Brief Definitive Report

$CD4^+CD25^+$ Immunoregulatory T Cells: New Therapeutics for Graft-Versus-Host Disease

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Abstract

$CD4^+CD25^+$ immunoregulatory T cells play a pivotal role in preventing organ-specific autoimmune diseases and in tolerance induction to allogeneic organ transplants. We investigated whether these cells could also control graft-versus-host disease (GVHD), the main complication after allogeneic hematopoietic stem cell transplantation (HSCT). Here, we show that the few $CD4^+CD25^+$ T cells naturally present in the transplant regulate GVHD because their removal from the graft dramatically accelerates this disease. Furthermore, the addition of freshly isolated $CD4^+CD25^+$ T cells at time of grafting significantly delays or even prevents GVHD. Ex vivo–expanded $CD4^+CD25^+$ regulatory T cells obtained after stimulation by allogeneic recipient-type antigen-presenting cells can also modulate GVHD. Thus, $CD4^+CD25^+$ regulatory T cells represent a new therapeutic tool for controlling GVHD in allogeneic HSCT. More generally, these results outline the tremendous potential of regulatory T cells as therapeutics.

Key words: regulatory T cells • hematopoietic stem cell transplantation • GVHD • tolerance • in vivo animal models

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many hematological malignancies and primary immunodeficiencies. GVHD, the life-threatening and frequent complication of allogeneic HSCT (1), is due to mature donor T cells present in the transplant. However, removal of these T cells before grafting is rarely envisaged because it leads to graft failure (2), prolonged immunosuppression (3), and leukemia relapse (4). To date, standard immunosuppressive treatments of GVHD, consisting in the administration of cyclosporin and methotrexate, are only partially effective (5, 6). This emphasizes the need to develop innovative therapeutic strategies to limit the pathological effects of donor-alloreactive T cells.

$CD4^+CD25^+$ immunoregulatory T cells play a major role in peripheral tolerance of autoreactive T cells. Mice that are rendered deficient for these cells develop multiple T cell–mediated organ-specific autoimmune diseases (7–13). The mechanism of action of these regulatory T cells is poorly understood and largely controversial. In vitro studies showed that these cells inhibit the activation of both $CD4^+$ and $CD8^+$ conventional $CD25^-$ T cells by acting either directly on target T cells or on APCs (13, 14). CTL antigen 4 or TGF-β have been suggested to play a critical role in their T cell suppressive functions (15, 16). These observations were not confirmed in other studies (13). Depending on the model of autoimmune disease, in vivo prevention of autoimmunity by regulatory $CD4^+CD25^+$ T cells has been shown to involve, or not involve, IL-10, IL-4, or TGF-β (13, 17–19). It is thus likely that more than one mechanism is involved in the immunosuppressive activity of these cells.

Three studies recently suggested that the $CD4^+CD25^+$ regulatory T cells could also control alloreactive responses. Taylor et al. (20) showed that these cells have a modest capacity to down-regulate the activation of alloreactive-specific $CD4^+$ T cells in vivo. In addition, the transfer of $CD4^+CD25^+$ regulatory T cells from mice tolerant to allografts can protect syngeneic recipients from rejection of allogeneic islets and skin transplantation (21, 22). The capacity of these cells to control pathogenic effects of alloreactive T cells in vivo leads us to investigate whether these regulatory T cells could also control GVHD after allogeneic HSCT.

In this study, we show that the few $CD4^+CD25^+$ T cells naturally present in the transplant during allogeneic HSCT regulate GVHD. The addition of $CD4^+CD25^+$

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T cells at the time of grafting delays or even prevents the disease. These therapeutic effects were obtained with either fresh cells or ex vivo–expanded cells specific to recipient-type alloantigens. Thus, $CD4^+CD25^+$ regulatory T cells represent a new feasible, therapeutic tool for controlling GVHD.

Materials and Methods

HSCT. C57Bl/6 (B6; H-2^b), BALB/c (H-2^d), (B6 × DBA/2 [D2])F1 (H-2^{bxd}), and C3H (H-2^k) mice were obtained from Charles River Laboratories. Mice were manipulated according to European Economic Community guidelines. Unless otherwise stated, experiments were performed as previously described (23). In brief, 24 h after lethal irradiation of (B6 × D2)F1 (11 Gy) and B6 (10 Gy) or C3H (9.5 Gy) mice, recipients were transplanted with cells from B6 or BALB/c donor mice, respectively. The transplants were constituted of 5×10^6 T cell–depleted bone marrow (BM) cells, 10×10^6 T cells collected from pooled spleen and peripheral LN (referred to as total T cells in the text), and when indicated, purified of $CD4^+CD25^+$ T cells. In control mice, the transplantation of only the T cell–depleted BM cells did not induce GVHD.

