DIPLOMARBEIT

IN VITRO INVESTIGATION OF TOLERANCE INDUCING EFFECTS OF CMV HYPERIMMUNOGLOBULIN CONTAINING DRUGS

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde
(Dr. med. univ.)

an der

Medizinischen Universität Wien

ausgeführt an der

Universitätsklinik für Chirurgie

Unter der Anleitung von
Univ.-Doz. Dr. HJ Ankersmit

eingereicht von

Konrad Hoetzenecker

Matr.Nr. 9871260
Neulerchenfelderstrasse 60/1/4
1160 WIEN

Wien am 20.12.07 ……..
(Unterschrift)

Die Wissenschaft fängt eigentlich erst da an interessant zu werden, wo sie aufhört.

Justus von Liebig (1803-73)
deutscher Chemiker

In diesem Sinne möchte ich mich bei allen bedanken, die zur Entstehung dieser Diplomarbeit durch fachliche oder persönliche Unterstützung beigetragen haben.

Besonderer Dank gilt dabei meinen Eltern, die mir dieses Studium ermöglicht haben.

Weiters möchte ich Univ.-Doz. Dr. HJ Ankersmit danken, der nicht nur diese Arbeit betreut hat, sondern mich seit dem Beginn meiner wissenschaftlichen Laufbahn begleitet.

Teile der vorliegenden Arbeit wurden in der Dezemberausgabe 2007 des *European Journal of Clinical Investigation* publiziert. Die vollständige Publikation befindet sich im Anhang am Ende dieser Diplomarbeit.

TABLE OF CONTENTS

ZUSAMMENFASSUNG

CMV Hyperimmunoglobuline (CMVIg) werden in der Herztransplantation routinemäßig zur Prophylaxe gegen CMV Erkrankungen eingesetzt. Ein Einfluss dieser Medikamentenklasse auf transplant-relevante Immunfunktionen ist nicht bekannt. Ziel dieser *in vitro* Studie war es, die Auswirkungen von CMVIg auf die zelluläre Immunantwort zu beschreiben und eine mögliche Wirkung auf toleranzinduzierende Mechanismen nachzuweisen.

Es konnte gezeigt werden, dass CMVIg sowohl in allogenen „mixed lymphocyte reactions“ als auch in T-Zell spezifischen Proliferationsansätzen anti-proliferative Eigenschaften besitzen. Diese Ergebnisse konnten mittel CFSE Dilutions-Experimenten und Zellzyklus-Analysen bestätigt werden. Weiters wurde ein bedeutender Einfluss von CMVIg auf die Zellviabilität beschrieben. Vor allem Effektorzellen (CD8+, CD56+) waren vulnerabel für eine CMVIg vermittelte Apoptoseinduktion. Im dritten Teil der Diplomarbeit wurde die Wirkung von CMVIg auf NK Zellen näher beschrieben. Hier konnte gezeigt werden, dass die Antikörper-vermittelte zelluläre Zytotoxizität von NK Zellen durch CMVIg massiv supprimiert wird.

Zusammenfassend kann gesagt werden, dass im Rahmen dieser Arbeit erstmals toleranzinduzierende Mechanismen von CMVIg nachgewiesen wurden. Die *in vitro* Ergebnisse erklären klinische Beobachtungen, die einen positiven Einfluss von CMVIg auf das Transplantatüberleben zeigen. Basierend auf den Daten dieser Arbeit kann nun das Konzept von CMVIg als rein anti-viral wirksame Substanzklasse auf ein Medikament mit breiterem, für die Transplantationsmedizin günstigem Wirkungsprofil erweitert werden.

ABSTRACT

Cytomegalovirus hyperimmunoglobulin containing drugs are routinely administered in solid organ transplantation for prophylaxis against CMV disease. Yet little is known about their influence on transplant relevant immune functions. The aim of this study was to evaluate the effect of CMVIg on cellular immunity *in vitro* and to define their role in tolerance inducing mechanisms.

CMVIg were shown to reduce proliferation in mixed lymphocyte reactions and T cell specific anti-CD3 blastogenesis assays. These anti-proliferative effects are associated with a cell-cycle arrest in the G0/G1 phase. Furthermore, CMVIg evidenced an induction of apoptosis primarily in cytotoxic T cells and natural killer cells. Co-incubation with CMVIg caused a downregulation of cell surface bound immunoglobulins and FcγRIII expression on natural killer cells. This led to an attenuation of antibody dependent cellular cytotoxicity effector functions.

Concluding, CMVIg induce immunological features on leukocytes *in vitro* that are known to be related to tolerance induction *in vivo*. These observations extend the current concept of CMVIg as passive CMV prophylaxis to a therapeutic drug compound capable to reduce allogeneic immune response.

BACKGROUND

Cytomegalovirus and its role in solid organ transplantation

CMV - definitions [1]:

Viremia is defined as a positive viral blood culture proved by standard or shell vial techniques.

Antigenemia: The detection of pp65, a CMV specific protein, in leukocytes is termed antigenemia.

Latency is the silent carriership of CMV genome causing a low grade infection without signs of active viral replication.

CMV infection is a state of active viral replication and defined by the isolation of either the whole virus, viral protein or viral deoxyribonucleic acid (DNA) from body fluids.

CMV disease is a symptomatic CMV infection.

Cytomegalovirus belongs to the *Betaherpesvirinae* family. Like other herpes viruses it is able to persist lifelong in healthy individuals unaffected by the immune system. Epidemiological data suggest that roughly 60 to 90 percent of the adult population in developed countries are infected [2, 3]. CMV antigens can be purified from urine, saliva, tears, semen, milk and liquor even years after first CMV contact. The virus is able to spread horizontally (transmission between members of the same social group that are not in a parent-child relationship) as well as vertically (from mother to child during the perinatal period). Breast feeding is the most common way of the vertical infection route [4]. However, transplancental transmission and infections during delivery are also described in literature [5, 6]. In a prospective study Vouchem *et al*. showed that the concentration of viral DNA in breast milk strongly correlates with the incidence of CMV infections in infants. Preterm infants were at a higher risk for CMV infection when compared to mature newborns [7].

For the horizontal infection route close contact seems to be necessary. However, the precise transmission route is still unknown. Histopathologic examinations of necropsy tissues indicate that the upper respiratory tract, the upper alimentary tract or the genitourinary tract are the most likely route of infection. Epidemiological data suggest that unhygienic conditions and low socioeconomic status are the major risk factors for a horizontal infection [8]. Rare cases of transmission through blood transfusions by

direct transfer of viral antigens in leukocytes (neutrophiles and monocytes) are also discussed in literature. However, this route only seems possible during acute infection [8], where a high virus load is present.

In healthy individuals CMV is kept under control by the immune system. Total clearance of the virus is rare resulting in latent, lifelong infections. Primary CMV infections normally proceed subclinical. In rare cases a symptomatic infection develops, becoming manifest in unspecific features e.g. persistent fever, myalgia, cephalea, lymphadenopathy and splenomegaly. However, a wide range of complications including gastrointestinal infections, hepatitis, cystitis, myocarditis, pneumonia, retinitis, encephalitis, polyradiculitis (Guillian-Barré syndrome) is known. Elevated liver enzymes and an increased serum bilirubin are often associated with CMV infections.

As most of the infections remain inapparent and complications are rare, CMV was of no great interest, traditionally. However, the impact of CMV infections has increased due to the growing importance of organ transplantation, immune deficiency syndromes (e.g. acquired immune deficiency syndrome - AIDS) and the extended use of immunosuppressive drugs.

The incidence of CMV infections in allograft recipients is considerable. Epidemiological data reveal that CMV is the second most infectious agent during post-transplant course.

Table 1 Incidence (%) of infectious diseases in solid organ transplant recipients (adapted form Patel *et al*. [9])

Type of infection	Liver	Kidney	Heart	Lung/Heart-Lung	Pancreas/Kidney-Pancreas
bacterial infections	33-68	47	21-30	54	35
CMV	22-39	8-32	9-35	39-41	50
HSV	3-14	53	1-42	10-18	6
VZV	5-10	4-12	1-12	8-15	9
Candida spp.	1-26	2	1-5	10-16	32
Mycelial fungi	2-4	1-2	3-6	3-19	3
Pneumocystis carinii	4-11	5-10	1-8	15	NA

CMV = cytomegalovirus; HSV = herpes simplex virus; VZV = varicella zoster virus; NA = not available

In other reviews CMV infections are even considered the most important infectious complication after solid organ transplantation [10, 11].

Post-transplant CMV disease is associated with an enormous financial strain resulting in a 40 to 80 percent increase in direct medical charges [12, 13]. The major risk factor for a later CMV disease is a serological CMV mismatch. Seronegative recipients receiving an organ from a seropositive donor are high risk patients [14]. Besides these immunological factors certain immunosuppressive agents also seem to have an influence. Induction therapy with antithymocyte globulin (ATG)-fresenius resulted in earlier and higher antigenemia when compared to induction therapy with thymoglobuline [15, 16]. In addition the administration of ATG during acute rejection periods in kidney transplantations was also associated with a consecutive development of a CMV disease [14, 16, 17].

CMV disease in the transplant patient can be caused by primary infection (if the patient has not been exposed to CMV before), re-activation or re-infection with CMV. It predominantly occurs during the first four months after transplantation [18]. Clinical symptoms range from general discomfort, fever, myalgia, arthralgia as well as organ involvement e.g. esophagitis, gastritis, enterocolitis (subsumed as CMV induced gastrointestinal diseases), hepatitis, pancreatitis, nephritis, cystitis, myocarditis, pneumonia, retinitis, CNS diseases (encephalopathy, polyradiculopathy) [1].
A generalized form of CMV disease, the CMV syndrome, must be assumed if the patient has a proven antigenemia or viremia and at least one of the following:
(i) temperature >38°C,
(ii) new or increased malaise,
(iii) leukopenia,
(iv) > 5 percent atypical lymphocytes,
(v) thrombocytopenia,
(vi) elevated liver enzymes.
The prognosis of a CMV syndrome is disastrous with a mortality of over 90 percent [13].

In addition to the above described deleterious effects of CMV infections, CMV can also damage the transplanted organ.
CMV infections were shown to promote acute graft rejections in heart [19] and kidney [20, 21] recipients. Data referring to lung allografts are contradictory [22, 23]. In liver

transplantation no significant correlation between CMV infections and graft rejection was found [24].

The reasons for these effects on early allograft failure are thought to be due to immunomodulatory properties of CMV. The virus is able to render the immunological state of a transplanted organ by upregulating major histocompatibility complex (MHC) class I and class II as well as boosting the nuclear factor kappa B (NfκB) pathway *in vitro* [25]. Furthermore, CMV can trigger superinfections with opportunistic infectious agents [26, 27].

Besides their ability to promote acute graft rejection, CMV infections were also shown to be a risk factor for chronic transplant dysfunction. A higher incidence of bronchiolitis obliterans syndrome in lung transplant recipients [22, 28], vanishing bile duct syndrome in liver transplantation [29-31] has been described following CMV disease.

In transplanted hearts an increased appearance of graft vasculopathy was associated with CMV infections [32, 33]. This phenomenon was studied by Lemstrom *et al*. in a rat transplant model [34]. The most prominent changes in the hearts of CMV infected rats were found in small intramyocardial arterioles. Immunohistochemical staining showed a CMV specific mononuclear cell infiltrate in tissue with replicating virus. This inflammatory infiltrate led to endothelial proliferation and intima-thickening – both relevant factors in graft vasculopathy. In addition CMV infections were shown to increase the amounts of atherosclerotic fatty streaks in endothelium [35].

Therapeutic strategies

In immunocompetent individuals normally no specific treatment of CMV infections is required. In immunocompromised patients there are three fundamental strategies: universal prophylaxis, pre-emptive therapy and treatment of an already manifest disease.

Universal prophylaxis

CMV disease, CMV-associated mortality and number of acute rejection periods can be effectively reduced by universal prophylaxis [36]. Pharmacoeconomical analysis revealed that the CMV prophylaxis is an effective way to save money in solid organ transplant medicine by significantly reducing the days spent in hospital and the overall costs after transplantation [37]. Therefore, CMV prophylaxis is recommended in most clinical guidelines [38].

Contrarily, universal anti-viral prophylaxis is expensive and most drugs used can cause bone marrow suppression and evidence renal toxic effects. In addition, the broad application of anti-viral drugs in universal prophylactic strategies creates drug resistances due to viral mutations [39, 40].

Regarding the different drugs available for universal prophylaxis ganciclovir, valganciclovir and valaciclovir proved to be more effective than aciclovir. Anti-viral prophylaxis also shows protective effects on CMV associated chronic allograft dysfunction [41]. This effect can be ameliorated by the addition of CMV hyperimmunoglobulin (CMVIg) to ganciclovir universal prophylaxis [13].

Pre-emptive therapy (selective prophylaxis)

Pre-emptive therapy was introduced in the early 90s to reduce adverse side effects to a minimum by constricting patient collective receiving anti-viral drugs [42]. It is also a prophylactic approach, however, anti-viral treatment is only initiated in patients with proven antigenemia. Antigenemia is thought to be a reliable marker for a later outburst of CMV disease (positive predictive value of 94%) [43]. Meta-analysis revealed that pre-emptive therapy with anti-viral drugs had the same positive effects on CMV disease, acute rejection, chronic rejection and all-cause mortality as the universal approach. On the other hand, adverse side effects were significantly lower in pre-emptive group compared to the universal prophylaxis [44]. The limiting factor of

the pre-emptive approach is a disaccordance in determinating cut-off values for clinical intervention. Furthermore, there is still a lack of laboratory assay standardization for the detection of antigenemia. Therefore pre-emptive therapy is only recommended in patients with low to intermediate risk for developing a CMV disease. The optimal drug regimen is either intravenous (iv) ganciclovir (5mg/kg per 12h) or valganciclovir (900mg/bid) [13].

The most recent revision of guidelines for prevention of CMV disease in solid organ transplantation has been published by the Canadian Society of Transplantation Consensus Workshop Group:

Table 2 Guidelines for CMV prevention in solid organ transplant recipients (adapted from Preiksaitis *et al*. [13])

Organ/CMV status	Relative merits of universal prophylaxis, pre-emptive therapy	Recommendations/options when applying universal prophylaxis
Kidney, liver, pancreas, heart D+/R-	Universal prophylaxis preferred over pre-emptive therapy as rapid rise in viral load in absence of previous immunity makes pre-emptive strategies logistically difficult	oral ganciclovir (3g/d) valganciclovir (900mg/d) iv ganciclovir (5mg/kg/d) valganciclovir (8g/d) is an alternative in kidney recipients
Kidney, liver, pancreas, heart R+	Either universal prophylaxis or pre-emptive therapy are acceptable options	oral ganciclovir (3g/d) valganciclovir (900mg/d)
Lung, heart-lung D+R-	In this high-risk group, universal prophylaxis is preferred rather than pre-emptive therapy	iv ganciclovir (5mg/kg/d) valganciclovir (900mg/d) +/- CMV hyperimmunoglobulin
Lung, heart-lung R+	Universal prophylaxis is preferred rather than pre-emptive therapy	iv ganciclovir valganciclovir (900mg/d) +/- CMV hyperimmunoglobulin

Abbr.: D - donor, R - recipient, CMV cytomegalovirus

Treatment of CMV disease

Ganciclovir, foscarnet and cidofovir have been proved beneficial for the treatment of a manifest CMV disease. However, the capacious clinical experience with the “old” substance ganciclovir and the described nephrotoxicity of foscarnet and cidofovir in combination with calcineurin inhibitors make iv ganciclovir (5mg/kg per 12h) the recommended fist-line drug for solid organ transplantation. Valganciclovir (a prodrug of ganciclovir with high oral bioavailability) has similar pharmacokinetics and can be used instead of the iv drug [45]. Addition of CMVIg is recommended for severe CMV

disease with lung and liver involvement. Immunosuppression should be reduced to a minimum during anti-viral treatment. When fulldose iv ganciclovir therapy for one week shows no effect (persisting viral loads or clinical symptoms) a ganciclovir-resistant CMV disease should be assumed. This often occurs in a seropositive donor/seronegative recipient setting. Rescue therapy with CMVIg, high dose foscarnet or cidofovir should be initiated for this ganciclovir insensitive CMV disease [13].

Passive immunization - CMV hyperimmunoglobulin

Passive immunization against CMV with high dose iv immunoglobulins can be added to a pre-existing therapeutical anti-viral approach (as described above) but is generally a prophylactic option. Two different drug preparations can be used: unspecific intravenous immunoglobulins (IvIg) and CMVIg.

IvIg are used in many clinical conditions (see Table 4) and are currently under discussion for their application in transplant medicine [46]. CMVIg are produced by pooling plasma from hundreds of healthy donors with a proven antibody titer greater than 1 in 7000. CMVIg were shown to be highly effective in preventing CMV disease when administered during the post-operative course [47-51]. CMVIg therapy is rather expensive, however, cost-effectiveness analysis show that CMVIg are comparable to other well-accepted medical treatments [52, 53].

Although beneficial effects of CMVIg in preventing CMV disease are well documented, little evidence exists describing underlying mechanisms. Andreoni *et al*. showed that CMVIg are able to neutralize CMV infectivity and can reduce the intracellular, pro-inflammatory response in a fibroblast cell line [54]. Furthermore, CMVIg could reduce cytopathic effects in infected fibroblasts in an antibody-dependent cell-mediated cytotoxicity assay *in vitro* [55].

Currently, two different CMVIg containing drugs are liscenced in Austria: Cytotect by Biotest and Cytoglobin by Bayer.

Table 3 Distribution of different Ig-subsets of Cytotect and Cytoglobin

	Cytotect	Cytoglobin
IgG	> 95%	> 98%
IgG1	62%	65%
IgG2	34%	29%
IgG3	0.5%	5.7%
IgG4	3.5%	1.1%
IgA	< 5mg/mL	< 0.21mg/mL
specific Ab against CMV	50 E	100 E

CMVIg containing drugs are considered “save” drugs as adverse side effects are rare. They are often caused by a too high infusion speed. Therefore, infusions should not run above 0.08mL/min for adults (about 20 drops per minute). Chills, cephalea, fever, nausea, reversible aseptic meningitis and arthralgia can occur. Hypersensitivity and anaphylactic reactions are extremely rare and often associated with an immunoglobulin A (IgA)-deficiency syndrome (anti-IgA antibodies). Furthermore, cases of renal failure have been described. Most of them were due to pre-existing renal insufficiency, diabetes mellitus, hypovolemia, hyperviscosity or co-administration of kidney-toxic drugs. Thrombo-embolic events can be triggered by CMVIg [56, 57]. The mechanism being responsible for this was shown to be a ligation of the low affinity Fcγ-receptor (FcγR; CD32) on thrombocytes resulting in platelet activation and subsequent aggregation [58].

The risk of transfection with pathogens is minimized by inactivation of possible infectious material during the manufacturing process. In addition the drug is tested routinely for human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV). No specific drug interactions have been reported yet. However, the effect of attenuated living vaccines (e.g. mumps, measles, roseola, chickenpox) can be reduced. The drug has no influence on pregnancy. In contrast, there are data indicating that CMVIg is a save and effective way to treat prenatal CMV disease [59].

Management of CMV at the Viennese Heart Transplant Center

All patients – unregarded of their or the donor’s serological status – undergoing heart transplantation at the Viennese Heart Transplant Center receive CMVIg (Cytotect, Biotest) as universal prophylaxis. Cytotect is given iv at a dose of 100mg/kg pre-operatively and on day 1, 7, 14, 21, 28 post-operatively. In addition high risk patients

receive valganciclovir at doses between 450 to 900mg/d depending on their renal function. This therapeutic concept was introduced in 1987 together with the application of rabbit antithymocyte globuline (ATG) as induction therapy. Since then, rejections rates are one of the lowest when compared to other transplantation centers (86 percent freedom of rejection ISHLT>2 – classified by the guidelines of the International Society for Heart and Lung Transplantation – within the first year after transplantation) [60]. In addition incidence of graft atherosclerosis is very low due to the aggressive CMV prophylaxis [61-63].

Intravenous immunoglobulins

The first therapeutical application of immunoglobulins can be dated 1891 when Emil von Behring treated two children suffering from diphtheria by injecting antisera. However, purification techniques for intravenous application were not developed before the 1970s. The first clinical indication of IvIg was the congenital Bruton agammaglobulinemia, applications in other immunodeficiency syndromes followed. By the 1980s IvIg were utilized in various autoimmune diseases and inflammatory disorders, administered in high intravenous doses. Interestingly, this new clinical effect was discovered incidentally during the treatment of two children with a congenital immune deficiency who coincidental suffered from idiopathic thrombocytopenic purpura. The pediatrist who treated the children observed that IvIg infusion also increased the number of thrombocytes in his patients [64]. Today, IvIg are suggested as first line therapy in several inflammatory disorders e.g. Kawasaki syndrome [65], Guillain-Barré syndrome [66], chronic inflammatory demyelinating polyneurophathy [67], Myasthenia gravis [68], idiopathic thrombocytopenic purpura [69], multifocal neuropathy [70], Epidermolysis bullosa [71].

Table 4 Proved beneficial effects of IvIg therapy (adapted from Emmi *et al*. [72])

Acquired immune thrombocytopenias
Autoimmune neutropenias
Autoimmune hemolytic anemia
Autoimmune erythroblastopenia
Autoimmune myocarditis [73]
Von Willebrand disease
Hemophilia A and B associated with antibodies to factors VIII and IX
Dermatomyositis
SLE [74, 75]

RA
Lambert-Eaton syndrome
ANCA positive systemic vasculitis
Goodpasture syndrome
Antiphospholipid syndrome
Chronic fatique syndrome
MS
Intractable childhood epilepsy (Lennox Gastaut and West syndrome)
Rasmussen encephalitis
Stiff-man syndrome
Thyroid-related eye disease
Therapy-refractory kidney and bone marrow graft rejections [76-79]
GvH disease [80]
Steroid dependent asthma
IBD

Abbr.: SLE - systemic lupus erythematosus, RA - rheumatoid arthritis, ANCA – anti-neutrophil cytoplasmatic antibodies, MS - multiple sclerosis GvH - graft versus host, IBS - inflammatory bowel diseases

Although the positive effects of IvIg have been proved in many controlled clinical trials as well as meta-analysis, their mode of action still remains unclear. The main theories are:

Natural antibodies

Natural antibodies are pre-existing antibodies not produced as a result of a prior immune response. They can be found during embryonic development and in animals held under sterile conditions. Their specificity, affinity and titer are low and they can not be transformed by affinity maturation. Natural antibodies were shown to be either directed against conserved auto-antigens or non-self antigens [81].

They are able to prevent early dissemination of viral pathogens into the brain and the kidney and therefore considered as part of the innate immune response. As some autoimmune disorders are associated with viral infections, natural antibodies can ameliorate these disorders by reducing viral loads [82].

Self-reacting natural antibodies were shown to have the property to regulate a ditched immune system by demonetising senescent or apoptotic cells *via* opsonisation [83, 84]. This is essential in order to prevent the leakage of auto-epitopes from aged or damaged cells.

However, as the vast majority of natural antibodies are subtype immunoglobulin M (IgM), their concentration in IvIg containing drugs is minimal.

