Immune responsiveness in immunosuppressed patients

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CHAPTER 7: IMMUNE RESPONSIVENESS IN IMMUNOSUPPRESSED RENAL TRANSPLANT RECIPIENTS

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submitted for publication
Abstract

Background: Current immunosuppressive drug treatment of renal transplant recipients result in high 1 year graft survival rates. Despite adequate suppression of the immune response directed to the allograft, the immune system frequently remains able to mount immune reactivity against infectious agents.

Methods: To define the influence of distinct immunosuppressive treatment protocols, primary and secondary cellular and humoral immune responses in groups of renal transplant recipients were studied: patient treated with prednisolone and cyclosporin A (P/CsA); with IgA CD3 monoclonal antibody as a rejection treatment superimposed on prednisolone and cyclosporin A, (IgA CD3 mAb+P/CsA); and with prednisolone, cyclosporin A and mycophenolate mofetil (P/CsA/MMF).

Results: Primary in vitro proliferative responses to the protein antigen keyhole limpet haemocyanin (KLH) were not significantly disturbed in P/CsA treated patients, nor in IgA CD3 mAb+P/CsA and P/CsA/MMF treated patients. In vitro proliferative responses to the recall antigen tetanus toxoid (TT) were similarly unaffected. Antigen specific antibody responses to immunization with KLH and TT were not affected by treatment with P/CsA, nor by IgA CD3 mAb+P/CsA, but severely disturbed in patients treated with P/CsA/MMF. All patients displayed a profound inhibition of the delayed type hypersensitivity skin reactivity to KLH and recall antigens. Nevertheless, in most patients with P/CsA treatment, T cell infiltrates were observed in skin biopsies from the site of KLH challenge, while expression of intercellular cell adhesion molecule-1 (ICAM-1) expression in challenged skin was significantly decreased in these patients. The balance between T helper 1 and T helper 2 cells was unaffected by immunosuppressive treatments during 1 year of follow up.

Conclusions: Immunosuppressive drug treatment with P/CsA inhibits delayed type hypersensitivity skin reactions to both primary and frequently encountered antigens. Histological studies indicate an effect on ICAM-1 expression, leaving the influx of CD3pos T cells unaffected. Administration of a 10 day course of IgA CD3 mAb does not add profound immunosuppressive effects on the measured parameters. In contrast, addition of treatment with MMF profoundly decreases both primary and secondary humoral immune responsiveness in vivo. Finally, no effect of the currently studied immunosuppressive drugs on Th1/Th2 balance in vivo was measured.
Introduction

We recently demonstrated that a substantial proportion of renal transplant recipients, receiving double immunosuppressive therapy consisting of prednisolone and cyclosporin A, mount readily detectable T helper cell responses to newly acquired human cytomegalovirus (hCMV)(1). Moreover, both the anti hCMV antibody response and the hCMV-specific CD8 responses rapidly followed the T helper cell response (Gamadia, unpublished). Recently, we found that protection against hCMV infections in immunosuppressed renal transplant recipients is achieved at the expense of increased frequencies of activated, cytotoxic effector cells (Gamadia et al, submitted and (2)). Thus, immunosuppressive drug treatment that is often sufficient to protect renal transplant recipients from rejection of the allograft, allows for effective cellular and humoral immune responses against infections.

In a previous study, the influence of cyclosporin A monotherapy on primary and secondary cellular and humoral immune responses was investigated in renal transplant recipients(3;4). Nowadays, the immunosuppressive drug regimen administered to renal transplant recipients consists of two and more frequently of three drugs, leading to better graft survival at the expense of more profound immunosuppression. In vitro, glucocorticoids affect Th1 polarizing properties of dendritic cells(5;6). The effect of glucocorticoids on human T cells are complex and depend on, amongst others the differentiation state of the T cells(7).

