Alloimmune Response Results in Expansion of Autoreactive Donor CD4+ T Cells in Transplants That Can Mediate Chronic Graft-versus-Host Disease

Dongchang Zhao, James S. Young, Yu-Hong Chen, Elizabeth Shen, Tangsheng Yi, Ivan Todorov, Peiguo G. Chu, Stephen J. Forman and Defu Zeng

*J Immunol* 2011; 186:856-868; Prepublished online 13 December 2010; doi: 10.4049/jimmunol.1002195
http://www.jimmunol.org/content/186/2/856
Alloimmune Response Results in Expansion of Autoreactive Donor CD4+ T Cells in Transplants That Can Mediate Chronic Graft-versus-Host Disease

Dongchang Zhao,*‡ James S. Young,*‡,† Yu-Hong Chen,*‡,§ Elizabeth Shen,* Tangsheng Yi,*‡ Ivan Todorov,* Peiguo G. Chu,‖ Stephen J. Forman,‡ and Defu Zeng*‡,†

Chronic graft-versus-host disease (cGVHD) is considered an autoimmune-like disease mediated by donor CD4+ T cells. This study demonstrates that the transplantation of donor spleen cells into thymectomized MHC-matched allogeneic BALB/c recipients induced autoimmune-like cGVHD, although not in control syngeneic DBA/2 recipients. The donor-type CD4+ T cells from the former but not the latter recipients induced autoimmune-like manifestations in secondary syngeneic BALB/c recipients as well as syngeneic DBA/2 recipients. Transfer of donor-type CD4+ T cells from secondary DBA/2 recipients with disease into syngeneic donor-type or allogeneic host-type tertiary recipients propagated autoimmune-like manifestations in both. Furthermore, TCR spectratyping revealed that the clonal expansion of the autoreactive CD4+ T cells in cGVHD recipients was initiated by an alloimmune response. Finally, hybridoma CD4+ T clones derived from DBA/2 recipients with disease proliferated similarly in response to stimulation by syngeneic donor-type or allogeneic host-type dendritic cells. These results demonstrate that the autoimmune-like manifestations in cGVHD can be mediated by a population of donor CD4+ T cells in transplants that simultaneously recognize Ags presented by both donor and host APCs. The Journal of Immunology, 2011, 186: 856–868.
required but de novo thymus-derived donor-type T cells, previously described extrathyrmic differentiated donor-type T cells (27), or residual host-type T cells are not required for disease induction. However, the mechanisms wherein donor CD4+ T cells become autoreactive in allogeneic recipients are still unclear.

In the current studies, using the MHC-matched mouse model of DBA/2 donor and thymectomized BALB/c host, we found that donor-type autoreactive CD4+ T cells in transplants were expanded after the allogeneic response and contributed to cGVHD pathogenesis; furthermore, cGVHD can be mediated by a population of donor CD4+ T cells in transplants that possess TCRs that can subsequently interact with host- and donor-type APCs.

Materials and Methods

**Mice**

Thymectomized DBA/2 (H-2b) and BALB/c (H-2s) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC II−/− DBA/2 or BALB/c mice were generated by back-crossing for eight generations with MHC II−/− C57BL/6 mice that have the whole MHC II segment deleted (28). All of the mice were maintained in a pathogen-free room at the City of Hope Research Animal Facilities (Duarte, CA). All of the animal protocols were approved by the City of Hope Research Animal Care Committee.

**mAbs, flow-cytometric analysis, and cell sorting**

The FITC-, PE-, allophycocyanin-, Cy7-allophycocyanin-, or biotin-conjugated mAbs to mouse CD5, CD4, CD8, CD25, CD11c, TCR Vβ panel (Vβ2, 3, 4, 5.1, 6, 7, 8.1, 8.3, 9, 10^6, 11, 12, 13, 14, 17), and TCR Vα panel (Vα2, 3, 8, 11) were all purchased from BD Pharmingen (San Diego, CA). Enrichment or depletion of cell subsets with a magnetic purification system from Miltenyi Biotec (Auburn, CA) and multiple-color FACs analysis and sorting were performed as described previously (12, 29).

**Proliferation assays**

Sorted CD4+ T cells together with irradiated allogeneic or syngeneic CD11c+ dendritic cells (DCs; 1 × 10^6 each) from spleen cells were cultured in a U-bottom 96-well plate for 5 d, and [3H]thymidine deoxyribose (TdR; 1 μCi/ml) was added 18 h before harvest. Background counts in the wells of responder T cells alone were <1000 cpm. The stimulating index was calculated using this formula: (cpm of culture of responder × stimulator − cpm of culture of responder alone)/(cpm of culture of responder alone).

