Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties

Mélanie Chesneau,*† Laure Michel,*†‡ Emilie Dugast,*† Alexis Chenouard,*† Daniel Baron,*† Annaïck Pallier,* Justine Durand,* Faouzi Braza,*†§ Pierrick Gueiff,‡ David-Axel Laplaud,*†‡ Jean-Paul Soulliou,*†‡ Magali Giral,*†‡ Nicolas Degauque,*† Elise Chiffleau,*† and Sophie Brouard*†‡

*Joint Research Unit 1064, French Institute of Health and Medical Research, Nantes, France; †Faculty of Medicine, Nantes University, Nantes, France; and ‡Institute of Transplantation Urology and Nephrology, Nantes University Hospital, Nantes, France

ABSTRACT
Whereas a B cell–transcriptional profile has been recorded for operationally tolerant kidney graft patients, the role that B cells have in this tolerance has not been reported. In this study, we analyzed the role of B cells from operationally tolerant patients, healthy volunteers, and kidney transplant recipients with stable graft function on T cell suppression. Proliferation, apoptosis, and type I proinflammatory cytokine production by effector CD4+CD25− T cells were measured after anti-CD3/anti-CD28 stimulation with or without autologous B cells. We report that B cells inhibit CD4+CD25− effector T cell response in a dosedependent manner. This effect required B cells to interact with T-cell targets and was achieved through a granzyme B (GzmB)–dependent pathway. Tolerant recipients harbored a higher number of B cells expressing GzmB and displaying a plasma cell phenotype. Finally, GzmB+ B-cell number was dependent on IL-21 production, and B cells from tolerant recipients but not from other patients positively regulated both the number of IL-21+ T cells and IL-21 production, suggesting a feedback loop in tolerant recipients that increases excessive B cell activation and allows regulation to take place. These data provide insights into the characterization of B cell–mediated immunoregulation in clinical tolerance and show a potential regulatory effect of B cells on effector T cells in blood from patients with operationally tolerant kidney grafts.


Tolerance in transplantation is defined as the maintenance of long-term, good, stable graft function in the absence of immunosuppression.1,2 Numerous studies have demonstrated that tolerance can easily be achieved in rodent models,3–5 including models for renal transplant.6 However, while it remains rare in human renal transplant it does exist, current estimates report roughly a hundred cases of operational tolerance, mainly patients not compliant with their immunosuppressive regimens.1,2,7 These patients, defined as “operationally tolerant,” are healthy, do not exhibit more infections or malignancies than healthy volunteers (HVs), and do not display clinical evidence of immune incompetence.2,8 Specific patterns have been associated with this operational tolerance; in particular, a B cell transcriptional signature that correlates with an increase in peripheral B cells has been reproducibly found.9–14 Several of these B cell markers are being currently tested and validated in multicenter studies around the world to predict patients who may benefit from immunosuppression withdrawal. In addition, some of these B cell markers are being examined for their involvement in the tolerance process; however, to date, none have been shown to have a role in tolerance.

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Correspondence: Dr. Sophie Brouard, Joint Research Unit 1064, Institute of Transplantation Urology and Nephrology, Nantes University Hospital, 30 Bd Jean Monnet, 44093 Nantes Cedex 01, France. Email: sophie.brouard@univ-nantes.fr

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induction or maintenance, and whether B cells are involved, or even have a potential role in tolerance, remains to be determined.

In transplantation, B cells are mainly known for their capacity to differentiate into plasma cells and produce antibodies that may be deleterious for the graft. However, B cells also have antibody-independent functions. They are able to produce cytokines and to present antigens, and thus to initiate or maintain an immune response. More recently, populations of regulatory B cells (Bregs) able to dampen the immune response have been highlighted as a “driving force” in autoimmune diseases, cancers, and transplantation. However, their nature, origin, phenotype, and mode of action in humans remain little known.

