$CD4^+CD25^+FOXP3^+$ regulatory T cells in allogeneic hematopoietic cell transplantation

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Abstract

$CD4^+CD25^+FOXP3^+$ regulatory T cells (T_{reg}) require activation through the T cell receptor for function. $CD4^+CD25^+FOXP3^+$ regulatory T cells are believed to be key players of the immune tolerance network and control the induction and effector phase of the immune system. Although these cells require antigen-specific activation, they are generally able to suppress bystander T cell responses once activated. This raises the possibility that antigen-specific T_{reg} may be useful therapeutically by localizing generalized suppressive activity to tissues expressing select target antigens. T_{reg} can exert a potent suppressive effect on immune effector cells reactive to host antigens and prevent graft versus host disease (GVHD) in allogeneic bone marrow transplantation (BMT). Here, we observed that co-transfer of $CD4^+CD25^+FOXP3^+$ T cells derived from donor type along with the donor bone marrow cells could control GVHD-like reactions by suppressing effectors cells of host responding to the donor hematopoietic compartment, and resulted in prevention of autoimmunity and rejection. We further demonstrate that $CD4^+CD25^+FOXP3^+$ regulatory T cells can control immune-based morbidity after allogeneic BMT by suppressing the development of granulocytes cells and increasing the level of B cell expression.

Keywords : Bone Marrow Transplantation, Regulatory T cells, $CD4^+CD25^+FOXP3^+$

Introduction

Graft-versus-host disease (GVHD continues to be a major life-threatening complication after allogeneic bone marrow transplantation. Some effort has been made to elucidate the patho-physiology of acute GVHD. Antigenic differences on antigen-presenting cells (APC) of the host was directly recognized by mature donor T cells transferred along with the marrow and donor antihost-specific T cells can mediate tissue destruction. Even though GVHD in allogeneic system always occurs until now we have no choice because for some disease highly dependent on bone marrow transplantation (BMT). BMT is the only curative treatment for myelofibrosis, and again allergy also a factor need more attention because evolved in final results. The risk for severe acute GVHD was increased after allo-SCT from mismatched donors. However, disease status at transplantation is the most important prognostic factor for transplantation success (1-6). The failure of clonally deleted of autoreactive T cells in the thymus is one of the reasons to initiate an autoimmune diseases and some symptoms look like GVHD. Clonal deletion in the thymus may be impaired either by damage to the thymic epithelium or by the use of immunosuppressive drugs that inhibit clonal deletion. Mature donor T cells transferred along with the marrow graft directly recognize antigenic differences on antigen-presenting cells of the host. Once activated, donor T cells that specifically respond to the host compartment can mediate tissue destruction (6-10). Induction of immunological tolerance to alloantigens would be the treatment of choice to prevent graft-versus-host disease (GVHD) and allograft rejection in transplantation medicine. The most potent self-tolerance mechanism is probably dominant tolerance assured by regulatory and suppressor T lymphocytes. The potential utilization of T_{reg} in preventing GVHD has been studied in several animal models. In some studies, purified populations of $CD4^+CD25^+$ cells from syngeneic mice infused together with the graft successfully prevented the development of GVHD. This strategy is based on the notion that the T_{reg} suppressive activity is best exploited when the anti-host response has not fully developed. In contrast, other studies demonstrated the capacity of T_{reg} cells infused later after transplant to control already

