Anti-B-cell therapy in renal transplantation

Aware!

Elena Kamburova
ANTI-B-CELL THERAPY IN RENAL TRANSPLANTATION

B AWARE!

Elena G. Kamburova
The research presented in this thesis was performed at the Department of Laboratory Medicine - Laboratory of Medical Immunology of the Radboud University Medical Centre, Nijmegen, The Netherlands.

This research project was funded by the Dutch Kidney Foundation (nr C09-2301)

Printing of this thesis was financially supported by the Radboud University Medical Centre Nijmegen and the Dutch Transplant Society


Cover design: IS Ontwerp - Ilse Schrauwers, Den Bosch - www.isontwerp.nl
Layout: Elena Kamburova
Printed by: Gildeprint Drukkerijen - www.gildeprint.nl

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ANTI-B-CELL THERAPY IN RENAL TRANSPLANTATION

B AWARE!

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann, volgens besluit van het college van decanen in het openbaar te verdedigen op

donderdag 25 september 2014 om 12.30 uur precies

door

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geboren op 2 oktober 1984 te Sofia (Bulgarije)
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ABMR</td>
<td>antibody mediated rejections</td>
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<tr>
<td>ABOi</td>
<td>ABO-incompatible</td>
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<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
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<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
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<td>AnxV</td>
<td>annexin V</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<td>ATG</td>
<td>anti-thymocyte globulin</td>
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<td>BAFF</td>
<td>B-cell-activating factor</td>
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<tr>
<td>BAFF-R</td>
<td>B-cell-activating factor-receptor</td>
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<td>BCMA</td>
<td>B-cell maturation antigen</td>
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<tr>
<td>BCR</td>
<td>B-cell receptor</td>
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<tr>
<td>Bm</td>
<td>mature B cells</td>
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<tr>
<td>BPAR</td>
<td>biopsy proven acute rejection</td>
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<tr>
<td>B&lt;sub&gt;REGS&lt;/sub&gt;</td>
<td>regulatory B cells</td>
</tr>
<tr>
<td>CAD</td>
<td>chronic allograft dysfunction</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CDC</td>
<td>complement-dependent cytotoxicity</td>
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<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
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<td>CLP</td>
<td>common lymphoid progenitors</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<td>CRS</td>
<td>cytokine release syndrome</td>
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<td>CSR</td>
<td>class switch recombination</td>
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<tr>
<td>CVID</td>
<td>common variable immunodeficiency</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DSA</td>
<td>donor specific antibodies</td>
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<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EM</td>
<td>effector memory</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FDC</td>
<td>follicular dendritic cells</td>
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<td>FOXP3</td>
<td>forkhead box P3</td>
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<td>HD</td>
<td>hemodialysis</td>
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<td>HLA</td>
<td>human leukocyte antigens</td>
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<td>HPS</td>
<td>human pooled serum</td>
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<td>IFNγ</td>
<td>interferon-γ</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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List of Abbreviations
CHAPTER 1

GENERAL INTRODUCTION
AND OUTLINE OF THE THESIS
Chapter 1
GENERAL INTRODUCTION

The immune system is a complex interplay of cells, tissues and molecules that protects us from foreign invaders, such as bacteria, viruses, as well as foreign antigens. The immune system can be divided into two arms, the innate and the adaptive immune system. The innate immune system, which is present from birth, forms the first line of defense and is designed to keep invaders out by physical barriers such as skin and mucosa. It consists of soluble proteins (e.g. complement) and a variety of cells including neutrophils, macrophages, monocytes, dendritic cells (DCs), and natural killer (NK) cells. Adaptive immunity refers to a specific immune response, which is acquired after exposure to a specific invader. Upon antigen exposure, an antigen-presenting cell (APC) displays the antigen to T helper (Th) cells which upon recognition of the antigen become activated and will initiate an immune response thereby activating also other immune cells, such as B cells and cytotoxic T cells. Activated B cells will mature to antibody-secreting plasma cells and activated antigen-specific cytotoxic T cells will directly kill the pathogens. This response takes a few days to develop completely. However, after exposure to the same antigen for a second time, this immune response will develop faster and be stronger due to the development of memory cells. Communication between the different types of immune cells critically depends on production of soluble mediators called cytokines and chemokines.

When functioning properly, our immune system is able to identify potential threats, and distinguish them from the body’s own (self) healthy tissue. In renal transplantation, the immune system will respond to the foreign donor antigens present on the kidney leading to graft rejection.

Renal transplantation

In the 20th century, transplantation medicine has overcome many limitations to become as successful as it is today. The first experimental organ transplantations were performed with animal organs and organs from deceased humans in the early 1900s. After the improvement of surgical techniques, the first kidney transplantation in humans was performed in 1950’s in the United States on a 44-year-old woman who received a kidney from an unrelated deceased donor (1). The donated kidney was rejected because no immunosuppressive therapy was available at that time. In 1954, the first successful kidney transplantation was performed between two monozygotic twin brothers (2). The need for immunosuppressive therapy was circumvented by the genetic identity between the donor and recipient. Studies on the mechanisms of rejection and the use of immunosuppressive drugs resulted in the first successful kidney transplantations between unrelated donors a few years later (3).

Nowadays, kidney transplantation is the most optimal treatment for patients with end stage renal failure. The discovery and development of potent
immunosuppressive drugs that are able to prevent or treat rejection of the transplanted graft has greatly improved the short-term graft survival after transplantation, however the long-term results have lagged behind (4). The 1-year graft survival is over 90%, but after 10 years this is only about 60% (5). The causes of graft loss will be discussed in the next sections.

Transplantation immunology

Transplantation of an organ from a genetically identical individual (monozygotic twin) does not lead to a detrimental immune response. In contrast, transplantation between genetically different (allogeneic) individuals will lead to rejection of the graft if no immunosuppressive treatment is given (6-8). Immune responses are triggered after recognition of the mismatched allogeneic major histocompatibility complex (MHC) antigens, which are also called human leukocyte antigens (HLA) (9). T cells play an important role in the pathogenesis of rejection via the recognition of these alloantigens on antigen-presenting cells (APCs). After transplantation, donor and recipient APCs migrate to the T-cell areas of secondary lymphoid organs. There, they activate naive and central memory T cells that recirculate between different lymphoid tissues (10).

Recognition of foreign HLA by T cells can take place via three pathways: direct, indirect or semidirect allorecognition. In the direct pathway, intact MHC on donor APCs is recognized by CD4+ and CD8+ T cells of the recipient (11). The indirect pathway is characterized by recipient APC uptake of allogeneic donor MHC that has been shed through apoptosis or necrosis. This is then processed, resulting in presentation of donor antigens via recipient MHC class II to recipient CD4+ T cells (12). Semidirect recognition results from the transfer of cellular membrane components, including intact donor MHC, from donor APCs to recipient APCs through cell-cell contact or the transfer of donor exosomes that fuse with recipient APC cell membranes (13). Recipient APCs are then chimeric for MHC and are able to stimulate recipient CD4+ and CD8+ T cells. Activated antigen-specific T cells undergo clonal expansion and differentiation to express effector functions.

The ability of our immune system to distinguish between self-antigens and foreign (non-self) antigens, and between harmful and harmless invaders, is critical to the maintenance of immune homeostasis. Failure to maintain tolerance to self- or harmless antigens results in development of autoimmune or allergic diseases, respectively. Following organ transplantation, it is essential to control both the innate immune response triggered by the injured tissue and the adaptive immune response stimulated by mismatched donor and recipient MHC to enable long-term graft survival. The ultimate goal in transplantation is to achieve transplant tolerance, where the recipients’ immune system is unresponsive to donor alloantigens, but is able to respond to all other antigens.
**Graft rejection**

In general, there are three forms of rejection: hyperacute, acute and chronic. Hyperacute rejection occurs within minutes after transplantation due to pre-existing antibodies, either toward blood group antigens (ABO) (14) or foreign HLA (15) present on the donor kidney (donor specific antibodies, DSAs). This rejection will result in organ failure within hours after transplantation. The pre-existing antibodies against non-self HLA can be formed after a previous transplantation, blood transfusion or pregnancy (16-20). To determine if the patient has pre-existing antibodies against foreign HLA, several techniques are currently in use: both solid phase methodology as well as the classic cytotoxicity method help in defining the so-called virtual panel reactive antibody (PRA) value, based on allele frequencies in the population (21). To determine whether the patient has detrimental pre-existing DSAs, serum from the patient is incubated with lymphocytes from the donor. When the test is positive, the transplantation will not be performed. Due to the introduction of these tests, hyperacute rejection is very uncommon (22).

Acute rejections can be classified as predominantly T-cell mediated (cellular), mainly antibody-mediated (humoral), or mixed types of rejection. Acute cellular rejection can occur at any time after transplantation, although it is most commonly seen within the first six months after transplantation. T cells play a central role in acute cellular rejection and the magnitude of the immune response is likely dependent on the frequency of effector T-cell precursors that recognize donor HLA molecules via the direct pathway of allorecognition (23, 24). Patients can also experience an acute humoral rejection in case they have been sensitized to a certain antigen but have undetectable DSAs levels at time of transplantation (25). After transplantation, donor-specific memory B cells can rapidly transform to plasma cells that produce antibodies against the donor kidney leading to rejection (26). Accordingly, patients with a high PRA value, referred to as sensitized, have a higher risk of developing rejection (27, 28). To reduce the incidence of antibody-mediated rejection in sensitized patients there are nowadays different treatment options (29).

Finally, chronic rejection or chronic allograft dysfunction (CAD) limits the long-term success of renal transplantation (30). It occurs years after transplantation, particularly by repeated inflammation and can be caused by a number of processes, both immunological and non-immunological (4, 31, 32). The origin and quality of the kidney, the degree of ischemia-reperfusion injury, but also the use of calcineurin-inhibitors are examples of non-immunological factors that might contribute to CAD. On the other hand, recipients’ T cells can recognize the alloantigen via the indirect pathway and initiate an immune responses associated with antibody formation against the kidney leading to a chronic type of humoral rejection (33).
T-cell differentiation and regulation

To achieve a state of immunological tolerance, the immune system has a variety of built-in mechanisms. The first line of self-tolerance is the elimination of self-reactive T-cell clones in the thymus, referred to as central tolerance induction (34). However, some self-reactive T cells escape this negative selection process and appear in the periphery. There are at least three mechanisms of peripheral tolerance: death of self-reactive T cells via negative selection, induction of anergy (unresponsiveness) and the induction of regulatory T cells (T_{REGS}). There are naturally occurring and acquired T_{REGS}, which regulate effector T-cell responses either directly through cell-cell contact, the production of immunosuppressive cytokines, or cytokine deprivation and/or indirectly by targeting dendritic cells (DCs) (35). Naturally occurring CD4^+ T_{REGS} can be characterized by the constitutive expression of the IL-2 receptor -chain (CD25), the transcription factor forkhead box P3 (FOXP3), and the absence of the IL-7 receptor -chain (CD127) (36). In contrast, conventional CD4^+ T cells have a low expression of CD25, are negative for FOXP3, and show high expression of CD127. However, upon activation CD4^+ T cells resemble the phenotype of T_{REGS}, which complicates the identification of T_{REGS} (37).

T-cell subsets

Once naive T cells (T_N) have left the thymus, they recirculate through the blood and secondary lymphoid organs in search of antigens presented by APCs. T_N cells are defined by the co-expression of CD45RA, the lymph node homing markers L-selectin (CD62L) and CCR7, and the co-stimulatory receptors CD27 and CD28 (38, 39). When T_N cells encounter their specific antigen, they get activated and subsequently proliferate and differentiate into effector and memory T cells (40). Effector T cells can enter peripheral tissues where they recognize antigens and generate inflammatory reactions. Protective memory is mediated by effector memory T cells (T_{EM}) that are able to migrate to inflamed peripheral tissues and display immediate effector function. In contrast, central memory T cells (T_{CM}) home to T-cell areas of secondary lymphoid organs, where antigenic stimulation leads to rapid proliferation and differentiation into effector cells. T_{CM} cells, like T_N cells co-express CD62L, CCR7, and CD27, but they have become CD45RO-positive and CD45RA-negative. The expression of CD45RA and CD45RO are regulated in a reciprocal manner: in the thymus T cells express CD45RO, which is converted to CD45RA at time of emigration to the periphery, and then switched back to CD45RO after antigen stimulation (41). T_{EM} cells have lost their expression of CCR7 and CD27, and are heterogeneous for CD62L expression (42).

CD4^+ T_N can differentiate into a variety of effector subsets, including T helper 1 (Th1), Th2, Th17, and follicular helper T (T_{FH}) cells, which have different roles in further activation of the immune system and the protection against pathogens (43). After activation of T_N by APCs, their differentiation is regulated predominantly
by the cytokines in the microenvironment (44). IL-12 produced by innate immune cells as well as IFNγ produced by both NK and T cells polarize cells toward Th1 differentiation through activation of T box transcription factor T-bet (45). Th1 cells are characterized by the production of IFNγ and IL-2, and are involved in cellular immunity against intracellular microorganisms. Th2 cells produce IL-4, IL-5 and IL-13 and are required for humoral immunity to control infections with helminths and other extracellular pathogens. Th2 cell differentiation requires the activation of the transcription factor GATA3 downstream of IL-4 (46). Th17 cells produce IL-17 and IL-22 and are needed to protect against extracellular microbes. In addition, they are associated with many inflammatory disorders. Th17 cell differentiation requires retinoid-related orphan receptor (ROR)γt, a transcription factor that is induced by the proinflammatory cytokines IL-6, IL-21, and IL-23 (47). Tfh cells are a subset of helper T cells that regulate the maturation of B cells in the germinal centers through the expression of CD40 ligand (CD40L) and the production of IL-21 (48). The various subsets of effector T cells can also be characterized by the expression of different chemokine receptors. The B cell-follicle-homing receptor CXCR5 identifies Tfh cells, whereas CXCR3 is mainly expressed on Th1 cells. In addition, CCR4 is expressed on Th2 cells, whereas Th17 cells are found within the CCR6+ memory T cells (49). The ligands for these receptors are inflammatory chemokines, which are expressed in inflamed tissues and mediate the selective recruitment of different types of effector cells (50).

B cells in renal transplantation

For years, the pathogenic role of B cells was thought to be limited to antibody production. The deleterious effects of pre-existing antibodies directed against HLA are well known. The presence of donor-specific anti-HLA antibodies before transplantation is associated with poorer allograft survival (28). However, additional functions of B cells have been shown in the pathogenesis of autoimmune diseases (51, 52), and are also increasingly recognized in the rejection of organ grafts (53, 54). After transplantation, B cells play a central role in humoral immunity via the production of antibodies, but they can also induce an immune response by acting as professional APCs (55), or by the production of various cytokines (Figure 1). As professional APCs, B cells can efficiently stimulate T cells and allow the optimal development of memory CD4+ T cells (56, 57). Studies in mice showed that B cells are required for systemic T-cell responses when antigen concentrations are low (58). Furthermore, B cells produce cytokines such as IL-6, IL-10 and IFNγ, which have regulatory effects on DCs and other lymphocytes (59). In animal models, cytokines produced by B cells are known to guide the differentiation of naive CD4+ T cells into Th1 or Th2 subsets (60), suggesting that the production of cytokines by B cells can regulate immune responses. B cells can both generate and respond to chemotactic factors responsible for leukocyte migration and therefore play a major role in mediating inflammatory cell-infiltration processes (61). In addition,
B cells with regulatory functions have been described (62, 63). Evidence for a regulatory effect of B cells in autoimmune diseases was first provided by Wolf et al. (64) who observed that mice lacking B cells suffered from a severe and chronic form of experimental autoimmune encephalomyelitis (EAE). Research on the underlying mechanism revealed that B cells regulate this autoimmune disease via the production of IL-10 (65). A suppressive function of IL-10 producing B cells has also been demonstrated in mouse models for inflammatory bowel disease (66) and collagen-induced arthritis (67). Therefore, they were named regulatory B cells (B\textsubscript{REGS}) (66). More recently, regulatory B cells have been identified in humans too (68). Their regulatory function appears to be mainly mediated by the production of IL-10, which inhibits proinflammatory cytokines and supports T\textsubscript{REG} differentiation (69). There is controversy regarding the surface markers used to characterize B\textsubscript{REGS}. Blair et al. describe CD24\textsuperscript{hi}CD38\textsuperscript{hi} B cells with regulatory capacities, which are functionally impaired in patients with systemic lupus erythematosus (70). Others indentify B\textsubscript{REGS} as CD24\textsuperscript{hi}CD27\textsuperscript{+} (71) or as CD1d\textsuperscript{hi} with or without co-expression of CD5 (72, 73). Interestingly, operationally tolerant renal transplant patients, who

**Figure 1. B-cell functions.**

B cells can induce immune responses by producing antibodies, secreting cytokines, or presenting antigens to T cells. In addition, they can regulate immune responses by secreting IL-10 which directly suppresses the activation and expansion of T cells, and indirectly via the differentiation of regulatory T cells (T\textsubscript{REG}) and suppression of dendritic cells (DCs).
have stable graft function despite receiving no immunosuppression for at least 1 year, have more B cells, including CD24<sup>hi</sup>CD38<sup>hi</sup> B cells, compared to patients on immunosuppression (74-76).

**B-cell development**

It is a long and hard way for a B-cell precursor to become a mature B cell. Only a small percentage of the B-cell precursors survive the maturation process. B-cell development takes place in several stages which are localized in different tissues: B-cell precursors differentiate from common lymphoid progenitors (CLP) to naive mature B cells in the bone marrow (77, 78) and the subsequent maturation to memory/effector cells takes place in secondary lymphoid tissues (lymph nodes, spleen, mucosa-associated lymphoid tissues (MALT)), and bone marrow (79, 80) (Figure 2). B cells constantly circulate in blood and lymphoid tissue until they encounter an antigen. Activated naive B cells generate antibody secreting plasma cells and memory B cells in response to a specific antigen.

**B-cell differentiation and maturation**

B cells differentiate from CLPs through various precursor stages in the bone marrow (81). Pre-B cells arise from progenitor (pro-B) cells that express neither the pre-BCR nor surface Immunoglobulin (Ig) (Figure 2). During the development of the pre-B-cell, the assembly of a correct BCR takes place in the bone marrow. The stromal cells in the bone marrow, which are crucial for the development of the pro- to pre-B cells, provide adhesive contacts and soluble factors like stem cell factor, CXCL12 and the cytokine BAFF (B cell-activating factor belonging to the TNF family) (82). Recombinant-activating genes-1 (RAG-1) and RAG-2 are enzymes crucial for the development of the BCR, and without these enzymes no lymphocytes can develop. If the B-cell fails to express the receptor, the cell is forced to undergo apoptosis. However, the cell can save itself by performing VDJ gene rearrangement of the BCR and if this is successful the B-cell can continue its development to become an immature B-cell. The receptor on the cell surface of the immature B-cell is a complete IgM molecule, which is important for the negative selection. An immature B cell that expresses an IgM, which strongly binds self-molecules, is eliminated via apoptosis. If that is the case, the cell gets a second chance to change its IgM molecule via a process called receptor editing, and if this is successful the cell survives. Besides apoptosis and gene rearrangement, the developing B cell has two other potential fates: a soluble self-molecule can induce anergy in the cell that eventually dies, and low-affinity non-crosslinking self-molecules can induce a clonally ignorant cell (which means that the antigen is present, but it will not be able to activate the cell). The B-cell that does not bind self-molecules continues its development as a transitional B-cell into a naive mature B-cell. The transitional B-cell expresses both IgM and IgD (83) on its cell surface and migrates through the blood to peripheral secondary lymphoid organs where
Chapter 1

Figure 2. B-cell differentiation.

Cell-surface expression during B-cell development in the bone marrow, periphery, lymph nodes, spleen and mucosa-associated lymphoid tissues (MALT). B cells develop from common lymphocyte progenitors (CLP) through various precursor stages in the bone marrow. After obtaining a functional B-cell receptor (BCR) they migrate to the periphery and become transitional B cells. Upon antigen encounter they mature and migrate to lymphoid tissues. Depending on the type of antigen, a T-cell dependent (TD) or T-cell independent (TI) B-cell response is initiated, resulting in the development of memory B cells and plasma cells with high affinity BCR due to somatic hypermutation (SHM) and class switch recombination (CSR). Adapted from Perez-Andres et al. (100) and van den Burg et al. (177).
the final maturation occurs. Once again, the B-cell is tested for self-reactivity and binding of self-antigens will lead to cell death. If the B-cell survives this final test, it has become a mature naïve B-cell, which is ready to enter the circulation in the search of pathogens. Similar to the early stages in the bone marrow, this phase of development in the spleen also requires the presence of a functional BCR and of a supporting microenvironment.

**B-cell activation**
As described in the previous section, transitional B cells are the earliest bone marrow emigrants. They rapidly differentiate into naïve mature B cells in the periphery, which form the majority of B cells in peripheral blood. Naive mature B
cells are short-lived and their survival is dependent on the interaction of BAFF and its receptor (BAFF-R) (84, 85). During B-cell maturation BAFF-R is expressed first by immature B cells in the bone marrow (86).

Most B-cell responses are T-cell dependent (87, 88). Upon antigen recognition with their specific BCR, naive mature B cells internalize the antigen which enters the MHC class II presentation pathway (89). After a subsequent co-stimulatory signal by T helper cells that recognize a MHC-peptide complex on the B-cell surface, the B cell is activated resulting in immunoglobulin class switch, memory B-cell and plasma cell formation, and antibody production (90, 91). This activation usually occurs in the T-cell zone of lymphoid organs by interaction with T helper cells. Next, B cells migrate into B-cell follicles, proliferate and establish germinal centers (92, 93). In these germinal centers, centroblasts form a network with follicular dendritic cells (FDCs), which present antigen to stimulate proliferation and survival of antigen-specific B cells (94). The proliferating centroblasts undergo somatic hypermutation (SHM), which involves random mutations in the genes encoding for Ig, giving rise to B cells with slightly different antigen-affinities (95). The centroblasts become resting centrocytes and can undergo class switch recombination (CSR). Only B cells that have gained an increased affinity for the antigen will survive after interaction with FDCs and germinal center T cells, the T<sub>FH</sub> (96). CSR recombination is dependent on the enzyme activation-induced cytidine deaminase (AID), which introduces single-strand DNA mutations in Ig genes (97, 98). Finally, B cells develop into memory B cells and plasma cells. Plasma cells are fully differentiated B cells that have lost their BCR and are only capable of producing large amounts of antibodies (99). Plasma cells are found mainly in the bone marrow, spleen and MALT (100).

B-cell responses can also occur independently of T cells in the marginal zone of the spleen or in the lamina propria of the gut (101, 102). T-cell independent antigens, which are represented predominantly by polysaccharide antigens, induce antibody responses in the absence of T-cell help (103). Alternatively, B cells can recognize antigens on pathogens, via toll-like receptors (TLRs) (104).

**B-cell subsets and their surface markers**

B cells can be characterized using a variety of molecules. Committed B cells induce the expression of the pan-B-cell marker CD19, which regulates intracellular signal transduction and is predominantly important in B-cell development (105, 106). CD20 is expressed on the majority of B cells from pre-B-cell stage, but is lost after differentiation into plasma cells (107). CD24 and CD38 are associated with intracellular signaling and upregulated during B-cell development in the bone marrow and the germinal center. CD24 is a cell-adhesion molecule and seems to be associated with B-cell activation via pattern recognition receptors, such as TLRs (108), while CD38 is a transmembrane glycoprotein that catalyzes the synthesis and hydrolysis of cyclic ADP-ribose involved in calcium signaling and signal transduction
General introduction and outline of the thesis

CD27 is a glycoprotein and a member of the tumor necrosis factor (TNF) receptor family, and was found to be a useful marker for B cells that have undergone somatic hypermutation and therefore a marker for memory B cells. B-cell subsets can be identified using flow cytometry using various classification schemes, of which the IgD/CD27 and the IgD/CD38 classification are most commonly used. Using the IgD/CD27 classification, B cells can be divided into four subsets: naïve (IgD+CD27-); unswitched memory (IgD-CD27+); switched memory (IgD-CD27+) and double-negative (IgD-CD27-). The expression level of IgD and CD38 distinguishes mature B (Bm) cells into sequential stages from Bm1 through Bm5 and was first used to characterize B cells in secondary lymphoid organs. Once activated in secondary lymphoid organs, naïve Bm1 (IgD+CD38-) cells become Bm2 (IgD+CD38+), and differentiate into germinal center Bm2' cells (IgD+CD38++). There they evolve into Bm3 centroblasts and Bm4 centrocytes (together IgD CD38++), which differentiate into either plasma cells (early Bm5; IgD-CD38+) or memory B cells (late Bm5; IgD-CD38-). Inside the germinal centers, a few cells of each subset escape into the circulation. Finally, CD24 and CD38 are especially used to characterize BREG, which are believed to be part of the transitional B-cell population (CD24+CD38++) while mature and memory B cells are defined as CD24+CD38+ and CD24+CD38-, respectively. Depending on the situation a different B-cell classification scheme might be used to characterize B cells.

**B-cell cytokines**

The cytokines that regulate human B-cell development are incompletely understood. The most important cytokines for B cells are BAFF, IL-2, IL-4, IL-6, IL-10 and IL-21. BAFF is a cell survival and maturation factor for B cells, and overproduction is associated with systemic autoimmune disease. BAFF is also known as BLys and also binds to 2 other receptors on B cells, namely the transmembrane activator and calcium-modulator and cytoplphilin ligand interactor (TACI) and the B-cell maturation antigen (BCMA). Activated B cells express CD25 (IL-2 receptor α-chain) and IL-2 is involved in the differentiation of B cells but it does not influence the isotype switch. IL-2 is mainly produced by activated T cells. IL-4 is produced mainly by Th2 cells, which are the biologically most active helper cells for B cells and also secrete IL-6. IL-4 promotes the proliferation and differentiation of activated B cells as well as the expression of MHC class II antigens and low affinity IgE receptors on resting B cells. IL-4 predominantly directs naïve B cells to express surface IgG1, with fewer cells expressing IgG2 and IgG3, but is unable to induce secretion of any IgG subclasses. IL-6 stimulates B cells to proliferate, differentiate, and to secrete immunoglobulin. However, it does not influence the isotype switch. The main sources of IL-6 production are macrophages, fibroblasts and endothelial cells, although B and T cells also can produce IL-6. IL-10 enhances B-cell survival, proliferation and differentiation. It also drives the isotype...
switch to IgG1-3 and IgA, and together with IL-4 to IgG4 and IgE. IL-10 is produced by T cells, NK cells, B cells, and monocytes. IL-21 provides co-stimulation to B cells, induces plasma cell differentiation, immunoglobulin secretion and class switch (123, 124). IL-21 is produced by activated peripheral T cells and spontaneously by CXCR5+ follicular helper T cells (48, 125, 126). Furthermore, at least 10 distinct transcription factors regulate the early stages of B-cell development (127). The transcriptional factor Pax5 is important for commitment of lymphoid progenitors to the B lymphocyte lineage. It fulfills a dual role by inhibiting non-B lineage genes and simultaneously activating B-cell lineage-specific genes (128).

