Characterization of B cell responses in relation to organ transplantation

Heidt, S.

Chapter 3

Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help

Sebastiaan Heidt, Dave L. Roelen, Chantal Eijsink, Michael Eikmans, Cees van Kooten, Frans H.J. Claas and Arend Mulder

Clinical and Experimental Immunology 2010; 159 (2): 199-207

ABSTRACT

In general, humoral immune responses depend critically on T cell help. In transplantation, prevention or treatment of humoral rejection therefore requires drugs that ideally inhibit both B cell and T helper cell activity. Here, we studied the effects of commonly used immunosuppressive drugs (tacrolimus, cyclosporin, MPA and rapamycin) on T cell helper activity and on T cell dependent B cell responses.

T cells were polyclonally activated in the presence of immunosuppressive drugs in order to analyse the effect of these drugs on T cell proliferation, costimulatory ligand expression and cytokines. The impact of immunosuppressive drugs on T cell dependent immunoglobulin production by B cells was addressed in T – B cell cocultures.

All drugs affected T cell proliferation and attenuated T cell costimulatory ligand (CD154 and CD278) expression when T cells were polyclonally activated. Tacrolimus, cyclosporin and rapamycin also attenuated B cell stimulatory cytokine mRNA levels in T cells. As a consequence, a decrease in immunoglobulin levels was observed in autologous T – B cell cocultures, where T cell help is essential for immunoglobulin production. In contrast, when preactivated T cells were used to stimulate autologous B cells, calcineurin failed to inhibit B cell immunoglobulin production, whereas MPA and rapamycin did show inhibition. From these studies it is evident that calcineurin inhibitors affect the humoral immune response by interfering with T helper signals, but not by targeting B cells directly. Our studies furthermore support the necessity of intervening in T cell helper function to attenuate humoral responses.

INTRODUCTION

Despite excellent one-year graft survival rates, graft rejection remains an issue in solid organ transplantation. Although hyperacute rejection is avoided by pre-transplant serological crossmatching (*1*) and acute rejection is treatable with current immunosuppressive drugs, rejection pathology still occurs and has shifted towards a later stage after transplantation. Typically, the cause of chronic organ failure is multi-factorial though, involving both immunologic and non-immunologic damage, termed chronic allograft vasculopathy (*2*). In recent years, the role of humoral immunity in the development of chronic rejection has become increasingly apparent, as anti-HLA antibodies are frequently detected prior to chronic kidney rejection (*3*). Staining for the complement split product C4d also revealed a strong correlation between chronic rejection and humoral immunity (*4*).

Medication for treatment of acute rejection is well defined. Steroids are administered to patients undergoing rejection which, in case of steroid resistance, are followed by anti-thymocyte globulin (ATG) (*5*). In contrast, therapy for chronic (humoral) rejection is less well defined. Besides standard immunosuppressive drugs, intervention strategies include administration of ATG, high dose IVIg, Rituximab and plasmapheresis (*6-10*).

Although terminology implies a clear division, cellular and humoral rejection are intertwined. B cells act as potent antigen presenting cells capable of activating T cells, thereby possibly enhancing cellular rejection (*11*). Conversely, most B cells will only get properly activated when T cell help is provided (*12*). Furthermore, T cells are needed for B cell class-switching and production of potentially harmful IgG antibodies (*13*). Besides cognate interaction via antigen, the T cell mediated activation of B cells takes place through CD40L (CD154) – CD40 and, inducible costimulator (ICOS; CD278) – ICOSL interaction, as well as through cytokine production and consumption. Therefore, drugs that preferentially act on T cells, such as calcineurin inhibitors, are likely to affect humoral immune responses.

Previously, we have reported that the function of highly purified B cells, upon CD40 driven activation, was inhibited by mycophenolic acid (MPA) and rapamycin, but not by calcineurin inhibitors tacrolimus and cyclosporin, especially when B cells were strongly stimulated (*14*). In the present study, we have investigated the effect of these immunosuppressive drugs on T cell help and addressed the question whether calcineurin inhibitor-induced inhibition of T cell help is sufficient for the prevention of immunoglobulin production by B cells in a T cell dependent culture system.

MATERIALS EN METHODS

Cells

Blood was obtained from healthy blood bank donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. Untouched $CD4^+$ T cells were obtained from PBMC by magnetic separation using the $CD4^+$ T cell isolation kit II (Miltenyi, Bergisch-Gladbach, Germany) and MS columns (Miltenyi). After separation, flow cytometric analysis (FCM) revealed >80% purity. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B and Detach-a-Bead CD19 (Invitrogen, Leek, the Netherlands), typically yielding >98% pure B cells, as assessed by FCM. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and insulin-tranferrin-selenium (ITS) (insulin 5 µg/ml, transferrin 5 µg/ml and selenium 5 ng/ml, Sigma-Aldrich).