**Measurement of autoantibodies in serum**

Anti-dsDNA IgG2a was measured with an ELISA as described previously (12, 29). Anti-dsDNA titers are expressed in units per milliliter, using a reference-positive standard of pooled serum from 6- to 7-mo-old NZB/W mice. A 1:100 dilution of this standard serum was arbitrarily assigned a value of 100 U/ml.

**TCR spectratyping**

The primers for TCR Vβs and TCR spectratyping were described previously (30). Briefly, CD4+ T cells (0.5–5 × 10^7) were recombined and lysed in TRIzol for RNA extraction. RNA (1 μg) was reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase. The cDNA was divided into separate wells and amplified using PCR primers for TCR Vβ genes, with the resulting product run for three to five additional cycles using primers labeled with FAM. The fluorescently labeled amplification products were then run on a sequencing gel and analyzed using a Hitachi AB model 3730 capillary DNA analyzer (Applied Biosystems, Los Angeles, CA). Analysis was performed with the aid of GeneMapper software (Applied Biosystems). Healthy DBA/2 CD4+ splenocytes were used to form a baseline for each Vβ spectrum, and samples from CD4+ recipient cells or cultured cells were compared with the baseline. A Vβ peak was considered to be skewed if a majority of the samples tested contained at least one peak that met the following criteria, based on the work of Friedman et al. (31): 1) the peak accounts for a minimum of 10% of the total peak area for the given Vβ which excludes minor clones, and 2) the relative use of the particular peak increased by 50% or more as compared with the baseline, indicating significant clonal expansion.

**Establishment of hybridoma T clones**

Thymoma BW5147.G.1.4 cell line was purchased from the American Type Culture Collection (catalog no. TIB 48) and cultured in 10% FBS RPMI 1640 complete medium (Invitrogen, Carlsbad, CA). Autoreactive hybridoma CD4+ T cells were generated by fusing the donor-reactive CD4+ T cells with the BW5147 cell line as described previously (32). After fusion, the cells were selected with hypoxanthine/aminopterin/thymidine (catalog no. 69-X; American Type Culture Collection) and hypoxanthine/thymidine (catalog no. 71-X; American Type Culture Collection). Two weeks later, hybridomas were expanded and TCR Vβ8.1 was screened by flow cytometry with anti-TCR Vβ8.1 Ab. The hybridoma TCR Vβ8.1+ cells were selected and expanded. Then, hybridoma Vβ8.1+CD4+ T cells were cloned with single-cell flow cytometry sorting.

**Measurement of IL-2 in supernatant**

To test the donor or host reactivity of hybridoma Vβ8.1+CD4+ T cell clones, cloned hybridoma T cells (0.5 × 10^6 per well) were stimulated by DBA/2 or BALB/c DCs (0.05 × 10^6 per well) sorted from spleen cells for 48 h. Then, supernatants were harvested, and IL-2 concentration was quantified by a Mouse IL-2 ELISA kit (catalog no. 555148; BD Biosciences).

**Histopathology**

Tissue specimens were fixed in formalin before embedding in paraffin blocks. Tissue sections were stained with H&E. Slides were examined at ×200 magnification and visualized with an Olympus and a Pixera (600CL) cooled charge-coupled device camera (Pixera, Los Gatos, CA). Tissue damage was blindly assessed on a scoring system described previously (29). In brief, skin GVHD was scored on the basis of tissue damage in epidermis and dermis and loss of s.c. fat; the maximum score is 10. Jejunum GVHD was scored based on the damage in intestine, including villous blunting, crypt regeneration, crypt epithelial cell apoptosis, crypt loss, luminal sloughing of cellular debris, lamina propria inflammatory cell infiltrate, and mucosal ulceration, and the maximum is 14, as described previously (15). Liver GVHD was scored on the number of involved tracts and the severity of disease in each tract, and the maximum score is 8. Lung GVHD was scored on the periluminal infiltrates, pneumonitis, and the severity of lung tissues involvement; the maximum score is 9, as described previously (33). Salivary GVHD was scored on mononuclear cell infiltration and follicular destruction, as described previously (34). The degree of inflammatory infiltrates was graded as follows. Grade 1: 1–5 foci of mononuclear cells were seen (>20 cells per focus). Grade 2: >5 foci of mononuclear cells were seen but without significant parenchymal destruction. Grade 3: multiple confluent foci were seen, with moderate degeneration of parenchymal tissue. Grade 4: extensive infiltration of the gland with mononuclear cells and extensive parenchymal destruction were seen. Kidney GVHD was graded by glomerular inflammation, proliferation, crescent formation, and necrosis, and the maximum score is 12. The mean ± SE of the score of six recipients in each group was calculated.

