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Immune reconstitution is preserved in hematopoietic stem cell transplantation coadministered with regulatory T cells for GVHD prevention

Aline Gaidot,[1,2] Dan Avi Landau,[3] Gaëlle Hélène Martin,[1,2] Olivia Bonduelle,[4,5] Yenkel Grinberg-Bleyer,[1,2,6] Diana Matheoud,[7,8] Sylvie Grégoire,[1,2,6] Claude Baillou,[1,2] Béhazine Combadière,[4,5,9] *Eliane Piaggio,[1,2,6] and *José Laurent Cohen[1,2]

[1]Immunology-Immunopathology-Immunotherapy (I3), Université Pierre et Marie Curie (UPMC), Université Paris 06, Unité Mixte de Recherche (UMR) 7211, Paris, France; [2]Immunology-Immunopathology-Immunotherapy (I3), Centre National de la Recherche Scientifique (CNRS), UMR 7211, Paris, France; [3]Department of Hematology, Yale Cancer Center, Yale University, New Haven, CT; [4]Inserm, U945, Paris, France; [5]Laboratory of Immunity and Infection, UPMC, Université Paris 06, Paris, France; [6]Immunology-Immunopathology-Immunotherapy (I3), Inserm, UMR S959, Paris, France; [7]Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France; [8]Inserm U1016, Paris, France; and [9]Interface, Assistance Publique–Hôpitaux Paris (AP-HP), Paris, France

Recipient-specific regulatory T cells (rsTreg) can prevent graft-versus-host disease (GVHD) by inhibiting donor T-cell expansion after hematopoietic stem cell transplantation (HSCT) in mice. Importantly, in adult humans, because of thymus involution, immune reconstitution during the first months after HSCT relies on the peripheral expansion of donor T cells initially present in the graft. Therefore, we developed a mouse model of HSCT that excludes thymic output to study the effect of rsTreg on immune reconstitution derived from postthymic mature T cells present within the graft. We showed that GVHD prevention with rsTreg was associated with improvement of the limited immune reconstitution compared with GVHD mice in terms of cell numbers, activation phenotype, and cytokine production. We further demonstrated a preserved in vivo immune function using vaccinia infection and third-party skin-graft rejection models, suggesting that rsTreg immunosuppression was relatively specific of GVHD. Finally, we showed that rsTreg extensively proliferated during the first 2 weeks and then declined. In turn, donor Treg proliferated from day 15 on. Taken together, these results suggest that rsTreg GVHD prevention is associated with improved early immune reconstitution in a model that more closely approximates the biology of allogeneic HSCT in human adults. (*Blood*. 2011;117(10):2975-2983)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many hematologic malignancies and disorders. In the graft, 2 major components intervene: HSCs which durably reconstitute the hematopoietic system and mature donor T cells that are essential for (1) engraftment,[1] (2) immune reconstitution,[2] and (3) graft-versus-leukemia (GVL) effect.[3] Nevertheless, donor T cells can also recognize the host histocompatibility antigens, proliferate, and induce graft-versus-host disease (GVHD),[4] a major cause of mortality and morbidity in HSCT. The most efficient preventive strategy for GVHD consists of an immunosuppressive regimen. Importantly, this treatment remains immunologically nonspecific and is only partially effective.[5] Thus, developing innovative therapeutic strategies to limit the pathologic effects of donor alloreactive T cells is crucial.

We and others proposed that naturally occurring CD4^{+}CD25^{+} regulatory T cells (Treg) could be used to prevent GVHD.[6-8] Treg represent a small (5%-10%) particular subpopulation of CD4^{+} T cells with the capacity to mediate suppression of conventional CD4^{+} or CD8^{+} T cells (Tcon).[9-11] This immunosuppressive property is of great interest as a possible new strategy for GVHD prophylaxis. We and others have demonstrated that control of GVHD in mice depends on the addition of an equivalent number of freshly isolated donor Treg to donor CD3^{+} T cells.[6,7] Thus,

obtaining a sufficient number of Treg from a single donor remains a major obstacle for which strategies for selection and expansion through purification and culture have been devised.[6] For this, we generated recipient-specific Treg (rsTreg) by culturing CD4^{+}CD25^{high}CD62L^{+} purified T cells in the presence of irradiated recipient-type splenocytes and interleukin 2 (IL-2). We have shown that rsTreg were able to control GVHD in mice more efficiently than polyclonal Treg, while maintaining the GVL effect.[12,13] An important caveat to these results is that they were obtained in models that include a functional thymus, unlike HSCT in adults where thymic involution is the rule and where, consequently, posttransplantation immune reconstitution relies on the peripheral expansion of postthymic donor T cells present in the graft.[14-16]

One of the most important parameters concerning any strategy to prevent GVHD is its effect on immune reconstitution, as opportunistic infections constitute a major post-HSCT risk. Little is known about immune reconstitution when GVHD is controlled by Treg or rsTreg. Previous work have shown that GVHD-protected mice were capable of rejecting tumoral cells.[12,17] Furthermore, Nguyen et al demonstrated that Treg enhanced immune reconstitution by preventing GVHD-induced damage of the thymic and secondary lymphoid microenvironment.[18] In addition, in this report, in thymectomized mice protected by Treg, an antiviral response was

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The online version of this article contains a data supplement.

observed (although reduced) compared with euthymic mice. These results imply that even in the absence of a functional thymus, the reconstituted immune system can still be functional. However, the impact of Treg on immune reconstitution derived only from postthymic donor T cells was not specifically studied.

For clinical use of rsTreg, it is therefore essential to determine their impact on donor T cells. To answer this question, we have developed a GVHD model in which donor T cells present in the graft were the sole source for T-cell reconstitution. In this model, [BALB/c × C57BL/6]F1 mice were lethally irradiated and grafted with bone marrow (BM) cells collected from CD3ε$^{-/-}$ C57BL/6 mice which do not have any T-cell precursors. GVHD was then induced by the transfer of donor C57BL/6 allogeneic CD3$^+$ T cells and was prevented by the infusion of rsTreg at a 1:1 ratio to donor T cells. This strategy more closely models HSCT in adults after thymic involution. We showed that in mice protected with rsTreg, the immune reconstitution derived from the donor postthymic T cells initially present in the graft is supported and the animals are capable of responding to viral and allogeneic antigens.

Methods

Mice

Six- to 10-week-old BALB/c (H-2^d), C3H (H-2^k), C57BL/6 (H-2^b) female mice were obtained from Charles River Laboratories. [BALB/c × C57BL/6]F1 (H-2^{dxb}) female mice were obtained from Harlan Laboratories. C57BL/6 CD3ε$^{-/-}$, C57BL/6 Thy1.1, and C57BL/6 Ly5.1 mice were bred in our animal facility under specific pathogen-free conditions. C57BL/6 knock-in mice expressing green fluorescent protein (GFP) under the control of the Foxp3 promoter (Foxp3-EGFP)[19] were kindly given by B. Malissen (CIML, Marseille, France). Experiments were performed according to the European Union guidelines and approved by our institutional review board (CREEA Ile de France no. 3).

