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Research Article

Frequency of natural regulatory T cells specific for factor VIII in the peripheral blood of healthy donors

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Tolerance to self-proteins involves multiple mechanisms, including conventional CD4⁺ T-cell (Tconv) deletion in the thymus and the recruitment of natural regulatory T cells (nTregs). The significant incidence of autoantibodies specific for the blood coagulation factor VIII (FVIII) in healthy donors illustrates that tolerance to self-proteins is not always complete. In contrast to FVIII-specific Tconvs, FVIII-specific nTregs have never been revealed and characterized. To determine the frequency of FVIII-specific Tregs in human peripheral blood, we assessed the specificity of *in vitro* expanded Tregs by the membrane expression of the CD137 activation marker. Amplified Tregs maintain high levels of FOXP3 expression and exhibit almost complete demethylation of the FOXP3 Treg-specific demethylated region. The cells retained FOXP3 expression after long-term culture *in vitro*, strongly suggesting that FVIII-specific Tregs are derived from the thymus. From eleven healthy donors, we estimated the frequencies of FVIII-specific Tregs at 0.17 cells per million, which is about 10-fold lower than the frequency of FVIII-specific CD4⁺ T cells we previously published. Our results shed light on the mechanisms of FVIII tolerance by a renewed approach that could be extended to other self- or non-self-antigens.

Keywords: Antigen-specific Treg · FVIII · Hemophilia A · Human regulatory T cells · Thymus-derived Treg



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Natural regulatory T cells (nTregs) constitute 5–10% of CD4⁺ T cells in healthy individuals and play a key role in maintaining immune tolerance to self-antigens and homeostasis. In contrast to conventional CD4⁺ T cells (Tconvs), nTregs constitutively

express high levels of CD25 [1], none or low levels of CD127 [2], and high expression of the master transcription factor FOXP3, which confers immunosuppressive activity [3, 4]. Mutations in the *FOXP3* gene cause systemic autoimmune disorders in mice and humans [5]. The majority of nTregs in the immune system are generated in the thymus (tTregs). Their development appears to be favored by high functional avidity T-cell receptor (TCR) interaction with endogenous self-antigens, which is essential for their immune regulatory capacity [6]. As shown in humans for

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environmental antigens [7, 8], nTregs can be specific for foreign antigens. A part of nTregs can differentiate outside the thymus, notably in the gut, chronically inflamed tissues, or transplanted tissues, from CD4⁺CD25⁻FOXP3⁻ Tconvs and they are called peripheral-generated Tregs (pTregs). Both tTreg and pTreg subsets are characterized by stable expression of FOXP3, which correlates with complete demethylation within the evolutionarily conserved region upstream of exon 1, named Treg-specific demethylated region (TSDR) [9]. Thereby, nTregs differ from activated CD4⁺ Tconvs [10] and induced Tregs (iTregs) [11], which can be produced *in vitro* in the presence of high levels of IL-2 and TGF- β [12]. Unlike tTregs, pTregs, and iTregs can lose FOXP3 expression in long-term *in vitro* cultures [7, 9, 10].

Although nTregs exert their suppressive activity upon the engagement of their TCR with an antigen [13, 14], antigen-specific Tregs in humans have been poorly investigated and their characterization so far remains limited to some aeroantigens and few autoantigens [7, 8, 15, 16]. This is due to the low frequency of these particular subsets in human peripheral blood, which raises particular challenges for their *ex vivo* isolation and *in vitro* expansion. However, their study is undoubtedly essential to evaluate their involvement in regulating the immune response.

For instance, the immune tolerance mechanisms to the blood coagulation factor VIII (FVIII) are still unclear. In patients with hemophilia A (HA), missense mutations or extensive gene alterations can result in bleeding due to lack of FVIII production or production of a nonfunctional protein, which is prevented by infusions of plasma-derived or recombinant human FVIII (rFVIII). These gene deficiencies make the therapeutic FVIII a partially or fully non-self-protein for HA patients and thus promote the recruitment of FVIII-specific CD4⁺ Tconvs [17, 18] while reducing the commitment of Tregs in the thymus. The appearance of neutralizing antibodies following FVIII treatments is more frequent in severe HA patients with none or low endogenous FVIII production, compared with patients with mild/moderate HA, in whom the endogenous, dysfunctional FVIII (cross-reacting material) [19] can still be recognized by the immune system and induce Tregs in the thymus. Nevertheless, tolerance to FVIII in healthy donors (HDs) also appears incomplete. Even though they express normal levels of functional endogenous FVIII, approximately 20% of the healthy population possess antibodies specific to FVIII [20]. Further, during their lifetime, individuals without altered hemostasis may develop acquired hemophilia A, a rare but severe autoimmune bleeding disorder caused by anti-FVIII autoantibody production [21].

