# Suppression by Allogeneic-Specific Regulatory T Cells Is Dependent on the Degree of HLA Compatibility

Joana Bianchi, Ana I. S. Vieira, Dário Ligeiro, Rita I. Azevedo and João F. Lacerda

*ImmunoHorizons* 2021, 5 (5) 307-321
doi: https://doi.org/10.4049/immunohorizons.2100021
http://www.immunohorizons.org/content/5/5/307

This information is current as of May 26, 2021.

**Supplementary Material** **http://www.immunohorizons.org/content/suppl/2021/05/12/immunohorizons.2100021.DCSupplemental**

**References** This article **cites 43 articles**, 23 of which you can access for free at:
http://www.immunohorizons.org/content/5/5/307.full#ref-list-1

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://www.immunohorizons.org/alerts

*ImmunoHorizons* is an open access journal published by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
All rights reserved.
ISSN 2573-7732.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

ImmunoHorizons

RESEARCH ARTICLE

Clinical and Translational Immunology

# Suppression by Allogeneic-Specific Regulatory T Cells Is Dependent on the Degree of HLA Compatibility

**Joana Bianchi,* Ana I. S. Vieira,† Dário Ligeiro,‡ Rita I. Azevedo,*,[1] and João F. Lacerda*,[1]**

*Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal; †Flow Cytometry Platform, Champalimaud Research, Champalimaud Centre for the Unknown, Lisbon, Portugal; and ‡Centro de Sangue e Transplantação de Lisboa, Instituto Português do Sangue e da Transplantação, Lisbon, Portugal

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

## ABSTRACT

Regulatory T cell (Treg) infusion for graft-versus-host disease treatment has been increasingly investigated. However, polyclonal Treg may suppress the desired graft-versus-leukemia effect. Although allogeneic-specific (allo-specific) Treg may provide a more-targeted graft-versus-host disease treatment, there is the need to develop easily translatable expansion protocols and to better characterize their specificity and mechanisms of suppression. In this article, we provide a robust protocol for human allo-specific Treg expansion and characterize their phenotype, potency, and specificity of suppression by testing different expansion conditions and suppression assay milieus. We found that higher concentrations of IL-2 during expansion with allogeneic APC yielded allo-specific Treg that were more-potent suppressors and displayed a more activated phenotype. Although responses to the same APC present during expansion were the most suppressed, responses to third-party APC partially matched to the expansion APC were still significantly more suppressed than responses to fully mismatched APC. Furthermore, suppression of responses to the expansion APC was strictly contact dependent, whereas suppression of responses to mismatched APC was partially independent of contact. Finally, distinct subsets in fresh and expanded Treg could be described using multidimensional visualization techniques. We propose that allo-specific Treg are HLA specific and that the mechanisms of suppression elicited depend on their compatibility with the stimulators. *ImmunoHorizons*, 2021, 5: 307–321.

## INTRODUCTION

Chronic graft-versus-host disease (cGvHD) is a major cause of morbidity and non–relapse-related mortality in recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1). The standard treatment for cGvHD is steroid based and has many side effects, with less than half of the patients achieving a complete response (2). Because the frequencies and numbers of regulatory T cells (Treg) have been shown to be reduced in cGvHD patients (3–5), we (www.tregeneration.eu) and others

Received for publication March 10, 2021. Accepted for publication March 19, 2021.

**Address correspondence and reprint requests to:** Prof. João F. Lacerda, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisbon, Portugal. E-mail address: jlacerda@medicina.ulisboa.pt

ORCIDs: 0000-0002-1964-547X (J.B.); 0000-0001-5306-3679 (D.L.).

[1]R.I.A. and J.F.L. share senior authorship.

This work was supported by Fundação para a Ciência e Tecnologia, Portugal, under the Harvard Medical School–Portugal Program Project Induction of Immune Tolerance in Human Allogeneic Hematopoietic Stem Cell Transplantation (HMSP-ICT/0001/2011) and Gilead Sciences, under Programa Gilead GÉNESE (FPJ001262). J.B. received a Ph.D. grant from Fundação para a Ciência e Tecnologia (PD/BD/105769/2014).

J.B. designed research, performed experiments, analyzed data, and wrote the manuscript. A.I.S.V. provided flow cytometry support. D.L. performed genotyping analysis. R.I.A and J.F.L. conceived and supervised the project and reviewed the manuscript.

**Abbreviations used in this article:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; allo-specific, allogeneic-specific; cGvHD, chronic graft-versus-host disease; CTV, CellTrace Violet; DC, dendritic cell; DC-match 3P, moDC from a third-party donor with partial HLA matches to the original DC donor; eTreg, expanded Treg; GvHD, graft-versus-host disease; GVL, graft-versus-leukemia; mismatch 3P, mismatched third-party donor; moDC, monocyte-derived dendritic cell; Resp, responder; SOM, self-organizing map; Tcon, conventional CD4 T cell; Treg, regulatory T cell; Treg-match 3P, moDC from a third-party donor with partial HLA matches to the Treg donor.

The online version of this article contains supplemental material.

This article is distributed under the terms of the CC BY 4.0 Unported license.

Copyright © 2021 The Authors

(6–8) are currently performing clinical trials of polyclonal donor Treg infusion in patients with steroid-resistant cGvHD. Although preclinical studies in mice suggest that infusion of polyclonal Treg can prevent cGvHD without compromising the desirable graft-versus-leukemia (GVL) effect (9), their lack of specificity still carries the risk of generalized immunosuppression. Allogeneic-specific (allo-specific) Treg have the potential to provide a more-potent and targeted suppression than polyclonal Treg (10, 11). In mice, allo-specific Treg of known Ag specificity have been shown to promote transplantation tolerance (12) and prevent graft-versus-host disease (GvHD), while sparing GVL (13). In humans, Treg may need to suppress responses to a plethora of mostly unknown minor histocompatibility Ags reportedly involved in GvHD (14). Thus, expansion of Treg with allogeneic APC will generate Treg specific for a pool of allogeneic minor histocompatibility Ags, which ultimately may provide a suitable solution for the treatment of cGvHD without compromising GVL.

Treg have also been described as suitable for the prevention of transplant rejection in solid organ transplantation, with demonstrated results in phase I clinical trials involving kidney and liver transplantation (15). A recent review by Atif et al. (16) lists the many current clinical trials assessing the potential of Treg therapy in solid organ transplantation while re-enforcing the advantages of Ag-specific Treg. Hence, the characterization of expanded Ag-specific Treg function and phenotype may also prove useful in solid organ transplantation applications.

The suppressive potency of allo-specific Treg can be assessed in vitro by suppression assays (17–20). Some studies have also tried to link the function and/or specificity of Treg to the expression of coinhibitory markers, namely CTLA-4 (21) and PD-1 (22), and T cell activation markers, such as HLA-DR (23), CD39 (24), and CD40-L and 4-1BB (25). Nevertheless, a comprehensive description of allo-specific Treg function and phenotype is still lacking.

The present work provides an in-depth characterization of expanded allo-specific Treg function and phenotype while establishing an expansion protocol that can be easily translated into a clinical setting. Treg were expanded in the presence of allogeneic monocyte-derived dendritic cells (moDC) using serum-free medium, with low or intermediate concentrations of IL-2. The ability of allo-specific Treg to suppress the proliferation of conventional CD4 T cells (Tcon) and CD8 T cells was assessed using allogeneic moDC with distinct HLA profiles as stimulators. In parallel, the mechanisms involved in the suppression of responses to these distinct stimulators, particularly the need for cell–cell contact, were investigated. To thoroughly characterize the phenotypic profile of fresh and expanded Treg (eTreg), an unbiased analysis of flow cytometry data was performed using multidimensional visualization techniques. We found that suppression by allo-specific Treg is contact dependent and seems to be HLA specific.

## MATERIALS AND METHODS

### *PBMC isolation and genotyping*

Buffy coats of healthy donors were provided by the local blood bank (Instituto Português do Sangue e Transplantação, Lisboa) with the approval of the Ethics Committee of the Lisbon Academic Medical Centre. PBMCs were isolated by Ficoll Paque PLUS (GE Healthcare) density gradient. Genomic DNA was isolated with NZY Blood gDNA Isolation Kit (NZYTech) before high-resolution genotyping at Instituto Português do Sangue e Transplantação.

### *Flow cytometry staining*

Cells were stained with the following anti-human monoclonal Abs: CD3 (OKT3), CD19 (HIB19), and CD56 (TULY45) FITC; CD3 (OKT3) PerCP-Cy5.5; CD86 (IT2.2) PerCPe710; CD4 (RPA-T4) APC; HLA-DR (LN3), CD8a (SK1) APCe780, and Fixable Viability Dye e506 (eBioscience); CD14 (MφP9) V450, CD11c (B-ly6) APC, CD127 (HIL-7R-M21) PE-Cy7, CD279 (EH12) BV605, CD152 (BNI3) PE-CF594, and CD39 (Tu66) BV650 (BD Biosciences); CD4 (RPA-T4) BV785, CD137 (4B4-1) APC, and CD154 (24–31) BV711 (BioLegend); and CD25 (2A3) PE (StemCell).

Intracellular staining for FOXP3 (PCH101) e450 (eBioscience), IL-2 (MQ1-17H12) APC, IL-10 (JES3-9D7) Alexa-Fluor 488 (BioLegend), and IL-4 (8D4-8) PE (BD Biosciences) was performed using fixation/permeabilization reagents from eBioscience. Data were analyzed using FlowJo10.

### *Differentiation of moDC*

Monocytes isolated with EasySep Human CD14 Positive Selection Kit II (StemCell) were cultured in X-Vivo 15 supplemented with L-glutamine (Lonza), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), IL-4 (40 ng/ml), and GM-CSF (50 ng/ml; Peprotech) at 37°C and 5% $CO_2$. Fresh medium was added on day 3. At day 5, differentiated moDC were activated for 48 h with IL-1β (10 ng/ml), IL-6 (10 ng/ml), TNF-α (20 ng/ml, Peprotech), and $PGE_2$ (1 μg/ml; Tocris Bioscience). Mature moDC were typically $CD14^{-}CD11c^{+}CD86^{2+}HLADR^{2+}CD80^{+}CD83^{+}$, as described previously (18).