Purification of $CD4^+CD25^+$ T Cells. Cells from the spleen and peripheral LN were sequentially incubated with saturating amounts of biotin-labeled anti-CD25 antibody (7D4; BD Biosciences) and streptavidin microbeads (Miltenyi Biotec) for 30 min on ice, followed by purification of magnetic cell separation using LS columns (Miltenyi Biotec) according to the manufacturer's instructions. To increase cell purification, the cells of the positive fraction were separated on another LS column. All steps were performed in PBS with 3% serum. The purity of the $CD4^+CD25^+$ T cells was of 80–85%. The CD25-depleted cells that did not bind to the anti-CD25–coated beads were harvested from the flow through and contained <0.3% $CD4^+CD25^+$ T cells. The fresh $CD4^+CD25^+$ T cells and the CD25-depleted cells were washed twice with PBS before injection in HSCT. For in vivo cell expansion, $CD4^+CD25^+$ T cells were additionally enriched. Cells were stained for 30 min on ice with FITC-labeled anti-CD4 (GK1.5), phycoerythrin-labeled anti-CD62L (MEL-14), and streptavidin-CyChrome (all from BD Biosciences), which bound to free biotin-labeled CD25 molecules uncoupled to beads. The $CD4^+CD25^+CD62L^{high}$ T cells were sorted on a FACStar^{Plus}™ (Becton Dickinson), giving a purity of 99%.

Culture of $CD4^+CD25^+CD62L^{high}$ T Cells. Highly purified $CD4^+CD25^+CD62L^{high}$ T cells from B6 or BALB/c mice were stimulated with total splenocytes from (B6 × D2)F1 or C3H and B6 mice, respectively. Cultures were performed in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL), L-glutamine, antibiotics, 10 mM Hepes, 5×10^{-5} M 2-β-mercaptoethanol, and 30 ng/ml mouse IL-2 (R&D Systems). At the beginning, 10^6 $CD4^+CD25^+CD62L^{high}$ T cells/ml were cocultured with 2×10^6 irradiated (20 Gy) splenocytes/ml. After 5 d of culture, cells were counted and cell density was adjusted to 10^6/ml with fresh medium if necessary. At day 8, cells were reseeded at 0.1×10^6/ml and restimulated with 2×10^6 irradiated splenocytes/ml. After 4 d, cells were counted and cell density was adjusted to 0.2×10^6/ml with fresh medium if necessary. Additional cycles of stimulation were similarly performed. Cells were analyzed by flow cytometry after staining with FITC-labeled anti-CD4 (GK1.5), phycoerythrin-labeled anti-CD62L (MEL-14), and streptavidin-CyChrome (all from BD Biosciences) on a FACSCalibur® (Becton Dickinson), or washed twice in PBS and used for HSCT.

Proliferation Assays. $CD4^+CD25^+CD62L^{high}$ cells purified from BALB/c mice were stimulated for 15 d by irradiated C3H or B6 splenocytes as described above. 10^5 T cells of both cultures were then restimulated by either 10^6 irradiated C3H or B6 splenocytes in the presence of 30 ng/ml IL-2 in flat-bottom 96-well plates for 48–72 h, and then pulsed with methyl-[^{3}H]thymidine for the last 15 h. $CD4^+CD25^+CD62L^{high}$ cells purified from BALB/c mice and stimulated by irradiated C3H splenocytes for 5 wk were also tested for their in vitro suppressive activity. After two washes to remove IL-2, different numbers of expanded regulatory T cells were added to the culture of 4×10^4 fresh, CD25-depleted T cells (purified from BALB/c spleen and LNs) stimulated by 10^5 irradiated C3H splenocytes without IL-2. Cells cultured in round-bottom 96-well plates for 72 h were pulsed with methyl-[^{3}H]thymidine for the last 6 h.

Statistical Analyses. Statistical analyses were performed using Statview software (SAS Inc.). Kaplan-Meier survival curves were established for each group. P-values for the log-rank test are indicated.

