Immunology and Cell Biology (2011), 1–10

www.nature.com/icb

ORIGINAL ARTICLE

Sclerodermatous chronic graft-versus-host disease induced by host T-cell-mediated autoimmunity

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Despite a long-standing hypothesis that chronic graft-versus-host disease (cGVHD) is an autoimmune disorder, most mouse models of cGVHD have been developed on the assumption that donor T cells are essential for its development. Here we show that cGVHD may be caused by autoreactive host T cells in mice that have been lethally irradiated and grafted with T-cell-depleted allogeneic bone marrow cells. In this chimera, host T cells derived from radioresistant intrathymic T-cell precursors caused dermal fibrosis and periportal inflammation, without the requirement for donor T cells. The lack of host DCs within the thymus after high-dose irradiation allowed autoreactive host T cells to escape thymic negative selection. Moreover, the homeostatic expansion of these T cells may augment their autoreactivity. These findings indicate that host T-cell-mediated cGVHD is an autoimmune process that occurs following the grafting of T-cell-depleted BM cells into hosts with functioning thymuses. We propose, based on the present data, that host T-cell-dependent autoimmunity is a potential mechanism by which cGVHD is induced.
Immunology and Cell Biology advance online publication, 31 May 2011; doi:10.1038/icb.2011.46

Keywords: autoimmunity; chronic graft-versus-host disease; host T cells

Graft-versus-host disease (GVHD), one of the most common complications of allogeneic bone marrow (BM) transplantation (allo-BMT), typically affects the skin, gastrointestinal tract, liver and lung.[1,2] The pathogenic mechanisms of acute GVHD have been extensively investigated, and a model comprising three sequential phases—activation of host antigen-presenting cells (APCs), activation/proliferation of donor T cells, and target tissue necrosis and destruction—is generally accepted.[1,3] By contrast, chronic GVHD (cGVHD) seems to develop by a quite different process, as evidenced by its autoimmune nature in clinical settings that leads to eventual target organ fibrosis and insufficiency.[4,5] Activation of donor T cells has been thought to be primarily responsible for the development of cGVHD (as well as acute GVHD).[1,5] These donor T cells stimulate host B cells to produce autoantibodies or activate macrophages and fibroblasts to induce collagen synthesis.[5] However, no single animal model reproduces all the pathologic features of human cGVHD, and its pathophysiology remains poorly understood.[1]

The appearance of host T cells in irradiated BM chimeras has long been recognized, and they are thought to develop from radioresistant thymic T-cell precursors.[6–9] Prior to donor T-cell development after irradiation and BMT, host radioresistant double-negative thymocytes differentiate into double-positive and single-positive T cells with functional T-cell receptor (TCR) gene rearrangements.[10–13] Given the possibility that host T cells escape thymic negative selection owing to a lack of host or donor APCs after high-dose irradiation,[14] these cells may be able to participate in pathologic processes because of their autoimmune nature. Based on this assumption, we analyzed the development and function of host T cells in T-cell-depleted allo-BMT models and found that host T cells could cause the development of cGVHD-like pathology in the skin and liver.

RESULTS

Induction of cGVHD by autoreactive host T cells after lethal irradiation

To assess the functional roles of host T cells in allogeneic BM chimeras, we transplanted T-cell-depleted BM cells (BMCs) from BALB/c (H-2^d) mice into lethally irradiated C57BL/6 (B6) hosts (H-2^b). During a follow-up period of approximately 50–60 days, chimeric mice developed symptoms of cGVHD in the skin and liver (Figure 1a, top). Skin lesions, showing pathologic features of interface dermatitis and thick dermal fibrosis accompanied by $CD3^+$ T-cell infiltration (Figure 1a, top), were found in approximately 50% of chimeras

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Received 26 October 2010; revised and accepted 20 April 2011

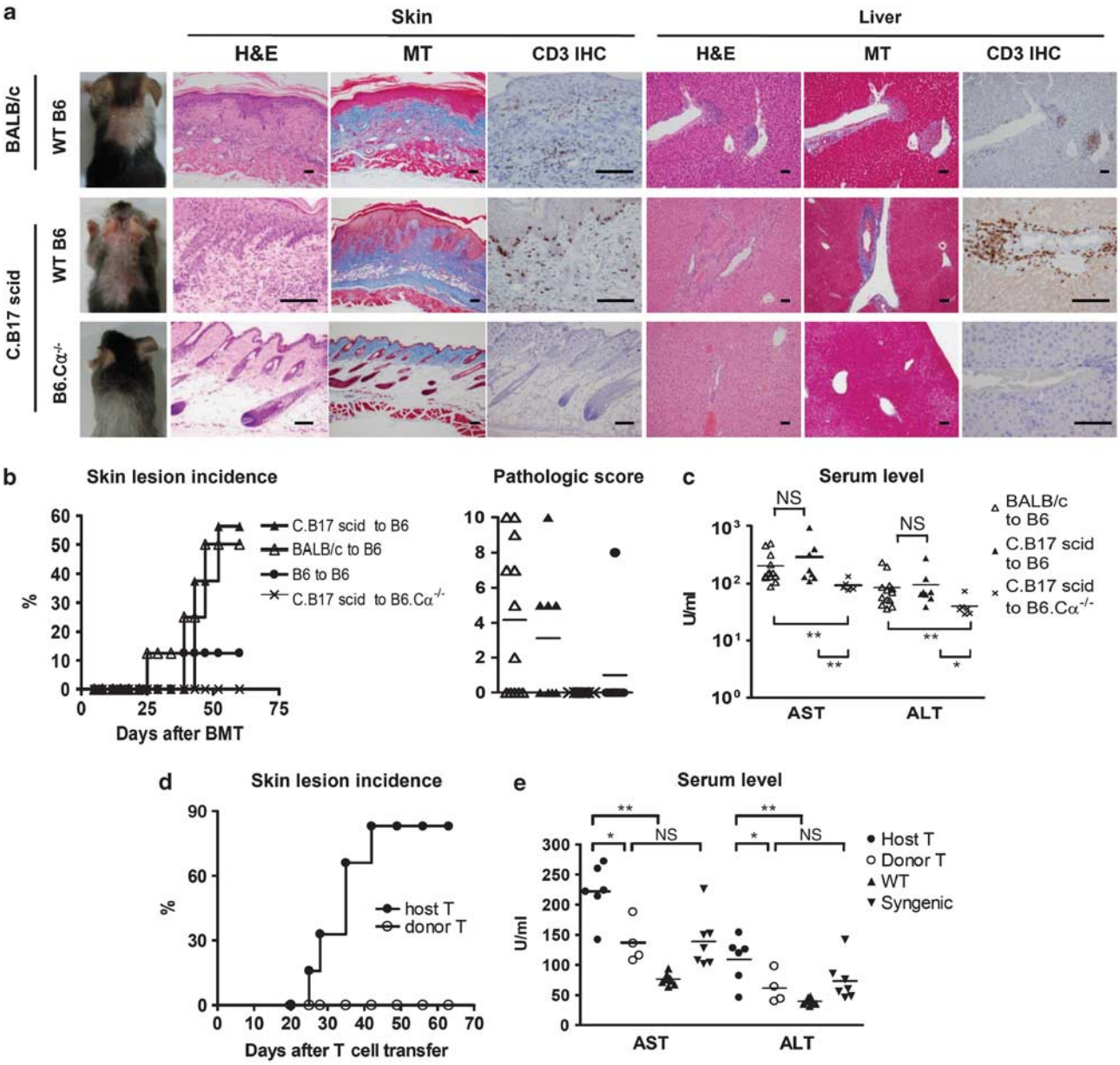

Figure 1 Host T cells are sufficient for the induction of cGVHD. (**a**) Wild-type (WT; top and middle) and TCR $C\alpha^{-/-}$ (bottom) B6 hosts were lethally irradiated and transplanted with T-cell-depleted BMCs from BALB/c (top) or C.B17 SCID (middle and bottom) mice, and monitored for 50 days. Representative histological sections of skin and liver subjected to hematoxylin and eosin, and Masson's trichrome staining, as well as CD3 immunostaining, are shown (bar=100 μm). (**b**, **c**) T-cell-depleted BMCs from C.B17 SCID, BALB/c and B6 mice were transplanted into lethally irradiated B6 or B6.TCR $C\alpha^{-/-}$ hosts. Summary of the incidence of inflammatory skin lesions (n=8), pathologic scores (n=6–12) (**b**), and serum AST and ALT concentrations (n=6–12) (**c**) 50 days after BMT are shown. The horizontal bars indicate mean values. (**d**, **e**) Thymectomized B6 mice were lethally irradiated and transplanted with T-cell-depleted BMCs from BALB/c mice. Five weeks later, they received donor (H-$2K^{d+}$) or host (H-$2K^{b+}$) splenic T cells (1.5×10^6) isolated from BALB/c-to-B6 BM chimeras (n=4–6). The incidence of inflammatory skin lesions (**d**) and serum AST and ALT concentrations (**e**) in recipient mice were monitored for 9 weeks. To compare serum AST and ALT levels, wild-type (WT) B6 and syngeneic B6-to-B6 BMT mice (50 days after BMT) that did not receive adoptive transfer were used as negative controls. The horizontal bars indicate mean values (**e**). NS, not significant. *P<0.05; **P<0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BM, bone marrow; BMC, BM cell; BMT, BM transplantation; cGVHD, chronic graft-versus-host disease; TCR, T-cell receptor.

