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Abstract

Apoptotic leukocytes are endowed with immunomodulatory properties that can be used to enhance hematopoietic engraftment and prevent graft-versus-host disease. This apoptotic cell-induced tolerogenic effect is mediated by host macrophages and not recipient dendritic cells or donor phagocytes present in the bone marrow graft as evidenced by selective cell depletion and trafficking experiments. Furthermore, apoptotic cell infusion is associated with TGF-β-dependent donor $CD4^+CD25^+$ T cell expansion. Such cells have a regulatory phenotype ($CD62L^{high}$ and intracellular $CTLA\text{-}4^+$), express high levels of Foxp3 mRNA and exert e*x vivo* suppressive activity through a cell-to-cell contact mechanism. *In vivo* CD25 depletion after apoptotic cell infusion prevents the apoptotic spleen cell-induced beneficial effects on engraftment and graft-versus-host disease occurrence. This highlights the role of regulatory T cells in the tolerogenic effect of apoptotic spleen cell infusion. This novel association between apoptosis and regulatory T cell expansion may also contribute to preventing deleterious auto-immune responses during normal turnover.

Keywords: Transplantation; macrophages; apoptosis; regulatory T cells

Abbreviations: Ab, antibody; APC, allophycocyanin; BM, bone marrow; BMT, bone marrow transplantation; CDC, complement-dependent cytotoxicity; DC, dendritic cells; DT, diphtheria toxin; Foxp3, forkhead-box transcription factor p3; GvHD, graft-versus-host disease; HC, hematopoietic cell; mAb, monoclonal antibody; MLR, mixed leukocyte reaction; PerCP, peridinin chlorophyll-a protein; QRT-PCR, quantitative real time PCR; TBI, total body irradiation; Tr1, T regulatory type 1 cells; Treg, regulatory T cells

Introduction

Immune tolerance is maintained by several mechanisms.[1] In addition to central tolerance, various peripheral mechanisms, including T cell anergy or peripheral T cell deletion, cooperate to prevent unwanted T cell immune responses directed against self antigens.[1] Recently, active suppression by $CD4^+CD25^+$ regulatory T cells (Treg) has been extensively explored as a mechanism involved in limiting auto-immunity[2,3] as well as a way to induce transplantation tolerance.[4] Thus, $CD4^+CD25^+$ Treg cells have been shown to prevent acute graft-versus-host disease (GvHD),[5,6] a frequent complication of allogeneic hematopoietic cell (HC) transplantation. Expression of CD25 is shared by different $CD4^+$ Treg subsets including $CD4^+CD25^+$ "suppressive" T cells which act by cell contact-dependent mechanisms,[3,7] IL-10 producing T regulatory type 1 (Tr1) cells[8] and TGF-β producing Th3 cells.[9] Other Treg-associated molecules such as CD62L or CTLA-4 (CD152) are commonly used to identify $CD4^+$ Treg cells.[4,5] Recently, forkhead-box transcription factor p3 (Foxp3, called also scurfin) has emerged as an adequate marker for "natural" $CD4^+CD25^+$ "suppressive" T cells[10-12] as well as other peripherally produced $CD4^+$ T cells endowed with regulatory properties.[13-15] However, the phenotypic characterization as well as suppressive mechanism of $CD4^+$ Treg cells may differ, depending on the experimental model used.[3]

Apoptosis is a physiological process which liberates intracellular proteins, rendering critical the maintenance of self-tolerance. Thus, following normal cellular turnover, billions of apoptotic cells are produced every day without systematically causing autoimmunity. Several peripheral tolerance mechanisms explaining the absence of immune responses against apoptotic cell-derived Ags have been proposed.[16-18] First, professional phagocytes, such as macrophages[19] or certain dendritic cells (DC) subsets,[20-23] quickly capture apoptotic bodies, thereby preventing the release of harmful products from dying cells.[24] In addition, apoptotic

cell death is frequently associated with an immunomodulatory milieu that may neutralize immune cells present in the environment of dying cells. Such milieu consists mainly of anti-inflammatory cytokines like IL-10 or TGF-β released by macrophages which phagocyte apoptotic cells[19,25,26] or by circulating monocytes in the absence of phagocytosis.[27] Furthermore, some cells like T cells may release TGF-β or IL-10 in the course of their apoptotic process.[28,29] Although the release of TGF-β or IL-10 during apoptosis has been extensively reported,[19,25-29] capacities of apoptotic cells to induce a $CD4^+$ T cell subset endowed with regulatory properties has not been demonstrated so far.

Allogeneic HC transplantation is characterized by the presence of immunocompetent cells of both host and donor origin and the subsequent alloreactive conflict. Donor graft rejection is mainly mediated by recipient T or NK cells that resist the conditioning regimen. In contrast, donor immune cells present in the HC graft are responsible for inducing GvHD. In some situations, GvHD can be beneficial to the host by limiting leukemia relapse (graft-versus-leukemia effect). Strategies are required to control this alloreactive conflict that follows allogeneic HC transplantation. Physiological mechanisms that maintain immune homeostasis and self-tolerance have been exploited for a long time to induce tolerance to allograft.[30] In this way, we used i.v. infusion of apoptotic leukocytes to enhance bone marrow (BM) engraftment across major histocompatibility barriers whatever their origin.[31] Here, we reported that after infusion, apoptotic spleen cells were engulfed by splenic dendritic cells and macrophages. Depletion of donor phagocytes present in the graft had no effect on apoptotic cell–induced engraftment. Conversely, depletion of host macrophages –but not host dendritic cells– before transplantation prevented apoptotic spleen cell-induced engraftment. Furthermore, apoptotic spleen cell infusion was associated with donor $CD4^+CD25^+$ T cell expansion which depends on TGF-β production. Such cells had a regulatory phenotype ($CD62L^+$, intracellular $CTLA4^+$)

and expressed high levels of Foxp3 mRNA. *Ex vivo*, these Treg exerted their suppressive activity through a cell-to-cell contact mechanism independently of IL-10 or TGF-β. *In vivo*, these Treg were involved in the protective effect of donor apoptotic spleen cell administration on GvHD occurrence.

RESULTS

The graft-facilitating effect of apoptotic spleen cell infusion requires host macrophages but not donor phagocytes present in the bone marrow graft.

Apoptotic cells are quickly removed by macrophages[19,26] or some DC subsets.[20-23] To examine which cell populations would engulf apoptotic cells, recipients were given donor fluorescent-labeled apoptotic spleen cells in addition to the BM graft. At various times post-BMT (30, 45 min, 1, 2, 4 and 8 h), the spleen, liver and lungs were analyzed to determine where and by which phagocytes apoptotic cells had been engulfed. Analysis of time kinetics revealed that apoptotic spleen cells were quickly removed mainly by splenic phagocytes, since there was no detectable fluorescent signal in the lung or in the liver at any time points after infusion (data not shown). Flow cytometric analysis indicated that splenic DC as well as macrophages were involved in apoptotic cell uptake (**Figure 1a**). A higher proportion of labeled phagocytes was observed 45 min after infusion, while no signal could be detected in the spleen tested 4 h after infusion (**Figure 1a**). Phagocytes involved in apoptotic spleen cell uptake may be resident host splenic phagocytes and/or phagocytes present in donor BM graft that migrate to the spleen after infusion. To evaluate the involvement of donor phagocytes, an enriched Sca-1^+ stem cell graft (3×10^5 cells) was used instead of a total BM graft. Despite significant depletion of $CD11c^+$ cells ($83 \pm 8\%$) and $F4/80^+$ cells ($95 \pm 9\%$) after Sca-1^+ cell enrichment, donor HC engraftment was conserved (**Figure 1b**). This suggests that donor phagocytes are not required for the graft facilitating effect of apoptotic spleen cells.

To deplete resident host phagocytes, liposomes loaded with clodronate[32, 33] were infused before BMT. Pretreatment of mice with clodronate-loaded liposomes reduced significantly both the number of splenic $CD11b^+$ macrophages and apoptotic cell-induced donor BM engraftment (**Figure 1c**). As previously described,[33] we observed that not all splenic DC subsets were depleted by clodronate-loaded liposome infusion due to their anatomic location

(**Supplementary Figure 1** online). To further explore the role of host DC in the graft-facilitating effect of apoptotic cells, a transgenic mouse model for *in vivo* DC ablation[34] was used. Administration of diphtheria toxin (DT) in such recipient transgenic mice allowed splenic DC depletion for 48 h (**Figure 1d** and ref.34). Despite significant depletion of $eGFP^+CD11c^+$ DC at the time of BMT, the graft-facilitating effect of apoptotic cells was maintained (**Figure 1d**). This suggests that host DC are not critical for the graft-facilitating effect induced by apoptotic spleen cell infusion. Altogether, these results show that recipient splenic phagocytes and in particular macrophages are involved in the graft-facilitating effect of i.v. apoptotic cell infusion.

