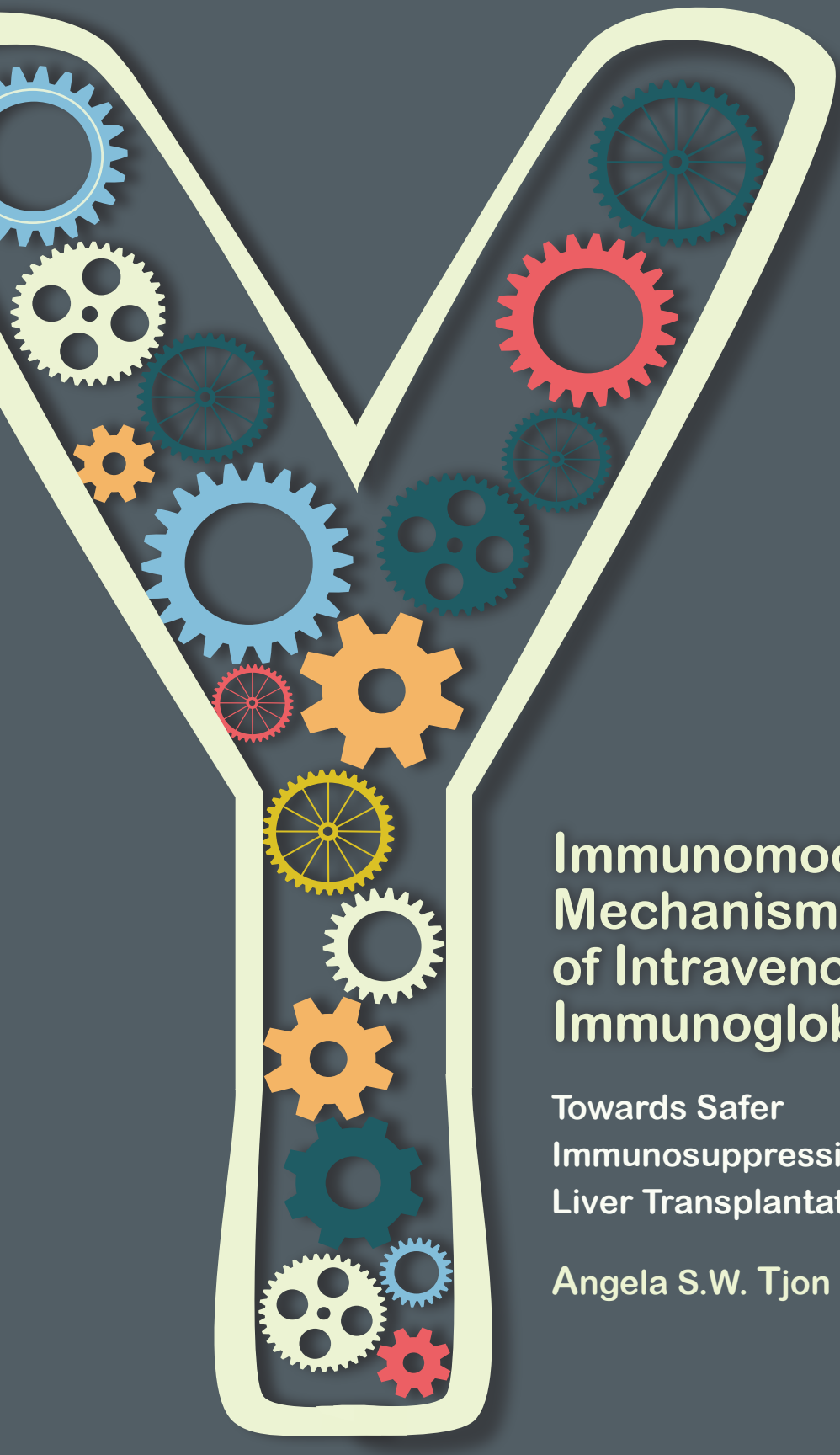

Immunomodulatory Mechanisms of Intravenous Immunoglobulin

Towards Safer Immunosuppression after Liver Transplantation

Angela S.W. Tjon

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The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands.

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Immunomodulatory Mechanisms of Intravenous Immunoglobulin

Towards Safer Immunosuppression after Liver Transplantation

De immunomodulatoire mechanismen van intraveneuze immuunglobuline

Op weg naar een veiligere immuunsuppressie na levertransplantatie

Proefschrift

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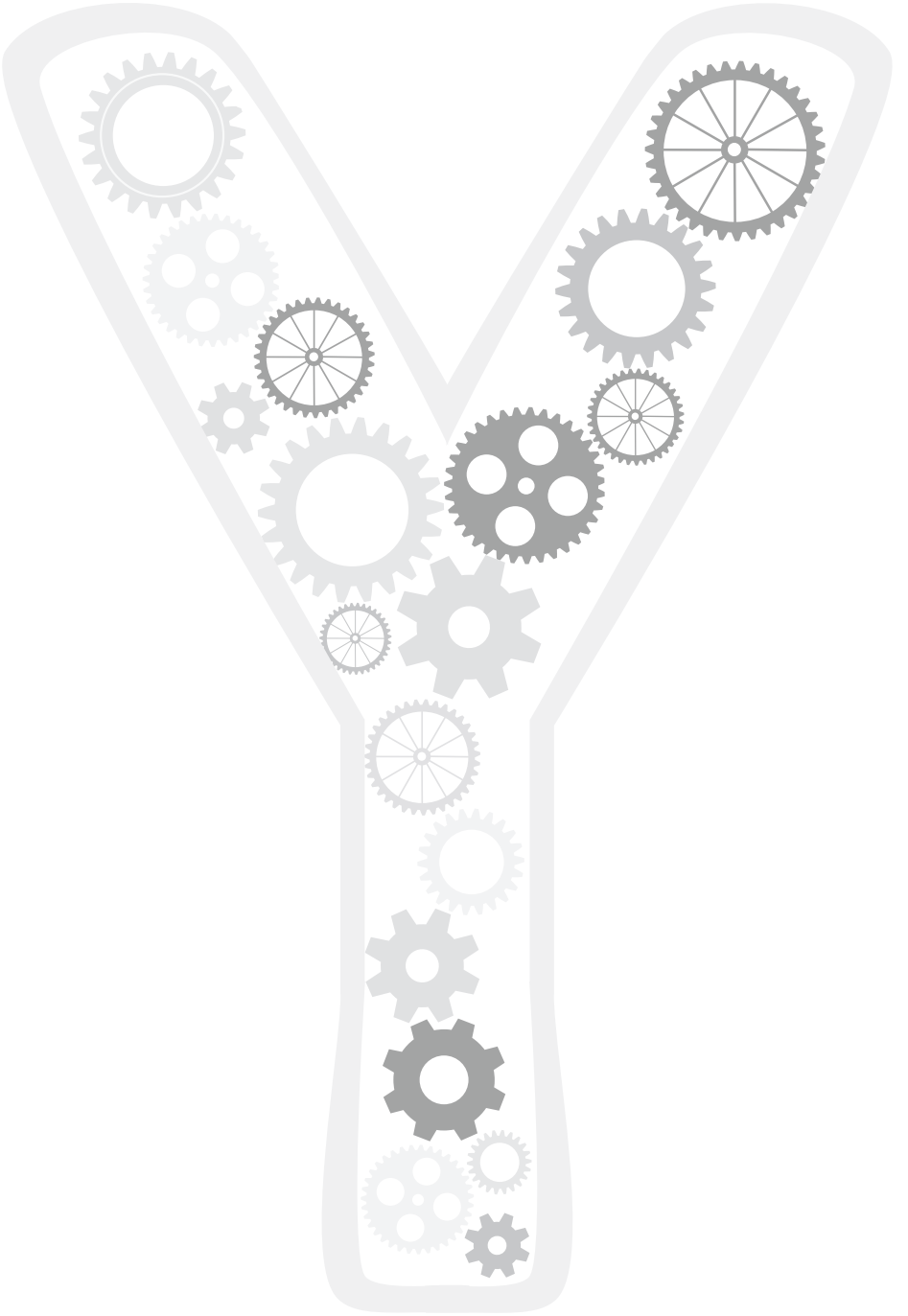
Voor mijn ouders

PART I

INTRODUCTION



1

General introduction and outline of the thesis

INTRODUCTION

1. The immune system

Immunity originates from the Latin term *immunis*, meaning "exempt", which refers to all the mechanisms used by the body as protection against invasion by agents that are foreign to the body. These agents may be infectious pathogens, foods, chemicals, drugs, and, in terms of transplantation, organ grafts from another individual (allograft). The immune system consists of two functionally distinct arms: the innate immunity arm and the adaptive immunity arm.

The innate immunity refers to all those elements with which an individual is born and that are always present and available at short term to protect the individual from challenges by foreign invaders. This response is nonspecific and occurs rapidly upon ligation to innate immune receptors (e.g. Toll-like receptors or C-type lectin receptors) (1). The cell types expressing these receptors and hence participate in the cellular innate immune response include dendritic cells (DCs), macrophages, e.g. Kupffer cells and splenic macrophages, granulocytes and natural killer (NK) cells (2-4). A fundamental defense mechanism carried out by these cells is phagocytosis, but also production of cytokines to affect other cells, or release of perforin/granzyme for extracellular killing. In humoral innate immunity, the complement system is the main component. Specific activation of complement by pathogens leads to a cascade of proteolytic reactions that coats pathogens with complement fragments. Complement-coated pathogens are recognized and bound by specific complement receptors on macrophages, taken up by phagocytosis, and destroyed (5).

The adaptive immunity is more specialized and is supplemental to the protection provided by the innate immune system. The advantages of this system are the ability to provide antigen-specific protection and the generation of long-lasting memory against the pathogen. During the first exposure to the pathogen, the induction of response is slow (can take up to days). However, by induction of memory cells, during a second exposure to the same pathogen a rapid and more effective response follows. Cells that are involved in the adaptive immune response include B cells and T cells. B cells can bind to antigens by their expression of B cell receptors, which are antigen specific membrane immunoglobulin (Ig) molecules. Once ligated, the B cell initiates an antibody response by secreting Ig molecules, whose purpose is to eliminate the antigen from the host. IgG facilitate the "clean up" of pathogens that they bind to, since cells from the innate immune system can recognize immune complexes by ligation of its Fcγ receptors (FcγRs) to the Fc part of IgG which stimulates phagocytosis. Unlike B cells, T cells recognize antigens when they are processed into peptides which are presented by an antigen presenting cell (APC) in association with MHC molecules. T cell activation by APC requires three signals. The first signal is the interaction between the peptide MHC molecules with T cell receptor. This interaction itself is insufficient

for T cell activation; it will need a second signal, which comprises the interaction of co-stimulatory molecules on APC, mainly CD80/CD86, with CD28 expressed on T cells. The third signal is the production of pro-inflammatory or anti-inflammatory cytokines by APCs. Upon activation, T cells release cytokines to either help B cells to produce antibody, activate other immune cells, or direct killing of pathogens by phagocytes (e.g. by IFN-γ secretion).

2. Liver transplantation

Liver transplantation (LTx) is a life-saving treatment that replaces a diseased and poorly functioning liver with either a whole or a portion of a healthy donated liver. The first successful transplantation of a liver in humans was performed by Thomasz Starzl in 1967 (6). The clinical use of transplantation has proceeded rapidly since a Consensus Development Conference of the National Institutes of Health concluded in June 1983 that liver transplantation had become an accepted treatment for end-stage liver diseases and was no longer an experimental procedure (7). This has made the management of both chronic and acute hepatic diseases possible. The most important indications for liver transplantation in Europe from 1986-2009 were cirrhosis (52% with 21% related to hepatitis B or C viral infections), primary liver tumors (14% with 12% of hepatocellular carcinoma), cholestatic disease (11%), and acute hepatic failure (8%) (8). With the improved survival rate after surgery achieved, the success of liver transplantation was not determined anymore by surgical techniques, but by the next challenge: immunological rejection of the allograft.

3. The process of allograft rejection

Upon transplantation, the allograft from another individual (=allogeneic) is recognized as non-self and thereby induces an immune response. The discrimination between self and non-self is mainly the result of genetic differences in expression of MHC molecules. The MHC locus in humans encodes two major classes of proteins: HLA class I and HLA class II. HLA class I molecules are expressed on the surface of all nucleated cells, whereas class II molecules are expressed mainly by DCs, macrophages and B cells. Upon IFN-γ stimulation, HLA class II can also be upregulated on a variety of cells, including epithelium, endothelium and T cells. Since the MHC locus is highly polymorphic, HLA molecules expression is quite unique for each individual. T cells that are responsive to allogeneic MHC molecules (1-10%) will rapidly react when encountering a non-self MHC molecule (9, 10).

The immunological response during rejection that causes damage to the graft is very complex and constitutes a multifaceted network among organ resident cells, infiltrating cells, cytokines, antibodies, and other molecular mediators contributing to the injury of the graft.

Clinically, allograft rejection fall into three major categories (see also **Table 1**):

1. **Hyperacute rejection.** This occurs within few minutes to a few hours of transplantation, since it is the result of preformed donor-specific antibodies, mainly directed against incompatible MHC antigens. Such antibodies are already produced in the recipient before transplantation, induced by a previous transplantation, by pregnancy or blood transfusion. These cytotoxic antibodies induce major graft damage by activation of the complement system and platelet activation and deposition, resulting in organ failure. After liver transplantation, hyperacute rejection is very rare, as it is believed that the liver absorbs and eliminates donor-derived antibodies by Kupffer cells and the presence of dual circulation providing immense vascular reserve (11-13). However, in the last decade, cases of acute antibody-mediated rejection after ABO-compatible liver transplant have been reported (14, 15).
2. **Acute rejection.** This occurs mostly within days or weeks after transplantation. This

TABLE 1. Types of liver allograft rejection

Type	Histological features	Infiltrating immune cells	Onset	Prevalence after LTx
Hyperacute rejection	Inflammation of portal tract, bile duct and portal vein	Lymphocytes (mainly T cells) 'Blast' cells Macrophages Neutrophils Eosinophils	Minutes	Rare
Acute rejection	Inflammation of portal and central vein branches, lymphocyte infiltration of portal tracts and bile duct injury	T cells Macrophages Eosinophils	Days to weeks, but incidentally also years after transplantation	20-40%
Chronic rejection	Loss of small bile ductuli, arteriopathy, presence of foam cells and absence of significant cellular infiltrate	T cells Macrophages B cells?	Months to years	~2%

response is mainly cell-mediated, in particular T cells (16). Histologically, intense infiltration of lymphocytes and macrophages at the rejection site is seen. This response is inhibited by immunosuppressive therapy, and can be treated successfully by high-dose corticosteroid therapy. Until recently, preformed donor-specific antibodies were considered to be clinically irrelevant to the outcome of liver allograft compared to other solid organ transplants (17). However, increasing evidence suggests that donor-specific antibodies also play a significant role in acute rejection of liver allograft (18). In liver biopsies of unexplained dysfunctional liver allograft in patients positive for donor-specific antibodies, the presence of C4d and anti-HLA antibodies deposition have been seen, suggesting involvement of antibodies in acute liver graft rejection (19). However, since not all patients with donor-specific antibodies show graft

failure, positive cross-match for donor-specific antibodies is not a strict contraindication for LTx. Evidence of effective anti-humoral agents in LTx recipients is quite limited, but in renal transplant recipients, antibody-mediated responses are managed with plasmapheresis, intravenous immunoglobulin (IVIg) or rituximab.

3. **Chronic rejection.** This occurs within months or years after transplantation. This response is mainly cell-mediated, but antibody-mediated reaction may also play a role (16, 20). Histologically, this response leads to inflammation of the small arteries and interstitial fibrosis and disappearance of small bile ductuli. Since the damage has already taken place, immunosuppressive treatment during this phase is not very helpful anymore.

For liver allograft rejection, the cell-mediated acute rejection is principally responsible (21). During such response two cell types play a pivotal role, which are APCs and T cells. APCs activate the immune response by presenting alloantigens (mainly HLA molecules) of the transplant to the recipient T cells. Allorecognition by T cells occurs by two different pathways. The first pathway is by direct allorecognition, the so called **direct pathway** (**Figure 1**). In this pathway, APCs that are present in the donor graft, also known as passenger leukocytes, leave the graft and migrate through the blood circulation to the recipient lymph nodes and spleen. Here, donor APCs directly activate recipient T cells by presenting intact non-self MHC molecules on their surface. Notably, select alloreactive T cells can recognize allo-MHC molecules regardless of which peptide is contained in the binding groove (22). Alloreactive T cells involved in the direct pathway may make up as many as 5% to 10% of the total pool of peripheral T cells. The second way of alloantigen recognition is the **indirect pathway**. In this pathway, donor alloantigens are shed from the graft, taken up and processed by recipient

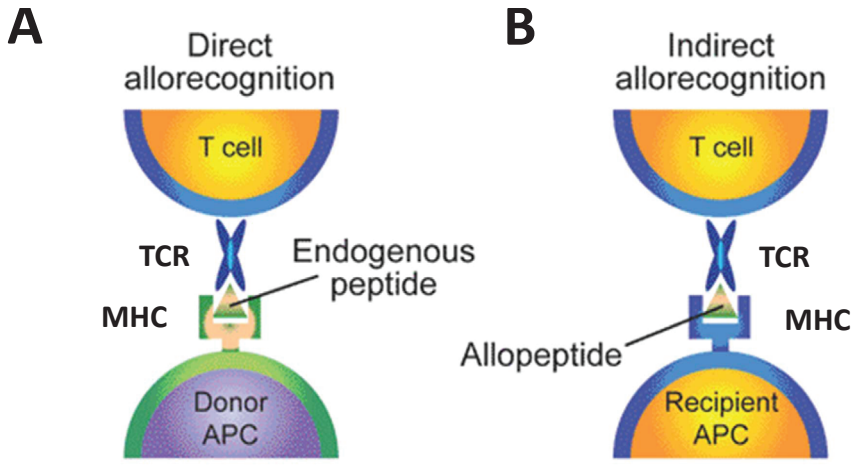


FIGURE 1. Pathways of allorecognition.
A) In the direct pathway, T cells recognize intact major histocompatibility molecules on donor antigen-presenting cells. B) In the indirect pathway, T cells recognize processed alloantigen in the form of peptides presented by recipient antigen-presenting cells. MHC is referred to MHC class I and MHC class II. *Adapted from the original figure of Briscoe DM and Sayegh MH, Nature Medicine, 2002.*

APCs, which then present them to T cells by their self-MHC molecules. Upon activation, naïve T cells proliferate and differentiate into effector T cells (Teff) which are specific for the alloantigens, and migrate via the circulation to the graft. Once in the graft they attack the graft tissue by direct lysis of graft cells, or by production of cytokines, among which IFN-γ, that stimulate macrophages to phagocytose graft tissue or B cells to produce antibodies (21, 23). Although both $CD4^+$ and $CD8^+$ T cells participate in acute cellular rejection, the rejection response is primary mediated by $CD4^+$ T cells (20). Important counterparts of Teff are regulatory T cells (Tregs). They suppress the alloreactive immune response by inhibiting Teff activation and proliferation, and by preventing DC maturation and their immunostimulatory properties (24-26).

The relative contributions of the two pathways in graft rejection are not fully known. The direct pathway is generally assumed to play a critical role in graft rejection during the early phase after transplantation, while the indirect pathway may become predominant later and presumably represents the driving force of graft destruction late after transplantation.

In the current thesis, we will mainly focus on two cell types that play a key role in inducing and controlling the allograft rejection response, which are DC and Treg, respectively.

3.1 Dendritic cells

Dendritic cells (DCs) are the most potent APCs and play a crucial role in both direct and indirect allorecognition. DCs arise from both myeloid and lymphoid progenitors within the bone marrow and are present in blood, lymph, lymphoid organs and non-lymphoid organs. There are two distinct types of DCs: Myeloid DCs (mDCs), which play an important role in antigen presentation and activation of naïve T cells, and plasmacytoid DCs, which are particularly involved in type I Interferon responses to viral infections. mDCs are activated though binding of their pattern recognition receptors (PRRs) to their respective ligands. Toll-like receptors and NOD-like receptors are well-described PRRs (27). The ligands of PRRs are either derived exogenously or endogenously. Exogenously derived molecules are called pathogen-associated molecular pattern (PAMPs), a well-known PAMP is lipopolysaccharide (LPS). Endogenous molecules are damage-associated molecular patterns (DAMPs), which are released from necrotic cells or from cells under stress (28). They function as important mediators of sterile inflammation, e.g. during graft rejection. In addition to direct recognition, mDCs can be indirectly activated by opsonized pathogens or antigens via ligation of immune-complexes to their activating Fcγ receptors (FcγRs), mainly FcγRIIa (CD32a). As a counter balance, DCs express the inhibitory Fcγ receptor IIb (CD32b) and a greater relative expression and ligation of FcγRIIb is sufficient to inhibit mDC activation induced by positive signaling through FcγRIIa (29, 30). Upon activation, mDCs increase their synthesis of MCH class II molecules and begin to express the costimulatory molecules CD80 and CD86 facilitating activation, expansion and differentiation of naive pathogen-specific T cells.

The importance of the mDC in graft rejection was demonstrated by early studies in rodents. Grafted tissue depleted of mDC showed prolonged or even indefinite graft survival in the absence of immunosuppression, proven an essential role for DCs (31, 32). Donor-derived mDC migrate from transplanted liver grafts to recipient lymphoid tissues, where they stimulate recipient T cells by presenting donor type HLA-molecules (33, 34).

3.2 Regulatory T cells

Important regulators of immune responses, e.g. transplant rejection, are regulatory T-cells (Treg). These "central regulators" have the ability to inhibit proliferation and production of cytokines of Teff, consequently limiting the damage caused by donor-specific Teff. Tregs are $CD4^+$ cells that also express CD25 (the α chain of the IL-2 receptor) and FOXP3 transcription factor. Inhibition of Teff or conventional T cells (Tconvs) by Tregs is mainly exerted in a contact-dependent fashion and by secretion of cytokines, e.g. IL-10 and TGF-β (24-26). In addition, Tregs can also inhibit DC activation and maturation and immunostimulatory capacity, which is mainly exerted in a contact-dependent manner (24, 35).

Interestingly, high numbers of Tregs in liver grafts are associated with graft acceptance (36). Hence, depending on the balance between activation of Teff and Treg, the graft is either rejected or accepted.

Importantly, experimental animal models have established that activated Treg not only temporarily prevent allograft rejection, but can even induce operational tolerance—defined as a state of permanent immunologic unresponsiveness to the allograft in the absence of immunosuppressive drugs (37). This implies that proper expansion and activation of Treg in human organ transplant recipients may durably protect against transplant rejection without the need for life-long treatment with immunosuppressive drugs. Therefore, finding a compound that activates Treg would greatly benefit patients. Unfortunately, current immunosuppressive drugs do not only suppress Teff, but they also have negative effects on Tregs by inhibiting FOXP3 expression and their suppression function (38).

3.3 Monocytes and macrophages

Other cells involved in allograft rejection are monocytes and macrophages. Monocytes are precursors of macrophages, but by themselves they play an important role in innate immunity by production of cytokines. During rejection, monocytes are recruited to the graft by local production of chemokines (e.g. MCP-1, MIP-1α, MIF) produced by infiltrating leukocytes and parenchymal cells (39). The numbers of monocytes within the allograft are dramatically increased when acute rejection develops (40). Monocytes can be classified into $CD14^{++}CD16^-$ (classical) and $CD14^+CD16^{++}$ (non-classical or "pro-inflammatory") subpopulations (41). The numbers of $CD14^+CD16^{++}$ monocytes are highly increased in patients with sepsis, rheumatoid arthritis or Kawasaki disease (41, 42). Recently, increased numbers of

$CD14^+CD16^+$ monocytes have also been reported after renal transplantation (43, 44), which is likely to be associated with acute rejection (44).

Macrophages are resident in almost all tissues and are the mature form of monocytes, which circulate in the blood and continually migrate into tissues, where they differentiate to macrophages. Tissue macrophages act as local surveillance and response cells to tissue damage and invading pathogens via several mechanisms, including phagocytosis, antigen presentation, cytokine production and secretion, and tissue repair. Macrophages are classified as M1 or classically activated cells with "pro-inflammatory" profile or M2 for alternatively activated cells with "regulatory" profile (45). While the M1/M2 differentiation is intriguing, the relative contribution of these cells in allograft rejection is not known. To date, numeral studies have indicated the accumulation of macrophages in both acute and chronic allograft rejection models, most of which are identified in renal transplant models. Damage to allograft is promoted by the production of pro-inflammatory cytokines, including IFN-γ, TNF-α and IL-1 (46-48). The production of reactive nitrogen and oxygen species by the inflammatory macrophage are also likely to contribute to tissue damage during acute rejection (49, 50). The precise mechanisms involved in tissue recognition and destruction by macrophages are largely unknown.

4. Current immunosuppressive drugs and side effects

The liver allograft is an immunologically privileged organ, which is illustrated by the low incidence of graft loss due to acute or chronic rejection and by its resistance to antibody-mediated injury (51). Approximately 20% of stable liver transplant recipients (> 2-3 years after LTx) can be withdrawn from all immunosuppressive therapy and still maintain normal graft function and not experience rejection (52-60). However, to date, there is no validated tool to identify these patients, and therefore, liver graft recipients still are mandated to use immunosuppressive drugs for life-long (61).

The current immunosuppressive drugs used in LTx act to inhibit the cellular rejection. Classical agents are corticosteroids, calcineurin inhibitors (CNI), mycophenolate mofetil (MMF; IMPDH inhibitor) and rapamycine (inhibitors of mTOR) (62). Most importantly, since their introduction in the 1980s, CNI have been the cornerstone of immunosuppressive treatment after organ transplantation (63). By inhibition of calcineurin signalling, IL-2 production by T cells is suppressed.

In the early years of clinical practice of LTx, acute rejection occurred in up to 75% of patients. Due to new immunosuppressive drugs as mentioned above, and improvements in treatment management, since 2000 the incidence of acute liver rejection has decreased to 15% (23). However, the need for life-long use of immunosuppressive drugs is accompanied by many side effects, such as hypertension, osteoporosis, cardiovascular diseases and renal

insufficiency (61, 62, 64). Moreover, by overall suppression of the immune system, patients suffer from major complications, such as cancer and infectious diseases.

One of the leading causes of late mortality in liver transplant recipients is the development of *de novo* malignancy (65). Growing evidence shows a higher risk for cancer in liver transplant recipients compared to the general population, with an incidence varying between 3 to 26% (64-69). With regard to the type of malignancy, unique types of malignancy occur more often after transplantation. Skin and lip tumors, and relatively rare tumors, such as post-transplant lymphoproliferative disease (PTLD), Kaposi's sarcoma, renal carcinomas, in situ carcinoma of the uterine cervix, hepatobiliary carcinomas, anogenital carcinoma and various sarcomas are prone to develop in transplant patients (70). In addition, recurrence of primary hepatic malignancy is well documented to occur after transplantation, and immunosuppression can accelerate metastatic tumor growth (68).

The pathophysiology underlying the increased cancer risk is not only suppression of immunosurveillance, but there is also evidence that CNIs have pro-carcinogenic effects that are independent of their effects on the host's immune response, including the inhibition of DNA repair, stimulation of tumor growth factor (TGF)-β and/or vascular endothelial growth factor (VEGF) synthesis, diminishing clearance of altered cells and transforming cancer cells into aggressive cancer cells (71-74).

Not all immunosuppressive regimens may give the same rise in cancer risk. However, so far, it is a matter of controversy which immunosuppressive regimen may be correlated with a higher cancer risk.

5. Intravenous immunoglobulin (IVIg): alternative safe immunosuppressive therapy?

IVIg is a therapeutic preparation of human IgG antibodies purified from pooled human plasma derived from 1,000-10,000 blood bank donors. This mixture of IgG molecules, containing traces of the IgM and IgA antibody isotypes, represents the entire antibody repertoire of numerous individuals. Consequently, IVIg antibodies recognize many different antigens.

IgG preparations were first used therapeutically in the 1950s as IgG replacement therapy by intramuscular administration. Due to technological advances in the fractionation of plasma, monomeric suspensions of IgG were developed that are suitable for intravenous use in the early 1980s. At that time, the only indication was for treatment of primary immunodeficiency disease (PID). PIDs are caused by mutations in any of the large number of genes that are involved in or control immune responses. Approximately 50% of PIDs are characterized by antibody deficiencies, including X-linked agammaglobulinemia (caused by Bruton tyrosine kinase deficiency), common variable immunodeficiency (CVID), and hyper IgM syndrome (75). CVID is the most frequent symptomatic PID in adults (76). In patients with CVID, the function of B and T cells is impaired, and there is usually a deficiency in IgG,

IgM and IgA. The cause of the disease is not entirely clear and is probably not uniform. For severe disease, with many recurrent or chronic infections, IVIg treatment is indicated. IVIg is believed to be beneficial as it contains a broad spectrum of antibody specificities against bacterial, viral, parasitic, and mycoplasma antigens that are capable both of opsonization and neutralization of microbes and toxins. The recommended initial dose of IVIg for PID is 400–600 mg/kg (77, 78). After the initial treatment, IVIg dosing is altered to achieve a targeted trough level of 500 mg/dl. Since the half-life of IVIg is 21 days, IVIg infusion is repeated approximately every 3-4 weeks. Current data increasingly indicate that optimal treatment of PID is based on individualizing the dose of immunoglobulin for each patient to prevent recurrent infection and pneumonia, rather than to achieve defined through levels (79, 80).

The use of IVIg to treat autoimmunity began after the first observation by Paul Imbach that IVIg administration to two patients who had agammaglobulinemia also improved their coexisiting thrombocytopenia. In 1981, he reported in several pediatric patients with immune thrombocytopenic purpura (ITP) receiving high-dose IVIg therapy a dramatic recovery from their thrombocytopenia (81, 82), which was also confirmed in adult patients (83). Since these first observations that IVIg exerts immunosuppressive properties, IVIg is increasingly used to treat various autoimmune diseases, including Guillain-Barré syndrome, Kawasaki disease and chronic inflammatory demyelinating polyneuropathy (CIDP) and other off-label indications (**Table 2**). The efficacy of administering IVIg in the various autoimmune

TABLE 2. Autoimmune diseases for which IVIg administration is recommended

Condition	Evidence	Grade	Usage (% total)	
			Patients	**Volume**
Neuromuscular			**31.54%**	**43.77%**
Chronic inflammatory demyelinating polyradiculoneuropathy	IA	A	10.28%	21.65%
Multifocal motor neuropathy	IA	A	7.58%	11.13%
Guillain-Barré syndrome	IA	A	4.27%	4.34%
Myasthenia gravis	IA	B	4.10%	3.27%
Inflammatory myopathies	IIB	B	1.53%	1.78%
Paraprotein-associated demyelinating neuropathy	IB	A	0.47%	0.78%
Stiff person syndrome	IB	A	0.42%	0.67%
Hematological			**17.46%**	**11.13%**
Immune thrombocytopenic purpura, acute and persistent	IIB	B	11.92%	7.10%
Autoimmune hemolytic anemia	III	C	NR	0.04%
Other autoimmune			**5.51%**	**3.27%**
Kawasaki disease	IA	A	2.69%	NR
Immunobullous diseases	III	C	2.02%	1.13%

Data derived from the 2011 clinical guidelines and the 2012 IVIg database report of the UK National Health System (NHS). The relative impact of each condition on IVIg usage in 156 NHS Trusts in years 2011–2012 are shown: numbers indicate the % of a total of 9458 patients (including patients with primary or secondary immunodeficiency) treated with a total volume of 2,613,076 grams IVIg. Sources: Clinical Guidelines for Immunoglobulin Use, 2nd Edition (2011), National Services Division of NHS Scotland Update, March 2012. *Adapted from the original table of Petta et al., Current Opinion in Pharmacology, 2014.*

diseases is well-established (84). Side effects of IVIg are mostly mild and nonanaphylactic. They are typically characterized by back or abdominal pain, nausea, vomiting, diarrhea, rhinitis, chills, low grade fever, myalgias, and/or headache (78, 85, 86). Slowing or stopping the infusion will reverse many reactions. Hence, IVIg is a relatively safe therapy for life-long treatment.

Interestingly, IVIg has also demonstrated to be effective in preventing allograft rejection. In our clinic it has been observed that prophylactic treatment of hepatitis B virus (HBV) recurrence with anti-HBs IVIg in patients receiving transplants for HBV-related cirrhosis resulted in significantly less risk of cell-mediated acute rejection (87). This finding was later confirmed in a multi-center study (88). Similar effects of IVIg have been observed in renal transplant patients (89). Interestingly, the mechanisms behind the immunosuppressive effects of IVIg are different from current immunosuppressive drugs. Our research group and other investigators have established that IVIg does not inhibit Teff directly, but indirectly by targeting mDC and Treg. IVIg effectively inhibits mDC function and promotes killing of mDC, thereby inhibiting presentation of allo-antigens to Teff (87, 90-92). Additionally, IVIg stimulates expansion of Treg and enhances their suppressive function *in vitro* and in animal models (93-95). Importantly, in the context of transplantation, our research group has shown that IVIg treatment can effectively prevent allogeneic skin graft rejection in mice (95).

In renal transplant patients, IVIg has already been used for decades in patients who are sensitized with high titers of donor-specific anti-HLA antibodies in order to prevent antibody-mediated allograft rejection (**Box 1**). These patients are often treated with IVIg in combination with plasmapheresis, anti-lymphocyte antibody (rituximab or bortezomib) or immunoadsorption techniques (96-99). The beneficial effect of IVIg in prevention or treatment (100) of antibody-mediated allograft rejection includes enhancement of auto-antibody clearance by blocking the neonatal FcR (FcRn), neutralizing anti-idiotypic effects and blocking of complement activation (101, 102). Interestingly, modulation of FcγRs may play a role in prevention of acute allograft rejection. In several antibody-mediated immune disease models, upregulation of the inhibitory FcγRIIb expression by IVIg on macrophages is an important effector mechanism (103-106). In an arthritis animal model, glycosylated IgG binding to macrophages in the spleen results in upregulation of FcγRIIb expression on effector macrophages, resulting in reduced activation via auto-antibody immune complexes (107, 108). Recent research showed that FcγRIIb upregulation was exerted by a cascade of enhanced IL-33 production in the spleen induced by IVIg, which in its turn stimulates IL-4 and IL-13 production by basophils, and thereby enhanced expression of the FcγRIIb on effector macrophages in the joint in an auto-antibody mediated mice model (109). In context of allograft rejection, upregulation of FcγRIIb on macrophages may lead to inhibited phagocytosis of donor-specific anti-HLA antibodies bound to allograft cells. In addition, upregulation of FcγRIIb on mDCs can result in reduced uptake of immune complexes of anti-donor IgG and donor HLA, resulting in decreased presentation of donor HLA peptides

by recipient mDCs and suppressed mDC activation (30). In a cardiac transplant model, it has recently been suggested that the loss of FcγRIIb expression leads to enhanced chronic alloantibody -mediated allograft arteriopathy (120).

Collectively, there is evidence that IVIg is able to modulate cells involved in graft rejection, including inhibition of APC functions, in particular of mDCs, and activation of Tregs. **Figure 2** summarizes some proposed mechanisms of action of IVIg on cells involved in graft

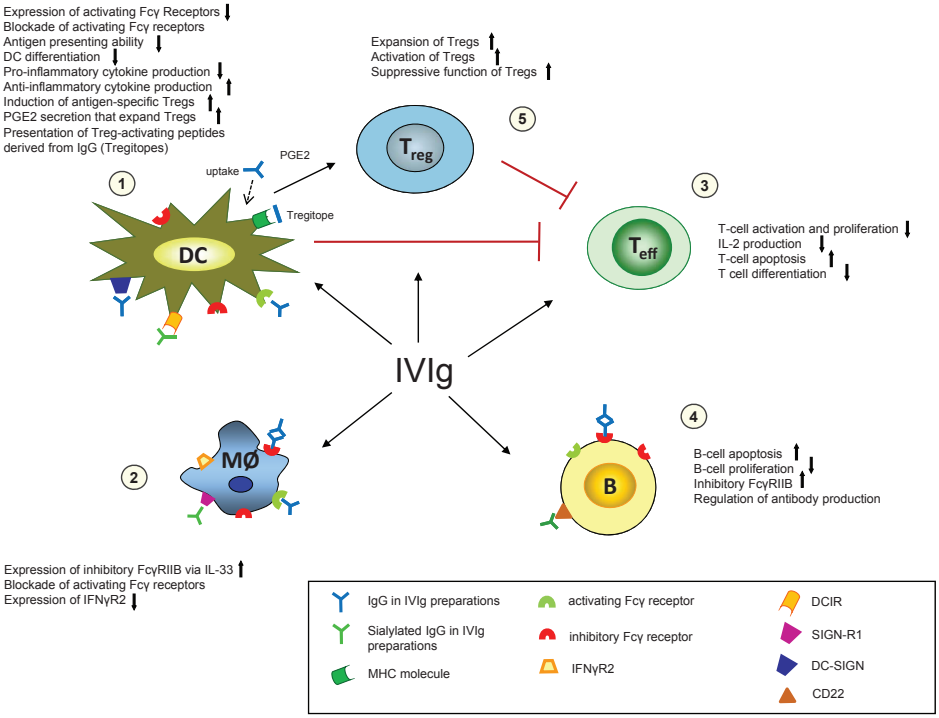


FIGURE 2. An overview of the proposed mechanisms of immunomodulation of IVIg on cells involved in allograft rejection.
IgG in IVIg preparations can modulate the expression of activating FcγR or block the binding of immune-complexes or cells opsonized by IgG to activating FcγR, thereby inhibiting endocytosis and phagocytosis by **1)** DCs and **2)** macrophages. Also, IVIg inhibits DC differentiation and pro-inflammatory cytokine production, while increasing anti-inflammatory cytokine secretion. In addition, IVIg inhibits **3)** T-cell activation by DCs via intracellular interference of IgG with presentation of antigens by MHC molecules. IgG binding to the C-type lectin SIGN-R1 on mouse splenic macrophages induces the expression of the inhibitory FcγRIIb on effector macrophages at sites of inflammation via increased IL-33 production. Via binding to FcγRIII, IVIg suppresses expression of IFNγR2 on macrophages, thereby inhibiting their functions. Furthermore, binding of IgG to CD22, a Siglec family member expressed on **4)** B cells, IVIg reduces B cell receptor signaling and enhances B cell apoptosis. IgG dimers suppress macrophage and B-cell functions by ligating the inhibitory FcγRIIb. Additionally, IVIg induces antigen-specific **5)** Tregs by tolerogenic DCs from non-Treg $CD4^+$ T cells by binding of sialylated IVIg to DCIR expressed on DCs, and induces PGE2 secretion by DCs which stimulates expansion of Tregs. The latter process is at least partially mediated by F(ab')2 binding to the human C-type lectin DC-SIGN. Presentation of Treg-activating peptides derived from the Fc part of IgG (Tregitopes) by antigen-presenting cells activates Tregs. The immunological effects depicted are not mutually exclusive and are likely to work in synergy. DC, dendritic cell; MØ, macrophage; Teff, effector T cell; Treg, regulatory T cell; SIGN-R1, specific ICAM-3 grabbing nonintegrin-related 1; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DCIR, dendritic cell immunoreceptor; PGE2, prostaglandin E2

rejection. These features are unique compared to conventional immunosuppressive drugs, which mainly act on Teffs.

However, most evidence for the immunomodulatory effects of IVIg on mDC and Treg are derived from *in vitro* or mouse model studies. Since humans and mice differ significantly in their immunology, it is highly questionable whether these mechanisms are translatable to humans. In addition, since few studies addressed the immunological effects of IVIg therapy in patients, it is largely unknown whether the mechanisms of action of the immunomodulatory effects of IVIg identified using human cells *in vitro* are operational in humans *in vivo*.

BOX 1

Current applications of IVIg in organ transplantation

- Treatment of sensitized patients with high titers of donor-specific anti-HLA antibodies, often in combination with plasmapheresis, immunoadsorption or anti-lymphocyte antibody (110, 111).
- Treatment of ABO-incompatible organ transplantation (in order to shorten the waiting time), often in combination with plasmapheresis, immunoadsorption or anti-lymphocyte antibody (112-114).
- Treatment of antibody-mediated rejection after organ transplantation (115).
- Treatment of steroid-resistant rejection in kidney transplant recipients (116, 117).
- Prophylactic HBV treatment in patients receiving liver transplants for HBV-related diseases using anti-HBV surface antigen-specific IVIg (anti-HBs IVIg) (118) .

AIMS AND OUTLINE OF THE THESIS

Since the major side effects of current immunosuppressive drugs counterbalance the success of LTx, there is a pressing clinical need for a safer immunosuppressive treatment in order to improve the long-term outcome. IVIg is a very promising option, since there is clinical evidence for protective effect against acute rejection. Importantly, long-term treatment with IVIg has no severe side effects (78, 85, 86). However, much knowledge still needs to be gained to understand the mechanisms behind the immunomodulatory effect of IVIg that are operational in humans. The current major drawback in the implementation of IVIg in standardized protocol post-transplantation is the predicted global shortage of human plasma and the high costs (119). Therefore, in order to enable long-term use of the immunosuppressive properties of IVIg, a major goal is to synthesize small molecules or biologicals that exert anti-inflammatory effects equivalent to IVIg preparations. Such compounds will obviate the need for human plasma. To accomplish this, we first need to understand the

cellular and molecular pathways by which IVIg suppress the immune system in humans *in vivo*.

The overall aim of this thesis was to identify the mechanisms by which intravenous immunoglobulin therapy modulates the immune system in humans, that could explain the clinical benefits seen in patients with autoimmune and inflammatory diseases as well as the prevention of graft rejection in transplant recipients. In addition, we identified an artifactual issue that influences results in studies on the effects of IVIg on mDC *in vitro*, as well as an artifact caused by one of the IVIg manufacturing processes to produce IVIg that affects its immunomodulatory capacity.

In the first part of this thesis (**chapter 2**), we first studied one of the emerging complications of immunosuppressive drugs in liver transplant recipients, which is the development of *de novo* cancer. We described the relative risk for development of *de novo* cancer in our LTx cohort and examined the risk factors associated with developing *de novo* cancer. Importantly, we determined whether there was a correlation with changes in immunosuppressive exposure over time.

In the second part of the thesis we studied possible mechanisms by which IVIg therapy exerts immunosuppressive effects in patients. mDCs are the most potent APCs and mediate both direct and indirect T-cell priming in alloreactive immune responses. In **chapter 3**, we studied the effect of IVIg therapy on mDC function in humans *in vivo*. We examined whether IVIg treatment stimulates production of IL-33 and Th2 cytokines and thereby modulates expression of FcγRs and IFN-γ receptor on mDCs in humans *in vivo*. In addition, we explored the effects of Th2 cytokines on the expression of FcγRs and IFN-γ receptor on mDCs and the functional consequences *in vitro*. Tregs are important immune regulators and possible strategies to induce and maintain transplant tolerance by Tregs is currently being investigated. In **chapter 4**, we studied the *in vivo* effect of IVIg therapy on activation of Tregs in patients. In addition we assessed whether IVIg therapy induces the suppressive capacity of Tregs *ex vivo*. In **chapter 5** we assessed whether high-dose IVIg therapy can modulate pro-inflammatory monocytes in patients, as has been recently suggested. To explore the dose-dependent effects, in all patient studies we have divided the patients into two groups: low-dose IVIg treatment (average 0.43 g/kg) and high-dose IVIg treatment (average 1.24 g/kg). In **chapter 6,** we reviewed several immunomodulatory mechanisms of action of IVIg in humans, and focus on important immunological differences between mice and men that differentially impact how IVIg exerts anti-inflammatory activities in both species. This review warrants careful interpretation of studies derived from mouse studies and illustrates the need for human *in vivo* studies to unequivocally address which mechanisms of action of IVIg are in fact operational upon treatment in humans.

In the third part, we focused on potential factors that might influence the immunomodulatory effects of IVIg or the examination of effects of IVIg on immune cells. In **chapter 7**, we highlighted discrepancies between current reports on the effect of IVIg on DC maturation

and cytokine production *in vitro*. We examined whether IgG absorption to culture plastic plate may artifactually mimic immune-complex mediated activation of FcγR-expressing cells. In **chapter 8**, we studied whether the manufacturing process may influence the biological activity of IVIg, demonstrated by comparing two hyperimmunoglobulin preparations, Cytotect® with Cytotect® CP, which are IVIg preparations enriched for CMV-specific antibodies. Both studies warn for artifactual influences in studying immunomodulatory effects of IVIg.

Finally, in **chapter 9**, we summarized and discussed our results and described future perspectives.

