Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.04051.x

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

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Accepted for publication 13 October 2009
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Summary

In general, humoral immune responses depend critically upon T cell help. In transplantation, prevention or treatment of humoral rejection therefore require drugs that ideally inhibit both B cell and T helper cell activity. Here, we studied the effects of commonly used immunosuppressive drugs [tacrolimus, cyclosporin, mycophenolic acid (MPA) and rapamycin] on T cell helper activity and on T cell-dependent B cell responses. T cells were activated polyclonally in the presence of immunosuppressive drugs in order to analyse the effect of these drugs on T cell proliferation, co-stimulatory ligand expression and cytokines. The impact of immunosuppressive drugs on T cell-dependent immunoglobulin production by B cells was addressed in T–B cell co-cultures. All drugs affected T cell proliferation and attenuated T cell co-stimulatory ligand (CD154 and CD278) expression when T cells were activated polyclonally. 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 co-cultures, where T cell help is essential for immunoglobulin production. In contrast, when pre-activated T cells were used to stimulate autologous B cells, calcineurin inhibitors 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. Furthermore, our studies support the necessity of intervening in T cell helper function to attenuate humoral responses.

Keywords: B cells, humoral rejection, immunoglobulin production, immunosuppression, T cell help

Introduction

Despite excellent 1-year graft survival rates, graft rejection remains an issue in solid organ transplantation. Although hyperacute rejection is avoided by pretransplant serological cross-matching [1] and acute rejection is treatable with current immunosuppressive drugs (ISD), rejection pathology still occurs and has shifted towards a later stage after transplantation. Typically, the cause of chronic organ failure is multi-factorial, involving both immunological and non-immunological 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-human leucocyte antigen (HLA) antibodies are detected frequently 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 ISD, 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 become properly activated when T cell

help is provided [12]. Furthermore, T cells are needed for B cell class-switching and production of potentially harmful immunoglobulin G (IgG) antibodies [13]. Besides cognate interaction via antigen, the T cell-mediated activation of B cells takes place through CD40L (CD154)–CD40 and inducible co-stimulator (ICOS; CD278)–ICOSL interaction, as well as through cytokine production and consumption. Therefore, drugs that act preferentially 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 stimulated strongly [14]. In the present study, we have investigated the effect of these ISD 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 and 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 isolated immunomagnetically 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 (Prograft, Astellas, Leiderdorp, the Netherlands, diluted in ethanol) and cyclosporin (Sandimmune; 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 ISD were diluted in culture medium.

Carboxyfluorescein succinimidyl ester (CFSE) assay

T cells (10^5) were CFSE (10 μM; Invitrogen), labelled for 10 min at 37°C and cultured with 5 μg/ml anti-CD28 monoclonal antibody (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 phycoerythrin (PE) labelled anti-CD4 mAb (BD Biosciences) and Sytox Red dead cell stain (Invitrogen, Paisley, UK) for dead cell exclusion. The proliferation index was calculated as follows (adapted from [20]):

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

Peak 1 represents the undivided peak. Data are expressed as percentage of the proliferation index relative to no addition of ISD. 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 mAb as described above in the presence or absence of ISD. 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 A_{260}/A_{280} 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 oligodeoxythymidylic acid (oligo-dT) primers, 500 ng of random primers and 1× 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,

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

IFN, interferon; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; RPL13a, ribosomal protein L13a; bp, base pairs.

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 programme consisted of one cycle of 10 min at 95°C, 40 cycles of 15 s 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, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, hypoxanthine-guanine phosphoribosyltransferase (HPRT), hydroxymethylbilane synthase (HMBS) and ribosomal protein L13a (RPL13a) served as a normalization factor to minimize general, if any, effects of ISD.

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 ISD. Cells were harvested and labelled with the following mAb conjugates: CD4-peridinin chlorophyll (PerCP), CD25-PE, CD154-PE, CD278-PE and CD69-fluorescein isothiocyanate (FITC) (all from BD Biosciences). Dead cells were excluded using Sytox Red. Cells were acquired using a fluorescence activated cell sorter (FACS)Calibur and analysed using CellQuest Pro software (BD Biosciences).

