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Hernandez M Silva,[1,2] Maisa C S Takenaka,[1,2] Pedro M M Moraes-Vieira,[1,2,3] Sandra M Monteiro,[1,2] Maristela O Hernandez,[1,2] Wahiba Chaara,[4,5,6] Adrien Six,[4,5] Fabiana Agena,[6] Patrícia Sesterheim,[2,7] Florencia Maria Barbé-Tuana,[2,7] David Saitovitch,[2,7] Francine Lemos,[8] Jorge Kalil,[1,2] and Verônica Coelho[1,2]

[1]Laboratory of Immunology, Heart Institute (InCor), University of São Paulo Medical School, São Paulo, Brazil; [2]Institute for Investigation in Immunology, iii, INCT (National Institute of Science and Technology), São Paulo, Brazil; [3]Department of Immunology, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil; [4]Université Pierre et Marie Curie (UPMC) University Paris 06, UMR 7211, Paris, France; [5]CNRS (Centre National de la Recherche Scientifique), Unité Mixte de Recherche (UMR) 7211, Paris, France; [6]Assistance Publique-Hôpitaux de Paris (AP-HP), Pitié Salpêtrière Hospital, Service de Biothérapie, Paris, France; [7]Division of Nephrology, São Lucas Hospital, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil; and [8]Division of Nephrology, University of São Paulo Medical School, São Paulo, Brazil

Transplanted individuals in operational tolerance (OT) maintain long-term stable graft function after completely stopping immunosuppression. Understanding the mechanisms involved in OT can provide valuable information about pathways to human transplantation tolerance. Here we report that operationally tolerant individuals display quantitative and functional preservation of the B-cell compartment in renal transplantation. OT exhibited normal numbers of circulating total B cells, naive, memory and regulatory B cells (Bregs) as well as preserved B-cell receptor repertoire, similar to healthy individuals. In addition, OT also displayed conserved capacity to activate the cluster of differentiation 40 (CD40)/signal transducer and activator of transcription 3 (STAT3) signaling pathway in Bregs, in contrast, with chronic rejection. Rather than expansion or higher activation, we show that the preservation of the B-cell compartment favors OT.

INTRODUCTION

Despite the great improvements in the immunosuppression regimens used to avoid allograft rejection (1–3), chronic rejection remains a major drawback for long-term organ transplantation. In addition, the lifelong use of nonspecific immunosuppressants leads to high susceptibility to infections (4), malignancies (5,6) and cardio- (7) and nephrotoxicity (8), which highly impairs the quality of life in transplanted individuals. Thus, the induction of immunological tolerance to the allograft is desirable in the transplantation field.

In recent years, researchers succeeded in inducing allograft tolerance in several experimental models (1,9–11); however, little has been translated in benefits to transplanted patients. Therefore, the development of new approaches to translate basic research in tolerance to the clinic is required. Nevertheless, transplantation tolerance does occur in the clinic. A rare group of transplanted individuals manage to develop a state called operational tolerance (OT), maintaining normal graft function and an immunocompetent immune system, after the complete withdrawal of immunosuppressive drugs (12). The study of individuals in OT may help to both understand the mechanisms involved in human allograft tolerance and determine biomarkers to discriminate this state among transplanted individuals. Indeed, some research groups, including ours, have been evaluating a variety of immunologic parameters in OT, mostly related to T-cell activity (13–18).

Despite the importance of B-cell–mediated immunity in the context of transplantation, there is limited information on the profile of the B-cell compartment in OT. It was reported that kidney-transplanted OT individuals present preserved numbers of total (19,20), naive (20) and memory B cells (19,20) compared with healthy individuals and a B-cell gene expression signature composed by a

Address correspondence to *Verônica Coelho, Laboratório de Imunologia,Instituto do Coração, Av Dr Enéas de Carvalho Aguiar, 44,bloco II,9 andar, Cerqueira César, São Paulo,SP 05403-000, Brazil.Phone: +55-11-3069-5905; Fax: +55-11-3069-5953; E-mail: vecoelho@usp.br.*

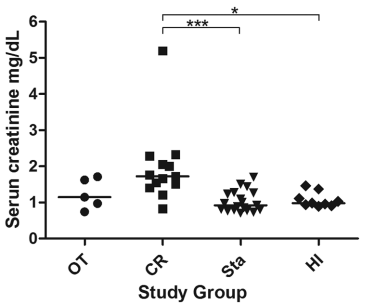

Figure 1. Renal function. One of the parameters used to evaluate the renal function was the median serum creatinine level. OT group, n = 5; CR group, n = 13; Sta group, n = 19; HI group, n = 9. *$p < 0.05$; ***$p < 0.001$.

higher expression of *IGKV4-1*, *IGLLA* and *IGKV1D-13* genes (20). However, the functional implication of this B-cell gene expression signature is unclear because these genes, which code for immunoglobulin light chains, have unknown immunoregulatory functions. Thus, despite the current enthusiasm about the role of B cells in OT, it is still unclear whether they comprise relevant functional players in this homeostatic state.

B cells are classically defined as antibody-producing cells; however, it is well established that these cells display several other functions, such as antigen presentation and cytokine production (21–25). Recently, some groups described, in experimental models, B-cell subsets that downregulate effector immune responses and help to control the development of autoimmune disorders (26–29). The suppressive activity of the so-called suppressive regulatory B cells (Bregs) was mostly related to the production of interleukin (IL)-10 (29,30) and to direct contact with $CD4^+$ T cells in mice (27,28). In humans, there are two reports on B-cell subpopulations displaying *in vitro* suppressive activity (31,32). It should be pointed that the immune phenotypic features of Bregs in humans are still controversial, and they are likely to be diverse, similarly to regulatory T cells (33). One group of investigators calls them B10 cells, highlighting their IL-10–dependent capacity to suppress monocyte proinflammatory cytokines (31). Another group defined a human Breg subpopulation with the $CD19^+CD24^{hi}CD38^{hi}$ phenotype, bearing suppressive activity also mediated by the production of IL-10, by activation of the cluster of differentiation 40 (CD40)/signal transducer and activator of transcription 3 (STAT3) signaling pathway and by engaging CD80 and CD86 (32). The immunoregulatory importance of the CD40/STAT3 signaling pathway was highlighted in the context of systemic lupus erythematosus, where Bregs presented impaired suppressive function. This impairment is probably related to the inadequate IL-10 production on CD40 activation, which is associated with low levels of STAT3 phosphorylation (32).