We previously demonstrated that FVIII-specific CD4⁺ T cells are present at a mean frequency of 1.7 per million total CD4⁺ T cells in the peripheral blood of HDs, thus escaping from thymic deletion [22]. The existence of FVIII-specific Tregs has been suggested *in vitro* and *ex vivo* by CD4⁺CD25^{hi} nTreg depletion experiments [23, 24]. However, they have never been formally isolated, and their specificity has never been studied. Therefore, we aimed to detect and quantify nTregs specific for FVIII in human peripheral blood to shed light on the mechanisms underlying T-cell tolerance to FVIII.

Results

In vitro expanded Tregs display stable FOXP3 expression

To detect low-frequency nTregs specific for FVIII in human peripheral blood, we set up a new protocol to favor their expansion. First, we purified CD4⁺ T cells using magnetic beads, sorted polyclonal CD3⁺CD4⁺CD25^{hi}CD127^{lo/-} Tregs by flow cytometry (Fig. 1A), and distributed them into multiple wells to limit the dilution of specific cells in each well. Then, Tregs were stimulated by four weekly round stimulations with autologous DCs previously loaded with rFVIII (Supporting information Fig. S1). As expected, purified Tregs seeded in culture expressed high levels of FOXP3 (Fig. 1B and Supporting information Fig. S2). Interestingly, we also observed a high level of FOXP3 expression for *in vitro* expanded Tregs (Fig. 1C and Supporting information Fig. S2). Accordingly, both day 0-purified and day 27-expanded Tregs displayed almost complete demethylation of FOXP3-TSDR (Fig. 2A). In addition, purified Tregs suppressed allogenic Tconv proliferation (Fig. 2B). Importantly, preliminary data showed that a day 27-expanded Treg cell line reduced allogenic Tconv proliferation and that this suppression was specific for FVIII (Supporting information Fig. S3). Altogether, these results strongly suggest that purified and expanded T cells displayed the characteristics of nTregs.

FVIII-specific Tregs can be detected by *in vitro* expansion

Following their *in vitro* culture, we investigated whether amplified Tregs included FVIII-specific Tregs. Each independent Treg cell line, that is, Tregs cultured in one well, was re-stimulated with rFVIII presented by autologous DCs and anti-CD28 antibody. Then we measured CD137 expression after a 24 h TCR activation on CD3⁺CD4⁺CD127^{lo/-}CD25^{hi} Tregs (Supporting information Fig. S4) as CD137 is expressed at cell surface following TCR activation and at its maximal level after 24 h [25]. We considered that a Treg cell line was FVIII-specific if the ratio between the CD137 MFI of the stimulated to the unstimulated Treg cell line was ≥ 2 . The percentage of CD137⁺ Tregs was obtained by subtracting the CD137⁺ background signal (non-stimulated Treg cell line). The lowest proportion of CD137⁺ cells was about 3%, and the highest was about 60% (Supporting information Fig. S5). The mean percentage of CD137⁺ Tregs for all the Treg cell lines from the eleven donors was 32.5. As exemplified for the same healthy individual (Fig. 3A), nonspecific Treg cell lines exhibit the same low level of CD137 expression comparing the rFVIII stimulated condition and the unstimulated condition. In contrast, high levels of CD137 expression were expressed by the FVIII-specific Treg cell line when stimulated by rFVIII, namely 32.5% of the cells expressing CD137 in the presence of rFVIII versus 4.3% in its absence. Notably, CD137⁻ Tregs expressed a low level of CD25, while the CD137⁺ Tregs expressed high levels of CD25 (pink dots)