**Immunosuppressive drugs**

Every transplant recipient is treated with immunosuppressive drugs to reduce the chance of rejection. Generally, these immunosuppressive drugs must be taken for the rest of the patients’ life. Today, a wide variety of immunosuppressive drugs is available, each interfering with different steps in the immune response (10, 129). The initial immunosuppressive therapy after transplantation usually consists of a combination of a calcineurin-inhibitor, a proliferation inhibitor, and corticosteroids (Figure 3). Depending on the transplant center, patients also receive induction therapy in the form of T-cell depleting antibodies (e.g. anti-thymocyte globulin, ATG) or antibodies against CD25 (130, 131). Most immunosuppressive drugs mainly target the T-cell response, while B-cell activation and subsequent antibody formation is more difficult to suppress. About 15-20% of renal transplant recipients undergo one or more acute rejection episodes, which can usually effectively be treated with additional immunosuppressive drugs. Cellular rejection is mostly treated with high dose of corticosteroids. In case of unresponsiveness to corticosteroids, ATG is given, which reduces the number of T cells. Treatment of humoral rejection is more difficult. Protocols using plasmapheresis, intravenous immunoglobulin (IVIG) (132), B-cell (133, 134) and plasma cell depleting (135) agents have all been described.

The use of powerful immunosuppressive drugs in organ transplantation has greatly improved the outcome, but is associated with the occurrence of many side effects such as increased susceptibility to infections (136), increased tumor incidence (137) drug toxicity (138) and cardiovascular disease (139). Therefore, minimizing the dose of immunosuppressive drugs is essential and more specific agents are needed (140, 141).

**Calcineurin-inhibitors**

Cyclosporin and tacrolimus (FK506) are powerful immunosuppressive drugs which inhibit T-cell activation via the inhibition of calcineurin. Upon T-cell activation via the TCR, the intracellular Ca\(^{2+}\) levels are increased and the phosphatase calcineurin gets activated. Subsequently, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) enabling it to enter the nucleus and to bind to the promoter of several genes, including the IL-2 gene. Cyclosporin binds to cyclophilin (142),
and tacrolimus to FK506-binding protein (FKBP-12) (143) thereby preventing the activation of calcineurin and the downstream signal transduction. The blockade of cytokine gene transcription impairs the activation and proliferation of T cells.

Tacrolimus has been associated with a lower incidence of acute rejection compared to cyclosporin, but one year graft survival rates are comparable with both drugs (144). An advantage of tacrolimus over cyclosporin is that it is associated with a lower incidence of hypertension and hyperliopidemia (145). An important disadvantage of tacrolimus is its diabetogenic potential.

**Corticosteroids**

Corticosteroids, such as prednisolone, are used in transplantation as maintenance therapy as well as in the treatment of acute rejection (146). Upon binding to intracellular glucocorticoid receptors, the complex is translocated to the nucleus where it inhibits the transcription of several genes (147), including proinflammatory cytokines (148). Corticosteroids are widely used due to their anti-inflammatory effects.

Figure 3. Targets of commonly used immunosuppressive drugs in renal transplantation.

Antigen-specific T-cell receptor activation results in calcineurin activation, which can be inhibited by cyclosporin and tacrolimus. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) enabling it to enter the nucleus binding to the promoter of several genes, including the IL-2 gene. Corticosteroids inhibit the transcription of different cytokines. Sirolimus acts on cytokine-signal transduction. The anti-proliferative agents mycophenolate mofetil (MMF) and azathioprine interrupt DNA replication by inhibiting purine synthesis.
Proliferation inhibitors

Anti-proliferative immunosuppressive drugs include mycophenolate mofetil (MMF) and azathioprine. MMF is converted to the active form mycophenolic acid (MPA), which inhibits an enzyme essential for de novo purine synthesis (149). This effect is rather specific for lymphocytes because other cells can obtain purines by the salvage pathway (150). Azathioprine is metabolized to 6-mercaptopurine (6-MP) and then converted to 6-thioguanine (6TG) (151). These compounds are incorporated in the DNA and prevent replication.

Monoclonal antibodies

The development of monoclonal antibodies (mAbs), directed against specific cell types or markers, has allowed more specific targeting of cells and their signaling pathways. MAbs are widely used in the treatment of malignancies, transplant rejections, as well as a range of autoimmune diseases such as rheumatoid arthritis. In transplantation, mAbs used include basiliximab, alemtuzumab and rituximab. Basiliximab is a chimeric mouse-human monoclonal antibody directed against the α-chain of the IL-2 receptor (CD25). Treatment with this agent does not result in depletion of CD25-positive cells but prevents full T-cell activation (152). Alemtuzumab (Campath-1H) is a humanized monoclonal depleting antibody directed against CD52 expressed on T cells, B cells, NK cells, monocytes, macrophages and DCs (153, 154).

Although treatment with mAbs is usually well tolerated, administration with some mAbs can be associated with acute infusion reactions, caused by various mechanisms including cytokine release syndrome (CRS) (155). Treatment with alemtuzumab leads to a first-dose CRS with elevated levels of IL-6, TNFα, and IFNγ, which appeared to be dependent on Fc-receptor (CD16) ligation on NK cells (156). After treatment with rituximab, especially patients with high B-cell counts such as those with a B-cell lymphoma, can develop a CRS (157). It is unknown which cells are responsible for this cytokine release and whether the cytokine release influences clinical outcome in terms of rejection or graft survival.

Rituximab

Rituximab (RTX) is a chimeric IgG1 monoclonal antibody that recognizes CD20 expressed on human B cells. This molecule is expressed throughout differentiation, but is lost during the final maturation of B cells into plasma cells (107; Figure 4). The antibody is constructed in such a way that the binding regions from a murine anti-human CD20 antibody, consisting of variable regions of immunoglobulin heavy and light chains, are fused to human IgG1 heavy-chain and human kappa light-chain constant regions. The Fc portion from human IgG1 was selected for its ability to fix complement and activate antibody-dependent cellular cytotoxicity (107). RTX was the first monoclonal antibody that was approved by the Food and Drug Administration (FDA) for the treatment of cancer, specifically B-cell non-Hodgkin’s
lymphoma (NHL) (158, 159). Since then it has been widely used in the treatment of malignancies, autoimmune diseases and in renal transplantation. RTX depletes B cells in three ways: through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and apoptosis (160). Although the clinical effectiveness of RTX is no longer in question, the exact contribution of each of these processes in vivo remains unclear. It is assumed that ADCC is the most dominant player, followed by CDC. Cell death through apoptosis is thought to be minimally involved (160-163). Recently, the mechanism of anti-CD20-mediated B-cell depletion was described in mice (164). Using intravital imaging, the authors revealed that after treatment of MAFIA mice with mouse anti-CD20 Ab, the Kupffer cells in the liver are responsible for the abrupt arrest and engulfment of B cells.

CD20 is a non-glycosylated transmembrane protein of 35 kDa (165) without a known ligand. It is expressed on the surface of human B cells and also weakly on a small subset of T cells (166). The CD20 protein is expressed at high levels, is not shed from the cell surface, and does not internalize upon antibody binding (167), which makes it an ideal target for the recruitment of effector cells expressing Fcγ receptors (168). In vitro studies suggest that CD20 is involved in the regulation of Ca^{2+} influxes across the plasma membrane (169) and in the regulation of B-cell activation and proliferation (170). Although treatment with CD20 mAbs depletes normal and malignant B cells in vivo, mAb crosslinking of CD20 alters Ca^{2+}

![Diagram of Rituximab binding to CD20](image)

**Figure 4. Rituximab.**

Rituximab targets B cells by binding specifically to CD20 on the cell surface.
homeostasis and could lead to apoptosis in vitro (171). The CD20 gene is strongly conserved between mouse and human. CD20-deficient mice were found to have normal B cells, however the CD19-induced calcium responses were reduced (172). More recently, Kuijpers et al. described that CD20 has a central role in the generation of T-cell-independent antibody responses (173). The observed CD20 deficiency in a 4 year old girl did not affect the differentiation of precursor B cells in the bone marrow, but did reveal a severe reduction of class-switched memory (IgD CD27+ ) B cells.

**AIM AND OUTLINE OF THIS THESIS**

Short-term allograft loss due to acute rejection has almost been eliminated by the use of effective immunosuppressive drugs, while long-term allograft survival can still be improved. Chronic rejection is one of the major causes of long-term allograft loss. T cells play a central role in the pathogenesis of rejection and therefore most immunosuppressive drugs are directed against alloreactive T cells. However, B cells can also play a major role in allograft rejection. Increased attention for the role of antibodies and B cells in the pathogenesis of rejection has been elicited by the association between the presence of pre-transplant anti-HLA antibodies in serum and B-cell clusters in renal allografts during acute rejection on the one hand and an increased risk for allograft failure on the other hand (28; 174). It is currently well recognized that B cells are not only involved in antibody production, but can also act as professional antigen presenting cells or produce of various cytokines leading to an increased immune response. Accordingly, anti-B-cell therapy has been shown to be effective in chronic inflammatory diseases mediated by T and B cells, such as rheumatoid arthritis (175) and multiple sclerosis (176). Consequently, depletion of B cells might be an important novel strategy to reduce the incidence of allograft rejection in renal transplant patients. The aim of this thesis is to investigate the effectiveness of the B-cell depleting agent RTX as induction therapy after renal transplantation and to explore the effects of RTX beyond peripheral B-cell depletion.

In chapter 2, we evaluated the efficacy and safety of RTX when added to standard triple immunosuppressive therapy after renal transplantation in a double-blind, placebo-controlled study. The peripheral depletion of B cells induced by RTX is followed by a repopulation phase during which a change in the subset distribution of B cells may occur. In addition, given the close interaction between B and T cells, B-cell depletion might also change the T-cell repertoire. Our study population provided the unique opportunity to perform a detailed study on the effects of RTX on the time course of phenotype and function of peripheral B and T cells in a homogeneous group of renal transplant patients who did not experience a rejection episode or CMV infection (chapter 3).
RTX has been used since 1993 to treat patients with malignant B-cells lymphoma, however still little is known about its effects on primary human B cells. Therefore, we studied the direct effects of RTX on the proliferation, activation and differentiation of purified peripheral blood B cells of healthy donors (chapter 4). From other studies, we learned that a single dose of RTX induces a nearly complete B-cell depletion in peripheral blood, however there often remains a residual B-cell population in secondary lymphoid organs. To study the functional properties of this non-depleted, but potentially modulated B cells that remain present in secondary lymphoid organs after treatment with RTX, we collected lymph nodes during renal transplant surgery in patients who received RTX 4 weeks earlier in preparation for an ABO-incompatible renal transplantation. In chapter 5 we compared the phenotypic and functional properties of B cells isolated from lymph nodes of RTX-treated patients to B cells isolated from lymph nodes collected from renal transplant patients not treated with RTX. In chapter 6, we focused on cytokine release which is known to be associated with anti-T-cell therapy and might also accompany the administration of RTX when given as induction therapy during renal transplantation. We examined the levels of various pro- and anti-inflammatory cytokines and chemokines after the start of the RTX infusion and compared this to levels in placebo-treated patients. Moreover, we performed in vitro studies to decipher the source of the cytokines that were released.

Finally, in chapter 7 the findings reported in this thesis are summarized and discussed, and future perspectives are outlined.
Chapter 1

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CHAPTER 2

RITUXIMAB AS INDUCTION THERAPY
AFTER RENAL TRANSPLANTATION;
A RANDOMIZED, DOUBLE-BLIND,
PLACEBO-CONTROLLED STUDY OF
EFFICACY AND SAFETY

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submitted for publication
ABSTRACT

Background. A variety of data indicates a role for B cells in acute renal allograft rejection. We evaluated the efficacy and safety of the anti-B-cell antibody rituximab when added to standard immunosuppression.

Methods. In a single centre, double-blind, placebo-controlled study, 280 adult renal transplant patients were randomized between a single dose of rituximab (375 mg/m²) or placebo during transplant surgery. Patients were stratified according to panel-reactive antibody (PRA) value and rank number of transplantation. Maintenance immunosuppression consisted of tacrolimus, mycophenolate mofetil, and steroids. The primary end point was the incidence of biopsy proven acute rejection (BPAR) within six months after transplantation. Secondary end points included the incidence of infections and malignancies within 24 months, graft function, and patient and graft survival. The study is registered with the ClinicalTrials.gov Register, number NCT00565331.

Findings. The incidence of BPAR was comparable between rituximab-treated (23/138, 16.7%) and placebo-treated patients (30/142, 21.2%, P=0.25 by log-rank test). Immunologically high risk patients (PRA >6% or re-transplant) receiving placebo had a significantly higher incidence of rejection (13/34, 38.2%) compared to other treatment groups (rituximab-treated immunologically high risk patients, and rituximab- or placebo treated immunologically low risk (PRA ≤6% or first transplant) patients (17.9%, 16.4%, and 15.7%, P=0.004 by log-rank test). Severity of rejection, patient and graft survival, and graft function, were not influenced by rituximab. Neutropenia (<1.5 x 10⁹/L) occurred more frequently in rituximab-treated patients (24.3% vs. 2.2%, P<0.001). After 24 months, the cumulative incidence of infections and malignancies was comparable.

Interpretation. A single dose of rituximab added to standard immunosuppression may reduce the incidence of BPAR in immunologically high risk patients. Treatment with rituximab is safe; the association with a higher incidence of neutropenia did not lead to more infections.
INTRODUCTION

With the current immunosuppressive treatment consisting of a calcineurin inhibitor, mycophenolate mofetil, and prednisolone, the incidence of acute rejection after renal transplantation is acceptably low. Since acute rejection is one of the main predictors of chronic transplant glomerulopathy (1). Further lowering of incidence of acute rejection, e.g. by the additional use of IL-2 receptor antagonists or polyclonal anti-T-cell antibodies, might improve long term outcome (2, 3). Increased attention for the role of antibodies and B cells in acute rejection has been elicited by the negative prognostic impact of donor-specific anti-HLA antibodies (4), the presence of B-cell clusters in biopsies of patients with severe rejection (5), as well as the frequent finding of capillary deposition of C4d in patients with acute rejection. In addition, anti-B-cell therapy has been shown to be effective in diseases that were considered to be mainly T-cell driven, like rheumatoid arthritis (6, 7).

Based on these considerations, we chose to investigate the effectiveness of the anti-B-cell monoclonal antibody rituximab (RTX) as induction therapy after renal transplantation. RTX induces long-lasting B-cell depletion in peripheral blood with limited short- and long-term toxicity (8, 9). Most experience with RTX in renal transplantation stems from its use in ABO-incompatible transplantation, where low rates of acute rejection were observed (10). At time of initiation of the current study, no data were available on the effect of RTX on acute rejection in ABO-compatible transplantation. We tested the hypothesis that adding a single dose of RTX to an immunosuppressive regimen consisting of tacrolimus, mycophenolate mofetil and steroids would reduce the incidence of biopsy proven acute renal allograft rejection (BPAR).

METHODS

Study design and patients

We performed a single centre, randomized, stratified, double-blind, placebo-controlled study at the Radboud University Medical Centre, Nijmegen, The Netherlands, from December 2007 to June 2012. All patients of 18 years or older who were scheduled to receive a renal allograft from either a living or deceased ABO compatible donor were screened for eligibility by the nephrologist on call. To be included, the immunosuppressive treatment had to consist of a combination of tacrolimus, mycophenolate mofetil, and prednisolone. Induction therapy with IL-2 receptor antagonists or anti T-cell antibodies was not part of our standard therapy, and was not allowed for this study. Other exclusion criteria were: a HLA-identical living donor; haemolytic uremic syndrome as original kidney disease; focal segmental glomerulosclerosis that had recurred in a previous graft; three or more previously failed grafts; a current or historic panel-reactive antibody (PRA)
value ≥85%; total white blood cell count <3.0 x 10⁹/L; platelet count <75 x 10⁹/L; active infection with hepatitis B, hepatitis C, or HIV; a history of tuberculosis; and previous treatment with RTX. Female patients at risk for pregnancy had a negative serum pregnancy test before randomisation and agreed to use contraception for 12 months. The PRA value was defined as the percentage of panel cells that reacts with patient serum in the complement-dependant cytotoxicity screening. The panel cells consisted of lymphocyte suspensions obtained from 60 different healthy individuals selected for HLA A, B, DR, and DQ as to achieve a maximum ability to detect anti-HLA antibodies (11).

All patients provided written informed consent before study entry. The study was approved by the Committee on Human-Related Research Arnhem–Nijmegen, conducted according to the Declaration of Helsinki and good clinical practice guidelines, and reported according to CONSORT guidelines (12).

Randomisation and study medication

Patients were randomized in a 1:1 ratio to treatment with RTX or placebo. Since we hypothesised that the effects of RTX could be unequal in immunologically high- and low- risk patients, we stratified for PRA according to the Eurotransplant cut-off value of 6% for allo-sensitization and history of prior transplantations (first vs. re-transplant). For allocation, a computer-generated list of random numbers was used for each of the four strata, prepared by an independent investigator. This list containing study number and treatment allocation was only accessible for authorised nurses, who signed confidentiality statements. For every new included patient, the lowest available study number was handled to one of the authorised nurses, who prepared study medication according to the randomisation list.

Patients randomized to RTX were treated with a single dose of 375 mg/m² intravenously during surgery. The required dose was diluted in a 500 ml bag of 0.9% sodium chloride. In placebo-treated patients infusion consisted of an identical 500 ml bag. Both bags had an identical appearance and were labelled ‘study medication’. At the start of the operation both groups received standard antibiotic prophylaxis next to 100 mg prednisolone and 2 mg clemastin intravenously to prevent allergic reactions to RTX. Infusion of study medication was started 30 minutes thereafter at a rate of 60 ml/hour, increased every 30 minutes to a maximum rate of 200 ml/hour. After the operation prednisolone was continued intravenously for three days at 100 mg/day, followed by 15–25 mg/day prednisolone orally, according to bodyweight, and tapered to 0.1 mg/kg/day. Tacrolimus (Prograft, Astellas Pharma) was given in a dose of 0.2 mg/kg/day, divided in two doses. The target trough levels were 15–20 ng/ml in the first two weeks post transplant, 10–15 ng/ml during weeks 3–6, and 5–10 ng/ml thereafter. Mycophenolate mofetil (CellCept, Hoffman-La Roche) was started at 2000 mg/day, divided in two doses, and reduced to 1500 mg/day after two weeks unless patients weighed more than 90 kg. Additional treatment consisted of trimethoprim/sulfamethoxazole 480 mg/day
for the first 3 months, and thrice weekly thereafter until one year post-transplant. Cytomegalovirus (CMV) seronegative patients who received a kidney from a CMV seropositive donor were treated prophylactically with valganciclovir during the first three months. Additionally, valganciclovir prophylaxis was given for two months after treatment with anti-T-cell antibodies when either the donor or recipient was CMV seropositive.

First-line anti-rejection therapy consisted of methylprednisolone for three consecutive days in a dose of 500-1000 mg/day intravenously. Steroid-resistant rejections were treated with anti-T-cell antibodies (Rabbit anti-thymocyte globulin [Thymoglobulin], Genzyme; Muromonab-CD3 [OKT3], Janssen-Cilag; alemtuzumab [Campath], Genzyme) according to local practice. Rejection was considered steroid-resistant if no stabilization or improvement of graft function occurred within five days after the first methylprednisolone dose.

Efficacy and safety

The primary end point was the incidence and severity of BPAR within the first six months after transplantation. For patients with more than one biopsy available during a single rejection episode, the biopsy score with the highest Banff grade was used for analysis. Borderline rejections were excluded. Biopsies were scored independently by two blinded pathologists according to the updated Banff 07 criteria (13). In case of different conclusions, biopsies were re-evaluated collectively. Protocol graft biopsies were not performed.

Secondary end points included the estimated glomerular filtration rate (eGFR) at six months (graft failure was recorded as 0 ml/min), cumulative incidence of infections and malignancies at six and 24 months, and patient and graft survival at six months and at end of follow-up. All serious adverse events were recorded during 24 months.

Number and phenotype of B cells

Blood was taken before and at several time points up to 60 months after transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Lymphoprep (Lucron, Dieren, The Netherlands) and stored in liquid nitrogen until further use. Cell phenotypes were analyzed by 10-color flow cytometry (Navios, Beckman-Coulter, Fullerton, USA). The following fluorochrome-conjugated monoclonal antibodies were used to study B cells: CD19(J3-119)-APC Alexa Fluor 750, CD27(1A4-CD27)-PeCy5.5, CD45(J.33)-Krome Orange and IgD(IADB6)-FITC (Beckman-Coulter). Isotype controls or unstained cells were used for gate settings. Data were analyzed using Kaluza software (Beckman-Coulter).

The B-cell phenotype at time of transplantation was analyzed in 26 immunologically high risk patients (for definition see below), based on availability
of the samples. For comparison we selected 28 immunologically low risk patients matched for age, gender, type of dialysis, and CMV status.

**Statistical analysis**

To detect a decrease in rejection incidence from 15% to 5% with a two-sided 5% significance level and a power of 80%, the required sample size was 140 patients per treatment arm. The trial was not powered to test superiority in the different strata. After 70 patients had reached a follow-up of six months, a planned interim safety analysis was performed to test the cumulative incidence of infections and malignancies (14). This interim analysis was not performed with the intention to test efficacy or futility.

Statistical testing was performed according to distribution and type of data (unpaired T test, Mann-Whitney U, or Fisher’s exact tests). Time to first BPAR, allograft loss, and death were analyzed with the Kaplan–Meier method, and differences were assessed by the log-rank test.

In a secondary analysis, the four strata were grouped as follows: patients with a re-transplant or PRA value >6% were considered to be immunologically high risk, and those with a first transplant and PRA value ≤6% were regarded as immunologically low risk. Comparison of subgroups was performed by one-way ANOVA, Kruskal-Wallis, and chi-square tests. All data were analyzed on an intention-to-treat basis. Analyses were performed with IBM SPSS Advanced Statistics 20.0. This study is registered with ClinicalTrials.gov register, number NCT00565331.

**Role of the funding source**

Funding for the clinical trial was provided by Hoffmann–La Roche and Astellas Pharma. RTX (MabThera, Hoffman-La Roche) was donated. Both companies were informed of the results and had no role in study design, data collection, analysis, interpretation, or writing of the report. The Dutch Kidney Foundation supported the ex vivo study of B cells.

**RESULTS**

Between December 2007 and June 2012, 488 adult renal transplant candidates were evaluated for eligibility and 281 patients were included, of whom 139 patients were randomized to RTX and 142 to placebo (Figure 1). One patient did not undergo transplantation and was therefore excluded from all analyses. Overall, the groups were well balanced with respect to demographic, clinical, and donor characteristics (Table 1).

Infusion with RTX was well tolerated and all but six patients received the full dose (Figure 1). One patient experienced an anaphylactic reaction during surgery, which was attributed to RTX. She recovered uneventfully. Temporary interruption
### Table 1. Baseline characteristics of the patients*

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients</th>
<th>Rituximab (n=138)</th>
<th>Placebo (n=142)</th>
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<td><strong>Age (yr)</strong></td>
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<td>49.8 ± 12.3</td>
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<tr>
<td>**Male sex (%)††</td>
<td></td>
<td>69.6</td>
<td>63.4</td>
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<tr>
<td>**White race (%)‡‡</td>
<td></td>
<td>94.9</td>
<td>96.5</td>
</tr>
<tr>
<td><strong>Cause of end-stage renal disease (%)</strong></td>
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<tr>
<td>Hypertension / vascular damage</td>
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<td>7.0</td>
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<tr>
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<tr>
<td><strong>Type of donor (%)</strong></td>
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<td><strong>Patients with re-transplant (%)</strong></td>
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<td>10.6</td>
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<td><strong>Cold-ischemia time — deceased donors only (hr)</strong></td>
<td></td>
<td>17.2 ± 5.1</td>
<td>17.4 ± 5.5</td>
</tr>
<tr>
<td><strong>Cytomegalovirus serologic status — donor positive, recipient negative (%)</strong></td>
<td>21.7</td>
<td>23.2</td>
<td></td>
</tr>
</tbody>
</table>

* Values are presented as mean ± standard deviation or median (range). Overall group differences were not significant.
†† *P* =0.02 for all strata calculated with the chi-square test.
‡‡ Race was determined by the investigator.
§§ *P* <0.01 for all strata calculated with the chi-square test.
Rituximab induction therapy in renal transplantation

<table>
<thead>
<tr>
<th>Immunologically low risk patients</th>
<th>Immunologically high risk patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>rituximab (n=110)</td>
<td>placebo (n=108)</td>
</tr>
<tr>
<td>51.0 ± 13.6</td>
<td>50.6 ± 11.8</td>
</tr>
<tr>
<td>71.8</td>
<td>69.4</td>
</tr>
<tr>
<td>94.5</td>
<td>96.3</td>
</tr>
<tr>
<td>28.2</td>
<td>33.3</td>
</tr>
<tr>
<td>4.5</td>
<td>5.6</td>
</tr>
<tr>
<td>7.3</td>
<td>5.6</td>
</tr>
<tr>
<td>6.4</td>
<td>7.4</td>
</tr>
<tr>
<td>22.7</td>
<td>26.9</td>
</tr>
<tr>
<td>16.4</td>
<td>13.0</td>
</tr>
<tr>
<td>14.5</td>
<td>8.4</td>
</tr>
<tr>
<td>60.9</td>
<td>61.1</td>
</tr>
<tr>
<td>10.0</td>
<td>11.1</td>
</tr>
<tr>
<td>29.1</td>
<td>27.8</td>
</tr>
<tr>
<td>54.7 ± 11.4</td>
<td>53.4 ± 9.1</td>
</tr>
<tr>
<td>3.26 ± 1.60</td>
<td>3.21 ± 1.49</td>
</tr>
<tr>
<td>0 (0 – 6)</td>
<td>0 (0 – 5)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16.7 ± 5.2</td>
<td>17.0 ± 5.3</td>
</tr>
<tr>
<td>21.8</td>
<td>26.9</td>
</tr>
</tbody>
</table>

of the infusion, mainly due to hypotension, occurred in seven RTX-treated patients (5.1%) compared to five placebo-treated patients (3.5%, \(P = 0.57\) by Fisher’s exact test).

