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. IVlg, also consisting of pooled immunoglobulins, are a drug class related to CMVIg. Immunomodulatory effects of IVlg 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, Cytooglobin 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 IVlg. Several mechanisms being responsible for the anti-proliferative effects of IVlg have been suggested. Nachbaur et al. showed that IVlg 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 IVlg preparations was suggested by other investigators [87, 121]. Vuist et al. demonstrated that IVlg contain antibodies against glycolipids expressed on lymphocytes leading to an IVlg-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 IVlg. 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 CMVlg on the FcγR system of NK cells. Two different observations were made:

1. **Discharging of membrane bound IgG.** Within the first three hours of CMVlg-incubation, density of membrane bound IgG proteins was massively reduced on NK cells. Furthermore, vacant FcγR were not filled up with IgG from CMVlg 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. **CMVlg 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 CMVlg. The ablation of FcγRIII could be seen mainly between 6 and 12 hours after CMVlg incubation. Therefore, the retraction of “oven” receptors seems to be a direct consequence of the losage of receptor bound IgG.

The effects of CMVlg on NK cells led to the hypothesis that CMVlg 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 CMVlg 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+/CD16bright to CD56+/CD16dim) 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 CMV Ig 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 CMV Ig 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 CMV Ig 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 CMV Ig 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 CMV Ig in the early post-transplant course has a beneficial effect on graft survival.
REFERENCES


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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
CMVlg  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
FcyR  Fcy-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
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
<table>
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<th>Abbreviation</th>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>R</td>
<td>Recipient</td>
</tr>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>TACAD</td>
<td>Transplant-associated coronary artery disease</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory cells</td>
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<tr>
<td>VZV</td>
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APPENDIX

Publication

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Cytomegalovirus hyperimmunoglobulin: mechanisms in allo-immune response in vitro


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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, interferon (IFN)-γ, 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 FcRRIII 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.

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 antithymocyte 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 'energy' in allograft recipients treated with poly or monospecific antibodies (rATG, OKT3, interleukin-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...
on postoperative days 1, 7, 14, 21 and 28 irrespective of the preoperative donorrecipient 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. CMVinduced morbidities in transplant recipients include gastrointestinal tract infection, lung infections, chorioretinitis and hepatitis-like syndrome [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 CMV Ig 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 CMV Ig is highly effective in preventing CMV disease [15,16]. CMV Ig 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, polyarteritis nodosa, Guillain-Barré syndrome and systemic lupus erythematosus. The modes of action of IVIg are complex and still the topic of ongoing research. Theories range from immunomodulation due to attenuation of complement-mediated tissue damage, down-regulation of B-cell response to Fcgβ receptors and blockade of FcRn-mediated catabolism [18]. Concerning allograft transplantation early reports found evidence that IVIg is able to decrease proliferation in allograft mixed lymphocyte reactions [19]. These in vitro experiments were recently complemented by the in vivo observation that IVIg are highly effective in treating steroid resistant allograft rejection [20,21].

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

Materials and methods
CMV Ig compounds and sample size
Two different CMV Ig preparations were used for experimental studies (Cytogen, kindly provided by Dr Schott, Bietest, 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). CMV Ig was added to all experiments in therapeutic concentrations as described elsewhere [22]. In control experiments CMV Ig was replaced by medium. Dialysis of CMV Ig 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).

[3H]-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 2.2% gentamicin sulphate (Sigma Chemical Co, St. Louis, MO, USA), 0.5% β-mercaptoethanol (Sigma, St Louis, MO, USA), 1% L-glutamin (Sigma, St Louis, MO, USA) and 150 μl medium containing 1 x 10⁶ 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 CMV Ig and then pulsed for 18 h with [3H]-thymidine (3.7 x 10⁵ Bq/well; Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were harvested and [3H]-thymidine incorporation was measured in a liquid scintillation counter.

Measuring of cell division
PBMC (1 x 10⁶) were labelled with 5 μM Carboxyfluorescin diacetate succinimidyl ester (CFSE, Prolia Biochromika, 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 x 10⁶) 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 immunosorbent 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 x 10^7) of purified subsets (CD4+ T-cells, CD8+ T-cells, B cells, NK cells) were incubated in a humidified atmosphere. Induction of apoptosis was measured by AnnexinV-fluorescine/propidium iodide (FITC/PE) 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 x 10^7) were incubated with or without CMV Ig in a humidified atmosphere 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 (OMU; Ultratome V), stained with uranyl acetate lead citrate and examined on a Jeol 1200 EXII electron microscope.

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

Purified NK cells (3 x 10^7) 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, Logan, UT, USA) and 0.2% gentamicin sulphate (Sigma Chemical Co) in a 5% CO2 humidified atmosphere at 37 °C.

Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC was measured using a europium release assay. In vivo 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 μg mL^-1^-1 of CMV Ig, 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 florescence (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 significance. 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

[1H]-thymidine incorporation is decreased in mixed lymphocyte reactions (MLR) and anti-CD3 blastogenesis assay after co-incubation with CMV Ig

To test the hypothesis whether CMV Ig 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 CMV Ig significantly decreases proliferation rates in a dose-dependent manner.

