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Characterization of B cell responses in relation to organ transplantation

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CHARACTERIZATION OF B CELL RESPONSES IN RELATION TO ORGAN TRANSPLANTATION

CHARACTERIZATION OF B CELL RESPONSES IN RELATION TO ORGAN TRANSPLANTATION

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Characterization of B cell responses in relation to organ transplantation

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Every little piece of your life
will mean something to someone
Editors – The Weight of the World

aan mijn ouders

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Chapter I



General introduction

THE IMMUNE SYSTEM

Pathogens like bacteria, viruses and parasites represent a constant threat to the human body. The immune system is the body's natural defense to these pathogens, as well as to other threats such as the outgrowth of tumor cells.

The immune system can be divided into two arms, the innate and the acquired immune system. The innate immune system consists of physical barriers such as the skin and the mucosa, but also a set of soluble factors in blood that are able to bind to pathogens and lead to inactivation of the pathogen, collectively called the complement system (1, 2). The innate immune system also includes receptors, such as Toll Like Receptors (TLR) that recognize patterns specific for pathogens (3). Moreover, there are cells within the innate immune system, such as phagocytic cells (granulocytes and macrophages), which internalize and kill pathogens (4) and natural killer (NK) cells, which respond to cells lacking a sign identifying them as being 'self' (5).

The acquired immune system comprises a repertoire of cells that is generated upon antigenic challenge and thus depends on the individual's exposure to pathogens. All cells that acquire memory after the encounter of a pathogen are considered to be of the acquired immune system. These cells bear receptors on their cell surface that provide specificity. Cells that encounter a certain pathogen for the first time are called naïve cells. These cells will expand and mature into effector cells and memory cells. These memory cells are capable of rapidly responding in case of a subsequent encounter with the same pathogen (6). Cells of the acquired immune system include T cells that provide cellular immunity and B cells that provide humoral immunity.

In case a person's immune system is not able to adequately respond to an infection, the individual can succumb to the infection. This is very well illustrated by patients suffering from AIDS, who do not have a functional immune system and often die of complications of (normally non life-threatening) infections such as pneumocystis pneumonia (7). On the other hand, if the immune system is responding too vigorously or fails to be regulated, this can lead to allergies and/or autoimmune diseases (8).

THE MAJOR HISTOCOMPATIBILITY COMPLEX

T cells constantly survey the body for the presence of pathogens. For T cells to be able to recognize the presence of foreign structures, they express a polymorphic receptor, called the T cell receptor (TCR). The TCR can recognize foreign antigens in the form of pep-

tides only when they are presented in molecules of the major histocompatibility complex (MHC) (9-11). This phenomenon, preventing T cells to respond in an uncontrolled fashion, is called MHC restriction. MHC molecules are present on virtually all cells of vertebrate animals. In humans, the polymorphic system of MHC molecules is called the human leukocyte antigen (HLA) system and was originally demonstrated by the use of agglutinating antibodies in the sera of patients who had undergone multiple blood transfusions and of multiparous women (12-14).

HLA class I

There are two classes of HLA molecules, both with similar, yet distinct functions. The HLA class I molecules, encoded by the HLA-A, HLA-B and HLA-C genes, are present on all nucleated cells and platelets. HLA class I molecules consist of a heavy chain, a light chain and a peptide. The 44kD heavy chain (α chain) is encoded on chromosome 6 (15) and is non-covalently associated with a 12 kD light chain called β_2 -microglobulin (β_2m), which is encoded on chromosome 15 (16, 17). The α chain consists of 3 extracellular domains (α_1 , α_2 and α_3), a transmembrane region and a cytoplasmic tail (Figure 1). The polymorphism of the HLA class I molecules is based on differences in amino acid sequences in the α_1 and α_2 domains (18). The function of HLA class I molecules is to present endogenously generated peptides (19). Proteins are degraded into peptides by the proteasome (20) and some of these peptides are subsequently loaded onto HLA molecules in the endoplasmic reticulum after which the HLA-peptide complexes are transported to the cell surface (19). These peptides are in general 8-13 amino acids in length (21-23).

In case of an infection, HLA class I molecules can present peptides originating from the intracellular pathogen to CD8⁺ cytotoxic T lymphocytes (CTL), which can subsequently eliminate the infected cell (24). Thus, HLA class I molecules enable the immune system to survey the intracellular space.

HLA class II

HLA class II molecules are less widely distributed than class I molecules and are mainly found on professional antigen presenting cells (APCs), such as monocytes, dendritic cells (DC), macrophages and B cells, but also on endothelial cells and activated T cells. They are encoded by the HLA-DP, HLA-DQ and HLA-DR genes. HLA class II molecules are formed by 2 non-identical chains, called the α chain (32 kD) and the β chain (28 kD), both encoded on chromosome 6 (25), and a peptide. HLA class II molecules consist of an extracellular part (with domains α_1 , α_2 , β_1 and β_2), a transmembrane region and a cytoplasmic part (Figure 1) (26, 27). The polymorphism of HLA-DR lies in the β_1 chain, while for HLA-DP

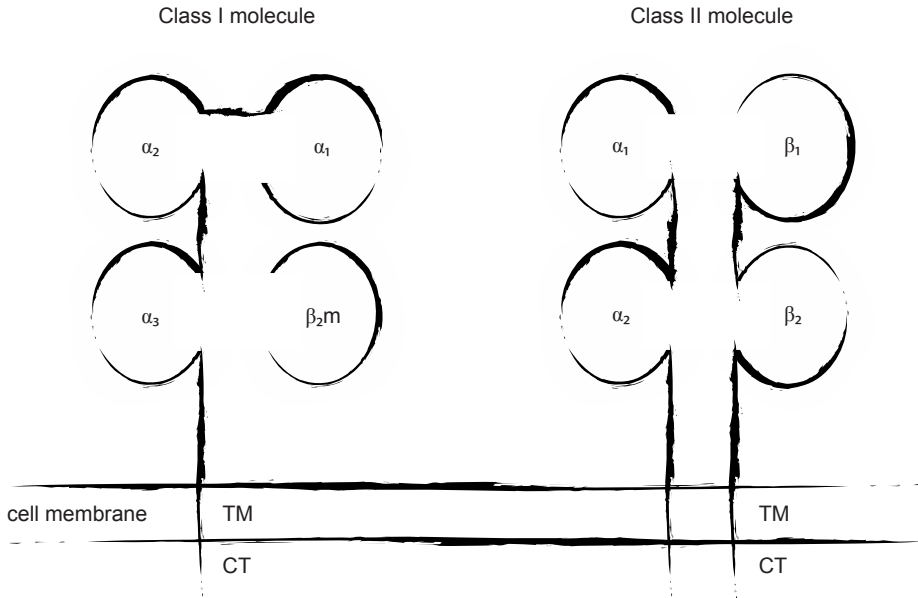


Figure 1. The structure of HLA molecules. HLA class I (left) consists of a heavy chain (α chain) and a light chain (β_2m). HLA class II (right) is a heterodimer consisting of an α chain and a β chain. CT: cytoplasmic tail, TM: transmembrane region.

and DQ the polymorphism resides in both the α_1 and the β_1 chain. The function of HLA class II molecules is to present peptides from proteins that were taken up from the extracellular environment to CD4⁺ helper T cells (28). To this end, proteins are digested into peptides in the lysosomal compartment and loaded into the HLA class II peptide binding cleft (19). These peptides are typically 13-25 amino acids long (29-32). The HLA-peptide complex is transported to the cell surface where it can be recognized by CD4⁺ T cells. This mechanism is utilized by the adaptive immune system to survey the extracellular space for pathogens.

ALLORECOGNITION

The enormous degree of polymorphism in HLA is essential for survival of the species. As a consequence of the diversity in HLA molecules (having distinct binding motifs), different peptides can be presented by different HLA molecules in different individuals. The diversity in pathogen-derived peptides that can be presented in the population ensures that in

case of an endemic infection, the chance of at least some individuals capable of coping with the infection is relatively high. For example, susceptibility to HIV and disease progression has been linked to certain HLA types (33). Furthermore, sustained presence of a pathogen can lead to a relative increase of certain HLA types in a population. This is nicely illustrated in relation to Malaria susceptibility. The class I allele HLA-B53 is associated with resistance to Malaria. Consequently, in West Africa, where Malaria infections have a high prevalence, a high frequency of HLA-B53 occurs (34).

A consequence of their polymorphism is that HLA molecules themselves can be recognized by the immune system as foreign, a phenomenon called allorecognition. Consequently, HLA molecules represent a huge hurdle in solid organ, islet and bone marrow transplantation as well as blood transfusion. Because of the high degree of polymorphism of HLA, the chance that unrelated donors and recipients have identical HLA molecules is very small (35). Recognition of foreign HLA by T cells can take place in two different ways as described below.

Direct allorecognition

When an organ is transplanted, donor immune cells bearing mismatched HLA class II molecules (mainly DC), can be recognized via the direct allorecognition pathway (Figure 2). In this route of allorecognition, T cells of the recipient directly recognize intact foreign HLA molecules and can initiate a vigorous immune response (36, 37). The peptide that is presented within the donor MHC may not necessarily be allogeneic, but often seems to be derived from donor MHC molecules and thereby increases immunogenicity (38, 39).

Indirect allorecognition

Later after transplantation, when donor antigen presenting cells are no longer present, peptides originating from the donor organ can only be presented by professional antigen presenting cells from recipient origin (Figure 2). Since this involves foreign peptides in the context of self-MHC, it is referred to as the indirect route of allorecognition (36, 40-42). This is the physiological way of the immune system to present and recognize foreign antigens. Evidently, indirect allorecognition will also take place early after transplantation. Given the great diversity in HLA molecules and knowing the consequences of the immune response following allorecognition, it is very important to achieve the highest degree of homology between donor and recipient as possible. Therefore, optimizing the HLA match of donor organs with prospective recipients has proven to be of great benefit in terms of graft survival (43-46). However, since a complete match can only be achieved in monozygotic twins, organ rejection remains a common issue.

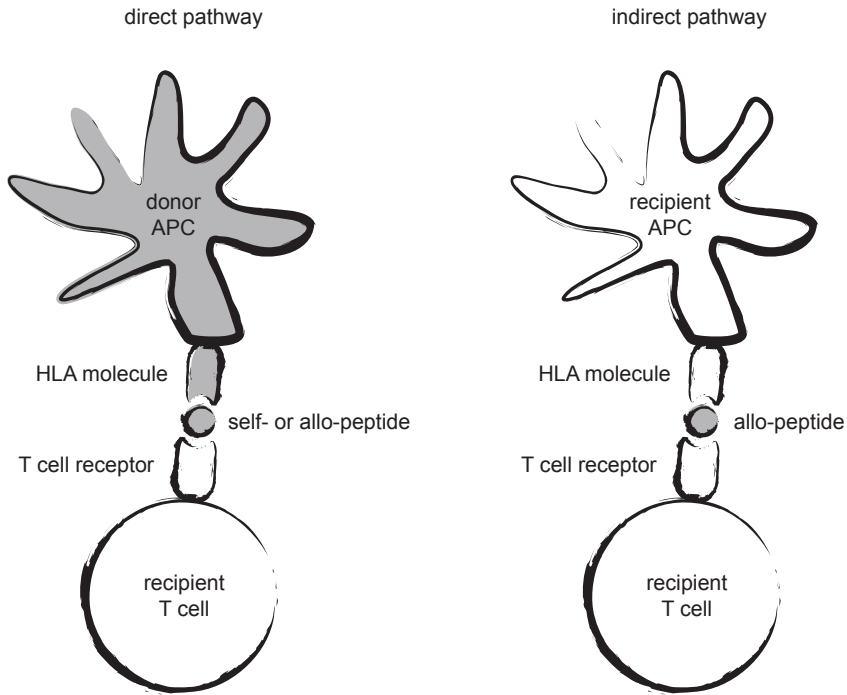


Figure 2. The direct and the indirect pathway of allorecognition. Direct allorecognition (left) involves recognition of mismatched donor HLA on donor antigen presenting cells (APCs), regardless of the peptide presented. Indirect allorecognition (right) involves presentation of peptides derived from donor cells in self HLA by the recipient APCs. HLA: human leucocyte antigen.

REJECTION

Hyperacute rejection

A transplanted organ can be subject of rejection throughout its presence in the recipient. Firstly, when there are specific pre-existing antibodies, either towards ABO blood group antigens (47) or foreign HLA (48) on the donor organ, it can be destroyed within the first 24 hours after transplantation. This phenomenon is called hyperacute rejection. The pre-existing antibody repertoire towards HLA can be either due to blood transfusions, previous transplants or pregnancies (49-52). To determine if the patient has preformed donor-specific antibodies, serum of the patient is routinely tested by incubation with cells from the donor prior to transplantation. When the test is positive, the transplantation will not be performed. Since the introduction of this pre-transplant serological crossmatching, hyperacute rejection is very uncommon (53).

Acute rejection

Acute cellular rejection occurs in the first months after transplantation and is mainly caused by the direct route of alloantigen presentation. The magnitude of this immune response is likely caused by the high precursor frequency of T cells that recognize intact HLA molecules (54, 55). The majority of patients receiving a graft undergo one or more acute rejection episodes, which, in general, are effectively treated with immunosuppressive drugs as discussed below.

Another form of acute rejection, acute humoral rejection, can occur in patients who have been sensitized to a certain antigen but have undetectable donor-specific antibody levels at time of transplantation. When transplanted, memory B cells can rapidly initiate the production of antibodies directed against the donor organ. Besides early acute rejection, both acute cellular and humoral rejection can occur late after transplantation, mostly due to noncompliance to medication by the transplanted patient (56). Both types of acute rejection can occur individually, but may coincide.

Chronic rejection

Finally, a transplanted organ can be subject to a process of slow deterioration, resulting usually from a multi-factorial process caused by a combination of non-immunological and immunological damage (57). The pathological image seen in kidney transplants that have been suffering from such multi-factorial damage, usually over a period of many years, is termed chronic allograft nephropathy (CAN). The term CAN indicates presence of chronic tissue damage, and is used in those cases where the underlying cause of the chronic transplant dysfunction is not clear (58). The term CAN has recently been replaced by interstitial fibrosis and tubular atrophy (IFTA). The major cause of non-immunologic organ damage is toxicity of immunosuppressive medication (59). The underlying immunologic process is characterized by a chronic alloreactive immune response, called chronic rejection. The main immunologic mechanism associated with antibody formation is thought to be the indirect pathway of alloantigen presentation (60). Treatment of CAN or chronic rejection is not as standardized as for acute rejection and appears to be less effective (61).

Cellular and humoral rejection

Classically, rejection has been considered to be a T cell mediated process (cellular rejection), mainly because in early mice studies, transfer of cells but not serum could initiate graft rejection (62). However, the contribution of humoral immunity is increasingly recognized (63, 64). When rejection is caused by alloantibodies, it is referred to as humoral rejection or antibody mediated rejection. The important role of antibodies in rejection was

shown in studies where formation of anti-donor antibodies regularly preceded chronic rejection of kidney transplants (65-67).

Next to serum alloantibodies, a hallmark for the diagnosis of humoral rejection is the presence of the complement split-product C4d in the kidney (68). When tissue-bound alloantibodies fix complement factor C1q, a cascade of reactions is initiated, resulting in binding of the split-product C4d to the tissue. There is a strong correlation between the presence of anti-donor HLA antibodies and C4d positivity (69), although C4d positivity without HLA-specific antibodies can occur in the case of non anti-HLA donor specific antibodies and anti-HLA specific antibodies without C4d positivity can be present in the case of non complement fixing alloantibodies. Peritubular capillary (PTC) deposition of C4d was a predictor of inferior 12-month graft function (70), underscoring the importance of humoral immunity in transplantation.

ANTIBODIES AND B CELLS

Antibodies

The importance of the humoral immune system is clear from inherited B cell deficiencies, such as X-linked agammaglobulinemia, where patients lack antibody production and therefore repeatedly acquire infections with certain bacteria and viruses, which can potentially be lethal (71).

Antibodies (soluble proteins, also known as immunoglobulins) consist of two identical light polypeptide chains and two identical heavy polypeptide chains. These chains form a homodimer with an antigen binding region (Fab) and a crystallizable region (Fc) per subunit (Figure 3) (72). The Fab fragment is responsible for binding to foreign structures and harbors antigenic specificity, whereas the Fc part determines the effector function of the antibody. There are nine different Fc parts, called isotypes (IgM, IgD, IgG₁-IgG₄, IgA₁, IgA₂ and IgE). Antibodies of the IgM isotype are most effective in activating a cascade of complement pro-enzymes, resulting in phagocytosis and killing of microorganisms (73). IgG antibodies (mainly IgG₁ and IgG₃) are effective in activating complement, as well as opsonization of antigens (74). Furthermore, binding of antibodies of IgG₁ and IgG₃ isotypes can result in target cell lysis by antibody dependent cytotoxicity (ADCC), brought about by NK cells upon engagement of their Fc receptor (FcγRIII) with these antibodies (63, 75). IgA antibodies are mainly present in mucous membranes to prevent infections via the intestinal and respiratory tract, whereas IgE antibodies are very efficient in triggering eosinophils and basophils, resulting in the release of histamine, which can eliminate parasitic

invaders (73).

Antibodies formed against a transplanted organ (mainly IgM and IgG) can have deleterious effects, as antibodies can cause damage of the endothelium lining the blood vessels of the transplanted organ (76). Platelets subsequently aggregate and this clotting will deprive the organ from oxygen, leading to necrosis of the organ (77).

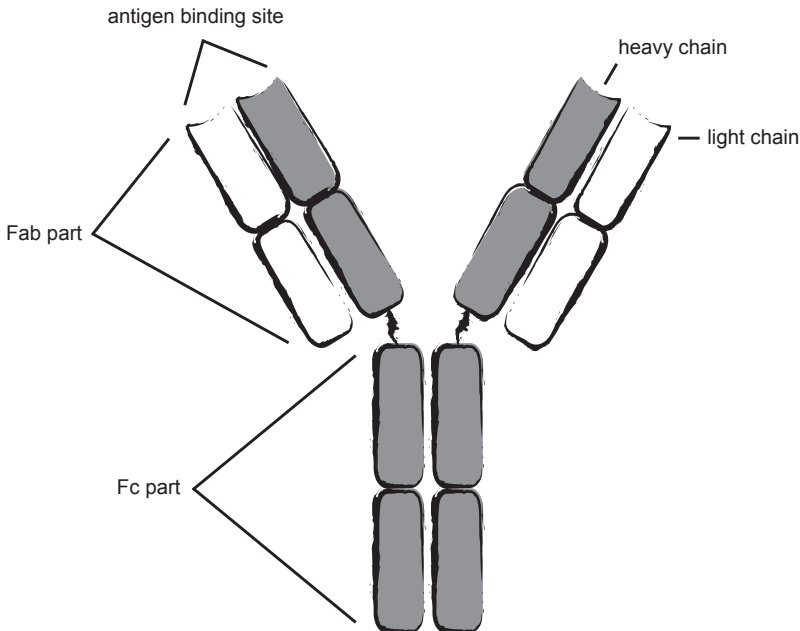


Figure 3. Schematic representation of an antibody. Antibodies consist of two heavy chains (grey) and two light chains (white). The variability of antibodies lies in the Fab part, which gives rise to the antigen-binding site. The functionality of the antibody lies in the Fc part, which determines the effector function. Fab: fragment antigen binding, Fc: fragment crystallizable.

B cells

B cells have the unique property of bearing antibodies on their cell membrane that act as receptors for recognition of foreign structures. This receptor is called the B cell receptor (BCR). Antigens are recognized by the BCR in a MHC unrestricted fashion, which is distinct from TCR recognition. Upon binding of the BCR, the bound antigen can be internalized and enter the MHC class II presentation pathway (78). This makes the B cell a potent antigen presenting cell capable of activating T cells (79-81). When a helper T cell

recognizes the presented peptide in this MHC restricted fashion, it gets activated. The T cell subsequently activates the B cell, resulting in immunoglobulin class switch, antibody production and memory B cell and plasma cell formation. Plasma cells are fully differentiated B cells that have lost their BCR and have the sole function of producing large quantities of antibodies.

Key molecules in the interaction between B cells and T cells are obviously HLA class II and the T cell receptor, but also costimulation by e.g. CD40 – CD40L interactions is essential (Figure 4) (82). Furthermore, cytokines produced by T cells play an important role in the activation and fate of the B cell. For example, the presence of IL-4 leads to isotype switching towards IgE producing B cells (83), whereas IL-5 skews towards IgA producing B cells (84) and IFN- γ towards IgG_{2a} (85).

B cells are formed in the bone marrow where early pro-B-cells carrying no antigen receptor develop into antigen receptor carrying mature B cells in an antigen independent fashion (73). Initial B cell activation can occur in various, non-exclusive, manners. Antigen recognition by the BCR will provide an activation signal to B cells, but also ‘danger’ signals in the form of TLR ligands play an important role in B cell activation (86). BCR crosslinking leads to rapid upregulation of TLR9 in naïve B cells, allowing for an additional activation signal (87, 88). This initial activation usually occurs in the T cell zone of lymphoid organs by interaction with T helper cells and dendritic cells (89). Following activation, B cells migrate into B cell follicles, proliferate and establish germinal centers (Figure 5) (90, 91). There, somatic hypermutation occurs, which involves random mutations in the genes encoding the recognition sites on antibodies, giving rise to B cells with a spectrum of antigen-affinities (92). Only B cells that have gained an increased affinity for the antigen will survive due to interaction with follicular dendritic cells (FDC) and germinal center T cells (93). Following this interaction, B cells will undergo several rounds of proliferation, mutation and selection. Furthermore, isotype switching can occur, leading to B cells that produce antibodies with specific functions (94). Finally the B cells develop into memory B cells (95) and plasma cells (96). The majority of plasma cells will migrate to the bone marrow, where they survive as long-lived plasma cells (97).

In vitro B cell activation

The *in vitro* activation of purified B cells can be achieved in various ways leading to phenotypically and functionally different cells. In principle, one can make use of (a combination of) the three natural stimulatory signals for B cells; BCR ligation, TLR signaling and T cell help.

For the first signal, inactivated *Staphylococcus aureus* bearing protein A, which has the

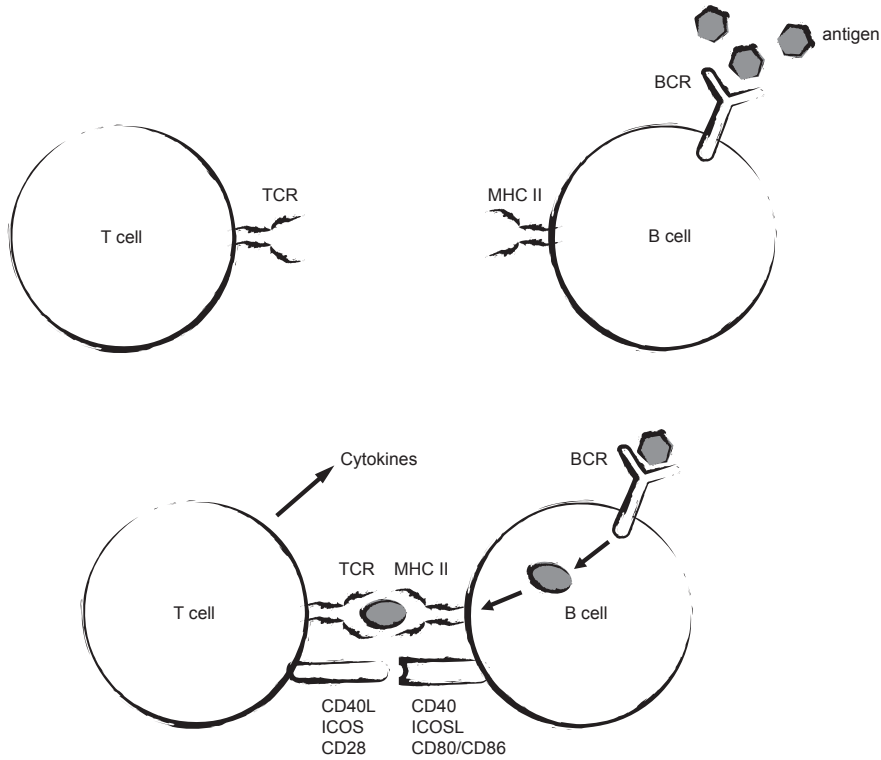


Figure 4. T – B cell interaction. Following antigen recognition, the B cell internalizes the antigen and, after processing, presents the antigen to T cells. Upon encounter of a T cell with the right specificity, the B stimulates the T cell to up-regulate stimulatory molecules at the cell surface and produce B cell activating cytokines. BCR: B cell receptor, ICOS: Inducible costimulator, TCR: T cell receptor.

property of binding the Fc part of the BCR, has been used frequently (98), as well as agonistic antibodies directed against the BCR (99). The addition of a TLR9 ligand, unmethylated CpG DNA, has been described to synergize with cognate T cell help and BCR triggering (88). Recombinant molecules, such as soluble CD40L or anti-CD40 monoclonal antibody (mAb), can mimic T cell help (100). Cell lines expressing CD40L on the cell surface are also used as surrogate for T cells (101, 102). Obviously, the addition of B cell stimulatory cytokines such as IL-2, IL-4, IL-10 and IL-21 is often used to get optimal B cell activation and differentiation (99, 103, 104). Furthermore, mitogenic signals activating lectin pathways such as Pokeweed Mitogen (PWM) have been frequently used, although this is a stimulus that requires T cell help (105).

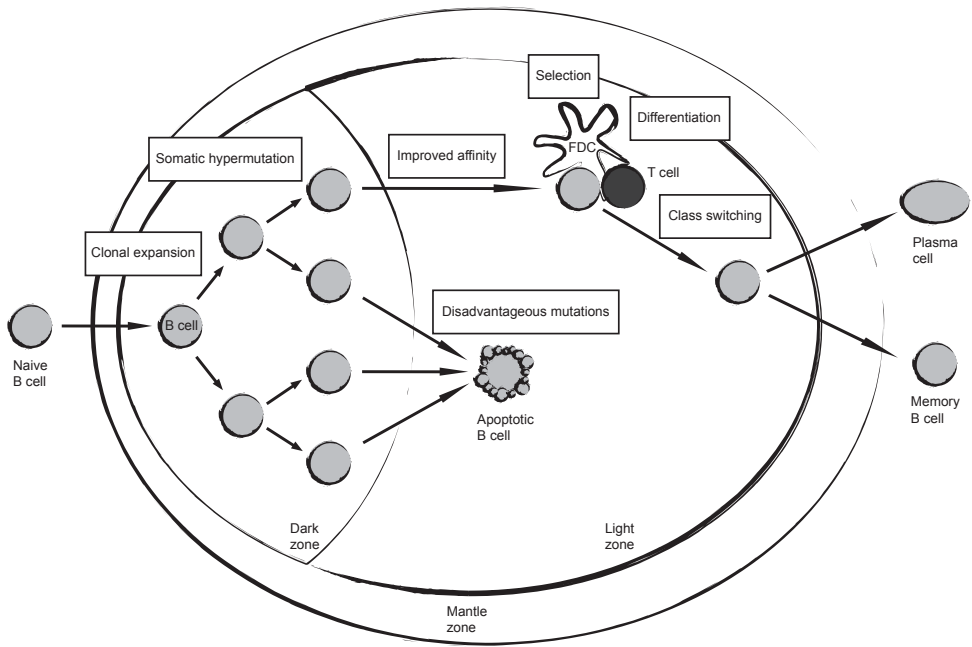


Figure 5. The germinal center reaction. In the dark zone of the germinal center, B cells undergo clonal expansion and somatic hypermutation. B cells with increased affinity for the antigen undergo further differentiation and class switching in the light zone. Finally the B cells become either memory B cells or plasma cells. FDC: follicular dendritic cell (adapted from Kuppers et al., *Nat Rev Immunol* 2003; 3 (10): 801).

IMMUNOSUPPRESSION

Every transplant recipient is treated with immunosuppressive drugs to minimize the chance of rejection. These immunosuppressive drugs must generally be taken for the rest of the patient's life. At time of transplantation, most patients are conditioned to make sure that the most vigorous immune response is prevented. The conditioning regiment varies between transplant centers. Often, conditioning is achieved by the use of depleting antibodies (e.g. anti-thymocyte globulin, ATG), leading to the depletion (of subsets) of lymphocytes (106, 107). After transplantation, patients receive maintenance immunosuppression typically consisting of a combination of a calcineurin inhibitor and corticosteroids with or without the addition of proliferation or mTOR (mammalian target of rapamycin) inhibitors. These immunosuppressive drugs mainly target the T cell response, while B cell priming and the subsequent antibody formation is more difficult to suppress. In case of rejection,

it is the type of the rejection that determines the treatment. Cellular rejection is mostly treated with corticosteroids, with ATG as a second-line therapy. Treatment of humoral rejection is more difficult and is still not standardized. Protocols including plasmapheresis, intravenous immunoglobulin (IVIg) and the B cell depleting chimeric mAb Rituximab have been described (108-111). More recently, a drug targeting plasma cells (bortezomib) has been described for treatment of humoral rejection (112).

The use of immunosuppressive drugs in organ transplantation is inevitable, and unfortunately has major side effects. Drug toxicity (59), the increased susceptibility to infections (113), increased tumor incidence (114) and cardiovascular disease (115) are major concerns when administering immunosuppressive drugs. Therefore, minimizing the dose of immunosuppressive drugs is essential and the search for more specific immunosuppressive drugs is needed (116, 117). In the following section, commonly used immunosuppressive drugs will be looked at in further detail, as well as experimental drugs that are described in this thesis.

Calcineurin inhibitors

The introduction of cyclosporin in the early 80's (in combination with the anti-CD3 mAb OKT3) into the clinic initiated a new era in solid organ transplantation. The one-year graft survival increased from 60% up to 90% (118, 119). Cyclosporin, as well as tacrolimus (FK506), is a calcineurin inhibitor, mainly affecting the activation of T cells.

When a T cell gets activated via its TCR, the intracellular Ca^{2+} levels are increased. Thereupon, the phosphatase calcineurin gets activated and subsequently activates the cytoplasmic form of members of the family of nuclear factors of activated T cells (NFAT). Activated NFAT enters the nucleus and initiates gene transcription of several genes, including the IL-2 gene. Cyclosporin binds to the intracellular protein cyclophilin (CyP), and tacrolimus to FK506-binding protein (FKBP). Both complexes subsequently bind to calcineurin, preventing it from activating NFAT (120, 121). In this manner, transcription of several genes that are expressed upon activation is suppressed.

Corticosteroids

Corticosteroids, such as prednisone, are used in transplantation as maintenance therapy as well as to revert (cellular) rejection. Corticosteroids are powerful anti-inflammatory drugs that bind to intracellular steroid receptors (122). This complex can cross the nuclear membrane and bind to specific gene regulatory sequences, and thereby modulate the transcription of numerous genes (123). This leads to a great number of effects, of which the anti-inflammatory effect is beneficial in transplant recipients.

