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Thymic Damage, Impaired Negative Selection, and Development of Chronic Graft-versus-Host Disease Caused by Donor CD4+ and CD8+ T Cells

Tao Wu,*†‡ James S. Young,*†§ Heather Johnston,*†§ Xiong Ni,*†‡ Ruishu Deng,*† Jeremy Racine,*†§ Miao Wang,*† Audrey Wang,‡ Ivan Todorov,* Jianmin Wang,‡ and Defu Zeng*†‡

Prevention of chronic graft-versus-host disease (cGVHD) remains a major challenge in allogeneic hematopoietic cell transplantation (HCT) owing to limited understanding of cGVHD pathogenesis and lack of appropriate animal models. In this study, we report that, in classical acute GVHD models with C57BL/6 donors and MHC-mismatched BALB/c recipients and with C3H.SW donors and MHC-matched C57BL/6 recipients, GVHD recipients surviving for >60 d after HCT developed cGVHD characterized by cutaneous fibrosis, tissue damage in the salivary gland, and the presence of serum autoantibodies. Donor CD8+ T cells were more potent than CD4+ T cells for inducing cGVHD. The recipient thymus and de novo–generated, donor-derived CD4+ T cells were required for induction of cGVHD by donor CD8+ T cells but not by donor CD4+ T cells. Donor CD8+ T cells preferentially damaged recipient medullary thymic epithelial cells and impaired negative selection, resulting in production of autoreactive CD4+ T cells that perpetuated damage to the thymus and augmented the development of cGVHD. Short-term anti-CD4 mAb treatment early after HCT enabled recovery from thymic damage and prevented cGVHD. These results demonstrate that donor CD8+ T cells cause cGVHD solely through thymic-dependent mechanisms, whereas CD4+ T cells can cause cGVHD through either thymic-dependent or independent mechanisms.

Donor CD8+ T cells are more potent than CD4+ T cells in facilitating stem cell engraftment and mediating graft-versus-lymphoma/leukemia (GVL) effects, but both CD4+ and CD8+ T cells mediate severe graft-versus-host disease (GVHD) in mice and humans (1–12). GVHD can be divided into acute (aGVHD) and chronic (cGVHD) based on different clinical manifestations and histopathology. aGVHD usually begins within 100 d after hematopoietic cell transplantation (HCT) and is characterized by acute tissue inflammation and infiltration of alloreactive lymphocytes in GVHD target organs such as colon, skin, and liver (13). cGVHD usually begins >100 d after HCT as an autoimmune scleroderma- and lupus-like syndrome characterized by autoantibody production, chronic inflammation, and collagen deposition in target tissues (14–18). cGVHD and aGVHD can both affect the skin, liver, and gastrointestinal tract, but cGVHD also affects prototypical target organs such as salivary gland (14–16). Although some cGVHD can occur without prior aGVHD, cGVHD often overlap with persistent, recurrent, and late aGVHD, and most cGVHD occurs after aGVHD (14–16, 19). Many murine models have been used to examine the pathophysiology of aGVHD or cGVHD (20–26), but none of these models clearly reflects the transition from aGVHD to cGVHD that typically occurs in humans. Additionally, the role of donor CD8+ T cells in cGVHD induction remains unclear, as all mouse cGVHD models focus on CD4+ T cells.

Medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) play important roles in central deletion of autoreactive T cells (27, 28). Because cGVHD often follows aGVHD, it has been proposed that cGVHD results from impaired negative selection in the thymus caused by alloreactive T cells during aGVHD, allowing for de novo generation of donor-derived T cells that recognize recipient tissues (29–33), but the role of damaging mTECs has not clearly been documented. Bone marrow cells from MHC class II (MHC II)x−/− mice give rise to autoreactive CD4+ T cells that mediate cGVHD in recipients conditioned with high-dose total body irradiation (TBI) owing to a defect in thymic DC-mediated negative selection (34). In this model, however, the role of thymic...
epithelial cells remains unknown, and the development of autoantibodies was not reported. These issues have not been addressed in other cGVHD models (20). In the present studies, we explore whether aGVHD mediated by donor CD4+ or CD8+ T cells can develop into characteristic cGVHD in murine models, and we explore the roles of thymic mTECs and DCs in the generation of autoreactive T cells early after HCT.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from the National Cancer Institute animal production program (Frederick, MD). Thymectomized and control euthymic BALB/c as well as CD4+ T cell– or CD8+ T cell–deficient C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Rag-2−/− BALB/c and Rag-2−/− C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Mice were maintained in a pathogen-free room in the City of Hope Animal Resource Center (Duarte, CA). All animal protocols were approved by the City of Hope Institutional Animal Care and Use Committee.

Statistical analysis

Clinical cutaneous damage scoring and survival in different groups were compared by using the rank sum test or log-rank test (Prism, version 5.0; GraphPad Software, San Diego, CA). Comparison of two means was analyzed using an unpaired two-tailed Student t test.

