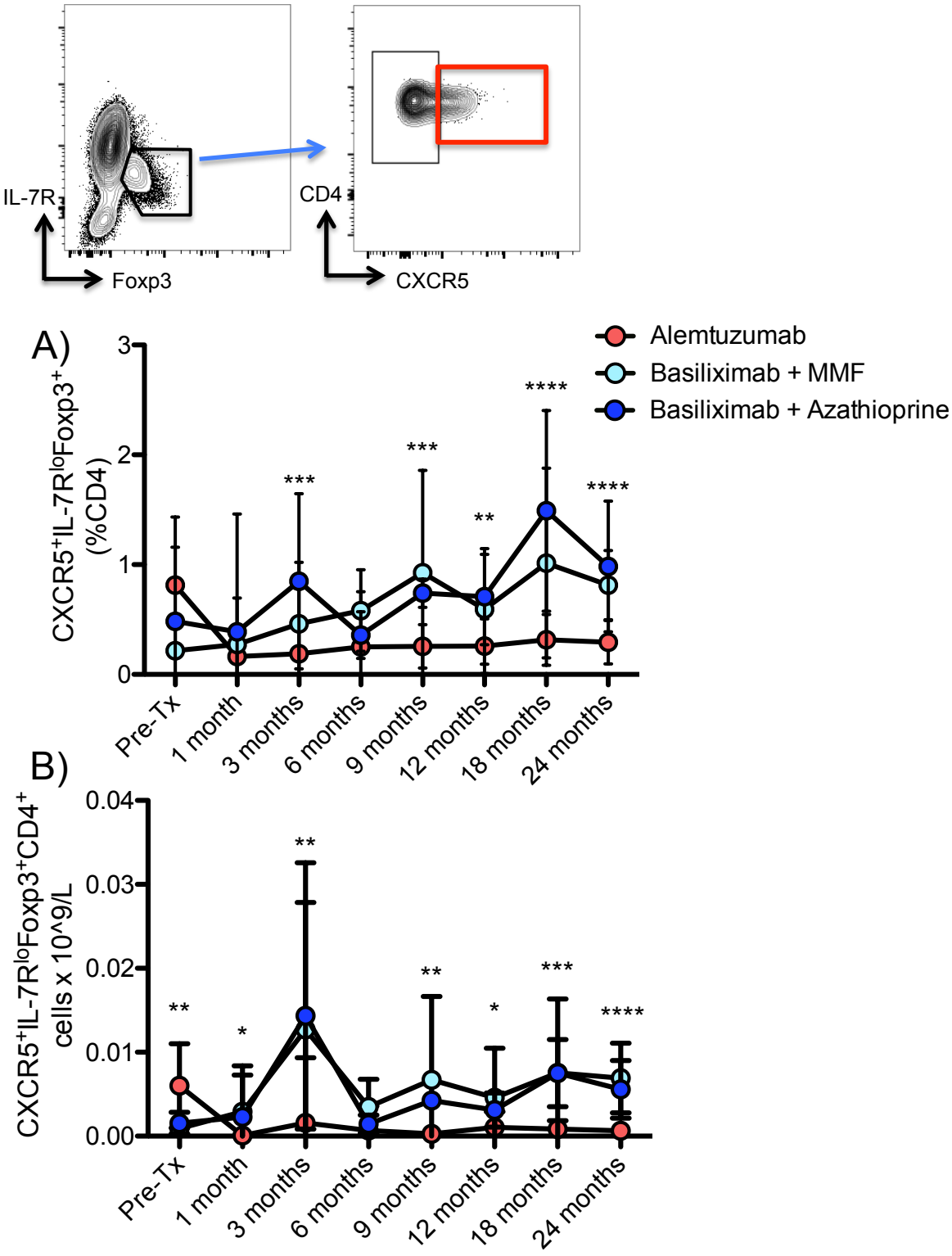


Figure 4.24 Circulating Tfr. CXCR5$^+$IL-7R^{lo}Foxp3$^+$CD4$^+$ Tfr as A) proportion of CD4$^+$ T cells and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9. Analysed with 1-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.4.13 Alterations seen in circulating Tfh cells

Given the work done in HIV and influenza showing that $CXCR3^{-}CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ and $CXCR3^{+}CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cells correlated with antibody formation depending on the immunising antigen, for the longitudinal studies Tfh cells were gated differently to pre-transplant LN samples. The gating strategy is shown in **fig 4.25**.

4.4.14 $CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells were similar in all treatment groups

Initially all $CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells were assessed, to establish the effects of immunosuppression on all $CXCR5^{+}$ cells within the circulation. These fell in all patients post-transplant both as a proportion of CD4 T cells (**fig 4.26Ai**) and absolute cell count (**fig 4.26Aii**). In all treatment groups they recovered steadily over the course of the study to around pre-transplant levels. Given the differences seen in patients receiving a week of tacrolimus prior to transplantation, this immediate reduction and slow recovery may reflect the impact of induction immunosuppression and early, higher dose maintenance therapy on cTfh cells.

4.4.15 $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells rose post-transplant

Given the association with $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ and development of antibodies in HIV patients, cTfh were split into $CXCR3^{+}$ and $CXCR3^{-}$ as per **fig 4.25.** We assessed the proportions and absolute count of these cells in transplant patients (**fig 4.27**). Interestingly proportions of these cells did not fall post-transplant, and began to rise both in proportion (**fig 4.27A**) and absolute cell count (**fig 4.27B**) post-transplant. The rise in proportion

occurred particularly in the alemtuzumab treated patients, but was seen in all treatment groups at later time points. After 12 months, the absolute cell count of $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh rose to above pre-transplant levels in all patients. It is not clear why this increase occurs, but it may be related to adjustment in immunosuppression levels. The first year of transplant is considered to hold the highest immunological risk, but clinical practice guidelines suggest that the reduction in immunosuppression can begin within the first few months post-transplant as long as the patient has not experienced rejection episodes[303]. It is possible that this late rise reflects alterations in the immunosuppression regimen, however patients were being followed up at peripheral centres at this point and our clinical data set is incomplete.

4.4.16 $CXCR3^{+}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh remained low post-transplant

In contrast to the $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cell subset associated with antibody production in HIV patients, those cells associated with acute antibody production in influenza, $CXCR3^{+}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ remained steady in both proportion (**fig 4.28A**) of Tfh and absolute count post-transplant (**fig 4.28B**). However there was a peak in the proportion of these cells at 1 month in alemtuzumab treated patients. This may reflect the extremely low CD4 count seen in these patients, as the absolute count remained low.

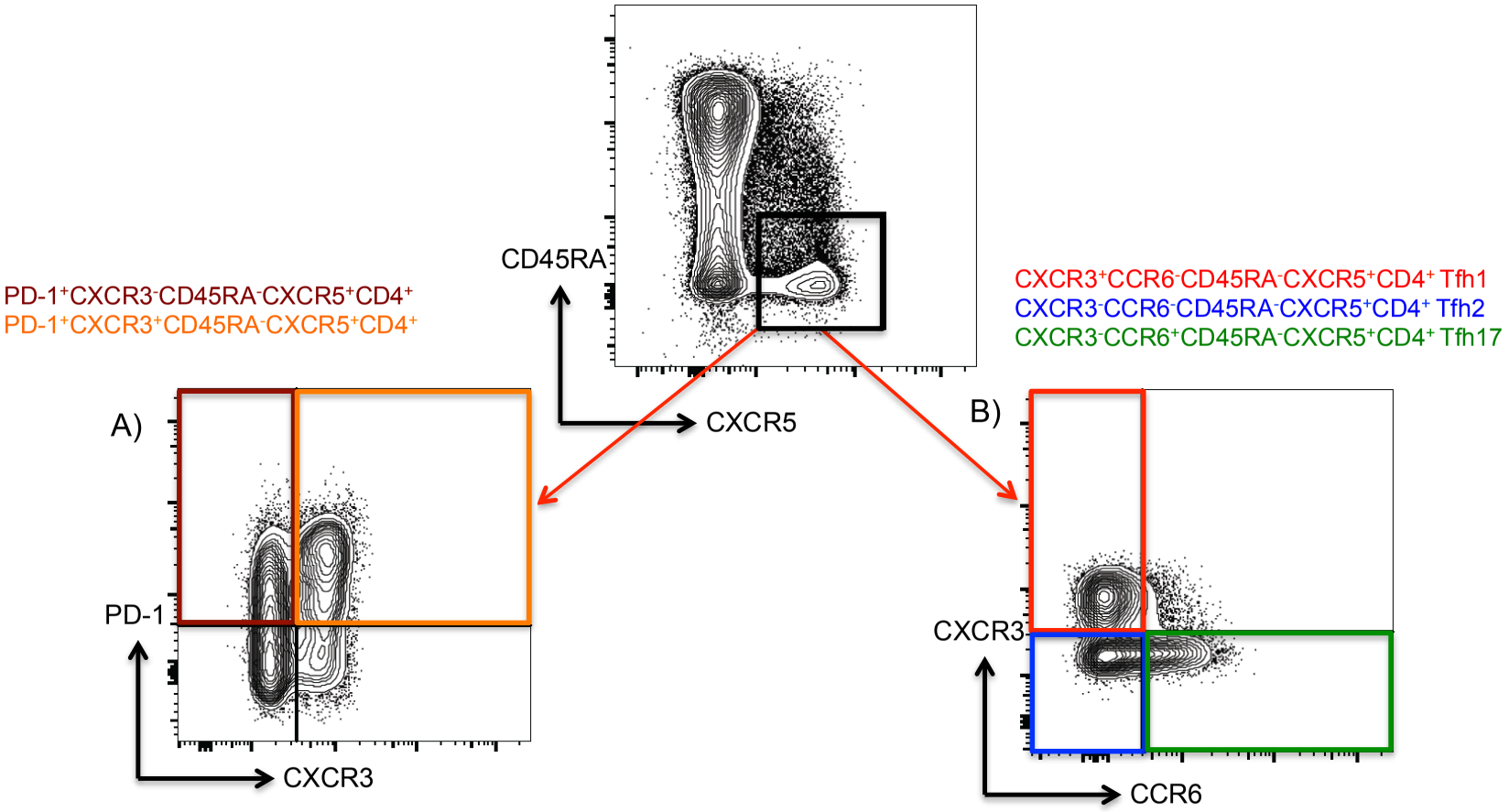


Figure 4.25: Gating for peripheral blood Tfh. Lymphocytes were gated on live CD4+ cells and then CD45RA-CXCR5+ Tfh, which were further divided into A) PD-1+CXCR3-CD45RA-CXCR5+CD4+ and PD-1+CXCR3+CD45RA-CXCR5+CD4+ and B) CXCR3+CCR6-CD45RA-CXCR5+CD4+ Tfh1, CXCR3-CCR6-CD45RA-CXCR5+CD4+ Tfh2 and CXCR3-CCR6+CD45RA-CXCR5+CD4+ Tfh17.

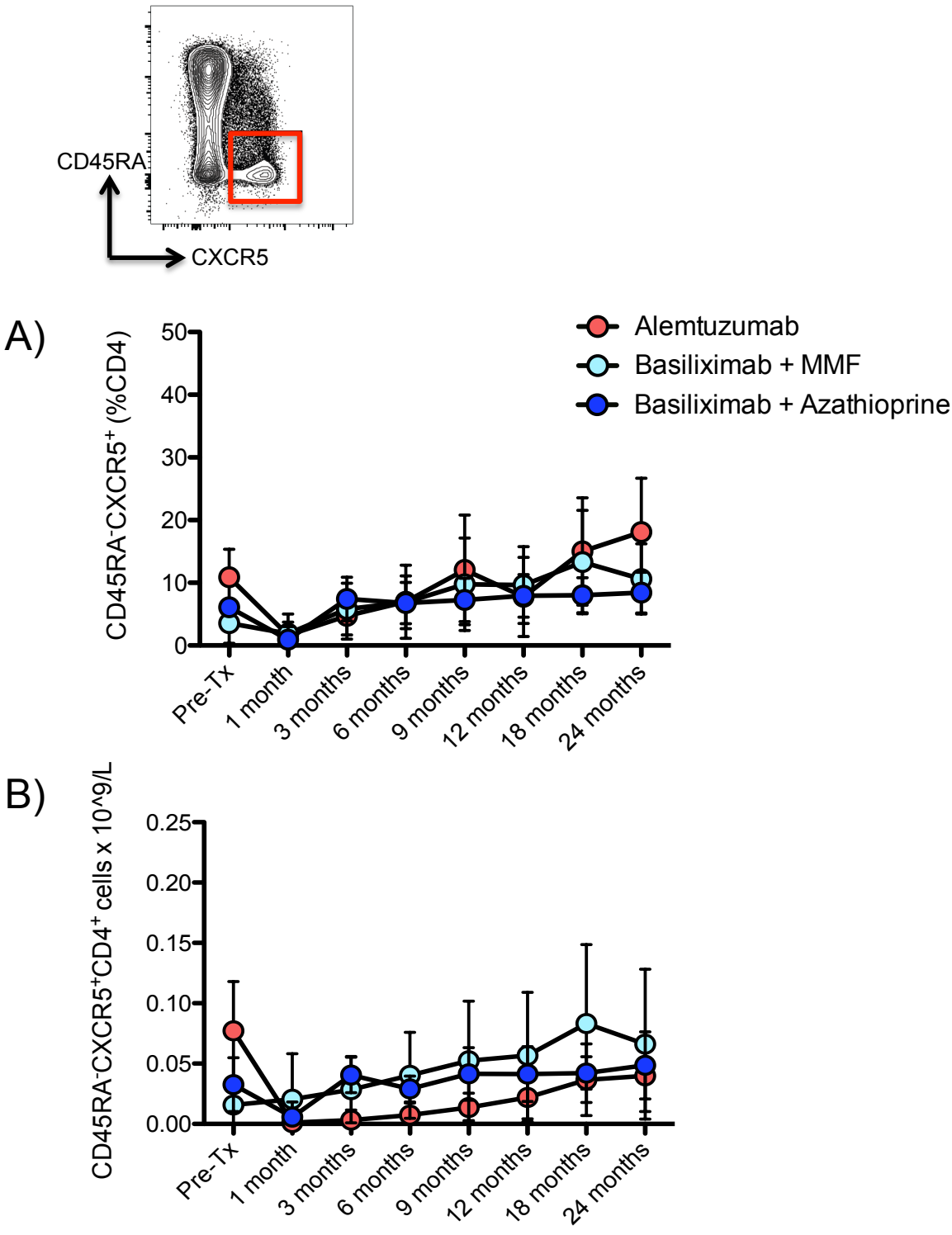


Figure 4.26: Circulating Tfh. $CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh as A) proportion of $CD4^{+}$ T cells and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

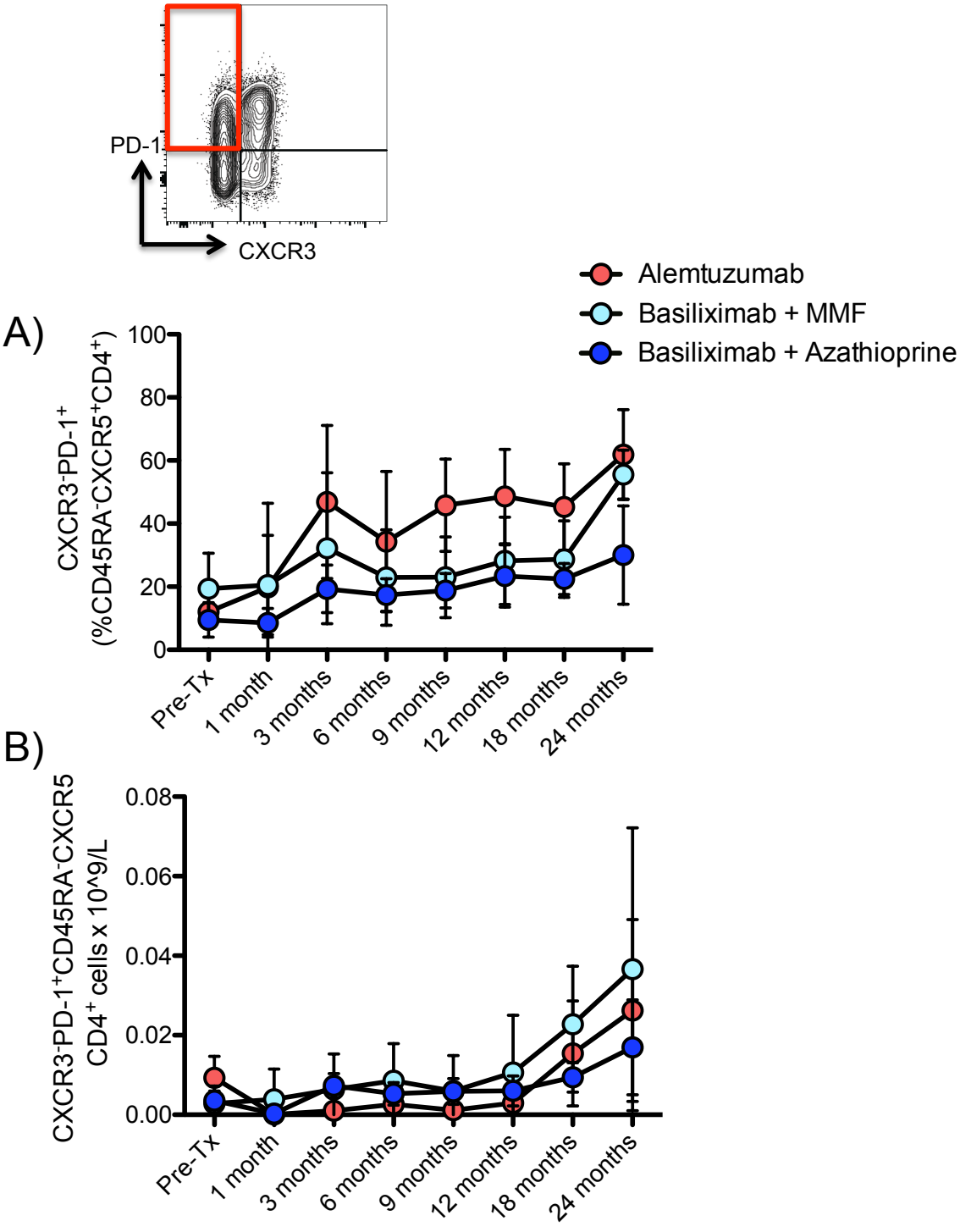


Figure 4.27: Circulating CXCR3$^-$PD-1$^+$ Tfh. CXCR3$^-$PD-1$^+$CD45RA$^-$CXCR5$^+$CD4$^+$ Tfh as A) proportion of CD4$^+$ T cells, and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

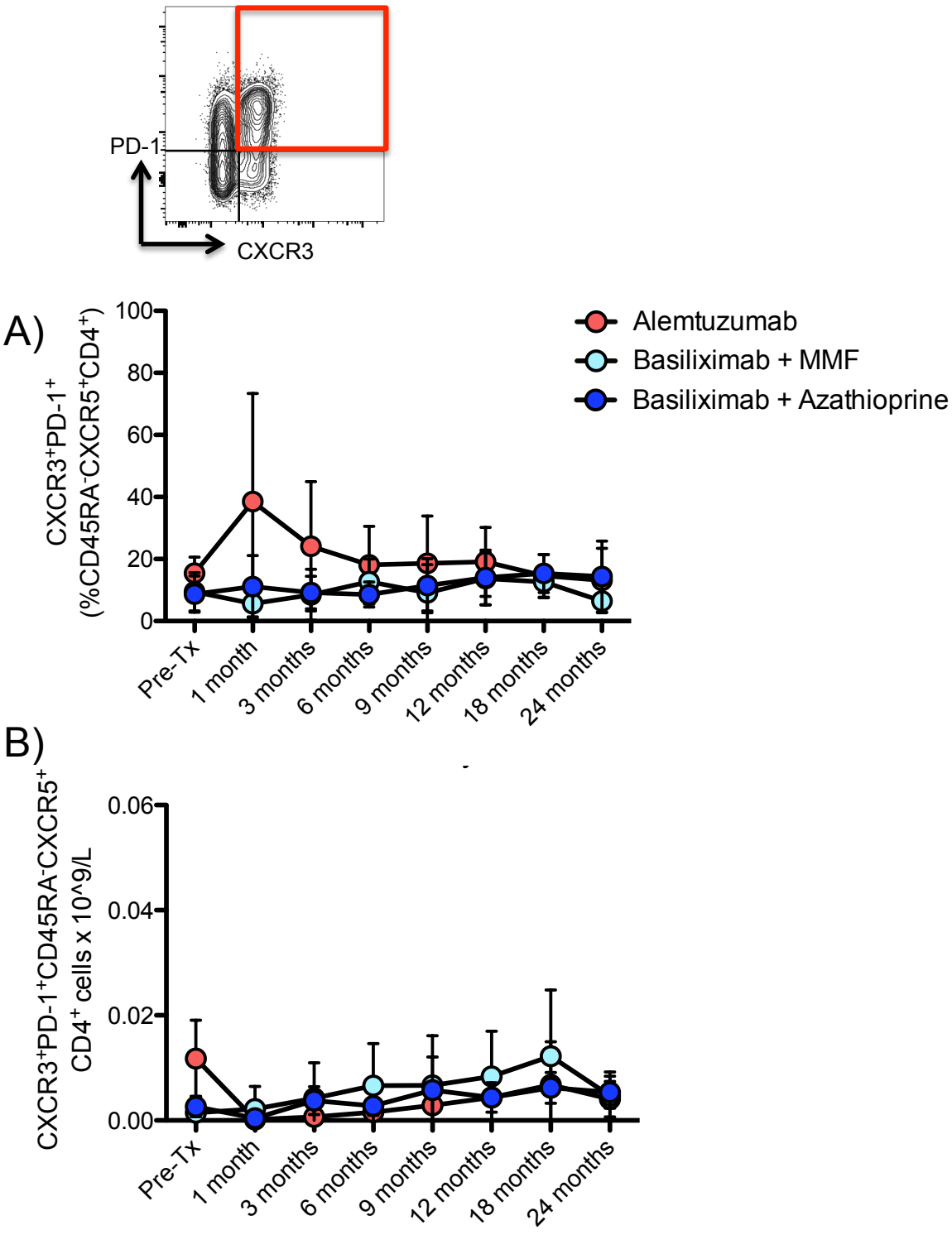


Figure 4.28: Circulating CXCR3⁺PD-1⁺ Tfh. CXCR3⁺PD-1⁺CD45RA⁻CXCR5⁺CD4⁺ Tfh as A) proportion of CD4⁺ T cells and B) absolute cell count, shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

4.4.17 Alterations in the proportions of Tfh1, Tfh2, Tfh17

Given the previously described alterations in CXCR3 and CCR6 expressing Tfh subsets in autoimmune disease, the proportions of these subsets were assessed after transplantation. Alemtuzumab treated patients showed a drop in the proportion of $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 and $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 one month post-transplant, with maintenance of the proportion of $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1. Following this, Tfh1 levels fell steadily over the two years of the study, and Tfh2 became the dominant subset (**fig 4.29A**). There was variability in the subset proportions of B+M and B+A treated patients (**figs 4.29B & 4.29C**) but all maintained a higher proportion of Tfh2 compared to Tfh1 or Tfh17.

4.4.18 Rising absolute cell count of all cTfh cell subsets post-transplant

In B+A treated groups, cTfh subsets fell post-transplant, along with all CD4 T cells, and then rose steadily across the 2 years of the study to around pre-transplant levels (**fig 4.30A**). In B+M treated patients numbers of all Tfh subsets rose post-transplant, continuing steadily to above pre-transplant levels for $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 at 2 years, with $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1 and $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 falling at 2 years to around pre-transplant levels (**fig 4.30B**). B+A treated patients showed an initial fall post-transplant and then recovery to around pre-transplant levels and stability of cell numbers across the 2 years of the study (**fig 4.30C**).

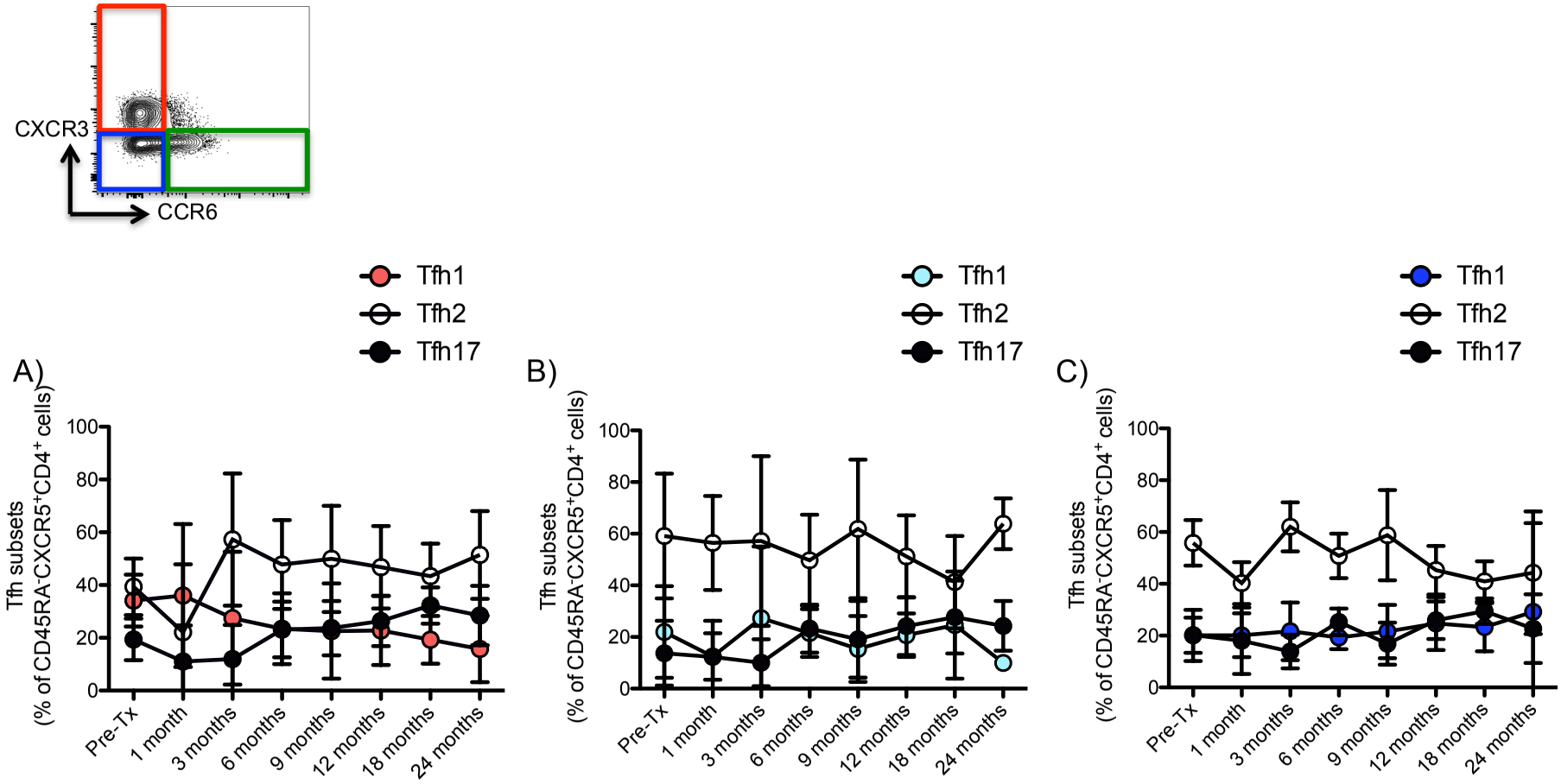


Figure 4.29: Circulating Tfh1, Tfh2, Tfh17. $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1, $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 and $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 as proportions of $CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh, showing A) alemtuzumab treated cohort, n42 B) basiliximab and MMF treated cohort (B+M, n=10), C) basiliximab and azathioprine treated cohort (B+A, n=9) . Each subset shown as mean and SD.

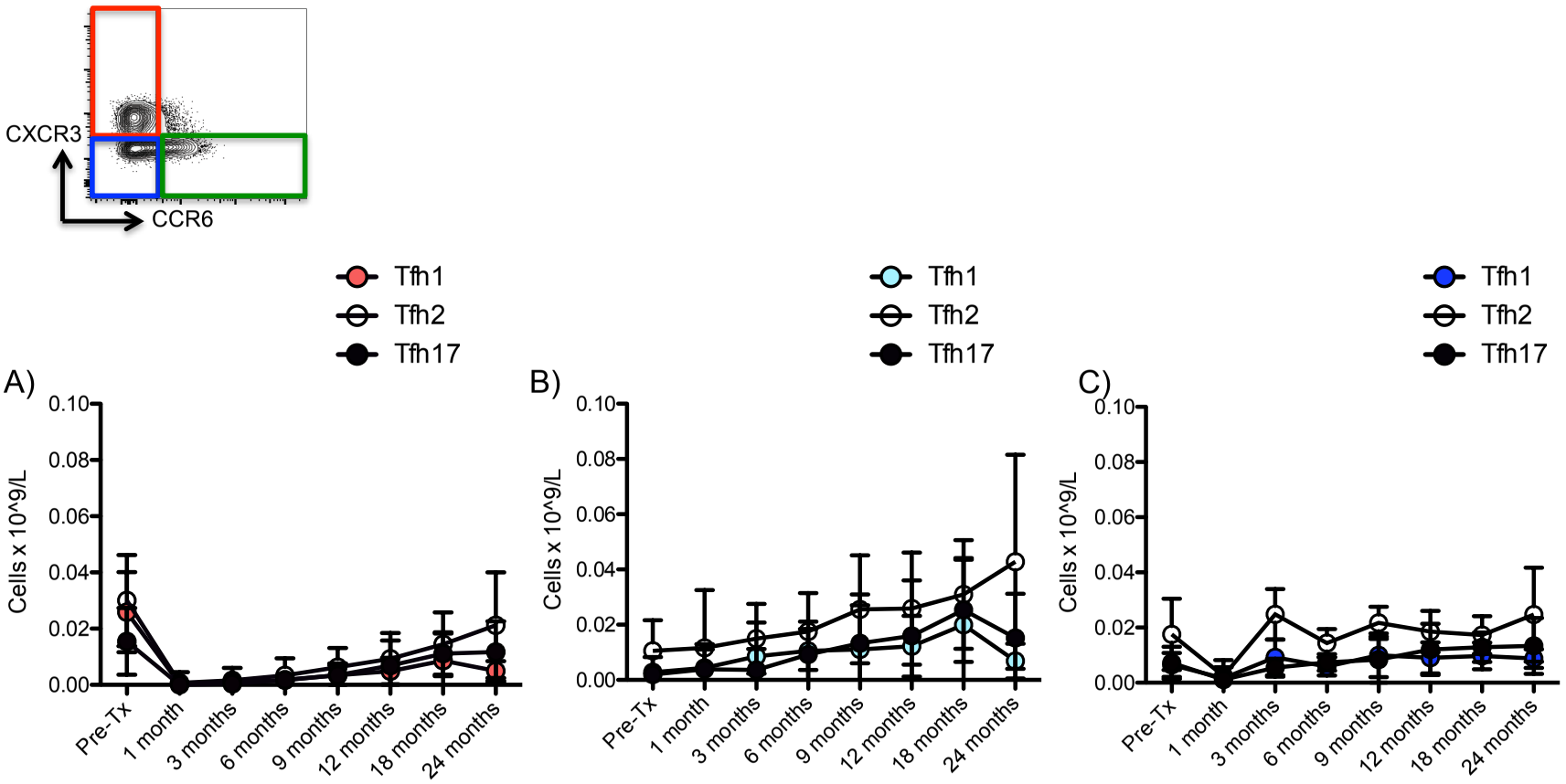


Figure 4.30: Circulating Tfh1, Tfh2, Tfh17. Absolute cell counts for $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1, $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 and $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 showing A) alemtuzumab treated cohort (n=42), B) basiliximab and MMF treated cohort (B+M, n=10), C) basiliximab and azathioprine treated cohort (B+A, n=9). Each subset shown as mean and SD.

4.4.19 CD45RA splice variants led to altered gating strategy

It is widely accepted that peripheral T cells alter expression of CD45 isoforms on activation. Naïve T cells express CD45RA, a high molecular weight isoform of CD45. On activation, this is altered and surface expression of CD45RA is replaced with expression of a low molecular weight isoform, CD45RO. This distinction divides naïve $CD45RA^+$ from memory $CD45RA^-$ cells, and is widely used to distinguish peripheral blood CD4 and CD8 T cell subsets. However, an autosomal dominant genetic variant has been recognised, where CD45RO is expressed alongside CD45RA, instead of the latter being downregulated[422-424]. This means that distinguishing naïve from memory cells in those with the genetic mutation is not possible using CD45RA expression. Three patients within the study had this mutation, a representative flow example is shown in **fig 4.31**

All previously assessed cTfh subsets in **figs 4.25-30** used CD45RA in the gating strategy, therefore a gating strategy that did not use CD45RA expression was sought. Activated $CXCR5^+PD\text{-}1^+$ Tfh were found to be altered in our pre-transplant analysis, and have been shown to be associated with antibody formation in autoimmune disease[134,140,406].

4.4.20 $CXCR5^+PD\text{-}1^+$ cTfh cells increased post-transplant

$CXCR5^+PD\text{-}1^+CD4^+$ cTfh cells were gated as shown in **fig 4.32A**. Both proportions and numbers fell post-transplant in all treatment groups (**fig 4.32B**) and rose in all treatment groups to above pre-levels. There were significant differences between the three groups on one-way ANOVA at 9 months ($p=0.045$), 18 months ($p=0.032$) and 24 months ($p=0.007$) with alemtuzumab

treated patients having higher proportions of $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$cTfh compared to B+M, which were higher than B+A.

These differences were not seen with absolute cell count, where there were significant differences on one-way ANOVA at early time points only. Pre-transplant results should be interpreted with caution, as this difference may reflect the fact that B+M and B+A treated patients had received the basiliximab infusion (anti-CD25) immediately prior to sampling, which may have affected circulating T cell levels, however alemtuzumab treated patients would not have received any induction therapy at the time of sampling. At one month ($p=0.005$), 3 months ($p<0.0001$), 6 months ($p=0.003$) and 9 months ($p=0.024$) there were differences between the treatment groups, which disappeared when alemtuzumab CD4 T cell counts began to recover at 12 months (see **fig 4.21**) and it is likely that these differences reflect the CD4 lymphopenia seen early after transplantation in alemtuzumab treated patients.

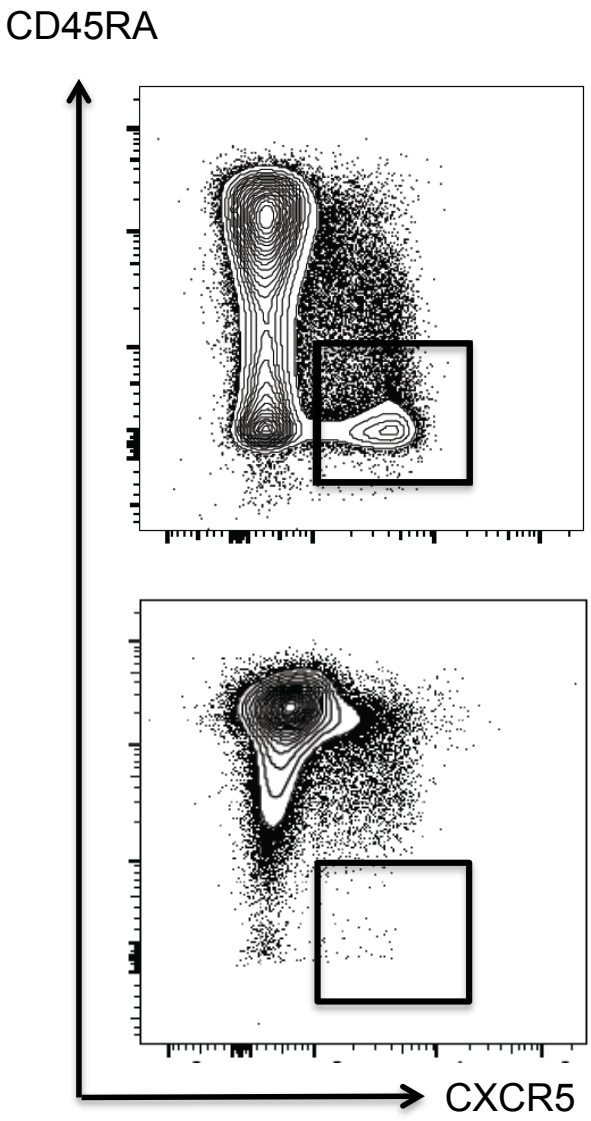


Figure 4.31 CD45RA gating. Example flow plots showing normal CD45RA downregulation (top panel) and splice variant with retention of CD45RA on the cell surface (bottom panel).