Experimental GVHD

After lethal irradiation (10 Gy), 8- to 12-week-old [BALB/c × C57BL/6]F1 recipient mice were injected intravenously in the retro-orbital sinus with 3×10^6 CD3ε$^{-/-}$ BM cells, 3×10^6 congeneic (Ly5.1) C57BL/6 CD3$^+$ T cells alone to induce GVHD, or with 3×10^6 cultured rsTreg. T cells were collected from lymph nodes of donor animals and the percentage of CD3$^+$ cells was determined by flow cytometry. GVHD was monitored regularly for diarrhea and skin lesions. Body weight loss of more than 30% of the initial weight led to euthanasia. Control groups were constituted of unmanipulated mice and mice grafted with 3×10^6 CD3ε$^{-/-}$ BM cells alone.

Ex vivo expansion of rsTreg

Cell suspensions were obtained from spleen and peripheral lymph node cells of C57BL/6 mice. Cells were first labeled with biotin-coupled anti-CD25 monoclonal antibody (mAb; 7D4; Becton Dickinson), followed with antibiotin microbeads (Miltenyi Biotec), and enriched in CD25$^+$ cells using magnetic cell large selection columns (Miltenyi Biotec). Cells were then stained with fluorescein isothiocyanate (FITC)–labeled anti-CD4 (GK1.5), phycoerythrin (PE)–labeled anti-CD62L (MEL-14) and streptavi-din-CyChrome (BD Pharmingen), which bound to free biotin-labeled CD25 molecules. The CD4$^+$CD25^{high}CD62L$^+$ T cells were sorted by flow cytometry using a FACSAria (Becton Dickinson), yielding a purity of 99%. Treg were cultured for 3 to 5 weeks, in the presence of 20-Gy–irradiated recipient-type BALB/c splenocytes and recombinant murine IL-2 (10 ng/mL; R&D Systems), as previously described.[6]

Flow cytometry

The following Abs were used for fluorescence-activated cell sorting (FACS) analysis: anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-B220, anti-

CD62L, anti-CD44, anti-CD45.1 (Ly5.1), anti-CD90.1 (Thy1.1), anti–IL-2, anti–tumor necrosis factor (TNF), and anti–interferon γ (IFNγ), labeled with FITC, PE, allophycocyanin (APC), peridinin chlorophyll protein (PerCP), or Alexa Fluor 700. All mAbs were purchased from BD Biosciences. The PE- or Pacific Blue–labeled anti-Foxp3 staining was performed using the eBioscience kit and protocol. EdU (5-ethynyl-2′-deoxyuridine) incorporation was measured by using the Click-iT EdU Flow Cytometry Assay Kit (Molecular Probes) after the FoxP3 staining. For intracellular cytokine staining, cells were restimulated with 1 μg/mL Phorbol 12-myristate 13 acetate (PMA; Sigma-Aldrich) and 0.5 μg/mL ionomicyn (Sigma-Aldrich) for 4 hours, in the presence of GolgiPlug (1 μL/mL; BD Biosciences). After cell-surface staining, intracellular staining was performed using the CytoFix/CytoPerm kit (BD Biosciences). Events were acquired on a LSRII (BD Biosciences) flow cytometer and analyzed using FlowJo (TreeStar) software.

Proliferation monitoring by EdU incorporation

To assess endogenous cell division, mice received 4 intraperitoneal injections every 12 hours of 1 mg of EdU (Molecular Probes), a nucleoside analog of thymidine that incorporates into dividing DNA, and killed 4 hours after the last EdU injection.

Vaccinia infection

Four months after HSCT, mice were infected intranasally with 5×10^4 PFU of live vaccinia virus (Copenhagen strain; Dr M.-P. Kieny, Transgene Laboratories, Strasbourg, France) and body weight was measured daily. BM-grafted and rsTreg-protected mice were infected 4 months posttransplantation and compared with unmanipulated mice.

Interferon-γ ELISPOT assay

The mouse-specific IFNγ ELISPOT kit (Diaclone) was used under the manufacturer's suggested conditions. Briefly, splenocytes (1.0×10^5 cells) were harvested at day 21 after vaccinia immunization. Cells were incubated with vaccinia (1 PFU/cell) for 36 hours at 37°C on a polyvinylidene difluoride plate (Millipore) coated with purified anti-IFNγ. The plates were then incubated with biotinylated anti-IFNγ mAb for 90 minutes, then with a secondary Ab conjugated to streptavidin–alkaline phosphatase for 1 hour and BCIP/NBT substrate buffer. The frequency of spot-forming cells (SFCs) was measured with an Axioplan 2 microscope using the KS-ELISPOT software (Zeiss).

Skin grafting

At day 30 after HSCT, tail-skin grafts from C57BL/6 and C3H 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. Rejection was defined as loss of viable donor epithelium.

Statistical analyses

Statistical significances were calculated using the 2-tailed unpaired Student t test. When statistically significant, P values were indicated.

Results

Prevention of GVHD by rsTreg is associated with improved donor postthymic immune reconstitution

To test the effect of rsTreg on immune reconstitution derived solely from mature donor T cells present in the graft, we developed a specific model of GVHD deprived of any thymus-derived T-cell production. For this, [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and transferred with 3×10^6 C57BL/6 CD3ε$^{-/-}$ BM cells. These mice showed a transient weight loss, then regained
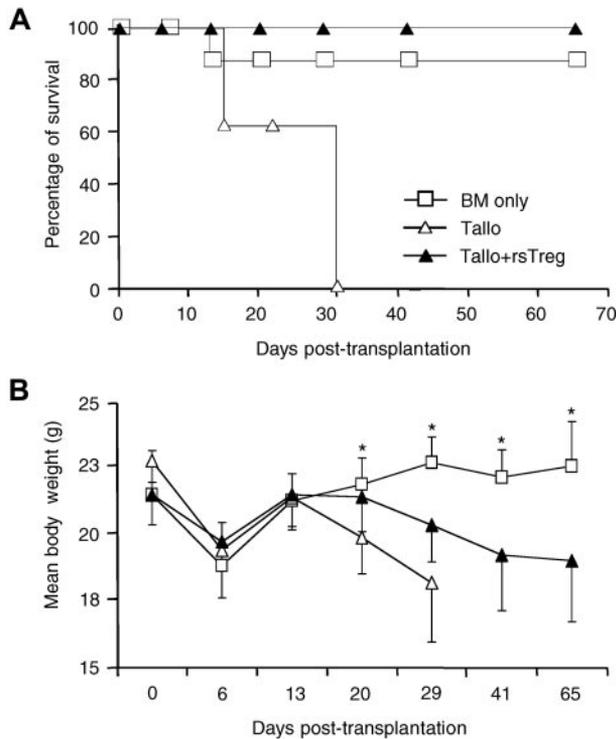
Figure 1. rsTreg prevent GVHD in C57BL/6→BALB/c × C57BL/6]F1 BM transplantation. [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3×10^6 CD3$\varepsilon^{-/-}$ BM cells (control group, BM only □; n = 8) plus 3×10^6 allogeneic Ly5.1$^+$ C57BL/6 T cells alone (Tallo △; n = 8) or with 3×10^6 specific rsTreg (Tallo+rsTreg ▲; n = 7). (A) Kaplan-Meier survival curves and (B) mean body weight curves were established for each group at different time points. Each symbol represents the mean value and bars are the SD. *$P < .05$ for BM vs Tallo at day 20 and for BM vs Tallo and BM only vs Tallo+rsTreg at days 29, 41, and 65.

weight which remained stable thereafter. This strategy thus allowed mice to survive without GVHD. In contrast, the cotransfer of 3×10^6 donor C57BL/6 allogeneic CD3$^+$ T cells resulted in GVHD characterized by progressive weight loss and death by day 30 (Figure 1A-B).