We evaluated the cellular and humoral immune responses in three groups of renal transplant recipients, with different immunosuppressive protocols. In renal transplant recipients and healthy control individuals, immunization protocols were performed and the effects of treatment consisting of prednisolone and cyclosporin A (P/CsA), IgA CD3 mAb as a rejection treatment in addition to prednisolone and cyclosporin A (IgA CD3 mAb+P/CsA) as well as prednisolone, cyclosporin A and mycophenolate mofetil (P/CsA/MMF) on primary and secondary, cellular and humoral immune responses were assessed in vivo as well as in vitro. In addition, after short term ex vivo stimulation, the frequencies of Th1 and Th2 cells in peripheral blood from these patients were evaluated.
Materials and Methods

Subjects
Twenty-one renal transplant recipients on basic immunosuppressive therapy consisting of prednisolone 10 mg per day orally and cyclosporin A microemulsions (Neoral®) orally in dosage scheme adjusted to blood trough levels of about 150 ng/mL (P/CsA-group) were included into this study (median age at entry: 43 (range: 21-71), female/male ratio: 10/11). In addition, six renal transplant recipients receiving a 10 day course of CD3 directed monoclonal antibody of mouse IgA subclass as a rejection treatment for biopsy proven acute rejection episodes were investigated (IgA CD3 mAb+P/CsA-group) (median age at entry: 47 (range:18-53), female/male ratio 2/4)(8). IgA CD3 mAb was administered as 5mg/day i.v. (bolus injection) preceded by promethazine 50 mg orally. On the first day of IgA CD3 mAb treatment, the patients received 500 mg of methyl prednisolone i.v. prior to IgA CD3 mAb administration. Moreover, seven renal transplant recipients on immunosuppressive drug therapy consisting of prednisolone, cyclosporin A, and mycophenolate mofetil entered this study (P/CsA/MMF-group, median age at entry: 50 (range:30-59) female/male ratio 1/6). In these patients, mycophenolate mofetil was given in a dose of 1 gram b.i.d. orally. Time of entry into the study is the day of transplantation for the P/CsA-group and the first day of rejection treatment for the IgA CD3 mAb+P/CsA-group, and at least three months after initiation of MMF administration for the P/CsA/MMF group. All blood samples were drawn just before intake of immunosuppressive drug medication. Finally, 10 voluntary healthy control individuals were included into this study (CI, median age at entry 47.5 (range: 38-57) female/male ratio 6/4). All patients and healthy control individuals consented with the study and the study was approved by the local medical ethical committee.

Reagents
Keyhole limpet haemocyanin was prepared as described(9). Tetanus Toxoid for intramuscular injection (containing at least 40 International Units (WHO)) was obtained from Pasteur Merieux MSD (Brussels, Belgium). For culture experiments, tetanus toxoid from the RIVM (Bilthoven, The Netherlands) was used in a final concentration of 19 limits of flocculation (Lf)/mL.

Immunizations, blood sampling, skin tests and skin biopsies
One mg of keyhole limpet haemocyanin was administered subcutaneously in the right arm. In the left arm, 1 mL of tetanus toxoid was administered, in the deltoid muscle. Before as well as 14 days after immunization, blood was drawn to analyze proliferation, frequencies of Th1 and Th2 cells and antibody production. Fourteen days after immunization, intracutaneous KLH
(100 μg) administration was performed in the gluteal region of the right leg. In addition, the CMI-multitest (intracutaneous administration of Tetanus Toxoid, Streptococcus, Diphtheria, Tuberculin, Candida, Trichophyton and Proteus, Pasteur Mérieux, Lyon, France) was applied at the frontal side of the antebrachium, according to the manufacturer’s instructions. Forty-eight hours later, local induration at the skin test area was palpated, the borders of the induration were marked off and its diameters were measured in two perpendicular directions. Results are expressed as either mean diameters of the indurations (keyhole limpet haemocyanin) or number of indurations equal to or larger than 5 mm (CMI multitest). In addition, a 4 mm diameter skin biopsy of the KLH-challenge site was performed under local anesthesia. Tissue was snap-frozen in liquid nitrogen and stored at -80°C.

Immunizations were performed at 6 months after entry into the study in case of the healthy control individuals. Renal transplant recipients from the P/CsA group were immunized at 3 months after transplantation, patients from the IgA CD3 mAb+P/CsA group were immunized at 3 months after the initiation of rejection treatment. Finally, patients from the P/CsA/MMF group were immunized at least 3 months after initiation of the triple immunosuppressive drug regimen.