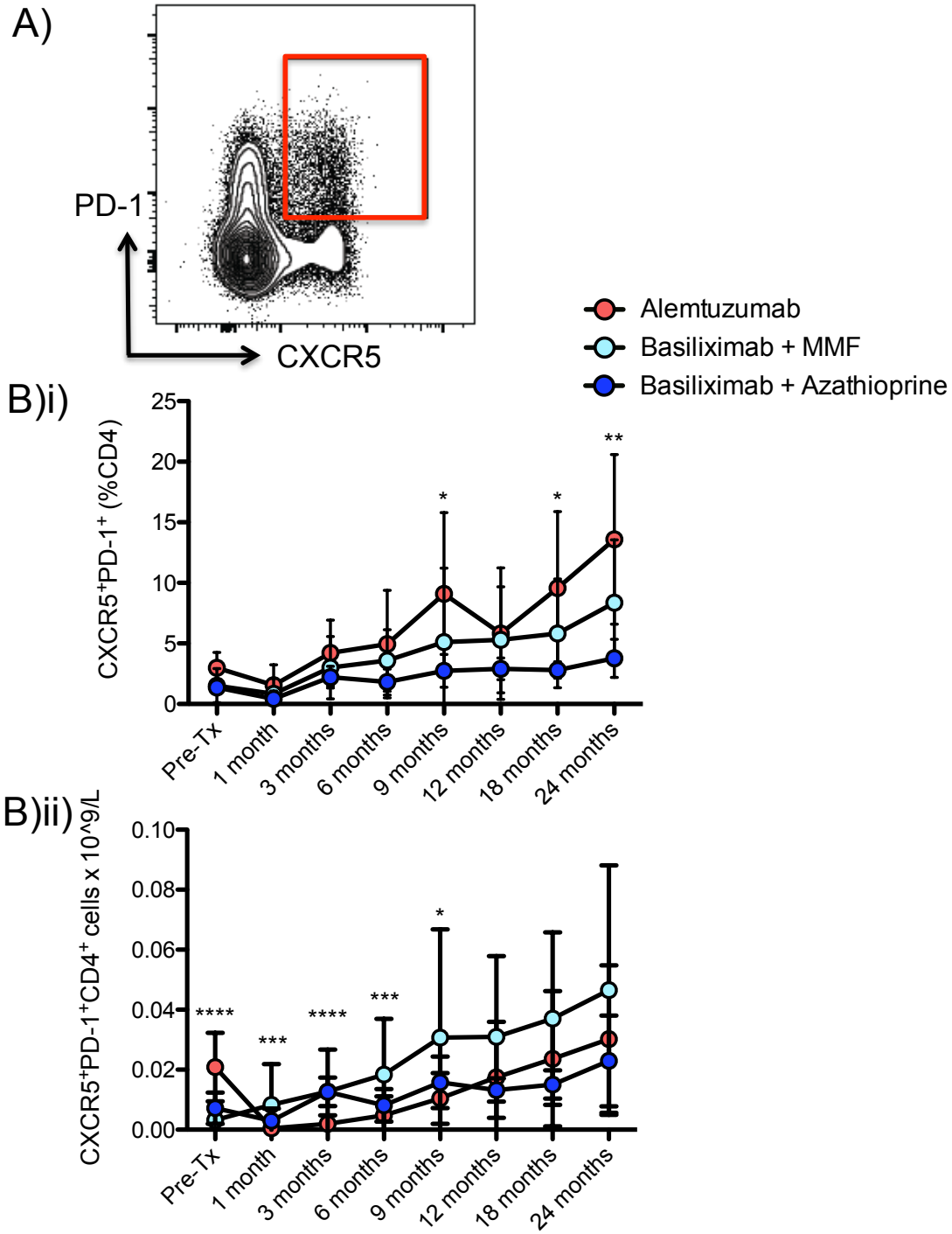


Figure 4.32: Circulating CXCR5⁺PD-1⁺ Tfh. A) Gating for CXCR5⁺PD-1⁺CD4⁺ Tfh, B) CXCR5⁺PD-1⁺CD4⁺ cells shown as i) proportion of CD4⁺ T cells with significant differences at 9 months (p=0.045), 18 months (p=0.032) and 24 months (p=0.007) on 1 way ANOVA testing and ii) absolute cell count, showing significant differences pre transplant (p<0.0001), at 1 month (p=0.005), 3 months (p<0.0001), 6 months (p=0.003) and 9 months (p=0.024). Shown as mean and SD for each treatment group. Alemtuzumab n=42, B+M n=10, B+A n=9.

Summary – Aim 2:

With this phenotyping data there were several key findings:

- Azathioprine maintenance therapy had a greater impact on B cells than MMF, regardless of which induction therapy was used in MMF treated patients (**figs 4.13-4.20**). However, switched memory B cells seemed relatively resistant to depletion by azathioprine (**fig 4.17**). The B cell changes found in this cohort were in keeping with previous data[412,413].
- Alemtuzumab induction had the greatest impact on CD4 T cells than either maintenance therapy, keeping both proportions and absolute counts of CD4 T cells low across the 2 years of the study (**fig 4.21**). This is in keeping with all previous reports. However, of note, alemtuzumab had a greater impact on Tfr than Tregs, keeping them at low levels despite recovery of Tregs (**figs 4.22-4.24**) [417,419-421]. This is a novel finding which may go some way towards explaining the high rates of autoantibody formation in alemtuzumab treated patients[420,421].
- Activated $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh and $CXCR3^{-}CXCR5^{+}CD45RA^{-}CD4^{+}$ cTfh were lower in azathioprine treated patients compared to other treatment groups (**figs 4.27, 4.32**) and rose at the later time points post-transplantation for all treatment groups. These two subsets have previously been associated with antibody formation in various disease states[127-129,134-138,140,146,405,406], which is of interest in the risk of *de novo* DSA formation.

With these findings, we were interested in whether any of the changes seen between drug treatment groups were also associated with antibody formation, in particular the alterations in cTfr and cTfh cell subsets. Therefore, to address **aim 3**, we planned to correlate the potential biomarkers identified in **aim 1** and **2** with the development of *de novo* DSAs. Given the alterations seen in different subsets with different treatment regimens, we also wondered if there was an altered risk of antibody formation with different treatment regimens.

4.4.21 Different drug regimens are associated with altered HLA antibody risk

The proportion of patients in each treatment group developing *de novo* HLA antibodies (**fig 4.33A**) was highest in the basiliximab induction with B+M maintenance group. The same held true for *de novo* DSA formation (**fig 4.33B**), however because the B+M and B+A treated groups were small, this did not reach statistical significance on chi-squared testing.

4.4.22 Low levels of cTfr cells are associated with de novo DSA formation

$CXCR5^{+}IL\text{-}7R^{lo}FOXP3^{+}CD4^{+}$ Tfr were lower in alemtuzumab treated patients compared to B+M or B+A treated patients. There was no difference in mean cTfr cells in patients developing *de novo* HLA antibodies compared to those who did not (**fig 4.34A**), however, patients developing *de novo* DSA had lower mean cTfr cells at all times until 2 years, when levels became comparable to those without *de novo* DSA (**fig 4.34B**). These differences did not reach statistical significance, which may be due to the small number of patients developing DSA (n=6).

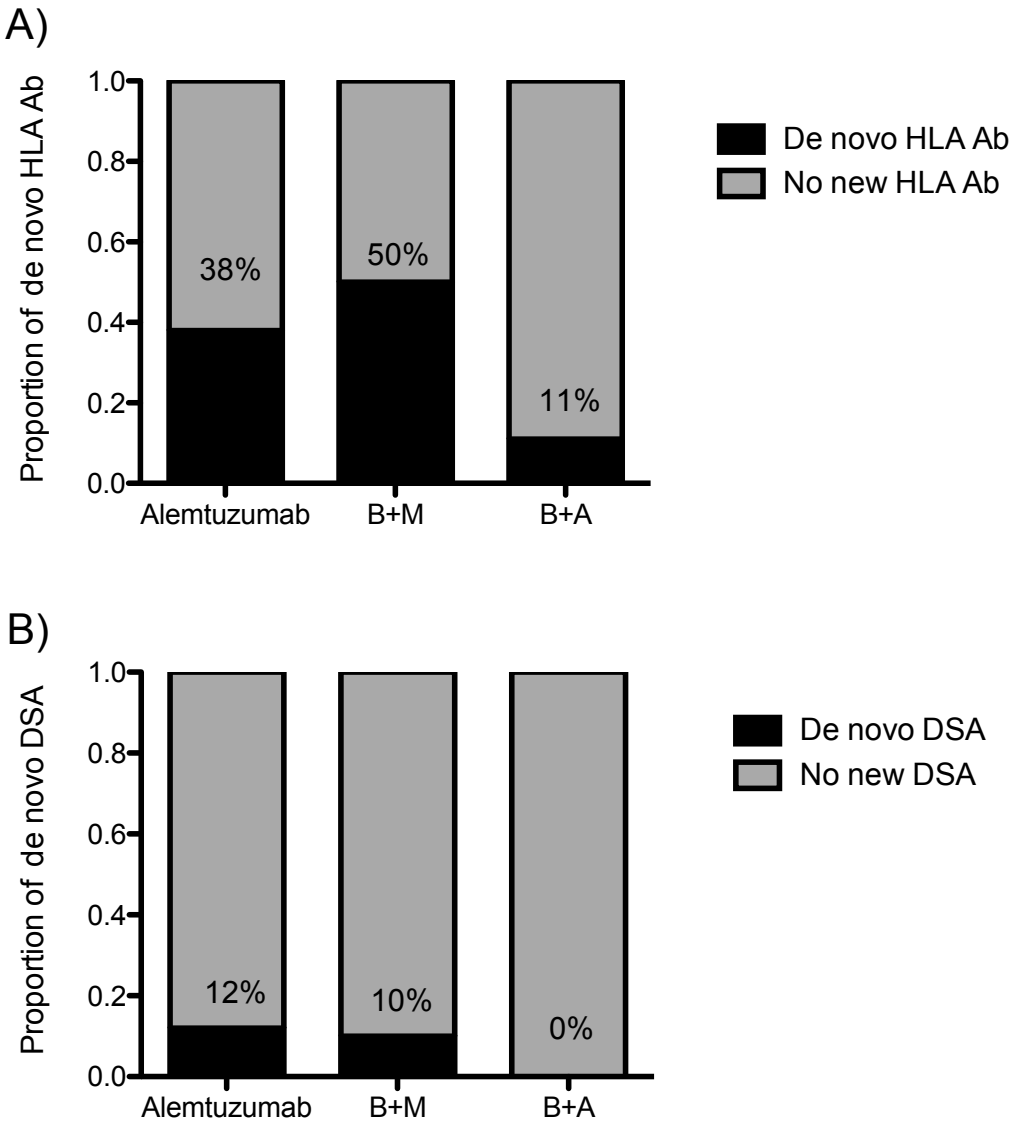


Figure 4.33: Impact of drug regimen on antibody risk. A) Proportion of patients developing de novo HLA antibodies by treatment group – alemtuzumab treated, B+M = basiliximab + MMF, B+A = basiliximab + azathioprine treatment. B) Proportion of patients developing de novo DSA by treatment group. There were no significant differences on Chi-square testing.

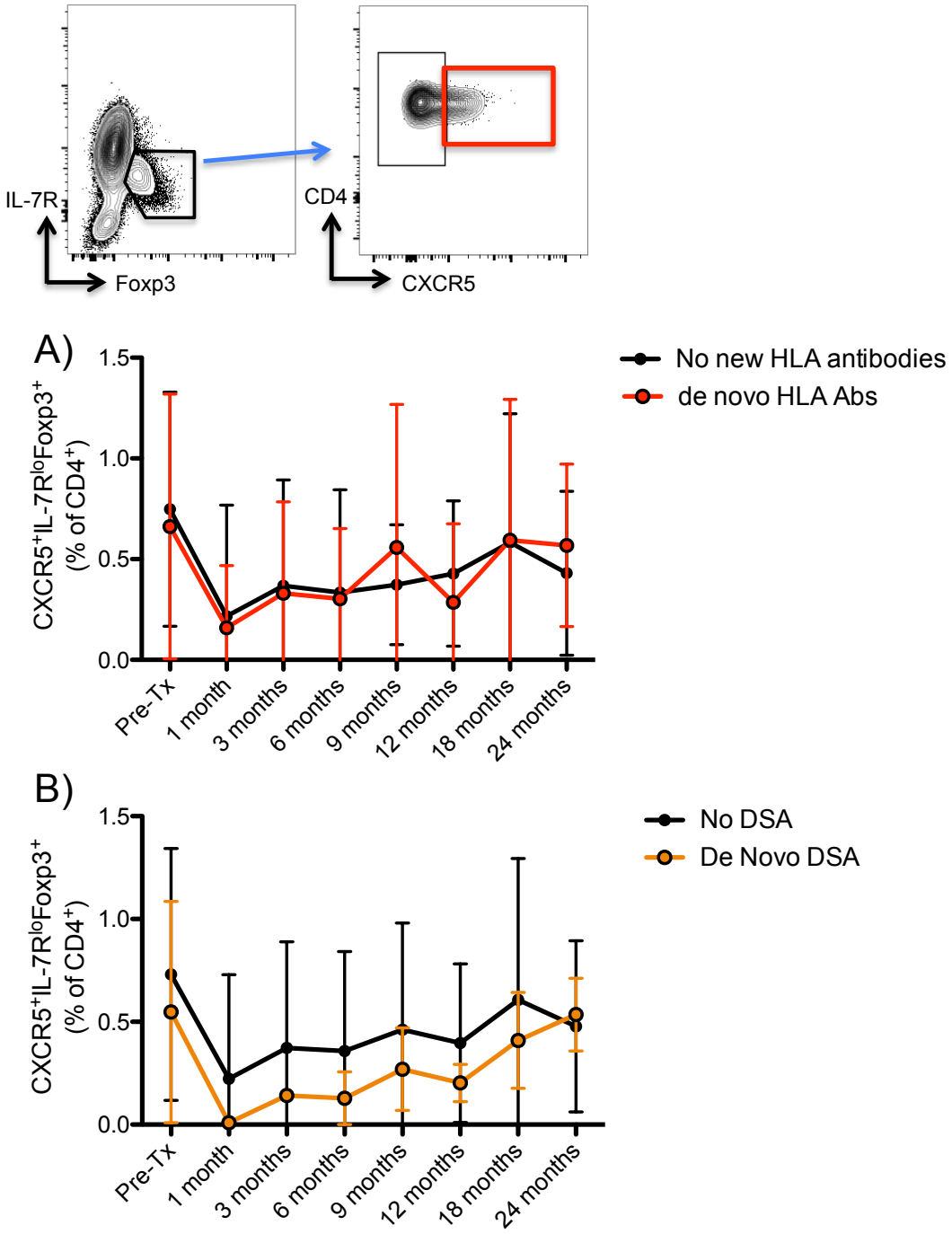


Figure 4.34 Circulating Tfr. A) CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr in patients developing de novo HLA antibodies compared to those with no new antibodies. B) CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr in patients developing de novo DSA compare to those with non-specific or no new antibodies. Shown as mean and SD. There were no statistically significant differences on Mann Whitney testing at any time point.

4.4.23 $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells were higher in patients with de novo DSA

$CXCR3^{-}PD\text{-}1^{+}$ $CD45RA^{-}$*CXCR5*$^{+}CD4^{+}$ cTfh cells rose post-transplant in all treatment groups. There was no difference in mean $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh in patients developing de novo HLA antibodies compared to those who did not (**fig 4.35A**) however, patients developing de novo DSA had higher mean $CXCR3^{-}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells at 1 month (p=0.026) and at 12 months (p=0.032) compared to those without *de novo* DSA (**fig 4.35B**). The differences did not reach statistical significance at other time points, and again this may be related to the small number of patients developing *de novo* DSA.

4.4.24 $CXCR3^{+}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh did not alter with DSAs

Despite their presence in vaccination studies suggesting active antibody formation, $CXCR3^{+}PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh cells were not significantly different with either *de novo* HLA antibody formation (**fig 4.36A**) or *de novo* DSA formation (**fig 4.36B**).

4.4.25 $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh1 cells fell early post-transplant in DSA patients

$CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ cTfh1 cells were low in all patients post-transplant, and mean levels were no different in patients who developed *de novo* HLA antibodies compared to those who did not (**fig 4.37A**). There was a reduction in mean $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1 one month post-transplant in patients who went on to develop *de novo* DSA

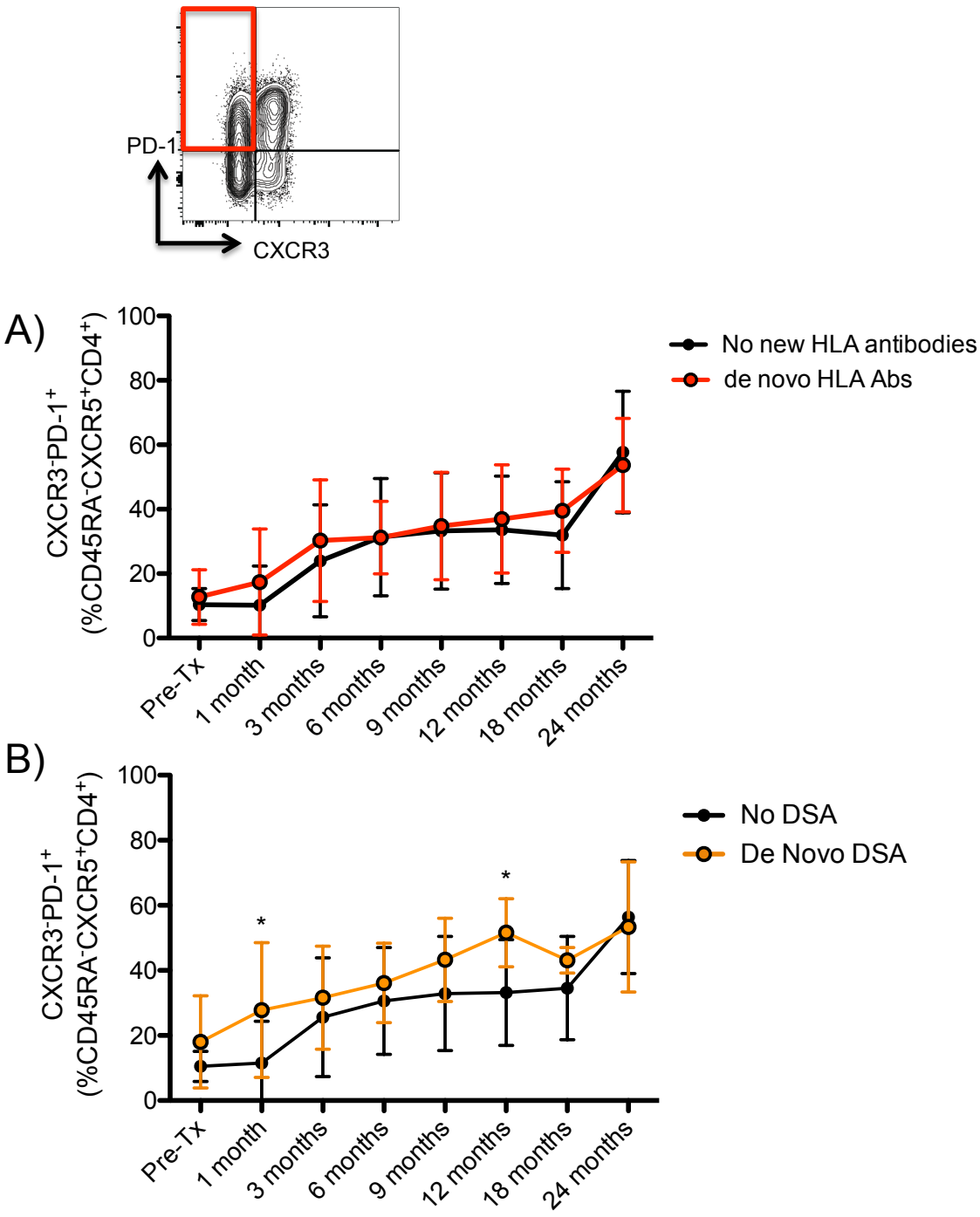


Figure 4.35: Circulating CXCR3$^-$PD-1$^+$ Tfh. A) CXCR3$^-$PD-1$^+$CD45RA$^-$CXCR5$^+$CD4$^+$ Tfh in patients developing de novo HLA antibodies compared to those with no new antibodies. B) CXCR3$^-$PD-1$^+$CD45RA$^-$CXCR5$^+$CD4$^+$ Tfh in patients developing de novo DSA compare to those with non-specific or no new antibodies. At 1 month (p=0.026) and 12 months (p=0.032) CXCR3$^-$PD-1$^+$CD45RA$^-$CXCR5$^+$CD4$^+$ Tfh were significantly higher in patients developing de novo DSA compared to those who did not. No other time points showed statistically significant differences on Mann Whitney testing. Shown as mean and SD.

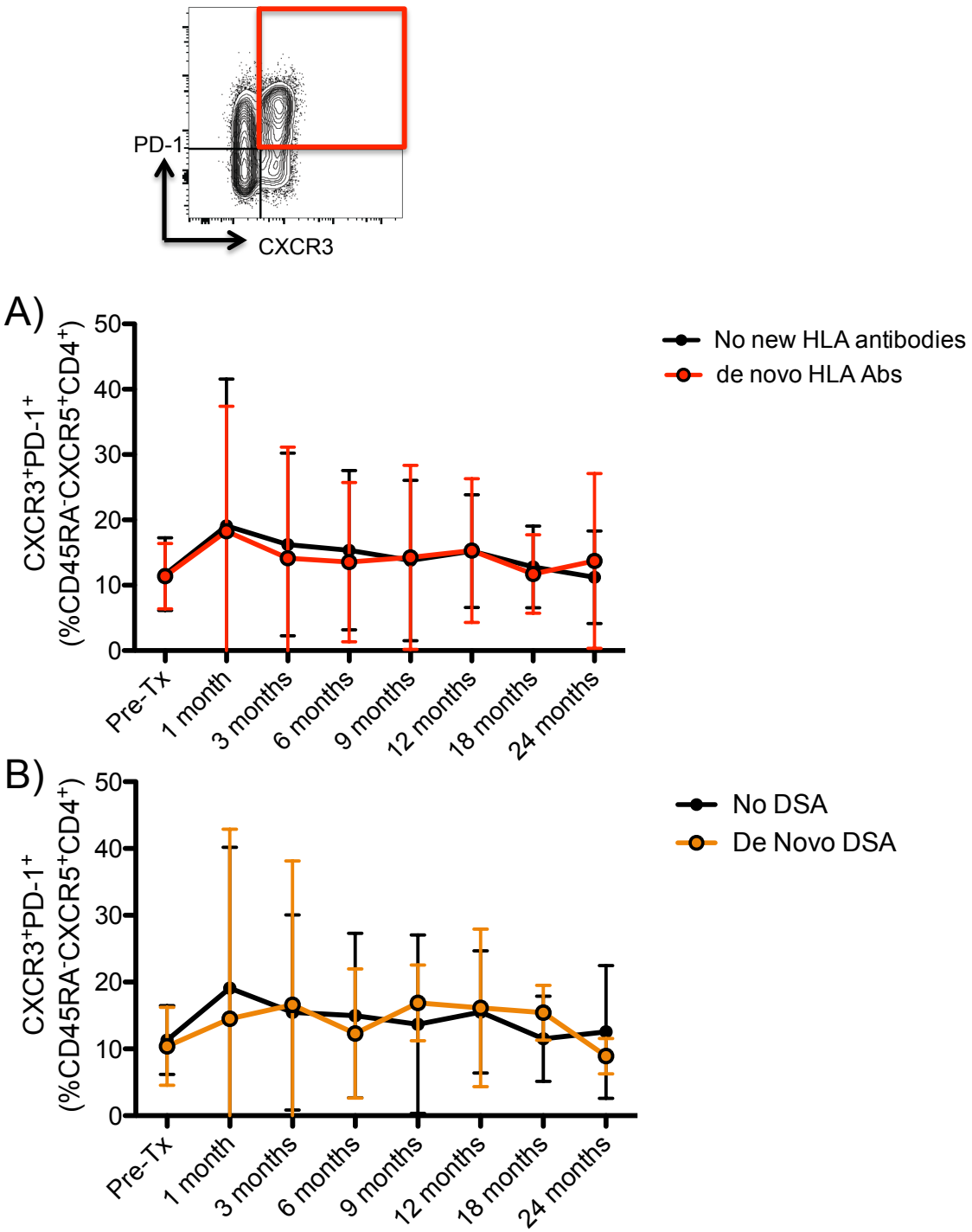


Figure 4.36: Circulating CXCR3⁺PD-1⁺ Tfh. A) CXCR3⁺PD-1⁺CD45RA⁻CXCR5⁺CD4⁺ Tfh in patients developing de novo HLA antibodies compared to those with no new antibodies. B) CXCR3⁺PD-1⁺CD45RA⁻CXCR5⁺CD4⁺ Tfh in patients developing de novo DSA compare to those with non-specific or no new antibodies. Shown as mean and SD. There were no statistically significant differences on Mann Whitney testing at any time point.

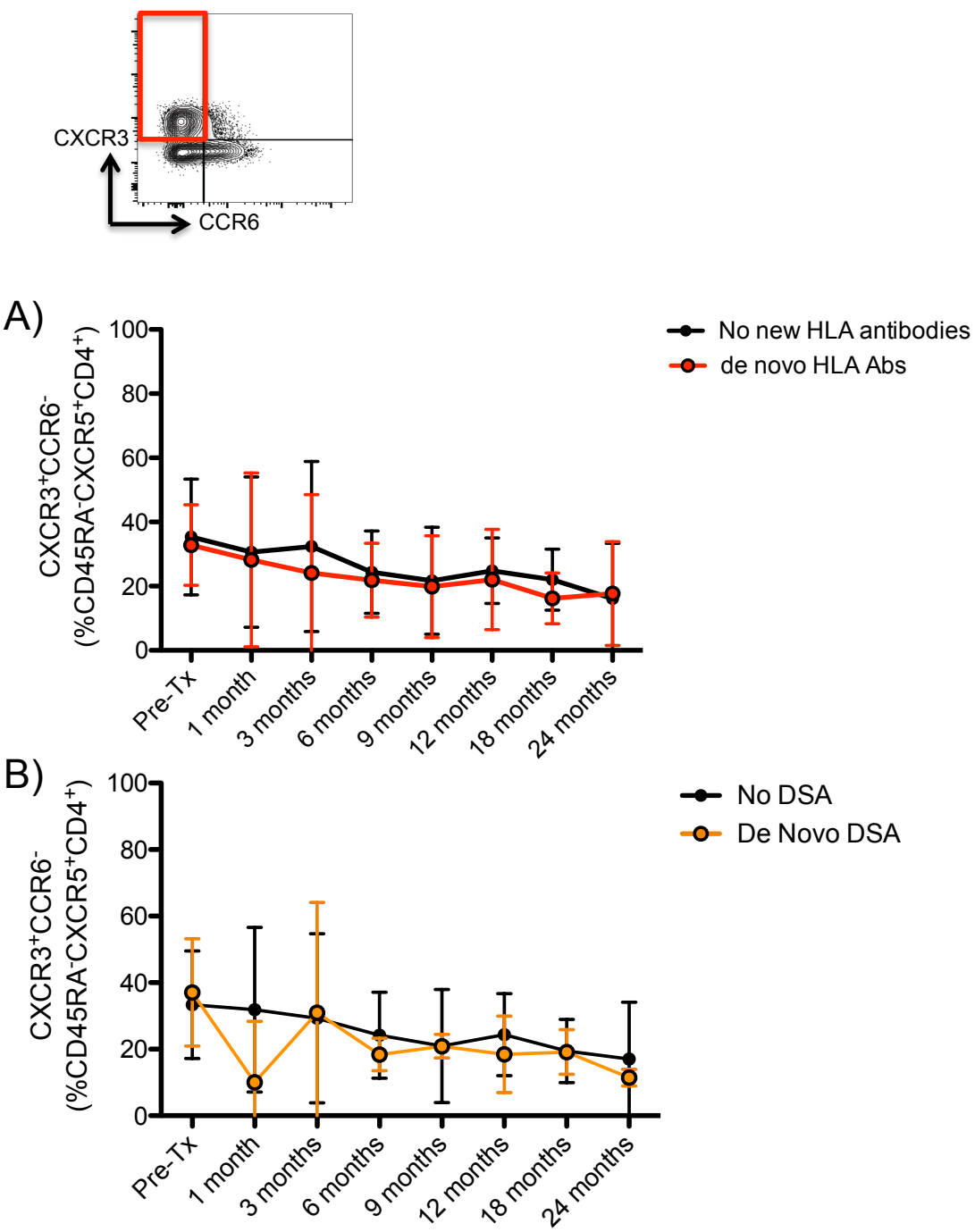


Figure 4.37: Tfh1 association with antibody formation. A) $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1 in patients developing de novo HLA antibodies compared to those with no new antibodies. B) $CXCR3^{+}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh1 in patients developing de novo DSA compare to those with non-specific or no new antibodies. Shown as mean and SD. There were no statistically significant differences on Mann Whitney testing at any time point.

compared to those who did not (**fig 4.37B**), but this did not reach statistical significance (p=0.093). This has been seen in autoimmune disease and it has been postulated that Tfh1-like cells are lower in the blood as they migrate to inflamed tissue, where they may set up local antibody production[425-428]. It is possible that a similar mechanism may account for this difference in transplant patients, however no biopsies were performed at this time in patients who developed DSA, therefore it is not possible to correlate tissue cell numbers with circulating cell levels.

4.4.26 $CXCR3^-CCR6^-CD45RA^-CXCR5^+CD4^+$ cTfh2 cells did not alter with antibody formation

$CXCR3^-CCR6^-CD45RA^-CXCR5^+CD4^+$ cTfh2 cells increased in alemtuzumab and B+M treated patients post-transplant, both groups have an increased risk of both general HLA antibody and DSA formation, however the mean proportion of Tfh2 was not significantly different with either *de novo* HLA antibody formation (**fig 4.38A**) or *de novo* DSA formation (**fig 4.38B**).

4.4.27 $CXCR3^-CCR6^+CD45RA^-CXCR5^+CD4^+$ cTfh17 cells were higher in patients developing de novo DSA

$CXCR3^-CCR6^+CD45RA^-CXCR5^+CD4^+$ Tfh17 have been associated with antibody formation in autoimmune diseases. There was no difference in the mean proportion of $CXCR3^-CCR6^+CD45RA^-CXCR5^+CD4^+$ Tfh17 in patients who developed *de novo* HLA antibodies compared to those who did not (**fig 4.39A**). However, mean $CXCR3^-CCR6^+CD45RA^-CXCR5^+CD4^+$ Tfh17 dropped at 3 months in patients developing *de novo* DSA, and then increased above non-DSA

patient levels from 6 months post-transplant. These differences did not reach statistical significance and the standard deviation was high in all groups analysed.

4.4.28 $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh cells were not associated with HLA or DSA formation

$CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ cTfh cells have been associated with antibody formation in autoimmune diseases, but were not associated with HLA antibody formation in this transplant cohort (**fig 4.40A**). There was a trend towards higher $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ Tfh in patients developing *de novo* DSA but there was considerable variation in cell numbers (**fig 4.40B**).

4.4.29 The ratio of $CXCR5^{+}IL\text{-}7R^{lo}FOXP3^{+}CD4^{+}$ Tfr to $CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh did not predict antibody formation

Animal models of autoimmune disease have suggested that, rather than absolute numbers, the ratio of Tfr to Tfh is more important, with a higher proportion of Tfh to Tfr being more likely to lead to antibody formation[429-431]. I assessed the ratio of $CXCR5^{+}IL\text{-}7R^{lo}FOXP3^{+}CD4^{+}$ firstly to $CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh and found no differences in patients developing *de novo* HLA antibodies to those who did not (**fig 4.41A**). There was considerable variation pre-transplant, but looking in detail at post-transplant time points (**fig 4.41B**) there were no significant differences between those developing *de novo* HLA antibodies and those who did not.

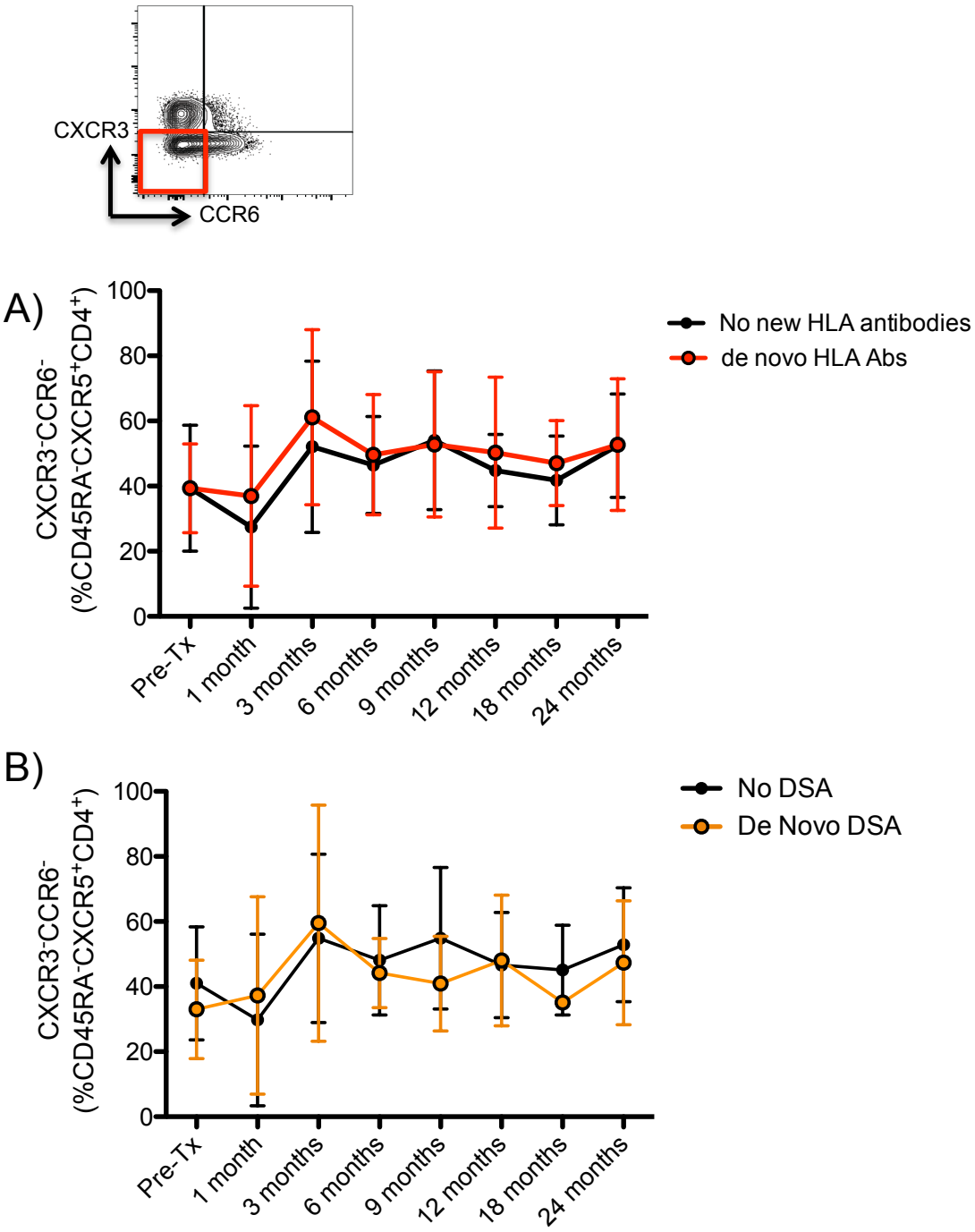


Figure 4.38: Tfh2 association with antibody formation. A) $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 in patients developing de novo HLA antibodies compared to those with no new antibodies. B) $CXCR3^{-}CCR6^{-}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh2 in patients developing de novo DSA compare to those with non-specific or no new antibodies. Shown as mean and SD. There were no statistically significant differences on Mann Whitney testing at any time point.