We previously reported that B cells from tolerant patients (TOLs) do not fully differentiate into plasma cells and that, during their differentiation, B cells from tolerant recipients produce higher levels of IL-10, suggesting that an imbalance between a lower number of deleterious plasma cells and a higher level of Bregs producing IL-10 may exist in these patients. In this article, we investigate the role of B cells in blood from this cohort of operationally TOLs, from patients with stable graft function under classic immunosuppression (STAs), and from HVs. We report that tolerant recipients exhibit a higher number of IL-21-dependent peripheral B cells that express granzyme B (GzmB), display a specific phenotype, and exhibit a contact- and GzmB-dependent, IL-10– and TGF-β–independent inhibition of effector T cell response. These results provide novel insights into the characterization of B cell–mediated immunoregulation in tolerance in the clinic.

RESULTS

CpG-CD40 Prestimulated B Cells Inhibit CD4⁺CD25⁻ T Cell Proliferation

Human B cells have been shown to exhibit regulatory activity through the inhibition of various cell types, in particular through the inhibition of T cells. CD4⁺CD25⁻ T cells were cocultured with CpG–CD40 stimulated or unstimulated autologous B cells, after polyclonal anti-CD3 and anti-CD28 activation, for 3 days and T cell proliferation was then analyzed, using CellTrace Violet staining. We found that prestimulated B cells from HVs, TOLs, and STAs significantly inhibit autologous CD4⁺CD25⁻ T cell proliferation (Figure 1A), whereas unstimulated B cells have a lesser effect (Figure 1B). No difference was found in the number of total B cells and GzmB⁺ B cells or for B cell inhibition between men and women.

CpG-CD40 Prestimulated B Cells Induce T Cell Apoptosis But Have No Effect on Proinflammatory Cytokine Production

Using Annexin V staining, apoptosis of CD4⁺CD25⁻ T cells was measured at day 3 after anti-CD3/anti-CD28 activation and addition of prestimulated B cells to the culture. Prestimulated B cells and a 1:2 T cell/B cell ratio were used in all of the experiments. The addition of prestimulated B cells to the coculture induces a significant increase in CD4⁺CD25⁻ T cell apoptosis in the three groups (Figure 1C). Interestingly, no difference was observed in apoptosis levels between cell trace⁺ and cell trace⁻ T cells, confirming that the increase in apoptosis was not due to inhibition of T cell proliferation (data not shown). Type I helper T cell (Th1) proinflammatory cytokines (IFN-γ and TNF-α) were analyzed using intracellular staining after 3 days of coculture. IFN-γ T cell production was slightly lower when prestimulated B cells from HVs were added to the culture, but this was due to a slightly higher level of IFN-γ production by CD4⁺CD25⁻ T cells from HVs only (Figure 1D). TNF-α production by T cells from the three groups of patients was unchanged when prestimulated B cells were added to the culture (Figure 1E). Representative pictures of IFN-γ and TNF-α production by T cells are displayed in Figure 1, F and G. Altogether, these data show that B cells from HVs, transplant TOLs, and STAs all inhibit T cell proliferation and induce T cell apoptosis but have no effect on Th1 proinflammatory cytokine production.

B Cell Inhibitory Effect on T Cells Is Dependent to GzmB and Is Contact Dependent

Having previously reported higher production of IL-10 by B cells from tolerant recipients during the differentiation process in vitro, as well as B cells having been shown to mainly display regulatory properties through IL-10, we decided to assess the role of IL-10 in our model. We looked at the frequency of IL-10–expressing B cells and the level of IL-10 expression by these B cells after 48 hours of CD40L and oligodeoxynucleotide (ODN) stimulation. As expected, although the resting B10 level was low, a significant and substantial increase in the frequency of B10 cells was found after activation (Figure 2A). No difference was observed in the frequency of B10 cells and in the relative amount of IL-10 expressed by B cells between the three groups of individuals (Figure 2, B and C). To assess the role of IL-10 in the coculture assay, we blocked its effect using anti-IL-10 antibody. We found that the blockade of IL-10 does not hinder the inhibitory effect of B cells on effector T cell proliferation (Figure 3A). Because other cytokines have been shown to play a role in the function of suppressive B cell populations, TGF-β and GzmB were similarly blocked by adding anti–TGF-β antibody and anti-GzmB peptide to the coculture at day 0. The blockade of TGF-β did not hinder the inhibitory effect of B cells on T cell proliferation (Figure 3B). However, for the three groups of patients, the addition of anti-GzmB peptide to the coculture significantly affects the suppressive effect of B cells on autologous CD4⁺CD25⁻ T cell proliferation (Figure 3C), whereas GzmB inhibitor has no effect on T cell proliferation in the absence of B cells (Figure 3D).