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established GVHD. These latter findings support the view that T_{reg} could also be utilized to correct chronic GVHD in humans. Interestingly, most studies report that T_{reg} expanded in vitro with IL-2 exhibit higher suppressive capabilities than freshly isolated T_{reg}. These data provides additional support for the use of in vitro expanded T_{reg} as a source for cellular immunotherapy. T_{reg} exert their suppressive function over a wide range of T cells including tumor specific effector T lymphocytes. It is therefore likely that the infusion of T_{reg} at the time of BMT would decrease the beneficial GVL effect promoted by donor T cells. Regulatory T cell may be one of the component to inhibit GVH-like reaction or autoimmune disorder. Multiple populations of cells is now claimed to participate in this process. However, $CD4^+$ regulatory T cells that innately express $CD25^+$ appear to be of the best component to control the immune response. Evidence for regulatory T cells in clinical bone marrow transplantation, however, remains unclear. The finding of $CD4^+CD25^+$ regulatory T cells that preferentially express the Foxp3 nuclear transcription factor and the development of molecular reagents to isolate antigen-specific T cells have provided unique opportunities to explore immunoregulatory mechanisms after clinical marrow trans-plantation. In recent studies we test the efficacy of $CD4^+CD25^+FOXP3^+$ cells whether they play a vital role in the regulatory control of GVH reactions mediated by both alloreactive and autoreactive lymphocytes. Immune reaction against virulent foreign objects could be harmful for self-organism. Therefore, a strict regulatory mechanism must exist in the immune system (11-16). $CD4^+CD25^+$ is now well documented as the key that control immune activity. However, other cells, especially those of $CD8^+$ T cell lineage, have also been suggested as regulatory T cells (17, 18). In previous study we have tested the capacity of $CD8^+CD122^+$ regulatory T cells to rescue an autoimmune disorder in IL-2R$\beta^{-/-}$ (CD122 knockout) mice. CD122 knockout exhibits severe autoimmune disorder characterized by increasing of activated T cells. The mice are also safer from severe anemia because of erythropoiesis suppression. Usually severe anemia progresses and the mice die in the ages of 3-4 months. We found that expansion of abnormally activated T cells is the underlying cause of all phenotypes (24). The mechanism of generation of activated memory-type T cells in CD122-deficient mice is controversial. In the initial investigations by Abbas and others (17-19), the defect of programmed cell death (apoptosis) in IL-2 or IL-2Rβ-deficient T cells could be applied to the mechanism for survival of activated CD122-deficient T cells, and such possibility has not been excluded. Because IL-2R molecule appear to include in vast major array in homeostasis system, we highlight $CD25^+$ (IL-2Rα) to investigate the function in allogeneic transplantation. We hypothesize that the lack of certain regulatory cells was responsible for the induction of both hematopoietic disorder and autoimmune diseases. In this study, however, direct evidence of the responsible subpopulation in $CD4^+$ cells was not provided, and it was still unclear whether the lack of regulatory system is sufficient to cause GVH-like reaction in allogeneic bone marrow transplantation. Here, we report a novel observation that transfer of highly purified $CD4^+CD25^+FOXP3^+$T cells from donor type adapted in F1 mice along with marrow graft in the allogeneic sytem of (C57BL/6 to BALB/c) resulted in normalization the ratio of Gr1 cell / B cell, inhibition of memory type development, and finally bone marrow recipient remain healthy two month post BMT. This result indicates that $CD4^+CD25^+FOXP3^+$ T cell population contains novel regulatory T cells that effectively control GVH-like reaction in allogeneic BMT with mismatch-MHC and also potent suppressive effect on immune effector cells reactive to host antigens and prevent graft versus host disease (GVHD).

MATERIAL AND METHOD

A. Mice

Breeding pairs of C57BL/$6^{CD45.1/CD45.1}$ congenic strain were maintained in our animal facility. BALB/c (H-2d) and CBF1 were use as host and T cells adaptation, respectively. Donor cells were obtained from C57BL/6 mice.

B. Antibodies

Biotin-conjugated anti-mouse FOXP3 (clone FJK-16s), phycoerythrin (PE)-conjugated anti-mouse B220 (clone RA3-6B2), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a (clone53-6.7), phycoerythrin (PE)- or allophycocyanin (APCn)-conjugated anti-mouse CD4 (clone GK1.5), biotin-conjugated anti-mouse, FITC-conjugated anti-mouse CD25 (clone PC61.5), FITC- or biotin-conjugated anti-mouse CD45.1 (clone A20), and biotin-

conjugated anti-mouse TER-119 (clone TER-119) antibodies were purchased from *e*Bioscience Inc. (San Diego, CA). FITC-conjugated anti-mouse CD69 (clone H1.2F3), FITC-conjugated anti-mouse CD44 (clone IM7), PE-conjugated anti-mouse CD62L (clone MEL-14), FITC-conjugated anti-mouse Gr-1 (clone RB6-8C5) antibodies were purchased from BD-Biosciences Pharmingen (San Diego, CA). Biotin-conjugated antibodies were visualized by streptavidin-PE-Cy5 (*e*Bioscience, San Diego, CA). CD45.1 is antibody to distinguish donor derived cells from recipient derived cells.