Considering the variety of immunoregulatory pathways to homeostasis, we hypothesized that the B-cell compartment and regulatory B cells are functional players in the state of OT. We found that tolerant individuals displayed a global preservation of the B-cell compartment and of the Breg activation status, whereas chronic rejection individuals displayed a significant impairment of Breg activation and B-cell numbers. We conclude that, rather than overactivation, the preservation of a healthy B-cell profile favors the development/maintenance of the homeostasis in OT.

MATERIALS AND METHODS

Experimental Design

We compared the B-cell compartment (circulating B-cell phenotypic characterization, B-cell receptor repertoire diversity and the activation status of the CD40/STAT3 signaling pathway) in (a) operational tolerance (OT, n = 5); (b) stable graft function and standard immunosupression (Sta, n = 19); (c) chronic rejection (CR, n = 13); and (d) healthy individuals (HI, n = 11). We also compared the B-cell parameters globally among groups.

Study Groups

The kidney graft recipients and healthy kidney donors enrolled in this study were followed at the Transplantation Unit of the Hospital das Clínicas, Universidade de São Paulo, and the Nephrology Unit of the Pontifícia Universidade Católica do Rio Grande do Sul, both part of the Brazilian Multicenter Study on Operational Tolerance, coordinated at the Heart Institute, School of Medicine, Universidade de São Paulo. Blind analyses were performed for all experiments. The ethics committee of both institutions approved this study (CAPPESQ [Comissão de Ética para Projetos de Pesquisa] 0476/08). All study subjects were recruited after obtaining informed consent.

The individuals were grouped according to the following classification: (a) OT: Transplanted individuals with stable graft function, in the absence of immunosuppression for at least 1 year (range 1.5–8 years); we have no biopsies of these individuals because they had normal and stable graft function, and this procedure is only clinically indicated when deterioration of graft function and/or proteinuria are observed (mean serum creatinine level: 1.2 mg/dL); (b) Stable graft function under conventional immunosuppression: Transplanted individuals with stable graft function, under standard immunosuppression (Azathioprine or mycophenolate mofetil/sodium and/or steroids and/or calcineurin inhibitor) and without clinical and laboratory features suggestive of rejection (mean serum creatinine level: 1.1 mg/dL); (c) Chronic rejection: Transplanted individuals under conventional immunosuppression with high serum creatinine concentration and progressive renal function deterioration. In all cases, chronic rejection was confirmed by biopsy according to Banff criteria (34,35) (mean serum creatinine level: 1.7 mg/dL). Three individuals presented C4d intragraft deposition, two of whom had circulating anti-human leukocyte antigen (anti-HLA) non–donor-specific antibodies. Two other individuals had anti-HLA antibodies without C4d intragraft deposition, one of whom presenting donor-specific HLA antibod-

Table 1. Demographic/clinical data of study individuals.

Study individual	OT1	OT2	OT3	OT4	OT5	CR Group (n = 13)	Sta group (n = 19)	HI group (n = 11)
Age (years)	53	42	43	49	32	44 (27–64)	50 (25–78)	41 (30–57)
Sex (F, M)	M	M	F	F	M	4, 9	10, 9	6, 5
Number of grafts	1	2	1	1	1	1 (n = 13) 2 (n = 1)	1 (n = 18)	NA
Time of transplantation (years)	23	5	7	23	13	8 (4–13)	8 (1–34)	NA
Induction therapy	NA	Anti-CD25	No	No	No	5 of 13 Anti-CD25 = 4 ATG = 1	14 of 19 Anti-CD25 = 13 ATG + IVIG = 1	NA
HLA (A, B, DR) mismatches	3	4	0	0	3	4.45 ± 1.75 (n = 11) NA (n = 2)	3.83 ± 1.30 (n = 18) NA (n = 1)	NA
Donor (living, deceased)	Living	Deceased	Living	Living	Living	6, 7	16, 3	NA
Blood transfusion (Y, N)	NA	Y	N	Y	N	6, 3 NA (n = 4)	11, 3 NA (n = 5)	NA
Acute rejection episode (Y, N)	N	Y	N	N	N	8, 5	6, 13	NA
Viral infections (Y, N)	NA	Y	NA	N	N	7, 5	4, 15	NA
Reason for immunosuppression interruption	Non-compliance	Non-compliance	Non-compliance	Non-compliance	Non-compliance	NA	NA	NA
Drug-free time (years)	6	1.5	4.1	8	4	NA	NA	NA

ATG, antithymocyte globulin; NA, not available or does not apply; IVIG, intravenous immunoglobulin. The numeric data are represented as mean ± standard deviation. Age (years): the age range for each group is shown in the parentheses. Time of transplantation: the time range is shown in the parentheses.

ies. The remaining five individuals were classified as displaying cellular-mediated rejection according to Banff criteria (34,35); and (d) Healthy individuals: healthy kidney donors with normal kidney function (mean serum creatinine level: 1.1 mg/dL).

Chronic rejection individuals displayed impaired renal function, assessed by higher serum creatinine (Figure 1). The demographic/clinical features of subjects in the study groups are shown in Table 1. We compared clinical parameters among all study groups to check for confounding factors and observed no clear association of clinical/demographic data (time after transplantation, age, sex and so on) with the allograft outcome and the immunologic results observed in the different study groups (Fisher exact test). We did not find statistical differences in the HLA sensitization among the study groups; 3 of 5 OT, 4 of 10 CR, 7 of 19 Sta, and 5 of 10 HI had detectable anti-HLA antibodies. Only two individuals (from Sta and CR groups) presented donor-specific anti-HLA antibodies.

