Intravenous Immunoglobulins after Liver Transplantation: new insights in mechanisms of action

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Intravenous Immunoglobulins after Liver Transplantation: New Insights in Mechanisms of Action

Intraveneuze Immunoglobulinen na levertransplantatie: nieuwe inzichten in de werkingsmechanismen

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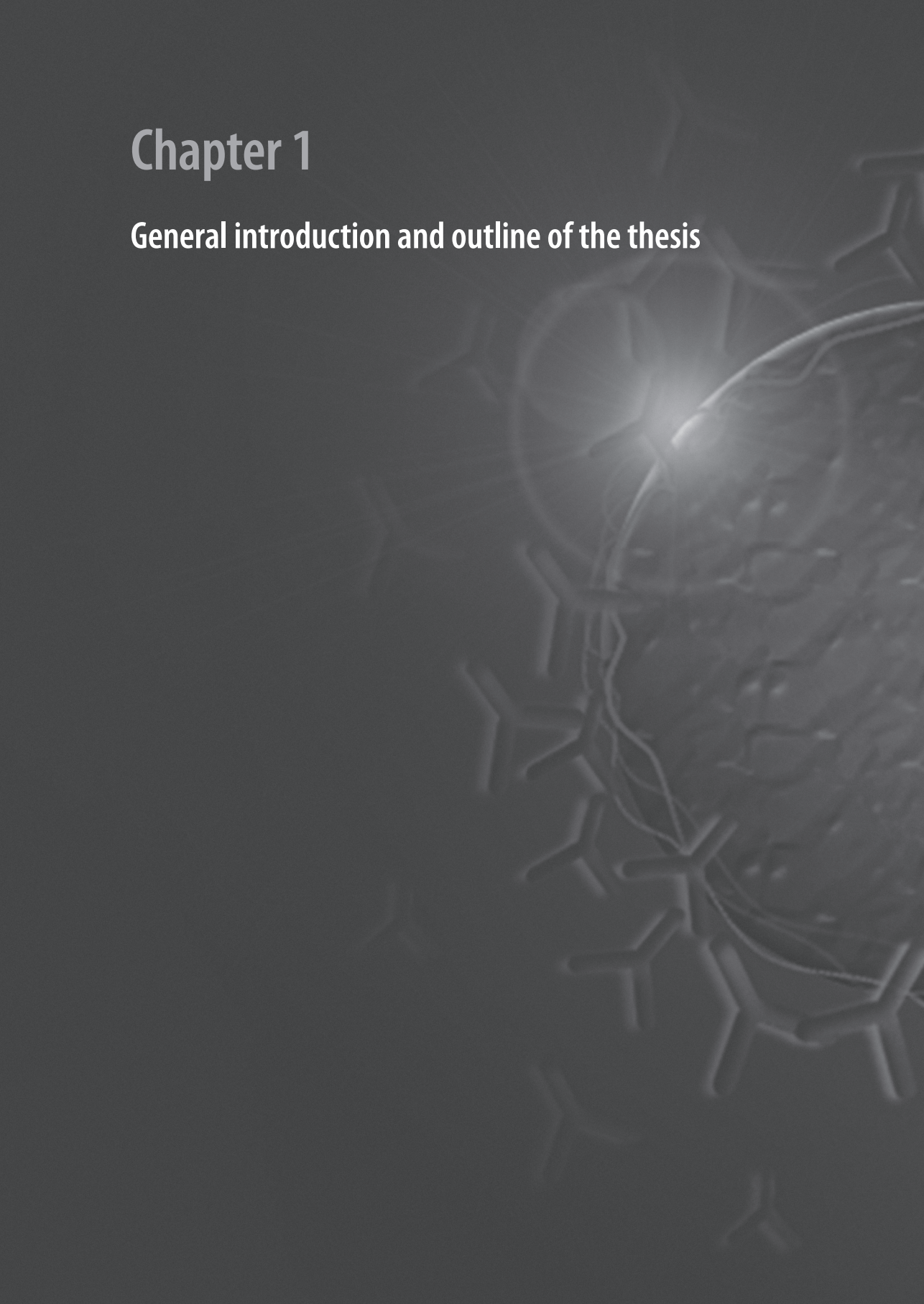

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Liver Transplantation

The principal concept of organ transplantation is the replacement of a diseased organ with a healthy one from another individual. In recent decades, transplantation has saved the lives of thousands of people who otherwise were condemned to death because of their life-threatening diseases. Currently, liver transplantation is the treatment of choice for both acute and chronic liver failure. The first successful transplantation of a liver in human was performed by Thomas Starzl in 1967 [1]. In 1983 the National Institutes of Health (NIH) declare liver transplantation as an accepted therapy for end-stage liver disease [2]. From the last two decades, most important indications for liver transplantation in Europe were cirrhosis (58%), cancer (13%), cholestatic diseases (11%) and acute hepatic failure (9%). Survival is excellent both in short term and long term transplant patients, with patient survival rates of approximately 81% one year after surgery, and 69% five years after transplantation (source: www.eltr.org).

Graft rejection

With the surgical improvements achieved, it has become apparent that graft rejection is the next major hurdle to be overcome after transplantation. If pre-existing antibodies to donor's blood group, HLA or other donorantigen are present in the recipient, an immunological cascade of *hyperacute* rejection can follow causing activation of complement and cytolytic cells, which finally result in necrosis of the vessel wall of the graft. Prior to kidney and heart transplantation, hyperacute rejection has largely been eliminated by routine cross match pre-screening [3]. Second, *acute cellular* rejection involves infiltration of the transplanted graft by different immune cells. HLA differences between recipient and donor trigger the recruitment of antigen presenting cells (APC) and T cells, follows by alloreactive T cell activation and proliferation, which result in rapid graft damage and destruction. In addition, different immune cells can infiltrate the graft involving granulocytes, macrophages, Natural Killer cells (NK cells), dendritic cells and T cells, all participating in the anti-donor response. In *chronic* rejection, graft damage by recipients' immune response occurs very slowly, unlike the more rapid type of acute rejection.

Comparing to other solid organs such as heart or kidney, the liver is an immune privileged organ with a lower incidence of graft rejection [4]. In addition, liver transplants do not require HLA matching of donor to recipients, and in contrast to recipients of heart or kidney transplants, it is estimated that about one third of liver transplant recipients with stable function can be totally withdrawn from immunosuppression [5-8]. Still, identification of stable patients suitable for withdrawn is complex, and therefore lifelong treatment with immunosuppressive drugs is required in the majority of liver transplant patients in order to prevent rejection of the liver graft [9].

In the early nineties, acute rejection occurred in up to 75% of transplants patients, which is reversible with vigorous immunosuppressive regimen to treat immune mediated graft rejection [10]. Lifelong dependency of immunosuppressive drugs comes with an enormous risk of serious side effects. These side effects are significant shortcomings of current anti-rejection therapies [11]. Common side effects are hypertension, osteoporosis, cardiovascular diseases and renal insufficiency [9,12,13]. Besides, by suppressing patients' immune system in order to prevent graft rejection, patients suffer from major complications, such as infectious diseases and cancer. By this, the life expectancy of transplant patients is impaired significantly and therefore counterbalances the success of transplantation.

The immune response to the transplanted liver grafts

During evolution, the immune system developed a highly specialized and tightly regulated series of responses, the main function of which is described as discriminating between self and non-self [4]. Transplantation of a liver graft is followed by activation of the immune system, which can be divided into two arms; namely innate and adaptive immunity.

The innate immunity serves as the initial warning signal for the adaptive immunity and involves NK cells and APC, such as macrophages, B cells and dendritic cells (DC). These cells can respond rapidly to foreignness, but their ability to remember the antigen is limited. Macrophages, B cells and DC are "professional" APC and have certain shared characteristics including hematopoietic origin, expression of major histocompatibility complex (MHC) class II molecules, and the ability to activate and provide co-stimulatory signals to T cells. DC are the most potent APC and play a central role in the initiation of allograft rejection [14,15]. However, liver resident cells, such as Kupffer cell, sinusoidal endothelial cells and stellate cells, with accessory immunological functions, may also play an important role in antigen presentation [16,17].

Furthermore, NK cells are lymphocytes of the innate immune system that are involved in early defences against autologous cells undergoing various forms of stress, such as malignant transformation or infections [18]. NK cell activation is the result of a balance between signals derived from activator and inhibitor receptors on NK cells [19]. The inhibitor receptors inhibit killing of target cells by specific interaction with MHC class I molecules, that are constitutively expressed by most healthy cells, but their expression may be lost upon stress, such as during malignant transformation [20]. By this pathway, NK cells are able to discriminate between normal and tumor cells and subsequently only kill the tumor cells without the necessity for priming ("the missing self hypothesis"). Recently, an increasing number of reports have suggested that the role of NK cells after transplantation is not as straight forward as was once thought. The traditional view is that NK cells are predominantly pro-inflammatory and are not sufficient to reject a solid allograft directly, but may contribute to early chemokine and cytokine production after transplantation supporting the rejection response [21,22]. In contrast, NK cells have been implicated as playing a role in the induction of tolerance to organ allografts [23,24].

Cross-talk between DC and NK cells can result in lysis, inhibition or maturation of DC by NK cells, and reciprocally, DC can activate or inhibit NK-cell functions. The final outcome of DC-NK cell interaction depends on the conditions in which both cell types encounter each other [25,26], and will subsequently determine the development of the following adaptive immune response.

The adaptive immunity, which involves T and B cells, is not able to respond rapidly during the first encounter with foreign antigens. However, during subsequent encounter of the antigen, the adaptive immunity can respond rapidly and with increased effectiveness due to the induction of memory T and B cells. One of the hallmarks identifying T cells is the T cell receptor (TCR) for antigens itself. The receptor is a heterodimer formed using two of four possible molecules: the α, β, γ or δ chain. Interestingly, the genes encoding the TCR chain are members of the immunoglobulin supergene family. This is a large family of genes, many of which are found on B, T and NK cells. Mature T cells are subdivided based on reciprocal expression of either the CD4 or CD8 glycoproteins. CD4+ T cells have MHC class II restricted TCR, and CD8+ T cells are MHC class I restricted in their antigen recognition [27]. This means that recognition of exogenous antigens processed by APC is almost entirely dependent on CD4+ T cells. T cells are described to be highly self –MHC-restricted, which means that, in general, T cells are only activated by peptides bound to self-MHC molecules, and not to foreign MHC molecules [28,29]. Still, important in transplantation setting, a very high proportion of T cells will respond to foreign MHC molecules [30]. In fact, the frequency of these cells responding to foreign MHC molecules is up to 1/100 T cells. This is partially due to the fact that different alleles of the MHC genetic locus can be up to 98% identical, varying in only a couple of amino acids [10]. As a result, foreign MHC can look remarkably like self-MHC. In our experiments, alloreactive response to foreign antigen is based on the activation of these alloreactive T cells responding to foreign MHC molecules directly, without antigen processing by the recipients own APC (direct pathway of allorecognition).

Different cell types of adaptive and innate immunity are involved in the rejection of allografts. Rejection is a direct result of the transfer of donor-antigens by APC to the recipient's lymphoid organs. Both donor DC and recipient DC can present alloantigen to T cells of the recipient. In case of interaction between donor DC and recipients T cell, the TCR of the recipient T cell will recognise the foreign MHC molecule on donor DC (direct pathway). In direct recognition, recipients' T cell recognizes foreign MHC directly, without processing via antigen presenting cells. When DC and T cells, both from the recipient, interact, the recipients' T cell will recognise the donor antigen displayed by the MHC of recipient DC (indirect pathway). (Figure 1)

It is generally accepted that the direct pathway predominates in the immediate aftermath of transplantation, when graft derived APC migrate to the surrounding lymphoid tissue, where they stimulate alloreactive T cells. As donor derived APC are relatively short lived [31], the indirect pathway of allorecognition is generally believed to predominates as the alloresponse progresses [32].

T cell activation by DC involves three important signals; i.e. signal 1, 2 and 3. All three signals are necessary for full T-cell activation, clonal expansion and attack of the graft. Signal 1 is the

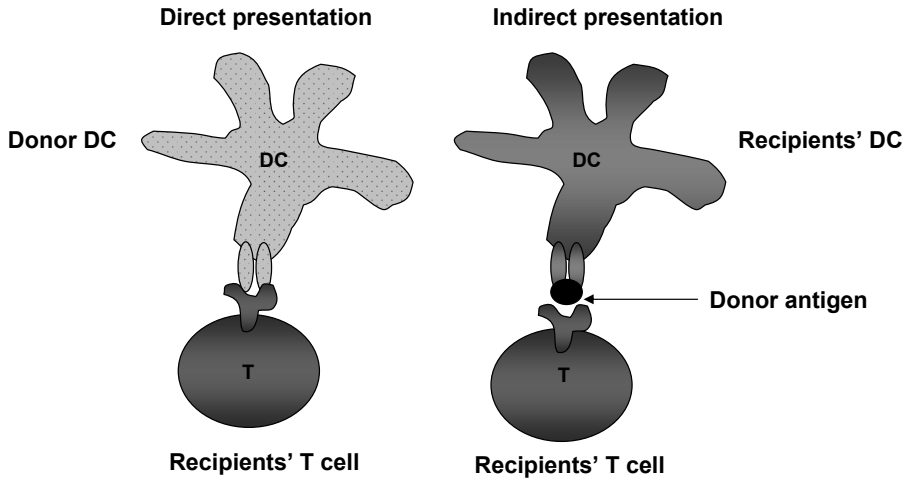

Figure 1: Direct and indirect pathway of antigen presentation
Both donor DC and recipient DC can present alloantigen to T cells of the recipient. Direct pathway: interaction between donor DC and recipient T cell in which the TCR of the recipient T cell will recognise the foreign MHC molecule on donor DC (left). Indirect pathway: DC and T cells, both from the recipient, interact, the recipients'T cell will recognise the donor antigen displayed by the MHC of recipient DC (right).

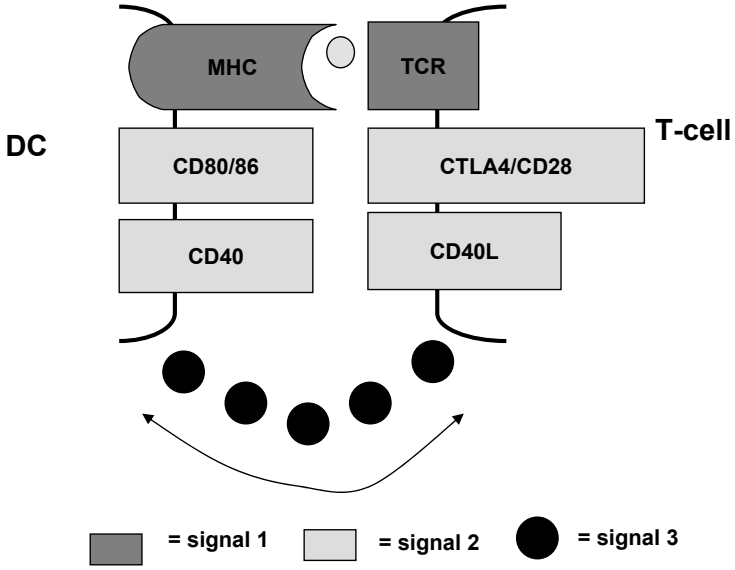

Figure 2: T cell stimulatory requirements
Stimulation of T cells by DC involves three important signals; i.e. signal 1, 2 and 3. All three signals are necessary for full T-cell activation, clonal expansion and attack of the graft. Signal 1 involves an antigenic peptide presented by a MHC on the DC to the TCR. Signal 2 is the co-stimulatory signals and signal 3 consist of cytokine production.

antigen-specific signal, which involves recognition of an antigenic peptide presented by a MHC on the DC to the T-cell receptor (TCR). The second signal (= Signal 2) is the co-stimulatory signal, which involves interaction of co-stimulatory molecules on DC and T cells [15]. Signal 3 consists of cytokines produced by the DC, which can alter the direction in which the naive T cell will differentiate [14,33]. (Figure 2)

In experimental animal studies it has been shown that graft derived DC, expressing donor allo antigens, migrate into the recipients lymph nodes and spleen, where they interact with recipients' T cells [10,34]. This hypothesis is supported by the fact that in a murine heart and liver transplant model augmentation of donor DC can induce rejection [35]. Once the APC encounter with naive alloreactive T cells, these T cells are activated and differentiate into effector T cells, which are primed to attack the transplanted liver graft. In addition, T cells with higher affinity for the donor antigens are being activated by APC, which results in development of highly reactive memory T cells [32]. As a counterpart of this pathway leading to rejection, regulatory T cells (Tregs) prevent uncontrolled activation of effector T cells. Regulatory T cells expressing the lineage marker Foxp3 are considered to be the "central immune regulator" of numerous immune processes [36,37]. Interestingly, Tregs play a critical role in induction and maintenance

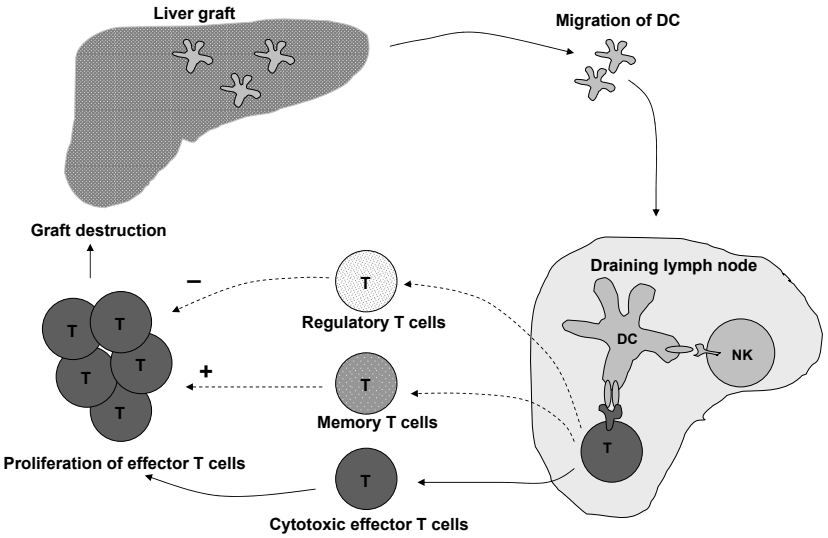

Figure 3: Mechanism of liver allograft rejection

An ordered sequence of events is thought to lead to rejection. Immature donor DC migrate from the transplanted liver to the recipients' lymphoid organs, including spleen and lymph nodes. In the lymphoid organs, donor DC and recipient T cell interact, and through its TCR the recipient T cell will recognise the foreign MHC molecule on donor DC directly leading to vigorous anti-donor T cell response with expansion of alloreactive effector T cells. T cells with higher affinity for the donor antigens will develop into highly reactive memory T cells. These cells can respond rapidly and with increased effectiveness to donor antigens, and thereby enhancing the rejection response. In contrast, some of the naïve T cell can evolve into regulatory T cells, which prevent uncontrolled activation of effector T cells, and thereby hampering the rejection response.

of transplantation tolerance in experimental animal models, and allograft acceptance can be transferred from tolerant animals to naive animals by transferring Tregs [38-40]. (Figure 3)

In summary, innate immunity contributes to the development of an appropriate adaptive immune response. Unfortunately, current immunosuppressive drugs mainly target adaptive immunity, and are less effective in altering innate immunity. Therefore, these drugs are only capable of controlling the effector immune response and are ineffective in shaping the regulatory part of the immune response.

Immunosuppressive drugs and tolerance induction in transplantation

A major challenge in transplantation immunology is to modulate the immune system of the recipient to tolerate the allograft in absence of immunosuppressive drugs. Current immunosuppressive regimens are effective in prevention of rejection as the incidence of acute liver rejection has decreased to approximately 15% [4]. However, the necessity of lifelong treatment and the occurrence of life threatening side effects are significant causes of morbidity and mortality after liver transplantation. In addition, the development of allograft acceptance is hindered by non-specific nature of these immunosuppressive drugs [41]. In experimental animal models, a few protocols have been developed to induce long term allograft acceptance in the absence of immunosuppression, however all these regimens were either not applicable or effective in humans. Protocol aimed at induction of allograft tolerance often entail aggressive ablation of recipients' immune system such as total body irradiation, massive depletion of peripheral T cells, and subsequent reconstitution with donor or mixed donor and recipient bone marrow [42-45]. Most approaches have severe side effects. In general, these protocols can save the graft, but harm the patient, and therefore unacceptable for wide spread use.

Classical immunosuppressive drugs used currently after liver transplantation are corticosteroids, calcineurin inhibitors (CNI), inhibitors of mTOR, various polyclonal or monoclonal antibodies [12]. Protocols based on these drugs can prevent graft rejection effectively, but induce global immunosuppression. (Table 1)

Table 1: Currently available immuosuppressants

Immunosuppressants agent	*Immunosuppressant class*
Corticosteroids	General immunosuppressants
Cyclosporin, Tacrolimus	Calcineurin inhibitors (CNI)
6-mercaptoprine, mycophenolate mofetil, azathioprine	Anti-metabolites
Sirolimus, Everolimus	Inhibitors of TOR
OKT3, IL-2R antibodies, Campath 1H	Antibodies
FTY720, leflunomide, FK778, FK779	Novel agents

TOR = target of rapamycine

IL-2R = interleukin 2 receptor

* adapted from Perry et al., Clinical and Experimental Immunology, 139, 2005

Calne and colleagues were the first to use cyclosporine to prevent graft rejection in human renal transplant patients [46]. In 1978, they reported that rejection episodes were mild to moderate degree after treatment with cyclosporine. The discovery of cyclosporine has changed transplantation practice as few other developments have. The cyclosporine dosing regimen based on body weight was effective in preventing acute rejection, but many patients developed severe infections or suffered from nephro- or hepatotoxicity, while other patients, using the same body weight-based dose, did not experience cyclosporine related side effects [47]. The cyclosporine whole blood concentration two hours after administration (C_2) has been shown to be the sampling point that correlates best with total drug exposure, and the probability of developing acute rejection after transplantation [48]. However, limited data exist on the benefits and safety of C_2 sampling strategies in stable patients long term transplant patients [49,50].

The acquisition of allograft tolerance, a state of permanent engraftment in absence of global immunosuppression, remains the "holy grail" in clinical transplantation. Transplantation tolerance is defined as acceptance of donor allograft despite cessation of immunosuppressive therapy, while retaining the ability to reject third party allografts. Central tolerance refers to a tolerant state established through clonal deletion during the development of allo-antigen reactive T cells in the thymus, while peripheral tolerance is a tolerant state that is primarily established without intrathymic selection [43,44]. Induction of peripheral tolerance involves modification in the activation process of mature alloreactive peripheral T cells. Peripheral allograft tolerance can be induced in various rodent experimental models using inbred donor and recipients strains. A variety of protocols including [9,10,29,41,45,51-56]: 1) administration of depleting monoclonal antibodies targeting cell surface molecules such as CD4, CD8, CD25, LFA-1 or the TCR; 2) blockade of the co-stimulatory pathways of T cell activation, notably CTLA-4Ig, anti-CD154 and anti-OX40L; 3) institution of pharmacological drug including steroids, rapamycin, cyclosporine; and 4) donor specific transfusion. Although these protocols can induce prolonged allograft survival, their impact on the acquisition of true allograft tolerance can be different [57]. In addition, the understanding of underlying mechanism is far from complete.

Intravenous Immunoglobulins: monomeric IgG, dimers, and aggregates

The history of potential therapeutic application of immunoglobulins in the treatment of human disease started in the 19th century by von Behring and Kitasato, who demonstrated that immune sera can ameliorate toxin mediated diseases [58]. In the World War II, Cohn developed an alcoholic fractionation method to separate human plasma in four fractions of plasma proteins [59]. Fraction II, containing the bulk of human antibodies, showed the greatest clinical merit, and has been used widely for multiple clinical purposes [60]. The immunoglobulin molecule, produced by B cells, consists of two light chain and two heavy chains. The distal ends of each light chain-heavy chain pair form an antigen binding site. The proximal ends of the heavy chain bind to Fc receptors on immune cells.

Intravenous immunoglobulin (IVIg) for therapeutic use is a polyspecific IgG preparation (> 95% IgG) purified from plasma pools of several thousand healthy donors. IVIg preparations primarily contain human IgG molecules, with small amounts of IgA and IgM. The distribution of IgG subclasses in IVIg is comparable to that of IgG in normal serum and the half-life of infused IVIg is approximately three weeks. IVIg are considered to be a safe preparation with no long term side effects. Although administration of IVIg can result in generalized reactions, such as headache, nausea and chills, in 1-15% of the patients. These adverse reactions are often mild and are relieved by NSAIDs or reducing the rate of infusion.

Due to the large donor pool and different manipulations during the purification process of IVIg, complexes of anti-idiotype antibodies with complementary idiotypes can be formed [61]. Since dimers and aggregates in IVIg preparations can induce severe side effects, several precautions are taken during the production process to keep the content of multimers in IVIg preparations minimal [62]. Excessive dimerization is prevented by methods such as keeping pH of the IVIg preparations low [61]. Therapeutic IVIg preparations contain less than 1% aggregates and 3 to 15% IgG dimers [61,62]. Nevertheless, IgG dimers [63] or multimers [64,65] present in the IVIg preparations have been shown to be responsible for the therapeutic efficacy of IVIg on macrophage-driven immune responses in different experimental models, such as Immune Thrombocytopenic Purpura (ITP) and Listeria monocytogenes infection. Moreover, the effects of IVIg in these models could be recapitulated by administration of antigen-antibody immune complexes, suggesting that complex IgG is responsible for the anti-inflammatory effects of IVIg [66]. Thus, while immune complexes are generally regarded as pro-inflammatory compounds, evidence is emerging that they can also mediate suppression of immune responses [67,68]. IgG dimers are one of the active anti-inflammatory components in IVIg, which are not present in the immune repertoire of a single individual, but are formed in plasma pools due to idiotype-anti-idiotype complex formation between IgG molecules from different individuals [61,63]. Yet, preparations with elevated levels of aggregates may cause severe side effects, such as anaphylactic reactions and thrombotic events. However, it is less likely that IgG dimers are responsible for the effects of IVIg on T cells. Moreover, some effects on DC *in vitro* could be recapitulated by F(ab)' fragments and are probably related to natural antibodies against cytokines

Fcγ receptors and neonatal Fc receptors

Fcγ receptors (FcγR) are immunoglobulin superfamily members recognizing the Fc part of IgG. FcγRI (CD64) binds IgG monomers with high affinity. FcγRI is an activating receptor expressed on macrophages, monocytes, neutrophils, eosinophils and DC. FcγRII (CD32) binds immune complexes with 1000-fold higher affinity for IgG-monomers. In humans two types of FcγRII have been identified: FcγRIIA, which is an activating receptor containing an ITAM motif in its cytoplasmic domain, and FcγRIIB which is an inhibitory receptor with in intracellular ITIM motif [69,70]. Macrophages, monocytes, neutrophils, eosinophils and DC express both isoforms, while B cells only express the inhibitory isoform FcγRIIB. In mice only the inhibitory variant of

FcγRII is present [71]. FcγRIII (CD16) binds immune complexes with much higher affinity than IgG monomers and is an activating receptor. Humans have two isoforms: FcγRIIIa, which is a transmembrane protein expressed on macrophages, monocytes, eosinophils, DC and NK-cells; and FcγRIIIb, which is a glycosyl-phosphatidylinositol (GPI)-linked form, expressed on human neutrophils. Mice express only FcγRIIIa. FcγRIV is a recently identified activating receptor involved in phagocytosis mediated by IgG2a and IgG2b antibodies [72]. This receptor is expressed in mice, but not in humans. Neonatal Fc receptor (FcRn) is expressed in the endosomal compartment of intestinal epithelium, vascular endothelium and macrophages [73]. It regulates serum IgG levels by binding pinocytosed IgG in the endosomes and recycling it to the cell surface, thereby rescuing it from degradation in lysosomes.[70,71,74-76]

Intravenous immunoglobulins and transplantation

IVIg were initially used as a substitution for immunoglobulins otherwise lacking in patients with primary immune deficiencies (PIDs) and secondary immune deficiencies. However, since the demonstration in 1981 that IVIg ameliorate ITP [77], IVIg are increasingly being used for the treatment of a wide range of autoimmune and systemic inflammatory diseases [78]. In addition to antibody-mediated diseases, IVIg are also effective in several disorders caused by derailment of cellular immunity, like Kawasaki disease, dermatomyositis, multiple sclerosis (MS), graft versus host disease (GVHD) in recipients of allogeneic bone marrow transplants, and treatment of cellular rejection after organ transplantation [78-83]. Clinically, the beneficial effects of IVIg extend beyond the half-life of infused IgG, therefore its effect is probably not merely due to a passive clearance or competition with pathogenic autoantibodies. Table 2 shows the reported immunomodulatory effects of IVIg [78]. By virtue of a broad spectrum of antibodies, which reflect the natural antibody repertoire of the human population, the mechanisms of action of IVIg are multifaceted and complex. Most likely, the immunological effects of IVIg are not mutually exclusive, and work in synergy to contribute to effective therapy in various clinical settings. Together, these observations evoke the possibility that IVIg therapy induces changes to the cellular immunity. (Table 2)

Cellular immunity is defined as an immune response that does not involve antibodies or complement, but rather involves the activation of macrophages, APC, NK cells, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Historically, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free bodily fluid or serum) and cellular immunity, for which the protective function of immunization was associated with cells [84].

In order to shorten the waiting time for solid organ transplantation and to improve graft survival, sensitized patients with high titers of donor-specific anti-HLA antibodies are treated with IVIg, often in combination with plasmapheresis, anti-lymphocyte antibody or immunoadsorption techniques [85-88]. Also, IVIg is used to facilitate transplantation of ABO incompatible organs

Table 2: Immunoregulatory effects of Immune Globulin

Effects on	Immunoregulatory effects
Fc receptors	Blockade of Fc receptors on macrophages and effector cells
	Induction of antibody dependent cellular toxicity
	Induction of inhibitory Fcγ receptor IIB
Inflammation	Attenuation of complement-mediated damage
	Decrease in immune complex mediated inflammation
	Inhibition of activation of endothelial cells
	Neutralization of microbial toxins
	Reduction of corticosteroid requirement
B cells and antibodies	Control of emergent bone marrow B cell repertoire
	Negative signalling through Fcγ receptors
	Selective down- and up regulation of antibody production
	Neutralization of circulating autoantibodies by antiidiotypes
T cells	Regulation of the production of T cell cytokines
	Neutralization of T cell superantigens
Cell growth	Inhibition of lymphocyte proliferation
	Regulation of apoptosis

* adapted from Kazatchkine and Kaveri, N Engl J Med, 345, 2001

[89]. After organ transplantation, IVIg has been used successfully for treatment of antibody mediated rejection [90]. In renal transplant patients, IVIg is effective as treatment of steroid-resistant cell mediated rejection [80,82].

To prevent re-infection of the liver graft with the hepatitis B virus (HBV) after liver transplantation, an anti-HBV surface antigen-specific IVIg (anti-HBs IVIg) is administered as prophylactics to HBV-positive liver transplant recipients [91]. Anti-HBs IVIg is a pooled hyperimmune immunoglobulin G preparation obtained from selected donors with high levels of reactivity to hepatitis B surface antigen [92]. Strikingly, treatment with anti-HBs IVIg proves to have an additional beneficial effect above preventing HBV recurrence, which forms the basic concept behind this thesis. Upon clinical observation, we observe that patients treated with anti-HBs IVIg had a two-until three-fold lower risk of liver graft rejection [93]. This suggests that IVIg may be effective in prevention of allograft rejection as suggested already in 1996 [94]. Since long term IVIg treatment has proved to have no side effects [95], using IVIg to substitute or replace current immunosuppressive drugs may benefit the long term survival of transplant patients in favour of a lower rate of complications. IVIg could be a powerful treatment for controlling the acute rejection process after liver transplantation. However, before clinical application can be implemented, the means by which IVIg exerts its therapeutic effects in immune mediated process of rejection involving complex cell-cell interactions needs to be verified.

AIMS AND OUTLINE OF THE THESIS

Since current immunosuppressive regimens have reduce the rejection rate significantly, the new priority is reduction in toxicity of global immunosuppression, while maintaining equivalent efficacy in controlling rejection. To reach this purpose, the development of safer and more effective protocols using the recently introduced immunosuppressive agents is needed.

Although, the established beneficial effects of IVIg in diseases mediated by autoantibodies may be explained by several mechanisms including neutralization and accelerated clearance of autoantibodies, and prevention of their binding to Fcγ receptors (FcγR) on phagocytes [78]. The question remains how IVIg exerts its effect on the different cells of the innate and adaptive immune system, which can explain the beneficial effects of IVIg in disorders caused by dysregulated cellular immunity. Better understanding of the effects of IVIg on the cellular rejection response may help us identify new therapeutic potential of IVIg, which form a basis to develop less toxic immunosuppressive protocols after liver transplantation.

Aim I: First, in order to develop a safer protocol using a conventional agent, cyclosporine, the benefits and drawbacks of cyclosporine dosing based on C_2 levels compared with conventional C_0 level monitoring was studied in stable liver transplant patients. (chapter 2)

Aim II: explore the therapeutic potential of non classical immunosuppressive drugs with fewer side effects; anti – HBs Ig. We determined whether anti – HBs Ig has an additional immunomodulatory effect, in that it can prevent acute allograft rejection in liver transplant recipients. (chapter 3)

Aim III: investigate in depth the immunomodulatory mechanisms of action of IVIg *in vitro* and *in vivo* in experimental animal models. Both the effects of IVIg on innate immunity (chapter 4, 5 and 6) and adaptive immunity (chapter 4 and 7) will be investigated. Focus will be on the IVIg effect on various cell types that play an important role in the cellular rejection response.

In chapter 8, the immunomodulatory effects of IVIg on both innate and adaptive cellular immunity are reviewed, and in chapter 9 the results of this thesis are summarized.

The results from this thesis may form an underlying principle or a discussion platform how to embrace IVIg in future immunosuppressive protocols after liver transplantation.

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Chapter 2

Clinical outcome after cyclosporine dose reduction based on C_2 levels in long term liver transplant patients

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SUMMARY

Recent studies suggest that cyclosporine dose adjustment based on C_2 levels results in improvement of renal function. This study investigates the effect on renal function after cyclosporine dose reduction based on the C_2 levels in long term liver transplant patients. In 60 patients (> 1yr after transplantation), C_2 levels were assessed (target 600 ng/ml ±20%). Dose reduction was performed when C_2 > 720 ng/ml. Serum creatinine concentrations were measured and creatinine clearance was calculated. Twenty-three patients (38%) had C_2 values > 720 ng/ml. After dose reduction, mean cyclosporine dose decreased by 25% ($p<0.01$). Mean C_2 value decreased by 42% ($p<0.01$). Serum creatinine concentrations remained stable. After dose reduction two patients experienced recurrence of primary biliary cirrhosis, in one patient auto immune hepatitis recurred and rejection was diagnosed in one patient. In conclusion, high cyclosporine C_2 concentrations above 720 ng/ml are common in long term liver transplant patients. Dose reduction of 25% did not improve kidney function and was accompanied by immune activation.

INTRODUCTION

Renal dysfunction after transplantation is considered a problem for transplant recipients as it may progress towards end-stage renal failure requiring dialysis or renal transplantation [1,2]. The incidence of chronic renal failure is reported to be as high as 7 to 21 percent in five years after transplantation of a non-renal organ. Forty-six percent of these patients in who end stage renal failure developed were placed on a waiting list for kidney transplantation [3]. In liver transplant patients, the incidence of chronic renal failure is 18 percent and seems to be higher compared with heart, lung and heart-lung transplant patients. Diabetes mellitus, hypertension and hepatitis C infection were independent risk factors for renal failure [3].

Monitoring cyclosporine blood levels to avoid under dosing or toxicity is one of the essential issues in the follow up of long term liver transplant patients. Traditionally, cyclosporine dose is adjusted based on the pre-dose level (C_0). However, there is accumulating evidence that the cyclosporine concentration 2 hours after administration (C_2) is a more sensitive tool for optimising cyclosporine dosing [4,5]. The C_2 level may be the most accurate predictor of the area under the curve (AUC) as a measure of total cyclosporine drug exposure [4]. Recent studies report a decreased incidence of acute rejection as well as a decrease in cyclosporine related side effects, in organ transplant patients by using C_2 monitoring instead of the conventional C_0 monitoring [6,7]. Dose reduction in overexposed patients according to C_2 levels is reported to result in improvements in renal function and blood pressure in stable long term renal, liver and heart transplant patients [8-10].

So far, one study has examined the effects of dose adjustment based on C_2 levels in liver transplant patients more than one year after transplantation [9]. In our center, the incidence of renal dysfunction increases after onset of treatment with calcineurin inhibitors. In 177 patients we observed that 50.1% of the patients had a glomerular filtration rate below 60 ml/min two years after liver transplantation (unpublished data). This is then followed by a slow but continuing deterioration of renal function.

Therefore, we analysed the effects of dosage individualisation by C_2 monitoring in liver transplant patients more than one year after transplantation. The aim of this study was twofold. First, we investigated the effects of dose reduction based on the C_2 levels on the renal function in long term liver transplant patients, and second, we evaluated the possible risks accompanying cyclosporine dose reduction.

PATIENTS AND METHODS

Patients

In February 2002, 60 stable liver transplant patients who received their first liver transplant between April 1988 and January 2001, were included in the study. The characteristics of the patients are presented in Table 1. Patients with stable allograft function, who had received their transplant at least one year earlier and who had microemulsion cyclosporine as maintenance immunosuppressive drug, were eligible to participate in this study. A written informed consent was signed by all participants. (Table 1)

Table 1: Patients characteristics

	all patients	*Group 1 ($C_2 \geq 720$ ng/ml)*	*Group 2 ($C_2 < 720$ ng/ml)*	*p-value*
Gender (M/F)	25/35	9/14	16/21	*NS*
Mean age at transplantation (yr)	47.11 ± 11.64	46.65 ±7.91	47.40 ± 13.55	*NS*
Mean time post-transplant (yr)	5.97 ± 3.20	5.09 ± 2.82	6.51 ± 3.34	*NS*
Liver disease (HBV, HCV, ALD, PBC, other) (n)	10/3/5/25/17	3/0/2/11/7	7/3/3/14/10	*NS*
Conversion from sandimmune to neoral (n)	20	8	12	*NS*

M: male, F: female, yr: years, HBV: hepatitis B, HCV: hepatitis C, ALD: alcoholic liver disease, PBC: primary biliary cirrhosis

Immunosuppression

Standardized immunosuppression protocols were used. All patients had previously been monitored by trough level (C_0). Cyclosporine was initiated within 24 hours post-reperfusion and adopted according to trough level between 200 and 400 ng/ml during the first 3 months and then between 100 and 200 ng/ml as maintenance therapy. Moreover, cyclosporine dosage was adjusted in case of rejection or cyclosporine related toxicity.

In September 1995, conversion from oil based cyclosporine, Sandimmune (Novartis, Basel, Switzerland), to the microemulsion formulation of cyclosporine, Neoral (Novartis), took place in our center. Before replacement of Sandimmune by Neoral, 20 patients were initially treated with Sandimmune. At the time of inclusion in this study, 38 patients (63.3 %) were on Neoral monotherapy, 13 patients (21.7 %) were treated with Neoral and prednisone, five patients (8.3 %) were treated with Neoral, prednisone and azathioprine, two patients were treated with Neoral and azathioprine, one patient was treated with Neoral and mycophenolate mofetil (MMF) and one patient was treated with Neoral, prednisone and MMF.

Study design

At the beginning of the study all sixty patients had their C_0 and C_2 levels assessed. For the purpose of this study, target C_0 level was defined as 125 ng/ml (± 20%). Target C_2 level was defined as 600 ng/ml (± 20%) [11]. Cyclosporine dose reduction was performed when C_2 value exceed 720 ng/ml using the formula: new dose = (old dose * 600) / actual C_2 level [12]. Cyclosporine dosage

was left unaltered in patients whom C_2 level was more than 20% below target (< 480 ng/ml) in order to avoid cyclosporine overexposure in stable patients. Patients were divided into two subgroups based on whether cyclosporine dose reduction is performed. Patients in group 1 had C_2 values above 720 ng/ml and the dose of cyclosporine was reduced. Patients in group 2 had C_2 values below 720 ng/ml and the dose of cyclosporine was left unchanged.

In order to assess changes in renal function, serum creatinine concentrations were collected six months before inclusion and measured at 2 weeks, 4 weeks, 8 weeks, 3 months and 6 months after cyclosporine dose reduction. In addition, the creatinine clearance was calculated using the Modification of Diet in Renal Disease Study (MDRD) formula [13]. Clinical outcome was monitored by routine biochemical measurements at similar visit. Graft rejection and recurrence of liver diseases were diagnosed by increased levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin and were confirmed by histological examination of a liver biopsy.

Cyclosporine concentration measurement

After drawing the pre dose whole blood sample (C0), patients were asked to take their cyclosporine. Then, blood was drawn at 2 hours ± 15 minutes after cyclosporine intake. After collection of the blood samples, cyclosporine concentrations were determined using the Emit 2000 assay (Syva company, Dade Behring inc., Cupertino, CA) on a Cobas Mira Plus analyzer (Roche diagnostic systems).

Statistical analysis

The statistical analysis was conducted using the SPSS statistics software SPSS/PC 11.1. The paired t-test was used. The Pearson's correlation coefficient between C_0 and C_2 was calculated. Differences in the distribution of gender, age at transplantation, time since transplantation, liver disease and conversion from Sandimmune® to Neoral® between group 1 and group 2 were assessed with the chi-2 test for categorical variables and student's t-test for continuous variables. P-values less than 0.05 were considered statistically significant. All data are represented as mean ± standard deviation.

RESULTS

The mean pre dose level (C_0) was 122.12 ± 54.92 ng/ml and the mean C_2 level was 652.13 ± 274.30 ng/ml. A wide range of C_2 values was detected between 188 and 1510 ng/ml). The correlation between C_0 and C_2 was weak (R = 0.587). Twenty-three patients had C_2 values above 720 ng/ml, 16 patients had C_2 values within the target range of 480 - 720 ng/ml and 21 patients had C_2 values below 480 ng/ml. Interestingly, eleven of the 23 patients (48%) with high C_2 had C_0 values below or within 100-150 ng/ml. (Table 2)

Accordingly, 23 patients detected with high C_2 level (> 720 ng/ml), were selected for cyclosporine dose reduction. However, three of 23 patients did not agree with dose reduction and were not included in prospective part of the study. Dose reduction was performed in twenty patients (group 1). In 37 patients, with the C_2 level within or below desired target range, cyclosporine dosage was not reduced (group 2).

After 6 months of follow up, the mean cyclosporine dose of group 1 decreased from 3.58 ± 0.95 mg/kg/day to 2.69 ± 0.91 mg/kg/day, representing a 25% reduction in cyclosporine dosage ($p< 0.01$). The corresponding mean C_2 decreased from 933.9 ± 209.0 ng/ml to 545.3 ± 228.3 ng/ml ($p< 0.01$), showing a 42% decrease in the mean C_2 value 6 months after dose reduction. Seventy-five percent of the patients were on target after 2 weeks. (Figure 1)

The mean creatinine concentration, creatinine clearance, systolic blood pressure and lipids level of group 1 remained stable during follow up. None of the patients had developed end stage renal failure. Mean creatinine concentration six months before inclusion was measured and did not differ from the mean creatinine concentration at time of inclusion (six months before inclusion: 1.4±0.4 mg/dL versus at time of inclusion: 1.3±0.4 mg/dL (NS)). Mean diastolic blood pressure decreased significantly after cyclosporine dose reduction ($p=0.01$). In group 2, the renal function, blood pressure and lipids level remained unchanged. Results of group 1 and 2 before and after follow up are presented in Table 3. Mean creatinine clearance of group 1 during the six months follow up is demonstrated in Figure 2. (Table 3 and Figure 2)

In group 1, patient 1 experienced cellular rejection after 3 months follow up, which was histologically classified as RAI 6. Corticosteroid pulse therapy was additionally given as treatment and cyclosporine dosage was not changed. The C_2 level at the time of diagnosis was 800 ng/ml. In patient 2 and 3 recurrence of primary biliary cirrhosis was diagnosed, respectively during the 8 weeks and 6 months visit. This was histologically confirmed. Both patients were treated with ursodeoxycholic acid and the cyclosporine dosage of patient 3 was increased. The C_2 levels at the time of diagnosis were respectively 555 ng/ml in patient 2 and 680 ng/ml in patient 3. In patient 4 autoimmune hepatitis recurred in week eight. Following, patient had an increase of cyclosporine dosage and allopurinol was given as treatment. The C_2 level at the time of diagnosis was 640 ng/ml. Patients 1, 2 and 3 were on cyclosporine monotherapy and patient 4 was treated with cyclosporine and prednisolone.

Patients 1, 2, and 3 had a C_2 value below the C2 target range during one visit. All events were before the diagnosis of rejection or recurrence. Respectively, patient 1 had a C_2 level of 180 ng/ml at week four, patient 2 had a C_2 level of 470 at week four and patient 3 had a C_2 level of 395 ng/ml at week eight after cyclosporine dose reduction.

In group 2, no rejection or recurrence of immune mediated liver diseases was observed.

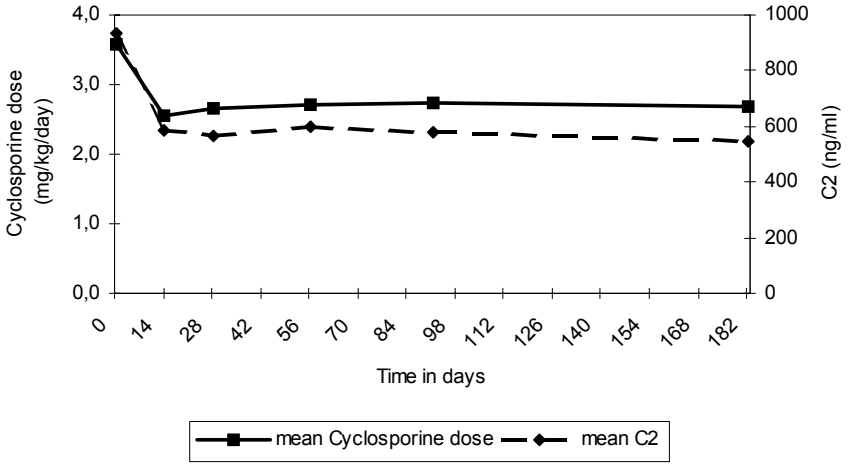

Figure 1: Cyclosporine dose (mg/kg/day) and C_2 level (ng/ml) of group 1

Table 2: C0 and C2

C_0/ C_2	*Low C_2 (≤ 480 ng/ml)*	*Normal C_2 (480-720 ng/ml)*	*High C_2 (≥720 ng/ml)*	**Total**
Low C_0	15	5	**1**	21
Normal C_0	5	8	**10**	23
High C_0	1	3	12	16
Total	21	16	23	60

C_0: trough level of cyclosporine

C_2: cyclosporine concentration 2 hours after administration

DISCUSSION

This study of C_2 monitoring in 60 stable liver transplant patients at more than one year after transplantation showed that high cyclosporine C_2 concentrations is common in this group of patients. Overexposure was observed in 23 of the 60 patients (38%). Eleven of the 23 patients (48%) had C_0 values below or within target range, indicating the limitations of C_0 monitoring in detecting drug overexposure. In addition, our findings confirm indicate that C_2 monitoring is a better measurement for cyclosporine overexposure in long term liver transplant patients.