T and B cell co-cultures

T cells ($1{\cdot}5 \times 10^3$) were stimulated for 9 days with anti-CD3 mAb/anti-CD28 mAb as described above in the presence of autologous B cells ($1{\cdot}5 \times 10^5$) 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 interleukin (IL)-2 (EuroCetus, Amsterdam, the Netherlands) for 2 days. After washing, 5×10^4 T cells were co-cultured with 5×10^4 B cells with the addition of CpG for 6 days instead of 9 days, because T cell were already activated in the preculture. Furthermore, cells were cultured in a 1:1 ratio, because co-cultures 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 enzyme-linked immunosorbent assay (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 phosphate-buffered saline (PBS-T). Fifty microlitres 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 colour reaction was obtained with 4·6 mM 2,2′-azine-bis(3-ethylbenzthiazoline-6-sulphonic 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 optical density $(OD)_{450\,nm}$ in an ELISA reader (Bio-Rad). Data were analysed using the Microplate Manager software version 4 (Bio-Rad).

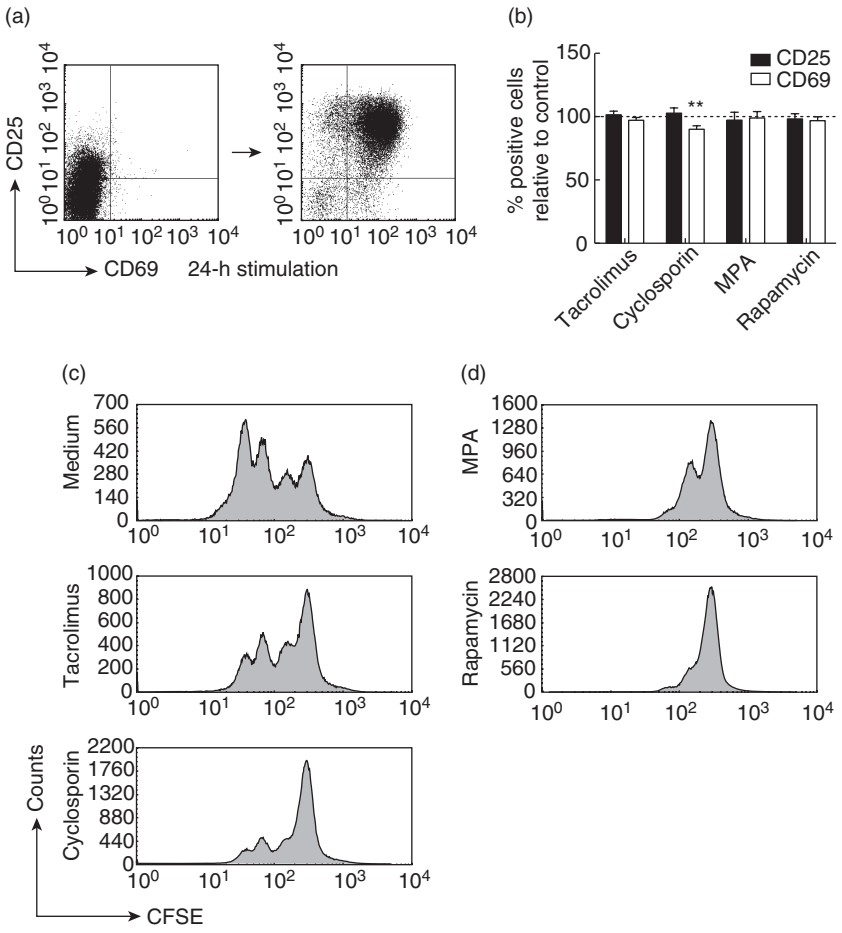

Fig. 1. Immunosuppressive drugs (ISD) did not alter T cell activation but did inhibit T cell proliferation. T cell activation with anti-CD3 monoclonal antibody (mAb)/anti-CD28 mAb for 24 h resulted in up-regulation of CD25 and CD69 (a). None of the ISD affected CD25 levels, whereas cyclosporin inhibited the expression of CD69. Drug concentrations were 1·0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml mycophenolic acid (MPA) and 8·0 ng/ml rapamycin. Data are expressed as percentage of cells expressing CD25 or CD69 compared to medium controls (dotted lines), $n = 4$ (b). Carboxyfluorescein succinimidyl ester (CFSE)-labelled T cells were activated with anti-CD3 mAb/anti-CD28 mAb for 3 days in the presence of ISD. Data from a representative experiment are shown. Similar results were obtained in three independent experiments (c). Percentage inhibition of the T cell proliferation index by ISD compared to medium controls (dotted lines) are depicted, $n = 3$ (d). Drug concentrations were: 0·3 ng/ml tacrolimus, 50 ng/ml cyclosporin, 100 ng/ml MPA and 4 ng/ml rapamycin. $^{**}P < 0{\cdot}01$.