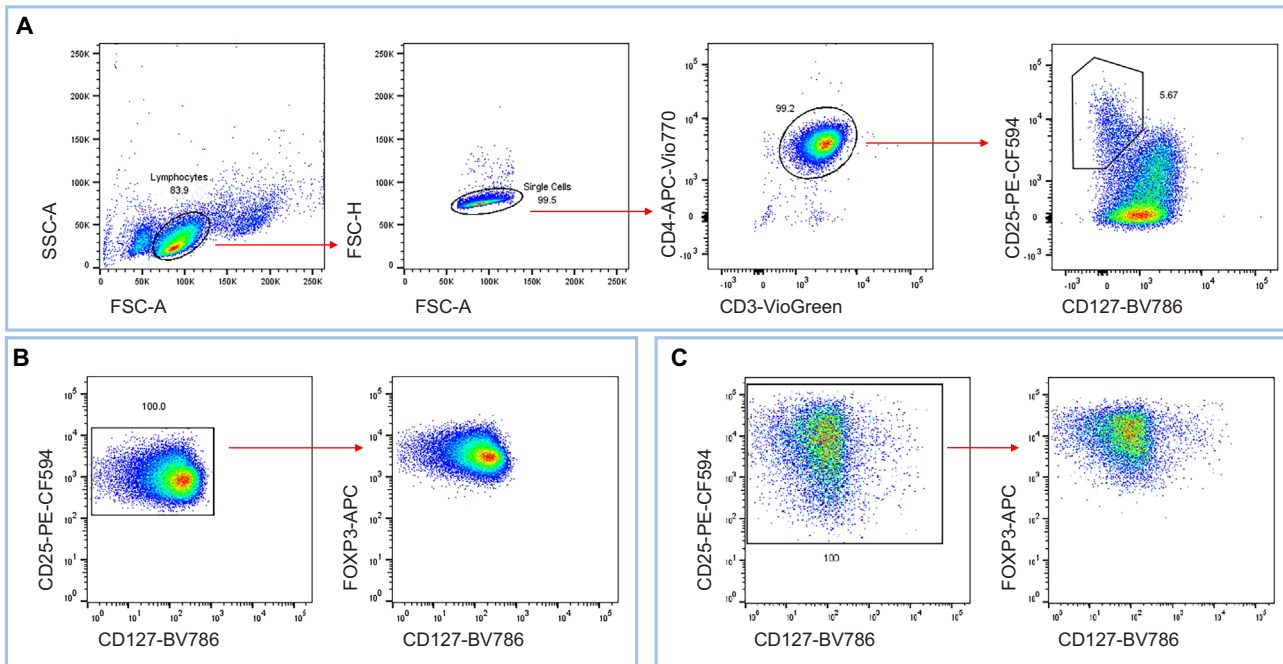


Figure 1. Gating strategy to sort Tregs and FOXP3 expression analysis by day 0- and day 27-Tregs. (A) Gating strategy used to identify and sort Tregs (day 0 of culture). A first gate was set on living lymphocytes through physical parameters (FSC-A vs. SSC-A) and on (FSC-H vs. FSC-A) to eliminate doublets, then on fluorescent parameters on $CD3^+CD4^+$ T cells and $CD127^{low/-}CD25^{hi}$ to identify Tregs (number of separate experiments corresponding to distinct healthy donors $n = 23$). (B) Representative flow cytometry analysis of FOXP3 expression by freshly sorted (day 0-culture) Tregs, same donor as in A. (C) Representative flow cytometry analysis of FOXP3 expression by expanded Tregs (day 27-culture). The FOXP3 expression by day 0- and day 27-expanded Tregs was evaluated from six healthy donors using the same cytometer settings.

(Fig. 3A). Both $CD137^-$ Tregs and $CD137^+$ Tregs, which are those specific for FVIII, expressed similar high levels of FOXP3 (Fig. 3B).

FVIII-specific Tregs are present at low frequency in the peripheral blood of healthy individuals

Altogether, we could identify FVIII-specific Tregs in 10 out of 11 donors, with 43 FVIII-specific Treg cell lines among 197 seeded Treg lines, counting for an average of four specific Treg cell lines from 18 Treg cell lines per donor (Supporting information Table S1). We explain that only a part of the wells gave rise to FVIII-specific Tregs by the low frequency of Tregs circulating in the donors' blood. At low frequency, some wells are seeded with a few FVIII-specific Tregs, while others do not receive any specific Tregs, the cell distribution following a Poisson distribution [26]. By enumerating the FVIII-specific Treg cell lines, we could evaluate the frequency of FVIII-specific Tregs in the blood of HDs. Frequencies ranged from 0.03 to 0.44 cells, with a mean of 0.17 cells per million total $CD4^+$ T cells among the 11 donors tested (Fig. 4), and approximately 3 FVIII-specific Tregs per million of total Tregs (Supporting information Table S1).