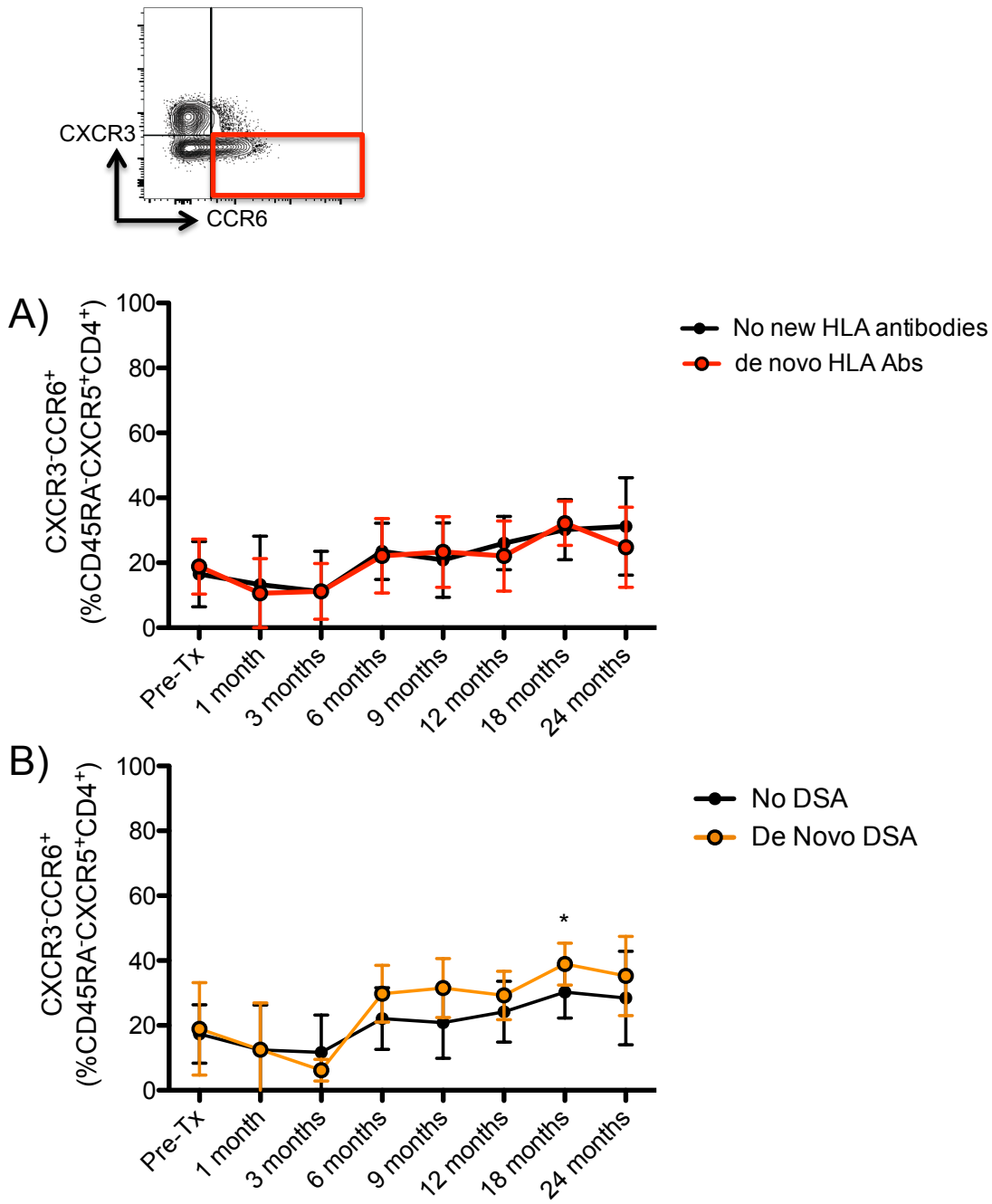


Figure 4.39: Tfh17 association with antibody formation. A) $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 in patients developing de novo HLA antibodies compared to those with no new antibodies. B) $CXCR3^{-}CCR6^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh17 in patients developing de novo DSA compare to those with non-specific or no new antibodies. From 6 months patients with de novo DSA had higher cTfh17, but this was only statistically significant at 18 months (p=0.043). There were no statistically significant differences on Mann Whitney testing at any other time point in either analysis. Shown as mean and SD.

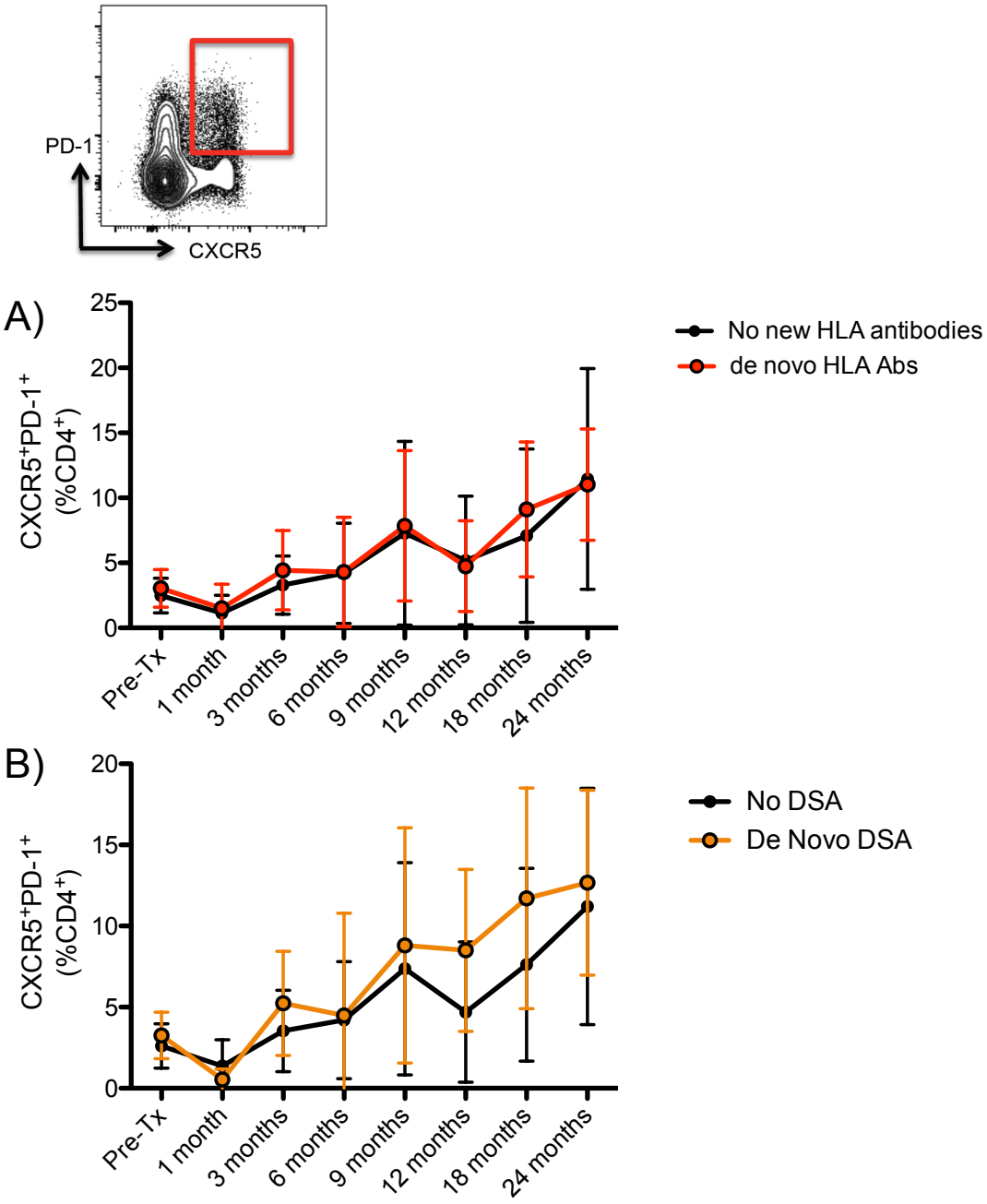


Figure 4.40: CXCR5⁺PD-1⁺ cTfh cells association with antibody formation. A) CXCR5⁺PD-1⁺ cTfh cells in patients developing de novo HLA antibodies compared to those with no new antibodies. B) CXCR5⁺PD-1⁺ cTfh cells in patients developing de novo DSA compare to those with non-specific or no new antibodies. There were no statistically significant differences on Mann Whitney testing at any time point. Shown as mean and SD.

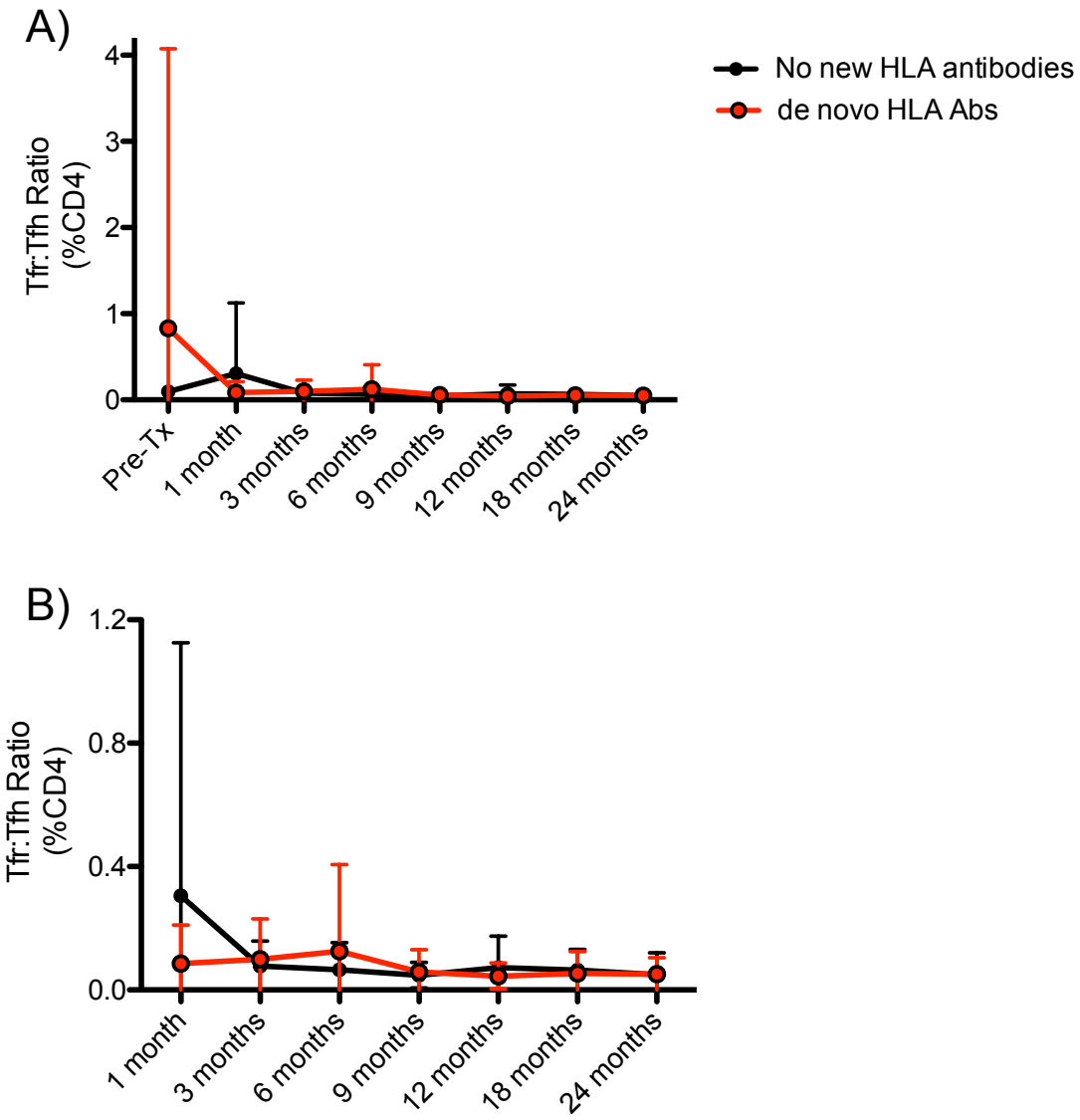


Figure 4.41: Ratio of CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr to CD45RA^{-} CXCR5^{+}CD4^{+} Tfh in patients developing de novo HLA antibodies. A) Tfr:Tfh ratio at all time points pre and post transplant in patients developing de novo HLA antibodies compared to those who did not. B) Detail of post-transplant ratio, shown as mean and SD. There were no statistically significant differences on Mann Whitney testing at any time point.

Comparing those who developed *de novo* DSA, with those who did not develop antibodies against their donor, there was again considerable variation pre-transplant (**fig 4.42A**) but a significantly higher ratio of Tfr to Tfh at 3 months in those developing *de novo* DSA (p=0.0062), seen in more detail in **fig 4.42B** of post-transplant time points. This is surprising, as studies in autoimmunity have suggested a low ratio is more associated with antibody formation. However, in transplantation, developing a response against the graft is a physiologically normal response to non-self, and Tfr cells are known to be important in preventing non-antigen specific B cell clones from receiving T cell help[117], it is therefore possible that an increased ratio of Tfr to Tfh means that those B cells with high affinity for donor antigen are more likely to receive help, rather than lower affinity clones, that might recognise shared epitopes instead of the antigen itself.

$CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh are a mixed group, containing recently activated and quiescent cells. I therefore compared the ratio of $CXCR5^{+}IL\text{-}7R^{lo}FOXP3^{+}CD4^{+}$ Tfr to $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ activated Tfh and found no significant differences between patients who developed *de novo* HLA antibodies compared to those who did not (**fig 4.43A**). However, patients developing *de novo* DSA had lower Tfr to Tfh ratios at all time points, both pre and post-transplant (**fig 4.43B**). This did not reach statistical significance, likely due to the low number of patients developing DSAs.

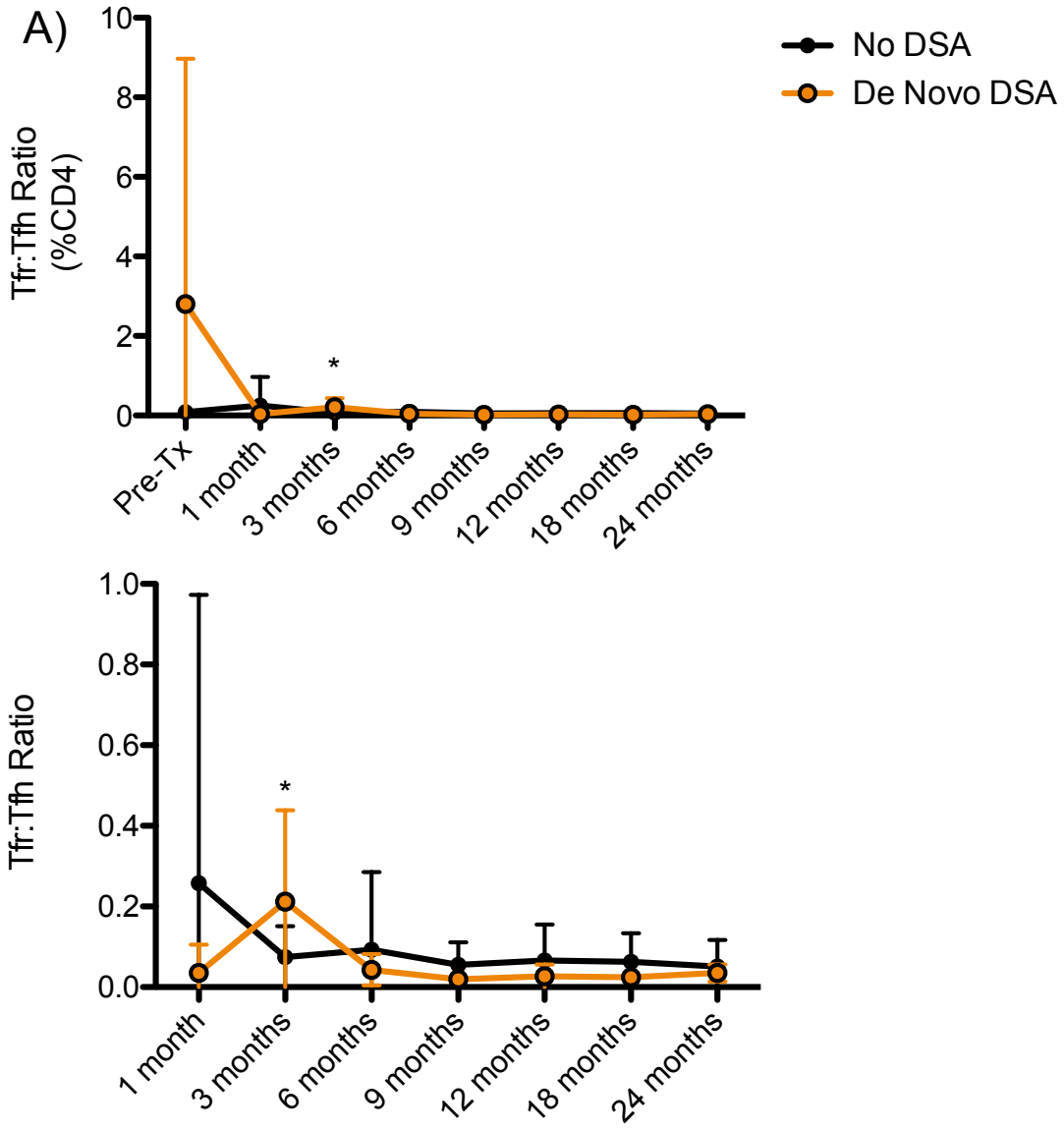


Figure 4.42: Ratio of CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr to CD45RA^{-}CXCR5^{+}CD4^{+} Tfh. A) Tfr:Tfh ratio at all time points pre and post transplant in patients developing de novo DSA compared to those who did not, shown as mean and SD. B) Detail of ratio at post-transplant time points in the same patients, showing significantly higher ratio at 3 months in patients developing DSA compared to those who did not (p=0.0062). There were no statistically significant differences on Mann Whitney testing at any other time point.

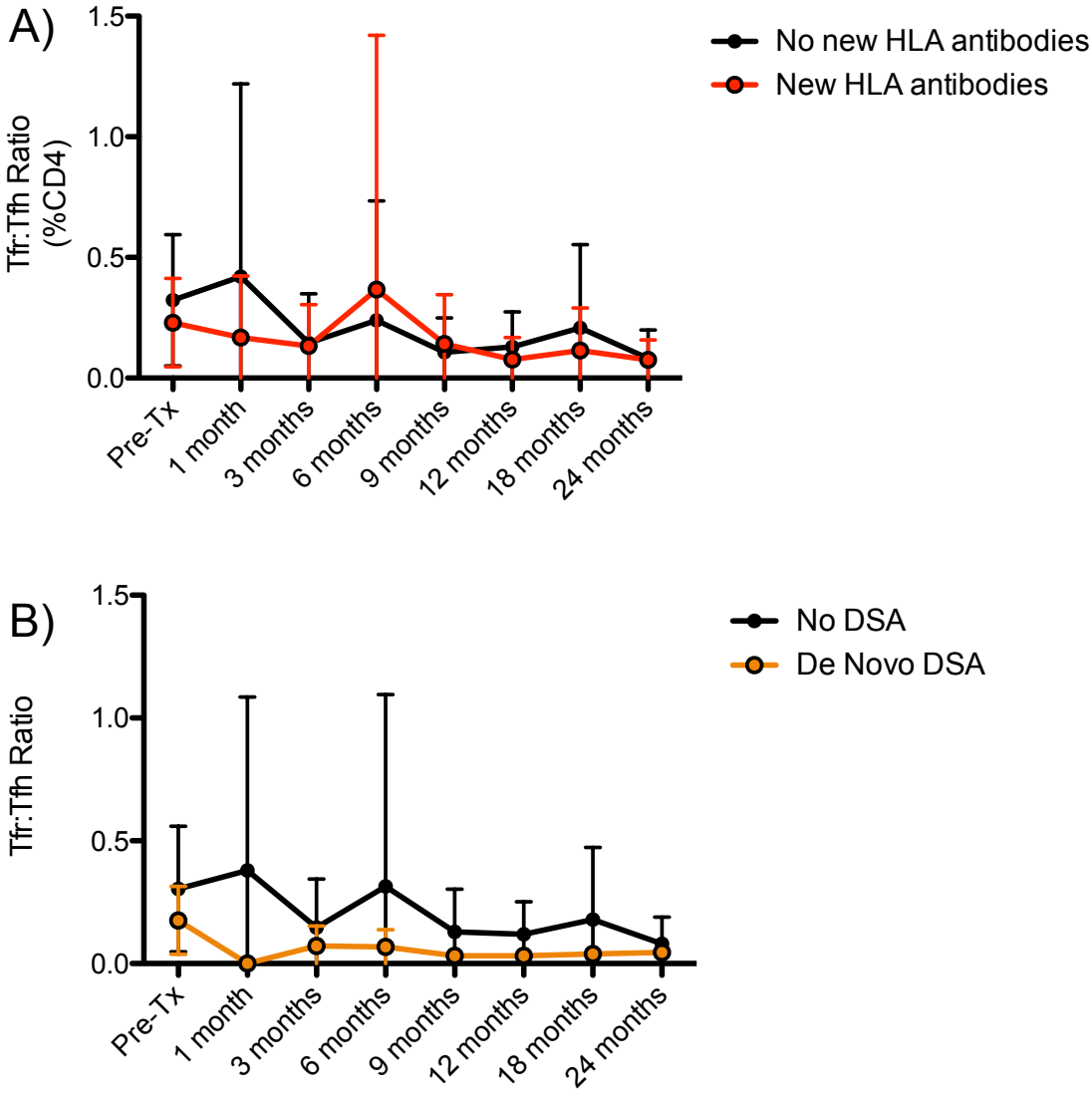


Figure 4.43: Ratio of CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr to CXCR5^{+}PD-1^{+}CD4^{+} Tfh. A) Ratio of CXCR5^{+}IL-7R^{lo}Foxp3^{+}CD4^{+} Tfr to CXCR5^{+}PD-1^{+}CD4^{+} activated Tfh pre and post transplant in patients with de novo HLA antibodies, shown as mean and SD with no significant differences at any time point on Mann Whitney testing. B) Ratio of Tfr to activated Tfh in patients developing de novo DSA compared to those who did not, shown as mean and SD. Although patients with DSAs had lower ratios at all time points, this did not reach significance on Mann Whitney testing.

Summary – aim 3

- There was a higher rate of *de novo* HLA (38% alemtuzumab, 50% B+M) antibody formation and of *de novo* DSA (12% alemtuzumab, 10% B+M) formation in both alemtuzumab treated and B+M treated patients compared to B+A patients (11% HLA, 0% DSA) – **fig 4.33**.
- The differences seen in peripheral blood with alemtuzumab and B+M treated patients (lower Tfr, higher $CXCR3^{-}PD\text{-}1^{+}$ Tfh) showed an association with *de novo* DSA formation, but this did not reach statistical significance (**figs 4.34, 4.35**).
- There was a low Tfr:Tfh ratio in patients developing *de novo* DSA at all time points, suggesting an imbalance that might promote antibody formation in these patients (**fig 4.43**).

4.5 Conclusions

There were clear alterations in all cell subsets analysed following transplantation; this is highly likely to be related to both induction and maintenance immunosuppressive drug therapy. The changes in B cell subsets observed here are consistent with previous studies[413]. Alemtuzumab treated patients showing an early spike of transitional B cells, followed by long term predominance of naïve B cells, is also consistent with previous studies[412]. Azathioprine treated patients showed suppressed B cells at all time points, and this was true for all subsets analysed. A lack of B cells might impact on LN Tfh cells, which are thought to rely on interactions with B cells to induce and maintain their

phenotype[432], but it is not clear if cTfh cells require the same interactions, as they are thought to be a memory subset of cells[128].

Alemtuzumab induction impacted on all CD4 T cell subsets, as has been well described in the literature. Interestingly, despite the high proportions of $FOXP3^+$ Tregs seen after alemtuzumab treatment in autoimmunity[417,419], we found that this was only true for $CXCR5^-$ Tregs; in contrast $CXCR5^+IL\text{-}7R^{lo}FOXP3^+$ Tfr fell in alemtuzumab treated patients. This was clearly an impact of alemtuzumab lymphodepletion, rather than maintenance therapy, as cTfr cell numbers rose in both B+M and B+A treated patients.

There was a general increase in Tfh post-transplant, and, with the exception of CXCR3 expressing cells, these rose to above pre-transplant levels in all treatment groups. In particular, cells expressing high levels of PD-1 seemed to be differentially affected, with higher levels in both alemtuzumab treated patients and those with MMF maintenance, in contrast to those on azathioprine maintenance therapy.

Alemtuzumab treatment has been associated with a number of autoimmune diseases, including Grave's thyroid disease, idiopathic thrombocytopenic purpura, autoimmune haemolytic anaemia and anti-GBM disease[414,420,433]. The common factor is the production of autoantibodies, which has been associated with excessive IL-21 in both mouse[434] and human studies[420]. We could not find reports of Tfh or cTfh levels following alemtuzumab treatment but IL-21 is produced by Tfh as well as Th17 cells, and it is conceivable that a disordered

ratio of Tfh to Tfr may contribute to the development of autoantibodies in alemtuzumab treated patients, in a similar way to that described in mouse models of autoimmunity[429-431] and human autoimmune disease[435]. With a disordered ratio of Tfh to Tfr following alemtuzumab induction in transplantation, this may also explain the increased risk of both donor-specific and non-specific HLA antibody development compared to patients receiving basiliximab induction.

Both cTfr cells and $CXCR3^{-}PD\text{-}1^{+}$ cTfh cells were associated with antibody formation, however the number of patients in these groups was too small to allow easy comparison of whether the association between drug regimen and antibody risk is consistent with the impact of drugs on cells. This led us to set up an *in vitro* model to test the impact of different immunosuppressive drugs on Tfh cells. As the effects on Tfr are likely due to the lymphodepleting action of alemtuzumab, it is not feasible to test these effects in an *in vitro* model.

5. In Vitro Assays and Impact of Immunosuppressive drugs

5.1 Introduction

The previous chapter demonstrated differences in the cellular composition of the immune system between kidney transplant and SPK transplant recipients treated with different immunosuppressive drug regimens. However, a question remained as to the impact of individual immunosuppressive drugs on antibody production. There has been considerable debate in the transplant literature as to which regimen is best able to prevent DSA formation, and therefore which regimen is most suitable for patients considered to be of high immunological risk. Irrespective of regimen, it is clear that under-immunosuppression, either from patient non-adherence[201,204,436,437] or planned weaning of drugs in conjunction with the medical team[438,439], is associated with *de novo* DSA formation. Adequate levels of CNIs are particularly important[440], especially after graft failure, where withdrawal of immunosuppression is strongly associated with DSA formation[207,209]. However, there remains a debate about the impact of immunosuppressive agent choice on long term DSA risk. Therefore I aimed to elucidate the effect of different immunosuppressive drugs on the cells that co-operate to produce antibody.

5.2 Immunosuppression in transplantation

Early transplants were performed without any immunosuppressive agents, and universally failed until the first successful transplant between monozygotic twins performed in 1954 by Murray, Merrill and Harrison[290,441]. Further transplants between twins were successful while those between less closely related individuals, even with extreme interventions such as total body irradiation[291], failed within the first few days or months. Roy Calne introduced the first immunosuppressive agents into transplantation following the discovery of 6-mercaptopurine (6-MP) and his experiments demonstrating its benefit in animal models of transplantation, albeit with significant side effects[321]. The discovery of azathioprine, an inactive pro-drug that is broken down to 6-MP following absorption, with fewer side effects as a consequence, allowed translation of his work into human transplantation[292] in the early 1960s. When this was combined with corticosteroids, successful transplants became possible outside of monozygotic twins[293]. Later in the 1960s, the development of anti-lymphocyte globulin (ALG) and its use for induction and treatment of rejection improved outcomes further, although 1 year graft survival was still only around 50%[442].

The major breakthrough came with the discovery of the CNI ciclosporin (cyclosporine) in 1976[294], and demonstration of effects in animal transplantation and, in the early 1980s, in human transplantation. While initially used alone[443], when combined with azathioprine and steroids, this triple therapy improved 1 year graft survival to above 80% and 5 year graft survival to over 50%[295,296]. Later in the 1980s another CNI - tacrolimus (FK506) - was discovered to be more

potent[297] and less nephrotoxic than ciclosporin[298]. In the 1990s MMF came to prominence as a more potent alternative to azathioprine[299]. Combining this with the advent of induction therapy with anti-CD25 monoclonal antibodies such as basiliximab[7] meant immunosuppression options for transplantation were becoming more sophisticated and multifaceted, targeting several aspects of the immune system[300,301].

During the 1990s and early 2000s, induction therapy with either a polyclonal antibody such as rabbit anti-thymocyte globulin (ATG) or a monoclonal antibody such as basiliximab, followed by triple therapy with a CNI, an antimetabolite and steroids, was felt to be the optimal therapy for all forms of solid organ transplant, and results improved year on year, particularly with regard to acute rejection episodes.

More recently alemtuzumab, an anti-CD52 lymphocyte depleting monoclonal antibody has been used in kidney[306-308], pancreas[309] and other solid organ transplants[310,311]. The function of CD52 is not clearly understood, but administration of alemtuzumab causes rapid and profound lymphocyte depletion with slow recovery first of B cells, then CD8 T cells, then CD4 T cells, the latter of which can remain suppressed for years after the initial dose[312,313]. Created by Herman Waldmann and colleagues for use in haematological malignancies[444,445] and then found to be of use in controlling autoimmune disease[433], there was much interest in the potential use of alemtuzumab in transplantation because of the reports that the recovering CD4 population contained a high proportion of $CD25^{hi}IL\text{-}7R^{lo}$ cells, markers associated with Tregs[417].

The role of Tregs in transplantation as both a marker of transplant tolerance[446] and as a potential therapy in transplantation had been extensively explored in animal models[447,448]. This work has recently translated into trials of *ex-vivo* generated Tregs as a cellular therapy for transplant recipients in an attempt to reduce immunosuppression requirements longer term[449]. This remains an experimental therapy at present, and prior to its advent the potential use of alemtuzumab as an alternative to both suppress acute rejection by depleting the key players, and potentially prevent longer-term rejection by increasing Treg numbers and hence induce tolerance, was an attractive prospect. There was a concern over the development of antibodies with alemtuzumab, as work in autoimmunity had shown a high risk of antibody-mediated autoimmune disease developing after treatment, with up to a third of patients developing autoimmune thyroid disease[433]. Doses were much higher in patients with autoimmunity, and they had a loss of self-tolerance that allowed the original disease to develop, therefore the risk was felt to be low in transplantation and this has been borne out with much lower rates of autoimmune disease in alemtuzumab treated transplant patients compared to patients treated for autoimmune disease[368,450].

5.3 Impact of immunosuppressive drugs on DSA risk

While induction therapy with alemtuzumab showed improved outcomes in terms of acute rejection compared to basiliximab[307,308,368], there was a trend towards an increased risk of HLA antibody development, and particularly DSA, in the longer term[309,451,452]. The largest study to date, the 3C study, compared basiliximab induction to alemtuzumab induction with reduction in maintenance

immunosuppression[367]. The study showed a significantly reduced rate of TCMR but no significant reduction in, and a trend towards a higher rate, of ABMR in the alemtuzumab treated cohort, although this did not reach significance[315]. The 3C study did not screen for HLA antibodies post-transplant, and it remains to be seen if this large study will show altered outcomes in the longer term.

The impact of CNIs in reducing DSA formation is clear from studies into non-adherence and withdrawal of immunosuppression after graft failure[207,209]. However the risks of DSA in azathioprine treated and MMF treated patients are not clear, with some studies suggesting an increased risk with azathioprine[439,453] and others with MMF[372,454].

5.4 Impact of immunosuppressive drugs on cells

While many studies have looked at the phenotype of circulating cells in patients, as discussed in chapter 4, the effects on isolated cells have been less well studied. Having noted the impact of different drug treatment regimens in transplant recipients, and particularly the increased association with both HLA antibody formation and specifically DSA formation with alemtuzumab and regimens using MMF maintenance rather than azathioprine, the impact of different drugs on antibody formation was highlighted as an important, as yet unanswered question.

In patients it is clearly not feasible to investigate the impact of individual drugs, as all patients are treated with at least two immunosuppressive drugs and will have had different induction regimens (see chapter 3, **table 3.1**). Given the

lymphocyte depletion associated with alemtuzumab, it is not possible to compare *in vitro* effects of basiliximab and alemtuzumab on antibody formation. It is also possible that the association of alemtuzumab with antibody formation is due to abnormalities in the repopulating cells[411,420,421,455], which may be influenced by maintenance immunosuppression. Therefore the choice of maintenance therapy is likely to be a significant factor in long term DSA risk.

5.5 Aims

To use an *in vitro* co-culture system to investigate the effects of tacrolimus, azathioprine and MMF on Tfh cells, B cells and antibody production.

I hypothesised that, given the results seen in transplant recipients; azathioprine would have a greater impact on antibody production than MMF.

5.6 Results and discussion

Many groups have shown that isolated Tfh cells can be co-cultured with B cells and are able to provide help for antibody production[127,132]. Tfh cells and B cells are flow-sorted from pre-enriched populations and co-cultured for 11 days with staphylococcal enterotoxin B (SEB) as a stimulant (please see detailed methods, chapter 2 sections 2.13-2.15). After 11 days of co-culture supernatants are tested to determine the quantity of IgG and IgM produced and the phenotype of the cells present in the co-cultures assessed by flow cytometry. By adding in immunosuppressive drugs to the culture medium, we could assess the impact of each drug both on plasmablast development and antibody production (**fig 5.1**).

Because of the previously described differences in Tfh cell subsets and their ability to help B cells, the sorting strategy was designed to allow comparison of the main subsets that have been previously described[127,130]. From PBMCs, CD4 T cells were pre-enriched and then flow sorted to compare T effectors ($CXCR5^{-}$ $CXCR3^{-/+}CD4^{+}$) as a control population, with Tfh-1 like cells ($CXCR5^{+}CXCR3^{+}CD4^{+}$), which have been described to be able to help memory, but not naïve, B cells and Tfh2/Tfh17 like cells ($CXCR5^{+}CXCR3^{-}CD4^{+}$)[127,132], which are able to help both memory and naïve B cells. B cells were also pre-enriched and sorted into memory ($CD27^{+}CD19^{+}$) and naïve ($CD27^{-}CD19^{+}$) populations (**fig 5.2**).

