T cell communication in kidney transplantation

T cel communicatie bij niertransplantatie

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Colofon

The research described in this thesis was performed at the Department of Internal Medicine, section Nephrology and Transplantation of the Erasmus University Medical Center, Rotterdam, The Netherlands.

ISBN 978-94-6375-321-0

Cover Peter de Leur
Layout Nikki Vermeulen - Ridderprint
Printing Ridderprint - www.ridderprint.nl

Publication of this thesis was financially supported by:
Nederlandse Transplantatie Vereniging
Erasmus Universiteit Rotterdam
Astellas Pharma B.V.
Chiesi Pharmaceuticals B.V.

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Proefschrift
ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. R.C.M.E. Engels

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 2 juli om 13:30 uur

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“Quaevis terra patria”

“Heel de wereld is mijn vaderland”

Desiderius Erasmus

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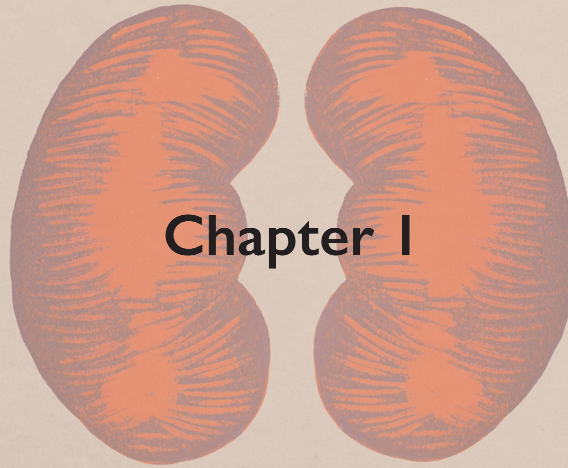

Chapter 1

General Introduction & Outline of the thesis

The immune system

For the protection against infections, the immune system must recognize and respond to different types of pathogens, such as bacteria, viruses and parasites. Immune recognition is exceptional by the capacity to distinguish foreign- from self-components. In general, two systems of immunity can be distinguished, innate and adaptive immunity. The innate immune response acts as a first line defense to recognize foreign components, but is not able to distinguish small molecular differences and lacks a memory for earlier encountered antigens. In contrast, the adaptive immune system is able to recognize, to respond and to provide increased protection (memory) against subsequent reinfection with the same pathogen. Both T and B cells are important players in the adaptive immune response. Depending on the type of T cell, different routes of activation are generated that ultimately lead to elimination of the pathogen (**Figure 1**). The CD4+ T helper cells respond to antigen by the production of cytokines and the expression of co-stimulatory molecules on their cell surface, which enables them to activate B cells and other immune cells. CD8+ cytotoxic T cells respond to antigen by secreting cytokines and cytotoxins such as granzymes and perforins. After penetration of the target cell, these cytotoxins trigger a caspase cascade that eventually leads to apoptosis of the target cell(1). T cells express a wide variety of T cell receptors (TCR) on their cell membrane by which they specifically recognize foreign antigens presented by human leukocyte antigen (HLA) molecules. These HLA molecules are polymorphic glycoproteins expressed on the cell membrane(2). Two major types of HLA molecules can be distinguished: HLA class I and HLA class II. The HLA class I molecules (HLA-A, HLA-B) are expressed by all nucleated cells and present peptides that are endogenously derived. Peptides presented by HLA class I molecules are predominantly recognized by CD8+ T cells, whereas HLA class II molecules (HLA-DR, HLA-DQ) are recognized by CD4+ T cells. These HLA class II molecules are restrictedly expressed by antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells. These APCs are able to take up, digest and load exogenous peptides on their cell membrane. Overall, TCR recognition of the cognate antigen, together with a co-stimulatory signal and cytokine secretion by the APC, results in the activation, proliferation and differentiation of CD4+ and CD8+ T cells(3).

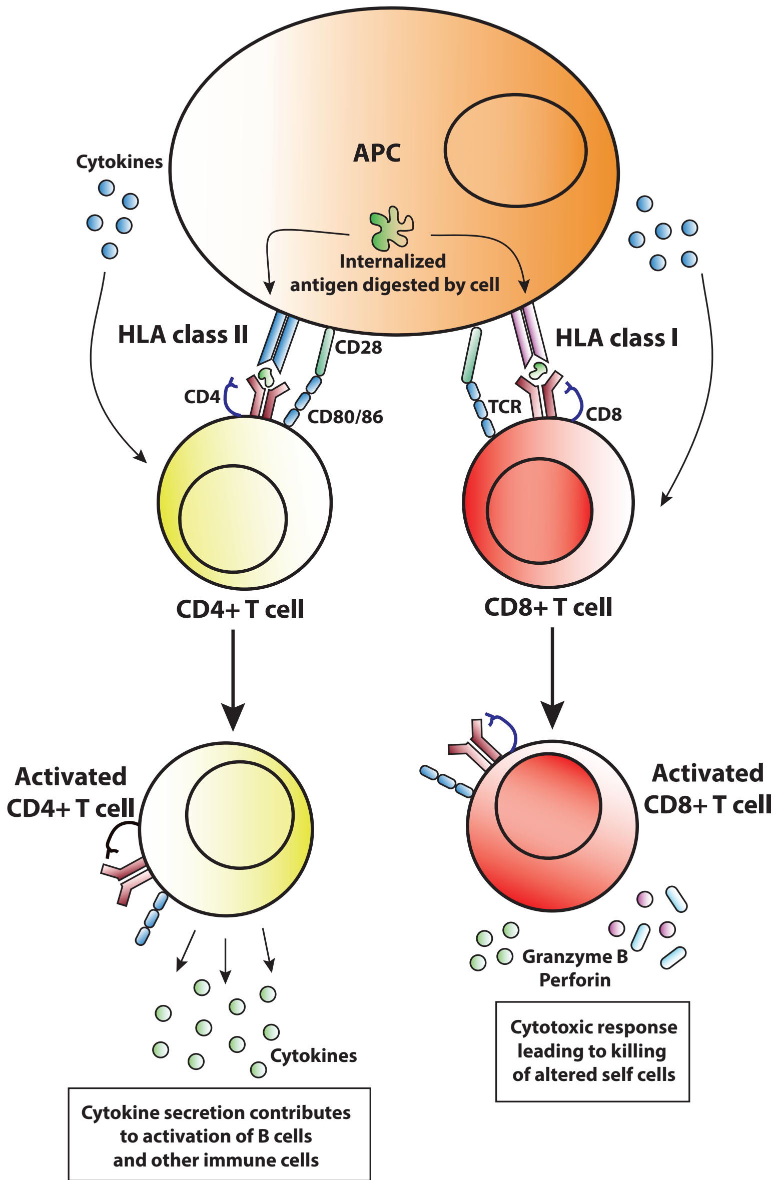

Figure 1. Activation pathways of CD4+ and CD8+ T cells.

Foreign antigen is digested by an antigen-presenting cell (APC) and presented by human leucocyte antigen (HLA) molecules. Molecules presented by HLA class I are predominantly recognized by the T cell receptor (TCR) of CD8+ T cells, followed by co-stimulation via CD28 binding to CD80/86 and cytokine stimulation leading to activation of the CD8+ T cell. Via a cytotoxic response the CD8+ T cell mediates killing of the altered self-cells. HLA class II molecules specifically present molecules that are recognized by CD4+ T cells. After TCR recognition, co-stimulation, and cytokine stimulation the CD4+ T cell is activated and starts secreting cytokines by which B cells and other immune cells are activated.

As described above, the immune system is highly capable of protecting us against foreign agents. However, in the setting of solid organ transplantation, the immune system also recognizes the donor organ as a foreign agent, which will lead to rejection of the transplanted cells or organ if not genetically identical to its own. With the discovery of immunosuppressive medication the process of donor-specific immune activation can now be often adequately suppressed(4). However, the immunosuppressive approaches to maintain survival of the allograft are not without complications. Rejection of the transplanted organ still occurs and life-long treatment with immunosuppressive drugs is associated side effects.

Kidney transplantation and rejection

Kidney transplantation is the treatment of choice for patients with end-stage renal disease (ESRD). The quality of life of ESRD patients improves after kidney transplantation and the mortality risk reduces in this group of patients (5). In addition, kidney transplantation is a cost-effective alternative to dialysis (6). Since the first kidney transplantation in 1954, allograft and patient survival significantly improved. This improvement relies on the careful HLA-matching between the donor and recipient, the development of potent immunosuppressive drugs and improved diagnostics (4, 7). Despite the promising short and long-term results after kidney transplantation, there is still room for improvement to better control the process of allograft rejection (8, 9).

Rejection of a kidney transplant is the consequence of genetic differences in the HLA system between the recipient and donor. Therefore, HLA matching is important in the organ-allocation process and matching is associated with fewer rejection episodes and increased graft survival rates (7, 10). Foreign HLA antigens expressed and presented by either recipient or donor-derived APCs initiate the activation of recipient T and B cells. Rejection of the allograft manifests as T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR) or a mixed rejection involving the histological features of both rejection types (11). These forms of rejection may occur in an acute or chronic fashion. Within TCMR, primed effector T cells are taking center stage. After activation of these T cells, clonal expansion occurs and these effector T cells migrate towards the allograft and cross the epithelial border to enter the allograft. Tubulo-interstitial inflammation is induced within the graft by the effector T cells via the production of pro-inflammatory cytokines (11-13). The process of ABMR is characterized by the presence of circulating donor-specific antibodies (DSA). These DSA interact with the vascular endothelium of the graft causing injury of the tissue (11, 12, 14).

Immunosuppressive medication

After transplantation, immunosuppressive reagents suppress the immune system of the transplant patient including the function of immune cells that are involved in the process of organ transplant rejection. The current standard combination of immunosuppressive reagents after kidney transplantation consists of mycophenolate mofetil (MMF), glucocorticoids, and calcineurin inhibitors (CNIs)(15). After intake, MMF is converted to the active metabolite mycophenolic acid (MPA) which eventually inhibits the synthesis of guanosine monophosphate nucleotides, necessary for the synthesis of DNA and thus leading to the prevention of T and B cell proliferation (4). Glucocorticoids are able to bind the glucocorticoid receptor present in T cells, but also in a variety of other immune cells. Afterwards, translocation of the glucocorticoids to the nucleus occurs where the complex interferes with the activity of pro-inflammatory transcription factors such as activator protein 1 and nuclear factor-κB (4, 16). Tacrolimus is currently the primary described CNI that inhibits T cell activation via blocking the calcineurin dependent dephosphorylation of NFAT, which among others results in diminished production of the pro-inflammatory cytokine IL-2 (4). As described above, current maintenance immunosuppressive reagents are rather general and have a wide biological effect on T cell activation, cell division, and suppressing inflammation (17). One consequence of this treatment combination is that the production of DSA is not completely inhibited, enhancing the process of ABMR followed by graft failure (18-20). Besides, treatment with tacrolimus is associated with different side effects such as nephrotoxicity and an increased risk for infections and malignancies (21-24).

Undoubtedly, we need to better understand the mode of action of currently prescribed immunosuppressive agents on cells of the immune system. That said, there is also a clear rationale for more specific drugs targeting dominant molecules or pathways involved in the anti-donor response. This will diminish the occurrence of drug-related side effects as well as reduce the incidence of rejection. In order to achieve this, the role of specific lymphocyte subsets on the alloimmune response needs to be studied in more detail. Two new subsets of T cells have been discovered in the last decade: T follicular helper (Tfh) cells and tissue-resident memory T (T_{RM}) cells. From experimental models we now know that both effector memory T cell populations play crucial roles in the interaction with effector B cells and cytotoxic T cells, and can accumulate in tissues during infection and in the allograft after transplantation. However, whether these newly identified T cell populations contribute in immune responses leading to allograft rejection and are targeted by immunosuppressive drugs is largely unknown. In this thesis we investigated the role of these two T cell subsets in the rejection process of immunosuppressed patients after kidney transplantation. Improved knowledge on the contribution of Tfh and T_{RM} cells in alloreactivity will be useful to develop new therapeutic strategies for the prevention and treatment of this severe complication.

The development and function of T follicular helper cells

Tfh cells are a subset of CD4+ T cells that provide help to B cells (25, 26). For this, the cytokine milieu is essential for T cell differentiation in a certain direction. Early differentiation towards the Tfh cell lineage is mediated by IL-12, IL-6 and TGF-β (27-29). Afterwards, maintenance of the Tfh cell phenotype is primarily dependent on the interplay between IL-6 and IL-21 (**Figure 2**)(30). Expression of CC-chemokine receptor 7 (CCR7) promotes migration of the T cell towards the secondary lymphoid organs (SLO). Within the SLO, CCR7 is downregulated, followed by upregulation of CXC-chemokine receptor 5 (CXCR5)(31). The expression of CXCR5 is crucial for localization of the Tfh cell at the border of CXCL13+ B-cell follicles (32, 33). Interaction of the Tfh cell with cognate antigen presenting DCs or B cells is required to fully activate the Tfh cell followed by migration inside the B-cell follicles, where Tfh cells orchestrate the formation, expansion and selection of high-affinity B cells within the germinal center (GC)(34). These activated Tfh cells are also characterized by the expression of inducible T-cell co-stimulatory molecule (ICOS) and programmed death 1 (PD-1)(26, 35). Expression of a master transcription factor is crucial in defining a T cell subset, in case of Tfh cells the expression of B cell lymphoma 6 (BCL6)(36). Circulating Tfh cells are known for their expression of CXCR5 and low expression of PD-1 and ICOS, but these cells lack expression of BCL6 (37-40). Findings in HIV patients indicate that circulating memory Tfh cells have strong transcriptional similarities to activated GC Tfh cells(39). These findings encourage the use of circulating Tfh cells for studying the function of these cells in patients as a biomarker for disease (37, 39, 41).

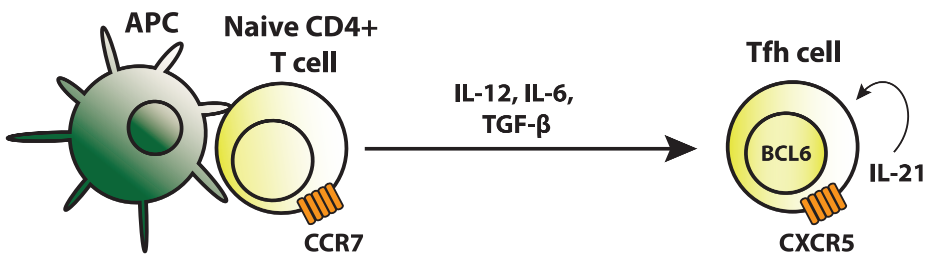

Figure 2. Differentiation of T follicular helper (Tfh) cells.

Naïve CD4+ T cells are activated by an antigen-presenting cell (APC) via their T cell receptor and a co-stimulatory signal. Subsequently, cytokines IL-12, IL-6, TGF-β and IL-21 are essential in the differentiation and maintenance towards the Tfh cell phenotype. Expression of CC-chemokine receptor 7 (CCR7) promotes migration towards the lymphoid organs followed by upregulation of CXC-chemokine receptor 5 (CXCR5) and the Tfh cell master transcription factor B cell lymphoma 6 (BCL6).

The role of Tfh cells in transplantation has been studied in several animal models. These models prove that the activation of allo-specific B cells is dependent on IL-21 producing

Tfh cells (42-44). The extent to which Tfh cells are able to communicate with B cells is dependent on the TCR interaction with cognate antigens presented by HLA class II molecules in combination with co-stimulatory molecules such as CD40 ligand (26, 32) (**Figure 3**). Thereafter, antigen-activated B cells differentiate into long-lived memory B cells or immunoglobulin producing plasma cells (45). In transplantation, it is suggestive that the formation of DSA relies on the interaction between Tfh and B cells (35). After transplantation, DSA can be formed and Tfh-B cell clusters can be detected in renal rejection biopsies (46). At present, B cell-depleting therapies such as alemtuzumab, anti-thymocyte globulin (ATG) and rituximab are used in patients diagnosed with ABMR, which according to the Banff 2017 rejection criteria depends on the presence of DSA (11, 47). However, these therapies are not optimal due to lack of efficacy. For instance, rituximab depletes CD20+ B cells but lacks the capacity to target the immunoglobulin producing CD20- plasma cells (47). Clearly, there is room for new strategies especially early in the activation cascade aiming to prevent the Tfh-dependent activation of alloantigen-stimulated B cells that leads to the formation of DSA. Of particular interest is the intervention of Tfh-B cell interaction via blockade of the IL-21/IL-21R signaling pathway. This strategy has been shown to be successful in B cell-mediated autoimmune diseases (48, 49).

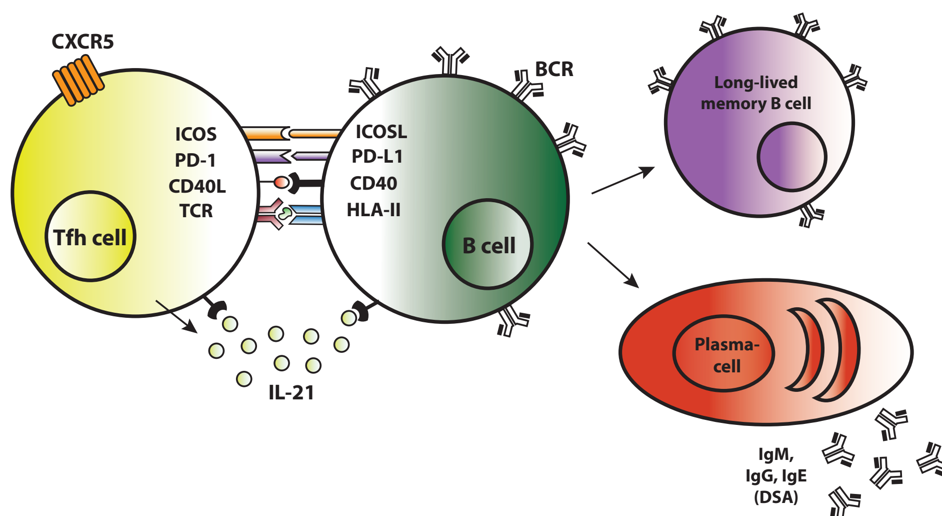

Figure 3. T follicular helper (Tfh) cell-dependent B cell differentiation.

B cell differentiation is initiated after cognate interaction of the T cell receptor (TCR) with the antigen presenting human leucocyte antigen (HLA) class II molecule followed by co-stimulation via for instance CD40L – CD40 interaction and interleukin-21 (IL-21) production by the Tfh cell. Binding between inducible T-cell co-stimulatory molecule (ICOS) and programmed death 1 (PD-1) and their respective ligands ICOSL and PD-L1 on the B cell strengthens the Tfh-B cell interaction. Ultimately, B cell differentiation towards either long-lived memory B cells or immunoglobulin producing plasma cells occurs.

Intervention of interleukin-21 signaling pathway: a new strategy to suppress alloimmune responses?

Because current maintenance therapies are less effective against the protection of humoral immune responses towards the allograft it is of interest to study early intervention of Tfh-B cell interaction. In this respect, selective blockade of Tfh cell help signals might prevent the activation of allo-activated B cells. A candidate of interest to target is the pleiotropic cytokine interleukin-21 (IL-21). This cytokine is produced by Tfh cells, Th17 cells and natural killer (NK) T cells(50). The IL-21 receptor (IL-21R) is among others expressed by CD4+ and CD8+ T cells, B cells and NK cells and consists of a common receptor γ-chain and a specific IL-21R part that activates downstream JAK1 and JAK3 signaling pathways, which allows the recruitment and phosphorylation of predominantly STAT3, but also the phosphorylation of STAT1 and STAT5(51, 52). IL-21 is a cytokine that influences the function of CD8+ T cells, Th17 cells and B cells(53-56). In the past decade, several studies presented the essential role of IL-21R signaling in autoimmune disorders, antiviral and antitumor responses and other inflammatory disorders (50, 57-59).

In transplantation, limited studies on the role of IL-21 in the human transplant setting have been performed (60, 61). Moreover, de Graav *et al.* presented that peripheral Tfh cell numbers remain stable after kidney transplantation. These Tfh cells still have the capacity to produce IL-21 (46). In the presence of the co-stimulatory inhibitor Belatacept the proportions of IL-21+ activated Tfh cells were only partially decreased in an allogeneic co-culture model (62). A study performed on cardiac allograft biopsies revealed that high IL-21 expression levels were present during acute rejection (63). However, the exact role of IL-21 in the immune activation after allogeneic stimulation is not fully understood. Thus, studies on the efficiency of targeting IL-21R pathway in an allogeneic setting are of high interest.

Lymphocytes present in the renal allograft

Effector T and B cells primed for alloantigen are able to migrate from the lymphoid organs to the allograft, where they locally contribute to the process of allograft rejection. Moreover, these intra-graft lymphocytes are able to form highly organized clusters that represent GC-like structures, normally present in the SLO. These lymphoid structures have also been detected in tissues affected by infection, autoimmunity and cancer and are referred to as ectopic lymphoid structures (ELSs) or tertiary lymphoid organs (TLOs)(64). ELSs are able to trigger a local antigen-specific response (64). In organ transplantation, ELSs are mainly associated with chronic rejection, but they are also detected in acutely rejected renal allografts (46, 65-67). Remarkably, in renal biopsies of acute TCMR the presence of intra-graft B cells is recognized (65, 68-70). Today, the exact composition and organization of

lymphocytes infiltrating the allograft is of interest, since these cells are directly located at the site of the alloantigen. The functional characteristics of ELSs are still debated, since these structures may have both protective and destructive capacities (67). In terms of influencing the T-cell dependent activation of B cells to prevent rejection of the allograft, it is of importance to study whether this T-B cell interaction locally occurs within the renal allograft and contributes to the process of rejection.

A recently discovered cell type residing within the non-lymphoid tissue are the tissue-resident memory T (T_{RM}) cells. These cells were first described by Masopust *et.al.* in 2001 (71). T_{RM} cells are a non-circulating subset of T cells that survey most non-lymphoid tissues and have the ability to respond rapidly to local antigens (72, 73). Several phenotypic and molecular markers define the T_{RM} cell subset, such as presentation of specific surface markers and up- or downregulation of genes involved in adhesion and migration (**Figure 4**) (74-76). The surface markers responsible for retention of the T_{RM} cells are CD69 and CD103. CD69 is able to bind and down-regulate the G-protein-coupled receptor sphingosine-1 phosphate (S1PR1), a receptor involved in stimulating the migration of the T cells from blood into the tissue (77). Another recognized T_{RM} cell marker, the αE integrin CD103, is able to bind E-cadherin, which is expressed on epithelial cells (78). After encounter with a cognate antigen, T_{RM} cells start to release cytokines including IFNγ and TNFα (79). The release of these pro-inflammatory factors results in the attraction of other immune cells including B cells and NK cells. Furthermore, T_{RM} cells exert effector function via the release of cytotoxic molecules such as granzyme B and perforin (80, 81).

So far, little is known about the presence and function of T_{RM} cells in transplanted organs, including the renal allograft (73). Different aspects of the T_{RM} cell are of interest to study from the perspective of transplantation, such as the potential of T_{RM} cells to protect the donor organ by controlling viral reactivation, while the T_{RM} cells may on the other hand contribute to the process of allograft rejection. Another phenomenon of interest is the origin of the T_{RM} cells within the allograft, since both donor- and recipient-derived T_{RM} cells may be present in the donor organ. Overall, the possible protective and destructive roles of T_{RM} cells within the renal allograft are of high significance and need further attention.

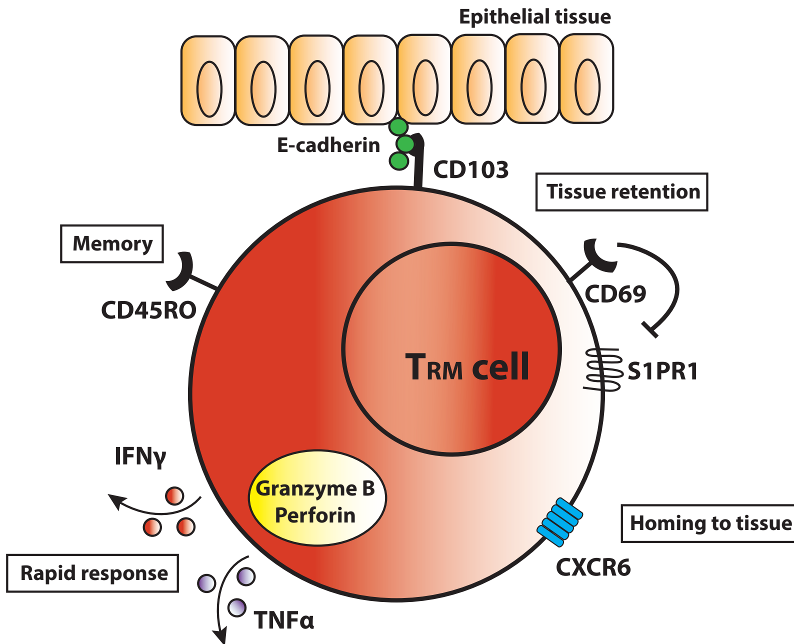

Figure 4. Schematic presentation of tissue-resident memory T (T_{RM}) cell characteristics.
General features of T_{RM} cells are presented. These include the presentation of retention markers CD69 and CD103, memory T cell marker CD45RO, and homing marker CXCR6. CD69 promotes residency within the tissue via interfering with the G-protein-coupled sphingosine-1 phosphate receptor (S1PR1). S1PR1 mediates migration of the T cells from the circulation into the tissues and vice versa. Furthermore, the αE integrin CD103 promotes TRM cell retention by binding to E-cadherin expressed by the epithelial tissue. T_{RM} cells are known for their rapid response after specific-antigen encounter, for instance via the production of interferon γ (IFNγ) and tumor necrosis factor α (TNFα) and via the secretion of cytotoxic molecules such as granzyme B and perforin.

Aim and outline of the thesis

Different T cell subsets are involved in the process of allograft rejection. Communication of these T cells with other lymphocyte populations is crucial in the development of a rejection response. For instance, studies in kidney transplantation plea for a key role for T cells in the process leading to chronic humoral allograft failure, traditionally thought to be orchestrated by B cells (82, 83). Current immunosuppressive treatments suppresses the immune system in a non-specific manner and outline the need for investigation of new strategies that only target specific parts of the well-defined alloimmune response, with which also some of the observed side effects can be prevented.

The overarching aim of this thesis is to better understand the role of Tfh and T_{RM} cells in alloreactivity by the characterization of the mechanisms by which both effector memory

T cell populations modulate anti-donor responses after kidney transplantation. This knowledge may help us to understand the contribution of these recently identified T cell populations to immune processes occurring after organ transplantation and will help us to design less toxic and more efficient immunosuppressive treatment strategies. For this, two relevant compartments were investigated: peripheral blood of the recipients and tissue biopsies of the transplanted kidney.

In the first part of this thesis the Tfh cell and its role in the alloimmune response takes center stage. In **Chapter 2** we provide an overview of the current knowledge on the effects of immunosuppressive medication on Tfh cell development and function and we describe new possible approaches to influence the function of Tfh cells. In addition, the potential role of the Tfh cell as pharmacodynamic biomarker to improve alloimmune-risk stratification is discussed. In the alloimmune response, the precise role of IL-21-producing Tfh cells on B cell differentiation is unknown. Therefore, **Chapter 3** focuses on whether Tfh cell-mediated differentiation of B cells is dependent on IL-21R signaling. This was performed in an allogeneic *in vitro* model in which we stimulated Tfh cells and memory B cells from patients pre-transplantation with their corresponding donor antigen. In **Chapter 4**, a humanized skin transplant mouse model with human T and B cell reconstitution is used to study the role of IL-21R signaling blockade in an *in vivo* transplant setting.

In the second part of this thesis, we focus on the characterization of lymphocytes within the renal allograft. The presence and activation status of T and B cells in organized ectopic lymphoid structures (ELSs) in different types of acute renal allograft rejection biopsies is studied in **Chapter 5**. We studied the presence of T and B cells with respect to GC features in acute/active antibody-mediated rejection (a/aABMR), acute T-cell mediated rejection grade I (aTCMRI) and acute T-cell mediated rejection grade II (aTCMRII). In **Chapter 6** we report about tissue-resident memory T (T_{RM}) cells in transplant nephrectomy specimens, a unique source of tissue to study the biology of this T cell population in the renal allograft. We are the first to study the presence, origin (donor or recipient) and functional properties of these CD8+ T_{RM} cells in the kidney allograft.

In **chapter 7 and 8** the results obtained in this thesis are summarized and discussed.

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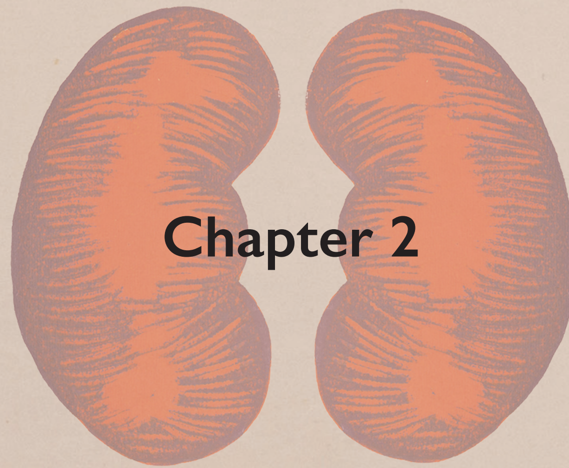

Chapter 2

T follicular helper cells as a new target for immunosuppressive therapies

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Frontiers in Immunology. November 2017; 8:1510

Abstract

Over the past decade antibody-mediated (humoral) rejection has been recognized as a common cause of graft dysfunction after organ transplantation and an important determinant for graft loss. In humoral alloimmunity, T follicular helper (Tfh) cells play a crucial role, because they help naïve B cells to differentiate into memory B cells and alloantibody-producing plasma cells within germinal centers. In this way, they contribute to the induction of donor-specific antibodies, which are responsible for the humoral immune response to the allograft. In this article we provide an overview of the current knowledge on the effects of immunosuppressive therapies on Tfh cell development and function, and discuss possible new approaches to influence the activity of Tfh cells. In addition, we discuss the potential use of Tfh cells as a pharmacodynamic biomarker to improve alloimmune risk stratification and tailoring of immunosuppression in order to individualize therapy after transplantation.

Introduction

Organ transplantation is the treatment of choice for end-stage organ failure. Although current immunosuppressive regimens are effective in the short-term, long-term allograft survival rates are still suboptimal with rejection being the leading cause of graft loss (1). Allograft rejection can develop from either cellular or humoral immune responses against the allograft, or from 'mixed rejection' involving both types of responses (2). In particular, humoral anti-donor reactivity via the formation of donor specific antibodies (DSA) is associated with poor allograft outcomes (3-5). Formation of DSA relies on antigen-activated T follicular helper (Tfh) cells, which are located in the germinal centers (GC) where they provide help to antigen-activated B cells, which in turn respond by differentiating into immunoglobulin-producing plasma cells and high affinity memory B cells (6, 7).

B cell depleting therapies have been used to control the formation of DSA in transplant recipients (8) but are not generally used as maintenance treatment because of the risk of side effects. Based on their pivotal role in regulating humoral immunity it can be postulated that Tfh cells, rather than B cells, could be targeted to inhibit the development of antibody-mediated anti-donor reactivity. Currently, no Tfh-specific agents have been evaluated in phase II or III trials. Several animal studies and a small number of clinical studies in organ transplant recipients have demonstrated the importance of Tfh cells in the process of alloantibody production (9). The specific effects of immunosuppressive therapies on Tfh cell activity, however, are less established and now subject to many ongoing research efforts. In this article we summarize current knowledge on the interplay between immunosuppressive drugs and the generation and function of Tfh cells, and consider new biological targets that might influence the proliferation, differentiation and activity of Tfh cells.

Biology of T follicular helper cells

Differentiation of Tfh cells

Differentiation of a human naïve CD4+ T cell into a Tfh cell is a complex and dynamic process involving multiple stages (10). A combination of signals determines whether the naïve T cell differentiates toward a Th1, Th2, Th17 or Tfh subset including the expression of specific transcription factors, signal transducer and activator of transcription (STAT) proteins, cytokines and chemokine receptors that allow the T cell to migrate to the site of inflammation. When a naïve T cell expresses C-C chemokine receptor 7 (CCR7), migration is promoted to T-cell rich zones in secondary lymphoid organs (SLO) and tertiary lymphoid structures present in chronically inflamed organs. Protein activin A (a member of the transforming growth factor-β (TGF-ß) superfamily) is present locally after the T cell

encounters an antigen-presenting dendritic cell (DC) and mediates downregulation of CCR7, followed by upregulation of CXC chemokine receptor 5 (CXCR5) (11). Expression of CXCR5 is essential for localization of the Tfh cells at the T-B border of B-cell rich follicles, where Tfh cells interact with B cells that recognize antigen via their B cell receptor (BCR) (Figure 1). Sequential antigen presentation by DCs and B cells is required for optimal differentiation of Tfh cells and the subsequent GC reaction (12). After cognate antigen recognition, Tfh cells migrate inside the B cell follicles and develop into activated GC Tfh cells which orchestrate the development of high affinity GC B cells. In addition to CXCR5, activated Tfh cells express the co-inhibitory protein programmed death 1 (PD-1) and inducible T cell costimulatory molecule (ICOS) (7, 9). Recently, it has been demonstrated in a conditional knock out mouse model that Tfh cells express the transcription factors Lymphoid Enhancer Binding Factor 1 (LEF-1) and T cell factor 1 (TCF-1), both of which are involved in regulation of the Tfh transcriptional repressor B cell lymphoma 6 (Bcl-6) (13). These transcription factors promote early Tfh cell differentiation by sustaining the expression of IL-6Rα and gp130, and by promoting upregulation of ICOS and expression of Bcl-6 which is also known as the master transcription factor for Tfh cells and represses transcription of among others *B lymphocyte-induced maturation protein-1* (*Blimp-1*), *T-box transcription factor* (*T-bet*) (Th1 development) and *RAR-related orphan receptor γt* (*RORγt*) (Th17 development) (14, 15). Apart from being present in SLOs, CXCR5+CD4+ Tfh cells are also present in blood, representing approximately 10% of human circulating memory CD4+ T cells (16, 17). Memory Tfh cells form a heterogeneous population based on the expression of the chemokine receptors CXCR3 and CCR6: $CXCR3^-CCR6^-$ represent Tfh2 cells, $CXCR3^+CCR6^-$ represent Tfh1 cells and $CXCR3^-CCR6^+$ represent Tfh17 cells (16). These subsets have distinct capacities to help B cells, the $CXCR3^-$ Tfh2 and Tfh17 cells promote B cell differentiation toward immunoglobulin producing cells via secretion of IL-21, while CXCR3+ Tfh1 cells lack this function (18, 19). In addition, the Tfh2 cells promote particularly IgG and IgE secretion, while Tfh17 cells are more efficient in promoting IgG and IgA secretion (16). Overall, an appropriate microenvironment is essential for coordination of Tfh cell lineage differentiation.

Cytokines involved in Tfh cell differentiation, activation and function

Coordinated activity by cytokines triggers specific transcription programs that stimulate the expression of molecules responsible for the effector function of Tfh cells (7). The differentiation of naïve human $CD4^+$ T cells in the SLOs toward a Tfh cell phenotype is primarily mediated by IL-12, IL-6 and TGF-β signaling. Activin A, in combination with IL-12, mediates an early shift toward the Tfh phenotype, including skewing toward expression of IL-21 (11, 20). IL-12 production is profoundly increased by activated DCs in the T-cell rich zone (21). TGF-β is another cytokine involved in human Tfh cell differentiation that after binding to its receptor, phosphorylates the transcription factors STAT3 and STAT4, key steps

in the Tfh cell differentiation process (22). As well as IL-12 and TGF-ß, IL-6 contributes to differentiation into Tfh cells. One clinical study, for example, showed that secretion of IL-6 by plasmablasts resulted in Tfh cell differentiation (23). Of note, interplay between IL-6 and IL-21 is required to achieve optimal Tfh-cell differentiation and function, although an absence of either IL-6 or IL-21 in a mouse model does not fully abolish Tfh cell formation (24, 25). In contrast, GC formation and the differentiation of B cells into immunoglobulin-producing plasmablasts are dependent on IL-21 producing Tfh cells (25, 26).

Inhibition of Tfh cells

Tfh cell function depends on the balance between pro-inflammatory and anti-inflammatory signals. Several factors have been reported to control Tfh cell activation. Recently it became evident that a subset of regulatory T cells – the follicular regulatory T (Tfr) cells – express Foxp3, Bcl-6 and CXCR5 and have the capacity to regulate the Tfh-driven GC reaction (27-29). However, the immunoregulatory mechanisms by which Tfr cell functions are controlled are largely unknown. Animal studies have shown that the co-inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which is highly expressed by Tfr cells and moderately expressed on Tfh cells is involved in the suppressive effects of these cells. Tfr cells lacking the co-inhibitory molecule CTLA-4 have an impaired ability to inhibit B cell antibody production (30, 31). Conversely, mice deficient for PD-1 on Tfr cells have increased suppressive activity, since PD-1 controls the activation of Tfr cells (32). In kidney transplant patients who received anti-CD20 rituximab induction therapy, both Tfh and Tfr cells remained in the lymph nodes despite disruption of the GC and elimination of B cells (33), underlining their independent mechanism of action. The role of Tfr cells in preventing rejection of the allograft is largely unknown. Recently, Chen *et al.* showed that the ratio of Tfr cells in peripheral blood and renal graft biopsies from patients with antibody-mediated rejection (AMR) was significantly lower than in non-AMR patients, while Tfh2 and Tfh17 ratios increased, suggesting that increased Tfh activation levels contribute to AMR (34).

Anti-inflammatory cytokines secreted by Tfr cells also influence Tfr cell function. The pleiotropic cytokine IL-10 inhibits antibody production via regulation of the quantity and quality of Tfh cells in mice immunized with sheep red blood cells (35). In contrast, lymphocytic choriomeningitis virus (LCMV)-infected mice deficient for IL-10 had lower frequencies of virus-specific Tfh cells but no decrease in GC B cells or LCMV-specific antibodies (36). IL-2 is a critical factor for the regulation of Tfh-B cell interaction *in vivo*. Although originally recognized as an essential T cell growth factor, IL-2 suppresses Tfh cell differentiation via activation of Blimp-1 resulting in a hampered formation of antigen-specific B cells and IgG responses in mice infected with the influenza virus (37, 38). Ray *et al.* recently demonstrated that IL-2-mediated activation of Akt kinase and mTORc1 was necessary to shift differentiation to Th1,

with less differentiation to Tfh cells (39). In contrast, IL-21 inhibits the expression of CD25 (part of the IL-2 receptor) in a Bcl-6 dependent manner (40).

Tfh-B cell crosstalk

Cytokines and co-stimulatory molecules secreted by B cells are able to encourage activation of Tfh cells within the GC (Figure 1). Moreover, apart from their role as an antigen presenting cell (APC), B cells contribute to the activation and regulation of the Tfh cells via secretion of cytokines like IL-6 and IL-10 (41, 42). Meanwhile, Tfh cells are involved in the GC reaction to promote B-cell activation. The GC consists of a polarized structure with two compartments that are designed for proliferation and affinity selection. Within the dark zone (DZ), B cells undergo several rounds of proliferation and somatic hypermutation (SHM) in the V-region of their BCR (43). The point mutations that are created during SHM allow affinity maturation and lead to antibody diversity. Afterwards, the DZ B cells migrates to the light zone (LZ), where they capture antigen presented by follicular dendritic cell networks and present it on MHC class II molecules to cognate Tfh cells (44). The amount of antigen captured by the B cell and presented to Tfh cells in the LZ directly corresponds to the amount of B-cell division and hypermutation in the DZ (45). Thus, T cell help and not direct competition for antigen is the limiting factor in GC selection (46). High affinity B cells present antigen to cognate Tfh cells, triggering a signaling pathway which allows (i) B-cell differentiation into long-living plasma cells, (ii) differentiation of long-lived memory B cells, or (iii) recirculation of B cells to the DZ for a new round of division and SHM (47). Activated Tfh cells produce IL-21 and IL-4, two cytokines which support B cell differentiation. Within the GC, Tfh cell function changes from IL-21^{+} Tfh cells, responsible for the selection of high-affinity B cell clones, toward IL-4^{+} Tfh cells that have high expression of CD40L and which direct B cell class switch recombination and differentiation toward antibody-producing plasma cells (48, 49). PD-1^{hi} Tfh cells are involved in this GC reaction while PD-1^{lo} Tfh cells represent precursor memory T cells with a Tfh-like phenotype (50). In a previous study, we found co-localization of T and B cells in cellular infiltrates of renal rejection biopsies, supporting a role for T-B cell interaction within the kidney allograft (51).

Genetic defects influencing human Tfh cell differentiation

Several heritable monogenic defects are known to affect the function and differentiation of Tfh cells. PBMCs of patients with primary immunodeficiencies (PID) have been characterized in various studies (52-57). In these studies, loss-of function (LOF) mutations in the genes encoding STAT3, ICOS, Bruton's tyrosine kinase (BTK), CD40L, NF-κB essential modulator (NEMO) and IL10R reduced the numbers of Tfh cells (52, 55, 56). LOF mutations in the genes encoding STAT3, IL21-R and gain-of function (GOF) mutation in the gene encoding STAT1 resulted in a phenotype with elevated levels of IFNγ and PD-1, both of which control Tfh-

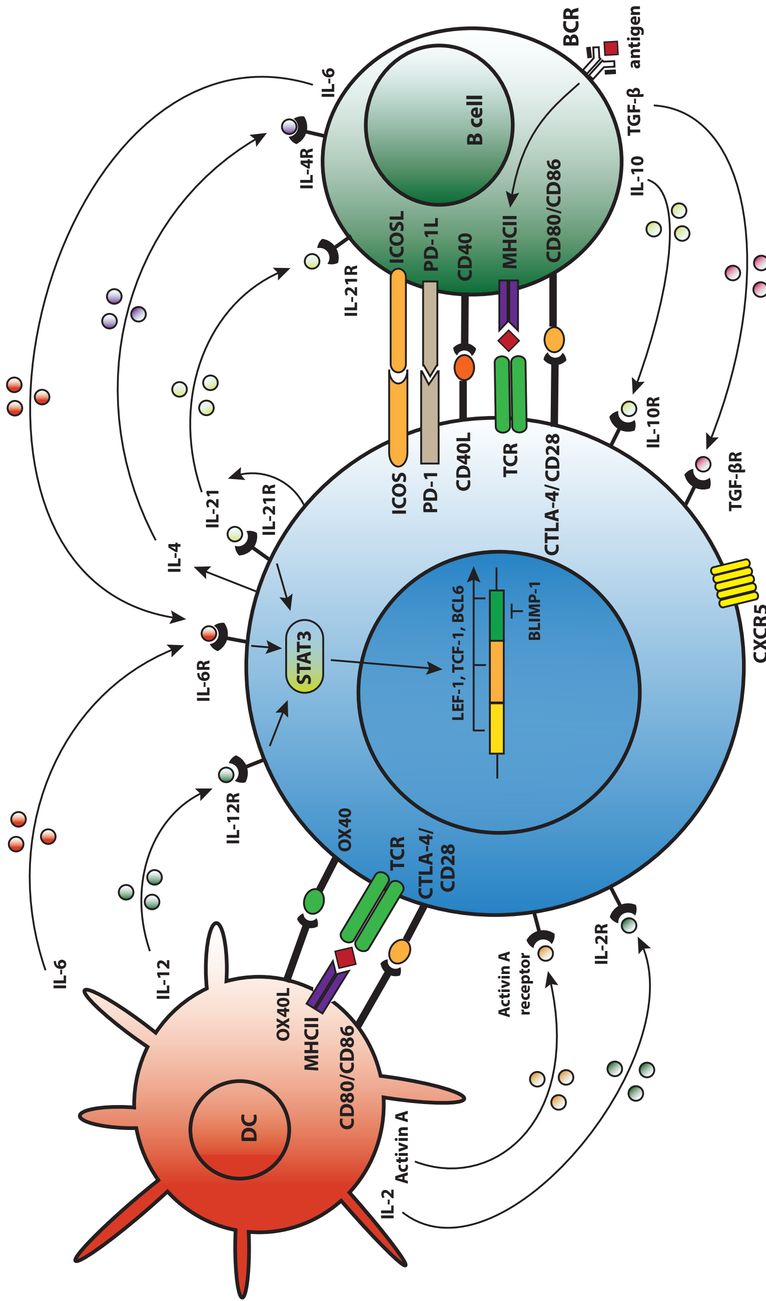

Figure 1. Tfh cell differentiation, activation and crosstalk.
Schematic overview of molecules involved in the differentiation of Tfh cells, the activation of Tfh cells by dendritic cells (DCs) and B cells and the crosstalk of Tfh cells with DCs and B cells.