Because the inhibitory functions of B cells involving GzmB have been shown to act through a direct interaction of B cells with their target, transwell cocultures were performed to determine whether contact was required by B cells to inhibit T cell proliferation. As shown in Figure 3E, the inhibitory B cell
effect disappeared when B and T cells were cultured in transwell, demonstrating that T cell–B cell interaction is necessary for the inhibitory B cell function. Altogether, these data show that B cells inhibit T cell proliferation via a GzmB pathway and depend on contact between the B and T cells.

**Tolerant Recipients Have a Higher Number of B Cells, Which Act in a Dose-Dependent Manner and Express GzmB**

On a cell-by-cell basis, B cells from TOLs added to the coculture have the same ability as B cells from HVs and STAs to regulate autologous effector T cell proliferation in a contact- and GzmB-dependent manner (Figure 3, C and E). We previously reported on a higher number of total B cells in blood from TOLs.10,12,24 Here we found that the absolute value of GzmB-producing B cells was significantly higher in TOLs compared with HVs and STAs \( (P < 0.05; \text{Figure 4A}) \) and the percentage of GzmB+ B cells is directly correlated with effector CD4+CD25 T cell proliferation inhibition (Figure 4B). Altogether, these data show that tolerant recipients have a higher number of peripheral B cells and GzmB+ B cells that are able to inhibit T cell response through a contact- and GzmB-dependent pathway and in a dose-dependent manner.

**Resting and Stimulated GzmB-Expressing B Cells Display a CD5+CD27+CD138+ Phenotype**

The GzmB+ B cell phenotype was analyzed by flow cytometry before and after 3 days of coculture using CD19, CD20, CD138, CD38, CD27, CD24, CD5, CD1d, IgD, IgG, and IgM cell surface markers. Unstimulated GzmB+ B cells express a higher level of CD138, CD27, CD5, and fewer IgD markers compared with unstimulated GzmB− B cells (Figure 4C). After stimulation, GzmB+ B cells have lower CD38 expression compared with GzmB− B cells (Figure 4D). Resting GzmB− B cells represented around 2.7% ± 1.45% of total B cells from TOLs. Twenty-four hours of activation with CD40L and ADN following by three days of coculture with activated effector T cells greatly increases the expression of GzmB (20% ± 2.5% after activation) (Supplemental Figure 1, A and B). Altogether, these data...
show that tolerant recipients have a higher number of GzmB-expressing B cells with a CD138+CD27+CD5+CD38+IgD2 phenotype.

**B Cell Regulatory Transcriptional Profile Is Not Different in TOLs, HVs, and STAs**

We investigated the expression of 60 markers selected either for their participation in the regulation of B cell functions (Supplemental Figure 2, A and B),26 or their implication in a tolerance-related B signature (Supplemental Figure 2, C and D)27 in purified and isolated B cells or PBMCs from samples from 32 individuals (10 TOLs, 10 HVs, and 12 STAs). Among these genes, only CD38 and CD1D exhibited a significant difference between STAs and HVs in B cells (Supplemental Figure 2A), whereas more than half were different in PBMCs (Supplemental Figure 2B). The second subset (Supplemental Figure 2, C and D) includes a panel of 35 markers previously linked to tolerance across blood transcriptomic studies and preferentially expressed in B cell subsets for 69% of them.27 Only two of these markers were different in B cells (EBF1 between TOLs and HVs; PLEKHG1 between STAs and HVs) (Supplemental Figure 2D), whereas all of them were different between TOLs and STAs in PBMCs (Supplemental Figure 2D).