Anti-idiotype antibodies

IvIg contain small amounts of antibodies directed against universal immunoglobulin structures e.g. the Fc fragment. These anti-antibodies are termed anti-idiotype antibodies. Anti-idiotype antibodies have the ability to neutralize disease-associated auto-antibodies [85]. However, the role of anti-idiotype antibodies is considered modest, suggesting that there must be additional mechanisms responsible for the immunomodulatory effects of IvIg containing drugs [72].

Modulation of complement

In some of the disease entities listed in Table 4 an overflowing compliment system mediating immune complex associated damage is deemed pivotal. The high immunoglobulin G (IgG) blood concentration after IvIg application is able to withdraw complement components C1, C3b, C4, C3b (reviewed in Stangel *et al*. [64]) and anaphylotoxins C3a and C5a [86] from circulation.

Auto-antibodies directed against CD4, MHC, CD95 and cytokines

IvIg contain small amounts of anti-CD4 and anti-MHC antibodies. In *in vitro* studies blocking of these membrane proteins resulted in a decreased function of CD4 and CD8 bearing cells [87, 88]. Blockage of CD95 (Fas-receptor, APO-1) on keratinocytes by antagonistic auto-antibodies present in IvIg preparations was described to be important in toxic epidermal necrolysis [89, 90]. Furthermore, IvIg contain physiological occurring auto-antibodies directed against interleukine (IL)-6, IL-1, tumor necrosis factor (TNF)-α, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) [89, 91]. The mechanism of action attributed to this theory is the neutralization of an inflammatory overshoot. However, contents of these auto-antibodies vary immensely between manufacturers and patches.

Triggering of Fcγ-receptors by IgG dimers

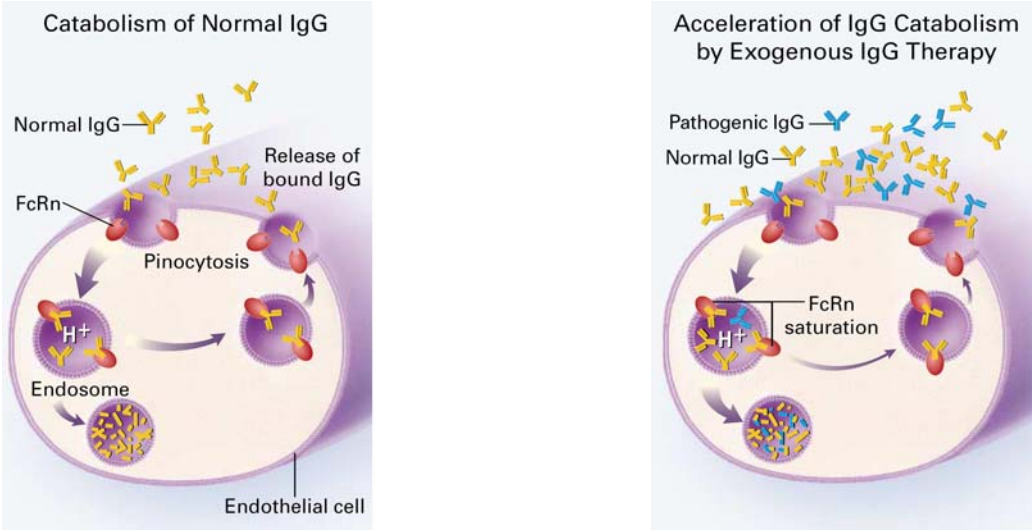
IgG are present as dimers in small quantities in IvIg containing drugs. These crosslinked IgG can trigger Fcγ-receptors resulting in a prolonged anti-inflammatory cytokine release subsequent to the initial release of pro-inflammatory peptides [92, 93]. Dimers were also described to bind to an inhibitory Fc-receptor, termed FcγRIIb. Activation of FcγRIIb leads to a direct release of anti-inflammatory cytokines, decreases proliferation of B cells and inhibits endogenous immunoglobulin

production [94]. Furthermore, IvIg themselves induce an upregulation of the FcγRIIb. FcγRIIb can crosslink with FcγRIII (CD16) and in this way reduce the pro-inflammatory effect of immune complexes.

Acceleration of auto-antibody catabolism by binding to FcRn

This theory addresses the ability of IvIg to accelerate IgG catabolism. Normally, IgG enter the cell through pinocytosis and are degraded by the endosome/lysosome system. However, IgG can escape their degradation by binding to FcRn. These receptors are initially internalized but can re-circulate to the cell membrane (Figure 1). IvIg application leads to an overflow of IgG in the circulation resulting in a saturation of FcRn. Therefore IvIg can promote the catabolism of pathognomonic auto-antibodies found in Myasthenia gravis or the Lambert-Eaton syndrome [95].

Figure 1 Mechanism of exogenous IgG therapy based on the concept of FcRn saturation (adapted from Yu *et al.* [96])



Fcγ-receptors and their functions

FcγR are classified through their affinity towards the IgG molecule. One high affinity (CD64 – FcγRI) and two low affinity receptors (CD32 – FcγRII, CD16 – FcγRIII) can be distinguished. Engagement of FcγR triggers a variety of biological functions ranging from phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC),

degranulation of preformed vesicles and transcriptional activation of cytokine coding genes [97]. FcγR are also responsible for demonetizing immunoglobulin complexes.

Table 5 Expression of human Fcγ-receptors (adapted from Takai *et al.* [97])

FcγRI	macrophage, monocyte, neutrophil, dendritic cell
FcγRIIa	macrophage, neutrophil, eosinophil, platelet, dentritic cell, NK cell
FcγRIIb	B cell, mast cell, dendritic cell, macrophage, eosinophil, neutrophil
FcγRIII	macrophage, monocyte, NK cell, mast cell, eosinophil, dendritic cell, T cell
FcRn	placenta, small intestine, monocyte, dendritic cell, endothelium

Table 6 Biological functions of human Fcγ-receptors (adapted from Flesch *et al.* [98])

	FcγRI	FcγRIIa	FcγRIIb	FcγRIII	FcRn
Endocytosis	+	+	-	+	-
Phagocytosis	+	+	-	+	-
ADCC	+	+	-	+	-
Superoxide production	+	+	-	+	-
Cytokine production	+	+	-	+	-
B cell downregulation	-	+	+	-	-
IgG regulation	-	-	-	-	+

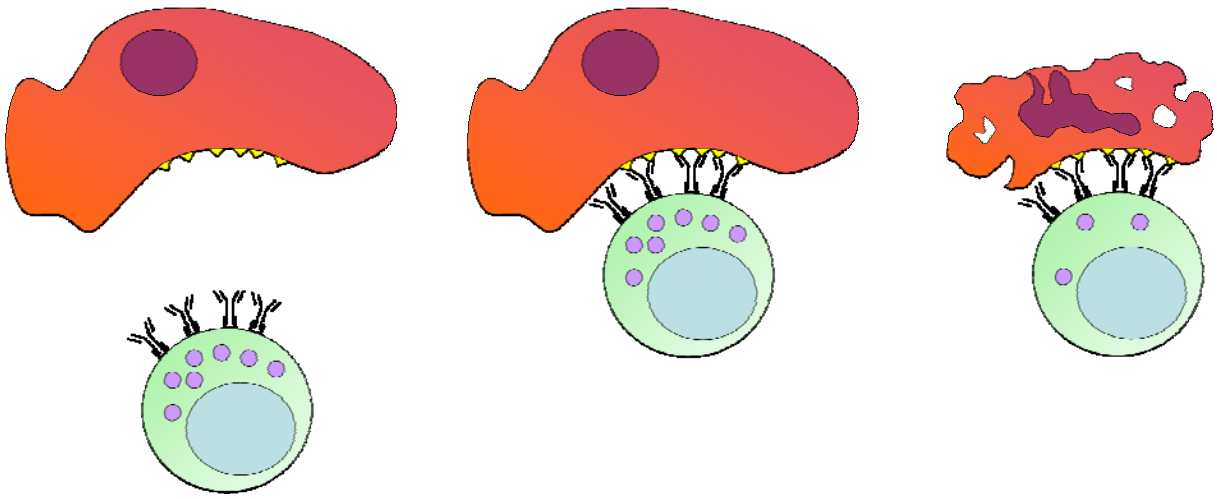
Antibody-dependent cell-mediated cytotoxicity

ADCC is an immune effector mechanism linking features of the innate and the adaptive immune system. Three components are required: a target antigen, an antigen-specific antibody and a Fc-receptor bearing effector cell. The antibody binds to the target cell's surface antigen leading to the crosslinking of Fc-receptors and a consecutive activation of effector cells (Figure 2) [99]. Natural killer (NK) cells, macrophages and neutrophils have been described to possess ADCC features. However, NK cells are considered most important. They kill their target cells mainly by releasing preformed granules containing granzymes and perforin.

Macrophages develop their ADCC effect by phagocytosis and by release of nitric oxide (NO), reactive oxygen radicals as well as a variety of proteases. They also use Fas/FasL interactions to lyse target cells [100]. Neutrophils have been described to exhibit ADCC through IgA receptors (CD89) leading to a direct injection of caspases into target cells [101-103].

ADCC in NK cells is mainly mediated through FcγRIII (CD16) and to a less content through FcγRII (CD32).

Figure 2 Mechanism of antibody-dependent cell-mediated cytotoxicity



CD4+CD25bright T cells (Tregs)

Treg (T regulatory) cells play a pivotal role in suppressing an immune response [104, 105]. Normally, they are produced in the thymus and therefore are also termed natural occurring Tregs (nTregs). However, they can also differentiate from naïve T cells in the periphery by certain stimuli (induced Tregs) [106, 107]. *In vitro* data suggest that T cell receptor triggering with a MHC bound self-peptide on cortical epithelial cells together with specific interactions of accessory molecules (CD28, B7, CD40) are imperative for the development of nTregs [108].

Tregs were found to be anergic *in vitro.* They cannot be stimulated by anti-CD3, or antigen presenting cells (APC). However, they are able to suppress proliferation of other responder T cells. nTregs do this in by close cell-cell contact, partly due to a perforin/granzyme-dependent cytolytic pathway [109-112]. Induced Tregs execute their anti-inflammatory properties mainly by secretion of high levels of IL-10 and transforming growth factor (TGF)-β [110, 113].

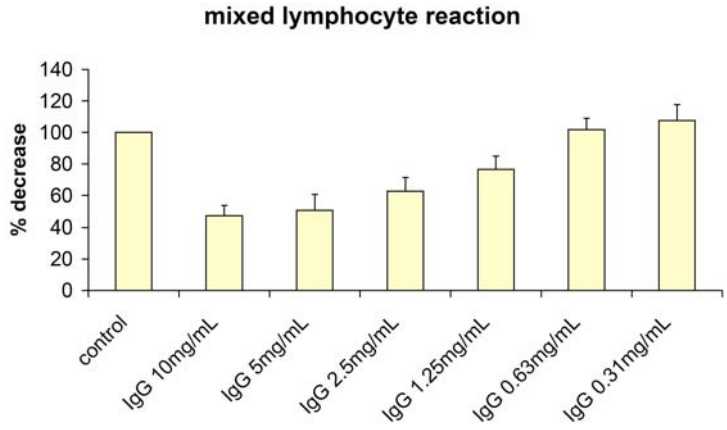
Tregs can be determined by their constitutively high expression of the IL-2Rα chain and stain positive for intracellular FoxP3, a transcriptional factor imperative for Treg development and function [114].

RATIONALE AND AIM OF THE STUDY

Previous findings of our research group

Pooled human IgG purchased from a biochemical company (Sigma, St. Louis, MO, USA) were tested in mixed lymphocyte cultures for their effects on cell proliferation. Addition of 10mg/mL of pooled IgG reduced the proliferation to 47% of maximal proliferative response (control). This reduction was dose-dependent and ceased at a IgG dose of 0.31mg/mL [115].

Figure 3 Anti-proliferative effects of pooled human IgG



Based upon this observation the zero hypothesis of this study was defined: Proliferation in blastogenesis assays is not influenced by the addition of CMVIg containing drugs.

The basis of this work was to evaluate anti-proliferative effects of CMVIg, the role of CMVIg on cell viability and the effect of CMVIg on NK cell effector functions. In detail the following parameters were important:

a) Proliferation assays were performed, stimulating PBMC with either anti-CD3 in blastogenesis assays or irradiated allogeneic PBMC in mixed lymphocyte reactions (MLR). Results obtained from the proliferation assays were verified by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution experiments and cell cycle analysis. To evaluate the role of cytokines in these experiments, supernatants obtained from the blastogenesis assays were analyzed for anti-inflammatory and pro-inflammatory cytokines.

b) To test apoptosis-inductive properties of CMVIg, PBMC were co-stained for Annexin/propidium iodide (PI) and assessed by flow cytometry. To determine the subpopulations most prone for apoptosis induction highly purified CD4+, CD8+, CD19+ and CD56+ were exposed to CMVIg.

c) Morphologic features of PBMC exposed to CMVIg were assessed by transmission electron microscopy.

d) The effect of CMVIg on NK effector cells was addressed by flow cytometric analysis of FcγRIII and cell membrane bound IgG. The functional relevance of these findings was confirmed by europium release ADCC assays.

e) To quantify the effect of CMVIg on the inflammatory response of monocytes, cytokine release (IL-8, TNFα) after lipopolysaccharide (LPS)-stimulation was evaluated.

f) To determine the ability of CMVIg to expand CD4+CD25bright cells *in vitro*, FACS analysis of CMVIg exposed PBMC were performed.

PROBAND SELECTION, SATISTICAL METHODS AND EXPERIMENTAL DESIGN

Proband selection

Experiments were performed with PBMC obtained from healthy, young volunteers. The study protocol was approved by the ethics committee of the Medical University of Vienna (EC-No: 083/2006). The main outcome variable was the reduction of proliferation rates in MLRs and blastogenesis assays. A power analysis based upon preliminary data revealed a necessary sample size of n=10 to get a statistically significant statement.

Inclusion criteria: age>18, age<40.
Exclusion criteria: Autoimmune diseases, acute or chronic infections, malignancies, medication with immunomodulatory agents e.g. steroids. Probands, unable to give permission or probands taking part in another clinical study at the same time were excluded.

Statistical methods

Statistical analysis were performed using SPSS 14.0 software (SPSSInc., Chicago, IL, USA). Data are given as mean ± standard error of the mean (SEM). Normal distribution was verified using the Kolmogorov-Smirnov test. Two sided students T-tests for paired and unpaired comparisons were used to calculate significances. P-values of 0.05 or lower were considered statistically significant. Bonferroni-Holm correction was applied to correct for multiple comparisons. For evaluation of ADCC data, areas under the curve (AUC), calculated by linear interpolation for each condition, were compared.

Blood samples and CMV hyperimmunoglobulin

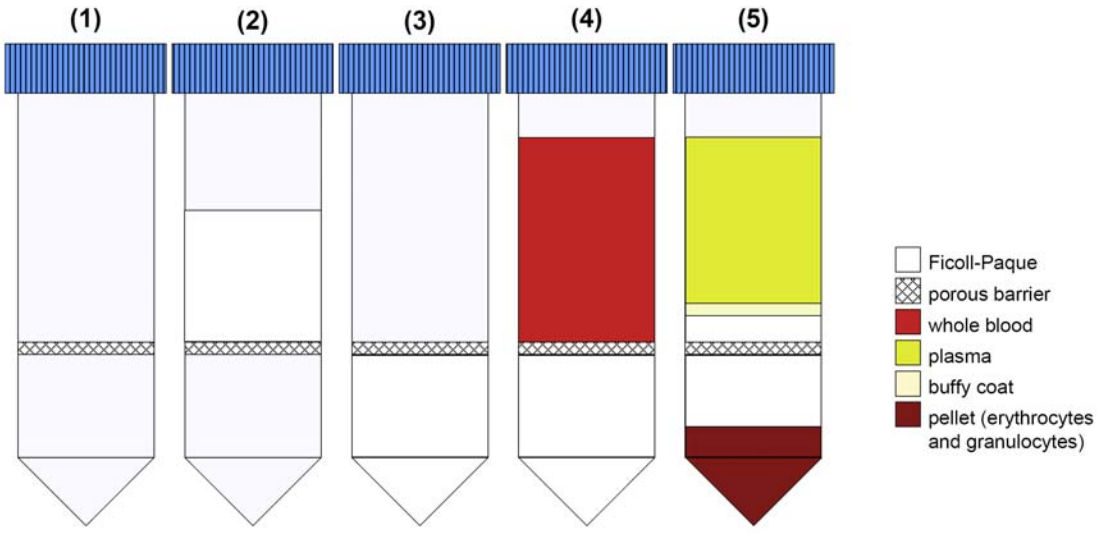
Samples of venous blood (anticoagulated by heparin) were freshly obtained from each proband. Experimental analysis were performed immediately after the blood draw in order to avoid loss of cell viability or functionality.

CMVIg were obtained from two different manufacturers (Cytotect by Biotest, Dreieich, Germany; Cytoglobin by Bayer, Leverkusen, Germany) and kept sterile at 4°C until used in the experiments. CMVIg were added to all experiments at therapeutic concentrations as described by Kwekkeboom *et al*. [116]. In control experiments CMVIg were replaced by medium. Dialysis of CMVIg with a 1kDa cut off for exclusion of stabilizing agents was additionally performed (Mini dialysis kit; Amersham Pharmacia Biotech, Uppsala, Sweden). This was due published data showing that stabilizing sugars had negative effects on cell viability and growth [117]. Dialysis of Cytotect and Cytoglobin had no effect in *in vitro* assays.

Separation of peripheral blood mononuclear cells

PBMC were separated by Ficoll densitiy gradient centrifugation. Freshly obtained whole blood samples were diluted 1:2 in Hanks' Balanced Salt Solution (HBSS). 15mL Ficoll-Paque (GE healthcare, Uppsala, Sweden) were transferred to a 50mL Leucosept tube (Greiner, Frickenhausen, Germany) and centrifuged for one minute at 1000g bringing Ficoll under the porous barrier. Diluted whole blood was shifted upon the porous barrier and tubes were centrifuged for 15min at 800g at room temperature. The buffy coat containing PBMC was lifted off carefully (Figure 4). Cells were washed two times in HBSS, stained for viability with trypan blue (Sigma, St Louis, MO, USA) and counted on a hematocytometer (KX-21, Sysmex, Norderstedt, Germany).

Figure 4 Ficoll density gradient centrifugation



^{3}[H]-thymidine proliferation assay

1*10^{5} PBMC were re-suspended in 150µL of serum free Ultra Culture Medium (Cambrex Corp., NJ, USA) containing 0.2% gentamycinsulfate (Sigma, St. Louis, MO, USA), 0.5% β-Mercapto-ethanol (Sigma, St Louis, MO, USA) and 1% L-Glutamin (Sigma, St Louis, MO, USA). For blastogenesis assays cells were stimulated with monoclonal antibodies (MoAb) to CD3 (10µg/mL, Becton Dickinson, Franklin Lakes, NJ, USA) for 48h at 37°C in a humidified atmosphere of 5% CO_2 in 96-well round-bottomed tissue culture plates (Figure 5a). For MLRs, 1*10^{5} responder cells were added to irradiated (60 Gy) stimulator cells at a 1:1 ratio and cultured for 120h in an incubator (Figure 5b). CMVIg were applied in various concentrations, medium served as control. Cells were pulsed for 18h with ^{3}[H]-thymidine (3,7*10^{4} Bq/well; Amersham Pharmacia Biotech, Uppsala, Sweden), harvested and thymidine uptake was measured in a liquid scintillation counter.

Figure 5a Model of an anti-CD3 blastogenesis assay

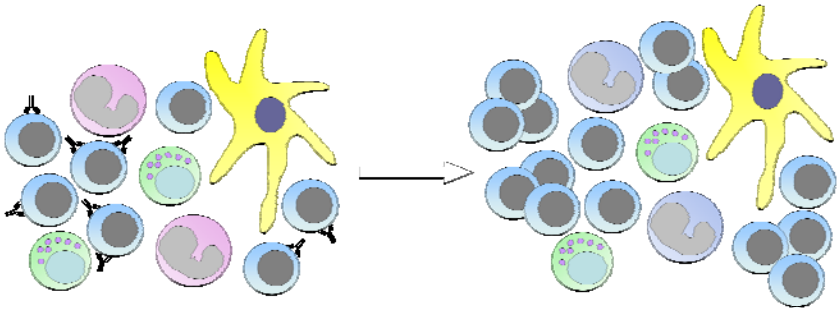
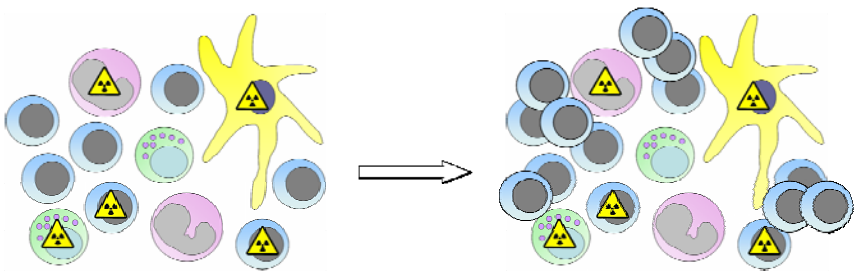


Figure 5b Model of a mixed lymphocyte reaction



CFSE dilution assay

$1*10^7$ PBMC were labelled with 5µM CFSE (Fluka BioChemika, Buchs, Switzerland) at room temperature for 10min. Labelling was stopped by addition of fetal calf serum (FCS). Cells were washed twice, resuspended in Ultra Culture Medium and stimulated with 10µg/mL anti-CD3 MoAb in a humidified incubator. CFSE dilution was measured after 48h on a flow cytometer FC500 (Beckman Coulter, Fullerton, CA, USA).

Cell cycle analysis

PBMC were stimulated with 10µg/mL anti-CD3 for 48h and stained for DNA content using a cell cycle test kit (Becton Dickinson, Franklin Lakes, NJ, USA); PI was utilized for DNA detection. Cells were measured on a flow cytometer and histograms were analyzed by ModFit LT software (Verity Software House, Topsham, ME, USA).

Apoptosis detection by flow cytometry

PBMC ($3*10^5$) in serum free Ultra Culture Medium were exposed to different CMVIg preparations and incubated in Cryovails (Simport, Beloeil, Canada) in a humidified atmosphere. For determination of apoptosis cells were transferred to 1.4mL Fluorescent-activated cell sorting (FACS) tubes (Micronic Systems, Lelystad, Netherlands), washed once in Annexin Binding Buffer (Becton Dickinson, Franklin Lakes, NJ, USA). Pre-experiments revealed 1µL of of Annexin-fluorescein (FITC) and 1µL of PI (both Becton Dickinson, Franklin Lakes, NJ, USA) *ad* 50µL Annexin Binding Buffer a suitable amount for $5*10^4$ cells to obtain optimal staining intensity. After addition of Annexin-FITC and PI tubes were incubated for 15min in the dark at room temperature. 500µL Annexin Binding Buffer were added and cells were analyzed on a Cytomics FC 500 (Beckman Coulter, Fullerton, CA, USA).

Morphologic analysis by transmission electron microscopy

$1*10^6$ PBMC were incubated with or without CMVIg for 12h. Cells were spun down at 270g for 7min and the material was processed according to standard electron

microscopical methods: fixation in 4% paraformaldehyde, pH 7.4, at 4°C, dehydration in a graded ethanol series and embedding in epoxy resin. Ultrathin sections (0,5µm) were cut on a Reichert ultra-microtome (OM U3, Ultracut S), stained with uranyl acetate/lead citrate and sections were examined on a Jeol 1200 EXII electron microscope.