Proliferative responses of peripheral blood mononuclear cells to keyhole limpet haemocyanin, tetanus toxoid, and CD3/CD28

PBMC were cultured in six replicates as 50,000 PBMC per well in IMDM, supplemented with 5% human pooled serum, penicillin and streptomycin and β-mercapto-ethanol in a final volume of 170 μL per well. The combination of CD3 (ascites, clone 1XE, CLB, Amsterdam, The Netherlands) and CD28(clone 15E8, CLB, Amsterdam, The Netherlands) was added in a final dilution of 1/1000 respectively 1 μg/mL (final concentration). KLH was added as 38 μg/mL (final concentration), and tetanus toxoid was added as 19 Lf/mL (final concentration). Thirty-seven MBq/ml [methyl-3H]Thymidine (Nycomed Amersham plc, Buckinghamshire, England) (20 μl/well) was added at day 4 of CD3/CD28 stimulated cultures and at day 6 of the KLH or tetanus toxoid stimulated cultures. The plates were incubated for either 4 hours (CD3/CD28) or 8 hours (KLH and tetanus toxoid) at 37ºC and 5% CO₂ in a humidified chamber. DNA was harvested on filter plates (UniFilter® GF/C™, Groningen, The Netherlands) using the Packard Harvester Filtermate 196 (Packard Instrument Company, Meriden, USA) and dried. Subsequently, scintillation fluid (Packard Bioscience, Groningen, The Netherlands) was added and filter plates were sealed (Packard Plate Sealer Micromate 496, Packard Instrument Company, Meriden, USA). Incorporated ³H-Thymidine was measured with a liquid scintillation counter (Packard Topcount microplate scintillation counter, Groningen, The Netherlands). Counts per minute were used as a readout for proliferation.
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Antibody response against keyhole limpet haemocyanin and against tetanus toxoid.
IgG anti KLH and IgG anti tetanus toxoid were measured by ELISA as described(3;9).

Flow cytometric determination of Th1 and Th2 subsets in peripheral blood
Frequencies of interferon-gamma (IFNγ) - or interleukin 4 (IL4) producing T helper cells were determined according to the method described by Picker et al.(10). Briefly, PBMC were incubated for 4 hours in the presence of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich Chemie GmbH, Deisenhofen, Germany, 1 ng/mL final concentration), ionomycin (Sigma, 1 μg/mL final concentration) and brefeldin A (Sigma, 10 μg/mL final concentration) in IMDM, containing 10% human pooled serum, β mercapto-ethanol and antibiotics, at 37°C, in a humidified chamber.

Cells were transferred to FACS tubes, surface stained with CD4-APC (BD) and CD8-PerCP, fixed in 2 ml per tube FACS lysing solution (BD), permeabilized in 0.5 ml per tube FACS permeabilizing solution (BD) followed by intracellular staining with anti IFNγ-FITC (BD) or anti IL4-PE (BD). To control for cellular stimulation, intracellular CD69 expression was analyzed. Flow cytometric analyses were performed on a FACS Calibur flow cytometer. Lymphocytes were gated based on forward scatter and sideward scatter. These events were further gated based on either positive CD4- or brightly positive CD8 expression. Within these gated events, the percentages of cells expressing either IFNγ or IL4 were evaluated.

Immunohistochemical staining of skin biopsies.
Frozen tissue sections were harvested onto slides, fixed in acetone and stained with either CD3 (OKT-3 ascites final dilution 1/1000), anti vascular cell adhesion molecule-1 (VCAM-1) (clone BBIG-V1(4B2)R&D systems 0.4 μg/mL final concentration), anti E-selectin (R&D Systems, clone BBIG-E4(5D11) 4 μg/mL final concentration), anti-CLA (HECA-452), and anti intercellular cell adhesion molecule-1 (ICAM-1)(R&D, clone BBIG-I1(11C81) 0.4 μg/mL final concentration) according to a previously described protocol(11). Infiltration of CD3pos cells as well as expression of E-selectin, ICAM-1 and VCAM-1 were scored semiquantitatively as described(12;13). CLA positive cells (i.e. cells binding the HECA-452 antibody) were scored as a percentage of mononuclear cells.
Results

*In vitro* proliferative responses to keyhole limpet haemocyanin after *in vivo* immunization are relatively intact in renal transplant recipients under immunosuppression.

Since most subjects are considered immunologically "naive" for keyhole limpet haemocyanin (KLH), we used this as a model antigen for the study of primary immune responses toward protein antigens. Proliferative responses to KLH were evaluated before and 14 days after subcutaneous immunization with 1 mg of KLH. In healthy individuals, the median ratio of these proliferative responses (i.e. counts per minute 14 days after immunization divided by counts per minute before immunization) was 5.1 (range 0.6-20.3) (Figure 1A.). In renal transplant recipients, the ratio of the *in vitro* proliferative response to KLH was not statistically different from the healthy control individuals (P/CsA-group: median: 2.7; range:1.7-8.1, IgA CD3 mAb+P/CsA-group: median:1.6; range: 0.2-18.1 and the P/CsA/MMF group: median:1.3; range: 0.4-4.4). The number of subjects responding to KLH immunization with a ratio of the proliferative response higher than 1 was calculated (table 1).