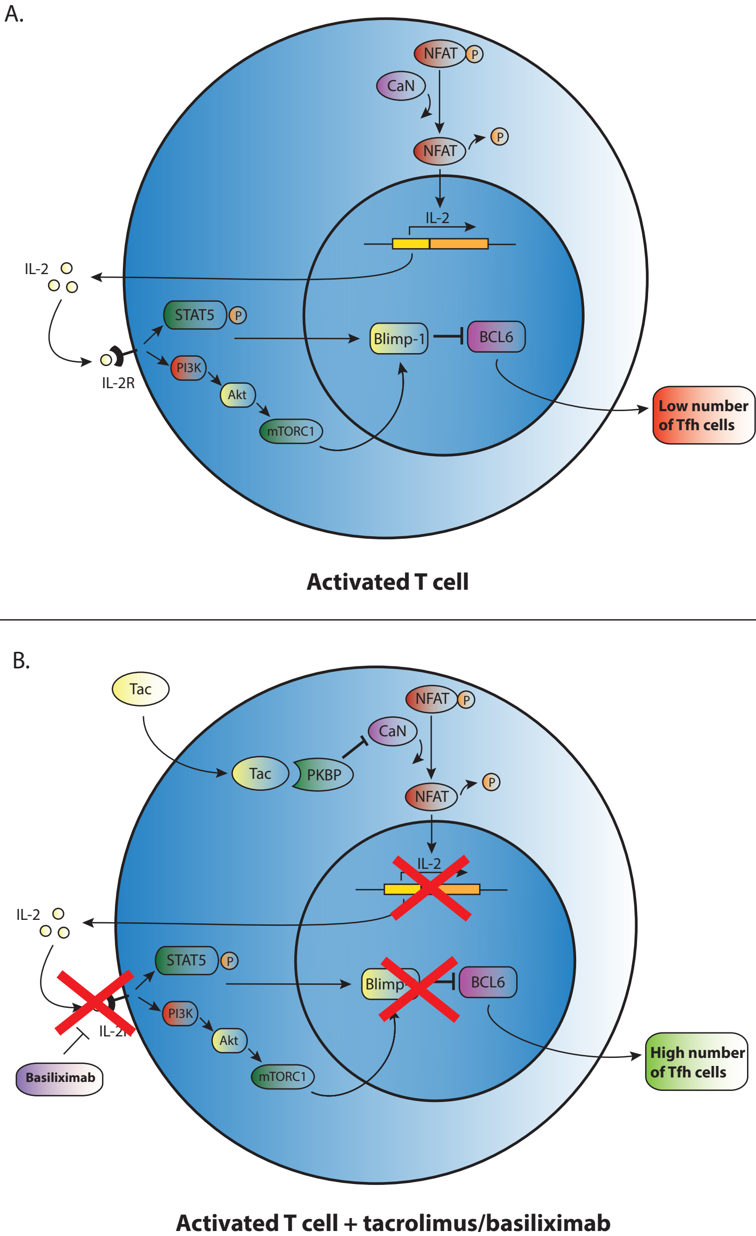

Figure 2. Possible effect of tacrolimus and basiliximab on Tfh cell differentiation and activation. (A.) An activated T cell is depicted in the upper panel. (B.) After addition of tacrolimus (Tac), calcineurin (CaN) is blocked and dephosphorylation of cytoplasmic NFAT is inhibited resulting in lower levels of IL-2 transcription. IL-2 promotes transcription of Blimp-1, a co-repressor of Bcl-6. In the absence of IL-2, lower transcription of Blimp-1 leads to increased expression of BCL6 and thus may enhance Tfh cell numbers. Basiliximab may promote the same effect of enhancing Tfh cell numbers via blocking the IL-2R.

mediated B cell differentiation. In contrast, LOF mutations in *IFNGR1/2, STAT1* and *IL12RB1* genes caused impaired function of IFNγ and thus promoted Tfh-B-cell interaction (52). Another study by Ma *et al.* reported that mutations in the genes encoding STAT3, IL-21R, CD40L, IFNGR1 LOF or STAT1 GOF inhibit the differentiation of Tfh cells via impairing the generation of IL-21 (53). Lower frequencies of Tfh cells were observed in patients with two transmembrane activator and CAML interactor (TACI) mutations compared to patients with a single mutation or without mutations (57). A study by Alroqi *et al.* described the presence of Tfh cells in patients with LPS-responsive beige-like anchor (LRBA) deficiencies. LRBA promotes the intracellular transport of CTLA4 toward the cell membrane, mostly expressed on regulatory T (Treg) cells and Th17 cells (58). In all patients studied increased frequencies of Tfh cells were measured associated with low CTLA4 expression levels on the Treg cells (54). When these patients were treated with CTLA4-Ig therapy the frequencies of Tfh levels significantly decreased (54). To this end, Tfh cell frequencies may be a useful readout in patients with LRBA and CTLA4 deficiencies to monitor the effect of CTLA4-Ig therapy. Taken together, the defects in differentiation of Tfh cells found in various PID patients provide strong evidence that the genes mutated in these diseases are essential for Tfh differentiation. Moreover, the finding of reduced Tfh cells in X-linked agammaglobulinemia patients with mutations in BTK shows that Tfh development is B cell dependent (52).

The effects of conventional immunosuppression on Tfh cells

Maintenance immunosuppression after solid organ transplantation typically consists of a calcineurin inhibitor (CNI), either tacrolimus or cyclosporin A, the T cell proliferation inhibitor mycophenolate mofetil (MMF) and steroids. An *in vitro* study analyzing the effect of methylprednisolone and CNI agents on T cells showed that these immunosuppressants could inhibit differentiation of human naïve CD4+ T cells into Tfh cells (59). *In vivo*, we found that Tfh-like cells are present in the circulation of kidney transplant recipients receiving tacrolimus-based immunosuppression. These circulating Tfh-like cells have the capacity to induce B cell differentiation into immunoglobulin-producing plasmablasts (51). A recent study from our group demonstrated that tacrolimus had a small inhibitory effect on Tfh-cell generation *in vitro* and could partially prevent Tfh-cell activation. The production of IL-21 was incompletely inhibited by high concentrations of tacrolimus, which may be why the remaining activated Tfh cells retained the potential capacity to assist B cells (60). In another study, methylprednisolone treatment of patients with systemic lupus erythematosus was found to reduce the number of peripheral $CD4^{+}CXCR5^{+}PD\text{-}1^{+}$ T cells, but without any evidence of altered Tfh function (61). Based on the available data, it can be concluded that conventional immunosuppressive therapies do not block Tfh cell activity completely and Tfh cell activity could usefully be examined in patients with allograft rejection or rapidly declining graft function.

This limited basis of evidence highlights that there is no clear understanding about the mechanisms by which immunosuppressants affect the development of Tfh cells. CNIs suppress IL-2 production through inhibiting the dephosphorylation of nuclear factor in activated T cells (NFAT), which is the key transcription factor for IL-2 and its receptor (Figure 2) (62). In a mouse model with acute viral infection, Ray *et al.* (39) demonstrated that IL-2 is able to enhance the expression of the transcriptional repressor Blimp-1 through the STAT5 and PI3K-Akt signaling pathway and promotes inhibition of Bcl-6 expression in activated T cells. It ultimately shifts the immune reaction during T cell differentiation away from a humoral response (Figure 2A). In theory, calcineurin inhibitors may influences Tfh differentiation through the control of IL-2 expression (Figure 2B). Basiliximab may also control Tfh differentiation in the same manner via blockade of the IL-2 receptor (Figure 2B). However, as proved in a mouse model, NFAT activity, which is suppressed by tacrolimus, is also a functional requirement for Tfh differentiation and may induce secretion of IL-21 by Tfh cells (63, 64). Since calcineurin inhibitors suppress NFAT activity and the corresponding expression of IL-2 and most likely also of IL-21, this class of agents may influence the generation of Tfh cells through regulating the balance of IL-2 and IL-21. However, further studies are needed to determine the mode of action of immunosuppressive agents on Tfh cells.

Tfh-targeted immunotherapy

Co-inhibitory pathways

As summarized in Figure 3, different strategies can be employed to target Tfh activation and/or function. CTLA4 could control B cell responses by modulating Tfh cell activity (30). Abatacept and belatacept are first and second generations of the fusion protein CTLA4-Ig. A study based on a mouse skin graft model by Kim *et al*. showed that abatacept reduced the number of activated $CD4^{+}PD\text{-}1^{+}CXCR5^{+}$ Tfh cells in the spleen, which was associated with suppression of antibodies directed against the skin transplant (65). CTLA4-Ig also inhibited the increase in circulating Tfh cells and B cell-mediated antibody production in a mouse heart transplant model (66). In primary Sjögren's syndrome patients, abatacept treatment attenuated circulating Tfh-cell numbers and Tfh-cell dependent B cell hyperactivity (67). In kidney transplant patients, we found that belatacept partially inhibited Tfh cell activation; the remaining activated Tfh cells were able to provide B cell help. Belatacept is less potent *in vitro* than tacrolimus in inhibiting Tfh cell-dependent plasmablast formation (60). Based on these preliminary data it seems that human circulating Tfh cells after transplantation are less susceptible to co-stimulation blockade than mouse Tfh cells. However, proof that lower susceptibility in humans leads to more extensive antibody-mediated anti-donor responses has not been established. New immune monitoring trials are needed to confirm the immunosuppressive effects of belatacept on human Tfh cells.

Co-stimulatory pathways

Tfh cells control the quality of long-lived humoral immunity through the ICOS/ICOSL signaling pathway (68). ICOS is able to directly promote Tfh cell recruitment toward the GC and acts as a linker between T and B cells, supporting positive selection for high-affinity bone-marrow plasma cells (68, 69). Based on these studies, targeting the ICOS/ICOSL signaling pathway might offer new opportunities to prevent production of DSA and to treat transplant patients with *de novo* DSA. A glyco-engineered antibody which depletes ICOS resulted in a significant reduction in anti-nucleosomal autoantibodies in a SLE lupus-prone mouse model (70). Sato *et. al.* demonstrated that ICOS expression was up-regulated on T cells in a canine hematopoietic cell transplantation model of graft rejection or chronic graft-versus-host disease. In this study, immunosuppressive effects were observed in mixed leukocyte reactions where anti-ICOS was combined with suboptimal concentrations of CLTA4-Ig or cyclosporine (71). Recently, another study showed that anti-ICOSL antibody did not impact the early Tfh differentiation in a mouse model with *Plasmodium chabaudi*AS infection, but ICOS is necessary for maintenance of a sustained high-affinity, protective Ab response (72). Targeting the ICOS pathway with biologicals is a promising new direction to control the function of Tfh cells and subsequently B cells. However, it is clear that more knowledge is warranted to better understand the reported discrepancies in above described *in vitro* and *in vivo* models. Therefore, the clinical use of anti-ICOS therapy is still in development.

The CD40L/CD40 signaling pathway is also important in the interaction between Tfh and B cells. There are several known anti-CD40 agents. One of these, 2C10R4, is currently being investigated in clinical trials. Kim *et al*. demonstrated in a rhesus macaques kidney transplant model that 2C10R4 prevented antibody-mediated rejection via affecting Tfh cells and IL-21 production in germinal centers and reducing production of early *de novo* DSA (73). In this study, belatacept was as effective as 2C10R4 in regulating Tfh cells and preventing acute rejection. More proof that blockade of the CD40-CD40L pathway is effective in inhibiting antibody production comes from studies analyzing the effect of CFZ533, a Fc-silencing and non-B cell depleting anti-CD40 monoclonal antibody. Non-human primates treated with this agent had prolonged kidney allograft survival rates and better kidney function parameters than the untreated control group. Treatment with CFZ533 prevented the production of alloantibodies in these animals (74). Hence, next to CTLA4-Ig, anti-CD40 agents represent a promising option for co-stimulatory blockade to inhibit both Tfh and B cells. An alternative strategy is the combined use of tacrolimus and new co-stimulatory blockers to inhibit allograft humoral immunity (73). However, the risk of over-immunosuppression should be considered.

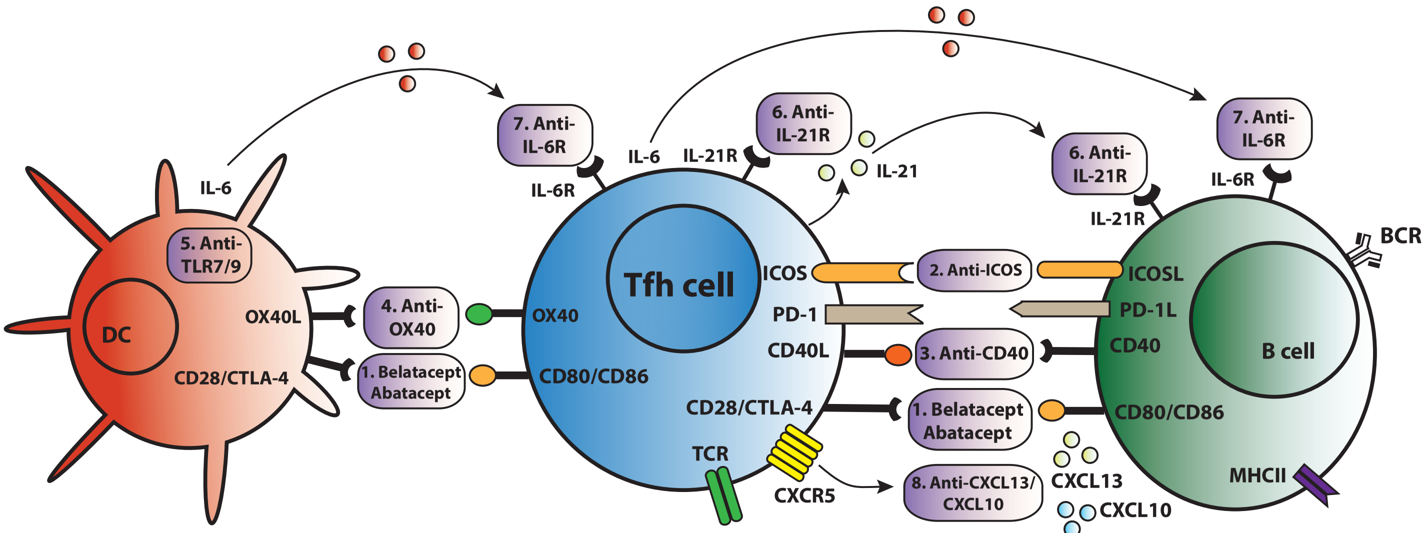

Figure 3. Tfh-targeted immunotherapy.

Blockage of the Tfh activation and function is established via several routes. An overview of these Tfh-targeted immunotherapies is summarized in this figure with (1.) Belatacept/abatacept, blocking co-stimulation of CD28/CTLA-4 and CD80/CD86, (2.) anti-ICOS, (3.) anti-CD40, (4.) anti-OX40, (5.) anti TLR7 or TLR9, (6.) anti-IL-21R, (7.) anti-IL-6R, and (8.) anti-CXCL13 and anti-CXCL10.

The tumor necrosis factor receptor OX40, activated by its cognate ligand OX40L, functions as a T cell costimulatory molecule. OX40L is expressed on DCs and myeloid APCs, but not on B cells. CD8α$^-$ DCs, known for their MHC class II presentation, are localized at the inter-follicular zone and play a pivotal role in the induction of antigen-specific Tfh cells by upregulating the expression of ICOSL and OX40L (75). The frequency of circulating OX40L-expressing myeloid APCs shows a positive correlation with disease activity and the frequency of ICOS$^+$ blood Tfh cells in SLE. This may result from the capacity of OX40 signals to stimulate naïve and memory CD4$^+$ T cells to express multiple Tfh cell molecules. Therefore, OX40 signals are adequate to induce these T cells to become functional B cell helpers (76). Blocking the OX40/OX40L pathway resulted in prolonged allograft survival in cardiac and skin transplantation mouse models (77, 78). However, the optimal timing for OX40 blockade is unclear and there are still potential problems with use of OX40/OX40L therapy for post-transplant autoimmunity (79). In addition, OX40L expression on myeloid APCs can be induced by immune complexes containing RNA via toll-like receptor 7 (TLR7) activation (76). Toll-like receptor 9 (TLR9) signaling in DCs led to higher numbers of Tfh and GC B cells, and accelerated production of broad-affinity anti-hapten IgG (80). Hence, TLR7/TLR9 might also offer a potential route to prevent antigen presentation of APC to Tfh cells.

Cytokine-dependent pathway

IL-21 acts as the dominate cytokine within the GC, and is a promising target to inhibit Tfh function. Treatment of SLE lupus-prone mice with an anti-IL-21 blocking antibody reduced titers of autoantibodies, delayed progression of glomerulonephritis and diminished renal-infiltrating Tfh and Th1 cells, whilst improving overall survival (81). In an allogeneic setting, we recently demonstrated through *ex vivo* experimentation that IL-21 produced by donor antigen-activated Tfh cells could simulate humoral immunity, while an IL-21 receptor antagonist (αIL-21R) could inhibit B cell differentiation and decrease the proportion of plasmablasts and production of IgM and IgG2 (26). Moreover, IL-21 has been shown capable of overcoming Tfr cell-mediated suppression and inhibiting Tfr cells, thus skewing the ratio of Tfh to Tfr cells (40, 82). Since IL-21 is more specific to the interaction of GC-Tfh cells than other cytokines and molecules, there is an urgent need to identify the efficiency and safety of IL-21 inhibition or IL-21R blockade in the suppression of humoral immunity. Both IL-21 receptor antagonists and IL-21 blocking antibodies have the potential to modulate the Tfh-mediated immune response.

IL-6 is a cytokine which offers another attractive option for targeting humoral immunity (23). Specific targeting of IL-6 receptor by Tocilizumab in patients with rheumatoid arthritis led to a significant reduction in the number of circulating Tfh cells and in IL-21

production by $CD4^+$ T cells (23). In transplantation, anti-IL-6 receptor treatment decreased the number of $IL21^+CD4^+$ and $CD4^+CXCR5^+$ Tfh cells in the spleens of allosensitized mice, as well as suppressing antibody recall responses and DSA levels (83, 84). A first study in kidney transplant patients proved that targeting the IL-6/IL-6R in highly sensitized patients could be a safe and new alternative in addition to currently used plasmapheresis with low-dose intravenous immunoglobulin (85). Given the interplay between IL-6 and IL-21, the combined blockade IL-6 and IL-21 might be a better option to inhibit the differentiation, expansion and function of Tfh cells at all stages.

TGF-ß appears to have multiple roles in transplantation. It promotes Tfh cells differentiation (22), while prevents Tfh cell accumulation, self-reactive B cell activation and autoantibody production in GCs (86). TGF-ß is also a vital fibrosis cytokine (87). A clear understanding of the effect of TGF-ß blockade on the entire humoral response and allograft fibrosis is required, however, before any therapeutic application.

Tfh migration-dependent pathways

Targeting chemokine receptors or ligands to prevent Tfh migration offers another possible route for intervention. C-X-C chemokine ligand 13 (CXCL13) is produced in abundance by follicular stromal cells within B cell follicles. Expression of CXCL13, the receptor for CXCR5, on B and Tfh cells is necessary for their migration to the center of follicles to establish GCs (88). In addition, circulating Tfh cells respond to CXCL13 and can move back to the secondary lymphoid organ GC (9). Although CXCL13 acts locally, it can also be detected in plasma and its level is associated with the extent of immune activity (89). Patients with active chronic graft versus host disease have a lower frequency of circulating Tfh cells but higher CXCL13 plasma levels, suggesting increased homing of Tfh cells to secondary lymphoid organs (90). Circulating Tfh cells are also able to migrate into allografts via other chemokines such as CXCL10, and take part in the formation of tertiary lymphoid organs (9), which have been found in kidney biopsies after rejection (51). Anti-CXCL10 has been used to treat rheumatoid arthritis and ulcerative colitis in phase II clinical trials (91, 92). Depletion of B cells by rituximab has no effect on tertiary lymphoid organs in chronic allograft dysfunction (93). Therefore targeting CXCL13 or CXCL10 to inhibit the homing and trafficking of Tfh cells might be a more efficient strategy than B cell depletion to prevent the accumulation of Tfh in secondary lymphoid organs and formation of tertiary lymphoid organs in the allograft.

Immune enhancing pathways

PD-1, which is highly expressed on activated GC-Tfh cells, plays an important role in Tfh cell differentiation and formation of GCs (50). In theory, anti-PD-1 could control Tfh-cell

activation. However, contradicting results are presented. In a PD-1-deficient mouse model, Kawamoto *et al.* found increased production of Tfh cells with altered phenotypes and dysregulation of GCs, eventually resulting in reduced antigen affinity of IgA (94). Good-Jacobson *et al.*, demonstrated that there were less plasma cells, GCs and Tfh cells in the absence of PD-1, however the remaining plasma cells were of a higher affinity (95). Others have also observed expansion of Tfh cells and enhanced humoral responses by blocking the PD-1 pathway (96, 97). In general, and thus also in organ transplantation patients, blockade of the PD-1 pathway boosts the immune response. These agents are approved for the treatment of solid tumors (98). A recent case report showed that a kidney transplant recipient given anti-PD-1 treatment for metastatic cutaneous squamous cell carcinoma had an acute cell-mediated allograft rejection two months after starting anti-PD-1, indirectly suggesting a role for PD-1 in regulation of transplant-related immune responses (99). There is no direct evidence that PD-1 agonists or antagonists would benefit transplant patients. The current observations might make PD-1 an unrealistic and ineffective target for achieving allograft humoral immunity.

Tfh cells as a biomarker in pharmacodynamics

Tfh cells may have a role in pharmacodynamic monitoring of immunosuppression, as a marker for the extent to which the antibody response is inhibited. Circulating Tfh cells could be the counterpart of GC-Tfh cells as they share functional properties with Tfh cells residing in SLOs (16). However, whether these circulating Tfh cells are formed in the circulation, or have left the GC after exposure to an antigen and reside as memory Tfh cells in the blood is unknown. He *et al.* demonstrated that the circulating $CCR7^{lo}PD\text{-}1^{hi}$ Tfh subset correlated with clinical indices in autoimmune diseases and suggested it might serve as a biomarker for pathogenic antibody responses in that setting (100). Bentebibel *et al.* found that the presence of $ICOS^{+}CXCR3^{+}CXCR5^{+}CD4^{+}$ T cells correlated with influenza vaccination antibody responses (101). Several papers have demonstrated that production of the neutralizing HIV antibody is dependent on Tfh cells, which could be a predictive biomarker for vaccine response (19, 102-104). Chenouard *et al.* suggested that a lack of Tfh cells, as seen in kidney transplant patients with operational tolerance, may induce a pro-tolerogenic environment with reduced risk of developing *de novo* DSA (105).

Individualized therapy after transplantation based on pharmacodynamic monitoring is the direction of immunosuppressant development. DSA, the traditional biomarker for humoral immunity, may not be the most sensitive indicator of the immune response after transplantation. As the launching cells for plasmablasts and memory B cells, the frequency of donor specific Tfh cells might also be a sensitive tool to identify patients

at risk for developing a DSA mediated immune response. These donor specific Tfh cells can be quantified by donor specific IL-21 Enzyme-Linked ImmunoSpot (ELISPOT) assay. Perhaps serum IL-21 measured by Enzyme Linked Immunosorbent Assay (ELISA) could also be a good alternative for pharmacodynamic monitoring. Of note, CXCL13 has also been suggested as a plasma biomarker for GC activity (106). Dose adjustments and switching immunosuppressive therapies based on monitoring of donor-specific IL-21^{+} Tfh numbers in combination with serum CXCL13 might be of benefit in alloimmune-risk stratification and for individualized therapy in transplantation.

Perspective and future directions

Graft failure in immunosuppressed patients is often a combination of mixed cellular and antibody-mediated rejection. Current immunosuppression seems to have limited capacity to control the allograft loss in recipients with high risk of rejection. Targeting the Tfh cell during maintenance therapy has the potential to prevent mixed clinical rejection early in the activation cascade, due to its pivotal role in conjugating the interaction of T and B cells. Suppression of both GC-Tfh cell formation and Tfh cell migration via two different agents – for example by blocking IL-21 and CXCL13 –probably provides more robust control of humoral immunity. In addition, treatment strategies for patients undergoing solid organ transplantation should be defined according to the individual's immune activity (*e.g.*, number of circulating donor-specific IL-21+ T cells) and risk of rejection. Monitoring donor-specific Tfh and memory B cells to guide the implementation of immunosuppression would help to tailor therapy appropriately. It would be of high interest to develop tools to easily investigate the specificity of these Tfh cells. In addition, immunosuppressive agents such as mTOR inhibitors with known Treg generating effects might also beneficially contribute to the generation of antigen specific Tfr cells in transplant patients (107). This recently described T cell population controlling B cell immune responses is of high interest to intervene in the humoral alloimmune response leading to improved graft survival in patients after organ transplantation (27).

Although many agents can influence Tfh cell development and function, only a few are likely to be suitable for clinical investigation. Successful immunosuppressive drug development has a lifecycle that includes the discovery of a target molecule, research into the mechanisms involved, animal experimentation, and validation in clinical trials. Clinical assessment requires comparison of the new drug versus current therapies and monitoring of adverse effects. Comparing the effects of new Tfh-targeting therapeutics against the conventional immunosuppressants used in transplantation, particularly in terms of Tfh dependent humoral immune activity, would be of considerable interest.

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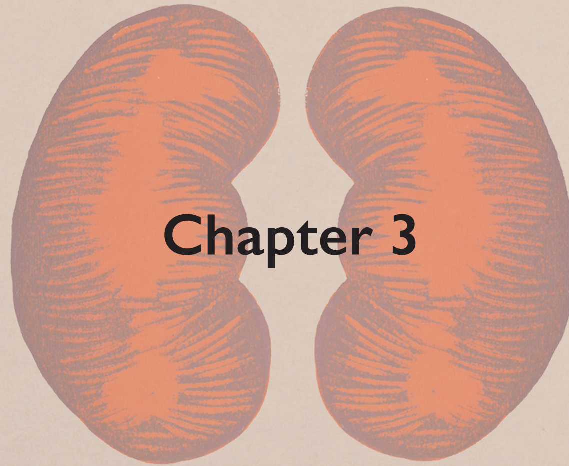

Chapter 3

IL-21 receptor antagonist inhibits differentiation of B cells toward plasmablasts upon alloantigen stimulation

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Frontiers in Immunology. March 2017; 8:306

Abstract

Interaction between T follicular helper (Tfh) cells and B cells is complex and involves various pathways, including the production of IL-21 by the Tfh cells. Secretion of IL-21 results in B cell differentiation toward immunoglobulin producing plasmablasts. In patients after kidney transplantation, the formation of alloantibodies produced by donor antigen activated B cells are a major cause of organ failure. In this allogeneic response, the role of IL-21 producing Tfh cells that regulate B cell differentiation is unknown. Here, we tested in an alloantigen driven setting whether Tfh cell help signals control B cell differentiation with its dependency on IL-21. Pre transplantation patient PBMCs were FACS sorted into pure $CD4^{pos}CXCR5^{pos}$ Tfh cells and $CD19^{pos}CD27^{pos}$ memory B cells and stimulated with donor antigen in the presence or absence of an IL-21R antagonist (αIL-21R). Donor antigen stimulation initiated expression of the activation markers inducible co-stimulator (ICOS) and programmed death 1 (PD-1) on Tfh cells, and a shift toward a mixed Tfh2 and Tfh17 phenotype. The memory B cells underwent class switch recombination (CSR) and differentiated toward IgM and IgG producing plasmablasts. In the presence of αIL-21R, a dose dependent inhibition of STAT3 phosphorylation was measured in both T and B cells. Blockade of the IL-21R did not have an effect on PD-1 and ICOS expression on Tfh cells but significantly inhibited B cell differentiation. The proportion of plasmablasts decreased by 78% in the presence of αIL-21R. Moreover, secreted IgM and IgG2 levels were significantly lower in the presence of αIL-21R. In conclusion, our results demonstrate that IL-21 produced by alloantigen activated Tfh cells controls B cell differentiation toward antibody producing plasmablasts. The IL-21R might therefore be a useful target in organ transplantation to prevent antigen driven immune responses leading to graft failure.

Introduction

After kidney transplantation the immunological barrier between organ donor and recipient still limits graft survival (1). In this setting, a large proportion of allograft recipients develop a donor-specific antibody response associated with an increased risk for chronic rejection (2-5). This complication accounts for more than 50% of chronic transplant failures leading to death, dialysis or re-transplantation of patients (3). Current immunosuppressive agents mainly aimed at T-cell-mediated alloimmunity, whereas agents that effectively target humoral effectors are still insufficient (6). Therefore, there is a need to develop new agents that specifically prevent the activation of B cell mediated immune responses.

Within humoral immunity, T cell-mediated help to B cells is required for the generation of antigen-specific antibody responses. This process is mainly driven via IL-21 secreting T follicular helper (Tfh) cells. Tfh cells are well known for their expression of CXC chemokine receptor 5 (CXCR5) (7). Sustained expression of CXCR5 helps Tfh cells localize to B cell follicles, where they interact with germinal-center (GC) B cells and produce IL-21 (8). Through autocrine and paracrine mechanisms, IL-21 amplifies and stabilizes Tfh cell-mediated responses, B cell proliferation, immunoglobulin class switch recombination (CSR), and B cell differentiation toward plasmablasts and long-living memory B cells (9, 10). In this respect, IL-21 directly effects B cell responses via IL-21R expressed on the B cells (11, 12). IL-21 signals through a receptor complex consisting of IL-21 receptor (IL-21R) and a common cytokine receptor γ-chain that activates downstream JAK/STAT pathways, predominantly by the phosphorylation of STAT3 (13, 14). Transcriptional repressor B-cell lymphoma 6 (Bcl-6) orchestrates the differentiation program of Tfh cells, while suppressing other T helper subset transcription factors (8, 15). The capacity of Tfh cells to interact with B cells is dependent on T-cell receptor interaction with antigens presented by MHC class II molecules and co-stimulatory molecules CD40ligand, inducible co-stimulator (ICOS) and programmed death 1 (PD-1) (7, 8). The circulating counterparts of the "GC-Tfh cells" in humans express CXCR5, low expression levels of PD-1 and ICOS and lack expression of transcription repressor Bcl-6 (16-18).

In transplantation, studies on peripheral Tfh cells and their role in IL-21 driven B cell differentiation are limited (19, 20). An increased frequency of circulating Tfh cells was found in patients with chronic antibody mediated allograft rejection after kidney transplantation (21). Furthermore, in patients with pre-existing donor-specific antibodies (DSA) an association was detected between pre-existing DSA's and the numbers of Tfh cells after transplantation (22). Co-stimulation blockade in a nonhuman primate kidney transplant model resulted in reduced IL-21 production in GCs and an attenuated antibody response (23). In addition, selective blockade of CD28 solely resulted in lower levels of IL-21 compared to CD80/86 co-stimulatory blocking therapy (24) For the development of immunosuppressive agents that

specifically target B cell mediated immune responses directed toward donor antigen early in the activation cascade a better understanding of Tfh biology is needed.

Kidney disease patients suffer from defective immune responses caused by decreased T and B cell activity (25, 26). Therefore, we have used patient materials to set up an *in vitro* system in which we studied whether Tfh cells instruct donor antigen-driven memory B cells to differentiate into immunoglobulin producing plasmablasts. Subsequently, we assessed whether this Tfh cell mediated differentiation and plasmablast formation is dependent on IL-21 by blocking the IL-21R with an antagonist (αIL-21R). Overall, our data define the role of IL-21/IL-21R signaling pathway in alloantigen driven and Tfh cell mediated B cell differentiation toward Ig producing plasmablasts.

Material and methods

Study population

For the *in vitro* assays, PBMCs of 17 kidney transplant recipients obtained one day pre-transplantation were analyzed and stimulated with the corresponding kidney donor PBMCs. Patient demographics are summarized in Table 1. The Medical Ethical Committee of our center approved this study (MEC-2010-022). All patients and donors gave written informed consent. B cell parameters were measured in all samples and T cell assays were performed when enough material was available for analysis. (Table 1)

Co-culture experiments of peripheral Tfh cells and memory B cells

Co-culture experiments with Tfh cells and memory B cells were conducted as schematically represented in supp. figure 1 to determine the functional interactions after donor antigen stimulation. PBMCs were thawed and $CD3^{pos}CD4^{pos}CXCR5^{pos}$ T cells (Tfh cells) and $CD19^{pos}CD27^{pos}$ (memory) B cells were FACS sorted by BD-FACSAria II $SORP^{TM}$ (purities ≥ 96%). Isolated Tfh cells ($2 \cdot 10^4$ cells/ well) were co-cultured with memory B cells ($2 \cdot 10^4$ cells/ well) in a 96-wells plate for 8 days in the presence of irradiated (40 Gy) donor PBMCs ($5 \cdot 10^4$/ well). At day 0 and after 8 days of culture, the Tfh cell phenotype, the B cell phenotype, and B cell differentiation toward Ig producing plasmablasts were measured with flow cytometry. The following MoAbs were used for the Tfh cell phenotype stainings: CD3 Brilliant Violet 510 (BV510, Biolegend, San Diego, CA), CD4 Brilliant Violet 421 (BV421, Biolegend), CXCR5 Alexa Fluor 647 (AF647, BD Biosciences, San José, CA), ICOS phycoerythrin-Cyanine7 (PE-Cy7, Biolegend), CCR6 PE (eBioscience, San Diego, CA), CXCR3 peridinin chlorophyll (PerCP, Biolegend) and PD-1 Allophycocyanin-Cy7 (APC-Cy7, Biolegend). MoAbs used for the B cell stainings: CD19 BV510 (Biolegend), CD38 BV421 (BD Biosciences), IgG APC (BD Biosciences),

CD27 Pe-Cy7 (eBioscience), IgM PE (BD Biosciences) and IgD APC-Cy7 (Biolegend). 7-aminoactinomycin (7-AAD) PerCP was included to measure cell viability. To define the role of IL-21/IL-21R signaling in alloantigen activated Tfh and memory B cells, the co-cultures were pre incubated for 30 minutes with humanized anti-IL-21R antibody ATR-107 (10µg/ml, kindly provided by Pfizer, New York, NY) or isotype-matched control (10 µg/ml, IgG1-Fc, R&D systems, Minneapolis, MN) at 37°C. Hereafter, the irradiated donor cells were added to the co-cultures and further incubated at 37°C for 8 days. Total IgM, IgG and IgG2 production was measured in the culture supernatants with a sandwich ELISA (eBioscience). All flow cytometry analyzes were performed with Kaluza Analysis 1.3 (Beckman Coulter, Fullerton, CA).

Table 1.

	Study group (n=17)
Patients age in years (median, range)	57 (33-74)
Recipient gender (% male)	76.5%
HLA-A mismatches (mean ± s.d.)	1.1 (± 0.7)
HLA-B mismatches (mean ± s.d.)	1.7 (± 0.5)
HLA-DR mismatches (mean ± s.d.)	1.5 (± 0.5)
Panel reactive antigen (median, range)	
• Current	0.0% (0.0-71%)
• Peak	4.0% (0.0-99%)
Previous kidney-transplantation	11.8% (2)
• Second kidney transplantation	5.9% (1)
• Third kidney transplantation	5.9% (1)
Renal replacement therapy before transplantation	88.3% (15)
• Haemodialysis	76.5% (13)
• Peritoneal dialysis	11.8% (2)
Cause of end-stage renal disease	
• Hypertensive nephropathy	35.3% (6)
• Diabetic nephropathy	41.2% (7)
• Focal segmental glomerulosclerosis	5.9% (1)
• IgA nephropathy	5.9% (1)
• Polyarteritis nodosa	5.9% (1)
• Unknown	5.9% (1)

Numbers between brackets represent patient number unless otherwise specified.

Phospho-specific flow cytometry

Phosphorylation of STAT3 by CD4+ T cells and CD19+ B cells was determined by phospho-specific flow cytometry. In brief, PBMCs were stained with CD3 BV510 (Biolegend) and CD19 Pe-Cy7 (Biolegend) for 30 minutes at RT in the dark. Next, the cells were incubated for 30 minutes at 37°C with various concentrations of the humanized anti-IL-21R antibody ATR-107 (Pfizer) or isotype-matched control (IgG1-Fc, R&D systems) followed by stimulation with recombinant human IL-21 (100 ng/ml, eBioscience) or recombinant human IL-6 (100 ng/ml, PeproTech, Rocky Hill, NJ) for 15 minutes at 37°C. Cells were fixed for 10 minutes with Cytofix buffer (BD Biosciences) at 37°C and permeabilized 30 minutes in 1ml methanol 90% at -20°C. Next, samples were stained with CD4 BV421 (Biolegend) and pSTAT3 PE (BD Biosciences). STAT3 phosphorylation was calculated as the median fluorescence intensity (MFI).

B cell stimulation assay

B cell stimulation with a minor cocktail of stimuli was performed to study the effect of IL-21, co-stimulation and BCR activation on plasmablast formation. CD19+ B cells were isolated via CD43 negative selection with CD43 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (purities ≥ 85%). B cells were incubated with anti-IL-21R antibody ATR-107 (10 µg/ml, Pfizer) or isotype-matched control (10 µg/ml IgG1-Fc, R&D systems). Next, cells were stimulated with 5 µg/ml soluble anti-CD40 (Bioceros, Utrecht, The Netherlands), 10 µg/ml goat-anti-human IgM (Jackson Immunoresearch, West Grove, PA) and human recombinant IL-21 (100 ng/ml, eBioscience). Subsequently, the presence of plasmablasts on day 0 and the differentiation of memory B cells into plasmablasts on day 8 were determined with flow cytometry. Plasmablasts were defined as $CD19^{pos}CD27^{high}CD38^{high}$ cells (16). The following monoclonal antibodies (MoAbs) were used: CD19 BV510 (Biolegend), CD27 Pe-Cy7 (eBioscience), IgD APC-Cy7 (Biolegend) and CD38 BV421 (BD Biosciences). In addition, viability staining with 7-AAD PerCP was performed (BD Biosciences).

Statistics

Statistical analyses were performed using Wilcoxon signed-rank test and Spearman's rank correlation test using GraphPad Prism 5 software (GraphPhad Software, San Diego, CA, http://www.graphpad.com). A two-tailed p-value <0.05 was considered statistically significant.

Results

Tfh cells are activated upon stimulation with alloantigen

We set up an *in vitro* system to study the functional interaction of $CD4^{pos}CXCR5^{pos}$ Tfh cells and $CD19^{pos}CD27^{pos}$ memory B cells upon alloantigen stimulation and the role of IL-21 in this response. Purified Tfh cells and memory B cells were co-cultured and stimulated with donor alloantigen. As a negative control, donor alloantigen was omitted (Supp. figure 2). For gating strategies see Figure 1A. Proportions of activation markers PD-1 and ICOS on the Tfh cells significantly increased after co-culture (Figure 1B, p=0.02 and p=0.008, respectively).

A heterogeneous Tfh cell population was observed at day 8 based on expression of chemokine receptors CCR6 and CXCR3. Three Tfh subsets can be distinguished: $CXCR3^{pos}CCR6^{neg}$ Tfh1 cells, $CXCR3^{neg}CCR6^{neg}$ Tfh2 cells and $CXCR3^{neg}CCR6^{pos}$ Tfh17 cells. Especially the Tfh2 and Tfh17 subsets ($CXCR3^{neg}$) are able to induce B cell differentiation and Ig CSR via IL-21 while the $CXCR3^{pos}$ Tfh1 cells lack this capacity (16, 27). The mean proportion of Tfh2 and Tfh17 cells at day 8 after co-culture was 71% (range: 38-80%), forming the majority of the different subsets (Figure 1C).

Tfh mediated B cell differentiation occurs upon stimulation with alloantigen

Over the 8 days of co-culture, the composition of surface immunoglobulins on the memory B cells changed. After co-culture CSR toward IgG occurred in part of the samples, with mean proportion surface IgG of 30% (range: 18-56%) before co-culture toward 35% (range: 12-89%) after co-culture (Figure 2A, p=0.04) while IgD proportions decreased from mean proportion of 18% (range: 4-26%) to 7% (range: 0.0-21%) after co-culture (Figure 2A, p=0.0009). The proportion of B cells expressing IgM did not significantly change after the co-culture (Figure 2A, p=0.09). A significant increase in plasmablast numbers was observed after co-culture, proving the robust capacity of alloantigen and Tfh cells in stimulating memory B cell differentiation (Maximum 28%, Figure 2B, p=0.003). This increase in plasmablast numbers was not observed in co-cultures without donor alloantigen stimulation (Supp. figure 2). Based on the median proportion (10%) at day 8, we observed a group of cultures with high plasmablast proportions (>10%, n=9) and a group with low plasmablast proportions after culture (<5%, n=8). No significant differences were found between the two groups based on baseline characteristics (Supp. Table 1). The immunoglobulin producing capacity of the generated plasmablasts was proven by the correlation between the proportion of plasmablasts and the concentration of IgM or IgG in the supernatants of all cultures (Figure 2C, p<0.0001 and p=0.0006, respectively). Overall, we showed that kidney transplant patient derived circulating Tfh cells before transplantation can be activated and are capable to stimulate memory B cell CSR and differentiation toward plasmablasts in the presence of alloantigen.