C. Flow cytometry and cell sorting

Analytical flow cytometry was performed using FACS Calibur™ flow cytometer (BD-Biosciences, San Jose, CA). Preparative cell sorting was performed using FACS Vantage cell sorter (BD-Biosciences, San Jose, CA).

D. Bone marrow transfer

Bone marrow transfer was done after recipient mice treated with lethal dose radiation of 850 RAD. Bone marrow cells ($1\sim2 \times 10^6$) from C57BL/6 were intravenously injected to BALB/c recipient mice.

E. Intracellular cytokine staining

Intracellular cytokine staining was performed using a Cytofix/Cytoperm kit (BD-Biosciences Pharmingen) according to the protocol provided by the manufacturer. Cells were incubated with biotin-conjugated anti-FOXP3 antibody and visualized with streptavidin-PE-Cy5.

RESULT AND DISCUSSION

$CD4^+CD25^+FOXP3^+$ cells prevent expansion activated T cells in recipient of BMT. In allogeneic bone marrow transplantation lethal dose radiation is needed to prevent immune attack specially from recipient immune system. Lethal dose radiation also provide a space in which donor cells have a big chance to develop with lower competition. Figure 1 showed that 850 RAD served as one factor in order donor cell can be accepted by recipient. Figure 1 showed that the donor cell can reconstitute recipient cell more than 90%. As shown in Figure 2 and Figure 3, transfer of bone marrow alone or in combination $CD4^+CD25^-FOXP3^-$ had high percentage of activated memory-type T cells with markers of $CD69^+$and $CD62L^-$. In contrast, recipient mice that received $CD4^+CD25^+FOXP3^+$ $2x10^5$ cells showed no such increase of activated $CD69^+$ T cells (Figure 2) and $CD62L^-$ activated memory-type T cells (Figure 3). Transfer of $CD4^+CD25^-FOXP3^-$ T cells into recipient did not prevent the development of activated $CD69^+$ T cells and $CD62L^-$ memory-type T cells. These results indicate that at least $2x10^5$ $CD4^+CD25^+FOXP3^+$ cells are sufficient to perform regulatory activity when transferred together with graft bone marrow. We analyzed both $CD4^+$ T and $CD8^+$ T cells to know the status of activated T cells.

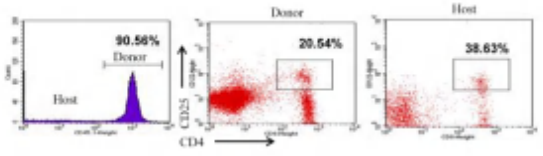

Figure 1. $CD4^+CD25^+$ regulatory T cells emerged from both donor and radiation resistance cells. After 8 wk post bone marrow transplantation, spleen cells obtained from recipient (host) mice were stained with anti-CD25, anti-CD4, and anti-CD45.1 antibodies, and analyzed by flow cytometry. Expression levels of CD25 are shown as dot plot for cells derived from both donor ($CD45.1^+$) and recipient ($CD45.1^-$). This pictures show that lethal dose radiation increase hematopoietic reconstitution in allogeneic system and most hematopoietic cells emerged from donor type. Data are mean ± SD values of three mice.