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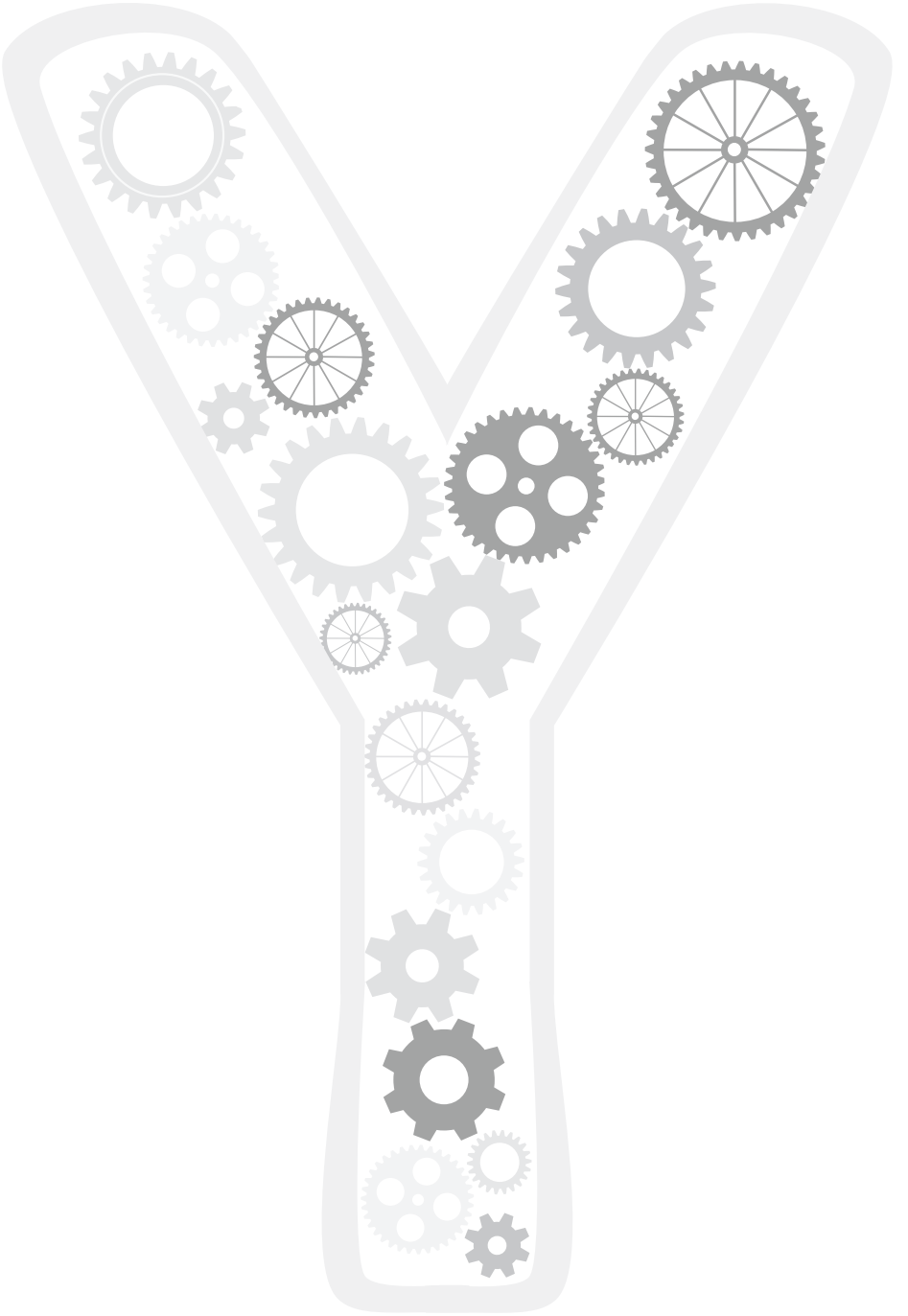

2

Increased incidence of early *de novo* cancer in liver graft recipients treated with cyclosporine: an association with C2 monitoring and recipient age

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ABSTRACT

The goal of the study was to determine the risk factors for *de novo* cancer after liver transplantation (LTx). Retrospective analyses were performed in 385 LTx patients transplanted between 1986 and 2007. In total, fifty (13.0%) recipients developed *de novo* malignancy. The cumulative incidence of *de novo* cancer at 1, 5, 10 and 15 years post-LTx was 2.9 (±0.9)%, 10.5 (±1.8)%, 19.4 (±3.0)% and 33.6 (±6.8)%, respectively. The standardized incidence ratio (SIR) of malignancy in LTx patients compared to the general population was 2.2 (95% confidence interval [CI]: 1.6–2.8). After excluding PTLD and skin cancer, *de novo* cancer patients had a significantly lower survival rate as compared to recipients who remained cancer-free. The identified univariate risk factors for *de novo* cancer were cyclosporine (CsA) treatment, time period of LTx, and recipient age. In multivariate analysis, only CsA treatment emerged to be independent risk factor for *de novo* cancer, which moreover attributed to more aggressive cancer types. A surprising finding was that CsA treatment specifically enhanced cancer risk in patients transplanted after 2004, when C2 monitoring was introduced. Additionally, these patients showed a significantly lower acute rejection rate, which might reflect a more robust immunosuppressive status caused by CsA-C2. Considering age, only patients ≤50 years had a higher cancer rate when treated with CsA compared to tacrolimus (TAC). Our data suggest that, compared to TAC treatment, CsA treatment with C2 monitoring or in younger patients of ≤50 years is associated with a higher early *de novo* cancer risk after LTx.

INTRODUCTION

The 1-year survival after liver transplantation (LTx) has dramatically increased in the past three decades. Nowadays, over 80% of the patients are alive 1 year after LTx (1, 2). In contrast, the long-term outcome has improved less impressively and its improvement is one of the main focuses in modern transplantation medicine (1). Importantly, malignancy is one of the major leading causes of late death after LTx (2-5). The reported risk for *de novo* cancer in liver transplant recipients is up to 2.1- till 4.3-times higher compared to the matched general population with an incidence varying between 3 and 26%.(3-10) The higher cancer risk after organ transplantation has been reported to be directly related to the intensity as well as the cumulative dose of immunosuppression (11). A dose reduction of cyclosporine (CsA) to maintain a trough blood level from 200 to 100 ng/ml in kidney transplant recipients resulted in a significant reduction in the *de novo* cancer incidence (12). Furthermore, a history of usage of immunosuppressive drugs prior to liver transplantation was a significant risk factor for development of *de novo* malignancy (13). The cancer pathogenesis induced by immunosuppressants includes direct damage to the host DNA and impairment of the recipients' immunosurveillance, which reduces their anti-tumor and anti-viral immunity (14, 15). Considering other risk factors, a higher age had been reported to play a significant role in the development of cancer after LTx (8, 13, 16).

Since their introduction in the eighties, calcineurin inhibitors (CNI) have been the cornerstone of immunosuppressive treatment after transplantation. The relationship between cancer and the use of different CNI, such as CsA or tacrolimus (TAC), is still to be elucidated (1). Several studies found no difference in the incidence of *de novo* cancer between CsA based and TAC based regimens (17-19), while other studies reported a higher *de novo* cancer risk for CsA (20, 21) or TAC (8) based immunosuppressive protocols. Thus, the role of different CNI in the occurrence of *de novo* cancer after LTx is still a matter of controversy.

As the therapeutic window between efficacy and toxicity of CsA is small, blood level monitoring to guide dosing is an essential tool (22). Traditionally, CsA dose adjustment was based on trough blood level (C_0). In more recent years, numerous studies have reported that 2 hours post-dose level monitoring (C_2) is a more effective monitoring strategy compared to C_0 level, as the absorption phase of CsA occurs within 2 hours and the concentration peak level is a better predictor of freedom from graft rejection (23, 24). Using CsA-C_2 monitoring, the overall incidence of biopsy-proven acute rejection (22-28%) appeared to be lower compared to CsA-C_0 monitoring (36-59%) (25-28). Therefore, in 2005, the CsA-C_2 monitoring strategy was introduced in our center.

In the present study, we describe the incidence of *de novo* cancer after LTx in our centre and determine the risk factors associated with *de novo* cancer risk. Since immunosuppressive regimens have changed over time and a delicate balance exists between effective prevention of rejection and over-suppression of immune surveillance, we additionally stud-

ied the incidence of *de novo* cancer over time and determined its correlation with changes in immunosuppressive regimens.

PATIENTS AND METHODS

Patients

Between October 1986 and December 2007, 565 liver transplantations were performed in 500 adults in the Erasmus MC University Medical Centre Rotterdam, The Netherlands. As malignancy is a time-dependent event, patients with a follow-up shorter than 4 months (n = 99) at the time of analysis were excluded. In addition, patients who underwent auxiliary LTx (n = 13), those who received a liver with the presence of occult malignant cells (n = 2) and those who had a cholangiocarcinoma in the native liver (n = 1) were excluded. Consequently, 385 patients were included in our cohort, which represented 3121 person years of follow-up. A retrospective analysis was performed using the LTx database and medical records of the recipients. Analyzed data included age of recipient at time of transplantation, gender of recipient, primary LTx indication, type of primary immunosuppressive therapy, *de novo* malignancy post transplantation, interval from LTx to diagnosis of malignancy, interval from LTx or diagnosis of cancer to death and interval from LTx to diagnosis of the first acute rejection, which was defined as biopsy-proven rejection (Snover-grade ≥ 2, or rejection activity index (RAI)-score ≥ 6) with a rise in transaminases and/or bilirubin, which responded to treatment with methylprednisolone, and in some patients with OKT3 or rabbit ATG. All patients were followed until December 2008.

The primary endpoint was *de novo* malignancy, which was defined as the development of cancer other than recurrent primary liver cancer. Recurrence cancer was not accepted as an endpoint, as this is not exclusively dependent on the immunosuppression state, but also on the aggressiveness of the primary liver cancer. Diagnosis of malignancy was confirmed histologically, and the date of histological diagnosis was taken as date of malignancy.

Data were collected from follow-up examinations in the outpatient clinic at month 6 and every 12 months after transplantation. Prior to LTx, all patients had undergone a routine tumor screening, which included chest radiography, mammography, abdominal ultrasound, gastroscopy and colonoscopy. After LTx, all patients participated in a yearly repeated clinical, laboratory and radiology (ultrasound and chest x-ray) study. Women participated in a routine cervical cytology study on a yearly basis. Other studies were done on an individual patient basis. Epstein-Barr virus (EBV) IgG in serum before LTx were recorded for 346 out of 385 patients.

All patients declared not to object for using their data in the study.

Primary immunosuppression

The choice of certain immunosuppressive protocols in our center over the years was based on the general development and new insights in immunosuppressive drugs (based on clinical trials) in the liver transplantation medicine. During the first 10 years of the study, dual and triple CsA-C_0 based maintenance immunosuppressive regimens have been routinely use. Dual therapy consisted of CsA and prednisolone, and in the triple regimen azathioprine was added. Between 1994 and 1999, the microemulsion formulation of CsA (Neoral; Novartis, East Hanover, NJ) has been altered with the oil-based CsA (Sandimmune; Novartis). From 1999, the microemulsion formulation of CsA was routinely used. After CsA was initiated within 24 hr post-reperfusion in a dose of 10-15 mg/kg/day, the dosage was adjusted to pre-dose level according to a range between 200 and 400 ng/mL during the first 3 months and thereafter between 100 and 200 ng/mL. Moreover, CsA dosage was adjusted in case of rejection or CsA-related toxicity as described previously (29). Since the introduction of TAC in 1990, a dual TAC-based regimen with prednisolone has been alternated with dual or triple CsA-based regimens. The monitoring of TAC was based on trough levels with a target range between 5 and 15 ng/ml. Started from 2000, dual therapy with TAC and prednisolone was used as standard regimen.

Since the introduction of CsA-C_2 level monitoring strategy in 2005, CsA-C_2 based immunosuppressive regimen was used as standard regimen interchanged with TAC based immunosuppressive regimen. The CsA-C_2 treated patients received an initial dose of 10-15 mg/kg/day, which was adjusted based on the C_2 target level of 800-1200 ng/ml during the first 3 months. Thereafter, target levels were maintained at 700-900 ng/ml at 4 till 6 months and 480-720 ng/ml thereafter. Dosing strategy of TAC remained unchanged.

In the induction immunosuppressive regimen, polyclonal antithymocyte globulin (ATG) (n=16) or monoclonal antilymphocyte antibody OKT3 (n=3) was used in the first decade of the study. Since 1998, these induction therapies have been replaced by IL2 receptor antagonists (IL2-RA).

Prior to LTx, all patients with autoimmune cirrhosis were treated with prednison and azathioprine.

Statistical analyses

Statistical analyses were performed using SPSS software package version 15.0 (SPSS, Chicago, IL). The cumulative incidences of *de novo* malignancy and survival were assessed by the Kaplan-Meier method. For the analysis of malignancy, the starting point was the first LTx and the endpoint was the first malignancy. When reviewing the interval from LTx to specific cancer types, we firstly categorized cancer into three groups based on the highest incidence in our center, which were non-melanoma skin cancer, PTLD and other cancer.

Then the end points were defined as (I) non-melanoma skin cancer, (II) PTLD and (III) other cancer. In all analyses, patients were censored in case of death and lost to follow-up. Underlying this approach was the assumption that patients were alive, therefore at risk, in the follow-up.

For survival analysis comparing patients who developed *de novo* cancer with those remained cancer-free, *de novo* cancer was considered as a time-dependent factor, since cancer developed at various follow-up times. This means that all patients entered at time 0 as cancer-free. At the time of the development of *de novo* cancer the patient was censored in the cancer-free group and entered the *de novo* cancer group. In this way, the period that a patient lived as cancer-free was calculated as the "event-free survival period" in the Kaplan-Meier analysis. The same method was used to estimate the association of different types of cancer with the patient survival. Patients could have more than one malignancy, but only the first malignancy was included in the survival analysis.

Univariate Cox regression and log-rank analyses were used to identify risk factors associated with *de novo* cancer development. To investigate whether these factors were independently associated with cancer development, all risk factors with $P \leq 0.05$ were used as a covariate in a multivariate Cox regression model. $P \leq 0.05$ was considered significant. The distribution of categorical variables was compared using the Chi-square test and differences between means were compared by Student's t-test.

In the analyses of the transplantation period as a risk factor for *de novo* cancer, malignancy incidence curves were made of the different LTx time periods containing 4 years-intervals. The recent LTx time period consisted of 3 years in order to provide more equal numbers in the groups, as a large number of LTx were performed in the latest years. The first LTx period starts at 1989, since all LTx performed in our centre before 1989 were auxiliary LTx.

For analyses of the mean daily dose of CsA in mg/kg at different time intervals within the first 12 months post-LTx, cumulative daily doses were divided by the mean body weight and the number of days in the same time interval. Differences between means were compared by Student's t-test.

The standardized incidence ratio (SIR) of malignancy was calculated by comparing the observed number of cancers in the study cohort with expected number of cancers based on age and gender specific rates for the Netherlands. The Dutch incidence rates of cancer were obtained from the Comprehensive Cancer Centre in The Netherlands (30) for the calendar year 1997, which is the median of our study period. We assume that the SIR estimation will not differ considerably when we matched our population with calendar-year specific rates in The Netherlands, as the Dutch cancer rates have not changed significantly over time (30). Then, the incidence rates were stratified into 5-year age groups per 100,000 person-years for both genders. The expected numbers of cases were calculated by the multiplication of the Dutch cancer incidence rate in both genders and age group by the

number of person-years in the corresponding period of observation. The standard error for hazard ratio was calculated for estimation of the 95% confidence interval (CI).

RESULTS

Incidence of *de novo* malignancy

Patient characteristics are summarized in **Table 1**. From the 385 liver graft recipients, who had at least 4 months of follow-up after the first LTx, 50 (13.0%) patients developed at least one *de novo* cancer. The cumulative incidence of *de novo* malignancy at 1, 5, 10 and 15 years after LTx was 2.9 (±0.9)%, 10.5 (±1.8)%, 19.4 (±3.0)% and 33.6 (±6.8)%, respectively (**Figure 1**).

In the Dutch population, the expected number of age- and gender- matched individuals with *de novo* malignancy in the same follow-up period as our cohort, was calculated to be 22.8. As our observed number of *de novo* cancer was 50, the standardized incidence ratio (SIR) of *de novo* malignancy in liver transplant patients in comparison with the general population was 2.20 (95% CI: 1.59 – 2.80).

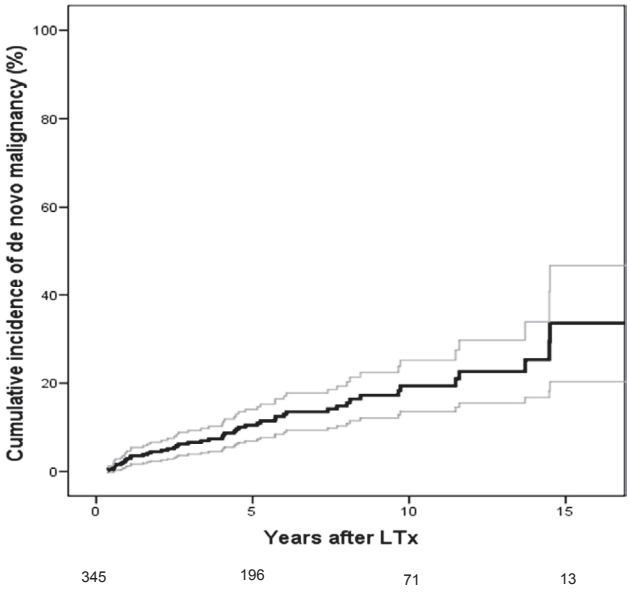

FIGURE 1. Cumulative incidence of de novo malignancy during the first 15 years after LTx in liver graft recipients transplanted in the Rotterdam LTx cohort (n = 385).
The grey lines represent the standardized error. Kaplan-Meier method was used under the assumption that patients were at risk during follow-up.

TABLE 1. Patient characteristics

		N	%
Median age at LTx (range)	49 (16-69)		
Median follow-up in years (range)	5.0 (0.3-19.1)		
Gender			
Male		220	57
Female		165	43
Primary LTx indication			
Viral cirrhosis [1]		65	17
Cholestatic disease[2]		96	25
Autoimmune cirrhosis		14	4
Alcoholic cirrhosis		39	10
Acute liver failure		62	16
Cancer[3]		44	11
Other		65	17
Time periods of LTx			
1989-1992		25	6
1993-1996		67	17
1997-2000		94	24
2001-2004		101	26
2005-2007		98	25
Primary immunosuppressive drug[4]			
Cyclosporin		167	52
Tacrolimus		202	43
Induction therapy			
ATG/OKT3		19	5
IL-2 RA		230	60
EBV IgG prior to LTx			
Positive		334	87
Negative		12	3
Unknown		39	10

LTx, liver transplantation; ATG, anti-thymocyte globulin; IL-2 RA, IL-2 receptor antagonist; EBV, Epstein-Barr virus; IgG, immunoglobulin G

[1]Viral cirrhosis: sum of hepatitis B cirrhosis (*n* =24), hepatitis BD cirrhosis (*n* =7) and hepatitis C cirrhosis (*n* =34).
[2]Cholestastic disease: sum of primary biliary cirrhosis (PBC; *n* =25), primary sclerosing cholangitis (PSC; *n* =64) and secondary biliary cirrhosis (*n* =7)
[3]Cancer: sum of hepatic cellular carcinoma (*n* =43) and hepatic epithelioid hemangioendothelioma (*n* =1)
[4]Sixteen patients did not have either cyclosporine or tacrolimus

Types of cancer

In total, 66 *de novo* malignancies appeared in 50 recipients. **Table 2** shows the distribution of the types of cancer in our study. Most common cancer types were non-melanoma skin cancer (55%) and PTLD (21%). Skin cancer included basal cell carcinoma (41%, 27/66) and

squamous cell carcinoma (14%, 9/66). The cumulative incidences of non-melanoma skin cancer, PTLD and other cancer are depicted in **Table 3**.

TABLE 2. Type of *de novo* cancer in patients after liver transplantation

Type	Number of tumors	Total (%)
Non-melanoma skin	36	55
PTLD	14	21
Gastrointestinal	4	6
Lung	3	5
Melanoma	2	3
Gynaecological	2	3
Kaposi sarcoma	1	2
Kidney	1	2
Liver	1	2
Other	2	3
Total	66	100

PTLD, post-transplant lymphoproliferative disorder

TABLE 3. Cumulative incidence of *de novo* cancer types in patients after liver transplantation

	Time after LTx			
Type of cancer	1 year	5 year	10 year	15 year
Non-melanoma skin	0.8%	5.1%	10.2%	19.7%
PTLD	1.3%	2.8%	6.4%	8.3%
Other cancer[1]	0.5%	2.6%	4.4%	10.1%

[1]Other cancer includes the remaining cancer types. PTLD, post-transplant lymphoproliferative disorder.

Influence of cancer on survival

Patients who developed *de novo* malignancy after LTx had a significantly shorter survival than those who remained cancer-free, which was at 10 year post-cancer diagnosis 50.8 (±8.6)% in the cancer group versus 79.0 (±2.9)% in the cancer-free group at 10 year post-LTx ($P < 0.001$).

With regards to the type of cancer, the survival was not impaired in patients who developed non-melanoma skin cancer and PTLD compared to patients who remained cancer-free ($P = 0.12$ and $P = 0.33$, respectively)(**Figure 2**). However, a highly reduced survival was found in patients who developed the other cancer types summed in **Table 2**, including the more aggressive gastrointestinal, lung and gynaecological tumors ($P <0.001$). The 1-year survival in these patients after diagnosis of cancer was 28.6% (±1.2)%. None of the patients who developed non-melanoma skin cancer and PTLD died from their disease, whereas 9 patients (64%, 9/14) who developed the remaining cancer types died due to cancer.

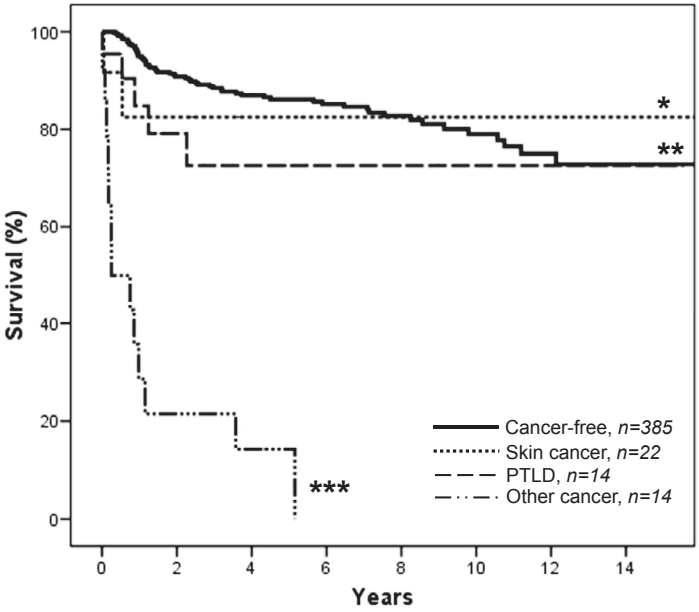

FIGURE 2. Estimated survival in patients who developed different types of cancer (after diagnosis of de novo cancer) and those who remained cancer-free (after LTx). All survival rates were compared with the survival rate of cancer-free recipients (*P = 0.122; **P = 0.330, ***P < 0.001).

Risk factors for malignancy after transplantation

Analyzing potential factors influencing the development of *de novo* cancer after LTx by univariate analyses, age > 50 year (*P* = 0.025), immunosuppressive treatment with CsA (*P* = 0.035), and the time period of LTx (*P* = 0.049) emerged to be significant risk factors (**Table 4**).

In order to see how the period of transplantation influenced the appearance of *de novo* cancer, malignancy incidence curves were made of the different LTx time periods containing 3 and 4 year-intervals (**Figure 3**). A high incidence was found in patients transplanted in the earliest LTx period (1989 till 1992). Comparing this period with the period 2001 till 2004, patients transplanted between 1989 and 1992 had a significantly higher risk for cancer (*P* = 0.004). After this earliest LTx period, a considerable decline of the incidence of *de novo* cancer was observed after each period of transplantation. In contrast, in patients transplanted in the most recent period of 2005 till 2007 an increase of the incidence of *de novo* malignancy was observed. Patients transplanted in this period had a significantly higher rate of *de novo* malignancy compared to those transplanted in the period of 2001 to 2004 during the first 4 years post-transplantation (*P* = 0.022). The rise in the *de novo* cancer incidence was particularly seen in the first year after LTx, which was 7.2 (±2.6)% in LTx period of 2005 till 2007 versus 1 (±1.0)% in LTx period of 2001 till 2004. Identifying which cancer types were increased

in recent period, we found that the incidence of all cancer types, rather than a specific cancer type, increased (data not shown).

In order to see which variables were independently associated with a higher cancer risk after LTx, a multivariate analysis was performed including factors that in the univariate analyses were associated with *de novo* cancer. In this Cox-regression analysis, CsA treatment remained a significant risk factor (P = 0.029), while LTx period (P = 0.401) and age > 50 years (P = 0.219) failed to show an independent association with the development of cancer. This data indicates that CsA treatment was the only independent risk factor for *de novo* cancer after LTx and that the effects of both LTx period and age on cancer risk were related to CsA treatment. Remarkably, when studying the types of cancer appearing in patients who were treated with CsA compared to TAC, we observed that CsA treated patients had a 2.5-times higher risk to develop more aggressive cancer types that do not belong to the non-melanoma skin cancer and PTLD summed in **Table 2** (data not shown). This data indicate that CsA is not only associated with a higher early *de novo* cancer risk, but also with cancer types having a worse prognosis.

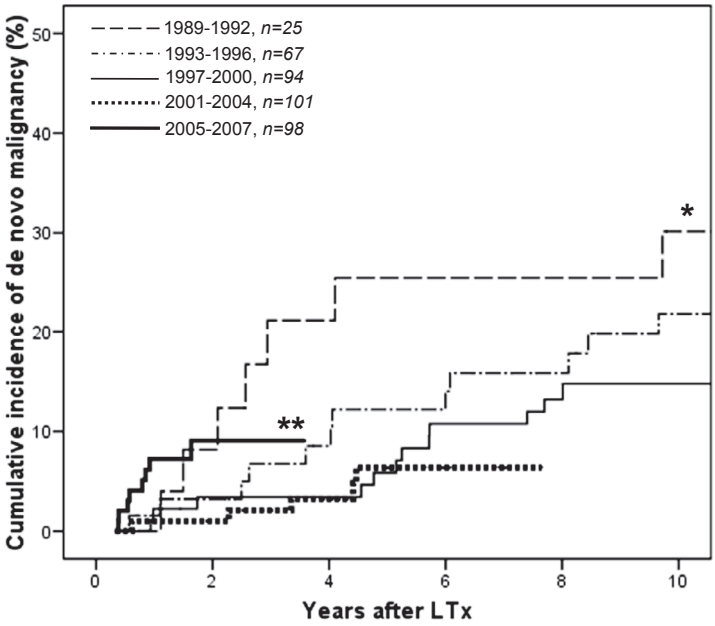

FIGURE 3. Cumulative incidence of de novo cancer of LTx patients transplanted in different time periods. A higher risk of de novo malignancy was observed in liver graft recipients transplanted in 1989-1992 and 2005-2007 compared to 2001-2004 (*P = 0.004 and **P = 0.022 compared to 2001-2004).

TABLE 4. Risk factors associated with *de novo* cancer development (univariate Cox regression model)

Risk factor	Hazard ratio (95% CI)	P- value
Age		
<50 year	1	**0.025**
>50 year	1.92 (1.09-3.38)	
Gender		
Male	1	0.431
female	0.79 (0.45-1.41)	
Indication for LTx		
Viral cirrhosis	1	0.753
Cholestatic disease	1.03 (0.45-2.35)	
Autoimmune cirrhosis	0 (0-∞)	
Alcoholic cirrhosis	0.99 (0.33-2.95)	
Acute liver failure	0.71 (0.27-1.86)	
Cancer	1.80 (0.63-5.11)	
Other	0.74 (0.26-2.10)	
Immunosuppressive regimen		
Dual	1	0.795
Triple	1.10 (0.62-1.89)	
Primary immunosuppressive drug		
Tacrolimus	1	0.035
Cyclosporine	2.02 (1.05-3.87)	
Induction immunosuppressive treatment		
Non- IL-2 receptor antagonist	1	0.070
IL-2 receptor antagonist	0.55 (0.29-1.05)	
Time period of LTx		
1989-1992	0.83 (0.28-2.45)	
1993-1996	0.51 (0.19-1.40)	
1997-2000	0.37 (0.13-1.02)	
2001-2004	0.22 (0.07-0.71)	
2005-2007	1	**0.049**
EBV IgG prior to LTx		
Positive	1	0.514
Negative	2.51 (0.89-7.07)	
Unknown	1.12 (0.52-2.42)	

LTx, liver transplantation; EBV, Epstein-Barr virus; IgG, immunoglobulin G

Increased cancer rate in CsA treated patients was only observed in recent years

To investigate how the influence of the LTx period on the cancer risk was related to CsA treatment, we compared the incidence of *de novo* cancer in CsA treated patients with TAC treated patients in the different LTx periods. As depicted in **Figure 4**, in the LTx period before 2005 we could not find a difference in the cancer rate between CsA and TAC treated patients. Strikingly, CsA treated patients transplanted from 2005 showed a significantly higher *de novo* cancer risk in the early phase after LTx compared to the TAC treated patients, which was 9.9-fold (95% CI 1.2 – 80.5, *P* = 0.032) higher compared to patients treated with TAC. These data indicate that only the specific CsA treatment used in recent years was associated with a higher risk for early development of *de novo* cancer.

In patients transplanted from 2005, 7 CsA treated patients developed *de novo* cancer, of which 3 non-melanoma skin cancer, 3 PTLD and 1 epithelioid sarcoma. In the TAC treated group, only one bone marrow metastasis from unknown origin occurred.

To see whether the CsA group was comparable with the TAC group in the LTx period from 2005, Chi-square tests were performed, showing that there were no differences in age (≤50

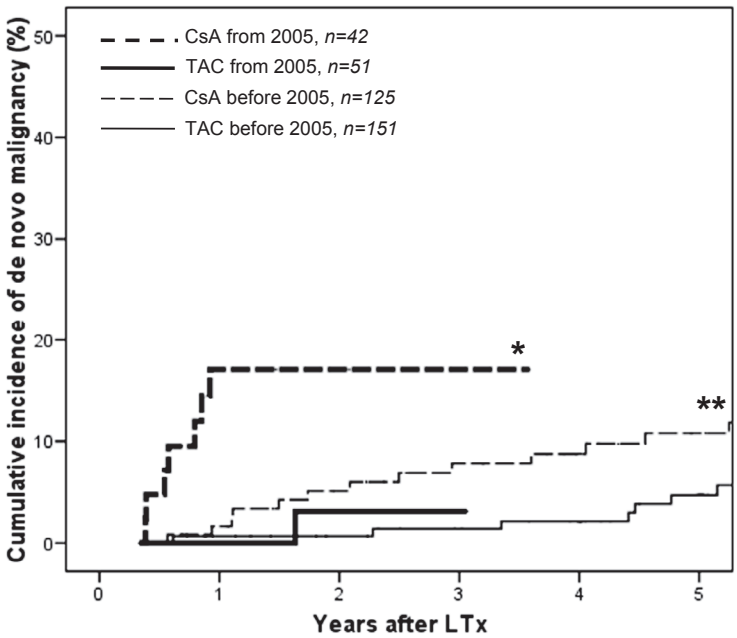

FIGURE 4. Cumulative incidence of de novo malignancy in CsA and TAC treated liver graft recipients transplanted before and from 2005.
Only CsA treatment in patients transplanted from 2005 showed a significantly higher early de novo cancer rate compared to TAC treatment. In this period, 7 de novo cancers occurred in the CsA treated group versus 1 de novo cancer in the TAC treated group. (*P = 0.009 compared to TAC from 2005, **P = 0.255 compared to TAC before 2005).

years of age: 41.5% in CsA group versus 58.5% in TAC group; *P* = 0.10), gender (male: 46.4% in CsA group versus 53.6% in TAC group; *P* = 0.18) and LTx indication (*P* = 0.35) between both treatment groups. Next, to determine whether CsA was more frequently used in a triple immunsuppressive regimen, a chi-square test was performed calculating the distribution of CsA and TAC in triple versus dual immunosuppressive regimens. We found that CsA and TAC were equally distributed in triple as well as in dual immunosuppressive regimens (data not shown).

From January 2005, CsA dosing based on the conventional C_0 level monitoring was replaced by dosing based on C_2 levels monitoring in all *de novo* LTx patients. As the CsA blood level monitoring was the only major change in the CsA treatment in the recent LTx period, our data suggests that the C_2 monitoring strategy was the reason for the increased early *de novo* cancer risk. In order to see whether CsA-C_2 treated patients received a higher dose that could have caused the higher cancer risk, the mean daily CsA doses of CsA-C_2 and CsA-C_0 treated patients were compared. Analysis was performed at different time intervals within the first 12 months after LTx, as all malignancies in CsA-C_2 treated patients occurred within the first year post-LTx. During the induction phase (first 14 days after LTx), CsA-C_2 treated patients received a significantly higher dose than CsA-C_0 treated patients, which was in average 11.76 mg/kg/day in CsA-C_2 treated patients versus 9.79 mg/kg/day in CsA-C_0 treated patients (*P* = 0.043). Thereafter, no differences in daily CsA dose were observed between patients treated with CsA-C_2 and CsA-C_0 up to 6 months post-LTx (5.26 mg/kg/day in CsA-C_2 versus 6.05 mg/kg/day in CsA-C_0, *P* = 0.157). However, at 12 months post-LTx, patients treated with CsA-C_2 received a significantly lower dose than patients treated with CsA-C_0 (3.69 mg/kg/day in CsA-C_2 versus 5.24 mg/kg/day in CsA-C_0, *P* < 0.001).

Nevertheless, CsA doses given to patients may not adequately represent drug exposure due to inter- and intrapatient variability in CsA absorption. A better surrogate marker for drug exposure is the rejection rate, which is strongly correlated with CsA bioavailability (31-34). Therefore, analyses were performed comparing acute rejection rates during the first year post-LTx between CsA and TAC treated patients transplanted before and after 2005. At 1 year post-LTx, CsA treated patients transplanted from 2005 showed a low incidence of acute rejection (9.6%), while this is significantly higher in CsA treated patients transplanted before 2005 (35.2%, *P* = 0.002), and TAC treated patients transplanted from 2005 (27.5%, *P* = 0.030) and before 2005 (23.9%, *P* = 0.047).

Increased cancer risk for CsA treatment was associated with younger recipient age

In order to see how recipient age was correlated with the difference in cancer rate between CsA and TAC treatment, we compared the incidence of *de novo* cancer between CsA and TAC treated patients in different age groups, of which 50 years was the best discriminating

cut-off value (data not shown). We found that CsA treated patients in both age groups (≤ 50 and >50 years) had a high *de novo* cancer risk (18.3% > 50 years versus 18.8% in ≤ 50 years, *P* = 0.560), which was statistically not different in TAC treated patients who were older than 50 years (9.2%, *P* = 0.459). In contrast, TAC treatment in patients who were 50 years or younger resulted in a significantly lower cancer risk as compared to CsA treatment in the same age group (5.2% in TAC versus 18.8% in CsA, *P* = 0.027). These data indicate that only in the younger patients a higher cancer risk was found for CsA treatment compared to TAC treatment.

Factors predisposing to the development of specific cancer types

We questioned whether the risks of the individual cancer types were associated with the same risk factors or might be influenced by confounding factors, like EBV-seronegativity for development of PTLD. Patients who were EBV-seronegative prior to LTx had a 4.95-fold (95% CI: 1.06 – 23.15, *P* = 0.042) higher risk to develop PTLD. By performing Chi-square test and Kaplan-Meier analysis, we found that there were no differences in the EBV status pre-LTx (P = 0.337) nor in the incidence of PTLD (P = 0.823) among the older and younger CsA treated patients. This suggests that the higher cancer risk in the younger CsA treated patients was not due to a negative EBV status pre-LTx. In addition, the rise of PTLD incidence in CsA treated patients transplanted from 2005 could not be explained by the influence of EBV serology status prior to LTx, since the EBV serology did not differ between the CsA and the TAC treated patients transplanted in this recent LTx period (P = 0.362). The risk for non-melanoma skin cancer appeared to be 3.08-fold (95% CI: 1.31 – 7.24, *P* = 0.01) higher in patients who were older than 50 years. For the development of the other cancer types summed in **Table 2**, we found that the only independent risk factor appeared to be CsA treatment (HR 4.79, 95%CI 1.01 – 22.66, *P* = 0.048).

DISCUSSION

In the present study, we found that CsA in comparison to TAC treatment is the most important risk factor for *de novo* malignancy after LTx. This higher cancer risk was, however, not observed in all CsA treated patients, but CsA specifically enhanced development of *de novo* cancer in patients transplanted in more recent years (2005-2007), and in younger patients (≤50 years of age). In addition, we showed that CsA treatment particularly resulted in more aggressive types of cancer compared to TAC, with a 1-year survival rate less than 30%. Interestingly, the incidence of *de novo* malignancies after LTx in our centre considerably decreased over time after the first LTx period (1989-1992), but increased again in liver graft recipients transplanted in the most recent years (2005-2007).

The high incidence of *de novo* cancer in patients transplanted in the earliest LTx period might be explained by the ATG induction treatment in 16 out of 25 LTx-patients, since ATG induction therapy has been described to be associated with post-transplantation cancer (17, 19, 35, 36). However, because the numbers of ATG treated patients were small, this association could not be confirmed in our cohort. Thereafter, since 2005, the trend of the decreasing *de novo* cancer incidence was noticeably turned into an increasing incidence, which was significantly related to CsA treatment. From January 2005, CsA dosing based on the conventional C_0 level monitoring was replaced by dosing based on C_2 levels monitoring in all *de novo* LTx patients. As the CsA blood level monitoring was the only major change in the CsA treatment in the recent LTx period, our data suggest that the C_2 monitoring strategy was the reason for the increased early *de novo* cancer risk.

We were not able to compare the incidence of *de novo* cancer risk in CsA-C_0 and CsA-C_2 treated patients head to head, since these dosing strategies were used in different time periods in our center. Therefore, we compared CsA-C_0 and CsA-C_2 treated patients, respectively, with TAC treated patients transplanted within the same time period, since TAC dosing strategy has not changed over time. This comparison corroborates that only in the period of C_2-based CsA dosing, patients treated with CsA had a higher risk of cancer compared to TAC treated patients (**Figure 4**).

Our finding that all malignancies in the CsA-C_2 treated patients occurred within the first year after LTx suggests that CsA-C_2 treated patients were exposed to a high dose during the early post-LTx phase. We found that CsA-C_2 treated patients received a significantly higher dose, but only during the induction phase after LTx. However, a far better correlate of drug exposure is the incidence of acute rejection, as CsA dose is dependent on drug absorption. Interestingly, we found a lower incidence of acute rejection during the first year after LTx in CsA treated patients transplanted from 2005 with C_2 based monitoring (9.6%), compared to CsA treated patients transplanted before 2005 with C_0 based monitoring (35.2%). Furthermore, to exclude a bias from the different LTx periods, the acute rejection rates were compared within the same LTx period (2005-2007) between CsA and TAC treated patients, showing that within this period the CsA group had a lower acute rejection incidence (9.6% in CsA versus 27.5% in TAC group). Corroborating our observations, several studies have shown that CsA-C_2 monitoring is associated with a lower acute rejection rate compared to CsA-C_0 (25, 26, 37). Collectively, these observations indicate that dosing of CsA based on CsA-C_2 monitoring results in more robust immunosuppression compared to CsA-C_0 based treatment, which consequently might impair immunosurveillance of developing malignancies. Regarding to the types of cancer arising in the CsA treated patients transplanted in recent years, we found a relative rise in the non-melanoma skin cancer and PTLD. The higher PTLD risk is not a consequence of negative EBV serology prior to LTx, since EBV status was not different in the CsA compared to TAC treated patients transplanted in this recent LTx period

Compared to TAC, CsA treatment was only unfavourable in younger patients with regards to *de novo* cancer development after LTx. This higher cancer risk in the younger CsA treated patients was not attributable to EBV seronegativity prior to LTx, since no difference in EBV status was present in the different age groups. Moreover, the younger CsA treated patients did not develop more PTLD.

Till now, it has been stated that the basis for the higher cancer incidence after LTx is the long-term immunosuppressive state, and not the specific immunosuppressive protocol that is used (9). Our data showed that CsA treatment is a strong determinant for *de novo* cancer development after LTx. First, we found that CsA compared to TAC treatment resulted in a higher *de novo* cancer risk in younger patients, of which is known that they have a low cancer risk (8, 13, 16). Secondly, we found that the dosing strategy does influence cancer development, since the intense dosing in the CsA-C_2 treatment protocol resulted in a more rapid development of *de novo* malignancy within the first year after LTx. Both observations suggest that not only the duration of immunosuppressive state, but also the immunosuppressive treatment protocol influences the development of cancer. Additionally, we observed that CsA treatment resulted in more aggressive types of cancer.

There is evidence that CsA has pro-carcinogenic effects that are independent of its effect on the host's immune response, including the inhibition of DNA repair, stimulation of tumor growth factor-β and/or vascular endothelial growth factor synthesis, diminishing clearance of altered cells and transforming cancer cells into aggressive cancer cells (15, 38, 39). While TAC has been reported to have similar pro-metastatic mechanism described for CsA (38, 40), there is also evidence that TAC has anti-metastatic effects not seen with CsA (41). Our observation that CsA treatment resulted in more diverse and aggressive types of cancer supports the notion that CsA may promote a higher level of carcinogenesis than TAC.

In contrast to other studies (5, 42) gender was not significantly associated with a higher cancer risk. Furthermore, we did not find pre-transplant use of immunosuppressive drugs to be correlated with a higher cancer risk, as all auto-immune cirrhotic patients received immunosuppressive drugs prior to LTx, which did not result in a higher *de novo* cancer risk. (13) In agreement with other reports, we found that IL-2RA as an induction therapy did not result in a higher incidence of cancer (19, 42, 43).

Overall, we found a 2.2-fold higher risk for *de novo* cancer development in the entire cohort of liver graft recipients compared to the age- and gender- matched Dutch population. Although this risk is high, it is within the lower part of the range of SIRs reported by other studies, which range from 2.1 to 4.3 (5, 6). The highest incidence of cancer was found for non-melanoma skin cancer and PTLD, which is consistent with other reports (5, 6, 9, 13, 14, 19, 20, 42, 44-47). It was not surprising that we found a relation between EBV seronegativity prior to LTx and PTLD development, as PTLD is mainly related to EBV infection (48), and transmission of EBV is frequent when the naïve transplant recipient receives an organ from an EBV-infected donor. Since a high frequency of the donor population (>85%) is EBV seroposi-

tive, mismatching for EBV is common (49). However, as discussed previously, EBV-negativity before LTx did not explain the higher cancer risk induced by CsA treatment. Considering the survival, it was remarkable that we did not find a higher mortality rate for recipients who developed PTLD compared to cancer-free recipients, while various studies showed a low survival for PTLD patients (40.8% at 5 year) (44, 50, 51). Our observed high survival rate of PTLD patients may be explained by successful treatment of PTLD by reduction of immunosuppressive drugs, rituximab and systemic chemotherapy. Indeed, disease management with adequate immunosuppression reduction is associated with improved survival of patients with PTLD (14).

In summary, we have shown that CsA treatment in comparison to TAC treatment is the most significant risk factor for development of *de novo* cancer in LTx patients. However, this effect was confined to CsA-C_2 treated patients and to patients of 50 years and younger. Furthermore, CsA treatment gave rise to more aggressive behaviour cancer types compared to TAC treatment. So far, to our knowledge, our study is the first study suggesting that CsA treatment based on C_2 monitoring in *de novo* LTx patients and CsA treatment in younger patients were associated with a significantly higher early *de novo* cancer risk compared to TAC. The limitations of our study include the retrospective study design, the limited numbers of patients and the lack of opportunity to compare C_0 and C_2 treatment within the same time period. So, larger randomized trials with long-term follow-up are needed to confirm this hypothesis. The ongoing international randomized trial comparing TAC with CsA-C_2 in hepatitis C related liver transplant patients might provide support to our finding. This study highlights the importance of re-evaluation and optimization of currently used immunosuppressive regimens.

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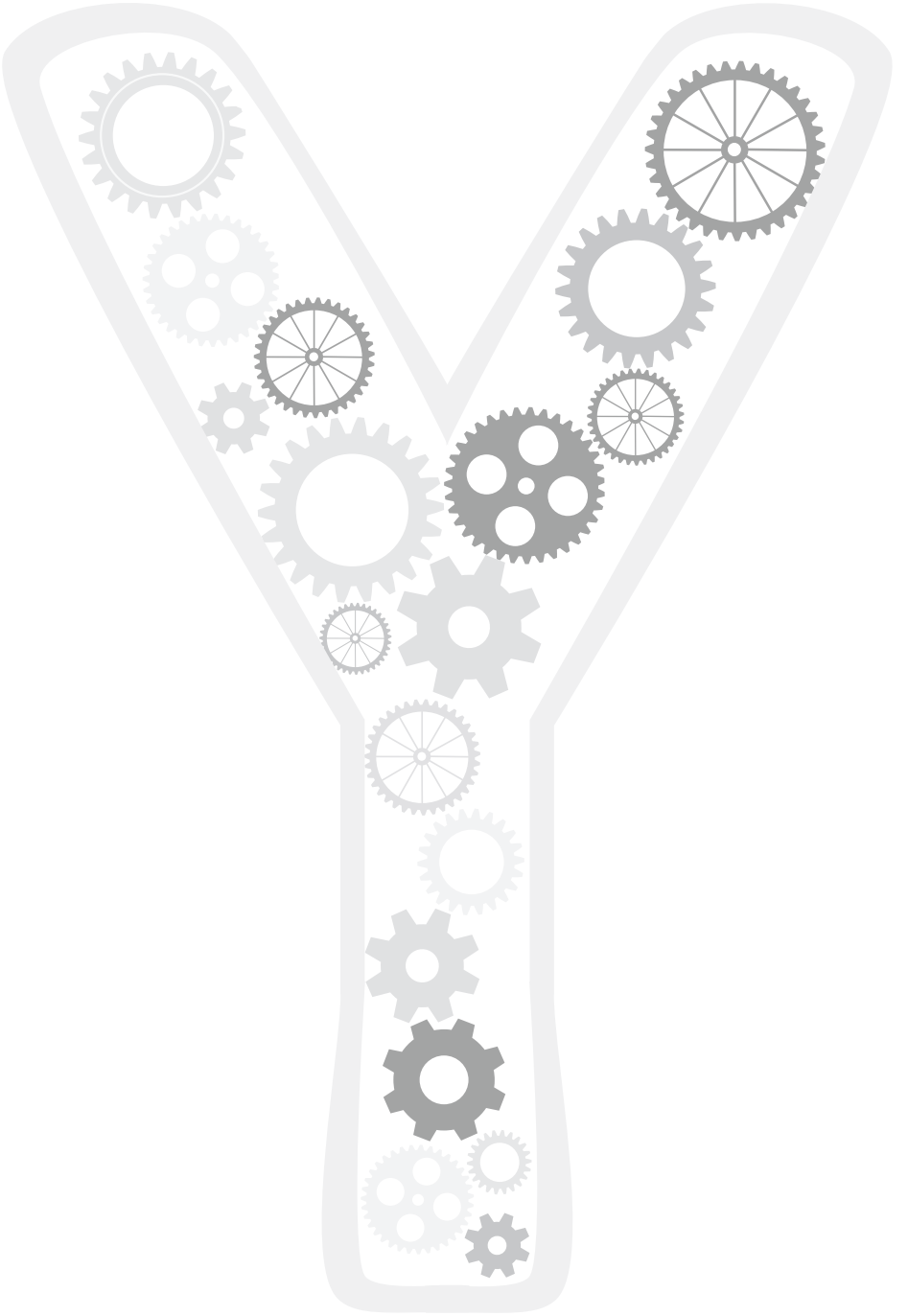
PART II

IMMUNOMODULATION BY IVIG IN PATIENTS



3

Intravenous immunoglobulin treatment in humans suppresses dendritic cell function via stimulation of IL-4 and IL-13 production

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ABSTRACT

High-dose intravenous immunoglobulin (IVIg) is a prominent immunomodulatory therapy for various autoimmune and inflammatory diseases. Recent mice studies suggest that IVIg inhibits myeloid cell function by inducing a cascade of IL-33-Th2 cytokine production causing upregulation of the inhibitory FcγRIIb, as well as by modulating IFN-γ signaling. The purpose of our study was to explore whether and how these mechanisms are operational in IVIg-treated patients. We show that IVIg in patients results in increases in plasma levels of IL-33, IL-4 and IL-13, and that increments in IL-33 levels correlate with rises in plasma IL-4 and IL-13 levels. Strikingly, no upregulation of FcγRIIb expression was found, but instead a decreased expression of the activating FcγRIIa on circulating myeloid dendritic cells (mDCs) after high-dose, but not after low-dose, IVIg treatment. In addition, expression of the signalling IFNγR2 subunit of the IFN-γR on mDCs was downregulated upon high-dose IVIg therapy. *In vitro* experiments suggest that the modulation of FcγRs and IFNγR2 on mDCs is mediated by IL-4 and IL-13, which functionally suppress the responsiveness of mDCs to immune complexes or IFN-γ. Human lymph nodes and macrophages were identified as potential sources of IL-33 during IVIg treatment. Interestingly, stimulation of IL-33 production in human macrophages by IVIg was not mediated by DC-SIGN. In conclusion, high-dose IVIg treatment inhibits inflammatory responsiveness of mDCs in humans by Th2 cytokine-mediated down-regulation of FcγRIIa and IFNγR2, and not by upregulation of FcγRIIb. Our results suggest that this cascade is initiated by stimulation of IL-33 production that seems DC-SIGN- independent.