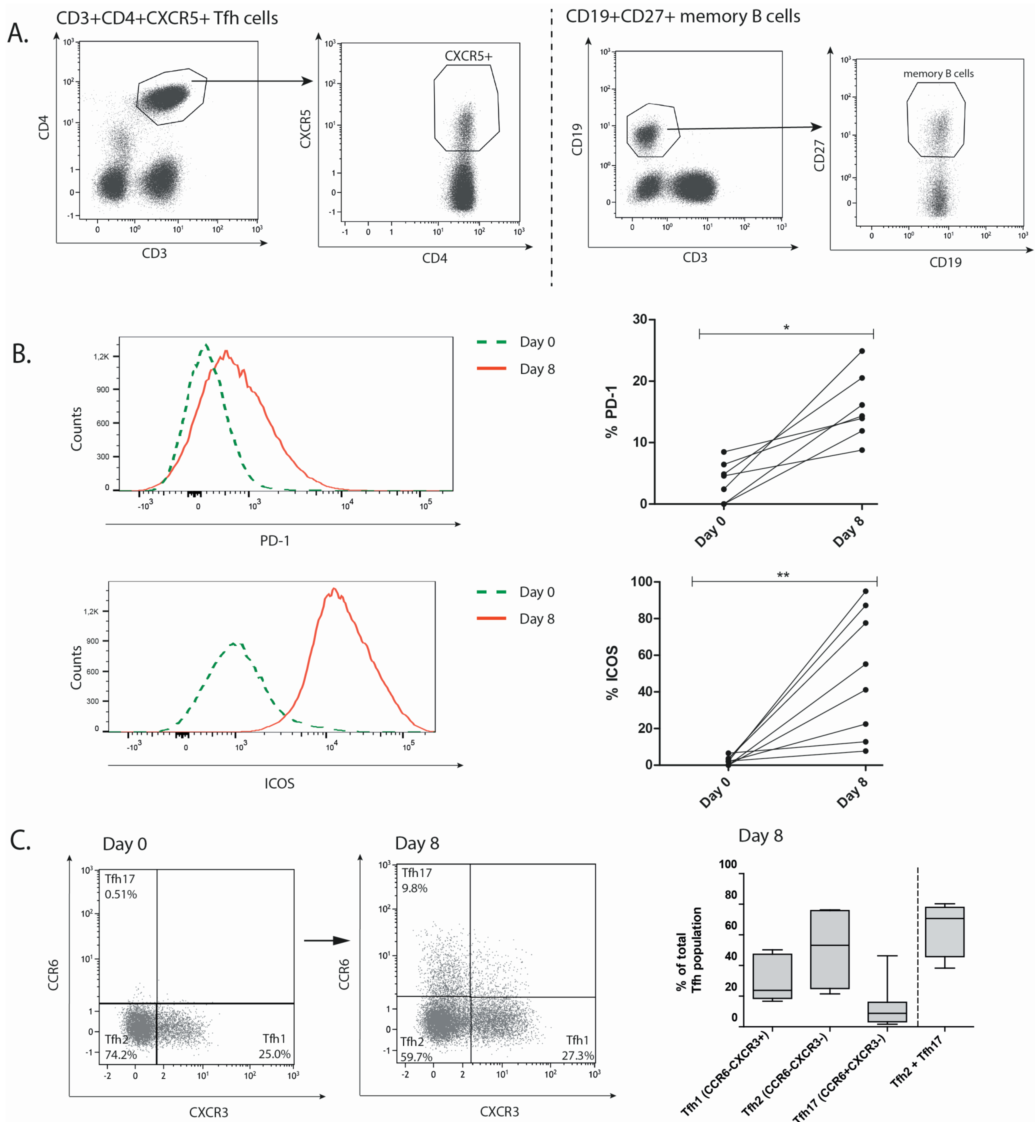


Figure 1. Tfh cells are activated upon stimulation with alloantigen.

Tfh cells and memory B cells from patients, pre kidney transplantation, were stimulated with alloantigen and co-cultured for 8 days. (A) Typical example of fluorescence activated cell sorting (FACS) gating strategy to obtain $CD4^{pos}CXCR5^{pos}$ Tfh cells and $CD19^{pos}CD27^{pos}$ memory B-cells. Cells were gated from viable (7-AAD negative) lymphocytes, defined by forward- and side-scatter. (B) Histogram overlays and quantified data of proportions PD-1 and ICOS within $CD4^{pos}CXCR5^{pos}$ Tfh cells at baseline (day 0) and day 8 after co-culture (PD-1 n=7; ICOS n=8). (C) Gating strategy and distribution of proportions $CCR6^{neg}CXCR3^{pos}$ Tfh1, $CCR6^{neg}CXCR3^{neg}$ Tfh2 and $CCR6^{pos}CXCR3^{neg}$ Tfh17 cells of the total Tfh population on day 0 and 8 after co-culture. N.B.: Box whiskers represent minimal and maximal values. The upper and lower border of the box represent the 25% and 75% percentile with the middle line representing the median (n=8).

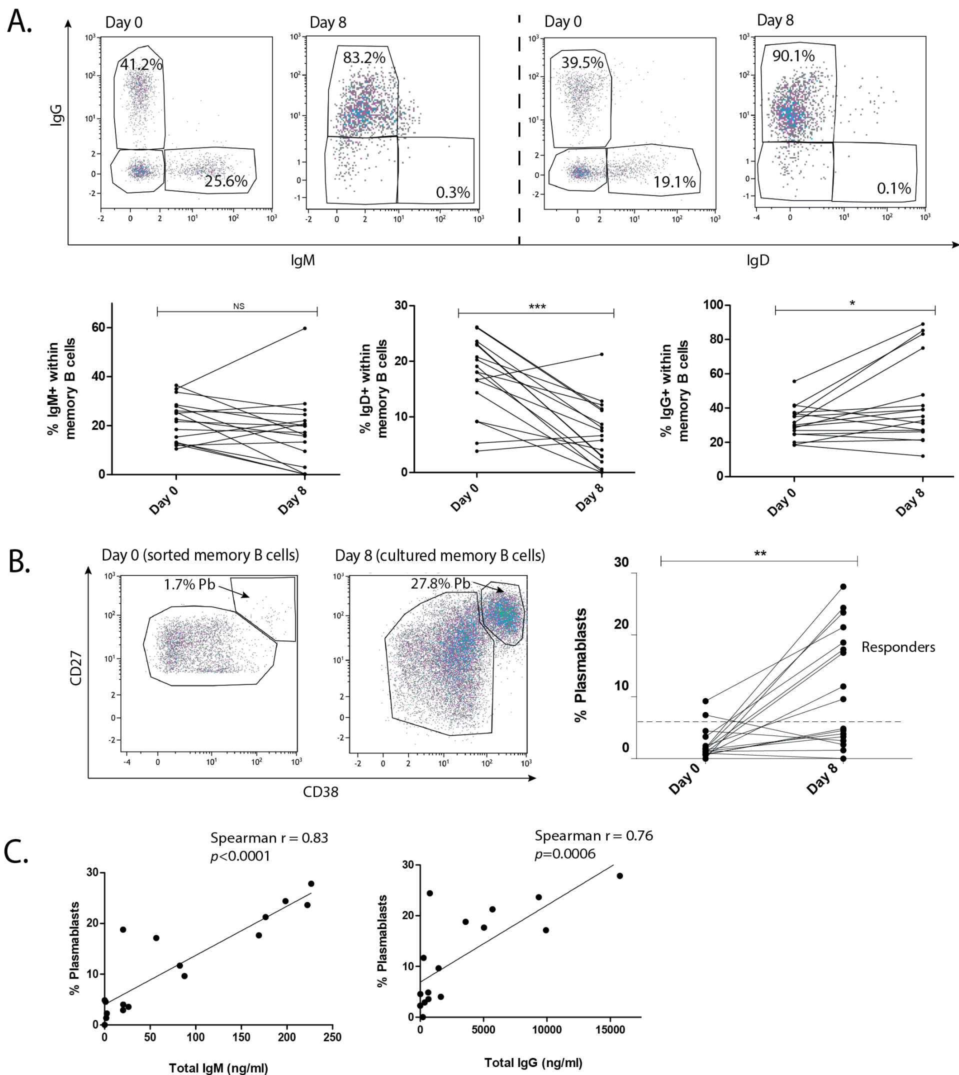


Figure 2. Tfh mediated B cell differentiation occurs upon stimulation with alloantigen

Tfh and memory B cells were stimulated with alloantigen and co-cultured for 8 days. (A) Gating strategy of surface immunoglobulins IgM, IgD and IgG within the memory B cell population before and after co-culture and corresponding quantified data (n=17). (B) Gating strategy of $CD27^{high}CD38^{high}$ plasmablasts and remaining memory B cells at baseline (day 0) and after 8 days co-culture is depicted on the left. Quantified data of plasmablast proportions at day 0 and after 8 days co-culture are depicted on the right. Dashed line distinguishes between cultures with a high plasmablast proportion (>10%) compared to a low plasmablast proportion (<5%) at day 8 (n=17). (C) Correlation between percentage plasmablasts and IgM or IgG (ng/ml) in the culture supernatant after 8 days co-culture (n=16). *p<0.05, **p<0.003, ***p<0.0009. Pb = plasmablast.

Phosphorylation of STAT3 is inhibited in the presence of Anti IL-21R antibodies

To determine the functionality of the IL-21R antibody (αIL-21R) in blocking IL-21R induced signaling events in T and B cells, we performed phosphor-specific flow cytometry. Phosphorylation of STAT3 was measured after stimulation with IL-21 or IL-6 (positive control). The specificity of αIL-21R was demonstrated by a 3.5 fold reduction in the phosphorylation of STAT3 on CD4+ T cells in the presence of 2 µg/ml αIL-21R onwards when comparing the IL-21 stimulated cells with the IL-6 stimulated cells (Figure 3A). Complete inhibition of STAT3 phosphorylation in the presence of αIL-21R was seen on both T and B cells (Figure 3B and 3C, p=0.002 and p=0.0005, respectively). In the presence of an isotype-matched control of αIL-21R the STAT3p levels were similar to the condition with only IL-21 stimulation. From these findings we conclude that αIL-21R efficiently blocks IL-21R induced signaling in T and B cells.

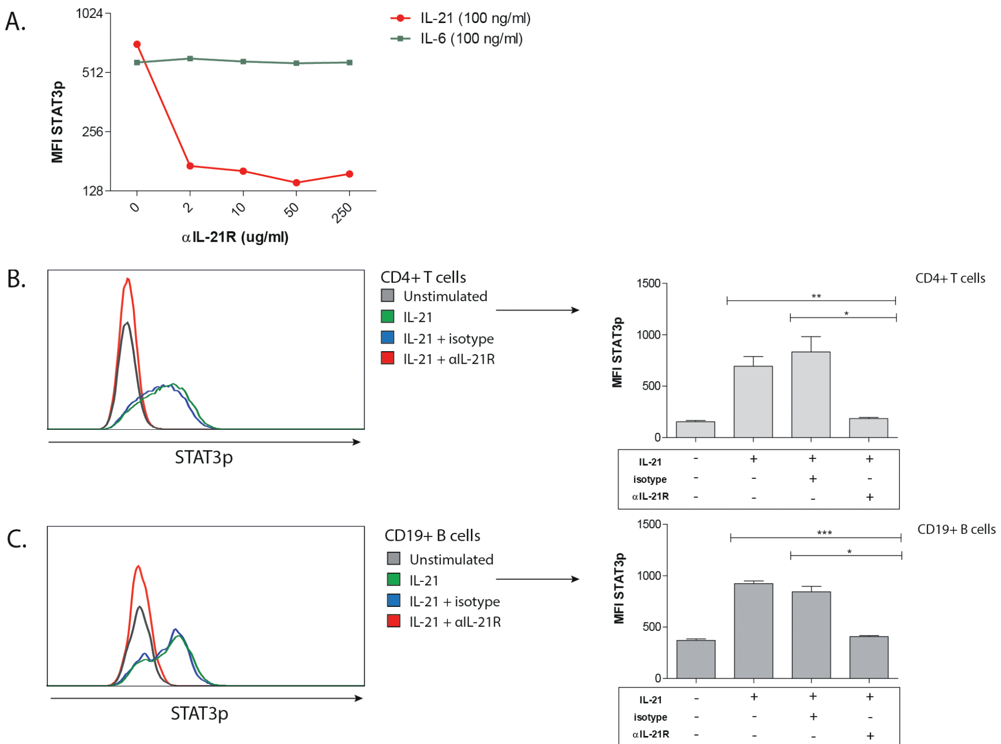


Figure 3. Phosphorylation of STAT3 is inhibited in the presence of αIL-21R antibodies

(A-C) PBMCs were stimulated with 100 ng/ml IL-21 or 100 ng/ml IL-6 for 15 minutes in the absence or presence of 10 ug/ml αIL-21R or 10 ug/ml IgG1 isotype. MFI values of phosphorylated STAT3 were measured directly afterwards. (A) Dose-response curve of STAT3p in CD4^{pos} T cells stimulated with 100 ng/ml IL-21 and different concentrations of αIL-21R. Stimulation with 100 ng/ml IL-6 was used as a positive control. (B-C) Histogram overlays and quantified data of phosphorylated STAT3 MFI values in CD4^{pos} T-cells (C) and CD19^{pos} B-cells (D) (n=7). *p<0.05, **p<0.005, ***p<0.0005. Upper line of the boxes represent mean with SEM represented by the whiskers.

Anti IL-21R inhibits memory B cell differentiation upon alloantigen stimulation

To explore the importance of the IL-21/IL-21R signaling pathway in an allogeneic system, αIL-21R was added to the co-cultures containing Tfh cells and memory B cells stimulated with alloantigen. Both cell populations expressed IL-21R (Supp. Figure 3). Prior to the co-cultures, we tested the capacity of αIL-21R to block the formation of plasmablasts in a culture system described by Ettinger and colleagues, where B cells were stimulated with anti-CD40, anti-IgM and IL-21 resulting in robust plasmablast proportions (28). Significant inhibition of plasmablast formation was found in the presence of αIL-21R in this setting (Supp. figure 4).

Next, we tested the efficacy of αIL-21R to block in our alloantigen Tfh-B cell co-culture system. To determine the inhibitory capacity of αIL-21R on B cell function we focused on the cultures with the highest proportion of plasmablasts. Cultures with low plasmablast proportions (<5%) were excluded. In these samples, after the culture period of 8 days, no differences were found regarding the phenotype of the Tfh cells. Moreover, proportions of PD-1, ICOS, and the distribution of CCR6 and CXCR3 expressed were comparable in the presence and absence of αIL-21R (Supp. figure 5). Thus, activated Tfh cells, and their Tfh1, Tfh2 or Tfh17 phenotype are not altered in terms of their phenotype when cultured with αIL-21R.

In the presence of αIL-21R, formation of plasmablasts was inhibited by 78% (p=0.004, Figure 4A). In parallel, IgM production was inhibited from mean production of 169 ng/ml (range: 20-226 ng/ml) to 38 ng/ml (range: 2-50 ng/ml) in the presence of αIL-21R (p=0.004, Figure 4B). Since αIL-21R is a fully humanized IgG1 compound we measured IgG2 levels in the culture supernatant. Although IgG2 is a noncomplement-fixing antibody, the IgG2 subclass of antibodies is the second most common Ig subclass present after immunization (29). Production of IgG2 decreased from mean production of 975 ng/ml (range: 116-6763 ng/ml) to 87 ng/ml (range: 22-540 ng/ml) in the presence of αIL-21R (p=0.004, Figure 4B). Taken together, these data show that the αIL-21R antibody has the capacity to inhibit B cell differentiation and subsequent immunoglobulin production.

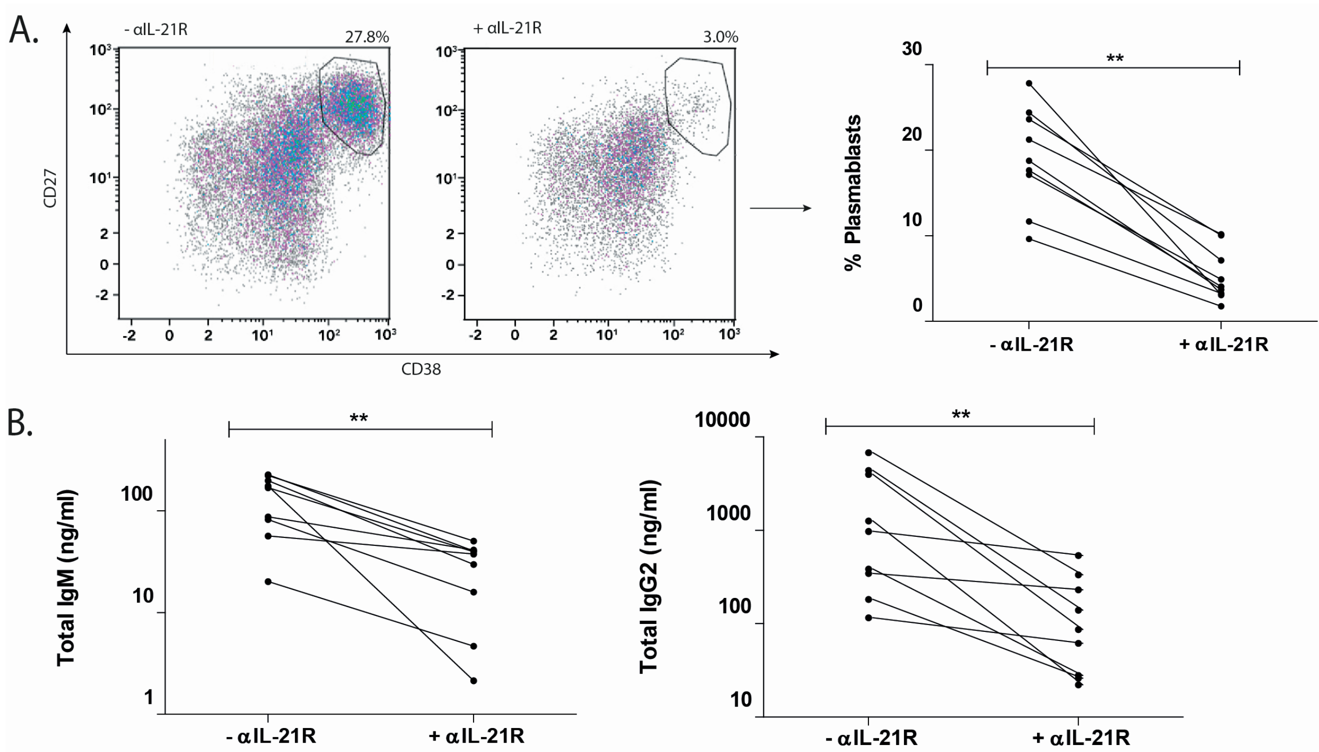


Figure 4. αIL-21R inhibits memory B cell differentiation upon alloantigen stimulation.

CD4^{pos}CXCR5^{pos} T-cells and CD19^{pos}CD27^{pos} memory B-cells stimulated with alloantigen were co-cultured for 8 days in the presence or absence of 10ug/ml αIL-21R. Only the cultures with >10% plasmablasts at day 8 (high responders) are depicted. (A) Representative dotplots and quantified data of proportions CD27^{high}CD38^{high} plasmablasts at day 8 after co-culture in the presence or absence of αIL-21R. (B) Total IgM and IgG2 measured in supernatants after 8 days co-culture. n=9. **p<0.004. *y*-axes for Ig production are scaled log-linearly.

Discussion

We studied the activation of Tfh and memory B cells upon stimulation with alloantigen and the role of IL-21 within this process. The present study demonstrates that stimulation of Tfh and memory B cells with donor alloantigen results in an activated Tfh2 and Tfh17 phenotype, CSR of memory B cells and differentiation of antigen driven memory B cells toward IgM and IgG producing plasmablasts. In the presence of αIL-21R, formation of plasmablasts and IgM and IgG2 production were significantly inhibited. Our *in vitro* system is of high value to test other interventions that might alter Tfh-B cell interaction after alloantigen response.

While αIL-21R nicely inhibited IL-21 dependent STAT3p, the Tfh cell phenotype was not changed (Sup. figure 5). This suggests that the autocrine effect of IL-21 on the Tfh cell and the presence of antigen is not essential for upregulation of activation markers PD-1, ICOS and chemokine receptors CCR6 and CXCR3. Our data are in line with *in vivo* and *in vitro* studies where the absence of either IL-21 or IL-6 did not affect Tfh differentiation, whereas combined absence of IL-21 and IL-6 led to a decreased Tfh frequency and Bcl6 gene expression (15, 30, 31). Thus, in our co-culture system the effect of IL-21 on the Tfh cell phenotype is redundant.

In our patient cohort, a high inter-individual variation in plasmablast numbers was observed after 8 days stimulation of Tfh and memory B cells with donor antigen (Figure 2B) reflecting the natural variation among patients. Response toward alloantigen apparently varied among the different co-cultures, even though no significant differences in baseline characteristics were found between the co-cultures with and without plasmablast formation (Supp. Table 1). Immunological variation, *e.g.* distinct expression rates of co-stimulation surface markers or distinct IL-21 production by the Tfh cells among the different co-cultures might contribute to this variation. Finally, effect of vaccination or viral infection might support the formation of alloantibody producing plasmablasts via cross-reactivity. This cross-reactivity results in heterologous immunity within the patient population, this may contribute to the variation in plasmablast numbers in our co-cultures (32).

In transplantation, the formation of complement fixing DSA and anti-HLA antibodies is associated with graft loss (2-5). In the presence of currently prescribed immunosuppressive drugs, *de novo* DSA and anti-HLA antibodies can be formed which contribute to the process of allograft rejection leading to graft loss (4, 33). A first hint that IL-21 producing Tfh cells are involved in processes leading to the production of alloantibodies comes from our study reporting that the absolute numbers of Tfh cells after transplantation are the highest in patients with pre-existing DSA (22). In addition, this study reports a decrease in plasmablast numbers when stimulating Tfh and memory B cells with staphylococcal enterotoxin B (SEB),

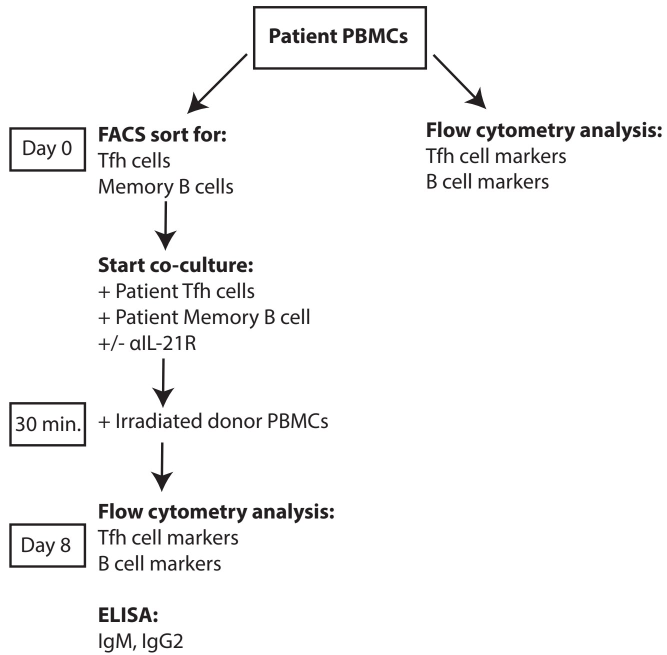
a strong polyclonal superantigen (22). In the present study, we determined the mechanisms involved by analyzing the IL-21+ Tfh and B cell activation pathway during activation by donor antigen solely. This study demonstrates that alloantigen and IL-21 are key factors in this response. Translating the outcome of our *in vitro* study by using patient materials, we speculate that also *in vivo* B cell differentiation might be mediated by IL-21 producing Tfh cells and that interaction with donor antigen stimulated B cells results in the formation of DSA. Future experiments, *e.g. in vivo* experimental transplantation studies, should reveal whether indeed IL-21 drives antibody mediated allogeneic immune responses.

In a phase I trial in healthy volunteers, 76% of the participants who received αIL-21R developed anti-drug antibodies (ADA) due to increased activity of the antigen presentation machinery (34, 35). Thus, identification of an IL-21R blocker with lower immunogenicity or a switch to an IL-21 cytokine antagonist may be an alternative. In addition, in sensitized patients αIL-21R may not work sufficiently since these patients have high levels of circulating DSA due to previous transplants or pregnancy. In these patients, the destructive effects of the existing plasma cells need to be neutralized, for instance via protease inhibitors, inhibition of IL-6 or inhibition of BAFF and APRIL (6). To date, B-cell depletion is achieved via treatment with the anti-CD52 antibody alemtuzumab, anti-thymocyte globulin (ATG) and anti-CD20 antibody rituximab. Compared to these treatments, IL-21R blocking therapy is of interest since it is a biological that mainly interferes the Tfh-B cell crosstalk, saving the presence of T and B cells with a resting or regulatory phenotype. The use of anti-IL6R treatment to decrease the formation of anti-HLA antibodies has also been widely studied. The first study in kidney transplant patients and experimental transplant models showed that anti-IL6R treatment affected the proportion Tfh cells and reduced B cell differentiation toward IgG producing plasmablasts (36-38). In these studies no data were reported on anti-IL21R agents and a next step would be to compare the functionality of these biologicals. We speculate that inhibition of both pathways *i.e.*, IL-6 and IL-21 may be of interest to test.

In this study, we focused on the interplay between peripheral Tfh cells and B cells. Low numbers of IL-21 producing T cells are present in the circulation, compared to high numbers found in inflamed tissues (39). In transplantation, the presence of Tfh cells that co-localized with B cells in follicular-like structures was confirmed in kidney biopsies taken during acute cellular rejection, suggesting IL-21 mediated Tfh-B cell interaction in local tertiary lymphoid structures in the kidney allograft (22, 23). In depth analysis of these graft infiltrating Tfh and B cells, *e.g.* via next generation sequencing or other single cell analysis, would be of great importance to improve our knowledge about molecular pathways involved in the anti-donor response.

Identifying the mode of action of Tfh-mediated activation of B cells upon alloantigen stimulation is important to further understand immunological processes that occur in transplant patients. In our allogeneic co-culture system IL-21 plays a non-redundant role in promoting B cell differentiation, as blockade of the IL-21/IL-21R signaling pathway resulted in almost complete inhibition of plasmablast formation as well as antibody production. In conclusion, our results demonstrate that IL-21 produced by alloantigen activated Tfh cells controls B cell differentiation toward antibody producing plasmablasts. The IL-21R might therefore be a useful target in organ transplantation to prevent rejection.

Supplemental figures

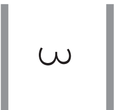


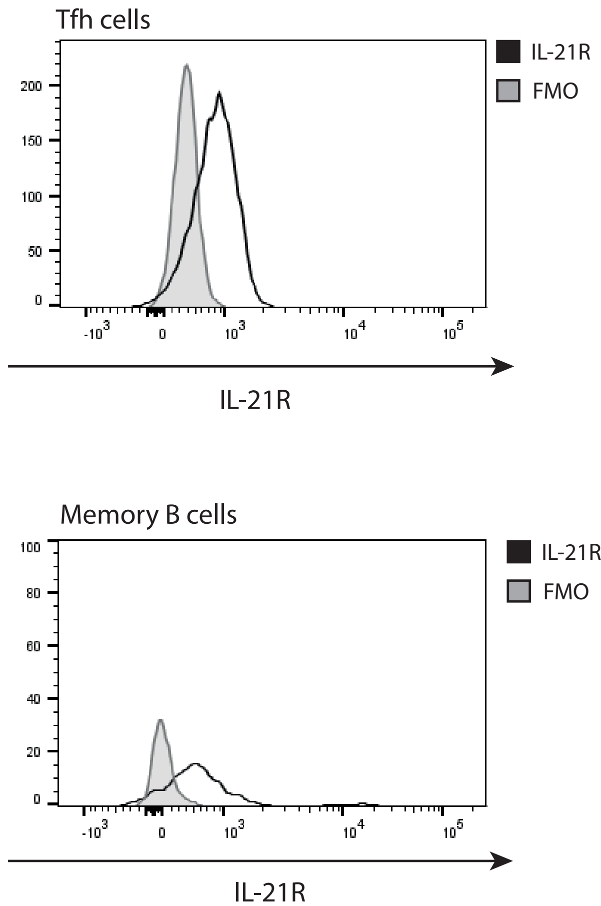
Supplemental Figure 1.

Schematic representation of the co-culture model and analytical approaches used in this study.

Supplemental Figure 2.

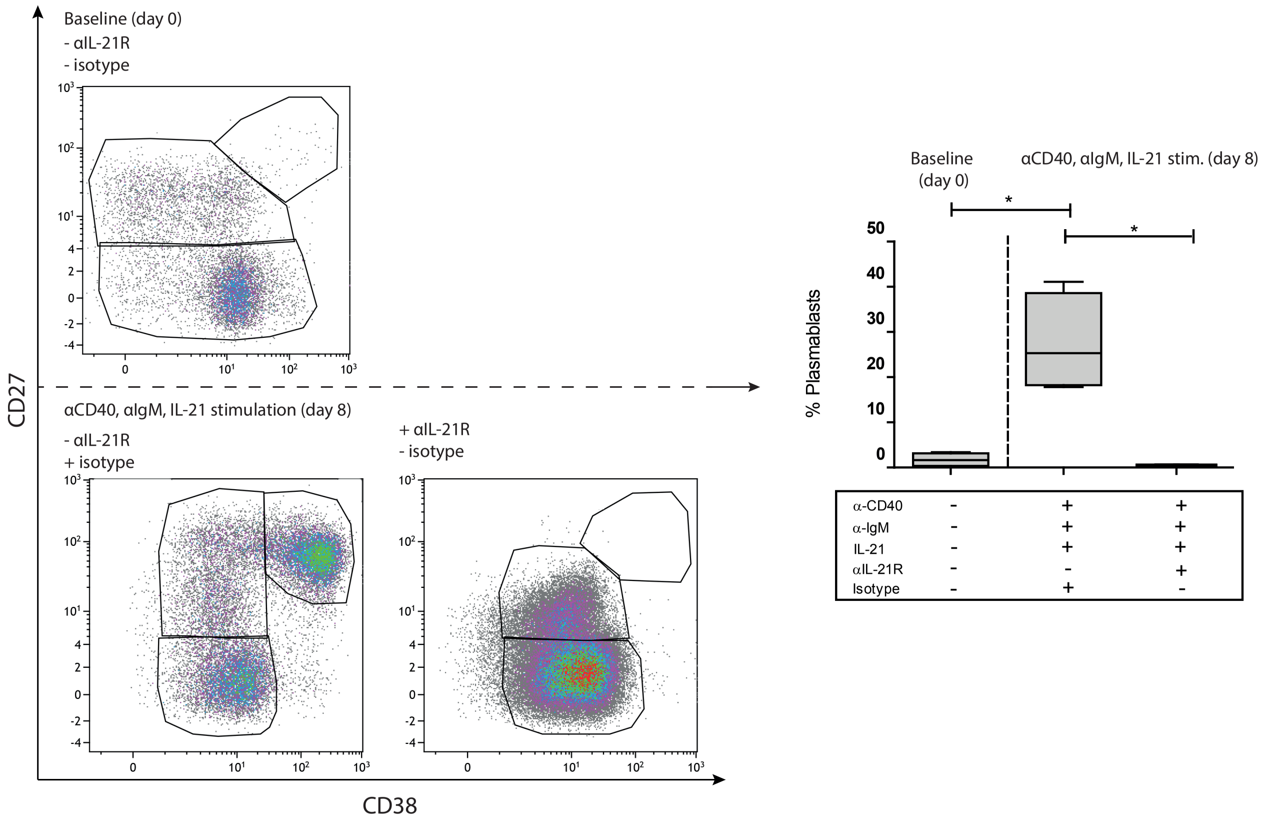
CD4posCXCR5pos Tfh cells and CD19posCD27pos memory B-cells were FACS sorted and cultured for 8 days in the absence (A) or presence (B) of donor antigen. Cells were gated by forward- and side-scatter and viability was determined by 7-AAD negative cells (viaprobe). Plasmablasts (CD27highCD38high) were gated from the total B cell population (CD19pos).





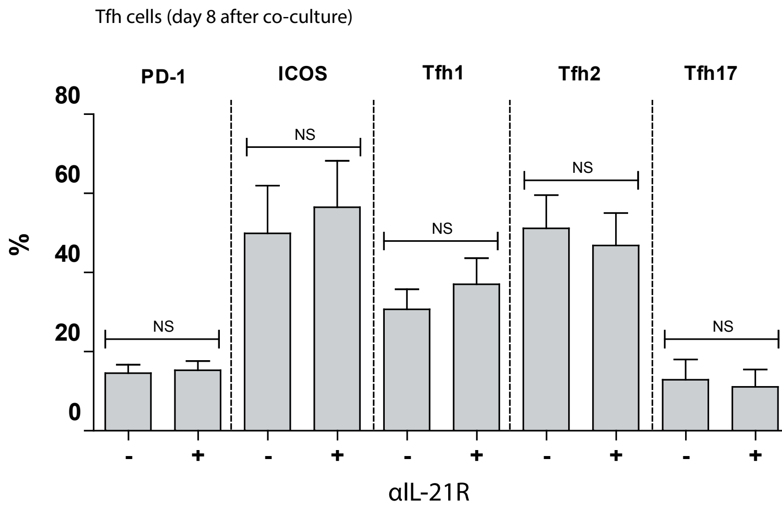
Supplemental Figure 3.

Histogram overlays of IL-21R MFI values (black) or FMO (grey) on Tfh cells and memory B-cells. MFI: mean fluorescence intensity, FMO: fluorescence minus one.



Supplemental Figure 4.

CD19pos B cells were stimulated with 5ug/ml α-CD40, 10ug/ml α-IgM and 100 ng/ml IL-21 for 8 days in the presence of 10ug/ml αIL-21R or 10ug/ml IgG1 isotype. Representative dotplots and quantified data of CD27highCD38high plasmablasts proportions at day 0 (baseline), and day 8 are shown. N.B.: Box whiskers represent minimal and maximal values. The upper and lower border of the box represent the 25% and 75% percentile with the middle line representing the median (n=4). *p<0.03.



Supplemental Figure 5.

Tfh and memory B-cells were co-cultured for 8 days in the presence (+) or absence (-) of 10ug/ml αIL-21R. (A) Percentages of PD-1pos, ICOSpos, CCR6negCXCR3pos Tfh1, CCR6negCXCR3neg Tfh2 and CCR6posCXCR3neg Tfh17 cells within the Tfh population after 8 days co-culture. Upper line of the boxes represent mean with SEM represented by the whiskers. n=8, NS=not significant.

Supplemental Table 1.

	Plasmablast formation		
	No (n=8)	**Yes (n=9)**	**p value (two-sided)**
Patients age in years (median, range)	50 (33-68)	62 (39-74)	0.89
Recipient gender (% male)	87.5%	66.7%	0.58
HLA-A mismatches (mean ± s.d.)	1,1 (±0.6)	1.0 (±0.9)	0.83
HLA-B mismatches (mean ± s.d.)	1.6 (±0.5)	1.7 (±0.5)	0.33
HLA-DR mismatches (mean ± s.d.)	1.5 (±0.5)	1.6 (±0.5)	0.35
Panel reactive antigen (median, range)			
• Current	8.9% (0.0-71%)	1.1% (0.0-5%)	0.80
• Peak	14,9% (0.0-99%)	1.7% (0.0-4%)	0.23
Previous kidney-transplantation	0% (0)	22.2% (2)	
• Second kidney transplantation	-	11.1% (1)	
• Third kidney transplantation	-	11.1% (1)	
Renal replacement therapy before transplantation			
• None	0% (0)	22.2% (2)	0.36
• Haemodialysis	87.5% (7)	66.7% (6)	
• Peritoneal dialysis	12.5% (1)	11.1% (1)	

Numbers between brackets represent patient number unless otherwise specified

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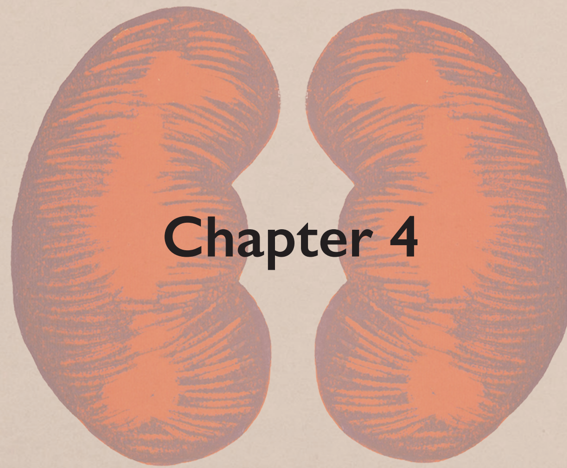

Chapter 4

The effects of an IL-21 receptor antagonist on the alloimmune response in a humanized mouse skin transplant model

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Transplantation. April 2019; accepted for publication

Abstract

Background

Interleukin 21 (IL-21) is involved in regulating the expansion and effector function of a broad range of leukocytes, including T cells and B cells. In transplantation, the exact role of IL-21 in the process of allograft rejection is unknown. To further explore this, the aim of this study is to test the effect of an IL-21 receptor (IL-21R) blocking antibody on the early phase of allograft rejection in a humanized skin transplantation model in mice reconstituted with human T and B cells.

Methods

Immunodeficient Balb/c IL2rγ-/-Rag2-/- mice were transplanted with human skin followed by adoptive transfer of human allogeneic splenocytes. Control animals were treated with a PBS vehicle while the other group was treated with a humanized anti-IL-21R antibody (αIL-21R).

Results

In PBS treated animals, human skin allografts were infiltrated with lymphocytes and developed a thickened epidermis with increased expression of the inflammatory markers Keratin 17 (Ker17) and Ki67. In mice treated with αIL-21R, these signs of allograft reactivity were significantly reduced. Concordantly, STAT3 phosphorylation was inhibited in this group. Of note, treatment with αIL-21R attenuated the process of T and B cell reconstitution after adoptive cellular transfer.

Conclusion

These findings demonstrate that blockade of IL-21 signaling can delay allograft rejection in a humanized skin transplantation model.

Introduction

In transplantation, both short- and long-term allograft survival rates remain suboptimal, illustrating the need for new immunosuppressive agents(1). In general, two types of renal allograft rejection are recognized: T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR). However, over the last years it has become clear that these two types of rejection responses are not as distinct as thought before. For example, in renal allograft biopsies, overlapping histological features of TCMR and ABMR are often detected(2). These rejections are characterized by allograft infiltration with both T and B cells as well as typical features of ABMR, for instance the presence of *de novo* donor-specific antibodies (DSA)(2, 3). Unsurprisingly, these cases are not responsive to treatment strategies that target either TCMR or ABMR alone(4).

Adaptive alloimmune responses are established via three essential signals: 1) donor antigen presentation, 2) co-stimulation, and 3) upregulation of cytokine receptors and cytokine production, resulting in T and B cell activation. Current immunosuppressive treatments include calcineurin inhibitors (CNIs) that mainly target the T cell activation cascade. CNIs are unable to prevent B-cell driven ABMR, which is normally addressed with B cell depleting therapies (*e.g.* rituximab and intravenous immunoglobulins). Therapies that target B cells are generally not used as maintenance therapy due to the side effect profile and their ineffectiveness in the prevention of TCMR(5). There is therefore a need for the development of immunosuppressive drugs with mechanisms of action that concurrently target T and B cells.

Interleukin-21 (IL-21) is a cytokine with a broad pattern of actions that affect the differentiation and function of several lymphoid cells(6). IL-21 binds to the IL-21 receptor (IL-21R), which forms a heterodimer with the common gamma chain (γc)(7). Upon IL-21/IL-21R signaling, downstream JAK/STAT pathways are activated, of which signal transducer and activator of transcription 3 (STAT3) is most predominant(6). IL-21 is produced by T follicular helper (Tfh) cells, Th17 cells, and natural killer T (NKT) cells and is important in the differentiation of functional Th17 and Tfh cells(6, 8-10). For the CD8+ T cell compartment, IL-21 is a potent regulator of expansion and effector function and prolongs CD8+ T cell effector function in chronically infected hosts(11-13). IL-21 is also crucial for naïve B cell differentiation to immunoglobulin-producing plasma cells after antigen recognition(14, 15).

In solid organ transplantation, IL-21 is expressed during the process of rejection(16-18). Upon alloantigen stimulation, IL-21-secreting Tfh cells are present in the circulation at three months after renal transplantation and provide help to B cells for their differentiation towards plasmablasts(19). In addition, IL-21-expressing cells have been detected in renal

rejection biopsies in acute TCMR, suggesting a local effect of IL-21 on cytotoxic CD8+ T cells in this type of rejection(20). We have previously demonstrated that IL-21 is a key factor in the Tfh-cell dependent differentiation of alloantigen-stimulated B cells towards immunoglobulin producing plasmablasts(21). Anti-human IL-21 mAb treatment has also been shown to suppress graft-versus-host (GVH)-associated disease in a humanized murine model(22). Therefore, IL-21R blockade may affect the process of allograft rejection. In the present study, we investigated whether blockade of the IL-21/IL-21R pathway can inhibit the process of allograft rejection in the early phase.

To investigate the effect of human IL-21 signaling blockade on allograft rejection, we used a humanized skin transplant model in which immunodeficient Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice are transplanted with human skin followed by adoptive transfer of human allogeneic splenocytes(23). When setting up a humanized transplantation model in mice, a skin transplant model is the first choice, since it is relatively easy to obtain human skin and with one piece of human skin several experiments can be performed, which improves reproducibility. The process of rejection of skin and a solid organ occurs via similar mechanism, making skin transplantation useful as a model for studying organ transplantation. Also, the use of the humanized skin transplant mouse model has proven successful in previous studies, in which the effect of several immune modulating therapies on skin inflammation was tested(23-26). In general, many of these compounds were shown to reduce the number of infiltrating T cells in the human skin(23-25). Using this model, we provide evidence for efficacy of IL-21 blockade in the control of the start of alloimmunity.

Material and methods

Mice

Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice were bred and housed under specific pathogen free (SPF) conditions at the Erasmus Medical Center Experimental Animal Facility. The animals were housed in individually ventilated cages with *ad libitum* access to water and food and a 12 hour light-dark cycle. Mice were aged between 8 and 14 weeks at the time of the first experimental procedure. All experiments were approved by the Central Committee Animal Experiments Ethical Committee (license number AVD101002016635) and complied with the 1986 directive 86/609/EC of the Council of Europe.

Human skin

Waste material from human abdominal skin surgeries performed for deep inferior epigastric artery perforator (DIEP) flap reconstruction was anonymously obtained as approved by the Medical Ethical Committee of our center (MEC-2014-347). The humanized skin allograft

transplant model is based on the model described by Issa *et al.* (23). Human skin grafts were harvested from discarded abdominal skin using an electric dermatome (Zimmer, Utrecht, the Netherlands) in strips with a thickness of 200 μm. From these strips, punch biopsies were taken with a diameter of 1cm and kept in ice-cold PBS. The human skin grafts were all transplanted within 16 hours.

