a case of hepatic GVHD with positive autoimmune serology that led to the definition of cytochrome P450 1A2 as a target Ag. This test may aid differential diagnosis and clinical management.

The standard therapy for AIH is corticosteroids, which have proved to be efficacious in 80% of the patients. However, autoimmune-like hepatitis following allo-SCT is sometimes resistant to steroid therapy. One case received liver transplantation[2] and the other case died during evaluation for liver transplantation.[5] Rituximab, a human–mouse chimeric MoAb against CD20 B-lymphocytes, has been reported to be effective in several cases of AIH.[6,7] Based on these reports, we treated this patient with four courses of rituximab. Sequential immunohistochemical studies proved that CD20+ B-cell and CD138+ plasmacytes had completely disappeared after rituximab administration, consistent with the observed improvement in clinical manifestations. Rituximab therapy could be a promising candidate for treatments of autoimmune-like hepatitis after allo-SCT.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929–938.

2 Habib S, Nalesnik MA, Ahmad J, BuchBarker D, Shakil AO. Alloimmune hepatitis following peripheral stem cell transplantation. *Bone Marrow Transplant* 2007; **39**: 573–575.

3 Ogose T, Watanabe T, Suzuya H, Kaneko M, Onishi T, Watanabe H *et al.* Autoimmune hepatitis following allogeneic PBSCT from an HLA-matched sibling. *Bone Marrow Transplant* 2003; **31**: 829–832.

4 Kamisako T, Tsubaki K, Adachi Y. Autoimmune hepatitis after cytomegalovirus infection in a bone marrow-transplanted patient. *Am J Gastroenterol* 1997; **92**: 1238–1239.

5 Granito A, Stanzani M, Muratori L, Bogdanos DP, Muratori P, Pappas G *et al.* LKM1-positive type 2 autoimmune hepatitis following allogenic hematopoietic stem-cell transplantation. *Am J Gastroenterol* 2008; **103**: 1313–1314.

6 Santos ES, Arosemena LR, Raez LE, O'Brien C, Regev A. Successful treatment of autoimmune hepatitis and idiopathic thrombocytopenic purpura with the monoclonal antibody, rituximab: case report and review of literature. *Liver Int* 2006; **26**: 625–629.

7 Evans JT, Shepard MM, Oates JC, Self SE, Reuben A. Rituximab-responsive cryoglobulinemic glomerulonephritis in a patient with autoimmune hepatitis. *J Clin Gastroenterol* 2008; **42**: 862–863.

8 Mori M, Tabata S, Hashimoto H, Inoue D, Kimura T, Shimoji S *et al.* Successful living donor liver transplantation for severe hepatic GVHD histologically resembling autoimmune hepatitis after bone marrow transplantation from the same sibling donor. *Transpl Int* 2010; **23**: e1–e4.

9 Mullighan CG, Bogdanos D-P, Vergani D, Bardy PG. Cytochrome P450 1A2 is a target antigen in hepatitic graft-verusus-host disease. *Bone Marrow Transplant* 2006; **38**: 703–705.

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ORIGINAL ARTICLE

Efficacy and safety of human adipose tissue-derived mesenchymal stem cells for supporting hematopoiesis

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Abstract We have demonstrated that adipose tissue-derived mesenchymal stem cells (ADSCs) from mice are capable of reconstituting the hematopoietic microenvironment, and facilitate hematopoiesis more effectively than bone marrow-derived mesenchymal stem cells (BMSCs) in mouse. The ready accessibility of fat tissue rich in MSCs and the higher hematopoiesis-supporting capacities of ADSCs suggest that ADSCs might represent a new therapeutic modality for the regeneration of impaired hematopoiesis. As a further step towards their use in clinical practice, we established human BMSCs and ADSCs from healthy volunteers of similar age, and compared their proliferation capacities, hematopoiesis-supporting properties, and safety. In vitro cell proliferation studies revealed that ADSCs have a higher population doubling number than BMSCs. In vitro co-culture assays showed that ADSCs not only support human $CD34^+$ peripheral blood stem cells (PBSCs), but also yield significantly more non-adherent hematic cells than BMSCs. In vitro progenitor assays revealed that ADSCs promote a higher frequency of early progenitors than do BMSCs. Interestingly, BM cellularity in irradiated mice that had received ADSCs tended to be higher than that of mice treated with BMSCs. When MSCs were injected into the BM cavity of tibiae, we observed no evidence of MSC-induced toxicity either during or after treatment. In addition, no microscopic abnormalities were observed in the bone marrow and major organs.

Keywords Human adipose tissue-derived mesenchymal stem cells · Hematopoiesis-supporting properties · Safety · Cell therapy

Introduction

Hematopoiesis is a dynamic process that involves self renewal of hematopoietic stem cells in the bone marrow, generation of lineage-committed cells, and mobilization of mature cells into the bloodstream. Mesenchymal stem cells (MSCs) present in bone marrow (BM) are thought to give rise to cells that constitute the hematopoietic microenvironment. MSCs produce a number of cytokines and extracellular matrix proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis [1].

The hematopoietic microenvironment can be damaged by various pathophysiological mechanisms such as chemotherapy, irradiation, aging and malignant disease [2, 3]. Not only intensive chemotherapy but also chemotherapeutic drugs alone disrupt the hematopoietic microenvironment [4, 5]. In the elderly, bone marrow structures tend to be replaced with adipocytes that can be negative regulators of the hematopoietic microenvironment [6].

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Long-lasting damage to the hematopoietic microenvironment impairs hematopoiesis, causing infection, bleeding, anemia and subsequent mortality.

The emerging field of regenerative medicine seeks to repair or restore lost or damaged tissue function due to the effects of injury, disease, and aging. Compelling studies showed that BM-derived mesenchymal stem cells (BMSCs), when directly injected into the BM, could reconstitute the hematopoietic microenvironment [7, 8]. These facts clearly suggest that BMSCs can be a new modality for regeneration of the hematopoietic microenvironment. However, there are several drawbacks in the use of BMSCs for clinical application. Even though it is desirable to establish MSCs from the patient to whom they will be administered due to the possibility that an immune response and subsequent adverse effects could be provoked by administration of allogeneic MSCs [9], BMSCs are only available in a limited number. To make matters worse, the number, differentiation potential, and maximal life span of BMSCs decline with increasing age [10, 11].

The discoveries that a large number of nonadipocyte stem cells exist in fat tissue (adipose tissue-derived MSCs; ADSCs) and that these cells can be rapidly expanded ex vivo suggested that ADSCs might be useful for clinical applications [12]. We recently reported that ADSCs are a better alternative to BMSCs for reconstitution of the hematopoietic microenvironment in a mouse model [13]. Since then, we have maintained an ongoing commitment to explore the potential of ADSCs for clinical applications. In this study, we provide evidence that human ADSCs support hematopoiesis better than human BMSCs.

Materials and methods

Animal studies

The animal experiments were approved by the institutional ethics committee for Laboratory Animal Research, Nagoya University School of Medicine, and were performed according to the guidelines of the institute.

Reagents and cells

RPMI 1640, heat-inactivated fetal bovine and horse serum, and α-minimal essential medium were purchased from Gibco-BRL (Carlsbad, CA, USA). Human BMSCs and ADSCs were established from healthy volunteers of similar age (20–30 years). Briefly, bone marrow cells and fat tissues were obtained with informed consent from four and five individuals, respectively, and were then processed as described elsewhere [14]. Before experimental use, we confirmed that the MSCs possessed the ability to differentiate into adipocytes and osteoblasts. Cultures between passages 4–8 were used. $CD34^+$ hematopoietic stem cells were mobilized by G-CSF into the periphery, collected and frozen at −130 °C until use.

In vitro cell proliferation studies

Human BMSCs and ADSCs were plated (5×10^3 cells/well, three independent determinations per MSC) onto 24-well plates. After 72-h incubation, the cells were trypsinized and viable cells were counted using trypan blue exclusion.

Co-culture of $CD34^+$ progenitor cells with BMSCs or ADSCs

1×10^5 human $CD34^+$ PBSCs suspended in long-term culture medium were applied (1×10^5 cells in 2 ml) to feeder layers comprising human BMSCs or human ADSCs, as described previously [13]. The co-cultures were incubated for 4 weeks with replenishment of the culture medium twice per week. Non-adherent viable cells were counted at the indicated time points and were analyzed by FACS at the end of incubation as described elsewhere [13]. Co-culture experiments were repeated three times.

In vitro progenitor assays

Effects of human MSCs on progenitor cells were analyzed using a colony-forming cell assay. The following human cells (5×10^2 each) were plated in 0.5 mL of methylcellulose media (Stemcell Technologies, Vancouver, Canada): BMSCs, ADSCs, $CD34^+$ PBSCs, PBSCs plus BMSCs and PBSCs plus ADSCs. Colonies of >50 cells were scored after 8-days of incubation. Experiments were repeated three times.

Intra-bone marrow transplantation

Human BMSCs, human ADSCs (1×10^5 cells each in 10 μl of RPMI 1640), or 10 μl of RPMI 1640 were injected into the right tibiae of irradiated (3.0 Gy) 6- to 8-week-old NOD/SCID mice (4–5 mice per subgroup, Chubu Kagaku Shizai, Nagoya, Japan) using a Hamilton syringe. All mice were killed 5 weeks after injection, and the tibiae and major organs were excised for histological evaluation. For quantitative analysis of BM cellularity, 4 fields were randomly selected and the number of nucleated cells was scored under a microscope.

Statistical analysis

Statistical significance of group differences was evaluated using Student's t test and Excel software (Microsoft, Redmond, WA, USA).

Results

Human ADSCs can be expanded faster than human BMSCs

An in vitro proliferation assay showed that the number of BMSCs increased 1.16- to 2.32-fold above the input cell number (5000 cells/well) after 72-h incubation. In contrast, the fold increase in the number of ADSCs (3.5–4.0) was significantly higher (Fig. 1a).

Human ADSCs support human $CD34^+$ PBSCs to a greater degree than human BMSCs

To analyze the ability of human ADSCs to induce granulocyte differentiation of human $CD34^+$ PBSCs, co-culture assays were performed. Human ADSCs yielded significantly more non-adherent cells from human $CD34^+$ PBSCs than human BMSCs (Fig. 1b, upper panel). As noted previously [13], round-shaped hematic cells grew in clusters, suspended in the culture supernatant or loosely attached to supportive stroma (Fig. 1b, lower right panel). FACS analysis of non-adherent cells showed that these cells differentiated into $CD33^+$ granulocytes derived from human $CD34^+$ PBSCs (Fig. 1b, lower right panel). These results suggest that human ADSCs significantly enhance proliferation of myeloid cells from human $CD34^+$ PBSCs compared with human BMSCs.

In vitro progenitor assays revealed that human ADSCs promoted a higher frequency of early progenitors than human BMSCs (Fig. 1c), whereas neither human BMSCs nor human ADSCs alone generated colonies (not shown). The major lineages of the colonies were colony-forming unit (CFU) granulocytes and CFU granulocyte-macrophages. Few erythrocyte colonies were observed. However, there was no significant difference in the percentage of CFU granulocytes or CFU granulocyte-macrophages within $CD34^+$ PBSCs in the presence or absence of BMSCs or ADSCs (data not shown). Representative results from three independent experiments are shown.

Safety of human ADSCs in vivo

To ensure the safety of human ADSCs in vivo, we injected human BMSCs or ADSCs (1×10^5 cells each) into the right tibiae of irradiated 6–8 weeks old NOD/SCID mice (one mouse per each individual MSC). We observed no evidence of MSC-induced toxicity (e.g., body weight loss or death) either during or after treatment. Histological evaluation 5 weeks after injection showed neither gross morphological nor microscopic change of heart, lung, liver, kidney or spleen (Fig. 2a). Similarly, no microscopic abnormalities such as fatty change or fibrosis were observed in the bone marrow of mice that received MSCs (Fig. 2b). Interestingly, BM cellularity in mice that had received human ADSCs was significantly higher than that of mice that had received human BMSCs (Fig. 2b).