Proliferation inhibitors

At the present time, the anti-proliferative drug commonly used in clinical protocols is Mycophenolate Mofetil (MMF). MMF is metabolized in the liver into Mycophenolic Acid (MPA), which inhibits the enzyme inosine-5'-monophosphate dehydrogenase (IMPDH). This enzyme is involved in the de novo pathway of purine synthesis. By inhibiting this enzyme, MPA is able to inhibit DNA synthesis of lymphocytes. This effect is rather specific for lymphocytes because cells other than lymphocytes can obtain purines by the salvage pathway (124).

mTOR inhibitors

Rapamycin, like tacrolimus, binds to FKBP. However, this complex does not bind to calcineurin, but to the mammalian target of rapamycin (mTOR). This is a serine/threonine protein kinase involved in a number of cellular processes, such as proliferation, cell survival and protein synthesis. mTOR is downstream of multiple signaling pathways and responds to nutrient concentrations, growth factors, mitogens and cytokines, like IL-2 (125). Blocking the mTOR pathway by rapamycin thus leads to a general suppression of lymphocyte activity.

IVIg

IVIg is a pool of plasma obtained from at least 1000 healthy donors. These large numbers of donors assure that IVIg consists of a repertoire of antibodies that represents the complete population (126). IVIg has originally been used as substitution therapy for patients with immunodeficiency diseases (127). The therapeutic area widened when it was discovered that IVIg was beneficial to patients suffering from various autoimmune diseases (128). Finally, IVIg is also used in transplantation settings in efforts to decrease the level of alloantibodies (129-131) and to treat humoral rejection (108, 109).

The mechanism of action of IVIg is incompletely understood, but its actions are thought to be manifold. First of all, because of the administration of high doses immunoglobulins, the natural metabolism of immunoglobulins through the neonatal Fc receptor (FcRn) may be enhanced (132). This can lead to clearance of pathogenic antibodies (133). Furthermore, anti-idiotypic antibodies contained within the IVIg can bind to autoantibodies, and therefore may limit tissue damage in certain autoimmune disorders (134). In transplantation, anti-idiotypic antibodies may interfere in the reaction of HLA-specific antibodies with their targets on HLA mismatched organs, thereby preventing humoral rejection (135, 136). Moreover, anti-idiotypic antibodies have been suggested to bind to B cell receptors resulting in stimulation of specific B cell clones (137, 138).

IVIg may also bind through the Fc portion of IgG to the inhibitory FcγRIIb (CD32) expressed on a variety of blood cells, including B cells, leading to the reduction of proliferation and the induction of apoptosis (139). IVIg may directly inhibit complement, and thereby modulate the effector function of antibodies (140, 141). Furthermore, IVIg may contain antibodies directed against cytokines and thereby limit their action (142). The possible presence of soluble HLA in IVIg may be of benefit for the immunized patient, as it may neutralize HLA-specific antibodies (143).

Bortezomib

The proteasome inhibitor bortezomib was originally used for the treatment of refractory multiple myeloma, a plasma cell neoplasia (144). Recently, the use of bortezomib as an agent for targeting allo-antibody producing plasma cells has received major interest (112, 145, 146). Bortezomib selectively inhibits the proteasome, an intracellular protease responsible for degrading misfolded proteins, as well as quick-turnover signaling proteins. When the proteasome is blocked, unfolded proteins accumulate, leading to apoptosis (147, 148). Moreover, the anti-apoptotic transcription factor NF-κB relies on the proteasome to be active (149). Since antibody producing cells have an extremely high protein turnover, these cells potentially are extremely sensitive to proteasome inhibition. Hence, bortezomib may present the long-sought after drug to treat humoral rejection.

AIM OF THIS THESIS

Whereas the short-term transplant survival is excellent at the present time, mainly because of potent immunosuppression, the long-term graft and patient survival is still a field in need of improvement. Two mechanisms are of importance regarding chronic rejection. Firstly, the involvement of the humoral arm of the immune system in rejection pathology is increasingly appreciated, particularly in chronic humoral rejection pathology. In 61% of renal transplant patients experiencing late allograft dysfunction a positive C4d staining was found, indicating the involvement of humoral immunity (150). Furthermore, in case of chronic rejection, HLA-specific antibodies are frequently detected prior to rejection, suggesting a causative role of antibody formation (65).

Secondly, in later phases after transplantation, the indirect route of allorecognition is the main pathway in which T cells get activated and can compromise graft integrity. Moreover, B cells involved in chronic rejection most likely receive T cell help from T cells that indirectly recognize alloantigens.

The studies described in this thesis are aimed to shed light on which immunosuppressive drugs, both standard drugs and experimental drugs, are suitable to inhibit humoral immune responses. Moreover, as described in this thesis, monitoring of both HLA-specific B cells and the indirect pathway of allorecognition are important to identify patients at risk for rejection and patients who are eligible for tapering of immunosuppressive drugs.

Since there is no specific treatment for (chronic) humoral rejection to date, it is generally treated with standard, non-specific immunosuppressive drugs. It is important to know which immunosuppressive drug is most potent in inhibiting humoral responses. Therefore, we studied the direct effects of standard immunosuppressive drugs on purified B cells *in vitro*, described in **chapter 2**, as well as the effects of immunosuppressive drugs on T cell help and B cells in the presence of T cell help, described in **chapter 3**.

One of the ways that is currently pursued to overcome the problems of pre-existing antibodies and humoral rejection is the use of IVIg (151, 152). However, little solid data exists on how IVIg would exert its beneficial effect in the setting of transplantation. We tested whether IVIg was able to affect key B cell responses in an *in vitro* culture system as described in **chapter 4**. In addition, we determined whether the anti-plasma cell agent bortezomib was also effective in targeting B cell responses, thereby broadening its therapeutic potential. These studies are described in **chapter 5**.

we have developed a novel technique to quantify the antigen specific peripheral B cell load in an individual, described in **chapter 6**. Using this technique, it is possible to monitor the HLA-specific B cell activity of a given patient in time, allowing for correlation with clinical parameters and aiding in the determination of tailor-made immunosuppressive regimens. Late after transplantation, monitoring of alloreactive T cells that indirectly recognize foreign HLA may provide insight on the humoral alloimmune status, since T cell help is important for developing B cell responses. However, the exact contribution of indirect allorecognition to rejection is not known and has proven to be difficult to study. In **chapter 7**, attempts at the development of a reliable assay to monitor indirect allorecognition are described, along with a critical literature review of the articles that claim to have identified indirect recognizing T cells *in vitro*.

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Chapter 2

Effects of immunosuppressive drugs on purified human B cells; evidence supporting the use of MMF and rapamycin

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ABSTRACT

Humoral immunity is increasingly recognized as an important factor in the rejection of organ transplants. In general, humoral rejection is treated with standard immunosuppressive drugs. The direct effect of these immunosuppressive drugs on B cells is not well known. Purified human B cells devoid of T cells were stimulated with either CD40L expressing L cells, or by anti-CD40 mAb with or without TLR triggering, all in the presence of B cell activating cytokines. These three protocols resulted in various degrees of B cell stimulation. We added four commonly used immunosuppressive drugs (tacrolimus, cyclosporin, MPA and rapamycin) to these cultures and tested a variety of parameters of B cell activity including proliferation, apoptosis induction and both IgM and IgG production.

Tacrolimus and cyclosporin marginally inhibited B cell proliferation and immunoglobulin production, and the extent of inhibition depended on the degree of the B cell stimulation. In contrast, MPA and rapamycin profoundly inhibited both B cell proliferation and immunoglobulin production, which was independent of the degree of B cell stimulation. Both drugs induced B cell apoptosis. Moreover, rapamycin caused a reduction of the number of B cells capable of producing immunoglobulins.

Our data show that MPA and rapamycin are capable of strongly inhibiting B cells responses. This provides a rationale for the use of both MPA and rapamycin to prevent and/or counteract humoral responses.

INTRODUCTION

When an organ is transplanted in a major histocompatibility complex noncompatible recipient, it can elicit an immune response. To achieve long-term graft survival with a fully functional graft, this immune reactivity needs to be suppressed. To this end potent immunosuppressive drugs have been developed. The downside of these non-specific immunosuppressants is their toxicity (1), the increased susceptibility to infections (2) and an increased tumor incidence (3). Therefore, minimizing the dose of immunosuppressive drugs is pivotal.

The impact of humoral immunity towards mismatched donor antigens was obvious from hyperacute rejection, where preformed anti-HLA or ABO antibodies destroyed the graft within minutes after transplantation (4). Such preformed antibodies are induced by pregnancies, blood transfusions or previous transplants. With the practice of pre-transplant serologic crossmatching, the occurrence of hyperacute rejection has become extremely rare (5). For a long time, T cells were considered the prime contributors to acute rejection. Therefore, immunosuppressive therapies for prevention or treatment of graft rejection so far have been mainly targeted towards T cells. However, both acute and chronic rejection can be antibody-mediated as well (6).

The only B cell specific agent that is currently in clinical use is Rituximab, a B cell depleting monoclonal antibody of which varying results in transplantation have been published (7, 8). Therefore, lacking B cell specific drugs with proven efficacy in transplantation, clinicians at the present time mostly treat antibody-mediated rejections with immunosuppressive therapies that are not specifically targeting the humoral immune system.

There are indications that standard immunosuppressive drugs have effects on humoral immunity. Cyclosporin was superior to azathioprine in suppressing the development of protective antibody titers following influenza vaccination post transplantation (9). Similar studies show a profound inhibitory effect of MMF and rapamycin on the formation of influenza-specific antibodies (10, 11). These studies provide information on the reduction of antibody production, but whether the drugs studied affect B cells directly is not known. The present study has been performed to gain insight on the direct effects of currently used immunosuppressive drugs on a variety of B cell parameters including proliferation, apoptosis and immunoglobulin production.

MATERIALS EN METHODS

Cells

Blood was obtained from blood bank donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B (Dyna, Oslo, Norway). The CD19-positive cells were released from the beads using Detach-a-Bead CD19 (Dyna). This typically yielded >98% pure B cells, as assessed by staining with a FITC-conjugated CD19 mAb (BD Biosciences, Breda, the Netherlands) followed by flow cytometric analysis (FCM).

Culture conditions

B cell cultures were done in culture medium consisting of IMDM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (insulin, transferrin and selenium, Sigma-Aldrich, 1000 fold diluted). B cells were cultured with 3 different stimuli at 37°C in a 5% CO₂ humidified incubator. The first stimulus consisted of irradiated (75 Gy) L-CD40L cells with 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D systems, Minneapolis, MN, USA) and 100 ng/ml IL-21 (Invitrogen, Leek, the Netherlands). The second stimulus consisted of an anti-CD40 mAb (1 µg/ml, R&D systems) combined with IL-2, IL-10, IL-21 (concentrations as above) and 2.5 µg/ml of the TLR9 ligand CpG ODN 2006 (Hycult Biotechnology, Uden, the Netherlands). Finally, the third stimulus was identical to the second, but lacking the ODN-2006 CpG.

Immunosuppressive drugs

Tacrolimus (Prograf, Astellas, Leiderdorp, the Netherlands, diluted in absolute ethanol) and cyclosporin (Sandimmune, obtained as a solution, was from Novartis, Arnhem, the Netherlands) were used at final concentration ranges of 0-1 ng/ml and 0-100 ng/ml, respectively. We based these concentration ranges on published plasma trough levels at 6 months post-transplantation (12-14). Calcineurin inhibitor concentrations reported for whole blood are considerably higher, but it should be noted that a substantial fraction of these drugs are bound to erythrocytes (15, 16), which are not present in our cultures. Mycophenolic acid (MPA, Sigma-Aldrich, Zwijndrecht, the Netherlands), the active metabolite of MMF, was dissolved in ethanol and used in concentrations up to 100 ng/ml, which is approximately 10-fold lower than used in the clinic. This concentration range was chosen because a maximal effect was already observed using 100 ng/ml in some experiments

and the limitation of low cell yields in other experiments. Rapamycin (Calbiochem, La Jolla, CA, USA) was dissolved in methanol and used in concentrations up to 8 ng/ml. Working solutions of all immunosuppressive drugs were made in culture medium.

Proliferation assay

B cells were seeded at 5×10^3 cells per well in 96-wells round bottom plates (BD Falcon, Breda, the Netherlands) and cultured with the stimuli described above (first stimulus with 10^3 L-CD40L cells) in the presence of increasing concentrations of immunosuppressive drugs, either added directly or after 48 h. At day 6, supernatants were collected for immunoglobulin detection and $1 \mu\text{Ci } ^3\text{H-TdR}$ (Amersham International, Amersham, United Kingdom) was added per well for the last 18 h of culture. $^3\text{H-TdR}$ incorporation was measured using a liquid scintillation counter (Wallac, Turku, Finland).

Immunoglobulin Production

Supernatants harvested from proliferation experiments were tested for IgM and IgG levels with a standard sandwich ELISA. Plates (Greiner, Alphen a/d Rijn, the Netherlands) were coated overnight with a goat anti-IgG or anti-IgM (Jackson ImmunoResearch, Westgrove, PA, USA) diluted in 10 mM Tris pH 9.0, then were blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.025% Tween-20 (Sigma-Aldrich) in PBS (PBS-T). Fifty μl of supernatants or standard human serum (Sanquin, Amsterdam, the Netherlands) in a serial dilution were incubated for 60 min at 37°C . After washing with PBS-T, biotin labeled goat anti-IgM or anti-IgG (Biosource, Camarillo, CA, USA) diluted in 1% BSA/PBS-T was incubated for 60 min at 37°C . After extensive washing, streptavidin horseradish peroxidase (Pierce, Rockford, IL, USA), diluted in 1% BSA in PBS-T was added and incubated for 60 min at 37°C . A color reaction was obtained with 4.6 mM 2,2'-azine-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) in a citric acid / PBS buffer at pH 4.2. The reaction was stopped with 250 mM oxalic acid (Sigma-Aldrich) and measured at OD450nm in an ELISA reader (Bio-Rad, Veenendaal, the Netherlands). Data were analyzed using Microplate Manager software version 4 (Bio-Rad). Sensitivities of the ELISA were 10 ng/ml for IgM and 5 ng/ml for IgG.

ELISPOT assay

To determine the number of IgM and IgG producing cells, we developed an ELISPOT assay. B cells were cultured at 10^4 cells per well in a 96-well round bottom plate (BD Falcon) with either 10^3 L-CD40L cells/well or anti-CD40 mAb + CpG in the presence of 100 ng/ml cyclosporin, 1 ng/ml tacrolimus, 50 ng/ml MPA or 1 ng/ml rapamycin. These concentrations

were chosen to yield sufficient numbers of cells for subsequent analysis. ELISPOT plates (Millipore, Amsterdam, the Netherlands) were prepared by coating with either goat anti-IgM or anti-IgG (Jackson ImmunoResearch) in PBS and blocked with 5% FCS/IMDM. At day 6, cells were harvested, washed, counted and seeded in the ELISPOT plates at 6×10^3 , 3×10^3 and 1.5×10^3 cells/well in 5% FCS/IMDM. These fixed cell concentrations were used to eliminate the potential effect of the immunosuppressive drugs on cell numbers during the first culture. A more extensive dilution series of B cells cultured without immunosuppressive drugs was included for a standard dilution curve. After 6 h of incubation at 37°C , cells were lysed with water. Following washing, the plates were incubated with biotinylated anti-IgM or anti-IgG (both from Biosource) overnight at 4°C . Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) for 1 h at RT followed by incubation of BCIP-NBT (Mabtech, Nacka Strand, Sweden) for 5 min. The reaction was stopped by rinsing with water. Plates were analyzed using a computer guided ELISPOT reader (Bioreader 3000, Bio-Sys GmbH, Karben, Germany). The cell number selected for analysis of drug effects was in the log phase of the standard dilution curve.

Apoptosis Assay

For determination of the percentage of apoptotic cells after culture, Propidium Iodide (PI) and Annexin-V-FITC (VPS Diagnostics, Hoeven, the Netherlands) were used following manufacturers' descriptions. In brief, $\text{CD}19^+$ cells were cultured at 5×10^5 cells per well in 24-well plates (Costar, Veenendaal, the Netherlands) with either 5×10^4 L-CD40L cells or anti-CD40 mAb + CpG, both in the presence of immunosuppressive drugs. After 3 days, cells were harvested and resuspended at 3×10^5 cells/ml. Annexin-V-FITC and PI were added and incubated for 10 min on ice. Cells were acquired using a FACS Calibur (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

Toxicity assay

To test whether the immunosuppressive drugs were toxic to B cells, a lactate dehydrogenase (LDH) activity assay (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer's instructions. Briefly, 2×10^5 B cells were cultured in 96-well round bottom plates in the presence of the immunosuppressive drugs in concentrations used in the proliferation assays. The culture was done in RPMI 1640 (Gibco) supplemented with 5% human serum (Sanquin Blood Supply, Leiden, the Netherlands) and 0.05 mM 2-mercaptoethanol. After 16 h incubation, cells were spun and 100 μl of cell-free supernatants were incubated with 100 μl of the kit's substrate for 30 min. The reaction was terminated with 1N HCl. Plates were read on the ELISA reader, and results were

expressed as OD_{490nm} minus OD_{620nm} .

Statistics

The paired T test was used for the analysis of the effects of single doses of immunosuppressive drugs on proliferation, immunoglobulin production and the number of immunoglobulin producing cells. Statistical level of significance was defined as $P < 0.05$.

RESULTS

Effects of the different stimuli on purified B cells

We tested if CD40 ligation in combination with B cell activating cytokines (17) was sufficient for activating human B cells. In mice, B cell receptor signaling is not required when antigen specific T cell help is provided (18). Similarly, we observed that CD40 ligation using either anti-CD40 mAb or L-CD40L cells in combination with cytokines is sufficient to obtain proliferative, immunoglobulin producing B cells (Figure 1). As CpG was shown to have an important role in B cell activation (19, 20), we also developed a culture condition including the TLR9 ligand CpG ODN 2006 (Figure 1). B cells cultured with L-CD40L cells proliferated vigorously and produced large quantities of both IgM and IgG. Culturing with

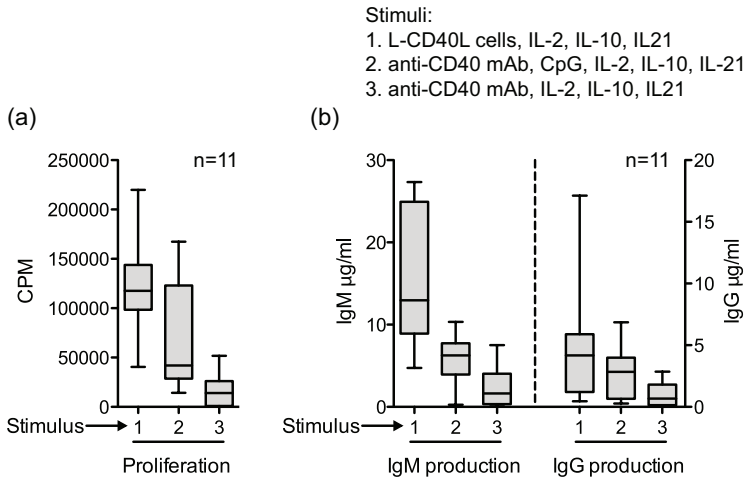


Figure 1. Proliferation, IgM and IgG production induced in three culture conditions. (a) Purified B cells were cultured for 7 days and ^3H TdR was added for the last 18 h for proliferation assessment. (b) Supernatants collected at day 6 were tested for the presence of IgM and IgG with ELISA. CPM: counts per minute.

anti-CD40 mAb + CpG resulted in intermediate levels of proliferation and immunoglobulin production. Stimulation with anti-CD40 mAb resulted in low B cell proliferation and immunoglobulin production. The latter stimulus was not used in some subsequent assays because of low cell yield. There was no correlation between the degree of responsiveness of individual donors and their susceptibility to the action of immunosuppressive drugs (data not shown).

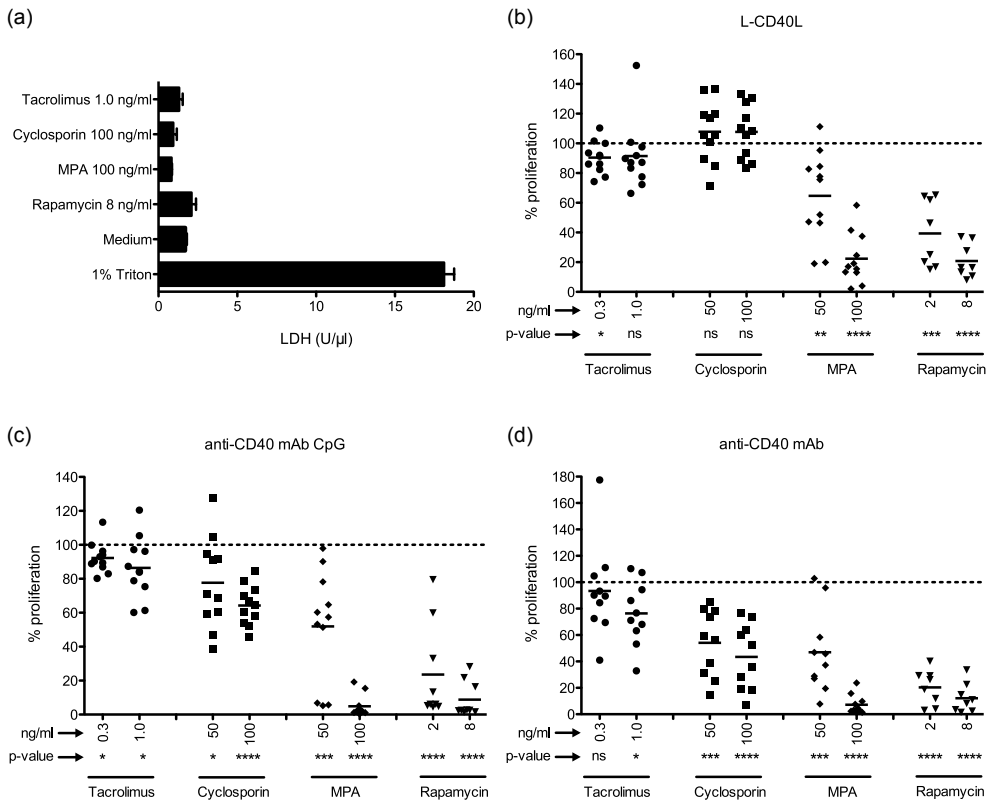


Figure 2. Immunosuppressive drugs are not toxic and have differential effects on the proliferation of B cells. (a) The LDH activity was measured in supernatants of B cells that were incubated with serial dilutions of immunosuppressive drugs. The effects of the highest concentrations of drugs are shown of a representative experiment ($n=3$). (b) Purified B cells were stimulated with L-CD40L cells in the presence of graded concentrations of immunosuppressive drugs. The percentage of proliferation relative to the addition of no immunosuppressive drugs is shown. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$. Horizontal bars indicate mean values. (c) Proliferation of anti-CD40 mAb + CpG stimulated B cells cultured with immunosuppressive drugs, legend as figure 2b. (d) Effect of immunosuppressive drugs on anti-CD40 mAb stimulated B cells, legend as figure 2b.

Effects of immunosuppressive drugs on B cell proliferation

The immunosuppressive drugs were not toxic to B cells in the concentrations used, as tested by an LDH release assay (Figure 2a). Solvents of the immunosuppressive drugs did not affect B cells, warranting that effects observed are solely caused by the immunosuppressive drugs (data not shown).

Tacrolimus in concentrations up to 1 ng/ml had minimal effects on the proliferation of B cells regardless of the stimulus (Figure 2b-d). It was previously shown that *Staphylococcus aureus* induced B cell proliferation was susceptible to high doses of tacrolimus (21). We therefore tested tacrolimus up to 16 ng/ml and found that high concentrations did not result in a stronger inhibition (data not shown).

Cyclosporin had no effect on proliferation when B cells were cultured with L-CD40L cells (Figure 2b). However, we observed a moderate inhibition of proliferation with B cells stimulated with anti-CD40 mAb + CpG (Figure 2c). Cyclosporin strongly inhibited the proliferation of anti-CD40 stimulated B cells (Figure 2d). No effect of either tacrolimus or cyclosporin on proliferation was observed when the drugs were added 48 h after culture initiation (data not shown). This is in concordance with the mechanism of action of calcineurin inhibitors on T cells, which is early in activation (22).

In contrast with the calcineurin inhibitors, both MPA and rapamycin were extremely potent inhibitors of B cell proliferation. MPA as well as rapamycin almost completely inhibited proliferation of B cells, regardless of the stimulus (Figure 2b-d). The inhibition was dose-dependent. Moreover, MPA or rapamycin were still effective when added 48 h after stimulation (data not shown).

MPA and Rapamycin induce apoptosis in B cells

A possible explanation for the inhibition of proliferation is the induction of apoptosis. To test this, B cells were cultured for 3 days with L-CD40L cells or anti-CD40 mAb + CpG in the presence of graded concentrations of immunosuppressive drugs after which B cell apoptosis was determined by Annexin-V and PI staining followed by FCM analysis (exemplified in Figure 3a). Tacrolimus and cyclosporin did not induce higher levels of apoptosis in the B cells when compared to controls. In contrast, addition of both MPA and rapamycin caused an increase of the percentage of apoptosis and this effect was dose dependent (Figure 3b).

Effects of immunosuppressive drugs on immunoglobulin production

Tacrolimus was marginally effective in inhibiting the production of both IgM and IgG with all culture conditions (Figure 4a-f). The degree of inhibition was dependent on the

strength of the B cell stimulation. Cyclosporin inhibited IgG production in all stimulatory conditions, and IgM in all except the most potent (L-CD40L) conditions (Figure 4a-f).

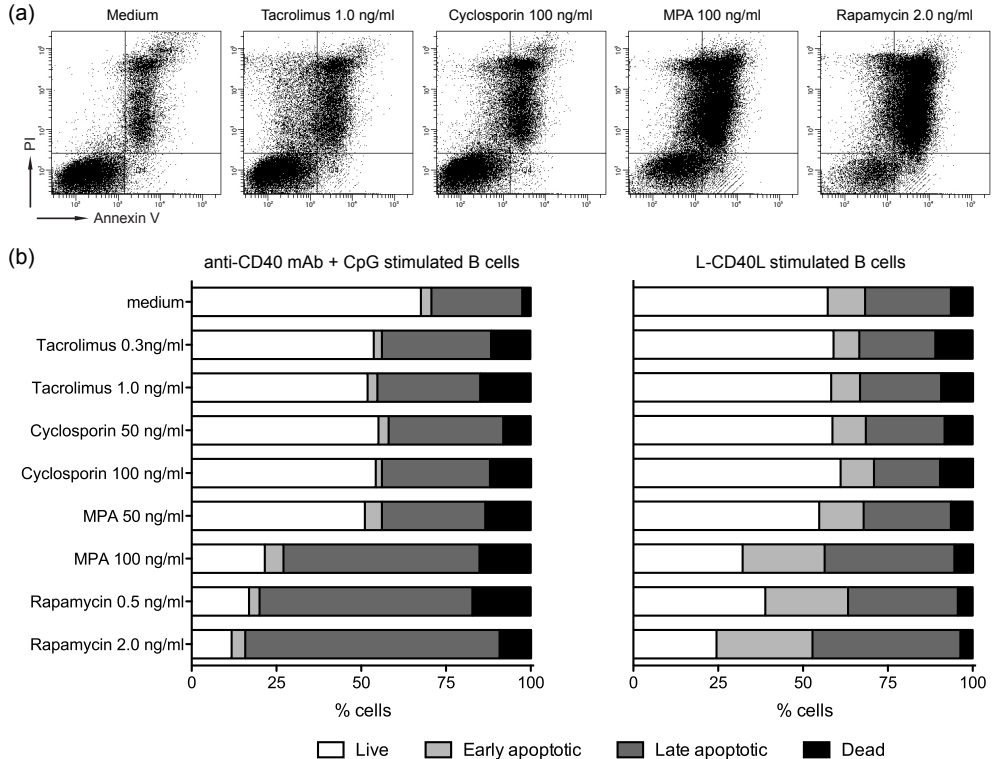


Figure 3. Apoptosis induction by immunosuppressive drugs. B cells were stimulated with either anti-CD40 mAb + CpG or L-CD40L in the presence of two concentrations immunosuppressive drugs. After 3 days cells were stained with Annexin-V and PI and analyzed by flow cytometry. (a) Effect of immunosuppressive drugs on the percentage of apoptotic cells stimulated with anti-CD40 + CpG. Depicted are dot plots of B cells treated with the highest concentration immunosuppressive drugs. The lower left quadrant are live cells, the lower right quadrant are early apoptotic cells, the upper right quadrant are late apoptotic cells and the upper left quadrant represents dead cells. (b) Percentage of apoptotic cells stimulated with anti-CD40 mAb + CpG (left panel) or L-CD40L (right panel) in the presence of two concentrations immunosuppressive drugs. Data from a representative experiment are shown.

Effects on immunoglobulin production of anti-CD40 mAb stimulated B cells were profound. Concurrent with the effects on proliferation, both tacrolimus and cyclosporin added 48 h after culture initiation failed to inhibit IgM as well as IgG production (Figure 5). In line with proliferation inhibition, both MPA and rapamycin were very potent in inhibiting immunoglobulin production. Addition of these immunosuppressive drugs led to

a virtually complete inhibition of IgM and IgG production in B cells cultured with any of the culture conditions (Figure 4a-f). These effects on immunoglobulin production were dose dependent. Addition of MPA and rapamycin 48 h after stimulation was equally effective in inhibiting IgM and IgG production compared to direct addition to the cultures (Figure 5).

Rapamycin decreases the number of immunoglobulin producing cells

To test whether the decrease in immunoglobulin production by the various immunosuppressive drugs was solely due to lower cell numbers caused by proliferation inhibition and/or induction of apoptosis, we developed an ELISPOT assay in which we enumerated the number of IgM and IgG producing cells. We normalized the effects on B cell division and apoptosis by adding fixed living cell numbers into ELISPOT plates, and therefore the effects of immunosuppressive drugs on the percentage of immunoglobulin producing cells reflected the effects within the live cell population only.

Tacrolimus, cyclosporin and MPA did not decrease the number of immunoglobulin producing cells (data not shown and Figure 6). MPA marginally inhibited the number of IgG producing cells when B cells were stimulated with anti-CD40 mAb + CpG, but this was not dose-dependent. In contrast, rapamycin significantly and dose dependently decreased the number of IgM and, to a lesser extent, IgG producing B cells with either L-CD40L cells or anti-CD40 mAb + CpG stimulus (Figure 6).

DISCUSSION

The impact of humoral immunity on transplant outcome is increasingly recognized, as antibodies are associated with various types of rejection. The development of HLA antibodies was shown to precede all chronic rejections of kidney transplants (23). Moreover, post-transplant donor HLA-specific antibodies were described to have a strong predictive value for transplant outcome (24). Peritubular capillary (PTC) deposition of C4d was associated with circulating antibodies and both chronic and acute rejections (25, 26). This deposition was shown to be a predictor of inferior 12-month graft function (27). In as many as 60% of all patients with chronic rejection a positive PTC staining for C4d was observed (25).