(Figure 1b). However, only one among the eight syngeneic (B6 to B6) BMT mice showed similar pathology in the skin (Figure 1b). In the liver, periportal inflammation, fibrosis and T-cell infiltration were the predominant histopathologic findings observed in 12 of the 13 mice examined microscopically (Figure 1a, top). Elevated serum concentrations of aspartate aminotransferase (AST) (BALB/c to B6: 234 ± 164 versus wild type: 76 ± 9.4) and alanine aminotransferase (ALT) (BALB/c to B6: 84 ± 83 versus wild type: 39 ± 5.3) reflected parenchymal liver damage (Figure 1c). By contrast, other organs, including lung, intestine, kidney and salivary gland, remained unaffected (Supplementary Figure 1). Inflammatory skin lesions and elevated serum levels of liver enzymes were also detected with varying

incidences and to different extents in B6 hosts, which received T-cell-depleted BMCs from mice of other haplotypes such as NOD (H-2^{g7}), DBA (H-2^{d}) and CBA (H-2^{k}) (Table 1), and even in mice reconstituted with major histocompatibility complex (MHC) class-II only (bm12) or minor antigen (BALB/b)-mismatched allogeneic BMCs (Supplementary Table 1). However, the pathologies were not detected in BALB/c (H-2^{d})-to-BALB/b (H-2^{b}) chimeras, indicating that the genetic background of host mice (aside from the MHC haplotype) may be important for the development of cGVHD (Table 1). When BMCs from C.B17 SCID or BALB/c RAG$^{-/-}\gamma c^{-/-}$ mice were transplanted into wild-type or TCR Cα-deficient B6 mice, similar pathology was observed in the wild-type recipients, whereas the TCR Cα-deficient mice did not suffer from GVHD (Figures 1a–c, Table 1 and Supplementary Table 1). These data indicate that host αβ T cells were responsible for the development of cGVHD.

Table 1 Development of cGVHD after MHC-mismatched BMT

Donor→Host	*Skin lesion incidence*	*AST (U ml^{-1} ± s.d.)*	*ALT (U ml^{-1} ± s.d.)*
C.B17 SCID (d)[a]→B6.TCR Cα$^{-/-}$	0/8	93 ± 21	40 ± 16
C.B17 SCID (d)→B6	4/8	291 ± 279[b]**	95 ± 77*
BALB/c (d)→B6	4/8	234 ± 164*	84 ± 83*
NOD (g7)→B6	4/8	173 ± 71*	55 ± 11*
DBA (d)→B6	2/8	162 ± 74 (NS)	47 ± 15 (NS)
CBA (k)→B6	4/8	175 ± 118*	71 ± 41 (NS)
BALB/c (d)→BALB/b (b)	0/6	80 ± 8 (NS)	68 ± 8 (NS)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMT, bone marrow transplantation; cGVHD, chronic graft-versus-host disease; MHC, major histocompatibility complex.
[a]MHC haplotype.
[b]*P*-values are compared with those of C.B17 SCID (d)→B6.TCR Cα$^{-/-}$ BM chimeras.
*$P<0.05$.
**$P<0.01$.
NS, not significant.

We next adoptively transferred donor or host T cells isolated from BALB/c-to-B6 chimeras into secondary recipients. In these experiments, BALB/c-to-thymectomized B6 BM chimeras were used as the secondary recipients because they provide a similar physiologic environment to BALB/c-to-B6 BM chimeras. Up to 9 weeks after adoptive transfer of donor T cells, recipient mice showed no clinical signs of GVHD (Figures 1d and e). By contrast, cGVHD-like pathology was detected in animals that received host T cells (Figures 1d and e), confirming that host T cells, but not donor T cells, had a critical role in the induction of sclerodermatous cGVHD in our allo-BMT model.

To determine whether these pathogenic T cells were reactive to donor or host cells, host T cells were isolated from BALB/c-to-B6 BM chimeras and stimulated with irradiated B6 or BALB/c splenocytes. Notably, Th1 and Th2 cytokine production was higher in host CD4^{+} T cells cultured with B6 APCs than in those cultured with BALB/c APCs, despite the proliferation rate of host CD4^{+} T cells not differing significantly between the two cultures (Figure 2). Particular attention

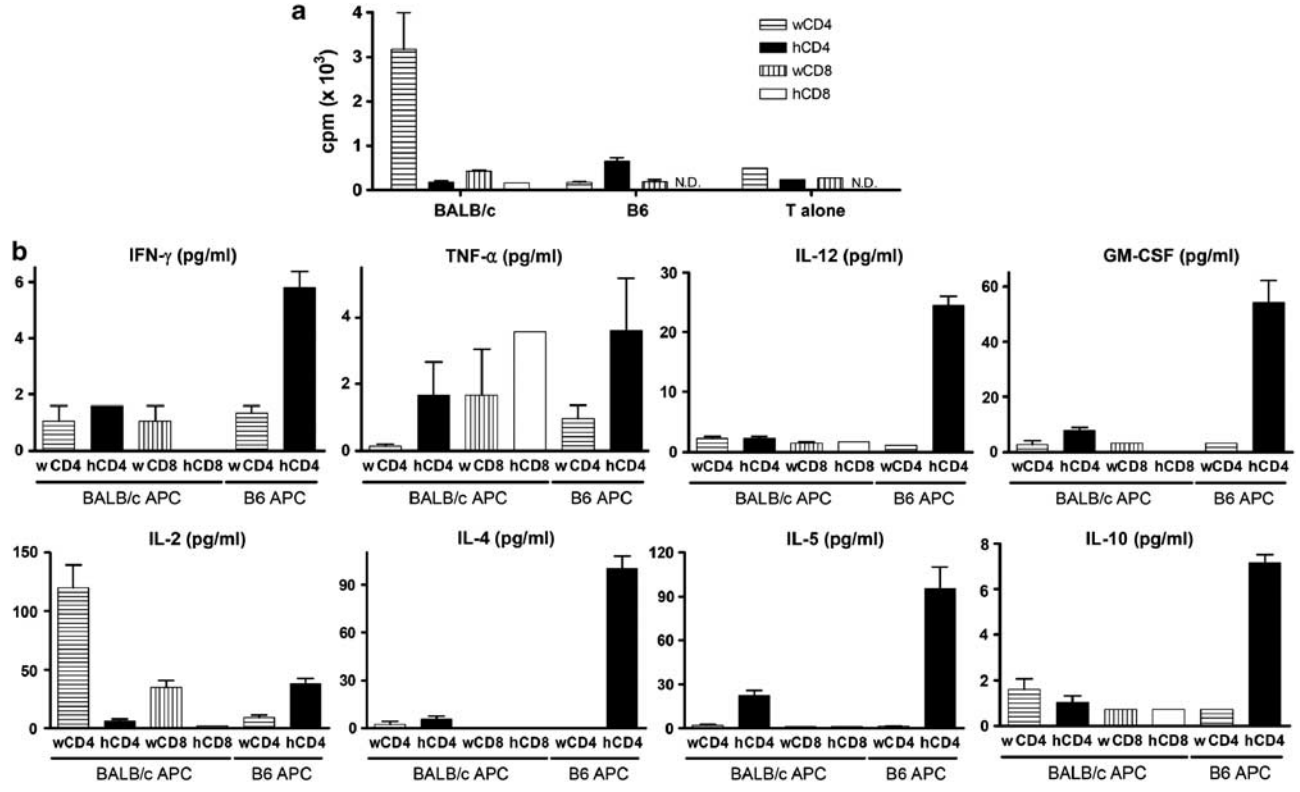

Figure 2 Host T cells do not respond to graft-derived BALB/c APCs, but are reactive to host-derived B6 APCs. Wild-type (WT) B6 CD4^{+} (wCD4) and CD8^{+} (wCD8) T cells, and host CD4^{+} (hCD4) and CD8^{+} (hCD8) T cells from BALB/c-to-B6 BM chimeras (obtained 5 weeks post-BMT), were sorted by fluorescence-activated cell sorting and co-cultured with irradiated BALB/c or B6 splenic APCs for 3 days. (**a**) [^{3}H]-thymidine uptake measurements show a modest proliferation of host CD4^{+} T cells in response to B6 or BALB/c APCs. ND, not done. (**b**) Host CD4^{+} T cells produce a range of cytokines in the presence of B6 APCs, whereas host CD4^{+} and CD8^{+} T cells are not reactive to BALB/c-derived APCs. Cytokine concentrations in culture supernatants were assayed in triplicate. The concentrations of cytokines in the supernatants from control cultures containing only irradiated APCs were below the limits of detection. The data are representative of three independent experiments. APC, antigen-presenting cell; BM, bone marrow.

was paid to the production of interleukin-4 (IL-4), which has been known to be related with tissue fibrosis.[5] Unexpectedly, secretion of IL-4 was about 20-fold higher in the syngeneic reaction (host T cell against B6 APC) as compared with that resulting from the reaction between host T cells and allogeneic APCs (host T cell against BALB/c APC) (Figure 2b). As the concentration of cytokines secreted from irradiated APCs cultured in the absence of T cells was below the level of detection, these results strongly suggest that host T cells may contribute significantly to the induction of cGVHD in BALB/c-to-B6 BM chimeras through an autoimmune process.

Generation of autoreactive host T cells in host DC-deficient thymuses

To investigate how autoreactive host cells are generated following allo-BMT, we first compared the reconstitution kinetics of host T cells with those of donor T cells in allo-BMT chimeras. Consistent with a previous report,[15] non-T-cell lineage splenocytes were almost completely replaced by those of donor origin by 4–6 weeks after BMT, irrespective of donor haplotype (as they were in congeneic BM chimeras) (Figure 3a and Supplementary Figures 2a and b). By contrast, host T cells outnumbered donor T cells in peripheral blood until 6 weeks after BMT (Figure 3b) and showed a similar TCR Vβ usage to that of donor T cells (Supplementary Figure 3). Host T cells were hard to detect in peripheral blood and lymphoid organs when host thymuses were removed prior to BMT, indicating that host T cells underwent intrathymic development (Figure 3c and Supplementary Figure 2c). To further exclude the possibility that radioresistant T precursors migrated from extrathymic tissues, $CD45.1^{-}CD45.2^{+}$ fetal thymuses (E15.5) were grafted under the kidney capsules of $CD45.1^{+}CD45.2^{+}$ congeneic mice, which were then irradiated and transplanted with $CD45.1^{+}CD45.2^{-}$ BMCs (Figure 3d). When we assessed the presence of host-derived thymocytes in both thoracic and grafted thymuses 16 days after BMT, we found that the grafted thymuses did not contain host-derived $CD45.1^{+}CD45.2^{+}$ cells, indicating that host T cells were generated from intrathymic T-cell precursors. Next, we also investigated whether the generation of host-derived autoreactive T cells is associated with the defect in the development of $Foxp3^{+}CD4^{+}$ regulatory T (Treg) cells. However, the Treg-cell proportion in host-derived $CD4^{+}$ T cells was not lower than that of donor-derived $CD4^{+}$ T cells (Figure 3e).

