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ARTICLE

High proportion of terminally differentiated regulatory T cells after allogeneic hematopoietic stem cell transplantation

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Abstract

It is now well-established that regulatory T cells (Treg) represent a heterogeneous group of $CD4^+$ T cells. Previous studies have demonstrated that Treg homeostasis was impacted by allogeneic hematopoietic cell transplantation (allo-HCT) and particularly so in patients with chronic graft-versus-host disease (GVHD). Here, we first assessed the ability of various Treg subsets to phosphorylate STAT5 in response to IL-2 or IL-7 stimulation in vitro. We then compared the frequencies of different Treg subtypes in healthy controls as well as in allo-HCT patients with or without chronic GVHD. The highest phosphorylated STAT5 (pSTAT5) signal in response to IL-2 was observed in the $CD45RO^+CD26^-CD39^+HLA\text{-}DR^+$ Treg fraction. In contrast, naive Treg were mostly less susceptible to IL-2 stimulation in vitro. Following IL-7 stimulation, most Treg subpopulations upregulated pSTAT5 expression but to a lesser extent than conventional T cells. Compared to healthy controls, allo-HCT patients had lower frequencies of the naive $CD45RA^{bright}CD26^+$ Treg subpopulation but higher frequencies of the most differentiated memory $CD45RO^+CD26^-CD39^+$ Treg subpopulations. Further, unbiased analysis revealed that six Treg clusters characterized by high expression of CD25, HLA-DR, and ICOS were significantly more frequent in patients with no or with limited chronic GVHD than in those with moderate/severe chronic GVHD.

Introduction

Allogeneic hematopoietic cell transplantation (allo-HCT) is frequently used as treatment of hematological malignancies [1, 2]. Its efficacy is based on a large part on graft-versus-tumor (GVT) effects mediated mainly by donor mature T cells contained in the graft [3–5]. Besides mediating GVT effects, donor immune cells can also target healthy recipient tissues, causing graft-versus-host disease (GVHD) [6]. GVHD includes two syndromes: acute GVHD, a deregulated inflammatory response causing skin, gastro-intestinal tract and/or liver damages, and a less inflammatory syndrome termed chronic GVHD, which share many clinical manifestations with auto-immune diseases and can evolve to tissue fibrosis [7–9]. Criteria for chronic GVHD definition and grading have been updated in 2014 and segregate patients between mild versus moderate/severe chronic GVHD who most often require systemic immunosuppressive treatments [10]. While chronic GVHD has a profound negative impact on the quality of life of affected patients, its occurrence has been associated with GVT effects and a lower incidence of relapse of hematological malignancies [4].

Treg (defined as $CD4^+CD25^+CD127^{dim}FOXP3^+$ T cells) are necessary for maintaining tolerance to self-antigens [11, 12] and play a crucial role for GVHD prevention/attenuation after allo-HCT [13–15]. Treg constitutively express the high-affinity α-subunit within αβγ form of IL-2 receptor CD25 and are mainly dependent on IL-2 for their homeostasis [16–18]. Specifically, IL-2 promotes Treg survival through regulation of antiapoptotic molecules such as MCL-1 and BCL-2 [17, 19]. Since Treg cannot produce IL-2, they are in need for IL-2 secreted by activated effector T cells for their homeostasis [17].

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Consequently, high concentration of IL-2 induces Treg proliferation during niche filling or when recombinant human IL-2 is administered [19]. In phase II study, administration of low doses of IL-2 in patients with advanced chronic GVHD led to meaningful clinical improvement in ~60% of them [20], although no complete remission (CR) was seen.

Despite Treg are characterized by a low expression of the IL-7 receptor CD127, several reports have suggested an IL-7 requirement for Treg homeostasis and function [21, 22]. This is relevant in the allo-HCT setting since it has been observed that patients suffering from chronic GVHD have a relative IL-2 functional deficiency together with elevated amounts of IL-7 (and IL-15), while high levels of IL-7 are alo seen early after transplantation [23–25].

Over the last decade it became evident that Treg represent a heterogeneous group of cells [17, 26–28]. First, Miyara et al. subdivided Treg into three populations based on their expression of CD45RA and the intensity of FOXP3 expression [26]. More recently, Hua et al. observed that Treg could be separated in 16 different subsets assessable by flow cytometry, which could then be classified in five distinctive genomic subgroups [29]. In addition, a recent paper demonstrated that Treg could also be subdivided into five stages of maturation based on their cell surface expression of CD45RA, CD26, and CD39 [30]. Specifically, naive Treg were defined as $CD45RA^{+}CD26^{+}CD39^{-}$ while memory Treg were divided into four major maturation subtypes: M1: $RA^{-}CD26^{+}CD39^{-}$, M2: $RA\text{-}CD26^{-}CD39^{-}$, M3: $RA^{-}CD26^{+}CD39^{+}$, and M4: $RA^{-}CD26^{-}CD39^{+}$.

Here, we aimed at assessing the impact of allo-HCT and of chronic GVHD on the proportion of various Treg subsets. Given the importance of IL-2 (and to a much lesser extent IL-7) in Treg homeostasis, we first assessed the impact of IL-2 and IL-7 on Treg subset activation in vitro in order to determine whether changes in Treg subsets observed after allo-HCT could be due to a relative lack of IL-2. We hypothesized that in a situation of a strong competition for available IL-2 as after allo-HCT, the Treg subsets the least able to activate pSTAT5 in response to IL-2 in vitro would be the most impacted. We then compared Treg subsets in healthy donors ($n = 27$) and in 70 patients after allo-HCT experiencing ($n = 31$) or not ($n = 39$) moderate/severe chronic GVHD. Finally, we assessed the impact of low-dose IL-2 administration on Treg subsets in a patient given low-dose IL-2 as treatment for severe refractory chronic GVHD.

Patients and methods

Impact of IL-2 and IL-7 on Treg subsets

Blood (30 ml) was collected from healthy volunteers, and peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation (GE Healthcare, Upsala, Sweden) and then resuspended in staining buffer PBS + 3% FBS (Lonza, Verviers, Belgium). Cell numbers were determined with an ABX diagnostics—Micros 60 (AxonLab, Baden, Switzerland), and $CD3^{+}$ T cells were isolated by immunomagnetic negative selection (EasySep™ Human T Cell Isolation Kit, Stemcell Technologies, Vancouver, Canada). T cells were stained with the extracellular antibodies (see below) and stimulated 15 min with different concentrations (0.01–0.1–1–10–100 ng/ml) of recombinant human IL-2 or IL-7 (PeproTech, Rocky Hill, New Jersey). T cells were then permeabilized with the PerFix EXPOSE (PFE, Beckman Coulter, Brea, California) method and stained for intracellular antigens. The following panel was used for the staining: CD4-BV786 (SK3, Becton Dickinson (BD), Franklin Lakes, New Jersey), CD127- APC R700 (HIL-7R-M21, BD), CD25-BUV395 (2A3, BD), CD45RA-BV510 (HI100, BD), CD45RO-BV650 (UCHL1, BD), CD196 (CCR6)-Biotin, Streptavidin-APC-eFluor780 (Thermo Fisher, Waltham, Massachusetts), CD183 (CXCR3)-PeCy5 (1C6, BD), CD39-PeCy7 (A1, Sony), CD26-PECF594 (M-A261, BD), skin-homing marker cutaneous lymphocyte Ag (CLA)-BV421 (HECA-452, BD), CD278 (ICOS)-BV711 (DX29, BD), HLA-DR- BV605 (L243, Sony Biotechnology, San Jose, California), FOXP3-AF488 (259D, Biolegend,, San Diego, California), STAT5-AF647 (pY694, BD). Data were acquired on a FACS LSRFortessa (BD) and were analyzed with FlowJo v10.6.1 (BD).