### *In vitro expansion of Treg*

CD4 T cells were isolated using EasySep Human CD4 Positive Selection Kit II (StemCell) and labeled with 0.5 μM CellTrace CFSE Cell Proliferation Kit (Invitrogen). Treg ($CFSE^{+}CD3^{+}CD4^{+}CD25^{high}CD127^{low}$) and Tcon ($CFSE^{+}CD3^{+}CD4^{+}CD25^{-}$) were then sorted on a BD FACSAria Fusion. Purity was ≥99% for both populations. Treg ($75 \times 10^3$ per well) were cocultured with irradiated allogeneic moDC (30 Gy) from HLA-mismatched donors at a Treg/moDC ratio of 4:1 in 96-well plates. Culture medium was TexMACS (Miltenyi Biotec) with recombinant human IL-2 (10 or 100 U/ml; R&D Systems), IL-15 (10 ng/ml; R&D Systems),

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

and rapamycin (100 ng/ml; Sigma-Aldrich). From day 7 onwards, culture medium was replenished every 2 d with TexMACS supplemented with only IL-2, until cells were harvested for functional assessment on day 14. As a control, sorted Tcon were expanded in the same conditions as Treg, except that culture medium was TexMACS supplemented with only recombinant human IL-2 (10 or 100 U/ml).

### *Suppression assays*

Fresh Treg and Tcon were isolated with EasySep Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation Kit, and $CD8^+$ cells were isolated with EasySep Human CD8 Positive Selection Kit II (StemCell), all from the same donor as eTreg. Purified Tcon or CD8 responders (Resp) were labeled with 2.5 μM CellTrace Violet (CTV) Cell Proliferation Kit (Invitrogen). Fresh or eTreg were titrated to indicated ratios of Treg/Resp in duplicate on 96-well plates with $25 \times 10^3$ CTV-labeled Resp and irradiated moDC at a Resp/moDC ratio of 4:1. Wells without Treg (0:1) were used as negative controls for suppression. Cells were incubated 6 d in RPMI 1640 with 10% heat-inactivated human AB serum (Sigma-Aldrich), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Invitrogen), after which cells were stained for CD3, CD4, and CD8 and acquired on BD LSRFortessa X-20. For analysis, cells from suppression assay wells were gated on a forward versus side scatter and then on $CD3^+CD8^+$ or $CD3^+CD8^-CD4^+$ Resp. To exclude Treg from the Tcon analysis, an additional gate was made on CTV versus CD4 plots to select CTV-labeled cells, excluding $CTV^-$ cells by comparison with wells containing only Treg or only Tcon (Treg/Resp 0:1). Within CTV-labeled cells (Tcon or CD8), a gate on unproliferated ($CTV^+$) cells was defined using unstimulated Resp cells, and the frequency of proliferated Resp was determined by gating on cells with diluted CTV labeling. Resp proliferation was calculated by normalizing the frequency of $CTV^-$ Resp in the presence of Treg to the frequency found in the absence of Treg.

### *Cytokine quantification by Multiplex*

At the end of suppression assays, cytokine concentration was measured in supernatants using the MILLIPLEX MAP Human High Sensitivity T Cell Panel (IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17A, IL-23, TNF-α), the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel IV (IL-35), and the MILLIPLEX MAP TGFβ1 Magnetic Bead Single Plex Kit (Millipore). Samples were acquired on a MAGPIX System (Luminex) and analyzed using the xPONENT software (Luminex).

### *Transwell suppression assays*

Tcon ($CD3^+CD4^+CD25^-$) and CD8 ($CD3^+CD4^-CD8^+$) Resp from the same donor as Treg were sorted on BD FACSAria Fusion and labeled with CTV. A total of $5 \times 10^4$ Resp and $1.25 \times 10^4$ irradiated moDC were cultured in duplicate in the bottom chamber of 96-well Transwell plates. Irradiated moDC were added to top and bottom chambers at a dendritic cell (DC)/T cell ratio of 1:4. In the top chamber, eTreg were cultured alone ($1 \times 10^4$) or with Resp cells at Treg/Resp ratio of 1:5. In standard suppression assay controls, eTreg ($1 \times 10^4$) were cultured with Resp in the bottom chamber, without cells in the top chamber.

At day 6, Resp proliferation was measured in bottom wells on BD FACSAria Fusion. Cells from top chambers of Transwell and from standard controls were restimulated for 4 h with Cell Stimulation Cocktail (eBioscience) prior to intracellular cytokine staining. Samples were acquired on BD LSRFortessa X-20. For analysis, Resp were distinguished from Treg based on the expression of CTV (similarly to previous suppression assays). Cutoffs for cytokine secretion were based on cytokine expression in fluorescence-minus-one controls.

### *Multidimensional analysis of cytometry data*

FlowSOM or k-means clustering algorithms were used to analyze flow cytometry data from three experimental replicates from each condition, as described previously (26). Briefly, data from each sample were manually compensated, gated on $CD3^+CD4^+$ cells, and downsampled (DownSampleV3 plugin) before being exported from FlowJo. The fcs files were then concatenated to a single fcs file (flowCore package). FlowSOM-based unsupervised analysis was then performed (27), in which each cell was assigned to a node in a $5 \times 5$ grid, building a self-organizing map (SOM) based on the expression of selected markers ("BuildSOM" function). A minimum spanning tree of the SOM was built and plotted ("BuildMST" function). Then, data from each condition (three concatenated fcs files each) were mapped to the same SOM ("NewData" function), and individual minimum spanning trees were built. For k-means clustering, data in a single concatenated file were preprocessed by transforming the fluorescence channels (28) (using the "flowTrans" function) and normalized by scaling. The optimal number of clusters (5) was determined by plotting sum of squares versus number of clusters, and then k-means clustering was performed ("kmeans" function). Individual histograms were created for each marker, showing mean expression for each cluster. Then, cells were labeled based on their condition of origin to observe cluster distribution per condition. Statistical analysis of the differences in frequency of cells assigned to each cluster was performed using ANOVA. Computational analysis was conducted using RStudio (version 1.2.5001, with R version 3.5.0) on a MacBook Pro running the OS X Mojave 10.14.6 with a 2.6 GHz Intel Core i5 processor.

### *Statistical analysis*

Statistical analysis was performed on GraphPad Prism 7.00 software using a confidence level of 0.95. The $p$ values $< 0.05$ were considered significant. For comparisons between conditions (e.g., different stimulators and conditions), Student paired $t$ tests with the Holm–Sidak method or Fisher least significant difference test were used. For comparisons within the same condition (e.g., different dilutions), two-way ANOVA was used, with Dunnett multiple comparison corrections.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

https://doi.org/10.4049/immunohorizons.2100021

## RESULTS

### *Allo-specific Treg are highly suppressive*

We sought to optimize an allo-specific Treg expansion protocol that could potentially be translated to the clinic because most GMP-suitable protocols focus on polyclonal Treg expansion (29, 30). As one of the hindrances for clinical translation of current protocols is the use of human serum (18, 31), we expanded allo-specific Treg in serum-free conditions. First, fresh $CD25^{+}CD127^{low}$ Treg were sorted from CFSE-labeled $CD4^{+}$ cells (Presort), resulting in a highly enriched population for FOXP3 (Fig. 1A, 1B, presort versus fresh Treg). $CFSE^{+}$ Treg were cocultured for 14 d with moDC from an allogeneic donor, henceforth referred to as original DC donor. To prevent the expansion of contaminating Tcon, rapamycin was added to the culture media (32), along with IL-2 and IL-15 (19). After 14 d, the frequency of proliferated Treg with different concentrations of IL-2 was similar (Fig. 1C), although Treg fold expansion was slightly higher with 100 U/ml of IL-2 (Fig. 1D).

To determine the suppressive potency of these eTreg, suppression assays were performed with moDC from the original DC donor as stimulators and Tcon (Fig. 1E, 1F) or $CD8^{+}$ (Fig. 1G, 1H) as Resp. When compared with control wells without Treg (Treg/Resp 0:1), Tcon proliferation was significantly reduced by Treg expanded with 10 U/ml of IL-2 at a Treg/Resp ratio of 1:10 (Fig. 1E). Interestingly, significant Tcon suppression was achieved by Treg expanded with 100 U/ml of IL-2 at just 1:50 Treg/Resp (Fig. 1E). When compared with fresh Treg, Treg expanded with 100 U/ml of IL-2 were significantly more suppressive of Tcon (Fig. 1F, $p = 0.045$). As for CD8 Resp, proliferation was significantly suppressed by eTreg from either expansion condition at a Treg/Resp ratio of just 1:50 (Fig. 1G). Furthermore, both types of eTreg were significantly more suppressive than fresh Treg (Fig. 1H, $p = 0.037$ and $p < 0.001$ for 10 U/ml and 100 U/ml of IL-2 eTreg, respectively). Thus, our serum-free protocol for allo-specific Treg expansion yielded highly suppressive Treg.

### *Allo-specific Treg and Tcon are phenotypically distinct*

The expression of CD25, CD127, and FOXP3 was analyzed by flow cytometry in both Treg and Tcon before expansion (fresh Treg and fresh Tcon, Fig. 2A) and then compared with the phenotype of these cells after expansion with different concentrations of IL-2 (Fig. 2B, 2C). eTreg had higher expression of CD25, CD127, and FOXP3 than fresh Treg (Fig. 2B). CD25 and FOXP3 expression in expanded Tcon was also increased in both settings but more so after expansion with 100 U/ml of IL-2 (Fig. 2C).

To better characterize the phenotype of eTreg and Tcon, we analyzed PD-1, CTLA-4, CD39, HLA-DR, CD40L, and 4-1BB expression with the multidimensional visualization technique FlowSOM (27). Briefly, using concatenated data from all samples being compared, a pseudo-randomized algorithm allocates each cell to a node on a user-defined $5 \times 5$ grid, based on its expression of all markers. The output is a minimum spanning tree with 25 nodes distributed spatially according to similarities between nodes. Then, data from each individual sample are projected onto the same minimum spanning tree to visualize its phenotype.

As the starting Treg population was mostly pure (Fig. 1B) and there was high potency of suppression by eTreg (Fig. 1E, 1H), the presence of contaminating Tcon within allo-specific Treg was unlikely. Nevertheless, as the phenotype of expanded Tcon might resemble that of Treg, we compared Treg and Tcon after expansion with 100 U/ml of IL-2. Interestingly, expanded Tcon and eTreg with the highest expression of activation markers (CD39, HLA-DR, CD40-L, and 4-1BB) were allocated to different nodes (Fig. 2D, 2E, orange arrow in Tcon and black arrow in Treg). Importantly, this visual representation of data analyzed in an unbiased way would not be possible through conventional flow cytometry data analysis.