Selection Criteria

All transplanted individuals included in this study had more than 1 year of transplantation. Renal function was evaluated by the estimated glomerular filtration rate (eGFR) by using the Cockroft-Gault formula (36), serum creatinine and proteinuria. We considered "conserved" or "stable" renal function when individuals presented an eGFR ≥40 mL/min and serum creatinine variation of ≤10%, during the last 6 months before study enrollment.

Blood Samples and Peripheral Blood Mononuclear Cell Isolation

Venous blood samples were collected in heparinized vacutainers. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation and then cryopreserved in a solution of 90% fetal calf serum/10% dimethyl sulfoxide in liquid nitrogen. For the signaling experiments, PBMCs were defrosted in RPMI medium supplemented with 10% of fetal calf serum (FCS). Cell viability was routinely ≥85%.

B-Cell and B-Cell Subpopulation Flow Cytometry Analysis

The PBMCs from renal transplanted individuals and healthy volunteers were incubated with allophycocyanin or phycoerythrin-cyanine dye 7 (PE-Cy7)-labeled anti-human CD19 (BD Biosciences, San Jose, CA, USA), fluorescein isothiocyanate–labeled anti-human CD24 (BD Biosciences) and phycoerythrin-cyanine dye 5 (PE-Cy5)-labeled anti-human CD38 (BD Biosciences). The FACS CantoII™ analyzer and DIVA software (BD Biosciences) were used for cell acquisition. At least 300,000 events were acquired in the lymphocyte gate. The data analysis was performed using the

Table 2. Primers for the B cell receptor heavy chain immunoscope analysis.

Primer	Sequence
IgVH family	
VH1	5′-TGGAGCTGAGSAGSCTGAGATCYGA-3′
VH2	5′-AACCCACASAGACCCTCAC-3′
VH3	5′-TCCCTKARACTCTCCTGTRCAGC-3′
VH4	5′-CTACAACCCSTCCCTCAAGAGT-3′
VH5	5′-CAGCACCGCCTACCTGCAGTGGAGC-3′
VH6	5′-TCCGGGGACAGTGTCTCT-3′
VH7	5′-CAGCACRGCATAYCTGCAGATCAG-3′
IgH γ chain	5′-6Fam-AAGTAGTCCTTGACCAGGCAGC-3′
IgH μ chain	5′-6Fam-GGAGACGAGGGGGAAAAGG-3′

The primers used to perform PCR in the immunoscope analysis of the 7 VH immunoglobulin families (VH1–VH7) for the IgG and IgM isotypes are shown.

FlowJo software 9.1 (Tree Star, Ashland, OR, USA). We used the following classification to analyze the B-cell subpopulations: $CD19^+$ for total B cells, $CD19^+CD24^{int}CD38^{int}$ for naive B cells, $CD19^+CD24^{hi}CD38^-$ for memory B cells (37,38) and $CD19^+CD24^{hi}CD38^{hi}$ for Bregs (32). The absolute numbers of B cells and subpopulations were calculated on the basis of the total blood cell counts obtained at the moment of blood collection for this study.

B-Cell Receptor Repertoire Analysis

RNA was isolated from 5×10^6 PBMCs using Trizol according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed with Superscript II reverse-transcriptase, using oligo(dT)12–18 primers and random primers (Invitrogen) in a final volume of 20 μL. cDNA was amplified in a standard polymerase chain reaction (PCR) reaction for 40 cycles with primers specific for the seven variable heavy chain (VH) immunoglobulin (Ig) families (Table 2) and with fluorescent reverse primers for the IgM and IgG isotypes (Table 2). Some of the primers were previously described in the literature (39,40). The denatured PCR products were then subjected to fragment analysis on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). CDR3 length profiles were obtained after analysis with GeneMapper software (Applied Biosystems). The data was extracted with GeneMapper software and submitted to analysis at the ISEApeaks® software, as described in the literature (41,42). To calculate the representativeness of each CDR3 length in relation to the total repertoire by using the ISEApeaks® software (42), for each VH analyzed, the software compared the area of each peak with the sum of the areas of all peaks expressed by each individual. We calculated the relative index of stimulation (43) by comparing the representativeness of each CDR3 length with the mean representativeness obtained in the HI group. We considered higher representativeness of a CDR3 length when the levels were ≥2 and lower representativeness when the levels were ≤0.5, in relation to levels observed in healthy individuals. Taking the reported correlation between the surface area of the peak and the number of transcripts for the respective CDR3 length (39,44,45), we correlated the expression levels of each CDR3 length with the peak area exhibited and evaluated the occurrence of expansions or decreases/deletions in the expression of specific CDR3 lengths among groups.

B-Cell STAT3 Signaling After CD40 Stimulation

The activation of the CD40/STAT3 signaling pathway was assessed as described before (32). PBMCs were thawed as mentioned above and allowed to rest for 1 h in RPMI/10% FCS. Subsequently, cells were washed with ice-cold PBS/1% FCS and then centrifuged for 5 min at 500*g* in a prechilled centrifuge. Cells were seeded in a 96-well round-bottom plate at a concentration of 5×10^5 cells/well. Cells were incubated on ice with 5 μg/mL purified stimulatory mouse anti-human CD40 monoclonal antibody (mAb) (clone-5C3; BD Biosciences) (0.5 μg CD40 mAb/10^6 cells) for 30 min in the dark. Cells were then washed, as described, and incubated with 10 μg/mL goat anti-mouse Ig (BD Biosciences) for 30 min at 4°C. Cells were then stained with appropriate antibodies for 30 min in ice-cold PBS/1% FCS on ice. For stimulation, cells were washed and resuspended in 37°C warmed PBS/1% FCS and kept at 37°C for 5 min. The reaction was stopped by the addition of 37°C warmed Cytofix™ (BD Biosciences). Cells were left to fix and permeabilize in Cytofix™ for 30 min at 37°C. Cells were washed by the addition of 50 μL Permwash™ (BD Biosciences) and incubated with 200 μL Permwash™ for 30 min on ice in the dark. Cells were then centrifuged and incubated with anti–pSTAT3-PE mAb Phosflow antibody (pY705; BD Biosciences) in Permwash™ for 30 min at 4°C in the dark. Cells were then washed twice with Permwash™ and once with PBS/2% FCS, and pSTAT3 expression was measured by flow cytometry. The fold of increase of STAT3 phosphorylation was calculated on the basis of the ratio between the median fluorescence intensity detected for stimulated and nonstimulated PBMCs. The fold increase in the number of pSTAT3-positive cells was calculated by the ratio between the percentages of pSTAT3-positive cells after anti-CD40 stimulation and before it.

