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# Ex Vivo-Expanded CD4<sup>+</sup>CD25<sup>+</sup> Immunoregulatory T Cells Prevent Graft-versus-Host-Disease by Inhibiting Activation/Differentiation of Pathogenic T Cells<sup>1</sup>

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CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory T cells (Tregs) can be administered to inhibit graft-vs-host disease (GVHD) while preserving graft-vs-leukemia activity after allogeneic bone marrow transplantation in mice. Preclinical studies suggest that it is necessary to infuse as many Tregs as conventional donor T cells to achieve a clinical effect on GVHD. Thus, it would be necessary to expand Tregs ex vivo before transplantation. Two strategies have been proposed: expansion of Tregs stimulated by anti-CD3/CD28-coated microbeads for polyclonal activation or by host-type allogeneic APCs for selecting Tregs specific for host Ags. In this study, we describe the mechanisms by which ex vivo-expanded Tregs act on donor T cells to prevent GVHD in mice. We demonstrate that expanded Tregs strongly inhibited the division, expansion, and differentiation of donor T cells, with a more pronounced effect with Tregs specific for host Ags. These latter cells permit the efficient and durable control of GVHD and favor immune reconstitution. *The Journal of Immunology*, 2006, 176: 1266–1273.

Although initially described for their crucial role in the induction and the maintenance of peripheral self-tolerance and the control of autoimmune diseases (1), there is now strong evidence that CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory T cells (Tregs)<sup>4</sup> also contribute to the control of alloreactive responses. Aluvihare et al. (2) demonstrated in mice that maternal Tregs suppressed the allogeneic response directed against the fetus during gestation, thus avoiding its rejection by the maternal immune system. In organ transplantation, it also was demonstrated that Tregs collected from mice rendered tolerant to an allograft could transfer this tolerance after their infusion in naive mice (3, 4). Tregs also are implied in allogeneic hemopoietic stem cell transplantation (HSCT), one of the most efficient approaches in treating hematological malignancies or disorders. In this setting, donor T cells present in the transplant favor engraftment (5) and immune reconstitution and can contribute to the graft-vs-leukemia (GVL) effect

by eliminating residual leukemic cells (6). However, donor T cells specific for recipient alloantigens also are responsible for graft-vs-host disease (GVHD), a life-threatening complication that frequently occurs after allogeneic HSCT (7). We and others have demonstrated that, if Tregs are depleted from the transplant, experimental GVHD is significantly accelerated (8, 9). This observation led to the development of new therapeutic strategies relying on the infusion of Tregs to control GVHD. Indeed, we and others demonstrated that the addition of an equivalent number of freshly isolated donor Tregs to donor CD3<sup>+</sup> T cells reduced GVHD in lethally irradiated mice (8, 10), whereas the GVL effect against certain types of leukemia model was maintained (11).

One difficulty in devising strategies for the clinical application of Tregs for the treatment of GVHD arises from their relative rarity. Tregs constitute only 5–10% of the whole CD4 T cell pool in mice (1) and <5% in humans (12). Thus, due to the necessity to infuse at least one Treg for every donor T cell to obtain a clinical effect on GVHD in mice, it is likely to be very difficult, if not impossible, to collect a sufficient number of Tregs from a single donor. To circumvent this potential barrier, purified Tregs can be expanded in the presence of immobilized anti-CD3 mAb plus IL-2 (9) or with anti-CD3/CD28-coated microbeads and IL-2 (13). This approach permits expansion of the entire population of Tregs while maintaining a diversified repertoire, now referred to as polyclonal Tregs (polyTregs). Ex vivo-expanded polyTregs infused at the time of grafting delay or even prevent GVHD in mice (9, 14). Alternatively, Tregs can be expanded and, at the same time, selected for their capacity to recognize allogeneic Ags so that a population of recipient-specific alloreactive Tregs, now referred to as recipient-specific Tregs (rsTregs), can be obtained. To achieve this, we purified Tregs and cultured them in the presence of host-type APCs and IL-2 (8). These ex vivo-expanded rsTregs infused either at the time of grafting or 2 days posttransplantation delay or even prevent GVHD in mice (8, 9, 15). Before the development of clinical trials, our aim in this work was to determine the mechanism by which expanded polyTregs and rsTregs, which efficiently

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<sup>4</sup> Abbreviations used in this paper: Treg, CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory T cell; HSCT, hemopoietic stem cell transplantation; GVL, graft-vs-leukemia; GVHD, graft-vs-host disease; polyTreg, polyclonal Treg; rsTreg, recipient-specific Treg; BM, bone marrow; LN, lymph node.

prevent GVHD, act on alloreactive donor T cells. We demonstrate that both types of Tregs block very early after transplantation, proliferation, and differentiation of donor T cells.

## Materials and Methods

### Mice and experimental GVHD

BALB/c (H-2<sup>d</sup>), C3H (H-2<sup>k</sup>), C57BL/6 (H-2<sup>b</sup>), and (BALB/c × C3H)<sub>F</sub><sub>1</sub> (H-2<sup>dkk</sup>) 5- to 9-wk-old female mice were obtained from Iffa Credo. Congenic Thy-1.1 BALB/c mice (H-2<sup>d</sup>) were bred in our animal facility. Mice were manipulated according to European Union guidelines. HSCT were performed as described previously (16), except as otherwise stated. Briefly, 24 h after lethal irradiation of 9-wk-old (BALB/c × C3H)<sub>F</sub><sub>1</sub> (9.5 Gy) or BALB/c (8.5 Gy) recipients, mice were transplanted with cells from BALB/c mice. The transplant comprised 5 × 10<sup>6</sup> bone marrow (BM) cells, pools of splenocytes, and lymph node (LN) cells, adjusted to give 10 × 10<sup>6</sup> T cells determined by an anti-CD3 staining and, when indicated, 10 × 10<sup>6</sup> BALB/c ex vivo-expanded Tregs. Control groups were constituted of non-grafted irradiated mice or irradiated mice receiving BM cells only.