Quantification of CD4+CD25bright cells

Amount of CD4+CD25bright cells was analyzed following a protocol from a publication by Lopez *et al*., who evidenced tolerance inducing effects of ATG [118]. $3*10^5$ PBMC were cultured in 450µL Ultra Culture Medium at 37°C with various concentrations of CMVIg. After 24h cells were stained for CD4 and CD25 expression (antibodies obtained from Becton Dickinson, Franklin Lakes, NJ, USA) and evaluated by flow cytometry.

Effect of CMV hyperimmunoglobulin on LPS-stimulated monocytes

Monocytes were separated from PBMC using CD14+ magnetic beads (positive selection, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purity obtained by this system was determined by flow cytometry as 96% CD14+ expressing cells (data not shown). $5*10^4$ purified monocytes were pre-incubated with therapeutic concentrations of CMVIg for 4h in 96-well round-bottomed plates. Cells were stimulated for 4h with 1ng/mL LPS (Sigma, St. Louis, MO, USA). Supernatants were collected and kept at -80°C till further tests were performed.

IL-2, IL-10, IFNγ, TNFα and IL-8 ELISA

Cytokines were measured utilizing ELISA kits (BenderMedSystems, Vienna, Austria) following the manufacturer's instructions. In detail plates were either supplied pre-coated or coated, sealed and incubated over night at 4°C. Plates were washed (Phosphate buffered saline (PBS) + 0.05% Tween20) and blocked with Assay buffer (PBS, 0.05% Tween20, 0.5% bovine serum albumin (BSA)) for two hours at room temperature. Standards or samples and a biotin conjugated detection antibody were added to each well and incubated for another two hours. Plates were washed and

streptavidin-horseradish peroxidase (HRP) conjugate was added. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as substrate solution (Sigma, St. Louis, MO, USA) and the enzymatic colour reaction was stopped with 1N sulphuric acid. Plates were read at 450nm on a Wallac Multilabel counter 1420 (PerkinElmer, Waltham, MA, USA). Cytokine concentrations were calculated by comparing optical density (OD) values of samples with OD of known concentrations of the standards.

Cell cultures

Jurkat (human, peripheral blood, leukemia T cells) and PANC-1 (pancreatic cancer cells) (both American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 (Cambrex Corp., NJ, USA) supplemented with 10% heat shock inactivated FCS and 0.2% gentamycinsulfate at 37°C in a humidified atmosphere of 5% CO_2. Cells were split twice per week; 0.25% trypsin (Sigma, St. Louis, MO, USA) was used to re-suspend adherent cells.

Measurement of ADCC by europium release assays

Target cells were counted and split one day before the killing assay to a concentration of $1*10^5$ cells per mL in order to obtain exponential growth.
Target cells: $5*10^6$ target cells were labelled with 1mL of labelling buffer supplemented with 20mM $Eu(CH_3C00)^{3+}$, 100mM diethylenetriaminopentaacetate and 0.5mg of dextran sulphate for 15 min at room temperature with occasional shaking. 20µL of stop solution (100mM $CaCl_2$) were added, cells were washed three times in repair buffer and re-suspended in Ultra Culture Medium. After incubation for 60min cells were stained for their viability using trypan blue and added to a 96-well round-bottomed assay plate.
Effector cells: Freshly obtained PBMC or highly purified NK cells were pre-incubated with CMVIg or Ultra Culture Medium (serving as control) for 4h. Cells were counted and cell number was corrected for viability before added to plates.
Effector/target ratio ranged from 50:1 to 1.6:1 each ratio arranged in triplets. The incubation period was 4h at 37°C in a humidified atmosphere of 5% CO_2. Then plates were centrifuged at 280g for 5min, 30µL supernatant were transformed to a 96-well flat-bottomed plate (Nunc, Roskilde, Denmark) and 100µL of an enhancement

solution (PerkinElmer, Boston, USA) were added. ADCC was measured by time-resolved fluorometry (PerkinElmer, Waltham, MA, USA). Specific cytotoxic activity was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100$$

Total release was determined by adding 2% Triton X-100 (Sigma, St Louis, MO, USA) to target cells.

RESULTS

Anti-proliferative effects of CMVIg

^{3}[H]-thymidine proliferation assay

To address the question if CMVIg possess anti-proliferative properties anti-CD3 blastogenesis assays and MLRs were performed. Both, Cytotect and Cytoglobin reduced proliferation rates in a dose-dependent manner. These effects ceased at concentrations of 0.08mg/mL. In the therapeutic concentration of 2.5mg/mL stimulation indices were reduced to a mean of 36±4.6% of the control value.

Figure 6 Anti-proliferative effects of CMVIg in MLRs

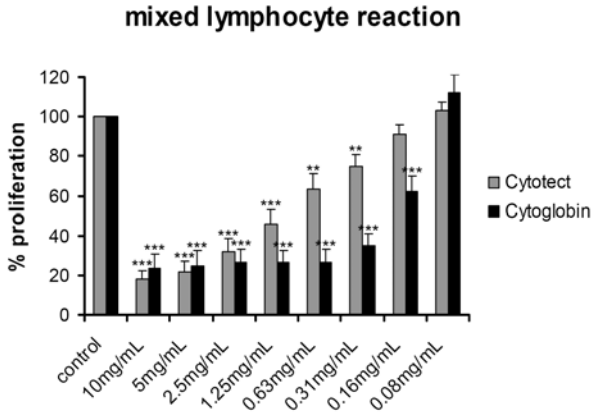


Figure 7 Anti-proliferative effects of CMVIg in blastogenesis assays

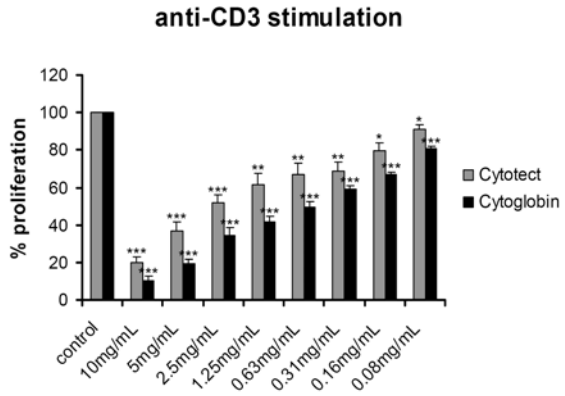


Figure 6 and 7 show anti-proliferative properties of Cytotect and Cytoglobin in MLRs and blastogenesis assays. Cytoglobin seems to be more potent in inhibiting clonal proliferation when compared to Cytotect (* $p<0.05$; ** $p<0.001$; *** $p<0.0001$; n=10).

To verify data obtained from the proliferation experiments, light microscopic evaluations were performed. PBMC cultures stimulated with MoAb to CD3 were exposed to therapeutic concentrations of CMVIg.

Figure 8 Light microscopic evaluations of anti-proliferative properties of CMVIg

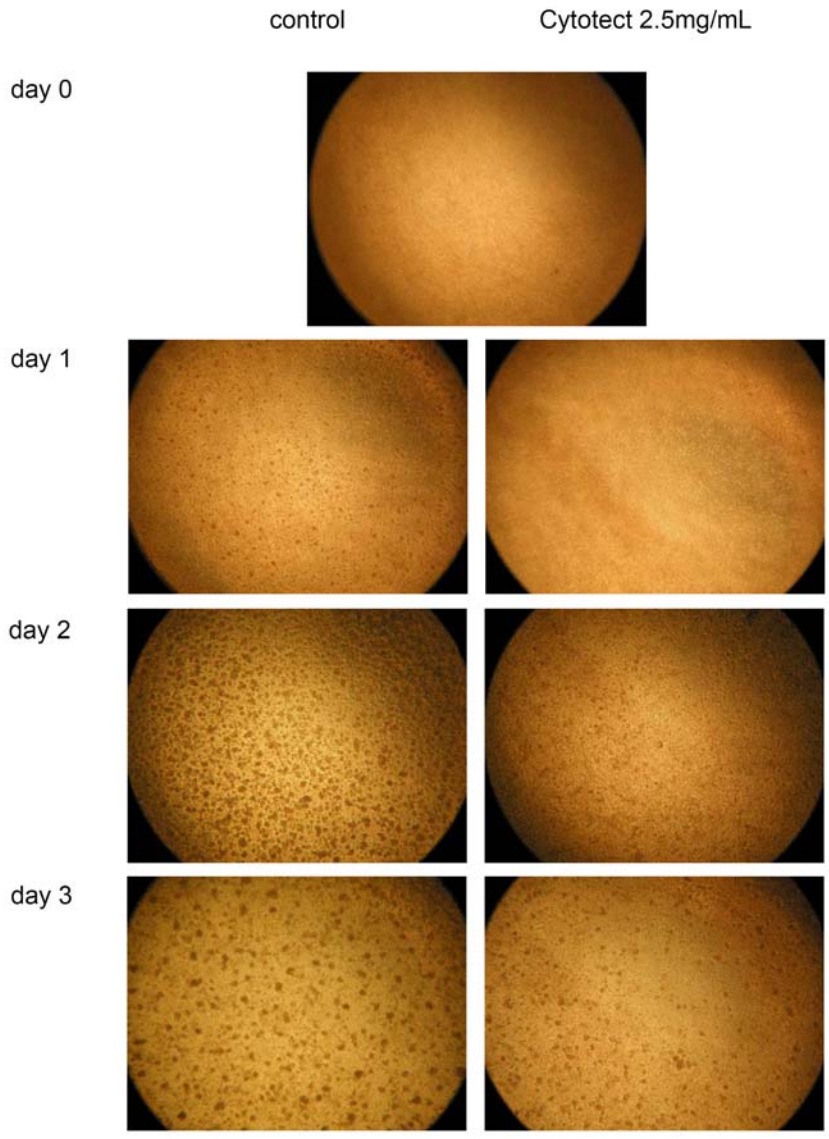


Figure 8 shows time resolved, light microscopic evaluations of one representative proband at a 40-fold magnification. The knobs indicate cell clusters of proliferating PBMC. Number of proliferation clusters considerably decreased by co-incubation with Cytotect. Similar results were obtained in MLRs and by adding Cytoglobin (data not shown).

CFSE dilution assays

CFSE dilution assays were performed to testify results obtained from the thymidine assays. Combined data of 10 probands demonstrated that the percentage of proliferating cells decreased from 24.1±2.5 (control) to 8.4±1.7 (Cytotect) and 5.3±1.0 (Cytoglobin).

Figure 9 CMVIg reduce proliferation upon anti-CD3 stimulation measured in CFSE dilution assays

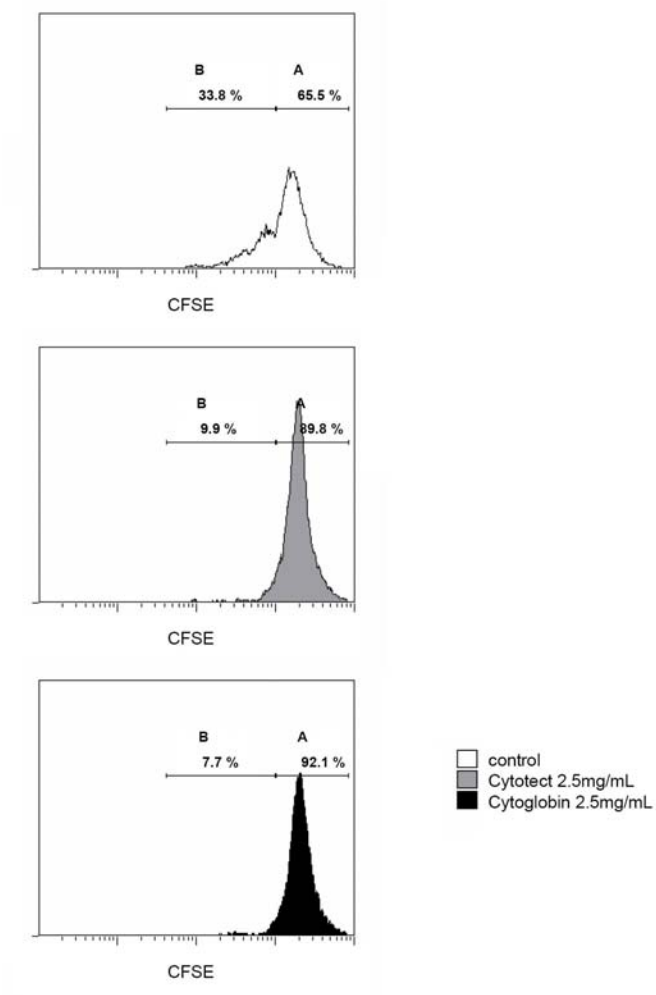


Figure 9 depicts a representative CFSE dilution experiment. The addition of CMVIg resulted in a nearly complete aberration of proliferation.

Table 7 Summarized results (n=10) of the CFSE dilution assays

	% resting cells	% proliferating cells
control	75.4 ± 2.5	24.1 ± 2.5 †‡
Cytotect 2.5mg/mL	91.3 ± 1.6	8.4 ± 1.7 †
Cytoglobin 2.5mg/mL	94.3 ± 1.0	5.3 ± 1.0 ‡

Summarized data of CFSE dilution assays are shown in Table 7. A significant reduction of proliferating cells was observed after co-incubation with CMVIg († $p<0.001$, ‡ $p<0.001$; n=10).

Cell cycle analysis

Cell cycle analysis of CMVIg co-incubated PBMC revealed a significant reduction of transition of resting G0/G1 cells to S and G2/M phase when compared to control cells.

Table 8 Cell cycle analysis of peripheral blood mononuclear cells (PBMC) exposed to CMVIg

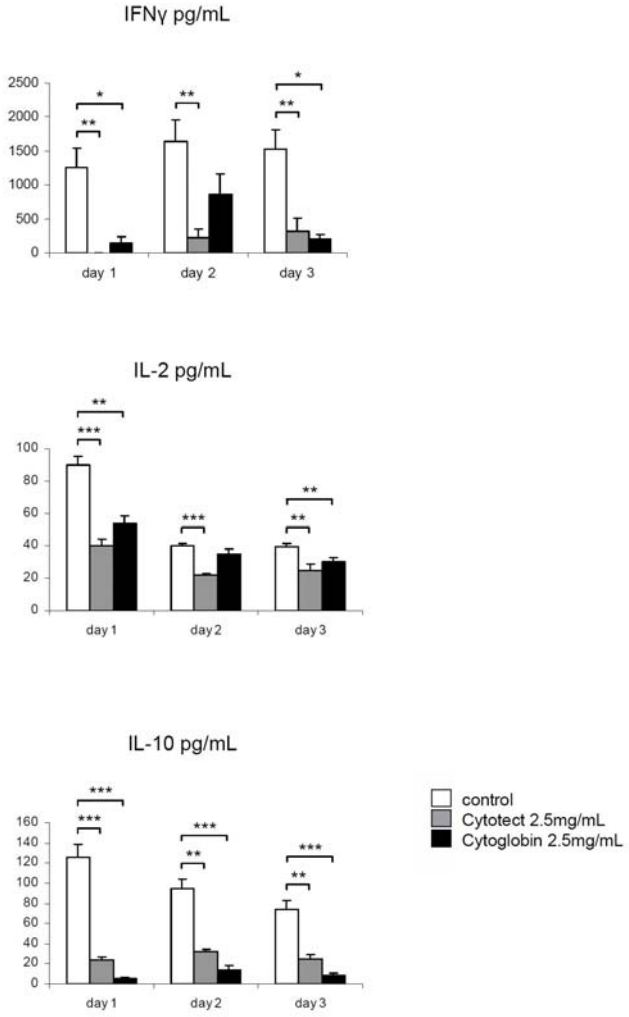
	% G0/G1	% S	% G2/M
control	85.3 ± 2.9	11.7 ± 2,3 †‡	3.0 ± 0,7 †‡
Cytotect 2.5mg/mL	98.8 ± 0.3	1.1 ± 0,3 †	0.1 ± 0,1 †
Cytoglobin 2.5mg/mL	95.9 ± 1.4	3.7 ± 1,1 ‡	0.4 ± 0,4 ‡

Table 8 shows results of cell cycle analysis performed in T cell triggered PBMC. Co-incubation with CMVIg led to a massive decrease of cells residing in S and G2/M-phase. These data confirm CFSE dilution experiments († $p<0.05$; ‡ $p<0.05$; n=10).

Reduction of cytokine release by CMVIg

To evaluate the influence of CMVIg on cytokine release supernatants of anti-CD3 simulated PBMC cultures exposed to CMVIg were collected. Samples were evaluated utilizing ELISA kits. Interestingly, cells treated with CMVIg significantly reduced cytokine secretion. This phenomenon was observed for pro-inflammatory – IL-2, interferon (IFN)γ – as well as for anti-inflammatory cytokines – IL-10.

Figure 10 Cytokine release of PBMC is reduced by co-incubation with CMVIg



Cytokines released by PBMC cultures co-incubated with Cytotect and Cytoglobin are shown in Figure 10. All three mainly T cell derived cytokines were significantly reduced (*p<0.05; **p<0.01; ***p<0.001; n=10).

CMVIg induce apoptosis in PBMC

To test the influence of CMVIg on cell viability flow cytometric analysis of CMVIg treated PBMC were performed. The percentage of Annexin positive cells was significantly increased in the CMVIg groups when compared to the control. A subsequent rise in the Annexin/PI double positive cells, indicating late apoptosis, was observed (data not shown). When comparing Cytotect and Cytoglobin data, Cytoglobin seems to display a greater effect on cell viability than Cytotect.

Figure 11 CMVIg induce apoptosis in resting PBMC

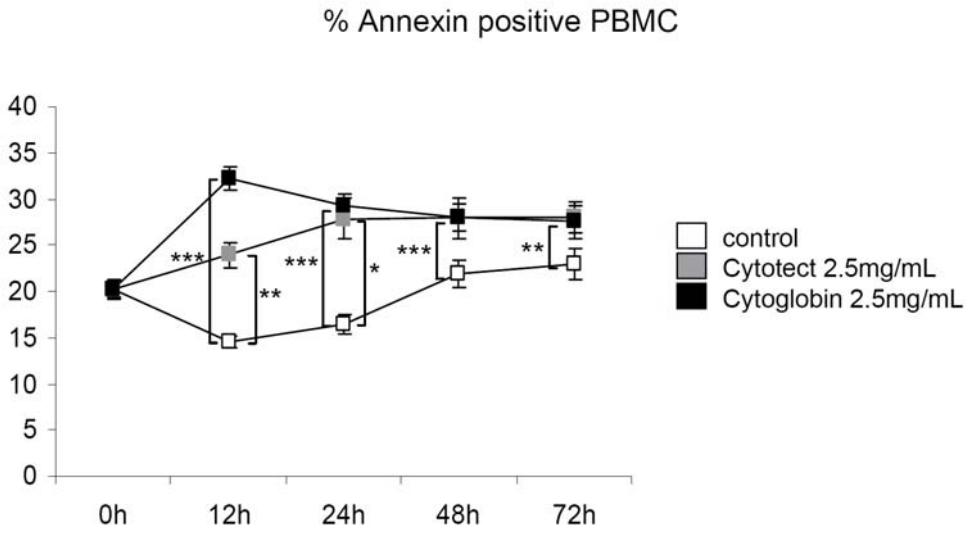
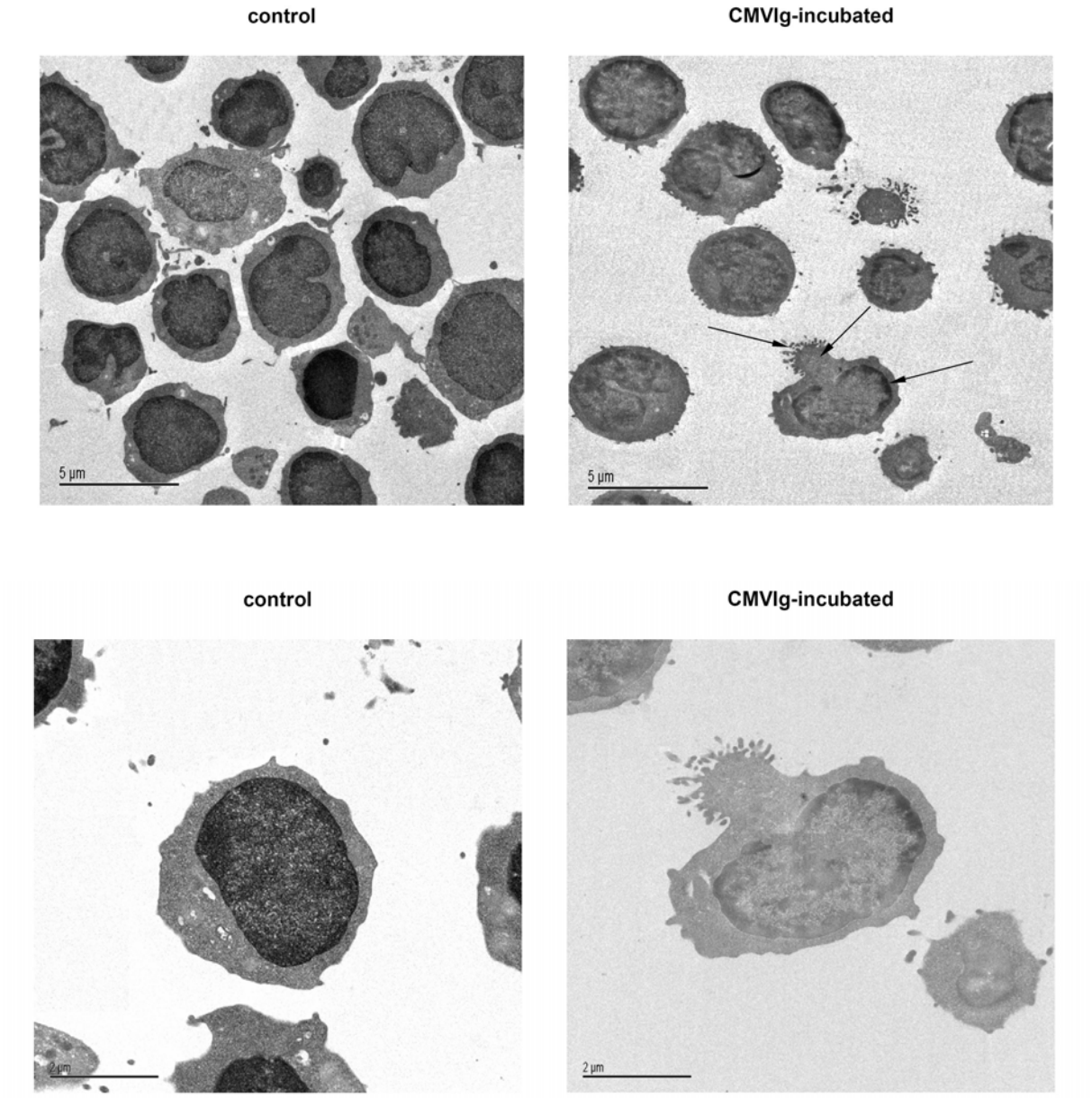


Figure 11 depicts the potency of CMVIg to induce apoptosis in resting PBMC. Percentage Annexin positive cells significantly increased by co-incubation of PBMC with CMVIg. This effect reached highest significance at the time point 12h (control: 14.5±0.8%; Cytotect: 23.9±1.3%; Cytoglobin: 32.3±1.3%).