Overall, this analysis indicates that allo-specific Treg were mostly free from activated Tcon, as there was virtually no overlap between the minimum spanning trees of expanded Tcon and eTreg.

### *IL-2 concentration during Treg expansion affects suppressive potency*

To verify the specificity of allo-specific Treg, the suppression of responses to moDC from the same donor used for expansion (original DC) was compared with the potency of suppression of responses to stimulators from a fully HLA-mismatched third-party donor (mismatch 3P). Proliferation in the presence of Treg was normalized to that found in control wells without Treg (0:1 Treg/Resp). In all settings, Resp were derived from the same donor as Treg.

Treg expanded with 10 U/ml of IL-2 did not suppress Tcon proliferation when mismatch 3P stimulators were used (Fig. 3A). In fact, at high Treg/Resp ratios, Tcon proliferation was significantly higher with mismatch 3P than with the original DC stimulators. Although CD8 proliferation was suppressed by Treg expanded with 10 U/ml of IL-2 in the presence of mismatch 3P stimulators, proliferation to original DC stimulators appeared more suppressed (Fig. 3B). In suppression assays with Treg expanded with 100 U/ml of IL-2, Tcon and CD8 proliferation also seemed higher with mismatch 3P than with original DC stimulators (Fig. 3C, 3D), indicating that the suppressive activity of allo-specific Treg was specific toward the original DC stimulator.

These results showed allo-specific Treg were capable of both allo-specific and nonspecific suppression and that both types of suppression were enhanced when higher IL-2 concentrations were used during Treg expansion.

### *Allo-specific Treg recognize cognate HLA alleles in third-party stimulators*

Next, we wanted to assess if these allo-specific Treg would be more suppressive of responses to third-party donors if there

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

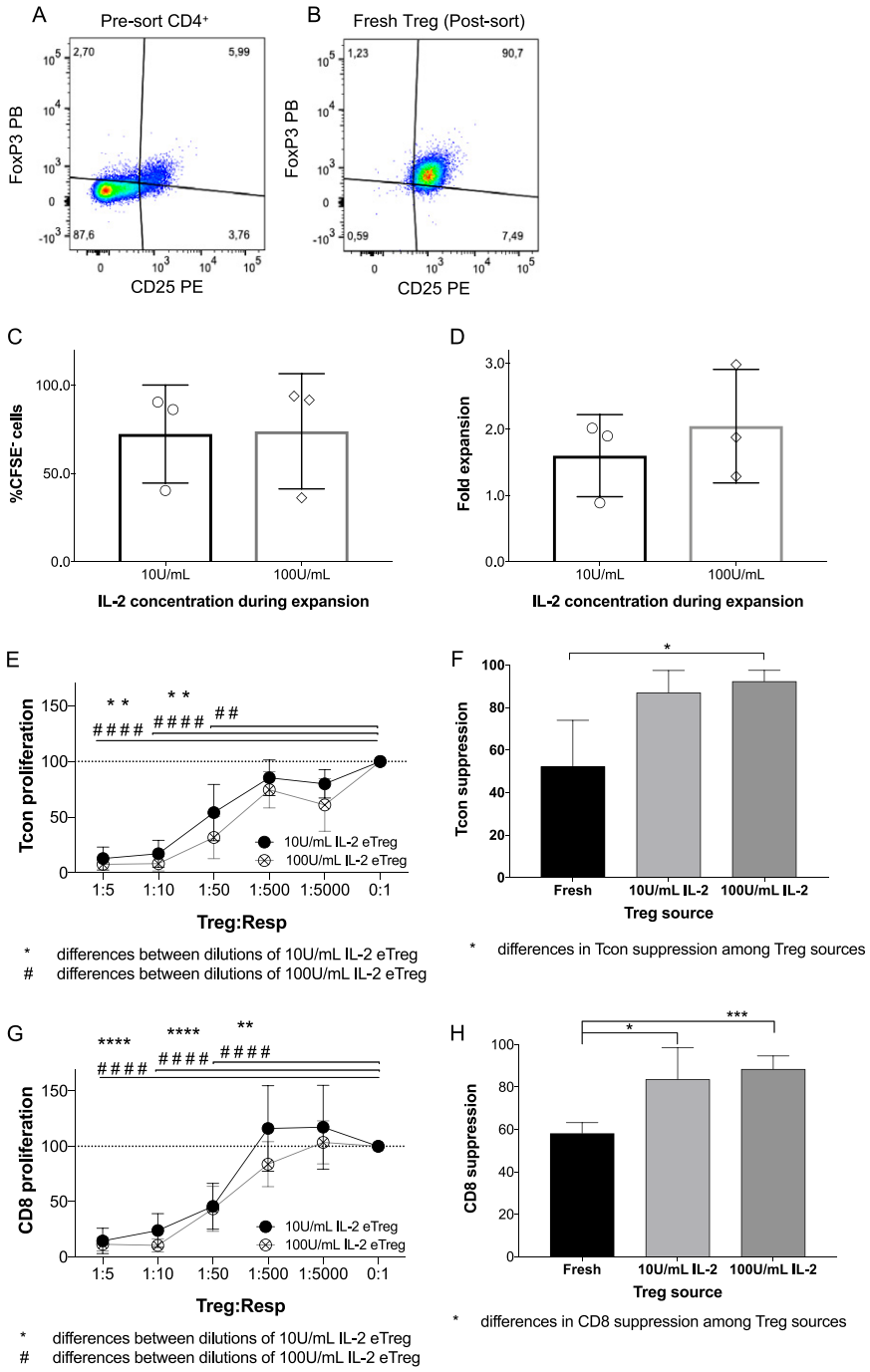


**FIGURE 1. Expansion of allo-specific Treg with 10 U/ml or 100 U/ml of IL-2 and respective potency of suppression of Tcon and CD8 Resp.** (**A** and **B**) Expression of CD25 and FOXP3 in $CD4^+$ cells used for sorting (A) and in fresh Treg after sorting (B). (**C** and **D**) Proliferation (C) and fold expansion (D) of Treg. (**E**) Normalized proliferation of Tcon in response to moDC from the original DC donor, in the presence of Treg expanded with 10 U/ml of IL-2 (black circles) or 100 U/ml of IL-2 (open circles) at several Treg/Resp ratios. (**F**) Degree of Tcon suppression by fresh Treg (black bar), Treg expanded with 10 U/ml of IL-2 (light gray bar), and Treg expanded with 100 U/ml of IL-2 (dark gray bar) at a Treg/Resp ratio of 1:5. (**G**) Normalized proliferation of CD8 cells in response to moDC from the original DC donor, in the presence of Treg expanded with 10 U/ml of IL-2 (black circles) or 100 U/ml of IL-2 (open circles) at indicated Treg/Resp ratios. (**H**) Degree of CD8 suppression by fresh Treg (black bar), Treg expanded with 10 U/ml of IL-2 (light gray bar), and Treg expanded with 100 U/ml of IL-2 (dark gray bar) at a Treg/Resp ratio of 1:5. Data were obtained from three independent experiments. (C–H) Values for average and SD are shown. Statistical analysis of differences between dilutions was performed with one-way ANOVA, and differences between conditions were evaluated with multiple *t* tests, by the Holm–Sidak method. Statistical significance was assumed for $p < 0.05$ ($^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.005$, $^{***,\#\#\#}p < 0.0005$, $^{****,\#\#\#\#}p < 0.0001$).

https://doi.org/10.4049/immunohorizons.2100021

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

were partial HLA matches between those donors and either the original DC donor or the Treg donor.

Thus, we performed suppression assays using, as stimulators, moDC from third-party donors either partially matched to the original DC donor in HLA class I and HLA class II (DC-match 3P) or partially matched to the Treg donor in HLA class II (Treg-match 3P). Stimulators from the original DC and mismatch 3P were used as controls for specific and nonspecific suppression, respectively. In matched donors, the degree of matching per class ranged from two to four out of six sequenced alleles. HLA high-resolution genotyping results can be found in Supplemental Table I.

In conditions with DC-match 3P and Treg-match 3P stimulators, Tcon proliferation was significantly suppressed by Treg expanded with either 10 U/ml or 100 U/ml of IL-2, when compared with the control without Treg (0:1 Treg/Resp; Supplemental Fig. 1A, 1B). When comparing the degree of proliferation detected with different stimulators at a Treg/Resp ratio of 1:10, Tcon responses to partially matched third-party stimulators seemed more suppressed than those to mismatch 3P (Fig. 4A). Interestingly, with 100 U/ml of IL-2 eTreg as suppressors, Tcon responses to DC-match 3P, but not to Tregmatch 3P, were more suppressed than those to mismatch 3P (Fig. 4A, right).

With CD8 Resp, there was statistically significant suppression of proliferation with all stimulators by 10 U/ml and 100 U/ml of IL-2 eTreg when compared with the control without Treg (Supplemental Fig. 1C, 1D). However, when comparing stimulators at a Treg/Resp ratio of 1:10, responses to DC-match 3P were more suppressed than those to mismatch 3P (Fig. 4B), as seen with Tcon Resp. Overall, Tcon and CD8 proliferation seemed most suppressed with original DC or DC-match 3P stimulators and least suppressed with Treg-match 3P and mismatch 3P, suggesting that allo-specific Treg are not only specific toward the stimulator used for their expansion but can also detect cognate HLA alleles in third-party stimulators, particularly those found in the original DC donor.