To control GVHD, we obtained rsTreg after 3-5 weeks of culture of purified CD4$^+$CD25$^+$CD62L^{high} Treg from C57BL/6

mice in the presence of irradiated recipient-type (BALB/c) splenocytes, as previously described.[12] At the time of transfer, more than 96% of the CD4$^+$ cells expressed FoxP3 (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Infusion of 3×10^6 rsTreg (1:1 ratio to donor T cells) protected animals from GVHD and related mortality (Figure 1A). After a short period of weight loss, treated mice regained weight but only transiently as after day 20, weight loss was again noticed (Figure 1B).

We then studied the effect of rsTreg on the reconstitution of the lymphoid compartment after HSCT (Table 1). The total number of CD4$^+$ and CD8$^+$ T cells and B cells in the spleen at days 7, 15, 30, and 60 postgraft was compared with nonmanipulated mice. As anticipated, mice with both transplant conditions (GVHD and protected mice) had a severe B- and T-cell lymphopenia accompanied by an inverted CD4/CD8 ratio compared with nonmanipulated mice. Although GVHD may be a strong drive for T-cell expansion early posttransplantation, surprisingly, when rsTreg were coadministered, T-cell numbers were comparable in GVHD and protected mice. Furthermore, in mice developing GVHD, B cells were virtually absent whereas rsTreg infusion seemed to have a beneficial effect on B-cell reconstitution. Indeed, in the spleen of protected mice, B cells were detectable at day 15 and remained present at more than 10×10^6 cells per spleen after day 30.

We also studied the naive/activated phenotype profile of T cells: naive (N), central memory (CM) and effector memory (EM) cells defined as CD62L^{hi}CD44^{low}, CD62L^{hi}CD44^{hi}, and CD62L^{low}CD44^{hi}, respectively.[20] More than half of the T-cell compartment (CD4$^+$ and CD8$^+$) of mice protected from GVHD by rsTreg showed a naive phenotype by day 7. Later on, most of the T cells were activated and acquired an EM phenotype. The CM compartment was weakly represented among CD4$^+$ T cells and was relatively more important among CD8$^+$ T cells (Figure 2A-B). Interestingly, CD8$^+$ T cells, which highly expressed the activation marker CD44 by day 15, showed a strong down-regulation of this marker afterward (Figure 2A).

Compared with the GVHD mice, it can be observed that the added rsTreg favored the maintenance of higher proportions of naive CD4$^+$ and CD8$^+$ T cells and central memory CD8$^+$ T cells along reconstitution. Conversely, higher proportions of CD4$^+$ and CD8$^+$ T EM cells were present in GVHD mice compared with protected animals (Figure 2A-B).

Table 1. Reconstitution of the lymphocyte compartment following allo-BMT

	Day 7	Day 15	Day 30	Day 60
CD4 T cell, $\times 10^6$				
n.m.	21.92 ± 2.16*	18.56 ± 1.10*	23.229 ± 1.74*	23.97 ± 3.98*
Tallo	0.18 ± 0.06†	1.77 ± 0.38	2.86 ± 2.11	
Tallo+rsTreg	0.38 ± 0.05	2.00 ± 0.78	2.95 ± 0.56	1.09 ± 0.15
CD8 T cell, $\times 10^6$				
n.m.	15.56 ± 1.42*	12.99 ± 1.30*	16.63 ± 1.70*	17.82 ± 3.00*
Tallo	0.16 ± 0.07	2.37 ± 0.44	3.11 ± 2.26	
Tallo+rsTreg	0.26 ± 0.04	3.35 ± 0.93	5.71 ± 1.30	1.63 ± 0.25
B cell, $\times 10^6$				
n.m.	69.26 ± 5.52*	78.24 ± 3.39*	70.50 ± 7.13*	83.77 ± 12.52*
Tallo	n.d.	0.05 ± 0.01†	0.49 ± 0.25†	
Tallo+rsTreg	0.04 ± 0.01	2.48 ± 1.00	15.25 ± 5.20	11.07 ± 4.39

[BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3×10^6 CD3$\varepsilon^{-/-}$ BM cells and 3×10^6 allogeneic Ly5.1$^+$ C57BL/6 T cells alone (Tallo) or with 3×10^6 Thy1.1$^+$ rsTreg (Tallo+rsTreg). Unmanipulated mice represent the control group (n.m.). Immune reconstitution of T- (CD4$^+$ and CD8$^+$) and B-cell compartments was evaluated in the spleen 7, 15, 30, and 60 days after transplantation. SD is indicated for each mean value. Data were cumulated from 4 independent experiments with 2 to 5 mice per group per time point.

n.d. indicates nondetectable.

*$P < .001$ for n.m. vs Tallo and for n.m. vs Tallo+rsTreg.
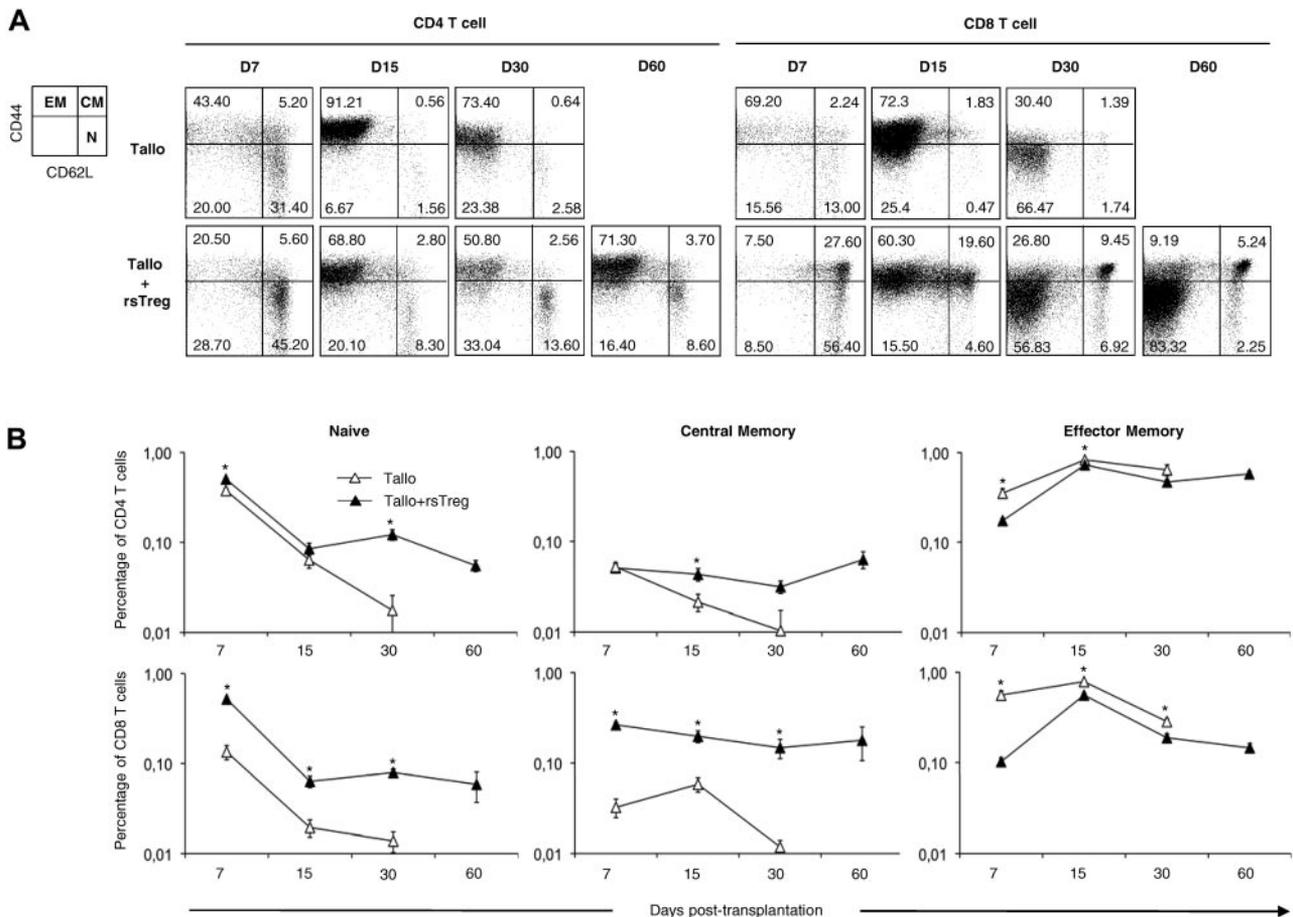
†$P < 0.05$ for Tallo vs Tallo+rsTreg.

