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Article **Immunology**

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Recipient-type specific CD4$^+$CD25$^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many hematological malignancies and hematological disorders. This approach relies on the elimination of the hematopoietic compartment, including malignant cells, by high-dose chemotherapy and irradiation and the reconstitution of a new hematopoietic system provided by the donor graft containing hematopoietic stem cells (1). The transplant also contains mature T cells capable of recognizing allogeneic host antigens. The graft-versus-host reaction induced by donor T cells is responsible for the main complication encountered after allogeneic HSCT: graft-versus-host disease (GVHD) (2). To date, an immunosuppressive regimen based on cyclosporin A associated with methotrexate and/or corticosteroids administered in the months following HSCT is the most efficient preventive treatment but remains only partially effective (3). Procedures in which T cells are depleted from the transplant can prevent GVHD but have also revealed the important role that donor T cells play in the prevention of graft rejection (4), in lowering risks of infection, and in the graft-versus-leukemia (GVL) and/or the graft-versus-tumor (GVT) effects (5). Thus, administering donor allogeneic T cells without causing GVHD remains the key challenge of allogeneic HSCT.

Recently, a particular subpopulation of CD4$^+$ T cells, which constitutively expresses the IL-2 receptor α chain (CD25) and which constitutes 5%–10% of the whole CD4$^+$ T cell pool in mice and humans, has been identified for its crucial role in the control of autoimmune processes (6, 7). In different models in which mice were rendered deficient for this CD4$^+$CD25$^+$ regulatory T cell (Treg) population, multiple T cell–mediated, organ-specific autoimmune diseases were observed (refs. 6, 8–10, and for review see refs. 11–13). One particularly important property of these Treg's is their capacity to mediate in vitro suppression of conventional CD4$^+$ or CD8$^+$ T cells (14–16). This property of the so-called Treg's was demonstrated in mice and humans.

Recent reports have shown that Treg's play a major role in tolerance induction to allogeneic transplants (17–20). We and others have also demonstrated that

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Nonstandard abbreviations used: hematopoietic stem cell transplantation (HSCT); graft-versus-host disease (GVHD); graft-versus-leukemia (GVL); graft-versus-tumor (GVT); bone marrow transplantation (BMT); CD4$^+$CD25$^+$ regulatory T cell (Treg); allophycocyanin (APC); phycoerythrine (PE); specific-Treg (sTreg); irrelevant-Treg (irTreg); alloantigen (allo-Ag).

Treg's can be used in strategies aimed at controlling GVHD following allogeneic bone marrow transplantation (BMT) in mice. Adding freshly isolated Treg's to the donor inoculum containing alloreactive T cells efficiently prevents GVHD in lethally irradiated mice (21, 22). Nevertheless, a major limitation for the clinical use of Treg's to prevent GVHD would be the difficulty of obtaining a sufficient number of these relatively rare cells from a single donor. This issue could potentially be resolved by cell culture. Indeed, we recently demonstrated that Treg's can be dramatically expanded by in vitro stimulation with allogeneic irradiated splenocytes plus IL-2. The administration of Treg's obtained after culture in the presence of irradiated recipient-type allophycocyanin (APC), at the time of grafting, can significantly delay or even prevent GVHD (21). These preclinical models have demonstrated that Treg's represent a feasible approach for controlling GVHD.

Even if the possibility of controlling GVHD using Treg's seems established, it is now essential to determine whether or not administering Treg's is compatible with immune reconstitution and GVL/GVT effects. Indeed, a possible nonspecific effect of Treg's on immune reconstitution has been reported (23). After HSCT, it could impair immune reconstitution and, consequently, result in general immunosuppression and loss of GVL/GVT effects. Here we showed that Treg's specific for recipient-type alloantigens (allo-Ag's), but not the ones specific for third-party allo-Ag's, controlled GVHD while it favored immune reconstitution. Importantly, under these conditions, a GVT effect against leukemia was also maintained.

Methods

Mice and experimental GVHD. C57Bl/6 (B6) (H-2^b), BALB/c (H-2^d), C3H (H-2^k), [B6 × DBA/2]F1 (H-2^{bxd}), and [BALB/c × C3H]F1 (H-2^{dxk}) 5- to 9-week-old female mice were obtained from Iffa Credo (L'Arbresle, France). Congenic Thy-1.1 BALB/c mice (H-2^d) were bred in our animal facility. Mice were manipulated according to European Union guidelines. HSCT were performed as previously described (21), unless otherwise stated. Briefly, 24 hours after lethal irradiation of 9-week-old [BALB/c × C3H]F1 recipients (9.5 Gy) or 9-week-old [B6 × DBA/2]F1 recipients (11 Gy) or BALB/c (8 Gy), mice were transplanted with cells from BALB/c mice for the first strain or B6 mice for the two other strains. The transplant comprised 5×10^6 T-depleted bone marrow (BM) cells, 10×10^6 T cells (BALB/c donor), or 0.5×10^6 T cells (B6 donor) collected from pooled spleen and peripheral LNs, and, when indicated, 10×10^6 BALB/c or 0.5×10^6 cells B6 ex vivo expanded Treg's. Control groups were constituted of nongrafted irradiated mice or irradiated mice receiving only BM cells.

Leukemia and tumor models. We injected 2×10^4 mastocytoma cells P815 (derived from DBA/2 mice) or 1×10^3 A20 cells (derived from BALB/c mice) i.v. in the retroorbital sinus at the time of BMT in lethally irradiated [B6 × DBA/2]F1 or BALB/c mice, respectively. P815 cells were identified in peripheral blood by flow cytometry by the surface expression of H-2^d (recipient type) but not H-2^b (donor type) histocompatibility antigens. A20 cells were identified by the surface coexpression of H-2^d histocompatibility antigens (recipient type) and B220 molecules.