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

To verify anti-proliferative effects CFSE dilution experiments and cell cycle analysis of anti-CD3 stimulated PBMC
CMV Ig in allo-immune response

Figure 1. (a) [H]-thymidine incorporation is decreased by co-incubation with Cytomegalovirus hyperimmunoglobulin (CMV Ig). This figure shows the effect of CMV Ig on [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 CMV Ig were compared to control experiments and are given as percentages of the maximal control proliferation (*P < 0.05; **P < 0.01; ***P < 0.001; n = 10). (b) Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assays confirm an antiproliferative effects of CMV Ig. A representative CFSE dilution experiment is depicted in this figure. The control setting demonstrates a distinct CFSE dilution whereas the addition of CMV Ig 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 CMV Ig. (P < 0.001, n = 10). (c) Release of interferon (IFNγ); interleukin (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 < 0.05; **P < 0.01; ***P < 0.001; n = 10).

Table 1: Cell cycle analysis evidence antiproliferative effects of CMVlg

<table>
<thead>
<tr>
<th></th>
<th>% G0-G1</th>
<th>% S</th>
<th>% G2-M</th>
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<tr>
<td>Control</td>
<td>65 ± 3</td>
<td>23 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Cytotoxic 2.5 mg/mL&lt;sup&gt;1&lt;/sup&gt;</td>
<td>93 ± 8</td>
<td>14 ± 0.3</td>
<td>4 ± 0.1</td>
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<tr>
<td>Cytoglobin 2.5 mg/mL&lt;sup&gt;1&lt;/sup&gt;</td>
<td>95 ± 6</td>
<td>3 ± 1</td>
<td>4 ± 0.1</td>
</tr>
</tbody>
</table>

This table shows results of cell cycle analysis performed in T-cell triggered PBMCs. Co-incubation with CMVlg results in a massive decrease of cells residing in S and G2/M-phase. These data confirm CMVlg dilution experiments (*P < 0.05; †P < 0.05; n = 10).

CMVlg was performed. Figure 1(b) shows a representative CFSE dilution experiment indicating that CMVlg 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 ± 1 ± 1.7 (Cytotoxic; P < 0.001) and 5 ± 1 ± 0.2 (Cytoglobin; P < 0.001). These findings were also confirmed by cell cycle analysis. Co-incubation of CMVlg significantly reduced the 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 CMVlg in anti-CD3 blastogenesis assays

To investigate whether cytokine profile is altered by CMVlg 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 CMVlg.

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

The effect of CMVlg on unstimulated PBMC was evaluated by Annexin-V/PI staining on a flow cytometer. Co-incubation of PBMC with CMVlg 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. CMVlg 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 CMVlg-induced apoptosis

To substantiate which subpopulations of PBMC are prone to undergoing apoptosis after incubation with CMVlg we exposed highly purified CD4+<sup>1</sup>, CD8+<sup>1</sup>, CD10+<sup>1</sup> and CD19+<sup>1</sup> cells to therapeutic CMVlg concentrations. CD4+<sup>1</sup> and CD19+<sup>1</sup> cells are hardly sensitive for induction of apoptosis as compared to cytotoxic CD8+<sup>1</sup> cells and NK cells (Fig. 2c,d).

CMVlg 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 CMVlg significantly reduces cell surface bound IgG and density of FcγRIII in purified NK cells. Data of 10 probands were summarized in Table 2. Both features, namely CD16 and cell surface bound immunoglobulin, are invariable to the performance of NK cell mediated ADCC.

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

Finally, based on the impact of CMVlg 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 CMVlg (Fig. 3b).

Discussion

Our findings represent the first demonstration that CMVlg 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 CMVlg preincubated PBMC and NK cells were decreased in ADCC assays. We deduce from our in vitro experiments that CMVlg has the potential to induce two immunological features that are known mechanisms for alllograft tolerance induction: (a) attenuation of allogeneic and T-cell receptor induced
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 CMVlg has similar effects in suppressing MLR as those published for IVlg. In these studies several investigators suggested responsible mechanisms for this in vivo observation. Nachbaur et al. showed that IVlg inhibited IL-2 protein synthesis with no effect on IL-2 mRNA expression [19]. Blockade of IFNγ or CD4 by antibodies found in IVlg preparations was suggested by other investigators [23,24]. Vuist et al. demonstrated that IVlg contain antibodies against glycolipids expressed on lymphocytes leading to the IVlg-induced growth attenuation [23]. 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 IVlg, which are directly involved in the alloantigen recognition process in the MLR. Moreover, IVlg was shown to also induce apoptosis in the setting of MLR, primarily in
Figure 7: (A) (a) FACScan histograms showing the decrease in FCM of NK and PBMC in PANC-1 cells. (B) FACScan histograms showing the decrease in FCM of NK and PBMC in Jurkat cells. A significant upregulation of NK cell surface molecules observed in the course of G1. FCM and PBMC in PANC-1 cells show a decrease in the antibody-dependent cytotoxicity against NK cells, as compared to NK cells from PBMC. The decrease in NK cell function in PANC-1 cells is significantly greater than in PBMC. (C) (a) FACScan histograms showing the decrease in FCM of NK and PBMC in PANC-1 cells. (B) FACScan histograms showing the decrease in FCM of NK and PBMC in Jurkat cells. A significant upregulation of NK cell surface molecules observed in the course of G1. FCM and PBMC in PANC-1 cells show a decrease in the antibody-dependent cytotoxicity against NK cells, as compared to NK cells from PBMC. The decrease in NK cell function in PANC-1 cells is significantly greater than in PBMC.
Table 2: CMV Ig reduces density of FcγRIII and cell surface bound IgG on NK cells