Although not statistically significant, the number of subjects responding, as well as the magnitude of the T helper cell response tend to be hampered by addition of either treatment with IgA CD3 monoclonal antibody or by addition of mycophenolate mofetil to prednisolone/cyclosporin A medication.

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<th>Table 1 KLH specific immune responses after immunization with KLH</th>
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²Chi-square test, not statistically significant
³Chi-square test, p<0.05
We conclude that, on average, T helper cell responses to KLH can be induced by immunization to KLH in renal transplant recipients. No effect on proliferation to CD3/CD28 was found by either of the immunosuppressive drug regimens, or the immunization procedure (not shown).

**Mycophenolate mofetil addition to prednisolone/cyclosporin A drug therapy abrogates IgG anti keyhole limpet haemocyanin production in most subjects.**

IgG anti KLH responses were analyzed before and 14 days after immunization with 1 mg KLH. Healthy individuals demonstrated a median 2.4 fold rise in IgG anti KLH antibody concentration (range 1.1-270.0) (figure 1B). In renal transplant recipients receiving prednisolone and cyclosporin A, the ratio of IgG anti KLH production was not affected (median ratio: 2.0; range:0.9-12.4). In addition, rejection treatment with the CD3 directed monoclonal antibody did not affect IgG anti KLH production (median ratio: 2.0, range:1.5-6.0). In contrast, in patients receiving triple immunosuppressive therapy consisting of prednisolone, cyclosporin A and mycophenolate mofetil, no B cell response was observed in 5 out of seven patients (table 1) (median ratio: 0.9; range 0.6-2.1). From these data, it may be inferred that addition of mycophenolate mofetil abrogates B cell responses toward a new protein antigen upon in vivo immunization, despite the presence of residual KLH specific T helper cell responses.

**Delayed type hypersensitivity reaction to KLH is markedly inhibited under systemic treatment with prednisolone/cyclosporin A and prednisolone/cyclosporin/mycophenolate mofetil**

Fourteen days after primary immunization with KLH, skin reactions to intracutaneous re-challenge with 100 µg of KLH were performed. Forty-eight hours thereafter, erythema and induration at the injection site were scored. Figure 1C demonstrates that, in all tested healthy control individuals, vigorous skin reactions were observed. In contrast, in none of the renal transplant recipients receiving IgA CD3 mAb rejection treatment in addition to prednisolone and cyclosporin A and in only two of the patients from the P/CsA- and P/CsA/MMF groups, indurations to intracutaneous KLH re-challenge were observed (figure 1C).

**Systemic immunosuppression with prednisolone/cyclosporin A reduces ICAM-1 expression in skin during delayed type hypersensitivity reactions to KLH; E-selectin expression is not affected.**

The observation of profoundly reduced skin reactions in the presence of residual proliferative T helper cell responses to the same antigens prompted us to investigate the influx of T cells into the skin test site. In most immunosuppressed individuals, we did observe perivascular infiltrates in the dermis in response to the vaccination (figure 2B).
Immune responsiveness in immunosuppressed renal transplant recipients

Figure 1 Cellular and humoral immune responses and delayed type hypersensitivity skin reactions to keyhole limpet haemocyanin.

A. Ratio of the proliferative response to keyhole limpet haemocyanin (KLH) of values from 14 days after immunization to values from before immunization. (Y-axis, ratio, log scale) in either healthy control individuals (C.l., open circles), renal transplant recipients receiving prednisolone and cyclosporin A (P/CsA, filled circles), renal transplant recipients receiving a rejection treatment with IgA CD3 monoclonal antibody, basic immunosuppressive treatment: prednisolone and cyclosporin A (IgA CD3 mAb+P/CsA, triangles) or renal transplant recipients receiving “triple” immunosuppressive regimen consisting of prednisolone, cyclosporin A and mycophenolate mofetil (P/CsA/MMF, diamonds).

B. Ratio of IgG anti keyhole limpet haemocyanin (KLH) production of values from 14 days after immunization to values from before immunization. (Y-axis, ratio, log scale) in individuals as in figure 1A.