Results and Discussion

$CD4^+CD25^+$ T cells represent 5–10% of the normal T cell compartment in mice and humans (7, 24). During allogeneic HSCT, donor T cells are present in the transplant. Consequently, when grafted, patients also receive $CD4^+CD25^+$ regulatory T cells. We first analyzed whether this population plays a role in the control of GVHD. In our murine model, $CD4^+CD25^+$ T cells represent 3–5% of the donor cells collected from the spleen and LN. The incidence of GVHD was compared after the allogeneic HSCT of lethally irradiated (B6 × D2)F1 mice receiving BM cells with either total donor T cells or CD25-depleted donor T cells from B6 mice. In this semiallogeneic combination between donor and recipient, the infusion of 10×10^6 total T cells induced lethal GVHD (Fig. 1). All mice had ongoing clinical signs of GVHD and were dead by day 41. When the mice were grafted with the same number of CD25-depleted T cells, the onset of clinical signs of GVHD such as weight loss, diarrhea, and hunching, appeared much sooner and all mice were dead by day 21 after transplantation (Fig. 1). This result revealed an unforeseen effect of $CD4^+CD25^+$ regulatory T cells present in the transplant, i.e., they play a major role in the control of GVHD.

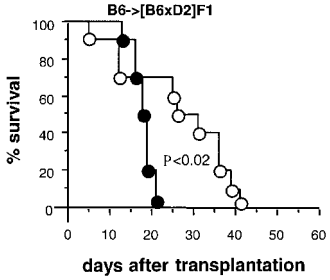

Figure 1. $CD4^+CD25^+$ regulatory T cells naturally present in the transplant modulate GVHD. Lethally irradiated (B6 × D2)F1 mice were grafted with semiallogeneic B6 BM cells supplemented with either 10×10^6 B6 T cells (○; $n = 10$) or 10×10^6 CD25-depleted B6 T cells (●; $n = 10$). Cumulative results from two independent experiments are shown. Kaplan-Meier survival curves were established for each group with the indicated P-values.

The effect of regulatory T cells on GVHD after HSCT suggested their potential use for therapeutic intervention. Therefore, we investigated whether GVHD would be delayed if additional numbers of $CD4^+CD25^+$ T cells were injected. First, we verified that $CD4^+CD25^+$ T cells did not induce GVHD. When lethally irradiated mice were grafted with a BM transplant supplemented with 5×10^6 $CD4^+CD25^+$ purified T cells, no GVHD was observed (unpublished data) in accordance with a previous report (20). We then grafted irradiated (B6 × D2)F1 mice with BM cells and 10×10^6 T cells supplemented with 5×10^6 $CD4^+CD25^+$ purified T cells from B6 mice. These mice remained healthy until about day 25, as opposed to the control mice (BM cells plus total T cells), which rapidly developed clinical signs of GVHD from days 8 to 10 (unpublished data). Significantly, two out of four mice receiving additional regulatory T cells survived without any additional treatment (Fig. 2 A). When these two mice were killed at day 60, we did not observe any histopathological signs of GVHD in the liver, a target organ of GVHD, and one mouse displayed moderate signs of GVHD in the spleen (unpublished data). We reproduced this experiment with a different genetic combination. When C3H mice were grafted with BALB/c donor cells, GVHD-related mortality occurred very fast in the control group transferred with BM cells and 10×10^6 T cells (100% of the mice died by day 10). The addition of 5×10^6 $CD4^+CD25^+$ purified T cells significantly delayed mortality compared with the control group. Clinical signs of GVHD were not observed before day 29 and no mice died until day 35 (Fig. 2 B). At day 60, three out of five mice did not display any clinical signs of GVHD. Altogether, these results demonstrate that the sole addition of fresh $CD4^+CD25^+$ regulatory T cells significantly delays or even prevents GVHD after allogeneic HSCT.