Ex vivo expansion of Treg's. Treg's were purified as previously described (21). Briefly, spleen and peripheral LN cells were first labeled with biotin-labeled, anti-CD25 mAb (7D4, Becton Dickinson, San Diego, California, USA) and streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified using magnetic cell large selection columns (Miltenyi Biotec). $CD25^+$ cells were then stained with FITC-labeled anti-CD4 (GK1.5), phycoerythrine-labeled (PE-labeled) anti-CD62L (MEL-14) and streptavidin-Cy-Chrome (Pharmingen, San Diego, California, USA), which bound to free biotin-labeled CD25 molecules, uncoupled to beads. The $CD4^+CD25^+CD62L^{high}$ T cells were sorted by flow cytometry using a FACStar^{Plus} (Becton Dickinson), yielding a purity of 99%. Treg's were cultured in the presence of 20-Gy-irradiated APCs and murine IL-2 (10 ng/ml, R&D Systems, Abingdon, United Kingdom), as previously described (21).

Proliferation assays. Treg's purified from BALB/c mice were stimulated for 3 weeks by 20-Gy-irradiated C3H or B6 splenocytes. After removal of dead cells by gradient of lymphocytes separation medium (Eurobio, Les Ulis, France), and five washes to remove residual IL-2, 1×10^5 expanded BALB/c Treg's were added to the culture of 1×10^5 fresh CD25-depleted T cells (purified from BALB/c spleen) stimulated by 1×10^5 irradiated C3H or B6 splenocytes. Cells cultured in round-bottom, 96-well plates for 96 hours were pulsed with [^{3}H] methyl-thymidine for the last 18 hours.

FACS analysis. The following Ab's were used for FACS analysis: PE-labeled anti-Thy-1.1 (clone OX-7, Becton Dickinson), anti-CD3 (clone 500A2), anti-Th1.2 (clone 30-H12) or anti-H-2Kb (clone AF6-88.5), FITC-labeled anti-CD8 (clone53-67) or anti-H-2Kd (clone SF1-1.1), biotinylated anti-CD25 (clone 7D4) revealed with streptavidin-Cy-Chrome (Pharmingen) and APC-labeled anti-CD4, and APC-labeled anti-B220 (clone RA3-6B2, Pharmingen). At least 10^5 events were acquired on a FACScalibur (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Histopathological examination. Livers were preserved in Bouin's fixative and embedded in paraffin. Spleens and LNs were embedded in Tissue-Tek (Sakura Finetek Europ B.V., Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen. 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. with minor modifications (24). 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 were scored in each spleen sample according to three items that were graded as follows: lymphoid atrophy (0, absent; 1, lymphoid periarteriolar sheath without follicle; 2, density of lymphoid periarteriolar sheath intermediate between score 1 and 3; 3, loss of lymphoid periarteriolar sheath); peliosis (0, absent; 1, involving less than one third of the spleen; 2, involving between one third and two thirds of the spleen; 3, involving more than 2/3 of the spleen) and fibrosis (0, absent; 1, mild interstitial fibrosis; 2, moderate interstitial fibrosis; 3, foci of diffuse fibrosis). The empty spleen was graded as 9. The sum of item scores for each organ defined a total score

Immunohistochemistry. Five-micrometer-thick spleen or LN sections were fixed in acetone for 5 minutes and then washed in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6 for 5 minutes. The sections were successively incubated with anti-Thy-1.1 (1:100; clone OX7, Pharmingen) or anti-Thy-1.2 (1:100; clone 30-H12, Pharmingen) biotinylated antibodies for 1 hour and streptABC complex/AP (code K0391, Dako, Trappes, France) for 30 minutes. Alkaline phosphatase activity was revealed using the Fast Red Substrate System kit (code K0699, Dako) according to the manufacturer's instructions. The sections were lightly counterstained with hematoxylin and mounted in Immu-Mount (Shandon, Pittsburgh, Pennsylvania, USA). Each sample was scored according to the number of Thy-1.1 or Thy-1.2 positive cells as follows: (0, no or rare cells; 1, low number; 2, intermediate number; 3, high number of stained cells).

Statistical analysis. Statview software (Abacus Concepts, Berkeley, California, USA) was used for statistical analyses. ANOVA was performed to compare weight curves (the protected least significant differences Fisher test). The nonparametric Mann-Whitney *U* test was used to compare GVHD scores and immune reconstitution. Kaplan-Meier survival curves were established for each group. Mice suffering from advanced stages of GVHD or leukemia were killed for histopathological examination and considered to be dead in the Kaplan-Meier analysis. *P*-values are indicated only when the difference between the two groups was statistically significant.

Results

Ex vivo expansion of Treg's. We first investigated, in a semiallogeneic BMT setting, the effect of Treg's rendered specific for recipient allo-Ag's (further referred to as sTreg's) and of Treg's rendered specific for third-party allo-Ag's, which are thus irrelevant in the genetic combination of BMT used in this work (further referred to as irTreg's). Because the irTreg population likely contained rare or no recipient-type, antigen-specific Treg's (21), their use as a control should allow an evaluation of any potential nonspecific effect of Treg's. Purified Treg's from BALB/c mice were stimulated by allogeneic irradiated C3H APCs (sTreg's) or B6 APCs (irTreg's). In these conditions, numbers of Treg's were dramatically increased by a factor of 4,500 for sTreg's or 13,000 for irTreg's, as previously described, using other genetic combinations and also maintained their $CD4^+CD25^+CD62L^{high}$ phenotype after expansion (21). We tested cells that had been cultured for 42 days and that had not received stimulation by fresh APCs since day 35 for their in vivo functionality in a semiallogeneic BMT setting.