To determine whether these host T cells underwent normal development in the thymus, we investigated their thymic ontogeny. The cortex and medullary architectures of thymuses in both allogenic and syngeneic BMT chimeras were easily identifiable as early as 2 weeks after BMT and comparable to those of wild-type thymus (Supplementary Figure 4a). In BALB/c-to-B6-CD45.1 chimeras, at 2 weeks after BMT, when most donor thymocytes were at the double-negative stage, host thymocytes began to differentiate into $CD24^{hi}$ single-positive cells, and most of them ultimately fully matured into $CD24^{lo}TCR^{hi}CD5^{hi}CD69^{-}$ single-positive cells[16,17] (Figure 4a and Supplementary Figure 4b). Notably, the numbers of host T cells in the thymus and peripheral blood were drastically reduced at 3 and 6 weeks, respectively, after BMT (Supplementary Figure 5 and Figure 3b), suggesting that host T-cell precursors have a limited capacity for self-renewal. To exclude the possibility of that host T cells developed independently of MHC expression on host thymic epithelial cells,[18] or that these T cells were selected by hematopoietic cells,[19–21] we transplanted T-cell-depleted BMCs from B6-CD45.1 mice into lethally irradiated B6 $\beta2m^{-/-}$ and MHC class-$II^{-/-}$ hosts. In both the thymus and spleen, $CD8^{+}$ and $CD4^{+}$ T cells failed to efficiently develop in B6 $\beta2m^{-/-}$ and the MHC class-$II^{-/-}$ hosts, respectively, indicating that the development of both host and donor T cells was largely dependent on the expression of MHC class-I and II molecules on host thymic epithelial cells (Figure 4b).

We next analyzed the development of intrathymic dendritic cells (DCs) and found that thymic $CD11c^{+}$ DCs were totally replaced by graft-derived cells as early as 2 weeks after BMT (Figure 4c and Supplementary Figure 6a), which was similar to DCs in the spleen (Figure 3a). It indicates that thymic DCs are radiosensitive, being different from thymic T-cell precursors, and suggests that radio-resistant thymic T-cell precursors are a different population from the common precursor of T cells and DCs that had been reported.[22,23] Of the donor-derived $CD11c^{+}$ cells present in the thymus, $CD11b^{+}$ myeloid, $B220^{+}$ plasmacytoid and $CD11b^{-}B220^{-}$ conventional DC populations[24,25] were easily identifiable in both congeneic (B6-CD45.1-to-B6-CD45.2) and allogenic (BALB/c-to-B6) recipients (Supplementary Figure 6b, upper). They also expressed MHC and B7 molecules at levels comparable to those in DCs from wild-type thymuses (Supplementary Figure 6b, lower). Given that DCs are a major contributor to the intrathymic depletion of autoreactive T cells[26] and that selective depletion of $CD11c^{+}$ cells leads to the spontaneous development of widespread autoimmunity,[27] these findings indicate that host T cells escape the negative selection mediated by host DCs and then become autoreactive to host APCs.

Homeostatic expansion of host T cells in the periphery

The essential role for host T cells in the development of cGVHD led us to investigate whether they had an effector/memory phenotype. As expected, host T cells in the spleens of BALB/c-to-B6 BM chimeras showed higher expression of CD44, CD11a, CD122 and Ly6C than donor T cells (Figure 5a). However, the expression of CD62L, CD25, CD69 and PD-1 was comparable to that of donor T cells (Supplementary Figure 7a), indicating that these cells have a memory phenotype. The $CD44^{hi}$ memory phenotype was also observed in T cells isolated from lymph nodes and liver. The liver contained more host T cells than the spleen and lymph nodes, reflecting induction hepatic inflammation by these cells (Supplementary Figure 7b). Upon *ex vivo* stimulation, host T cells also robustly produced various cytokines, including interferon-γ (IFN-γ), IL-2, IL-13 and IL-17 (Figure 5b), which have been known to induce inflammation and fibrosis.[5] We next investigated whether the acquisition of memory markers by host T cells was because of allostimulation. In congeneic BM chimeras (B6-CD45.1-to-B6-CD45.2), in which cGVHD did not develop (Figure 1b), host T cells showed a memory phenotype similar to those of allo-BMT mice (Figures 5a and b), indicating that T-cell activation upon GVHD-associated antigenic stimulation was not responsible for the acquisition of memory markers. Notably, the $CD44^{hi}$ memory subset in host T-cell population was drastically expanded, resulting in about a threefold increase of host T-cell number in the spleen of these BMT chimeras during the period from the second to the fourth week after BMT (Figure 5c). Considering that the surface markers of T cells undergoing homeostatic expansion were identical to those of host T cells,[28] homeostatic expansion may be the main mechanism by which the host T cells acquire a memory phenotype. This notion was further supported by results of experiments performed using TCR-transgenic recipients. When the activation status of host T cells in B6-CD45.1-to-OT-I B6 and B6-CD45.1-to-OT-II B6 BM chimeras was compared, only OT-I T cells showed a memory phenotype in terms of CD44 expression, proliferation and cytokine production (Figure 6). Considering that OT-I T cells are able to undergo homeostatic proliferation in a

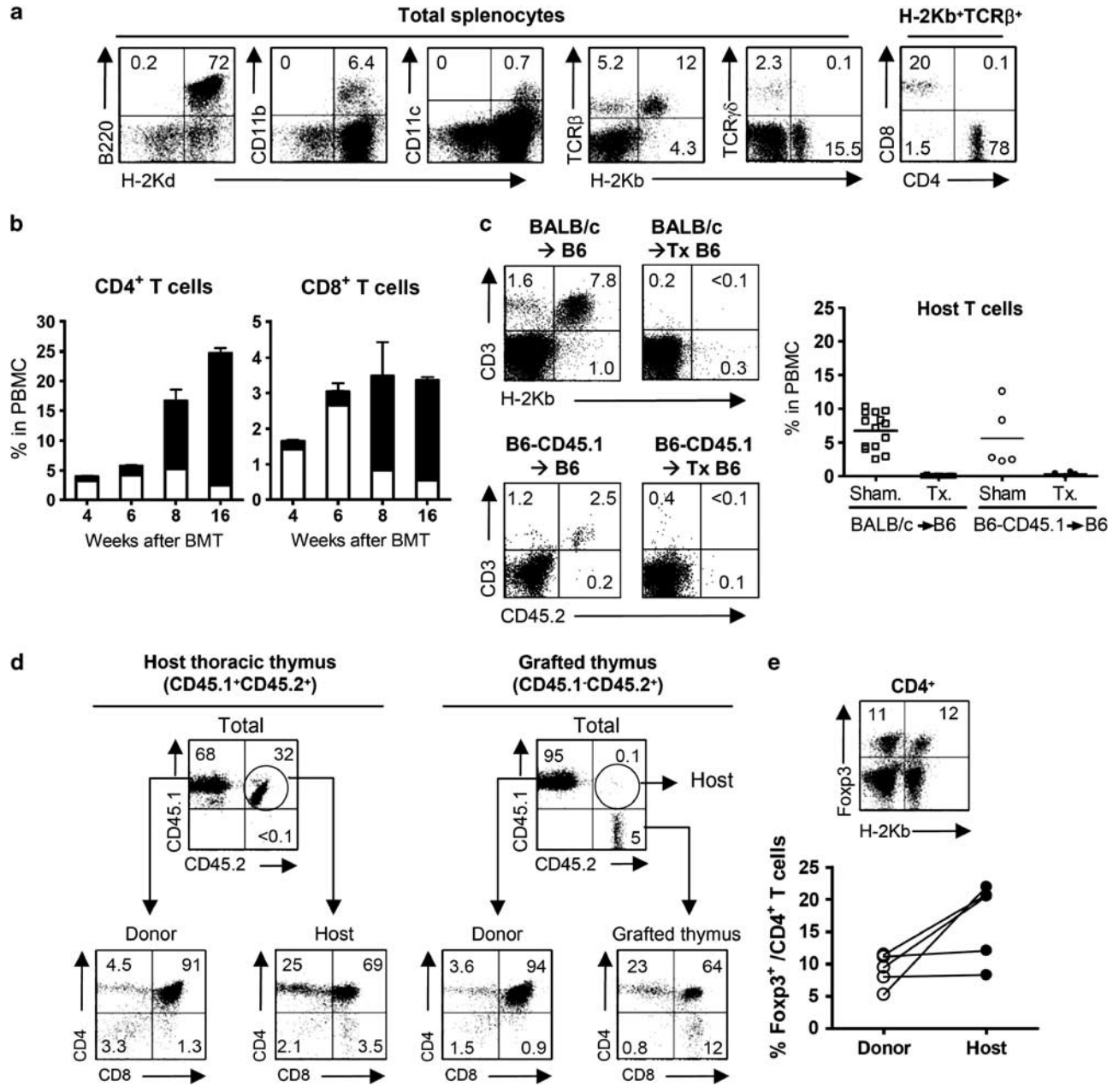

Figure 3 Generation of host T cells from intrathymic precursors after high-dose irradiation. (**a**) Host and donor T cells coexist, whereas B cells, macrophages and DCs are almost completely replaced by graft-derived cells after allo-BMT. Splenocytes from BALB/c-to-B6 BM chimeras were analyzed 6 weeks after BMT. The numbers indicate the percentage of cells in each quadrant. (**b**) Host T cells outnumber donor T cells until 6 weeks after BALB/c-to-B6 BMT. Host (open bar) and donor (closed bar) $CD4^+$ and $CD8^+$ T-cell numbers are presented as percentages of the overall peripheral blood mononuclear cell (PBMC) population, in BALB/c-to-B6 BM chimeras (n=3–8). (**c**) Thymectomized (Tx) and sham-operated B6 mice were lethally irradiated and transplanted with T-cell-depleted BALB/c or B6-CD45.1 BMCs. Four weeks later, donor and host T-cell development in PBMC samples was assessed by flow cytometry (n=4–14). The horizontal bars indicate mean values. (**d**) *De novo* host T cells developed in grafted thymuses. $CD45.1^+CD45.2^+$ (CD45.1/2) B6 mice grafted with $CD45.1^-CD45.2^+$ (CD45.2/2) B6 fetal thymuses (E15.5) were lethally irradiated and received T-cell-depleted BMCs from $CD45.1^+CD45.2^-$ (CD45.1/1) B6 mice. Sixteen days later, thoracic and grafted thymuses were analyzed for expression of CD45.1, CD45.2, CD4 and CD8. (**e**) Host-derived $Foxp3^+CD4^+$ Treg cells were generated in a comparable fraction to that of donor-derived Treg cells. At 6 weeks after BMT, splenocytes from BALB/c-to-B6 BM chimeras were stained with anti-H-$2K^b$, anti-CD4 and Foxp3 antibodies, and $Foxp3^+$ populations were analyzed by flow cytometry after gating on $CD4^+$ cells. The numbers in dot blot indicate the percentage of $Foxp3^+$ cells in the donor (H-$2K^{b-}$) and host (H-$2K^{b+}$) $CD4^+$ T-cell population, and summarized results from four mice are presented. BM, bone marrow; BMT, BM transplantation; DC, dendritic cell; Treg, regulatory T.

lymphopenic host, whereas OT-II T cells are not,[28–30] these results strongly suggest that the memory phenotype of host T cells in the periphery was acquired as a consequence of homeostatic expansion.