INTRODUCTION

Intravenous immunoglobulin (IVIg) is used for the treatment of several autoimmune and systemic inflammatory diseases caused by autoantibodies and/or derailment of the cellular immune system (1-7). Importantly, the anti-inflammatory effects of IVIg require treatment with high doses, which exceed those used for replacement therapy in immune deficiencies (3, 8-10). Although the clinical benefit of IVIg is evident, the mechanisms by which IVIg can suppress different types of immune responses have not yet been fully elucidated. Effects of IVIg on antibody-mediated immune responses include prevention of immune complex binding to activating Fcγ receptors (FcγRs), and enhanced clearance of autoantibodies by saturation of the neonatal Fc receptor (4, 5, 8). The beneficial effects of IVIg on diseases caused by hyperactivity of cellular immunity are mediated by modulation of regulatory T cells (5, 11-14), macrophages (15, 16) and dendritic cells (12, 17, 18).

In several mouse models of antibody-induced inflammatory diseases, the anti-inflammatory effects of IVIg are initiated by binding of 2,6-sialylated IgG molecules to SIGNR1 on splenic marginal zone macrophages, resulting in upregulation of the inhibitory FcγRIIb on macrophages at the site of inflammation, thereby preventing their activation by pathogenic immune complexes (IC) (19). Recent research has uncovered the missing link between these two types of macrophages in a serum-induced arthritis model by showing that IVIg stimulates IL-33 production in the spleen, which induces production of the Th2 cytokines IL-4 and IL-13 by basophils, which on their turn up-regulate expression of the inhibitory FcγRIIb on effector macrophages in the joints (20). However, the question whether and how this anti-inflammatory mechanism of IVIg also plays a role in humans is actually far from trivial. Firstly, the structural organization of the human spleen differs from the murine spleen, especially with regard to the marginal zone. The marginal zone macrophage, the postulated source of IL-33 production upon binding of 2,6-sialylated IgG molecules to SIGNR1, is absent in the human spleen (21). Secondly, splenectomized ITP patients do still respond to IVIg therapy, arguing against an indispensable role for the spleen in mediating the protective effects of IVIg in humans (22). Thirdly, recent studies in ITP mouse models demonstrated that the inhibitory effects of 2,6-sialylated IgG molecules were not impaired upon splenectomy (23, 24), and were even independent of IL-33 and IL-4 signaling (23). Collectively, these observations warrant a study to determine whether IVIg stimulates the IL-33-Th2-FcγRIIb pathway in humans.

Therefore, the first aim of the current study was to investigate the effects of IVIg therapy on IL-33 and Th2 cytokines as well as its effects on the expression of FcγRs on myeloid cells in humans *in vivo*. Since IVIg treatment has been shown to down-regulate expression of the signaling IFN-γ receptor 2 unit (IFNγR2) on murine macrophages, thereby rendering them refractory to activation by IFN-γ (16), we also studied the effects of IVIg-treatment on IFNγR expression on circulating myeloid cells. To discern whether the effects of IVIg depend on

treatment dose, we included patients treated with high or low dose of IVIg in our study. In the second part of the study, we investigated whether a causative relation may exist between IVIg, IL-33, Th2 cytokines, receptor expression on myeloid cells, and the functional responsiveness of these cells, using human cells *in vitro*.

MATERIAL AND METHODS

Patients

Twenty-nine patients (22 female/7 male) with either immunodeficiency or autoimmune disease and treated with IVIg were included in this study, and subdivided into two groups: those who received "low-dose" IVIg (LD) and those who received "high-dose" IVIg (HD). Since supplemental dose treatment started initially with 0.4-0.6 g/kg, we defined "low dose" IVIg as ≤0.6 g/kg, and "high dose" IVIg as >0.6 g/kg. The indications for IVIg treatment in these patients are depicted in **Table 1A, B**. Twenty-two patients were on IVIg monotherapy, and 6 patients received additional corticosteroid treatment. All patients showed clinical improvement after treatment. After approval by the local institutional ethical review board, written informed consent was obtained from all participants.

TABLE IA. Patient characteristics of low-dose IVIg-treated patients

IVIg treatment indication	Number of patients	Age in years, median (range)	IVIg dose in g/kg, median (range)
Common variable immunodeficiency	5	33 (20-57)	0.38 (0.30-0.48)
Hypogammaglobulinemia	7	60 (40-77)	0.39 (0.25-0.59)
Agammaglobulinemia	1	30	0.25

TABLE IB. Patient characteristics of high-dose IVIg-treated patients

IVIg treatment indication	Number of patients	Age in years, median (range)	IVIg dose in g/kg, median (range)
Refractory polymyositis*	3	41 (33-56)	1.00 (0.67-1.67)
Hypogammaglobulinemia	3	68 (40-69)	0.86 (0.66-0.86)
Common variable immunodeficiency	2	40 (37-43)	0.68 (0.65-0.70)
Immune trombocytopenic purpera	2	68 (65-71)	1.00
Acquired von Willebrand syndrome*	2	52 (40-63)	0.95 (0.93-0.98)
Polyserositis e.c.i.*	1	24	0.78
Polychondritis*	1	68	0.69
Refractory dermatomyositis*	1	46	1.71
Systemic vasculitis e.c.i.*	1	64	1.4

* IVIg treatment was indicated based on unresponsiveness to conventional treatment.

Intravenous immunoglobulins

The IVIg preparations received by the patients were Nanogam (n= 13; Sanquin, Amsterdam, The Netherlands), Kiovig (n= 11; Baxter, Deerfield, IL), Flebogamma (n= 4, Grifols, Barcolona, Spain) and Octagam (n= 1; Octapharma, Lachen, Switzerland).

For *in vitro* experiments, we used Intratect (Biotest Pharma, Dreieich, Germany), which was a kind gift from the company. IVIg was dialyzed against large volumes of RPMI at 4˚C using Slide-A-Lyzer gamma-irradiated dialysis cassettes (Pierce, Rockford, USA) to remove stabilizing agents and to obtain neutral pH. In all experiments IVIg was used at a concentration of 10 mg/ml, which is similar to the median increment in serum IgG concentration we observed in the IVIg-treated patients. To avoid artificial immune-complex mediated activation of mDCs due to adsorption of the IgG molecules to the culture plates, we pre-coated round-bottom 96-wells cell culture plates (Greiner Bio-One , The Netherlands) with fetal calf serum (FCS) to prevent IgG binding (25). After 24 hours, the plates were extensively washed with PBS, remaining liquid was removed and mDCs, splenocytes or lymph node cells were cultured as described below. To control for the elevated levels of protein upon addition of IVIg to cell cultures, we used equimolar amounts of human serum albumin (HSA, Sanquin) as described previously (6).

Sample collection and preparation

Heparin-decoagulated blood samples were collected from healthy blood donors and from patients immediately before IVIg infusion, immediately after IVIg infusion (LD: 4-6 hours and HD: 24-30 hours after the start of the infusion), and 7 days after infusion. Plasma and PBMCs were isolated from whole blood by density gradient sedimentation using Ficoll-Paque (GE healthcare, Uppsala, Sweden). Until further analysis, plasma samples were stored at -80 degrees and PBMC samples at -135 degrees. To minimize possible inter-assay variation, measurements on PBMCs and plasma obtained at different time points from the same patient were performed on the same day. Since we did not have sufficient PBMCs from all patients, measurements of different receptor expression on mDCs were performed on 10 LD and 11 HD patient samples.

Human splenic tissue (n=6) and hepatic lymph nodes (n=6) were obtained during organ procurement from liver donors. Lymph nodes were dissected from the hepatoduodenal ligament. Spleen tissue and lymph nodes were sliced into small pieces and passed over a 100µm nylon mesh filter to obtain a single cell suspension. Mononuclear cells were then obtained by Ficoll-Paque density centrifugation.

Antibodies and flow cytometry

For identification of $CD20^{-}BDCA1^{+}$ mDC in blood of patients, PBMCs were stained with anti-CD20-Pacific Blue (eBioscience, San Diego, CA) and anti-BDCA1-PE (Miltenyi, Bergisch Gladbach, Germany), and for identification of monocytes with anti-CD14 PE-Cy7 (eBioscience). To detect expression of FcγRIIa and FcγRIIb on their surface, we used FITC-conjugated or Alexa Fluor 488-conjugated anti-CD32a/FcγRIIa mAb (clone IV.3, StemCell Technologies, Grenoble, France) and human-mouse chimeric anti-CD32b/FcγRIIb-FITC (clone ch2B6N297Q (26, 27) which was kindly provided by MacroGenics, Rockville, Maryland, USA). IFNγR chain 2 was stained by using anti-IFNGR chain 2 mAb (clone MMHGR-2, PBL biomedical laboratories, Piscataway, NJ) followed by FITC-conjugated rabbit anti-mouse IgG1 (DAKO, Glostrup, Denmark). To prevent non-specific binding, cells were incubated for 15 minutes with rabbit serum prior to FITC-conjugated rabbit anti-mouse IgG1 staining, followed by incubation with mouse serum prior to staining with mouse antibodies specific for leukocyte markers. The rather low expression of IFNγR2 on circulating immune cells was not due to insensitivity of the anti-IFNγR2 mAb (clone MMHGR-2) used, since another mAb (clone 2HUB-159, Biolegend, San Diego, CA) showed similar results, and is in agreement with previous studies (28). IFNγR chain 1 was stained by using anti-IFNGR chain 1 mAb (Biolegend).

In order to assess the maturation status of mDCs in *in vitro* experiments, cells were stained with anti-HLA-DR-APC-Cy7, CD38-APC (BD Pharmingen), CD83-APC, CD86-Pacific blue (both from Biolegend), CD80-FITC (Beckman, Brea, CA), and CCR7-FITC (R&D systems).

For surface labeling, cells ($1x10^{6}$) were incubated with mAB in 50µl PBS (Lonza, Verviers, Belgium) $^{+}$ 1% bovine serum albumin $^{+}$ 0.02% sodium azide (both from Sigma, Brooklyn, NY) for 30 min on ice and protected from light. Then, cells were washed and resuspended in 100µl PBS supplemented with 7-amino actinomycin D (7-AAD; BD Biosciences) for measurement by flow cytometry (FACsCanto, BD Biosciences, San Jose, CA). A minimum of 3×10^{5} MNC were acquired. Analyses were performed by FACS Diva software (BD Biosciences, San Jose, CA). Viable MNC were gated based on forward/side scatter and exclusion of 7-AAD. For each sample we used matched isotype control mAB to set gates for analysis.

ELISA

Plasma and/or culture media concentrations of IL-33, IL-5 (Biolegend, San Diego, CA, USA), IL-4, IL-13, IL-6 (Pelikine, Sanquin, Amsterdam, The Netherlands), IFN-γ (Abcam, Cambridge, UK), TNF-α, IL-10 (Quantikine HS, R&D systems, MN, USA) and IP-10 (Invitrogen, Toronto, Ontario, Canada) were measured by ELISA according to the manufacturer's instructions. IVIg present in plasma did not interfere with cytokine detection, since addition of IVIg (10 mg/ml) to samples with known cytokine levels did not alter the measured concentrations.

mDC isolation and macrophage generation

BDCA1^{+}CD20^{-}mDCs were purified from fresh heparinized blood of healthy volunteers by positive selection with PE-conjugated anti-CD1c mAb and anti-PE MACS-beads, after depletion of B cells with CD19-conjugated MACS-beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (18). The purity of mDCs as analyzed by flow cytometry was 92±8%. Macrophages were generated *in vitro* by culturing human monocytes, which were purified by positive selection using anti-CD14 MACS-beads (Miltenyi Biotec), with M-CSF (20ng/ml) for 6 days. Fresh medium and cytokines were replenished every 2 days.

Effect of cytokines on receptor expression and function *in vitro*

To analyze the effects of IVIg, IL-33, IL-4 and IL-13, mDCs or macrophages (1x10^{5}/200µl) were cultured in 96-well round-bottom plates (Greiner Bio-One, The Netherlands) with IVIg (10mg/ml), human rIL-33 (50ng/ml, Enzo Life Sciences, Farmingdale, NY), rIL-4 (10ng/ml, eBioscience, San Diego, CA) or rIL-13 (100ng/ml, Invivogen, San Diego, CA) in RPMI supplemented with penicillin, streptomycin and 10% fetal bovine serum for 24 hours.

To assess the responsiveness of mDCs to IC, IgG was immobilized by overnight incubation of IVIg (1mg/ml) in 200µl PBS in wells of 96-well round bottom plates at 4°C for 24 hours (26), and mDCs either or not pre-treated with IL-4 or IL-13 were cultured in these plates for another 48 hours. To determine their responsiveness to IFN-γ, pre-treated mDCs were re-cultured for 24 hours in the presence of rIFN-γ (250U/ml, Miltenyi Biotec). Maturation of DCs was stimulated by addition of 50ng/ml LPS (derived from *S. Minnesota*; Ultrapure, Invivogen, San Diego, California).

Effect of IVIg on cytokine gene expression *in vitro*

To identify the source of IL-33 or IL-4 production induced by IVIg, freshly isolated human splenocytes or lymph node cells (2x10^{6}/2ml), or M-CSF-induced monocyte-derived macrophages (1x10^{6}/2ml) were cultured with or without IVIg (10mg/ml) in 6-wells plates in RPMI supplemented with penicillin, streptomycin and 10% fetal bovine serum. LPS was used at 1µg/ml to stimulate the cells. Cells were harvested after 24 hours and relative *IL-33* mRNA levels were determined by quantitative RT-PCR. RNA was isolated from the cells using a Machery-Nagel NucleoSpin RNA II kit (Bioké, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). TaqMan gene expression assays were performed to measure the mRNA levels of *IL-33* or *IL-4* as the gene of interest, and *GAPDH* as the housekeeping gene using primer/probe sets from Applied Biosystems (Foster City, Calif., USA). As positive control for IL-33, we used RNA isolated

from tonsils obtained during routine tonsillectomies, which was a kind gift from Dr. T. Cupedo (Hematology Department, Erasmus MC, Rotterdam, The Netherlands). As positive control for IL-4, we used RNA isolated from $CD3^+$ T cells stimulated with PMA and ionomycin. All reactions were performed in duplicate. Relative expression was calculated by the cycling threshol

Statistical analyses

Differences in measured variables in blood between time points before and after IVIg treatment were pairwise analyzed using the Wilcoxon Signed Rank test. Differences in measured variables obtained from cell cultures were pairwise analyzed using the Student's *t*-test, since the differences were normally distributed. Regression analyses were performed by using the Spearman correlation test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). For all tests, P-values <0.05 were considered as significant.

RESULTS

Increased IL-33, IL-4 and IL-13 plasma concentrations after IVIg treatment *in vivo*

LD patients, defined as patients who were treated with IVIg dose ≤0.6 g/kg, received a median dose of 0.38 g/kg (range: 0.25-0.59) and HD patients, defined as patients who were treated with IVIg dose >0.6 g/kg, received a median dose of 0.90 g/kg (range: 0.65-1.71). Plasma IgG levels increased to significantly higher concentrations in HD patients compared to LD patients (data not shown).

Following IVIg treatment, plasma IL-33 significantly increased for at least 7 days in both LD patients (+215% immediately after, +169% at day 7) and HD patients (+152% immediately after, +70% at day 7) (**Figure 1A**). However, the rise of plasma IL-33 in LD patients did not exceed that of the baseline levels of HD patients, and post-treatment levels were significantly higher in the HD patients compared with those in LD patients. The higher baseline IL-33 level in HD patients (3-fold compared to LD patients) may be explained by the active inflammation in these patients. Likewise, plasma IL-4 concentrations significantly increased for at least 7 days after IVIg infusion in both LD (+115% immediately after, +52% at day 7) and HD (+227% immediately after, +136% at day 7) patients (**Figure 1B**), and also reached significantly higher post-treatment concentrations in HD patients than in LD patients. IL-13 plasma levels were significantly elevated in HD patients after IVIg therapy (+574% immediately after, and +524%

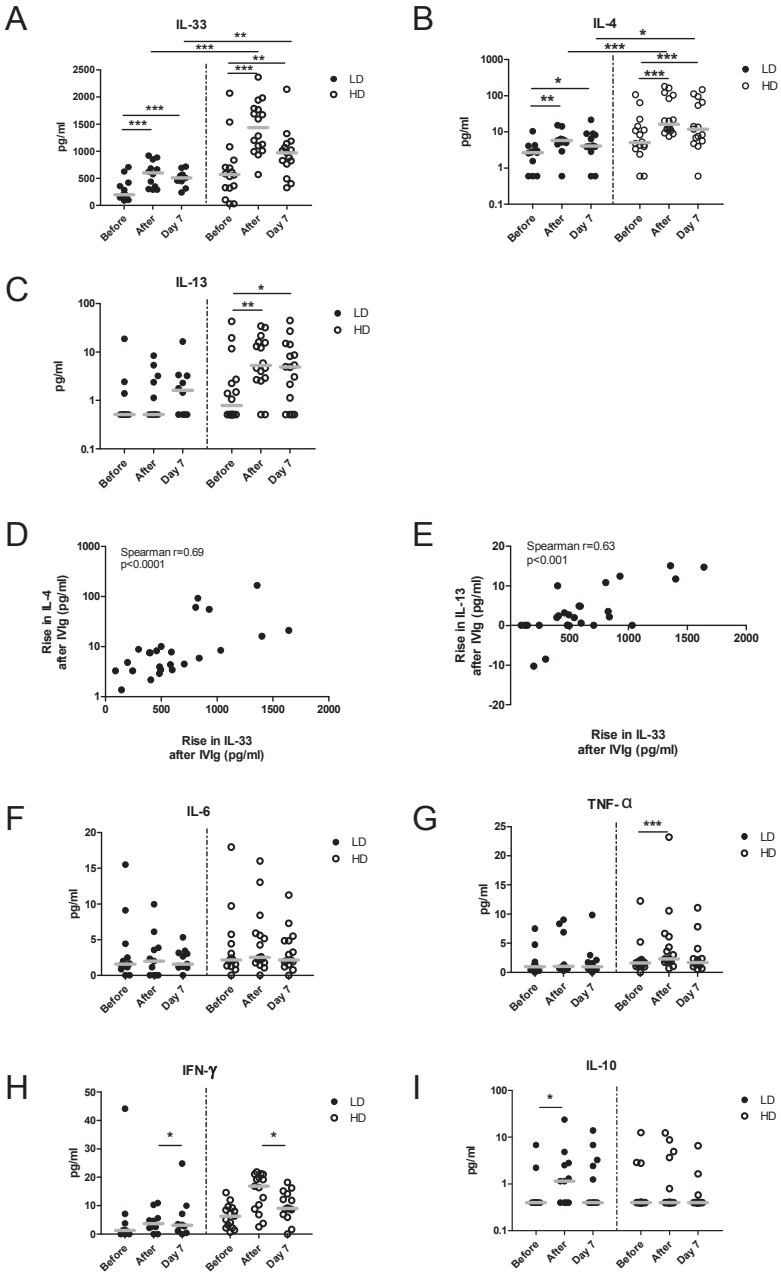

FIGURE 1. IVIg treatment enhances and sustains IL-33, IL-4 and IL-13 levels in plasma.
In plasma samples collected from LD (n=13) and HD (n=16) patients before, after, and at day 7 after IVIg treatment, (A) IL-33 (B) IL-4 and (C) IL-13 were determined by ELISA. Spearman regression analyses were performed to investigate the correlations between the rise in IL-33 plasma levels and the rise in plasma levels of (D) IL-4 and (E) IL-13 immediately after IVIg treatment. Th1 cytokines (F) IL-6, (G) TNF-α, (H) IFN-γ and (I) the anti-inflammatory cytokine IL-10 in plasma samples, similar to those in (A-C). *P <0.05, **P < 0.01, ***P < 0.001. Horizontal lines represent median.

at day 7) but not in LD patients (**Figure 1C**). In contrast, we could not detect IL-5 in plasma in any of the patients (Human IL-5 ELISA, Biolegend; sensitivity 2 pg/ml). Among HD patients, 11 were treated with IVIg for autoimmune disease and 5 for immunodeficiency, and elevations in IL-33 and Th2 cytokine levels were observed for both patient groups (data not shown).

The rise in plasma IL-4 and IL-13 significantly correlated with the rise in IL-33 levels (**Figures 1D-E**), supporting the hypothesis that IL-33 stimulates Th2 cytokine production in humans *in vivo*. To elucidate whether IVIg therapy selectively enhances plasma IL-33 and Th2 cytokine concentrations, we measured plasma levels of Th1 cytokines and of the anti-inflammatory cytokine IL-10. IL-6 plasma level did not increase upon IVIg treatment, while transient increases were observed for TNF-α in HD patients, IFN-γ in both LD and HD patients and IL-10 levels in LD patients, but all these cytokines returned to baseline levels at day 7 after treatment (**Figures 1 F-I**). Together, these data show that IVIg therapy upregulates IL-33, IL-4 and IL-13 plasma levels up to at least one week after IVIg infusion, while not or only transiently enhancing plasma levels of Th1 cytokines and IL-10.

IVIg treatment reduces expression of activating FcγRIIa and IFNγR2 on circulating mDCs *in vivo*

We hypothesized that the sustained increase in Th2 cytokine levels induced by IVIg treatment might modulate the expression of FcγRIIb or FcγRIIa on myeloid cells, as has been previously shown in mice (20, 29) and for human monocytes *in vitro* (30, 31). Therefore, we measured the expression of the inhibitory FcγRIIb and activating FcγRIIa on circulating mDCs and monocytes, defined as $CD20^{-}BDCA1^{+}$ and $CD14^{+}$ leukocytes, respectively. IVIg treatment did not affect FcγRIIb or FcγRIIa expression on circulating monocytes (data not shown). However, clear effects were observed on the expression of these receptors on circulating mDCs in HD patients, but not in LD patients. FcγRIIb expression decreased directly after infusion, but expression was restored at day 7 (**Figures 2A-B**). In contrast, a stepwise decline of the activating FcγRIIa expression was observed, which became statistically significant (-30%) at day 7 after IVIg treatment (**Figures 2A, C**). These decreases were not attributable to interference of IVIg with anti-FcγRIIa or anti-FcγRIIb mAB binding (**Supplemental Figures 1A, B**).

Next, we studied IFNγR expression on mDCs and monocytes. A median of 97% (range: 84-100) of mDCs expressed the ligand-binding IFNγR1 subunit at baseline, which did not change after IVIg treatment (data not shown). In contrast, expression of the signaling IFNγR2 subunit, which was restricted to 4.9% of total mDCs at baseline, stepwise declined after treatment in HD patients (-51% at day 7), but not in LD patients (**Figures 2A, D**). This decrease was not attributable to interference of IVIg with anti-IFNγR2 mAB binding (**Supplemental Figure 1C**). A trend towards reduction of IFNγR2 expression was also observed for monocytes on day 7, but did not reach statistical significance (P=0.11) (data not shown). Similar modulation

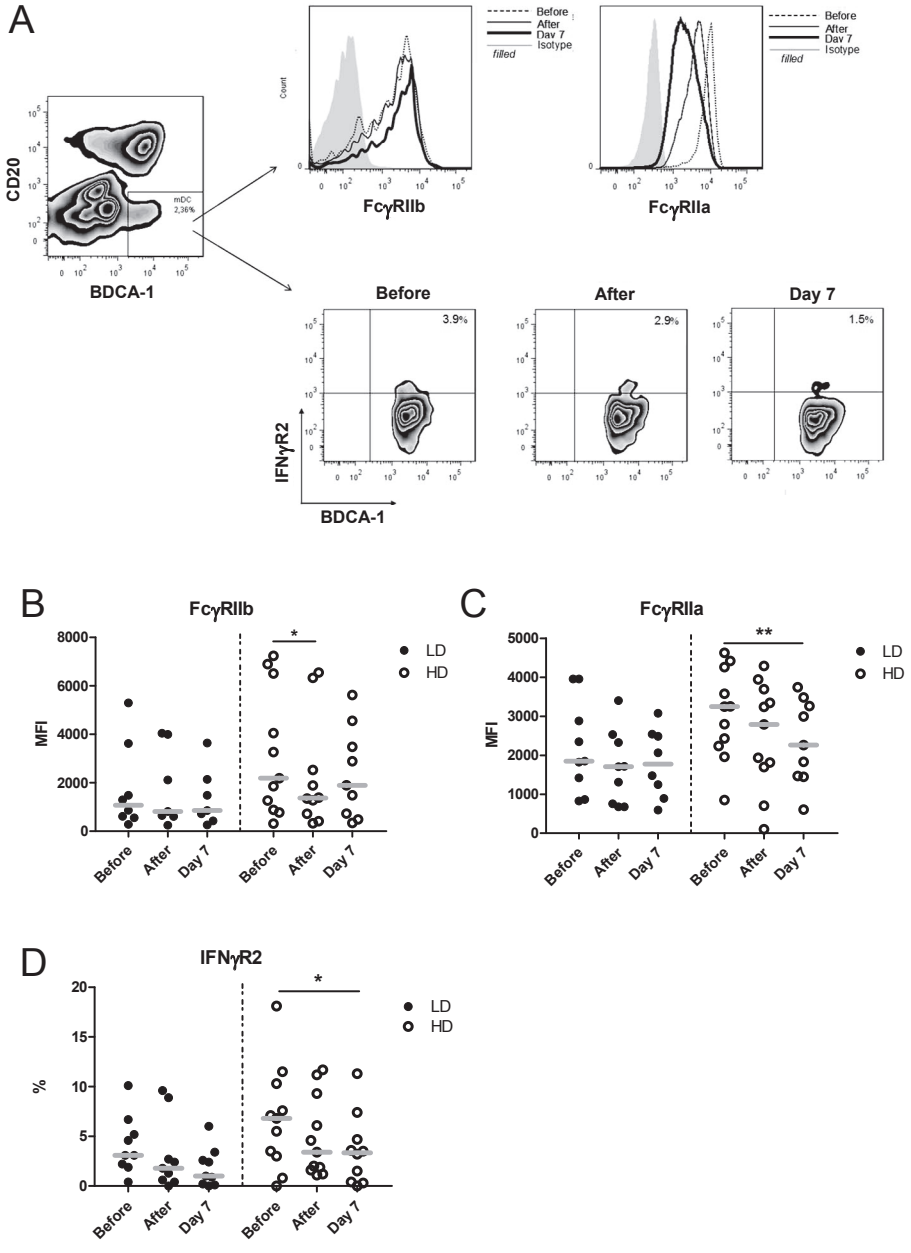

FIGURE 2. High-dose IVIg treatment modulates expression of Fcγ receptor IIb (FcγRIIb), Fcγ receptor IIa (FcγRIIa) and IFN-γ subunit 2 (IFNγR2) on circulating mDCs. mDCs were identified by first gating on 7AAD- cells, followed by BDCA1^{+}CD20^{-} cells.
(A) FcγRIIa and FcγRIIb (histograms) and IFNγR2 (density plots) expression on BDCA1^{+}CD20^{-} mDCs of a representative patient before, after, and at day 7 after IVIg treatment. Mean of Fluorescence Intensities (MFI) of (B) FcγRIIb and (C) FcγRIIa on circulating mDCs and (D) percentages of circulating mDCs expressing IFNγR2 in LD (n=10) and HD (n=11) patients. *P < 0.05, **P < 0.01. Horizontal lines represent medians.

of FcγRIIb, FcγRIIa and IFNγR on mDCs was observed in HD patients that received IVIg for either autoimmune or immunodeficient conditions (**Supplemental Figures 1D-F**). Prednisone treatment did not influence the effect of IVIg on FcγRIIb, FcγRIIa and IFNγR expression on mDCs, since all 6 patients that were co-treated with prednisone (low-dose n=2, high-dose n=4) showed comparable patterns as were observed in patients without prednisone treatment (data not shown).

Together these data show that upon high-dose, but not upon low-dose IVIg treatment, FcγRIIa and IFNγR2 expression on circulating mDCs is sustainably suppressed, while FcγRIIb expression is only temporary reduced.

IL-4 and IL-13 reduce FcγRIIb, FcγRIIa, and IFNγR2 expression on human mDCs *in vitro*

To assess whether the decreased expression of FcγRIIb, FcγRIIa and IFNγR2 on circulating mDCs upon IVIg therapy may have been caused by the observed increase in IL-33, IL-4 or IL-13 levels, or by IVIg itself, we cultured mDCs purified from blood of healthy donors with IVIg, rIL-33, rIL-4 or rIL-13 *in vitro* for 24 hours. Interestingly, FcγRIIb, FcγRIIa and IFNγR2 expression was reduced on mDCs cultured with rIL-4 or rIL-13, while their expression was not affected by rIL-33 or by IVIg (**Figures 3A-C**, **Supplemental Figures 2A-C**). IL-4 and IL-13 showed redundant roles in the modulation of these receptors as their expression did not further decrease in the presence of both cytokines in comparison to either cytokine alone (**Supplemental Figures 3A**). Next, we assessed whether IL-4 and IL-13 could support downregulation of these receptors during LPS-induced mDC maturation. mDCs were cultured for 24 hours in the presence of IL-4 or IL-13, followed by LPS stimulation for an additional 24 hours. LPS down-regulated the expression of FcγRIIb, FcγRIIa and IFNγR2 expression on mDCs, and we found that IL-4, IL-13 and LPS additively downregulate FcγRIIb, FcγRIIa and IFNγR2 expression (**Supplemental Figures 3B**). Collectively, these data suggest that the enhanced levels of IL-4 and IL-13 after IVIg infusion *in vivo* are primarily responsible for the modulation of FcγIIb, FcγIIa and IFNγR2 expression on circulating mDCs.

Since the beneficial effects of IVIg in mice have been attributed to Th2 cytokine-mediated upregulation of FcγRIIb expression on macrophages (20), we also cultured human monocyte-derived macrophages with rIL-4 or rIL-13 for 24 hours. However, Th2 cytokines did not significantly affect FcγRIIa or FcγRIIb expression on human macrophages (**Supplemental Figures 4A, B**).

IL-4 and IL-13 reduce responsiveness of human mDCs to IC and IFN-γ *in vitro*

The balance between the expression of FcγRIIa and FcγRIIb establishes a threshold for mDC activation by IC (26). In order to determine the functional effects of the downregulation of both activatory FcγRIIa and inhibitory FcγRIIb by IL-4 or IL-13, we cultured purified mDCs with rIL-4 or rIL-13 for 24 hours, and subsequently re-cultured them in the presence of immobilized IgG for 48 hours. Both rIL-4 and rIL-13 pre-treatment suppressed IC-mediated maturation, as assessed by CD83 upregulation (**Figure 4A**) and repressed pro-inflammatory IL-8 (**Figure 4B**) and TNF-α (**Figure 4C**) production after IC challenge. To determine the role of FcγRIIa and FcγRIIb in this effect, we blocked FcγRIIa or FcγRIIb ligation. Blockade of FcγRIIa prevented activation of mDCs by IC. Interestingly, blocking of FcγRIIb during IC stimulation of IL-4- or IL-13- pre-treated mDCs resulted in a greater rise in CD83 expression (**Figure 4A**) and cytokine production (**Figures 4B, C**) compared to FcγRIIb blockade of control mDCs, indicating that FcγRIIb is relatively dominant over FcγRIIa in regulating the response of IL-4- or IL-13-treated mDCs to IC. Thus, downregulation of FcγRIIa by IL-4 or IL-13 reduces the responsiveness of mDCs to IC, despite simultaneous suppression of inhibitory FcγRIIb expression.

To establish the functional effect of IFNγR2 downregulation by IL-4 and IL-13, we cultured purified mDCs with rIL-4 or rIL-13 for 24 hours, followed by IFN-γ stimulation for an additional 24

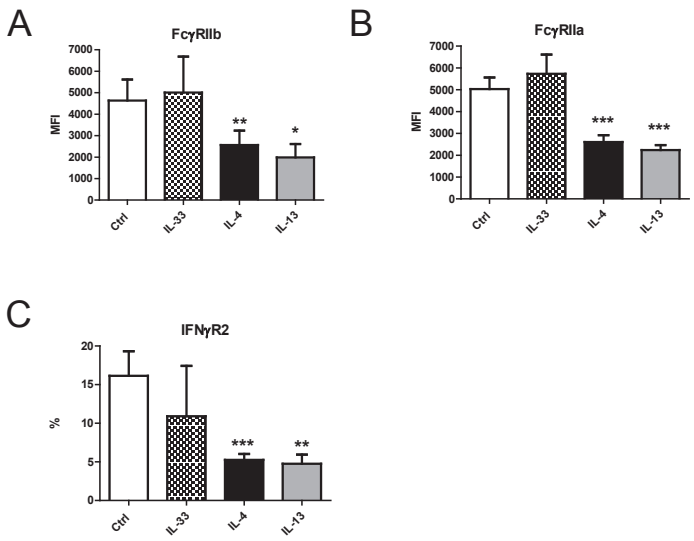

FIGURE 3. IL-4 and IL-13 treatment reduces FcγRIIb, FcγRIIa and IFNγR2 expression on mDCs in vitro. Freshly-isolated BDCA1$^+$CD20$^-$ mDCs (1x10^5/200µl) from blood of healthy volunteers were cultured in 96-wells round bottom plates with human rIL-33 (50ng/ml), rIL-4 (10ng/ml) or rIL-13 (100ng/ml) for 24 hours. The expression of (A) FcγRIIb, (B) FcγRIIa and (C) IFNγR2 on mDCs was measured by flow cytometry as indicated in Fig. 2A. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to CTRL. Bars represent mean±SEM of 8 independent experiments.

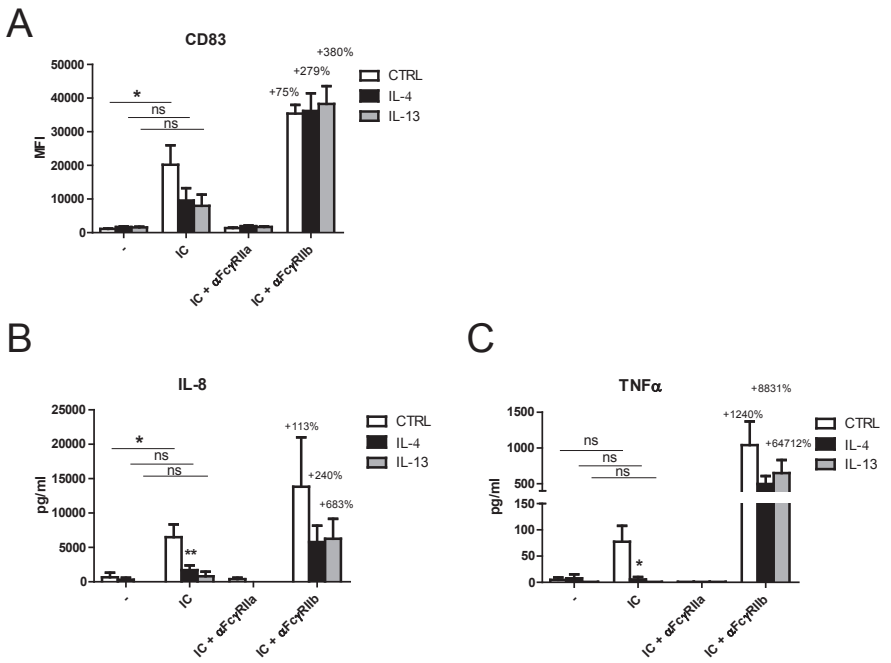

FIGURE 4. IL-4- and IL-13-treated human mDCs are less responsive to immune complexes in vitro. Purified BDCA1^{+}CD20^{-} mDCs (1x10^{5}/200µl) from blood of healthy volunteers were cultured with medium or rIL-4 (10ng/ml) or rIL-13 (100ng/ml) for 24 hours, extensively washed and re-cultured in 96-wells round bottom plates in the presence of immobilized human IgG for another 48 hours. After culture (A) the expression of maturation marker CD83 on mDCs was determined by flow cytometry, and secretion of (B) IL-8 and (C) TNF-α in the supernatants was assessed by ELISA. Percentages depict upregulation of described marker when comparing conditions treated with anti-FcγRIIb + IC to IC alone. *P < 0.05, **P < 0.01 as compared to CTRL + IC, ns = non-significant. Bars represent mean±SEM of 4 independent experiments.

hours. In contrast to mDCs pre-incubated in medium, mDCs pre-treated with rIL-4 and rIL-13 could not upregulate CD86 (**Figure 5A**), CCR7 (**Figure 5B**) and CD38 (**Figure 5C**) expression after IFN-γ stimulation. Moreover, the production of interferon-induced protein-10 (IP-10)/CXCL10, an important mediator of chemotaxis, apoptosis, cell growth and angiostasis (32) was significantly reduced in mDCs in response to IFN-γ after treatment with rIL-4 or rIL-13 (**Figure 5D**). Of note, IFN-γ stimulation did not affect the expression of FcγRIIa, FcγRIIb and IFNγR2 on mDCs, suggesting that the transient increase of IFN-γ in plasma directly after IVIg administration in our patient cohort was not the cause for the observed modulation of these receptors on circulating mDCs (data not shown).

Collectively, our data indicate that IL-4 and IL-13 treatment suppresses IC- and IFN-γ-mediated mDC maturation and cytokine/chemokine production by suppression of FcγRIIa and IFNγR2 expression, respectively.

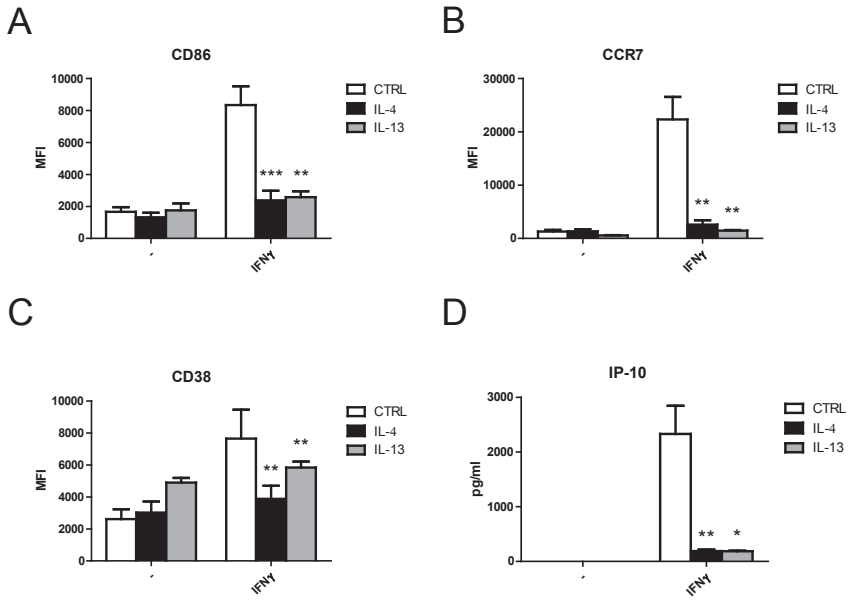

FIGURE 5. IL-4- and IL-13-treated human mDCs are less responsive to IFN-γ stimulation in vitro. Purified BDCA1$^+$CD20$^-$ mDCs (1x10^5/200µl) from blood of healthy volunteers were treated with medium or rIL-4 (10ng/ml) for 24 hours and re-cultured with or without human rIFN-γ (250U/ml) for another 24 hours. The expression of maturation markers (A) CD86, (B) CCR7 and (C) CD38 on mDCs was determined by flow cytometry, and secretion of (D) IP-10 in the supernatant was assessed by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001, as compared to CTRL + IFN-γ. Bars represent mean±SEM of 8 independent experiments.

Human lymph node is a potential source for IL-33 production

We made a first attempt to identify the source of the enhanced IL-33 plasma levels upon IVIg infusion in humans. We hypothesized that IVIg may induce IL-33 production in secondary lymphoid tissues, since IL-33 is expressed in human secondary lymphoid tissues (33), and up-regulation of this cytokine has been shown in murine spleen upon IVIg treatment (20). Therefore, we cultured freshly isolated human splenocytes or lymph node cells *in vitro* with or without IVIg. Since IL-33 expression can be induced by TLR4 ligation (34-36), cells were cultured in the absence or presence of LPS. We detected no *IL-33* mRNA in splenocytes, but in lymph node cells *IL-33* mRNA was detectable and markedly increased after incubation with IVIg in the presence of LPS (**Figure 6A**). However, IL-33 protein could not be detected in supernatants from the cell cultures by ELISA (Biolegend, sensitivity 4 pg/ml), indicating no or very low IL-33 secretion by the cells *in vitro.* No *IL-4* mRNA was found in splenocytes or lymph node cells upon IVIg treatment (data not shown).

Stimulation of IL-33 production in human macrophages by IVIg is DC-SIGN independent

We hypothesized that a potential source of IL-33 production in lymph nodes could be macrophages, as it was shown that macrophages in human placenta are IL-33 producers (37). Since isolation of macrophages from lymph nodes was practically unattainable, we differentiated human macrophages from monocytes in the presence of M-CSF *in vitro,* and assessed whether these cells produced *IL-33* mRNA. Similar to the observations on human lymph node cells, we found that human macrophages produced high levels of *IL-33* mRNA in the presence of LPS and IVIg, but hardly any production was observed with LPS or IVIg alone (**Figure 6B**). As these macrophages express DC-SIGN (data not shown), a previously-identified IVIg-binding target (20), we wondered whether the IVIg-mediated IL-33 production was dependent on DC-SIGN. Using an approved DC-SIGN blocking antibody (AZN-D1) (38), we surprisingly found that *IL-33* mRNA levels in human macrophages in the presence of IVIg and LPS were not affected when DC-SIGN was blocked (**Figure 6B**). Collectively, these data identify human macrophages as a potential source of IVIg-induced IL-33 production that is independent of DC-SIGN.

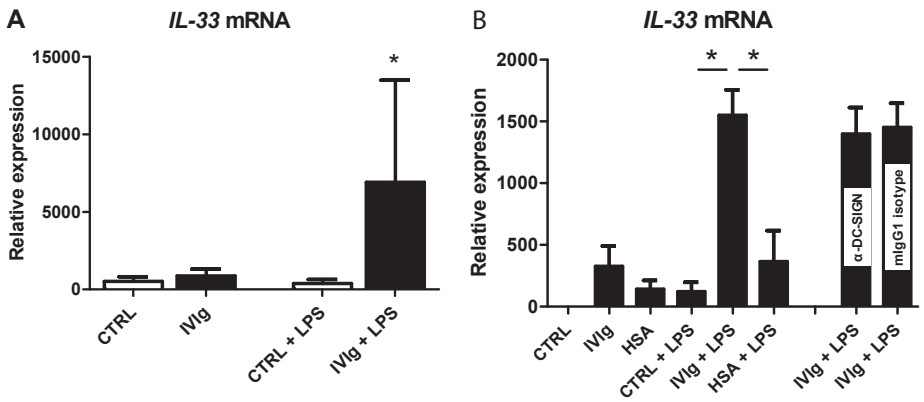

FIGURE. 6. IVIg treatment induces IL-33 mRNA in human lymph node cells and macrophages. (A) Mononuclear cells isolated from fresh human hepatic lymph node cells ($2x10^6$/2ml) were cultured with medium or IVIg (10mg/ml) with or without LPS (1µg/ml) for 24 hours. RNA was isolated from the cells and mRNA levels of IL-33 were assessed by quantitative RT-PCR. Relative expression was compared to GAPDH housekeeping gene. *P < 0.05 as compared to CTRL + LPS. Bars represent mean±SEM of 6 independent experiments. (B) M-CSF-induced monocyte-derived macrophages ($1x10^6$/2ml) were cultured with medium, IVIg (10mg/ml), or HSA (4.5mg/ml) for 1 hour, after which LPS (100ng/ml) was added for 24 hours. Blocking of DC-SIGN was performed by pre-incubating macrophages for 30' at 4°C with 10µg/ml anti-DC-SIGN antibody (AZN-D1). An irrelevant mouse IgG1 antibody was used an isotype control. Assessment of IL-33 mRNA levels was performed as in (A). *P < 0.05; Bars represent mean±SEM of 4 independent experiments.

DISCUSSION

Although high-dose IVIg is increasingly being used to treat various autoimmune and inflammatory diseases, its mode of action is not fully understood. Recent mice studies have shown an essential role for a pathway involving IVIg-induced IL-33-IL-4 production that ultimately leads to upregulation of FcγRIIb expression on effector macrophages (20). However, whether this pathway is operational in humans is unknown, and is also questionable due to several fundamental physiological differences between mice and men. In the current study, we demonstrate that IVIg treatment in human results in a robust and selective increase of plasma levels of IL-33 and the Th2 cytokines IL-4 and IL-13, that lasts for at least one week. Although a causative relationship between IL-33 and Th2 cytokine production in humans *in vivo* is difficult to establish, the observed associations between the rise of IL-33 and the increases in Th2 cytokines plasma levels support the hypothesis that IVIg can stimulate the IL-33-Th2 axis in humans, as has been previously observed in mice (20).

In contrast to mouse studies showing a stimulatory effect of IVIg treatment on inhibitory FcγRIIb expression on myeloid cells (4, 19, 20), we observed a rapid but transient decrease of FcγRIIb expression on circulating mDCs upon high-dose IVIg treatment in humans. Several studies have shown that FcγRIIb expression on circulating monocytes was not affected by IVIg treatment in humans (39-41), which we were able to confirm. In one human study an increase of FcγRIIb expression was observed on monocytes and B cells after IVIg treatment. It has to be noted that in this study FcγRIIb expression levels prior to IVIg treatment were in most cases diminished in comparison to healthy individuals, suggesting that IVIg treatment may enhance expression levels of FcγRIIb only when they were initially decreased (42).

However, high-dose IVIg therapy reduced expression of the activatory FcγRIIa and of IFNγR2 on mDCs till at least 1 week after treatment. The discrepancies between the effect of IVIg on FcγRII expression between humans and mice may be related to the presence of both FcγRIIa and FcγRIIb in humans while mice have only FcγRIIb (43, 44). Thus, due to major structural differences in FcγR expression between mice and humans, the mechanism of FcγR modulation found in mice may not be fully recapitulated in humans. Receptor down-regulation was only observed in patients treated with high-dose IVIg, which corresponds with the insufficiency of low-dose IVIg therapy for effective anti-inflammatory treatment. The plasma IL-4 levels reached in LD patients did not exceed the baseline levels of HD patients, and may therefore have been insufficient to down-regulate receptor expression on mDCs.