### Ex vivo expansion of Tregs

CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> Tregs were purified from spleen and peripheral LN cells by flow-cytometric cell sorting as described previously (16), yielding a purity of 99%. Tregs were cultured in the presence of anti-CD3- and anti-CD28-coated 4.5-μm paramagnetic microbeads (Xcyte Therapies) and human IL-2 (600 U/ml) (13) to yield polyTregs or with irradiated allogeneic splenocytes and murine IL-2 (10 ng/ml; R&D Systems) to yield rsTregs.

### Flow cytometric analysis

The following Abs were used for FACS analysis: PE-labeled anti-Thy1.1 (clone OX-7), anti-Thy1.2 (clone 30-H12), and anti-IFN-γ. FITC-labeled H-2K<sup>d</sup> CyChrome-labeled CD8, biotinylated anti-H2K<sup>k</sup>, anti-CD25 (clone 7D4), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.2F3), and anti-CD44 (clone IM7) revealed with streptavidin-CyChrome or streptavidin-allophycocyanin and allophycocyanin-labeled anti-B220 (clone RA3-6B2) and anti-CD4 (RM4-5), all from BD Pharmingen. For intracellular cytokine staining, splenocytes were isolated 3.5 days after transplantation and were restimulated for 6 h with anti-CD3 (1 mg/ml) in the presence of GolgiPlug (BD Biosciences) and stained with the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. FACSCalibur analyses were performed with either CellQuest (BD Biosciences) or FlowJo (Tree Star) software.

### Histopathological examination

Livers and lungs were preserved in Bouin's fixative and embedded in paraffin. For these organs, 5-μm-thick sections were stained with H&E for histological examination. One pathologist analyzed slides in a blinded fashion to assess the intensity of GVHD. GVHD lesions in each sample were scored according to a semiquantitative scoring system described by Hill et al. (17) with minor modifications. GVHD lesions were scored in each liver sample according to seven parameters that were graded as follows: portal inflammation (0, absent; 1, lymphocytic aggregates in some portal tracts; 2, lymphocytic aggregates in all portal tracts; 3, dense lymphocytic aggregates in all portal tracts); bile duct inflammation (0, absent; 1, lymphocytes infiltrating bile duct in less than one-third of portal tracts; 2, lymphocytes infiltrating bile duct between one-third and two-thirds of portal tracts; 3, lymphocytes infiltrating bile duct in more than two-thirds of portal tracts); periportal necroinflammatory activity (0, absent; 1, focal alteration of the periportal plate in some portal tracts; 2, diffuse alteration of the periportal plate in some portal tracts; 3, diffuse alteration of the periportal plate in all portal tracts); lobular necroinflammatory activity (0, absent; 1, less than one necroinflammatory foci per lobule; 2, at least one necroinflammatory foci per lobule; 3, more than one necroinflammatory foci per lobule); confluent necrosis (0, absent; 1, present in one lobule; 2, present in some lobules; 3, present in most lobules); endothelialitis, defined as attachment of lymphocytes to the endothelium of portal or centrilobular venules (0, absent; 1, focal in some portal or centrilobular venules; 2, focal in most portal or centrilobular venules; 3, heavy lymphocytic infiltration in at least one portal or centrilobular venule); and sinusoidal lymphocytic infiltrate (0, absent; 1, present in one lobule; 2, present in some lobules; 3, present in most lobules). GVHD lesions in the lung were evaluated as described previously (18) according the following score, which is the sum of a periluminal (around airways and vessels) lymphocytic infiltrate index (0, absence; 1, lymphocytic cuff of 1–3 cells in diameter; 2, lymphocytic cuff of 4–10 cells in diameter; 3, lymphocytic cuff >10 cells in diameter), an

alveolar/interstitial lymphocytic infiltrates index (0, absence; 1, increased number of cells only visible at high magnification; 2, interstitial thickening; 3, interstitial thickening and alveolar infiltrates), and an index of lung-injury extension quantitated according the percentage of tissue involved (1, 5–25%; 2, 25–50%; and 3, >50%).

### Statistical analysis

StatView software (Abacus Concepts) was used for statistical analyses. ANOVA *t* tests were performed to compare numbers and percentage of cells. Kaplan-Meier survival curves were established for each group of grafted mice. Mice suffering from an advanced stage of GVHD were sacrificed for histopathological examination and considered dead in the Kaplan-Meier analysis. *p* values are indicated in figures only when the difference between the two groups was statistically significant. \*, *p* < 0.05; \*\*, *p* < 0.005 when mice grafted with CD3<sup>+</sup> T cells plus Tregs were compared with mice grafted with CD3<sup>+</sup> T cells alone. \*, *p* < 0.05; \*\*, *p* < 0.005 when mice grafted with CD3<sup>+</sup> T cells plus rsTregs were compared with mice grafted with CD3<sup>+</sup> T cells plus polyTregs.

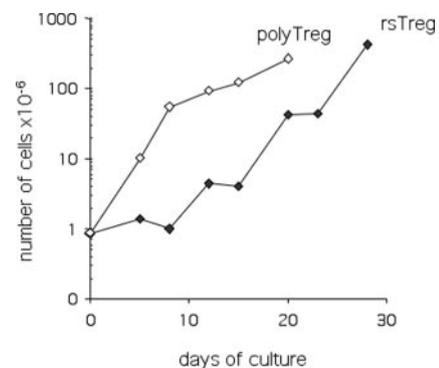
### Skin grafting

At day 80 after BM transplantation, tail-skin grafts from C57BL/6 mice were transplanted onto the lateral thoracic wall of the recipients under ketamine (75 mg/kg) and xylazine (15 mg/kg) anesthesia. Skin grafts were monitored regularly by visual and tactile inspection, and rejection was defined as complete loss of viable donor epithelium.