Abs, flow cytometry analysis, and cell sorting

FITC-Ly51 (6C3), FITC-CD45.1 (A20), FITC-1A1/1-2E (2G9), FITC-Vβ3 (K25), FITC-Vβ4 (KT4), FITC-Vβ5.1/5.2 (MR9-4), FITC-Thy1.2 (30-H12), PE-B220 (RA3-6B2), allophycocyanin-Cy7-CD8α (53-6.7), allophycocyanin-Cy7-CD4 (GK-1.5), and PE-streptavidin were purchased from BD Pharmingen (San Diego, CA). eFluor 450-epithelial cellular adhesion molecule (EpCAM) (G8.8), eFluor 450-CD4 (RM4-5), PE-Cy7-CD8α (53-6.7), allophycocyanin-TCRβ (H57-597), allophycocyanin-B20 (RA3-6B2), PE-H-2Kb (AF6-88.5.5.3), allophycocyanin-CD11c (N418), and FITC-CD11c (N418) were purchased from eBioscience (San Diego, CA). Biotinylated Ulex europaeus agglutinin (UEA) I was purchased from Vector Laboratories (Burlingame, CA). All ophycocyanin-CD45 (clone 30F11) was purchased from Miltenyi Biotec (Auburn, CA). Aqua fluorescent reactive dye for viability analysis was purchased from Invitrogen (Carlsbad, CA). Flow

FIGURE 1. Low dose of donor spleen cells induced cGVHD. Lethally irradiated BALB/c recipients were transplanted with titrated numbers of spleen cells (5–1.25 × 10⁶) and TBCD-BM cells (2.5 × 10⁶) from C57BL/6 donors. Control recipients were given TBCD-BM cells alone. Recipients were monitored for clinical GVHD, including body weight changes, diarrhea, hair loss, and survival († indicates death of all recipients in a group). Fifteen and sixty days after HCT, recipient serum samples were tested for presence of autoantibodies by staining donor-type Rag-2−/− C57BL/6 skin and salivary gland tissues. Sixty days after HCT, recipients were measured for percentage and yield of CD4+CD8+ thymocytes. (A–D) Percentage of body weight changes, percentage of recipients without diarrhea, clinical cutaneous GVHD score, and percentage of survival. Each group contained 12 recipients combined from three replicate experiments. (E) Representative photomicrographs (original magnification ×200) of GVHD recipient serum autoantibody staining of Rag-2−/− skin and salivary gland tissues. DAPI staining is shown in blue, and autoantibody staining is shown in green. One representative result is shown from eight samples evaluated in each group. (F) One representative of CD4+CD8+ thymocytes staining pattern is shown of eight recipients examined in each group. The mean ± SE of yield of CD4+CD8+ thymocytes (n = 8).

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cytometric data were analyzed with FlowJo software (Tree Star, Ashland, OR) as described in our previous publications (35–37).

**Induction and assessment of GVHD**

Mice were exposed to 850 cGy TBI with the use of a [$^{137}$Cs] source 8 h before HCT. Recipients were injected with T and B cell–depleted donor bone marrow (TBCD-BM) cells and whole splenocytes or sorted CD4+ T or CD8+ T cells. T and B cells in BM were depleted with the use of biotin-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-B220 mAb and streptavidin-conjugated micromagnetic beads, followed by passage through an autoMACS cell sorter (Miltenyi Biotec). The purity of depletion was >99%. CD4+ and CD8+ T cells were isolated from donor spleen suspensions with the use of biotin-conjugated anti-CD4 or anti-CD8 mAb and streptavidin-conjugated micromagnetic beads, and passage through an autoMACS cell sorter (Miltenyi Biotec). The purity of the positive fraction was >95%. The assessment and scoring of clinical cutaneous GVHD were described in our previous publications (35, 37, 38).

**Histopathology**

Tissue specimens were fixed in formalin before embedding in paraffin blocks, cut, and stained with H&E. Slides were examined at ×200–400 magnification and visualized with an Olympus BX51 and a Pixera (600CL) cooled charge-coupled device camera (Pixera, Los Gatos, CA). Tissue damage was blindly assessed according to a scoring system described previously (35, 37).

**Proliferation assays**

De novo–generated CD4+ T cells were sorted with flow cytometry with anti-CD45.1 (marker for de novo–generated donor cells), anti-CD4, and anti-Thy1.2. Proliferating CD4+ T cells were measured as previously reported (35, 37). Briefly, sorted CD4+ T cells (2×10^5) were incubated with irradiated DCs (1×10^6) in complete RPMI 1640 media for 4 d, with tritiated thymidine deoxyribose added to the culture 18 h before harvest. Cultures were established in triplicates. The stimulation index was calculated according to the formula: [(cpm of culture of responder with stimulator) − (cpm of culture of responder alone)]/(cpm of culture of responder alone).

**Thymic epithelial cell staining**

Thymic epithelial cell isolation was performed according to a protocol from Dr. Marcel van den Brink’s Laboratory (39). In brief, the thymus was cut into small (<0.25 cm^2) pieces and placed in complete RPMI 1640 media with collagenase D and DNase I. Thymic fragments were rapidly pipetted through the aperture of a 1000-μl pipette tip and incubated in a 37°C water bath to digest the thymus and release epithelial cells from the extracellular matrix. Supernatants were harvested every 15 min, and the process was repeated three additional times. For the fourth digestion, dispase was added to release additional thymic epithelial cells. Fractions were stained with anti-EpCAM, CD45, UEA, and Ly-51. Cortical epithelial cells were defined as EpCAM+CD45+UEA−Ly-51highLy-51low, whereas medullary epithelial cells were defined as EpCAM+CD45+UEA−Ly-51highLy-51low. Cells from six thymi were combined to provide sufficient cells for analysis.

