

Dynamic regulation of effector IFN- γ -producing and IL-17-producing T cell subsets in the development of acute graft-versus-host disease

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Abstract. Graft-versus-host disease (GVHD) as the predominant complication of allogeneic hematopoietic stem cell transplantation remains to be fully understood. It is known that the cytokines produced by allogeneic reactive effector CD4⁺ and CD8⁺ T cells are involved in GVHD. However, the regulation and coordination of IFN- γ -producing and IL-17-producing effector T cells remain unclear. The present study aimed to investigate the dynamic changes of alloantigen-specific effector CD4⁺ T and CD8⁺ T cell subsets by flow cytometry, which produce inflammatory cytokines involved in the multistep GVHD pathogenesis progress. The results demonstrated that IL-17-producing CD8⁺ T (Tc17) cells and IFN- γ ⁺CD8⁺ T (Tc1) cells were detected in the early stage of GVHD. The differentiation of CD4⁺ T cells into Th1 cell (IFN- γ ⁺CD4⁺ T) and Th17 (IL-17⁺CD4⁺ T) cells was later than that of the Tc1 and Tc17 cells. The effector CD4⁺ T and CD8⁺ T cell subsets either became exhausted or became memory cells, exhibiting a CD62L⁺CD44⁺ phenotype following marked expansion during GVHD. Furthermore, T cell-associated type I (IL-2 and IFN- γ) and type II (IL-4 and IL-10) classical cytokines exhibited coordinated dynamic regulation. It was concluded that the differentiation of cytokine-producing Tc1 and Tc17 cells may be the key step in the initiation of GVHD, whereas CD4⁺ effector Th1 and Th17 cells are considered to be pathophysiological factors leading to the continuous aggravation of GVHD.

Introduction

Graft-versus-host disease (GVHD), mediated by mature T cells in the donor graft, remains a major complication following allogeneic bone marrow transplantation. Alloreactive T cells involved in the generation of acute GVHD can be derived from a naïve T cell pool (1). The core of the GVHD reaction is donor T cell activation, in which donor T cells proliferate, differentiate and produce cytokines. Several studies have focussed more attention on the role of the CD4⁺ T cell subsets involved in the development of acute GVHD. Th1 cells secreting pro-inflammatory cytokines, including interferon (IFN)- γ and tumor necrosis factor (TNF)- α , which are considered to be responsible for driving cellular immune responses, have been demonstrated to be etiological factors in the induction of GVHD (2,3). Th17 cells, which are characterized by the production of interleukin (IL)-17A, IL-17F, IL-21 and IL-22, have been demonstrated to be sufficient, but not necessary, to induce GVHD (4,5). In addition experimental data in mice and humans have demonstrated the potential of Th cell subsets to exhibit plasticity, shifting between phenotypes (6). The above observations suggest that the Th1 and Th17 cells involved in GVHD have roles in the network. Although efforts have been focussed on investigating the solitary function of these cells, the correlation of Th1 and Th17 cells in GVHD process remain to be fully elucidated.

It is important to consider that, despite the prominent role of CD4⁺ T cells in the pathogenesis of GVHD, the function of CD8⁺ T cells in GVHD cannot be ignored.

Several studies have reported the function of CD8⁺ T cells as cytotoxic T lymphocytes in the final cellular and inflammatory effector phase III of GVHD. It has been reported that donor cytotoxic double deficient (perforin^{-/-} and FasL^{-/-}) CD8⁺ T cells expand continuously and cause life-threatening GVHD (7,8). However, during phase II of GVHD, characterized by T cell activation and polarization, evidence of the effects of CD8⁺ T cells remains limited. Furthermore, certain findings support the hypothesis that donor anti-host cytotoxicity via the two major pathways (perforin and FasL) is not a prerequisite for the induction of GVHD (9). Therefore, the role of donor CD8⁺

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T cells in the pathogenesis of GVHD is not only limited to the perforin or FasL pathways.

Classically, CD8⁺ T cells have been assigned to Tc1 or Tc2 lineages, based on the cytokines produced by these cells. Notably, it has been reported that naive CD8⁺ T cells can also differentiate into IL-17-producing T cells, termed Tc17 cells, in the same culture conditions as CD4⁺ T cells (10). However, there are no associated reports regarding the generation and role of Tc17 cells during GVHD. In addition, although a common idea regarding the activation of CD4⁺ T subsets and CD8⁺ T subsets, the pathophysiological link between them during the process of GVHD remains poorly understood (11). Therefore, the involvement of alloreactive donor T-cell responses, and whether T cell polarization leads to distinct targeted tissue damage, remain to be fully elucidated and require investigation.

In the present study, the dynamic changes of alloantigen specific effector CD4⁺ T and CD8⁺ T cell subsets, which produce inflammatory cytokines involved in the multistep GVHD pathogenesis progress were investigated. These cells and cytokines act as critical links between the occurrence and progression of GVHD. The present study may provide novel information to clarify the mechanism of GVHD.

Materials and methods

Mice. Male C57BL/6 (H-2Kb) mice, as donors, and BALB/c (H-2Kd) mice, as recipients, aged between 6-8 weeks were purchased from SLRC Laboratory Animal Centre, Co., Ltd. (Shanghai, China). The mice were housed in individual cages (5 mice/cage) under controlled temperature (21-23°C) and relative humidity (60-65%). The mice underwent a 12/12 h light/dark cycle, and had *ad libitum* access to a standard mouse diet and water. The mice were bred in a specific pathogen-free facility at Xuzhou Medical College (Xuzhou, China). All experiments were performed according to the guidelines of the Institutional Animal Committee of Xuzhou Medical College. The study was approved by the Laboratory Animal Ethics Committee of Xuzhou Medical College (Jiangsu, China).