Discussion

We reveal in this study the existence of FVIII-specific Tregs and quantify their frequency. Frequencies of FVIII-specific Tregs

ranged from 0.03 to 0.44 cells per million total $CD4^+$ T cells. We estimated the frequency of FVIII-specific Tregs by a new approach relying on the weekly stimulation with rFVIII of $CD3^+CD4^+CD127^{low/-}CD25^{hi}$ Tregs and assessment of their specificity by the upregulation of the CD137 activation marker in the presence of rFVIII. To our knowledge, only one previous study attempted to isolate FVIII-specific Tregs by stimulating $CD4^+CD25^+$ cells with $CD4^+$ T-cell epitopes from the C2 domain followed by tetramer staining [27]. However, they failed to detect any specific Tregs, probably because of their low frequency, which increments the difficulty of their characterization *ex vivo*. Moreover, our approach gives the advantage of full-length protein applications and not single epitopes only.

In agreement with the mechanisms of thymic T-cell selection and differentiation, it is probable that the FVIII-specific Tregs we describe in this study have been committed to the Treg lineage within the thymus and therefore correspond to thymic Tregs [6, 28]. In fact, the Tregs specific for this self-protein did not lose Foxp3 expression in long-term *in vitro* cultures at variance to pTregs and iTregs [7, 9, 10]. Therefore, FVIII appears to be part of the category of endogenous antigens leading to both thymic escape and tTreg differentiation [28]. Although no study reports FVIII expression level in the thymus, the Human Protein Atlas reveals 3.1 FVIII transcripts per million in the thymus, which is associated with a presumably low protein level [29]. This limited thymic expression was shown to lead to partial intrathymic deletion and impaired generation of effector T cells but enhanced Treg generation [28].