Comparison of the effect of sTreg's and irTreg's on clinical GVHD. In order to be able to analyze the effects of sTreg's and irTreg's on GVHD and immune reconstitution, we used a model of semiallogeneic BMT in which GVHD did not induce rapid mortality. When 9.5-Gy-irradiated [BALB/c × C3H]F1 mice were grafted with 5×10^6 bone marrow cells supplemented with 10×10^6 T cells from BALB/c mice, the mice developed severe clinical signs of GVHD, such as hunching, dull fur, skin lesions (Figure 1a, upper panel), weight loss (Figure 1b), and strong diarrhea but did not die during the first 45 days after transplantation. When 10×10^6 sTreg's (BALB/c Treg's cultured in the presence of C3H APC) were added to the inoculum, no clinical signs of GVHD were observed for the duration of the experiment (Figure 1a, lower panel). The mean weight curve of these mice was undistinguishable from that of control mice receiving BM cells alone and not developing GVHD. Thus in this experimental model, adding sTreg's efficiently and durably prevented the occurrence of clinical GVHD. When mice received irTreg's (BALB/c Treg's cultured in the presence of B6 APC), the clinical outcome was different. After a short period of weight gain (days 5–10), weight curves declined rapidly and continually, as observed in mice of the GVHD control group (Figure 1b), although hunching, dull fur, skin lesions, and strong diarrhea were not observed (not shown).

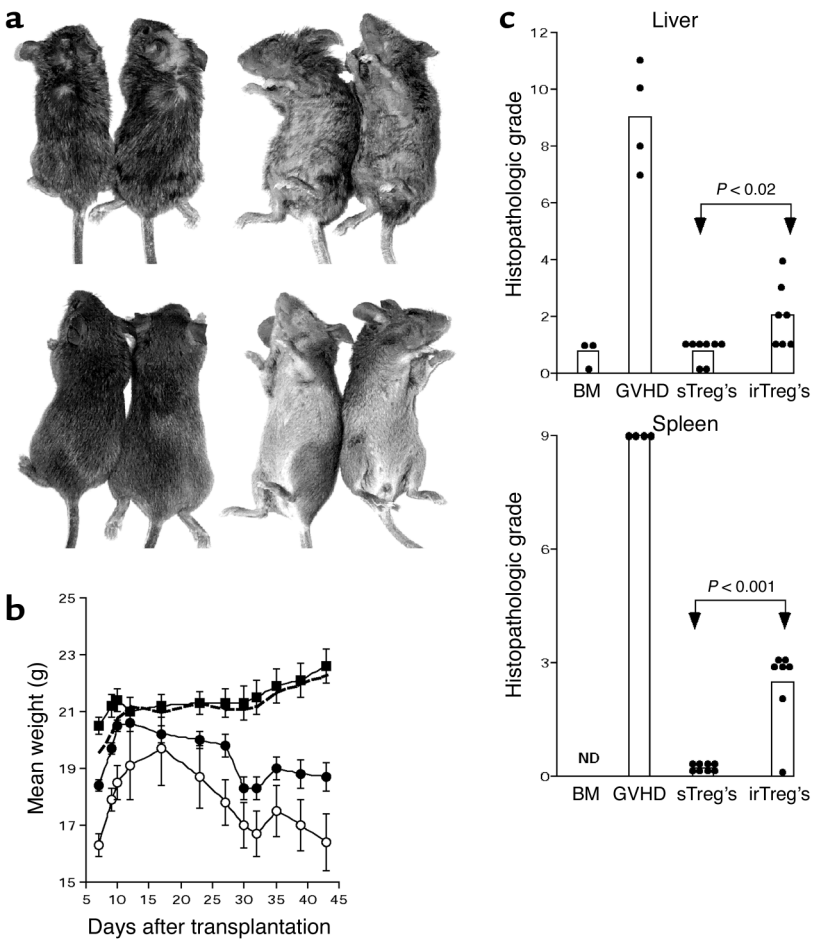

Figure 1
Regulation of GVHD by the addition of ex vivo–expanded Treg's. At the end of the culture, Treg's were tested for their capacity to control GVHD in the BALB/c → [BALB/c × C3H]F1 combination. (**a**) The picture illustrates the skin lesions and general status of grafted [BALB/c × C3H]F1 mice undergoing GVHD (upper pairs) or mice protected from GVHD by adding sTreg's (lower pairs). (**b**) Mice were weighed at different time points prior to sacrifice at day 45. Mean weight curves were established for mice receiving BM cells alone (dashed line, $n = 3$), BM cells supplemented with 10×10^6 conventional T cells (open circles, $n = 5$) in addition to either 10×10^6 sTreg's (filled squares, $n = 15$) or irTreg's (filled circles, $n = 14$). $P < 0.05$ between all groups except for BM cells alone versus sTreg's. (**c**) Histopathologic score of liver and spleen after semiallogeneic BMT. Grading of GVHD was performed 45 days after transplantation in liver and spleen. BM control mice infused with BM cells alone did not develop GVHD ($n = 3$). ND, not done. GVHD control mice received BM cells plus T cells and represented the maximum intensity of GVHD in this model ($n = 4$). Experimental mice received BM cells plus T cells and either sTreg's ($n = 8$) or irTreg's ($n = 7$). Points correspond to histopathological scores of individual mice; histograms show the mean histopathological score for each group. $P < 0.05$ between all groups for all tissues except for BM cells alone versus sTreg's and BM cells alone versus irTreg's in the liver.