Figure 2. Phenotype of donor T cells that have reconstituted the host. [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3×10^6 CD3$\varepsilon^{-/-}$ BM and 3×10^6 allogeneic Ly5.1$^+$ C57BL/6 T cells alone (Tallo) or with 3×10^6 specific rsTreg (Tallo+rsTreg). The activation profile of donor Ly5.1$^+$ CD4$^+$ and CD8$^+$ T cells was evaluated in the spleen 7, 15, 30, and 60 days after transplantation. (A) Naive (N), central memory (CM), and effector memory (EM) cells are defined as CD62L^{hi}CD44^{low}, CD62L^{hi}CD44^{hi}, and CD62L^{low}CD44^{hi}, respectively. Representative dot plots of the activation profile of Ly5.1$^+$ CD4$^+$ and CD8$^+$ T cells are shown 7, 15, 30, and 60 days posttransplantation. Numbers in each quadrant indicate the percentage of cells. (B) The mean percentage of naive, CM, and EM Ly5.1$^+$ CD4$^+$ and CD8$^+$ T cells is represented at 7, 15, 30, and 60 days posttransplantation for Tallo ($\triangle$) and Tallo+rsTreg ($\blacktriangle$). Symbols represent the mean percentage of cells and bars are the SD. At day 7, n = 5 from 1 experiment; at day 15, n = 5 to 7 from 2 independent experiments; at day 30, n = 2 to 8 from 3 independent experiments; at day 60, n = 8 from 2 independent experiments. *$P < .05$ for Tallo vs Tallo+rsTreg.

We also studied the capacity of T cells to secrete cytokines on nonspecific ex vivo restimulation along reconstitution. We observed that the ability of CD4$^+$ T cells to produce the proinflammatory cytokines IL-2 and TNF, was significantly reduced in protected mice compared with GVHD mice at day 7. In accordance with our previous results,[13] we also observed a diminution of IFNγ production, although not statistically significant in the present work. However, at day 30, both groups of mice showed comparable levels of IL-2, TNF and IFNγ production which remained stable at day 60 (Figure 3). In CD8$^+$ T cells, IL-2, TNF, and IFNγ production was similar in both GVHD and protected mice from day 7 to day 30 (data not shown).

Taken together, these results indicate that in the presence of rsTreg, donor T cells display a less activated phenotype, with lower proportions of EM cells and persistence of naive and CM cells. In addition, this donor T-cell compartment reconstituted in the presence of rsTreg is capable of producing proinflammatory cytokines later posttransplantation.

Donor T-cell function is maintained after control of GVHD by rsTreg

We next assessed whether GVHD protected mice were able to mount an immune response in vivo. We used the vaccinia (poxvirus) model because this type of infection involves strong CD4$^+$ and CD8$^+$ T-cell responses and production of neutralizing antibodies.[21] Because recipient-type T cells are present at 2.3% ± 1.3% of total T cells in mice grafted with rsTreg at day 15, and decline to 0.35% ± 0.04% at day 30, and are also detected in mice receiving donor T cells alone (0.24% ± 0.02% at day 15), we decided to delay to month 4 immune function experiments to ensure that the immune response observed was because of donor but not recipient-type T cells (supplemental Figure 2). Four-month–reconstituted mice were intranasally infected with vaccinia virus and the effect on body weight was assessed. Twenty-one days postinfection, we evaluated the lymphoid compartment and the development of virus-specific T- and B-cell responses by IFNγ production by ELISPOT and neutralizing antibody production.