Analysis of B cells in peripheral blood in 20 CMV-negative patients without BPAR, confirmed nearly complete depletion in RTX-treated patients as compared to placebo-treated patients at six months after transplantation (median CD19+ B cells and range; 0.6/µl (0/µl – 16.4/µl) vs. 141/µl (31/µl – 458/µl); \(P < 0.001\).
Chapter 2

488 Patients were assessed for eligibility

141 Were ineligible
18 Received a HLA identical kidney
9 Had HUS as primary renal disease
11 Had a history of primary FSGS with recurrence
6 Had a PRA >85%
3 Had received ≥ three previous transplants
7 Were previously treated with rituximab or had participated in the current trial before
10 Received cyclosporine instead of tacrolimus
5 Had a history of HIV, Hepatitis B, or Hepatitis C
7 Had a history of tuberculosis
30 Participated in another clinical trial
11 Underwent an ABO-incompatible transplantation
27 Were unable to give informed consent (either due to language problems or impaired cognition)
7 Were started on an incompatible immunosuppressive regimen (mostly basiliximab induction)

281 Underwent randomization

139 Were assigned to receive rituximab
1 Did not undergo transplantation

138 Underwent transplantation

6 Did not receive the full dose
3 Had side effects during infusion
2 Had problems with anesthesia before infusion
In 1 infusion was stopped after finding a mass in the caecum

138 Were included in the analysis
0 Were lost to follow-up

142 Were assigned to receive placebo

142 Underwent transplantation

1 Did not receive the full dose
1 Had side-effects during infusion

141 Received the full dose

0 Did not undergo transplantation

6 Did not receive the full dose
3 Had side effects
2 Had problems with anesthesia before infusion
1 Infusion was stopped after finding a mass in the caecum

142 Were included in the analysis
0 Were lost to follow-up

Figure 1. Trial profile of all patients.
The primary outcome, BPAR within six months after transplantation, occurred in 23 of the 138 RTX-treated patients (16.7%), compared to 30 of 142 placebo-treated patients (21.1%, \( P = 0.25 \) by log-rank test, Figure 2A). Based on the stratification according to PRA value and rank number of transplantation, we grouped the four strata to form a immunologically low risk group (n=218) and an immunologically low risk group (n=218) and an immunologically

**Figure 2.**
(A) Cumulative probability of biopsy proven acute rejection (BPAR) in all patients. (B) Cumulative probability of BPAR in immunologically low vs. high risk patients.
high risk group (n=62). Immunologically high risk patients receiving placebo had a significantly higher incidence of acute rejection compared to immunologically low risk patients (RTX- or placebo-treated) and RTX-treated immunologically high risk patients (38.2% vs. 16.4%, 15.7%, and 17.9%, P =0.004 by log-rank test, Figure 2B). When the group of immunologically high risk patients was analyzed separately, there was a clear trend towards a lower incidence of BPAR with RTX treatment (17.9% vs. 38.2%, P =0.06 by log-rank test).

Since RTX appeared to be more effective in immunologically high risk patients, we studied B-cell number and phenotype in a peripheral blood sample of immunologically high risk and immunologically low risk patients obtained immediately before transplantation. The number of CD19$^+$ B cells, especially CD27$^+$ memory B cells, was higher in the immunologically high risk patients (CD19$^+$CD27$^+$ memory B cells 35 cells/µl [12-152] vs. 24 cells/µl [5-78], P =0.02 by Mann-Whitney U test, Figure 3).

Most rejections were T-cell mediated according to the Banff classification (Table 2). RTX-treated patients tended to have less antibody mediated rejections (ABMR), compared to placebo-treated patients (4/138, 2.9% vs. 11/142, 7.7% P =0.11 by Fisher’s exact test). At six months, there were small but significant differences in maintenance immunosuppression between RTX and placebo-treated patients (Table 3).

Figure 3. Pre-transplant levels of B cells in immunologically low vs. high risk patients.

Absolute numbers (with medians) of total CD19$^+$, naive (CD19$^+$IgD$^+$CD27$^-$) and memory (CD19$^+$CD27$^+$) B cells in pre-transplantation blood samples of 26 immunologically high risk and 28 matched immunologically low risk patients. P values are calculated with the Mann-Whitney U test.
Patient and graft survival (at six months, and after a median duration of follow-up of 3.2 years, range 1.3–5.7 years) as well as graft function were comparable between the RTX and placebo group (Table 4). One RTX-treated patient was diagnosed with progressive multifocal leukoencephalopathy and died shortly thereafter. The overall incidence of infections or malignancies was not higher after treatment with RTX compared to placebo.

During the first six months after transplantation, treatment with RTX was associated with a significantly higher cumulative incidence of grade 2 or more severe leucopenia (19.0% vs. 1.4%, \( P < 0.001 \) by Fisher’s exact test) and neutropenia (24.3% vs. 2.2%, \( P < 0.001 \) by Fisher’s exact test, Table 4).

**DISCUSSION**

Our data show that induction therapy with a single dose of RTX at the time of renal transplantation is safe but ineffective to reduce the incidence of BPAR in the general population of renal transplant patients. The stratification of our patients according to re-transplantation and PRA, enabled comparison of immunologically high and low risk patients. We observed that within the whole population, immunologically high risk patients who did not receive RTX had the highest incidence of BPAR. Furthermore, a separate analysis of the immunologically high risk patients showed a clear trend towards a lower incidence of BPAR in RTX-treated patients. Notably, the study was not sufficiently powered for this secondary analysis within a subgroup of 62 patients. Interestingly, the incidence of ABMR tended to be lower after RTX, especially in immunologically high risk patients. Altogether, these results suggest a protective effect of RTX against acute rejection in this specific group of transplant recipients. With the current median duration of follow-up of 3.2 years, this has not resulted in improved graft function or graft survival.

In a recent study with 140 patients, Tydén et al. showed no significant effect of RTX on the incidence of BPAR within six months after transplantation, although a tendency toward fewer and milder rejection episodes in the RTX group was observed (11.8% vs. 17.6%, \( P = 0.32 \)) (15). In the current study, we included twice as many patients and stratified for immunological risk.

It is tempting to speculate that a protective effect of RTX in immunologically high risk patients could be explained by reducing the relatively high pre-transplant levels of memory B cells in our immunologically high risk patients. Indeed, an increase in circulating memory B cells has been associated with acute rejection in paediatric renal transplant recipients (16), while heart transplant recipients with higher percentages of naive B cells had a lower risk of acute rejection (17). However, we and others have previously shown that memory B cells are more resistant to depletion by RTX than naive B cells (18, 19). Interestingly we have noticed that RTX can affect B-cell phenotype and function, resulting in an altered outcome of
### Table 2. Incidence and type of biopsy proven acute rejection (BPAR)*

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients</th>
<th>Immunologically low risk patients</th>
<th>Immunologically high risk patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rituximab (n=138)</td>
<td>Placebo (n=142)</td>
<td>P value</td>
<td>Rituximab (n=110)</td>
</tr>
<tr>
<td>Patients with biopsy proven rejection (no.)†</td>
<td>23</td>
<td>30</td>
<td>0.25</td>
<td>18</td>
</tr>
<tr>
<td>Patients with steroid-resistant biopsy proven rejection (no. – % of biopsy proven rejections)‡</td>
<td>10 (43.5)</td>
<td>15 (50.0)</td>
<td>0.64</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>T-cell mediated rejection (no.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IA</td>
<td>5</td>
<td>6</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Type IB</td>
<td>2</td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Type IIA</td>
<td>10</td>
<td>9</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Type IIB</td>
<td>2</td>
<td>2</td>
<td></td>
<td>2</td>
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<tr>
<td>Antibody mediated rejection (ABMR; no.)</td>
<td>1</td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Combined rejections (no.)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ABMR + Type IIA</td>
<td>2</td>
<td>9</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>ABMR + Type IIB</td>
<td>1</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

* Biopsies were independently scored by two pathologists according to the Banff 07 classification (13). If patients experienced multiple rejection episodes, the most severe rejection was reported. P values calculated with the log rank test.
† Calculated with the T test.
‡ Calculated with the one-way ANOVA test.
Table 3. Maintenance immunosuppression*

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients</th>
<th>Immunologically low risk patients</th>
<th>Immunologically high risk patients</th>
<th>Rituximab (n=138)</th>
<th>Placebo (n=142)</th>
<th>P value</th>
<th>Rituximab (n=84)</th>
<th>Placebo (n=81)</th>
<th>P value</th>
<th>Rituximab (n=20)</th>
<th>Placebo (n=26)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At three months after transplantation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trough levels of tacrolimus – ng/ml</td>
<td>8.3 ± 2.7</td>
<td>8.4 ± 2.1</td>
<td>0.78</td>
<td>8.0 ± 2.5</td>
<td>8.4 ± 2.1</td>
<td>9.2 ± 3.2</td>
<td>8.1 ± 2.1</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycophenolate mofetil dose – mg/day</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2500)</td>
<td>0.081</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2500)</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2500)</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid dose – mg/day</td>
<td>11.7 ± 3.3</td>
<td>11.7 ± 4.1</td>
<td>1.0</td>
<td>11.7 ± 3.3</td>
<td>11.9 ± 4.2</td>
<td>11.8 ± 3.5</td>
<td>11.1 ± 4.1</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>At six months after transplantation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trough levels of tacrolimus – ng/ml</td>
<td>7.4 ± 1.7</td>
<td>8.0 ± 2.2</td>
<td>0.047</td>
<td>7.4 ± 1.7</td>
<td>7.9 ± 2.4</td>
<td>7.5 ± 1.6</td>
<td>8.1 ± 1.6</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycophenolate mofetil dose – mg/day</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2500)</td>
<td>0.037</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2500)</td>
<td>1500 (0 – 2000)</td>
<td>1500 (0 – 2000)</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid dose – mg/day</td>
<td>10.4 ± 5.0</td>
<td>9.3 ± 3.0</td>
<td>0.029</td>
<td>10.0 ± 3.5</td>
<td>9.5 ± 3.3</td>
<td>11.9 ± 8.5</td>
<td>8.5 ± 1.4</td>
<td>0.013</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are presented as mean ± standard deviation or median (range). Data are shown for patients on tacrolimus, mycophenolate mofetil and steroids.
† Calculated with the T test, except for the mycophenolate mofetil dose for which the Mann-Whitney U test was used.
‡ Calculated with the one-way ANOVA test, except for the mycophenolate mofetil dose for which the Kruskal-Wallis test was used.
Table 4. Secondary end points*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rituximab (n=138)</th>
<th>Placebo (n=142)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated GFR at six months – ml/min†</td>
<td>48.7 ± 20.0</td>
<td>45.6 ± 22.1</td>
</tr>
<tr>
<td>Proteinuria at six months – g/10 mmol creatinine</td>
<td>0.16 (0.10 – 6.5)</td>
<td>0.18 (0.10 – 6.7)</td>
</tr>
<tr>
<td>Allograft survival at six months (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Censored for death of patients with functioning graft</td>
<td>96.4</td>
<td>93.0</td>
</tr>
<tr>
<td>Uncensored for death of patients with functioning graft</td>
<td>94.9</td>
<td>90.1</td>
</tr>
<tr>
<td>Allograft survival at end of follow-up (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Censored for death of patients with functioning graft</td>
<td>92.0</td>
<td>88.0</td>
</tr>
<tr>
<td>Uncensored for death of patients with functioning graft</td>
<td>81.2</td>
<td>82.4</td>
</tr>
<tr>
<td>Patient survival at six months (%)</td>
<td>97.8</td>
<td>95.8</td>
</tr>
<tr>
<td>Patient survival at end of follow-up (%)</td>
<td>88.4</td>
<td>89.4</td>
</tr>
<tr>
<td>Cause of death (no.)</td>
<td></td>
<td>number of patients</td>
</tr>
<tr>
<td>Infection related</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Malignancy related</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Other causes</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Patients with ≥ 1 infection within six months (%)</td>
<td>63.8</td>
<td>62.0</td>
</tr>
<tr>
<td>Patients with CMV disease within six months (%)</td>
<td>14.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Patients with ≥ 1 infection within 24 months (%)</td>
<td>79.0</td>
<td>76.1</td>
</tr>
<tr>
<td>Patients with CMV disease within 24 months (%)</td>
<td>15.9</td>
<td>12.7</td>
</tr>
<tr>
<td>Patients with ≥ 1 malignancies within 24 months (%)</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Type of malignancies (no.)</td>
<td></td>
<td>number of patients</td>
</tr>
<tr>
<td>Skin</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Non-skin</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Patients with ≥1 serious adverse event (SAE) within 24 months (%)</td>
<td>70.3</td>
<td>64.8</td>
</tr>
<tr>
<td>Incidence of grade ≥ 2 leucopenia within six months (leucocytes &lt; 3.0 x 10^9/L) (%)‡</td>
<td>19.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Incidence of grade ≥ 2 neutropenia within six months (neutrophils &lt; 1.5 x 10^9/L) (%)‡</td>
<td>24.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Haemoglobin level at six months (mmol/L)</td>
<td>7.6 ± 1.0</td>
<td>7.6 ± 1.0</td>
</tr>
<tr>
<td>Thrombocyte count at six months (x10^9/L)</td>
<td>220 ± 69</td>
<td>222 ± 72</td>
</tr>
</tbody>
</table>
B-T-cell interaction (20). Moreover, after treatment with one dose of RTX a B-cell population remains in secondary lymphoid organs, despite complete depletion in peripheral blood (19). These remaining B cells mainly consist of switched memory (IgD−CD27+) B cells, and have different functional capacities as compared to B cells obtained from lymph nodes of patients not treated with RTX. Taken together, these data indicate that RTX can have effects beyond B-cell depletion.

Our study confirmed previous observations of a high incidence of leucopenia and neutropenia after treatment with RTX (21). The cause of this so called late-onset neutropenia remains incompletely understood. The higher incidence of neutropenia did not lead to more infections, which is in accordance with the results of other studies (22-27). Potentially worrisome are the recently published 3-year follow-up data from Tydén’s study, suggesting an increased mortality in RTX-treated patients (25). We could not confirm these results in our study.

In conclusion, our study has demonstrated that addition of RTX induction therapy to a standard immunosuppressive triple drug regimen does not reduce the incidence of acute rejection in immunologically low risk patients. Therefore, we do not recommend the use of RTX induction therapy in a population of unselected renal transplant recipients. However, our data suggest that treatment with RTX may reduce the incidence of BPAR in immunologically high risk patients to a level comparable to that in immunologically low risk patients, thereby representing a further step in improving the treatment of this specific group of patients. The results need to be confirmed in a multicenter clinical trial focused on immunologically high risk patients, with special attention for the combination or comparison with other induction agents, like basiliximab and anti-thymocyte globulin.

Acknowledgement
We thank Judith Kal-van Gestel for monitoring the data collection and analysis throughout the study. The study was performed at the nephrology unit of the Radboud University Medical Centre and we acknowledge the contribution of all physicians and nurses involved in the study. We thank the participating patients for their confidence and contribution. We acknowledge Jan van den Brand for contribution to the statistical analysis.

* Values are presented as mean ± standard deviation or median (range). For patients who have not reached follow-up of 24 months, data available until the last visit were used. Except for the incidence of leucopenia/neutropenia, group differences were not statistically significant as calculated with the T test, except for the number of serious adverse events, total days spend in hospital, and proteinuria, for which the Mann-Whitney U test was used.
† For the calculated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (ml/min/1.73 m²) = 175 x (serum creatinine/88.4)^1.54 x (Age)^0.203 x (0.742 if female) x (1.212 if African American) (30).
‡ P<0.001 calculated with the Fisher’s exact test.
REFERENCES


CHAPTER 3

LONGITUDINAL ANALYSIS OF T- AND B-CELL PHENOTYPE AND FUNCTION IN RENAL TRANSPLANT RECIPIENTS WITH OR WITHOUT RITUXIMAB INDUCTION THERAPY

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* these authors contributed equally to this work

submitted for publication
ABSTRACT

Background. Prevention of rejection after renal transplantation requires treatment with immunosuppressive drugs. Data on their in vivo effects on T- and B-cell phenotype and function are limited.

Methods. In a randomized double-blind placebo-controlled study to prevent renal allograft rejection, patients were treated with tacrolimus, mycophenolate mofetil (MMF), steroids, and a single dose of rituximab or placebo during transplant surgery. We analyzed the number and phenotype of peripheral T and B cells by multiparameter flow cytometry before transplantation, and at 3, 6, 12, and 24 months after transplantation.

Results. In patients treated with tacrolimus/MMF/steroids the proportion of central memory CD4+ and CD8+ T cells was higher at 3 months post-transplant compared to pre-transplant levels. In addition, the ratio between the percentage of central memory CD4+ and CD4+ regulatory T cells was significantly higher up to 24 months post-transplant compared to pre-transplant levels. Interestingly, treatment with tacrolimus/MMF/steroids resulted in a shift toward a more memory-like B-cell phenotype post-transplant. Addition of a single dose of rituximab resulted in a long-lasting B-cell depletion. At 12 months post-transplant, the small fraction of repopulated B cells consisted of a high percentage of transitional B cells. Rituximab treatment had no effect on the T-cell phenotype and function post-transplant.

Conclusions. Renal transplant recipients treated with tacrolimus/MMF/steroids show an altered memory T and B-cell compartment post-transplant. Additional B-cell depletion by rituximab leads to a relative increase of transitional and memory-like B cells, without affecting T-cell phenotype and function.
Life-long use of immunosuppressive drugs is required to prevent rejection after renal transplantation. Nevertheless, the continuous use of immunosuppressive drugs does not preclude the development of chronic rejection, which is a major cause of long-term allograft loss (1). T cells play an important role in the pathogenesis of rejection via the recognition of alloantigens, resulting in T-cell activation, proliferation, and differentiation into CD8+ cytotoxic T cells and CD4+ T helper cells (2). Therefore, the most commonly used immunosuppressive drugs in transplantation are directed against T cells to inhibit these processes (3). On the other hand, regulatory T cells are able to suppress the immune response and prevent allograft rejection (4). The balance between memory and regulatory T cells during the course after transplantation can be used to predict renal graft rejection following the reduction of immunosuppressive therapy (5). Next to T cells, B cells can be involved in graft rejection (6). The presence of B-cell clusters in renal grafts during acute rejection or the presence of anti-HLA antibodies before transplantation is associated with poorer graft survival (7-9). Notably, B cells can induce alloimmune responses by acting as professional antigen presenting cells, or by the production of various (pro-)inflammatory cytokines (10). Therefore, depletion of B cells in renal transplant recipients might help to prevent allograft rejection. Current immunosuppressive regimens consisting of steroids, a calcineurin-inhibitor, and mycophenolate mofetil (MMF) inhibit B-cell function directly due to inhibition of their proliferation and indirectly via the inhibition of T-cell help. B cells can also be selectively depleted by rituximab (RTX), an anti-CD20 monoclonal antibody. RTX is successfully used in the treatment of B-cell malignancies and autoimmune disorders mediated by T and B cells (11, 12).

Although the major target of RTX-based treatment was to reduce the levels of circulating autoantibodies, additional B-cell functions may be affected, such as antigen presentation and cytokine production (13). Furthermore induction of regulatory T cells (T_{REGS}) was reported after RTX treatment in patients with lupus nephritis (14). Therefore, next to its effect on B cells, RTX might decrease the chance of rejection after transplantation by affecting the T-cell compartment. Remarkably little is known about the effects of the currently used immunosuppressive strategies on the phenotype and function of T and B cells during the course after renal transplantation. Advancements in multiparameter flow cytometry have made it possible to analyze the effects of immunosuppressive agents on various T- and B-cell subsets in more detail. We had the opportunity to study the effects of standard immunosuppression (tacrolimus, MMF and steroids), with or without the addition of RTX induction therapy on the phenotype and function of T and B cells over time in renal transplant recipients participating in a randomized placebo-controlled trial, studying the efficacy and safety of RTX added to standard immunosuppression. To avoid bias by other immunological events as much as possible, we analyzed only...
Cytomegalovirus (CMV) seronegative patients who received a kidney from a CMV seronegative donor, did not experience a rejection episode, and were not treated with additional immunosuppressive drugs during the follow-up period.

MATERIALS AND METHODS

Patients

Patients were selected from a clinical trial with RTX in renal transplantation at our hospital (ClinicalTrials.gov, NCT00565331). This study investigated the effectiveness and safety of RTX for prophylaxis of acute rejection after renal transplantation. Patients were randomized between treatment with a single dose of RTX (375 mg/m$^2$) or placebo during transplant surgery. Concomitant immunosuppression consisted of tacrolimus, MMF, and steroids. Patients received 100 mg of prednisolone intravenously during the first 3 days after transplantation and subsequently an oral dose of 15-25 mg/day, tapered to a maintenance dose of 0.1 mg/kg/day. Tacrolimus was started at 0.2 mg/kg/day and the dose was subsequently adjusted to achieve whole-blood trough levels of 15-20 ng/ml during the first 2 weeks, 10-15 ng/ml during weeks 3-6, and 5-10 ng/ml from week 7. MMF was administered at 1000 mg twice daily with a dose reduction to 750 mg twice daily at 2 weeks after transplantation, and discontinued after 6 months.

To avoid bias by other immunological events as much as possible, we investigated only Cytomegalovirus (CMV) seronegative patients who received a kidney from a CMV seronegative donor (and thus did not develop CMV infection), did not experience a rejection episode, and were not treated with other immunosuppressive drugs during the follow-up period. 14 patients were included in the group treated with tacrolimus, MMF and steroids, and 12 patients in the group additionally treated with RTX. Peripheral blood mononuclear cells (PBMCs) of 4 standard-treated patients and 3 RTX-treated patients were not available at 24 months after transplantation. Table 1 summarizes the characteristics of all patients. The study was approved by the Institutional review board of the Radboudumc Nijmegen. Written informed consent was obtained from all participants.

Cells

Peripheral blood samples were obtained before transplantation, and up to 24 months after transplantation. Whole blood counts were performed and PBMCs were isolated by density gradient centrifugation using Lymphoprep (Lucron, Dieren, The Netherlands). PBMCs were cryopreserved in liquid nitrogen until analysis. For each patient longitudinal flow cytometric analysis was performed for all available samples at the same time.
Flow cytometry

For cell surface staining, the following fluorochrome-conjugated monoclonal antibodies were used: CD3(UCHT1), CD4(13B8.2), CD8(B9.11), CD19(J3-119), CD24(ALB9), CD38(LS198-4-3), CD45(J.33), CD45RO(UCHL1), CD127(R34.34) and IgD(IADB6) (all from Beckman-Coulter, Mijdrecht, The Netherlands), CD25(M-A251), CCR4(1G1), CCR6(11A9) and CXCR3(1C6/CXR3) (BD Biosciences, Erembodegem, Belgium), and BAFF-R(11C1) (BioLegend, Uithoorn, The Netherlands). Intracellular analysis of IL-2(MQ1-17H12) (BD Biosciences), IL-4(8D4-8), IL-17(EBIO64CAP17), IFN-γ(4S.B3) (eBioscience, San Diego, CA, USA), and TNFα(Mab11) (Dako, Glostrup, Denmark) was performed after fixation and permeabilization, using Fix and Perm reagent (eBioscience). Before intracellular cytokine measurement, the cells were stimulated for 4 hours with PMA (12.5 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands).

The cell phenotype was analyzed by five-color (FC500) or ten-color flow cytometry (Navios™), and data were analyzed using Kaluza® software (all from Beckman-Coulter). Isotype controls or unstained cells were used for gate settings. Cell populations >0.1% of the CD45+ lymphocyte population with a threshold of more than 50 cells were considered reliable.

Table 1. Patient characteristics.