Induction and assessment of GVHD. The procedures used for the induction of GVHD were as described as our previous study (12). Briefly, the BALB/c recipients underwent 7.5 Gy total body irradiation (TBI) from a ⁶⁰Co source (Beijing Kang Keda Technology Co., Ltd., Beijing, China), and bone marrow transplantation (BMT) was performed via injecting the mice intravenously with bone marrow cells (5×10⁶/mouse) from the donor mice 4 h post-irradiation. Bone marrow was flushed from the medullary cavity of the femur and tibia using phosphate-buffered saline (PBS). For the induction of GVHD, donor splenic T cells, which were isolated using flow cytometry (FACSCalibur; BD Pharmingen, San Diego, CA, USA), were co-transferred (5×10⁵/mouse) with the bone marrow cells to the recipient mice (BS; bone marrow cells+splenic T cells). The transplantation day was set as day 0. The experimental and control groups were established at the same time and experiments were replicated 2-3 times. The recipients were monitored daily to assess survival, and every 2-3 days to assess changes in body weight. Mice were sacrificed by cervical dislocation and specimens (1 cm x 1 cm x 5 mm) of the liver, gut and lung

of the recipients were fixed in formalin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) prior to being embedded in paraffin (Sinopharm Chemical Reagent Co., Ltd.) blocks. The tissue sections were stained with hematoxylin and eosin (H&E; Sinopharm Chemical Reagent Co., Ltd.) for histopathological detection. Liver GVHD was evaluated on the severity of liver cell necrosis and inflammatory infiltration. Gut GVHD was evaluated on the level of inflammation in the epithelium and lamina propria. Lung GVHD was evaluated on the severity of peri-bronchic infiltration and pneumonitis.

Media and antibodies. RPMI-1640 media was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.1% 2-methoxyestradiol, 100 U/ml penicillin and 100 µg/ml streptomycin. Monoclonal rat anti-mCD8-fluorescein isothiocyanate (FITC; cat. no. 553031), rat anti-mCD4-peridinin chlorophyll (PerCP; cat. no. 553052), rat anti-mIFN-γ-allophycocyanin (APC; cat. no. 554413), and isotype control antibodies were purchased from BD Pharmingen. The rat anti-mIL-17a-APC (cat. no. 506916), rat anti-mCD8-phycoerythrin (PE; cat. no. 100707), rat anti-mCD4-FITC (cat. no. 100510), rat anti-mCD3-PerCP-Cy5.5 (cat. no. 100328), rat anti-mCD45-PerCP (cat. no. 103130), rat anti-H-2Kb-PE (cat. no. 116507) and rat anti-mCD44-PerCP-Cy5.5 (cat. no. 103031) were obtained from BioLegend, Inc. (San Diego, CA, USA). Rat anti-mCD62L-PE (cat. no. 12-0621) was provided by eBioscience, Inc. (San Diego, CA, USA). The concentration of all antibodies used was 1 µg/ml (1:200).

Cytometric bead array analysis for cytokines. Plasma from each of the each groups was obtained 7, 14, 40 and 50 days following transplantation, respectively. The production of IL-6, TNF-α, IL-2, IFN-γ, IL-4 and IL-10 cytokines were measured with a cytometric bead array (CBA) using flow cytometry (FacsCalibur, BD Pharmingen), according the manufacturer's instructions. Briefly, six bead populations specific for IL-6, TNF-α, IL-2, IFN-γ, IL-4 and IL-10 with distinct fluorescence intensities were coated with capture antibodies specific for different cytokines. Following incubation of the beads with 50 µl diluted plasma (2-fold dilution), different cytokines in the sample were captured by their corresponding beads. The cytokine-captured beads were then mixed with PE-conjugated detection antibodies to form sandwich complexes. Following incubation for 20 min at room temperature in the dark, the fluorescent samples were washed, harvested and analyzed using CellQuest version 6 software (BD Pharmingen).

Intracellular cytokine and cell surface staining. The mice were sacrificed by cervical spine fracture 7, 14, 28, 40, 50 and 60 days following transplantation. The spleens and bone marrow cells from each mouse were harvested, and lymphocytes from the spleen were isolated using mouse lymphocyte separation medium (Dakewe, China), with a purity of 90%. The single lymphocyte suspension was incubated with or without 50 ng/ml phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO, USA) with 750 ng/ml ionomycin (Sigma-Aldrich) in the presence of 10 µg/ml brefeldin A (Invitrogen Life Technologies) at 37°C for 5 h. The cells were washed, fixed with 4% paraformaldehyde (Sinopharm Chemical Reagent