In earlier publications, Cole et al. reported cyclosporine overexposure of 49% in renal transplant patients more than three months after transplantation [8]. In liver transplant patients, an overexposure of 68% was documented in patients more than six months after transplantation [14]. In two studies on stable renal transplant patients more than one year after transplantation, one described a C_2 level exceeding 800 ng/ml in 29% of the patients and another observed C_2

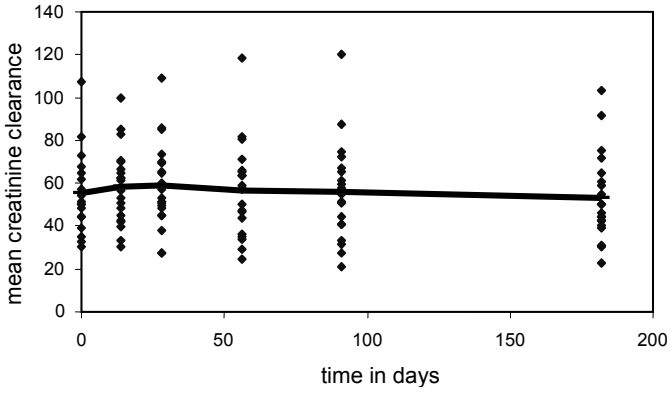

Figure 2: Mean creatinine clearance of group 1 (ml/min)

Table 3: Renal function, bloodpressure and lipids level before and after follow-up

		T=0	*T=6 months*	*p-value*
Group 1	*serum creatinine (mg/dL)*	1.3±0.4	1.4±0.4	*NS*
(C_2 ≥720 ng/ml)	*creatinine clearance (ml/min/1.73m^2)*	55.2±18.1	53.0±19.1	*NS*
(n=20)	*systolic bloodpressure (mmHg)*	148±20	144±21	*NS*
	diastolic bloodpressure (mmHg)	92±8	83±11	*0.01*
	Total cholesterol (mmol/l)	5.3±0.7	5.6±1.6	*NS*
	Triglycerides(mmol/l)	2.0±1.1	1.9±1.4	*NS*
Group 2	*serum creatinine (mg/dL)*	1.4±0.4	1.4±0.4	*NS*
(C_2 <720 ng/ml)	*creatinine clearance (ml/min/1.73m^2)*	53.2±20.2	53.1±21.1	*NS*
(n=37)	*systolic bloodpressure (mmHg)*	143±19	139±16	*NS*
	diastolic bloodpressure (mmHg)	85±14	85±9	*NS*
	Total cholesterol (mmol/l)	5.4±1.1	5.5±1.5	*NS*
	Triglycerides(mmol/l)	1.9±0.6	1.9±1.4	*NS*

above 850 ng/ml in 18% of the patients [15,16]. Our study showing cyclosporine overexposure of 38% in stable liver transplant patients is in line with these findings.

Despite the linear relation in the used formula, a discrepancy was observed in our study between the cyclosporine dose and the measured C_2 level. In renal transplant patients after dose reduction of 27.5%, Cole et al. observed a 37.3% decrease in mean C_2 value [8]. In liver transplant patients, Langers et al. reported that a dose reduction of 26.9% resulted in a 25.8% decrease in mean C_2 value measured on day 2 after dose reduction [14]. We noticed that the discrepancy between the cyclosporine dose and the measured C_2 level in our study is considerable compared to studies mentioned above. This may be due to high within-patient variability.

In addition, differences in studied patient population where the time post transplantation and the use of co-medication differ between groups should be taken in consideration.

After dose reduction we observed a decline in mean diastolic blood pressure in group 1. This was in line with findings of previous studies in long term renal, heart and liver transplant patients [8-10]. The mean serum creatinine concentration and mean creatinine clearance remained unchanged in our study. Since the mean creatinine concentration six months before enrolment is comparable to those at the time of inclusion, we consider that there was no slope decline of renal function in group 1 post-transplantation. Thus, the stabilised renal function during follow up was probably not due to cyclosporine dose reduction. In contrast to other studies in non-renal transplant patients, we did not observe improvement in kidney function six months after cyclosporine dose reduction based on C_2 levels. In liver transplant patients, Langers et al reported a significant improvement of 11.6% ($p = 0.016$) in creatinine clearance at more than six months after transplantation (target C_2 was 600 ± 15%) [14]. In two studies of Cantarovich et al., in one study a 5.1% ($p = 0.006$) decrease in mean serum creatinine level was observed after cyclosporine dose reduction based on C_2 level in liver transplant patients at more than one year after transplantation. Their target C_2 was between 300 and 600 ng/ml [9]. A second study of Cantarovich et al in heart transplant patients at more than one year after transplantation, a 2.3% decrease of serum creatinine was reported after dose reduction based on C_2 level aiming between 300 and 600 ng/ml [10]. The lack of improvement in renal function in our study may be related to the C_2 target range we used, as this was higher than in the mentioned studies. Consistent with this idea Cantarovich et al. reported an increase of mean serum creatinine level by 16% when a C_2 target level between 700 and 1000 ng/ml was used [9]. Therefore, the selected C_2 range is crucial in order to observe effects of dose adjustment based on C_2 levels performed in long term liver transplant patients. Furthermore, our population had received a liver transplant at least 1 year before the enrolment. Consequently, irreversible damage to the kidney, and its management by the reduction of cyclosporine dose already took place at the discretion of the attending hepatologist. The reversibility of cyclosporine nephrotoxicity is most likely to be more prominent early after transplantation. Cole et al. reported a significant decrease of serum creatinine level in 54% of renal transplant patients at more than three months after transplantation by performing cyclosporine dose reduction based on C_2 levels [8]. Therefore, early introduction of dose adjustment based on C_2 is probably more effective.

However, during follow up we observed a brief period of increase in mean creatinine clearance in the first four weeks after cyclosporine dose reduction. These improvements in renal function were temporary. We hypothesize that this event is an acute response of the kidney to a lower cyclosporine dose, which can not be maintained due to irreversible damage in the kidney. Therefore, we doubt the necessity of a longer follow-up.

It is noteworthy that cyclosporine dose reduction in long term liver transplant patients may lead to allograft rejection and recurrence of autoimmune hepatitis or primary biliary cirrhosis.

This is attributable to a period of cyclosporine underexposure, which could not be confirmed, as the C_2 levels at the time of diagnosis were within the target range. However, a short period of low immune suppression can not be excluded. In addition, patients using no or a small amount of co-medication are probably susceptible to experience complications of low immune suppression. Fluctuation of C_2 levels during follow up may easily cause immune activation as the patients are using only cyclosporine or cyclosporine and prednisolone as immunosuppressive therapy. Therefore, the use of co-medication should be taken in consideration when cyclosporine dose adjustment is performed. When a patient is on cyclosporine monotherapy dose reduction should be done with caution, and it is probable that the target C_2 levels for this group of patients is higher than 600 ng/ml (±20%).

So far, none of the related studies in stable renal transplant patients reported such side effects [8,16]. After late conversion from C_0 to C_2 monitoring of cyclosporine in paediatric living donor liver transplant recipients, rejection was reported in one patient (1,7%) [17]. In adult stable liver transplant patients, rejection was reported in two patients after dose reduction [14]. The area under the curve (AUC) in these patients was below target. The authors performed cyclosporine dose reduction in liver transplant patients at more than six months after transplantation with 19.4% of the patients on cyclosporine monotherapy [14]. In our study 63.3% (38/60) of the patients (more than one year after transplantation) are on cyclosporine monotherapy and 6.7% (4/60) of the patients had complications of immune activation. Hence, the problem of underexposure to immune suppressive drug after cyclosporine dose reduction based on C_2 levels is expected to be more prominent in long term transplant patients, since the number of patients who are using no co-medication is enlarging as the post transplantation time increase.

In conclusion, cyclosporine dose reduction based on C_2 levels in liver transplant patients at more than one-year post transplantation is less effective than in earlier stages, and renal function may not be influenced. We realise that the risks of rejection and recurrence of autoimmune liver diseases are not to be underestimated, especially in patients on cyclosporine monotherapy. By using C_2 target range of 600 ng/ml (±20%) no benefit in renal function was observed after cyclosporine dose reduction in long term liver transplant patients.

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Chapter 3

Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation

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SUMMARY

The efficacy of anti-viral intravenous immunoglobulins (anti-HBs IVIg and anti-CMV IVIg) in preventing acute rejection after liver transplantation was assessed in a retrospective analysis, and correlated to their effects on immune cells in vitro. HBs Ag-positive liver graft recipients (n = 40) treated prophylactically with anti-HBs IVIg had a significantly lower incidence of acute rejection compared with recipients without viral hepatitis (n = 147) (12% versus 34%; p = 0.012), while the incidence of rejection in HCV-positive recipients (n = 29) was similar to that in the control group. Treatment with anti-CMV IVIg (n = 18) did not protect against rejection. In vitro, anti-HBs IVIg suppressed functional maturation of and cytokine production by human blood-derived dendritic cells (DC) at concentrations similar to the serum concentrations reached during anti-HBs IVIg treatment of liver graft recipients. In addition, anti-HBs IVIg inhibited allo-antigen- and lectin-stimulated proliferation of peripheral T cells. Anti-CMV IVIg suppressed functional DC maturation and alloantigen-stimulated T-cell proliferation, but not lectin-driven T-cell proliferation. In conclusion, anti-HBs IVIg protects against acute rejection after liver transplantation, probably by functional inhibition of the two principal immune cells involved in allograft rejection, DC and T cells.

INTRODUCTION

Intravenous immunoglobulins (IVIg) are therapeutic preparations of IgG derived from plasma pools of either healthy blood donors or individuals with high titers of antibodies against certain viruses. IVIg containing specific antibodies against CMV (anti-CMV IVIg) are used to prevent transplantation-associated CMV infection, and anti-HBV surface antigen specific IVIg (anti-HBs IVIg) are administered as prophylaxis to Hepatitis B Virus (HBV) positive liver transplant recipients to prevent infection of the graft with HBV. Non-specific IVIg have anti-inflammatory properties, which are exploited in their application in the treatment of autoimmune disorders (reviewed in [1]). Among the disorders responding to IVIg treatment are not only autoantibody mediated diseases, but also T-cell mediated immuno-pathological conditions, like multiple sclerosis [2], indicating that IVIg may suppress T-cell activation or function.

In the context of transplantation, prophylactic treatment with non-specific IVIg was found to decrease the incidence of acute graft versus host diseased (GVHD) after clinical bone marrow transplantation [3], and in an experimental settings, in which GVHD was induced by lymphocyte transfer [4]. In addition, IVIg are effective in the treatment of steroid resistant acute rejection after kidney transplantation [5,6]. So far, It is unknown whether IVIg are effective as prophylaxis for acute rejection after organ transplantation. In line with this possibility, two papers have reported that treatment of liver graft recipients with anti-HBs IVIg or anti-CMV IVIg protects against acute rejection [7,8], but a study from another liver transplant center failed to confirm the mentioned protective effect [9].

Non-specific IVIg have been shown to inhibit the in vitro equivalent of acute rejection, the mixed lymphocyte reaction (MLR) [10,11]. One report suggests that virus specific IVIg have MLR-suppressive capacity too [12]. Inhibition of MLR by IVIg may be due to suppression of the T cells or the Antigen Presenting Cells (APC) or both. It has been observed that non-specific IVIg suppress proliferation [13] and cytokine production [13-15] by human T-cells in vitro. In addition, non-specific IVIg have been found to inhibit the differentiation of dendritic cells (DC) from human monocytes , and their maturation in vitro [16]. As DC migrating from the organ graft are considered as the principal APC priming T-cell reactivity against donor antigens after organ transplantation [17], this finding merits further study as a potential explanation for the beneficial effect of IVIg on allograft rejection. Whether anti-viral IVIg have similar effects on DC is so far unknown.

The aims of the present study were to determine whether the anti-viral IVIg used in our center protect against acute rejection after liver transplantation, and whether these hyperimmune anti-viral IVIg affect the function of principal immune cells involved in allograft rejection, i.e. DC and T-cells.

PATIENTS AND METHODS

Intravenous immunoglobulins

Anti-HBs IVIg (Hepatect® or Hepatect CP®) and anti-CMV IVIg (Cytotect®) were obtained from Biotest Pharma, Dreieich, Germany. For all three preparations only healthy individuals are accepted as donors. Individuals selected for donation of anti-CMV plasma have been naturally infected by CMV, and have high titers of CMV-IgG (9-15 Paul-Ehrlich-CMV-Units/ml plasma). For Hepatect and Hepatect CP plasma from healthy individuals who are vaccinated with a standard HBV vaccine are selected for donation (40 - 60 IU anti-HBsAg IgG/ml). When indicated these IVIg were dialyzed twice against large volumes of culture medium (RPMI) at 4°C to remove stabilizing agents and to obtain neutral pH. After dialysis, IgG concentration was determined by the Tina-quant® immuno-turbidimetric assay (Roche Diagnostics, Mannheim, Germany).

Study population

A retrospective analysis was performed on recipients of a primary orthotopic liver graft within the Erasmus Medical Center between January 1992 and October 2002. Patients treated with ATG or OKT-3 as part of their induction immunosuppressive therapy (n = 21) were excluded from the study cohort. In addition, patients with a follow-up of less than 5 days (n = 30) were excluded. The remaining 234 liver transplant recipients were grouped into four categories (Table 1):

1. HBs Ag-positive patients transplanted for either liver cirrhosis due to chronic HBV infection (n = 31) or for fulminant (n = 9) HBV infection. All these patients had been treated with Hepatect® or Hepatect CP® as prophylaxis;
2. CMV IgG negative recipients transplanted with a graft from a CMV IgG positive donor, and were treated with Cytotect® as a prophylaxis for CMV-infection (n = 18);
3. patients transplanted for chronic HCV infection (n = 29);
4. all other recipients (n = 147).

Table 1: Patient and donor characteristics of the study groups.

	p-value	*1.anti-HBs Ig*	*2. Anti-CMV Ig*	*3. HCV*	*4. other*
Number of patients		40	18	29	147
sex (m/f)	0.047	27/13	7/11	19/10	71/76
age (mean±sd)	0.017	47±10	43±14	53±8	46±13
donor sex (m/f)	0.727	23/17	11/7	15/14	73/73
donor age (years)	0.123	44±13	37±15	39±13	38±14
Graft ischemia time (hours)	0.089	10.3±3.0	12.3±3.3	9.9±3.6	10.4±3.0
Year of transplantation	<0.001	1999 (1993- 2002)	1995 (1992-1998)	1998 (1994-2002)	1998 (1992-2002)
Immunosuppresssion (with IL-2 rec. mAb)	0.003	20/40 (50%)	0/18 (0%)	11/29 (38%)	59/147 (40%)

Clinical data were gathered from an existing computerized database. Two of the anti-HBs IVIg-treated patients had co-infection with HCV, and two other anti-HBs IVIg-treated patients had also been treated with anti-CMV IVIg. These patients were classified into the anti-HBs IVIg-treated category. Median follow-up of the patients was 38 months (range: 5 days – 118 months).

Treatment with anti-HBs IVIg and anti-CMV IVIg, and acute rejection

Anti-HBs IVIg (10,000 IU) was administered intravenously during the anhepatic phase of the transplantation procedure, and on days 1 and 2 after liver transplantation. During the next 5 days, anti-HBs IVIg was given i.v. once daily until serum HBsAg was not detectable. Thereafter anti-HBs IVIg treatment aimed at maintaining serum anti-HBs IVIg concentrations above 500 IU/l. Anti-CMV IVIg was given intravenously during the anhepatic phase (150 mg/kg), and post-operatively on day 2, week 1, 2, 5, 8, 11. Doses of 100 mg/kg were given. As primary end point of the analysis, clinically evident acute rejection, was defined as biopsy proven rejection (Snover-grade ≥ 2, or RAI-score ≥ 6) with a rise in transaminases and/or bilirubin, which responded to treatment. Rejection was treated with methylprednisolone, and in some patients OKT3 or rabbit ATG was given additionally.

Determination of serum level of anti-HBs IVIg and anti-CMV IVIg

Serum anti-HBs IVIg were determined by AUSAB AxSYM (Abbott Laboratories, North Chigaco, IL, USA). Complete serial data of post-transplant serum anti-HBs IVIg titers, i.e. measured at daily intervals during the first 3 weeks after transplantation, and weekly during the next 12 weeks were available from 17 anti-HBs IVIg treated liver transplant recipients. Since these patients were anti-HBs IVIg negative before transplantation, it was assumed that increments in anti-HBs IVIg level reflected the anti-HBs IVIg treatment. Hepatect CP concentrations (mg/ml) were calculated from the anti-HBs IVIg titers (IU/l) by using the mean specific activity of Hepatect CP(0.5 IU/mg). Anti-CMV IVIg titers were determined by VIDAS (BioMérieux, Marcy-l'Etoile, France) in archival sera obtained at weekly intervals during the first month after transplantation from 8 Cytotect treated liver graft recipients. Since these patients were anti-CMV IVIgG-negative before transplantation, it was assumed that serum anti-CMV IVIgG post-transplantation reflected the Cytotect titers. Cytotect concentrations were calculated using the relative fluorescence values of a standard curve of serial dilutions of Cytotect (50 – 0.78 mg/ml).

Effect of anti-HBs IVIg and anti-CMV IVIg on DC

DC were purified from fresh heparinized blood of healthy volunteers by positive selection with PE conjugated anti-CD1c mAb and anti-PE MACS beads using MS columns, after depletion of B cells with CD19 conjugated MACS beads, and separation over LD-columns (Miltenyi Biotec, Bergisch Gladbach, Germany), as described previously [18]. The purity of DC (defined as $CD1c^{+}CD20^{-}$ cells) as analyzed by flow cytometry was 97±2%. To analyze the effects of anti-HBs IVIg on DC maturation, DC ($1x10^{4}$/200µl) were stimulated for 24 hours at 37°C with TNFα (25 ng/

ml) and IL-1β (50 ng/ml) (both from Strathmann Biotech, Hannover, Germany) in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Gibco BRL Life Technologies, Breda, The Netherlands), 10% fetal bovine serum (Hyclone, Logan, UT, USA) and GM-CSF (500U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands). Anti-HBs IVIg, anti-CMV IVIg, or human serum albumin (HSA, CLB, Amsterdam, The Netherlands) were added in different concentrations to the cultures. For determination of their maturation status, DC were labeled with CD1c-PE, anti-HLA-DR-FITC, CD20-PERCP, CD86-APC (all from BD Biosciences, Heidelberg, Germany), CD80-FITC (Beckman Coulter Immunotech, Marseille, France), CD83-APC (Caltach, Burlingame, CA, USA) or appropriate isotype control mAb, and analyzed by flow-cytometry.

To determine the effects of anti-viral IVIg on the acquisition of allogeneic T-cell stimulatory capacity, different numbers of DC (5, 2.5, and 1.25 x 10^3 cells per well of a 96-well flat bottom plate) were stimulated to mature with TNF-α, IL1-β and GM-CSF for 24 hours in the absence or presence of anti-HBs IVIg, anti-CMV IVIg or HSA. The next day, the culture medium was aspirated, DC were washed two times with culture medium to remove additives, and 1.5 x 10^5 allogeneic T cells were added. In all experiments with DC, one T-cell preparation was used, which was enriched by Ficoll gradient centrifugation and nylon wool filtration from a buffy coat of one healthy blood donor. This preparation contained 83% $CD3^+$ cells. After 5 days, T-cell proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) during 18 hours. Each condition was tested in triplicate, from which means were calculated. These means were used in the analyses.

For the analysis of cytokine production, purified DC were cultured at a concentration of 4x10^4 cells per 200 μl RPMI supplemented with 10% fetal bovine serum and GM-CSF, and stimulated with either polyriboinosinic-polyribocyticylic acid (poly I:C); 20 μg/ml; Sigma-Aldrich, St. Louis, MA) and recombinant human IFN-γ (1000 U/ml), or with Staphylococcus aureus Cowan strain I (SAC; 75 μg/ml, Calbiochem, San Diego, CA), in the absence or presence of anti-HBs IVIg or HSA. After 24 hours cell-free supernatants were harvested and the levels of TNF-α and IL-10 were determined by specific sandwich ELISA using pairs of mAb and recombinant cytokine standards from Biosource International (Camarillo, CA, USA). The concentration of IL-12 was determined using a commercial ELISA-kit (Diaclone, Besancon, France).

Effect of anti-HBs IVIg and anti-CMV IVIg on T cells

T cells were enriched from buffy coats of healthy blood donors by Ficoll gradient separation and immunomagnetic depletion of monocytes, NK cells and B cells by labelling the cells with CD14-PE, CD56-PE (both from BD Biosciences) and CD19-PE (from Beckman Coulter Immunotech) followed by incubation with anti-PE MACS beads and separation over MS columns. The four independent T-cell preparations used in these experiments contained on the average 68 ± 11% $CD3^+$ cells. Spleen APC were enriched from human spleen tissue obtained from a multi organ donor by Ficoll gradient separation and immunomagnetic depletion of T cells and NK cells by labelling the cells with CD3-PE and CD56-PE (both from BD Biosciences) followed by

incubation with anti-PE MACS beads and separation over MS columns. T cells (1.5 x 10^4 per 200 μl RPMI supplemented with 10% FCS) were either stimulated with phytohemagglutinin (PHA, 5 μg/ml; Murex, Paris, France) or with splenic APC (1.5 x 10^4) in the presence or absence of anti-HBs IVIg (Hepatect CP), anti-CMV IVIg or HSA. After 5 days, T-cell proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) during 18 hours. Each condition was tested in triplicate from which means were calculated. These means were used in the analyses.

Statistical analysis

Differences in immunosuppressive therapy or gender between the study groups were analyzed by the Chi-square test and differences in numerical values by the Kruskal Wallis test. The actuarial incidence of acute rejection was determined by the Kaplan Meier technique, using the time to the first rejection episode as end-point, and censoring for graft failure or death. Multivariate analysis was performed using the Cox's proportional hazard model. The effects of anti-viral IVIg in vitro were analysed by the wilcoxon test for paired data or the student t-test for paired data. A p-value < 0.05 was considered as indicating significant difference.

RESULTS

Patient characteristics

The effect of treatment with anti-viral IVIg on the occurrence of acute rejection was analysed retrospectively by grouping the liver transplant recipients (n=234) into two categories. One that had been treated with anti-viral IVIg and two as control categories. The two anti-viral IVIg treated categories were: HBsAg positive liver graft recipients, which had been treated with anti-HBs IVIg as prophylaxis for infection of the graft with HBV (group 1), and recipients that had been treated with anti-CMV IVIg as a prophylaxis for CMV infection (group 2). HCV positive recipients served as a control group for viral infection in the liver as original liver disease (group 3). The incidence of acute rejection the mentioned three groups was compared with that in the control group, which was formed by all other recipients (group 4). Characteristics of the patients and their donors are summarized in Table 1.

The study groups differed significantly in the distributions of recipient gender and age, year of transplantation and immunosuppressive therapy and, with borderline significance in graft ischemia time. The difference in year of transplantation between the groups was due to the fact that all patients treated with anti-CMV IVIg had been transplanted in the early study period, before 1999, because CMV prophylaxis with anti-CMV IVIg had been replaced in 1998 by Ganciclovir. With regard to immunosuppressive induction therapy, the patients were grouped in those who had been treated with a calcineurin inhibitor (either Cyclosporin A or Tacrolimus), low-dose corticosteroids, either or not supplemented with azathioprine (n=144), and those

who had been treated additionally with an anti-IL-2 receptor monoclonal antibody (mAb), either Basiliximab (Novartis Pharma, Basel, Switzerland) or Daclizumab (Roche Pharmaceuticals, Basel, Switzerland) (n=90). The difference in immunosuppressive therapy between the patient categories is again detected in the anti-CMV IVIg treated group. None of the patients included in this category had received anti-IL-2 receptor mAb, because the use of anti-CMV IVIg was stopped just before anti-IL-2 receptor mAb treatment was introduced.

Anti-HBs IVIg and anti-CMV IVIg concentrations in serum of liver transplant recipients

Treatment of HBsAg positive liver transplant patients with anti-HBs IVIg resulted in relatively high concentrations of anti-HBs IVIg in their serum (Figure 1), which peaked at day 4 after transplantation to a mean level of 8 mg/ml, than declined to about 2 mg/ml at day 9, and subsequently slowly decreased to about 0.5 mg/ml at week 8. No sera were available from the first six post-transplant days of the anti-HBs IVIg-treated patients, but at day 7 post-transplantation the anti-CMV IVIg serum concentrations were about two times lower compared to anti-HBs IVIg concentrations in anti-CMV IVIg-treated recipients. (Figure 1)

Incidence of acute rejection in the study groups

The overall three months cumulative incidence of acute rejection was 31%, but differed significantly between the patient groups (overall log rank p-value = 0.026). Figure 2A shows that the HBsAg-positive patient treated with anti-HBs IVIg treated had a significant lower incidence of acute rejection compared to control group 4 (12% versus 34%; p=0.012, log rank test), while the anti-CMV IVIg-treated and HCV positive recipients did not differ from control group (group 4)

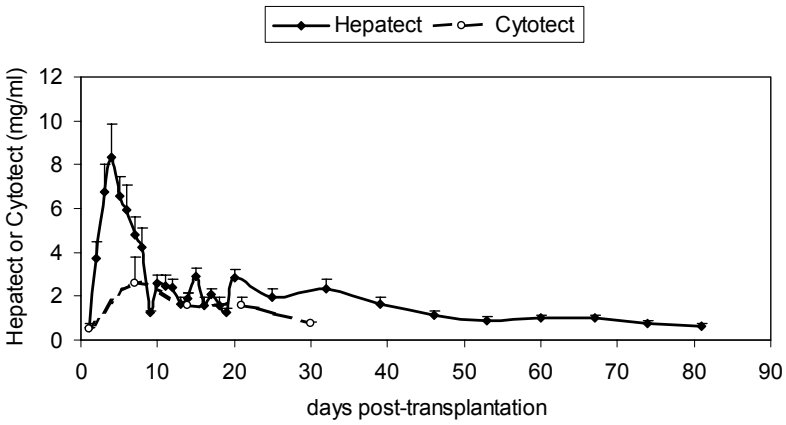

Figure 1 Anti-HBs IVIg and anti-CMV IVIg serum concentrations in liver graft recipients.
Mean (± SEM) post-transplant serum concentrations of anti-HBs IVIg (Hepatect CP) in HBsAg-positive liver graft recipients treated with Hepatect CP(n=17) and of anti-CMV IVIg (Cytotect) in recipients treated with Cytotect (n=8).

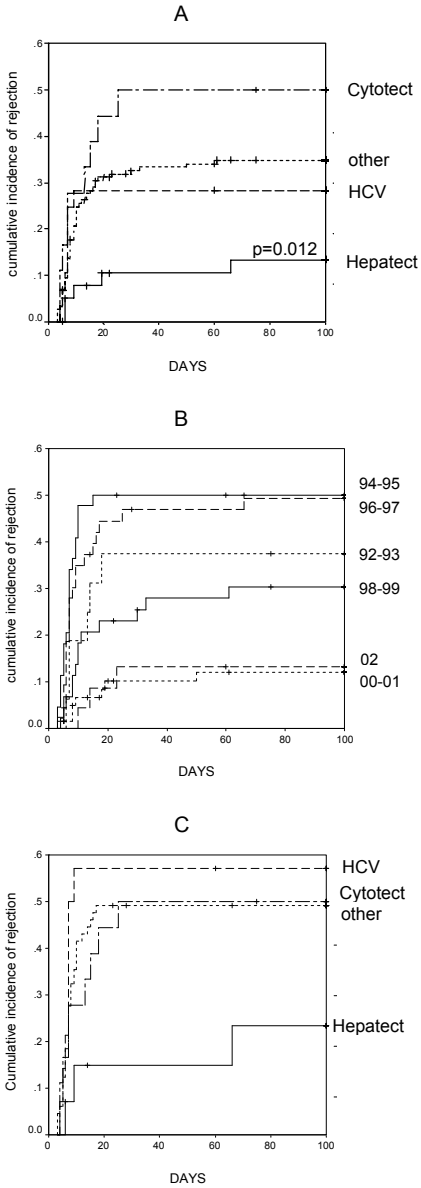

Figure 2 Cumulative incidence (Kaplan-Meier curves) of acute rejection in different categories of liver graft recipients.

A. Cumulative incidence of acute rejection during the first three months after liver transplantation in HBsAg-positive recipients treated with anti-HBs IVIg (n=40), anti-CMV IVIg-treated recipients (n=18), HCV positive recipients (n=29), and all other recipients (other; n=147).

B. Influence of the period in which the transplantation was performed on the cumulative incidence of acute rejection. Patients were grouped into two-year periods, except the last period which was the year 2002.

C. Cumulative incidence of acute rejection in different groups of liver graft recipients transplanted before September 1998. HBs Ag-positive recipients treated with anti-HBs IVIg (n=14), anti-CMV IVIg-treated recipients (n=18), HCV-positive recipients (n=14), other recipients (n=65). P-values depicted are from paired comparisons with control group 4 (log-rank test).

(50%; p = 0.19, 28%; p = 0.65, respectively). The incidence of rejection within the HBsAg-positive/anti-HBs IVIg-treated category was reduced, both in recipients transplanted for liver cirrhosis due to chronic HBV infection (4 out of 31 patients experienced rejection) as in patients transplanted for fulminant acute HBV infection (1 out of 9 experienced rejection), indicating that the lowered incidence of rejection was not confined to patients with persistent chronic infection.

As is shown in Figure 2B, the incidence of acute rejection decreased significantly during the study period (log rank $p_{trend} < 0.001$). All anti-CMV IVIg-treated patients had been transplanted in the early years of the study period. In order to exclude the bias introduced by the difference in transplantation period between the study groups, a subgroup analysis on the effect of anti-CMV IVIg was performed on patients transplanted before August 1998. This subgroup (n = 111) consisted of 14 HBsAg-positive/anti-HBs IVIg-treated, all 18 anti-CMV IVIg-treated-, 14 HCV-positive, and 65 control recipients. As is shown in Figure 2C, in this subgroup analysis the cumulative incidence of rejection in the anti-CMV-treated category was equal to that of control group 4 (50% versus 49% p = 0.93), while the incidence of acute rejection of anti-HBs IVIg treated recipients differed with almost statistical significance from that of the control group. Figure 2 A-C)

All patients treated with anti-CMV IVIg were CMV IgG negative recipients receiving livers from CMV IgG positive donors. A possible protective effect of anti-CMV IVIg on acute rejection might have been masked if these patients would have been more susceptible to acute rejection. To investigate this possibility, the association between this recipient/donor combination and acute rejection was analyzed in patients of control group 4, and transplanted after August 1998, when anti-CMV IVIg prophylaxis was no longer given to patients. In this period 19 liver transplantations from CMV IgG positive donors into CMV IgG negative recipients was performed within control group 4. The incidence of rejection in these patients was comparable to that in patients of control group 4 not belonging to this specific recipient/donor combination (5/19 (26%) versus 13/63 (21%); p=0.75, Fisher's exact test). Together, these data show that treatment with anti-CMV IVIg did not protect against acute rejection.

Multivariate analysis of factors influencing the incidence of acute rejection

To investigate whether the differences in risk of acute rejection between the patients categories were independent of other factors in which the study groups differed (immunosuppressive therapy, year of transplantation, recipient sex and age, and graft ischemia time), a Cox regression analysis was performed in which these variables were entered together with the patient categories. Table 2 shows that of these factors only the HBs Ag-positive/anti-HBs IVIg-treated patient group and the year of transplantation were independently and significantly associated with the risk of acute rejection. In HBsAg-positive/anti-HBs IVIg treated patients the rate of acute rejection was reduced by 61% as compared to patients in control group 4, while in each year between 1992 and 2002 the risk of rejection was reduced by a factor 13% compared to the year before. (Table 2)

Table II: Multivariate analysis of factors univariately associated with acute rejection.

	Hazard ratio	*95% CI*	*p value*
Patient categories			
group 1 (anti-HBs Ig-treated)	0.39[a]	0.16 - 0.99	0.047
group 2 (anti-CMV Ig-treated)	0.91[a]	0.43 - 1.95	0.807
group 3 (HCV-positive)	1.16[a]	0.54 - 2.51	0.707
Other variables			
Year of transplantation	0.87	0.78 - 0.98	0.017
Immunosuppression (+ anti-IL-2 rec mAb)	0.57[b]	0.26 - 1.28	0.177
Recipient sex (female)	1.15[c]	0.72 - 1.84	0.562
Recipient age	0.98	0.97 – 1.00	0.088
Graft ischemia time (hours)	0.95	0.88 – 1.03	0.185

Reference categories: [a]group 4 (all other patients); [b]no anti-IL-2 rec. mAb; [c]male recipients.

Effect of anti-viral IVIg on DC

To establish whether anti-viral IVIg suppress functional maturation of DC, immature DC isolated from blood of healthy volunteers were stimulated in vitro with pro-inflammatory cytokines (IL-1β and TNF-α) to mature in the presence or absence of anti-HBs IVIg or anti-CMV IVIg. After 24 hours, T cells from an unrelated donor were added to the DC to assess their allogeneic T-cell stimulatory capacity. Freshly isolated immature blood DC had a poor allogeneic T-cell stimulatory capacity, which was strongly upregulated during cytokine-driven maturation (data not shown). Figure 3A shows that the addition of anti-HBs IVIg during maturation in a concentration comparable to the peak concentration reached in the serum of HBsAg positive liver graft recipients (9 mg/ml) significantly suppressed the acquisition of allogeneic T-cell stimulatory capacity in DC. An equal amount of another human serum protein, HSA, slightly enhanced the capacity of DC to stimulate allogeneic T-cell proliferation. To exclude that the observed inhibition of DC function was due to stabilizing agents present in, or to the low pH of the anti-HBs IVIg preparation, anti-HBs IVIg were first dialyzed against a large volume of culture medium. The dialyzed preparation showed similar suppression of allogeneic T-cell stimulatory capacity (data not shown).

The suppressive effect of anti-HBs IVIg on functional DC maturation was concentration dependent. A concentration of 3.5 mg/ml anti-HBs IVIg suppressed the acquisition of allostimulatory activity in DC less potently, while a concentration of 0.5 mg/ml had no effect (Figure 3B). Anti-CMV IVIg also suppressed the acquisition of allogeneic T-cell stimulatory capacity in DC (Figure 3C).

To investigate the mechanism by which anti-HBs IVIg suppressed allogeneic T-cell stimulatory capacity of DC, expressions of the DC maturation marker CD83, HLA-DR, and co-stimulatory molecules were determined. Expressions of all four molecules were upregulated

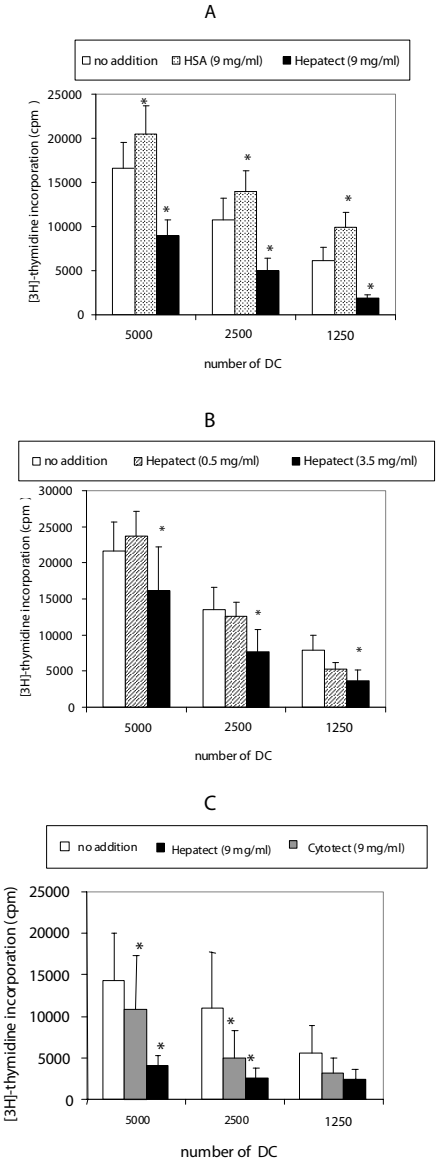

Figure 3 Effect of anti-viral IVIg on the acquisition of allogeneic T-cell stimulatory capacity in DC upon stimulation with pro-inflammatory cytokines. Purified blood DC were stimulated in vitro with IL-1β, TNF-α and GM-CSF for 24 hours in the presence or absence anti-HBs IVIg (Hepatect CP), anti-CMV IVIg (Cytotect), or HSA. After 24 hours, the additions were removed, and T cells from an unrelated healthy individual were added to determine the allostimulatory capacity of DC. Data are depicted as means ± SEM from n independent experiments with DC from different donors.

A. Effect of anti-HBs IVIg and HSA both at 9 mg/ml (n = 9)

*= p-values <0.03; **=p-values <0.01; paired t-test.

B. Effect of 0.5 and 3.5 mg/ml anti-HBs IVIg (n = 6). *= all p-values <0.04; paired t-test

C. Effect of anti-CMV IVIg at 9 mg/ml (n = 5). *= all p-values <0.02; paired t-test

during DC-maturation. Anti-HBs IVIg suppressed the upregulation of CD80 and CD83, but not of HLA-DR and CD86 (data not shown). (Figure 4)

Anti-HBs IVIg also suppressed cytokine production by DC. As is shown in Figure 5, anti-HBs IVIg suppressed the production of TNF-α, IL10 and IL12 by DC stimulated with poly I:C and IFN-γ, or with SAC, in a dose-dependent fashion, while HSA had no effect. To exclude the fact that the reduced concentrations of cytokines in the supernatants of DC incubated with anti-HBs IVIg were due to an blocking effect of anti-HBs IVIg on cytokine detection in ELISA, anti-HBs IVIg

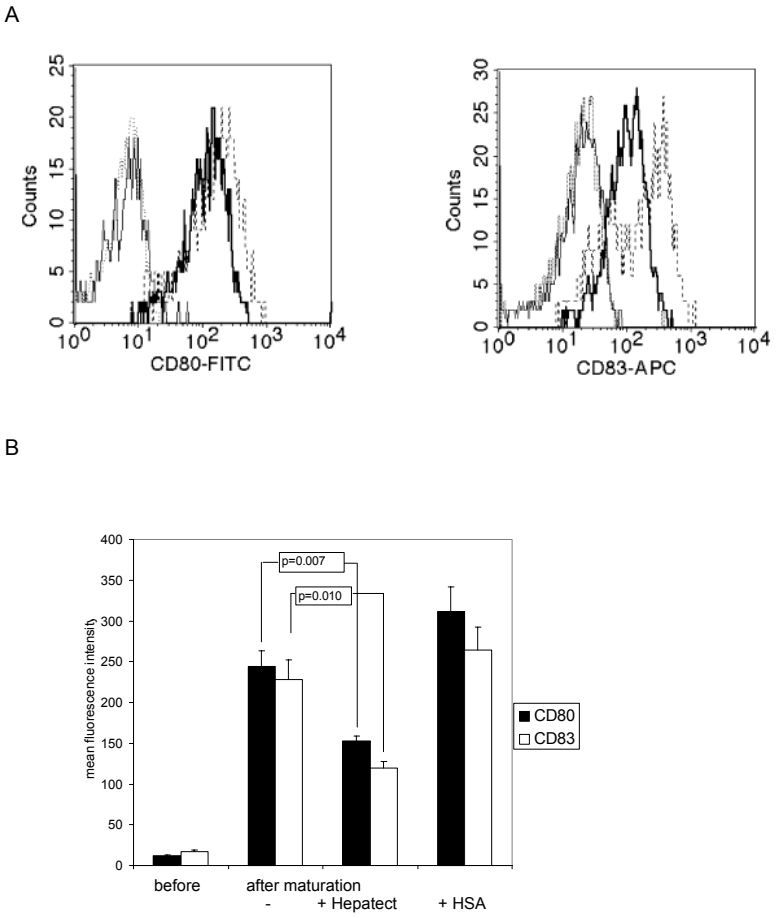

Figure 4 Effect of anti-HBs IVIg on the upregulation of CD80 and CD83 on DC during stimulation with pro-inflammatory cytokines.
DC purified from blood of healthy volunteers were stimulated in vitro with IL-1β, TNF-α and GM-CSF for 24 hours in the absence or presence of anti-HBs IVIg (Hepatect CP) or HSA (both 9 mg/ml).
A. Flow cytometry histograms showing the upregulation of CD80 and CD83 during DC-maturation. Dotted lines represent IgG isotype control binding, thin solid lines CD80- or CD83-expression on freshly isolated DC, dashed lines expression after maturation in the absence of anti-HBs IVIg, and thick solid lines expression after maturation in the presence of anti-HBs IVIg.
B. CD80 and CD83-expression before and after maturation in the absence or presence of anti-HBs IVIg or HSA. Results are expressed as mean fluorescence intensities ± SEM from eight experiments with DC from different individuals.

was added to recombinant IL-10, IL-12 or TNF-α in the ELISA-plates and subsequently cytokines were quantified. No inhibitory effect of anti-HBs IVIg on the detection of these cytokines was observed compared to cytokines measured in the absence of anti-HBs IVIg. (Figure 5 A and B)

Effect of anti-viral IVIg on T cells

Anti-HBs IVIg, both as pharmaceutical formulation and after dialysis against culture medium, strongly suppressed T-cell proliferation induced by stimulation with PHA (Figure 6A). In contrast,

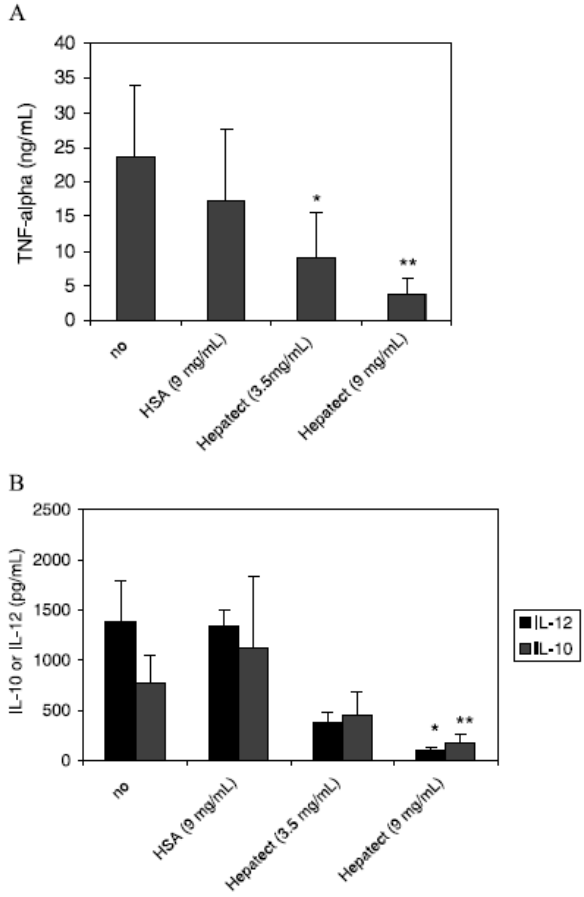

Figure 5: Effect of anti-HBs IVIg on cytokine production by DC.

A. TNF-α was determined in culture supernatants of purified DC ($4x10^4$ per 200 μl) stimulated with SAC for 24 hours in the presence or absence of anti-HBs IVIg (Hepatect CP) or HSA.

*: p=0.056; and **: p=0.018 compared with TNF-α-production in cultures without additions (t-test for paired data after log-transformation of the data)

B. IL-12 was determined in supernatant of DC ($4x10^4$ per 200 μl) stimulated with poly (I:C) and IFN-γ for 24 hours, and IL-10 in culture supernatants of DC stimulated with SAC.

*: p=0.025; and **: p=0.053 (t-test for paired data after log-transformation of the data) .

Data are depicted as means ± SEM from three independent experiments with DC from different donors.

anti-CMV IVIg did not inhibit PHA induced T-cell proliferation (Figure 6B). However, when T-cells were stimulated with allogeneic splenic APC, both anti-HBs IVIg and anti-CMV IVIg suppressed T-cell proliferation (Figure 6C).

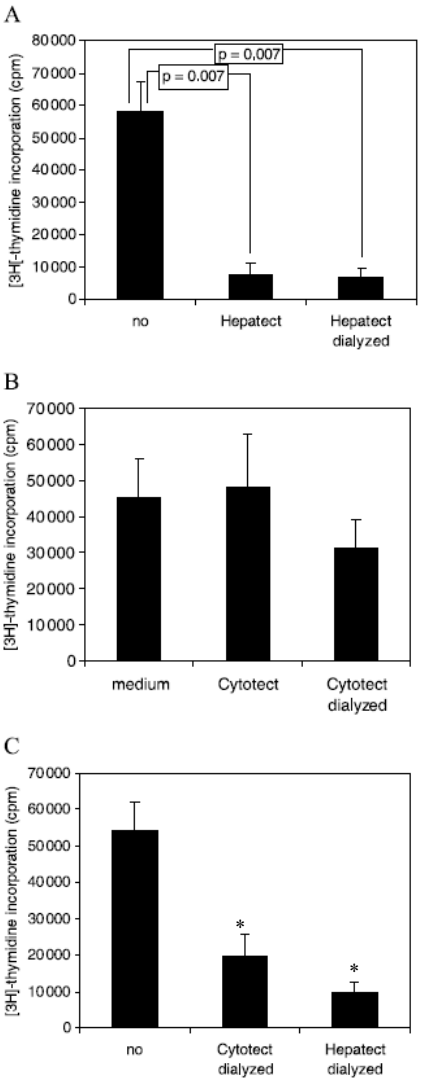

Figure 6: Effect of anti-viral IVIG on T-cell proliferation stimulated by PHA or allogeneic APC.

A. Peripheral blood T-cells were stimulated with PHA in the presence or absence of anti-HBs IVIg (Hepatect CP; 9 mg/ml) as a pharmaceutical preparation or after dialysis and [^{3}H]-thymidine incorporation was determined 5 days later (n=4).

B. The effect of anti-CMV IVIg (Cytotect; 9 mg/ml) as a pharmaceutical preparation or after dialysis on PHA-stimulated T-cell proliferation (n=4).

C. Peripheral blood T-cells were stimulated with allogeneic splenic APC in a ratio of 1:1 in the presence or absence of dialyzed anti-HBs IVIg or Cytotect (both at 9 mg/ml). [^{3}H]-thymidine incorporation was determined 5 days later (n=3).

Data are depicted as means ± SEM from n independent experiments with T-cells from different donors.

DISCUSSION

This study shows that the incidence of acute rejection in HBsAg-positive liver transplant patients treated with anti-HBs IVIg was significantly lower as compared to patients transplanted with non-viral liver diseases. This phenomenon may be due to the large proportion of patients with chronic HBV infection within this category. Patients chronically infected with HBV or HCV have certain immune dysfunctions, like functional impairments in their DC [18,19]. However, we consider this explanation highly unlikely. First, the HCV-positive liver graft recipients, which all had a chronic infection, showed a similar risk of rejection as patients without viral hepatitis. Secondly, the reduced incidence of rejection was not confined to patients with chronic HBV-infection, but was also observed in patients transplanted for acute fulminant HBV infection, which are not immunocompromised. Thirdly, in a study on risk factors for acute rejection in liver transplant recipients, Wiesner et al [9] did not find a lower incidence of acute rejection in HBV infected patients. However, the patients included in that study were transplanted in three USA centers between 1990 and 1994, and published data show that during this period in at least two of these centers the $HBsAg^+$ recipients were either not treated with anti-HBs IVIg [20] or were treated with a low dosage regimen [21]. In contrast, two other studies [7,8] in which HBsAg+ liver transplant recipients were treated with the same high-dose regimen of anti-HBs IVIg as our patients [22], confirm our data and report a reduced risk of acute rejection in these patients. Therefore, we propose that the reduced risk of acute rejection in $HBsAg^+$ recipients is not due to chronic infection with the HBV virus, but due to the treatment with anti-HBs IVIg.