Human skin transplantation

Animals were subcutaneously injected with 0.05 mg/kg buprenorphine 0.5 hours prior and 12 hours after the surgery as analgesic. During the surgical procedure mice were kept under complete anesthesia via isoflurane inhalation (5% isoflurane initially followed by 2% to 2.5% with 1:1 air/oxygen mixture for maintenance) and the animals were kept warm on 37°C heating pads. The left dorsal flank of the mice was shaved and a circular piece of skin with a diameter of 1 cm was removed. A human skin graft was placed in the circular incision and stitched on the mouse with absorbable 5/0 suture (Safil®, B. Braun, Melsungen, Germany). The skin grafts were covered with a povidone-iodine non-adherent mesh (Johnson & Johnson, Amersfoort, the Netherlands) and pressure dressing wound and fixed with surgical tape. Ten days after the transplant procedure, the tape was removed under general anesthesia. Engraftment of the human skin graft was achieved 35 days after transplantation.

Preparation and adoptive transfer of human splenocytes

Human splenocytes were adoptively transferred 35 days after skin transplantation. Human splenocytes were obtained from a segment of the spleen from deceased kidney donors. Splenocytes were anonymously used for research purposes as described in article 13 of The Netherlands law of organ donation (Wet op Orgaandonatie, WOD). The spleen segments were disrupted and filtered through a 70μm cell strainer (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) to obtain a single-cell suspension. Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient was used to collect mononuclear cells. An aliquot of splenocytes was used to isolate quiescent B cells via negative selection with CD43 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (purities ≥ 90%). $25x10^6$ splenocytes were mixed with $12.5x10^6$ enriched B cells and suspended in 1mL of PBS. 35 days post skin transplantation $5x10^6$ splenocytes and $2.5x10^6$ enriched B cells were administered in 200μl PBS via intraperitoneal (IP) injection. The composition of T and B cells within the total infusion is depicted in Supplemental Figure 1.

αIL-21R therapy

Humanized anti-IL-21R antibody (αIL-21R; also referred to as ATR-107) was kindly provided by Pfizer (New York, NY, USA). αIL-21R was administered via IP injection at a dose of 10mg/kg

(recommended by Pfizer) on days 7, 14 and 21 after adoptive transfer of splenocytes(27, 28). We started the administration of αIL-21R at day 7 after adoptive transfer in order to allow the splenocytes to first engraft and proliferate in order to reflect a healthy functioning immune system in the mice. αIL-21R antibody was infused three times to build up a significant level of antibody. PBS was used as a vehicle control.

Monitoring of the human skin graft

All mice were sacrificed 30 days after adoptive transfer of splenocytes in order to assess for evidence of skin rejection and inflammation at a single time point(23).

Flow cytometry

Human splenocytes were characterized by flow cytometry prior to adoptive transfer and from mouse blood and spleens 30 days after adoptive transfer. Cells were stained with antibodies specific for CD45 allophycocyanin (APC, BD), CD3 Brilliant Violet 510 (BV510, Biolegend, San Diego, CA, USA), CD4 Brilliant Violet 421 (BV421, Biolegend), and CD19 BV510 (Biolegend) and the viability marker 7-AAD (BD Biosciences).

In order to test whether αIL-21R effectively blocked the IL-21R, phosphorylation of STAT3 downstream of IL-21R was measured on CD4+ T cells by phospho-specific flow cytometry. Briefly, 200μl of blood from treated animals was stained with anti-CD3 BV510 (Biolegend, San Diego, CA, USA) and subsequently stimulated with recombinant human IL-21 (100 ng/ml, eBioscience, San Diego, CA, USA) or recombinant human IL-6 (100 ng/ml, PeproTech, Rocky Hill, NJ) for 15 minutes at 37°C. Cells were fixed for 10 minutes with Cytofix buffer (BD Biosciences, San José, CA, USA) at 37°C and permeabilized for 30 minutes in 1 ml 90% methanol at -20°C. Samples were then stained with anti-CD4 BV421 (Biolegend) and anti-pSTAT3 phycoerythrin (PE, BD Biosciences). All samples were measured on the FACSCanto II (BD Bioscience). Flow data were analyzed with Kaluza Analysis 5.1 software (Beckman Coulter, Brea, CA, USA).

Immunohistochemistry

Human skin grafts were removed at day 30 post adoptive cell transfer, formalin fixed and paraffin-embedded (FFPE), and processed following a standardized diagnostic protocol. Three μm sections were cut and stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed on the Benchmark Ultra Stainer (Ventana, Basel, Switzerland). The following human mAbs were used: CD45 (Cell Marque, ref. V0000963, Rocklin, CA, USA), CD4 (Ventana, ref. 790-4423), CD8 (Ventana, ref. Y04591), CD20 (Ventana, ref. 790-2531), Keratin 17 (Ker17, Ventana, ref. G03114), Ki67 (Ventana, ref. 790-4286). Antibodies were incubated

on the tissue sections for 30 minutes together with anti-rabbit or anti-mouse amplifiers. 3.3'-diaminobenzidine (DAB) was used as chromogen and sections were counterstained with hematoxylin. Analysis of the proportions of positive cells in a representative region of interest (including the epidermis and dermis) was performed by setting a fixed threshold followed by calculating the positive area with the ImageJ software (NIH, http://imagej.nih.gov/ij/).

Measurement of the epidermal thickness

Epidermal thickness was measured at twenty consecutive points in H&E stained skin slides from which the mean epidermal thickness was calculated.

Statistical testing

GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA, USA, http://www.graphpad.com) was used for statistical analysis. Differences between unpaired groups were analyzed with the nonparametric Mann-Whitney test. A two-tailed p-value of <0.05 was considered to be statistically significant.

Results

αIL-21R antibody effectively blocks STAT3 phosphorylation

In order to assess the efficacy of αIL-21R to block IL-21R signaling, phosphorylation of STAT3 downstream of the IL-21R was measured on human T cells after adoptive transfer of human splenocytes in mice(29). We tested the duration of efficient blockade of the IL-21R by measuring STAT3 phosphorylation at multiple time points after cellular infusion (Figure 1A). Mice were treated with the αIL-21R compound on days 7, 14 and 21 after adoptive transfer of human splenocytes and sacrificed at specific time points as depicted in Figure 1A (n=1 per time point). From day 42 after adoptive cellular transfer onwards the degree of human leukocyte chimerism within the blood was sufficient to perform the phospho-specific flow cytometry protocol to measure STAT3 phosphorylation (STAT3p). Whole blood was stimulated with IL-21 or IL-6 to induce STAT3p, followed by measurement of STAT3p. IL-6 stimulation was used as a positive control to induce STAT3p through bypassing the IL-21R. Figure 1B shows a representative flow cytometry plot of STAT3p staining. In this example, only 1.4% of CD3+ T cells were positive for STAT3p after IL-21 stimulation in the presence of αIL-21R, compared with 65.9% in control animals that did not receive αIL-21R treatment. These cells responded to IL-6 stimulation thereby demonstrating that αIL-21R specifically targets the IL-21R. Cells from a vehicle treated animal expressed pSTAT3 after both IL-21 and IL-6 stimulation (Figure 1B). STAT3p remained suppressed until 49 days after the last infusion

of αIL-21R (56 days after adoptive cellular transfer, Figure 1C). These data confirm efficient blockade of the IL-21 signaling pathway by αIL-21R treatment. For subsequent assays, we selected day 30 after adoptive cellular transfer for analysis to ensure IL-21R blockade.

αIL-21R mAb treatment delays rejection of the human skin

We next assessed the *in vivo* immunosuppressive effects of αIL-21R using a humanized mouse skin transplantation model. Mice were transplanted with a human skin allograft, reconstituted with human splenocytes, and subsequently treated with αIL-21R or PBS control as described above (Figure 2A). Mice were sacrificed at day 30 after adoptive cellular transfer for cross-sectional analysis by measurement of several hallmarks of skin rejection (Figure 2A). As expected, there was clear epidermal dyskeratosis in skin allografts from mice treated with PBS control. By contrast, mice that received αIL-21R treatment had markedly reduced evidence of epidermal damage (Figure 2B). Mean epidermal thickness was 370.9 µm in animals treated with vehicle control (n=7) compared with 90.6 µm in the αIL-21R-treated animals (n=8; $p<0.05$, Figures 2B and D). Infiltrates of mononuclear cells were detected in the human dermis of vehicle-treated animals. These infiltrates were absent in the human dermis of αIL-21R-treated animals (Figure 2C). Overall, these results show that the epidermal changes indicative of severe rejection that occur in vehicle-treated animals are delayed upon infusion of αIL-21R.

IL-21R blockade reduces lymphocyte infiltration in skin grafts

Human skin graft sections were stained by immunohistochemistry in order to phenotype the infiltrating mononuclear cells. The mean area of CD45 cell positivity in the dermis was 10.2% in the vehicle-treated animals compared to 2.2% in the αIL-21R-treated animals ($p<0.001$, Figure 3A). CD3 cell positivity correlated with the staining patterns for CD45, with 9.0% in the control group compared to 1.4% in the αIL-21R group ($p<0.05$, Figure 3B). CD4 cell positivity was 6.2% in the control group compared to 1.2% in the αIL-21R group ($p<0.05$, Figure 3C). CD8+ T cell numbers in the dermis and epidermis were low in both the control group and αIL-21R group (Figure 3D; $p<0.05$). Some CD8+ T cells resided within the epidermis in the control group animals while the CD4+ cells in these animals were mainly detected within the dermis (Figures 3C and D). CD20+ B cells were low within the control animals and barely detectable within the skin of αIL-21R-treated animals ($p=0.07$; Figure 3E).

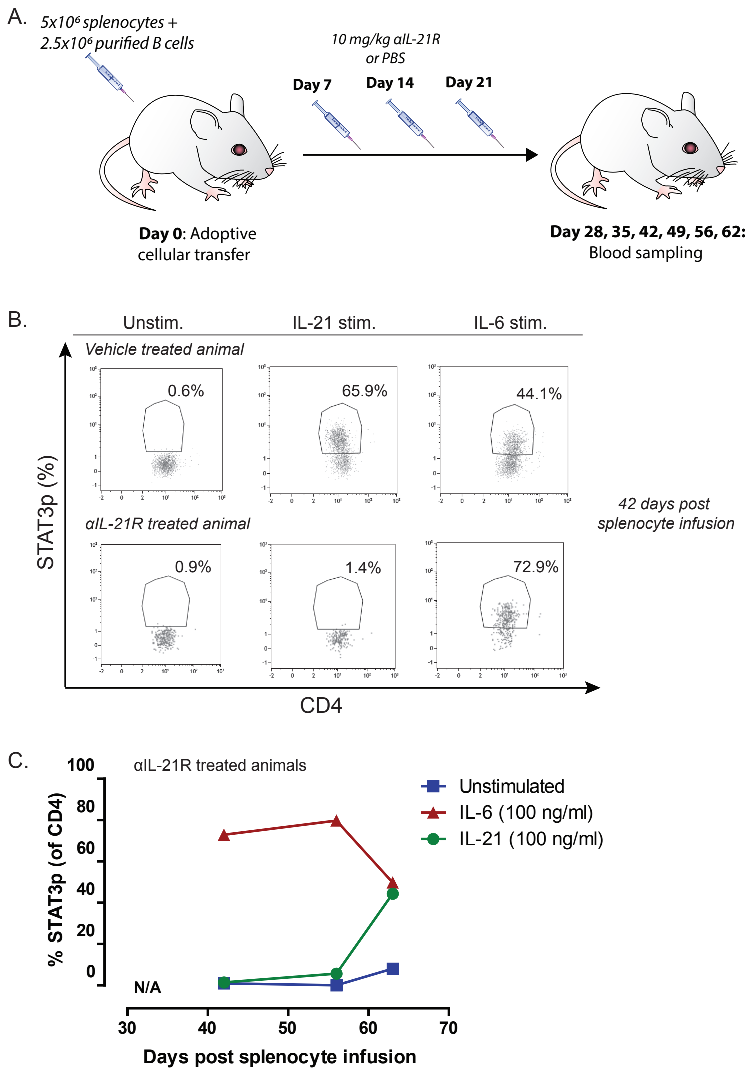


Figure 1. STAT3 phosphorylation is effectively blocked by Anti IL-21R

(A) Schematic representation of the treatment strategy to measure blockade of IL-21R dependent STAT3 phosphorylation (STAT3p). Mice were adoptively transferred with $5x10^6$ splenocytes plus $2.5x10^6$ enriched quiescent B cells. αIL-21R was administered via intraperitoneal injection at a concentration of 10mg/kg at day 7, 14 and 21 after adoptive transfer of splenocytes. PBS was used as a vehicle control. Blood was sampled on days 28, 35, 42, 49, 56, and 62 after adoptive cellular transfer (*n*=1 per time point). (B) Typical example dot plots of STAT3p analysis of a vehicle-treated animal and a αIL-21R-treated animal. 42 days after adoptive cellular transfer blood was sampled and stimulated for 15 minutes with 100 ng/ml recombinant human IL-21, 100 ng/ml recombinant human IL-6 as a positive control, or not stimulated (unstim.). Subsequently, cells were stained for CD3, CD4 and STAT3p. Numbers within the dot plots indicate proportions of the STAT3p positive population. (C) Proportions of STAT3p of CD4+ T cells after stimulation with IL-21, IL-6, or control at different time points after adoptive cellular transfer and αIL-21R treatment. Before day 42, the human lymphocyte numbers in the blood were not sufficient for STAT3p measurements.

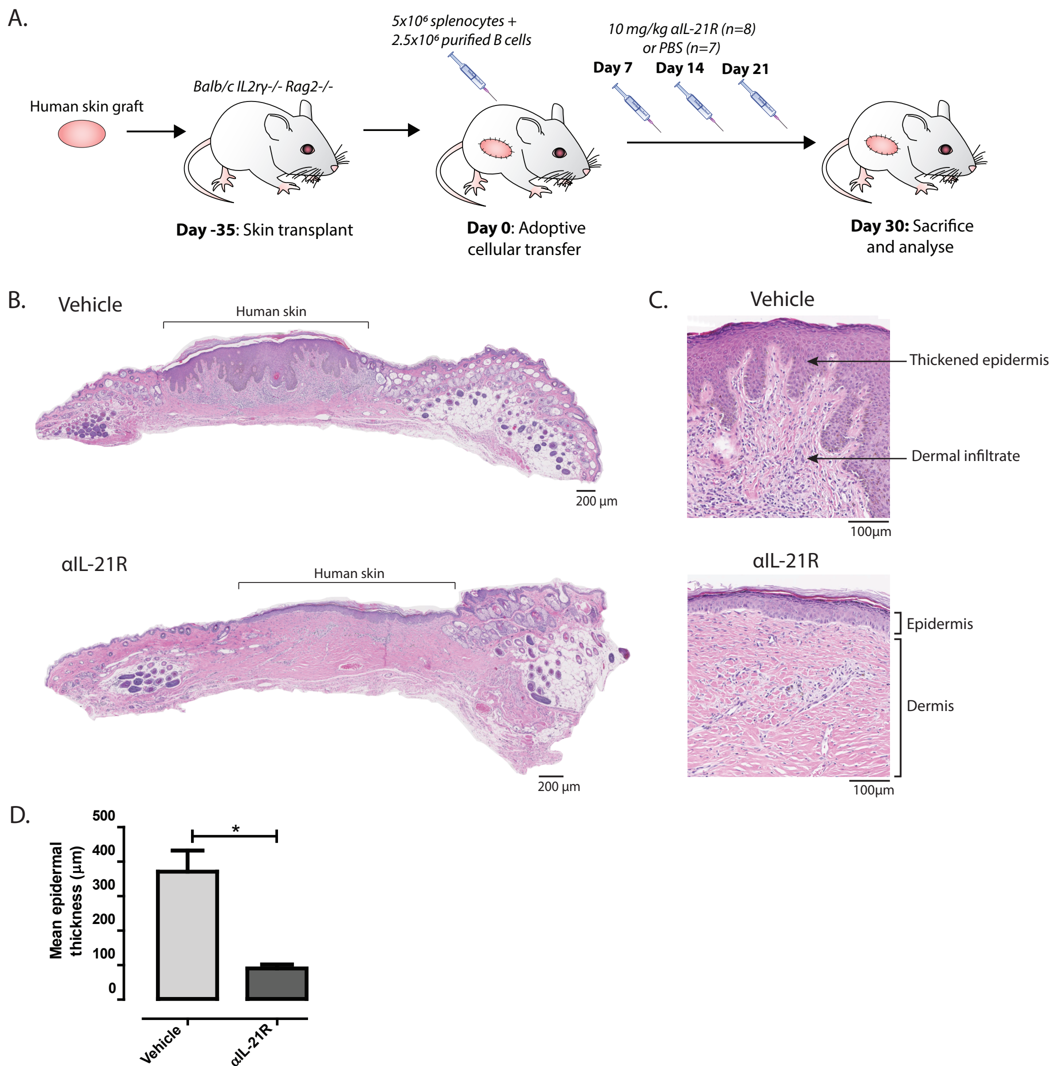


Figure 2. αIL-21R mAb treatment delays rejection of human skin grafts

(A) Treatment strategy used to explore the effect of αIL-21R on rejection of human skin grafts. A circular piece of human skin with a diameter of 1 cm was stitched on the mouse (day -35). After 35 days the human skin was engrafted and mice then received $5x10^6$ splenocytes with $2.5x10^6$ enriched quiescent B cells (day 0). αIL-21R was administered at a concentration of 10mg/kg on days 7, 14 and 21 after adoptive cellular transfer (*n*=8 mice). PBS was used as a vehicle control (*n*=7 mice). 30 days after adoptive cellular transfer mice were sacrificed for cross-sectional analysis. (B) Hematoxylin and eosin (H&E) staining of the complete human skin graft of a vehicle-treated animal and an αIL-21R-treated animal. (C) 10x magnification of the H&E staining of the human skin graft of a vehicle and αIL-21R-treated animal. In the vehicle-treated animal (upper panel) thickening of the epidermis and dermal infiltrate was detected in contrast to the αIL-21R-treated animal (lower panel). (D) Quantified data of epidermal thickness. Epidermal thickness is presented in µm as a mean of twenty consecutive measurements with the standard error of the mean (SEM). Vehicle group *n*=7, αIL-21R group *n*=8. (* $p<0.05$).

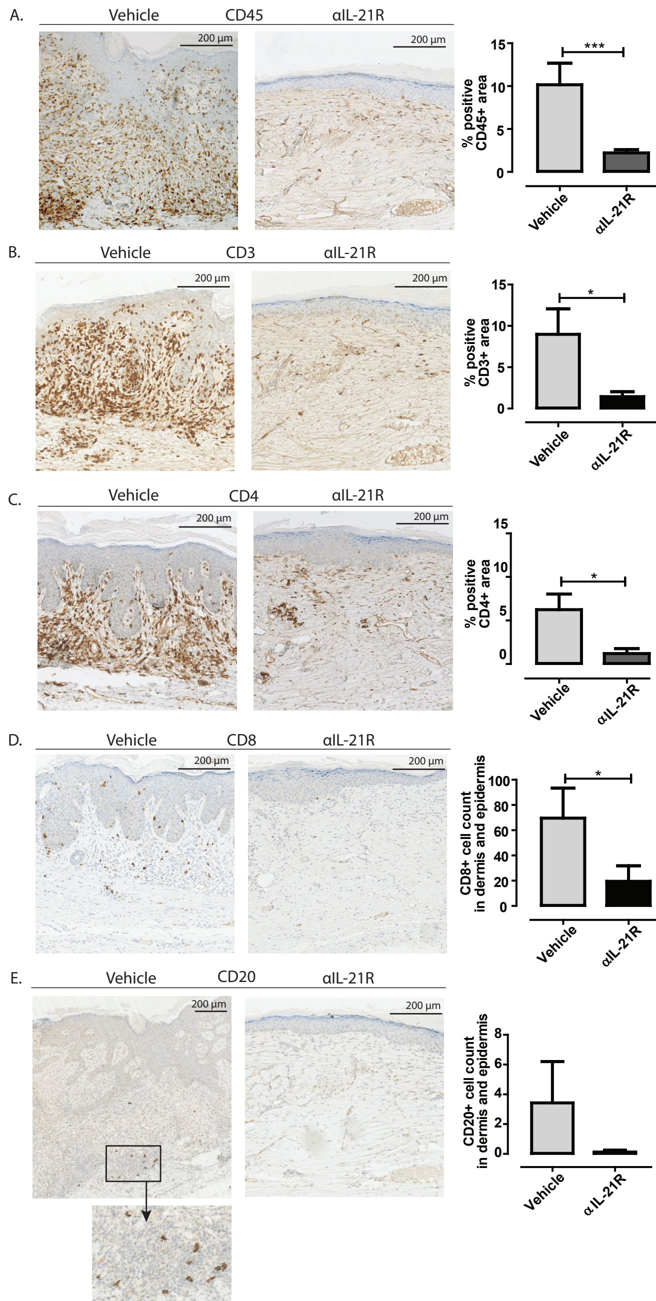


Figure 3. IL-21R blockade reduces lymphocyte infiltration in skin grafts

Typical examples of immunohistochemical stainings of human skin grafts for (A) human CD45, (B) human CD3, (C) human CD4, (D) human CD8, and (E) human CD20. Increased infiltration of human lymphocytes was detected in skin of vehicle-treated animals compared to αIL-21R-treated animals. Quantified data are presented as mean proportions of the positive area with SEM or as mean positive cells counted within the total dermis and epidermis with SEM. Magnification: 10x. Vehicle group n=7, αIL-21R group n=8. (* p<0.05, *** p<0.001).

IL-21R blockade reduces human skin inflammation markers

A consequence of the infiltration of mononuclear cells is the induction of local inflammation measured among others by increased cell proliferation (epidermal hyperplasia) and the activation of several keratines. In order to further assess this, we stained human skin graft sections with two epidermal proliferation markers. Upon injury, *de novo* production of Keratin 17 (Ker17) is associated with epidermal formation after skin injury(30). Indeed, significantly higher levels of Ker17 were found in the vehicle-treated animals (mean positive area: 30.5%) compared to the αIL-21R-treated animals (mean positive area: 7.6%, $p<0.05$, Figure 4A). Proliferation of the epidermal cells was assessed by staining with the proliferation marker Ki67. The mean area of dividing epidermal cells of the vehicle-treated animals was 4.6% compared to 1.9% in the αIL-21R-treated animals ($p<0.05$, Figure 4B). Ki67 positive cells were mainly detected in the basal layers of the epidermis, suggesting active hyperplastic activity in vehicle-treated animals. These findings suggest that IL-21R signaling contributes to the generation of a pro-inflammatory climate in the transplanted skin.

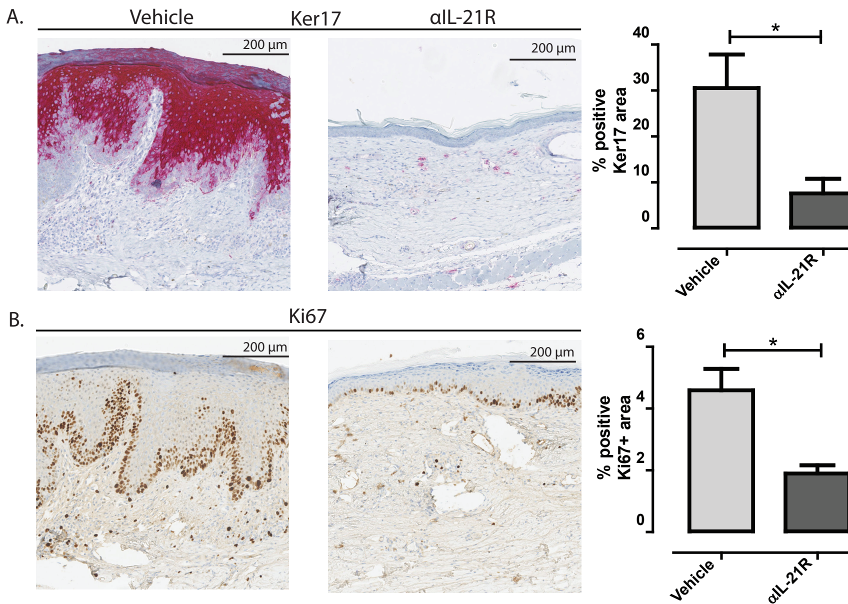


Figure 4. IL21R blockade reduces human skin inflammation markers

(A) Immunohistochemistry for skin inflammation marker keratin 17 (Ker17). Typical example of the human skin graft of a vehicle-treated animal and an αIL-21R-treated animal are depicted combined with the quantified data. (B) Immunohistochemistry for proliferation marker Ki67. A typical example of the human skin graft of a vehicle-treated animal and an αIL-21R-treated animal are depicted combined with the quantified data. Quantified data are presented as mean proportions of the positive area with SEM. Magnification: 10x. Vehicle group $n=7$, αIL-21R group $n=8$. (* $p<0.05$).

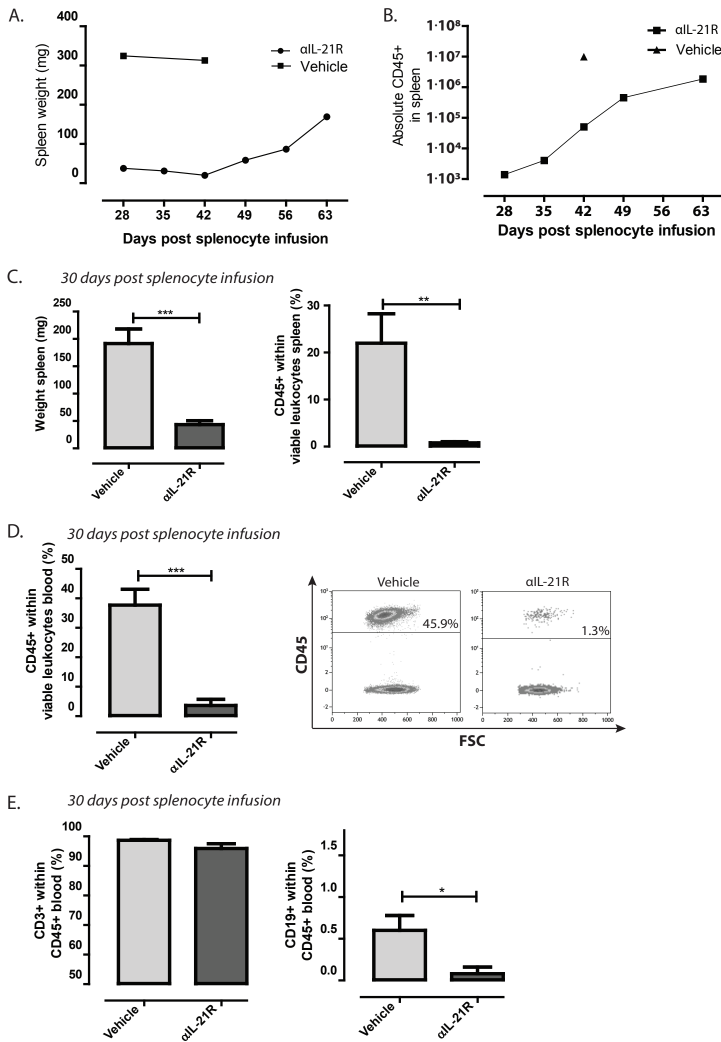


Figure 5. αIL-21R mAb treatment affects human leukocyte engraftment

(A) Spleen weight in mg of mice treated following the schedule depicted in Figure 1A Mice were sacrificed on days 28, 35, 42, 49, 56, and 63 post adoptive cellular transfer. Two vehicle-treated animals were sacrificed on days 28 and 42 post adoptive cellular transfer. *n*=1 per time point. (B) Absolute numbers of CD45+ lymphocytes of mice treated following the schedule depicted in Figure 1A. Mice were sacrificed on days 28, 35, 42, 49, 56, and 63 post adoptive cellular transfer. One vehicle-treated animal was sacrificed on day 42 post adoptive cellular transfer. *n*=1 per time point. (C) Spleen weight in mg and proportions of CD45+ human lymphocytes of the total viable leukocytes in the spleen. Measurements were performed 30 days post adoptive cellular transfer (D) Proportions of human CD45+ lymphocytes within the total viable leukocytes within blood of the vehicle-treated animals versus the αIL-21R-treated animals 30 days after adoptive cellular transfer. A typical example of a dot plot from a vehicle-treated animal and an αIL-21R-treated animals is depicted on the right-hand side. (E) Proportions of CD3+ T cells and CD19+ B cells within human CD45+ lymphocytes in the blood. Vehicle-treated animals were compared to the αIL-21R-treated animals 30 days after adoptive cellular transfer. Vehicle group *n*=7, αIL-21R group *n*=8. (N.S. = not significant, * p<0.05, ** p<0.01, *** p<0.001).

αIL-21R mAb treatment reduced human leukocyte engraftment

In order to investigate the mechanisms underlying decreased infiltration of lymphocytes and reduced inflammation of human skin grafts in αIL-21R-treated animals, we measured the numbers of lymphocytes in blood and spleen at different time points after adoptive transfer of human splenocytes in animals without a skin graft. The mean spleen weight (as a measure of cell engraftment) of two vehicle-treated animals was 319 mg at day 28 and 42 post splenocyte infusion (Figure 5A). Spleen weights of the αIL-21R-treated animals (at day 7, 14 and 21; protocol Figure 1A) were 38 mg at day 28 and increased to 169 mg at day 63 after cell infusion (Figure 5A). Absolute numbers of human CD45+ cells in the spleen of αIL-21R-treated animals also increased over time (Figure 5B).

In animals that received a skin graft, similar effects of αIL-21R mAb on human splenocyte engraftment were observed. The average spleen weight was 192 mg in the vehicle-treated animals compared to 42 mg in the αIL-21R-treated animals at day 30 after administration of splenocytes ($p<0.001$, Figure 5C) and the proportions of human CD45+ within total viable lymphocytes were 22% in the vehicle group versus 0.8% in the αIL-21R group ($p<0.01$, Figure 5C). In the blood we detected the same trend with a significant difference in human CD45+ lymphocyte chimerism levels when comparing the vehicle group and the αIL-21R group (mean: 3.6% versus 38% of total viable cells within the lymphocyte gate, respectively, $p<0.001$, Figure 5D). The majority of the human CD45+ lymphocytes measured within the blood consisted of CD3+ T cells, with only minor amounts of CD19+ B cells (Figure 5E). This was observed for both the vehicle-treated group and the αIL-21R-treated group and correlated with the low level of infiltrating human B cells in the human skin (Figure 3D). Overall, blockade of IL-21R signaling hampers engraftment of human lymphocytes in Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice, suggesting an effect of IL-21R blockade on human leukocyte survival and proliferation.

Discussion

Evidence from previous studies presents a role for the pleiotropic cytokine IL-21 in the process of allograft rejection(16, 17). Therefore, we hypothesized that blockade of the IL-21R in a humanized skin transplant model affects the early phase of rejection of the human skin. In the present study we demonstrate that rejection of a human skin graft occurs after adoptive transfer of human splenocytes in Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice. In the presence of αIL-21R mAb, phosphorylation of STAT3 downstream the IL-21R is effectively blocked. Blockade of IL-21R signaling led to a significant inhibition of epidermal thickening, reduced infiltration of lymphocytes and decreased expression of inflammation markers Ker17 and Ki67 in human skin allografts.

The inhibition of human skin rejection through IL-21R blockade was accompanied by impaired engraftment of human lymphocytes. Blockade of IL-21R influenced the homeostatic proliferation of the infused cells and therefore their engraftment within the host. Indeed, previous studies present a role for IL-21 in lymphocyte homeostasis(31-33). This impaired homeostatic proliferation may be a result of impaired cross talk between different lymphocyte subsets upon IL-21R blockade. For instance, IL-21R signaling is involved in the generation of Tfh cells and mediates Tfh cell help to activate B cell differentiation towards immunoglobulin producing plasma cells(21, 34, 35). Moreover, IL-21 is involved in sustaining CD8+ T cell responses, which is in line with the low levels of CD8+ T cells in the human skin that we detected in the presence of αIL-21R(36, 37). Furthermore, there is evidence that activation of Th17 cells is impaired by αIL-21R(10), which may contribute to the reduced allograft rejection upon IL-21R blockade. Despite the low engraftment of human lymphocytes following IL-21R inhibition, we succeeded to reach a level of human lymphocyte chimerism that is acknowledged in the literature as a reconstituted human lymphocyte system(38, 39). To allow some degree of immune cell engraftment to occur, we decided to delay blockade of IL-21R signaling until day seven after infusion of human lymphocytes. Further delay of αIL-21R treatment or administration of higher numbers of immune cells to enhance engraftment of the human immune system was limited by the risk for graft-versus-host disease (GVHD) in this model.

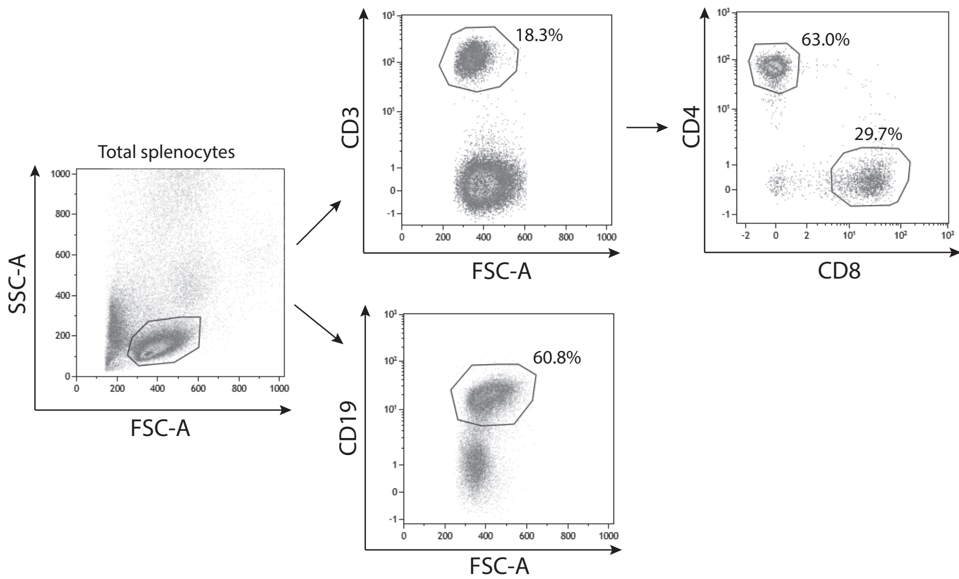
In the present study, B cell engraftment was detected with marginal B cell numbers in the peripheral blood and less than expected B cells in the spleens of control mice. In αIL-21R-treated animals, B cell engraftment was negligible. It is known that the engraftment of B cells in humanized mouse models is challenging, as described by others(40). One technical obstacle is the long maturation time of B cells after infusion, which can take up to five to six months after administration of human hematopoietic stem cells in mice(41). The model used in the present study does not allow such long engraftment times as after three months the risk for GVHD caused by human T cells that recognize mouse antigens rapidly increases(42). In addition, we have seen that infusion of higher splenocyte doses as well leads to an increased incidence of GVHD. In our study, infusion of αIL-21R at a later time point in order to diminish the effect of the IL-21R blockade on T and B cell repopulation is therefore an unsuitable option. An alternative might be to infuse mature B cells instead of the CD43- quiescent B cell population in order to accelerate the process of B cell activation and reconstitution.

As described above, T cells are necessary in the process of B cell expansion(41). Thus, a decreased T cell homeostastic proliferation caused by αIL-21R might lead to impairment of B cell reconstitution as we have seen in our model. Since we found an effect of αIL-

21R on T and B cell engraftment within the host due to the effects of IL-21R signaling on homeostatic proliferation, blockade of this receptor may also impair maintenance of both arms of the immune system. The inhibition of allograft rejection through reduced engraftment of reconstituted immune cells by IL-21R blockade that we detected in our study is in line with a previous study by Oliviera *et al.*(25). In this study, cyclosporine-A and rapamycin were introduced in a similar humanized skin transplant mouse model which resulted in lower numbers of peripheral T cells combined with a decrease in T cell infiltration and inflammation of the human skin(25). The humanized skin transplant model is also used to study the effect of regulatory T (Treg) cells on reducing rejection of human skin (23, 24). These studies demonstrated that Treg cells may have an inhibiting effect on the engraftment of human lymphocytes. The effect of immune modulating compounds on the engraftment of lymphocytes forms the main limitation in this study. For future studies, local infusion of the compound into the transplanted skin may be an alternative to overcome the systemic effect of the immune modulating therapies on lymphocyte reconstitution (43). In addition, the use of a reference drug alongside the αIL-21R compound during future experiments will further clarify the effectiveness of blockade of IL-21R signaling.

The humanized mouse model represented in this study forms a valuable preclinical model to evaluate human-specific reagents such as humanized monoclonal antibody therapies. However, since the infusion of these reagents might also affect the reconstitution of the infused human lymphocytes one should take into account that reduced rejection of the skin may occur as a result of the impaired engraftment of the human lymphocytes. In conclusion, we prove that blockade of IL-21R signaling delays the start of rejection of a human skin graft, but also influences T and B cell reconstitution in Balb/c IL2rγ$^{-/-}$Rag2$^{-/-}$ mice. These preclinical data using an *in vivo* humanized system demonstrate significant promise for blockade of IL-21R signaling in transplantation.

Supplemental Figures



Supplemental figure 1. Composition of splenocyte subsets used for adoptive cellular transfer

Dot plots depicting the proportions of CD3+ T cells, CD4+ and CD8+ T cells within the total CD3+ T cell population, and CD19+ B cells. Proportions of the different lymphocyte populations are depicted within the dot plots.

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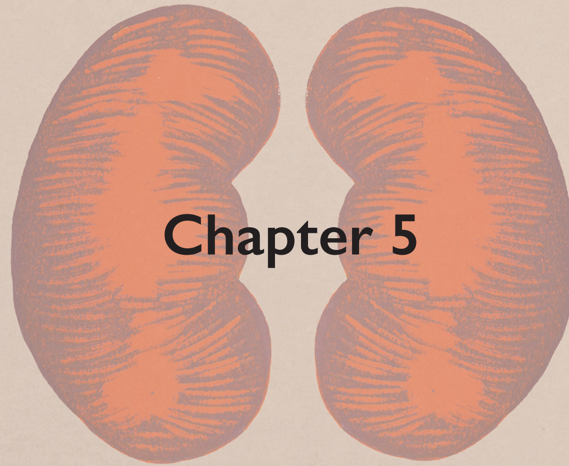

Chapter 5

Characterization of ectopic lymphoid structures in different types of acute renal allograft rejection

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Clinical and Experimental Immunology. May 2018; 192(2):224-232

Summary

We hypothesize that T cells such as interleuking (IL)-21+ B cell lymphoma 6 (BCL6)+ T follicular helper cells can regulate B cell-mediated immunity within the allograft during acute T cell-mediated rejection; this process may feed chronic allograft rejection on long term. To investigate this mechanism we determined the presence and activation status of organized T and B cells in so-called ectopic lymphoid structures (ELSs) in different types of acute renal allograft rejection. Biopsies showing the following primary diagnosis were included: acute/active antibody-mediated rejection, C4d+ (a/aABMR), acute T cell-mediated rejection grade I (aTCMRI), and acute T cell-mediated rejection grade II (aTCMRII). Paraffin sections were stained for T cells (CD3 and CD4), B cells (CD20), follicular dendritic cells (FDCs, CD23), activated B cells (CD79A), immunoglobulin (Ig)D, cell proliferation (Ki67), and double immunofluorescent stainings for IL-21 and BCL6 were performed. Infiltrates of T cells were detected in all biopsies. In aTCMRI, B cells formed aggregates surrounded by T cells. In these aggregates, FDCs, IgD and Ki67 were detected, suggesting the presence of ELSs. In contrast, a/aABMR and aTCMRII showed diffuse infiltrates of T and B cells but no FDCs and IgD. IL-21 was present in all biopsies. However, co-localization with BCL6 was observed mainly in aTCMRI biopsies. In conclusion, ELSs with an activated phenotype are found predominantly in aTCMRI where T cells co-localize with B cells. These findings suggest a direct pathway of B cell alloactivation at the graft site during T cell mediated rejection.

Introduction

Current immunosuppressive regimens in renal transplantation are directed primarily against T cell-mediated alloimmunity. However, many allograft recipients still develop allograft rejection through both T cell-mediated and/or antibody-mediated alloimmune responses (1, 2). Allograft rejection is a consequence of the activation of the recipient's immune system and includes the interaction between T and B cells via antigen presentation, co-stimulation and cytokine production. The local interaction in the renal allograft between T and B cells and its potential contribution to graft damage is still debated (3). Recent studies by Loupy *et al*. and Holloran *et al.* plea for a key role for T cells in the mechanism leading to chronic allograft rejection, traditionally the outcome of B cell mediated immunity (4, 5). Thus, studying the presence, organization and functional hallmarks of T and B cells within grafts of acute renal allograft rejection is essential for gaining a better understanding of the *in situ* immunological interactions in renal allograft rejection.

The formation of antibodies is a result of the interaction between T follicular helper (Tfh) cells and B cells (6). This Tfh-B-cell interaction is complex and involves various activation and regulatory pathways, including interleukin (IL)-21 signaling (6, 7). Activated Tfh and B cells reside in germinal centers (GCs) and both express transcriptional repressor B-cell lymphoma 6 (BCL6). Expression of BCL6 is essential for GC maintenance and BCL6 represses transcription factors of other lymphocyte subsets (8). The organized structure of the GC -a T-cell zone surrounding the active center where B cells and follicular dendritic cells (FDCs) reside- is crucial for correct B cell affinity maturation (9). After antigen recognition, Tfh cells initiate the differentiation of B cells into antibody-producing plasma cells via secretion of IL-21 (10). The maintenance of the GCs as part of secondary lymphoid organs (SLOs) is preserved by the presence of FDC networks (11). These FDC networks are involved in antigen priming of T cells and stimulation of B-cell affinity selection and maturation. Antigen-dependent Tfh-B-cell interaction and GC formation primarily takes place in SLOs such as draining lymph nodes. However, chronic persistence of antigen leads to the formation of highly organized leukocyte aggregates that resemble SLOs. These ectopic lymphoid structures (ELSs) or also called tertiary lymphoid organs (TLOs) initiate antigen-specific responses locally, *i.e.* at the site of the antigen (12). The formation of ELSs has been recognized in inflamed tissue caused by infection, auto-immunity and cancer (12). In organ transplantation ELSs are mainly associated with chronic rejection, though these structures are also recognized in acute renal allograft rejection (13-15). In 2003 Sarwal *et al.* described the presence of CD20+ B cell clusters in kidneys of renal transplant patients with acute rejection (13). The potential functions of these T and B cells in ELSs in acute renal allograft rejection and in which types of acute renal rejection these ELSs are particularly formed is largely unknown.