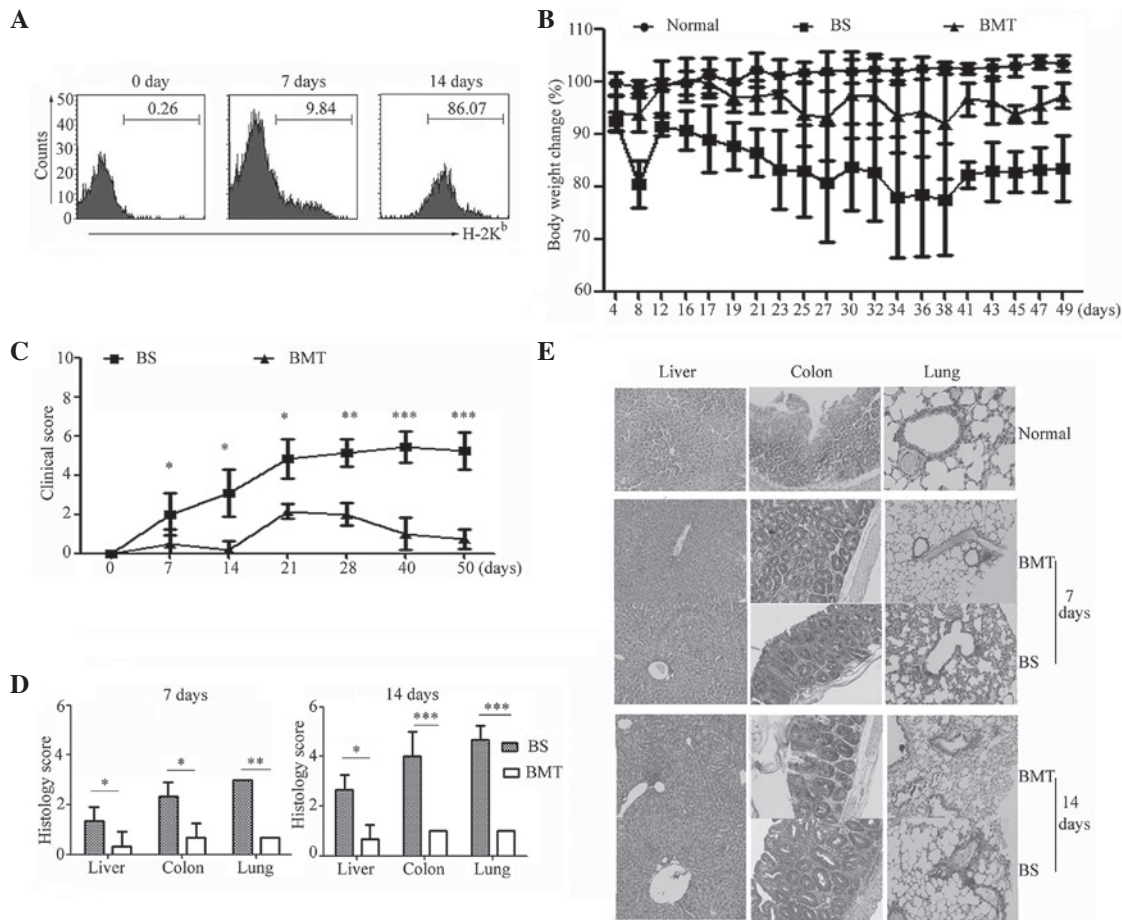


Figure 1. Establishment and evaluation of the GVHD mouse model. GVHD was induced in BALB/c mice by transfusion of bone marrow cells and splenic lymphocytes from C57BL/6 donors. (A) Donor and host chimerism of bone marrow cells was measured using flow cytometry following transplantation. Animals were monitored for (B) body weight changes, (C) clinical score and (D) histological score. (E) Histological evidence of GVHD in the liver, colon and lung sections were obtained using hematoxylin and eosin staining and visualized using an Olympus BX-51 microscope (magnification, x200). There were 3-6 mice in each group, and two replicate experiments were performed. Data are expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. BMT, bone marrow transplant; BS; bone marrow + splenic cells; PMA, phorbol 12-myristate 13-acetate.

Co., Ltd.) and permeabilized in PBS containing 0.1% saponin (Sigma-Aldrich), 0.1% bovine serum albumin and 0.05% NaN₃ overnight at 4°C. The cells were then stained with conjugated monoclonal antibodies for the CD3, CD4, CD8, CD62L and CD44 surface markers and IFN- γ and IL-17a intracellular cytokines for 20 min in the dark. The cells were acquired using a flow cytometer (FacsCalibur, BD Pharmingen) and data were analyzed using CellQuest Pro software (version 6.0; BD Pharmingen). Isotype controls were included for each staining.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Comparisons were performed using a non-parametric t-test for two-group comparisons, or one-way analysis of variance for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GVHD evaluation. GVHD was routinely induced in BALB/c mice (H-2K^d) by transplantation of bone marrow cells combined with splenic T cells from C57BL/6 mice (H-2K^b). The reconstituted immune system was assessed 7 and 14 days following transplantation using flow cytometry. Bone marrow cells were

collected and stained with anti-mouse CD45 and anti-mouse H-2K^b antibodies. As shown in Fig. 1A, the results demonstrated that a low level of donor-derived cells was detected on day 7, whereas the CD45⁺ cells were almost all donor origin (H-2K^b) on day 14 in the BS groups, indicating a successful reconstituted immune system in the recipients. Subsequently, the onset and progression of GVHD were observed. As shown in Fig. 1B, the results revealed that the recipients in the BS group exhibited more severe weight loss, compared with the recipients in the BMT and normal groups. As shown in Fig. 1C and D, the clinical score and histopathology score of the GVHD targets, including the liver, colon and lung, were analyzed on day 7 and 14. A higher score was detected from day 7 in the BS mice, compared with the BMT mice, and statistical analysis demonstrated that this was a significant difference ($P < 0.05$). As shown in Fig. 1E, marginal perivascular infiltration was detected in the lung of the BS mice 7 days post-transplantation. As time progressed, higher levels of severe tissue damage and higher histopathology scores were detected in the BS mice than the BMT mice on day 14. In the BS groups, the liver exhibited marked portal and lobular inflammation with mononuclear and granulocytic infiltrates. The colon specimens from the BS mice exhibited marked infiltration of mononuclear and