In contrast to Farges et al [7] we did not observe any effect of anti-CMV IVIg treatment on the incidence of acute rejection. This discrepancy may be related to differences in treatment regimens and/or the anti-CMV IVIg preparations used in both studies. The patients included in the study of Farges received a higher dose of anti-CMV IVIg [23] as compared to our patients. Unfortunately, resulting serum levels were not reported, and therefore cannot be compared with those reported in our study. In our patients, the serum concentrations of anti-CMV IVIg were considerably lower as compared to serum anti-HBs IVIg concentrations, and did not exceed 2.5 mg/ml. This concentration is probably too low to be effective, since comparable concentrations of anti-HBs IVIg had only mild effects on functional DC maturation in vitro. In addition, although the anti-CMV IVIg preparation used in the present study inhibited functional DC maturation and APC stimulated T-cell proliferation, it did not suppress T-cell proliferation induced by a polyclonal stimulus. This indicates that the anti-CMV IVIg preparation may not inhibit T cells directly, but probably only via APC. In the study of Farges et al anti-CMV IVIg from another manufacturer was used [23], which probably may have a stronger capacity to suppress T-cells directly.

To investigate the mechanism behind the reduced risk of acute liver graft rejection in anti-HBs IVIg treated recipients, we focused on the influence of anti-HBs IVIg on the key cells of the immune system involved in allograft rejection, i.e. DC and T cells. The present study shows that

anti-HBs IVIg, in concentrations similar those reached in the serum of liver graft recipients, suppresses functional cytokine-driven maturation of immature blood DC. The resulting DC showed a reduced capacity to stimulate allogeneic T-cell proliferation. This was not due to an effect of anti-HBs IVIg on the T cells added to the DC, since the anti-HBs IVIg was thoroughly washed away before the T cells were added. Given the importance of CD80 as a co-stimulatory molecule in T-cell activation, the reduced upregulation of this molecule may be at least partly responsible for the reduced allostimulatory capacity of DC maturated in the presence of anti-HBs IVIg. In addition anti-HBs IVIg also inhibited production of cytokines by DC upon stimulation by microbial components. Bayry et al [16] showed similar data using DC derived from monocytes in vitro. However, such DC differ in several aspects from DC present in vivo [24]. The present study is the first to show that an anti-viral IVIg-preparation inhibits critical functions of ex vivo derived DC.

The mechanism by which IVIg suppress DC function is at present unclear. Upregulation of the inhibitory Fcγ receptor IIB after, which binding of anti-HBs IVIg to this receptor invokes inhibitory signals to the DC, a mechanism which has been shown to be involved in the beneficial effects of non-specific IVIg in an experimental animal model of immune trombocytopenia [25], is an attractive hypothesis.

Anti-HBs IVIg also strongly suppressed T-cell proliferation stimulated by both PHA as by allogeneic APC. It is reported that non-specific IVIg have an inhibitory effect on mitogen- and antigen driven T-cell proliferation and cytokine production [13,15,26]. Our data show that an anti-HBs IVIg preparation has similar effects on T cells, but that the anti-CMV IVIg preparation tested inhibited T-cell proliferation primed by an allo-antigen, but not by PHA, which is a non-specific polyclonal T-cell activator. This indicates that anti-CMV IVIg does not inhibit T-cell proliferation directly. The different immunomodulatory properties of these two preparations of anti-viral IVIg may be related to different production procedures. The anti-HBs IVIg preparation (Hepatect CP) used in the present study is purified by HPLC and virus-inactivated by treatment with caprylic acid, tri-n-butyl phosphate and polysorbat 80. The anti-CMV IVIg (Cytotect) is not purified by HPLC and is treated with beta-propiolacton and subjected to nanofiltration for virus-inactivation. Especially the treatment with beta-propiolactone influences the molecular structure of the IgG molecules by alkylation and acylation of certain amino acids in the IgG molecules [27]. According to information from the manufacturer 18% of the IgG molecules in Cytotect are modified during the production process. We think that the lack of a direct effect on T cells by anti-CMV IVIg has to do with the mentioned chemical modification. Apparently, this does not negatively influence it's Fab-mediated activity as Cytotect has excellent CMV-neutralizing properties [28].

In conclusion, prophylactic treatment with anti-HBs IVIg protects against acute rejection after liver transplantation, probably by targeting the two principal immune cell types involved in graft rejection, DC and T cells. IVIg, if slowly infused, have almost no serious side effects [29]. Therefore, anti-HBs IVIg and, if these have similar immunomodulatory efficacy, also non-specific IVIg, may be attractive agents for immunosuppressive therapy after liver transplantation.

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Chapter 4

Superior immunomodulatory effects of intravenous immunoglobulins on human T cells and dendritic cells

- Comparison to calcineurin inhibitors-

Transplantation 2006:81:1725-1734

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SUMMARY

Prophylactic administration of anti-HBs intravenous immunoglobulins (anti-HBs IVIg) in hepatitis B infected liver transplant patients protects against acute rejection. To explore the suitability of intravenous immunoglobulins (anti- HBs IVIg) as prophylaxis of acute rejection and Graft versus Host Disease (GVHD) after allograft transplantation, the effects of IVIg and calcineurin inhibitors (CNI) on human blood-derived T cells and DC were compared. After mitogenic and allogeneic stimulation, T cell proliferation and cytokine production were determined in presence or absence of IVIg or CNI. In addition, immature blood dendritic cells (DC) were stimulated, and allogeneic T-cell stimulatory capacity, cell death and phenotypic maturation were established in presence or absence of IVIg or CNI. We observed that both IVIg and CNI equally inhibited proliferation and IFN-γ production of T cells after stimulation. CD8+ T cells were preferentially affected by both IVIg and CNI. Like CNI, addition of IVIg at later time points after T-cell activation suppressed mitotic progression of responding T cells. Furthermore, IVIg treated DC were suppressed in their capacity to stimulate allogeneic T-cell proliferation by 73±12%, whereas DC function was not affected by CNI. The decreased allogeneic T-cell stimulatory capacity of IVIg treated DC correlated to induction of cell death in DC and decreased up-regulation of CD40 and CD80. From these observations, we concluded that IVIg functionally inhibit the function of two principal immune cell types involved in rejection and GVHD, i.e. T-cells and DC, in vitro, while CNI only suppress T-cell function. By targeting both T cells and DC, IVIg may be a promising candidate for immunosuppressive treatment after allograft transplantation.

INTRODUCTION

To prevent Graft versus Host Disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT), treatment with immunosuppressive drugs is needed. In addition, solid organ transplant recipients require lifelong immunosuppressive treatment in order to prevent graft rejection. In these patients, calcineurin inhibitors (CNI), Cyclosporin (CsA) and Tacrolimus (TAC), are the cornerstones of current immunosuppressive therapy. Common adverse effects of these drugs are hypertension, diabetes, hypercholesterolemia, nephrotoxicity, infections and malignancies [1-7]. These drug related morbidities impair the life expectancy of transplant recipients significantly. Therefore, one of the main focus in transplantation research is to find immunosuppressive regimens, which have no or reduced side effects.

Recently we have reported that prophylactic administration of anti-HBs intravenous immunoglobulins in hepatitis B-infected liver transplant patients protected against acute rejection [8]. Intravenous immunoglobulins (IVIg) are pharmaceutical preparations of human IgG molecules prepared from pools of plasma. Non-specific IVIg are prepared from plasma pools of healthy blood donors, and anti-viral types of IVIg from plasma pools of individuals with high titers of antibodies against particular viruses. Clinically, IVIg exert a broad range of immunomodulatory functions, and are increasingly being used for the treatment of autoimmune and systemic inflammatory diseases (reviewed in [9]). In the context of transplantation, IVIg were reported to be effective as treatment of steroid-resistant acute rejections after kidney transplantation [10,11]. In addition, prophylactic treatment with non-specific IVIg was found to decrease the incidence of GVHD after bone marrow transplantation [12,13]. Still, optimal treatment regimens and mechanisms of action of IVIg in the transplantation setting are poorly defined.

Optimal immunosuppression should target the different immune cells involved in the rejection response. Initiation of the immune response against alloantigens is due to a course of action in which dendritic cells (DC) serve as potent antigen presenting cells (APC), and activate resting T cells [14-17]. In vitro, it has been reported that non-specific IVIg suppress proliferation and cytokine production by human T cells stimulated by mitogens or alloantigens [18-21]. In addition, non-specific IVIg have been found to inhibit the differentiation and function of human monocyte-derived DC (Mo-DC) in vitro [22]. Thus, IVIg may have the potential to suppress the function of the two most important cell types, i.e. DC and T cells, which are involved in immune activation after transplantation.

Since long term IVIg treatment has no significant side effects [9], inclusion of IVIg in immunosuppressive protocols after transplantation may enable dose reduction of CNI, and thereby contribute to a lower rate of complications. However, the immune suppressive activities of IVIg have never been compared to that of CNI, which are the first of choice in current immunosuppressive regimens. CNI are extremely potent inhibitors of T-cell proliferation and cytokine production. Whether CNI also inhibit DC function is less clear. Most reports show that CNI do not affect DC differentiation from human monocytes, and that functional activities of treated

Mo-DC were unaltered [23,24]. However, whether these results can be translated to the in vivo situation is unclear, since these studies were performed with Mo-DC which differ in several aspects from DC present in situ [25].

To explore the suitability of IVIg as a potential immunomodulatory agent to be used as immunosuppressive agent after HSCT or solid organ transplantation, we compared the immunomodulatory effects of IVIg and CNI on human blood-derived T-cells and DC in detail.

MATERIALS AND METHODS

Intravenous immunoglobulins (IVIg) and calcineurin inhibitors (CNI)

Human IVIg (Intraglobin®CP) were a kind gift from Biotest Pharma (Dreieich, Germany). In the experiments two different batches of non-specific type of IVIg were used. IVIg preparations were dialyzed twice against large volumes of culture medium (RPMI) at 4°C using Slide-A-lyzer® gamma irradiated dialysis cassette (Pierce, Rockford, USA). This is to remove stabilizing agents and to obtain neutral pH. After dialysis, IgG concentration was determined by the Tina-quant® immuno-turbidimetric assay (Roche Diagnostics, Mannheim, Germany). CsA and TAC were provided by Novartis Pharma AG, Basel, Switzerland and by Fujisawa Holland BV, Houten, The Netherlands, respectively. CsA was dissolved in a 1:1 mixture of ethanol and 10% Tween-20 in water. Clinically relevant peak levels of IVIg and calcineurin inhibitors were selected for the experiments: IVIg 10 mg/ml, CsA 600 ng/ml and TAC 60 ng/ml. The concentration of the IVIg selected for the experiments was within range of serum anti-HBs immunoglobulins in liver transplant patients treated with anti-HBs IVIg in our center. This concentration (10 mg/ml; 0.06mM) protects against acute rejection 8. Similar increments in serum IgG concentration are obtained in patients treated with IVIg at 1-2 g/kg for autoimmune disorders [26-28]. In addition, clinical relevant peak levels of calcineurin inhibitors were selected for the experiments; CsA 600 ng/ml [29,30] and TAC 60 ng/ml [31].

Isolation of immune cells

DC were purified from fresh heparinized blood of healthy volunteers. After Ficoll density gradient separation and depletion of B cells with CD19-conjugated immunomagnetic beads and separation over Large Depletion (LD)-columns (Miltenyi Biotec, Bergisch Gladbach, Germany), DC were isolated by positive selection with PE-conjugated anti-CD1c mAb and anti-PE immunomagnetic beads using Medium Seperation-columns, as described previously [32]. The purity of DC (defined as CD1c+ CD20- cells) as analyzed by flow cytometry was 91 ± 5 %.

For T-cell experiments, T cells were enriched from the residual B- and DC-depleted cells left over after DC isolations. Immunomagnetic depletion of monocytes and NK cells was performed by labelling the cells with CD14-PE and CD56-PE (both from BD Biosciences) followed by

incubation with anti-PE immunomagnetic beads, and separation over Large Seperation (LS)-columns. These T-cell preparations contained on the average 92 ± 5% CD3+ cells. Contaminating cells consisted largely of granulocytes. For determination of allogeneic T-cell stimulatory capacity of DC, a batch of T cells was prepared from a buffy coat of a healthy blood bank donor by nylon wool filtration. This preparation contained 83% CD3+ cells and 10% CD56+ cells. Spleen antigen presenting cells (APC) were enriched from human spleen tissue obtained from a multi-allograft donor by Ficoll gradient separation and immunomagnetic depletion of T cells and NK cells by labelling the cells with CD3-PE and CD56-PE (both from BD Biosciences) followed by incubation with anti-PE immunomagnetic beads and separation over LD columns. The mixture of APC used in all experiments consisted of 67% B-cells, 7% monocytes and 5.4% DC.

Effects of IVIg and CNI on T-cell proliferation and cytokine production

Triplicate incubations of T cells (1.5 x 10^5) in 200 μl RPMI per well supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Gibco BRL Life Technologies, The Netherlands) and 10% fetal bovine serum (FCS; Hyclone, UT, USA) were either stimulated with phytohemagglutinin (PHA, 5 μg/ml; Murex, France) or with allogeneic spleen APC (1.5 x 10^5) and cultured in the presence or absence of IVIg, human serum albumin (HSA, Sanquin, The Netherlands), CsA, or TAC in flat bottom plates. After 5 days, T-cell proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H] thymidine (Radiochemical Center, Little Chalfont, UK) during 18 hours.

To determine the effects of IVIg and CNI on T-cell expansion and frequency of responding precursors, T cells were labeled with 2μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) per 5 X 10^6 cells for 10 minutes at 37 °C. Labelled T cells (1.5 x 10^5) were stimulated with PHA (5 μg/ml) or with allogeneic spleen APC (1.5 x 10^5), and cultured in the presence or absence of IVIg, CsA, or TAC. After 6 days, T-cell divisions were analyzed by flow cytometry after labeling the cells with CD3-PE (Beckman Coulter Immunotech, France) and CD8-APC (DAKO, Glostrup, Denmark). CFSE-flow cytometry data were analyzed by ModFit TM software version 3.0 (Verity Software House, Topsham, USA). Spacing between the generations was set using the most distinct peaks, and was always about 19 channels, and CFSE fluorescence intensity histograms of CD3+, CD3+CD8+ or CD3+CD8- cells were made by the program. The software calculated the proliferation Index (PI), which is the sum of the cells in all generations, divided by the computed number of original parent cells, thus indicating the extent of T-cell expansion. If PI is one, this indicates that no T-cell division took place during the course of the culture. In addition, precursor frequency (%prec) was calculated, which is the percentage of T cells, which underwent at least one division during the course of the experiment.

To determine whether ongoing division of T cells can be inhibited by IVIg or CNI, CFSE labelled T cells were stimulated with PHA (5 μg/ml) and the tested agents were added to culture at day 0, 1, 2, 3, 4 or 5. T-cell apoptosis was determined with Annexin V-APC (BD Biosciences, Germany) staining.

To study T-cell activation and cytokine production, T cells were stimulated with PHA (5 μg/ml) or with spleen APC (1.5 x 10^5) in the presence or absence of IVIg, CsA, or TAC. At day 3 and day 6 of culture, cell-free supernatants were harvested and the levels of interferon-γ (IFN-γ) and IL-4 were determined by ELISA (U-CyTech, Utrecht, The Netherlands). To exclude blocking effects by IVIg on IFN-γ detection in the ELISA, IVIg were added to recombinant IFN-γ in the ELISA plates. No effect of IVIg on IFN-γ detection was observed. In addition, T cells were harvested at day 6 and re-stimulated with PMA and ionomycin calcium during 6 hours at 37° degrees in the presence of Brefeldin A (all from Sigma; Missouri, USA). Cells were labelled with CD2-RD mAb (Beckman Coulter Immunotech, France) and CD8-PerCp (BD Biosciences, Germany) and, after permeabilization with Intraprep reagent (Beckman Coulter Immunotech, France), with FITC-conjugated anti-IFN-γ mAb (BD Biosciences, Germany), and analyzed by flow cytometry. Furthermore, T cells harvested on day 6 were analyzed for their expression of CD25 using a CD25-PE mAb (BD Biosciences)

Effects of IVIg and CNI on allogeneic T-cell stimulatory capacity of DC

To determine the effects of IVIg and CNI on the acquisition of allogeneic T-cell stimulatory capacity of DC, different numbers of immature blood DC (5, 2.5, and 1.25 x 10^3 cells per well of a 96-well flat bottom plate) (Greiner, Netherlands) were stimulated to maturate with TNFα (25 ng/ml) and IL-1β (50 ng/ml) (both from Strathmann Biotech, Hannover, Germany) in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% FCS and GM-CSF (500U/ml; Leucomax, Novartis Pharma, The Netherlands) for 18 hours in the absence or presence of IVIg, CsA or TAC. The next day, the culture medium was aspirated, DC were washed two times with culture medium to remove additives, and 1.5 x 10^5 nylon wool-enriched allogeneic T-cells were added. In all experiments with DC, one T-cell batch obtained from one healthy blood donor was used. Cell free supernatants were collected at 72 hours after start of the culture, and the levels of IFN-γ were determined by ELISA. After 5 days, T-cell proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H] thymidine during 18 hours.

Cell death analysis and maturation of DC

DC (4x10^4/200μl) were stimulated for 18 hours at 37°C with TNFα, IL-1β and GM-CSF in the presence or absence of IVIg. After 18 hours DC were washed, and 1.5 x 10^5 allogeneic T-cells were added. Using flow cytometry, the percentages of cell death were determined by analyzing the proportions of 7-aminoactinomycin D (7AAD)+ cells (BD Biosciences, Germany) within CD1c+ cells before culture, and 18, 48 and 72 hours after start of the culture. To determine the maturation kinetics DC were labeled with CD1c-PE, anti-HLA-DR-PerCp, CD86-APC, CD40-APC (all from BD Biosciences, Germany), CD80-FITC (Beckman Coulter Immunotech, France), or biotinylated anti-PD-L1 (eBioscience, San Diego, USA) followed by streptavidin-APC (BD Biosciences, Germany), or appropriate isotype control mAb. CD1c+ 7AAD- DC were analyzed for the expression of surface molecules using flow cytometry and data were processed using CellQuest software.

Statistical analysis

In each individual experiment, proliferation and cytokine production was tested in triplicate from which means were calculated. The means from independent experiments were used in the analyses. Statistical analyses were performed by the two-sided student t-test for paired data using software package SPSS version 10.1 (SPSS, Chicago, IL). P-values < 0.05 was considered significant.

RESULTS

Effects of IVIg and CNI on T-cell proliferation

IVIg inhibited PHA stimulated T-cell proliferation in a concentration dependent manner, while HSA had no effect. Maximal suppression by IVIg was observed at 10 mg/ml, by CSA at 800 ng/ml and by TAC at 60 ng/ml (Figure 1A). The IVIg concentration of 10 mg/ml was used in further experiments. IVIg suppressed PHA induced T-cell proliferation on the average by 87%, CsA (600 ng/ml) by 92%, and TAC (60 ng/ml) by 93% (Figure 1B). After allogeneic stimulation IVIg inhibited T-cell proliferation by 81%, CsA by 93% and TAC by 91% (Figure 1C).

To study whether IVIg and CNI interfere with the recruitment of precursor cells or with the expansion of already recruited T cells, T-cell division was monitored by CFSE-staining. IVIg and CNI equally suppressed the number of divisions and the expansion of the T cells after PHA stimulation (Figure 2A and B). In addition, IVIg and CNI equally reduced the proportion of precursor T cells (%prec) responding to PHA (Figure 2C). Likewise, after stimulation with allogeneic splenic APC, IVIg and CNI inhibited T-cell expansion (Figure 2D) and the proportion of precursor T cells responding to allogeneic stimulation (Figure 2E) to a similar extent.

To determine whether IVIg or CNI preferentially affect proliferation of CD4+ or CD8+ T-cell subsets, CFSE dilution was determined for CD3+/CD8- and CD3+/CD8+ T cells separately. This analysis revealed that IVIg and CNI inhibited equally CD3+/CD8- and CD3+/CD8+ T-cell division after PHA stimulation (data not shown). However, after allogeneic stimulation both IVIg and CNI preferentially inhibited proliferation of CD8+ T cells (PI: 2.49 ± 0.49 in control, 1.10 ± 0.1 in IVIg, 1.13 ± 0.2 in CsA and 1.08 ± 0.1 in TAC treated conditions ($p < 0.05$) (% precursors: 14.39 ± 3.9 in control, 0.8 ± 0.3 in IVIg, 0.9 ± 0.8 in CsA and 1.2 ± 0.5 in TAC treated conditions ($p < 0.05$). Expansion of CD8+ T cells was completely inhibited, while some CD3+/CD8- helper T cells divided and reached similar numbers of generations as in the untreated conditions (Figure 3). This resulted in an increased CD4/CD8-ratio at the end of the cultures in the presence of IVIg (CD4/CD8: 4.5±0.9), CsA (CD4/CD8: 5.2±1.1) or TAC (CD4/CD8: 4.4±0.8) compared with control cultures (CD4/CD8: 2.7±0.6; $p < 0.03$). (Figure 3)

To compare the ability of IVIg and CNI to inhibit mitotic progression of already proliferating T cells, IVIg or CNI were added at different time points after PHA stimulation. Suppression of T-cell

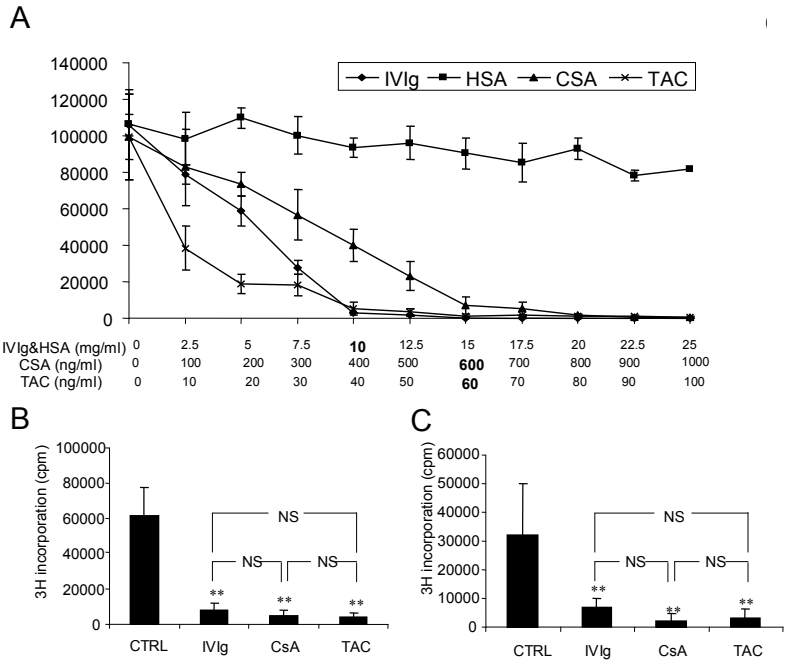

Figure 1: Effects of IVIg and CNI on T-cell proliferation

A. Human T cells were stimulated with PHA in presence of various concentrations of IVIg, CsA, TAC or HSA. After 5 days [3H]thymidine incorporation was determined (n = 3).

B. Comparison of the effects of IVIg (10 mg/ml), CsA (600 ng/ml) and TAC (60 ng/ml) on T-cell proliferation after PHA stimulation (n=7).

C. Comparison of the effects of IVIg (10 mg/ml), CsA (600 ng/ml) and TAC (60 ng/ml) on T-cell proliferation upon stimulation with allogeneic spleen antigen presenting cells (APC) (n=7).

Data are depicted as means ± SD from independent experiments.

** $P < 0.01$ compared to control conditions

division was maximal when IVIg or CNI were added at the initiation of the cultures, but also addition at later time points resulted in suppression. Later addition affected T-cell expansion and the proportions of precursor T cells (%prec) responding to the stimulus significantly when added at day 0, day 1 or day 2 (Figure 4 A and B). A possible explanation for the reduced T cell proliferation when cultured with IVIg is induction of apoptosis of T cells. Annexin V staining was conducted on day 5 after T cells were stimulated with PHA in presence or absence of IVIg. Of IVIg treated T cells, 45.9 ± 4.1% were Annexin V positive compared to the control in which 28.4 ± 4.7 % of T cells were Annexin V positive ($p < 0.05$; n=3). The suppression of T-cell proliferation by IVIg is at least partially due to induction of apoptosis.

Effects of IVIg and CNI on T-cell cytokine production

The mode of action of IVIg on T-cell activation includes inhibition of T-cell activation, since IVIg suppressed the up-regulation of CD25 (Figure 5A). To compare the effects of IVIg and

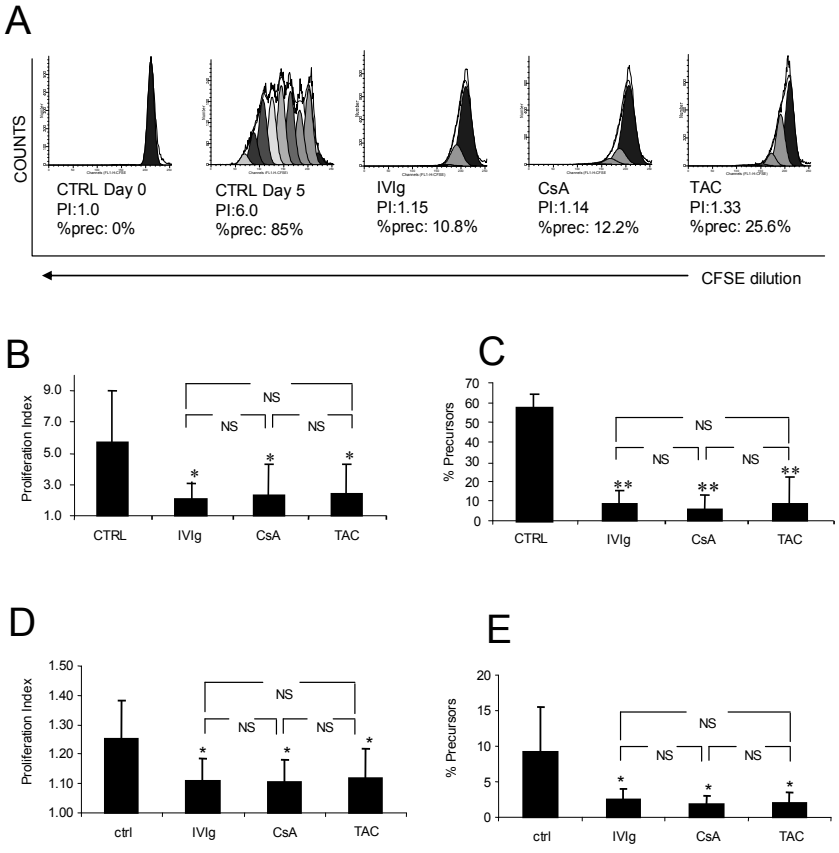

Figure 2: Effects of IVIg and CNI on T-cell recruitment and expansion

T-cell division was monitored by flow cytometric analysis of CFSE-dilution after 6 days, and analyzed by ModFitTM software.

A-C: The effects of IVIg (10 mg/ml), CsA (600 ng/ml) or TAC (60 ng/ml) on proliferation of T-cells stimulated with PHA. A: Histograms of CFSE dilution from one representative experiment. Day 0: all T-cells are in parent peak (black). Day 5: T-cell division visualized by dilution of CFSE over daughter cells in absence or presence of tested agents. B: Proliferation Index (PI), which indicates the extent of T-cell expansion as calculated by ModFit TM software. C: Precursor frequency (%prec), which is a measure for the percentage of T cells responding to the stimulus. The results are means ± SD of five independent experiments.

* $P < 0.05$ ** $P < 0.01$ compared to control conditions

D-E: T cells were stimulated with allogeneic antigen presenting cells (APC) in presence or absence of IVIg (10 mg/ml), CsA (600 ng/ml) or TAC (60 ng/ml). D: Proliferation Index (PI), and E: Precursor frequency (%prec). The results are means ± SD of five independent experiments.

* $P < 0.07$ compared to control conditions

CNI on cytokine secretion by T cells, IFN-γ and IL-4 concentration ware determined in cell free supernatants of stimulated T cells at day 3 and day 6. As is shown in Figures 5 B and C, both after stimulation with PHA and with allogeneic APC IFN-γ synthesis was suppressed by IVIg. The extent of suppression was comparable to that of CNI. In allogeneically stimulated T-cell cultures the secretion of the Th2 cytokine IL-4 was determined. IL-4 production was reduced compared

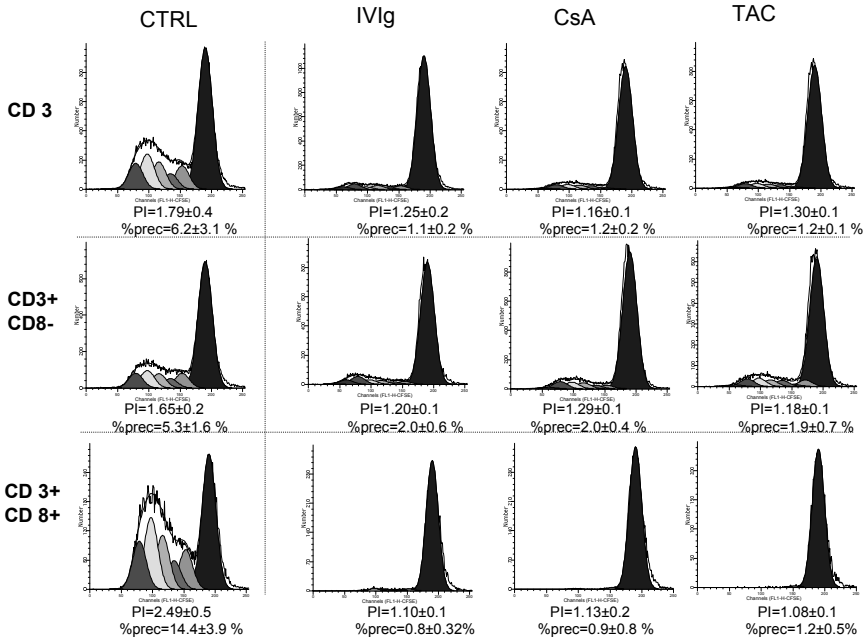

Figure 3: Effects of IVIg and CNI on cytotoxic and helper T-cell division

The effects of IVIg, CsA or TAC on CFSE-dilution in helper CD3+/CD8- and cytotoxic CD3+/CD8+ T-cells after stimulation with allogeneic APC. The histograms are from one experiment representative for four independent experiments.

to IFN-γ production (1.17 ± 0.77 ng/ml in control conditions), but was also inhibited by IVIg (0.42 ± 0.28 ng/ml) and TAC (0.16 ± 0.06) to a comparable extent (n = 3).

To determine whether the tested agents inhibited only the rate of IFN-γ secretion, or the proportion of T cells producing IFN-γ, PHA and allogeneic stimulated T cells were harvested at day 6 and re-stimulated with PMA and ionomycine. Thereafter, IFN-γ production was analyzed by flow cytometry. IVIg and CNI both suppressed the numbers of T cells producing IFN-γ after PHA stimulation (Figure 5D). Since we had observed that after allogeneic APC stimulation IVIg and CNI preferentially affected CD8+ T cell proliferation, the proportions of cells producing IFN-γ were analyzed separately for the CD2+/CD8- and CD2+/CD8+ T cell subsets. Figure 5E shows that IVIg and CNI reduced the numbers of helper, as well as the numbers of cytotoxic T cells producing IFN-γ. These data show that IVIg and CNI inhibit not only the production rate similarly, but also the number of T cells capable of producing IFN-γ.

Effects of IVIg and CNI on allostimulatory capacity of DC

To establish the effects of IVIg and CNI on the function of DC, immature blood DC were stimulated in vitro with pro-inflammatory cytokines (IL-1β and TNF-α) in the presence or absence

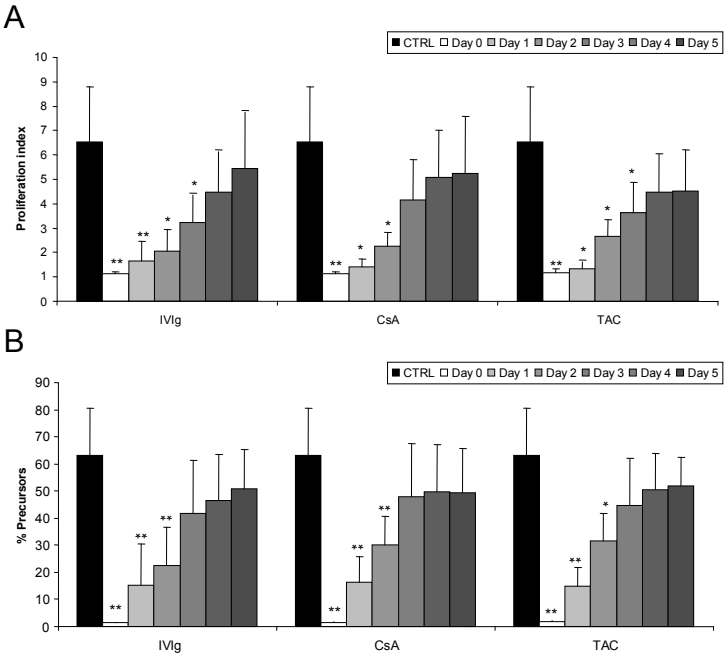

Figure 4: Effects of IVIg and CNI on mitotic progression of proliferating T cells

CFSE labelled T cells were stimulated with PHA, and IVIg (10 mg/ml), CsA (600 ng/ml) or TAC (60 ng/ml) were added to culture at day 0,1,2,3,4 and 5. CFSE-dilution was determined after 6 days and proliferation Index (PI) (A) and precursor frequency (%prec) (B) were calculated. The results are means ± SD of four independent experiments.

* $P < 0.05$ ** $P < 0.01$ compared to control conditions

of IVIg or CNI. After 18 hours, T cells from an unrelated donor were added to the DC to assess their allogeneic T-cell stimulatory capacity. Freshly isolated immature blood DC had a poor allogeneic T-cell stimulatory capacity, which was strongly up-regulated during cytokine driven maturation (data not shown). Addition of IVIg to DC during their maturation suppressed their acquisition to stimulate allogeneic T cell proliferation by 73 ± 12% ($n = 6$; $p < 0.01$). A lower concentration of IVIg inhibited DC function in a lesser extent (data not shown). In contrast, DC matured in presence of CsA (600 ng/ml) or TAC (60 ng/ml) were not affected in their stimulatory capacity of allogeneic T cells (Figure 6A). Determination of IFN-γ in supernatants harvested 3 days after addition of T cells to pre-treated mature DC, revealed that co-cultures of T cells with IVIg treated DC produced significantly less IFN-γ in comparison to the co-cultures of T cells with control DC (2.0 ± 1.2 ng/ml in IVIg treated condition versus control 4.1± 0.8 ng/ml; $p < 0.05$, $n = 4$), supporting the reduced allogeneic T-cell activation by IVIg treated DC.

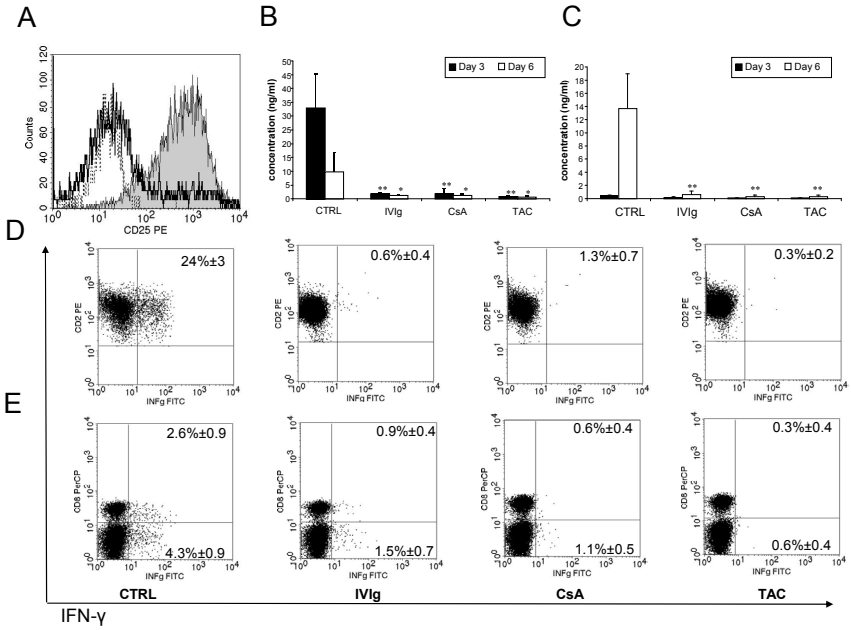

Figure 5: Effects of IVIg and CNI on T-cell activation and cytokine production

A. T cells were stimulated with PHA in the presence or absence of IVIg (10 mg/ml), and on day 6 CD25 expression was determined. Depicted are: CD25-expression on T cells stimulated in the absence of IVIg (gray histogram), presence of IVIg (open histogram with solid line), and isotype-matched control histogram (open histogram with dotted line).

B and C: T cells were stimulated with PHA (B) (N=6) or allogeneic splenic APC (N=4) (C), in presence or absence of IVIg, CsA or TAC, and after 3 and 6 days IFN-γ concentration was determined in the supernatant. * $P < 0.05$ ** $P < 0.01$ compared to control conditions.

D and E. Six days after PHA-stimulation (D) or allogeneic APC stimulation (E) in presence or absence of IVIg, CsA or TAC the numbers of IFN-γ producing T cells was determined. T cells were harvested and re-stimulated with PMA and ionomycin and the proportion of T cells that produced IFN-γ was analyzed by flowcytometry. The percentages depicted in the pictures are means ± SD of three independent experiments.

Effects of IVIg on DC viability and immunophenotypic maturation

To analyze whether the reduced T-cell stimulatory capacity of IVIg treated DC was due to IVIg induced cell death of DC, percentages of 7AAD+ cells within the CD1c+ DC population and expression of maturation markers were determined in freshly isolated DC (= time point 0) and at time points 18, 48 and 72 hours. Until time point 18 hr the DC were matured with TNFα, IL-1β and GM-CSF in the presence or absence of IVIg, but without T cells present. Thereafter, supernatants were removed, and allogeneic T cells were added. In short, the conditions were comparable to the conditions in the allogeneic T-cell stimulation experiments described above. As is shown in Figures 6 B and C, immediately after maturation no difference in DC viability was observed between IVIg-treated DC and control DC. But at 48 hours, which is 30 hours after IVIg had been removed and allogeneic T cells had been added, a significant increase of cell death was observed in IVIg maturated DC. On the contrary, when untreated DC were cultured with T

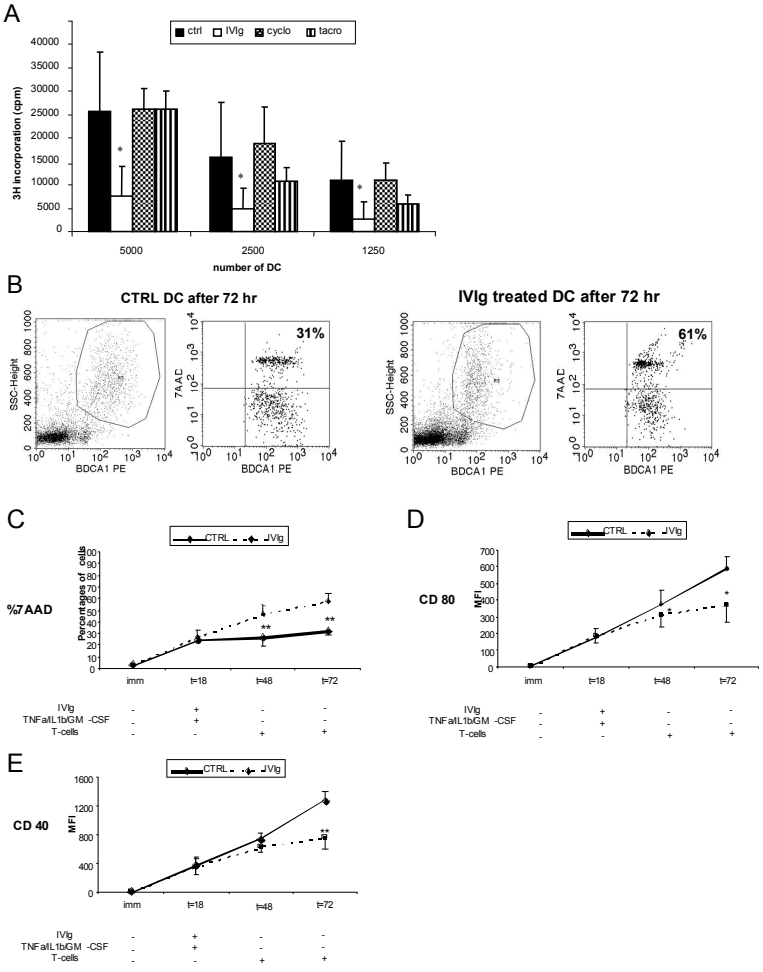

Figure 6: Effects of IVIg and CNI on DC

A. Effect on allostimulatory capacity. Different numbers of immature blood DC (5, 2.5, and 1.25 x 103 cells per well) were stimulated with TNF-α (25 ng/ml) and IL-1β (50 ng/ml) and GM-CSF for 18 hours in the absence or presence of IVIg (10 mg/ml), CsA (600 ng/ml) or TAC (60 ng/ml). Subsequently, the culture medium was aspirated, DC were washed two times with culture medium to remove additives, and 1.5 x 105 allogeneic T cells were added. After 5 days, T-cell proliferation was assessed by determination of the incorporation of 0.5 μCi [3H]thymidine during 18 hours. Each condition was tested in triplicate from which means were calculated. These means were used in the analyses. The results are means ± SD of six independent experiments. * $P<0.05$ compared to control conditions.

B and C. Effect on cell death. DC (40 x 103 cells) were stimulated with TNF-α, IL-1β and GM-CSF for 18 hours in the absence or presence of IVIg (10 mg/ml). Then, DC were washed two times to remove additives, and 1.5 x 105 allogeneic T-cells were added. B: Representative dot plots of 7AAD incorporation into CD1c+ DC maturated in the presence or absence of IVIg, two days after addition of the T cells. DC were gated on CD1c+ against side scatter. C: Percentages of 7AAD+ cells of CD1c+ cells determined before culture (imm), immediately after maturation (t = 18 hrs), one day (t = 48 hrs), and two days after addition of T cells (t = 72 hrs). Note that after t = 18 h, no IVIg was present in the cultures. Data are means ± SD of six independent experiments.

D and E. Effect on expression of co-stimulatory molecules. Expression of CD80 (D) and CD40 (E) on DC during the culture. Data are means ± SD of four independent experiments, respectively.

* $P < 0.05$ ** $P < 0.01$ compared to control conditions.

cells the viability remained stable. At 72 hours of culture, IVIg treated CD1c+ DC incubated with allogeneic T cells were 58 ± 6% 7AADpositive compared to the control in which 32 ± 4 % of CD1c+ DC were 7AAD+ ($p < 0.01$, $n = 6$). In addition, IVIg suppressed the up-regulation of CD80 and CD40 molecules on DC (Figure 6D and 6E). The expression of HLA-DR, CD 86 and PDL-1 on IVIg treated DC did not differ from control DC (data not shown).

DISCUSSION

This is the first study that compares the immunomodulatory effects of IVIg and CNI on the two principal human immune cell types involved in acute rejection and GVHD after allogeneic transplantation, i.e. T cells and DC.

Firstly, using two methods to determine T-cell proliferation, we found that the inhibitory effect of a clinically achievable concentration of IVIg on T-cell proliferation is similar to the effect of therapeutic peak concentrations of CNI. Similar concentrations of HSA had no effect on T-cell proliferation. This excludes that the suppressive effect of IVIg is merely due to an elevated protein concentration in the cultures. After PHA or allogeneic stimulation, both IVIg and CNI inhibited the recruitment of precursor cells and their expansion. Experiments in which IVIg and CNI were added at later time point after initiation of the T-cell cultures showed that both agents were capable of suppressing mitotic progression of already recruited T cells. In addition, IVIg and CNI were equally effective in suppression of IFN-γ and IL-4 production by T cells. Like CNI, IVIg inhibits T-cell activation, as shown by the suppression of CD25 up-regulation. Collectively, these findings show that IVIg can control T-cell activation after alloantigen presentation as effectively as CNI, even after its onset.

The mechanism of action by which IVIg interfere with T-cell proliferation probably involves a direct effect of IVIg on the T cells, since not only alloantigen stimulated, but also PHA stimulated T cells were inhibited in their proliferation. The mechanism by which IVIg suppress activation of T cells is at present unknown. The direct inhibitory effect of IVIg on T cell proliferation is at least partly due to induction of apoptosis in T cells. However, the increased rate of apoptosis cannot totally explain the strong inhibition of approximately 87% by IVIg. After allogeneic stimulation both IVIg and CNI preferentially inhibited the proliferation of CD8+ T cells. Since CD8+ T cells are the actual cells that destroy the transplanted allograft [33], this finding supports the notion that IVIg may be equally able to control rejection as CNI. Secondly, we observed that immature human blood-derived myeloid DC stimulated in presence of IVIg were suppressed in their capacity to stimulate allogeneic T cells. Our group reported similar results with anti-HBs IVIg [8]. The present study shows that the suppression of DC function is not restricted to the anti-viral type of IVIg, but is also the case of non-specific type of IVIg. The suppressive effect of IVIg on DC was not due to the elevated protein concentration in the cultures, since addition of an equal concentration of HSA during DC-maturation had no effect on T-cell stimulatory capacity of DC

[8]. Bayry et al. showed that IVIg inhibit the differentiation, maturation and T-cell stimulatory capacity of human Mo-DC in vitro 9. Mo-DC differ in several aspects from myeloid blood CD1c+ DC present in situ [25]. Nevertheless, the present report shows that the suppressive effect of IVIg on the allogeneic T-cell stimulatory capacity of blood DC is comparable to that on Mo-DC. However, whereas IVIg suppressed the up-regulation of co-stimulatory molecules on Mo-DC during maturation [22], IVIg had no immediate effects on immunophenotypic maturation of blood DC. We observed only a delayed effect on the up-regulation of CD40 and CD80, which became apparent after IVIg had been removed and allogeneic T cells had been added. The reduced T-cell stimulatory capacity of IVIg treated blood DC may also be caused by the induction of cell death in DC. Also this effect of IVIg was not observed immediately after DC maturation, but became apparent at later time points after IVIg had been removed and allogeneic T-cells had been added. To exclude non viable T cells from the analysis, we selected CD1c+ cells to identify the proportion of non-viable 7AAD+ DC. Therefore, underestimation of percentage of cell death in these cultures cannot be excluded, since DC will finally lose their CD1c expression when they lose their membrane integrity. Whether IVIg treatment itself induces delayed DC death, or whether IVIg interfere with T-cell regulated DC-survival is presently unclear. Supporting the possibility that IVIg directly induces death of DC, IVIg has been reported to induce apoptosis in B cells [26,34].

Taken together, we propose that the impaired T-cell stimulatory capacity of blood DC, when treated with IVIg, is caused by induction of cell death and reduced up-regulation of CD40 and CD80. The present study shows that IVIg inhibit T-cell activation and suppress DC function. Interestingly, simultaneous targeting of T-cells and DC has been shown to result in tolerance induction in macaques [35]. Therefore, IVIg-treatment may be able to contribute to graft tolerance. However, this subject merits further investigation.

Most reports showed that CNI do not affect DC differentiation from monocytes [23,36]. However, studies of the influence of CNI on the maturation of human DC have led to discrepant observations. While a few studies reported that the presence of CNI during maturation of human DC suppressed their capacity to stimulate allogeneic T cells [36,37], others observed no effect [23]. Most of these studies were performed using Mo-DC. In this study we found that CNI do not suppress the acquisition of allogeneic T-cell stimulatory capacity during cytokine induced maturation of blood DC.