## Results

### Ex vivo expansion of Tregs

To study how expanded Tregs act on donor T cells after transplantation, we produced a high number of either rsTregs ex vivo after purification of fresh BALB/c Tregs and their culture in the presence of allogeneic irradiated splenocytes plus IL-2 or polyTregs after culture in the presence of anti-CD3/CD28-coated microbeads plus IL-2. In the presence of irradiated C3H splenocytes, as well as by polyclonal activation, Tregs could be expanded >500-fold. However, the initial expansion of Tregs differed according to the stimulation. In the presence of anti-CD3/CD28-coated microbeads and IL-2, cells continuously expanded from the beginning of the culture, and their number increased 60-fold at day 9 and 300-fold at day 21. Compared with this polyTreg expansion, 1 additional week of culture with irradiated splenocytes was needed to reach the same number of rsTregs (Fig. 1). Additionally, the growth of rsTreg cells displayed plateau phases approximately every week. This is probably due to the disappearance of irradiated APCs after 4–5 days of culture. At the end of the culture, both polyTregs and rsTregs maintained their



**FIGURE 1.** Highly purified Tregs expanded rapidly in vitro. BALB/c-purified CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> T cells were stimulated in the presence of murine IL-2 with allogeneic irradiated splenocytes from C3H mice to generate rsTregs. To generate polyTregs, BALB/c-purified CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> Tregs were stimulated with anti-CD3/CD28-coated microbeads and human IL-2 (600 U/ml). The graph is representative of four independent experiments and depicts the number of Tregs during the culture.

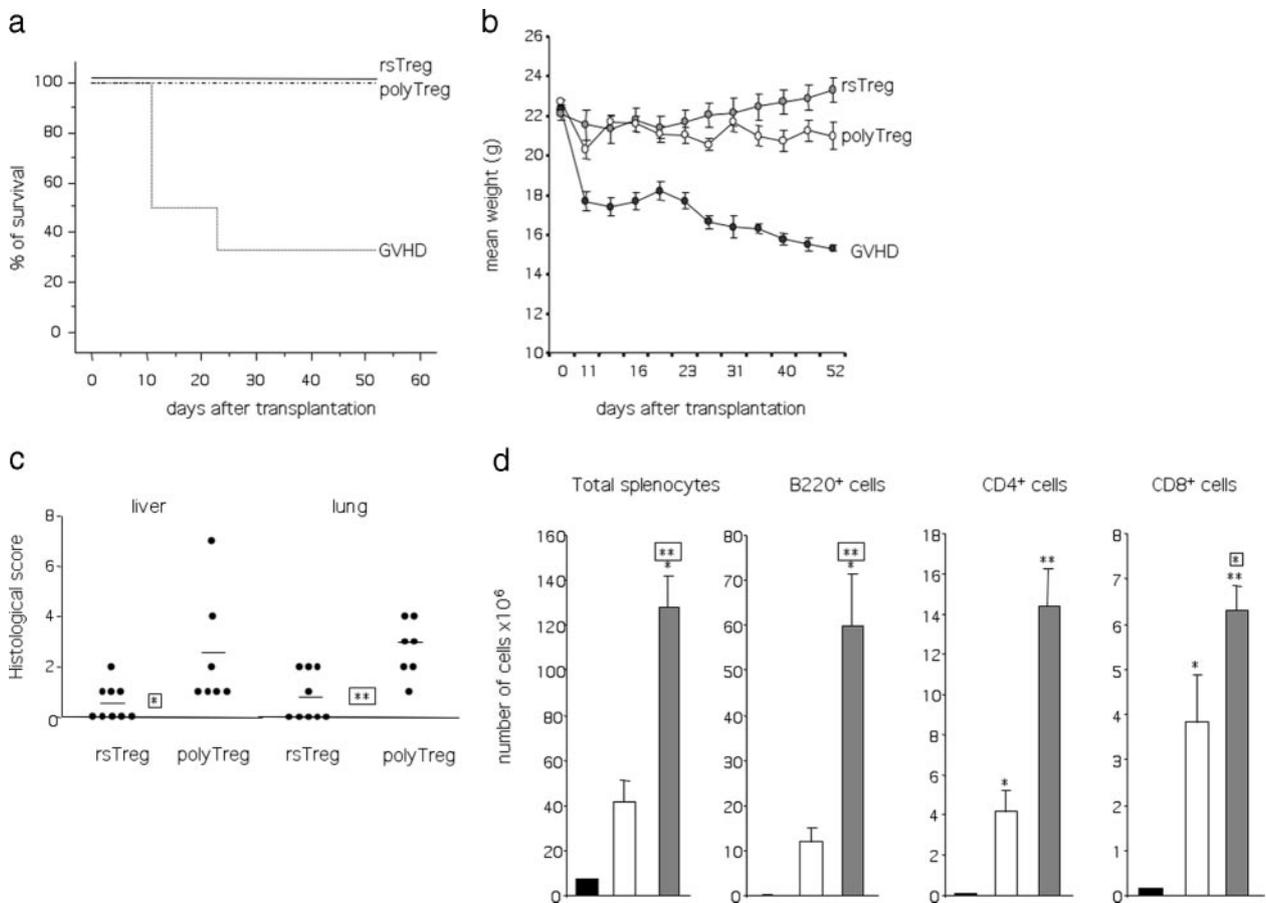
CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> phenotype together with their capacity to suppress T cell proliferation in vitro (Ref. 8 and data not shown). Additionally, real-time RT-PCR analysis revealed that polyTregs and rsTregs expressed a high level of Foxp3 (data not shown), as described previously for freshly purified Tregs (19, 20).

#### *PolyTregs and rsTregs prevent GVHD*

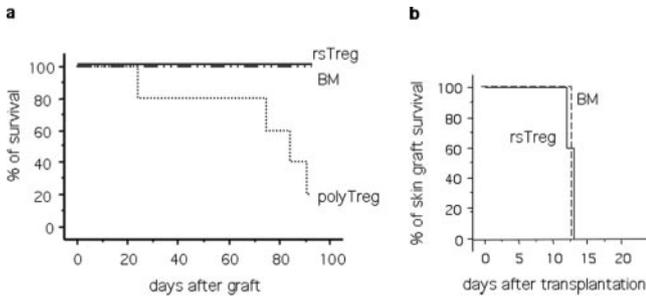
Next, we evaluated the capacity of both types of expanded Tregs to inhibit GVHD and promote immune reconstitution. During the 52 days following HSCT, no clinical signs of GVHD were observed in mice receiving donor T cells supplemented with rsTregs. In contrast, during this period, 65% of mice grafted with donor T cells alone died with characteristic signs of GVHD, such as hunching, dull fur, skin lesions, and severe diarrhea. In mice receiving polyTregs, clinical signs of GVHD also were absent (Fig. 2a). Additionally, the mean weight curves of animals receiving rsTregs or polyTregs were comparable, whereas mice grafted with donor T cells alone displayed important weight loss (Fig. 2b). At day 52, mice were sacrificed to evaluate histological signs of GVHD in target organs and immune reconstitution by measuring the number of total splenocytes, and B and T cells. In mice protected with rsTregs, no or residual histological signs of GVHD were observed (mean scores were 0.5 and 0.8 for liver and lung, respectively),