Intravenous apoptotic spleen cell infusion leads to the TGF-β dependent expansion of $CD4^+CD25^+$ regulatory T cells.

We recently reported that i.v. administration of apoptotic spleen cells, simultaneously with BM grafts, favored engraftment via a TGF-β-dependent mechanism.[35] Because TGF-β can be implicated in the generation of Treg sub-populations[9,13-15] and as fluorescent apoptotic bodies within splenic phagocytes could be found (**Figure 1a**), we first analyzed the spleen for the presence of $CD4^+CD25^+$ T cells at week 7-9 post-BMT. Analysis of splenic T cell populations in mice given apoptotic cells revealed a $CD4^+CD25^+$ T cell increase in engrafted versus non-engrafted recipients (8.8 $\pm$ 0.6% vs. 3.9 $\pm$ 0.4%, mean $\pm$ SEM, 10 mice per group, $P < 0.01$, **Figure 2a**). All these recipient mice exhibited a similar splenic cellularity (between 70-100 x 10^6 cells). Expansion of $CD4^+CD25^+$ T cells was restricted to engrafted mice and specific to apoptotic cell infusion, since no such increase was observed in recipient mice engrafting after infusion of a higher number of BM cells (3 x 10^6 BM cells, 1.0 $\pm$ 0.2%, $n = 8$, $P < 0.05$) or after an increased conditioning regimen (total body irradiation, TBI dose: 7 Gy, 1.5 $\pm$ 0.2%, $n = 5$, $P < 0.05$) in the absence of apoptotic cells (**Figure 2a**). Such $CD4^+CD25^+$ T cell increase

–observed 7-9 weeks after apoptotic cell infusion– was not systematically found in the blood nor in other organs tested, including BM, cervical as well as inguinal lymph nodes (data not shown). To determine the role of TGF-β in this apoptotic cell-induced increase, $CD4^+CD25^+$ T cells were analyzed at week 7-9 after BMT and TGF-β neutralization. Mice treated with anti-TGF-β antibody (Ab) did not exhibit any increase in splenic $CD4^+CD25^+$ T cells (**Figure 2b**). In contrast, mice given irrelevant Ab plus apoptotic cells had similar splenic $CD4^+CD25^+$ T cell levels as mice receiving apoptotic cells (**Figure 2b**). TGF-β neutralizing effect could be also observed in peripheral blood (62 ± 7 $CD4^+CD25^+$ T cells/µL in anti-TGF-β Ab treated mice vs 288 ± 46 cells/µL in control Ab-treated mice; $P < 0.01$). Overall, this suggests that TGF-β production is involved in apoptotic cell-induced $CD4^+CD25^+$ T cell increase.

Further phenotypic and functional characterization of splenic $CD4^+CD25^+$ T cells detected in mice given apoptotic spleen cells was performed to know whether such cells displayed regulatory functions. The $CD4^+CD25^+$ T cells present in engrafted mice were of donor origin; they presented a regulatory T cell-like phenotype ($CD45RB^{low}CD62L^{high}$ intracellular CTLA-4^{high}) and expressed high levels of Foxp3 mRNA (**Figure 3a-b**). In contrast, $CD4^+CD25^+$ T cells found in non-engrafted mice exhibited a phenotype rather consistent with an activated T cell phenotype (intracellular CTLA-4^{low}, $CD62L^{low}$) and were, as expected, of host origin (**Figure 3a**). Purified $CD4^+CD25^+$ T cells from non-engrafted mice expressed low levels of Foxp3 mRNA (**Figure 3b**). Increase of splenic $CD4^+CD25^+$ T cells 7-9 weeks after apoptotic cell infusion was confirmed by direct quantification of FoxP3 mRNA in recipient whole spleen. A 9-fold increase of FoxP3 mRNA expression was detected at week 7-9 after BMT in mice given apoptotic cells plus BM in comparison to mice given BM alone (**Figure 3c**).

Finally, $CD4^+CD25^+$ T cells were functionally characterized in *in vitro* assays. CD25 depletion restored allogeneic proliferation of splenic $CD4^+$ T cells from engrafted mice that had received apoptotic spleen cells, whereas CD25 depletion did not affect the proliferation of

splenic $CD4^+$ T cells from non-engrafted mice (**Figure 3d**). In addition, suppressive assays with FACS-sorted $CD4^+CD25^+$ T cells demonstrated that apoptotic cell-induced $CD4^+CD25^+$ T cells exerted their suppressive activity through cell contact-dependent mechanisms. Their suppressive activity could only be inhibited by adding a transwell but not by IL-10, TGF-β, or IL-10 and TGF-β neutralization (**Figure 3e**). These results show that simultaneous apoptotic spleen cell and BM infusion induced a TGF-β-dependent splenic expansion of $CD62L^{high}CD4^+CD25^+$ T cells endowed with regulatory properties at week 7-9 after BMT.

Host macrophage but not dendritic cell depletion is critical for the induction of splenic $CD62L^+CD4^+CD25^+$ T cells after apoptotic spleen cell infusion.

We then considered which host cells phagocyting apoptotic cells (macrophages and/or DC) were responsible for splenic $CD62L^{high}CD4^+CD25^+$ T cell induction. Effects of DC depletion at time of transplantation were analyzed in CD11c/DTR/eGFP transgenic recipient. No significant modification of $CD62L^{high}C4^+CD25^+$ T cell population was observed when DC were depleted at time of transplantation in comparison to non-CD11c depleted transgenic mice, even after apoptotic spleen cell infusion (**Figure 4**). Clodronate-liposome infusion at the time of BMT and apoptotic cell infusion induced the failure of both BM engraftment and donor $CD4^+CD25^+$ T cell expansion (data not shown). It was thus difficult to know whether donor $CD4^+CD25^+$ T cells participated to BM engraftment or whether donor $CD62L^+CD4^+CD25^+$ T cell increase was related to BM engraftment. To answer to this question, splenic $CD62L^{high}CD4^+CD25^+$ T cells were analyzed by cytometry at different early time points after BMT and macrophage depletion (using clodronate-loaded liposome). This phenotype ($CD62L^+CD4^+CD25^+$) was chosen since we showed that cells with such phenotype express high levels of Foxp3 mRNA (**Figure 3b**) and exert suppressive activity (**Figure 3d-e**). Early after apoptotic spleen cell infusion (day 6 and day 8 after BMT), a significant

increase of $CD62L^+CD4^+CD25^+$ T cells was observed in the spleen (**Figure 5a**). This increase was not found in irradiated mice (data not shown), in mice receiving BM alone as well as in mice pretreated with clodronate-loaded liposomes before apoptotic spleen cell and BM infusion (**Figure 5a**). This shows that macrophage depletion prevents apoptotic spleen cell-induced $CD62L^+CD4^+CD25^+$ T cell increase. In addition, these experiments showed that increase of splenic $CD62L^+CD4^+CD25^+$ T cells was detected early in all mice receiving apoptotic cells independently of whether they engrafted or not and that this increase implicated both donor and recipient $CD62L^+CD25^+CD4^+$ T cells (**Figure 5b**). This suggests that the $CD62L^+CD4^+CD25^+$ T cell increase is independent of BM engraftment and that these cells are induced peripherally after apoptotic cell infusion in the presence of recipient macrophages.

***In vivo* CD25 depletion after donor apoptotic spleen cell infusion limits the graft facilitating effect of apoptotic cells.**

To determine the role of splenic $CD62L^+CD4^+CD25^+$ T cell expansion after apoptotic spleen cell infusion, anti-CD25 monoclonal antibody (mAb) was infused at day 0 and day 3 after BMT and engraftment was evaluated at week 7 post-BMT. Despite a significant reduction of peripheral blood $CD4^+CD25^+$ T cells one week after BMT (227 $\pm$ 25 cells/µL in irrelevant mAb treated mice vs. 12 $\pm$ 3 cells/µL in CD25 treated mice), the administration of anti-CD25 mAb did not significantly alter the proportion of engrafted mice 7-9 weeks post-BMT (50% vs. 42% in mice having received irrelevant mAb, **Figure 6**). However, CD25 depletion significantly decreased the levels of circulating donor-derived cells in engrafted mice at time of engraftment analysis (24.8 $\pm$ 6.4% donor cells after anti-CD25 mAb administration vs. 72 $\pm$ 15% after control mAb treatment, $P < 0.05$; **Figure 6**). This result was not surprising since CD25 is not a specific marker of Treg and is expressed by activated T cells.[4] Thus, anti-CD25

mAb might deplete both apoptotic cell-induced recipient and donor-derived Treg as well as host activated T cells that limit engraftment. This latter effect could favor donor engraftment explaining why similar proportions of engrafted mice were found after CD25 depletion. Altogether with kinetic studies showing an early increase of $CD62L^+CD4^+CD25^+$ T cells after apoptotic cell infusion, these *in vivo* results suggest that $CD62L^+CD4^+CD25^+$ T cells are involved in the graft facilitating effect observed after apoptotic spleen cell infusion.