Discussion

We have recently demonstrated that ADSCs from mice can facilitate hematopoiesis more effectively than BMSCs [13]. These data suggested that ADSCs possess clinical potential to facilitate hematopoiesis. However, human cells and mouse cells do not always behave similarly. To determine the hematopoiesis-supporting properties of human ADSCs, we established BMSCs and ADSCs from a number of individuals of similar age, because the number, differentiation potential, and maximal life span of BMSCs decline with increasing age [11] while ADSCs are abundant even in the elderly [15]. Although we were not able to establish BMSCs and ADSCs from the same individual, in vitro coculture and progenitor assays clearly showed that human ADSCs generated significantly more granulocytes and progenitor cells from human hematopoietic stem cells (HSCs) than human BMSCs (Fig. 1b, c). Intra-bone marrow transplantation experiments revealed that BM cellularity in mice that had received human ADSCs was significantly higher than that of mice that had received human BMSCs (Fig. 2b). These data clearly suggest that human ADSCs are superior to human BMSCs in terms of hematopoiesis-supporting properties. Bone marrow failure is a heterogeneous disease that is caused by various pathophysiological mechanisms including immune destruction of hematopoiesis, quantitative and qualitative defects in hematopoietic stem cells in addition to perturbation of the hematopoietic microenvironment [2, 16]. ADSCs are useful, not only for supporting hematopoiesis, but also for modulation of immunoreactions [17, 18]. In addition, ADSCs freshly isolated from fat tissue (adipose-derived stem and regenerative cells) using the Celution® system (Cytori Therapeutics, Inc. http://www.cytori.com/Home.aspx) may contain hematopoietic stem and progenitor cells [19]. Again, our data and these facts suggest that fat tissue is a good source of cells that can be used for therapy to reconstitute impaired hematopoiesis.

Analysis of the proliferation capacities showed that ADSCs possessed higher population doubling numbers than BMSCs, which is consistent with a previous report [20]. A prospective randomized study using MSCs as first-line therapy for graft failure after hematopoietic stem cell transplantation (HSCT) showed that two out of six patients with poor hematopoietic recovery after HSCT responded to the infusion of BMSCs (1×10^6/kg) and their blood cell counts increased [21]. The authors speculated that

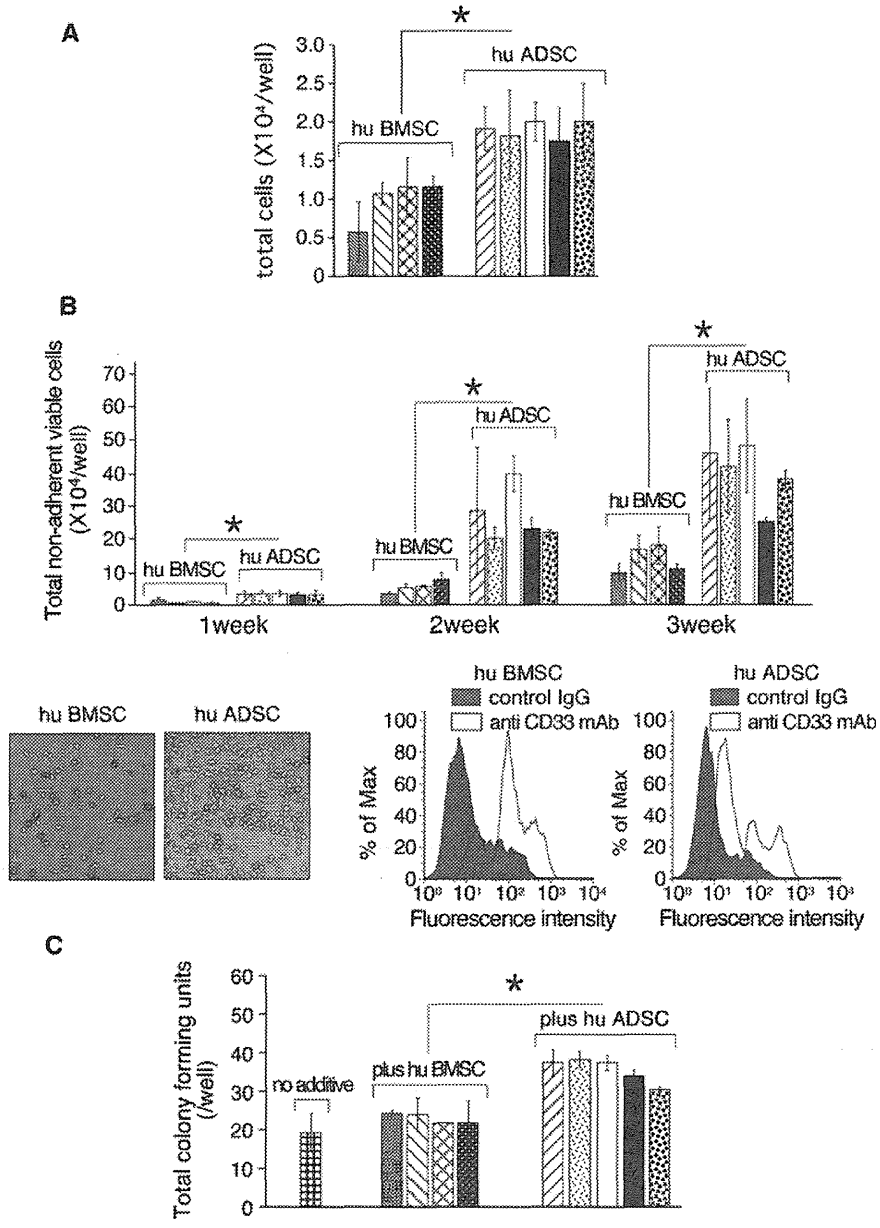

Fig. 1 In vitro comparison of human BMSCs and ADSCs. **a** In vitro proliferation of human BMSCs and ADSCs. Human BMSCs and human ADSCs were plated (5×10^3 cells/well, three independent determinations per MSC) onto 24-well plates. After 72-h incubation the cells were trypsinized and viable cells were counted using trypan blue exclusion. *Each bar* represents cells from one individual. The results are presented as the mean ± SD. The *asterisk* denotes statistical significance (*$P < 0.05$). **b** Co-culture of $CD34^+$ PBMCs with human MSCs. BMSCs and ADSCs were established from four and five individuals, respectively. Cell layers of BMSCs and ADSCs were established on 0.5 % gelatin pre-coated 24-well plates (80 % confluent). $CD34^+$ PBSCs were applied onto the stromal layers (4 wells/each MSC). The cocultures were incubated for 3 weeks with replenishment of the culture medium twice a week. Non-adherent viable cells were counted at the indicated time points (*upper panel*), photographed (*lower left panel*, representative photographs), and analyzed by FACS at the end of the incubation (*lower right panel*, representative results). Each point represents the mean (±SD) of four replicates. The *asterisk* denotes statistical significance (*$P < 0.05$). **c** In vitro progenitor assays using human MSCs. Human $CD34^+$ peripheral blood stem cells (PBSCs: 500 cells, no additive), PBSCs plus BMSCs (500 cells each) or PBSCs plus ADSCs (500 cells each) were plated in 0.5 mL of methylcellulose media containing human recombinant IL-3, SCF, and Epo (3 wells/each MSC). The plates were incubated for 8 days following which progenitors were scored. The results represent the mean (±SD) of three replicates. The *asterisk* denotes statistical significance (*$P < 0.05$)

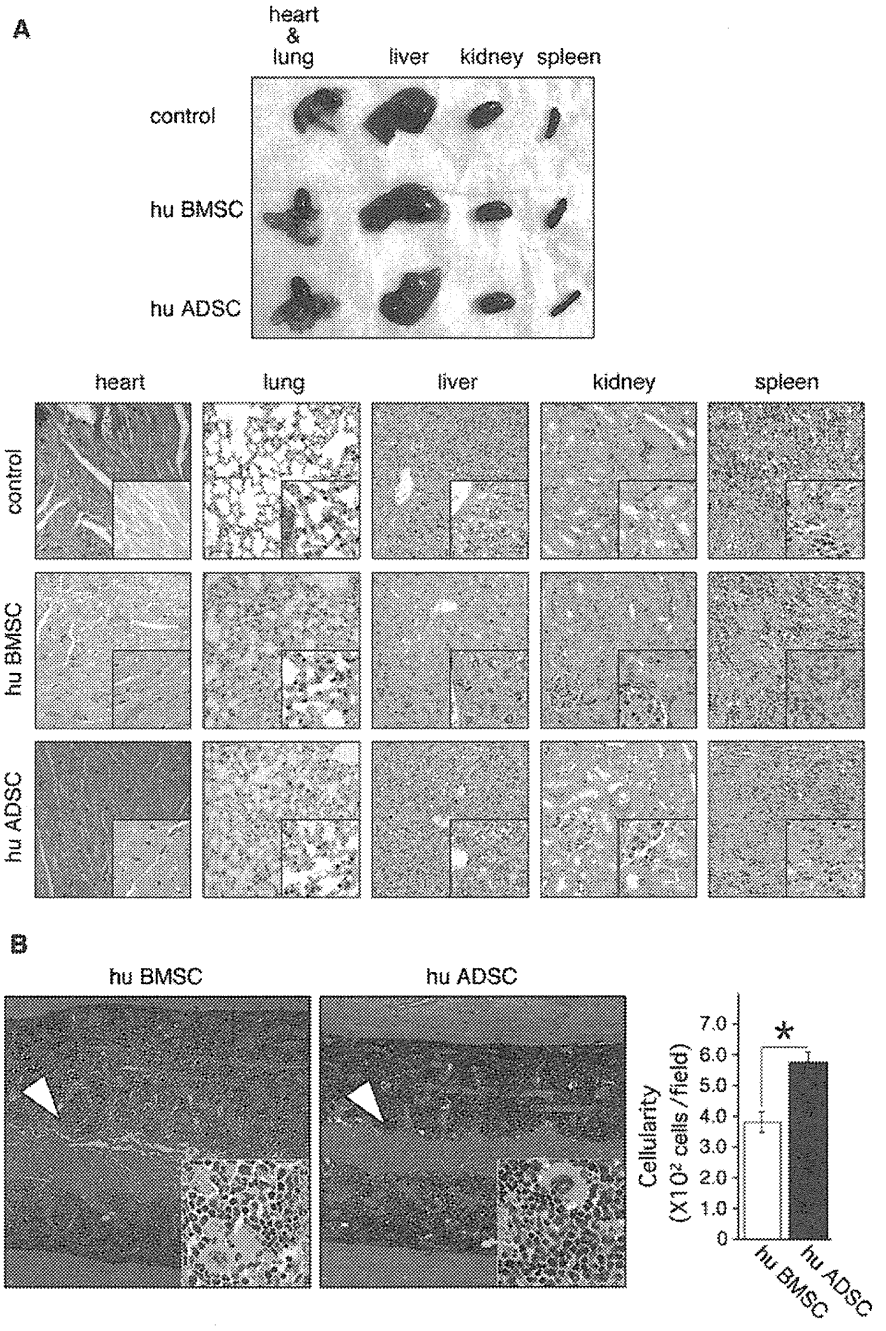

Fig. 2 Intra-bone Marrow Transplantation of human MSCs. Human (*hu*) BMSCs (1×10^5 cells), hu ADSCs (1×10^5 cells) or RPMI 1640 (control) were injected into the right tibiae of irradiated (3.0 Gy) 6- to 8-week-old NOD/SCID mice (one mouse per each MSC). All mice were humanely killed 5 weeks after injection, and the tibiae and major organs were excised for histological evaluation. **a** Representative gross morphology (*upper panel*) and microscopic histology (*lower panel* ×20; *inset* ×40) of heart, lung, liver, kidney, and spleen from control, BMSC- and ADSC-treated mice. **b** Representative microscopic histology of the BM stained with H&E stain (×10). *Inset* BM cellularity in mice that had received human BMSCs or ADSCs (×60). The cavities into which the 31-gauge needle had been inserted were filled with red blood cells (*arrowheads*). For quantitative analysis of BM cellularity, 4 fields were randomly selected and the number of nucleated cells was scored under a microscope. The mean nucleated cells per field ± SD is shown for each group. Statistical significance: $^*P < 0.05$

administration of 1×10^6 cells/kg body weight might be insufficient to restore a functional hematopoietic microenvironment. According to our recent findings, 1×10^5 ADSCs per mouse (about 25 g in weight) were required to facilitate hematopoiesis [13], which would be equivalent to 4×10^6 ADSCs per kilogram for adult humans. These data suggest that large quantities of infused cells are required for treatment. Thus, the rapid expansion capacity of ADSCs will also be advantageous for their clinical exploitation.

We observed no evidence of human ADSC-induced toxicity either during or after treatment. Histological evaluation 5 weeks after injection showed neither gross morphological nor microscopic changes in major organs of mice that received human ADSCs, which are sometimes a clue to the presence of latent adverse effects [22] (Fig. 2a). It has been reported that MSCs that persist in vivo may be tumorigenic [23]. However, we found that human ADSCs alone formed no colonies in semi-solid cultures (not

shown) and no tumors in vivo (Fig. 2). These data indicate the clinical safety of human ADSCs.