A substantial number of *in vitro* studies have been performed on effects of immunosuppressive drugs on B cells with either tonsillar or peripheral blood B cells stimulated with a variety of agents (28-32). Diversity in study parameters makes comparison of results difficult. To our knowledge, this is the first study comparing the effects of four different immunosuppressive

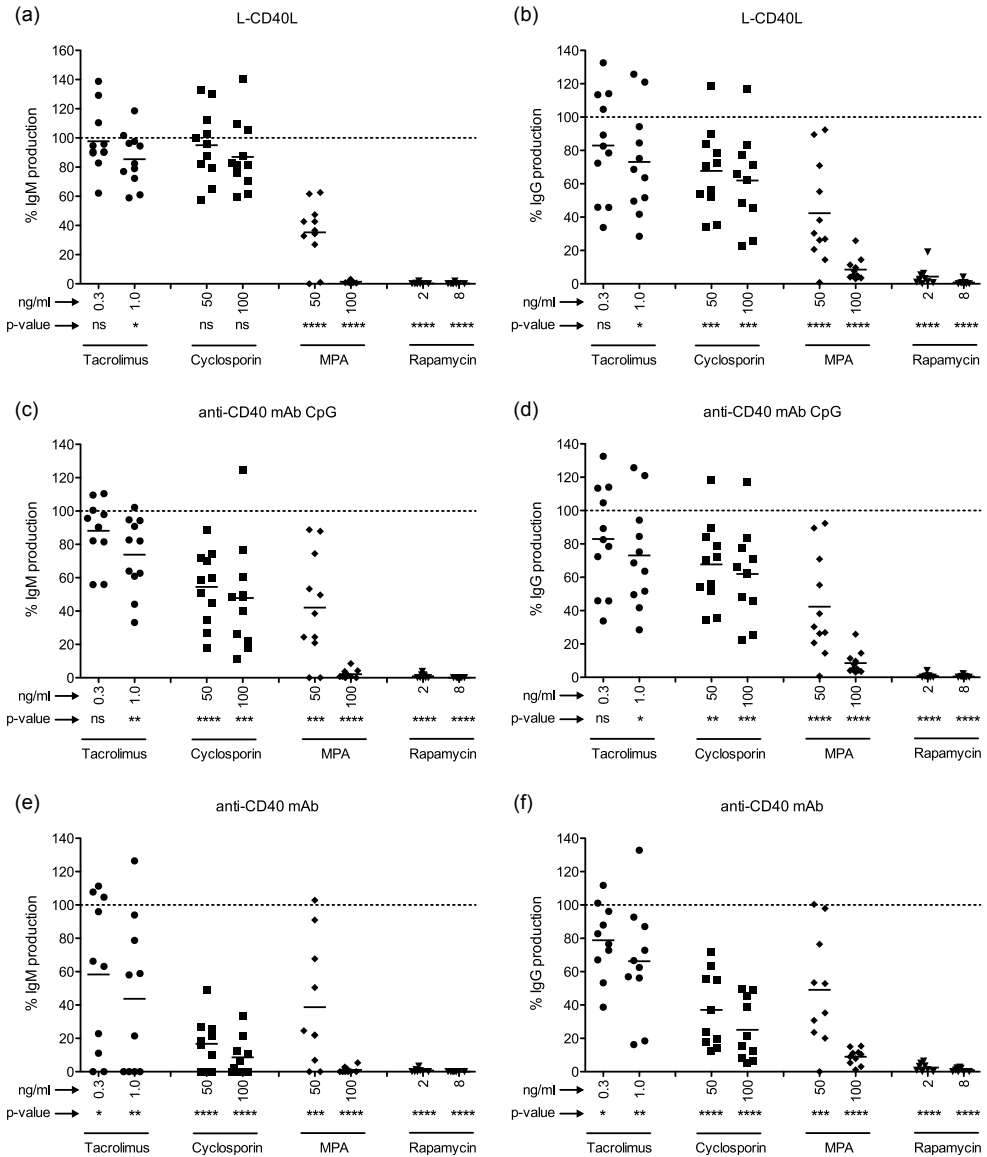


Figure 4. Effects of immunosuppressive drugs on immunoglobulin production. Supernatants from 6-day B cell cultures with immunosuppressive drugs were examined for the amount of IgM and IgG produced. (a-c) Percentage of IgM production with graded concentrations immunosuppressive drugs relative to the addition of no immunosuppressive drugs for B cells stimulated with L-CD40L cells, anti-CD40 mAb + CpG and anti-CD40 mAb, respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$, horizontal bars indicate mean values. (d-f) Percentage of IgG production of L-CD40L cell, anti-CD40 mAb + CpG and anti-CD40 mAb stimulated B cells, respectively, treated with immunosuppressive drugs. Legend as figure 4a-c.

drugs on purified B cells in a well-defined experimental system.

We made use of three methods to stimulate B cells into proliferating, immunoglobulin producing cells. In this manner, we were able to investigate whether the effect of immunosuppressive drugs depended on the strength and type of stimulus. These stimuli are polyclonal and can therefore not be extrapolated to antigen-specific humoral immune responses.

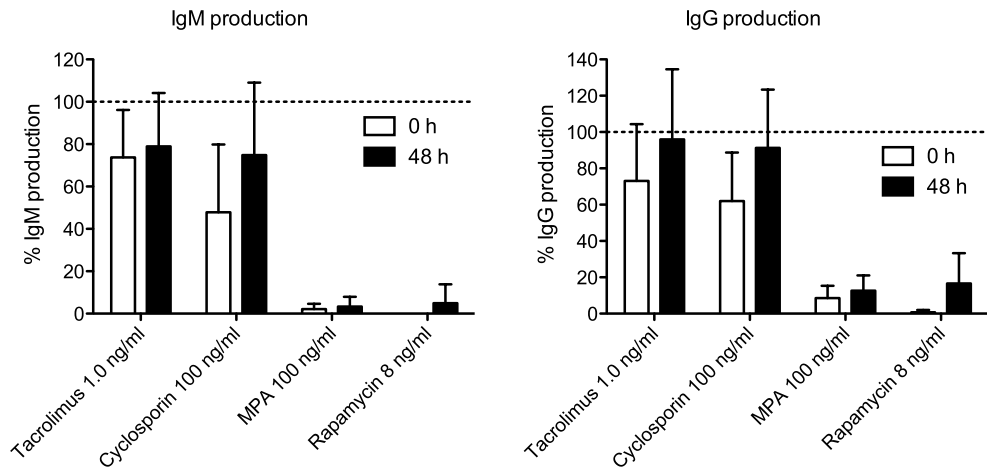


Figure 5. Calcineurin inhibitors are not effective in inhibiting IgM and IgG production when added 48 h after the start of the stimulation whereas MPA and rapamycin are still effective. Shown are effects of the highest concentrations of immunosuppressive drugs added to anti-CD40 mAb + CpG stimulated B cells. The percentage of inhibition relative to no addition of immunosuppressive drugs is depicted.

Calcineurin inhibitors are included in most immunosuppressive regimens, often combined with steroids. As a major proportion of chronic rejections in patients receiving Cyclosporin-based immunosuppression appears to be antibody-mediated, this drug clearly is not able to prevent humoral rejection (25). We tested the effect of both tacrolimus and cyclosporin on B cell responses. We have taken the binding of calcineurin inhibitors to erythrocytes into consideration for selecting drug concentrations to be tested. Our *in vitro* data support cyclosporin's relative ineffectiveness in directly inhibiting B cell responses. Likewise, tacrolimus had only marginal effects on B cell parameters. While multiple reports showed that in case of acute or chronic humoral rejection rescue therapy with tacrolimus and MMF was effective in reverting the rejection episodes (33-35), our data suggest that MMF

is likely to be the main drug responsible for the effect. In a multi-center study, MMF in combination with cyclosporin resulted in significantly lower frequencies of HLA antibodies as compared with azathioprine and cyclosporin treatment (36). Moreover, MMF was described to be very effective in inhibiting primary antigen-specific antibody responses in renal transplant patients (37).

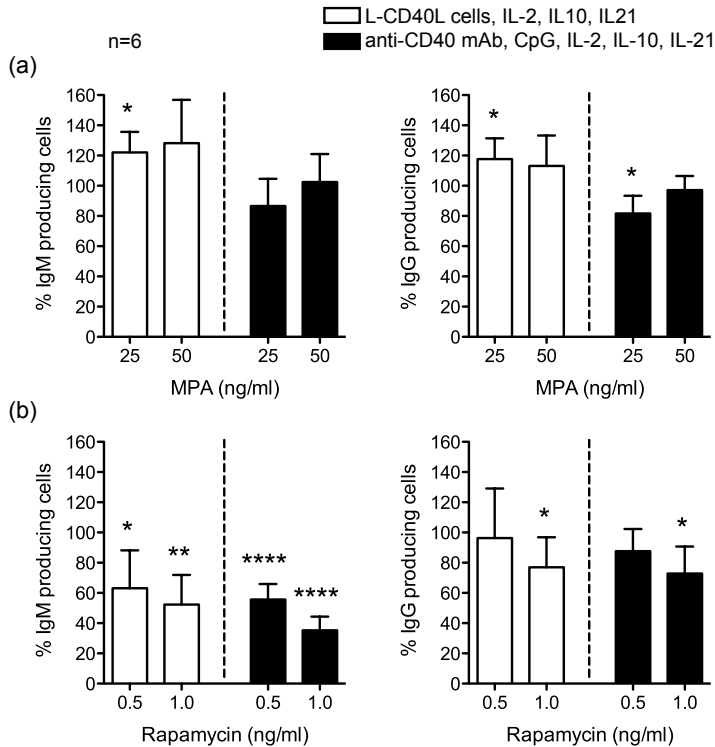


Figure 6. Rapamycin diminishes the number of immunoglobulin producing cells, whereas MPA does not. Open bars represent B cells stimulated with L-CD40L cells and filled bars represent B cells stimulated with anti-CD40 mAb + CpG. (a) MPA did not dose-dependently inhibit the percentage of both IgM and IgG producing cells. (b) Rapamycin dose-dependently inhibited both the percentage of IgM and IgG producing cells. The effect on IgM producing cells was more profound than on IgG producing cells. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$.

In our experiments, MPA was extremely potent in inhibiting both proliferation and immunoglobulin production. Moreover, these effects persisted when MPA was added to already activated B cells, implying that an ongoing B cell response may be dampened by MPA, while calcineurin inhibitors are ineffective. MPA levels used are lower than levels that

are usually achieved physiologically. In the experiments describing proliferation and total IgG and IgM levels in the supernatant of B cell cultures, we found in preliminary experiments already a complete inhibition when using these suboptimal MPA concentrations. Any higher dose would not result in a dose-dependent effect and would therefore raise the question whether the effect would be biologically relevant. In subsequent experiments such as the apoptosis and ELISPOT tests, we needed sufficient numbers of cells to analyze. Since MPA profoundly induced apoptosis already at 100 ng/ml, we were not able to perform these tests with higher concentrations than 50 ng/ml. Therefore, the effects observed might therefore even be an underestimation of the suppression of B cell function *in vivo*. Furthermore, an effect of MPA, in clinically relevant concentrations, on the number of immunoglobulin producing cells cannot be excluded.

Rapamycin is generally not included in maintenance immunosuppressive protocols from the day of transplantation onwards because it causes delayed graft function (38), increases surgical complications and impairs wound healing (39). However, rapamycin, like MMF, was described to be extremely potent in inhibiting humoral responses. For example, *in vivo* data comparing cyclosporin in combination with either azathioprine, MMF or rapamycin on the formation of antibodies against equine antithymocyte globulin after renal transplantation show that both MMF and rapamycin are capable of reducing anti-equine antibody formation (40). In our *in vitro* experiments rapamycin was the most effective drug tested, as it inhibited not only B cell proliferation and immunoglobulin production, but also inhibited the number of immunoglobulin producing cells. None of the other drugs tested were capable of decreasing the number of immunoglobulin producing cells.

An important effector mechanism of immunosuppressive drugs is apoptosis induction. We found that MPA and rapamycin induced B cell apoptosis. Since rapamycin induced apoptosis in dendritic cells as well, it is likely that this is a general effect of rapamycin (41). Since we stimulated the B cells polyclonally, we were not able to identify specific populations of B cells susceptible or resistant to apoptosis induction.

To get more insight in the possible mechanism of action on B cells, we performed cytokine production analyses on supernatants of B cells cultured in the presence of immunosuppressive drugs, but no correlations with the outcome of the proliferation and immunoglobulin data were found (data not shown). The antigen presenting function of B cells may play an important role in the process of allograft rejection (42). We therefore performed phenotypic analysis of B cells stimulated with anti-CD40 mAb and CpG treated with immunosuppressive drugs, but observed no alterations in the expression levels of HLA-DR, CD80 and CD86 (data not shown), suggesting that antigen presentation is not altered. However, studies using antigen specific interactions between B cells and T cells

are needed to address the influence of immunosuppression on the APC function of B cells in more detail.

Besides the effects of rapamycin described here, there are additional effects that would argue in favor of the use of rapamycin. Data on favorable effects of rapamycin on regulatory T cell subsets are emerging. A selective survival of both CD4⁺CD25⁺Foxp3⁺ as well as CD103⁺CD8⁺ regulatory T cells was recently described (43, 44). These effects are not observed for other immunosuppressive drugs such as cyclosporin (45, 46). Furthermore, rapamycin has been described to inhibit angiogenesis and might therefore be anti-tumorigenic (47).

In the present study, the effects of immunosuppressive drugs on T cell help are not taken into account. Whereas the prime target of calcineurin inhibitors is the T cell, the effect of calcineurin inhibitors might be underestimated. However, baseline calcineurin inhibitor therapy appears not to be very effective to prevent humoral rejection (25). To get more insight into this, studies on the *in vitro* effects on T cell dependent B cell responses are currently performed in our laboratory.

Many clinical studies are currently focused on drug tapering and withdrawal. Tapering or sparing of calcineurin inhibitors is widely performed to minimize their side effects (reviewed in (48)). Our data suggest that tapering of calcineurin inhibitors will most likely not increase the risk of humoral rejection.

Taken together, evaluation of highly purified, polyclonally stimulated B cells shows that MPA and rapamycin were superior in inhibiting B cell functionality *in vitro*, which underscores the clinical usefulness of these drugs for respectively prevention and treatment of humoral rejection. However, to predict accurately which immunosuppressive regimen will be optimal for treating humoral rejections, *in vitro* studies with combinations of immunosuppressive drugs are essential. Studies of this kind are currently underway in our laboratory, and are intended to define a combination regimen that can be preferentially used to treat humoral rejection.

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Chapter 3

Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help

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ABSTRACT

In general, humoral immune responses depend critically on T cell help. In transplantation, prevention or treatment of humoral rejection therefore requires drugs that ideally inhibit both B cell and T helper cell activity. Here, we studied the effects of commonly used immunosuppressive drugs (tacrolimus, cyclosporin, MPA and rapamycin) on T cell helper activity and on T cell dependent B cell responses.

T cells were polyclonally activated in the presence of immunosuppressive drugs in order to analyse the effect of these drugs on T cell proliferation, costimulatory ligand expression and cytokines. The impact of immunosuppressive drugs on T cell dependent immunoglobulin production by B cells was addressed in T – B cell cocultures.

All drugs affected T cell proliferation and attenuated T cell costimulatory ligand (CD154 and CD278) expression when T cells were polyclonally activated. Tacrolimus, cyclosporin and rapamycin also attenuated B cell stimulatory cytokine mRNA levels in T cells. As a consequence, a decrease in immunoglobulin levels was observed in autologous T – B cell cocultures, where T cell help is essential for immunoglobulin production. In contrast, when preactivated T cells were used to stimulate autologous B cells, calcineurin failed to inhibit B cell immunoglobulin production, whereas MPA and rapamycin did show inhibition. From these studies it is evident that calcineurin inhibitors affect the humoral immune response by interfering with T helper signals, but not by targeting B cells directly. Our studies furthermore support the necessity of intervening in T cell helper function to attenuate humoral responses.

INTRODUCTION

Despite excellent one-year graft survival rates, graft rejection remains an issue in solid organ transplantation. Although hyperacute rejection is avoided by pre-transplant serological crossmatching (1) and acute rejection is treatable with current immunosuppressive drugs, rejection pathology still occurs and has shifted towards a later stage after transplantation. Typically, the cause of chronic organ failure is multi-factorial though, involving both immunologic and non-immunologic damage, termed chronic allograft vasculopathy (2). In recent years, the role of humoral immunity in the development of chronic rejection has become increasingly apparent, as anti-HLA antibodies are frequently detected prior to chronic kidney rejection (3). Staining for the complement split product C4d also revealed a strong correlation between chronic rejection and humoral immunity (4).

Medication for treatment of acute rejection is well defined. Steroids are administered to patients undergoing rejection which, in case of steroid resistance, are followed by anti-thymocyte globulin (ATG) (5). In contrast, therapy for chronic (humoral) rejection is less well defined. Besides standard immunosuppressive drugs, intervention strategies include administration of ATG, high dose IVIg, Rituximab and plasmapheresis (6-10).

Although terminology implies a clear division, cellular and humoral rejection are intertwined. B cells act as potent antigen presenting cells capable of activating T cells, thereby possibly enhancing cellular rejection (11). Conversely, most B cells will only get properly activated when T cell help is provided (12). Furthermore, T cells are needed for B cell class-switching and production of potentially harmful IgG antibodies (13). Besides cognate interaction via antigen, the T cell mediated activation of B cells takes place through CD40L (CD154) – CD40 and, inducible costimulator (ICOS; CD278) – ICOSL interaction, as well as through cytokine production and consumption. Therefore, drugs that preferentially act on T cells, such as calcineurin inhibitors, are likely to affect humoral immune responses.

Previously, we have reported that the function of highly purified B cells, upon CD40 driven activation, was inhibited by mycophenolic acid (MPA) and rapamycin, but not by calcineurin inhibitors tacrolimus and cyclosporin, especially when B cells were strongly stimulated (14). In the present study, we have investigated the effect of these immunosuppressive drugs on T cell help and addressed the question whether calcineurin inhibitor-induced inhibition of T cell help is sufficient for the prevention of immunoglobulin production by B cells in a T cell dependent culture system.

MATERIALS EN METHODS

Cells

Blood was obtained from healthy blood bank donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. Untouched CD4⁺ T cells were obtained from PBMC by magnetic separation using the CD4⁺ T cell isolation kit II (Miltenyi, Bergisch-Gladbach, Germany) and MS columns (Miltenyi). After separation, flow cytometric analysis (FCM) revealed >80% purity. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B and Detach-a-Bead CD19 (Invitrogen, Leek, the Netherlands), typically yielding >98% pure B cells, as assessed by FCM. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and insulin-tranferrin-selenium (ITS) (insulin 5 µg/ml, transferrin 5 µg/ml and selenium 5 ng/ml, Sigma-Aldrich).

Immunosuppressive drugs

Tacrolimus (Prograf, Astellas, Leiderdorp, the Netherlands, diluted in ethanol) and cyclosporin (Sandimmune obtained from Novartis, Arnhem, the Netherlands) were used at final concentration ranges of 0-1 ng/ml and 0-100 ng/ml, respectively, based on plasma levels measured at 6 months post-transplantation (15-17). Calcineurin inhibitor concentrations reported for whole blood are considerably higher, but it should be noted that *in vivo* a substantial fraction of these drugs is bound to erythrocytes (18, 19), which are not present in our cultures. Mycophenolic acid (MPA, Sigma-Aldrich), the active metabolite of mycophenolate mofetil (MMF), was dissolved in ethanol and used in concentrations up to 100 ng/ml, which is approximately 10-fold lower than used in patients. This concentration range was chosen because maximal effects were already observed using 100 ng/ml. Rapamycin (Calbiochem, La Jolla, CA, USA) was dissolved in methanol and used in concentrations up to 8 ng/ml, which is within the clinical range. Solutions of immunosuppressive drugs were diluted in culture medium.

CFSE assay

T cells (10⁵) were carboxyfluorescein succinimidyl ester (CFSE from Invitrogen, 10 µM) labelled for 10 min at 37°C and cultured with 5 µg/ml anti-CD28 mAb (CLB-CD28/1, Sanquin, Amsterdam, the Netherlands) in 24-well plates (Costar, Veenendaal, the Netherlands) that had been coated with 5 µg/ml anti-CD3 mAb (UCHT1, BD Biosciences,

Breda, the Netherlands). Cells were harvested at day 3 and stained with CD4-PE (BD Biosciences) and Sytox Red dead cell stain (Invitrogen, Leek, the Netherlands) for dead cell exclusion. The proliferation index was calculated as follows (adapted from (20)):

$$\text{total events} / \sum(\text{events in peak}[n]/2^{(n-1)})$$

Peak 1 represents the undivided peak. Data are expressed as percentage of the proliferation index relative to no addition of immunosuppressive drugs. To calculate this percentage, data were transformed such that a proliferation index of zero represents no division.

Cytokine mRNA detection

T cells (5×10^5) were stimulated for 8 h with anti-CD3 mAb / anti-CD28 as described above in the presence or absence of immunosuppressive drugs. Cells were harvested and preserved in RNeasy lysis solution (Qiagen, Chatsworth, CA, USA). RNA was extracted using the RNeasy® mini kit (Qiagen) following the manufacturer's instructions. RNA was treated with DNase (Qiagen) on the spin columns and RNA quantity was assessed with a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All samples showed A260/A280 ratios between 1.9 and 2.1. cDNA was synthesized by incubating 12.8 µl RNA solution with 7.2 µl cDNA mix containing 2'-deoxynucleosides 5' triphosphate (dNTPs) (final concentration of 0.5 mM), 2 U reverse transcriptase-avian myeloblastosis virus (RT-AMV), 20 U rRNase inhibitor, 100 ng oligo-dT primers, 500 ng of random primers, and 1x reverse transcriptase buffer (all from Promega, Leiden, the Netherlands).

Primer sets (Table 1) for quantitative polymerase chain reaction (Q-PCR) were selected using Beacon Designer Software (version 7.02, Premier Biosoft International, Palo Alto, CA, USA) and were obtained from Eurogentec (Liège, Belgium). PCR mixes contained 1 µM of forward and reverse primers, 3 mM MgCl₂, and 1x iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands). PCR was performed using an iCycler MyiQ (Bio-Rad). The PCR program consisted of one cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, and was finalized with a melting curve analysis. Reactions were carried out in optical 96-well plates (Bio-Rad) covered with Microseal 'B' Film (Bio-Rad). The mean signal of the stably expressed reference genes 18S rRNA, GAPDH, β-actin, HPRT, HMBS and RPL13a served as a normalization factor to minimize general, if any, effects of immunosuppressive drugs.

Flow cytometry

T cells (5×10^5) were stimulated for 24 h with anti-CD3 mAb / anti-CD28 mAb as described above in the presence of graded concentrations of immunosuppressive drugs. Cells were harvested and labelled with the following mAb conjugates: CD4-PerCP, CD25-PE, CD154-PE, CD278-PE and CD69-FITC (all from BD Biosciences). Dead cells were excluded using Sytox Red. Cells were acquired using a FACS Calibur and analyzed using CellQuest Pro software (BD Biosciences).

Table 1. Sequences for primers used in quantitative polymerase chain reaction (Q-PCR).

Transcript	Forward primer	Reverse primer	Amplicon
IFN- γ	AGCTCTGCATCGTTTTGGGT	GTTCCATTATCCGCTACATCTGAA	118 bp
IL-2	AGGATGCTCACATTTAAGTTTAC	GAGGTTTGAGTTCTTCTTCTAGACACTGA	85 bp
IL-4	GTCTCACCTCCCAACTGCTT	GTTACGGTCAACTCGGTGCA	157 bp
IL-5	AGCCAATGAGACTCTGAGGATTC	GACTCTCCAGTGTGCCTATTCC	95 bp
IL-10	GCGCTGTATCGATTTCTTCC	GTAGATGCCTTTCTCTTGAGCTTA	94 bp
IL-13	TCCTCTCCTGTTGGCACTG	AGCGGAGCCTTCTGGTTC	165 bp
IL-21	AAACCACCTTCCACAAATGC	AGAGGACAGATGCTGATGAATC	147 bp
18S rRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	68 bp
GAPDH	ACCCACTCCTCCACCTTTGAC	TCCACCACCCTGTTGCTGTAG	110 bp
HPRT-I	AGATGGTCAAGGTCGCAAGC	TCAAGGGCATATCCTACAACAAAC	115 bp
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	64 bp
RPL13a	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	126 bp
β -actin	ACCACACCTTCTACAATGAG	TAGCACAGCCTGGATAGC	161 bp

bp: base pairs.

T and B cell cocultures

T cells (1.5×10^3) were stimulated for 9 days with anti-CD3 mAb / anti-CD28 mAb as described above in the presence of autologous B cells (1.5×10^5) with 2.5 μ g/ml of the Toll-like receptor ligand cytosine-guanine dinucleotide oligodeoxynucleotide (CpG ODN) 2006 (Hycult Biotechnology, Uden, the Netherlands). A T – B cell ratio of 1:100 was chosen because of strong proliferation of T cells after polyclonal stimulation, resulting in a 1:1 ratio after 9 days of culture (data not shown).

For some experiments, T cells (5×10^5) were prestimulated with anti-CD3 mAb / anti-CD28 mAb and 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands) for 2 days. After washing, 5×10^4 T cells were cocultured with 5×10^4 B cells with the addition of CpG for 6

days instead of 9 days, since T cell were already activated in the preculture. Furthermore, cells were cultured in a 1:1 ratio, since cocultures were performed without continued T cell stimulation, resulting in only minor T cell proliferation.

Immunoglobulin levels

Supernatants were tested for IgM and IgG levels with a standard sandwich ELISA. Plates (Greiner, Alphen a/d Rijn, the Netherlands) were coated overnight with a goat anti-IgG or anti-IgM (Jackson ImmunoResearch, Westgrove, PA, USA) diluted in 10 mM Tris pH 9.0, and then blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.025% Tween-20 (Sigma-Aldrich) in PBS (PBS-T). Fifty microliters of supernatants or standard human serum (Sanquin, Amsterdam, the Netherlands) in a serial dilution were incubated for 60 min at 37°C. After washing with PBS-T, biotin labelled goat anti-IgM or anti-IgG (Biosource, Camarillo, CA, USA) diluted in 1% BSA/PBS-T was incubated for 60 min at 37°C. After extensive washing, streptavidin horseradish peroxidase (Pierce, Rockford, IL, USA), diluted in 1% BSA/PBS-T was added and incubated for 60 min at 37°C. A color reaction was obtained with 4.6 mM 2,2'-azine-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) in a citric acid/PBS buffer at pH 4.2. The reaction was stopped with 250 mM oxalic acid (Sigma-Aldrich) and measured at OD_{450nm} in an ELISA reader (Bio-Rad). Data were analyzed using the Microplate Manager software version 4 (Bio-Rad).

Statistics

The one-sample T test was used for the analysis of immunoglobulin levels induced by different conditions within one donor. The paired T test was used for the analysis of single doses of immunosuppressive drugs. Statistical level of significance was defined as $P < 0.05$.

RESULTS

Effects of immunosuppressive drugs on T cell activation and proliferation

T cell activation with anti-CD3 mAb / anti-CD28 mAb led to a substantial increase of CD25⁺ and CD69⁺ cells (Figure 1a). None of the immunosuppressive drugs altered the percentage of CD25⁺ cells, whereas only cyclosporin slightly inhibited the percentage of CD69⁺ cells (Figure 1b). Additionally, polyclonal T cell activation resulted in a strong proliferative response, as measured by CFSE (Figure 1c, upper left panel). Since immunosuppressive drugs profoundly inhibit T cell proliferation, high doses of immunosuppressive drugs

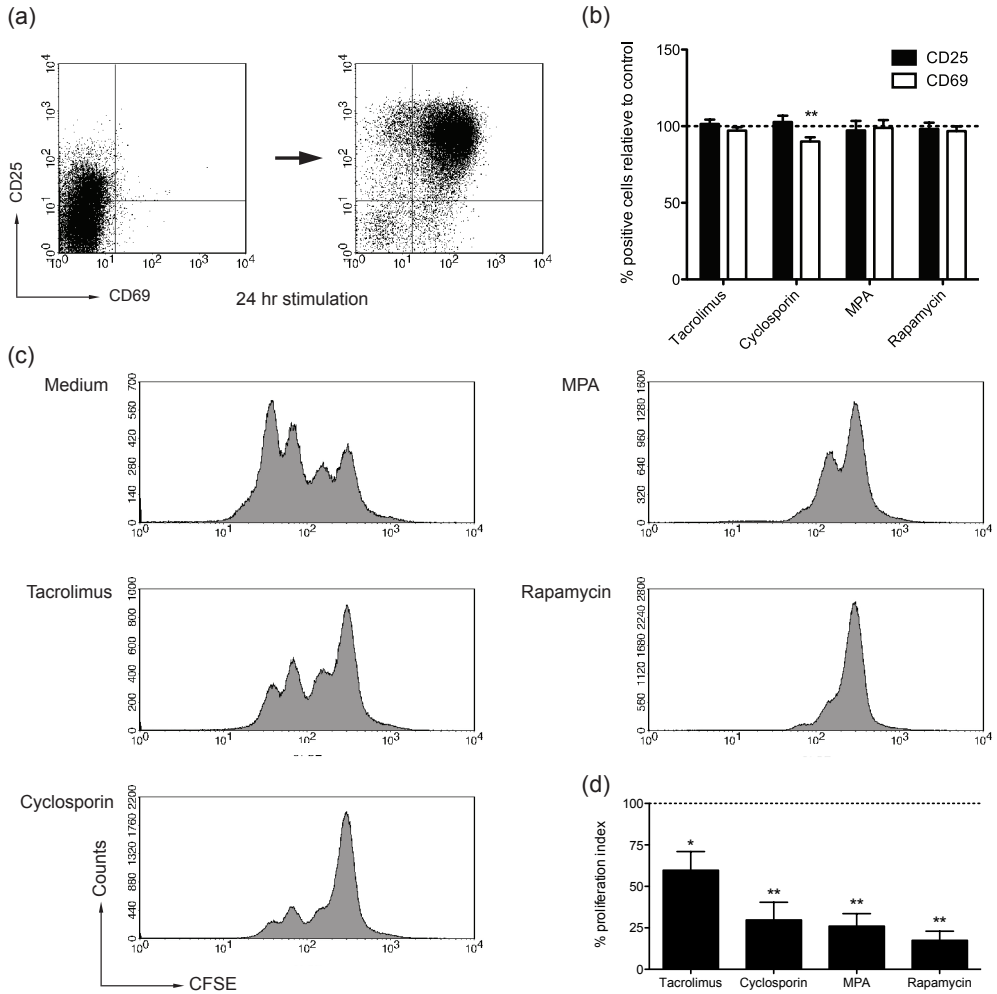


Figure 1. Immunosuppressive drugs did not alter T cell activation but did inhibit T cell proliferation. (a) T cell activation with anti-CD3 mAb / anti-CD28 mAb for 24 h resulted in upregulation of CD25 and CD69. (b) None of the immunosuppressive drugs affected CD25 levels, whereas cyclosporin inhibited the expression of CD69. Drug concentrations were 1.0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml MPA and 8.0 ng/ml rapamycin. Data are expressed as percentage of cells expressing CD25 or CD69 as compared to medium controls (dotted lines), $n=4$. (c) CFSE labelled T cells were activated with anti-CD3 mAb / anti-CD28 mAb for 3 days in the presence of immunosuppressive drugs. Data from a representative experiment are shown. Similar results were obtained in three independent experiments. (d) Percentage inhibition of the T cell proliferation index by immunosuppressive drugs compared to medium controls (dotted lines) are depicted, $n=3$. Drugs concentrations were: 0.3 ng/ml tacrolimus, 50 ng/ml cyclosporin, 100 ng/ml MPA and 4 ng/ml rapamycin. * $P<0.05$ and ** $P<0.01$.

resulted in low cell yields, insufficient for analysis. Therefore, we used suboptimal drug concentrations to address the effect of immunosuppressive drugs on T cell proliferation (Figure 1c). These relatively low, but clinically relevant, drug concentrations caused strong inhibition of the T cell proliferation index (Figure 1d).