These data clearly show that B cells are not intrinsically different between the groups of patients and that, at least for these selected genes, the signature of B cells in blood from tolerant recipients is mainly the result of a higher number of B cells in PBMCs (Supplemental Figure 2).

**B Cells from Tolerant Recipients Regulate IL-21–Producing T Cell Levels; IL-21 Production by T Cells Regulates the Number of GZMB+ B cells**

GzmB-expressing B cells have been shown to depend on T cell–produced IL-21, and IL-21 is also an important factor in B cell homeostasis. We found that in TOL stimulation with anti-CD3/anti-CD28 of CD4+CD25+ T cells is associated with a lower number of IL-21–producing T cells compared with HVs and STAs (P<0.05). However, when B cells are added to the coculture, IL-21–producing T cells significantly increased in TOLs (P<0.01), whereas no difference, or even decreased IL-21 production, was observed in HVs and STAs (Figure 5, A and B). This correlates with a higher production of IL-21 by T cells stimulated by B cells from TOLs compared with those from HVs and STAs (P<0.05) (Figure 5C). Altogether, these data show that B cells from TOLs regulate the number of IL-21+ T cells and IL-21 production in vitro.

Finally, to assess the role of IL-21 on GzmB-expressing B cells, we blocked its effect using anti–IL-21 antibody in the coculture assay. Increasing doses of anti–IL-21 (0, 2, 4, 6, and 8 µg/ml) significantly decreased the number of GzmB-expressing B cells (Figure 5D).

**DISCUSSION**

B cells may have a dual effect, acting as a driver and as a regulator of the immune system.15–18,28–32 We recently showed that tolerant recipients are characterized by a higher number of circulating B cells,10,12 a defect in terminal differentiation,24 and a B cell–transcriptional profile with overexpression of molecules associated with regulation.9,12 In animal models, that B cells have a role in tolerance is clearly suggested by the accumulation of B cells and formation of germinal centers in tolerant allografts and the ability of B cells to prolong graft survival.33 However, the role and nature of Bregs in the modulation of immune response in alloimmunity and in tolerance in humans remains unclear. Although tolerant kidney recipients clearly display a strong B-cell signature, with a higher
number of B cells, their potential role in establishing and/or maintaining tolerance remains to be determined.

It is tempting to speculate that this increase in B cells with a specific inhibitory profile may be linked to the tolerance of these rare patients. Haynes et al. recently proposed an indirect pathway model in which a decrease in immunosuppression is associated with an increased number of indirect pathway regulatory T cells (Tregs) and of B cells, possibly Bregs, but the mechanisms have not been established.34 Silva et al. showed that peripheral B cells from tolerant recipients maintain the capacity to activate CD40-CD40L signaling, phosphorylate STAT3, and preserve B cell compartment diversity, suggesting a role for these B cells in tolerance.14 In this article, we explore the potential regulatory role of B cells in such patients. Because of the difficulty in identifying a unique Bregs population with a unique phenotype in humans, we analyzed the in vitro suppressive properties of B cells overall. We report a higher number of B cells with dose-dependent suppressive properties in blood from patients with a tolerant kidney graft. The inhibitory effect of B cells is dependent on GzmB and on the interaction of B cells with their T cell targets.

Much evidence suggests that activation is instrumental in Bregs activity.35 In this article, we show that prestimulated B cells are more efficient at suppressing effector T cell proliferation than nonstimulated B cells, a result in accordance with previous data showing that maturation and B cell activation were important parameters in this process.28,30–32,36 It may be a little counterintuitive to say that B cells need such activation to regulate the immune system because activated B cells are usually associated with stimulatory activity on other cell types. However, this has been extensively discussed and can be explained as the participation of B cells in a general feedback loop that both induces an immune process and prevents excessive inflammation or unwanted autoaggressive T cell response.37 Moreover, we clearly show that the inhibitory effect of B cells is dependent on GzmB, the expression of which occurs in resting B cells in vivo and is increased in stimulated B cells. Interestingly, as already described for the pro-B10 regulatory cell population, this leads to the conclusion that B cell–mediated regulation is probably inducible and that the effect of B cells is greatly influenced by the nature of the microenvironment.35,36,38–40

