



Inhibition of T Helper Cell Differentiation by Tacrolimus or Sirolimus Results in Reduced B-Cell Activation: Effects on T Follicular Helper Cells

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ABSTRACT

The effect of immunosuppressive drugs on the generation of T follicular helper (Tfh) cells, specialized in supporting B-cell differentiation, is largely unknown. We examined whether the calcineurin inhibitor tacrolimus (TAC) and the mammalian target of rapamycin (*mtor*) inhibitor sirolimus (SRL) inhibit Tfh cell differentiation, and affect subsequent B-cell functions. Isolated naive T cells were polarized into Tfh-like cells in the presence of TAC or SRL. To demonstrate their functionality, we co-cultured these cells with isolated B cells in the presence of alloantigen and studied the activation and differentiation of these B cells. Tfh-like cells were defined as CD4⁺CXCR5⁺ T cells, expressing immunoinhibitory programmed death protein 1 (*pd1*) and inducible T-cell costimulator (*icos*). We found that TAC and SRL significantly inhibited Tfh-like cell differentiation. Therapeutic concentrations of TAC and SRL reduced the percentage of *pd1*⁺ and *icos*⁺ Tfh cells compared to controls. In addition, T cells grown in the presence of TAC or SRL expressed less IL-21 and provided less B-cell help. TAC and SRL both inhibited Tfh-dependent alloantigen-activated B-cell proliferation and differentiation into plasma cells and transitional B cells. In conclusion, TAC and SRL inhibited the differentiation of naive T cells into functional Tfh-like cells, a finding that can be extrapolated to immunosuppressive regimens in transplant patients.

WORLDWIDE, over 100,000 transplantations are performed each year, with numbers rising annually. In spite of this increase, the number of patients requiring a transplant is rising even more rapidly and exceeds the number of organs available for transplantation. Although the lifespan of a transplanted organ has increased over the last decade due to the introduction of new pharmaceutical agents and improved diagnostics, the loss of organ transplants over 10 years is still at 30%, of which 30% to 50% is caused by chronic humoral rejection (ie, antibody-mediated rejection, or ABMR) [1,2]. Current strategies to target ABMR are generally not used as maintenance therapy and have several side effects. Improved treatment strategies are therefore needed to more specifically prevent and treat this type of rejection [3].

After transplantation, a graft can not only be rejected due to humoral immunity, but also cellular immunity (ie, T cell-mediated rejection, or TCMR). And although the names suggest that these rejections are mediated by only antibodies or T cells, the opposite is quite true. Previous

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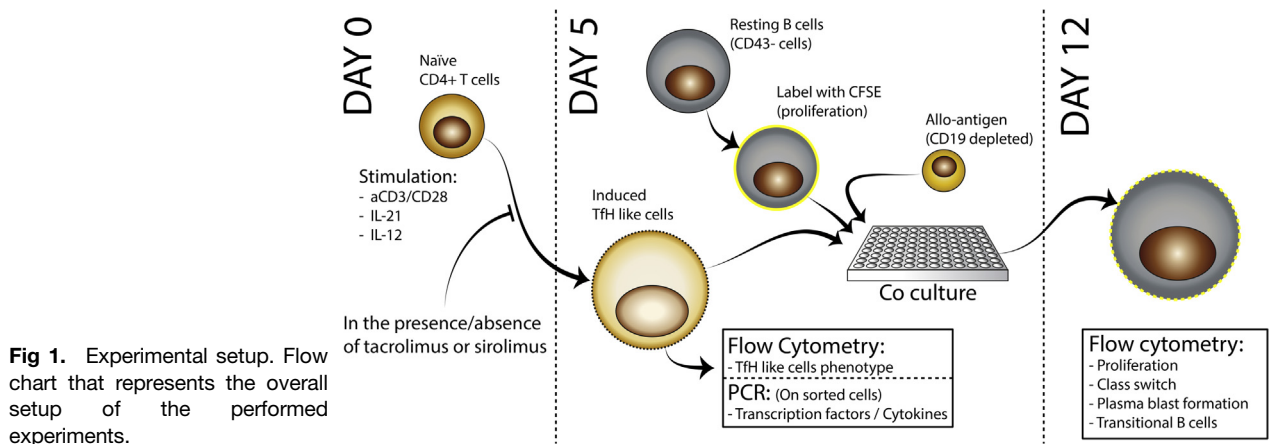


Fig 1. Experimental setup. Flow chart that represents the overall setup of the performed experiments.

studies reported that in TCMR, as well as ABMR, and both infiltrating B cells and T cells, can be found [4]. Different T helper subsets, including T helper 1 (Th1), T helper 2 (Th2), regulatory T (Treg) cells, and T follicular helper (Tfh) cells fulfill different roles in transplant alloreactivity. For example, whereas Th1 cells are mostly mediators of TCMR, Tfh cells are important mediators of B cell-mediated humoral immunity [5,6]. Activation of these Tfh cells consists of 3 signals: (1) the interaction of T-cell receptors with HLA-presented alloepitope; (2) costimulation molecules, such as *pd1* and *icos*; and (3) the cytokine interleukin (IL) 21. Peripheral blood CD4⁺CXCR5⁺ T cells represent the circulating memory compartment of Tfh-lineage cells [7]. Importantly, circulating CD4⁺CXCR5⁺ T cells expressing high levels of the coinhibitory molecule *pd1* reflect active Tfh cell differentiation in lymphoid tissues. This CD4⁺ T-cell subtype is

specialized in supporting B-cell activation and differentiation (plasma blasts and transitional B cells) [8–11]. In organ transplantation, this can lead to the secretion of donor-specific antibodies [6,10–12], resulting in ABMR in patients with a transplant who are receiving lifelong immunosuppression [12,13]. Tfh cells are thought to be derived from naive T cells, and differentiation toward the Tfh phenotype is most likely initiated by dendritic cell interaction. This is primarily mediated by IL-6, IL-12, IL-21, and transforming growth factor β [10–14]. These naive T cells will then upregulate B-cell lymphoma 6 (*bcl6*) protein, which is an important transcription factor for Tfh cell differentiation. In contrast, IL-2 and IL-10 have been described as negative regulators of Tfh differentiation. These cytokines activate the transcription factor B lymphocyte-induced maturation protein 1 (*blimp1*), which inhibits *bcl6* expression [15–17].