Assessment of Treg subsets in healthy controls as well as patients with or without moderate/severe chronic GVHD

Blood samples were collected in 27 healthy controls and 70 allo-HCT recipients (cross sectional study of allo-HCT recipients without progressive disease and beyond 100 days after transplantation). Allo-HCT recipients were subdivided in two groups according to the absence ($n = 39$) or presence ($n = 31$) of moderate/severe chronic GVHD according to the 2014 NIH classification [10]. Median age of healthy controls was 46 years (25th percentile 27 years and 75th percentile 54 years). Among patients classified in the no/mild GVHD group, two had prior moderate/severe chronic GVHD. One of them had eye, non-sclerotic skin and mouth chronic GVHD starting 217 days after transplantation that was treated by topical medications. At the time of the sample analysis, the patient was on day 1473 after transplantation with sequel of eye chronic GVHD, without anti-GVHD medication. The second patient was diagnosed with severe chronic GVHD affecting the skin (non-sclerotic) and the liver. She was treated by corticosteroids that were discontinued on day 620. At the time of the analysis, the patient was on day 1089 without chronic GVHD and without systemic immunosuppression. Thirty of 31 patients in the moderate/severe

chronic GVHD group were on systemic immunosuppression at the time of the analysis. This include 18 patients with steroids with ($n = 16$; steroids + mTor inhibitor ($n = 1$), steroids + mTor inhibitor + photophoresis ($n = 2$), steroids + photophoresis ($n = 2$), steroids +tacrolimus ($n = 3$), steroids + mycophenolate mofetil ($n = 1$), steroids + mycophenolate mofetil + tacrolimus + photophoresis ($n = 1$); steroids plus mycophenolate mofetil plus photophoresis ($n = 2$); steroids + ruxolutinib ($n = 1$); steroids plus ruxolutinib plus photophoresis ($n = 1$), steroids plus cyclophosphamide ($n = 1$), and steroids plus tacrolimus plus photophoresis ($n = 1$)) or without ($n = 2$) other systemic medication, 7 with mTOR inhibitors (combined with photophoresis in 2 patients), 2 with mycophenolate mofetil, 2 with tacrolimus, and 1 with ruxolutinib plus photophoresis. The remaining patient had moderate chronic GVHD and was started on steroids 1 week after the analysis of his blood sample. T cells were isolated as described above with Ficoll density gradient centrifugation and immunomagnetic negative selection with the EasySep™ Human T Cell Isolation Kit. For flow cytometry analyses, T cells (2×10^6/sample) were incubated with surface antibodies for 20 min at 4 °C in the dark then washed with staining buffer and the same procedure was used to stain cells with streptavidin for 15 min. For intracellular staining, cells were fixed and permeabilized with FOXP3 Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples were stained with two panels of fifteen colors with the following antibodies: CD4-BV786 (SK3, BD), CD127-APC-eFluor® 780 (eBioRDR5, Thermo Fisher), CD25-BUV395 (2A3, BD), CD45RA-BV510 (HI100, BD), CD45RO-PeCy5 (UCHL1, Thermo Fisher), CD196 (CCR6)-Biotin, Streptavidin-BV650 (Sony), CD183 (CXCR3)-APC (G025H7, Biolegend), CD39-PerCP-eFluor® 710 (eBioA1, Thermo Fisher), CD26-PECF594 (M-A261, BD), CLA-BV421 (HECA-452, BD), CD278 (ICOS)-BV711 (DX29, BD), HLA-DR- BV605 (L243, Sony), FOXP3-PE-Cy7 (236A/E7, Thermo Fisher); for panel 1, BCL-2-PE (BD) and Ki67-FITC (BD); for panel 2, CD120b (TNFR2)-PE (BD) and HELIOS-AF488 (22F6, Biolegend). Absolute numbers were obtained with staining made directly on 50 µl of blood. After red blood cell lysis (Thermo Fisher), cells were washed with staining buffer and stained with CD45-PECy7 (HI30, Thermo Fisher), CD3-V500 (SP34-2, BD), and CD4-APC (RPA-T4, Thermo Fisher). Absolute $CD4^+$ and Treg numbers were calculated by multiplying absolute lymphocyte counts by the percentages of $CD4^+$ T cells and Treg, respectively. Data were acquired on a FACS LSRFortessa (BD) and were analyzed with FlowJo v10.6.1 (BD).

Gating strategy and definition of Treg subsets

In all experiments, Treg were defined as $CD4^+CD25^+CD127^{dim}FOXP3^+$ cells among the $CD4^+$ T cells. The subsets were defined based on the paper by Hua et al. [29] (Supplementary Fig. 1), on the differentiation classification by Schiavon et al. [30], and by using 2 FlowJo v10.6.1 plugins: t-distributed stochastic neighbor embedding (t-SNE). Data from a subset of patients with or without moderate/severe chronic GVHD were also analyzed with the Citrus algorithm. This software performs hierarchical clustering based on the expression of different markers and generates a tree map that groups phenotypically similar cellular subsets (Citrus V0.8, code available through GitHub (https://github.com/nolanlab/citrus)), as previously reported [31]. The analysis was made on 65,357 cells with a minimal cluster size of 5% of the total cell number. For the nodes created, Citrus calculated a score of abundance related to the percentage of cells associated with each group of individuals. Correlative statistical model (SAM) was used to detect associations between calculated cluster properties and an experimental endpoint, in this case a difference of frequency between allo-HCT recipients with no or limited chronic GVHD versus those with moderate/severe chronic GVHD.

Treatment of a patient with chronic GVHD with low-dose IL-2

A 39-year-old patient with steroid-refractory severe chronic GVHD was included in a prospective study assessing sirolimus, donor Treg infusion, and low-dose IL-2 administration (clinicaltrials.gov # NCT01903473; https://www.tregeneration.eu/). The patient was started on sirolimus but could not receive the Treg product because the purity of the product did not meet the release criteria. He was, however, treated with low dose of IL-2 (Proleukin®, 1 million IU/day, subcutaneous injections) according to the protocol. Blood samples were collected the day of the injection, as well as on days 7, 14, and 30 after IL-2 initiation. The same antibodies panel used for in vitro IL-2 and IL-7 stimulation was used here (see above).