Mousepox disease is associated with high mortality in the susceptible BALB/c, and DBA/2 strains of mice, but causes an unapparent infection in the C57BL/6 mouse strain.[22] Indeed, weight loss depends on the genetic background of mice. In C57BL/6 mouse strain, weight loss reflects the transient vaccinia infection. Because of the different susceptibility of mice strain, we screened unmanipulated mice and reconstituted mice for their susceptibility to vaccinia virus infection. Unmanipulated mice were of [BALB/c × C57BL/6]F1 background whereas grafted mice were [BALB/c × C57BL/6]F1 reconstituted with B6 bone marrow.
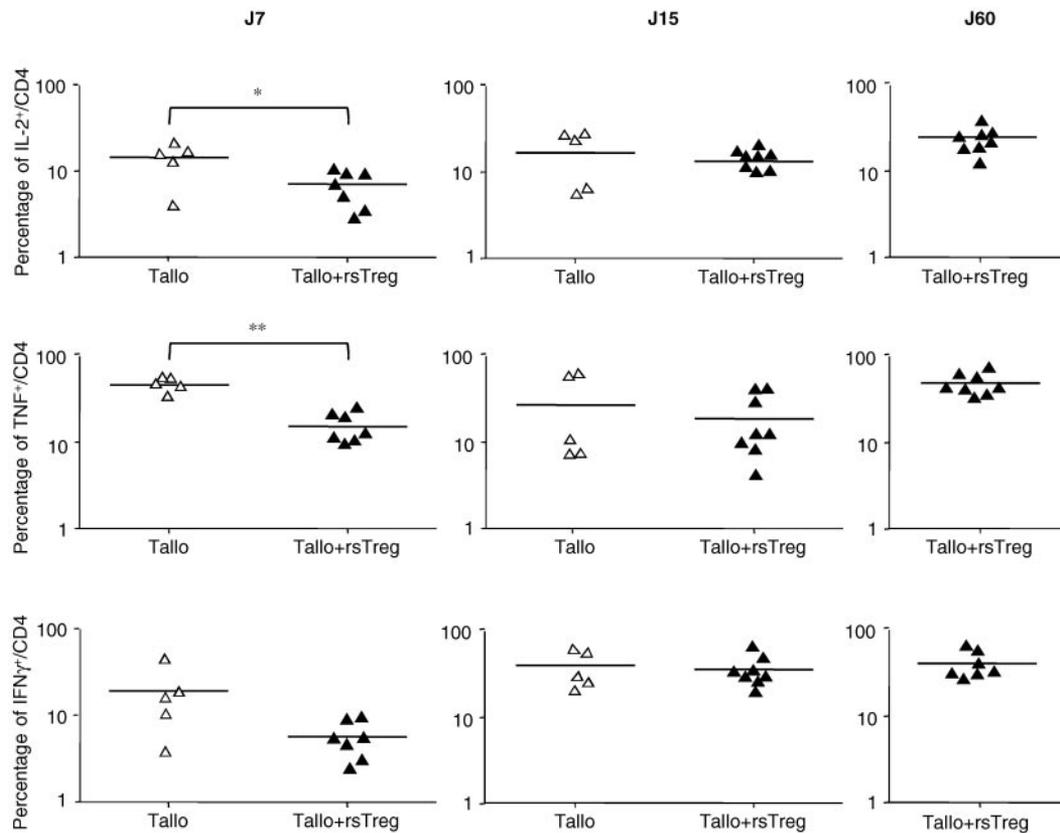
Figure 3. Early inhibition of proinflammatory cytokine production by rsTreg. [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3×10^6 CD3ε$^{-/-}$ BM cells and 3×10^6 allogeneic Ly5.1$^+$ C57BL/6 T cells alone (Tallo △) or with 3×10^6 specific rsTreg (Tallo+rsTreg ▲). Cytokine production was quantified after ex vivo restimulation with PMA/ionomycin. The percentage of Ly5.1$^+$ CD4$^+$ T cells that produce IL-2, TNF, and IFNγ was evaluated in the spleen 7, 15, and 60 days after transplantation. Cytokine profiles at day 30 (not shown) were comparable with day 15. Each symbol represents an individual mouse and bars are the mean. For each time point, data were combined from 2 independent experiments. *$P < .05$ for Tallo vs Tallo+rsTreg. **$P < .001$ for Tallo vs Tallo+rsTreg.

As described in the literature,[21] in nonmanipulated as well as in C57BL/6 CD3ε$^{-/-}$ BM-grafted controls, infection with the vaccinia virus was associated with progressive weight loss until day 7 when mice began to regain weight. Recipient GVHD-protected mice also survived vaccinia infection and showed a similar weight variation profile (Figure 4A). However, it is of note that CD3ε$^{-/-}$ BM-grafted mice, in the absence of GVHD, were reconstituted from radio-resistant recipient-type T cells, as depicted in supplemental Figure 2. On the contrary, GVHD or GVHD-protected mice were devoid of recipient-type T cells (supplemental Figure 2), implying that in these cases, donor T cells participate in protection from vaccinia.

At day 21 postinfection, the absolute numbers of total CD4$^+$ and CD8$^+$ T cells were similar in GVHD-protected mice uninfected versus vaccinia infected (Figure 4B). Regarding the generation of a viral-specific T-cell response, vaccinia-infected nonmanipulated and GVHD-protected mice were able to produce IFNγ after restimulation with the cognate virus, although to varying degrees (Figure 4C). As expected, the noninfected GVHD-protected control group did not produce IFNγ. Of note, in rsTreg-protected animals, we could distinguish 2 IFNγ-producing populations with different strength of response which positively correlated with the level of T-cell reconstitution (Figure 4B-C).

Concerning the B-cell compartment, cell numbers were similar in infected and uninfected mice (Figure 4B) and no production of neutralizing antibodies was detected. As expected, neutralizing antibodies were present in the infected unmanipulated group (data not shown).

T-cell function was also evaluated in a third-party allogeneic skin-graft model. One-month–reconstituted GVHD-protected mice were grafted on the thoracic wall with donor-type C57BL/6 or allogeneic C3H tail skin. All protected mice reconstituted with cells provided by C57BL/6 donors accepted C57BL/6 grafts. For third-party C3H graft, at day 10, 1 of 5 mice rejected the graft and at day 20, all C3H skin grafts were rejected (Figure 5). Because rsTreg are still present at day 30, these experiments indicate that rsTreg do not interfere with allogeneic immune response. Kinetics of rejection was slightly delayed compared with C57BL/6 mice where C3H skin grafts were rejected between day 10 and day 15 (not shown).

In conclusion, in the 2 experimental settings used to study the function of the reconstituted immune system in the presence of rsTreg, we observed a maintained capacity to mount an immune response. The immune response manifested mainly by normal IFN-γ production (Figure 4C), though neutralizing antibodies were not formed. Because CD8$^+$ cells are the main source of IFN-γ, a plausible explanation would be that CD8 T-cell responses are preserved but the function of CD4 T cells and/or B cells is deficient. These results showing that rsTreg immunosuppression is relatively specific of GVHD, suggested that the GVL reaction could also be spared.