attesting to the efficiency of these cells. In comparison, although clinical GVHD was prevented, we observed histological signs of GVHD in the lung (mean score, 3) and liver (mean score, 2.5) of mice grafted with polyTregs (Fig. 2c). Comparable results were obtained in a second C57BL/6 → (C57BL/6 × BALB/c)F<sub>1</sub> combination of HSCT in which Tregs were collected from C57BL/6 mice, confirming that Ag specificity of Tregs is critical for their capacity to suppress GVHD (data not shown). Surviving control mice receiving donor T cells alone had very poor immune reconstitution attested by profound splenic atrophy cells ( $0.54 \times 10^6$  and  $0.4 \times 10^6$  cells in B and T cell compartments, respectively), compatible with severe GVHD. When rsTregs were added, lymphopenia was prevented, as evidenced by spleens that contained  $\sim 130 \times 10^6$  cells consisting of 50% B cells and 16% T cells. In contrast, mice receiving polyTregs displayed partial splenic atrophy (Fig. 2d), which could be due to a GVHD affecting specific organs such as liver, lung (Fig. 2c), and spleen.

The differences observed on target organs of GVHD and in immune reconstitution between rsTregs and polyTregs prompted us to evaluate the long-term effect of these two types of Tregs on GVHD. Interestingly, in a long-term follow-up of GVHD, we observed that mice treated with polyTregs displayed late clinical signs of GVHD, such as weight loss and skin lesions, and four of



**FIGURE 2.** GVHD can be controlled by the addition of ex vivo-expanded Tregs. Tregs were tested for their capacity to control GVHD in the BALB/c → (BALB/c × C3H)F<sub>1</sub> combination. *a*, Kaplan-Meier survival curves were established in mice receiving  $10 \times 10^6$  donor T cells alone (GVHD group;  $n = 7$ ) or coinfused with  $10 \times 10^6$  rsTregs ( $n = 9$ ) or  $10 \times 10^6$  polyTregs ( $n = 7$ ). *b*, Mean weight curves were established after mice were weighted at different time points during the experiments. *c*, Histopathologic scores of liver and lung after semiallogeneic BM transplantation. Grading of GVHD was performed 52 days after transplantation. Points correspond to histopathological score of individual mouse, and horizontal bars show the mean histopathological score for each group. *d*, Immune reconstitution after allogeneic HSCT was evaluated 52 days after transplantation. Total splenocytes were counted and stained with appropriate mAbs. The number of B, CD4<sup>+</sup>, and CD8<sup>+</sup> cells was evaluated after analysis by flow cytometry for GVHD control mice (■;  $n = 2$ ), mice receiving  $10 \times 10^6$  rsTregs (▣;  $n = 9$ ) or  $10 \times 10^6$  polyTregs (□;  $n = 7$ ). Histograms display the mean number  $\pm$  SEM of cells for each group.



**FIGURE 3.** Mice protected from GVHD with rsTregs rejected allogeneic skin graft. Tregs were tested for their capacity to control GVHD in the BALB/c  $\rightarrow$  (BALB/c  $\times$  C3H)F<sub>1</sub> combination in long-term experiments. *a*, Kaplan-Meier survival curves were established in mice receiving mice receiving bone marrow cells alone (BM alone group;  $n = 4$ ) or coinjected with  $10 \times 10^6$  donor T cells with  $10 \times 10^6$  rsTregs ( $n = 5$ ) or  $10 \times 10^6$  polyTregs ( $n = 5$ ). *b*, rsTreg-treated mice were tested for their capacity to reject skin grafts from C57BL/6 background at day 80. Kaplan-Meier survival curves were established in mice receiving BM cells alone (BM-alone group;  $n = 3$ ) or BM supplemented with  $10 \times 10^6$  donor T cells and coinjected with  $10 \times 10^6$  rsTreg ( $n = 5$ ).

five mice died by day 90 posttransplant. In comparison, no mice treated with rsTregs displayed clinical signs of GVHD at the same time point (Fig. 3*a*). To test the functionality of the reconstituted T cell compartment in mice protected from GVHD, third-party C57BL/6 allogeneic skin grafts (different from both donor and recipient genetic background) were performed in five mice treated with rsTregs at day 80. All skin grafts were clearly rejected at day 13 (Fig. 3*b*). Together, these results revealed that rsTregs, more efficiently than polyTregs used at equivalent cell doses, durably prevent GVHD and support immune reconstitution.

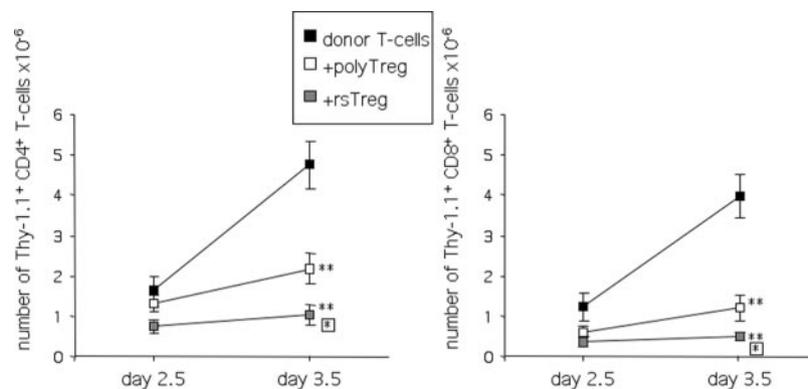
#### Expanded Tregs inhibit expansion of donor T cells in vivo

We and others have described previously that division and differentiation of donor T cells take place within the first few days after allogeneic HSCT in mice (21–23). In this study, we analyzed the early effect of ex vivo-expanded Tregs on coinjected donor T cells at days 2.5 and 3.5 after grafting. We used the Thy-1 congenic marker to distinguish donor T cells (Thy-1.1<sup>+</sup> cells) from cultured Tregs and recipient cells (Thy-1.2<sup>+</sup> cells). We transferred  $5 \times 10^6$