Immunosuppressive drugs

Tacrolimus (Prograf, Astellas, Leiderdorp, the Netherlands, diluted in ethanol) and cyclosporin (Sandimmune obtained from Novartis, Arnhem, the Netherlands) were used at final concentration ranges of 0-1 ng/ml and 0-100 ng/ml, respectively, based on plasma levels measured at 6 months post-transplantation (*15-17*). Calcineurin inhibitor concentrations reported for whole blood are considerably higher, but it should be noted that *in vivo* a substantial fraction of these drugs is bound to erythrocytes (*18, 19*), which are not present in our cultures. Mycophenolic acid (MPA, Sigma-Aldrich), the active metabolite of mycophenolate mofetil (MMF), was dissolved in ethanol and used in concentrations up to 100 ng/ml, which is approximately 10-fold lower than used in patients. This concentration range was chosen because maximal effects were already observed using 100 ng/ml. Rapamycin (Calbiochem, La Jolla, CA, USA) was dissolved in methanol and used in concentrations up to 8 ng/ml, which is within the clinical range. Solutions of immunosuppressive drugs were diluted in culture medium.

CFSE assay

T cells (10^5) were carboxyfluorescein succinimidyl ester (CFSE from Invitrogen, 10 µM) labelled for 10 min at 37°C and cultured with 5 µg/ml anti-CD28 mAb (CLB-CD28/1, Sanquin, Amsterdam, the Netherlands) in 24-well plates (Costar, Veenendaal, the Netherlands) that had been coated with 5 µg/ml anti-CD3 mAb (UCHT1, BD Biosciences,

Breda, the Netherlands). Cells were harvested at day 3 and stained with CD4-PE (BD Biosciences) and Sytox Red dead cell stain (Invitrogen, Leek, the Netherlands) for dead cell exclusion. The proliferation index was calculated as follows (adapted from (*20*)):

$$\text{total events} / \sum(\text{events in peak}[n]/2^{(n-1)})$$

Peak 1 represents the undivided peak. Data are expressed as percentage of the proliferation index relative to no addition of immunosuppressive drugs. To calculate this percentage, data were transformed such that a proliferation index of zero represents no division.

Cytokine mRNA detection

T cells (5×10^5) were stimulated for 8 h with anti-CD3 mAb / anti-CD28 as described above in the presence or absence of immunosuppressive drugs. Cells were harvested and preserved in RNAlater solution (Qiagen, Chatsworth, CA, USA). RNA was extracted using the RNeasy® mini kit (Qiagen) following the manufacturer's instructions. RNA was treated with DNase (Qiagen) on the spin columns and RNA quantity was assessed with a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All samples showed A260/A280 ratios between 1.9 and 2.1. cDNA was synthesized by incubating 12.8 µl RNA solution with 7.2 µl cDNA mix containing 2'-deoxynucleosides 5' triphosphate (dNTPs) (final concentration of 0.5 mM), 2 U reverse transcriptase-avian myeloblastosis virus (RT-AMV), 20 U rRNase inhibitor, 100 ng oligo-dT primers, 500 ng of random primers, and 1x reverse transcriptase buffer (all from Promega, Leiden, the Netherlands).

Primer sets (Table 1) for quantitative polymerase chain reaction (Q-PCR) were selected using Beacon Designer Software (version 7.02, Premier Biosoft International, Palo Alto, CA, USA) and were obtained from Eurogentec (Liège, Belgium). PCR mixes contained 1 µM of forward and reverse primers, 3 mM $MgCl_2$, and 1× iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands). PCR was performed using an iCycler MyiQ (Bio-Rad). The PCR program consisted of one cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, and was finalized with a melting curve analysis. Reactions were carried out in optical 96-well plates (Bio-Rad) covered with Microseal 'B' Film (Bio-Rad). The mean signal of the stably expressed reference genes 18S rRNA, GAPDH, β-actin, HPRT, HMBS and RPL13a served as a normalization factor to minimize general, if any, effects of immunosuppressive drugs.

Flow cytometry

T cells (5×10^5) were stimulated for 24 h with anti-CD3 mAb / anti-CD28 mAb as described above in the presence of graded concentrations of immunosuppressive drugs. Cells were harvested and labelled with the following mAb conjugates: CD4-PerCP, CD25-PE, CD154-PE, CD278-PE and CD69-FITC (all from BD Biosciences). Dead cells were excluded using Sytox Red. Cells were acquired using a FACS Calibur and analyzed using CellQuest Pro software (BD Biosciences).

Table 1. *Sequences for primers used in quantitative polymerase chain reaction (Q-PCR).*

Transcript	Forward primer	Reverse primer	Amplicon
IFN-γ	AGCTCTGCATCGTTTTGGGTT	GTTCCATTATCCGCTACATCTGAA	118 bp
IL-2	AGGATGCTCACATTTAAGTTTTAC	GAGGTTTGAGTTCTTCTTCTAGACACTGA	85 bp
IL-4	GTCTCACCTCCCAACTGCTT	GTTACGGTCAACTCGGTGCA	157 bp
IL-5	AGCCAATGAGACTCTGAGGATTC	GACTCTCCAGTGTGCCTATTCC	95 bp
IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA	94 bp
IL-13	TCCTCTCCTGTTGGCACTG	AGCGGAGCCTTCTGGTTC	165 bp
IL-21	AAACCACCTTCCACAAATGC	AGAGGACAGATGCTGATGAATC	147 bp
18S rRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	68 bp
GAPDH	ACCCACTCCTCCACCTTTGAC	TCCACCACCCTGTTGCTGTAG	110 bp
HPRT-1	AGATGGTCAAGGTCGCAAGC	TCAAGGGCATATCCTACAACAAAC	115 bp
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	64 bp
RPL13a	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	126 bp
β-actin	ACCACACCTTCTACAATGAG	TAGCACAGCCTGGATAGC	161 bp

bp: base pairs.