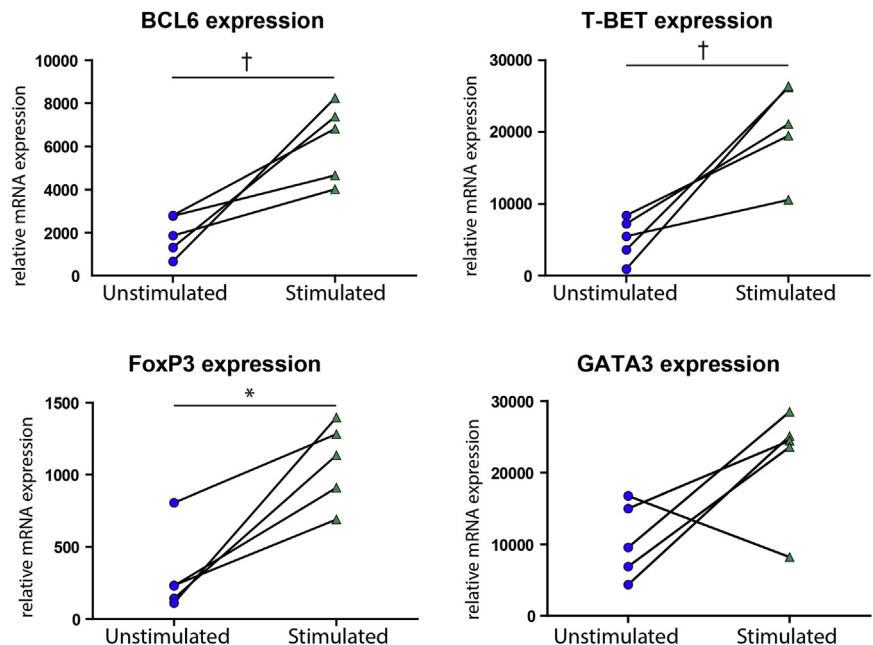


Fig 2. Upregulation of transcription factors. After stimulating CD4⁺ naive T helper cells for 5 days with anti-CD3/28 antibodies, goat antimouse antibodies, and IL-12 and IL-21, we found an upregulation of the transcription factors *bcl6*, *tbet*, *gata3*, and *foxp3*. Expression of *ror γ t* was not detected in both conditions and therefore not shown (n = 5). Note. Mann-Whitney testing was used. *Indicates $P < .05$. †Indicates $P < .01$.