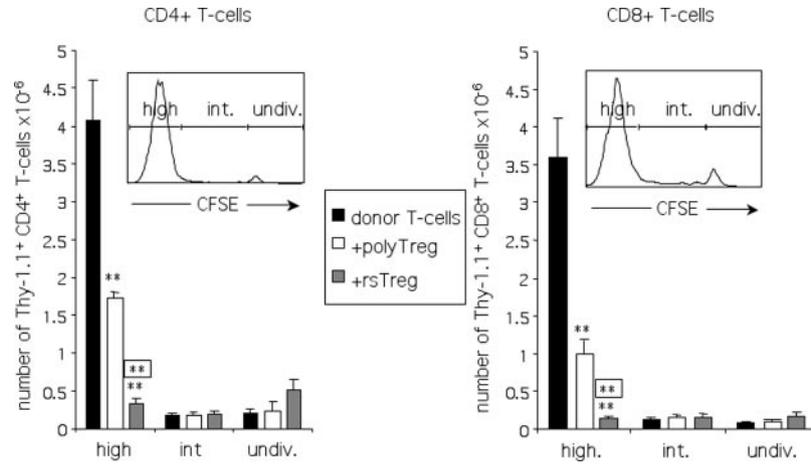
BM cells and  $10 \times 10^6$  donor T cells isolated from BALB/c mice into semiallogeneic (BALB/c  $\times$  C3H)F<sub>1</sub> lethally irradiated recipients. Only spleens were collected for analysis, because LN were too small to be analyzed at these early time points. When donor T cells were infused alone, the number of donor T cells present in the spleen rapidly expanded between days 2.5 and 3.5 (Fig. 4). It reached  $4.7 \times 10^6$  for CD4<sup>+</sup> and  $3.9 \times 10^6$  CD8<sup>+</sup> T cells at day 3.5. When rsTregs were coinjected, this expansion was strongly inhibited. The number of donor T cells diminished to  $1 \times 10^6$  and  $0.5 \times 10^6$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells at day 3.5, respectively. PolyTregs also significantly inhibit expansion of donor T cells. Noteworthy, at equivalent cell doses, the inhibitory effect of rsTregs was significantly more pronounced than that observed for mice receiving polyTregs.

#### Expanded Tregs inhibit cell division of donor T cells in vivo

After HSCT, alloreactive donor T cells will divide after encountering host Ags. To assess the inhibitory activity of expanded Tregs, we evaluated their ability to inhibit donor T cells' division in vivo. We first labeled donor T cells from BALB/c (Thy-1.1) mice with CFSE before infusion. Because the inhibitory effect of expanded Tregs was greater at day 3.5, subsequent analyses were restricted to this time point. In the absence of Tregs, CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells massively and rapidly divided by day 3.5. Approximately 90% of CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells present at this time point had more than seven divisions and represented  $4 \times 10^6$  and  $3.5 \times 10^6$  donor T cells, respectively, of each population in the spleen. In the presence of  $10 \times 10^6$  rsTregs, the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells that had undergone more than seven divisions were reduced to  $0.3 \times 10^6$  and  $0.15 \times 10^6$ , respectively, per spleen. Concurrently, the numbers of T cells that had undergone fewer or no divisions were not statistically modified (Fig. 5). In mice in which polyTregs were added,  $>1.7 \times 10^6$  CD4<sup>+</sup> and  $1 \times 10^6$  CD8<sup>+</sup> T cells had undergone more than seven divisions. The numbers of T cells that had undergone fewer or no divisions also were unchanged, compared with mice receiving donor T cells alone. Thus, rsTregs effectively inhibit alloreactive donor T cells' division, resulting in reduced numbers of donor T cells in the spleen. This effect was more pronounced, compared with that of polyTregs.



**FIGURE 4.** Tregs inhibit donor T cell expansion in vivo. A total of  $10 \times 10^6$  donor T cells isolated from BALB/c-Thy-1.1 mice were grafted into semiallogeneic (BALB/c  $\times$  C3H)F<sub>1</sub> lethally irradiated recipients (Thy-1.2). At days 2.5 and 3.5 posttransplantation, spleens were harvested. Splenocytes were counted, and donor T cells were quantified by flow-cytometric gating on Thy-1.1<sup>+</sup> cells. The expansion of donor T cells was evaluated in the absence or in presence of Thy-1.2 rsTregs, or Thy-1.2 polyTregs. At day 2.5,  $n = 6$  for mice receiving  $10 \times 10^6$  donor T cells alone,  $n = 6$  for mice receiving  $10 \times 10^6$  donor T cells plus  $10 \times 10^6$  rsTregs,  $n = 4$  for mice receiving  $10 \times 10^6$  donor T cells plus  $10 \times 10^6$  polyTregs. At day 3.5,  $n = 9$  for mice receiving  $10 \times 10^6$  donor T cells alone,  $n = 10$  for mice receiving  $10 \times 10^6$  donor T cells plus  $10 \times 10^6$  rsTregs,  $n = 8$  for mice receiving  $10 \times 10^6$  donor T cells plus  $10 \times 10^6$  polyTregs. Data are represented as the mean of the number of cells  $\pm$  SEM. Data were pooled from three to seven independent experiments.