**Serum staining of Rag-2−/− mouse skin or salivary gland tissues**

Serum autoantibodies were measured by staining Rag-2−/− mouse skin and salivary gland tissues as previously described (40). In brief, cryosections from Rag-2−/− C57BL/6 and BALB/c mice were prepared by soaking tissues in 10% formalin for 1 h followed by dehydration in a solution of 30% sucrose in PBS. Tissue was then frozen in OCT gel and cryosectioned. Cryosections were placed in acetone at −20°C for 15 min and then rehydrated in PBS containing MgCl2. Tissues were then incubated overnight with 5×-diluted serum and washed again. Tissues were then stained with anti-mouse IgG-Alexa Fluor 488 and DAPI for 2 h and washed. Representative photomicrographs were taken of each tissue at ×200 with the use of an Olympus BX51 and a Pixera (600CL) cooled charge-coupled device camera (Pixera).

**Results**

**Low-dose donor spleen cells enable recipient survival and development of cGVHD**

Transplantation of BM and spleen cells from C57BL/6 donor into lethally irradiated BALB/c recipients has been considered an aGVHD model for more than three decades (21, 41), and autoreactive CD4+ T cells were detected in the recipients early after HCT (42–44). To test whether cGVHD developed in BALB/c recipients transplanted with C57BL/6 donor BM and spleen cells, TBCD-BM cells (2.5×10^6) were injected with or without titrated dose of donor spleen cells (5×1.25×10^6) into lethally irradiated recipients. Recipients were monitored for body weight change, clinical manifestations of diarrhea and hair loss, and survival. Additionally, at days 15 and 60 after HCT, the presence of autoantibodies in the recipient was measured by staining the skin and salivary gland tissues of donor-type Rag-2−/− C57BL/6 mice and recipient-type Rag-2−/− BALB/c mice, as previously described (40). Histopathologies of colon, jejunum, skin, and salivary gland, as well as percentage and yield of CD4+ CD8+ thymocytes, were also compared.

The recipients given TBCD-BM alone showed gradual recovery of body weight and no signs of diarrhea or hair loss, and all survived for >60 d. In contrast, recipients given 5 or 2.5×10^6 spleen cells showed severe body weight loss and diarrhea and all died by 30 or 45 d after HCT (Fig. 1A–D). Approximately 60% of recipients given low-dose (1.25×10^6) spleen and TBCD-BM cells showed mild diarrhea, and 85% survived for >60 d after HCT, but they showed gradual body weight loss and hair loss beginning at ~45 d after HCT (Fig. 1A–D).

Our recent report showed that in a cGVHD model with DBA/2 donors and BALB/c recipients, cGVHD characteristic features...
became obvious at ~45 d after HCT. Other manifestations included serum autoantibodies, expansion of dermis, loss of fat, increased collagen deposition in the skin, loss or blunting of intestinal crypts in the jejunum, and infiltration and loss of secretory follicles in the salivary gland (35, 37). We tested whether similar manifestations developed in the classical aGVHD model using C57BL/6 donors and BALB/c recipients. Sera from control GVHD-free BALB/c recipients at 60 d after HCT and sera from GVHD recipients at 15 d after HCT showed little diarrhea, and most survived for 30 d. Recipients given 0.1 \times 10^6 CD8^+ T cells showed more severe weight loss and hair loss 40 d after HCT (p < 0.01). The skin tissues of GVHD recipients showed epidermal hyperplasia and tissue infiltration at 15 and 30 d after HCT, but these manifestations appeared to be reduced by 60 d after HCT (Fig. 2C). Instead, we observed significant expansion of the dermis with collagen deposition together with loss of hair follicles and fat tissues (Fig. 2C, Supplemental Fig. 1B). Compared to GVHD-free control TBCD-BM recipients, salivary gland tissues of GVHD recipients were mildly damaged at day 15, moderately damaged by day 30, and severely damaged by day 60 after HCT (p < 0.01, Fig. 2D). Taken together, BALB/c recipients given low-dose donor C57BL/6 spleen cells developed typical clinical and histopathological features of cGVHD by 60 d after HCT.

aGVHD in C57BL/6.SJL (H-2K^b, CD45.1) recipients induced by CD8^+ T cells from MHC-matched but minor histocompatibility Ag-mismatched C3H.SW (H-2K^b, CD45.2) donors has been described as evolving to cGVHD at late time points after HCT (33), but the characteristic features of cGVHD were not reported. In the present study, recipients given CD8^+ T cell–enriched spleen cells showed gradual body weight loss and hair loss starting at ~30 d after HCT (Supplemental Fig. 2A, 2B), whereas control recipients given TBCD-BM did not develop these changes. By 60 d after HCT, the GVHD recipients developed characteristic cGVHD features with serum autoantibody production, loss of CD4^+CD8^+ thymocytes, and chronic tissue damage in the jejunum, skin, and salivary gland (Supplemental Fig. 2D–F).