Mice were killed at day 45 after transplantation for histopathological studies, a time point at which all the mice of the GVHD control group developed strong clinical signs of GVHD. In control mice grafted with semiallogeneic BM cells and T cells, severe histological signs of GVHD were observed in the small and large bowel, skin, liver, and spleen. When sTreg's were added to the transplant containing BM cells and T cells, no histological signs of GVHD (grade 0 or 1) were detected in the small bowel, skin, liver, and spleen Treg's, although two of the eight mice exhibited mild signs of GVHD in the large bowel (Figure 1c and not shown). This confirmed, at the infraclinical level, the potent effect of sTreg's for the prevention of GVHD. In contrast, in mice receiving irTreg's instead of sTreg's, histological analysis of target organs clearly showed signs of GVHD in the spleen for six out of seven mice, in the liver for four out of seven mice (Figure 1c), and in the large bowel but not in the skin for three out of seven mice (not shown). These histological signs of GVHD were of lower intensity than those seen in the GVHD control group but were significantly higher than in mice receiving sTreg's or BM cells alone. Together, these results show that sTreg's can efficiently prevent GVHD, whereas irTreg's only provide a partial protection.

Enhanced immune reconstitution after semiallogeneic BMT with sTreg's as compared with irTreg's. The foregoing experiments were performed after infusion of similar proportions of total T cells and Treg's to obtain a clinical effect. Since it has been demonstrated in vitro that Treg's can mediate bystander suppression of conventional $CD4^+$ or $CD8^+$ T cells (15, 25), general immunosuppression could be a possible drawback of this strategy. We thus tested the effects of Treg injections on subsequent immune reconstitution by evaluating the number of total splenocytes, together with B and T cell reconstitution. In mice receiving BM cells alone, good immune reconstitution was achieved by 45 days after transplantation, with spleens containing about 80×10^6 cells, approximately 50% of which were B cells and 15% T cells. In contrast, control mice receiving BM cells plus T cells displayed strong lymphopenia characterized by profound splenic atrophy and an absence of both the B and T cell compartments (Figure 2), compatible with severe GVHD (26). Strikingly, adding sTreg's efficiently prevented lymphopenia, since spleens contained about 150×10^6 cells, with approximately 55% B cells and 15% T cells. Interestingly, the numbers of B and T cells (both $CD4^+$ and $CD8^+$) were increased in comparison with mice receiving BM cells alone. Thus, adding sTreg's in the transplant along with donor T cells favored immune reconstitution.

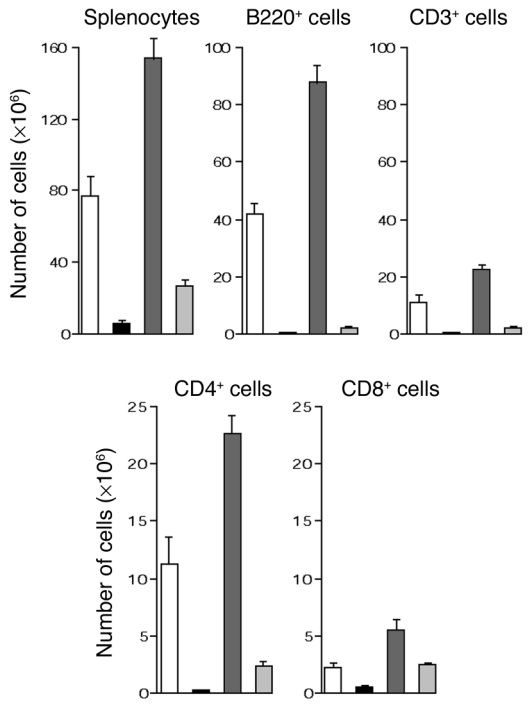

Figure 2
Regulation of GVHD by sTreg's is associated with good immune reconstitution. Immune reconstitution was evaluated 45 days after transplantation in the spleen of mice grafted as described in Figure 1. Total splenocytes were counted and stained with appropriate mAb's. The number of $B220^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells was evaluated after analysis by flow cytometry for BM control mice (white bars; $n = 3$), GVHD control mice (black bars; $n = 4$), mice receiving sTreg's (dark gray bars; $n = 5$), or irTreg's (light gray bars; $n = 5$). Histograms indicate the mean number ± SEM of cells for each group. $P < 0.05$ between all groups and for all cell populations except for BM alone versus irTreg's for $CD8^+$ cells.

In contrast, mice receiving irTreg's displayed splenic atrophy compatible with an infraclinical GVHD and had slightly more total splenocytes and B and T cells (both $CD4^+$ and $CD8^+$ subpopulations) than mice of the GVHD control group. Together, these results revealed that sTreg's favor immune reconstitution and irTreg's do not.