These apoptosis-inducing properties of CMVIg were confirmed by ultrastructural electron microscopic analysis. CMVIg treated PBMC showed typical apoptotic features whereas control cells displayed normal morphology of resting PBMC.

Figure 12 Electron microscopic evaluation of the apoptosis-inductive potency of CMVIg



Ultrastructural changes in cell morphology of CMVIg treated PBMC are shown in Figure 12. Upon CMVIg incubation cells developed typical apoptotic features (e.g. formation of apoptotic bodies, cell membrane blebbing, chromatin condensation - arrows). Control cells remained unchanged.

Purified NK cells and CD8+ cells are sensitive to CMVIg-induced apoptosis

To identify cell types being most sensitive to CMVIg-induced apoptosis CD4+, CD8+, CD19+ and CD56+ cells were purified by a magnetic bead system. Interestingly, viability of “effector” cells (cytotoxic T cells and NK cells) was most affected by CMVIg.

Figure 13 Evaluation of the sensitivity of PBMC subpopulations for apoptosis induction by CMVIg

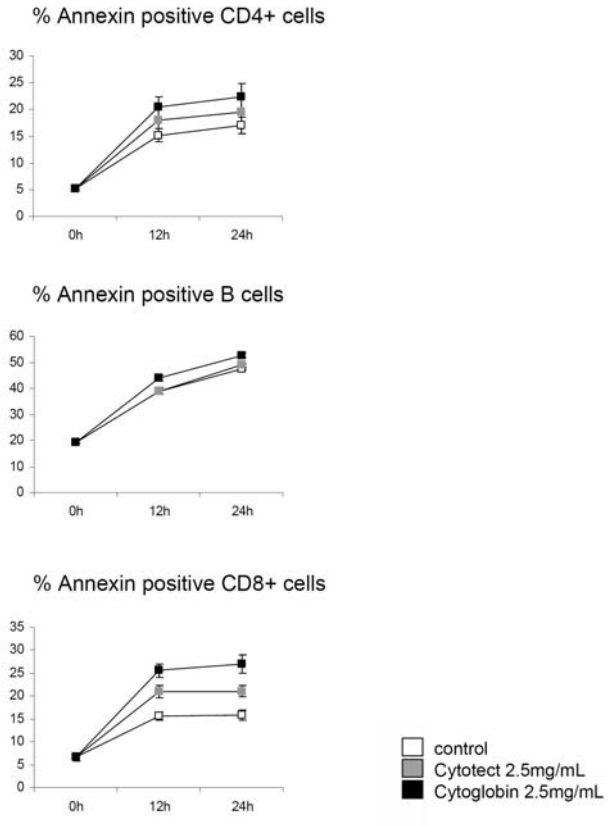
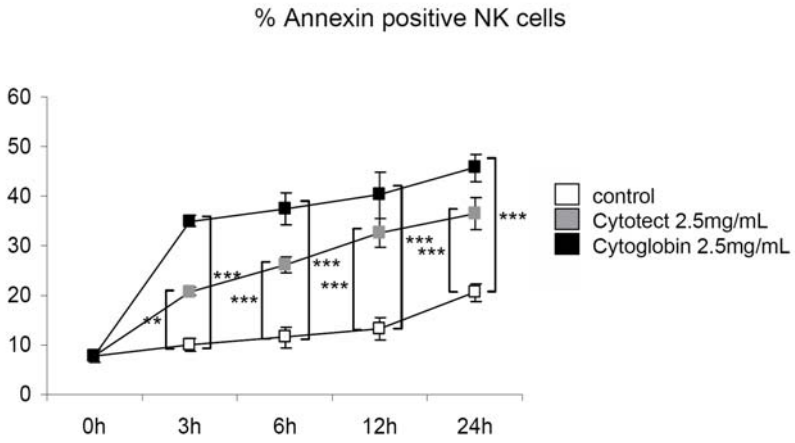


Figure 13 demonstrates the sensitivity of CD4+, CD19+ and CD8+ cells to undergo apoptosis upon CMVIg incubation. CD8+ cells were most prone for CMVIg-induced apoptosis whereas CD4+ and B cells were hardly affected.

Figure 14 CMVIg induce apoptosis in highly purified NK cells



CMVIg induced apoptosis of highly purified NK cells is shown in Figure 14. Both Cytotect and Cytoglobin were potent inducers of programmed cell death when compared to untreated control NK cells (*p<0.05; **p<0.01; ***p<0.001; n=10).

FcγRIII and cell surface bound IgG is downregulated by CMVIg

Based on the apoptosis data obtained for NK cells, we sought to evaluate the role of CMVIg on NK cell effector functions. Therefore, the influence of CMVIg on cell surface bound IgG and on the expression of the low affinity FcγRIII (CD16) was evaluated by flow cytometric analysis. Both surface proteins were shown to be downregulated.

Figure 15 CMVIg lead to losing of cell surface bound IgG and FcγRIII in purified NK cells

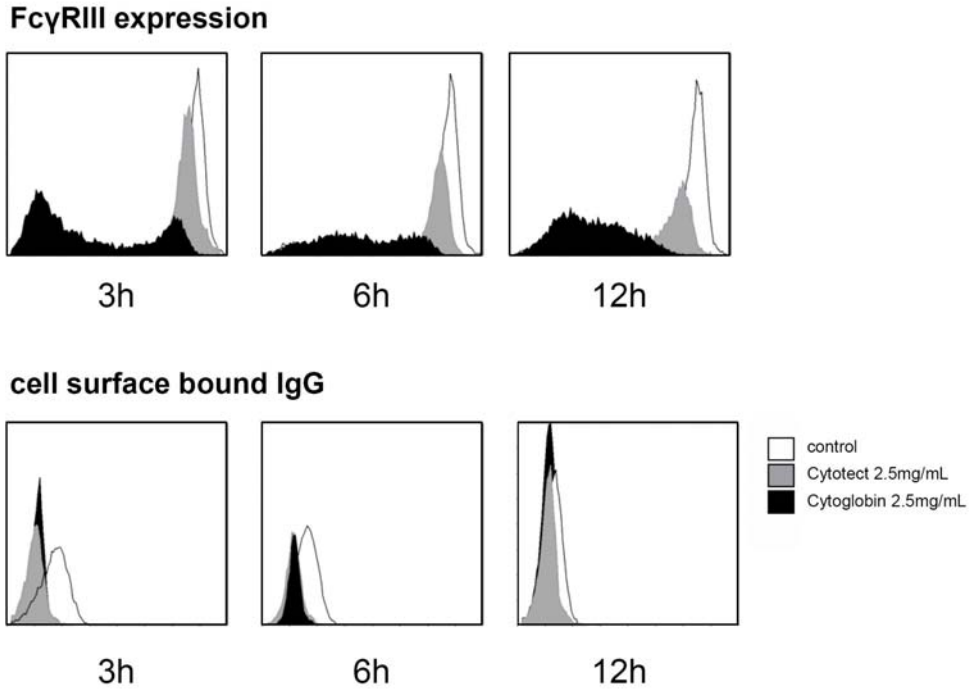


Figure 15 shows flow cytometric evaluation of a representative proband indicating that CMVIg significantly reduced cell surface bound IgG and density of FcγRIII in purified NK cells.

Table 9 Cell surface bound IgG and FcγRIII on purified NK cells are downregulated by CMVIg (n=10)

cell bound IgG - mean fluorescence

	control	Cytotect 2.5mg/mL	Cytoglobin 2.5mg/mL
0h	1.32 ± 0.50	1.32 ± 0.50	1.32 ± 0.50
3h	0.62 ± 0.16† ‡	0.38 ± 0.12†	0.47 ± 0.10‡
6h	0.50 ± 0.13	0.38 ± 0.03	0.39 ± 0.02
12h	0.39 ± 0.04	0.34 ± 0.03	0.39 ± 0.06

CD16 expression - mean fluorescence

	control	Cytotect 2.5mg/mL	Cytoglobin 2.5mg/mL
0h	286.00 ± 66.11	286.00 ± 66.11	286.00 ± 66.11
3h	224.38 ± 54.23# §	131.90 ± 52.71#	39.66 ± 20.30§
6h	200.25 ± 52.43# §	91.89 ± 44.42#	19.26 ± 8.97§
12h	163.63 ± 61.93# §	57.01 ± 39.22#	8.65 ± 5.53§

Summarized data of the CD16 and FcγRIII FACS evaluations are shown in Table 9. The losing of membrane bound IgG reached significance at the 3h time point whereas the downregulation of FcγRIII draged behind († $p<0.05$; ‡ $p<0.05$; # $p<0.001$; § $p<0.001$).

CMVIg reduce ADCC of PBMC and purified NK cells

Finally, based on the impact of CMVIg on NK cell viability and downregulation of FcγRIII, we sought to extend these findings by quantifying ADCC against PANC-1 and Jurkat target cells. Immune function against these two tumor cell lines was significantly reduced when effector cells were pre-incubated with CMVIg.

Figure 16 PBMC and purified NK cells evidence a decrease in antibody dependent cellular cytotoxicity

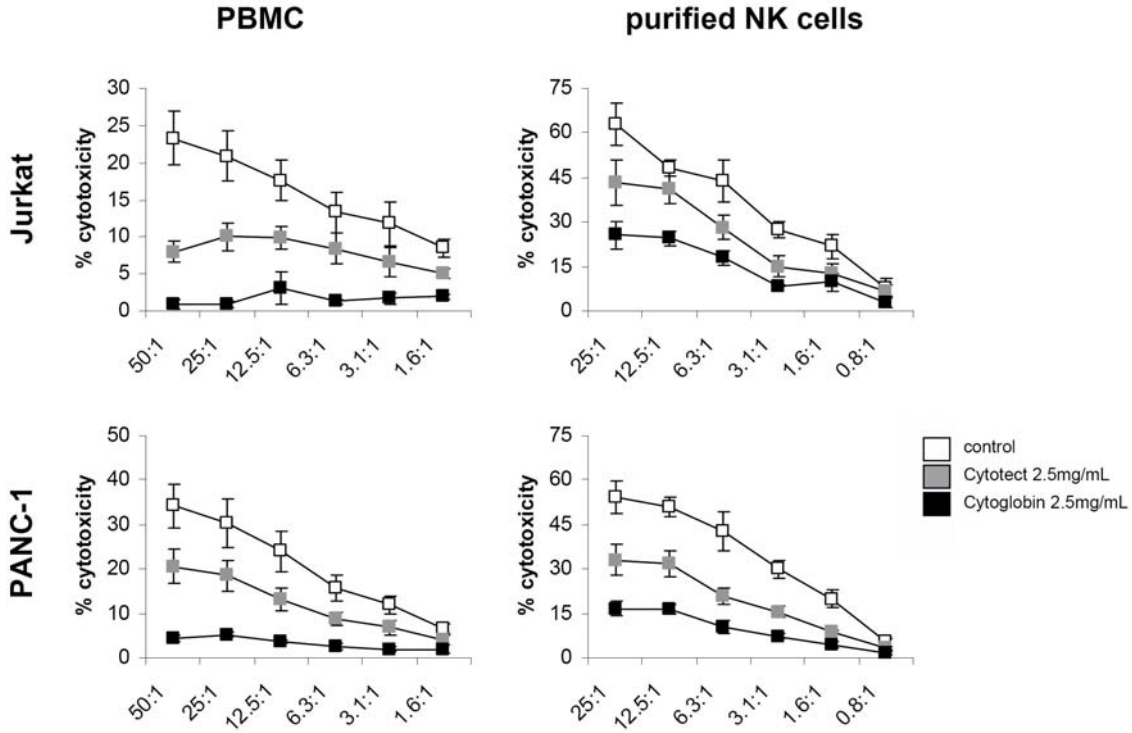
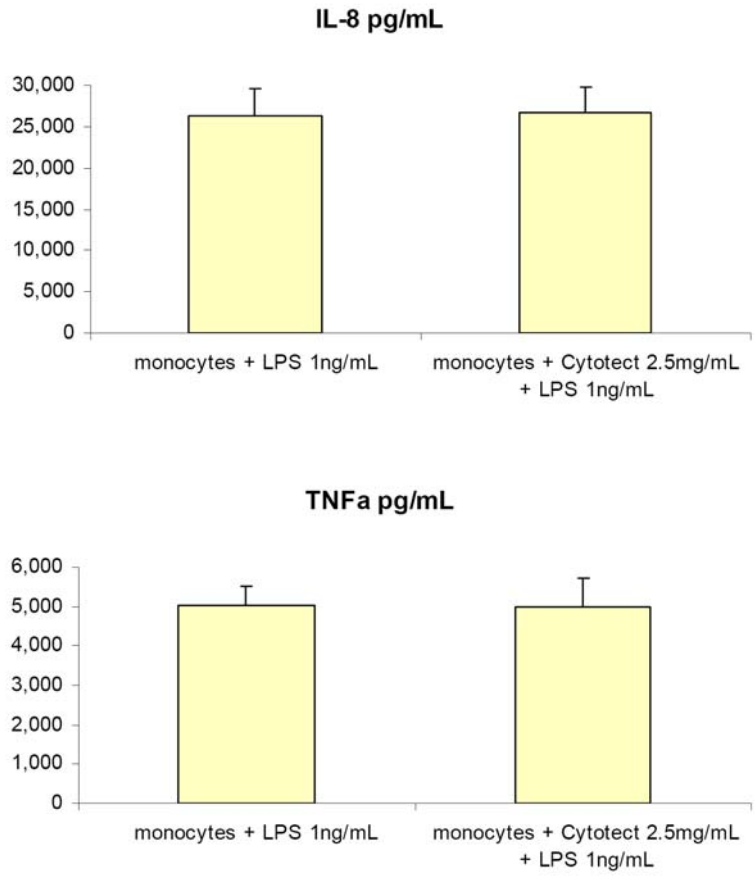


Figure 16 shows natural occurring cytotoxicity of PBMC against Jurkat and PANC-1 after pre-incubation with therapeutical CMVIg concentrations. ADCC was significantly reduced when cells were pre-incubated with Cytotect and Cytoglobin. This effect was seen at all different effector/target ratios ranging from 50:1 to 1.6:1 and was comparable at both target cell lines. Significances refer to comparison between AUC as described in the Material and Methods section. PBMC-Jurkat: control 944.9±148.5 Cytotect: 434.7±71.0 ($p<0.01$) Cytoglobin: 68.7±37.9 ($p<0.05$); PBMC-PANC-1: control: 1327.2±220.9, Cytotect: 785.0±145.2 ($p<0.05$), Cytoglobin: 205.2±23.8 ($p<0.001$); NK cells-Jurkat: control 1138.5±82.4, Cytotect: 844.1±98.2 ($p<0.05$), Cytoglobin: 510.2±57.0 ($p<0.05$); NK cells-PANC-1: control 1120.6±101.8, Cytotect: 653.9±85.7 ($p<0.01$), Cytoglobin: 331.6±44.8 ($p<0.001$) (n=10).

CMVIg do not interfere with NfκB dependent pathway of monocytes

A minor objective of this study was to evaluate the impact of CMVIg on NfκB dependent signal pathway in monocytes. Therefore LPS-stimulated monocytes were exposed to CMVIg and supernatants were evaluated for NfκB associated cytokines.

Figure 17 CMVIg do not influence cytokine release of stimulated monocytes

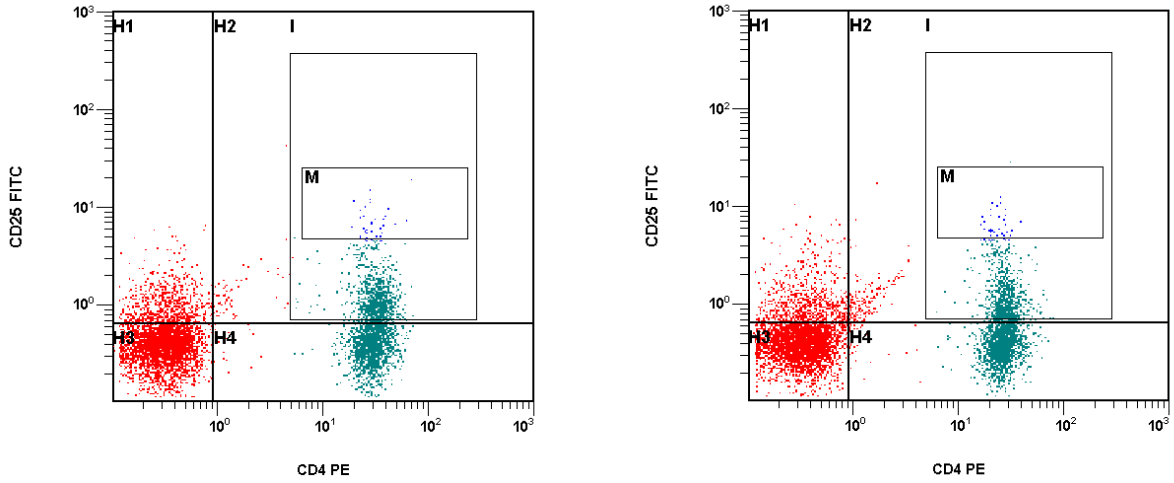


To evaluate if CMVIg interfere with NfkB pathway, LPS-stimulated purified monocytes were analyzed for their NfkB dependent cytokine release. Levels of TNFα and the chemokine IL-8 were not affected by the addition of Cytotect to the experiment indicating that CMVIg do not influence NfκB pathway in monocytes.

CD4+CD25brigtht cells are not expanded by CMVIg

Recently, the ability of antithymocyte globuline (ATG) to expand Tregs *in vitro* has been described [118]. As the effective components of ATG are polyclonal antibodies we sought to evaluate if CMVIg preparations also have the capacity to induce CD4+CD25bright cells *in vitro*.

Figure 18 CMVIg do no expand CD4+CD25bright cells *in vitro*



Percent cells of all CD4+ cells

	pos control	Cytotect 2.5mg/mL
CD4+CD25+	33.3±1.1	32.0±1.1
CD4+CD25bright	1.0±0.3	1.9±0.3

Figure 18 and the incorporated table show summarized data of 10 probands indicating that Cytotect did not induce Treg differentiation. Hence there is strong evidence that the anti-proliferative effects seen in the MLRs and in the anti-CD3 stimulations are not due to the induction of suppressor T cells but are either a direct cellular effect of CMVIg or an indirect effect caused by modulation of cytokine profiles.

DISCUSSION

CMVIg have been routinely administered at the Viennese Heart Transplant Center since 1987 as prophylaxis against CMV disease during the post-transplant period. Their anti-viral effects are sufficiently described in literature and have been confirmed in several meta-analysis [47-51].
CMVIg are produced by pooling immunoglobulins of hundreds seropositive donors. IvIg, also consisting of pooled immunoglobulins, are a drug class related to CMVIg. Immunomodulatory effects of IvIg are well known and have been addressed in many studies (reviewed in [119]).
The aim of this study was to evaluate if CMVIg also possess such immunomodulatory properties making them an additional useful tool for immunosuppressive drug regimen in the early post-transplant course.
Both purchasable CMVIg compounds, currently licensed in Austria were analyzed in this study (Cytotect from Biotest, Cytoglobin from Bayer).

We could show for the first time that CMVIg exhibit remarkable anti-proliferative activity in *in vitro* stimulation assays. Similar effects have been published for IvIg. Several mechanisms being responsible for the anti-proliferative effects of IvIg have been suggested. Nachbaur *et al*. showed that IvIg inhibited IL-2 protein synthesis, whereas no influence on IL-2 mRNA expression was observed [120]. A blockade of IFNγ or CD4 by neutralizing antibodies found in IvIg preparations was suggested by other investigators [87, 121]. Vuist *et al.* demonstrated that IvIg contain antibodies against glycolipids expressed on lymphocytes leading to an IvIg-induced growth attenuation [122]. More recently, Jordan *et al*. evidenced that antibodies directed against MHC class I and II as well as T cell receptor molecules are found in IvIg. They were shown to directly interfere with the allo-antigen recognition process in MLRs [123].

The attenuation of proliferation by CMVIg, either upon T cell triggering or in an allogeneic MLR, was associated with an increased apoptotic turnover proved by Annexin/PI co-staining in flow cytometric analysis. Morphologic studies underlined these findings by showing that typical apoptotic features can be found in CMVIg treated PBMC cultures.

To answer the question which cells are most affected by the addition of CMVIg we purified different cell populations with a magnetic bead separation system. Interestingly, cytolytic effector cells are most affected by CMVIg – namely CD8+ T cells and NK cells. Both cell populations are known to play a pivotal role in acute rejection episodes.

The role of CD8+ cells in acute allograft rejection has been known for a long time [124, 125]. Cytotoxic T cells amount half of all graft infiltrating cells in biopsies obtained from patients experiencing an acute rejection period [126]. CD8+ cells need pre-activation *via* IL-2 and IFNγ, secreted by CD4+ cells, to exhibit their cytolytic properties. The two main mechanisms thereby are: (1) pore formation resulting in a colloid osmotic collapse of the target cell and (2) the release of pro-apoptotic proteins (granzyme B, FasL).

The role of NK cells in acute allograft rejection has traditionally not been a highlight in transplant research. Although early morphological studies of biopsies obtained during rejection periods have shown that NK cells are part of the inflammatory infiltrate [127], the role of NK cells in allo-immune response has only been addressed recently [128, 129]. Maier *et al*. reported evidence that NK cells provide an alternative source of T cell help thus contributing to the acute rejection of wild-type hearts transplanted into CD28-deficient mice [130].

Madsen and colleagues found that NK cells also play an important role in chronic graft dysfunction. They demonstrated that NK cells were able to be activated in the absence of self MHC class I molecules on donor endothelium and by that mechanism participate in the pathogenesis of transplant-associated coronary artery disease (TACAD) [131].

An important question arising from the data on anti-proliferation and apoptosis induction is whether the reduction of clonal proliferation is a sole effect of CMVIg or the consequence of an increased apoptotic turnover, leaving fewer cells to proliferate. Transmission electron microscopy revealed that anti-proliferation and apoptosis are most likely two independent mechanisms. Cells featuring typical apoptotic morphology were found in direct neighbourhood to resting cells – unaffected by the proliferative stimuli.

To specify these findings we performed CFSE dilution assays and cell cycle analysis. Co-incubation with CMVIg resulted in significantly increased cell numbers remaining

in G0/G1 phase as compared to the control condition. These data demonstrate that anti-proliferative effects and induction of apoptosis primarily in CD8+ and NK cells are two clearly independent mechanisms.

Another important aspect of this work was the influence of CMVIg on the FcγR system of NK cells. Two different observations were made:

(1) ***Discharging of membrane bound IgG.*** Within the first three hours of CMVIg-incubation, density of membrane bound IgG proteins was massively reduced on NK cells. Furthermore, vacant FcγR were not filled up with IgG from CMVIg preparations. These findings were rather surprising because they are in conflict to the concept of IvIg exhibiting their immunomodulatory effect partly through the blockade of Fc-receptors [72].