DISCUSSION

In most animal models of GVHD, infused donor T cells are known to be indispensable for the induction of both acute and cGVHD.[1,3,5]

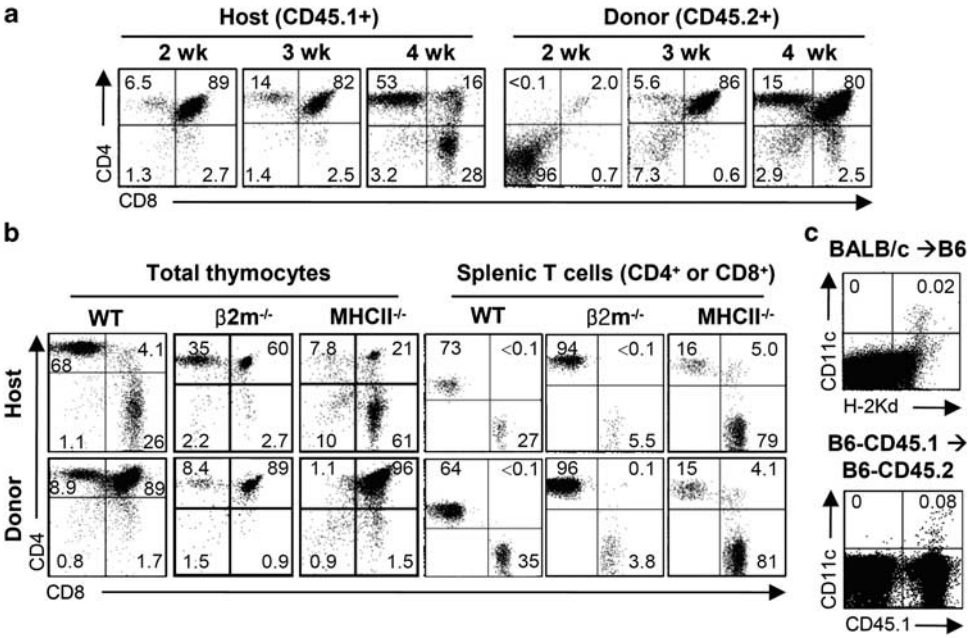

Figure 4 The developmental profile of host T cells in the thymus. (**a**) B6-CD45.1 mice were lethally irradiated and transplanted with T-cell-depleted BALB/c BMCs. Chimeric mice were killed in weeks 2, 3 and 4 after BMT, and thymocytes were analyzed for their expression of CD4 and CD8. The numbers indicate the percentage of cells in each quadrant. The data are representative of two independent experiments. (**b**) The development of both host and donor T cells is dependent on the expression of MHC molecules on thymic epithelial cells. Wild-type (WT) B6, B6.β2m$^{-/-}$ and B6.MHC class-II$^{-/-}$ mice were lethally irradiated and transplanted with T-cell-depleted BMCs from B6-CD45.1 congeneic mice. Thymocytes and splenocytes were analyzed 21 days and 6 weeks after BMT, respectively. The numbers indicate the percentage of cells in each quadrant. (**c**) Intrathymic host DCs are completely replaced by graft-derived cells as early as 2 weeks after BMT. Thymocytes from BALB/c-to-B6 or CD45.1 B6-to-CD45.2 B6 BM chimeras were subjected to Fc receptor blocking, stained with anti-CD11c and anti-H-2K^{d} or anti-CD45.1 antibodies, and analyzed using a flow cytometer. The numbers indicate the percentage of cells in each quadrant. The data are representative of three independent experiments. BM, bone marrow; BMC, BM cell; BMT, BM transplantation; DC, dendritic cell; MHC, major histocompatibility complex.

In this study, we showed that allotransplantation of T-cell-depleted BMCs induced cGVHD-like pathology in recipient B6 mice. Unexpectedly, the development of sclerodermatous cGVHD depended on host T cells derived from radioresistant thymic T-cell precursors under conditions of major or minor antigen-mismatched allo-BMT, irrespective of the MHC haplotype of the donor mouse. We also observed these features following the transplantation of LSK stem cells from BALB/c mice BM (Supplementary Table 1 and Supplementary Figure 2a). The finding that no host DCs remained in the thymus, presumably allowing host T cells to escape negative selection and ultimately resulting in an autoimmune reaction, provided an explanation for this phenomenon.

The development of cGVHD after T-cell-depleted BMT seems to be dependent on two factors: the conditioning regimen for BMT and the host factors. In the present study, high-dose irradiation prior to allo-BMT induced the near-total replacement of host intrathymic DCs with donor DCs. As a result, autoreactive host T cells, which escaped negative selection by host DCs, survived and expanded homeostatically to recognize radioresistant host APCs in the skin and liver. Unlike DCs in the peripheral blood and lymphoid organs, which are mostly derived from radiosensitive precursors, the homeostasis of cutaneous Langerhans cells and dermal DCs is fairly well maintained by local radioresistant precursors under steady-state conditions in mice and humans.[31–34] A recent report further showed that, in the liver, a subset of Kupffer cells is radioresistant and survives for more than 12 weeks following BMT.[35] In the intestine, where cGVHD is also prone to develop, most DCs are replaced by graft-derived cells,[36] and a few remaining host DCs do not have a major role in the development of cGVHD in the gut.[37] Data from our laboratory showed that the pathology became evident when host T cells developed in BALB/c-to-B6 BM chimeras were adoptively transferred to BALB/c-to-thymectomized B6 BM chimeras. These data strongly suggest that the interaction between autoreactive host T cells and self-APCs is critical for the development of these lesions. The idea that intrathymic DCs have an essential role in the generation of autoreactive T cells was further supported by the relatively rare development of the cGVHD-like pathology in syngeneic BM chimeras, in which tolerance to self (host)-APCs was acquired by donor-derived syngeneic DCs.

Host factors also seem to have causal roles in the development of cGVHD. In contrast to B6-to-BALB/c BM chimeras, in which cGVHD does not develop in the skin,[37] this pathology was found in B6 recipient mice, irrespective of donor MHC haplotype. Moreover, we found no evidence of GVHD in BALB/c (H-2^{d})-to-BALB/b (H-2^{b}) chimeras, despite the presence of host T cells with a memory phenotype (Table 1 and Supplementary Figure 2a). This suggests that host factors other than MHC haplotype may contribute to the induction of GVHD, although they remain to be further identified.

Detailed analysis of intrathymic maturation further confirmed that host T cells develop normally within the thymus in terms of MHC restriction and the sequence of surface marker expression. Therefore, the acquisition of memory characteristics in host T cells shown here is likely to be a peripheral event. Although the possibility exists that alloreactive memory T cells could persist in recipients with GVHD,[38,39] the present data showed that host T cells acquired a memory phenotype during the early period after BMT. This result was