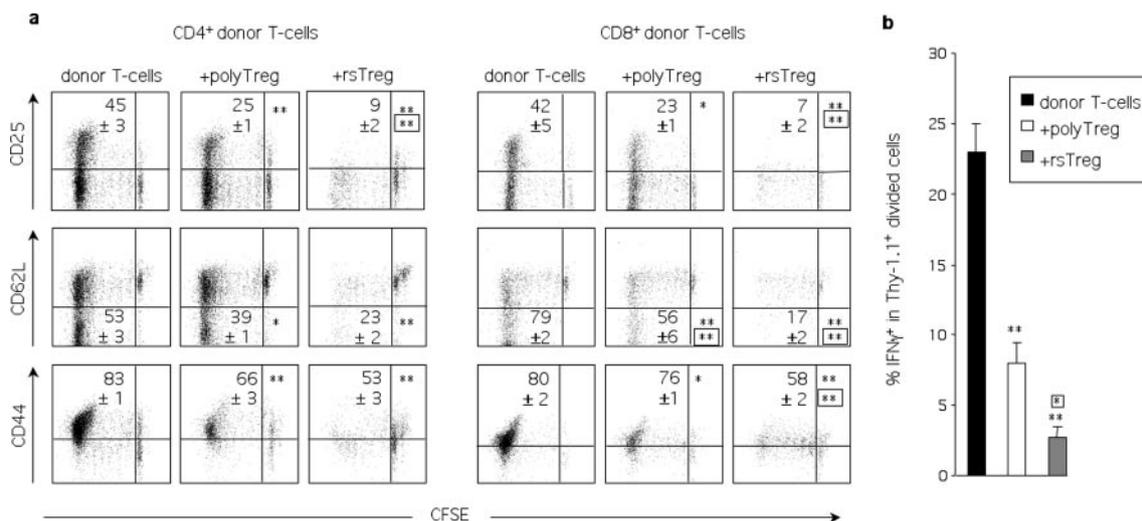


**FIGURE 5.** Tregs inhibit donor T cell division in vivo. A total of  $10 \times 10^6$  donor T cells isolated from BALB/c Thy-1.1 mice were labeled with CFSE and then infused into semiallogenic (BALB/c  $\times$  C3H) $F_1$  lethally irradiated recipients (Thy-1.2) alone (donor T cells,  $n = 13$ ) or in the presence of  $10 \times 10^6$  rsTregs ( $n = 14$ ),  $10 \times 10^6$  polyTregs ( $n = 8$ ). At day 3.5, splenocytes from grafted animals were collected. The donor T cells were detected by gating on expression of the Thy-1.1 congenic marker. Cell proliferation was measured as the sequential loss of CFSE. CFSE histograms were representative examples from mice that received no Treg marker. The numbers of highly divided, intermediate-divided, and undivided donor CD4<sup>+</sup> or CD8<sup>+</sup> T cells are expressed as the mean  $\pm$  SEM. Data were pooled from three to seven independent experiments.

#### Expanded Tregs inhibit differentiation of donor T cells into effector T cells

To investigate whether the inhibitory effect of Tregs on the expansion and proliferation of donor T cells correlated with an inhibition of effector T cell differentiation, we evaluated the surface expression of different markers on dividing donor T cells. In mice receiving donor T cells alone, cell division was associated with increased expression of activation markers for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells: CD25 was up-regulated, and by day 3.5, >40% of donor T cells that had divided were CD25<sup>+</sup>. In marked contrast, only a small fraction of donor T cells that had divided up-regulated CD25 when rsTregs were added (9% of the CD4<sup>+</sup> T cells and 7% of the CD8<sup>+</sup> T cells). The addition of polyTregs generated an intermediate effect (Fig. 6a). CD69 is a very early activation

marker, which becomes highly expressed, even before the first cell division, and then down-regulates after several divisions. For this reason, CD69 expression was analyzed at day 2.5 post-HSCT and, consequently, was not shown in Fig. 6. At this time point, we observed the inhibition of CD69 induction on dividing donor T cells in the presence of Tregs. Their proportion was reduced by 84% with rsTregs and 68% with polyTregs, compared with control mice not receiving Tregs. We also analyzed markers commonly used to distinguish naive (CD62L) and activated/memory (CD44) T cells (Fig. 6a). In mice grafted without Tregs, CD62L was down-regulated on half of CD4<sup>+</sup>, and most of CD8<sup>+</sup> donor T cells that had undergone division. When rsTregs were added, only a small fraction of cells that had divided down-modulated CD62L. The effect of polyTregs on donor T cells was less marked. Additionally,



**FIGURE 6.** The presence of Tregs modifies the surface expression of activation markers and cytokine production by donor T cells. Donor T cells were labeled with CFSE before their infusion into semiallogenic (BALB/c  $\times$  C3H) $F_1$  lethally irradiated recipients (Thy-1.2). *a*, Surface expression of CD25, CD62L, and CD44 was analyzed on CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells (Thy-1.1<sup>+</sup>) 3.5 days after transplantation when they were either infused alone ( $n = 11$ ), in the presence of  $10 \times 10^6$  rsTregs ( $n = 14$ ), or in the presence of  $10 \times 10^6$  polyTregs ( $n = 8$ ). *b*, The percentage of IFN- $\gamma$ -producing cells in the pool of dividing CD3<sup>+</sup> donor T cells was determined at day 3.5 in mice receiving  $10 \times 10^6$  donor T cells alone ( $n = 8$ ) or concomitantly infused with  $10 \times 10^6$  rsTregs ( $n = 8$ ) or  $10 \times 10^6$  polyTregs ( $n = 4$ ). Data are expressed as the mean of the percentage of producing cells  $\pm$  SEM. Data were pooled from three to seven independent experiments.

the memory CD44 marker was up-regulated upon division on donor T cells. In this study, mice coinjected with rsTregs or polyTregs had the percentage of CD4<sup>+</sup>CD44<sup>high</sup> donor T cells reduced among cells that had undergone division. On CD8<sup>+</sup> T cells, the addition of rsTregs reduced the percentage of CD8<sup>+</sup>CD44<sup>high</sup> T cells among cells that had undergone division. Little or no effect was observed when polyTregs were added. We also analyzed the production of IFN- $\gamma$  in donor T cells by flow cytometry using intracellular staining after *in vitro* restimulation with an anti-CD3 Ab. At day 3.5, in the absence of Tregs, 23% of divided donor T cells produced IFN- $\gamma$ . In the presence of rsTregs, 2.7% of divided donor T cells produced IFN- $\gamma$ , whereas in the presence of polyTregs, 8% of divided donor T cells made IFN- $\gamma$  (Fig. 6*b*).