(2) ***CMVIg evidenced a downregulation of low affinity FcγRIII.*** Ichiyama *et al*. could show in a monocyte cell line (U-937) that IvIg application leads to a downregulation of the high-affinity FcγRI [132]. This work confirms their findings in PBMC cultures (data not shown) and extends their observations to NK cells. Density of the low affinity FcγRIII (CD16) on NK cells was diminished by co-incubation with CMVIg. The ablation of FcγRIII could be seen mainly between 6 and 12 hours after CMVIg incubation. Therefore, the retraction of “oven” receptors seems to be a direct consequence of the losage of receptor bound IgG.

The effects of CMVIg on NK cells led to the hypothesis that CMVIg reduce ADCC, the main NK cell effector mechanism against an allograft. We could prove this theory by standard europium release cytotoxicity assays against Jurkat and PANC-1 cell lines. Pre-incubation of PBMC as well as highly purified NK cells with different CMVIg preparations resulted in a dose dependent reduction of ADCC. Moreover, these results corroborate work showing that co-incubation of NK cells with anti-CD16 led to NK cell apoptosis, alteration of NK phenotype (CD56+/CD16^{bright} to CD56+/CD16^{dim}) and decreased NK cell effector function [133].

Anti-proliferative effects and apoptosis induction are accepted mechanisms to obtain an immunological anergic state – the so-called functional graft tolerance [134]. The standard drug regimen to achieve such functional graft tolerance is a triple

immunosuppressive therapy consisting of an anti-proliferative drug (azathioprin, mycophenoic acid), a calcineurin inhibitor (cyclosporine, tacrolimus) and glucocorticoids (prednisolone). In addition to this standard regimen an induction therapy with monoclonal antibodies directed against T cell epitopes (antithymocyte globulin, OKT-3, anti-IL2-receptor antibodies) is performed in most heart transplant centers worldwide.

By showing that CMVIg also exhibit anti-proliferative effects and induce apoptosis in effector cells, their application could help achieving functional graft tolerance and could therefore have a beneficial influence on acute graft rejection and formation of TACAD.

The *in vitro* findings of this work are underlined by clinical data of the Viennese Heart Transplant Center. Since CMVIg prophylaxis with Cytotect has been introduced in the 80s, rejections rates are one of the lowest reported in the world. The observations made in the Viennese center is confirmed by data from clinical trials indicating that the application of CMVIg is beneficiary in regard to graft survival and overall mortality when compared to CMV immune prophylaxis with anti-viral drugs [135, 136].

The results of this work extend the current concept of CMVIg as passive CMV prophylaxis to a therapeutic drug compound capable of reducing allogeneic immune response. In respect to the clinical observation of low incidence in allograft rejection rates and TACAD formation it is tempting to suggest that administration of CMVIg in the early post-transplant course has a beneficial effect on graft survival.

REFERENCES

1. Ljungman P, Griffiths P, Paya C: **Definitions of cytomegalovirus infection and disease in transplant recipients.** *Clin Infect Dis* 2002, **34:**1094-1097.
2. Cainelli F, Vento S: **Infections and solid organ transplant rejection: a cause-and-effect relationship?** *Lancet Infect Dis* 2002, **2:**539-549.
3. Harari A, Zimmerli SC, Pantaleo G: **Cytomegalovirus (CMV)-specific cellular immune responses.** *Hum Immunol* 2004, **65:**500-506.
4. Dworsky M, Yow M, Stagno S, Pass RF, Alford C: **Cytomegalovirus infection of breast milk and transmission in infancy.** *Pediatrics* 1983, **72:**295-299.
5. Pass RF: **Epidemiology and transmission of cytomegalovirus.** *J Infect Dis* 1985, **152:**243-248.
6. Reynolds DW, Stagno S, Hosty TS, Tiller M, Alford CA, Jr.: **Maternal cytomegalovirus excretion and perinatal infection.** *N Engl J Med* 1973, **289:**1-5.
7. Vochem M, Hamprecht K, Jahn G, Speer CP: **Transmission of cytomegalovirus to preterm infants through breast milk.** *Pediatr Infect Dis J* 1998, **17:**53-58.
8. Landolfo S, Gariglio M, Gribaudo G, Lembo D: **The human cytomegalovirus.** *Pharmacol Ther* 2003, **98:**269-297.
9. Patel R, Paya CV: **Infections in solid-organ transplant recipients.** *Clin Microbiol Rev* 1997, **10:**86-124.
10. Rubin RH: **Prevention of cytomegalovirus infection in organ transplant recipients.** *Transpl Infect Dis* 2000, **2:**99-100.
11. Razonable RR, Emery VC: **Management of CMV infection and disease in transplant patients. 27-29 February 2004.** *Herpes* 2004, **11:**77-86.
12. Kanj SS, Sharara AI, Clavien PA, Hamilton JD: **Cytomegalovirus infection following liver transplantation: review of the literature.** *Clin Infect Dis* 1996, **22:**537-549.
13. Preiksaitis JK, Brennan DC, Fishman J, Allen U: **Canadian society of transplantation consensus workshop on cytomegalovirus management in solid organ transplantation final report.** *Am J Transplant* 2005, **5:**218-227.

14. Hassan-Walker AF, Kidd IM, Sabin C, Sweny P, Griffiths PD, Emery VC: **Quantity of human cytomegalovirus (CMV) DNAemia as a risk factor for CMV disease in renal allograft recipients: relationship with donor/recipient CMV serostatus, receipt of augmented methylprednisolone and antithymocyte globulin (ATG).** *J Med Virol* 1999, **58:**182-187.
15. De Santo LS, Romano G, Mastroianni C, Roberta C, Della Corte A, Amarelli C, Maiello C, Giannolo B, Marra C, Ragone E, et al: **Role of immunosuppressive regimen on the incidence and characteristics of cytomegalovirus infection in heart transplantation: a single-center experience with preemptive therapy.** *Transplant Proc* 2005, **37:**2684-2687.
16. Krogsgaard K, Boesgaard S, Aldershvile J, Arendrup H, Mortensen SA, Petterson G: **Cytomegalovirus infection rate among heart transplant patients in relation to anti-thymocyte immunoglobulin induction therapy. Copenhagen Heart Transplant Group.** *Scand J Infect Dis* 1994, **26:**239-247.
17. Boland GJ, Hene RJ, Ververs C, de Haan MA, de Gast GC: **Factors influencing the occurrence of active cytomegalovirus (CMV) infections after organ transplantation.** *Clin Exp Immunol* 1993, **94:**306-312.
18. van der Bij W, Speich R: **Management of cytomegalovirus infection and disease after solid-organ transplantation.** *Clin Infect Dis* 2001, **33 Suppl 1:**S32-37.
19. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE: **Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis.** *Jama* 1989, **261:**3561-3566.
20. von Willebrand E, Pettersson E, Ahonen J, Hayry P: **CMV infection, class II antigen expression, and human kidney allograft rejection.** *Transplantation* 1986, **42:**364-367.
21. Reinke P, Fietze E, Ode-Hakim S, Prosch S, Lippert J, Ewert R, Volk HD: **Late-acute renal allograft rejection and symptomless cytomegalovirus infection.** *Lancet* 1994, **344:**1737-1738.
22. Keller CA, Cagle PT, Brown RW, Noon G, Frost AE: **Bronchiolitis obliterans in recipients of single, double, and heart-lung transplantation.** *Chest* 1995, **107:**973-980.

23. Arbustini E, Morbini P, Grasso M, Diegoli M, Fasani R, Porcu E, Banchieri N, Perfetti V, Pederzolli C, Grossi P, et al: **Human cytomegalovirus early infection, acute rejection, and major histocompatibility class II expression in transplanted lung. Molecular, immunocytochemical, and histopathologic investigations.** *Transplantation* 1996, **61:**418-427.
24. Teixeira R, Pastacaldi S, Davies S, Dhillon AP, Emery VC, Rolles K, Davidson B, Patch D, Burroughs AK: **The influence of cytomegalovirus viraemia on the outcome of recurrent hepatitis C after liver transplantation.** *Transplantation* 2000, **70:**1454-1458.
25. Singh N: **Cytomegalovirus infection in solid organ transplant recipients: new challenges and their implications for preventive strategies.** *J Clin Virol* 2006, **35:**474-477.
26. Schooley RT, Hirsch MS, Colvin RB, Cosimi AB, Tolkoff-Rubin NE, McCluskey RT, Burton RC, Russell PS, Herrin JT, Delmonico FL, et al: **Association of herpesvirus infections with T-lymphocyte-subset alterations, glomerulopathy, and opportunistic infections after renal transplantation.** *N Engl J Med* 1983, **308:**307-313.
27. George MJ, Snydman DR, Werner BG, Griffith J, Falagas ME, Dougherty NN, Rubin RH: **The independent role of cytomegalovirus as a risk factor for invasive fungal disease in orthotopic liver transplant recipients. Boston Center for Liver Transplantation CMVIG-Study Group. Cytogam, MedImmune, Inc. Gaithersburg, Maryland.** *Am J Med* 1997, **103:**106-113.
28. Keenan RJ, Lega ME, Dummer JS, Paradis IL, Dauber JH, Rabinowich H, Yousem SA, Hardesty RL, Griffith BP, Duquesnoy RJ, et al.: **Cytomegalovirus serologic status and postoperative infection correlated with risk of developing chronic rejection after pulmonary transplantation.** *Transplantation* 1991, **51:**433-438.
29. Freese DK, Snover DC, Sharp HL, Gross CR, Savick SK, Payne WD: **Chronic rejection after liver transplantation: a study of clinical, histopathological and immunological features.** *Hepatology* 1991, **13:**882-891.
30. Demetris AJ, Qian SG, Sun H, Fung JJ: **Liver allograft rejection: an overview of morphologic findings.** *Am J Surg Pathol* 1990, **14 Suppl 1:**49-63.

31. Evans PC, Soin A, Wreghitt TG, Taylor CJ, Wight DG, Alexander GJ: **An association between cytomegalovirus infection and chronic rejection after liver transplantation.** *Transplantation* 2000, **69:**30-35.
32. Weill D: **Role of cytomegalovirus in cardiac allograft vasculopathy.** *Transpl Infect Dis* 2001, **3 Suppl 2:**44-48.
33. Rubin RH: **The indirect effects of cytomegalovirus infection on the outcome of organ transplantation.** *Jama* 1989, **261:**3607-3609.
34. Lemstrom K, Koskinen P, Krogerus L, Daemen M, Bruggeman C, Hayry P: **Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection.** *Circulation* 1995, **92:**2594-2604.
35. Span AH, Grauls G, Bosman F, van Boven CP, Bruggeman CA: **Cytomegalovirus infection induces vascular injury in the rat.** *Atherosclerosis* 1992, **93:**41-52.
36. Hodson EM, Barclay PG, Craig JC, Jones C, Kable K, Strippoli GF, Vimalachandra D, Webster AC: **Antiviral medications for preventing cytomegalovirus disease in solid organ transplant recipients.** *Cochrane Database Syst Rev* 2005**:**CD003774.
37. Das A: **Cytomegalovirus infection in solid organ transplantation: economic implications.** *Pharmacoeconomics* 2003, **21:**467-475.
38. Jassal SV, Roscoe JM, Zaltzman JS, Mazzulli T, Krajden M, Gadawski M, Cattran DC, Cardella CJ, Albert SE, Cole EH: **Clinical practice guidelines: prevention of cytomegalovirus disease after renal transplantation.** *J Am Soc Nephrol* 1998, **9:**1697-1708.
39. Drew WL, Paya CV, Emery V: **Cytomegalovirus (CMV) resistance to antivirals.** *Am J Transplant* 2001, **1:**307-312.
40. Limaye AP, Corey L, Koelle DM, Davis CL, Boeckh M: **Emergence of ganciclovir-resistant cytomegalovirus disease among recipients of solid-organ transplants.** *Lancet* 2000, **356:**645-649.
41. Valantine HA, Gao SZ, Menon SG, Renlund DG, Hunt SA, Oyer P, Stinson EB, Brown BW, Jr., Merigan TC, Schroeder JS: **Impact of prophylactic immediate posttransplant ganciclovir on development of transplant atherosclerosis: a post hoc analysis of a randomized, placebo-controlled study.** *Circulation* 1999, **100:**61-66.

42. Rubin RH: **Preemptive therapy in immunocompromised hosts.** *N Engl J Med* 1991, **324:**1057-1059.
43. Niubo J, Perez JL, Martinez-Lacasa JT, Garcia A, Roca J, Fabregat J, Gil-Vernet S, Martin R: **Association of quantitative cytomegalovirus antigenemia with symptomatic infection in solid organ transplant patients.** *Diagn Microbiol Infect Dis* 1996, **24:**19-24.
44. Strippoli GF, Hodson EM, Jones CJ, Craig JC: **Pre-emptive treatment for cytomegalovirus viraemia to prevent cytomegalovirus disease in solid organ transplant recipients.** *Cochrane Database Syst Rev* 2006**:**CD005133.
45. Razonable RR, Paya CV: **Valganciclovir for the prevention and treatment of cytomegalovirus disease in immunocompromised hosts.** *Expert Rev Anti Infect Ther* 2004, **2:**27-41.
46. Leroy F, Sechet A, Abou Ayache R, Thierry A, Belmouaz S, Desport E, Bauwens M, Bridoux F, Touchard G: **Cytomegalovirus prophylaxis with intravenous polyvalent immunoglobulin in high-risk renal transplant recipients.** *Transplant Proc* 2006, **38:**2324-2326.
47. Davis CL: **The prevention of cytomegalovirus disease in renal transplantation.** *Am J Kidney Dis* 1990, **16:**175-188.
48. Snydman DR, Werner BG, Heinze-Lacey B, Berardi VP, Tilney NL, Kirkman RL, Milford EL, Cho SI, Bush HL, Jr., Levey AS, et al.: **Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients.** *N Engl J Med* 1987, **317:**1049-1054.
49. Zamora MR, Fullerton DA, Campbell DN, Leone S, Diercks MJ, Fisher JH, Badesch DB, Grover FL: **Use of cytomegalovirus (CMV) hyperimmune globulin for prevention of CMV disease in CMV-seropositive lung transplant recipients.** *Transplant Proc* 1994, **26:**49-51.
50. Wittes JT, Kelly A, Plante KM: **Meta-analysis of CMVIG studies for the prevention and treatment of CMV infection in transplant patients.** *Transplant Proc* 1996, **28:**17-24.
51. Glowacki LS, Smaill FM: **Use of immune globulin to prevent symptomatic cytomegalovirus disease in transplant recipients--a meta-analysis.** *Clin Transplant* 1994, **8:**10-18.

52. Arbo MD, Snydman DR, Wong JB, Goldberg HS, Schmid CH, Pauker SG: **Cytomegalovirus immune globulin after liver transplantation: a cost-effectiveness analysis.** *Clin Transplant* 2000, **14:**19-27.
53. Tsevat J, Snydman DR, Pauker SG, Durand-Zaleski I, Werner BG, Levey AS: **Which renal transplant patients should receive cytomegalovirus immune globulin? A cost-effectiveness analysis.** *Transplantation* 1991, **52:**259-265.
54. Andreoni KA, Wang X, Huong SM, Huang ES: **Human CMV-IGIV (CytoGam) neutralizes human cytomegalovirus (HCMV) infectivity and prevents intracellular signal transduction after HCMV exposure.** *Transpl Infect Dis* 2001, **3 Suppl 2:**25-30.
55. Forthal DN, Phan T, Landucci G: **Antibody inhibition of cytomegalovirus: the role of natural killer and macrophage effector cells.** *Transpl Infect Dis* 2001, **3 Suppl 2:**31-34.
56. Okuda D, Flaster M, Frey J, Sivakumar K: **Arterial thrombosis induced by IVIg and its treatment with tPA.** *Neurology* 2003, **60:**1825-1826.
57. Marie I, Maurey G, Herve F, Hellot MF, Levesque H: **Intravenous immunoglobulin-associated arterial and venous thrombosis; report of a series and review of the literature.** *Br J Dermatol* 2006, **155:**714-721.
58. Pollreisz A, Hacker S, Hoetzenecker K, Wliszczak T, Volf I, Ankersmit HJ: **CMVIg and IVIg induce CD32-mediated platelet aggregation in vitro: implication of therapy induced thrombocytopenia and thrombosis in vivo.** *Eur Surg* 2007, **39:**28.
59. Negishi H, Yamada H, Hirayama E, Okuyama K, Sagawa T, Matsumoto Y, Fujimoto S: **Intraperitoneal administration of cytomegalovirus hyperimmunoglobulin to the cytomegalovirus-infected fetus.** *J Perinatol* 1998, **18:**466-469.
60. Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, Andersen CB, Angelini A, Berry GJ, Burke MM, et al: **Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection.** *J Heart Lung Transplant* 2005, **24:**1710-1720.
61. Zuckermann AO, Grimm M, Czerny M, Ofner P, Ullrich R, Ploner M, Wolner E, Laufer G: **Improved long-term results with thymoglobuline induction therapy after cardiac transplantation: a comparison of two different rabbit-antithymocyte globulines.** *Transplantation* 2000, **69:**1890-1898.

62. Zuckermann A, Dunkler D, Deviatko E, Bodhjalian A, Czerny M, Ankersmit J, Wolner E, Grimm M: **Long-term survival (>10 years) of patients >60 years with induction therapy after cardiac transplantation.** *Eur J Cardiothorac Surg* 2003, **24:**283-291; discussion 291.
63. Ankersmit HJ, Moser B, Zuckermann A, Roth G, Taghavi S, Brunner M, Wolner E, Boltz-Nitulescu G: **Activation-induced T cell death, and aberrant T cell activation via TNFR1 and CD95-CD95 ligand pathway in stable cardiac transplant recipients.** *Clin Exp Immunol* 2002, **128:**175-180.
64. Stangel M, Pul R: **Basic principles of intravenous immunoglobulin (IVIg) treatment.** *J Neurol* 2006, **253 Suppl 5:**v18-v24.
65. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, Shulman ST, Bolger AF, Ferrieri P, Baltimore RS, et al: **Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association.** *Pediatrics* 2004, **114:**1708-1733.
66. van der Meche FG, Schmitz PI: **A randomized trial comparing intravenous immune globulin and plasma exchange in Guillain-Barre syndrome. Dutch Guillain-Barre Study Group.** *N Engl J Med* 1992, **326:**1123-1129.
67. Vermeulen M, van der Meche FG, Speelman JD, Weber A, Busch HF: **Plasma and gamma-globulin infusion in chronic inflammatory polyneuropathy.** *J Neurol Sci* 1985, **70:**317-326.
68. Arsura EL, Bick A, Brunner NG, Grob D: **Effects of repeated doses of intravenous immunoglobulin in myasthenia gravis.** *Am J Med Sci* 1988, **295:**438-443.
69. Letsky EA, Greaves M: **Guidelines on the investigation and management of thrombocytopenia in pregnancy and neonatal alloimmune thrombocytopenia. Maternal and Neonatal Haemostasis Working Party of the Haemostasis and Thrombosis Task Force of the British Society for Haematology.** *Br J Haematol* 1996, **95:**21-26.
70. Nobile-Orazio E: **Treatment of dys-immune neuropathies.** *J Neurol* 2005, **252:**385-395.
71. Gourgiotou K, Exadaktylou D, Aroni K, Rallis E, Nicolaidou E, Paraskevakou H, Katsambas AD: **Epidermolysis bullosa acquisita: treatment with**

intravenous immunoglobulins. *J Eur Acad Dermatol Venereol* 2002, **16:**77-80.

72. Emmi L, Chiarini F: **The role of intravenous immunoglobulin therapy in autoimmune and inflammatory disorders.** *Neurol Sci* 2002, **23 Suppl 1:**S1-8.
73. George J, Barshack I, Malka E, Goldberg I, Keren P, Laniado S, Keren G: **The effect of intravenous immunoglobulins on the progression of experimental autoimmune myocarditis in the rat.** *Exp Mol Pathol* 2001, **71:**55-62.
74. Levy Y, Sherer Y, Ahmed A, Langevitz P, George J, Fabbrizzi F, Terryberry J, Meissner M, Lorber M, Peter JB, Shoenfeld Y: **A study of 20 SLE patients with intravenous immunoglobulin--clinical and serologic response.** *Lupus* 1999, **8:**705-712.
75. Goodfield M, Davison K, Bowden K: **Intravenous immunoglobulin (IVIg) for therapy-resistant cutaneous lupus erythematosus (LE).** *J Dermatolog Treat* 2004, **15:**46-50.
76. Luke PP, Scantlebury VP, Jordan ML, Vivas CA, Hakala TR, Jain A, Somani A, Fedorek S, Randhawa P, Shapiro R: **Reversal of steroid- and anti-lymphocyte antibody-resistant rejection using intravenous immunoglobulin (IVIG) in renal transplant recipients.** *Transplantation* 2001, **72:**419-422.
77. Jordan SC, Vo AA, Toyoda M, Tyan D, Nast CC: **Post-transplant therapy with high-dose intravenous gammaglobulin: Applications to treatment of antibody-mediated rejection.** *Pediatr Transplant* 2005, **9:**155-161.
78. Guglielmo BJ, Wong-Beringer A, Linker CA: **Immune globulin therapy in allogeneic bone marrow transplant: a critical review.** *Bone Marrow Transplant* 1994, **13:**499-510.
79. Latremouille C, Genevaz D, Hu MC, Schussler O, Goussef N, Mandet C, Bruneval P, Haeffner-Cavaillon N, Carpentier A, Glotz D: **Normal human immunoglobulins for intravenous use (IVIg) delay hyperacute xenograft rejection through F(ab')2-mediated anti-complement activity.** *Clin Exp Immunol* 1997, **110:**122-126.
80. Orlandi C, Frassetto A, Gnucci E, Lehmann JR, Neri R, Melandri D: **Treatment of acute post-transfusion- graft-versus-host disease (GVHD)**

with intravenous human immunoglobulins: a case report. *J Eur Acad Dermatol Venereol* 2006, **20:**760-761.

81. Avrameas S: **Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'.** *Immunol Today* 1991, **12:**154-159.
82. Horwitz MS, Sarvetnick N: **Viruses, host responses, and autoimmunity.** *Immunol Rev* 1999, **169:**241-253.
83. Rettig MP, Low PS, Gimm JA, Mohandas N, Wang J, Christian JA: **Evaluation of biochemical changes during in vivo erythrocyte senescence in the dog.** *Blood* 1999, **93:**376-384.
84. Kim SJ, Gershov D, Ma X, Brot N, Elkon KB: **I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation.** *J Exp Med* 2002, **196:**655-665.
85. Kazatchkine MD, Kaveri SV: **Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin.** *N Engl J Med* 2001, **345:**747-755.
86. Basta M, Van Goor F, Luccioli S, Billings EM, Vortmeyer AO, Baranyi L, Szebeni J, Alving CR, Carroll MC, Berkower I, et al: **F(ab)'2-mediated neutralization of C3a and C5a anaphylatoxins: a novel effector function of immunoglobulins.** *Nat Med* 2003, **9:**431-438.
87. Hurez V, Kaveri SV, Mouhoub A, Dietrich G, Mani JC, Klatzmann D, Kazatchkine MD: **Anti-CD4 activity of normal human immunoglobulin G for therapeutic use. (Intravenous immunoglobulin, IVIg).** *Ther Immunol* 1994, **1:**269-277.
88. Kaveri S, Vassilev T, Hurez V, Lengagne R, Lefranc C, Cot S, Pouletty P, Glotz D, Kazatchkine MD: **Antibodies to a conserved region of HLA class I molecules, capable of modulating CD8 T cell-mediated function, are present in pooled normal immunoglobulin for therapeutic use.** *J Clin Invest* 1996, **97:**865-869.
89. Viard I, Wehrli P, Bullani R, Schneider P, Holler N, Salomon D, Hunziker T, Saurat JH, Tschopp J, French LE: **Inhibition of toxic epidermal necrolysis by blockade of CD95 with human intravenous immunoglobulin.** *Science* 1998, **282:**490-493.