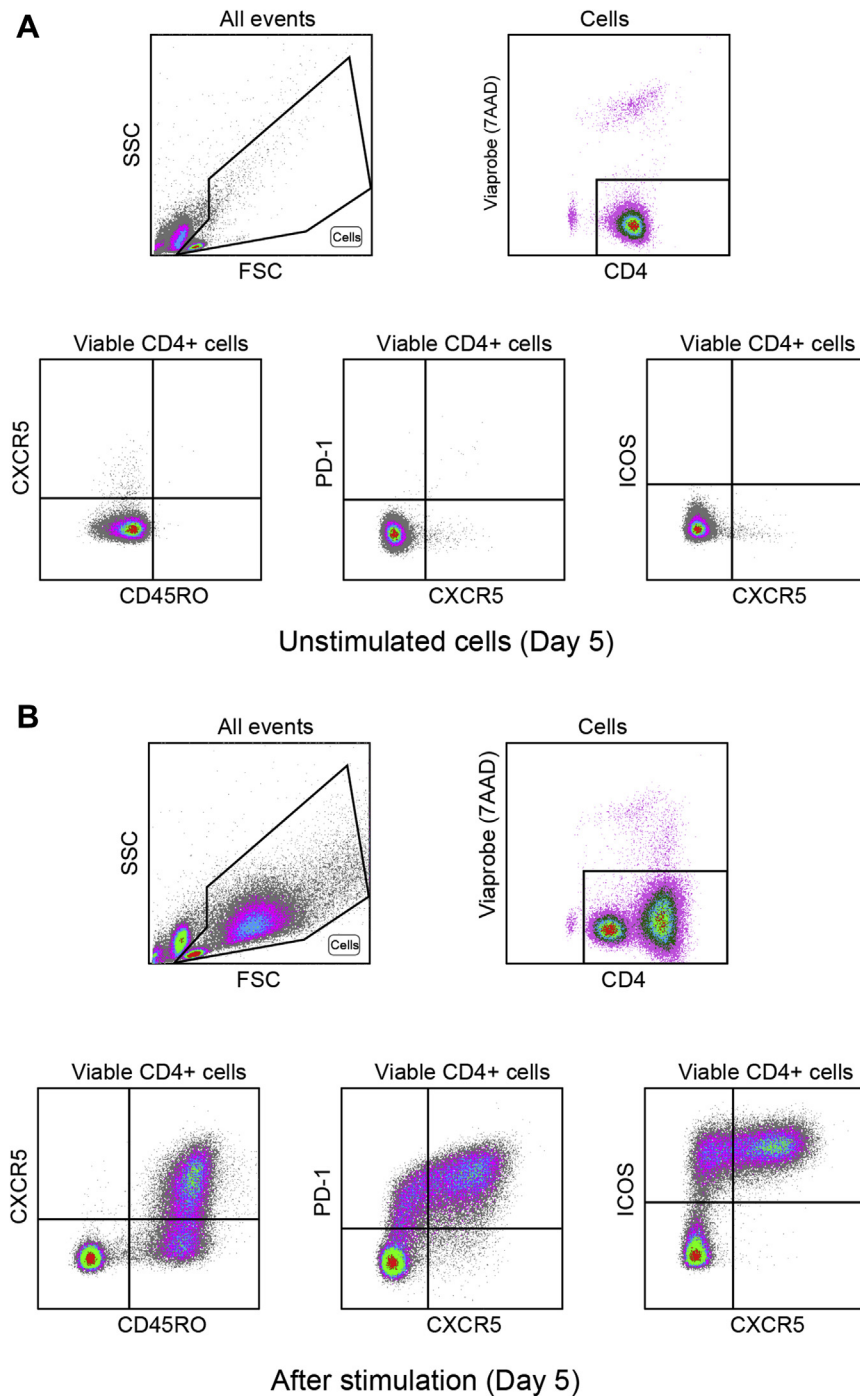


Fig 3. Representative FACS plots showing the gating strategy to determine the frequency of Tfh ($CD4^+CXCR5^+PD1^{+hi}$ and $CD4^+CXCR5^+ICOS^{+hi}$ T cells). **(A)** Flow cytometry plots of the frequency of $CD45RO^+$ memory $CD4^+$ T cells, $CD4^+CXCR5^+$ cells, $CXCR5^+pd1^{+hi}$ cells, and $CXCR5^+ICOS^{+hi}$ cells after 5-day culture of $CD4^+CD45RA^+$ naive T cells without stimulation. **(B)** Flow cytometry plots of the expression of $CD45RO^+$ memory cells, $CD4^+CXCR5^+$ cells, $CXCR5^+pd1^{+hi}$ cells, and $CXCR5^+ICOS^{+hi}$ cells after 5-day culture of $CD4^+CD45RA^+$ naive T cells with the stimulation of anti-CD3/28 antibodies, goat antimouse antibody, IL-21, and IL-12. FACS, fluorescence-activated cell sorting; Tfh, T follicular helper.

After organ transplantation, patients must remain on lifelong immunosuppression, such as tacrolimus (TAC) and sirolimus (SRL), to prevent transplant rejection. TAC inhibits the calcineurin pathway, thereby blocking the transcription of T-cell growth factors, such as IL-2 [18–20]. SRL is a mammalian target of rapamycin (*mTOR*) inhibitor that inhibits multiple cellular processes, such as IL-2 mediated

T-cell proliferation [21]. These agents might affect Tfh as well as other T helper subtype cell activity and subsequent B-cell responses in different ways. This can be explained by the fact that IL-2, a cytokine inhibited by TAC, antagonizes Tfh cell differentiation [22]. TAC has been shown to prevent B cell-mediated humoral alloreactivity in patients with transplant by inhibiting B-cell proliferation [23,24].

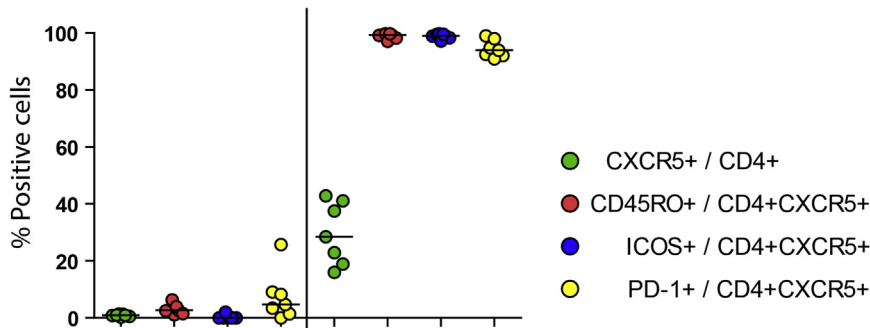


Fig 4. The differentiation of naive T helper cells into T follicular helper cells. The percentage of CXCR5⁺ within CD4⁺ T cells and the percentage of CD45RO⁺, CXCR5⁺, *icos*^{+hi}, and *pd1*^{+hi} cells within CD4⁺CXCR5⁺ T cells are depicted, with and without stimulation, proving that all CXCR⁺ CD4⁺ T cells generated are highly positive for these markers. Note. Data are presented as the median of the percentage. *icos*, inducible T-cell costimulator; *pd1*, immunoinhibitory receptor programmed death 1.

However, its effect on T cell-dependent antibody production has been inconclusive [25,26]. In contrast, SRL affects both T cell-dependent and T cell-independent B-cell proliferation and antibody production, while also increasing B-cell apoptosis [26].

In our previous clinical study in renal transplant recipients, we found that the frequency of CD4⁺CXCR5⁺*pd1*^{+hi} Tfh cells was significantly higher in the TAC-treated group than in the SRL-treated cohort, suggesting that SRL might suppress the generation of Tfh cells more effectively than TAC in vivo [26]. It is unknown whether this observation is the result of the combination of immunosuppressive therapy given to transplant recipients. This makes it difficult, if not impossible, to draw conclusions

about the impact of TAC and SRL on Tfh cell differentiation and their benefit to B cells in vivo.

In this study, we reported the effect of TAC and SRL on Tfh-like cell generation using an in vitro co-culture model [27]. Isolated pure naive human CD4⁺ T cells were stimulated with anti-CD3/28, IL-12, and IL-21 in the presence of different concentrations of TAC or SRL. The Tfh-like cells generated in vitro were studied for their frequency, phenotype, and capacity to help alloantigen-activated B cells.

MATERIAL AND METHODS

Polarization of Tfh-Like Cells

Peripheral blood of 7 healthy controls was collected according to the biobank protocol, which was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, The Netherlands (MEC-2010-022). All individuals gave written informed consent before the start of the study. The work was performed in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (VWR, Amsterdam, the Netherlands). Naive CD4⁺ T cells were isolated using the CD4⁺ Naive T-Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with an AutoMacs (Miltenyi Biotec). Purity of the isolated population was determined by flow cytometry by staining with CD4-FITC (BD Biosciences, Vianen, the Netherlands) and CD45RA-BV421 (Biolegend, London, United Kingdom) and was > 95% for all samples.