Because Treg expanded with higher concentrations of IL-2 seemed more potent in both specific and nonspecific settings, subsequent assays were focused on allo-specific Treg expanded with 100 U/ml of IL-2. To ascertain if the presence of Treg affected cytokine concentrations during the suppression assay, the levels of Th1 (Fig. 4C–E) and Th2 (Fig. 4F, 4G) cytokines were measured in the supernatants. Control wells without Treg (0:1 Treg/Resp) had quantifiable concentrations of IL-2, TNF-α, and IFN-γ in their supernatant (Fig. 4C–E), yet these cytokines were nearly undetectable in wells with allo-specific Treg at a 1:5 Treg/Resp ratio. Interestingly, similar trends were found for IL-2 and IFN-γ with CD8 Resp, albeit at much lower ranges, because cytokine secretion by CD8 Resp alone was very low (Supplemental Fig. 1E, 1F). Furthermore, IL-5 (Fig. 4F) and IL-4 (Fig. 4G) were only detected in the supernatant of control wells with Tcon (0:1 Treg/Resp), but only IL-5 seemed to be affected by the presence of allo-specific Treg (Fig. 4F). Of note, we also measured the concentration of IL-10 and TGF-β, usually associated with Treg function, but we did not find their concentrations to be increased by the presence of allo-specific Treg (Supplemental Fig. 1G, 1H). Although it cannot be excluded that Treg produce IL-10, this observation suggests that other mechanisms may be driving suppression by allo-specific Treg.

The suppressive mechanisms employed by Treg depend on both Resp and DC. Thus, we compared the degree of suppression achieved by Treg to the expression of CD86 in each DC population at the beginning of suppression assays. Interestingly, we found that higher initial CD86 median fluorescence intensity was strongly correlated to lower suppression of both Tcon and CD8 (Supplemental Fig 2A, 2B). Because it has been shown that CTLA-4 may capture CD86 from the surface of DC, we hypothesized that the association of lower suppression with higher CD86 expression could be a result of limiting levels of CTLA-4 on Treg. However, CTLA-4 blockade did not impair suppression by Treg (Supplemental Fig 2C, 2D). Moreover, regardless of the CD86 expression, responses to the original DC donors were always the most suppressed, indicating that HLA specificity is more relevant for Treg suppression than CD86 levels on moDC.

In summary, suppression of Resp proliferation by allo-specific Treg seemed most influenced by the HLA profile of the stimulators, suggesting that allo-specific Treg are HLA specific in their suppression.

### *Specific suppression by allo-specific Treg requires cell–cell contact*

To assess whether T–T cell contact was required for suppression by allo-specific Treg, transwell experiments were performed in which Treg were cultured in the top chamber alone or with Resp at a 1:5 Treg/Resp ratio. CTV-labeled Tcon or CD8 Resp were cultured in the bottom chamber. In both settings, cells in top and bottom chambers were stimulated by either original or mismatch 3P moDC. Standard suppression assays, with cell–cell contact between Resp and allo-specific Treg, were used as a positive control. HLA high-resolution genotyping of all donors is available in Supplemental Table II. To assess the importance of contact for allo-specific Treg function, fold suppression of Resp proliferation in bottom chambers was evaluated, by normalizing suppression in top chambers to that found in equivalent conditions of standard suppression assays.

With moDC from the original DC donor as stimulators, suppression of Tcon was significantly lowered by the lack of contact between Treg and Resp in the bottom well, when compared with the control (Fig. 5A, black bars). In this setting, the presence of Tcon together with allo-specific Treg in the top chamber (Top well 1:5 Treg/Tcon) did not increase the suppression of Resp cells in the bottom chamber, when compared with Treg alone in the top well, suggesting that allo-specific

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

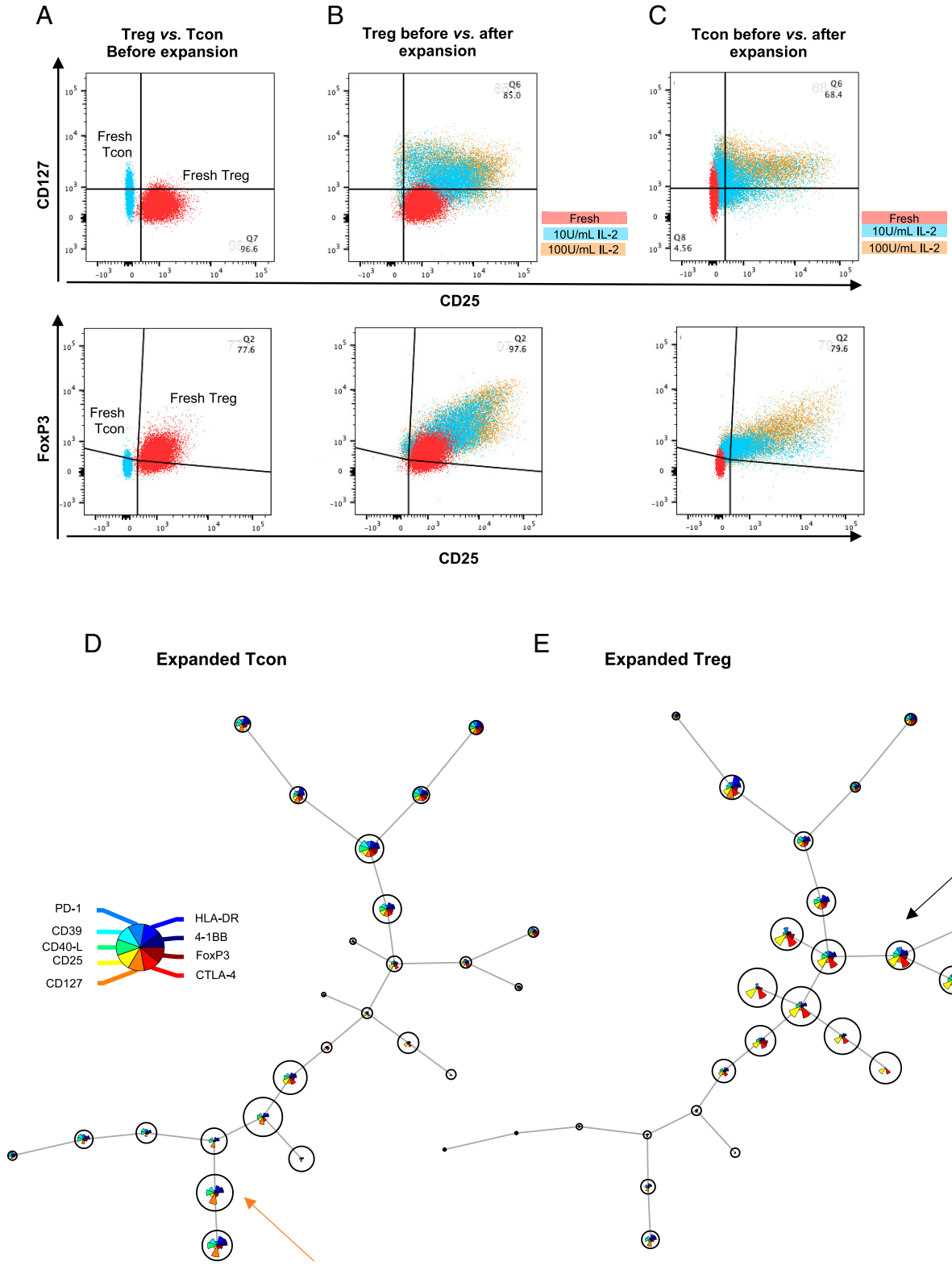


**FIGURE 2. Phenotype of eTreg and expanded Tcon samples.**

(**A**–**C**) Dot plots depicting the expression of CD25 and CD127 (top) and CD25 and FOXP3 (bottom) on Treg and Tcon before expansion (A), on Treg before and after expansion with 10 or 100 U/ml of IL-2 (B), and on Tcon before and after expansion with 10 or 100 U/ml of **(Continued)**