C. Mean diameter of induration of the skin-area, 48 hours after intracutaneous skin testing with 100 μg KLH (Y-axis, millimeters) of the same individuals as in figure 1A.

n.s.: not statistically significant p=0.055 Kruskal-Wallis test
*p<0.05 Mann-Whitney

Compared to healthy individuals, CD3 expression in the challenged skin area from renal transplant recipients was not significantly depressed (figures 2A., B. and E.). Expression of E-selectin on endothelium of challenged skin was similar in healthy individuals and renal
transplant recipients (figure 2G.), as well as the frequency of mononuclear cells expressing the E-selectin ligand CLA. In contrast, in comparison with healthy individuals, renal transplant recipients expressed lower levels of ICAM-1 in challenged skin (figure 2C, D and F.). No effect of any of the immunosuppressive drug treatments on VCAM-1 expression was observed (not shown).

Figure 2. Reduced ICAM-1 expression in delayed type hypersensitivity skin of patients treated with prednisolone/cyclosporin A
A. and B. CD3 expression in challenged skin, 48 hours after intracutaneous challenge with 100 μg KLH from either a representative healthy control individual (A.) or a representative renal transplant recipient receiving prednisolone and cyclosporin A (B).
C. and D. ICAM-1 expression in challenged skin, 48 hours after intracutaneous challenge with 100 μg KLH from the same subjects as in figure 3A. respectively 3B.
E., F., and G. Frequency distributions of the semi-quantitative score after immunohistochemical staining of KLH challenged skin biopsies with CD3 (E.), anti-ICAM-1 (F.) or anti E-selectin (G.) in skin biopsies at 48 hours after KLH challenge from either healthy control individuals (CI, open bars) or renal transplant recipients receiving prednisolone and cyclosporin A (P/CsA, filled bars).
H. Frequencies of mononuclear cells expressing the cutaneous lymphocyte associated antigen (CLA) as detected by the HECA-452 antibody (Y-axis) in either healthy control individuals (CI, open circles) or renal transplant recipients receiving prednisolone and cyclosporin A (P/CsA, filled circles) 
n.s.: not statistically significant (figures 2E-G chi-square test, figure 2H Mann-Whitney test)
**Immune responsiveness in immunosuppressed renal transplant recipients**

Proliferative responses to tetanus toxoid are relatively intact in renal transplant recipients under immunosuppression.

In addition to KLH vaccination, all subjects were simultaneously immunized with tetanus toxoid intramuscularly. Proliferative responses to tetanus toxoid were measured before and 14 days after immunization and are presented as a ratio of the proliferative response after immunization to the proliferative response before immunization. The tetanus toxoid induced proliferative capacity of peripheral blood mononuclear cells in response to immunization did not differ among the healthy individuals and the renal transplant recipients in three treatment groups. Median ratio’s of the proliferative responses were 2.9 (range: 0.5-85.6), 3.9 (range: 2.3-62.3), 2.6 (range: 0.2-13.3) and 1.9 (range: 0.8-8.5) in respectively the healthy control individuals, the P/CsA-, the IgA CD3 mAb+P/CsA- and the P/CsA/MMF groups (figure 3A). Importantly, after immunization, the majority of the patients from the IgA CD3 mAb+P/CsA and P/CsA/MMF groups responded with an enhanced proliferative response to tetanus toxoid (table 2).

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<th>Table 2 Tetanus toxoid specific immune responses after immunization with tetanus toxoid</th>
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¹Ratio of values from 14 days after immunization to values from before immunization. The figures represent numbers of subjects within each treatment group, either not responding (ratio ≤ 1) or responding (ratio >1).

²CI: healthy control individuals, P/CsA: renal transplant recipients receiving immunosuppressive drug therapy consisting of prednisolone and cyclosporin A, IgA CD3 mAb+P/CsA: renal transplant recipients receiving a rejection treatment of IgA CD3 monoclonal antibody, basic immunosuppressive drug therapy: prednisolone and cyclosporin A, P/CsA/MMF: renal transplant recipients receiving immunosuppressive drug treatment consisting of prednisolone, cyclosporin A and mycophenolate mofetil.