The expression of IL-10 has been shown to be a frequent characteristic of Bregs.41–43 Such cells, referred to as B10,32,44 are involved in the initiation, the onset, and the severity of various autoimmune diseases, and in transplantation.45–47 We show here that the suppressive properties of B cells are not dependent on IL-10. Anti–IL-10, used at doses able to efficiently block an IL-10 T cell–dependent response (data not shown), does not diminish the inhibitory effect of B cells on T cell proliferation. These data are in agreement with the work of Deng et al., who reported that anti-CD45RB treatment
Figure 4. Tolerant recipients have a higher number of B cells expressing GzmB and act in a dose-dependent manner. (A) Number of GZMB+ B cells per microliter of blood from HVs (n=6), TOLs (n=6), and STAs (n=6) (mean±SEM; *P<0.05). (B) Linear regression of percentage of GzmB+ B cells and percentage of inhibition of CD4+CD25+ T cell proliferation (P<0.04). (C) Representative histograms for CD138, CD27, CD5, CD38, CD24, CD1d, IgD, IgG, and IgM expression within the GzmB+ (thin line) and GzmB- (thick line) in CD19+ B cells before stimulation. (D) Representative histograms for CD38 and CD138 within the GzmB+ (thin line) and GzmB− (thick line) in CD19+ B cells after stimulation.

induces strong and antigen-specific tolerance, which is dependent on the presence of B lymphocytes and is independent from IL-10. In this model, IL-10 clearly counter-regulates tolerance induction, and even exerts a negative effect and causes histologic lesions of rejection. The same observations have been reported in other models. Interestingly, it has been reported that B cells from patients with chronic antibody-mediated rejection have a defect in suppressive properties, in contrast with B cells from STAs, which inhibit T cell proliferation and induce Treg generation through a TGF-β and IDO-dependent pathway (personal communication, Nouël et al. Ann Rheum Dis abstract A8.32, 2014). In our situation, blocking TGF-β has no effect on the suppressive function of B cells, suggesting that TGF-β is not involved in B cell regulation either.

Interestingly, other regulation pathways have been shown to be involved in B cell regulatory activity. We found that GzmB blockade diminishes the B cell inhibitory effect and restores T cell proliferation. This is associated with a higher number of GzmB-producing B cells in blood from tolerant recipients and B cells that act in a contact- and dose-dependent manner. GzmB is a 32-kD serine protease mainly known as a component of the cytotoxic granule of T cells and natural killer cells but is also produced by other cell types, such as B cells. Tregs, involved in B cell regulation in our present situation. To our knowledge, nothing has yet been reported on the effect of GzmB on IFN-γ and/or TNF-α production by B cells.

Several microenvironmental factors have been shown to be instrumental in B cell homeostasis. These factors include molecules such as IL-21. We first demonstrated that GzmB-expressing B cells are dependent on IL-21. Increasing doses of anti–IL-21 decrease the number of GzmB-expressing B cells in coculture. Moreover, when B cells are added to the coculture, IL-21–producing T cells increase significantly and there is greater production of IL-21 by T cells from TOLs. IL-21 is a key cytokine for GzmB gene transcription, which suggests that the increase in GzmB+ B cells in blood from tolerant recipients may be due to a direct effect of IL-21. These data are reinforced by the effective STAT3 phosphorylation in B cells from tolerant recipients, a key signal for the generation of GzmB-activated B cells. Finally, the fact that tolerant recipients have a lower level of IL-21+ CD4+ T cells, and that B cells from these patients alone (and not HVs and STAs) increase IL-21 production by CD4+ T cells, strongly suggests a negative feedback loop in these patients, increasing excessive B cell activation and inflammation and allowing regulation to take place.
Bregs are able to control the immune response, but an excessive reaction from these cells may also promote tumor cell growth or chronic infection. We hypothesize that this fine-tuning of regulation by B cells and IL-21 production by T cells might be a key factor in tolerance maintenance.