In vitro experiments revealed that both IL-4 and IL-13, but not IL-33 or IVIg itself, can reduce the expression of FcγRIIb, FcγRIIa, and IFNγR2 on human mDCs. To our knowledge, we are the first to show an effect of Th2 cytokines on FcγR and IFNγR expression on human mDCs. Direct suppression of IFNγR2 expression on macrophages as was observed in a previous study (16), was probably caused by adsorbance of IgG to the culture plastic (25) which was prevented in our experiment by pre-coating of the culture wells with FCS. Our results

reveal that IVIg represses IFNγR2 on mDCs indirectly via stimulation of Th2 cytokine production. Down-regulation of IFNγR2 expression on mDCs prevented upregulation of CD86, CCR7 and CD38 as well as production of IP-10 upon IFN-γ stimulation, implicating reduced T cell co-stimulation, chemotaxis and migration.

Although *in vitro* expression of both inhibitory FcγRIIb and activatory FcγRIIa were downregulated, IC-stimulated maturation and pro-inflammatory cytokine production of mDCs were strongly suppressed upon IL-4 or IL-13 treatment. These results, together with the experiments in which we blocked FcγRIIb, suggest that inhibitory FcγRIIb signaling becomes dominant over activatory FcγRIIa signaling when expression of both FcγRII isoforms is reduced. It would have been highly interesting to study the functional effects of modulated receptor expression on mDCs isolated from IVIg-treated patients *ex vivo*. However, this was not feasible, since numbers of circulating mDCs were very low (median mDC%: 0.67% of PMBC in our patients) and not sufficient PBMC from the patients were available to isolate the amounts of mDC required for functional experiments. Since our study cohort is rather heterogeneous, clinical outcomes cannot be standardized for all patients and a correlation study associating outcomes with cytokine or receptor levels is not reliable. However, in all patients included in the high-dose group, clinical improvement was reported, while increases of IL-33, IL-4 and IL-13 (except for one patient) were consistently observed in these patients. Although we cannot perform direct correlation testing, contribution of the increased IL-33 and Th2 cytokines to the clinical improvement of patients is not unlikely.

Previous studies have reported that human basophils stimulated with IL-33 are able to produce IL-4 and IL-13 *in vitro* (45-47), and we have found similar results (data not shown). Moreover, basophils in mice can also produce IL-4 and IL-13 when stimulated with IL-33 (20). T cells (48), mast cells (49) and macrophages (50) are a less likely sources, since IL-33 induces production of IL-5 and IL-13, but not IL-4, in these cell types.

Although it has been shown in mice that IgGs having 2,6-sialylated Fc induce IL-33 production by marginal zone macrophages in the spleen, recent studies show that the anti-inflammatory effects mediated by these IgG molecules in mouse ITP models were independent of IL-33 and the presence of the spleen (23). Furthermore, the spleen has been found dispensable for the anti-inflammatory effects of IVIg in humans, while a human counterpart of the murine marginal zone macrophage has not been identified (21, 22). Nevertheless, we found that IVIg infusion in humans enhanced IL-33 levels in plasma. In a first attempt to identify the source of IL-33 production, we found that IVIg induces IL-33 gene expression in human lymph node cells, but not in human splenocytes *in vitro*, although additional TLR4 ligation by LPS was needed. We hypothesized that macrophages in lymph nodes may potentially be able to produce IL-33 in response to IVIg since recent data on human placenta revealed that placental and decidual macrophages are able to produce IL-33 (37). Since it was not feasible to isolate sufficient numbers of macrophages from lymph nodes, we differentiated human monocytes towards macrophages *in vitro*, and found that these cells produced high levels of *IL-33*

mRNA upon culture with IVIg and LPS. Hence, we suggest that macrophages are potential sources of IVIg-induced IL-33 in humans, although fibroblasts (51) and epithelial cells (52, 53) may be other sources of IL-33 production. Surprisingly, IL-33 production in our macrophage experiments was not dependent on DC-SIGN. In concert with recent findings by others (54-56) these data ask for a re-evaluation of the role for DC-SIGN and 2,6-sialylated Fc in the anti-inflammatory effects of IVIg in humans. Moreover, further research is required to establish the role for and source of IL-33 production upon IVIg treatment in humans.

In the present study, we show that the recently identified IL-33-Th2 pathway by which IVIg inhibits myeloid cells functions in mice may also, with some variations, be operational in humans. Instead of upregulating FcγRIIb, as has been observed in mice, IVIg downregulates FcγRIIa and IFNγR2 on circulating mDCs in humans, and stimulates IL-33 production by human macrophages probably via a DC-SIGN-independent mechanism. The current study, together with our previous report showing that high-dose IVIg therapy activates Tregs in humans (14), provide evidence that important anti-inflammatory mechanisms of action of IVIg identified in animal models are operational in humans. These modes of action observed in patients may at least partially explain the beneficial effects of high-dose IVIg treatment in various autoimmune diseases

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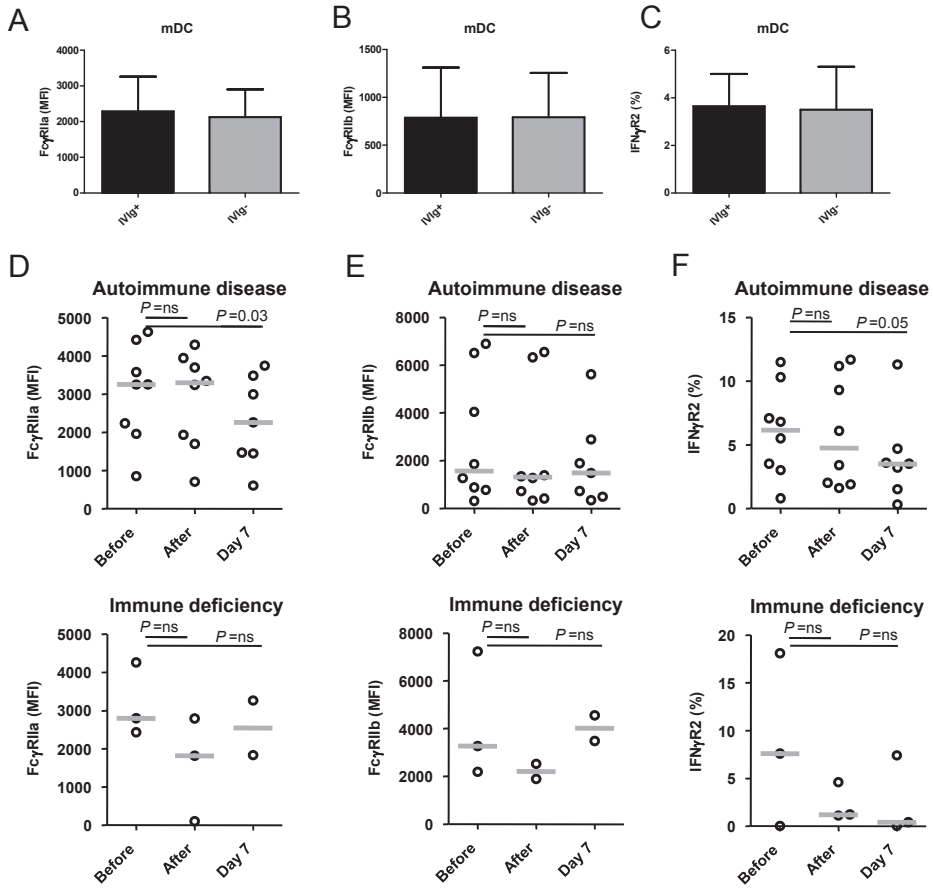
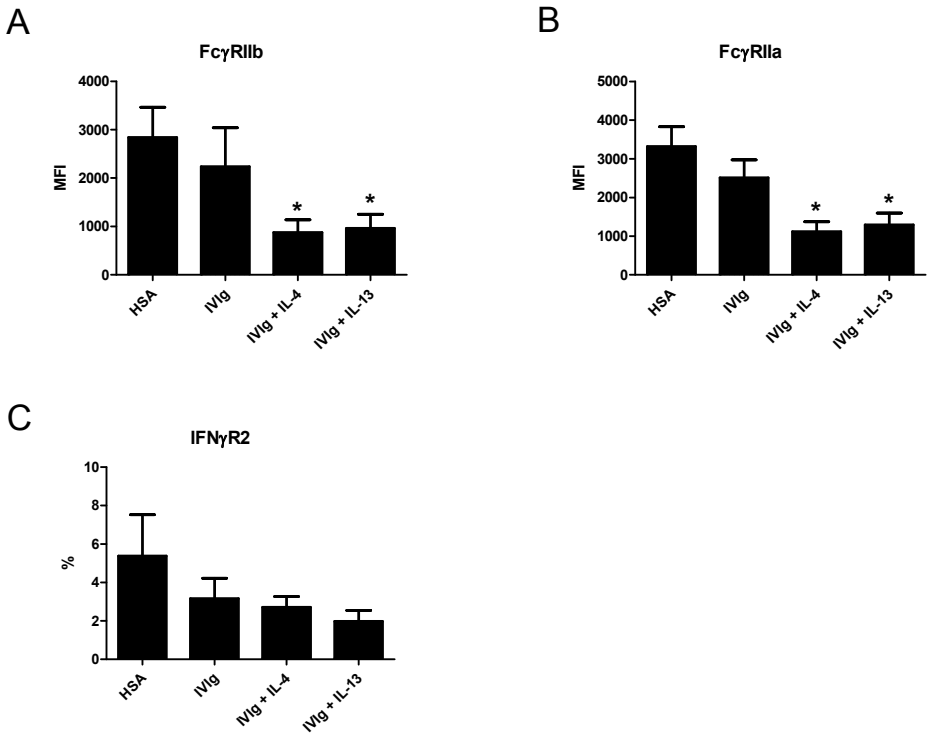
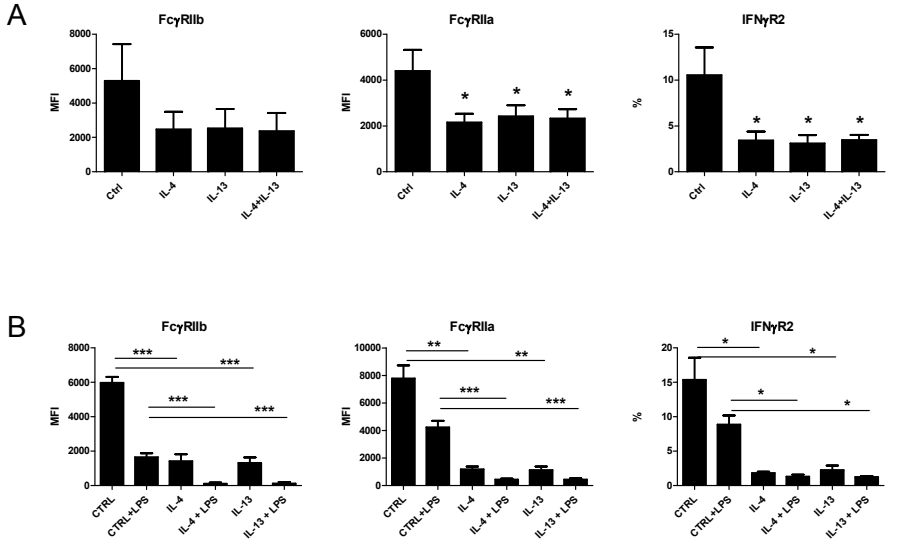


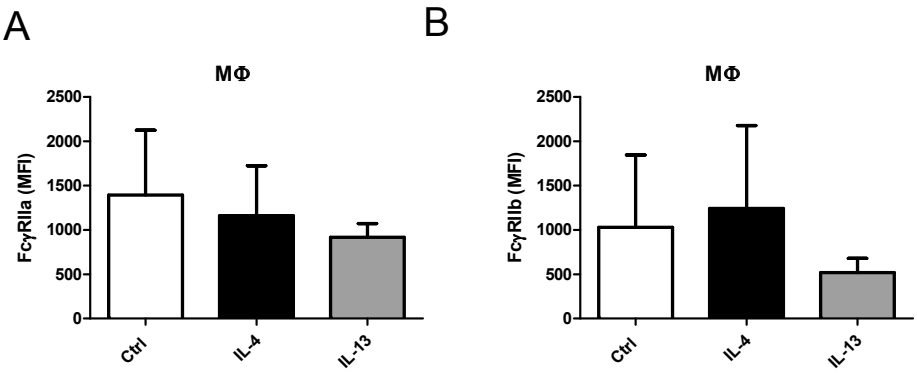
Supplementary Figure 1.
Freshly-isolated BDCA1^{+}CD20^{-} mDCs (1x10^{5}) from blood of healthy volunteers were incubated without or with 10mg/ml IVIg for 30' at 4°C. The expression of (A) FcγRIIa, (B) FcγRIIb and (C) IFNγR2 on mDCs was measured by flow cytometry as indicated in Fig. 2A. Bars represent mean±SEM of 3 independent experiments. mDCs from HD IVIg-treated autoimmune disease (n=11) and immune deficiency (n=5) patients were analyzed for surface expression of (D) FcγRIIa, (E) FcγRIIb and (F) IFNγR2 by flow cytometry as in (A-C). Horizontal lines represent medians. ns = not significant



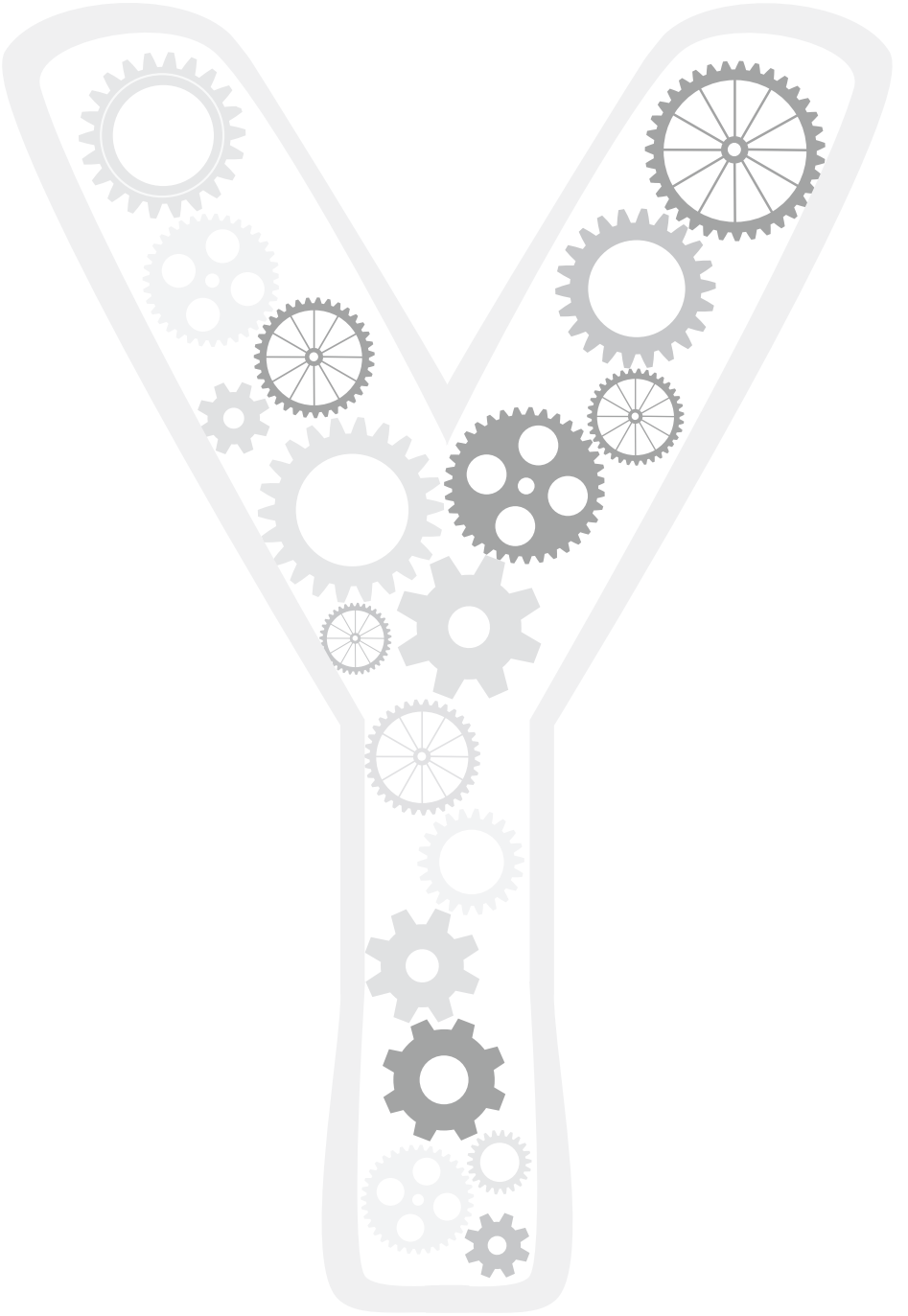
Supplementary Figure 2.
Freshly-isolated BDCA1^{+}CD20^{-} mDCs (1x10^{5}/200µl) from blood of healthy volunteers were cultured in FCS-precoated 96-wells round-bottom plates with human serum albumin (HSA) as negative control, IVIg or IVIg with human rIL-4 (10ng/ml) or rIL-13 (100ng/ml) for 24 hours. The expression of (A) FcγRIIb, (B) FcγRIIa and (C) IFNγR2 on mDCs was measured by flow cytometry as indicated in Fig. 2A. *P < 0.05 as compared to HSA. Bars represent mean±SEM of 8 independent experiments.



Supplementary Figure 3.
The expression of FcγRIIb, FcγRIIa and IFNγR2 on freshly-isolated BDCA1^{+}CD20^{-} mDCs from blood of healthy volunteers mDCs were measured by flow cytometry after indicated incubation time.
(A) mDCs (1x10^{5}/200µl) were cultured in 96-wells round bottom plates with human rIL-4 (10ng/ml) and rIL-13 (100ng/ml), either cytokine alone or added both, for 24 hours. Bars represent mean±SEM of 6 independent experiments.
(B) mDCs (1x10^{5}/200µl) were cultured in 96-wells round-bottom plates with human rIL-4 (10ng/ml) or rIL-13 (100ng/ml). After 24 hours, cells were cultured for another 24 hours with or without LPS (1µg/ml). Bars represent mean±SEM of 5 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplementary Figure 4.
Monocyte-derived macrophages (MΦ) were cultured in 96-wells round-bottom plates (1x10^{5}/200µl) with medium, human rIL-4 (10ng/ml) or rIL-13 (100ng/ml) for 24 hours. The expression of (A) FcγRIIa and (B) FcγRIIb on MΦ was measured by flow cytometry. Bars represent mean±SEM of 6 independent experiments.



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Patients treated with high-dose intravenous immunoglobulin show selective activation of regulatory T cells

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ABSTRACT

Intravenous immunoglobulin (IVIg) is used to treat autoimmune and systemic inflammatory diseases caused by derailment of humoral and cellular immunity. Here we investigated whether IVIg treatment can modulate regulatory T cells (Tregs) in humans *in vivo*. Blood was collected from IVIg-treated patients with immunodeficiency or autoimmune disease who were treated with low-dose (n=12) or high-dose (n=15) IVIg before, immediately after, and at 7 days after treatment. Percentages and activation status of circulating $CD4^+CD25^+FOXP3^+$ Tregs and of conventional $CD4^+FOXP3^-$ T-helper cells (Tconv) were measured. The suppressive capacity of Tregs purified from blood collected at the indicated time points was determined in an *ex vivo* assay. High-dose, but not low-dose, IVIg treatment enhanced the activation status of circulating Tregs, as shown by increased FOXP3 and HLA-DR expression, while numbers of circulating Tregs remained unchanged. The enhanced activation was sustained for at least 7 days after infusion, and the suppressive capacity of purified Tregs was increased from 41% to 70% at day 7 after IVIg treatment. The activation status of Tconv was not affected by IVIg. We conclude that high-dose IVIg treatment selectively activates Tregs and enhances their suppressive function in humans *in vivo*. This effect may be one of the mechanisms by which IVIg restores imbalanced immune homeostasis in patients with autoimmune and systemic inflammatory disorders.

INTRODUCTION

Intravenous immunoglobulin (IVIg) was initially introduced as a replacement therapy for patients with immune deficiencies, but high-dose IVIg is now widely used for the treatment of autoimmune and systemic inflammatory diseases caused by auto-antibodies and/or derailment of the cellular immune system (1, 2). Moreover, IVIg has shown efficacy in prevention and treatment of organ allograft rejection and Graft-versus-Host Disease (GvHD) (3-6). Several possible mechanisms of action that explain the beneficial effects of high-dose IVIg in autoantibody- and immune complex-mediated diseases have been unraveled during the last decades. These mechanisms include inhibition of the binding of immune complexes or cell-bound IgG to activating Fcγ receptors, saturation of the neonatal FcR resulting in an enhanced clearance of auto-antibodies, and up-regulation of the inhibitory FcγRIIb (1, 2, 7). However, the mechanisms by which high-dose IVIg is effective in diseases which are mainly caused by cell-mediated immune responses, such as Kawasaki disease, dermatomyositis, GvHD, and acute transplant rejection, have not yet been fully elucidated. Recent *in vitro* and mice studies suggest that one of the mechanisms by which IVIg suppresses cellular immunity is by activating $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) (8, 9).

Tregs are critical regulators of cell-mediated immune responses and suppress pathogenic immune responses in autoimmune diseases, transplantation, and GvHD (10). Current immunosuppressive drugs used to treat or to prevent these diseases exert generalized inhibition of the immune system, thereby disabling protective immunity against pathogens and malignancies. Therapeutic modalities that stimulate Treg-mediated immune regulation without affecting global immune functions are attractive. Recently, we found that IVIg can activate both human and mouse $CD4^+CD25^+FOXP3^+$ Tregs *in vitro* and increase their ability to suppress allogeneic T cell proliferation (11). By triggering functional activation of Tregs, IVIg prevented graft rejection in a fully mismatched skin transplant model. In line with our data, it has been shown that IVIg can prevent mice from developing experimental autoimmune encephalomyelitis (12) and herpes simplex virus-induced encephalitis (13) by enhancing the suppressive capacity and stimulating the expansion of Tregs. In addition, IVIg was found to enhance the suppressive capacity of human Tregs *in vitro* (14).

These data suggest that IVIg treatment can stimulate Treg expansion and function, and may therefore be an attractive tool to re-establish Treg-mediated immune regulation. However, the data on the effects of IVIg on Tregs are restricted to *in vitro* and mice experiments and it is unknown whether IVIg treatment actually affects Treg expansion and function in patients. Therefore, in the present study, we systematically analyzed the effects of either low- or high-dose IVIg treatment on the percentages, activation status and suppressive capacity of circulating Tregs in autoimmune and immunodeficient patients. We observed increased activation of Tregs after high-dose IVIg treatment, which was not observed for conventional

T cells (Tconv). Additionally, Tregs isolated after high-dose IVIg treatment showed enhanced suppressive capacity *ex vivo*.

MATERIAL AND METHODS

Patients

Twenty seven patients (21 female/6 male) who were treated with IVIg were included in this study. To assess whether IVIg had a dose-dependent effect on Treg activation, we subdivided the patients into two groups: those who received "low-dose" IVIg and those who received "high-dose" IVIg. Since patients with immunodeficiency started initially with a supplementary dose of 0.4-0.6 g/kg, we defined "low-dose" IVIg as a dose ≤0.6 g/kg, and "high-dose" IVIg as a dose >0.6 g/kg. The median dose of IVIg received by low-dose patients was 0.39 g/kg (range: 0.25-0.59) and that of high-dose patients was 0.98 g/kg (range: 0.65-1.71). High-dose IVIg-treated patients showed a significantly higher rise in plasma IgG levels ($^{+}$17.3 mg/ml) compared to low-dose IVIg-treated patients ($^{+}$8.2 mg/ml) immediately after treatment ($p<0.001$), as quantified by immunoturbometric analysis (Roche Diagnostics GmbH, Mannheim, Germany). At day 7, plasma IgG levels dropped again in both groups (data not shown). The indications for IVIg treatment in these patients are depicted in **Table 1A-B**. In the high-dose treatment group (**Table 1B**), five patients received IVIg for licensed indications, including common variable immunodeficiency (n=2), hypogammablobulinemia (n=1) and idiopathic thrombocytopenic purpura (n=2). One patient with Good's syndrome received high-dose IVIg as supplementary therapy for hypogammaglobulinemia. Other patients included in **Table 1B** received high-dose IVIg as anti-inflammatory therapy "off-label", since they did not respond to conventional immunosuppressive treatment. The IVIg preparations used for treatment were Nanogam (n= 13; Sanquin, Amsterdam, The Netherlands), Kiovig (n= 9; Baxter, Deerfield, IL), Flebogamma (n= 4, Grifols, Barcolona, Spain) and Octagam (n= 1; Octapharma, Lachen, Switzerland). With the exception of 3 patients, all patients had previously been treated with IVIg, with an average 28 days (range: 21-35 days) before this study. Twenty-one patients were on IVIg monotherapy and six patients received additional corticosteroid treatment.

Ethics statement

The medical ethics committee of the Erasmus University Medical Centre approved the study, and written informed consent was obtained from all patients participating in the study.

TABLE 1A. Patient characteristics in low-dose IVIg-treated patients

IVIg treatment indication	Number of patients	Age in years, median (range)	IVIg dose in g/kg, median (range)
Common variable immunodeficiency	5	41 (20-57)	0.38 (0.30-0.48)
Hypogammaglobulinemia	6	60 (45-77)	0.39 (0.25-0.59)
Agammaglobulinemia	1	30	0.25

TABLE 1B. Patient characteristics in high-dose IVIg-treated patients

IVIg treatment indication	Number of patients	Age in years, median (range)	IVIg dose in g/kg, median (range)
Polymyositis*	3	41 (33-56)	1.00 (0.67-1.67)
Common variable immunodeficiency	2	40 (37-43)	0.68 (0.65-0.70)
Immune trombocytopenic purpera	2	68 (65-71)	1.00
Acquired von Willebrand syndrome*	2	52 (40-63)	0.95 (0.93-0.98)
Hypogammaglobulinemia	1	40	0.66
Good's syndrome/ Hypogammaglobulinemia	1	69	0.86
Polyserositis e.c.i.	1	24	0.78
Polychondritis*	1	68	0.69
Dermatomyositis*	1	46	1.71
Systemic vasculitis e.c.i.*	1	64	1.4

* IVIg treatment was indicated based on unresponsiveness to conventional treatment

Sample collection and preparation

Heparin-decoagulated blood samples were collected from healthy blood bank donors, and from patients immediately before IVIg infusion, immediately after IVIg infusion (4 to 24 hours after the start of the infusion) and 7 days after infusion. Due to practical issues we were unable to obtain blood samples from 1 patient immediately after IVIg treatment and from 2 patients 7 days after treatment. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient sedimentation using Ficoll-Paque (GE healthcare, Uppsala, Sweden). Plasma was collected from the gradient and centrifuged at 3000 rpm for 10 min at 4°C to remove thrombocytes and debris. For storage of PBMC, the cells were cryopreserved in RPMI 1640 (Gibco BRL Life technologies, Breda, The Netherlands) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MA). Until further analysis, the PBMC samples were stored at -135 degrees and plasma at -80 degrees. To minimize possible effects of inter-assay variation, measurements in plasma and on PBMC obtained at different time points from the same patient were performed on the same day.

Antibodies and flow cytometry

For identificiation of T cell subsets, PBMCs were stained with anti-CD3-AmCyan, anti-CD4-APC-H7 and CD8-Pacific blue (all from BD Biosciences, San Jose, CA). Percentages and activation status of Tregs were determined by surface labeling with anti-CD4-APC-H7, anti-CD25-PE-Cy7 (both from BD Biosciences), anti-CD127-PE (Beckman Coulter, Immunotech, Marseille, France), anti-CD38-Pacific Blue (ExBio antibodies, Czech Republic), anti-HLA-DR-PerCP (eBioscience, San Diego, CA) mAb and proper isotype controls. For the detection of memory ($CD45RO^+$) Tregs, we additionally labelled the cell surface using CD45RO-FITC mAb (Beckman Coulter). Cells ($1x10^6$) were incubated with the mAb in 50µl PBS (Lonza, Verviers, Belgium) for 30 min on ice and protected from light. Subsequently, intracellular FOXP3 staining was performed using anti-FOXP3-APC mAb (or rat IgG2a isotype control mAb) purchased from eBioscience according to manufacturer's instructions. To study proliferation, intracellular labeling with anti-Ki-67-FITC (or mIgG1 isotype control mAb) from BD Biosciences was performed. After staining, the cells were washed and resuspended in 100µl PBS for measurement by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA). A minimum of $1.5x10^5$ MNC were acquired. Analyses were performed by FACS Diva software (BD Bioscience). Viable MNC were gated based on forward/side scatter. For the calculation of percentages of cells expressing a certain marker, or for calculation of the geometric mean of fluorescence intensities (MFI), fluorescence of isotype-matched control mAb was subtracted from fluorescence of specific mAb.

Suppression assay

To determine the effect of IVIg treatment on the suppressive capacity of Tregs *ex vivo*, we collected additional blood from 7 patients in the study who received high-dose IVIg. To purify Tregs and T responder cells (Tresp), we first purified non-touched $CD3^+$ cells from thawed PBMCs from each time point by performing immunomagnetic depletion of non-T cells using PE-conjugated antibodies against BDCA1 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD14, CD56 (both from BD Bioscience) CD19, CD56, CD123 (all from Beckman Coulter Coulter) as well as CD15- and CD235-microbeads (both from Miltenyi Biotec). The purified $CD3^+$ cells were then stained with anti-CD3-AmCyan, anti-CD4-APC-H7, anti-CD25-APC, 7-AAD (all from BD Bioscience) and anti-CD127-PE mAb (Beckman Coulter Coulter). Subsequently, Tregs were obtained by flow cytometric sorting $7\text{-}AAD^-CD3^+CD4^+CD127^-CD25^+$ cells using FACS Aria (BD Biosciences). Purity of Tregs (defined as $CD4^+CD25^+CD127^-$), as determined by flow cytometry, was 98±1%. Tresp were obtained by sorting both $7\text{-}AAD^-CD3^+CD4^-$ and $7\text{-}AAD^-CD3^+CD4^+CD127^+CD25^-$ cells, which resulted in a purity of 99±0.4% (defined as $CD3^+CD127^+CD25^-$ cells), as determined by flow cytometry. Cells were recovered after cell sorting by incubation in culture medium (RPMI 1640 supplemented with penicillin (100U/ml),

streptomycin (100μg/ml) and 10% fetal bovine serum (Hyclone, Logan, UT, USA)) at 37°C for at least 3hrs prior to co-culture.

To compare their suppressive capacity, Tregs purified from the patient blood collected before IVIg treatment, immediately after treatment, and at day 7 after treatment, were co-cultured with Tresp ($5x10^4$) obtained before treatment that were stimulated with 1μg/ml phytohemagglutinin (PHA) in the presence of 40Gy irradiated autologous PBMC ($5x10^4$) at Treg:Tresp ratio's of 1:4 in culture medium in round-bottom 96-well plates (Greiner Bio-One, The Netherlands). After 5 days, supernatants were collected and the cumulative IFN-γ secretion by Tresp was quantified by ELISA (Ready-SET-Goâ, eBioscience) according to manufacturer's instructions. Each condition was tested at least in triplicate from which means were calculated. These means were used to calculate the percentage of suppression using the formula: $((\text{IFN-}\gamma\ \text{level}_{Tresp}\text{-IFN-}\gamma\ \text{level}_{Tresp+Treg})/\text{IFN-}\gamma\ \text{level}_{Tresp}) \times 100\%$.

Statistical analyses

Differences in measured variables between time points before and after IVIg treatment were pair-wise analyzed by using the Wilcoxon signed rank test. For analysis of the Treg suppression assay, we used the paired Student's T-test as the differences in IFN-γ production by Tresp collected at different time points before and after IVIg treatment were normally distributed. Two-sided P-values <0.05 were considered as significant. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). In accordance to the statistical test that is used, all data presented in dot plots show median values, but data on the suppressive capacity of Treg (**Figure 2B**) are depicted as means ± SEM.

RESULTS

IVIg selectively enhances the activation status of Tregs *in vivo*

IVIg treatment had no effect on the numbers of circulating $CD4^+$ and $CD8^+$ T cells (**Figure 1A**). To study the effects of IVIg treatment on Tregs, we measured the percentages and immunophenotype of circulating Tregs. The gating strategy used to determine $CD4^+CD25^+FOXP3^+$ cells is shown in **Figure 1B**. At baseline, we observed comparable percentages of circulating Tregs in patients who were treated with low-dose IVIg (n=12) or high-dose IVIg (n=15), and in healthy controls (n=10) (**Figure 1C**). In addition, baseline activation status of the Tregs in the low-dose and high-dose patients were comparable to healthy controls, as assessed by expression levels of FOXP3 (**Figure 1D**) and HLA-DR (**Figure 1E**).

IVIg treatment had no effect on the percentages of circulating Tregs in both low- and high-dose IVIg-treated patients (**Figure 1F**). Although low-dose patients showed a slight increase of Treg percentages, this increase was not significant. Additionally, Ki-67 staining in Tregs showed no increased proliferation of Tregs after IVIg treatment in both groups (data not shown). However, in patients treated with high-dose IVIg, we did observe an increased expression level of FOXP3 in $CD4^+CD25^+FOXP3^+$ Tregs immediately after IVIg treatment, with an average increase of 29% (**Figure 1B** and **G**, P=0.04). This increased FOXP3 expression level was sustained on day 7 after infusion (+35%, P=0.02).

Next, we analyzed the expression of the activation marker HLA-DR on $CD4^+CD25^+FOXP3^+$ Treg, gated as shown in **Figure 1B**. We found that the proportions of HLA-DR expressing Treg gradually increased after IVIg treatment, again only in high-dose treated patients, and was 37% higher immediately after and 39% at day 7 after treatment (**Figure 1B** and **H**). Prednisone treatment did not influence the expression of FOXP3 and HLA-DR, since all 6 patients that were co-treated with prednisone (low-dose n=3, high-dose n=3) showed the same patterns as patients without prednisone treatment (data not shown). In addition, the same trends of Treg activation were found in patients with autoimmune disease and immunodeficient patients who were treated with high-dose IVIg (**Supplementary Figure 1**).

To determine whether IVIg also affects the activation status of Tconv, we measured HLA-DR expression on $CD4^+FOXP3^-$ Tconv (**Figure 1B** and **I**). Compellingly, HLA-DR expression remained low on Tconv after IVIg treatment, even in high-dose IVIg-treated patients, which may suggest that IVIg treatment selectively activates $CD4^+CD25^+FOXP3^+$ Tregs, but not $CD4^+FOXP3^-$ Tconv in humans *in vivo*.

To study the effect of IVIg on the differentiation stage of Tregs, we measured CD45RO expression on Tregs. We found no effect of IVIg treatment on numbers of $CD45RO^+$ Tregs in peripheral blood in both groups, suggesting that IVIg did not drive conversion from naïve into memory Treg within the time frame of the study (data not shown).

IVIg increases the suppressive capacity of Tregs *ex vivo*

To investigate whether IVIg treatment stimulates the suppressive capacity of Tregs, we isolated $CD3^+CD4^+CD25^+CD127^-$ Tregs and autologous $CD3^+CD25^-CD127^+$ responder T cells (Tresp) from PBMCs of seven patients who received high-dose IVIg that were collected before, after and at day 7 after IVIg treatment. The gating strategy for flow cytometric purification of these cells is depicted in **Figure 2A**. Tresp isolated from blood collected before IVIg infusion were stimulated with PHA and co-cultured with Tregs purified from blood samples collected before, after and at day 7 after treatment in a Treg:Tresp ratio of 1:4. After 5 days of culture, cumulative IFN-γ production by Tresp was determined. Tregs that were isolated before treatment showed a suppression of IFN-γ production of 41%, while Tregs isolated at day 7 demonstrated a suppression of 70% (P=0.001, **Figure 2B**). Thus, in accordance with the

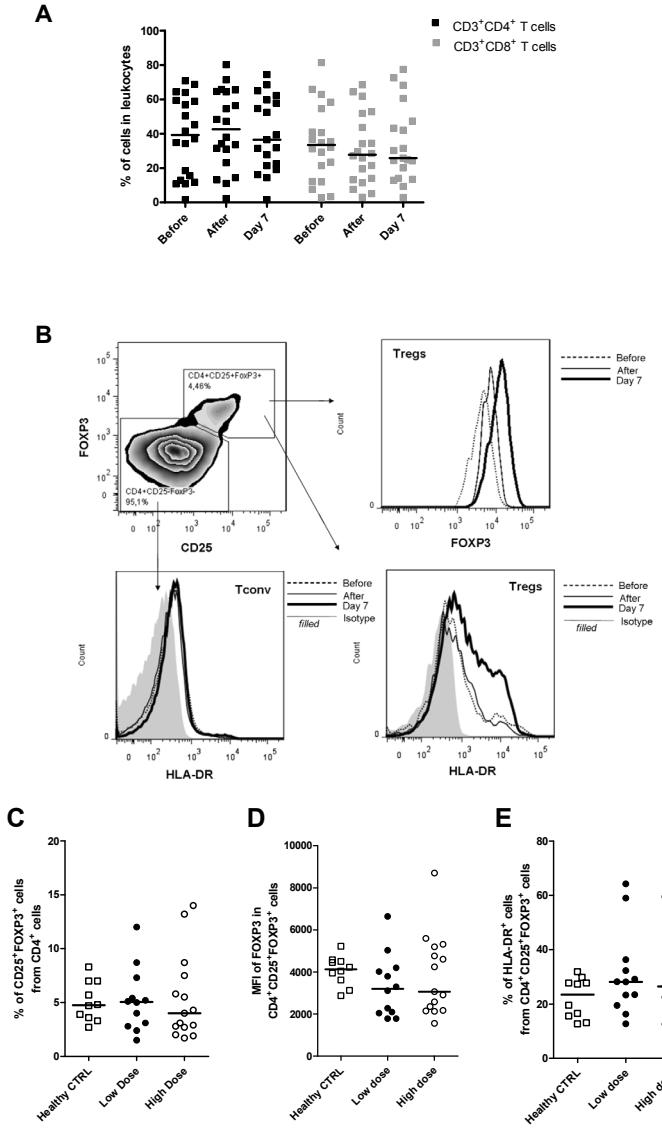

FIGURE 1. IVIg treatment selectively activates circulating Tregs, but not conventional T helper cells (Tconv). A) Percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ in PBMCs did not alter after IVIg treatment. B) Density plot shows the gating strategy of Tregs and Tconv after gating on $CD4^+$ cells. Histograms show intracellular FOXP3 expression in Tregs and surface expression of HLA-DR on Tregs and Tconv obtained before, immediately after, and at day 7 after IVIg treatment of a representative patient. C) Baseline percentages of $CD25^+FOXP3^+$ Tregs within circulating $CD4^+$ T cells, D) MFI of FOXP3 in Tregs and E) percentages of Tregs expressing HLA-DR from healthy blood donors (n=10) and patients treated with low-dose IVIg (n=12) or high-dose IVIg (n=15). F) Percentages of $CD25^+FOXP3^+$ Tregs within the $CD4^+$ population before, immediately after, and 7 days after IVIg treatment in patients who received low-dose (n=12) or high-dose IVIg (n=15). (G-I): G) MFI of FOXP3 in Tregs, H) percentages of Tregs, and I) percentages of Tonv expressing HLA-DR before, immediately after, and 7 days after IVIg treatment in patients who received low-dose (n=12) or high-dose IVIg treatment (n=15). Horizontal lines represent median. *P <0.05.

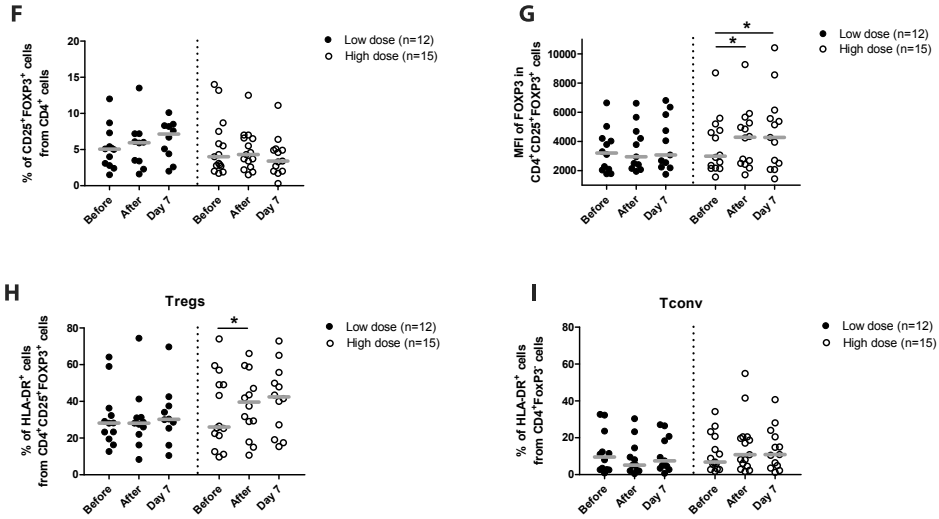

FIGURE 1 continued

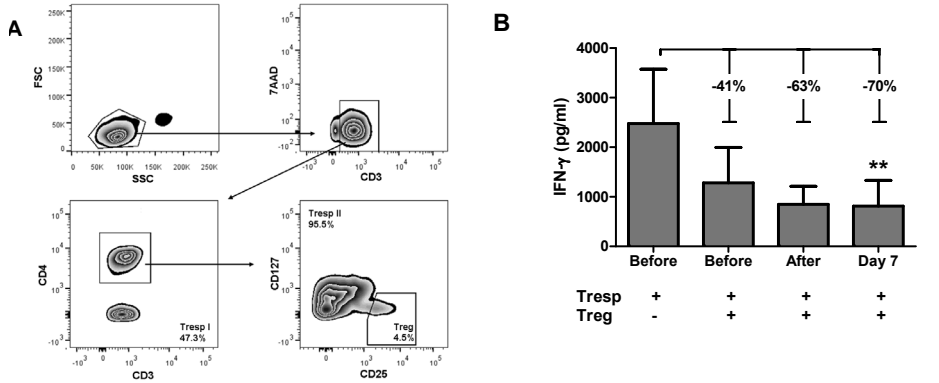

FIGURE 2. Enhanced suppressive activity of Tregs purified from blood after IVIg treatment.
A) Tregs were obtained by flow cytometric sorting on 7-AAD$^-$CD3$^+$CD4$^+$CD127$^-$CD25$^+$ cells (Treg) from PBMC collected from 7 high-dose IVIg-treated patients before treatment, immediately after treatment, and at day 7 after treatment, and Tresp were obtained by sorting both 7-AAD$^-$CD3$^+$CD4$^-$ (Tresp I) and 7-AAD$^-$CD3$^+$CD4$^+$CD127$^+$CD25$^-$ (Tresp II) cells from autologous PBMC collected before IVIg-treatment. B) Tregs purified from the patients before, after, and at day 7 after treatment were co-cultured with Tresp ($5x10^4$) obtained before treatment that were stimulated with 1μg/ml phytohemagglutinin (PHA) in the presence of 40Gy irradiated autologous PBMCs ($5x10^4$) at a Treg:Tresp ratio of 1:4 in culture medium in round bottom 96-well plates. After 5 days, supernatants were collected and the cumulative IFN-γ production by Tresp was quantified by ELISA. Each condition was tested at least in triplicate from which means were calculated. From each patient, these means were used to calculate the percentage of suppression using the formula: (IFN-γ $level_{Tresp}$-IFN-γ $level_{Tresp+Treg}$)/IFN-γ $level_{Tresp}$ x 100%. Bar graphs represent mean ± SEM. *P <0.05, as compared to Tresp alone.

observed increased Treg activation status at day 7 after IVIg treatment, Tregs isolated at 7 days also showed a higher suppressive capacity. These data suggest that IVIg treatment may stimulate the suppressive function of circulating Tregs up to at least 1 week after treatment.

DISCUSSION

Multiple on-going studies are being performed to induce Treg expansion or stimulate Treg function in order to control T cell-mediated disorders. Interestingly, the current study suggests that high-dose IVIg treatment may be useful to enhance Treg activation and suppressive function *in vivo*. We show that IVIg treatment enhances both the activation status and suppressive function of Tregs in patients. Upon high-dose IVIg treatment, a gradual rise in FOXP3 and HLA-DR expression in circulating $CD4^+CD25^+FOXP3^+$ Tregs was found, with the highest expression on day 7 after infusion. This finding indicates that IVIg treatment activates Tregs for at least 1 week, when IVIg levels already declined. Treg activation was clearly dependent on the IVIg dose, since patients who received low-dose IVIg (≤0.6g/kg) did not show significant effects. Activation of Treg was not restricted to patients with autoimmune disease, as immunodeficient patients who received high-dose IVIg also showed a rise in FOXP3 and HLA-DR expression on Tregs. Thus, our data support the generally accepted idea that high-dose treatment regimen is required in order to gain anti-inflammatory activity (7).

Our *ex vivo* functional assays showed that IVIg treatment may not only stimulate the activation status, but may also enhance the suppressive function of circulating Tregs. We observed an increased suppressive function of Tregs that was highest on day 7 after IVIg treatment, which is in line with the time-dependent increase of the activation status after treatment. Supporting our data, previous studies have shown that Tregs with a higher HLA-DR (11) and FOXP3 (15, 16) expression exert higher suppressive capacity. Further studies are warranted to confirm this finding, since suppressive function was studied in a small numbers of patients. Moreover, it will be highly interesting to monitor the activation status and suppressive function of Tregs after high-dose IVIg therapy over a longer time period to study the durability of this effect.

In order to assess the suppressive capacity of Tregs, we did not use proliferation as the classical read-out, since IL-2 produced by Tresp in the co-culture system may influence proliferation of Tregs, hence affecting overall ^{3}H-thymidine incorporation (17). Therefore, we analyzed IFN-γ secretion by Tresp, which is independent of the extent of Tregs hyporesponsiveness and may therefore be more unbiased.

While a few previous studies have shown an increase of circulating Treg numbers in patients treated with IVIg (18-21), we show an increase in both the activation status and functional suppressive capacity of Treg upon high-dose IVIg therapy in humans *in vivo*. Although the ef-

fects are moderate, this finding may be of importance, since in several autoimmune diseases the numbers of Tregs are normal, yet these cells display a functional defect (22-24). A possible explanation for the fact that we did not find an increase in the circulating Treg numbers after IVIg treatment is that our patients did not have a deficit of Treg numbers compared to healthy subjects (**Figure 1C**), while the previous studies included patients with acute inflammatory diseases which had decreased numbers of circulating Tregs before IVIg treatment (18-20).

Compellingly, high-dose IVIg treatment selectively activated $CD4^+CD25^+FOXP3^+$ Tregs, but not $CD4^+FOXP3^-$ Tconv. This observation may be interesting for the clinic, as conventional immunosuppressive drugs used for treating autoimmune diseases and allograft rejection, in particular calcineurin inhibitors, do not only suppress Tconv, but also Tregs (25-32). By selective activation of $CD4^+CD25^+FOXP3^+$ Tregs, but not Tconv, IVIg may be superior to classical immunosuppressive drugs. Similar observations were also found *in vitro* and in mice models (11, 12, 14). In addition, IVIg selectivity for Tregs has also been suggested by Maddur and colleagues (33), who showed that IVIg inhibited Th17 cell differentiation and amplification, while it increased the numbers of Tregs among memory T cells.