³Chi-square test, not statistically significant

⁴Chi-square test, p<0.05

**IgG anti tetanus toxoid production cannot be boosted by immunization under systemic treatment with and prednisolone/cyclosporin A/mycophenolate mofetil**

In most healthy control individuals, tetanus toxoid specific IgG antibodies could on average be induced by the immunization protocol (median anti TT ratio: 8.7; range: 1.0-67.7). In renal transplant recipients from both the P/CsA- and the IgA CD3 mAb+P/CsA groups, IgG anti tetanus toxoid antibodies were elevated after immunization with median ratios of 7.0 (range: 103
Figure 3. Cellular and humoral immune responses to tetanus toxoid and delayed type hypersensitivity skin reactions to recall antigens.

A. Ratio of the proliferative response to tetanus toxoid (TT) of values from 14 days after immunization to values from before immunization. (Y-axis, ratio, log scale) in either healthy control individuals (C.I., open circles), renal transplant recipients receiving prednisolone and cyclosporin A (P/CsA, filled circles), renal transplant recipients receiving a rejection treatment of IgA CD3 monoclonal antibody, basic immunosuppressive treatment: prednisolone and cyclosporin A (IgA CD3 mAb+P/CsA, triangles) or renal transplant recipients receiving "triple" immunosuppressive regimen consisting of prednisolone, cyclosporin A and mycophenolate mofetil (P/CsA/MMF, diamonds).

B. Ratio of IgG anti tetanus toxoid (TT) production of values from 14 days after immunization to values from before immunization. (Y-axis, ratio, log scale) in the same subjects as in figure 2A.

C. Skin test to recall antigens: frequency distributions of number of positive indurations 48 hours after intracutaneous challenge with 7 different protein antigens (>5 mm mean diameter of induration, X-axis) versus number of subjects (Y-axis) in healthy control individuals and three groups of immunosuppressed individuals as in figure 2A.

1.1-56.2), respectively 2.8 (range: 1.5-9.3)(figure 3B). In contrast, in the renal transplant recipients treated with prednisolone, cyclosporin A and mycophenolate mofetil, an antibody response to tetanus toxoid was completely absent in four out of six patients treated (table 2).
In the other two patients from this treatment group, the IgG anti tetanus toxoid response was very small (complete P/CsA/MMF group: median ratio: 0.94; range: 0.7-1.3).

![Bar graph showing immune responsiveness in immunosuppressed renal transplant recipients](image)

**Figure 4.** Long term systemic immunosuppression with prednisolone/cyclosporin A does not affect the ex vivo IFNy- or IL4 production profiles of T cells from peripheral blood. A. Frequencies of IFNy producing cells within peripheral blood CD4^{pos} T cells (Y-axis, % of CD4^{pos} T cells) versus time after entry into the study (X-axis, months). Open circles represent healthy control individuals (C.I.), filled circles represent renal transplant recipients receiving prednisolone and cyclosporin A (P/CsA), and triangles represent renal transplant recipients receiving a rejection treatment of IgA CD3 monoclonal antibody, basic immunosuppressive treatment: prednisolone and cyclosporin A (IgA CD3 mAb+P/CsA). The horizontal bar in the graph of IgA CD3 mAb+P/CsA represents the time of the rejection treatment with IgA mouse anti human CD3. Time of entry into the study is the day of transplantation for the P/CsA-group and the first day of rejection treatment for the IgA CD3 mAb+P/CsA-group. Small arrows indicate the time points of immunization with tetanus toxoid and keyhole limpet haemocyanin.

B. Frequencies of IL4 producing cells within peripheral blood CD4^{pos} T cells (Y-axis, % of CD4^{pos} T cells) versus time after entry into the study (X-axis, months), symbols as in figure 4A.
Chapter 7

Systemic immunosuppression with prednisolone/cyclosporin A based medication reduces the skin reactions to recall antigens

To test whether memory T cells that have been generated in the absence of immunosuppression are also defective in inducing delayed type hypersensitivity reactions, intracutaneous administration were performed with tetanus toxoid and a panel of six additional antigens, i.e. Proteus, Trichophyton, Candida, Streptococcus, Tuberculin and Diphtheria. Figure 3C. displays the frequency distributions of the number of significant skin reactions to any of these antigens in healthy control individuals and in the three groups of renal transplant recipients. The number of positive skin reactions was different among the groups. Since the different groups of immunosuppressed individuals had very similar low responses, the CMI multitest data from these groups of renal transplant recipients were pooled. Compared to healthy control individuals, the relative risk for these renal transplant recipients for scoring no positive skin reactions as opposed to 1 or more positive skin reactions was calculated as 3.0 (95% confidence interval 1.5-6.1).