We show that TOLs have more B cells in absolute value with regulatory properties, but on a cell-per-cell basis, their B cells have the same suppressive activity as B cells from STAs and HVs. These results are supported by transcriptional analysis that shows that the B cells are not intrinsically different between the groups of patients and that, at least for these selected genes, the B cell signature in blood from tolerant recipients’ results mostly from a higher number of B cells in PBMCs. It is not surprising that the greater suppressive effect in blood of tolerant recipients is due to a higher number of circulating GzmB+ cells, if we consider that clinical outcomes in numerous situations are mainly driven by the quantity of infiltrating and circulating cells, more than their quality. Interestingly, we find that GzmB+ B cells express a specific phenotype with upregulation of CD5, CD27, CD38, and CD138 markers. This is in accordance with our previous data reporting a higher level of memory CD27+ B cells and CD5+ B cells in blood from tolerant recipients. These data are also in accordance with the fact that CD5+ B cells constitutively express GzmB in an IL-21–dependent pathway and that CD5+ B cells express higher levels of IL-21–R than CD5− B cells. We also found that these cells express CD138 and CD38 and are IgD−, markers expressed by B cells secreting IL-10 and IL-35 and involved in suppression mechanisms, and also associated with plasma cell maturation. Interestingly, CD138 has also been shown to be associated with GzmB production, regulatory activity, and IL-21 dependence.

In conclusion, we have demonstrated that TOLs have a higher absolute number of GzmB+ B cells with a plasma cell–like phenotype and with dose-dependent suppressive properties through the GzmB pathway. B cells decrease T cell proliferation and induce their apoptosis, two processes that may contribute to a tolerogenic environment. GzmB-producing B cells are under the control of IL-21. The fact that there is more IL-21 in vitro in TOLs after B cell stimulation correlates with a greater number of IL-21–dependent circulating GzmBs and a higher inhibitory effect of B cells, which may act as pro-B10 that need to be activated to display an increased effect. In previous articles, we reported on a decreased number of circulating plasma cells and we showed that B cells from tolerant recipients do not fully differentiate into plasmablasts and are more sensitive to apoptosis. Here again, this is in accordance with the fact that in vitro unstimulated T cells from TOLs produce less IL-21, a molecule that directly acts on B cell differentiation. We hypothesize that these properties may contribute to a favorable tolerogenic environment and favorable inversion of the B effector–plasma cell/Bregs balance in these patients and their lower antibody production. These data support a role for B cells in patients with operational tolerance. They also raise the question of whether it would be possible to trigger an in vivo or in vitro increase to encourage any potential therapeutic effects, such as counteracting the alloimmune response or even promoting tolerance. These data also question the effectiveness of depleting B cells in order to control antibody-mediated rejection, rather than developing new strategies to maintain the fragile balance between the negative and beneficial effects of B cells in transplantation.
CONCISE METHODS

Patients and HVs
Forty-one patients took part in the study and signed informed consent as follows: (1) TOLs with stable kidney graft function (creatinemia < 150 μmol/L and proteinuria < 1 g/24 h) for at least 1 year (n = 12) except for 2 who have creatinemia > 150 μmol/L±20% but were stable over time, (2) STAs with stable kidney graft function (creatinemia < 150 μmol/L and proteinuria < 1 g/24 h) for at least 3 years under standard immunosuppression (calcineurin inhibitors and corticosteroids) (n = 17), and (3) HVs without pathologies or infectious episodes in the previous 6 months (n = 17) (Table 1).

Cell Culture
PBMCs were stimulated at 2 × 10^6 cells/ml for 48 hours in complete RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing L-glutamine, penicillin/streptomycin (Life Technologies, Carlsbad, CA), and 10% FCS (Lonza, Verviers, Belgium) in 96-well plates (Nunc, Langenselb, Germany) at 37°C, 5% CO2. Stimulation was performed using CpG oligonucleotide (ODN 2006, 10 μg/ml; InvivoGen, San Diego, CA), washed, and stimulated for 24 hours with CD40L (1 μg/ml) and CpG-ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. As a control, a proportion of PBMCs were kept at 4°C in complete medium for 24 hours. B cells were purified using the same technique, without stimulation. CD4^+CD25^- responding T cells were purified by negative selection using successively CD4^+ T Cell Isolation Kit II and an autoMACS PRO Separator, with purity > 95% (Miltenyi Biotech, Gladbach, Germany) and stimulated for 24 hours with CD40L (1 μg/ml) and CpG-ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. A control, a proportion of PBMCs were kept at 4°C in complete medium for 24 hours. B cells were purified using the same technique, without stimulation. CD4^+CD25^- responding T cells were purified by negative selection using successively CD4^+ T Cell Isolation Kit II and CD25^+ Microbeads II (Miltenyi Biotech), according to the manufacturer’s instructions with a purity > 95%.