<table>
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<tr>
<td>Peritoneal dialysis</td>
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<td>4</td>
<td></td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Median PRA, % (range)</td>
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<td>0 (0-10)</td>
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<td>Living donor, no. (%)</td>
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<td>8 (67%)</td>
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<td>HLA mismatches total, median (range)</td>
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<td>3 (1-5)</td>
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</table>
Peripheral T and B cells in renal transplant recipients

**Statistical analysis**
Continuous data are expressed as mean ± SD. Nonparametric tests were used to compare variables. One-way ANOVA was used to test differences within the triple immunosuppression-treated group over time. To test the difference between RTX- and placebo-treated patients a Mann-Whitney U-test was performed. \( P<0.05 \) was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

**RESULTS**

Phenotype and function of T cells in renal transplant recipients after treatment with tacrolimus, MMF and steroids
CD4\(^+\) and CD8\(^+\) T cells can be divided into naive, central and effector memory, and highly differentiated memory cells based on CD27 and CD45RO expression. (15). The absolute numbers of CD4\(^+\) and CD8\(^+\) T cells in peripheral blood did not change during the use of triple drug immunosuppression after transplantation (Figure 1A-C). However, the percentages of central memory (T\(_{\text{CM}}\); CD27\(^+\)CD45RO\(^+\)) CD4\(^+\) and CD8\(^+\) T cells were significantly higher at 3 months after transplantation compared to before transplantation, while the percentages of effector memory (T\(_{\text{EM}}\); CD27\(^-\)CD45RO\(^-\)) CD4\(^+\) and CD8\(^+\) T cells, and regulatory (T\(_{\text{REGS}}\); CD25\(^{hi}\)FOXP3\(^+\)) CD4\(^+\) T cells were significantly decreased. The percentages of naive (T\(_{\text{N}}\); CD27\(^+\)CD45RO\(^+\)) and highly differentiated memory (T\(_{\text{EMRA}}\); CD27\(^-\)CD45RO\(^-\)) CD4\(^+\) and CD8\(^+\) T cells were comparable to pre-transplant levels. Next to the T-cell subset distribution, the ratio between memory and regulatory T cells might be an important determinant of the risk of rejection (16). Interestingly, the CD4\(^+\) T\(_{\text{CM}}\) / CD4\(^+\) T\(_{\text{REGS}}\) ratio significantly increased from 3 months after transplantation and remained elevated compared to the pre-transplant ratio (Figure 1D). The CD4\(^+\) T\(_{\text{EM}}\) / CD4\(^+\) T\(_{\text{REGS}}\) ratio after transplantation was comparable to pre-transplant levels. Finally, we determined the expression of chemokine receptors associated with T helper (Th) 1 (CXCR3), Th2 (CCR4), and Th17 (CCR6) cells (Figure 1E). The percentage of CXCR3\(^+\) and CCR6\(^+\) CD4\(^+\) T cells increased during immunosuppressive treatment, whereas triple drug immunosuppression did not affect the percentage of CCR4\(^+\) CD4\(^+\) T cells. Also, the percentage of CD4\(^+\) T cells expressing co-stimulatory (CD28) or co-inhibitory (PD-1) receptors was comparable to before transplantation (data not shown).

Although triple drug immunosuppression had minimal effects on the phenotype of peripheral T cells, this did not preclude an effect on functional capacities. To assess the cytokine producing capacity of circulating T cells, PBMCs were ex vivo stimulated for 4 hours with PMA, ionomycin and Brefeldin A. Effector cytokine production was assessed using intracellular cytokine staining in both CD4\(^+\) and CD8\(^+\) T cells. The percentage of IL-2, IL-4, IL-17, and TNFa producing CD4\(^+\) cells decreased at 3 months after transplantation compared to pre-transplant levels.
Figure 1. Subset distribution of circulating T cells in renal transplant recipients after treatment with tacrolimus, MMF and steroids over time.

(A) Representative dot plots for a renal transplant recipient showing CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells within the CD45⁺ lymphocyte population. Circulating CD4⁺ and CD8⁺ T cells can be characterized as naive (T_N; CD27⁺CD45RO⁻), central memory (T_CM; CD27⁺CD45RO⁺), effector memory (T_EM; CD27⁻CD45RO⁺) and highly differentiated memory (T_EMRA; CD27⁺CD45RO⁻) cells. Furthermore, CD4⁺ T cells can be characterized as regulatory T cells (T_REG; CD25⁺FOXP3⁺). (B) Shown are the absolute numbers of CD4⁺ T cells and the percentages of T_N, T_CM and T_EM within the CD4⁺ T-cell population for 14 triple immunosuppression-treated patients before transplantation (t=0) and at 3, 6, 12 and 24 months after transplantation (n=10 at 24 m). (C) As described under B, for CD8⁺ T cells. (D) The ratio between the percentage of CD4⁺ T_CM or T_EM and the percentage of T_REG. (E) Longitudinal analysis of the percentages of CCR4⁺, CCR6⁺, and CXCR3⁺ cells within the CD4⁺ T-cell population of 14 triple immunosuppression-treated patients (n=10 at 24 m). Bars show standard deviation (SD). Significant differences are indicated compared to pre-transplant levels: *P<0.05, **P<0.01, ***P<0.001.
Figure 2. *Ex vivo* cytokine production by circulating T cells in renal transplant recipients after treatment with tacrolimus, MMF and steroids.

Peripheral blood mononuclear cells (PBMCs) were stimulated for 4 hours in the presence of PMA, ionomycin and Brefeldin A. Shown are the percentages IL-2, IL-4, IL-17, IFNγ or TNFα-producing cells within the CD4+ or CD8+ T-cell population of 14 triple immunosuppression-treated patients before transplantation (t=0) and at 3, 6, 12, and 24 months after transplantation (n=10 at 24 m). Bars show standard deviation (SD). Significant differences are indicated compared to pre-transplant levels: *P<0.05, **P<0.01, ***P<0.001.

Hereafter, the production of all cytokines but TNFα returned to baseline levels within 24 months after transplantation (Figure 2). *Ex vivo* IFNγ production was not affected by triple drug immunosuppression, nor was cytokine producing capacity of CD8+ T cells. Taken together, within the T-cell compartment, the most notable changes were found in the effector CD4+ T-cell pool.

Under treatment with tacrolimus, MMF and steroids, renal transplant recipients develop a memory-like B-cell phenotype

During immunosuppressive treatment, the absolute B-cell numbers in peripheral blood were comparable to pre-transplant levels up to 24 months after transplantation (Figure 3A). To define whether the composition of the peripheral B-cell compartment was affected, we characterized CD19+ B cells using the Bm1-Bm5 classification (17). Compared to pre-transplant levels, the percentages of naive Bm2 (IgD–CD38+) and transitional Bm2' (IgD–CD38++) CD19+ cells were lower, and the percentage of late memory Bm5 (IgD CD38–) cells was higher after transplantation (Figure 3B–C). This shift to a more memory-like phenotype was accompanied by an increase in the percentage of virgin naive Bm1 cells, probably needed for B-cell renewal. Accordingly, using an alternative terminology for B cells, the percentages of transitional CD24+CD38–, mature CD24+CD38–, and naive IgD–CD27– B cells were decreased, while there was a relative increase in
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CD24<sup>++</sup>CD38<sup>-</sup> memory and IgD<sup>-</sup>CD27<sup>+</sup> switched memory B cells (data not shown). At 12 and 24 months after transplantation, there was a higher percentage of CD80<sup>+</sup> and CD95<sup>+</sup> B cells as compared to before transplantation, supporting the more complete differentiation towards memory cells within the B-cell compartment.

Figure 3. Longitudinal analysis of circulating B cells in renal transplant recipients after treatment with tacrolimus, MMF and steroids.

(A) Shown are the absolute numbers of CD19<sup>+</sup> B cells of 14 triple immunosuppression-treated patients before transplantation (t=0) and at 3, 6, 12, and 24 months after transplantation (n=10 at 24 m). (B) Representative dot plots for a renal transplant recipient over time using the Bm1-Bm5 classification: Bm1 (IgD<sup>-</sup>CD38<sup>-</sup>), Bm2 (IgD<sup>-</sup>CD38<sup>+</sup>), Bm2' (IgD<sup>-</sup>CD38<sup>++</sup>), Bm3+4 (IgD<sup>-</sup>CD38<sup>+-</sup>), Early Bm5 (IgD<sup>-</sup>CD38<sup>+</sup>) and Late Bm5 (IgD<sup>-</sup>CD38<sup>-</sup>) cells within the CD19<sup>+</sup> B-cell population. (C) Shown are the percentages of the different B-cell subsets using the Bm1-Bm5 classification over time. (D) Shown are the percentages of CD80<sup>+</sup>, CD95<sup>+</sup>, and BAFF-receptor<sup>+</sup> (BAFF-R) cells within the CD19<sup>+</sup> B-cell population. (E) Overlay plot of the BAFF-R expression (MFI: median fluorescence intensity) within the CD19<sup>+</sup> B-cell population of one patient before transplantation (pre-Tx) and 24 months (24 m) after transplantation under treatment with tacrolimus, MMF and steroids. Gray line shows unstained cells. (F) Summary graph showing the BAFF-R MFI of 14 triple immunosuppression-treated patients before over time (n=10 at 24 m). Bars show standard deviation (SD). Significant differences are indicated compared to pre-transplant (pre-Tx; t=0) levels: *P<0.05, **P<0.01, ***P<0.001.
Peripheral T and B cells in renal transplant recipients

(Figure 3D and data not shown). The majority of B cells remained positive for B-cell activating factor receptor (BAFF-R) after transplantation. Interestingly, the expression level of BAFF-R increased after transplantation, as documented by an increase in median fluorescence intensity (MFI; Figure 3E). Overall, renal transplant recipients treated with tacrolimus, MMF and steroids developed a more memory-like B-cell phenotype.

A single dose of RTX results in a long-lasting B-cell depletion in peripheral blood without affecting the T-cell compartment

The participation of renal transplant recipients in a randomized double-blind placebo-controlled study evaluating the efficacy and safety of RTX when added to triple drug immunosuppression gave us the opportunity to study the effects of additional B-cell depletion on the phenotype and function of T and B cells. RTX treatment resulted in a nearly complete depletion of B cells from the peripheral lymphocyte population up to 12 months after transplantation. Remarkably, the B-cell depletion after a single dose of RTX was long lasting. The absolute numbers of B cells remained quite low at 24 months after RTX treatment with a median of 9.7 B cells/µl (range 3.0-294.8) compared to a median of 116.4 B cells/µl (range 49.7-379.7) in patients not treated with RTX (Figure 4A-B). Interestingly, at 12 months the percentage of Bm2' (IgD**CD38**+) and Bm3+4 (IgD‘CD38**’) cells was significantly higher in RTX-treated patients, while the percentages of Bm1 (IgD‘CD38’+) and Bm2 (IgD‘CD38’-) cells were lower in RTX-treated patients (Figure 4C). Accordingly, the percentages of CD24**CD38** transitional and IgD‘CD27’ switched memory B cells were higher in RTX-treated patients, while the percentages of IgD‘CD27’ naïve B cells were lower in RTX-treated patients (data not shown). Fitting with the relative increase of Bm3+4 (IgD‘CD38’+) cells, there was an increase in the percentage of CD80’ and CD95’ B cells at 12 and/or 24 months after transplantation (Figure 4D). Remarkably, the percentage of BAFF-R’ B cells was lower in RTX-treated patients at 3 and 12 months after transplantation. In addition, the BAFF-R expression (MFI) on B cells of RTX-treated patients was lower up to 24 months after transplantation (Figure 4D).

Next, we analyzed whether the long-lasting B-cell depletion with a relative increase of transitional B cells resulted in changes in the T-cell compartment. There was no significant effect of RTX treatment on the absolute numbers and percentages of CD4+ and CD8+ T cells or on the subset distribution and phenotype of these cells (Figure 4E and data not shown). In addition, in vivo B-cell depletion with RTX had no effect on the production of IL-2, IL-4, IL-17, IFNγ and TNFα by ex vivo stimulated T cells (Figure 4F).
Chapter 3

A

pre-Tx 3 m 12 m 24 m

CD3

CD19

triple IS

triple IS + RTX

B

CD19⁺ B cells

cells/µl

months after Tx

0 3 12 24

CD19

CD3

triple IS

triple IS + RTX

C

pre-Tx 3 m 12 m 24 m

triple IS

triple IS + RTX

D

CD19⁺

CD80⁺

CD95⁺

BAFF-R⁺

BAFF-R

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

E

CD4⁺

TREG

Central memory

Effector memory

CXCR3⁺

CCR6⁺

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

0 3 12 24

F

CD4⁺

IL-2⁺

IL-4⁺

IL-17⁺

IFNγ⁺

TNFα⁺

months after Tx

0 3 12 24

months after Tx

0 3 12 24

months after Tx

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months after Tx

0 3 12 24

months after Tx
Peripheral T and B cells in renal transplant recipients

**Discussion**

Despite the extensive clinical experience with currently used immunosuppressive drug regimens, there are limited data available regarding their effects on the peripheral lymphocyte compartment after kidney transplantation. One study describes the effects of cyclosporine, MMF, steroids, and anti-CD25 monoclonal antibody therapy on T and B cells of mainly CMV seropositive renal transplant recipients at 6, 24, and 60 months after transplantation (18). This therapy resulted in an increased percentage of CD4+CD25+ T_{REGS} and CD27+ memory B cells in renal transplant recipients compared to healthy donors (18), but the data were not compared with pre-transplant levels. In contrast, we performed a longitudinal analysis of T- and B-cell phenotype and function in CMV seronegative patients who received a kidney from a CMV seronegative donor and did not experience a rejection episode up to 24 months after transplantation. In this homogeneous patient population, not affected by major immunological events, we showed that treatment with the combination of tacrolimus, MMF and steroids had no effects on the total number of T and B cells. Nevertheless, these patients had a

![Figure 4. Longitudinal analysis of circulating B cells in renal transplant recipients after treatment with tacrolimus, MMF and steroids, and a single dose of rituximab (RTX) during transplant surgery.](image)
higher proportion of central memory CD4+ and CD8+ T cells at 3 months after transplantation compared to pre-transplant levels. Interestingly, the triple drug immunosuppression resulted in a shift toward a more memory-like phenotype in the B-cell population. Addition of a single dose of RTX resulted not only in a long-lasting B-cell depletion, but also in a higher percentage of transitional B cells upon B-cell recovery at 12 months post-transplant. The additional RTX treatment had no effect on the T-cell phenotype.

Although tacrolimus, MMF, and steroids mainly target T-cell activation, proliferation, and differentiation (3, 19), we found that treatment with a combination of tacrolimus, MMF, and steroids, induced only marginal changes in the peripheral T-cell phenotype. These changes were mainly present within the first 6 months after transplantation, which suggests a role for MMF, as this drug was discontinued at 6 months after transplantation. Ex vivo, the T cells collected from patients treated with triple immunosuppressive therapy were functional, suggesting that they are only suppressed when the drug is present (during treatment). In addition, we found that the ratio between CD4+ central memory and TREGS was increased under triple drug immunosuppressive therapy. Concomitantly, we observed a relative increase of CXCR3+ and CCR6+ CD4+ T cells, chemokine receptors associated with memory or activated Th1 and Th17 cells, respectively. This expression enables them to migrate toward inflammatory sites that express their cognate chemokines (20), such as observed in the graft during rejection (21) and on activated human primary tubular epithelial cells (22).

With respect to B cells, mycophenolic acid, but not tacrolimus, has been shown to inhibit the proliferation and immunoglobulin production in vitro (23). However, in patients with systemic lupus erythematosus who were treated with MMF, the number and phenotype of B cells were similar to that in controls without immunosuppressive therapy (24). In our cohort, discontinuation of MMF at 6 months after transplantation resulted in a relative increase of virgin naive Bm1 cells, while naive Bm2 cells were decreased compared to pre-transplant levels. Transitional Bm2 cells remained low up to 24 months after transplantation, suggesting that their development is mainly suppressed by treatment with tacrolimus and/or steroids. Finally, following the discontinuation of MMF, the percentage of memory B cells became comparable to levels before transplantation. Steroids were also found to have clear effects on B cells; ex vivo immunoglobulin production by PBMC was decreased during treatment with a high dose of prednisolone (60 mg) while a lower dose (30 mg) resulted in an increased production after stimulation (25). Others have described that steroids have an effect on B-cell activation, while proliferation and activation are less affected (26). Under combined treatment with tacrolimus, MMF, and steroids, our renal transplant recipients had a more memory-like B-cell phenotype compared to before transplantation. This relative increase of memory B cells was also found in a patient cohort treated with cyclosporine, MMF, steroids, and an anti-CD25 monoclonal antibody (18). The observed memory-like B-cell
phenotype was accompanied by an increased percentage of CD80+ and CD95+ B cells, which may be explained by the preferential expression of these molecules on memory-like B cells (17).

Treatment with RTX provides a highly efficient means for the (temporary) depletion of B cells, with potential suppression of B cell-associated anti-graft responses. Adding a single dose of RTX to the combination of tacrolimus, MMF, and steroids in our patients indeed resulted in a long lasting B-cell depletion in peripheral blood. A remarkable characteristic of the returning B cells was a decreased expression of the receptor for B-cell activating factor (BAFF), an essential growth factor for B cells (27). The decreased percentage of BAFF-R+ B cells after RTX treatment, which was also found in patients with rheumatoid arthritis (28) may be due to a relative increase of memory-like B cells, which have lower or no BAFF-R expression (data not shown). In addition, treatment with B-cell depleting agents has previously been shown to elevate BAFF levels (29-31), and increased BAFF levels in turn were inversely correlated with BAFF-R expression during B-cell repopulation (30, 32). Another interesting observation was an increase in the percentage of transitional B cells at 12 months after treatment with RTX compared to triple immunosuppression therapy alone. Interestingly, BAFF-R deficiency in patients with common variable immunodeficiency (CVID) was associated with B-cell lymphopenia and a relative increase in the number of transitional B cells (33). Taken together, an increase in BAFF level, reduced BAFF-R expression, and an increase in the proportion of transitional B cells appear to be interrelated phenomena which are associated with RTX treatment.

Upon activation, B cells are able to proliferate, produce various cytokines and process antigen for presentation to T cells (34-36). Previously, we showed that in vitro RTX treatment can affect B-cell phenotype and function, resulting in an altered outcome of B-T-cell interaction upon stimulation (37). However, in contrast, we did not observe any changes in the T-cell compartment in our patients treated with RTX. It should be noted that we were only able to analyze peripheral blood T cells. From a previous study in renal transplant patients, we know that a single dose of RTX leads a to nearly complete B-cell depletion in peripheral blood, but not in secondary lymphoid organs, and that these remaining B cells have different functional capacities (38). From our current data, it seems that this population of mostly memory type B cells residing in lymphoid organs does not noticeably affect the peripheral blood T-cell compartment as compared to transplant recipients on triple immunosuppression without RTX. Interestingly, several studies on patients with autoimmune disease revealed that the T-cell compartment was affected upon RTX treatment (12, 39, 40). However, in most of these patients the cumulative dose of RTX was higher than in our patients, who received only a single, relatively low dose (39).

In summary, we have demonstrated that treatment of renal transplant recipients with tacrolimus, MMF and steroids leads to alterations in the T- and
B-cell compartments. This detailed longitudinal analysis provides more insight into the immune status of renal transplant recipients with stable graft function and may be used as a reference in the monitoring of renal transplant patients.
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Chapter 4

In Vitro Effects of Rituximab on the Proliferation, Activation and Differentiation of Human B Cells

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ABSTRACT

Rituximab is a chimeric anti-CD20 monoclonal antibody used in B-cell malignancies, various autoimmune disorders, and organ transplantation. Although administration of a single dose of rituximab results in full B-cell depletion in peripheral blood, there remains a residual B-cell population in secondary lymphoid organs. These nondepleted B cells might be altered by exposure to rituximab, with subsequent immunomodulatory effects. Therefore, we analyzed in vitro the effects of rituximab on proliferation, activation, and differentiation of CD19+ B cells by means of carboxyfluorescein succinimidyl ester (CFSE)-based multiparameter flow cytometry. Rituximab inhibited the proliferation of CD27- naive, but not of CD27+ memory B cells. Interestingly, upon stimulation with anti-CD40 mAb and IL-21 in the presence of rituximab there was an enrichment of B cells that underwent only one or two cell divisions, and displayed an activated naive phenotype (CD27-IgD+CD38-/+). The potency of prestimulated B cells to induce T cell proliferation was increased by exposure of the B cells to rituximab. Of note, after stimulation with rituximab-treated B cells, proliferated T cells displayed a more Th2-like phenotype. Overall, these results demonstrate that rituximab can affect human B-cell phenotype and function, resulting in an altered outcome of B-T cell interaction.
INTRODUCTION

Rituximab (RTX) is a chimeric IgG1 monoclonal antibody that recognizes human CD20, a cell-surface glycoprotein, expressed on B cells throughout differentiation and lost during maturation to plasma cells (1, 2). RTX depletes B cells through three mechanisms: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis. Although the exact contribution of these mechanisms in vivo remains unclear, it is assumed that ADCC is the most dominant player, followed by CDC. Cell death through apoptosis is thought to be minimal (2-5).

Anti-B-cell therapy has an important place in the treatment of patients with malignant B-cell lymphoma (5). Furthermore, RTX is used to reduce the level of auto-antibodies in various auto-immune disorders (6, 7). However, treatment has also beneficial effects on T-cell mediated diseases, such as rheumatoid arthritis (RA), multiple sclerosis, and type 1 diabetes, which suggests that the therapeutic effects cannot be solely ascribed to effects on antibody production (7-10). In renal transplantation, RTX is used in ABO-incompatible transplantation, in desensitization protocols, and for treatment of antibody-mediated rejection (11-13). Because the presence of CD20+ B cells in the graft is associated with poor outcome (14), RTX may be of additional value in the treatment of steroid resistant acute rejection with intragraft B-cell infiltration (15, 16). The exact effects of RTX on B cells, and probably indirectly on T cells, in these various clinical conditions remains to be elucidated.

Following activation by antigen recognition, B cells are able to proliferate, produce various cytokines, and process antigen for presentation to T cells (17-19). T follicular helper cells provide help to B cells via expression of CD40L and production of IL-21 (20). Depending on the activating conditions, B cells will eventually differentiate into plasma cells or will acquire a memory phenotype, which allows for rapid responses upon subsequent encounter of cognate antigen (17, 18, 21, 22). The cell surface molecules CD27 and IgD are used to identify the two main B-cell populations in peripheral blood: naive B cells are CD27-IgD+, while circulating memory B cells are CD27+IgD- (23). Recently, a regulatory B-cell subset has been identified, which is predominantly found within the CD24highCD27+ B-cell population, and produces IL-10. This population is very small in healthy individuals, but was found to be increased in patients with autoimmune disease (24, 25).

In RTX-treated patients, the duration of B-cell depletion in the peripheral blood appears to depend on the disease, in combination with the number of RTX infusions. After a single dose of RTX there is a nearly complete B-cell depletion in peripheral blood. However, it should be appreciated that there remains a residual B-cell population in secondary and tertiary lymphoid organs (26-28).

So far it is not clear what the exact functional capacity of these persisting, but potentially modulated B cells might be, and how they could influence the immune
response after treatment with RTX. To address this issue we set up an in vitro, non-depleting B-cell stimulation model, and investigated the effects of RTX treatment on the phenotypic and functional characteristics of B cells.

MATERIALS AND METHODS

Cell isolation
Buffy coats from healthy donors, who gave written informed consent for scientific use of the buffy coats, were purchased from Sanquin Blood Bank, Nijmegen, The Netherlands. PBMC were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). CD19+ B cells were positively selected using anti-CD19 magnetic microbeads (Miltenyi Biotec, Utrecht, The Netherlands). This resulted in a CD19+ B-cell enrichment of more than 97%. CD19+CD27+ and CD19+CD27- B cells were isolated by high-purity fluorescence-activated cell sorting on an ALTRA cell sorter (Beckman-Coulter, Mijdrecht, The Netherlands). A rerun confirmed that the purity of the sorted cells was more than 98%.

CD4+CD25- T cells were obtained by sequential negative and positive selection of CD25+ cells and CD4+ cells, respectively, with anti-CD25 and anti-CD4 magnetic microbeads (Miltenyi Biotec). This resulted in a 98% pure CD4+CD25- population.

Culture conditions
Freshly isolated B cells (5 x 10^4 cells per well) were cultured in 200 µl medium in the presence of anti-CD40 monoclonal antibody (αCD40 mAb, 1 µg/ml, Bioceros, Utrecht, The Netherlands) and recombinant human IL-21 (25 ng/ml, ZymoGenetics, Seattle, WA) for 6 days in 96-well round bottom plates (Greiner, Frickenhausen, Germany) in a 37°C, 95% humidity, 5% CO₂ incubator. The culture medium consisted of RPMI-1640 supplemented with pyruvate (0.02 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Gibco, Paisley, United Kingdom), and 10% heat-inactivated pooled human serum (HPS). Where indicated, RTX was added in a concentration of 5 µg/ml.

In separate experiments, the effects of RTX and complement on viability of the B cells was assessed by incubating B cells (5 x 10^4 cells per well, 100 µl medium) for 30 min at 37°C with or without RTX (5 µg/ml), and subsequently with 10-30% of HLA-ABC/DR rabbit complement (Invitrogen, Breda, The Netherlands) or culture medium only. After an additional 60 min the cell viability was assessed by flow cytometry using Annexin V-FITC (AnxV) and propidium iodide (PI, 5 µg/ml; Bender Medsystems, Vienna, Austria) according to the manufacturer’s instructions.

In some experiments, the cells that remained after incubation with RTX and complement were washed and subsequently incubated for six days with medium containing αCD40 mAb, IL-21 and RTX, as described above.
Flow cytometry and CFSE labeling

For cell surface staining, the following fluorochrome-conjugated mAbs were used: CD19(SJ25C1)-PeCy7, CD70(Ki-24)-PE, CD80(L307.4)-PeCy5, CD86(233-(fun-1))-PE, IgD(IA6-2)-biotin, CCR4(1G1)-PeCy7, CCR6(11A9)-PE, HLA-DR(L243(G46-6))-PE (BD Biosciences, Erembodegem, Belgium), CD27(1A4-CD27)-PeCy5, CD62L(DREG56)-ECD, CD69(TP1.55.3)-ECD (Beckman-Coulter, Mijdrecht, The Netherlands), CD38(HIT2)-PE (Immunotools, Friesoythe, Germany), CXCR3(1C6/CXCR3)-PeCy5 (eBioscience, Uithoorn, The Netherlands), CD27(M-T271)-PE, CD95(DX2)-PE (Dako, Glostrup, Denmark), CD138(B-B4)-PeCy5 (IQ Products, Groningen, The Netherlands), CD25(4E3)-biotin (Miltenyi Biotec), CD27(M-T271)-biotin (Ancell, Bayport, MN), IgM-PE (Southern Biotech, Birmingham, AL), BAFF-R(11C1) (BioLegend, San Diego, CA). The detection of biotinylated antibodies was performed with streptavidin conjugated to Quantum dots (Qdot 605, Invitrogen, Breda, The Netherlands). Isotype-matched antibodies were used to define marker settings. Intracellular analysis of IL-2(MQ1-17H12)-PE, IL-6(AS12)-PE, IL-10(JES3-19F1)-PE, TNFa (Mab11)-PE (BD Biosciences), GATA3(TWAJ)-Alexa fluor 647, RORγt(AFKJS-9)-PE, FOXP3(PCH101)-Alexa fluor 647, IL-4(8D4-8)-PeCy7, IL-17(EBI064DEC17)-Alexa fluor 647, IL-21(eBIO3A3-N2)-PE, IFNγ(4S.B3)-PeCy7 (eBioscience), Tbet(4B10)-PE (Santa Cruz Biotech, Heidelberg, Germany) and IL-22(142928)-PE (R&D Systems, Oxon, UK) was performed after fixation and permeabilization, using Fix and Perm reagent (eBioscience). Before intracellular cytokine measurement, the cells were stimulated for 4 hours with PMA (12.5 ng/ml) plus ionomycin (500 ng/ml) in the presence of Brefeldin A (5 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands).

