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ORIGINAL ARTICLE

Regulatory/inflammatory cellular response discrimination in operational tolerance

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ABSTRACT

Background. Antigen-specific cellular response is essential in immune tolerance. We tested whether antigen-specific cellular response is differentially modulated in operational tolerance (OT) in renal transplantation with respect to critical antigenic challenges in allotransplantation—donor antigens, pathogenic antigens and self-antigens.

Methods. We analysed the profile of immunoregulatory (REG) and pro-inflammatory (INFLAMMA) cytokines for the antigen-specific response directed to these three antigen groups, by Luminex.

Results. We showed that, in contrast to chronic rejection and healthy individuals, OT gives rise to an immunoregulatory deviation in the cellular response to donor human leucocyte antigen DR isotype peptides, while preserving the pro-inflammatory response to pathogenic peptides. Cellular autoreactivity to the N6 heat shock protein 60 (Hsp60) peptide also showed a REG profile in OT, increasing IL4, IL-5, IL-10 and IL-13.

Conclusions. The REG shift of donor indirect alloreactivity in OT, with inhibition of interleukin (IL)-1B, IL-8, IL-12, IL-17, granulocyte colony-stimulating factor, Interferon-γ and monocyte chemoattractant protein-1, indicates that this may be an important mechanism in OT. In addition, the differential REG profile of cellular response to the Hsp60 peptide in OT suggests that REG autoimmunity may also play a role in human transplantation tolerance. Despite cross-reactivity of antigen-specific T cell responses, a systemic functional antigen-specific discrimination takes place in OT.

Keywords: antigen-specific cellular response, HLA-DR peptide, immunoregulation, operational tolerance, renal transplantation

INTRODUCTION

Our immune system is constantly dealing with various antigenic challenges throughout life, namely, self-antigens and microbial antigens—both from commensals and pathogens—as well as airborne and food antigens, continuously activating and downregulating inflammation and repairing damaged tissues, while dynamically maintaining homeostasis. In addition to these antigenic challenges, transplanted individuals, on a daily basis, have to deal with robust pro-inflammatory (INFLAMMA) stimuli induced by alloantigens.

The immunobiology of allotransplantation involves immune response not only to donor alloantigens, but also to self-antigens such as cellular response [1] to heat shock protein 60 (Hsp60) [2, 3] and antibody response to collagen in lung transplantation [4] and to angiotensin II receptor in renal

transplantation [5]. Autoimmunity has been reported to display both INFLAMMA and immunoregulatory (REG) roles [6] in allotransplantation, and it is likely that its REG/INFLAMMA functions depend on factors such as the time point of transplantation, interactions with different immune cell types, the immunological microenvironment and the effect of immunosuppressants. It is believed that the inflammatory context of allotransplantation and tissue damage leads to exposure to various self-antigens, favouring the expansion and activation of autoreactive effector T cells that escape REG control and contribute to graft aggression. On the other hand, autoreactive regulatory T cells may play a role in controlling inflammatory autoimmunity in this very context of allotransplantation.

Among self-antigens involved in the immunobiology of transplantation, Hsp60 is a particularly interesting molecule due to its involvement in homeostasis. Various immunologic roles for Hsp60 and its peptides have been reported in pathological autoimmunity [7, 8], transplantation [2, 3, 9] and neurologic protection [10]. Our group has designed various Hsp60 peptides and determined that some display predominantly REG or INFLAMMA activities [3, 6], pointing to potential therapeutic applications.

Transplantation tolerance remains a great challenge in the clinic, despite successful protocols to induce tolerance in experimental models [11]. Nevertheless, the existence of a special group of transplanted patients who do not reject and manage to keep good graft function, after complete withdrawal of immunosuppressive drugs is, by itself, significant evidence that transplantation tolerance is possible in the clinic. Although operational tolerance (OT) is quite rare, much can be learned from this phenomenon and the scientific community has been putting a lot of effort into understanding the potential underlying mechanisms, as well as determining reliable biomarkers of tolerance [12–14].

To date, it is believed that OT is an active process involving multiple REG pathways, initially facilitated by immunosuppressive drugs, which allow overcoming the initial inflammatory storm, providing control of graft rejection and building a state of homeostasis with the graft. Much data have been generated on OT in renal transplantation, with a recent focus on the potential role of B cells [15–17]. Our group has suggested the occurrence of a type 2 helper T cell (Th2) immune deviation, due to the higher expression of the transcription factor GATA-3 in OT, compared with both chronic rejection (CR) and healthy individuals (HIs), suggesting that this is an immunosuppressant-independent feature [18].

Antigen-specific cellular response is critical in immune tolerance, though it has not been much explored in the context of human transplantation tolerance. Our aim was to determine whether antigen-specific cellular response is differentially modulated in OT. To this end, we compared the immunologic functional profile of the antigen-specific cellular response to three major antigenic challenges in the context of transplantation: donor antigens [human leucocyte antigen DR isotype (HLA-DR) peptides], pathogenic antigens [cytomegalovirus (CMV) peptides] and self-antigens [Hsp60 peptides—with REG (N6 pep) or INFLAMMA (C9 pep) properties]. We analysed the production of cytokines displaying predominantly REG or INFLAMMA activities in the context of transplantation in response to these three antigens, comparing OT with different clinical groups and to HIs. We show here that OT in renal transplantation involves the immune deviation of indirect alloreactivity to donor HLA-DR peptides, possibly also involving the participation of REG autoimmunity to the N6 Hsp60 peptide, while the inflammatory cellular response to pathogens is maintained, providing preserved immunocompetence to deal with infectious challenges.

MATERIALS AND METHODS

Experimental design

We compared the profile of the antigen-specific cellular response to three critical antigenic challenges in the context of transplantation: donor antigens (HLA-DR peptides), pathogenic antigens (CMV peptides) and self-antigens [Hsp60 peptides bearing predominantly REG (N6 pep) or INFLAMMA (C9 pep) properties]. We analysed the production of cytokines classified as displaying predominantly REG or INFLAMMA activities in the context of allotransplantation or in other contexts, in response to the three antigenic stimuli. We analysed the levels of different cytokines and the frequency of a REG or INFLAMMA type of response to antigenic stimuli, comparing first OT to its opposing clinical outcome CR, then to HIs and transplanted individuals with stable graft function on conventional immunosuppression (STA). We also analysed the baseline condition with no antigenic stimuli (i.e. spontaneous cytokine production) in order to evaluate the *in vivo* ongoing systemic process. Differential profiles between OT and CR were considered, with a view to determining potential underlying mechanisms, and HIs with a view to identifying up- or downregulation in comparison to the physiologic state (Supplementary data, Figure S1).