T and B cell cocultures

T cells (1.5×10^3) were stimulated for 9 days with anti-CD3 mAb / anti-CD28 mAb as described above in the presence of autologous B cells (1.5×10^5) with 2.5 µg/ml of the Toll-like receptor ligand cytosine-guanine dinucleotide oligodeoxynucleotide (CpG ODN) 2006 (Hycult Biotechnology, Uden, the Netherlands). A T – B cell ratio of 1:100 was chosen because of strong proliferation of T cells after polyclonal stimulation, resulting in a 1:1 ratio after 9 days of culture (data not shown).

For some experiments, T cells (5×10^5) were prestimulated with anti-CD3 mAb / anti-CD28 mAb and 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands) for 2 days. After washing, 5×10^4 T cells were cocultured with 5×10^4 B cells with the addition of CpG for 6

days instead of 9 days, since T cell were already activated in the preculture. Furthermore, cells were cultured in a 1:1 ratio, since cocultures were performed without continued T cell stimulation, resulting in only minor T cell proliferation.

Immunoglobulin levels

Supernatants were tested for IgM and IgG levels with a standard sandwich ELISA. Plates (Greiner, Alphen a/d Rijn, the Netherlands) were coated overnight with a goat anti-IgG or anti-IgM (Jackson Immunoresearch, Westgrove, PA, USA) diluted in 10 mM Tris pH 9.0, and then blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.025% Tween-20 (Sigma-Aldrich) in PBS (PBS-T). Fifty microliters of supernatants or standard human serum (Sanquin, Amsterdam, the Netherlands) in a serial dilution were incubated for 60 min at 37°C. After washing with PBS-T, biotin labelled goat anti-IgM or anti-IgG (Biosource, Camarillo, CA, USA) diluted in 1% BSA/PBS-T was incubated for 60 min at 37°C. After extensive washing, streptavidin horseradish peroxidase (Pierce, Rockford, IL, USA), diluted in 1% BSA/PBS-T was added and incubated for 60 min at 37°C. A color reaction was obtained with 4.6 mM 2,2'-azine-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) in a citric acid/PBS buffer at pH 4.2. The reaction was stopped with 250 mM oxalic acid (Sigma-Aldrich) and measured at OD_{450nm} in an ELISA reader (Bio-Rad). Data were analyzed using the Microplate Manager software version 4 (Bio-Rad).

Statistics

The one-sample T test was used for the analysis of immunoglobulin levels induced by different conditions within one donor. The paired T test was used for the analysis of single doses of immunosuppressive drugs. Statistical level of significance was defined as $P<0.05$.

RESULTS

Effects of immunosuppressive drugs on T cell activation and proliferation

T cell activation with anti-CD3 mAb / anti-CD28 mAb led to a substantial increase of $CD25^+$ and $CD69^+$ cells (Figure 1a). None of the immunosuppressive drugs altered the percentage of $CD25^+$ cells, whereas only cyclosporin slightly inhibited the percentage of CD69+ cells (Figure 1b). Additionally, polyclonal T cell activation resulted in a strong proliferative response, as measured by CFSE (Figure 1c, upper left panel). Since immunosuppressive drugs profoundly inhibit T cell proliferation, high doses of immunosuppressive drugs