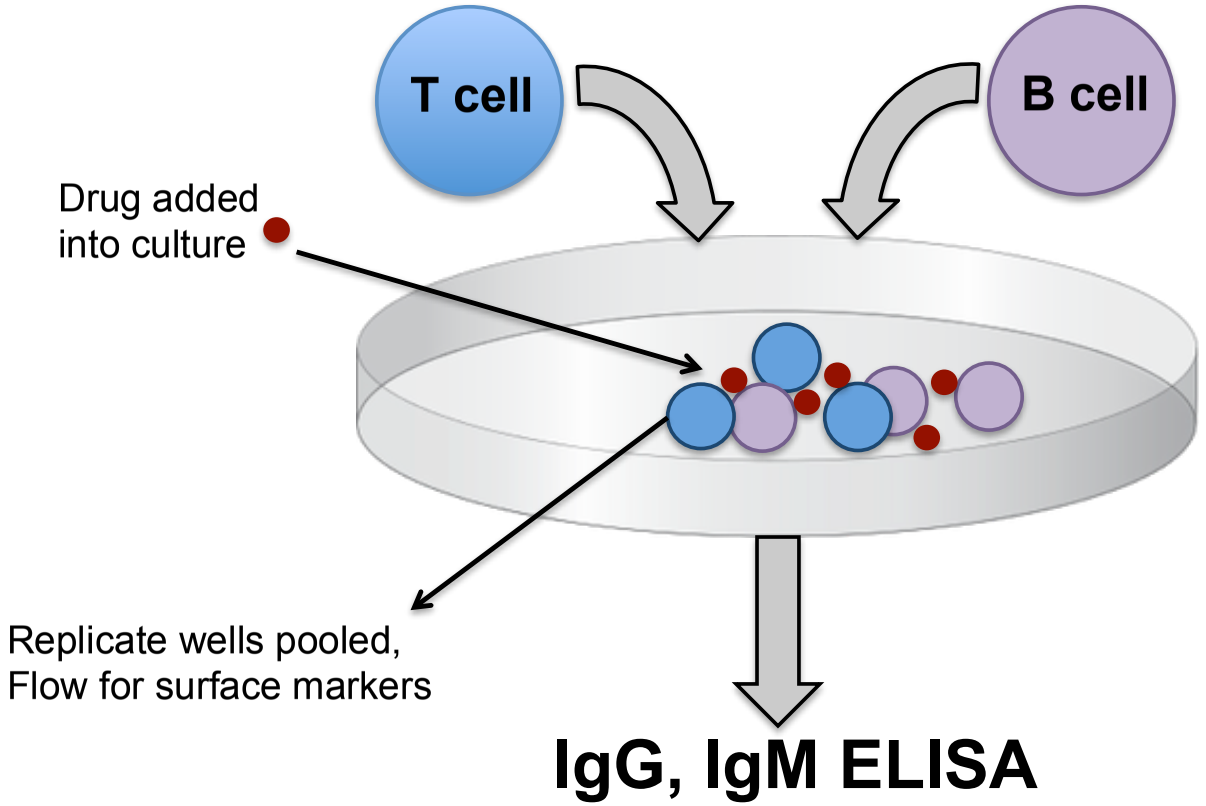


Figure 5.1: Coculture set up. Isolated Tfh and B cells were flow sorted and cultured in 1:1 proportions with staphylococcal enterotoxin B (SEB) stimulation, with or without drugs or vehicle control. At day 11, supernatants were removed and assessed for antibody production by ELISA, replicate wells were then combined to assess cells for alterations in cell number or phenotype by flow cytometry.

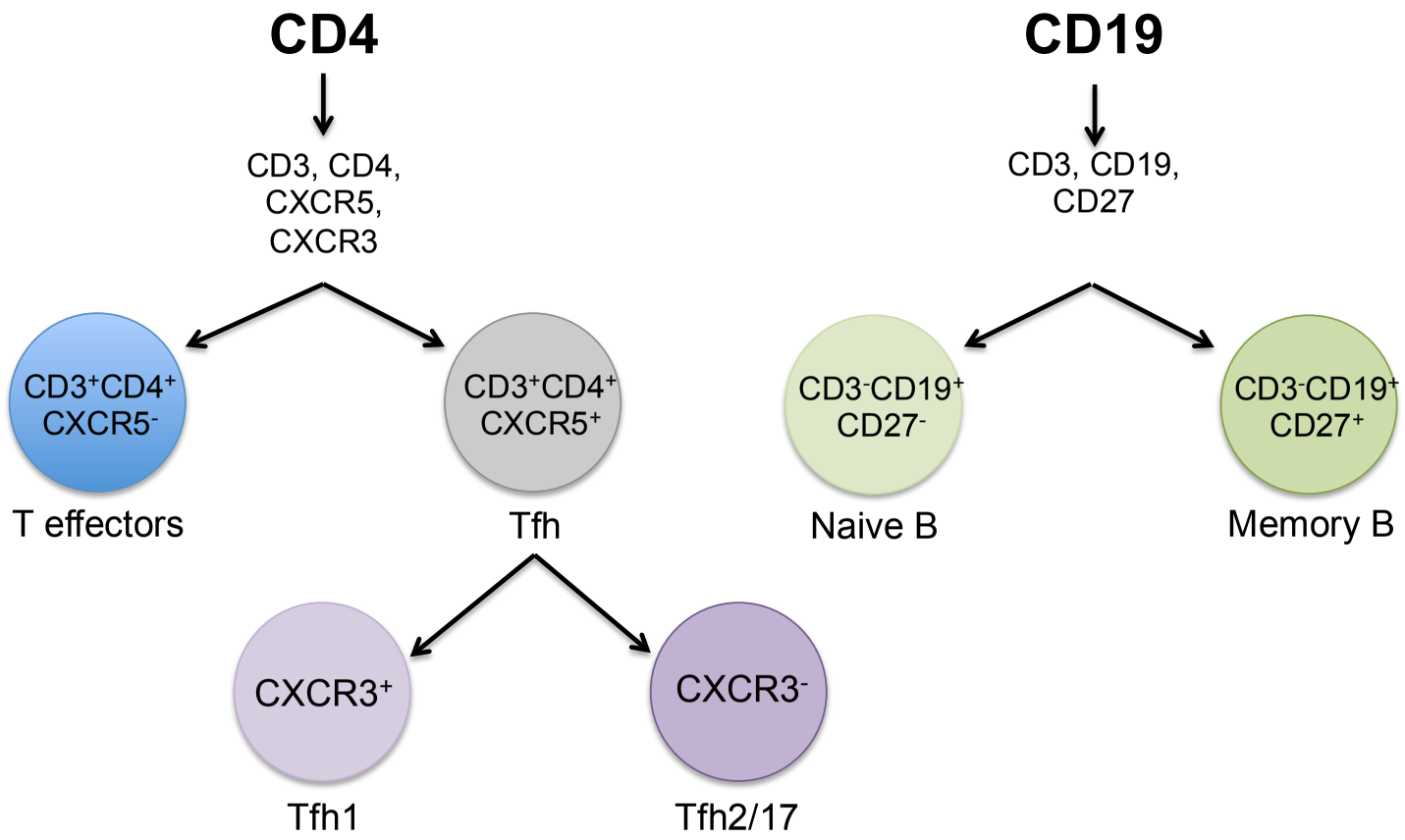


Figure 5.2: Flow sorting strategy. Cells were pre-enriched with magnetic beads to isolate CD4 T cells and CD19 B cells from PBMCs. Isolated cells were then fluorescently labelled and flow sorted as shown above.

5.6.1 Pre-assessment of leucocyte cones prior to culture

The cells for culture were taken from leucocyte cones, which come from anonymous donors with no additional information. To ensure that there were sufficient memory B cells for our assays, which tend to increase in proportion with age[456,457], a sample from each cone was tested to determine if there were sufficient cells for the planned analyses. Additionally, the proportion of Tfh subsets was assessed, as the proportions of $CXCR3^+$ and $CXCR3^-$ cells in the circulation can vary between donors[127,130]. Seven leucocyte cones were tested by flow cytometry using the proposed sorting panel, the Tfh immunophenotyping panel and the B cell immunophenotyping panel (see chapter 2, sections 2.6, 2.14). Cell frequencies were used to determine which cones contained sufficient cells to allow comparison of naïve B cells and memory B cells with $CXCR3^+$ and $CXCR3^-$ Tfh, as well as control samples of B cells with Teffectors (Teff) and both T and B cells in isolation. Samples with sufficient cell numbers to be used in co-culture were chosen for analysis (**fig 5.3**).

5.6.2 Sorting strategy

Samples were flow sorted as described above and gates were set to maximise both the purity and yield of individual subsets**.** Test sorts were performed before each bulk sort to check purity, which was confirmed to be greater than 99% for each population (**figs 5.4, 5.5**). Cells were then cultured for 11 days; both naïve and memory B cells were co-cultured with $CXCR3^+$ Tfh (referred to as Tfh1 cells) and $CXCR3^-$ Tfh (referred to as Tfh2/17 cells).

Cone	Lymphs	CD4	CXCR3^{+} Tfh	CXCR3^{-} Tfh	CD19	CD27^{-} B cells	CD27^{+} B cells
1	61.5%	48%	3.5%	0.4%	14.4%	13.3%	0.8%
2	54.8%	52%	2.7%	4.1%	10.0%	6.2%	3.6%
3	52.3%	42%	1.4%	2.1%	9.0%	1.9%	7.0%
4	61.0%	57%	1.6%	3.0%	8.6%	4.0%	4.5%
5	44.9%	50%	6.6%	1.2%	7.9%	3.7%	4.1%
6	55.0%	52%	2.6%	3.4%	10.2%	7.3%	2.8%
7	53.1%	49%	1.8%	5.7%	10.2%	7.6%	2.5%

Figure 5.3: Cone test results. Cones were assessed with three flow panels and an average number of cells was calculated for each cone. Cones with cell proportions (shown as percentage of lymphocytes) too low to provide sufficient subsets for duplicates of each culture condition were discarded, highlighted in red.

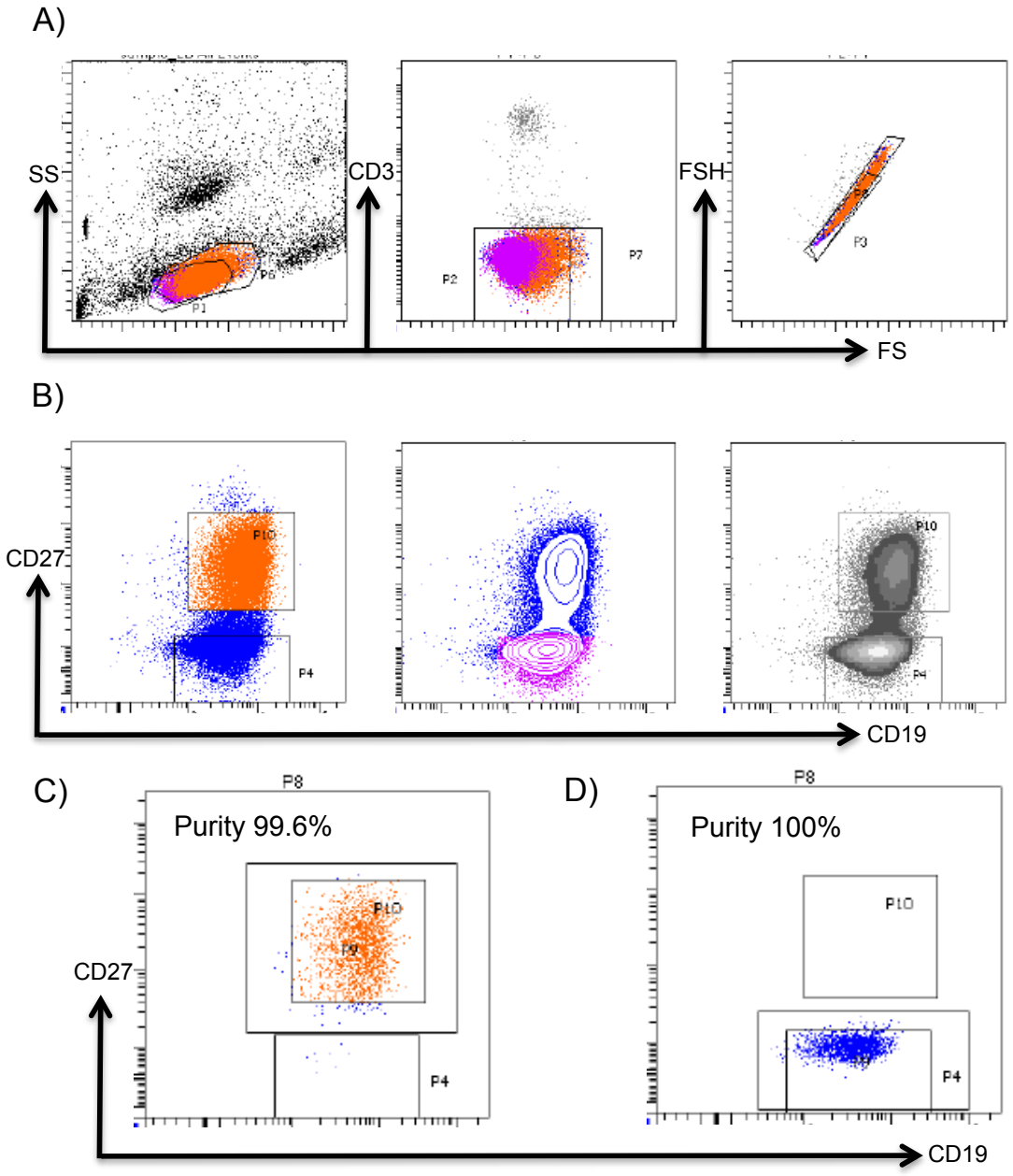


Figure 5.4: Representative flow cytometric plots showing sorting strategy for B cells. Pre enriched B cells were gated on A) lymphocytes, CD3 negative singlets and then sorted on B) $CD27^{+}$ memory cells and $CD27^{-}$ naïve cells. Test sorts were performed prior to each bulk sort and showed high purity of C) $CD27^{+}$ memory cells and D) $CD27^{-}$ naïve cells.

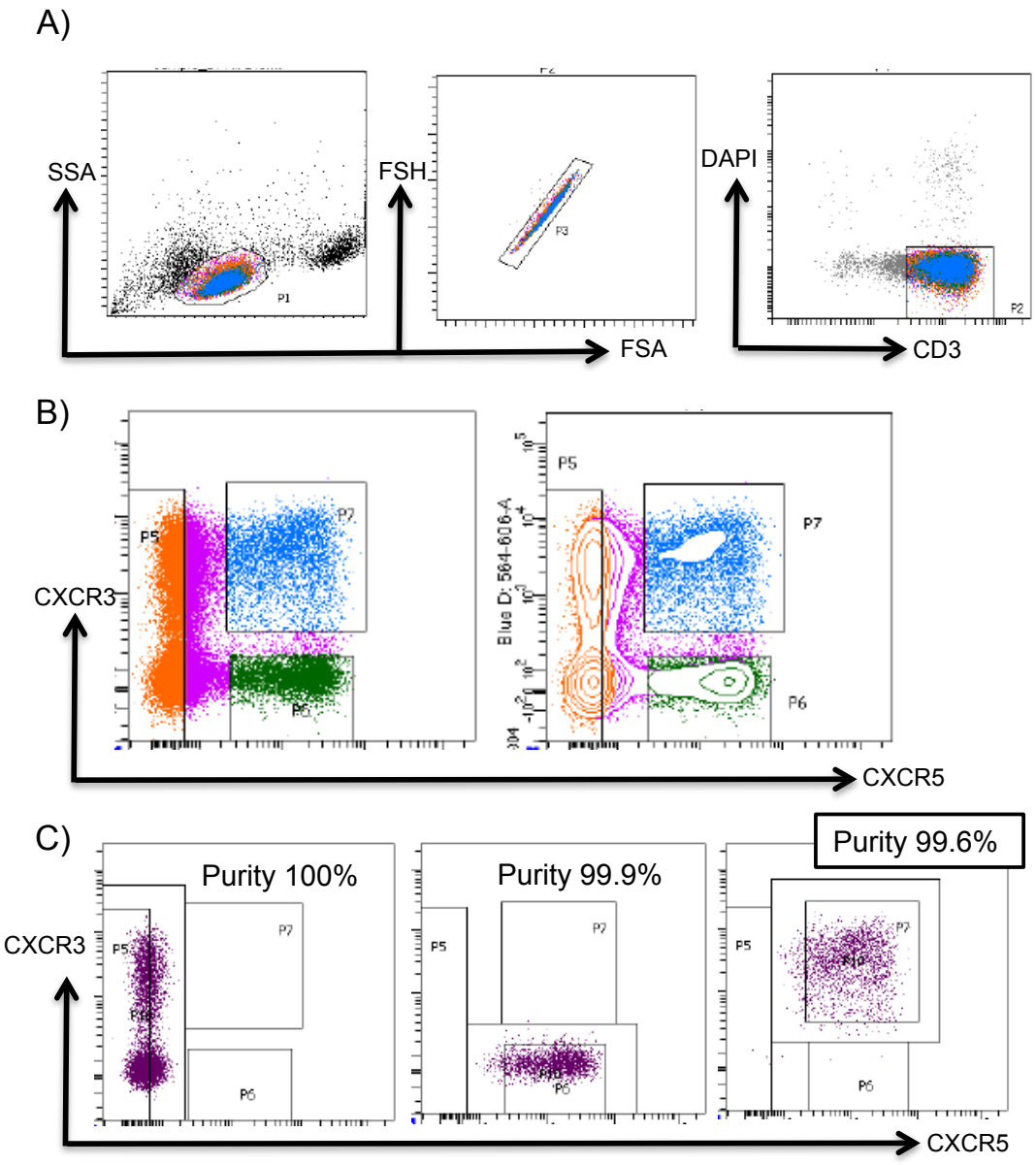


Figure 5.5: Representative flow cytometric plots showing sorting strategy for T cells. Pre enriched $CD4^+$ T cells were gated on A) lymphocytes, singlets, and DAPI negative $CD3^+CD4^+$ and then sorted on B) $CXCR5^-CXCR3^{+/-}$ Teffectors, $CXCR5^+CXCR3^+$ Tfh1 and $CXCR5^+CXCR3^-$ Tfh2/17 cells . Test sorts were performed prior to each bulk sort and showed C) high purity of all subsets.

Control conditions were included for each assay; B cells without T cells, B cells with CXCR5$^-$ Teff, and each T cell subset without B cells.

At 11 days, supernatants were removed for ELISA and technical replicates were pooled to assess for plasmablast formation by flow cytometry. The gating strategy with representative B/Tfh co-culture flow plots is shown in **fig 5.6**.

5.6.3 Tfh cell subsets supported plasmablast development in co-culture

Initial results showed good plasmablast formation at day 11 in all cones (**fig 5.7**), although both proportion and numbers varied, consistent with previous reports[132]. Confirming previous observations, Teff cells showed little to no plasmablast development regardless of memory or naïve B cell culture. Tfh1 cells were able to support plasmablast formation when co-cultured with memory B cells, but not with naïve B cells (p=0.0001). Tfh2/17 were able to support plasmablast formation in both B cell subsets, but leading to significantly higher proportions in memory B cell co-cultures than naïve B cell co-cultures (p=0.0001), shown in **fig 5.7A**[127,132].

Counting beads were included to determine absolute cell counts from each culture condition. Initial culture required 30 000 cells of each subset per well, therefore combined wells show the results from 60 000 starting B cells. Recovered plasmablast numbers were low in naïve B (NB) cell cultures (**fig 5.7B**) but showed significantly higher plasmablast numbers in NB with Tfh2/17 cells compared to NB and Tfh1 cells (p=0.0002) or NB with Teff (p<0.0001). Plasmablast numbers were much higher in memory B (MB) cell cultures **fig 5.7C**,

and showed significantly higher numbers in MB and Tfh2/17 co-cultures compared to MB and Tfh1 cells (p=0.004) or MB and Teff (p=0.0003).

Reflecting the degree of plasmablast formation, levels of IgG (**fig 5.8Aii**) and IgM (**fig 5.8Aii**) were low but detectable in NB cell cultures and significantly higher when NB were co-cultured with Tfh2/17 compared to Tfh1 cells (p=0.009) or Teff (p=0.001). Tfh1 cells were able to induce IgM production by NB cells to a greater extent than IgG production, but still at lower levels than Tfh2/17 cells (p=0.03). In contrast both IgG (**fig 5.8Bi**) and IgM (**fig 5.8Bii**) production was detected at high levels in memory B cell cultures with both Tfh1 and Tfh2/17 cells.

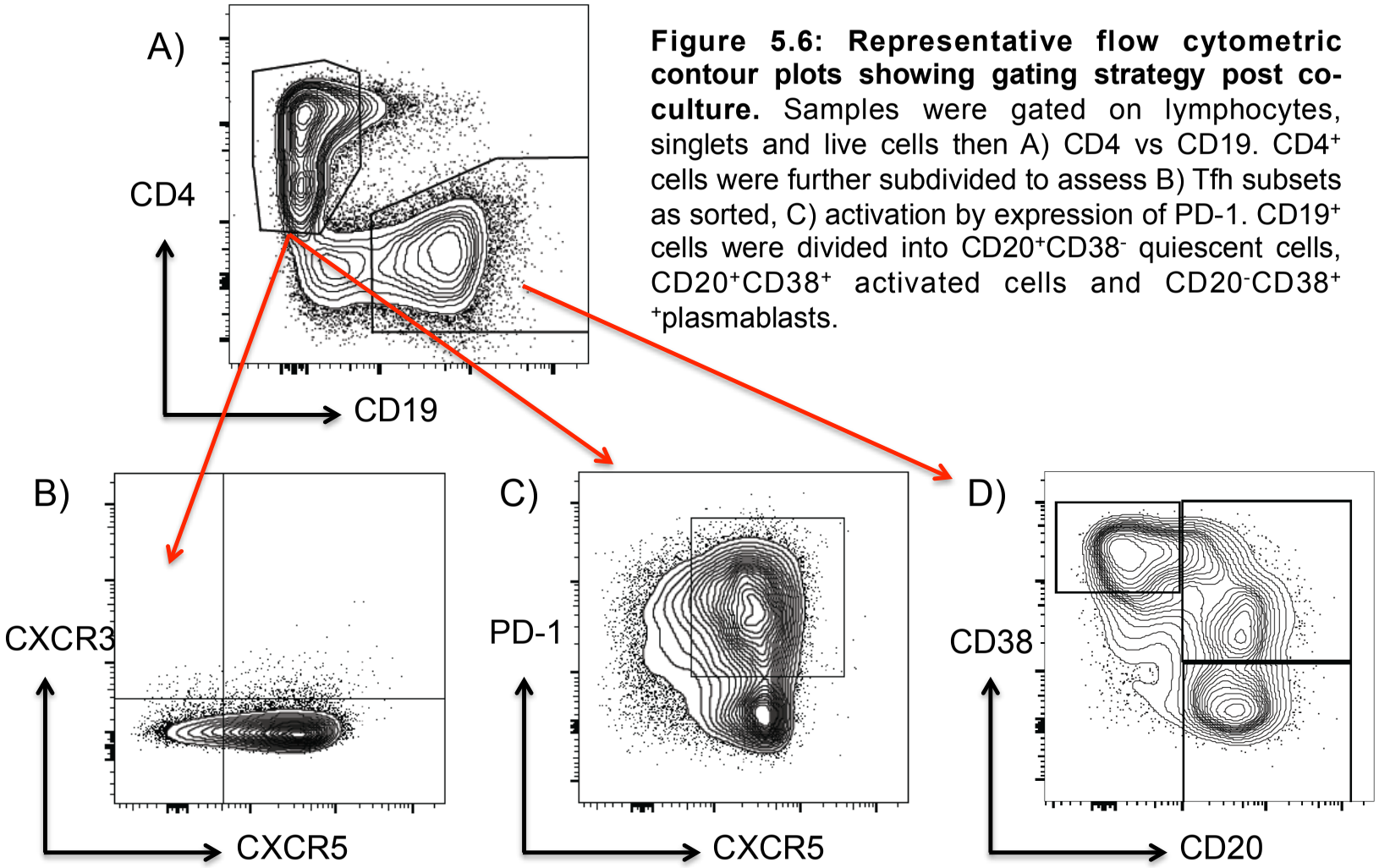


Figure 5.6: Representative flow cytometric contour plots showing gating strategy post co-culture. Samples were gated on lymphocytes, singlets and live cells then A) CD4 vs CD19. $CD4^+$ cells were further subdivided to assess B) Tfh subsets as sorted, C) activation by expression of PD-1. $CD19^+$ cells were divided into $CD20^+CD38^-$ quiescent cells, $CD20^+CD38^+$ activated cells and $CD20^-CD38^+$ $^+$plasmablasts.

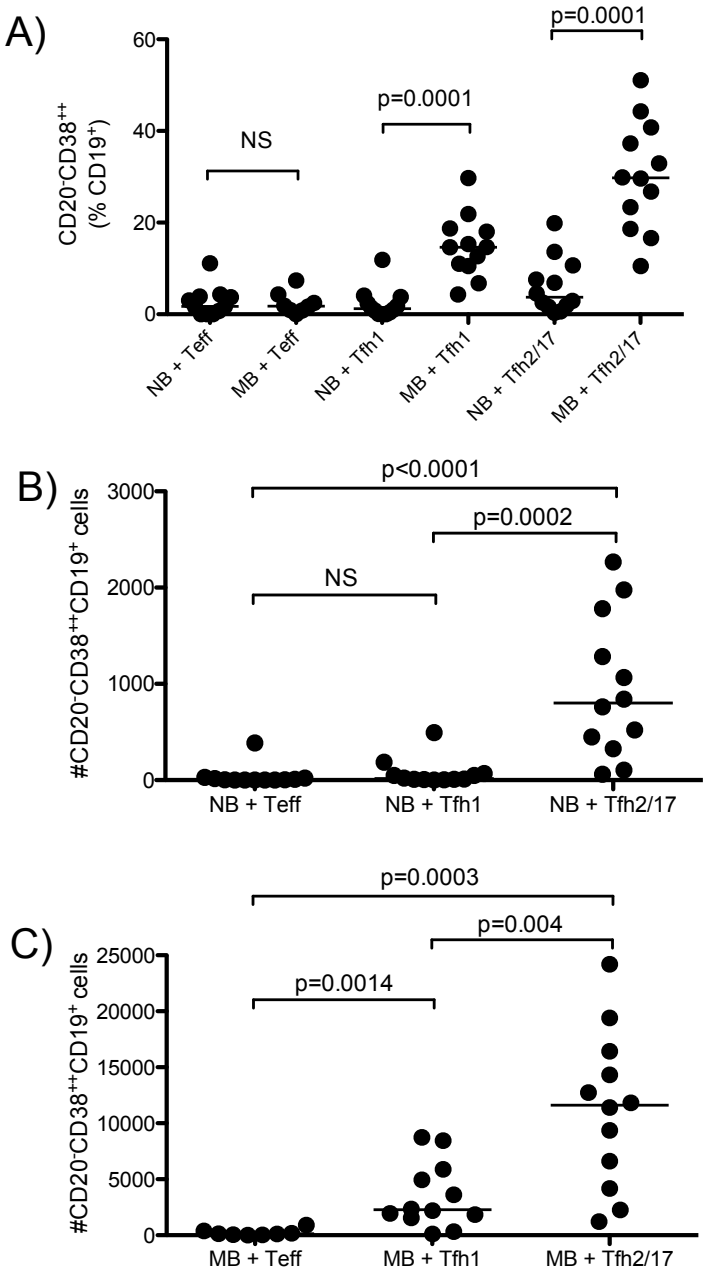


Figure 5.7: Plasmablast development from untreated cocultures. A) Plasmablast development after 11 day coculture as percentage of live B cells. Plasmablast cell count in B) naïve B cell cultures and C) memory B cell cultures. Each point marks a combined duplicate, with median at line. Samples analysed with Mann Whitney U test. 4 cones were tested with 3 independent experiments per cone, n=12.

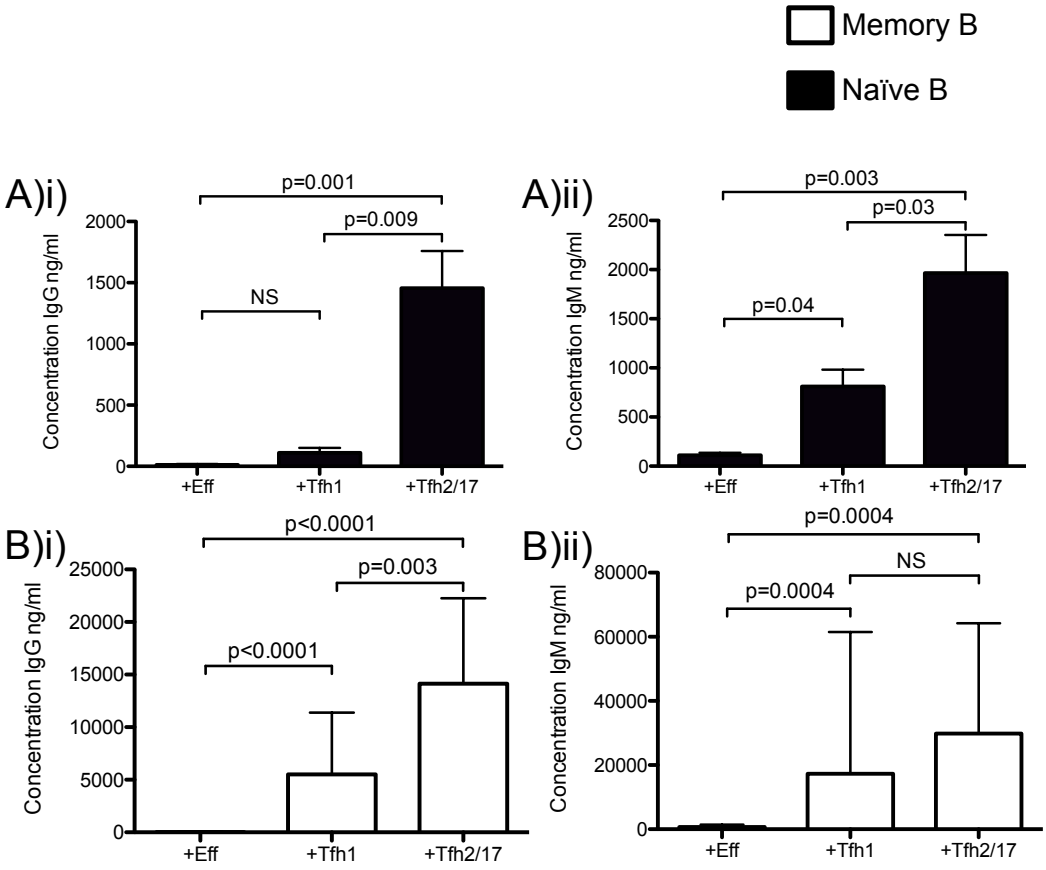


Figure 5.8: Antibody production from untreated co-cultures. A) ELISAs from naïve B (NB) cell co-cultures showing (i) IgG production in co-cultures A)ii) IgM production in co-cultures. B) ELISAs from memory B (MB) cell co-cultures showing (i) IgG production in co-cultures B)ii) IgM production in co-cultures. Bar represents median with range. 4 cones were tested with 3 replicate cultures and 2 wells per sample, total n=24. Samples analysed with Mann Whitney U test.

5.6.4 Immunosuppressive drugs reduce plasmablast production

In order to assess the impact of drugs on antibody production, each immunosuppressive drug was added into the culture medium in turn.

In vivo, azathioprine is absorbed from the gut with around 80-90% bioavailability. It is a pro-drug, which is broken down slowly and non-enzymatically by reductive removal of a thioether, converting it to the active metabolite, 6-MP. This takes place on the intestinal wall, in the liver and on red blood cells, and therefore azathioprine cannot be converted to 6-MP *in vitro*. For these co-cultures 6-MP was used as the active metabolite at a dose of 50μM to correlate with human dosing and in keeping with previous *in vitro* work[347].

MMF is also a pro-drug, which is absorbed with >90% bioavailability, and then rapidly hydrolysed to MPA in the intestine, liver and blood. This leads to the same issues as with azathioprine, and hence MPA was used in these co-cultures at 50μg/ml to correlate with human dosing and in keeping with previous *in vitro* work[348].

Tacrolimus was used at 8ng/ml, aiming for the mid-range of recommended dosing for patients, which is 5-15ng/ml[315,349] and in keeping with previous *in vitro* work[350].

Samples were co-cultured for 11 days in the presence of drug or vehicle control. Because of high B cell death rates seen in untreated test cultures, media was topped up at day 6 to ensure sufficient nutrients remained to support cell growth

throughout. The media for top up contained drug but not SEB (see section 2.15, chapter 2).

Results showed a significant impact of all drugs on plasmablast formation in all culture conditions. In cultures of MB cells with Tfh cell subsets there was no significant difference between treated and untreated co-culture with Teff (**fig 5.9A, B**), likely due to the lack of plasmablast induction by this T cell subset. There was a significant reduction in the proportion (**fig 5.9A**) ($p<0.0001$) and total number (**fig 5.9B**) ($p=0.0091$) of plasmablasts in all co-cultures of MB+Tfh1 cells with immunosuppressive drugs. A similar pattern was seen with MB+Tfh2/17 as a proportion of plasmablasts ($p<0.0001$, **fig 5.9A**) and cell count per well ($p=0.0005$, **fig 5.9B**).

The differences in NB cell co-cultures were smaller and not significant for cultures with Teff or Tfh1 cells. Co-culture with Tfh2/17 cells showed a significant reduction in the proportion (**fig 5.9C**, $p=0.04$) and total number (**fig 5.9D**, $p=0.005$) of plasmablasts with all drugs.