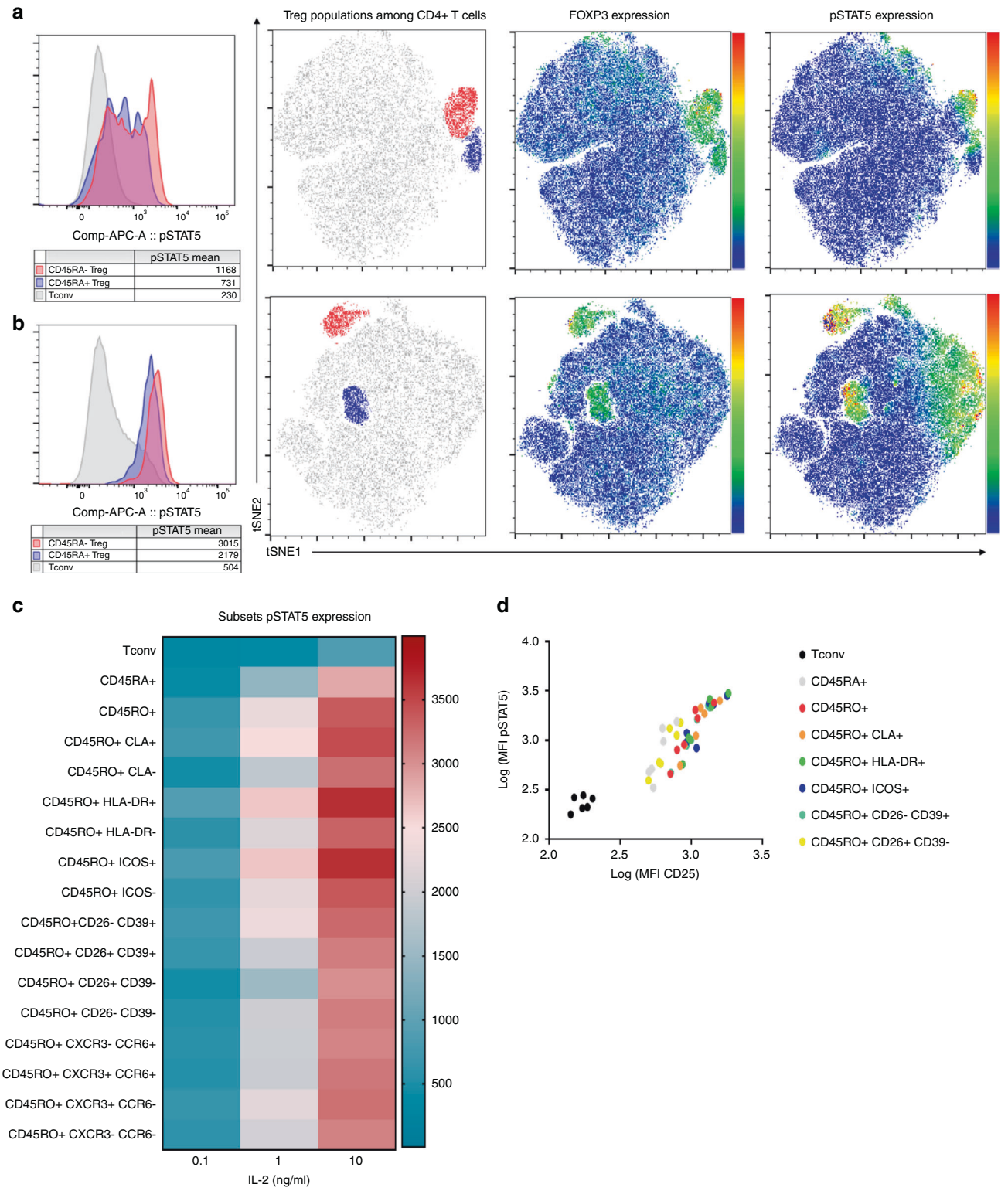
Statistical analyses

All statistical analyses were performed with GraphPad.Prism (Graphpad Software, San Diego, California). The Kruskal–Wallis and Dunn's multiple comparison tests were used to compare data for the different groups or different subsets. Analyses of potential correlations were made with the Spearman test. Heatmaps were created based on the mean intensity fluorescence (MFI). P values < 0.05 were defined as statistically significant. All P values were two-sided.

Results

Response of Treg subtypes to IL-2 and IL-7

We first assessed the ability of IL-2 and IL-7 to phosphorylate STAT5 in various Treg subtypes as well as in $CD4^+$



conventional T cells (Tconv) isolated from the peripheral blood of six healthy volunteer donors. Indeed, it is well-established that IL-2 and IL-7 both induce phosphorylation of JAK1 and JAK3, leading preferentially to STAT5 phosphorylation [32, 33]. We observed that IL-2 at a dose of 1-ng/mL phosphorylated STAT5 nearly exclusively in Treg (Supplementary Fig. 2 and Fig. 1a). Interestingly, however, only a fraction of naive Treg responded to these low dose of

◀ **Fig. 1 Impact of IL-2 on Treg subests in vitro.** Human T cells from six healthy donors were isolated by ficoll density gradient centrifugation followed by an immunomagnetic negative selection of CD3+ T cells. Cells were stained with extracellular antibodies and stimulated 15 min at 37 °C with different IL-2 concentrations, then washed and stained for intracellular antigens. t-SNE showing FOXP3 and pSTAT5 expression (Geometric mean) in naive (blue) and memory (red) Treg as well as in CD4 + Tconv (gray) after stimulation with 1 ng/mL (**a**) or 10 ng/mL (**b**) of IL-2. The t-SNE was created with 5000 $CD4^+$ cells/donor and includes CD25, CD127, FOXP3, CD45RA, and CD45RO (to determine naïve and memory Treg populations) and pSTAT5 markers among $CD4^+$ T cells. The population of Tconv positive for pSTAT5 signaling in **b** corresponds to activated Tconv ($CD45RO^+CD127^{bright}CD25^{medium}CD26^+CXCR3^+CCR6^+$). **c** Heatmap of pSTAT5 expression (MFI) in Treg subpopulations (as defined by Hua et al. [29]) according to the dose of IL-2. **d** Correlation between pSTAT5 expression and CD25 expression by Treg subpopulations after stimulation with 1 ng/mL of IL-2. (Color figure online).

IL-2. At 10 ng/mL of IL-2 (Fig. 1a), pSTAT5 was mainly increased in Treg while a small fraction of $CD4^+$ Tconv (with an activated phenotype: $CD45RO^+CD127^{bright}CD25^{medium}CD26^+CXCR3^+CCR6^+$) also upregulated pSTAT5 (Fig. 1b). The highest Treg/Tconv ratio of pSTAT5 MFI was observed with 1 ng/mL of IL-2 (Supplementary Fig. 3). As expected, IL-7 induced higher pSTAT5 levels in Tconv than in Treg at each concentration assessed (Supplementary Fig. 4).

We then looked at the susceptibility of various Treg subsets to IL-2. Using the classification of Hua et al. [29], we observed that the highest pSTAT5 signals in response to IL-2 were observed in the most activated/differentiated Treg fractions such as $CD45RO^+CLA^+$, $CD45RO^+HLA\text{-}DR^+$, $CD45RO^+ICOS^+$, and M4 $CD45RO^+CD26^-CD39^+$ Treg subpopulations (Fig. 1c and Supplementary Fig. 4). In contrast, as mentioned above, naive ($CD45RA^+$) Treg were generally less susceptible to IL-2 in term of STAT5 phosphorylation. The activated/differentiated Treg subpopulations had also the highest expression of both CD25 and FOXP3. Accordingly, there was a correlation between the expression of pSTAT5 after stimulation with 1 ng/mL and CD25 expression by Treg subsets (Spearman $R = 0.8634$, $P < 0.0001$) (Fig. 1d).

We further subclassified Treg following their expression of CD25, FOXP3, CD45RA, CD45RO, CD26, CD39, CLA, and HLA-DR using t-SNE analyses (Fig. 2a). We first observed that there was hotspots of pSTAT5 expression following stimulation with IL-2 in naïve, M1, M2, and M4 Treg maturation stages (Fig. 2b). We also observed two subpopulations of naïve Treg: one $CD45RA^{bright}CD26^+$ somewhat responding to IL-2 (pSTAT5 MFI = 1037) and another $CD45RA^{dim}CD45RO^{neg}CD26^+$ not stimulated by IL-2 (pSTAT5 MFI = 433). In contrast, the subset of RO^+CD39^+ Treg (M4) that further expresses HLA-DR and CLA had the highest expression of pSTAT5 following IL-2 activation (Fig. 2c).

Following IL-7 stimulation, most Treg subpopulations upregulated pSTAT5 expression but to a lesser extent than conventional T cells (Supplementary Fig. 5).

Impact of allo-HCT on Treg subsets and maturation stages

Samples from 70 allo-HCT recipients without ($n = 39$) or with ($n = 31$) moderate/severe chronic GVHD were analyzed. Median age at transplantation was 56 versus 57 years, respectively ($P = 0.78$) (Table 1). Median time from transplantation to sample analysis was 746 versus 1059 days in patients without or with moderate/severe chronic GVHD, respectively ($P = 0.21$). Among GVHD patients, 23 had severe chronic GVHD and 8 moderate chronic GVHD. Among the remaining patients, 6 had mild chronic GVHD, 2 had resolved chronic GVHD, and the remaining 31 patients had no current as well as no history of chronic GVHD.