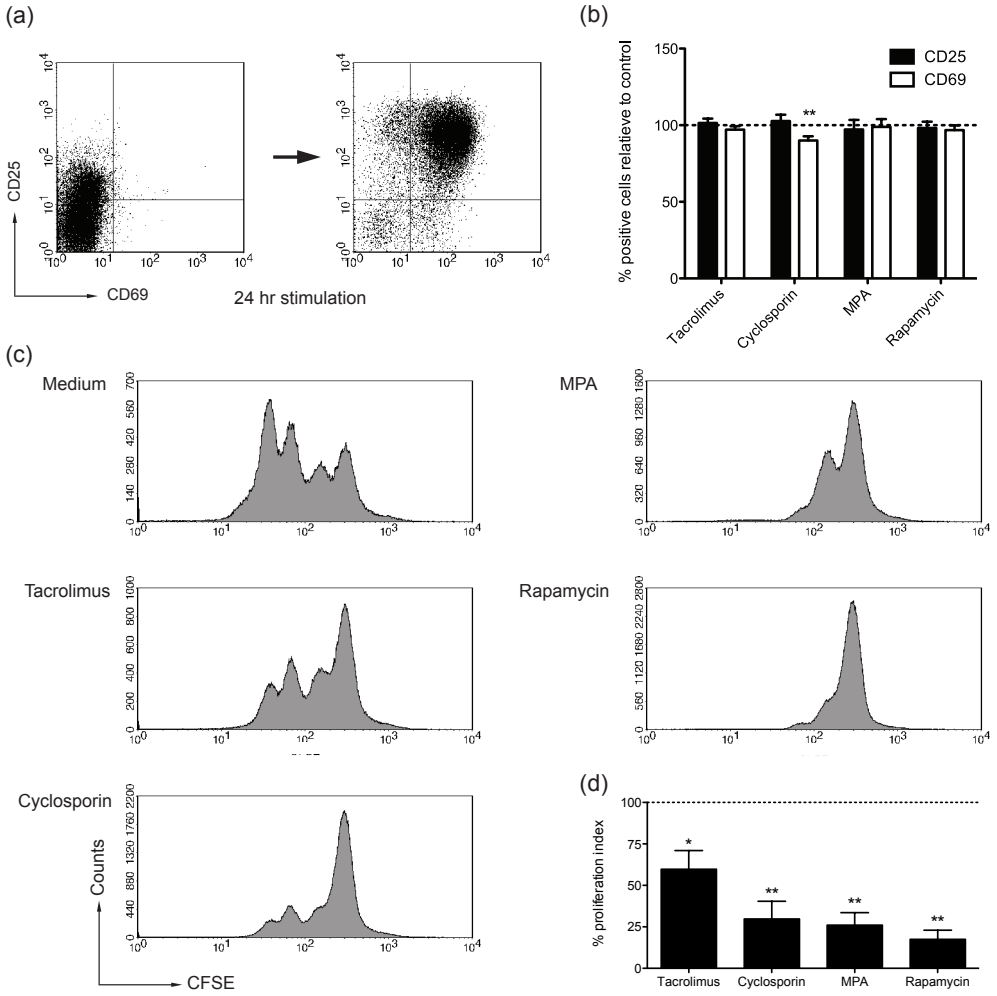

Figure 1. *Immunosuppressive drugs did not alter T cell activation but did inhibit T cell proliferation. (a) T cell activation with anti-CD3 mAb / anti-CD28 mAb for 24 h resulted in upregulation of CD25 and CD69. (b) None of the immunosuppressive drugs affected CD25 levels, whereas cyclosporin inhibited the expression of CD69. Drug concentrations were 1.0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml MPA and 8.0 ng/ml rapamycin. Data are expressed as percentage of cells expressing CD25 or CD69 as compared to medium controls (dotted lines), n=4. (c) CFSE labelled T cells were activated with anti-CD3 mAb / anti-CD28 mAb for 3 days in the presence of immunosuppressive drugs. Data from a representative experiment are shown. Similar results were obtained in three independent experiments. (d) Percentage inhibition of the T cell proliferation index by immunosuppressive drugs compared to medium controls (dotted lines) are depicted, n=3. Drugs concentrations were: 0.3 ng/ml tacrolimus, 50 ng/ml cyclosporin, 100 ng/ml MPA and 4 ng/ml rapamycin. *P<0.05 and **P<0.01.*

resulted in low cell yields, insufficient for analysis. Therefore, we used suboptimal drug concentrations to address the effect of immunosuppressive drugs on T cell proliferation (Figure 1c). These relatively low, but clinically relevant, drug concentrations caused strong inhibition of the T cell proliferation index (Figure 1d).

Effect of immunosuppressive drugs on T cell helper function

Next, we investigated the effect of immunosuppressive drugs on T cell helper signals, by addressing costimulatory ligand expression and helper cytokines. Polyclonal T cell activation led to increased numbers of $CD154^+$ (CD40L) cells and, to a lesser extent, of $CD278^+$ (ICOS) cells (Figure 2a). All immunosuppressive drugs attenuated the percentage of $CD154^+$ cells to a similar extent, although cyclosporin inhibition did not reach statistical significance. The effects on CD278 were more profound. Cyclosporin was superior in inhibiting the percentage of $CD278^+$ cells, as compared to tacrolimus, MPA and rapamycin (Figure 2b).

We determined effects of immunosuppressive drugs on cytokines by their mRNA levels at 8 h post stimulation, rather than by their presence in culture supernatants to avoid confounding effects due to proliferation and cytokine consumption. Polyclonal T cell activation led to increased IFN-γ, IL-2, IL-4, IL-5 and IL-13 mRNA levels, whereas IL-10 mRNA remained stable and IL-21 mRNA was undetectable (data not shown). Tacrolimus inhibited all cytokines tested, although IL-5 inhibition did not reach statistical significance (Figure 2c). Likewise, cyclosporin inhibited all cytokines tested, although not reaching statistical significance for IL-4, IL-5 and IL-10. MPA did not inhibit any of the cytokines tested although the effect of MPA on cytokine mRNA expression varied highly between subjects. Rapamycin inhibited all cytokines tested except IL-13, but not to the same extent as the calcineurin inhibitors.

All immunosuppressive drugs affect T cell dependent B cell activation

To test the effects of immunosuppressive drugs on T cell dependent B cell activation, we developed a culture system in which B cells were activated in a T cell dependent manner, using polyclonal T cell activation in the presence of autologous B cells. In this test system T cells were necessary for B cell activation, since cultures of purified B cells alone with anti-CD3 mAb / anti-CD28 mAb and CpG resulted in low IgM and IgG levels, whereas high levels of IgM and IgG were produced in the presence of autologous T cells (Figure 3a). In these T – B cell cocultures, CpG was added to increase immunoglobulin production (*21*), however, CpG alone did not induce immunoglobulin production (data not shown). B cell activation was T cell contact dependent, since co-cultures of B cells with pre-stimulated T