To study cell division by flow cytometry, 1-10 x 10^6 cells of interest were labeled with 0.5-1 μM carboxyfluorescein succinimidy ester (CFSE; Molecular Probes, Leiden, The Netherlands) prior to stimulation. The cell phenotype was analyzed by five-color flow cytometry (FC500, Beckman-Coulter), and data were analyzed using CXP software (Beckman-Coulter). In some experiments, cells were counted by flow cytometry using Flow-Count fluorospheres (Beckman-Coulter).

Cytokine detection

After 6 days of culture, B cells were labeled with CD27(M-T271)-PE. CFSE^{int}CD27{^low} and CFSE^{low}CD27^{high} were isolated on an ELITE cell sorter (Beckman-Coulter). Human IL-1β, IL-2, IL-4, IL-6, IL-10, IFNγ and TNFα were determined in the supernatant of these B-cell cultures using a human cytokine multiplex kit (Invitrogen) according to the manufacturer’s instructions. Before cytokine measurement, B cells (2.5-5 x 10^4 cells per well) were stimulated overnight with PMA (12.5 ng/ml) plus ionomycin (500 ng/ml; Sigma-Aldrich). Supernatants were collected and stored at -20°C until analysis.
**In vitro T-cell proliferation assay**

B cells harvested at day 6 of culture were washed, counted and added to $5 \times 10^4$ CFSE-labeled allogeneic CD4⁺CD25⁻ T cells in a 1:1 ratio for an additional 6 days of culture. CD4⁺ T cells were analyzed by flow cytometry.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Data in box-and-whisker plots represent the median, lower and upper quartiles, and minimum and maximum values. Paired t-tests were used to compare results obtained with cells cultured in the presence or absence of RTX. The Wilcoxon matched-pairs signed rank test was used for non-normally distributed data. P-values <0.05 were considered statistically significant and are indicated with asterisks.

**RESULTS**

RTX inhibits B-cell proliferation, without apoptosis induction

In order to mimic the *in vivo* situation in lymphoid organs, where B cells are exposed to RTX but not depleted, we first characterized a non-depleting *in vitro* B-cell stimulation model. Freshly isolated B cells were incubated in the presence or absence of RTX for 30 min, followed by an additional 60 min in the presence or absence of complement. Next, cell viability was assessed by flow cytometry using AnxV and PI staining. RTX alone did not increase the frequency of apoptotic (AnxV⁺PI⁻) or necrotic (AnxV⁺PI⁺) cells (Figure 1A). The addition of complement to the RTX-treated cells resulted in a dose-dependent necrosis of B cells, while complement alone had a negligible effect on the percentage and absolute number of viable cells (AnxV⁻PI⁻; Figure 1B). Notably, the phenotype of the viable B cells that remained after exposure to RTX and complement was largely similar to that of the cells that were exposed to RTX only (Figure 1C).

To assess the effect of RTX on the proliferative capacity of B cells, fresh B cells were stimulated with αCD40 mAb and IL-21, in the presence or absence of RTX. Dose-response kinetics, using 0, 2.5, 5, and 10 μg/ml RTX revealed optimal inhibition of proliferation at a concentration of 5 μg/ml (data not shown). In the absence of RTX, about 70% of the cells had divided at day 6 of culture, whereas in the RTX-treated condition this percentage was significantly lower (60%, $P<0.01$) (Figures 1D and 1E, upper and middle panels). The same experiment was repeated with the small proportion of B cells that remained after incubation with RTX and complement as starting population. In this case a similar inhibition of proliferation by RTX was observed (Figures 1D and 1E, lower panels). As the effects of RTX on the total B-cell population and on the fraction remaining after exposure to RTX
Figure 1. Characterization of an in vitro nondepleting system to establish the functional effects of RTX on B cells.

(A) Freshly isolated CD19+ B cells were incubated with (bottom row) or without (top row) 20% rabbit HLA-ABC/DR complement in the presence (right panel) or absence (left panel) of 5 μg/ml RTX. Annexin V (AnxV) and propidium iodide (PI) staining were analyzed using...
flow cytometry. The percentage of viable (AnxV⁻PI⁻), apoptotic (AnxV⁺PI⁻) and necrotic (AnxV⁺PI⁺) cells is indicated. One representative experiment of 4 is presented. (B) The percentages and absolute numbers of viable cells (AnxV⁻PI⁻) are shown after 30 min incubation in the absence (W/O; open) or presence of RTX (grey) with subsequent addition of various concentrations of complement for 60 min. Shown are the results for 4 different donors. (C) The proportions of IgD⁺, CD27⁺ and CD38⁺ B cells within the population of viable cells harvested after incubation with RTX and 20% complement. Shown are the results for 4 different donors. (D) CFSE-labeled CD19⁺ B cells were incubated first in the absence (W/O) or presence of RTX and complement. After washing, the cells were stimulated with αCD40 mAb and IL-21 in the presence or absence of RTX. Histograms show the CFSE division patterns at day 6 of total B-cell population (top) or the remaining B cells after RTX and complement mediated lysis (bottom). (E) Summary plot showing the percentages of proliferated B cells (data from 4 independent experiments, using 4 different blood donors); *P<0.05; **P<0.01; ns: not significant. W/O: without addition of RTX or complement.

and complement appeared similar, we continued to use the non-depleted B-cell population in further experiments for practical reasons (cell numbers).

**B-cell stimulation in the presence of RTX results in a population shift toward an activated naive phenotype**

Following the finding that RTX inhibited B-cell proliferation, we studied the phenotype of the RTX-treated B cells by means of CFSE-based multiparameter flow cytometry. Typically, stimulation of B cells with αCD40 mAb and IL-21 for 6 days resulted in the loss of IgD, and the gain of CD27 expression during sequential cycles of cell division (Figure 2A, middle panels). The inhibition of cell division by RTX resulted in a relative increase of IgD⁺CD27⁻ cells. Based on the CFSE content and the expression of CD27, IgD, and CD38, we defined four B-cell subsets after αCD40 mAb and IL-21 stimulation: undivided CFSE⁺⁺CD27⁻IgD⁻CD38⁻ cells (from here on referred to as subset I), undivided CFSE⁺⁺CD27⁻IgD⁻⁻CD38⁻⁻ cells (subset II), cells that underwent one or two cell divisions (CFSE⁻⁻CD27⁻IgD⁻⁻CD38⁻⁻; subset III) and cells that underwent three or more cell divisions (CFSE⁻⁻⁻CD27⁻IgD⁻⁻⁻CD38⁻⁻⁻; subset IV). A similar subset distribution was observed when the fraction of B cells remaining after incubation with RTX and complement was used as starting population for stimulation with αCD40 mAb and IL-21 (data not shown). The presence of RTX during B-cell stimulation resulted in a relative increase of both the percentage and absolute cell numbers in subset III and a decrease of cells in subset IV (Figure 2B and 2C).

Next, the phenotype of the four different subsets was assessed in more detail. Freshly isolated B cells were positive for BAFF-R and the majority of these cells expressed CD24 and CD62L. Approximately half of this population expressed IgD, around 25% expressed CD27 and IgM, whereas expression of CD38, CD138, CD25, CD69, CD95, CD70, CD80, and CD86 was virtually lacking (Figure 3, left panel).
Figure 2. Effect of RTX on B-cell maturation.

(A) A representative experiment showing flow cytometric analysis of IgD and CD27 expression of freshly isolated CD19+ B cells (start day 0). Subsequently CD19+ B cells were CFSE-labeled and stimulated with αCD40 mAb and IL-21 in the presence or absence of 5 μg/ml RTX. Plots show the IgD and CD27 expression at day 6 of culture against the CFSE expression. (B) Upper panel: Based on the CFSE content and cell surface expression of CD27, IgD, and CD38 on day 6 of culture with αCD40 mAb and IL-21, four B-cell subsets can be defined: CFSE++CD27-IgD-CD38- (subset I), CFSE++CD27-IgD+-CD38- (subset II), CFSE++CD27-IgD+-CD38- (subset III), and CFSE CD27-IgD-CD38++ (subset IV). Lower panel: Percentages of the different B-cell subsets after stimulation in the presence or absence of 5 μg/ml RTX. One representative experiment of 8 is presented. (C) Summary plot showing the percentages (n=14) and absolute numbers (n=6) of the different subsets within the total CD19+ B-cell population; *P<0.05, **P<0.01, ***P<0.001. W/O: without addition of RTX.
Upon stimulation there was an increased expression of the activation marker CD25 in all subsets, and CD69 was expressed in subsets I, II, and III (Figure 3). Subset II was characterized by a relatively low expression of IgD. CD138 was most strongly

![Figure 3. Phenotypic characterization of RTX-treated B cells.](image)

The expression of various markers on freshly isolated CD19+ B cells (start) and on B cells that were CFSE-labeled and stimulated with αCD40 mAb and IL-21 in the presence or absence of 5 μg/ml RTX. Data are shown from 6-8 independent experiments, with different donors; *P<0.05, **P<0.01. W/O: without addition of RTX.
expressed by cells in subset III, while CD38 and CD95 were typically expressed by cells in subset IV.

Addition of RTX resulted in a significantly higher percentage of IgD⁺ cells in all subsets, except for subset IV, which lacked IgD⁺ cells. Furthermore, there was a lower percentage of CD24⁺ cells in subsets I and II. The expression of other cell surface markers was less markedly changed in the presence of RTX (Figure 3).

RTX inhibits the proliferation of CD19⁺CD27⁻ naive, but not of CD19⁺CD27⁺ memory B cells
As we noticed that the starting population of freshly isolated B cells consisted of both naive CD27⁻ and memory-type CD27⁺ cells, we examined which of these populations was most affected by RTX. Sorted CD19⁺CD27⁻ and CD19⁺CD27⁺ B cells were labeled with CFSE and cultured with αCD40 mAb and IL-21 in the presence or absence of 5 μg/ml RTX for 6 days (Figure 4A). In this system, the CD27⁺ cells proliferated much stronger than the CD27⁻ cells (Figure 4B). The same gating strategy as in Fig. 2B was then used to define four B-cell subsets. Upon stimulation, part of the CD19⁺CD27⁻ B cells divided and differentiated into subset III, and to a lesser extent into subset IV type cells, whereas the majority of memory CD19⁺CD27⁺ B cells rapidly turned into subset IV-type cells. So, RTX inhibited the proliferation and differentiation of naive CD19⁺CD27⁻, but not of memory CD19⁺CD27⁺ B cells.

As for the total CD19⁺ population, we also examined the detailed phenotype of isolated CD19⁺CD27⁻ and CD19⁺CD27⁺ B cells before and after stimulation, in the presence or absence of RTX (Figures 4C and 4D, respectively). The main finding was that in the presence of RTX, stimulation of CD19⁺CD27⁻ B cells resulted in a larger fraction of IgD⁺ cells in subsets I and III, similarly as previously observed for the total CD19⁺ population. Similar to CD19⁺ B cells, subset III, differentiated from CD19⁺CD27⁻ B cells, expressed significantly less CD69 and more CD95 after exposure to RTX, which resulted in a heterogeneous activation status. As expected, RTX had no effect on the phenotype of subset IV, differentiated either from CD19⁺CD27⁻ or from CD19⁺CD27⁺ B cells. Again, RTX increased the percentage of IgD positive B cells in all subsets, except subset IV. Thus, RTX inhibits the proliferation of naive CD19⁺CD27⁻, but not memory of CD19⁺CD27⁺ B cells, with a population shift into a more naive (IgD⁺) phenotype.

RTX has no major effects on the B-cell-cytokine profile
We wondered whether the RTX-induced B-cell population shift was associated with changes in cytokine production. At day 6 of culture, CFSEintCD27⁻ (subset III) and CFSElowCD27high (subset IV) cells were sorted, restimulated with PMA/ionomycin and cytokines were measured in the supernatant. TNFa, IL-2, IL-6, and IL-10 were exclusively produced by CFSEintCD27⁻ cells, but not by CFSElowCD27high cells. RTX slightly increased the production of IL-6 by CFSEintCD27⁻ cells, but did not change the production of TNFa, IL-2, and IL-10 (Figure S1A).
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We also measured the intracellular cytokine production of TNFα, IFNγ, IL-2, IL-4, IL-6, and IL-10 in the B-cell subsets. After exposure to RTX, the fraction of TNFα-producing cells in subsets I+II was slightly enlarged, while the fraction of IL-4 producing cells was smaller (Figure S1B). RTX had no effect on the percentage of IL-2 or IL-6 expressing B cells.
B cells exposed to RTX enhance CD4+ T cell proliferation and induce a more Th2-like phenotype

Next, we studied the effect of RTX on the antigen-presenting function of B cells, after having established that RTX had no effect on HLA-DR expression (Figure S2). B cells stimulated with αCD40 mAb and IL-21 for 6 days in the presence or absence of RTX were added to CFSE-labeled allogeneic CD4+CD25− T cells for an additional 6 days of culture. B cells that were stimulated in the presence of RTX induced considerably stronger T cell proliferation than B cells stimulated in the absence of RTX (Figures 5A and B).

T cells that proliferated after stimulation with RTX-treated B cells contained a smaller fraction of CD27+ cells and a larger fraction of CD70+ cells, however

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Figure 5. T-cell proliferation upon stimulation with B cells.

(A) CFSE division patterns of CD4+CD25− T cells after coculture with αCD40 mAb and IL-21 prestimulated B cells in the presence or absence of RTX. Numbers denote the percentage of proliferated cells.

(B) Summary plot showing the percentage of proliferated cells from 8 different donors; **P<0.01

(C) The surface expression of the indicated molecules, the intracellular expression of several transcription factors, and the intracellular cytokine staining within the proliferated T cell population were analyzed on day 6 of culture.

(D) The Th1/Th2 ratio of the proliferated T cell population, represented as IL-2/IL-4 and T-bet/GATA3 ratios. Shown are the results of 4-5 different donors; *P<0.05. W/O: without addition of RTX.
there was no effect on T cell activation (CD25 and CD69) or the ratio of naive/memory T cells (CD62L; Figure 5C). Interestingly, after stimulation of T cells with RTX-treated B cells, the proliferated T cells showed an increased expression of the Th2-associated chemokine-receptor CCR4, transcription factor GATA3, and increased production of cytokine IL-4. The expression of Th1-associated (CXCR3, Tbet, IL-2, IFNγ, TNFa) and Th17-associated (CCR6, RORγt, IL-17) markers in these T cells was not influenced by exposure of the B cells to RTX prior to their use as T cell stimulators. Consequently, the proliferated T cells displayed a decreased Th1/Th2 ratio, as represented by decreased IL-2/IL-4 and T-bet/GATA3 ratios (Figure 5D).

DISCUSSION

In this study, we showed that RTX inhibited the proliferation of stimulated human B cells, which was associated with a relative increase of B cells with an activated naive phenotype. Aside from this population shift, there were no major changes in phenotype or cytokine profile of the various B-cell subsets as such. B cells stimulated in the presence of RTX induced stronger T-cell proliferation, compared to B cells stimulated in the absence of RTX. Moreover, the resulting T-cell population showed a more Th2-like phenotype.

Studies on the immune processes involved in transplant rejection have been mainly focused on T-cell-mediated mechanisms. Accordingly, most immunosuppressive drugs especially target T cells. Interestingly, anti-B-cell therapy appears to be of additional value in ABO-incompatible transplantation, desensitization protocols, and for treatment of antibody-mediated rejection (11-13). Moreover, renal transplant patients treated with a single-dose of RTX as an induction therapy, together with standard immunosuppressive treatment, showed a tendency toward fewer and milder rejection episodes compared to the placebo-group (29). However, the relative contribution of B cells in these conditions, and the exact mechanism of action of RTX remain to be elucidated. In renal transplant patients, a single dose of RTX effectively depleted all peripheral B cells, as well as B cells in the renal allograft, but spared up to 50% of B cells in lymph nodes (26). Notably, RTX appeared to be bound to these B cells (26). Also in patients with RA, the number of synovial B cells was significantly decreased at 4 weeks after RTX-treatment, but there was no complete depletion (30). These important observations suggest that treatment with RTX may affect the phenotype or function of a residual population of B cells. Our in vitro culture system mimicked this scenario, since B cells were exposed to RTX, but survived when no complement was added to the culture medium. Moreover, the small fraction of B cells that remained after complement mediated lysis, resembled the total B-cell population with respect to phenotype and effects of RTX. To imitate the in vivo stimulation of
B cells in the lymph nodes, we added αCD40 mAb and IL-21 to the culture medium. Furthermore, the culture medium was supplemented with pooled human serum, resulting in IgG1 concentrations of about 500-1000 μg/ml. We therefore consider the observed effects of RTX (used in a concentration of 5 μg/ml) specific for its CD20 binding property and not a consequence of non-specific binding of the IgG1 molecule.

CD20 is a tetraspanin-like protein involved in lipid raft formation (31). Binding of RTX to CD20 interferes with B-cell receptor (BCR) signaling by preventing BCR relocalization into the lipid rafts and inhibition of the downstream BCR signaling cascade (32). These effects of RTX can explain the inhibition of B-cell proliferation that we observed. Interestingly, we found that the antiproliferative effect of RTX was limited to the CD27− naive B-cell population. In agreement with these findings, it has been reported that RTX administered in vivo as part of a desensitization protocol decreased the number of splenic naive B cells, but had no effect on the number of CD27+ memory B cells (33). These discordant effects of RTX on naive and memory B cells do not seem to be directly related to the level of CD27 expression, since Franke et al. found no relationship between the RTX induced changes in gene expression and the surface expression of CD27 in various B-cell lines (34). The antiproliferative effect of RTX during stimulation of the B cells was accompanied by a shift in the distribution among B-cell subsets, as defined by the CFSE content in combination with the expression of CD27, IgD, and CD38. After 6 days of culture in the presence of RTX, there was an enrichment of CD27−IgD+CD38−/+ cells representing an activated naive phenotype. These cells underwent only one or two cell divisions (subset III), but did not go into apoptosis which can be explained by the lack of cross-linking of the Fc parts of RTX in our in vitro culture system (31).

A striking finding was that B cells stimulated with αCD40 mAb and IL-21 in the presence of RTX induced stronger T cell proliferation than B cells stimulated in the absence of RTX. Since the phenotype and cytokine profile of the various B-cell subsets only marginally differed, we believe that the altered distribution among the B-cell subsets was mainly responsible for this observation. There was a relative increase of activated naïve B cells (subset III) and a decrease of plasmablast-like cells (subset IV). Plasmablasts (and plasma cells) are terminally differentiated B cells specialized in producing antibodies while activated B cells can have antigen presenting functions (35). A relative increase of these latter cells after B-cell stimulation in the presence of RTX can explain the stronger induction of T-cell proliferation.

The enhanced T-cell proliferation induced by RTX-treated B cells might implicate that anti-B-cell therapy can also have deleterious effects, as has been reported for renal transplant patients (36). On the other hand, we observed that the proliferated T cells displayed a differentiation toward a Th2-like phenotype, which is usually associated with graft acceptance rather than rejection (37). Interestingly, a recent study showed the accumulation of B cells with an inhibited profile in a rat
model of allograft tolerance (38). This changed B-cell profile was accompanied by a deviation toward the Th2-related IgG1 isotype alloantibodies. A similar shift in the B-cell population has also been observed in renal transplant patients with stable kidney graft function in the absence of immunosuppression (39).

In summary, we have demonstrated that RTX can affect B-cell proliferation and differentiation, leading to an altered distribution among defined B-cell subsets. Exposure of B cells to RTX has effects on subsequent interaction between B and T cells in vitro. In organ transplantation, anti-B-cell therapy should therefore be applied with caution, and preferably be accompanied by additional studies that give more insight into the effects of RTX on the alloimmune response in vivo.

Acknowledgments
The authors thank R. Woestenenk (Dept. of Laboratory Medicine – Laboratory of Hematology) for cell sorting. This study was supported by a grand of the Dutch Kidney Foundation (nr C09-2301).
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Supplementary Figure 1. Effects of RTX on the cytokine profile of stimulated B cells.

(A) CFSE-labeled B cells were stimulated with αCD40 mAb and IL-21 in the presence or absence of RTX. On day 6, CFSE<sup>low</sup>CD27<sup>high</sup> (subset III) and CFSE<sup>low</sup>CD27<sup>low</sup> (subset IV) cells were sorted and re-stimulated with PMA plus ionomycin. Culture supernatants were analyzed for the production of IL-1β, IL-2, IL-4, IL-6, IL-10, IFNγ, and TNFα by Luminex. IL-1β, IL-4, and IFNγ remained undetectable. Data are shown from 5 independent experiments, with different donors. (B) Intracellular expression of TNFα, IFNγ, IL-2, IL-4, IL-6, and IL-10 was measured in the B-cell subsets as defined in Fig. 2B at day 6 of culture. Intracellular expression of IFNγ and IL-10 remained below 0.1% and are therefore excluded from this analysis. After the fix and perm treatment, CD27 expression was changed in such a way that subsets I and II could no longer be distinguished from one another. Therefore, these subsets were taken together for intracellular cytokine measurement. Data are shown from 5-8 different experiments with different donors. W/O: without addition of RTX.
Supplementary Figure 2. HLA-DR expression of stimulated B cells.

Expression of HLA-DR by αCD40 mAb plus IL-21 stimulated B cells in the absence (W/O) or presence of RTX for 6 days. The percentage positive cells (A) and the mean fluorescence intensity (B) are depicted.
A single dose of rituximab does not deplete B cells in secondary lymphoid organs, but alters phenotype and function

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ABSTRACT

A single dose of the anti-CD20 monoclonal antibody rituximab induces a nearly complete B-cell depletion in peripheral blood, but not in secondary lymphoid organs. Modulation of this remaining B-cell population due to rituximab treatment may contribute to the therapeutic effects of rituximab. To assess the in vivo effects of rituximab we used lymph nodes collected during renal transplant surgery in patients who had received rituximab four weeks earlier in preparation for an ABO-incompatible transplantation. Rituximab treatment resulted in a lower percentage of naive (IgD<sup>+</sup>CD27<sup>-</sup>) and a higher percentage of switched memory (IgD<sup>-</sup>CD27<sup>+</sup>) B cells. Remarkably, transitional (CD24<sup>++</sup>CD38<sup>++</sup>) B cells were virtually lacking in the lymph nodes of rituximab-treated patients. Moreover, lymph node-derived B cells from rituximab-treated patients produced different amounts of various Ig-subclasses after anti-CD40/IL-21 stimulation ex vivo. Finally, after stimulation of allogeneic T cells with lymph node-derived B cells from rituximab-treated patients, the proliferated T cells showed a decreased production of IL-17. In conclusion, after treatment with rituximab there remains a B-cell population with different functional capacities. Consequently, the effect of rituximab on the immune response will not only be determined by the extent of B-cell depletion, but also by the functional properties of the remaining B cells.
INTRODUCTION

After transplantation of a solid organ graft, B cells can play a major role in graft rejection via the production of alloantibodies, but they can also induce an immune response by acting as professional antigen presenting cells, or by the production of various cytokines (1). It has recently been shown that a subset of human B cells can display regulatory function (B_{REG}). This subset, which is predominantly found within the CD24^{++}CD38^{++} B-cell population and produces IL-10, is very small in healthy individuals but was found to be increased in patients with autoimmune diseases (2, 3) and in tolerant transplant patients who had stable graft function despite receiving no immunosuppression for at least one year (4). Since B cells can play such a variety of roles in the immune response, the effects of anti-B-cell therapy have to be analyzed carefully.

The chimeric anti-CD20 monoclonal antibody rituximab (RTX) triggers B-cell lysis through antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, or apoptosis induction (5, 6). RTX is used to reduce autoantibody levels in various autoimmune disorders (7). However, RTX can also ameliorate chronic inflammatory diseases mediated by T and B cells, such as rheumatoid arthritis (8) and multiple sclerosis (9), suggesting that the therapeutic effect of RTX not solely depends on inhibition of antibody production. In renal transplantation, RTX is used successfully in ABO-incompatible (ABOi) transplantation (10), in desensitization protocols (11), and for treatment of antibody-mediated rejection (12).

Although a single dose of RTX induces a nearly complete B-cell depletion in peripheral blood, there often remains a residual B-cell population in secondary lymphoid organs (13-15). We have previously shown that exposure of human peripheral blood B cells to RTX in an in vitro nondepleting stimulation model affects B-cell phenotype and function, resulting in an altered outcome of B-T-cell interaction (16). This triggered us to study the functional properties of the nondepleted but potentially modulated B cells that remain present in secondary lymphoid organs after treatment with RTX. To this end, we collected lymph nodes (LNs) during renal transplant surgery in patients who had received RTX four weeks earlier in preparation for an ABO-incompatible renal transplantation (17). The phenotypic and functional properties of B cells isolated from these LNs were compared with B cells isolated from LNs collected from renal transplant patients not treated with RTX.

MATERIALS AND METHODS

Collection and preparation of samples
Peripheral blood (PB) samples were obtained before renal transplantation and buffy coats from healthy donors were purchased from Sanquin Blood Bank (Nijmegen, The
Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Lucron, Dieren, The Netherlands). Iliac LN were obtained during transplant surgery. Spleen cells (SPL) were obtained from deceased organ donors. LN and SPL samples were first homogenized and subsequently forced through 75 µm netwell filters (Costar, Corning International, Amsterdam, The Netherlands) to obtain single-cell suspensions. Mononuclear LN and SPL cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Roskilde, Denmark). All samples were cryopreserved in liquid nitrogen until analysis.