Effect of immunosuppressive drugs on T cell helper function

Next, we investigated the effect of immunosuppressive drugs on T cell helper signals, by addressing costimulatory ligand expression and helper cytokines. Polyclonal T cell activation led to increased numbers of CD154⁺ (CD40L) cells and, to a lesser extent, of CD278⁺ (ICOS) cells (Figure 2a). All immunosuppressive drugs attenuated the percentage of CD154⁺ cells to a similar extent, although cyclosporin inhibition did not reach statistical significance. The effects on CD278 were more profound. Cyclosporin was superior in inhibiting the percentage of CD278⁺ cells, as compared to tacrolimus, MPA and rapamycin (Figure 2b).

We determined effects of immunosuppressive drugs on cytokines by their mRNA levels at 8 h post stimulation, rather than by their presence in culture supernatants to avoid confounding effects due to proliferation and cytokine consumption. Polyclonal T cell activation led to increased IFN- γ , IL-2, IL-4, IL-5 and IL-13 mRNA levels, whereas IL-10 mRNA remained stable and IL-21 mRNA was undetectable (data not shown). Tacrolimus inhibited all cytokines tested, although IL-5 inhibition did not reach statistical significance (Figure 2c). Likewise, cyclosporin inhibited all cytokines tested, although not reaching statistical significance for IL-4, IL-5 and IL-10. MPA did not inhibit any of the cytokines tested although the effect of MPA on cytokine mRNA expression varied highly between subjects. Rapamycin inhibited all cytokines tested except IL-13, but not to the same extent as the calcineurin inhibitors.

All immunosuppressive drugs affect T cell dependent B cell activation

To test the effects of immunosuppressive drugs on T cell dependent B cell activation, we developed a culture system in which B cells were activated in a T cell dependent manner, using polyclonal T cell activation in the presence of autologous B cells. In this test system T cells were necessary for B cell activation, since cultures of purified B cells alone with anti-CD3 mAb / anti-CD28 mAb and CpG resulted in low IgM and IgG levels, whereas high levels of IgM and IgG were produced in the presence of autologous T cells (Figure 3a). In these T – B cell cocultures, CpG was added to increase immunoglobulin production (21), however, CpG alone did not induce immunoglobulin production (data not shown). B cell activation was T cell contact dependent, since co-cultures of B cells with pre-stimulated T

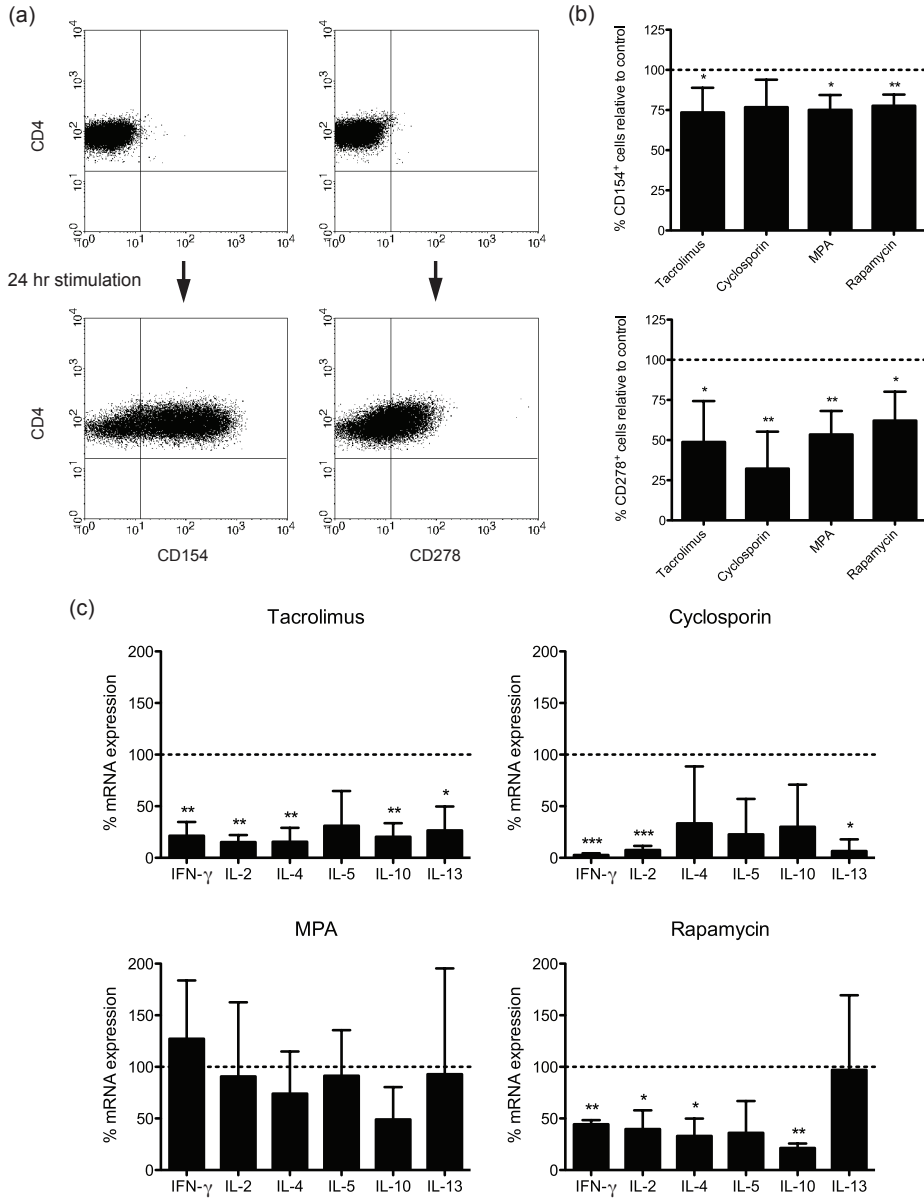


Figure 2. Effect of immunosuppressive drugs on T cell stimulatory signals. (a) T cell stimulation with anti-CD3 mAb / anti-CD28 mAb for 24 h resulted in upregulation of CD154 and CD278. (b) All immunosuppressive drugs inhibited the expression of CD154 and CD278. Drug concentrations were 1.0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml MPA and 8.0 ng/ml rapamycin. Data are expressed as percentage of cells expressing CD154 or CD278 as compared to medium controls (dotted lines), $n=4$. (c) Immunosuppressive drugs inhibit the mRNA levels of B cell stimulatory cytokines produced by T cells to various extents. Data are expressed as percentage of cytokine mRNA inhibition compared to medium controls, $n=3$. Drugs concentrations are identical to those of Figure 2b. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

cells in transwell plates resulted in low immunoglobulin production, whereas co-cultures performed in standard 24-well plates resulted in high IgM and IgG production (Figure 3b). To address the effects of immunosuppressive drugs on T cell dependent B cell cultures, freshly isolated T and B cells were cocultured with immunosuppressive drugs for 9 days in the presence of polyclonal T cell stimulation, after which supernatants were tested for immunoglobulin levels. As expected from their direct effects on B cells, MPA and rapamycin profoundly inhibited immunoglobulin levels in T cell dependent B cell cultures. Interestingly, tacrolimus and cyclosporin were equally potent in inhibiting T cell dependent immunoglobulin production as compared to MPA and rapamycin, indicating that inhibition of T cell help by calcineurin inhibitors is sufficient to prevent immunoglobulin production (Figure 4a and 4b).

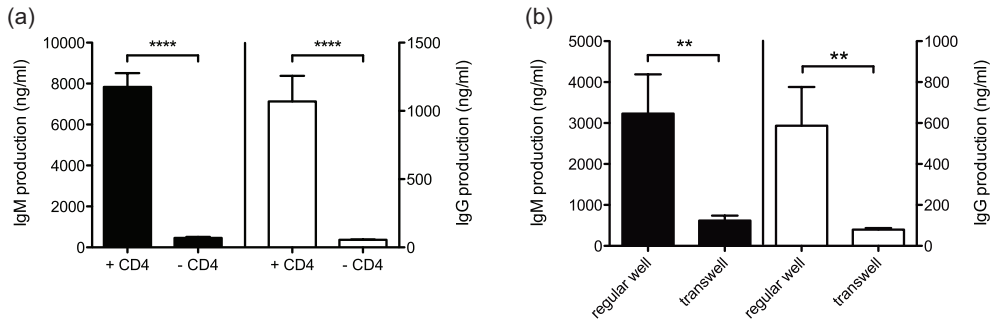


Figure 3. T cells were necessary for B cell activation in a cell-cell contact dependent fashion. (a) B cells were cultured in anti-CD3 mAb coated wells with soluble anti-CD28 mAb in the absence or presence of autologous T cells. After 9 days supernatants were harvested for immunoglobulin assessment. (b) Co-cultures of B cells and pre-stimulated T cells were performed in either regular or transwell plates (B cells in lower compartment). Supernatants were harvested at day 6 for detection of immunoglobulins. ** $P < 0.01$ and **** $P < 0.0001$.

Tacrolimus and cyclosporin fail to inhibit B cells directly

To corroborate that the calcineurin inhibitor-induced inhibition of immunoglobulin production was due to the inhibition of T cell help, we developed a modification of the culture system described above in which T cells were already activated prior to coculture with autologous B cells. MPA and rapamycin almost completely inhibited immunoglobulin levels, ensuring that the B cells in this culture system were susceptible to inhibition by immunosuppressive drugs (Figure 4c and 4d). In contrast, tacrolimus and cyclosporin failed to

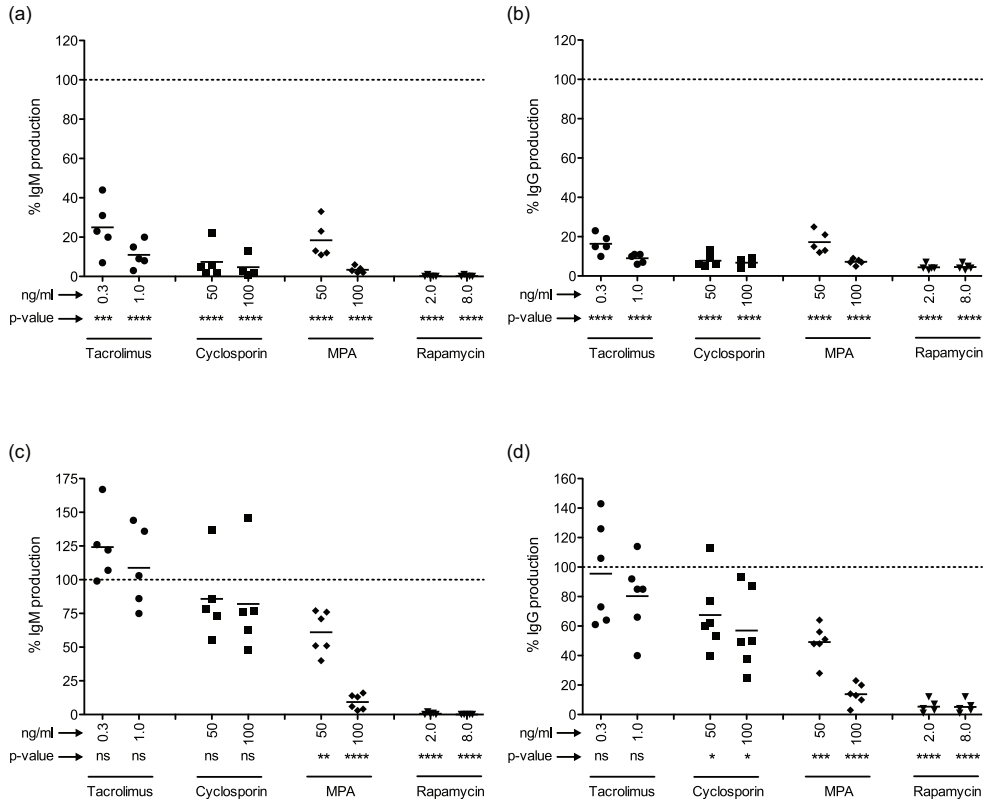


Figure 4. All immunosuppressive drugs were capable of inhibiting immunoglobulin production when B cells are cultured with non pre-activated T cells, but calcineurin inhibitors failed to inhibit immunoglobulins levels when pre-activated T cells were used to stimulate B cells. B cells were cultured with fresh, autologous T cells and anti-CD3 mAb / anti-CD28 mAb with CpG in the presence of graded concentrations of immunosuppressive drugs for 9 days, whereupon supernatants were tested for IgM (a) and IgG levels (b). B cells were cultured with immunosuppressive drugs for 6 days with CpG in the presence of pre-stimulated T cells. Depicted are the percentages of IgM (c) and IgG (d) levels compared to medium controls (dotted lines). Horizontal bars indicate mean values (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

inhibit IgM production (Figure 4c), whereas cyclosporin marginally inhibited IgG levels (Figure 4d). Thus, the inhibition of B cell responses by calcineurin inhibitors that was shown in Figure 4a and 4b appears solely due to inhibition of T cell help.

DISCUSSION

Chronic damage to transplanted organs can be caused by a variety of mechanisms, and in some of these, the binding of anti-HLA antibodies to the endothelium of the graft is implicated (3). For effective prevention and treatment of humoral rejection, immunosuppressive drugs should preferably affect the function of B cells as well as helper T cells, since T cell help by ligand interactions and cytokines activates B cells (12). Inhibition of T cell proliferation by calcineurin inhibitors and suppression of the transcription of the IL-2 gene, amongst others, is well known (22). Furthermore, calcineurin inhibitors attenuate T cell dependent Pokeweed Mitogen activation of B cells (23, 24). However, from these studies it is unclear whether the inhibition is due to inhibition of T cell help.

We have previously shown that calcineurin inhibitors are not efficient in directly inhibiting B cells, in cultures of purified B cells, activated in the absence of T cells (14). These data raised the question whether calcineurin inhibitors inhibit humoral immune responses by the inhibition of T cell help. We therefore set out to determine whether, by inhibition of T cell help, calcineurin inhibitors were capable of inhibiting B cell immunoglobulin production. Therefore, we developed a culture system in which B cells were activated in a T cell dependent fashion. Furthermore, we examined whether inhibition of T cell help was sufficient to completely abrogate immunoglobulin production.

The number of T cells available to provide help towards B cells may be reduced by inhibition of proliferation, or induction of apoptosis. We showed that, although T cells did get highly activated, all immunosuppressive drugs tested were capable of inhibiting proliferation. As expected, MPA and rapamycin, well known for their anti-proliferative effect (25, 26), were more potent in inhibition of T cell proliferation than the calcineurin inhibitors. Nonetheless, calcineurin inhibitors did inhibit T cell proliferation, which, at least partly, resulted in insufficient generation of activated T cells. None of the immunosuppressive drugs induced apoptosis in polyclonally activated T cells (data not shown).

The level of costimulation and cytokines mainly determines the strength of T cell help towards B cells. Thus a possible mechanism of drug-induced suppression of T cell dependent humoral immune responses is the reduction of B cell stimulatory signals, either as ligand interaction or as soluble mediators. All immunosuppressive drugs decreased the number of cells expressing the costimulatory ligands CD40L and ICOS, reducing the ability of T cells to activate B cells. This is reminiscent of patients with defective CD40L, who suffer from hyper-IgM syndrome (27) and patients with a homozygous deletion of the ICOS gene, who are severely antibody deficient (28). As expected, calcineurin inhibitors, but also rapamycin profoundly inhibited T cell IL-2 mRNA levels, which in turn can contribute

to the failure of B cell responses (29). Additionally, calcineurin inhibitors and rapamycin inhibited several other B cell differentiation cytokines, thereby abrogating B cell signals necessary for activation and class switching.

We performed T cell dependent B cells cultures to investigate whether the inhibition of T cell help by calcineurin inhibitors was of sufficient magnitude to cause downstream inhibition of B cell activation. Activation of T cells by immobilized anti-CD3 mAb eliminated the need for an antigen specific culture system to obtain immunoglobulin producing B cells (30). In the cocultures that we employed here, B cell activation was achieved via T cell activation and was cell contact dependent. In contrast to our previous findings in T cell free B cell cultures (14), tacrolimus and cyclosporin almost completely inhibited T cell dependent immunoglobulin production. The magnitude of inhibition was comparable to that caused by MPA and rapamycin, indicating that by inhibition T cell help, activation of B cells can be prevented.

It has previously been published that calcineurin inhibitors are incapable of inhibiting already activated T cells (31-33). This is in line with our current observations in cocultures of B cells with preactivated T cells, where tacrolimus and cyclosporin failed to inhibit immunoglobulin production. In contrast, MPA and rapamycin, which are able to inhibit activated T cells (26, 33) as well as to directly inhibit B cells (14), completely abrogated immunoglobulin production. The marginal inhibition of IgG levels by cyclosporin suggests minor inhibition of B cells directly, which however, is far less potent than its effect on T cell help. Taken together, our data show that calcineurin inhibitors can only prevent humoral responses by inhibiting T cell help. Consequently, these data stress the importance of targeting the T cell compartment to affect humoral immune responses.

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A large, stylized graphic of a Y-shaped antibody molecule, rendered in black and white with a thick, hand-drawn style. It is positioned on the left side of the page, partially overlapping the chapter title.

Chapter 4

Intravenous immunoglobulin preparations have no direct effect on B cell proliferation and immunoglobulin production

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ABSTRACT

Intravenous immunoglobulin (IVIg) is used for treatment of a variety of immunological disorders and in transplantation. As one of its applications in transplantation is the reduction of donor specific antibodies in the circulation, we examined the direct effect of IVIg on essential parameters of human B cell responses *in vitro*. Purified human B cells, human B cell hybridomas and T cells were cultured in the presence of graded concentrations of IVIg to test its effect on their proliferative capacity. To address the effect of IVIg on immunoglobulin production, we designed a novel technique making use of quantitative PCR to assess IgM and IgG levels. IVIg failed to inhibit proliferation of human B cells and human B cell hybridomas. In contrast, when IVIg was added to T cell cultures, a dose-dependent reduction of the proliferative capacity was observed. IVIg did not affect the levels of IgM and IgG mRNA of activated B cells. Our data show that IVIg is not capable of directly inhibiting key B cell responses. Direct B cell inhibition by IVIg seems therefore unlikely, implying that alteration in humoral immunity by IVIg is due to indirect effects on T cells and/or interactions with circulating antibodies and complement factors.

INTRODUCTION

Intravenous immunoglobulin (IVIg) is a preparation of pooled human plasma prepared from over a thousand donors and was originally used as a substitution therapy for patients with immunodeficiencies (1). IVIg is increasingly used as treatment for autoimmune and systemic inflammatory diseases, and in the field of solid organ- and bone marrow transplantation. In organ transplantation settings, IVIg is used, among other treatments, to reduce plasma HLA antibody levels prior to transplantation (2-4) and in attempts to reverse acute humoral rejection (5).

The present study focuses on the effect of IVIg on humoral immunity. Several mechanisms have been proposed for its mode of action. Firstly, anti-idiotypic antibodies in IVIg may interfere in the reaction of HLA-specific antibodies with their targets on HLA mismatched organs, thereby preventing humoral rejection (6). Secondly, IVIg may bind through the Fc portion of IgG to the inhibitory FcγRIIb expressed on a variety of blood cells, including B cells, leading to the reduction of proliferation and apoptosis induction (7). Thirdly, IVIg may inhibit complement, and thereby modulate the effector function of antibodies (8). Fourthly, changes in monocyte Fc receptor functions can inhibit antibody-mediated immune damage (9). Lastly, clearance of pathogenic antibodies may be accelerated by saturation of the FcRn receptor (10).

We used a robust culture system, devoid of auxiliary cells, for stimulating purified human B cells, as well as autonomously proliferating human B cell hybridomas to study the direct effect of IVIg on B cell proliferation and immunoglobulin production. The latter was assessed by a novel, PCR based, technique for quantifying immunoglobulin production.

MATERIALS AND METHODS

Cells

Blood was collected from healthy blood bank donors with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B (Invitrogen, Leek, the Netherlands) and released from the beads using Detach-a-Bead CD19 (Invitrogen). This yielded >98% pure B cells, as assessed by flow cytometric analysis. Human B cell hybridomas producing a variety of HLA monoclonal antibodies (mAbs) were established

as described previously (11). The hybridomas used were SN607D8 (HLA-A2/A28, IgG1), BVK5B10 (HLA-B8, IgM), VTM1F11 (HLA-B27/B7/B60, IgG1) and SN66E3 (HLA-A2/A28, IgM).

T cells were purified from PBMC by magnetic separation using the Pan T cell isolation kit II (Miltenyi, Bergisch-Gladbach, Germany) and MACS MS columns (Miltenyi). Flow cytometric analysis revealed >85% purity.

Proliferation experiments

B cells were cultured for 7 days at 5×10^3 cells/well in 96-well roundbottom plates (BD Falcon, Breda, the Netherlands) in culture medium consisting of IMDM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (insulin 5 μ g/ml, transferrin 5 μ g/ml and selenium 5 ng/ml, Sigma-Aldrich) at 37°C and 5% CO₂. B cells were stimulated with anti-CD40 mAb (1 μ g/ml, R&D systems, Minneapolis, MN, USA), 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D Systems), 100 ng/ml IL-21 (Invitrogen) and 2.5 μ g/ml of the TLR9 ligand CpG ODN 2006 (Hycult Biotechnology, Uden, the Netherlands). T cells were stimulated with 1 μ g/ml phytohemagglutinin (PHA, Remel, Lenexa, KS, USA) and cultured at 10^4 cells/well for 3 days in culture medium without ITS. Autonomously growing B cell hybridomas were cultured at 10^3 cells/well for 5 days in IMDM with 5% FCS, 0.05 mM 2-mercaptoethanol and 2 mM L-glucyl-L-glutamine (Sigma-Aldrich).

All cultures were performed in the absence or presence of graded concentrations of IVIg (Sanquin, Amsterdam, the Netherlands). Multiple lot numbers of IVIg were used, giving comparable results. Proliferation experiments were also performed with Gammagard (Baxter, Utrecht, the Netherlands), which gave similar results. Physical properties of the IVIg preparations are shown in Table I. All data shown were obtained with IVIg from Sanquin.

Table I. Physical properties of IVIg preparations used.

Product	Manufacturer	Method of preparation	% IgG	Stabilizing agent
Immunoglobulin i.v.	Sanquin	Cohn's cold ethanol fractionation	> 95%	Glucose
Gammagard S/D	Baxter	Cohn-Oncley-fractionation, ion-exchange, ultrafiltration, chromatography	> 90%	Glucose, glycine, albumin, PEG

i.v.: intravenous, *PEG*: polyethylene glycol.

All IVIg preparations were dialyzed prior to use unless otherwise stated. Dialysis was performed in 10 kDa Slide-A-Lyzer cassettes (Pierce, Rockford, IL, USA) against large volumes of IMDM. The presence of intact IgG after dialysis was confirmed by ELISA (data not shown). Where appropriate, cell culture grade BSA (Calbiochem, La Jolla, CA, USA) was used as a control for the high protein concentrations and D(+) glucose (Merck, Amsterdam, the Netherlands) was used as a control for the stabilizing agent of IVIg. Proliferation was determined by the incorporation of ^3H -TdR (1 μCi /well, Amersham International, Amersham, UK), added 18 hours before termination of culture.

Quantitative PCR

For IgM and IgG mRNA detection, B cells were cultured at 5×10^5 cells per well for 7 days in 24-well plates (Costar, Veenendaal, the Netherlands) and stimulated as described above in the presence of graded concentrations of IVIg. Rapamycin was used as a control for the inhibition of immunoglobulin production (12). Cells were harvested and preserved in RNAlater solution (Qiagen, Chatsworth, CA, USA). RNA was extracted using the RNeasy® mini kit (Qiagen) following the manufacturer's instructions. RNA was treated with DNase (Qiagen) on the spin columns. RNA quantity was assessed with a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All samples showed A260/A280 ratios between 1.9 and 2.1. cDNA was synthesized by incubating 12.8 μl RNA solution with 7.2 μl cDNA mix containing dNTPs (final concentration of 0.5 mM), 2 U reverse transcriptase-avian myeloblastosis virus (RT-AMV), 20 U rRNase inhibitor, 100 ng oligo-dT primers, 500 ng random primers, and 1 $\times$ reverse transcriptase buffer (all from Promega, Leiden, the Netherlands).

Primer sets (Table 2) for quantitative polymerase chain reaction (Q-PCR) were selected using Beacon Designer Software (version 7.02, Premier Biosoft International, Palo Alto, CA, USA) and obtained from Eurogentec (Liège, Belgium). For the amplification of total IgG transcript two forward primers, one hybridizing to the IgG₁ sequence and the other to IgG₂, IgG₃ and IgG₄ sequences, were used at a 1:1 ratio. The IgG reverse primer detected all four IgG variants. PCR mixes contained 1 μM of forward and reverse primers, 3 mM MgCl₂, and 1 $\times$ iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands). The PCR was performed using an iCycler MyiQ (Bio-Rad). The PCR program consisted of one cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C and was finalized with a melting curve analysis. Reactions were carried out in optical 96-well plates (Bio-Rad) covered with Microseal 'B' Film (Bio-Rad). The signal of the stably expressed household gene 18S rRNA served as a normalization factor.

Table 2. Sequences for primers used in quantitative polymerase chain reaction (Q-PCR).

Transcript	Forward primer	Reverse primer	Amplicon
IgG ₁	CATCTCCAAAGCCAAAGG	ATGTCGCTGGGATAGAAG	126 bp
IgG _{2,4}	CATCTCCAAAGCCAAAGG	ATGTCGCTGGGGTAGAAG	126 bp
IgM	CAGGGCACAGACGAACAC	CGGCAATCACTGGAAGAGG	85 bp
18S rRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	68 bp

bp: base pairs.

RESULTS

IVIg fails to inhibit B cell and B cell hybridoma proliferation

IVIg (at concentrations up to 35 mg/ml) failed to affect the proliferative capacity of both purified *in vitro* stimulated B cells from different donors (n=5) as well as four autonomously growing B cell hybridomas (Figure 1a and 1b). In contrast, non-dialyzed IVIg did inhibit the proliferation of B cell hybridomas (Figure 1c), which is in agreement with published data (13). However, inhibition was not due to the immunoglobulin component, as is clear from the lack of proliferation inhibition with dialyzed IVIg, but rather caused by the stabilizing agent glucose that is present in the IVIg solution (Figure 1d).

Since IVIg has been described to inhibit mitogen-induced lymphocyte proliferation (14), we tested its capacity to inhibit PHA induced T cell proliferation (n=4). The IVIg preparation dose-dependently inhibited T cell proliferation, indicating that the IVIg preparation was functional (Figure 1e). This inhibition was not due to high protein concentration, as shown by the inability of comparable concentrations of BSA to inhibit proliferation (Figure 1f).

IVIg does not inhibit immunoglobulin mRNA levels

Previously, we showed that B cells cultured in the presence of anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 were capable of producing large quantities of both IgM and IgG (12). This stimulation protocol induced vast elevations in mRNA levels for both IgM and IgG when compared to unstimulated B cells (Figure 2a and 2b), which is in concordance with immunoglobulin levels measured in the supernatants of these B cell cultures by ELISA (data not shown).

To test the effect of IVIg on immunoglobulin production, B cells were stimulated in the presence of IVIg at graded concentrations up to 35 mg/ml. Rapamycin was used as positive control for the reduction of immunoglobulin production. Q-PCR with primers specific for IgM and IgG revealed that IVIg did not affect the levels of IgM or IgG mRNA, whereas rapamycin dose-dependently reduced IgM and IgG mRNA levels (Figure 3a and 3b).

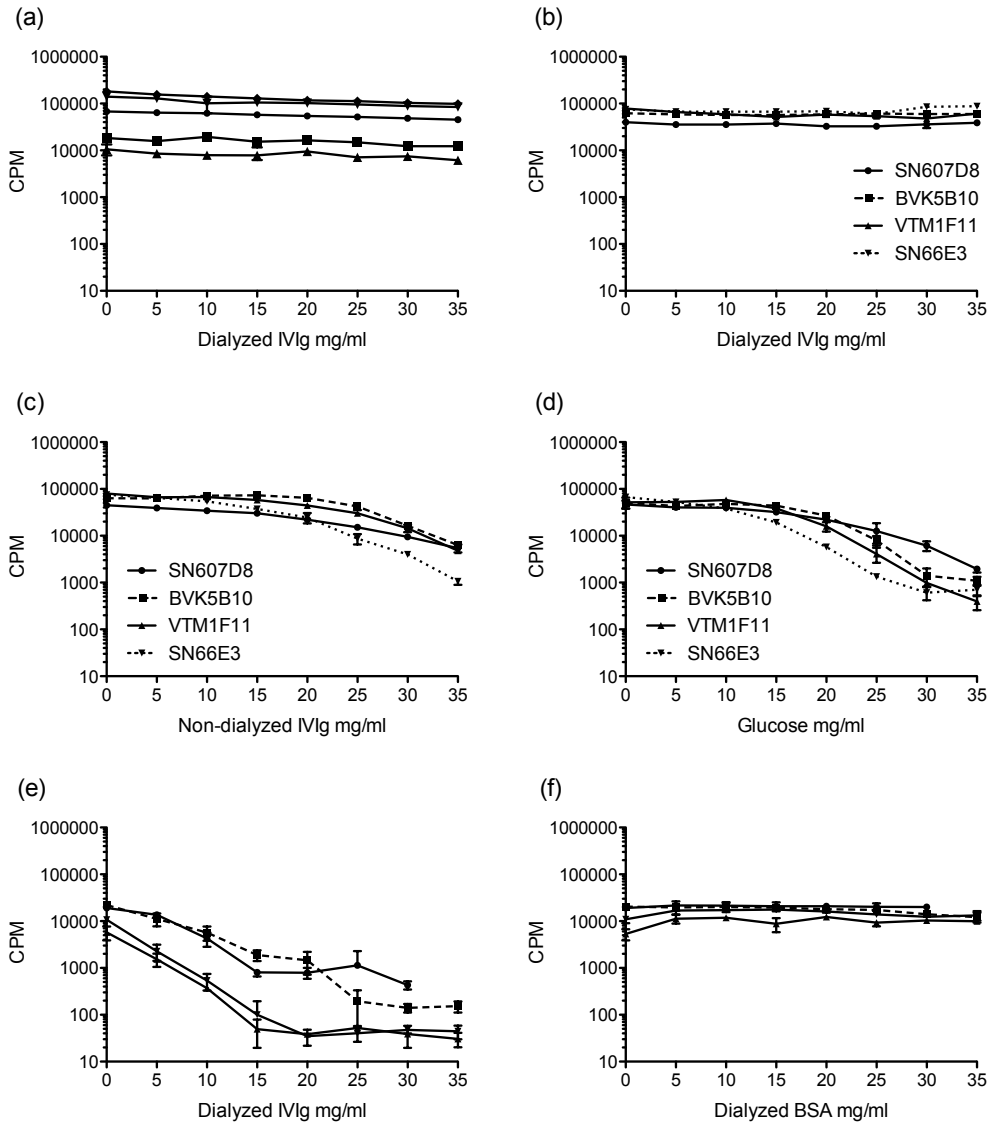


Figure 1. IVIg does not reduce the proliferative capacity of B cells and B cell hybridomas, whereas mitogen-induced T cell proliferation is inhibited. Non-dialyzed IVIg does inhibit hybridoma proliferation, caused by the stabilizing agent glucose. (a) B cells were cultured at 5×10^3 cells/well with the addition of anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 in the presence of graded concentrations of IVIg. Connecting lines represent the individuals donors ($n=5$) tested. (b) Autonomously growing B cell hybridomas were cultured at 10^3 cells/well in the presence of graded concentrations of IVIg. (c) non-dialyzed IVIg or (d) glucose in concentrations similar to the IVIg solution. Connecting lines represent individual hybridomas. Representative experiments are shown. (e) T cells were isolated by MACS isolation and cultured at 10^4 cells per well with 1 mg/ml PHA in the presence of graded concentrations of IVIg or (f) BSA in concentrations equal to the IVIg solution. Connecting lines represent the individuals donors ($n=4$) tested. Proliferation was measured by ^3H -TdR incorporation, CPM: counts per minute. Depicted are mean values of triplicate measurements.