Low-dose C57BL/6 donor CD8^+ T cells are more potent than CD4^+ T cells in mediating cGVHD, although high-dose CD4^+ T cells were more potent at mediating aGVHD

The GVHD model of C57BL/6 donor to BALB/c recipient has been characterized as a CD4^+ T cell–dependent model (21). We com-

**FIGURE 3.** High doses of donor CD4^+ T cells induced more severe aGVHD but low doses of donor CD8^+ T cells induced more severe cGVHD. Lethally irradiated BALB/c recipients were injected with titrated numbers (5, 0.5, and $0.1 \times 10^6$) of sorted donor CD4^+ or CD8^+ T cells and TBCD-BM cells from C57BL/6 donors. Recipients given TBCD-BM alone were used as controls. Recipients were monitored for body weight change, hair loss, diarrhea, and survival ($\uparrow$ indicates death of all recipients in a group). There were eight recipients in each group, combined from two replicate experiments. (A) Recipients given $5 \times 10^6$ donor CD4^+ T cells developed diarrhea, and all died before development of hair loss; in contrast, recipients given $5 \times 10^6$ CD8^+ T cells showed little diarrhea, and most survived for >60 d, but all developed severe hair loss. (B) Recipients given $0.5 \times 10^6$ donor CD4^+ T cells developed diarrhea and hair loss, although most survived for >60 d. In contrast, recipients given $0.5 \times 10^6$ CD8^+ T cells showed severe hair loss without diarrhea, and most survived for >60 d. (C) Recipients given $0.1 \times 10^6$ donor CD4^+ T cells showed mild weight loss with little hair loss. In comparison, recipients given $0.1 \times 10^6$ CD8^+ T cells showed more severe weight loss and hair loss 40 d after HCT (p < 0.01).
pared the capacity of C57BL/6 donor CD4+ and CD8+ T cells to induce aGVHD and cGVHD in BALB/c recipients. Lethally irradiated recipients were transplanted with donor TBCD-BM with or without titrated numbers (5–0.1 × 10^6) of donor CD4+ or CD8+ T cells. Donor CD4+ T cells (5 × 10^5) induced severe aGVHD with body weight loss and diarrhea, and all recipients died within 20 d after HCT. In contrast, 5 × 10^5 donor CD8+ T cells induced little diarrhea. All recipients developed severe hair loss by 45 d after HCT, although most of the recipients survived for >60 d (p < 0.01, Fig. 3A). Reduction of the donor CD4+ T cell dose decreased early mortality in the recipients. With 0.5 × 10^5 CD4+ or CD8+ donor T cells, most recipients survived for >60 d after HCT. Recipients in both groups showed similar body weight loss and hair loss (Fig. 3B). Diarrhea was induced with donor CD4+ cells but not with CD8+ T cells (p < 0.01, Fig. 3B). With 0.1 × 10^6 donor CD4+ or CD8+ T cells, body weight loss and hair loss were more severe with CD8+ cells than with CD4+ T cells beginning at 45 d after HCT (p < 0.05, Fig. 3C).

We also measured serum autoantibody, percentage and yield of thymic CD4+CD8+ T cells, and histopathology of skin and salivary gland 60 d after HCT in recipients given low numbers (0.1 or 0.5 × 10^5) of donor CD4+ or CD8+ T cells. Sera from control TBCD-BM recipients showed no autoantibody staining, and sera from recipients given 0.1 × 10^6 CD4+ T cells showed very weak autoantibody staining (Fig. 4A). In contrast, sera from recipients given 0.1 × 10^6 CD8+ T cells showed strong autoantibody staining of donor-type Rag-2^−/− skin and salivary gland tissues (Fig. 4A). The recipients given 0.1 × 10^6 donor CD8+ T cells developed clinical cGVHD and had tiny thymus with markedly reduced yield of CD4+CD8+ thymocytes, although the percentage of CD4+CD8+ thymocytes was only ~2-fold lower as compared with recipients given 0.1 × 10^6 CD4+ T cells (p < 0.01, Fig. 4B). The skin and salivary gland tissue damage was also markedly more severe in CD8+ T recipients than in CD4+ T recipients (p < 0.01, Fig. 4C). Alternatively, the recipients given 0.5 × 10^5 CD4+ or CD8+ T cells developed similar severity in cGVHD features (data not shown). Taken together, these results indicate that 1) low numbers of donor CD8+ T cells are more potent than CD4+ T cells in causing cGVHD, although high numbers of donor CD4+ T cells are more potent in causing aGVHD; and 2) cGVHD mediated by low numbers of donor CD8+ T cells is associated with thymic damage.

cGVHD caused by donor CD8+ but not CD4+ T cells in transplant requires the presence of the recipient thymus

We evaluated the role of the recipient thymus in the pathogenesis of cGVHD caused by donor CD4+ and CD8+ T cells. Lethally irradiated euthymic or thymectomized BALB/c recipients were injected with TBCD-BM with CD4+ or CD8+ T cells (0.5 × 10^5). To avoid the contamination of mature CD4+ or CD8+ T cells in transplants, CD4+ T cells from CD8+ T cell–deficient donors or CD8+ T cells from CD4+ T cell–deficient donors were coinjected with TBCD-BM from wild-type donors. Donor CD8+ T cells induced mild body weight loss without signs of diarrhea in either euthymic or thymectomized recipients early after HCT. Approximately 30 d after HCT, both recipients started to show gradual body weight loss and hair loss (Fig. 5A–C). At ~45 d after HCT, euthymic recipients showed worsening clinical GVHD, but the thymectomized recipients showed gradual recovery, and clinical manifestations of GVHD disappeared by 60 d after HCT (p < 0.01, Fig. 5A, 5C). Sera from euthymic recipients showed strong