We first compared the absolute numbers of $CD4^+$ T cells and of Treg in the three groups of patients along with healthy controls. We observed that absolute $CD4^+$ T-cell numbers were significantly higher in healthy controls (median 1058 (25th percentile 826 and 75th percentile 1361) $+/- \times 10^3$ cells/mL) than in patients without moderate/severe chronic GVHD (median 473 (25th percentile 134 and 75th percentile 611) $\times 10^3$ cells/mL) ($P < 0.001$), while patients with moderate/severe chronic GVHD had the lowest $CD4^+$ T-cell counts (median 262 (25th percentile 64 and 75th percentile 609) $\times 10^3$ cells/mL) ($P < 0.001$ compared to healthy controls and $P = 0.25$ compared to patients without GVHD). Similarly, absolute Treg counts were significantly higher in healthy controls (median 71 (25th percentile 49 and 75th percentile 84) $\times 10^3$ cells/mL) than in patients without moderate/severe chronic GVHD (median 24 (25th percentile 8 and 75th percentile 46) $\times 10^3$ cells/mL) ($P < 0.001$), while patients with moderate/severe chronic GVHD had the lowest Treg counts (median 16 (25th percentile 2 and 75th percentile 33) $\times 10^3$ cells/mL) ($P < 0.001$ compared to healthy controls and $P = 0.049$ compared to patients without GVHD) (Supplementary Fig. 6).

Using the Treg classification by Hua et al. [29], we observed that compared to healthy controls, allo-HCT patients had a lower proportion of naive (RA^+) Treg but a higher proportion of terminaly differentiated M4 ($CD45RO^+CD26^-CD39^+$) Tregs (Fig. 3a). Among the $CD45RO^+$ subpopulations of Treg, we observed that allo-HCT patients (either with or without chronic GVHD) had higher frequencies of $CD45RO^+CLA^+$, $CD45RO^+HLA\text{-}DR^+$, and $CD45RO^+ICOS^+$ Treg subpopulations. Interestingly, there was no statistically significant difference in the frequency of the different Treg subtypes between patients with or without chronic GVHD.

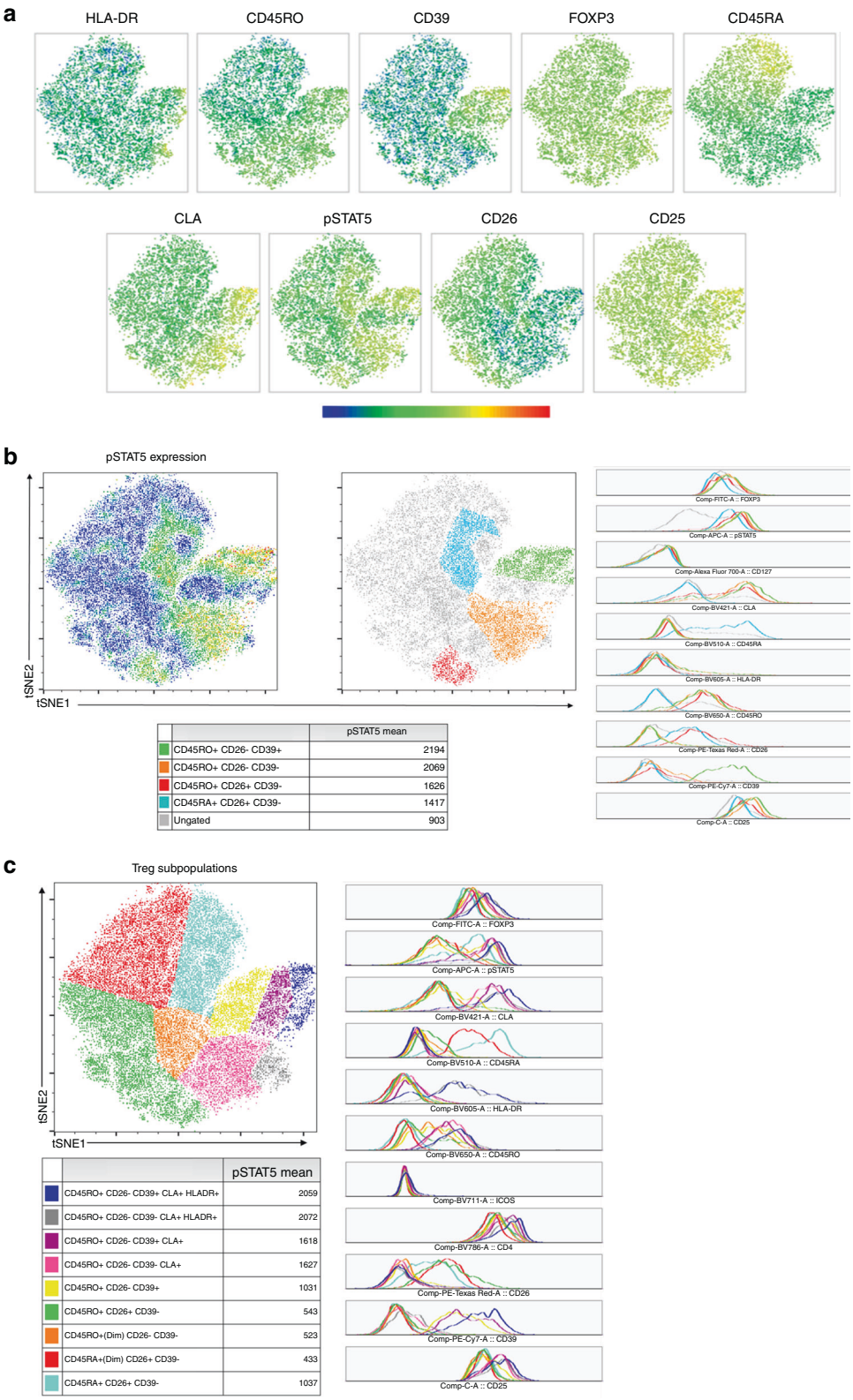


Fig. 2 Impact of IL-2 on Treg subsets in vitro using T-SNE analyses. Human T cells from six healthy donors were isolated by ficoll density gradient centrifugation followed by an immunomagnetic negative selection of $CD3^+$ T cells. Cells were stained with extracellular antibodies and stimulated 15 min at 37 °C with different IL-2 or IL-7 concentrations, then washed and stained for intracellular antigens. T-SNE was created on 15349 Treg (total Treg number from the 6 donors) and includes FOXP3, CD25, CD45RA, CD45RO, CD26, CD39, CLA, HLA-DR and pSTAT5 markers. **a** Visualization of marker expression (MFI) among Treg cells after stimulation with 1 ng/ml. **b** Four Treg populations were determined based on pSTAT5 expression (geometric mean), Treg CD45RA + CD26 + CD39− (blue, pSTAT5 mean = 1417), Treg M1 CD45RO + CD26 + CD39− (red, pSTAT5 mean = 1626), Treg M2 CD45RO + CD26 − CD39− (orange, pSTAT5 mean = 2069), Treg M4 CD45RO + CD26 − CD39+ (green, pSTAT5 mean = 2194). Histogram show subpopulations marker expression. **C** Treg cells were manually divided into subpopulations based on the marker expression (MFI). PSTAT5 of each subject is shown. (Color figure online).

We next compared the expression of FOXP3, CD25, KI67, BCL-2, and TNFR2 between Treg subtypes (classified using the strategy of Hua et al. [29]) and Tconv (Fig. 3b). As expected, Tconv did not express FOXP3, CD25, and TNFR2. They also had a higher expression of BCL-2 than Treg. The highest KI67 expression was observed among the $CD45RO^+$

Table 1 Patient charateristics.