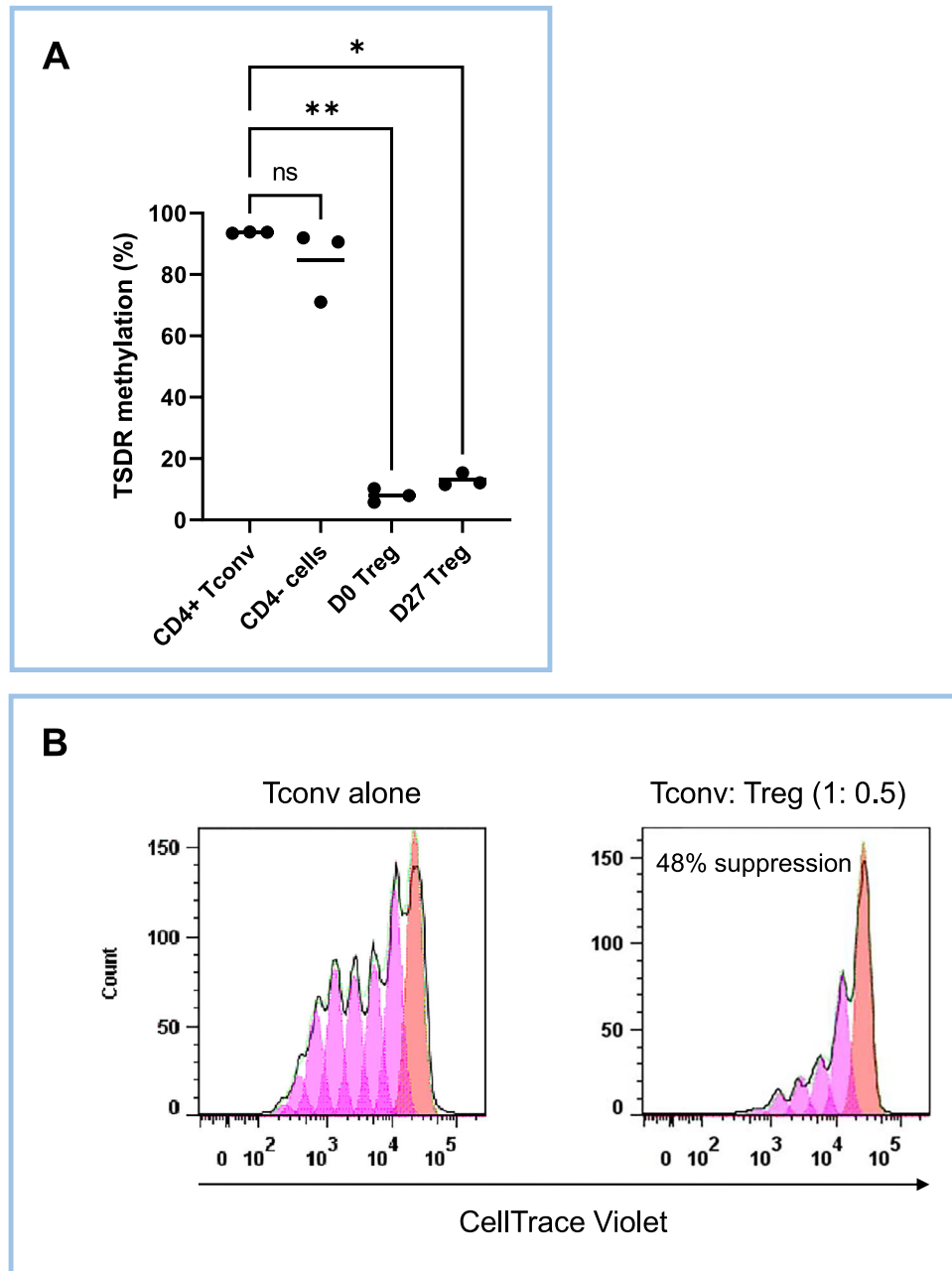


Figure 2. Analysis of the suppressive capacity of flow cytometry-sorted Tregs and FOXP3 TSDR DNA demethylation of day 0- and day 27-Tregs. (A) Analysis of DNA demethylation at the FOXP3 TSDR in Tregs. DNA was extracted from Tregs purified by flow cytometry as described in Figure 1A (D0 Treg) and from cells recovered at the end of the culture (D27 Treg). CD4⁻ cells and CD3⁺CD4⁺CD25⁻CD127⁺ T cells purified by flow cytometry (CD4⁺ Tconv) (the best control) were used as positive DNA methylation control. DNA methylation was evaluated at 10 CpG sites from TSDR of the FOXP3 gene for each cell subset, and results were expressed as a mean value. We analyzed the FOXP3 TSDR DNA methylation of CD4⁺ Tconvs, CD4⁻ cells, day 0 Tregs, and day 27 Tregs from three HD. Scatter plots indicate means. The Kruskal–Wallis test one-way ANOVA was performed to test whether the difference in the percent of FOXP3 TSDR methylation was statistically significant. ns: nonsignificant, **p* < 0.05, ***p* < 0.005. (B) Representative analysis of the suppressive capacity of the purified polyclonal Tregs seeded in culture (*n* = 4). Suppression assays were performed using freshly purified CD3⁺CD4⁺CD25^{hi}CD127^{lo/-} Tregs, autologous DCs, and allogeneic Tconvs as described in Methods. Allogeneic Tconvs were labeled with CellTrace Violet (CTV) dye before stimulation. On day 3, cells were stained by mAbs to CD3, CD4, CD25, and CD127, and flow cytometry analysis was performed by gating on the CTV-labeled cells (Tconv) and excluding Treg autofluorescence signal. The dilution of CTV reveals distinct generations of proliferating cells. Tconv cell proliferation was evaluated using FlowJo's proliferation platform. The orange peak (brightest CTV fluorescence signal) represents the non-proliferating Tconv cells, while the pink peaks represent successive generations of live Tconv cells (CTV dye dilution). The percentage of suppression by Treg was calculated using the formula: % Suppression = 100 - (division index [Treg:Tconv]/division index Tconv alone) × 100.