![Figure 4](http://www.jimmunol.org/Downloadedfrom)
FIGURE 5. Donor CD8+ but not CD4+ T cell induction of cGVHD required recipient thymus. Lethally irradiated euthymic and thymectomized BALB/c recipients were transplanted with 0.5 × 10^6 CD4+ T cells from CD8+ T cell–deficient or CD8+ T cells from CD4+ T cell–deficient C57BL/6 and TBCD-BM cells from wild-type C57BL/6 mice. The use of CD4+ or CD8+ T cell–deficient donors avoided contamination of CD4+ or CD8+ T cells in injected CD8+ or CD4+ T cells. Recipients were monitored for body weight change, diarrhea, hair loss, and survival after HCT. Data are combined from two replicate experiments (n = 8). At day 60 after HCT, recipient sera were tested for autoantibodies. Recipient skin and salivary gland tissues were used for histopathology. (A and D) Percentage body weight changes. Compared with euthymic recipients, thymectomized recipients given donor CD8+ T cells showed body weight recovery 45 d after HCT (p < 0.01), but no difference was observed between thymectomized or euthymic recipients given donor CD4+ T cells. (B and E) Percentage of mice without diarrhea. Recipients given donor CD8+ T cells showed no diarrhea, but recipients given donor CD4+ T cells all showed diarrhea. (C and F) Hair loss. Thymectomized recipients that were transplanted with donor CD8+ T cells showed transient weak hair loss and then regrowth of hair at ∼45 d after HCT, whereas euthymic recipients showed worsening hair loss (p < 0.01). Both thymectomized (Figure legend continues)
autoantibody staining, but the sera from thymectomized recipients had no autoantibody staining of donor-type Rag-2−/− skin and salivary gland (Fig 5G). Whereas the former recipients had severe tissue damage in the skin and salivary gland, the latter had little tissue damage by 60 d after HCT (p < 0.01, Fig 5H).

In contrast, donor CD4+ T cells (0.5 × 10^6) caused early body weight loss and diarrhea in both euthymic and thymectomized recipients (Fig. 5D, 5E), and both types of recipients started to show increasing hair loss at ~30 d after HCT, with no significant difference between the two groups (Fig 5F). Sera from both types of recipients showed strong autoantibody staining of the donor-type Rag-2−/− skin and salivary gland tissues (Fig. 5I). Both types of recipients showed similar severe damage in the skin and salivary gland (Fig 5J). Taken together, these results indicate that 1) cGVHD caused by donor CD8+ but not CD4+ T cells requires thymic damage in the recipients; 2) donor CD4+ T cells are sufficient to induce cGVHD in the absence of recipient thymus; and 3) although CD8+ T cells in transplants are sufficient to induce aGVHD, the subsequent development of cGVHD requires help from de novo–generated, donor-derived T cells developing in the GVHD-damaged thymus.

De novo–generated donor-derived CD4+ T cells in recipients given low-dose donor CD8+ T cells play an important role in thymic damage

Low-dose donor CD8+ T cells appeared to be more potent than low-dose donor CD4+ T cells in damaging the recipient thymus (Fig. 4). To explore the mechanisms that account for this difference, we kinetically compared CD4+CD8+ thymocyte regeneration at days 15, 30, 45, and 60 after HCT in recipients given low numbers of donor CD4+ or CD8+ T cells (0.1 × 10^6).

At day 15 after HCT, the percentage of CD4+CD8+ thymocytes was higher in CD8+ T recipients than in CD4+ T recipients, although it was lower than in control TBCD-BM recipients (p < 0.01, Fig. 6A). The percentage of CD4+CD8+ thymocytes increased in both types of recipients by 30 d after HCT as compared with day 15 (p < 0.01). The percentage of CD4+CD8+ thymocytes in CD4+ T cell recipients continued to increase and reached a plateau by 45 d after HCT, which was similar to control TBCD-BM recipients. In contrast, the percentage of CD4+CD8+ thymocytes in CD8+ T recipients gradually decreased, starting 30 d after HCT, and it was ~3-fold lower compared with CD4+ T cell recipients by 60 d after HCT (p < 0.01, Fig. 6A). This indicates a second-phase thymic damage in the CD8+ T recipients. Additionally, anti-donor and anti-recipient reactivity by de novo–generated, donor-derived CD4+ T cells from the spleen was stronger in CD8+ T recipients than in CD4+ T cell recipients (p < 0.01, Fig. 6A). These results indicate that 1) thymic regeneration early after HCT is more robust in CD8+ T cell recipients than in CD4+ T cell recipients; and 2) CD8+ T cell recipients showed a second phase of thymic damage, which was associated with de novo generation of donor-derived autoreactive CD4+ T cells.