	Allo-HCT		*P* value
	No/mild cGVHD ($n = 39$)	Moderate/severe cGVHD ($n = 31$)	
Age (yrs); median (p25, p75)	56 (45–75)	57 (37–67)	$P = 0.78$
Sex (# males/# females)	17/22	17/14	$P = 0.47$
Donor type (# MSD/MUD/MMUD/ Haplo)	7/23/7/2	6/19 / 6 / 0	$P = 0.54$
Conditioning intensity (# MAC/RIC/ Sequential)	14/23/2	9/21/1	$P = 0.70$
ATG (# yes/no)	20/19	12/19	$P = 0.29$
Day after allo-HCT; median (p25, p75)	746 (371–1642)	1059 (490–2073)	$P = 0.22$
cGVHD			
None/mild	33/6	0	
Moderate/severe	0[a]	8/23	

[a]Two patients had resolved moderate/severe chronic GVHD

cGVHD chronic graft-versus-host disease (GVHD), *MSD* HLA-identical sibling donor, *MUD* HLA-matched unrelated donor, *MMUD* HLA-mismatched unrelated donor, *Haplo* HLA-haploidentical transplantation, *MAC* myeloablative conditioning regimen, *RIC* reduced-intensity conditioning regimen (either fludarabine + 8 mg/kg busulfan + ATG or fludarabine + 140 mg/m2 melphalan + ATG or fludarabine + 2 Gy total body irradiation), *Sequential* sequential conditioning combining clofarabine + ara-C followed by cycplophosphamide + 4 Gy TBI.

ICOS$^+$ Treg subpopulation while the highest TNFR2 expression was found in CD45RO$^+$ICOS$^+$ and CD45RO$^+$HLA-DR$^+$ Treg subpopulations. Interestingly, allo-HCT patients had a lower KI67 expression in two Treg subpopulations (CD45RO$^+$ HLA-DR$^+$ and CD45RO$^+$ICOS$^+$ Treg) than healthy controls (Supplementary Fig. 7).

We next performed a t-SNE analysis using CD25, FOXP3, CD45RA, CD45RO, CD26, CD39, CLA, HLA-DR, and ICOS markers (Fig. 4). These analyses confirmed that, compared to controls, allo-HCT patients had lower frequencies of the CD45RA^{bright}CD26$^+$ naive Treg subpopulation but an increase in the terminally differentiated M4 (CD45RO$^+$CD26$^-$CD39$^+$ Treg) subpopulations. Among the M4 subpopulations, in comparison to healthy controls, allo-HCT patients had significantly higher proportions of CD45RO$^+$CD26$^-$CD39$^+$HLA-DR$^+$ ICOS$^+$, CD45RO$^+$CD26$^-$CD39$^+$CLA$^+$HLA-DR$^+$ICOS$^+$, and CD45RO$^+$CD26$^-$CD39$^+$CLA$^+$ Treg subpopulations. This indicates that allo-HCT patients had accumulated more terminally differentiated Treg than controls. In addition, these Treg had higher expression of activation markers such as HLA-DR and ICOS.

Impact of chronic GVHD on Treg subsets and Treg maturation stages

To further dissect whether chronic GVHD impacted Treg subtypes we compared the expression of the different markers between patients with or without moderate/severe chronic GVHD using Citrus. Unbiased Citrus analyses revealed that six Treg clusters characterized by a high expression of CD25, HLA-DR, and of ICOS (and by a high FOXP3 expression for four of the six clusters) were significantly more frequent in patients without or with limited chronic GVHD than in those with moderate/severe chronic GVHD (Fig. 5 and Supplementary Fig. 8).

Impact of low-dose IL-2 on Treg subtypes after allo-HCT

We finally assessed the impact of the administration of low-dose IL-2 (one million units s.c. per day) on Treg subtypes in a patient with chronic GVHD. This 39-year-old patient had severe chronic GVHD involving the skin, the mouth, the lung, and the eyes. At the time of IL-2 initiation (day 0), the patient was on prednisolone 8 mg/day, sirolimus, extracorporeal photopheresis, and fluticasone, azithromycine plus montelukast (FAM). At day 0 (before start of IL-2), the patient had low Treg levels (Fig. 6). Further, Treg had mainly a CD45RO$^+$CLA$^+$ or CD45RO$^+$CD39$^+$ phenotype with or without additional HLA-DR expression with very few naive Treg. Interestingly, low-dose IL-2 administration resulted in a dramatic Treg increase (in term of Treg frequencies among total CD4$^+$ T cells) and a major change in Treg phenotype with increasing proportions of naive CD45RA + Treg and of activated M1 (CD45RO + CD26 + HLA-DR +) Treg. This suggested that in this patient low-dose IL-2 administration resulted in higher Treg levels in a large part through neogeneration of Treg by the thymus.

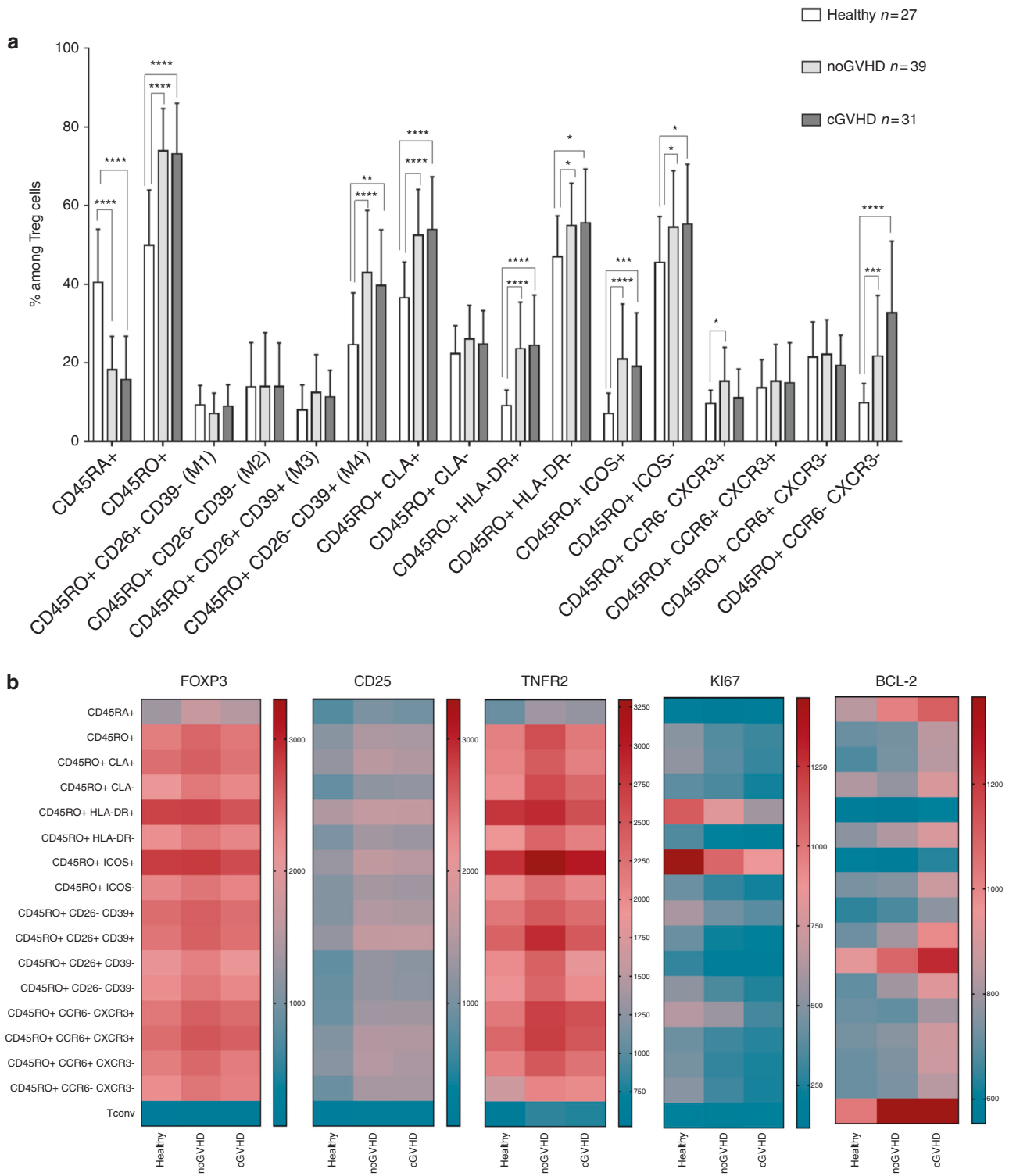
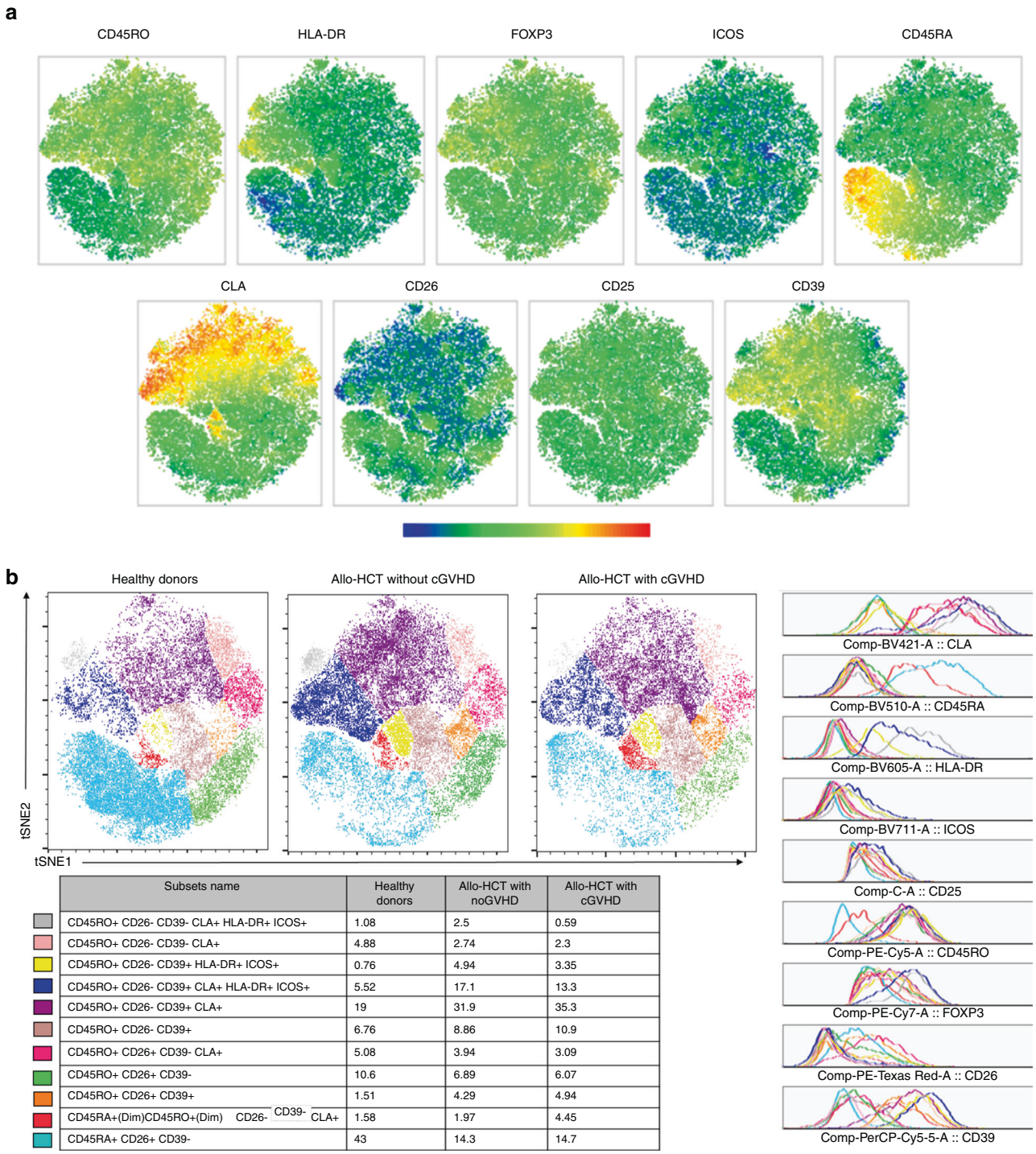