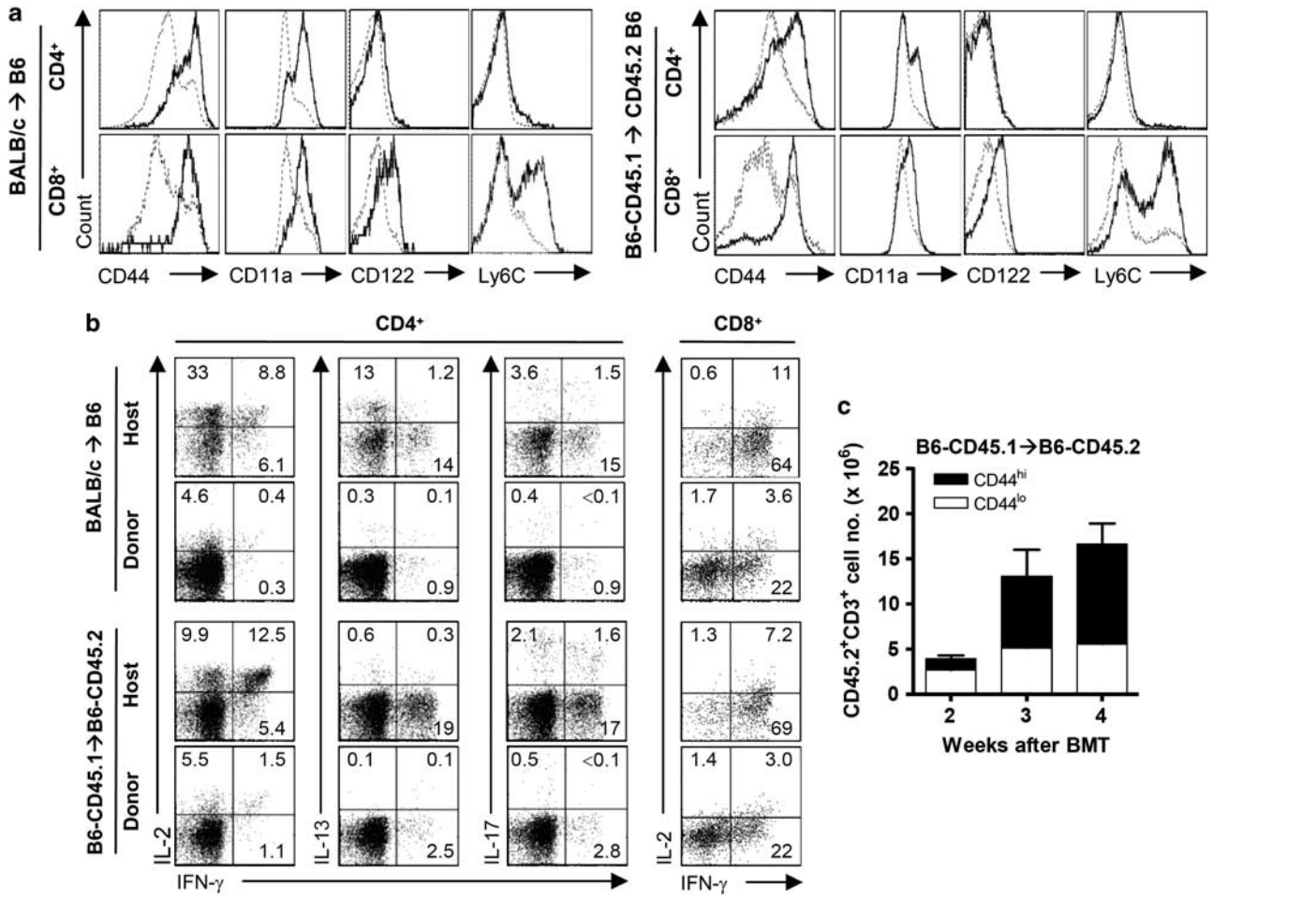

Figure 5 Acquisition of a memory phenotype by T cells in the periphery. (**a**) Host T cells have an activated memory phenotype in the periphery. Splenocytes from BALB/c-to-B6 and CD45.1 B6-to-to CD45.2 B6 BM chimeras were stained 6 weeks after BMT and expression of activation markers on host T cells (thick line) was compared with that of donor T cells (dotted line) after gating on $CD4^+$ or $CD8^+$ cells. Donor and host T cells were identified based on the expression of H-$2k^b$ (BALB/c-to-B6) or CD45.1 (CD45.1 B6-to-CD45.2 B6). The data are representative of four independent experiments, each involving two mice. (**b**) Host T cells secreted multiple cytokines following *ex vivo* stimulation. Total splenocytes from BMT chimeras were activated with phorbol 12-myristate 13-acetate and ionomycin, and stained with anti-IL-2, anti-IFN-γ, anti-IL-13 and anti-IL-17 antibodies. Cell populations secreting each cytokine were analyzed after gating on $CD4^+$ or $CD8^+$ cells, and donor and host T cells were identified based on the expression of H-$2k^b$ (BALB/c-to-B6) or CD45.1 (CD45.1 B6-to-CD45.2 B6). The numbers indicate the percentage of cells in each quadrant. The data are representative of three independent experiments. (**c**) Host T-cell numbers, especially of the memory phenotype population, rapidly increased in spleens during the early period after BMT. Absolute numbers of naïve (open bar) and memory (closed bar) phenotype host T cells in spleens of CD45.1 B6-to-CD45.2 B6 BM chimeras (n=3–5) are presented. BM, bone marrow; BMT, BM transplantation; IFN, interferon; IL, interleukin.

observed even in syngeneic chimera where alloreactions do not occur, strongly suggesting that homeostatic proliferation rather than allostimulation drives host T cells to acquire a memory phenotype. This notion is supported by the finding that the acquisition of a memory phenotypic by host T cells was closely correlated with the potential for homeostatic expansion in TCR-transgenic recipients such as OT-I and OT-II mice. BMT conditioning for therapeutic purposes renders recipients lymphopenic, affording room for expansion of residual and newly generated T cells. Opportunities for homeostatic expansion are likely to favor host T cells (in terms of competition for space), as host T cells repopulated the lymphoid organs prior to the generation of mature donor T cells in our irradiated BM chimeras. Due to the limited self-renewal of thymic T-cell precursors following BMT, the numbers of newly formed thymic emigrants that entered the periphery drastically declined 4 weeks after irradiation. This may explain why the T-cell ratio is reversed (on a population basis) between the donor and host.

Because homeostatic expansion represents T-cell proliferation in response to endogenous self-peptide/MHC ligands,[30] T cells expanded in this way are prone to develop autoimmune characteristics.[40] Recent data support the idea that homeostatic expansion is associated with the higher susceptibility to various autoimmune diseases in murine models and human patients.[41–43] In our cGVHD model, only host T cells caused the pathology after adoptive transfer, despite the fact that both host and donor T cells escaped negative selection mediated by host DCs. Thus, our results raise the possibility that preferential expansion of autoreactive host T cells soon after allo-BMT, by homeostatic proliferation, increases the likelihood of the development of cGVHD.

Until now, the established models of cGVHD have been developed on the assumption that donor T cells play an essential role in its development through the activation of host B cells or fibroblasts.[5] In another proposal, the autoreactivity behind cGVHD stems from a mutual interaction between donor APCs and donor T cells that have

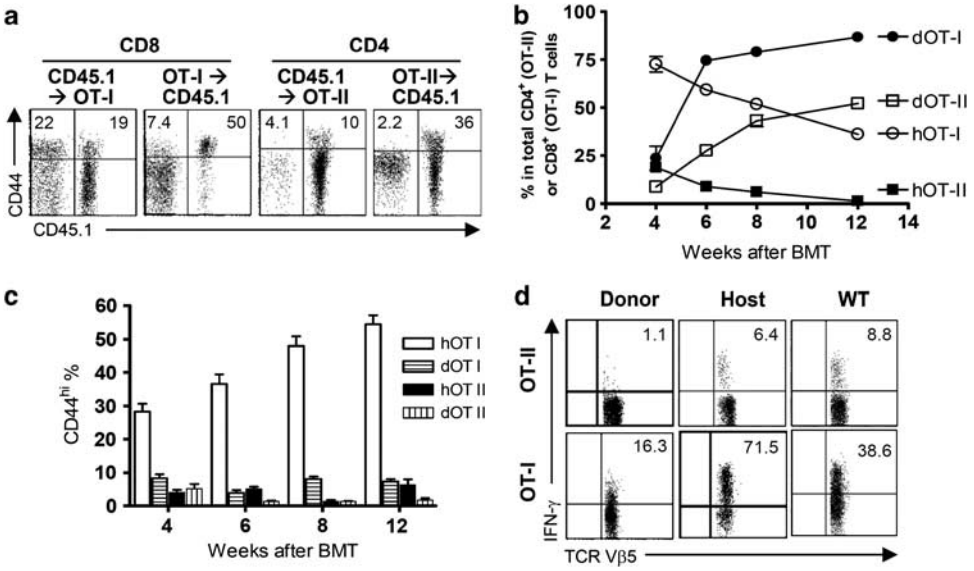

Figure 6 Comparative analysis between OT-I and OT-II host T cells suggests that their expansion and acquisition of a memory phenotype depend on homeostatic proliferation. (**a**) Host OT-I T cells, but not OT-II T cells, have a memory phenotype. B6-CD45.1, B6-CD45.2 OT-I and B6-CD45.2 OT-II mice were used as donors or hosts as indicated. Splenocytes from chimeric mice were stained for CD45.1, CD4, CD8 and CD44, and expression of CD44 on $CD4^+$ and $CD8^+$ T cells was analyzed 12 weeks after BMT. Donor and host cells were identified based on the expression level of CD45.1. The numbers indicate the percentages of $CD45.1^+$ and $CD45.1^-$ cells positive for $CD44^{hi}$. (**b**, **c**) Percentages of host OT-I and OT-II cells (**b**) in the overall $CD8^+$ and $CD4^+$ T-cell populations in PBMCs, and percentages of donor and host OT-I and OT-II cells expressing $CD44^{hi}$ (**c**) (n=7). (**d**) The $CD44^{hi}$ phenotype was correlated with IFN-γ secretion. Eight weeks after BMT, OT-I and OT-II cells were isolated from wild-type (WT) and chimeric mice, and activated with phorbol 12-myristate 13-acetate and ionomycin. Their secretion of IFN-γ was then analyzed. The numbers indicate the percentage of cells in each quadrant. The data are representative of two independent experiments. BM, bone marrow; BMT, BM transplantation; IFN, interferon; PBMC, peripheral blood mononuclear cell.

escaped thymic negative selection.[26,44] Our model of cGVHD shares some similarities with this suggestion in terms of the contribution of post-thymic T cells to the autoimmune pathology. However, the present data have some distinctive features: host T-cell precursors and APCs surviving high-dose irradiation were the key culprits in the development of cGVHD; and homeostatic expansion followed by the development of an autoimmune state, rather than an alloresponse, seems to be the major pathogenic mechanism inducing cGVHD; finally, none of these processes shows any relationship with humoral immunity (Figure 1).

In summary, cGVHD may have resulted from interactions between post-thymic host T cells and radioresistant host APCs in the periphery under particular conditions. T-cell-depleted BMCs should be transplanted into hosts with functioning thymuses and the preconditioning protocol should involve complete depletion of mature T cells and intrathymic DCs in the host, with the preservation of intrathymic radioresistant T-cell precursors and peripheral DCs. Unidentified host factors are also suggested to contribute to the development of GVHD. Under the allo-BMT conditions in humans, preconditioning protocols, genetic background and the presence of mature T cells in donor grafts may contribute to the development of GVHD.[45] We propose, based on the present data, that host T-cell-dependent autoimmunity is one of the potential mechanisms by which cGVHD develops.