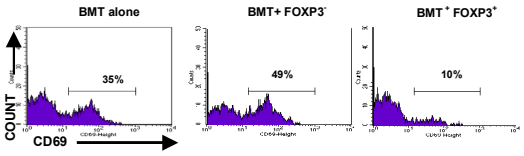

Figure 2. $CD4^+CD25^+FOXP3^+$ $_{Treg}$ inhibites T cell activation *in vivo*. T cell-depleted bone marrow cells obtained from C57BL/6 mice ($2x\ 10^6$) were intravenously injected into irradiated (8.5 Gy)BALB/c mice. BM cells alone or those in combination with $CD4^+CD25^+FOXP3^+$ or $CD4^+CD25^-FOXP3^-$ cells (2×10^5) were injected as indicated in the figure. Two months post BMT, spleen cells were analyzed by flow cytometry to assess the CD69 T cells. The result showed that the percentages of $CD69^+$ in $CD8^+$ population decrease in spleen of mice that received allogeneic BM cells together with $CD4^+CD25^+FOXP3^+$ T_{reg}.

$CD4^+CD25^+FOXP3^+$ cells normalize granulocytes, B cells, and erythrocytes in recipient mice. Examination of granulopoiesis showed that the number of $Gr\text{-}1^+$ cells in the bone marrow was markedly increased in recipient accepted BM alone or in combination with $CD4^+CD25^-FOXP3^-$ T cells (Figure 4). This increase was normalized by transferring $2x10^5$ $CD4^+CD25^+FOXP3^+$ cells into recipient but not by transferring $2x10^5$ $CD4^+CD25^-FOXP3^-$

cells T cells (Figure 4). Changes in the number of Gr-1$^+$ cells in the bone marrow matched those of leukocyte numbers in peripheral blood (data not shown). Increased leukocyte number in peripheral blood of recipient mice was normalized by the transfer of 2x10^5 CD4$^+$CD25$^+$FOXP3$^+$ cells into recipient but neither by the transfer of BM alone nor by that of 2x10^5 CD4$^+$CD25$^-$FOXP3$^-$. Hair lost and skin injure caused by a combination of autoimmune hemolysis and suppression of erythropoiesis is another striking feature of BMT without manipulation using Treg. Examination of erythroid-lineage cells by expression of TER-119 antigen, an erythroid specific marker, showed marked reduction in the number of TER-119$^+$ cells in the bone marrow of recipient BMT lacking of regulatory T cells. This erythroid suppression was also rescued by the transfer of 2x10^5 CD4$^+$CD25$^+$FOXP3$^+$ cells into recipient, whereas the transfer of 2x10^5 CD4$^+$CD25$^-$FOXP3$^-$ cells was ineffective, resulting in the same decrease of TER-119$^+$ cells as that in transfer of BM alone (Figure 5).

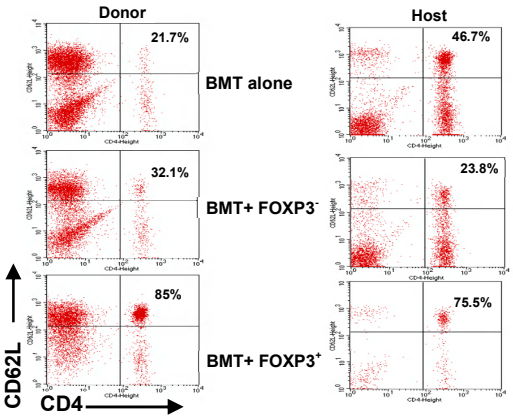

Figure 3. T cell-depleted bone marrow cells obtained from C57BL/6 mice (2 x 10^6) were intravenously injected into irradiated (8.5 Gy)BALB/c mice. BM cells alone or those in combination with CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^-$FOXP3$^-$ cells (2 x 10^5) were injected as indicated in the figure. Two months post BMT, spleen cells were analyzed by flow cytometry to assess the CD62L T cells. The result showed that CD62L$^+$ T cells increase in mice that received allogeneic BM cells together with CD4$^+$CD25$^+$FOXP3$^+$ T$_{reg}$.

It is now well documented from experimental in animal model that T$_{regs}$ represent a potential new therapeutic modality for the control of GVHD. Unfortunately T$_{reg}$ remain anergy by TCR mediated stimulation. In individual, the frequency of T$_{reg}$ is very low in the T cell compartment. To achieve a clinical benefit in the setting of murine GVHD, a phase of ex vivo expansion of T$_{reg}$ would probably be mandatory. This approach may also be effective in humans. After confirming the preventive effects of CD4$^+$CD25$^+$FOXP3$^+$ we recommended to combine both CD4 and CD8 derived T$_{reg}$ in allogeneic BMT.