90. Prasad NK, Papoff G, Zeuner A, Bonnin E, Kazatchkine MD, Ruberti G, Kaveri SV: **Therapeutic preparations of normal polyspecific IgG (IVIg) induce apoptosis in human lymphocytes and monocytes: a novel mechanism of action of IVIg involving the Fas apoptotic pathway.** *J Immunol* 1998, **161:**3781-3790.
91. Bendtzen K, Svenson M, Hansen M: **Autoantibodies to cytokines in IVIG.** *J Rheumatol* 1993, **20:**2176-2177.
92. Farber CM, Crusiaux A, Schandene L, van Vooren JP, Goldman M, Dupont E, Tasiaux N: **Tumor necrosis factor and intravenous gammaglobulins in common variable immunodeficiency.** *Clin Immunol Immunopathol* 1994, **72:**233-236.
93. Aukrust P, Froland SS, Liabakk NB, Muller F, Nordoy I, Haug C, Espevik T: **Release of cytokines, soluble cytokine receptors, and interleukin-1 receptor antagonist after intravenous immunoglobulin administration in vivo.** *Blood* 1994, **84:**2136-2143.
94. Samuelsson A, Towers TL, Ravetch JV: **Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor.** *Science* 2001, **291:**484-486.
95. Rabinovitch N, Gelfand EW, Leung DY: **The role of immunoglobulin therapy in allergic diseases.** *Allergy* 1999, **54:**662-668.
96. Yu Z, Lennon VA: **Mechanism of intravenous immune globulin therapy in antibody-mediated autoimmune diseases.** *N Engl J Med* 1999, **340:**227-228.
97. Takai T: **Fc receptors and their role in immune regulation and autoimmunity.** *J Clin Immunol* 2005, **25:**1-18.
98. Flesch BK, Neppert J: **Functions of the Fc receptors for immunoglobulin G.** *J Clin Lab Anal* 2000, **14:**141-156.
99. Iannello A, Ahmad A: **Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies.** *Cancer Metastasis Rev* 2005, **24:**487-499.
100. Boyle JJ: **Human macrophages kill human mesangial cells by Fas-L-induced apoptosis when triggered by antibody via CD16.** *Clin Exp Immunol* 2004, **137:**529-537.
101. Stockmeyer B, Beyer T, Neuhuber W, Repp R, Kalden JR, Valerius T, Herrmann M: **Polymorphonuclear granulocytes induce antibody-**

dependent apoptosis in human breast cancer cells. *J Immunol* 2003, **171:**5124-5129.

102. Otten MA, Leusen JH, Rudolph E, van der Linden JA, Beelen RH, van de Winkel JG, van Egmond M: **FcR {gamma}-Chain Dependent Signaling in Immature Neutrophils Is Mediated by Fc{alpha}RI, but Not by Fc{gamma}RI.** *J Immunol* 2007, **179:**2918-2924.
103. Otten MA, Rudolph E, Dechant M, Tuk CW, Reijmers RM, Beelen RH, van de Winkel JG, van Egmond M: **Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors.** *J Immunol* 2005, **174:**5472-5480.
104. Akbari O, Stock P, DeKruyff RH, Umetsu DT: **Role of regulatory T cells in allergy and asthma.** *Curr Opin Immunol* 2003, **15:**627-633.
105. Chinen J, Shearer WT: **Advances in asthma, allergy and immunology series 2004: basic and clinical immunology.** *J Allergy Clin Immunol* 2004, **114:**398-405.
106. Fontenot JD, Rudensky AY: **A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3.** *Nat Immunol* 2005, **6:**331-337.
107. Sakaguchi S: **Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.** *Annu Rev Immunol* 2004, **22:**531-562.
108. Fehervari Z, Sakaguchi S: **CD4+ Tregs and immune control.** *J Clin Invest* 2004, **114:**1209-1217.
109. Shevach EM, Piccirillo CA, Thornton AM, McHugh RS: **Control of T cell activation by CD4+CD25+ suppressor T cells.** *Novartis Found Symp* 2003, **252:**24-36; discussion 36-44, 106-114.
110. von Boehmer H: **Mechanisms of suppression by suppressor T cells.** *Nat Immunol* 2005, **6:**338-344.
111. Umetsu DT, Akbari O, Dekruyff RH: **Regulatory T cells control the development of allergic disease and asthma.** *J Allergy Clin Immunol* 2003, **112:**480-487; quiz 488.
112. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ: **Human T regulatory cells can use the perforin pathway to cause autologous target cell death.** *Immunity* 2004, **21:**589-601.

113. O'Neill E J, Sundstedt A, Mazza G, Nicolson KS, Ponsford M, Saurer L, Streeter H, Anderton S, Wraith DC: **Natural and induced regulatory T cells.** *Ann N Y Acad Sci* 2004, **1029:**180-192.

114. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S: **Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells.** *Int Immunol* 2004, **16:**1643-1656.

115. Hoetzenecker K, Hacker S, Hoetzenecker W, Polreisz A, Bohle B, Roth G, Moser B, Krenn C, Grimm M, Bolz-Nituescu G, et al: **Pooled human IgG and IgM has immunesupressive properties in vitro and is partly triggered by Fc blockade.** *Eur Surg* 2005, **37:**26.

116. Kwekkeboom J, Tha-In T, Tra WM, Hop W, Boor PP, Mancham S, Zondervan PE, Vossen AC, Kusters JG, de Man RA, Metselaar HJ: **Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation.** *Am J Transplant* 2005, **5:**2393-2402.

117. Williams MA, Rhoades CJ, Provan D, Newland AC: **In vitro cytotoxic effects of stabilizing sugars within human intravenous immunoglobulin preparations against the human macrophage THP-1 cell-line.** *Hematology* 2003, **8:**285-294.

118. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N: **A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells.** *J Am Soc Nephrol* 2006, **17:**2844-2853.

119. Negi VS, Elluru S, Siberil S, Graff-Dubois S, Mouthon L, Kazatchkine MD, Lacroix-Desmazes S, Bayry J, Kaveri SV: **Intravenous immunoglobulin: an update on the clinical use and mechanisms of action.** *J Clin Immunol* 2007, **27:**233-245.

120. Nachbaur D, Herold M, Eibl B, Glassl H, Schwaighofer H, Huber C, Gachter A, Pichl M, Niederwieser D: **A comparative study of the in vitro immunomodulatory activity of human intact immunoglobulin (7S IVIG), F(ab')2 fragments (5S IVIG) and Fc fragments. Evidence for post-transcriptional IL-2 modulation.** *Immunology* 1997, **90:**212-218.

121. Toungouz M, Denys C, Dupont E: **Blockade of proliferation and tumor necrosis factor-alpha production occurring during mixed lymphocyte reaction by interferon-gamma-specific natural antibodies contained in intravenous immunoglobulins.** *Transplantation* 1996, **62:**1292-1296.
122. Vuist WM, Van Schaik IN, Van Lint M, Brand A: **The growth arresting effect of human immunoglobulin for intravenous use is mediated by antibodies recognizing membrane glycolipids.** *J Clin Immunol* 1997, **17:**301-310.
123. Jordan SC, Tyan D, Czer L, Toyoda M: **Immunomodulatory actions of intravenous immunoglobulin (IVIG): potential applications in solid organ transplant recipients.** *Pediatr Transplant* 1998, **2:**92-105.
124. Binah O: **Immune effector mechanisms in heart transplant rejection.** *Cardiovasc Res* 1994, **28:**1748-1757.
125. Frisman DM, Fallon JT, Hurwitz AA, Dec WG, Kurnick JT: **Cytotoxic activity of graft-infiltrating lymphocytes correlates with cellular rejection in cardiac transplant patients.** *Hum Immunol* 1991, **32:**241-245.
126. Carlquist JF, Greenwood JH, Hammond EH, Anderson JL: **Phenotype and serine esterase production of human cardiac allograft-infiltrating lymphocytes.** *J Heart Lung Transplant* 1993, **12:**748-755.
127. Marboe CC, Knowles DM, 2nd, Chess L, Reemtsma K, Fenoglio JJ, Jr.: **The immunologic and ultrastructural characterization of the cellular infiltrate in acute cardiac allograft rejection: prevalence of cells with the natural killer (NK) phenotype.** *Clin Immunol Immunopathol* 1983, **27:**141-151.
128. Kitchens WH, Uehara S, Chase CM, Colvin RB, Russell PS, Madsen JC: **The changing role of natural killer cells in solid organ rejection and tolerance.** *Transplantation* 2006, **81:**811-817.
129. McNerney ME, Lee KM, Zhou P, Molinero L, Mashayekhi M, Guzior D, Sattar H, Kuppireddi S, Wang CR, Kumar V, Alegre ML: **Role of natural killer cell subsets in cardiac allograft rejection.** *Am J Transplant* 2006, **6:**505-513.
130. Maier S, Tertilt C, Chambron N, Gerauer K, Huser N, Heidecke CD, Pfeffer K: **Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-*/*- mice.** *Nat Med* 2001, **7:**557-562.
131. Uehara S, Chase CM, Kitchens WH, Rose HS, Colvin RB, Russell PS, Madsen JC: **NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts.** *J Immunol* 2005, **175:**3424-3430.

132. Ichiyama T, Ueno Y, Hasegawa M, Niimi A, Matsubara T, Furukawa S: **Intravenous immunoglobulin inhibits NF-kappaB activation and affects Fcgamma receptor expression in monocytes/macrophages.** *Naunyn Schmiedebergs Arch Pharmacol* 2004, **369:**428-433.
133. Warren HS, Kinnear BF: **Quantitative analysis of the effect of CD16 ligation on human NK cell proliferation.** *J Immunol* 1999, **162:**735-742.
134. Fehr T, Sykes M: **Tolerance induction in clinical transplantation.** *Transpl Immunol* 2004, **13:**117-130.
135. Potena L, Holweg CT, Chin C, Luikart H, Weisshaar D, Narasimhan B, Fearon WF, Lewis DB, Cooke JP, Mocarski ES, Valantine HA: **Acute rejection and cardiac allograft vascular disease is reduced by suppression of subclinical cytomegalovirus infection.** *Transplantation* 2006, **82:**398-405.
136. Valantine HA, Luikart H, Doyle R, Theodore J, Hunt S, Oyer P, Robbins R, Berry G, Reitz B: **Impact of cytomegalovirus hyperimmune globulin on outcome after cardiothoracic transplantation: a comparative study of combined prophylaxis with CMV hyperimmune globulin plus ganciclovir versus ganciclovir alone.** *Transplantation* 2001, **72:**1647-1652.

ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immune deficiency syndrome
ANCA	Anti-neutrophil cytoplasmatic antibodies
APC	Antigen presenting cells
ATG	Antithymocyte globuline
AUC	Area under the curve
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CMVIg	CMV hyperimmunoglobulin
CTLA	Cytotoxic T-lymphocyte antigen
D	Donor
DNA	Deoxyribonucleic acid
E	Reference units of the Paul-Ehrlich institute
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FcγR	Fcγ-receptors
FITC	Fluorescein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvH	Graft versus host
HBSS	Hanks' balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IBS	Inflammatory bowel diseases
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukine
iv	Intravenous
IvIg	Intravenous immunoglobulin
ISHLT	International Society for Heart and Lung Transplantation
Jurkat	human, peripheral blood, leukemia T cells
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MoAb	Monoclonal antibodies
MS	Multiple sclerosis
NA	Not available
NfκB	Nuclear factor kappa B
NK cells	Natural killer cells
NO	Nitric oxide
OD	Optical density
PANC-1	Pancreatic cancer cells

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI	Propidium iodide
R	Recipient
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
TACAD	Transplant-associated coronary artery disease
TGF	Transforming growth factor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
Tregs	T regulatory cells
VZV	Varicella zoster virus

APPENDIX

Publication

European Journal of Clinical Investigation (2007) **37**, 978–986 DOI: 10.1111/j.1365-2362.2007.01881.x

Cytomegalovirus hyperimmunoglobulin: mechanisms in allo-immune response *in vitro*

K. Hoetzenecker*, S. Hacker*, W. Hoetzenecker‡, K. Sadeghi*, M. Sachet*, A. Pollreisz*, A. Mangold*, T. Wliszczak*, E. Bielek†, F. Muehlbacher*, W. Klepetko* and H. J. Ankersmit*

**Department of Surgery, Medical University of Vienna, Austria, †Center of Anatomy and Cell Biology, Medical University of Vienna, Austria, ‡Department of Dermatology, Medical University Tübingen, Germany*

Abstract

Background Cytomegalovirus hyperimmunoglobulin (CMVIg) containing drugs are routinely administered in cardiac transplantation for prophylaxis against CMV disease. Yet little is known about their influence on transplant relevant immune functions. The aim of this study was to evaluate the effect of CMVIg on cellular immunity in *in vitro* experiments and to define their role in tolerance inducing mechanisms.

Materials and methods/results CMVIg reduces proliferation in mixed lymphocyte reactions and anti-CD3 blastogenesis assays and is related to decreased production of immune modulating cytokines interleukin (IL)-2, interferonr (IFNγ), IL-10. This antiproliferative effect is associated with a cell-cycle arrest in the G0/G1 phase and induction of apoptosis in CD8+ and natural killer cells. Co-incubation with CMVIg causes down-regulation of cell bound immunoglobulin and FcγRIII surface expression on natural killer cells and leads to attenuation of antibody dependent cellular cytotoxicity effector functions.

Conclusions We conclude that CMVIg induces immunological features on leukocytes *in vitro* that are known to be related to tolerance induction. Our observations extend the current concept of CMVIg as passive CMV prophylaxis to a therapeutic drug compound capable of reducing allogeneic immune response.

Keywords Allo-immune response, apoptosis, CMV hyperimmunoglobulin, heart transplantation, NK cell function.
Eur J Clin Invest 2007; 37 (12): 978–986

Introduction

Cardiac transplantation has evolved as the best treatment option for patients with advanced heart failure remaining refractory to medical treatment. The advances made in transplant management, surgical techniques and modifications of immunosuppression have resulted in significant improvements in patient survival [1]. Recently, our heart transplant programme reported a conspicuously low incidence of acute rejection and development of transplant-associated coronary artery disease (TACAD) in heart allograft recipients [2–4]. Induction therapy with rabbit anti-thymocyte globulin (rATG), in addition to a standard drug scheme based on cyclosporine, mycophenolate mofetil and steroids, is held responsible for this striking clinical observation. The immunological mechanism responsible for this clinical state of 'anergy' in allograft recipients treated with poly or monoclonal antibodies (rATG, OKT3, interleukine-2 receptor blocker) is thought to be related to its potential to cause apoptosis/activation induced cell death (AICD) in allogeneic activated T cell [5–8].

In addition to the immunosuppressive regime described above, cytomegalovirus (CMV) hyperimmunoglobulin (CMVIg) has been routinely applied to all heart recipients as passive prophylaxis against CMV in our transplant centre since 1987. CMVIg is administered intravenously

Department of Surgery, Medical University of Vienna, Vienna, Austria (K. Hoetzenecker, S. Hacker, M. Sachet, A. Pollreisz, A. Mangold, T. Wliszczak, F. Muehlbacher, W. Klepetko, H. J. Ankersmit); Department of Pediatrics, Medical University of Vienna, Vienna, Austria (K. Sadeghi); Center of Anatomy and Cell Biology, Medical University of Vienna, Vienna, Austria (E. Bielek); Department of Dermatology, Medical University Tübingen, Tübingen, Germany (W. Hoetzenecker).

Correspondence to: H. J. Ankersmit, Department of Cardiothoracic Surgery, Medical University of Vienna, Währinger Gürtel 18–20, 1090 Vienna, Austria. Tel.: +43-1-40400-6966; fax: +43-1-40400-6968; e-mail: hendrik.ankersmit@meduniwien.ac.at and www.applied-immunology.org

Received 8 July 2007; accepted 11 September 2007

on postoperative days 1, 7, 14, 21 and 28 irrespective of the preoperative donor/recipient CMV status. CMV, a virus belonging to the herpes family, was identified as the most important pathogen affecting morbidity and mortality in the immediate postoperative course after transplantation. CMV induced morbidities in transplant recipients include gastrointestinal tract infection, lung infections, chorioretinitis and hepatitis–like syndromes [9–12]. Moreover, CMV infection was also shown to be associated with incidence of TACAD, a clinical entity describing morphological and functional changes in the small coronary arteries of the transplanted heart [13,14].

The CMVIg drug compound is produced by pooling plasma from hundreds of healthy donors with a proven antibody titre greater than 1 : 7000. Several reports have found evidence that CMVIg is highly effective in preventing CMV disease [15,16]. CMVIg is pharmacologically related to pooled intravenous immunoglobulin (IVIg). The systemic application of IVIg is shown to be highly beneficial in several autoimmune and inflammatory disorders including Kawasaki disease, dermatomyositis, lupus erythematosus, polyradiculoneuritis, Guillain–Barré syndrome and toxic epidermal necrolysis [17]. The modes of action of IVIg are complex and are still the topic of ongoing research. Theories range from immunomodulation due to attenuation of complement-mediated tissue damage, down-regulation of B-cell response by FcγR blockade and acceleration of FcRn-mediated catabolism [18]. Concerning allogeneic transplantation early reports found evidence that IVIg is able to decrease proliferation in allogeneic mixed lymphocyte reactions [19]. These *in vitro* experiments were recently complemented by the *in vivo* observation that IVIg were highly effective in treating steroid resistant allograft rejection [20,21].

Since there is little published evidence relating CMVIg and immunosuppression we sought to investigate (a) the effect of CMVIg on proliferation in mixed lymphocyte reaction (MLR) and anti-CD3 blastogenesis assays; (b) the potential of CMVIg to induce apoptosis in peripheral blood mononuclear cells (PBMC) and highly purified CD4+, CD8+, CD19+ and CD56+ cells; (c) the effect of CMVIg on natural killer (NK) cell effector function utilizing antibody dependent cellular cytotoxicity (ADCC) assays.

Materials and methods

CMVIg compounds and sample size

Two different CMVIg preparations were used for experimental studies (Cytotect, kindly provided by Dr Schrot, Biotest, Frankfurt, Germany and Cytoglobulin, Bayer AG, Leverkusen, Germany). The study protocol was approved by the Ethics Committee of the Medical University of Vienna (EC-No: 083/2006). Cells used for the experiments were obtained from healthy volunteers. All experiments were conducted with a sample size of ten ($n = 10$). CMVIg was added to all experiments in therapeutic concentrations as described elsewhere [22]. In control experiments CMVIg was replaced by medium. Dialysis of CMVIg with a 1 kDa cutoff for exclusion of stabilizing agents was additionally performed (Mini dialysis kit; Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions. Dialysis had no effect in *in vitro* assays (data not shown).

^{3}[H]-thymidine proliferation assay

PBMC were separated by Ficoll density gradient centrifugation. They were resuspended in serum free Ultra Culture Medium (Cambrex Corp., North Brunswick, NJ, USA) containing 0·2% gentamycinsulfate (Sigma Chemical Co, St. Louis, MO, USA), 0·5% β-mercapto-ethanol (Sigma, St Louis, MO, USA), 1% L-glutamin (Sigma, St Louis, MO, USA) and 150 µL medium containing 1×10^5 cells per well were added to 96-well round-bottomed tissue culture plates. Responder cells were either stimulated with irradiated allogeneic PBMC at a 1 : 1 ratio (for MLR) or by monoclonal antibodies (MoAb) to CD3 (10 µg mL^{-1}, Becton Dickinson, Franklin Lakes, NJ, USA). Plates were incubated at 37 °C in a humidified incubator for 48 h or 5 days together with CMVIg and then pulsed for 18 h with ^{3}[H]-thymidine ($3{\cdot}7 \times 10^4$ Bq/well; Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were harvested and ^{3}[H]-thymidine incorporation was measured in a liquid scintillation counter.

Measuring of cell division

PBMC (1×10^7) were labelled with 5 µM Carboxyfluorescein diacetate succinimidyl ester (CFSE, Fluka BioChemika, Buchs, Switzerland) at room temperature for 10 min. Labelling was stopped by the addition of fetal calf serum (FCS). Cells were washed, resuspended in Ultra Culture Medium and stimulated with 10 µg mL^{-1} MoAb to CD3 in a humidified incubator. CFSE dilution was measured after 48 h on a flow cytometer FC500 (Beckman Coulter, Fullerton, CA, USA).

Cell cycle analysis

PBMC were stimulated with 10 µg mL^{-1} MoAb to CD3 for 48 h and stained for DNA content using a cell cycle test kit (Becton Dickinson, Franklin Lakes, NJ, USA) utilizing propidium iodide for DNA detection. Cells were measured on a flow cytometer and histograms were analysed by ModFit LT software (Verity Software House, Topsham, ME, USA).

Cytokine profiles

PBMC (1×10^6) were incubated with 10 µg mL^{-1} anti-CD3. Supernatants were collected and frozen immediately at –80 °C until further tests were performed. Content of

cytokines was measured by commercially available enzyme linked immunosorbant assays (ELISA, BenderMedSystems, Vienna, Austria). Assays were performed according to the manufacturer's instructions. All plates were read at 450 nm on a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA).

Isolation of purified CD4+, CD8+, B cells, NK cells

CD4+, CD8+, B cells, NK cells were isolated from prior obtained PBMC by negative selection using magnetic labelling isolation kits (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Purity of separated cells measured by flow cytometry was determined to be higher than 95%.

Detection of apoptosis

PBMC (3×10^5) of purified subpopulations (CD4+ T-cells, CD8+ T-cells, B cells, NK cells) were incubated in a humidified atmosphere. Induction of apoptosis was measured by AnnexinV-fluorescein/propidium iodide (FITC/PI) costaining (Becton Dickinson, Franklin Lakes, NJ, USA) on a flow cytometer at different time points (0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h).

Electron microscopy

PBMC (1×10^6) were incubated with or without CMVIg at 37 °C in an incubator for 12 h. Cells were spun down and the material was processed according to standard electron microscopical methods: fixation in 4% paraformaldehyde, pH 7·4, at 4 °C followed by dehydration in a graded ethanol series and embedding in epoxy resin (glycidether). Ultrathin sections (0·5 μm) were cut on a Reichert ultra-microtome (OM U3, Ultracut S), stained with uranyl acetate lead^{-1} citrate and examined on a Jeol 1200 EXII electron microscope.

Evaluation of FcγRIII (CD16) and cell surface bound IgG content

Purified NK cells (3×10^5) were incubated in a humidified atmosphere. Cells were costained with anti-CD16 and anti-IgG (Serotec, Oxford, UK) following a standard staining protocol. CD16 expression and amount of cell surface bound IgG were evaluated by flow cytometry.

Tumour cell lines

JURKAT (human, peripheral blood, leukaemia T cells) and PANC-1 (pancreatic cancer cells) (both American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (HyClone; Perbio, Logan, UT, USA) and 0·2% gentamycinsulfate (Sigma Chemical Co) in a 5% CO_2 humidified atmosphere at 37 °C.

Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC was measured using a europium release assay. *In vitro* cultured target cells (Jurkat, PANC-1) were harvested and labelled with europium for 15 min. PBMC or purified NK cells serving as effector cells were pre-incubated for 4 h with 2·5 mg mL^{-1} of CMVIg, washed twice, resuspended serum free Ultra Culture Medium and incubated with target cells at ratios ranging from 50 : 1 to 0·8 : 1 for 4 h in round bottom 96-well plates. Europium release to the supernatant was measured by time resolved flourometry (Wallac Multilabel counter 1420, PerkinElmer, Boston, MA, USA). Cytotoxic activity was calculated with the following formula:

$$\%\ \text{cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100$$

Total release was determined by adding 2% Triton X-100 to target cells.

Statistical methods

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). All data are given as mean ± standard of the mean. Normal distribution was verified using the Kolmogorov–Smirnov test. Paired two-sided *T*-tests for dependent variables were utilized calculating significances. For evaluation of ADCC data, areas under the curve (AUC), calculated by linear interpolation for each condition were compared. Bonferroni–Holm correction was used to adjust *P*-values for multiple testing. *P*-values < 0·05 were considered statistically significant.

Results

^{3}[H]-thymidine incorporation is decreased in mixed lymphocyte reactions (MLR) and anti-CD3 blastogenesis assay after co-incubation with CMVIg

To test the hypothesis whether CMVIg contributes to a decreased allo-immune response and a decreased response after T-cell triggering, we employed established models of MLR and anti-CD3 blastogenesis assays. Figure 1(a) shows that addition of CMVIg significantly decreases proliferation rates in a dose-dependent manner.

Anti-proliferative effect determined by CFSE dilution and cell cycle analysis

To verify antiproliferative effects CFSE dilution experiments and cell cycle analysis of anti-CD3 stimulated PBMC

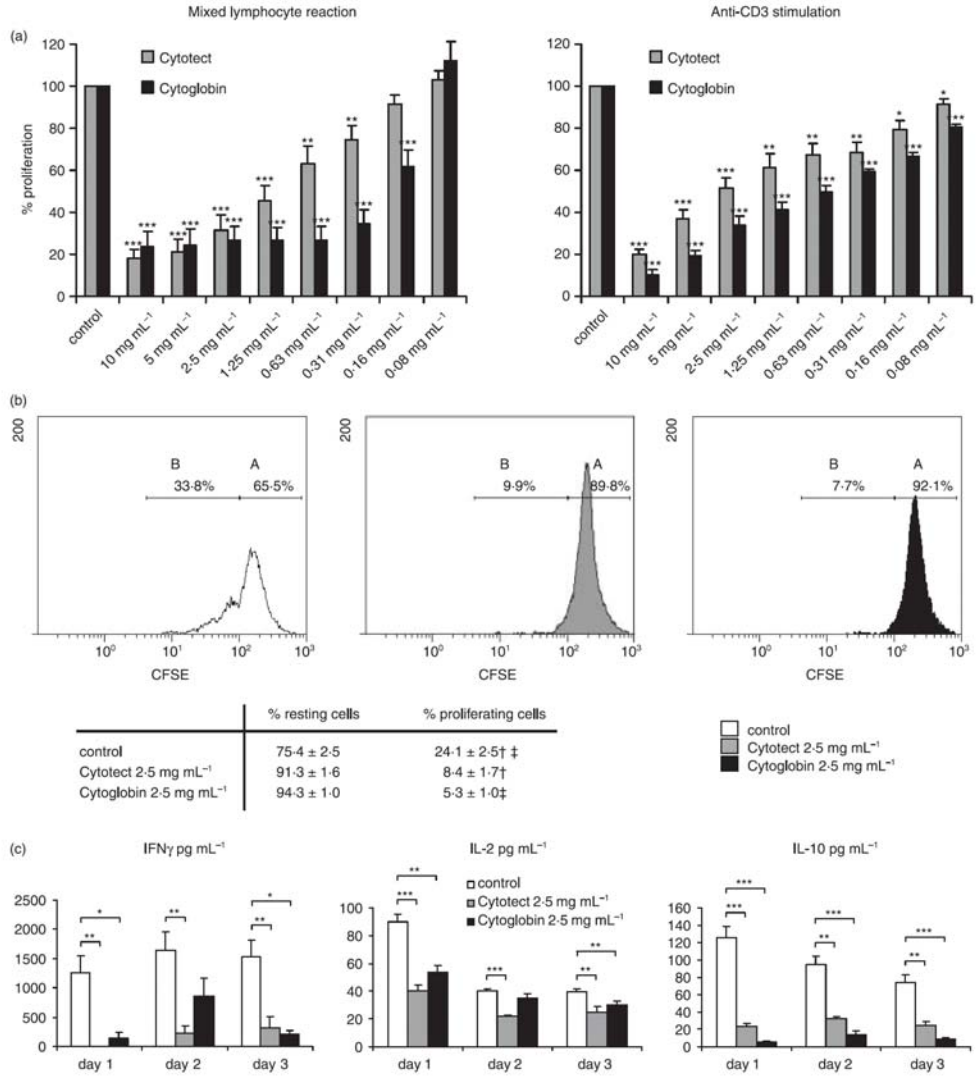


Figure 1 (a) ^{3}[H]-thymidine incorporation is decreased by co-incubation with Cytomegalovirus hyperimmunoglobulin (CMVIg). This figure shows the effect of CMVIg on ^{3}[H]-thymidine incorporation in an allogeneic stimulation and in a T-cell specific triggering by monoclonal antibodies (MoAb) against CD3. Proliferation rates of experiments containing different concentrations of CMVIg were compared to control experiments and are given as percentages of the maximal control proliferation (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$; $n = 10$). (b) Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assays confirm antiproliferative effects of CMVIg. A representative CFSE dilution experiment is depicted in this figure. The control setting demonstrates a distinct CFSE dilution whereas the addition of CMVIg resulted in strongly reduced proliferation. Summarized data are shown in the incorporated table. A significant reduction of proliferating cells was observed after co-incubation with CMVIg (†$P < 0{\cdot}001$, ‡$P < 0{\cdot}001$; $n = 10$). (c) Release of interferon (IFN)γ, interleukin (IL)-2 and IL-10 in anti-CD3 blastogenesis assays is reduced. This figure displays the evaluation of supernatants for IFNγ, IL-2 and IL-10 of anti-CD3 stimulated peripheral blood mononuclear cells (PBMC) in a time resolved manner. All three T-cell derived cytokines were significantly reduced (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$; $n = 10$).

Table 1 Cell cycle analysis evidence antiproliferative effects of CMVIg

Cell cycle analysis of peripheral mononuclear cells (PBMC) exposed to CMVIg			
	% G0-G1	% S	% G2-M
Control	85·3 ± 2·9	11·7 ± 2·3*†	3·0 ± 0·7*†
Cytotect 2·5 mg mL^{-1}	98·8 ± 0·3	1·1 ± 0·3*	0·1 ± 0·1*
Cytoglobin 2·5 mg mL^{-1}	95·9 ± 1·4	3·7 ± 1·1†	0·4 ± 0·4†

This table shows results of cell cycle analysis performed in T-cell triggered PBMC. Co-incubation with CMVIg results in a massive decrease of cells residing in S and G2/M-phase. These data confirm CFSE dilution experiments (*$P < 0·05$; †$P < 0·05$; $n = 10$).

were performed. Figure 1(b) shows a representative CFSE dilution experiment indicating that CMVIg has clear antiproliferative properties. Combined data of 10 probands demonstrated that the percentage of proliferating cells decreased from 24·1 ± 2·5 in the control to 8·4 ± 1·7 (Cytotect; $P < 0·001$) and 5·3 ± 1·0 (Cytoglobin; $P < 0·001$). These findings were also confirmed by cell cycle analysis. Co-incubation of CMVIg significantly reduces transition of resting G1 cells to S and G2/M phase as shown in Table 1.

Decreased production of IFNγ, IL-2 and IL-10 in the presence of CMVIg in anti-CD3 blastogenesis assays

To investigate whether cytokine profile is altered by CMVIg we employed ELISA technique to quantify IFNγ, IL-2 and IL-10 cytokine content in the supernatant after T-cell specific triggering. Results are depicted in Fig. 1(c). All measured cytokines were significantly reduced by the addition of CMVIg.

Resting PBMC exposed to CMVIg undergo apoptosis and evidence characteristic morphology as determined by electron microscopy

The effect of CMVIg on unstimulated PBMC was evaluated by Annexin-V/PI staining on a flow cytometer. Co-incubation of PBMC with CMVIg resulted in a significant increase in Annexin-V binding cells, indicating early apoptosis, as shown in Fig. 2(a). Subsequent increase in PI staining cells evidencing late apoptosis was observed at time point 48 h (data not shown).

Flow cytometric data were confirmed by electron microscopy. CMVIg treated PBMC showed typical apoptotic features (e.g. formation of apoptotic bodies, cell membrane blebbing, chromatin condensation) whereas control cells displayed normal morphology of resting PBMC (Fig. 2b).

Purified NK cells and CD8+ cells are sensitive to CMVIg–induced apoptosis

To substantiate which subpopulations of PBMC are prone to undergoing apoptosis after incubation with CMVIg we exposed highly purified CD4+, CD8+, CD19+ and CD56+ cells to therapeutic CMVIg concentrations. CD4+ and CD19+ cells are hardly sensitive for induction of apoptosis as compared to cytotoxic CD8+ cells and NK cells (Fig. 2c,d).

CMVIg evidences a down-regulation of low affinity FcγRIII (CD16) resulting in a decrease of cell surface bound IgG in purified NK cells

Figure 3(a) shows that CMVIg significantly reduces cell surface bound IgG and density of FcγRIII in purified NK cells. Data of 10 probands are summarized in Table 2. Both features, namely CD16 and cell surface bound immunoglobulin, are inalienable to the performance of NK cell mediated ADCC.

PBMC and purified NK cells evidence a decrease in ADCC after preincubation with CMVIg

Finally, based on the impact of CMVIg on apoptosis in PBMC and NK cells and down-regulation of FcγRIII expression, we sought to extend these findings by quantifying ADCC against PANC-1 and Jurkat target cells. Immune function against these two tumour cell lines was significantly reduced when effector cells were preincubated with CMVIg (Fig. 3b).

Discussion

Our findings represent the first demonstration that CMVIg countered proliferation in allogeneic and T-cell receptor stimulated lymphocytes *in vitro*. A significant induction of apoptosis in PBMC (predominantly CD8+ and NK cells) was verified, accompanied by loss of cell surface bound immunoglobulin and low-affinity Fc receptor (FcγRIII; CD16) expression. Moreover, effector functions of CMVIg preincubated PBMC and NK cells were decreased in ADCC assays. We deduce from our *in vitro* experiments that CMVIg has the potential to induce two immunological features that are known mechanisms for allograft tolerance induction: (a) attenuation of allogeneic and T-cell receptor induced

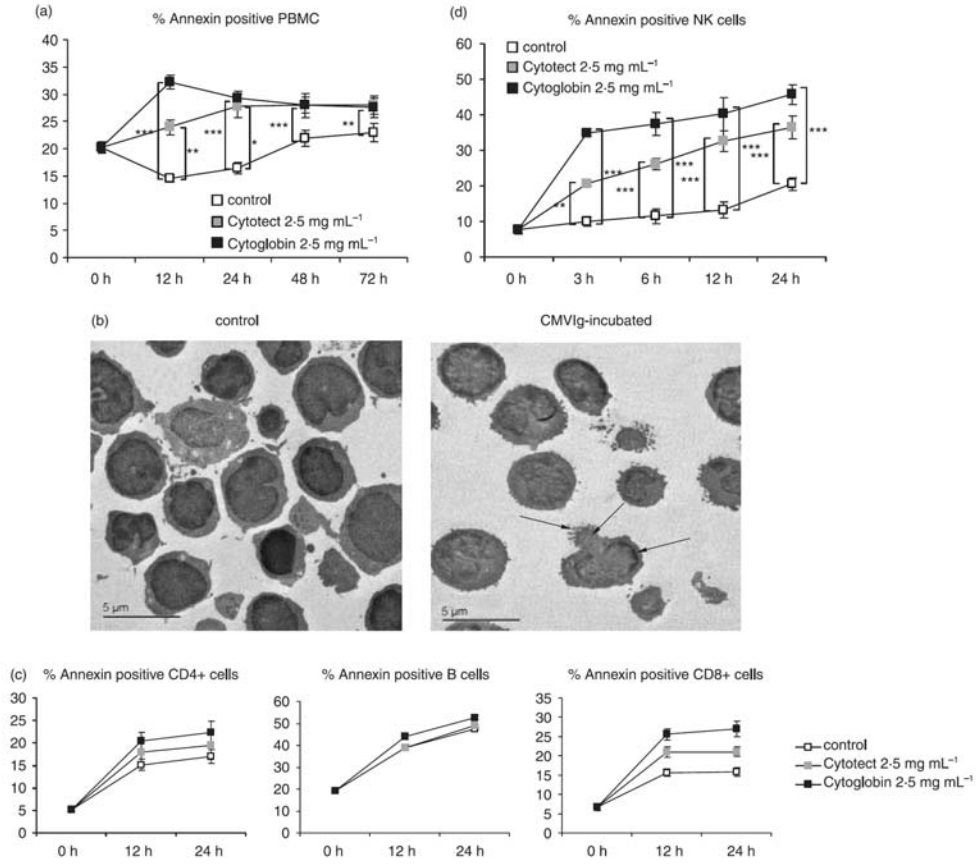


Figure 2 (a) CMVIg induces apoptosis in PBMC. The potential of CMVIg to induce apoptosis in resting PBMC is shown in this figure. Co-incubation of PBMC with CMVIg results in a massive increase of Annexin-V binding, indicating early phases of apoptosis. This increase reached significance after 12 h ($n = 10$). (b) Electron microscopy reveals typical morphological changes in PBMC after incubation with CMVIg. This figure shows the influence of CMVIg on cell morphology as evaluated by electron microscopy. Cells treated with therapeutic concentrations of Cytotect developed typical apoptotic features whereas control cells remained unchanged. Similar results were found for Cytoglobin (data not shown). (c) Evaluation of the sensitivity of subpopulations for apoptosis induction by CMVIg. The sensitivity of purified CD4+, CD19+ and CD8+ cells to undergo apoptosis after CMVIg triggering is shown in in this figure. CD4+ and B cells are hardly affected for programmed cell death whereas cytotoxic T-cells are prone to apoptosis induction by CMVIg ($n = 10$). (d) Natural killer (NK) cells are most prone for CMVIg induced programmed cell death. This figure demonstrates cell viability of highly purified NK cells. Both Cytotect and Cytoglobin are potent inducers of programmed cell death when compared to untreated control NK cells (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$; $n = 10$).

proliferation; (b) induction of apoptosis in cytotoxic CD8+ and NK cells and reduction of NK effector function.

We provide evidence for the first time that CMVIg has similar effects in suppressing MLR as those published for IVIg. In these studies several investigators suggested responsible mechanisms for this *in vitro* observation. Nachbaur *et al.* showed that IVIg inhibited IL-2 protein synthesis with no effect on IL-2 mRNA expression [19]. Blockade of IFNγ or CD4 by antibodies found in IVIg preparations was suggested by other investigators [23,24]. Vuist *et al.* demonstrated that IVIg contain antibodies against glycolipids expressed on lymphocytes leading to the IVIg-induced growth attenuation [25]. More recently, Jordan *et al.* evidenced that antibodies directed against MHC class I and II as well as T-cell receptor molecules are found in IVIg, which are directly involved in the alloantigen recognition process in the MLR. Moreover, IVIg was shown to also induce apoptosis in the setting of MLR, primarily in

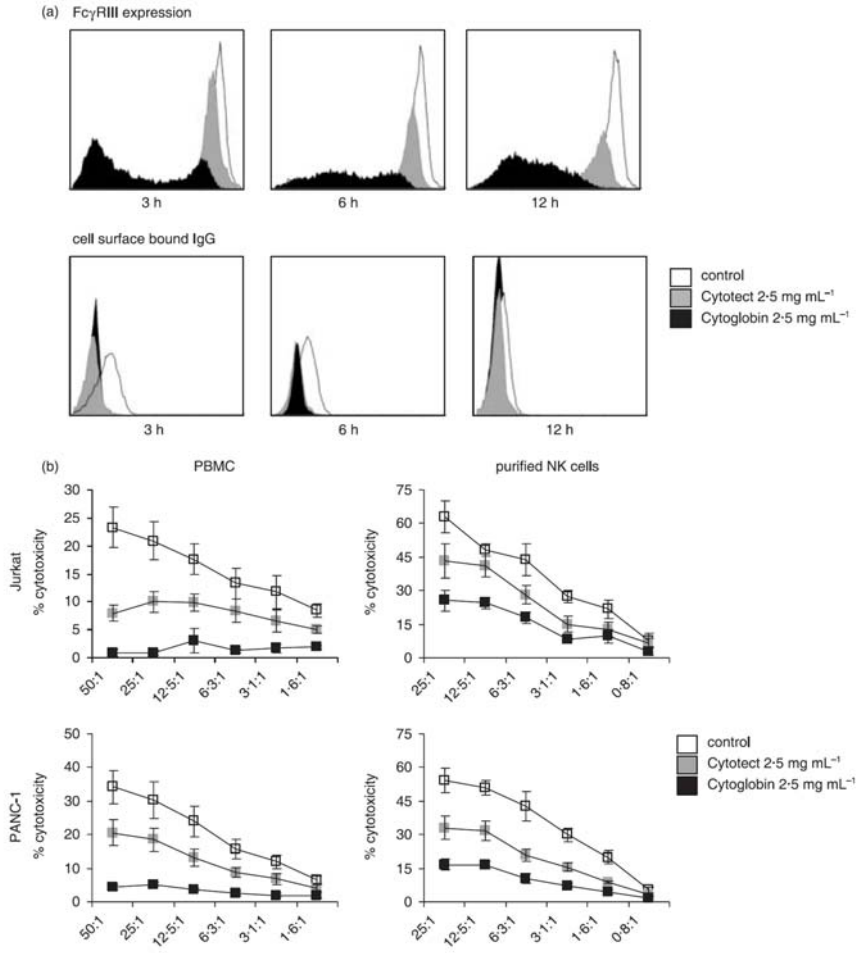


Figure 3 (a) FcγRIII (CD16) and cell surface bound IgG is down-regulated in purified NK cells by CMVIg. This figure displays histograms of a representative experiment showing the density of FcγRIII and surface bound IgG on purified NK cells. A massive down-regulation of both surface proteins is observed in the course of time. (b) PBMC and purified NK cells evidence a decrease in antibody dependent cellular cytotoxicity (ADCC) against two tumour cell lines. ADCC of PBMC and purified NK cells against Jurkat and PANC-1 tumour cell lines are depicted in this figure. Both Cytotect and Cytoglobin are potent inhibitors of ADCC *in vitro*. Significances refer to comparison between AUC as described in the material and methods section. PBMC-Jurkat: control 944·9 ± 148·5, Cytotect: 434·7 ± 71·0 ($P < 0·01$), Cytoglobin: 68·7 ± 37·9 ($P < 0·05$), PBMC-PANC-1: control 1327·2 ± 220·9, Cytotect: 785·0 ± 145·2 ($P < 0·05$), Cytoglobin: 205·2 ± 23·8 ($P < 0·001$); NK cells-Jurkat control 1138·5 ± 82·4, Cytotect: 844·1 ± 98·2 ($P < 0·05$), Cytoglobin: 510·2 ± 57·0 ($P < 0·05$), NK cells-PANC-1: control 1120·6 ± 101·8, Cytotect: 653·9 ± 85·7 ($P < 0·01$), Cytoglobin: 331·6 ± 44·8 ($P < 0·001$) ($n = 10$).

B cells [26–28]. These *in vitro* findings of IVIg-induced leukocyte apoptosis were corroborated by the clinical observation of transient lymphopenia after infusion of IVIg [29].

In addition to the MLR data we were able to show that CMVIg also causes a decrease of proliferation in T-cell triggered blastogenesis assays. An important question arising from our proliferation data is whether reduced proliferation rates were real antiproliferative effects or only masked effects due to increased cell death. To dissect these mechanisms we performed CFSE dilution assays and cell cycle analysis. Co-incubation with CMVIg revealed that a significantly increased number of cells were in G0/G1 phase as compared

Table 2 CMVIg reduces density of FcγRIII and cell surface bound IgG on NK cells

Cell bound IgG and FcγRIII on purified NK cells are down-regulated by CMVIg	Control	Cytotect 2·5 mg mL^{-1}	Cytoglobin 2·5 mg mL^{-1}
Cell bound IgG – mean fluorescence			
0 h	1·32 ± 0·50	1·32 ± 0·50	1·32 ± 0·50
3 h	0·62 ± 0·16*†	0·38 ± 0·12*	0·47 ± 0·10†
6 h	0·50 ± 0·13	0·38 ± 0·03	0·39 ± 0·02
12 h	0·39 ± 0·04	0·34 ± 0·03	0·39 ± 0·06
CD16 expression – mean fluorescence			
0 h	286·00 ± 66·11	286·00 ± 66·11	286·00 ± 66·11
3 h	224·38 ± 54·23‡§	131·90 ± 52·71‡	39·66 ± 20·3§
6 h	200·25 ± 52·43‡§	91·89 ± 44·42‡	19·26 ± 8·97§
12 h	163·63 ± 61·93‡§	57·01 ± 39·22‡	8·65 ± 5·53§

This table demonstrates the summarized flow cytometric data of surface bound IgG and FcγRIII in purified NK cells. Density of surface bound IgG is down-regulated within a few hours subsequently followed by the reduction of FcγRIII (*$P < 0·05$; †$P < 0·05$; ‡$P < 0·001$; §$P < 0·001$; $n = 10$).

to control condition. These *in vitro* results indicated that antiproliferative effects and induction of apoptosis in CD8+ and NK cells were two clearly independent mechanisms. This observation seems to be of particular interest in the setting of allo-immmune response as cytotoxic CD8+ cells are known to be primarily responsible for acute allograft rejection [30,31].

The relevance of our data on NK cell function has to be seen in context to recent reports evidencing that NK cells are vital players in the initiation of transplant-associated coronary artery disease (TACAD) and allo-immune response [32,33]. Maier *et al.* reported evidence that NK cells provide an alternative source of T-cell help and thus contribute to the acute rejection of wild type hearts transplanted into CD28-deficient mice [34]. With their experimental design, Madsen and colleagues extended that the adaptive immune system is not solely responsible for TACAD formation/allograft rejection in an animal transplant model. They demonstrated that NK cells were able to be activated in the absence of self MHC class I molecules on donor endothelium and thus participate in the pathogenesis of TACAD [35].

The investigation leading us to NK cell relevant experiments were initiated by our primary findings that incubation of PBMC with CMVIg lowered content of cell surface bound immunoglobulin on PBMC (data not shown) and purified NK cells. In addition to cell surface bound immunoglobulin the presence of FcγRIII (CD16) is mandatory for NK cell recognition and subsequent ADCC effector functions. Therefore we sought to investigate whether CMVIg has the ability to influence FcγRIII expression on purified NK cells. We found that CMVIg attenuated FcγRIII receptor density on NK cells within a few hours. The relevance of both findings was proven by attenuation of NK cell function in ADCC assays. Moreover, our results corroborate work showing that co-incubation of NK cells with anti-CD16 led to NK cell apoptosis, alteration of NK phenotype (CD56+/CD16^{bright} to CD56+/CD16^{dim}) and decreased NK effector function [36].