Isolated naive CD4⁺ T cells were stimulated with a cocktail consisting of anti-CD3 antibody (2 µg/mL; BD Biosciences), anti-CD28 antibody (2 µg/mL) (BD Biosciences), goat antimouse antibody (2 µg/mL) (BD Biosciences), IL-21 (20 ng/mL; R&D systems, Abingdon, United Kingdom), and IL-12 (20 ng/mL; PeproTech, London, United Kingdom) in a 96-well plate at 100,000 CD4⁺ T cells per well for 5 days [27]. This stimulation was performed in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) in the presence of different concentrations of TAC (0, 0.5, 1, 5, 10, and 20 ng/mL) or SRL (rapamycin) (0, 1, 2, 5, and 10 ng/mL) diluted in culture medium. After 5 days, the stimulated T cells were harvested and stained for CD3-BV510 (BioLegend), CD4-FITC (BD Biosciences), CXCR5-AF647 (BD Biosciences), ICOS-PE-

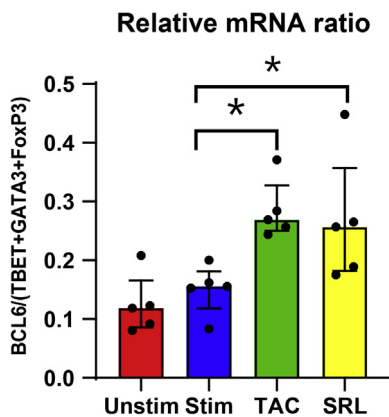


Fig 5. Effect of TAC and SRL on *bcl6/tbet* ratio. This figure shows the ratio of the relative mRNA expression levels of the transcription factor *bcl6* with *tbet*, *gata3*, and *foxp3* combined by CD4⁺ naive T helper cells before stimulation (day 0) and sorted viable CD3⁺CD4⁺ T cells after stimulation (day 5) in the presence or absence of TAC (1 ng/mL) or SRL (1 ng/mL). The data show the effect TAC on the polarization of Tfh cells in favor of Th1 cells. (n = 5) Note. Different conditions were compared using Mann-Whitney testing. SRL, sirilimus; TAC, tacrolimus; Tfh, T follicular helper; Th1, type 1 T helper cell. *Indicates $P < .01$.

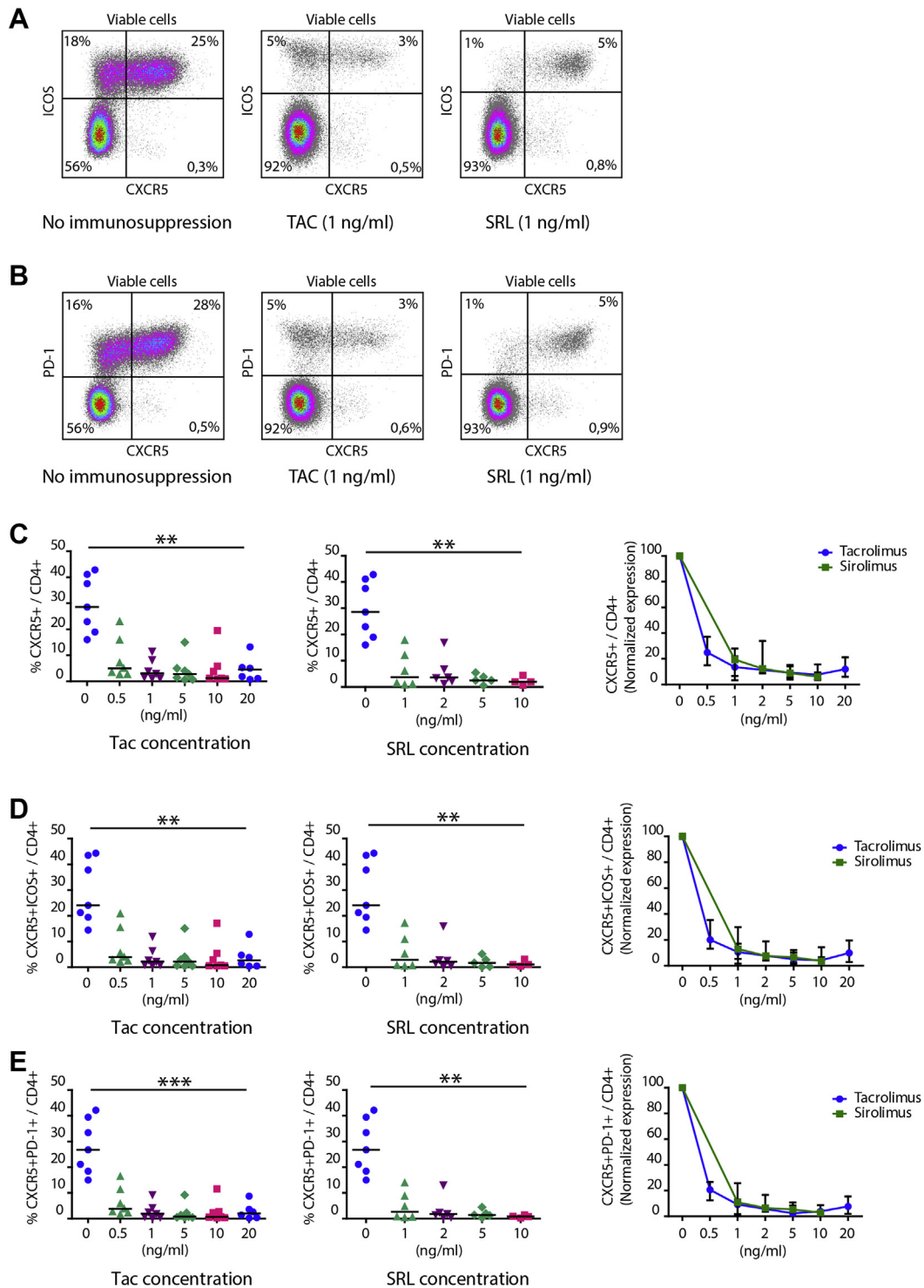


Fig 6. Effect of TAC and SRL on Tfh cell differentiation. Flow cytometry plots of the differentiation into **(A)** CXCR5⁺icos⁺ and **(B)** CXCR5⁺pd1⁺ Tfh cells in the presence of TAC (1 ng/mL) and SRL (1 ng/mL). **(C)** The percentage of CXCR5⁺ Tfh cells in the presence of different concentrations of TAC (0.5, 1, 5, 10, 20 ng/mL) and SRL (1, 2, 5, 10 ng/mL). The right graph shows the normalized data, as compared to no inhibition. **(D)** The percentage of CXCR5⁺icos⁺ Tfh cells. **(E)** The percentage of CXCR5⁺pd1⁺ Tfh cells. Note. Data are presented as the median of the percentage and error bars show interquartile range. Kruskal-Wallis testing was used. icos, inducible T-cell costimulator; pd1, immunoinhibitory receptor programmed death 1; SRL, sirolimus; TAC, tacrolimus; Tfh, T follicular helper. *Indicates $P < .05$.