In conclusion, IVIg have strong suppressive effects on human T cells and DC, while CNI only inhibit T cells. Therefore, by targeting both T cells and DC, we assume that IVIg will be superior in the control of acute rejection as GVHD in comparison to CNI. As IVIg are registered and safe drugs, we consider IVIg as a promising candidate for prophylaxis of rejection and GVHD prophylaxis in order to ensure a safe patient survival after allogeneic HSCT and organ transplantation.

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Chapter 5

Intravenous Immunoglobulins suppress T-cell priming by modulating the bi-directional interaction between Dendritic Cells and Natural Killer cells

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SUMMARY

The modes of action of intravenous immunoglobulins (IVIg) in exerting their immunomodulatory properties are broad and not fully understood. IVIg can modulate the function of various immune cells, including suppressing the capacity of dendritic cells (DC) to stimulate T cells. In the present study, we showed that DC matured in presence of IVIg (IVIg-DC) activated NK cells, and increased their IFN-γ production and degranulation. The activated NK cells induced apoptosis of the majority of IVIg-DC. In consequence, only in the presence of NK cells, IVIg-DC were 4-fold impaired in their T-cell priming capacity. This was due to NK-cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC) to IVIg-DC, probably induced by IgG-multimers, which could be abrogated by blockade of CD16 on NK cells. Furthermore, IVIg-DC down regulated the expression of NKp30 and KIR receptors, and induced the generation of $CD56^{bright}CD16^{-}CCR7^{+}$ lymph node type NK cells. Our results identify a novel pathway, in which IVIg induce ADCC of mature DC by NK cells, which downsizes the antigen presenting pool and inhibits T-cell priming. By influencing the interaction between DC and NK cells, IVIg modulate the ability of the innate immunity to trigger T-cell activation, a mechanism that can "cool down" the immune system at times of activation.

INTRODUCTION

Intravenous immunoglobulins (IVIg) are pharmaceutical preparations of human IgG purified from pools of plasma of thousands of donors. For decades IVIg have been established as treatment of autoantibody mediated [1] and T-cell mediated inflammatory disorders [2]. The distribution of IgG subclasses in IVIg is comparable to that of IgG in normal human serum, however, unlike IgG purified from a single individual, therapeutic IVIg preparations contain substantial amounts of IgG dimers and traces of multimers, due to the idiotype-anti-idiotype complex formation between IgG molecules from different individuals [3,4]. In general, IVIg were shown to be effective in conditions in which the immune system is hyperactive, but still the mechanisms of action by which IVIg correct immune dysregulation are not fully understood. Various reports showed that the mode of actions of IVIg is multifaceted and complex, involving interference with different components of the immune system. Clinical and immunological improvements induced by IVIg treatment are reported to be profound and to extend the half-life of infused IgG, suggesting that IVIg can modify cellular immune reactivity for prolonged periods [1,5].

Recently, we observed that hyperimmune IVIg against hepatitis B surface antigen (anti-HBs IVIg) protect against acute rejection after liver transplantation, indicating that IVIg treatment can modulate the T-cell mediated immune response against allo-antigens [6]. We and others found that in vitro both anti-HBs IVIg and non-specific IVIg are able to suppress T-cell proliferation and cytokine production, and to impair the allogeneic T-cell stimulatory capacity of blood-derived and monocyte-derived DC [6-9], demonstrating that IVIg can suppress T-cell responses, both during the priming phase as well as in the effector phase. The importance of DC as a cellular target of IVIg in vivo was recently shown in a murine model of immune thrombocytopenic purpura (ITP), in which treatment with IVIg could be replaced by adoptive transfer of IVIg-treated DC [10]. With regard to the mechanism by which IVIg affects DC function, we found that the decreased T-cell stimulatory capacity of IVIg-treated DC (IVIg-DC) was associated with induction of cell death in mature DC. Interestingly, IVIg treatment itself did not induce DC death directly, as the increased death of IVIg-DC only occurred when cultured with other immune cells, i.e. T cells and NK cells [9]. As activated NK cells are capable of killing DC in a number of circumstances [11,12], we hypothesized that NK cells may induce apoptosis of IVIg-DC during the initial phase of DC-NK cell encounter, i.e. before the T-cell activation.

Interactions between DC and NK cells have been documented in a variety of settings, shedding light on the complexity of the bi-directional interaction between these two cell types. Bajenoff et al. showed that NK cells are present in the medulla and the paracortex of lymph nodes, where they closely interact with DC. Upon receiving an inflammatory signal, NK cells interact with DC, and regulate co-localized T-cell responses [13]. Cross-talk between DC and NK cells can result in lysis, inhibition or maturation of DC by NK cells, and reciprocally, DC can activate or inhibit NK-cell functions. The final outcome of DC-NK cell interaction depends on the

conditions in which both cell types encounter each other [12,14], and will subsequently determine the development of the following adaptive immune response.

In this study, we investigated the mechanism by which IVIg modulate the interaction between DC and NK cells, and how this consequentially shapes T-cell priming. Our observations form the basis of a model to clarify how administration of IVIg may "cool down" hyperactivity of the cellular immune system for extended periods.

MATERIALS AND METHODS

Reagents

Human IVIg (Intraglobin® CP) were a kind gift from Biotest Pharma (Dreieich, Germany) and the humanized monoclonal antibody Herceptin® (Trastuzumab) was kindly provided by Roche Pharma (Mijdrecht, The Netherlands). Both preparations were dialyzed against large volumes of culture medium (RPMI) at 4°C using Slide-A-lyzer® gamma irradiated dialysis cassettes (Pierce, Rockford, USA) to remove stabilizing agents and to obtain neutral pH. After dialysis, IgG concentration was determined by the Tina-quant® immuno-turbidimetric assay (Roche Diagnostics, Mannheim, Germany). In all experiments IVIg were used in a concentration of 10 mg/ml (0.06M). This is similar to increments in serum IgG concentration in patients treated with IVIg at 1-2 g/kg for autoimmune disorders [15-17]. To detect binding of IVIg, biotinylated IVIg or rabbit $F(ab')_2$ anti-human IgG-FITC (DAKO Cytomation, Denmark) were used. The biotinylation was performed using D-biotinoyl-ε-aminocaproic Acid N-hydroxysuccinimide ester (Roche Diagnostics, Indianapolis) according to manufacturer's instructions. To exclude that the suppressive effects of IVIg were merely due to an elevated protein concentration in the cultures, Human Serum Albumin (HSA) (Sanquin, Amsterdam, The Netherlands) was used as a negative control.

Isolation of immune cells

DC, NK cells and T cells were purified from fresh heparinized blood of healthy individuals. After Ficoll density gradient separation and depletion of B cells with CD19-conjugated immunomagnetic beads and separation over Large Depletion (LD)-columns (Miltenyi Biotec, Germany), DC were isolated by positive selection with PE-conjugated anti-CD1c mAb and anti-PE immunomagnetic beads using Medium Separation-columns, as described previously [6]. The purity of DC (defined as $CD1c^+$ $CD20^-$ cells) as analyzed by flowcytometry was above 95 %.

NK cells were negatively selected by the NK cell isolation Kit (Miltenyi Biotec) using LD-columns. The purity of NK cells (defined as $CD56^+$ $CD3^-$ cells) as analyzed by flowcytometry was above 95%.

T cells were enriched by immunomagnetic depletion of B cells, monocytes, DC, and NK cells. From the same healthy blood donor T cells were also isolated without NK cell depletion. The

cells were labeled with CD19-conjugated immunomagnetic beads, PE-conjugated anti-CD1c mAb (both from Miltenyi Biotec), CD14-PE, and either or not CD56-PE (both from BD Biosciences, Belgium), followed by incubation with anti-PE immunomagnetic beads and separation over LD columns. The purified T-cell preparations contained 92% ± 4% $CD3^+$ cells and < 0.3% $CD56^+$ cells. Contaminating cells consisted of granulocytes. The T-cell preparation from which NK cells were not depleted contained 80% $CD3^+CD56^-$ T cells and 12% $CD3^-CD56^+$ NK cells.

Effects of IVIg and NK cells on allogeneic T-cell stimulatory capacity of DC

If not otherwise mentioned, in all experiments with DC, immature human blood DC were stimulated to mature with TNF-α (25 ng/ml) and IL-1β (50 ng/ml) (both from Strathmann Biotech, Germany) in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% FCS (Hyclone, UT, USA) and GM-CSF (500U/ml; Leucomax, The Netherlands) for 18 hours in the absence or presence of IVIg or HSA.

To determine the effects of IVIg on the acquisition of allogeneic T-cell stimulatory capacity of DC, and the role of NK cells in this process, different numbers of immature blood DC (5, 2.5, 1.25 and 0.75 x 10^3 cells per well of a 96-well flat bottom plate) (Greiner, Netherlands) were stimulated with TNF-α and IL-1β for 18 hours in the absence (CTRL-DC) or presence of 10 mg/ml IVIg (IVIg-DC). The next day, DC were washed, and either allogeneic T cells, which contained 12% NK cells (i.e. 1.2 x 10^5 plus T cells 0.18 x 10^5 NK cells), or 1.2 x 10^5 pure allogeneic T cells, were added. In all experiments with allogeneic T cells, one T cell and one T + NK cell batch obtained from the same healthy blood donor were used. For addition of autologous NK cells (Figure 5D), 0.18 x 10^5 NK cells from the same donor as the DC, combined with 1.2 x 10^5 allogeneic T cells were added per well. After 5 days, proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H] thymidine (Radiochemical Center, UK) during 18 hours. In addition, to determine the effects of IVIg on T cell and NK cell expansion separately, T cells (with or without NK cells) were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, OR) per 5 x 10^6 cells for 10 minutes at 37 °C. Labeled T cells with or without NK cells from the same donor were cultured with allogeneic CTRL-DC and IVIg-DC. After 6 days, T and NK-cell divisions were analyzed by flowcytometry after labeling the cells with CD3-PE and CD56-APC (both from Beckman Coulter Immunotech, France). CFSE-flowcytometry data were analyzed by ModFit ^{TM} software version 3.0 (Verity Software House, USA). The software calculated the proliferation Index (PI), which indicates the extent of cell expansion, and the precursor frequency (% prec) which is the percentage of cells, which underwent at least one division.

To block the FcγRIII on NK cells, we added CD16 blocking antibody (clone 5D2; 10 μg/ml [18]), which was kindly provided by Dr. M. de Haas (Sanquin Research, The Netherlands) to the cultures.

Detection of binding of IVIg to DC, DC apoptosis and NK-cell activation

To detect binding of IVIg to DC, DC were matured in the presence or absence of biotinylated IVIg for 18 hours in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Ultraglutamine with 4.5 g/l Glucose (BioWhittaker™, Belgium), penicillin, streptomycin, 10% FCS and GM-CSF, TNF-α and IL-1β. DMEM was used instead of RPMI, since RPMI contains biotin. After DC were washed extensively to remove all non-bound IVIg-biotin, biotinylated IVIg on the DC membrane was detected using strepavidin-APC (BD Biosciences). To detect internalization of IVIg-biotin, first surface-bound biotinylated IVIg was detected using streptavidin-PerCP (BD Biosciences). Subsequently, residual free biotinylated IVIg on the cell surface was blocked using biotin-blocking reagent (Dako Cytomation, Denmark), DC were fixed and permeabalized with Intraprep permeabilization reagens (Beckman Coulter Immunotech), and intracellular IVIg-biotin was detected using streptavidin-APC. Alternatively, FITC-conjugated rabbit anti-human IgG $F(ab)_2$ was used to detect binding of non-biotinylated IVIg on the DC surface.

After maturation, the culture medium was aspirated, DC were washed two times with culture medium, and 2.4 x 10^5 allogeneic or autologous NK cells were added. The DC : NK ratio was 1:6, which is between the ratio used in the mixed lymphocyte reaction performed with 5.000 and 2.500 DC (Figure 1A). To determine apoptosis of stimulated DC in these DC-NK co-cultures, the number of active caspase-3 expressing $CD1c^+$ DC was determined after 8 hours incubation with allogeneic NK cells by intracellular labeling with anti-active caspase-3-FITC mAb (BD Pharmingen) using Intraprep permeabilization reagens, after membrane labeling with PE-conjugated anti-CD1c mAb. Secondly, cell death of DC at 18 hours of co-culture of DC with NK cells was detected by Annexin V-APC staining combined with 7-AAD uptake in $CD1c^+$ DC (both BD Biosciences, Germany), and analyzed by flowcytometry. In addition, we determined the absolute numbers of viable DC in the cultures at 18 and 48 hours after co-culture of mature DC with allogeneic NK cells by adding a fixed number of Calibrite™ 3 beads (BD Biosciences) to the cells and determining the ratio of $7AAD^-$ DC to beads by flowcytometry. Absolute numbers of viable DC were calculated by multiplying this ratio by the absolute number of beads added to the cells [19].

After 48 hours of DC-NK cell co-culture, supernatants were collected for cytokine production, and NK cell activation was established using CD56-PE, CD69-APC and CD25-APC (all from BD Biosciences). Degranulation of NK cells was determined in 6-hours co-cultures of NK cells and DC by adding PE-conjugated CD107a mAb (BD Pharmingen) to the cultures, according to the protocol described in literature [20,21]. To detect spontaneous degranulation, a control sample of NK cells without DC was included in all experiments. IFN-γ concentration in the supernatants was quantified by ELISA (U-CyTech, The Netherlands).

Effect of IVIg-DC on NK-cell phenotype

To verify whether IVIg-DC promote phenotypical changes of NK cells, we cultured 1.0 x 10^4 DC with 4.8 x 10^5 allogeneic T and 1.2 x 10^6 NK cells (ratio DC: T: NK is 1: 48: 12), and after

5 days we determined the expression of FcγRIII (CD16), Natural Cytotoxicity receptors (NCR), killer immunoglobulin like receptors (KIR) and lymph node homing chemokine receptors using CD16-PE (clone 3G8), NKG2A-PE, NKp30-PE, CD158a-APC, CD158b-APC (all from Beckman Coulter Immunotech, France), CXCR3-APC and CCR7-FITC (both from R&D systems, USA) on $CD56^{+}CD3^{-}$ cells. We have checked whether IVIg prevented binding of the 3G8 mAb to NK cells by pre-incubating NK cells with 10 mg/ml IVIg at 4° C, and found that 3G8 binding was not affected by IVIg.

Statistical analysis

In each individual experiment, proliferation and cytokine production was tested in triplicate from which means were calculated. In the results, means with SD from independent experiments are depicted. Statistical analyses were performed by the wilcoxon test for paired data or the student t-test for paired data using software package SPSS version 10.1 (SPSS, Chicago, IL) as indicated in the legend. A two-sided p-value < 0.05 was considered as indicating significant difference.

RESULTS

Impaired allogeneic T-cell priming by IVIg-DC occurs only in presence of NK cells

To study whether NK cells were involved in reduction of the capacity of IVIg-treated DC (IVIg-DC) to prime allogeneic T cells, we matured DC by addition of IL-1β and TNF-α in presence or absence of 10 mg/ml IVIg for 18 hours. Then the additions were washed out, and either allogeneic T cells, which contained 12% NK cells, or pure allogeneic T cells, where the NK cell contamination level was below 0.3%, were added. When IVIg-DC were co-cultured with allogeneic T and NK cells, their capacity to prime allogeneic T cells was 4-fold impaired ($p < 0.05$, $N = 4$). However, this effect was not observed when NK cells were absent in the cultures (Figure 1A). Using CFSE labeled cells, it was confirmed that the effect of IVIg treatment on the allogeneic T-cell stimulatory capacity of DC is dependent on the presence of NK cells, and that the reduced ^{3}H thymidine incorporation observed in Figure 1A indeed reflected lowered T-cell proliferation. (Figure 1A-C)

IVIg were present on the surface and in the cytoplasm of DC after maturation, and increase DC susceptibility for NK-cell mediated killing

Using biotinylated IVIg allowed clear detection of IVIg in the cytoplasm and on the surface of DC with strepavidin fluorochromes. Binding of IVIg-biotin was detected on the surface of 92 ± 3 % of DC matured in the presence of biotinylated IVIg ($N = 3$). Untreated DC (CTRL-DC) or DC matured in the presence of HSA (HSA-DC) did not bind strepavidin-APC (Figure 2A). A second method using FITC-conjugated rabbit-anti-human IgG $F(ab)_2$ to detect IVIg binding on DC,

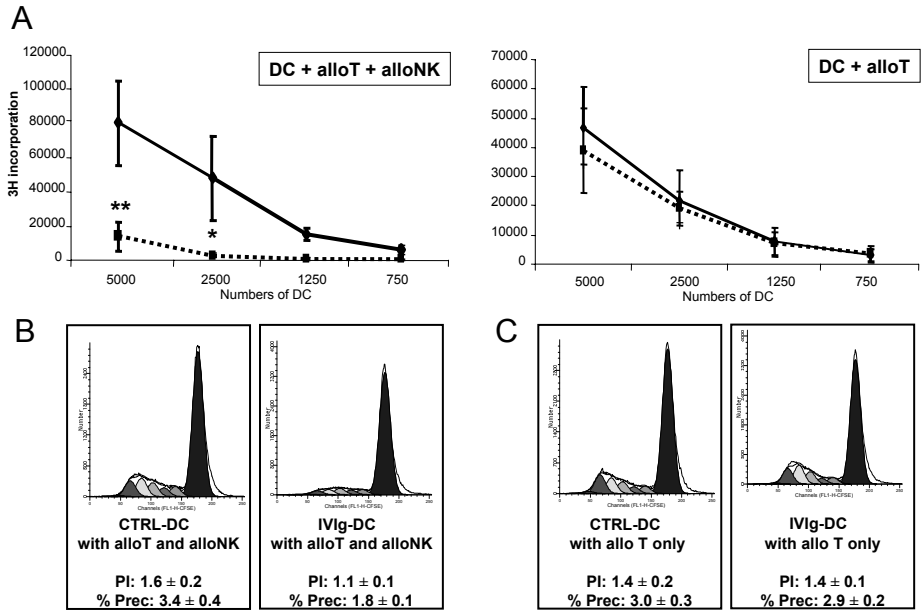

Figure 1: Impaired allogeneic T-cell priming by IVIg-DC occurs only in presence of NK cells

A: Indicated numbers of immature blood DC were stimulated with TNFα and IL-1β for 18 hours in the absence (CTRL-DC, solid line) or presence of 10 mg/ml IVIg (IVIg-DC, dotted line). The next day, DC were washed, and allogeneic T-cells, which contained 12% NK cells, or the same number of allogeneic T-cells without NK cells were added. Proliferation was assessed after 5 days by determination of the incorporation of [^{3}H] thymidine (N=4, * $p < 0.05$, ** $p < 0.01$; paired student t test)

B: Purified allogeneic T-cells and NK cells with CFSE were added and subsequently cultured with CTRL-DC (left) or IVIg-DC (right) at a DC: T: NK ratio of 1:48:12. At day 6 of co-culture, cells were onomyci with CD3-PE and CD56-APC, and the CFSE-dilution profile of $CD3^+CD56^-$ T-cells was analyzed using ModFit^{TM} software. CFSE analysis was also performed on CD56+CD3- NK cells, which is shown in figure 7A separately.

C: The same experiment, as depicted in B, but CFSE-labeled allogeneic T-cells without NK cells, were cultured with CTRL-DC (left) or IVIg-DC (right) at a DC: T ratio of 1 : 48. Proliferation index (PI) and percentage of precursor T-cells (% Prec) depicted in figures B and C are means ± SD from 4 independent experiments.

showed equivalent findings, as 91 ± 9 % of the IVIg-DC were positively stained (N = 4) (Figure 2B).

To determine whether DC internalized IVIg during maturation, we first detected biotinylated IVIg on the DC surface by strepavidin-PerCP. Thereafter, intracellular biotinylated IVIg was detected using strepavidine-APC (Figure 2C). We observed that 90 ± 4% of DC stained for both strepavidin-PerCP and strepavidin-APC, indicating that IVIg was present on the surface as well in the cytoplasm of the DC (N = 3) (Figure 2C). (Figure 2 A-C)

When allogeneic NK cells were cultured with IVIg-DC, the NK cells induced apoptosis of IVIg-DC, as shown by increased intracellular expression of active caspase 3 (CTRL-DC: 7 ± 2%, IVIg-DC: 14 ± 3%, HSA-DC: 7 ± 3%, $p < 0.01$, N = 6) after 8 hours of DC-NK cell encounter (Figure 3A). Subsequently, after 18 hours of co-culture we observed increased DC death, as indicated by enhanced 7-AAD uptake in IVIg-DC (CTRL: 17 ± 8%, IVIg: 33 ± 9%, HSA: 18 ± 6% $p < 0.01$, N

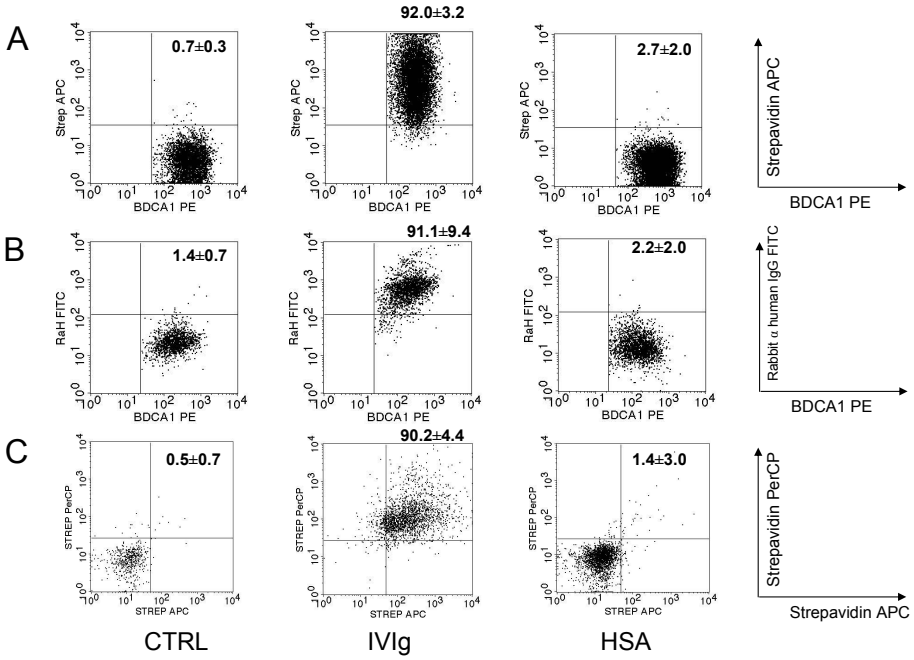

Figure 2: Presence of IVIg on the surface and in the cytoplasm of DC after maturation

A: DC were stimulated for 18 hours in absence (left plot) or presence of biotinylated IVIg (middle plot) or HSA (right plot). Using streptavidin-APC, binding of IVIg-biotin was detected on 92 ± 3 % of the surface of IVIg-DC after maturation (N = 3).

B: DC were stimulated for 18 hours in absence (left plot) or presence of IVIg (middle plot) or HSA (right plot). Rabbit-anti-human IgG-FITC $F(ab)_2$ was used to detect IVIg binding on the DC membrane. This showed equivalent results (N = 4).

C: To determine internalization of IVIg during maturation, biotinylated IVIg on the DC surface was detected by strepavidin-PerCP. Thereafter, residual free biotinylated IVIg on the cell surface was blocked using biotin blocking reagent, DC were fixed and permeabilized and intracellular biotinylated IVIg was detected using strepavidin-APC. We observed that IVIg was present on the surface as well in the cytoplasm of the DC (N=3).

= 9) (Figure 3B). The increased DC death was not the result of allogeneic differences, as autologous NK cells were also able to induce enhanced killing of IVIg-DC (CTRL: 16 ± 9%, IVIg: 35 ± 10%, HSA: 17 ± 8% 7-AAD^+ DC, $p < 0.05$, N = 4).

In addition, Annexin V staining combined with 7AAD was performed. The proportion of AnnexinV^+7AAD^+ cells was increased in the IVIg-DC population (CTRL-DC: 15 ± 6 %, IVIg-DC: 30 ± 15%, $p < 0.05$, N = 6), while the proportion of Annexin V^-7AAD^- IVIg-DC was reduced compared to CTRL-DC (CTRL-DC: 55 ± 12%, IVIg-DC: 40 ± 5%, $p < 0.08$, N = 6). To exclude that the effects of IVIg-treatment of DC were specific for DC stimulated with pro-inflammatory cytokines, we repeated the experiments with DC stimulated with LPS. Indeed, IVIg treatment during DC stimulation with LPS resulted in equivalent increases of DC death after addition of NK cells (data not shown). (Figure 3C and D)

Furthermore, after 18 and 48 hours of co-culture with allogeneic NK cells, we have determined the absolute numbers of viable $7AAD^-$ DC in the cultures by flowcytometry after addition

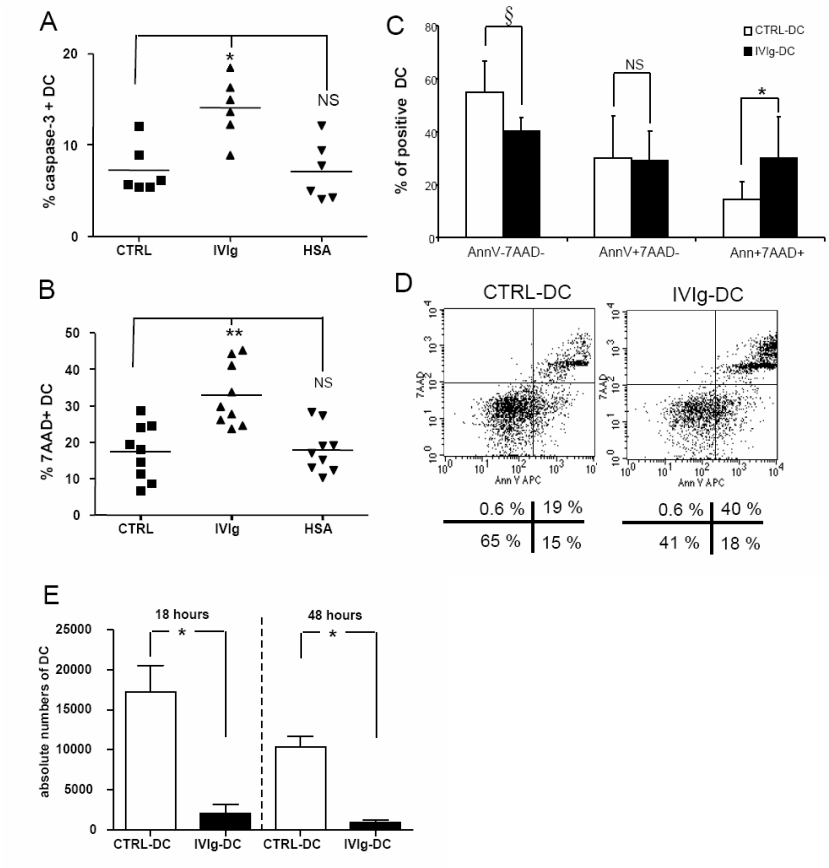

Figure 3: IVIg treatment increases the susceptibility of matured DC for NK-mediated killing

A: After maturation with IL-1β and TNF-α, DC were cultured with allogeneic NK cells at the ratio 1:6. Eight hours thereafter, intracellular expression of active Caspase-3 in CTRL-DC (■), IVIg-DC (▲) and HSA-DC (▼) was determined (N = 6, wilcoxon test for paired data * $p < 0.05$ compared to CTRL-DC, NS = not significant).

B: After 18 hours of co-culture, DC-death was monitored by 7-AAD uptake in CTRL- DC (■), IVIg-DC (▲) and HSA-DC (▼) (N = 9, wilcoxon test for paired data ** $p < 0.01$, compared to CTRL-DC, NS = not significant).

C and D: After 18 hours of co-culture of matured DC with allogeneic NK cells, DC-apoptosis was monitored by Annexin V staining combined with 7-AAD (N = 6, wilcoxon test for paired data § $p < 0.08$, * $p < 0.05$ compared with CTRL-DC, NS = not significant). Panel D shows plots of one representative experiment out of 6 experiments.

E: After 18 and 48 hours of co-culture of 40.000 matured DC with allogeneic NK cells (DC: NK ratio 1:6) cells were harvested from the cultures and fixed numbers of Calibrite™ beads were added. Absolute numbers of viable DC were calculated by determining the ratio of $7AAD^-$ DC to beads and then multiplying this ratio by the number of beads in the tube. Data is depicted as mean with SE, wilcoxon test for paired data * $p < 0.05$, N=7)

of known numbers of Calibrite™beads. After co-culture with NK cells the absolute numbers of viable IVIg-DC were profoundly reduced compared to CTRL-DC (18 hours: IVIg-DC: 2046 ± 1173, CTRL-DC: 17181 ± 1266, 48 hours: IVIg: 968 ± 266, CTRL: 10318 ± 1363, $p < 0.05$, N = 7) (Figure 3E).

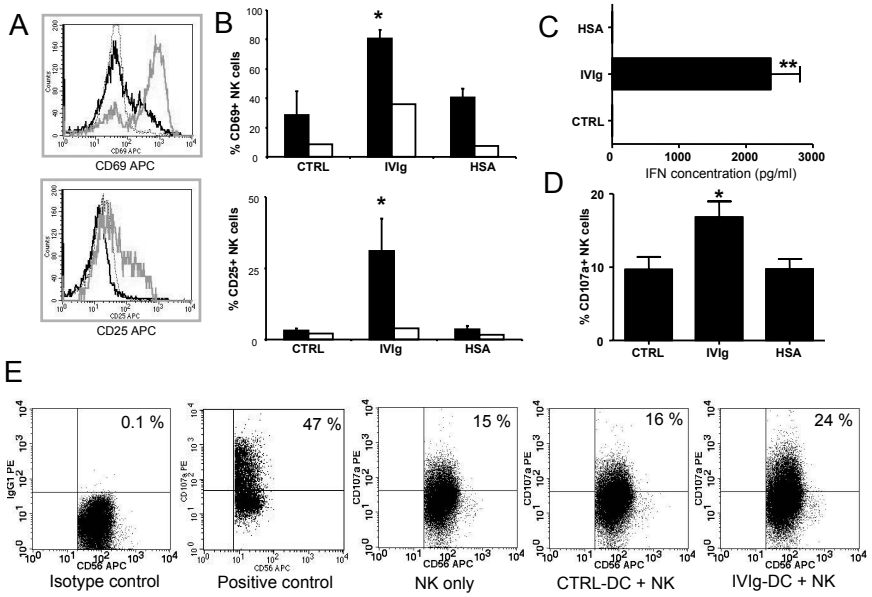

Figure 4: IVIg-DC trigger NK-cell activation, IFN-γ production and degranulation

A: IVIg-DC, HSA-DC or CTRL-DC were cultured for 48 hours with allogeneic NK cells (ratio DC: NK was 1:6) after which CD69 (top plot) and CD25-expression (lower plot) on $CD56^+$ cells were determined. Depicted are NK cells activated by IVIg-DC (grey histogram), by CTRL-DC (black histogram), and staining with an irrelevant isotype control mAb of NK cells activated by IVIg-DC (dotted histogram). Similar NK-activation was observed when DC: NK ratio was changed to 1:3 or 1:1 (data not shown).

B: IVIg-DC, HSA-DC or CTRL-DC were washed to remove additives, and cultured for 48 hours with allogeneic NK cells (black bars). CD69 expression (* $p < 0.05$, wilcoxon for paired data, $N = 6$) and CD25 expression (* $p < 0.05$, wilcoxon for paired data, $N = 5$) on NK cells were significantly enhanced after co-culture with IVIg-DC. Treatment of NK cells with 10 mg/ml IVIg or HSA in absence of DC (white bars) had only minor activating effects on NK cells.

C: IFN-γ concentration was determined in cell-free supernatants of DC-NK co-cultures. In the supernatants of NK cells stimulated with CTRL-DC or HSA-DC, no IFN-γ could be detected, while in the supernatants of NK cells stimulated with IVIg-DC, high IFN-γ levels were detected (** $p < 0.01$, wilcoxon for paired data, $N = 7$).

D: Expression of the lytic granule membrane protein CD107a on the NK-cell surface after 6 hours of co-culture of matured allogeneic CTRL-DC, IVIg-DC or HSA-DC (* $p < 0.05$, wilcoxon test for paired data, $N = 5$).

E. Representative dot plots showing CD107a expression on NK cells cultured for 6 hours without DC (negative control), NK cells stimulated with PMA and ionomycine (2.5 ug/ml and 0.5 ug/ml) (positive control), NK cells co-cultured with CTRL-DC, and NK cells co-cultured with IVIg-DC.

IVIg-DC trigger NK-cell activation, IFN-γ production and degranulation

To investigate the effect of IVIg-DC on NK-cell activation and cytokine production, allogeneic NK cells were co-cultured with IVIg-DC, HSA-DC or CTRL-DC. After 48 hours of co-culture, we observed that IVIg-DC promoted activation of allogeneic NK cells as determined by CD69 expression (CTRL: 29 ± 16 %, IVIg: 81 ± 6 % and HSA: 41 ± 6 %, $p < 0.01$, $N = 6$) and CD25 expression (CTRL: 3 ± 0.8%, IVIg: 31 ± 11% and HSA: 4 ± 1 %, $p < 0.01$, $N = 5$) on NK cells (Figure 4A and 4B).

In contrast, 48 hours treatment of NK cells with 10 mg/ml of IVIg in absence of DC had marginal activating effects on NK cells, showing that full NK-cell activation requires both DC and the IVIg-treatment of DC prior to co-culture with NK cells. Moreover, supernatants collected at 48 hours from the co-cultures of IVIg-DC and NK-cells contained high levels of interferon γ (IFN-γ) (IVIg-DC and NK-cells: 2.4 ± 0.2 ng/ml), while in the supernatants of NK cells cultured with CTRL-DC or HSA-DC, no IFNγ could be detected (N = 7) (Figure 4C). IL10 production was not detectable in all collected supernatants. Again, the increased activation and cytokine production of NK cells was not due to allogeneic differences, since experiments performed with autologous NK cells showed equivalent results (data not shown).

To elucidate whether IVIg-DC stimulate NK-cell degranulation, we determined the expression of the lytic granule membrane protein CD107a on the NK cell surface. About 10% of NK cells cultured without DC spontaneously expressed CD107a, and CTLR-DC did not induce degranulation above this background. We observed an increased surface expression of CD107a on NK cells only after they had been co-cultured with IVIg-DC (CTRL-DC: 10 ± 4 %, IVIg-DC: 17 ± 5%, HSA-DC: 10 ± 3 %, $p < 0.05$, N = 5) (Figure 4D and E). The enhanced degranulation of lytic granules by NK cells after co-culture with IVIg-DC supports the conclusion that NK cells are responsible for the enhanced killing of IVIg-DC.

Impaired allogeneic T-cell priming is due to ADCC of IVIg-DC by NK cells

To investigate whether NK-cells recognize IVIg treated DC via their FcγRIII (CD16), a CD16-blocking antibody (clone 5D2) was added into DC-NK cell cultures. The increased NK-cell-mediated killing of IVIg-DC, as assessed by 7-AAD uptake in DC, was significantly reduced from 37 ± 7 % to 28 ± 3 % of $CD1c^+$ DC ($p < 0.05$, N = 5) when the CD16-blocking antibody was added, while CD16 blockade had no effect on NK-cell mediated lysis of CTRL- and HSA-DC (Figure 5A). In addition, by blocking CD16, more viable IVIg-DC were present after co-culture with NK cells (Figure 5B). This indicates that the increased NK-cell mediated apoptosis of IVIg-DC by NK cells is largely due to FcγRIII-mediated Antibody Dependent Cellular Cytotoxicity (ADCC).

ADCC of IVIg-DC by NK cells was indeed the main cause of the reduced T-cell priming capacity of IVIg-DC. When the 5D2 antibody was added to block the FcγRIII on NK cells during co-cultures of IVIg-DC and allogeneic T- and NK cells, the difference in allogeneic priming capacity between IVIg-DC and CTRL-DC was almost completely abolished (Figure 5C). Again, the impairment in T-cell priming of IVIg-DC was not the result of allogeneic differences between DC and NK cells, as autologous NK cells also killed IVIg-DC from the same donor and reduced the allogeneic T-cell priming. In addition, impairment of IVIg-DC function by autologous NK cells could be abrogated by addition of the 5D2 mAb (Figure 5D). Addition of an equivalent concentration of mouse-IgG2a mAb as a control for the 5D2 blocking antibody to the cultures did not restore the impaired T-cell stimulatory capacity IVIg-DC (data not shown).

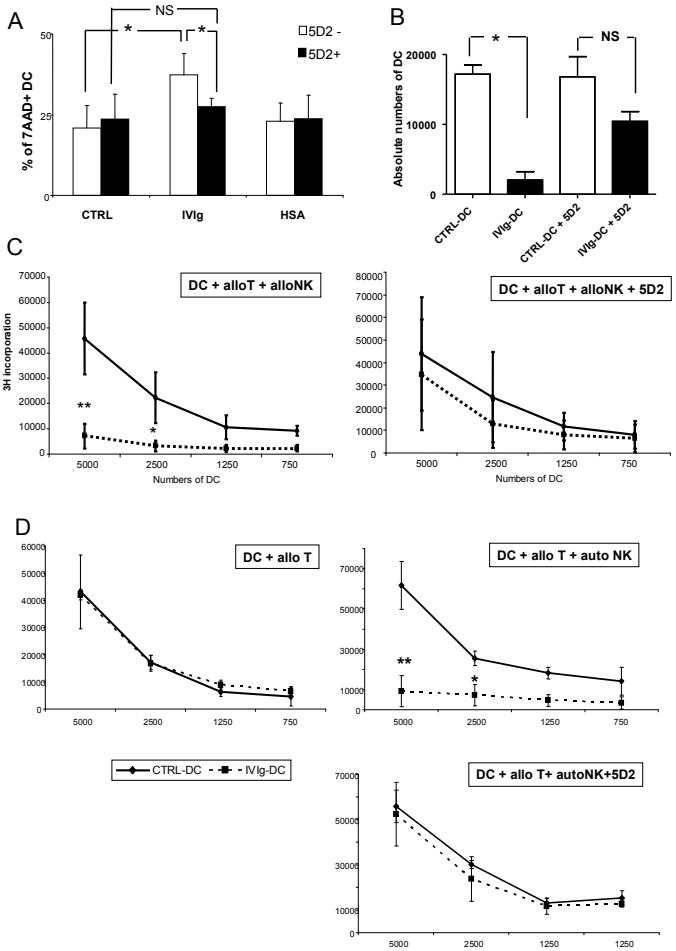

Figure 5: Impaired allogeneic T-cell priming is due to ADCC of IVIg-DC by NK cells

A: Matured CTRL-DC, IVIg-DC or HSA-DC were cultured for 18 hours with allogeneic NK cells (ratio 1:6) with (black bars) or without (white bars) 10 µg/ml CD16-blocking antibody (5D2). DC-death was determined by 7AAD uptake. The increased death of IVIg DC was abrogated by blocking the FcγRIII on NK cells (* $p < 0.05$, NS = not significant, N = 5, wilcoxon test for paired data).

B: Matured CTRL-DC (white bars) and IVIg-DC (black bars) were cultured for 18 hours with allogeneic NK cells (ratio 1:6) with or without 10 µg/ml 5D2 antibody. Absolute numbers of viable DC were calculated by determining the ratio of $7AAD^-$ DC to detected beads and then multiplying this ratio by the number of beads in the tube. Data is depicted as mean with SE, wilcoxon test for paired data * $p < 0.05$, NS = Not significant ($p = 0.14$), N=7)

C: Matured IVIg-DC (dotted line) or CTRL-DC (solid line) were cultured for 5 days with allogeneic T-cells and allogeneic NK cells from the same donor in the absence (left graph) or presence (right graph) of CD16-blocking antibody (5D2) (10 µg/ml). Addition of 5D2 restored, the capacity of IVIg-DC to stimulate allogeneic T-cells (N = 6, wilcoxon test for paired data, ** $p< 0.01$, * <0.05).

D: Matured IVIg-DC (dotted line) or CTRL-DC (solid line) were cultured for 5 days with allogeneic T-cells alone (left graph), or with allogeneic T-cells and autologous NK cells (right graph). In the latter case DC and NK cells are from the same donor. In addition, DC was cultured with autologous NK cells and allogeneic T-cells and CD16-blocking antibody (5D2) (10 µg/ml) was added to the culture (right below graft). Addition of 5D2 restored the capacity of IVIg-DC to stimulate allogeneic T-cells (** $p< 0.01$, * <0.05, N=3).

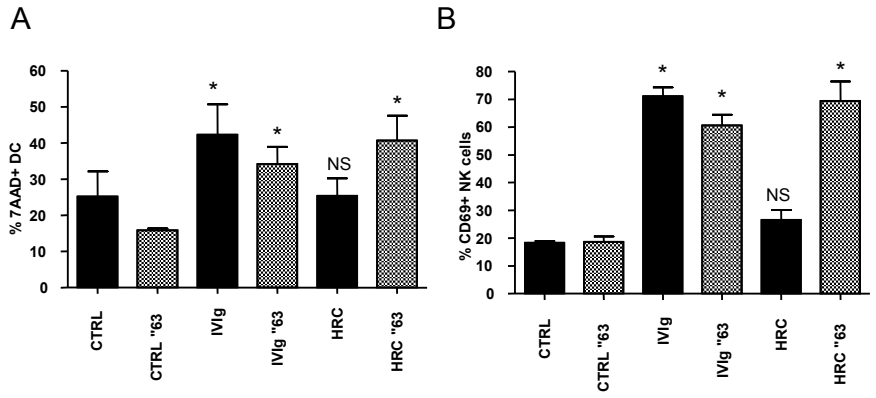

Figure 6: IgG multimers, and not monomers, cause ADCC of IVIg-DC by NK cells

A and B: Culture medium (CTRL), IVIg, and humanized monoclonal antibody Herceptin (HRC), were heated at 63° C for 30 minutes (grey bars) or left untreated (black bars) before addition to immature blood DC during their maturation with pro-inflammatory cytokines. IVIg and HRC were added to the DC in a concentration of 10 mg/ml. After 18 hours of maturation, all additions were removed, and allogeneic NK cells were added at ratio DC: NK 1:6. Percentages of $7AAD^+$ DC (A) and CD69 expression on NK cells (B) were determined after 18 and 48 hours of DC-NK co-culture respectively. ($N = 4$, * $p < 0.05$, wilcoxon test for paired data)

IgG multimers, and not monomers, cause ADCC of IVIg-DC by NK cells

Using the fully humanized and glycosylated monoclonal antibody Herceptin (HRC), which contains only the complementary determining regions of a murine mAb that binds to human epidermal growth factor receptor 2 [22,23], we asked whether human IgG monomers were able to promote DC lysis by NK cells. Herceptin treatment of maturing DC (HRC-DC) did not increase NK cell activation and DC lysis in co-cultures of DC and NK cells. However, when Herceptin was aggregated at 63° C for 30 minutes, which induces the formation of IgG aggregates [24], maturation of DC in presence of the aggregated form of Herceptin promoted DC lysis by NK cells, and induced NK-cell activation (Figure 6A and 6B). This observation suggests that the stimulation of the reciprocal interaction between DC and NK cells by IVIg is not mediated by IgG monomers present in the therapeutic IVIg preparations, but by IgG multimers and/or dimers.

IVIg-DC promote expansion of NK cells and induce $CD56^{bright}CD16^{-}CCR7^{+}CXCR3^{+}$ lymph node type NK cells

We showed that IVIg-DC, when co-cultured with allogeneic T cells and NK cells, have a decreased T-cell stimulatory capacity, and that this effect is dependent on the presence of NK cells. Moreover, looking at the proliferation of NK cells in these cultures using CFSE-dilution, we observed that IVIg-DC did not only activate the NK cells, but also promoted their proliferation. When IVIg-DC were co-cultured with T and NK cells 88 ± 3 % of all $CD56^+$ cells, 91 ± 3 % of CD56 ^{bright} cells and 86 ± 4 % of CD56 ^{dim} cells had undergone more than one round of

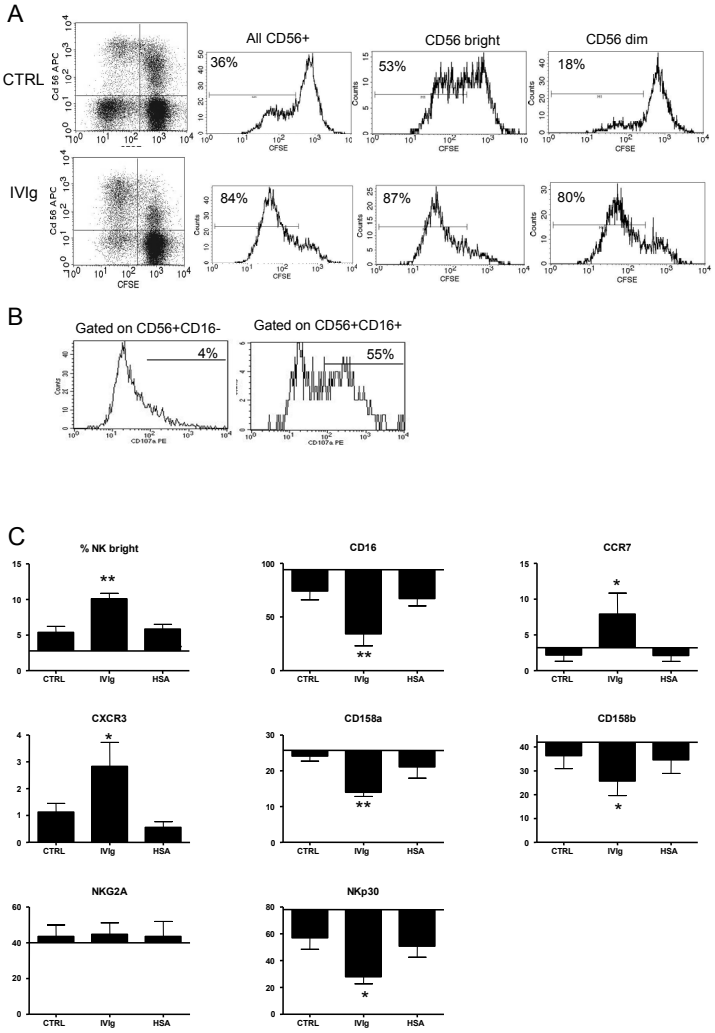

Figure 7: IVIg-DC promote expansion of NK cells and induce $CD56^{bright}CD16^{-}CCR7^{+}CXCR3^{+}$ lymph node type NK cells

A: CTRL-DC (upper plots) or IVIg-DC (lower plots) were cultured with CFSE-labeled allogeneic T-cells and NK cells at the ratio DC: T: NK 1: 48: 12 and after 5 days proliferation was determined. Dotplots: the two upper quadrants show proliferation of NK cells ($CD56^{+}CD3^{-}$), the two lower quadrants show proliferation of the T-cells ($CD56^{-}CD3^{+}$). Histograms: proliferation of all NK cells (left histogram), $CD56^{bright}$ (middle histogram) and $CD56^{dim}$ NK cells (right histogram). The plots are representative for one out of four experiments. Percentages indicate proportions of NK cells that have undergone at least one division.

B. After the 5 days culture of NK cells with IVIg-DC, NK cells were stimulated with PMA and ionomycine (2.5 ug/ml and 0.5 ug/ml) and CD107a expression on $CD56^{+}CD16^{+}$ and $CD56^{+}CD16^{-}$ subsets was determined.