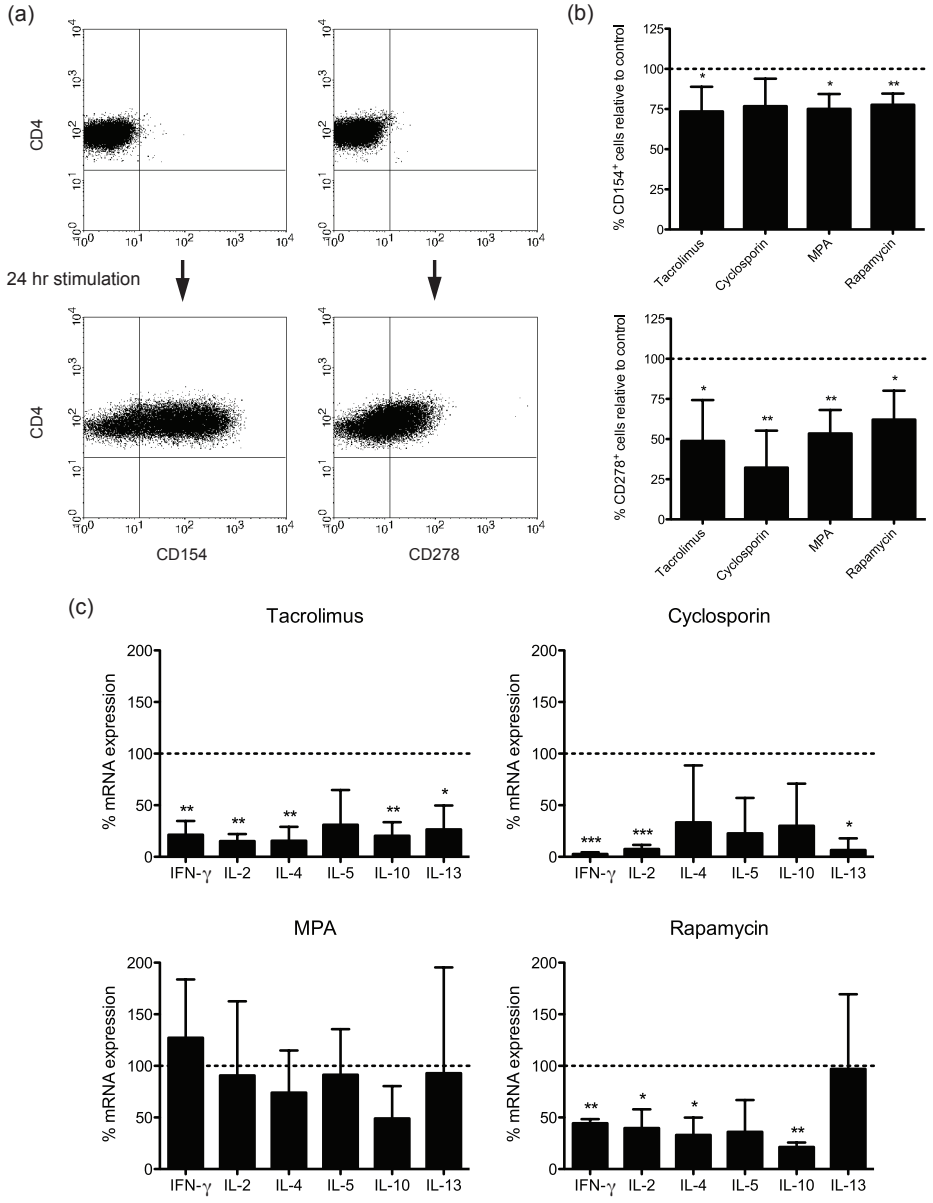

Figure 2. *Effect of immunosuppressive drugs on T cell stimulatory signals. (a) T cell stimulation with anti-CD3 mAb / anti-CD28 mAb for 24 h resulted in upregulation of CD154 and CD278. (b) All immunosuppressive drugs inhibited the expression of CD154 and CD278. Drug concentrations were 1.0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml MPA and 8.0 ng/ml rapamycin. Data are expressed as percentage of cells expressing CD154 or CD278 as compared to medium controls (dotted lines), n=4. (c) Immunosuppressive drugs inhibit the mRNA levels of B cell stimulatory cytokines produced by T cells to various extents. Data are expressed as percentage of cytokine mRNA inhibition compared to mediums controls, n=3. Drugs concentrations are identical to those of Figure 2b. *P<0.05, **P<0.01 and ***P<0.001.*

cells in transwell plates resulted in low immunoglobulin production, whereas co-cultures performed in standard 24-well plates resulted in high IgM and IgG production (Figure 3b). To address the effects of immunosuppressive drugs on T cell dependent B cell cultures, freshly isolated T and B cells were cocultured with immunosuppressive drugs for 9 days in the presence of polyclonal T cell stimulation, after which supernatants were tested for immunoglobulin levels. As expected from their direct effects on B cells, MPA and rapamycin profoundly inhibited immunoglobulin levels in T cell dependent B cell cultures. Interestingly, tacrolimus and cyclosporin were equally potent in inhibiting T cell dependent immunoglobulin production as compared to MPA and rapamycin, indicating that inhibition of T cell help by calcineurin inhibitors is sufficient to prevent immunoglobulin production (Figure 4a and 4b).