In summary, these data provide an important step in the regeneration of a perturbed hematopoietic microenvironment by ADSCs in a clinical setting.

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References

1. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000;100(1):157–68.
2. Scopes J, Bagnara M, Gordon-Smith EC, Ball SE, Gibson FM. Haemopoietic progenitor cells are reduced in aplastic anaemia. Br J Haematol. 1994;86(2):427–30.
3. Nakayama T, Mutsuga N, Tosato G. Effect of fibroblast growth factor 2 on stromal cell-derived factor 1 production by bone marrow stromal cells and hematopoiesis. J Natl Cancer Inst. 2007;99(3):223–35.
4. Carlo-Stella C, Tabilio A, Regazzi E, Garau D, La Tagliata R, Trasarti S, Andrizzi C, Vignetti M, Meloni G. Effect of chemotherapy for acute myelogenous leukemia on hematopoietic and fibroblast marrow progenitors. Bone Marrow Transplant. 1997;20(6):465–71.
5. Kemp K, Morse R, Wexler S, Cox C, Mallam E, Hows J, Donaldson C. Chemotherapy-induced mesenchymal stem cell damage in patients with hematological malignancy. Ann Hematol. 2010;89(7):701–13.
6. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature. 2009;460(7252):259–63.
7. Muguruma Y, Yahata T, Miyatake H, Sato T, Uno T, Itoh J, Kato S, Ito M, Hotta T, Ando K. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood. 2006;107(5):1878–87.
8. in 't Anker PS, Noort WA, Kruisselbrink AB, Scherjon SA, Beekhuizen W, Willemze R, Kanhai HH, Fibbe WE. Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol. 2003;31(10):881–9.
9. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood. 2006;108(6):2114–20.
10. Nishida S, Endo N, Yamagiwa H, Tanizawa T, Takahashi HE. Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. J Bone Miner Metab. 1999;17(3):171–7.
11. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cell Biochem. 2001;82(4):583–90.
12. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7(2):211–28.
13. Nakao N, Nakayama T, Yahata T, Muguruma Y, Saito S, Miyata Y, Yamamoto K, Naoe T. Adipose tissue-derived mesenchymal stem cells facilitate hematopoiesis in vitro and in vivo. Advantages over bone marrow-derived mesenchymal stem cells. Am J Pathol. 2010;177(2):547–54.
14. Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, Kikuchi Y, Saito Y, Tamai K, Ogihara T, Kaneda Y. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol. 2005;25(12):2542–7.
15. Rigotti G, Marchi A, Sbarbati A. Adipose-derived mesenchymal stem cells: past, present, and future. Aesthetic Plast Surg. 2009;33(3):271–3.
16. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. Blood. 2006;108(8):2509–19.
17. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol. 2005;129(1):118–29.
18. Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA. Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells. 2006;24(11):2582–91.
19. Han J, Koh YJ, Moon HR, Ryoo HG, Cho CH, Kim I, Koh GY. Adipose tissue is an extramedullary reservoir for functional hematopoietic stem and progenitor cells. Blood. 2010;115(5):957–64.
20. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24(5):1294–301.
21. Meuleman N, Tondreau T, Ahmad I, Kwan J, Crokaert F, Delforge A, Dorval C, Martiat P, Lewalle P, Lagneaux L, Bron D. Infusion of mesenchymal stromal cells can aid hematopoietic recovery following allogeneic hematopoietic stem cell myeloablative transplant: a pilot study. Stem Cells Dev. 2009;18(9):1247–52.
22. Satoh H, Uesugi Y, Kawabata T, Mori K, Fujii F, Kashimoto Y, Kajimura T, Furuhama K. Morphological classification of dental lesions induced by various antitumor drugs in mice. Toxicol Pathol. 2001;29(3):292–9.
23. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. Cancer Res. 2005;65(8):3035–9.

Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17

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Chronic GVHD (cGVHD) is a main cause of late death and morbidity after allogeneic hematopoietic cell transplantation, but its pathogenesis remains unclear. We investigated the roles of Th subsets in cGVHD with the use of a well-defined mouse model of cGVHD. In this model, development of cGVHD was associated with up-regulated Th1, Th2, and Th17 responses. Th1 and Th2 responses were up-regulated early after BM transplantation, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were infiltrated in the lung and liver from allogeneic recipients than those from syngeneic recipients. We then evaluated the roles of Th1 and Th17 in cGVHD with the use of IFN-γ–deficient and IL-17–deficient mice as donors. Infusion of IFN-$\gamma^{-/-}$ or IL-$17^{-/-}$ T cells attenuated cGVHD in the skin and salivary glands. Am80, a potent synthetic retinoid, regulated both Th1 and Th17 responses as well as TGF-β expression in the skin, resulting in an attenuation of cutaneous cGVHD. These results suggest that Th1 and Th17 contribute to the development of cGVHD and that targeting Th1 and Th17 may therefore represent a promising therapeutic strategy for preventing and treating cGVHD. (*Blood.* 2012; 119(1):285-295)

Introduction

GVHD is a result of immune attack of host tissues, such as the skin, gut, liver, and lung, by donor T cells in transplants.[1,2] On the basis of the differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic types. Chronic GVHD (cGVHD) is the main cause of late death and morbidity after allogeneic hematopoietic stem cell transplantation.[3-5] cGVHD often presents with clinical manifestations that resemble those observed in autoimmune diseases, such as systemic lupus erythematosus, Sjögren syndrome, lichen planus, and scleroderma. It has traditionally been assumed that the predominant cytokines produced during acute GVHD are Th1 cytokines, whereas those produced during cGVHD are Th2 cytokines. Although recent studies have suggested that cGVHD could be caused by cytokines secreted by Th1 cells,[6] Th17 cells,[7] or autoantibodies,[8] or both, the immune mechanisms leading to the development of cGVHD are not completely understood.

Th17 cells are a third subset of polarized effector T cells characterized by their expression of proinflammatory cytokine IL-17 and other cytokines.[9] IL-17 belongs to a family of 6 members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F. Of these, IL-17A and IL-17F are the best characterized cytokines and form heterodimers. IL-17 plays an important role in the control and clearance of various pathogens.[9] In addition, Th17 cells have been implicated in allograft rejection of solid organs and several autoimmune diseases.[10,11] Although a number of studies have addressed how Th17 cells contribute to GVHD[12] and have reported that Th17 cells are sufficient but not necessary to induce acute GVHD,[13,14] the functional role of Th17 in cGVHD is unclear.

Retinoic acid, the active metabolite of vitamin A, has multiple effects on cell differentiation and survival by ligating the receptors from 2 families, retinoic acid receptors (RARs) and retinoid X receptors, each of which exists in multiple isoforms.[15] All-*trans*-retinoic acid (ATRA) has been reported to inhibit IFN-γ synthesis by Thl cells and to suppress the differentiation of Th17 cells by down-regulating the orphan nuclear receptor RORγt, a key regulator of Th17 differentiation.[16-19] Am80 is a novel RARα/β-specific synthetic retinoid that shows ~ 10-fold more potent biologic activity than ATRA by binding to RARα and RARβ but not to RARγ.[20] Am80 also inhibits IL-6 signaling[20,21] and reduces the severity and progression of inflammatory disease models.[20-23]

In the present study, we used the B10.D2 (H-2^d) into BALB/c (H-2^d) MHC-compatible, multiple minor histocompatibility Ag (miHA)–incompatible model of cGVHD to address the contribution of Th1/Th17 cells and the effects of retinoids on cGVHD with the use of IFN-$\gamma^{-/-}$ mice and IL-$17^{-/-}$ mice as donors. We also tested the hypothesis that the administration of Am80 ameliorates cGVHD by reducing the levels of Th1 and Th17 inflammatory cytokines and the fibrosis factor TGF-β.

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Methods

Mice

Female B10.D2 (H-2^d) mice were purchased from Japan SLC. BALB/c (H-2^d) recipient mice were purchased from Charles River Japan. IL-17A–deficient (IL-17$^{-/-}$) mice with the BALB/c background were generated previously.[24] IFN-γ–deficient (IFN-γ$^{-/-}$) mice were purchased from The Jackson Laboratory. IL-17$^{-/-}$ and IFN-γ$^{-/-}$ mice with the B10.D2 background were backcrossed for 8-10 generations from the original knockout mice. All experiments involving animals were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BM transplantation

Mice received transplants according to the standard protocols described previously.[25] Briefly, BALB/c mice received a single dose of 6.75 Gy x-ray total body irradiation. Recipient mice were injected with 2×10^6 spleen T cells and 8×10^6 T cell–depleted BM (TCD-BM) cells from B10.D2 donors. T-cell depletion and purification were performed with anti-CD90.2 Microbeads, pan T-cell isolation kit, and CD25 isolation kit and an AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Evaluation of cGVHD

After BM transplantation (BMT), animals were weighed every 3 days and scored for skin manifestations of GVHD. The following scoring system was used[25]: healthy appearance, 0; skin lesions with alopecia < 1 cm^2 in area, 1; skin lesions with alopecia 1-2 cm^2 in area, 2; skin lesions with alopecia > 2 cm^2 in area, 3. In addition, animals were assigned 0.3 points each for skin disease (lesions or scaling) on the ears, tails, and paws. The minimum score was 0, and the maximum score was 3.9.

Tissue histopathology

Shaved skin from the interscapular region (~ 2 cm^2), the left lung, liver, and colon specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with H&E. Slides were scored by a pathologist blind to experimental group (K.T.) on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop-out (0-2 for each category; the maximum score was 10).[25] Lung, liver, and colon slides were scored by a pathologist blind to the experimental group (T.T.). Lung slides were scored according to periluminal infiltrates, pneumonitis, and the extent of injury (0-3 for each category), and the maximum score was 9.[26] Liver slides were scored according to bile duct injury and inflammation (0-4 for each category), and the maximum score was 8.[27] Colon slides were scored according to crypt apoptosis and inflammation (0-4 for each category), and the maximum score was 8.[27]

Intracellular cytokine staining and cytokine analysis

Organs from mice were removed, processed into single-cell suspensions, and stimulated in vitro with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 100 ng/mL ionomycin (Sigma-Aldrich) at 37°C for 3 hours. Cells were then incubated with GolgiStop (BD PharMingen) for an additional 2 hours. mAbs conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein complexes, allophycocyanin, or Alexa Fluor 488 were used to assess the cell populations and were purchased from BD PharMingen or eBioscience. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (both from Becton Dickinson) or MACS Quant flow cytometer (Miltenyi Biotec) with FlowJo software (TreeStar); both were housed in the Central Research Laboratory, Okayama University Medical School. Total peripheral lymph node (PLN) cells were adjusted to 1×10^6/mL in cultures. Supernatants were removed, and cytokine levels were measured with a BD Cytometric Bead Array (CBA) or by ELISA (R&D Systems) according to the respective manufacturer's protocol.

IFN-γ neutralization

Anti–mouse IFN-γ mAbs for in vivo experiments were prepared from mouse ascites from clones R4-6A2. Mice were treated intraperitoneally with anti–IFN-γ mAbs or rat IgG (160 μg/mouse; Sigma-Aldrich) on days 0, 5, 10, and 15 after BMT.

Administration of ATRA and Am80

Recipients were orally administered ATRA (200 μg/mouse; Wako), Am80 (1.0 mg/kg body weight; Nippon Shinyaku), or vehicle solutions daily from day 0.

Real-time RT-PCR

Total RNA was isolated from homogenized ear tissue with the use of an RNeasy mini kit (QIAGEN). cDNA synthesis was initiated by application of oligo dT primers and TaqMan Reverse Transcription Reagents (Applied Biosystems). Target cDNA levels were quantified by real-time PCR. The TaqMan Universal PCR Master Mix and the following Assay-on-Demand mouse gene-specific fluorescently labeled TaqMan MGB probes were used in an ABI Prism 5300 sequence detection system (Applied Biosystems): Mm01178820_m1 (TGF-β1). The mRNA expression of individual genes was normalized relative to GAPDH with the use of the equation $dCt = Ct_{target} - Ct_{GAPDH}$. The samples were obtained at room temperature using light microscopy (BX51; Olympus) with an objective lens (10×/0.40 NA, or 20×/0.70 NA; Olympus) and a camera (DP-70; Olympus). The images were acquired with image processing software (DP2-BSW Version 1.2; Olympus).