To better understand the pathophysiology of rejection after organ transplantation we studied the organization of T- and B-cells with respect to GC features (*i.e.* positive for FDCs, IgD (indicating active Ig production), Ki67 (indicating proliferation) and co-localization of BCL6 and IL-21)(16) in human renal allografts with acute rejection. To assess whether T cells regulate B cell mediated immunity within the grafts of T cell mediated rejection, we studied for the first time the presence of these structures in various categories of acute rejection: acute T cell-mediated rejection grade I (aTCMRI), acute T cell-mediated rejection grade II (aTCMRII) and acute/active antibody-mediated rejection (a/aABMR). Improved characterization of the immunological features of the ELSs in the renal allograft may provide a deeper understanding of the mechanisms underlying different types of acute rejection.

Materials and methods

Patient population

In this retrospective study we analysed a total of fifteen renal transplant biopsies from fifteen different patients with an acute allograft rejection. The patients were selected based upon the primary rejection diagnosis based on routine histopathological stainings. The primary diagnoses were acute/active antibody-mediated rejection (a/aABMR, *n*=5), in which, according to the Banff, C4d was positive and donor specific antigens (DSA) were present; acute T cell-mediated rejection grade I (aTCMRI, *n*=5), and acute T cell-mediated rejection grade II (aTCMRII, *n*=5). The different pathological features of the renal allograft rejections were classified according to the Banff '15 classification (17). Patient characteristics are summarized in Table 1. The Medical Ethical Committee of the Erasmus MC approved this study (MEC-2010-022). Renal biopsy material used was that what was available after routine diagnostics and this resides under the Dutch law on medical research and is not WMO compliant and is regulated by the Dutch Code of Conduct.

Immunohistochemical stainings

All renal biopsies were formalin fixed and paraffin-embedded (FFPE). Biopsies were processed according to a standardized diagnostic protocol. For histomorphological assessment, 3 μm sections were cut and stained with hematoxylin and eosin (H&E), periodic acid-schiff (PAS) and Jones' silver stain. In addition, an immunohistochemical C4d staining was performed on a 4 μm paraffin section. Subsequently, immunohistochemical stainings were performed on the Benchmark Ultra Stainer (Ventana, Basel, Switzerland). The following ready to use mAbs were used: CD3 (Ventana, ref. 290-4341), CD4 (Ventana, ref. 790-4423), CD20 (Ventana, ref. 790-2531), CD23 (Ventana, ref. 790-4408), CD79A (Ventana, ref. 790-4432), IgD (Ventana, ref. 760-4444) and Ki67 (Ventana, ref. 790-4286). Antibodies were incubated on the tissue sections for 30 minutes and anti-rabbit or anti-mouse amplifiers were used.

3.3'-diaminobenzidine (DAB) was used as chromogen. Sections were counterstained with hematoxylin. The quantification method used for these stainings was based on a numerical scale ranging from negative (0) to severe (3). High-power fields (HPF) with 0-25 positive cells were scored as mild (1), 26-100 positive cells were scored as moderate (2) and >100 positive cells were scored as severe (3). When biopsy material on the slide was insufficient we did not score and noted the staining as not applicable (N/A).

Table 1. Patient baseline characteristics

							Number of mismatches		
Case number	BANFF	Gender	Age at KT	L(U)R/ PM	Rejection (days post Tx)	Previous Ktx	HLA-A	HLA-B	HLA-DR
a/aABMR									
1	a/aABMR C4d+	M	53	PM	10	1	1	2	1
2	a/aABMR C4d+	F	61	LUR	2	0	0	2	1
3	a/aABMR C4d+	M	38	LUR	7	1	0	1	2
4	a/aABMR C4d+	F	32	LR	34	0	1	1	1
5	a/aABMR C4d+	M	54	LUR	65	1	2	2	1
aTCMR I									
1	aTCMR IB	M	65	LUR	117	0	2	2	1
2	aTCMR IB	M	25	LR	94	0	1	1	1
3	aTCMR IA	M	55	LR	63	0	1	2	2
4	aTCMR IA	F	25	LR	408	0	1	1	1
5	aTCMR IB	M	42	LUR	589	0	1	2	2
aTCMR II									
1	aTCMR IIB	M	60	LUR	146	0	2	1	1
2	aTCMR IIA	M	50	LUR	5	1	1	1	1
3	aTCMR IIB	M	59	LR	1268	0	0	0	0
4	aTCMR IIB	M	55	PM	11	1	2	1	0
5	aTCMR IIA	F	53	LR	4	0	1	0	1

a/aABMR = acute/active antibody-mediated rejection; aTCMR I = acute T cell-mediated rejection grade I; aTCMR II = acute T cell-mediated rejection grade II; L(U)R = living (un)related; PM = post mortal; Ktx = kidney transplantation; HLA = human leukocyte antigen.

Immunofluorescence

Four µm FFPE sections were deparaffinized up to 100% EtOH. Endogenous peroxidase was blocked for 10 minutes in 0.5% H_2O_2 in methanol. Afterwards, sections were heated in a microwave in a Tris-EDTA buffer to obtain antigen retrieval. In order to bypass non-specific antibody binding, sections were incubated for 30 minutes with 10% human serum

and 10% goat serum in a Tris-EDTA buffer enriched with 0.15M sodiumchloride, 0.25% gelatin and 0.05% Tween-20 at 4ºC. Primary antibodies BCL6 (mouse monoclonal; 1:200 dilution; Novocastra; Newcastle upon Tyne, UK) and IL-21 (rabbit polyclonal; 1:100 dilution; LS Bio; Seattle, WA) were incubated at 4ºC overnight. Subsequently, secondary antibodies Dyelight®594 (1:200 dilution; Vector laboratories; Burlingame, CA, USA) to visualize BCL6 expression in red and Dyelight®488 (1:200 dilution; vector laboratories) to visualize IL-21 expression in green were incubated for 1 hour at room temperature. Finally, sections were incubated with DAPI (Sigma-Aldrich, St. Louis, MO) for 30 minutes and enclosed with mowiol and a coverslip. As a positive control for IL-21 and BCL6 co-localization, duodenal biopsy sections obtained from a patient with celiac disease were used (Supp. figure 1). Images were taken and a double blind scoring on the images was performed on the presence of single and double positive cells with a ranked scale ranging from negative (0) to severe (3) presence of positive cells.

Results

Organized ELSs predominantly present in aTCMRI

All fifteen renal rejection biopsies were stained with H&E to assess cellular infiltrates within the renal allograft (a representative example is shown in Figure 1). In total, six out of fifteen biopsies (40%) contained dense cellular aggregates. Remarkably, cellular aggregates were detected in four out of five aTCMRI biopsies (80%) (black arrows, Figure 1, Table 2). In contrast, only one out of five biopsies (20%) in both the aTCMRII and a/aABMR groups contained cellular aggregates (Table 2).

Analyses of the immunohistochemical stainings are depicted in Table 2 and representative examples are presented in Figure 2 and 3. In all 15 biopsies, diffuse cellular infiltrates of $CD3^{pos}$ T cells were detected in the tubulointerstitial compartment and $CD4^{pos}$ cells were present which might be a mixture of CD4+ T cells and monocytes (Figure 2, Table 2)(18, 19). Within the aTCMRI biopsies, with aggregate formation, the T cells formed clear clusters compared to a more diffuse localization of T cells in the biopsies with aTCMRII or a/aABMR (Figure 2). As expected, biopsies showing a/aABMR only had moderate to mild infiltration of T cells in contrast to extensive T cell infiltration in the biopsies with either aTCMRI or aTCMRII.

$CD20^{pos}$ B cells were detected in a diffuse manner in four out of five a/aABMR biopsies (80%) and three out of five aTCMRII biopsies (60%). In contrast, all biopsies with aTCMRI revealed a moderate to severe presence of B cells, which on consecutive sections co-localized with the T-cell infiltrates (Figure 2), representing a B- and T-cell architecture of an ELS. In addition, Five out of six biopsies with aggregates (83%) showed the presence of CD23-positive FDC

networks, of which 4 (80%) were biopsies with an aTCMRI. The FDCs formed clear networks in the center of the B- and T-cell aggregates, with overlap with the B-cell nodules (Figure 2).

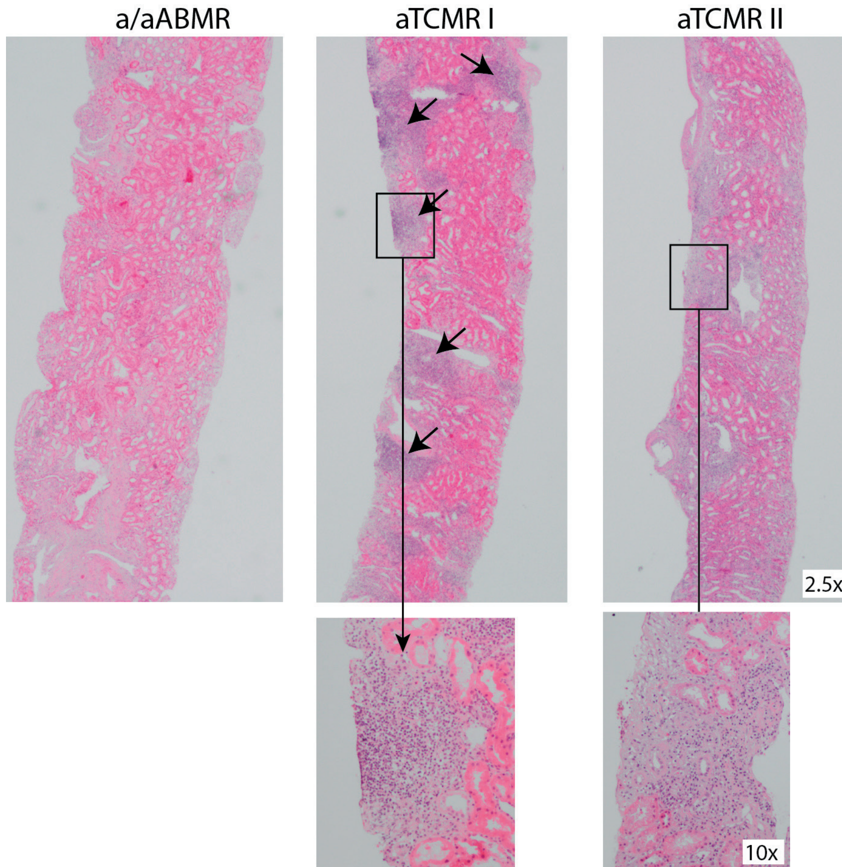


Figure 1. Representative haematoxylin and eosin (H&E) stainings of the different renal allograft rejection types.

Black arrows indicate cellular infiltrates referred to in table 2 as aggregates. Cropped images represent an aggregate in the acute T cell-mediated rejection grade I (aTCMRI) and a more diffuse cellular infiltrate in the aTCMRII. a/aABMR = acute/active antibody-mediated rejection, aTCMRI = acute T cell-mediated rejection grade I, aTCMRII = acute T cell-mediated rejection grade II.

In terms of activation, a subset of the B cells was positive for CD79A in all biopsies with aTCMRI, demonstrating an activated B-cell phenotype, up until differentiation to plasma cells (Figure 3) (20). The presence of IgD in all aTCMRI biopsies –mainly within the aggregates- confirmed local immunoglobulin production supporting a GC reaction (Figure 3). In all aTCMRI biopsies Ki67+ cells were present pointing towards active structures with

proliferating cells. As expected, in the aTCMRII biopsies infiltrating cells stained positive for Ki67 due to the high number of infiltrating cells in this type of rejection. In contrast, the majority of the a/aABMR stained negative for Ki67. The follow up of renal graft function after the rejection episode revealed that the patients with aTCMRI biopsies positive for T cells, B cells, FDC networks and IgD did not present differences in graft function at 3 months after the rejection biopsy compared to the other patients (Table 3).

Co-localization of BCL6 and IL-21 in ELSs

Simultaneous staining for BCL6 and IL-21 was performed to determine GC features. IL-21^{pos} cells were found in all biopsies. Within the aggregates, the majority of cells were IL-21^{pos}, whereas outside the aggregates and in biopsies without aggregates, lower numbers of IL-21^{pos} cells were found. Co-localization of IL-21 and BCL6 expression was predominantly observed within the B- and T-cell aggregates (Figure 4) in the aTCMRI biopsies (Table 2).

Table 2. Amount of renal biopsy stainings

Case number	Aggregates (ELSs)	CD3	CD4	CD20	CD79A	CD23	IgD	Ki67	IL-21	IL-21+BCL6
a/aABMR										
1	Yes	2	3	1	1	1	1	2	2	1
2	No	2	3	1	1	0	0	0	n.a.	n.a.
3	No	1	2	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	No	2	2	1	1	0	0	0	1	0
5	No	1	2	1	1	0	0	0	1	0
aTCMR I										
1	No	3	3	3	1	1	1	2	2	1
2	Yes	3	3	2	2	1	1	1	2	1
3	Yes	2	2	1	1	1	3	2	3	1
4	Yes	2	2	1	1	1	1	1	3	1
5	Yes	3	3	3	3	2	3	1	3	2
aTCMR II										
1	Yes	3	3	3	0	0	0	1	1	0
2	No	3	3	0	0	1	0	1	1	0
3	No	3	3	2	0	n.a.	n.a.	n.a.	1	0
4	No	2	2	0	0	0	0	1	n.a.	n.a.
5	No	3	3	1	1	0	1	2	1	0

a/aABMR = acute/active antibody-mediated rejection; aTCMR I = acute T cell-mediated rejection grade I; aTCMR II = acute T cell-mediated rejection grade II; 3, severe (>100 positive cells/HPF); 2, mild (26-100 positive cells/HPF); 1, moderate (0-25 positive cells/HPF); 0 = negative; n.a. = not applicable; HPF = high-power field; Ig = immunoglobulin; IL = interleukin; BCL6 = B cell lymphoma 6; ELSs = ectopic lymphoid structures.

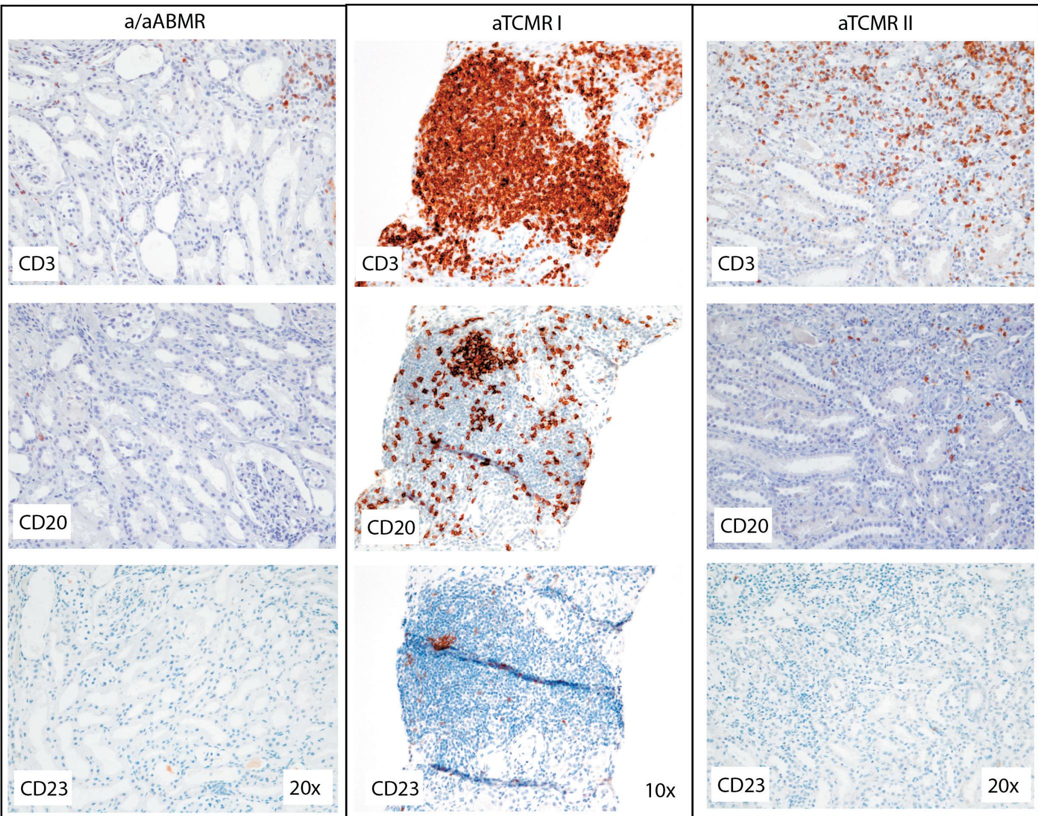


Figure 2. Immunohistochemistry for CD3, CD20, and CD23 to determine the organization of T cells, B cells and follicular dendritic cells (FDCs), respectively.

Stainings were performed in three types of renal allograft rejection: acute/active antibody-mediated rejection (a/aABMR), acute T cell-mediated rejection grade I (aTCMRI) and aTCMRII. Magnification: a/aABMR and aTCMRII x20, aTCMRI x10. aTCMRI biopsies were analysed with a lower magnification than the a/aABMR and aTCMRII biopsies in order to visualize the complete aggregate structures.

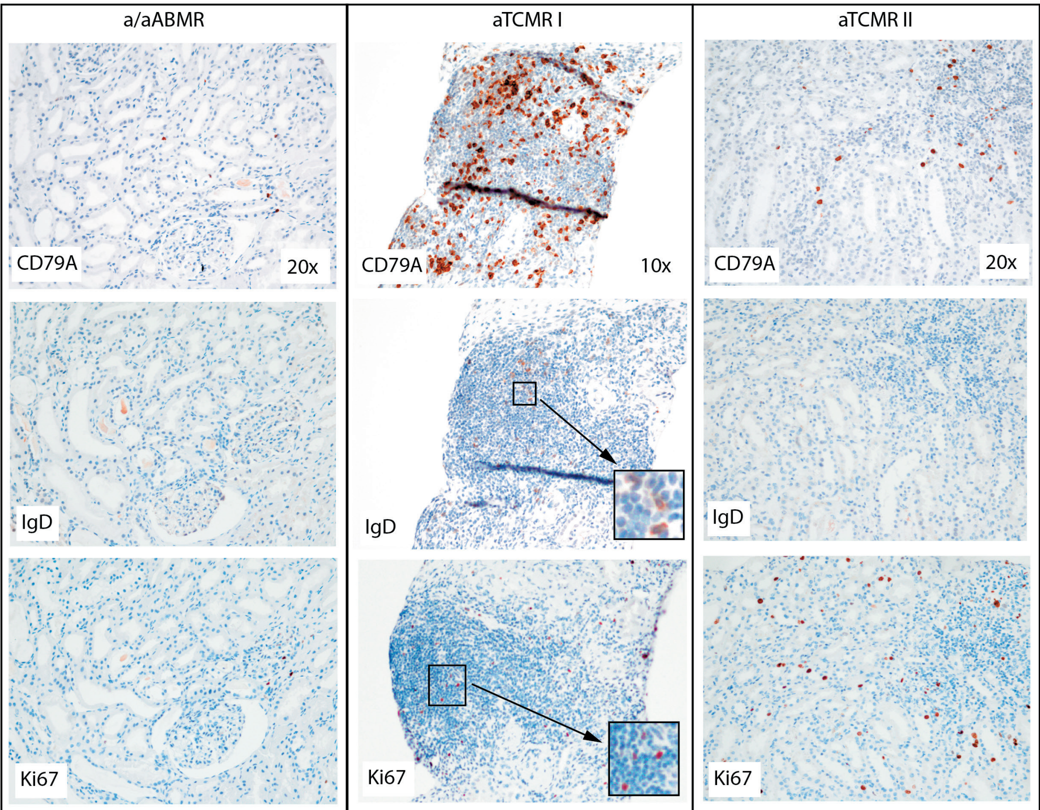


Figure 3. Immunohistochemistry for CD79A (B cell activation), immunoglobulin (Ig)D (immunoglobulin production) and Ki67 (cell proliferation).

Stainings were performed in three types of renal allograft rejection: acute/active antibody-mediated rejection (a/aABMR), acute T cell-mediated rejection grade I (aTCMRI) and aTCMRII. Magnification: a/aABMR and aTCMRII x20, aTCMRI x10. aTCMRI biopsies were analysed with a lower magnification than the a/aABMR and aTCMRII biopsies in order to visualize the complete aggregate structures.

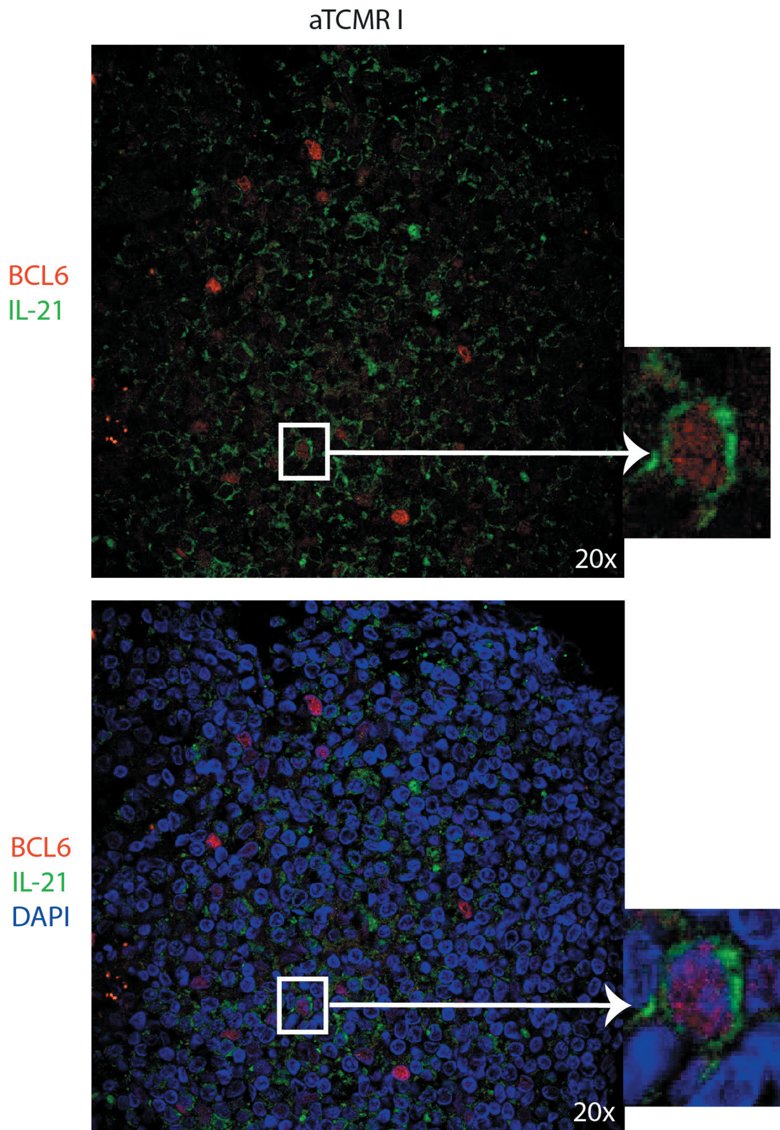


Figure 4. Immunofluorescent stainings for transcription factor B cell lymphoma 6 (BCL6) (red) and cytokine interleukin (IL)-21 (green) were performed.

4',6-diamidino-2-phenylindole (DAPI) (blue) was used to stain the chromatin of the cells. Magnification: x20. aTCMRI = acute T cell-mediated rejection grade I.

Table 3. Follow-up renal allograft function

Case number	Lowest creatinine before rejection	Creatinine rejection	Creatinine M3	Rejection therapy					Graft loss (Months after biopsy)
				Methyl pred-nisolon	ATG	IVIg	Alemtu-zumab	Rituxi-mab	
a/aABMR									
1	556	556	149	Yes	No	Yes	No	No	No
2	313	313	134	Yes	No	Yes	Yes	No	No
3	312	312	462	Yes	No	Yes	No	Yes	No
4	148	254	190	Yes	No	No	No	No	No
5	191	249	183	Yes	No	Yes	Yes	No	Yes (29)
aTCMR I									
1	142	229	235	Yes	No	No	No	No	No
2	109	148	112	Yes	No	No	No	No	No
3	106	155	95	Yes	No	No	No	No	Yes (37)
4	91	188	185	Yes	No	No	Yes	No	No
5	135	222	207	Yes	Yes	No	No	No	No
aTCMR II									
1	122	269	431	Yes	Yes	No	No	No	Yes (3)
2	157	157	142	Yes	No	No	No	No	No
3	135	327	331	Yes	Yes	No	No	No	No
4	1083	1083	106	Yes	Yes	No	No	No	No
5	303	303	200	Yes	Yes	No	No	No	No

Follow-up renal allograft function with lowest creatinine (µmol/L) between renal transplantation and renal allograft rejection, creatinine at time-point rejection and creatinine 3 months (M3) after rejection. a/aABMR = acute/active antibody-mediated rejection; aTCMR I = acute T cell-mediated rejection grade I; aTCMR II = acute T cell-mediated rejection grade II; ATG = anti-thymocyte globulin; IVIg = intravenous immunoglobulin

Discussion

T and B cell aggregates are formed locally in the renal allograft upon persistent alloantigen exposure (3, 7, 13). The role of these aggregates in alloreactivity is still debated. A growing body of evidence suggests that these structures are also present in more acute responses in which T cells are dominating (21). Here, we characterized lymphoid aggregates present in renal allografts and describe for the first time, to our knowledge, differences in the composition and formation of ELSs among three types of renal allograft rejection, namely aTCMRI, aTCMRII and a/aABMR. We found that organized lymphoid aggregates are mainly present in biopsies diagnosed as aTCMRI. We believe these represent active ELSs with FDC networks, IgD and BCL6^{pos}IL-21^{pos} cells, possibly Tfh cells, that provide B cell help.

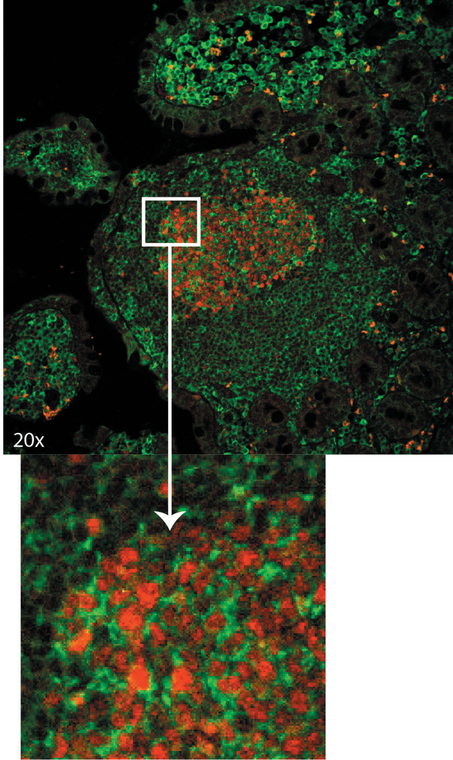
B-cells were found in all biopsies in which aggregates were seen. In accordance with previous work by Sarwal *et al.* and de Graav *et al.*, high numbers of B-cells were found in biopsies showing aTCMRI (13, 15). The role of these intragraft B-cell infiltrates is debated. Some studies have found that B-cell clusters are associated with poorer graft outcomes (22, 23), whereas other studies did not find an association between $CD20^{pos}$ B-cell infiltrates and graft survival (24-26). The presence of B cells in combination with FDC networks, IgD and Ki67 suggests humoral activity including the production of antibodies. However, all cases of aTCMRI were negative for C4d and DSA. Although it is possible that these B-cells produced donor-specific non-HLA antibodies we believe this is not the most likely explanation since such antibodies are rare. B-cell infiltrates in the setting of aTCMRI more likely have an antibody-independent function. B cells are known for their antigen-presenting capacity that may facilitate T cell-mediated alloreactivity and aTCMRI. From mouse studies we know that antigen presentation by B cells does indeed contribute to acute T cell-mediated rejection (27, 28). In addition, B cells may also contribute to T-cell activation via the secretion of cytokines like IFNγ, IL-10, TNF and IL-6 (29). Another hypothesis is that the clustered, intragraft B-cells found in aTCMRI have a resting phenotype. However, all aTCMRI biopsies were double positive for BCL6 and IL-21, markers associated with an activated state of Tfh cells and B cells. Finally, in different mouse models ELSs are also associated with donor-specific tolerance rather than rejection (30, 31), suggesting that the B cells may have a regulatory function. The presence of transitional $CD19^{+}CD24^{hi}CD38^{hi}$ B cells, also known as regulatory B cells, is associated with lower acute rejection rates after renal transplantation (32-34). In addition, in a mouse model for multiple sclerosis, maturation of regulatory B cells requires IL-21 and CD40 interaction with T cells (35), both present in ELSs in our aTCMRI biopsies. In our study, analyzing biopsy specimens from patients, the function of the aggregates in aTCMRI renal allografts has not been determined. Though given that aTCMRI is considered to be less detrimental to the graft than the other rejection types it is tempting to speculate that these aggregates might have a resting or regulatory function rather than a harmful one. In contrast, on long term the T-cell dependent activation of B cells within the allograft may contribute to the process of chronic allograft rejection.

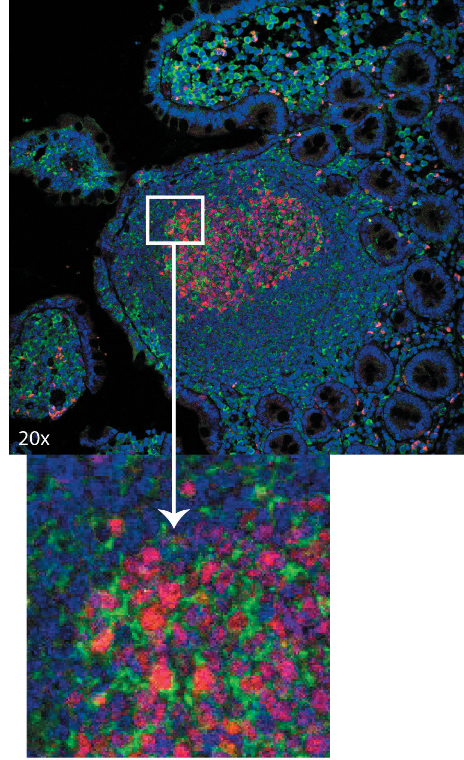
All aggregates contained numerous IL-21 single positive cells of which only a few were positive for BCL6, the hallmark of GC Tfh cells and B-cells. The presence of $BCL6^{pos}IL\text{-}21^{pos}$ cells in the aggregates in combination with Ki67 positivity points towards GC activity and suggests the presence of activated Tfh cells. In addition to B-cell help, the IL-21 positive T cells in aTCMRI might also enhance IFNγ production and cytotoxic activity of CD8+ effector T cells, as observed in an allogeneic system by Kasaian *et al.* (36). Another possibility is that the IL-21 single positive cells represent Th17 cells that are known to be involved in ELS formation (37, 38). In the biopsies with either aTCMRII or a/aABMR a diffuse presence of T

and B cells was observed and no FDC networks, IgD and co-localization of BCL6 and IL-21 was present in the majority of the cases. These findings suggest that the peripheral DSA present in the a/aABMR cases were not produced locally but outside the allograft (*e.g.* in draining lymph nodes). The effect of the presence of ELSs on renal allograft outcome is debated, with conflicting data that present both harmful and protective function. A recent study by Xu *et al*. found an association between the presence of ELSs and well-functioning kidney grafts pointing toward a regulatory role for these ELSs (39). The small number of samples in this study does not allow us to draw conclusions about the impact of ELS on kidney function and warrant further investigation.

In this study, we focused on T-B cell interaction and their functional activation markers in different types of acute renal allograft rejection. We found organized lymphoid structures in aTCMRI, implicating an important role for T- and B-cell interactions in this type of renal allograft rejection. Apparently, more cells are involved in aTCMRI than meets the eye, with presumably more mechanisms occurring than the classical alloreactive CD8+ T cell infiltrates such as providing help to allo-activated B cells. In conclusion, various compositions of infiltrating lymphocytes are present in the setting of aTCMRI, aTCMRII and a/aABMR with ELS formation predominantly in aTCMRI. These findings suggest a direct pathway of B cell allo-activation at the graft site during aTCMRI.

Supplemental Figures





Supplemental Figure 1.

Positive control for interleukin (IL)-21 (green) and B cell lymphoma 6 (BCL6) (red) immunofluorescent double staining of a duodenal biopsy from a patient with celiac disease. 4',6-diamidino-2-phenylindole (DAPI) (blue) was used to stain the chromatin of the cells. Magnification: x20.

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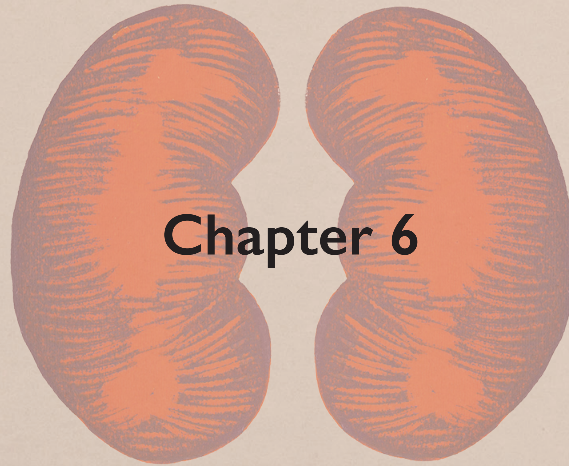

Chapter 6

Characterization of donor and recipient CD8+ tissue-resident memory T cells in transplant nephrectomies

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Scientific Reports. Arpil 2019; 12;9(1):5984

Abstract

Tissue-resident memory T (T_{RM}) cells are characterized by their surface expression of CD69 and can be subdivided in CD103+ and CD103- T_{RM} cells. The origin and functional characteristics of T_{RM} cells in the renal allograft are largely unknown. To determine these features we studied T_{RM} cells in transplant nephrectomies. T_{RM} cells with a CD103+ and CD103- phenotype were present in all samples (*n*=13) and were mainly CD8+ T cells. Of note, donor-derived T_{RM} cells were only detectable in renal allografts that failed in the first month after transplantation. Grafts, which failed later, mainly contained recipient derived T_{RM} cells. The gene expression profiles of the recipient derived CD8+ T_{RM} cells were studied in more detail and showed a previously described signature of tissue residence within both CD103+ and CD103- T_{RM} cells. All CD8+ T_{RM} cells had strong effector abilities through the production of IFNγ and TNFα, and harboured high levels of intracellular granzyme B and low levels of perforin. In conclusion, our results demonstrate that donor and recipient T_{RM} cells reside in the rejected renal allograft. Over time, the donor-derived T_{RM} cells are replaced by recipient T_{RM} cells which have features that enables these cells to aggressively respond to the allograft.

Introduction

Over the last two decades, the presence and importance of a non-migrating subset of memory T cells surveying immune responses in non-lymphoid tissues has been recognized: the so-called tissue-resident memory T (T_{RM}) cells (1, 2). Their restricted anatomical localization in combination with their effector memory phenotype enables T_{RM} cells to rapidly respond to local antigens. Today, two distinct subsets of T_{RM} cells have been identified: CD69+CD103+ and CD69+CD103- T_{RM} cells (hereafter referred to as CD103+ T_{RM} cells and CD103- T_{RM} cells, respectively)(3-6). CD69 is a C-type lectin that was originally identified as a marker of activated T cells but is also involved in tissue retention of T_{RM} cells (2, 7-9). CD69 binds to and down-regulates the G protein-coupled receptor sphingosine 1 phosphate (S1PR1) expressed on the T cell membrane, resulting in a decreased ability of T_{RM} cells to sense the S1P gradient that promotes migration of memory T cells from the blood into peripheral tissue (7-9). A unique T_{RM} cell gene expression profile has recently been identified by Kumar *et al.* with 31 core genes that are differentially up- or downregulated in CD69+ T_{RM} cells isolated from human lung and spleen(10). The other recognized T_{RM} marker, CD103, is an αE integrin that binds E-cadherin which is expressed on epithelial tissues (11). Today, no clear consensus exists about the functional differences between CD103+ and CD103- T_{RM} cells.

6

Although the presence and antigen specificity of T_{RM} cells is recognized in several non-lymphoid human tissues (*e.g.* skin, liver, lungs, intestine and brain), the presence and function of T_{RM} cells in the human kidney is currently unknown (4, 5, 12-16). In experimental mouse models, it was demonstrated that T_{RM} cells homed to the kidney where they resided, and that this migration was promoted by TGF-β (17, 18). Otherwise, little is known about the functional properties of these kidney T_{RM} cells (19).

Transplantation of a renal allograft is accompanied by the transfer of donor leucocytes. It is probable that these leucocytes also include donor-derived T_{RM} cells. It is, however, unknown if these cells persist after transplantation or whether they are replaced by T_{RM} cells of recipient origin. It is therefore informative to identify the donor or acceptor origin of these cells and thus the degree of chimerism. This will help us to understand if donor T_{RM} cells are present in the allograft to control local viral and bacterial responses (20) and whether these T_{RM} cells might be enriched for graft-*versus*-host (GvH) reactive clones, as seen in intestinal transplant patients (21-23). The presence of recipient T_{RM} cells will shed light on their potential role in alloreactivity and will show if recipient T cells differentiate into T_{RM} cells in the renal allograft.

Here, we postulate that T_{RM} cells are present in the renal allograft and that these cells are primarily from recipient origin and are capable of mounting an allo-reactive response. To this end, we used the unique tissue resource of transplant nephrectomies from

immunosuppressed patients to study the presence, provenance (donor or recipient), and the effector phenotype of CD103+ T_{RM} cells (CD69+CD103+), CD103- T_{RM} (CD69+CD103-), and recirculating T cells not expressing the tissue retention markers CD69 and CD103 (CD69-CD103-). We performed similar control experiments with spleen cells of organ donors, a lymphoid cell population in which T_{RM} cells are known to be present (3, 10).

Materials and Methods

Study population

Thirteen transplant nephrectomy specimens and two kidneys that were discarded for transplantation were studied. The characteristics of the patients from whom the transplant nephrectomies were derived are described in Table 1. Of these nephrectomies, the pathological features corresponding to the type of renal allograft rejection were classified according to the Banff'17 classification(43). In this study, residual material previously used for histopathological diagnosis was analysed. Residual materials were used in accordance with non-WMO compliant research that is regulated by the Dutch Code of Conduct (Federa). Splenocytes were obtained from deceased organ donors and peripheral blood mononuclear cells (PBMCs) were from healthy controls. The Medical Ethical Committee of the Erasmus MC, University Medical Center, approved this work (MEC-2010-022). All experiments were performed in accordance with relevant guidelines and regulations as described by our institution. All patients gave written informed consent. No organs were procured from (executed) prisoners.

Isolation of lymphocytes

Half of the renal allograft (cortex and medulla) was processed towards a single cell suspension, the other half was used for routine diagnostic assessment. The renal allograft was thoroughly rinsed with PBS to remove peripheral cells and afterwards dissected into small pieces (<0.5 cm^3) and incubated for 60 minutes at 37°C with 1.1 mg/ml collagenase IV (Serva, Heidelberg, Germany). The collagenase treatment was stopped by the addition of heat-inactivated fetal bovine serum with an end concentration of 10%. The tissue suspension was filtered through different sieves up to a 100μm sieve (Greiner Bio-One, Kremsmünster, Austria) followed by a Ficoll-Paque Plus procedure (GE healthcare, Uppsala, Sweden). Isolated cells were stored at -190°C until further use. Afterwards, cells were thawed and analysed by flow cytometry and reverse transcription-qPCR (RT-qPCR) to determine their phenotype and gene expression profile.

Human splenocytes were disrupted and filtered through a 70µm cell strainer (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) to obtain a single-cell suspension. Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient was used to collect mononuclear cells. Human PBMCs were isolated with the Ficoll-paque density gradient method.

Flow cytometric and functional analysis

Renal lymphocytes, splenocytes and PBMCs were stained with the following antibodies to characterize the T_{RM} phenotype: CD3 brilliant violet 510 (BV510; Biolegend, San Diego, CA, USA), CD8 Allophycocyanin-Cy7 (APC-Cy7; Biolegend), CD4 fluorescein isothiocyanate (FITC; BD, Franklin Lakes, New Jersey, USA) CD69 brilliant violet 421 (BV421; BD), CD103 phycoerythrin-cyanine7 (PE-Cy7; Biolegend). Viability was measured with the live-dead marker 7-aminoactinomycin (7-AAD; BD). FACS flow enriched with bovine serum albumin was used to wash the cells and block a-specific antibody interactions.

The donor or acceptor origin of the cells was determined with monoclonal antibodies (mAb) directed against human leukocyte antigen (HLA) class I antigens of the donor or acceptor. mAb used are listed in Supplemental Table 1, and were developed at Leiden University Medical Centre. We performed single labelling with either an antigen for which solely the donor was positive or solely the acceptor was positive.

The cytotoxic potential of T_{RM} cells was analysed by measuring intracellular granzyme B and perforin. Cells were stained as described above for T_{RM} cells. Afterwards, the cells were immediately fixed with FACS lysing solution (BD) and permeabilized with PERM II (BD). Subsequently, cells were stained intracellularly with granzyme B Allophycocyanin (APC) (Biolegend) and perforin phycoerythrin (PE; Biolegend).

The cytokine-producing capacity of the cells was measured after the cells were stimulated for four hours with 50 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C. Monensin and Brefeldin A (GolgiStop and GolgiPlug, BD Biosciences, Franklin Lakes, NJ, USA) were used to promote intracellular accumulation of the cytokines. PMA/ionomycin stimulation was stopped by adding Ethylene-diamine-tetra-acetic acid. Subsequently, cells were stained with the T_{RM} surface staining mixture and fixed and permeabilized as described above. The following mAb were used to measure cytokine production: interferon-γ (IFNγ) FITC (BD Biosciences), and tumour necrosis factor-α (TNFα) PE (Biolegend). Cell samples were measured on the FACSCanto II (BD) and analysed with Kaluza Analysis 1.5a software (Beckman Coulter, Brea, CA, USA).

Allogeneic reactivity

Mixed lymphocyte reactions (MLR) were performed in order to study T cell-reactivity of the renal lymphocytes following stimulation with donor cells, 3rd party cells, or recipient PBMCs. Renal lymphocytes were thawed and labelled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes®, Leiden, The Netherlands). Next, the renal lymphocytes were stimulated at 5 x 10^4 cells per well with irradiated donor cells, 3rd party cells, or recipient PBMCs (40 Gray) from which the CD3+ T cells had been depleted with magnetic-activated cell sorting (MACS). The cells were cultured at a ratio 1:1 in human culture medium (HCM) and after 7 days the CFSE dilution was measured on the FACSCanto II, indicating the amount of proliferation. Degranulation was measured by APC-labelled anti-CD107a (BD).