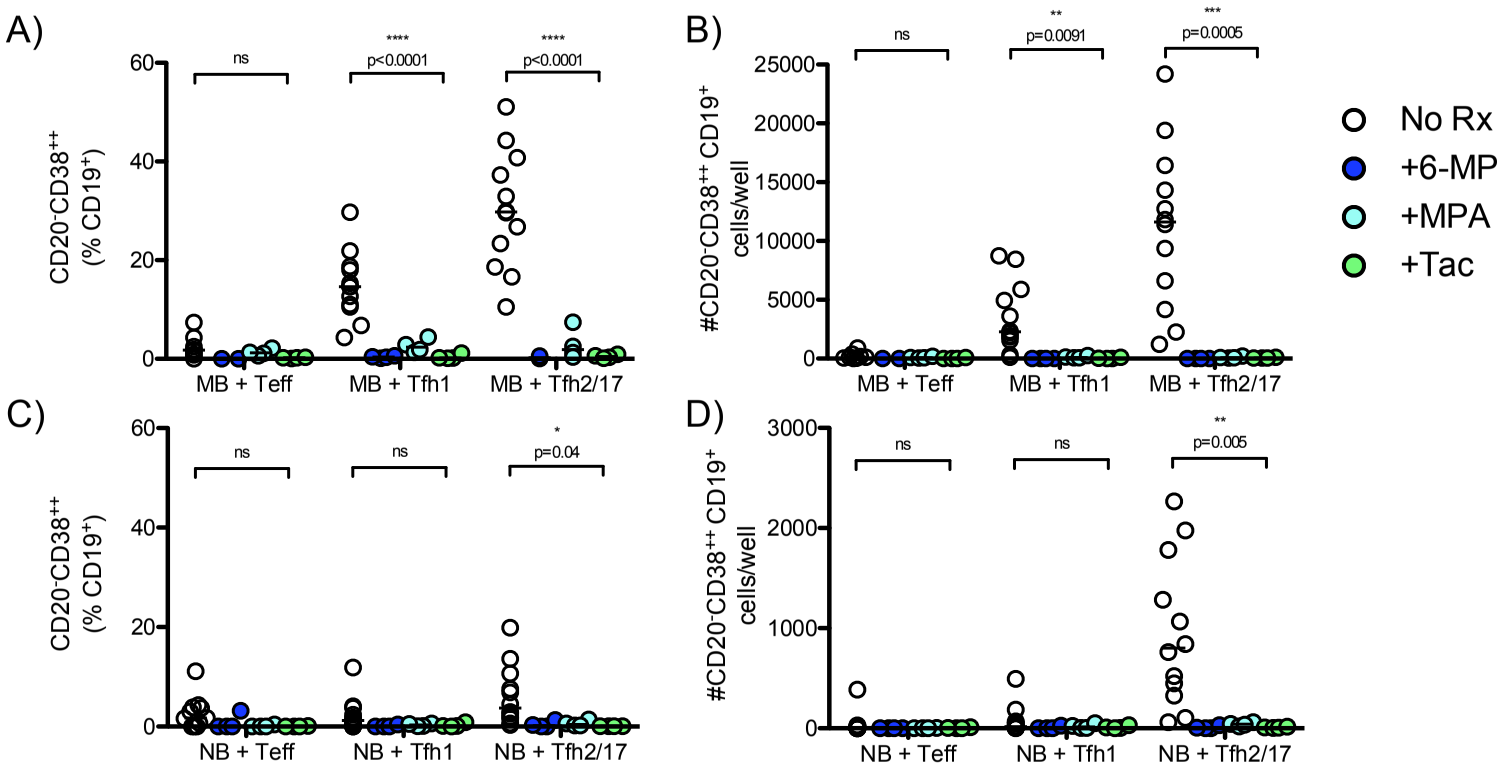


Figure 5.9: Impact of drugs on plasmablast production. Co-cultures with drugs showing an abrogation of plasmablast development in all culture conditions. Plasmablasts as A) proportion of B cells in memory co-cultures, B) cell count in memory co-cultures; C) plasmablasts as proportion of B cells in naïve B co-cultures and D) cell count in naïve co-cultures. Each point marks a combined duplicate with median at line. 4 cones were tested with one co-culture for each drug, each with an untreated control, therefore n = 4 for drug-treated, n=12 for untreated. All samples analysed with 1 way ANOVA comparing treated to untreated as control.

5.6.5 Immunosuppressive drugs reduced antibody production in $CD27^+CD19^+$ memory B cell co-cultures

The impact on plasmablast production was reflected in the reduction of antibody production. This was most pronounced in memory B cell co-cultures. MPA treatment significantly reduced IgG production in both MB+Tfh1 ($p<0.05$) and MB+Tfh2/17 ($p<0.0001$) cell co-cultures compared to untreated or vehicle control co-cultures (**fig 5.10A**). The impact on IgM production was less pronounced, but still significantly reduced in MB+Tfh2/17 co-cultures in MPA treated compared to untreated or vehicle control co-cultures ($p<0.05$, **fig 5.10B**).

Tacrolimus had a similar impact on IgG production in both MB+Tfh1 ($p<0.01$) and MB+Tfh2/17 ($p<0.0001$) co-cultures compared to untreated or vehicle control co-cultures (**fig 5.11A**). Tacrolimus also reduced IgM production in MB+Tfh1 ($p<0.05$) and MB+Tfh2/17 ($p<0.001$) co-cultures compared to untreated or vehicle control co-cultures (**fig 5.11B**).

6-MP similarly significantly reduced IgG production in both MB+Tfh1 ($p<0.05$) and MB+Tfh2/17 ($p<0.01$) co-cultures compared to untreated or vehicle control co-cultures (**fig 5.12A**); and significantly reduced IgM production in MB+Tfh1 ($p<0.05$) and MB+Tfh2/17 ($p<0.0001$) co-cultures compared to untreated or vehicle control co-cultures.

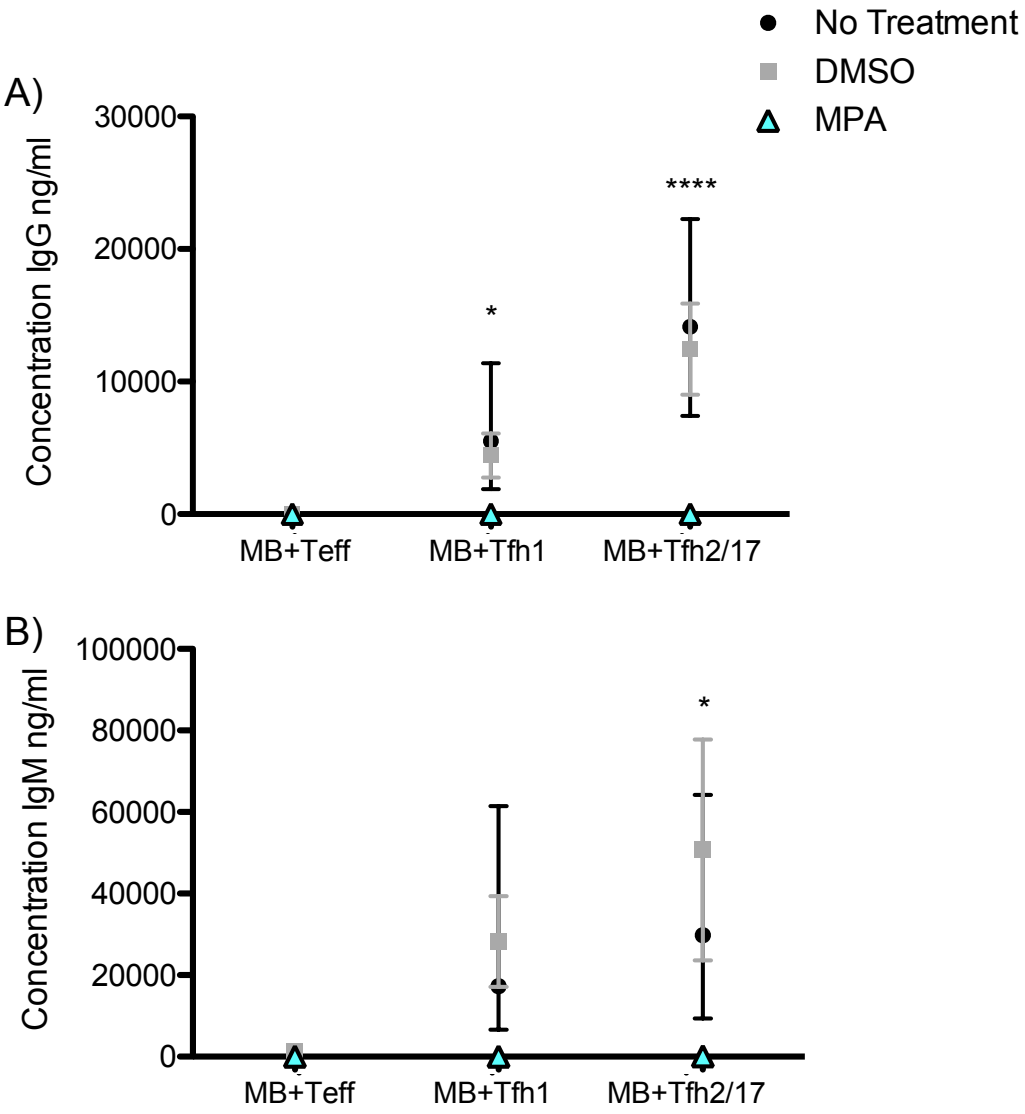


Figure 5.10: Impact of mycophenolic acid (MPA) on antibody production by CD27^{+}CD19^{+} memory B cultures. A) ELISA showing significant reduction in IgG production in MPA treated compared to untreated or vehicle control in memory B (MB) + Tfh1 ($p<0.05$) and MB +Tfh2/17 ($p<0.0001$) but no significant change in MB+Eff co-culture. B) ELISA showing significant reduction in IgM production in MPA treated compared to untreated or vehicle control in MB+Tfh2/17 ($p<0.05$) but no significant reduction in other culture conditions. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

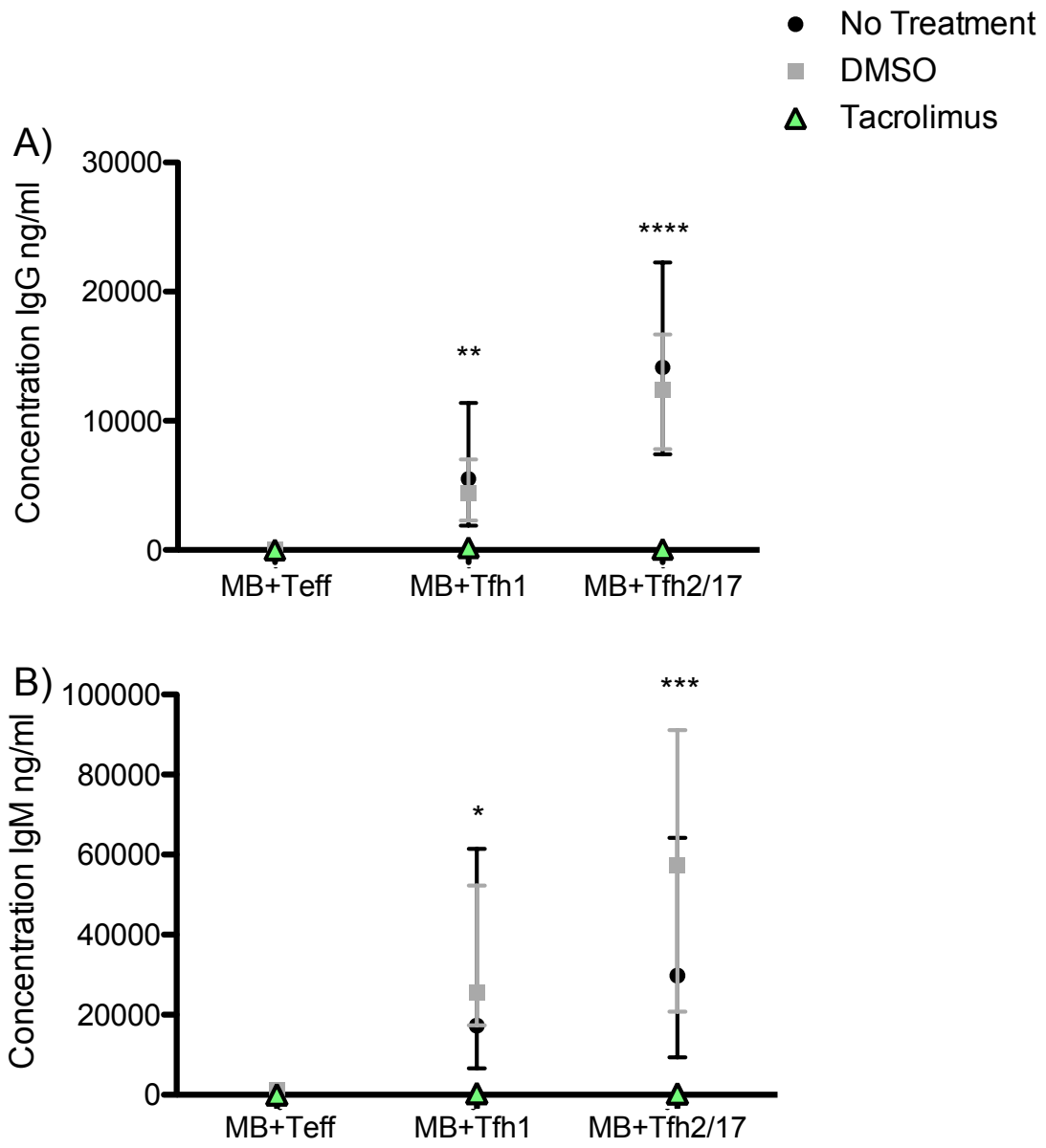


Figure 5.11: Impact of tacrolimus on antibody production by CD27^{+}CD19^{+} memory B cultures. A) ELISA showing significant reduction in IgG production in tacrolimus treated compared to untreated or vehicle control in memory B (MB) + Tfh1 ($p<0.01$) and MB+Tfh2/17 ($p<0.0001$) but no significant change in MB+Eff co-culture. B) ELISA showing significant reduction in IgM production in tacrolimus treated compared to untreated or vehicle control in MB+Tfh1 (<0.05) and MB +Tfh2/17 ($p<0.001$) but no significant reduction in MB+Eff co-culture. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

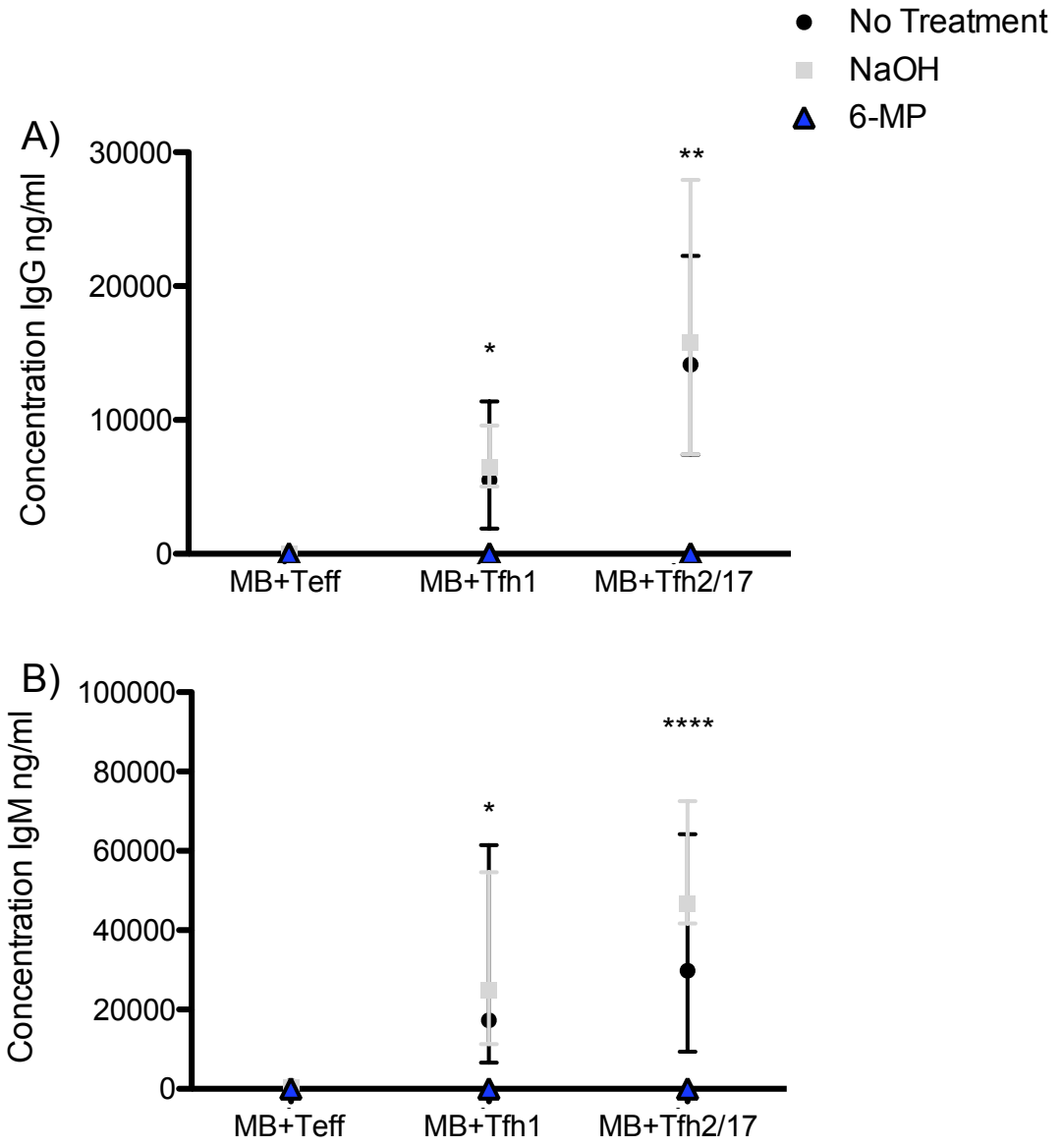


Figure 5.12: Impact of 6-mercaptopurine (6-MP) on antibody production by CD27^{+}CD19^{+} memory B cultures. A) ELISA showing significant reduction in IgG production in 6-MP treated compared to untreated or vehicle control in memory B (MB) + Tfh1 ($p<0.05$) and MB +Tfh2/17 ($p<0.01$) but no significant change in MB+Eff co-culture. B) ELISA showing significant reduction in IgM production in 6-MP treated compared to untreated or vehicle control in MB+Tfh1 (<0.05) and MB +Tfh2/17 ($p<0.0001$) but no significant reduction in MB+Eff co-culture. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

5.6.6 MPA had less effect on $CD27^+CD19^+$ memory B cell co-cultures

Comparing only drug-treated co-cultures it was clear that both MPA and tacrolimus treatment allowed plasmablast formation in memory B cell co-cultures, albeit at much lower levels than untreated co-cultures (as previously shown in **fig 5.9**). MPA induced significantly higher plasmablast proportions in both MB+Tfh1 ($p=0.007$) and, surprisingly in MB+Teff ($p=0.011$) compared to 6-MP or tacrolimus (**fig 5.13Ai**). Absolute numbers of plasmablasts were also higher in MPA treated co-cultures compared to 6-MP or tacrolimus treated co-cultures, reaching significance in MB+Tfh1 ($p=0.040$) and MB+Tfh2/17 ($p=0.049$). There was a trend towards higher plasmablast levels in tacrolimus treated co-cultures but this did not reach significance (**fig 5.13Aii**).

Antibody production in all drug-treated co-cultures was 1-2 log-folds lower than untreated co-cultures. However, IgG production was higher in MPA treated co-cultures compared to tacrolimus treated or 6-MP treated co-cultures, and was significantly higher in MB+Tfh1 co-cultures ($p<0.05$, **fig 5.13Bi**). There was a trend towards higher IgM production in both MB+Tfh1 and MB+Tfh2/17 co-cultures with both MPA and tacrolimus compared to 6-MP, but this did not reach statistical significance (**fig 5.13Bii**).

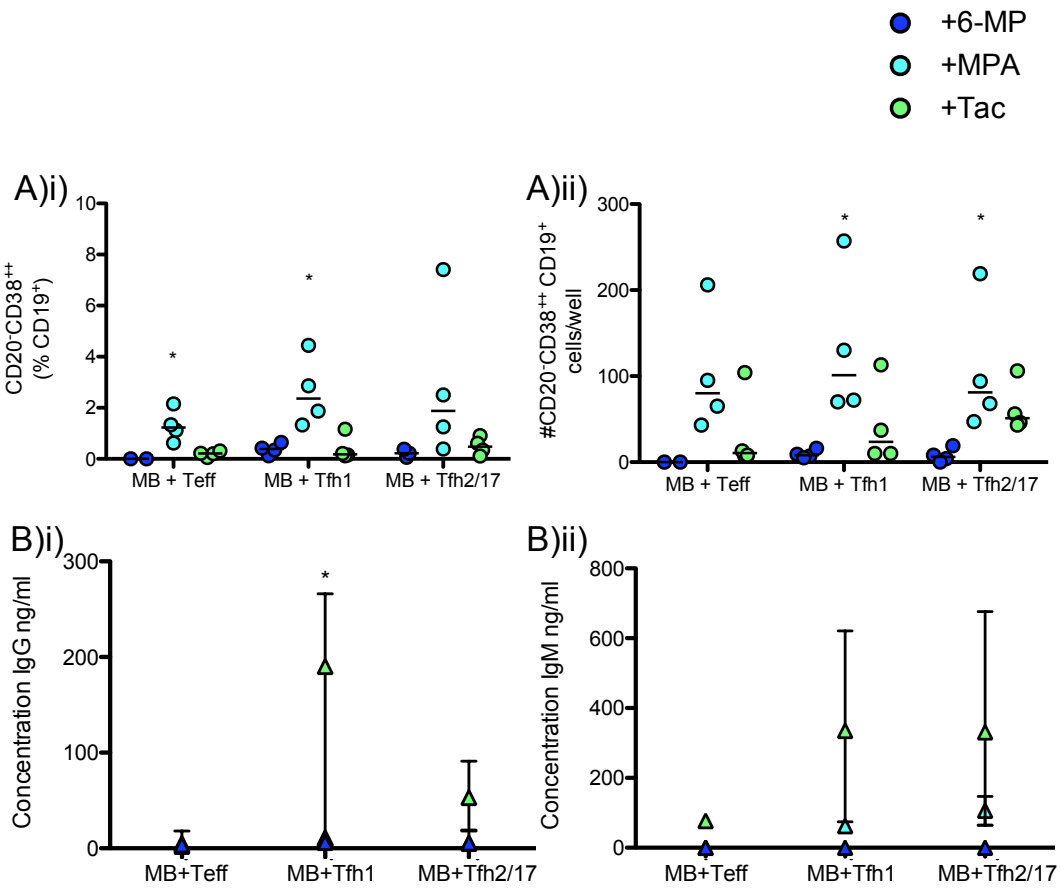


Figure 5.13: Comparison of drug-treated CD27⁺CD19⁺ memory B cell co-cultures. A) plasmablast differentiation in 6-mercaptopurine (6-MP), tacrolimus and mycophenolic acid (MPA) treated memory B (MB) co-cultures showing (i) significant increase in plasmablast proportions in MPA treated co-cultures compared to 6-MP or tacrolimus in MB+Eff (p=0.011), MB+Tfh1 (p=0.007) but no significant difference in MB+Tfh2/17. A)ii) Absolute cell count showing no significant difference in MB+Eff co-culture but a significant increase in plasmablast numbers in MPA treated co-cultures compared to 6-MP or tacrolimus in MB+Tfh1 (p=0.040) and MB+Tfh2/17 (p=0.049). Each point represents one combined duplicate with line at median, n=4 per drug. Analysed via 1-way ANOVA comparing all samples to 6-MP treatment. B) ELISAs for drug-only co-cultures showing (i) a significant increase in IgG production in MPA treated compared to 6-MP or tacrolimus in MB+Tfh1 co-cultures (p<0.05) but no significant change in other co-culture conditions. B)ii) IgM ELISA showing no significant alterations in IgM production with any drug. Median and range shown, each replicate run separately, therefore n=8 per sample.

5.6.7 Immunosuppressive drugs reduced antibody production in $CD27^{-}CD19^{+}$ naïve B cell co-cultures

MPA also reduced IgG and IgM production in NB cell co-cultures. There was no significant IgG production from NB+Teff or NB+Tfh1 co-cultures in untreated controls. Both untreated and vehicle control showed higher IgG production compared to MPA treated (**fig 5.14A**) but this did not reach statistical significance. There was a small increase in IgM production in NB+Tfh1 cell co-cultures but this was not significantly different from treated or untreated controls. IgM production in MB+Tfh2/17 co-cultures was significantly reduced ($p<0.05$) in MPA treated co-cultures compared to untreated or vehicle control (**fig 5.14B**).

There was a trend towards reduced IgG (**fig 5.15A**) and IgM (**fig 5.15B**) production with tacrolimus treated naïve B co-cultures compared to untreated or vehicle control co-cultures but this did not reach statistical significance.

6-MP reduced IgG production in NB+Tfh2/17 co-cultures compared to untreated or vehicle control (**fig 5.16A**), there was negligible IgG or IgM production in NB+Tfh1 or NB+Teff co-cultures. IgM production was significantly reduced by 6-MP treatment compared to untreated or vehicle control ($p<0.01$, **fig 5.16B**).

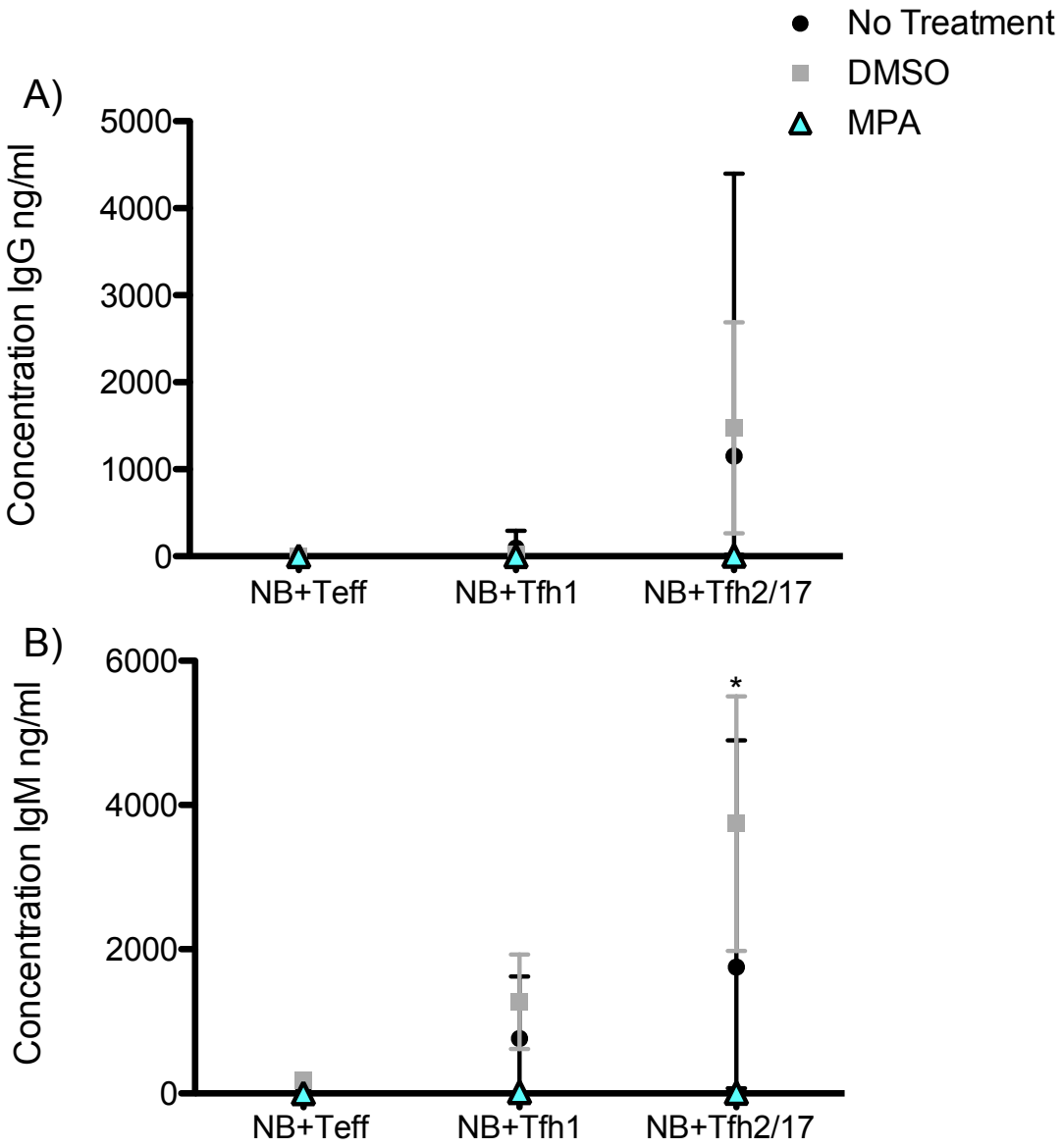


Figure 5.14: Impact of mycophenolic acid (MPA) on antibody production by CD27$^-$CD19$^+$ naïve B cultures. A) ELISA showing no IgG production in naïve B (NB) co-cultures with Tfh1 or Teff but reduction in IgG production in MPA treated compared to untreated or vehicle control in naïve B (NB) +Tfh2/17, this did not reach significance on 2-way ANOVA. B) ELISA showing significant reduction in IgM production in MPA treated compared to untreated or vehicle control in NB+Tfh2/17 ($p<0.05$) but no significant change in other culture conditions. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

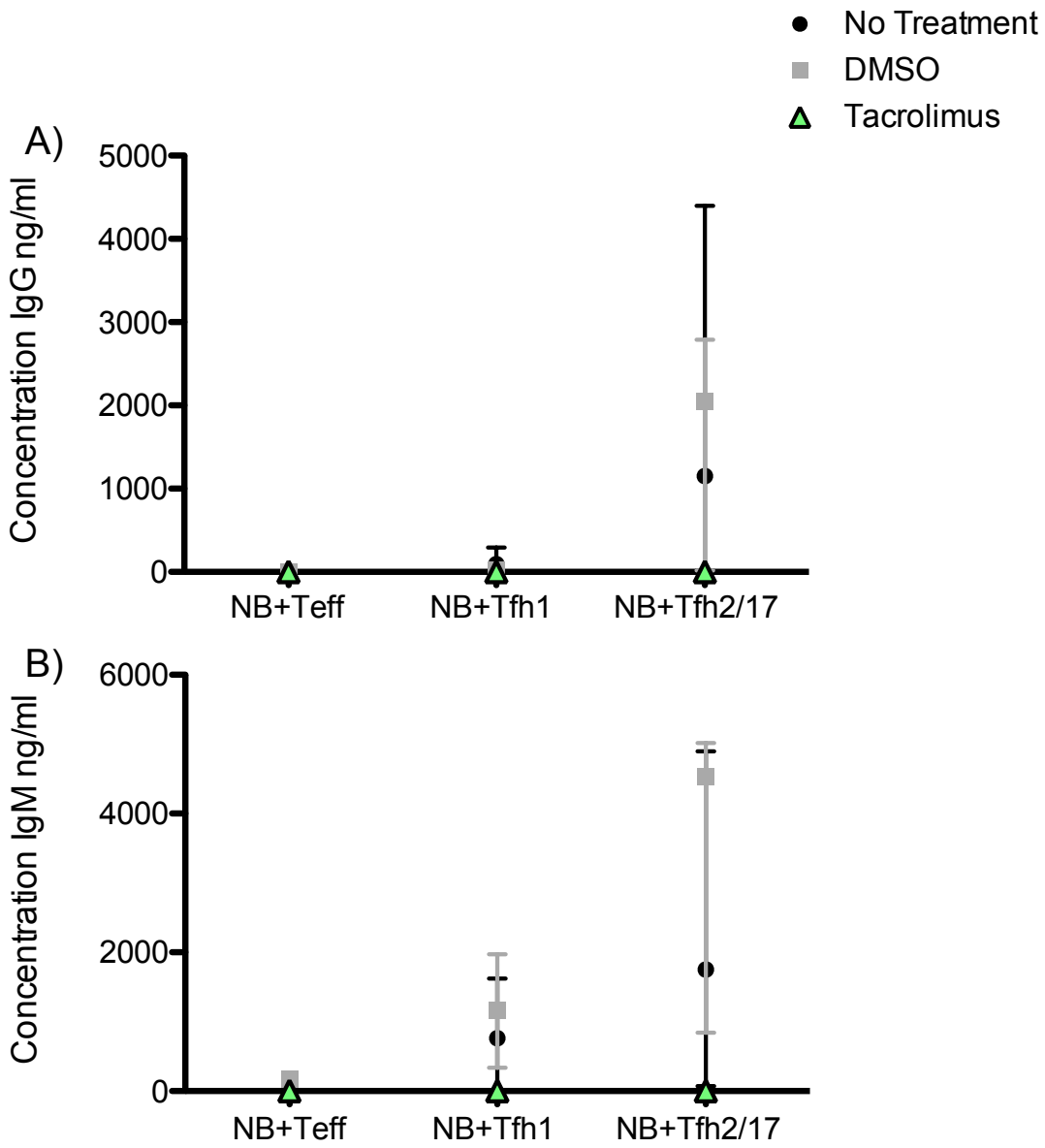


Figure 5.15: Impact of tacrolimus on antibody production by CD27$^-$ CD19$^+$ naïve B cultures. A) ELISA showing production of IgG production in tacrolimus treated compared to untreated or vehicle control in naïve B (NB) co-cultures, with no statistically significant alterations. B) ELISA showing production of IgG production in tacrolimus treated compared to untreated or vehicle control in NB co-cultures, with no statistically significant alterations. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

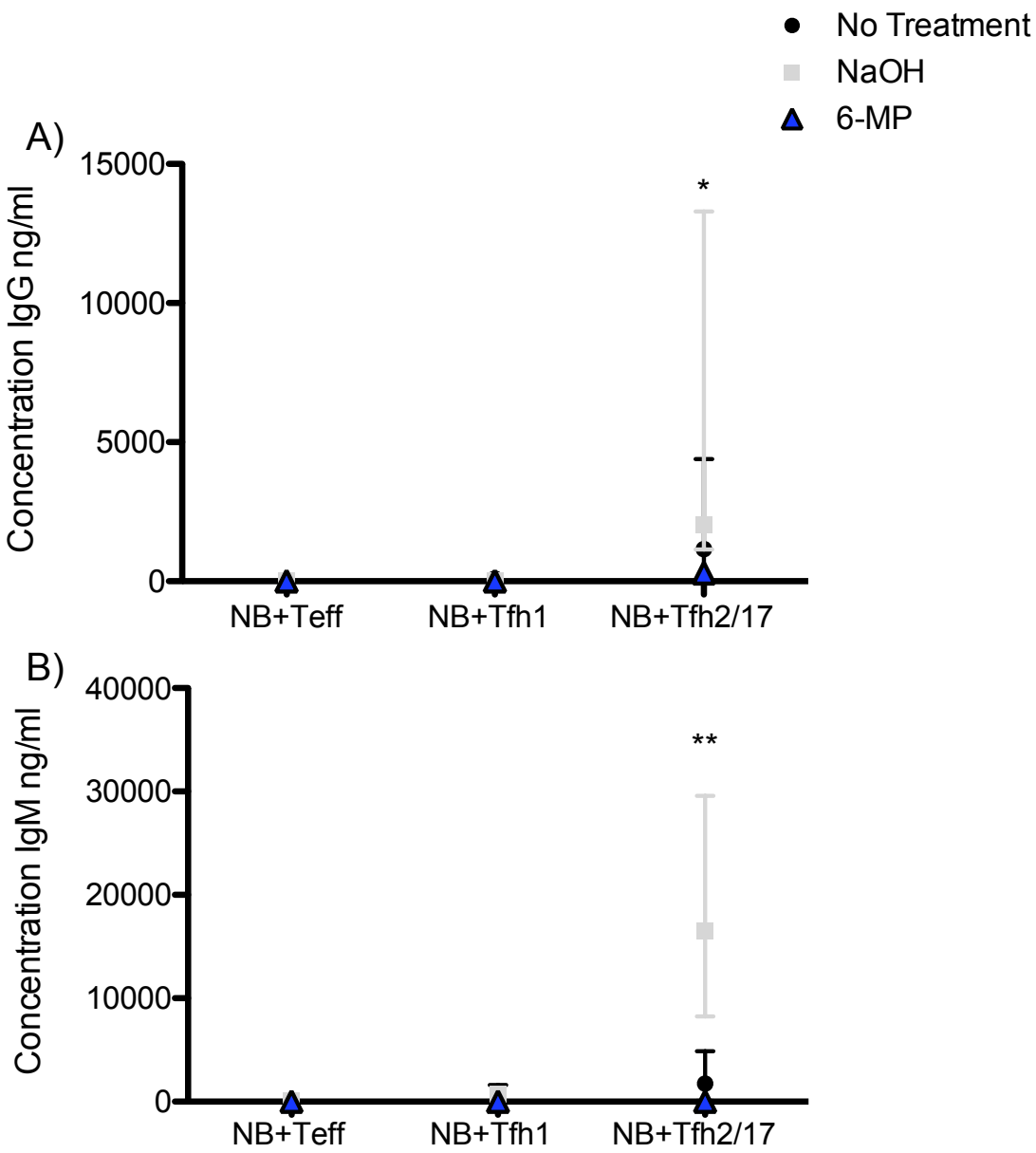


Figure 5.16: Impact of 6-mercaptopurine (6-MP) on antibody production by $CD27^{-}CD19^{+}$ naive B cultures. A) ELISA showing significant reduction in IgG production in 6-MP treated compared to untreated or vehicle control in naïve B (NB) +Tfh2/17 ($p<0.05$) but no significant IgG production in other co-culture conditions. B) ELISA showing significant reduction in IgM production in 6-MP treated compared to untreated or vehicle control in NB+Tfh2/17 ($p<0.01$) but no significant IgM production in other co-culture conditions. Shown as median and range for each sample. 4 cones were tested with one co-culture per drug, and duplicate wells were run for each sample, therefore n=8 for each point of each graph. Analysed with 2-way ANOVA comparing treatment groups with culture conditions.