DISCUSSION

IVIg is known to have a wide range of clinical effects. When used *in vitro*, IVIg reduced the reactivity of serum HLA antibodies (15, 16). It also inhibited mixed lymphocyte responses, and mitogen induced PBMC and T cell proliferation (14), as well as T cell dependent B cell responses (17-20).

The present study shows that IVIg does not affect the proliferative capacity of polyclonally stimulated purified B cells. This lack of inhibition is not due to extremely high B cell stimulation that is no longer amenable to interference, since we have previously shown that relatively low levels of standard immunosuppressive drugs can completely abolish proliferation, as well as immunoglobulin production of B cells stimulated with the same protocol (12). In contrast to the lack of B cell proliferation inhibition by IVIg, mitogen-induced T cell proliferation was significantly inhibited by the same IVIg preparation, indicating that the IVIg preparation was effective. This inhibition was not due to high protein levels, indicating that immunoglobulins present in the IVIg preparation were the cause of proliferation inhibition.

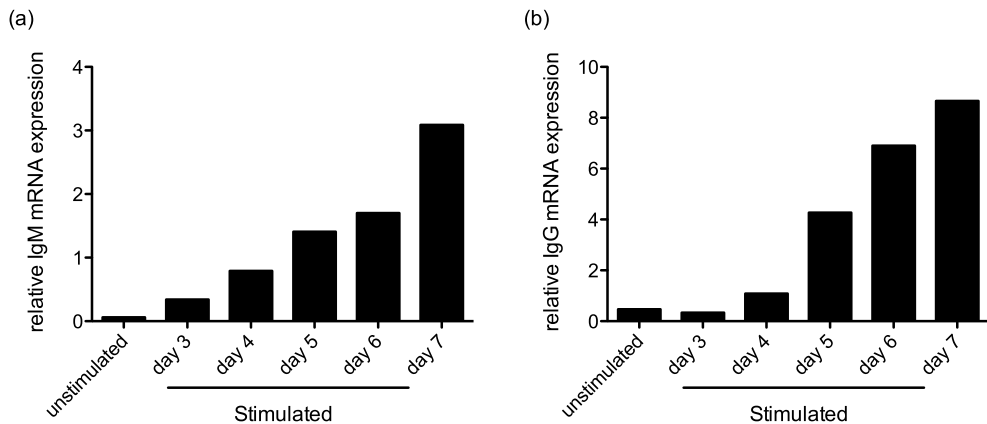


Figure 2. B cell activation induces IgM and IgG mRNA levels. B cells were activated with anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 and the cells were harvested at different time-points after activation. IgM (a) and IgG (b) mRNA levels were assessed by quantitative PCR. Unstimulated cells were harvested at day 0 and served as negative control. The results of one representative experiment are shown. Similar results were obtained in 4 independent experiments.

B cell hybridomas were previously shown to be inhibited by a non-dialyzed IVIg preparation (13). We added non-dialyzed IVIg to similar B cell hybridomas and observed a comparable

inhibition. However, when we dialyzed the IVIg prior to use to remove stabilizing agents such as glucose from the solution, the inhibition was abrogated. In agreement with previous studies (21), addition of glucose in concentrations similar to those in the IVIg preparation did inhibit the proliferation of B hybridomas. These data underscore the importance of dialysis of the IVIg preparation for *in vitro* studies.

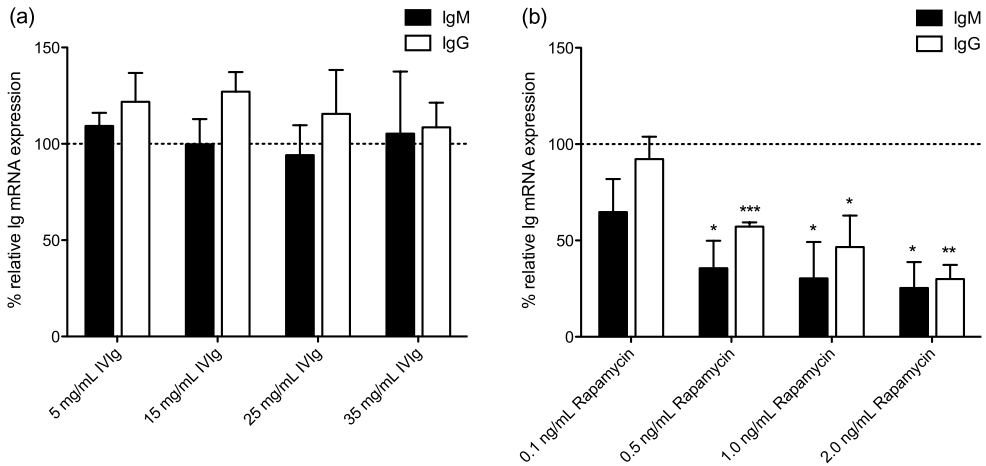


Figure 3. IVIg does not affect the mRNA synthesis of IgM and IgG, whereas rapamycin does inhibit IgM and IgG mRNA levels. B cells were stimulated with anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 and cultured in the presence of graded concentrations of IVIg (a). Control cultures were performed with graded concentrations of rapamycin. mRNA levels for IgM and IgG were quantified using quantitative PCR (b). The dotted lines represent the mRNA steady state level in stimulated, non-treated B cells. Data from 3 experiments with different donors are shown. Depicted are the percentages of IgM and IgG mRNA expression, relative to the mRNA expression of untreated controls. Statistics: paired T test, *P<0.05; **P<0.01 and ***P<0.001.

Besides B cell proliferation, studying the effects of IVIg on immunoglobulin production is of particular interest, since anti-idiotypic antibody binding and binding to inhibitory Fc receptors are candidate mechanisms for interference with immunoglobulin production (6, 7).

Unfortunately, the high IgG content of IVIg precludes the detection of newly synthesized IgG by standard ELISA techniques. De Grandmont *et al.* circumvented this issue by radioactive labeling of secreted immunoglobulins followed by capture with anti-IgG antibodies (22). But even then, competition by IVIg for capture antibody cannot be excluded. Therefore, we designed a novel approach to indirectly measure immunoglobulin production by detection of IgM and IgG mRNA levels. We found that IVIg does not affect the mRNA synthesis of

both IgM and IgG by stimulated B cells. It seems therefore highly unlikely that IVIg directly influences immunoglobulin production.

This conclusion is at variance with other studies that do show an inhibitory effect of IVIg on immunoglobulin production (17-20). These studies, which describe B cells as targets for IVIg, were performed with Pokeweed Mitogen stimulation, which is a model of T cell dependent antibody production by B cells (23). However, it cannot be ruled out that in that culture system IVIg interferes with the actual stimulus, *i.e.* Pokeweed Mitogen, rather than that it truly inhibits B cell functions. In another study, inhibition of proliferation and IgE secretion of purified tonsillar B cells cultured with IL-4 and anti-CD40 was described (24). However, these results were obtained using a non-dialyzed IVIg preparation, and are likely an effect of the stabilizing agents in the IVIg preparation.

IVIg preparations differ greatly in terms of production process and Ig content. While B cell inhibitory effects were not found in two different IVIg preparations, it cannot be ruled out that other preparations will show inhibitory effects, particularly when other isotypes are present, as in Pentaglobulin.

In line with our results, a series of *in vivo* studies show the lack of direct effects on immunoglobulin producing cells. IVIg was unable to reduce B cell and plasma cell numbers in human splenic follicles (25). Furthermore, IVIg failed to reduce the number of bone marrow residing long-lived plasma cells producing HLA-specific antibodies (26).

The inhibitory effect of IVIg on humoral immunity may therefore be explained by alternative mechanisms, such as inhibition of T cell help, interaction with circulating antibodies and interaction with complement factors. Inhibitory effects of IVIg on T cell priming by dendritic cells have been described (27), as well as cytokine-dependent T cell proliferation (28). Inhibition of T cell function may lead to insufficient generation of helper T cells, which affects B cell responses. Future research on the effect of IVIg on T cell help directly, and on T cell dependent B cell responses in co-culture systems will provide more insight in the effect of IVIg on humoral immune responses.

Interaction of the variable regions of IVIg with variable regions of autoantibodies of multiple specificities has been described (29, 30), suggesting anti-idiotypic activity of IVIg. Circulating anti-HLA antibodies may be subject to the same mechanism of suppression. In addition, interference of IVIg with complement activation (8, 31) and impairment of monocyte Fc receptor functions (9) have been suggested. These mechanisms may affect the action of deleterious complement binding antibodies and/or phagocytosis, leading to protection from immune damage. Alternatively, saturation of the FcRn receptor may be involved in clearance of pathogenic antibodies (10).

Taken together, our results show that IVIg fails to directly act on immunoglobulin producing

cells. The capability of IVIg to interfere with humoral immunity is therefore more likely due to effects on T cells and interactions with circulating antibodies and/or complement factors.

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Chapter 5

Bortezomib affects the function of human
B cells: possible implications for humoral
rejection therapy

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INTRODUCTION

The proteasome inhibitor bortezomib is a potent inducer of apoptosis in malignant (1) as well as non-malignant human plasma cells (2). For that reason, bortezomib has recently come to the attention of the transplantation community for the treatment of humoral rejection. Bortezomib was shown to reduce donor specific antibody levels and effectively revert rejection episodes (3, 4), as well as attenuate HLA antibody levels in non-rejecting transplant patients (5). Since proteasomes are present in all eukaryotic cells, bortezomib, besides its effects on plasma cells, likely affects other cell types of the immune system as well. Additional drug effects towards B cells may be beneficial for the treatment of humoral rejection. Therefore, we tested whether, in addition to plasma cell depletion, bortezomib was capable of inhibiting the functionality of human B cells upon CD40 or B cell receptor mediated stimulation.

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll Hypaque density gradient centrifugation from blood of healthy blood bank donors after informed consent. B cells were immunomagnetically isolated by positive selection (Invitrogen, Leek, the Netherlands). These CD19⁺ B cells were cultured in Iscove's Modified Dulbecco Medium (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (Sigma-Aldrich) at 37°C and 5% CO₂. B cells were activated with 1 µg/mL of anti-CD40 mAb (R&D systems, Minneapolis, MN, USA), 100 U/mL of IL-2 (EuroCetus, Amsterdam, the Netherlands), 25 ng/mL IL-10 (R&D systems), 100 ng/ml of IL-21 (Invitrogen) and 2.5 µg/ml of the TLR9 ligand CpG ODN 2006 (Hycult Biotechnology, Uden, the Netherlands), or with 1:10.000 diluted *Staphylococcus aureus* (Calbiochem, La Jolla, CA, USA) with 5% v/v supernatant of phytohaemagglutinin (PHA) activated T cells, prepared as described before (6). At day 6, supernatants were collected for IgM and IgG detection by ELISA (7). The incorporation of ³H-TdR (Amersham International, Amersham, UK, 1 µCi/well added during the last 18 h of a 7 day culture) was measured for determining B cell proliferation (7).

RESULTS AND DISCUSSION

Bortezomib completely abrogated IgM and IgG production as well as proliferation in a dose dependent fashion. Complete inhibition of immunoglobulin production was achieved at 1.0 ng/ml of bortezomib with IgM and IgG levels equally influenced (Figure 1a). Proliferation was already completely inhibited at 0.75 ng/ml of bortezomib. Additionally, when added to B cells that were already activated for 2 days, bortezomib completely inhibited proliferation, albeit at slightly higher doses (Figure 1b). Bortezomib also inhibited immunoglobulin production and proliferation of *Staphylococcus aureus* stimulated B cells (data not shown), indicating that this is a general effect of bortezomib on B cells, regardless of the mode of stimulation.

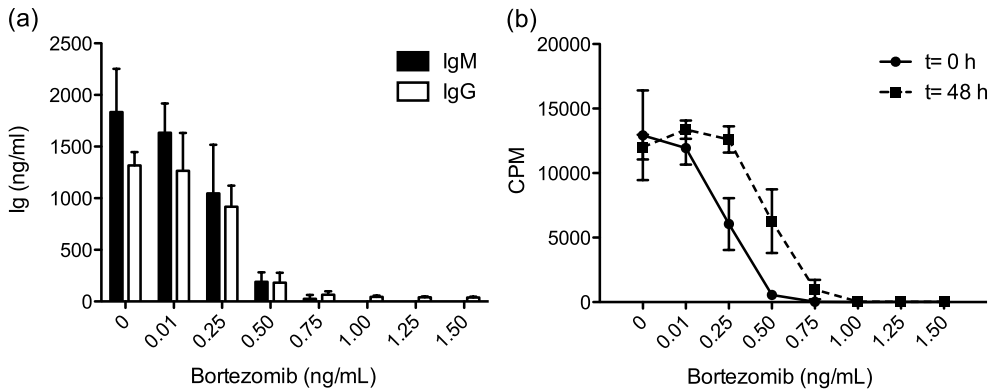


Figure 1. Bortezomib effectively inhibits immunoglobulin production and proliferation of activated human B cells. Isolated B cells were cultured at 5×10^3 cells per well in 96-wells round bottom plates for 7 days in the presence of graded concentrations of bortezomib. At day 6, supernatants were collected for immunoglobulin (Ig) detection. Bortezomib dose-dependently inhibited IgM and IgG levels in B cell cultures (a). After 7 days of culture, B cell proliferation was measured by ^3H -TdR incorporation. Bortezomib added either directly ($t = 0$ h), or after 2 days ($t = 48$ h) dose-dependently inhibited B cell proliferation (b). Data are depicted as means of at least triplicate wells with standard deviation. Representative data from one experiment are shown; similar results were obtained in four independent experiments. CPM: counts per minute.

We conclude that, in addition to depleting plasma cells, bortezomib profoundly abrogates B cell function. Thus, bortezomib may prevent the *de novo* emergence of HLA antibodies as well as deplete an important source of antigen presenting cells. Our findings suggest that, when bortezomib is used for desensitization or for the treatment of humoral rejection, there is no need for additional anti-B cell therapy, such as anti-CD20 mAb treatment.

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Chapter 6

A novel ELISPOT assay to quantify HLA-specific B cells in HLA-immunized individuals

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Manuscript in preparation

ABSTRACT

Quantification of the humoral alloimmune response is generally achieved by measuring serum HLA antibodies, which provides no information about the cells involved in the humoral immune response. Therefore, we have developed an HLA-specific B cell ELISPOT assay allowing for quantification of B cells producing HLA antibodies.

We used recombinant HLA monomers as target in the ELISPOT assay. Validation was performed with human B cell hybridomas producing HLA antibodies. Subsequently, we quantified B cells producing HLA antibodies in HLA-immunized individuals, non HLA-immunized individuals and one transplant patient with serum HLA antibodies.

B cell hybridomas exclusively formed spots against HLA molecules of corresponding specificity with similar sensitivity to total IgG ELISPOT assays. HLA immunized healthy individuals had up to 182 HLA-specific B cells per million total B cells. In contrast, non-immunized individuals did not form spots. A kidney transplant patient who was immunized by an HLA-A2-mismatched graft had 143 HLA-A2-specific B cells per million total B cells.

In conclusion, we have developed a highly specific and sensitive HLA-specific B cell ELISPOT assay, which can be used to determine the frequency of peripheral HLA-specific B cells in transplant patients. This technique provides an alternative way to quantify humoral immune responses.

INTRODUCTION

In organ transplantation, B cells can be harmful to a transplanted organ mostly by their antibody producing capacity. The presence of HLA antibodies is associated with allograft rejection, since their development frequently precedes chronic rejection in renal transplantation (1, 2).

Techniques for the analysis of donor-specific serum HLA antibodies are widely available and include complement dependent cytotoxicity (CDC) (3), ELISA (4, 5), flow cytometry (6) and Luminex-based assays (7). These assays are valuable for determination of donor-specific antibodies in the circulation, but are likely to underestimate the magnitude of the humoral immune response in the case of antibody absorption by the graft (8). Therefore, assessing the frequency of circulating HLA specific B cells may provide a more accurate estimate of the humoral immune status of a patient. Moreover, the demonstration of an increment of B cell numbers with anti-donor specificity may be taken as a predictor of antibody formation in the case that an incipient B cell response is not yet detectable as serum HLA antibodies.

Assays to quantify the number of cells contributing to alloantibody production are scarce. We previously described a technique to estimate the B cell precursor frequency (BCPF) of B cells with HLA-specificity (9). This laborious technique only allows for the quantification of B cells producing complement-fixing antibodies. However, non-complement-fixing antibodies may play a role in humoral rejection (10, 11). The inability to detect complement-fixing antibodies is circumvented by an alternative technique in which HLA-specific B cells from CD19-enriched cell populations are stained with tetramers, quantifying B cells harboring a B cell receptor (BCR) directed against certain HLA molecules (12, 13). A disadvantage of this technique, which relies on binding of the BCR to HLA tetramers, is that it does not readily provide information on the proportion of cells actually capable of producing antibodies. Alternatively, an elegant technique for the quantification of bone-marrow residing plasma cells producing HLA antibodies has been described (14). However, bone marrow is not regularly available from transplant patients, which makes this technique unsuitable for routine screening purposes.

Here, we describe a novel technique to detect and enumerate HLA-specific memory B cells that are capable of producing antibodies. The assay is performed on B cells from peripheral blood, which is routinely collected before and after transplantation in many transplant centers. The HLA-specific B cell ELISPOT enables longitudinal post-transplant monitoring of humoral HLA-specific immunity, and may assist in tailoring the immunosuppressive regimen for the individual patient.

MATERIALS AND METHODS

Subjects

The subject population consisted of 10 HLA-A2-immunized, 3 HLA-B7-immunized and 3 HLA-A1-immunized multi- and uniparous healthy individuals, 11 healthy control blood donors and 1 kidney transplant patient. The immunized healthy individuals had developed serum HLA-A2, HLA-B7 or HLA-A1 antibodies by pregnancy, as determined by CDC. The control blood donors either had an HLA-type similar to the HLA molecule to which they were tested, or an HLA-type different from the HLA molecule to which they were tested, but not sensitized to HLA. The kidney transplant patient (HLA-A2⁻) was on the waiting list for a transplant after having rejected a previous, HLA-A2 mismatched, graft. This patient had developed serum antibodies towards HLA-A2 during the rejection, which were still present at time of sampling.

Cells

Blood was obtained with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. To retrieve as many B cells as possible, we partially depleted T cells from PBMC, rather than positively isolating the B cell fraction (12). To this goal, we incubated PBMC with magnetically labeled CD2 Microbeads (Miltenyi, Bergisch-Gladbach, Germany), followed by magnetic cell separation. Flow cytometry (FCM), using CD19-PE (BD Biosciences, Breda, the Netherlands) revealed, on average, a 6-fold enrichment of CD19⁺ B cells. The CD2⁻ cell population will be referred to as enriched B cell population.

The human monoclonal antibody (mAb) producing B cell hybridomas GV5D1 (IgG, HLA-A1/A9), SN607D8 (IgG, HLA-A2/A28), MUL2C6 (IgG, HLA-A3/A11/A24), VTMIF11 (IgG, HLA-B27/B7/B60) and BVKF9 (IgG, HLA-B8) were developed as described previously (15) and used for optimization and validation of the HLA-specific ELISPOT assays. The human CD40L expressing murine fibroblast cell line L-CD40L was used to activate B cells (16).

Monomeric HLA molecules

The HLA-A*0101 monomer was constructed around the melanoma associated MAGE3 peptide (sequence: EVDPIGHLY), the HLA-A*0201 monomer was constructed around the melanoma associated MART-I peptide (sequence: ELAGIGILTV), the HLA-A*301 monomer around the MAGE1 melanoma peptide (sequence: SLFRAVITK), the HLA-A*1101 monomer around the EBV derived EBNA3B peptide (sequence: AVFDRKSDAK)

and the HLA-B*0702 monomer around the hCMV derived pp65 peptide (sequence: RPHERNGFTV) as described previously (17).

Cell culture

Enriched B cells were cultured for 7 days at 2.5×10^5 cells per well in 24-well plates (Costar, Veenendaal, the Netherlands) in culture medium consisting of IMDM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (insulin 5 µg/ml, transferrin 5 µg/ml and selenium 5 ng/ml, Sigma-Aldrich) at 37°C and 5% CO₂. Cells were stimulated with irradiated (75 Gy) L-CD40L cells with 40 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands), 10 ng/ml IL-10 (R&D systems, Minneapolis, MN), 10 ng/ml IL-21 (Invitrogen, Leek, the Netherlands) and 1 µg/ml of the TLR-9 ligand ODN-2006 CpG (Hycult Biotechnology, Uden, the Netherlands). At day 7, supernatants were collected and frozen at -20°C until further use and cells were harvested for FCM and for use in the ELISPOT assay.

ELISPOT assay

We used a previously established total IgG ELISPOT assay for detecting total numbers of IgG producing B cells (18) and developed an HLA-specific ELISPOT assay for enumerating the number of HLA-specific B cells. For measuring total IgG spots, ELISPOT plates (Millipore, Amsterdam, the Netherlands) were coated with goat anti-IgG (Jackson ImmunoResearch, Suffolk, UK) in PBS overnight at 4°C and blocked with 5% FCS/IMDM at 37°C for 2 h. For detecting HLA-specific B cells, ELISPOT plates were coated with Streptavidin (Pierce, Rockford, IL, USA) in 10 mM Tris pH 9.0 overnight at 4°C, followed by 4 h incubation of a biotin labeled HLA class I monomer at 500 ng/well as capture matrix in PBS at RT and blocking with 5% FCS/IMDM at 37°C for 1 h. To make sure no aspecific background spots were counted in the ELISPOT, we included wells coated with streptavidin, but without monomer. Spots, if any, in these control wells were of low intensity, and spot numbers in these control wells were deducted from spot numbers counted in wells with monomer present.

B cell hybridomas were harvested, thoroughly washed, diluted in a range of cell concentrations in 5% FCS/IMDM with 200 U/ml penicillin and 200 µg/ml streptomycin, and transferred to wells of anti IgG- and HLA-monomer coated ELISPOT plates. Activated enriched B cells were similarly treated but seeded at 2.5×10^5 cells per well in multiple wells of ELISPOT plates. After overnight incubation at 37°C, plates were thoroughly washed and incubated for 2 h at RT with HRP conjugated goat anti-IgG (Southern Biotech, Birmingham, AL). Plates were washed and incubated with TMB substrate (Mabtech, Nacka Strand,

Sweden) for 5 min. The reaction was stopped by rinsing with water. Plates were analyzed using a computer guided ELISPOT reader (iSPOT system, AID, Strassberg, Germany).

To calculate the ratio of HLA-specific B cells per million total input B cells, the percentage live B cells (based on forward/sideward scatter and CD19 FCM positivity) present in the pre-culture at day 7 was used to calculate the ratio of HLA-specific B cells per million total B cells as follows:

$$\left(\frac{(\# \text{ specific spots}) \times (1 \times 10^6)}{(\# \text{ specific wells}) \times (\# \text{ cells/well})} - \frac{(\# \text{ background spots}) \times (1 \times 10^6)}{(\# \text{ background wells}) \times (\# \text{ cells/well})} \right) \times \left(\frac{100}{\% \text{ B cells}} \right)$$

HLA-specific antibody detection

Supernatants from enriched B cell pre-cultures were tested for the presence of antibodies against HLA-A2 in a Luminex assay using LABScreen beads (OneLambda, Canoga Park, CA) following the manufacturer's description. Beads carrying A*0201 molecules (batch LSIA04NC8_005_00) were used to detect HLA-A2 antibodies. As negative control we used the LABScreen negative control serum (LS-NC, OneLambda). Samples were measured using a Luminex Bio-plex 100 reader (Bio-Rad, Veenendaal, the Netherlands).

Statistics

To compare the difference between spot numbers of HLA-immunized and non-immunized individuals, the Wilcoxon Rank test was used. Statistical level of significance was defined as $P < 0.05$.

RESULTS

ELISPOT specificity and detection level

Mixing experiments of HLA-A2 specific B cell hybridomas with hybridomas of other specificity were performed to determine the specificity of the ELISPOT assay with HLA-A2 monomers as capture matrix. We observed titratable spots when using HLA-A2-specific hybridomas, whereas background levels of spots were observed when using a hybridoma with other specificity (Figure 1a). Additionally, we tested the specificity of the assay using an HLA-A1 specific hybridoma mixed with a hybridoma of other specificity in an ELISPOT assay with a HLA-A1 monomers as capture matrix. Similar to the HLA-A2 ELISPOT, HLA-

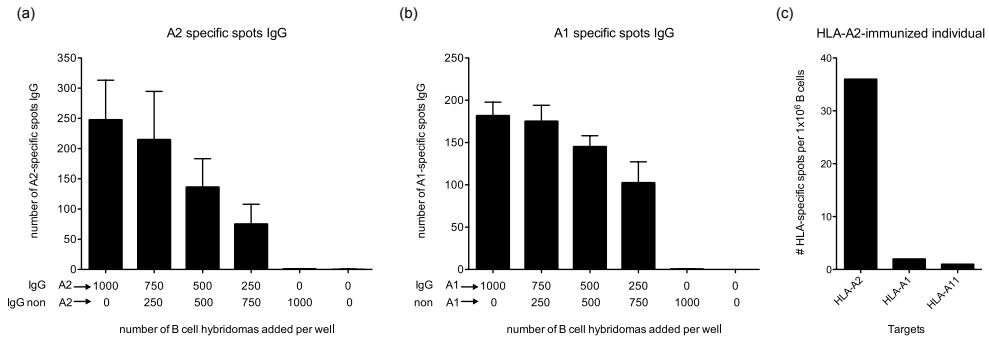


Figure 1. The HLA-specific ELISPOT assay is highly specific. (a) An IgG producing HLA-A2 specific B cell hybridoma (SN607D8) was mixed with an IgG producing HLA-B27/B7/B60 specific B cell hybridoma (VTMIF11) in various ratios. (b) An IgG producing HLA-A1 specific B cell hybridoma (GV5D1) was mixed with an IgG producing HLA-B8 specific B cell hybridoma (BVK1F9) in various ratios. (c) B cells from a healthy individual (HLA class I typed A3, B7, B15, Cw3, Cw7), who was immunized against HLA-A2, but not HLA-A1 or HLA-A11, formed spots against HLA-A2, and no spots against HLA-A1 or HLA-A11.

A1 specific hybridomas generated spot formation, whereas hybridomas of other specificity did not (Figure 1b). Specificities of ELISPOT assays using HLA-A3 and HLA-A11 monomers as capture matrix were similar to those of HLA-A2 and HLA-A1 (data not shown). We corroborated the specificity of the ELISPOT assay by testing HLA-A2- healthy individuals who were immunized by pregnancy towards HLA-A2, but not towards HLA-A1 or HLA-A11, in ELISPOT assays with these HLA molecules as targets. We observed spots against HLA-A2, but not against HLA-A1 or HLA-A11, warranting that the ELISPOT assay is highly specific (Figure 1c).

To make sure that the HLA-specific ELISPOT assay was able to detect all cells producing HLA-specific immunoglobulins, we compared the spot count of the total IgG ELISPOT with spot count of the HLA-specific ELISPOT within the same experiment. The HLA-specific ELISPOT with HLA-A2 as antigen was able to detect all HLA-A2 antibody producing cells, since the number of spots detected in the total Ig ELISPOT and the HLA-A2 specific ELISPOT were comparable (Figure 2). Identical results were obtained when using HLA-A11 specific hybridomas in an HLA-A11 ELISPOT assay (data not shown).

HLA-specific B cells are detected in HLA-immunized individuals

To determine whether the HLA-specific ELISPOT assay was capable of detecting human B cells producing HLA-specific immunoglobulins, we made use of PBMC from healthy women who were HLA-A2-immunized by pregnancy. HLA-A2⁺ individuals and HLA-A2⁻, non-HLA-A2 immunized individuals were used as negative controls. In individuals who were HLA-immunized by pregnancy, we found HLA-A2 specific B cells ranging from 0 to 125 cells per million total B cells (median=14, n=10), whereas in HLA-A2⁺ (n=3) and HLA-A2⁻ non-immunized individuals (n=3) the number of spots ranged from 0 to 1 per million total B cells (median=0, Figure 3). In HLA-A1-immunized individuals we observed spots

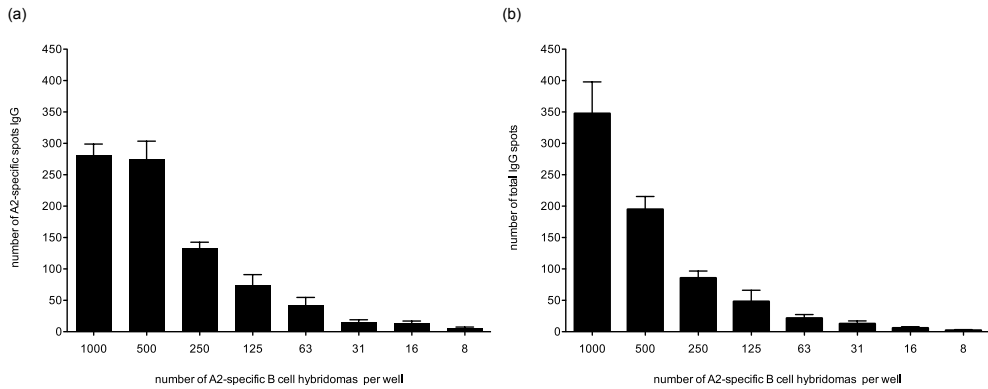


Figure 2. The HLA-specific ELISPOT is able to recognize all antibody-producing cells of corresponding specificity. The IgG producing HLA-A2 specific B cell hybridoma SN607D8 was titrated to be able to compare the HLA-specific ELISPOT (panel a) and the total IgG ELISPOT (panel b) for the number of spots.

ranging from 5 to 42 spots per million B cells (median=40, n=3), and no spots in HLA-A1⁺ and HLA-A1⁻, non-immunized individuals (n=3). Finally, in HLA-B7-immunized individuals we observed frequencies ranging from 9 to 182 spots per million B cells (median=109, n=3), whereas in control experiments with HLA-B7⁺ individuals and HLA-B7⁻, non sensitized individuals we observed no spot formation (n=3).

To ensure that spots detected in the ELISPOT assay were truly formed against corresponding HLA molecules, we performed a Luminex assay to detect HLA-A2 specific antibodies in the supernatants of the pre-culture of selected individuals who developed spots in an HLA-A2-specific ELISPOT assay. In those cases where we found spots against HLA-A2, we detected HLA-A2 specific antibodies in corresponding supernatants (Table 1).

Interestingly, in the one pregnancy-immunized individual where we did not observe spots against HLA-A2, we also did not find HLA-A2 specific antibodies in the supernatant of the pre-culture. No correlation between the number of spots and the mean fluorescence intensity (MFI) was observed.