C: After 5 days of culture with CTLR-DC, IVIg-DC or HSA-DC, percentages of $CD56^{bright}$ NK cells, and percentages of NK cells expressing CD16, KIR receptors CD158a and CD158b, lymph node homing chemokine receptors CCR7 and CXCR3, and Natural Cytotoxicity Receptors NKG2A and NKp30, were determined. NK cells cultured with IVIg-DC upregulated CCR7 and CXCR3, and downregulated CD16, the KIR receptors and NKp30 ($N = 4$, * $p < 0.05$ compared to NK cells co-cultured with CTRL-DC, student t test for paired data). The percentages of cells are displayed within reference to the baseline percentage of expression of the markers on the NK cells before addition to culture. No expression of CXCR3 on NK cells was detected at this time point.

division, compared to CTRL-DC co-cultures where 48 ± 13 % of all $CD56^+$ cells, 59 ± 5 % of $CD56^{bright}$ cells and 35 ± 10 % of $CD56^{dim}$ started to proliferate ($p < 0.05$ for comparison of the proliferation of $CD56^+$ NK cells, $CD56^{bright}$ cells and $CD56^{dim}$ cells upon stimulation with IVIg-DC compared to CTRL-DC; N = 4; Figure 7A). Thus, while CTRL-DC stimulated proliferation of $CD56^{bright}$ NK cells more strongly than proliferation of $CD56^{dim}$ cells, IVIg-DC promoted similar expansion of $CD56^{bright}$ and $CD56^{dim}$ NK cells. NK-cell proliferation was not observed in co-cultures of DC and NK cells without T cells, showing that T cells, although dispensable for NK activation, were indispensable for NK cell proliferation. To dissect differences in cytotoxicity, we harvested NK cells from the cultures, and determined CD107a expression on both $CD56^+CD16^+$ and $CD56^+CD16^-$ NK cell subtypes upon PMA/ionomycine stimulation. Indeed, a difference in CD107a-expression between the two NK subtypes, gained after 5 days of culture with IVIg-DC and T-cells, was detected. The $CD16^+$ subset express a higher level of CD107a than the $CD16^-$ NK cells after stimulation (Figure 7B). We concluded that, while IVIg enhances the proliferation of both subsets, their cytotoxic profile is unchanged.

In addition, the 5 days co-cultures of NK cells with IVIg-DC and T cells resulted in higher proportions of $CD56^{bright}$ NK cells in comparison with CTRL-DC. After cultures of CTRL-DC with T and NK cells, 5.4 ± 2 % of $CD56^+$ cells were $CD56^{bright}$, while upon cultures with IVIg-DC, 10.1 ± 2 % of the $CD56^+$ cells were $CD56^{bright}$ (Figure 7C). Moreover, upon stimulation with IVIg-DC higher percentages of NK cells expressed the lymph node homing chemokine receptors CCR7 and CXCR3. Furthermore, NKp30, which is reported to be one of receptors involved in DC-NK cell interaction [25], was down-regulated when NK cells were co-cultured with IVIg-DC. The expression of CD16 and of the KIR-receptors, CD158a and CD158b, on NK cells was also reduced when NK cells are cultured with IVIg-DC and T cells. Together, these results show that in presence of T cells, IVIg-DC stimulated vigorous proliferation of NK cells, and promoted immunophenotypic changes resulting in an enrichment of $CD56^{bright}CD16^-CCR7^+$ $CXCR3^+$ lymph node type NK cells.

DISCUSSION

The present study unravels the main mechanism of action by which IVIg hamper the process of T-cell priming by DC, i.e. by inducing Antibody Dependent Cellular Cytotoxicity (ADCC) of IVIg-treated DC by NK cells. By binding to the DC surface, IVIg facilitate recognition of mature DC by NK cells via FcγRIII, resulting in activation of the NK cells. These NK cells acquire the capacity to kill the IVIg-DC by ADCC, thereby reducing in numbers the total stimulatory pool of mature DC. Interestingly, multimers, but not monomers of human monoclonal IgG, could recapitulate the effects of IVIg in our culture systems, suggesting that IgG multimers and/or dimers in IVIg preparations are the active components that induce NK-cell activation and DC lysis. In addition, IVIg-DC stimulate the proliferation of NK cells and induce $CD56^{bright}$ NK cells with lymph node homing properties, which can further shape the adaptive immune response.

Clinically, IVIg have been shown to be effective for the treatment of a variety of immune-mediated inflammatory diseases [1], but still its actual mode of action on the cellular components of the human immune system remains unclear. We showed that by modulating the interaction between two cell types of innate immune system, i.e. DC and NK cells, IVIg can affect the development of the linked adaptive immune response.

First, we demonstrated that IVIg bind to the surface of maturing DC. By using a blocking mAb for FcγRIII, we demonstrated that ADCC of IVIg-DC occurs due to binding of the Fc-region of cell-bound IgG to FcγRIII on NK cells. More specifically, since humanized monoclonal antibody multimers, but not monomers, could recapitulate the effects of IVIg on DC-NK interaction, death of DC is induced upon recognition of DC-bound IgG multimers by FcγRIII on NK cells. Indeed, binding of immune complexes to FcγRIII has been reported to induce target cell lysis by NK cells, through activation via an immunoreceptor tyrosine based activation motif [26]. CD69 is an early marker for NK cell activation, and was enhanced profoundly when NK cells were cultured with IVIg-DC. IVIg treatment of NK cells alone was not able to induce the same level of activation of NK cells. Although also the matured CTRL-DC alone were able to activate NK cells to some extent, which is in agreement with numerous reports [12,14,27,28], only NK activated by IVIg-DC secreted substantial amounts of IFN-γ, and degranulated their cytotoxic granules. Therefore, both IVIg and DC were needed for full NK-cell activation. NK-cell activation and degranulation resulted in killing of the majority of DC matured in presence of IVIg, which could be largely abrogated by FcγRIII blockade. Therefore killing of IVIg-DC is predominantly attributable to ADCC mediated by activated NK cells. Since death of IVIg-DC was not completely abrogated by CD16-blockade, additional cytotoxic mechanisms may contribute to the profound killing of IVIg-DC in the presence of NK cells, e.g. cytotoxic effect of IFN-γ produced by the NK cells [29].

So far to our knowledge, this phenomenon has not been described as one of the potential modes of action of IVIg on the cellular immune system. Supporting our finding, administration of high doses of IVIg in patients with Kawasaki disease has been reported to increase the numbers of circulating NK cells, and to elevate ADCC activity of NK cells [30]. However, this does not exclude that also other mechanisms may contribute to the impaired T-cell stimulatory capacity of IVIg-treated DC. In a previous study we observed that IVIg-treated blood DC, when cultured with T and NK cells, were suppressed in their up-regulation of co-stimulatory molecules CD40 and CD80 [9]. In addition, IVIg have been shown to suppress differentiation of human monocytes to DC, and to inhibit maturation of monocyte-derived DC [8]. Moreover, IVIg have been shown to induce regulatory activity in murine DC via activating Fcγ-receptors [10]. However, according to the dramatic effect of CD16 blockade on the T-cell stimulatory capacity of IVIg-DC (Figure 5C and 5D), we reckon that NK-mediated DC killing is the most important mechanism of action by which IVIg reduces the T-cell stimulatory capacity of DC.

In vivo NK cells interact with DC in lymph nodes and in inflamed peripheral tissues [13,31-35], and the conditions in which DC and NK cells encounter can determine the following T-cell responses [12-14]. Equipped to perform "DC editing", NK cells can induce killing of immature DC,

that fail to undergo proper maturation. Since mature DC are resistant to lysis by NK cells, this is believed to provide help to create fully potent adaptive responses [32,36]. However, while performing their DC editing task, NK cells may, upon IVIg therapy, also recognize the mature IVIg-DC as a target cell, as it is fully coated with IgG. In this case, the NK cell may kill the IVIg-coated mature DC, and thereby reduce the pool of immunogenic antigen presenting cells migrating from inflamed tissue to the lymph node, or present in the lymph node, and thereby dampen the following T-cell responses.

It is intriguing, that our own IgG exerts predominantly pro-inflammatory properties, e.g. as inducer of complement- or cell-mediated cytotoxicity, while high-dose therapeutic IgG treatment has anti-inflammatory effects. Our finding that humanized monoclonal IgG only caused NK-cell mediated ADCC of DC after heat-aggregation, may elucidate this discrepancy. We propose that the active anti-inflammatory components in IVIg are IgG-multimers and dimers, which are not present in human serum from one individual, but are formed in plasma pools due to idiotype-anti-idiotype complex formation between IgG molecules from different individuals [3,4]. This hypothesis is supported by studies that described IgG polymers in commercial IVIg preparations to be the active component in various immune mediated disease models [4,37]. In addition, during normal physiological immune responses, IgG immune complexes can be formed, which may also stimulate NK-cell mediated lysis of DC, and thereby inhibiting T-cell activation. In the line of this thinking, accumulating evidence supports an immune regulatory role of immune complexes in physiological immune responses. In two different IVIg-treatable autoimmune disease models in rodents, i.e. ITP and arthritis, Siragam et al. demonstrated that both small soluble and large particulate immune complexes can mimic the therapeutic effects of IVIg treatment [38]. Recently, it was shown that IgG immune complexes induce an IFN-γ refractory state in macrophages via binding to FcγRIII on their surface, thereby providing a potential mechanism for the immunosuppressive effects of immune complexes in IFN-γ driven inflammatory responses [39].

Human $CD56^{bright}CD16^{-}$ NK cells (NK^{bright}) and $CD56^{dim}CD16^{+}$ NK cells (NK^{dim}) are reported to have distinct functions. The NK^{dim} subset exhibits primarily a cytotoxic effector function, whereas NK^{bright} cells can produce immunoregulatory cytokines such as IFN-γ, tumor necrosis factor β (TNF-β), IL10, IL13 and GM-CSF [40,41]. Surprisingly, IVIg-DC stimulated immunophenotypic changes in NK cells resulting in an increased proportion of NK^{bright} cells, with reduced expression of CD16, NKp30 and the KIR receptors, CD158a and CD158b, and enhanced expression of the chemokine receptors CCR7 and CXCR3, which can mediate NK-cell migration to secondary lymphoid organs [31,42,43]. Together, these immunophenotypic changes confirm the induction of lymph node type NK cells [31,44,45]. To date the mechanism of action by which IVIg-DC promote enrichment of the NK^{bright} population is not understood. Since IVIg-DC stimulate proliferation of NK^{dim} and NK^{bright} cells to a similar extent, the enrichment of NK^{bright} cells is not due to differential expansion. Most likely, it is due to differentiation from NK^{dim} cells. NK^{bright} cells maintain interactions with DC in lymph nodes for extended periods, are not cytotoxic, but

have immunomodulatory functions [13,34,46]. Induction of NK^{bright} cells may, therefore, constitute a mechanism of action by which immunomodulatory effects of IVIg treatment remain after IVIg have been cleared.

Therefore, we speculate that in vivo IVIg treatment exerts its effect on cellular immunity by affecting NK-DC crosstalk. An immediate immunosuppressive effect is caused by promoting the interaction between cytotoxic NK^{dim} cells and DC resulting in killing of mature DC. This is expected to occur mainly in inflamed non-lymphoid tissues [35]. Recent evidence confirms that NK cells can prevent immune activation in vivo. NK cells regulated T-cell priming in a skin transplant model by killing donor antigen presenting cells [47], and NK-cell alloreactivity after hematopoietic stem cell transplantation from unrelated donors is associated with decreased incidence of Graft-versus-Host Disease [48,49].

In the light of our findings, we propose a model to clarify how administration of IVIg may dampen hyperactivity of the cellular immune system for extended periods. IgG multimers and dimers present in IVIg preparations stimulate NK cell mediated ADCC of mature DC, and thereby prevent proper T-cell priming. This model has also physiological relevance, in that it predicts that immunoglobulin-antigen immune complexes may contribute to the termination of the T-cell responses during clearance of infections and to maintain tolerance to self-antigens. As immune complexes emerge at the end of the humoral responses, physiological immune complexes as well as immune complexes in IVIg preparations may be the essential element that contributes to the termination or "cooling down" of the immune system after prolonged activation.

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Chapter 6

Functional dichotomy of Natural Killer cells in organ transplantation

Expert Review in Clinical Immunology 2007:3:3: 261-266

Evaluation of the paper: NK cells promote transplant tolerance by killing donor antigen-presenting cells. Yu G., Xu X., Vu M. et al. The Journal of Experimental Medicine 203, 1851-1858 (2006)

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SUMMARY

Natural killer (NK) cells have the potential to display different functional activities after transplantation. The traditional view is that NK cells have the capacity to contribute to rejection by facilitating the activation/differentiation of leukocytes that destroy the graft. In contrast, in the manuscript under review a novel role for NK cells was identified in the setting of co-stimulation blockade where alloreactive NK cells of recipient origin were found to have the capacity to kill donor derived antigen presenting cells (APC), thereby reducing T cell priming and promoting long term skin graft acceptance.

INTRODUCTION

A major challenge in transplantation immunology is to modulate the functional activity of different types of leukocytes, both to prevent allograft rejection and to promote the development of unresponsiveness/tolerance to the allograft. Immune cells, such as dendritic cells (DC) and T cells, can either be modified to express a regulatory phenotype or subsets of cells with regulatory activity can be identified. These regulatory populations can control or regulate rejection and, in some experimental models, induce long term graft acceptance. The investigation under review explored the role of Natural killer cells (NK cells) in regulating T cell priming by donor antigen presenting DC in a skin transplant model [1].

Recently, an increasing number of reports have suggested that the role of NK cells after transplantation is not as straight forward as was once thought. The traditional view is that NK cells are predominantly pro-inflammatory and are not sufficient to reject a solid allograft directly, but may contribute to early chemokine and cytokine production after transplantation supporting the rejection response. In liver transplantation, the presence of NK cells has been shown to exacerbate rejection [2], and in a chronic rejection model NK cells can trigger allograft vasculopathy in T cell deficient recipients, but only after receiving adoptively transferred CD4+ T cells [3].

In contrast, NK cells have been implicated as playing a role in the induction of tolerance to organ allografts [1,4], while in hematopoietic stem cell transplantation (HSCT) the presence of NK cell alloreactivity in the graft-versus-host direction has been associated with a reduced incidence of leukemia relapse without causing graft-versus-host disease (GVHD) [5]. Due to this functional dichotomy in the potential functional activities of NK cells, interest in understanding more precisely the interaction between antigen presenting cells (APC), NK cells and T cells in more detail to elucidate their role in responses to alloantigens has been stimulated.

The challenge, unique for transplantation, is the allogeneic mismatch between donor and recipient. When major histocompatibility complex antigens are mismatched, donor derived APC can migrate from the graft and initiate T cell mediated rejection via the direct pathway of allorecognition. NK cell alloreactivity derives from a mismatch between inhibitory receptors for self-major histocompatibility complex (MHC) class I molecules expressed by the NK cells and the MHC class I ligands on the APC. In humans, killer cell Ig-like receptors (KIRs) on NK cells discriminate between self and non-self HLA class I molecules, and in rodents the diverse repertoires of Ly49 receptors recognize groups of MHC class I. Without prior antigen priming, NK cells sense the missing expression of the self-MHC class I alleles on the allogeneic target, and are activated (missing self recognition) [6].

The investigation reported in the manuscript under review identified a novel role for NK cells by demonstrating that they can influence the survival of donor derived APC, and thereby regulate T cell priming [1]. The data presented demonstrate that by killing donor derived dendritic cells thus NK cells can prevent rather than stimulate, rejection.

METHODS AND RESULTS

Full-thickness skin grafts from DBA/2 ($H2^d$) mice were transplanted to C57BL/6 ($H2^b$) $Rag^{-/-}$ and C57BL/6 ($H2^b$) ($Rag^{-/-}$ common $\gamma c^{-/-}$; $Rag^{-/-}\gamma c^{-/-}$) double knockout mice. T cells and B cells are absent in both types of recipient used in the study as a result of the Rag mutation. $Rag^{-/-}\gamma c^{-/-}$ mice are also deficient for NK cells.

The first observation reported in the manuscript demonstrated that the presence of NK cells prevented the survival of skin graft derived APC. This suggested that NK cells present in the recipient could kill donor APC. Thirty days after transplantation of a DBA/2 skin graft to $Rag^{-/-}$ recipients, no donor APC were detected in the spleen, while in $Rag^{-/-}\gamma c^{-/-}$ mice that have no NK cells, APC of donor origin could still be detected. When (DBA/2xB6)F1 DC were purified and adoptively transferred into $Rag^{-/-}$, these DC survived even though NK cells were present. The (DBA/2xB6)F1 DC express both donor ($H2^b$) and recipient ($H2^d$) alloantigens and therefore express the self-MHC class I allele that can interact with the $H2^b$-specific Ly49C/I inhibitory receptor on recipients NK cells inhibiting NK cell activation.

It is important to note that NK alloreactivity in solid organ transplantation varies to some extent from the situation that arises after allogeneic haematopoietic stem cell (HSC) transplantation. After HSC transplantation, alloreactive NK cells, emerging from the donor stem cell graft, can control leukemia relapse and GvHD due to the graft-versus-leukemia effect and graft-versus-recipients-APC effect respectively [5]. In the setting of organ transplantation, the role of recipient derived NK alloreactivity against donor-graft derived APC is probably more prominent, since the percentage of donor NK cells migrating out of the graft, which can cause alloreactions to recipient APC, is minimal. (Figure 1) Second, NK cells are fully capable of rejecting HSC even in absence of T or B cells, while after solid allograft transplantation NK cells are thought to have a minor role in acute rejection, facilitating rejection by alloreactive T cells, but not playing a critical role.

Next, to determine if NK alloreactivity to graft derived APC affected T cell priming, DBA/2 DC were purified and transferred in to $Rag^{-/-}$ and $Rag^{-/-}\gamma c^{-/-}$. Two weeks later, the recipient mice were reconstituted with CFSE-labelled syngeneic C57BL/6 ($H2^b$) T cells. Vigorous T cell proliferation was detected in the spleen and extralymphoid sites in Rag $^{-/-}\gamma c^{-/-}$, but not in $Rag^{-/-}$ mice.

Furthermore, the authors studied the effect of triple costimulation blockade comprising treatment with CTLA-4Ig, anti-CD154 and anti-OX40L, on T cell activation and skin graft survival in the presence and absence of NK cells. Depletion of NK cells from the recipient mice using a depleting anti-NK1.1 mAb rendered the triple costimulation blockade treatment completely ineffective at preventing the rejection response. All recipients depleted of NK cells and treated with triple costimulation blockade rejected their DBA/2 skin graft with a mean survival time of 22 days, compared to survival of >100 days when NK cells were present. The intriguing question is: How NK cells influence the efficacy of triple costimulation blockade?

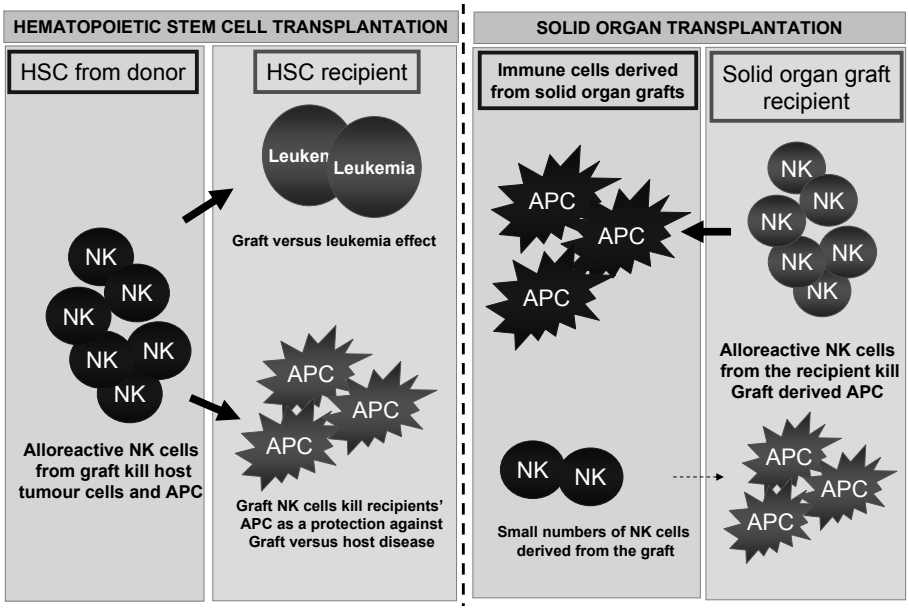

Figure 1: Differences between HSC transplantation and solid organ transplantation regarding NK cell alloreactivity
After solid organ transplantation, the percentage of donor NK cells migrating out of the graft, which can cause alloreactions to recipient APC, is minimal. While after HSC transplantation, alloreactive NK cells, emerging from the donor stem cell graft can kill leukemic cells and host APC.

To demonstrate that the requirement for NK cells to enable skin allograft survival is confined to MHC mismatched organ transplants, the authors transplanted both MHC mismatched DBA/2 ($H2^d$) and minor mismatch 129/svJ ($H2^b$) skin allografts on to the same C57BL/6 ($H2^b$) recipients with or without triple costimulation blockade treatment. When recipient NK cells were depleted, DBA/2 skin was again rejected promptly, while survival of the 129/svJ skin was not affected. By examining the survival of the two different grafts in the same recipient, the authors showed convincingly that the recipient derived alloreactive NK cells can indeed affect the process of T cell priming during tolerance induction through costimulation blockade.

Discussion and significance

The paper under review highlights the importance of DC and NK cells interaction after organ transplantation. Interactions between DC and NK cells have been documented in a variety of settings previously, elucidating the complexity of the bi-directional interaction between these two cell types. Bajenoff et al. showed that NK cells are present in the medulla and the paracortex of lymph nodes, where they closely interact with DC [7]. Cross-talk between DC and NK cells can result in lysis, inhibition or maturation of DC by NK cells, and reciprocally, DC can activate or inhibit NK cells. The final outcome of DC-NK cell interaction depends on the conditions in which the encounter between the two cell types takes place, and it will subsequently direct the development of the adaptive immune response.

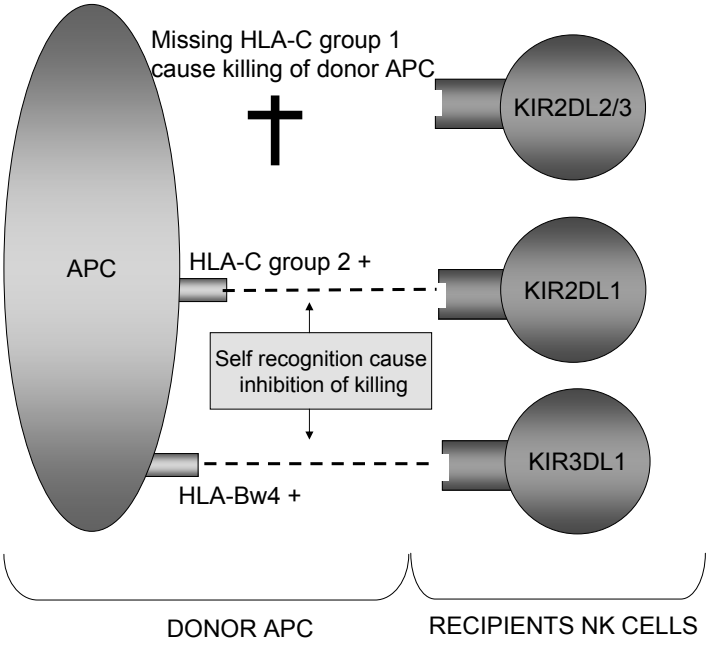

Figure 2: NK cell alloreactivity between donor APC and recipients NK cells
The KIR2DL2/3+ NK cell can not detect its ligand HLA-C (group 1) on the donor APC and according to the missing self hypothesis, this KIR2DL2/3+ NK cell will get activated and kill the donor APC. The table illustrate the variety of human inhibitory NK cell receptor and its ligand. The table is adapted from [8]

In the setting of solid organ transplantation, NK cell alloreactivity derives from a mismatch between the inhibitory receptors expressed by NK cells that recognise self-MHC class I molecules and the MHC class I ligands expressed by donor-derived APC. NK inhibitory killer cell Ig-like receptors (KIRs) discriminate between groups of HLA class I molecules. KIR2DL1 recognizes HLA-C alleles characterized by the lysine residue at position 80 (HLA-Cw4 and related group 2 alleles), whereas KIR2DL2 and KIR2DL3 recognize HLA-C that have an asparagine residue at this position (HLA-Cw3 and related group 1 alleles). KIR3DL1 is the receptor for HLA-Bw4 alleles. (Figure 2) NK cells that do not express KIRs express the CD94/NKG2A receptor instead. These NK cells generally do not display alloreactivity and recognize the poorly polymorphic HLA-E molecules [8]. Most individuals possess a full complement of inhibitory KIRs. The KIR2DL2 and KIR2DL3 receptors are present in all individuals, while the KIR2DL1 receptor for HLA-C group 2 and the KIR3DL1 receptor are found in approximately 97% and 90% of individuals respectively. Therefore, only a small percentage of the population has a limited NK cell KIR receptor repertoire, and cannot mount NK alloreactions due to the specific KIR that is missing (reviewed in [9]). NK cells are highly cytotoxic to target cells, however, evidence for NK cell mediated cytotoxicity to the parenchymal cells present in the graft itself is marginal. Therefore, it is assumed that NK alloreactivity may be restricted to lymphohematopoietic targets, such as APC. (Figure 2)

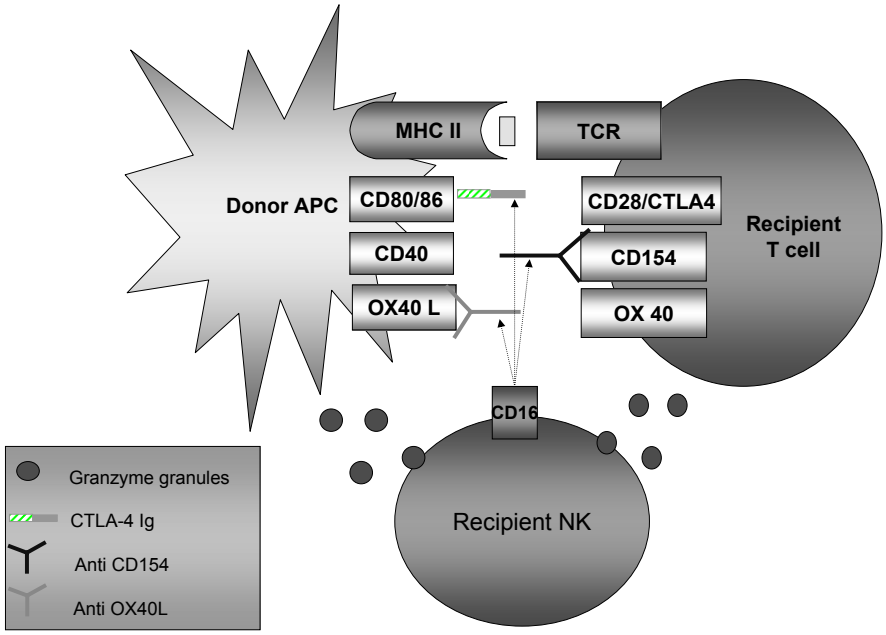

Figure 3: The triple costimulation blockade treatment can render susceptibility of DC and T cells to ADCC killing by NK cells.
By using peri-transplant CTLA4-Ig + anti-CD154 + anti OX40L therapy, the NK cells can mediate ADCC as the CD16 (FcγRIII) on NK cells can bind to the Fc region of the anti-CD154, anti OX40L antibody or CTLA-4 Ig as indicated by arrows. Thus, the NK cells can deplete both donor APC and recipient T cells by release of granzymes, and therefore NK cell mediated ADCC can be at least part of the explanation for the long-term graft survival of the skin grafts shown in the paper under review.

One outstanding question from the data presented is whether killing of donor-derived APC in skin graft recipients treated with triple costimulation blockade, is entirely attributable to NK allorecognition and activation. NK cells also express Fcγ receptor III (CD16), which recognizes IgG1 and IgG3 subclasses. It is well established that NK cells can cause destruction of antibody coated target cell through antibody-dependent cell-mediated cytotoxicity (ADCC). Each component of the triple co-stimulation blockade treatment, anti-CD154, CTLA-4Ig and anti-OX40L has an Fc region and can bind to either the T cell or APC, and thus has the potential render the cells susceptible to ADCC killing by NK cells. Thus, it is quite possible that at least part of the explanation for the long-term graft survival of the skin grafts induced by peri-transplant CTLA4-Ig + anti-CD154 + anti OX40L therapy, is that NK cells mediate ADCC, and thus, deplete both donor APC and recipient T cells. Given the fact that CD154 on T cells is induced upon T cell activation, it is possible that this ADCC mediated by NK cells is relatively selective for those cells responding to alloantigen. (Figure 3) This hypothesis is entirely consistent with the data shown in Figure 4A in the manuscript since it is predicted that in the absence of NK cells, costimulation blockade would be ineffective.

Supporting this hypothesis is the finding that costimulation blockade through CD154 specific antibody is reported to be mediated by Fc-dependent depletion of activated T cells rather than costimulation blockade itself [10]. However, for the survival of minor mismatched grafts, NK cells are not needed. This speculates that ADCC by NK cells will probably not be the only pathway in operation to induce graft acceptance. Costimulation blockade, itself may just prevent T cell priming by APC and induce graft acceptance. However, this does not exclude the possibility that ADCC can play a role when the skin grafts are mismatched for MHC antigens. Possibly, a combination of costimulation blockade and ADCC by NK cells is exerting such pronounced effect on graft tolerance, when the role of NK cell is studied.

Another interesting question would be, whether antigen specific regulatory T cells can be generated when the process of T cell priming is inhibited by NK cell mediated killing of the donor APC. As regulatory T cells can actively promote long term graft acceptance, it is interesting to know whether T cells with regulatory function can be generated in this model.

Expert commentary

NK cell alloreactivity against APC, both in host-versus-graft and graft-versus-host responses, is important and unique for the field of transplantation. Recipient NK cells have the potential to kill graft-derived APC, thereby inhibiting the process of T cell priming via the direct pathway of allorecognition. In addition, the indirect pathway may also be triggered, when NK cells derived from the transplant exert cytotoxicity against the APC of the recipient. The latter is less significant for small grafts, however, for liver grafts where large numbers of viable donor lymphocytes migrate into the recipient after transplantation, NK alloreactivity in the graft-versus-host direction could become important. In support of this idea, the characteristics of liver-associated lymphocytes in liver perfusates are predominantly activated NK cells and $CD8^+$ T cells [11]. In this case, NK cells of donor origin may interact with recipient APC, thereby inhibiting antigen presentation by the indirect pathway of allorecognition.

In the light of findings reported in this paper, immunosuppressive therapies that enhance NK cell survival and activity may facilitate the induction of tolerance to donor alloantigens by killing donor-derived APC after transplantation. Moreover, the data presented suggest that the addition of agents that enhance NK cell function may be of value. So far, current clinical immunosuppressive agents, such as cyclosporine, tacrolimus, mycophenolate mofetil and azathioprine, have little effect on NK cell function (reviewed in [12]).

In contrast to conventional thinking, it is important to note that NK cells can be "the good" or "the bad" in different settings after organ transplantation and as yet, the role of NK cell subtypes is not well understood.

Human $CD56^{bright}CD16^-$ NK cells (NK^{bright}) and $CD56^{dim}CD16^+$ NK cells (NK^{dim}) are reported to have distinct functions. The NK^{dim} subset exhibits primarily a cytotoxic effector function, whereas NK^{bright} cells can produce immunoregulatory cytokines such as IFN-γ, tumor necrosis factor β (TNF-β), IL-10, IL-13 and GM-CSF [8]. In addition, NK cells are innate immune effector

cells expressing stimulatory receptors, such as NKG2D and NKp46, which recognize antigens on virus infected cells and tumour cells, while sparing normal cells. In immunocompromised transplant patients, it is essential to try and maintain these useful functions of NK cells on the one hand, while on the other the effects of immunosuppressive therapies on NK cell function should considered.

Key issues

- Natural killer cells (NK cells) can display different functional activities after transplantation.
- The role of NK cell alloreactivity after solid organ transplantation and after haematopoietic stem cell transplantation varies in some extent.
- In the setting of costimulation blockade, NK cells of the recipient can kill donor derived antigen presenting cells (APC), thereby reducing T cell priming.
- Antibody-dependent cell-mediated cytotoxicity (ADCC) killing of APC mediated by NK cells may be a pathway to induce graft acceptance by costimulation blockade.
- Immunosuppressive therapies that enhance NK cell activity may facilitate the induction of transplant tolerance.

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Chapter 7

Intravenous Immunoglobulins trigger functional activation of human and mouse CD4+CD25+Foxp3+ T cells and promote skin allograft acceptance

Submitted for publication

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SUMMARY

Intravenous Immunoglobulins (IVIg) therapy is effective as a treatment for T cell mediated immune diseases, but how IVIg suppress T cell responses is largely unknown. In this study, we investigated whether IVIg enhance the suppressive function of CD4+CD25+Foxp3+ regulatory T cells, and if so, whether IVIg could prevent allograft rejection. We demonstrate that IVIg bind to both human and mouse regulatory T cells. In both human and mouse, IVIg treatment enhances the activation of CD4+CD25+Foxp3+ regulatory T cells (Tregs), as detected by increased expression of surface activation markers and ZAP70 phosphorylation. Pre-incubation of human CD4+CD25+ T cells with IVIg enhanced their ability to suppress allogeneic T cell proliferation, while depletion of human CD25+ T cells resulted in a twofold reduction in IVIg mediated suppression of allogeneic T cell proliferation. In vivo, administration of IVIg prevented T cell mediated rejection of fully mismatched skin grafts in immunodeficient mice reconstituted with total CD4+ T cells. Importantly, this IVIg effect was lost when CD4+CD25+ cells were depleted from transferred T cells, indicating that IVIg activation induces dominant allograft protection mediated by Tregs. Our data show that IVIg suppress allogeneic T cell responses by direct binding and activation of Tregs, high-lighting an additional immunomodulatory property of IVIg. Importantly, the data underline that the therapeutic potential for IVIg may be broader than the current application in organ transplantation.

INTRODUCTION

Intravenous Immunoglobulins (IVIg) are therapeutic preparations of human IgG purified from large donor plasma pools that contain a broad spectrum of natural antibodies and are widely used for the treatment of autoimmunity and inflammation. The mechanisms of action of IVIg are multifaceted and complex, involving interactions with several components of the human immune system [1]. Clinically, the beneficial effects of IVIg extend beyond the half-life of infused IgG. Therefore, its effects cannot merely be a result of a enhanced passive clearance of pathogenic antibodies or competition with their binding to Fcγ receptors (FcγR), but probably involve changes in cellular immunity. In addition, in past decades clinical evidence has emerged that IVIg are effective in the treatment of T cell mediated autoimmune diseases [2,3], and that hyperimmune IVIg can protect against T cell mediated graft rejection after liver transplantation [4]. In vitro, IVIg can inhibit human T cell proliferation and cytokine production [5,6], but the mechanisms by which IVIg attenuate T cell responses are not fully elucidated.

Regulatory T cells (Tregs) expressing the lineage marker Foxp3 are considered to be the "central immune regulator" of numerous immune processes [7]. Due to their versatility and adaptability, Tregs can police the immune system and control a wide-range of immune responses [8]. Tregs have been demonstrated to be involved in controlling autoimmunity, inflammation and anti-tumor responses. In addition, Tregs play a critical role in the induction and maintenance of transplantation tolerance in experimental animal models [9,10]. The suppressive activity of Tregs requires prior activation through the T cell receptor (TCR), but once activated, Tregs can suppress in an antigen non-specific manner referred to as "bystander regulation" [11,12]. Due to the limited number of Tregs in vivo and their need for prior activation to become suppressive, successful application of Tregs in prevention of allograft rejection requires ex vivo or in vivo stimulation and expansion [13].

Several compounds have been identified that stimulate the expansion and/or the suppressive capacity of Tregs. For example, rapamycine has been shown to stimulate the activity of Tregs and their survival in vitro [14-17] and in vivo in patients [18]. In addition, therapeutic antibodies such as anti-CD4 mAb and rabbit anti-thymocyte globulin (rATG) can enhance the activation status and the expansion of CD4+CD25+Foxp3+ cells [19-21]. Although all these compounds are suited for in vitro expansion and stimulation of Tregs, their use to activate Tregs in vivo may be accompanied by severe side effects upon administration to patients. Recently, Ephrem et al. reported that IVIg treatment protects against disease in an experimental autoimmune encephalomyelitis (EAE) model by promoting expansion and enhancing the suppressive capacity of murine CD4+CD25+Foxp3+ Tregs [22]. Activation of Tregs in vivo by IVIg treatment may be an attractive option for prophylaxis of transplant rejection, since IVIg administration is safe and has moderate side effects. However, the mechanisms through which IVIg exert their effect

on Tregs, and whether IVIg treatment alone, can influence acute allograft rejection, are largely unknown.

In this study, we have investigated the direct interaction of IVIg with human and mouse Tregs, and determined the effects of IVIg on their activation and regulatory function both in vitro and in vivo. Our observations provide an immunological insight into the mechanisms of IVIg and clarify how IVIg may control T-cell mediated immune responses in diseases involving T-cell hyperactivity .

MATERIALS AND METHODS

IVIg preparations

Human IVIg (Intratect®) were a kind gift from Biotest Pharma (Dreieich, Germany). IVIg preparations were dialyzed against large volumes of culture medium (RPMI) at 4°C using Slide-A-lyzer® gamma irradiated dialysis cassettes (Pierce, Rockford, USA) to remove stabilizing agents and to obtain a neutral pH prior to use in vitro. In all in vitro experiments IVIg was used at a concentration of 10 mg/ml (0.06M), to correspond with reported serum IgG concentrations in patients treated with IVIg at 1-2 g/kg for autoimmune disorders [23-25]. Biotinylation of IVIg was performed using D-biotinoyl-ε-aminocaproic Acid N-hydroxysuccinimide ester (Roche Diagnostics, Indianapolis) according to manufacturer's instructions. To control for the elevated protein concentration in the in vitro cultures, Human Serum Albumin (HSA) (Sanquin, Amsterdam, The Netherlands) was used as a negative control.

Mice

CBA.Ca (CBA, H2k), C57BL/10 (B10, H2b) and CBA-recombination-activating gene 1 knockout mice (CBA-Rag -/-, H2k; kindly provided by Dr D. Kioussis, Division of Molecular Immunology, National Institute for Medical Research, Mill Hill, London, UK) were obtained and housed in the Biomedical Services Unit, John Radcliffe Hospital (Oxford, UK). Sex matched mice between 8 and 12 weeks of age at the time of first experimental procedure were used in all experiments.

Flowcytometry and monoclonal antibodies

In human in vitro experiments, the following monoclonal antibodies were used for flowcytometry: CD4 PerCP-Cy5.5, CD25 PE-Cy7, HLA-DR PE, CD32 FITC, CD45RO PE, CD64-biotin (all from BD Biosciences, CA, USA), FoxP3 APC (eBiosciences, CA, USA), CD16 FITC, CD38 PE and CD69 PE (all from Beckman Coulter).

The following reagents were used in mouse experiments for flowcytometry and cell isolation: YTS 169 (anti-CD8, hybridoma kindly provided by Prof. H. Waldmann, Sir William Dunn School of Pathology, Oxford, UK), TIB120 (anti- class II), M1/70 (anti - CD11b) and RA3.6B2

(anti-B220) were from the American Type Culture Collection, Manassas, VA. CD4 PerCp and Foxp3 APC were from eBioscience. CD16/32 FITC or PE, CD16/32 blocking antibody, CD64 PE, CD25 PE, CD25 FITC, CD44 PE, goat anti human IgG-bio and anti-TCRβ mAb were all from BD Biosciences, Oxford, UK.

To detect binding of IVIg to T cells, biotinylated IVIg and strepavidin-PE (Caltag, Burlingame), biotinylated goat-anti human IgG and strepavidin-PE, or rabbit F(ab')2 anti-human IgG-FITC (DAKO Cytomation, Denmark) were used.

Flow cytometry was performed using either FACSCanto or FACSscan instruments (BD Biosciences).

Cell isolation

Human PBMC were obtained from fresh heparinized blood of healthy individuals. After Ficoll (Ficoll-Paque plus, Pharmacia, UK) density gradient separation, CD4+ T cells were purified by negative selection using the untouched CD4 isolation kit (Miltenyi Biotec, Germany). CD4+CD25+ T cells (typical purity >90%) were isolated by positive selection from CD4+ T cells using anti-CD25-microbeads (Miltenyi Biotec) [26]. The CD4+CD25- fraction, containing 0.7 ± 0.5% CD4+Foxp3+ cells, was collected and used as a responder population. The isolations were performed according to manufacturer's instructions. Spleen antigen presenting cells (APC) enriched by immunomagnetic depletion of T cells and NK cells from human spleen tissue obtained from a multi-allograft donor, were used as stimulators [5].

Mouse CD4+ cells were isolated from splenocytes of naive CBA mice by positive selection using anti-mouse CD4-microbeads (Miltenyi Biotec, Germany). The purity of CD4+ T cell fraction was > 95%. CD4+CD25- T cells were obtained by enrichment of CD4+ cells using YTS 169, TIB120, B220 and M1/70 rat mAb to deplete CD8+, MHC class II+ cells, B cells and macrophages, respectively and depletion with sheep anti-rat IgG coated Dynabeads (Dynal Biotech, Norway) followed by depletion of CD25+ cells using anti-mouse CD25-PE and anti-PE beads (Miltenyi Biotec, Germany). The percentage of CD4+Foxp3+ cells in the CD4+CD25- fraction was below 0.5 %.

Bone marrow-derived DC (BMDC) from C57BL/10 mice were generated according to a published method [27]. DC precursor enriched BM cells were cultured with 20 ng/ml rmGM-CSF (PeproTech, London, UK). At day 6, DC were harvested, washed and counted prior to use.

Mixed lymphocyte reactions

In all in vitro experiments with human cells, cells were cultured in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS (Hyclone, UT, USA). To determine to what extent IVIg inhibits human T cell proliferation and whether the inhibitory effect on T cells is mediated through CD4+CD25+ Tregs, we stimulated human CD4+ T cells or the same population depleted of CD25+ T cells with allogeneic spleen APC in the presence of IVIg or HSA (10 mg/ml). Responder T cells (5×10^4) were cultured with 25×10^3 allogeneic APC per well in

round bottom 96 well plates (Greiner, Netherlands), in the absence or presence of IVIg or HSA. To determine whether IVIg directly enhance the inhibitory capacity of Tregs, 2 x 10^5 purified CD4+CD25+ T cells were cultured for 18 hours at 37 °C in the presence of IVIg or HSA. The cells were then washed twice to remove the excess and unbound IVIg, counted and added to 5 x 10^4 CD4+CD25- T cells stimulated by 25 x 10^3 allogeneic APC at a CD25+ to CD25- T cell ratio of 1:1. After 4 days, proliferation was assessed by determination of the incorporation of 0.5 μCi [^{3}H] thymidine (Radiochemical Center, UK) during the final 18 hours of the culture. Percentage of suppression was determined by the formula: $((CPM_{no\_Tregs} - CPM_{IVIG\ or\ HSA}) / CPM_{no\_Tregs}) \times 100\%)$.

Analysis of ZAP70 phosphorylation

To determine ZAP70 phosphorylation, 5 x 10^4 human CD4+ T cells were activated with allogeneic spleen APC (25 x 10^3) in the presence or absence of IVIg (10 mg/ml), while mouse splenocytes were incubated in presence or absence of IVIg (10 mg/ml) or phytohemagglutinin (PHA, 5 μg/ml; Murex, France) as positive control. Mouse splenocytes were incubated for 30 minutes, while human cells were incubated for 18 hours, as a timeline experiment with human T cells showed that ZAP70 phosphorylation induced by IVIg was the highest at the chosen time point (data not shown). After incubation cells were fixed immediately at 37ºC, stained for surface markers, permeabilized and stained with PE-labeled isotype control antibody or antiphospho-ζ-associated protein 70 (ZAP70) (BD Pharmingen) for 30 minutes using the BD phosflow kit (BD Pharmingen). Cells were washed with staining buffer before analysis by flowcytometry. For specific inhibition of src tyrosine kinases cells were incubated with 10 μM PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazole [3,4-d] pyrimidine, Calbiochem) [28].

Expression of Fc gamma receptors on mouse cells

2 x 10^6 CBA.Ca murine splenocytes were cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.5 mM 2-mercaptoethanol (Sigma) and 100 U/mL of penicillin and streptomycin (Sigma) in a six-wells plate and stimulated with phytohemagglutinin (PHA, 5 μg/ml; Murex, France) or 1.5 x 10^4 bone marrow derived DC (BMDC). Cells were harvested at several time points (day 1, day 3 and day 5) and expression of Fc gamma receptors determined by flow cytometry.

Cell adoptive transfer, skin transplantation and in vivo treatment protocol

CBA-Rag -/- mice were reconstituted intravenously with 1 x 10^5 total CD4+ or CD4+CD25- T cells from unmanipulated CBA/Ca mice and transplanted one day later with a full-thickness C57BL/10 skin graft. Twenty-five mg of undialyzed IVIg was administered i.v. through the tail vein on day 1, 3, 7, 10 and 14. Human Serum Albumin (HSA) i.v. was used as a negative control. Human IgG concentrations in serum of mice treated with IVIg were measured by an in-house ELISA using goat anti human IgG – HRP (Sigma- Aldrich, St. Louis, MO).

Statistical analysis

In each in vitro experiment, proliferation and cytokine production was tested in triplicate from which means were calculated. In the results, means with SD from independent experiments are depicted. Statistical analyses were performed by the wilcoxon test for paired data using SPSS version 10.1 (SPSS, Chicago, IL). In vivo graft survival analyses were performed using the log-rank test (GraphPad Prism). A P-value < 0.05 was considered as statistically significant

RESULTS

IVIg enhance the suppressive capacity of human CD4+CD25+ T cells

We have previously shown that IVIg inhibit human T-cell proliferation and cytokine production after allogeneic stimulation as effectively as calcineurin inhibitors [5]. To determine whether this inhibitory effect is mediated through regulatory CD4+CD25+ T cells, we used as responders in an MLR, either total CD4+ T cells or the same population depleted of naturally occurring Tregs. IVIg inhibited the proliferation of total CD4+ T cells by 68 ± 9 % (p = 0.03, N=6), whereas in contrast, the proliferation of CD4+CD25- effector T cells was not significantly reduced in the presence of IVIg (p=0.22, N = 6, inhibition by 16 ± 5 % (Figure 1A). To dissect whether IVIg exert their inhibitory effect directly via Tregs, we cultured purified CD4+CD25+ T cells for 18 hours in presence of IVIg or HSA. Tregs were then washed to remove the unbound IVIg and

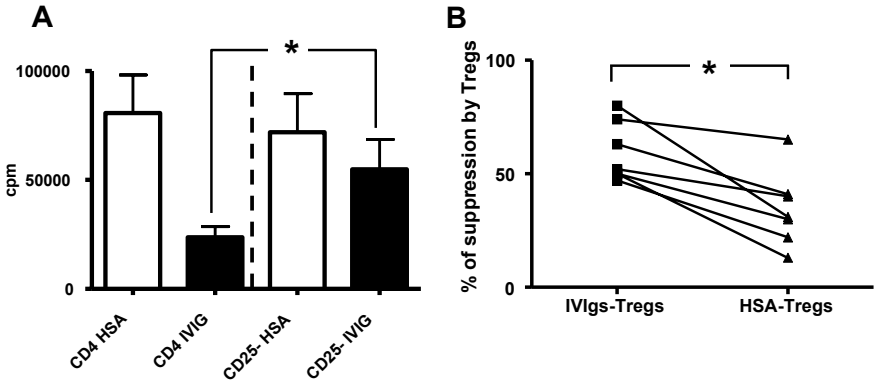

Figure 1: IVIg enhance the suppressive capacity of human CD4+CD25+ T cells

A. Human CD4+ T cells or CD4+CD25- effector T cells (5 x 10^4 per well) were stimulated with allogeneic spleen antigen presenting cells (depleted from T and NK cells; 25 x 10^3 per well) in presence or absence of IVIg or HSA (10 mg/ml). After 4 days, T cell proliferation was measured using [^{3}H] thymidine incorporation (* $p < 0.05$, $N = 6$).