Patients scheduled for an ABO-incompatible renal transplantation received a single dose of 375 mg/m² RTX (Mabthera, Roche Pharma AG, Grenzach-Wyhlen, Germany) intravenously four weeks before transplantation. Prior to RTX administration, 25 mg prednisolone was given intravenously. Two weeks before transplantation, treatment with tacrolimus (0.2 mg/kg/day), mycophenolate mofetil (2 g/day) and prednisolone (10 mg/day) was started. Intravenous immunoglobulin (IVIG, Nanogam, Sanquin, Amsterdam, The Netherlands; 0.5 g/kg) was administered the day before surgery. Patients scheduled for a regular, ABO-compatible living donor kidney transplantation were used as controls. Table 1 summarizes the characteristics of all patients. None of the patients had a systemic auto-immune disease as cause of renal insufficiency and none of them received any immunosuppressive drug during the last three months prior to transplantation (or the administration of RTX in the ABO-incompatible group). The study was performed in accordance with the regulations set by the Medical Ethics Committees of the participating hospitals. Informed consent was obtained from all participants.

Cell isolation
B cells were purified from SPL and LN cells by negative selection using monoclonal antibodies directed against CD3(UCHT1), CD8(RPA-T8), CD14(M5E2), CD16(3G8), CD33(P67.6), CD56(B159), and CD235a(GA-R2(HIR2)) (BD Biosciences, Erembodegem, Belgium) combined with sheep anti-mouse Ig-coated magnetic beads (Dynal, Denmark). This resulted in a CD19⁺ B-cell enrichment of more than 90%. CD3⁺ T cells were positively selected from PBMCs of healthy donors using anti-CD3 magnetic microbeads (Miltenyi Biotec, Utrecht, The Netherlands) resulting in a purity of more than 95%.

Culture conditions
For functional studies, 5 x 10⁴ cryopreserved LN and SPL cells were cultured in RPMI-1640 medium supplemented with pyruvate (0.02 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Gibco, Paisley, United Kingdom), and 10% heat-inactivated pooled human serum (HPS) in 96-well round
bottom plates (Greiner, Frickenhausen, Germany) in a 37°C, 95% humidity, 5% CO₂ incubator. In selected conditions, 5 μg/ml RTX was added to the culture medium.

**Flow cytometry and CFSE labeling**

For cell surface staining, the following fluorochrome-conjugated mAbs were used: CD19(SJ25C1)-PeCy7, CD20(2H7)-PeCy7, CD25(M-A251)-PE (BD Biosciences), CD3(UCHT1)-ECD, CD4(13B8.2)-PeCy5, CD5(BL1a)-APC Alexa Fluor 700, CD8(SFC121Thy2D3)-ECD, CD19(J3-119)-ECD, CD19(J3-119)-APC Alexa Fluor 750, CD24(ALB9)-APC, CD27(1A4-CD27)-PeCy5.5, CD38(LS198-4-3)-PeCy7, CD45(J.33)-FITC, CD45(J.33)-Krome Orange, CD45RO(UCHL1)-ECD, CD56(N901)-PeCy5, IgD(IADB6)-FITC, IgM(SA-DA4)-PE (Beckman-Coulter, Mijdrecht, The Netherlands), CD127(EBIORDR5)-PeCy7 (eBioscience, Uithoorn, The Netherlands), CD8(DK25)-PE and CD27(M-T271)-PE (Dako, Glostrup, Denmark). Isotype-matched antibodies were used to define marker settings. Intracellular analysis of IL-2(MQ1-17H12)-PE (BD Biosciences), IL-4(8D4-8)-PeCy7, IL-17(EBIO64CAP17)-PE, and IFN-γ(4S.B3)-PeCy7 (eBioscience) was performed after fixation and permeabilization, using Fix and Perm reagent (eBioscience). Before intracellular cytokine measurement, the cells were stimulated for 4 hours with PMA (12.5 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands).

To study cell division by flow cytometry, 8 x 10⁶ cells were labeled with 0.5 μM CFDA-SE (Molecular Probes, Leiden, The Netherlands) prior to stimulation. The cell phenotype was analyzed by five-color flow cytometry (FC500) or ten-color flow cytometry (Navios™), and data were analyzed using CXP or Kaluza® software, respectively (all from Beckman-Coulter).

**Immunoglobulin isotyping assay**

CD19⁺ LN B cells (5 x 10⁴ cells) were cultured in the presence of anti-CD40 monoclonal antibody (αCD40 mAb, 1 μg/ml, Bioceros, Utrecht, The Netherlands) and recombinant human IL-21 (100 ng/ml, ZymoGenetics, Seattle, WA) for 8 days in culture medium supplemented with 10% fetal bovine serum (FBS). Supernatants were stored at -20°C until analysis. Human IgG1, IgG2, IgG3, IgG4, IgM, IgA, and IgE were determined using a human Bio-Plex Pro™ immunoglobulin isotyping assay (BioRad, Veenendaal, The Netherlands) according to the manufacturer’s instructions.

**Real-time quantitative PCR of activation-induced cytidine deaminase (AID)**

CD19⁺ SPL B cells (5 x 10⁴ cells) were cultured in the presence or absence of 5 μg/ml RTX with or without addition of 1 μg/ml αCD40 mAb and 50 ng/ml IL-21. After 4 days of culture, total RNA was extracted, cDNA was synthesized and transcripts were quantified as described previously(18). Probes with the following identification
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number were used: AID, Hs00757808_m1 (Applied Biosystems, Foster City, CA, USA). Results were normalized using the human HPRT1 Endogenous Control (4333768T; Applied Biosystems) and expressed as the relative fold change compared to the control condition.

**In vitro T-cell proliferation assay**

CD19⁺ LN B cells were added to 5 x 10⁴ CFSE-labeled allogeneic CD3⁺ T cells in a 1:1 ratio. Intracellular cytokine production by proliferating (CFSE<sub>low</sub>) CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells was analyzed by flow cytometry at day 7.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.03. Paired t-tests were used to compare results obtained with SPL cells cultured in the presence or absence of RTX. To test differences between both patient groups, a Mann-Whitney U-test or an unpaired t-test was performed. P-values <0.05 were considered statistically significant and are indicated with asterisks.

**Table 1. Patient characteristics.**

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ADPKD = autosomal dominant polycystic kidney disease; FSGS = Focal segmental glomerulosclerosis; HD = hemodialysis; PD = peritoneal dialysis; RTX = Rituximab; TIN = tubulo-interstitial nephritis; Tx = transplantation; *= not further specified. NA = not available
RESULTS

A single dose of RTX induces a nearly complete B-cell depletion in PB, but not in LNs of renal transplant recipients

RTX treatment resulted in a nearly complete depletion of B cells from the peripheral lymphocyte population (CD19^+; 0.12±0.05% versus 4.9±1.1%; P=0.002) while T-cell and NK-cell percentages were not affected by RTX (Figure 1A). Importantly, the percentage of CD19^+ B cells in LNs of RTX-treated patients did not differ from that of the untreated patients (35.1±8.5% versus 40.3±11.0%; P=0.61; Figure 1A). Likewise, RTX treatment had no effect on the percentages of other lymphocyte populations in the LNs.

To exclude that LN B cells were not depleted by RTX treatment because they lack CD20 expression we first demonstrated that CD19^+ LN B cells of untreated patients express CD20 (Figures 1B and 1C). Remarkably, LN B cells of RTX-treated patients were CD19^+ but CD20^− suggesting that RTX influenced detection of CD20 on LN B cells. However, we were unable to detect RTX on the surface of the LN B cells of RTX-treated patients using an anti-idiotype antibody (19; Figure S1).

B cells remaining in the LNs after RTX treatment are predominantly of an IgD^−CD27^+ switched memory phenotype associated with an altered Ig-isotype production

Since RTX treatment failed to deplete CD19^+ B cells in the LNs, we examined the remaining CD19^+ LN B cells in more detail. RTX treatment resulted in significant reduction of the percentages of naive B-cells (IgD^+CD27^−, 1.9±1.0% versus 31.0±14.7%; P=0.01), transitional B-cells (CD24^++CD38^++ , 0.1±0.05% versus 2.3±1.4%; P=0.01) and CD24^+CD38^− (mature) B-cells (8.4±4.4% versus 28.6±16.5%; P=0.02), and in an increase in the percentages of switched memory B-cells (IgD CD27^+, 86.1±8.2% versus 53.0±17.0%; P=0.02) percentages and CD24^+CD38^− (memory) B-cells (85.5±6.2% versus 67.5±17.2%; P=0.07). Accordingly, the percentage of B cells positive for the naive B-cell markers CD5 and IgM was also reduced after RTX treatment (1.3±0.5% versus 9.1±4.5%; P=0.01 and 13.5±5.9% versus 70.3±12.4%; P=0.01, respectively; Figures 2A and 2B). Plasma cells (CD19lowCD20^−CD38^high) were virtually lacking in the LNs, irrespective of treatment with RTX (data not shown). Similar results were obtained after 3 days exposure of human SPL B cells to RTX in vitro (Figure S2).

To define whether the RTX-induced shift of LN B cells from a naive to a switched memory phenotype influenced Ig-isotype production, LN B cells were stimulated ex vivo with αCD40 mAb and IL-21 to mimic the in vivo help of follicular helper CD4^+ T cells (20). After 8 days Ig-isotypes were measured in the culture supernatant. LN B cells from RTX-treated patients produced lower amounts of IgM (0.6±0.2 µg/ml versus 14.1±7.8 µg/ml; P=0.01) and IgG2 (0.9±0.3 µg/ml versus 2.3±1.5 µg/ml; P=0.02), with a trend toward a higher IgG1 production compared to LN B cells from
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Untreated patients (77.8±33.0 µg/ml versus 44.3±15.7 µg/ml; P=0.17; Figure 3A). The production of IgA, IgE, IgG3, and IgG4 was not affected by RTX treatment.

Figure 1. Distribution of the lymphocyte population in peripheral blood (PB) and lymph nodes (LNs) of renal transplant recipients after RTX treatment.

(A) Shown are the percentages of T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) and NK cells (CD3-CD56+) within the total lymphocyte (CD45+) population of renal transplant patients treated with RTX (Tx RTX+; grey bars) and untreated control patients (Tx RTX-; white bars) in PB (n=3) and LNs (n=4-6). (B) Expression of CD19 and CD20 in peripheral blood (PB) and lymph nodes (LNs) of renal transplant recipients after RTX treatment. Shown are the results of two representative patients. (C) Summary plot showing the percentages CD19 and CD20 positive cells within CD45+ lymphocytes in PB and LNs of RTX-treated (Tx RTX+; n=3-4; grey bars) and untreated (Tx RTX-; n=3-6; white bars) patients; Significant differences are indicated by asterisks: **P<0.01.
Chapter 5

**In vitro** exposure to RTX enhances the mRNA expression of activation-induced cytidine deaminase in stimulated human splenic B cells

To determine whether the RTX-induced population shift from a naive to a switched memory phenotype was accompanied by class-switch recombination (CSR), we studied expression of activation-induced cytidine deaminase (AID) which plays a key role in CSR (21). CD19+ SPL B cells were cultured in the presence or absence of RTX with or without addition of αCD40 and IL-21. After 4 days of culture AID mRNA expression was determined by quantitative PCR (Figure 3B). AID mRNA expression levels tended to be higher when SPL B cells were stimulated with αCD40/IL-21 and exposed to RTX compared to the control condition ($P=0.07$).

**Figure 2. Phenotypic and functional characterization of lymph node (LN) B cells.**

(A) A representative example of naive (IgD+CD27-), unswitched memory (IgD-CD27+), switched memory (IgD-CD27-), memory (CD24++CD38+), mature (CD24+CD38+), transitional (CD24++CD38+), CD5+ and IgM+ B cells within the total CD19+ B-cell population of RTX-treated (Tx RTX+, right panel) and untreated (Tx RTX-, left panel). (B) Shown are the results from RTX-treated (grey box-plots; n=4) and untreated (white box-plots; n=6) patients. Significant differences are indicated by asterisks: *$P<0.05$, **$P<0.01$. 

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T cell stimulation with LN B cells from RTX-treated patients resulted in a weaker Th17 response
Based on our previous finding that in vitro exposure of human PB B cells to RTX altered the B-T-cell interaction (16), we analyzed the intracellular cytokine production by T cells after stimulation with LN B cells. LN B cells from RTX-treated or untreated patients were added to allogeneic CFSE-labeled CD3+ T cells. After 7 days of culture, we measured the intracellular cytokine production of IL-2, IL-4, IL-17, and IFNγ by proliferating (CFSElow) T cells. Interestingly, the percentage of CFSElow CD4+ T cells that produced IL-17 was lower upon stimulation with LN B cells from RTX-treated patients as compared to LN B cells from untreated patients (2.6±0.6% versus 4.6±1.4%; P=0.003; Figure 4).

Figure 3. RTX affects the Ig-isotype production and enhances the mRNA expression of activation-induced deaminase (AID).

(A) Production of IgM, IgA, IgE, IgG1, IgG2, IgG3 and IgG4 in the supernatant by lymph node (LN) B cells of renal transplant recipients after ex vivo stimulation with αCD40 mAb and IL-21 for 8 days. Shown are the results for RTX-treated (Tx RTX+; n=4; grey bars) and untreated patients (Tx RTX-; n=6; white bars); *P<0.05, **P<0.01. (B). Real-time quantitative PCR of the AID mRNA expression in human splenic CD19+ B cells after 4 days of culture in the presence or absence of RTX with or without addition of 1 μg/ml αCD40 mAb and 50 ng/ml IL-21. Results were normalized using the human HPRT1 endogenous control and the expression in unstimulated control condition was set at 1.0. Shown are the relative fold changes in B cells obtained from 3 different donors.
Figure 4. Intracellular cytokine production by T cells upon stimulation with lymph node (LN) B cells from renal transplant recipients

(A) The intracellular cytokine production of IL-2, IL-4, IL-17 and IFN\(\gamma\) within the proliferated CD4\(^+\) (upper panel) and CD8\(^+\) (lower panel) T-cell population was analyzed on day 7 of culture. (B) Shown are the percentages of the cytokines produced by the proliferated CD4\(^+\) and CD8\(^+\) T cells after stimulation with CD19\(^+\) LN B cells of RTX-treated (Tx RTX\(^+\); grey box-plots) and untreated (Tx RTX\(^-\); white box-plots) from 8 to 12 different donors; Significant differences are indicated by asterisks: **\(P<0.01\).
DISCUSSION

In this study, we showed that at four weeks after administration of a single dose of RTX there is a nearly complete B-cell depletion in PB, but not in LNs of renal transplant recipients. Exposure of human B cells to RTX resulted in a lower percentage of naive (IgD+CD27-) and a higher percentage of switched memory (IgD-CD27+) B cells. Concomitantly, there was a change in the production of Ig-subclasses after ex vivo stimulation with αCD40 mAb and IL-21. Finally, CD4+ T cells showed lower IL-17 production upon stimulation with LN B cells from RTX-treated patients as compared to LN B cells from untreated patients in an in vitro stimulation assay.

The observed persistence of B cells in lymphoid tissues after RTX treatment, has been reported by others before (8, 13, 15, 22-26). However, in contrast to our finding of unaffected numbers of LN B cells, these studies described at least some degree of B-cell reduction in synovial tissue, spleen or lymph nodes. Renal transplant recipients treated with RTX for antibody-mediated rejection, showed a complete depletion of circulating B cells with a 50% reduction of B cells in tertiary lymphoid organs (24). RTX therapy of patients with autoimmune thrombocytopenia resulted in complete B-cell depletion in PB and in a reduction in SPL B cells (13). In patients with rheumatoid arthritis, the number of synovial and bone marrow B cells were decreased after RTX treatment, but there was no complete depletion (8, 22, 23). Genberg et al. (15) reported an average of 50% reduction of the percentage of CD19+ B cells in LNs after treatment with RTX as induction therapy in renal transplant patients. The variation in B-cell depletion might be explained by differences in RTX treatment regimen, choice of immunosuppressive agents, and heterogeneity of the patient populations. The majority of the patients were treated with at least two doses of 375 mg/m², or 1000 mg for RA patients, with different time intervals before obtaining secondary lymphoid tissue (8, 22-24), while our patients only received a single dose of 375 mg/m² four weeks before collecting the lymph nodes. Notably, there is a trend to use less intensive dosing regimens of RTX for auto-immune diseases, and like in our study, a single dose of RTX is currently applied in several conditions (27, 28).

Why there is such a wide discrepancy between depletion of B cells in PB and LN after RTX treatment is an intriguing question. A possible explanation could be an inability of RTX to reach the B cells in the LNs. However, we clearly demonstrate that LN B cells that remain after RTX treatment were CD19+CD20+, probably due to modulation of CD20 by the binding of RTX (29-32), which indicates that the LN B cells had been exposed to RTX. Exhaustion of the complement system is another possible explanation, which could not be further addressed in the current study (33). Finally, a high concentration of the B-cell activation factor (BAFF) in LNs, might favor the survival of B cells (24).
Although treatment with RTX had minimal effect on the proportion of LN B cells, it induced a striking population shift from a naive to a switched memory phenotype, which can be the result of two non mutually exclusive processes. First, RTX might selectively deplete and/or inhibit naive but not memory B cells leading to a relative increase of memory B cells. Second, binding of RTX to naive B cells might induce differentiation into memory B cells. Using an in vitro nondepleting B-cell stimulation model, we have previously shown that RTX inhibited the proliferation of CD27 naive, but not of CD27+ memory B cells (16). In agreement with these findings, it has been reported that RTX administered in vivo as part of a desensitization protocol in kidney transplantation decreased the number of splenic naive B cells, but had no effect on the number of CD27+ memory B cells (14). In general, as compared to naive cells, memory B cells are characterized by a high expression of activation and prosurvival molecules, which allows them to respond quickly during an immune response and to persist for long time (34). These properties may also result in a relative resistance of memory B cells to depletion by RTX. In summary, these observations suggest that selective inhibition of naive, but not of memory B cells can indeed contribute to the observed shift from a naive to a switched memory B-cell phenotype. Next to its effect on the phenotype of B cells, RTX also affected the Ig-isotype production with a decrease in IgM production. Combined with a trend towards increased AID mRNA expression, this suggests that the population shift might be accompanied by class-switch recombination (CSR). Taken together, the LN B-cell population shift from a naive to a switched memory phenotype after treatment with RTX might be due to both a selective depletion of naive B cells and a direct effect on signaling cascades resulting in CSR and transition from a naive to a switched memory phenotype.

Treatment with RTX can be effective in chronic inflammatory diseases, such as rheumatoid arthritis (8, 35) and multiple sclerosis (9). The improvement of these conditions by RTX has been associated with a reduced Th17 response (35). In the current study, we found that LN B cells obtained from RTX treated patients resulted in a weaker Th17 response when used as stimulators in an allogeneic mixed lymphocyte reaction. Likewise, it has been observed that RTX reduces the IL-17 production by PBMCs after stimulation with Candida albicans in vitro (35). Moreover, the Th17-cell frequency has been shown to correlate with the frequency of both switched memory B cells and serum BAFF levels (36). These findings suggest a close relationship between Th17-cell homeostasis and B-cell maturation which can be affected by RTX. In a previous study, we observed that B cells that were treated with RTX in vitro, were able to induce a Th2-like shift in proliferating T cells. However, it should be noted that in the present study we investigated lymph node derived B cells, whereas in the previous study peripheral blood B cells were used. The B-cell subset distribution between the two is clearly different, which might explain a different T-cell response regardless of any further treatment (e.g. RTX), as
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was observed by comparing CD27− and CD27+ B cells to stimulate allogeneic CD4+ T cells (37).

We acknowledge that the changes in the B-cell repertoire that we observed at four weeks after RTX treatment, could theoretically also have been caused by steroids that were given at the time of RTX administration, or by the treatment with immunosuppressive drugs (tacrolimus, mycophenolate mofetil, and prednisone from 2 weeks before transplantation), or IVIG (1 day before transplantation). However, none of these drugs directly targets B cells. It has been shown that IVIG does not affect B-cell responses in vitro (38), and treatment with IVIG as part of desensitization protocols had no effect on B cells in the spleen (14). In SLE patients treated with mycophenolate mofetil, the number and phenotype of B-cells were similar to that in controls without immunosuppressive therapy (39). Tacrolimus had minimal effect on B-cell proliferation and survival after stimulation in vitro (38).

Finally, treatment of healthy volunteers with a single dose of prednisolone (30 mg) resulted in a decrease of the absolute counts of total lymphocytes, B cells, T cells and NK cells, but the counts returned to baseline levels within 13 to 26 hours after administration (40). Moreover, the only differences between RTX-treated and untreated patients were those found in the B-cell compartment. The fact that LN B cells from treated patients were negative for CD20, strongly indicates that RTX was involved in the observed changes in B-cell phenotype and function.

In summary, we have demonstrated that a single dose of RTX depletes B cells in PB, but not in LNs at four weeks after administration. Exposure of human B cells to RTX results in a relative increase of B cells with a switched memory phenotype. Consequently, the effect of rituximab on the immune response will not only be determined by the extent of B-cell depletion, but also by the functional properties of the remaining B cells.

Acknowledgments

This study was supported by research funding from the Dutch Kidney Foundation (nr C09-2301). The authors thank L. Boon (Bioceros, Utrecht) for kindly providing the αCD40 mAb. Technical support was kindly provided by F.W. Preijers and H. Tijssen (Radboud University Medical Centre). We also thank M.C. Warlé (Radboud University Medical Center), M.M. Idu and E.B. Remmerswaal (Academic Medical Centre, Amsterdam) for harvesting the lymph nodes.
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Rituximab alters B cells in human lymph nodes

CD45+ lymphocytes

Lymph nodes

Spleen

Tx RTX-

Tx RTX+

- RTX

+ RTX

CD20

anti-RTX

CD19

isotype anti-RTX

Supplementary Figure 1. Anti-RTX antibody MB2A4 for the detection of RTX on the surface of cells.

Lymph node (LN) cells from RTX-treated (Tx RTX+) and untreated patients (Tx RTX-) were stained for the expression of CD19, CD20 and the presence of RTX (anti-idiotype antibody MB2A4) on the surface of the cells. Isotype control for the anti-RTX antibody was used to define gate settings. As a positive control condition, spleen cells from organ donors were first incubated in the presence (+ RTX) or absence (- RTX) of 5 µg/ml RTX for 30 min in vitro. Subsequently, cells were stained for the expression of CD19, CD20 and the presence of RTX. In this control setting all CD19+ cells were CD20- and anti-RTX+, indicating that RTX was bound to CD20 and blocked binding of the staining anti-CD20 antibody. In contrast, LN B cells from patients who were treated with RTX four weeks earlier, were also negative for CD20, but the presence of RTX on these cells could not be detected.
Supplementary Figure 2. Human splenic B-cell subsets at day 3 of culture after exposure to RTX in vitro.

Cryopreserved spleen (SPL) cells were incubated with or without RTX in the presence or absence of CpG for 3 days. A representative experiment showing flow cytometric analysis of IgD and CD27 expression (A) within the CD19\(^+\) SPL B-cell population. (B) Summary plot showing the percentages of naive (IgD\(^+\)CD27\(-\)), unswitched (IgD\(^+\)CD27\(\pm\)) and switched memory (IgD\(^-\)CD27\(\pm\)) cells within the CD19\(^+\) SPL B-cell population with (grey box-plots) or without (white box-plots) RTX in the absence (n=9) or presence (n=6) of CpG. Significant differences are indicated by asterisks: \(*P<0.05, ***P<0.001.\)
Rituximab alters B cells in human lymph nodes
Cytokine release after treatment with rituximab in renal transplant recipients

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ABSTRACT

Background. Treatment with rituximab may be accompanied by a systemic cytokine release. We studied the effects of a single dose of rituximab on cytokine levels in transplant patients and examined the underlying mechanism.

Methods. Twenty renal transplant recipients (10 rituximab-treated, 10 placebo-treated) were recruited from a randomized clinical trial. Rituximab or placebo was infused during surgery and blood samples were taken before, during, and after surgery and analyzed for IL-2, IL-6, IL-10, IL-12, IL-17, TNFα, IFNγ, and MIP-1β. In vitro, healthy donor peripheral blood mononuclear cells, purified B cells, monocytes, NK cells, or combinations thereof were incubated with rituximab, rituximab-F(ab′)2, or medium and MIP-1β, IL-10, IFNγ, and TNFα levels were measured in the supernatant.

Results. Rituximab-treated patients had higher serum levels of IL-10 (101±35 pg/ml versus 41±9 pg/ml; P<0.01) and MIP-1β (950±418 pg/ml versus 125±32 pg/ml; P<0.001) compared to placebo-treated patients at 2 hours after start of infusion. There was no difference in the level of other cytokines. In vitro, significant levels of MIP-1β were only detected when rituximab was added to a co-culture of B and NK cells. Incubation with rituximab-F(ab′)2 did not result in increased MIP-1β levels. Levels of MIP-1β were higher in patients with a high affinity Fc-receptor compared to those with a lower affinity FcYRIIIa (1356±184 pg/ml versus 679±273 pg/ml; P<0.01).

Conclusions. Next to B-cell depletion, rituximab can modulate the immune response by inducing cytokine secretion, especially IL-10 and MIP-1β. NK cells appear to be responsible for the MIP-1β secretion in a B-cell and Fc-receptor dependent manner.
INTRODUCTION

Monoclonal antibodies (mAbs) are widely used in the treatment of malignancies, transplant rejections, as well as a range of autoimmune diseases. For several of these mAbs, administration has been associated with acute infusion reactions, caused by various mechanisms, including systemic inflammatory response syndrome (1). Rituximab (RTX), a chimeric anti-CD20 mAb, is an effective treatment for malignant lymphomas and various autoimmune diseases (2). It is also used in organ transplant patients for desensitization and treatment of antibody-mediated rejection (3). RTX can deplete B cells via antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis (4). The observation that polymorphisms in the FcRIIIa gene affect the effectiveness of RTX, indicates that ADCC plays an important role (5). NK cells express the Fc-receptor FcRIIIa (CD16) which has a high affinity for binding to IgG1 (6), the isotype of RTX. FcRIIIa appears in two allelic forms that differ by the amino acid on position 158. FcRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcRIIIa with phenylalanine at that position (VF or FF) (7). Patients with the high affinity receptor show a better clinical response to RTX (8-10).