Regulatory T cell expansion is involved in the protective effect of donor apoptotic spleen cell administration on graft-versus-host disease occurrence

Apoptotic spleen cell-induced Treg expansion may also be implicated in prevention of GvHD. We have therefore studied the effects of donor apoptotic spleen cell infusion in an experimental model of GvHD. BALB/c recipient mice were lethally irradiated and the following day received a graft containing donor BM cells plus viable donor splenic T cells (to induce GvHD) with or without apoptotic spleen cells from the BM donor origin. Apoptotic cell infusion significantly delayed the onset of lethal GvHD in a dose-dependent manner (**Figure 7a-b**). We then addressed whether donor $CD4^+$ $CD25^+$ T cells expansion was involved in this delayed GvHD lethality observed after donor apoptotic spleen cell infusion. Depleting anti-CD25 mAb was administrated at days 3 and 6 post-BMT. This treatment abolished completely the protective effect of donor apoptotic cell infusion on GvHD (**Figure 7c**). Overall, these data strongly suggest that donor apoptotic spleen cell infusion induces the expansion of donor-derived $CD4^+$ $CD25^+$ Treg minimizing anti-recipient immune responses involved in GvHD lethality.

Discussion

New therapeutic approaches are required to modulate alloreactivity in HC transplantation. We recently described an alternative cell-based therapy using i.v. apoptotic leukocyte infusion to favor BM engraftment across MHC barriers.[31,35] We have previously shown that the apoptotic feature only and not the cell origin is implicated in favoring BM engraftment.[31] Here, we provide evidence that i.v. infused apoptotic spleen cells were quickly captured by host splenic macrophages and DC. Donor phagocytes present in the BM graft were not implicated in the graft facilitating effect of apoptotic spleen cell infusion. In contrast, depletion of host macrophages –but not host dendritic cells– before transplantation prevented apoptotic cell-induced engraftment. Furthermore, apoptotic spleen cell infusion was associated with donor Treg expansion. *Ex vivo,* these Treg exerted their suppressive activity through a cell-to-cell contact mechanism independently of IL-10 or TGF-β. Both host macrophages and TGF-β production were implicated in this splenic Treg expansion. *In vivo* CD25-depletion after donor apoptotic spleen cell infusion reduced the rate of donor-derived cells and prevented the protective effect of donor apoptotic cell administration on GvHD occurrence. Altogether, these *in vivo* results confirm that Treg induced by the simultaneous infusion of donor apoptotic spleen cells and allogeneic grafts are involved in the immunomodulatory effects of such apoptotic cells on alloreactivity.

Apoptotic cells are endowed with immunomodulatory properties.[16,17] Macrophages involved in apoptotic cell capture have been described to release TGF-β both *in vitro*[19] and *in vivo.*[26] A previous *in vitro* study reported that TGF-β was specifically released by macrophages ingesting apoptotic cells but not during any other type of phagocytosis.[19] TGF-β production is due mainly to the ligation of phosphatitylserines exposed on apoptotic cells to their receptor expressed on macrophages.[36] This immunomodulatory milieu created by macrophages

participated in the neutralization of unwanted immune responses against apoptotic cell derived Ag.[16,26] This may explain why autoimmunity is controlled although billions of cells die during physiological turnover. Moreover, we have reported the absence of autoimmunity after long-term follow-up of mice that had received a single infusion of apoptotic spleen cells simultaneously with their BM grafts.[37] TGF-β was reported by several groups to induce $CD4^+CD25^+$ T cells endowed with regulatory functions.[13-15] Several $CD4^+CD25^+$ T cell subsets have been described to exert regulatory functions.[2-4] Sagakuchi's group first demonstrated that $CD4^+$ T cells expressing CD25 molecules controlled autoimmunity in naive mice.[2] Here, we strongly suggest a direct link between macrophages, apoptotic spleen cell uptake, TGF-β and Treg-like cell expansion. This Treg induction after massive apoptotic primary tissue cell death –that was mimicked in our model by i.v. infusion of five millions of apoptotic spleen cells– may constitute an additional mechanism that takes over from TGF-β secretion to maintain homeostasis. Since TGF-β activation from TGF-β latent complex is known to be tightly regulated and lead to a transient effect restricted to neighbor cells,[38] Treg expansion after massive apoptotic cell death may be responsible for long-term autoimmunity control (either locally or in other sites). Interestingly, induction of Treg cells in the spleen after random blood product transfusion has been previously reported[39] and non-specific immunomodulatory effects of preoperative blood product transfusion has been proposed to be related to the presence of apoptotic leukocytes present in stored blood products.[16,40,41] The immunomodulatory role of apoptotic leukocytes can be also extended to the prevention of allo-immune responses. Sun et al. have recently reported that i.v. infusion of donor apoptotic spleen cells prolongs heart allograft survival. Interestingly, these authors demonstrated that this effect required phagocytosis by host cells.[42] We reported that apoptotic spleen cell infusion with an allogeneic graft prevented anti-BM donor allo-immunization even in mice rejecting their grafts.[35] Treg expansion after apoptotic cell infusion could be beneficial for

engraftment as well as to the host (*i.e.*, absence of anti-donor immunization or GvHD control).

Dendritic cell subsets have also been implicated in apoptotic cell uptake[17,20-23] and subsequent T cell cross-tolerization.[20-22] In our model, we found fluorescent apoptotic bodies in splenic DC, suggesting that apoptotic cell-derived Ag can also be processed by some DC subsets. However, selective DC depletion did not significantly affect either the graft-facilitating effect or splenic Treg expansion after apoptotic spleen cell infusion. Macrophages were thus sufficient to uptake apoptotic cells and possibly released TGF-β after phagocytosis.[19,26] Macrophages were more efficient than immature DC to uptake apoptotic cells[43] and were found in higher numbers than DC in the spleen. Macrophages may create an immunomodulatory milieu that neutralizes DC Ag-presenting functions. TGF-β has been reported to inhibit the ability of DC to present antigen, stimulate IFN-γ production by T cells, and migrate to draining lymph nodes.[44] Our model made it possible to determine *in vivo* the respective implications of macrophages and DC after massive apoptotic leukocyte death in an inflammatory situation (i.e., after TBI): macrophages appear as the key players. This sustains the results of Sun *et al.*.[42]

The thymus and the periphery (*i.e.*, where regulation takes place) are both involved in generating Treg. Regulatory T cells induced by apoptotic cell infusion can be generated from a conversion of peripheral naive $CD4^+$ T cells into a regulatory phenotype or by an increased production of natural suppressive T cells after engraftment. In our model, several arguments are in favor of the first hypothesis: preferential expansion in the spleen (where fluorescent apoptotic bodies were found), early induction of both donor and recipient populations (as attested by kinetic studies) and TGF-β dependency. Regulatory $CD4^+$ T cells induced by *in*

vitro TGF-β exposure arise from "conventional" naive $CD4^+CD25^-$ T cell precursors.[13-15] They acquire Foxp3 mRNA expression and exert their suppressive activity mainly through TGF-β.[13-15] In our study, despite TGF-β dependency of Treg induction, such cells induced after apoptotic cell infusion expressed high levels of Foxp3 mRNA and exerted *ex vivo* suppressive activities mainly through a cell contact-dependent activity. However, we cannot exclude that the observed Treg exert their activity through membrane-bound TGF-β. Membrane TGF neutralization with mAb is indeed not easy to perform.[7] Human and murine Treg generated in the periphery from naive $CD25^-CD4^+$ T cells can exert suppression trough a cell-to-cell contact dependent and cytokine-independent mechanism.[45-47] Such findings suggest that apoptotic cell-induced Treg that exert cell-to-cell suppression are generated peripherally from $CD4^+$ T cells in the presence of TGF-β. "Spontaneous" conversion of $CD4^+CD25^-$ T cells into $CD62L^+$ Foxp3 $mRNA^+$ Treg after in vivo transfer in syngeneic irradiated recipient has been recently reported.[45] Thus, *in vivo* administration of apoptotic cells at time of infusion of $CD4^+$ T cells may increase the conversion of such cells into Treg.