Analysis of Intracellular IL-10 Production by B Cells

For intracellular IL-10 analysis, B cells were isolated from PBMCs of four healthy individuals by magnetic separation (B-cell isolation kit II; Miltenyi Biotec, Gladbach, Germany) (purity was on average ≥90%). Purified B cells (4×10^5) were cultured in 96-well flat-bottom plates in 0.2 mL medium (RPMI/10% FCS) for 48 h. B cells were stimulated by

incubation in precoated wells with 10 μg/mL mouse anti-human CD40 mAb (clone-5C3; BD Biosciences) and 1 μg/mL soluble CD40L (Invitrogen, Grand Island, NY, USA). GolgiPlug (BD Biosciences) was added for the last 5 h along with phorbol myristic acid (50 ng/mL) and ionomycin (500 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). Cells were then stained with LIVE/DEAD Fixable Far Red Dead Cell Stain Kits (Invitrogen, NY) for 30 min according to the manufacturer instructions. Subsequently, cells were stained with appropriate antibodies for 30 min in ice-cold PBS/1% FCS on ice in the dark. Cells were fixed and permeabilized in Cytofix™ (BD Biosciences) for 30 min on ice in the dark. Cells were washed by the addition of Permwash™ (BD Biosciences) and incubated with anti–IL10-PE antibody (BD Biosciences) in Permwash™ for 30 min at 4°C in the dark. Cells were then washed twice with Permwash and once with PBS/2% FCS, and intracellular IL-10 production was measured by flow cytometry. At least 100,000 events were acquired for analysis in the gate of lymphocytes.

Statistical Analysis

The results of each group were compared using the nonparametric Kruskal-Wallis test with the Dunn post test using Prism 5.0 software (GraphPad Software, San Diego, CA, USA) and one-way analysis of variance with the Newman-Keuls post test for the analysis of the B-cell receptor repertoire. $p < 0.05$ was considered significant. Significant differences are shown in the figures as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The values are expressed as mean ± standard error of the mean (SEM) in the column bar graphs. In scatter plots, the medians are exhibited.

RESULTS

Operational Tolerance Displays Preserved Numbers of B Cells, Naive B cells and Bregs

We analyzed total B-cell numbers and their subpopulations using the following classification: $CD19^+$ for total B cells (Figure 2), $CD19^+CD24^{int}CD38^{int}$ for naive B cells, $CD19^+CD24^{hi}CD38^-$ for memory B cells and $CD19^+CD24^{hi}CD38^{hi}$ for Bregs, as described in Materials and Methods (Figure 3A). CR individuals presented a decrease in the percentage and absolute numbers of circulating B cells in relation to HI ($p < 0.05$) (Figures 2A, B), whereas OT individuals presented preserved numbers of B cells (see Figures 2A, B). The reduction in the percentage of B cells in CR was frequently accompanied by decreased frequency of the Breg subpopulation, whereas the other subpopulations remained unchanged (Figures 3B, D, F). Absolute numbers of B cells were also lower in CR individuals who exhibited lower numbers of circulating memory B cells in relation to HI ($p < 0.01$) and Bregs in relation to OT and HI ($p < 0.05$) (Figures 3C, E, G).

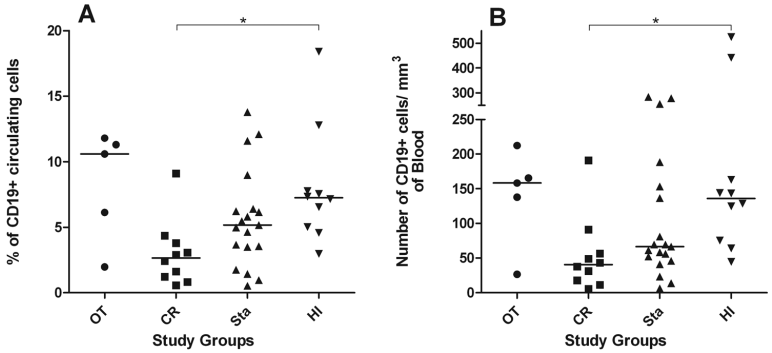

Figure 2. Quantitative analysis of total circulating B cells in the study groups. CR individuals display lower frequencies (A) and absolute numbers (B) of B cells in relation to HI. The absolute cell numbers were calculated on the basis of the complete blood count of each individual performed at the same moment of study sample. OT group, n = 5; CR group, n = 10; Sta group, n = 19; HI group, n = 10. $*p < 0.05$.

OT Displays Preserved B-Cell Receptor Diversity and Expansion of Specific CDR3 Lengths

To analyze if the OT group exhibits preferential use of the B-cell receptor (BCR) repertoire, we used the immunoscope methodology, as described in the literature (39). As shown in representative graphs for the IgM isotype (Figure 4A), the BCR repertoire was diverse for the majority of the families for all study groups. We first evaluated the diversity of the BCR repertoire by assessing the number of different CDR3 lengths expressed in each of the 7 VH immunoglobulin families (VH1–VH7), for the IgM and IgG isotypes, for each individual. The number of different CDR3 lengths found for each individual gives the complexity score (46). The more diverse the BCR repertoire, the higher the complexity score. CR displayed a lower BCR diversity for the IgM isotype in relation to HI ($p < 0.05$) (Figure 4B) and in relation to Sta and HI for the IgG isotype ($p < 0.01$) (Figure 4C). OT individuals showed BCR diversity with no significant difference in relation to both HI and Sta.