Treg compartment reconstitution

We first analyzed the fate of the therapeutic rsTreg during immune reconstitution. We observed that in the spleen of grafted animals,
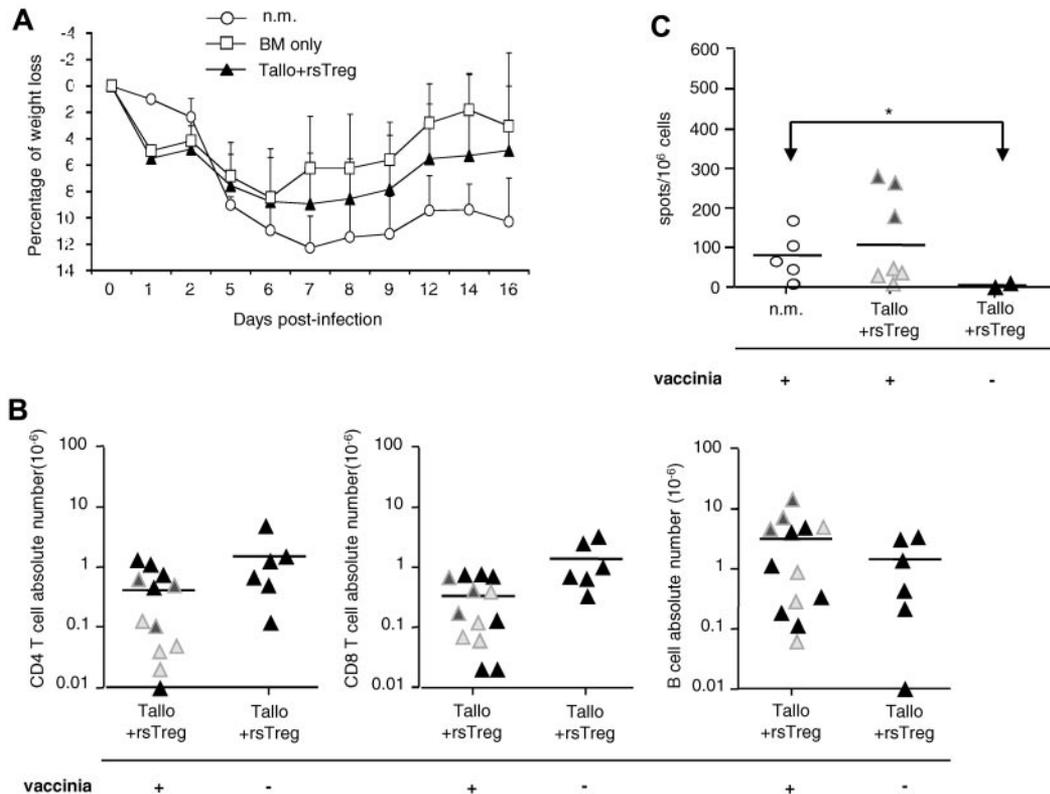
Figure 4. Response to vaccinia infection in the presence of rsTreg. [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3 × 10⁶ CD3ε⁻/⁻ BM cells (BM only □) and 3 × 10⁶ allogeneic Ly5.1⁺ C57BL/6 T cells with 3 × 10⁶ specific rsTreg (Tallo+rsTreg ▲, , and). Nonmanipulated mice represent the control group (n.m. ○). At 4 months postreconstitution mice were infected intranasally with 5 × 10⁴ plaque-forming units (PFU) of vaccinia virus and (A) body weight loss was determined at different time points postinfection up to day 16. Symbols represent the mean value and bars are the SD. (B) Absolute number of Ly5.1⁺ CD4⁺ and CD8⁺ T cells and B cells in the spleen, at day 21 postinfection, is indicated for Tallo+rsTreg mice (▲). As a control, a Tallo+rsTreg group was not infected with vaccinia. and , in infected Tallo+rsTreg group, represent spleens that were tested in ELISPOT. (C) Elispot for the production of IFNγ by splenocytes was done at day 21 postinfection on splenocytes. In the Tallo+rsTreg group infected with vaccinia, high and low IFNγ producers were identified by and , respectively. As a control, a Tallo+rsTreg (▲) group was not infected with vaccinia. Each symbol represents an individual mouse and the horizontal bars are the means. Data were combined from 2 independent experiments with 2 to 7 mice per group per time point. *P < .05.

the rsTreg compartment strongly expanded from day 7 to day 15 and then importantly declined from day 30 to day 60 (Table 2). At day 30, 98% of the rsTreg identified by the CD4⁺Thy1.1⁺ phenotype still expressed FoxP3 (supplemental Figure 1b). This retrospectively attested that not only were the expanded rsTreg population used to graft mice of high purity, but also the phenotype of this population is stable over time, as 1 month posttransplantation they continue to be 98% Foxp3⁺. Besides rsTreg, the transplant contains a fraction of Treg naturally present among the donor CD4⁺ T cells; the donor Treg (dTreg). To study the impact of rsTreg on the dTreg compartment, we used as donor T cells, CD3⁺ T cells collected from mice expressing the congeneic marker Ly5.1. For GVHD and rsTreg protected groups, Ly5.1⁺ dTreg content in grafted mice represented ~ 5% of the donor CD4⁺ T cells at day 7 and day 15. However, while for GVHD mice the percentage of dTreg remained constant, in mice protected by rsTreg, it increased from day 15 to reach more than 30% at day 60 (Figure 6A).

This suggested that the development of the dTreg compartment was favored among the overall CD4⁺ T-cell reconstitution in protected mice. We also evaluated the impact of rsTreg on dTreg division by analysis of EdU incorporation. In the GVHD group, dTreg extensively divided from day 7 to day 30 but their percentage among CD4⁺ cells remained low. In contrast, when rsTreg were added, dTreg division was significantly reduced while their representation among CD4⁺ cells increased, suggestive of increased survival of dTreg (Figure 6B-C).

Knoechel et al have demonstrated in a GVHD-like model that naive T cells transferred in a lymphopenic context could differentiate into induced Treg (iTreg).[23] We next assessed whether dTreg present in protected mice originated from natural dTreg initially present in the graft or alternatively were iTreg converted from donor Tcon. To answer this question, we used allogeneic donor T cells from FoxP3-EGFP mice,[19] which allow for the detection of conversion of CD4⁺FoxP3⁻GFP⁻ Tcon into CD4⁺FoxP3⁺GFP⁺

Table 2. rsTreg reconstitution

	rsTreg numbers, ×10⁶			
	Day 7	**Day 15**	**Day 30**	**Day 60**
Tallo+rsTreg	0.45 ± 0.11	1.72 ± 0.23	0.64 ± 0.12	0.09 ± 0.01
Percentage of rsTreg/CD4	n = 5	n = 5	n = 5	n = 4
Tallo+rsTreg	32.26 ± 4.32	31.88 ± 0.67	12.26 ± 2.30	8.69 ± 1.90

[BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3 × 10⁶ CD3ε⁻/⁻ BM cells and 3 × 10⁶ allogeneic Ly5.1⁺ C57BL/6 T cells and with 3 × 10⁶ Thy1.1⁺ rsTreg (Tallo+rsTreg). Immune reconstitution of rsTreg was evaluated in the spleen 7, 15, 30, and 60 days after transplantation.

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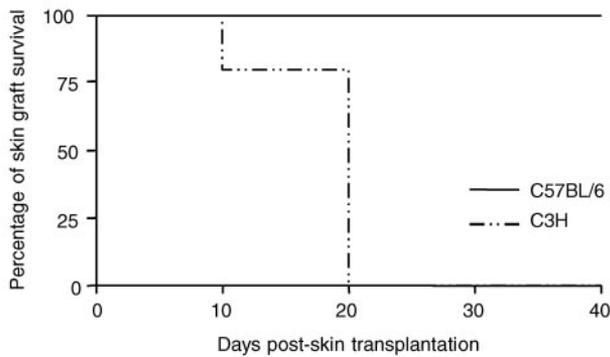
Figure 5. Rejection of a third-party allogeneic skin graft in the presence of rsTreg. [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3 × 10⁶ CD3ε⁻/⁻ BM and 3 × 10⁶ allogeneic Ly5.1⁺ C57BL/6 T cells with 3 × 10⁶ rsTreg. At day 30, tail-skin grafts from C57BL/6 (n = 10; solid line) and C3H (n = 5; dashed line) mice were transplanted onto the lateral thoracic wall of reconstituted mice. Skin-graft survival curves are shown for each group of 1 representative experiment.

iTreg in vivo.[19] In this experiment, control mice were grafted with allogeneic T cells obtained from FoxP3-EGFP (GFP⁺/⁻ group) mice, thus containing the physiologic proportion of Tcon (GFP⁻)

and Treg (GFP⁺). In the experimental group, allogeneic T cells were obtained from FoxP3-EGFP mice, in which GFP⁺ cells were depleted and replaced by an equivalent number of natural Treg from nontransgenic mice (GFP⁻ group).