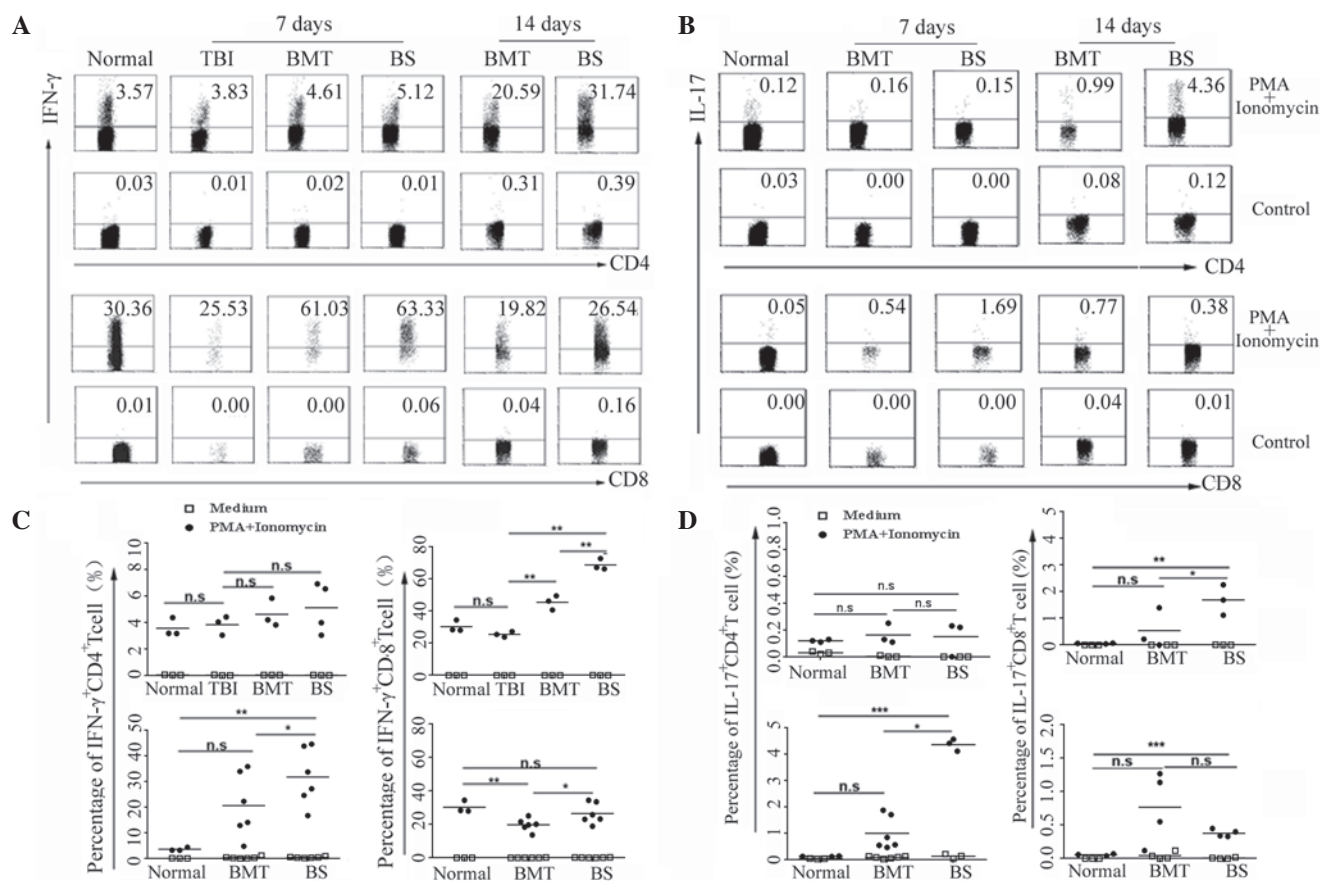


Figure 2. Detection of effector T cell subsets following transplantation. Splenic lymphocytes from each group were cultured with or without PMA + ionomycin for 5 h, and were then harvested and stained for flow cytometric analysis. (A and B) Representative results from one of two independent experiments showing the expression levels of IFN- γ and IL-17 in each group, respectively. The number in the corner of each square represents the mean percentage of positive cells in the gated T cells. (C and D) Statistical analysis of two independent experiments was performed. Each symbol represents one data point from one mouse. The horizontal bars represent the mean values. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., no significance ($P > 0.05$). IFN, interferon; IL, interleukin; BMT, bone marrow transplant; BS, bone marrow + splenic T cells; PMA, phorbol 12-myristate 13-acetate.

granulocytic cells into the lamina propria, with extensive goblet cell depletion. More severe perivascular cuffing, vasculitis and peribronchiolar cuffing were observed in the lung specimens of the BS recipients, compared with the BMT mice (Fig. 1E). The tissue sections from the normal control mice exhibited no abnormality. Taken together, these results demonstrated that the GVHD model was successfully established in the BS mice in the present study, and that more severe tissue lesions were detected on day 14 following allogeneic transplantation.