https://doi.org/10.4049/immunohorizons.2100021

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

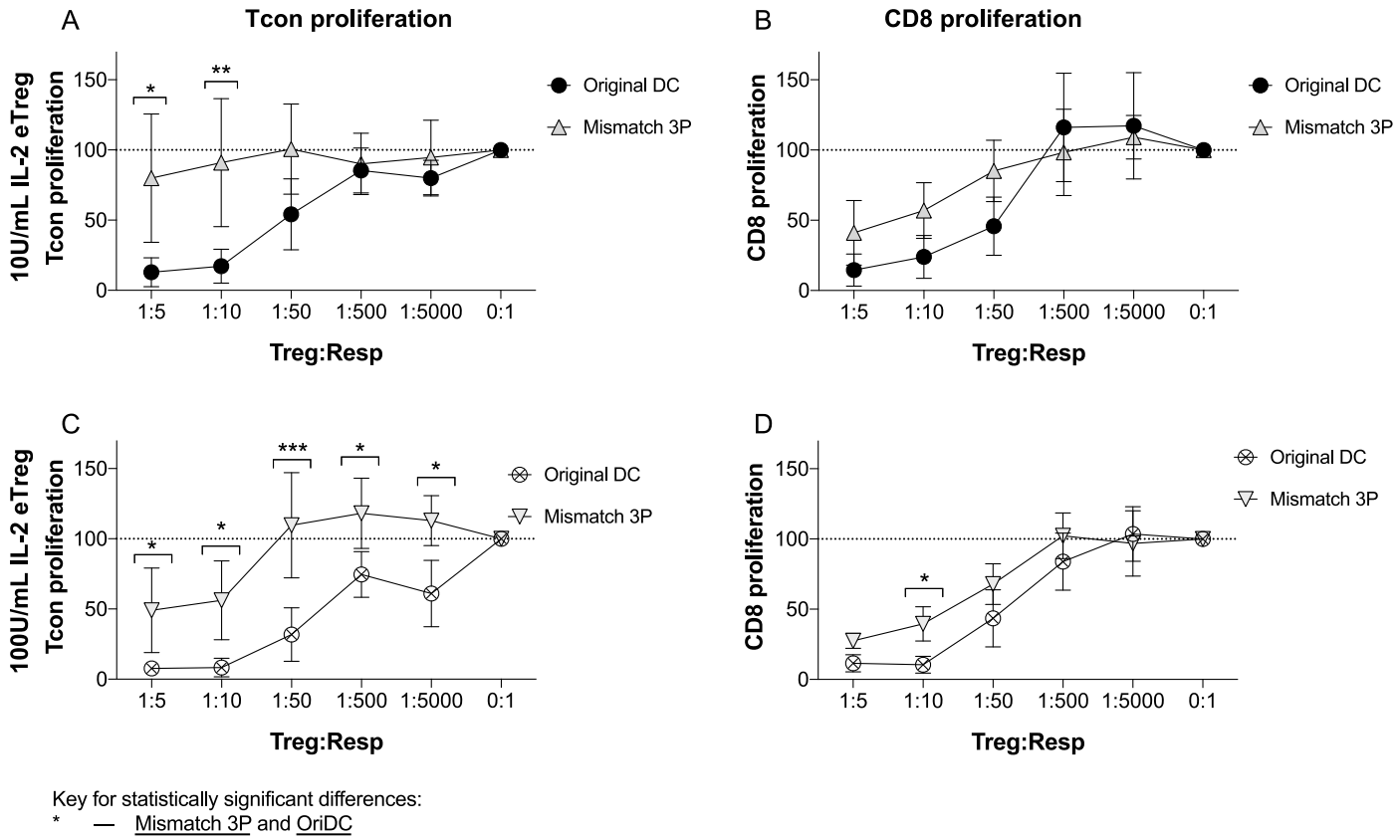


**FIGURE 3. Specificity of suppression of different Resp by Treg expanded with 10 U/ml or 100 U/ml of IL-2.**
(**A** and **B**) Proliferation of Tcon (A) or CD8 (B) in the presence of Treg expanded with 10 U/ml of IL-2 at several Treg/Resp ratios, in response to moDC from the original DC donor (black circles) or from a mismatch 3P (gray triangles). (**C** and **D**) Proliferation of Tcon (C) or CD8 (D) in the presence of Treg expanded with 100 U/ml of IL-2 at several Treg/Resp ratios, in response to moDC from the original DC donor (open circles with cross) or from a mismatch 3P (gray inverted triangles). Data were obtained from four independent experiments. Statistical analysis of differences between dilutions was performed with one-way ANOVA, and differences between conditions were evaluated with multiple *t* tests, by the Holm–Sidak method. Statistical significance was assumed when $p < 0.05$ (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). Error bars show SD.

Treg do not release suppressive cytokines nor stimulate Resp to produce suppressive factors (Fig. 5A, black bars). Similar results were observed with CD8 Resp, as significantly lower fold suppression was found when allo-specific Treg were cultured alone in the top well than in control wells with Resp (Fig. 5B, black bars).

Furthermore, fold suppression in the absence of contact was higher in conditions with mismatch 3P than with original DC stimulators, being closer to the suppression found in controls with contact (dotted line), suggesting that the suppression of nonspecific responses was only partially inhibited by the lack of contact between allo-specific Treg and Resp in the bottom well (Fig. 5A, 5B; gray bars). Interestingly, the presence of Resp together with allo-specific Treg in the top well seemed to enhance the suppression of CD8 Resp, when compared with the control (Top well 1:5 Treg/CD8, Fig. 5B). Overall, these results suggest that distinct mechanisms of suppression could be at play in specific and nonspecific settings.

To ascertain if allo-specific Treg modulate the maximal capacity for cytokine production in Resp, we

IL-2(C). Data from one representative experiment are shown. (**D** and **E**) Minimum spanning trees of the phenotype expressed by Tcon expanded with 100 U/ml of IL-2 (D) and by Treg expanded with 100 U/ml of IL-2 (E). The diameter of each node is proportional to the total number of cells allocated to it, and each node displays the relative intensity of all markers on that node. Nodes with highest expression of activation markers are signaled by an orange arrow for Tcon and a black arrow for Treg. The legend for each marker can be found in a pie chart. Each phenotype shows cumulative data from three independent experiments, after down sampling.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

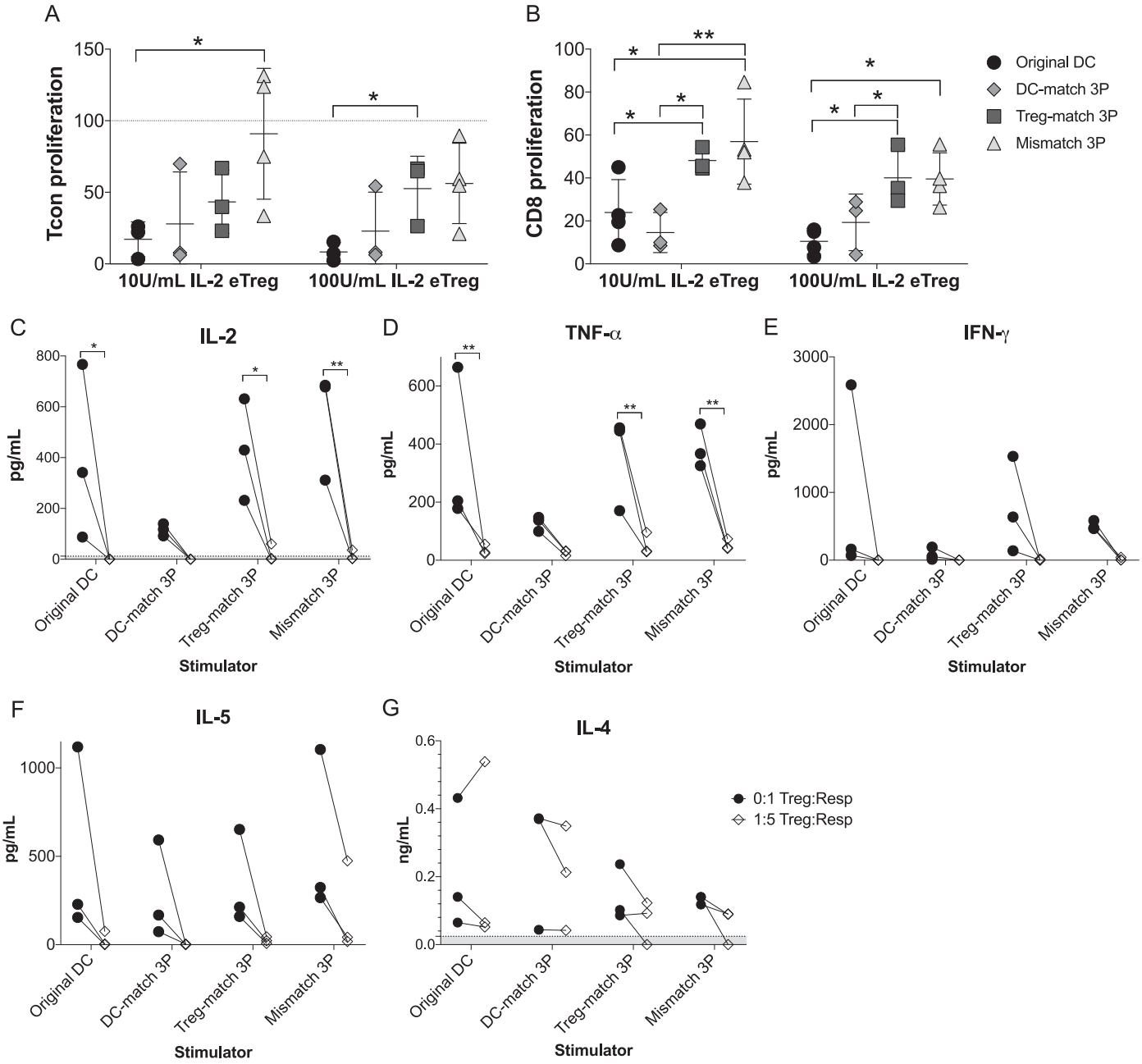


**FIGURE 4. Suppression of proliferation and cytokine secretion by allo-specific Treg in the presence of moDC from differently HLA-mismatched donors.**

(**A** and **B**) Average of normalized proliferation of Tcon (A) and CD8 (B) in the presence of allo-specific Treg expanded with 10 U/ml of IL-2 (10 U/ml of IL-2 eTreg, left) or 100 U/ml of IL-2 (100 U/ml of IL-2 eTreg, right) at a Treg/Resp ratio of 1:10. Stimulators are moDC from the donor used in Treg expansion (original DC donor; black circles), DC-match 3P (gray diamonds), Treg-match 3P (dark gray squares), or mismatch 3P (gray triangles). (**C**–**G**) Concentration of IL-2 (C), TNF-α (D), IFN-γ (E), IL-5 (F), and IL-4 (G) measured by Multiplex in the supernatant of suppression assay wells without Treg (0:1 Treg/Resp; black circles) or with Treg expanded with 100 U/ml of IL-2 at a Treg/Resp ratio of 1:5 (1:5 Treg/Resp; open diamonds). Data were obtained from three to four independent experiments. Statistical differences were evaluated with multiple *t* tests, by the Holm–Sidak method, with $p < 0.05$ (*$p < 0.05$, **$p < 0.005$). Error bars show SD.

restimulated the cells with PMA and ionomycin at the end of suppression assays and measured the frequency of IL-2$^+$ and IL-4$^+$ Resp. These frequencies were then normalized to those found in control wells without Treg (control 0:1). The normalized frequency of IL-2$^+$ Tcon and CD8 was similar with original DC or mismatch 3P stimulators and seemed unaltered by culture with allo-specific Treg (Fig. 5C, 5D). As for IL-4, the capability of

https://doi.org/10.4049/immunohorizons.2100021

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

Tcon and CD8 to produce IL-4 upon restimulation was maintained or slightly lowered after culture with allo-specific Treg and original DC stimulators (Fig. 5E, 5F). Surprisingly, this capability seemed increased after culture with allo-specific Treg if the stimulators were mismatch 3P (Fig. 5E, 5F), suggesting culture with allo-specific Treg may modulate the cytokine production potential of Resp.

Overall, we found that the mechanisms of suppression by allo-specific Treg were dependent on their specificity toward stimulators, as suppression of responses to the same stimuli used during expansion was strictly contact dependent, whereas suppression of responses to unknown stimuli was partially independent of contact.

### *Allo-specific Treg express multiple phenotypes*

To thoroughly characterize the phenotype of allo-specific Treg subsets after expansion, multidimensional visualization techniques were applied based on the expression of CTLA-4, PD-1, CD39, HLA-DR, 4-1BB, and CD40-L. By applying k-means clustering to fresh and allo-specific Treg samples, five clusters could be identified (Fig. 6A). Cluster phenotypes are described in histograms for each marker (Fig. 6B).