Analyses of IL-10 Production by Stimulated B Cells
After 48 hours of culture, viability staining was performed using the aqua Live/Dead cell staining kit (Invitrogen/Life Technologies). B lymphocytes were stained with anti-CD19-PC7 (BD Biosciences, San Diego, CA), washed, fixed, and permeabilized using a permeabilization/fixation kit (BD Biosciences). Fc receptor inhibitor (eBiosciences, Oslo, Norway). After 72 hours of coculture, brefeldin-A was added at 10 μg/ml for 4 hours. Viability of T and B cells was checked by 4',6-diamidino-2-phenylindole staining. The proliferation of CD4^+CD25^- responding T cells was measured after staining with CellTrace Violet (Invitrogen). T cell IFN-γ, IL-21, and TNF-α secretion was measured after permeabilization of responding T cells and staining with anti–CD4-PE (BD Biosciences), anti–IFN-γ–allophycocyanin (APC) and anti–TNF-α–fluoresceine isothiocyanate (FITC) (BD Biosciences) of fresh blood samples. B cells were purified by negative selection using a Human B Cell Isolation Kit II and an autoMACS PRO Separator, with purity > 95% (Miltenyi Biotech, Gladbach, Germany) and stimulated for 24 hours with CD40L (1 μg/ml) and CpG-ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. As a control, a proportion of PBMCs were kept at 4°C in complete medium for 24 hours. B cells were purified using the same technique, without stimulation. CD4^+CD25^- responding T cells were purified by negative selection using successively CD4^+ T Cell Isolation Kit II and CD25^+ Microbeads II (Miltenyi Biotech), according to the manufacturer’s instructions with a purity > 95%.

Coculture Experiments
Coculture assays were performed for 72 hours by adding 1 × 10^5 autologous prestimulated B cells or unstimulated B cells to 0.5 × 10^5 CD4^+CD25^- responding T cells, stimulated with anti-CD3 and anti-CD28 dynabeads (at a 1:1 ratio of dynabeads/T cells) (Invitrogen, Oslo, Norway). After 72 hours of coculture, brefeldin-A was added at 10 μg/ml for 4 hours. Viability of T and B cells was checked by 4',6-diamidino-2-phenylindole staining. The proliferation of CD4^+CD25^- responding T cells was measured after staining with CellTrace Violet (Invitrogen). T cell IFN-γ, IL-21, and TNF-α secretion was measured after permeabilization of responding T cells and staining with anti–CD4-PE (BD Biosciences), anti–IFN-γ–allophycocyanin (APC) and anti–TNF-α–fluoresceine isothiocyanate (FITC) (BD Biosciences) of fresh blood samples. B cells were purified by negative selection using a Human B Cell Isolation Kit II and an autoMACS PRO Separator, with purity > 95% (Miltenyi Biotech, Gladbach, Germany) and stimulated for 24 hours with CD40L (1 μg/ml) and CpG-ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. As a control, a proportion of PBMCs were kept at 4°C in complete medium for 24 hours. B cells were purified using the same technique, without stimulation. CD4^+CD25^- responding T cells were purified by negative selection using successively CD4^+ T Cell Isolation Kit II and CD25^+ Microbeads II (Miltenyi Biotech), according to the manufacturer’s instructions with a purity > 95%.