Alloreactive effector IFN- γ ⁺ and IL-17⁺ T cell subsets are involved at different time-points. To examine the correlation between IFN- γ -producing and IL-17-producing T cells following allogeneic transplantation, the present study isolated splenic lymphocytes from normal, TBI, BMT and BS mice on days 7 and 14 respectively. Lymphocytes were cultured for 5 h with or without phorbol 12-myristate 13-acetate and ionomycin, and were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies to set gates. Subsequent intracellular cytokine staining was performed using anti-mouse-IFN- γ antibody. The CD4⁺ IFN- γ ⁺ T cells represented Th1 cells and the CD8⁺ IFN- γ ⁺ T cells represented Tc1 cells. The percentages of Th1 cells and Tc1 cells were compared 7 days post-transplantation. As shown in Fig. 2A and B, the percentages of the Th1 cells in the normal, TBI, BMT and BS mice were at a

similar level, and statistical analysis revealed no significant differences ($P > 0.05$). However, the percentage of Tc1 cells in the BS mice was significantly higher than those in the normal, TBI and BMT mice ($P < 0.01$). An increased percentage of Th1 cells and a reduced percentage of Tc1 cells were observed in the BS mice on day 14 post-transplantation, which were higher than those in the BMT mice ($P < 0.05$). These data suggested that Tc1 cells were induced in the initial phase of GVHD, and the involvement of Th1 cells in the process of GVHD was later than that of the Tc1 cells.

With the exception of Th1 cells, the Th17 cell as a novel Th cell subset has been demonstrated to be crucial in the induction and development of GVHD. A novel population of IL-17-expressing CD8⁺ effector T cell (Tc17) has been identified in several diseases (13), however, its involvement in acute GVHD have not been reported. The present study aimed to detect Th17 cells and Tc17 cells at different time following transplantation. At 7 days post-transplantation for, the proportion of Th17 cells in the BS mice was at a low level and was not significantly different to the normal or BMT mice ($P > 0.05$; Fig. 2C and D). However, the percentage of Th17 cells in the BS mice increased markedly on day 14, whereas no marked IL-17⁺CD4⁺ T cell involvement was observed in the normal mice or BMT mice without GVHD. As shown in Fig. 2D, increased production of IL-17 in CD8⁺ T cells was observed

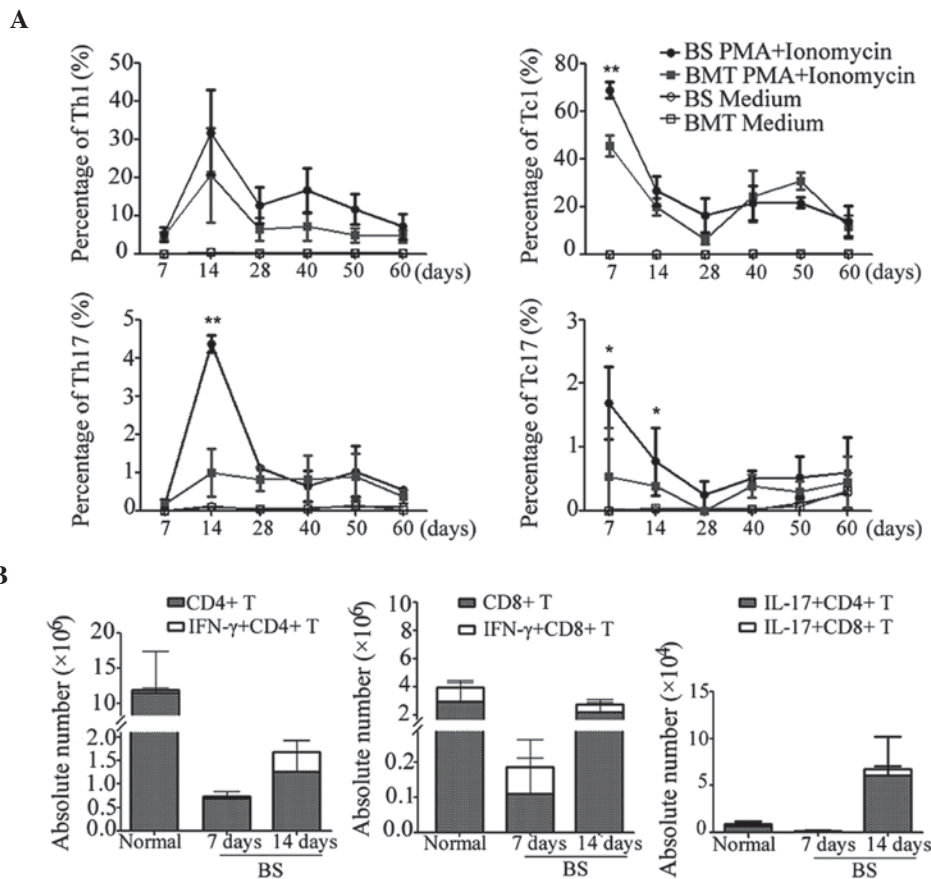


Figure 3. Dynamic changes in effector CD4⁺ T and CD8⁺ T cells. Splenic lymphocytes from the BS mice were harvested and stimulated with or without PMA and ionomycin at different time-points following transplantation. Cells from BMT mice were treated in the same manner as the control mice. (A) Based on the intracellular cytokine staining, the percentages of CD4⁺ T and CD8⁺ T cell subsets were analyzed using flow cytometry. The lines indicate connections between mean values. (B) Summaries of the absolute numbers of each T cell subpopulation. The bars represent the mean \pm standard deviation. There were 3-9 mice in each group and three replicate experiments were performed. * $P < 0.05$; ** $P < 0.01$, BS PMA+Ionomycin group vs. BMT PMA+Ionomycin group. IFN, interferon; IL, interleukin; BMT, bone marrow transplant; BS, bone marrow + splenic T cells; PMA, phorbol 12-myristate 13-acetate.

in the BS mice, but not in the BMT mice, on day 7. Compared with the normal and BMT mice, the percentage of Tc17 cells induced in the BS mice was higher ($P < 0.05$). Further investigation demonstrated that the proportion of Tc17 cells decreased marginally with the development of GVHD, although it remained higher than that in the normal mice (Fig. 2D). The above results indicated that Th17 and Tc17 cells were induced in GVHD at different phases and the predominant cellular source of IL-17 may change during GVHD by Tc17 and Th17 cells.