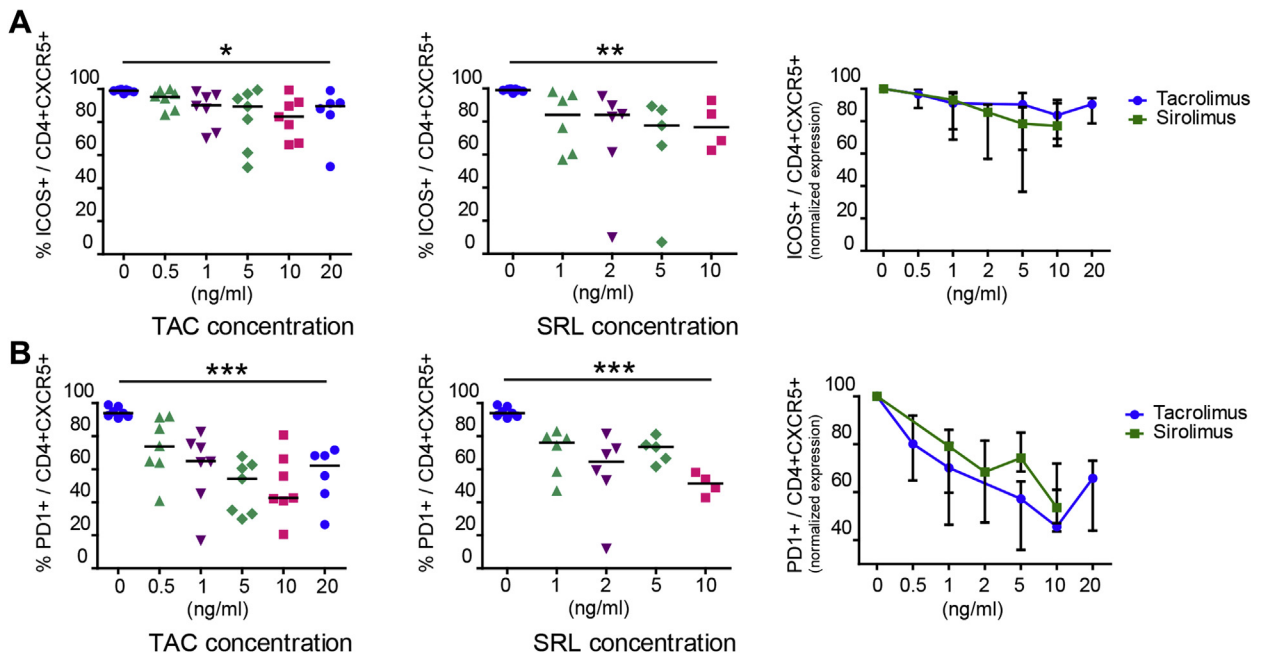


Fig 7. Effect of TAC and SRL on *icos* and *pd1* expression on CD4⁺CXCR5⁺ T cells. **(A)** The percentage of *icos*^{hi}/CD4⁺CXCR5⁺ T cells in the presence of different concentrations of TAC (0.5, 1, 5, 10, 20 ng/mL) and SRL (1, 2, 5, 10 ng/mL) **(B)** *pd1*^{hi}/CD4⁺CXCR5⁺ in the presence of different concentrations of TAC (0.5, 1, 5, 10, 20 ng/mL) and SRL (1, 2, 5, 10 ng/mL). Note. Each dot represents one sample. Data are presented as the median of the percentage and error bars show interquartile range. Kruskal-Wallis testing was used. *icos*, inducible T-cell costimulator; *pd1*, immunoinhibitory programmed death protein 1; SRL, sirolimus; TAC, tacrolimus. *Indicates $P < .05$. †Indicates $P < .01$.

Cy7 (BioLegend), PD1-APC-Cy7 (BioLegend), CD45RO-BV421 (BioLegend), and 7-AAD ViaProbe (BD Biosciences) for 30 minutes at room temperature and measured on a fluorescence-activated cell sorting (FACS) Canto 2 (BD Biosciences).

We repeated this experimental setup ($n = 5$) to determine mRNA expression of IL-21 and the transcription factors of 5 different T helper subsets, that is, *tbet* (Th1), *gata3* (Th2), *foxp3* (Treg), *roryt* (Th17), and *bcl6* (Tfh). The stimulation was performed as described above in the presence or absence of 1 ng/mL TAC or 1 ng/mL SRL. Next, we stained these stimulated cells for CD3-BV510 (BioLegend), CD4-BV421 (BioLegend), CXCR5-AF647 (BD Biosciences), PD1-APC-Cy7 (BioLegend), CD45RO-PE-Cy7 (BD Biosciences), and 7AAD ViaProbe (BD Biosciences) for 30 minutes. Viable CD3⁺CD4⁺ T cells were isolated with FACSAria II (BD Biosciences). RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany) for the collection of high-quality RNA. Total RNA was subsequently reverse transcribed with oligo-deoxythymine primer. We studied mRNA expression levels by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) using the following PCR primers: IL-21 (HS 00222327-M1), *bcl6* (HS 00153368-M1), *tbet* (HS 00894392-M1), *foxp3* (HS 00203958-M1), *roryt* (HS 00172858-M1), and *gata3* (HS 00231122-M1) (ThermoFisher Scientific, Waltham, Mass, United States). The expression level of each target gene was quantified by measuring the threshold cycle (Ct). This was then transformed on a TaqMan Real-Time PCR system (ThermoFisher Scientific) to the number of cDNA copies ($2^{(40-Ct)}$) (When the threshold is exceeded, every cycle before the last cycle of 40 means a doubling of the amount of copies) and their relative concentrations were normalized to relative

concentrations of 18S (HS 99999901-S1) the housekeeping gene present in each sample. [28]

T- and B-Cell Interaction Assays

The B-cell activating properties of the induced Tfh-like cells after Tfh polarization conditions were studied in co-culture experiments with isolated B cells [8,29]. B cells were isolated by depletion of CD43⁺ cells by using a CD43 isolation kit in combination with an AutoMacs (Miltenyi Biotec) Afterwards the cells were labeled with CellTrace CFSE (Invitrogen, Paisley, United Kingdom). B cells (50,000 per well) were co-cultured for 7 days with induced Tfh-like cells (25,000 per well) in a 96-well plate in the presence of 50,000 irradiated (40 Gy) allogeneic PBMCs [11] PBMCs from which the B cells were depleted (CD19 isolation kit in combination with AutoMacs) [11] in RPMI-1640 with 10% FBS. At day 7 the cells were harvested and stained with 7AAD ViaProbe (BD Biosciences), CD4-PerCP (BD Biosciences), CD19-BV510 (Biolegend), CD27-PE-Cy7 (BD Biosciences), CD38-BV421 (BD Biosciences), IgM-PE (BD Biosciences), IgD-APC-Cy7 (BioLegend), and measured using a FACSCanto II (BD Biosciences) flow cytometer. A flow chart summarizing the experimental setup can be found in Fig 1.