Detection of HLA-A2 specific B cells in transplant patients

Since the HLA-immunized individuals we tested in the ELISPOT assay were immunized by pregnancy, we wanted to confirm the ability to detect HLA specific B cells in individuals who had developed HLA antibodies due to allograft rejection. One HLA-A2⁺ kidney transplant patient who was on the waiting list for a transplant after having rejected a previous,

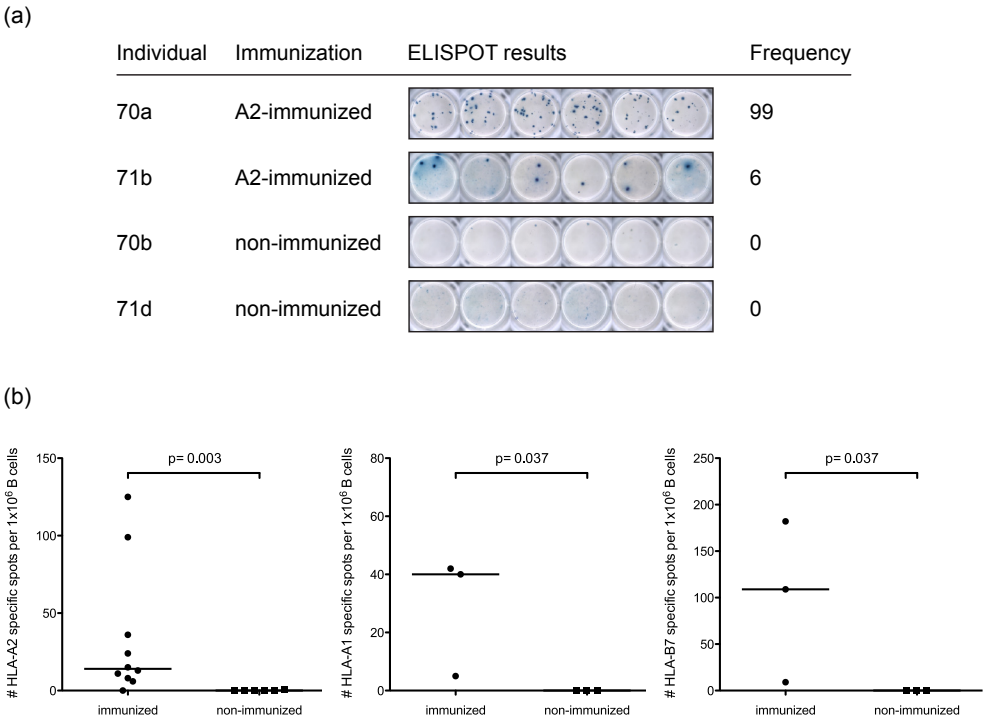


Figure 3. HLA-specific B cells are detected in HLA-immunized individuals, but not in non-immunized individuals. (a) Raw ELISPOT data of 2 HLA-A2 immunized and 2 non-immunized individuals. Frequency is the number of spots per million B cells. (b) Enriched B cells were activated for 7 days and seeded into ELISPOT plates coated with HLA-A2 monomers. Depicted are the number of HLA-A2 specific antibody producing B cells per million total B cells of HLA-A2 immunized and non-immunized healthy individuals, as well as the number of HLA-A1 spots measured in HLA-A1-specific ELISPOT assays and HLA-B7 spots measured in HLA-B7-specific ELISPOT assays. Horizontal bars indicate median values.

HLA-A2 mismatched, graft was tested for the presence of HLA-A2 specific B cells. We found a frequency of 143 HLA-A2-specific B cells per million total B cells (data not shown).

Table 1. HLA-A2 spot numbers and MFI values of HLA-A*0201 specific Luminex.

Individual	Immunization status	Spot number ^a	MFI supernatant	MFI negative control
70a	A2-immunized	99	59.79	0.98
71a	A2-immunized	0	0.00	0.76
71b	A2-immunized	6	61.80	0.75
73a	A2-immunized	8	112.18	1.48
73b	A2-immunized	24	140.22	1.00
70b	non-immunized	0	0.00	1.14
71c	non-immunized	1	0.00	2.91
71d	non-immunized	0	0.00	1.10
73d	non-immunized	0	3.90	0.90

^aPer million total B cells.

MFI: mean fluorescence index.

DISCUSSION

Monitoring of the immune status after transplantation can be of great benefit to patients, since it may be of help in determining tailor-made immunosuppressive protocols. Techniques for the assessment of the cellular immune status are manifold and have been in use for a considerable time. These include the mixed lymphocyte reaction (MLR), the cytotoxic T cell precursor assay (CTLp) (19), IFN- γ and IL-10 ELISPOT assays (20) as well as phenotypic analysis by flow cytometry. For humoral immunity, the main techniques used are to detect serum antibody levels (3-7), but not the (potential) activity of alloantibody-producing cells.

Upon encounter with antigen, B cells can form germinal centers in which memory B cells and plasma cells are generated. Plasma cells subsequently migrate to the bone marrow where they produce antibodies. A fraction of memory B cells circulate in the peripheral blood, where they make up about 30% of total CD19⁺ B cells (21, 22). The use of an ELISPOT technique was already described for quantifying human antigen-specific memory B cells from peripheral blood specific for an antigen of an Anthrax vaccine (22) and for the analysis of B cell memory towards antigens of the Malaria causing parasite *Plasmodium falciparum* (23) and HIV antigens (24). Here we present a validated technique to quantify

HLA-specific memory B cells of several specificities from peripheral blood.

To be able to detect HLA-specific B cells with specificity towards certain HLA molecules, we used recombinant monomeric HLA molecules as a source of pure HLA antigen. We previously showed the capability of HLA-specific B cells to recognize MHC class I tetramers, as well as the specific recognition of HLA-A2 tetramers by monoclonal B cell hybridomas producing HLA-A2 antibodies (25). This provided a rationale for using recombinant monomeric MHC class I molecules as antigen in the ELISPOT assay. The specificity of the assay was determined by using B cell hybridomas, which produce antibodies towards defined HLA molecules. The unambiguous specificity of hybridomas for their corresponding MHC class I monomer in the HLA-specific B cell ELISPOT assay is in concordance with previous data from our laboratory, in which tetrameric MHC class I molecules were used to stain a panel of beads that were coated with mAbs derived from these hybridomas (26). Recombinant MHC molecules are routinely loaded with peptides from a single specificity, allowing for the detection of specific T and/or B cell subsets in a variety of assays. We have previously shown that the embedded peptide affects binding of human HLA-specific mAbs (27). In contrast, Barnardo *et al.* did not observe an effect of different peptides on the binding of sera from HLA-immunized individuals to HLA monomers in an ELISA assay (28). Additionally, Zachary and colleagues have shown no effect of the HLA-bound peptide on tetramer staining of polyclonal B cells (12). Similarly, in the ELISPOT assay we found in preliminary tests that the use of a single monomeric HLA-A2 molecule resulted in the same number of spots as compared to a pool of 3 monomeric HLA-A2 molecules with different peptides (data not shown). It is therefore likely that a B cell alloresponse includes sufficient numbers of clones to surpass the contribution of single clones. As such, to be able to identify HLA-specific B cells in an HLA-immunized individual, the use of single recombinant monomeric MHC molecules is likely to be sufficient.

The demonstration that the detection of HLA-specific B cells by the ELISPOT corresponded to the presence of HLA-antibodies in the supernatant of pre-cultures warrants that the B cells truly produce anti-HLA antibodies. As expected from the relatively low HLA-specific B cell frequency, Luminex MFI values were low compared to positive control serum generated values (mean MFI: 3012.75), but considerably elevated compared to the negative control. Furthermore, the data showing that HLA-A2⁺ non-immunized individuals lack spot formation against HLA-A2 monomers as well as HLA-A2 specific antibodies in the pre-culture ensures that positive signals are due to memory, and not naïve, B cell responses. The lack of correlation between the number of A2-specific spots in the ELISPOT assay and the A2-specific immunoglobulin levels in the Luminex assay suggest that antibody producing B cell clones differ in the amount of immunoglobulin produced.

HLA-specific B cells in transplant patients under immunosuppression can be detected using the current ELISPOT assay, indicating that this technique is suited for assessing the HLA-specific humoral immune status in transplant patients. Additional studies on PBMC from transplant patients collected at set time-points are currently performed to extend these data and to determine whether the HLA-specific ELISPOT can predict humoral rejection. Moreover, we are rapidly expanding the number of HLA antigens that can be used in our ELISPOT assay to provide a comprehensive panel of HLA-specific B cells to be detected.

Recently, Wadia *et al.* published an article containing preliminary data claiming the development of an HLA-specific B cell ELISPOT (29). However, no data were shown on the specificity and sensitivity of this assay. Therefore, to our knowledge, ours is the first report of a well-validated, reproducible HLA-specific B cell ELISPOT. This assay may be a useful tool for monitoring transplant patients for their HLA-specific B cell load. More importantly, it may allow the detection humoral rejection at an early stage, preceding the emergence of serum HLA antibodies. Whether this is the case, will be subject to further studies.

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Chapter 7

Monitoring of indirect allorecognition: wishful thinking or solid data?

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ABSTRACT

Monitoring of T cells involved in an alloimmune response requires the presence of *in vitro* assays, which can detect T cells with direct as well as indirect allospecificity. While generally accepted assays exist to measure helper and cytotoxic T cells involved in direct allorecognition, consensus about an assay for monitoring indirect T cell allorecognition in clinical transplantation is lacking. Many studies claim a relationship between the reactivity of T cells with indirect allospecificity and allograft rejection, but different approaches are used and often, essential controls are lacking. In this review, the disadvantages and pitfalls of the different approaches used so far are discussed and supported by our own *in vitro* experiments. We conclude that an international workshop is necessary to establish and validate a uniform, robust and reliable assay for the monitoring of transplant recipients and to study the actual role of indirect allorecognition in acute and chronic rejection.

INTRODUCTION

Transplantation, blood transfusion or pregnancy may result in activation of alloreactive T cells leading to rejection, graft versus host disease and/or induction of IgG alloantibodies. These alloreactive T lymphocytes recognize non-self antigens, mostly derived from the highly polymorphic major histocompatibility complex (MHC) on allogeneic cells or tissue. The way by which T lymphocytes recognize alloantigens has been the focus of many studies and can occur by two distinct, not mutually exclusive pathways: the direct and indirect pathway.

Direct allorecognition refers to the recognition of intact allo-MHC molecules expressed on the surface of donor antigen-presenting cells (APCs). Allorecognition in this way results in a vigorous immune response, due to the high precursor frequency of T cells involved in this pathway (1). Two hypotheses have been proposed for this T cell activation, referred to as the 'high determinant density model' and the 'multiple binary complex model'. In the high determinant density model it is presumed that every MHC molecule on the cell surface, irrespective of the bound peptide, can serve as a ligand for host alloreactive T cells. The high antigen density may thus account for the vigorous immune response. In the multiple binary complex model, the peptide bound in the groove of the allo-MHC molecule is the decisive entity. Here, each peptide-allo-MHC complex is recognized by a unique host alloreactive T cell, also leading to a strong immune response. It is obvious that direct allorecognition only occurs after transfer of tissues or cells between genetically different individuals. In contrast, the mechanism of indirect allorecognition is basically not different from MHC restricted recognition of virally encoded antigens.

Indirect allorecognition is the stimulation of recipient T lymphocytes by processed donor antigens presented as peptides in the context of self-MHC. Evidence for this alternative pathway of T cell allorecognition came from observations that graft rejection still occurred in the absence of immunogenic donor-derived passenger cells in the graft (2). Alloantigens shed from the graft are internalized and processed in the same way as exogenous antigens and presented by recipient APCs as peptides in self-MHC class II molecules to CD4⁺ T cells. The frequency of T cells with indirect allospecificity is two orders of magnitude lower than T cells directly recognizing alloantigens, and the maximal response in the indirect pathway develops later (3).

To gain more insight into these alloimmune responses and eventually control them, it is essential to develop reliable *in vitro* assays to monitor the magnitude and specificity of a T cell alloimmune response. Although assays such as the mixed lymphocyte culture (MLC), the cytotoxic T cell precursor (CTLp) assays and HLA antibody screening have shown to

be useful for monitoring transplant recipients, we question in this review the reliability of assays used so far for the detection of indirect allorecognition in clinical transplantation.

ALLORECOGNITION AND ALLOGRAFT REJECTION

Both the direct and indirect pathway of allorecognition can lead to graft rejection. Direct recognition of alloantigens predominates in the first weeks to months after transplantation and is generated by donor-derived antigen presenting cells bearing allogeneic MHC class II molecules. CD4⁺ T cells with exclusively direct allospecificity can mediate allograft rejection (4).

With elapsing time after transplantation, donor APCs fade away and the indirect pathway becomes more important. Evidence that the indirect pathway is sufficient to mediate graft rejection was provided by Auchincloss *et al.* (5), who used MHC class II deficient mice as donors in a skin allograft model. Indirect allorecognition of donor MHC class I antigens by host CD4⁺ T cells could initiate rapid skin rejection. Moreover, allospecific CD4⁺ T cells induced by the indirect pathway were involved in the generation of cytotoxic T cells against donor MHC class I and the induction of IgG alloantibody production (5, 6). The availability of knock out mice and depletion of specific cell subsets enabled the study of individual cell types involved in indirect allorecognition. Combined with results from other experimental studies (2, 7-13), this provides circumstantial evidence that T cells exclusively activated by the indirect pathway are able to mediate acute and chronic allograft rejection.

Many clinical studies in humans support the notion that an increased frequency or reactivity of T cells with indirect anti-donor allospecificity is associated with chronic graft dysfunction or rejection.

INDIRECT ALLORECOGNITION AND TRANSPLANTATION TOLERANCE

Indirect allorecognition can occur throughout the lifetime of a graft, since recipient APCs continuously migrate through the graft and encounter donor-derived peptides. Graft rejection can be caused by indirect allorecognition. However, a protective role of T cells with indirect allospecificity is also likely, as the presence of regulatory T cells with indirect allospecificity is associated with transplantation tolerance. Immunological tolerance involves central and peripheral mechanisms (14). An example of the role of indirect allorecognition in inducing a state of central tolerance is the prolongation of graft survival after intrathy-

mic administration of donor MHC peptides in rodents (15, 16). In the periphery, tolerance can be achieved by various mechanisms, including deletion and regulation of effector cells. Several studies described a state of peripheral tolerance caused by indirect allorecognition in presence of suboptimal stimulation (17-19), negative feedback (20) or regulation (21-24). It is also believed that the beneficial effect of pre-transplant blood transfusions (25-28) is mediated by regulatory T cells with indirect allospecificity (29, 30). Considering the central role of indirect recognition in graft rejection and tolerance, the availability of a reliable *in vitro* assay for monitoring indirect allorecognition is crucial.

IN VITRO ASSAYS TO MONITOR INDIRECT ALLORECOGNITION

Studies that have established key factors for measuring indirect allorecognition *in vitro* are listed in Table 1. They show that *in vitro* allogeneic T cell responses require the presence of APCs, and that CD4⁺ cells recognize an allogeneic peptide in the context of a self-HLA class II molecule (31, 32). T cells described to respond to allogeneic peptides via the indirect pathway recognized synthetic HLA peptides, as well as endogenous peptides (33). Moreover, autologous dendritic cells (DCs) pulsed with cellular fragments were able to trigger T cells with indirect allospecificity (34, 35). Dominant epitopes on common HLA molecules were not limited to the hypervariable region of the HLA molecule, but could also be derived from the α_3 and the transmembrane domains (36). Quantitative analysis showed that T cells with indirect allospecificity have a significantly lower frequency than cells involved in the direct allorecognition pathway (3) and could react by both proliferation and cytotoxicity (37).

As chronic allograft rejection is thought to be mediated mainly by recipient T cells with indirect allospecificity, most *in vitro* assays aiming to detect indirect allorecognition have been performed after solid organ transplantation. As depicted in Table 2, recipient T cell reactivity to donor-like or donor-derived antigens was associated with acute as well as chronic rejection in numerous studies, except for some (38-43). However, in the following sections it will become evident that test conditions vary considerably among studies, while often essential controls are lacking. The different problems will be discussed, supported by our own *in vitro* experiments in order to underline the need to develop a robust and reproducible test system.

Table 1. Publications that have established key factors in indirect allorecognition *in vitro*.

Goal	Priming <i>in vitro</i>	Responders	Alloantigens	Read out	Outcome
Recognition of HLA class II peptides by Th cells (33)	7 day coculture of HLA-DP3 ⁺ PBMC with HLA-DR3 ⁺ synthetic peptides (U6)	TCL/TCC	HLA-DR3 ⁺ synthetic peptides; HLA-DR3 ^{+/+} ; HLA-DP3 ^{+/+} allogeneic PBMC	3 day proliferation in presence of autologous PBMC	Recognition of synthetic HLA-DR3 as well as endogenous, denatured HLA-DR3 in context of HLA-DP3 by TCC
T cell - APC interaction involved in IAR (31)	No	PBMC	Allogeneic PBMC depleted and non depleted of APCs	Presence of IL-2 in supernatant after 7 day proliferation	Allogeneic T cell responses require presence of APCs; only CD4 ⁺ T cells can respond in a self-restricted fashion
Ability of T cells to recognize allogeneic HLA-DR peptides (32)	14 day coculture of HLA-DR1 ^{+/12+} PBMC with HLA-DR1 ⁺ synthetic peptides	T cells	HLA-DR1 ⁺ synthetic peptides	7 day LDA: proliferation in presence of autologous PBMC	Allogeneic HLA-DR peptides are recognized as nominal antigens by CD4 ⁺ T cells; response is self-HLA-DR restricted
Contribution of IAR and DAR to alloreactivity (3)	11 day coculture of DR1 ^{+/12+} PBMC with allogeneic HLA-DR1 ⁺ PBMC	TCL/TCC	HLA-DR1 ⁺ synthetic peptides	3 day proliferation in presence of autologous PBMC	Frequency of T cells involved in IAR is 100-fold lower than in DAR; dominant epitope: residue 21-42 of HLA-DR1, restricted by HLA-DR12
Role of IAR in B cell stimulation (73, 74)	Coculture of HLA-DR7 ^{+/11+} PBMC with HLA-DR4 ⁺ protein or synthetic peptides	TCL	sHLA-DR4 protein; HLA-DR4 ⁺ synthetic peptides	3 day proliferation in presence of autologous HLA-DR1 ⁺ cells; alloantibody production by autologous B cells in RIA	Dominant epitope: residue 69-88 of HLA-DR4; high concentrations of peptide can suppress IAR response; TCL provides specific B cell help
Ability of T cells to mediate IAR (37)	7 day coculture of HLA-DR1 ^{+/DR4+} PBMC with HLA-A1 ⁺ and HLA-B8 ⁺ synthetic peptides	TCL/TCC	HLA-A1 ⁺ and HLA-B8 ⁺ synthetic peptides	3 day proliferation in presence of autologous PBMC; 5hr ⁵¹ Cr-release CTL assay	T cells with IAR specificity are both proliferative and cytolytic

Table 1. Continued.

Goal	Priming <i>in vitro</i>	Responders	Alloantigens	Read out	Outcome
Use of DCs in monitoring IAR (35)	Coculture of T cells with alloantigen pulsed DCs	T cells	HLA-DRI ⁺ ; HLA-DR13 ⁺ synthetic peptides; DCs pulsed with necrotic cells	6 day proliferation; 24 h IFN- γ ELISPOT	DCs can be used to monitor IAR T cells with IAR specificity secrete predominantly Th1 cytokines
Optimal kinetic conditions for IAR (34)	No	T cells	Apoptotic cells; necrotic cells; sonicated cells	5 day MLC: proliferation in presence of autologous DCs; 48 h IFN- γ ELISPOT	It takes 16-20 h for processing, intracellular routing and peptide presentation by DC sonicated cells are more potent than apoptotic cells

APCs: antigen presenting cells, CTL: cytotoxic T lymphocyte, DAR: direct allorecognition, DC: dendritic cell, ELISPOT: enzyme-linked immunosorbent spot, HLA: human leukocyte antigen, IAR: indirect allorecognition, IFN- γ : interferon- γ , IL-2: interleukin-2, LDA: limiting dilution analysis, MLC: mixed lymphocyte culture, PBMC: peripheral blood mononuclear cells, RIA: radioimmunoassay, TCC: T cell clone, TCL: T cell line, Th: T helper.

SOURCE OF ALLOANTIGEN

Indirect allorecognition refers to the recognition of a foreign peptide in the context of an HLA class II molecule on self APCs by recipient CD4⁺ cells. The shedding of soluble HLA molecules during homeostasis or attack on graft tissue during inflammatory processes or other pathological conditions *in vivo* will lead to presence of allogeneic cells or cellular fragments for presentation to the recipients' immune system. They may end up in APCs, transported to the lymph nodes and presented to recipient T cells via the indirect pathway. The strength of this type of alloimmune response is dependent on the amount and source of antigen as well as on the strength and duration of the interaction between recipient APC and T cell. To ensure proper investigation of indirect alloimmune responses, the selection of alloantigens is crucial. Alloantigen can be derived from any polymorphic protein present in the donor and not in the recipient, but in the transplantation setting donor HLA molecules, which can be presented via the direct and indirect way, account for the vigorous alloimmune response. As alloantigen source, one can use donor cells or cellular fragments with the advantage that all possible alloantigens are available for indirect presentation. Alternatively, peptides from alloantigens can be generated synthetically for use in *in vitro* assays. The advantages and disadvantages of different sources of alloantigens will be discussed in more detail.

Allogeneic cells depleted of APCs

Theoretically, depletion of allogeneic APCs will prevent the response of recipient CD4⁺ T cells towards foreign HLA class II molecules, referred to as the direct way of allorecognition. As shown in Table 2a, the stimulation of recipient CD4⁺ T cells with APC-depleted allogeneic peripheral blood mononuclear cells (PBMC) correlated with acute rejection in two studies (44, 45), whereas other studies found no correlation between indirect allorecognition and acute allograft rejection (39, 41, 46). However, it cannot be ruled out that direct allorecognition still interferes in the immune reaction as small numbers of residual donor APCs may remain present. Therefore, the use of cellular fragments lacking intact HLA molecules seems to be a more safe way of alloantigen delivery for indirect recognition.

Cellular fragments

A big advantage of the use of fragments derived from cells of the organ donor is that theoretically the full repertoire of alloantigens is covered. After natural processing, peptides derived from the HLA class I and class II molecules but also from other (minor) transplan-

tation antigens are presented by the recipients' antigen presenting cells. A disadvantage is that the specificity of the alloreactive T cells is not known. Many different techniques have been used for the fragmentation of cells, which makes it difficult to compare results obtained by different studies. Nevertheless, all studies listed in Table 2b found a positive association between patient response to donor derived cellular fragments and acute or chronic allograft rejection (47-52). However, it should be noted that not all studies included proper negative controls such as third party cellular fragments.

Fragmentation of cells or cell membranes has some pitfalls. First, without further analysis, the content of a suspension after fragmentation is unknown with respect to the size of the particles. The suspension may contain whole membrane-derived HLA molecules or much smaller components similar in size to the synthetic peptides used in other experimental designs. Few studies performed Western blot analysis or ELISA to demonstrate the presence of HLA molecules after fragmentation, but even then it is unknown whether the HLA molecules need to be intact in order to be processed and presented by an APC (48,51). When membrane fragments still contain intact HLA molecules and costimulatory molecules, these can cause allorecognition via the direct pathway by patient responder cells. Recipient APCs may also acquire and integrate intact donor-derived HLA molecules, a process called trogocytosis (53). Thus, the absence of cells in the preparation does not warrant absence of direct allorecognition. Second, the concentration of relevant constituents is unknown. A wide range of cellular fragments was used (equivalents of 5×10^4 to 2×10^6 cells), compared to the final concentration of synthetic peptides (10-20 $\mu\text{g/ml}$ per synthetic peptide) used in most studies. The concentration of relevant peptides in a fragmented cell preparation is not known and may be much lower compared to the concentrations usually applied for synthetic peptides.

The availability of many different protocols for fragmentation of cells to obtain donor-derived antigens, underscores the lack of consensus on a robust and reliable test system. To further illustrate this, we tested the reactivity of two CD4^+ T cell clones against APCs with the specific restriction element after incubation with different preparations of the cellular fragments. Clone 4.1 recognizes an HLA-A2 peptide in the context of HLA-DR1. Clone 2014, derived from the ThoU6 cell line (33), recognizes an HLA-DR3 peptide in the context of HLA-DP3. Fragmentation of HLA-A2⁺ and HLA-DR3⁺ cells was performed using methods described by various studies (overview in Table 3). The reactivity of the clones towards natural ligand, synthetic peptides and cellular fragments was determined in proliferation and ELISPOT assays. Both clone 4.1 and clone 2014 recognized the natural ligand and the specific synthetic peptide but none of the cellular fragments (Figure 1).

Table 2. *In vitro* assays measuring indirect allorecognition after solid organ transplantation^a.

Goal	Patients	Responders	Alloantigens	Read out	Outcome
(a) Removal of allogeneic APCs					
<i>In vitro</i> assay predictive for kidney graft rejection (44)	With (n=23) and without (n=19) acute rejection	PBMC	APC-depleted allogeneic PBMC	MLC	Rejection only occurs in patients who retain their self-restricted pathway of alloreactivity (IAR)
Role of IAR after kidney transplantation (45)	With (n=10) and without (n=29) rejection	PBMC	APC-depleted allogeneic PBMC	IL-2 in supernatant after 7 day MLC	Activation of IAR pathway correlates with risk of acute rejection
Role of IAR in heart transplantation (39)	Before and after transplantation	PBMC	APC-depleted donor spleen cells	6 day MLC; IL-2 in supernatant after 3 day proliferation in LDA	No IAR of alloantigens; MLC and LDA are not usable to measure IAR
Role of IAR after kidney transplantation (46)	With (n=2) and without (n=11) chronic rejection	PBMC	APC-depleted allogeneic PBMCs	5 day MLC	IAR of alloantigens is present in patients with and without chronic rejection
Role of IAR after kidney transplantation (41)	With (n=2) and without (n=6) chronic rejection	PBMC	APC-depleted donor or 3P PBMCs	5 day / 9 day MLC	IAR is present in transplanted patients irrespective of rejection
(b) Cellular fragments					
Most effective way of alloantigen delivery for IAR (47)	No	TCC (EL26)	HLA-A2* synthetic peptides (residues 92-120, final conc: 10 µg/ml); HLA-A2* ⁺ frozen/thawed PBMC (equivalent of 5×10 ⁴ cells/well)	3 day proliferation in presence of HLA-DRI 5 ⁺ APCs	Frozen/thawed donor cells are most efficient for alloantigen delivery
Role of IAR in chronic rejection after heart transplantation (47)	With (n=7) and without (n=4) chronic rejection	T cells	Frozen/thawed donor spleen cells; Frozen/thawed 3P antigens; equivalent of 5×10 ⁴ cells/well	3 day LDA; proliferation in presence autologous APCs	Elevated frequencies of donor specific T cells with IAR in patients with chronic rejection

Table 2. Continued.

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of DAR and IAR in chronic rejection after kidney transplantation (48, 52)	With (n=9) and without (n=13) chronic rejection	PBMC	Frozen/thawed donor PBMC	5 day proliferation	Higher T cell reactivity to donor antigens in patients with chronic rejection
Role of DAR and IAR in chronic rejection after lung transplantation (49)	With (n=8) and without (n=11) BOS	T cells	Frozen/thawed donor spleen cells; frozen/thawed 3P antigens; equivalent of 5×10^4 cells/well	Presence of IL-2 in supernatant after 3 day LDA proliferation in presence of autologous APCs	Higher T cell reactivity to donor antigens in patients with BOS
Role of DAR and IAR in acute rejection after heart transplantation (50)	Before, during and after acute rejection (total: n=13)	PBMC	Frozen/thawed donor spleen cells; equivalent of 2×10^5 cells/well	40 h IFN- γ ELISPOT	Higher T cell reactivity to donor antigens during acute rejection ELISPOT: sensitive method to measure DAR and IAR
A non-invasive, immune monitoring tool to measure DAR and IAR after kidney transplantation (51)	With (n=9) and without (n=10) acute rejection	PBMC	Frozen/thawed donor PBMC or spleen cells; frozen/thawed 3P antigens; equivalent of 10^6 cells/well	15 h IFN- γ FCCS in presence of autologous APCs	Higher T cell reactivity to donor antigens in patients with acute rejection; FCCS: clinically useful method to measure DAR and IAR
(c) Synthetic peptides					
Role of IAR in allograft rejection after heart transplantation (54-59)	With and without acute or chronic rejection	PBMC	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 μ M of each peptide	3 day proliferation (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor allo-peptides correlates with acute and chronic rejection; frequency of T cells involved in IAR is 10-50 fold higher in graft than in blood; epitope spreading exists in patients with chronic rejection
		Graft T cells	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 μ M of each peptide	3 day blastogenesis assay after 7 day expansion in presence of autologous APCs	

Table 2. Continued.

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of IAR in acute or chronic rejection after heart/lung transplantation (38)	With acute rejection (n=12) and chronic rejection (n=3)	PBMC	Corresponding to donor and 3P; HLA class I derived; 15-mer peptides; final concentration: 20 µg/ml	4 day proliferation	No response to incompatible donor HLA class I peptides or syngeneic peptides
Role of IAR in acute and chronic rejection after liver transplantation (60-62)	With and without rejection	PBMC	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 µM of each peptide	3 day proliferation (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor allo-peptides correlates with acute and chronic rejection; activation of IAR occurs early after transplantation
A clinically useful assay to study IAR in chronic rejection after kidney transplantation (63)	With (n=16) and without (n=28) chronic rejection	PBMC	Corresponding to donor and 3P; HLA-DRI, -DRI5, -DR3; 20-mer; β -chain hypervariable regions; final concentration: 3.125-100 µg/ml	7 day proliferation; proliferation (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor peptides in patients with chronic rejection; epitope switching occurs in some patients
Quantitate and characterize the IAR in chronic rejection after kidney transplantation (64,65)	One HLA-A2- patient with chronic rejection of HLA-A2 ⁺ kidney allograft	PBMC TCC (EL26)	HLA-A2; residues 57-84, 92-120, 138-170; final conc: 10 µg/ml HLA-A2; residues 57-84, 92-120, 138-170; HLA-A2 analogues with single aa substitutions; final concentration: 10 µg/ml	Presence of IL-2 in supernatant after 3 day proliferation in LDA 3 day proliferation in presence of B-LCLs	T cells with indirect anti-HLA-A2 specificity are present <i>in vivo</i> Recipient APCs are responsible for maintaining these cells <i>in vivo</i> Dominant epitope: residue 92-120 of HLA-A2, restricted by HLA-DR15; altered peptides induce tolerance in T cells with IAR specificity
Role of IAR of HLA class I derived peptides in lung transplantation (66)	With (n=5) and without (n=4) BOS Healthy individuals (n=3)	PBMC	Corresponding to donor and 3P; cocktail of HLA-A1, -A2, -B8, -B44; residue 60-84 (α_1 domain); final concentration: 50 µg/ml	7 day proliferation; 3 day proliferation (after 7 day LDA) in presence of autologous PBMC	Higher proliferative response and precursor frequency of T cells towards donor HLA class I peptides in patients with BOS

Table 2. Continued.