B. 2 x 10^5 purified CD4+CD25+ Tregs were cultured for 18 hours in presence of IVIg or HSA (10 mg/ml). The day after, pre-treated CD4+CD25+ Tregs were extensively washed and added to 5 x 10^4 autologous CD4+CD25- T cells stimulated with 25 x 10^3 allogeneic spleen APC per well at a Tregs : Teff ratio of 1:1. After 4 days, proliferation was assessed using [^{3}H] thymidine incorporation. The suppression by Tregs was determined by the formula: $((CPM_{no\_Tregs} - CPM_{IVIG\ or\ HSA}) / CPM_{no\_Tregs}) \times 100\%)$. (* $p < 0.05$, $N = 7$)

added to CD4+CD25- T cells stimulated by allogeneic APC. As shown in Figure 1B, the suppressive effect of CD4+CD25+ T cells pre-treated with IVIg (IVIg-Tregs) on the proliferation of CD4+CD25- responders was significantly enhanced compared to the suppressive effect of CD4+CD25+ T cells pre-cultured with HSA (HSA-Tregs). This enhanced suppression applied to Tregs from all the donors tested (inhibition by IVIg-Tregs: 59 ± 13% versus HSA-Tregs: 35 ± 17% at CD25+:CD25- T cell ratio of 1:1 ($p<0.05$, N = 7) (Figure 1B).

IVIg bind to and activate human CD4+CD25high Foxp3+ regulatory T cells

In the light of the observation that IVIg enhances the suppressive capacity of naturally occurring Tregs, we asked whether IVIg binds to and activates human Tregs. Purified human total CD4+ T cells were incubated for 18 hours with IVIg or HSA, either without stimulation or stimulated with allogeneic spleen APC. Rabbit F(ab')2 anti-human IgG-FITC was used to detect IVIg binding. As shown in Figure 2A, IVIg bound to human CD4+CD25high Foxp3+ regulatory T cells. This binding occurred regardless whether the cells were stimulated by APC or not (unstimulated: 83 ± 13 %, and stimulated by APC: 83 ± 4 % IVIg+ CD4+ Foxp3+ T cells, N = 3). Human IgG bound also to conventional CD4+Foxp3- T cells. However, IVIg binding to Tregs was higher (mean fluorescence intensity; MFI = 7063) than that to conventional T cells (MFI = 3197) (Figure 2B). This has been reported earlier for mouse T cells [22].

After 18 hours of culture, we determined the surface expression of activation markers HLA-DR, CD69 and CD38 on CD4+CD25high Foxp3+ Tregs, both in unstimulated and by allogeneic APC stimulated conditions. Comparing the conditions with and without stimulation by allogeneic APC, we detected activation of Tregs induced by allogeneic APC. However, addition of IVIg to allogeneically stimulated T cells enhanced expression of HLA-DR, CD69 and CD38 on CD4+CD25high Foxp3+ Tregs on top of the baseline allogeneic activation. (Figure 2C)

In contrast, no effect of IVIg on Tregs activation was observed in the absence of APC, suggesting an important role of APC in activation of Tregs by IVIg. CD45RO expression on human CD4+CD25+Foxp3+ T cells remained stable in all conditions. In addition, the CD4+CD25high Foxp3+ T cells to which IVIg was bound had a higher expression of the activation markers than the IVIg-negative CD4+CD25high Foxp3+ population, suggesting preferential activation as a direct consequence of IVIg binding (Figure 2D).

Upon TCR ligation of CD4+ T cells, the intracellular domain of CD4 interacts with the src tyrosine kinase p56lck leading to activation and phosphorylation of Tyr^{319} within the linker region of ZAP70 [29]. This in turn leads to activation of ZAP70 and results in downstream activation of the T cell [20]. Thus, the level of ZAP70 Tyr^{319} phosphorylation provides an independent assessment of the activation status of T cells. As shown in Figure 2E, in the presence of allogeneic stimulation, IVIg treatment resulted in enhanced Tyr^{319} phosphorylation of ZAP70 in human CD4+CD25+ T cells relative to controls. Moreover, inhibition of lck tyrosine kinase activity by the src tyrosine kinase-specific inhibitor PP1 during incubation with IVIg prevented the phosphorylation of Tyr^{319} of ZAP70 in CD4+CD25+ cells in the presence of IVIg (Figure 2E), verifying

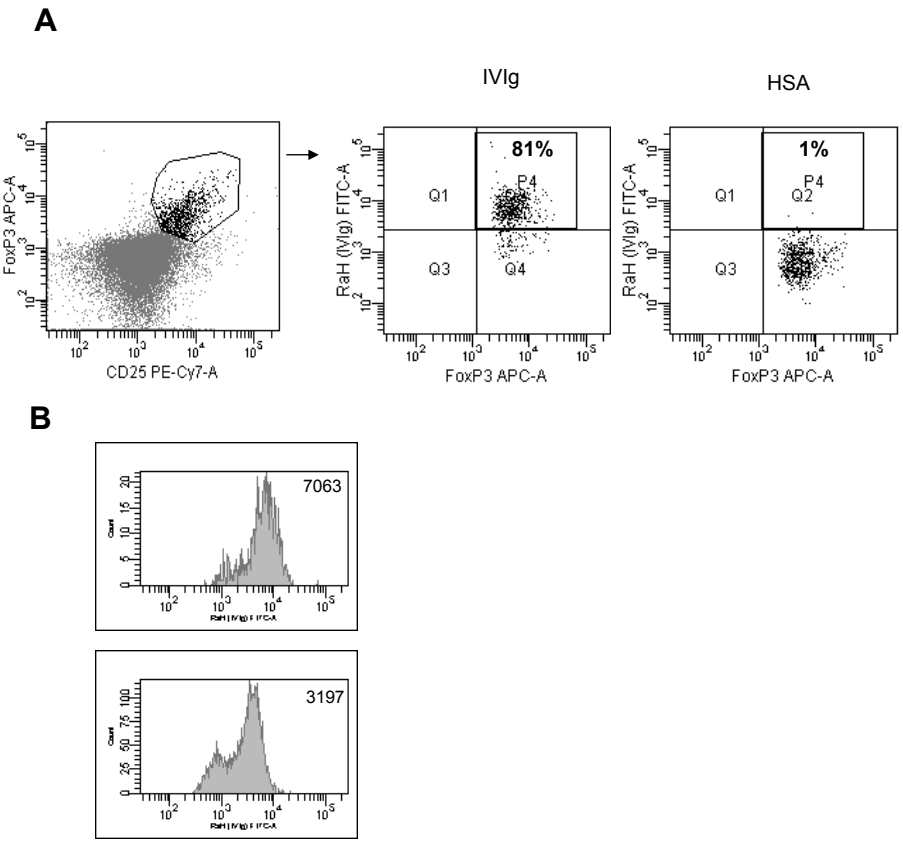

Figure 2: IVIg bind to and activate human CD4+CD25+ FoxP3+ regulatory T cells

A. Purified human total CD4+ T cells (5 x 10^4 per well) were incubated for 18 hours with IVIg or HSA, either without stimulation or stimulated with allogeneic spleen APC (25 x 10^3 per well). The day after, the binding of IVIg to and the activation status of human CD4+CD25+Foxp3+ T cells were determined by flowcytometry.

B. Rabbit F(ab')2 anti-human IgG-FITC was used to detect IVIg binding. 84 ± 4% of CD4+CD25+Foxp3+ T cells stimulated by spleen APC have bound IVIg on their surface. As antibodies can adhere non-specifically when cells are activated, we used HSA-Treg as negative control. FACS plots of one representative experiment out of 3 experiments are shown.

Rabbit F(ab')2 anti-human IgG-FITC was used to detect IVIg binding on CD4+Foxp3+ cells (top plot) and on conventional CD4+Foxp3- T cells (bottom plot). Binding of IVIg was analyzed based on the mean fluorescence intensity (MFI).

that this IVIg dependent activation is mediated through p56lck, as the inhibition of p56lck using the inhibitor PP1 abolished the increase on ZAP70 phosphorylation. Together, these data show that binding of IVIg to human CD4+CD25high Foxp3+ regulatory T cells in the presence of APC leads to intracellular ZAP70-signaling, enhanced expression of activation markers and increased suppressive capacity.

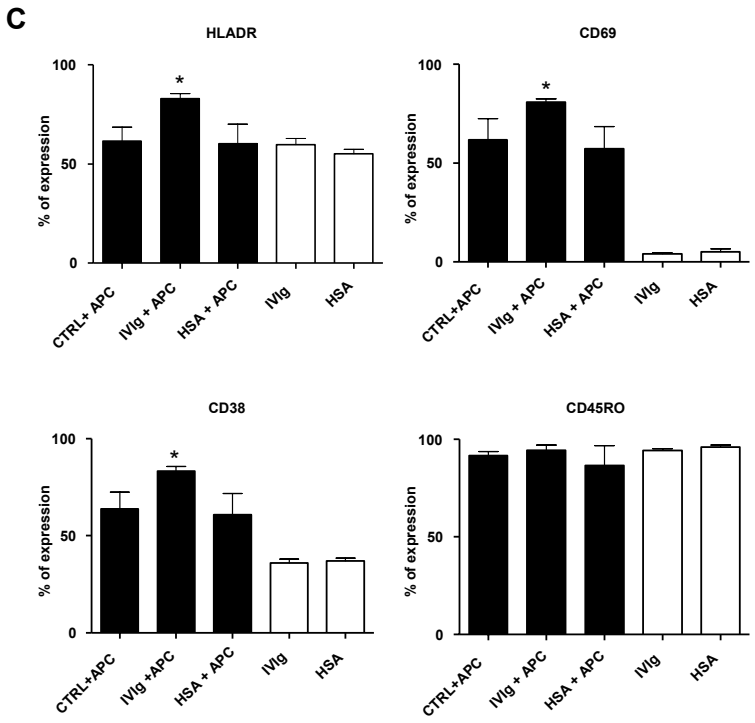

Figure 2: IVIg bind to and activate human CD4+CD25+ FoxP3+ regulatory T cells

C. CD4+ T cells were incubated with IVIg or HSA without APC (□ white bars) or stimulated with allogeneic spleen APC without (= CTRL + APC) or with IVIg or HSA (with APC, ■ black bars) for 18 hours in presence of IVIg or HSA. After incubation, the expression of the activation markers HLA-DR, CD69 and CD38 on CD4+CD25+Foxp3+ T cells was determined by flowcytometry. After incubation with IVIg in the presence of allogeneic APC, higher percentages of CD4+CD25+Foxp3+ T cells expressed HLA-DR, CD69 and CD38, while CD45RO expression was comparable to the untreated and HSA treated conditions. The enhanced expression of activation markers in the presence of IVIg occurred only when stimulated by allogeneic APC. (* $p < 0.05$, N =3)

IVIg bind to and activate mouse CD4+CD25high Foxp3+ regulatory T cells

To establish whether human IVIg can bind to mouse Tregs, IVIg were injected intravenously in CBA.Ca mice. Twenty-four hours later binding of human IgG to mouse spleen CD4+Foxp3+ and CD4+Foxp3- T cells was determined. IVIg preferentially bound to a subpopulation of mouse CD4+Foxp3+ T cells (16 ± 2%) (N = 4), while binding to CD4+Foxp3- T cells was minimal (Figure 3A). This finding was confirmed using biotinylated IVIg (data not shown). In addition, as shown in Figure 3B, an increased level of IVIg-binding was observed on CD4+Foxp3+ cells that were also positive for CD44, a marker of antigen experience [30], suggesting that IVIg binds preferentially to antigen experienced cells. (Figure 3B)

To investigate whether IVIg treatment could induce phosphorylation of ZAP70 in mouse CD4+CD25+ cells, mouse splenocytes were incubated for 30 minutes with or without IVIg or

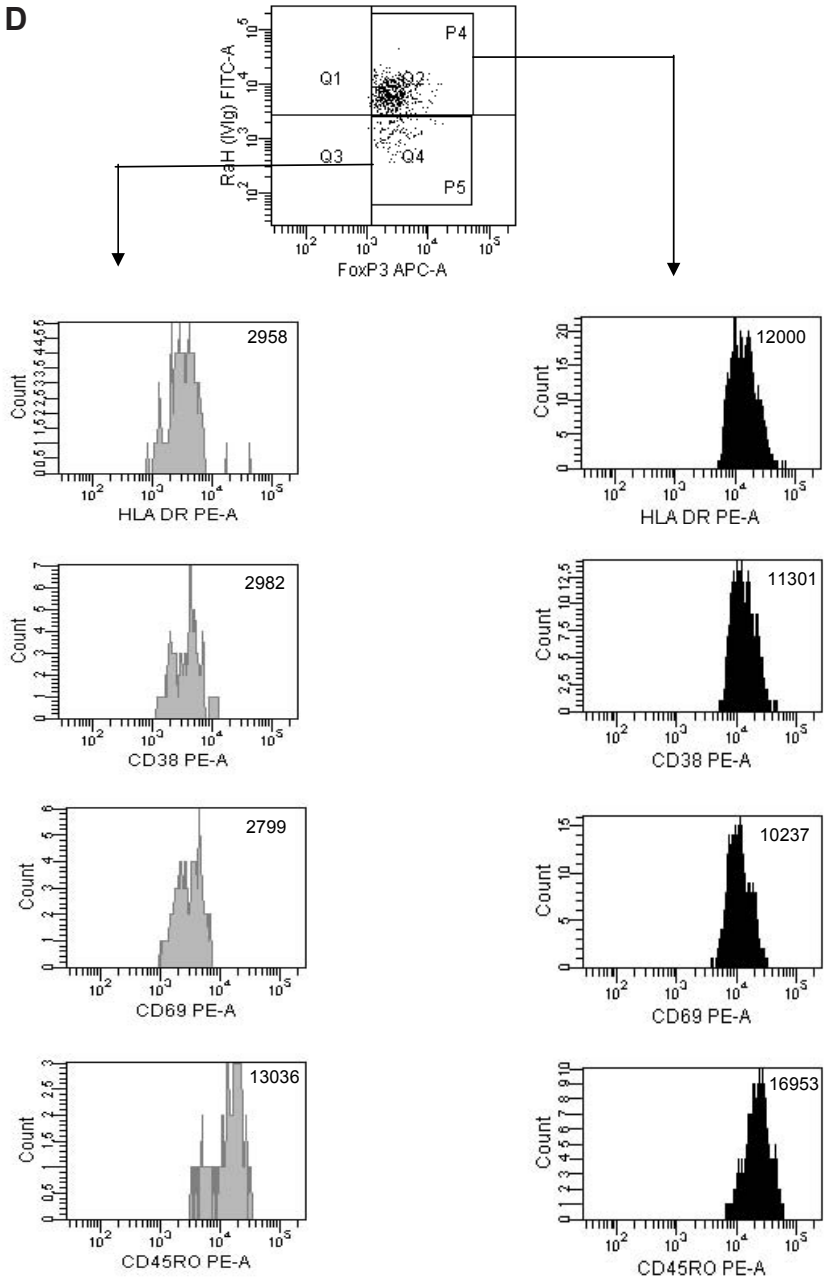

Figure 2: IVIg bind to and activate human CD4+CD25+ FoxP3+ regulatory T cells

D. The CD4+CD25+Foxp3+ T cells that bound IVIg showed enhanced expression of the activation markers HLA-DR, CD69 and CD38 compared to CD4+CD25+Foxp3+ T cells that did not bind IVIg. This while the expression of CD45RO did not differ between he two populations. Mean fluorescence intensity (MFI) is depicted in the plots. Experiments are representative of 1 out of 3 experiments.

E

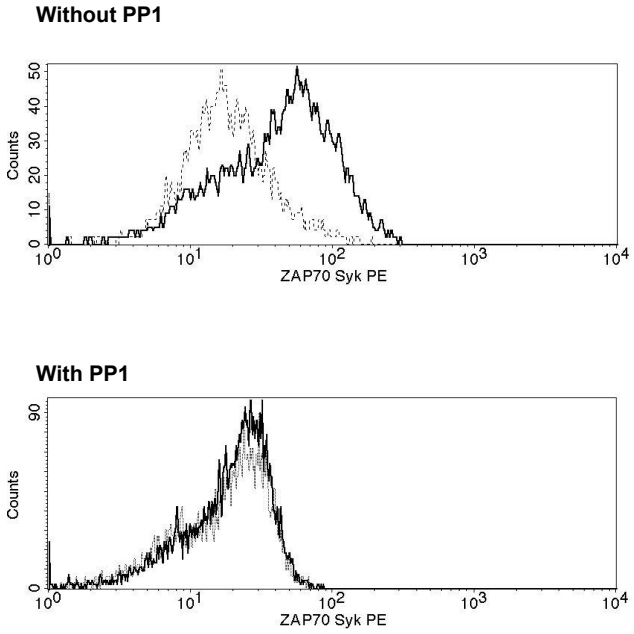

Figure 2: IVIg bind to and activate human CD4+CD25+

E. After CD4+ T cells were incubated with allogeneic spleen APC for 18 hours in presence or absence of IVIg (dotted line = control, black line = IVIg), ZAP70 phosphorylation in CD4+CD25+ Tregs was determined. Foxp3 staining could not be combined with the phosphorylated ZAP70 staining (data not shown). We detected an increased phosphorylated ZAP70 expression in CD4+CD25+ cells cultured in presence of IVIg. For specific lck tyrosine kinase inhibition, 10 μM PP1 was added, and phosphorylated ZAP70 expression in CD4+CD25+ cells was determined. The histograms are representative of 1 out of 3 experiments.

HSA and the level of ZAP70 phosphorylation determined by FACS analysis. The presence of IVIg resulted in a significant up-regulation in phosphorylated ZAP70 expression (IVIg: 36 ± 8 % versus no treatment 18 ± 9 % versus HSA: 23 ± 7 %; $p < 0.05$, N = 6). PHA (5 ng/ml) was used as a positive control. Similar to human cells, phosphorylation of ZAP70 was inhibited by specific lck tyrosine kinase inhibition (Figure 3 C).

IVIg bind to mouse CD4+Foxp3+ T cells: exploration of the mechanism of binding

Several reports have suggested that that Fc receptors may be present on activated mouse T lymphocytes [31-34], but their functional significance is unclear. Santana et al. reported that aggregated human IgG binds to mouse thymocytes and T cells through receptors for the Fc region [35], which prompted us to ask whether CBA CD4+Foxp3+ T cells express Fc receptors, which might bind human IgG. We explored the expression of FcγR I, II and III on resting and on mitogenically and allogeneically activated CD4+Foxp3+ cells at different time points. Mouse FcγR II (CD32) and III (CD16) are approximately 90% homologous [36], therefore, we used a rat monoclonal antibody which reacts with an epitope shared by mouse CD16 and CD32.

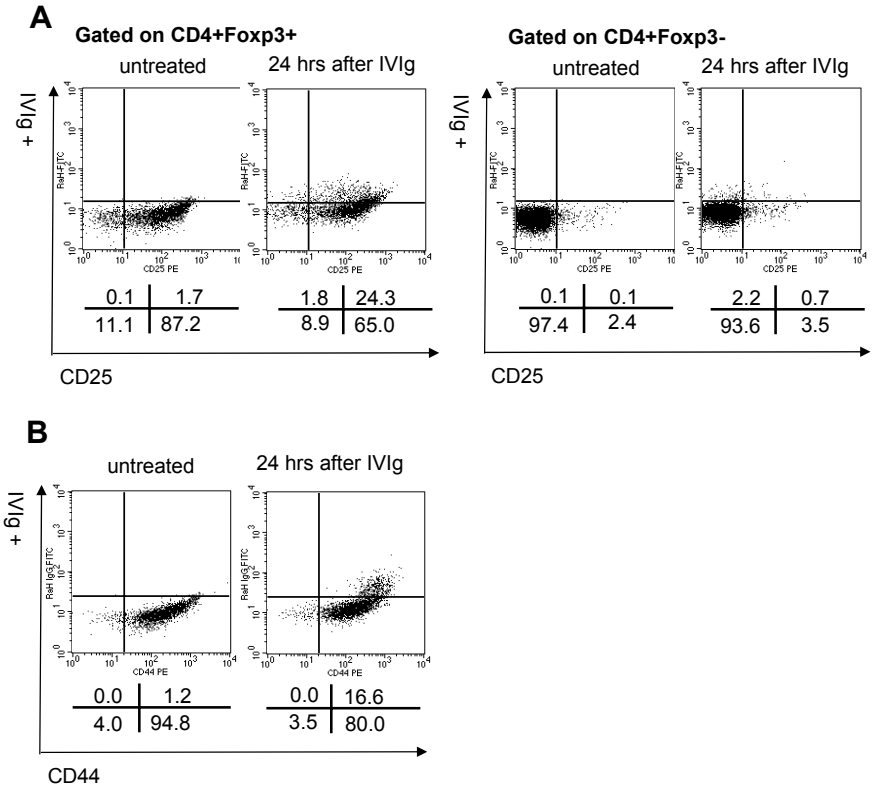

Figure 3: IVIg bind to and activate mouse CD4+CD25high Foxp3+ regulatory T cells

A. IVIg (25 mg) was injected intravenously into naïve WT CBA.Ca mice. The day after, the spleen was harvested, single cells were prepared and used in flowcytometry analysis. Rabbit F(ab')2 anti-human IgG-FITC was used to detect IVIg binding to mouse T cells. Splenocytes from untreated naïve WT CBA.Ca mice were used as control. The left panel shows IVIg-binding to CD4+Foxp3+ cells and the right panel to CD4+Foxp3- cells. In this experiment IVIg bound to 24.3% of the mouse spleen CD4+ CD25+Foxp3+ T cells 24 hours after intravenous administration, while binding to CD4+Foxp3- cells was minimal. The binding did not increase when it was measured after 48 hours (data not shown)

B. To determine the activation status of CD4+Foxp3+ cells in mice that were treated with IVIg, we determined CD44 expression. The plots are representative of 1 of 4 experiments.

Stimulation of splenocytes with PHA resulted in a dramatic increase in the proportion of TCRβ+CD4+Foxp3+ cells that expressed FcγR II/III. This up-regulation was rapid (37 ± 5% at day +1,$p < 0.01$ compared to day 0, $N = 4$) but most significantly, was much less marked on Foxp3- cells where expression was lower than CD4+Foxp3+ cells at all time points (Figure 4A). More importantly, allogeneic stimulation with bone marrow-derived dendritic cells (BMDC) also resulted in a striking up-regulation of FcγR II/III expression on Foxp3+ T cells, but not on Foxp3- T cells (Figure 4B). No expression of FcγR I (CD64) was detected on CD4+Foxp3+ T cells (data not shown).

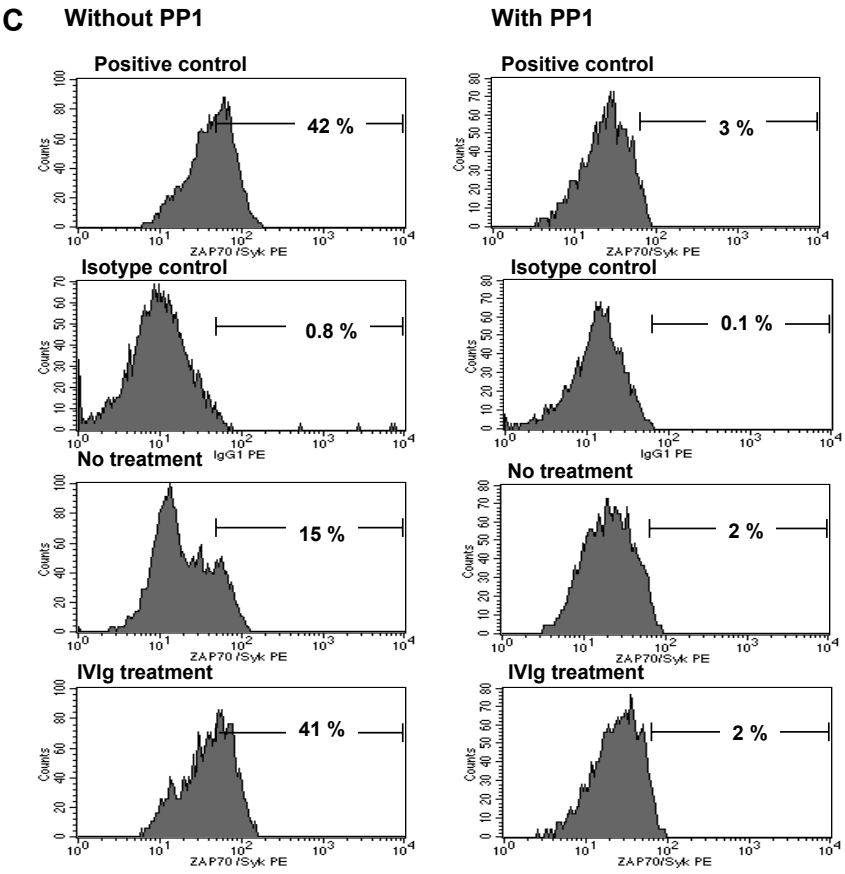

Figure 3: IVIg bind to and activate mouse CD4+CD25high Foxp3+ regulatory T cells

C. As the phosphorylation of ZAP70 is a transient event and difficult to determine in T cells from mice after intravenous administration of IVIg, we cultured naïve CBA.ca splenocytes in presence or absence of IVIg or HSA (10 mg/ml) for 30 minutes at 37° C, after which ZAP70 phosphorylation in CD4+CD25+ Tregs was determined. The isotype control is depicted for control Treg, but was similar for HSA- and IVIg-Treg. PHA (5 ng/ml) was used as a positive control. Histograms shown are representative of 6 experiments. By specific inhibition of lck tyrosine kinase with 10 μM PP1 during the 30 minutes cultures, phosphorylation of ZAP70 was inhibited.

To determine whether the binding of IVIg to mouse CD4+Foxp3+ T cells is mediated through FcγR II/III, we studied the binding of IVIg after in vivo injection and after in vitro activation of T cells. Twenty-four hours after injection into naïve CBA.Ca mice, IVIg binding to CD16/32positive CD4+Foxp3+ T cells could be detected, but interestingly, the vast majority of IVIg was detected on the surface of CD16/32negative Foxp3+ cells (Figure 4C). Presumably, it is because the natural occurring Foxp3+ were not activated in these mice, and only 2% to 3% of the CD4+Foxp3+ cells express FcγR II/III. To verify whether the binding to activated CD4+Foxp3+ T cells is mediated by FcγR II/III, we activated CD4+ T cells with PHA and blocked FcγR II/III with an FcγR II/III-specific antibody before addition of IVIg in vitro. Indeed, binding of IVIg to CD16/32+CD4+Foxp3+ T

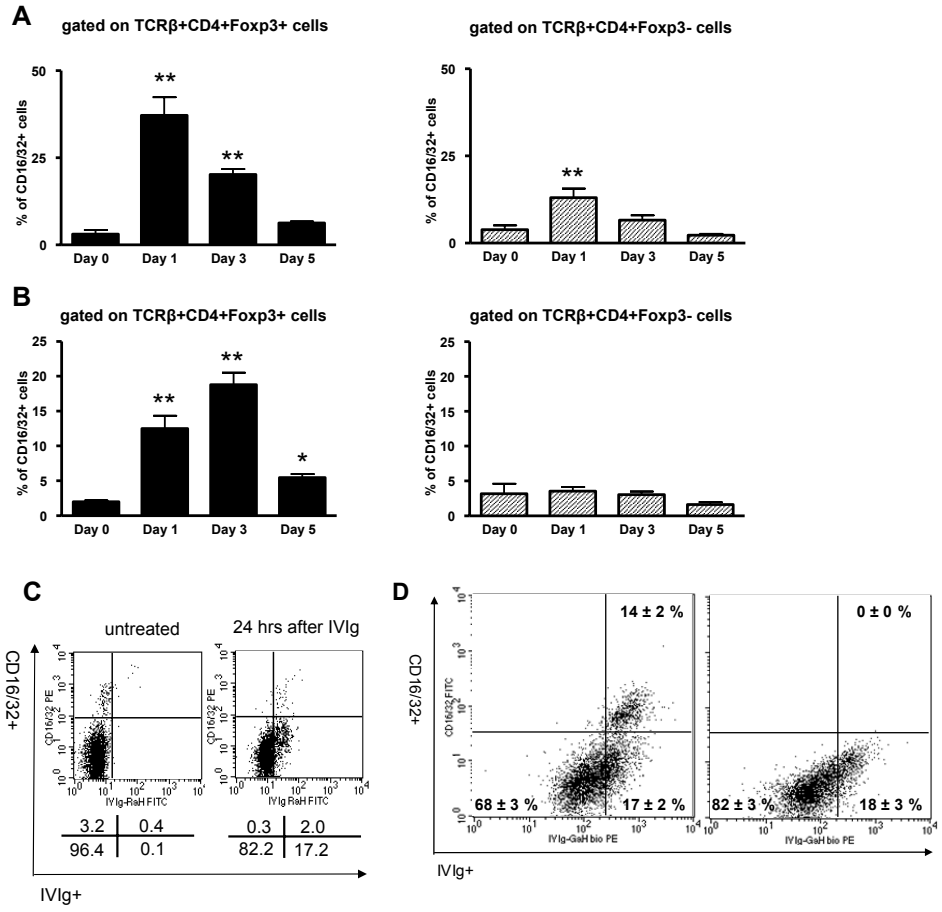

Figure 4: IVIg bind to mouse CD4+Foxp3+ T cells: exploration of the mechanism of binding

A. WT CBA.Ca splenocytes were stimulated with PHA (5 ng/ml) and expression of FcγR II/III (CD32/16) was determined on CD4+TCRβ+Foxp3+ cells (left panel; black bars) and CD4+TCRβ+Foxp3- cells (right panel; grey bars) before culture and at day 1, 3 and 5 of culture. (** $p < 0.01$ in comparison to day 0, $N = 4$)

B. WT CBA.Ca splenocytes were stimulated with B10 BMDC at the ratio of 1 : 1 and expression of FcγR II/III was determined on CD4+TCRβ+Foxp3+ cells (left panel; black bars) and CD4+TCRβ+Foxp3- cells (right panel; grey bars) before culture and at day 1, 3 and 5 of culture. (** $p < 0.01$, * $p < 0.05$ compared to day 0, $N = 3$)

C. 25 mg of IVIg was injected intravenously in naïve WT CBA.Ca mice. The day after, binding of IVIg to spleen CD4+Foxp3+ CD16/32+ T cells and CD4+Foxp3+ CD16/32- T cells was determined. Representative plots from 1 out of 3 experiments.

D. To investigate whether FcγR II/III mediate binding of IVIg to activated Tregs, we activated CBA.Ca splenocytes with PHA (5 ng/ml). After 6 hours IVIg (1 mg/ml) were added and 30 minutes thereafter the activated cells were extensively washed and stained for IVIg binding using biotinylated goat anti human IgG and strepavidin-PE (left plot). To perform blockade of FcγR II/III, CD32/16 blocking antibody (2,5 µg in 50 µl) was added 30 minutes before IVIg was added (right plot). Depicted are FACS-plots or one representative experiment out of 3.

cells was prevented by the blocking antibody, while IVIg binding to CD16/32-CD4+Foxp3+ T cells remained unaltered (Figure 4D). Taken together, these data indicate that IVIg binds to mouse CD4+Foxp3+ T cells via both FcγR II/III dependent and independent pathways.

IVIg treatment promotes fully MHC-mismatched skin allograft acceptance

In order to determine whether IVIg mediated binding and activation of naturally occurring regulatory cells has a functional consequence in vivo, CBA-Rag -/- mice were reconstituted on day -1 with 1 x 10^5 CBA total CD4+ T cells from wild type CBA.Ca (H2k) mice, or the same population depleted of CD25+ cells, and transplanted one day later with allogeneic full thickness C57BL/10 (H2b) skin allografts. IVIg (25 mg) was administered i.v. on days 1, 3, 7, 10 and 14 after transplantation. Control animals were treated with equal doses of HSA (Figure 5A). Serum samples collected at day 15 showed that human immunoglobulin concentrations in serum increased from 4 ± 2 mg/ml on day 7 to a peak level of 11 ± 3 mg/ml on day 15 (N = 5), which is comparable to those in patients treated with IVIg [24]. Mice reconstituted with total CD4+ T cells that received no further treatment or were treated with HSA, rejected their grafts acutely (median survival times of 14 and 16 days respectively). However, in clear contrast, mice reconstituted with the same population of CD4+ T cells and treated with IVIg all accepted their skin grafts long-term (MST >100 days) and showed no signs of tissue necrosis (Figure 5A, $p<0.01$).

Importantly, the same IVIg regimen had no effect on graft outcome in mice reconstituted with CD4+ T cells depleted of naturally occurring Tregs. Indeed, these mice rejected their grafts at exactly the same time as reconstituted mice given no further treatment (Figure 5B). These data suggest that that IVIg exerts an inhibitory effect on T cell mediated graft rejection by enhancing the suppressive capacity of CD4+CD25+ regulatory T cells.

DISCUSSION

IVIg treatment is increasingly being used for the control of T cell mediated autoimmunity and inflammation and more recently, also in transplantation. Whilst its application in clinical transplantation has been limited largely to the rapid removal of antibodies in highly sensitised patients [37-39], IVIg may also have significant effects on T cell mediated graft rejection [4,40]. In the current study, we demonstrate that IVIg is effective in prevention of CD4+ T cell-mediated graft rejection in a fully MHC mismatched skin transplant model by triggering functional activation of CD4+CD25+Foxp3+ regulatory T cells. Interestingly, through direct binding of IgG present in IVIg preparation to Tregs, IVIg activate not only mouse, but also human Tregs, thereby enhancing their suppressive function. Especially, the Tregs that bind IVIg to their surface, show increased expression of activation markers. Our findings extend recent data from other groups showing IVIg mediated activation of Tregs [22,41], by demonstrating that IVIg induce functional activation of Tregs via direct binding to their surface and triggering the src kinase ZAP70 signaling pathway.

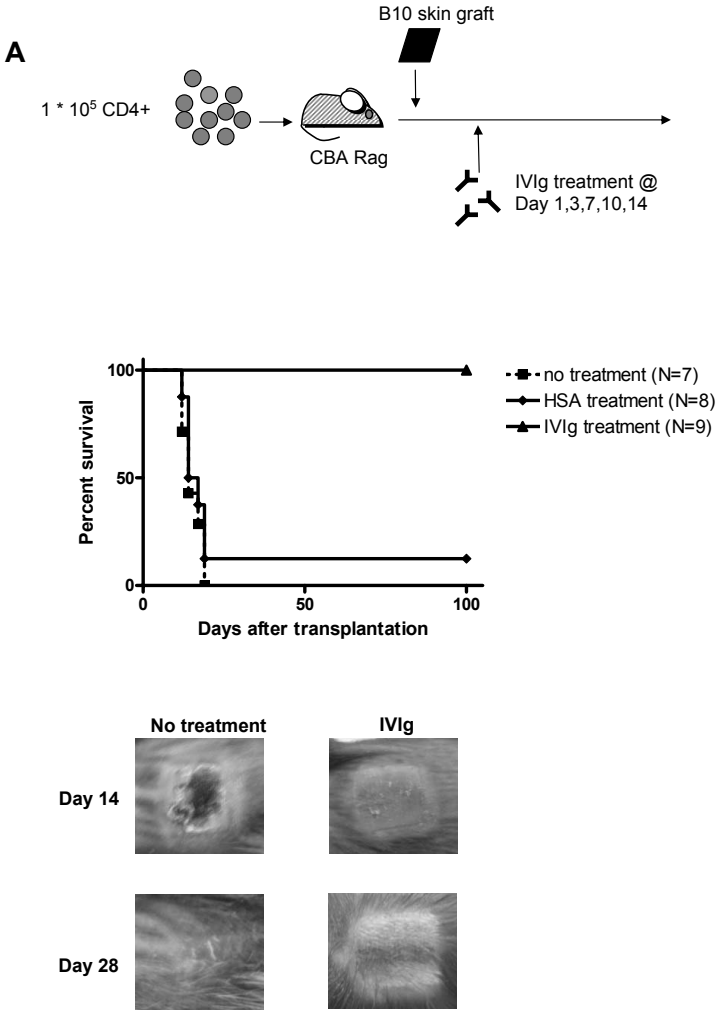

Figure 5: IVIg treatment promotes acceptance of fully MHC-mismatched skin allografts

A/B. CBA-Rag -/- mice were reconstituted intravenously with 1 x 10^5 total CD4+ (A) or CD4+CD25- T cells (B) harvested from naive CBA.Ca spleen. One day later a full thickness tail skin graft of C57BL/10 mice was transplanted on the flanks of the reconstituted mice. Twenty-five mg of IVIg was administered i.v. on day 1,3,7,10 and 14 after transplantation. Human Serum Albumin (HSA) was administered in the same dose to control mice. Skin graft survival in mice reconstituted with total CD4+ T cells: MST in mice treated with IVIg >100 vs HSA 16 days, $p<0.01$, $N = 9$); in mice reconstituted with CD4+CD25- T-cells : MST in IVIg-treated and non-treated mice were both 17 days ($p = NS$, $N = 8$).

Binding of IVIg to mouse Tregs was partly mediated by FcγR, but the majority was bound in a FcγR independent fashion. We detected expression of FcγR II and III on mouse T cells which increased upon their activation, but found no expression of Fc receptors on activated human T cells (data not shown). Therefore, binding of IVIg to human Tregs seems to be FcγR independent.

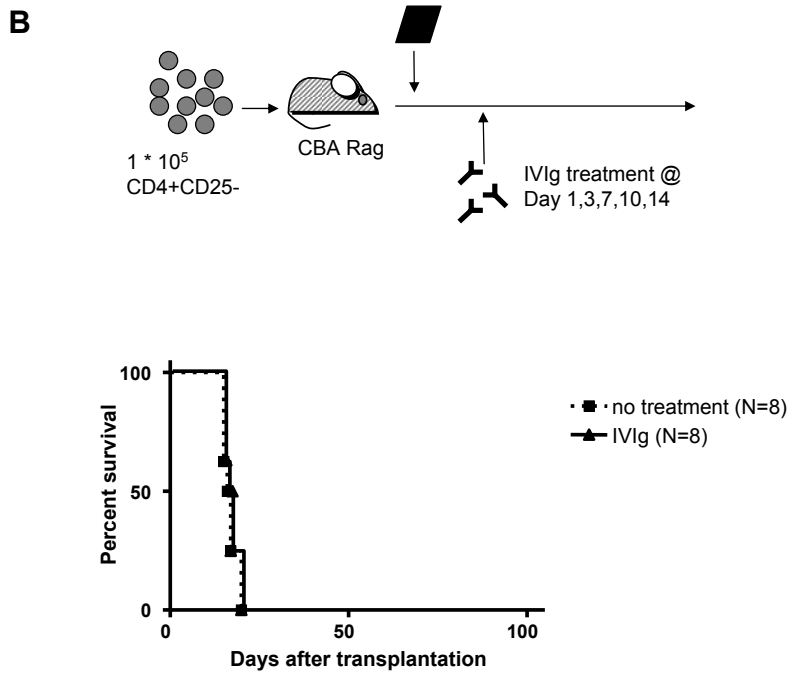

Figure 5: IVIg treatment promotes acceptance of fully MHC-mismatched skin allografts
A/B. CBA-Rag -/- mice were reconstituted intravenously with 1 x 10^5 total CD4+ (A) or CD4+CD25- T cells (B) harvested from naive CBA.Ca spleen. One day later a full thickness tail skin graft of C57BL/10 mice was transplanted on the flanks of the reconstituted mice. Twenty-five mg of IVIg was administered i.v. on day 1,3,7,10 and 14 after transplantation. Human Serum Albumin (HSA) was administered in the same dose to control mice. Skin graft survival in mice reconstituted with total CD4+ T cells: MST in mice treated with IVIg >100 vs HSA 16 days, $p<0.01$, $N = 9$); in mice reconstituted with CD4+CD25- T-cells : MST in IVIg-treated and non-treated mice were both 17 days ($p = NS$, $N = 8$).

Several studies have shown that IVIg contain natural antibodies that recognize different surface molecules on T cells, such as TCRβ, CD4 and CD95 [42-44]. Possibly, binding of IgG to human Tregs is mediated via its F(ab)2 region, which recognises surface molecules, such as TCR or CD4 on Tregs. Still, the binding of the Fc region of IgG to an unknown Fc receptor or a glycoprotein-recognizing molecule on Tregs is a possibility, that can not be excluded. Moreover, we showed that IVIg treatment leads to src-kinase-mediated ZAP70 phosphorylation of in both mouse and human CD4+CD25+ T cells, which supports the hypothesis that IVIg may trigger activation of Tregs via TCR or other ITAM motif bearing activating receptors, like (unknown) activating Fc receptors, on Tregs.

Recently, two major histocompatibility complex class II T cell epitopes in the Fc fragment of IgG have been identified, which are capable of activating natural regulatory T cells [45]. Considering that these two epitopes are present in IVIg preparations, activation of Tregs by these epitopes is a possible alternative explanation for activation of Tregs by IVIg. However, since we observed

that activation of Tregs was a direct consequence of IVIg binding, and that Tregs that did not bind IVIg were less activated than IVIg positive Tregs, we believe that MHC restricted presentation of IVIg derived epitopes to Tregs is not the only explanation for the activation of Tregs in the co-cultures of CD4+ T-cells with APC.

Interestingly, activation of human Tregs by IVIg occurred only in presence of allogeneic APC. The need of APC and the specific activation of Tregs that have bound IVIg to their surface reflect the complexity of Tregs activation by IVIg. We propose a model in which both binding and cross linking of IgG are necessary to induce Tregs activation. Probably, APC serve in this situation as an intermediate to crosslink the Fc regions of IgG present on the Tregs surface, thereby inducing Tregs activation. Still, further studies need to dissect the functional differences between IVIg positive and negative Tregs.

Our data indicate that there are striking discrepancies between human and mouse cells in their response to IVIg treatment. Human IgG in IVIg preparations bound to not only human CD4+Foxp3+ T cells but also to CD4+Foxp3- T cells, while binding to mouse conventional T cells was negligible. Since we have preliminary evidence to suggest that IVIg bind more strongly to human CD4+CD25+Foxp3+ T cells than that to CD4+CD25-Foxp3- effector T cells, it seems likely that this preferential binding of IVIg alters the functional balance between effector and Tregs, leading to suppression. Moreover, the inhibitory effect of IVIg on human CD4+CD25- T cells (depleted for CD4+CD25+ T cells) was small, and not statistically significant, showing that binding of IVIg to human CD4+CD25- has only a minor suppressive effect. Second, we detected increased expression of FcγR II and III on activated mouse T cells, while no expression of Fc receptors on activated human T cells was detected. These discrepancies between human and mouse data underline the difficulty to interpret and correlate both. Further research is warranted to unravel the mechanistic basis of the effect of IVIg on both human and mouse Tregs.

We demonstrated that IVIg protected against T cell mediated rejection of fully mismatched skin grafts only when total CD4+ T cells, but not CD4+CD25- T cells, were adoptively transferred. It is worth noting that IVIg treatment resulted in allograft survival that persisted well beyond 100 days even though the IVIg treatment was discontinued on day 14. Thus, a short period of IVIg therapy appears to lead to stable and robust prolonged graft survival despite the presence of CD4+CD25- T cells. However, we have been unable to achieve prolongation of allogeneic skin allograft survival in immunologically intact wild-type mice using IVIg treatment (data not shown). This almost certainly reflects the large total number of alloreactive T cells, including CD8+ T cells, in intact mice compared to the limited numbers of CD4+ T cells in the adoptive transfer experiments described in the current study. This observation suggests that the potential of IVIg to prevent allograft rejection may be seen only in combination with strategies that induce partial T cell depletion and attenuation. Of the potential candidates that exist, depletion

using CAMPATH-1H [46] or modulation using humanised anti-CD3 antibodies [47] appear to be promising.

Our results identify a novel pathway through which IVIg treatment induces functional activation of both mouse and human regulatory T cells. Direct binding and activation of regulatory T cells is one of the mechanisms in the immunomodulatory repertoire of IVIg, which allows rapid inhibition of allogeneic responses, and therefore can be a valuable tool after organ transplantation. The identified pathway by which antibodies influence the function of the "central immune regulator", the Tregs, may also be an important physiological phenomenon, and may form a basis for production of synthetic or biological alternatives that will enable us to selectively target pathogenic immune activity.

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Chapter 8

Modulation of the cellular immune system by Intravenous Immunoglobulins

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SUMMARY

Intravenous Immunoglobulins (IVIg) are therapeutically used in a variety of immune-mediated diseases. The beneficial effects of IVIg in autoantibody-mediated diseases can be explained by neutralization, accelerated clearance, and prevention of Fcγ receptor binding of autoantibodies. However, the means by which IVIg exert therapeutic effects in disorders mediated by cellular immunity has remained an enigma. Clinical improvements followed by IVIg treatment often extend beyond the half-life of infused IgG, suggesting that IVIg modify the cellular immune compartment for a prolonged period. We discuss here recent advances in our understanding of different, mutually non-exclusive mechanisms of action of IVIg on cells of the innate and adaptive immune system. These mechanisms might explain the beneficial effects of IVIg in certain autoimmune and inflammatory diseases.

INTRAVENOUS IMMUNOGLOBULINS THERAPY

Intravenous immunoglobulins (IVIg) for therapeutic use are polyspecific IgG preparations purified from plasma pools of several thousand healthy donors. IVIg preparations primarily contain human IgG molecules, with small amounts of IgA and IgM. The distribution of IgG subclasses in IVIg is comparable to that of IgG in normal serum and the half-life of infused IVIg is approximately three weeks. IVIg are safe preparations with no long term side effects. Although administration of IVIg can result in generalized reactions, such as headache, nausea and chills, in 1-15% of the patients, these adverse reactions are often mild and are relieved by NSAIDs or reducing the rate of infusion.

IVIg were initially used as a substitution for immunoglobulins otherwise lacking in patients with primary immune deficiencies (PIDs) and secondary immune deficiencies. However, since the demonstration in 1981, that IVIg ameliorate immune thrombocytopenic purpura (ITP) [1], IVIg are increasingly being used for the treatment of a wide range of autoimmune and systemic inflammatory diseases [2]. In addition to antibody mediated diseases, IVIg are also effective in several disorders caused by derailment of cellular immunity, like Kawasaki disease, dermatomyositis, multiple sclerosis (MS), graft versus host disease (GvHD) in recipients of allogeneic bone marrow transplants, and treatment of cellular rejection after organ transplantation [2-5]. Clinically, the beneficial effects of IVIg extend beyond the half-life of infused IgG, therefore its effect is probably not merely due to a passive clearance or competition with pathogenic autoantibodies. Together, these observations evoke the possibility that IVIg therapy induces lasting changes in the cellular compartment of the immune system.

The established beneficial effects of IVIg in diseases mediated by auto-antibodies can be explained by several mechanisms including neutralization by naturally occurring anti-idiotype antibodies present in IVIg, accelerated clearance (by saturation of the neonatal Fc receptor), and prevention of auto-antibody binding to Fcγ receptors (FcγR) on phagocytes [2]. By virtue of a broad spectrum of antibodies, which reflect the natural antibody repertoire of the human population, the mechanisms of action of IVIg are multifaceted and complex. Most likely, the immunological effects of IVIg are not mutually exclusive, and work in synergy to contribute to effective therapy in various clinical settings. For a long time, the means by which IVIg exert their therapeutic effects in complex immune mediated disorders involving cell-cell interactions had remained a mystery. However, the results of several recent studies have shed light on the mechanistic aspects of IVIg therapy in these diseases. In this review, we discuss recent advances in our understanding of the effects of IVIg on the different cells of the innate and adaptive immune system that together explain the beneficial effects of IVIg in disorders caused by dysregulated cellular immunity.