Increased BM engraftment linked to Treg induction has only been recently reported.[48-50] For example, $CD8^+TCR^-$ facilitating cells, derived from BM, induced regulatory T cell genes (including Foxp3) 28 days after transplantation.[48] This was observed at the time of full donor engraftment suggesting that these putative $CD4^+CD25^+$ Treg cells were of donor origin. In this study, up-regulation of regulatory T cell genes was associated with the absence of GvHD despite donor effector cell administration.[48] All these data including those on the prevention of GvHD[5-6] and our findings highlight the *in vivo* donor Treg induction/expansion as a promising approach to prevent the deleterious alloreactivity after HC transplantation.

In conclusion, apoptotic cell infusion enhances BM engraftment by a TGF-β-dependent mechanism that leads to Treg expansion. For the first time to our knowledge, a link between apoptotic cells and Treg expansion is reported. This could be an additional mechanism to immunomodulatory cytokine production[19,25-29] or cross-tolerization[20-22] to prevent deleterious responses against dying cells during normal turnover. We propose to take advantage of this physiological mechanism that maintains self-tolerance to improve HC transplantation outcomes.

MATERIALS AND METHODS

Mice

Specific pathogen-free 5- to 6-week old male FVB (H-2^q), C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (Janvier, Genest-St-Isle, France) were kept in quarantine for at least one week before BM transplantation (BMT). CD11c/DTR/eGFP transgenic mice from a BALB/c background (kindly provided by S. Jung, The Weizmann Institute of Science, Rehovot, Israel) were bred in our animal facility. This transgenic model allows short-term ablation of DC *in vivo* after administration of diphtheria toxin (DT, 100 ng per mouse, Sigma, Saint Quentin Fallavier, France) without affecting macrophages.[34] Mice were given *ad libitum* access to food and water. Neomycin sulfate (1.1 g/L, Demavic, Longvic, France) was added to their drinking water as of d–1 of BMT to reduce the risk of infection. Experiments were performed according to institutional guidelines.

Antibodies

The following mAb were used (all obtained from BD PharMingen, San Diego, CA, except when stated): FITC-labeled anti-H-2^q (KH114, mouse IgG2a), CD8 (53-6.7, rat IgG2a), CD11c (HL3, Armenian hamster IgG), CD19 (1D3, rat IgG2a), CD25 (7D4, rat IgM), CD45RB/B220 (RA3-6B2, rat IgG2a), Sca-1 (D7, Rat IgG2a), PE-labeled anti-I-A^b (M5/114.15.2, rat IgG2a, reacts also with I-A^d, I-E^d, I-E^k and I-A^q), H-2^d (SF1-1.1, mouse IgG2a), CD25 (3C7, rat IgG1 or PC61, rat IgG1), CD62L (MEL-14, rat IgG2a), CD117 (2B8, rat IgG2b), CD152 (UC10-4F10-11, Armenian hamster IgG), PE-cyanin 5-labelled F4/80 (A3-1, rat IgG2b, Serotec, Oxford, UK), peridinin chlorophyll-a protein (PerCP)-conjugated anti-CD4 (RM4-5, rat IgG2a), anti-CD3 (145-2C11, hamster IgG1), PerCP cyanin 5.5-conjugated anti-H-2^d (SF1-1.1), CD11b (M1/70, rat IgG2b) and allophycocyanin (APC) conjugated-anti-CD3 (145-2C11). Azide-free low endotoxin purified anti-CD25 (3C7, rat

IgG2b or PC61, rat IgG1) or control (irrelevant) Abs (BD PharMingen) and anti-pan TGF-β (rabbit IgGs) or rabbit IgGs (Sigma) were used *in vivo*.

Cell preparation

Donor BM cells from FVB or C57BL/6 mice and splenocytes from BALB/c, C57BL/6 or FVB mice were prepared as described.[31,35] After isolation procedures, cell viability was always greater than 90% by Trypan Blue (Sigma) dye exclusion. Sca-1^+ cell-enriched grafts were obtained by immunomagnetic cell sorting (Miltenyi Biotec, Paris, France) from C57BL/6 BM cells according to the manufacturer's instructions. Purity assessed by Sca-1 mAb staining was > 80% and donor BM phagocyte depletion assessed by CD11c and F4/80 mAb staining was > 83%. Splenocyte apoptosis was induced by a 40-Gray dose of γ-irradiation 6 h before infusion to allow apoptotic changes to occur.[31] Apoptosis was confirmed by FITC-conjugated Annexin-V (Beckman Coulter Immunotech, Marseille, France) and flow cytometry as well as Hoechst 33342 (Molecular Probes, Eugene, OR) staining and UV microscopy. Absence of secondary necrosis was documented using Trypan Blue staining just before infusion. At time of infusion, spleen cells were mainly early-stage apoptotic cells (70-85% of cells were Annexin-V positive and propidium iodide negative; less than 10% of cells were propidium iodide positive).[31] Splenic $CD62L^+CD4^+CD25^+$ T cells were sorted using an EPICS ALTRA cell sorter (Beckman Coulter Immunotech). The purity achieved was >95% and staining these cells did not alter their suppressive function. These purified $CD4^+CD25^+$ T cells were used for *in vitro* assays and RNA extraction for quantitative real time PCR (QRT-PCR).

Bone marrow transplantation

BALB/c recipients were exposed to a sublethal single dose (6 Gy at a rate of 2.7 Gy/min) of TBI 16 h before BMT. After, they were given a single i.v. infusion, via a caudal vein, containing a limited number of FVB or C57BL/6 BM cells (10^6) or Sca-1^+ cell-enriched graft (10^5) alone or with apoptotic splenocytes (5×10^6) of various origins (recipient, donor or third-party mice). Both hematopoietic grafts and conditioning regimens were defined to favor autologous reconstitution and not engraftment.[31] Preliminary experiments indicated that a minimum of 10^6 apoptotic spleen cells were required to favor engraftment. The number of apoptotic spleen cells used exceeds this minimum number of cells by a factor of 5. In these conditions (i.e., i.v. infusion of 5×10^6 early-stage apoptotic spleen cells simultaneously with BM grafts), no autoimmunity was observed.[37] To study the effects of donor apoptotic cell infusion on the prevention of acute GvHD, we used a lethal dose of TBI (8 Gy) and a higher number of donor BM cells (10^7) as well as donor viable splenic T cells (3×10^6 to trigger GvHD).[51] Increasing numbers of donor apoptotic spleen cells (2×10^6, 2×10^7 or 10^8) was infused. No immunosuppressive agents were given at any time.

Antibody treatment

Mice were given 150 µg anti-pan TGF-β Ab or rabbit IgGs by tail vein injection on d0 (simultaneously with the graft) and on day 3.[35] CD25 cells were depleted by anti-CD25 mAb infusion (8.5 mg/kg or 250 µg/mouse) on day 0 and day 3. Treatment efficiency was confirmed by a significant decrease in circulating $CD4^+CD25^+$ T cells (12 ± 3 cells/µL, n=3) as compared to control mAb-treated mice (227 ± 25 cells/µL, n=3, P<0.01, Student t test).

Host phagocyte depletion

Host phagocyte depletion was performed using clodronate-loaded liposome administration. Liposome preparation and i.v. infusion were performed as described.[32,33] Briefly, liposomes

consisting of phosphatidyl choline and cholesterol (Sigma) in 6:1 molar ratio were resuspended in PBS and injected via the tail vein (final volume of 0.2 ml containing about 2 mg of clodronate-loaded liposomes). The day after, mice were irradiated and grafted as described above. Host phagocyte depletion was controlled 20 h after treatment, on two treated mice in each experiment, by immunohistochemistry and cytometry analysis. Spleens were removed, cut (5-µm sections), mounted on microscope slides and air dried for staining. After endogenous phosphatase quenching by levamisole solution (DakoCytomation, Glostrup, Denmark) and nonspecific site blocking, sections were stained with purified CD11b (M1/70), CD11c (HL3) or control Ab (BD PharMingen) and revealed by biotinylated-conjugated goat anti-rat or anti-Armenian hamster Ab (Jackson ImmunoResearch, West Grove, PA) followed by a streptavidin/alkaline phosphatase complex (DakoCytomation). Then, a solution of fuchsine substrate chromogen (DakoCytomation) was added. Hematoxylin was used as a counterstain.