5.6.8 MPA has less effect on $CD27^{-}CD19^{+}$ naïve B cell co-cultures

There were no significant differences in the proportion of plasmablasts in MPA, 6-MP or tacrolimus treated cultures (**fig 5.17Ai**) however there were higher numbers of plasmablasts in both NB+Tfh1 and NB+Tfh2/17 co-cultures, with MPA plasmablasts significantly higher than 6-MP or tacrolimus co-cultures in NB+Tfh2/17 ($p=0.015$, **fig 5.17Aii**).

Antibody production was much lower in drug-treated naïve B cell co-cultures, in the region of 5-15ng/ml, compared to 100-600ng/ml in drug-treated memory B cell co-cultures. There was negligible IgG production in any naïve B co-culture (**fig 5.17Bi**) and similarly low levels of IgM production (**fig 5.17Bii**) although a trend towards higher IgM production in MPA treated co-cultures compared to 6-MP or tacrolimus treated co-cultures.

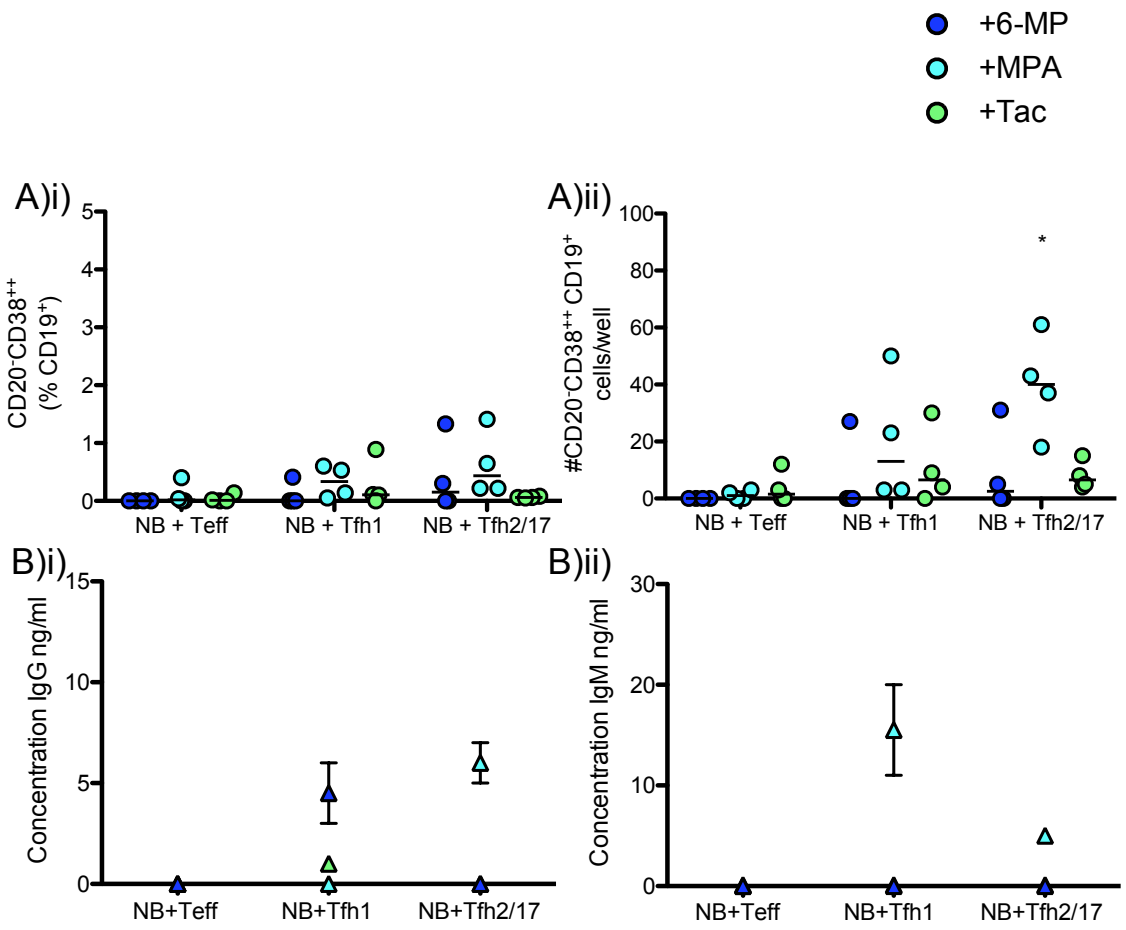


Figure 5.17: Comparison of drug-treated CD27$^-$CD19$^+$ naïve B cell co-cultures. A) plasmablast differentiation in 6-mercaptopurine (6-MP), tacrolimus and mycophenolic acid (MPA) treated naïve B (NB) co-cultures showing (i) no significant change in plasmablast proportions in any drug-treated co-cultures. A)ii) Absolute cell count showing no significant difference in NB+Eff or NB+Tfh1 co-cultures but a significant increase in plasmablast numbers in MPA treated co-cultures compared to 6-MP or tacrolimus in NB +Tfh2/17 (p=0.015). Each point represents one combined duplicate with line at median, n=4 per drug. Analysed using 1-way ANOVA for each culture condition comparing all samples to 6-MP treatment. B) ELISAs for drug-only naïve B co-cultures showing no significant differences in (i) IgG or (ii) IgM production in any co-culture condition. 2-way ANOVA comparing treatment with T cell culture condition. Median and range shown, each replicate run separately, therefore n=8 per sample.

5.6.9 Immunosuppressive drugs impacted T cell activation status

In these co-cultures, the effect of drug on plasmablast formation and antibody production may reflect the effects on B cells. But, as antibody production is T cell dependent in this system, these results may be explained by the action of the drug directly on T cell function. To determine whether these drugs have an impact on T cells, alterations in T cell subsets were also examined. Activated Tfh cells up-regulate PD-1[127,129] so post-culture samples were assessed for the proportion of PD-1^{+} cells compared to pre-culture samples (**fig 5.18A**).

Untreated and NaOH control co-cultures showed significantly higher PD-1 expression on all T cell subsets compared to pre-culture levels ($p<0.001$, **fig 5.18B**), but 6-MP abrogated this upregulation, with Teff, Tfh1 and Tfh2/17 cells expressing PD-1 at similar levels to pre-culture levels.

In MPA co-cultures, PD-1 upregulation was seen in untreated ($p<0.01$) and DMSO controls ($p<0.001$) and to a lesser extent in MPA treated co-cultures. This was significantly higher than pre-culture levels ($p<0.05$) suggesting that MPA does not limit PD-1 up-regulation to the same extent as other immunosuppressive drugs. Tacrolimus treated T cells did not up-regulate PD-1 compared to pre-culture levels.

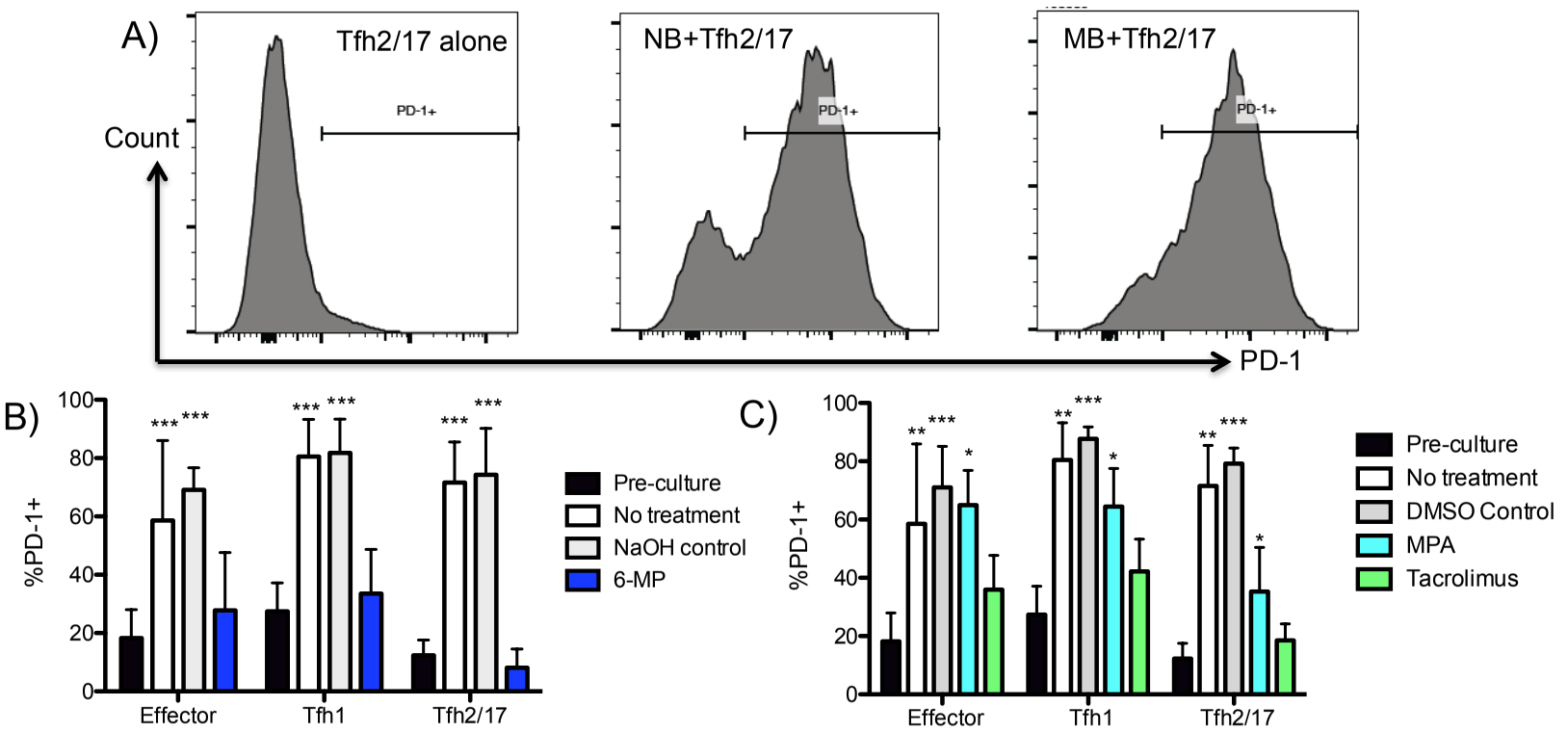


Figure 5.18: Impact of drugs on activation of Tfh. After co-culture all T cells up-regulated PD-1 as an activation marker. Representative flow histograms are shown in A) of T cells without B cells, Tfh1 with naïve B cells and Tfh2/17 with memory B cells. B) PD-1 was significantly increased in untreated ($p<0.001$) and NaOH control ($p<0.001$) compared to pre-treatment, but there was no significant change in 6-MP treated co-cultures. C) PD-1 was significantly increased in untreated ($p<0.01$), DMSO control ($p<0.001$) and MPA treated ($p<0.05$) compared to pre-treatment, but there was no significant change in tacrolimus treated co-cultures. 4 cones were tested with one co-culture per drug per cone, n=4 for drug-treated and vehicle control, n=12 for untreated and pre-culture.

5.6.10 There was no impact of drugs on CXCR5 expression

Early in the post-transplant period there were very few $CXCR5^{+}CD4^{+}$ T cells in the circulation of patients (see chapter 4, **fig 4.26**) and it was not clear if this was an impact of induction therapy, steroid therapy or maintenance therapy. Therefore Tfh cell phenotype was analysed pre and post co-culture to see if individual drugs had any impact on the phenotype of Tfh cells at the end of the 11 day stimulation.

There was a reduction in both CXCR5 and CXCR3 expression on Tfh1 cells (**fig 5.19A**) and in CXCR5 expression on Tfh2/17 cells (**fig 5.19B**) in both memory and naïve B cell co-cultures, this down-regulation was reduced by immunosuppressive therapy, possibly due to lack of proliferation.

In 6-MP treated co-cultures, CXCR5 expression (**fig 5.20A**) and CXCR3 expression on Tfh1 cells (**fig 5.20B**), and CXCR5 expression on Tfh2/17 cells (**fig 5.20C**) were not significantly different between 6-MP treated, NaOH treated or untreated co-cultures, although there was a trend towards preservation of CXCR5 expression on both Tfh1 and Tfh2/17 cells with 6-MP treatment, which may reflect reduced proliferation of cells.

Tacrolimus treatment showed preservation of CXCR5 expression on Tfh1 cells compared to MPA treatment, DMSO control or untreated controls ($p<0.01$) but this was still lower than pre-culture levels ($p<0.05$, **fig 5.21A**). Both tacrolimus and MPA reduced the proportion of $CXCR3^{+}CXCR5^{+}$ Tfh1 cells, and this

reduction trended to be greater than in untreated or vehicle controls, although this did not reach statistical significance (**fig 5.21B**).

It may be that the Th1-like skewing of $CXCR3^{+}CXCR5^{+}$ cells is more dependent on interaction with B cells, as all Tfh1-cell alone co-cultures showed greater loss of $CXCR3^{+}CXCR5^{+}$ compared to Tfh1 cells co-cultured with naïve or memory B cells (**figs 5.20B, 5.21B**), although again this did not reach statistical significance.

Both MPA and tacrolimus significantly reduced CXCR5 expression on Tfh2/17 cells compared to pre-culture levels ($p<0.001$) but at similar levels to untreated and vehicle control (**fig 5.22**).

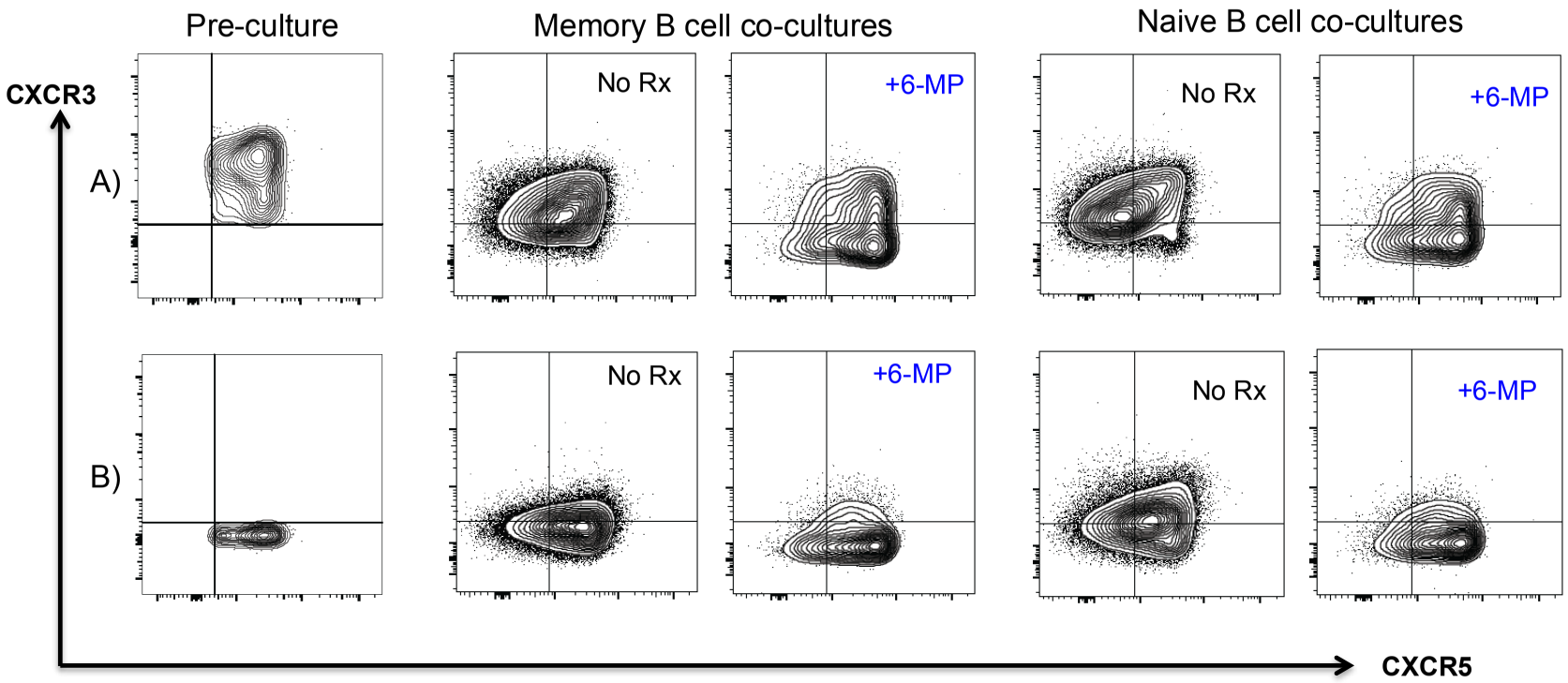


Figure 5.19: Representative flow cytometric contour plots showing alteration of Tfh phenotype before and after 11 days of co-culture. A) Impact of co-culture on Tfh1 cells with alterations in both CXCR3 and CXCR5 expression with untreated cultures and 6-MP treated cultures. B) Impact of co-culture on Tfh2/17 cells with alteration of CXCR5 expression and upregulation of CXCR3 expression in untreated and 6-MP cultures. Memory B cell cultures shown in top panels, naïve in bottom panels.

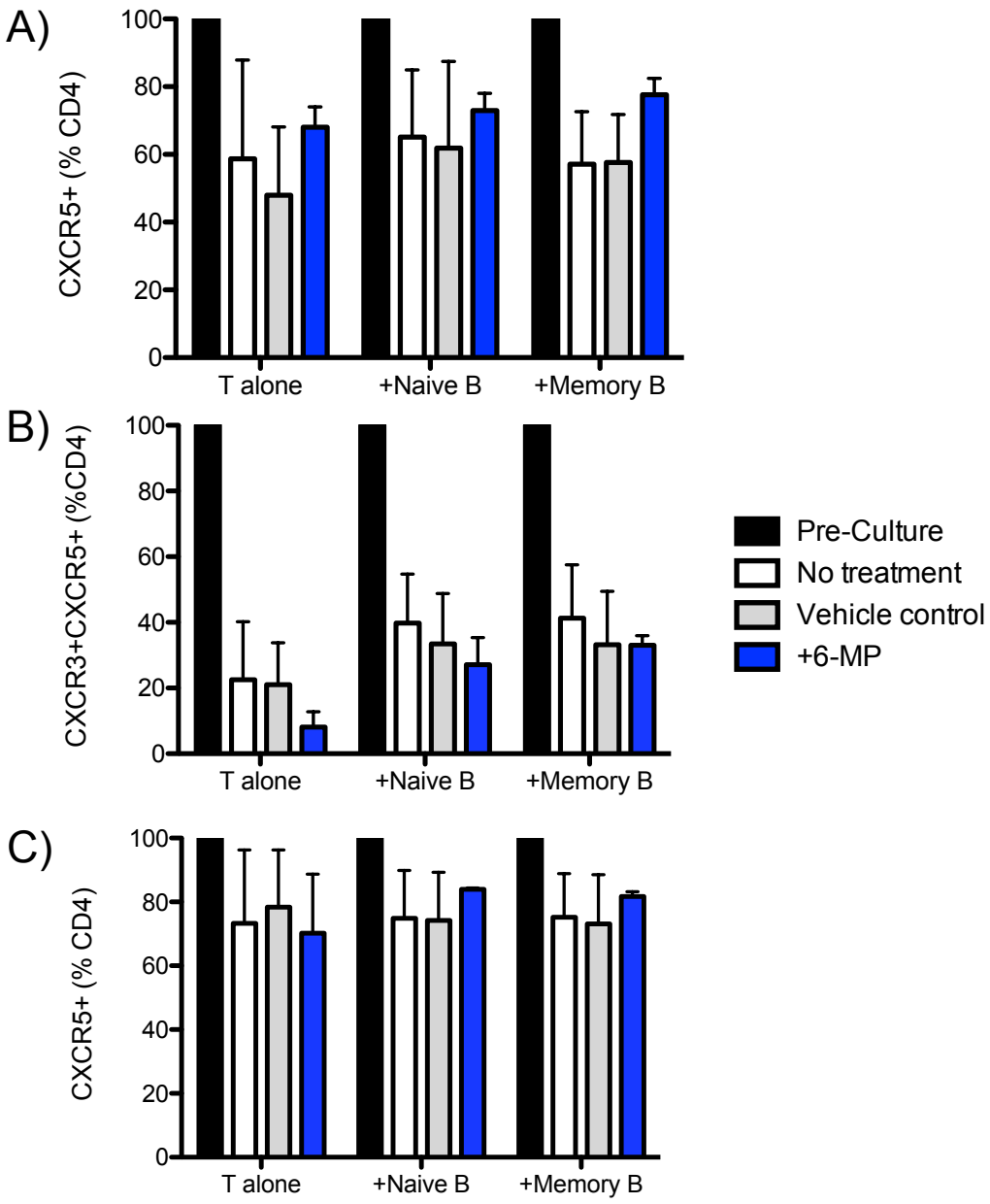


Figure 5.20. Impact of co-culture with 6-MP on Tfh phenotype. A) All Tfh1 cultures showed significant reduction in CXCR5 expression compared to pre-treatment ($p<0.001$) but there were no significant differences between untreated, vehicle control and 6-MP treated. B) All Tfh1 cultures showed significant reduction in $CXCR3^{+}CXCR5^{+}$ expression compared to pre-treatment ($p<0.001$) but there were no significant differences between untreated, vehicle control and 6-MP treated. C) All Tfh2/17 cultures showed significant reduction in CXCR5 expression compared to pre-treatment ($p<0.0001$) but there were no significant differences between untreated, vehicle control and 6-MP treated. Each bar represents mean with SD. 4 cones were tested with one experiment per cone, total n=4 per bar. Analysed using 1-way ANOVA with Bonferroni multiple-test correction.

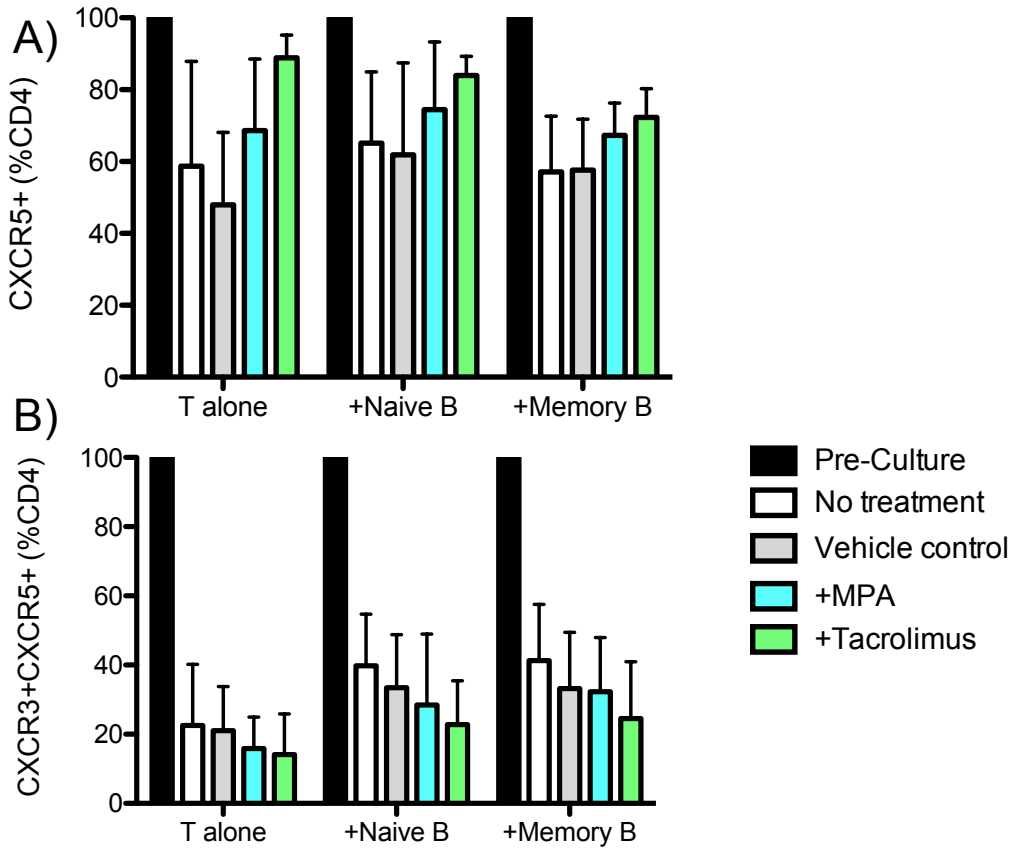


Figure 5.21. Impact of co-culture with MPA or tacrolimus on Tfh1 phenotype. A) Untreated, vehicle control and MPA treated Tfh1 cultures showed significant reduction of CXCR5 expression compared to pre-culture ($p<0.001$) but no significant differences between treatment groups. Tacrolimus treated co-cultures showed significantly reduced CXCR5 expression compared to pre-culture ($p<0.05$), but this remained significantly higher than in untreated, vehicle control and MPA treated co-cultures ($p<0.01$). B) All Tfh1 cultures showed significant reduction in $CXCR3^{+}CXCR5^{+}$ expression compared to pre-culture ($p<0.001$) but there were no significant differences between untreated, vehicle control, MPA or tacrolimus treated cultures.

Each bar represents mean with SD. 4 cones were tested with one experiment per cone, total n=4 per bar. Analysed using 1-way ANOVA with Bonferroni multiple-test correction.

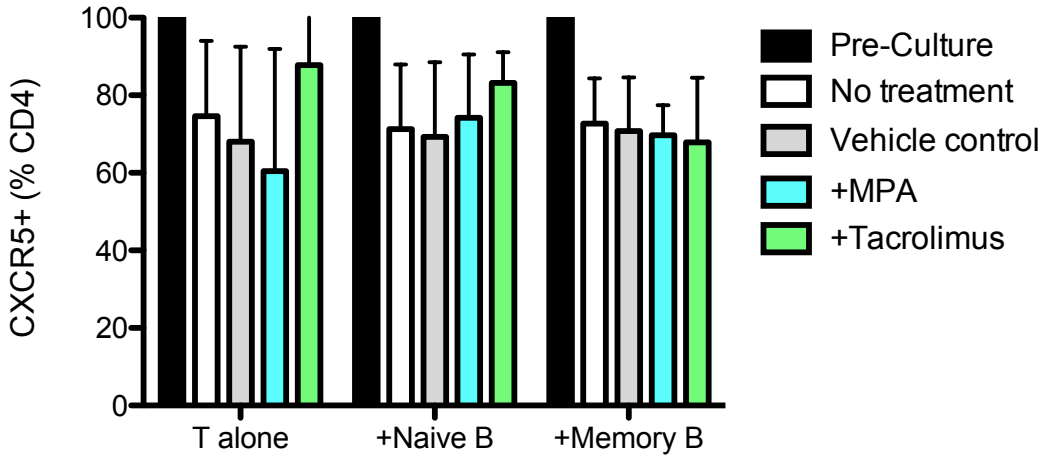


Figure 5.22. Impact of co-culture with MPA or tacrolimus on Tfh2/17 phenotype. A) All Tfh2/17 cultures showed significant reduction of CXCR5 expression compared to pre-culture ($p<0.001$) but no significant differences between treatment groups. Each bar represents mean with SD. 4 cones were tested with one experiment per cone, total n=4 per bar. Analysed using 1-way ANOVA with Bonferroni multiple-test correction.

5.6.11 Mechanism of 6-MP action

Given the altered phenotype of Tfh with 6-MP and the reduced plasmablast formation and absent antibody production, further investigation of the impact of 6-MP was undertaken. 6-MP is broken down into 6-thiouric acid, 6-methyl-MP and 6-thioguanine, which are the active compounds and are integrated into DNA on replication, blocking proliferation, as well as blocking *de novo* purine synthesis. The latter effect is particularly significant in lymphocytes, which, unlike other cell types, do not have a salvage pathway for purines.

To determine whether 6-MP was mainly inhibiting proliferation or inducing apoptosis in these co-cultures a timed assay was undertaken, retrieving cells at day 1, day 3 and day 5 to look at both B and T cell proliferation and apoptosis in the presence or absence of 6-MP or vehicle control (NaOH). Samples were sorted according to the usual protocol (section 5.6.2 above) and then T cells were stained with violet proliferation dye (VPD), and B cells with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured in duplicates to assess proliferation. At fixed intervals cells were recovered and stained with annexin V and 7AAD then assessed by flow cytometry to assess the levels of proliferation (**fig 5.23**) and apoptosis (**fig 5.25A**) respectively.

As expected, there was no proliferation of B or T cells on day 1, low levels on day 3 and good proliferation on day 5 in untreated and vehicle control samples (**fig 5.23**). Samples were assessed using division index, which is the average number of divisions that a cell in the original population has undergone, and includes the undivided peak. Proliferation index is calculated using the number of

divisions divided by the original number of cells that went into culture and therefore includes only those cells that have divided, not the undivided peak. The proliferation index therefore more accurately reflects how many divisions the average dividing cell has undergone, but the division index reflects the activity in the culture system as a whole.

In T cells labelled with VPD, there was a significant reduction in both division index (**fig 5.24Ai**) and proliferation index (**fig 5.24Aii**) in 6-MP treated compared to untreated or NaOH control ($p=0.0008$). This suggests 6-MP is both reducing the number of divisions per cell, but also the number of cells dividing.

B cells labelled with CFSE showed poor proliferation in naïve B cultures, but an increase in both division index (**fig 5.24Bi**) and proliferation index (**fig 5.24Bii**) in memory B cell cultures. Both were reduced by 6-MP treatment, but these results reflect one experiment only and therefore it was not possible to assess the significance of these alterations.

Levels of apoptosis were not significantly different at day 1 (**fig 5.25Bi**) or day 3 (**fig 5.25Bii**), but there was a significant difference in late apoptotic cells at day 5 in 6-MP co-cultures compared to untreated co-cultures on one-way ANOVA (**fig 5.25Biii**). Taken together, these results demonstrate that 6-MP acts by both inhibiting cellular proliferation and increasing apoptosis, resulting in reduced cell number, and responsiveness.

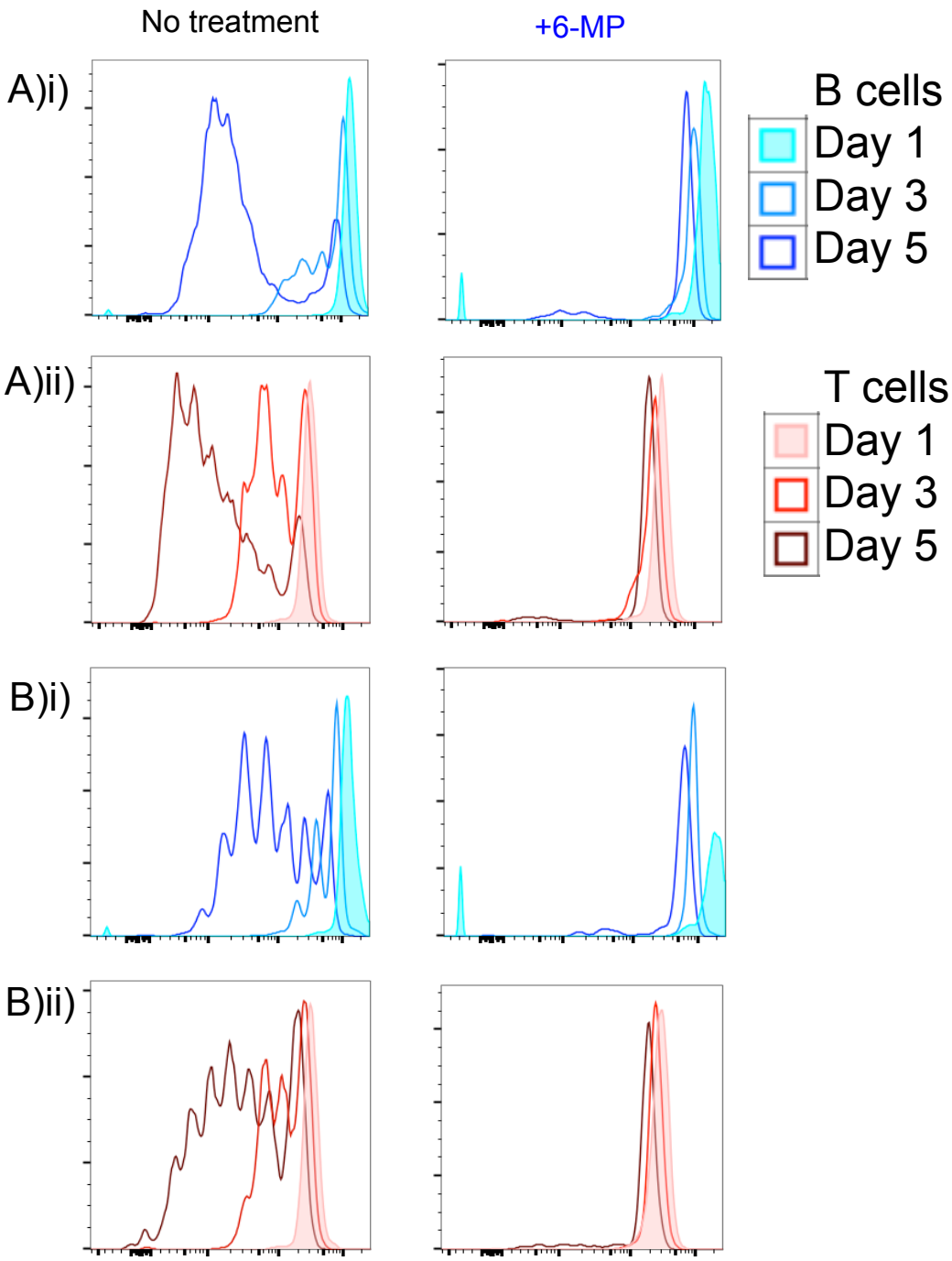


Figure 5.23: Representative flow histograms showing proliferation of B and T cells. A) Memory B cell and Tfh2/17 cocultures showing proliferation of i) B cells with CFSE dilution and ii) T cells with VPD dilution, with untreated (left) and 6-MP treated (right). B) Naïve B cell and Tfh2/17 cocultures showing proliferation of i) B cells with CFSE dilution and ii) T cells with VPD dilution with untreated (left) and 6-MP treated (right).