Data Analyses

All flow cytometry results were analyzed using Kaluza Software version 1.5 (Beckman Coulter, Woerden, the Netherlands). Statistical analyses were performed using GraphPad Prism, software version 5.0.1 (GraphPad Software Inc, San Diego, Calif, United States). Mann-Whitney testing was used to determine significant differences in relative mRNA expression. Kruskal-Wallis testing

was used to compare the changes under the 2 drug treatments with different concentrations in the Tfh differentiation experiments. Wilcoxon signed-rank testing was used to determine significant differences in the T- and B-cell co-culture experiments. *P* values less than .05 were considered significantly different.

RESULTS

In Vitro Differentiation of Naive T Cells

CD4⁺ naive T cells were stimulated with anti-CD3/CD28 antibodies, goat antimouse antibody, IL-21, and IL-12 for 5 days (Fig 1). Next, the viable CD3⁺ CD4⁺ T cells were isolated. In these isolated samples the expression of the different transcription factors was measured. After stimulation, mRNA levels of *bcl6*, *tbet*, *foxp3*, and *gata3* were increased (Fig 2). In this culture system no positive signal was found for *roryt*.

Fig 3 depicts the gating strategy of unstimulated CD4⁺ (naive) T cells (Fig 3A) and their differentiation into Tfh-like cells after a 5-day stimulation (Fig 3B). After stimulation, a substantial proportion of the naive T cells differentiated into CD4⁺CXCR5⁺ T cells. These cells also express CD45RO (for memory T cells), and the costimulatory markers *pd1* and *icos* (Fig 4).

Effect of TAC and SRL on T-Cell Differentiation

The effect of TAC and SRL on the expression of transcription factor *bcl6* within the Tfh-like cells was examined on sorted

and stimulated CD3⁺CD4⁺ T cells (Fig 5). Of interest are our findings that in the presence of subtherapeutic concentrations of TAC (1 ng/mL) and SRL (1ng/mL). Of the residual, low number of SRL and TAC nonsensitive T cells the balance between *bcl6* and the other non-Tfh cell transcription factors was studied. In the T cells, a shifted balance toward a higher proportion of *bcl6* mRNA was found in both the TAC and SRL experiments (Fig 5). Next, we defined the effect of TAC and SRL on CD4⁺CXCR5⁺ T cells. Typical examples of these Tfh-like cells in the presence or absence of TAC and SRL are shown in Fig 6A and 6B. Within the CD4⁺ T cells 29% (median; range 16%-43%) were CXCR5⁺ cells (Fig 6C), 27% (15%-42%) of these CD4⁺ T cells expressed both CXCR5 and *pd1* cells, and 24% (15%-44%) of the CD4⁺ T cells expressed both CXCR5 and *icos* (Fig 6D). Due to the low yields of CD4⁺ naive T cells after isolation, not all of the conditions have been met for a number of samples. In the presence of both TAC and SRL, even at subtherapeutic doses of 1 ng/mL, the differentiation of naive T cells toward CD4⁺CXCR5⁺, CD4⁺CXCR5⁺*icos*⁺ and CD4⁺CXCR5⁺*pd1*⁺ T cells was inhibited by > 90% (Fig 6D-E). The expression of *pd1* and *icos* was reduced within the residual CD4⁺CXCR5⁺ T cells (both *P* < .05; Fig 7).

TAC and SRL Inhibit Tfh-Like Cell Help to B Cells

A typical example of B-cell proliferation and differentiation is shown in Fig 8. After allogeneic stimulation, the T

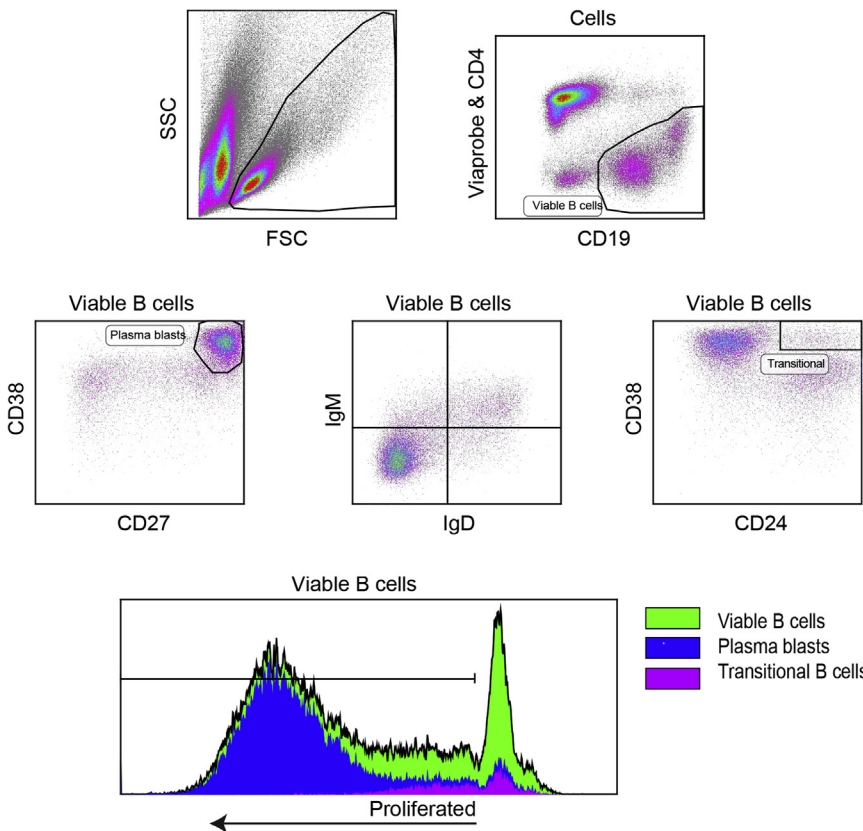


Fig 8. Analysis of B cells after co-culture with induced Tfh cells and alloantigen for 7 days. This figure shows representative FACS plots with an example of the gating strategy used to identify the frequency of plasmablasts, class-switched B cells, transitional B cells, and the level of B-cell proliferation. FACS, fluorescence-activated cell sorting; Tfh, T follicular helper.