Study groups

Renal transplant recipients from Hospital das Clínicas–Universidade de São Paulo and the Nephrology Unit of the Pontifícia Universidade Católica do Rio Grande do Sul were enrolled in the Brazilian Multicenter Study on Operational Tolerance, which is coordinated at the Heart Institute, School of Medicine, University of São Paulo. This study was approved by the ethics committee of both institutions and was conducted in accordance with good clinical practice and with the Declaration of Helsinki, as required by our Ethics Committee (Medical School, University of São Paulo-HCFMUSP (CAPPesq - Comissão de Ética para Projetos de Pesquisa—number 0476/ 08). All patients provided signed informed consent. In Brazil, no organs/tissues are procured from prisoners and the entire process of organ procurement, harvesting and distribution is controlled, regulated and supervised by the Brazilian Health Ministry.

Study groups (all transplanted subjects with >1 year of transplantation) comprised the following: (i) OT group: six subjects with stable graft function, off immunosuppressants for at least 1 year, mean serum creatinine level 1.12 mg/dL; (ii) STA group: four subjects with stable graft function, on

standard immunosuppression, mean serum creatinine level 0.98 mg/dL; (iii) CR group: seven subjects on standard immunosuppression, with progressive renal function impairment and biopsy-proven CR [19], mean serum creatinine level 2.19 mg/dL; and (iv) HI group: seven HIs (kidney donors or not) with normal kidney function, mean serum creatinine level 1.05 mg/dL (Supplementary data, Table S3). There were no significant differences in age and frequency of females and males among subjects, but as expected, there was a higher frequency of previous acute rejection episodes and higher creatinine levels in the CR group (Supplementary data, Table S2). All individuals agreed to participate and provided signed informed consent.

Biological samples

Whole blood was collected in heparinized vacutainer tubes (Becton Dickinson, San Diego, CA, USA), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (density 1.077 g/L) gradient and cryopreserved in liquid nitrogen until use.

Synthetic peptides

The HLA-DR peptides were selected and designed as follows: (i) we selected one HLA-DR donor mismatch, when present, for the transplanted individuals or any allo-HLA-DR molecule for HIs; (ii) we selected the peptide displaying the best binding prediction to recipients' HLA-DR molecules within the entire sequence, using the prediction programme Immune Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org). HLA-DR peptide sequences are shown in the Supplementary data (Supplementary Table S1).

The Hsp60 peptides had been previously designed by our group and displayed predominantly REG (N6 pep) or INFLAMMA (C9 pep) activities. Hsp60 and HLA-DR 25-mer peptides, with 90% purity, were synthesized by GL Biochem (Shanghai, China) and diluted to a concentration of 1 mg/mL and stored at −80°C until use. Sequences of the Hsp60 peptides are not described in the article due to ongoing patent issues.

The CMV peptides are a pool of mainly 15-mer peptides covering the complete sequence of the pp65 CMV protein (PepTivator CMV pp65; UniProt ID: P06725; Miltenyi Biotec, Bergisch Gladbach, Germany).

Cytokine quantification: in the basal condition and in response to alloantigens, pathogenic antigens and self-antigens

PBMCs (5×10^5 cells/well) were cultured for 48 h in 48-well plates in 0.5 mL of complete Roswell Park Memorial Institute medium (Gibco, Waltham, MA, USA) with 10% Hyclone foetal bovine serum (GE Healthcare, Chicago, Illinois, USA) with the different peptides at previously tested optimal concentrations, based on a dose–response curve [HLA-DR peptides (50 µg/mL), CMV peptides (2.5 µg/mL), and N6 and C9 peptides from Hsp60 (20 µg/mL)] as well as with 1 µg/mL anti-CD3 antibody (OKT3, Janssen-Cilag, Breda, The Netherlands) as positive control and without antigenic stimulus (basal or spontaneous cytokine production) in a humidified incubator at 37°C in 5% carbon dioxide. We determined cytokine concentrations in the 48-h culture supernatants using a panel of 17 cytokines, classified as predominantly REG [interleukin (IL)-4, IL-5, IL-7, IL-10, IL-13] or INFLAMMA [IL-1B, IL-6, IL-8, IL-12, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1B (MIP-1B)] by Luminex assay (Bio-Plex Pro Human Cytokine 17-Plex Panel; BioRad, Hercules, CA, USA), according to the manufacturer's instructions. Classification of cytokines as predominantly REG or INFLAMMA was based on literature data on transplantation and autoimmune diseases. In these contexts, there is a significant amount of data supporting a predominantly REG role of the Th2 immune response [18, 20–22], contributing to the control of inflammation, both in experimental models and in human transplantation. In contrast, the Th1 response has been reported to have a predominantly INFLAMMA role, contributing to allograft aggression [22, 23]. Some cytokines, such as the homeostatic cytokine IL-7, despite the limited availability of information, have been recently reported to play an important role in promoting regulatory T cell activity, including in the context of transplantation tolerance [24, 25]. IL-2 was not classified as predominantly REG or INFLAMMA, due to its clear dual activity. We considered the occurrence of spontaneous cytokine production when the detected cytokine concentration was above the lower detection limit for each cytokine, as defined by the manufacturer's curve. The effect of the antigenic stimuli on cytokine production was calculated as follows: (detected cytokine concentration in the presence of antigen) minus [cytokine concentration obtained for PBMCs cultured without antigens (spontaneous cytokine production)]. For normalization purposes in this calculation, all values below the detection limit for a particular cytokine, found in the basal condition (without antigens), were considered as the exact value of the detection limit. We defined the following: (i) induction of cytokine production: there was no spontaneous cytokine production and antigen stimulation led to an increase in cytokine concentration of $\geq$1 pg/mL, above the detection limit; (ii) increase in cytokine production: spontaneous cytokine production was detected and antigenic stimulation led to an increase in cytokine production of $\geq$1 pg/mL; (iii) inhibition of cytokine production: spontaneous cytokine production was detected and antigen stimulation led to a decrease (partial or total) in cytokine production of at least 1 pg/mL; and (iv) no effect: there was no change in cytokine concentration ($\geq$1 pg/mL) compared with no antigenic stimulation.

Analysis of REG and INFLAMMA types of response

Cellular responses to peptides were classified as REG or INFLAMMA, according to the criteria shown in Table 1. For each study group, we calculated the frequency (%) of REG and INFLAMMA types of responses for both predominantly REG and INFLAMMA cytokines, separately; the global frequency (%) of REG responses and INFLAMMA responses, as well as of no response, taking into account all cytokines studied; and the REG/INFLAMMA ratio for predominantly REG and INFLAMMA cytokines, as well as globally.