**Statistical analysis**

Body weight changes, proteinuria incidences, and survival in different groups were compared using the log-rank test (Prism, version 4.0; GraphPad Software, San Diego, CA). Comparison of two means was analyzed using an unpaired two-tail Student t test.

**Results**

**Donor CD4+ T cells in transplants mediated autoimmune-like manifestations of cGVHD via interaction with host-type APCs in allogeneic recipients**

We previously reported that transplantation of DBA/2 donor spleen cells into sublethally irradiated MHC-matched euthymic or athymic BALB/c recipients induced severe autoimmune-like cGVHD (12, 29). However, the origin of the autoreactive T cells in the recipients is still unclear. In the current study, we explored the mechanisms how the mature donor T cells in transplants led to the development of autoimmune-like disease in allogeneic recipients. First, as shown in Fig. 1A of the experimental scheme, sublethally irradiated (800 rad) thymectomized allogeneic BALB/c and thymectomized syngeneic DBA/2 recipients were transplanted with donor CD25+ TCD spleen (CD25−SPL) cells (50 × 10^6). A majority of the donor CD4+ T cells in the CD25−SPL
cells had the CD62L\textsuperscript{hi}CD44\textsuperscript{lo} naive phenotype (Supplemental Fig. 1). We found that, although none (0/12) of the syngeneic DBA/2 recipients (1˚ Syn-Rec) showed any signs of GVHD, all (12/12) of the allogeneic BALB/c recipients (1˚ Allo-Rec) showed high serum levels of IgG2a anti-dsDNA, hair loss, and severe proteinuria and died by 30 d after HCT ($p$, 0.01; Fig. 2 A–C). In addition, we observed that the cGVHD BALB/c recipients had severe tissue damage in the skin, jejunum, liver, lung, salivary gland, and kidney, although little tissue damage was observed in the syngeneic DBA/2 recipients ($p$, 0.01; Fig. 2 D,2 E). The identification of tissue damage in the jejunum and salivary gland of the cGVHD mouse recipients is of interest, because recent reports show that tissue damage in jejunum and salivary gland is often associated with cGVHD in patients (1, 7).

It was proposed that autoimmunity in cGVHD recipients was mediated by thymus-derived donor-type T cells that interact with donor-type APCs (13, 20). Next, we tested whether donor-type CD4\textsuperscript{+} T cells derived from mature T cells in transplants and their interaction with host-type APCs could mediate autoimmune-like manifestations. Accordingly, as shown in Fig. 1B of the experimental scheme, 15 d after HCT, sorted CD4\textsuperscript{+} T cells (5 $\times$ 10\textsuperscript{6}) plus TCD-SPL cells (45 $\times$ 10\textsuperscript{6}) from the primary thymectomized BALB/c recipients were injected into sublethally irradiated thymectomized BALB/c recipients (1˚ Allo-BALB/c) or control primary DBA/2 recipients (1˚ Syn-DBA/2). Donor DBA/2 T cells express CD5.1 and host BALB/c T cells express CD5.2; donor CD5.1$^{+}$CD4\textsuperscript{+} T cells were sorted by flow cytometry after CD4\textsuperscript{+} cell enrichment with magnetic microbes as described in our previous publications (12, 29) and Supplemental Fig. 2. The majority of the donor-type CD4\textsuperscript{+} T cells from the primary allogeneic recipients had the CD62L\textsuperscript{lo}CD44\textsuperscript{hi} effector memory phenotype (Supplemental Fig. 1). We found that all (12/12) of the secondary BALB/c recipients given the CD4\textsuperscript{+} T cells and TCD-SPL cells developed weight loss, proteinuria, high serum levels of IgG2a anti-dsDNA, and hair loss and died by 40 d after cell transfer. In contrast, the recipients given TCD-SPL cells only showed no signs of cGVHD ($p$, 0.01; Fig. 3 A–E). Histopathology showed that, although recipients given TCD-SPL cells only showed minimum signs of tissue damage, the recipients given CD4\textsuperscript{+} T cells had severe tissue damage in skin, jejunum, liver, lung, salivary gland, and kidney ($p$, 0.01; Fig. 3 F,3 G). These results indicate that mature donor-type CD4\textsuperscript{+} T cells from transplants and their interaction with host-type APCs can result in autoimmune-like manifestations in the allogeneic recipients.