Statistical analyses

Group comparisons of skin cGVHD scores and pathology scores were performed using the Mann-Whitney U test or Kruskal-Wallis test. Cell populations, cytokine levels, mean weights, and gene expression data were analyzed with the unpaired 2-tailed Student t test. In all analyses, $P < .05$ was taken to indicate statistical significance.

Results

Th17 cells are increased in lymphoid organs during cGVHD development

We first assessed the kinetics of Th1/Th2/Th17 cytokine production of donor T cells generated during cGVHD. We used the most common cGVHD model: the MHC-compatible, multiple miHA-incompatible allogeneic BMT model (B10.D2 into BALB/c). Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 B10.D2 spleen T cells and 8×10^6 B10.D2 TCD-BM cells. Ly9.1 was used as a marker to distinguish donors from recipients; B10.D2 and BALB/c are negative and positive for Ly9.1, respectively. Flow cytometric analysis of the spleens and PLNs on days 14 and 28 indicated that donor chimerism as determined by the negativity for Ly9.1 was > 95%. The allogeneic recipients showed pathologic damage to the skin, salivary glands, lung, and liver, as reported previously (Figure 1A).[25,27] Cells isolated from PLNs were harvested on days 14 and 28 after BMT and analyzed for cytokine expression. In the early phase (day 14), IL-17$^+$ T cells were detected more frequently in the PLNs of recipients of syngeneic BMT, whereas in the late phase (day 28), IL-17$^+$ T cells in allogeneic recipients increased and were detected significantly more frequently than in syngeneic recipients (Figure 1B). We detected consistently higher percentages of donor T cells expressing IFN-γ and IL-13 in PLNs from allogeneic recipients than from syngeneic recipients (Figure 1B). Intracellular staining showed that most of the IL-17–producing cells were CD4$^+$ T cells (Figure 1C) and that IFN-γ/IL-17 double-positive cells (Th1/Th17

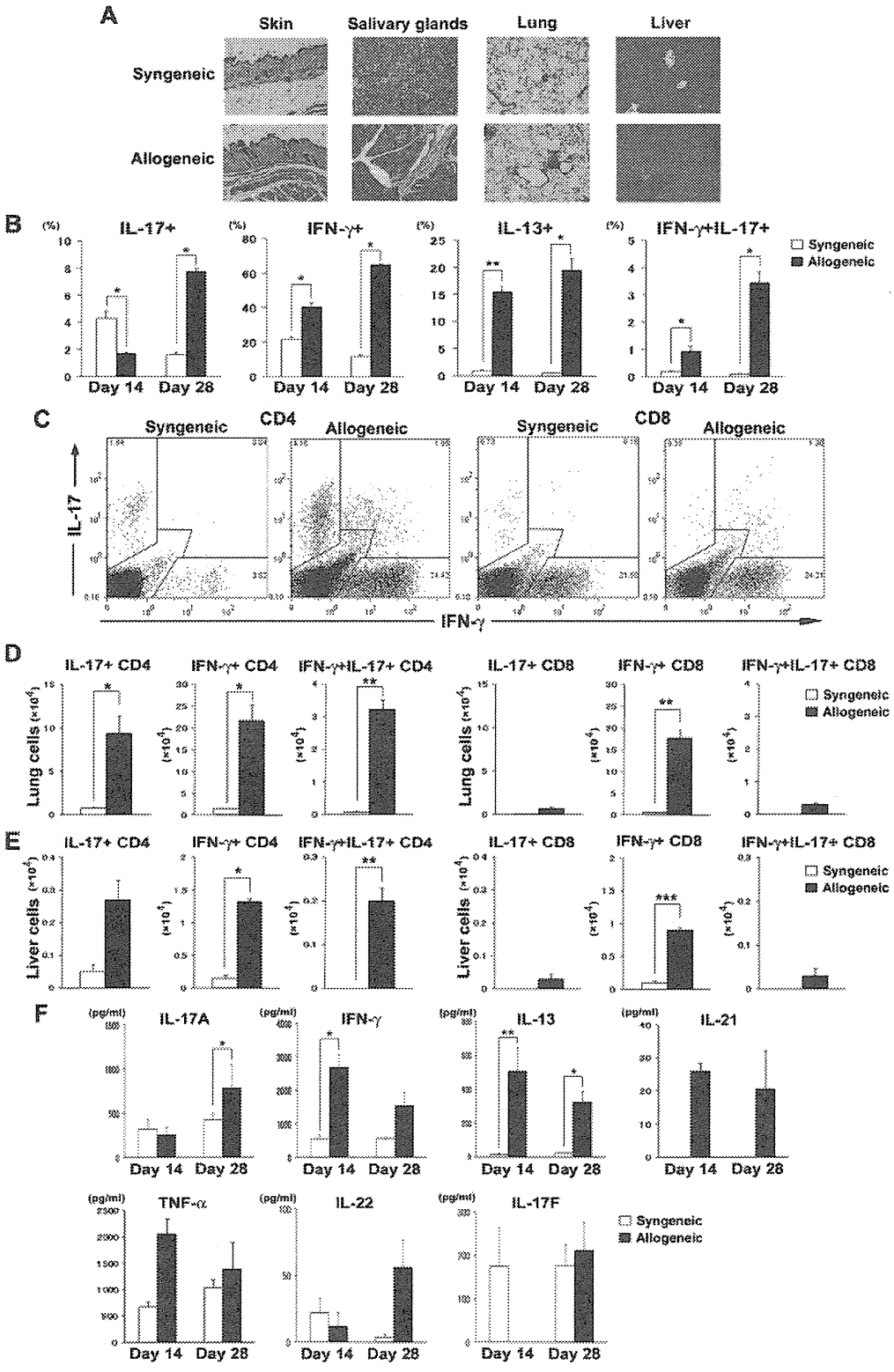

Figure 1. Th17 cells are increased in lymphoid organs during the late phase of cGVHD. Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells plus 8×10^6 TCD-BM from WT B10.D2 mice (allogeneic group; black bars). The syngeneic group (white bars) received a transplant of the same dose of splenocytes and TCD-BM from BALB/c mice. (A) Histopathology of skin, salivary glands, lung, and liver of syngeneic and allogeneic recipients 35 days after BMT. (B) The percentages of donor-derived $CD3^+$ T cells expressing IL-17, IFN-γ, IL-13, and IFN-γ/IL-17 on days 14 and 28 are shown. (C) Representative staining for intracellular IFN-γ and IL-17 on $CD4^+$ and $CD8^+$ T cells on day 28 for syngeneic and allogeneic mice. (D-E) Absolute numbers of IL-17–, IFN-γ–, and IFN-γ/IL-17–producing $CD4^+$ and $CD8^+$ T cells in recipient lung (D) and liver (E). (F) PLN cells from syngeneic and allogeneic recipients on days 14 and 28 were stimulated with PMA and ionomycin in vitro. Five hours later, the supernatants were collected to determine cytokine levels by ELISA or CBA. Graphs indicate the levels of cytokines secreted per 1×10^6 total stimulated PLN cells. Three to 6 mice per group were used. The means (± SE) of each group are shown. Data are from 1 representative of ≥ 2 independent experiments. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

cells) were exclusively detected in allogeneic recipients (Figure 1B-C). As allogeneic recipients developed GVHD-induced lymphopenia on day 28; absolute numbers of IFN-γ^+ T and IL-17^+ T cells in PLNs from allogeneic recipients were not greater than those from syngeneic recipients (IFN-γ^+ T, $51.8 \pm 17.5 \times 10^4$ vs $49.4 \pm 4.2 \times 10^4$, $P = .92$; IL-17^+ T, $5.9 \pm 2.2 \times 10^4$ vs $6.9 \pm 0.59 \times 10^4$, $P = .16$). Numbers of Th1 and Th17 cells from allogeneic recipients were significantly greater than those from syngeneic recipients in the lung (Figure 1D) and liver (Figure 1E). Cells isolated from PLNs of allogeneic recipients secreted significantly greater amounts of IL-17, IFN-γ, and IL-13 after stimulation with PMA and ionomycin (Figure 1F) or without stimulation (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). These cytokine levels were also elevated in serum from allogeneic recipients 28 days after BMT (supplemental Figure 2). To confirm that our observations were not strain dependent or model dependent, we performed similar experiments in the DBA/2 into BALB/c model of cGVHD. We confirmed the up-regulated Th1 and Th17 responses in this model (supplemental Figure 3).

IL-$17^{-/-}$ donor T cells ameliorate cGVHD

We next used IL-$17^{-/-}$ mice with the B10.D2 background as donors to evaluate whether Th17 contributes to cGVHD. On transfer of IL-$17^{-/-}$ B10.D2 donor T cells into allogeneic BMT models, weight loss was mild and fur loss was clearly ameliorated in comparison to that seen in recipients of wild-type (WT) T cells (Figure 2A-B). Clinical cGVHD severity was assessed with a standard scoring system (see "Methods"). Allogeneic IL-$17^{-/-}$ BMT recipients showed significantly less skin cGVHD than WT controls ($P < .05$; Figure 2C). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IL-$17^{-/-}$ donors (3.17 ± 1.09 vs 8.50 ± 0.84; $P < .01$; Figure 2D). A dry mouth is one of the distinctive features of cGVHD, and lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of WT BMT recipients. Histopathologic examination of the salivary glands showed reduced cGVHD pathology in the recipients of IL-$17^{-/-}$ donors (Figure 2E). Atrophy of the salivary glands as determined by their size was significantly reduced in recipients of IL-$17^{-/-}$ donors (4.21 ± 0.13 vs 3.54 ± 0.11; $P < .01$; Figure 2E). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IL-$17^{-/-}$ and WT donors (lung, 2.6 ± 1.04 vs 0.8 ± 0.44, $P = .19$; liver, 1.5 ± 0.87 vs 1.83 ± 0.37, $P = .75$; colon, 1.6 ± 0.36 vs 2.8 ± 0.33, $P = .06$). Thus, IL-$17^{-/-}$ BMT recipients showed less cGVHD in the skin and salivary glands than did the WT controls. Flow cytometric analysis of the PLNs in the early phase (day 14) showed no differences in frequency of IFN-g^+ cells between IL-$17^{-/-}$ and WT recipients, whereas recipients of IL-$17^{-/-}$ showed fewer IFN-γ^+ cells in the late phase (day 35, $4.3\% \pm 0.8\%$ vs $18.9\% \pm 3.5\%$; $P = .01$; Figure 2F). As allogeneic WT recipients developed more severe GVHD-induced lymphopenia on day 35 than IL-$17^{-/-}$ recipients, absolute numbers of IFN-g^+ cells in PLNs from allogeneic WT recipients were not greater than those from IL-$17^{-/-}$ recipients (IFN-γ^+ T cells, $6.08 \pm 0.87 \times 10^4$ vs $4.83 \pm 1.23 \times 10^4$; $P = .48$). As expected, IFN-γ/IL-17 double-positive cells were not detected in recipients of IL-$17^{-/-}$ donors on days 14 and 35 (Figure 2G-H). No differences were observed in the IL-13^+ cells or Foxp3^+ cells between the groups (data not shown). These data suggest that donor IL-17 contributes to the pathogenesis of cGVHD.

Donor Th1 differentiation is responsible for the development of cGVHD

To test whether donor Th1 differentiation is responsible for cGVHD, we used IFN-$\gamma^{-/-}$ mice with the B10.D2 background as donors. BMT from IFN-$\gamma^{-/-}$ donors compared with WT donors significantly improved the clinical cGVHD score ($P < .05$; Figure 3A). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IFN-$\gamma^{-/-}$ donors (4.75 ± 0.54 vs 7.80 ± 0.52; $P = .02$; Figure 3B). Salivary gland atrophy was also reduced in recipients of IFN-$\gamma^{-/-}$ donors (3.81 ± 0.05 vs 2.87 ± 0.19; $P < .05$; Figure 3C). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IFN-$\gamma^{-/-}$ and WT donors (lung, 2.4 ± 0.61 vs 3.2 ± 0.52, $P = .4$; Figure 3B; liver, 1.0 ± 0.4 vs 1.6 ± 0.32, $P = .21$; colon, 0.75 ± 0.21 vs 1.6 ± 0.67, $P = .36$). Intracellular staining of PLNs showed no differences in IL-13– or IL-17–producing cells between IFN-$\gamma^{-/-}$ and WT recipients (data not shown), although significantly greater numbers of Foxp3^+ cells were detected in the IFN-$\gamma^{-/-}$ recipients (day 35; $P < .05$; Figure 3D). To examine whether an increase in numbers of Treg cells was responsible for the reduced cGVHD in the absence of donor IFN-$\gamma^{-/-}$, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 3E, depletion of CD25^+ cells from the donor inoculum exacerbated skin scores ($P < .05$). However, CD25-depleted T cells from IFN-$\gamma^{-/-}$ mice caused less severe skin GVHD than those from WT mice ($P < .05$). These findings suggest that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Next, we evaluated the role of IFN-γ in the development of skin cGVHD by administering anti–IFN-γ mAbs to recipients of WT or IL-$17^{-/-}$ donors. Anti–IFN-γ mAb treatment significantly reduced skin scores and pathology scores in recipients of WT donors (Figure 3F-G). Recipients of IL-$17^{-/-}$ donors again showed reduced skin scores, and treatment with anti–IFN-γ mAbs further reduced skin scores (Figure 3H). These findings suggest that IFN-γ contributes to cGVHD pathogenesis.