Looking for potential deletion or expansion of B-cell populations using specific CDR3 lengths, we used the ISEApeaks® software (42) to calculate the representativeness of each CDR3 length of the whole repertoire. The OT group displayed two CDR3 lengths differentially expressed in relation to all study groups. In the IgM isotype, the OT group exhibited an overrepresentation of the 16–amino acid (aa) CDR3 length, in the VH3 Ig family, when compared with CR ($p < 0.01$) and Sta/HI ($p < 0.05$) (Figure 4D). In addition, in the IgG isotype, the OT group exhibited an overrepresen-

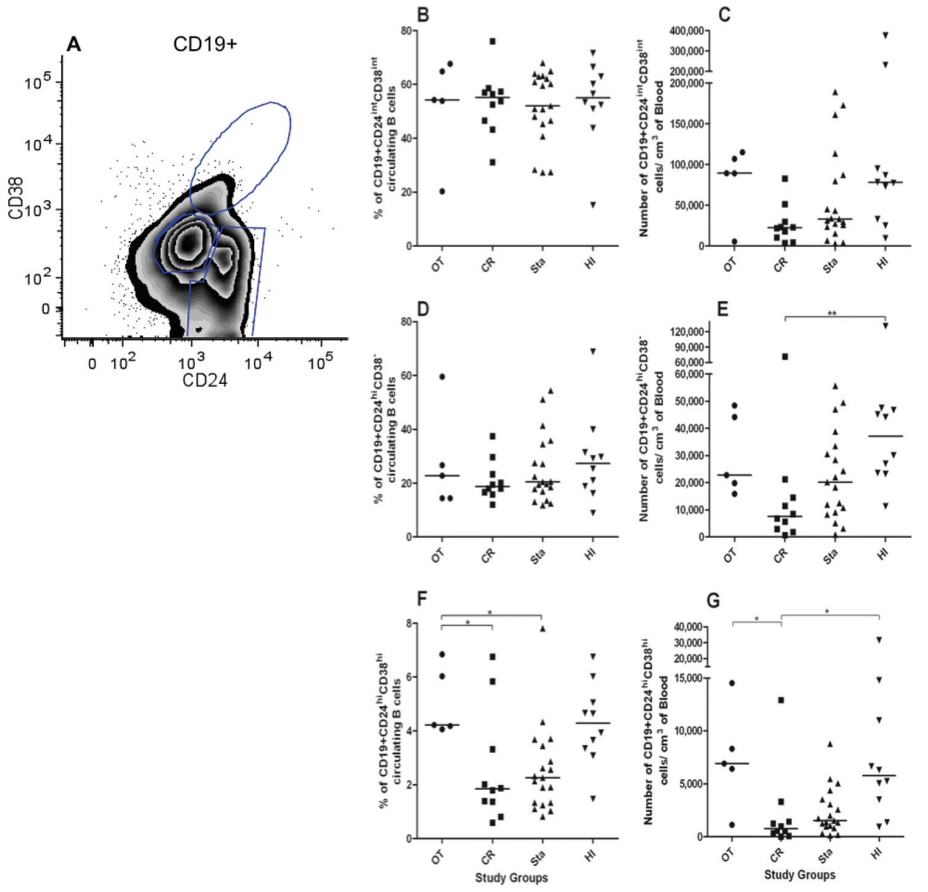

Figure 3. OT display preserved numbers of circulating Bregs. (A) Representative gate strategy used to analyze the following B-cell subpopulations: naive B cells ($CD19^+CD24^{int}CD38^{int}$), memory B cells ($CD19^+CD24^{hi}CD38^-$) and Bregs ($CD19^+CD24^{hi}CD38^{hi}$). Percentage (B) and absolute number (C) of naive B cells ($CD19^+CD24^{int}CD38^{int}$) are shown. Percentage (D) and absolute number (E) of memory B cells ($CD19^+CD24^{hi}CD38^-$) are shown. Percentage (F) and absolute number (G) of Bregs ($CD19^+CD24^{hi}CD38^{hi}$) are shown. The absolute cell numbers were calculated on the basis of the complete blood count of each individual performed at the same time the blood was collected. OT group, n = 5; CR group, n = 10; Sta group, n = 19; HI group, n = 10. *$p < 0.05$; **$p < 0.01$.

tation of the 5-aa CDR3 length, in the VH1 Ig family, when compared with all other study groups ($p < 0.001$) (Figure 4E). For all other CDR3 sizes, we detected no statistical differences among the groups.

Activation of CD40/STAT3 Signaling Pathway in Bregs Is Preserved in OT

We evaluated whether the different study groups displayed differences in the IL-10 activation pathway by analyzing the pattern of STAT3 phosphorylation (pSTAT3) and the capacity to activate this signaling pathway in the Bregs and naive and memory B-cell subpopulations upon CD40 engagement. In Figure 5A, we illustrate representative histograms of these analyses.

We performed two types of analysis. In the first (Figures 5B–D), we evaluated the intensity of STAT3 phosphorylation poststimulus by calculating the increase in the pSTAT3 fluorescence intensity (median of fluorescence intensity). In the second one, (Figures 5E–G), we analyzed the capacity to activate the CD40/STAT3 pathway, by observing the capacity of pSTAT3-negative cells to become positive following anti-CD40 stimulation. This is shown by the fold increase in the number of pSTAT3 after stimulus.

The CD40/STAT3 pathway activation with the agonistic CD40 mAb *in vitro* exhibited conserved and similar levels of STAT3 phosphorylation for all study groups, in all B-cell subpopulations (Figures 5B–D). Nevertheless, OT individuals exhibited a higher capacity to generate Bregs positive for pSTAT3 in relation to CR ($p < 0.05$) and similar to HI individuals (Figure 5E). We did not observe any differences among all groups in the fold increase of $pSTAT3^+$ after stimulation, for the naive and memory B-cell subpopulations (Figures 5F, G).