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 ISD. Statistical level of significance was defined as $P < 0{\cdot}05$.

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 (Fig. 1a). None of the ISD altered the percentage of $CD25^+$ cells, whereas only cyclosporin slightly inhibited the percentage of $CD69^+$ cells (Fig. 1b). Additionally, polyclonal T cell activation resulted in a strong proliferative response, as measured by CFSE (Fig. 1c, upper left panel). Because ISD profoundly inhibit T cell proliferation, high doses of ISD resulted in low cell yields, insufficient for analysis. Therefore, we used suboptimal drug concentrations to address the effect of ISD on T cell proliferation (Fig. 1c). These relatively low, but clinically relevant, drug concentrations caused strong inhibition of the T cell proliferation index (Fig. 1d).

Effect of immunosuppressive drugs on T cell helper function

Next, we investigated the effect of ISD on T cell helper signals, by addressing co-stimulatory 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 (Fig. 2a). All ISD 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, compared to tacrolimus, MPA and rapamycin (Fig. 2b).

We determined the effects of ISD 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 interferon (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

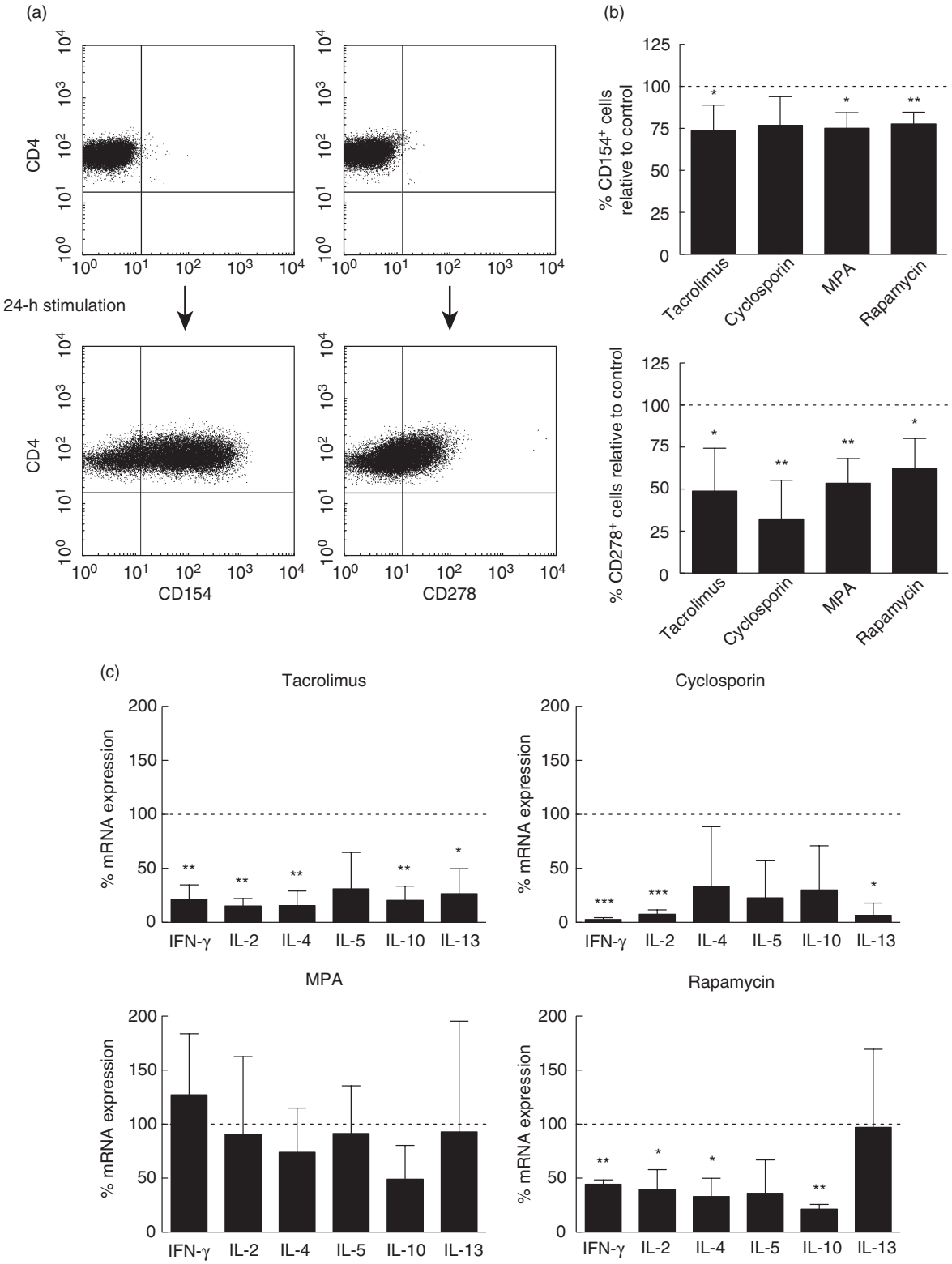

Fig. 2. Effect of immunosuppressive drugs (ISD) on T cell stimulatory signals. T cell stimulation with anti-CD3 monoclonal antibody (mAb)/anti-CD28 mAb for 24 h resulted in up-regulation of CD154 and CD278 (a). All ISD inhibited the expression of CD154 and CD278. Drug concentrations were 1·0 ng/ml tacrolimus, 100 ng/ml cyclosporin, 100 ng/ml mycophenolic acid (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), $n = 4$ (b). ISD 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, $n = 3$. Drug concentrations are identical to those of Fig. 2b (c). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