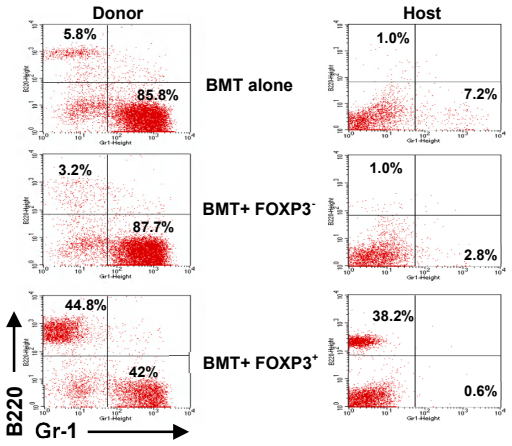

Figure 4. CD4$^+$CD25$^+$FOXP3$^+$ $_{Treg}$ maintain normal ratio of B cells and granulocyte cells. T cell-depleted bone marrow cells obtained from C57BL/6 mice (2 x 10^6) were intravenously injected into irradiated (8.5 Gy)BALB/c mice. BM cells alone or those in combination with CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^-$FOXP3$^-$ cells (2 x 10^5) were injected as indicated in the figure. Two months post BMT spleen cells were analyzed by flow cytometry to assess the B cell / Granulocyte ratio. The result showed normalization of B cell / Granulocyte ratio in mice that received allogeneic BM cells together with CD4$^+$CD25$^+$FOXP3$^+$ cells.

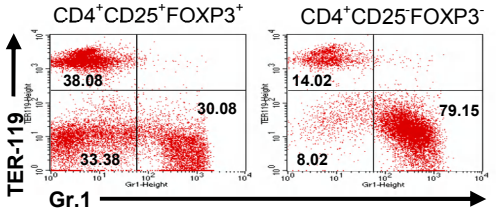

Figure 5. Increased erythropoiesis and reduced granulopoiesis in recipient mice that received CD4$^+$CD25$^+$FOXP3$^+$. Bone marrow cells obtained from mice that had received allogeneic BMT in combination with CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^-$ FOXP3$^-$ cells were stained with anti-Gr-1 and anti-TER-119 antibodies. Percentages of cells in myeloid lineage (Gr-1$^+$TER-119$^-$) and cells in erythroid lineage (Gr-1$^-$TER-119$^+$) are shown.

In a model of GVHD prevention using freshly purified T$_{regs}$, Edinger et al. (22) showed a strong inhibition of expansion of donor T cells. However, in a cell-per-cell analysis of donor T cells, the addition of freshly purified T$_{regs}$ had minimal or no effect on the acquisition of activation markers and IFN-γ production. Here we observed that CD4$^+$CD25$^+$FOXP3$^+$ T$_{regs}$ strongly inhibited the division and expansion of

host remaining T cells. It is clear that CD4$^+$CD25$^+$FOXP3$^+$ T_{regs} promote the donor engraftment and replaced host derived T cells (Figure7). CD4$^+$CD25$^+$ FOXP3$^+$ expanded T_{regs} additionally inhibit the development of donor toward Gr1 cells (Figure3). The function of CD4$^+$CD25$^+$FOXP3$^+$ T_{reg} could be mediated by IL-10 and TGFβ production. In the system of in vitro, CD4$^+$CD25$^+$FOXP3$^+$ can suppress T cells proliferation under anti-CD3 stimulation and in the presence of antigen-presenting cell (APC) (Figure5). This property of T_{regs} could explain why expanded T_{regs} seem to have a more profound suppressive effect on the activation of donor T cells in an allogeneic BMT setting. Here we propose that GVHD is not due to the early expansion and differentiation of alloreactive donor T cells. In this case we have an evident that absolute exclusion of mature donor T cells from donor marrow could not inhibit such activation, this explains that may be T_{reg} is one component necessary to prevent the disease. It had been demonstrated that CD4$^+$CD25$^+$ T_{regs} efficiently prevent GVHD (17, 18). Other group demonstrated that CD4$^+$CD25$^+$ can ameliorate GVHD.