METHODS

Mice

MHC class-$II^{-/-}$, $\beta2m^{-/-}$, TCR $C\alpha^{-/-}$, CD45.1-congenic B6 (B6-CD45.1) and OT-I or OT-II TCR-transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C.B17 SCID mice were obtained from the Animal Resource Center (Canning Vale, WA, Australia), and CBA and DBA mice were acquired from the Shizuoka Laboratory Center (Hamamatsu, Japan). (Note: the C.B17 mouse has the same genetic background as BALB/c mice except in the IgH chain locus.) NOD mice were purchased from the Korea Research Institute of Bioscience and Biotechnology (Ochang, Korea), and BALB/c mice were obtained from KOATECH (Pyeongtaek, Korea). BALB/c $RAG^{-/-}\gamma c^{-/-}$ mice were provided by CW Kim (Seoul National University, Seoul, Korea) with permission from the Central Institute for Experimental Animals (CIEA) in Japan. All mice were maintained under specific pathogen-free conditions in the animal facility at the Center for Animal Resource Development, Seoul National University College of Medicine. Experiments were performed under the approval of the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University.

Antibodies and flow cytometric analysis

The following fluorochrome- or biotin-labeled monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA, USA), eBioscience (San Diego, CA, USA), Miltenyi Biotec (Auburn, CA, USA) or Dinona (Seoul, Korea): anti-mouse CD3 (145-2C11), CD4 (RM4.5), CD5 (53-7.3), CD8 (53-6.7), CD11a (M17/4), CD11b (M1/70), CD11c (HL3), CD24 (M1/69), CD25 (PC61 or 7D4), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD80 (16-10A1), CD86(GL1), CD117 (3C1), Sca-1 (D7), B220 (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), CD122 (TM-β1), NK1.1 (PK136), TCRβ (H57-597), H-$2K^b$ (AF6-88.5), H-$2K^d$ (SF1-1.1), I-$A^{b/d}$ (M5/114.15.2), TCR γδ (GL3), Ly6C/G (RB6-8C5), PD-1 (J43), IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-13 (JES10-5A2), IL-17 (TC11-18H10) and foxp3 (FJK-16s). Anti-mouse Vβ2 (B20.6), Vβ3 (KJ25), Vβ4 (KT4), Vβ5.1 and 5.2 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8 (F23.1), Vβ10 (B21.5), Vβ11 (RR3-15), and Vβ13 (MR12-3) antibodies were purchased from BD Pharmingen. Cell preparations from spleen, thymus and lymph node were resuspended in flow cytometry buffer (phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% sodium azide). Hepatic lymphocytes were prepared as described

previously.[46] Briefly, liver samples were homogenized and hepatocyte-rich matrix was removed by centrifugation for 1 min at 30 *g*. The supernatants were harvested and lymphocytes were separated by density-gradient centrifugation in Lymphocyte-M solution (Cedarlane, Burlington, ON, Canada). After staining with fluorophore-conjugated antibodies for 30 min at 4 °C, live cells (gated as the propidium iodide-negative population) were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) in conjunction with the CellQuest Pro software (Becton Dickinson).

Intracellular staining

Total splenocytes were stimulated with 50 ng ml^{-1} phorbol 12-myristate 13-acetate and 1.5 μM ionomycin (Sigma, St Louis, MO, USA) for 5 h at 37 °C. Brefeldin-A (10 μg ml^{-1}) (Sigma) was applied for the final 3 h of stimulation. Stimulated cells were then surface-stained, fixed using a Cytofix/Cytoperm kit (BD Pharmingen) and stained with antibodies specific for intracellular cytokines.

BM chimeras and adoptive transfer

Recipient mice were irradiated with 1300 rad (two doses of 650 rad applied 4 h apart) from a ^{137}Cs source and then rested for approximately 4–24 h before receiving BMCs.[47] Total BMCs were prepared from the femurs and tibiae of donor mice, and mature T cells were then depleted through magnetic sorting, performed using a cocktail of CD4 and CD8 microbeads (MCAS; Miltenyi Biotec), and purity was routinely >99% (Supplementary Figure 8a). LSK (lin$^-$sca-1$^+$c-kit$^+$) stem cells were obtained by fluorescence-activated cell sorting of c-kit$^+$ and Sca-1$^+$ cells from lineage-negative BMCs that were purified by MACS (magnetic activated cell sorter, Lineage Cell Depletion Kit; Miltenyi Biotec). Each recipient mouse received approximately 3–5×10^6 T-cell-depleted BMCs or 5000 LSK cells, in 300 μl of phosphate-buffered saline, through lateral tail vein injection.

For adoptive transfer of donor or host splenic T cells, donor (H-2K^{d+}CD4$^+$ and H-2K^{d+}CD8$^+$) and host (H-2K^{b+}CD4$^+$ and H-2K^{b+}CD8$^+$) T cells were isolated from BALB/c-to-B6 BM chimeras by flow cytometric sorting and a purity of >95% was achieved (Supplementary Figure 8b). Each 1.5×10^6 cells were transferred to thymectomized B6 mice that were lethally irradiated and transplanted with T-cell-depleted BMCs from BALB/c mice five weeks ago.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were dewaxed in xylene, rehydrated using a graded alcohol series and incubated in an endogenous peroxide-blocking solution for 5 min. Antigen retrieval was performed through incubation in 6 mM citrate buffer at 99 °C for 20 min using the Leica Bond Max system (Leica, Wetzlar, Germany), and nonspecific staining was prevented by treating tissue sections with rabbit serum (1% in phosphate-buffered saline) for 30 min. Anti-mouse CD3 (SP7) (Abcam, Cambridge, UK), diluted 1:300, was applied for 30 min and antibody binding was detected using a VECTASTAIN Elite ABC kit (PK6101; Vector Laboratories, Burlingame, CA, USA).

Histopathologic examination of tissue sections

Formalin-fixed, paraffin-embedded tissues were sectioned to a thickness of 4 μm and stained with hematoxylin and eosin. Pathologic scoring of skin lesions was subsequently performed as described previously.[48] Masson's trichrome staining was performed using a Trichrome Stain kit (Sigma).

Assessment of GVHD

The incidence of skin lesions was evaluated by the presence of obvious areas of denudation on the head and back that were confirmed by pathologic examination. Serum AST and ALT levels were measured using an automated chemistry analyzer (model 7070; Hitachi, Tokyo, Japan).

Thymectomy and thymus graft

Thymectomy and thymus grafting were performed as described previously.[44] Briefly, mice were anesthetized with isoflurane (Choongwae Pharma Corp., Hwaseong, Korea) and their parotid glands were bisected along the midline through midline skin incisions. Thymic tissues were aspirated using a 2-mm-diameter glass pipette through the suprasternal notch and the skin was then sutured with 5-0 nylon (Silkam; Aesculap AG, Tuttlingen, Germany). Complete thymectomy was confirmed by the absence of CD4 CD8 double-positive cells in mediastinal soft tissue at the end of the experiment. Incompletely thymectomized mice were excluded from the results. Embryonic day 15.5 fetal thymuses were grafted under the kidney capsule through left flank incisions under anesthesia. The presence of thymic tissues in the kidneys was confirmed histologically.

Mixed-lymphocyte reaction and cytokine analysis

T cells (2×10^5) purified by fluorescence-activated cell sorting or MACS were stimulated for 3 days with T-cell-depleted BMCs and irradiated (2000 cGy) B6 or BALB/c splenocytes (6×10^5) in RPMI medium (Gibco, Carlsbad, CA, USA). Cultures were pulsed with 1 μCi well^{-1} [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ, USA) for the final 16 h of the incubation period and mean incorporation of thymidine was measured in triplicate wells. Culture supernatants were analyzed using a Mouse Group I Cytokine 23-Plex Panel in conjunction with a Bio-Plex instrument (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were analyzed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Bar graphs showing the percentage of each cell type or concentration of each cytokine represent the mean ± s.d. Data were compared by unpaired Student's *t*-tests.

ACKNOWLEDGEMENTS

We thank DH Chung (Department of Pathology, Seoul National University, College of Medicine) for helpful discussion and criticism of our manuscript. This work was supported by grants from the National Research Foundation (NRF) through the Tumor Immunity Medical Research Center at Seoul National University College of Medicine, Korea (R13-2002-025-01003-0).

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)

Supplementary Table 1. Development of chronic GVHD in major and minor antigen mismatched conditions

Donor → Host	Skin lesion incidence	AST (U/ml±SD)	ALT (U/ml±SD)
BALB/b (b)[a] → B6	6/8[b]	156±61**[c]	97±59*
B6.bm12 (b) → B6	4/8	146±66 (n.s.)	60±25*
BALB/c-RAG$^{-/-}\gamma c^{-/-}$ (b) → B6	5/8	n.d.	n.d.
BALB/c-RAG$^{-/-}\gamma c^{-/-}$ → B6.TCR C$\alpha^{-/-}$	0/8	n.d.	n.d.
BALB/c (d) LSK → B6[d]	3/6	n.d.	n.d.