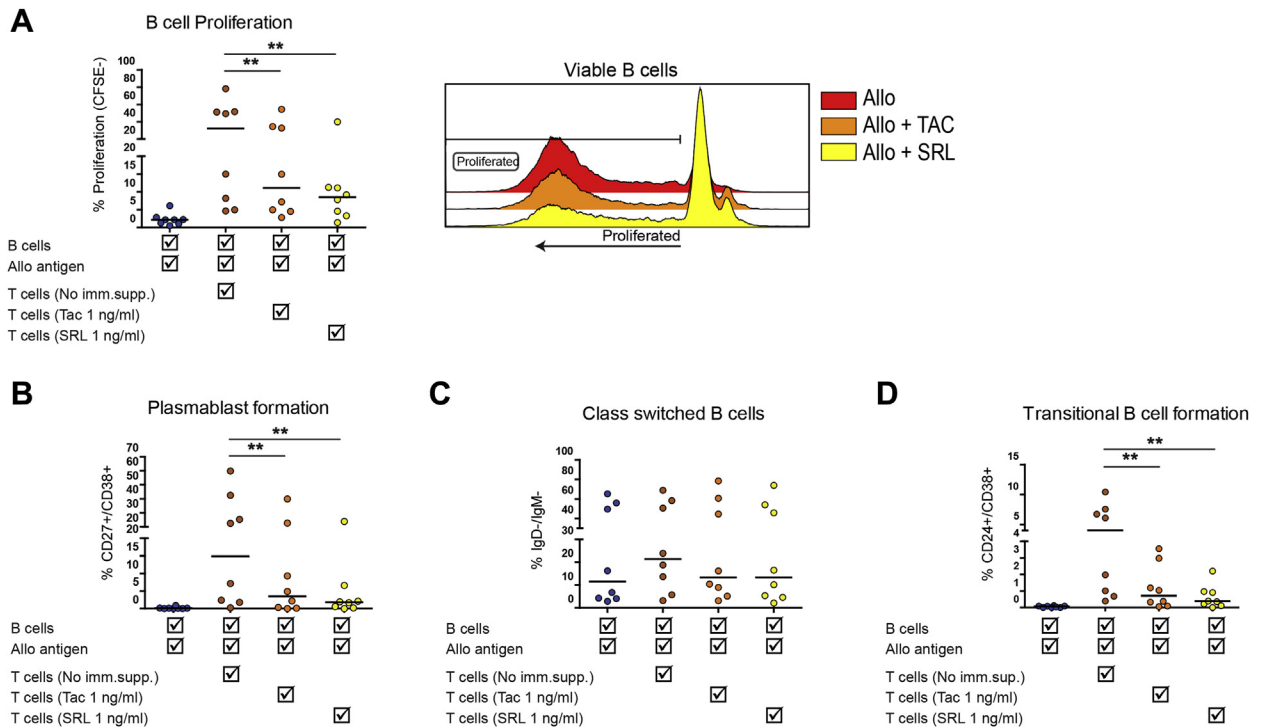


Fig 9. Differentiation of B cells into different subsets. Functional effect of the induced follicular helper-like cells on the activation and differentiation of B cells when generated in the absence and presence of TAC (1 ng/mL) and SRL (1 ng/mL). **(A)** The frequency of proliferating B cells. **(B)** A typical example of the proliferation of the 3 different stimulation conditions is depicted. **(C)** The frequency of plasmablasts. **(D)** The frequency of class-switched B cells. **(E)** The frequency of transitional B cells. Tfh cells generated from naive CD4⁺ T cells and resting B cells were obtained from 7 healthy controls. Note. The median is shown. Wilcoxon matched pairs test was used. SRL, sirolimus; TAC, tacrolimus; Tfh, T follicular helper. [†]Indicates $P < .01$.

cell-mediated B-cell proliferation and differentiation toward transitional B cells, class-switched B cells, and plasmablasts was determined. In the presence of alloantigen, a substantial proportion of B cells proliferated (20.5%) and differentiated into plasmablasts (median: 4.8%).

We found that T-cell help function was impaired when induced in the presence of TAC or SRL (Fig 9). T cells including Tfh-like cells generated in the absence of any immunosuppression nicely demonstrated the help they provided to B cells, which proliferated and differentiated into diverse B cell subsets. In contrast, Tfh-like cells generated in the presence of TAC or SRL could not activate B cells effectively. Decreased proliferation and differentiation into both transitional B cells and plasmablasts were found, while the frequency of class-switched B cells was unaffected. SRL inhibited B-cell proliferation by 60% (median) and TAC by 38% (both $P < .01$) (Fig. 9A). In Fig 10A we depicted the mRNA expression level of IL-21 after Tfh-like cell differentiation measured in samples generated in the absence and presence of TAC and SRL. The lower graph (Fig 10B) depicts the normalized data. In the immunosuppressed samples we found lower levels of IL-21 mRNA expression. These data suggest that the impaired T cell-dependent B cell help might also be the result of lower

IL-21 production by these TAC and SRL Tfh-like generated cells.