Am80 inhibits donor Th1 and Th17 cells both in vitro and in vivo

ATRA has been reported to suppress the differentiation of Th17 cells with a reciprocal induction of Treg cells.[28] Am80, a novel RARα/β-specific synthetic retinoid, has a biologic activity ~ 10 times more potent than that of ATRA[20] and directly inhibits Th1 cytokine production.[20,22,29] Therefore, we hypothesized that ATRA or Am80 down-regulates both Th1 and Th17 differentiation in donor T cells, resulting in attenuation of cGVHD. To clarify whether retinoids directly inhibit the production of cytokines, PLNs were isolated from mice 14 days after allogeneic BMT and cultured with Am80 for 24 hours to determine cytokine production. Am80 inhibited IFN-γ (Figure 4A) and IL-17 (Figure 4B) production in a dose-dependent manner. Next, BMT recipients were orally administered Am80 at a dose of 1.0 mg/kg of body weight or vehicle daily from day 0 of BMT, and cytokine expression was assessed in PLNs harvested on day 35. We detected significantly fewer IFN-γ^+ T cells in Am80-administered recipients (Figure 4C). In addition, PLNs from Am80-treated recipients produced significantly less IFN-γ after stimulation with PMA and ionomycin ($P < .01$; Figure 4D). No difference was observed in the percentage of IL-17–producing donor cells, although PLN cells from Am80-treated recipients produced significantly less IL-17 ($P < .05$) and IL-21 ($P < .01$) after stimulation with PMA and ionomycin (Figure 4D). Taken together, these data suggest that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo.

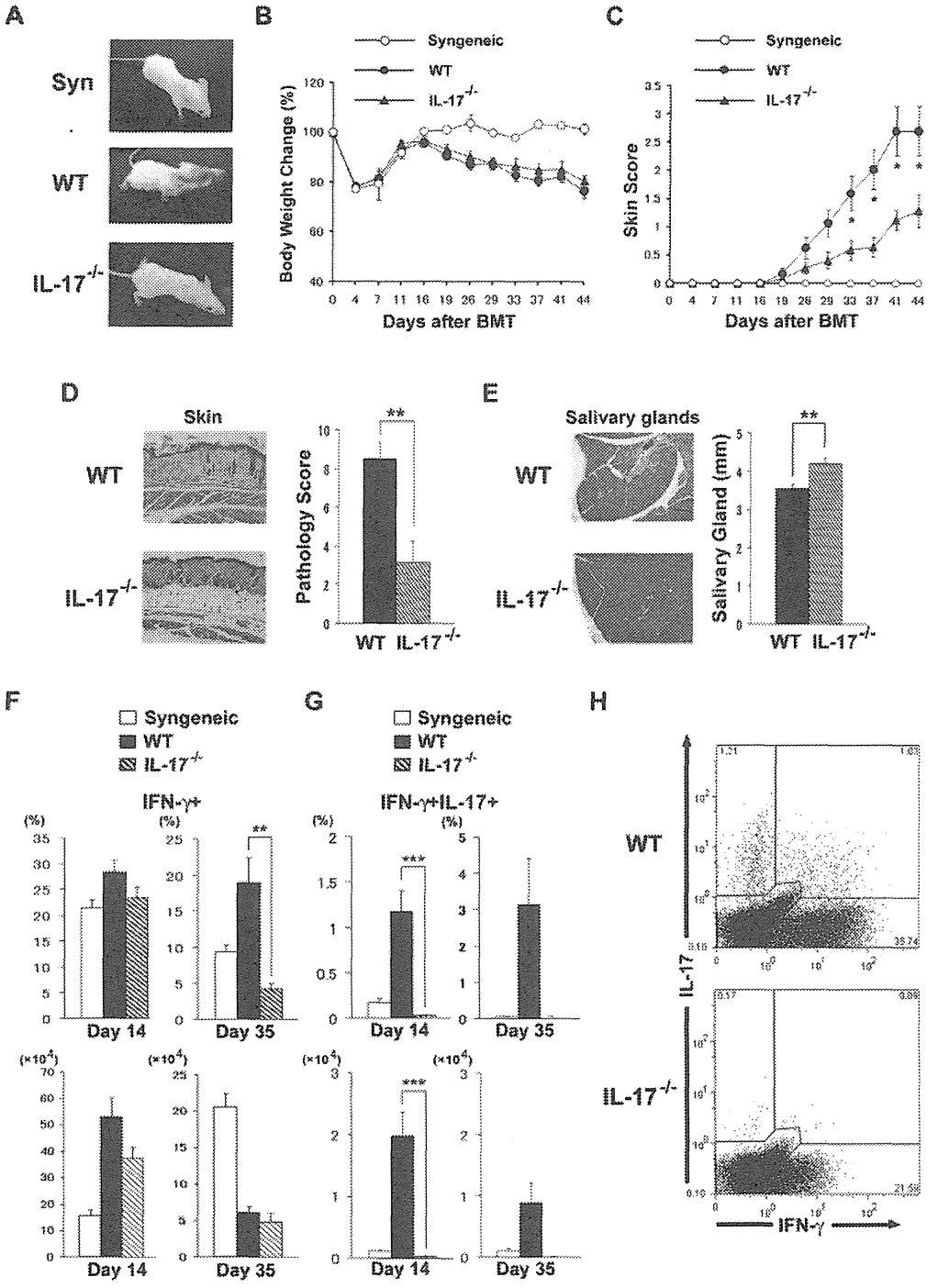

Figure 2. IL-17$^{-/-}$ donor T cells ameliorate cGVHD. Sublethally irradiated BALB/c recipients were transplanted from WT, IL-17$^{-/-}$ B10.D2, or syngeneic BALB/c donors. (A) Gross observation of the skin lesions from recipients of syngeneic, WT, and IL-17$^{-/-}$ donors 28 days after BMT. The recipients were analyzed for body weight (B) and cGVHD skin scores (C); data from 2 independent experiments were combined (n = 14 per group). Pathology score of skin (D) and the longest diameter of the salivary gland (E) on day 35 of BMT are shown. Four to 6 recipients were examined in each group. (F-G) PLN cells of the recipients of syngeneic (white bar), WT (black bar), or IL-17$^{-/-}$ (striped bar) donors were stained for intracellular IFN-γ and IL-17 on days 14 and 35 after BMT. The percentages and absolute numbers of IFN-γ$^+$ cells (F) and IFN-γ$^+$/IL-17$^+$ cells (G) are shown. Data from 2 replicated experiments were combined (n = 6-11 per group). (H) Representative staining for intracellular IFN-γ and IL-17 on CD4$^+$ T cells of WT or IL-17$^{-/-}$ mice on day 35 is shown. Data represent the means ± SEs. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$.

Administration of Am80 ameliorates cGVHD

Next, we examined whether ATRA or Am80 can down-regulate cGVHD. BALB/c recipients were orally administered ATRA (200 μg/mouse) or Am80 from day 0 of BMT. We found that ATRA tended to decrease the clinical cGVHD score (Figure 5A), whereas Am80 significantly ameliorated the clinical score compared with controls ($P = .01$; Figure 5B). Histopathologic examination of the skin on day 16 showed significantly reduced cGVHD damage in Am80-treated animals (day 16, 4.8 ± 0.4 vs 7.4 ± 0.4; $P < .01$; Figure 5C), No differences were observed in pathology scores of the lung, liver, or colon between the 2 groups (Figure 5C). Because it has been reported that Am80 can induce Treg cells,[29] we

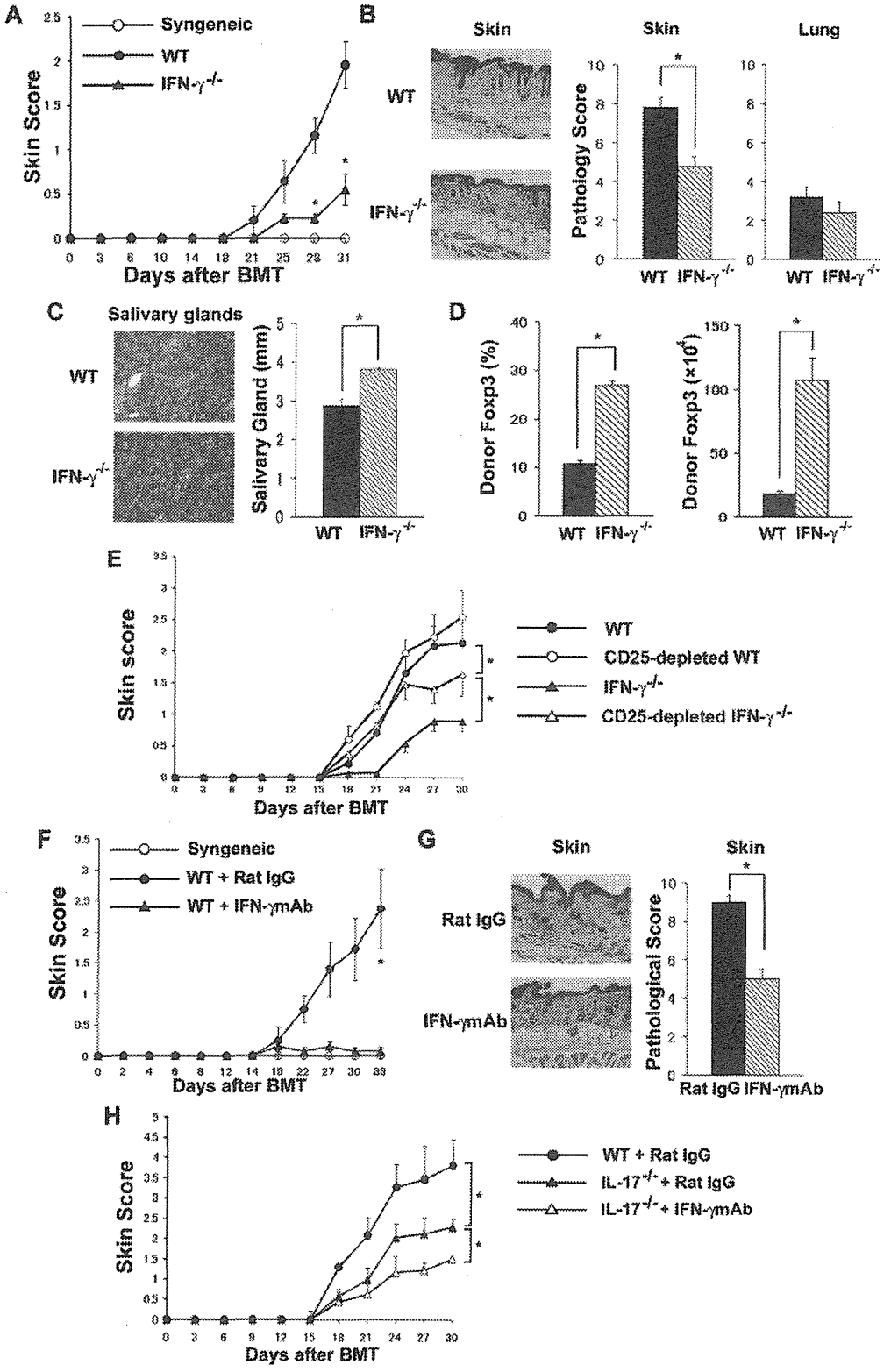

Figure 3. Donor Th1 differentiation and IFN-γ production are responsible for exacerbated cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. Clinical skin cGVHD scores (A), pathology score of skin and lung (B), and the longest diameter of the salivary gland (C) on day 35 after BMT are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 3 independent experiments. (D) PLN cells of the recipients on day 35 were stained for intracellular Foxp3. The percentages and the absolute number of $CD4^+Foxp3^+$ Treg cells are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. The skin cGVHD scores are shown ($n = 6$ per group). Data are from 1 representative of ≥ 2 independent experiments. (F-H) Sublethally irradiated BALB/c recipients were transplanted from WT or IL-$17^{-/-}$ B10.D2 donors. The recipients were injected with anti–IFN-γ mAbs or rat IgG (160 μg/mouse) on days 0, 5, 10, and 15 after BMT and were assessed for the clinical signs of cGVHD every 3 days. The clinical skin cGVHD scores (F), histopathology, and pathology score of the skin (G) on day 35 of BMT from WT donors. Four mice per group were used. Data are from 1 representative of ≥ 2 repeated experiments. (H) The clinical skin cGVHD scores after BMT from WT or IL-$17^{-/-}$ donors are shown. Six mice per group were used. Data are from 1 representative of 2 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$.