Effects of IVIg on innate cellular immunity

Interactions with various Fcγ receptors on innate immune cells: blockade of Fcγ-receptors

Immune cells recognise the Fc part of IgG via Fcγ receptors (FcγR). FcγR are expressed on a variety of immune cells, and can generate intracellular activating or inhibitory signals, thereby modulating the function of these cells. In autoantibody-mediated pathologies, the beneficial effects of high-dose IVIg therapy implicate at least in part the blockade of activating FcγR by monomeric IgG, thereby preventing the binding of opsonised antigens and induction of effector functions, like phagocytosis and secretion of pro-inflammatory cytokines by macrophages [6,7] and degranulation of granulocytes [8]. Active suppression of inflammation: interference with activating Fcγ receptors. Modulation of activating FcγR expression can contribute to the observed sustained unresponsiveness of phagocytes to autoantibodies following IVIg therapy. Thus, IVIg treatment has been shown to down-regulate expression of activatory FcγRI and FcγRIII on human monocytes in Kawasaki's disease patients [9], and FcγRIV on kidney-infiltrating macrophages in a murine nephritis model [10]. In vitro, IVIg suppress expression of FcγRIIA on human dendritic cells (DC), thereby preventing their activation by immune complexes [11]. In addition, by binding to FcγRIII, IVIg render murine and human macrophages refractory to IFNγ driven phagocytosis and inflammation. The mechanism includes suppression of the expression of the Interferon-γ (IFN-γ) receptor 2 subunit [12]. This suggests that engagement of the activatory FcγRIII can paradoxically trigger an inhibitory cellular program. The notion that non-conventional inhibitory signals can be generated by ligation of activatory FcγR has recently gathered support in several studies, although the mechanistic basis is still unclear (reviewed in: [13]).

Active suppression of inflammation: modulation of expression of inhibitory Fcγ receptor IIB

In humans, two types of FcγRII exist: the activatory FcγRIIA and the inhibitory FcγRIIB, the latter contains an intracellular ITIM motif, while in mice only the inhibitory variant is present. Evidence is accumulating in favour of an important role of the inhibitory FcγRIIB in the anti-inflammatory properties of IVIg in animal models. IVIg treatment induces FcγRIIB expression on murine effector macrophages in spleen [14] and kidney [10], and FcγRIIB blocking antibody abrogates the protective effect of IVIg in animal models [14]. In addition, as shown in experimental ITP, arthritis or nephritis, mice that are deficient for FcγRIIB do not respond to IVIg treatment [10,14,15]. The mechanisms by which IVIg alter the balance in expression of activating and inhibitory FcγR on murine cells are at present unclear. One possibility might be by neutralization of complement factor C5a [16]. C5a induces FcγRIII on murine macrophages, while inhibiting FcγRIIB expression [17]. IVIg are able to inhibit these effects of C5a on FcγR expression [18]. Another way might be via a two-step mechanism, in which IVIg interact with an unknown receptor on "regulatory macrophages" in the marginal zone of the spleen, which up-regulates the expression of FcγRIIB on "effector macrophages" so resulting in their inactivation (as discussed in the macrophages

section)[15]. However, whether modulation of FcγRIIB expression contributes to the beneficial effects of IVIg therapy in humans is questionable, since IVIg do not alter FcγRIIB expression on human DC in vitro [11], and IVIg treatment does not change the level of FcγRIIB transcripts in human monocytes [9].

Interaction with FcRn on innate immune cells

The role of neonatal Fc receptors (FcRn) in accelerating the clearance of pathogenic autoantibodies upon IVIg treatment has been thoroughly investigated. FcRn is expressed in the endosomal compartment of intestinal epithelium, vascular endothelium and macrophages. It regulates serum IgG levels by binding pinocytosed IgG in the endosomes and recycling it to the cell surface, thereby rescuing it from degradation in lysosomes. High dose IVIg therapy leads to saturation of this receptor by IgG, resulting in enhanced clearance of pathogenic autoantibodies (reviewed in: [13]). However, this receptor is also expressed on the surface of human monocytes and macrophages [19]. It would be interesting to investigate whether binding of IVIg to FcRn on these cells influences their function.

Interaction of IVIg with dendritic cells

Dendritic Cells (DC) are involved at several levels in the pathogenesis of autoimmune diseases, inflammatory disorders, and allograft rejection. High dose IVIg (at least 10 mg/ml) inhibit the differentiation and maturation of human DC, the up-regulation of co-stimulatory molecules CD80 and CD86, that are critical in DC-T cell crosstalk, and the ability to uptake autoantigens such as nucleosomes. Moreover, IVIg abrogate the capacity of mature DC to secrete pro-inflammatory cytokine IL-12 upon activation, while enhancing the production of anti-inflammatory cytokine IL-10, and suppress DC-mediated activation and proliferation of auto- and allo-reactive T cells [20-23]. Both Fc- and F(ab')2 fragments of IVIg are able to mediate the suppression of DC, thus suggesting the involvement of FcγR and non-Fc-receptor mediated signalling events in IVIg mediated modulation of DC function [20]. Moreover, IVIg differentially modulate the antigen presenting molecules on DC. While the expression of MHC class II is down regulated upon IVIg treatment of DC [20], the expression of CD1d and activation of CD1d-restricted NKT cells are enhanced [24]. In experimental models, IVIg suppress DC function in an autoimmune giant cell myocarditis model [25], and DC treated with IVIg ex vivo ameliorate ongoing autoimmune disease in vivo upon adoptive transfer, indicating that IVIg induce so-called "regulatory" activity in DC [26].

Interaction of IVIg with dendritic cells: primary immune deficiencies

Patients with primary immune deficiencies (PIDs), like common variable immunodeficiency (CVID) and X-linked agammaglobulinemia (XLA), are characterized by deficiency of circulating antibodies and recurrent infections. In addition, these patients also display defective functioning of the cellular immune compartment such as DC, macrophages and T cells [27-29].

To prevent recurrent infections, IVIg are used as a replacement therapy in these patients. The effect of substitution of IgG with broad specificities in patients with IgG deficiency is evident in protection against infections. However, we found that IVIg replacement therapy is not merely a substitution; rather, it can also rectify defective functioning of the cellular compartment. In vitro, reconstitution of patients' plasma with IVIg corresponding to the concentration reached in patients immediately following infusion (replacement dose; 5 mg/ml), could partially restore some of the defective expressions of markers on their DC [27,28]. This process is mediated at least in part by self-reactive anti-CD40 Abs within IVIg, and is accompanied by an increased IL-10 and decreased IL-12 production. This effect of IVIg on DC is mediated by the activation of the CREB-1 pathway that is implicated in the secretion of IL-10 by DC [28]. Therefore, the recovery of "regulatory" DC functions might explain the remission from Th1 immunopathologies in patients with PIDs upon receiving IVIg. Concisely, physiological concentrations of IgG attained in patients after IVIg replacement therapy can stimulate maturation of impaired DC from patients with PIDs, while supra-physiological concentrations reached upon high dose IVIg therapy of patients with auto-immune diseases, but normal plasma IgG concentrations, can inhibit maturation and function of healthy DC.

Interaction of IVIg with monocytes and macrophages

IVIg treatment suppresses the activation of monocytes and macrophages by altering transcription of various inflammatory genes [9], and lowers circulating levels of monocyte/macrophage inflammatory cytokines TNF-α and IL-1β [30]. Conversely, IVIg trigger monocyte production of IL-1 receptor antagonist (IL-1ra), a potent anti-inflammatory cytokine that counteracts IL-1. However, in mice this mechanism seems not to contribute to the beneficial effect of IVIg treatment in experimental ITP [31]. IVIg are able to transiently block the function of FcγR on splenic macrophages as demonstrated by a decrease in clearance of anti-D-coated autologous erythrocytes in patients [6]. In addition, peripheral blood monocytes from IVIg treated patients with ITP exhibit a decreased ability to form rosettes with IgG coated erythrocytes, and the administration of Fc fragments of IVIg to patients with ITP exhibit similar effects to those of IVIg [7]. As mentioned earlier, IVIg increase expression of FcγRIIB on the surface of "effector" macrophages in mice [10,14,15,32,33]. However, the IVIg mediated beneficial effect was not observed in mice lacking colony-stimulating factor (CSF)-1 dependent macrophages [15]. Thus, it has been suggested that CSF-1–dependent "regulatory" macrophages in the marginal zone of the spleen regulate IVIg mediated therapeutic effects by enhancing the expression of FcγRIIB on the surface of "effector" macrophages [33,34]. However, this hypothesis is inconsistent with a recent report showing that overexpression of FcγRIIB on spleen macrophages does not influence the pathogenesis of autoimmune diseases in mice [35]. The effects of IVIg on FcγRIIB expression on human tissue macrophages have not been studied, but IVIg treatment does not alter FcγRIIB mRNA expression in circulating human monocytes [9]. Therefore, the role of FcγRIIB in the effects of IVIg treatment, especially in humans, is still unclear.

Effects of IVIg on granulocytes

Neutrophil activation by immune complexes is antagonized by monomeric IgG present in IVIg by blocking activating FcγR [8]. Under inflammatory conditions, high doses of IVIg can also induce human neutrophil and eosinophil death, by virtue of antibodies against the sialic acid-binding Ig-like lectin 9 (Siglec-9) and Siglec-8, respectively, present in IVIg [36,37]. In addition, by inhibiting neutrophil adhesion to endothelium IVIg reduce neutrophil recruitment to inflamed areas in a mouse model of sickle cell disease. The molecular mechanism of action of IVIg in this model has still to be elucidated [38]. Conversely, anti-neutrophil cytoplasmic antibodies (ANCA) and IgG-dimers present in IVIg can enhance human neutrophil activation and thereby contribute to the adverse effects of IVIg therapy in some patients [39,40].

Effects of IVIg on natural killer cells

Contradictory observations have been published on the effect of IVIg on peripheral blood natural killer cells (NK cells). First, in women with recurrent spontaneous abortions accompanied with elevated NK cell levels, treatment with IVIg is associated with a favourable pregnancy outcome as IVIg suppress the percentage of NK cells in peripheral blood and reduce their functional activity [41-43]. On the other hand, beneficial effects of IVIg treatment in patients with Kawasaki disease and seizure disorders are accompanied by increased NK cell activity in peripheral blood [44]. In addition, IVIg stimulate the production of IL-12 by mononuclear cells, which leads to increased anti-tumor activity of NK cells, thereby reducing tumor spread in mice [45]. However, differences between NK-cell subtypes and the site at which the NK cells exert their function might explain the apparent contradictions between these studies. The enhanced NK-cell activity in blood of Kawasaki patients might in fact reflect reduced recruitment to the site of infection [44]. It is also important to note that NK cells are capable of interacting with other innate immune cells. We have reported that multimers present in IVIg enhance antibody dependent cell-mediated cytotoxicity (ADCC) of DC by NK cells. Thus, by promoting the interaction between NK cells and DC, IVIg might reduce the antigen-presenting pool and inhibit T-cell priming induced by DC [46].

Effects of IVIg on adaptive cellular immunity

Effects of IVIg on T-cell subsets

IVIg inhibit human T cell proliferation and cytokine production after mitogenic and allogeneic stimulation in vitro as effectively as calcineurin inhibitors [22,47]. The mode of action by which IVIg inhibits T-cell activation is so far not completely elucidated and likely involves multiple pathways. Inhibition might occur by natural autoantibodies against TCR, CD4 and MHC class I that are present in IVIg preparations [2]. The inhibitory effect of IVIg on T-cell activation is partly due to suppression of antigen presenting cells (as discussed above), but is certainly also mediated by a direct interaction of IVIg with T cells. IVIg suppress proliferation and cytokine production

of purified T cells [47,48] by inhibition of IL-2 production by T cells at a post-transcriptional level [47] and induction of lymphocyte apoptosis via Fas (CD95) and activation of caspases [49]. Pre-treatment of activated T cells with IVIg has been reported to attenuate neurotoxicity of human T cells, possibly through an effect on LFA-1 expression, and by lowering the level of Fas and FasL expression on T cells [50].

In rat models, IVIg have been shown to be protective against two distinct T cell mediated autoimmune diseases, i.e. uveoretinitis and encephalomyelitis [51-53]. Interestingly, IVIg can directly bind to activated, but not to resting rat T cells, thus suggesting FcγR independent interaction of IVIg with T cells [53]. In addition, IVIg treatment protects against GVHD in rats, which was associated with decreased proliferation of lymphocytes, reduced production of IFN-γ and enhancement of apoptosis of activated T cells [54]. Reduced proliferative responses of T cells might also explain the beneficial effect in lupus like syndrome and atherosclerosis in mice [55,56]. Yet IVIg treatment is not effective in T cell-mediated concanavalin A-induced hepatitis in mice [57].

Although the suppressive effects of IVIg on T cells are significant, how IVIg affect the function of distinct T-cell subsets is unclear. Interestingly, IVIg have been shown to expand and enhance the functions of human and murine Foxp3+ regulatory T cells (Tregs) both in vitro and in vivo [58-61]. Thus, beneficial effects of IVIg in patients are associated with an increase in Tregs both functionally and numerically [59,60], while IVIg failed to protect against encephalomyelitis in mice that were depleted of the Tregs population [58]. The mechanisms underlying the IVIg mediated expansion, and enhanced suppressive properties of Tregs remain unclear and are in need of further investigation. Interestingly, both CD4+CD25+ Treg and CD4+CD25- non-Treg bind IVIg, but Tregs better that non-Treg [58]. We speculate that most of the direct effects of IVIg on T cells published before are mediated by activation of Tregs. It is at present unknown to which surface molecules on Tregs IgG binds. It is tempting to speculate that self reactive natural autoantibodies present in IVIg preparations may play an important role.

Interaction of IVIg with B cells

The therapeutic effects of IVIg in several antibody-mediated diseases are not merely due to passive effects, such as neutralization and FcγR blockade. They also include suppression of the expansion of autoreactive B lymphocytes through signalling via FcγRIIB, idiotypes mediated inhibition of B-cell receptors, and neutralization of cytokines such as the B-cell survival factors BAFF and APRIL [49,62-67]. Binding of the anti-idiotypic antibodies to the surface IgG or IgM of B cells can transmit negative signals and result in down modulation of pathogenic auto-antibody production [2]. In addition, IVIg can induce the secretion of IgG reacting against various self- and non-self antigens from a unique subset of human B lymphocytes. These de novo induced antibodies might further help in controlling reactivities of pathogenic autoantibodies either by idiotype-mediated mechanisms or by sequestering autoantigens [67]. Analogous to these in vitro findings, IVIg therapy in women with recurrent spontaneous abortion is accompanied by decrease in the peripheral blood B cell numbers [41].

THE IMMUNOMODULATORY EFFECTS OF IVIG IN VIVO: RESULTS FROM CLINICAL EXPERIENCE

IVIg have major clinical impact in the treatment of various diseases caused by hyperactivity of the cellular arm of the immune system. Both in autoimmune diseases and transplantation, considerable progress has been made in recent years in elucidation of the effects of IVIg on cellular immunity in experimental animal models, but these models might not always reproduce the complexity of the diseases in the human situation. Limited data are available on the mechanistic effects of IVIg treatment on cellular immunity in patients.

IVIg in immune thrombocytopenic purpura (ITP)

The precise mechanisms of action of IVIg in patients with ITP remain controversial, and include at least inhibition of platelet phagocytosis by FcγR blockade (as discussed in the monocytes section above). Treatment with IVIg also results in a rapid, but transient increase of anti-inflammatory cytokines [68], which are thought to further reduce the phagocytic activity of the reticuloendothelial system. Stable remission and good prognosis after IVIg therapy has been reported to be associated with a Th0 or Th2 cytokine pattern, whereas sustained high expression of IFN-γ reflects poor prognosis for ITP [69]. A strong association between FcγRIIB polymorphisms and development of chronic disease in children with acute ITP suggests involvement of this receptor in ITP pathogenesis in humans [70]. However, the importance of inhibitory FcγRIIB receptor or inhibition of IFN-γ signalling in mediating the therapeutic effects of IVIg, as suggested by animal studies, remains to be determined in patients.

IVIg in Kawasaki Disease

Serum levels of pro-inflammatory cytokines are elevated in patients with Kawasaki disease, which stimulate de novo production of acute phase proteins causing thrombocytosis and fever in response to tissue injury. Following IVIg therapy, the expression of adhesion molecule CD11b on neutrophils, and of inflammatory cytokines and chemokines including IL-6, G-CSF and C-reactive protein, are significantly decreased [31,71,72], while IL1Ra is increased. More recently, gene array analysis has revealed that IVIg down regulate transcripts of activating FcγRI and FcγRIII receptors and the chemokine receptor CCR2 in monocytes. In addition the production of monocyte derived inflammatory proteins adrenomedullin, S100 A8, A9 and A12 were suppressed [9]. Interestingly, FCGR2B transcripts were not increased following IVIg therapy, although the staining intensity for CD32 was unchanged [9]. These results suggest that IVIg in patients can balance the expression of activating and inhibitory FcγR but, unlike mouse models, this modulation might not involve up-regulation of FcγRIIB.

IVIg in Guillain-Barré syndrome

Cellular interactions mediated through the release of cytokines play a role in the pathogenesis of GBS. The majority of the patients with GBS show elevated serum levels of TNF and IL-1β, inflammatory cytokines that can induce demyelination of peripheral nerves. Following IVIg therapy, these cytokines decline significantly [73,74]. Further, a recent study has demonstrated that the number of Tregs in acute stage GBS patients is significantly decreased and attains the level of healthy controls in stable patients following IVIg therapy [60]. In addition, soluble TNF receptor II is increased in the serum of patients following IVIg therapy, which may inhibit the demyelinating effect of TNF in the peripheral nerves [74].

IVIg in transplantation

Following allogeneic hematopoietic stem cell transplantation, a potential beneficial effect of IVIg in preventing GVHD has been reported, but it requires a high dose of IVIg [75]. The beneficial effects of IVIg in GVHD were not confirmed in other studies, but there were differences in dosing strategy in various studies [4]. The utility of targeted trough levels for IVIg dosing in GVHD is a potential area of investigation.

To shorten the waiting time for solid organ transplantation and to improve graft survival, sensitized patients with high titers of donor-specific anti-HLA antibodies are treated with IVIg, often in combination with plasmapheresis, anti-lymphocyte antibody or immunoadsorption techniques. In addition, IVIg are used to facilitate transplantation of ABO incompatible organs. After organ transplantation, IVIg have been used successfully for treatment of antibody mediated rejection [76]. In renal transplant patients, IVIg are effective as treatment of steroid resistant cell mediated rejection [3,5]. In addition, prophylactic administration of hyperimmune anti-HBV surface antigen specific IVIg (anti-HBs IVIg) in hepatitis B-infected liver transplant patients protects against acute rejection [23,77].

Together, IVIg could be a powerful treatment for controlling the acute rejection process after organ transplantation. However, clinical trials are needed to determine whether IVIg administration allows reduction or discontinuation of the maintenance immunosuppressive treatment (e.g. cyclosporine) in order to reduce the long term side effects after transplantation.

Future perspectives

Antibodies are the "first line defence" mechanism of our immune system, with a broad repertoire of activities, and exert a wide range of effector functions. Therapeutically administered antibodies in the form of IVIg, as a physiological means to modulate the immune system, have proven over the years to be effective. IVIg targets the cellular immune compartment at multiple levels, including innate and adaptive immune cells (Figure 1). Although increasing numbers of animal models have provided us with new insights, the reported mechanisms of action of IVIg are either not confirmed in patients or have shown discrepancies. Future studies in humans must focus on immune cells at the site of inflammation or in lymphoid tissues. Due to the

high costs and the upcoming shortage of IVIg as a consequence of shortage in donated blood plasma, it is important to identify the active components in IVIg in various diseases. Although IgG dimers and sialylated IgG have shown promising results in experimental models, their efficacy in patients needs to be established. Purification of the anti-inflammatory compound or production of synthetic or biological alternatives of IVIg will enable us to selectively target pathogenic immune activity.

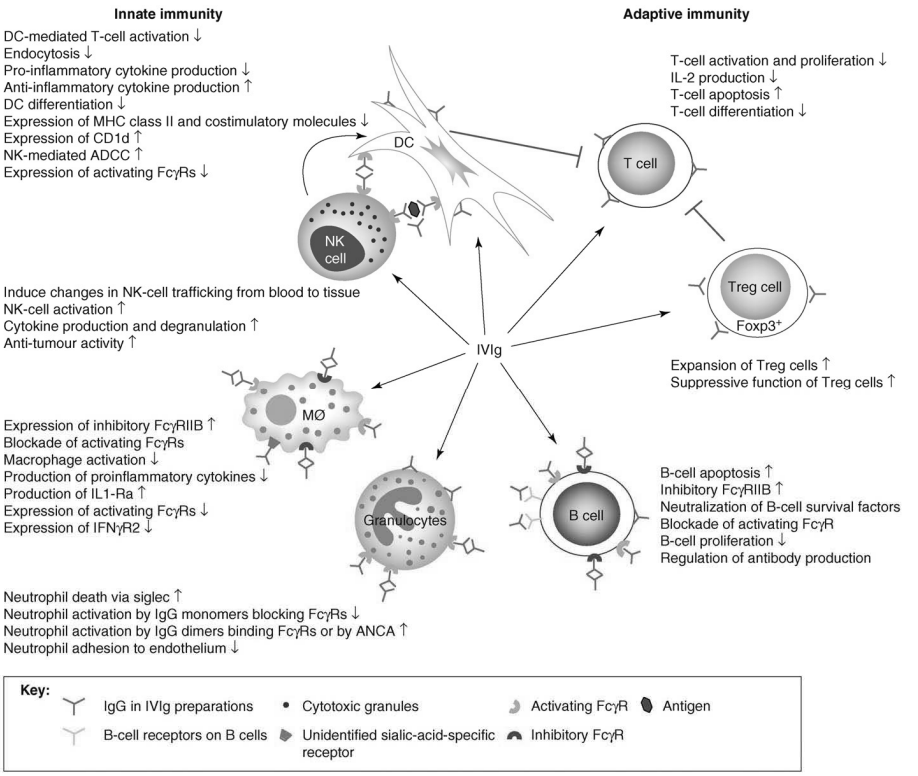

Figure 1: A schematic representation of the proposed mechanisms of action of Intravenous Immunoglobulin (IVIg) on cellular immunity.
IVIg target the cellular immune compartment at multiple levels, including innate and adaptive immune cells. IVIg interact with DC, macrophages (MØ) and granulocytes mainly via activating and inhibitory Fcγ receptors. Monomeric IgG in IVIg preparations can block the interaction of immune complexes with activating Fcγ receptors, thereby inhibiting endocytosis and phagocytosis by DC and macrophages, and activation of granulocytes. IgG dimers in IVIg preparations binding to activating Fcγ receptors on macrophages induce the expression of the inhibitory FcγRIIB and suppress expression of IFNγR2, thereby inhibiting macrophages functions. In addition, IgG dimers promote ADCC of DC by NK cells, resulting in reduced T-cell activation. IgG dimers suppress macrophage and B-cell functions by ligating FcγRIIB. In addition, F(ab')2-mediated effects of natural antibodies present in IVIg have been described for DC activation (mainly via CD40), granulocytes (induction of apoptosis via Siglec) and B cells (idiotype-mediated inhibition of B-cell receptors). Interactions between IVIg and Tregs via yet unknown mechanisms lead to expansion and increased suppressive function of Tregs.

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Chapter 9

Summary and discussion

Conclusions and future directions

Dutch summary (Samenvatting)

Summary and discussion

Introduction

Over de last four decades, liver transplantation has evolved from an experimental therapy for a limited number of selected patients to the treatment of choice of patients with end stage liver diseases. This remarkable success is in part attributable to the development of immunosuppressive drug therapy. The introduction of calcineurin inhibitors and other immunosuppressive agents has led to a dramatic reduction in the incidence and the severity of acute rejection, and consequently to a significantly improved graft and patient survival during the first post-operative years. Still, the long term outcome of liver transplantation has not improved in similar degree [1,2]. Today, the main obstacles to successful long term patient survival are the high incidence of cancer, infection, renal failure, and cardiovascular diseases, due to life long use of immunosuppressive drugs [3-8]. Currently, the main challenge of transplant physicians is to improve the long term outcomes after liver transplantation by optimising the current immunosuppressive regimen and dosing strategy in order to reduce toxicity, or by exploring therapeutic potentials of new immunosuppressive drugs with moderate side effects [2].

Aim of this dissertation

The overall aim of this dissertation was to explore ways of reducing long term side effects of immunosuppressive drugs in liver transplant recipients by either optimising the current dosing strategy of cyclosporine, and to investigate the therapeutic potential of alternative immunosuppressive drugs, in particular intravenous immunoglobulins (IVIg). Our final goal is to develop a less toxic immunosuppressive protocol to be used as therapy to prevent liver allograft rejection. The basic concept behind our approach is based upon the clinical observation that liver transplant patients treated with anti-HBs IVIg, a form of hyperimmune IVIg, as prophylaxis, had a two-until three-fold lower risk of rejection (chapter 3) [9-11]. Based on this finding, we investigated the effect of IVIg on different cell types mediating innate and adaptive immunity, searching for the explanation for the beneficial effects of IVIg in hampering the process of cell mediated rejection. Better understanding of the effects of IVIg on the cellular immune response, may help us to identify the therapeutic potential of IVIg in preventing transplant rejection, which can form a basis to develop a less toxic immunosuppressive regimen after liver transplantation.

Main findings

Benefits and drawbacks of cyclosporine dosing based on C_2 levels

In chapter 2, we investigated the effect of cyclosporine dose reduction based on cyclosporine whole blood concentration two hours after administration (C_2 levels) on renal function in long term liver transplant recipients. Cyclosporine C_2 levels were measured in 60 stable liver

transplant recipients more than 1 year after transplantation, and dose reduction was performed if C_2 levels exceeded the recommended target range of 600 ng/ml ± 20 % [12]. In twenty three patients (38%), C_2 levels were above the upper limit of the target range, whereas C_2 levels were within the target range in 27% of the patients. In twenty of the 23 patients with high C_2 levels, the cyclosporine dose was reduced. Although cyclosporine target levels were reached rapidly in these patients, at 6 months follow up, no improvement of renal function, lipid levels or systolic blood pressure was observed. However, after cyclosporine dose reduction, one patient experienced an episode of acute cellular rejection, in 2 patients primary biliary cirrhosis recurred, and one patient had a recurrence of autoimmune hepatitis. We concluded that according to current recommendations [12,13], cyclosporine overexposure is common among long term liver transplant patients. However, cyclosporine dose reduction based on recommended C_2 levels did not result in an improvement in renal function, and was accompanied with a risk of immune activation, especially in patients on cyclosporine monotherapy.

The therapeutic potential of anti – HBs IVIg

In order to explore the therapeutic potential of other immunosuppressive drugs with fewer side effects, chapter 3 became the cornerstone of this thesis, as it forms the basis for a concept to investigate the immunomodulatory effects of anti-viral, and non-specific IVIg on the cellular rejection response. In this chapter, the efficacy of anti-viral intravenous immunoglobulins (anti-HBs IVIg and anti-CMV IVIg) [10,11] in preventing acute rejection after liver transplantation was assessed in a retrospective analysis, and the effects of anti-HBs IVIg was correlated to their effects on immune cells in vitro. We observed that HBsAg positive liver transplant patients (n = 40), who were treated with anti-HBs IVIg as prophylaxis to prevent HBV re-infection of the graft, had a significant lower incidence of acute rejection compared to recipients without viral hepatitis (n = 147) (12% versus 34%; p = 0.012). The low rejection rate was not due to a possible immune compromised status of HBsAg positive patients before transplantation, as the incidence of rejection in HCV positive recipients (n = 29) was similar to that in the control group. Treatment with anti-CMV IVIg (n = 18), which resulted in lower anti-CMV IVIg concentrations in serum compared to anti-HBs IVIg-treatment, did not protect against rejection. In vitro, anti-HBs IVIg suppressed functional maturation and cytokine production by human blood-derived dendritic cells (DC) at concentrations similar to the serum concentrations reached during anti-HBs IVIg treatment of liver graft recipients. In addition, anti-HBs IVIg inhibited allo-antigen and lectin stimulated proliferation of peripheral T cells. We concluded that anti-HBs IVIg protects against acute rejection after liver transplantation, probably by functional inhibition of the two principal immune cells involved in allograft rejection, DC and T cells. The mechanism by which IVIg suppress DC and T-cell function was so far unidentified. In the next chapters, we investigated in depth the immunomodulatory effect of IVIg on immune cells of, both from innate and adaptive origin [14], which play altogether a pivotal role in the cellular rejection process.

The immunomodulatory mechanisms of action of IVIg

Comparison to calcineurin inhibitors

In chapter 4, we compared the suppressive effects of IVIg and the calcineurin inhibitors (CNI), Cyclosporin (CsA) and Tacrolimus (TAC), on human blood derived T cells and DC. This is the first study to compare the immune suppressive activities of IVIg to that of CNI, which are the first treatment of choice in current immunosuppressive regimens [5,8]. CNI are extremely potent inhibitors of T-cell proliferation and cytokine production. Whether CNI also inhibit DC function was less clear [15]. We observed that IVIg and CNI were equally effective in inhibiting proliferation and IFN-γ production of T cells after polyclonal or allogeneic stimulation. CD8+ T cells were preferentially affected by both IVIg and CNI. Like CNI, addition of IVIg at later time points after T-cell activation suppressed mitotic progression of responding T cells. Furthermore, IVIg treated DC were suppressed in their capacity to stimulate allogeneic T-cell proliferation by 73 ± 12%, whereas DC function was not affected by CNI. The decreased allogeneic T-cell stimulatory capacity of IVIg treated DC correlated to induction of DC apoptosis, and decreased up-regulation of CD40 and CD80. The direct inhibitory effect of IVIg on T cell proliferation was at least partly due to induction of apoptosis in T cells. From these observations, we concluded that IVIg have strong suppressive effects on both human T cells and DC, while CNI only inhibit T cells. Therefore, by simultaneous targeting both T cells and DC, we assume that IVIg will be superior in the control of acute rejection and GVHD in comparison to CNI. Since long term IVIg treatment has no significant side effects [16,17], inclusion of IVIg in immunosuppressive therapy after transplantation may enable dose reduction of CNI, and thereby contribute to a lower rate of complications. The exact mechanisms of action of the inhibitory effects of IVIg on DC and T cells were further investigated in chapter 5 and 7.

Effects of IVIg on dendritic cell function: ADCC by NK cells

IVIg can modulate the function of various immune cells, including suppressing the capacity of DC to stimulate T cells. In chapter 5, we focused on the mechanism by which IVIg affect DC function [18,19]. We found that the decreased T-cell stimulatory capacity of IVIg treated DC was associated with induction of cell death in mature DC. Interestingly, IVIg treatment itself did not induce DC death directly, as the increased death of IVIg treated DC only occurred when cultured together with other immune cells, i.e. NK cells. Cross-talk between DC and NK cells can result in lysis, inhibition or maturation of DC by NK cells, and reciprocally, DC can activate or inhibit NK-cell functions. The final outcome of DC-NK cell interaction depends on the conditions in which both cell types encounter each other [20-23], and will subsequently determine the development of the following adaptive immune response. We showed that DC matured in presence of IVIg (IVIg-DC) activated NK cells, and increased their IFN-γ production and degranulation. The activated NK cells induced apoptosis of the majority of IVIg-DC. In consequence, only in the presence of NK cells, IVIg-DC were 4-fold impaired in their T-cell priming capacity. This was

due to NK-cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC) to IVIg-DC. By binding to the DC surface, IVIg facilitate recognition of mature DC by NK cells via FcγRIII, resulting in activation of the NK cells. These NK cells acquire the capacity to kill the IVIg-DC by ADCC, thereby reducing in numbers the total stimulatory pool of mature DC. Interestingly, multimers, but not monomers of human monoclonal IgG, could replicates these effects of IVIg, suggesting that IgG multimers and/or dimers in IVIg preparations are the active components that induce NK-cell activation and DC lysis. Furthermore, IVIg-DC down regulated the expression of NKp30 and KIR receptors, and induced the generation of $CD56^{bright}$CD16-CCR7+ lymph node type NK cells. These results identify a novel pathway, in which IVIg induce ADCC of mature DC by NK cells, which downsizes the antigen presenting pool, and inhibits T-cell priming [24]. This model has also physiological relevance, in that it predicts that immunoglobulin-antigen immune complexes may contribute to the termination of T-cell responses during for example clearance of infections. As immune complexes emerge when antigen-specific antibodies have been formed, physiological immune complexes as well as immune complexes in IVIg preparations may be the essential element that contributes to the termination or "cooling down" of the immune system after prolonged activation [25-27]. ADCC, identified as the pathway by which IVIg exert one of their immunomodulatory capacities, is probably not restricted to DC killing. Any cell type that expresses Fcγ receptors [28,29], that are able to bind IgG complexes, can be identified as "target cells", and is therefore susceptible to ADCC by NK cells. On the other hand, in anti-rejection protocol in which therapeutic antibodies such as anti-CD4 mAb, rabbit anti-thymocyte globulin (rATG) or co-stimulation blockade are used [30-33], NK cells may cause destruction of antibody coated target cells through antibody-dependent cell-mediated cytotoxicity. In chapter 6, we elaborate further on this hypothesis.

The changing role of Natural Killer cells in transplantation

Natural killer cells (NK cells) have the potential to display different functional activities after transplantation. The traditional view is that NK cells are predominantly pro-inflammatory and although they are not sufficient to reject a solid allograft directly, they may contribute to early chemokine and cytokine production after transplantation, and thereby supporting the rejection response [34-36]. In contrast, NK cells have been implicated as playing a role in the induction of tolerance to organ allografts [24,37], while in hematopoietic stem cell transplantation (HSCT) the presence of NK cell alloreactivity in the graft-versus-host direction has been associated with a reduced incidence of leukemia relapse without causing graft-versus-host disease (GVHD) [38,39]. In chapter 6, we reviewed a manuscript [24], in which a novel role for NK cells in the setting of co-stimulation blockade was identified. Alloreactive NK cells of recipient origin were found to have the capacity to kill donor derived antigen presenting cells (APC), thereby reducing T cell priming and promoting long term skin graft acceptance in mice with co-stimulation blockade therapy. Depletion of NK cells from the recipient mice using a depleting anti-NK1.1 mAb rendered the triple costimulation blockade treatment completely ineffective at preventing the

rejection response. We went further and tried to provide an answer for the intriguing question: How do NK cells influence the efficacy of triple costimulation blockade? We proposed a model in which the cells to which the co-stimulation blockade antibodies have bound are rendered susceptible to ADCC killing by NK cells. NK cells express Fcγ receptor III (CD16), which recognizes IgG1 and IgG3 subclasses [40]. Each component of the triple co-stimulation blockade treatment, anti-CD154, CTLA-4Ig and anti-OX40L can bind with either T cells or APC [41]. Their Fc region can be recognized by Fcγ receptor III on the NK cell, which can lead to ADCC of both donor APC and recipient T cells. In addition, another pathway of alloreactive killing may also be triggered, when NK cells derived from the transplant exert cytotoxicity against the APC of the recipient. The latter is less significant for small grafts, however, for liver grafts where large numbers of viable donor lymphocytes migrate into the recipient after transplantation, NK alloreactivity in the graft-versus-host direction could become important [38,39,42,43].

NK cells are innate immune effector cells expressing stimulatory receptors, which recognize antigens on virus infected cells and tumour cells, while sparing normal cells [44]. In immunocompromised transplant patients, it is essential to try maintaining these useful functions of NK cells. Therefore, the effects of immunosuppressive therapies on NK cell function should be considered.

Effects of IVIg on regulatory T cells

In the past decades clinical evidence has emerged that IVIg are effective in the treatment of T cell mediated autoimmune diseases [16,45,46], and that hyperimmune IVIg can protect against T cell mediated graft rejection after liver transplantation [9]. In vitro, IVIg can inhibit human T cell proliferation and cytokine production as mentioned in chapters 3 and 4 [47,48]. Chapter 7 focus on the mechanisms by which IVIg attenuate T cell responses. We investigated whether IVIg might enhance the suppressive function of CD4+CD25+Foxp3+ regulatory T cells, and if so, whether IVIg could prevent allograft rejection. Regulatory T cells (Tregs), expressing the lineage marker Foxp3, are considered to be the "central immune regulator" of numerous immune processes, including transplant rejection [49]. Importantly, Tregs play a critical role in induction and maintenance of transplantation tolerance in experimental animal models [50-52]. Several compounds have been identified that stimulate the expansion and/or the suppressive capacity of Tregs. For example, rapamycin has shown to stimulate Tregs activity and survival in vitro [53-57] and in vivo in patients [57]. In addition, therapeutic antibodies such as anti-CD4 mAb and rabbit anti-thymocyte globulin (rATG) can enhance the activation status and the expansion of CD4+CD25+Foxp3+ cells [32,58-60]. Although all these compounds are suited for in vitro expansion and stimulation of Tregs, their use to activate Tregs in vivo will be accompanied by severe side effects upon administration to patients. Therefore, activation of Tregs in vivo by IVIg treatment may be an attractive option for prophylaxis of transplant rejection, since IVIg administration is safe and has moderate side effects.

We demonstrated that IVIg bind to both human and mouse regulatory T cells. As IVIg contain natural antibodies that recognize different surface molecules such as TCR-β, CD3 and CD4 on cells of the immune system [61,62], binding of IgG to human Tregs may be mediated via its variable F(ab)2 region, which recognise surface molecules on Tregs. Still, the binding of the Fc region of IgG to an unknown Fc receptor on Tregs is a possibility [63-66]. In addition, in both human and mouse, IVIg treatment enhanced the activation of CD4+CD25+Foxp3+ regulatory T cells, as detected by increased expression of surface activation markers and ZAP70 phosphorylation. Pre-incubation of human CD4+CD25+ T cells with IVIg enhanced their ability to suppress allogeneic T cell proliferation, while depletion of human CD25+ T cells resulted in a twofold reduction in IVIg mediated suppression of allogeneic T cell proliferation. Interestingly, both direct binding of IVIg to Tregs and the presence of APC were necessary to induce Tregs activation. Probably, APC may serve in this situation as an intermediate to crosslink the Fc regions of IgG present on the Tregs surface, and thereby induce Tregs activation. In vivo, administration of IVIg prevented T cell mediated rejection of fully mismatched skin grafts in immunodeficient mice reconstituted with total CD4+ T cells. Significantly, this IVIg effect was lost when CD4+CD25+ cells were depleted from T-cells which were transferred to the mice, suggesting that IVIg mediate their effect through Tregs. IVIg was able to induce allograft survival that persisted well beyond 100 days even though the IVIg treatment was discontinued after day 14. Unfortunately, we have been unable to achieve prolongation of skin allograft survival in immunologically intact wild-type mice using IVIg treatment. This is probably due to the large total number of alloreactive T cells in intact recipients compared with that in the adoptive transfer experiments described in our study. This observation suggests that the full potential of IVIg to modulate adaptive T cell responses may be seen only in combination with strategies that induce partial T cell depletion and attenuation. Of the potential candidates that exist, depletion using CAMPATH-1H [67] or modulation using humanised anti-CD3 antibodies [68] appear to be promising. In this chapter, we demonstrated that through direct binding and activation of regulatory T cells, IVIg can mediate suppression of allogeneic T cell responses, highlighting an additional, important property of these compounds. Importantly, the data underline that the therapeutic potential for IVIg may be broader than today's application in organ transplantation considers.

Modulation of the cellular immune system by IVIg

In chapter 8, we discuss recent advances in our understanding of different, mutually non-exclusive mechanisms of action of IVIg on cells of innate and adaptive immunity. The means by which IVIg exert therapeutic effects in disorders mediated by cellular immunity has remained an enigma. Clinical improvements followed by IVIg treatment often extend beyond the half-life of infused IgG, suggesting that IVIg modify the cellular immune compartment for a prolonged period.

Both in autoimmune diseases and transplantation, considerable progress has been made in recent years in elucidation of the effects of IVIg on cellular immunity in experimental animal models, but these models might not always reproduce the complexity of the diseases in the human situation. Limited data are available on the mechanistic effects of IVIg treatment on cellular immunity in patients.

In the light of organ transplantation, it is of importance to study the effects of IVIg on the indirect pathway of allorecognition as donor derived APC are probably deceased long after transplantation [69,70]. The indirect pathway in which the recipients' T cells recognise the donor antigen displayed by the MHC of recipients' APC will therefore become more prominent late after transplantation. In this thesis, we focus on the effect of IVIg on the direct pathway of allorecognition. How IVIg alter the process of antigen processing and antigen presentation by recipients' APC, and how IVIg modify recipient' APC-T cell interaction, remain important questions to be answered by future studies.

Antibodies have a broad repertoire of activities and exert a wide range of effector functions. Therapeutically administered antibodies in the form of IVIg, as a physiological means to modulate the immune system, have proven over the years to be effective in various immune mediated diseases [16,17,26,71-73]. IVIg target the cellular immune compartment at multiple levels, including innate and adaptive immune cells. Although increasing numbers of animal models have provided new insights, the reported mechanisms of action of IVIg are either not confirmed in patients or there are discrepancies between human and animal data.

Potential active components in IVIg

Due to the high costs and the upcoming shortage of IVIg as a consequence of shortage in donated blood plasma, it is important to identify the active components in IVIg in various diseases. IgG dimers and sialylated IgG are promising candidates with encouraging results in experimental models [26,74].

Due to the large donor pool and different manipulations during the purification process of IVIg, complexes of anti-idiotype antibodies with complementary idiotypes can be formed [25]. Since dimers and aggregates in IVIg preparations can induce severe side effects, several precautions are taken during the production process to keep the content of multimers in IVIg preparations minimal [75]. Immune complexes are generally regarded as pro-inflammatory compounds, still, evidence is emerging that they can also mediate suppression of immune responses [76,77]. IgG dimers can be one of the active anti-inflammatory components in IVIg, which are not present in the immune repertoire of a single individual, but are formed in plasma pools due to idiotype-anti-idiotype complex formation between IgG molecules from different individuals [25,78,79]. However, it is less likely that IgG dimer is the only component of IVIg that is responsible for the immunomodulatory effects. Moreover, some effects on DC in vitro can be recapitulated

by F(ab)' fragments and are probably related to natural antibodies against cytokines present in IVIg [80].

Accumulating evidences suggest that the immune system is able to make distinctions between different antibody glycoforms. Interactions between human IgG and Fc receptors depend on the sugar structures on both the IgG as on the receptor [81]. G0 glycoforms (no galactoses and no sialic acids) correspond to the pro-inflammatory effects of IgG [74]. In two animal models, Kaneko et al. illustrated that sialylated S2 glycoforms are responsible for the anti-inflammatory effects of IVIg [82]. The precise sialylation pattern that is responsible for the anti-inflammatory activity of IVIg in experimental models is the 2,6-linkage of sialic acid to galactose on the glycan found at Asn^{297} in the CH2 region of human IgG [83]. Since sialylation at this residue reduced FcγR binding, it was proposed that this 2,6-sialylated IgG binds to a currently unidentified sialic acid-specific receptor on macrophages in the marginal zone of the spleen. These marginal zone macrophages in turn enhance the expression of FcγRIIB on conventional macrophages by an unknown mechanism. Whether the effects of IVIg on human immune cells are mediated by this minor sialylated component of IVIg (1-2 %) is at present unknown.

Overall, the efficacy of IgG dimers and sialylated IgG in patients needs to be established in future research. Purification of the anti-inflammatory compound or production of synthetic or biological alternatives of IVIg will enable us to selectively target pathogenic immune activity in future.

Liver transplantation and IVIg

The liver is an intriguing organ. Spontaneous acceptance of liver allografts occurs in several species. However, tolerance is rare in human transplant patients even though liver graft rejection is relatively easily reversed [84-87]. Histological features of acute rejection in liver transplantation are similar to those in other organs. Nevertheless, mechanisms of rejection of liver transplants may differ in degrees and cellular involvement. Liver-specific cell populations, such as Kupffer cells, liver sinusoidal epithelial cells, and hepatic stellate cells, may contribute to liver tolerogenicity. Other mechanisms, such as microchimerism, soluble MHC molecules, and regulatory T cells, may also participate in inducing tolerance [88]. Understanding the mechanisms of liver transplant rejection and tolerance, and the availability of better immune monitoring could help develop strategies to recognize tolerance and reduce rejection.

In the setting of liver transplantation, physicians still face the challenge to improve the long term outcomes after transplantation by either optimising current immunosuppressive regimens or exploring therapeutic potentials of immunosuppressive drugs. In this thesis, we investigated the effects of IVIg on the different cells of the innate and adaptive immune system, which altogether play a pivotal role in the cellular rejection response. Based on the reported findings from our group and others, we consider IVIg as a promising candidate for prevention and attenuation of the acute cellular rejection process after liver transplantation.

It is important to note that IVIg treatment will probably not be able to create "true allograft tolerance", defined as acceptance of donor allograft despite cessation of immunosuppressive therapy, while retaining the ability to reject third party allografts [33]. As IVIg contain a broad spectrum of antibodies and can exert various immunomodulatory properties, most of the identified immunomodulatory mechanisms appear to be antigen non-specific, and therefore do not specifically target or modulate alloreactive T cells. However, IVIg is still a compound that can induce strong suppression of alloreactive responses at multiple levels, while having moderate side effects. Therefore, IVIg should be considered as a serious candidate to be included in future immunosuppressive regimens. First, clinical trials are needed to determine optimal dosing strategy, and to assess whether IVIg administration allows reduction or discontinuation of the maintenance immunosuppressive treatment in order to reduce the long term side effects after liver transplantation.

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CONCLUSIONS

Chapter 2: Cyclosporine dose reduction based on recommended C_2 levels does not result in an improvement in renal function in stable liver transplant patients, and is accompanied with the risk of immune activation, especially in patients on cyclosporine monotherapy.

Chapter 3: Anti-HBs IVIg protects against acute rejection after liver transplantation, probably by functional inhibition of the two principal celtypes involved in allograft rejection, i.e. dendritic cells and T cells.

Chapter 4: Since IVIg can simultaneously target both human T cells and DC, while calcineurin inhibitors only inhibit T cells, inclusion of IVIg in future immunosuppressive therapy after transplantation may enable dose reduction of calcineurin inhibitors, and thereby contribute to a lower rate of complications.

Chapter 5: By influencing the bi-directional interaction between DC and NK cells, IVIg induce ADCC of mature DC by NK cells, and thereby hamper the ability of the innate immunity to trigger proper T-cell activation.

Chapter 6: Antibody-dependent cell-mediated cytotoxicity (ADCC) killing of APC mediated by NK cells may be a pathway by which costimulation blockade induces graft acceptance in a skin transplant model.

Chapter 7: Through immediate binding and activation of regulatory T cells, IVIg can mediate suppression of allogeneic T cell responses, high-lighting an additional, important therapeutic potential of IVIg in organ transplantation.

Chapter 8: With the novel immunomodulatory mechanisms of IVIg identified and the upcoming shortage of IVIg, it is important to identify the active components in IVIg in various diseases.