Table 1. Criteria for the classification of REG and INFLAMMA responses to peptides and strategies for the calculations of the frequency of REG and INFLAMMA responses for individual cytokines and predominantly REG and INFLAMMA cytokines in each study group

Classification of REG and INFLAMMA responses for peptide stimulations	Frequency (%) of REG responses in each study group: calculations for individual cytokines and for each cytokine type (REG or INFLAMMA)	Frequency (%) of INFLAMMA responses in each study group: calculations for individual cytokines and for each cytokine type (REG or INFLAMMA)	REG:INFLAMMA ratios	Global REG or INFLAMMA profile classification
REG response: induction or increase of predominantly REG cytokines, or inhibition of predominantly INFLAMMA cytokines	Frequency of REG responses calculated in each study group considering each cytokine individually; predominantly REG cytokines and predominantly INFLAMMA cytokines separately; all cytokines: global frequency in each study group	Frequency of INFLAMMA responses was calculated in each study group considering each cytokine individually; predominantly REG cytokines and predominantly INFLAMMA cytokines separately; all cytokines: global frequency in each study group	REG:INFLAMMA ratios for peptide responses were calculated in each study group, for individual cytokines and globally, considering all cytokines classified as predominantly REG or INFLAMMA	Global profile of responses to each group of antigenic stimulus was classified as predominantly REG or INFLAMMA for each study group. Predominantly REG profile: value of REG:INFLAMMA ratio ≥ 1.3
INFLAMMA response: induction or increase of predominantly INFLAMMA cytokines or inhibition of predominantly REG cytokines	REG response frequency: calculated by counting the number of REG responses within the specific study group × 100 and divided by the total possible responses considered for (i) each cytokine: total possible responses = number of subjects in the study group; (ii) (a) predominantly REG cytokines: total possible responses = number of subjects in the study group × number of REG cytokines tested, (b) predominantly INFLAMMA cytokines: total possible responses = number of subjects in the study group × number of INFLAMMA cytokines tested; (iii) global frequency per group: total possible responses = number of subjects in the study group × number of all cytokines tested, classified as predominantly REG or INFLAMMA	INFLAMMA response frequency: calculated by counting the number of INFLAMMA responses within the specific study group × 100 and divided by the total possible responses, considered for (i) each cytokine: total possible responses = number of subjects in the study group; (ii) (a) predominantly REG cytokines: total possible responses = number of subjects in the study group × number of REG cytokines tested, (b) predominantly INFLAMMA cytokines: total possible responses = number of subjects in the study group × number of INFLAMMA cytokines tested; (iii) global frequency per group: total possible responses = number of subjects in the study group × number of all cytokines tested classified as predominantly REG or INFLAMMA	Calculation of REG:INFLAMMA ratios: for each cytokine: percentage of REG responses divided by percentage of INFLAMMA responses for the specific cytokine within the study group; global: percentage of REG responses divided by percentage of INFLAMMA responses considering all cytokines classified as predominantly REG or INFLAMMA	Predominantly INFLAMMA profile: value of REG:INFLAMMA ratio ≤ 0.7

Spontaneous cytokine production: detection of cytokine above the limit of detection for the specific cytokine in the condition with no antigenic stimulus. Predominantly REG cytokines (cytokines exhibiting predominantly REG activities, especially in the context of transplantation): IL-4, IL-5, IL-7, IL-10 and IL-13; predominantly INFLAMMA cytokines (cytokines exhibiting predominantly INFLAMMA activities, especially in the context of transplantation): IL-1B, IL-6, IL-8, IL-12, IL-17, G-CSF, GM-CSF, IFN-γ, TNF-α, MCP-1 and MIP-1B. The absolute numbers of REG and INFLAMMA responses were used for statistical analysis (chi-squared and Fisher) for comparisons between study groups for each type of antigenic stimulus.

Statistical analyses

We used the chi-squared test to determine whether the frequency of REG and INFLAMMA responses was significantly different, either globally or within the set of predominantly REG and INFLAMMA cytokines, for each antigenic stimulus, comparing OT and CR, OT and HI and finally CR and HI. The non-parametric Kruskal–Wallis Dunn's multiple comparison test was used for comparison of cytokine concentrations (pg/mL) among study groups and the Mann–Whitney test was used to compare the same between two study groups. We used Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$. Significant differences are shown as $^{*}P < 0.05$, $^{**}P \leq 0.001$ and $^{***}P \leq 0.0001$.

RESULTS

OT has a REG differential profile of spontaneous cytokine production

Spontaneous cytokine production (no antigen stimulus) may provide relevant information on the immunologic activities ongoing *in vivo*. Thus we compared the profiles of spontaneous cytokine production among study groups (Supplementary data, Table S4). Although spontaneous cytokine production was detected in all groups (above the detection limit for each cytokine) (Supplementary data, Figures S2A and S2B), the OT group displayed a differential global profile for the frequency of predominantly REG and predominantly INFLAMMA cytokines detected compared