Finally, to confirm if Bregs present a higher predisposition to produce IL-10, as reported in the literature (32), we stimulated purified B cells, *in vitro*, from four healthy individuals with plate-bound anti-CD40 plus sCD40L for 48 h and evaluated intracellular IL-10 production. Bregs displayed a higher percentage of IL-10–producing cells when compared with memory and naive B cells (Figures 5H, I).

DISCUSSION

In the recent years, a lot of effort has been put forth to better understand the multiple immunoregulatory pathways potentially involved in the state of OT in human transplantation. Although some attention has been directed to the potential importance of the B-cell compartment in the state of OT, the functional implication of B cells for OT is not so clear. We have attempted to tackle this question using a global quantitative and functional B-cell analysis. Kidney transplanted individuals in OT presented a global preservation of the B-cell compartment parameters, regarding the percentage and the absolute numbers of B cells, naive B cells and Bregs; the B-cell receptor diversity; and the Breg activation status, all similar to the one observed in healthy individuals. In addition, the OT group presented higher Ig transcript levels or expansions of B cells

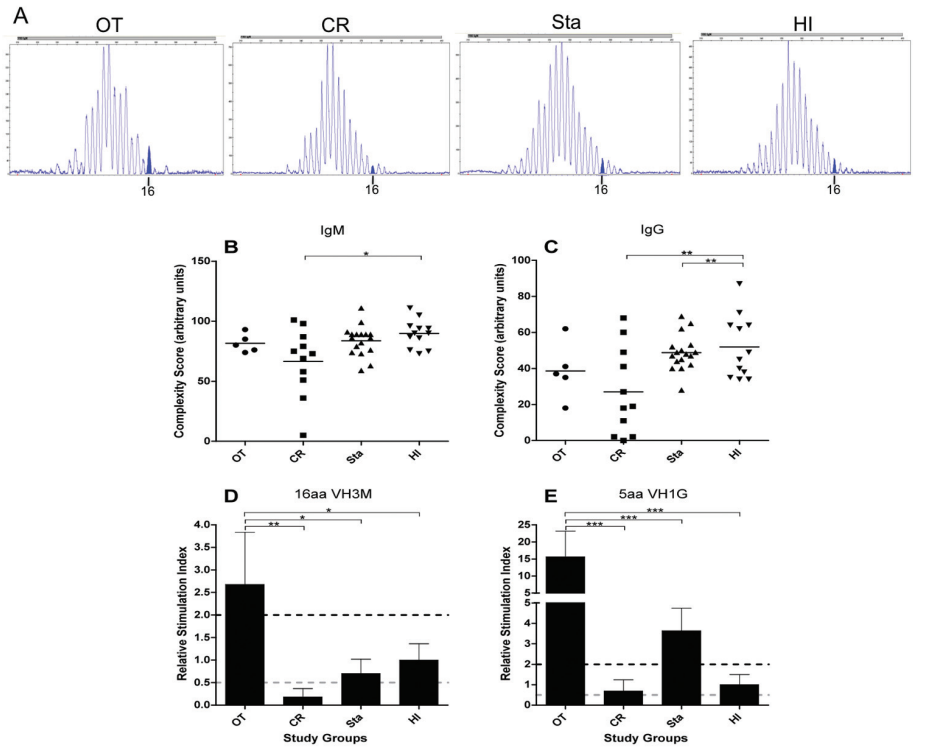

Figure 4. OT displays higher representativeness of specific B-cell receptor CDR3 lengths. (A) Representative profiles of the BCR CDR3 region of the VH3 Ig family, for the IgM isotype, showing higher representativeness for the 16-aa CDR3 length. Analyses were performed using PBMC cDNA of study individuals, after fragment analysis of fluorescent PCR products on a 3730xl DNA Analyzer (Applied Biosystems). Profiles were obtained after analysis with GeneMapper software (Applied Biosystems). The IgM (B) and IgG (C) repertoire diversity was calculated by the sum of the number of different BCR CDR3 lengths expressed in each individual. The OT group exhibited expansion of clones expressing the BCR CDR3 lengths of the 16-aa VH3 family, for the IgM isotype (D), and 5-aa VH1 family, for the IgG isotype (E). The black dashed line corresponds to the point in which we considered higher gene expression than in healthy individuals (>2). The gray dashed line corresponds to the point at which we considered expression lower than in healthy individuals. OT group, n = 5; CR group, n = 11; Sta group, n = 17; HI group, n = 11. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

bearing B-cell receptors with 16 aa in the VH3 Ig family (for the IgM isotype) and 5 aa in the VH1 Ig family (for the IgG isotype), differentiating this group in relation to all other study groups, including healthy individuals. In contrast, the CR group exhibited major quantitative and qualitative alterations in the B-cell compartment. These data allow us to conclude that rather than inducing an overactivation or augmentation of B cells, the state of OT essentially involves the capacity to preserve a healthy B-cell compartment profile.

Our quantitative data corroborate previous works in which OT presented similar percentage and absolute numbers of circulating B cells to HI individuals (13,19,20), although the authors often refer to the OT group as displaying increased numbers of circulating B cells (19). The state of OT does not seem to induce a global expansion of total circulating B cells or any of their subpopulations, but rather involves the quantitative maintenance of B cells similar to that observed in HI. This result could favor the control of the inflammatory process directed to the allograft or even just reflect the achieved homeostatic state. In contrast, the drop in the numbers of circulating B cells could reflect the uncontrolled ongoing inflammatory process in CR individuals.

One possible explanation for the decreased numbers of B cells in CR is the recruitment of circulating B cells to the graft. Indeed, it was reported that during rejection, the kidney allografts express chemokines (C-X-C motif chemokine 13 [CXCL13]) (47–49) and integrins (vascular cell adhesion molecule [VCAM] and intracellular adhesion molecule [ICAM]-1) (50), which could favor the recruitment of circulating B cells to the graft and induce a reduction of circulating B cells, as we observed in CR. It could also be argued that the immunosuppressive drugs could affect the number of B cells in the periphery. Although this result cannot be completely excluded, it does not seem to be the case because the Sta group, which is also under the same immunosuppressive regimen, did not show B-cell quantitative differences in relation to the other groups. This scenario suggests that other active processes are likely to be involved in the reduction of circulating B cells in CR.