However, administration of RTX can be followed by acute infusion reactions due to the release of cytokines (1), especially in patients with high B-cell counts like lymphoma patients (11), although not every patient with elevated levels of cytokines develops clinical symptoms (12, 13). After stimulation, B cells can produce virtually any cytokine (14). Although the role of these cytokines in the regulation of other cell types is not completely understood, a rise in the level of (proinflammatory) cytokines could lead to activation of the immune system and therefore be of clinical relevance. When using RTX as induction therapy to prevent acute rejection, a cytokine release could potentially contribute to the risk of rejection.

Currently, it is unknown to which extent cytokine release occurs in patients with normal B-cell numbers, and the cell type responsible for the cytokine release has not been identified. In a double-blind placebo-controlled study, we measured the release of various cytokines after administration of RTX to renal transplant recipients. In addition, we studied the mechanism of this cytokine release in vitro.

MATERIALS AND METHODS

Patients
For this study, patients were recruited from the RTX in Renal Transplantation trial (ClinicalTrials.gov, NCT00565331), which evaluated the efficacy and safety of RTX (MabThera, Hoffmann-La Roche, Basel, Switzerland) when added to standard immunosuppression in renal transplant recipients. At the start of transplant surgery, patients received 1000 mg acetaminophen, 100 mg prednisolone and 2 mg
Cytokine release after treatment with rituximab
clemastin i.v. next to the standard treatment with 2000 mg ceftriaxone as antibiotic prophylaxis. After 30 minutes, RTX (375 mg/m²) or placebo was administered at an increasing infusion rate. The total infusion time was approximately 4 hours.

For logistic reasons, only recipients of a living donor kidney were selected for this study. Peripheral blood samples were collected from 20 patients (10 RTX-treated, 10 placebo-treated) a few hours before the transplantation (baseline), at 2 and 4 hours after starting the RTX infusion (t=2 h and t=4 h), and the next morning (t=24 h). Sera were stored at -80°C until analysis. Table 1 summarizes the patient characteristics.

Healthy donors and cell isolation
For in vitro experiments, peripheral blood samples were obtained from healthy donors after written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). CD14⁺ Monocytes and CD19⁺ B cells were positively selected using specific-magnetic microbeads, and NK cells were negatively selected using the NK-cell isolation kit II (all from Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in a purity of more than 95% for all lymphocyte subsets.

Culture conditions
Whole blood culture was used to determine the cytokine production in vitro. Therefore, whole blood was diluted 1:5 with culture medium in 24-well plates (Greiner Bio-One, Frickenhausen, Germany). To study the cytokine production by different lymphocyte subsets, 2 x 10⁵ PBMCs, B cells, NK cells, and/or monocytes were cultured in RPMI-1640 medium supplemented with pyruvate (0.02 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Gibco, Paisley, United Kingdom), and 10% fetal bovine serum (FBS) in 96-well round bottom plates (Greiner Bio-One) in a 37°C, 95% humidity, 5% CO₂ incubator. In selected conditions, 250 µg/ml RTX (MabThera, Hoffmann-La Roche) or 250 µg/ml RTX-F(ab’)₂ (provided by Genmab, Utrecht, The Netherlands) was added to the culture medium. Supernatant was collected after 14 hours and/or 24 hours and stored at -20°C until analysis.

Cytokine measurements
Serum and culture supernatant levels of IL-2, IL-6, IL-10, IL-12, IL-17, TNFα, IFNγ (eBioscience, San Diego, CA), and MIP-1β (Invitrogen, Carlsbad, CA) were determined by ELISA according to the manufacturer’s instructions.

FcγRIIIa-158 genotype analysis
DNA was extracted from peripheral blood of 10 RTX-treated patients using a DNA isolation kit (Qiagen, Valencia, CA). Genotyping of FcγRIIIa-158 (rs396991) was performed using the TaqMan-Allelic discrimination method with a specific probe
for rs396991 designed for single nucleotide polymorphism (SNP) of FcγRIIIa and results were analyzed using the Allelic Discrimination software program according to the manufacturer’s instructions (all from Applied Biosystems, Foster City, CA).

Statistical analysis
Cytokine concentrations are presented as mean ± SD. Nonparametric tests were used to compare variables. One-way ANOVA was used to compare the different groups over time, followed by Dunn’s multiple comparison test for post-testing. To test the difference between the cultured cells with rituximab or without (culture medium) the Wilcoxon signed rank test was performed. \( P<0.05 \) was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

RTX infusion is associated with specific cytokine release
The infusion of RTX induced a temporary rise in the levels of IL-10 and MIP-1β. At 2 hours after the start of the infusion, the serum levels of these cytokines were significantly higher in RTX-treated patients than in placebo-treated patients (IL-10 (101±35 pg/ml versus 41±9 pg/ml; \( P<0.01 \)) and MIP-1β (950±418 pg/ml versus 125±32 pg/ml; \( P<0.001 \); Figure 1). However, RTX infusion did not increase the levels of IL-2, IL-6, IL-12, IL-17, IFNγ, or TNFα levels as compared to placebo treatment, suggesting that RTX infusion is associated with a specific cytokine release. None of the patients experienced any clinical symptoms associated with cytokine release.

Table 1. Patient characteristics*.

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Rituximab (n=10)</th>
<th>Placebo (n=10)</th>
</tr>
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<tr>
<td>Age; mean ± SD</td>
<td>46.7 ± 12.5</td>
<td>44.7 ± 12.5</td>
</tr>
<tr>
<td>Sex; Male : female, n</td>
<td>6 : 4</td>
<td>9 : 1</td>
</tr>
<tr>
<td>HLA mismatches; mean ± SD</td>
<td>2.9 ± 1.3</td>
<td>3.3 ± 1.3</td>
</tr>
<tr>
<td>Immunosuppression before transplantation; n</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Patients with panel reactive antigen &gt; 6%; n</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Renal replacement therapy</td>
<td>none / hemodialysis / peritoneal dialysis; n</td>
<td>3 / 6 / 1</td>
</tr>
</tbody>
</table>

* There were no statistical differences
Cytokine release after treatment with rituximab

Exposure to RTX induces a B-cell dependent secretion of MIP-1β by NK cells

Using in vitro studies with blood from healthy donors, we next analyzed which cells were responsible for the cytokine production after exposure to RTX. We used whole blood cultures next to PBMC, to stay as close to the in vivo situation as possible (15). In a 24 hours whole blood culture as well as isolated PBMC culture, addition of RTX induced elevated MIP-1β levels as compared to the medium alone (whole blood culture: 592±218 pg/ml versus 79±68 pg/ml; P=0.06 and PBMC culture: 500±450 pg/ml versus 23±6 pg/ml; P<0.05), whereas IL-10, IFNγ, and TNFα were not detectable in this system (Figure 2A).

To pinpoint the cells that produced MIP-1β after RTX administration, MIP-1β levels were measured in the culture supernatant of freshly isolated PBMC, B cells, NK cells, or monocytes that were exposed to RTX for 14 hours. In addition, MIP-1β was measured in co-cultured B-NK-cells and co-cultured B-cell-monocytes upon RTX treatment. RTX did not increase the MIP-1β levels in cultures of B cells, NK cells or monocytes alone, nor in co-cultures of B cells-monocytes. In contrast, RTX-

Figure 1. In vivo cytokine release after RTX infusion.

Serum levels of IL-2, IL-6, IL-10, IL-12, IL-17, IFNγ, MIP-1β and TNFα in patients who underwent renal transplantation. 20 patients received a single dose of RTX (375 mg/m²) or placebo during transplant surgery. Concomitant immunosuppression consisted of tacrolimus, mycophenolate mofetil (MMF) and steroids. Blood samples were taken a few hours before transplantation (t=baseline), 2 and 4 hours after the start of infusion of RTX or placebo, and the next morning after transplantation (t=24 hours). Results are depicted as mean ± SD. Significant differences between placebo and RTX-treated patients at the different time points are indicated by asterisks: **P<0.01, ***P<0.001.
treated co-cultures of B cells and NK cells revealed significantly enhanced MIP-1β levels as compared to the medium control (597±321 pg/ml versus 30±23 pg/ml; \(P<0.05\); Figure 2B). Taken together, this suggests that after exposure to RTX MIP-1β is secreted by NK cells in a B cell-dependent manner.

**MIP-1β secretion after exposure to RTX is Fc-receptor dependent**

To establish if the MIP-1β secretion caused by exposure to RTX is Fc-receptor dependent, PBMCs were incubated with RTX, RTX-F(ab')₂ or in culture medium alone. PBMCs cultured with RTX-F(ab')₂ did not show increased MIP-1β secretion (Figure 2C), indicating that MIP-1β secretion is Fc-receptor dependent.

It is known that single nucleotide polymorphisms (SNP) of the FcγRIIIa gene at position 158 result in an altered Fc-receptor function, where FcγRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcγRIIIa with phenylalanine at that position (VF or FF) (7). We therefore wondered whether the MIP-1β secretion after treatment with RTX in vivo could be correlated to the genotype of the Fc-receptor FcγRIIIa (CD16) present on NK cells. To this end, we determined the SNP at position 158 in 10 RTX-treated renal transplant patients. Interestingly, RTX-treated patients in the high affinity group (VV) revealed higher serum levels of MIP-1β as compared to the lower affinity group (VF/VV) at 2 hours after the start of the RTX infusion (1356±184 pg/ml versus 679±273 pg/ml; \(P<0.01\); Figure 2D). Although not significant, a similar trend was observed for IL-10 levels (288±111 pg/ml versus 184±35 pg/ml; \(P=0.17\)). These findings support our conclusion from the in vitro studies that the cytokine release is Fc-receptor dependent.

**DISCUSSION**

In this study, we showed that RTX infusion leads to a specific cytokine release in renal transplant recipients. At 2 hours after the start of the infusion, IL-10 and MIP-1β serum levels were significantly higher in RTX-treated patients as compared to placebo-treated patients, whereas the levels of IL-2, IL-6, IL-12, IL-17, IFN and TNFα remained unaffected. Additional in vitro data revealed that NK cells were largely responsible for the MIP-1β release in a B-cell and Fc-receptor dependent manner.

Although RTX is usually well tolerated, the first infusion may be accompanied by severe side-effects, which are correlated with complement activation (16) and with the number of circulating B cells (11), as more side-effects occur in patients with high B-cell counts, like lymphoma patients. There is limited data on the cytokine releasing effects of RTX in patients with B-cell counts in the normal range, like renal transplant recipients or patients with autoimmune diseases. In our study cohort, we found elevated cytokine levels after RTX infusion. The type of cytokines secreted was different from that observed in other cohorts treated with distinct monoclonal
Cytokine release after treatment with rituximab

antibodies (6, 17). The cytokine release syndrome associated with OKT3 treatment is characterized by the release of the inflammatory cytokines IL-2, TNFα, and IFNγ (17), while treatment with the humanized anti-CD52 mAb alemtuzumab was accompanied with elevated levels of IL-6, TNFα, and IFNγ (18). In these cases, it is believed that the release of inflammatory cytokines is due to direct T-cell activation

Figure 2. Cytokine release after exposure to RTX is Fc-receptor mediated.

(A) Production of MIP-1β, IL-10, TNFα and IFNγ from whole blood cultures (n=5) and PBMCs of healthy donors (n=4) incubated with medium or 250 μg/ml RTX for 24 hours. (B) Production of MIP-1β by PBMCs, isolated CD19+ B cells, CD3 CD56+ NK cells, CD14+ Monocytes, and a co-culture of B cells with NK cells or Monocytes of healthy donors (n=6) incubated with medium or 250 μg/ml RTX after 14 hours. (C) Production of MIP-1β by PBMCs of healthy donors 24 hours after incubation with medium, 250 μg/ml RTX or RTX-F(ab’)2. (D) Shown are the serum levels of MIP-1β and IL-10 in patients with the high affinity receptor SNP-158VV (n=4) and the low affinity receptor SNP-158VV/VF group (n=6) 2 hours after the start of the RTX infusion. Significant differences are indicated by asterisks: *P<0.05, **P<0.01.
Chapter 6

(17) or dependent on Fc-receptor ligation on phagocytic cells, such as monocytes, macrophages, and NK cells (19). We found that exposure to RTX, without further stimulus, only led to significant MIP-1β levels in co-cultures of purified B and NK cells. As single NK-cell cultures did reveal some, albeit limited, MIP-1β production, and the levels of MIP-1β secreted by B cells alone were below detection level, we suggest that the cytokine release observed was coming from NK cells and largely dependent on the binding of these cells to RTX-coated B cells. Finally, incubation of PBMCs with RTX-F(ab')2 did not lead to an increased MIP-1β secretion, indicating that the cytokine release is indeed Fc-receptor dependent.

NK cells express the Fc-receptor FcγRIIIa (CD16) which has a high affinity for binding to IgG1 (6), the isotype of RTX. FcγRIIIa appears in two allelic forms that differ by the amino acid on position 158. Single nucleotide polymorphisms (SNPs) at position 158 result in an altered Fc-receptor function. FcγRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcγRIIIa with phenylalanine at that position (VF or FF) (7). Interestingly, we found that MIP-1β levels in patients treated with RTX correlated to the FcγRIIIa-158 SNP. Patients with the high affinity SNP produced higher levels of MIP-1β. This observation fits with previous data showing that the degree of NK cell activation and the clinical response upon RTX treatment are influenced by the FcγRIIIa-158 SNP (20).

The observed rise in IL-10 and MIP-1β could be of clinical relevance, since it may well modulate the (allo)immune response influencing the transplantation outcome. IL-10 is a well known anti-inflammatory cytokine which plays an important role in immune regulation (21). In a mouse model, MIP-1β was shown to be the most potent chemoattractant for regulatory T cells within a range of chemokines (22), suggesting that increased secretion might down modulate the immune response. On the other hand, MIP-1β can be produced by lymphocytes that are involved in inducing immune reactivity, including NK cells and B cells. (23). Moreover, MIP-1β recruits monocytes, T cells, and dendritic cells to the site of injury or inflammation via the chemokine receptor CCR5, which is highly expressed by monocytes and has a lower expression on T cells and dendritic cells. Therefore, in renal transplantation increased MIP-1β levels might contribute to activation of the immune response, and thus graft injury. Indeed, in a previous study with RTX as induction therapy in renal transplantation, an increased rate of acute rejection was associated with elevated levels of proinfl ammatory cytokines in some of the patients (24). In that report no data are provided on MIP-1β levels.

In summary, results from this study indicate that RTX does not only lead to B-cell depletion, but also results in the release of cytokines, especially IL-10 and MIP-1β, which might modulate the immune response. NK cells appear to be responsible for MIP-1β secretion in a B-cell and Fc-receptor dependent manner.
Acknowledgments

This study was supported by research funding from the Dutch Kidney Foundation (nr C09-2301). We would like to thank Jan van de Winkel (Genmab, Utrecht, The Netherlands) for providing the RTX-F(ab’)₂ fragment. Technical support was kindly provided by H. Tijssen (Radboud University Medical Center).
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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION
Organ transplantation is the preferred treatment for patients with end-stage renal disease. It improves quality of life and increases life expectancy compared to dialysis treatment. The short-term transplantation outcome is greatly increased over the last 50 years, mainly due to the introduction of potent immunosuppressive drugs. However, the long-term success of renal transplantation is limited by chronic allograft dysfunction (1, 2). This term refers to a clinical syndrome of slowly deteriorating graft function due to multiple causes, of which chronic rejection is probably the most important.

T cells play an important role in the pathogenesis of rejection via the recognition of alloantigens, resulting in T-cell activation, proliferation, and differentiation into CD4+ T helper cells and CD8+ cytotoxic T cells (3). Therefore, the most commonly used immunosuppressive drugs in transplantation are directed against T cells to inhibit these processes (4). Next to T cells, B cells can be involved in graft rejection via the production of alloantibodies, but they can also induce an immune response by acting as professional antigen presenting cells, or by the production of various cytokines (5). The presence of anti-HLA antibodies before or after transplantation, as well as the presence of B-cell clusters in renal grafts during acute rejection, are associated with poorer graft survival (6-8). Therefore, depletion of B cells in renal transplant recipients might help to prevent allograft rejection. On the other hand, B cells with regulatory functions have been described in tolerant transplant patients who had stable allograft function despite not receiving immunosuppression for at least one year (9). Since B cells can play such a variety of roles in the immune response, the effects of anti-B-cell therapy have to be analyzed carefully.

In the studies included in this thesis, we aimed to gain more knowledge on anti-B-cell therapy in renal transplantation. The basis of these studies was formed by a randomized clinical trial, in which the effects of a single dose of the B-cell depleting monoclonal antibody rituximab (RTX) added to standard immunosuppression in renal transplant recipients were evaluated (chapter 2). In a selected subgroup of participants of this clinical trial, we analyzed the phenotypic and functional characteristics of peripheral T and B cells before and after transplantation (chapter 3). Although RTX leads to a nearly complete B-cell depletion in peripheral blood, B cells do remain in secondary lymphoid organs after RTX treatment. Therefore, we wondered whether RTX had any direct effects on B cells besides inducing B-cell depletion. In chapter 4, we studied an in vitro non-depleting model to establish the effects of RTX on the proliferation, activation, and differentiation of purified B cells. In chapter 5, we assessed the in vivo effects of RTX on B cells in secondary lymphoid organs. Additionally, we showed that RTX infusion leads to a specific cytokine release in renal transplant recipients (chapter 6).
Targeting B cells in renal transplantation

Current immunosuppressive regimens consisting of steroids, a calcineurin-inhibitor, and MMF reduce B-cell function directly by inhibition of their proliferation and indirectly via the inhibition of T-cell help. An alternative approach is specific B-cell depletion by treatment with RTX.

In chapter 2, we evaluated the safety and efficacy of RTX when added to a standard immunosuppressive drug regimen consisting of tacrolimus, MMF, and steroids. We showed that induction therapy with a single dose of RTX at the time of transplantation is safe, but ineffective to reduce biopsy-proven acute rejection (BPAR) in the total population of renal transplant recipients. Our data also indicate that in immunologically high risk patients, RTX induction therapy may reduce the incidence of acute rejection to a level comparable to that in immunologically low risk patients. Therefore, induction therapy with RTX might improve the long-term graft survival in this specific group of transplant patients. However, this hypothesis needs to be confirmed in a multicenter clinical trial focused on immunologically high risk patients. In addition, it is important to monitor mortality rates of RTX-treated patients in the future, since increased mortality has been reported in long term follow-up of renal transplant recipients treated with RTX (10-11).

Interestingly, we found that immunologically high risk patients had more memory B cells before transplantation compared to immunologically low risk patients. It is tempting to speculate that a protective effect of RTX in the immunologically high risk patients could be explained by the higher pre-transplant levels of memory B cells in these patients, which may then be depleted after RTX treatment. Upon re-exposure to antigen after transplantation, these memory B cells could quickly differentiate into anti-HLA antibody-producing plasma cells (12). Indeed, an increase in circulating memory B cells has been associated with acute rejection in paediatric renal transplant recipients (13), while heart transplant recipients with higher percentages of naive B cells had a lower risk of acute rejection (14). It should be noted however that memory B cells appear more resistant to depletion by RTX than naive B cells, especially in secondary lymphoid organs (15), which leaves the possibility that the protective effect of RTX might rather be due to the depletion of the naive donor antigen-specific B-cell pool, thus preventing further expansion and differentiation of these cells into memory B cells and/or antigen-presenting cells.

In general, B-cell repopulation in autoimmune disease patients treated with RTX starts within 6 to 9 months (16, 17). However, in our cohort of renal transplant recipients a single dose of RTX added to triple immunosuppressive drug regimen resulted in a long lasting B-cell depletion in peripheral blood (chapter 3). The majority of the RTX-treated patients had lower B-cell numbers, as compared to pre-transplant levels, up to 24 months after transplantation. Our findings, combined with previously published studies on renal transplant recipients (18), suggest that the delayed repopulation of B cells might be related to the
maintenance immunosuppression. However, also in the absence of maintenance immunosuppression, in sensitized patients awaiting renal transplantation and treated with a single dose of RTX, B-cell repopulation was slower compared to autoimmune disease patients (19). At 12 months after treatment, B-cell numbers were lower compared to pre-treatment levels, but returned to baseline after 24 months. Therefore, the type of underlying condition may also influence the rate of B-cell recovery.

Although RTX does not target antibody-producing plasma cells, but rather their B-cell precursors, prolonged B-cell depletion might lead to a decrease in the concentration of donor-specific anti-HLA antibodies (DSA), which have been associated with poorer kidney graft survival (8). To determine whether the higher pre-transplant levels of memory B cells in immunologically high risk patients can be correlated to DSA and long-term graft survival several issues remain to be resolved. There is still controversy on which type of plasma cells (short or long-lived) is responsible for the DSA production (12), what the precursors are of those DSA-producing plasma cells, and whether or not anti-B-cell therapy might be effective in the prevention of pre-existing and/or de novo DSA production. So far, RTX has been successfully used in the treatment of antibody-mediated rejections (2), which in some (20, 21), but not all (5) cases was associated with decreased DSA levels. Although this might suggest that DSAs are produced by short-lived plasma cells, it cannot be excluded that additional IVIG treatment was responsible for the observed reduction. In sensitized patients treated with RTX it was observed that the repopulation of memory B cells was more delayed for donor-specific B cells than those specific for third-party HLA (22). In the near future, the effect of RTX treatment on the titers of pre-existing DSA and on the formation of de novo DSA will be measured in sera collected from patients who participated in our prospective clinical trial.

Prevention of rejection after renal transplantation requires treatment with immunosuppressive drugs that inhibit the function of alloreactive T and B cells. In spite of the wide use of immunosuppressive drugs, there is limited data available regarding their in vivo effects on the peripheral T and B cells after renal transplantation. In chapter 3, we performed a longitudinal analysis of T- and B-cell phenotype and function in a homogeneous patient population consisting of CMV seronegative patients who received a kidney from a CMV seronegative donor and did not experience a rejection episode up to 24 months after transplantation. This strict selection criteria were chosen to exclude any potential effects on the immune system of a CMV infection or rejection. Our main finding was that renal transplant recipients treated with the combination of tacrolimus, MMF and steroids showed an altered memory T- and B-cell compartment as compared to pre-transplantation, without changes in the total numbers of peripheral T and B cells.

Recent data indicate that the composition of the B-cell compartment is related to the clinical outcome of transplantation. Tolerant renal transplant recipients, who
have stable graft function despite not receiving immunosuppression for at least one year, show a higher number of total B cells, of which especially the naive and transitional B cells are increased (9). It has been suggested that this increase might lead to tolerance induction, since regulatory B cells, which produce the regulatory cytokine IL-10, can be found within the transitional B-cell compartment (23). In addition, in animal models naive B cells have been shown to be tolerogenic for naive T cells (24) and to induce a regulatory phenotype in naive T cells upon stimulation (25). Interestingly, in patients with autoimmune disease higher numbers of memory B cells were associated with worse clinical outcome (26, 27). Also, in pediatric renal transplant recipients increasing counts of circulating memory B cells were seen at time of acute rejection (13). This could imply that in transplantation patients the balance between naive and memory B cells might be associated with the degree of acceptance of the graft.

Currently, there is limited data on the course of B-cell subset distribution after renal transplantation. It is also unclear whether maintenance immunosuppression or the presence of an allograft has an effect on the B-cell phenotype. We found that under triple immunosuppressive drug regimen renal transplant recipients showed a shift toward a more memory-like phenotype in the B-cell population (chapter 3). The explanation for this phenomenon is not obvious yet. In vitro, steroids and MMF, but not tacrolimus have been found to inhibit B-cell activation and proliferation (28-30). However, none of these studies described an effect on the B-cell subset distribution. Alternatively, the presence of an allograft and therefore the introduction of novel antigens, might induce an immune response resulting in the differentiation of antigen-specific naive B cells into memory B cells. Also, viral infections can have a pronounced effect on the immune system (31). However, we tried to avoid that effect by analyzing only CMV seronegative patients who received a CMV seronegative donor (and thus did not develop CMV infection).

In our patients, addition of induction therapy with RTX to the standard immunosuppressive treatment resulted in long term B-cell depletion, but eventually in the enrichment of CD24\textsuperscript{hi}CD38\textsuperscript{hi} transitional B cells in the reconstituted B-cell compartment. In several conditions, a preferential repopulation of transitional and naive B cells after RTX treatment has been associated with better clinical response and outcome, due to the relative increase of regulatory B cells present in the transitional B-cell compartment (13, 26, 32-34). Although we did not find a clinical effect of RTX in our patient cohort, this observed change in the B-cell compartment might be beneficial for the long term outcome. However, for the short term, B-cell depletion and thus the removal of B cells with regulatory properties may have adverse effects. In an autoimmune disease animal model, depletion of regulatory B cells was associated with disease progression (35). Therefore, future anti-B-cell therapies should focus on reducing or blocking effector B-cell activation rather than depleting all B cells, including regulatory B cells. Another possibility might be enhancing the number of regulatory B cells by specific therapeutic antibodies.
or by cell therapy with regulatory B cells. First, further research is needed on the characterization of regulatory B cells in humans since there is still controversy about the exact phenotype of these cells.