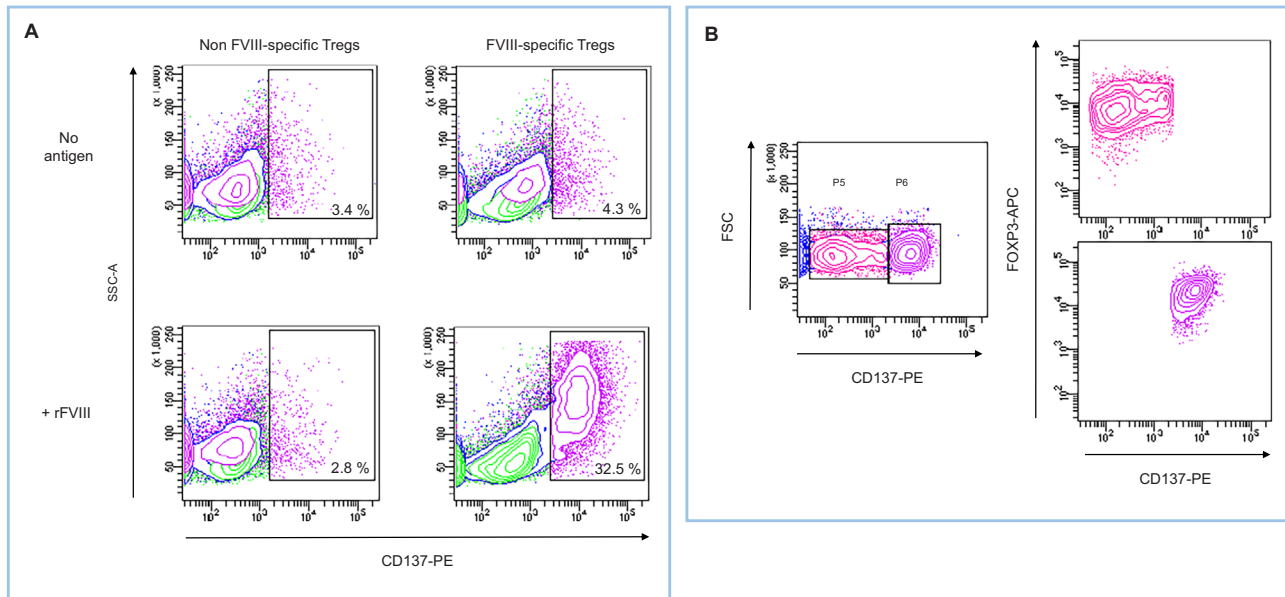


Figure 3. Expression analysis of the CD137 activation marker and FOXP3 by expanded Tregs. (A) Example of CD137 staining on non-FVIII-specific (left panel) and FVIII-specific Treg cell lines (right panel) for one Treg cell line displaying 32.5% of CD137⁺ Tregs, this percentage corresponding to the mean of CD137⁺ Tregs for all the Treg cell lines of the ten donors. Top contour plots correspond to unstimulated Treg cell lines, and bottom contour plots correspond to those stimulated. CD137 expression was analyzed by combining two gates: the first on CD3⁺CD4⁺ T cells and the second on two CD127^{lo/-} Treg subsets, CD25^{lo} (green contour plots) and CD25^{hi} (pink contour plots), respectively. (B) Day 28-analysis of FOXP3 expression on CD137^{lo/-} Tregs (gate P5 pink) and CD137⁺ Tregs (gate P6 purple) on all live CD3⁺CD4⁺CD25⁺CD127⁻ Tregs (n = 2).

In a previous study, we quantified the frequency of FVIII-specific CD4⁺ T cells in HDs and highlighted their escape from negative selection, probably due to the low level of expression of this self-protein in the thymus [22]. Although comparing frequencies calculated in two different studies and hence from distinct donors must be considered with caution, the mean frequency of FVIII-specific Tregs is 10-fold lower than that of FVIII-specific CD4⁺ T cells. Frequencies of FVIII-specific CD4⁺ T cells and Tregs

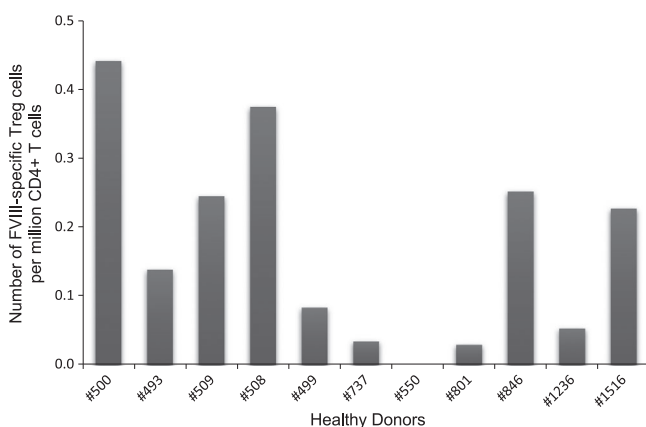


Figure 4. Frequencies of FVIII-specific Tregs in 11 healthy individuals. (A) Frequencies were expressed as the number of FVIII-specific Tregs (i.e. CD137⁺ Tregs) per million CD4⁺ T cells. They were calculated by multiplying the number of FVIII-specific Tregs per million Treg by the percentage of Treg per 1 CD4⁺ T cell. Frequencies of FVIII-specific Tregs per million Treg were calculated using Poisson distribution according to the following formula: Frequency = $-\ln(\text{number of CD137-negative wells}/\text{total number of wells}/\text{number of Tregs per well})$.