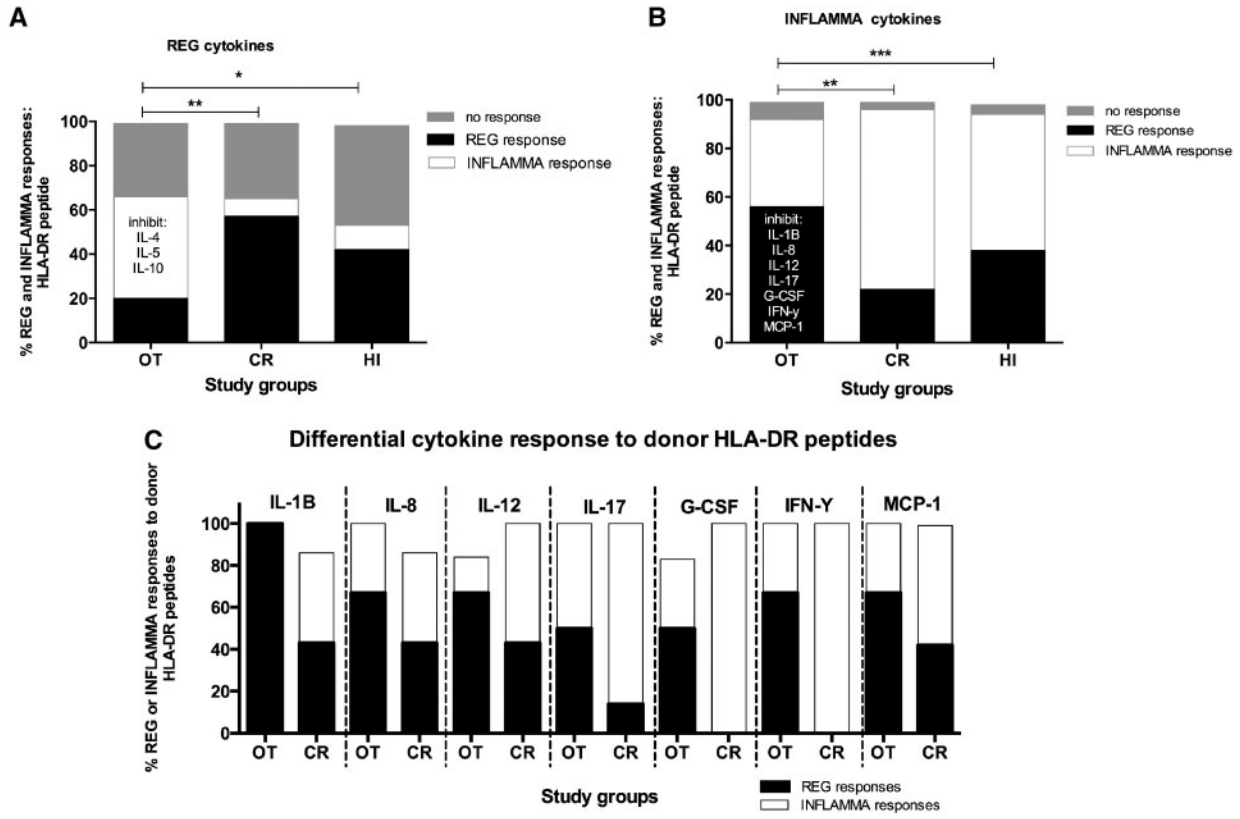

FIGURE 1: Frequency (%) of REG and INFLAMMA cytokine production in response to HLA-DR peptides. Predominantly REG cytokines analysed include IL-4, IL-5, IL-7, IL-10 and IL-13; predominantly INFLAMMA cytokines analysed include IL-1B, IL-6, IL-8, IL-12, IL-17, G-CSF, GM-CSF, IFN-γ, TNF-α, MCP-1 and MIP-1B (Luminex). Study groups: OT ($n = 6$), CR ($n = 7$) and HI ($n = 7$). REG response (black bars): increase/induction of REG cytokines or decrease of INFLAMMA cytokines; INFLAMMA response (white bars): increase/induction of INFLAMMA cytokines or decrease of REG cytokines; no response (grey bars): no effect on baseline cytokine production. The graphs were plotted using the percentages of subjects presenting REG and INFLAMMA types of response (Table 1) to HLA-DR peptides in relation to predominantly REG or predominantly INFLAMMA cytokines for each study group. (**A**) Frequency of REG and INFLAMMA responses in relation to REG cytokines. The OT group had a significantly different profile: higher frequency of inhibition of IL-4, IL-5 and IL-10; OT versus CR, $P = 0.0007$; OT versus HI, $P = 0.005$ (chi-squared test). (**B**) Frequency of REG and INFLAMMA responses in relation to INFLAMMA cytokines. The OT group had a significantly different profile: higher frequency of inhibition of IL-1B, IL-8, IL-12, IL-17, G-CSF, IFN-γ and MCP-1; OT versus CR, $P = 0.0001$; OT versus HI, $P = 0.0005$ (chi-squared test). (**C**) Frequency of REG (black bars) and INFLAMMA (white bars) responses to HLA-DR peptides in relation to INFLAMMA cytokines: IL-1B, IL-8, IL-12, IL-17, IFN-γ, G-CSF and MCP-1; OT versus CR: higher frequency of REG responses in OT; $P = 0.0001$ (chi-squared test). OT ($n = 6$) and CR ($n = 7$).

with both the CR ($P < 0.0001$) and HI groups ($P = 0.002$) (Supplementary data, Figure S2A), mostly due to a higher frequency of predominantly REG cytokines—IL-4, IL-5 and IL-13 production in the OT group.

The REG:INFLAMMA ratios for spontaneous cytokine production were OT 0.8, CR 0.5, STA 0.8 and HI 0.6; hence a predominantly INFLAMMA profile for the CR and HI groups and a more balanced profile for the OT and STA groups (Supplementary data, Table S4). In contrast to the OT group, no IL-5 production was detected in the CR group, and the OT:CR ratios for the frequency of REG cytokine production, namely IL-4, IL-7 and IL-13, were, respectively, 1.8-, 1.8- and 2.3-fold higher in the OT group. For IL-2, the OT group showed 1.5-fold higher frequency compared with the CR group. Despite significant differences in the global profile of spontaneous cytokine production, we found no significant differences in cytokine levels among the study groups.

REG deviation of cellular response to donor HLA-DR peptides in OT

As mentioned in the Materials and methods section, we classified the effect of HLA-DR peptide stimulation on cytokine production as either REG or INFLAMMA responses, depending on whether there is an increase in or inhibition of predominantly REG or INFLAMMA cytokines (Table 1). The frequency profile of REG and INFLAMMA responses was significantly different between the OT and CR groups in terms of both predominantly REG cytokines (greater INFLAMMA response in the OT group and greater REG response in the CR group; $P = 0.0007$) and predominantly INFLAMMA cytokines (greater REG response in the OT group and greater INFLAMMA response in the CR group; $P < 0.0001$) (Figure 1A and B). Significant differences were also found between the OT and HI groups in terms of predominantly REG cytokines (greater INFLAMMA response in the OT group and mostly no

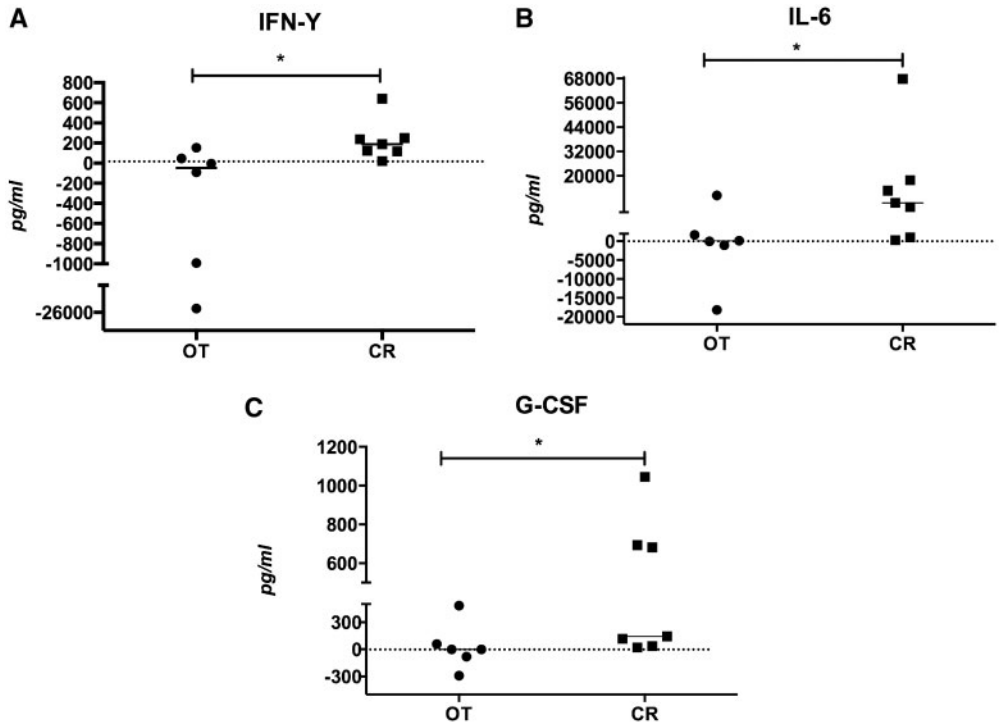

FIGURE 2: Levels of selected INFLAMMA cytokines in response to HLA-DR peptides. Predominantly INFLAMMA cytokines: IL-6, G-CSF and IFN-γ in response to HLA-DR peptides (Luminex). Study groups: OT ($n = 6$) and CR ($n = 7$). (**A**) Levels (pg/mL) of IFN-γ, OT: lower levels than in CR, P = 0.01 (Mann–Whitney test); (**B**) levels (pg/mL) of IL-6, OT: lower levels than in CR, P = 0.03 (Mann–Whitney test); (**C**) levels (pg/mL) of G-CSF, OT: lower levels than in CR, P = 0.03 (Mann–Whitney test). *P < 0.05, **P ≤ 0.001, ***P ≤ 0.0001.

response and REG response in the HI group; P = 0.005) and predominantly INFLAMMA cytokines (greater REG response in the OT group and greater INFLAMMA response in the CR group; P = 0.0005) (Figure 1A and B), indicating a REG deviation in the OT group compared with to the physiologic state, which has a predominantly global INFLAMMA profile in response to HLA-DR allopeptides, particularly in relation to INFLAMMA cytokines.