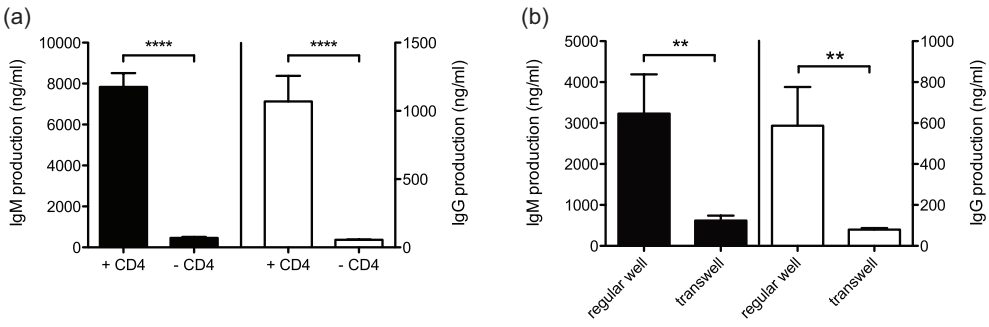

Figure 3. *T cells were necessary for B cell activation in a cell-cell contact dependent fashion. (a) B cells were cultured in anti-CD3 mAb coated wells with soluble anti-CD28 mAb in the absence or presence of autologous T cells. After 9 days supernatants were harvested for immunoglobulin assessment. (b) Co-cultures of B cells and pre-stimulated T cells were performed in either regular or transwell plates (B cells in lower compartment). Supernatants were harvested at day 6 for detection of immunoglobulins. **P<0.01 and ****P<0.0001.*

Tacrolimus and cyclosporin fail to inhibit B cells directly

To corroborate that the calcineurin inhibitor-induced inhibition of immunoglobulin production was due to the inhibition of T cell help, we developed a modification of the culture system described above in which T cells were already activated prior to coculture with autologous B cells. MPA and rapamycin almost completely inhibited immunoglobulin levels, ensuring that the B cells in this culture system were susceptible to inhibition by immunosuppressive drugs (Figure 4c and 4d). In contrast, tacrolimus and cyclosporin failed to

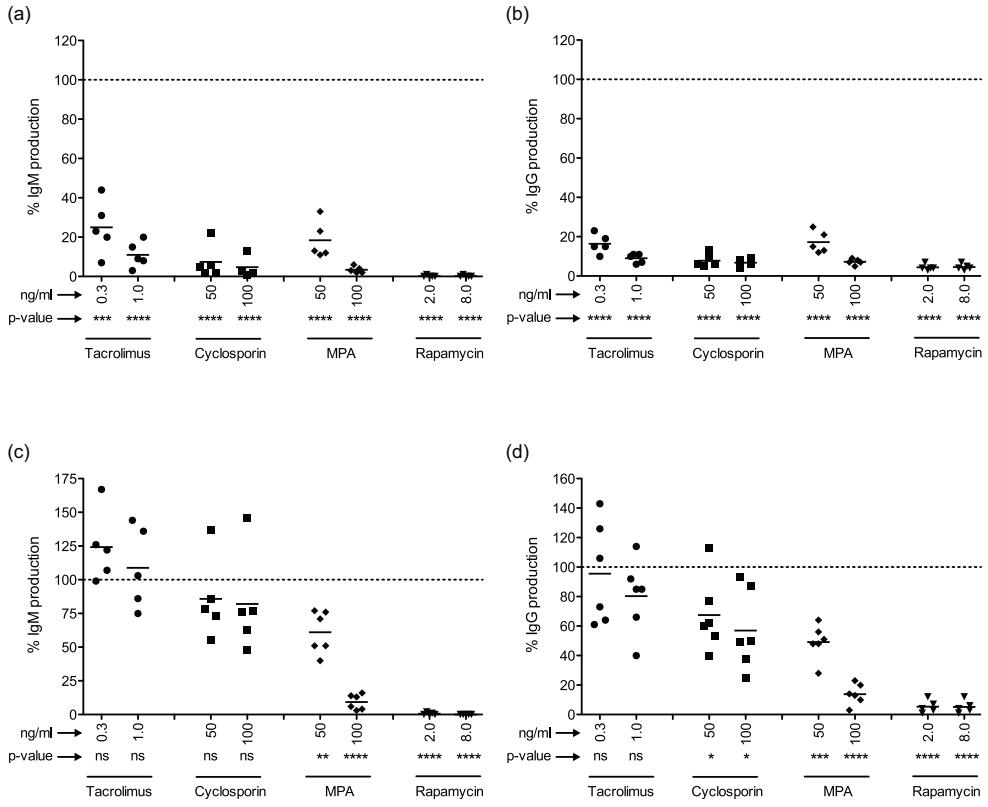

Figure 4. *All immunosuppressive drugs were capable of inhibiting immunoglobulin production when B cells are cultured with non pre-activated T cells, but calcineurin inhibitors failed to inhibit immunoglobulins levels when pre-activated T cells were used to stimulate B cells. B cells were cultured with fresh, autologous T cells and anti-CD3 mAb / anti-CD28 mAb with CpG in the presence of graded concentrations of immunosuppressive drugs for 9 days, whereupon supernatants were tested for IgM (a) and IgG levels (b). B cells were cultured with immunosuppressive drugs for 6 days with CpG in the presence of pre-stimulated T cells. Depicted are the percentages of IgM (c) and IgG (d) levels compared to medium controls (dotted lines). Horizontal bars indicate mean values (n=5). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.*

inhibit IgM production (Figure 4c), whereas cyclosporin marginally inhibited IgG levels (Figure 4d). Thus, the inhibition of B cell responses by calcineurin inhibitors that was shown in Figure 4a and 4b appears solely due to inhibition of T cell help.