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of IAR after kidney transplantation (42)	Patients with stable graft function (n=10)	T cells	Corresponding to donor; HLA-A2; overlapping 5-15 mer; final concentration: 2.5-10 µg/ml	5 day proliferation in presence of autologous APCs Presence of IL-2 in supernatant	T cell reactivity to donor alloantigen in patients without clinical signs of rejection
Role of IAR of HLA class II derived peptides in lung transplantation (67)	With (n=9) and without (n=9) BOS	PBMC	Corresponding to donor and 3P; cocktail of HLA-DR1, -DR3, -DR15; β -chain hypervariable region; final concentration: 6.25 to 100 µg/ml	7 day proliferation; 3 day proliferation (after 7 day LDA) in presence of autologous PBMC	Higher proliferative response and precursor frequency of T cells towards donor HLA class II peptides in patients with BOS
Role of IAR of HLA-DR peptides before and after kidney transplantation (40)	With (n=18) and without (n=10) acute rejection Healthy individuals (n=20)	PBMC	Corresponding to donor and 3P; HLA-DR; 14-21 mer; final concentration: 50 µg/ml	7 day proliferation Cytokine ELISA of culture supernatant	No association between proliferation and acute rejection; also IAR in healthy individuals; predominant IL-10 production in patients with IAR
A sensitive method to measure IAR in patients after kidney transplantation (68)	Stable (n=12) and high risk (at least one acute rejection episode) patients (n=15)	PBMC	Corresponding to donor and 3P; HLA-DR; final concentration: 10 µg/ml	48 h IL-5, IL-10 or IFN- γ ELISPOT	More IFN- γ producing T cells upon peptide stimulation in high risk patients compared to stable patients; ELISPOT assay is useful for monitoring IAR
Alloreactivity after kidney transplantation (43)	With and without chronic rejection	PBMC	Corresponding to donor; HLA-A, -B, -DR alleles; final concentration: 1-30 µg/ml	24 h IFN- γ ELISPOT	No association between indirect alloreactivity and chronic rejection
Antigenic properties of HLA-A2 derived peptides (36)	Patients on dialysis, awaiting for a kidney transplant	PBMC	HLA-A2; overlapping: 15- or 16-mer; final concentration: 4 or 10 µg/ml	HLA-DR peptide binding assay; 48 h IFN- γ ELISPOT assay	Dominant epitopes: also in α_3 and transmembrane domain Positive anti-HLA-A2 antibody history associated with response to A2 peptides; Also response to peptides identical to self

Table 2. Continued.

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Activity of cells through IAR after kidney transplantation (69)	With (n=8) and without (n=3) acute rejection	PBMC	Corresponding to donor; HLA-DR; I4- to 2I-mer; final concentration: 10-50 µg/ml	7 day proliferation	No association between proliferation and acute rejection
		TCL	Corresponding to donor; HLA-DR; I4- to 2I-mer; 10 µg/ml	Proliferation inhibition ELISA/CBA of culture supernatants; Foxp3 flow cytometric analysis and real-time PCR	Indirect alloreactive TCL produces inflammatory and regulatory cytokines; CD4 ⁺ CD25 ⁺ Foxp3 ⁺ TCL can suppress both IAR and DAR

^aThe listed references in Table 2 are in vitro studies measuring indirect allorecognition in transplanted human individuals corresponding to the following MeSH terms: indirect allorecognition, indirect presentation and alloantigens, indirect recognition and alloantigens, indirect presentation and allogeneic, indirect and alloantigen and rejection, synthetic peptides and rejection, T helper and allogeneic and rejection.

3P: third party, aa: amino acid, APCs: antigen presenting cells, B-LCL: B lymphoblastoid cell line, BOS: bronchiolitis obliterans syndrome, CBA: cytometric bead array, DAR: direct allorecognition, ELISA: enzyme-linked immunosorbent assay, ELISPOT: enzyme-linked immunosorbent spot, FCCs: flow cytometry cytokine secretion assay, HLA: human leukocyte antigen, IAR: indirect allorecognition, IFN- γ : interferon- γ , IL-2: interleukin-2, LDA: limiting dilution analysis, MLC: mixed lymphocyte culture, PBMC: peripheral blood mononuclear cells, PCR: polymerase chain reaction, TCC: T cell clones, TCL: T cell lines.

To exclude that the lack of proliferation of the T cell clones was due to fragmentation induced toxicity, T cell clones and the specific allopeptide were cultured with (supernatants of) cellular fragments. Fragmentation did not result in inhibitory or toxic substances and the response to the specific peptide remained intact (data not shown).

Synthetic peptides

The studies presented in Table 2c used overlapping, synthetic peptides as source of donor antigen (36, 38, 40, 42, 43, 54-69). Both HLA class I and class II peptides corresponding to the donor phenotype, were used to stimulate patient cells. However, it can be assumed that donor-HLA class I-derived peptides are the major inducers of recipient T cell activation leading to chronic allograft rejection, since the expression of HLA class I molecules in the graft is far more abundant than that of HLA class II, especially after donor passenger cells bearing HLA class II molecules have disappeared. In that perspective, it is odd that several studies aim at the detection of a T cell response to synthetic HLA-DR peptides rather than to HLA class I peptides (54-63, 67).

The advantage of using synthetic peptides is the exact knowledge of the antigen (amino acid length and concentration), resulting in a reproducible assay. By varying the amino acid length, immunodominant epitopes were identified for several HLA antigens. However, the indiscriminate dissection of a protein into synthetic peptides has disadvantages, one of them being the creation of new epitopes (neo-epitopes). Peptides may be synthesized which do not occur *in vivo*, due to absence of natural splicing sites at relevant positions on the full protein. On the other hand, when working from the encoded sequence, one does not take into account that several posttranslational modification mechanisms can occur *in vivo*. This includes glycosylation of proteins to improve protein folding and stability of the peptide-HLA complex (70). By exogenously offering 15- to 30-mer synthetic peptides to recipient APCs, they may end up directly in an HLA molecule in unglycosylated form. In addition, posttranslational splicing of peptides has been shown to occur (71). In peptide synthesis that is purely based on amino acid sequence of the full protein the latter two possibilities are simply not considered. Apart from this, and more importantly, a major flaw in many studies is the lack of appropriate peptide controls. To support the results of indirect T cell activation studies, inclusion of control peptides based on self-HLA sequences, seems to be a logical corollary, but is rarely performed.

Half of the studies used only peptides corresponding to the donor phenotype and based their conclusive remarks on the response to the specific peptide. These studies should be considered non-conclusive as responses to synthetic peptides corresponding to self-HLA molecules do occur frequently. Since the adaptive antigen-specific immune system distin-

Table 3. Overview of different methods to fragment cells.

Fragmentation method	PBMC				SALs
	1 (47,50)	2	3 (48,51)	4 ^a	
Cells	20×10 ⁶ PBMC	20×10 ⁶ PBMC	PBMC	PBMC	20×10 ⁶ SALs
Lysis	3× N ₂ /37°C ^b RPMI	3× N ₂ /37°C RPMI	3× N ₂ /37°C Tris-EDTA- based buffer; 1/5000 NP-40	3× N ₂ /37°C in Tris-EDTA- based buffer; 1/8000 NP-40	4× N ₂ /37°C RPMI
Protease inhibitors	–	–	0.1 mM PMSF 1/200 mixture 5 ng/ml SBTI	0.1 mM PMSF 1/800 mixture 5 µg/ml SBTI	–
1 st centrifugation step	–	20 min, 16,000 g, RT	2 min, 1000 g, 4°C	2 min, 2000 g, 4°C	–
Sample	Whole solution	Supernatant	Supernatant	Supernatant	Whole solution ^c
2 nd centrifugation step	–	–	45 min 14,000 g, RT	2 min, 3000 g, 4°C	–
Sample	–	–	Pellet	Supernatant	–
3 rd centrifugation step	–	–	–	60 min, 100,000 g, 4°C	–
Sample	–	–	–	Pellet	–

^aPersonal communication Dr. Maria Hernandez-Fuentes, King's College London, UK.

^bFreezing/thawing cycle in liquid nitrogen and waterbath, respectively.

^cBefore use, cell suspension was filtered to remove clots.

EDTA: ethylenediaminetetraacetic acid, PBMC: peripheral blood mononuclear cells, PMSF: phenylmethylsulphonyl fluoride, RT: room temperature, SALs: single antigen cell lines, SBTI: soybean trypsin inhibitor.

guishes self from non-self, starting with deletion of T cells in the thymus with a high affinity for self-antigens, it is not likely that individuals recognize a natural self peptide presented in the context of their own HLA class II. Figure 2 shows that reactivity was observed to synthetic peptides based on self-HLA molecules using cells from a healthy non-primed individual or umbilical cord blood (UCB). The peptides used here probably differ from naturally processed peptides and are seen as neo-epitopes by the autologous T cells. A logical consequence is that in some cases the reactivity towards synthetic allopeptides is interpreted incorrectly. This type of reactivity is not restricted to T cell responses to synthetic HLA peptides but also accounts for responses towards synthetic RhD peptides in RhD-positive individuals, as tested in our laboratory (data not shown). Our observations are in agreement with others. Hanvesakul *et al.* showed recognition of peptides with a sequence identical to self in patients awaiting a renal transplant, as determined in IFN- γ ELISPOT assay (36). Barker *et al.* described proliferation of naïve T cells of RhD-positive individuals to synthetic RhD peptides and concluded that this was presumably due to cor-

respondence of the peptides to cryptic epitopes normally not presented *in vivo* (72). Two other studies also demonstrated that non-primed T cells are able to respond to allogeneic synthetic peptides (40, 42). Two possibilities arise for the response to self: absence of regulatory mechanisms or induction of neo-epitopes. Natural regulatory mechanisms, which prevent occurrence of an autoimmune response *in vivo*, may be absent in *in vitro* test systems. A more plausible explanation lies in the synthetic character of the peptides. A synthetic peptide does not use the normal route of processing and may end up as a new amino acid sequence in an HLA molecule of an APC. Responder T cells will recognize such

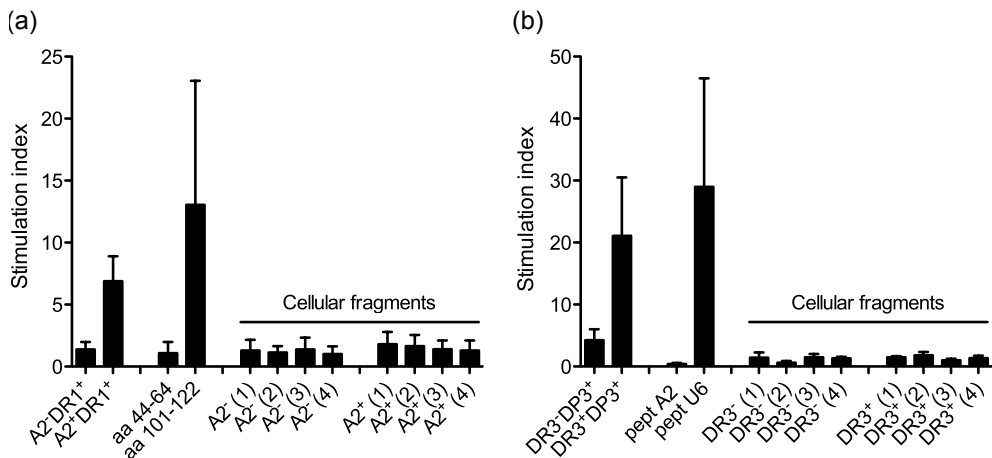


Figure 1. Response of Clone 4.1 (panel a) and Clone 2014 (panel b) towards natural ligand, synthetic peptides and cellular fragments. Clone 4.1 (recognizes HLA-A2 in HLA-DRI) proliferated upon stimulation with natural ligand (A2*DR1⁺ cell) or specific A2⁺ peptide (aa 101-122), but not upon stimulation with A2⁺ or A2⁻ cellular fragments, produced by method 1, 2, 3 or 4 ($n=3$). Clone 2014 (recognizes HLA-DR in HLA-DP3) produced IFN- γ as measured in ELISPOT upon stimulation with natural ligand (DR3*DP3⁺ cell) or specific DR3⁺ peptide (U6), but not upon stimulation with DR3⁺ or DR3⁻ cellular fragments, produced by method 1, 2, 3 or 4 ($n=2$). Stimulation index panel a: counts per minute (CPM) of stimulated clone divided by CPM of unstimulated clone (medium control). Stimulation index panel b: IFN- γ production of stimulated clone divided by IFN- γ production of unstimulated clone (medium control).

a new epitope (neo-epitope) in the context of self HLA class II; however this type of recognition is not necessarily related to indirect recognition of a naturally processed allopeptide. To our opinion, positive tests obtained with synthetic peptides should be confirmed by reactivity against the natural ligand.

EXPERIMENTAL SET UP

Besides the antigenic stimulus, the responder cell population, as well as the experimental setup of an *in vitro* assay, are crucial. The optimal location to detect recipient T cells with indirect allospecificity would be in or near the graft, since T cells migrate towards the graft after they have been triggered by recipient APCs presenting a donor derived peptide. In the graft they are supposed to exert their effector functions, which eventually may cause graft rejection. Although the frequency of T cells with indirect allospecificity during rejection may be 10-50 fold higher in the graft than in the periphery (54), for practical reasons most studies have been performed with PBMC.

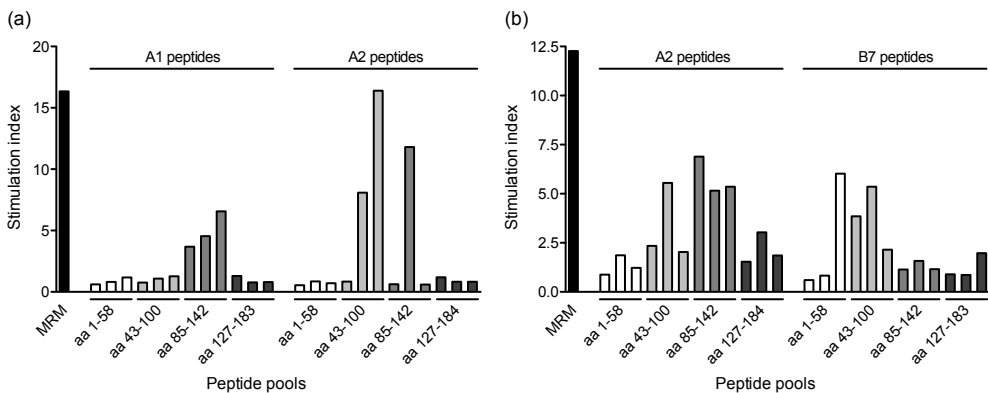


Figure 2. Proliferative response (in triplicate) of healthy control PBMC (panel a) and umbilical cord blood (UCB; panel b) towards foreign synthetic peptides (HLA-A1 and HLA-A2 respectively) and peptides corresponding to self (HLA-A2 and HLA-B7 respectively). HLA type of healthy control: A2, A25, B7, B18, Cw4, Cw12, DRI, DRI5, DQ5, DQ6. HLA type of UCB: A3, A32, B7, B35, Cw4, Cw7, DRI1, DRI5, DQ3, DQ6. Peptides were added in pools of 3 overlapping 30-mer peptides. Proliferation was measured at day 7. MRM: memory recall mix. Stimulation index: counts per minute (CPM) of stimulated cells divided by CPM of unstimulated cells (medium control).

In some studies donor antigens were added to PBMC, by which the encounter of antigen, processing and presentation by autologous APCs occurred in the same environment as presentation to T cells. Only a few studies used a more sophisticated system, in which autologous APCs were purified by T cell depletion, incubation with antigen and finally added to responder T cells (42, 47, 49). To refine the antigen presenting cell population, we performed costaining experiments of fragments of single antigens lines (SALs) and different cell populations (Figure 3). Only CD14⁺ cells (monocytes) showed costaining of PKH26

labeled SAL fragments and may be able to internalize the fragments, process them and finally present them in the context of HLA class II. This allows for selection of the proper APC population and control the ratio between CD14⁺ APCs and T cells. In order to develop and validate a reliable *in vitro* system, standardization and quality controls within the different phases leading to indirect allorecognition are inevitable.

As read out, most studies focused on the capacity of CD4⁺ cells to proliferate upon stimulation, but differ in other test characteristics. Proliferative capacity is often measured upon coculture with synthetic peptides or cell fragments directly or after stimulation in limiting dilution analysis (LDA). LDA quantifies the response by measuring the frequency of alloreactive cells, but involves prolonged *in vitro* culture systems, which may affect estimations of the true *in vivo* frequencies. More recently, ELISPOT and flow cytometric approaches are used to estimate the number and phenotype of alloreactive T cells respectively. The ELISPOT is more sensitive than other techniques, such as MLC and ELISA, but represents a snapshot of the T cell response. Some of the cytokine-producing cells may not proceed

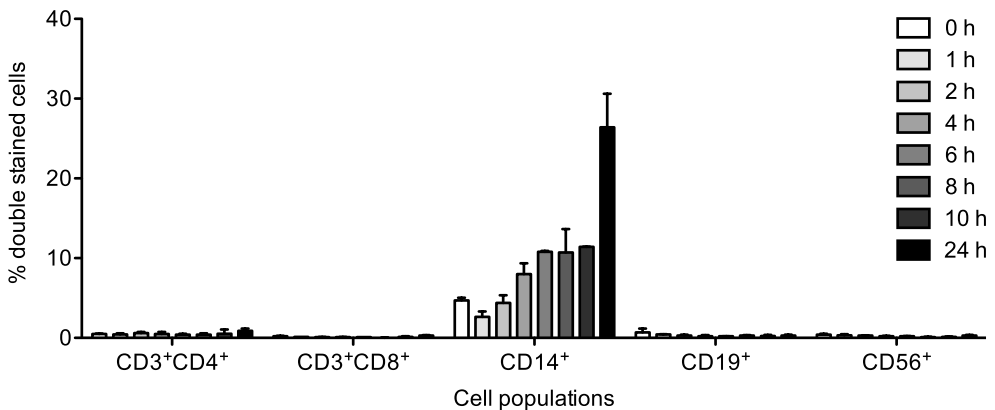


Figure 3. Costaining of SAL fragments on different cell populations. SAL fragments (stained with PKH26) were incubated for various lengths of time with PBMC (stained for different cell populations) ($n=2$). The percentage of double stained cells was determined in flowcytometric analysis.

to proliferation or may undergo activation-induced cell death (68). The flow cytometric approach allows for the individual characterization of cells and the subsequent staining for cytokines to refine the categorization of specific cell subsets, rather than just surface markers. Because a major feature of T cells with indirect allospecificity is their low fre-

quency, a sensitive technique is required. In this respect, the phenotypic characterization of alloreactive T cells has an additional benefit above standard proliferation assays.

When monitoring patients, it is essential to distinguish the response of naïve cells from that of primed donor reactive T cells. To develop an *in vitro* assay able to detect T cells with primed indirect allospecificity, one should focus on the detection of memory cells. Therefore, knowledge about the kinetics of a response is crucial. In our above-described experiment using stained SAL fragments cocultured with stained PBMC, it was possible to monitor the double positive-stained cells at different time points (Figure 3). Within 24 h after coculture, 25% of the CD14⁺ cells showed costaining of SAL fragments. This time span is in agreement with results of a recently performed study in which a time period of 16-20 h was found to be necessary for processing, intracellular routing and peptide presentation by APCs (34). Considering the use of primed responder cells and the uptake and processing of antigen within 1 day, one may question the need for a 7 day incubation period to detect memory cells as is used in most studies. Because cytokine secretion precedes proliferation upon stimulation, incubation of recipient PBMC with donor antigens in ELISPOT was usually much shorter. Nevertheless, a study focusing on the kinetics of a memory is required to shed light on this.

CONCLUDING REMARKS

Various studies claim a relationship between the reactivity of T cells with indirect allospecificity and graft rejection. However, many different approaches are used and often, essential controls are lacking. Hardly any study confirms the presence of indirect recognition using different approaches including the natural ligand. This is essential as all assays used so far clearly have disadvantages and pitfalls.

The use of donor cells for fragmentation has our preference as antigen source for *in vitro* assays because they contain the full HLA phenotype. However, no method to fragment cells has emerged as optimal, which is emphasized by the lack of consensus. Additionally, one should include control experiments when using fragmented cells to exclude recognition of alloantigens via the direct pathway. The exact knowledge of the alloantigen when using synthetic peptides is overshadowed by the ability to create neo-epitopes. This turned out to be a big obstacle in defining a specific response and requires at least the use of control peptides. Besides different antigenic sources, additional differences exist in test characteristics between studies in which most of them do not distinguish a naïve response from a memory response. To monitor the low frequency of T cells with indirect

allospecificity *in vitro*, it is essential to determine the kinetics and optimal conditions to detect a memory response.

Detailed knowledge about the indirect allorecognition is required and it would be the right time to set up an international workshop to establish and validate a proper and robust assay to measure T cells with indirect allospecificity.

MATERIALS AND METHODS

Subjects and sample collection

PBMC from heparinized peripheral blood from healthy individuals or mononuclear cells from umbilical cord blood (UCB) were isolated by standard Ficoll density-gradient centrifugation. PBMC were used immediately as responder cells in proliferation assays or were cryopreserved in RPMI 1640 (Gibco, Paisley, UK) with 20% fetal calf serum (FCS, Gibco) and 10% dimethyl sulfoxid (DMSO, Fluka, Buchs, Switzerland). Cells were frozen at -70°C and stored in liquid nitrogen until further use.

T cell clones

The T cell line ThoU6 was obtained as described before (33). In brief, PBMC of individual Tho (DPBI*0301⁺, DRBI*0301⁻) were primed in the presence of an HLA-DR3 peptide (aa 67-85: LLEQKRGRVDNYCRHNYGV) in RPMI 1640 supplemented with 15% pooled human serum and gentamycin. After 7 days, the cultures were collected and restimulated with irradiated autologous feeder cells at a ratio of 10:1 and synthetic HLA-DR3 peptide. The line was cloned by limiting dilution in the presence of T cell growth factor (TCGF, Biotest, Germany) and irradiated autologous feeder cells. The CD4⁺ clone 2014 was kept in culture by weekly stimulation with irradiated allogeneic PBMC with IL-2 in RPMI 1640 supplemented with 10% pooled human serum.

The CD4⁺ T cell clone 4.I recognizes an HLA-A2 peptide (aa 101-122: CDVGSDWRFLR-GYHQYAYDGKD) presented in HLA-DRI. The clone was maintained in culture by weekly stimulation with irradiated allogeneic PBMC and allogeneic B cells, PHA and IL-2 in IMDM (Gibco) with 10% pooled human serum. Both T cell clones were used in functional assays between 1 and 2 weeks after restimulation.

Synthetic peptides

Synthetic 20-mer overlapping peptides corresponding to residues 45-66 and 101-122 of α_1 and α_2 domain from HLA-A2 alleles and synthetic 30-mer overlapping peptides represent-

ing the α_1 and α_2 domain of HLA-A1, -A2, -B7 and -B8 were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II; MultiSynTech, Witten, Germany). The purity of the peptides was determined by analytical reversed-phase high performance liquid chromatography (HPLC).

Cellular fragments

Fresh or thawed PBMC or SALs were used for fragment preparation. Fragmentation of PBMC was performed using 4 different methods:

- Method 1: PBMC were lysed by three cycles of freezing (liquid N₂) and thawing (waterbath: 37°C) in RPMI 1640 complete medium at a concentration of 2×10⁷ cells/ml. The absence of whole intact cells was confirmed by microscopy (47, 50).
- Method 2: PBMC were lysed by three cycles of freezing (liquid N₂) and thawing (waterbath: 37°C) in RPMI 1640 complete medium at a concentration of 2×10⁷ cells/ml, centrifuged at 16.000 g for 20 min and the supernatant was collected.
- Method 3: PBMC were lysed by three cycles of freezing (liquid N₂) and thawing (waterbath: 37°C) in a Tris-EDTA-based buffer containing 1/5000 NP-40 (Fluka, 74385), 0.1mM PMSF (Sigma P7626; Buchs, Switzerland), 1/200 protease inhibitor mixture (Sigma, P8340) and 5 ng/ml soybean trypsin inhibitor (Sigma, T6522), centrifuged at 1000 g for 2 min, and the supernatant was collected and further centrifuged at 14.000 g for 45 min at 4°C (48, 51).
- Method 4: PBMC were lysed by three cycles of freezing (liquid N₂) and thawing (waterbath: 37°C) in a Tris-EDTA-based buffer containing 1/8000 NP-40 (Fluka, 74385), 0.1mM phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma, Buchs, Switzerland), 1/800 protease inhibitor mixture (Sigma, 8340) and 5 µg/ml soybean trypsin inhibitor (Sigma, T6522), centrifuged at 2000 g for 2 min (4°C), followed by a centrifugation step of the supernatant at 3000 g for 2 min (4°C). Supernatant was harvested and further centrifuged at 100.000 g for 60 min (4°C) in the buffer described above. The pellet was resuspended in 10-20 µl of solubilising solution: urea (Sigma, U0631), 2% CHAPS (Calbiochem, San Diego, CA, USA) and protease inhibitor cocktail (Roche 1836153; Basel, Switzerland) (personal communication Dr. Maria Hernandez-Fuentes, King's College London, UK).

Fragmentation preparations were tested for protein concentrations using BCA protein assay kit (Pierce, Rockford, IL, USA) and stored at -70°C until further use.

Fragmentation of SALs was similar to method 1, but one additional round of freeze/thawing was performed. Before use, the cell suspension was filtrated through a 70 µm cell

strainer (Falcon; BD Bioscience, Bedford, MA, USA) to remove cell cloths.

Proliferation assays: healthy control and UCB

A total of 2×10^5 PBMC of healthy individuals or mononuclear UCB cells were incubated with a pool of three synthetic HLA-derived peptides at a final concentration of 10 $\mu\text{g/ml}$ per peptide. As a positive control a memory recall mix (MRM) was used containing tetanus toxoid (SVM, Bilthoven, the Netherlands), mycobacterium tuberculosis sonicate (Statens Serum Institute, Copenhagen, Denmark) and Candida albicans (HAL Allergy, Haarlem, the Netherlands). The cells were plated out in round-bottom 96-well plates (Costar, Cambridge, MA, USA) in a total volume of 200 μl of RPMI 1640 complete medium supplemented with 5% autologous serum. After 6 days, wells were pulsed with 1 μCi of $^3\text{H-TdR}$ (Amersham International, Amersham, UK) and the cultures were harvested onto glass fiber filters 18 h later. Proliferation was measured by $^3\text{H-TdR}$ incorporation using a beta-plate counter (Wallac, Turku, Finland).

Proliferation assay: clone 4.1

A total of 10^5 irradiated (30 Gy) A2:DRI^+ PBMC were pulsed overnight with A2^+ synthetic peptides at a final concentration of 10 $\mu\text{g/ml}$ per peptide or $\text{A2}^{+/-}$ cellular fragments at a final concentration of 1 $\mu\text{g/well}$ in IMDM complete medium with 5% pooled human serum. Also, intact A2:DRI^+ , $\text{A2}^+\text{DRI}^+$ or A2:DRI^- cells were kept overnight in Iscove's medium. T cell clone 4.1, which was kept overnight at 37°C in Iscove's medium containing 100 U/ml IL-2, was then added at 5×10^3 cells per well. After 48 h, wells were pulsed with 1 μCi of $^3\text{H-TdR}$ and proliferation was measured as described above.

Enzyme linked immunospot (ELISPOT) assay: clone 2014

A total of 10^5 irradiated (30 Gy) DR3:DP3^+ PBMC were pulsed overnight with DR3^+ synthetic peptides at a final concentration of 10 $\mu\text{g/ml}$ per peptide or cellular fragments at a final concentration of 1 $\mu\text{g/well}$ in IMDM with 5% pooled human serum. T cell clone 2014, which was kept overnight at 37°C in Iscove's medium containing 20 U/ml IL-2, was then added at 5×10^3 cells per well, centrifuged for a short time to concentrate the cells and stored for 2 h at 37°C . Then, cells were harvested and transferred to an ELISPOT plate (MAHA S45 10, Millipore, Amsterdam, the Netherlands) coated with a mouse anti-human IFN- γ mAb (Mab 1-DIK; Mabtech, Nacka, Sweden) followed by blocking for 1 h at 37°C with RPMI 1640 containing 5% pooled human serum. Cells were incubated in the ELISPOT plate for 24 hr at 4°C to allow spot formation. Plates were washed with PBS supplemented with Tween20 and an IFN- γ detection antibody (Mab 7-B6-1-biotin; Mabtech, Nacka,

Sweden) was added for 2 h at room temperature (RT). The conjugate (extravidin alkaline phosphatase, Sigma E2636) was added for 1 h at RT, followed by a washing step and addition of the substrate (BCIP-NBT tablet, Sigma B5655) for 5 min at RT. The reaction was stopped with tap water. The resulting spots were counted using a Bioreader 3000 Pro (BioSys, Karben, Germany).

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Chapter 8



Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

Organ transplantation is a life saving procedure for many end-stage diseases. Generally, short-term transplantation outcome is excellent, mainly due to progress that has been made within the last 50 years. The discovery of HLA in the late 1950s (1-3) has allowed for the development of tissue typing techniques to ensure the best possible match between donor and recipient (4). Particularly in the era where powerful immunosuppressive drugs were not yet available, the impact of matching for HLA proved to be highly significant (5), and facilitated the establishment of clinical organ transplantation.

Introduction, in the late 1960s, of the serologic cross-match technique for the detection of pre-transplant donor specific antibodies has had tremendous impact on short term graft survival, since hyper-acute rejection could now be prevented (6). The serologic cross-match technique involves *in vitro* incubation of donor cells and patient serum to screen for pre-existent humoral immune reactivity towards the graft *in vivo*. A positive cross-match remains to this day, at least in most European transplant centers, a contra-indication for transplantation.

Finally, the introduction of the potent immunosuppressive drug cyclosporin in the early 1980s was instrumental for the prevention of acute rejection (7) and opened a new era in organ transplantation in which the discovery and development of new immunosuppressive drugs became an important field of investigation.

In contrast to the vast improvement of one-year graft survival rates in the last decades, long-term graft survival rates have made little progress (8). For the most part this is due to chronic organ failure, a multifactorial process caused by both immunological and non-immunological damage to the graft (9). Distinct from acute rejection, the immunological damage resulting in chronic graft failure affects the transplanted organ in a slow, continuous fashion and appears to be relatively insensitive to current immunosuppressive drugs (10). It is important to understand the mechanism of the chronic rejection process to be able to interfere with it. The humoral immune response appears to be a major factor contributing to chronic rejection, since development of anti-HLA antibodies regularly precedes chronic rejection (11-13) and C4d staining revealed a strong correlation between chronic rejection and humoral immunity (14).

Maintenance immunosuppressive drugs

Since the majority of immunosuppressive drugs do not specifically target the humoral immune system, humoral rejection episodes have proven to be difficult to treat, leading to a significantly reduced long-term graft survival (15). Nonetheless, maintenance immunosup-

pressive drugs are likely to have an effect on B cells due to similarities between activation pathways of lymphocyte subsets (16). Moreover, inhibition of T cell help may lead to a diminished humoral allo-immune reactivity. Therefore, the composition of the maintenance immunosuppressive regimen may affect the susceptibility to, and outcome of, humoral rejection episodes.