<table>
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<tr>
<th>Cell bound IgG and FcγRIII on purified NK cells are down-regulated by CMV Ig</th>
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<th>Cytoglobin 2.5 mg mL⁻¹</th>
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<tr>
<td>0 h</td>
<td>1.32 ± 0.50</td>
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<tr>
<td>3 h</td>
<td>0.38 ± 0.12</td>
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<td>12 h</td>
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<tr>
<td>Cytotoxic expression - mean fluorescence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>286.00 ± 66.11</td>
<td>286.00 ± 66.11</td>
</tr>
<tr>
<td>3 h</td>
<td>224.38 ± 54.23</td>
<td>224.38 ± 54.23</td>
</tr>
<tr>
<td>6 h</td>
<td>209.25 ± 52.43</td>
<td>209.25 ± 52.43</td>
</tr>
<tr>
<td>12 h</td>
<td>163.63 ± 61.93</td>
<td>163.63 ± 61.93</td>
</tr>
</tbody>
</table>

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, following the reduction of FcγRIII. *P < 0.05; **P < 0.01; ***P < 0.001; n = 10.

This observation seems to be of particular interest in the setting of allogeneic 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 viral players in the initiation of transplant-associated coronary artery disease (TACAD) and allogeneic 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/alloantigen 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 to an NK cell relevant experiments were initiated by our primary findings that incubation of PBMC with CMV Ig lowered content of cell surface bound immunoglobulin on PBMC (data not shown) and purified NK cells. In addition, 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 CMV Ig has the ability to influence FcγRIII expression on purified NK cells. We found that CMV Ig 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+CD16neg to CD56+CD16dim) and decreased NK effector function [36].

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

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References


globulin prophylaxis in 377 heart transplant recipients.


# Curriculum vitae

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**Education**
gr graduated 1998 at the Bundesrealgymnasium Aufhof with distinction
Anton Bruckner University for music, drama, and dance
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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 hyperimmunglobuline study 2006
Stipend of the Medical University of Vienna for outstanding study performance 2005/06
Original papers:


**Heightened levels of circulating 20S proteasome in critically ill patients.**


**Soluble ST2 protein in cardiac surgery: a possible negative feedback loop to prevent uncontrolled inflammatory reactions.**


**Recovery from giant cell myocarditis with ECMO support and utilisation of polyclonal antithymocyte globulin: a case report.**


**Apoptosis-specific activation markers in on- versus off-pump coronary artery bypass graft (CABG) patients.**

Ankersmit HJ, Hacker S, Hoetzenecker K, Moser B, Wolner E.

**The impact of hypogammagammaglobulinemia on infection outcome in patients undergoing ventricular assist device implantation.**


**Elevated levels of interleukin-1beta-converting enzyme and caspase-cleaved cytokeratin-18 in acute myocardial infarction.**

*both authors contributed equally – shared first authorship


**Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration.**


**Cytomegalovirus hyperimmunoglobulin: Mechanisms in Allo-Immune Response in vitro.**

*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


**Heat Shock Proteins (HSP27/60/70/90a) and 20S Proteasome in On- versus Off-Pump Coronary Artery Bypass Graft (CABG) Patients.**

*both authors contributed equally – shared first authorship
Abstracts:


Pooled human IgG and IgM has immunesuppressive properties in vitro and is partly triggered by Fc-blockade. 

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.

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

Heat shock proteins 27/60/70/90α and 20S proteasome in on- versus off-pump coronary artery bypass graft patients. 

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Gigantic coronary fistula: rare finding without clinical symptom. 

Degenerative cardiac pigment lipofuscin contains cytokeratin-18 and caspase-cleaved cytokeratin 18. 

Hoetzenecker K, Hacker S, Hoetzenecker W, Sachet M, Klepetko W, Ankersmit HJ. 

Mangold A, Hoetzenecker K, Hacker S, Szerafin T, Auer J, Ankersmit HJ. 
Alpha-Gal specific humoral immune response after implantation of biopropheses in cardiac surgery. 
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. 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. Cytotec (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 properties and reduces 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

In Vitro Investigation of Immunosuppressive Effects of CVM hyperimmunoglobulin (Hoetzenecker K, Hacker S, 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

Clinical 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 efficacy, 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