In conclusion, our experiments extend the currently accepted CMVIg function as passive virus prophylaxis in allograft recipients. If we interpret our *in vitro* data correctly, CMVIg administration could have a beneficial influence on graft rejection and formation of TACAD. Previous clinical observatory studies have indicated that the application of CMVIg is beneficiary in regard to graft survival and overall mortality [37,38].

Our *in vitro* findings extend the current concept of CMVIg as passive CMV prophylaxis to a therapeutic drug compound capable of reducing allogeneic immune response. In respect to our clinical observation of low incidence in allograft rejection rates and TACAD formation it is tempting to suggest that co-administration of rATG and CMVIg has a synergistic effect in the early post-transplant course.

Acknowledgements

This work was supported by FOLAB Chirurgie (Prof F Muehlbacher, Prof E Roth), and private funding (H.J.A.). K Hoetzenecker and HJ Ankersmit designed the study.

References

1 Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: eighteenth Official Report-2001. *J Heart Lung Transplant* 2001;**20**:805–15.
2 Zuckermann A, Dunkler D, Deviatko E, Bodhjalian A, Czerny M, Ankersmit J *et al.* Long-term survival (> 10 years) of patients > 60 years with induction therapy after cardiac transplantation. *Eur J Cardiothorac Surg* 2003;**24**:283–91; discussion 91.
3 Syeda B, Roedler S, Schukro C, Yahya N, Zuckermann A, Glogar D. Transplant coronary artery disease. Incidence, progression and interventional revascularization. *Int J Cardiol* 2005;**104**:269–74.
4 Kocher AA, Bonaros N, Dunkler D, Ehrlich M, Schlechta B, Zweytick B *et al.* Long-term results of CMV hyperimmune

globulin prophylaxis in 377 heart transplant recipients. *J Heart Lung Transplant* 2003;**22**:250–7.
5 Chiffoleau E, Walsh PT, Turka L. Apoptosis and transplantation tolerance. *Immunol Rev* 2003;**193**:124–45.
6 Ankersmit HJ, Moser B, Zuckermann A, Roth G, Taghavi S, Brunner M *et al*. Activation-induced T cell death, and aberrant T cell activation via TNFR1 and CD95-CD95 ligand pathway in stable cardiac transplant recipients. *Clin Exp Immunol* 2002;**128**:175–80.
7 Woodside KJ, Hu M, Meng T, Hunter GC, Sower LE, Daller JA. Differential effects of interleukin-2 blockade on apoptosis in naive and activated human lymphocytes. *Transplantation* 2003;**75**:1631–5.
8 Woodside KJ, Hu M, Liu Y, Song W, Hunter GC, Daller JA. Apoptosis of allospecifically activated human helper T cells is blocked by calcineurin inhibition. *Transpl Immunol* 2006;**15**:229–34.
9 Rubin RH. Cytomegalovirus disease and allograft loss after organ transplantation. *Clin Infect Dis* 1998;**26**:871–3.
10 Shuster LD, Cox G, Bhatia P, Miner PB Jr. Gastric mucosal nodules due to cytomegalovirus infection. *Dig Dis Sci* 1989;**34**:103–7.
11 Chien JW, Johnson JL. Viral pneumonias. Infection in the immunocompromised host. *Postgrad Med* 2000;**107**:67–70,3–4,7–80.
12 Fishburne BC, Mitrani AA, Davis JL. Cytomegalovirus retinitis after cardiac transplantation. *Am J Ophthalmol* 1998;**125**:104–6.
13 Lemstrom K, Koskinen P, Krogerus L, Daemen M, Bruggeman C, Hayry P. Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection. *Circulation* 1995;**92**:2594–604.
14 Span AH, Grauls G, Bosman F, van Boven CP, Bruggeman CA. Cytomegalovirus infection induces vascular injury in the rat. *Atherosclerosis* 1992;**93**:41–52.
15 Wittes JT, Kelly A, Plante KM. Meta-analysis of CMVIG studies for the prevention and treatment of CMV infection in transplant patients. *Transplant Proc* 1996;**28**:17–24.
16 Glowacki LS, Smaill FM. Use of immune globulin to prevent symptomatic cytomegalovirus disease in transplant recipients – a meta-analysis. *Clin Transplant* 1994;**8**:10–8.
17 Sapir T, Shoenfeld Y. Facing the enigma of immunomodulatory effects of intravenous immunoglobulin. *Clin Rev Allergy Immunol* 2005;**29**:185–99.
18 Kazatchkine MD, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med* 2001;**345**:747–55.
19 Nachbaur D, Herold M, Eibl B, Glassl H, Schwaighofer H, Huber C *et al*. A comparative study of the in vitro immunomodulatory activity of human intact immunoglobulin (7S IVIG), F (ab') 2 fragments (5S IVIG) and Fc fragments. Evidence for post-transcriptional IL-2 modulation. *Immunology* 1997;**90**:212–8.
20 Luke PP, Scantlebury VP, Jordan ML, Vivas CA, Hakala TR, Jain A *et al*. Reversal of steroid- and anti-lymphocyte antibody-resistant rejection using intravenous immunoglobulin (IVIG) in renal transplant recipients. *Transplantation* 2001;**72**:419–22.
21 Luke PP, Scantlebury VP, Jordan ML, Vivas CA, Hakala TR, Jain A *et al*. IVIG rescue therapy in renal transplantation. *Transplant Proc* 2001;**33**:1093–4.
22 Kwekkeboom J, Tha-In T, Tra WM, Hop W, Boor PP, Mancham S *et al*. Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation. *Am J Transplant* 2005;**5**:2393–402.
23 Toungouz M, Denys C, Dupont E. Blockade of proliferation and tumor necrosis factor-alpha production occurring during mixed lymphocyte reaction by interferon-gamma-specific natural antibodies contained in intravenous immunoglobulins. *Transplantation* 1996;**62**:1292–6.
24 Hurez V, Kaveri SV, Mouhoub A, Dietrich G, Mani JC, Klatzmann D *et al*. Anti-CD4 activity of normal human immunoglobulin G for therapeutic use. (Intravenous immunoglobulin, IVIg). *Ther Immunol* 1994;**1**:269–77.
25 Vuist WM, Van Schaik IN, Van Lint M, Brand A. The growth arresting effect of human immunoglobulin for intravenous use is mediated by antibodies recognizing membrane glycolipids. *J Clin Immunol* 1997;**17**:301–10.
26 Jordan SC, Tyan D, Czer L, Toyoda M. Immunomodulatory actions of intravenous immunoglobulin (IVIG): potential applications in solid organ transplant recipients. *Pediatr Transplant* 1998;**2**:92–105.
27 Toyoda M, Zhang XM, Petrosian A, Wachs K, Moudgil A, Jordan SC. Inhibition of allospecific responses in the mixed lymphocyte reaction by pooled human gamma-globulin. *Transpl Immunol* 1994;**2**:337–41.
28 Toyoda M, Pao A, Petrosian A, Jordan SC. Pooled human gammaglobulin modulates surface molecule expression and induces apoptosis in human B cells. *Am J Transplant* 2003;**3**:156–66.
29 Koffman BM, Dalakas MC. Effect of high-dose intravenous immunoglobulin on serum chemistry, hematology, and lymphocyte subpopulations: assessments based on controlled treatment trials in patients with neurological diseases. *Muscle Nerve* 1997;**20**:1102–7.
30 Frisman DM, Fallon JT, Hurwitz AA, Dec WG, Kurnick JT. Cytotoxic activity of graft-infiltrating lymphocytes correlates with cellular rejection in cardiac transplant patients. *Hum Immunol* 1991;**32**:241–5.
31 Vaessen LM, Baan CC, Ouwehand AJ, Jutte NH, Balk AH, Mochtar B *et al*. Acute rejection in heart transplant patients is associated with the presence of committed donor-specific cytotoxic lymphocytes in the graft but not in the blood. *Clin Exp Immunol* 1992;**88**:213–9.
32 Kitchens WH, Uehara S, Chase CM, Colvin RB, Russell PS, Madsen JC. The changing role of natural killer cells in solid organ rejection and tolerance. *Transplantation* 2006;**81**:811–7.
33 McNerney ME, Lee KM, Zhou P, Molinero L, Mashayekhi M, Guzior D *et al*. Role of natural killer cell subsets in cardiac allograft rejection. *Am J Transplant* 2006;**6**:505–13.
34 Maier S, Tertilt C, Chambron N, Gerauer K, Huser N, Heidecke CD *et al*. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28–/– mice. *Nat Med* 2001;**7**:557–62.
35 Uehara S, Chase CM, Kitchens WH, Rose HS, Colvin RB, Russell PS *et al*. NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts. *J Immunol* 2005;**175**:3424–30.
36 Warren HS, Kinnear BF. Quantitative analysis of the effect of CD16 ligation on human NK cell proliferation. *J Immunol* 1999;**162**:735–42.
37 Potena L, Holweg CT, Chin C, Luikart H, Weisshaar D, Narasimhan B *et al*. Acute rejection and cardiac allograft vascular disease is reduced by suppression of subclinical cytomegalovirus infection. *Transplantation* 2006;**82**:398–405.
38 Valantine HA, Luikart H, Doyle R, Theodore J, Hunt S, Oyer P *et al*. Impact of cytomegalovirus hyperimmune globulin on outcome after cardiothoracic transplantation: a comparative study of combined prophylaxis with CMV hyperimmune globulin plus ganciclovir versus ganciclovir alone. *Transplantation* 2001;**72**:1647–52.

Curiculum vitae

Name	Konrad Hoetzenecker, cand. med.
Born	19th September, 1980, Linz, Austria
Parents	Dipl.Ing. Klaus Hoetzenecker Dr. Brigitte Hoetzenecker, maiden name: Weigand
Siblings	DDr. Wolfram Hoetzenecker Katharina Hoetzenecker
Citizen	Austrian
Home address	Neulerchenfelderstrasse 60/1/4 1160 Vienna Austria/Europe
email	konrad.hoetzenecker@meduniwien.ac.at
Education	graduated 1998 at the Bundesrealgymnasium Aufhof with distinction Anton Bruckner University for music, drama, and dance October 1998 – May 2002 Teaching certificate for classical piano 5/2002 Medical University of Vienna Since 10/2003
Postgraduate training	Austrotransplant 2005, Alpbach, Austria Österreichischer Chirurgenkongress 2006, Wien, Austria Austrotransplant 10/2006, Hof, Austria Kardiovaskuläre Forschungstage 2007, Weissensee, Austria ISHLT, 27th Annual Meeting 2007, San Francisco, USA Medical University of Vienna, Core Unit for Medical Statistics and Informatics, Section of Clinical Biometrics Biometrie I: Beschreibung und Visualisierung medizinischer Daten Biometrie II: Statistische Tests und Lebensdaueranalyse bei medizinischen Fragestellungen Österreichischer Chirurgenkongress 2007, Graz, Austria Austrotransplant 2007, St.Wolfgang, Austria ÖGAI 2007, Alpbach, Austria
Membership and Organizations	Austrian Transplant Society International Society for Heart & Lung Transplantation Austrian Society of Allergology and Immunology
Grants	Stipend of the Medical University of Vienna for outstanding study performance 2003/04 Stipend of the Medical University of Vienna for outstanding study performance 2004/05 Stipend of the Medical University of Vienna for the CMV hyperimmunoglobuline study 2006 Stipend of the Medical University of Vienna for outstanding study performance 2005/06

Original papers:

Roth GA, Moser B, Krenn C, Roth-Walter F, Hetz H, Richter S, Brunner M, Jensen-Jarolim E, Wolner E, Hoetzenecker K, Boltz-Nitulescu G, Ankersmit HJ.
Heightened levels of circulating 20S proteasome in critically ill patients.
Eur J Clin Invest. 2005 Jun;35(6):399-403.

Szerafin T, Brunner M, Horvath A, Szentgyorgyi L, Moser B, Boltz-Nitulescu G, Peterffy A, Hoetzenecker K, Steinlechner B, Wolner E, Ankersmit HJ.
Soluble ST2 protein in cardiac surgery: a possible negative feedback loop to prevent uncontrolled inflammatory reactions.
Clin Lab. 2005;51(11-12):657-63.

Ankersmit HJ, Ullrich R, Moser B, Hoetzenecker K, Hacker S, German P, Krenn C, Horvat R, Grimm M, Wolner E, Zuckermann A.
Recovery from giant cell myocarditis with ECMO support and utilisation of polyclonal antithymocyte globulin: a case report.
Thorac Cardiovasc Surg. 2006 Jun;54(4):278-80.

Szerafin T, Horvath A, Moser B, Hacker S, Hoetzenecker K, Steinlechner B, Brunner M, Roth G, Boltz-Nitulescu G, Peterffy A, Wolner E, Ankersmit HJ.
Apoptosis-specific activation markers in on- versus off-pump coronary artery bypass graft (CABG) patients.
Clin Lab. 2006;52(5-6):255-61.

Ankersmit HJ, Hacker S, Hoetzenecker K, Moser B, Wolner E.
The impact of hypogammagammaglobulinemia on infection outcome in patients undergoing ventricular assist device implantation.
J Heart Lung Transplant. 2006 Nov;25(11):1387-8.

Adlbrecht C*, Hoetzenecker K*, Posch M, Steiner S, Kopp C, Hacker S, Auer J, Horvath R, Moser B, Roth G, Wolner E, Lang IM, Ankersmit HJ.
Elevated levels of interleukin-1beta-converting enzyme and caspase-cleaved cytokeratin-18 in acute myocardial infarction.
Eur J Clin Invest. 2007 May;37(5):372-80.

*both authors contributed equally – shared first authorship

Hetz H, Hoetzenecker K, Hacker S, Faybik P, Pollreisz A, Moser B, Roth G, Hoetzenecker W, Lichtenauer M, Klinger M, Krenn CG, Ankersmit HJ.
Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration.
J Clin Lab Anal. 2007;21(5):277-81.

Hoetzenecker K, Hacker S, Hoetzenecker W, Sadeghi K, Sachet M, Pollreisz A, Mangold A, Wliszczak T, Bielek E, Muehlbacher F, Klepetko W, Ankersmit HJ.
Cytomegalovirus hyperimmunoglobulin: Mechanisms in Allo-Immune Response in vitro.
Eur J Clin Invest. 2007 Dec;37(12):978-86.

Hoetzenecker W, Zipfel M, Hoetzenecker K, Röcken M.
Vasculitic leg ulcers in a patient with mixed myelodysplastic and myeloproliferative syndrome.
JEADV 2007. Jun. accepted for publication; ahead of print

Szerafin T*, Hoetzenecker K*, Hacker S, Horvath A, Pollreisz A, Peterffy A, Mangold A, Wliszczak T, Moser B, Seitelberger R, Wolner E, Ankersmit HJ.
Heat Shock Proteins (HSP27/60/70/90α) and 20S Proteasome in On- versus Off-Pump Coronary Artery Bypass Graft (CABG) Patients.
Ann Thorac Surg. 2007. Jul. accepted for publication; ahead of print

*both authors contributed equally – shared first authorship

Abstracts:

Ankermit HJ, Moser B, Hoetzenecker K, Steinlechner B, German P, Krenn C, Horvat R, Wieselthaler G, Grimm M, Wolner E, Zuckermann A.
Recovery Of Giant Cell Myocarditis By Means Of ECMO Support And Utilization Of Polyclonal Antithymocyte Globuline: A Case Report.
***Acta Chirurgica Austriaca**, 2005, Alpbach, Austria.*

Hoetzenecker K, Hacker S, Hoetzenecker W, Pollreisz A, Bohle B, Roth G, Moser B, Krenn C, Grimm M, Bolz-Nituescu G, Klepetko W, Zuckermann A, Wolner E, Ankersmit HJ.
Pooled human IgG and IgM has immunesuppressive properties in vitro and is partly triggered by Fc-blockade.
***Acta Chirurgica Austriaca**, 2005, Alpbach, Austria.*

Hoetzenecker K, Hacker S, Hoetzenecker W, Sadeghi K, Pollreisz A, Mangold A, Moser B, Grimm M, Zuckermann A, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
CMV hyperimmunoglobulin evidence anti-proliferative activity in vitro triggered by the induction of apoptosis: Possible role in tolerance induction in allograft recipients.
***Acta Chirurgica Austriaca**, 2006, Hof, Austria.*

Hoetzenecker K, Hacker S, Hoetzenecker W, Sadeghi K, Pollreisz A, Mangold A, Moser B, Grimm M, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
Anti-proliferative properties of CMV hyperimmunoglobulin are related to activation induved cell death in vitro: possible role in tolerance induction.
***J Heart Lung Transplant**. 2007 Feb;26(2S):195*

Hoetzenecker K, Szerafin T, Hacker S, Pollreisz A, Mangold A, Wliszczak T, Moser B, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
Heat shock proteins 27/60/70/90α and 20S proteasome in on- versus off-pump coronary artery bypass graft patients.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 215, 2007

Hoetzenecker K, Hacker S, Hoetzenecker W, Sachet M, Sadeghi K, Pollreisz A, Mangold A, Wliszczak T, Moser B, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
CMV Hyperimmunoglobulin evidences anti-proliferative porperties and reduves natural occuring cell mediated cytotoxicity in vitro.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 215, 2007

Ankersmit HJ, Hacker S, Hoetzenecker K, Klepetko W, Wolner E, Wollenek G, Grimm M.
Gigantic coronary fistula: rare finding without clinical symptom.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 215, 2007

Hacker S, Soeiman A, Lukschal A, Hoetzenecker K, Horvat R, Ankersmit HJ.
Degenerative cardiac pigment lipofuscin contains cytokieratin-18 and caspase-cleaved cytokeratin 18.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 218, 2007

Hoetzenecker K, Hacker S, Hoetzenecker W, Sachet M, Klepetko W, Ankersmit HJ.
CMV hyperimmunoglobulin: mechanisms in allo-immune response in vitro.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 218, 2007

Mangold A, Hoetzenecker K, Hacker S, Szerafin T, Auer J, Ankersmit HJ.
Alpha-Gal specific humoral immune response after implantation of bioprotheses in cardiac surgery.
European Surgery, Acta Chirurgica Austriaca, Volume 39, Suppl. 218, 2007

Presentations:

Austrotransplant 2005, Alpbach. Case report: giant cell myocarditis - ECMO and rATG. (oral presentation)

Austrotransplant 2005, Alpbach. Pooled human Ig and Immunosuppression. (oral presentation)

Posterausstellung anlässlich der vfwf-Universitätsvorlesung "Die Medizinische Universität der Zukunft" 2006, Wien. Caspase-cleaved cytokeratin 18 and 20S proteasome in liver degeneration. (poster presentation)

Posterausstellung anlässlich der vfwf-Universitätsvorlesung "Die Medizinische Universität der Zukunft" 2006, Wien. Case report: rabbit antithymocyte globulin (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis. (poster presentation)

Österreichischer Chirurgenkongress 2006, Wien. Case report: rabbit antithymocyte globulin (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis. (poster presentation)

Austrotransplant 2006, Hof. Cytotect (CMV hyperimmunoglobuline) – passive immunisation, active immune suppression (oral presentation)

Kardiovaskuläre Forschungstage 2007, Weissensee. Elevated levels of interleukin-1β-converting enzyme and caspase-cleaved cytokeratin-18 (ccCK-18) in acute myocardial infarction (oral presentation)

International Society for Heart and Lung Transplantation 27th Annual Meeting 2007, San Francisco. Anti-proliferative properties of CMV hyperimmunoglobulin are related to activation induced cell death in vitro: Possible role in tolerance induction (poster presentation, mini oral presentation)

Morning lectures of the Dept of Cardiothoracic Surgery, Medical University of Vienna 2007. CMV hyperimmunglobulin - Role In Allo-Immune Response (oral presentation)

Österreichischer Chirurgenkongress 2007, Graz. Gigantic coronary fistula: rare finding without clinical symptom (poster presentation)

Österreichischer Chirurgenkongress 2007, Graz. Heat shock proteins 27/60/70/90α and 20S proteasome in on- versus off-pump coronary artery bypass graft patients (oral presentation)

Österreichischer Chirurgenkongress 2007, Graz. CMV hyperimmunoglobulin evidences anti-proliferative porperties and reduves natural occuring cell mediated cytotoxicity in vitro (oral presentation)

Austrotransplant 2007, St. Wolfgang. CMV hyperimmunoglobulin influence NK cell viability and function in vitro (poster presentation)

Austrotransplant 2007, St. Wolfgang. CMV hyperimmunglobulin role in allo-immune response (invited lecture)

Studies approved by the institutional review board of the Clinical Research Ethics Committee of the Medical University of Vienna:

Search for apoptosis specific activation marker and TH2 cytokine profile as surrogate markers for bronchiolitis Obliterans (BO) syndrome: implications for treatment and early detection of BO in lung transplant recipients (Klepetko W, Pollreisz A, Hoetzenecker K, Brunner M, Roth G, Ankersmit HJ) – 2004

Investigation of T-Cell Response to Industrial Glutaraldehyde Fixed Porcine Valves: The Role of Specific Immune System in Degeneration During Adult Aortic Valve Replacement Therapy (Hoetzenecker K, Pollreisz A, Ankersmit HJ) – 2004

Investigation of T Lymphocytes in COPD Patients and Healthy Smokers: Role of CD4+CD28null Cells in the Pathogenesis of COPD (Lambers C, Hacker S, Hoetzenecker K, Pollreisz A, Ankersmit HJ) – 2006

In Vitro Investigation of Immunosuppressive Effects of CVM hyperimmunoglobulin (Hoetzenecker K, Hacker S, Ankersmit HJ) – 2006

Investigation of T Lymphocytes and Cytokines in Cystic Fibrosis Patients: Search for Specific Diagnostic Markers (Jaksch P, Klepetko W, Pollreisz A, Hacker S, Hoetzenecker K, Ankersmit HJ) – 2006

Endothelial Activation and Soluble Death Receptors in Coronary Artery Bypass Surgery: Cardiopulmonary Bypass versus "Off-pump" techniques (Hacker S, Hoetzenecker K, Pollreisz A, Ankersmit HJ) – 2006

Studies approved by the Animal Experiment Committee according to the animal experiment ordinance of the Austrian Federal Ministry for Education, Science and Culture:

Immunomodulation after MI – investigated in a rat model (Podesser BK, Hoetzenecker K, Ankersmit HJ) – 2006

Teaching:

VO AHA Guidelines in der Cardiovaskulären Chirurgie 2007 – invited lecture
VO+SE Vom Textbuch der Herzchirurgie zur PowerPoint-Präsentation 2007 – invited lecture

Clincal Investigations:

K Hoetzenecker coordinated the Viennese Site (Dept. of Cardiothoracic Surgery – Prof. Seitelberger) of a clinical investigation **"Multi-center, double-blind, randomized, placebo-controlled, parallel-group study to assess the efficiacy, safety and tolerability of tezosentan in patients with pre-operative pulmonary hypertension, due to left heart disease, undergoing cardiac surgery"** assigned by Actelion – 2007.

Review activity:

American Journal of Transplantation