IFNγ ELISPOT

The frequencies of IFNγ producing cells upon stimulation with donor cells, 3rd party cells, or recipient PBMCs were measured with an Enzyme-Linked ImmunoSpot (ELISPOT) assay (U-CyTech Biosciences, Utrecht, The Netherlands). Briefly, anti IFNγ coated plates (U-CyTech Biosciences), were seeded with 100,000 renal lymphocytes and 100,000 irradiated donor cells, 3rd party cells or recipient PBMCs. Upon overnight incubation, plates were washed and incubated with biotinylated anti-human IFNγ detection antibody and streptavidin-HRP conjugate followed by the addition of AEC substrate (U-CyTech Biosciences). Spots were analysed using the ELISPOT reader (Bioreader®-600V, BIO-SYS GmbH, Karben, Germany).

Cell sorting of T_{RM} cells and RT-qPCR analysis

To determine the expression levels of a set of T_{RM} key genes of the CD103+ and CD103- T_{RM} cells and CD69-CD103- recirculating T cells, we performed RT-qPCR analysis on FACS-sorted, pure (>95%) T cell populations. Renal lymphocytes and splenocytes were sorted with the BD-FACSAria II SORP™. Subsequently, cells were pelleted and snap frozen and stored at -190°C until further use.

RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany) for the collection of high-quality RNA. Total RNA was subsequently reverse transcribed with oligo-dT. We used RT-qPCR to quantify the amount of *ITGA1*, *IL10*, *CXCR6*, *CX3CR1*, *KLF2*, *KLF3*, *SELL*, *S1PR1*, and the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). Assay-on-demand products for the detection and quantification of the different genes were used and are listed in Supplemental Table 2 (ThermoFisher, Waltham, MA, USA). The amount of each target gene was quantified by measuring the threshold cycle (Ct), which was transformed on a TaqMan Real-Time PCR system to the number of cDNA copies ($2^{(40-Ct)}$). The relative concentrations of the analysed genes were normalized to the relative concentration of the

housekeeping gene GAPDH present in each sample. Heatmapper software was used to cluster the cell-sorted samples based on the expression of the above described genes(44).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software; San Diego, CA, USA). Differences between paired groups were analysed with the Wilcoxon signed-rank test. A two-tailed *p*-value of <0.05 was considered statistically significant.

Results

T cells of donor and recipient origin are present in the renal allograft

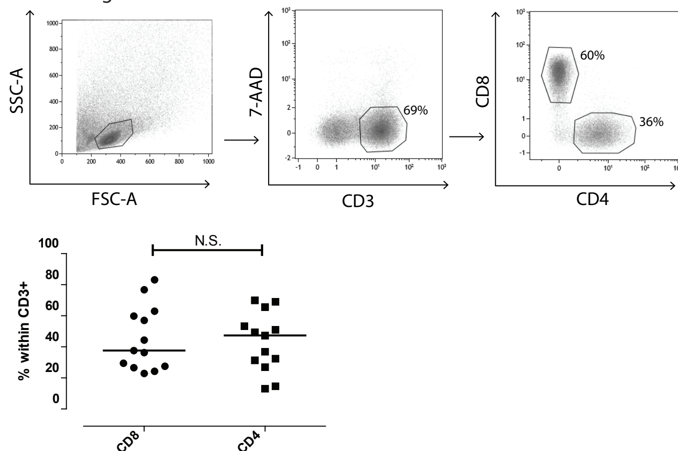
Thirteen transplant nephrectomy specimens were studied. Patient demographics are listed in Table 1. These kidney allografts failed either acute ($n = 4$) or chronically ($n = 9$) as a result of humoral, cellular or mixed-type rejection and were removed after a mean time of 6.1 years (range: 8 days – 26 years).

After the isolation procedure, CD3+ T cells were detected in all renal allografts with a median proportion among the total viable lymphocytes of 72.1% (range: 30.1 – 78.5%; Fig. 1A). Of the CD3+ T cells, 37.6% (22.9 – 83.2%) were CD8+ and 47.4% (13.1 – 70.0%) were CD4+ T cells (Fig. 1A). The CD3+ T cells isolated from nephrectomy number three and four (Table 1) were capable of mounting an allogeneic response, since both the proportions of proliferating cells and the expression of the degranulation marker CD107a increased in the presence of donor antigen, while a negligible response was measured after stimulation with irradiated recipient PBMCs (Supp. Fig. 1A, B). In addition, the cells of patient number three reacted to a fully HLA mismatched 3rd party and responded to donor antigen by the production of IFNγ (Supp. Fig. 1A-C).

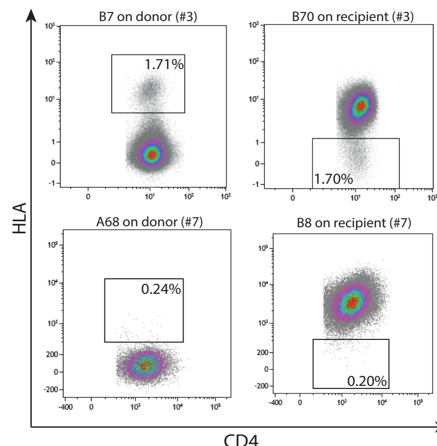
Next, the degree of chimerism was studied in the explanted renal allografts. Conjugated HLA-mAbs were available to distinguish recipient from donor cells in ten renal lymphocyte samples. In the example depicted in Figure 1B (upper panel), the proportions of donor cells measured by a mAb against HLA-B7 (donor positive) and a mAb against HLA-B70 (acceptor positive) resulted in similar donor proportions of 1.71% and 1.70% respectively, showing chimerism in this sample. Three out of the ten renal allografts studied were removed within the first month after transplantation (Table 1). In these renal allografts, clear populations of CD4+ and CD8+ T cells from donor origin were detected (Fig. 1C). The renal allografts removed five months or later after transplantation only contained marginal proportions (<0.5%) of donor-derived T cells, and we could not distinguish a clear positive and negative

fraction in these samples as depicted in the dot plots of patient number 7 in figure 1B (lower panel). Thus, high proportions of donor-derived T cells were only seen in early rejection nephrectomies.

A. Renal allograft



B. CD4+ T cells



C.

Renal allograft # table 1.	Time to explantation (days)	% Donor of total: % CD8*	% CD4*	
1.	8	**3.9**	**4.9**	
2.	12	**17.4**	**14.9**	
3.	15	**3.6**	**1.7**	
4.	150	0.38	0.36	<0.5%
5.	270	N/D	N/D	
6.	390	0.17	0.15	
7.	2268	0.095	0.22	
8.	2340	N/D	N/D	
9.	2520	0.32	0.37	
10.	3240	0.1	0.15	
11.	3780	0.08	0.04	
12.	4320	N/D	N/D	
13.	9360	0.05	0.08	

* Mean of two different HLA mAb measurements

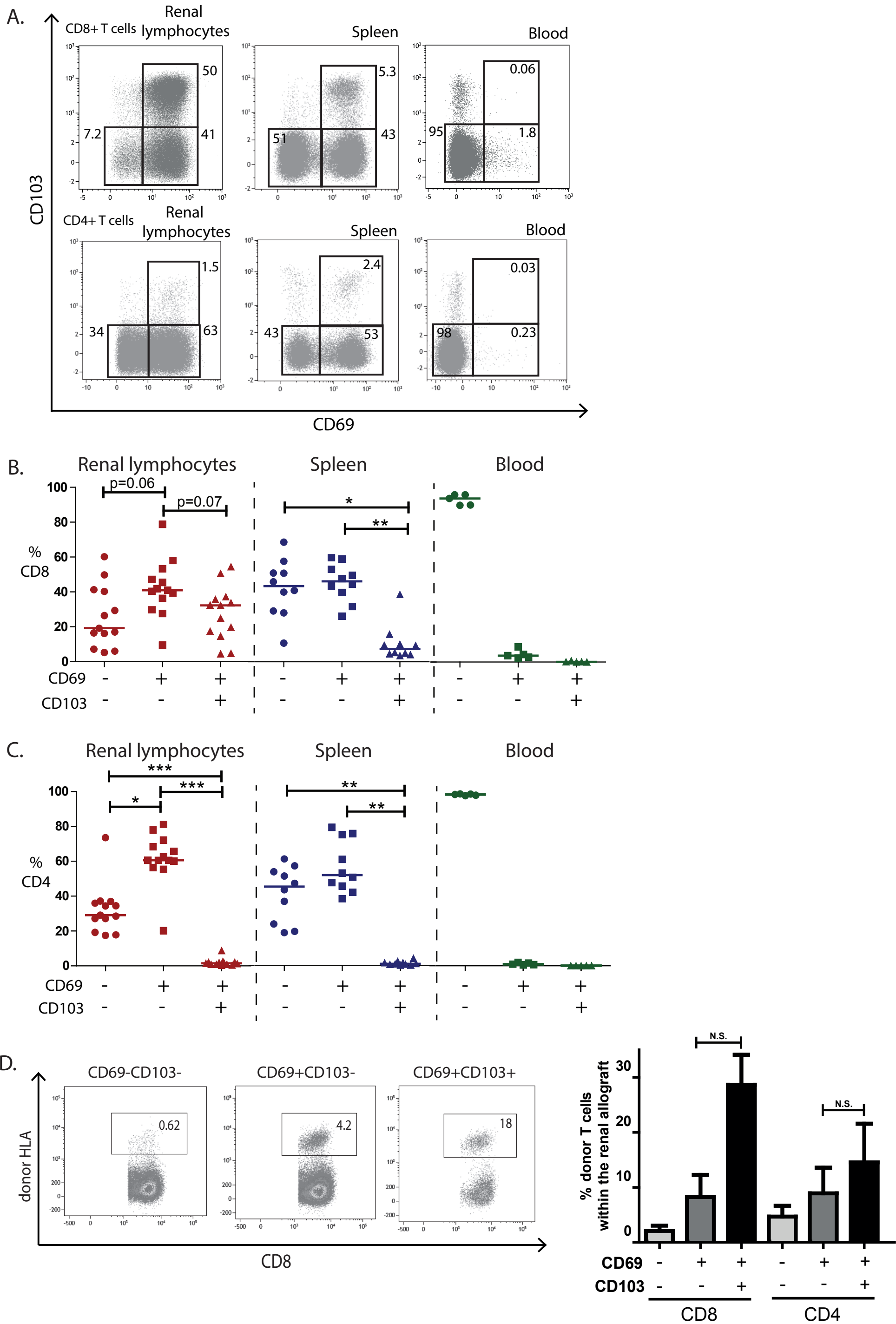
Figure 1. CD8+ and CD4+ T cells of donor and recipient origin are present in the renal allograft

Lymphocytes were isolated from the rejected renal allografts and subsequently stained and analysed by flow cytometry. (A) Gating strategy used to detect CD3+ T cells within the total viable lymphocytes, of which the CD8+ and CD4+ T cells were selected. (B) Typical examples of renal lymphocyte samples of patient number three and seven stained with mAb against human leukocyte antigen (HLA) class I antigens within the CD4+ T cells. Proportions of cells originating from the donor are depicted. (C) Table with numbers referring to the renal allografts described in Table 1, the time to explantation in days, and the proportions of donor cells detected within the CD8+ and CD4+ T cell compartment. Frequencies of the cells are presented as individual proportions with medians. HLA = human leukocyte antigen; N.S. = not significant; N/D = not determined.

Table 1. Patient baseline characteristics

	Recipient				Transplantation						
	Gender	Age at nephrec-tomy (y)	Cause of ESRD	Number of previous renal transplants	Donor type	CMV status donor*	CMV status acceptor*	Maintenance IS	Anti-rejection treatment	Time to explantation (days)	Cause of graft failure
1.	M	30	Hypertensive nephropathy	0	L	-	+	Tac, MMF, Pred	MP, Ivlg, plasmaferese, Alemtuzumab	8	aTCMR2B and aABMR and pyelonephritis
2.	F	48	ADPKD	0	L	-	+	Bela, Tac**, MMF, Pred, Basiliximab	MP, Alemtuzumab	12	aTCMR2B and aABMR
3.	M	63	Hypertension nephropathy	0	D	+	+	Tac, MMF, Pred, Basiliximab	MP, IVIg	15	aTCMR 2B and aABMR
4.	F	28	GPA	1	L	+	-	Tac, MMF, Pred	IVIg, Alemtuzumab	150	aTCMR3 and aABMR
5.	M	46	ADPKD	0	L	+	-	Tac, MMF, Pred	MP	270	aTCMR3
6.	F	67	Hypertension nephropathy	0	D	-	+	None#	MP	390	aTCMR3 and aABMR
7.	M	71	Diabetic nephropathy	1	D	+	+	Tac, MMF	MP	2268	aTCMR3 and c-aABMR
8.	M	29	HUS	0	D	-	-	Tac, MMF	None	2340	c-aABMR
9.	M	24	FSGS	0	L	+	+	MMF, Pred, Ecu***	MP, IVIg	2520	c-aABMR
10.	M	51	Congenital hydronephrosis	0	D	-	-	Tac	None	3240	aTCMR2B and aABMR
11.	F	51	poststreptococcal glomerulo-nephritis	0	D	+	-	None#	None	3780	c-aABMR and c-aTCMR
12.	M	53	Hypertension nephropathy	1	L	+	+	Tac, Pred	None	4320	aTCMR3
13.	M	57	Hypertension nephropathy	0	D	+	+	Aza, Pred	None	9360	End-stage kidney

*CMV status prior to transplantation, ** Switch from Belatacept to Tacrolimus due to rejection, *** No tacrolimus due to thrombotic microangiopathy, # Decreased IS and restarted dialysis. aABMR = acute antibody mediated rejection; ADPKD = autosomal dominant polycystic kidney disease; aTCMR = acute T cell-mediated rejection; Aza = azathioprine; CMV = cytomegalovirus; D = deceased; Ecu = eculizumab; ESRD = end-stage renal disease; FSGS = focal segmental glomerulosclerosis; GPA = granulomatosis with polyangitis; HUS = hemolytic uremic syndrome; IS = immunosuppression; IVIg = intravenous immunoglobulin; L = living; MP = methylprednisolone; MMF = mycophenolate mofetil; Pred = prednisone; Tac = tacrolimus



A.
CD8+ T cells
Renal lymphocytes
Spleen
Blood
50
41
7.2
5.3
43
51
0.06
1.8
95
CD4+ T cells
1.5
63
34
2.4
53
43
0.03
0.23
98
CD103
CD69
B.
Renal lymphocytes
Spleen
Blood
p=0.06
p=0.07
*
**
% CD8
CD69
CD103
C.
Renal lymphocytes
Spleen
Blood
***
***
*
**
**
% CD4
CD69
CD103
D.
CD69-CD103-
CD69+CD103-
CD69+CD103+
0.62
4.2
18
donor HLA
CD8
N.S.
N.S.
% donor T cells within the renal allograft
CD69
CD103
CD8
CD4

Figure 2. T_{RM} cells are present within CD8+ and CD4+ T cells in the renal allograft

Lymphocytes from rejected renal allografts, spleens of organ donors, and blood from healthy individuals were stained with mAb against CD69 and CD103. (A) Representative examples of the gating strategy of CD69 and CD103 of lymphocytes originating from the renal allograft, spleen, and blood. Proportions of the gated areas are depicted within the dot-plots. (B-C) Quantified data of the recirculating T cells (CD69-CD103-), CD103- T_{RM} cells (CD69+CD103-), and CD103+ T_{RM} cells (CD69+CD103+) subsets within the CD8+ T cell compartment (B) and CD4+ T cell compartment (C) of the renal allograft, spleen, and blood (blood $n = 5$, spleen $n = 10$, renal allograft $n = 13$). Frequencies of the cells are presented as individual proportions with medians. Significant differences were calculated and presented (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (D) mAb against HLA class I antigens were used to discriminate between donor and recipient lymphocytes. Typical example dot plots and quantified data of proportions of donor-derived cells within the CD103+ T_{RM} cells, CD103- T_{RM} cells and recirculating T cells are depicted of the transplant nephrectomies removed within the first month after transplantation (Table 1, patient 1 to 3). Frequencies of the positive cells are depicted within the dot plots. Frequencies of positive cells are shown as mean with the SEM ($n = 3$).

T_{RM} cells are present in the renal allograft

The absence of T_{RM} cells in PBMC fractions and the presence of T_{RM} cells in the spleen was recognized in previous studies(3, 10). Subsequently we used PBMCs from healthy controls as a negative control and splenocytes of deceased organ donors as a positive control for T_{RM} cell identification. CD69+ T_{RM} cells were present in all transplant nephrectomy specimens with a median proportion of 73.2% in the CD8+ T cell compartment and 62% in the CD4+ T cell compartment (Fig. 2A). We subdivided the T_{RM} cells in CD103+ T_{RM} (CD69+CD103+) cells and CD103- T_{RM} cells (CD69+CD103-). The median proportion of CD103+ T_{RM} cells within the CD8+ T cell compartment was 32.3% (range: 4.5 - 54.4%) compared to 1.4% (0.7 – 8.8%) within the CD4+ T cell compartment. In contrast, CD103- T_{RM} cells were more evenly distributed among the CD8+ (40.9%, 9.4 – 78.8%) and CD4+ (60.6%, 20.2 – 81.2%) T cell compartments (Fig. 2B, C). As expected, minimal proportions of CD103+ T_{RM} cells and CD103- T_{RM} cells were found in peripheral blood, while both T_{RM} cell subsets were present in the spleen (Fig. 2). Similar proportions of CD4+ and CD8+ T_{RM} cells were detected in two native kidneys that were discarded for transplantation (Supp. Fig. 2). No significant differences were detected when we subdivided the CD103+ and CD103- T_{RM} cells of the transplant nephrectomies based on donor type, Banff 2017 category and time to explantation (Supp. Fig. 3 A-C). Of interest might be the observation that in chronic-active antibody-mediated rejection (c-aABMR) specimens the proportion of CD103- T_{RM} cells was low (Supp. Fig 3B). However, this observation is only based on two c-aABMR cases.

We questioned whether T_{RM} cells of donor or recipient origin were present in the kidneys explanted in the first month after transplantation (Fig. 1C). For that we stained the renal lymphocytes of the transplant nephrectomies removed within the first month after transplantation (patients 1-3, Table 1) again with antibodies recognizing either donor or recipient HLA molecules. Remarkably, donor-derived lymphocytes were most prominent

within the CD103+ T_{RM} cell population, with lower levels of donor cells in the CD103- T_{RM} cells and recirculating T cell compartments (Fig. 2D). This was observed for both CD8+ and CD4+ donor T cells. These data suggest the increased ability of donor-derived CD103+ T_{RM} cells to reside in the renal allograft compared to the remaining donor-derived T cells.

Because the proportion of CD103+ T_{RM} cells was very low among the CD4+ T cells, not allowing further analysis, we focused on the CD8+ T_{RM} cells for subsequent experiments. In addition, the three renal allografts explanted within the first month after transplantation were excluded in order to study a pure population of recipient T_{RM} cells that may mediate and control the local immune response.

Expression of T_{RM} signature genes

To define the gene expression profile of the T_{RM} cells, we selected 8 genes from a set of signature genes described by Kumar *et al.* that are differentially expressed between CD69- and CD69+ CD8+ T cells isolated from the spleen and lung (10). We compared the expression of these genes in cells isolated form the renal allograft and in splenocytes in the following FACS-sorted CD8+ T cell populations: CD69+CD103+ T_{RM} cells, CD69+CD103- T_{RM} cells, and CD69-CD103- (recirculating) T cells. The gating strategy for the FACS sort experiments is depicted in Figure 3A.

We used RT-qPCR to quantify the expression of genes that are involved in pathways that mainly control T cell migration, adhesion and activation. Separate heat maps were created of the gene expression levels of the renal allograft and spleen (Fig. 3B). Overall, two clusters were identified in the heat maps of both the renal allograft and spleen: a cluster of genes upregulated in the total T_{RM} cells (CD103+ and CD103- T_{RM} cells) and a cluster of genes downregulated in the total T_{RM} cells, compared to the recirculating T cells (Fig. 3B). The gene expression levels of the adhesion marker ITGA1 (CD49a) were especially high in the CD103+ T_{RM} cells, with lower expression levels in the CD103- T_{RM} cells and recirculating T cells. Chemokine receptor CXCR6 and cytokine IL-10 were clearly expressed at a higher level in the total T_{RM} cell subsets compared to the recirculating T cells. In both the spleen and renal allograft samples, the T cell trafficking and homing markers S1PR1, Kruppel-like transcription factor 2 (KLF2), SELL (CD62L), KLF3 and CX3CR1 were expressed at a lower level in the total T_{RM} cells compared to the expression levels in the recirculating T cells. When we clustered the FACS-sorted samples of the renal allograft and spleen, the CD103+ T_{RM} cells and CD103- T_{RM} cells clearly clustered together as opposed to the recirculating T cells (Supp. Fig. 4). In summary, the total population of T_{RM} cells can be clearly distinguished from the recirculating T cells based on their gene expression profile. In addition, similar

gene expression levels were found in the different T cell subsets when comparing the renal allograft and the spleen samples.

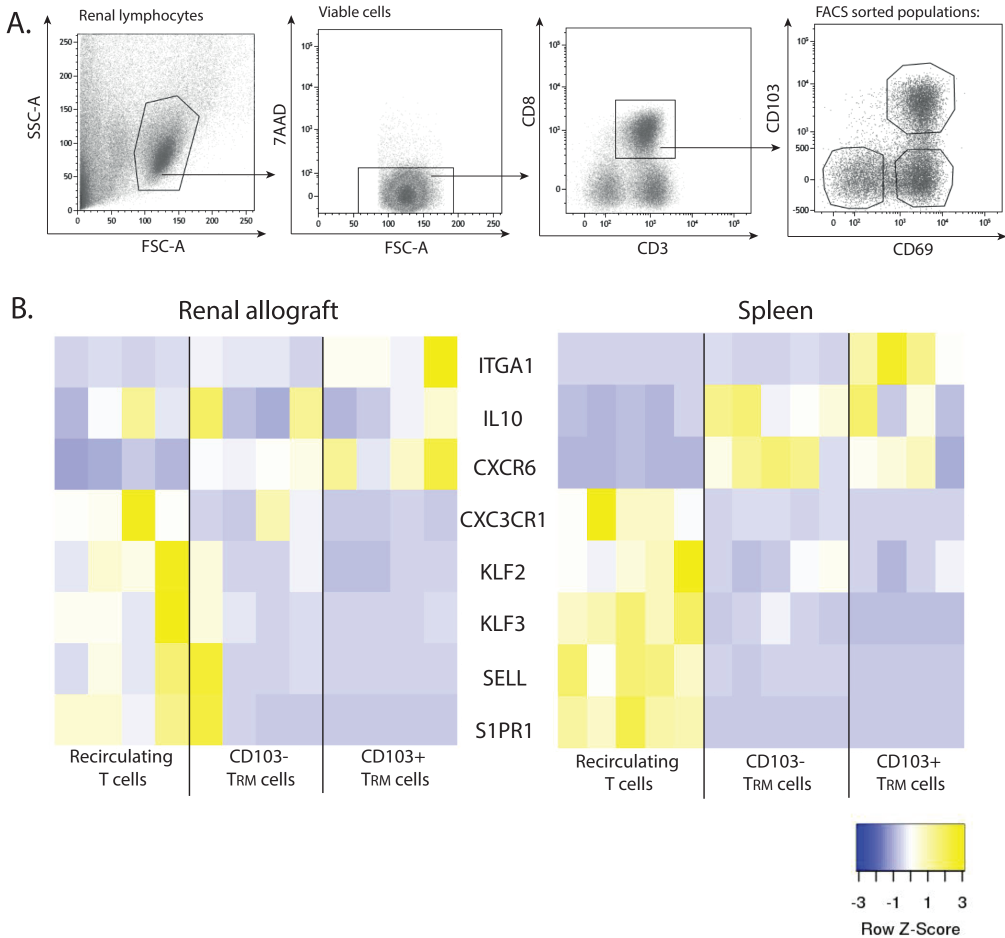


Figure 3. Expression of T_{RM} signature genes

(A) Typical example of the gating strategy used after fluorescence activated CD8+ cell sorting to obtain recirculating T cells (CD69-CD103-), CD103- T_{RM} cells (CD69+CD103-), and CD103+ T_{RM} cells (CD69+CD103+). Cells were gated by forward- and side-scatter followed by 7-AAD negative (viable) gating. (B) Heatmaps depicting the normalized gene expression of eight core genes known to be upregulated (yellow) or downregulated (blue) in T_{RM} cells of the renal allograft and spleen. Spleen *n* = 5, renal lymphocytes *n*=4.

T_{RM} cells in the renal allograft have an effector memory phenotype

To demonstrate that the CD8+ T_{RM} cells found in the renal allograft have an effector phenotype we stained the cells for the surface molecules CCR7 and CD45RO. With these markers naïve (CCR7+CD45RO-), central memory (CM, CCR7+CD45RO+), effector memory (EM, CCR7-CD45RO+) and highly-differentiated effector memory (EMRA, CCR7-CD45RO-) T cells were discriminated (see Fig. 4A for a typical example). While the recirculating T cells in the renal allograft and spleen were more divergent in terms of CCR7 and CD45RO expression, the majority of the CD103- T_{RM} cells and CD103+ T_{RM} cells were CCR7-CD45RO+ and thus EM T cells (Fig. 4B). This finding is in line with previous studies in non-lymphoid tissues where the majority of T_{RM} cells was also of an EM phenotype(3, 10).

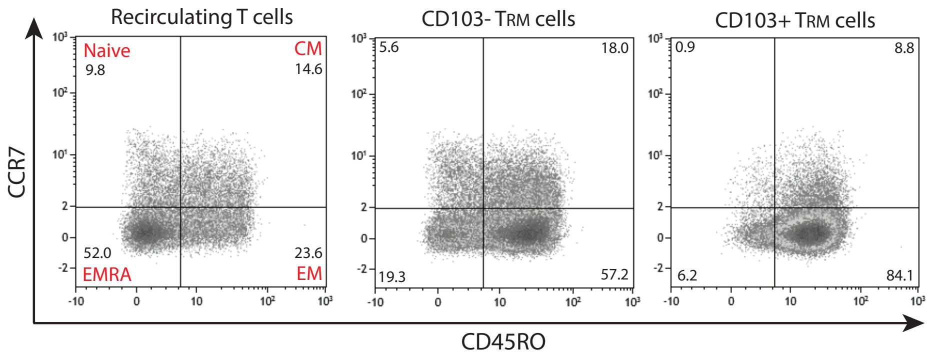
T_{RM} cells are capable of producing effector molecules

The capacity of the cells to produce the pro-inflammatory cytokines IFNγ and TNFα was measured after polyclonal stimulation. No change in the expression and proportion of CD69+ cells before and after three hours of polyclonal T cell stimulation was observed, in contrast to an altered expression of CD69 and CD103 on the CD8+ T cells after seven days allostimulation (Supp. Fig. 5, 6). Subsequently, we compared the T_{RM} cell subsets and the recirculating T cells after stimulation for their cytokine production capacity.

stimulation, all resident and recirculating CD8+ T cell subsets expressed high levels of IFNγ (Fig. 5A). A slightly higher expression in the IFNγ production capacity was observed in the CD103+ T_{RM} cells in both renal allograft and spleen compared to the recirculating and CD103- T_{RM} cell subsets (Fig. 5A). For TNFα, a different profile was found with the highest expression levels in the recirculating T cells, lower levels in the CD103- T_{RM} cells and the lowest levels in the CD103+ T_{RM} cells (Fig. 5B). Within the spleen, the differences in TNFα proportions were significantly different between the different subsets (Fig. 5B). Highly effector CD8+ T cells that were concurrently positive for IFNγ and TNFα were present in both the renal CD103- and CD103+ T_{RM} cells (Supp. Fig. 7).

To determine the degranulation capacity of the different CD8+ T_{RM} cell subsets, intracellular granzyme B and perforin expression were measured (Fig. 5C, D). In the renal allograft, the intracellular granzyme B levels did not significantly differ between the recirculating T cells, the CD103- T_{RM} cells and CD103+ T_{RM} cells (Fig. 5C). The granzyme B levels within the spleen were significantly lower in the CD103+ T_{RM} cells compared to both the recirculating T cells and the CD103- T_{RM} cells. With regard to the intracellular levels of perforin, the same trend between the different subsets was found in the renal allograft samples compared to the spleen samples (Fig. 5D). The perforin levels were significantly lower in the CD103+ T_{RM} cells

compared to the recirculating T cells (Fig. 5D). Together, these data show that T_{RM} cells, but also the recirculating T cells, are capable of mounting and effector response.



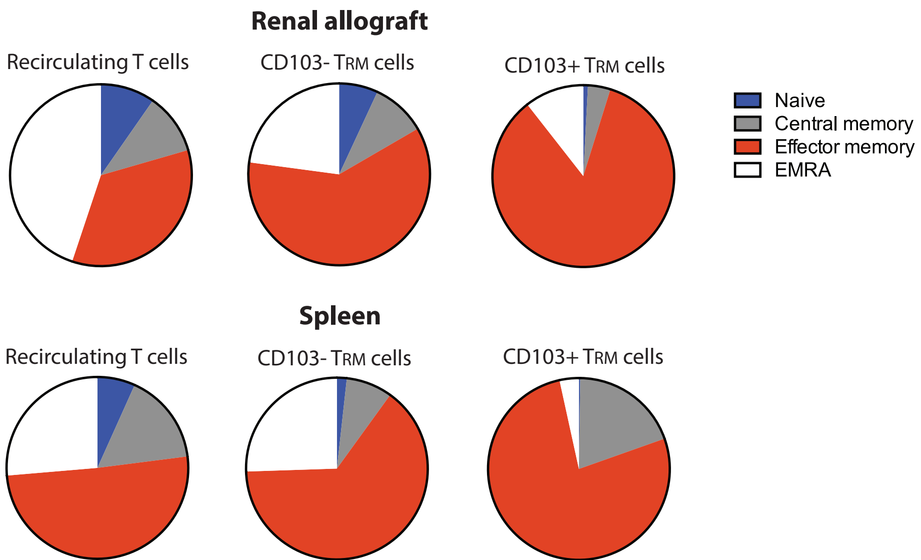


Figure 4. T_{RM} cells in the renal allograft have an effector memory phenotype

(A) Typical examples of dot plots presenting the distribution of naïve T cells (CCR7+CD45RO-), central memory T cells (CM; CCR7+CD45RO+), effector memory T cells (EM; CCR7-CD45RO+), and EMRA T cells (CCR7-CD45RO-) within the CD8+ recirculating T cells, CD103- T_{RM} cells and CD103+ T_{RM} cells of the renal allograft. Numbers within the dot plots indicate proportions of the different cell subsets. (B) Pie charts representing the median proportion of naïve, central memory, effector memory, and EMRA T cells within the CD8+ recirculating T cells, CD103- T_{RM} cells and CD103+ T_{RM} cells of the renal allograft and spleen (renal lymphocytes *n*=6, spleen *n*=8).

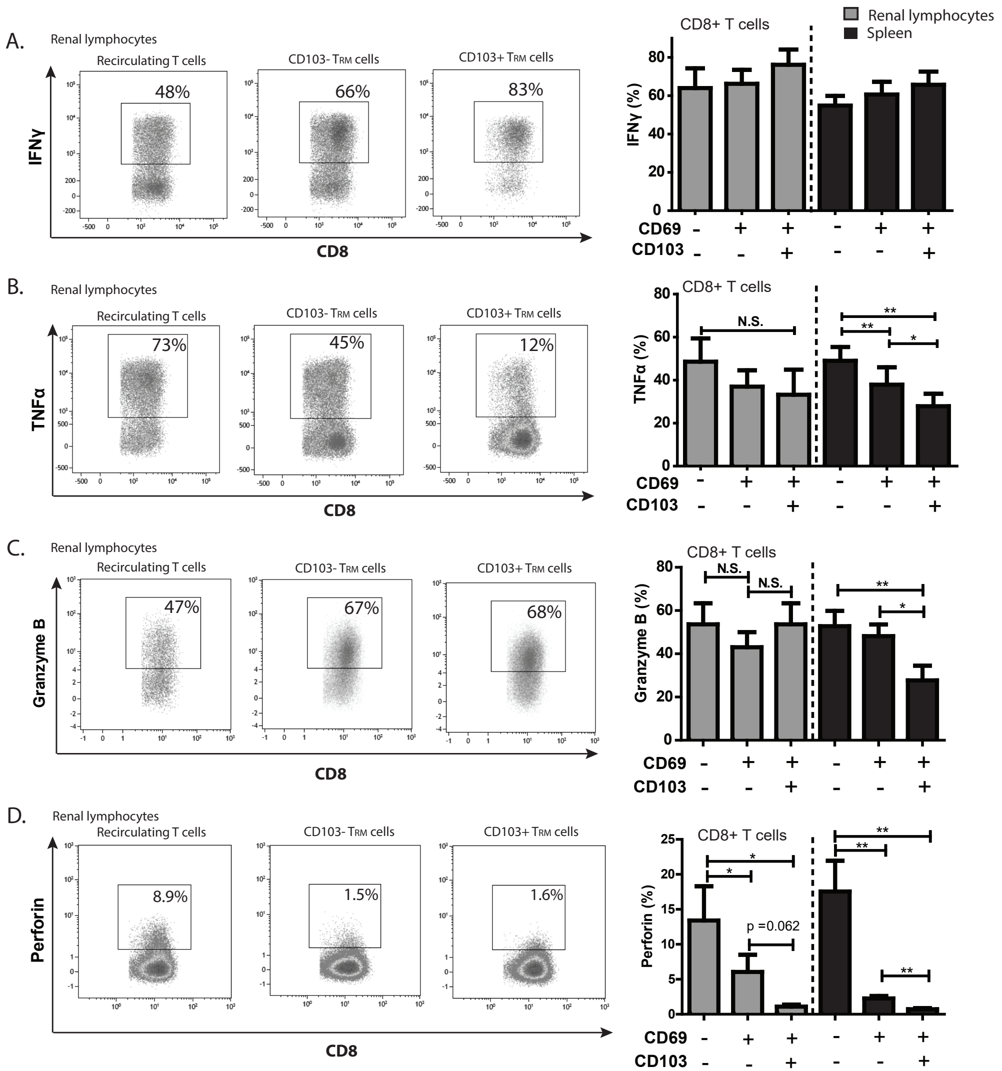


Figure 5. T_{RM} cells are capable of producing effector molecules

(A-B) Proportions of IFNγ (A) and TNFα (B) producing cells are depicted upon 4 hours PMA/ionomycin stimulation in the presence of monensin and brefeldin A. Cytokine proportions were measured in renal lymphocytes and splenocytes within the recirculating T cells, CD103- T_{RM} cells, and CD103+ T_{RM} cells of the CD8+ T cell compartment. (C-D) Frequencies of granzyme B (C) and perforin (D) levels were measured in the recirculating T cells, CD103- T_{RM}, and CD103+ T_{RM} cells of the CD8+ T cell compartment. Frequencies of positive cells were shown as mean with the SEM (renal lymphocytes *n*=6, spleen *n*=10). Significant differences were calculated and depicted (N.S. = not significant, * $p<0.05$, ** $p<0.01$).

Discussion

In this study, we demonstrate that 1) T cells with a resident memory phenotype and gene expression profile are present in the renal allograft, 2) T_{RM} cells in the renal allograft have strong immunostimulatory capacity, and 3) the donor-derived cells present are mainly CD103+ T_{RM} cells and are replaced by recipient-derived T cells within five months after transplantation. An overview of these findings is depicted in Figure 6.

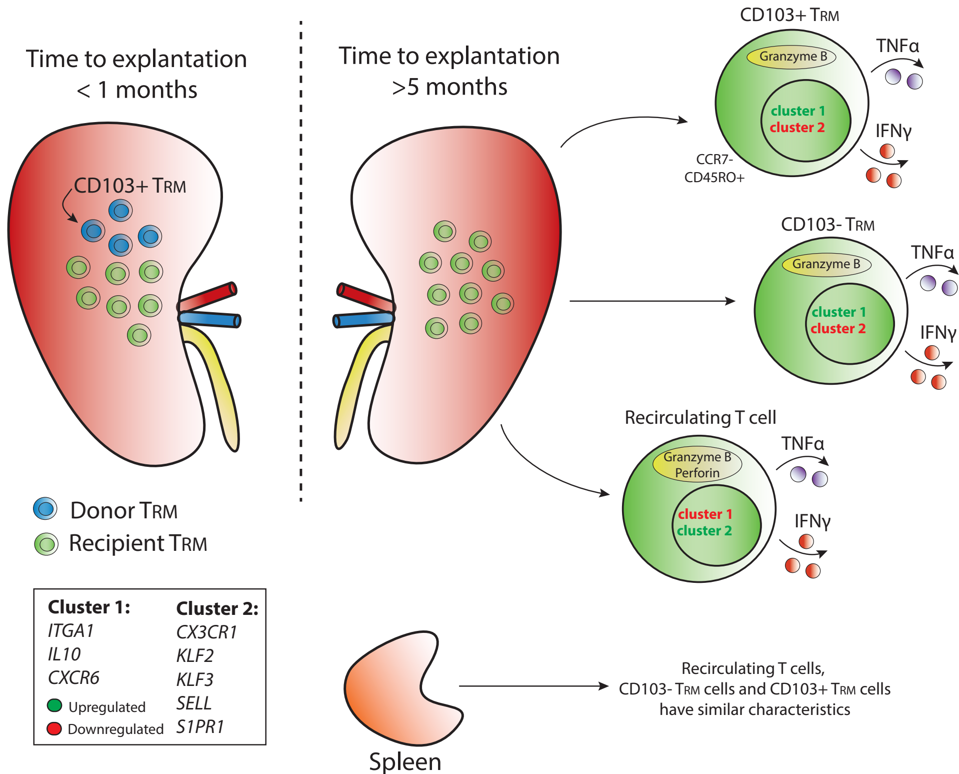


Figure 6. Schematic overview of T_{RM} cell characteristics in the renal allograft

Distribution of donor-derived and recipient-derived tissue-resident memory (T_{RM}) cells and the phenotypic and functional characteristics of the recipient-derived T_{RM} cells in the explanted renal allograft are depicted in a schematic overview. Cluster one and cluster two indicate T_{RM} core genes of which the expression was measured. Cluster one consists of genes involved in T cell activation (*ITGA1*, *IL10*, and *CXCR6*) and cluster two consists of genes involved in T cell migration (*CX3CR1*, *KLF2*, *KLF3*, *SELL*, *S1PR1*).

No major differences were found in gene expression signature and functional profiles between the CD103+ T_{RM} and CD103- T_{RM} cells, highlighting that these two T_{RM} cell subtypes have comparable characteristics. Previous studies report that CD103+ T_{RM} cells reside more

predominantly within the barrier tissues while CD103- T_{RM} cells are more common within non-barrier tissues(18). In transplantation, CD8+CD69-CD103+ T cells are involved in the effector mechanism of chronic and acute renal allograft rejection(24-27). In addition, in urinary samples, the mRNA levels of CD103 predicted acute renal allograft rejection(28). However, in the context of kidney transplantation the exact difference in CD103+ and CD103- T_{RM} cells is unknown and of interest for further analysis.

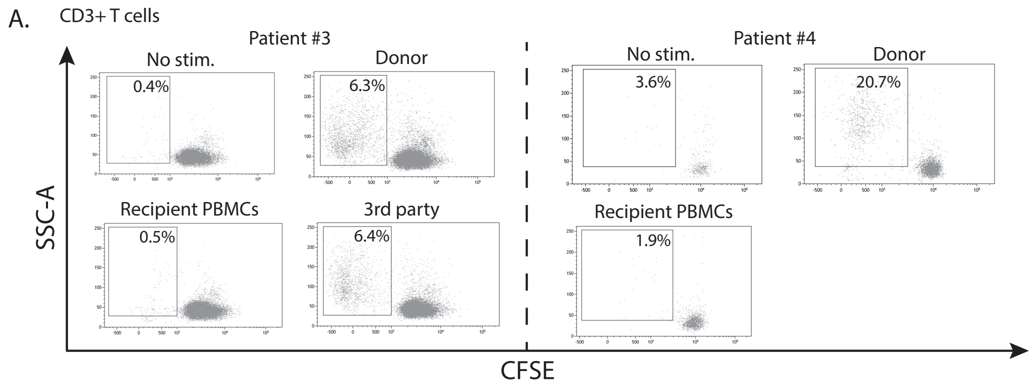
The majority of the T_{RM} cells detected in the renal allograft were of an effector memory phenotype. Therefore, these cells have been antigen challenged and are able to rapidly exert immunological responses. Both the CD103+ and CD103- T_{RM} cell subsets had the capacity to produce TNFα, a cytokine involved in, among others, the activation of endothelial cells, thereby attracting other T cells to the site of inflammation(29). The capacity of the T_{RM} cells to produce IFNγ and the presence of preloaded granzyme B positive granules underlines their cytotoxic phenotype. Low levels of perforin were measured in the T_{RM} cells which is in line with previous studies(30, 31). In these studies, perforin was rapidly upregulated upon antigen stimulation, delineating a dynamic process(30, 32). Here we found that T_{RM} cells present in renal allografts are potentially harmful cells that might contribute to the process of allograft rejection. However, additional experiments need to clarify the exact roles of the T_{RM} cell subsets in the alloimmune response and show causality. For future studies, it would be of interest to include the number of cells per gram tissue. The functional profiles of the T_{RM} subtypes found in the renal allograft were comparable to those found in the T_{RM} subsets residing in the spleen. This supports that we have identified T_{RM} cells in the renal allograft.

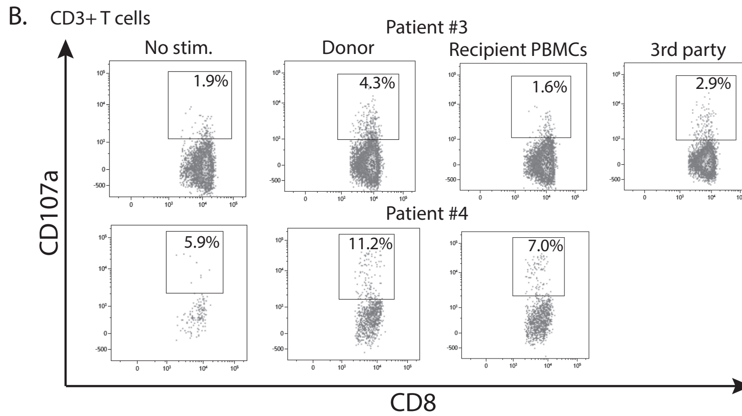
Memory T cell signalling is still occurring under immunosuppression because these cells are less reliant on co-stimulatory signals(33-36). For this reason, we can hypothesize about the long lasting persistence of T_{RM} cells within the renal allograft. Also, the distribution of immunosuppressive agents into the tissue may influence the presence of T_{RM} cells. For instance, alemtuzumab depletes circulating CM T cells in leukemic cutaneous T cell lymphoma patients without completely compromising the immune response to infection, since the skin resident memory cells are spared(37). The samples analysed in this study are a heterogeneous group with different types of end-stage immunological transplant failure. Further study on the differentiation and function of T_{RM} cells upon transplantation, would benefit from similar studies on cells harvested from healthy renal tissue or grafts undergoing an evolving rejection, which for obvious reasons is not possible in transplant recipients. The best alternative for healthy renal tissue would be the use of kidneys discarded for transplantation. Our first findings showed the presence of T_{RM} cells in these kidneys with comparable frequencies of CD103- and CD103+ T_{RM} cells as found in the transplant nephrectomies.