A major limitation in the potential use of regulatory T cells for preventing GVHD is the difficulty in obtaining a sufficient number of these relatively rare cells. Therefore, we tested whether they could be expanded while retaining their functional properties. We chose to stimulate these cells by allogeneic APCs in the presence of IL-2 with the aim to increase their number (24–27) and specificity to recipient-type alloantigens. We started with highly purified populations of $CD4^+CD25^+CD62L^{high}$ T cells constituting the major fraction of the $CD4^+CD25^+$ regulatory T cells (26) to limit the contamination with conventional activated $CD4^+CD25^+CD62L^{low}$ T cells (28). The cells purified from BALB/c or B6 mice were then cocultured with irradiated C3H or (B6 × D2)F1 splenocytes, respectively. In both cultures, regulatory T cells rapidly expanded. From 5.5×10^6 BALB/c $CD4^+CD25^+$ T cells, we were able to produce 100×10^6 regulatory T cells (20-fold expansion) after 15 d of culture. In the same manner, the number of B6 $CD4^+CD25^+$ T cells was increased 10-fold during the first 2 wk and 100-fold during the next 2 wk of culture (Fig. 3 A). Similar expansion was observed in another genetic combination, in which BALB/c $CD4^+CD25^+$ T cells were stimulated by B6 splenocytes (unpublished data). Importantly, these cells kept the phenotype of regulatory T cells because they expressed even higher levels of CD25 and most of them maintained high levels of CD62L expression (Fig. 3 B). Interestingly, the absence of down-regulation of CD62L expression after repeated activation could be an intrinsic characteristic of these regulatory T cells. Because regulatory T cells were stimulated by allogeneic splenocytes, we tested whether this population was enriched in cells responding preferentially to these alloantigens. After 2 wk of culture of BALB/c regulatory T cells stimulated by irradiated C3H APCs, these cells did not respond to B6 APCs after short-term stimulation, although they continued to proliferate to C3H APCs. Similar findings were observed when using B6 APCs instead of C3H APCs (Fig. 3 C). We then analyzed whether these ex vivo–expanded regulatory T cells maintained their in vitro–suppressive properties. When added to a culture of fresh $CD25^-$ T cells stimulated by allogeneic APCs, regulatory T cells strongly inhibited T cell proliferation (Fig. 3 D).

To test the capacity of the ex vivo–expanded $CD4^+CD25^+CD62L^{high}$ T cells to regulate GVHD, we performed experiments similar to those presented in Fig. 2 using cultured $CD4^+CD25^+CD62L^{high}$ T cells instead of freshly isolated $CD4^+CD25^+$ T cells. In the B6→B6 × D2)F1 combination, the addition of 7×10^6 $CD4^+CD25^+$ T cells cultured in the presence of recipient-type alloantigens to BM cells and 10×10^6 T cells remarkably prolonged mouse survival compared with the control group (Fig. 4 A). This observation was confirmed when BALB/c regulatory T cells cultured in the presence of C3H alloantigens were used to modulate GVHD in the BALB/c→C3H combination. We then tested whether the regulation of GVHD required the use of regulatory T cells specific to recipient-type alloantigens. In the BALB/c–B6 combination,

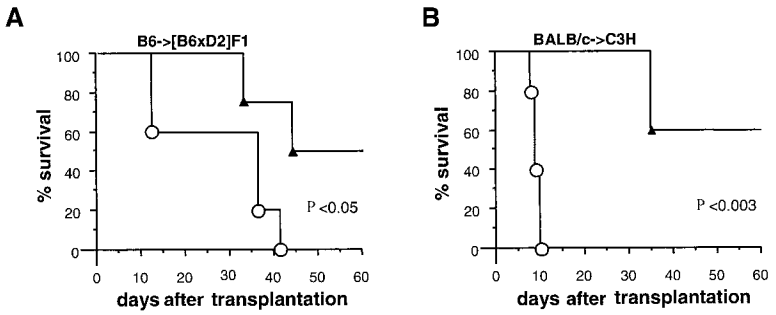

Figure 2. Prevention of GVHD by the addition of fresh $CD4^+CD25^+$ regulatory T cells. Lethally irradiated mice were grafted with allogeneic BM cells supplemented with either 10×10^6 T cells (○; $n = 5$) or 10×10^6 T cells and 5×10^6 freshly isolated $CD4^+CD25^+$ T cells (▲; $n = 4$). (A) Survival of (B6 × D2)F1 recipients transplanted with semiallogeneic B6 cells. (B) Survival of C3H recipients transplanted with fully allogeneic BALB/c cells. Kaplan-Meier survival curves were established with the indicated P-values.