Interestingly, we found that some phenotypes were expressed mostly by fresh Treg, whereas others were only expressed by allo-specific Treg (Fig. 6A). Fresh Treg expressed cluster 2 (CTLA-4$^-$CD40-L$^-$4-1BB$^-$PD-1$^+$) and cluster 4 (CTLA-4$^+$CD40-L$^+$4-1BB$^+$PD-1$^-$) phenotypes

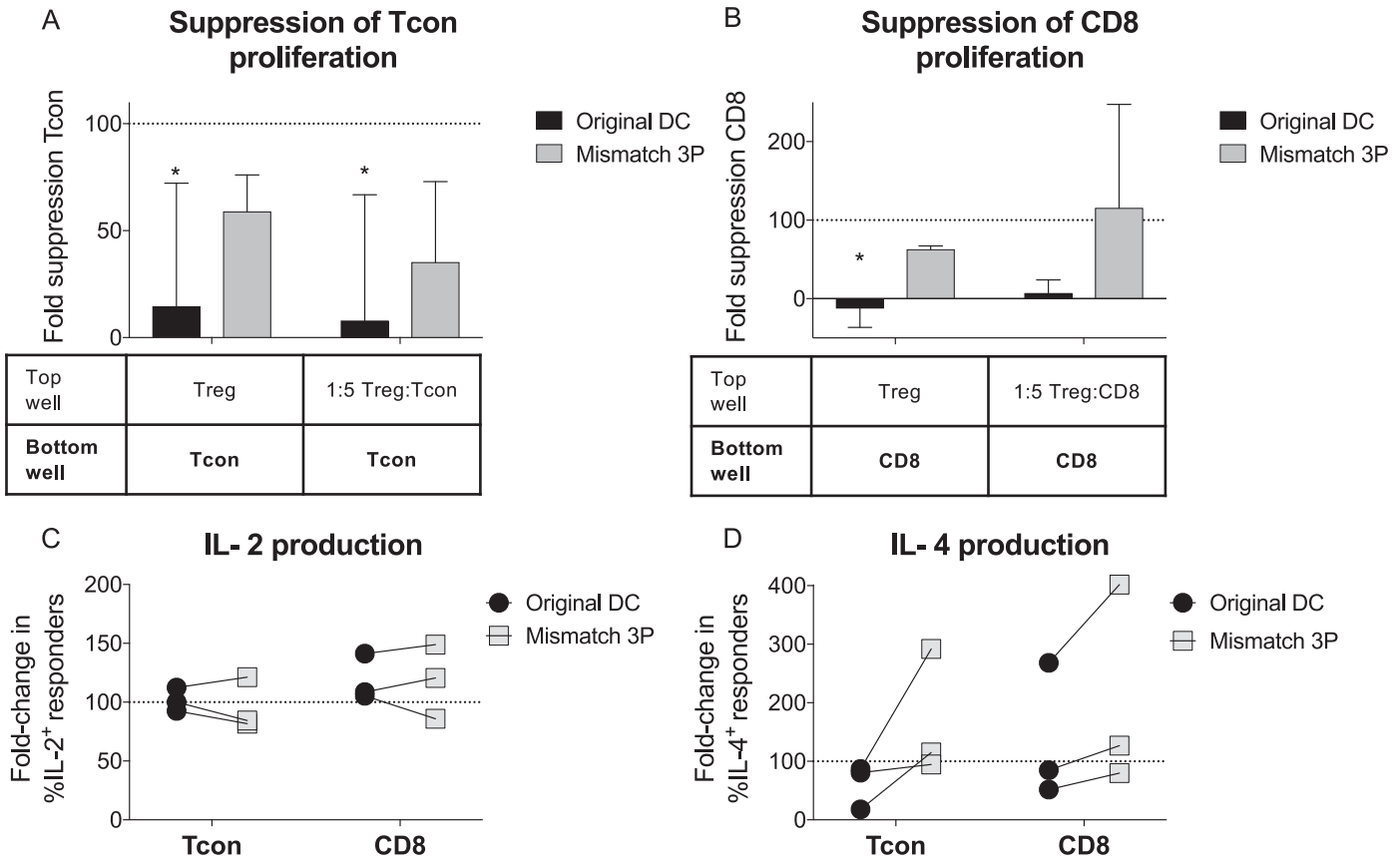


**FIGURE 5. Potency and mechanisms of suppression by allo-specific Treg in cognate and noncognate settings.**

(**A** and **B**) Fold suppression of proliferation of Tcon (A) and CD8 (B) cultured in the bottom chamber, when Treg expanded with 100 U/ml of IL-2 were cultured in the top chamber of Transwell plates alone (bars on the left) or with Resp, at a Treg/Resp ratio of 1:5 (bars on the right). Black bars show results when original DC were used as stimulators in top and bottom chambers, and gray bars show results for mismatch 3P. Data were normalized to the suppression found when both Resp and Treg were cultured in the bottom chamber (standard suppression assay controls), represented by the dotted line. (**C** and **D**) Fold change in the frequency of IL-2– (C) and IL-4– (D) producing Tcon (left) or CD8 (right) upon restimulation with PMA and ionomycin, after culture with Treg expanded with 100 U/ml of IL-2 at a Treg/Resp ratio of 1:5 in the bottom chamber of standard suppression assay controls. Data were normalized to controls without Treg (0:1), represented by the dotted line. Data were obtained from two to three independent experiments. Statistical analysis of differences in Resp proliferation or cytokine concentration was evaluated with multiple *t* tests, by the Holm–Sidak method. Statistical significance was assumed when $p < 0.05$. The asterisk (*) represents statistically significant differences between a condition and the control with $p < 0.05$.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

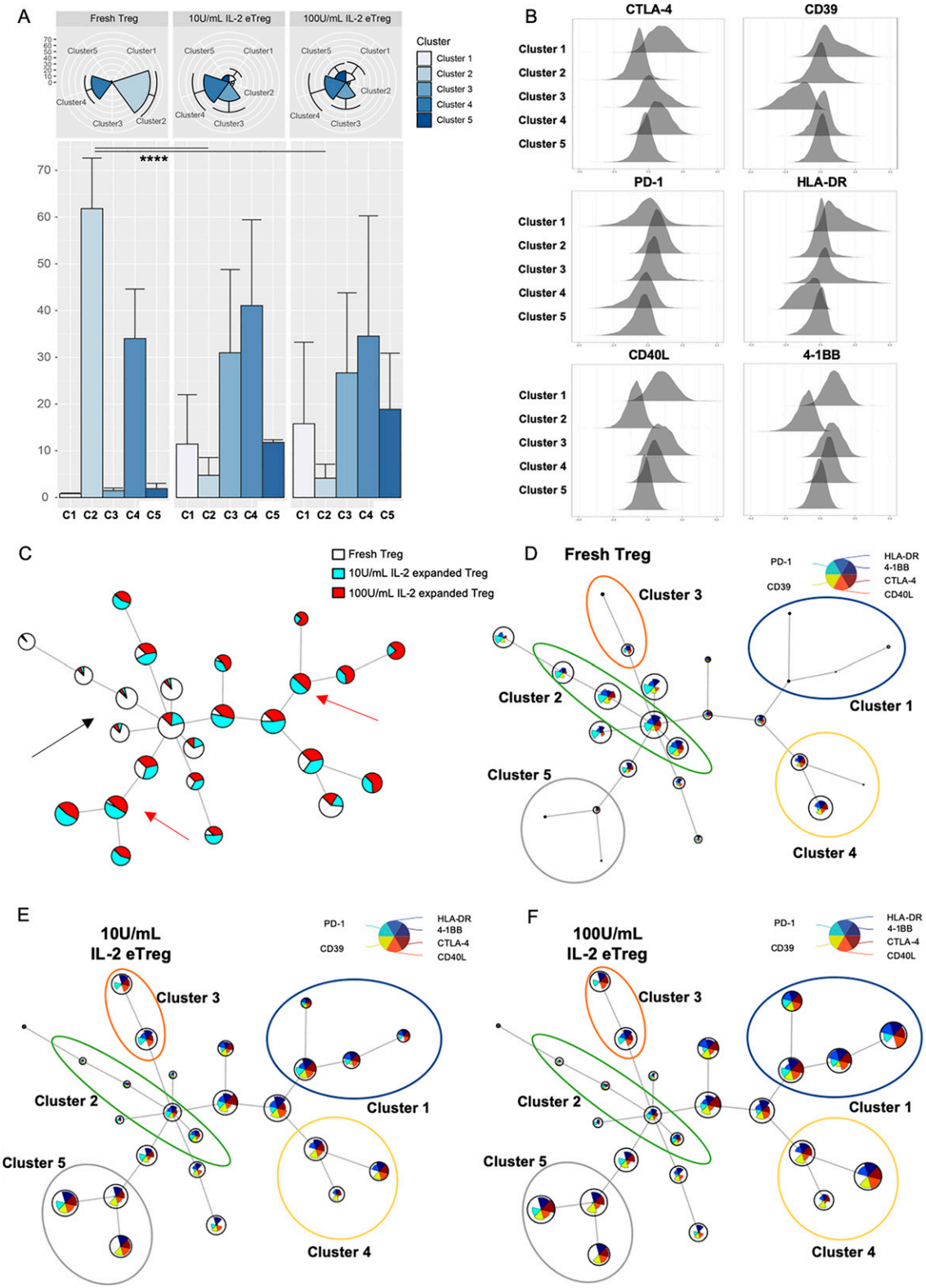


**FIGURE 6. Phenotype of fresh and eTreg.**

(**A**) The k-means clustering analysis. The frequency of cells allocated to each cluster is represented on polar plots (top) or bar plots (bottom). The radius of a cluster or the height of a bar represents the mean percentage of cells allocated to that cluster, and the error bar represents SD. Statistical analysis was performed by multiple *t* tests on R Stats package. (**B**) Histograms with the relative expression of each marker **(Continued)**

https://doi.org/10.4049/immunohorizons.2100021

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

(Fig. 6A), which had converse expression of CTLA-4, CD40-L, 4-1BB, and PD-1 yet were similar in their low expression of HLA-DR and relatively low CD39, a typical phenotype of ex vivo cells.

After expansion, allo-specific Treg had a similar frequency of cells expressing cluster 4 to fresh Treg, yet cluster 2 was significantly reduced. In addition to cluster 4, allo-specific Treg expressed three unique subsets with distinct levels of PD-1, CD39, and HLA-DR: clusters 1, 3, and 5. Clusters 1 (PD-$1^{low}$ $CD39^{+}HLA\text{-}DR^{+}$) and 3 ($PD\text{-}1^{+}CD39^{-}HLA\text{-}DR^{+}$) had converse expression of PD-1 and CD39, and when compared with cluster 4, cluster 1 (PD-$1^{low}$ $CD39^{+}HLA\text{-}DR^{+}$) and cluster 3 ($PD\text{-}1^{+}CD39^{-}HLA\text{-}DR^{+}$) had higher expression of HLA-DR, CD40-L, and 4-1BB, which was expected after culture. In contrast, cluster 5 (PD-$1^{low}$ $CD39^{low}HLA\text{-}DR^{low}$) had low expression of all activation markers.