We studied the direct effect of four immunosuppressive drugs, widely used as maintenance immunosuppressive therapy (tacrolimus, cyclosporin, MPA and rapamycin), on *in vitro* humoral immune responses in great detail. Data presented in **chapter 2** show that not all immunosuppressive drugs are equally capable of directly inhibiting B cell responses. Purified B cells were polyclonally activated with stimuli of various strength. MPA and rapamycin completely inhibited immunoglobulin production, even after strong B cell stimulation, in contrast to the calcineurin inhibitors cyclosporin and tacrolimus, whose effect was only marginal and dependent on the strength of B cell activation. Addition of MPA and rapamycin to already activated B cells resulted in strongly decreased immunoglobulin levels, whereas calcineurin inhibitors were ineffective at this stage. MPA and rapamycin inhibited B cell proliferation and induced B cell apoptosis. Moreover, rapamycin inhibited the number of IgM and IgG producing cells. From this chapter, we conclude that MPA and rapamycin are superior in directly inhibiting B cell responses, and may therefore be preferred to treat humoral rejection.

To establish whether calcineurin inhibitors can inhibit humoral immune responses indirectly, we investigated their effect on T cell help. **Chapter 3** describes the effect of tacrolimus, cyclosporin, MPA and rapamycin on various aspects of T cell help as well as *in vitro* humoral immune responses consisting of B cells stimulated by helper T cells. To address the effect of immunosuppressive drugs on T cell help, we polyclonally activated T cells in the absence and presence of immunosuppressive drugs and measured proliferation, expression of general T cell activation markers, as well as T cell help in the form of costimulation and cytokines. Calcineurin inhibitors mainly affected the levels of T cell costimulatory molecule expression and cytokines, whereas MPA and rapamycin strongly inhibited T cell proliferation in addition to the attenuation of costimulatory molecule expression. Rapamycin affected the production of multiple B cell stimulatory cytokines, whereas MPA did not affect cytokines. Subsequently, in T cell dependent B cell cultures, all immunosuppressive drugs tested were capable of completely inhibiting immunoglobulin production. From these data we conclude that all immunosuppressive drugs are capable of inhibiting T cell help, albeit to different extents. We confirmed that calcineurin inhibitors affect B cell immunoglobulin production by interfering with T cell help in a culture system using already activated T cells to stimulate B cells.

Since the relative contributions of T cell proliferation, costimulation and cytokines to B cell activation in this culture system are not known, we are unable to conclude that one of the drugs is superior in inhibiting T cell help towards B cells. Nonetheless, among the drugs we have tested, rapamycin had the broadest range of effects on T cell help, suggesting that this drug is most potent in inhibiting T cell dependent B cell responses. Figure 1 provides a simplified overview of the action of the immunosuppressive drugs described in this thesis.

It is highly unlikely that the problem of humoral rejection will be solved using the current standard immunosuppressive drugs. However, until an effective therapy becomes available, the impact of standard immunosuppressive drugs on humoral immunity remains important. Based on our data, we hypothesize that the choice of immunosuppressive drugs included in the maintenance immunosuppressive protocol affects the propensity of a patient to develop humoral rejection and may also determine the outcome of the rejection. From our data, it is evident that MPA and, even more clearly, rapamycin are superior in inhibiting humoral immune responses, and thus may be the drugs of choice in maintenance immunosuppressive protocols.

Experimental immunosuppressive protocols

In addition to standard immunosuppressive drugs, many experimental immunosuppressive protocols are used in efforts to treat humoral rejection, including high dose IVIg, Rituximab and plasmapheresis (17-19), as well as bortezomib (20, 21). IVIg has been widely used for treatment of humoral immunodeficiency and autoimmune diseases, and its use in transplantation is on the rise. However, the mechanism of action of IVIg, especially in the setting of organ transplantation, has not been fully elucidated. Therefore, in studies described in **chapter 4**, we questioned whether IVIg affected the activation of highly purified B cells. We stimulated B cells with recombinant stimulatory agents, resulting in the induction of proliferation and immunoglobulin production. Unexpectedly, we found that IVIg (in concentrations up to 35 mg/ml) failed to affect B cell proliferation. In contrast, IVIg did inhibit the proliferation of mitogen activated T cells, warranting that the IVIg solution was effective. Additionally, we tested whether IVIg altered immunoglobulin production by addressing the mRNA levels of IgM and IgG. Similar to the lack of effects on B cell proliferation, IVIg did not alter immunoglobulin mRNA levels.

Since IVIg has been described to inhibit humoral immune responses *in vivo*, we hypothesize that IVIg interferes with T cell help. This hypothesis is supported by our data on IVIg-induced inhibition of mitogen-induced T cell proliferation, as well as data from others showing inhibition of T cell activation and proliferation by IVIg (22, 23). Besides effects of

IVIg on cells involved in humoral immune responses, IVIg may also influence humoral immune responses by interfering with the complement system (24, 25), or by anti-idiotypic interactions with pathogenic antibodies (26). However, the contribution of these mechanisms to the inhibition of humoral allo-immunity remains unknown and requires further investigation.

To be able to address the question whether IVIg has an effect on humoral rejection, *in vivo* studies will be extremely informative. However, performing studies in which transplant patients are randomized between IVIg therapy and placebo will raise ethical concerns. On the other hand, testing the effect of a human plasma product on the immune system of laboratory animals will likely not provide relevant information for the human situation. Instead, the use of humanised mouse models, in which human tissues can be transplanted, may allow investigating the effect of IVIg on the human immune system *in vivo*.

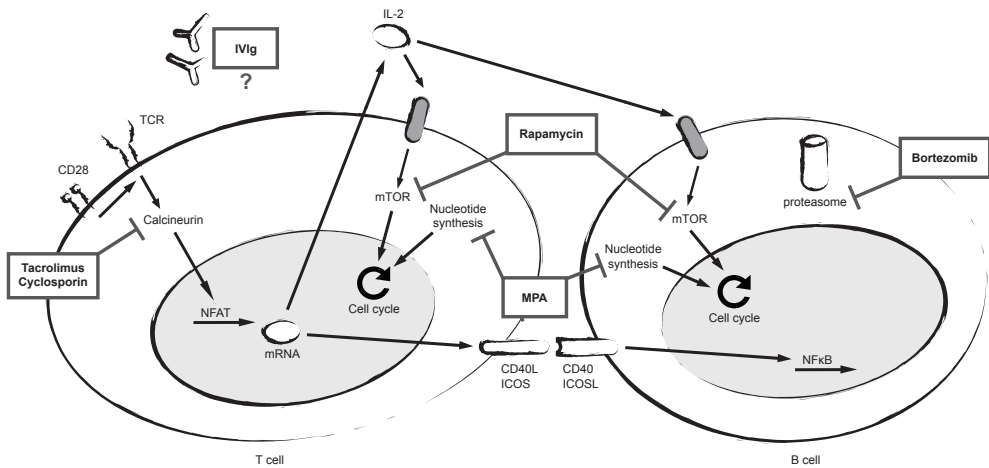


Figure 1. Simplified scheme of the effects of immunosuppressive agents on T cell dependent B cell activation. The calcineurin inhibitors cyclosporin and tacrolimus potentially inhibit T cell help towards B cells by interfering with T cell proliferation, cytokine production and costimulatory molecule expression, but lack direct effects on B cells. Both MPA and rapamycin inhibit T cell help towards B cells as well as B cell function directly. MPA and rapamycin inhibit T cell proliferation and costimulatory molecule expression, with rapamycin also inhibiting T cell cytokines. Both drugs inhibit B cell proliferation and induce B cell apoptosis. Additionally, rapamycin directly inhibits B cell antibody production. IVIg does not directly inhibit B cells when tested in a T cell free culture system. However, IVIg directly inhibits T cell proliferation by a mechanism yet to be determined. Bortezomib inhibits B cell proliferation and antibody production when tested in a culture system lacking T cells. We hypothesize that, in analogy with its effect on plasma cells, Bortezomib affects B cells by inhibition of the proteasome.

To prevent development of novel HLA antibodies after transplantation, B cells can be depleted from the circulation. Since B cells are also potent in activating T cells by antigen presentation (27), an additional advantage of B cell depletion is the removal of this important source of antigen presenting cells. The commonly used chimeric anti-CD20 mAb Rituximab leads to profound B cell depletion, which lasts over 6 months (28). Several studies have shown beneficial effects of Rituximab on graft survival (18, 19), stressing the important role of B cells in rejection. In addition to Rituximab, novel B cell depletion strategies are under investigation in the field of autoimmunity, such as anti-CD79 mAb, which ameliorates murine auto-immune disease (29) and epratuzumab (an anti-CD22 mAb), which, besides B cell depletion, affects B cell activation in SLE patients (30).

Unfortunately, Rituximab treatment appears not to be effective in all instances, which is illustrated by reports on the failure of Rituximab to prevent humoral rejection (31, 32). Most likely, this is caused by ongoing antibody production by plasma cells. Bone marrow residing plasma cells are responsible for the preponderance of antibodies present in the circulation, but are not affected by Rituximab treatment, since they lack CD20 expression. This is evidenced by unaltered normal serum immunoglobulin concentrations during Rituximab induced B cell depletion (33). Alternative strategies, such as IVIg or ATG administration and plasmapheresis, also leave plasma cells unaffected (34, 35). Lack of inhibition of plasma cell activity presents a major impediment to the treatment of humoral rejection. Recently, the proteasome inhibitor bortezomib, originally designed for the treatment of multiple myeloma, has been described as an effective therapy for both humoral and cellular rejection in a study involving a small patient cohort (20). Additionally, HLA antibody levels in non-rejecting patients were attenuated (21). Immunosuppressive effects of bortezomib are caused by induction of plasma cell apoptosis (35). However, it is to be expected that induction of apoptosis by proteasome inhibition is a general effect on cells with high protein turnover. Therefore, we have investigated the effect of bortezomib on activated B cells, as described in **chapter 5**. Bortezomib potently inhibited B cell proliferation and immunoglobulin levels. The dose at which bortezomib exerted maximal effects was approximately a 1000-fold lower than described for plasma cells (35), suggesting that B cells are even more susceptible to the effect of bortezomib than plasma cells. The mechanism of action of bortezomib towards human B cells has yet to be defined, although it is likely that the effects are caused by the induction of apoptosis, analogous to plasma cells. Induction of B cell apoptosis has already been demonstrated in murine B cells (36).

Preliminary results on the use of bortezomib are promising. The finding that human B cells are affected by bortezomib suggests that the use of additional B cell specific drugs, such as Rituximab, may not be necessary. Monitoring of B cell numbers and subsets in transplant

patients receiving bortezomib therapy will provide more insight on whether B cells are sufficiently affected to make B cell specific therapy superfluous.

However, due to the apparent lack of specificity, side effects may be expected. Large multicenter studies will be needed to determine whether bortezomib is truly the potent anti-humoral rejection drug it appears to be and what side effects can be expected in transplant patients.

Monitoring the immune system

Once an organ has been transplanted, monitoring of the alloimmune response is of great importance to tailor immunosuppressive drugs for the minimization of side effects. Historically, the humoral alloimmune response is monitored by quantification of the level of donor-specific serum antibodies. However, there are several reasons why the detection of serum alloantibodies may not be adequate. Firstly, alloantibodies may not be detected in the periphery because of antibody adsorption by the graft, as has been demonstrated by studies on serum alloantibody negative patients who experienced graft failure. In these patients, only after transplantectomy serum alloantibodies were detectable, indicating antibody adsorption by the graft (37, 38). Secondly, at the time that alloantibodies are detected in the circulation, the humoral immune response is already ongoing, and intervention is likely too late to prevent organ damage. Finally, when only antibody levels are measured, the proportion of the B cell clones capable of producing the alloantibodies or differentiating into plasma cells remains unknown.

To get around these problems, we developed a novel ELISPOT technique to quantify the number of B cells capable of producing IgG antibodies against defined HLA molecules. Development of this ELISPOT assay, which is described in **chapter 6**, was possible by using recombinant monomeric HLA molecules as target molecules for the HLA antibodies to bind. We determined the frequency of HLA antibody producing B cells of several HLA-immunized individuals and found frequencies of 0 to 182 HLA-specific B cells per million B cells. In contrast, B cells from non-immunized controls and from individuals whose HLA type corresponded to the HLA of the test did not form spots. Moreover, we were able to detect HLA-specific B cells in a patient who had developed HLA antibodies due to allograft rejection.

We hypothesize that the production of alloantibodies of certain specificity will be preceded by an increase of B cells clones of the same specificity. Therefore, by monitoring the frequency of HLA-specific B cells longitudinally, we may be able to detect a humoral allo-immune response in the early phase. Whether the increase of peripheral HLA-specific B cells will actually precede the formation of antibodies may be subject of future investiga-

tions in which samples from transplant patients, who developed humoral rejection, will be retrospectively tested for their HLA-specific B cell load. Once these studies have been performed, the HLA-specific B cell ELISPOT may be added to the current repertoire of techniques to monitor transplant patients.

The HLA-specific B cell ELISPOT may also be used to determine the effect of immunosuppressive drugs on HLA-specific B cells. However, the present culture method prior to the ELISPOT assay is based on artificial T cell help by irradiated L-CD40L cells. This precludes studies on the effect of immunosuppressive drugs on the frequency of HLA-specific B cells, since immunosuppressive drugs may act on L-CD40L cells. Further development of the assay to eliminate L-CD40L cells from the pre-culture may render the assay suitable for the investigation of immunosuppressive drugs.

The previously mentioned T cell help is pivotal for B cells to differentiate into memory B cells or IgG producing plasma cells. T cell help is, by definition, provided by CD4⁺ T cells. Early after transplantation, CD4⁺ T cells will predominantly recognize foreign HLA directly on the cell surface of donor DCs. In contrast, late after transplantation, when donor DCs are no longer present, CD4⁺ T cells will recognize allogeneic structures as peptides in the context of self-MHC, a pathway known as the indirect pathway of allorecognition. To be able to predict late transplant outcome and monitor the chronic alloimmune response, proper techniques are required to quantify T cells capable of indirectly recognizing donor HLA. **Chapter 7** summarizes the current literature describing assays to quantify the indirect allo-recognition, accompanied by data from our own laboratory showing the pitfalls of such techniques. Many papers cited in this chapter lack proper controls, rendering interpretation of the data extremely difficult. Our own data underscore the necessity of proper control samples and the difficulty to define and implement such controls.

Selection of the source of alloantigen to test indirect alloreactivity is critical and potentially induces errors. A major problem in assays using cell lysates to detect alloreactivity is the inability to formally exclude any direct alloreactivity. Detailed analysis of cell lysates by, for example, mass-spectrometry may be required to ensure no intact HLA molecules are present in the lysate, ruling out direct alloreactivity. Alternatively, when using synthetic peptides, the induction of reactivity against neo-epitopes cannot be excluded, which is a problem that is extremely difficult to overcome. Reactivity towards peptides should be validated by using other sources of antigen, which is seldomly done. We conclude from this systematic literature scan that, to date, there is no reliable assay to determine the human T cell repertoire with indirect allospecificity. However, development of reliable assays to monitor T cells that indirectly recognize donor HLA remains worthwhile to pursue.

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Chapter 9



Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

De natuurlijke afweer van het lichaam wordt verzorgd door het immuunsysteem. Zonder immuunsysteem zouden pathogenen zoals virussen en bacteriën vrij spel hebben. Het immuunsysteem is onder te verdelen in twee onderdelen, de aangeboren immuniteit en de verworven immuniteit. De aangeboren immuniteit bestaat onder andere uit fysieke barrières zoals de huid en de slijmvliezen. Ook zijn er een aantal celtypen en moleculen die op een niet-specifieke manier (onderdelen) van pathogenen kunnen herkennen en deze kunnen neutraliseren.

De verworven immuniteit wordt, zoals de naam al doet vermoeden, opgebouwd naarmate het lichaam in aanraking komt met pathogenen. Cellen uit deze tak van het immuunsysteem hebben specifieke receptoren op hun celoppervlak waarmee ze pathogenen kunnen herkennen. Deze cellen zijn ook in staat geheugen op te bouwen zodat bij een tweede aanval van hetzelfde pathogeen er sneller actie ondernomen kan worden. Het verworven immuunsysteem bestaat ruwweg uit twee celtypen; de T cellen die zorgen voor cellulaire immuniteit en de B cellen die zorgen voor de humorale immuniteit. Het cellulaire immuunsysteem surveilleert voor pathogenen binnenin cellen, waarbij geïnfecteerde cellen door T cellen opgeruimd worden. Lichaamsvreemde eiwitten worden hiervoor door de geïnfecteerde cel op speciale receptoren gepresenteerd. Het humorale immuunsysteem houdt de ruimte tussen cellen vrij van pathogenen, voornamelijk door het maken van antistoffen. Dit zijn moleculen die binden aan pathogenen, welke vervolgens op verschillende manieren kunnen worden vernietigd. De twee armen van het verworven immuunsysteem zijn nauw met elkaar verbonden. Zo hebben de meeste B cellen T cellen nodig om volledig geactiveerd te raken, de zogenaamde T cel hulp, en kunnen B cellen T cel activatie bevorderen door lichaamsvreemde eiwitten aan T cellen aan te bieden.

Het immuunsysteem beschouwt een getransplanteerd orgaan als lichaamsvreemd (behalve wanneer het een orgaan van een ééneiige tweeling betreft) en zal daarom tot de aanval overgaan, wat kan leiden tot afstoting van het transplantaat. Afstoting van een getransplanteerd orgaan is dus eigenlijk een normale reactie van het lichaam op een 'pathogeen'. Als niet de juiste selectiecriteria voor donoren en voorzorgsmaatregelen in de vorm van medicatie zouden worden genomen, zouden de meeste getransplanteerde organen door het immuunsysteem van de ontvanger worden afgestoten.

Sinds het begin van de orgaantransplantatie is door onderzoek grote vooruitgang geboekt op de korte termijn transplantaat overleving. Ten eerste is de ontdekking van weefsel groepen (de HLA moleculen), die bij ieder uniek individu verschillend zijn, zeer belangrijk

geweest voor het selecteren van de juiste donor. Deze ontdekking heeft de basis gevormd voor de weefseltypering. Zonder juiste medicatie veroorzaakt een verschil in de opmaak van HLA moleculen tussen donor en ontvanger vrijwel zeker afstoting van het getransplanteerde orgaan.

Als tweede mijlpaal in de transplantatie immunologie is de ontwikkeling van de zogenaamde kruisproef van groot belang geweest om versnelde, hyperacute afstoting te voorkomen. Bij deze techniek wordt serum van de potentiële ontvanger in contact gebracht met cellen van de donor. Als de patiënt reeds antistoffen in het serum heeft die reageren tegen de donor zullen in deze test de donorcellen vernietigd worden. Dergelijke antistoffen kunnen in de patiënt reeds aanwezig zijn door zwangerschap (bloed uitwisseling met het ongeboren kind), bloedtransfusies of eerdere transplantaties. Een positieve kruisproef is een contra-indicatie voor transplantatie.

Ten slotte heeft de introductie van sterke immunosuppressieve medicatie een groot effect gehad op de uitkomst van transplantaties. Door deze medicatie werd het mogelijk organen van (gedeeltelijk) niet gematchte donoren toch succesvol te transplanteren. De huidige immunosuppressieve medicatie werkt vooral op de cellulaire arm van het immuunsysteem. Afstoting kort na transplantatie kan met deze medicatie goed voorkomen of, wanneer nodig, behandeld worden. Helaas blijkt het gunstige effect van deze medicatie alleen de korte termijn overleving van het transplantaat ten goede te komen.

Hoewel de korte termijn transplantaat overleving door verschillende ontdekkingen dus zeer is verbeterd, is op het gebied van de lange termijn overleving nauwelijks vooruitgang geboekt. Afstoting veroorzaakt door het humorale immuunsysteem lijkt hierbij een belangrijke rol te spelen. Deze manier van afstoting is jarenlang overschaduwd door afstoting veroorzaakt door het cellulaire immuunsysteem. Dankzij de krachtige onderdrukking van de cellulaire immuniteit met immunosuppressieve medicatie wordt meer en meer de rol van de humorale immuniteit duidelijk. Het exacte mechanisme van humorale afstoting is niet volledig bekend, evenals de beste manier van behandeling. Dit proefschrift beschrijft verschillende aspecten van humorale immuniteit in relatie tot orgaantransplantatie met als doel meer inzicht te krijgen in de behandeling en monitoring van humorale afstoting. Allereerst wordt in hoofdstuk 1 een algemene inleiding gegeven om de verschillende hoofdstukken van dit proefschrift in de juiste context te plaatsen. Tevens wordt het doel van de beschreven studies duidelijk gemaakt. Vervolgens beschrijft het eerste deel van dit proefschrift het effect van verschillende immunosuppressieve medicatie op humorale immuniteit. **Hoofdstuk 2** beschrijft het effect van vier verschillende immunosuppressieve medicijnen (tacrolimus, cyclosporine, MPA en rapamycine) op gezuiverde B cellen in een

kweekstelsel met B cel stimulatie via activerende reagentia met als doel het directe effect van immunosuppressieve medicijnen op B cellen te onderzoeken. In dit kweekstelsel zijn alle immunosuppressieve effecten te herleiden tot directe effecten op B cellen. De resultaten beschreven in dit hoofdstuk laten duidelijk zien dat tacrolimus en cyclosporine (de zogenaamde calcineurine remmers) een marginaal direct effect hebben op B cellen, terwijl MPA en rapamycine sterke remmende eigenschappen hebben.

In **hoofdstuk 3** hebben we gebruik gemaakt van B cellen die op een T cel afhankelijke manier zijn gekweekt. Hierbij is gekeken naar het effect van immunosuppressieve medicatie op B cellen en T cellen, en naar het effect op de activatiesignalen die T cellen geven als hulp aan B cellen. Het blijkt dat alle vier de geteste medicijnen in staat zijn T cel hulp aan B cellen te remmen, zij het in verschillende mate. Tacrolimus en cyclosporine kunnen alleen via hun effect op T cel hulp de humorale immuunrespons remmen, terwijl MPA en rapamycine zowel T cel hulp als B cellen direct remmen. Deze bevinding benadrukt tevens het belang van het onderdrukken van T cel hulp om B cel responses te remmen.

Uit deze twee hoofdstukken blijkt rapamycine het medicijn te zijn met het meest brede immunosuppressieve effect op het humorale immuunsysteem. Uit andere studies is reeds gebleken dat rapamycine ook andere onderdelen van de afweer betrokken bij afstoting remt, terwijl het gunstige, regulerende cellen van het immuunsysteem spaart. De conclusie van deze hoofdstukken is dan ook dat rapamycine een geschikte kandidaat is als standaard immuunsuppressief medicijn na transplantatie.

De volgende twee hoofdstukken beschrijven studies met experimentele immunosuppressieve medicatie die mogelijk gebruikt kunnen worden om humorale afstoting te voorkomen of te behandelen. **Hoofdstuk 4** beschrijft het effect van Intraveneuze Immunoglobuline (IVIg) op de activatie van zuivere B cellen. IVIg is gepoold humaan plasma van tenminste 1000 bloeddonoren en werd oorspronkelijk gebruikt om patiënten met antistof deficiëntie te behandelen, omdat het hoge concentraties antistoffen met allerlei specificiteiten bevat. Uit studies bij patiënten met auto-immuunziekten is gebleken dat IVIg ook in staat is het immuunsysteem te beïnvloeden, alhoewel de exacte manier waarop onduidelijk is. Door deze immuun-modulerende eigenschappen wordt IVIg tegenwoordig ook gebruikt om het humorale immuunsysteem te dempen in transplantatiepatiënten. Omdat het mechanisme waarop IVIg het humorale immuunsysteem beïnvloedt nog steeds onduidelijk is hebben wij het effect van IVIg op B cellen getest. Onze studies laten zien dat IVIg geen direct effect heeft op enkele belangrijke B cel parameters. Wij concluderen hieruit dat IVIg niet direct op B cellen aangrijpt, maar via inhibitie van de eerder genoemde T cel hulp zijn effecten op cellen van het humorale immuunsysteem uitoefent.

Hoofdstuk 5 beschrijft het effect van het experimentele medicijn Bortezomib op B

cellen. Het is reeds bekend dat Bortezomib in staat is geprogrammeerde celdood te induceren in bepaalde celtypen van het immuunsysteem en mogelijkwijs humorale afstoting kan onderdrukken, maar het effect op humane B cellen is nog niet eerder beschreven. Onze studies laten zien dat relatief lage concentraties Bortezomib B cel activatie remmen, hetgeen leidt tot verminderde celdeling en antilichaam productie. Het eindresultaat is een verminderde antistofproductie, hetgeen relevant kan zijn in de context van humorale afstoting. De beschreven data leveren additioneel bewijs dat bortezomib een potentiële kandidaat is voor het voorkomen en behandelen van humorale afstoting.

Het tweede deel van dit proefschrift beschrijft manieren om het immuunsysteem van patiënten na transplantatie te monitoren. Dit is van groot belang om de dosis van immuon-suppressieve medicijnen zo laag mogelijk te houden, en zo bijwerkingen te minimaliseren. Momenteel bestaan er weinig bruikbare technieken om de cellen van het humorale immuunsysteem te monitoren. In **hoofdstuk 6** beschrijven we een nieuw ontwikkelde techniek om de frequentie van B cellen die specifiek gericht zijn tegen de donor te kwantificeren. Deze zeer gevoelige techniek stelt ons in staat zeer lage aantallen B cellen gericht tegen de donor per miljoen B cellen te detecteren. Met behulp van deze techniek verwachten we humorale afstoting vroeg op te kunnen sporen, waardoor adequate behandeling mogelijk is.

Zoals gezegd is T cel hulp van groot belang voor de activatie van B cellen. Kort na transplantatie herkennen T cellen complete cellen van het immuunsysteem van de donor die mee zijn getransplanteerd (directe herkenning). Lang na transplantatie zijn immuuncellen van de donor opgeruimd en zullen de T cellen moleculen van het getransplanteerde orgaan alleen nog maar herkennen op receptoren van lichaamseigen cellen, net zoals bij een infectie met een virus of bacterie (indirecte herkenning). De frequentie van deze indirect herkende T cellen is heel erg laag en daarom zijn deze cellen moeilijk te traceren. **Hoofdstuk 7** beschrijft de problemen waar men tegenaan loopt bij het monitoren van deze T cellen. Vele wetenschappers beweren technieken te hebben ontwikkeld voor het kwantificeren van indirect herkende T cellen, maar essentiële controles missen vaak. Gegevens uit ons eigen laboratorium laten zien wat de problemen zijn bij de beschreven technieken.



Abbreviations

ABBREVIATIONS

³ H-TdR	³ H-tritiated thymidine
3P	Third party
aa	Amino acid
ADCC	Antibody dependent cytotoxicity
APCs	Antigen presenting cells
ATG	Anti-thymocyte globulin
BCPF	B cell precursor frequency
BCR	B cell receptor
B-LCL	B lymphoblastoid cell line
BOS	Bronchiolitis obliterans syndrome
bp	Base pairs
BSA	Bovine serum albumin
CAN	Chronic allograft nephropathy
CBA	Cytometric bead array
CDC	Complement dependent cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
CTLp	Cytotoxic T lymphocyte precursor
CpG DNA	Cytosine-guanine dinucleotide oligodeoxynucleotide
CPM	Counts per minute
CyP	Cyclophilin
DAR	Direct allorecognition
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fc receptor
FcRn	Neonatal Fc receptor
FCS	Fetal calf serum
FCCS	Flow cytometry cytokine secretion assay

FCM	Flow cytometric analysis
FDC	Follicular dendritic cell
FKBP	FK506-binding protein
Foxp3	Forkhead box P3
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IAR	Indirect allorecognition
ICOS	Inducible costimulator
IFN- γ	Interferon- γ
IFTA	Interstitial fibrosis and tubular atrophy
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
IMDM	Iscove's modified Dulbecco's medium
IMPDH	Inosine-5'-monophosphate dehydrogenase
IVIg	Intravenous immunoglobulin
LDA	Limiting dilution analysis
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
mRNA	Messenger RNA
mTOR	Mamalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor- κ B
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PMSF	Phenylmethanesulphonyl fluoride
PWM	Pokeweed mitogen

Abbreviations

PI	Propidium Iodide
PRA	Panel reactive antibodies
PTC	Peritubular capillary
Q-PCR	Quantitative polymerase chain reaction
RhD	Rhesus D
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-AMV	Reverse transcriptase-avian myeloblastosis virus
SAL	Single antigen line
TCL	T cell line
TCC	T cell clone
TCR	T cell receptor
Th	T helper
TLR	Toll like receptor
UCB	Umbilical cord blood



Publications

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Curriculum Vitae

CURRICULUM VITAE

Sebastiaan Heidt was born on the 12th of April 1979 in Zoeterwoude, the Netherlands. He attended secondary school at the Vlietland College in Leiden. After graduating in 1997 he started his study of Biomedical Sciences at the University of Leiden. During this study he did his practical training at the Department of Experimental Microbiology of the Leiden University Medical Center (LUMC) under supervision of Drs. D. Koppers-Lalic and Prof. Dr. E.J.H.J. Wiertz on the subject of the interference of varicellaviruses with MHC class I presentation. After this he continued his practical training at the immunological laboratory of the Department of Paediatrics of the LUMC under supervision of Drs. B. Heemskerk and Dr. M.J.D. van Tol where he carried out a research project on adenoviruses infection after stem cell transplantation. He wrote his doctoral thesis on the subject of stem cells in regenerative medicine under supervision of Dr. M.W. Schillham. In 2003 he received his doctoral degree in Biomedical Sciences.

From July 2003 until October 2009 he worked as a PhD student under supervision of Dr. A. Mulder, Dr. D.L. Roelen and Prof. Dr. F.H.J. Claas at the Department of Immunohaematology and Bloodtransfusion of the LUMC, where the research that is presented in this thesis was carried out.

From October 1st 2009 until present he has been working as a post-doctoral scientist in the laboratory of Prof. Dr. K.J. Wood at the Nuffield Department of Surgery of the University of Oxford, United Kingdom.

CURRICULUM VITAE

Sebastiaan Heidt werd geboren op 12 april 1979 in Zoeterwoude. Hij behaalde zijn VWO diploma in 1997 aan het Vlietland College te Leiden. Vervolgens ging hij Biomedische Wetenschappen studeren aan de Universiteit van Leiden. Tijdens deze studie liep hij stage bij de Afdeling Experimentele Microbiologie van het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van Drs. D. Koppers-Lalic en Prof. Dr. E.J.H.J. Wiertz met als onderwerp de interferentie van varicellavirussen met MHC klasse I presentatie. Vervolgens liep hij stage op het immunologisch laboratorium van de Afdeling Kindergeneeskunde van het LUMC onder begeleiding van Drs. B. Heemskerk en Dr. M.J.D. van Tol waar hij onderzoek deed naar adenovirus infecties na stamcel transplantatie. Zijn afstudeerscriptie schreef hij onder begeleiding van Dr. M.W. Schilham en had als onderwerp stamcellen in regeneratieve geneeskunde. In 2003 behaalde hij het doctoraalexamen Biomedische Wetenschappen.

Van juli 2003 tot oktober 2009 was hij werkzaam als onderzoeker in opleiding op de Afdeling Immunohaematologie en Bloedtransfusie van het LUMC onder begeleiding van Dr. A. Mulder, Dr. D.L. Roelen en Prof. Dr. F.H.J. Claas, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Vanaf 1 oktober 2009 is hij werkzaam als postdoctoraal onderzoeker in het laboratorium van Prof. Dr. K.J. Wood in het Nuffield Department of Surgery van de Universiteit van Oxford, Verenigd Koninkrijk.