Bone marrow engraftment

Detection of engraftment was performed by flow cytometry[31,35] using H-2 specific mAb on peripheral blood cells and at week 7 or 9 post-BMT on splenic cells. Analysis was performed with a FACSCalibur™ (BD Biosciences, San Jose, CA) using CellQuest® software (Becton Dickinson) or a CyAn LX™ (DakoCytomation) using Summit® software (DakoCytomation). Recipients were considered as engrafted when they presented at least 15% of cells expressing BM donor H-2.[31]

Tracking of fluorescent apoptotic cells

Apoptotic cells (5 x 10^6) were labeled using either green PKH67 dye (Sigma) or 5,6-carboxyfluorescein diacetate succimidyl ester[52] (CFSE, Molecular Probes, Eugene, OR)

before apoptosis induction according to the manufacturer's instructions. After staining and induction of apoptosis (40 Gy), fluorescent-labeled apoptotic cells were transplanted as previously described with the BM graft in 6-Gy-irradiated recipients. After 30, 45 min, 1, 2, 4 and 8 hrs, mice were CO_2-euthanized; spleen, liver and lungs were harvested to perform fluorescent-labeled apoptotic cell tracking. Cells from each organ were stained using PE-conjugated anti-CD11c and PE-cyanin 5-conjugated anti-F4/80 mAb and analyzed by cytometry.

Phenotypic analysis of T-cell subsets

T-cell subsets from blood, spleen, thymus, BM and LN were analyzed after BMT using specific mAb staining and cytometry. For intracytoplasmic staining, cells were stained with CD3, CD4 and CD25 mAb, fixed in 0.25% paraformaldehyde (Sigma) in PBS and permeabilized in saponin solution (Sigma) prior to staining with CD152 mAb. Absolute counts of circulating lymphocyte subsets were determined using a single-platform approach based on cytometry and tubes containing a defined number of microbeads (TruCOUNT®, BD Biosciences).[53]

***Ex vivo* functional analysis of $CD4^+CD25^+$ T cells**

Functional characterization of $CD4^+CD25^+$ T cells was performed using two mixed leukocyte reaction (MLR) assays due to limited material and to avoid pooling spleens from separate experimented mice. In the first assay, we depleted $CD25^+$ cells among $CD4^+$ T cells using complement-dependent cytotoxicity (CDC) and compared CD25-depleted vs non-depleted splenic $CD4^+$ T cell proliferation after allogeneic stimulation. In the second assay, we added FACS-sorted splenic $CD4^+CD25^+$ T cells to MLR assays. Spleens from naive FVB (donor origin) or C57BL/6 (third-party origin) mice were 40-Gy irradiated and used as stimulator

cells in both assays. In the first assay, splenic $CD4^+$ T cells from BMT recipients (isolated at week 7 post-BMT) were used as responder cells and cultured for 2 h in complete medium containing DMEM supplemented with 10% of heat-inactivated FCS (BioWhittaker, Verviers, Belgium), 5 x 10^{-5} M 2-ME, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma). Non-adherent responder cells were washed and incubated for 20 min with purified either anti-CD25 mAb (3C7) or control IgG2a and then with newborn rabbit complement (EFS B/FC, Besançon, France) for 1 h under agitation at room temperature. After washing, lysis was repeated for 30 min. CD25 depletion was confirmed by cytometry (< 0.7 % $CD4^+CD25^+$ T cells after CDC). CD25-depleted or control responder cells were stimulated in MLR assays in 96-well round-bottom plates (Sarstedt, Orsay, France) in triplicates for 5 days in complete medium at 37°C in a 5% CO_2 incubator. After a 5-d stimulation, cells were pulsed with 1µCi of [methyl-^{3}H]-thymidine (NEN life Science Products, Boston, MA). After approximately 16 h of additional culture, cells were harvested and radioactivity was counted in a beta scintillation counter (Wallac Betaplate counter, Perkin-Elmer, Norwalk, CT). In the second assay, FACS-sorted $CD4^+$ $CD25^+$ T cells were added to CD25-depleted responder cells from naive BALB/c mice stimulated by allogeneic cells, as described above. To determine the mechanism(s) by which FACS-sorted $CD4^+CD25^+$ T cells exert their suppressive activity, we added $CD4^+CD25^+$ T cells above a cell culture insert (0.3 µm, BD Biosciences). We also investigated the neutralizing effect of various Abs at a final concentration of 5 µg/ml: anti-pan TGF-β polyclonal Ab, its control Ab (Sigma), anti-IL-10R (1B1.2, kindly provided by K. Moore, DNAX Research Institute, Palo Alto, CA, via H. Groux, Nice, France), its control mAb (Rat IgG1 mAb, GL113).

Quantitative Real-Time PCR (QRT-PCR) for Foxp3 mRNA

Total RNA was extracted using RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and reverse transcribed using random hexamers and M-MLV reverse transcriptase (Life Technologies, Rockville, MD) to use as a template for QRT-PCR. QRT-PCR reactions were then performed in duplicates using gene specific probes and universal master mix (Qiagen) on an iCycler iQ thermocycler (Bio-Rad Laboratories, Marnes-la-Coquette, France). Primers and dual labeled fluorescent probes were designed using Primer Express® software (Applied Biosystems, Forster City, CA) to be specific for RNA but not for genomic DNA. Primer pairs and related probes were as follow (sense, anti-sense and probe respectively): Foxp3: 5'-CCCACCTACAGGCCCTTCTC-3', 5'-GGCATGGGCATCCACAGT-3' and 5'-FAM-GGACAGACCACACTTCATGCATCAGCTC-TAMRA-3'; GAPDH (used as endogenous reference): 5'-TTCACCACCATGGAGAAGGC-3', 5'-GGCATGGACTGTGGTCATGA-3' and 5'-FAM-TGCATCCTGCACCACCAACTGCTTAG-TAMRA-3'. For each sequence, a standard curve was generated with a dilution series of a known copy number of the target. The relative amount of each unknown sample was determined automatically with the iCycler iQ® software, using the threshold cycle (Ct). Data were expressed as normalized Foxp3 mRNA expression, which was obtained by dividing the relative amount of Foxp3 mRNA for each sample by the relative amount of GAPDH mRNA of the same sample.

Statistical analysis

Group comparisons of continuous data were made by Student's t or Mann-Whitney Rank Sum test. Survival data were analyzed using Kaplan-Maier survival analysis. *P*-values < 0.05 were considered as significant.

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FIGURE LEGENDS

Figure 1 Recipient macrophages are mainly involved in the graft-facilitating effect of apoptotic spleen cell infusion. (**a**) Green-PKH- or CFSE-labeled apoptotic cells (detectable in the FL1 channel) were used to explore phagocytes involved in the apoptotic cell uptake. Left panel: 45 min after BMT, fluorescent apoptotic cells infused simultaneously with BM graft were found in splenic DC ($F4/80^{low}CD11c^{+}FL1^{+}$; white arrows) as well as in macrophages ($F4/80^{high}CD11c^{-}FL1^{+}$; black arrows). Results from a representative experiment out of 4. Middle panel: non-fluorescent apoptotic cells used as controls. Right panel: kinetics of the appearance of $CFSE^{+}CD11c^{+}$ DC (■) and $CFSE^{+}F4/80^{+}$ macrophages (●) after infusion of CFSE-labeled apoptotic cells. The percentage of $CFSE^{+}$ cells contained within the indicated splenic cell population is listed on the y axis. $n = 3$ mice per time point. Bars represent S.D. (**b**) Sca-1 cell sorting was used to deplete phagocytes from the BM (left panel: a representative sorting experiment out of 4). Right panel: donor engraftment after infusion of total C57BL/6 BM (10^6 cells) or sca-1-enriched graft (Sca-1, 10^5 cells) with (Apo cells, hatched bars) or without (white bars) apoptotic cells. Pooled results from 3 independent experiments (at least 20 mice/group) expressed in % of engrafted mice. * $P < 0.05$. (**c**) Clodronate-loaded liposomes were infused before BMT to deplete host splenic phagocyting cells. Left panel: flow cytometric CD11b staining shows the effective depletion of $CD11b^{+}$ macrophages at day 3 after clodronate-liposome infusion. CD11b staining in PBS loaded-liposome treated mice was shown as control. Right panel: donor engraftment after infusion of total BM (10^6 cells) with (hatched bars) or without (white bars) apoptotic cells in mice treated by PBS-liposomes or clodronate-liposomes. (**d**) Recipient $CD11c^{+}$ DC were depleted at day-1 before BMT using a single infusion of diphtheria toxin (DT). Top panel: a representative depletion of $eGFP^{+}CD11c^{+}$ cells after a 20 h DT treatment. Bottom panel: donor engraftment after infusion of total BM (10^6 cells) with (hatched bars) or without (open bars) apoptotic cells

in DT-treated transgenic mice. Pooled results from 2 independent experiments expressed in % of engrafted mice. * $P < 0.05$.