GVHD mice grafted with GFP⁺/⁻ cells had ∼ 4% GFP⁺ cells among the CD4⁺ T-cell population at days 15 and 30 (Figure 6D top panels). In contrast, GVHD mice grafted with GFP⁻ cells had almost no detectable GFP⁺ cells, indicating that conversion of Tcon into iTreg was minimal in the GVHD groups. When protected animals received GFP⁺/⁻ cells, the percentage of GFP⁺ cells progressively grew from ∼ 4% at day 15 to ∼ 19% at day 60 (Figure 6D bottom panels). Interestingly, in the GFP⁻ group, iTreg only represented ∼ 3.5% of the total CD4 population. The near absence of GFP⁺ cells in protected mice one month after transplantation indicated that the generation of iTreg was a late event.

Overall, these results indicate that at 2 months posttransplantation in protected mice, rsTreg are no longer detectable in the spleen of grafted animals (Table 1) and the dTreg population assumes a significant proportion (> 30%) of the total CD4⁺ T-cell compartment (Figure 4). It is noteworthy that this dTreg compartment is composed of ∼ 80% of the Treg initially present in the graft that have proliferated and ∼ 20% of iTreg.
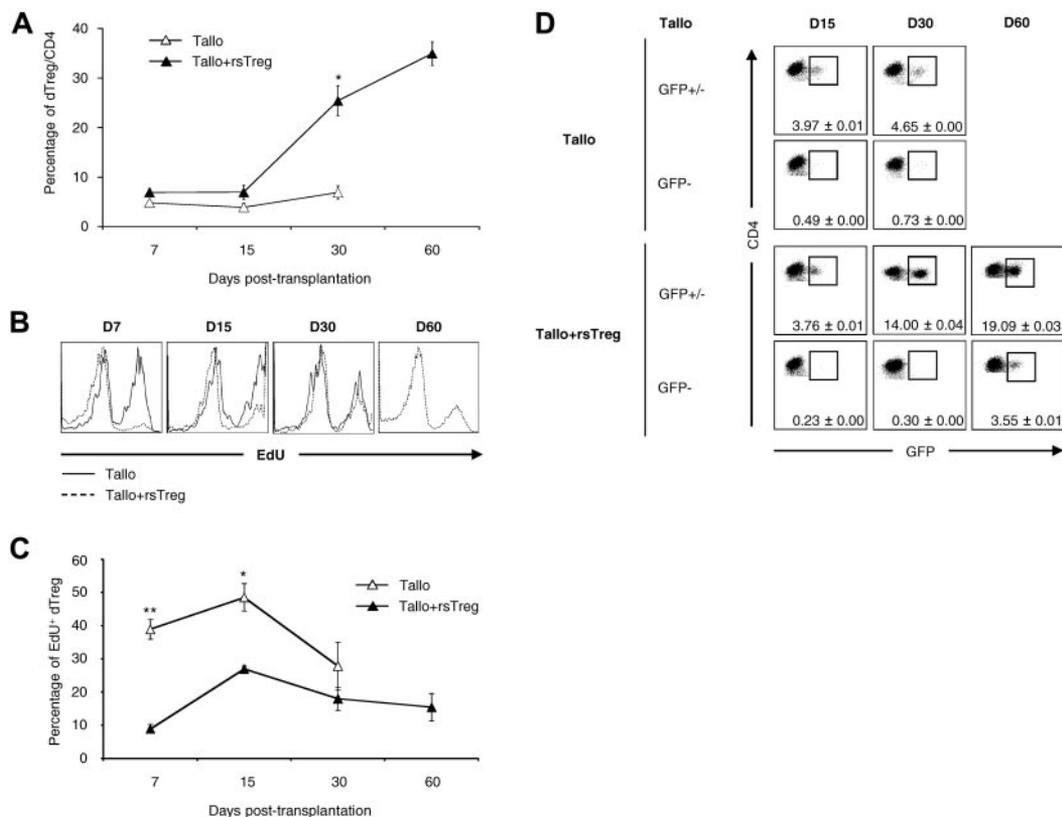

Figure 6. Reconstitution of the Treg compartment after allo-BMT. (A) [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3 × 10⁶ CD3ε⁻/⁻ BM cells and 3 × 10⁶ allogeneic Ly5.1⁺ C57BL/6 T cells alone (Tallo △) or with 3 × 10⁶ specific Thy1.1⁺ rsTreg (Tallo+rsTreg ▲). The percentage of CD4⁺FoxP3⁺ dTreg among the Ly5.1⁺CD4⁺ T cells was evaluated at days 7, 15, 30, and 60 posttransplantation. Each symbol represents the mean value and bars are the SD. For each time point, data were combined from 2 independent experiments with n = 5 to 8 mice per group. *P < .05 for Tallo vs Tallo+rsTreg. (B-C) To compare the proliferation of dTreg from Tallo group (solid line) and Tallo+rsTreg group (dashed line), mice were injected with EdU to assess T-cell division during the last 48 hours before sacrifice. (B) Histograms depict EdU incorporation in CD4⁺FoxP3⁺ dTreg, at different time points. (C) The percentage of EdU⁺ dTreg among the CD4⁺ T cells is indicated for Tallo (△) and Tallo+rsTreg (▲) groups, at different time points. Symbols represent the mean percentage and bars are the SD. *P < .05. **P < .001. Data were cumulated from 2 independent experiments with n = 5 mice for each group. (D) [BALB/c × C57BL/6]F1 recipient mice were lethally irradiated and then grafted with 3 × 10⁶ CD3ε⁻/⁻ BM cells and 3 × 10⁶ C57BL/6 T cells from FoxP3-EGFP mice. These T cells were GFP-depleted (GFP⁻) or not (GFP⁺/⁻) and were coinjected with 3 × 10⁶ specific rsTreg (Tallo+rsTreg) or not (Tallo). The expression of GFP among the CD4⁺ T cells was monitored at days 15, 30, and 60 posttransplantation. Dot plots are from 1 representative experiment of 2. Each experiment comprises 2 to 5 mice per group per time point. Numbers in each quadrant represent the percentage of CD4⁺GFP⁺ ± SD among the CD4⁺ T cells.

Discussion

The use of Treg is a promising new strategy under development for the control of GVHD, with extensive support in mice models.[6-8,12,17,18,24,25] An important theoretical advantage of rsTreg for the control of GVHD is that suppression may be specific to alloreactive T cells, unlike conventional immunosuppressive medications, allowing for functional immune system reconstitution.

An important limitation to existing mouse models is the presence of a thymic source for immune reconstitution, which poorly approximates human HSCT in adults, where early immune reconstitution relies primarily on peripheral expansion of T-cell contained in the graft. Indeed, in previous models of Treg-based GVHD prophylaxis, it was difficult to distinguish whether immune responses were due to expansion of T cells present within the graft or rather to newly generated thymus-derived T cells. This is a crucial question because donor T cells present within the graft after allogeneic HSCT are essential for engraftment,[1] immune reconstitution,[2] and GVL effect[3] in humans. If Treg are used as therapeutic tools to prevent GVHD, their effect on postthymic donor T-cell reconstitution needs to be examined. In addition, because of the lack of reliable immunosuppressive medication models protecting mice from the uniform fatality of GVHD, most of what is known about immune reconstitution is derived from studies performed in syngeneic models. However, immune reconstitution in syngeneic and allogeneic contexts are markedly different from the biologic perspective. In this work, we present results from an animal model without thymic contribution, engineered to study immune reconstitution in mice protected from GVHD by rsTreg. Our main objective was to study the effect of rsTreg on immune reconstitution derived from postthymic mature T cells present within the graft. By using CD3-ε knockout BM instead of thymectomized recipients, we excluded residual thymic production because of residual thymic tissue that may remain in thymectomized mice.