Increased number of sTreg's as compared to irTreg's in vivo. We next tested whether the differences observed between sTreg's and irTreg's in modulating GVHD and in promoting immune reconstitution were associated with their capacity to proliferate and/or to survive in vivo after their infusion into recipient mice. In our model, only Treg's expressed the congenic marker Thy-1.1, which was thus used to trace them. When Thy-1.2 mice received 10×10^6 sTreg's, 0.3×10^6 Thy-1.1^+ cells were detected by flow cytometry in the spleen at day 45 after transplantation. Most of these Thy-1.1^+ cells still expressed both CD4 and CD25 markers (Figure 3a). In contrast, when the same number of irTreg's were used, only 0.006×10^6 Thy-1.1^+ cells were detected in the spleen at day 45. This observation was confirmed by immunohistochemistry performed in the spleens and LNs of grafted mice. In mice treated with sTreg's, numerous Thy-1.2^+ T-cells were detected in the T cell zone of the spleen, attesting to the good T cell reconstitution in these mice protected from GVHD (Figure 3c) and consistent with the flow cytometry data (Figure 3a). In these mice, Thy-1.1^+ Treg's were easily detected in the spleen of protected mice (Figure 3b,c). In contrast, lower numbers of Thy-1.2^+ T-cells were detected in the spleens of mice receiving irTreg's (Figure 3c), attesting to poor T cell reconstitution and confirming flow cytometry data (Figure 3a). In these tissues, few or none of the Thy-1.1^+–infused Treg's were still present. Comparable results were also observed in the LNs of grafted animals (not shown).

We tested whether the increased proportion of sTreg's as compared with irTreg's resulted from a difference in their proliferation to specific allo-Ag's. We previously observed, using the same genetic combinations, that BALB/c sTreg's, generated by a 2-week-culture in the presence of C3H APCs, proliferated very strongly when restimulated in vitro with C3H APCs, as compared with irrelevant B6 APCs (21). Here, we tested whether similar differences in the proliferation of cultured Treg's to specific allo-Ag's can also be observed in vivo. First, we addressed this point by injecting CFSE-stained Treg's into nonirradiated, semiallogeneic recipient mice. The choice of using nonirradiated recipient mice was driven by previous reports showing that homeostatic expansion of Treg's occurs in lymphopenic syngeneic hosts (27, 28). When C3H-specific cultured BALB/c Treg's were transferred into [BALB/c × C3H]F1 recipients, they rapidly proliferated, with three to four rounds of division already evidenced by day 3 after infusion. In contrast, irTreg's, which were in vitro selected for their capacity to respond to B6 APC, but not C3H APC, did not divide. An increased proportion of divided sTreg's as compared with irTreg's was still observed at day 14 but not at day 28 (Figure 4a). The presence of divided irTreg's at day 28 was likely due to the extensive division of autoreactive Treg's in the steady state, as we reported recently (29). Early proliferation of sTreg's resulted in an increased absolute number of sTreg's as compared with irTreg's (Figure 4b). Then, we tested whether such observations could also be made in a BMT setting. Here again, the number of divided sTreg's was increased at day 3 as compared with the number of irTreg's when infused in lethally irradiated recipients (Figure 4c). These results likely explain the better potential of sTreg's to durably control GVHD as compared with irTreg's.

Nevertheless, we also observed a partial beneficial effect against GVHD with irTreg's, as illustrated by general clinical status and histopathological analysis observed in grafted mice (Figure 1). We thus tested whether irTreg's could suppress activation of T cells stimulated by third-party allo-Ag's in vitro. When BALB/c $CD25^-$ T cells were stimulated by irradiated allogeneic C3H splenocytes, adding sTreg's strongly