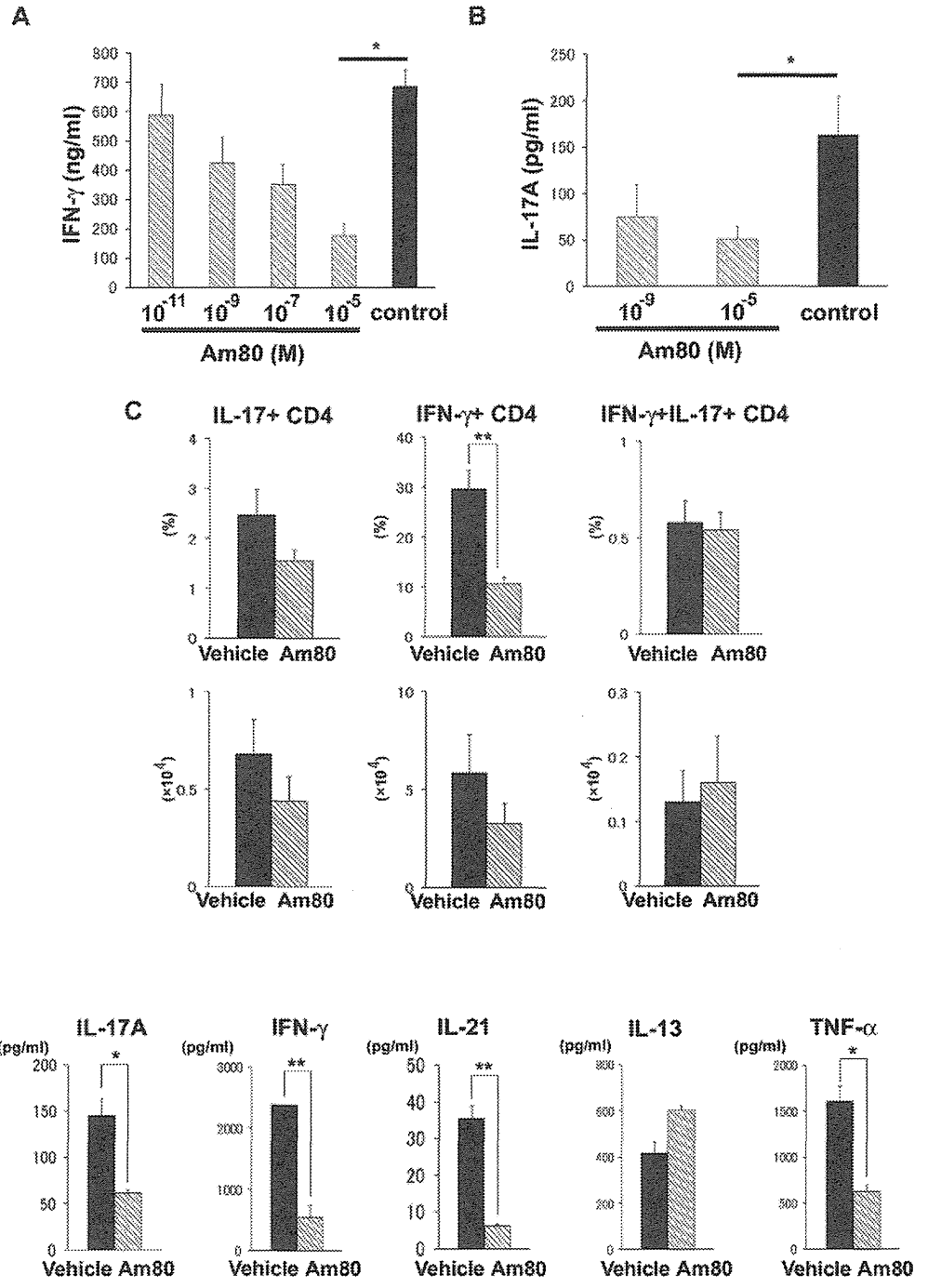

Figure 4. Am80 inhibits donor Th1 and Th17 cells in vitro and in vivo. Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. (A-B) PLN cells from recipients (n = 3-6 per group) on day 14 were treated with Am80 or vehicle solution for 24 hours, the supernatants were collected, and ELISA was performed to determine the cytokine levels. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. (C-D) After BMT, recipients (n = 4-6 per group) were administered oral Am80 (1.0 mg/kg of body weight) or vehicle solution daily from day 0. PLNs of the recipients were stained for intracellular IFN-γ and IL-17. (C) The percentage and absolute number of IFN-γ$^+$ and IL-17$^+$–producing CD4$^+$ T cells. Data are from 1 representative of ≥ 2 repeated experiments. (D) PLN cells from recipients (n = 3-6 per group) treated with Am80 or vehicle on day 16 were stimulated with PMA and ionomycin. Five hours later, the supernatants were collected to determine cytokine levels by CBA. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$ and $^{**}P < .01$.

quantified the frequency of Foxp3-expressing CD4$^+$ T cells in the PLNs after BMT. Recipients administered Am80 showed a decreased frequency of Foxp3$^+$ cells (day 17, 12.3% ± 2.5% vs 23.5% ± 2.6%; P = .02; Figure 5D). Foxp3 mRNA expression of the target organ (the ear) was also decreased in the Am80 recipients (data not shown). To confirm that the effects of Am80 are independent of Treg cells, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 5E, depletion of CD25$^+$ cells from the donor inoculum did not exacerbate skin cGVHD in Am80-treated mice, thus suggesting that the effects of Am80 treatment are not associated with Treg cells.

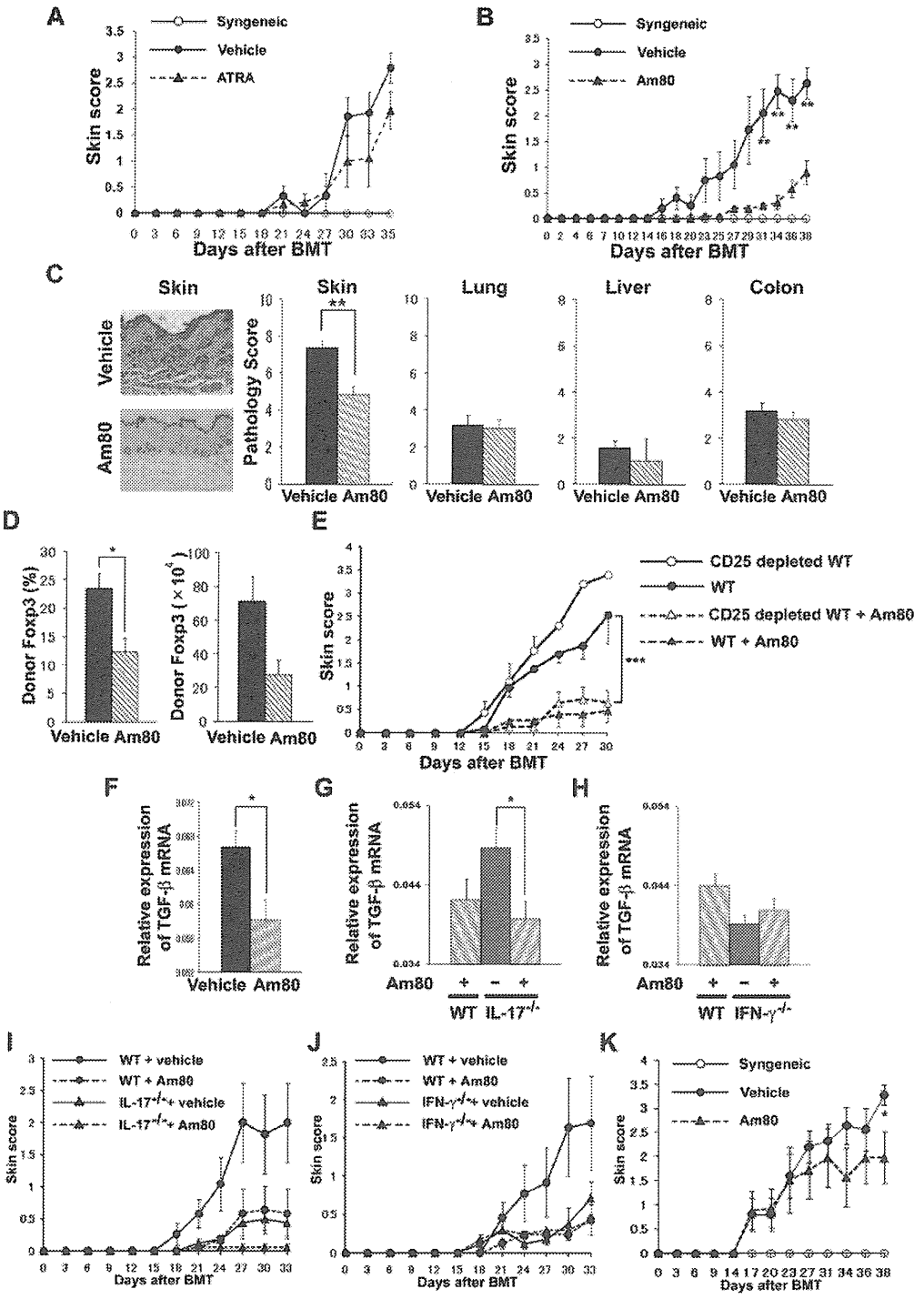

Figure 5. Administration of Am80 ameliorates cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. The recipients received daily administration of ATRA (200 μg/mouse; A), Am80 (1.0 mg/kg of body weight; B), or vehicle solution orally after BMT and were assessed for clinical signs of cGVHD every 3 days. The skin cGVHD scores are shown. (C) Representative histopathology of skin and pathology score of skin, lung, liver, and colon in each group (n = 5-6 per group) on day 16 after BMT are shown. (D) PLN cells of the recipients on day 16 were stained for intracellular Foxp3. The percentages and absolute numbers of $CD4^+Foxp3^+$ Treg cells are shown. Data are from 1 representative of ≥ 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted with 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. After BMT, recipients were given Am80 or vehicle solution. The skin cGVHD scores are shown. There were 6 recipients in each group; the data are from 1 representative of ≥ 2 independent experiments. (F-K) Sublethally irradiated BALB/c recipients were transplanted from WT (F), IL-$17^{-/-}$ (G), and IFN-$\gamma^{-/-}$ (H) donors. After BMT, recipients were given Am80 or vehicle solution. TGF-β mRNA expression in the ears on day 35 after BMT (F-H) and skin cGVHD scores (I-J) are shown. Data are from 1 representative of ≥ 2 independent experiments (n = 5 per group). (K) The skin cGVHD scores of BMT recipients treated with Am80 or vehicle solution orally daily after day 21 of BMT; data from 3 independent experiments were combined (n = 12-14 per group). *$P < .05$, **$P < .01$, and ***$P < .005$.

TGF-β is a critical mediator of fibrosis in cGVHD skin lesions.[30] TGF-β mRNA expression was decreased in the ear of the Am80 recipients (day 17, $P = .02$; Figure 5F). We then assessed TGF-β mRNA expression in recipients of IL-$17^{-/-}$ or IFN-$\gamma^{-/-}$

donors treated with Am80. Am80 further reduced skin scores and TGF-β expression in recipients of IL-17$^{-/-}$ donors (Figure 5G-I) but not in recipients of IFN-γ$^{-/-}$ donors (Figure 5H,J). These results suggest that the effects of Am80 are more dependent on IFN-γ than on IL-17.

Finally, we examined whether Am80 could be used for the treatment of cGVHD. Am80 was orally administered to mice from day 21 of BMT, when mice had developed clinical signs of cGVHD. Am80 significantly improved clinical scores ($P = .016$; Figure 5K).