Figure 2 Apoptotic spleen cell infusion simultaneously with a bone marrow graft induces an *in vivo* TGF-β-dependent splenic expansion of $CD4^+CD25^+$ T cells. (**a**) Splenic $CD4^+CD25^+$ T cells increase after apoptotic spleen cell infusion. These cells were evaluated by cytometry in different groups: naive mice, engrafted and non-engrafted mice given apoptotic cells, engrafted mice given only a higher number of BM cells (3×10^6) and engrafted mice given a higher conditioning regimen (7 Gy). Boxes represent mean $\pm$ SEM, bars the S.D. and circles the highest and lowest values. *: $P < 0.01$. (**b**) TGF-β neutralization inhibits apoptotic spleen cell-induced splenic $CD4^+CD25^+$ T cell increase. The percentage of splenic $CD4^+CD25^+$ T cells was evaluated 7-9 weeks post-graft in TGF-β mAb-treated mice ($n = 7$) vs in irrelevant isotype Ab-treated mice ($n= 9$). Pooled results of 2 independent experiments. *: $P < 0.01$.

Figure 3 Apoptotic spleen cell infusion simultaneously with a bone marrow graft induces *in vivo* splenic expansion of $CD4^+CD25^+$ regulatory T cells expressing high levels of Foxp3 mRNA and exhibiting *in vitro* cell-to-cell contact-dependent suppression. (**a**) Expanded splenic $CD4^+CD25^+$ T cells from engrafted and non-engrafted mice were characterized 7-9 weeks post-BMT. CD25, $H\text{-}2^d$ and CD62L expressions were evaluated on the $CD4^+CD3^+$ T cell gate (CD3+ ; CD4+ ; SSC vs FSC), whereas intracellular CTLA-4 expression was evaluated on the $CD3^+CD4^+CD25^+$ T cell gate. (**b**) Seven to nine weeks post-BMT, cell-sorted splenic $CD4^+CD25^+$ T cells were analyzed for Foxp3 mRNA expression in engrafted ($n = 2$, hatched bars) and non-engrafted ($n = 1$, open bar) mice that had been given apoptotic cells plus BM cells. Relative Foxp3 mRNA expression was obtained by dividing relative Foxp3 expression in the $CD4^+CD25^+$ T cell fraction by that of the $CD4^+CD25^-$ T cell fraction.

(**c**) Foxp3 mRNA expression is assessed in whole spleen of either mice given BM alone (left open bars, *n*= 4), mice given apoptotic cells plus BM (hatched bars, *n*= 6) 7-9 week post-BMT. Foxp3 mRNA expression in the spleen of naive BALB/c mice (right open bars, *n*= 8) was shown as control. Results are expressed as mean ± SEM of normalized Foxp3 expression (obtained by dividing the relative amount of Foxp3 mRNA for each sample by the relative amount of GAPDH mRNA of the same sample). (**d**) Splenic $CD4^+$ T cells from engrafted (left panel, *n* = 3) or non-engrafted (right panel, *n* = 2) BALB/c mice given donor FVB BM and apoptotic cells were cultured with or without allogeneic (FVB donor or C57BL/6 third party) stimulator cells. T cells were CD25-depleted (open bars) or not (hatched bars). MLR proliferation was assessed by [^{3}H]thymidine uptake (cpm). *: $P < 0.01$. (**e**) Cell-sorted $CD4^+CD25^+$ T cells were added to a MLR between stimulator irradiated splenic FVB donor cells and CD25-depleted responder BALB/c recipient $CD4^+$ T cells (*n* = 2) with or without (Ø) anti-IL10R, anti-TGF-β mAb or both. A transwell was also used to separate the MLR from sorted $CD4^+CD25^+$ T cells. Results are expressed as relative proliferation obtained by dividing the $CD4^+$ T cell proliferation in response to allogeneic stimulators by the proliferation obtained in similar conditions but in the presence of blocking Abs or transwell. [^{3}H]thymidine uptake > 9000 cpm. *: $P < 0.05$.

Figure 4 Depletion of $CD11c^+$ dendritic cells has no effect on $CD62L^{high}CD4^+CD25^+$ T cell increase induced by apoptotic spleen cell infusion. Recipient $CD11c^+$ DC were depleted at day -1 before BMT using a single infusion of diphtheria toxin (DT). CD11c/eGFP/DTR transgenic mice given donor apoptotic cells plus BM were treated (left box, *n*= 8) or not (right box, *n*= 5) with DT. $CD62L^+CD4^+CD25^+$ T cells were analyzed by cytometry 7-9 weeks post-BMT. Boxes represent mean ± SEM, bars the SD.

Figure 5 Apoptotic spleen cell infusion simultaneously with a bone marrow graft induces an early increase of $CD62L^+CD4^+CD25^+$ T cells of both host and donor origin. (**a**) Host macrophages are involved in the early increase of $CD62L^+CD4^+CD25^+$ T cells induced by apoptotic spleen cell infusion. Splenic $CD62L^+CD4^+CD25^+$ T cells were analyzed by cytometry at different time points after the infusion of FVB BM alone (□, *n*=5), or FVB BM plus FVB apoptotic cells (■, *n*=5). Such cells were also analyzed in BALB/c mice pre-treated with clodronate-liposomes and grafted with FVB BM plus FVB apoptotic cells (▲, *n*=3) and in irradiated BALB/c mice (Δ, *n* = 3). *: $P < 0.05$. (**b**) Percentage of donor $CD62L^+CD4^+CD25^+$ T cells in the spleen of mice given FVB BM alone (□, *n*=5) or BM plus apoptotic spleen cells (■, *n*=5) at different early time points after BMT. *: $P < 0.05$.

Figure 6 $CD25^+$ cell depletion significantly reduces the number of circulating donor-derived cells after apoptotic spleen cell infusion. Percentage of donor-derived cells was evaluated 7-9 week post-graft in the spleen of mice given only BM (●, *n*=10), BM and apoptotic cells plus anti-CD25 mAb (▲, *n*=10) or plus irrelevant mAb (■, *n*=12). Number of engrafted mice in each group was shown. Dotted line indicates 15% of donor-derived cells corresponding to engraftment. Pooled results of 2 independent experiments. *: $P < 0.05$.

Figure 7 $CD25^+$ cell depletion prevents the protecting effect of donor apoptotic spleen cell administration on graft-versus-host disease occurrence.(**a**) Cumulative survival was assessed in the GvHD model in mice receiving BM plus splenic T cells and donor apoptotic spleen cells (▲) or not (control GvHD group; □). Mice receiving BM alone (●) or only lethally irradiated (○) were also used as control. Results of a representative experiment out of 4 are expressed in % of cumulative survival. (**b**) Donor apoptotic spleen cells delays lethal GvHD occurrence in a dose-dependent manner. Median survival time was assessed in mice receiving

increasing number of apoptotic cells (2 x 10^6, 2 x 10^7 or 10^8 apoptotic spleen cells; hatched bars) or not (GvHD Ctrl group; open bar) in addition to BM and splenic T cells. Pooled results from 4 independent experiments (10 mice per group, excepted for ctrl group *n*= 5) are expressed in median survival time (day) $\pm$ SD. (**c**) Delayed $CD25^+$ cell depletion abolishes the protective effects of donor apoptotic spleen cell infusion on lethal GvHD occurrence. Cumulative survival was assessed in mice receiving BM plus splenic T cells (GvHD Ctrl group, □); with apoptotic cells and treated at day 3 and 6 by anti-CD25 mAb (●) or irrelevant IgG1 control (■). Results of a representative experiment out of 3 are expressed in % of cumulative survival. 10 mice per group.