(data not shown). Tacrolimus inhibited all cytokines tested, although IL-5 inhibition did not reach statistical significance (Fig. 2c). Similarly, 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 ISD 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, as 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 (Fig. 3a). In these T–B cell co-cultures, 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, as co-cultures of B cells with prestimulated T 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 (Fig. 3b).

To address the effects of ISD on T cell-dependent B cell cultures, freshly isolated T and B cells were co-cultured with ISD 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 compared to MPA and rapamycin, indicating that inhibition of T cell help by calcineurin inhibitors is sufficient to prevent immunoglobulin production (Fig. 4a and b).

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 co-culture with autologous B cells. MPA and rapamycin inhibited immunoglobulin levels almost completely, ensuring that the B cells in this culture system were susceptible to inhibition by ISD (Fig. 4c and d). In contrast, tacrolimus and cyclosporin failed to inhibit IgM production (Fig. 4c), whereas cyclosporin marginally inhibited IgG levels (Fig. 4d). Thus, the inhibition of B cell responses by calcineurin inhibitors that is shown in Fig. 4a and b appears due solely to inhibition of T cell help.

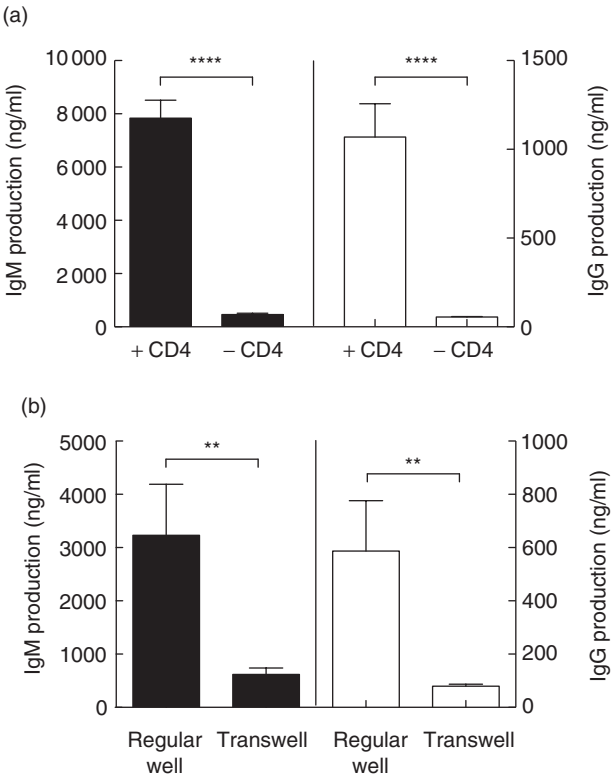

Fig. 3. T cells were necessary for B cell activation in a cell–cell contact-dependent fashion. B cells were cultured in anti-CD3 monoclonal antibody (mAb)-coated wells with soluble anti-CD28 mAb in the absence or presence of autologous T cells. After 9 days supernatants were harvested for immunoglobulin assessment (a). Co-cultures of B cells and prestimulated 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 (b). $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$.

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, ISD should preferably affect the function of B cells as well as helper T cells, as T cells help by ligand interactions and cytokines activate B cells [12]. Inhibition of T cell proliferation by calcineurin inhibitors and suppression of the transcription of the IL-2 gene, among 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.