Another interesting observation is that the OT and HI groups exhibited similar numbers of circulating B cells with the regulatory phenotype $CD19^{+}CD24^{hi}CD38^{hi}$, previously described in the literature (32), whereas CR displayed an expressive reduction of these cells. Thus, we may interpret that the Breg population may be an important regulator of the anti-donor inflammatory immune response and that Breg deficiency could be involved in the process of chronic allograft rejection.

In experimental models, Bregs have been associated with the control of exacerbation/perpetuation of a variety of inflammatory responses (26–28,51). In humans, it was observed that these cells suppress Th1 responses *in vitro* (32). Nevertheless, there is still some controversy in the scientific community as to whether to consider these cells as "bona

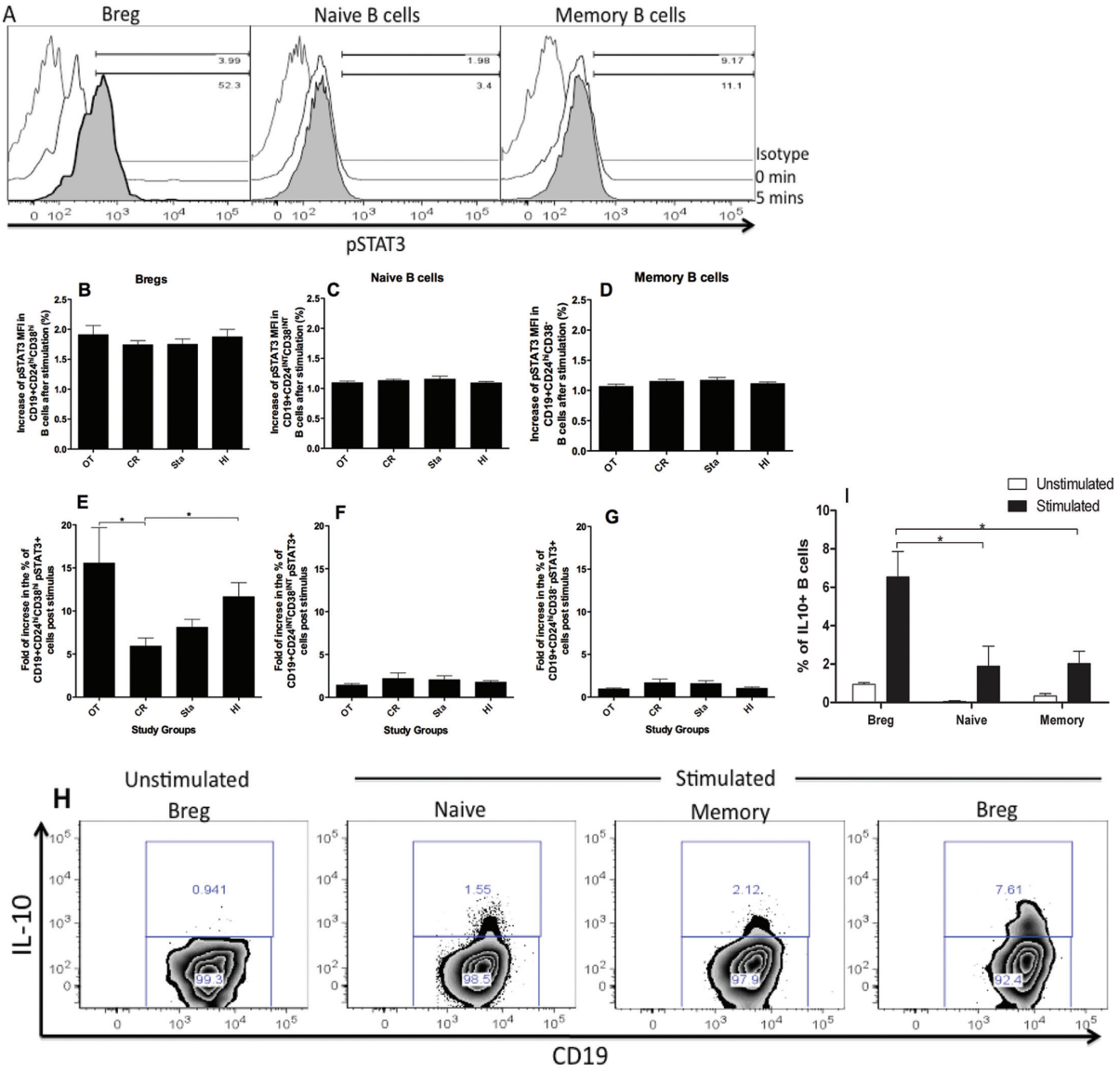

Figure 5. OT preserves the capacity to activate the CD40 signaling pathway. (A) Representative histograms of STAT3 phosphorylation in Bregs ($CD19^{+}CD24^{hi}CD38^{hi}$), naive B cells ($CD19^{+}CD24^{int}CD38^{int}$) and memory B cells ($CD19^{+}CD24^{hi}CD38^{-}$) at 0 and 5 min after stimulation with CD40 mAb. (B), (C) and (D) display the percentage of increase of the median of fluorescence intensity (MFI) of pSTAT3 in Bregs, naive B cells and memory B cells after stimulation, respectively. (E), (F) and (G) show the fold increase in the percentage of pSTAT3-positive cells after stimulation in Bregs, naive B cells and memory B cells, respectively. (H) Representative zebra plot for one healthy individual showing the frequency of IL-10–positive cells after stimulation within the B-cell subpopulations studied. Purified B cells were stimulated for 48 h with plate-bound anti-CD40 mAbs plus soluble CD40L. Phorbol myristic acid and ionomycin were added for the last 5 h of culture. $CD19^{+}IL\text{-}10^{+}$ B cells were measured by intracellular cytokine staining. (I) IL-10 production by the B-cell subpopulations in four healthy individuals. Bar graphs indicate mean ± SEM percentages of Breg, naive and memory B cells that produced IL-10. OT group, n = 5; CR group, n = 10; Sta group, n = 17; HI group, n = 10. *$p < 0.05$.