Immune reconstitution is a complex process after HSCT (for review, see Cavazzana-Calvo et al[26]), affected by thymus involution, thymic damage, and modification of the immunohematopoietic niches both because of GVHD and cytokine storm. The influence of GVHD on the fate of grafted postthymic T cells was tackled a decade ago by Perreault et al. Using an original mouse model that distinguished alloreactive and nonalloreactive T cells in the same recipient mice, they observed that alloreactive T cells underwent a massive Fas-dependent activation-induced cell death. This was accompanied by bystander lysis of grafted non–host-reactive T cells, leading to abrogated immune reconstitution by donor-derived postthymic T lymphocytes. However, even in the absence of host-reactive T cells, the number of T cells remained limited,[27] in accordance with our results. Furthermore, Dulude et al demonstrated that the reduced size of the postthymic compartment was not because of an intrinsic T-cell defect but rather to an extrinsic microenvironment abnormality.[28] More recently, the same group suggested that IFNγ produced by alloreactive T cells may cause a severe graft-versus-host reaction, responsible for the lymphopenia associated with GVHD.[29] These insights may explain the observations made in the present work: rsTreg may have a preferential suppressive effect on alloreactive T cells, leading to a qualitative as well as a quantitative improvement in immune reconstitution. The decrease in the fulminant alloactivation may lead to better survival of immune cells as well as promotion of a more "benevolent" cytokine environment.

In this work, we observed an overall limited reconstitution of the CD4+ T-cell compartment. These data are not surprising considering the lack of thymopoiesis. In this setting, young mice thymectomized never recover a full repertoire of T cells and remain chronically lymphopenic. Similar findings were made in human where absence of thymopoiesis is typically associated with chronic CD4+ T-cell lymphopenia.[2] Recently, Guimond et al have proposed that high IL-7 level in lymphopenic mice may be responsible for MHC class II down-modulation on dendritic cells, indirectly inhibiting the homeostatic proliferation of CD4+ T cells.[30]

We also showed that the CD8+ T cells compartment is poorly reconstituted, with an inverted CD4/CD8 T-cell ratio. This is also the case in humans, where the CD8+ T-cell compartment is reconstituted early (within a month) whereas the reconstitution of the CD4+ T-cell compartment can take up to 2 years.[14] Interestingly, we observed in protected mice that CD8+ T cells initially proliferated and up-regulated CD44 expression, but by day 30 it was again down-modulated, compatible with dTreg proportion increase. It has been previously shown that CD8+ T cells can acquire a memory-like phenotype characterized by an up-regulation of CD44 during homeostatic expansion and then revert to a naive one at steady state.[31,32] Thus, it may be hypothesized that in our model, in the presence of rsTreg, some CD8+ T cells returned to a naive phenotype after prolonged stimulation even in an incomplete T-cell reconstitution, an important improvement from a therapeutic perspective.

Limited B-cell compartment reconstitution is another characteristic of HSCT in humans, taking up to 2 years after transplantation (for review, see Seggewiss and Einsele[33]). Moreover, absence of immune reconstitution of the B-cell compartment is typically a good marker of GVHD. A recent publication has shown that during GVHD, CD4+ T-cell mediated damage of the bone marrow niche could be responsible for the impaired B-cell hematopoiesis.[34] In this sense, our findings of a rapid B-cell recovery in the setting of rsTreg-based therapy suggested that GVHD was controlled, and are highly encouraging.

We were also able to examine Treg reconstitution after allogeneic HSCT to greater detail. We first observed that rsTreg extensively proliferated in the first 15 days, associated with an efficient control of donor T-cell proliferation and cytokine production. After day 15, rsTreg proliferated less and cytokine production was no longer controlled. Interestingly, the presence of rsTreg allowed for an important expansion of the dTreg compartment, which was not associated with enhanced proliferation of these cells. Indeed, in the presence of rsTreg, dTreg divided less than in their absence while being present in higher numbers. This could be due for instance to reduced activation-induced cell death.[27,28] These results highlight that the impact of rsTreg on graft-host tolerance may exceed rsTreg lifespan. Twenty days after transplantation, protected mice displayed decreasing numbers of rsTreg and concomitantly lost weight. These results confirm our previous observation that the effect of rsTreg to control GVHD is essential at an early time posttransplantation,[13] when the developing dTreg compartment is unable to control donor T-cell division. Thus, the abrogated immune reconstitution observed from day 30 onward could also be a result of a nonlethal and delayed GVHD, which is classically associated with lymphopenia.[27] Interestingly, we did not observe any signs of GVHD in our previous studies using rsTreg to control GVHD in mice grafted with unmanipulated BM. Mice were healthier and displayed a better immune reconstitution[12,13] compared with GVHD-protected mice grafted with CD3ε−/− BM in the present work. This may be caused by T-cell activation because of prolonged lymphopenia or a decrease in thymic derived Treg, leading to decreased numbers of peripheral Treg as well as a decrease of their diversity and their host tissue specificity. Moreover, Matsuoka et al have shown in humans that CD4+ T-cell lymphopenia after HSCT was associated with an increased Fas-mediated Treg apoptosis, secondary to a strong homeostatic drive, possibly contributing to the high incidence of extensive chronic GVHD.[35] Although there are many hypothesized mechanisms that may explain this interesting finding, it once more underlines the

importance of using mouse models that approximate the conditions in human adults, namely models with limited thymic contribution.

In conclusion, these findings support the use of Treg for GVHD prophylaxis as it enables alloreactive specific suppression, thus allowing a qualitative and quantitative improvement in immune reconstitution. This was further supported by 2 different in vivo experimental settings demonstrating a preserved immune function. Consequently, rsTreg may be a safer therapeutic option to prevent GVHD, avoiding generalized immunosuppression early posttransplantation until the grafted HSC reconstitute a functional immune system. However, our study also revealed that in certain conditions the effect of infused rsTreg is time-limited, an important consideration as Treg-based therapy is making its way from the bench to the bedside.

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Authorship

Contribution: A.G. performed research, analyzed the data, and wrote the paper; D.A.L analyzed data and wrote the paper; G.H.M., O.B., D.M., S.G. and C.B. performed research; Y.G.-B. analyzed the data; B.C. designed research and analyzed data; and E.P. and J.L.C designed research, analyzed the data, and wrote the paper.

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Correspondence: José L. Cohen, Centre d'Investigation Clinique en Biothérapie, CHU Henri Mondor, 51 av du Maréchal de Lattre de Tassigny, 94010 Créteil cedex, France; e-mail: jose.cohen@hmn.aphp.fr.

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