Donor-type autoreactive CD4\textsuperscript{+} T cells in transplants were expanded in allogeneic BALB/c but not in syngeneic DBA/2 recipients

Because we observed that DBA/2 donor spleen cells induced autoimmune-like manifestations in thymectomized allogeneic but not syngeneic recipients, we tested whether autoreactive donor-type T cells were expanded in the allogeneic recipients. Accordingly, as shown in Fig. 1C of the experimental scheme, sorted CD5.1$^{+}$CD4\textsuperscript{+} T cells (5 $\times$ 10\textsuperscript{6}) and/or TCD-SPL cells (45 $\times$ 10\textsuperscript{6}) from primary allogeneic (1˚ Allo-BALB/c) recipients or primary syngeneic (1˚ Syn-DBA/2) recipients were transplanted into secondary DBA/2 recipients (2˚ Syn-DBA/2 or 1˚ Syn-DBA/2, respectively. As mentioned above, the majority of the donor-type CD4\textsuperscript{+} T cells from the primary allogeneic recipients had the CD62L\textsuperscript{lo}CD44\textsuperscript{hi} effector memory phenotype

FIGURE 1. Experimental scheme. A, DBA/2 donor spleen cells were transplanted into sublethally irradiated thymectomized MHC-matched allogeneic BALB/c recipients or syngeneic DBA/2 recipients. B, Sorted donor-type CD4\textsuperscript{+} T cells with TCD-SPL cells from the primary thymectomized BALB/c recipients (1˚ Allo-BALB/c) were transferred into secondary thymectomized BALB/c recipients (2˚ Allo-BALB/c). C, Sorted donor-type CD4\textsuperscript{+} T cells and TCD-SPL cells from the primary thymectomized recipients (1˚ Allo-BALB/c) or control primary DBA/2 recipients (1˚ Syn-DBA/2) were transferred into the secondary syngeneic thymectomized BALB/c recipients (2˚ Syn-DBA/2). D, Sorted donor-type CD4\textsuperscript{+} T cells from secondary allogeneic BALB/c (2˚ Allo) or syngeneic DBA/2 (2˚ Syn) recipients were further expanded by host-type BALB/c DCs or donor-type DBA/2 DCs to generate host- or donor-reactive CD4\textsuperscript{+} T cells. Thereafter, the host- or donor-reactive CD4\textsuperscript{+} T cells were transferred into tertiary syngeneic DBA/2 (3˚ Syn) or allogeneic BALB/c (3˚ Allo) recipients.
Consistent with a previous report that T cells that go through homeostatic expansion upregulate CD44 and downregulate CD62L (35), the majority of the donor-type CD4+ T cells in the primary syngeneic recipients appeared to have the CD62LloCD44hi phenotype (Supplemental Fig. 1). We found that the recipients given the CD4+ T cells from the primary allogeneic recipients (1° Allo-BALB/c) showed weight loss, fur dyspigmentation, and increased serum anti-dsDNA IgG, as compared with recipients given CD4+ T cells from the primary syngeneic recipients (1° Syn-DBA/2) (Fig. 4A–C). In addition, histopathology showed that, compared with recipients given CD4+ T cells from the 1° Syn-DBA/2 recipients, the recipients given CD4+ T cells from the 1° Allo-BALB/c recipients had much more severe tissue damage, including increased cell infiltration in the dermis, increased apoptotic cells in hair follicles, increased regeneration in the small intestine [a sign of cGVHD as described previously (15)], and increased infiltration in the liver, salivary gland, and kidney glomeruli (p < 0.01; Fig. 4D, 4E). The DBA/2 secondary recipients given TCD-SPL cells alone from either allogeneic or syngeneic recipients showed no signs of disease (data not shown).