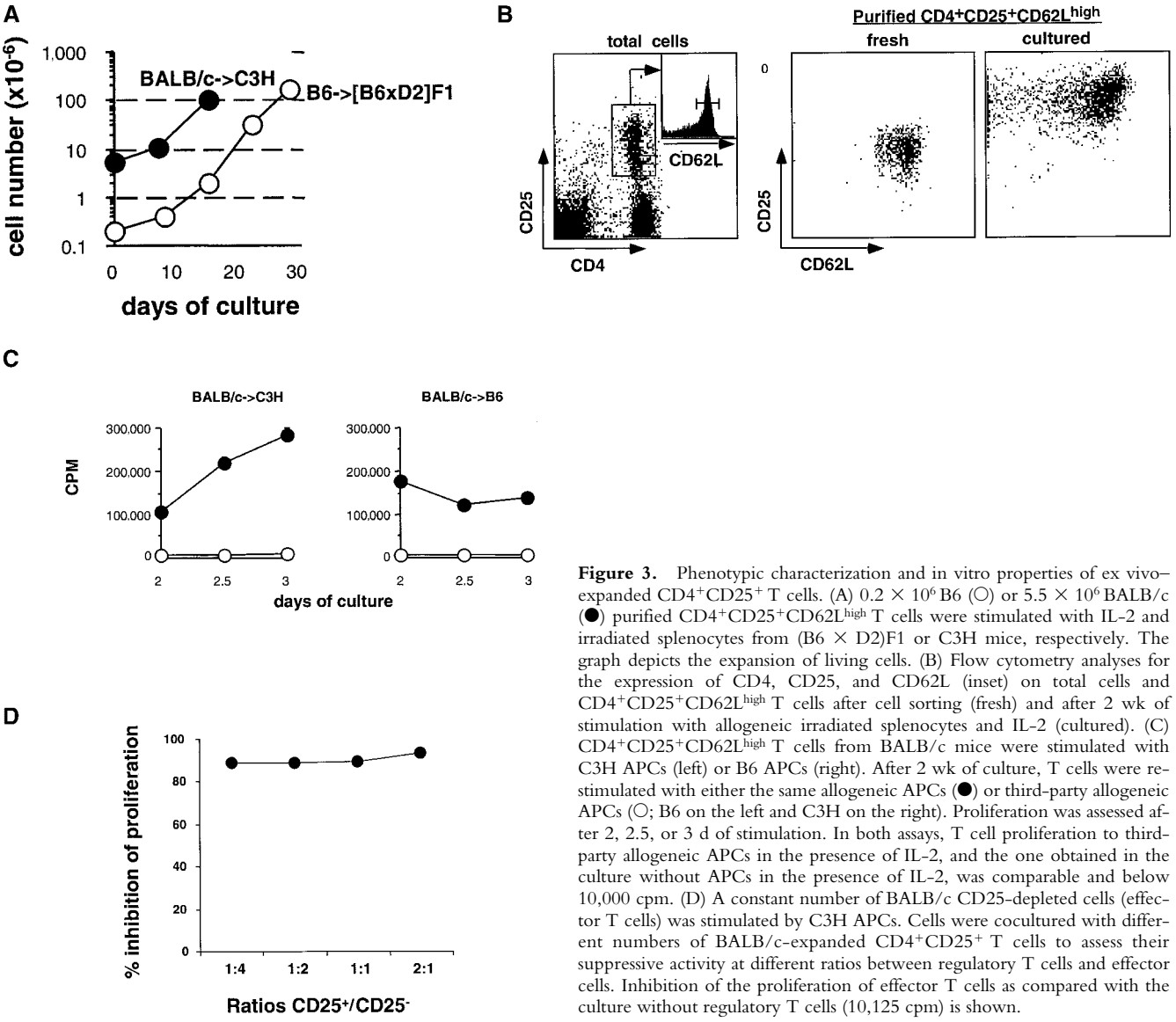

Figure 3. Phenotypic characterization and in vitro properties of ex vivo–expanded $CD4^+CD25^+$ T cells. (A) 0.2×10^6 B6 (○) or 5.5×10^6 BALB/c (●) purified $CD4^+CD25^+CD62L^{high}$ T cells were stimulated with IL-2 and irradiated splenocytes from (B6 × D2)F1 or C3H mice, respectively. The graph depicts the expansion of living cells. (B) Flow cytometry analyses for the expression of CD4, CD25, and CD62L (inset) on total cells and $CD4^+CD25^+CD62L^{high}$ T cells after cell sorting (fresh) and after 2 wk of stimulation with allogeneic irradiated splenocytes and IL-2 (cultured). (C) $CD4^+CD25^+CD62L^{high}$ T cells from BALB/c mice were stimulated with C3H APCs (left) or B6 APCs (right). After 2 wk of culture, T cells were restimulated with either the same allogeneic APCs (●) or third-party allogeneic APCs (○; B6 on the left and C3H on the right). Proliferation was assessed after 2, 2.5, or 3 d of stimulation. In both assays, T cell proliferation to third-party allogeneic APCs in the presence of IL-2, and the one obtained in the culture without APCs in the presence of IL-2, was comparable and below 10,000 cpm. (D) A constant number of BALB/c CD25-depleted cells (effector T cells) was stimulated by C3H APCs. Cells were cocultured with different numbers of BALB/c-expanded $CD4^+CD25^+$ T cells to assess their suppressive activity at different ratios between regulatory T cells and effector cells. Inhibition of the proliferation of effector T cells as compared with the culture without regulatory T cells (10,125 cpm) is shown.