The mechanism by which IVIg selectively activates Tregs is still unclear. Our previous data (11) together with the study of Ephrem et al. (12) suggest that the activation may be mediated by direct binding of IVIg to an unknown "IVIg receptor" present on Tregs but not on Tconv. Since we observed that human Tregs do not express classical Fcγ receptors (11), this receptor must be a non-classical IgG-binding molecule. Alternatively, IVIg may bind to specific surface receptors by its F(ab')2 fragment, as suggested by Maddur et al (33), or IVIg may contain Tregitopes that can activate Tregs by presentation on MHC II^+ antigen presenting cells towards Tregs, as proposed by De Groot and colleagues (34, 35).

The current study focused on patients treated with IVIg for different indications. Although the underlying diseases are variable, the positive effect of high-dose IVIg on Treg activation is consistently seen in patients treated with high-dose IVIg. These results suggest that the effects of IVIg on Treg activation are not restricted to a certain disease, but to high-dose treatment. It would, however, be interesting to confirm the dose-dependent effect of IVIg in patients with the same treatment indication in the future.

In summary, we have demonstrated that high-dose IVIg therapy in humans *in vivo* can selectively activate Tregs and enhance their suppressive capacity. Since the effects in our patient cohort were moderate, we propose that Treg stimulation may be one of the mechanisms by which IVIg controls inflammation in patients with autoimmune and systemic inflammatory disorders, but other mechanisms are probably also required to restore imbalanced immune homeostasis. The data presented in this study underline the multifaceted character of the immunomodulatory mechanisms of IVIg.

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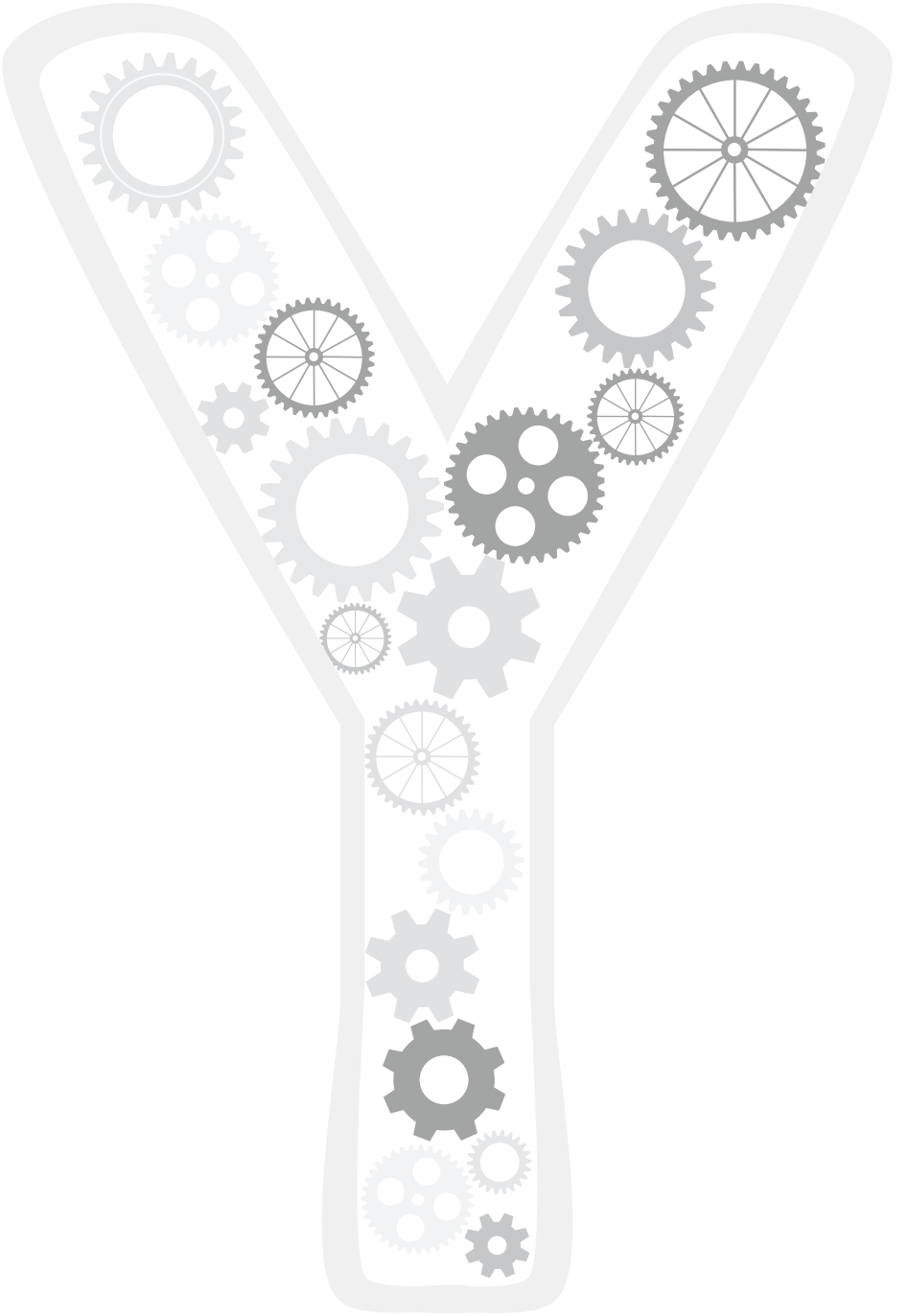

5

High-dose intravenous immunoglobulin does not reduce the numbers of circulating $CD14^{+}CD16^{++}$ monocytes in patients with inflammatory disorders

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To the Editor:

With great interest we have read the paper of Siedlar et al (1), in which a new mechanism of action of intravenous immunoglobulins (IVIg) is presented. This mechanism includes a reduction of the numbers of "pro-inflammatory" $CD14^{+}CD16^{++}$ monocytes and suppression of their TNF-α production. Modulating the numbers of $CD14^{+}CD16^{++}$ monocytes is of particular clinical importance in inflammatory diseases (e.g. sepsis, hemophagocytic syndrome and Kawasaki disease), where the numbers of this subset are dramatically increased. Therefore, the authors suggest that declining the numbers of $CD14^{+}CD16^{++}$ monocytes may contribute to the anti-inflammatory effects of high-dose IVIg treatment (1-2 g/kg) in patients with inflammatory conditions. However, the study of Siedlar et al (1) was not performed in patients receiving the "anti-inflammatory" dose of 1-2 g/kg, but in patients with common variable immunodeficiency (CVID) that received a replacement dose of 0.4 g/kg IVIg.

Therefore, to explore the effects of high-dose IVIg on the numbers of $CD14^{+}CD16^{++}$ monocytes in patients with inflammatory diseases, we performed a comparative study including patients with inflammatory disorders treated with "high dose" IVIg (average 1.24 g/kg ±0.34, n=7) and patients with immunodeficiency disorders receiving a "low dose" (average 0.43 g/kg ±0.16, n=7). The indications for high-dose IVIg treatment were: acquired von Willebrand syndrome (n=2), immune thrombocytopenic purpura (n=1), dermato(poly)myositis (n=2), CREST syndrome (n=1), vasculitis (n=1). Treatment with low-dose IVIg was given for the following indications: CVID (n=5) and immunoglobulin IgG1 and IgG3 subclass deficiency (n=2). Blood was collected before infusion and 4 to 24 hours after infusion. In addition, to study the effect of IVIg on longer term, we collected blood 7 days after IVIg infusion. By flow cytometry, we determined the percentages of inflammatory $CD14^{+}CD16^{++}$ monocytes and the classical $CD14^{+}CD16^{-}$ monocytes, according to the gating strategy used by Siedlar et al., excluding $CD56^{+}$ cells.

At baseline, the numbers of $CD14^{+}CD16^{++}$ cells were similar in patients receiving high dose (11.7%±11.5) and low dose (10.4%±6.1) IVIg (p=0.81). In line with the observations of Siedlar et al, we found a significant decline of the numbers of $CD14^{+}CD16^{++}$ cells immediately after IVIg infusion in patients that received low-dose IVIg (**Figure 1A**, average decrease: 42%, $P<0.05$). After 7 days, the numbers of inflammatory monocytes were still reduced in 4 of 6 patients measured. To our surprise, in patients treated with high-dose IVIg, no significant reduction of the numbers of $CD14^{+}CD16^{++}$ monocytes was observed (**Figure 1B**, before versus after IVIg: P=0.69; before versus day 7: P=1.00). Of the patients receiving low-dose IVIg, 4 from 7 patients showed a decline, of which all had CVID. Therefore, the described effect by Siedlar et al. appears to be more specific for CVID patients and the amount of IVIg administered. Similar to Siedlar et al., we did not observe changes in the classical $CD14^{+}CD16^{-}$ monocytes after IVIg treatment in both groups.

As Dr. M. Berger mentioned in his editorial (2), the relationship between the observed decrease in numbers of pro-inflammatory monocytes in blood of IVIg-treated CVID patients

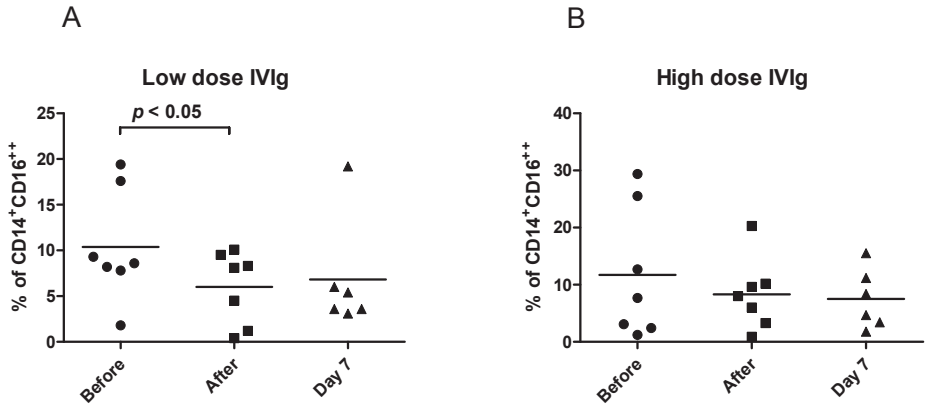

FIGURE 1. The percentages of CD14+CD16++ monocytes were significantly reduced immediately after IVIg infusion in patients receiving low-dose IVIg (A) (n=7), but not in patients receiving high-dose IVIg (B) (n=7). Horizontal lines represent means.

and the decreased production of TNF-α was not clear in the paper of Siedlar et al [1]. Therefore, to study whether there may be an effect of the reduced numbers of the non-classical monocytes on the levels of TNF-α, we quantified plasma TNF-α levels in our patients. In patients treated with low-dose IVIg, in which numbers of $CD14^{+}CD16^{++}$ monocytes decreased after IVIg treatment, we did not find a decrease in plasma TNF-α levels immediately after IVIg (+45%, p= n.s). Therefore, we do not think that IVIg treatment results in a decreased TNF-α production by $CD14^{+}CD16^{++}$ monocytes *in vivo*. Of note, when we combined patients treated with low-dose and high-dose IVIg (and thereby increased the power), we found a significantly higher plasma TNF- α level immediately after IVIg treatment (+37%, p< 0.05).

Together, similar to Siedlar et al (1), we found that low-dose IVIg diminishes the numbers of circulating pro-inflammatory $CD14^{+}CD16^{++}$ monocytes in CVID patients. However, this effect was not observed in patients with inflammatory disorders treated with high "anti-inflammatory doses" of IVIg. We therefore postulate that this mechanism does not explain the beneficial clinical effect of high-dose IVIg treatment in patients with inflammatory disorders.

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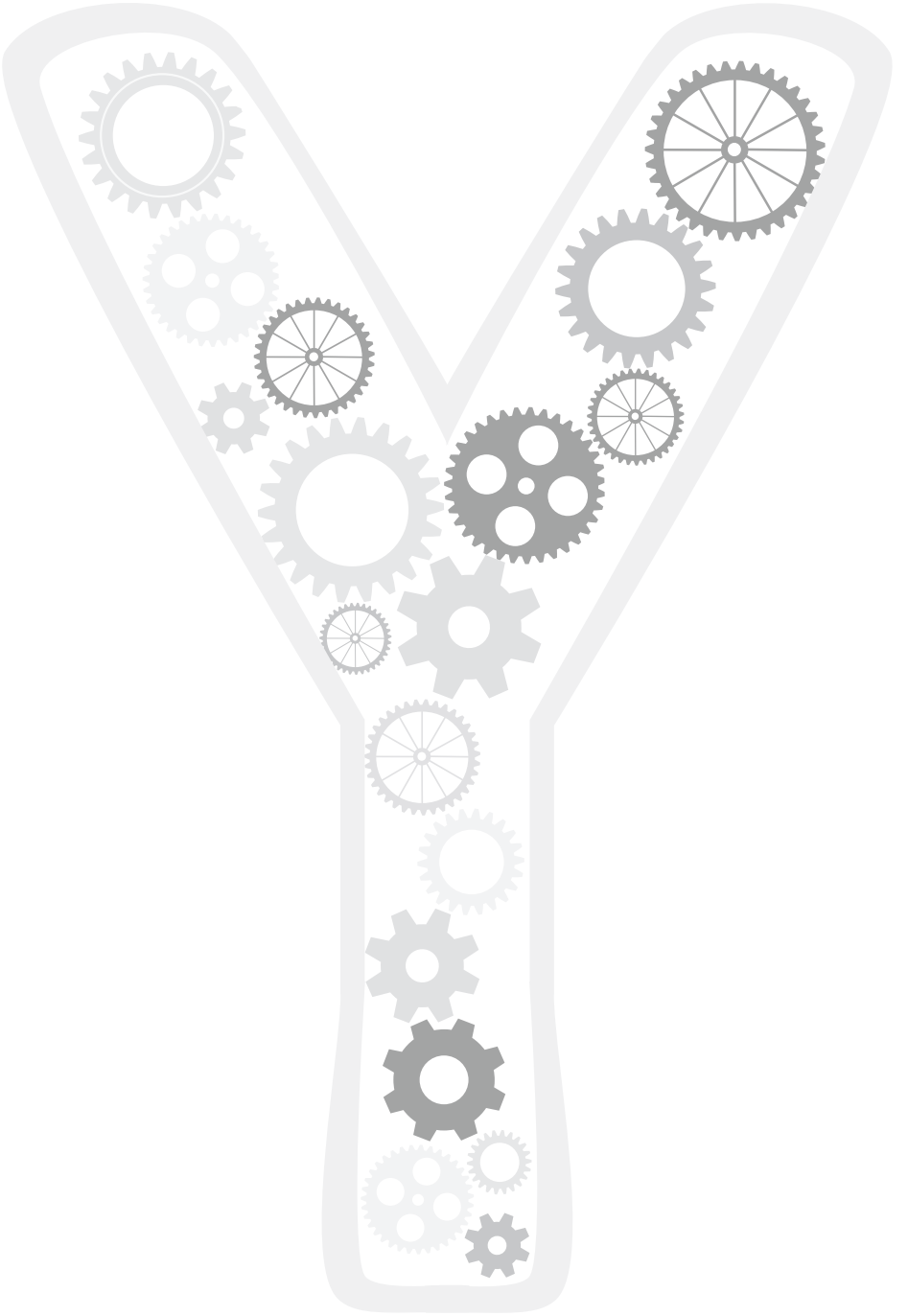

6

The anti-inflammatory actions of intravenous immunoglobulin in mice and men: a critical review

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ABSTRACT

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of polyspecific human IgGs prepared from plasma pooled from thousands of individuals. When administered at a high dose, IVIg is able to inhibit inflammation and has proven beneficial in the treatment of various autoimmune and systemic inflammatory diseases, as well as allograft rejection. Importantly, IVIg therapy can ameliorate both auto-antibody-mediated and T-cell mediated immunopathologies. In the last decades, extensive research in murine disease models has resulted in the elucidation of several anti-inflammatory mechanisms of action of IVIg. Surprisingly, it has only been limitedly studied whether these mechanisms are operational in humans treated with IVIg. In this perspective, we focus on the similarities and differences of the anti-inflammatory properties of IVIg in mice and humans. We examine two anti-inflammatory mechanisms that have been extensively studied in recent years: the IL-33-Th2-FcγRIIb pathway and stimulation of regulatory T cells. We demonstrate that both pathways are activated in humans treated with IVIg, but that incorrect extrapolations from mice to men have been made on the molecular and cellular components involved in these cascades that warrant for critical re-evaluation of these anti-inflammatory mechanisms of IVIg in humans.

INTRODUCTION

As IVIg contains a wide spectrum of antibody specificities, representative for the natural antibody repertoire of the adult human population, IVIg was initially administered to restore humoral function of patients with primary or secondary immunodeficiency. After it had been shown that high-dose IVIg treatment (4-fold higher than supplementation dose) (1) could ameliorate idiopathic thrombocytopenic purpura (ITP) (2), its anti-inflammatory properties have increasingly been exploited to treat various autoimmune and systemic inflammatory diseases.

Several non-exclusive mechanisms by which IVIg exerts its anti-inflammatory effects have been elucidated over the past decades. These include neutralization of autoantibodies by anti-idiotype interactions, increased clearance of pathogenic antibodies by saturation of the neonatal FcR (FcRn), prevention of binding of pathogenic immune complexes (ICs) to activating Fcγ-receptors (FcγR), modulation of FcγR expression, inhibition of the complement cascade, reduced pro-inflammatory cytokine production, inhibition of dendritic cells (DCs) and B cells, inhibition of T-helper (Th)-1 and Th-17 differentiation, and expansion and enhanced suppressive function of $CD4^+FOXP3^+$ regulatory T cells (Treg). All these anti-inflammatory mechanisms of IVIg have been excellently reviewed elsewhere (1, 3-8). It is important to realize, that most of these anti-inflammatory mechanisms have been established in murine studies, and that extrapolation of these mechanisms from mice to humans is far from trivial due to fundamental differences between the murine and human immune system that affect the mode-of-action of IVIg within these species. In this perspective, we focus on the similarities and differences between the anti-inflammatory mechanisms activated by IVIg in mice and humans, but due to size limitations, we cannot cover all the anti-inflammatory mechanisms as listed above. Therefore, we will focus on two most advertised anti-inflammatory mechanisms in recent years: the IL-33-Th2-FcγRIIb pathway and stimulation of regulatory T cells. We will examine the evidence for the involvement of their proposed cellular and molecular components in immunomodulation by IVIg therapy in humans and, when validation in humans is still lacking or incomplete, discuss their translatability from murine to human studies considering human and murine biology.

Sialylated IVIg and the IL-33-Th2-FcγRIIb pathway

In the last decade, a number of landmark studies has revealed that IgGs with α2,6-sialic acid-containing N-linked glycans attached to the IgG Fc-part, display potent anti-inflammatory activity in antibody-mediated inflammation. Identification of the anti-inflammatory properties of this IgG fraction started with a study in a murine ITP-model in which the protective effects of IVIg appeared dependent on 1) upregulation of FcγRIIb expression on effector macrophages, thereby limiting IC-mediated activation, and 2) the IgG Fc-part (9).

In subsequent studies, the protective effect of IVIg in a mouse model of IC-mediated (K/BxN) arthritis was shown to be mediated by CSF-1-dependent macrophages that act as sensors for IVIg and are involved in the induction of inhibitory FcγRIIb expression on CSF-1-independent effector macrophages, thereby raising the threshold for activation of these cells by auto-antibody-IC (10, 11). These CSF-1-dependent IVIg-sensor macrophages were identified as splenic SIGN-R1^{+} marginal zone macrophages (MZM) which were able to bind a specific fraction of IgGs having α2,6-sialic acid-containing N-linked glycans attached to the IgG Fc (12). Parallel studies meanwhile had demonstrated that these IgGs were essential for the anti-inflammatory activity of IVIg in the K/BxN arthritis model in a FcγRIIb-dependent manner (13-15). Infusion of α2,6-sialic acid Fc (sialylated Fc, sFc) protected wild type, but not SIGN-R1$^{-/-}$ mice from arthritis, suggesting that binding of sFc to SIGN-R1 on MZM was required (12). A human orthologue of SIGN-R1, DC-SIGN, was also able to bind sFc *ex vivo* (12). Indeed, the protective activity of sFc and sialylated IVIg (sIVIg) was retained upon induction of arthritis in SIGN-R1$^{-/-}$ mice that transgenically expressed DC-SIGN (15). These data suggested that DC-SIGN might be able to mediate the anti-inflammatory properties of sIVIg in humans *in vivo*. Subsequently, it was shown that sIVIg induced the production of IL-33 in the spleen of wild-type but not SIGN-R1$^{-/-}$ mice. IL-33 subsequently promoted the production of IL-4 and IL-13 by basophils, which enhanced the expression of FcγRIIb expression on macrophages and monocytes, thereby providing a link between IVIg-sensor macrophages and induction of FcγRIIb expression on effector myeloid cells (**Figure 1**) (15).

The elucidation of this mechanism has profoundly improved our understanding of the protective effects of IVIg in antibody-mediated inflammation in mice. However, what is the current evidence that a similar mechanism mediates the anti-inflammatory effects of high-dose IVIg-therapy in humans? In the following sections, we will address the evidence for activation of this anti-inflammatory pathway upon high-dose IVIg therapy in humans. Moreover, we will discuss the biological differences between mice and men which are related to a number of unresolved issues regarding involvement of FcγR modulation, the role splenic MZM, the dependence on DC-SIGN, and the importance of IgG sialylation in the therapeutic efficacy of IVIg in humans.

IVIg and the IL-33-Th2 cytokine-Fcγ receptor pathway in humans

Upregulation of inhibitory FcγRIIb expression by IVIg as an effector mechanism by which antibody-mediated immune diseases are prevented, has been demonstrated in various animal models (9, 10, 13, 16), although in murine studies on ITP and antigen-driven allergic airway disease, the beneficial effect of IVIg was also observed in FcγRIIB$^{-/-}$ mice (17, 18). Is there evidence for the involvement of FcγRIIb in IVIg-treated humans? Recently, we demonstrated no increase of FcγRIIb expression on circulating monocytes and DCs in patients treated with IVIg for diverse autoimmune pathologies (19). These data corroborated findings in IVIg-treated patients with ITP (20), Kawasaki disease (21), or common variable immuno-

deficiency (CVID) (22) that showed no changes in FcγRIIb protein or mRNA expression of circulating monocytes. In addition, IVIg did not induce upregulation of FcγRIIb expression on human myeloid DCs *in vitro* (19, 23). In contrast to these studies, a majority of patients with chronic inflammatory demyelinating polyneuropathy (CIDP) showed increased expression of FcγRIIb on monocytes and B cells after IVIg treatment (24). It has to be noted that the untreated CIDP patients in this study showed reduced FcγRIIb expression and the observed increase may have reflected a normalization of expression levels upon reduction of overall inflammation upon IVIg therapy. So strikingly, whereas IVIg treatment in mice consistently has shown to stimulate expression of inhibitory FcγRIIb on myeloid cells (9, 10, 25, 26), this effect has not been observed in human studies.

Do these observations imply that IVIg treatment in humans does not inhibit IC-mediated immunity via modulation of FcγRs? Although we did not find increase of FcγRIIb expression, after high-dose IVIg treatment in patients with autoimmune diseases, we did find down-regulation of the activating FcγRIIa expression on circulating myeloid DCs (19). In addition,

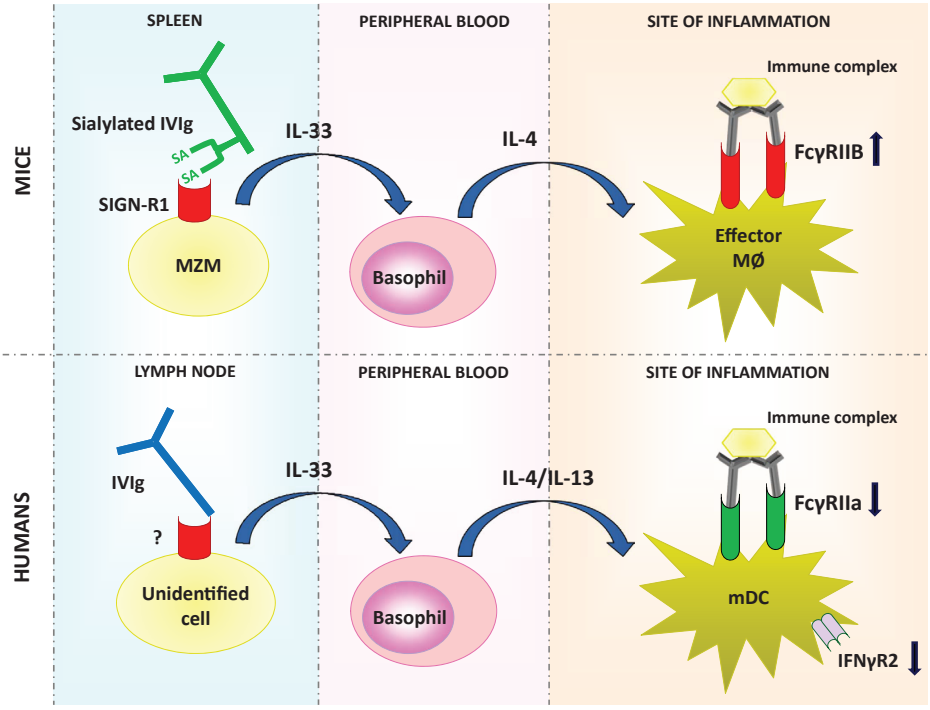


FIGURE 1. IVIg-IL-33-Th2-FcγRIIB pathway in mice and humans.
In mice, sialylated IVIg binds to SIGN-R1 expressed on splenic marginal zone macrophages and induces IL-33 production. IL-33 subsequently promotes the production of IL-4 by basophils, which enhances the expression of FcγRIIB expression on effector macrophages at the site of inflammation. In humans, IVIg binds to an, as of yet, unidentified cell type in lymph nodes. This induces IL-33 production, which in turn enhances IL-4 and IL-13 production by basophils resulting in decreased expression of FcγRIIa and IFNγR2 on myeloid dendritic cells. MZM, marginal zone macrophages; SA, sialic acid; MØ, macrophage; mDC, myeloid dendritic cell.

we observed an increase in plasma levels of IL-33 and the Th2 cytokines IL-4 and IL-13 upon high-dose IVIg treatment in these patients, showing homology between mice and humans. Enhanced IL-33 plasma levels were also observed in another cohort of autoimmune disease patients treated with IVIg, although IL-4 in plasma was hardly detectable (27). *In vitro* experiments on human myeloid DCs suggested that FcγRIIa downregulation was not directly caused by IVIg, but rather indirectly by the elevated levels of IL-4 and IL-13, and resulted in suppressed responses of myeloid DCs to IC-stimulation (19). Thus, in humans IVIg does stimulate the IL-33-Th2 pathway, but reduces IC-mediated myeloid DC activation by downregulation of the activating FcγRIIa, instead of upregulation of the inhibitory FcγRIIb on myeloid cells as observed in mice (**Figure 1**). Interestingly, we found that the same cytokine cascade also downregulates expression of the IFN-γ receptor 2 subunit on myeloid DCs in humans (19), which may contribute to suppression of cellular immunity by IVIg (28).

Given the differences in expression of FcγRs between mice and men, it is not entirely surprising that the effects of IVIg treatment on FcγR modulation in mice are distinct from those in humans. Humans have six different FcγRs, namely FcγRIa, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb, while mice have four: FcγRI, FcγRIIb, FcγRIII and FcγRIV (29-31). Thus, FcγRIIa, which we showed to be downregulated by IVIg treatment (19), is not present in mice.

IVIg-sensing cell types in humans

Murine studies have demonstrated the importance of splenic MZM in the IL-33-Th2-FcγRIIb pathway. Because the protective effect of sIVIg was lost upon splenectomy or specific deletion of MZM, this macrophage subset was suggested to be responsible for binding of, and initiating the protection mediated by sIVIg or sFc (10, 12), although it is important to note, that it has not been formally proven that MZM are the source of IL-33 production. One would expect a similar role for this cell type in IVIg-treated humans, as IL-33 plasma levels in humans increased upon IVIg treatment (19, 27). However, the human and murine spleen differ to a major extent, both in morphology and the presence of specific cell types. MZM are diffusely spread within the marginal zone of the murine spleen (32), they express SIGN-R1 and cooperate with marginal zone B cells to mount T-cell-independent IgM responses (33). Importantly, MZM are not present in the human spleen, suggesting that a different cell type is responsible for the IVIg-induced IL-33 production in humans (34). In contrast to the spleen in mice, the human spleen contains an additional zone, called the perifollicular zone. It is located between the marginal zone and the red pulp (35). In the perifollicular zone, branches of the central arteriole terminate without endothelial lining. It contains a special subset of macrophages, of which some express DC-SIGN, that form sheats around the capillaries (36). Although their anatomical location in the human spleen is different, it is tempting to speculate that these perifollicular macrophages are the counterparts of murine MZM.

However, arguing against an indispensable role for the spleen in mediating the anti-inflammatory effects of IVIg in humans is the observation that IVIg treatment of splenec-

tomized ITP patients is still effective (37). Therefore, cell type(s) producing IL-33 upon IVIg treatment in humans may be present outside the spleen. Indeed, IVIg induces *IL-33* mRNA expression in primary human lymph node cells *in vitro* (19), but not in human splenocytes (19, 27). In human lymph nodes, medullary sinus macrophages (MSM) (38), which function to filter lymph for microorganisms, as well as subcapsular sinus macrophages (SSM), a macrophage subset which is in direct contact with afferent lymph, are likely candidates for the IVIg-induced IL-33 production in humans *in vivo* as they express DC-SIGN (39, 40). Although we were not yet able to determine whether MSM an SSM are the cellular sources of IL-33 *in vivo*, we did establish that human macrophages produce IL-33 upon IVIg exposure *in vitro* (19). Alternatively, epithelial cells and fibroblasts may also be likely sources as they can produce IL-33 (41, 42).

Recent studies using two different murine models of ITP showed that splenectomy in mice did not impair the protective effect of IVIg (17, 43). In one of these models, the protective effect of IVIg was even independent of IL-33 and IL-4 signaling, although still dependent on SIGN-R1 and sFc (43). This suggests that, as in humans, cells at anatomical locations outside the spleen may alternatively mediate the anti-inflammatory properties of sIVIg in mice.

Collectively, these data indicate the need to study which human tissues and cells are involved in stimulation of the IL-33-Th2 cytokine pathway by IVIg. Obviously, availability of tissues from IVIg-treated patients is limited, but *in vitro* studies using human tissues and cells can help to clarify which human cell types are able to bind IVIg and produce IL-33.

Contribution of DC-SIGN and Fc sialylation to the anti-inflammatory activity of IVIg in humans

Currently, the only evidence that DC-SIGN might be involved in the anti-inflammatory response to IVIg in humans is the observation that DC-SIGN can replace its murine orthologue SIGN-R1 in IL-33-mediated protection from serum-induced arthritis in mice upon sFc treatment (15). However, *in vitro* studies on human DC-SIGN expressing cells did not provide any indication for involvement of DC-SIGN in the protective effects of sFc. We found that stimulation of IL-33 production of human macrophages by IVIg was not inhibited by blocking of DC-SIGN (19). Moreover, human splenocytes and monocyte-derived DCs (moDCs), that both abundantly express DC-SIGN, did not produce IL-33 upon exposure with IVIg (19, 27). Although DC-SIGN was partially responsible for the IVIg-mediated induction of regulatory T cells by prostaglandin E2 (PGE2)-producing moDCs, this effect was not Fc- but $F(ab')_2$-dependent and therefore not mediated by sFc binding to DC-SIGN (44).

Collectively, these data ask for a re-evaluation of the involvement of DC-SIGN in mediating the anti-inflammatory activity of IVIg in humans. Whether DC-SIGN is the proper human homologue of SIGN-R1 can in fact be argued. L-SIGN, another C-type lectin, shares stronger homology to SIGN-R1 with regard to cellular expression pattern than DC-SIGN. SIGN-R1 and L-SIGN are both expressed by liver sinusoidal and lymph node endothelial cells (45-47).

SIGN-R1 is also expressed on MSM in lymph nodes (48) and MZM in the spleen (32). MSM in humans do not express L-SIGN (45) and whether L-SIGN is expressed in human spleen is unclear, as no expression was detected in human spleen on mRNA or protein level (46, 47), although recently a few scattered L-SIGN-expressing cells were observed in the perifollicular zone (15). In contrast, shared DC-SIGN and SIGN-R1 expression has only been observed for human and murine MSM in lymph nodes, respectively (32, 40). DC-SIGN is also expressed on subsets of monocytes (15), DCs (46), some macrophages in the perifollicular zone of the human spleen (36), and SSM in human lymph nodes (39, 40). All three C-type lectins have similar glycan binding capabilities, as they are all able to bind high-mannosylated structures and fucosylated glycans, with the exception that L-SIGN does not bind Lewis^x-containing glycans (49, 50). L-SIGN is also able to bind to sFc *in vitro*, although with reduced affinity compared to DC-SIGN, which suggests a possible role of L-SIGN in sensing sIVIg in humans. In our opinion, the published results of selective blockade of DC-SIGN in SIGN-R1$^{-/-}$ mice that express a transgene containing both DC-SIGN and L-SIGN, do not completely exclude involvement of L-SIGN in the anti-inflammatory effects of sFc (15). Therefore, additional studies using human cells are required to demonstrate whether L-SIGN is involved in mediating anti-inflammatory effects of IVIg in humans.

In addition, whether DC-SIGN or SIGN-R1 can bind sIVIg or sFc with sufficient affinity is still a matter of debate. Importantly, binding of sFc to SIGN-R1- or human DC-SIGN-expressing cells *in vivo*, e.g. upon infusion of fluorescently-labeled sFc fragments, has never been shown. SIGN-R1 and DC-SIGN do not bind sialylated glycans or glycoconjugates (50-52), suggesting that binding of sFc by these lectins must involve non-canonical interactions. A recent study suggested that sialylation of the N-linked Fc glycan structurally affects the IgG Cγ2 domain, causing a so-called 'closed' state of sFc which would enable interaction with DC-SIGN, while asialylated Fc has an 'open' state, resulting in preferential binding to FcγRs (53). However, this model of sFc-DC-SIGN interaction is controversial and has initiated an ongoing scientific debate as contradictory evidence shows that Fc sialylation does not induce alterations in the Fc conformation (54, 55). Moreover, binding affinity of engineered IgG glycoforms that were either hyper-α2,6-sialylated, asialylated or deglycosylated to tetramerized DC-SIGN was shown not to differ and, strikingly, was Fab- but not Fc-dependent (56).

In addition to the above-mentioned unresolved issues on binding of sIVIg to DC-SIGN, there are now various reports that question whether SIGN-R1 and (Fc) sialylation are required for the anti-inflammatory effects of IVIg in mice. SIGN-R1 was found to be dispensable for therapeutic amelioration of ITP as well as prevention of antigen-driven airway disease in mice (16, 18). In murine models of ITP (57-59) (in contrast to other studies on ITP reporting a role for sialylation (13, 16, 43)), arthritis (60) and herpes simplex virus (HSV)-induced encephalitis (26) the anti-inflammatory properties of IVIg, either administered prophylactically or therapeutically, were sialylation-independent. Therefore, the beneficial effects of sIVIg (in mice) may be disease- and model-dependent, suggesting that clinical application of

sialic acid-enriched IVIg in humans may not *per se* be superior to conventional IVIg regimens. Taken together, there is still limited evidence for a prominent role of DC-SIGN and Fc sialylation in mediating the anti-inflammatory activity of IVIg in humans.

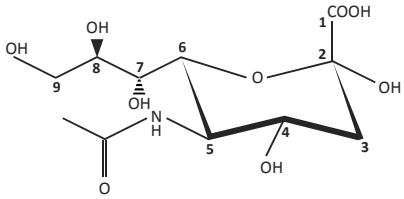
Alternative sialylated IgG-binding molecules

CD22 is a sialic acid-binding Ig-like lectin (Siglec-2) family member expressed on murine and human B cells and murine DCs (61), and binds α2,6-sialic acid-containing glycans with high specificity (62). sIVIg, but not asialyated IVIg, was shown to bind to CD22 on human B cells *in vitro*, resulting in reduced BCR signaling and enhanced apoptosis upon stimulation with anti-IgM (63). As in this study sIVIg was enriched by *Sambucus nigra agglutinin* (SNA) lectin fractionation which mainly enriches for F(ab')$_2$-sialylated IgG, it is likely that the inhibitory effects of sIVIg mediated by CD22 are not Fc-dependent (57, 64).

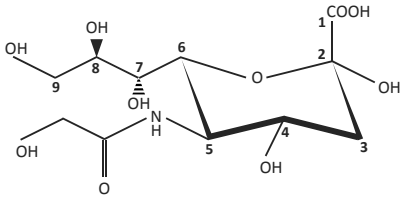
Arguing against a role for CD22 in mediating the anti-inflammatory effects of IVIg is the observation that CD22$^{-/-}$ mice were still protected from ITP and serum-induced arthritis by IVIg (65). However, it should be noted that murine studies on the involvement of CD22 in the effects of IVIg are hampered by the differences between sialic acids in humans and mice. Sialic acids are nine-carbon sugars which contain a carbon ring (C2-C6) and an exocyclic side chain (C7-C9). One group of sialic acids, called neuraminic acids, are N-acetylated at C5 in the carbon ring, yielding N-acetyl neuraminic acid (Neu5Ac). In many vertebrates, including mice, this N-acetyl group can be converted by the enzyme CMP-N-acetyl hydroxylase (CMAH) to a N-glycolyl group resulting in N-glycolyl neuraminic acid (Neu5Gc). Importantly, CMAH is non-functional in humans (66). Therefore, terminal sialic acids attached to N-linked glycans like those in human IgG molecules are Neu5Ac and not Neu5Gc. While human CD22 binds both Neu5Ac and Neu5Gc, murine CD22 can only bind Neu5Gc ligands (**Figure 2**) (67). Therefore, it can be expected that sIVIg cannot bind to murine CD22, and on basis of murine experiments it can therefore not be ruled out that sIVIg modulates immune responses in humans via binding to CD22.

Recently, another C-type lectin, called dendritic cell immunoreceptor (DCIR), was shown to specifically bind sIVIg. As a result, DCIR-expressing tolerogenic DCs induced expansion of regulatory T cells (Tregs) which attenuated ovalbumin-induced airway hyperresponsiveness in mice (18). Whether DCIR is involved in the anti-inflammatory effects of IVIg in humans is still unexplored. Finally, sFc also binds to human CD23, the low-affinity IgE receptor (53), but whether this interaction has functional effects has not been studied yet. It is tempting to speculate that CD22, DCIR, CD23 or other still unknown receptors for IVIg mediate the SIGN-R1-independent anti-inflammatory effects of prophylactic IVIg treatment observed in mice, as well (16). In addition, elucidation of the functional role of these alternative 'IVIg receptors' in the immunomodulatory effect of IVIg in humans deserves scientific effort.

A



N-acetyl neuraminic acid (Neu5Ac)



N-glycolyl neuraminic acid (Neu5Gc)

B

	synthesized in	
	humans	mice
Neu5Ac	yes	yes
Neu5Gc	no	yes

C

	binds CD22 in	
	humans	mice
Neu5Ac	yes	no
Neu5Gc	yes	yes

FIGURE 2. Sialic acids and binding to CD22.
A) A schematic view of sialic acid structure. The carbon at position 5 is either N-acetylated (left) or N-glycolylated (right). B) Biosynthesis of sialic acids in humans and mice. C) Binding properties of sialic acids to human or murine CD22.

Stimulation of regulatory T cells

It has now firmly been established both in mice and humans that IVIg exerts its anti-inflammatory effects also by stimulating expansion and suppressive function of $CD4^+Foxp3^+$ Tregs. Tregs are key players in the maintenance of self-tolerance, involved in controlling autoimmunity, inflammatory disorders, and immune responses to allogeneic transplants, cancer and various infectious agents. Numerous murine studies on a variety of disease models have shown an indispensable role for Tregs in mediating the protective effects of IVIg treatment, as they expand from normal to supranormal levels and show enhanced suppressive capacity (18, 26, 68-74). A similar expansion of Tregs was observed in humans who were treated with IVIg for various diseases, e.g. in Kawasaki disease (75, 76) Guillain Barré Syndrome (77), rheumatoid arthritis (78) and eosinophilic granulomatosis with polyangiitis (79), except in a study in CVID patients in which no expansion of Tregs was observed upon IVIg-treatment (80). We demonstrated that circulating Tregs in patients with various auto-immune diseases or immunedeficiencies are selectively activated upon IVIg therapy, as these cells showed increased FOXP3 and HLA-DR expression and enhanced suppressive capacity *ex vivo*, while T helper cells were not affected. However, our study did not show an increase of circulating Tregs upon IVIg treatment (81). Comparison of the human studies reveals that expansion of Treg upon IVIg therapy was only observed in patients with inflammatory diseases in which

the levels of Tregs were reduced prior to IVIg infusion, while Treg did not expand in patients without Treg deficit. Collectively, these studies demonstrate that IVIg in humans enhances the suppressive capacity of Tregs, while expansion only occurs in conditions with deficit numbers of circulating Tregs. The latter observation differs from the findings in murine studies, which may be explained by the different compartments in which Treg are measured, i.e. Treg levels in humans were determined in peripheral blood, and in mice in spleen, lymph nodes and inflamed tissues. Collectively, expansion and enhanced suppressive activity of Tregs is a common feature of IVIg treatment in mice and humans.

Interestingly, Treg expansion and enhanced suppressive capacity may be related to induction of IL-33 production upon IVIg treatment. Three recent murine studies have shown that IL-33 directly stimulates $CD4^+Foxp3^+$ Treg expansion (82-84). In allogeneic heart transplant models IL-33 administration resulted in expansion of recipient Tregs in cardiac grafts and spleen and in prolonged allograft survival, while depletion of Tregs from recipients eliminated any therapeutic benefit from IL-33 therapy (82, 83). In a chronic colitis model, administration of IL-33 induced Treg proliferation *in vivo*, promoted Treg accumulation in the spleen and in inflamed tissues, and prevented loss of Foxp3 expression in the inflammatory environment (84). Stimulation of Tregs by IL-33 was dependent on expression of ST2, the IL-33 receptor, on Tregs (82, 84). *In vitro* experiments showed that IL-33 can serve as a cofactor in TGF-β-mediated Treg differentiation (84).

Several other mechanisms by which IVIg may modulate Treg function and expansion have been postulated, and these have extensively reviewed elsewhere (**Figure 3**)(7, 8, 85). The contribution of IgG sialylation on Treg expansion has been recently addressed in several studies. In a murine model of antigen-driven allergic airway disease, induction of antigen-specific Treg differentiation was dependent on binding of sIVIg to DCIR on DCs (18, 71, 72). Contrastingly, in HSE and EAE mouse models IgG sialylation was not required for functional activation of Tregs (26, 59). *In vitro*, IVIg induced expansion of human Tregs by moDCs partially via DC-SIGN in a $F(ab')_2$-dependent manner, but it is unknown whether IgG sialylation was involved (44). In addition, expansion and activation of Tregs upon recognition of IgG-derived peptides called Tregitopes that are presented by antigen-presenting cells is sialylation-independent (86, 87). Collectively, further study is warranted to determine the contribution of IgG sialylation and the IL-33-Th2 cytokine pathway on IVIg-driven human Treg expansion and function, both in antibody-mediated inflammatory diseases as well as in T-cell-mediated autoimmune pathologies.

Towards studying the cellular and molecular pathways required for IVIg-mediated immunosuppression in humans

Despite the vast knowledge that has been gained on the anti-inflammatory activity of IVIg in mice, it is surprising how little we know of its immunosuppressive mechanisms of action

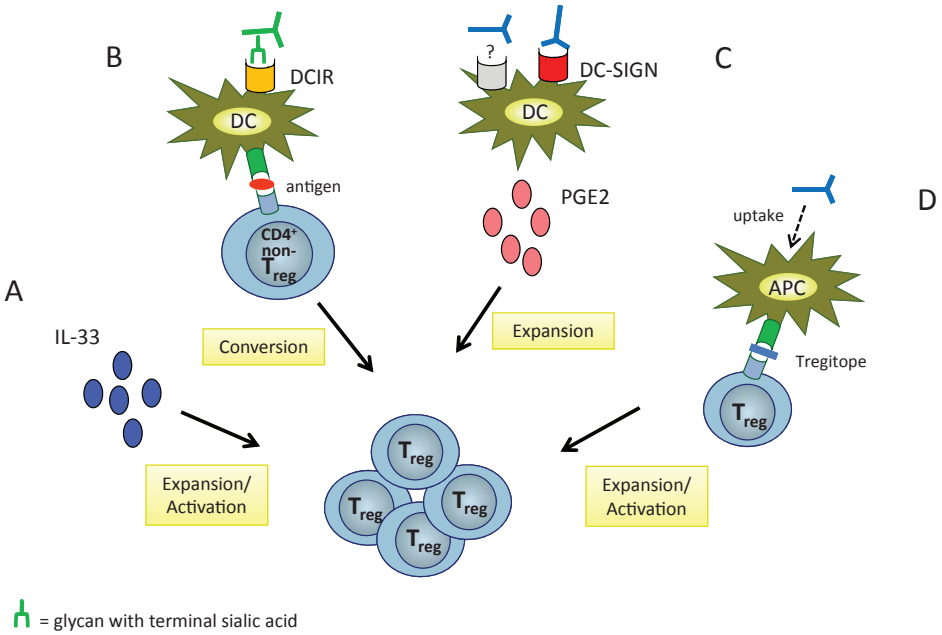


FIGURE 3. Proposed mechanisms of action by which IVIg modulates regulatory T cells. A) IVIg-mediated IL-33 production induces Treg proliferation and activation. B) IVIg induces antigen-specific Tregs by tolerogenic DCs from non-Treg $CD4^+$ T cells by binding of sialylated IVIg to DCIR expressed on DCs. C) IVIg induces prostaglandin E2 secretion by DCs, partly via DC-SIGN in a $F(ab')_2$-dependent manner. This leads to expansion of Tregs. The other "IVIg-receptor" involved in the secretion of PGE2 has yet to be identified. D) Presentation of Treg-activating peptides derived from conserved epitopes of IgG (Tregitopes) by antigen-presenting cells activates and expands Tregs. APC, antigen-presenting cell; DCIR, dendritic cell immunoreceptor; PGE2, prostaglandin E2; Treg, regulatory T cell.

in humans. The IL-33-Th2 pathway identified in mice is also active in humans treated with IVIg and results in reduced sensitivity of DCs to activation by ICs and IFN-γ, but there are significant differences in cellular and molecular components of this pathway between mice and men. In contrast, induction of Treg expansion and suppressive capacity by IVIg is a common pathway exploited by IVIg in mice and men. However, the molecular pathway(s) used by IVIg to stimulate these anti-inflammatory cascades in humans are unresolved. Several murine studies have shown that the anti-inflammatory properties of IVIg reside in the minor fraction of Fc-sialylated IgG molecules present in IVIg, but Fc-sialylation was shown to be dispensable in others studies. It is however unknown whether sIVIg also has immunomodulatory effects in humans, and priority should be given to investigate this important question. Moreover, whether the identified sIVIg-binding molecules DC-SIGN, DCIR, CD22 and CD23 are involved in the anti-inflammatory activity of IVIg in humans *in vivo* should become subject of scrutiny as current evidence thereon is very limited.