Similar results were found using FlowSOM, as some nodes comprised mostly cells from fresh Treg samples (Fig. 6C, black arrow) and others comprised cells from allo-specific Treg samples (Fig. 6C, red arrows). Overall, the phenotype of fresh Treg (Fig. 6D) was very different from that of eTreg (Fig. 6E, 6F). Particularly, allo-specific Treg had more cells allocated to nodes representing a more-activated phenotype, as had been detected by k-means clustering analysis, whereas fresh Treg had more cells allocated to nodes with low expression of most markers (Fig. 6D–F). Furthermore, we were able to identify groups of nodes with subphenotypes similar to the clusters defined by k-means clustering and label them accordingly (Fig. 6D–F). Of note, the concentration of IL-2 during expansion did not seem to affect the range of phenotypes expressed by Treg, just the frequency of cells allocated to each cluster.

Ultimately, using these innovative techniques for multidimensional visualization of flow cytometry data, five unique Treg subsets could be defined. Particularly, ex vivo Treg expressed two major phenotypes, one of which was no longer expressed after expansion. The other subset was expressed by allo-specific Treg, together with three other activated subsets, distinguishable through the combined expression of PD-1, CD39, and HLA-DR.

## DISCUSSION

The present work demonstrates that allo-specific Treg can be expanded in serum-free conditions with low and intermediate IL-2 concentrations, without compromising the potency or specificity of suppression. To our knowledge, this is also the first report of eTreg displaying distinct mechanisms of suppression in specific and nonspecific settings.

Treg expanded with our serum-free expansion protocol were more-potent suppressors of Tcon and CD8 proliferation than fresh Treg, as has been described for Treg expanded in the presence of human serum (19, 33). Significant suppression of responses to the stimulator used during expansion was consistently found at Treg/Resp ratios of 1:10 and even at Treg/Resp ratios of 1:50, whereas suppression by polyclonally eTreg has been shown to require Treg/Resp ratios of at least 1:2 (34, 35). Thus, allo-specific Treg were found to be more potent than polyclonally eTreg. Allo-specific Treg expansion is usually carried out with low IL-2 concentration to prevent polyclonal Treg expansion and the recruitment of non-Treg (19). However, there are also reports of allo-specific Treg being expanded with high IL-2 concentration (10, 11, 36). In our study, intermediate IL-2 concentration during allo-specific Treg expansion seemed to increase the potency of Tcon suppression.

Treg with a particular specificity have been shown to also suppress nonspecific responses (37). Nevertheless, Treg are usually considered specific when they are more-potent suppressors of responses to their original stimulator than to fully mismatched donors (10, 19, 38–40) or to mitogenic beads (25). In this work, we show that allo-specific Treg are always more suppressive of responses to their original stimulators. However, there is still some nonspecific suppression, particularly when CD8 cells are used as Resp. Based on the cytokine quantification studies performed in this study, it seems that the suppression mechanisms may be distinct for CD4 and CD8 T cells. In fact, we show that modulation of cytokine production may be one of the mechanisms by which Treg suppress Resp, particularly Tcon. Furthermore, the downregulation of the production of cytokines, such as IL-2, TNF-α, and IFN-γ, seems to have a higher impact on the suppression of Tcon responses than on that of CD8.

The Ag specificity in allo-specific Treg is usually unknown. In fact, it is expectable that they are specific for a plethora of Ags, rather than to a particular one. Until now, it had not been ascertained whether allo-specific Treg could suppress responses to partially matched donors and to what extent. We found that, when a third-party donor is partially HLA matched to the DC donor used in expansion, there is similar suppression of responses to both stimulators, suggesting that allo-specific

within clusters defined by k-means clustering. (**C**–**F**) FlowSOM analysis of phenotypes expressed by Treg before and after expansion. (C) Minimum spanning tree of all analyzed samples. The diameter of each node is proportional to the total number of cells attributed to it, and each node displays the frequency of cells from each sample allocated to that node. Black arrow indicates nodes comprising mostly cells from fresh Treg, whereas red arrows indicate nodes comprising predominantly expanded cells. (D) Minimum spanning tree of fresh Treg, (E) Treg expanded with 10 U/ml of IL-2, and (F) Treg expanded with 100 U/ml of IL-2. Groups of nodes with similar phenotype to the clusters found by k-means clustering were identified: cluster 1 is represented by a blue circle, cluster 2 is represented by a green circle, cluster 3 is represented by an orange circle, cluster 4 is represented by a yellow circle, and cluster 5 is represented by a gray circle. Each node displays the relative intensity of all markers on that node, and the legend for markers can be found in a pie chart in the lower right corner.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

Treg can recognize cognate HLA alleles. This may prove relevant for future development of Treg therapies, as in cases in which patient-derived APC are not available, donor-derived Treg could be expanded with APC from donors partially matched to the patient.

In this article, we show, to our knowledge for the first time, that different mechanisms are involved in suppression of responses to specific and nonspecific stimuli. We showed that suppression of responses to the original expansion stimulators was strictly contact dependent, whereas there was partial contact-independent suppression of responses to mismatch 3P. In fact, even when allo-specific Treg were cultured with Resp and stimulated by the original expansion DC, it did not seem to result in the production of suppressive cytokines capable of contact-independent suppression.

It is of relevance that the suppression mediated by allo-specific Treg is strictly contact dependent and apparently HLA specific, as this finding may have important clinical implications. In the setting of allo-HSCT, the main potential use of these cells would be in the prevention and/or treatment of GvHD. Going forward, we plan to generate donor-derived recipient-specific Treg, using real-life donor/recipient allo-HSCT samples. Our ultimate goal is to ascertain that donor-derived allo-specific Treg suppress responses against normal recipient-derived DC but not against recipient-derived DC presenting leukemia Ags. Thus, the current study is an essential first endeavor, to our knowledge, toward generating donor-derived allo-specific Treg that suppress GvHD while sparing GVL responses.

IL-10 is reportedly involved in contact-independent suppression by Treg (23). However, we did not find IL-10 concentration to be increased by the presence of allo-specific Treg in the supernatants of suppression assays (Supplemental Fig. 1G, 1H) nor did we detect the production of IL-10 by Treg or Resp upon restimulation with PMA and ionomycin at the end of suppression assays (data not shown). Thus, suppression seemed independent of IL-10, which is in accordance with previous reports (33, 41), and suggests that other mechanisms may be at play in suppression by allo-specific Treg.

Importantly, allo-specific Treg seemed to modulate the capability for IL-4 production in Resp, possibly eschewing them to express an anti-inflammatory phenotype. The induction of IL-4 secretion in Tcon has been described by others in suppression assays using mitogenic beads (23, 42), but the observation that the capability to produce IL-4 seems increased only in nonspecific suppression settings constitutes a to our knowledge novel finding. It would be interesting to block IL-4 in suppression assays, but, as Ag-specific Treg have been shown to rely on IL-4 signaling (43), this could also impair Treg function.

Looking into the effect of DC phenotype in suppression by Treg, we found higher CD86 expression on moDC was correlated to lower suppression. Because CTLA-4 blockade did not impair suppression by Treg, this correlation does not seem derived from a limiting amount of CTLA-4 on Treg surface. One possibility is that higher CD86 expression allows for Resp to escape Treg-mediated suppression, as it has been shown that costimulation with CD80 and CD86 allowed for Th cells to evade suppression in the presence of high Ag dose (44).

Finally, HLA-DR (23), CD39 (24, 45), and 4-1BB (25) have been individually linked to the suppressive ability of Treg. However, no report has described the simultaneous expression of these markers on Treg subsets. After multidimensional analysis of ex vivo and eTreg, our data suggest that the coexpression of HLA-DR, CD39, and PD-1 can identify Treg subsets that might share not only the same phenotype but possibly also similar functional mechanisms.

Overall, our data show that Treg expanded in serum-free conditions with allogeneic moDC are allo-specific and may recognize matched HLA alleles in third-party donors. Furthermore, we provide new insights on the mechanisms of suppression displayed by allo-specific Treg, as specific stimulation prompts contact-dependent suppression, whereas nonspecific stimuli elicit both contact-dependent and -independent suppression.

## DISCLOSURES

The authors have no financial conflicts of interest.

## REFERENCES


1. Ferrara, J. L., J. E. Levine, P. Reddy, and E. Holler. 2009. Graft-versus-host disease. *Lancet* 373: 1550–1561.
2. Lee, S. J., J. P. Klein, A. J. Barrett, O. Ringden, J. H. Antin, J. Y. Cahn, M. H. Carabasi, R. P. Gale, S. Giralt, G. A. Hale, et al. 2002. Severity of chronic graft-versus-host disease: association with treatment-related mortality and relapse. *Blood* 100: 406–414.
3. Zorn, E., H. T. Kim, S. J. Lee, B. H. Floyd, D. Litsa, S. Arumugarajah, R. Bellucci, E. P. Alyea, J. H. Antin, R. J. Soiffer, and J. Ritz. 2005. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* 106: 2903–2911.
4. Soares, M. V. D., R. I. Azevedo, I. A. Ferreira, S. V. Bucar, A. C. Alho, E. L. Espada, J. Clara, N. Camacho, C. M. Martins, J. A. Carmo, F. Lourenço, R. Moreno, C. Vaz, A. M. Campos, F. Campilho, R. Ferreira, J. Ritz, and J. F. Lacerda. 2016. TREG and Tcon dynamics after Allo-HSCT: Cgvhd is associated to decreased naive and stem cell memory subsets with a concomitant increase in terminally differentiated T cell subsets. *Blood*. DOI: 10.1182/blood.V128.22.2229.2229.
5. Alho, A. C., H. T. Kim, M. J. Chammas, C. G. Reynolds, T. R. Matos, E. Forcade, J. Whangbo, S. Nikiforow, C. S. Cutler, J. Koreth, et al. 2016. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD. *Blood* 127: 646–657.
6. Theil, A., S. Tuve, U. Oelschlägel, A. Maiwald, D. Döhler, D. Oßmann, A. Zenkel, C. Wilhelm, J. M. Middeke, N. Shayegi, et al. 2015. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* 17: 473–486.
7. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, et al. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117: 1061–1070.
8. Di Ianni, M., F. Falzetti, A. Carotti, A. Terenzi, F. Castellino, E. Bonifacio, B. Del Papa, T. Zei, R. I. Ostini, D. Cecchini, et al. 2011. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 117: 3921–3928.


Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

9. Del Papa, B., L. Ruggeri, E. Urbani, S. Baldoni, D. Cecchini, T. Zei, R. Iacucci Ostini, B. Crescenzi, A. Carotti, A. Pierini, et al. 2017. Clinical-grade-expanded regulatory T cells prevent graft-versus-host disease while allowing a powerful T cell-dependent graft-versus-leukemia effect in murine models. *Biol. Blood Marrow Transplant*. 23: 1847–1851.
10. Mathew, J. M., J. H. Voss, S. T. McEwen, I. Konieczna, A. Chakraborty, X. Huang, J. He, L. Gallon, R. S. Kornbluth, and J. R. Leventhal. 2018. Generation and characterization of alloantigen-specific regulatory T cells for clinical transplant tolerance. *Sci. Rep.* 8: 1136.
11. Sagoo, P., N. Ali, G. Garg, F. O. Nestle, R. I. Lechler, and G. Lombardi. 2011. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci. Transl. Med.* 3: 83ra42.
12. Golshayan, D., S. Jiang, J. Tsang, M. I. Garin, C. Mottet, and R. I. Lechler. 2007. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* 109: 827–835.
13. Li, J., J. Heinrichs, K. Haarberg, K. Semple, A. Veerapathran, C. Liu, C. Anasetti, and X. Z. Yu. 2015. HY-specific induced regulatory T cells display high specificity and efficacy in the prevention of acute graft-versus-host disease. *J. Immunol.* 195: 717–725.
14. Falkenburg, J. H. F., L. van de Corput, E. W. A. Marijt, and R. Willemze. 2003. Minor histocompatibility antigens in human stem cell transplantation. *Exp. Hematol.* 31: 743–751.
15. Mathew, J. M., J. H-Voss, A. LeFever, I. Konieczna, C. Stratton, J. He, X. Huang, L. Gallon, A. Skaro, M. J. Ansari, and J. R. Leventhal. 2018. A phase I clinical trial with ex vivo expanded recipient regulatory T cells in living donor kidney transplants. *Sci. Rep.* 8: 7428.
16. Atif, M., F. Conti, G. Gorochov, Y. H. Oo, and M. Miyara. 2020. Regulatory T cells in solid organ transplantation. *Clin. Transl. Immunology* 9: e01099.
17. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636–645.
18. Litjens, N. H. R., K. Boer, J. M. Zuijderwijk, M. Klepper, A. M. A. Peeters, E. P. Prens, W. Verschoor, R. Kraaijeveld, Z. Ozgur, M. C. van den Hout-van Vroonhoven, et al. 2015. Allogeneic mature human dendritic cells generate superior alloreactive regulatory T cells in the presence of IL-15. *J. Immunol.* 194: 5282–5293.
19. Veerapathran, A., J. Pidala, F. Beato, X. Z. Yu, and C. Anasetti. 2011. Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. *Blood* 118: 5671–5680.
20. Collison, L. W., and D. A. A. Vignali. 2011. In vitro Treg suppression assays. *Methods Mol. Biol.* 707: 21–37.
21. Friedline, R. H., D. S. Brown, H. Nguyen, H. Kornfeld, J. Lee, Y. Zhang, M. Appleby, S. D. Der, J. Kang, and C. A. Chambers. 2009. $CD4^+$ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. *J. Exp. Med.* 206: 421–434.
22. Asano, T., Y. Meguri, T. Yoshioka, Y. Kishi, M. Iwamoto, M. Nakamura, Y. Sando, H. Yagita, J. Koreth, H. T. Kim, et al. 2017. PD-1 modulates regulatory T-cell homeostasis during low-dose interleukin-2 therapy. *Blood* 129: 2186–2197.
23. Baecher-Allan, C., E. Wolf, and D. A. Hafler. 2006. MHC class II expression identifies functionally distinct human regulatory T cells. *J. Immunol.* 176: 4622–4631.
24. Gu, J., X. Ni, X. Pan, H. Lu, Y. Lu, J. Zhao, S. Guo Zheng, K. L. Hippen, X. Wang, and L. Lu. 2017. Human $CD39^{hi}$ regulatory T cells present stronger stability and function under inflammatory conditions. *Cell. Mol. Immunol.* 14: 521–528.
25. Schoenbrunn, A., M. Frentsch, S. Kohler, J. Keye, H. Dooms, B. Moewes, J. Dong, C. Loddenkemper, J. Sieper, P. Wu, et al. 2012. A converse 4-1BB and CD40 ligand expression pattern delineates activated regulatory T cells (Treg) and conventional T cells enabling direct isolation of alloantigen-reactive natural Foxp3+ Treg. *J. Immunol.* 189: 5985–5994.
26. Liu, M., E. S. Barton, R. N. Jennings, D. G. Oldenburg, J. M. Whirry, D. W. White, and J. M. Grayson. 2017. Unsupervised learning techniques reveal heterogeneity in memory $CD8^+$ T cell differentiation following acute, chronic and latent viral infections. *Virology* 509: 266–279.
27. Van Gassen, S., B. Callebaut, M. J. Van Helden, B. N. Lambrecht, P. Demeester, T. Dhaene, and Y. Saeys. 2015. FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A* 87: 636–645.
28. Finak, G., J.-M. Perez, A. Weng, and R. Gottardo. 2010. Optimizing transformations for automated, high throughput analysis of flow cytometry data. *BMC Bioinformatics* 11: 546.
29. Safinia, N., T. Vaikunthanathan, H. Fraser, S. Thirkell, K. Lowe, L. Blackmore, G. Whitehouse, M. Martinez-Llordella, W. Jassem, A. Sanchez-Fueyo, et al. 2016. Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. *Oncotarget* 7: 7563–7577.
30. Voskens, C. J., A. Fischer, S. Roessner, C. Lorenz, S. Hirschmann, R. Atreya, C. Neufert, I. Atreya, M. F. Neurath, and G. Schuler. 2017. Characterization and expansion of autologous GMP-ready regulatory T cells for TREG-based cell therapy in patients with ulcerative colitis. *Inflamm. Bowel Dis.* 23: 1348–1359.
31. Veerapathran, A., J. Pidala, F. Beato, B. Betts, J. Kim, J. G. Turner, M. K. Hellerstein, X. Z. Yu, W. Janssen, and C. Anasetti. 2013. Human regulatory T cells against minor histocompatibility antigens: ex vivo expansion for prevention of graft-versus-host disease. *Blood* 122: 2251–2261.
32. Strauss, L., M. Czystowska, M. Szajnik, M. Mandapathil, and T. L. Whiteside. 2009. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. *PLoS One* 4: e5994.
33. Collison, L. W., M. R. Pillai, V. Chaturvedi, and D. A. A. Vignali. 2009. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J. Immunol.* 182: 6121–6128.
34. Hoffmann, P., R. Eder, L. A. Kunz-Schughart, R. Andreesen, and M. Edinger. 2004. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood* 104: 895–903.
35. Hippen, K. L., S. C. Merkel, D. K. Schirm, C. M. Sieben, D. Sumstad, D. M. Kadidlo, D. H. McKenna, J. S. Bromberg, B. L. Levine, J. L. Riley, et al. 2011. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci. Transl. Med.* DOI: 10.1126/scitranslmed.3001809.
36. Putnam, A. L., N. Safinia, A. Medvec, M. Laszkowska, M. Wray, M. A. Mintz, E. Trotta, G. L. Szot, W. Liu, A. Lares, et al. 2013. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am. J. Transplant.* 13: 3010–3020.
37. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164: 183–190.
38. Koenen, H. J. P. M., E. Fasse, and I. Joosten. 2005. CD27/CFSE-based ex vivo selection of highly suppressive alloantigen-specific human regulatory T cells. *J. Immunol.* 174: 7573–7583.
39. Peters, J. H., L. B. Hilbrands, H. J. P. M. Koenen, and I. Joosten. 2008. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4posCD25high T cells for immunotherapy. *PLoS One* 3: e2233.
40. Tawara, I., W. D. Shlomchik, A. Jones, W. Zou, E. Nieves, C. Liu, T. Toubai, R. Duran-Struuck, Y. Sun, S. G. Clouthier, et al. 2010. A crucial role for host APCs in the induction of donor CD4+CD25+ regulatory T cell-mediated suppression of experimental graft-versus-host disease. *J. Immunol.* 185: 3866–3872.
41. Oberle, N., N. Eberhardt, C. S. Falk, P. H. Krammer, and E. Suri-Payer. 2007. Rapid suppression of cytokine transcription in human CD4+CD25 T cells by CD4+Foxp3+ regulatory T cells: independence of IL-2 consumption, TGF-beta, and various inhibitors of TCR signaling. *J. Immunol.* 179: 3578–3587.
42. Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M. J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine


Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021

deprivation-mediated apoptosis of effector CD4+ T cells. *Nat. Immunol.* 8: 1353–1362.

43. Verma, N. D., K. M. Plain, M. Nomura, G. T. Tran, C. Robinson, R. Boyd, S. J. Hodgkinson, and B. M. Hall. 2009. CD4+CD25+ T cells alloactivated ex vivo by IL-2 or IL-4 become potent alloantigen-specific inhibitors of rejection with different phenotypes, suggesting separate pathways of activation by Th1 and Th2 responses. *Blood* 113: 479–487.
44. George, T. C., J. Bilsborough, J. L. Viney, and A. M. Norment. 2003. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur. J. Immunol.* 33: 502–511.
45. Fletcher, J. M., R. Lonergan, L. Costelloe, K. Kinsella, B. Moran, C. O Farrelly, N. Tubridy, and K. H. G. Mills. 2009. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J. Immunol.* 183: 7602–7610.

Downloaded from http://www.immunohorizons.org/ at Universidade de Lisboa on May 26, 2021