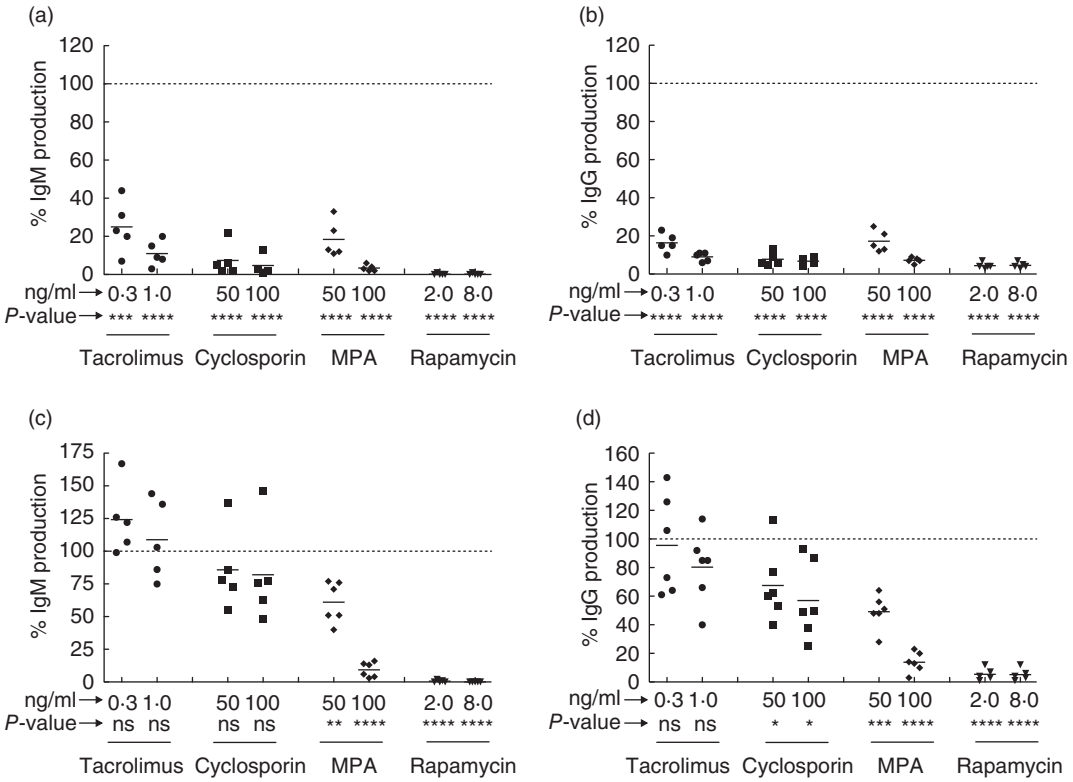

Fig. 4. All immunosuppressive drugs (ISD) were capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but calcineurin inhibitors failed to inhibit immunoglobulins levels when pre-activated T cells were used to stimulate B cells. B cells were cultured with fresh, autologous T cells and anti-CD3 monoclonal antibody (mAb)/anti-CD28 mAb with cytosine-guanine dinucleotide (CpG) in the presence of graded concentrations of ISD for 9 days, whereupon supernatants were tested for immunoglobulin M (IgM) (a) and IgG levels (b). B cells were cultured with ISD for 6 days with CpG in the presence of prestimulated T cells. Depicted are the percentages of IgM (c) and IgG (d) levels compared to medium controls (dotted lines). Horizontal bars indicate mean values ($n = 5$). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$; ****$P < 0{\cdot}0001$.

We have shown previously 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 of 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 abrogate immunoglobulin production completely.

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 become highly activated, all ISD 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. None the less, calcineurin inhibitors inhibited T cell proliferation which, at least partly, resulted in insufficient generation of activated T cells. None of the ISD induced apoptosis in polyclonally activated T cells (data not shown).

The level of co-stimulation 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 ISD decreased the number of cells expressing the co-stimulatory 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 cell 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 co-cultures 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 inhibited T cell-dependent immunoglobulin production almost completely. The magnitude of inhibition was comparable to that caused by MPA and rapamycin, indicating that by inhibition of T cell help, activation of B cells can be prevented.

It has been published previously that calcineurin inhibitors are incapable of inhibiting already activated T cells [31–33]. This is in line with our current observations in co-cultures of B cells with pre-activated 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 inhibit B cells directly [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 Center for Histocompatibility Testing. The authors thank Jacqueline Anholts for technical assistance in the q-PCR assays and Anneke Brand and Frits Koning for critical reading of the manuscript.

Disclosure

The authors have no financial conflict of interest.

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