Discussion

The results of the present study showed that Th1 and Th17 cells contribute to cGVHD with the use of a MHC-compatible, miHA-incompatible model of cGVHD. In addition, we demonstrated that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD.

For many years, the best defined subsets of effector T cells of the CD4$^+$ Th lineage were the Th1 and Th2 cells. A third subset of CD4$^+$ effector cells was identified and named Th17 cells, because the signature cytokine that they produce is IL-17.[31] Although the role of Th17 in acute GVHD has been evaluated by several groups with inconsistent results,[32-35] few studies have addressed the role of Th17 in cGVHD. Initially, cGVHD was hypothesized to be a Th2-mediated disease on the basis of the results in a nonirradiated P→F1 model of cGVHD. cGVHD in this model is mediated by host B-cell autoantibody production stimulated by donor Th2 cells. Th1 polarization of donor T cells activates donor CD8$^+$ CTLs to kill host B cells, resulting in amelioration of cGVHD.[36] However, the relevance of this model is unclear in clinical BMT in which host B cells are eliminated by conditioning. Such different effector mechanisms between the models may be associated with distinct requirement of Th subsets for cGVHD between the studies. In the present study, we assessed the kinetics of Th1, Th2, and Th17 cells during the development of cGVHD in the B10.D2→BALB/c model. Th1 and Th2 responses were up-regulated early after BMT, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were detected in the lung and liver from allogeneic recipients than in those from syngeneic recipients. We then evaluated the role of Th17 in cGVHD with the use of IL-17$^{-/-}$ mice as several groups had used,[32-34,37,38] although interpretation of the results deserves caution because the Th17 lineage is uniquely regulated by RORγt,[13,14] and other cytokines such as IL-21 and IL-22 produced by Th17 cells may also contribute to Th17-mediated GVHD. On transfer of IL-17$^{-/-}$ B10.D2 donor T cells, cGVHD was significantly ameliorated compared with that in recipients of WT T cells, suggesting that Th17 contributes to cGVHD in this model. In particular, Th17 plays a significant role in skin cGVHD. This agrees with the recent observation by Hill et al[37] that donor pretreatment with G-CSF induces Th17 differentiation of donor T cells and induces skin GVHD after peripheral blood stem cell transplantation. In an adoptive transfer model of autoimmune cGVHD, Th17 cells infiltrated target tissues.[39] However, a subsequent study showed the absence of donor Th17 cells did not abrogate GVHD pathology,[38] in contrast to our results. In the absence of donor IL-17, Th1 responses were preserved in that study but were reduced in our study. Such difference in Th1 responses may produce different outcomes between the studies. In mouse models of acute GVHD, Yi et al showed enhanced Th1 differentiation of donor T cells by increased production of IL-12 from dendritic cells in the absence of IL-17.[33] By contrast, Kappel et al showed reduced numbers of IFN-γ–positive CD4$^+$ T cells and IFN-γ secretion in culture in the absence of IL-17.[34] These results together with our results suggest that IL-17 may induce IFN-γ, although such a hierarchy of Th1/Th17 pathways may be context or model dependent or both and will need to be studied in the future. Nonetheless, it should be noted that cGVHD still developed in the absence of donor IL-17 cells in our study. Taken together, it is probable that Th17 is not an absolute requirement for cGVHD, and either Th1 or Th17 is sufficient to cause cGVHD.

We demonstrated that IFN-γ$^{-/-}$ donor mice and injecting anti–IFN-γ mAb ameliorated cGVHD. Thus, Th1 and Th17 responses play a pathogenic role in cGVHD in this model. These results were consistent with a recent study reporting that cGVHD is mediated by Th1 and Th17 responses because of the progressive loss of CD4$^+$CD25$^+$Foxp3$^+$ T cells during acute GVHD in mice.[39] These results were also consistent with clinical studies showing that Th1 cells and Th17 cells increased in patients with active cGVHD.[40-43] Increased transcription of IFN-γ has also been detected in the affected skin and oral mucosa of patients with cGVHD.[41,44] In this study, we found no differences in Th17 cells between IFN-γ$^{-/-}$ and WT recipients, although significantly greater numbers of Treg cells were detected in IFN-γ$^{-/-}$ recipients. CD25-depleted T cells from IFN-γ$^{-/-}$ mice induced more severe skin cGVHD compared with CD25-replete IFN-γ$^{-/-}$ T cells, but still less severe cGVHD compared with CD25-depleted T cells from WT mice (Figure 3E), suggesting that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Neutralization of IFN-γ ameliorated cGVHD in the absence of donor IL-17 (Figure 3H), suggesting again that both Th1 and Th17 responses contribute to the pathogenesis of cGVHD.

We found that donor-derived Th17 cells were generated in recipients of syngeneic transplantation in addition to allogeneic transplantation. However, the kinetics of Th17 development differed between the syngeneic and allogeneic settings; Th17 cells developed in the early phase after syngeneic transplantation. Kappel et al speculated that Th17 development may be the result of increased immune reconstitution of syngeneic hosts compared with allogeneic hosts with GVHD.[34] We additionally identified a population of donor-derived IFN-γ$^+$IL-17$^+$ cells after allogeneic BMT. It has been shown that a subset of IL-17–producing cells can also produce IFN-γ in vivo.[34,45] Such CD4$^+$IFN-γ$^+$IL-17$^+$ T cells have been postulated to play a causative role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE).[46] IFN-γ$^+$IL-17$^+$ cells were only detected after allogeneic BMT, but not after syngeneic BMT, suggesting that this population is generated by allogeneic stimulation, but not because of lymphopenia-induced proliferation. Further investigations are required to clarify the difference in function between IL-17 single-positive and IFN-γ/IL-17 double-positive cells.

ATRA suppresses Th17 differentiation and effector function by RARα signaling,[18] but ATRA can also bind to RARβ and RARγ, which can form a variety of homodimers and heterodimers with 3 retinoid X receptors.[15] Nonselective receptor binding is thought to be a main cause of the side effects associated with the administration of ATRA and other pan-RAR agonists. Am80 is a synthetic RAR agonist that shows high affinity to RARα/β. In addition to a greater specificity for RARα, Am80 offers several other advantages over ATRA as a therapeutic agent, including less toxicity, greater stability, fewer potential side effects, and superior bioavailability. Am80 is effective in autoimmune disease models of collagen-induced arthritis,[20,47] EAE,[21,29] 2,4-dinitrofluorobenzene–

induced contact dermatitis,[22] and atherosclerosis.[23] Because retinoids can down-regulate Th1 and Th17 cells and can ameliorate autoimmune diseases, we hypothesized that these retinoids would attenuate cGVHD. We demonstrated that Am80 down-regulated Th1 and Th17 differentiation of donor T cells in BALB/c recipients of B10.D2 donors, resulting in reduced cGVHD. Our results suggest that combined blockade of Th1 and Th17 responses may represent a promising strategy to prevent or treat cGVHD, as has been suggested for acute and chronic GVHD.[32,39,48] Most recently, Yu et al used mice deficient for both T-bet and RORγt as T-cell donors and clearly showed that blockade of both Th1 and Th17 differentiation is required to prevent acute GVHD.[14] In addition, TGF-β mRNA expression in the skin decreased in the Am80 recipients of WT and IL-17$^{-/-}$ but not IFN-γ$^{-/-}$ donors. These results suggest that Am80 down-regulates TGF-β and that this effect is more dependent on IFN-γ than on IL-17. Unexpectedly, those recipients administered Am80 had a significantly lower frequency of Foxp3^{+} cells. These results differ from those of in vitro studies performed by Mucida et al,[28] in which retinoic acids were shown to be capable of inhibiting the IL-6–driven induction of Th17 cells and to promote Treg cell differentiation. Thus, retinoic acids enhance Treg differentiation and inhibit both Th17 and Th1 in vitro; however, the effects of retinoids may be more complex in vivo, because retinoids can affect not only T cells but also other immunoregulatory cells. For example, previous in vivo studies reported that Am80 suppressed Treg cells in experimental models of EAE[29] and collagen-induced arthritis,[47] similar to our study. In our study, Am80 suppressed TGF-β expression, a key cytokine in Treg development, which may have resulted in the suppression of Treg.

In conclusion, both Th1 and Th17 contribute to the development of cGVHD. Am80 down-regulates TGF-β and also regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD. Thus, administration of Am80, which is currently available as medication for acute promyelocytic leukemia in Japan,[49] may represent effective strategy for prevention and treatment of cGVHD.

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Authorship

Contribution: H.N. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; H.S., K.K., Y.Y., S.K., and H.U. performed the research; K.T., T. Tanaka, and T.Y. performed histopathologic analyses of the organs; Y.I. provided vital new reagents for the study; and T. Teshima and M.T. supervised the research.

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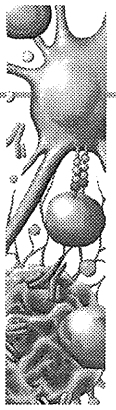
References

1. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet.* 2009; 373(9674):1550-1561.
2. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol.* 2007;7(5):340-352.
3. Teshima T, Wynn TA, Soiffer RJ, Matsuoka K, Martin PJ. Chronic graft-versus-host disease: how can we release Prometheus? *Biol Blood Marrow Transplant.* 2008;14(1 suppl 1):142-150.
4. Socie G, Stone JV, Wingard JR, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. *N Engl J Med.* 1999;341(1): 14-21.
5. Baker KS, Gurney JG, Ness KK, et al. Late effects in survivors of chronic myeloid leukemia treated with hematopoietic cell transplantation: results from the Bone Marrow Transplant Survivor Study. *Blood.* 2004;104(6):1898-1906.
6. Zhou L, Askew D, Wu C, Gilliam AC. Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J Invest Dermatol.* 2007;127(2):281-292.
7. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med.* 2006;203(13):2785-2791.
8. Zhang C, Todorov I, Zhang Z, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood.* 2006;107(7):2993-3001.
9. Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol.* 2008;8(5):337-348.
10. Yuan X, Paez-Cortez J, Schmitt-Knosalla I, et al. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med.* 2008;205(13):3133-3144.
11. Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol.* 2007;8(9):950-957.
12. Teshima T, Maeda Y, Ozaki K. Regulatory T cells and IL-17-producing cells in graft-versus-host disease. *Immunotherapy.* 2011;3(7):833-852.
13. Iclozan C, Yu Y, Liu C, et al. T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. *Biol Blood Marrow Transplant.* 2010;16(2):170-178.
14. Yu Y, Wang D, Liu C, et al. Prevention of GVHD while sparing GVL by targeting Th1 and Th17 transcription factor T-bet and ROR{gamma}t in mice [published online ahead of print August 19, 2011]. *Blood.* doi:10.1182/blood-2011-03-340315.
15. Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol.* 2006; 46(1):451-480.
16. Cantorna MT, Nashold FE, Chun TY, Hayes CE. Vitamin A down-regulation of IFN-gamma synthesis in cloned mouse Th1 lymphocytes depends on the CD28 costimulatory pathway. *J Immunol.* 1996;156(8):2674-2679.
17. Schambach F, Schupp M, Lazar MA, Reiner SL. Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. *Eur J Immunol.* 2007;37(9):2396-2399.
18. Elias KM, Laurence A, Davidson TS, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood.* 2008;111(3): 1013-1020.
19. Xiao S, Jin H, Korn T, et al. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol.* 2008; 181(4):2277-2284.
20. Nagai H, Matsuura S, Bouda K, et al. Effect of Am-80, a synthetic derivative of retinoid, on experimental arthritis in mice. *Pharmacology.* 1999; 58(2):101-112.
21. Wang T, Niwa S, Bouda K, et al. The effect of Am-80, one of retinoids derivatives on experimental allergic encephalomyelitis in rats. *Life Sci.* 2000; 67(15):1869-1879.
22. Niwa S, Ochi T, Hirano Y, et al. Effect of Am-80, a retinoid derivative, on 2, 4-dinitrofluorobenzene-induced contact dermatitis in mice. *Pharmacology.* 2000;60(4):208-214.
23. Takeda N, Manabe I, Shindo T, et al. Synthetic retinoid Am80 reduces scavenger receptor expression and atherosclerosis in mice by inhibiting IL-6. *Arterioscler Thromb Vasc Biol.* 2006;26(5): 1177-1183.
24. Nakae S, Komiyama Y, Nambu A, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity.* 2002; 17(3):375-387.
25. Anderson BE, McNiff JM, Matte C, Athanasiadis I, Shlomchik WD, Shlomchik MJ. Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood.* 2004;104(5): 1565-1573.

26. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood.* 1996; 88(8):3230-3239.
27. Kaplan DH, Anderson BE, McNiff JM, Jain D, Shlomchik MJ, Shlomchik WD. Target antigens determine graft-versus-host disease phenotype. *J Immunol.* 2004;173(9):5467-5475.
28. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science.* 2007;317(5835):256-260.
29. Klemann C, Raveney BJ, Klemann AK, et al. Synthetic retinoid AM80 inhibits Th17 cells and ameliorates experimental autoimmune encephalomyelitis. *Am J Pathol.* 2009;174(6):2234-2245.
30. Chu YW, Gress RE. Murine models of chronic graft-versus-host disease: insights and unresolved issues. *Biol Blood Marrow Transplant.* 2008;14(4):365-378.
31. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature.* 2006; 441(7090):231-234.
32. Yi T, Chen Y, Wang L, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood.* 2009;114(14):3101-3112.
33. Yi T, Zhao D, Lin CL, et al. Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood.* 2008;112(5):2101-2110.
34. Kappel LW, Goldberg GL, King CG, et al. IL-17 contributes to CD4-mediated graft-versus-host disease. *Blood.* 2009;113(4):945-952.
35. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, Serody JS. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood.* 2009;113(6):1365-1374.
36. Shustov A, Luzina I, Nguyen P, et al. Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. *J Clin Invest.* 2000;106(6): R39-R47.
37. Hill GR, Olver SD, Kuns RD, et al. Stem cell mobilization with G-CSF induces type 17 differentiation and promotes scleroderma. *Blood.* 2010; 116(5):819-828.
38. Chen X, Das R, Komorowski R, van Snick J, Uyttenhove C, Drobyski WR. Interleukin 17 is not required for autoimmune-mediated pathologic damage during chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* 2010;16(1):123-128.
39. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. *Blood.* 2007;110(10):3804-3813.
40. Dander E, Balduzzi A, Zappa G, et al. Interleukin-17-producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem-cell transplantation. *Transplantation.* 2009;88(11):1261-1272.
41. Ochs LA, Blazar BR, Roy J, Rest EB, Weisdorf DJ. Cytokine expression in human cutaneous chronic graft-versus-host disease. *Bone Marrow Transplant.* 1996;17(6):1085-1092.
42. Ritchie D, Seconi J, Wood C, Walton J, Watt V. Prospective monitoring of tumor necrosis factor alpha and interferon gamma to predict the onset of acute and chronic graft-versus-host disease after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant.* 2005;11(9):706-712.
43. Korholz D, Kunst D, Hempel L, et al. Decreased interleukin 10 and increased interferon-gamma production in patients with chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 1997;19(7): 691-695.
44. Imanguli MM, Swaim WD, League SC, Gress RE, Pavletic SZ, Hakim FT. Increased T-bet+ cytotoxic effectors and type I interferon-mediated processes in chronic graft-versus-host disease of the oral mucosa. *Blood.* 2009;113(15):3620-3630.
45. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature.* 2007;448(7152):484-487.
46. Axtell RC, Xu L, Barnum SR, Raman C. CD5-CK2 binding/activation-deficient mice are resistant to experimental autoimmune encephalomyelitis: protection is associated with diminished populations of IL-17-expressing T cells in the central nervous system. *J Immunol.* 2006;177(12): 8542-8549.
47. Sato A, Watanabe K, Kaneko K, et al. The effect of synthetic retinoid, Am80, on T helper cell development and antibody production in murine collagen-induced arthritis. *Mod Rheumatol.* 2010; 20(3):244-251.
48. Tawara I, Maeda Y, Sun Y, et al. Combined Th2 cytokine deficiency in donor T cells aggravates experimental acute graft-vs-host disease. *Exp Hematol.* 2008;36(8):988-996.
49. Tobita T, Takeshita A, Kitamura K, et al. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. *Blood.* 1997;90(3):967-973.



Regulatory T cells and IL-17-producing cells in graft-versus-host disease

Graft-versus-host disease (GvHD), a major complication following allogeneic hematopoietic stem cell transplantation, is mediated by donor-derived T cells. On activation with alloantigens expressed on host antigen-presenting cells, naive CD4+ T cells differentiate into T-helper cell subsets of effector T cells expressing distinct sets of transcriptional factors and cytokines. Classically, acute GvHD was suggested to be predominantly related to Th1 responses. However, we now face a completely different and complex scenario involving possible roles of newly identified Th17 cells as well as Tregs in GvHD. Accumulating data from experimental and clinical studies suggest that the fine balance between Th1, Th2, Th17 and Tregs after transplantation may be an important determinant of the severity, manifestation and tissue distribution of GvHD. Understanding the dynamic process of reciprocal differentiation of regulatory and T-helper cell subsets as well as their interactions will be important in establishing novel strategies for preventing and treating GvHD.

KEYWORDS: graft-versus-host disease graft-versus-leukemia helper T cell hematopoietic stem cell transplantation immunotherapy regulatory T cell Th17

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Allogeneic hematopoietic stem cell transplantation (SCT) is a curative modality in a substantial number of patients with hematologic malignancies, bone marrow failure, immunodeficiency syndromes and certain congenital metabolic disorders. The disadvantages of this procedure are the immunological consequences of crossing the major and minor histocompatibility barrier (i.e., graft-versus-host disease [GvHD] and graft rejection). In particular, GvHD remains a major obstacle to the success of allogeneic SCT, whereas graft rejection can usually be overcome with intensive conditioning regimens and infusion of increased number of stem cells. The pathophysiology of acute GvHD is complex, involving donor T-cell responses to host alloantigens expressed by host antigen-presenting cells (APCs) and dysregulation of inflammatory cytokine cascades [1–3]. Experimental and clinical studies suggest that pretransplant conditioning regimens (i.e., total body irradiation and/or chemotherapy) play a critical role in amplifying systemic GvHD [4–6].

Peripheral T cells can be broadly divided into naive T cells and antigen-experienced T cells, including effector, central memory, effector memory and possibly memory progenitor cells [7]. Among these subsets, naive T cells are primarily responsible for experimental GvHD induction, whereas memory T cells contribute to GvHD persistence [8,9]. Generation of distinct functional subsets of effector or Treg cells from naive precursors of antigenic specificity is a fundamental strategy by which the adoptive immune system orchestrates tolerogenic or destructive responses to host alloantigens. T-cell activation requires the following: interaction of the T-cell receptor (TCR) with antigenic peptides bound to the MHC expressed on APCs (signal 1) in secondary lymphoid organs; and costimulatory signals provided by APCs (signal 2) [10,11]. Upon activation, CD4+ T cells can differentiate into T-helper subsets that display distinct effector functions by expressing distinct sets of transcriptional factors and cytokines (FIGURE 1) [12]. Expression of many cell surface molecules, such as adhesion molecules and chemokine receptors, also changes the ability of T cells to traffic, thereby resulting in further recruitment of diverse effector cells into the inflamed tissue. Innate immune systems can direct such CD4+ T-cell development chiefly through cytokine cues, thereby ensuring appropriate coordination between the innate and adoptive immune responses after allogeneic SCT.

For many years, the best defined subsets of effector T cells of CD4+ Th lineages are Th1 and Th2 cells [12]. Numerous studies have addressed how Th1 and Th2 cells contribute to GvHD. Recently, a third subset of CD4+ effector cells have been identified; this subset of cells is designated Th17, because the signature cytokine produced by this subset is IL-17 [13–15]. Tregs are another subset of the immunosuppressive

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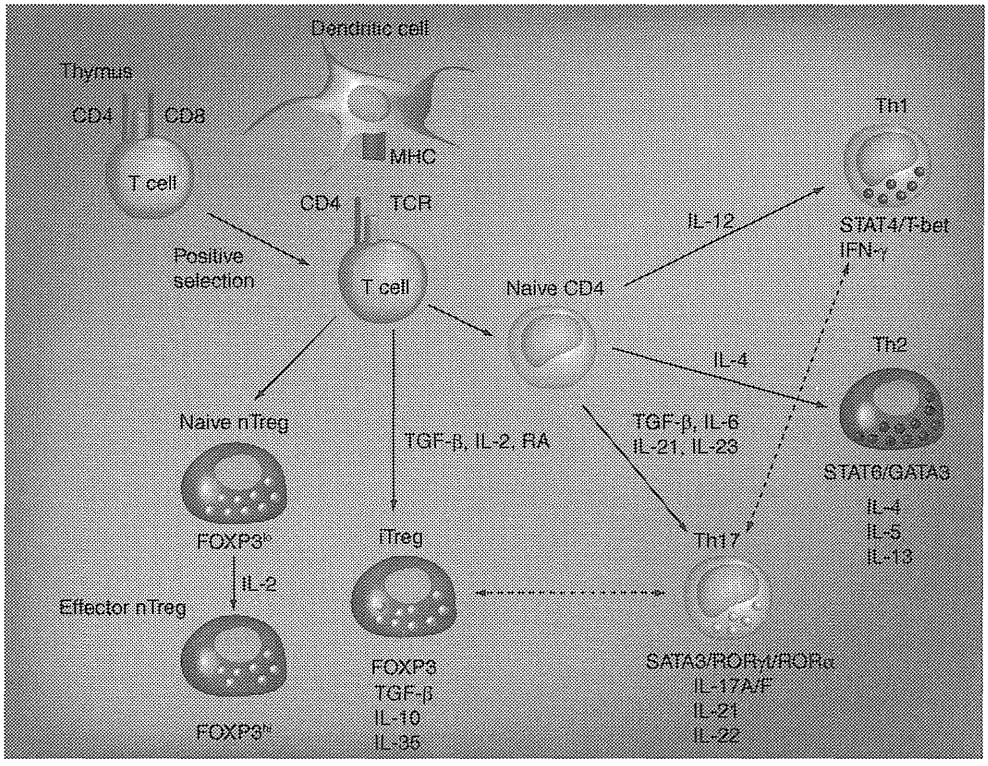

Figure 1. Naive CD4⁺ T cells and naturally occurring Tregs are generated in the thymus.
Naive CD4 T cells can give rise to at least four distinct helper subsets of effector T cells at periphery (Th1, Th2, Th17 and iTreg) depending on the presence of cytokines upon mitogenic stimulation. However, these committed T cells preserve flexibility to alter their cytokine program according to the stimuli they received (indicated by a broken line).
iTreg: Induced Treg; nTreg: Naturally occuring Treg; TCR: T-cell receptor.

CD4⁺ T-cell lineage that expresses the forkhead-winged helix transcription factor FOXP3 [16,17]. Thus, we now face a completely different and complex scenario, involving the possible roles of Th17 cells and Tregs in GvHD.

The Th1/Th2 paradigm in GvHD

Th1 and Th2 cells are distinguished most clearly by the cytokines they produce. IFN-γ is the defining cytokine produced by Th1 cells, whereas IL-4, IL-5 and IL-13 are the signature cytokines produced by Th2 cells [12]. They are cross-regulatory *in vitro*, and their balance determines the character of cell-mediated immune and inflammatory responses *in vivo*. Differential activation of Th1 or Th2 cells has been suggested to play an important role in GvHD development. Based on results from nonirradiated P→F_1 models of GvHD, acute GvHD was hypothesized to be a Th1-mediated disease, whereas chronic GvHD was hypothesized to be a Th2-mediated disease [18]. This assumption was soon supported in clinically relevant, irradiated models of GvHD. Combined Th2 cytokine deficiency in donor T cells aggravates GvHD [19]. Infusion of Th1 cells polarized *in vitro* induces more severe GvHD than that of Th2 cells [20]. Infusion of *in vitro*-polarized donor Th2 cells ameliorates GvHD when infused with naive T or Th1 cells [21–23]. A 'cytokine storm' characteristics related to the Th1 phenotype has been found to correlate with the development of acute GvHD in mice and humans [1].

Paradoxically, T cells from mice deficient in a Th1 cytokine such as IFN-γ, IL-2, IL-12 or IL-18 induce more severe GvHD, whereas those from IL-4-deficient mice induce less severe disease [24–26]. Signal transducer and activator of transcription (STAT)4-deficient T cells that impair Th1 differentiation and STAT6-deficient T cells that impair Th2 differentiation can cause injury to distinct GvHD target organs [27]. These results argue against the simplified Th1/Th2 paradigm in GvHD. To evaluate the feasibility and biological properties of IL-4-polarizing Th2 cells, a Phase I clinical trial was conducted in patients who underwent allogeneic peripheral blood SCT (PBSCT) from a HLA-matched sibling donor [28]. Various doses of Th2 cells were infused 1 day after PBSCT. Although drawing