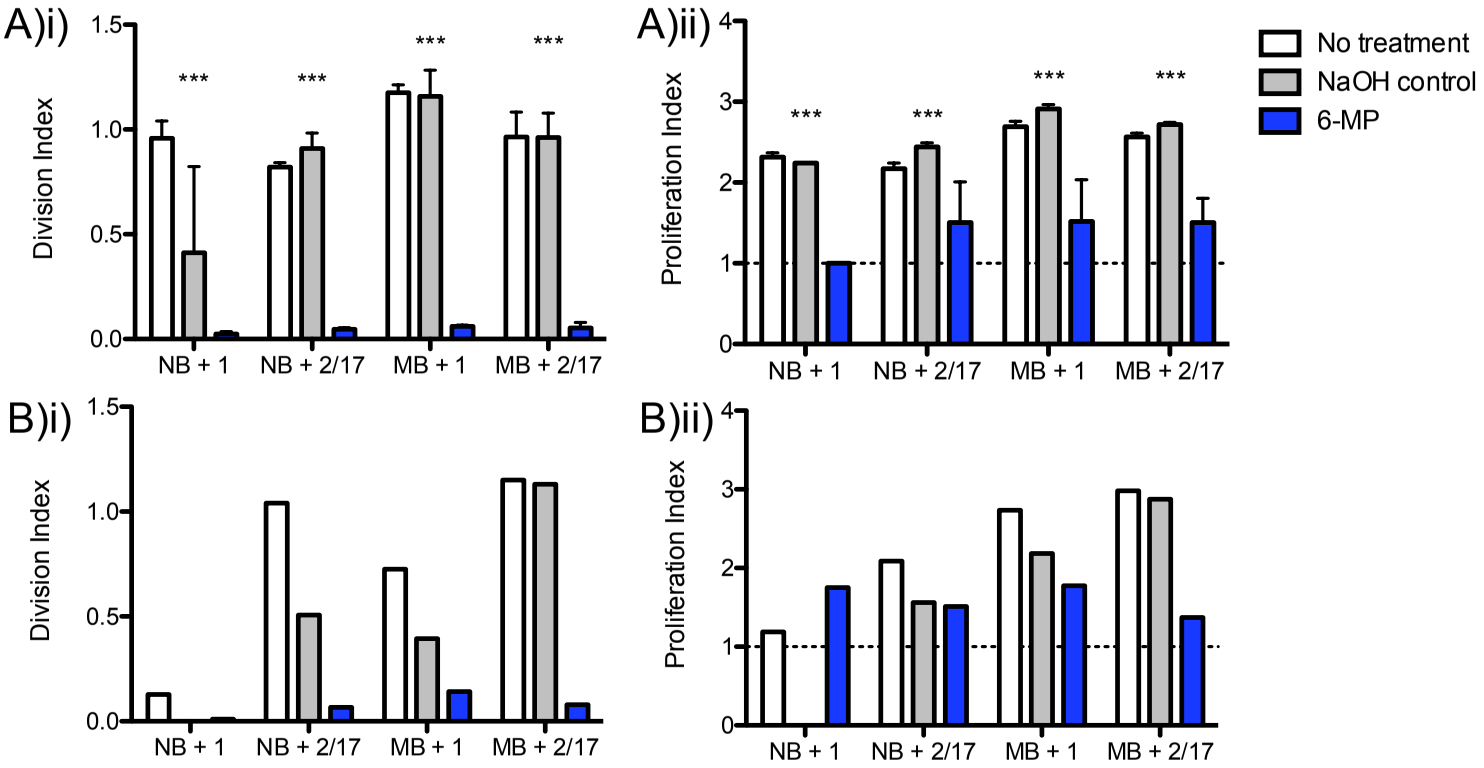


Figure 5.24: Division and proliferation of cells. A) Proliferation of T cells after 5 days of co-culture enumerated from dilution of VPD. One way ANOVA showed significant reduction in both (i) division index ($p=0.0008$) and (ii) proliferation index ($p=0.0008$) in all culture conditions. Data from 2 independent experiments with 2 pooled replicates per bar. Shown as median and range. B) Proliferation of B cells after 5 days of co-culture enumerated from dilution of CFSE showing i) division index and ii) proliferation index. Data from one experiment. No statistics shown as only one data point per culture condition (replicates pooled for flow cytometry).

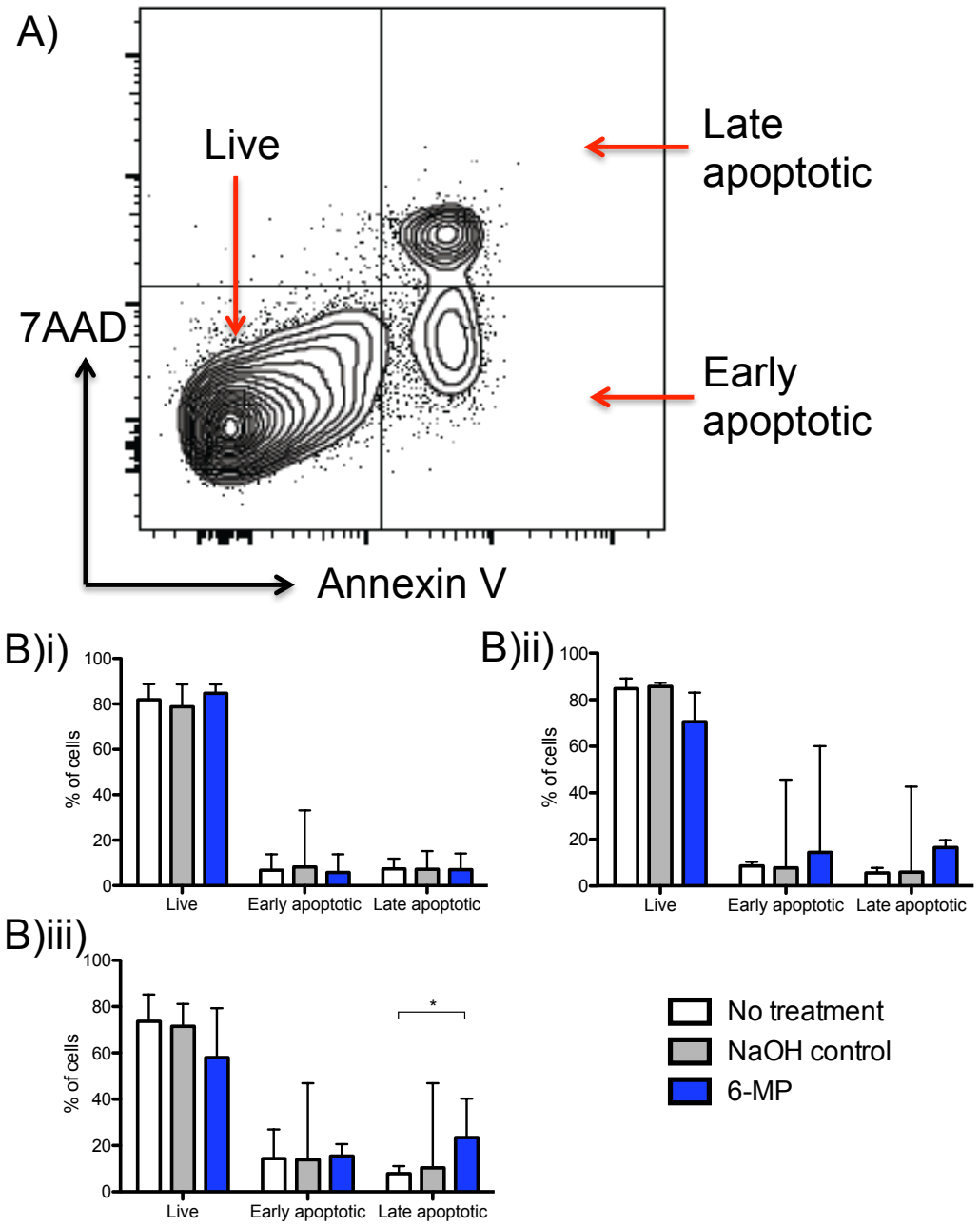


Figure 5.25: Apoptosis in treated vs untreated samples. A) Representative flow cytometric plot showing gating strategy for apoptosis, B) differences between untreated, vehicle treated and 6-MP co-cultures showing apoptosis at i) day 1, ii) day 3, iii) day 5, where late apoptotic cells in 6-MP treated were significantly different to untreated samples ($p<0.05$). Each bar represents mean + SD of combined duplicates, with n=16 per bar. Analysed with one way ANOVA comparing treated to untreated.

5.6.12 Reduction in antibody production appears to be mediated through 6-MP effects on Tfh cells

Assessment of surface markers in dead or dying cells is unreliable due to auto-fluorescence and non-specific staining. It was not clear if the increased apoptosis was due to B cells or T cells, and which cell type was most affected by 6-MP. Clinical data and previous studies have suggested a disproportionate effect of azathioprine on B cells compared to MPA[413,458] and interaction between B and Tfh cells is required to maintain the Tfh cell phenotype[144,459,460]. It is possible that the impact of 6-MP on B cells is the main reason for the alterations in Tfh cell phenotype seen, and the lack of PD-1 upregulation (**fig 5.18**).

To try and investigate the impact of 6-MP on T cells alone, cells were sorted as previously described, and then T cells were pre-incubated in complete media or media containing 6-MP or NaOH vehicle control. Although azathioprine is broken down very slowly to 6-MP *in vivo*, 6-MP is taken up by cells very rapidly, with high intracellular levels seen *in vitro* within 15-20 minutes and peak levels by 60 minutes[461]. With this in mind, T cells were incubated at 37^{o}C for 1 hour to ensure reasonable uptake of 6-MP, and then washed extensively to remove any residual drug. T and B cells were then incubated as previously described and assessed at 11 days. In these cultures, where 6-MP is only acting in T cells, plasmablast development in pre-incubated cultures was abrogated both as proportion of B cells ($p<0.01$, **fig 5.26A**) and total number in naïve B cell cultures (**fig 5.26Bi**) and memory B cell cultures (**fig 5.26Bii**).

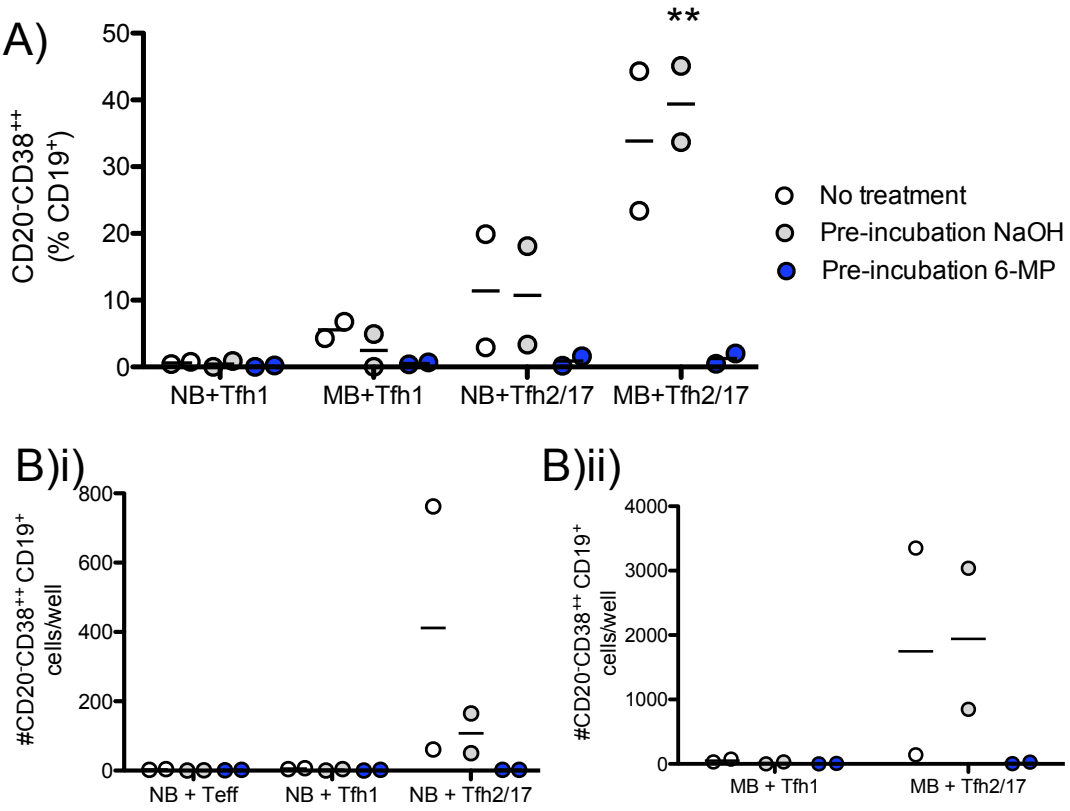


Figure 5.26: Effects of pre-incubating T cells alone with 6-MP prior to co-culture with B cells. A) Plasmablast development as a proportion of $CD19^+$ cells. No statistically significant differences seen when Tfh1 were co-cultured with either $CD27^+$ memory B cells or $CD27^-$ naïve B cells, or when Tfh2/17 were co-cultured with naïve B cells. Significant decrease in plasmablast development with 6-MP pre-incubated Tfh2/17 compared to untreated or NaOH pre-incubated Tfh2/17 when co-cultured with memory B cells ($p<0.01$) on 1 way ANOVA with Bonferroni correction. B) Plasmablast development shown as absolute cell count for (i) naïve B cell co-cultures and (ii) memory B cell co-cultures. Results did not reach statistical significance on ANOVA testing. For each graph, each point represents a combined duplicate, 2 cones were tested in two independent experiments, so n=2 per point.

There was a concordant decrease in the production of antibody, which was absent in the 6-MP pre-incubated cultures but present in untreated and vehicle control samples. IgG was significantly reduced in memory B co-cultures with 6-MP pre-incubated Tfh2/17 cells compared to untreated or NaOH pre-incubated ($p<0.001$, **fig 5.27Ai**), and IgM was similarly significantly lower ($p<0.001$, **fig 5.27Aii**). Negligible IgG production was seen in naïve B co-cultures in this experiment, however IgM production was significantly reduced in NB co-cultures with 6-MP pre-incubated Tfh2/17 compared to untreated or NaOH pre-incubated ($p<0.0001$, **fig 5.27B**).

While isolated T cell treatment with 6-MP seems to have a significant impact on both plasmablast production and antibody output, there are only two data points for this co-culture, with two cones tested in one preliminary experiment, it is therefore difficult to draw firm conclusions. Of note, in this culture there was very poor plasmablast production with Tfh1 and memory B cell co-cultures, but as seen from previous experiments, there is significant variation in plasmablast production and antibody output even with different aliquots of the same cone (see **fig 5.7**).

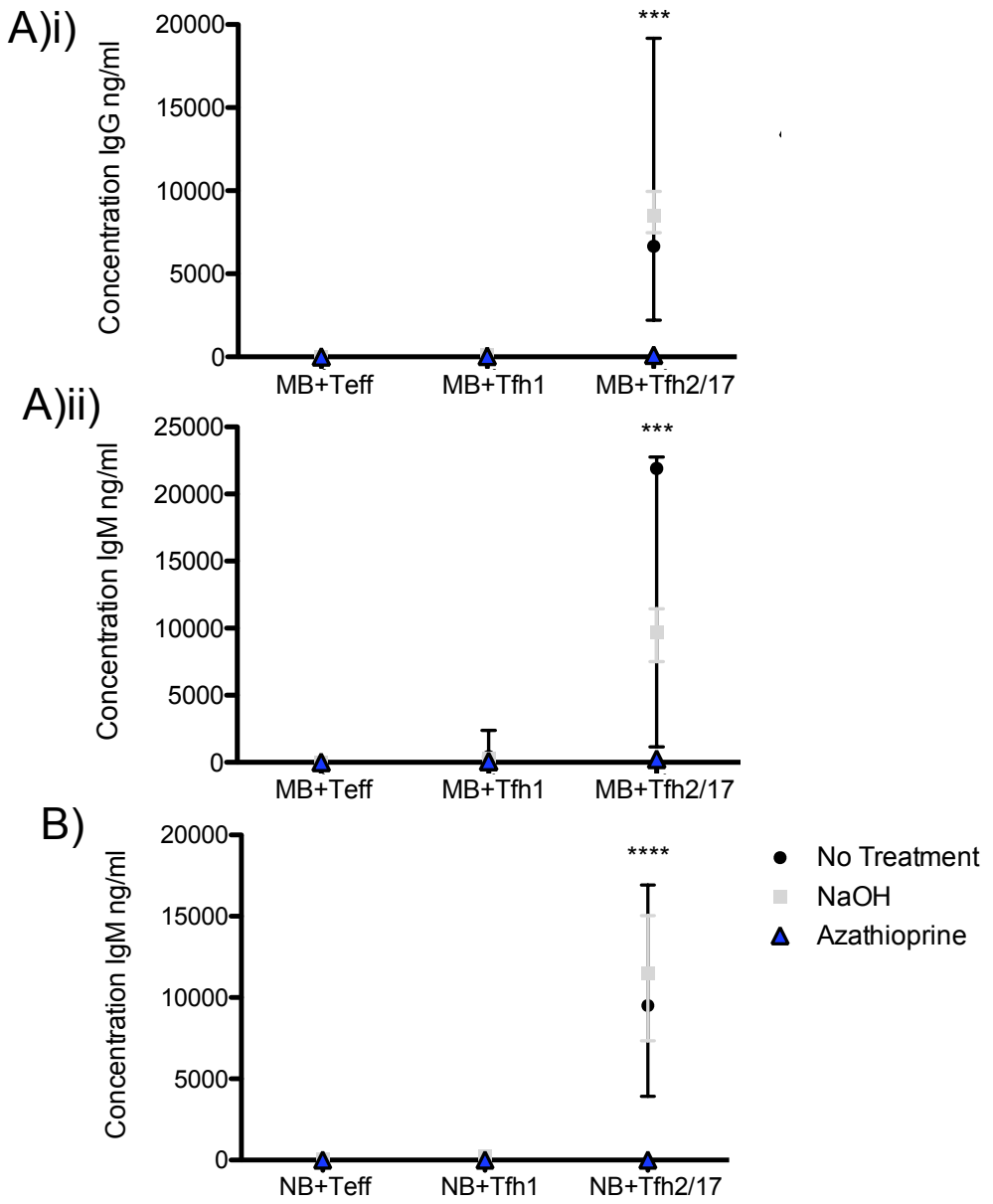


Figure 5.27: Antibody outputs following pre-incubation of T cells with 6-MP. A) Outputs from memory B (MB) cell co-cultures showing (i) a significant reduction in IgG production from MB co-cultured with Tfh2/17 pre-incubated with 6-MP ($p<0.001$) compared to untreated or NaOH pre-incubation. A)ii) similar pattern with IgM production, showing a significant reduction in IgM production when Tfh2/17 were pre-incubated with 6-MP ($p<0.001$) compared to untreated or NaOH pre-incubation. B) IgM production from naïve B cell co-cultures showing significant reduction in antibody production in MB co-cultured with Tfh2/17 pre-incubated with 6-MP ($p<0.0001$) compared to untreated or NaOH pre-incubation. 2 cones were analysed in one experiment with replicates run separately, n=6 per condition. Analysed with 2-way ANOVA.

5.6.13 Upregulation of PD-1 on Tfh cells was similar in untreated and pre-incubated 6-MP cultures

Interestingly the reduction in PD-1 expression on Tfh seen with combined B and T cell 6-MP treatment was not observed (**fig 5.28**), with a significant increase in PD-1 expression compared to pre-culture in untreated ($p<0.001$), NaOH pre-incubated ($p<0.001$) and 6-MP pre-incubated ($p<0.01$) T cells. This provides some support for the idea that PD-1 upregulation is due to continuous TCR activation by interactions with B cells[144,459,460] as the highest levels are seen in GCs, and peripheral blood Tfh cells express lower levels of PD-1 when resting[127]. This may explain some of the reduction in PD-1 expression seen in mixed co-cultures (**fig 5.18**), as 6-MP inhibited B cell proliferation and therefore limited B cell-Tfh interaction. However, Tfh pre-incubated with 6-MP had PD-1 expression comparable to untreated cells and were still unable to provide help to B cells to produce antibody, so the absence of stimulation from B cells is clearly not the only mechanism limiting Tfh cell function.

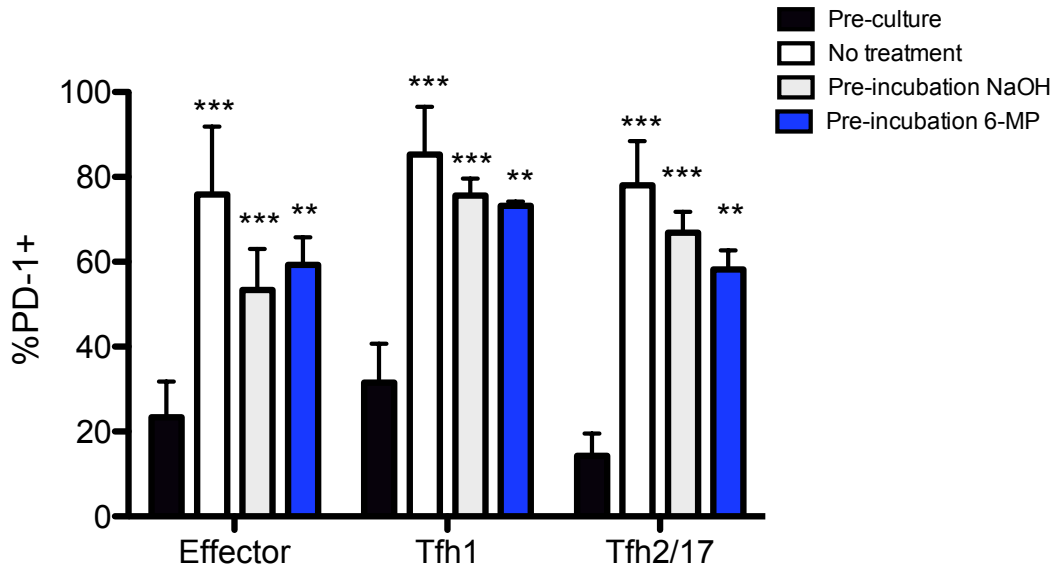


Figure 5.28: Impact of pre-incubating T cells with 6-MP on PD-1 expression. There was a significant difference in PD-1 expression on all T cell subsets after co-culture when compared to pre-culture expression levels. This was significant for untreated T cells ($p<0.001$), T cells pre-incubated with NaOH ($p<0.001$) and T cells pre-incubated with 6-MP ($p<0.01$). Samples compared to pre-culture PD-1 expression with 1 way ANOVA. Data from 2 cones from 2 independent experiments. Duplicate wells combined for flow cytometry, n=8 for pre-culture and untreated samples, n=4 for cells pre-incubated with NaOH or 6-MP.

These preliminary experiments suggested a differential effect of 6-MP compared to MPA or tacrolimus. However, further work is required to further elucidate the detailed mechanisms of action of these drugs. In particular, given the difficulty in finding equivalent doses of antimetabolites, and therefore the difficulty comparing MPA with 6-MP either *in vitro* or *in vivo*, further experiments would be important to assess the differences between the drugs. The doses used for these *in vitro* studies were selected to mimic those seen in the clinic and for MPA are higher than used in previous *in vitro* systems. A dose escalation study would be useful to determine the concentration at which each drug has its maximal impact, however the drawback of this strategy is that higher doses may simply lead to the death of B cells. Repetition of the pre-incubation study with MPA and varied doses of 6-MP would add weight to these findings and allow some elucidation of mechanism.

However, one of the biggest limitations with the *in vitro* system is the lack of drug binding proteins. In serum, only a portion of drug is available in an unbound state, up to 99% of MPA can be bound to plasma proteins[462], and around 30% of azathioprine is protein bound. It is therefore difficult to find an equivalent dose for direct comparison *in vitro* even with the use of serum in the media. In order to bridge the gap between isolated cells *in vitro* and clinical data, experimental animal models can provide a more physiological system but with manipulation that would be impossible in human studies. We were keen to see if any of the results seen *in vitro* translated into an *in vivo* system, to add weight to the clinical data, which had been suggestive but not reached statistical significance.

To try and address this, an *in vivo* model of skin transplantation was set up, using techniques familiar to the laboratory with well-established protocols for the use of immunosuppression (TRIG lab unpublished data, Blaha *et al.*[463]). This means animals can be treated with equivalent doses to those used in humans (see chapter 2, sections 2.22-23), allowing translation of the differences between individual drugs seen in the *in vitro* system into a more physiological set up.

5.6.14 In Vivo experiments

The model used was an HLA mismatched, full-thickness tail skin transplant from C57BL/6 ($H2^b$) donors to CBA.Ca ($H2^k$) recipients. Under normal circumstances mice with allogeneic grafts reject their grafts by 14-17 days, shown here compared to syngeneic CBA to CBA transplants, in which grafts are maintained long-term (**fig 5.29**). At fixed intervals, groups of mice were sacrificed and the draining left axillary lymph node (DLN) removed and compared to the contralateral LN (CLN). Nodes were stained to assess for Tfh (**fig 5.30A**), defined as $CXCR5^+PD\text{-}1^+CD4^+$. These were seen at day 7 post-transplant, when they peaked and began to decline (**fig 5.30B**). GC B cells were defined as $Ki67^+Bcl6^+B220^+$ cells (**fig 5.30C**) and, while some were seen by day 7 post-transplant, numbers peaked at days 10-14 before declining after graft rejection (**fig 5.30D**). In contrast to Tfh cells, where no significant difference was seen between DLN and CLN, GC B cells were significantly higher in DLN compared to CLN at day 10 ($p=0.01$), day 14 ($p=0.046$) and day 21 ($p=0.017$). This demonstrates that allogenic skin grafts induce a GC response, and therefore provide an *in vivo* model to study the effects that immunosuppressive drugs have on this response in the context of transplantation.

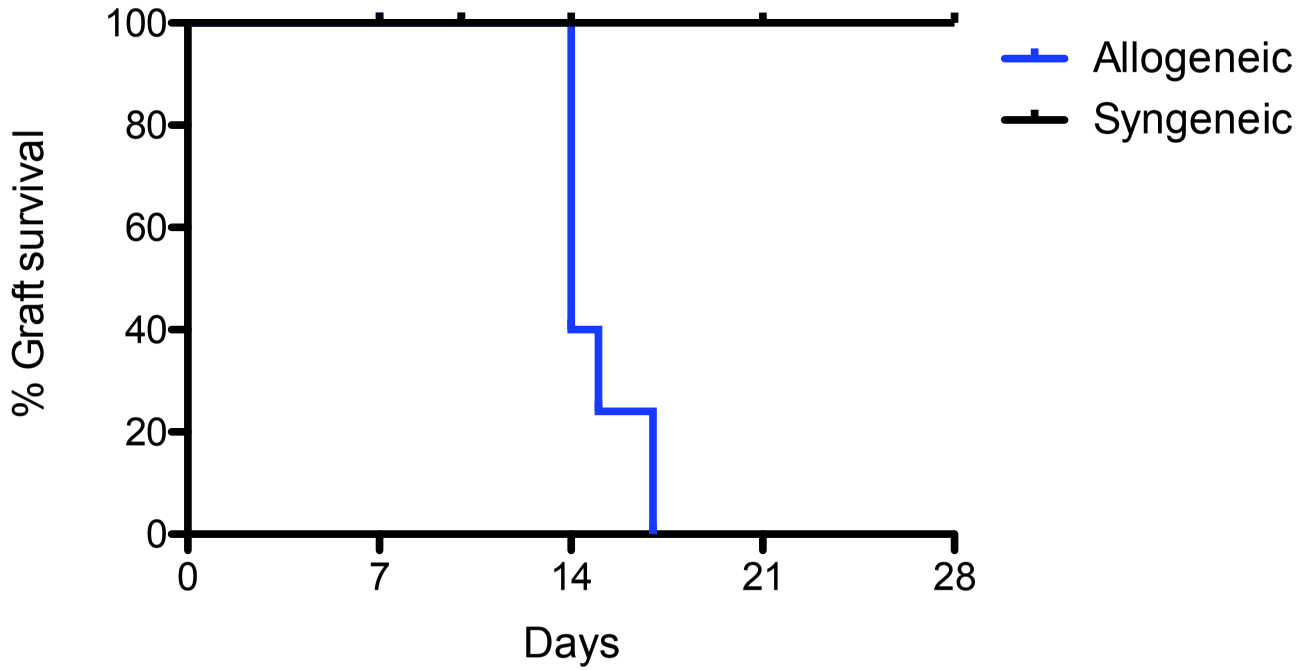


Figure 5.29: Survival of murine skin transplants. Syngeneic transplants from CBA donor to CBA recipient show long term survival, allogeneic transplants from C57BL/6 donor to CBA recipient all reject between days 14-17. n= 15 syngeneic, n=35 allogeneic. Pooled data from 3 independent experiments.

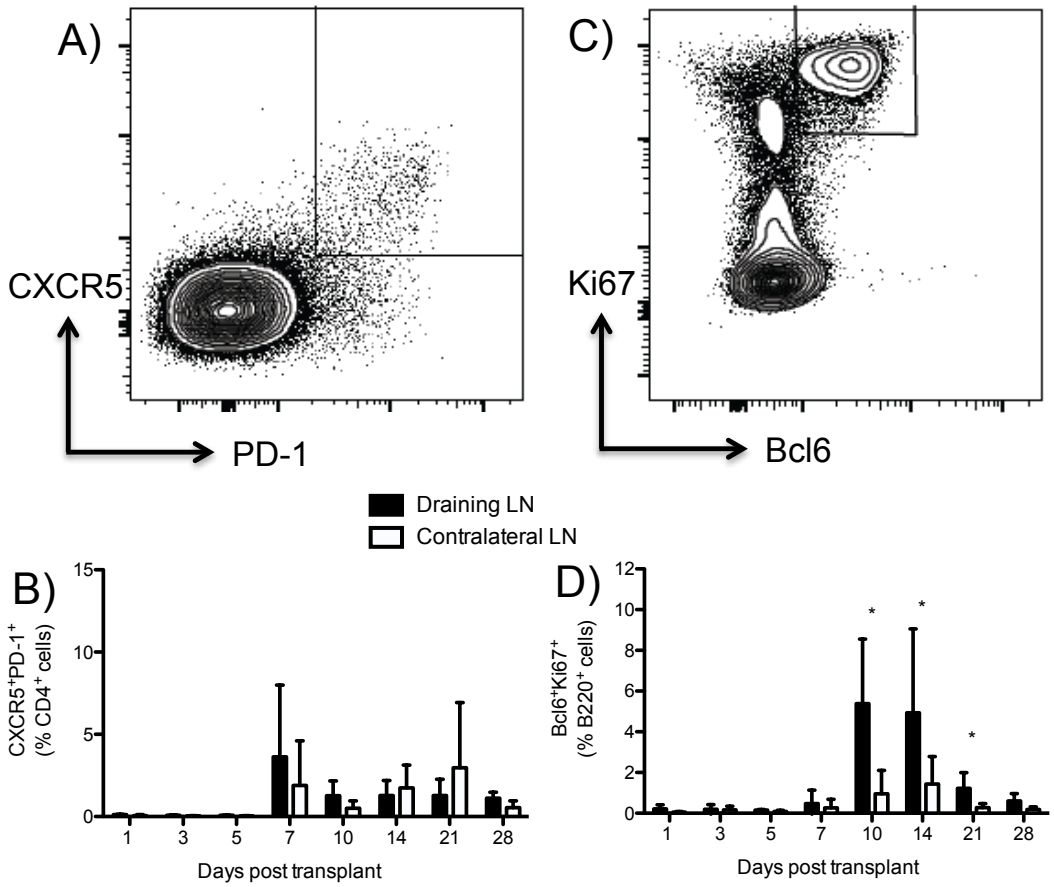


Fig 5.30 Mouse skin transplant. All samples were gated on lymphocytes and then CD4 vs B220. A) Representative flow cytometric contour plot showing gating for Tfh, defined as $CXCR5^{+}PD\text{-}1^{+}$ in the $CD4^{+}$ gate. B) Tfh as proportion of $CD4^{+}$ cells, shown as mean and SD. There was no statistically significant difference between draining lymph node (DLN) and contralateral lymph node (CLN) on paired T-testing at any time point. C) Representative flow cytometric contour plot showing gating for germinal centre (GC) B cells, defined as $Ki67^{+}Bcl6^{+}$ within the $B220^{+}$ gate. D) GC B cells as proportion of $B220^{+}$ cells, shown as mean and SD. GC B cells were significantly higher on paired T test in DLN compared to CLN at days 10 ($p=0.010$), 14 ($p=0.046$) and 21 ($p=0.017$), but did not reach significance for other time points. Pooled data from 3 independent experiments, n=4 days 1-5; n=12 day 7; n=8 days 10-28. All mice rejected skin grafts between days 14-17.

5.6.15 MMF-treated in vivo model

On the basis of this, we set up a skin transplant model with MMF treatment. Previous work with immunosuppression in our lab had suggested that MMF dosed at 1mg/kg on day 0, 1, 3 and 5 post-transplant increased the median survival time (MST) from 8 days to 80 days in C57BL/6 to CBA heterotopic heart transplants (A. Whatcott, unpublished data, see chapter 2, section 2.23). A preliminary experiment was therefore set up using this dosing regimen of MMF in CBA mice transplanted with a C57BL/6 skin graft and mice sacrificed at days 7, 14 and 21. At all time points there were 5 untreated mice and 7 treated mice.

Unfortunately, in skin transplantation the MST was not prolonged with the addition of MMF treatment at this dosing level (**fig 5.31A**). However there were alterations in Tfh and GC B cells. At day 7 Tfh in the DLN were higher in untreated than MMF treated mice, although this did not reach statistical significance (**fig 5.31B**). Tfh cell numbers were maintained at day 14 in treated mice but had fallen from their peak in untreated mice. Surprisingly, GC B cell numbers were unaffected, with equivalent numbers at day 14. At day 21 there was a trend towards higher numbers of GC B cells in MMF treated compared to untreated mice, although this did not reach significance on Mann-Whitney U test p=0.06 (**fig 5.31C**).

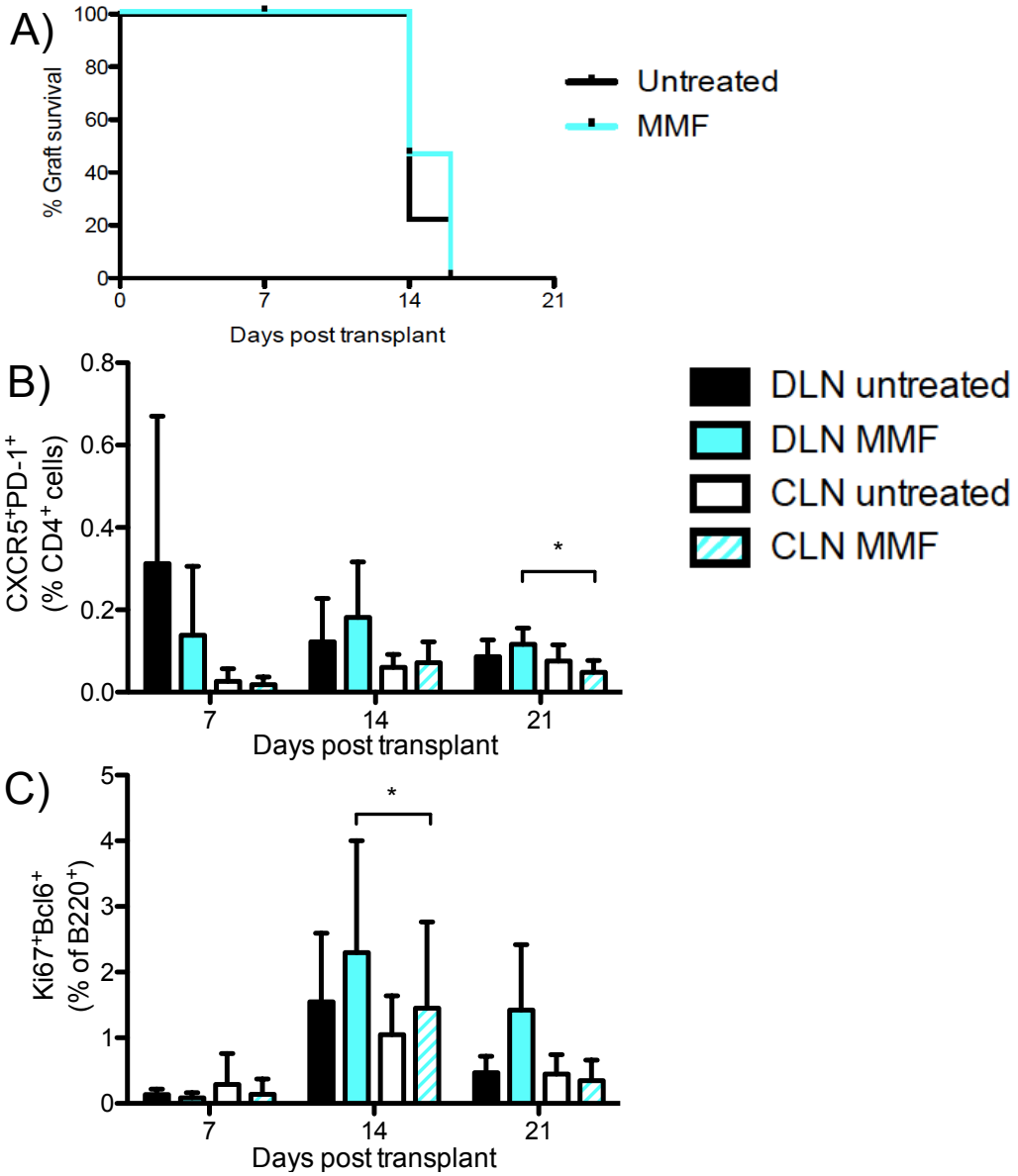


Figure 5.31 Impact of MMF on skin transplantation outcomes. A) Graft survival was unchanged in MMF treated compared to untreated mice. B) Tfh cells showing significantly higher Tfh in DLN compared to CLN from MMF treated mice at 21 days (p=0.03, paired T test) but no other significant differences between MMF treated and untreated (unpaired T test). Shown as mean and SD. C) GC B cells showing significantly higher GC B cells in DLN compared to CLN from MMF treated mice at 14 days (p=0.02, paired T test) but no other significant differences between CLN and DLN or MMF treated and untreated. Shown as mean and SD. Data from one experiment, n = 36 mice total, n=5 per untreated time point, n=7 per MMF-treated time point. Samples from DLN compared to CLN of same mouse with paired T test, samples from treated mice compared to untreated mice with unpaired T test.