are therefore in a similar proportion in their respective T-cell subset, CD4⁺ [22] and CD4⁺CD25^{hi}CD127^{lo/-}. Half of the FVIII-specific CD4⁺ T cells exhibited a memory phenotype, suggesting their recruitment in vivo by the endogenous FVIII under physiological conditions in HDs. However, their frequencies were in the range of the naïve T cells and hence at low frequency for memory cells, suggesting they have been recruited but have poorly proliferated. The discovery of FVIII-specific Tregs in HDs suggests their role in counteracting the FVIII-specific CD4⁺ Tconv cell response. Accordingly, CD4⁺CD25^{hi} T-cell depletion from peripheral blood mononuclear cells (PBMCs) was shown to increase FVIII-specific responses, suggesting their active role in suppressing T-cell response to FVIII, in both healthy donors [23] and hemophilia A patients [24].

While many antigen models have been designed in mice to analyze the generation and function of antigen-specific nTregs, few studies have been published on antigen-specific nTregs in humans [7, 15, 16, 30]. Two of these publications investigated the T-cell subsets sorted with autoantigens tetramers and highlighted the role of nTregs in the control of autoimmune diseases and to account for HLA association with autoimmune diseases [15, 16]. The CD137 activation marker was used to reveal high frequencies of Tregs specific for food and aeroantigens in allergic and healthy donors [7, 8]. Frequencies of nTregs specific for these exogenous antigens were 300–1700 fold higher than FVIII-specific Tregs. This difference might result from the foreign origin of these antigens, as the corresponding nTregs should have differentiated upon T-cell activation in the periphery into memory pTregs. Furthermore, the repeated inhalation or ingestion of these environmental

antigens is expected to maintain Treg survival through frequent activation.

In conclusion, we revealed the existence of human FVIII-specific nTregs circulating in the blood of healthy donors. To date, no studies have been able to evaluate FVIII-specific Tregs in hemophilia A patients, but their possible role in controlling inhibitor onset has been suggested [31]. The remaining cross-reacting material of FVIII resulting from missense mutations or some other gene alterations influences the prevalence of neutralizing antibodies and might influence the differentiation of FVIII-specific Tregs. Further studies in hemophilia A patients should clarify the mechanisms of FVIII peripheral tolerance and the role of FVIII-specific Tregs in reducing rFVIII immunogenicity.

Study limitations and future perspectives

As stated above, it would be interesting to evaluate FVIII-specific Treg numbers generated from various groups of hemophilia A patients, for example, those receiving initial FVIII infusions, multiply infused patients, or patients with inhibitors undergoing immune tolerance induction via intensive FVIII replacement therapy, or patients with different hemophilia A severities. However, the volume of blood samples collected from hemophilia patients might be too limited to recover a sufficient number of cells to derive FVIII-specific Treg cells. Other future perspectives include autoimmune diseases and malignancies.

Materials and Methods

Cell preparation

PBMCs were isolated from buffy coats of adult anonymous HDs who gave informed consent (Etablissement Français du Sang). Monocytes from PBMCs were isolated by positive magnetic selection using CD14 microbeads (Miltenyi Biotec). They were subsequently differentiated into immature DCs in 5-day culture with 1000 IU/mL of human IL-4 and 1000 IU/mL of human GM-CSF (R&D Systems). Immature DCs were differentiated into mature DCs by incubating overnight at 37°C 5% CO₂ with 1 μg/mL LPS (eBioscience) in the presence of the same amounts of IL-4 and GM-CSF. CD4⁺ cells were isolated from thawed CD14⁻ cells using magnetic CD4 microbeads (Miltenyi Biotec).

Treg cell sorting

Purified CD14⁻ cells were stained in PBS1X supplemented with 2% human AB-serum for 20 min at +4°C in the dark using the following anti-human antibodies: CD3-VioGreen (REA613), CD4-APC-Vio770 (M-T466) (Miltenyi Biotec), CD25-PE-CF594 (M-A251), and CD127-BV786 (HIL-7R-M21) (BD Biosciences). CD3⁺CD4⁺CD25^{hi}CD127^{lo/-} Tregs were purified by fluorescence-

activated cell sorting (FACS) using a BD FACSAria III flow cytometer and the FACSDiva software (BD Biosciences).