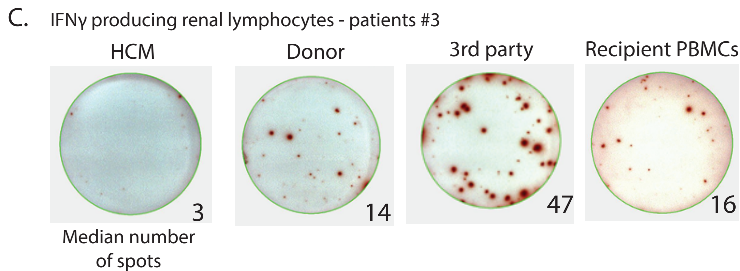
The results of our study reveal that donor-derived lymphocytes are replaced by their recipient counterparts within five months after transplantation and that the recipient-derived T cells differentiate locally towards a resident memory phenotype. The rapid presence of recipient T_{RM} cells in these specimens sheds light on the important role of this cell type in the process of alloreactivity. The repopulation by recipient lymphocytes has also been observed in lung and intestinal allografts(38, 39). The prolonged retention of the CD103+ T_{RM} cells compared to the other donor-derived T cells supports that this subtype of T_{RM} cells is highly capable to bind to epithelial cells of the renal allograft (Fig. 6). The retention of donor-derived T_{RM} cells might have a protective role in the renal allograft since irradiation of donor cells in rodent models resulted in rejection of liver transplants(40, 41). Also, in visceral transplant patients, T cell chimerism is observed in the absence of graft-*versus*-host disease (GVHD)(21). Donor-derived cells with graft specific TCR clones are even thought to slow down the constant threat of recipient-derived T cells, illustrating that the balance between graft-*versus*-host (GvH) and host-*versus*-graft (HvG) clones *in situ* influences lymphocyte turnover and development of rejection(22). A rapid clearance of the donor-derived cells may thus contribute to the rejection of the renal allograft. The low IFNγ-producing response of the renal lymphocytes to recipient PBMCs that we measured might be due to the mixed population of donor and recipient cells within the renal lymphocytes. This observation might be a reflection of the potential GvH response which may also explain a lower response to the donor cells, in line with the findings of Zuber *et. al.*(22). For future experiments, it is of interest to compare the GvH and HvG T cell balance in protocol biopsies at different time points after transplantation and compare this balance between patients with and without rejection. In addition to the response to the allograft, T_{RM} cells also recognize and clear pathogens. Therefore, it is tempting to speculate that the T_{RM} cells are both friends and foes to the renal allograft. Virus-specific T_{RM} cells are known to be able to quickly exert their effector function within the peripheral tissue, and thus the T_{RM} cells present in the renal allograft may also exert this function(20, 42). Therefore, the precise contribution of donor and recipient T_{RM} cells to alloreactivity is of high interest for future studies.

In conclusion, our results demonstrate that T_{RM} cells are present in the human renal allograft and that donor-derived T_{RM} cells are replaced within the first months after transplantation by recipient T_{RM} cells, which have the capacity to aggressively respond to the allograft. Understanding the potentially destructive or protective roles of the T_{RM} cells in the renal allograft is of high interest to enhance renal transplant outcomes.

Supplemental Figures







D. HLA-typing patient #3

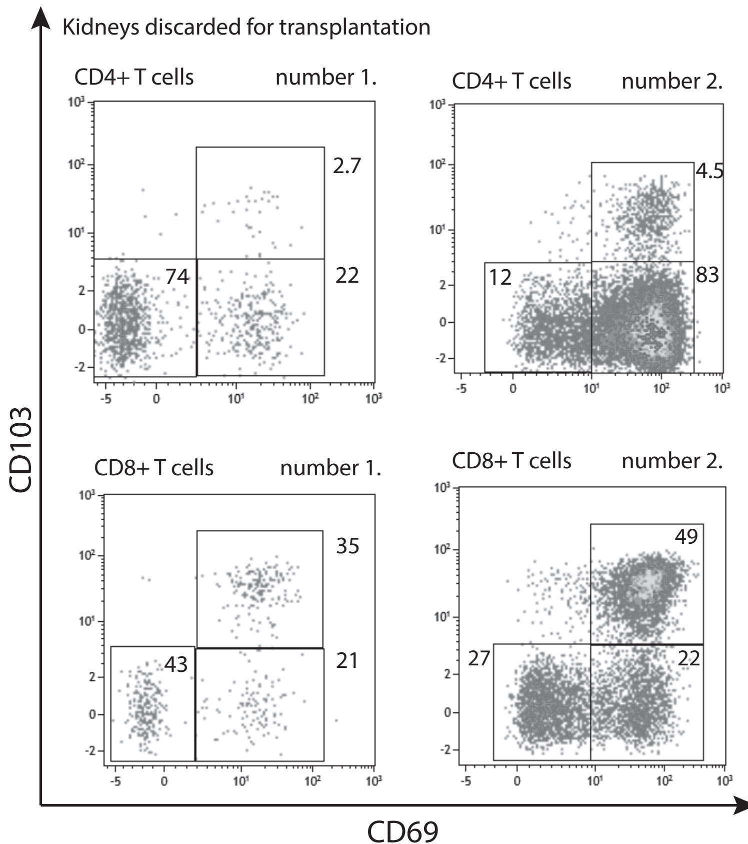
Recipient:	A3 A23(9)	B8 B72(70)	DR17(3) DR7
Donor:	A3 A1	B8 B7	DR17 (3) DR4
3rd party:	A2 A33(19)	B44(12) B58(17)	DR11(5) DR13(6)

HLA-typing patient #4

Recipient:	A3 A25(10)	B7 B44(12)	DR15(2) DR4
Donor:	A1	B8	DR3 DR4

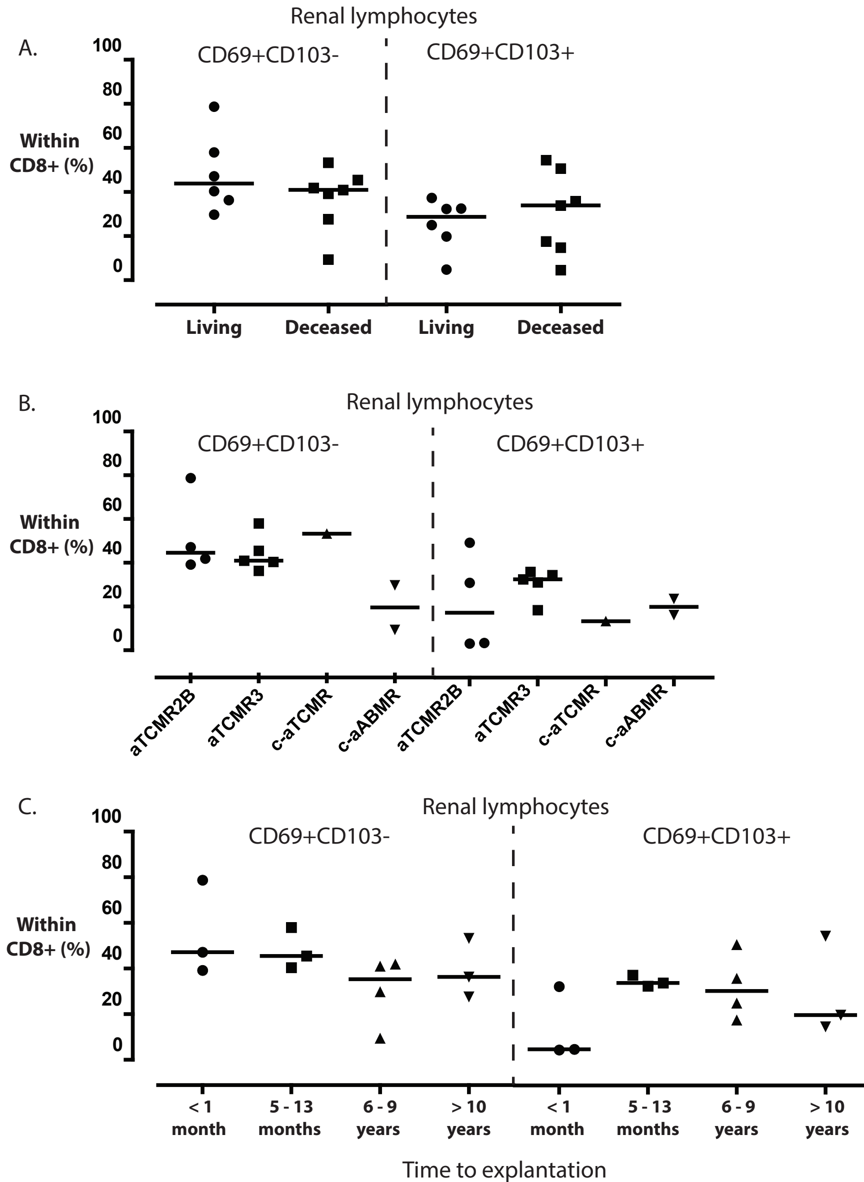
Supplemental figure 1. Renal lymphocytes do have specificity for donor cells

(A) Renal lymphocytes of transplant nephrectomy number three and four (Table 1, Fig. 1C) were labelled with CFSE and stimulated with only human culture medium (HCM), donor cells, 3rd party cells, or recipient PBMCs for seven days. Afterwards, the proportion of proliferating cells was measured by gating the CFSE negative T cell fraction. (B) Degranulation of the renal lymphocytes after seven days of stimulation with HCM, donor cells, 3rd party cells, or recipient PBMCs was measured by measuring the CD107a+ T cell fraction. (C) Numbers of IFNγ producing cells were measured with the ELISPOT assay after stimulating the renal lymphocytes with HCM, donor cells, 3rd party cells, or recipient PBMCs. (D) HLA-typing of the renal allograft recipients, donor and 3rd party cells used for the mixed lymphocyte reaction and ELISPOT assay. The number of renal lymphocytes of patient four was not sufficient to include a 3rd party control and to perform an ELISPOT assay.



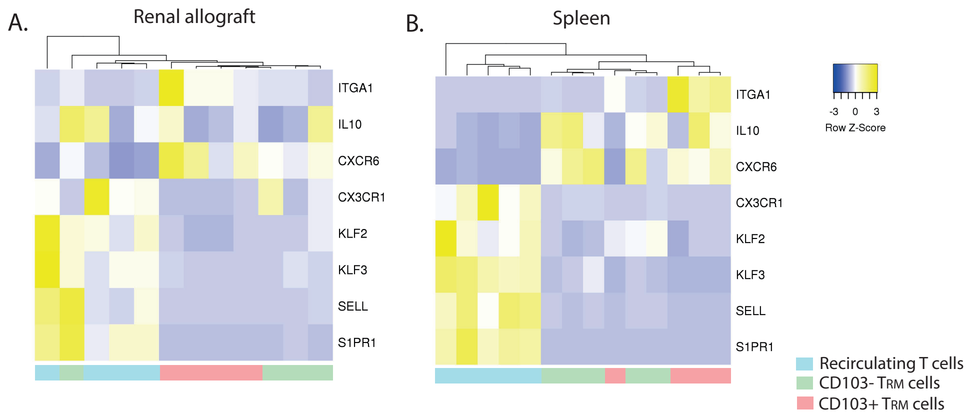
Supplemental figure 2. T_{RM} cells are present in two kidneys that were discarded for transplantation

Lymphocytes from two kidneys that were discarded for transplantation were stained with mAb against CD69 and CD103. Frequencies of CD69+ and CD103+ T cells within the CD4+ and CD8+ T cell compartment are depicted within the figure.



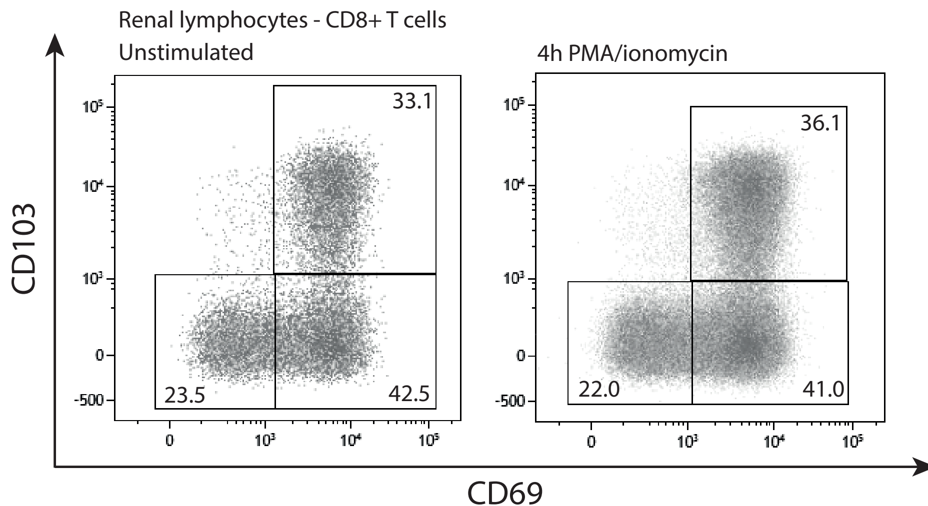
Supplemental figure 3. No significant differences in T_{RM} cell frequencies when subdividing on donor type, Banff 2017 category, and time to explantation.

We subdivided the CD103- and CD103+ T cells within the CD8+ compartment based of donor type (A), Banff 2017 category (B), and time to explantation (C). No significant differences in T_{RM} cell frequencies were detected between the different subgroups.



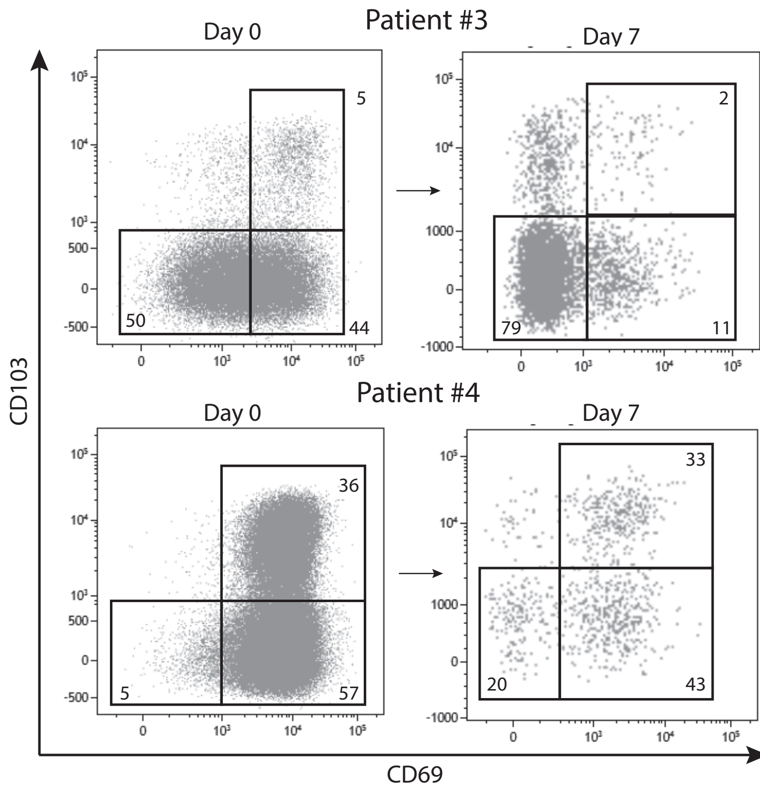
Supplemental figure 4. Clustered expression of T_{RM} signature genes

Heatmaps depicting the normalized gene expression of eight core genes in the renal allograft (A) and spleen (B). The genes depicted are known to be upregulated (yellow) or downregulated (blue) in T_{RM} cells. Gene expression was measured in recirculating T cells (blue line), CD103- T_{RM} cells (green line), and CD103+ T_{RM} cells (red line) within the CD8+ compartment. Spleen *n*=5, renal lymphocytes *n*=4. Average linkage method was used to cluster the different samples and the Euclidean method was used to calculate the distance between the different samples



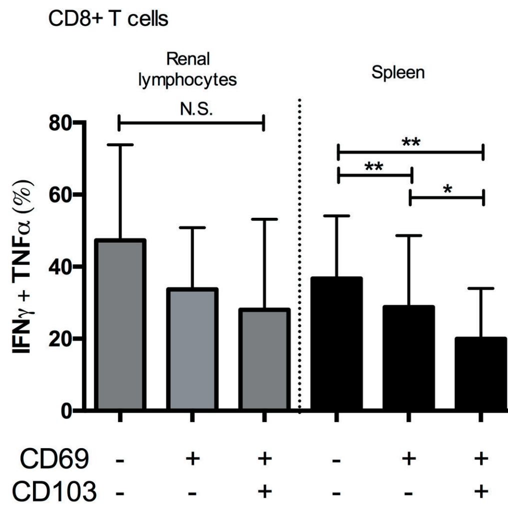
Supplemental figure 5. No difference in CD69 and CD103 expression before and after PMA/ ionomycin stimulation

Renal lymphocytes were stimulated for 4 hours with PMA/ionomycin in order to study cytokine producing capacities. Before and after stimulation the expression of CD69 and CD103 was measured with flow cytometry. Proportions of gated areas are depicted within the figure.



Supplemental figure 6. Decreased expression of CD69 and CD103 on renal CD8+ T cells after allostimulation

Renal lymphocytes of patient number three and four (Table 1) were stimulated with the corresponding donor cells for seven days. Subsequently, cells were stained for CD8+ T cells. Within the CD8+ T cells, proportions of CD69 and CD103 were measured. Numbers in the figure represent the proportions of the gated cells.



Supplemental figure 7. Proportions of different T_{RM} subsets that produce both IFNγ and TNFα

Proportions of IFNγ and TNFα producing cells are depicted upon 4 hours PMA/ionomycin stimulation in the presence of monensin and brefeldin A. Cytokine proportions were measured in renal lymphocytes and splenocytes within the recirculating T cells, CD103- T_{RM} cells, and CD103+ T_{RM} cells of the CD8+ T cell compartment. Frequencies of positive cells were shown as mean with the SEM (renal lymphocytes n = 6, spleen n = 10). Significant differences were calculated and depicted (N.S. = not significant, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$).

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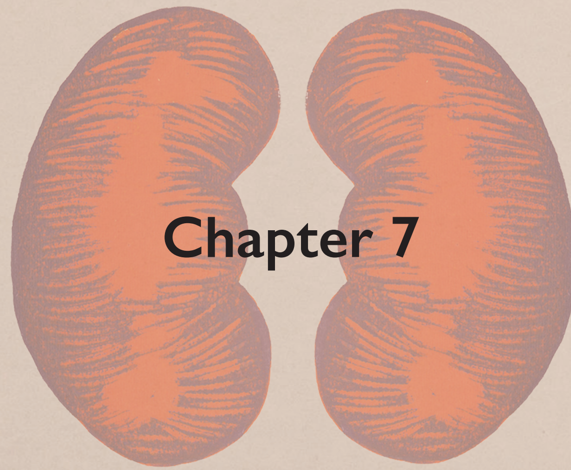

Chapter 7

Summary and general discussion

Summary

Kidney transplantation has become the treatment of choice for patients with end-stage renal disease (ESRD). Currently, the five-year survival time of recipients with a donor kidney is over 90%. Unfortunately, acute rejection is still a barrier on the short-term while chronic rejection is causing late graft failure (1, 2). At present, the cornerstone immunosuppressant after kidney transplantation is calcineurin inhibition by tacrolimus (3). The efficacy and safety of this treatment is well recognized. The ultimate goal in transplantation is achieving long-term graft and patient survival, both of which are currently negatively influenced by side effects of the given immunosuppressive drugs and by uncontrolled anti-donor responses in which T cells and B cells of the recipient play a crucial role (1, 4). Acute rejection is often dominated by T cell-mediated rejection (TCMR), in which effector helper CD4+ and cytotoxic CD8+ T cells infiltrate the transplanted kidney, are activated and express pro-inflammatory soluble factors such as interferon-gamma (IFNγ) and granzyme B (5). Importantly, alloantigen-challenged T cells provide help to antigen-activated B cells that in turn differentiate into immunoglobulin secreting plasma cells and are responsible for antibody-mediated rejection (ABMR) in which donor-specific antibodies (DSA) play an important role (6). The presence of DSA is associated with poor transplant outcomes. Eight to ten percent of the kidney transplant recipients develop *de novo* DSA within the first year, and 15% to 30% within 10 years after transplantation (7, 8). Another challenge is the short- and long-term toxicity profile of tacrolimus or other calcineurin inhibitor (CNI)-based treatments. Patients on CNI suffer from an increased risk for nephrotoxic, metabolic, and cardiovascular side effects (9-11). There is therefore an interest in finding new directions to intervene in the allogeneic response in a more safe and specific way. In this respect, T cells that support differentiation of alloantigen-activated B cells and the formation of DSA secreting plasma cells are of interest. So far, the precise functions of T cells within the alloimmune response are unknown. A better understanding about the features and the mechanisms by which alloreactive T and B cells mediate the anti-donor response may contribute to the development of new immunosuppressive strategies and the discovery of new biomarkers.

In this thesis, we aim to better understand the role T follicular helper (Tfh) cells and tissue-resident memory T (T_{RM}) cells in the process leading to and mediating alloimmunity. We characterized the mechanisms by which these effector memory T cell subsets modulate the alloimmune response *in vivo* and *in vitro* by following three complementary approaches. First, we determined the helper function of patient Tfh cells to B cells, whereby we focused on the actions of the pleiotropic cytokine IL-21. Apart from determining the fate of B cells this particular cytokine is also of importance for the function of cytolytic T cell responses. Second, we studied the contribution of IL-21 in the rejection process using an experimental

humanized mouse skin transplant model and third, we characterized T cell infiltrates in kidney biopsies and rejected kidneys. Insights into these immune mechanisms can help to fine-tune treatment strategies and design less toxic and more efficient immunosuppressive drugs, thereby increasing the quality of life of kidney transplant recipients. In **part I** of this thesis the role of Tfh cells and the cytokine IL-21 is studied in different allogeneic settings. **Part II** of this thesis focuses on the characterization and functional properties of ectopic lymphoid structures (ELSs) and T_{RM} cells in the kidney allograft.

Part I: The role of T follicular helper cells and IL-21 signaling in the alloimmune response

Tfh cells play a crucial role in T cell-dependent activation of B cells, in which naïve B cells differentiate into memory B cells and antibody producing plasma cells. In this way, Tfh cells contribute to the formation of DSA after transplantation, which are involved in the process of ABMR. In **Chapter 2**, we reviewed the current knowledge on the effects of immunosuppressive drugs on Tfh cell differentiation and function. With the existing evidence described in the literature we discuss new possible approaches to influence the functions of Tfh cells, which can yield new therapeutic targets in the transplantation field. Available evidence suggests that conventional immunosuppressive therapies, including tacrolimus, only partially block Tfh-cell differentiation and activity. We propose a model in which the IL-2 blocking compounds tacrolimus and basiliximab may influence the generation of Tfh cells through disturbing the balance between IL-2 and IL-21. Tfh-cell activity may be altered at different stages in the activation, effector and differentiation pathways, such as blocking co-stimulatory signaling, cytokine-dependent pathways, and migration-dependent pathways. Lastly, we speculate about the potential of Tfh cells to act as a pharmacodynamic biomarker to improve alloimmune-risk stratification. In this perspective, the measurement of donor-specific Tfh cell frequencies by donor-specific IL-21 ELISPOT assays might be a sensitive tool to identify patients at risk for rejection.

As described above, blockade of cytokine-dependent pathways is of interest to affect Tfh-cell activity. In **Chapter 3** we tested the role of IL-21R signaling in an allogeneic co-culture model. Through autocrine and paracrine mechanisms IL-21 is able to support Tfh-cell activation and subsequent Tfh-dependent differentiation of B cells. We used pre-transplantation patient peripheral blood mononuclear cells (PBMCs) and isolated pure Tfh cells and memory B cells. Subsequently, the Tfh and memory B cells were stimulated with corresponding donor antigen in the absence or presence of an IL-21R antagonistic antibody (αIL-21R). Stimulation of the Tfh cells and memory B cells resulted in an activated Tfh cell phenotype corresponding to Tfh2 and Tfh17 cells, as well as the induction of antigen-driven differentiation and class switch recombination (CSR) of memory B cells resulting in the

formation of IgM and IgG-producing plasmablasts. The formation of plasmablasts and IgM and IgG synthesis were significantly inhibited in the presence of the αIL-21R compound. Because we did not find evidence for effects of IL-21R blockade on the activation of the Tfh cells, we concluded that IL-21 produced by alloantigen-stimulated Tfh cells acted on B cells to support the formation of antibody-producing plasmablasts. Therefore, the IL-21R might be a potent target to prevent the generation of DSA.

To further investigate the role of IL-21 signaling in an *in vivo* model of alloreactivity we performed a study in a humanized skin transplant mouse model (**Chapter 4**). We transplanted immunodeficient Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice, which lack B, T and NK cells, with human skin and infused them with human allogeneic splenocytes. Control animals were treated with PBS, while the treatment group received the αIL-21R compound. In the control animals, STAT3 was phosphorylated downstream of the IL-21R and we observed a thickened epidermis, infiltration of T and B cells in the graft and increased expression of the inflammation marker keratin 17 (Ker17) and proliferation marker Ki67 in the epidermal cells. In contrast, in the αIL-21R-treated animals phosphorylation of STAT3 was efficiently blocked downstream of IL-21R and this resulted in inhibition of the epidermal thickening, reduced infiltration of T and B cells, and decreased expression of inflammation markers Ker17 and Ki67. This reduction in histological signs of rejection might be explained by the observed reduction in T and B cell reconstitution in the presence of αIL-21R.

Part II: Composition of lymphocytes within the renal allograft

After renal transplantation, recipient-derived lymphocytes have the capability to infiltrate the donor organ. These lymphocytes are directly located at the graft site and are therefore a cell population of high interest to study. Due to chronic persistence of the allograft the infiltrating lymphocytes may form highly organized ELSs, resembling germinal centers with B cells and follicular dendritic cells (FDCs) and surrounded by T cells. The exact function of these ELSs in the local tissue is largely obscure. In **Chapter 5** we therefore aimed to determine the presence and activation status of ELSs in three different types of acute renal allograft rejection. For this, paraffin sections of acute/active antibody-mediated rejection (a/aABMR), acute T cell-mediated rejection grade I (aTCMRI), and acute T cell-mediated rejection grade II (aTCMRII) biopsies were stained for different markers related to GC-features. Remarkably, in the aTCMRI rejection biopsies we detected B cell aggregates that were surrounded by T cells. These aggregates were also positive for FDCs, IgD, Ki67, Bcl6 and IL-21, which are all features of ELSs. In contrast, in the a/aABMR and aTCMRII biopsies we found diffuse infiltration of T and B cells. We speculate that the T cells found in the ELSs regulate B cell-mediated immunity during acute T cell-mediated rejection, which on the long-term may feed the process of chronic humoral rejection of the allograft.

Understanding the pathophysiology of rejection after kidney transplantation is of high interest. However, studies on T_{RM} cells in the kidney allograft and their local role in the process of rejection are currently lacking. In **Chapter 6**, results are presented on the origin and functional characteristics of T_{RM} cells in a set of transplant nephrectomy specimens. T cells with a tissue-resident memory phenotype and gene expression profile were detected in all transplant nephrectomies, with the highest proportions of T_{RM} cells within the CD8+ T cell compartment. Moreover, these CD8+ T_{RM} cells had strong immunostimulatory capacity measured by the production of pro-inflammatory cytokines IFNγ and TNFα, as well as granzyme B, but harbored low levels of intracellular perforin. Within the first months after transplantation, the donor-derived T cells were replaced by recipient-derived T cells. Remarkably, prolonged retention of CD103+ T_{RM} cells was detected, when compared with other donor-derived T cells. We are the first to describe the presence and effector phenotype of this recently discovered T cell population in the renal allograft.

Taken together, in this thesis we explored the functional characteristics of Tfh cells and T_{RM} cells within the alloimmune response. Our study demonstrates that Tfh cells and T_{RM} cells communicate to other immune competent cells to improve alloreactivity via among others the formation of DSA, cytotoxins such as granzyme B and perforin, and pro-inflammatory cytokines (**Table 1**). Modeling these cell-to-cell communication networks will help us to design less toxic and more efficient immunosuppressive treatment strategies.

Table 1.

Overall conclusions based on this thesis:
• The activation of Tfh cells by alloantigen does not rely on IL-21R signaling
• IL-21 is a non-redundant factor in alloantigen-driven B cell differentiation towards immunoglobulin producing plasmablasts
• Blockade of IL-21R signaling in a humanized mouse skin transplantation model prevents rejection of the human skin, but also effects reconstitution of the human T and B cells in this model
• Ectopic lymphoid structures that resemble activated germinal-center structures are predominantly found in aTCMRI renal allograft biopsies, with more diffuse patterns of infiltrating lymphocytes detected in aTCMRII and a/aABMR biopsies
• T cells with a tissue-resident memory cell-surface phenotype and gene expression profile are present in transplant nephrectomy specimens
• T_{RM} cells of donor-origin are completely replaced by their recipient counterparts within the first five months after transplantation

Tfh cell = T follicular helper cell; IL-21R = IL-21 receptor; aTCMRI = acute T-cell mediated rejection grade I; aTCMRII = acute T-cell mediated rejection grade II; a/aABMR = acute/active antibody-mediated rejection; T_{RM} = tissue-resident memory T cell

General Discussion

The main challenge after kidney transplantation is to establish long-term graft and patient survival without the occurrence of rejection and drug-related side effects. Despite the use of conventional immunosuppressive treatments, T-cell alloimmune responses are still a major problem after kidney transplantation. Activated CD4+ T cells are thought to feed the process of CD8+ T cell-mediated immune responses such as TCMR and B cell-mediated rejection leading to ABMR (12, 13). Therefore, it is of importance to understand the immunological mechanisms that are responsible for these anti-donor responses. In this thesis, we explored the role of two recently discovered T cell subsets; the Tfh cells and T_{RM} cells. We studied their role in alloreactivity by the characterization of the mechanisms by which these effector memory T cell populations modulate anti-donor responses after transplantation. This will help us to design more safe and efficient immunosuppressive treatment strategies.

The role of Tfh cells in the alloimmune response

Formation of DSA requires the interaction between Tfh cells and B cells (14-16). Consequently, an intriguing question is how to interfere with this Tfh-B cell interaction in order to diminish the formation of *de novo* DSA. As described in **Chapter 2**, Tfh cell function can be inhibited in different ways via blockade of for instance co-stimulatory signaling, cytokine-dependent pathways, or Tfh cell migration-dependent pathways. In this thesis, we demonstrate that IL-21 production by activated Tfh cells controls the differentiation of alloantigen-activated B cells towards antibody producing plasmablasts (**Chapter 3**). This was not dependent on the autocrine effect of IL-21. Moreover, in the presence of an anti-IL-21R mAb the expression of Tfh cell activation markers ICOS and PD-1 after alloantigen stimulation was upregulated. This is in line with other studies that prove the redundant effect of IL-21 on maintenance of the Tfh cell phenotype. These studies report that only combined blockade of IL-21 and IL-6 is effective in reducing Tfh cell frequencies (17, 18). This combined IL-21 and IL-6 blocking therapy was also proven successful in the suppression of disease onset in a collagen-induced arthritis mouse model and might be an interesting approach to study in an allogeneic setting (19). One could question whether complete blockade of Tfh cell activation is truly necessary, since reduction of the end product, the DSA in organ transplantation, is the ultimate goal. To this end, interfering with Tfh cell function by blocking IL-21R signaling prevents the formation of *de novo* DSA. However, pre-formed circulating DSA and long-living plasma cells formed during previous sensitization can still exert their destructive effects during IL-21R blockade. These cells may be neutralized via other interventions, such as the use of the protease inhibitor bortezomib (20).

When we stimulated patient Tfh and memory B cells with corresponding donor antigen, we detected a high inter-individual variation in the proportions of plasmablasts that were formed after 8 days of stimulation (**Chapter 3**). No significant differences in patient baseline characteristics were detected between the co-cultures with high and low plasmablast numbers. Therefore, it is likely that the observed differences in plasmablast formation reflected the natural variation among patients, established for instance via cross-reactivity, differences in IL-21 levels, and distinct expression rates of co-stimulatory surface markers. In the last decade, a regulatory counterpart of the Tfh cell has been described, the T follicular regulatory (Tfr) cell. These Tfr cells have the ability to enter B-cell follicles and temper the production of antibodies. Tfr cells are characterized by the expression of Bcl6 alongside Foxp3 and other Tfh markers such as CXCR5, PD-1 and ICOS (21-23). In our co-culture model, we used total CD4+CXCR5+ Tfh cells. Within this T cell population we did not determine the ratio between Tfr and Tfh cells. Fluctuations in Tfr:Tfh ratio might also explain the variable numbers of plasmablasts formed after our Tfh-B cell co-cultures. Moreover, during an immune response the Tfh and Tfr cells both start to proliferate, but the Tfh cells proliferate faster and skew towards a pro-inflammatory response (24). When the antigen is cleared Tfh cell numbers start to drop again, while the Tfr cells continue proliferating and thus the Tfr:Tfh ratio is increasing and returns to a resting state (25). In tissues with continuous antigen exposure the Tfr:Tfh ratio is constantly low. This low Tfr:Tfh ratio is for example detected in Peyer patches in which constitutive IgA production occurs, and in the spleen where ongoing fast responses towards blood born antigens are needed (26, 27). Interestingly, in a pilot experiment in which we quantified the Tfr:Tfh ratios in age and gender-matched PBMCs of healthy individuals, PBMCs of patients pre-kidney transplantation, and PBMCs of patients six months post kidney transplantation a significantly lower Tfr:Tfh ratio was detected in the PBMCs of patients six months post transplantation compared with PBMCs of patients prior to transplantation (**Figure 1**). This might be explained by the constant threat of donor antigen that is skewing the Tfr:Tfh balance in favor of helper capacity. Overall, future research should aim to clarify whether the balance between Tfr and Tfh cells is affecting the process of B cell differentiation and formation of anti-donor antibodies and if this Tfr:Tfh balance can be shifted by blocking IL-21R signaling.

The formation of Tfh cells is highly dependent on nuclear factor of activated T-cells (NFAT) signaling (28, 29). Activated calcineurin promotes NFAT dephosphorylation followed by translocation of NFAT to the nucleus (30). The use of CNI-based treatment during transplantation inhibits the calcineurin-dependent activation of NFAT and thus in theory the formation of Tfh cells as well. In contrast, previous studies describe that high levels of IL-2, of which the expression is dependent on NFAT signaling, limit the differentiation of Tfh cells (31-33). A recent study by Wallin *et al.* shows that tacrolimus specifically inhibits lymph

node and circulating Tfh cells (34). The production of IL-2 promotes Blimp-1 expression, which subsequently prevents upregulation of the Tfh master transcription factor Bcl6. IL-2 production and Blimp-1 expression are inhibited during CNI treatment, followed by enhanced expression of Bcl6, which is in favor of Tfh cell development. These contradicting theories on the effect of CNI-based treatment on Tfh differentiation urge to further study this phenomenon to be able to better manipulate the formation of donor-specific Tfh cells, ultimately leading to a decrease in DSA formation.

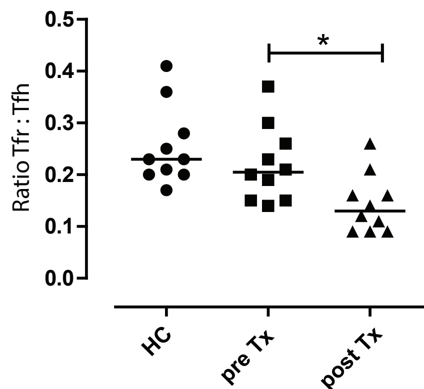

Figure 1. Tfr:Tfh ratio in PBMCs is significantly reduced in patients at six months after transplantation.

PBMCs of healthy controls (HC), patients prior to kidney transplantation (pre Tx), and patients six months post kidney transplantation (post Tx) were isolated and directly stained with CD4, CXCR5 and Foxp3. The ratio of CD4+ CXCR5+ Foxp3+ T follicular regulatory (Tfr) cells to CD4+ CXCR5+ T follicular helper (Tfh) cells was determined. Tfr:Tfh ratios are presented as individual ratios with medians. n=10 for each group; *p<0.05.

Optimizing current immunosuppressive treatment: a role for IL-21R blockade?

The pleiotropic cytokine IL-21 orchestrates the differentiation of multiple CD4+ and CD8+ T cell subsets (35). In this thesis we studied the role of IL-21R signaling in both *in vitro* and *in vivo* transplantation models. As described above, in **Chapter 3** we found a non-redundant role for IL-21 in Tfh-dependent differentiation of alloantigen activated B cells towards immunoglobulin producing plasmablasts. However, this *in vitro* model is designed to study the Tfh-B cell interaction after an alloantigen response. The effects of IL-21R blockade are complex and may depend on the experimental system used (35). Therefore, we set up a humanized mouse skin transplantation model with both human T and B cell reconstitution to explore the effect of IL-21R signaling blockade in an *in vivo* transplantation setting (**Chapter 4**). This humanized mouse model forms a valuable preclinical tool to study human-specific reagents. We demonstrated that blockade of IL-21R signaling inhibits the

alloimmune response and inflammation of the human skin transplant, but also influences T and B cell engraftment in the Balb/c IL2rγ$^{-/-}$ Rag2$^{-/-}$ mice. From these data, it is evident that IL-21R signaling is involved in the homeostatic proliferation of T and B cells, consistent with previous studies that implicated IL-21 in homeostatic T and B cell proliferation, especially of the CD8+ T cell subset (36, 37). And thus, this *in vivo* study provides evidence that IL-21 exerts effects on both the cellular and humoral arm of the immune system. A technical obstacle of the humanized skin transplant mouse model is the long engraftment time of B cells, which can last five to six months from the moment of cellular infusion (38). This long B cell maturation time is not feasible in this model since after three months the risk of graft-versus-host disease caused by the human T cells that recognize mouse antigen is rapidly increasing (39). An alternative *in vivo* transplant model that bypasses the human lymphocyte engraftment might be skin transplantation on CD19-hBtk mice. These transgenic mice have increased expression of Bruton's tyrosine kinase (Btk) within the B cell compartment, resulting in spontaneous germinal center formation and elevated levels of Tfh cells (40). Since IL-21R displays a high degree of homology compared to mouse IL-21R, transplantation of skin derived from C57BL/6 wild type mouse on CD19-hBtk mice in the presence and absence of the anti-IL-21R (αIL-21R) compound is specifically of interest to study the effect of IL-21R signaling within the Tfh-B cell interaction in an *in vivo* allogeneic setting (41). Overall, additional *in vivo* transplant studies are needed that shed light on the exact mechanisms by which IL-21 is contributing to the process of the alloimmune response, preferably *in vivo* models with a constituted immune system.

IL-21 plays a role in the communication between T and B cells in the allograft during rejection, as we found that IL-21+ T cells were present in organized T- and B-cell aggregates, also known as ectopic lymphoid structures (ELSs). We predominantly detected ELSs in biopsies diagnosed with aTCMRI, in contrast to aTCMRII and a/aABMR rejection biopsies (**Chapter 5**). In these ELSs, both IL-21 single positive and IL-21 Bcl6 double positive cells were detected. The presence of IL-21^{+}Bcl6^{+} cells in combination with Ki67^{+} cells implies the presence of activated Tfh cells that promote a germinal center-like response. However, all aTCMRI cases were negative for C4d and DSA, which suggests a role for IL-21 in the IFNγ dependent cytotoxic activity of CD8+ effector T cells (42). Indeed, other studies present a role for IL-21 in sustaining CD8+ T cell responses during chronic infection, which may also occur in the graft at the time of an ongoing alloimmune response. In these studies, IL-21 directly interacts with CD8+ T cells to maintain the cytotoxic response and to limit exhaustion of the cells during chronic LCMV infection (43, 44). The IL-21 positive cells that we detected in the ELSs may also resemble Th17 cells, which are known to secrete IL-21 and to be involved in the formation of these lymphoid structures (45, 46).

As described above, IL-21 is taking a center stage during various effector immune processes after organ transplantation. In addition, our work shows that this broad role of IL-21 is exerted by peripheral T cells, as well as T cells that infiltrated the renal allograft. So far, only a phase I trial in healthy volunteers was conducted to assess the safety of the same IL-21-receptor antagonist (αIL-21R) as was used in our study. However, 76% of the healthy volunteers that received the αIL-21R compound developed anti-drug antibodies, which resulted in rapid clearance and thus a low bioavailability of the compound (47, 48). In the autoimmune field, phase I and II trials have been performed to study the role of IL-21-specific antibodies in rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus. However, the results of these trials have not been published so far. Clearly, further studies are necessary to identify an IL-21R antagonist or an anti-IL-21 antibody with high efficacy and safety. Ultimately, because IL-21R blockade affects the functions of various T and B cell populations this intervention might be of specific interest as a form of induction therapy. Especially for sensitized patients, induction therapy with an IL-21R antagonist may hamper the formation of donor-specific Tfh cells that are contributing to the formation of *de novo* DSA.

Lymphocytes infiltrating the renal allograft: friends or foes?

When studying T cell communication with other cell types in transplantation it is of high importance to consider the context. Peripheral T cells mainly bear a resting naïve or memory phenotype, in contrast to the cells that migrate to the lymphoid tissues where activation of the T cells takes place. In addition, inflammatory mediators produced by local stromal cells are able to control recruitment, activation and survival of lymphocytes locally in primary tissues (49). Despite the use of immunosuppressive therapies, T and B cells are able to infiltrate the kidney allograft. Moreover, organized lymphocyte structures have been detected within rejection biopsies of kidney allografts (50). Remarkably, we predominantly detected ELSs with an activated phenotype in rejection biopsies diagnosed with aTCMRI, while more diffuse T- and B-cell structures were detected in most of the aTCMRII and a/aABMR biopsies, with no FDC networks, IgD and IL-21^{+}Bcl6^{+} cells. This indicates that there may be more cells involved during an aTCMRI rejection on top of the infiltration of classical CD8+ T cells. The findings in **Chapter 5** argue that ELSs are present in acute responses in which T cells are dominating. However, the function of the ELSs in the aTCMRI rejection biopsies is not fully elucidated, with contradicting evidence in literature that these structures have both protective and destructive immune properties (51-53). For this reason, it would be of high interest to study the inflammatory patterns of the ELSs which we detected in the aTCMRI rejection biopsies in more depth. The nCounter Analysis System (NanoString Technologies, Seattle, WA) platform for multiplex analysis of RNA expression on formalin-fixed paraffin-embedded (FFPE) tissue might be an interesting approach to further study the ELSs in aTCMRI. The advantage of this technique is the direct and sensitive measurement

of mRNA expression in the absence of enzymatic or signal amplification (54). This technique has been applied to identify gene expression heterogeneity in different rejection types in a recent publication by Smith *et al.* (55). Overall, we plea that TCMR is ultimately leading to a chronic antibody-mediated rejection, which is also described by Loupy *et al.* and Halloran *et al.* (12, 13).