FUTURE DIRECTIONS

Mechanisms of action

- Identify the active components in IVIg preparations. IgG dimers and sialylated IgG are promising candidates.
- IVIg treatment is accompanied by increased NK cell activity and cytokine production *in vitro* and *in vivo* in peripheral blood of treated patients. NK cells are innate immune effector cells expressing receptors, which recognize antigens on virus infected cells and tumour cells, while sparing normal cells. We would like to study the potential anti-tumor and anti-viral effects of IVIg, first in vitro, and later in liver transplant patients.
- Study the immunomodulatory effects of IVIg on liver-specific cell populations, such as Kupffer cells, liver sinusoidal epithelial cells, and hepatic stellate cells.
- Study the effect of IVIg on the indirect pathway of donor antigen processing and presentation by recipients' APC.

Ex vivo immunomonitoring in patients

- Investigate the immunomodulatory effects of IVIg *ex vivo* in patients receiving IVIg therapy or anti-HBs IVIg therapy. Focus will be on the changes in cellular immunity, cytokine profile and expression of inhibitory Fc receptors.

Include IVIg in the immunosuppressive protocol after liver transplantation

- Determine with clinical trials the optimal dosing strategy for IVIg
- Assess whether IVIg administration allows reduction or discontinuation of the maintenance immunosuppressive treatment in order to reduce the long term side effects after liver transplantation.

SAMENVATTING EN DISCUSSIE

Introductie

Levertransplantatie als therapie is de afgelopen veertig jaar geëvolueerd van een experimentele behandeling voor een beperkt aantal patiënten naar de aangewezen behandeling voor patiënten met eindstadium leverfalen. Dit succes is mede te danken aan de ontwikkeling van immunosuppressieve geneesmiddelen, die de afweer tegen de getransplanteerde lever kunnen onderdrukken. De introductie van calcineurineremmers en andere immunosuppressieve geneesmiddelen leidde tot een sterke afname van het aantal acute afstotingen na levertransplantatie. Dientengevolge is er een aanzienlijke verbetering opgetreden van de overleving in de eerste vijf jaar na transplantatie van zowel de getransplanteerde lever als de patiënt. De lange termijn overleving na levertransplantatie is ondanks deze ontwikkelingen echter niet verbeterd [1,2]. De hoge incidentie van bijwerkingen veroorzaakt door levenslang gebruik van immunosuppressieve medicijnen. Bijwerkingen, zoals maligniteiten, infecties, nierfalen en cardiovasculaire ziekten [3-8], vormen heden de grootste belemmering voor een optimaal lange termijn resultaat na levertransplantatie. De grootste uitdaging voor transplantatie geneeskundigen is dan ook het verbeteren van de lange termijn overleving na levertransplantatie door het huidige immunosuppressieve regime te optimaliseren met betere dosering om de toxiciteit te verminderen, of de therapeutische mogelijkheden van nieuwe immunosuppressieve geneesmiddelen met minder bijwerkingen te onderzoeken [1].

Doel van het onderzoek

Het doel van het onderzoek zoals beschreven in dit proefschrift was om te onderzoeken hoe de lange termijn bijwerkingen van de huidige immunosuppressieve behandeling kan worden gereduceerd. Het uiteindelijke streven is om een minder toxisch immunosuppressief protocol te ontwikkelen als behandeling *van afstoting* na levertransplantatie. Hierbij werd de mogelijkheid onderzocht om de dosering van ciclosporine, de hoeksteen van de huidige immunosuppressieve behandeling, te verlagen met als doel bijwerkingen te verminderen. Tevens werden de therapeutische mogelijkheden van het immunosuppressieve geneesmiddel, intraveneuze immunoglobulinen (IVIg), onderzocht. Het idee om IVIg te gebruiken als immunosuppressief geneesmiddel komt voort uit een klinische observatie. Het risico op acute afstoting bleek twee tot driemaal verlaagd te zijn bij levertransplantatie patiënten die werden behandeld met antiviraal IVIg (hoofdstuk 3) [9-11]. Deze patiënten werden behandeld met anti-Hepatitis B surface IVIg (anti-HBs IVIg) om re-infectie van het getransplanteerde orgaan door het hepatitis B virus te voorkomen. Op basis van deze waarneming, werden we geïnspireerd om verder de immunologische effecten van IVIg op verschillende celtypen, van zowel het innate als adaptieve immune systeem, te onderzoeken. Hierdoor wilden wij inzicht verkrijgen in hoe IVIg het immunologische proces van cellulaire afstoting kan remmen en of IVIg een nieuwe therapeutische benadering kan zijn om afstoting na levertransplantatie te voorkomen.

Belangrijkste bevindingen

Voor- en nadelen van ciclosporine dosering op basis van C_2 spiegels

In hoofdstuk 2, onderzochten wij de mogelijkheden om de doseringsstrategie van ciclosporine te verbeteren. Hierbij werden de effecten van ciclosporine dosis reductie, op geleide van bloed spiegels twee uur na ciclosporine inname (C_2 spiegels) op de nierfunctie van stabiele levertransplantatie patiënten onderzocht. Hiertoe werden de C_2 spiegels gemeten in 60 stabiele ontvangers van een levertransplantaat (>1 jaar na levertransplantatie). De ciclosporine dosis werd vervolgens gereduceerd als de C_2 spiegel boven het aanbevolen niveau van 600 ng/ml ± 20% lag [12]. Bij 23 patiënten (38%) werd een C_2 spiegel boven de bovengrens van het aanbevolen niveau gemeten. In 27% van de gevallen lag C_2 binnen de normaalwaarde. Twintig van de 23 patiënten met een "te hoge" blootstelling aan ciclosporine stemden in met het verlagen van de ciclosporine dosis. Het aanbevolen C_2 niveau werd snel bereikt met een gemiddelde ciclosporine dosis reductie van 25%. Echter, deze dosis reductie leidde na 6 maanden follow up niet tot een verbetering van het belangrijkste eindpunt; de nierfunctie, of secundaire eindpunten; het lipidenspectrum of de systolische bloeddruk. Met betrekking tot de immunosuppressieve effectiviteit kreeg één patiënt een acute afstoting en bij drie andere patiënten keerde de oorspronkelijke ziekte terug in het transplantaat (twee gevallen van primaire biliaire cirrose en één geval van autoimmune hepatitis). Wij concludeerden daarom dat, uitgaande van huidige aanbevelingen [12,13], een te hoge blootstelling aan ciclosporine frequent voorkomt onder stabiele levertransplantatie patiënten. Een dosis reductie met als doel het realiseren van de aanbevolen C_2 spiegels, leidt echter niet tot verbetering van de nierfunctie en geeft mogelijk zelfs een risico op immuunactivatie.

Therapeutische mogelijkheden van anti-HBs IVIg

Hoofdstuk 3 is de hoeksteen van dit proefschrift, die als doel heeft de therapeutische mogelijkheden van immunosuppressieve geneesmiddelen met minder bijwerkingen te onderzoeken. Het fungeert als basis concept om de immunomodulatoire effecten van anti–virale, en later non-specifieke IVIg op de cellulaire immune response te onderzoeken. In dit hoofdstuk, werden de effecten van anti-virale intraveneuze immunoglobulinen anti-HBs IVIg [9,10] en anti-cytomegalovirus IVIg (anti-CMV IVIg) in het voorkomen van acute afstoting na levertransplantatie onderzocht in een retrospectieve analyse en gecorreleerd aan hun *in vitro* effecten op immuuncellen. Hepatitis B surface antigeen (HBsAg) positieve levertransplantatie patiënten (n = 40), die worden behandeld met anti-HBs IVIg als profylaxe om HBV-reïnfectie van het transplantaat te voorkomen bleken een lagere incidentie van afstoting te hebben vergeleken met patiënten zonder virale hepatitis (n = 147) (12% versus 34%; p = 0.012). De lagere incidentie van afstoting was niet toe te schrijven aan een mogelijke immuungecompromitteerde status van de HBsAg positieve patiënten voor transplantatie, gezien de incidentie van afstoting in de HCV positieve patiënten (n = 29) gelijk is aan de controle groep zonder virale hepatitis. De behandeling met

anti-CMV IVIg (n = 18) bood geen bescherming tegen acute afstoting. Anti-HBs IVIg remden *in vitro* de functionele maturatie en cytokine productie van humane dendritische cellen (DC) bij een anti-HBs IVIg concentratie vergelijkbaar met het gemeten serum concentratie in de met anti-HBs IVIg behandelde levertransplantatie patiënten. Tevens, remde anti-HBs IVIg de proliferatie van perifere T cellen gestimuleerd door lectine of allo-antigenen. Wij concludeerden dat anti-HBs IVIg beschermt tegen acute afstoting na levertransplantatie, mogelijk door functionele inhibitie van twee belangrijke celtypen van het immuunsysteem die betrokken zijn bij het afstotingsproces; DC en T cellen. Het mechanisme van DC en T cel remming door IVIg was voor dit proefschrift nog niet bekend. De volgende hoofdstukken beschrijven ons diepgaand onderzoek naar de immunomodulatoire effecten van IVIg op immuuncellen van zowel innate als adaptieve origine [14], die samen een cruciale rol spelen in het cellulaire afstotingsproces.

Immunomodulatoire mechanismen van IVIg

Een vergelijking met calcineurineremmers

De bruikbaarheid van non-specifieke IVIg als profylaxe voor acute afstoting en graft versus host disease (GVHD) na transplantatie wordt in hoofdstuk 4 beschreven. De immunomodulatoire effecten van IVIg en calcineurineremmers (CNI), ciclosporine (CsA) en tacrolimus (TAC), op humane T cellen en DC werden vergeleken. Dit is de eerste studie die de immunosuppressieve activiteiten van IVIg vergelijkt met die van de CNI. In het huidige immunosuppressieve regime zijn CNI de eerste keuze om acute afstoting na orgaan transplantatie te voorkomen [6,8]. CNI remmen potent de proliferatie en de cytokine productie van T cellen, maar of CNI de functie van DC kunnen beïnvloeden is nog onduidelijk [15]. In onze studie waren IVIg en CNI even effectief in het remmen van de proliferatie en IFN-γ productie door T cellen. Vooral de proliferatie van $CD8^+$ T cellen werd geremd door zowel IVIg als CNI. Zelfs bij toevoeging van IVIg of CNI op een later tijdstip na T cel stimulatie, kon de proliferatie van reagerende T cellen worden geremd. Naast een effect op T cellen, remde IVIg ook het vermogen van DC om allogene T cellen te stimuleren met 73 ± 12%, terwijl behandeling met CNI de DC functie niet beïnvloedde. Het door IVIg geïnduceerde verminderde vermogen van DC om allogene T cellen te stimuleren bleek gerelateerd te zijn aan de inductie van DC apoptose en verminderde expressie van CD40 en CD80. Door de lagere expressie van de co-stimulatoire moleculen zijn de overgebleven DC minder goed in staat op T cellen te activeren. Het remmend effect van IVIg op T cellen was ook toe te schrijven aan de inductie van apoptose van behandelde T cellen. IVIg heeft dus een sterk suppressief effect op zowel humane T cellen als DC, terwijl CNI alleen T cellen remmen. Omdat IVIg tegelijkertijd zowel DC als T cellen kunnen attaqueren, postuleren wij dat IVIg mogelijk beter het afstotingsproces onder bedwang kunnen houden dan CNI. Uit eerdere onderzoeken, weten we dat lange termijn gebruik van IVIg geen ernstige bijwerkingen heeft [16,17], derhalve kan toevoeging van IVIg aan de huidige immunosuppressieve behandeling mogelijk leiden tot dosis reductie van CNI, met als mogelijke gevolg een lagere incidentie van aan CNI gerelateerde

bijwerkingen. De exacte mechanismen van de remmende effecten van IVIg op DC en T cellen zullen verder uiteen gezet worden in de hoofdstukken 5 en 7.

Effecten van IVIg op DC functie: ADCC door NK cellen

In hoofdstuk 5 focussen wij op het remmende effect van IVIg op de DC functie om T cellen te stimuleren [18,19]. Opmerkelijk, is het feit dat IVIg niet op een directe manier de eerder beschreven celdood van DC induceerde. De toename in celdood trad pas op wanneer de door IVIg behandelde DC werden gekweekt samen met natural killer cellen (NK cellen). NK cellen kunnen DC remmen door DC te lyseren, hun uitrijping te voorkomen of hun activiteit stop te zetten. Omgekeerd kunnen DC het functioneren van NK cellen remmen of juist stimuleren. De uiteindelijke uitkomst van de interactie tussen DC en NK cellen is afhankelijk van de conditie waarin de beide celtypen elkaar treffen [20-23] en bepaalt uiteindelijk welke soort adaptieve immuunrespons hierna wordt gevormd. Wij hebben aangetoond dat DC die behandeld zijn met IVIg (IVIg-DC) NK cellen konden activeren en daarmee de IFN-γ productie en het degranulerende vermogen van NK cellen verhoogden. De geactiveerde NK cellen konden op hun beurt apoptose van de IVIg-DC bewerkstelligen. De verhoogde DC apoptose trad alleen maar op in aanwezigheid van NK cellen. Hierdoor waren de IVIg-DC minder goed in staat om T cellen te stimuleren. De T cel stimulatoire capaciteit van IVIg-DC was viermaal verlaagd vergeleken met de onbehandelde DC. Dit effect was toe te schrijven aan de door antilichaam geïnduceerde cellulaire cytotoxiciteit (ADCC) geïnduceerd door NK cellen. IVIg, gebonden aan het DC celoppervlak, resulteerde in herkenning van de mature IVIg-DC door NK cellen via Fc gamma receptor III (FcγRIII), met als gevolg dat de NK cellen geactiveerd raakten. De geactiveerde NK cellen konden op hun beurt de IVIg-DC doden met het ADCC mechanisme. Hiermee werd het aantal mature DC in de T cel stimulatoire pool verlaagd. Opvallend was dat de alleen multimeren, en niet de monomeren van humaan IgG, dit effect induceerden. Dit suggereert dat de IgG multimeren de actieve componenten zijn in IVIg, die NK cel activatie en DC lysis bewerkstelligden. Daarnaast hebben wij aangetoond dat IVIg-DC de expressie van NKp30 en KIR receptoren op NK cellen verlaagde en dat IVIg-DC de aanmaak van $CD56^{bright}CD16^{-}CCR7^{+}$ lymfeklier type NK cellen gunstig beïnvloedde. Deze resultaten onderscheiden een nieuw mechanisme, waarin IVIg therapie leidt tot ADCC van mature DC door NK cellen. Dit heeft tot gevolg dat het aantal mature DC dat T cellen kunnen primen wordt verlaagd [24]. Dit model is fysiologisch relevant, aangezien niet alleen multimeren in IVIg, maar ook immuuncomplexen van IgG en antigenen, T cel reacties kunnen beëindigen ten tijde van bijvoorbeeld klaring van infecties. Omdat immuuncomplexen worden gevormd aan het eind van het humorale immuunrespons, kunnen zowel fysiologische immuuncomplexen als immuuncomplexen in IVIg preparaten bijdragen tot het "afkoelen" van actieve immuunprocessen [25-27]. ADCC, hier geïdentificeerd als het mechanisme waarmee IVIg haar immunomodulatoire capaciteiten beoefent, remt waarschijnlijk niet alleen DC. Waarschijnlijk kan elk celtype dat Fcγ receptoren tot expressie brengt [28,29] en dus IgG complexen kan binden op dezelfde wijze als doelwit fungeren voor ADCC gemedieerde celdoding. Tevens

is het belangrijk om op te merken dat therapeutische antilichamen, gebruikt in verscheidene protocollen tegen afstoting na orgaan transplantatie, antilichamen zoals anti-CD4 mAb, rabbit anti-thymocyte globulin (rATG) of costimulatie blokkade [30-33], ook ADCC van de door de antilichamen gebonden cellen kunnen induceren. In hoofdstuk 6, zetten wij de discussie over deze hypothese en de rol van NK cellen in transplantatie voort.

Rolverandering van de NK cel in transplantatie

NK cellen hebben verscheidene functionele activiteiten na transplantatie. De gebruikelijke opvatting is dat NK cellen voornamelijk pro-inflammatoir zijn, maar dat zij niet zelfstandig een transplantaat kunnen afstoten. Wel kunnen NK cellen het afstotingsproces bevorderen door vroege chemokine en cytokine productie na transplantatie [34,35]. Uit recente publicaties, komt naar voren dat NK cellen ook een belangrijke rol spelen in tolerantie inductie na orgaan transplantatie [24,35-37]. Tevens kan na hematogene stamcel transplantatie (HSCT), de NK-cel alloreactiviteit van donor richting ontvanger leiden tot verminderde terugkeer van leukemie [38,39].

In hoofdstuk 6 bespraken wij een publicatie [24], die een nieuwe rol van NK cellen na het toepassen van costimulatie blokkade hadden vastgesteld. Co-stimulatie blokkade met, anti-CD154, CTLA-4Ig en anti-OX40L, voorkwam dat een huidtransplantaat wordt afgestoten in een muizen model. Ze hadden in deze publicatie aangetoond dat alloreactieve NK cellen van de ontvanger de antigeen presenterende cellen (APC) van de donor kunnen doden bij gebruik van co-stimulatie blokkade. Door hun behandeling met anti-CD154, CTLA-4Ig en anti-OX40L konden zij het proces van T cel priming remmen en induceerden langdurige acceptatie van het huidtransplantaat. Na depletie van NK cellen met anti-NK1.1 mAb bleek de triple costimulatie blokkade therapie echter geheel ineffectief te zijn in het voorkomen van afstoting. Wij bespraken verder hoe de NK cellen de effectiviteit van costimulatie blokkade kunnen beïnvloeden. Hierbij introduceerden wij de hypothese dat costimulatie blokkade antilichamen, anti-CD154, CTLA-4Ig and anti-OX40L, mogelijk aan hun target cellen binden en hiermee de gebonden cellen gevoelig maken voor ADCC killing door NK cellen. Elke component van de costimulatie blokkade therapie, anti-CD154, CTLA-4Ig en anti-OX40L, kan binden aan APC of T cellen [40], met als gevolg dat costimulatie blokkade antilichamen zowel ADCC killing van zowel donor APC als ontvanger T cellen door NK cellen kan induceren. Daarnaast kunnen ook donor NK cellen, die migreren uit het transplantaat, ontvanger APC doden. Dit geldt voornamelijk voor grote orgaantransplantaten zoals de lever, waarbij na transplantatie grote aantallen donor lymfocyten in de ontvanger migreren. In dit geval, kan de NK-alloreactiviteit in de graft versus host richting relevant worden [38,39,41,42].

NK cellen zijn innate effector immuuncellen die stimulatoire receptoren tot expressie brengen. Hiermee herkennen ze door virus geïnfecteerde cellen en tumorcellen, terwijl normale cellen worden gespaard [43,44]. In immuungecompromitteerde transplantatie patiënten, is het belangrijk om deze bruikbare functie van de NK cellen te behouden. Daarom dienen de effecten van verscheidene immunosuppressieve geneesmiddelen op NK cellen verder te worden onderzocht.

Effecten van IVIg op regulatoire T cellen

In toenemende mate wordt er gerapporteerd dat IVIg effectief is in de behandeling van, door T cellen gemedieerde, auto-immuunziekten [17,45,46]. Tevens hebben wij waargenomen dat anti-HBs IVIg beschermt tegen T cel gemedieerde afstoting na levertransplantatie [11]. IVIg remt *in vitro* de humane T cel proliferatie en cytokine productie zoals besproken in hoofdstuk 3 en 4 [47,48]. In hoofdstuk 7 ligt de focus op het mechanisme waarmee IVIg de T cel respons beïnvloedt. Wij onderzochten of IVIg de suppressieve functie van CD4+CD25+Foxp3+ regulatoire T cellen verhoogt en of IVIg de T cel gemedieerde afstoting kan voorkomen in een transplantatie model met muizen. Regulatoire T cellen (Tregs) brengen de lineage marker Foxp3 tot expressie en zijn de "centrale immuunregulator" van verscheidene immuunprocessen waaronder transplantaatafstoting [49]. Tregs spelen een cruciale rol in de inductie en handhaving van transplantaat acceptatie in verschillende experimentele diermodellen [50-52]. Verschillende middelen, die Tregs kunnen stimuleren en expanderen, zijn geïdentificeerd. Van rapamycine is bijvoorbeeld aangetoond dat dit de overleving en de activiteit van Tregs gunstig kan beïnvloeden, zowel *in vitro* [53-56], als *in vivo* in transplantatie patiënten [57]. Daarnaast kunnen therapeutische antilichamen zoals anti-CD4mAb en rATG een verhoogde activatie status en een toename van het aantal Tregs induceren [32,58-60]. Hoewel deze geneesmiddelen geschikt zijn voor in vitro expansie kan het gebruik van deze geneesmiddelen om Tregs *in vivo* te activeren in patiënten gepaard gaan met serieuze bijwerkingen. Indien behandeling met IVIg leidt tot een verhoging van de suppressieve functie van Tregs en daarmee T cel gemedieerde afstoting kan worden verminderd in hevigheid of zelfs voorkomen, dan is dit een aantrekkelijke optie, aangezien IVIg behandeling weinig bijwerkingen heeft.

Wij hebben aangetoond dat IVIg kon binden aan Tregs van muizen en mensen. IVIg bevat antilichamen die van nature voorkomen in het lichaam en deze natuurlijke antilichamen kunnen verschillende oppervlakte moleculen zoals TCR-β, CD3 en CD4 op immuuncellen kunnen herkennen [61,62]. Mogelijk bindt IVIg aan Tregs via haar variabele F(ab')2 fragment door herkenning van een van de oppervlakte moleculen op Tregs. Ook binding van de Fc regio van het IgG aan een onbekende Fc receptor op Tregs behoort tot de mogelijkheid [63-66]. IVIg therapie leidde tot activatie van $CD4^+CD25^+Foxp3^+$ Tregs in zowel experimenten met humane cellen als in een muizen model. Deze activatie werd geobjectiveerd door verhoogde expressie van activatie markers en ZAP70 fosforylatie. Voorbehandeling van humane Tregs met IVIg leidde tot een toename van hun suppressieve capaciteit vergeleken met onbehandelde Tregs. Dus IVIg oefende haar effect op T cellen uit via Tregs. Na depletie van humane CD25+ Tregs nam de suppressieve capaciteit van IVIg op humane T cel proliferatie met tweevoud af, dit in vergelijking met condities waarbij Tregs wel aanwezig zijn. Opmerkelijk is dat zowel binding van IgG als de aanwezigheid van APC noodzakelijk was om Tregs te activeren. Waarschijnlijk fungeerden de APC in deze situatie als een intermediair die de Fc regio's van het IgG op het Tregs oppervlak crosslinkten waardoor de activatie van Treg werd geïnduceerd. *In vivo* beschermde de IVIg behandeling tegen T cel gemedieerde afstoting van een huidtransplantaat

in een muizenmodel, waarbij immuundeficiënte muizen werden gerepopuleerd met totale CD4+ T cellen. Opvallend is dat het beschreven IVIg effect geheel afwezig was wanneer de muizen werden gereconstitueerd met CD4+CD25- conventionele T cellen zonder Tregs. In aanwezigheid van Tregs, induceerde IVIg therapie een transplantaat overleving van langer dan 100 dagen. Dit terwijl de behandeling alleen werd toegepast gedurende de eerste 14 dagen na transplantatie. Helaas konden wij met IVIg therapie geen transplantaat acceptatie induceren in een transplantatie model met immuun competente wild type muizen. Dit werd mogelijk veroorzaakt door de grote aantal alloreactieve T cellen in immuun competente muizen. Om de maximale therapeutische potentie van IVIg in het moduleren van T cel reacties te kunnen realiseren, zal IVIg therapie daarom waarschijnlijk gecombineerd moeten worden met een T cel depleterende strategie. Mogelijk kan IVIg therapie worden gecombineerd CAMPATH-1H [67] of gehumaniseerde anti-CD3 antilichamen [68]. In dit hoofdstuk hebben we aangetoond dat IVIg T cel reacties kunnen remmen door het suppressieve effect van Tregs te verhogen via directe binding en activatie. Toepassingsmogelijkheden van IVIg als therapie na orgaan transplantatie zijn daarom mogelijk groter dan in de huidige richtlijnen is vastgelegd.

Modulatie van het cellulaire immuunsysteem door IVIg

In hoofdstuk 8 vatten we samen wij de recente ontwikkelingen in werkingsmechanismen van IVIg op cellen van het innate en adaptieve immuunsysteem. Hiermee proberen we de therapeutische effecten van IVIg op verscheidene ziekten veroorzaakt door verstoringen op cellulair niveau te doorgronden. Klinische verbeteringen veroorzaakt door IVIg duren veel langer voort dan de tijd dat IVIg na behandeling in het lichaam aanwezig is. Dit suggereert dat IVIg langdurige effecten kan induceren op het cellulaire immuunrespons.

Zowel op het gebied van auto-immuunziekten en transplantatie zijn er recente bevindingen die de effecten van IVIg op het cellulaire immuunrespons kan verklaren in diermodellen. Echter, deze waarnemingen komen niet altijd overeen met werkingsmechanismen van IVIg in het complexe humane immuunsysteem. Tevens zijn er onvoldoende gegevens voorhanden om de effecten van IVIg om de cellulaire immuun response in patiënten te verklaren.

Na orgaantransplantatie is het verder van belang om de effecten van IVIg op de indirecte antigeenpresentatie te onderzoeken. Het proces van indirecte antigeen presentatie is in principe het proces waarbij de ontvanger APC het donor antigen presenteren aan de ontvanger T cellen [69,70]. Vroeg na transplantatie kunnen donor APC, die uit het transplantaat migreren, T cellen van de ontvanger activeren (directe antigeenpresentatie), echter, lang na transplantatie zullen de donor APC waarschijnlijk niet meer aanwezig zijn. De ontvanger APC zullen dan zelf het proces van T cel priming induceren. Langer na transplantatie zal dit proces van antigeen presentatie een prominentere rol gaan innemen. Hoe IVIg dit proces van antigeenprocessing en presentatie door ontvanger APC beïnvloedt en hoe IVIg de DC-T cel interactie moduleert, is een belangrijke vraag voor verder onderzoek.

Antilichamen vormen de frontlinie van ons afweersysteem. Ze hebben een breed repertoire aan activiteiten en een variëteit aan effector functies. Het therapeutisch toedienen van antilichamen in de vorm van IVIg om ons immuunsysteem te moduleren is over de jaren effectief gebleken [16,17,27,71-73]. IVIg attaqueert het immuunsysteem op meerdere niveaus, waaronder het innate en adaptieve immuunsysteem. Hoewel veel studies in diermodellen nieuwe werkingsmechanismen van IVIg rapporten, zijn deze mechanismen vaak niet bevestigd in patiënten en kennen veel tegenstrijdigheden.

Mogelijke actieve componenten in IVIg

Het is belangrijk om de actieve componenten in IVIg preparaten te identificeren, gezien de hoge kosten van de behandeling en de opkomende tekorten aan plasma donoren. IgG dimeren en gesialyleerd IgG zijn potentiële kandidaten, maar hun effectiviteit in patiënten dient nog te worden onderzocht [27,74].

Door het grote donor aantal en door verscheidene bewerkingsmethoden tijdens het zuiveren van IVIg, worden er complexen gevormd tussen anti-idiotype antilichamen met hun complementaire idiotype-antilichaam [25]. Dimeren en aggregaten in IVIg preparaten kunnen ernstige bijwerkingen induceren. De producenten van IVIg proberen om bovenstaande reden het percentage aggregaten laag te houden [75]. Immuuncomplexen worden dan ook gezien als pro-inflammatoire elementen, echter, in toenemende mate zijn er aanwijzingen dat immuuncomplexen ook anti-inflammatoir kunnen werken [76,77]. IgG dimeren kunnen een van de actieve componenten zijn van IVIg. Dimeren komen niet voor in het immuun repertoire van een individu, maar wordt juist gevormd door het idiotype-anti-idiotype complex formatie tussen IgG moleculen van verscheidene individuen [25,78,79]. IgG dimeer is waarschijnlijk niet de enige component verantwoordelijk voor de immunomodulatoire effecten van IVIg. Sommige effecten van IVIg op DC kunnen in vitro ook worden bewerkstelligd door F(ab)' fragmenten. Dit is waarschijnlijk gerelateerd aan natuurlijke antilichamen tegen cytokinen, die aanwezig zijn in IVIg [80].

Het immuunsysteem maakt onderscheid tussen verschillende glycosylatie vormen van een antilichaam. Interactie tussen een IgG en een Fc receptor is afhankelijk van suikergroepen aan zowel het antilichaam als aan de receptor [81]. "G0 glycoforms" (no galactoses and no sialic acids) hangt samen met de pro-inflammatoire effecten van IgG [74]. Dit terwijl "sialylated S2 glycoforms" gecorreleerd zijn aan de anti-inflammatoire effecten van IgG [82,83]. Of de anti-inflammatoire effecten van IVIg gerelateerd zijn aan de gesialyleerde vorm van IgG, die 1-2% aanwezig is in IVIg, is onbekend.

Effecten van IgG dimeren en gesialyleerde IgG in patiënten dienen te worden onderzocht. Het zuiveren van de componenten met anti-inflammatoire activiteit of productie van synthetische of biologische alternatieven van IVIg kan er mogelijk voor zorgen dat pathologische immuunactivatie in verschillende ziekten selectief worden aangepakt.

Levertransplantatie en IVIg

De lever is een fascinerend orgaan. Spontane acceptatie van een levertransplantaat komt voor bij sommige diersoorten. Acute afstoting van de lever is bij mensen vaak goed behandelbaar, maar tolerantie komt weinig voor bij levertransplantatie patiënten [84-87]. Het feit dat de lever een relatief tolerogeen orgaan is, doet ons vermoeden dat het mechanisme van afstoting wat genuanceerder is vergeleken met de afstotingsreactie van andere getransplanteerde organen zoals hart en nier. Leverspecifieke cellen, zoals Kupffer cellen, lever sinusoïdale cellen en stellate cellen, kunnen een belangrijke rol spelen bij het tolerogene karakter van de lever. Andere mechanismen zoals microchimerisme, "soluble" MHC moleculen en Tregs, kunnen ook bijdragen tot tolerantie inductie [88]. Door de mechanismen van lever afstoting en tolerantie te doorgronden en door immunomonitoring na levertransplantatie tot te passen, zullen we in de toekomst in staat zijn strategieën te ontwikkelen om tolerantie te herkennen en afstoting te reduceren.

Op het gebied van transplantatie, hebben artsen nog steeds de uitdaging om de lange termijn uitkomsten en overleving na transplantatie te verbeteren. Men probeert dit te bereiken door het optimaliseren van de huidige immunosuppressieve regimes of door de therapeutische mogelijkheden van andere geneesmiddelen te onderzoeken. In dit proefschrift, onderzochten wij de effecten van IVIg op verscheidene cellen van zowel het innate als het adaptieve immuunsysteem. In het bijzonder DC, T cellen en NK cellen spelen een cruciale rol in cellulaire afstoting. Op basis van onze resultaten en die van andere onderzoekers, kunnen wij concluderen dat IVIg een potente behandeling is om cellulaire afstoting na levertransplantatie te voorkomen.

Het is van belang op te merken dat IVIg therapie waarschijnlijk niet zal leiden tot "echte transplantaat acceptatie" gedefinieerd als orgaan acceptatie na het staken van de behandeling, terwijl de ontvanger de potentie behoudt om een transplantaat van de derde partij af te stoten. IVIg kent een breed spectrum aan antilichamen en heeft verscheidene immunomodulatoire activiteiten. Omdat de meeste mechanismen, die geïdentificeerd zijn, antigeen-specifiek blijken te zijn, is het onwaarschijnlijk dat IVIg specifiek "echte transplantaat acceptatie" kan realiseren door het moduleren van alloreactieve T cellen. Echter, IVIg therapie kan de alloreactieve immuun response sterk remmen, terwijl de behandeling meestal weinig bijwerkingen heeft. IVIg dient daarom als een serieuze kandidaat te worden beschouwd om te worden toegevoegd aan toekomstige immunosuppressieve protocollen na transplantatie. Eerst zijn klinische trials echter nog noodzakelijk om de optimale dosering te bepalen. Ook dient te worden onderzocht of IVIg therapie daadwerkelijk kan leiden tot dosisverlaging of zelfs stoppen van de huidige toxische immunosuppressieve geneesmiddelen en hiermee de lange termijn bijwerkingen na orgaantransplantatie kan verlagen.

CONCLUSIES

Hoofdstuk 2: Ciclosporine dosis reductie op basis van de aanbevolen C_2 spiegels leidt niet tot verbetering van de nierfunctie in stabiele levertransplantatie patiënten en geeft mogelijk zelfs een risico op immuunactivatie, met name bij patiënten met ciclosporine monotherapie.

Hoofdstuk 3: Anti-HBs IVIg beschermt tegen acute afstoting na levertransplantatie, mogelijk door functionele inhibitie van twee belangrijke immune celtypen die betrokken zijn in het afstotingsproces; DC en T cellen.

Hoofdstuk 4: Omdat IVIg tegelijkertijd zowel DC als T cellen kan attaqueren in het afstotingsproces, kan toevoeging van IVIg aan de huidige immunosuppressieve behandeling leiden tot dosis reductie van CNI, met als gevolg een lagere incidentie van aan CNI gerelateerde bijwerkingen.

Hoofdstuk 5: Door de interactie tussen DC en NK cellen te beïnvloeden, leidt IVIg therapie tot antilichaam geïnduceerde cellulaire cytotoxiciteit (ADCC) doding van mature DC door NK cellen. Het gevolg is dat het aantal mature DC in de T cel stimulatoire pool wordt verlaagd en daarmee ook het proces van T cel priming.

Hoofdstuk 6: Antilichaam geïnduceerde cellulaire cytotoxiciteit (ADCC) doding van APC door NK cellen kan een mechanisme zijn, waarbij costimulatie blokkade leidt tot transplantaat acceptatie in een transplantatie model.

Hoofdstuk 7: IVIg kan T cel responsen remmen door het suppressieve effect van Tregs te verhogen via directe binding en activatie, hiermee worden de toepassingsmogelijkheden van IVIg als therapie na orgaan transplantatie mogelijk vergroot.

Hoofdstuk 8: Het is belangrijk om de actieve componenten in IVIg preparaten te identificeren gezien de recent geïdentificeerde werkingsmechanismen een de opkomende tekorten aan plasma donoren.

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Chapter 10

Abbreviations

Contributing authors

Dankwoord/ Acknowledgements

PhD Portfolio

List of publication

Curriculum vitae auctoris

ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity;
Anti-HBs IVIg	anti-HBV surface antigen-specific IVIg
APC	Antigen presenting cells;
AUC	area under the curve;
BMDC	bone marrow-derived dendritic cells;
C_0	cyclosporine whole blood concentration before administration
C_2	cyclosporine whole blood concentration two hours after administration
CFSE	Carboxyfluorescein diacetate Succinimidyl Ester;
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CNI	Calcineurin inhibitors;
CsA	Cyclosporine;
CTRL-DC	DC stimulated with pro-inflammatory cytokines;
DC	Dendritic Cells;
EAE	Experimental Autoimmune Encephalomyelitis;
FcγR	Fc gamma receptor;
FcRn	Neonatal Fc Receptor
GBS	Guillain Barré syndrome
GVHD	Graft versus Host Disease;
HBV	Hepatitis B Virus
HBsAg	Hepatitis B surface antigen;
HLA	Human Leukocyte Antigen;
HRC	humanized monoclonal antibody against human epidermal growth factor receptor 2 (Herceptin)
HRC-DC	DC stimulated with pro-inflammatory cytokines in presence of 10 mg/ml Herceptin;
HSA	Human Serum Albumin;
HSA-DC	DC stimulated with pro-inflammatory cytokines in presence of 10 mg/ml Human Serum Albumin;
HSA-Tregs	Regulatory T cells pre-treated with HSA;
HSCT	Hematopoietic Stem Cell Transplantation;
IFN-γ	Interferon γ
ITP	Immune Thrombocytopenic Purpura;
IVIg	Intravenous immunoglobulins;
IVIg-DC	DC stimulated with pro-inflammatory cytokines in presence of 10 mg/ml Intravenous Immunoglobulins;
IVIg-Tregs	Regulatory T cells pre-treated with IVIg;
MDRD	Modification of Diet in Renal Disease Study formula;

MHC	Major Histocompatibility complex;
MLR	Mixed lymphocyte reaction;
MMF	mycophenolate mofetil;
Mo-DC	Monocyte-derived DC;
MS	Multiple Sclerosis
NCR	Natural Cytotoxicity Receptors;
NK cells	Natural Killer cells;
PHA	Phytohemagglutinin;
PIDs	Primary Immune Deficiencies
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazole [3,4-d] pyrimidine;
rATG	rabbit anti-thymocyte globulin (rATG);
Siglecs	Sialic Acid Binding Ig-like Lectins
TAC	Tacrolimus ;
TCR	T cell Receptor;
Tregs	Regulatory T cells;
ZAP70	phophorylated ζ-associated protein 70

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DANKWOORD / ACKNOWLEDGEMENTS

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Professor Metselaar, beste Herold, met de woorden "Ik weet niet of je wat kan, maar je bent wel lekker enthousiast", heb je mij aangenomen als onderzoeksstudent in 2002. En hiermee zette ik ongemerkt de eerste stap van mijn wetenschappelijke carrière. Ik wist toen nog niet goed wat mij te wachten stond, maar al snel raakte ik in de ban van de levertransplantatie groep. Je directe manier van begeleiden is een verademing. Vaak zeg je dingen, die ik absoluut niet wil horen, maar die wel het gewenste effect opleveren. Dank voor je begeleiding, je morele steun en alle mogelijkheden die je mij hebt gegeven. De vrijheid in de opzet en ontwikkeling van het onderzoek heb ik heel erg gewaardeerd. Ik heb zo ontzettend veel van je geleerd en het is voor mij een eer om je als promotor te mogen hebben.

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Professor IJzermans, ik bewonder uw klinische en wetenschappelijke vaardigheden. U, als opleider, weet heel goed anderen te inspireren en daardoor het beste uit mensen te halen. Ik verheug mij erop om mijn opleiding straks voort te zetten in het Erasmus MC.

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Drie jaar lang heb ik mijn "ups en downs" beleefd op het MDL lab, ik heb zeer prettig met iedereen samengewerkt. Hartelijk dank daarvoor. Naast het harde werken, is de L-vleugel een waar "soap serie". Als ik af en toe mijn "power nap" deed, gebeurde er weer van alles in de koffiekamer en op het lab. Altijd weer de eeuwige "strijd" tussen lever en GE, en tussen "LTx" en "Hepatitis". Maar op vrijdagmiddag was het gewoon weer samen borrelen bij Dizzy. Vele (oud)collega's hebben heel veel voor mij betekend en zijn ondertussen vrienden geworden. Graag wil ik in het bijzonder noemen:

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PhD PORTFOLIO

Name PhD Student:	Thanyalak Tha-In
Erasmus MC Department:	Gastroenterology and Hepatology
PhD Period:	November 2004 – June 2008
Promotor:	Prof. dr. H.J. Metselaar
Copromotor:	Dr. J. Kwekkeboom

PhD TRAINING

General academic and research skills	*year*
Basic radiation protection 5A/B	2005
Statistics: classical methods for data analysis	2005
Bioscientific course Module 1-4 (certificate for personal licence)	2005

(Inter)national conferences	*year*
Annual Meeting NTV [1], Rotterdam, The Netherlands	2003
Annual Meeting NTV [1], Texel, The Netherlands	2004
International Transplantation Society Congress, Vienna, Austria	2004
Stafdag Heelkunde, Rotterdam, The Netherlands	2004
Basic science symposium of TTS [2], Nantes, France	2005
Stafdag Heelkunde, Rotterdam, The Netherlands	2005
ESOT [3] Congress, Geneva, Switzerland	2005
Annual Meeting NVVI [4], Noordwijkerhout, The Netherlands	2006
Annual Meeting NTV [1], Zeewolde, The Netherlands	2006
International Congress of ILTS [5], Milan, Italy	2006
Molmed annual meeting, Rotterdam, The Netherlands	2006
Stafdag Heelkunde, Rotterdam, The Netherlands	2006
Wetenschapsdag Erasmus MC, Rotterdam, The Netherlands	2006
World Transplant Congress, Boston, USA	2006
Annual Meeting NTV [1], Zeewolde, The Netherlands	2007
European Society for Surgical Research, The Netherlands	2007
International Congress of ILTS [5], Rio de Janeiro, Brasil	2007
Najaarsvergadering NVGE/NVH [6], The Netherlands	2007
Sanquin Symposium, Amsterdam, The Netherlands	2007
Stafdag Heelkunde, Rotterdam, The Netherlands	2007
Wiser, NWO [6], Maastricht, The Netherlands	2007
Annual Meeting NTV [1], Zeewolde, The Netherlands	2008

Basic science symposium of TTS [2], Brussel, Belgium	2008
International Congress of ILTS [5], Paris, France	2008
American Transplant Congress, Toronto, Canada	2008

[1] Nederlandse Transplantatie Vereniging
[2] The Transplantation Society
[3] European Society of Transplantation
[4] Nederlandse Vereniging voor Immunologie
[5] International Liver Transplantation Society
[6] Nederlandse vereniging van Gastroenterologie en Hepatologie

Membership

2008- present	Dutch Association for Surgery (NvvH)
2004- present	Dutch Association for Gastroenterology (NVGE)
2004- present	Dutch Association for Hepatology (NVH)
2004- present	Dutch Association for Gastrointestinal Surgery (NVGIC)
2003- present	Dutch Association for Transplantation (NTV)
2006-2007	American Society of Transplantation

Teaching activities	*year*
Lecture for second year medical students	2005
Lecture for fourth year medical students, teaching laboratory skills	2008

Scientific Awards and Grants

- NWO-Mosaic 2005 (The Netherlands Organization for Scientific Research (NWO))
- Travel Grant Vereniging Trustfonds Erasmus University Rotterdam
- Travel Grant Dutch Association for Transplantation
- De Zilveren Kreeft prijs 2005 (best presentation, Stafdag Heelkunde)
- Biotest Research grant 2006-2007
- Young Investigator Award TTS and ATC 2006
- Genzyme Speakers Award 2006
- Rising Star Award ILTS 2007
- Novartis Transplantation Grant 2008 (best paper published in 2007)
- Dr. W.H. Posthumus- Stipend 2008 (promising female PhD student)
- Young Investigator Travel Award ILTS 2008
- Basic Science ESOT Travel Grant 2009 (best abstract)

Invited Speaker	*year*
Meeting werkgroep Immunodeficiëntie, Amsterdam, Netherlands	2007
Immunology Review Club, Leiden, Netherlands	2008

LIST OF PUBLICATION

Modulation of the cellular immune system by intravenous immunoglobulins
T. Tha-In *, J. Bayry *, H. J. Metselaar, S. V Kaveri ξ, Jaap Kwekkeboom ξ
*TT and JB are co-first authors, and ξSK and JK contributed equally as senior authors to the conception of the manuscript.
Trends in Immunology 2008:29:2: 608-615

Intravenous immunoglobulins suppress T-cell priming by modulating the bidirectional interaction between dendritic cells and natural killer cells
T. Tha-In, H.J. Metselaar, H.W. Tilanus, E.J. Kuipers, R.A. de Man, J. Kwekkeboom
Blood 2007, 110:3253

Functional Dichotomy of Natural Killer cells in organ transplantation
T. Tha-In, S. Luo, A.R. Bushell, K.J. Wood
Expert Review in Clinical Immunology 2007:3:3: 261

Superior immunomodulatory effects of intravenous immunoglobulins on human T-cells and dendritic cells
T. Tha-In, H.J. Metselaar, H.W. Tilanus, P.P.C. Boor, S. Mancham, E.J. Kuipers, R.A. de Man, J. Kwekkeboom
Transplantation 2006, 81:1725

Therapeutic anti-HBs immunoglobulins inhibit dendritic cell maturation and T-cell proliferation and protect against acute rejection after liver transplantation
J. Kwekkeboom, **T. Tha-In**, W.M. Tra, W. Hop, P.P.C. Boor, S. Mancham, P.E. Zondervan, A.C.T.M. Vossen, R.A. de Man, H.J. Metselaar
American Journal of Transplantation 2005, 5; 10:2393

Clinical outcome after cyclosporine dose reduction based C2 levels in long term liver transplant patients
T. Tha-In, D.A. Hesselink, H.W. Tilanus, L. Elshove, A.L. Wilschut, B.E. Hansen, T. van Gelder, H.J. Metselaar
Clinical Transplantation 2005, 19; 4: 537

Intravenous immunoglobulins trigger functional activation of human and mouse CD4+CD25+Foxp3+ T cells and promote skin allograft acceptance
T. Tha-In , H. J. Metselaar, A. R. Bushell, J. Kwekkeboom #, K. J. Wood #
#: JK and KJW contributed equally as senior investigators to this study
Submitted for publication

Increased incidence of early de *novo* cancer in liver graft recipients transplanted in recent years: an association with cyclosporine by C_2 monitoring
S.W. Tjon, J. Sint Nicolaas, J. Kwekkeboom, R.A. de Man, G. Kazemier, H.W. Tilanus, B.E. Hansen, L.J.W. van der Laan, **T. Tha-In**, H.J. Metselaar
Submitted for publication

Parotidectomy for benign and malignant disease of the parotis: Harmonic scalpel versus conventional cold knife
T. Tha-In, B.M.E. Mees, M.M.F. Franken, I.H. Oei
In preparation

CURRICULUM VITAE AUCTORIS

The author was born on December 28th , 1979 in Wiang Papao, Thailand. In 1990, at ten years of age, she moved to Rotterdam, The Netherlands. After graduating from high school at the Einstein Lyceum in 1998, she attended medical school at the Erasmus University Rotterdam. During medical school, she worked as a student assistant at the Department of Cardiology, Medium Care and Heart transplantation Unit, and performed research electives at the Department of Gastroenterology and Hepatology, Liver Transplantation Unit, Erasmus Medical Center (Prof. dr. H.J. Metselaar, Prof. dr. E.J. Kuipers). In 2003 and 2004, she performed a clinical subinternship at the Department of Surgery, Hepatobiliary Surgery Unit, Chiang Mai Rai University, Thailand (Dr. Chotirosniramit). In 2004, she graduated from medical school cum laude.

After graduation she was offered a position as a PhD student at the Department of Gastroenterology and Hepatology, Liver Transplantation Unit of the Erasmus Medical Center. Under supervision of Prof. dr. H.J. Metselaar and Dr. J. Kwekkeboom, she focused on the immunomodulatory effect of intravenous immunoglobulins after liver transplantation. During her PhD training, she was a member of the liver transplantation operating theatre and laboratory support team (Prof.dr. H.W. Tilanus). In 2005, she received the Mosaic grant of The Netherlands Organization for Scientific Research (NWO) and an unrestricted grant of Biotest Seralc, Soest, The Netherlands, to perform her research. In 2006 she was awarded with the Young Investigator Award at the World Transplant Congress, Boston, U.S.A. and in 2007 she received the prestigious Rising Star Award of the International Liver Transplantation Society (ILTS), Rio de Janeiro, Brasil. From September 2006 until August 2007, she studied the immunomodulatory effects of IVIg in vivo and performed skin allograft transplantation in mice. This was performed in close collaboration at the Nuffield Department of Surgery, University of Oxford, Oxford, United Kingdom (Dr. A.R. Bushell and Prof. K.J. Wood).

In July 2008 she started her residency in General Surgery at the Reinier de Graaf Gasthuis in Delft (Dr. L.P.S. Stassen and Dr. M. van der Elst) and she will complete her residency at the Erasmus Medical Center (Prof.dr. J.N.M. IJzermans en Prof.dr. J.J.B. van Lanschot). She remains close involved with the IVIg project and is passionate about research.

A portret of me by Sir Roy Calne, a british surgeon, a true pioneer in organ transplantation and a wellknown artist, during the International Congress of ILTS, Milan, Italy (5th of May 2006)