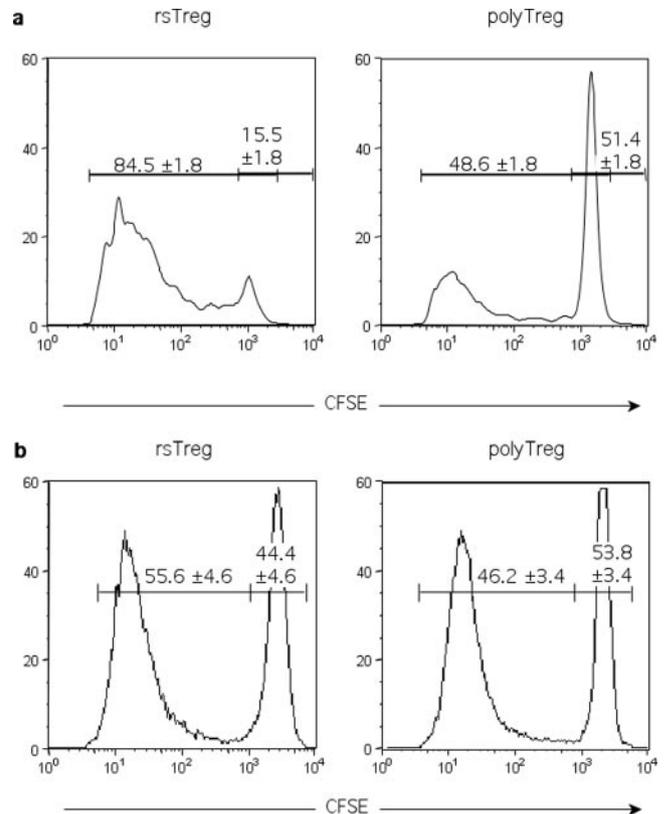
In conclusion, on a cell-per-cell basis, both expanded Tregs inhibit acquisition of activation markers by donor T cells and their differentiation of IFN- $\gamma$ -producing T cells, with a more pronounced effect of rsTregs, compared with polyTregs used at equivalent cell doses.

#### *In vivo* increased division of rsTregs, compared with polyTregs

We tested whether the differences observed between rsTregs and polyTregs in modulating donor T cell activation correlated with the capacity of Tregs to proliferate *in vivo* early after infusion into recipient mice. In our model, Tregs expressing the congenic marker Thy-1.2 were used to facilitate distinction from their non-Treg counterparts. Following BM transplant, mice were injected with CFSE-labeled Thy-1.2<sup>+</sup> Tregs. To identify infused Tregs by flow cytometry, we gated on Thy-1.2<sup>+</sup>CFSE<sup>+</sup> double-positive cells to distinguish them from residual host Thy-1.2<sup>+</sup>CFSE<sup>-</sup> T cells. Because Tregs that had divided >7-fold could not be formally distinguished from recipient T cells due to diminished CFSE signal, this procedure likely underestimated the percentage of divided Tregs. However, even under these circumstances, we observed that >80% of rsTregs had divided at least once at day 3.5, with a majority of cells dividing five or more times (Fig. 7*a*). The percentage of divided polyTregs was significantly lower. The increased capacity of rsTreg to proliferate in these conditions could be related either to their specificity or, alternatively, to intrinsic properties due to culture conditions. Thus, we compared the capacity of polyTregs and rsTregs to divide *in vivo* under the unique driving force of homeostatic expansion. For this, lethally irradiated BALB/c mice were grafted with BALB/c donor T cells supplemented with rsTregs or polyTregs. At day 3.5, we did not observe any significant differences in Treg division between rsTregs and polyTregs (Fig. 7*b*). Thus, after culture, these two types of expanded Tregs have similar capacity to proliferate *in vivo*. Interestingly, in this experiment, ~50% of rsTregs and polyTregs had divided at least once at day 3.5. The same proportion of divided polyTreg was observed when they were infused in semiallogeneic recipients. This suggests that the division of polyTregs in semiallogeneic recipients could be due to homeostatic expansion. This also shows that the level of suppression exerted by Tregs on donor T cell proliferation may correlate with the *in vivo* level of Treg activation and/or degree of Treg proliferation.

## Discussion

It is now well documented from experimental data obtained in mice that Tregs represent a potential new therapeutic modality for the control of GVHD. Due to 1) the low frequency of Tregs in the T cell compartment and 2) the likely requirement that at least one Treg would need to be infused for every infused donor T cell to achieve a clinical benefit in the setting of murine GVHD, a phase of *ex vivo* expansion of Treg would probably be mandatory for this approach to be effective in humans. For this, at least two clinically



**FIGURE 7.** rsTregs expanded more rapidly *in vivo* than their polyTreg counterparts in semiallogeneic recipients. *a*, Before their coinfusion with donor T cells in lethally irradiated semiallogeneic recipients, rsTregs ( $n = 10$ ), polyTregs ( $n = 8$ ) were labeled with CFSE. At day 3.5, splenocytes from grafted animals were collected. The injected Tregs were detected in the spleen of grafted animals by gating on CFSE<sup>+</sup>Thy-1.2<sup>+</sup> cells. Cell proliferation was measured as the sequential loss of CFSE within the Thy-1.2<sup>+</sup> cell population by flow cytometry. Data are expressed as the mean of the percentage of dividing cells  $\pm$  SEM. Data were pooled from four to seven independent experiments. *b*, The same experiment was reproduced in lethally irradiated syngeneic recipients with rsTregs ( $n = 2$ ) or polyTregs ( $n = 2$ ) collected at day 3.5 or 5.5 (data not shown).

relevant approaches can be envisaged. Tregs with a diversified repertoire can be obtained after culture in the presence of anti-CD3 Ab or with anti-CD3/CD28-coated microbeads. Alternatively, Tregs can be expanded and, at the same time, selected for their capacity to recognize allogeneic Ags so that to expand a population of recipient-specific alloreactive Tregs. We and others have demonstrated that both polyTregs and rsTregs efficiently prevent GVHD (14, 16). After confirming the preventive effects of both types of expanded Tregs in this work, we described their mode of action on donor T cells.