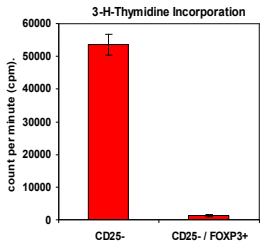

Figure 6. CD4$^+$CD25$^-$ T cells cocultured with CD4$^+$CD25$^+$FOXP3$^+$ $_{Treg}$ become unresponsive to anti-CD3 stimulation. Purified CD4$^+$CD25$^-$ T cells were cultured either alone or cocultured with CD4$^+$CD25$^+$FOXP3$^+$ T_{reg} at ratio 2:1. Cultures were performed in 96-well round bottom plates in the presence of T cell-depleted and gamma irradiated (3000Rad) spleen cells as an APC source and soluble anti-CD3. Incubation was performed 72 hrs in a medium containing rIL-2 (50 ng ml-1). After culture, cells were harvested and analyzed using standard method of ^{3}H- Thymidine uptakes. Proliferation rate of T cells is presented in the panels.

The better effect of T_{regs} could rely on their increased ability to rapidly become activated in vivo, and exert their immunosuppressive effect early after infusion. In further support of this concept, it was recently showed that in fully MHC-mismatch, GVHD usually occur when T_{regs} from adoptive transfer are rejected (20). Early after injection, most of T_{reg} are likely activated by their cognate allogeneic Ags in the system of syngeneic BMT, but will be rejected in fully MHC-mismatch. Another point is that T_{regs} also could display a suppressive effect mainly targeted to alloreactive donor T cells, whereas non-alloreactive donor T cells could be partially spared. Here, we show that CD4$^+$CD25$^+$FOXP3$^+$ T_{regs} were not rejected by recipient and have a capacity to prevent both donor and host type T-cells from developing become activated memory type (Figure 2, 3). This data suggest that T_{regs} contribute to silencing T cell reactivity between donor and host. In a previous report, we showed the function of T_{reg} to ameliorate autoimmune destruction in syngeneic model experiment (21).

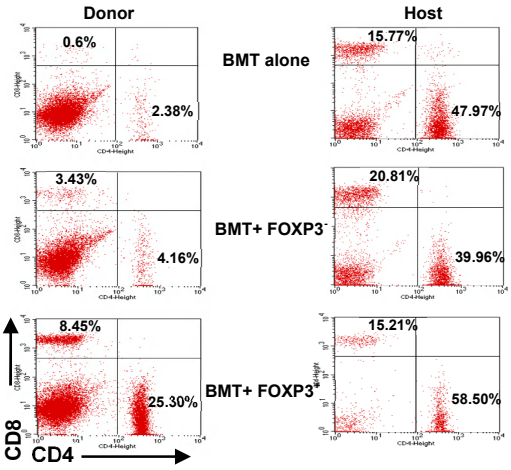

Figure 7. CD4$^+$CD25$^+$FOXP3$^+$ T_{reg} promote host remaining T cell replacement. T cell-depleted bone marrow cells obtained from C57BL/6 mice (2 x 10^6) were intravenously injected into irradiated (8.5 Gy)BALB/c mice. BM cells alone or those in combination with CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^-$FOXP3$^-$ were injected as indicated in the figure. Two months after BMT, spleen cells were analyzed by flow cytometry to assess T cell replacement. The result indicated that CD4$^+$CD25$^+$FOXP3$^+$ T cells promoted T cell replacement from host type to donor type.