In contrast, REG deviation did not occur in the CR group. The global REG/INFLAMMA ratios for HLA-DR peptide cytokine responses were predominantly INFLAMMA for the HI and CR groups, in contrast to a more balanced response found in the OT and STA groups (OT 1.1, CR 0.6, STA 0.9, HI 0.6) (Supplementary data, Table S5). The differential REG profile in the OT group, compared with both the CR and HI groups, was mainly due to inhibition of the INFLAMMA cytokines IL-1B, IL-8, IL-17, IL-12, G-CSF, IFN-γ and MCP-1 (Figure 1C).

It should be noted that these cytokines were predominantly increased (INFLAMMA response) in response to HLA-DR peptides in the CR and HI groups, with REG/INFLAMMA ratios ≤0.7, in contrast to ratios ≥1.3 in the OT group (except for IL-17, in which case the ratio was 1). In addition to the predominantly REG profile in the OT group, the levels of some of these INFLAMMA cytokines were significantly higher in the CR group compared with the OT group: IFN-γ (P = 0.01; Figure 2A), IL-6 (P = 0.03; Figure 2B) and G-CSF (P = 0.03; Figure 2C). The CR and HI groups exhibited a significantly higher frequency of REG indirect alloresponse in terms of REG cytokines and there were higher levels of IL-4 (P = 0.005) and IL-10 (P = 0.03) in the CR group compared with the OT group, despite low levels of IL-4 (Supplementary data, Table S5). Nevertheless, the CR and HI groups displayed predominantly INFLAMMA global profiles of HLA-DR alloreactivity.

Preservation of INFLAMMA cellular response to CMV pathogenic peptides in OT

The response to CMV peptides led to both inhibition and induction/increase of REG and INFLAMMA cytokine production in all groups, but mostly an increase in INFLAMMA cytokines (Supplementary data, Table S6). The REG/INFLAMMA ratios of the response to CMV peptides were predominantly INFLAMMA in all groups, but especially in the CR and STA groups (OT 0.7, CR 0.4, STA 0.4, HI 0.6). The OT and HI groups showed similar profiles, with no significant differences in the frequencies of REG or INFLAMMA responses, both in terms of INFLAMMA (P = 0.11) and REG cytokines (P = 0.05) (Figure 3A and B). IL-10 and IL-13 production was essentially increased as a result of CMV response in the HI group (also in the CR and STA groups), in contrast to some inhibition in the OT group. Nevertheless, the overall cytokine response to CMV peptides in the OT group was essentially preserved INFLAMMA type, compared with the physiologic state. We found no significant differences in cytokine levels between the OT and HI groups as a result of CMV response. Preservation of the INFLAMMA profile in the OT group involved the induction/increase of IL-1B, IL-8, IL-17, IFN-γ, MCP-1 and MIP-1B and some inhibition of REG cytokines (Supplementary data, Table S6).