In a model of GVHD prevention using freshly purified Tregs, Edinger et al. (11) showed a strong inhibition of expansion of donor T cells. However, in a cell-per-cell analysis of donor T cells, the addition of freshly purified Tregs had minimal or no effect on the acquisition of activation markers and IFN- $\gamma$  production. In contrast, we observed in this study that expanded Tregs strongly inhibited the division and expansion of donor T cells. However, expanded Tregs additionally inhibit the capacity of residual activated donor T cells to express activation markers and to produce IFN- $\gamma$ . It was demonstrated that the *in vitro* activation of Tregs led to an increase of their suppressive activity (24). This property of Tregs could explain why expanded Tregs seem to have a more profound suppressive effect on the activation of donor T cells in an

HSCT setting. GVHD is due to the early expansion and differentiation of alloreactive donor T cells. Because expanded Tregs potentially inhibit such activation, this explains their capacity to prevent the disease.

Whereas both types of expanded Tregs prevent GVHD, we observed differences in the intensity of the effect of rsTregs and polyTregs. The better effect of rsTregs could rely on their increased ability to rapidly become activated *in vivo*, as shown in the present study, and exert their immunosuppressive effect early after infusion. In further support of this concept, it was recently showed that delaying Treg injection after day 4 was ineffective in treating GVHD (15). Due to the procedure of culture, rsTregs are selected for their ability to recognize host alloantigens. Early after injection, most of them are likely activated by their cognate allogeneic Ags.

Another point is that rsTregs also could display a suppressive effect mainly targeted to alloreactive donor T cells, whereas nonalloreactive donor T cells could be partially spared. In a previous report, we compared the characteristics of allogeneic vs homeostasis-driven T cell proliferation by analyzing the kinetics of cell division and phenotypic changes that occur in donor CD8<sup>+</sup> and CD4<sup>+</sup> T cells after transfer in either semiallogeneic or syngeneic lymphopenic mice. After T cell transfer in lymphopenic recipients, we observed that alloreactive T cells rapidly divided, up-regulated CD69 and CD25 molecules, and down-regulated CD62L. In contrast, during homeostatic expansion, syngeneic T cells started to divide later and did not up-regulate CD69 and CD25, whereas they lost CD62L only after eight only divisions. Thus, alloreactive and nonalloreactive T cells can be discriminated by the expression of these different markers (23). In this study, we observed that T cells that divide in the presence of rsTregs behaved as nonalloreactive T cells. Indeed, rsTregs dramatically reduced the expansion/differentiation of donor T cells, and most of the remaining dividing donor T cells neither expressed CD25 nor up-regulated CD44 while CD62L expression was maintained. Additionally, the percentage of IFN- $\gamma$ -producing cells was strongly reduced with rsTregs. This suggests that, when using rsTregs, activation and differentiation of alloreactive T cells in effector T cells involved in GVHD are inhibited, whereas nonalloreactive T cells can expand. After allogeneic HSCT, good immune reconstitution requires 1) an absence of GVHD, because lymphoid organs are usually attacked during GVHD and 2) homeostatic proliferation of donor T cells. Because we observed a better immune reconstitution when rsTregs were used, it is likely that, in this situation, nonalloreactive donor T cells expand significantly in the recipient and participate to the improved immune reconstitution.

The use of polyTregs vs rsTregs for the control of GVHD in allogeneic HSCT is an area of debate of intense interest. However, to date, nothing is known regarding the respective therapeutic benefit of these two types of Tregs in humans. In a clinical setting, the primary difference between these two types of Tregs is the method used to activate and drive the expansion of the cells; in one case using irradiated APCs for specific activation (rsTregs) and in the other case using anti-CD3/CD28-coated microbeads for polyclonal activation (polyTregs). The generation of high numbers of polyTregs is a rapid and simple process when purified Tregs are cultured in the presence of anti-CD3/CD28-coated microbeads, IL-2, and feeder cells (25). Alternatively, purified polyTregs also could be obtained after culture in the presence of donor APCs plus anti-CD3. However, this procedure seems to be more complicated without the benefit of the specificity of the Tregs obtained.

The generation of rsTregs would probably raise more technological issues because of the necessity to use APCs, as opposed to clinically standardized beads. The necessity to select nonmalignant

APCs when HSCT is performed for the treatment of leukemia could be an additional major hurdle. Furthermore, the duration of the culture to achieve clinically relevant cell numbers could be longer with rsTregs. Indeed, in mice, to obtain similar high numbers of Tregs, polyTregs were generated over 21 days and rsTregs over 28 days. An additional difference is that murine IL-2 was used for the generation of rsTregs, and human IL-2 was used for the generation of polyTregs. It is not clear whether these differences could affect the *in vivo* potential of the *ex vivo*-expanded Tregs.

In this study, we used equivalent numbers of polyTregs and rsTregs and observed that generally rsTregs are more efficient than polyTregs in controlling GVHD. It is quite possible that infusion of higher numbers of polyTregs might achieve the same level of GVHD inhibition and promotion of immune reconstitution. In fact, studies by Taylor et al. (14) demonstrated that by increasing the number of polyTreg 3-fold (three Tregs per infused allogeneic T cell), nearly complete inhibition of overt GVHD was durably achieved despite the presence of histopathological signs of GVHD in target organs (>10 mo). Compared with mice receiving  $10 \times 10^6$  polyTregs, when we injected  $50 \times 10^6$  polyTregs, immune reconstitution was improved. However, in this latter group, immune reconstitution was less efficient as compared with mice receiving  $10 \times 10^6$  rsTregs (preliminary data not shown). However, to be clarified, this point deserves specific experiments, such as comparing the effect of rsTregs and polyTregs expanded by the same techniques (using syngeneic irradiated APCs and anti-CD3) together with performing Treg dose-response experiments.

Our work demonstrates that rsTregs are very efficient in providing durable protection from GVHD and facilitate immune reconstitution after allogeneic HSCT in mice. Thus, we propose that rsTregs should be explored as a viable modality for treating human GVHD in the setting of allogeneic transplantation. Although preclinical data have demonstrated that both rsTregs and unselected Tregs help to maintain the GVL effect (11, 16), this still remains to be studied in clinical trials. Thus, we suggest that patients eligible for allogeneic HSCT for the treatment of nonmalignant hematological disorders represent, in a first step, a preferable application for this new therapeutic strategy. Such a clinical setting should permit bypassing the question of the separation of GVHD and the GVL effect with Tregs, together with the capacity to dispose of nonmalignant APCs in patients with leukemia to generate rsTregs.

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## Disclosures

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