Table 1. Summary of clinical and demographic characteristics of patients and HVs

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<th>Time between Graft and Analysis (mo)</th>
<th>Creatinemia (μmol/L)</th>
<th>Proteinuria (g/24 h)</th>
<th>Time between Imunosuppression Withdrawal and Analysis (yr)</th>
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<td>Maximum</td>
<td>85.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>STA (n = 17)</td>
<td>5/17</td>
<td>1/17</td>
<td></td>
<td></td>
<td>120.4</td>
<td>124.0</td>
<td>0.1</td>
<td>1</td>
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<tr>
<td>Median</td>
<td>57.6</td>
<td></td>
<td></td>
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<tr>
<td>SD</td>
<td>10.8</td>
<td></td>
<td></td>
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<tr>
<td>Minimum</td>
<td>40.4</td>
<td></td>
<td></td>
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<tr>
<td>Maximum</td>
<td>74.9</td>
<td></td>
<td></td>
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</tbody>
</table>

F, female; M, male; LD, living donor; NLV, nonliving donor.
Biosciences). For measurement of T cell apoptosis, cells were stained with CD4-APC, CD19-PE-C7, and Annexin V-APC (BD Biosciences). To investigate whether B cells have a dose-dependent effect on T cells, the number of B cells was progressively increased in the coculture (50,000, 100,000, and 200,000), whereas the number of 50,000 T cells remained fixed.

**Coculture Experiments Blockade**

These coculture assays were performed under the same conditions using transwll polycarbonate inserts (0.4 μm; Corning, Inc.) or using anti–IL-10 (BD Biosciences), anti–TGF-β1 (Abcam, Inc., Cambridge, UK), or anti-GzmB, a peptide that irreversibly inhibits GzmB activity (Ac–IEPD–CHO; BioVision, San Francisco, CA). All antibodies were used at 10 μg/ml concentration.

**Microarrays**

Purified B cell samples from 32 individuals (10 TOLs, 10 HVs, and 12 STAs) were analyzed with whole-genome Agilent human microarray as previously described according to the manufacturer’s instructions (Agilent Technologies). Hybridization signals were normalized using a Lowess procedure.64 Probe conversion was performed using a Lowess procedure.64 Probe conversion was performed using MADGene65 and those pertaining to the same gene were averaged. The expression of 60 markers selected either for their participation in the regulation of B cell functions26 and/or their implication in a tolerance-related B signature27 was then investigated. Significance of differential expression was evaluated using a one-tailed t test and results were compared with those obtained on PBMCs.27

**GzmB+ B Cell Phenotyping**

Anti–human mAbs included the following: CD19–V450 CD38–FITC, CD24–PE, CD5–APC, CD1d–PE, and GzmB–Alexa Fluor 700 from BD Biosciences. CD20–FITC and CD138 PE from Miltenyi Biotech were used for PBMC staining. PBMC suspensions were stained on ice using a predetermined optimal concentration of each antibody for 15–30 minutes. PBMCs were then fixed and permeabilized using a permeabilization/fixation kit (BD Biosciences). For detection of intracellular GzmB, staining was performed using anti–GzmB-A700.

**IL-21 Measurement and Inhibition**

Coculture assays were performed for 72 hours by adding $1 \times 10^5$ autologous prestimulated B cells or unstimulated B cells to 0.5 × 10^5 CD4–CD25– responding T cells stimulated with anti-CD3 and anti-CD28.2 dynabeads (at a 1:1 ratio of dynabeads/T cells) (Invitrogen). Cytokine production was measured in culture supernatants at 72 hours. IL-21 level was determined using BD CBA Human IL-21 FLEX SET as recommended by the manufacturer (BD Biosciences). GzmB production by B cells in B cell/T cell cocultures was measured at day 3 by blocking IL-21 with increasing doses (0, 2, 4, 6, and 8 μg/ml) of anti–IL-21 blocking molecule (recombinant IL-21R Fc chimera) (R&D Systems).

**Statistical Analyses**

Data are expressed as the means±SEM. Statistical analyses were performed using GraphPad Prism software, and P values were calculated by the Mann–Whitney test.

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**DISCLOSURES**

None.

**REFERENCES**


CLINICAL RESEARCH


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