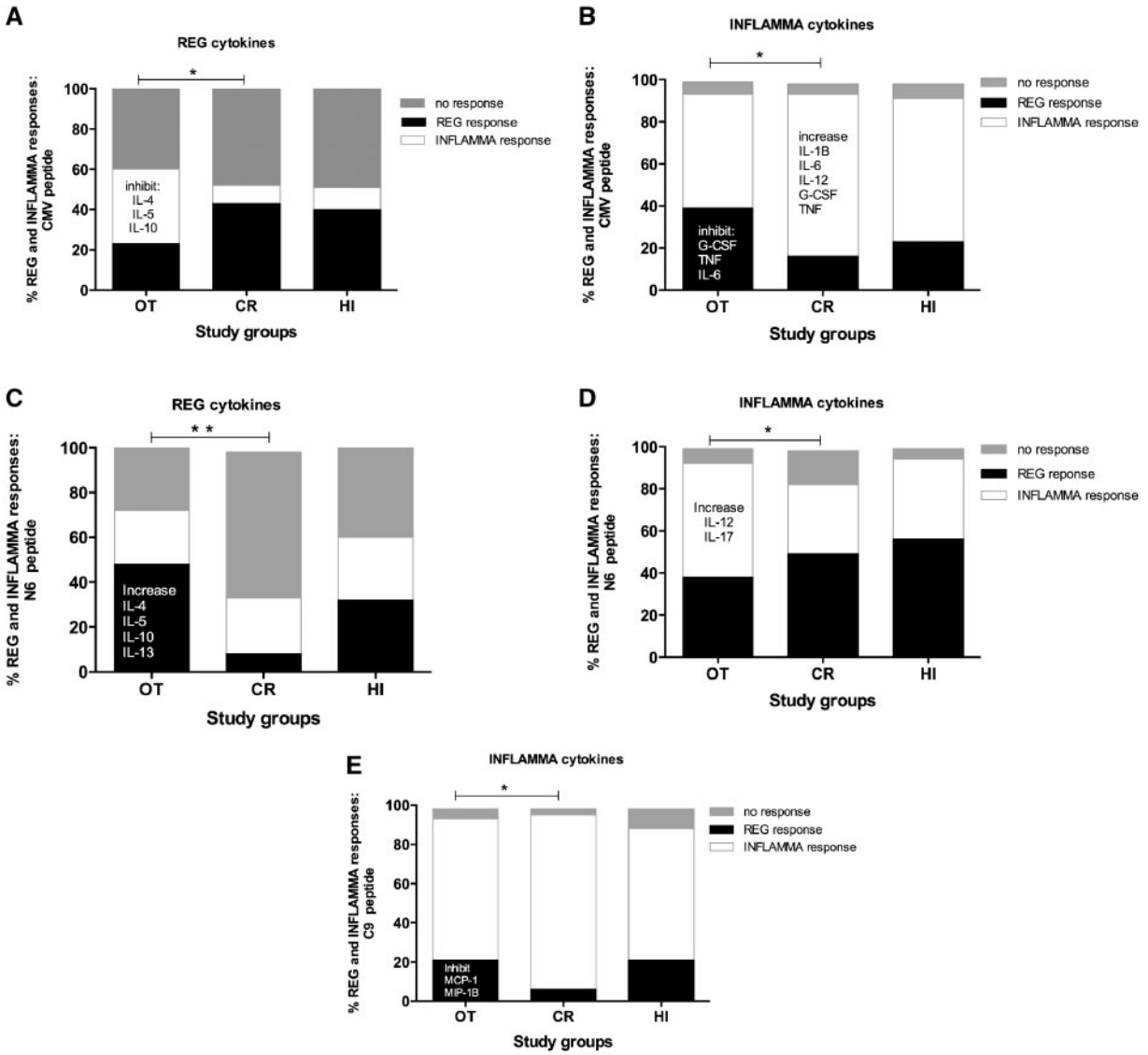

FIGURE 3: Frequency (%) of REG and INFLAMMA cytokine responses to CMV and N6 and C9 Hsp60 peptides. Predominantly REG cytokines analysed: IL-4, IL-5, IL-7, IL-10 and IL-13; predominantly INFLAMMA cytokines analysed: IL-1B, IL-6, IL-8, IL-12, IL-17, G-CSF, GM-CSF, IFN-γ, TNF-α, MCP-1 and MIP-1B (Luminex). Study groups: OT ($n = 6$), CR ($n = 7$) and HIs ($n = 7$). REG responses (black bars): increase/induction of REG cytokines or decrease of INFLAMMA cytokines; INFLAMMA responses (white bars): increase/induction of INFLAMMA cytokines or decrease of REG cytokines; no response (grey bars): no effect on cytokine production. The graph was plotted using the percentages of subjects presenting REG and INFLAMMA types of response (Table 1) to CMV and Hsp60 peptides in relation to predominantly REG or to predominantly INFLAMMA cytokines for each study group. (**A**) Frequency of REG and INFLAMMA responses to CMV peptides in relation to REG cytokines. The OT group had a significantly different profile in relation to CR: higher frequency of INFLAMMA responses with inhibition of IL-4, IL-5 and IL-10; OT versus CR, P = 0.02 (chi-squared test), OT versus HI, P = not significant (NS). (**B**) Frequency of REG and INFLAMMA responses to CMV peptides in relation to INFLAMMA cytokines. The OT group had predominantly REG responses in comparison to the CR group: higher frequency of inhibition of G-CSF, TNF and IL-6; OT versus CR, P = 0.008 (chi-squared test); OT versus HI, P = NS. (**C**) Frequency of REG and INFLAMMA responses to the N6 Hsp60 peptide in relation to REG cytokines. The OT group had a predominantly REG profile compared with the CR group: higher frequency of induction/increase of IL-4, IL-5, IL-10 and IL-13; OT versus CR, P = 0.001 (chi-squared test), OT versus HI, P = NS. (**D**) Frequency of REG and INFLAMMA responses to the N6 Hsp60 peptide in relation to INFLAMMA cytokines. The OT group had a significantly different profile compared with the CR group: higher frequency of induction/increase of IL-12 and IL-17; OT versus CR, P = 0.04 (chi-squared test), OT versus HI, P = NS. (**E**) Frequency of REG and INFLAMMA responses to the C9 Hsp60 peptide in relation to INFLAMMA cytokines. The OT group had a significantly different profile compared with CR: higher frequency of MCP-1 and MIP-1B inhibition; OT versus CR, P = 0.02 (chi-squared test). *$P < 0.05$, **$P \leq 0.001$.

In contrast, the profile of REG and INFLAMMA responses to CMV significantly differed between the OT and CR groups in terms of both REG (greater INFLAMMA response in the OT group; P = 0.02) and INFLAMMA cytokines (greater INFLAMMA response in the CR group; P = 0.008) (Figure 3A and B). Also, the levels of IFN-γ were significantly higher in the CR group compared with the OT group (P = 0.03). The difference in the REG versus the INFLAMMA profile as a result of

the CMV response between the OT and CR groups was mostly due to inhibition of REG cytokines in the OT group (INFLAMMA type of response) and an increase in INFLAMMA cytokines in the CR group (INFLAMMA type of response). Despite some degree of REG deviation in the CMV cytokine response in relation to pro-inflammatory cytokines (inhibition of G-CSF, TNF and IL-6), the profile in the OT group was not significantly different from that in the HI group (P = 0.44).

Differential REG autoimmunity to N6 Hsp60 peptide in OT

We tested the cellular response to two peptides previously studied by our group, whose immune functions were predominantly REG (N6 pep) or INFLAMMA (C9 pep) (Martello, Coelho *et al.*, unpublished data). These two peptides induced various effects on cytokine production, confirming the predominantly REG or INFLAMMA activities for N6 pep and C9 pep, respectively. The REG:INFLAMMA ratios for the frequency of cytokine responses for N6 pep were OT 0.9, CR 1.2, STA 1.2 and HI 1.4 (Supplementary data, Table S7); hence mostly REG under physiologic conditions and balanced for all other study groups. The profile of cytokine response was significantly different between the OT and CR groups in terms of both REG cytokines (greater REG response in the OT group and greater 'no response' in the CR group; P = 0.001; Figure 3C) and INFLAMMA cytokines (greater REG response and greater 'no response' in the CR group; P = 0.04; Figure 3D). The differences for the REG cytokines involved an increase/induction of IL-4, IL-5, IL-10 and IL-13 in the OT group, but for INFLAMMA cytokines, we found a greater INFLAMMA type of response in the OT group in relation to two cytokines (IL-12 and IL-17). In addition, we found a high frequency of 'no response' in the CR group (32.1%) globally. IL-4 production in response to N6 pep was distinct in the OT (REG:INFLAMMA ratio for IL-4 = 2) and CR groups (REG:INFLAMMA ratio for IL-4 = 0.5), despite overall low levels. In addition, the frequency profile of REG and INFLAMMA cytokine responses to N6 pep was similar in the OT and HI groups in relation to REG (P = 0.4) and INFLAMMA cytokines (P = 0.1), indicating a preservation of the physiologic response profile to N6 pep in the OT group. We found no differences in cytokine levels in response to N6 pep among the study groups.

The cytokine response to C9 pep was predominantly INFLAMMA in all groups, except for the STA group (more balanced). The REG:INFLAMMA ratios for the frequency of cytokine responses were OT 0.5, CR 0.3, STA 0.9 and HI 0.6 (Supplementary data, Table S8). The REG type of response was predominant for REG cytokines and the INFLAMMA type of response was predominant for INFLAMMA cytokines. Despite a mostly INFLAMMA type of response, we found significant differences in the profile of REG and INFLAMMA frequencies with respect to INFLAMMA cytokines (P = 0.02), hence more INFLAMMA response in the CR group (Figure 3E), but not REG cytokines (P = 0.43) in the OT and CR groups. We found no differences between the OT and HI groups for both REG (P = 0.43) and INFLAMMA (P = 0.77) cytokines. MCP-1 was the only INFLAMMA cytokine displaying a different response to C9 pep between the OT (REG:INFLAMMA ratio for MCP-1 = 1.5) and CR groups (REG:INFLAMMA ratio for MCP-1 = 0.2) (Supplementary data, Table S8). The levels of IL-10 were higher in the CR group than in the OT group (P = 0.005), although both OT and CR groups displayed an increase in production in response to C9 pep. We found no other significant differences among the other study groups.