Coordinated and dynamic changes in IFN- γ ⁺ and IL-17⁺ T cells. The present study subsequently detected the dynamic changes in cytokine positive effector T cells through the progression of GVHD. The percentage of effector CD4⁺ T cell and CD8⁺ T cell subpopulations in the recipient mice were investigated at different time-points. As shown in Fig. 3A, the percentage of Th1 cells in the BS mice increased continuously between days 7 and 14, whereas the Tc1 cells exhibited a decreasing trend. In the BS mice, the Tc17 cells were sustained at a marginally higher level than in the BMT mice, whereas the Th17 cells were markedly upregulated on day 14. Subsequently, both of the effector CD4⁺ T cells and CD8⁺ T cells began to decline following expansion as time progressed.

The above data confirmed that self-reactive effector T cells were induced and proliferated in the early phase of GVHD. Therefore, the absolute number of T cell subsets were assessed in the BS mice. As shown in Fig. 3B, the number of total CD4⁺ and CD8⁺ T cells increased between days 7 and 14, indicating T cell expansion. The number of T cells in the BS mice was markedly lower than that in the normal mice due to the pre-conditioning irradiation. However, the numbers of effector T cells, including IFN- γ ⁺ Th1 and Tc1 cells, and IL-17⁺ Th17 and Tc17 cells, were higher than those in the normal mice, suggesting that more activated T cells were generated in GVHD development. In addition, compared with the CD4⁺ T cells, which were predominantly activated on day 14, the CD8⁺ T cells exhibited activity earlier.

Phenotype of CD4⁺ T and CD8⁺ T cells in GVHD mice. The above data indicated that the donor-derived T cells were activated and produced cytokines in the BS mice. The present study subsequently evaluated the expression of T cell activation markers in donor T cells 14 days post-transplantation. Splenic lymphocytes from the BS mice were isolated and stained, and the normal and BMT mice were set as controls. CD62L, which is a lymphocyte homing receptor, is regarded as a marker for naïve or memory T cells. CD44 has been reported to be a valuable marker for the detection of activated T cells and adhesion molecules at the sites of inflammation (14). As shown in Fig. 4,

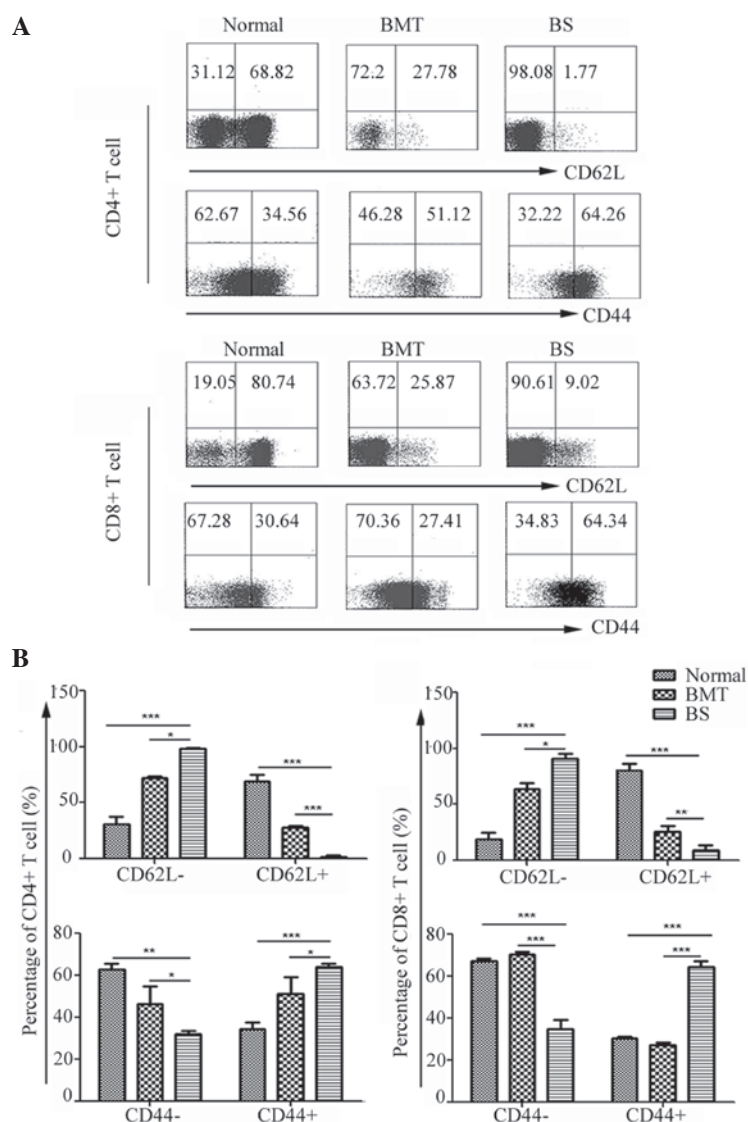


Figure 4. Characteristics of CD4⁺ T and CD8⁺ T cells. Splenic lymphocytes from each group were collected and stained with cell surface anti-mCD62L and anti-mCD44 antibodies. (A) Representative results from two similar independent experiments of the expression of CD62L and CD44 by CD4⁺ and CD8⁺ T cells. The numbers at the upper left and upper right corner represent the mean percentage of positive cells in the lower left and lower right quadrants, respectively. (B) Data are presented as the mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. no significance ($P > 0.05$). BMT, bone marrow transplant; BS; bone marrow + splenic T cells.