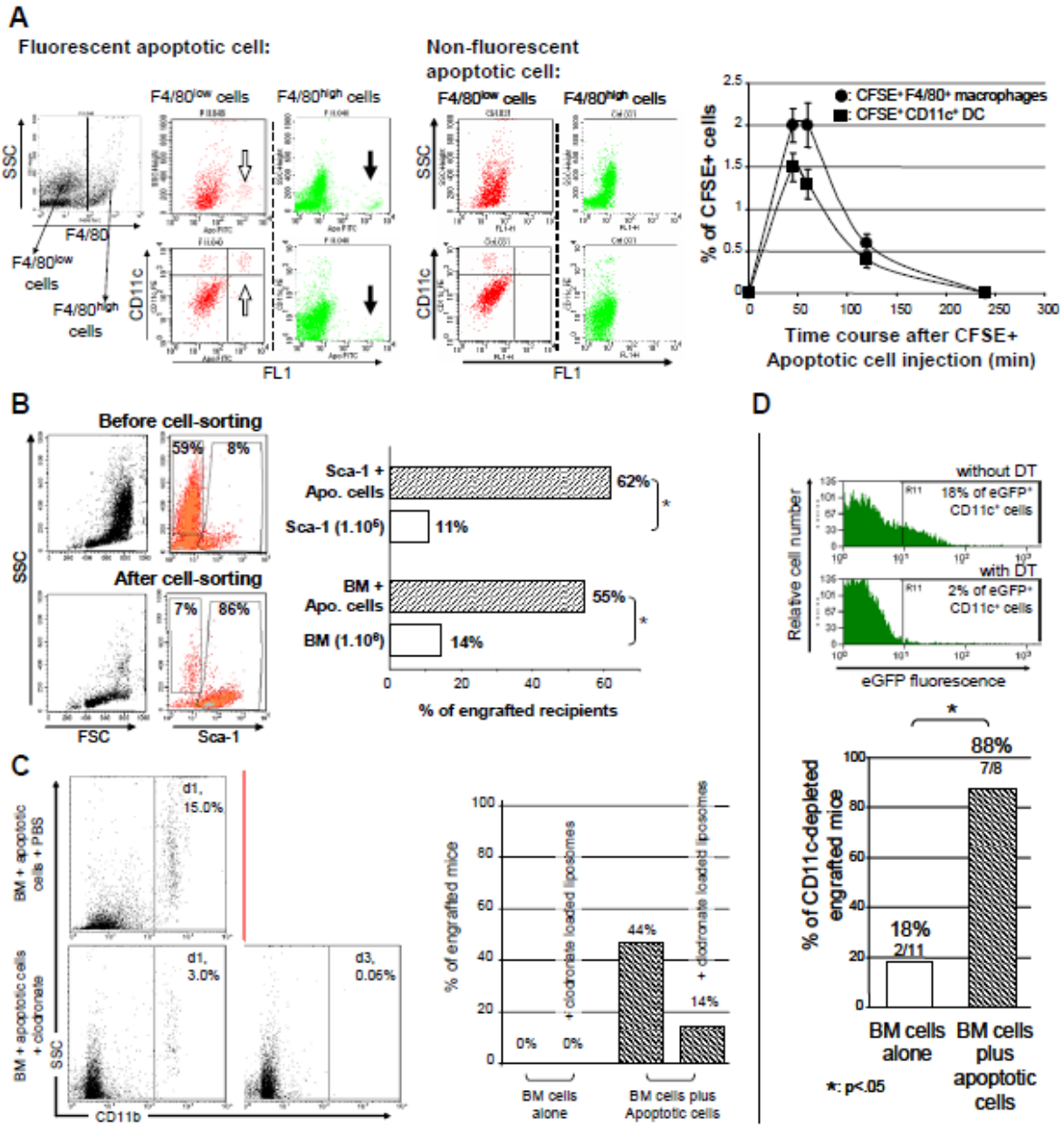

A
Fluorescent apoptotic cell:
Non-fluorescent apoptotic cell:
F4/80low cells
F4/80high cells
SSC
F4/80
CD11c
FL1
% of CFSE+ cells
●: CFSE+F4/80+ macrophages
■: CFSE+CD11c+ DC
Time course after CFSE+ Apoptotic cell injection (min)
B
Before cell-sorting
After cell-sorting
59%
8%
7%
86%
FSC
Sca-1
Sca-1 + Apo. cells
62%
Sca-1 (1.10⁵)
11%
BM + Apo. cells
55%
BM (1.10⁶)
14%
% of engrafted recipients
C
BM + apoptotic cells + PBS
BM + apoptotic cells + clodronate
d1, 15.0%
d1, 3.0%
d3, 0.05%
CD11b
% of engrafted mice
+ clodronate loaded liposomes
44%
14%
0%
0%
BM cells alone
BM cells plus Apoptotic cells
D
without DT
18% of eGFP+ CD11c+ cells
with DT
2% of eGFP+ CD11c+ cells
Relative cell number
eGFP fluorescence
88%
7/8
18%
2/11
% of CD11c-depleted engrafted mice
BM cells alone
BM cells plus apoptotic cells
*: p<.05

Figure 1.

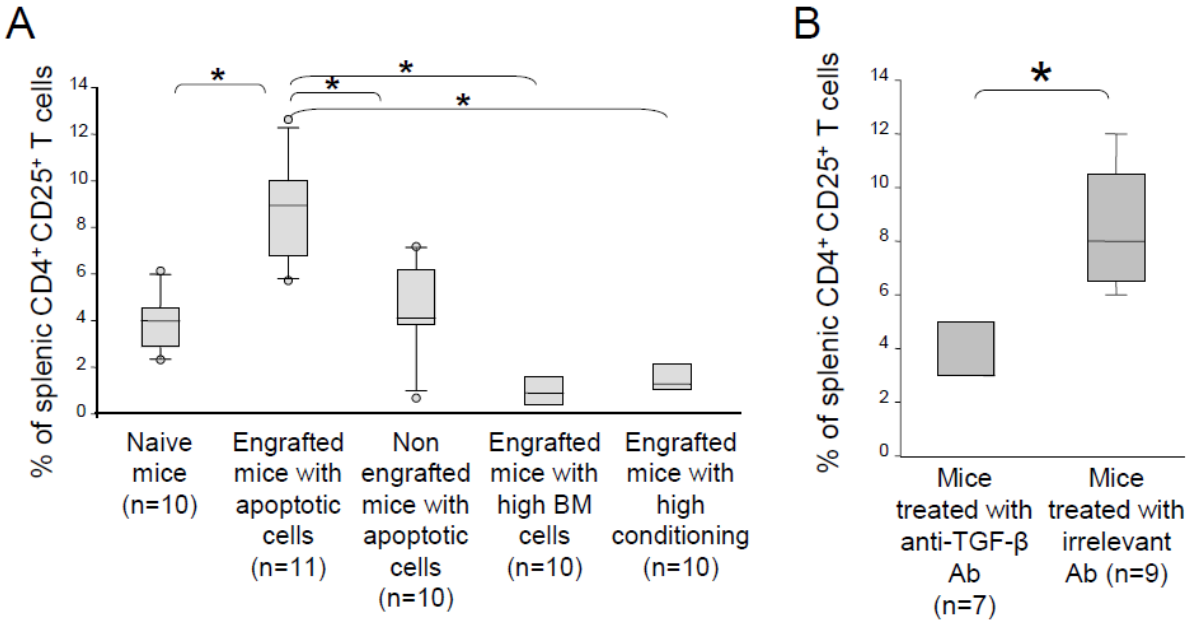


Figure 2.