DISCUSSION

It is well recognized that developing and maintaining immune tolerance critically involve the functional modulation of antigen-specific immune responses [26–28]. We tested the hypothesis of whether antigen-specific cellular response is differentially modulated in OT in renal transplantation, directed to three critical antigenic challenges in allotransplantation—donor antigens, pathogenic antigens and self-antigens. Indeed, we found a significantly differential profile in OT compared with both CR and HIs.

The major novelty of our study is raising that the ongoing REG mechanisms in OT modulate the antigen-specific cellular response directed to relevant antigenic challenges in allotransplantation, leading to a REG deviation in the response to donor HLA-DR peptides, while preserving the INFLAMMA response to pathogens. Additionally, given the differential predominant REG profile of cytokine response to the N6 Hsp60 peptide, we suggest that REG autoimmunity could also play a role in OT.

We highlight the interest of studying simultaneously the cellular responses to these three antigenic universes that constantly challenge transplanted individuals, first because they are critical to homeostasis, and second because antigen-specific cellular responses share overlapping T cell repertoires and therefore are likely to affect one another due to cross-reactivity. In fact, cross-reactivity is argued to be an essential functional feature of the immune system [29, 30]. Nonetheless, cross-reactivity and molecular mimicry—mostly between pathogenic antigens and self-antigens, but also between pathogenic antigens and alloantigens [31]—have also been implicated in INFLAMMA deviation and the breakdown of tolerance, and consequently in the development of autoimmune diseases [32] or in rejection in the context of transplantation [33]. This highlights the importance of understanding the interactive nature of these antigen-specific cellular responses and their potential INFLAMMA and REG functional activities.

Our data (summarized in Figure 4) show a clear shift towards a REG profile of indirect alloresponse to donor HLA-DR peptides in OT, while preserving an INFLAMMA profile in response to pathogenic antigens, despite ongoing robust immunoregulation. This argues in favour of a systemic functional antigen-specific discrimination taking place in OT, though the mechanisms of such discrimination are largely unknown. This is especially interesting if we take into account that in the physiologic state (HIs), the cellular response to HLA-DR allopeptides is markedly INFLAMMA for cytokine production, while in the OT state, there is a striking immunodeviation to a REG profile. In addition, such immunodeviation does not occur in CR that has a predominant INFLAMMA profile of cellular response to donor HLA-DR peptides.

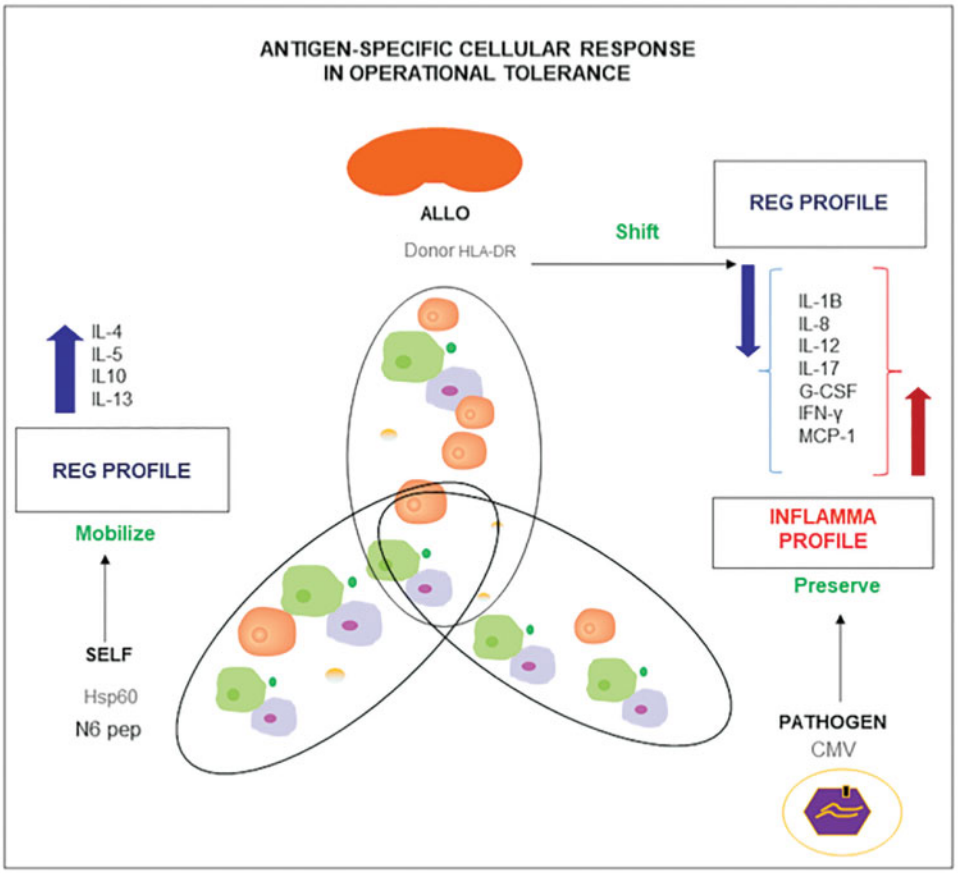

FIGURE 4: Antigen-specific cellular response is essential in immune tolerance in human transplantation. In contrast to CR and HIs, OT gives rise to a REG immunodeviation in cellular response to donor HLA-DR peptides and to N6 Hsp60 peptide, while preserving the INFLAMMA response to pathogenic peptides (CMV).

Considering that OT and CR are opposing clinical outcomes, this strongly suggests that REG deviation in indirect allorecognition of donor HLA-DR peptides contributes to the OT state. The REG shift in donor alloreactivity in OT involved mostly inhibition of the INFLAMMA cytokines IL-1B, IL-8, IL-12, IL-17, G-CSF, IFN-γ and MCP-1. Accordingly, the levels of IL-6, G-CSF and IFN-γ were also significantly lower in the OT group compared with the CR group. Indeed, several of these cytokines have been reported to be involved in graft aggression in CR [34, 35]. Thus partial inhibition of these cytokines is likely to contribute to avoiding graft rejection, together with other mechanisms such as inhibition of both effector and effector memory T cells, which are critical in triggering and sustaining graft rejection [36–38]. This REG immunodeviation in anti-donor response in OT is in concordance with our previous data showing an increase in the expression of the Th2 transcription factor GATA-3 (mRNA and protein) in both CR and HIs [18].