the addition of 7×10^6 BALB/c regulatory T cells cultured in the presence of B6 alloantigens significantly delayed the occurrence of GVHD, which confirmed the capacity of specific regulatory T cells to regulate GVHD in a third genetic combination. In comparison, the addition of 7×10^6 BALB/c regulatory T cells cultured in the presence of third-party C3H alloantigens had no effect on GVHD mortality in the BALB/c–B6 combination. This control culture also shows that the sole injection of ex vivo–expanded $CD4^+$ T cells was not sufficient to regulate GVHD. Remarkably, in the three genetic combinations, the mice that had received $CD4^+CD25^+$ regulatory T cells cultured in the presence of recipient-type alloantigens appeared completely healthy for several weeks. Their clinical status then suddenly and rapidly deteriorated and they finally developed clinical signs of severe GVHD. Thus, although the use of ex vivo–expanded $CD4^+CD25^+$ T cells significantly delayed GVHD, it did not preclude the occurrence of a delayed severe GVHD. This suggests that ex vivo–expanded regulatory T cells have a limited half-life after adoptive transfer and sequential injection of these cells should be required to induce long-term protection from GVHD. Nevertheless, we observed that the clinical status of mice receiving cultured regulatory T cells improved compared with mice receiving fresh regulatory T cells injected in comparable proportions (5 and 7 million for fresh and cultured cells, respectively) during the first few weeks after transfer (unpublished data). In sum, these results demonstrate that a high number of $CD4^+CD25^+$ T cells can be generated ex vivo without altering their phenotype nor their regulatory property toward GVHD.

So far, $CD4^+CD25^+$ regulatory T cells have been shown to regulate both autoimmune diseases (7, 8) and the rejection of allogeneic solid organ transplantation (21, 22). In this study, we show that the few regulatory T cells naturally present in the inoculum during allogeneic HSCT signifi-