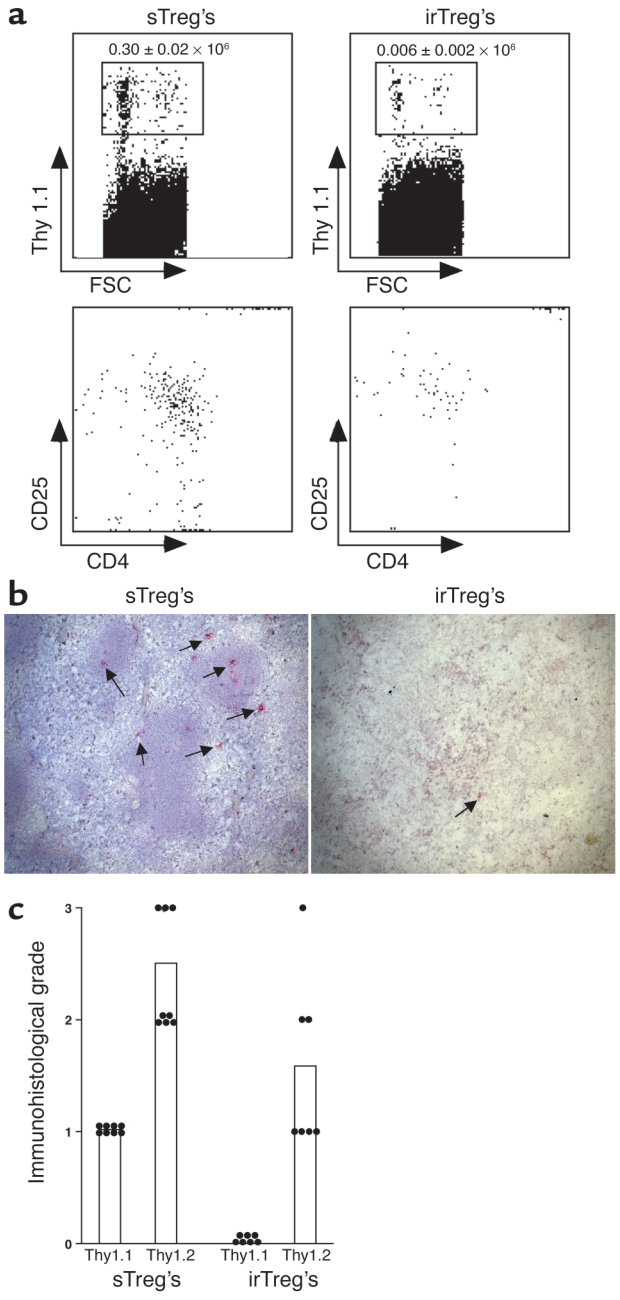

Figure 3
Increased survival of sTreg's as compared to irTreg's in the spleen of grafted mice. The injected Treg's were detected in the spleen of animals grafted as described in Figure 1 by the expression of the Thy-1.1 congenic marker 45 days after transplantation. (**a**) Upper panels show proportions of Thy-1.1$^+$ cells after they received either sTreg's (n = 5) or irTreg's (n = 5). Values indicate mean ± SEM of the absolute number of Thy-1.1$^+$ cells. $P < 0.05$ between the two groups. Lower panels show the CD4 CD25 phenotype of cells gated on Thy-1.1$^+$ cells. FSC, forward scatter. (**b** and **c**) The presence of injected Thy-1.1$^+$ Treg's was also evaluated in the spleen of grafted animals by immunohistochemistry. (**b**) Arrows indicate Thy-1.1–positive cells. (**c**) Each spleen was scored for the presence of injected Treg's (Thy-1.1) or other T cells (Thy-1.2) in grafted mice receiving either sTreg's (n = 8) or irTreg's (n = 7). The y-axis indicates the intensity of staining ranging from 0 to 3. Each point corresponds to the histopathological score of an individual mouse; histograms indicate the mean histopathological score for each group. $P < 0.05$ between sTreg's versus irTreg's for Thy-1.1.

inhibited T cell proliferation, as previously described (21). Surprisingly, irTreg's inhibited T cell proliferation as well. Similar findings were reproduced in another genetic combination (Figure 4d). These results indicate that irTreg's specific for particular allo-Ag's maintains suppressive activity on conventional T-cells stimulated by third-party allo-Ag's.

GVL/GVT effects after prevention of GVHD using sTreg's. We finally tested whether GVL/GVT effects could be maintained when sTreg's were used to control GVHD. We first used the A20 leukemia cells of BALB/c origin. We thus developed a new model of GVHD in which lethally irradiated BALB/c mice were grafted with BM and T cells collected from B6 mice. In this experiment, sTreg's were obtained after culture of purified B6 Treg's stimulated by BALB/c-irradiated splenocytes. When mice received BM cells plus A20 cells, four out of five mice died from day 25 to day 42 from leukemia, as attested by the presence of A20 cells in the blood at days 15, 22 and 37. Mice receiving BM cells alone remained healthy (Figure 5a and not shown). Mice receiving BM and T cells together with A20 cells died with characteristic clinical signs of GVHD. Except for one mouse at day 22, A20 cells were never detected in the blood, attesting to an efficient GVL effect. In the experimental group in which sTreg's were added to BM cells, T-cells, and A20 cells, four out of five mice were still alive at day 60. As compared with the two control groups, the presence of sTreg's protected mice from lethal GVHD, whereas the GVL effect was maintained. Indeed, leukemic cells were not detected in these mice at days 15, 22, and 37, except for one mouse that died at day 34 (Figure 5a), probably from leukemia (not shown).

The GVT effect was also analyzed using another tumor cell line, the P815 mastocytoma, that we have previously used in an allogeneic BMT setting (30). Since P815 cells derived from DBA/2 mice, we tested the GVT effect in the B6→[B6 × D2]F1 combination of BMT, a situation in which sTreg's significantly delayed GVHD (21). In the control group receiving BM and P815 cells, the presence of growing tumors at the site of injection was observed in all mice, and four out of five mice died between days 14 and 25. At the end of the experiment (day 35), a large tumor detected at the site of injection was still observed in the remaining mouse. In mice receiving allogeneic T cells in addition to BM and P815 cells, development of tumor cells was not detected at the site of injection, attesting to an efficient GVT effect. However, all mice developed clinical signs of severe GVHD, and two out of five died at days 19 and 29. As compared with these two control groups, the presence of sTreg's in addition to BM cells, T cells, and P815 cells efficiently controlled GVHD. However, the GVT effect was lost, since all mice displayed tumors at