However, we should point out the higher number of HLA-DR disparities in the CR group compared with the OT group. One could therefore argue that this would have a major impact on the REG profile in OT, contrasting with the INFLAMMA profile in CR. Nevertheless, two contrasting observations between OT and CR strongly support the occurrence of a REG shift in response to donor HLA-DR peptides exclusively in the OT group, likely to integrate ongoing REG mechanisms in OT. First, Subject 87 in the CR group displayed no donor HLA disparities but displayed a predominantly INFLAMMA profile in response to HLA-DR peptides, in contrast to OT subjects who displayed a predominantly REG profile, especially regarding the INFLAMMA cytokines. That is, the shared donor/self HLA-DR peptides induced opposing functional profiles in OT (predominantly REG) and CR (predominantly INFLAMMA), suggesting the emergence of distinct allo-/auto-REG/INFLAMMA interplay, in relation to HLA-DR antigens, in these opposing clinical outcomes. Second, Subject 3 in the OT group, despite displaying three donor HLA disparities, also presented a predominantly REG profile in response to donor HLA-DR peptide, indicating that the REG profile was not related to the absence of HLA disparity only. This contrasts with the CR group as a whole and with Subject 79 (CR), who also displayed three donor HLA disparities and presented a predominantly INFLAMMA profile (differing mostly in regard to IL-12, IL-17, IFN-γ, TNF and MIP-1B).

Our data underscore the role of donor HLA-DR peptides in this REG shift, although we cannot exclude that other peptides from donor HLA antigens may be similarly important. Classical studies on experimental transplantation tolerance have shown that major histocompatibility complex (MHC) alloantigens are involved in transplantation tolerance [39], but differential participation of HLA class I and II

peptides in human transplantation tolerance is still unclear. Nonetheless, disparities of HLA-DR molecules have been differentially associated with the development of CR [40], suggesting that downregulating inflammatory alloreactivity to HLA-DR may be harder to achieve. Although donor indirect alloreactivity was previously reported by our group [41] and others [27] to play a REG role, we found no data on the indirect pathway of alloreactivity to HLA-DR peptides in OT. In addition, the fact that donor-specific alloreactive Tregs are more effective in controlling inflammation in allotransplantation [42] further supports the relevance of developing REG donor indirect alloreactivity. In concordance with our data, donor indirect alloreactivity was previously reported to have an INFLAMMA profile in CR, with the production of IFN-γ [27].

The cellular response to CMV pathogenic peptides in OT was not significantly different from that in HIs, indicating a preservation of the overall INFLAMMA profile in response to a pathogen. Although the CR group had more infections and the OT group had no infection, all subjects in our study presented immunoglobulin G antibodies against CMV, indicating previous infection. No active CMV infection was observed at the time of sample collection. The INFLAMMA response to CMV in OT involved an increase in/induction of IL-1B, IL-8, IL-17, IFN-γ, MCP-1 and MIP-1B and some inhibition of predominantly REG cytokines, suggesting that this is an important mechanism in the reported preservation of immunocompetence in OT [43], also observed in our study. The capacity to induce IFN-γ in response to CMV peptides in OT seems particularly important in preserving immunocompetence, as it is an important cytokine in successfully dealing with CMV infection in renal transplantation [44].

It is interesting to highlight that some of the INFLAMMA cytokines, namely IL-1B, IL-8, IL-17, IFN-γ and MCP-1, whose production was increased/induced in response to the pathogen-derived peptides, suffered the opposite effect in the cellular alloresponse to donor HLA-DR peptides; they were partially inhibited. This underscores the intriguing antigen-specific functional discrimination taking place in OT.

Hsp60 was the third antigen tested in our triad of antigenic challenges relevant to transplantation. The N6 Hsp60 peptide induced a differential profile of cytokine response in OT compared with CR, mostly due to the induction/increase of IL-4, IL-5, IL-10 and IL-13 in OT, suggesting that this peptide may contribute to ongoing REG mechanisms in OT, thus promoting the production of Th2 cytokines, which is well documented to play a REG role in transplantation [18–21]. This is in accordance with the REG role of Hsp60 and some of its peptides, such as in autoimmune diseases [45, 46] and in transplantation [6], described by our group and others [9]. Since soluble Hsp60 is found in blood and Hsp-derived peptides can be eluted from MHC molecules in the physiologic state [47], they may be considered as endogenous physiologic homeostatic molecules. Thus we can presume that Hsp60 peptides could also be involved in the underlying mechanisms in OT. It is interesting to mention that OT also showed a predominantly REG profile of spontaneous cytokine production, mostly due to the production of IL-4, IL-5 and IL-13, that is significantly different from CR and HIs. Given that the spontaneous production of cytokines may reflect the *in vivo* ongoing process, we may interpret that the state of OT involves active REG deviation, perhaps also with the contribution of endogenous Hsp60 peptides such as N6 inducing the production of the REG cytokines IL-4, IL-5 and IL-13. In contrast, the Hsp60 C9 peptide induced a predominantly INFLAMMA cytokine profile in all study groups, including in HIs, confirming our previously observed INFLAMMA immune response profile for this peptide (Martello, Coelho *et al.*, unpublished data). The significantly higher INFLAMMA C9 peptide response in CR suggests that it could contribute to further amplifying the ongoing inflammation in CR.

A relevant feature of our results is that the immunological REG shift for donor HLA-DR cytokine response observed in OT is distinct not only from its opposing clinical outcome, i.e. CR, but also from the response observed in HIs. As previously shown in the context of tolerance to skin graft, animals tolerized at birth to transplantation antigens by injection of semi-allogeneic cells contain very high numbers of activated T and B lymphocytes in their spleen [48, 49]. In addition, lymphoid hyperactivity correlates with the tolerant state; it is present only in animals accepting skin allografts. Our data are in agreement with these results, showing that although tolerant individuals present a state of homeostasis, their immunological profile regarding antigen-specific cellular responses indicates that they achieve homeostasis by highly active mechanisms and a REG shift in cytokine production. This suggests that the immune system develops redundant ways to maintain homeostasis and can build diverse homeostatic states in different contexts, which may or not be similar to the physiologic state, finding novel homeostatic points or basins of attraction, as previously discussed [50].

A significant contribution of our study is to bring the antigen-specific cellular response directed to a triad of major antigenic challenges in allotransplantation to the setting of OT, highlighting that, despite the potential cross-reactivity of the T cell repertoires directed to these three groups of antigens, a functional antigen-specific discrimination takes place in OT. We believe the interest in this phenomenon goes beyond the field of transplantation, for it embraces the very challenge of understanding homeostasis from the antigen-specific perspective, while facing various antigenic challenges simultaneously.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

All authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, revising critically for important intellectual content and final approval of the version to be published. P.C. contributed to acquisition and interpretation of data for the work; conception of the work, edited, analysed and interpreted data for the work; drafted the manuscript; revised critically for important intellectual content and gave final approval of the version to be published. Y.M.-A. contributed to the interpretation of data for the work, edited the manuscript, revised critically for important intellectual content and gave final approval of the version to be published. A.C., S.M.M., S.G.F., F.L., D.S. and I.d.L.N. contributed to analysis of the data, revised critically for important intellectual content and gave final approval of the version to be published. J.K. contributed to the conception and design of the work, revised critically for important intellectual content and gave final approval of the version to be published. V.C. was a mentor; provided substantial contribution to the conception, design, analysis and interpretation of the work; wrote the manuscript; revised critically for important intellectual content and gave final approval of the version to be published.

CONFLICT OF INTEREST STATEMENT

None declared.

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