After T_{reg} cell transfer in lymphopenic recipients, we observed that activated T cells are down-regulated. This down-regulation looks very similar with T_{reg} cells transfer in allogeneic system. T_{reg} cells could suppress early activated T cells and inhibit CD69 expression then keep the existence of CD62L on cell surface (Figure 2, 3). In contrast, in the absent of T_{reg} the mice model of autoimmune disease will die because of activated T cell reaction. Alloreactive and nonalloreactive T cells can be discriminated by the expression of CD62L and also CD69 (22-24). In this study, we observed that T cells that express CD62L in the presence of T_{regs} behaved as nonalloreactive T cells. Indeed, T_{regs} dramatically reduced the expansion and differentiation of host remaining T cells, and

most of the remaining dividing host T cells expressed CD69 and loss the CD62L.This suggests that, when using $CD4^+CD25^+$ $FOXP3^+$, activation and differentiation of alloreactive T cells in effector T cells involved in GVHD are inhibited by these T_{regs} whereas nonalloreactive T cells can expand. In allogeneic BMT, GVHD may contribute to the destruction of lymphoid organ. In this report we observe that GVHD also related to donor rejection and remaining host T cell development. Because we observed a better immune reconstitution when T_{regs} were used, it is likely that, in this situation, nonalloreactive donor T cells expand significantly in the recipient and participate to the improved immune reconstitution and T cell replacement from recipient increased.

The use of T_{regs} for the control of GVHD in allogeneic BMT is an area of debate of intense interest. However, to date, nothing is known regarding the respective therapeutic benefit of the most effective T_{regs} in humans. In a clinical setting, the primary difference between $CD4^+CD25^+$ and $CD8^+CD28^-$ has not been tested yet. We hypothesize that combination of both CD4 and CD8 T_{reg} may effectively protect the body weigh loss in allogeneic BMT. These T_{regs} seem to be more complicated without knowing the molecules which is recognized in the regulatory of action. The generation of T_{regs} would probably raise more technological issues because of the necessity to use APCs, as opposed to clinically standardized beads. The necessity to select nonmalignant APCs when BMT is performed for the treatment of leukemia could be an additional major hurdle. Furthermore, the duration of the culture to achieve clinically relevant cell numbers could be longer with T_{regs}. Indeed, in mice, to obtain high numbers of T_{regs}, take over 28 days. In this study, we used equivalent numbers of T_{regs} and suspected non T_{regs} ($CD4^+CD25^-FOXP3^-$) and observed that T_{regs} are more efficient than non T_{regs} in controlling GVHD. It is quite possible that infusion of higher numbers of T_{regs} promote better of GVHD inhibition and promotion of immune reconstitution. In fact, some studies (26) demonstrated that by increasing the number T_{reg} 3-fold (three T_{regs} per infused allogeneic T cell), nearly complete inhibition of overt GVHD was durably achieved despite the presence of histopathological signs of GVHD in target organs. Again our study empesis that T_{reg} function not only inhibit alloreactive T cells but also promote donor engraftment. However, to be clarified, this point deserves specific experiments, such as comparing the effect of T_{regs} in very small number of BM cells and huge number of BM cells in allogeneic BMT system. Our work demonstrates that $CD4^+CD25^+FOXP3^+$ T_{regs} are efficient in providing durable protection from GVHD and facilitate immune reconstitution after allogeneic bone marrow transplantation. Since only donor derived $CD4^+CD25^+FOXP3^+$ T_{reg} could be effective in allogeneic BMT, thus, we propose that T_{regs} should be explored and adapted again host as a viable modality for treating human GVHD in the setting of allogeneic bone marrow transplantation. Because so far there are no preclinical data have demonstrated in human to maintain the GVHDL effect (21, 27-30), this still remains to be studied in clinical trials.

CONCLUSION

$CD4^+CD25^+FOXP3^+$ T_{reg} cells contribute to inhibit GVH-like reaction. It is important to find a strategy to isolate and expand $CD4^+CD25^+FOXP3^+$ regulatory T cells. $CD4^+CD25^+FOXP3^+$ T_{regs} actively inhibit the development of GVH-like reaction by increasing graft engraftment and preventing T cell activation. Emerging data provide a new evident to change old paradigm that GVHD is problem of mature T cell contamination from donor type. Here, we postulate that GVHD is not problem of mature T cell contamination from donor type but rather of lacking or non functional of T_{reg}. Finally we recommend to combine $CD4^+CD25^+FOXP3^+$ T_{reg} with $CD8^+CD28^-$ T_{reg} in allogeneic bone marrow transplantation.

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