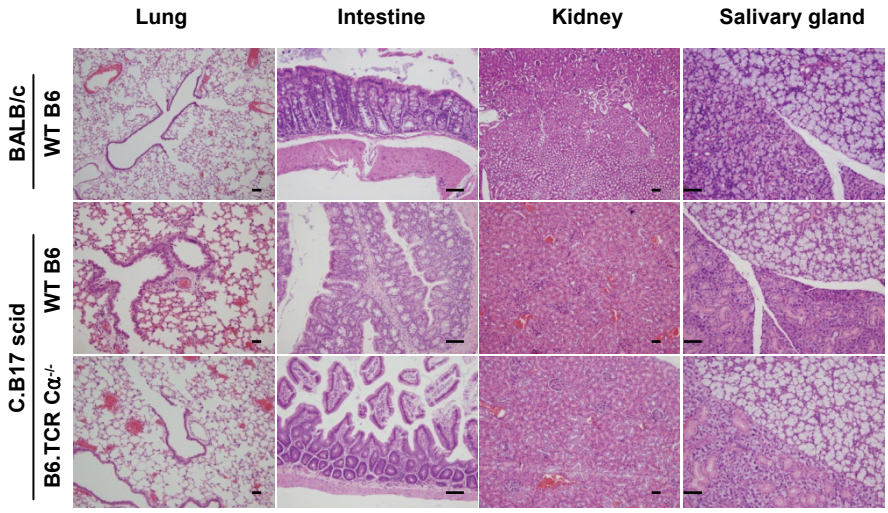
[a]: MHC haplotype

[b]: four mice died in the course of the experiments

[c]: *p* values are compared with those of C.B17 SCID (d) → B6.TCR C$\alpha^{-/-}$ BM chimeras in Table 1. *p<0.05, **p<0.01, n.s. not significant

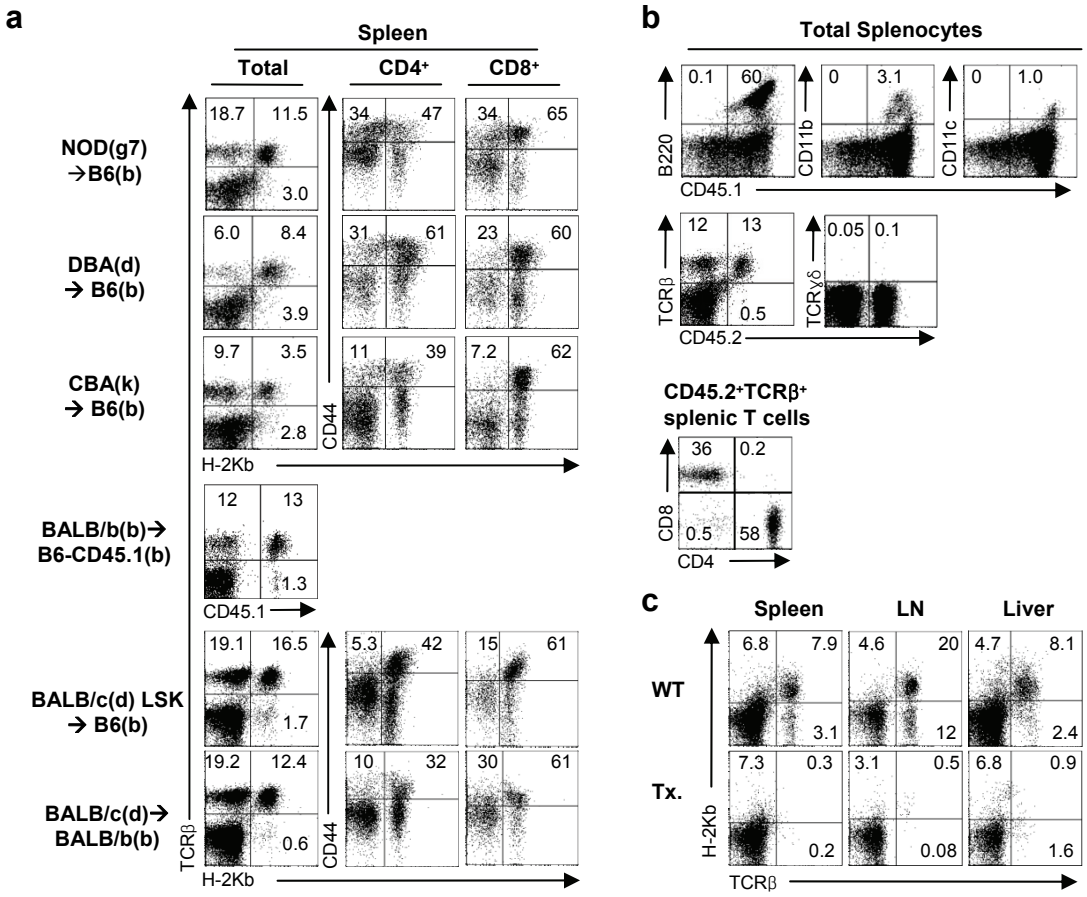
[d]: mice were all died before day 50, n.d., not done

Supplementary Figure 1.



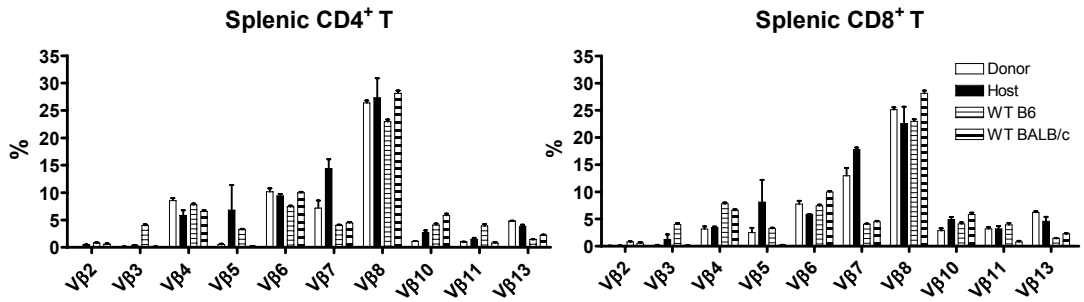
Chronic GVHD-like pathology is not found in the lung, intestine, kidney and salivary gland. Wild type (WT, top and middle) or TCR $C\alpha^{-/-}$ (bottom) B6 mice were lethally irradiated and received T cell-depleted BM cells from BALB/c (top) or C.B-17 SCID (middle and bottom) mice; they were then followed for 60 days. Representative histologic sections of lung, intestine, kidney and salivary gland are shown (bar = 100 μm).

Supplementary Figure 2.



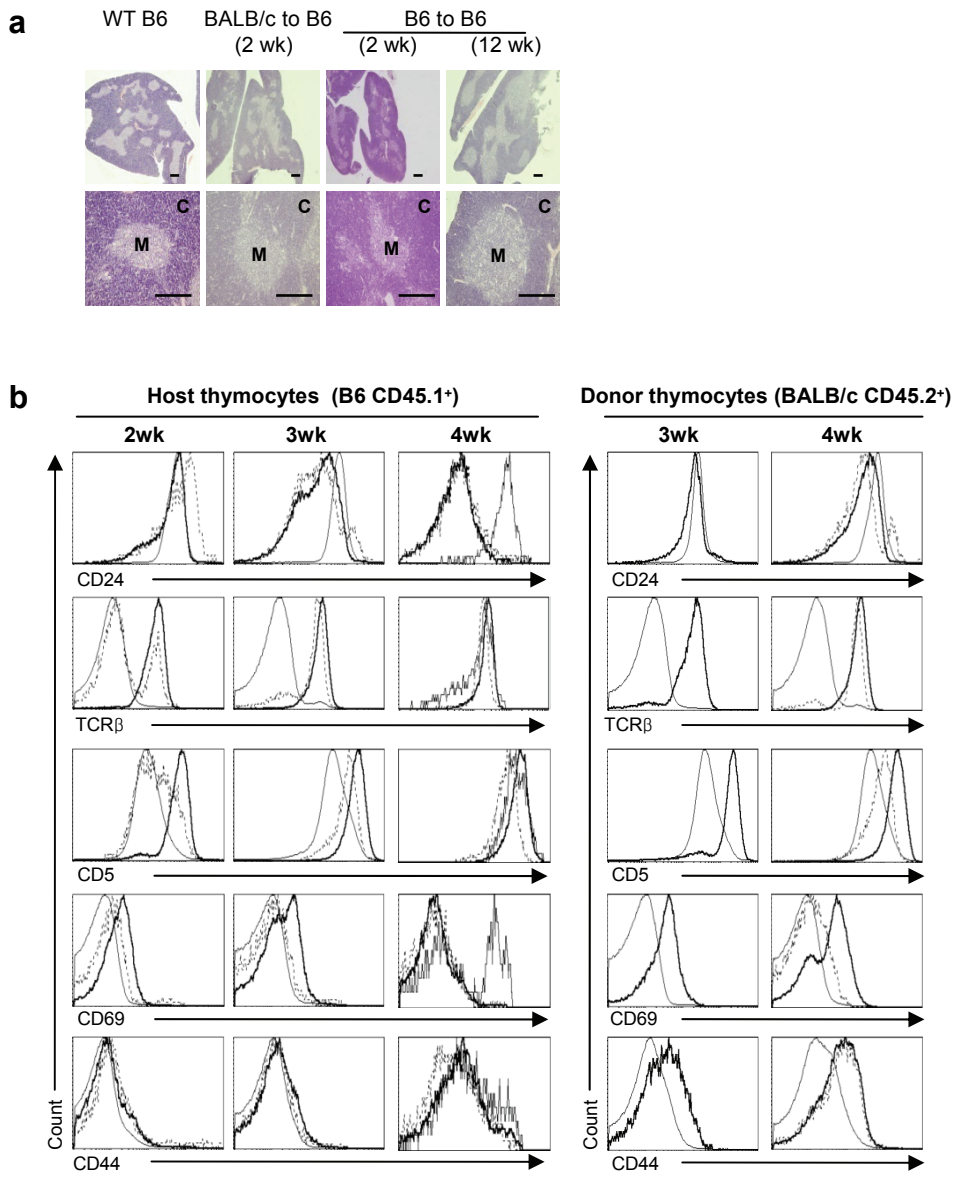
Developmental profile of host T cells in congeneic, major and minor allo-antigen mismatched and stem cell grafted BMT conditions. (a) B6 mice were lethally irradiated and transplanted with T cell-depleted BM cells of NOD (H-2^{g7}), DBA (H-2^{d}), CBA (H-2^{k}) and BALB/b (H-2^{b}) mice or BALB/c stem cell (LSK). In one group, T cell-depleted BM cells from BALB/c (H-2^{d}) mice were transplanted into irradiated BALB/B (H-2^{b}) mice. Six weeks later, mice were sacrificed, and splenocytes were analyzed to evaluate the repopulation status of host and donor cells. Numbers indicate the percentage of cells in each quadrant among the total cells (left column) or the percentage of CD44^{hi} cells among H-2K^{b} positive (host) or negative (donor) populations after gating of CD4^{+} or CD8^{+} T cells (middle and right columns). (b) Representative flow cytometric profile of splenocytes (6 weeks after BMT) and thymocytes (2 weeks after BMT) from B6-CD45.1 → B6-CD45.2 BM chimeras. Numbers indicate the percentage of cells in each quadrant. Representative data of three independent experiments are shown. (c) Generation of host T cells in spleen, lymph node (LN) and liver is dependent on thymi of host mice. WT or thymectomized (Tx) B6 mice were lethally irradiated and grafted with T cell depleted BM cells of BALB/c mice and analyzed the development of donor and host T cells 5 weeks after BMT. Numbers indicate percentage of cells in each quadrant.