One particular subset of T cells infiltrating the non-lymphoid tissues has been acknowledged over the past decade: the tissue-resident memory T (T_{RM}) cells. This subset of T cells rapidly responds to local antigens and forms a first line of defense against re-circulating pathogens (56). In this thesis, we identified T_{RM} cells with an effector phenotype in transplant nephrectomies (**Chapter 6**). When we stimulated the total population of renal lymphocytes with corresponding donor cells, these cells were able to mount an alloreactive response. We also found that donor-derived T cells are replaced by recipient-derived T cells in the first months after kidney transplantation. The retention of donor-derived cells might have a protective role in the renal allograft, as observed in other studies (57, 58). Moreover, in visceral transplants, donor-derived T cells with graft-specific TCR clones are thought to decrease the constant threat of recipient-derived T cells, thus slowing down the *in situ* development of rejection (59). It is of high interest to study the exact function of the donor- and recipient-derived T_{RM} cells in the renal allograft. When we identify the balance between protective and destructive T_{RM} cells we will be able to better tailor immunosuppressive strategies. Assuming that recipient-derived T_{RM} cells contribute to the alloimmune response, strategies to diminish lymphocyte repopulation within the renal allograft are of interest. The immunomodulating drug FTY720 is an antagonist for the sphingosine-1-phosphate receptor (S1PR) and able to block T cell homing to the tissue. In this respect the results of phase I and II trials in multiple sclerosis patients are promising, however, after renal transplantation FTY720 has limited effects on migration profiles of T cells and subsequently did not decrease acute rejection incidence in combination with conventional immunosuppressants (60-62). A recent study by Tian *et al.* showed that IL-21R$^{-/-}$ mice had lower levels of T_{RM} cells in the small intestine. The remaining CD8+ T cells in these IL-21R$^{-/-}$ mice still expressed the typical CD69+CD103+ T_{RM} cell phenotype but the levels of granzyme B produced by these cells are lower compared to the IL-21R$^{+/+}$ mice (36). Overall, future studies need to clarify via which mechanisms we will be able to tailor the migration of T_{RM} cells in favor of the donor organ.

T cell communication in organ transplantation: what do we have to take into account?

The findings described in this thesis provide new perspectives in the mechanisms by which Tfh cells and T_{RM} cells modulate an alloimmune response after kidney transplantation as summarized in **Table 1**. Both cell types have an effector memory phenotype and are able

to mount an alloreactive response in which the function of IL-21 is involved. Moreover, these IL-21 driven T cells are active in the kidney allograft where they directly contribute to the process of rejection via the communication to CD8+ cytotoxic T cells and B cells. Furthermore, we found a broad effect of IL-21R blockade in our *in vitro* and *in vivo* transplant models all pointing to the same direction. Namely, blockade of IL-21R signaling has inhibiting effects on both the humoral and cellular arm of the alloimmune response. A next step is to translate the knowledge obtained in this thesis to the clinical setting. To this end, a randomized controlled trial with IL-21R antagonistic antibodies is needed that ultimately leads to new strategies to improve short- and long-term outcomes after kidney transplantation. This randomized controlled trial should be designed to treat an ongoing rejection episode or prevent the rejection by induction therapy to hamper the function of IL-21 responsive T and B cells. Moreover, the presence of potentially harmful Tfh cells and T_{RM} cells during a rejection episode might be an indication to use an IL-21R antagonistic antibody to reduce the activity of these cells. Another interesting future approach is to measure the pre-transplantation IL-21-producing memory T cells with specificity for the donor that might form a predictive marker to identify patients at risk for rejection. Currently, no routine tests are performed to measure pre-transplant-donor-specific T cells. It is tempting to speculate that patients with high numbers of IL-21+ T cells directed toward donor antigens should receive IL-21R antagonistic antibodies as a desensitization therapy. The findings described in this thesis might be used to also move away from a 'one size fits all' approach for the use of immunosuppressive treatment and care of patients after kidney transplantation. The outstanding questions for future research are summarized in **Table 2**.

Overall, in this thesis we functionally characterized Tfh cells and T_{RM} cells to gain knowledge about their role in alloreactivity after organ transplantation in patients. Our studies show that these T cell subsets communicate to other immune competent cells to enhance the alloimmune response via the formation of DSA, pro-inflammatory cytokines, and cytotoxins such as granzyme B and perforin. Modeling cell-to-cell communication networks of the T cells might help us to improve immunosuppressive treatment strategies, ultimately leading to enhanced kidney transplant outcomes.

Table 2.

Outstanding questions for future research
Fundamental questions:
• What is the balance between Tfh cells and Tfr cells during an allogeneic response and are we able to modify this balance via the use of immunosuppressive drugs?
• What are the exact mechanisms by which current maintenance immunosuppressive drugs alter the function of Tfh cells?
• What is the number of alloantigen-specific T_{RM} cells in the renal allograft after transplantation compared to the number of T_{RM} cells with antigen specificity for viral and bacterial pathogens in a resting state and during a rejection episode?
• Are the donor-derived T_{RM} cells within the renal allograft slowing down the threat of recipient-derived T_{RM} cells that are involved in the process of rejection?
New experimental approaches:
• Is the CD19-hBtk skin transplant mouse model a suitable *in vivo* model to study the impact of IL-21 signaling on the T-B cell interaction during an allogeneic response?
• Can we identify the functional characteristics of ELSs in the aTCMRI biopsies by NanoString RNA expression analysis on FFPE tissue?
New treatment strategies:
• Are we able to specifically target the donor specific T_{RM} cells and spare the recipient-derived T_{RM} cells that are specific for viral or bacterial pathogens?

Tfh cell = T follicular helper cell; Tfr cell = T follicular regulatory cell; TRM cell = tissue-resident memory T cell; ELSs = ectopic lymphoid structures; aTCMRI = acute T-cell mediated rejection grade I; FFPE = formalin-fixed paraffin-embedded

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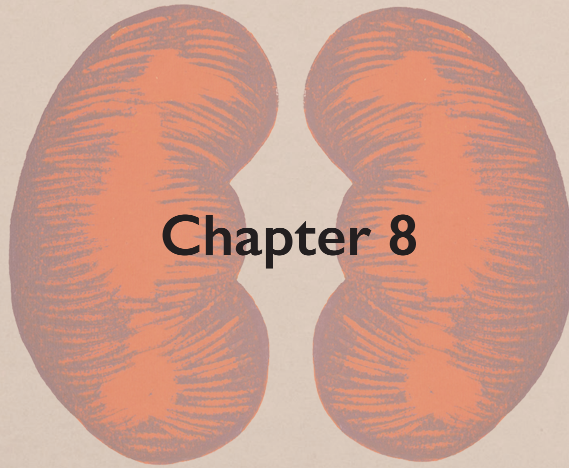


Chapter 8

Nederlandse samenvatting

Nederlandse samenvatting

Het immuunsysteem beschermt het lichaam tegen het binnendringen van ziekteverwekkers zoals bacteriën en virussen. Het is continu actief om deze ziektekiemen te herkennen en uit te schakelen. De T en B lymfocyten vormen hierbij belangrijke spelers binnen het immuunsysteem. Er bestaan verschillende subtypen T cellen, die via de T-cel receptor (TCR) op hun celoppervlak lichaamsvreemde eiwitten (antigenen) zeer specifiek kunnen herkennen. De belangrijkste typen zijn de CD4+ T helper cellen en de CD8+ cytotoxische T cellen. De CD4+ T helper cellen worden gekenmerkt door hun 'helper' activiteit waarmee ze cytotoxische CD8+ T cellen en B cellen kunnen activeren. De B cellen kunnen ook specifiek antigenen herkennen en na activatie zich ontwikkelen tot plasmacellen, die verantwoordelijk zijn voor de productie van antistoffen (immuunglobulines). In deze activatie processen is - naast de specifieke antigen binding via de TCR - ook ligand binding van een co-receptor (co-stimulatie) en de uitscheiding van signaalstoffen (cytokines) van essentieel belang. CD8+ cytotoxische T cellen produceren naast cytokines ook toxische moleculen zoals granzyme B en perforine die met ziektekiemen besmette cellen kunnen uitschakelen. De T folliculaire helper (Tfh) cellen zijn een subtype CD4+ T cellen die specifiek betrokken zijn bij het activeren van B cellen in de lymfeklieren. Door middel van verschillende signalen, waaronder cel-cel contact (via CD40 ligand op de T cel en CD40 op de B cel) en uitscheiding van het cytokine IL-21, stimuleren de Tfh cellen de B cel differentiatie naar antilichaam-producerende plasmacellen. Het cytokine IL-21 is daarnaast ook betrokken bij de ontwikkeling van CD8+ cytotoxische T cellen. De T en B cellen bevinden zich in de bloedbaan en de lymfeklieren en migreren na activatie naar organen zoals de darmen, longen, en nieren. Een recent ontdekt type T cellen dat zich nestelt in organen zijn de zogenoemde tissue-resident memory T (T_{RM}) cellen, die niet zozeer circuleren maar zich in de weefsels vestigen. Deze T_{RM} cellen kunnen een snelle respons genereren nadat ze lokaal in contact komen met ziektekiemen.

Zoals hierboven beschreven vormt het immuunsysteem de essentiële verdedigingslinie tegen ziekteverwekkers. Echter, het immuunsysteem zal ook worden geactiveerd als het na orgaantransplantatie het donororgaan herkent als lichaamsvreemd. Indien de donor niet genetisch identiek is aan de ontvanger, zal dit in principe leiden tot afstoting van het donororgaan. Dit wordt ook wel een allogene respons genoemd.

Een niertransplantatie levert de beste uitkomst voor patiënten met eindstadium nierfalen. Na de niertransplantatie zijn patiënten niet meer afhankelijk van dialyse waardoor hun kwaliteit van leven en de levensverwachting sterk verbetert. Ondanks het gebruik van immuunonderdrukkende medicatie kan afstoting van de donornier optreden. Dit afstotingsproces kent verschillende vormen. Kort na transplantatie is het meest voorkomende

type afstoting de T-cel gemedieerde afstoting (TCMR) waarbij voornamelijk CD8+ cytotoxische T cellen de donornier aanvallen. De activatie van T cellen na niertransplantatie zorgt er ook voor dat B cellen geactiveerd worden. Activatie van de B cel vindt plaats door antigeen herkenning via de B-cel receptor, waardoor een cyclus van B-T cel interacties ontstaat. De geactiveerde B cellen differentiëren vervolgens naar plasmacellen die donor-specifieke antilichamen produceren, hetgeen leidt tot een antilichaam gemedieerde afstoting (ABMR). Om het aantal afstotingen na niertransplantatie te verminderen is het essentieel om de huidige immuunonderdrukkende medicatie te verbeteren. Wanneer we de processen van de T cellen en B cellen die betrokken zijn bij het afstotingsproces beter begrijpen, zullen we in staat zijn therapieën te ontwikkelen die het immuunsysteem op een efficiëntere manier onderdrukken en tevens een lager bijwerkingen profiel hebben. Tezamen geeft dit een langere halfwaardetijd van het getransplanteerde orgaan en een langere overleving met een betere kwaliteit van leven voor de patiënt.

Het doel van dit proefschrift is om de functie van twee recent ontdekte subtypen T cellen - de Tfh cel en de T_{RM} cel - in het proces van een donor-specifieke immuunrespons beter te begrijpen. Een verbeterd inzicht in de werking van deze twee typen T cellen in het proces van donororgaan afstoting zal bijdragen aan een verbeterde afstemming van immuunonderdrukkende geneesmiddelen en het ontwikkelen van geneesmiddelen met minder bijwerkingen en een efficiëntere werking. Dit proefschrift bestaat uit twee delen. In **Deel I** wordt de rol van de IL-21 producerende Tfh cellen beschreven aan de hand van studies in verschillende allogene modellen. **Deel II** richt zich op de werking van ectopische lymfoïde structuren en T_{RM} cellen die zich in de donornier bevinden.

In **hoofdstuk 2** wordt de huidige kennis van immuunonderdrukkende medicatie op de ontwikkeling en werking van Tfh cellen uiteengezet. Op basis van deze kennis bespreken we mogelijke nieuwe strategieën waarbij de functie van Tfh cellen beïnvloed kan worden. Deze strategieën dragen bij aan de ontwikkeling van betere en meer specifieke immuunonderdrukkende geneesmiddelen. De activiteit van Tfh cellen kan op verschillende punten beïnvloed worden, zoals door het blokkeren van co-stimulatie signalen, cytokine signalen, of signalen die de migratie van de Tfh cellen naar de lymfeklieren beïnvloeden. Daarnaast hebben we op basis van de bestaande literatuur de mogelijkheden in kaart gebracht om de Tfh cel te gebruiken als biomarker om een immuunrespons tegen het donororgaan te voorspellen.

In **hoofdstuk 3** wordt de rol van het cytokine IL-21 binnen de interactie tussen Tfh cellen en B cellen bestudeerd in een allogeen kweekmodel. Hierbij hebben we gebruik gemaakt van geïsoleerde Tfh cellen en B cellen van niertransplantatie patiënten. Deze Tfh cellen en

B cellen zijn gestimuleerd met de cellen van de donor zowel in de aan- als afwezigheid van een antagonistisch antilichaam dat de IL-21 receptor blokkeert. Hieruit bleek dat Tfh cellen in combinatie met de allogene stimulus de B cellen konden laten differentiëren naar immunoglobuline producerende plasmablasten. In de aanwezigheid van het anti-IL-21 receptor antilichaam werden de plasmablastformatie en immunoglobulineformatie significant geremd. Het remmen van de IL-21 receptor signalering had geen effect op de activatiestatus van de Tfh cellen. We kunnen hieruit concluderen dat het blokkeren van IL-21 signalering een potentiële manier is om na transplantatie de formatie van donor-specifieke antilichamen te remmen.

Om de rol van het cytokine IL-21 binnen de alloimmuunrespons verder te bestuderen wordt in **hoofdstuk 4** gebruik gemaakt van een gehumaniseerd transplantatiemodel. In dit model zijn immuundeficiëntie muizen getransplanteerd met een stukje menselijke huid. Vervolgens zijn deze muizen geïnjecteerd met lymfocyten van een ander individu. De muis is in dit model dus de drager van een humane anti-donor immuunrespons. De controle dieren werden behandeld met fysiologisch zout terwijl bij de behandelde groep een blokkerend anti-IL-21 receptor antilichaam werd toegediend. In de controle dieren was de humane huid sterk verdikt en was er infiltratie van T en B cellen in de huid zichtbaar. Ook was de huid van de controle dieren ontstoken en konden we aantonen dat de expressie van de ontstekingsmarkers keratine 17 en Ki67 verhoogd was. Daarentegen bleek dat in de behandelde dieren de humane huid niet verdikt was, er nauwelijks T en B cellen geïnfiltreerd waren in de humane huid, en de ontstekingsmarkers keratine 17 en Ki67 nauwelijks tot expressie kwamen. Deze verminderde ontsteking van de huid in de behandelde dieren kan deels verklaard worden doordat het nestelen van de humane lymfocyten in de muis ook verstoord werd door de aanwezigheid van het blokkerende anti-IL-21 receptor antilichaam.

Deel II van het proefschrift richt zich op de werking van ectopische lymfoïde structuren (ELS) en T_{RM} cellen die zich in de donornier bevinden. ELS worden gedefinieerd als georganiseerde structuren van immuuncellen, die veel overeenkomsten vertonen met lymfeklieren, maar die zich in weefsels bevinden waar zich een immuunreactie afspeelt. Na een niertransplantatie wordt het lichaam van de ontvanger continu blootgesteld aan de lichaamsvreemde donornier. Ondanks het gebruik van immuunonderdrukkende medicatie zijn de lymfocyten van de ontvanger in staat om naar de donornier te migreren waar ze zich nestelen en organiseren tot ELS. De rol van ELS formatie in de donornier is niet geheel bekend.

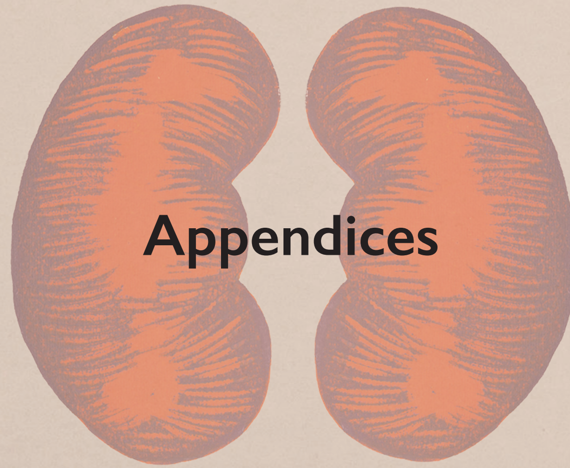
In **hoofdstuk 5** wordt de aanwezigheid en activatie status van ELS in nierbiopten van drie verschillende typen acute afstoting onderzocht. Hierbij hebben we gebruik gemaakt van

paraffine coupes van acute/actieve antilichaam-gemedieerde rejectie (a/aABMR), acute T-cel gemedieerde rejectie type I (aTCMRI) en acute T-cel gemedieerde rejectie type II (aTCMRII). Deze weefselcoupes van nierbiopten zijn gekleurd voor verschillende markers gerelateerd aan ELS formatie. Hieruit bleek dat voornamelijk in de aTCMRI biopten cel aggregaten aanwezig waren bestaande uit B cellen omringd door T cellen. Deze T-B cel aggregaten waren ook positief voor verschillende ELS markers. In tegenstelling hiermee werd in de a/aABMR en aTCMRII biopten een diffuser beeld gedetecteerd van geïnfiltreerde T en B cellen, in de afwezigheid van ELS formatie. We speculeren dat T cellen in ELS de aanwezige B cellen kunnen stimuleren tijdens een acute T-cel gemedieerde rejectie. Dit proces van T-B cel interactie kan op lange termijn leiden tot een chronische antilichaam gemedieerde afstoting tegen de donornier.

In **Hoofdstuk 6** wordt de lokale rol van T_{RM} cellen in de donornier onderzocht. De T_{RM} cellen nestelen zich in de organen waar ze vanwege hun memory status lokaal een snelle immuunrespons kunnen genereren. Voor deze studie is gebruik gemaakt van donornieren die vanwege eindstadium afstoting verwijderd zijn, ook wel explantaatnieren genoemd. In alle explantaatnieren waren T cellen aanwezig met het T_{RM} fenotype en genexpressie profiel, met de hoogste frequentie T_{RM} cellen binnen het CD8+ T cel compartiment. Deze CD8+ T_{RM} cellen hadden de capaciteit om de pro-inflammatoire cytokines IFNγ en TNFα te produceren, waren positief voor granzyme B en zwak positief voor perforine. De aanwezige T cellen in de explantaatnieren kunnen in principe zowel van de donor als van de ontvanger afkomstig zijn. Indien zowel donor als ontvanger cellen aanwezig zijn spreekt men van chimerisme. Op basis van analyse van de explantaatnieren in deze studie kan geconcludeerd worden dat in de donornier binnen de eerste vijf maanden na transplantatie de T cellen afkomstig van de donor worden vervangen door T cellen afkomstig van de ontvanger. Dit chimerisme is dus van korte duur. Een interessante bevinding was dat de T_{RM} cellen afkomstig van de donor in vergelijking met de overige donor-T cellen langer in het donororgaan konden verblijven.

Concluderend, in dit proefschrift worden verschillende nieuwe manieren beschreven waarop Tfh cellen en T_{RM} cellen een bijdrage leveren aan de anti-donor respons na niertransplantatie. Beide typen T cellen hebben een effector memory fenotype en zijn in staat om een alloimmuunrespons te initiëren. Ook wordt beschreven dat IL-21 gestuurde T cellen lokaal actief zijn in de donornier, waar ze direct bijdragen aan het afstotingsproces door te communiceren met CD8+ cytotoxische T cellen en met B cellen. Met een blokkerend anti-IL-21R antilichaam werd bevestigd dat de functie van allogeen geactiveerde T en B cellen onderdrukt werd in de afwezigheid van IL-21R signalering. Een volgende stap is om de uitkomsten beschreven in dit proefschrift te vertalen naar een klinische setting. Een

klinische trial waarbij de effectiviteit van een anti-IL-21R antilichaam wordt onderzocht zou hierbij een goed vervolg kunnen zijn. Deze zou uiteindelijk een belangrijke bijdrage kunnen leveren aan nieuwe therapieën om de korte en lange termijn uitkomsten na niertransplantatie te verbeteren. Patiënten waarbij voorafgaande aan de transplantatie hoge waarden IL-21 positieve donor-specifieke T cellen circuleren zouden als inductie therapie behandeld kunnen worden met een anti-IL-21R antilichaam. Ook patiënten die ten tijde van afstoting hoge Tfh cel aantallen hebben zouden baat kunnen hebben bij een IL-21R blokkerende therapie die de activiteit van deze agressieve cellen vermindert. Samenvattend zouden de bevindingen uit dit proefschrift gebruikt kunnen worden om in de toekomst patiënten op een meer persoonsgerichte manier te behandelen die past bij het specifieke type afstoting dat zij ondergaan. Hierbij is het in kaart brengen van cel-cel communicatie tussen verschillende type T cellen en andere immuuncellen van groot belang om nieuwe geneesmiddelen te ontwikkelen die uiteindelijk zullen leiden tot verbeterde uitkomsten na niertransplantatie.



Appendices

Curriculum Vitae
PhD portfolio
List of publications
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Curriculum Vitae

Kitty de Leur was born on the 30th of October 1988 in The Hague, the Netherlands. In 2007 she completed her secondary education at the Dalton Den Haag. In the same year she started with the bachelor Biomedical Sciences at Utrecht University. During her bachelor degree she went on a half-year exchange to follow courses at Lund University in Sweden. Afterwards, she enrolled in the master program Infection and Immunity at Utrecht University. As part of this program she did her nine-month internship at the Laboratory of Translational Immunology at the UMC Utrecht under the supervision of dr. Ellen Wehrens in the group of prof. dr. Berent Prakken. During this internship she studied the role of monocytes in joint inflammation. Subsequently, she went to the Estación Experimental del Zaidín (EEZ-CSIC) in Granada, Spain, for her second internship under the supervision of dr. Marian Llamas. During this internship, she studied the PUMA3 cell-surface signaling system of the *Pseudomonas aeruginosa* bacterium. In 2013, she participated as orchestral manager in the board of the Dutch Student Orchestra (NSO). After obtaining her master degree in June 2014, she started in the same year as a PhD candidate under the supervision of prof. dr. Carla Baan, prof. dr. Rudi Hendriks, dr. Frank Dor (2014-2015) and prof dr. Luc van der Laan (2016-2018) at the department of Internal Medicine, Division of Nephrology and Transplantation, at the Erasmus MC, Rotterdam, the Netherlands. The results of her research are described in this thesis. In December 2018, Kitty started her training as a Clinical Chemist in the Franciscus Gasthuis en Vlietland hospital in Rotterdam. In her spare time, Kitty is playing the violin in various ensembles and orchestras.

PhD portfolio

Name PhD student: Kitty de Leur
Erasmus MC department: Internal Medicine, Section Nephrology and Transplantation
Research school: Postgraduate School Molecular Medicine
PhD period: December 2014 – December 2018
Supervisors: Prof. dr. Carla C. Baan, Prof. dr. Rudi W. Hendriks, Prof. dr. Luc J.W. van der Laan
Total workload: 34.9 ECTS

Courses		**Workload (ECTS)**
2015	Artikel 9 supplemental animal course	0.3
2015	Biostatistical Methods I: Basic Principles (CC02)*	3
2015	Scientific Integrity*	0.3
2016	Photoshop and Illustrator CS6 workshop*	0.3
2016	Advanced Immunology course*	4
2016	Scientific speed reading*	0.3
2017	Biomedical English writing and communication*	4
2017	Career Guidance Program*	1
2018	Indesign CS6 workshop*	0.3

*Erasmus MC, Rotterdam

Attended meetings		
2014-2018	Lab meetings internal medicine department, transplantation lab, Erasmus MC, Rotterdam	2
2014-2018	Journal Club internal medicine department, Erasmus MC, Rotterdam	1

Conferences		
2015	Science days internal medicine	0.5
2015	BOOT conference (Dutch transplant society), Bournemouth UK	0.5
2016	Science days internal medicine (Poster)	1
2016	14th B Lymphocyte Forum, Zeist (Poster)	1
2016	Keystone symposium Tfh Cells and GCs, Monterey USA (Poster)	1
2016	BOOT conference (Dutch transplant society), Groningen (Oral)	1
2016	Congress of the Transplantation Society, Hong Kong (Poster)**	0.1
2017	Science days internal medicine (Oral)	1
2016	BSI/NVVI conference, Liverpool (Poster)	1

2017	BTS meeting, Harrogate, UK (Oral)**	0.1
2017	BOOT conference (Dutch transplant society), Utrecht (Oral+Poster)	1
2017	BANFF-SCT conference (Poster)**	0.1
2017	ATC 2017, Chicago (Two orals)	1
2017	ESOT, Barcelona (Pitch + oral)	1
2018	Science days internal medicine (Poster)	1
2018	BOOT conference (Dutch transplant society), Rotterdam (Oral)	1
2018	TTS, Madrid (Oral)	1
2018	ESOT Basic Science Meeting, Rotterdam (Poster)**	0.1
	** Presented by colleagues	

Teaching activities

Counseling and teaching a Master student Immunity and Infection and a visiting PhD student from Chengdu University with setting up experiments and writing a review	4

Miscellaneous

2017	Organization labday Internal Medicine department, Erasmus MC	0.5
2017 - 2018	Board member of the NTV Young Professionals Network	0.5

Travel grants

2016	Travel grant EUR trust fund
2016	Bootbeurs NTV (annual meeting NTV)
2017	NTV scholingsbeurs
2018	Bootbeurs NTV (annual meeting NTV)
2018	Travel grant EUR trust fund

Awards

2017	Young investigators award American Transplant Conference (ATC)
2017	Poster award Mol Med postgraduate school day

Memberships

Dutch Society of Immunology (Nederlandse Vereniging voor Immunologie, NVVI)
Dutch Transplantation Society (Nederlandse Transplantatie Vereniging, NTV)
The European Society of Organ Transplantation (ESOT)
The Transplant Society (TTS)

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Kraaijeveld R, Li Y, Yan L, **de Leur K**, Dieterich M, Peeters AMA, Wang L, Shi Y, Baan CC. Inhibition of follicular helper T cell polarization by tacrolimus or sirolimus results in reduced B cell activation. Submitted to International Journal of Molecular Sciences

Acknowledgements / Dankwoord

Net als bij een wielerwedstrijd heeft een promotie een kop en een staart. Er zijn momenten van rustig doortrappen en van sprinten. Er zijn steile beklimmingen, en lange afdalingen. Maar bovenal is het niet opgeven tot de finish. Zonder teamgenoten, coaches, mecaniciens en publiek is het behalen van de eindstreep vrijwel onmogelijk. Bij promoveren is dit precies zo, je kunt het niet alleen. Onderstaande mensen hebben ervoor gezorgd dat ik een onvergetelijke rit heb gehad!

Prof. dr. Baan, beste Carla, vanaf de eerste dag van mijn promotie heb je mij gestimuleerd om kritisch na te denken. In het begin vond ik dat spannend en moeilijk, maar naarmate de tijd vorderde werden onze discussies steeds makkelijker en diepgaander. De wijze woorden "de wens is de vader van de gedachten" zal ik niet snel vergeten. Je wist op tijd in te grijpen wanneer ik meer wilde dan mogelijk was. Hartelijk dank voor jouw vertrouwen in mij en jouw aanstekelijke enthousiasme voor het onderzoek.

Prof. dr. Hendriks, beste Rudi, jouw visie op onderzoek was erg waardevol voor mij. Ook al zagen we elkaar niet vaak, de momenten dat we inhoudelijk overleg hadden, waren inspirerend. Daar wil ik je voor bedanken. Naast het onderzoek delen we ook de passie voor klassieke muziek. Hopelijk komen we elkaar nog vaak tegen bij mooie concerten.

Prof. dr. van der Laan, beste Luc, sinds het tweede jaar ben jij betrokken geweest bij mijn promotietraject. Bedankt dat je tijd wist vrij te maken om mijn project te begeleiden en op de juiste momenten kritische vragen wist te stellen. Heel veel succes als jonge professor!

Dr. Dor, beste Frank, jij stond aan het prille begin van mijn promotietraject. Ik vind het bijzonder dat we, ondanks jouw vertrek naar Londen, het project ook samen afronden. Jouw enthousiasme voor wetenschappelijk onderzoek in combinatie met jouw toegankelijkheid zijn unieke eigenschappen. Hartelijk dank voor alle support.

Dr. Clahsen-van Groningen, beste Marian, wanneer ik de pathologie afdeling verliet deed ik dit altijd met een grote glimlach. Je was immer bereid om coupes te bekijken of materiaal voor de GIL-studie te verwerken ("alles voor de wetenschap!"). Ik bewonder jouw drive voor het uitvoeren van onderzoek naast jouw klinische taken. Dank voor alle hulp.

Dr. Hesselink, beste Dennis, jouw gedrevenheid ten aanzien van wetenschappelijk (lab)onderzoek naast de patiëntenzorg is bijzonder. Bedankt voor het meedenken met verschillende projecten en je oog voor precisie bij het lezen van mijn manuscripten.

Ook alle andere leden van de kleine en grote commissie bedank ik voor de tijd en bereidheid om mijn proefschrift te beoordelen.

Lieve **paranimfen**, Lilian en Marjolein, vanaf het begin van mijn promotie tot de feestelijkheden rond de afronding hebben jullie mij enorm geholpen en gemotiveerd. Ook wanneer het tegen zat, stimuleerden jullie mij om door te zetten. Met als resultaat dit boekje. Dank dat jullie op deze bijzondere dag naast mij staan! **Lilian**, als klein meisje vond ik het al heel erg interessant dat jij als mijn tante in een laboratorium in een groot ziekenhuis in Rotterdam werkte. Wie had ooit gedacht dat we jaren later beiden in datzelfde ziekenhuis zouden werken? De lunchafspraakjes met jou waren gezellig en zeer waardevol. Ze gaven vaak precies het steuntje in de rug dat ik nodig had. De vele activiteiten 'buiten de stad' waren altijd fijn en ik hoop dat we in de toekomst nog veel mooie tripjes gaan maken. **Marjolein**, jouw bijdrage aan dit proefschrift was onmisbaar! Van lange sort-experimenten tot prak-festijnen en ingewikkelde FACS kleuringen. Jij deinsde nergens voor terug. Naast al deze ingewikkelde experimenten hebben we ook ontzettend veel gelachen op het lab en daarbuiten. Als derde Bela-AIO van jou vlieg ik nu uit. Heel veel dank voor alle steun en hulp bij mijn project en vooral de fijne vriendschap.

Team Bela, het dreamteam waar ik altijd op terug kon vallen tijdens dagen met onmogelijke experimenten, deadlines, een tekort aan suiker/koffie en andere lab struggles. **Gretchen**, nooit had ik verwacht dat ik op mijn eerste werkdag zo warm ontvangen zou worden in ons kantoor gevuld met Hello-Kitty gadgets. De eerste periode van mijn PhD heb je mij enorm geholpen om mijn weg binnen het transplantatielab te vinden. Dit heeft geresulteerd in een heleboel flawless data ;) Al onze congressen en andere uitjes, met als hoogtepunt Chicago, zal ik niet snel vergeten. Dank voor al je humor, gezelligheid en steun. **Nynke**, als Bela-zusjes konden we altijd veel met elkaar delen en zeker in de laatste fase van mijn PhD kon ik altijd bij je aankloppen voor advies. Ook was het een hele eer om jouw paranimf te mogen zijn. Het was fijn om met jou samen te werken en ik hoop dat we elkaar nog vaak blijven zien. **Rens**, naast het handschoenen schieten en stoelen sjoelen waren er ook de eindeloze discussies over hoe die Tfh cel en B cel precies samenwerken. Jouw nieuwsgierigheid en enthousiasme voor de wetenschap (en Game of Thrones!) zijn aanstekelijk. Dank voor alle goede grappen en voor al je hulp bij mijn onderzoek. **Marieke**, als overburen hadden we al snel een klik. Het bespreken en verwezenlijken van onze carrièredromen, het delen van lekkere recepten, fantaseren over mooie vakantiebestemmingen maar ook uitwisseling van klinische en lab-gerelateerde zaken. Jouw hulp en fijne gesprekken hielpen mij altijd in de goede richting. Gaan we deze zomer nog een keertje racefietsen?

All the other PhD's, thank you for making my PhD time an inspiring and great time! **Burç**, buur! Wat heb ik ontzettend genoten van jouw droge humor in ons kantoor. We hebben heel wat afgelachen, maar daarnaast kon ik bij jou ook altijd terecht met serieuze vragen. Ik weet zeker dat jij een super huisarts gaat worden! **Samantha**, Sam, jouw open en lieve houding zorgden ervoor dat ik mij al snel thuis voelde op het lab. Dankjewel voor alle hulp en support bij mijn experimenten en vooral dat je daarbij de knisper-sokken wilde trotseren. Ook dank voor alle glitters, snoepjes, (brandweerman)posters en andere grapjes die mijn PhD zoveel leuker hebben gemaakt. Heel veel succes met de opleiding tot klinisch embryoloog. **Franka**, Fluk, mijn partner in crime bij het skin-transplant project. Wat hebben we veel getrotseerd, van een bezoekje aan de slager om onze dermatoom-skills te oefenen tot aan de eindeloze experiment-sessies in de stal. Jij laat je niet snel uit het veld slaan en blijft altijd positief. Dankjewel dat je altijd voor mij klaar staat! **Ling**, you always made me smile. Our late afternoon conversations in the office were always stimulating and inspiring for me. Thank you for being my happy friend and colleague and I hope we will meet again in China or in the Netherlands in the future. **Lin**, writing the review with you was a great experience! Every week you came up with new ideas that resulted in a really nice publication. I would like to wish you all the best with your career and life in Chengdu. **Fleur**, onze PhD's gingen vrijwel gelijk op waardoor we veel konden delen. Daarnaast zaten we samen in het Young Professionals bestuur. Als pionier op het gebied van het epigenetica onderzoek binnen het transplantatieveld hield jij je staande als een gedreven onderzoekster. Gaaf dat je nu in jouw eigen Amsterdam aan de slag bent gegaan als Postdoc. Dankjewel voor alle leuke momenten en succes met jouw wetenschappelijke carrière. **Thierry**, jij stond altijd voor mij klaar om in vliegende vaart coupes te kleuren en mij de fijne kneepjes van de confocal microscopie te leren. Daarnaast zal ik onze congres week in Chicago niet snel vergeten. Veel succes met jouw werkzaamheden bij de Pathologie. **Jesus**, our Spanish beard man! Thank you for all the funny moments in and outside the lab. Especially during several conferences we spend together (Spain!). The cold beers alongside the pool after a long conference day were always good. **Anusha**, jij draait je hand niet om voor een ingewikkeld organoïd project. Ook bood je altijd een luisterend oor, zeker als we samen op kantoor waren en te lang bleven hangen ;) Heel veel succes met jouw promotietraject! **Jeroen**, koning van de graslelies! Als een lichtelijk chaotische wervelwind kwam jij bij ons op het lab. Met jouw doorzettingsvermogen en enthousiasme weet ik zeker dat je het ver gaat schoppen. Dankjewel voor alle fijne en grappige momenten en alle mooie posters (Ryan!) in ons kantoor. **Wouter**, niet alleen bracht jij veel reuring op het lab, je bent ook creatief. Deze creatieve en nieuwsgierige blik op wetenschap is inspirerend! Daarnaast delen we ook de liefde voor muziek en lekkere biertjes. Dank voor de goede tijd en succes met jouw PhD. **Aleixandra**, altijd vrolijk en in voor een koffiemoment beneden onder het mom van een Tfh/IL-21 werkoverleg. Rustig en gericht ga jij op je doel af. Veel plezier en succes de

komende jaren op het Tx lab! **Steven**, although we only worked together for a short period you were always interested in my work. Good luck with your PhD and enjoy your time in the Netherlands!

Alle **Postdocs** wil ik bedanken voor de input op mijn werk en uiteraard voor alle leuke momenten. **Karin**, jouw scherpe en doortastende blik heeft een belangrijke bijdrage geleverd aan mijn proefschrift. Onze sessies in het lab/microscoophok zal ik niet snel vergeten. Dankjewel voor al je support de afgelopen jaren en de fijne vriendschap. **Martin**, voor elk probleem had jij een ingenieuze oplossing. Onze samenwerking heeft geresulteerd in een mooie publicatie over het skin-transplant muismodel. Bedankt voor jouw geduld en hulp gedurende mijn PhD. **Nicolle**, er was altijd ruimte om een ingewikkeld experiment met jou door te nemen of gewoon even te kletsen. Dank daarvoor! Ik vind het leuk dat we elkaar als 'bijna buren' af en toe nog zien. **Nicole**, altijd geïnteresseerd in het reilen en zeilen van de verschillende projecten. Heel veel succes met jouw ELISPOT studies. **Ana**, me flipa como eres!! Your happy and crazy character always made me laugh. But also your enormous lab-knowledge and critical questions were valuable. Thank you for inviting us to your home in Cordóba, an experience I will never forget. **Fabiany**, it was a big surprise to welcome you again to the Tx lab as a Postdoc and a pleasure to spend some weeks together. I wish you all the best with your career and life in Rotterdam.

De Analisten, ook wel de mecaniciens van het laboratorium. Zonder jullie waren mijn experimenten in het lab simpelweg niet mogelijk geweest. **Annemiek**, dankjewel voor je hulp bij de RT-qPCR experimenten en voor onze fijne gesprekken. **Derek**, altijd in voor nieuwe avonturen op het lab en tripjes naar de Efteling of het foute feest. Dank voor jouw hulp bij de muizenexperimenten. **Ronella**, praten over Lowlands, motorrijden of andere stoere dingen, de tijd vloog voorbij als ik naast je in de kweek zat. Dank voor je support! **Wenda**, heldin van de FACS sorts en vele andere praktische lab-zaken. Bedankt voor al je hulp en gezelligheid de afgelopen jaren. **Mariska**, wanneer het einde van de week naderde was jij vaak weer achter de FACS te vinden, een vertrouwd moment om even bij te praten. Dank daarvoor! **Sander**, jouw droog-ijs kunsten op het lab waren altijd een welkome afleiding. Dank voor alle leuke momenten. **Jeroen**, naast een harde werker op het lab ook altijd in voor een gezellig avondje (met speciaal biertjes). Dank voor alle grappige momenten en de inspirerende verhalen over de mooie reizen die je maakt.

Alle andere oud-collega's van het transplantatielab bedank ik voor de betrokkenheid bij mijn promotietraject: **Joke, Marcel.la, Ruud, Yongkang Wu, Rocio, Tanja, Bastiaan, Elly, Ruben, Frieda, Thea.**

Ook mijn nieuwe collega's van het Franciscus Gasthuis en Vlietland wil ik bedanken voor de fijne werksfeer en support tijdens de start van mijn opleiding en de laatste fase van mijn promotietraject.

Jaarclub Femidabel, lieve **Fiore, Tessa, Marjoleine, Sophia, Merel, Marlotte, Charlie, Machteld, Lorena en Elbrich.** Van etentjes in onze kleine studentenkamers tot het daadwerkelijk realiseren van onze dromen. We hebben elkaar zien groeien en ontwikkelen. Dank jullie wel voor de nodige afleiding de afgelopen jaren en de hechte vriendschap.

Jasmijn, Esther en Nienke, wat een cursus 'career guidance' allemaal teweeg kan brengen! De ontmoetingen met jullie zijn altijd een verrijking en bovenal ontspannend.

Inez en Elisah, immer in voor avontuur en op zoek naar nieuwe uitdagingen. Wat ben ik dankbaar dat we elkaar na het NESKO project beter hebben leren kennen. Jullie zien mij zoals ik ben en dat waardeer ik enorm. **Anouk en Marianne**, Alle mooie tournees die we hebben meegemaakt en georganiseerd, de gekke schnabbels en de recente start van onze oranjebitter distributie. Niets is te gek! Het feit dat jullie altijd voor mij klaarstaan koester ik. **Maaike**, de studie en orkest zijn twee gemeenschappelijke delers. Het uitwisselen van ervaringen tijdens eindeloze koffie afspraakjes is iets waar ik altijd van geniet. **Liesbeth**, mijn aller-langste orkest vriendinnetje. Ondanks dat jij nu in Singapore woont blijven we elkaar op de hoogte houden van onze bezigheden, dat vind ik waardevol. **Pieter**, lieve Dini, op het moment van schrijven schiet ik al in de lach. Het contrast tussen onze diepgaande en wat minder diepgaande gesprekken is enorm. Je daagt me uit om verder te kijken dan de oppervlakte, een gave die niet veel mensen hebben. Dank voor jouw interesse en onze mooie vriendschap.

Sytha en Jos, al snel voelde ik mij thuis tussen de Vinken. Naast lachen over wat we allemaal meemaken zijn jullie ook erg geïnteresseerd in mijn onderzoek maar ook in alles wat ik daarbuiten beleef. Heel veel dank daarvoor! **Marlou, Jasper en Thomas**, het is iedere keer weer een feest om naar jullie mooie stukje boerengrond aan de Lekdijk toe te komen. Dank voor alle lachstuipen en ontspanning de afgelopen jaren!

Piet, als oom en tevens mede-levensgenieter ben je altijd geïnteresseerd in waar ik allemaal mee bezig ben. Dankjewel voor al je interesse en de fijne familie etentjes. Lieve **Oma van Capelle**, een gedeelte van mijn PhD tijd heb jij nog meegemaakt. Jij hebt mij altijd gestimuleerd om verder te leren maar bovenal ook plezier te maken en te lachen. Wat ben ik dankbaar voor alle mooie herinneringen die we hebben.

Mam en Pap, zonder jullie onvoorwaardelijke liefde en steun was dit boekje er niet geweest. Jullie hebben mij altijd gestimuleerd om datgene te doen waar ik gelukkig van word. Fietsen door de duinen, gek doen in Miggelenberg en naar mooie concerten of musea gaan. We delen veel met elkaar, en dat is van onschatbare waarde voor mij.

Floris, lieve Flo, wat ben ik gek op jou! Snel nadat ik naar Rotterdam verhuisde begon ons grote avontuur. Jij maakt mij aan het lachen wanneer ik verdrietig ben en zet mij met twee benen op de grond wanneer ik ergens niet uit kom. En bovenal, je ziet mij zoals ik ben. Bij jou ben ik gelukkig!