DISCUSSION

Chronic damage to transplanted organs can be caused by a variety of mechanisms, and in some of these, the binding of anti-HLA antibodies to the endothelium of the graft is implicated (*3*). For effective prevention and treatment of humoral rejection, immunosuppressive drugs should preferably affect the function of B cells as well as helper T cells, since T cell help by ligand interactions and cytokines activates B cells (*12*). Inhibition of T cell proliferation by calcineurin inhibitors and suppression of the transcription of the IL-2 gene, amongst others, is well known (*22*). Furthermore, calcineurin inhibitors attenuate T cell dependent Pokeweed Mitogen activation of B cells (*23, 24*). However, from these studies it is unclear whether the inhibition is due to inhibition of T cell help.

We have previously shown that calcineurin inhibitors are not efficient in directly inhibiting B cells, in cultures of purified B cells, activated in the absence of T cells (*14*). These data raised the question whether calcineurin inhibitors inhibit humoral immune responses by the inhibition of T cell help. We therefore set out to determine whether, by inhibition of T cell help, calcineurin inhibitors were capable of inhibiting B cell immunoglobulin production. Therefore, we developed a culture system in which B cells were activated in a T cell dependent fashion. Furthermore, we examined whether inhibition of T cell help was sufficient to completely abrogate immunoglobulin production.

The number of T cells available to provide help towards B cells may be reduced by inhibition of proliferation, or induction of apoptosis. We showed that, although T cells did get highly activated, all immunosuppressive drugs tested were capable of inhibiting proliferation. As expected, MPA and rapamycin, well known for their anti-proliferative effect (*25, 26*), were more potent in inhibition of T cell proliferation than the calcineurin inhibitors. Nonetheless, calcineurin inhibitors did inhibit T cell proliferation, which, at least partly, resulted in insufficient generation of activated T cells. None of the immunosuppressive drugs induced apoptosis in polyclonally activated T cells (data not shown).

The level of costimulation and cytokines mainly determines the strength of T cell help towards B cells. Thus a possible mechanism of drug-induced suppression of T cell dependent humoral immune responses is the reduction of B cell stimulatory signals, either as ligand interaction or as soluble mediators. All immunosuppressive drugs decreased the number of cells expressing the costimulatory ligands CD40L and ICOS, reducing the ability of T cells to activate B cells. This is reminiscent of patients with defective CD40L, who suffer from hyper-IgM syndrome (*27*) and patients with a homozygous deletion of the ICOS gene, who are severely antibody deficient (*28*). As expected, calcineurin inhibitors, but also rapamycin profoundly inhibited T cell IL-2 mRNA levels, which in turn can contribute

to the failure of B cell responses (*29*). Additionally, calcineurin inhibitors and rapamycin inhibited several other B cell differentiation cytokines, thereby abrogating B cell signals necessary for activation and class switching.

We performed T cell dependent B cells cultures to investigate whether the inhibition of T cell help by calcineurin inhibitors was of sufficient magnitude to cause downstream inhibition of B cell activation. Activation of T cells by immobilized anti-CD3 mAb eliminated the need for an antigen specific culture system to obtain immunoglobulin producing B cells (*30*). In the cocultures that we employed here, B cell activation was achieved via T cell activation and was cell contact dependent. In contrast to our previous findings in T cell free B cell cultures (*14*), tacrolimus and cyclosporin almost completely inhibited T cell dependent immunoglobulin production. The magnitude of inhibition was comparable to that caused by MPA and rapamycin, indicating that by inhibition T cell help, activation of B cells can be prevented.

It has previously been published that calcineurin inhibitors are incapable of inhibiting already activated T cells (*31-33*). This is in line with our current observations in cocultures of B cells with preactivated T cells, where tacrolimus and cyclosporin failed to inhibit immunoglobulin production. In contrast, MPA and rapamycin, which are able to inhibit activated T cells (*26, 33*) as well as to directly inhibit B cells (*14*), completely abrogated immunoglobulin production. The marginal inhibition of IgG levels by cyclosporin suggests minor inhibition of B cells directly, which however, is far less potent than its effect on T cell help. Taken together, our data show that calcineurin inhibitors can only prevent humoral responses by inhibiting T cell help. Consequently, these data stress the importance of targeting the T cell compartment to affect humoral immune responses.

ACKNOWLEDGEMENTS

This work was supported by The Landsteiner Foundation for Blood Transfusion Research and the National Reference Centre for Histocompatibility Testing. The authors thank Jacqueline Anholts for technical assistance in the Q-PCR assays and Anneke Brand and Frits Koning for critically reading the manuscript.