In further experiments, we tested whether de novo-generated CD4+ or CD8+ T cells play a role in causing thymic damage and cGVHD induction. Lethally irradiated BALB/c recipients were transplanted with CD8+ T cells (0.5 × 10^6) in combination with TBCD-BM (2.5 × 10^6) from CD4+ or CD8+ T cell–deficient donors. As expected, recipients given CD4+ T cell–deficient donor BM cells showed de novo–generated CD8+ T cells with very few de novo–generated CD4+ T cells, whereas recipients given CD8+ T cell–deficient donor BM cells showed de novo–generated CD4+ T cells with very few de novo–generated CD8+ T cells (Supplemental Fig. 3A). The recipients that had only de novo–generated, donor-derived CD8+ T cells showed only mild weight loss at 30–45 d after HCT and then recovered. In contrast, the recipients that had only de novo–generated, donor-derived CD4+ T cells showed increasing body weight loss and hair loss, especially beyond 45 d after HCT (p < 0.01, Fig. 6B, Supplemental Fig. 3B). These recipients also developed serum autoantibodies and severe damage in the skin and salivary gland (Supplemental Fig. 3C, 3D). Consistently, thymocyte yield in the recipients that had only de novo–generated, donor-derived CD8+ T cells was 20-fold lower than in the recipients that had only de novo–generated, donor-derived CD8+ T cells (p < 0.01, Fig. 6B). These results indicate that de novo–produced CD4+ T cells play an important role in causing thymic damage and cGVHD in recipients given low-dose donor CD8+ T cells, whereas de novo–generated CD8+ T cells do not.

We also tested whether injection of anti-CD4 mAb could prevent cGVHD development in recipients given low-dose donor CD8+ T cells. Lethally irradiated recipients were transplanted with donor CD8+ T cells (0.5 × 10^6) and TBCD-BM cells. Fifteen and 30 d after HCT, recipients were injected with anti-CD4 mAb or control rat IgG (500 μg/mouse). As compared with rat IgG control, anti-CD4 mAb treatment depleted de novo–generated CD8+ but not CD8+ T cells early after HCT, but the CD4+ T cells recovered by 60 d after HCT (Supplemental Fig. 4A). Anti-CD4 mAb treatment significantly increased body weight and decreased hair loss starting at ~45 d after HCT (p < 0.01, Fig. 6C, Supplemental Fig. 4B). Anti-CD4 mAb treatment markedly decreased serum autoantibody production (Supplemental Fig. 4C) and tissue damage in the skin and salivary gland (Supplemental Fig. 4D). Anti-CD4 treatment also significantly increased the total thymocyte yield (p < 0.01, Fig. 6C), and percentage and yield of CD4+CD8+ thymocytes (Supplemental Fig. 4E). Anti-CD4 mAb treatment markedly reduced the allo- and autoreactivity of donor-derived CD4+ T cells (p < 0.01, Fig. 6D). These results demonstrate that de novo–generated, donor-derived CD4+ T cells can mediate thymic damage and cGVHD development, and a short-term depletion of the de novo–generated CD4+ T cells enables thymic recovery from initial damage mediated by donor CD8+ T cells, with consequent prevention of cGVHD.

Donor CD8+ T cells preferentially damage thymic mTEC and cause defective thymic selection early after HCT

Because recipients given low-dose donor CD8+ T cells appeared to have more de novo–generated autoreactive CD4+ T cells (Fig. 4), we tested whether donor CD8+ T cells preferentially damaged mTECs or reduced thymic DCs early after HCT. Indeed, 15 d after HCT, the percentage and yield of mTECs in the thymus of recipients given donor CD8+ T cells (0.1 × 10^6) was 2- to 3-fold lower than in recipients given donor CD4+ T cells (0.1 × 10^6).
Although the mTEC yield in both recipients was significantly lower than that in control TBCD-BM recipients ($p < 0.01$, Fig. 7A, 7B), the yield of CD11c+ DCs by day 15 after HCT was significantly increased in CD8+ T cell recipients as compared with CD4+ T recipients or control TBCD-BM recipients ($p < 0.01$, Fig. 6).

**FIGURE 6.** Low-dose donor CD8+ T cells were more potent than CD4+ T cells for inducing thymic damage and development of cGVHD. (A) Lethally irradiated BALB/c recipients were transplanted with $0.1 \times 10^6$ CD4+ or CD8+ T cells in addition to TBCD-BM from donor C57BL/6 mice. Recipients given TBCD-BM alone were used as controls. Percentage of CD4+CD8+ thymocytes was measured on 15, 30, 45, and 60 d after HCT. Means ± SE of four recipients at each time point from each group were combined from two replicate experiments. At 60 d after HCT, de novo–generated donor-derived CD4+ T cells ($0.2 \times 10^6$) from recipients given donor CD4+ or CD8+ T cells were stimulated with recipient- or donor-derived CD11c+ DCs ($0.1 \times 10^6$). Proliferation of donor CD4+ T cells was measured with [3H]thymidine deoxyribose incorporation. Mean ± SE of the stimulation index was combined from four replicate experiments. (B) Lethally irradiated BALB/c recipients were transplanted with donor CD8+ T cells ($0.5 \times 10^6$) with TBCD-BM cells ($2.5 \times 10^6$) from CD4+ T cell– or CD8+ T cell–deficient C57BL/6 donors. Recipients were monitored for body weight changes and hair loss. Thymocyte yield was also compared at 60 d after HCT. As compared with recipients given CD8+ T cell–deficient BM cells, recipients given CD4+ T cell–deficient BM cells showed body weight increase beginning at ∼45 d after HCT ($p < 0.01$). There were eight recipients in each group, combined from two replicate experiments. (C) Lethally irradiated recipients were transplanted with donor CD8+ T cells ($0.5 \times 10^6$) and TBCD-BM cells from wild-type C57BL/6 donors. On days 15 and 30 after HCT, the recipients were injected with anti-CD4 mAb or control rat IgG (500 μg/mouse). Recipients were monitored for body weight changes and hair loss. At 60 d after HCT, total thymocyte yield was compared. Data were combined from eight recipients in each group from two replicate experiments. As compared with recipients treated with rat IgG, recipients treated with anti-CD4 mAb showed body weight increase at ∼45 d after HCT ($p < 0.01$). There were eight recipients in each group, combined from two replicate experiments. (D) De novo–generated CD4+ T cell proliferation in response to recipient- or donor-derived CD11c+ DCs at 60 d after HCT. Mean ± SE of the stimulation index was combined from four replicate experiments.