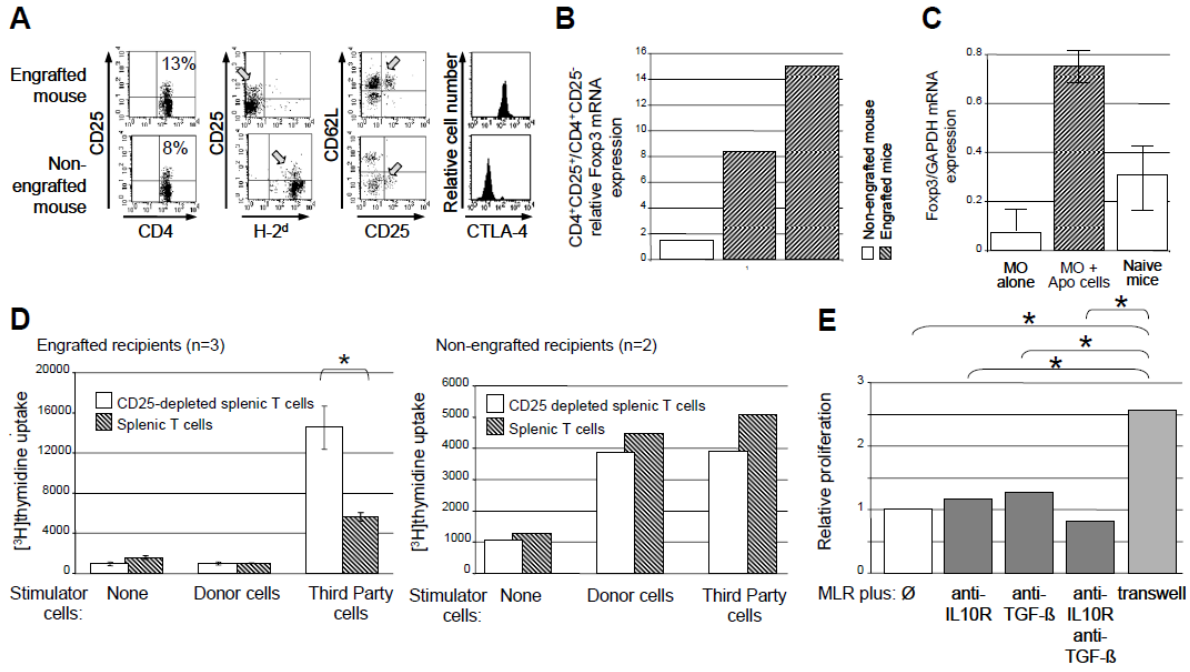


Figure 3

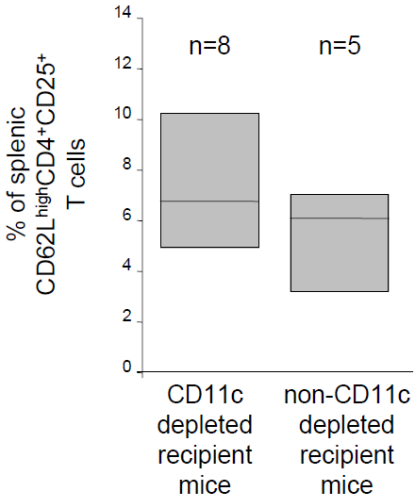


Figure 4.

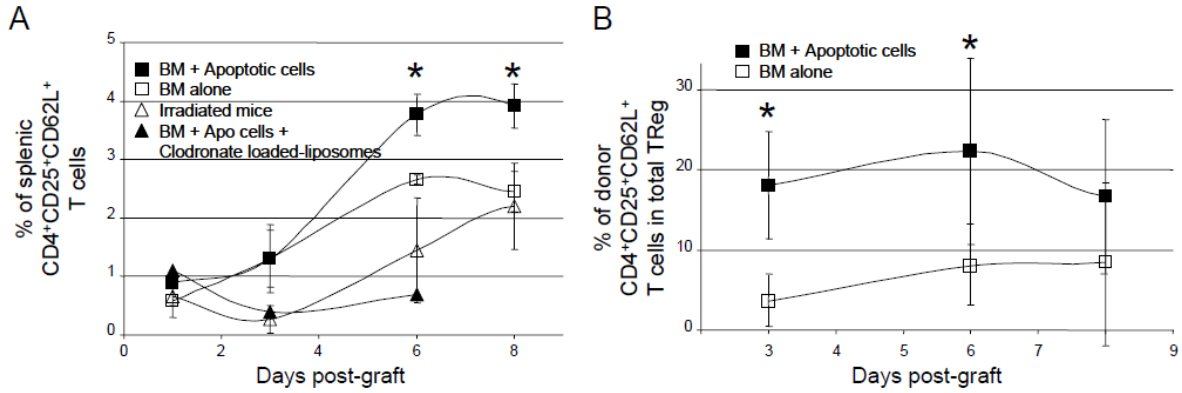


Figure 5

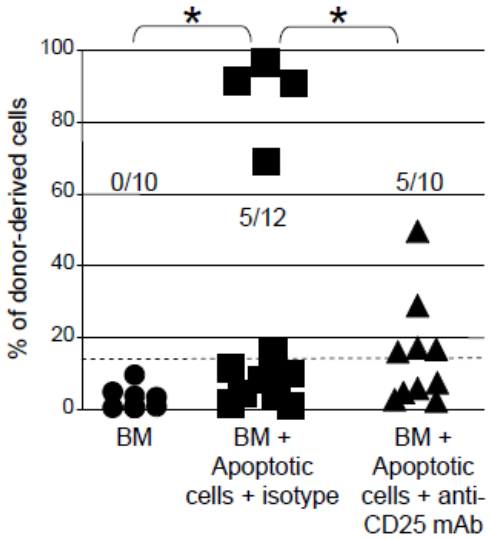


Figure 6.

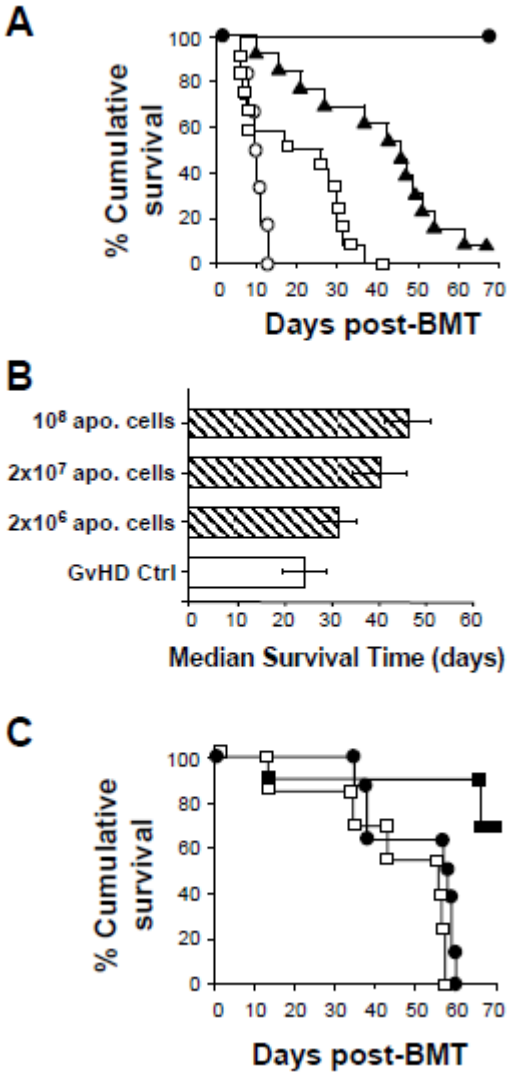
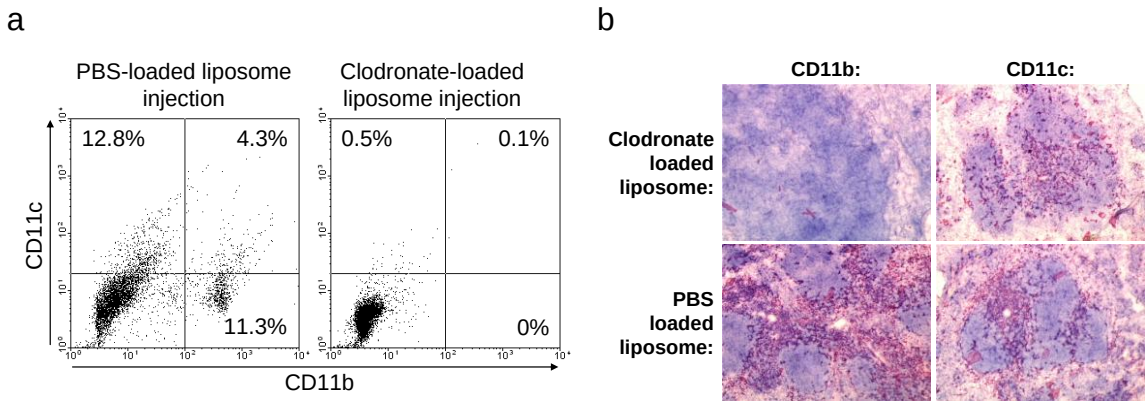

A
% Cumulative survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Days post-BMT
B
10^8 apo. cells
2x10^7 apo. cells
2x10^6 apo. cells
GvHD Ctrl
0 10 20 30 40 50 60
Median Survival Time (days)
C
% Cumulative survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Days post-BMT

Figure 7.



Supplement Figure 1:

Clodronate-loaded liposomes infused before BMT deplete host splenic phagocytes. (**a**) A representative staining of splenic phagocytes using anti-CD11c and CD11b antibodies, 24 hours after the infusion of PBS-loaded liposomes or clodronate-loaded liposomes. (**b**) Left panels: immuno-histological CD11b staining shows the effective depletion of $CD11b^{+}$ macrophages in the recipient spleen 12 h after clodronate-liposome infusion (top panel). A CD11b staining was observed when PBS-liposomes were infused (bottom panel) (magnified: 10X). Right panels: immuno-histological CD11c staining reveals that not all splenic DC subsets were depleted by clodronate-loaded liposome infusion, due to their anatomic location, as previously reported (Leenen, P. J., K. Radosevic, J. S. Voerman, B. Salomon, N. van Rooijen, D. Klatzmann, and W. van Ewijk. 1998. Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J Immunol 160:2166*).