Fig. 3 Treg subsets in healthy controls and in patients with or without moderate/severe chronic GVHD. Human T cells from all participants were isolated by ficoll density gradient centrifugation followed by an immunomagnetic negative selection of CD3+ T cells. Cells were stained with a 15-color panel to determine Treg subsets as classified by Hua et al. **a** Frequencies of Treg subsets (as classified by Hua et al. [29]) in healthy controls ($n = 27$), and in allo-HCT patients with ($n = 31$) or without ($n = 39$) moderate/severe chronic GVHD. Data show median values with interquartile range (one-way ANOVA, $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$). **b** Heatmap of FOXP3, CD25, TNFR2, KI67, and BCL-2 expression (MFI) among CD4 + Tconv as well as Treg subsets (as classified by Hua et al. [29]) in healthy controls ($n = 27$), and in allo-HCT patients with ($n = 31$) or without ($n = 39$) moderate/severe chronic GVHD.



Subsets name	Healthy donors	Allo-HCT with noGVHD	Allo-HCT with cGVHD
CD45RO+ CD26- CD39- CLA+ HLA-DR+ ICOS+	1.08	2.5	0.59
CD45RO+ CD26- CD39- CLA+	4.88	2.74	2.3
CD45RO+ CD26- CD39+ HLA-DR+ ICOS+	0.76	4.94	3.35
CD45RO+ CD26- CD39+ CLA+ HLA-DR+ ICOS+	5.52	17.1	13.3
CD45RO+ CD26- CD39+ CLA+	19	31.9	35.3
CD45RO+ CD26- CD39+	6.76	8.86	10.9
CD45RO+ CD26+ CD39- CLA+	5.08	3.94	3.09
CD45RO+ CD26+ CD39-	10.6	6.89	6.07
CD45RO+ CD26+ CD39+	1.51	4.29	4.94
CD45RA+(Dim)CD45RO+(Dim) CD26- CD39- CLA+	1.58	1.97	4.45
CD45RA+ CD26+ CD39-	43	14.3	14.7

Fig. 4 T-SNE of Treg subpopulations in healthy controls ($n = 20$) and in allo-HCT patients without ($n = 20$) or with ($n = 15$) moderate/severe chronic GVHD. Tregs (1000/sample) from the three groups were concatenated together (55,000 Tregs) to create the t-SNE and includes FOXP3, CD25, CD45RA, CD45RO, CD26, CD39, CLA, HLA-DR, and ICOS markers. **a** Visualization of marker expression (MFI) among Treg cells. **b** Treg cells from allo-HTC patients with ($n = 31$) or without ($n = 39$) cGVHD and healthy controls ($n = 20$) were manually divided into subpopulations based on marker expression (MFI). Histogram show subpopulations marker expression. Table shows frequencies of Treg subsets in each group.