**Effects of RTX beyond B-cell depletion**

Upon activation, B cells are able to proliferate, produce various cytokines and process antigen for presentation to T cells (36-38). All these processes can be affected by RTX, resulting in an altered immune response after treatment with RTX. To study the direct effects of RTX on B cells, we used an *in vitro* non-depleting stimulation model. In *chapter 4*, we describe that *in vitro* RTX treatment can affect B-cell phenotype and function, resulting in an altered outcome of B-T-cell interaction upon stimulation in a mixed lymphocyte reaction. However, similar changes in the T cell phenotype were not observed *ex vivo* in our renal transplant recipients treated with RTX (*chapter 3*). This is remarkable, as several studies on patients with autoimmune disease revealed that the T-cell compartment was indeed affected upon RTX treatment (39-41). Systemic lupus erythematosus patients had relatively decreased percentages of memory T cells 2 years after RTX treatment (39), and patients with idiopathic thrombocytopenic purpura, particularly responders showed increased levels of regulatory T cells upon treatment. Also, B-cell depletion in patients with multiple sclerosis resulted in diminished Th1 and Th17 responses (41). This is of interest as in an *in vitro* stimulation assay, we did observe a lower IL-17 production in CD4$^+$ T cells upon stimulation with B cells from RTX-treated patients compared to B cells from untreated patients. However, in most of these autoimmune disease patients the cumulative dose of RTX was higher than in our patients, who received only a single, relatively low dose (39). Another explanation might be that the immune system in the patients with autoimmune disease is reset after treatment with RTX by depleting autoreactive B cells, while renal transplant recipients have in general a less affected immune system compared to patients with autoimmune disease, which is likely to be less influenced by depleting (some of the) B cells. It should also be noted that we were only able to analyze peripheral blood T cells. We have demonstrated that in renal transplant patients a single dose of RTX leads to nearly complete B-cell depletion in peripheral blood, but not in secondary lymphoid organs, and that these remaining B cells have different functional capacities (*chapter 5*). The B cells remaining in the lymph nodes after RTX treatment were predominantly of a switched memory (IgD CD27+) phenotype. It appeared that this population of mostly memory type B cells residing in lymphoid organs did not noticeably affect the peripheral blood T-cell compartment. Notably, lymph nodes from RTX-treated patients contained no transitional B cells, including B cells with a regulatory phenotype, which contrasts with the enrichment of transitional B cells after repopulation of the peripheral blood. Theoretically, this relative increase of memory B cells and lack of regulatory B cells in lymph nodes
might contribute to an enhanced alloimmune response and thus be undesirable in transplant patients.

Even though the majority of studies report an effective B-cell depletion in peripheral blood, it remains remarkable why there are such discrepancies between different studies on the percentage of remaining B cells in lymphoid organs (18), bone marrow (42), or synovial tissues (43) after RTX treatment. In contrast to our finding of unaffected percentages of lymph node B cells (chapter 5), the previous studies described at least some degree of B-cell reduction. There are several theoretical explanations for this lack of complete B-cell depletion in lymphoid organs. First, RTX may not be able to reach the B cells in the lymph nodes. This seems to be unlikely, because CD19 positive B cells in lymph nodes of transplant recipients that did not receive RTX were all CD20 positive, while the CD19 positive B cells that remain in the lymph nodes of renal transplant recipients after RTX treatment were all CD20 negative, indicating that RTX was bound to these cells. Next, there may be an exhaustion of complement dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC), or both (44). Also, the microenvironment in the lymphoid organs may play a crucial role in determining the chances of survival versus depletion of B cells upon RTX treatment. For instance, increased expression of B-cell activating factor (BAFF) in lymphoid tissues may provide a survival signal for the B cells (45). Finally, the differences in RTX treatment regimen, choice of immunosuppressive drugs and heterogeneity of the patient population might also explain the variation in B-cell depletion across different studies.

Based on the considerations above, we exposed B cells in vitro to RTX in the absence of complement (chapter 4), which mimics the in vivo situation insofar that B cells are exposed to RTX but are spared from cytotoxic effector mechanisms, whatever the reason(s) for the latter might be. Addition of complement to the in vitro system resulted in an increase of the proportion of dying cells, however there were no clear differences in the expression of relevant cell surface markers between the cells remaining after incubation with RTX and complement versus the cells exposed to RTX only. Taking into account that an in vitro model can never fully mimic the in vivo environment, our in vitro non depleting model gave us the opportunity to study the direct effects of RTX on B cells more in depth compared to the in vivo situation.

The findings described in chapters 4 and 5 demonstrate that RTX especially affects naive, but not memory B cells. The relative resistance of memory B cells to RTX has also been reported by others (15), but the exact reasons for this difference remains to be determined. An obvious explanation would be a variation in the level of CD20 expression, however this was similar in both subsets and therefore does not seem to be involved. Remarkably, peripheral blood B cells stimulated in the presence of RTX induced stronger T-cell proliferation, compared to B cells stimulated in the absence of RTX. Although this might suggest that anti-B-cell therapy could have adverse effects in renal transplant recipients by enhancing T cell
alloreactivity, in our study the resulting T-cell population showed a more Th2-like phenotype, which is usually associated with graft acceptance rather than rejection (46). In contrast in chapter 5, we described that lymph node B cells obtained from RTX treated patients resulted in a weaker Th17 response when used as stimulators in an allogeneic mixed lymphocyte reaction. It should be noted that this difference in the T-cell responses might be due to the different origin of the B cells; in one study we used lymph nodes obtained from renal transplant recipients while in the other study we used peripheral blood from healthy donors. The B-cell subset distribution between the two clearly differs with the majority of peripheral blood B cells being naive (IgD⁺CD27⁻), while lymph node B cells are predominantly of an IgD⁻CD27⁺ switched memory phenotype. This difference in B-cell subsets potentially leads to a different T-cell response regardless of any further treatment (e.g. RTX), as was described using CD27⁻ and CD27⁺ B cells to stimulate allogeneic CD4⁺ T cells in vitro (47). Importantly, we showed that RTX inhibits specifically naive, but not memory B cells.

Finally, another effect of RTX beyond B-cell depletion is that administration of RTX can be followed by an acute infusion reaction due to the release of cytokines (48), especially in patients with high B-cell counts, like lymphoma patients (49). In chapter 6, we studied the effect of RTX infusion on the cytokine levels in renal transplant recipients, who exhibit B-cell counts in the normal range. RTX infusion led to a specific cytokine release, with increased IL-10 and MIP-1β serum levels in RTX-treated patients as compared to placebo-treated patients at 2 hours after start of the infusion. Using in vitro cultures with blood from healthy donors, we found that the highest MIP-1β levels were measured in the RTX-treated co-cultures of B cells and NK cells. MIP-1β was most likely produced by NK cells after binding to RTX-coated B cells via Fc-receptors. Single nucleotide polymorphisms (SNP) in FcγRIIIa (CD16) at position 158 are known to affect the binding affinity for IgG1, the isotype of RTX. Notably, the MIP-1β release was more pronounced in patients with a high affinity Fc-receptor compared to those with a lower affinity FcγRIIIa. The observed rise in cytokines could be of clinical relevance, since it may modulate the (allo)immune response influencing the outcome of renal transplantation. For instance, the temporary increase of IL-10 might dampen the inflammatory response during the early post transplant period. In this respect, it would be interesting to compare the clinical outcome in RTX-treated patients with SNP-158VV (high affinity receptor) and those with the low affinity receptor.
CONCLUDING REMARKS

The long term success of renal transplantation depends on tricking the immune system into thinking that the kidney is not harmful, preferably by inducing tolerance. The role of B cells in transplant rejection and tolerance, but also in several autoimmune diseases has been increasingly appreciated. The wide use of anti-B-cell agents offers accumulating evidence for their beneficial effects in a wide range of diseases. Even though we have increased our knowledge about the role of B cells in renal transplantation, further research is needed. Currently, it is still difficult to predict whether anti-B-cell therapy will be beneficial for the long term transplantation outcome or not. Also the mechanism of action of RTX may not be as straightforward as first was expected. Although RTX depletes B cells, the precursors of antibody-producing cells, clinical experience with RTX in autoimmune diseases suggests that antibody levels are not always affected by RTX treatment. Altered T-cell stimulation by the B-cell pool, either by a change in antigen presentation or by the production of immunomodulatory cytokines might be important mechanisms of action of RTX as well. This would mean that the therapeutic effects go beyond simple B-cell depletion. In this thesis, we described that RTX can directly affect B-cell phenotype and function. Therefore, the effect of RTX on the immune response will not only be determined by the extent of B-cell depletion, but also by the functional properties of the remaining B cells. Moreover, the relative increase of regulatory B cells in the repopulating B-cell compartment after B-cell depletion might potentially promote tolerance. Eventually, the therapeutic effect of B-cell depletion will depend on the resulting balance between effector and regulatory B cells next to the timing of treatment and the type of disease or condition. Consequently, future therapy should be more directed at selective depletion or modulation of the B-cell compartment.
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CHAPTER 8

NEDERLANDSE SAMENVATTING
Niertransplantatie is de eerste keus behandeling voor patiënten die geen eigen nierfunctie meer hebben. Patiënten die een succesvolle niertransplantatie hebben ondergaan, leven gemiddeld langer dan patiënten die worden behandeld met dialyse. Ook leidt niertransplantatie tot een sterk verbeterde kwaliteit van leven. Een transplantatie heeft het beste kans van slagen als de donor nier goed past bij de ontvanger. Hoe beter de match, hoe minder kans op afstoting. Ons afweersysteem (immuunsysteem) is ontwikkeld om op efficiënte wijze lichaamsvreemde indringers, zoals virussen en bacteriën, op te sporen en ze onschadelijk te maken. Het immuunsysteem beschouwt de getransplanteerde nier als iets wat niet in het lichaam thuis hoort. Als reactie gaan de witte bloedcellen van het immuunsysteem de nier aanvallen. De T cellen, die onderdeel uitmaken van de witte bloedcellen, spelen een belangrijke rol in de herkenning van lichaamsvreemde indringers. Activering van T cellen die specifiek de getransplanteerde nier herkennen, zogenaamde alloreactieve T cellen, leidt tot een snelle vermeerdering (proliferatie) van deze cellen en differentiatie waarna ze ontstekingswitten (cytokines) gaan produceren (CD4+ helper T cellen) of direct in staat zijn om andere cellen te vernietigen (CD8+ cytotoxicke ‘killer’ T cellen). Helper T cellen geven hulp aan andere witte bloedcellen, zoals de B cellen die antilichamen gaan maken. Antilichamen kunnen een ziekteverwekker of andere lichaamsvreemde indringers herkennen en door er aan te binden vormen ze een belangrijke schakel in het onschadelijk maken ervan. B cellen worden gedurende het hele leven aangemaakt in het beenmerg, waar ze zich ontwikkelen uit bloedstamcellen. Tijdens de voorloper B cel ontwikkeling in het beenmerg creëert elke voorloper B cel een uniek antilichaamsmolecuul, ook wel immunglobuline (Ig) genoemd. Vervolgens verlaat deze B cel het beenmerg via de bloedbaan en migreert tussen de verschillende lymfoïde organen, zoals de milt en lymfklieren, op zoek naar antigenen. Wanneer een B cel zijn specifieke antigeen herkent, zal deze worden geactiveerd en zichzelf vermenigvuldigen. De dochtercellen rijpen vervolgens uit tot plasmacellen, die antilichamen produceren, of tot geheugencellen, die zeer snel in actie kunnen komen zodra hetzelfde antigeen nog een keer het lichaam binnenkomt. Naast de productie van antilichamen zijn B cellen in staat om een immuunrespons op gang te zetten door antigenen aan T cellen te presenteren, waardoor de T cellen geactiveerd raken, of door de productie van verschillende cytokines.

Om activering van het immuunsysteem en afstoting van de nier te voorkomen, moet iemand met een getransplanteerde nier levenslang afweeronderdrukkende middelen (immunosuppressiva) gebruiken. Omdat immunosuppressiva het gehele immuunsysteem onderdrukken, hebben transplantatiepatiënten een verhoogde kans op infecties en het ontwikkelen van tumoren. Om de kans op afstoting te verminderen wordt er gekeken naar bloedgroep en weefseltypering (witte bloedcellen) om vast te stellen of er sprake is van een goede match tussen donor en ontvanger. De kans op afstoting van een donor nier is kleiner als de weefselkenmerken van donor en ontvanger meer op elkaar lijken. Die kenmerken zijn erfelijk bepaald en
worden Humaan Leukocyten Antigenen (HLA) genoemd. Met de zich steeds weer verbeterende ontwikkeling op het gebied van immuunosuppressiva, is de levensduur van een getransplanteerde nier sterk verbeterd. Optimisering van het gebruik van verschillende immuunosuppressiva tegelijkertijd, heeft ertoe geleid dat 90% van de nieren 1 jaar na transplantatie nog steeds functioneert, terwijl dit na 10 jaar ongeveer 60% is. Huidige veelgebruikte immuunosuppressiva zijn met name gericht om T cel activering te voorkomen, maar zoals eerder genoemd kan B cel activering en differentiatie ook nadelige gevolgen hebben voor de transplantaatoverleving. De aanwezigheid van anti-HLA antilichamen, die geproduceerd worden door B cellen, voor en na transplantatie, evenals de aanwezigheid van B cellen in de nier ten tijde van een afstotingsreactie, zijn geassocieerd met kortere transplantaatoverleving. Daarom zou het verwijderen (depletie) van B cellen bij niertransplantatiepatiënten mogelijk kunnen helpen om afstoting te voorkomen. Anderzijds, zijn er B cellen met regulerende (afstotingsremmende) functies beschreven bij patiënten die een stabiele nierfunctie hebben ondanks dat ze al meer dan een jaar gestopt zijn met het gebruik van immuunosuppressiva. Omdat B cellen een verscheidenheid van functies kunnen hebben bij een immuunrespons, moeten de effecten van het verwijderen van B cellen (anti-B-cel therapie) zorgvuldig geanalyseerd worden. Een voorbeeld van anti-B-cel therapie is het B cel depleterende monoklonale antilichaam rituximab. Rituximab bindt aan het CD20 eiwit, dat zich aan het oppervlak van B cellen bevindt. Na binding van rituximab aan de B cellen worden deze laatste door het eigen afweersysteem herkend en vernietigd. Er worden weer nieuwe B cellen gevormd uit de stamcellen in het beenmerg, maar het kan maanden tot jaren duren totdat weer normale aantallen in het bloed aanwezig zijn. Omdat het CD20-eiwit alleen voorkomt op B cellen zal rituximab niet aan andere cellen in het lichaam binden.

**B cellen als doelwit bij niertransplantatie**

Het doel van het onderzoek beschreven in dit proefschrift is het verkrijgen van meer kennis over anti-B-cel therapie bij niertransplantatie. De basis werd gevormd door een gerandomiseerde, dubbelblinde, placebocontroleerde klinische studie waarin de effecten van rituximab gegeven aan niertransplantatie patiënten werden geëvalueerd (**hoofdstuk 2**). In deze studie werden twee patiëntengroepen met elkaar vergeleken. De ene groep kreeg rituximab in de operatiekamer gedurende de niertransplantatie, de andere een placebo (nepmiddel, in dit geval zoutoplossing). De toewijzing van patiënten aan één van beide groepen gebeurde willekeurig (gerandomiseerd) en daarnaast wisten zowel de onderzoeker, als de patiënt niet wie welk middel kreeg (dubbelblind). Beide patiënten groepen kregen de standaard immuunosuppressieve behandeling bestaande uit tacrolimus, mycofenolaat mofetil en steroïden. Uit de resultaten bleek dat één dosis van rituximab veilig is, maar niet leidt tot vermindere afstoting in de totale populatie niertransplantatiepatiënten. Bij geïmmuniseerde patiënten, zij die een eerdere transplantatie en/of anti-HLA
antilichamen vóór transplantatie hadden, lijkt rituximab het aantal afstotingen te verminderen tot een niveau dat vergelijkbaar is met dat bij niet geëxcontinueerde patiënten (geen eerdere transplantatie en geen anti-HLA antilichamen). Een verklaring voor het beschermende effect van rituximab in de geëxcontinueerde patiënten kan zijn dat deze patiënten meer geheugen B cellen hebben vóór de transplantatie in vergelijking met niet geëxcontinueerde patiënten. Na herkenning van het specifieke antigeen, bijvoorbeeld in de nier, kunnen deze geheugen B cellen snel differentiëren in antilichaamproducerende plasma cellen. Door deze B cellen te verwijderen met rituximab kunnen er geen antilichamen geproduceerd worden, waardoor een afstoting voorkomen kan worden.

Om afstoting na niertransplantatie te voorkomen moeten patiënten levenslang immunsupressiva gebruiken die de functie van T en B cellen remmen. Ondanks het veelvuldige gebruik van immunsupressiva is er niet veel bekend over de effecten van deze medicijnen op de aantallen en functies van T en B cellen in het bloed van niertransplantatiepatiënten. In hoofdstuk 3 hebben we in een groep niertransplantatiepatiënten een aantal karakteristieken van T en B cellen in het bloed onderzocht. Behandeling met combinatietherapie van tacrolimus, mycofenolaat mofetil en steroïden leidt tot een verandering in de geheugen T en B cellen in vergelijking met vóór de transplantatie. Toevoeging van rituximab naast de standaard combinatietherapie resulteerde in een langdurige afwezigheid van B cellen in het bloed. Wanneer na verloop van tijd nieuwe B cellen in het bloed terugkomen, zien we een verrijking van CD24hiCD38hi transitionele B cellen. In bepaalde situaties wordt de aanwezigheid van relatief meer transitionele en naïeve B cellen na rituximab behandeling geassocieerd met een beter klinisch resultaat, waarschijnlijk omdat er B cellen met regulerende functie aanwezig zijn binnen de transitionele B cellen.

Effecten naast B cel depletie

B cellen hebben verschillende functies. Na activering kunnen B cellen prolifereren en differentiëren in cellen die verschillende ontstekings eiwitten kunnen produceren, antigenen kunnen presenteren aan T cellen of antilichamen produceren. Al deze processen kunnen door rituximab beïnvloed worden. Om de directe effecten van rituximab op B cellen te bestuderen, hebben we in hoofdstuk 4 B cellen geïsoleerd uit bloed van gezonde vrijwilligers en gekweekt met of zonder rituximab. Uit de analyses bleek dat rituximab de proliferatie van naïeve maar niet van geheugen B cellen remt, waardoor de verhoudingen tussen de verschillende B cellen veranderen. Wanneer de aan rituximab blootgestelde B cellen gebruikt werden om antigenen te presenteren aan T cellen, waren er meer geactiveerde T cellen die het cytokine IL-4 produceerden dan wanneer onbehandelde B cellen antigenen presenteerden aan T cellen. Uit eerder onderzoek was al bekend dat behandeling van rituximab de B cellen in het bloed goed kan depleteer, maar dat er in lymfoïde organen B cellen overblijven. Hoofdstuk 5 beschrijft het effect van rituximab op de
B cellen in lymfeknopen van niertransplantatiepatiënten. De overgebleven B cellen in de lymfeknopen waren met name geheugen B cellen. Na stimulatie van allogene T cellen met B cellen uit lymfeknopen van rituximab-behandelde patiënten, waren er minder geactiveerde T cellen die het cytokine IL-17 produceerden. Er blijven dus na behandeling met rituximab B cellen over die andere functionele eigenschappen hebben. Dit betekent dat het effect van rituximab op de immuunrespons niet alleen bepaald zal worden door de mate van B cel depletie, maar ook door de functionele eigenschappen van de overgebleven B cellen.

Een bijwerking na behandeling met rituximab van patiënten met B cel kanker kan zijn dat er kort na de infusie grote hoeveelheden van bepaalde cytokines geproduceerd worden. Deze verhoging kan tot een immuunreactie kunnen leiden, wat nadelige gevolgen zou kunnen hebben. In hoofdstuk 6 is dit voor niertransplantatiepatiënten geëvalueerd. Toediening van rituximab leidde tot een specifieke cytokine productie, met verhoogde concentraties van het regulerende cytokine IL-10 en het cel-aantrekkende eiwit MIP-1β. Deze eiwitten kunnen de immuunrespons beïnvloeden.

In hoofdstuk 7 worden de bevindingen samengevat en bediscussieerd vanuit een breder oogpunt. De resultaten besproken in dit proefschrift tonen aan dat rituximab direct de B cellen kan beïnvloeden. Hierdoor zal het effect van rituximab op het immuunrespons niet alleen bepaald worden door de mate van B cel depletie, maar ook door de functionele eigenschappen van de overgebleven B cellen. De relatieve verhoging van het aantal regulatoire B cellen na rituximab behandeling kan positieve gevolgen hebben op de lange termijn. Uiteindelijk zal het klinische effect van anti-B-cel bepaald worden door de balans tussen effector en regulatoire B cellen. Daarom zou toekomstige therapie zich meer moeten richten op selectieve depletie of verandering van de B cel subtypes.
CHAPTER 9

DANKWOORD
Het laatst geschreven, maar het meest gelezen hoofdstuk van dit proefschrift... Toch kan ik iedereen aanbevelen om de hoofdstukken hiervoor ook te lezen, of in ieder geval door te bladeren om een idee te krijgen waar ik me de afgelopen jaren mee bezig heb gehouden 😊.

Meer dan bij alle andere hoofdstukken flitsen er allerlei gedachten, herinneringen en gezichten door mijn hoofd. Allereerst gaat mijn dank uit naar iedereen die direct dan wel indirect heeft bijgedragen aan het tot stand komen van dit proefschrift. Een aantal mensen wil ik in het bijzonder noemen:

Mijn promotoren: Irma en Luuk, bedankt dat jullie mij de mogelijkheid en vertrouwen hebben gegeven om dit promotietraject in te gaan! Irma, door de juiste vragen te stellen, wist jij altijd de lijn weer helder te krijgen. Luuk, je scherpe blik, oog voor detail en vaak snelle reacties op manuscripten hebben me erg geholpen. Door jullie verschillende achtergronden bleef de brede context en de klinische relevantie altijd in beeld, wat ik heel prettig vond. Ik heb veel van jullie geleerd. Zonder jullie was dit proefschrift er nooit geweest!

Ook Hans en Marije, mijn copromotoren, wil ik ontzettend bedanken voor de fijne samenwerking. Hans, als mijn stagebegeleider, heb je me als eerste enthousiast gemaakt over de effecten van rituximab. Ik ben heel blij dat Irma, Luuk en jij een projectvoorstel bij de Nierstichting hebben ingediend wat heeft geresulteerd tot sponsoring van dit promotietraject. Marije, bedankt voor je kritische commentaar op onze resultaten en manuscripten. Door jou zijn de manuscripten iets “sexier” geworden 😊.

Bram en Marina, mijn paranimfen, heel erg bedankt voor jullie ondersteuning. Bram, jouw enorme brede kennis heeft me enorm geholpen door de jaren heen, zelfs nu ik in Utrecht werk 😊. Daarnaast heb je me ontzettend geholpen in de laatste fase. Marina, bedankt voor het opwerken en organiseren van (bijna) alle samples van de rituximab-studie. Ook kon ik altijd met een andere (praktische) vragen bij je terecht.

Mijn kamergenoten door de jaren heen: Jorieke, Diana, Sip, Vivian, Xuehui, Claire, Clive en mijn (oud) collega’s van het kweeklab: Bram, Marina, Esther van Rijsen, Esther Fasse, Ronald, Jos. Bedankt voor de goede sfeer op het lab, alle hulp en de gezelligheid!!

Samenwerken is heel belangrijk om tot een mooi (wetenschappelijk) eindresultaat te leiden. Alle coauteurs bedankt voor jullie bijdrage!

Ook dank aan de andere collega’s. Binnen het LMI, in het bijzonder het DNA lab, Henk en Kjeld bedankt voor de SNP analyses deels beschreven in hoofdstuk 6. Maar
ook alle collega's van het flow cytometrie unit van het hematologie lab. Bedankt voor de gezelligheid, hulp en tips tijdens de vele uren die ik achter de FACS heb doorgebracht.

De mensen van afdeling Nierziekten ben ik dankbaar voor het voor mijn onderzoek broodnodige patiëntengegevens en -materiaal en in het bijzonder, Martijn. De rituximab-studie die (grotendeels) door jou werd georganiseerd is onmisbaar geweest voor het tot stand komen van dit proefschrift. Ook de medewerking van de patiënten en donoren is hier natuurlijk onmisbaar.

De masterstudenten die ik heb mogen begeleiden tijdens mijn promotietraject: Kyra en Ellen. Het is mooi om te zien hoe enthousiast jullie waren en ik hoop dat jullie dat nog steeds zijn. Kyra, je hebt de start gemaakt met het project naar de effecten van complement en rituximab op milt B cellen, wat uiteindelijk geresulteerd heeft in een mooie publicatie. Ellen, ook al staan geen van je stageresultaten in dit proefschrift beschreven, heb ik veel van je geleerd over B cel stimulaties. Ik wens jullie veel succes met jullie promoties!

Ontspanning is tijdens een promotietraject ook heel belangrijk! Lieve vriend(inn)en bedankt voor jullie steun, gezelligheid en ontelbaar veel mooie herinneringen. Dat de feestjes, weekendjes weg en andere gezamenlijke activiteiten vooral zo door mogen gaan!

Tot slot wil ik het thuisfront bedanken: mijn familie (in Nederland en Bulgarije) en schoonfamilie. Iedereen heeft op zijn eigen manier iets bijgedragen. Bedankt voor de interesse en vragen die jullie hadden de afgelopen jaren. Mijn ouders wil ik bedanken voor hun steun en vertrouwen. Татко, баба и дядо: въпреки че ви е трудно да си представите и разберете с какво точно се съм занимавала последните години, винаги се интересувахте за мен и моята работа. Благодаря за вашият интерес и подкрепа!

Ben, bedankt voor al je steun, liefde en eindeloos geduld! Maar vooral ook voor de afgelopen 14 jaar en de leuke momenten die we samen al hebben mogen delen. Dat we nog lang samen mogen genieten van het leven. Ik hou van je!

Elena
CHAPTER 10

LIST OF PUBLICATIONS
CURRICULUM VITAE
LIST OF PUBLICATIONS


Elena G. Kamburova, Hans J.P.M. Koenen, Martijn W.F. van den Hoogen, Marije C. Baas, Irma Joosten* and Luuk B. Hilbrands*. Longitudinal analysis of T- and B-cell phenotype and function in renal transplant recipients with or without rituximab induction therapy. 2014; Submitted.

Elena G. Kamburova, Martijn W.F. van den Hoogen, Hans J.P.M. Koenen, Marije C. Baas, Luuk B. Hilbrands* and Irma Joosten*. Cytokine release after treatment with rituximab in renal transplant recipients. 2014; Submitted.


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