(As compared with recipients treated with rat IgG, recipients treated with anti-CD4 mAb showed body weight increase at ∼45 d after HCT ($p < 0.01$). There were eight recipients in each group, combined from two replicate experiments. (D) De novo–generated CD4+ T cell proliferation in response to recipient- or donor-derived CD11c+ DCs at 60 d after HCT. Mean ± SE of the stimulation index was combined from four replicate experiments.)

Alternatively, the yield of CD11c+ DCs by day 15 after HCT was significantly increased in CD8+ T cell recipients as compared with CD4+ T recipients or control TBCD-BM recipients ($p <
FIGURE 7. Low numbers of donor CD8+ T cells preferentially damaged mTECs in the recipient thymus and caused defective thymic negative selection. Lethally irradiated BALB/c recipients were transplanted with 0.1 × 10^6 CD4+ or CD8+ T cells and TBCD-BM cells from C57BL/6 donors. Recipients given TBCD-BM cells alone were used as controls. At 15 d after HCT, recipient thymus was measured for the percentage and yield of mTECs, percentage and yield of thymic CD11c+ DCs, and expression of MHC II and CCR9. At 30 d after HCT, recipient spleen cells were measured for mouse mammary tumor virus–mediated clonal deletion of Vß3, Vß4, and Vß5. Additionally, flow cytometry–sorted, de novo–generated, donor-derived CD4+ T cells were measured for donor and recipient reactivity. (A) Gated thymic epithelial cells are shown in UEA I (mTEC marker) versus Ly51 (cortical TEC marker). A representative flow cytometry pattern and mean ± SE of percentage and yield of mTECs are shown from one of four replicate experiments. In each experiment, six thymi were combined from each group to obtain a sufficient number of thymic epithelial cells. (B) Percentage (Figure legend continues)
stimulated with donor- or recipient-type DCs (0.1–3). Expression of MHC II and CCR9+ DC subset has been proposed to mediate thymic negative selection (46, 47). We found no reduction of MHC II expression by DCs or decrease in the percentage of CCR9+ DCs in the thymus of CD8+ T cell recipients by days 15 or 30 after HCT (Fig. 7D, 7E and data not shown), although we observed a marked reduction by day 45 (data not shown). These results indicate that low numbers of donor CD8+ T cells preferentially damage mTECs in the recipients early after HCT.

In further experiments, we tested whether the reduced numbers of mTECs early after HCT in the recipient thymus was associated with defective negative selection of recipient- and donor-reactive T cells by measuring mouse mammary tumor virus–mediated clonal deletion (48) and T cell proliferation in response to donor- or host-type DCs. At day 15 after HCT, the number of de novo–generated, donor-derived CD4+ T cells in the spleen was ∼0.4–0.8% and then increased to ∼3–12% by day 30. Vβ3+ and Vβ5+ but not Vβ4+ T cells were deleted in BALB/c mice but not in C57BL/6 mice. At day 30 after HCT, C57BL/6 donor-derived Vβ3+ and Vβ5+ cells were deleted in GVHD-free BALB/c recipients given TBCD-BM alone or low-dose CD4+ T cells, but they were not deleted in GVHD recipients given donor CD8+ T cells (p < 0.01, Fig. 7F). Additionally, the de novo–generated, donor-derived CD4+ T cells from the spleen of CD8+ T cell recipients showed much stronger proliferation in response to stimulation by donor- or recipient-type DCs as compared with CD4+ T cells from recipients given TBCD-BM or CD4+ T cells (p < 0.01, Fig. 7G). Taken together, these results indicate that damage to thymic mTECs caused by donor CD8+ T cells leads to a defective negative selection of de novo–generated autoreactive T cells early after HCT, as depicted in the diagram (Fig. 8).