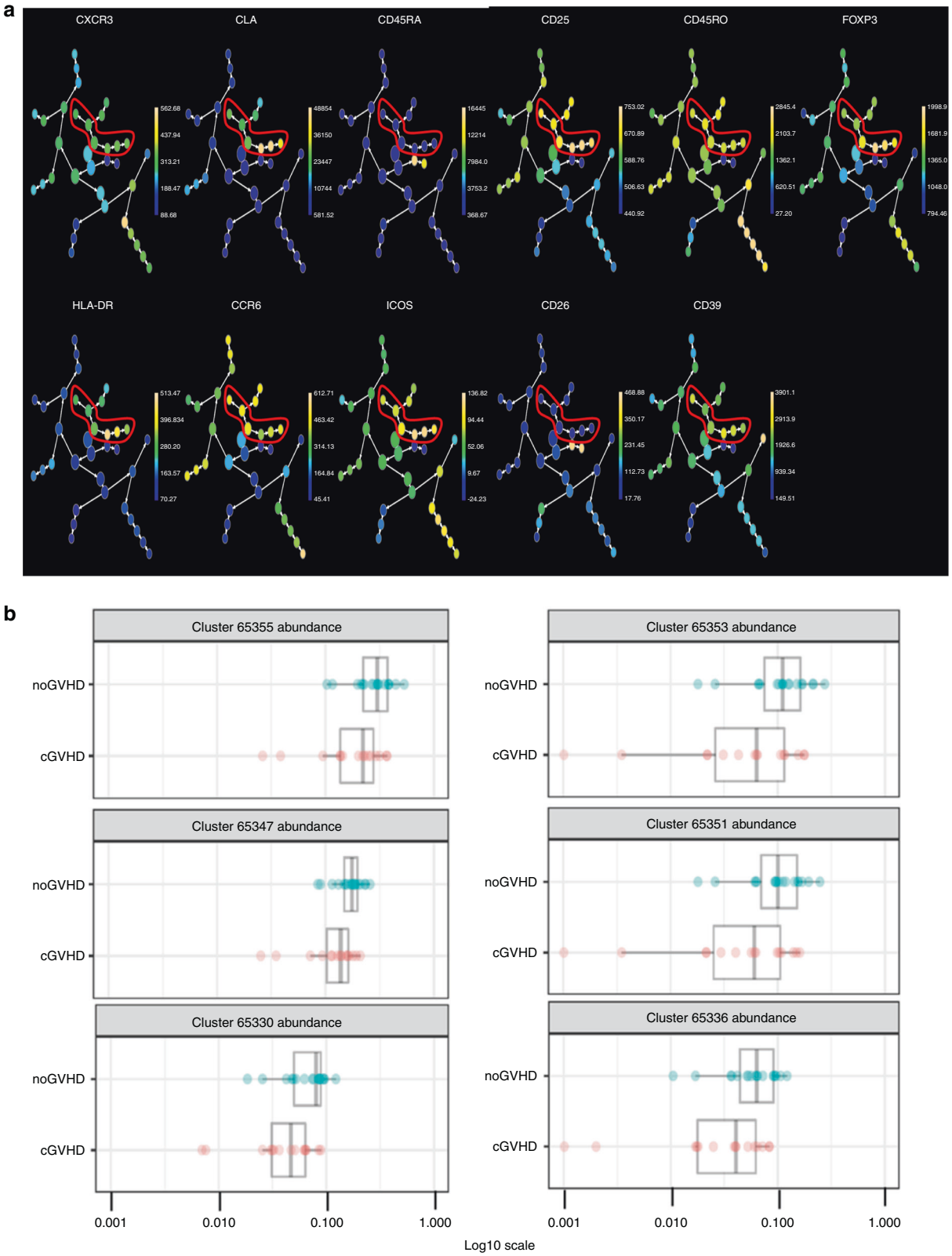


Fig. 5 Citrus analyses of Treg (2000/sample) from allo-HCT recipients without ($n = 20$) or with ($n = 15$) moderate/severe chronic GVHD. Citrus created a hierarchical clustering of Treg cells based on the marker MFI (FOXP3, CD25, CD45RA, CD45RO, CLA,CD26, CD39,HLA-DR,ICOS, CXCR3, and CCR6). **a** Plots show the expression of each marker in the different nodes (minimal size: 3300 cells). **b** Cluster manually surrounded in red in plot A characterized by a high expression of CD25, CLA, CD39, HLA-DR, and ICOS differ in abundance between the two groups (FDR = 0.05) and are more abundant in allo-HCT patient without cGVHD. (Color figure online).

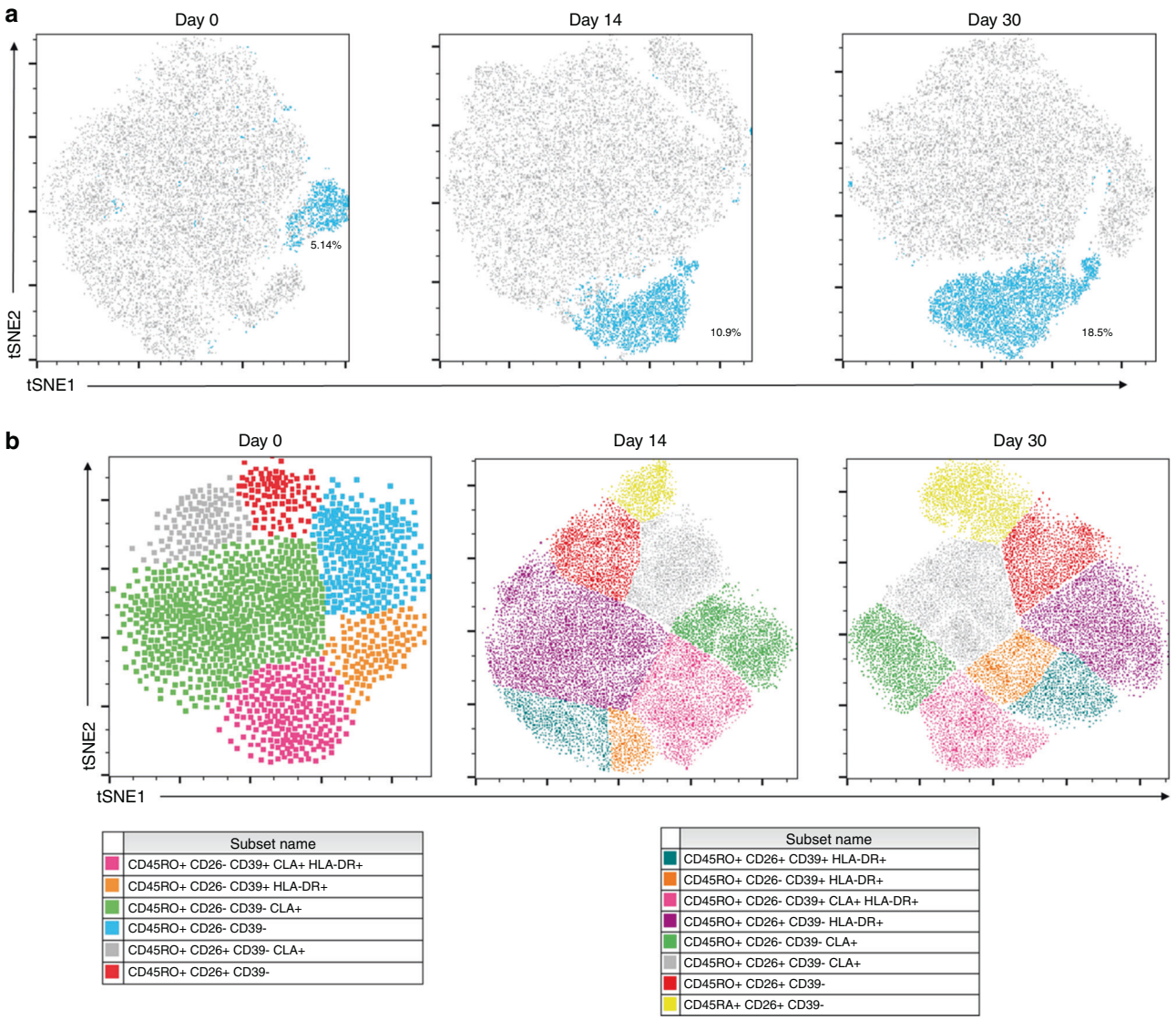


Fig. 6 Impact of injection of low-dose IL-2 in a patient with severe steroid-refractory chronic GVHD. Blood samples were collected at day 0, 14, and 30. **a** t-SNE showing the frequency of Treg (in blue) among $CD4^+$ T cells before and after IL-2 injection. t-SNE was created on 20,000 $CD4^+$ T cells and includes CD25, CD127, FOXP3 markers to determine Treg populations. **b** t-SNE of Treg subpopulations among Treg created with CD25, FOXP3, CD26, CD39, CLA, and HLA-DR; subpopulations were defined based on the expression (MFI) of each marker and on the manual gating created individually for each marker. After IL-2 injection, new subpopulations appeared. (Color figure online).