the expression of CD62L in CD4⁺ T cells from the BS mice was markedly downregulated, with higher levels of CD44 detected in the CD4⁺ T cells. It was observed that the majority of the CD4⁺ T cells in the BS mice were activated. Regarding the CD8⁺ T cells, the expression of CD62L in the BS mice was lower than that in the normal and the BMT mice, and a statistically significant difference in the percentage of CD44⁺CD8⁺ T cells between the BS mice and BMT mice was observed (Fig. 4). These data indicated that the CD4⁺ and CD8⁺ T cells were activated and involved in different stages of GVHD.

Changes in systemic T cell-associated cytokines in GVHD mice. To investigate the levels of systemic T cell-associated cytokines in the process of GVHD, plasma was collected from each group and cytokines were detected using a CBA. As shown in Fig. 5A, plasma levels of IL-6 and TNF- α in the BS mice were higher than in the normal, TBI and BMT mice, and increased to a peak on day 14. The levels of IL-6

and TNF- α began to decrease in the later phase of GVHD. IL-2 and IFN- γ , which are considered classical cytokines of Th1 or Tc1 cells were at a high level in the early phase, followed by a continuous reduction (Fig. 5B). However, IL-4 and IL-10, as immunosuppressive cytokines of Th2 or Tc2 cells, increased in a step-wise manner with the development of GVHD (Fig. 5C). These results indicated that, during the whole process of GVHD alloantigenic specific effector T cells, considered key for the pathogenesis of GVHD, were regulated within the dynamically changing environment.

Discussion

The present study investigated the characteristics of transplanted CD4⁺ and CD8⁺ T cells and the subsequent immune responses in the recipients. Based on the analysis of cytokine production, the results demonstrated that diverse, but coordinated, T cell immune responses were induced during

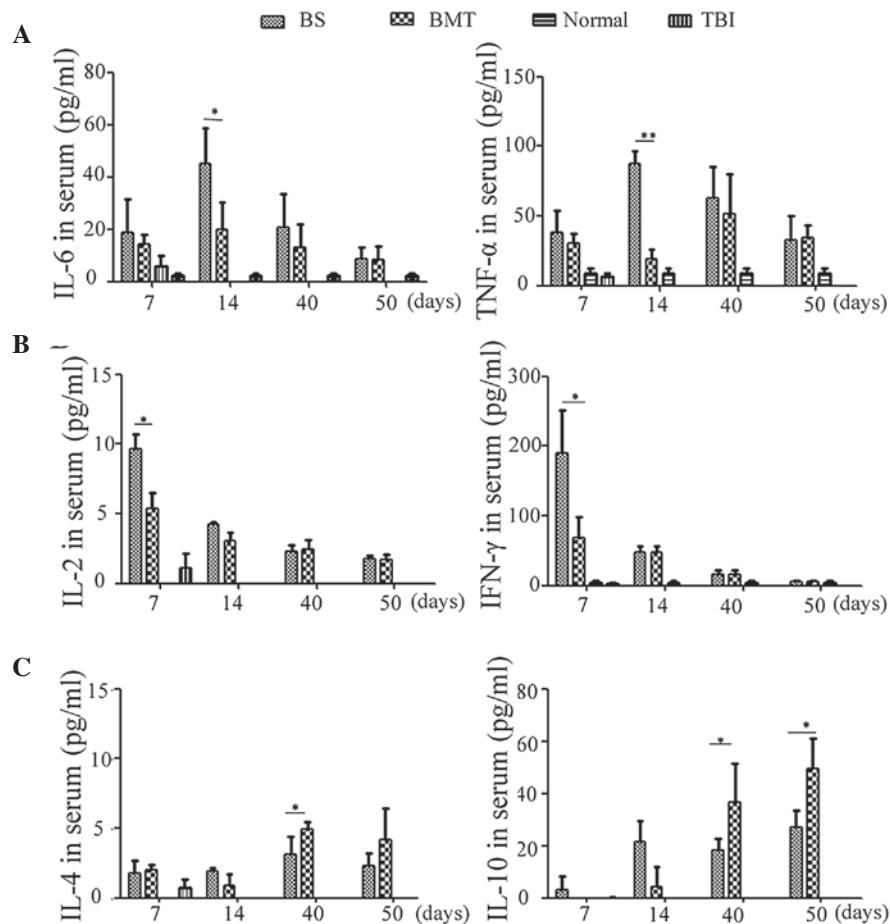


Figure 5. Cytokine levels in the plasma at different time-points. The levels of cytokines in the plasma from each group (n=3-6) were analyzed using a CBA with a flow cytometer. The data were generated in graphical format using the CBA software. (A) Concentration of IL-6 and TNF- α in the plasma. (B) Expression levels of Th1 and Tc1 cell-associated IL-2 and IFN- γ cytokines and (C) Th2 and Tc2 cell-associated IL-4 and IL-10 cytokines. Data are expressed as the mean \pm standard deviation. *P<0.05, **P<0.01. CBA, cytometric bead array; IFN, interferon; IL, interleukin; BMT, bone marrow transplant; BS, bone marrow + splenic T cells; TBI, total body irradiation.