Discussion

Most cGVHD patients have aGVHD that is controlled with immunosuppressants (49, 50). Separate models of aGVHD and cGVHD have been developed with specific donor/recipient strain combinations (20, 21), but the link between aGVHD and cGVHD has not been well established, and no single model reflects all characteristics of human cGVHD (20). Our current results demonstrate that, similar to in humans, aGVHD can evolve into characteristic cGVHD in mice, as long as the donor T cell dose is appropriate. The key for establishing cGVHD mouse model is not the special donor/recipient combination; instead, the key is allowing the recipient to survive aGVHD, giving time for autoreactive T cells to expand and cause cGVHD.

We observed that donor CD8+ T cell–mediated thymic mTEC damage allowed autoreactive CD4+ T cells to escape negative selection in the thymus. Kinetic differences between the presence of autoreactive CD4+ T cells and changes in the numbers of donor DCs or tolerogenic CCR9+ DCs or MHC II expression in DCs suggest that these DC changes do not initiate cGVHD. These results indicate that mTEC damage is sufficient to initiate the generation of autoreactive CD4+ T cells and the development of cGVHD. This observation appears to be inconsistent with a previous report that MHC II–deficient TCD-BM cells led to development of autoreactive CD4+ T cells in recipients exposed to high-dose TBI (34). In fact, we observed that MHC II–deficient C57BL/6 or DBA/2 TCD-BM gave rise to cGVHD in BALB/c recipients conditioned with 950 cGy TBI but not in recipients conditioned with 850 cGy TBI (J.S. Young and D. Zeng, unpublished data). Therefore, it is possible that irreversible severe damage to mTECs also contributes to cGVHD development in high-dose TBI-conditioned recipients transplanted with MHC II–deficient donor BM. The mechanisms of the preferential damage of mTECs by donor CD8+ T cells remain unclear. A previous publication showed that donor T cell damage of thymic epithelial cells required cognate cytotoxicity (51); another report showed that thymic mTECs could directly activate the alloreactive T cells (32). Additionally, donor CD8+ T cells usually have stronger cytotoxic activity than do CD4+ T cells. As T cells enter the thymus in the medullary area adjacent to cortical area (52), it is possible that the preferential damage of mTECs by donor CD8+ T cells is due to the fact that donor T cells that enter the thymus are anatomically closer to mTECs. The interaction between donor DCs and host mTECs in GVHD recipients and the role of donor DC abnormality in induction of cGVHD remain unclear and are under investigation.

It is of interest that donor CD8+ T cells induced only transient aGVHD that did not develop into cGVHD in thymectomized recipients. This finding is consistent with our observation that de novo–generated, donor-derived CD4+ T cells were required for induction of cGVHD in recipients given donor CD8+ T cells alone. The mechanisms explaining why donor CD8+ T cell–mediated GVHD does not evolve into cGVHD remain to be explored.
We recently showed that the expansion of donor CD8+ T cells in GVHD target tissues was coincident with the presence of de novo–generated CD4+ T cells (53). These observations suggest that purified donor CD8+ T cells could be used to facilitate engraftment and mediate GVL effects without cGVHD in elderly patients with little thymic de novo T cell production.

We observed that two injections of anti-CD4 mAb early after HCT depleted de novo–generated donor CD4+ T cells for a short period of time, and the donor-derived CD4+ T cells recovered at ~1 mo after the last Ab injection. Anti-CD4 mAb treatment restored thymic production, prevented production of de novo–generated autoreactive T cells, and prevented cGVHD. This observation is of important potential clinical significance. This short-term anti-CD4 mAb treatment spares donor CD8+ T cells that are needed to facilitate engraftment and mediate GVL effects and prevents cGVHD development.

In summary, this report has provided 1) a new approach to establish murine models that reflect the pathogenesis of cGVHD in humans; 2) novel observations that donor CD4+ T cells induce cGVHD in the presence or absence of recipient thymus, whereas donor CD8+ T cells induce cGVHD only in the presence of the recipient thymus; 3) a demonstration that damage to thymic mTECs early after HCT is sufficient to allow the development of autoreactive T cells and cGVHD; and 4) depletion of de novo–generated autoreactive CD4+ T cells can allow recovery of thymic negative selection and prevent the development of cGVHD. Our recent studies also showed that replacing wild-type with Igμ knockout donor TBCD-BM cells that do not give rise to donor B cells markedly reduced cGVHD induced by donor CD8+ T cells (T. Wu, J. Young, X. Ni, and D. Zeng, unpublished observations). Combining these observations with our previous publications showing that donor B cells play important roles in augmenting cGVHD (22, 35, 37), we propose the pathophysiology of cGVHD. As described in Fig. 8, donor CD4+ and CD8+ T cells are activated and expanded by host APCs after allogeneic HCT. The autoreactive T cells migrate into the thymus and damage mTECs, resulting in production of autoreactive CD4+ T cells. Autoreactive CD4+ T cells can also derive from CD4+ T cells in transplants, probably through recognizing nonpolymorphic Ags (35, 37, 43). Autoreactive T cells interact with donor-derived DCs and B cells, resulting in mutual expansion and autoantibody production as well as cGVHD development. These new insights into cGVHD pathogenesis could guide the development of novel therapies for prevention of cGVHD. For example, strategies that deplete donor CD4+ T cells and/or donor B cells in the graft and de novo–generated CD4+ T cells early after HCT would allow donor CD8+ T cells to facilitate engraftment and mediate GVL effects without causing cGVHD.

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Disclosures

The authors have no financial conflicts of interest.

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