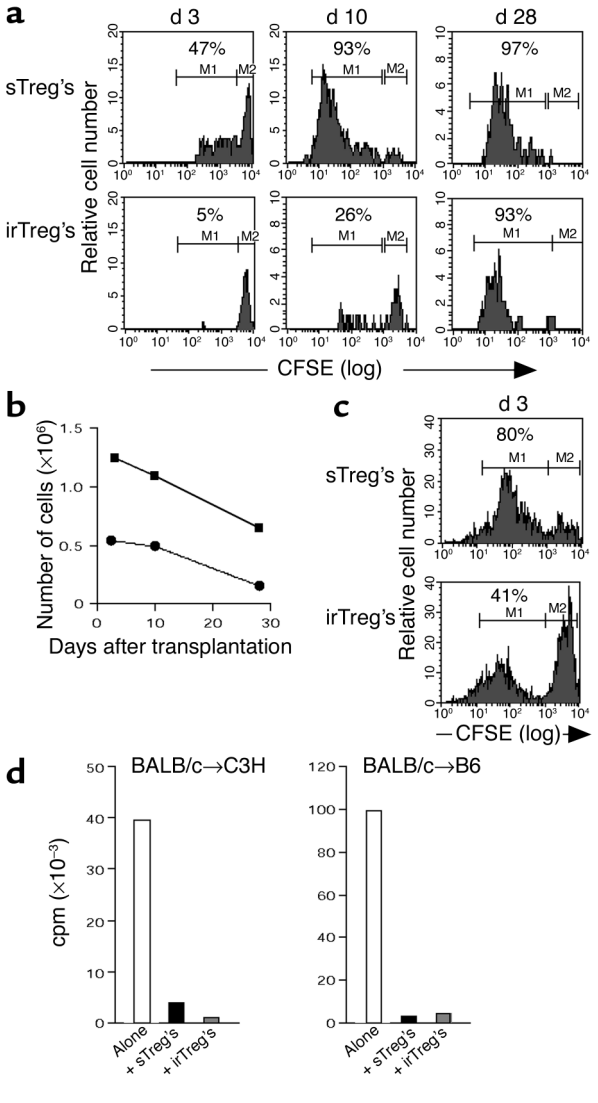

Figure 4
Comparison of in vitro and in vivo properties of cultured sTreg's and irTreg's. (**a** and **b**) 1×10^6 sTreg's or irTreg's were labeled with CFSE and injected into semiallogeneic, nonirradiated [BALB/c × C3H]F1. At days 3, 10, and 28, splenocytes from grafted animals were collected. The injected Treg's were detected in the spleen of grafted animals by the expression of the Thy-1.1 congenic marker. Cell proliferation was measured as the sequential loss of CFSE within the Thy-1.1$^+$ cell population by flow cytometry (**a**) and by the count of the absolute number of Thy-1.1$^+$ cells in the spleen (magnitude ×100) (**b**). (**c**) 1×10^6 sTreg's or irTreg's were labeled with CFSE and injected into semiallogeneic irradiated [BALB/c × C3H]F1. At day 3, splenocytes from grafted animals were collected and cell division of donor cells was evaluated. (**d**) The in vitro suppressive properties of cultured Treg's were tested after 3 weeks of culture. BALB/c CD25-depleted cells (effector T cells, white bar) were stimulated either by C3H APCs (left panel) or B6 APCs (right panel). Cells were cocultured with BALB/c sTreg's (black bar) or irTreg's (gray bar) in order to assess their suppressive activity. This figure is representative of three independent experiments.

the site of injection and four out of five mice died between days 19 and 28 (Figure 5b). The presence of tumors correlated with the detection of P815 cells in the blood (not shown). Inhibition by sTreg's of GVT activity against P815 was also observed in another experiment in which mice received high numbers of P815 cells 2 days before irradiation.

Discussion

The possibility of using Treg's as a new therapeutic for the control of GVHD was recently proposed and has been demonstrated in mice by several groups (21, 22, 31). Although the experimental models of these different studies varied in several parameters, such as the genetic combination between donors and recipients, the origin of lymphopenia in recipient mice (irradiation or use of SCID mice) or the source of Treg's (fresh or cultured cells from spleen, LN or BM), in each case, prevention of GVHD has required the use of similar proportions of total donor T cells and Treg's. Such high numbers of Treg's are necessary to expend them in vitro. We have previously demonstrated and confirmed here that after 4 weeks of culture with allogeneic APC, the number of Treg's increases by more than 1,000-fold, regardless of the genetic combinations used, while preserving their CD4$^+$CD25$^+$CD62L^{high} phenotype.

Although the possibility of controlling GVHD with Treg's has been described, the immune reconstitution, which still represents a major challenge for allogeneic HSCT, has not been analyzed. After allogeneic BMT in irradiated recipients, in addition to thymic production, immune reconstitution is due to both alloreactive and homeostatic-driven expansions of donor T cells (32). Thus, one could fear that using Treg's to control GVHD affects immune reconstitution for two reasons. First, even if the suppressive activity of Treg's is strictly dependent on their specific activation via their T cell receptor, once activated, they suppress proliferation of conventional T cells in vitro, regardless of the antigeneic specificity of the latter cells (15, 25). Second, it has been suggested that Treg's could nonspecifically limit homeostatic expansion (23, 27, 33). In this work, we demonstrate that the use of sTreg's not only permits, but also favors, immune reconstitution in these mice when compared with mice receiving BM cells alone. We can thus speculate that the injection of recipient-type, allo-specific sTreg's preferentially inhibits the expansion of T cells responsible for GVHD while partially preserving expansion of nonalloreactive T cells.

Whether or not GVT is maintained when Treg's are envisaged for the control of GVHD is also a critical point. A recent publication demonstrated that the use of unselected freshly isolated Treg's permits us to control GVHD without affecting the GVT effect against A20 leukemic cells or the BCL1 lymphoma (34). With the A20 leukemia, we made the same observation using cultured sTreg's. Thus, the possibility of controlling GVHD without compromising GVT effect is now demonstrated in two different laboratories using either sTreg's or unselected Treg's. However, we also