Unfortunately, due to time constraints, I was unable to repeat this experiment to find an optimal dose of MMF to prolong MST in skin transplantation. Additionally, it was not possible to complete *in vivo* experiments for azathioprine or tacrolimus.

Along with *in vitro* dose escalation studies, these would be important next steps to further elucidate the effects of different immunosuppressant drugs.

5.7 Conclusions

The aim of these experiments was to elucidate the effects of the immunosuppressive agents azathioprine, MMF and tacrolimus on Tfh and B cells. In the *in vitro* system all drugs abrogated plasmablast formation and reduced antibody production by B cells. Mycophenolic acid, the active form of MMF, had a lesser impact than 6-mercaptopurine, the active form of azathioprine, despite both drugs co-opting the purine synthesis pathway. MMF is also thought to be more potent and to have a more lymphocyte-specific action, but at the dose used it allowed both plasmablast development and antibody production.

Tacrolimus has a different mechanism of action compared to azathioprine or MMF, and while not reaching significance, seemed to allow more plasmablast formation and antibody production than 6-MP, but less than MPA.

This may be due, in part, to the impact of individual drugs on B cells. Tacrolimus is a T-cell targeted immunosuppressant, while both MMF and azathioprine will

target all cycling lymphocytes. Azathioprine seems to have a greater impact on B cells in both this study (chapter 4, **figs 4.13-4.20**) and previous clinical[413] studies, and this *in vitro* work provides evidence to support this.

These experiments show that the impact of azathioprine on PD-1 expression, seen in the clinical work and early *in vitro* experiments, seems to be due to its impact on B cells, supported by the pre-incubation data where only T cells were treated and PD-1 expression was equivalent to untreated. However, further experiments would be required to elucidate this further. I would plan to repeat the time-course experiment with more donors, as well as with MPA and tacrolimus. For future experiments, I would also plan to pre-incubate T cells alone with all immunosuppressive drugs to separate the impact of immunosuppressive agents on T cells from any impact on B cells, as a block in B cell proliferation will prevent antibody formation in any co-culture condition.

Given the differences seen in azathioprine compared to MMF, it would be important to carry out a dose-escalation study, as the doses used were equivalent in Treg expansion studies, but may not be so in co-culture studies.

While the *in vivo* work did not provide much evidence of the impact of immunosuppressive drugs on antibody producing cells, it has the advantage over *in vitro* work by providing a more physiological system, therefore it would be useful to optimise this experimental set up.

The murine model used for *in vivo* work did not give clear separation of MMF treated and untreated animals. It is possible that the doses used for cardiac transplantation are not sufficient for skin transplantation, and a dose escalation study to establish when MST can be prolonged would be important. A power calculation using the means and SDs generated by this experiment suggested that 36 mice per group would be needed to see a significant difference between MMF treated and untreated Tfh cells in DLN, giving a total of 216 mice per experiment. This would be far in excess of recommended numbers, and therefore refinement of the process by finding the optimal dose to prolong MST would be a more appropriate next step.

It is also possible that, while cell numbers were not significantly altered, antibody levels may have been affected by immunosuppression. However, I was not able to optimise this assay within the time available.

In summary, *in vitro* work supported the clinical work, which suggested a differential effect of azathioprine on Tfh cells compared to MMF. This may be mediated by the effects of azathioprine on B cells, rather than on the Tfh cells themselves, but further work would be required to elucidate this.

6. Discussion and future directions

6.1 Aims of study

The aim of this study was to recruit a prospective cohort of kidney and SPK transplant patients using paired blood and LN samples, and longitudinal immunophenotyping to assess the potential of cTfh and cTfr as biomarkers predicting *de novo* DSA formation.

A secondary aim was to investigate the effects of commonly used immunosuppressive drugs on both the development of *de novo* DSA and the cells that cooperate to produce antibodies, with the objective of identifying better therapeutic strategies for the management of patients who do develop *de novo* DSA.

Key findings from this study

6.2 Circulating cells as biomarkers of *de novo* DSA formation

De novo DSA formation occurs in up to 30% of kidney transplant recipients over the lifetime of the graft, both shortening graft survival and also making re-transplantation more difficult[13,199,464-466]. In human and mouse studies cTfh have been suggested as biomarkers of GC activity[125,128], with particular focus on those expressing high levels of PD-1 as being markers of active Tfh differentiation, and therefore markers of an on-going GC reaction[125,130,467]. In many human studies,

the immunising antigen is either unknown (autoimmune disease) or the timing of immunisation is not known (HIV infection, autoimmunity). In contrast, in the setting of transplantation the timing of alloantigen exposure is clearly defined, and the 'adjuvants' of inflammation around surgery well explored. Unfortunately the time to development of DSA after transplantation is variable[11,437,468], depending on factors such as immunosuppressive drugs, concordance with therapy, inflammatory complications such as infection or acute TCMR, and the degree of mismatch between donor and recipient[198,209,372,464,469]. There are no currently accepted biomarkers for DSA formation in transplantation, and, although small studies have suggested that $CXCR5^{+}CD4^{+}$ T cells are higher in kidney transplant patients with preformed DSA[273] and in those with chronic ABMR[407], no comprehensive studies have been undertaken investigating the correlation between cTfh and risk of *de novo* DSA formation in transplantation.

The study was designed to test the hypothesis that cTfh or subsets of cTfh would be higher in patients developing *de novo* DSA compared to those who did not.

In this study, as seen in chapter 3, 6 patients developed *de novo* DSA. These patients were more likely to have poorly HLA-matched grafts (4/6 poorly matched, 2/6 moderately mismatched) but there was significant heterogeneity between patients developing *de novo* DSA in terms of age, gender, donor type and treatment regimen. While this is not surprising given wider clinical experience and previously reported data[13,242,403,464,470,471] it makes interpretation of the data from this study more difficult, as there are many confounding factors when correlating DSA development with circulating cell populations.

Comparison of pre-transplant samples showed that tacrolimus treatment did impact on cTfh and, importantly, that the changes seen in circulating cells were reflected in LN cells. This suggests that cTfh are a reasonable biomarker of LN Tfh as seen in animal models and previous data[125,128],[8,9] and post-transplant phenotyping of peripheral blood in transplant patients could use the surface markers that correlated with LN cells.

With longitudinal phenotyping, the data obtained showed that $CXCR3^{-}$ $CXCR5^{+}PD\text{-}1^{+}$ cTfh were higher at all time points in patients developing *de novo* DSA (**fig 4.35**). However, as the numbers of patients developing DSA in the study were small the finding did not reach statistical significance. Nevertheless, these findings are exciting and suggest that further work in a larger cohort of patients would be worth pursuing.

Other cTfh subsets that have been associated with antibody formation were not significantly different between patients developing *de novo* DSA and those who did not. There was a trend towards higher cTfh17 at later time points in patients developing *de novo* DSA (**fig 4.39**), and it remains to be seen whether the inclusion of CCR6 as a marker of cTfh17 in future studies will be useful as a predictor of later DSA development.

No cTfh cell subset was higher in patients developing *de novo* HLA antibodies that were not donor directed (**figs 4.35-40**). This may be because a GC response is not always generated in the case of non-donor directed HLA antibodies. Many of these non-donor directed antibodies were detected early post-transplant, and

may be either transfusion related or memory responses triggered by the inflammation around surgery. Transfusion related HLA antibody formation is often transient, antibody levels drop with time[195] and memory B cells are rarely found[194]. This suggests these antibodies may reflect an extra follicular B cell response, particularly in patients who are T cell depleted at induction, as was the case for the group of patients treated with alemtuzumab. Studies have also suggested that memory B cells seem relatively resistant to depletion[451] with alemtuzumab. The acute inflammatory milieu around transplantation may lead to recall responses against antigens to which the recipient has been previously sensitised, but titres should decline over time without on-going antigenic stimulation[201,472,473].

A novel finding was that cTfr were lower in patients developing *de novo* DSA compared to those who did not (**fig 4.34**). Again this did not reach statistical significance, but numbers were small. A small number of studies have been done looking at cTfr in human autoimmunity with mixed results, some showing higher proportions[474] while others demonstrated lower levels[147]. Another study in chronic viral infection showed that higher levels of cTfr correlated positively with higher levels of hepatitis B or C viral DNA[475], suggesting that in the presence of chronic antigen stimulation, having higher levels of Tfr prevents the formation of antibodies that might help clear the infection. The situation in transplantation may be similar as parallels can be drawn between chronic viral infection and chronic exposure to donor alloantigens.

Extensive animal work supports this hypothesis, showing that the Tfr:Tfh ratio is important for control of antibody responses. Before antigen stimulation, the balance of Tfr to Tfh is high in steady state SLOs. With immunisation, Tfh differentiate more rapidly than Tfr and skew the balance, allowing the GC response to form. After Tfh numbers begin to decline, the proportion of Tfr to Tfh falls back to baseline levels, suggesting dynamic regulation of both cell numbers and GC responses[128,148,476]. This work suggests that a low Tfr to Tfh ratio limits the suppression of GC responses, and, combined with the work showing high levels of cTfr correlate with impaired clearance of chronic viral infections[475] and are memory counterparts of LN Tfr[128], suggests that the balance of cTfr and cTfh may reflect the balance of Tfr and Tfh in the SLOs.

In this cohort of transplant patients, those developing *de novo* DSA had lower levels of Tfr at all time points, and a low ratio of Tfr to Tfh at all time points (**fig 4.43**), although again this did not reach statistical significance. It has been proposed that cTfr may prevent reactivation of circulating memory B cells and cTfh in the context of low-level antigen[128,477]. This is particularly relevant in the context of transplantation, where many patients will have immunological memory to HLA antigens[227], and shared epitopes in the context of acute inflammation may provide a low-level trigger to these memory cells.

While small patient numbers and significant heterogeneity within the cohort limited this study, it did highlight the potential for cTfh and cTfr as biomarkers predicting *de novo* DSA formation. This data, while not conclusive, suggests that

a larger study with carefully selected patients would be worth pursuing. This is addressed in more detail in Future Directions, below.

6.3 Impact of immunosuppressive drugs

Some of the key findings from this project were the differences seen in peripheral blood cells with different induction and maintenance regimens of immunosuppressive drugs. Many of the circulating cell changes described above in association with DSA formation, were also seen in particular treatment groups. For example, $CXCR5^{+}IL7\text{-}R^{lo}FOXP3^{+}$ cTfr were low in both proportion and number at all time points in alemtuzumab treated patients compared to those receiving basiliximab induction (**fig 4.24**). This is an intriguing finding, and is the first description of cTfr in alemtuzumab treated patients. Previous studies have reported higher proportions of $FOXP3^{+}$ Tregs in the recovering CD4 T cell population after alemtuzumab treatment[312,417] but separating these into $CXCR5^{-}$ Tregs and $CXCR5^{+}$ Tfr showed the discrepancy (**figs 4.23-24**) within this cohort, suggesting that regulation of GC responses may be impaired in alemtuzumab treated patients. This may go some way towards explaining the phenomenon of post-alemtuzumab antibody-mediated autoimmune disease, which has been well described in patients with multiple sclerosis[433], but is much less common in the transplant setting[315,368]. Even with a higher proportion of Tregs, without the specific suppression of B cell/Tfh GC interactions provided by Tfr, non-specific antibody responses could be generated; as this study shows that both B cells and cTfh cells recover following lymphocyte depletion with alemtuzumab, while cTfr cell remain suppressed.

However, caution must be exercised in interpreting these findings as the patient cohort was heterogeneous, and the alemtuzumab-treated group includes both SPK recipients, who have an underlying autoimmune disease as their cause of ESRF, and kidney transplant recipients. It also includes some patients who also received basiliximab, and patients receiving variable doses of corticosteroids. Further studies with larger patient groups and in different disease settings would be helpful to confirm this observation.

$CXCR3^{-}CXCR5^{+}PD\text{-}1^{+}$ cTfh cells were higher in proportion in alemtuzumab treated patients compared to either group receiving basiliximab induction (**fig 4.27**), and rose over the 2 years following transplantation. Uncontrolled Tfh responses lead to spontaneous autoantibody production as well as increased antigen-specific antibodies in mouse models[109,117,478,479] and autoimmunity following lymphocyte depletion with alemtuzumab is driven by IL-21 in patients with MS[420]. These findings combined with the results from this study suggest that the rising proportion of cTfh cells in the recovering cell population, with a lack of balancing cTfr cells, could underlie the increased risk of autoantibodies seen in autoimmune disease and, in the context of transplantation, an increased risk of alloantibody formation. However, this would require further investigation and ideally animal models to test this hypothesis.

Alemtuzumab is increasingly used as first line induction therapy for kidney transplantation in the UK following results of the 3C study, which, along with other studies showed lower rates of acute TCMR with no significant increase in adverse events[307,308,315,367,368]. However, a reasonable proportion of patients still

receive induction therapy with basiliximab, and in the longer term, the choice of maintenance therapy may influence the development of DSAs. The first choice of maintenance CNI in the UK is tacrolimus, however both azathioprine and MMF are widely used in combination with tacrolimus. There is no clear consensus in the literature as to which antimetabolite is associated with a greater risk of DSA formation in the longer term[372,439,453,454], although there are moves within the UK to reduce azathioprine usage and move towards MMF as the maintenance therapy of choice.

This study showed that $CXCR5^{+}PD\text{-}1^{+}$ cTfh cells were higher in alemtuzumab treated patients, but also those receiving basiliximab induction with MMF maintenance, compared to those receiving azathioprine maintenance (**fig 4.32**). This correlates with the risk of *de novo* HLA antibody formation in this patient cohort, as patients receiving alemtuzumab induction had higher rates of both *de novo* HLA antibody formation (16/42, 38%) and *de novo* DSA formation (6/42, 12%) compared to those treated with basiliximab induction, both in terms of HLA antibody formation (6/19, 32%) and DSA formation (1/19, 5%). When looking at the difference in maintenance therapy, patients receiving MMF after basiliximab had a higher risk of both HLA antibody (5/10, 50%) and DSA (1/10, 10%) compared to those receiving azathioprine after basiliximab (HLA – 1/9, 9%; DSA – 0/9, 0%) – **fig 4.33**. These data led to questions about the impact of different maintenance therapies on cTfh and cTfr cells.

Comparing azathioprine treated patients with MMF treated patients there were lower levels of both cTfh and B cells in azathioprine treated patients, with a

relative preservation of switched memory B cells (**fig 4.17**). This suggested a greater impact of azathioprine on cTfh and B cells, and therefore potentially on the risk of antibody formation. However, it must be noted that azathioprine in this cohort was chosen as a maintenance agent for those patients considered at 'low immunological risk' and therefore the lack of antibody formation with azathioprine may be a coincidence due to this lower immunological risk rather than a causative relationship. Nonetheless, the results warranted further investigation, and the *in vitro* system was designed to assess the impact of individual drugs in more detail.

The experiments showed that all immunosuppressive drugs impacted on plasmablast development, and hence antibody production, but that 6-MP/azathioprine had a greater impact than MPA/MMF at these doses. Interestingly, 6-MP appeared to prevent upregulation of PD-1 on cTfh cells. This was mediated, at least in part, through its effect on B cells, as PD-1 was upregulated to similar levels as untreated cells when only cTfh cells were treated with 6-MP (**fig 5.28**). However, 6-MP treatment of cTfh cells still abrogated plasmablast development and antibody production, which was at least in part due to inhibition of proliferation (**fig 5.24**).

Interestingly, PD-1^{+} cTfh cells were lower in the circulation of azathioprine treated patients (**fig 4.32**), and it is possible that this was also mediated through the effects of azathioprine on B cells, as B cell-Tfh interaction is thought to be required to maintain PD-1 expression on Tfh in GCs[144,459,460]. It is not clear

whether this is also the case for circulating cells, or whether another mechanism underlies the lack of PD-1^{+} cells in azathioprine treated patients.

The function of PD-1 on Tfh and cTfh cells is not entirely clear. Evidence from *Pdcd1-/-* mouse models suggests that a lack of PD-1 leads to increased proportions of Tfr in draining LNs after immunisation[148]. LN Tfh were lower in these mice, but cTfh cells higher in proportion, which Sage *et al.* postulated was due to higher suppression of LN Tfh by LN Tfr, but a lack of suppression in the blood, where cTfr cells are not able to target cTfh[148,477]. Blockade of PD-1 signalling has been suggested as a strategy in cancer immunotherapy, where high PD-1 expression denotes exhausted cells[480]. However, blockade of PD-1 in animal models of immunisation showed increased B helper function and increased antibody levels in treated mice[481]. It has been postulated that PD-1 limits interaction of both Tfh and Tfr with GC B cells expressing PD-L1, and therefore blockade of PD-1 would allow longer interactions of Tfh with GC B cells, allowing time for B cells to receive help early after GC formation, when Tfh are proliferating faster than Tfr[477]. However Tfr are also influenced by PD-1 expression and blockade can also lead to increased Tfr interactions with GC B cells, thus limiting their interactions with Tfh and reducing antibody levels[148]. If azathioprine is able to limit PD-1 expression, whether by effects on B cells, or through another mechanism, this may lead to increased Tfr function and hence suppression of GC B cell/Tfh responses. In conjunction with tacrolimus treatment, which seems from pre-transplant studies to disproportionately affect cTfh over cTfr (**figs 4.9-4.12**) the use of azathioprine may reduce the likelihood of *de novo* DSA development.

This is potentially a very exciting development, as there are limited options for treatment of chronic ABMR associated with *de novo* DSA development[338]. Given the associations seen in this study and others that suggest an increased risk of *de novo* DSA development with alemtuzumab induction[309,451,452], rather than combining alemtuzumab with tacrolimus and MMF maintenance therapy, which is the current standard in the UK, combining tacrolimus with azathioprine may reduce the risk of *de novo* DSA development, by limiting PD-1 signalling and allowing the remaining Tfr to mediate suppression of Tfh responses. The combination of the suggestive clinical data and the supportive *in vitro* work is exciting and warrants further studies, both laboratory based and larger clinical cohorts, as well as more epidemiological data collection and analysis.

6.4 Limitations of this study

While this study has established several novel findings, it has a number of limitations, the most significant being the small number of patients developing DSA, as well as the heterogeneity of the patients between and within treatment groups.

Limitations of Clinical Data Collection

While numbers for transplantation for the previous three years in Oxford suggested that the proposed period would allow recruitment of a large enough cohort based on power calculations, the months during which recruitment occurred had lower transplant numbers compared to previous years, and thus

limited the size of the cohort. This is an unfortunate consequence of clinical studies, particularly those recruiting based on transplantation, which is by its nature an unpredictable operation with unpredictable timing. To address this, previous clinical trials have had much longer recruitment periods, often over several years, to allow sufficient numbers and more control over patient selection to reduce heterogeneity. However, this was not feasible within the time constraints of my clinical fellowship, and therefore both numbers and patient choice were restricted to the transplants that occurred within a fixed time period. This has led to a small and very heterogeneous cohort, including both kidney and SPK patients, who differ in a number of ways:

- SPK recipients have an autoimmune cause of ESRF and therefore may have a different immunological risk for antibody development, as seen in patients with MS who are more prone to develop autoantibodies after alemtuzumab compared to kidney transplant recipients, perhaps reflecting an underlying tendency to break self tolerance. Kidney recipients have a wide range of causes of ESRF, as seen in this cohort.
- Studies suggest SPK recipients are more likely to develop *de novo* DSAs than kidney alone recipients
- SPK recipients will receive a fixed induction and maintenance regimen including alemtuzumab, tacrolimus, MMF and no corticosteroids, whereas kidney alone recipients receive very variable regimens, as seen in this cohort
- Donors for SPK are always deceased, whereas kidney donors are split between live and deceased donors, with different inflammatory factors surrounding surgery, including intra-peritoneal transplantation of the

pancreas. This is likely to alter the immunological risk profile, as SPK recipients will have a more pro-inflammatory environment at the time of surgery and be more primed to generate a response against the graft.

Alongside these issues, there was also heterogeneity introduced by the immunosuppressive regimens. At the time of recruitment, there were several routinely used regimens, and the choice of regimen was based partly on local protocols for immunological risk, but also on clinician choice. For example, patients considered to be at risk of non-compliance were given alemtuzumab induction with azathioprine maintenance, rather than the regimen dictated by their immunological risk profile. The decision to give a second dose of alemtuzumab was based partly on laboratory lymphocyte count, but also clinician judgement. Additionally, the timing of steroid weaning, whether to give pulsed methylprednisolone for presumed rejection while awaiting biopsy results, and alteration of doses were all based on clinician judgement. While these decisions and changes are entirely appropriate in the context of managing patients, they contributed to the heterogeneity of the cohort and made interpretation of data more difficult.

Finally, as the patients were drawn from a large referral area, follow up of patients was difficult. Many patients were lost to follow up after return to their referral centre, and others had samples delayed or missed because of change in follow up arrangements at local centres. This meant that, at later time points particularly, the number of samples for patients developing *de novo* DSA was too small to allow robust statistical analysis.

In the context of this, while there were associations between DSA development and cTfr and cTfh cells, as well as associations between treatment group and DSA development, and treatment group and cell numbers, there were no statistically significant differences. We sought assistance from a statistician to see if the associations identified were significant on multivariate analysis, but due to the heterogeneity of the cohort and small numbers of patients developing *de novo* DSA, it was not possible to identify factors that differed significantly between those developing *de novo* DSA and those who did not.

To address these issues, a more structured approach to future studies would be important.

Limitations of Clinical Data Analysis

The choice of sample methodology was determined by the need to minimise inter-sample variation both between patients, and between samples for each patient. A decision was made to freeze samples and batch process to minimise variation between groups. This meant that overnight, when there were potentially multiple transplants happening, all samples could be quickly processed and frozen. It also meant that follow up samples, which would travel back from peripheral centres and may not be processed until 3 hours after sampling, would also have been frozen and thawed, thus minimising variation in processing. However this methodology introduces an extra variable in the form of freeze-thawing samples. While several studies show that the impact on T cells is minimal if samples are handled appropriately, the impact on B cells is less clear

and studies suggest that the freeze-thaw process may alter B cell numbers, particularly if the B cells are activated at the time of freezing. This was also the case with LN samples, which had poor recovery after thawing, which may be due to the activation from the recent inflammatory insult of surgery, or may be due to the large quantities of fat in most human lymph nodes.

When processing samples for flow cytometry, protocols were strictly adhered to with regards to staining for markers, however each batch was processed separately with varied antibody lots. In retrospect, a control sample, for example from a leucocyte cone, could have been included with each batch to compare and control for inter sample variation. Control of variation between batches was attempted by compensating each batch with single colour beads and examining the change in compensation between batches, however there is a degree of human error that should be considered.

Additionally, drawing inferences between proportions assessed in frozen samples and absolute cell counts from comparison with laboratory lymphocyte counts, which are processed fresh, should be viewed with caution. This is a widely accepted practice and has evidence suggesting that freezing below -150^{o}C allows preservation of both surface markers and transcription factors[482-484] however, thawing may lead to disproportionate cell death amongst some subsets compared to others[485,486] which may alter results. Studies suggest that with liquid nitrogen storage and strict adherence to thawing protocols, cell viability and phenotype is preserved[487,488], however it is not clear whether immunosuppressive drugs might exert an additional influence on cell death or

viability from thawing, and this is a question that should be addressed in future studies.

At the time of study initiation, there were limited options for unbiased data analysis, and all samples were analysed using Flow Jo and internal controls. Subsequently, several options have been developed for unbiased data analysis using statistical packages such as R to undertake principal component analysis, or through the online Cytobank, which provides several open-source, free to access packages such as SPADE (spanning-tree progression analysis of density-normalized events)[489,490] and viSNE[491] amongst several others. These require a degree of bioinformatics expertise, but would be of benefit in providing consistent and operator-independent analysis of large data sets such as this[492,493].

Limitations of supportive work

In addition to the problems with clinical data, due to time constraints I was not able to complete all the planned *in vitro* experiments that would have allowed comparison of the different immunosuppressive drugs in all co-culture conditions. Therefore these data must also be viewed with caution, as the greater impact of azathioprine may be mediated by its effects on B cells, not on Tfh, and the experiment that allows separation of these effects was not able to be performed with MMF. The doses of 6-MP and MPA may not be comparable, particularly given the lack of protein binding in an *in vitro* setting, and a dose escalation study would be important for formal comparison of the effects of these two drugs.

While attempts were made in the early part of this fellowship to set up a detailed animal model of alloantibody formation using a model antigen system in murine kidney transplantation, the complexity and technical difficulty of this model led us to seek an alternative *in vivo* experimental set up. Unfortunately, due to the timing of this change, the associations observed *in vitro* were not repeatable with *in vivo* experiments. However, it would be important to pursue this research avenue further.

6.5 Future directions

6.5.1 Epidemiological studies

One of the biggest limitations of this study was the size of the cohort and the number of patients developing DSA. In order to investigate the association between DSA development, induction therapy and maintenance therapy, much larger cohorts of patients would be required. Several large studies have been carried out in the UK looking at different induction and maintenance immunosuppressive therapies, and many of these studies have stored serum from patients, as well as detailed clinical information. To undertake HLA antibody screening for these patients would be straightforward, as consent to further testing of samples was taken at the time of study entry. To test, for example, the serum from participants in the 3C study would potentially allow comparison of 852 transplant recipients, with up to 5 years worth of data[315,367]. If rates of *de novo* DSA development were consistent with previous studies at 10-20% in the first 5 years[199] this study would potentially find 80-160 patients developing *de*

novo DSA, allowing for much more robust statistical associations. Assessing other large scale studies where there are biobanked serum and PBMC samples may allow additional data gathering. These studies would also reduce heterogeneity as inclusion criteria are controlled and drug regimens protocolised, therefore allowing more standardisation between groups and cleaner data sets.

It would also be possible to gather historical data from samples stored in tissue typing laboratories, and to test this for DSA formation, however this would be more difficult to coordinate and raises ethical issues in the processing of stored samples without specific patient consent to research. However, this approach would add to the data on azathioprine, which is now used much less frequently, but was previously the antimetabolite of choice for all transplants. Comparing *de novo* DSA formation in a historical cohort of patients where all were treated with azathioprine, to current cohorts where all are treated with MMF may allow elucidation of the risk of *de novo* DSA development when the confounders of clinical assessment of immunological risk and compliance are removed. However, this data would also need correlation with outcomes, as improvements have been made in surgical techniques, donor selection and allocation, and HLA matching, which may add additional confounding factors when comparing a historical cohort with current cohorts.

6.5.2 Prospective immunophenotyping studies

Taken as a pilot study, this work has shown that cTfh and cTfr cells are relevant in the formation of DSA, however associations were not statistically significant, and would require prospective immunophenotyping studies on a larger scale.

Given the lack of experimental evidence suggesting which subsets might be relevant, this work has provided a framework for future immunophenotyping and indicated that it would be important to include PD-1, CXCR3 and CCR6 in future staining panels for cTfh cells in transplantation. It would also be important to divide $CXCR5^+$ cells into those with helper and those with regulatory capacity by the inclusion of FOXP3 as an intracellular marker, as the ratio of cTfr to cTfh cells is likely to be relevant in predicting *de novo* DSA development.

Addressing the limitations of this study I would suggest that a future phenotyping study should be linked to a larger transplant trial where drug regimens are controlled by protocol and recruitment is phased over several years to allow careful patient selection. It would be important to separate live donor recipients from deceased donor recipients, perhaps focussing on recruiting only one donor type, and to recruit only kidney transplants, to avoid the confounding factors from SPK transplantation.

Ideally the study would gather similar numbers to the 3C study, aiming for 800-1000 participants with follow up of at least 5 years per participant. To collect these numbers would require a minimum of 4 years of recruitment, depending on the number of centres involved, and would therefore require 9-10 years for the study initiation and completion. This would need to be a multi-centre study and therefore would require strict protocols for processing and analysing samples. The ONE study (http://www.onestudy.org/), which is currently completing follow up, ran parallel immunophenotyping, processing samples and running flow cytometric phenotyping locally according to standardised protocols agreed

across the centres. However, an additional aliquot of cells was sent frozen to the reference laboratory and processed with the same protocols, to minimise variation between centres[494].

There is already excellent co-operation between UK centres, as seen with the 3C study, and this type of trial would be feasible to set up, providing agreement on treatment protocols could be established. However, the time and manpower required would make the study expensive. Therefore prior to a large scale study, supplementing the data gathered in this small cohort with a larger cohort of 100-200 deceased donor transplant patients, large cohort epidemiological data, such as that from the 3C study, and further *in vitro* and *in vivo* data would be essential to provide funding bodies with sufficient information to decide if a large scale study is worth supporting. Addressing concerns over the analysis of flow data, fresh and frozen sample comparison, and methods for standardising sample collection and processing would be important precursors to this work.

6.5.3 Further in vitro and in vivo work

Given the associations seen in the immunophenotyping work, key next steps that would not require recruitment of additional patients or ethical approval for use of historical samples, would be to expand the *in vitro* experiments to include comprehensive dose escalation studies to find the doses at which 6-MP and MPA have maximal and minimal effects to try and determine equivalent doses for comparison. Additionally it would be important to carry out pre-incubation experiments with MPA to remove the impact of the drug on B cells, and look in isolation at its effect on cTfh cells.

Supportive *in vivo* work using well defined animal models would be essential. The original plan for *in vivo* work in this study was to use ovalbumin (OVA) and hen egg lysozyme (HEL) as a combined model antigen, by using mice expressing OVA-HEL under the nephrin promotor (NOH mice)[495] or under the rat insulin promotor (ROH mice)[496] to perform either a kidney or islet transplant. The NOH and ROH genotypes are on a C57BL/6 background, and by transplanting into a wild type C57BL/6 mouse, only the OVA-HEL is a foreign antigen. This would allow tracking of OVA-HEL antibodies, and could be manipulated by controlling the number of OVA and HEL specific B and T cells, adoptively transferring SW_{HEL} B cells and OTII T cells, which are specific for these antigens. However, the technical difficulties associated with these models meant that I was unable to complete experiments using these techniques. Using the same model-antigen approach it would be possible to use KLK mice, which express membrane-bound HEL under the MHC Class I promoter, therefore in all tissues. Performing a skin transplant as described in this study, would allow a technically simpler approach to the surgical procedure, but still allow titration of B and T cell numbers, and potentially cTfh and cTfr proportions, by adoptive transfer.

However, these experiments would also require optimising the skin transplant model to find doses of MMF, azathioprine and tacrolimus that led to a prolonged MST, to be able to analyse the effects on LN Tfh, Tfr and GC B cells in drug treated compared to untreated mice.

6.6 Summary

The novel findings from this study, while limited by the small data set and confounding factors, are relevant for future research into *de novo* DSA formation and chronic ABMR, as previous studies of cTfh cells in human transplantation have been limited, focussing on all $CXCR5^+$ cells within the circulation and either early time points[273], or late stage chronic rejection[407]. This is the first prospective study looking at the first two years post-transplant, which is the highest risk period for alloantibody formation. Animal work in transplantation has been more detailed but has focussed only on GC located Tfh[392,396,497], not cTfh cells. This is also the first description of the role of cTfr cells in human transplantation and the formation of alloantibodies.

In combination with this, the *in vitro* and *in vivo* experiments support the clinical findings, and go some way towards elucidating a potential mechanism by which azathioprine may have greater protective effect against *de novo* DSA formation in patients, by detailing the effects of the drug on cTfh and B cells.

These data therefore suggest potential biomarkers for the prediction of *de novo* DSA formation, and risk factors for antibody formation in terms of immunosuppressive drug choices. The study has a number of limitations but the findings warrant further investigation as they suggest that widely used induction and maintenance regimens for transplantation may paradoxically increase rather than decrease the risk of *de novo* DSA formation post transplant.

7. Appendix 1: Updated 2015 Banff Classification Categories

Category 1: Normal biopsy or non specific changes

Category 2: Antibody mediated changes

Acute/active ABMR – requires 3 features for diagnosis

1. Histological evidence of acute tissue injury including one or more of:
    - **a.** Microvascular inflammation (glomeruli or peritubular capillaries)
    - **b.** Intimal or transmural arteritis
    - **c.** Acute thrombotic microangiopathy in the absence of any other cause
    - **d.** Acute tubular injury in the absence of any other apparent cause
2. Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
    - **a.** Linear C4d staining in peritubular capillaries
    - **b.** At least moderate microvascular inflammation (more if TCMR coexists)
    - **c.** Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
3. Serological evidence of DSAs, either HLA or other antigens

Chronic-active ABMR – requires all three features for diagnosis

1. Histologic evidence of chronic tissue injury, including one or more of:
    a. Transplant glomerulopathy, if no evidence of chronic TMA
    b. Severe peritubular capillary basement membrane multilayering on EM
    c. Arterial intimal fibrosis of new onset, excluding other causes. Leucocytes within the sclerotic intima favour chronic ABMR if there is no previous biopsy proven TCMR
2. Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
    - **a.** Linear C4d staining in peritubular capillaries
    - **b.** At least moderate microvascular inflammation (more if TCMR coexists)
    - **c.** Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
3. Serological evidence of DSAs, either HLA or other antigens

Category 3: Borderline changes suspicious for aTCMR

- Foci of tubulitis with minor intersitital inflammation, or interstitial inflammation with mild tubulitis
- No intimal arteritis

Category 4: T cell mediated rejection

IA Significant interstitial inflammation and foci of moderate tubulitis
IB Significant interstitial inflammation and foci of severe tubulitis
IIA Mild to moderate intimal arteritis +/- interstitial inflammation + tubulitis

IIB Severe intimal arteritis of >25% luminal area +/- interstitial inflammation + tubulitis

III Transmural arteritis +/- arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation

Chronic active TCMR - 'Chronic allograft arteriopathy' – arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo intima. NB This may represent chronic, active ABMR as well as TCMR, and it may manifest as tubulointerstitial inflammation

Category 5: Interstitial fibrosis and tubular atrophy

I Mild <25% cortical area

II Moderate 26-50% cortical area

III Severe >50% of cortical area

Category 6: Other changes not thought to be due to acute or chronic rejection

- BK virus nephropathy
- Post transplant lymphoproliferative disorders
- CNI nephrotoxicity
- Acute tubular injury
- Recurrent disease
- *de novo* glomerulopathy (other than transplant glomerulopathy)
- Pyelonephritis
- Drug induced interstitial nephritis

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