DISCUSSION

We used an in vitro co-culture model to study Tfh-dependent B-cell activation in the presence of TAC or SRL. We found that TAC and SRL inhibited the generation of human Tfh-like cells (CD4⁺CXCR5⁺icos⁺ and CD4⁺CXCR5⁺pd1⁺) by 90% to 95% at therapeutic or even lower concentrations. We presume that in this in vitro system, the effect of these immunosuppressive drugs are more profound than in vivo, because these drugs are not able to be caught up in another compartment (eg, red blood cells will capture a great part of TAC in vivo). In this study, in the remaining T cells, the balance shifted toward *bcl6*. Tfh cells have been reported to be regulated by multiple signals [30,31]. Research reports have demonstrated that the *icos-PI3K/Akt/mTOR* signaling pathway plays a critical role in promoting Tfh cell differentiation [32–34]. Both *mTOR* complex 1 and *mTOR* complex 2 contribute to Tfh cell differentiation via different mechanisms. *Mtor* complex 1 mainly promotes proliferation of CD4⁺ T cells to achieve the required division for Tfh cell differentiation, while *mTOR*

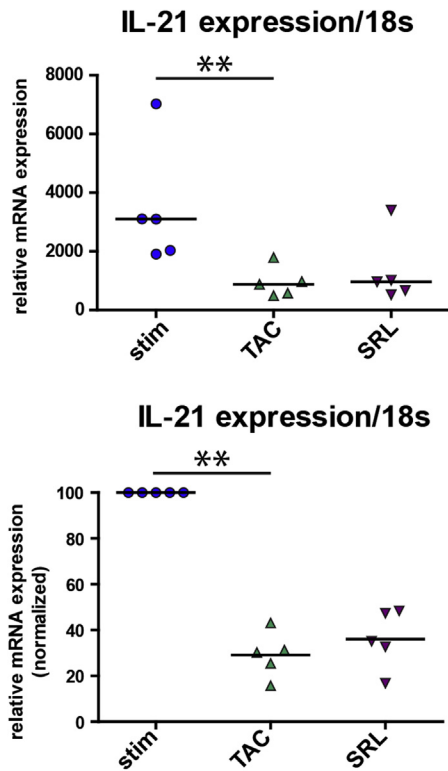


Fig 10. IL-21 mRNA expression. In this figure, the effect of TAC and SRL on the relative mRNA expression of IL-21 of pure FACS-sorted CD3⁺CD4⁺ T cells is depicted. The relative mRNA expression of IL-21 after of stimulation with anti-CD3/28, IL-12, and IL-21 in the presence or absence of TAC or SRL is shown in the top figure. Normalized data (Stim = set as 100%) is depicted within the lower figure. Note. Mann-Whitney testing was used. FACS, fluorescence-activated cell sorting; IL, interleukin; SRL, sirolimus; TAC, tacrolimus. [†]Indicates $P < .01$.

complex 2 assists Tfh cell differentiation by activating *Akt* and *tefl* [35,36]. Furthermore, it is well established that SRL promotes the generation of regulatory T cells [37,38], which was recently reported to play an important role in controlling the expansion of Tfh cell numbers [39]. This explains why in the presence of SRL the generation of Tfh cells was inhibited. However, the few T cells that escape from *mtor* inhibition by SRL can have a Tfh-like profile. Despite the inhibition of this Tfh-like cell subtype, we found that in the presence of TAC, the transcription factor *bcl6* was abundantly present in these cells. Although this finding is in line with observations from previous study in TAC-treated patients [40], mRNA levels and cell numbers cannot be directly translated to cellular functions. Despite the high presence of *bcl6*, lower mRNA expression of IL-21 was found, and fewer Tfh cells were generated when in the presence of this agent [20,41], which is most likely the result of less T-cell activation and proliferation. Where our previous studies showed that SRL has a more potent effect on inhibition of Tfh differentiation, in this in vitro study we

were unable to find a significant difference between these 2 immunosuppressive drugs. This can be attributed to a difference in results between in vivo and in vitro studies as well as that the TAC- and SRL-treated patients in the former in vivo study are also given other immunosuppressive drugs [26].

Ballesteros-Tato et al [42] and Johnston et al [43] both reported that IL-2 was a negative regulator of Tfh cell differentiation via a *stat5/blimp1*-dependent mechanism, which then inhibited the B-cell response in vivo. When IL-2 is scarce, subsequent Tfh cell differentiation may be enhanced, ultimately reinforcing the humoral response [44]. However, this differentiation did not result in direct B-cell help as these T cells had a lower capacity to provide B-cell help [20,41].

Apart from IL-2 production, TAC also prevents the induction of other cytokines. These soluble mediators, for example, IL-6 and IL-21, deliver signals through *stat3*-mediated pathways and are critical for the expansion and function of Tfh cells [45]. Thus, even in the presence of in vitro added IL-12, IL-21, and T-cell receptor activation by anti-CD3/28 antibodies, TAC still inhibited Tfh differentiation.

Study Limitations

This study has some limitations. This study was performed on a limited PBMC samples from volunteers without a significant medical history ($n = 4$ to $n = 7$). Therefore, these findings must be considered as preliminary data. Secondly, our study showed that only a minute population still differentiated toward Tfh-like cells. To study the functional properties of these few cells, isolation of the cells is required. These cells should then be co-cultured with B cells in the same way as currently described. But since the inhibitory effect of TAC and SRL both interfere with T-cell proliferation and differentiation, we found that isolation of these cells was practically impossible. Despite this limitation, we can conclude that the direct effect TAC and SRL has on Tfh differentiation still affects B-cell proliferation and differentiation in an indirect manner. This is of relevance because in vivo, in immunosuppressed transplant patients, the number of Tfh cells that escape from TAC, SRL, or other immunosuppressive treatments may still provide help to B cells. For example, after T-cell depletion therapy, which triggers homeostatic T-cell proliferation [46] in patients with kidney transplant, an association was found between the risk of developing ABMR and the frequency of Tfh cells in the circulation [40,47].

Where this study solely focused on the differentiation of Tfh-like cells originating from a naive T helper subtype, the effect of these immunosuppressive drugs or depletion therapy by anti-thymoglobulin on memory Tfh-like cells was not tested and is therefore an interesting and relevant subject for future studies. In addition, other immune cell populations, such as follicular natural killer T cells, can trigger B-cell responses [48].

CONCLUSIONS

In conclusion, in vitro generated human Tfh-like cells provide the required signals to help B cells. Immunosuppression, in our case TAC and SRL, affects this process by halting the differentiation into Tfh-like cells, effectively canceling their ability to activate B cells. These findings suggest that in patients with a history of transplant, immunosuppressive drugs may similarly influence both the differentiation and function of Tfh cells.

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