Discussion

Chronic GVHD has remained one of the most serious limitation of allo-HCT [8]. Over the last decade, several observations in pre-clinical models have unraveled an important role for Treg in the pathogenesis of chronic GVHD [34, 35]. Further, other reports have demonstrated that Treg number and phenotype are impacted by allo-HCT and particularly so in patients with prior acute or chronic GVHD [25, 36, 37]. This has been attributed at least in part to high IL-7/IL-2 ratio in chronic GVHD patients favoring Tconv over Treg homeostasis [25]. Accordingly, a phase II study has shown that administration of low dose of IL-2 restored Treg homeostasis and resulted in clinical improvement in ~60% of patients suffering from advanced chronic GVHD, although unfortunately no CR was observed [20, 38]. Comparable observations were made in another phase II trial combining IL-2 with photophoresis [39]. Recent observations have unraveled that the Treg population was remarkably heterogeneous and that several Treg subtypes could be identified by flow cytometry [26, 29, 40]. Here, we hypothesized that Treg subsets the least able to activate pSTAT5 in response to IL-2 in vitro would be the most affected by clinical situations involving a

strong competition for available IL-2 such as allo-HCT (and particularly in case of chronic GVHD). Indeed, it has been previously demonstrated that in severe immunological disorders various T-cell subsets compete for IL-2 and that T cells with the highest CD25 expression consumed the available IL-2 leading to depletion of other T-cell population with a lower CD25 expression. To challenge this hypothesis, we first assessed the impact IL-2 and IL-7 in Treg subtypes in vitro. We then assessed the impact allo-HCT and chronic GVHD on Treg subtype frequencies. We finally assessed whether treatment with low-dose IL-2 could restore Treg homeostasis (and correct Treg subset distribution) in a patient suffering from severe chronic GVHD. Several observations were made.

A first observation was that the most activated Treg subtypes were the ones with the highest phosphorylation of STAT5 following ex vivo IL-2 administration. Further, we observed a strong correlation between CD25 MFI and pSTAT5 MFI following IL-2 administration. Interestingly, when very low doses of IL-2 (1 ng/mL) were added, only a fraction of Treg phosphorylated STAT5. As already observed by other investigators, Tconv were much less responsive to low-dose IL-2 than Treg [32]. However, a small fraction of Tconv also expressing low levels of CD25 activated pSTAT5 following addition of low doses of IL-2. This observation is of clinical importance since current techniques of clinical-grade Treg enrichment (generally based on a 2-step process of CD8/CD19 depletion followed by CD25 selection) result in the presence of a fraction of Tconv with a comparable phenotype [15], which thus could be activated in vivo if donor Treg infusion is combined with IL-2.

Despite their low CD127 expression, another observation of our study was that IL-7 administration resulted in STAT5 phosphorylation in several Treg subtypes, although to a much lesser extend than what was observed in Tconv. These observations are in line with the recent findings showing that IL-7Rα$^{-/-}$ mice have a reduced Treg population [41] while selective peripheral IL-7 deficiency in the presence of a fully functional thymus not only resulted in a general paucity of T cells, but also in a dramatic reduction of the Treg pool, which further exhibited low FOXP3 expression [21]. Further, IL-7Rα–deficient Treg exhibited impaired proliferation and lower sensitivity to IL-2 and were unable (in contrast to wild-type Treg) to induce skin allograft tolerance in mice [22].

A second observation of our study was that allo-HCT had a strong impact on the proportions of the different Treg subsets, with an important decrease in the proportion of naive Treg (as previously reported [36]) and higher frequencies of the most differentiated (M4) Treg subpopulation as well as of activated Treg subpopulations such as CD45RO$^+$HLA-DR$^+$ and CD45RO$^+$ICOS$^+$. Interestingly, these two last Treg subpopulations had lower expression of KI67 in allo-HCT patients than in controls.

Comparing Treg subtypes from allo-HCT patients with no or mild chronic GVHD versus from those with moderate/severe chronic GVHD, we first observed that patients with moderate/severe chronic GVHD had lower absolute Treg counts than those without. This observation is in line with previous reports [37, 42] but probably reflect in a large part the immunosuppressive medications given to control chronic GVHD (such as steroids). Further, interestingly, we observed that six Treg clusters characterized by a high expression of CD25, HLA-DR, and of ICOS were significantly more frequent in patients without or with limited chronic GVHD than in those with moderate/severe chronic GVHD. Although these results should be taken with caution given the heterogeneity of our dataset for GVHD prophylaxis and timing after allo-HCT, they suggest a role for ICOS$^+$ Treg in the control of chronic GVHD. These findings, if confirmed, would be in line with the recently established important role of ICOS in the maintenance and function of tissue-resident Treg and establishment/maintenance of tissue-specific tolerance [43].

Finally, we assessed the impact of low dose of IL-2 (one million IU/day) administration on Treg subtypes in a patient suffering from advanced steroid-refractory chronic GVHD. We observed that low-dose IL-2 resulted in a dramatic Treg change in Treg phenotype with increasing proportion of naive CD45RA$^+$ Treg and activated M1 (CD45RO$^+$CD26$^+$ HLA-DR$^+$) Treg. This could be in line with our hypothesis that the low number of naïve Treg observed after allo-HCT could be in part due to a lack of available IL-2. Prior reports by the Dana-Farber group have also shown that low-dose IL-2 administration dramatically expanded Treg and particularly naïve Treg [38]. This has been attributed to multiple mechanisms including increased naïve Treg proliferation, conversion of Tconv into Treg (evidenced by clone tracking), and perhaps by increasing thymic Treg output (as suggested by an increase in recent-thymic-emigrant Treg (defined in that study by CD45RA + CD31 + Treg) following IL-2 administration) [38].

Two approaches are currently investigated to restore Treg function/phenotype in patients with advanced chronic GVHD. One consists of infusing fresh donor Treg. The other consists of low-dose IL-2 administration that results in peripheral Treg expansion as well as probably in neogeneration by Treg in the thymus. While the efficacy of donor Treg infusion in patients with refractory chronic GVHD is currently under evaluation (www.tregeneration.eu), it is now well-established that low-dose IL-2 administration provides clinical benefit in >60% of the patients [20]. Importantly, these two approaches are not exclusive and the combination of donor Treg infusion and IL-2 administration

might be a promising way to restore Treg homeostasis after allo-HCT, perhaps particularly in older chronic GVHD patients who are known to have particularly poor thymic function [24, 44]. While preliminary results of a study combining donor Treg infusion and low-dose IL-2 suggest clinical benefit in up to 60% of the patients, a possible limitation of this strategy might be that current clinical-grade Treg enrichment procedures also enrich for a fraction of $CD25^{dim}$ Tconv that, according to our in vitro observations, could respond to IL-2 stimulation. Combination of donor Treg infusion, IL-2, and sirolimus administration thus seems particularly of interest as recently demonstrated in nonhuman primates [45]. This approach is also under clinical evaluation in patients with steroid-refractory chronic GVHD (www.tregeneration.eu).

In summary, these data indicate that allo-HCT patients have a high frequency of terminally differentiated Treg. While low-dose IL-2 activates preferentially the most activated Treg subpopulations in vitro, low-dose IL-2 administration in a patient with severe chronic GVHD resulted in the generation of naive and M1 Treg subpopulations. Finally, unbiased analyses suggested that six Treg clusters characterized by a high expression of CD25, HLA-DR, and ICOS (and by high FOXP3 expression for four of the six clusters) were significantly more frequent in patients with no or with limited chronic GVHD than in those with moderate/severe chronic GVHD.

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Author contributions CR performed the experiments, analyzed the data, and wrote the manuscript; FB wrote the manuscript, designed the study, and interpreted the data; GE performed the experiments, helped in the study design and data interpretation, and edited the manuscript; CG and CD performed the experiments and edited the manuscript; YB helped in the study design, provided clinical data, and edited the manuscript; EW and SS provided clinical data and edited the manuscript. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest FB has received travel grants and/or speaker honoraria from Celgene, AbbVie, Novartis, Pfizer, and Sanofi. The other authors declare that they have no conflict of interest.

Ethics approval The study was approved by the Ethics Committee of the CHU of Liège (protocol number TJB1609). All volunteers and patients provided their written informed consent.

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