In light of the biological differences between mice and men, we recommend studying the immunomodulatory pathways of IVIg in humans using *ex vivo* measurements, as well

as *in vitro* studies on human cells. To address causality, studies should be initiated using immunodeficient mice reconstituted with a human immune system. In the light of the growing demand for IVIg concomitant with the predicted shortage of human plasma in the future and the high costs of IVIg therapy, it is of utmost importance to unravel the molecular interactions between IVIg and the human immune system, as such knowledge may enable the design of biologicals or small molecule drugs that mimic the anti-inflammatory effects of IVIg.

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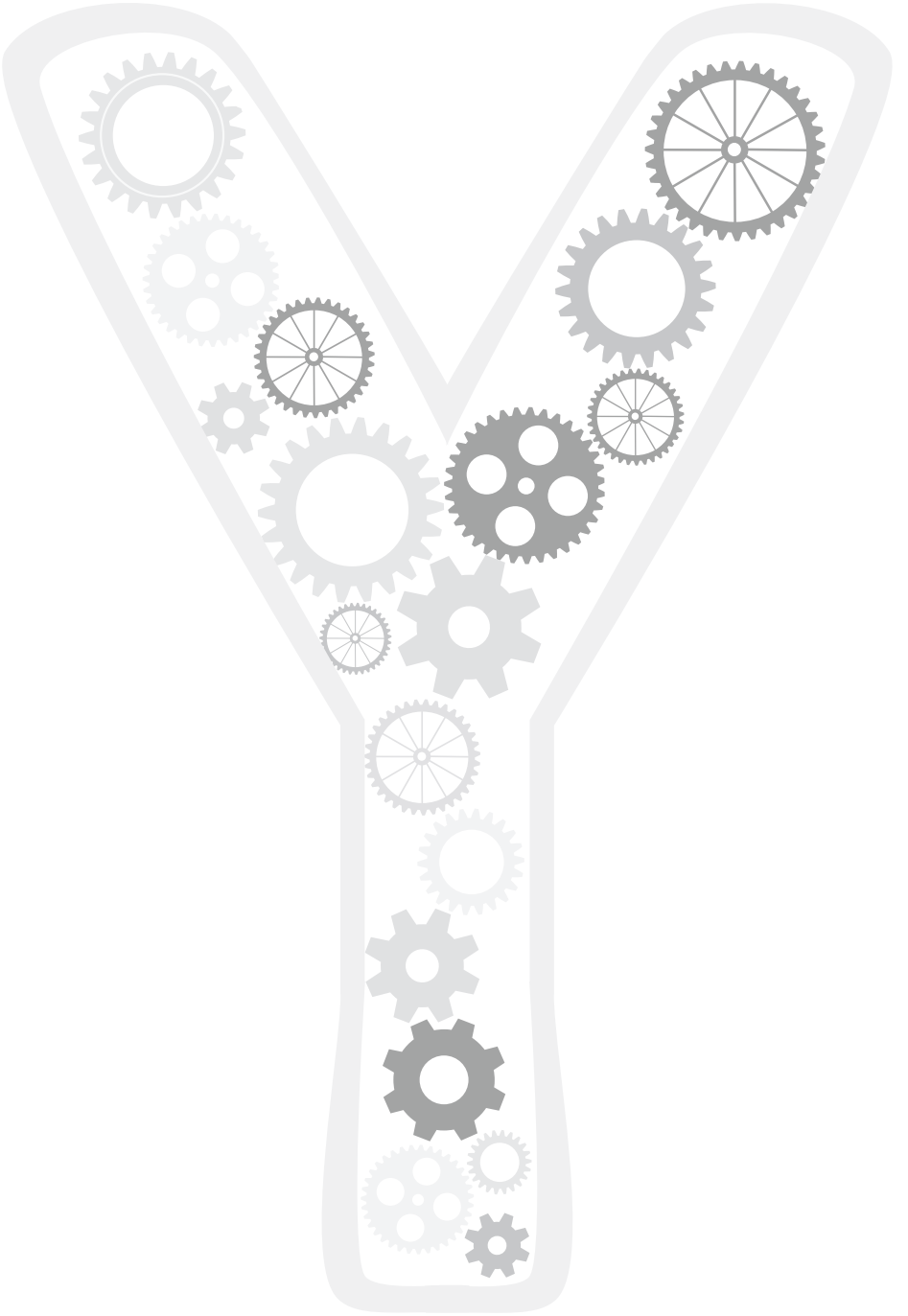
PART III

IMMUNOMODULATION BY IVIG *IN VITRO*



7

Prevention of IgG immobilization eliminates artifactual stimulation of dendritic cell maturation by intravenous immunoglobulin *in vitro*

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ABSTRACT

Intravenous immunoglobulin (IVIg), a therapeutic preparation containing pooled human immunoglobulin G (IgG), has been suggested to inhibit differentiation and maturation of dendritic cells (DCs), however controversies exist on this issue. We aimed to re-investigate the effects of IVIg on human DC maturation and cytokine production, and to determine whether an artifactual determinant is involved in the observed effects. Human monocyte-derived DCs or freshly isolated blood myeloid DCs were cultured in the presence of IVIg *in vitro*, and the expression of maturation markers CD80, CD86, CD83 and HLA-DR were determined by flow cytometry, while production of IL-12 and IL-10 was measured by enzyme-linked immunosorbent assay (ELISA) and T-cell stimulatory capacity was determined in co-cultures with allogeneic $CD4^+$ T cells. Interestingly, we observed that IVIg did not inhibit, but instead stimulated spontaneous maturation and T-cell stimulatory ability of human DC, while leaving lipopolysaccharides (LPS)-induced DC maturation and cytokine production unaffected. Strikingly, prevention of IVIg binding to culture plate surface, or blocking of the activating FcγRIIa on DC, abrogated the stimulatory effect of IVIg on co-stimulatory molecule expression and on T-cell stimulatory capacity of DC, suggesting that IVIg activates DC upon IgG adsorption to the plastic surface. This study warrants for careful study design when performing cell culture studies with IVIg in order to prevent artifactual effects, and shows that IVIg does not directly modulate co-stimulatory molecule expression, cytokine production, or allogeneic T-cell stimulatory capacity of human DC.

INTRODUCTION

Dendritic cells (DCs) are professional APCs that play a crucial role in initiation of T-cell immunity (1). Human DCs express FcγRIIa (CD32a) by which they can sense multimeric IgG complexed to antigens or bound to cells and thereby become activated (2-4).

Intravenous immunoglobulins are preparations of IgG isolated from pooled human plasma. For its anti-inflammatory properties, IVIg is widely used to treat autoimmune and systemic inflammatory diseases. Bayry and colleagues (5) reported that IVIg can inhibit differentiation and maturation of human monocyte-derived DCs (moDCs), while in concert reducing IL-12 and enhancing IL-10 production. Interestingly, using monocytes from patients with X-linked agamaglobulinemia displaying defective DC differentiation, the same group found that IVIg promoted moDC differentiation (6). More recently, two reports (7, 8) likewise observed stimulation of moDC differentiation by IVIg, particularly when monocytes were "primed" with traces of LPS (8). Thus, the effects of IVIg on human DC differentiation are controversial.

Interestingly, a recent study demonstrates that IVIg can activate neutrophils to release elastase by artifactual adsorbance of IgG to plastic plate surface. By blocking IgG surface adsorbance, elastase release was prevented (9). Since DCs express the activating Fc-receptor FcγRIIa, which has low affinity for monomeric IgG and can be activated only in response to multimeric IgG (4), we speculated that DC may be susceptible for activation by immobilized IgG rather than by soluble, monomeric IgG within IVIg preparations, and that artifactual immobilization of IgG to the culture plate may account for the contradictory reports of the effect of IVIg on DCs *in vitro*.

Here, we sought to determine whether IVIg affects human DC maturation, T-cell stimulatory capacity, and cytokine production, using both moDCs and freshly isolated blood myeloid DCs (mDCs). Given that IgG can be adsorbed to plastic solid surfaces, we studied whether immobilization of IVIg to the culture plate surface might artificially influence DC maturation by crosslinking FcγRIIa.

METHODS

Reagents

Human IVIg preparations used were Intratect (kind gift from Biotest Pharma, Dreieich, Germany), Nanogam (Sanquin, Amsterdam, The Netherlands) and Kiovig (Baxter, Deerfield, IL). Human serum albumin (HSA, Sanquin) was used as protein control. Preparations were dialyzed against RPMI medium at 4°C to remove stabilizing agents and to obtain neutral

pH. Unless otherwise stated, IVIg concentration used was 10 mg/mL (0.06 M), which is similar to increments in serum IgG concentration in patients treated with IVIg at 1 to 2 g/kg for autoimmune disorders (10, 11).

Generation of monocyte-derived DCs and isolation of mDCs

$CD14^+$ monocytes were purified from blood of healthy volunteers using a negative selection immunomagnetic bead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. The purity of $CD14^+$ monocytes as analyzed by flow cytometry, defined as 7AAD-$CD14^+$ cells was 97±3%. $CD14^+$ cells were then cultured in 6-wells plate (Greiner Bio-One, The Netherlands) with 10 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 days in RPMI supplemented with penicillin, streptomycin and heat-inactivated 10% fetal calf serum (FCS), and were replenished every 2 days. At day 5, the cells exhibited the phenotypic characteristics of DC ($CD1a^+$DC-$SIGN^+CD14^-$). $BDCA1^+CD20^-$ mDCs were purified from blood of healthy volunteers by positive selection with PE-conjugated anti-CD1c mAb and anti-PE MACS-beads, after depletion of B cells with CD19-conjugated MACS-beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (12). The purity of mDCs as analyzed by flow cytometry was 92±8%.

Cell cultures

Immature moDCs harvested at day 5 or freshly isolated blood mDCs were cultured (80.000 cells/200 µl) in medium (RPMI supplemented with penicillin, streptomycin and 10% FCS), either or not supplemented with IVIg (10 mg/ml) or HSA (4.5 mg/ml), for 24 hours in a 96-wells round bottom plate (Corning Costar, Sigma Aldrich, St. Louis, MO). When indicated, LPS (1 µg/ml, Ultrapure, Invivogen, San Diego, CA) was added to the cultures after 3 hours of initial treatment with IVIg or HSA. To prevent IgG binding to the plate surface, wells were pre-incubated with 200 µl 0.05% poloxamer 407 (Sigma, St. Louis, MO) or heat-inactivated FCS at 4 °C for 24 hours and aspirated before cells were transferred into the wells. To assess the role of activating FcγRIIa on DC maturation by IVIg, we blocked FcγRIIa by pre-incubating the cells for 30 min with anti-CD32a blocking antibody (5 µg/ml; Clone IV.3, StemCell technology), after which medium, IVIg or HSA were added for the final 24 hour incubation.

Antibodies and flowcytometry

Cells were labeled with anti-CD1a-PE, anti-CD14-PE-Cy7 (both eBioscience), anti-HLA-DR-APC-Cy7 (BD Biosciences, San Jose, CA), anti-CD83-APC, anti-CD86-Pacific blue (both from Biolegend, San Diego, CA), anti-CD80-FITC (Beckman, Brea, CA), and 7-AAD (BD

Biosciences) and measured by flow cytometry (FACsCanto, BD Biosciences). Analyses were performed by FACS Diva software (BD Biosciences).

ELISA

Cytokines were quantified in cell-free supernatants using IL-10 and IL-12 ELISA kits (Ready-set-go, eBioscience).

Allogeneic T-cell stimulation

To determine their T-cell stimulatory ability, moDC were harvested, extensively washed to remove IVIg, and co-cultured in 96-wells plates with $1.5x10^5$ allogeneic $CD4^+$ T cells at DC:$CD4^+$ T cell ratios of 1:10, 1:20 or 1:40. After 4 hours, 5µg/ml brefeldin A was added, and 16 hours later cells from several wells were pooled and stained for surface expression of CD3 and CD4, fixed and permeabilized (Fix and Perm cell permeabilization kit, An der Grub, Vienna, Austria), and stained intracellularly for IL-2, IFNγ and CD137. At least $4x10^5$ $CD4^+$ T cells were measured by flowcytometry to reliably assess intracellular expression of IL-2, IFNγ and CD137. $CD4^+$ T cells were purified from PBMC isolated from a buffy coat obtained from a healthy blood bank donor using the negative CD4 selection kit of Miltenyi Biotec.

Statistical analysis

Differences between medium, IVIg or HSA were pair-wise analyzed by using the Student's T-test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data are expressed as mean±SEM.

RESULTS

IVIg stimulates human DC maturation

IVIg (10 mg/ml) added to immature moDC for 24 hours significantly enhanced CD80, CD86, CD83 and HLA-DR expression compared to control conditions without IVIg or in the presence of HSA (**Figure 1A**). Using 25 mg/ml IVIg, we found similar effects (data not shown). Expression of the co-stimulatory molecules was further increased after 48 hours and the stimulatory effects of IVIg disappeared (data not shown). However, IVIg did not modulate LPS-induced maturation of moDC (**Figure 1B**) or LPS-stimulated IL-12 or IL-10 production (**Figure 1C**). The observed suppression of IL-12 secretion was not specific for IVIg since it was also observed

upon addition of HSA, and should therefore be regarded as non-specific inhibition caused by addition of a high amount of protein. When lowering the LPS concentrations to 0.5 - 0.001 µg/ml, we still did not find any effect of IVIg on LPS-mediated moDC maturation (data not shown). Stimulation of spontaneous moDC maturation was also found when moDC were cultured with other IVIg brands (**Figure 1D**). Similar to its effects on moDCs, IVIg stimulated freshly isolated CD1c$^+$ mDC maturation (**Figure 1E**). Our data demonstrate that IVIg does not inhibit spontaneous or LPS-induced maturation or cytokine production of human DC, but instead stimulates spontaneous maturation.

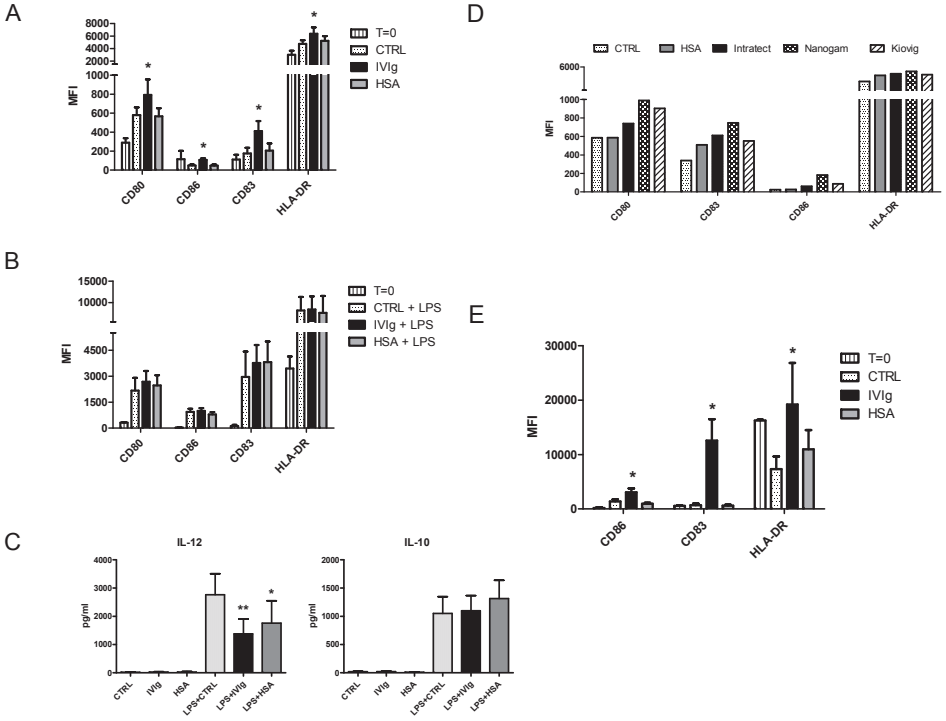

FIGURE 1. IVIg stimulates maturation of human mDC, but does not affect LPS-driven maturation. A) moDCs were generated by culturing human monocytes with 10 ng/ml GM-CSF and 10 ng/ml IL-4. At day 5, cells were harvested and 80,000 cells/200 µl were cultured in medium alone, with IVIg (10 mg/ml = 0.06 M), or with an equivalent molar concentration of HSA (4.5 mg/ml = 0.06 M), for 24 hours in a 96-wells round bottom plate (n=9). B) moDCs were stimulated with LPS (1 µg/ml) after 3 hours of pre-culture with medium, IVIg or HSA, and cultured for an additional 24 hours (n=4). Supernatants were harvested thereafter and C) IL-12 and IL-10 production were measured by ELISA (n=10). D) moDCs were treated with Intratect, Nanogam or Kiovig (all at 10 mg/ml), HSA (4.5 mg/ml) for 24 hours. E) CD19$^-$CD1c$^+$ mDCs were purified from blood of healthy donors and cultured with medium alone, IVIg (10 mg/ml), or HSA (4.5 mg/ml) for 24 hours. Maturation markers were measured by flow cytometry (n=9). * $p<0.05$, ** $p<0.01$, compared to CTRL and HSA, Student's paired t-test. Data are shown as means ± SEM from the indicated numbers of independent experiments.

Artifactual IgG surface binding induces DC maturation

In order to study whether IVIg stimulates DC maturation by binding to the plastic surface of the culture plates and therefore mimics immune complexes, we have first blocked the wells with poloxamer 407 at 0.05%, which is the lowest concentration capable of preventing IgG binding to plastic (9). However, we found that poloxamer 407 strongly induces DC maturation after 24 hours of culture (**Figure 2**), and therefore we continued by pre-coating the wells with FCS overnight to block IgG adsorption. FCS was used because of its high protein content and its presence in the culture medium in all conditions. Pre-treatment of

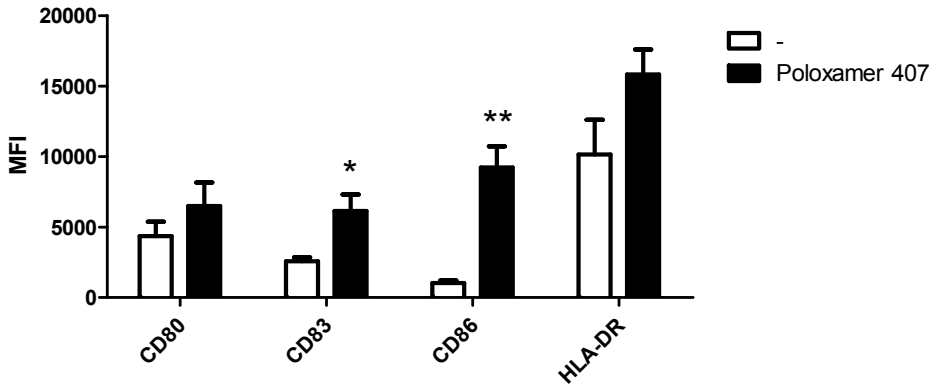

FIGURE 2. Poloxamer 407 induces DC maturation.
To prevent IgG binding to the plate surface, wells from 96-wells round bottom plate were pre-coated with 0.05% poloxamer 407 in PBS overnight at 4 °C after which PBS was removed. moDCs were generated by culturing human monocytes with 10 ng/ml GM-CSF and 10ng/ml IL-4. At day 5, cells were harvested and 80.000 cells/200 µl were added to wells with or without pre-coating with 0.05% poloxamer 407 and culture for 24 hours. * p<0.05, **< 0.01 compared to CTRL, Student's paired t-test. Data are shown as means ± SEM of 4 independent experiments.

the wells with FCS did not directly affect moDC maturation (**Figure 3A**). However, FCS coating abrogated induction of co-stimulatory molecule expression by IVIg, although expression of HLA-DR was still somewhat enhanced, suggesting that moDC maturation was largely caused by artifactual binding of IVIg to the culture plastic. Since DC recognizes immobilized IgG via FcγRIIa (13), we investigated the effect of blocking FcγRIIa. FγRIIa blocking on moDC cultured in non-FCS-pre-coated wells significantly abrogated the stimulatory effect of IVIg on moDCs, supporting that stimulation of moDC maturation by IVIg was exerted by binding of immobilized IgG to the activating FcγRIIa on moDCs (**Figure 3A**).

Finally, we determined the effect of IVIg on the T-cell stimulatory ability of moDC. MoDC were cultured in the presence or absence of IVIg for 24 hours, and after washing moDC to remove IVIg, allogeneic $CD4^+$ T cells were added. To detect allo-reactive $CD4^+$ T cells, expression of activation-induced CD137 was determined after 24 hours of co-culture (14, 15). MoDC stimulated allogeneic $CD4^+$ T cells in a dose-dependent manner (**Figure 3B**),

and the cytokine-producing T cells resided within the CD137-expressing fraction, confirming that CD137 expression adequately distinguishes allo-responding $CD4^+$ T cells (**Figure 3C**). Concordant with the effects of IVIg on co-stimulatory molecule expression, we found that moDC that were pre-cultured in the presence of IVIg activated allogeneic $CD4^+$ T cells markedly better compared to moDC that were pre-cultured in the absence of IVIg (**Figure 3B**). In contrast, moDC pre-cultured with IVIg in FCS-coated wells did not show enhanced allogeneic $CD4^+$ T-cell stimulatory ability. Collectively, these data show that upregulation of

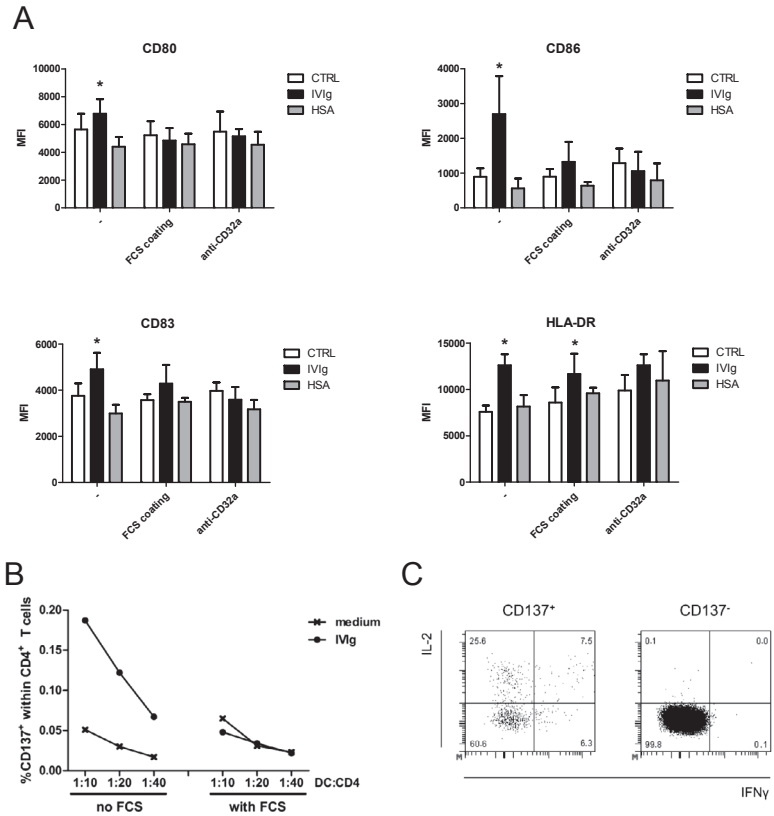

FIGURE 3. The stimulatory effect of IVIg on DC maturation is due to immobilization of IgG. MoDCs were generated by culturing human monocytes with 10 ng/ml GM-CSF and 10 ng/ml IL-4. At day 5, cells were harvested and 80.000 cells/200 µl were either or not pre-cultured with anti-CD32a (10 µg/ml) for 30 minutes, after which IVIg (10mg/ml) or HSA (4.5mg/ml) were added to the culture for an additional 24 hours. To prevent IgG binding to the plate surface, wells were pre-coated with 100% fetal calf serum (FCS) overnight at 4 °C after which FCS was removed before DCs were transferred to these wells and cultured in the presence or absence of IVIg or HSA (denoted in the graphs by 'FCS-coating'). A) After 24 hours maturation markers were measured by flow cytometry. * $p<0.05$, compared to CTRL and HSA, Student's paired t-test. Data are shown as means ± SEM of 4 independent experiments. B) After 24 hours moDC were harvested, washed, and thereafter co-cultured for 20 hours in 96-wells plates with $1.5x10^4$ allogeneic $CD4^+$ T cells at DC:$CD4^+$ T cell ratios of 1:10, 1:20 or 1:40. During the last 16 hours brefeldin A was added to enable intracellular cytokine detection. Allogeneic $CD4^+$ T cell activation was determined by measuring activation-induced CD137-expression. A representative experiment is shown. C) Intracellular cytokine staining demonstrates that all $CD4^+$ T cells that produce IL-2 and IFNγ in response to allogeneic moDC reside within the CD137-expressing cells.

co-stimulatory molecule expression and stimulation of allogeneic T-cell stimulatory capacity of DC by IVIg in cell culture results from artifactual binding of IgG to the cell culture plate.

DISCUSSION

In the current study, we re-investigated the effects of IVIg on human DC maturation, cytokine production, and allogeneic T-cell stimulatory capacity reported by Bayry and colleagues (5). Consistent with recent studies on the effects of IVIg on DC differentiation (7, 8), we did not observe inhibition of DC, but instead stimulation of the spontaneous maturation of both moDCs and blood-derived mDCs by IVIg. In addition, IVIg enhanced the capacity of moDC to stimulate allogeneic T cells, similar to the observations of Ballow et al who added IVIg to DC during the differentiation from monocytes (8). However, we found no evidence for inhibition of LPS-stimulated DC maturation. Interestingly, the stimulatory effect of IVIg on the expression of co-stimulatory molecules on DC was abrogated when IVIg binding to plastic surface was blocked, suggesting that immobilization of IgG to plate surface caused crosslinking of activating FcγRIIa on DCs. Supporting this, when we blocked FcγRIIa ligation, stimulation of DC maturation by IVIg was completely abrogated. Apparently, although FcγRIIa and FcγRIIb are both expressed on human DC and are probably simultaneously ligated by immobilized IgG, the net effect is DC activation, similar to the observations made by Boruchov et al (13). Likewise, when binding of IVIg to the culture plate was prevented, IVIg did not modulate the T-cell stimulatory capacity of moDC. Collectively, our results indicate that adsorption of IgG to the culture plastic significantly influences studies on the effects of IVIg on DC maturation.

In agreement with Bayry et al. (5), we observed suppression of LPS-stimulated IL-12 production by IVIg. However, this effect appeared to be non-specific, as HSA showed similar inhibition. Moreover, we did not observe any modulation of LPS-driven IL-10 secretion, as has been previously shown.

Differences between the study of Bayry et al. (5) and our study, such as the use of different IVIg brands, different concentrations of IVIg, and different brands of cell culture plates, may account for the opposite results. Bayry et al. (5) added 25 mg/ml IVIg to moDC *in vitro*, while we used 10 mg/ml IVIg, since the increments in serum IgG concentration we observed in patients treated with IVIg at 1 to 2 g/kg are about 10 mg/ml (10, 16). However, when we tested the effect of 25 mg/ml IVIg on DC maturation, we also found stimulation, and not inhibition, of DC maturation. In addition, we and Bayry et al. (5) both tested several brands of IVIg. However, it is unclear whether different brands of culture plastic were used, and if IgG adsorption to plastic surfaces differs between different polystyrene based surfaces (9).

Speculations

Our results show that IgG surface binding may be a potential artifact when studying the effects of IVIg on DC. This warrants for careful study design and critical interpretation of results of cell culture studies on the effects of IVIg on cells expressing FcγRs. In most previous *in vitro* studies concluding that IVIg activates human or murine DC (6, 8, 17-20), macrophages (21), or neutrophils (22), no precaution was taken to prevent IgG-adsorption to the culture plastic. Therefore, these studies did not distinguish between activation caused by soluble IgG present within IVIg either in monomeric, dimeric, or polymeric form, and artifactual activation by surface-bound IgG. Interestingly, several of these studies showed that activation of the cells by IVIg was mediated by activating FcγRs (17-19, 21, 22), like in the present study. Our observations demonstrate that prevention of IgG binding to the solid surface of the culture plates is required to obtain conclusive results from *in vitro* studies on the effects of IVIg on cells that express activating FcγRs. For this purpose, proteins like human serum albumin (23) or bovine serum albumin, protein-containing solutions like FCS or human serum, or surfactants such as poloxamer 407 (9), can be used. Importantly, it should first be determined which of the potential blocking agents is compatible with the cell type studied. Supporting our recommendation, studies that took precautions to prevent adsorption of IgG to the culture plastic demonstrated that IVIg does not directly stimulate neutrophils functions *in vitro* (9, 23).

The final conclusion from our study regarding the direct effect of IVIg on human DC must be that IVIg in itself does not modulate their co-stimulatory molecule expression, allogeneic T-cell stimulatory capacity, or cytokine production. This conclusion is supported by a recent study showing that IVIg does not modulate expression of co-stimulatory molecules on murine bone marrow-derived DC, but instead inhibits antigen presentation resulting in reduced antigen-specific T cell responses, by interference of IVIg with immune complex binding to activating FcγRs expressed on DCs (24).

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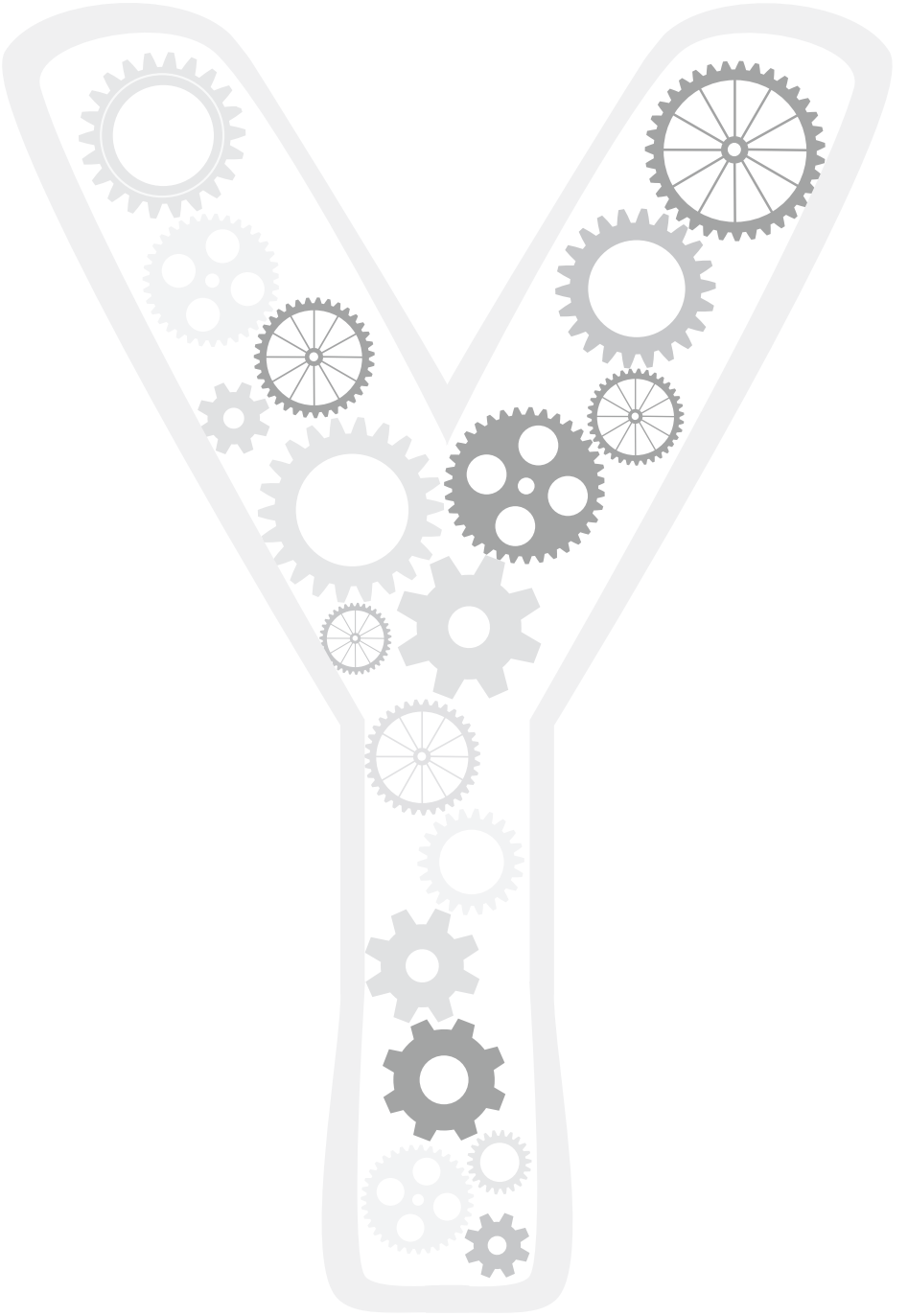

8

T-cell inhibitory capacity of hyperimmunoglobulins is influenced by the production process

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ABSTRACT

Intravenous immunoglobulin (IVIg) preparations are widely used for anti-inflammatory therapy of autoimmune and systemic inflammatory diseases. Hyperimmunoglobulins enriched in neutralizing antibodies against viruses can, in addition to their virus-neutralizing activity, also exert immunomodulatory activity. Previously, we observed that Cytotect®, an anti-CMV hyperimmunoglobulin, was less effective in suppressing human T-cell responses *in vitro* compared to Hepatect® CP, an anti-HBV hyperimmunoglobulin. We hypothesized that the poor immunomodulatory activity of Cytotect® results from treatment with β-propiolactone during the manufacturing process. The manufacturer of these hyperimmunoglobulins has now introduced a new anti-CMV hyperimmunoglobulin, called Cytotect® CP, in which β-propiolactone treatment is omitted. Here we show that Cytotect® CP inhibits PHA-driven T-cell proliferation and cytokine production with similar efficacy as Hepatect® CP, whereas the former Cytotect® does not. In addition, Cytotect® CP inhibits allogeneic T-cell responses better than Cytotect®. Our results advocate the use of hyperimmunoglobulins that have not been exposed to β-propiolactone in order to benefit from their immunomodulatory properties.

INTRODUCTION

Intravenous immunoglobulins (IVIg) have anti-inflammatory properties and are commonly used for the treatment of autoimmune and systemic inflammatory diseases (1, 2). IVIg preparations enriched in neutralizing anti-viral antibodies (hyperimmunoglobulins) are used for prevention of viral infections. We (3) and others (4) have previously shown that hyperimmunoglobulins enriched in neutralizing antibodies against CMV or HBV also exert immunomodulatory activity in addition to their virus-neutralizing activity. However, we observed that Cytotect®, an anti-CMV hyperimmunoglobulin, was less effective in suppressing human T-cell responses *in vitro* compared to Hepatect® CP, an anti-HBV hyperimmunoglobulin, both manufactured by Biotest AG (3). We assumed that the difference in immunomodulatory capacity between these two hyperimmunoglobulins was related to differences in their manufacturing processes. While Hepatect® CP is virus-inactivated by treatment with caprylic acid, tri-n-butyl phosphate and polysorbat 80, virus-inactivation of Cytotect® includes treatment with β-propiolactone, which influences the conformation of IgG by covalent modification of amino-groups (5).

Biotest AG has now introduced a new anti-CMV hyperimmunoglobulin, called Cytotect® CP, which is manufactured according to the same process as Hepatect® CP, omitting β-propiolactone treatment. The aim of the current study is to evaluate the T-cell inhibitory capacity of Cytotect® CP in comparison to the former Cytotect® and to Hepatect® CP.

MATERIALS AND METHODS

Hyperimmunoglobulins and human serum albumin

Cytotect®, Hepatect® CP and Cytotect® CP were obtained from Biotest AG, Dreieich, Germany and Human serum albumin (HSA) was purchased from Sanquin (Amsterdam, The Netherlands). All agents were dialyzed against RPMI culture medium (Gibco) at 4°C to remove stabilizing agents and to obtain physiological pH.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation from buffy coats of six different blood bank donors. Human splenocytes were isolated from splenic tissue derived from a multi-organ donor, and mononuclear cells (MNC) were isolated by Ficoll gradient centrifugation. PBMC ($5x10^4$) in 200 µl RPMI supplemented with

10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, were stimulated by addition of phytohemagglutinin (PHA; Sigma) at a final concentration of 5 µg/ml, or by addition of $5x10^4$ human splenic MNC. After 5 days of culture in the absence or presence of dialyzed hyperimmunoglobulins (5, 10 or 15 mg/ml) or HSA (7 mg/ml; which controls for the addition of protein in a molar concentration similar to 15 mg/ml of the dialyzed hyperimmunoglobulins), 50 µl of culture supernatant was harvested to determine IFN-γ production, and 0.5 µCi [^{3}H]-thymidine was added. Eighteen hours later, T-cell proliferation was assessed by [^{3}H]-thymidine incorporation into the cells. IFN-γ levels in supernatants were determined by ELISA (Invitrogen Life Technologies) according to the manufacturer's instructions. Each condition was tested in triplicate from which means were calculated. These means were used in statistical analyses.

Statistical analysis

Results are depicted as means and SEM of 6 independent experiments with PBMC from 6 different subjects. Differences in measured variables between culture conditions were pairwise analyzed by the Wilcoxon Signed-Rank Test using SPSS version 20. *p*-values <0.05 were considered to indicate significant differences.

RESULTS

Effects of hyperimmunoglobulins on human T-cell responses to PHA

PBMC were stimulated by PHA for 5 days in the presence of either Cytotect®, Hepatect® CP or Cytotect® CP, and proliferation of T cells was assessed by [^{3}H]-thymidine incorporation. In accordance with our previous findings (3), Cytotect® did not inhibit PHA-driven human T-cell proliferation, while Hepatect® CP significantly suppressed PHA-stimulated T-cell proliferation at all concentrations tested (5-15 mg/ml) (**Figure 1A**). Interestingly, the new anti-CMV hyperimmunoglobulin formulation (Cytotect® CP) inhibited PHA-stimulated T-cell proliferation to a similar extent as Hepatect® CP did. Importantly, human serum albumin (HSA) did not inhibit T-cell proliferation, implying that inhibition of T-cell proliferation is not solely caused by the addition of protein to the cell cultures. The differences in T-cell suppressive ability between the old and new anti-CMV hyperimmunoglobulin formulations were confirmed at the level of IFN-γ production, which was significantly suppressed by Hepatect® CP and Cytotect® CP, but not by Cytotect® (**Figure 1B**). Collectively, these data show that Cytotect® CP can successfully inhibit mitogen-stimulated human T-cell activation.

Effects of hyperimmunoglobulins on human T cells upon allogeneic stimulation

Subsequently, we compared the effect of the hyperimmunoglobulins on T-cell activation after allogeneic stimulation by splenocytes. After a 5 day co-culture of PBMC and allogeneic human splenocytes, we found that all three hyperimmunoglobulin reduced T-cell proliferation (**Figure 1C**). Thus, while Cytotect® did not inhibit PHA-stimulated T-cell proliferation and cytokine production, it did suppress allogeneic T-cell activation, which was in accordance with our previous findings (3). However, at all concentrations tested (5-15 mg/ml) Cytotect® CP inhibited allogeneic T-cell proliferation significantly stronger than Cytotect® (**Table 1**). Likewise, Cytotect® CP suppressed IFN-γ production by T cells upon allogeneic stimulation as effectively as Hepatect® CP did (**Figure 1D**), while Cytotect® inhibited IFN-γ secretion only partially when added at concentrations of 10 and 15 mg/ml, and no significant suppression was observed at a concentration of 5 mg/ml. These data therefore show that Cytotect® CP more potently inhibits allogeneic T-cell responses than the previous anti-CMV formulation, Cytotect®.

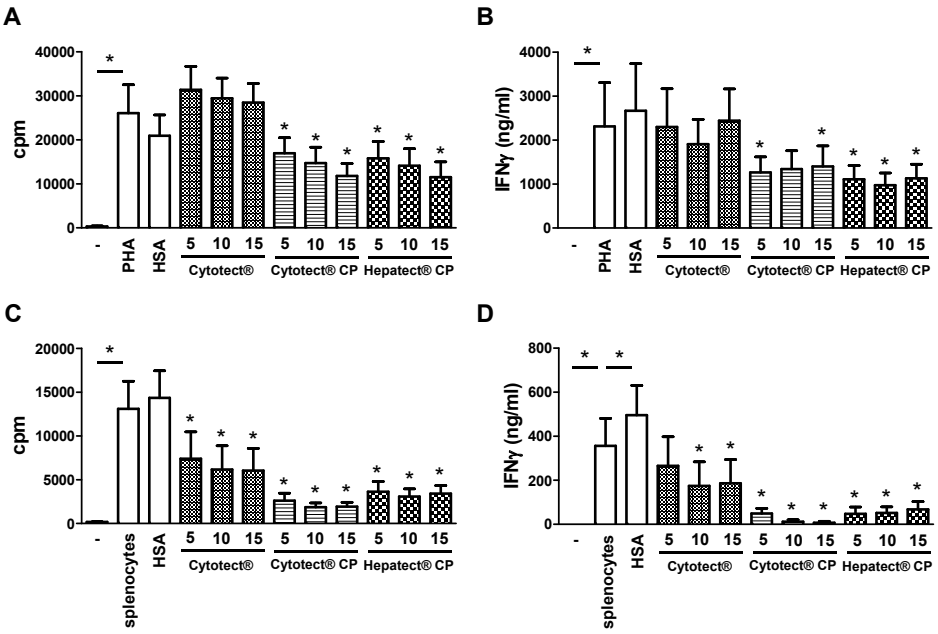

FIGURE 1. Effect of hyperimmunoglobulins on human T-cell responses.
Human PBMC ($5x10^4$) were stimulated with 5 µg/ml PHA (A-B) or by addition of $5x10^4$ human splenic MNC (C-D) in the presence or absence of hyperimmunoglobulins or HSA. (A, C) After 6 days proliferation was assessed by [^{3}H]-thymidine incorporation. (B, D) IFN-γ was determined in culture medium collected at day 5 of the cultures. Data are depicted as means and SEM of 6 independent experiments with PBMC from different donors. * $p < 0.05$ compared to PHA-stimulated PBMC (A-B]) or splenocyte-stimulated PBMC (C-D) in the absence of hyperimmunoglobulins or HSA.

TABLE 1. Effects of Cytotect® CP and Cytotect® on allogeneic T-cell proliferation

	proliferation upon allogeneic stimulation	
Cytotect® CP vs Cytotect®	% inhibition[1]	P-value[2]
5 mg/ml	66%	0.028
10 mg/ml	72%	0.046
15 mg/ml	70%	0.046

[1] The percentage of inhibition is defined as the average drop in proliferation when cultured in the presence of Cytotect® CP compared to Cytotect®.

[2] Depicted P-values were calculated in paired comparisons of [^{3}H]-thymidine incorporation between cultures of allogeneic splenocytes and PBMC in the presence of the indicated concentration of either Cytotect® CP or Cytotect®.

DISCUSSION

The current study shows that the ability of Cytotect® CP to inhibit T-cell proliferation and IFN-γ secretion is equivalent to Hepatect® CP and significantly better compared to the former anti-CMV hyperimmunoglobulin formulation Cytotect®. In a previous study, we reported that a polyvalent IVIg formulation (Intraglobin® CP) has a similar potent T-cell inhibitory capacity (6). We propose that the differences in T-cell inhibitory effect between Cytotect® on the one hand and Hepatect® CP or Intraglobin® CP on the other hand are related to differences in their production process. In order to reduce viral contamination, Cytotect® is treated with β-propiolactone during the production process (7), while β-propiolactone treatment is not included in the production of Hepatect® CP and Intraglobin® CP. β-Propiolactone modifies IgG by covalent modification of amino-groups, thereby disturbing Fc-dependent functions, like Fcγ-receptor binding, protein A binding, and complement fixation (5).

Recent studies suggest that IVIg has no direct effect on T cells, but rather modulates the activity of antigen-presenting cells (APCs) (8). IVIg can interfere with antigen uptake and presentation of antigenic peptides in MHC-molecules (9, 10). However, in allogeneic mixed-lymphocyte reactions (MLR), as were used in the present study, T cells do not so much respond to antigenic peptides presented on MHC-molecules, but rather to intact allogeneic MHC molecules, arguing against interference with antigen presentation as the main mechanism by which the hyperimmunoglobulins suppressed T-cell activation in MLR (11). In a previous study, we found that a main mechanism by which IVIg inhibits MLR responses is by stimulating depletion of APCs via stimulation of NK cell-mediated antibody-dependent cellular cytotoxicity (12). This mechanism requires binding of the IgG Fc-parts to Fcγ-receptor III on NK cells. The superiority of Cytotect® CP and Hepatect® CP compared to Cytotect® in suppressing allogeneic T-cell activation may therefore be due to reduced binding of β-propiolactone-treated IgG to Fcγ-receptor III on NK cells. Another mechanism by which IVIg can suppress allogeneic T- cell responses by IVIg involves activation of regulatory T cells (13), which is hypothesized to be mediated by specific peptides derived from the IgG Fc-

part, called Tregitopes, that are recognized by regulatory T cells via MHC class II (14, 15). This mechanism may also be dysfunctional in case of Cytotect® due to chemical modifications of the Fc-part.

Recently, it was shown that inhibition of PHA-induced T-cell activation by IVIg is caused by binding of immunoglobulins to PHA. IVIg thereby blocks the interaction between PHA and T cells, resulting in diminished T-cell activation (15). Likely, the altered conformation of the immunoglobulins present in Cytotect® diminishes their interaction with PHA, resulting in the observed inability of Cytotect® to inhibit PHA-driven T-cell proliferation.

In conclusion, Cytotect® CP and Hepatect® CP have superior capacities to suppress human T-cell responses *in vitro* compared to Cytotect®. This difference is probably related to omission of β-propiolactone treatment from their production process. High-dose therapy with non-specific IVIg is effective in prevention of antibody-mediated rejection (16) and in treatment of acute rejection after kidney transplantation (17, 18). Likewise, treatment with hyperimmunoglobulins against HBV or CMV with the aim to prevent viral re-infection after organ transplantation is associated with less rejection episodes (3, 19-22). Based on our current data, we advocate the use of hyperimmunoglobulins that have not been exposed to β-propiolactone for clinical management of CMV and HBV infection after organ transplantation in order to benefit from the immunomodulatory properties of these hyperimmunoglobulins.