Long term systemic immunosuppression with prednisolone/cyclosporin A does not affect the ex vivo IFNγ- or IL4 production profiles of T cells from peripheral blood

Delayed type hypersensitivity reactions are generally considered to be T helper 1 type cell mediated immune reactions. Moreover, T helper 1 cells are more likely to enter inflamed skin(14). Therefore, we examined whether immunosuppressive regimens influence the Th1/Th2 balance in humans. In both healthy control individuals and renal transplant recipients, some variation in the frequencies of IFNγ and IL4 producing T helper cells was found. However, these changes were not statistically significant during follow up of 1 year(figure 4A. and B.) Moreover, no effect on the ratio of IFNγ producing to IL4 producing cells was observed in these patients (not shown.)

Discussion

The present study shows that priming of T cells, specific for new protein antigens still occurs in the presence of double immunosuppressive drug treatment consisting of prednisolone and cyclosporin A and can even occur in the presence of triple immunosuppressive therapy with prednisolone, cyclosporin A and mycophenolate mofetil. In contrast effector T cell responses are severely reduced: delayed type hypersensitivity reactions were hampered, even if the antigen specific T cells were educated in the absence of immunosuppression.

In a previous study, van der Heijden et al. found no effect of cyclosporin A monotherapy on the in vivo delayed type hypersensitivity responses to recall antigens(3). The discrepancy with our data may be explained by the difference in pharmacological formulation of cyclosporin A.
In the study by van der Heijden et al., Sandimmune was administered as opposed to Neoral, which was used in the present study. Neoral administration is known to result in higher drug level area under the curves, which may contribute to the differences in results between the two studies(15). Moreover, the addition of 10 mg per day of prednisolone in our study, may have exerted additional inhibitory effects on the delayed type hypersensitivity reactions. A similar depressed skin reactivity was found in renal transplant recipients treated with prednisone in conjunction with azathioprine(16).

Histological studies on the delayed type hypersensitivity reaction to KLH indicate an effect of prednisolone/cyclosporin A treatment on ICAM-1 expression, leaving the influx of T cells unaffected. Presumably, ICAM-1 expression is directly inhibited by the complex of the glucocorticoid receptor and prednisolone(17;17). In addition, it may be speculated that tethering of lymphocytes on E-selectin in skin is unaffected by this drug regimen. Immunosuppressive treatment with prednisolone and cyclosporin A did not significantly affect the frequencies of peripheral blood derived Th1 or Th2 cells or the balance between these subsets. Differentiation of naive T cells into Th1 or Th2 cells is mainly driven by the cytokine environment in which naive to memory transition occurs and is accompanied by switching on specific genetic programs while at the same time other genetic programs are switched off(18). In vitro, treatment of dendritic cells with glucocorticoids impairs their Th1 skewing and enhances their Th2 skewing capacity(5;6). However, upon glucocorticoid treatment in vivo, it may require many naive to memory transitions of T cells before peripheral blood Th2 deviation becomes apparent, i.e. requiring more prolonged follow up of patients. Alternatively, co-medication may have inhibited the Th2 skewing effects of prednisolone treated dendritic cells.

Administration of a 10 day course of IgA CD3 monoclonal antibody does not add profound immunosuppressive effects on cellular or humoral immune responsiveness either in vivo or in vitro. In contrast, addition of treatment with mycophenolate mofetil to prednisolone and cyclosporin A profoundly decreases both primary and secondary humoral immune responsiveness in vivo. In response to KLH, three out of seven patients did not display a T helper cell response, which may in part explain the absence of the switch to IgG production. However, in response to immunization with tetanus toxoid, augmentation of the T helper cell responses were detectable in most subjects, whereas 4 out of 6 patients did not respond with increased antigen specific IgG production. These results are in accordance with previously described impairments in antibody production under mycophenolate mofetil treatment. In patients treated with prednisolone, cyclosporin A and mycophenolate mofetil, significant impairment of antibody formation to influenza A virus was observed when compared to patients treated with azathioprine in stead of MMF(19). In a comparison among patients with similar drug treatment protocols, antibody formation directed against equine derived
polyclonal anti-thymocyte globulin or against the murine CD3 monoclonal antibody OKT-3 was significantly impaired in MMF treated patients\(\text{\textsuperscript{20,21}}\). Taken together, our results imply that addition of MMF to a drug regimen consisting of prednisolone and cyclosporin A acts directly on the humoral immune response while T helper cell responses are relatively spared.

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