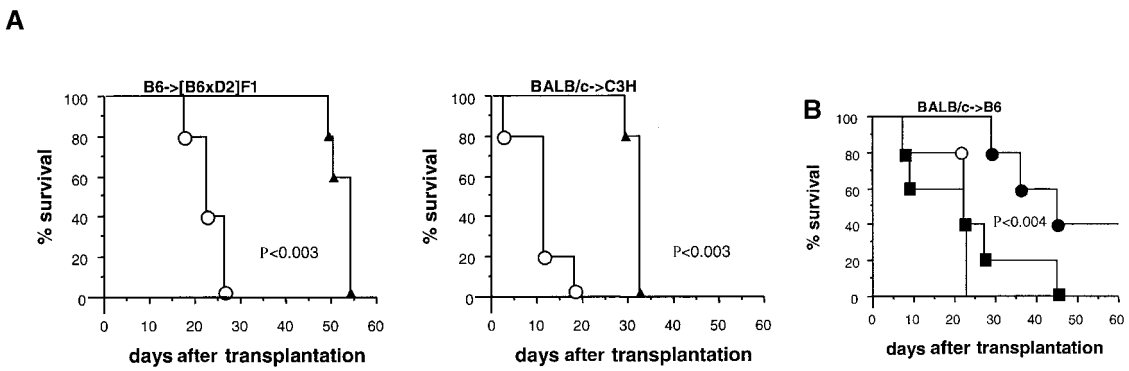

Figure 4. Regulation of GVHD by the addition of expanded $CD4^+CD25^+$ regulatory T cells. At the end of the culture (days 15 and 28 for regulatory T cells from BALB/c and B6 mice, respectively), expanded regulatory T cells were tested for their capacity to control GVHD. (A) Lethally irradiated mice were grafted with allogeneic BM cells supplemented with either 10×10^6 fresh T cells (○; $n = 5$ per group) or 10×10^6 fresh T cells and 7×10^6 expanded $CD4^+CD25^+$ T cells (▲; $n = 5$ per group). For both genetic combinations, the addition of expanded $CD4^+CD25^+$ T cells statistically increased the survival of mice. (B) Lethally irradiated B6 mice were grafted with BALB/c BM cells and 10×10^6 fresh BABL/c T cells (○, GVHD control group; $n = 5$) supplemented with 7×10^6 expanded regulatory T cells derived from cultured $CD4^+CD25^+$ T cells stimulated by C3H splenocytes (■, nonspecific regulatory T cells; $n = 5$), or B6 splenocytes (●, specific regulatory T cells; $n = 5$). The difference in survival between the GVHD control group and mice receiving nonspecific $CD4^+CD25^+$ T cells is statistically insignificant. When statistically significant, Kaplan-Meier survival curves were established with the indicated P-values.

cantly delay the occurrence of GVHD and associated mortality, and can be used in cell therapy. It should be noted that even if these cells are regarded as having a major therapeutic potential in autoimmune diseases, such effect has only been demonstrated to date in CD25-deficient animals (7–9). $CD4^+CD25^+$ regulatory T cells have also been demonstrated to efficiently prevent the rejection of allogeneic solid organ transplants, but this effect was obtained with cells purified from mice that had previously received an in vivo treatment for tolerance induction (21, 22). Thus, our work is the first report demonstrating that the addition of freshly isolated regulatory T cells from unmanipulated animals can control an immunopathology in a model mimicking a clinical setting.

In GVHD, we obtained a therapeutic effect after the addition of regulatory T cells in similar proportions to donor T cells. Furthermore, our results suggest that repeated injections of regulatory T cells could be required for long-term protection from GVHD. Thus, the purification of sufficient numbers of regulatory T cells could be a bottleneck for applying this strategy to humans. Indeed, 3 billion T cells are usually present in the infused transplant, whereas a maximum of 100 million cells of fresh regulatory T cells could be collected from the blood of the same donor. To date, the only realistic use of these cells in humans would be to expand them ex vivo. This led us to test the functionality of $CD4^+CD25^+$ regulatory T cells in GVHD after their ex vivo expansion. Previous reports demonstrated that cultured regulatory T cells from both mice and humans remain functional after expansion, but their suppressor activity was only shown in in vitro assays (24–27). Here, we show for the first time that extensively expanded regulatory T cells can still be used to modulate an immunopathological process in vivo and could consequently be envisaged as a new therapeutic tool when a large number of regulatory T cells is required. The ex vivo expansion of regulatory T cells stimulated by recipient-type alloantigens presents three additional advantages. First, the repertoire of regulatory T cells specific to recipient alloantigens can be selected, whereas nonalloreactive cells die during the culture in the absence of TCR-mediated activation as suggested in this study. In this case, the regulatory effects of these expanded cells could be preferentially targeted to the pathogenic donor T cells specific to the recipient alloantigens. As a result, GVHD would be controlled without altering the immune reconstitution after allogeneic HSCT. Second, the extensive proliferation of regulatory T cells during culture is compatible with retroviral gene transfer. This offers the possibility to transduce these cells with suicide genes, for example, to control or eliminate them in case of significant side effects after their injection (29). Finally, the possibility to produce high numbers of regulatory T cells should provide versatility in designing therapeutic schemes adapted to different clinical setting of allogeneic HSCT. Our results suggest that the therapeutic use of $CD4^+CD25^+$ regulatory T cells could not only be envisaged in putative pathologies linked to a deficiency of these cells, but also for the treatment of multiple T cell–dependent immunopathologies.

We acknowledge Sébastien Maury, Sylvie Bruel, and Guillaume Gavori for technical assistance, Micaël Yagello for cell sorting, and Gilbert Boisserie and François Baillet for the irradiation of mice. We thank Laurence Zitvogel, Jeffrey Bluestone, Ricardo Cibotti, Nuria Serrano, and Olivier Boyer for critical review of the manuscript.

This work was supported in part by the Association Française contre les Myopathies, Université Pierre et Marie Curie (Paris VI), Genopoïetic, Association de Recherche contre le Cancer, and the Fondation de France.

Submitted: 17 January 2002
Revised: 28 May 2002
Accepted: 5 June 2002

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