Supplementary Figure 3.



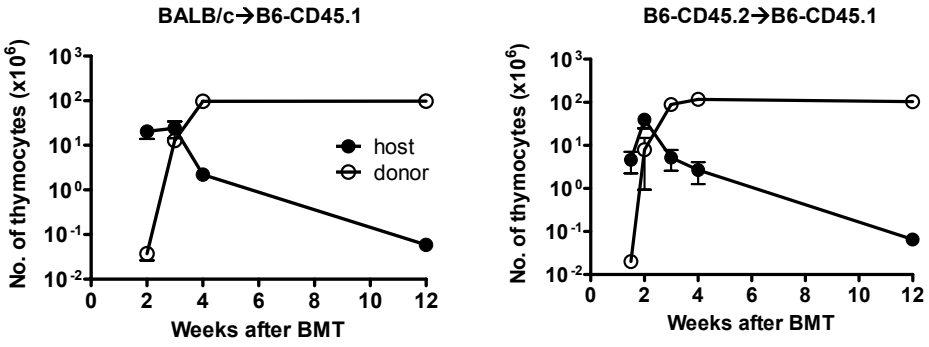
TCR Vβ usage of donor and host splenic T cells. Splenic $CD4^+$ or $CD8^+$ T cells from BALB/c → B6 BM chimeras were analyzed for their usage of TCRβ chains 6 weeks after BMT, and compared to those of WT B6 and BALB/c mice (n=3).

Supplementary Figure 4.



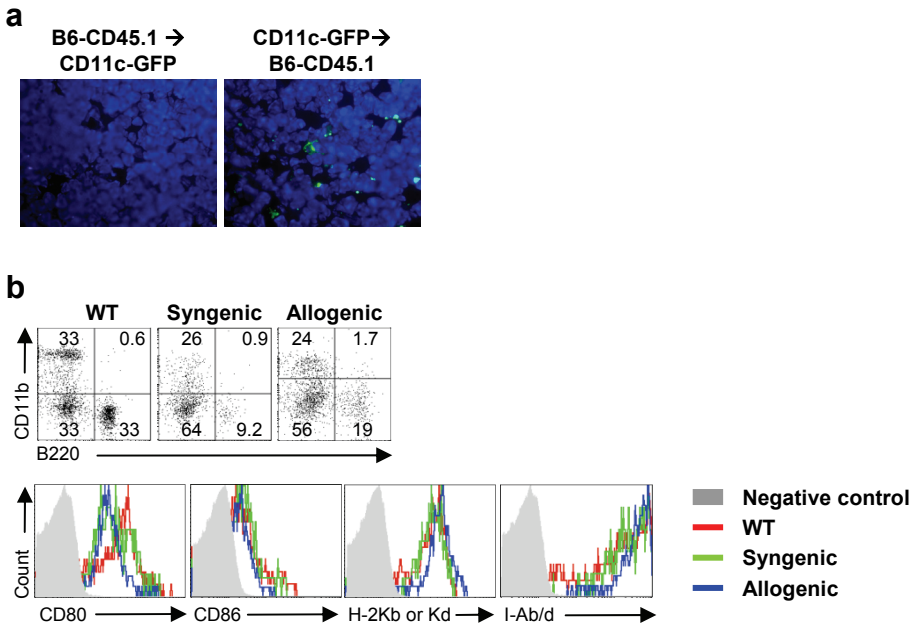
Comparison of intrathymic maturation process between donor and host thymocytes and analysis of intrathymic DCs. (a) Intact thymic architecture of after BMT. Cortex (C) and medullary (M) structures are well organized in thymi of allogenic (2 weeks after BMT) and syngenic BM chimera (2 and 12 weeks after BMT) compared to those of 8 weeks old wild type (WT) B6 mouse (bar = 100 μm). (b) B6-CD45.1 mice were lethally irradiated and received T cell-depleted BM cells from BALB/c mice. Host mice were sacrificed in the 2nd, 3rd and 4th weeks after BMT, and thymocytes were analyzed for their expression of CD24, TCRß, CD5, CD69 and CD44 after gating of double-positive (thin line) and $CD4^+$ (thick line) and $CD8^+$ single-positive (dotted line) thymocytes. Host and donor cells were identified based on their expression of CD45.1 and CD45.2, respectively. Representative data of two independent experiments are shown.

Supplementary Figure 5.



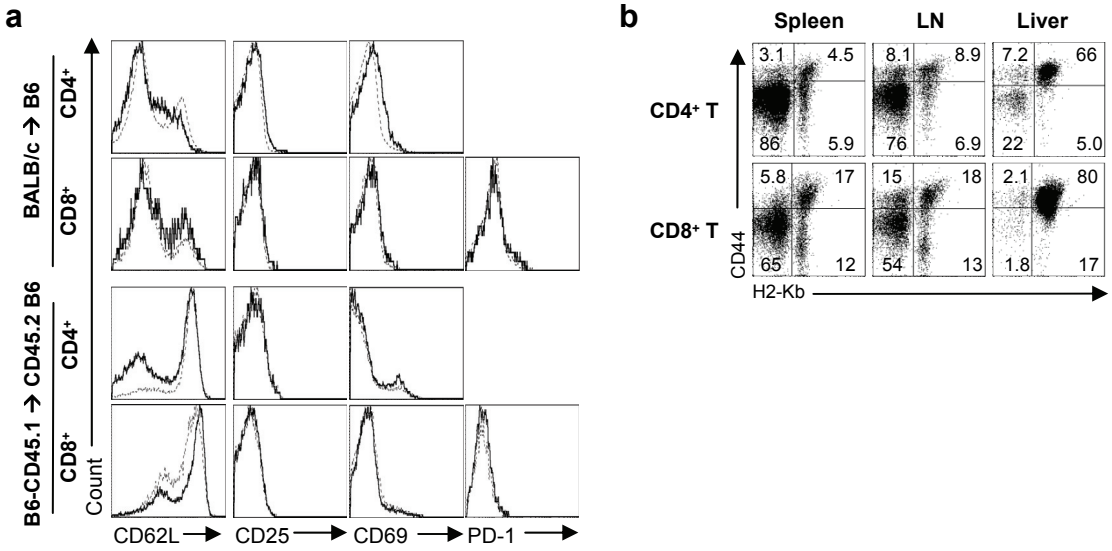
Developmental kinetics of intrathymic host and donor T cells. B6-CD45.1 mice were lethally irradiated and received T cell-depleted BM cells from BALB/c or B6-CD45.2 mice. Mice were sacrificed at each time point (n=2~5) and analyzed for the absolute number of donor and host thymocytes. No., number.

Supplementary Figure 6.



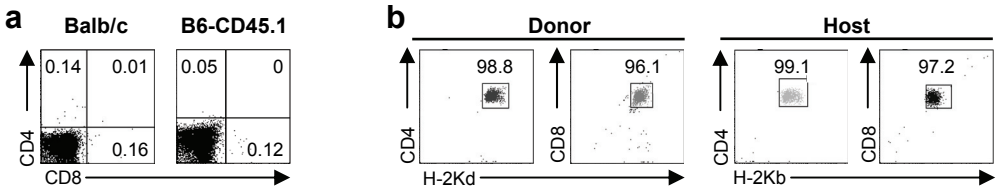
Analysis of DC population in BM chimeras. (a) To visualize the DC population in the thymus of BM chimeras, CD11c-GFP transgenic mice that express GFP molecules in CD11c^{+} DCs were used recipients (B6-CD45.1→CD11c-GFP^{tg}) or donor (CD11c-GFP^{tg}→B6-CD45.1) for BMT. At 5 week post-BMT, thymus were extracted and representative fluorescent microscopic images are shown after DAPI staining. (b) Comparative analysis of intrathymic DC subsets in BM chimeras and 6~8 weeks old WT B6 mouse. Donor CD11c^{+} cells were analyzed according to B220 and CD11b expression profiles in wild type (WT) and congenic and allogenic BM chimera after 3 weeks of BMT (top). DC maturation markers such CD80, CD86, MHC I (H-2Kb in congenic or H-2K^{d} in allogenic BM chimera) and MHC II (I-A$^{b/d}$) expression were also compared among thymic CD11c^{+} cells of WT, congenic and syngenic BM chimera mice. Representative data of two independent experiments are shown.

Supplementary Figure. 7



Memory phenotype of host T cells in spleen, lymph node and liver. (a) Splenocytes from BALB/c-to-B6 and CD45.1 B6-to-CD45.2 B6 BM chimeras were stained 6 weeks post-BMT and the expression of activation markers on host T cells (thick line) was compared with that of donor T cells (dotted line) after gating on $CD4^+$ or $CD8^+$ cells. Donor and host T cells were identified based on the expression of $H\text{-}2k^b$ (BALB/c-to-B6) or CD45.1 (CD45.1 B6-to-CD45.2 B6). Data are the representative of four independent experiments, each involving two mice. (b) To compare the host T cell population showing memory phenotype in spleen, lymph node (LN), and liver, mononuclear cells were isolated from each organ of BALB/c-to-B6 chimera after 8 weeks of BMT. Memory phenotype of $CD4^+$ or $CD8^+$ T cells was analyzed by their expression of CD44. Numbers indicate percentage of cells in each quadrant.

Supplementary Figure 8.



Sorting purity. (a) Magnetic depletion of mature $CD4^+$ and $CD8^+$ T cells from donor bone marrow cells. Each dot plot represents bone marrow cells after magnetic depletion and numbers indicate the percentage of cells in each quadrant. (b) Flow cytometric sorting of donor and host T cells from splenocytes of BALB/c-to-B6 BM chimera. Each dot plot represents the sorted population of donor ($H\text{-}2k^{d+}CD4^+$ and $H\text{-}2k^{d+}CD8^+$) and host ($H\text{-}2k^{b+}CD4^+$ and $H\text{-}2k^{b+}CD8^+$) T cells. Numbers indicate the purity of sorted cells.