the occurrence and development of GVHD. T-cell activation, proliferation and differentiation in response to host APCs are core components of the immune reaction of GVHD, and donor CD4⁺ and donor CD8⁺ T cells are crucial in the pathogenesis of GVHD (15). During the progress of GVHD, it is hypothesized that CD4⁺ T cells are activated by major histocompatibility complex (MHC) class II molecules, and that naive CD8 T cells require priming by activated APCs with the assistance of CD4⁺ T cells to proliferate and differentiate into effector T cells (16-18). In the present study, recipient mice were exposed to 7.5 Gy total body irradiation and were transplanted with MHC class I mismatched donor cells. The results revealed that CD8⁺ T cell alloimmune responses were induced faster and earlier than CD4⁺ T cells following allogeneic transplantation. Consistent with this data, it has been reported that in heavily-irradiated MHC class I-mismatched mice, purified CD8⁺ T cells initiate GVHD without the assistance of CD4⁺ T cells (19,20). This suggests that cooperation between CD4⁺ T and CD8⁺ T cells may not be required for the effective activation of primary alloimmune responses. Therefore, the present study hypothesized that pre-conditioning regimens and mismatched cell transplantation trigger the production of proinflammatory cytokines in CD8⁺ T cells.

In the present study, when the phenotype of alloreactive CD4⁺ and CD8⁺ T cells were assessed 14 days post-transplantation, >90% of the donor CD4⁺ T cells were activated, as demonstrated by low expression levels of CD62L and the upregulated level of CD44 in the BS mice. These results suggested that CD4⁺ T cells were well activated on day 14. However, regarding the CD8⁺ T cells, the expression of CD44 in the BS mice was lower than that in the normal and BMT mice 14 days post-transplantation, which was consistent with the previous results that the percentages of Tc1 and Tc17 cells reduced between days 7 and 14. It is known that effector T cells, defined by their secretion of IFN- γ , are short lived (21). Recent advances have indicated the presence of a subset of postmitotic, self-renewing CD44^{lo}/CD62L^{hi}/CD8⁺ T cells, which can generate and sustain all allogeneic T-cell subsets in GVHD reactions, including central memory, effector memory and effector CD8⁺ T cells (22). Thus, the present study hypothesized that a large quantity of alloreactive CD8⁺ T cells secreting high levels of IFN- γ may be exhausted and a small number persist, becoming central memory or effector memory cells. Alternatively, it is possible that, following activation in secondary lymphoid organs, alloreactive CD8⁺ T cells migrate into target tissues, thereby amplifying the GVHD response locally.

IL-17-secreting CD8⁺ T cells, termed Tc17 cells have been recently identified as a novel subset of CD8⁺ T cells, and have been observed to promote inflammation and mediate immunity to influenza (13). In addition, Tc17 cells can also be found in mice deficient in T-bet alone, where they appear to be involved in allograft rejection (23). However, whether IL-17-secreting CD8⁺ T cells can be induced in GVHD remains to be fully elucidated. In an effort to enhance current understanding of GVHD pathophysiology, the present study performed investigations and demonstrated for the first time, to the best of our knowledge, that a large percentage of Tc17 cells were generated in BS mice, which have received allogeneic transplantation. The results demonstrated that Tc17 cells had a functional role in the initiation of GVHD. It has been reported that recipients of Tc17 cells exhibit infiltration, hemorrhage, widened alveolar septae and peribronchiolar thickening, and blinded-scoring of these sections confirmed that inflammation was significantly increased in the mice receiving the IL-17 secreting Tc17 cells (24). Previous studies have suggested that IL-17 is critical for GVHD lung pathology (5,25). Thus, the present study hypothesized that, during the process of GVHD, lung lesions may be the consequence of not only Th17 cells, but also Tc17 cells.

The pathophysiological process of GVHD is well known to cause lesions in host organs to activated donor T cells, which is caused by an imbalance in cytokine profile following allogeneic transplantation (26). To compare the profile of cytokines in GVHD, the present study quantified the expression of cytokines using CBA, to confirm the expression of each cytokine in the plasma. The results demonstrated that IL-2 and IFN- γ were enriched in the early stage following transplantation, while low levels of IL-4 and IL-10 were detected. It was suggested that IL-2 and IFN- γ have a prominent role in initiating GVHD, however, a shift from Th1 to Th2 cytokines indicated the development of tolerance. Previously, it was reported that the effect of IFN- γ on acute GVHD may depend on the timing of its production, as IFN- γ can have immunosuppressive effects when it is present immediately following HSCT, but can exacerbate disease via its pro-inflammatory properties at later stages (15). Lai *et al* reported that IFN- γ -secreting T cells were enriched in all murine GVHD target tissue lesions, and Tc1 and Tc2 cells were predominant in human GVHD colon and liver sections, respectively. However, the IFN- γ ⁺ Th1, IL-17⁺ Th17, IFN- γ ⁺ Tc1 and IL-17⁺ Tc17 cells were more frequent in human skin lesions, compared with the IL-4⁺ Th2 and IL-4⁺ Tc2 cells (26). Taken together, the results of the present study indicated that the regulation of GVHD pathogenesis involves complex cytokine interactions and variations in cytokine functionality. Cytokine imbalance may, therefore, be a critical factor in the pathophysiology of GVHD. To elucidate the various roles of the complex cytokines and their possible interaction in the GVHD process, further investigations are required.

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