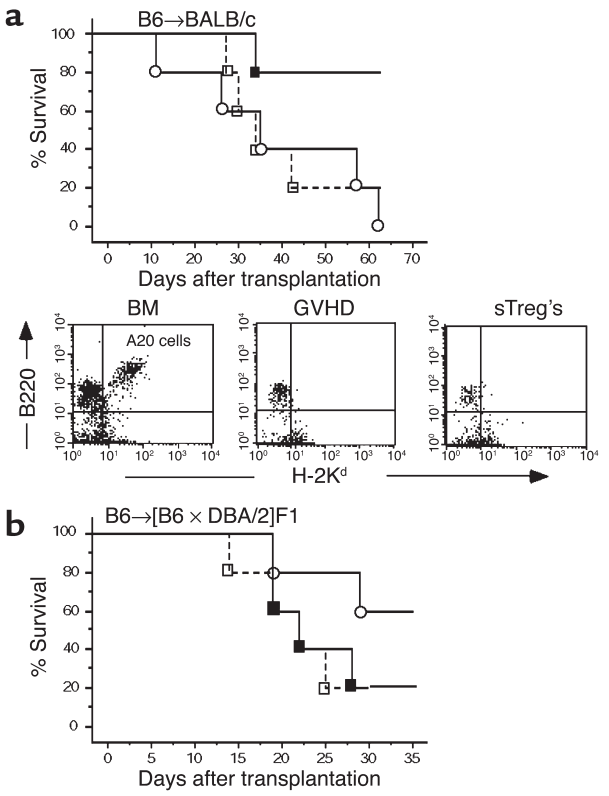

Figure 5

GVL/GVT effects after control of GVHD by sTreg's. (**a**) A20 leukemic cells were injected into irradiated mice at time of BMT. Results are presented as a Kaplan-Meier survival curve for mice receiving BM cells alone (dashed line, open squares, $n = 5$), BM cells supplemented with 0.5×10^6 conventional T cells (open circles, $n = 5$), in addition to 0.5×10^6 sTreg's (filled squares, $n = 5$). $P < 0.05$ between the last two groups. GVL effect is also evaluated by the presence of A20 cells in the blood of mice detected by the coexpression of B220 and H-2K^d Ag, and also by their large size. (**b**) A similar experiment was reproduced using P815 cells. Results are presented as a Kaplan-Meier survival curve for mice receiving BM cells alone (dashed line, open squares, $n = 5$), or BM cells supplemented with 10×10^6 conventional T cells (open circles, $n = 5$), in addition to 10×10^6 sTreg's (filled squares, $n = 5$). Because of severe morbidity due to the presence of tumor in all mice of the experimental group, the experiment was stopped at day 35.

observed that the GVT effect was abolished using the P815 mastocytoma. Different hypotheses could be made to explain whether or not, depending on the tumor model, the GVT is maintained when Treg's are used in therapy. First, Edinger et al. demonstrated that the mechanisms leading to GVHD were rather due to an excessive donor T cell proliferation and accumulation of proinflammatory cytokines, a process controlled by Treg's, whereas the latter cells did not affect GVT activity mediated mainly through the perforine-killing pathway (34). Treg's could inhibit a GVT activity against some tumors in cases where GVHD and GVT would be mediated by the same process. Second, we previously observed that the GVT effect that permits the elimination of P815 cells is strongly dependent on alloreactive donor T cells (30). Diminishing the alloreactivity of donor T cells using sTreg's could explain the complete loss of GVT effect that we observed with the P815 mastocytoma. Other parameters such as the tumor burden, its aggressive characteristics, or the localization of its growth could also determine whether GVT activity would be lost or not in the presence of Treg's.

In this study, we observed that sTreg's more efficiently control GVHD as compared with irTreg's. Two hypotheses could be made to explain this difference. First, the immunoregulatory effect obtained with sTreg's could be mostly targeted to donor T cells specific for recipient-type allo-Ag's. In favor of this hypothesis, we observed that mice receiving sTreg's, but not the ones receiving irTreg's, had high numbers of T cells in lymphoid tissues in the absence of any signs of GVHD 45 days after transplantation. Alternatively, the higher efficacy of sTreg's, compared with irTreg's, could be due to the former's increased capacity to expand and/or survive in vivo. Indeed, we observed that 45 days after BMT, irTreg's were barely detectable in the spleen and LN, whereas sTreg's were present in these tissues at greater than 50-fold higher levels. We also showed that Treg's specific for allo-Ag's strongly and rapidly proliferated following adoptive transfer in both irradiated and nonirradiated recipients only if these allo-Ag's were expressed by the recipient cells. This suggests that the ability of Treg's to recolonize the host depends on their reactivation by cognate allo-Ag's. Thus, at least in this particular context of allogeneic HSCT, ex vivo–expanded Treg's must be restimulated via their T cell receptors to survive and to exert their protective effect on GVHD.

In this study, we observed a weak but significant effect on GVHD with irTreg's, selected because of their capacity to proliferate in vitro in the presence of third-party APCs. This effect could be explained by our data demonstrating that irTreg's in vitro displayed a nonspecific immunosuppressive effect on conventional T cells. Thus, when reinfused in mice, irTreg's could exert a time-limited, but efficient, protective effect against GVHD.

The use of sTreg's to control GVHD is particularly suitable for clinical application for several reasons. Treg's have now been described in mice and humans. The sTreg's share remarkable similarities between the two species, and no important phenotypic or functional difference has been described to date (35, 36). Allogeneic BMTs are rarely performed in an urgent manner. Thus, it should be possible to collect Treg's from the donor of BM several weeks before transplantation, to cultivate them in the presence of irradiated peripheral blood recipient cells and IL-2, and to test their suppressive activity prior to their clinical use.

The therapeutic potential of Treg's for controlling GVHD without affecting immune reconstitution and GVT effect shown in preclinical experiments strongly supports the development of future clinical trials.

Nevertheless, whether this strategy is suitable in each case where HSCT is performed for the treatment of malignant hematological disorders remains to be studied further. In addition, patients eligible for allogeneic BMT for the treatment of nonmalignant hematological disorders (37) or autoimmune disease (38), which do not necessitate a GVL effect but which are confronted by the risk of developing GVHD due to the presence of donor T cells in the transplant, also represent possible applications for this strategy.

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