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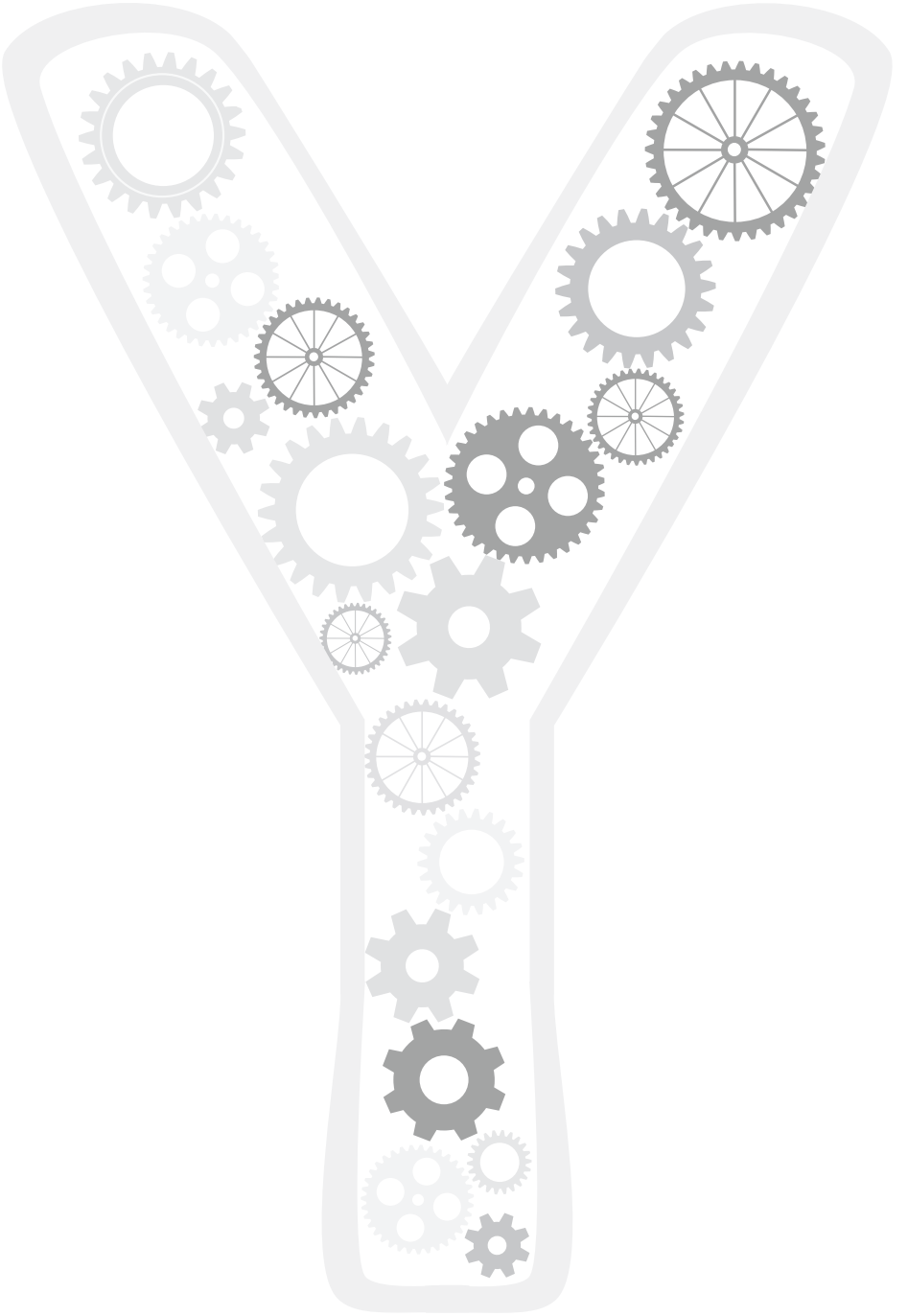
PART IV

SUMMARY AND GENERAL DISCUSSION



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Summary and general discussion

Liver transplantation (LTx) has developed from an experimental treatment in the sixties to a standardized procedure for patients with end-stage liver disease since 1983. In the early years, most of the effort was put in improvement of short-term survival after transplantation. Thanks to improvements in terms of surgical techniques, procurement, allocation and preservation of donor liver, risk stratifications of LTx candidates and peri-operative care after LTx, an excellent short-term survival has been achieved (one-year survival of >80%) (1, 2). In addition, advances in immunosuppressive drugs have contributed to a better short-term survival. However, with the better short-term survival, long-term complications of immunosuppressive agents have been uncovered, which include cardiovascular disease, renal insufficiency and malignancy. The increased risk for *de novo* cancer is of particular concern and in the first part of this thesis we have studied the incidence of this complication and in particular potential influences of implementation of different immunosuppressive regimens.

1. *De novo* malignancy after LTx

The higher risk for the development of *de novo* cancer is a direct consequence of relative oversuppression of immune surveillance in order to prevent acute rejection. To assess whether the increased risk of cancer is related to a certain immunosuppressive regimen, in **chapter 2** we have performed a retrospective study in our LTx cohort. First we explored the incidence of *de novo* cancer in our LTx patients and calculated the standardized incidence ratio. Here we found that the risk for developing cancer after LTx is 2.2-times higher compared to the general Dutch population. This ratio is relatively low compared to other studies. The most common cancer types occurring after LTx were non-melanoma skin cancer and post-transplant lymphoproliferative disease (PTLD). The overall survival rate of patients who developed cancer was significantly lower compared to cancer-free patients (at 10 years: 51% vs. 79%). However, patients who developed non-melanoma skin cancer and PTLD did not show a significantly lower survival rate compared to cancer-free patients. The rather good prognosis of PTLD in our center is possibly due to adequate treatment with rituximab, chemotherapy and reduction of immunosuppression. In contrast, patients who developed other cancer types, such as pulmonal, gastrointestinal or gynaecological cancer had a dramatically impaired survival rate (1-year survival rate of <30%).

Since there is a delicate balance between effective prevention of rejection and overimmunosuppression, we studied whether improvement of immunosuppressive regimens over the years has influenced the risk of *de novo* cancer over time. According to our expectations, the first period of LTx in our center (1989-1992) showed the highest incidence of *de novo* cancer. This may be due to aggressive immunosuppressive treatments used in the early years. After this initial period, a considerable decline of *de novo* cancer incidence was observed after each period of transplantation (1989-1992 > 1993-1996 > 1997-2000 > 2001-2004). Strikingly, patients who were transplanted in the most recent time period included in

this study (2005-2007) showed a significant rise in the incidence of *de novo* cancer. Interestingly, we found that the factor that was significantly associated with this increased cancer risk in recent years was the use of cyclosporine. Since cyclosporine therapy was also used in the earliest time periods, this finding suggests a change in the period after 2005 regarding the use of cyclosporine. Indeed, from 2005, a significant change has been made in terms of dosing strategy of cyclosporine compared to earlier time periods, which was dosing adjustment based on 2-hour postdose levels (C2) instead of predose level (C0) monitoring. C2 dosing strategy was introduced since C2 level was considered to be a better predictor of the area under the curve (AUC) of total cyclosporine drug exposure. However, our findings suggest that this dosing strategy might have led to overdosing of the immunosuppressant. Indeed, patients treated with cyclosporine-C2 showed significantly less rejection, suggesting that C2 dosing strategy leads to relatively higher immunosuppression with consequently higher cancer rate. This effect was mainly seen during the induction phase (< 1 year). Importantly, cyclosporine does not only lead to increased incidence of *de novo* cancer, but also to more aggressive cancer with a higher mortality. Moreover, mainly younger patients were affected by cyclosporine in terms of cancer development, while younger patients in general have a lower risk. Therefore, our data suggest that cyclosporine treatment is a significant determinant for *de novo* cancer development after LTx. Much more attention is needed for improving the long-term outcome of LTx patients by means of minimizing side effects of immunosuppressive drugs. Current drugs are very effective in suppressing immune responses; however, the balance towards oversuppression is unfortunately delicate, as can be seen from the results described in chapter 2. The principal goal is to use an immunosuppressive treatment that minimizes the risk of acute rejection while maximizing the long-term survival. The aim of current thesis was to investigate the immunomodulatory mechanisms of an alternative immunosuppressive agent: intravenous immunoglobulin (IVIg).

2. IVIg as alternative safe immunomodulating treatment

The idea to study the immunosuppressive properties of IVIg was born after the observation in our center that liver transplant patients treated with anti-HBs IVIg had significantly lower risk of cell-mediated acute rejection (3), which was later on confirmed in a multi-center study (4). Similar effects of IVIg have been observed in renal transplant patients (5). The ultimate aim of studies in this field is to unravel the immunomodulatory pathways stimulated by IVIg and to develop compounds that target these pathways. Such compounds may be suitable for safe immunosuppressive maintenance therapy after transplantation.

In recent years, puzzle pieces have slowly come together regarding the mechanisms responsible for suppression of the cellular graft rejection. Inhibition of myeloid dendritic cells (mDCs) (3, 6, 7) and stimulation of regulatory T cells (Tregs) (8-10) seem to form the basis of the graft protective effect of IVIg. However, today, most of the evidence is derived from *in*

vitro and mouse studies, and evidence from patient studies is limited. Therefore, in the next part of the thesis we mainly focus on the effect of IVIg therapy on DC and Treg functions in patients *in vivo* and provide novel mechanistic insights.

3. IVIg modulates mDC function via IL-33-mediated Th2 cytokine secretion

A major breakthrough in our understanding of the mechanisms by which IVIg modulates antibody-mediated immune responses in the last decade is the finding that sialylated IgG binding to macrophages in the mouse spleen results in upregulation of inhibitory FcγRIIb receptors on effector macrophages, and thereby prevents their activation by auto-antibody immune complexes (11). Recent research has uncovered the missing link between these two types of macrophages by showing that IVIg stimulates IL-33 production in the spleen, which in turn induces IL-4 and IL-13 production by basophils, and thereby enhances expression of the FcγRIIb on effector macrophages in the joint in an auto-antibody mediated mice arthritis model (12) (see **chapter 6**, **Figure 1**). The question raised from this animal study is whether this mechanism plays a role in humans *in vivo*, since there are relevant physiological differences between mice and men, among which differences in FcγR expression patterns. In **chapter 3**, we investigated whether IVIg therapy affects IL-33, Th2 cytokine and receptor expression in patients, and explored the causative links between these phenomena using human cells *in vitro*. We showed that immediately after IVIg infusion, IL-33 and the Th2 cytokines IL-4 and IL-13 plasma levels significantly increased in patients. Interestingly, the increase in plasma IL-33 was correlated with the rise in plasma IL-4 and IL-13. This finding suggests that IVIg stimulates a similar IL-33-Th2 cytokine pathway in humans as in mice. However, in contrast to mice, we did not find an increase in inhibitory FcγRIIb on myeloid cells, but a decrease in the activatory FcγRIIa on mDCs.

To investigate whether there is a causal relation between the rise in IL-4 or IL-13 and the decrease in the activatory FcγRIIa on circulating mDCs, we treated mDCs with IL-4 or IL-13 *in vitro*, which resulted in significantly decreased FcγRIIa expression. The reduced FcγRIIa resulted in a decreased responsiveness of mDCs to immune complexes in terms of maturation and pro-inflammatory cytokine (IL-8, TNF-α) production. Thus, in patients, we found evidence that stimulation of the IL-33-IL-4/IL-13 pathway may play a role in the clinical benefits seen after IVIg treatment in antibody-mediated inflammation, e.g. antibody-mediated rejection. However, the final effect of this pathway is not enhancement of inhibitory FcγRIIb, as has been shown in mice, but reduction of the activating FcγRIIa expression. The observed difference in FcγR modulation by IVIg treatment in humans compared to mice is not surprising, since there are major fundamental differences in FcγRs expression pattern between mice and men. We further elaborated on this subject in **chapter 6**.

An important activator of cellular immune responses is the Th1 cytokine IFN-γ. In terms of allograft rejection, IFN-γ contributes to graft injury by direct cytotoxicity and indirectly by inducing migration and activation of macrophages and mDC. Moreover, IFN-γ increases MHC-class II molecules on APCs, which thus increases the effectiveness of antigen recognition and enhances graft rejection (13). Previously it has been demonstrated in mice that IVIg down-regulates the IFN-γ receptor subunit 2, IFNγR-2, on macrophages, thereby rendering the cells refractory to activation by IFN-γ (14). Interestingly, in **chapter 3** we also provide evidence that this immunomodulatory mechanism is involved in the anti-inflammatory effects of IVIg in humans. Soon after IVIg treatment, IFNγR2 expression on circulating mDCs in patients is diminished. Inhibition of IFNγR2 expression on mDC by treatment with IL-4 and IL-13 was demonstrated *in vitro*. Upon suppression of IFNγR2 expression by IL-4 or IL-13, mDC maturation and the production of chemokine IP-10 (CXCL10) was dramatically hampered. In the context of allograft rejection, the latter effect is interesting, since intragraft production of IP-10 has been shown in cardiac (15), lung (16) and renal (17) allograft rejection. Increased IP-10 resulted in attraction of activated T cells and macrophages bearing the corresponding chemokine receptor, CXCR3. Inhibition of chemokine production may result in prolonged allograft survival, even when rejection already has begun. Additionally, IFN-γ not only induces IP-10, but also other chemokines, such as monokine induced by IFN-γ (Mig/CXCL9) and IFN-inducible T-cell-α chemoattractant (I-TAC/CXCL11), that may also play a role in alloresponsive T cell attraction to the graft (18).

Since we found that IFNγR2 is downregulated by Th2 cytokines IL-4 and IL-13 *in vitro*, we show for the first time that IFNγR modulation is another effect of the IVIg-IL-33-Th2 cytokine pathway. Together with our finding of FcγR modulation, we have demonstrated in patients and by using human cells *in vitro* that IVIg stimulates IL-33 production leading to a cascade of IL-4 and IL-13 production resulting in downregulation of FcγRIIa and IFNγR expression on mDC (see **chapter 6**, **Figure 1**). This cascade may ultimately suppress antibody-mediated as well as cellular immune reactions, which both contribute to acute and chronic rejection after LTx. Also, some cases of hyperacute rejection after LTx in ABO-incompatible liver transplant recipients have been described, which were successfully treated with high-dose IVIg (19). The underlying mechanism is not fully clear, but enhancement of autoantibody clearance by blocking FcRn, neutralizing anti-idiotypic effects and blocking of complement activation probably play a role (11, 20). In addition, our findings as demonstrated in **chapter 3** suggest that inhibition of immune-complex-mediated activation by FcγR modulation via IL-33-IL-4/IL-3 cascade may have an important contribution to this effect.

Interestingly, it may be likely that the IVIg-IL-33 cytokine pathway is also (partially) responsible for Treg expansion and enhanced suppressive capacity. This will be further discussed in the next paragraph.

4. IVIg enhances Treg activation and suppressive capacity in patients

Tregs are the key players in the maintenance of self-tolerance. Many studies have established the involvement of natural Tregs in controlling autoimmunity, inflammatory disorders, and immune responses to tumors and various infectious agents (21). Importantly, a critical role for Tregs in the induction and maintenance of transplant tolerance by suppressing alloreactive T cells has been established in experimental transplantation models (22-32) and patient studies (33-36). Interestingly, over the last few years, accumulating evidence has shown that Treg activation and suppressive function can be induced by IVIg *in vitro* and in experimental animal models, and this effect may contribute to the anti-inflammatory effects of IVIg. The first evidence of a positive effect of IVIg on Tregs was provided by *in vitro* experiments. By culturing human $CD4^+$ cells with IVIg *in vitro*, increased expression of FOXP3, TGF-β and IL-10 in $CD4^+CD25^+$ Tregs and increased suppressive function were found (9). Also, IVIg enhances the number of $FOXP3^+$ T cells among memory $CD4^+$ T cells *in vitro* (37). Following these *in vitro* observations, several studies in animal models have shown that IVIg treatment activates Tregs, stimulating their expansion and/or suppressive capacity and thereby preventing the development of inflammatory diseases. These animal models include skin transplant rejection (38), experimental autoimmune encephalomyelitis (8) and herpes simplex virus-induced encephalitis (39). However, whether this phenomenon holds true in humans is largely unknown. To explore this clinically important question, in **chapter 4**, we have studied whether IVIg can induce Treg activation in patients and whether it can enhance Treg suppressive capacity *ex vivo* by collecting blood from IVIg-treated patients before and after IVIg treatment at different time points. We did not include LTx patients who received anti-HBs IVIg, since those patients are on conventional immunosuppressive drug treatment, known to inhibit Tregs (40). Instead, we have included patients who were treated with IVIg as mono-therapy for several autoimmune diseases and immunodeficient patients (see **chapter 4**, **Table 1A** and **1B**). In exceptional cases, these patients were co-treated with corticosteroids; but we did not see any significant influence of this drug on Treg activation compared to patients not treated with corticosteroids. In this study, we found that IVIg enhanced selectively the activation status of circulating Tregs, as reflected by increased FOXP3 and HLA-DR expression, while it did not affect conventional T cells. *Ex vivo*, we demonstrated that IVIg enhanced the suppressive capacity of circulating Tregs nearly two-fold one week after IVIg infusion. Interestingly, a stimulatory effect of IVIg on Tregs was only found in patients who were treated with high-dose IVIg, and not low-dose. This observation confirms the generally accepted hypothesis that a high-dose IVIg treatment regimen is required in order to gain anti-inflammatory activity. The high-dose requirement supports the idea that there is only a minor fraction within IVIg that has immunosuppressive activity. From this study we conclude that IVIg not only enhances Treg function in mice, but also in humans *in vivo*. Thus, there is growing evidence that IVIg can enhance Treg function

in experimental animal models, *in vitro* and, as we now have shown, in patients, which may contribute to the clinical benefits seen in patients with inflammatory disorders treated with IVIg. Since conventional immunosuppressive drugs used for treating autoimmune diseases and allograft rejection, in particular calcineurin inhibitors, suppress both Teff and Tregs (40-47), selective activation of Tregs by IVIg, but not Teff, suggests that IVg may be superior to classical immunosuppressive drugs.

Next, we have performed pilot studies *in vitro* aimed at investigating the mechanism by which IVIg stimulates Tregs. We have isolated human $CD4^+CD25^+$ Tregs from peripheral blood of healthy subjects and treated them with IVIg or HSA with or without the presence of allogeneic monocyte-derived DCs (moDCs). Interestingly, from our pilot study we have demonstrated that IVIg did not directly stimulate Tregs, but significantly enhanced early-activation marker CD69 expression on Tregs in the presence of moDC (**Figure 1**, unpublished data). These preliminary data suggest that IVIg can activate Tregs via an mDC-dependent manner. Indeed, several recent studies using mouse models or in vitro studies have shown a DC-dependent activation of Tregs by IVIg (see **chapter 6**, **Figure 3**). A recent study by Trinath

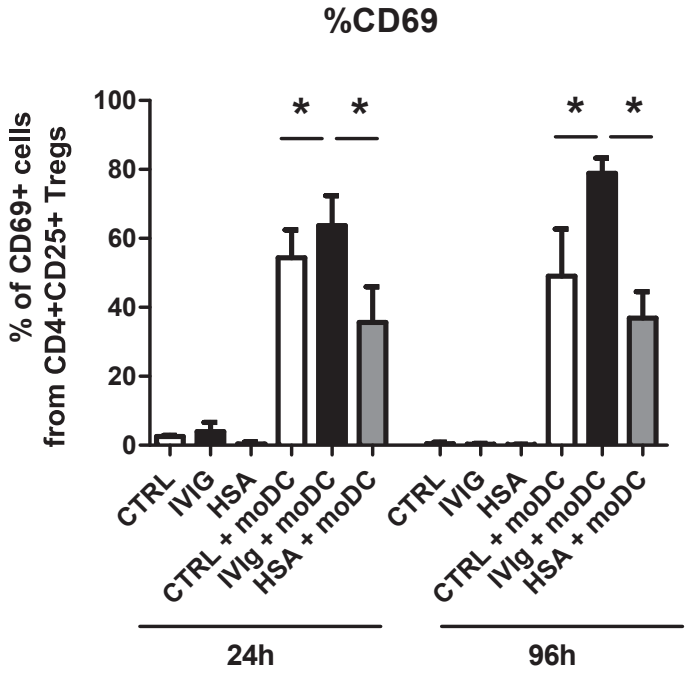

FIGURE 1. IVIg activates Tregs via a moDC-dependent manner.
$CD4^+CD25^+$ Tregs were isolated from healty blood donors and incubated with medium, IVIg (10 mg/ml) or HSA (4.5 mg/ml) with or without the presence of immature monocyte-derived dendritic cells (moDCs), which were generated by culturing human monocytes with 10 ng/ml GM-CSF and 10 ng/ml IL-4 and cultured for 5 days. Cells were co-cultured in a ratio of Tregs: moDC = 50,000:25,000. After 24 h or 96 h, cells were harvested and CD69 activation marker was measured on $CD4^+CD25^+$ cells by using flow cytometry. N=4, * $P<0.05$, Student's paired t-test

et al. has shown a central role for mDCs in expanding Tregs in an EAE mouse model (48). The authors showed that IVIg enhances prostaglandin E2 synthesis by moDCs via induction of cyclooxygenase 2, which subsequently stimulates expansion of Tregs. This mechanism partially depended on interaction of IVIg with DC-SIGN in a $F(ab')_2$-dependent manner. Another study showed that IVIg induced generation of antigen-specific Tregs from non-Tregs by inducing tolerogenic DCs in an antigen-driven allergic airway model (49). Recently, an immunoreceptor tyrosine-based inhibition motif (ITIM)-linked C-type lecin receptor, the so called DC immunoreceptor, has been identified to be responsible for the induction of tolerogenic DC via sialylated IVIg (50). Alternatively, an interesting hypothesis for DC-dependent Treg activation by IVIg is the identification of Tregitopes in human IgG. De Groot et al. have demonstrated that Tregs can be activated and expanded via the presentation of Treg-activating peptides derived from IgG, the so called Tregitopes, by APCs (51). However, arguing against the presence of Tregitopes, in studies in the HSE model, the authors showed that purified sialylated IgG was unable to induce Treg, even though sialylated IgG should contain Tregitopes (39). Therefore, other mechanisms are probably involved in Treg stimulation by IVIg (see **chapter 6**, **Figure 3**). In our pilot study, we studied whether crosslinking of natural antibodies within IVIg that may react to molecules on Tregs, such as CD4, CD5, CD95, TCR and MHC (52, 53), by FcγR ligation on moDCs is a possibility. Therefore, we have co-cultured Tregs with monocytes that also express FcγRs. However, since co-culture with monocytes did not show similar activation, crosslinking is less likely to be the responsible mechanism.

With regard to the selectivity of IVIg for the activation of Treg, results from *in vitro* and animal model studies by other groups corroborate our finding (8, 9, 37, 38). Several hypotheses can be postulated why IVIg selectively activates Tregs, but not Teff. First, presentation of Tregitopes by APCs towards Tregs might explain the selectivity (51). Second, cytokines that specifically activate Tregs may play a role, for example TGF-ß. IL-10 is probably not involved in this mechanism, as we did not find increased IL-10 after IVIg treatment as described in **chapter 3**. Another interesting cytokine that may play a role is IL-33. It is conceivable that Treg expansion and enhanced suppressive capacity are linked to the IVIg-IL-33-Th2 cytokine pathway, as described in **chapter 3** and reviewed in **chapter 6**. Three recent studies (54-56) in mice have shown that IL-33 not only induces Th2 cytokine production, but also expands $CD4^+FOXP3^+$ Tregs. Using an MHC-mismatched allogeneic heart transplant model (54, 55), it was shown that IL-33 treatment after transplantation resulted in prolonged cardiac allograft survival. This protection was dependent on host ST2 expression, the receptor for IL-33 (54). Interestingly, heart grafts (54, 55) and spleens (55) from IL-33-treated mice had increased numbers of $FOXP3^+$ cells, and depletion of natural Tregs from recipients prior to transplantation eliminated any therapeutic benefit from IL-33 therapy, implying that Tregs are indispensable for IL-33-mediated protection from allograft rejection (54). In a chronic colitis model, it was demonstrated that IL-33 possibly serves as a cofactor for TGF- β-mediated Treg

differentiation (56). Since our group previously found that protection against skin allograft rejection by IVIg depends on Treg activation and expansion (38), and we now observed that IVIg induced IL-33 production in humans *in vivo* (**chapter 3**), induction of IL-33 by IVIg may potentially cause the enhanced Treg suppressive capacity or expansion leading to decreased allograft rejection. It should be studied in the future whether this link indeed exists.

5. The effects of IVIg on pro-inflammatory $CD14^{+}CD16^{++}$ monocytes

Throughout the years, investigators are continuously searching for new mechanisms of action by which IVIg modulate immune responses. One of the mechanisms that has been recently presented is the reduction of the number of pro-inflammatory $CD14^{+}CD16^{++}$ monocytes (57). The amount of these cells are dramatically increased in some inflammatory diseases (e.g. Kawasaki disease, sepsis) and therefore reducing the numbers of these cells may be an important mechanism that contributes to the benefits of IVIg therapy seen in these patients. However, the authors have only studied patients who were treated with low "supplementary" doses of IVIg, while this mechanism should be studied in patients receiving high "immunomodulatory" doses. In **chapter 5** we investigated the effect of IVIg on the numbers of circulating pro-inflammatory $CD14^{+}CD16^{++}$ monocytes in IVIg-treated patients and divided the patients into those who received low-dose (average 0.43 g/kg) and those who received high-dose (average 1.24 g/kg) IVIg. Consistent with Siedlar et al., we observed a decrease in the number of inflammatory $CD14^{+}CD16^{++}$ monocytes who received low-dose IVIg. However, strikingly, we did not observe a significant effect on the numbers of pro-inflammatory monocytes in patients who received high-dose IVIg, while this patient group actually requires immunomodulation by IVIg. In addition, the authors suggested that reduced TNF-α production as a consequence of lowered number of $CD14^{+}CD16^{++}$ monocytes is the mechanism behind the anti-inflammatory effect of IVIg. However, in contrast, when measuring TNF-α level in plasma, we observed that the level was not decreased after IVIg treatment. Therefore, from our observation we postulate that the beneficial clinical effect of high-dose IVIg treatment seen in patients with inflammatory disorders cannot be explained by a reduction of the frequency of pro-inflammatory $CD14^{+}CD16^{++}$ monocytes.

6. Novel insights into the mechanisms of action of IVIg in mice: translatable to humans?

Growing evidence for several mechanisms of action of immunomodulation by IVIg exist. However, the majority of these findings have been identified in *in vitro* experiments or in animal studies. In **chapter 3**, **4** and **5**, we explored several pathways by which IVIg modulates the immune system in humans *in vivo*. We found some similarities, but also some important differences. This latter is not surprising, since there are fundamental differences between the

immune systems of mice and men, and animal models may not represent the complexity of disease in the human situation. Remarkably, the consequences of these differences on the translation of the mechanistic insights in the anti-inflammatory activity of IVIg from mice to humans have not been thoroughly explored. In **chapter 6** we reviewed several recently characterized anti-inflammatory mechanisms of IVIg, and explored whether evidence is available for involvement of these anti-inflammatory mechanisms of IVIg in humans. In addition, we discussed biological differences between mice and humans that may determine the translatability of these mechanisms from mice to men. The first evidence that the IL-33-Th2 pathway might be stimulated in IVIg-treated humans comes from the observation by Anthony et al. who demonstrated that human DC-SIGN could replace its murine orthologue SIGN-R1 in the serum-induced arthritis mouse model (12). Importantly, in this chapter we questioned whether DC-SIGN is indeed the proper human orthologue of murine SIGN-R1, since the expression pattern of human DC-SIGN is highly different from that of murine SIGN-R1. In addition, the question is whether human DC-SIGN can really recognize sialylated Fc-molecules, as few recent studies have argued against an interaction (58, 59). Furthermore, we showed evidence that the postulated source of IL-33 upon IVIg administration in mice, marginal zone macrophages in the spleen, is absent in humans. We continued the discussion we started in **chapter 3** where we discovered that IVIg did not upregulate the expression of inhibitory FcγRIIb in patients, which has been one of the key conclusions from recent murine studies on IVIg (12, 60, 61). Instead, we found that IVIg downregulated the expression of activating FcγRIIa. Importantly, mice do not possess an orthologue of FcγRIIa. The functional results of the downregulation of this activating receptor in humans are discussed in this chapter. Also, we describe similarities and disparities regarding the possible mechanisms of stimulation of Treg expansion and suppressive capacity by IVIg between mice and humans. In this chapter we recommend that, although murine studies on IVIg are highly valuable to identify causal relationships and to attain mechanistic insights, findings in animal models should be verified in humans treated with IVIg in order to draw clinically relevant conclusions. Because IVIg is applied in the clinic, studying the immunomodulatory effects of IVIg in humans *in vivo* is a feasible option and *ex vivo* functional assays are achievable.

As illustrated, fundamental differences between the immune systems of men and mice may explain discrepancies found in the mechanism of action of IVIg using animal models and human materials. However, also contradicting data have emerged from *in vitro* studies using human cells. In the following chapters, we have uncovered some of the possible explanations for these controversies.

7. Studies on the effects of IVIg on monocyte-derived DCs (moDCs) *in vitro* can be highly influenced by artifactual IgG immobilization

In **chapter 3** we described an *indirect* effect of IVIg on DC activation and function by means of stimulation of Th2 cytokine secretion that modulates the expression of activation receptors. In order to study the *direct* effect of IVIg on DC, in **chapter 7** we investigated the effect of IVIg on maturation, cytokine production and T-cell stimulatory capacity *in vitro*. It has been demonstrated by Bayry et al. that IVIg directly inhibit differentiation and maturation of monocyte-derived DCs (moDCs), while reducing IL-12 production and T-cell stimulatory capacity (6). In contrast to Bayry et al., in our experiments we did not find suppression of moDC activation, but instead stimulation of its maturation and T-cell priming capacity. The same effect could be found for freshly isolated mDCs. Our findings corroborate another report that showed stimulation of moDC differentiation by IVIg instead of inhibition (62). So, how can these controversial observations on the effects of IVIg on human DC maturation be explained? Given that IgG can be adsorbed to plastic surfaces and given that moDCs express FcγRs abundantly, we asked whether immobilization of IVIg to the culture plate surface might artifactually influence DC maturation by binding to FcγRs. To examine this we have used fetal calf serum to block potential binding sites for IgG to the plate surface. Indeed, when IgG immobilization was blocked, the stimulatory effect of IVIg on moDC maturation was abrogated. Since the previous *in vitro* studies did not take any precaution to prevent IgG adsorption to the culture plastic, the stimulatory effects reported may be a consequence of artifactual activation. When blocking FcγRIIa, we found that the artifactual activation of moDCs was also abrogated, which suggests activation of moDCs by binding of immobilized IgG to FcγRIIa. So for a better interpretation of the data and in order to provide consistency, we would like to recommend blocking binding sites of the solid plate surface for IgG when studying the effects of IVIg on cells expressing FcγRs. Regarding the direct effect of IVIg on human DCs, from this study we conclude that IVIg itself does not modulate the maturation of DCs directly.

8. The effects of IVIg on T-cell inhibitory capacity is influenced by the production process

In **chapter 8** we explored another potential factor that can influence the immunomodulatory effect of IVIg, which is the manufacturing process. This might explain the differences between various brands of IVIg preparations. In a previous study, hyperimmunoglobulins against HBV Hepatect® CP showed a strong T-cell suppressive capacity, while an anti-CMV hyperimmunoglobulin, Cytotect®, did not (3). An important difference between Hepatect® CP and Cytotect® is the process of virus inactivation. While Hepatect® CP is virus-inactivated by treatment with caprylic acid, tri-n-butyl phosphate and polysorbate 80, virus-

inactivation of Cytotect® includes treatment with β-propiolactone, which influences the conformation of IgG by covalent modification of amino-groups. Recently, a new anti-CMV hyperimmunoglobulin has been introduced, named Cytotect® CP, which is not exposed to β-propiolactone and undergoes the same manufacturing process as Hepatect CP®. In order to study the influence of omitting β-propiolactone treatment on the immunomodulatory effect of IVIg, we have compared the effects of Cytotect® CP to the former Cytotect® and to Hepatect® CP on human T-cell proliferation and cytokine production. Here, we show that the ability of Cytotect® CP to inhibit T-cell proliferation and IFN-γ secretion is equivalent to Hepatect® CP and significantly better compared to the former anti-CMV hyperimmunoglobulin formulation Cytotect®. One of the mechanisms behind this difference might be the modulation of the Fc-binding site of IgG by β-propiolactone, which may prevent its binding to FcγRs, for example FcγRIII on NK cells, which was previously shown to be responsible for depletion of antigen presenting cells *in vitro* (7). Thus, our current results show that the manufacturing processes can significantly influence the immunomodulatory effects of IVIg. This may explain discrepancies in published reports on the immunomodulatory effect of IVIg using different IVIg brands.

9. Overall conclusion and future perspectives

Liver transplantation is a life-saving treatment for patients with end-stage liver disease. While over 50 years ago these patients were condemned to death, nowadays LTx is a standardized treatment for this significant patient group. Since patient's short-term survival has strongly improved owing to improved surgical techniques and medical care, the next challenge in transplantation medicine is to improve the long-term survival. One major obstacle for long-term survival improvement is the major side effects of current immunosuppressive drugs. Although approximately 20% of stable liver transplant recipients can be withdrawn from all immunosuppression therapy and still maintain normal graft function and do not experience rejection (63-71), to date, there is no validated tool to identify these patients. Therefore, liver graft recipients are still mandated to use life-long immunosuppressive drugs. Because most of the long-term complications of LTx are due to side effects of immunosuppressive drugs rather than rejection, treatment strategies focus on maintaining a delicate balance between avoiding immunosuppressive drug toxicity and reducing the risk of acute rejection.

A promising therapeutic candidate for reducing the toxicity of current immunosuppressive agents is the implementation of IVIg as immunosuppressive treatment after LTx. It is firmly established that IVIg has potent anti-inflammatory activity that can modulate autoimmune and systemic inflammatory responses. Interestingly, increasing evidence suggests that IVIg is effective in preventing allograft rejection. Since long-term administration of IVIg has proven not to be accompanied by major side effects (72-74), including IVIg in immunosuppressive protocols after LTx is very attractive. In addition to available studies in animal models provid-

ing mechanistic insights underlying the protection against graft rejection by IVIg, in this thesis we have provided new perspectives on the mechanisms of immunomodulation by IVIg in human situation.

In this thesis we suggest that IVIg therapy represents a promising avenue for the development of a safe immunosuppressive protocol after LTx. However, implementation of IVIg in maintenance immunosuppressive protocols after LTx still faces two obstacles: 1) there is a predicted global shortage of donated human plasma due to an increased plasma demand (75), and 2) IVIg treatment is accompanied by high costs. Therefore, in order to enable wide use of the therapeutic benefits from IVIg, it is of high importance to unravel the molecular interactions between IVIg and the human immune system. This may enable the design of biologicals or small molecule drugs that mimic the anti-inflammatory effects of IVIg that may be suitable for long-term immunosuppressive therapy after transplantation. In current thesis, we show that IVIg enhances Treg activation and suppressive capacity in patients. We and others show that Treg activation by IVIg is exerted via an indirect route, possibly via DCs (as discussed in subparagraph 4). In addition, via binding to cells present in lymph nodes, e.g. macrophages, IVIg induces IL-33 production, which eventually leads to FcγR modulation on mDCs. Understanding the molecular interaction between IVIg and the Treg-modulating cells as well as IL-33-producing cells may enable the identification and purification of the compound(s) of IVIg that induce(s) Treg activation and DC inhibition, respectively. For Treg stimulation, DC immunoreceptor (DCIR) is interesting (40), as described in **chapter 6**, but there is to date no evidence that this molecule is involved in the mechanism of action in humans. DC-SIGN is likely excluded as a candidate receptor via which IVIg induces IL-33 production, as shown in **chapter 3**. Immunoprecipitation and mass spectrometry techniques may be used in order to identify the IVIg-binding receptor via which IVIg activates Tregs and inhibits DCs. Since the interactions are probably complex, it is likely that multiple interacting molecules may be identified. Identification of these receptors may enable the design of biological or small molecule drugs targeting these receptors.

With regard to clinical implementation of currently available IVIg preparations, clinical trials on high-dose IVIg therapy in liver transplant recipients should be the next focus. One of the first steps is to determine the optimal dose. Studies in PID patients suggest that serum IgG level needed to prevent infections may vary substantially from one patient to another (76, 77). In addition, the rise in serum IgG after high-dose IVIg treatment varies significantly between patients, as has been demonstrated in patients with Guillain-Barré (78) and CIPD (79). Therefore, determining the optimal serum IgG required for prevention of allograft rejection and the required IVIg dose to achieve that specific IgG level in liver transplant patients are important subjects to study.

Furthermore, clinical trials on IVIg should include protocols minimizing current immunosuppressive drugs. Whereas IVIg may not entirely replace current immunosuppressive drugs,

it may allow dose reduction of conventional drugs such as tacrolimus and MMF. This may reduce the side effects of the current drugs.

Another growing aspect in current studies on IgG therapy is the replacement of intravenous administration of IgG by subcutaneous administration. Subcutaneous IgG (SCIg) preparations were already introduced in the 1980s in the US and Europe. However, the slow infusion technique and the low IgG concentrations in the preparations available at that time made SCIg less attractive and were therefore replaced by IVIg. However, owing to recent technical advances in IgG formulation, pure and highly concentrated SCIg preparations have been developed that have relatively low viscosity, and can therefore be infused relatively rapidly (80, 81). SCIg has advantages compared to IVIg, since it is easier in self-administration and therefore can be administered at home, which improves quality of life and treatment satisfaction (82). Therefore, in future clinical studies using SCIg therapy in liver transplant recipients should be considered.

In transplantation medicine, the ultimate goal is to acquire operational tolerance, defined as acceptance of donor allograft without the need for immunosuppressive drugs, while retaining the ability to reject third party allografts (83). Since IVIg is able to modulate DCs and Tregs, which both play key roles in operational tolerance after organ transplantation (22, 84-86), IVIg may not only be capable to suppressing allograft responses, but also in promoting operational tolerance. Recent reports showing that IVIg can induce tolerogenic DCs that are able to induce antigen-specific Tregs in mice are promising (49, 50). Whether donor-specific tolerance can be achieved by IVIg therapy in liver allograft recipients is a subject for future studies.

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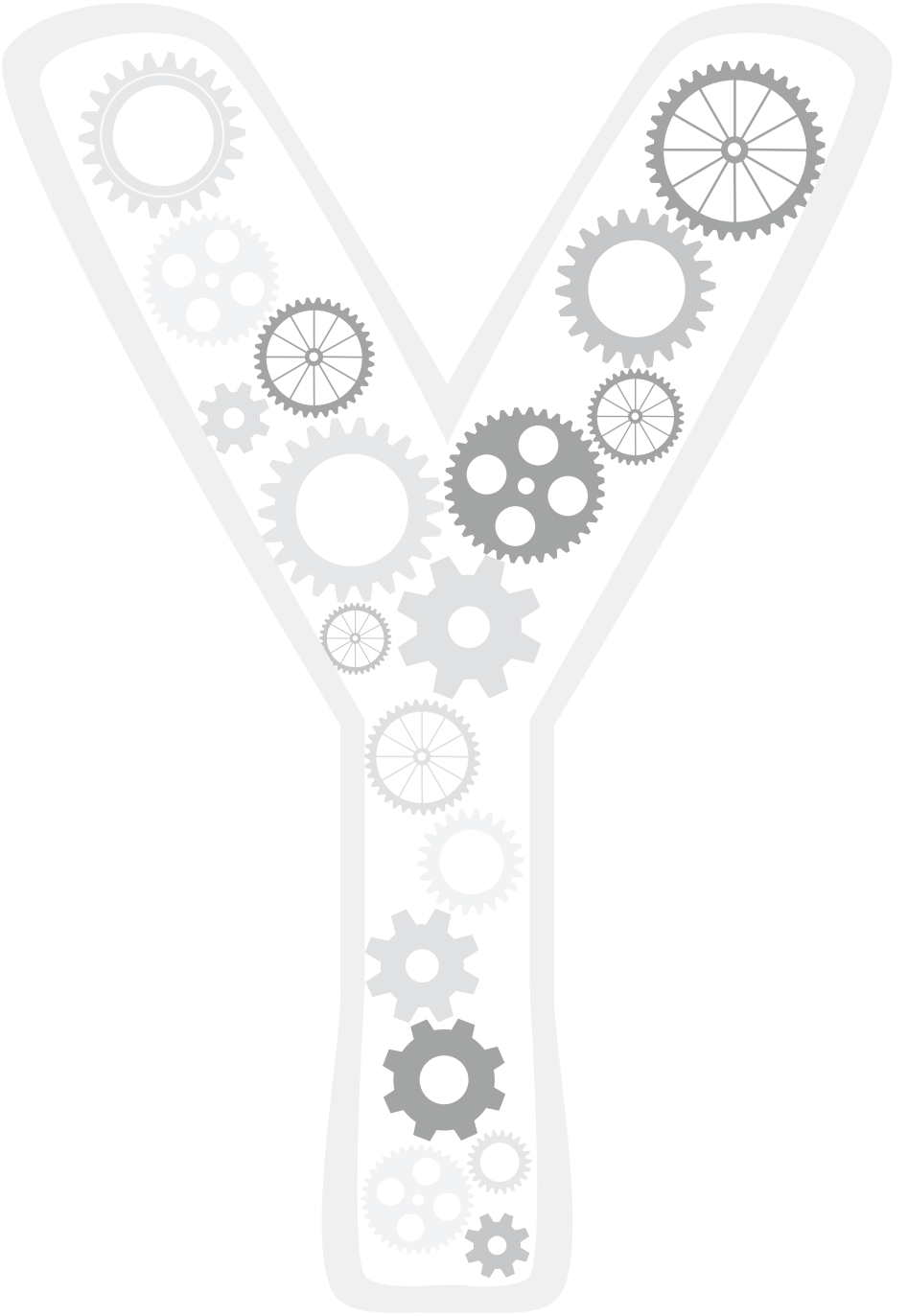

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Nederlandse samenvatting/ Dutch summary

Sinds de eerste succesvolle levertransplantatie (LTx) in de jaren 80 is LTx uitgegroeid van een experimentele behandeling tot een gestandaardiseerde behandelingsprocedure voor patiënten met eindstadium leverfalen. In de beginjaren werd er veel inspanning verricht om de korte termijn overleving na LTx te verbeteren. Dankzij een aantal verbeteringen in o.a. chirurgische technieken en perioperatieve zorg is de korte termijn overleving significant verbeterd (1, 2). Echter, met deze verbetering kwamen al snel complicaties op de lange termijn aan het licht. Deze zijn met name het gevolg van het levenslange gebruik van immunosuppressiva wat gepaard gaat met ernstige bijwerkingen, waaronder hart- en vaatziekten, nierinsufficiëntie en maligniteit. De verhoogde kans op het ontwikkelen van maligniteit is een belangrijke complicatie. In de eerste deel van dit proefschrift hebben wij onderzoek gedaan naar de incidentie van deze complicatie en in het bijzonder is onderzocht of het gebruik van verschillende immunosuppressiva protocollen hierop invloed hebben gehad.

1. *De novo* maligniteit na levertransplantatie

De verhoogde kans op het ontwikkelen van kanker is een direct gevolg van een relatieve oversuppressie van het immuunsysteem om acute afstoting te voorkomen. Om te onderzoeken of deze verhoogde kans op maligniteit gerelateerd is aan een bepaald immunosuppressiva protocol, hebben we in **hoofdstuk 2** een retrospectieve studie verricht in ons LTx cohort. Wij hebben gevonden dat LTx patiënten een 2,2-maal verhoogde kans hebben op het ontwikkelen van kanker na transplantatie in vergelijking met de algehele Nederlandse populatie. De meest voorkomende maligniteiten zijn de huidkanker (niet-behorend tot melanoom) en post-transplant lymphoproliferative disease (PTLD). De overlevingskans van patiënten die een maligniteit ontwikkelde was significant lager vergeleken met LTx patiënten die geen maligniteit ontwikkelden (10-jaars overleving: 51% vs. 79%).

Gezien er bij het gebruik van immunosuppressiva een delicate balans bestaat tussen het voorkomen van afstoting en over-immuunsuppressie, hebben wij bestudeerd of veranderingen van de gebruikte immunosuppressiva protocollen door de jaren heen, waarbij doseringen worden geoptimaliseerd, hebben geleid tot een afname van de ontwikkeling van kanker. Wij toonden aan dat LTx patiënten getransplanteerd in de eerste periode van LTx in ons centrum (1989-1992) inderdaad een verhoogde incidentie hadden op *de novo* kanker, maar dat de incidentie geleidelijk afnam in patiënten die getransplanteerd werden in de daaropvolgende perioden. Echter, een verrassende bevinding was dat patiënten die getransplanteerd waren in de meest recente periode van deze studie, namelijk tussen 2005 en 2007, juist weer een verhoogde incidentie van maligniteit vertoonden na LTx. Onderzoek naar risicofactoren wees uit dat de verhoogde kans op maligniteit geassocieerd bleek te zijn met het gebruik van cyclosporine. Gezien cyclosporine ook gebruikt werd in de voorgaande LTx perioden, suggereerde onze bevinding dat er in de periode van 2005-2007 een wijziging in het gebruik van cyclosporine was gebracht. Inderdaad, vanaf 2005 is de dosering

van cyclosporine veranderd en werd deze gebaseerd op het monitoren van bloedspiegelwaarden van dit immunosuppressivum. Oorspronkelijk werden doseringen van cyclosporine aangepast aan de bloedspiegel dat direct vooraf aan de volgende dosering is gemeten (C0). Echter, omdat de bloedspiegel 2 uur na dosering (C2) een betere voorspelling zou geven van de blootstelling aan het medicijn, is het protocol gewijzigd in het aanpassen van cyclosporine dosering op basis van de C2 bloedspiegel. Gezien patiënten die via deze methode cyclosporine behandeling kregen verhoogde kans op maligniteit vertoonden, vermoeden wij dat deze methode resulteert in een relatief hogere mate van immuunsuppressie. Deze hypothese wordt ondersteund door onze bevinding dat patiënten behandeld met cyclosporine-C2 een verlaagde incidentie van afstoting vertoonden. Een andere belangrijke bevinding is dat de gewijzigde cyclosporine dosering niet alleen resulteerde in een verhoogd risico op kanker, maar ook in agressievere tumoren met hogere mortaliteit. Daarnaast vonden wij dat met name jongere patiënten die cyclosporine gebruikten een verhoogde kans hebben op het ontwikkelen van maligniteit. Onze data suggereert dan ook dat cyclosporine een belangrijke determinant is in het ontwikkelen van kanker na LTx.

Er behoeft meer aandacht te komen voor het verbeteren van de lange-termijn uitkomst na LTx door de bijwerkingen van immunosuppressiva te minimaliseren. Het algemene doel van de behandeling is het minimaliseren van de kans op acute afstoting en het maximaliseren van de lange termijn overleving. Huidige medicijnen zijn erg effectief in het onderdrukken van de afweerrespons, maar de keerzijde is dat over-immuunsuppressie gevaren met zich meebrengt, zoals gedemonstreerd in hoofdstuk 2. Het doel van het huidige proefschrift is het onderzoeken van de immunomodulerende mechanismen van een alternatief immunosuppressivum: intraveneuze immuunglobulinen (IVIg).