fide" Bregs. Indeed, cells displaying the $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype are not a homogeneous subpopulation and may include more than one type of B cell, such as transitional B cells, as previously described in the literature (38). In addition, other investigators have proposed IL-10 production as the best phenotypic marker for human Bregs, because of the difficulty in determining specific surface markers for this B-cell subpopulation (31). It should be mentioned that the IL-10–producing B cells, described in the cited work, exhibited some phenotypic differences in relation to

the $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs, such as the expression of CD27, which is absent in $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs (32,52). This result suggests the existence of different suppressive B-cell subpopulations, as well the establishment of regulatory T cells (33). It is not presently known whether different Breg subpopulations in fact exist and if they could eventually play differential roles in OT. Nevertheless, given our data, we suggest that the state of OT has a systemic repercussion and the maintenance of the Breg subpopulation displaying the $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype may, indeed, have an important role in this homeostatic state.

In addition to the data on the numbers of circulating B cells, we observed that the CR group also exhibited reduced B-cell receptor repertoire diversity in PBMCs, whereas OT, Sta and HI presented similar BCR repertoire diversity. These data reinforce the idea that an imbalance in the B-cell compartment may, indeed, be related to an impaired capacity to control inflammation, favoring the development of chronic rejection. In addition, the higher Ig transcript levels or expansions of B cells expressing specific BCR CDR3 lengths (16 aa in the VH3 Ig family [IgM isotype] and 5 aa in the VH1 Ig family [IgG isotype]), in the OT group, differentiated this state from all other study groups, including HI individuals. This result indicates that besides the global quantitative preservation of the B-cell compartment, the achievement and/or maintenance of the OT involves a differential expansion of particular B-cell clones, bearing specific CDR3 lengths, which could eventually contribute to tolerance. However, we do not have any functional information on these cells expressing specific CDR3 lengths.

To evaluate whether the specific CDR3 lengths expanded in OT were contained within the Breg population, we sorted $CD19^{+}CD24^{hi}CD38^{hi}$ and analyzed the B-cell receptor repertoire (data not shown). The B-cell receptor repertoire of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was diverse and included the CDR3 lengths expanded in OT. However, the $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype apparently showed no CDR3 length expansion (data not shown). Therefore, we can say that the CDR3 lengths found to be expanded in OT are indeed part of the $CD19^{+}CD24^{hi}CD38^{hi}$ Breg population. Unfortunately, there are no VH family–specific antibodies available that could be used to purify these cells and test their functional activity. Nevertheless, we favor that the differential expression of these specific BCR CDR3 lengths has a potential to be used as biomarkers for OT, if confirmed in another cohort of patients.

Another important observation is that the Sta group exhibited individuals with B-cell profiles similar to either CR or OT. This result is noteworthy because this group of individuals is a clinically relevant target to search for potential OT individuals who could benefit from immunosuppression minimization. Thus, the identification of individuals in the Sta group with a B-cell profile similar to OT could indicate a potential evolution to OT, whereas a B-cell profile similar to CR could suggest progression to chronic rejection and loss of allograft function.

Looking for possible B-cell signaling pathways involved in OT, we analyzed the STAT3 phosphorylation pathway upon CD40 engagement. We observed that the OT and HI groups exhibited a similar capacity to activate the Breg STAT3 phosphorylation, whereas the CR group displayed an impaired capacity to activate this pathway. This Breg functional alteration, together with the decreased numbers of circulating Bregs seems to create a favorable context for the development to chronic rejection. In contrast, the preservation of a healthy B-cell compartment profile, including Breg numbers and functional activity, in OT, together with maintenance of other immune system parameters described in the literature, such as regulatory T cell (Treg) numbers (53,54), Toll-like receptor 4 expression (55) and CD4 and CD8 T-cell numbers (13), probably act synergically to compose a favorable context for the development of transplantation tolerance. We believe that the experimental design we used, which included a comparative analysis of OT with its opposite clinical outcome (chronic rejection), was critical for our data interpretation.

One question that emerges from our data on CD40/STAT3 signaling pathway is whether OT individuals display a differential profile for IL-10 production by Bregs in relation to CR. Unfortunately, because of technical problems involving the appropriate CD40 stimulus for a prolonged culture experiment (48 h) and limited biological material from OT individuals, we were unable to answer this question. Nevertheless, we did find a higher percentage of IL-10–producing cells within the Breg population (see Figures 5H, I) in comparison to naive and memory phenotypes in healthy individuals. However, only small percentages (10–15%) of Bregs are IL-10 producers, indicating that other mechanisms should be involved in their suppressive activity. Indeed, it was also reported that the regulatory activity mediated by these cells also depends on cell contact mediated by CD80/86 interactions (32). These are important issues to be investigated in the context of OT.

We believe it is time to reexamine and integrate all relevant immunologic data available in the literature on OT at this point. This step will allow us to have an integrated global view of the various mechanisms potentially involved in OT, which may be relevant for the development of new immunoregulatory strategies. We propose that the B-cell profile found in chronic rejection, combined with other immunologic features observed in this state, should be tested for their predictive value regarding the progression to chronic rejection. If confirmed to be predictive, they could be used as a tool to identify potential chronic rejection progressors that could benefit from additional immunosuppression and/or immunoregulatory stimuli.

CONCLUSION

It is important to highlight that OT individuals are quite rare worldwide. De-

spite the limited numbers of OT individuals in the present study, we clearly defined a differential B-cell profile in relation to the CR group. We conclude that OT induces a significant systemic repercussion and that, rather than inducing expansion or higher activation, the preservation of the B-cell compartment seems to favor the achievement and/or maintenance of transplantation homeostasis.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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