REFERENCES

1. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med 1969; 280 (14): 735.
2. Joosten SA, Sijpkens YW, van Kooten C, Paul LC. Chronic renal allograft rejection: pathophysiologic considerations. Kidney Int 2005; 68 (1): 1.
3. Lee PC, Terasaki PI, Takemoto SK, *et al.* All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. Transplantation 2002; 74 (8): 1192.
4. Mauiyyedi S, Pelle PD, Saidman S, *et al.* Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. J Am Soc Nephrol 2001; 12 (3): 574.
5. Hardy MA, Nowygrod R, Elberg A, Appel G. Use of ATG in treatment of steroid-resistant rejection. Transplantation 1980; 29 (2): 162.
6. Rocha PN, Butterly DW, Greenberg A, *et al.* Beneficial effect of plasmapheresis and intravenous immunoglobulin on renal allograft survival of patients with acute humoral rejection. Transplantation 2003; 75 (9): 1490.
7. Ibernon M, Gil-Vernet S, Carrera M, *et al.* Therapy with plasmapheresis and intravenous immunoglobulin for acute humoral rejection in kidney transplantation. Transplant Proc 2005; 37 (9): 3743.
8. Faguer S, Kamar N, Guilbeaud-Frugier C, *et al.* Rituximab therapy for acute humoral rejection after kidney transplantation. Transplantation 2007; 83 (9): 1277.
9. Becker YT, Becker BN, Pirsch JD, Sollinger HW. Rituximab as treatment for refractory kidney transplant rejection. Am J Transplant 2004; 4 (6): 996.
10. Shah A, Nadasdy T, Arend L, *et al.* Treatment of C4d-positive acute humoral rejection with plasmapheresis and rabbit polyclonal antithymocyte globulin. Transplantation 2004; 77 (9): 1399.
11. Noorchashm H, Reed AJ, Rostami SY, *et al.* B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. J Immunol 2006; 177 (11): 7715.
12. Blanchard D, Gaillard C, Hermann P, Banchereau J. Role of CD40 antigen and interleukin-2 in T cell-dependent human B lymphocyte growth. Eur J Immunol 1994; 24 (2): 330.
13. Steele DJ, Laufer TM, Smiley ST, *et al.* Two levels of help for B cell alloantibody production. J Exp Med 1996; 183 (2): 699.
14. Heidt S, Roelen DL, Eijsink C, van Kooten C, Claas FH, Mulder A. Effects of immunosuppressive drugs on purified human B cells: evidence supporting the use of MMF and rapamycin. Transplantation 2008; 86 (9): 1292.
15. Wood AJ, Lemaire M. Pharmacologic aspects of cyclosporine therapy: pharmacokinetics. Transplant Proc 1985; 17 (4 Suppl 1): 27.
16. Winkler M, Ringe B, Baumann J, Loss M, Wonigeit K, Pichlmayr R. Plasma vs whole blood for therapeutic drug monitoring of patients receiving FK 506 for immunosuppression. Clin Chem 1994; 40 (12): 2247.
17. Backman L, Nicar M, Levy M, *et al.* FK506 trough levels in whole blood and plasma in liver transplant recipients. Correlation with clinical events and side effects. Transplantation 1994; 57 (4): 519.
18. Lemaire M, Tillement JP. Role of lipoproteins and erythrocytes in the *in vitro* binding and distribution of cyclosporin A in the blood. J Pharm Pharmacol 1982; 34 (11): 715.
19. Nagase K, Iwasaki K, Nozaki K, Noda K. Distribution and protein binding of FK506, a potent immunosuppressive macrolide lactone, in human blood and its uptake by erythrocytes. J Pharm Pharmacol 1994; 46 (2): 113.
20. Diener KR, Moldenhauer LM, Lyons AB, Brown MP, Hayball JD. Human Flt-3-ligand-mobilized dendritic cells require additional activation to drive effective immune responses. Exp Hematol 2008; 36 (1): 51.
21. Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. J Exp Med 2007; 204 (13): 3095.
22. Wiederrecht G, Lam E, Hung S, Martin M, Sigal N. The mechanism of action of FK-506 and cyclosporin A. Ann N Y Acad Sci 1993; 696: 9.

23. Paavonen T, Hayry P. Effect of cyclosporin A on T-dependent and T-independent immunoglobulin synthesis *in vitro.* Nature 1980; 287 (5782): 542.
24. Stevens C, Lempert N, Freed BM. The effects of immunosuppressive agents on *in vitro* production of human immunoglobulins. Transplantation 1991; 51 (6): 1240.
25. Quemeneur L, Flacher M, Gerland LM, Ffrench M, Revillard JP, Bonnefoy-Berard N. Mycophenolic acid inhibits IL-2-dependent T cell proliferation, but not IL-2-dependent survival and sensitization to apoptosis. J Immunol 2002; 169 (5): 2747.
26. Kay JE, Kromwel L, Doe SE, Denyer M. Inhibition of T and B lymphocyte proliferation by rapamycin. Immunology 1991; 72 (4): 544.
27. Aruffo A, Farrington M, Hollenbaugh D, *et al.* The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. Cell 1993; 72 (2): 291.
28. Warnatz K, Bossaller L, Salzer U, *et al.* Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood 2006; 107 (8): 3045.
29. Johnson-Leger C, Christenson JR, Holman M, Klaus GG. Evidence for a critical role for IL-2 in CD40-mediated activation of naive B cells by primary CD4 T cells. J Immunol 1998; 161 (9): 4618.
30. Hirohata S, Jelinek DF, Lipsky PE. T cell-dependent activation of B cell proliferation and differentiation by immobilized monoclonal antibodies to CD3. J Immunol 1988; 140 (11): 3736.
31. Kay JE, Benzie CR. Effects of cyclosporin A on the metabolism of unstimulated and mitogen-activated lymphocytes. Immunology 1983; 49 (1): 153.
32. Kay JE, Benzie CR, Goodier MR, Wick CJ, Doe SE. Inhibition of T-lymphocyte activation by the immunosuppressive drug FK-506. Immunology 1989; 67 (4): 473.
33. Strauss G, Osen W, Debatin KM. Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs. Clin Exp Immunol 2002; 128 (2): 255.