2. IVIg: een alternatieve en veilige immunomodulerende behandeling

Het idee om de immunomodulerende mechanismen van IVIg te onderzoeken is geboren in ons centrum na de constatering dat LTx patienten die behandeld zijn met anti-HBs IVIg een significant verlaagde kans hadden op acute afstoting (3, 4). Soortgelijke effecten van IVIg waren ook gevonden in niertransplantatiepatiënten (5). Het grote doel van studies op dit gebied is om de onderliggende immunomodulerende mechanismen van IVIg te ontrafelen. Met deze kennis is het mogelijk om recombinante antilichamen of andere factoren te ontwikkelen die deze anti-inflammatoire mechanismen op een soortgelijke wijze in gang kunnen zetten. Zulke recombinante factoren zouden gebruikt kunnen worden als veilige onderhoudsbehandeling ter voorkoming van afstoting na LTx. Gedurende de laatste jaren komen steeds meer puzzelstukken bij elkaar over het mechanisme dat verantwoordelijk is voor het afremmen van het cellulaire afstotingsproces. De inhibitie van myeloïde dendritische cellen (mDCs) (3, 6, 7) en het stimuleren van regulatoire T cellen (Tregs) (8-10) lijken de basis te vormen voor het beschermend effect tegen afstoting. Echter, hedendaagse

studies naar deze mechanismen zijn vooral uitgevoerd *in vitro* of in muizenstudies. Bewijzen in IVIg-behandelde patiënten zijn echter maar beperkt. Om die reden hebben we ons in het volgende deel van dit proefschrift gericht op het bestuderen van de effecten van IVIg op DC en Treg functies in patiënten *in vivo* en brengen wij meer inzicht in nieuwe mechanismen van IVIg.

3. IVIg moduleert de functie van mDC door stimulatie van IL-33-gemedieerde Th2 cytokine secretie

In het laatste decennium is er een grote doorbraak gemaakt in ons begrip van het mechanisme waarop IVIg antilichaam-gemedieerde immuunreacties moduleert. Studies in muizen wezen uit dat door binding van gesialyleerde IgG aan "regulatoire" macrofagen in de milt de expressie van inhibitoire FcγRIIb receptoren op perifere "effector" macrofagen wordt verhoogd, wat uiteindelijk leidt tot verminderde activatie door auto-antilichaam immuuncomplexen (11). Recent onderzoek heeft de ontbrekende schakel tussen deze twee cellen geïdentificeerd. Doordat IVIg IL-33 productie door myeloïde cellen stimuleert in de milt, worden basofiele granulocyten aangezet tot de productie van IL-4 en IL-13, wat uiteindelijk resulteert in verhoogde FcγRIIb op effector macrofagen (zie **hoofdstuk 6, Figuur 1**). Hierdoor krijgen muizen minder symptomen van artritis doordat macrofagen in het gewricht niet meer geactiveerd kunnen worden door auto-immuun antilichamen (12). Een belangrijke vraag die hieruit voortkomt is of dit mechanisme ook daadwerkelijk plaats vindt in de mens. Dit is triviaal omdat expressiepatronen van receptoren, waaronder FcγRs, significant verschillen tussen de mens en de muis. Om deze vraag te beantwoorden hebben we in **hoofdstuk 3** het effect van IVIg op IL-33, Th2 cytokinen en receptor expressie in patiënten onderzocht. Daarnaast hebben we het oorzakelijk verband tussen deze fenomenen onderzocht door gebruik te maken van humane cellen *in vitro*. Wij toonden aan dat IL-33 en de Th2 cytokinen IL-4 en IL-13 direct stijgen na IVIg behandeling in het plasma van patiënten en de stijging van deze cytokinen was gecorreleerd aan elkaar. Deze bevinding suggereert dat IVIg, net als in muizen, een IL-33-Th2 cytokine cascade stimuleert in de mens. Echter, in tegenstelling tot de muis, vinden wij in patiënten geen stijging van de remmende FcγRIIb expressie, maar een afname in de activerende FcγRIIa expressie op mDCs. Dit verschil zou kunnen voortkomen uit verschillen in FcγR expressiepatronen tussen beide soorten en hierop wordt verder gediscussieerd in **hoofdstuk 6**.

Vervolgens hebben wij onderzocht of er een causaal verband zou kunnen zijn tussen de stijging van de Th2 cytokinen en de modulatie van FcγRs op circulerende mDCs. *In vitro* vonden we inderdaad een daling van FcγRIIa als we mDCs behandelen met IL-4 of IL-13. Functioneel toonden we aan dat deze daling resulteert in verminderde gevoeligheid voor activatie van mDCs na blootstelling aan immuuncomplexen. Dus ook in patiënten zou de IL-33-Th2 cytokine route een rol kunnen spelen in het klinisch effect van IVIg behandeling in

antilichaam-gemedieerde ontsteking, waaronder antilichaam-gemedieerde afstoting. Echter het effect van deze cytokinenproductie leidt niet tot toename van de inhibitoire FcγRIIb expressie, zoals in muizen, maar tot afname van expressie van de activerende FcγRIIa.

Een belangrijke activator van de cellulaire immuunrespons is het Th1 cytokine IFN-γ. Tijdens het proces van transplantaatafstoting speelt IFN-γ een belangrijke rol door directe cytotoxiciteit, maar ook indirect door het induceren van macrofaag en mDC activatie en migratie (13). Eerder is er in muizen aangetoond dat IVIg de activatie door IFN-γ kan afremmen door de expressie van een onderdeel van zijn receptor, IFN-γ receptor subunit 2 (IFNγR2), op macrofagen te verminderen (14). Interessant is dat we ook aanwijzingen hebben gevonden dat dit immunomodulerend effect in de mens plaatsvindt, zoals beschreven in **hoofdstuk 3**. Een dag na IVIg infusie vinden wij een daling van de IFNγR2 expressie op circulerende mDCs afkomstig van IVIg-behandelde patiënten. *In vitro* vonden we dat IL-4 en IL-13 inderdaad voor afname van IFNγR2 op mDCs kunnen zorgen. Functioneel resulteerde deze afname van IFNγR2 expressie tot een verminderde gevoeligheid voor IFN-γ gemedieerde DC maturatie en productie van chemokine IP-10 (CXCL10). In de context van transplantaatafstoting is dit laatste een interessant gegeven, gezien hoge productie van IP-10 een grote rol speelt tijdens de afstotingsreactie van hart (15), longen (16) en nier (17). Door verhoogde IP-10 productie worden geactiveerde T cellen en macrofagen aangetrokken tot het transplantaat, die daar hun cytotoxiciteit uitoefenen. Daarnaast induceert IFN-γ niet alleen IP-10, maar zou het ook andere chemokinen als Mig (CXCL9)en I-TAC (CXCL11) kunnen induceren die een rol spelen bij het aantrekken van alloresponsieve T cellen naar het transplantaat (18). Het effect van modulatie van de IVIg-IL-33-Th2 cytokine cascade op de expressie van IFN-γ receptor is nog niet eerder aangetoond.

Kortom, door het effect van de IVIg-IL-33-Th2 cytokine route op de expressie van FcγR en IFNγR (zie ook **hoofdstuk 6, Figuur 1**), tonen wij aan dat deze cascade zowel een rol kan spelen bij antilichaam-gemedieerde als bij cellulaire immuniteit, die allebei betrokken zijn bij acute en chronische afstoting na LTx.

Een interessant gegeven is dat de IVIg-IL-33 cytokine cascade mogelijk ook een rol zou kunnen spelen in de expansie en inductie van de suppressieve capaciteit van Tregs. Dit wordt verder bediscussieerd in de volgende paragraaf.

4. IVIg induceert Treg activatie en suppressieve capaciteit in patiënten

Tregs zijn de hoofdrolspelers in het onderhouden van immunologische zelf-tolerantie. Verscheidene studies hebben aangetoond dat Tregs betrokken zijn in het reguleren van autoimmuniteit, inflammatoire aandoeningen en immuunrespons tegen tumoren en verschillende pathogenen (21). Daarnaast is het aangetoond dat Tregs een kritische rol spelen in de inductie en behouden van transplantaat tolerantie door het onderdrukken

van alloreactieve T cellen in experimentele transplantatie modellen (22-32) en patiënten studies (33-36). Zeer interessant is dat er in de afgelopen jaren steeds meer bewijzen zijn dat de Treg activatie en suppressieve functie geïnduceerd kunnen worden door IVIg *in vitro* en in muizenstudies. Deze effecten zouden bij kunnen dragen aan de anti-inflammatoire effecten van IVIg. Echter, de vraag of deze effecten daadwerkelijk in de mens plaatsvinden bleef nog onbeantwoord. Om die reden hebben wij in **hoofdstuk 4** onderzocht of IVIg behandeling in patiënten resulteert in verhoogde Treg activatie en bestuurden wij het functioneel effect van IVIg behandeling *ex vivo*. Wij hebben in deze studie geen LTx patiënten geïncludeerd die behandeld zijn met anti-HBs IVIg, gezien deze patiënten ook andere immunosuppressiva nemen die negatieve effecten hebben op Tregs (40). In plaats daarvan hebben wij patiënten geïncludeerd die behandeld zijn met IVIg in verband met immuundeficiënties, autoimmuun- of inflammatoire aandoeningen (zie **hoofdstuk 4, tabellen 1A** en **1B**). In deze studie toonden wij aan dat IVIg selectief Tregs kan activeren, gedemonstreerd door verhoogde FOXP3 en HLA-DR expressie, maar dat het de conventionele T cellen niet activeert. *Ex vivo* studie laat zien dat IVIg de functionele suppressieve capaciteit van de circulerende Tregs nagenoeg verdubbelt. Opvallend is dat de stimulatoire effecten van IVIg op Tregs enkel te zien is bij patiënten die behandeld zijn met hoge dosis IVIg. Deze bevinding bevestigt de algemeen geaccepteerde hypothese dat hoge dosis IVIg behandeling nodig is om een anti-inflammatoire effect te bereiken. Uit de studie beschreven in hoofdstuk 4 concluderen wij dat IVIg niet alleen Treg functie kan stimuleren in muizen, maar ook in de mens *in vivo*. Gezien conventionele immunosuppressiva die gebruikt worden in patiënten met autoimmuunziekte en in transplantatiepatiënten om afstoting te voorkomen zowel conventionele T cellen als Tregs onderdrukken (40-47), zou gebruik van IVIg superieur kunnen zijn aan de klassieke immunosuppressiva.

Een belangrijke vraag is wat het mechanisme is waarop IVIg Tregs kan stimuleren. Om deze vraag te beantwoorden hebben wij enkele pilotstudies *in vitro* verricht door Tregs te isoleren uit gezonde individuen en deze te kweken met IVIg. Wij toonden dat Tregs die behandeld zijn met IVIg de activatiemarker CD69 verhoogd tot expressie brengen. Echter, dit gebeurde alleen in aanwezigheid van monocyt-afkomstige DC (moDCs) (zie **hoofdstuk 9**, **Figuur 1**, ongepubliceerde data). Deze data suggereert dat IVIg Tregs kan activeren op een DC-afhankelijke wijze. Deze bevinding sluit aan bij enkele recent geïdentificeerde mechanismen waarop IVIg Tregs kan induceren in muismodellen (zie hoofdstuk 6, Figuur 3). In een EAE muismodel is er gedemonstreerd dat IVIg Tregs kan expanderen door middel van het aanzetten van prostaglandine E2 (PGE2) productie door DCs (48). Dit mechanisme is deels afhankelijk van het $F(ab')_2$ gedeelte van het IgG molecuul. Een andere studie toonde aan dat IVIg antigeen-specifieke Treg kan laten differentiëren uit non-Tregs door het induceren van tolerogene DCs in een antigen-gemedieerde allergische luchtwegmodel (49). Recent is de receptor geïdentificeerd via welk IVIg dit effect mogelijk veroorzaakt, de immunoreceptor tyrosine-based inhibition motif (ITIM)-linked C-type lectin receptor, de zoge-

naamde DC immunoreceptor, DCIR (50). Een andere hypothese is dat de DC-afhankelijke Treg activatie verklaard wordt middels de aanwezigheid van Tregitopes in humane IgG. De Groot et al. hebben gedemonstreerd dat Tregs geactiveerd en geëxpandeerd kunnen worden via de presentaties van Treg-activerende peptiden die afkomstig zijn van IgG, de zogenaamde Tregitopes, door APCs (51). Gezien gesialyleerde IgGs, die ook Tregitopes moeten bevatten, niet leidt tot Treg activatie (39), zullen ook andere mechanismen een rol spelen. Door middel van onze pilotstudies achten wij het minder waarschijnlijk dat DCs nodig zijn voor crosslinking van IgG via hun FcγRs die gebonden zijn aan Tregs via het $F(ab')_2$ gedeelte, aangezien het kweken met monocyten, die ook FcγRs tot expressie brengen, niet tot hetzelfde resultaat leidt.

Betreffende de selectiviteit van IVIg voor Treg activatie sluiten onze bevindingen aan op andere studies die verricht zijn *in vitro* en in diermodellen (8, 9, 37, 38). Verschillende hypotheses kunnen worden gepostuleerd met betrekking tot Treg selectiviteit. Zoals eerder besproken kunnen presentatie van Tregitopes door APCs specificiek Tregs activeren (51). Daarnaast kunnen cytokinen die speciaal Tregs activeren een rol spelen, bijvoorbeeld TGF-β. IL-10 is minder waarschijnlijk, gezien wij geen verhoging zagen van IL-10 na IVIg behandeling. Een andere cytokine wat een interessante kandidaat is, is IL-33. Het is goed mogelijk dat er een link bestaat tussen de Treg expansie en verhoogde suppressieve capaciteit en de IVIg-IL-33-Th2 cytokine pathway, zoals beschreven in **hoofdstuk 3** en uiteengezet in **hoofdstuk 6**. Drie recente studies in muizen (54-56) hebben laten zien dat IL-33 niet alleen Th2 cytokinen productie kan aanzetten, maar ook $CD4^+FOXP3^+$ Tregs kunnen expanderen. In harttransplantatie muismodellen (54, 55) wordt er aangetoond dat IL-33 behandeling leidt tot verbeterde transplantaat overleving. Deze bescherming was afhankelijk van de expressie van ST2, de receptor voor IL-33 (54). Interessant is dat in het harttransplantaat (54, 55) en in de milt (55) in muizen behandeld met IL-33 een verhoogd aantal $FOXP3^+$ cellen vertoont, en dat depletie van Tregs van de ontvanger vooraf aan de transplantatie het effect van IL-33 therapie volledig laat verdwijnen. Deze resultaten laten het belang zien van IL-33 in het voorkomen van afstoting en dat inductie van Tregs daarbij een belangrijke rol speelt. In een recent onderzoek is er ook in een chronische colitis model aangetoond dat IL-33 de proliferatie van Treg kan induceren (56). Mogelijk dient de IL-33 als cofactor in TGF-β-gemedieerde Treg differentiatie. Gezien er in onze onderzoeksgroep eerder is aangetoond dat IVIg de afstoting van huidtransplantaat kan voorkomen op een Treg-afhankelijke wijze (38) en het gegeven dat IVIg de productie van IL-33 kan aanzetten in mensen *in vivo* (**hoofdstuk 4**), is het goed voor te stellen dat IL-33 inductie door IVIg een potentiële oorzaak kan zijn voor de verhoogde Treg suppressieve capaciteit of expansie. In de toekomst zou deze link verder onderzocht moeten worden.

5. De effecten van IVIg op de proinflammatoire $CD14^{+}CD16^{++}$ monocyten

Door de jaren heen zijn onderzoekers voortdurend op zoek naar nieuwe mechanismen waarop IVIg de immuunrespons kan moduleren. Een van de mechanismen die recentelijk gepresenteerd is, is de inductie van het aantal $CD14^{+}CD16^{++}$ monocyten (57). Gezien in bepaalde inflammatoire aandoeningen (bijvoorbeeld in de ziekte van Kawasaki of sepsis) deze aantallen sterk verhoogd kunnen zijn, is een verlaging in aantallen van deze cellen mogelijk een belangrijk mechanisme waarop IVIg immunomodulatie geeft in patiënten. Echter, de auteurs van dit artikel hebben alleen de patiënten geïncludeerd die IVIg in een "supplementaire" dosis geven, terwijl de modulerende effecten bestudeerd moeten worden in de "immunomodulatoire" doseringen. In **hoofdstuk 5** hebben wij daarom het effect van IVIg onderzocht op het aantal pro-inflammatoire $CD14^{+}CD16^{++}$ monocyten in IVIg-behandelde patiënten en deze opgedeeld in patiënten die een lage dosering ontvingen (gemiddeld 0,43 g/kg) en patiënten die een hoge dosering kregen (gemiddeld1,24 g/kg). Evenals Siedlar et al., vonden wij dat het aantal $CD14^{+}CD16^{++}$ monocyten waren gedaald in patiënten die een lage dosering IVIg ontvingen. Echter, opmerkelijk is dat patiënten die een hoge dosering IVIg kregen geen daling vertoonden in het aantal pro-inflammatoire monocyten, terwijl deze groep patiënten juist die immunomodulatie nodig hebben. Verder vonden wij ook geen verlaging van TNF-α in het serum van IVIg-behandelde patiënten, ofschoon er gesuggereerd werd door Siedlar et al. dat afremming van TNF-α productie door monocyten wordt bewerkstelligd met IVIg behandeling. Concluderend zijn wij van mening dat de gunstige effecten van hoge dosering IVIg die bij patiënten worden gevonden niet veroorzaakt worden door verlaging van pro-inflammatoire monocyten.

6. Nieuwe inzichten in de werkingsmechanismen van IVIg in muizen: is het te vertalen naar de mens?

Er komen steeds meer nieuwe aanwijzingen voor verschillende werkingsmechanismen van immunomodulatie door IVIg. Echter, het merendeel van deze bevindingen is geïdentificeerd in dierenstudies. In **hoofdstukken 3**, **4** en **5** hebben wij verschillende wijzen van immunomodulatie door IVIg in de mens *in vivo* onderzocht. Wij vonden enige overeenkomsten, maar vonden ook belangrijke verschillen. Dit laatste is niet onverwacht, gezien er fundamentele verschillen bestaan in het immuunsysteem tussen de mens en dat van de muis. Opvallend is dat de consequenties van deze verschillen in de vertaling van de anti-inflammatoire mechanismen van IVIg van de muis naar de mens nooit helemaal uitgekristaliseerd is. In **hoofdstuk 6** zetten wij enkele recent gevonden anti-inflammatoire mechanismen van IVIg uiteen en onderzochten of er ook bewijzen zijn dat deze mechanismen in de mens een rol spelen. Daarnaast bediscussiëren wij biologische verschillen tussen de muis en de mens. Het

eerste bewijs dat de IL-33-Th2 pathway gestimuleerd kan worden door IVIg in de mens is afkomstig uit de waarneming van Anthony et al. die demonstreerde dat humaan DC-SIGN het muizenortholoog SIGN-R1 in het serum-geïnduceerde artritis muismodel kan vervangen om het anti-inflammatoire effect van gesialyleerd IVIg te bewerkstelligen (12). In dit hoofdstuk vragen wij ons af of DC-SIGN inderdaad het juiste humane ortholoog is van SIGN-R1, omdat het expressiepatroon van DC-SIGN in mensen zeer verschillend is van dat van SIGN-R1 in muizen. Daarnaast is het de vraag of de DC-SIGN echt gesialyleerde Fc-moleculen kan herkennen, gezien enkele recente studies tegen een mogelijk interactie hebben betoogd (58, 59). Verder tonen we aan dat de gepostuleerde bron van IL-33 na IVIg toediening bij muizen, de marginale zone macrofagen in de milt, afwezig is in mensen. We vervolgen onze discussie die we begonnen zijn in **hoofdstuk 3**, waar we ontdekten dat IVIg de expressie van de inhibitoire FcγRIIb bij patiënten niet verhoogt, terwijl dit één van de belangrijkste conclusies is uit recente IVIg studies in muizen (12, 60, 61). In plaats daarvan vonden we dat IVIg de expressie van de activerende FcγRIIa verlaagt. Belangrijk in deze discussie is dat muizen geen ortholoog van de FcγRIIa bezitten. Verder worden de functionele resultaten van de downregulatie van deze activerende receptor bij de mens in dit hoofdstuk besproken. Ook beschrijven we de overeenkomsten en verschillen met betrekking tot de mogelijke mechanismen van stimulatie van Treg expansie en suppressieve capaciteit door IVIg tussen de muis en de mens. In dit hoofdstuk adviseren wij, hoewel muizenstudies over IVIg zeer waardevol zijn om causale relaties te identificeren en mechanistische inzichten te verkrijgen, om bevindingen in diermodellen te verifiëren in patiënten behandeld met IVIg om klinisch relevante conclusies te kunnen trekken. Omdat IVIg wordt toegepast in de kliniek, is het bestuderen van de immunomodulerende effecten van IVIg bij mensen *in vivo* realiseerbaar en zou het uitvoeren van *ex vivo* functionele assays mogelijk moeten zijn.

Zoals in dit hoofdstuk geïllustreerd wordt, kunnen fundamentele verschillen tussen het immuunsysteem van de mens en de muis de discrepanties uit verschillende onderzoeken naar het werkingsmechanisme van IVIg, die gebruik maken van diermodellen en menselijke materialen, verklaren. Er zijn echter ook tegenstrijdige gegevens naar voren gekomen uit *in vitro* onderzoek met menselijke cellen. In de volgende hoofdstukken hebben we een aantal van de mogelijke verklaringen voor deze discrepanties beschreven.

7. Studies naar de effecten van IVIg op moDCs *in vitro* kunnen aanzienlijk beïnvloed worden door IgG immobilisatie als artifact

In **hoofdstuk 3** hebben we een *indirect* effect van IVIg op DC activatie en functie beschreven door middel van stimulatie van Th2 cytokine secretie die de expressie van de activerende receptoren moduleert. Met het oog op de *directe* werking van IVIg op DC, bestudeerden wij in **hoofdstuk 7** het direct effect van IVIg op de rijping, cytokine productie en T-cel stimulerende capaciteit van DCs *in vitro*. Het is reeds aangetoond door Bayry et

al. dat IVIg de differentiatie en maturatie van moDCs direct afremt. Daarnaast remt het ook IL-12 productie en T-cel stimulerende capaciteit van moDCs (6). In tegenstelling tot Bayry et al., vonden wij in onze experimenten geen inhibitie van moDC activatie, maar juist stimulatie van moDC maturatie en zijn T-cel-activerende capaciteit. Hetzelfde resultaat werd bereikt door gebruik te maken van vers geïsoleerde mDCs. Onze bevindingen bevestigen een andere studie die ook stimulatie van moDC differentiatie door IVIg toonde in plaats van inhibitie (62). Hoe kunnen deze controversiële resultaten betreffende het effect van IVIg op de maturatie van humane DCs worden verklaard? Gezien IgG moleculen kunnen worden geadsorbeerd aan kunststof oppervlakken en het feit dat moDCs uitgebreid FcγRs tot expressie brengen, vroegen we ons af of immobilisatie van IVIg aan het kunststof plaatoppervlakte tijdens het kweken de DC rijping zou kunnen beïnvloeden door binding aan FcγRs. Om aan te tonen dat dit artefact daadwerkelijk optreedt, hebben wij gebruik gemaakt van het eiwitrijke foetaal kalfsserum om potentiële bindingsplaatsen voor IgG aan het plaatoppervlak te blokkeren. Inderdaad, als IgG immobilisatie werd voorkomen, was het stimulerende effect van IVIg op moDC maturatie verdwenen. Wanneer we FcγRIIa blokkeerden, vonden we dat de activatie van moDCs ook verdween. Deze bevindingen suggereren dat moDCs *in vitro* geactiveerd kunnen worden door IVIg vanwege binding van geïmmobiliseerd IgG aan FcγRIIa op moDCs. Gezien er in de voorgaande onderzoeken geen voorzorgsmaatregelen zijn genomen om IgG adsorptie aan de kunststof kweekplaat te voorkomen, kunnen de gerapporteerde effecten van moDC stimulatie het gevolg zijn van artificiële activering. Dus voor een betere interpretatie van de gegevens en met het oog op consistentie, zouden we willen adviseren om bindingsplaatsen voor IgG in de kweekplaat te blokkeren bij het bestuderen van de effecten van IVIg op cellen die FcγRs tot expressie brengen. Ten aanzien van het directe effect van IVIg op de humane DCs kunnen we uit deze studie concluderen dat IVIg op zichzelf de DC maturatie niet direct beïnvloedt.

8. De inhibitoire effecten van IVIg op T-cel activatie wordt beïnvloed door het productieproces

In **hoofdstuk 8** hebben we een andere mogelijke factor gevonden die het immunomodulerende effect van IVIg kan beïnvloeden, namelijk het fabricageproces van IVIg. Deze bevinding kan mogelijk de verschillen in gepubliceerde artikelen verklaren die gebruikmaken van diverse merken van IVIg preparaten. In een eerdere studie is er aangetoond dat hyperimmunoglobulinen tegen HBV Hepatect® CP een sterke T-cel onderdrukkende capaciteit kan bewerkstelligen, terwijl een anti-CMV hyperimmunoglobulin, Cytotect®, dit effect niet had (3). Een belangrijk verschil tussen Hepatect® CP en Cytotect® is het proces van de virus inactivatie tijdens de productie van deze preparaten. Bij Hepatect® CP wordt de virusinactivatie verkregen door middel van behandeling met caprylzuur, tri-n-butylfosfaat en polysorbaat 80. Echter, bij Cytotect® wordt de virusinactivatie ook verkregen door middel

van β-propiolacton behandeling, die de conformatie van IgG beïnvloedt door covalente modificatie van aminogroepen. Onlangs is een nieuw anti-CMV hyperimmunoglobuline ontwikkeld, genaamd Cytotect® CP, dat niet is blootgesteld aan β-propiolacton en verder hetzelfde fabricageproces ondergaat als Hepatect® CP. Om de invloed van de weglating van β-propiolacton behandeling op het immunomodulerende effect van IVIg te bestuderen, hebben we de effecten vergeleken van Cytotect® CP, Cytotect® en Hepatect® CP op humane T-cel proliferatie en cytokine productie *in vitro*. Hier tonen we aan dat het vermogen om T-celproliferatie en IFN-γ secretie af te remmen van Cytotect® CP gelijk is aan dat van Hepatect® CP, en dat deze significant beter is dan de voorgaande anti-CMV hyperimmunoglobuline formulering Cytotect®. Een van de mechanismen die dit verschil mogelijk verklaart is de modulatie van de Fc staart van IgG door β-propiolacton, waardoor de binding aan FcγRs wordt aangetast. Dit zou mogelijk een rol spelen bij binding aan de FcγRIII op NK cellen, welke eerder was aangetoond betrokken te zijn bij depletie van APCs *in vitro* (7). Kortom, dit resultaat toont aan dat de productieprocessen de immunomodulerende effecten van IVIg aanzienlijk kunnen beïnvloeden.

9. Algemene conclusie en toekomstperspectieven

LTx is een levensreddende behandeling voor patiënten met eindstadium leverziekte. De korte termijn overleving van patiënten is sterk verbeterd dankzij verbeterde chirurgische technieken en medische zorg, en de volgende uitdaging in orgaantransplantatie is dan ook het verbeteren van de lange termijn overleving. Een belangrijk obstakel voor de verbetering hiervan is de levenslange behoefte aan immunosuppressiva, die gepaard gaat met ernstige bijwerkingen. Over het algemeen is het levertransplantaat relatief gezien een immunologisch tolerogeen orgaan. Dit wordt geïllustreerd door de lage incidentie van levertransplantaat verlies door acute of chronische afstoting en het bestand zijn tegen antilichaam-gemedieerde schade (87). Ongeveer 20% van LTx patiënten kunnen stoppen met alle immuunsuppressieve behandeling en een normale transplantaatfunctie behouden zonder dat afstoting plaatsvindt. Echter, gezien er tot op heden geen gevalideerde test is om deze patiënten te identificeren, zijn LTx patiënten nog steeds genoodzaakt om levenslang immuunsuppressieve medicatie te gebruiken. Omdat het merendeel van de complicaties op lange termijn na LTx te wijten is aan de effecten van immunosuppressiva, zijn de therapeutische strategieën gericht op het handhaven van een delicaat evenwicht tussen het vermijden van toxische effecten van immunosuppressiva en het reduceren van het risico op acute afstoting.

Een veelbelovende therapeutische kandidaat voor het verminderen van de toxiciteit van de huidige immunosuppressiva is de implementatie van IVIg als immunosuppressieve behandeling na LTx. Het is bewezen dat IVIg sterke anti-inflammatoire effecten heeft die autoimmuunziekten en systemische ontstekingsreacties kunnen moduleren. Interessant is

dat er steeds meer aanwijzingen zijn dat IVIg ook effectief is in het voorkomen van transplantaatafstoting. Aangezien het gebruik van IVIg op lange termijn geen ernstige bijwerkingen geeft (52), is het includeren van IVIg in het immunosuppressiva protocol na LTx zeer aantrekkelijk. In dit proefschrift leveren wij, in aansluiting op de beschikbare mechanistische inzichten van studies in diermodellen, nieuwe perspectieven op de mechanismen van immunomodulatie door IVIg in de mens, die ten grondslag liggen aan de bescherming tegen afstoting.

In dit proefschrift pleiten we voor het gebruik van IVIg therapie als een veilige immunosuppressieve behandeling na LTx. Echter, de implementatie van IVIg in een standaard immunosuppressiva protocol na LTx stuit op twee hindernissen: 1) er is een voorspeld wereldwijd tekort aan IVIg als gevolg van een toename van de vraag naar humaan plasma (75), en 2) IVIg behandeling gaat gepaard met hoge kosten. Teneinde de brede toepassing van IVIg mogelijk te maken, is het van groot belang de moleculaire interacties tussen IVIg en het menselijke immuunsysteem te ontrafelen. Dit maakt het ontwerp van alternatieve of recombinante moleculen mogelijk die de anti-inflammatoire effecten van IVIg kunnen nabootsen en het geschikt maakt voor langdurige immunosuppressieve therapie na transplantatie. In dit proefschrift laten we zien dat IVIg Treg activatie en de suppressieve capaciteit kan stimuleren in patiënten. Samen met anderen laten we zien dat Treg activatie door IVIg waarschijnlijk indirect wordt uitgeoefend, mogelijk via DCs (zoals beschreven in paragraaf 4). Daarnaast kan IVIg via binding aan cellen in lymfeknopen, bijvoorbeeld aan macrofagen, IL-33 productie induceren, wat uiteindelijk leidt tot het moduleren van FcγR expressie op DCs. Inzicht in de moleculaire interactie tussen IVIg en Treg en IL-33-producerende cellen maakt het wellicht mogelijk een component van IVIg te identificeren die Treg activeert en DCs afreemt, en deze op te zuiveren. Voor Treg stimulatie is DCIR een interessante kandidaat (40), zoals beschreven in **hoofdstuk 6**, maar er is nog geen aanwijziging dat dit molecuul betrokken is bij de werking van IVIg in de mens. DC-SIGN lijkt uitgesloten als kandidaat receptor voor IVIg-gemedieerde IL-33 productie, zoals bestudeerd in **hoofdstuk 3**. Immuunprecipitatie en massaspectrometrie kunnen worden gebruikt om de IVIg-bindende receptor waarmee IVIg Tregs stimuleert en DCs inhibeert te identificeren. Het is zeer waarschijnlijk dat er meerdere betrokken moleculen worden geïdentificeerd. Identificatie van deze receptoren kan het ontwerp van een alternatieve behandelingstherapeutica mogelijk maken die op deze receptoren aangrijpen.

Met betrekking tot de klinische toepassing van de huidige beschikbare IVIg preparaten moeten er eerst klinische trials worden uitgevoerd naar het gebruik van hoge dosis IVIg in LTx patiënten. Een van de eerste stappen is om de optimale dosis te bepalen. Huidige onderzoeken in PID patiënten suggereren dat een bepaald serum IgG spiegel nodig is om infecties te voorkomen en dat de benodigde spiegel aanzienlijk kan verschillen tussen patienten (76, 77). Daarnaast is de toename van serum IgG na hoge doses IVIg sterk variërend tussen patiënten, zoals is aangetoond bij Guillain-Barre (78) en CIPD (79) patiënten. De

bepaling van de optimale serum IgG die nodig is ter voorkoming van transplantaatafstoting en de vereiste dosis IVIg om deze IgG spiegel te bereiken in LTx patiënten dient daarom eerst onderzocht te worden.

Daarnaast dienen er klinische trials naar IVIg uitgevoerd te worden met het oog op het minimaliseren van de huidige immunosuppressieve geneesmiddelen. Het is mogelijk dat de huidige immunosuppressiva niet geheel vervangen kunnen worden door IVIg. In dat geval kan IVIg het mogelijk maken om de dosering van huidige immunosuppressiva te reduceren en daarmee de toxische effecten van de medicatie, zoals in het geval van tacrolimus en MMF, af te doen nemen.

Een ander aspect in huidige studies omtrent IgG therapie is de verandering van intraveneuze naar subcutane toediening van IgG. Subcutane IgG (SCIg) preparaten waren al in de jaren 1980 in de VS en Europa geïntroduceerd. Echter, de langzame infusie en de lage concentratie in de beschikbare preparaten maakten SCIg minder aantrekkelijk waardoor ze uiteindelijk vervangen werden door IVIg. Als gevolg van recente technische ontwikkelingen in IgG formulering is het mogelijk om zuivere en geconcentreerde SCIg preparaten te ontwikkelen die een relatief lage viscositeit hebben en die dus relatief snel kan worden toegediend (80, 81). SCIg heeft voordelen ten opzichte van IVIg, omdat het gemakkelijker is zelf toe te dienen en dus thuis gebruikt kan worden. Dit verbetert de kwaliteit van leven en de tevredenheid over de behandeling (82). Daarom zou het gebruik van SCIg therapie in toekomstige klinische trials in LTx patiënten overwogen moeten worden.

Op het gebied van orgaantransplantatie-immunologie is het uiteindelijke doel om operationeel tolerantie te verkrijgen, gedefinieerd als de acceptatie van het donor -orgaan zonder het gebruik van immunosuppressieve middelen, maar wel met behoud van afstotingsreactie tegen het donororgaan van een derde partij (83). Aangezien IVIg DCs en Tregs kan moduleren, die beiden een sleutelrol spelen bij operationele tolerantie na orgaantransplantatie (22, 84-86), zou het gebruik van IVIg niet alleen kunnen leiden tot het onderdrukken van de afstotingsreactie, maar ook tot het bevorderen van operationele tolerantie. Recente studies die aantonen dat IVIg tolerogene DCs kunnen induceren die in staat zijn antigeen-specifieke Tregs te induceren in muizen zijn zeer veelbelovend (49, 50). Of donor-specifieke tolerantie kan worden bereikt door IVIg therapie in LTx patiënten zal moeten blijken uit toekomstige studies.

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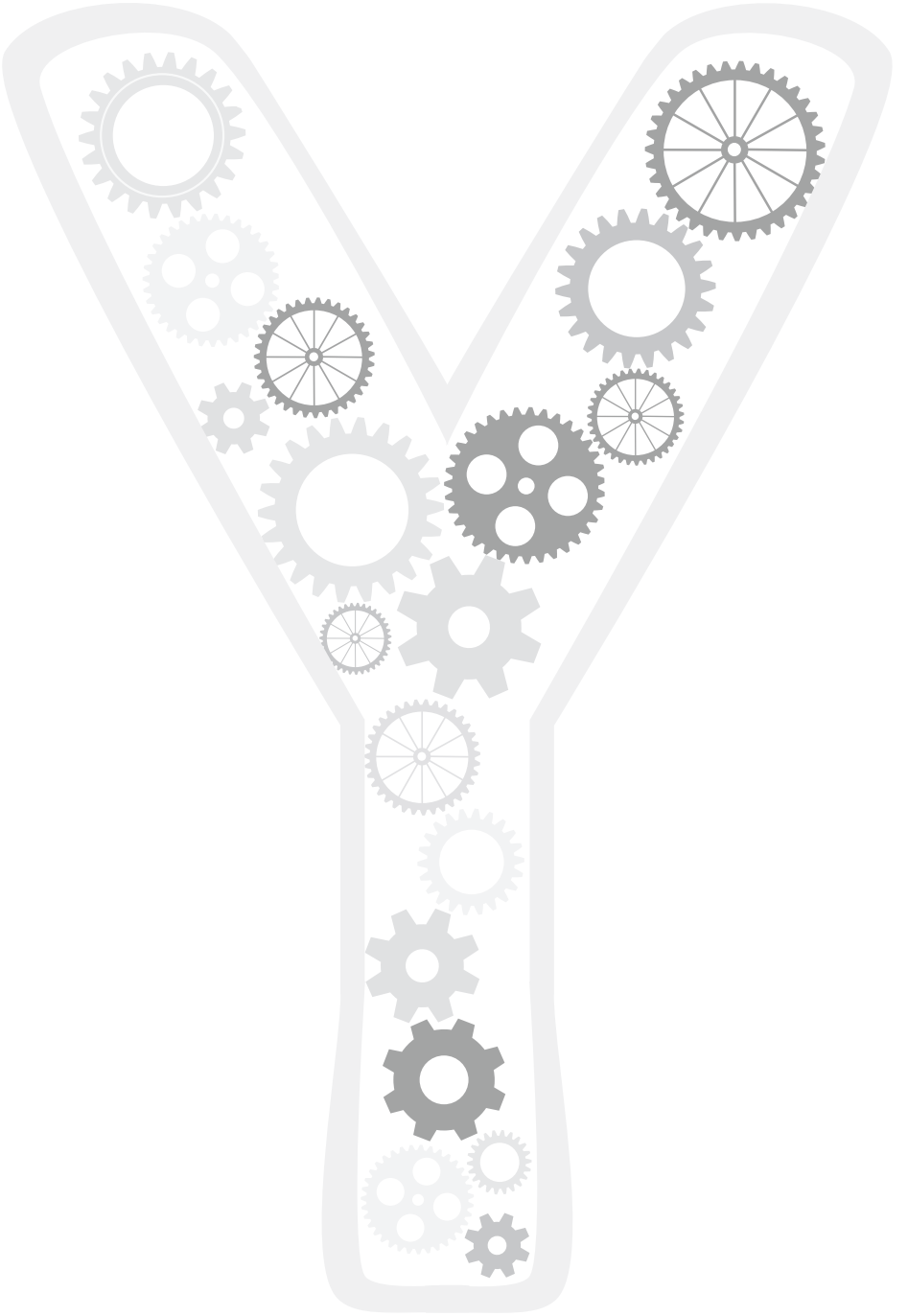

11

Appendix

Dankwoord/
Acknowledgement

List of publication

PhD Portfolio

About the author

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LIST OF PUBLICATION

1. Sint Nicolaas J, **Tjon AS**, Metselaar HJ, Kuipers EJ, de Man RA, van Leerdam ME. Colorectal cancer in post-liver transplant recipients.
   Dis Colon Rectum. 2010 May;53(5):817-21.

2. **Tjon AS**, Sint Nicolaas J, Kwekkeboom J, de Man RA, Kazemier G, Tilanus HW, Hansen BE, van der Laan LJ, Tha-In T, Metselaar HJ.
   Increased incidence of early de novo cancer in liver graft recipients treated with cyclosporine: an association with C2 monitoring and recipient age.
   Liver Transpl. 2010 Jul;16(7):837-46.

3. **Tjon AS**, Metselaar HJ, te Boekhorst PA, van Hagen PM, Kwekkeboom J.
   High-dose intravenous immunoglobulin does not reduce the numbers of circulating CD14($^{+}$)CD16($^{++}$) monocytes in patients with inflammatory disorders.
   Clin Immunol. 2012 Oct;145(1):11-2.

4. **Tjon AS**, Tha-In T, Metselaar HJ, van Gent R, van der Laan LJ, Groothuismink ZM, te Boekhorst PA, van Hagen PM, Kwekkeboom J.
   Patients treated with high-dose intravenous immunoglobulin show selective activation of regulatory T cells.
   Clin Exp Immunol. 2013 Aug;173(2):259-67.

5. van Gent R, Jaadar H, **Tjon AS**, Mancham S, Kwekkeboom J.
   T-cell inhibitory capacity of hyperimmunoglobulins is influenced by the production process.
   Int Immunopharmacol. 2014 Mar;19(1):142-4.

6. **Tjon AS**, Jaadar H, van Gent R, van Kooten PJ, Achatbi N, Metselaar HJ, Kwekkeboom J. Prevention of immunoglobulin G immobilization eliminates artifactual stimulation of dendritic cell maturation by intravenous immunoglobulin in vitro.
   Transl Res. 2014 Jun;163(6):557-64.

7. **Tjon AS**, van Gent R, Jaadar H, Martin van Hagen P, Mancham S, van der Laan LJ, te Boekhorst PA, Metselaar HJ, Kwekkeboom J.
   Intravenous immunoglobulin treatment in humans suppresses dendritic cell function via stimulation of IL-4 and IL-13 production.
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8. Verhaar AP, Hoekstra E, **Tjon AS**, Utomo WK, Deuring JJ, Bakker ER, Muncan V, Peppelenbosch MP.
   Dichotomal effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity.
   Sci Rep. 2014 Jun 27;4:5468.

9. **Tjon AS**, van Gent R, Geijtenbeek TB, Kwekkeboom J.
   The anti-inflammatory actions of intravenous immunoglobulin in mice and men: a critical review.
   Submitted for publication.

10. Chen K, Hernanda P, Li J, **Tjon AS**, Sideras K, Hansen BE, Kong X, van der Laan LJW, Man K, Sprengers D, Kwekkeboom J, Metselaar HJ, Peppelenbosch MP, Pan Q. IMPDH2-Targeted Constraint of Hepatocellular Carcinoma by Mycophenolic Acid in Experimental Models and in Liver Transplant Patients.
    Submitted for publication.

PhD PORTFOLIO

Name PhD student:	Angela S.W. Tjon
Erasmus MC department	Gastroenterology and Hepatology
PhD period:	January 2009- April 2013
Promotor:	Prof. dr. H.J. Metselaar
Co-promotor:	Dr. J. Kwekkeboom

General courses

2009	CPO Minicursus: Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen, Erasmus MC, Rotterdam
2010	Stralingshygiene, deskundigheidsniveau 5B, Erasmus MC, Rotterdam
2010	Biomedical English Writing and Communication, Erasmus MC, Rotterdam
2010	Photoshop CS3 Workshop, Erasmus MC, Rotterdam
2010	Basic Immunology, Erasmus MC, Rotterdam
2011	Biostatistics for Clinicians, Netherlands Institute for Health Sciences (NIHES), Erasmus MC, Rotterdam
2011	Regression Analysis for Clinicians, Netherlands Institute for Health Sciences (NIHES), Erasmus MC, Rotterdam

National Conferences- Presentations

2009	Bootcongres 2009, Nederlandse Transplantatie Vereniging (NTV), Zeewolde, Nederland. (Oral presentation)
2009	Voorjaarsvergadering 2009, Nederlandse Vereniging voor Gastroenterologie (NVGE), Veldhoven, Nederland. (Oral presentation)
2010	Bootcongres 2010, Nederlandse Transplantatie Vereniging (NTV), Rotterdam, Nederland. (Oral presentation, plenary session)
2011	Voorjaarsvergadering 2011, Nederlandse Vereniging voor logie (NVGE), Veldhoven, Nederland. (Oral presentation)
2013	Bootcongres 2013, Nederlandse Transplantatie Vereniging (NTV), Duiven, Nederland. (Oral presentation)

International Conferences- Presentations

2009	European Society for Organ Transplantation (ESOT) 14th Congress, Paris, France. (Oral presentation)
2009	International Liver Transplantation Society (ILTS) 15th Annual International Congress 2009, New York, USA. (Poster presentation)
2010	XXIII International Congress of the Transplantation Society (TTS) 2010, Vancouver, Canada. (Oral presentation)
2010	International Liver Transplantation Society (ILTS) 16th Annual International Congress 2010, Hong Kong, China. (Poster presentation, 'poster of distinction')
2011	European Society for Organ Transplantation (ESOT) 15th, Glasgow, United Kingdom. (Oral presentation)
2012	European Congress of Immunology (ECI) 2012, Glasgow, United Kingdom. (Oral presentation, selected for the 'Bright Sparks in Immunology')
2012	International Liver Transplantation Society (ILTS) 18th Annual International Congress San Francisco, USA. (Poster presentation)

2012	American Association for the Study of Liver Diseases 2012, Boston, USA. (2 Poster presentations)

National Conferences- Participation

2009	Symposium "Sensing and Signalling by the Immune System". NVVI. Lunteren, The Netherlands.
2011	Bootcongres 2011, Nederlandse Transplantatie Vereniging (NTV), Amsterdam, The Nederlands.

Scientific Awards and Grants

2008	Mark van Blankensteijn "Stichting Lever Onderzoek" (SLO) Studentenprijs 2008 (Erasmus MC)
2009	Genzyme Speakers Award (Nederlandse Transplantatie Vereniging)
2009	ILTS Young Investigator Travel Award 2009 (International Liver Transplantation Society)
2009	Travel Grant Novartis
2009	Travel Grant Erasmus Trustfonds
2010	NWO Mosaic 2010
2010	International Basic Science Mentee/Mentor Travel Award (International Congress of the Transplantation Society)
2010	Travel Grant Biotest
2010	Travel Grant Novartis
2010	Travel Grant Nederlandse Vereniging voor Immunologie
2010	Travel Grant Erasmus Trustfonds
2011	Travel Grant Novartis
2012	Travel Grant Erasmus Trustfonds (awarded on Talent Day)

Supervising activities

February- July 2011	Supervising master student: 'Master in Infection and Immunity'
December 2011- May 2012	Supervising master student: 'Master in Infection and Immunity'

Invited speaker

2010	Radio program 'NOS- Met het oog op morgen', Radio 1
2010	Radio program 'KRO-De ochtend van 4', Radio 4
2010	NWO- Mosaic Award Day 2010
2012	NWO- Mosaic workshop 'How do I present my research?'

Other activities

2012	Research member, space project 'MASER 12 launch campaign- The effects of microgravity on the immune system', Esrange Space Center, Sweden

ABOUT THE AUTHOR

Angela Tjon is born on September 24th 1983 in Hong Kong, China. At 5th year of age, she moved with her parents and older brother to The Netherlands, where she grew up in Spijkenisse. In 2002, she completed her secondary school at the Blaise Pascal in Spijkenisse and started her medical school at the Erasmus University in Rotterdam. In 2008, she performed her graduation research on "Incidence of malignancy after liver transplantation and the role of NK cell modulation by intravenous immunoglobulin" at the department of Gastroenterology and Hepatology. For this work she received the "Mark van Blankensteijn SLO student award" for best student research. She got interested in basic science and decided to perform a PhD. After obtaining her medical degree, in 2009 she started her PhD project under supervision of prof. dr. H.J. Metselaar and dr. J. Kwekkeboom at the department of Gastroenterology and Hepatology. The results of this project are presented in this thesis. In 2010, she honorary received the "Mosaic grant" of the Netherlands Organization for Scientific Research (NWO). From September 2013, she has started her residency training in Internal Medicine at the Sint Franciscus Gasthuis, Rotterdam under supervision of dr. A.P. Rietveld. The second part of her residency will be continued at the Erasmus MC in Rotterdam under supervision of prof. dr. J.L.C.M. van Saase.