FOXP3 expression analysis

Tregs were stained using the anti-human FOXP3 staining set APC (eBioscience) containing the buffers and the PCH101 monoclonal antibody (mAb) conjugated to APC necessary to stain and identify human FOXP3⁺ cells. Briefly, we first stained cell surface CD3, CD4, CD25, and CD127 antigens with the monoclonal antibodies used for Treg cell sorting, and then we fixed, permeabilized, and stained intracellular FOXP3 according to the supplier's protocol (eBioscience). We subsequently analyze FOXP3 expression on the BD FACSAria III with the FACSDiva software.

FOXP3 TSDR methylation analysis

Tconvs or CD4⁻ cells and Tregs from male HDs were frozen as soon as purified. Genomic DNA was isolated using the QIAamp DNA Micro or Mini Kit (QIAGEN) depending on cell numbers and bisulfite-treated with the EpiTect Fast 96 DNA Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. The FOXP3 TSDR region was amplified by PCR (forward primer: 5'-TGGGTTAAGTTTGTGTAGGATAGG; reverse primer: 5'-Biotin-TCCCTTTCTAACTAAATTTCTCAAAAAC) in the bisulfite-treated samples and analyzed on a Q24 pyrosequencer (QIAGEN) using PyroMark Q24 Advanced CpG Reagents (QIAGEN), as described previously [32].

Suppression assay

First, allogeneic Tconvs were stained with CellTrace Violet (CTV) dye. Then, CD3⁺CD4⁺CD25^{hi}CD127^{lo/-} Tregs freshly purified with the BD FACSAria III and allogeneic CTV⁺ Tconvs were stimulated separately by immobilized anti-CD3 and anti-CD28 mAbs on day -1. On day 0, Tregs and allogeneic Tconvs (0.5:1 ratio) were seeded on mature DCs autologous to Tregs. On day 3, T cells were stained by mAbs to CD3, CD4, CD25, and CD127, and analysis was performed using the BD FACSAria III. Tconv proliferation was evaluated using FlowJo's proliferation platform (v7.6 software).

Enrichment and culture of FVIII-specific Tregs

FVIII-specific Treg-cell lines were generated by four once-per-week stimulations of approximately 100,000 Tregs per well of a 96-well round-bottom plate with autologous matured DCs (30,000 per well). Mature DCs were obtained through incubation overnight at 37°C with LPS in the presence of IL-4 and GM-CSF, loaded with 0.2 μM recombinant FVIII (Kogenate), and then used as antigen-presenting cells. Cells were cultured in 200 μL per well of CTS OpTimizer T-cell expansion SFM (Gibco, Thermo-Fisher),

supplemented with 2% human AB-serum, Glutamax (Gibco), 100 IU/mL Penicillin, 100 µg/mL Streptomycin, 100 IU/mL human IL-2 (premium grade; Miltenyi Biotec), and 10 ng/mL human IL-15 (R&D Systems). The CTS OpTimizer T-cell expansion SFM is a serum-free, xeno-free medium supporting the growth of human T cells. Medium was replenished every 3–4 days and Tregs were split as needed.

Assessment of Treg cell specificity

Tregs were left to rest for three days in OpTimizer 2% human AB-serum. On day 27, each Treg cell line was stimulated with autologous DCs previously loaded with rFVIII or with unloaded autologous DCs in the presence of 1 µg/mL CD28 functional-grade pure Ab (15E8 clone) (Miltenyi Biotec) in 96-well flat-bottom plates. On day 28, Tregs were stained with the same antibodies used for FACS-sorting, and recombinant human CD137-PE antibody (REA765) (Miltenyi Biotec). Samples were acquired on a BD FACSAria III flow cytometer and analyzed using FACSDiva software.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author Contributions: Catherine Menier designed research, performed research, analyzed data, and wrote the paper; Valeria Porcheddu participated in designing the specific suppression test and correcting the manuscript; Sylvain Meunier, Laurène Romano, and Evelyne Correia performed some experiments; Florence Busato performed the experiments to analyze the methylation of *FOXP3* TSDR; Jorg Tost designed the methodology to analyze the methylation of *FOXP3* TSDR and Bernard Maillère supervised the project and wrote the paper.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: CTV: CellTrace Violet dye · FACS: fluorescence-activated cell sorting · HA: hemophilia A · HDs: healthy donors · nTregs: natural regulatory T cells · PBMCs: Peripheral blood mononuclear cells · pTregs: peripheral-generated Tregs · rFVIII: recombinant factor VIII · Tconvs: conventional CD4⁺ T cells · TCR: T-cell receptor · Tregs: regulatory T cells · TSDR: Treg-specific demethylated region · tTregs: thymus-derived Tregs

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