In addition, 15 d after HCT, donor-type CD4+ T cells from syngeneic DBA/2 and allogeneic BALB/c recipients were sorted...
and stimulated in vitro with CD11c+ DCs from DBA/2 or BALB/c mice. We found that the proliferation of CD4+ T cells from allogeneic BALB/c recipients with cGVHD was 4-fold stronger than that of the CD4+ T cells from syngeneic recipients without cGVHD when they were stimulated with syngeneic DBA/2 DCs (p < 0.01; Fig. 4F). The proliferation was DBA/2 MHC II-restricted, because they did not proliferate in response to stimulation by MHC II−/− DBA/2 DCs (Fig. 4F). In contrast, their proliferation was not significantly different when they were stimulated with allogeneic BALB/c DCs: both proliferated vigorously in a BALB/c MHC II-dependent manner (Fig. 4G). Taken together, these results indicate that autoreactive CD4+ T cells in transplants are expanded in allogeneic but not in syngeneic recipients and that the autoreactive CD4+ T cells also contribute to the development of autoimmune-like manifestations in the allogeneic recipients with cGVHD.

Both donor- and host-reactive CD4+ T cells derived from mature T cells in transplants induced autoimmune-like manifestations in allogeneic BALB/c as well as syngeneic DBA/2 recipients

To further dissect the roles of donor- and host-reactive CD4+ T cells derived from mature T cells in transplants in mediating autoimmune-like manifestations in allogeneic hosts, we used serial in vivo transfer and in vitro culture to further expand the donor- and host-reactive CD4+ T cells. Accordingly, as shown in Fig. 1D of the experimental scheme, 15 d after HCT, the CD4+ T cells from the primary allogeneic (1° Allo) BALB/c recipients with GVHD were transferred into secondary (2° Allo) BALB/c recipients or (2° Syn) DBA/2 recipients. Fifteen days after secondary transfer, the donor-type CD4+ T cells from the (2° Allo) BALB/c recipients were sorted and then repeatedly stimulated...
FIGURE 4. Donor-type CD4⁺ T cells from cGVHD BALB/c recipients induced autoimmune manifestations in syngeneic thymectomized DBA/2 recipients. Sorted CD4⁺ T cells (5 × 10⁶) and/or TCD-SPL cells (45 × 10⁶) from the primary allogeneic (1˚ Allo) recipients or sorted CD4⁺ T cells (5 × 10⁶) and/or TCD-SPL cells (45 × 10⁶) from the primary syngeneic (1˚ Syn) recipients were transplanted into sublethally irradiated thymectomized secondary DBA/2 recipients (2˚ Syn), respectively. The recipients were monitored for survival daily and proteinuria, body weight, and clinical GVHD twice a week for up to 50 d. Fifteen days after cell transfer, the serum levels of anti-dsDNA IgG2a were measured. Fifty days after cell transfer, the recipients were euthanized for histopathology study. A, Percentage of body weight changes after cell transfer (n = 12). B, Serum levels of anti-dsDNA IgG2a (n = 12). C, A representative photo of mice given donor-type CD4⁺ T cells from primary syngeneic recipients (1˚ Syn → Syn), which had a healthy appearance, or mice given donor-type CD4⁺ T cells from primary allogeneic recipients (1˚ Allo → Syn), which had depigmented fur. D, A representative histopathology photo of the skin, jejunum, liver, lung, salivary, and kidney tissues in secondary recipients 50 d after cell transfer. E, Mean ± SE of histopathology scores.
with host-type BALB/c CD11c+ DCs in complete medium with 100 U/ml IL-2 for 15 d. The expanded CD4+ T cells were designated host-reactive CD4+ T cells. Similarly, the donor-type CD4+ T cells from the (2˚ Syn) DBA/2 recipients were sorted and in vitro-expanded by donor-type DBA/2 CD11c+ DCs, and the expanded CD4+ T cells were designated donor-reactive CD4+ T cells. The CD4+ T cells in both cultures were expanded ∼20-fold.

The donor- or host-reactive CD4+ T cells were measured for their donor or host reactivity in a proliferation assay with donor-type DBA/2 or host-type BALB/c DC stimulation. Surprisingly, we found that the expanded donor- or host-reactive CD4+ T cells proliferated similarly in response to donor- or host-type DC stimulation (Fig. 5A), although the stimulation index was 2- to 3-fold higher when stimulated with host-type DCs (p < 0.01; Fig. 5A).

Next, we tested whether the expanded donor- and host-reactive CD4+ T cells could induce autoimmune-like manifestations in allogeneic BALB/c recipients. Accordingly, the in vitro-expanded donor- or host-reactive CD4+ T cells (10 × 10⁶) were injected with TCD-SPL cells (50 × 10⁶) from the primary allogeneic BALB/c recipients into sublethally irradiated thymectomized BALB/c recipients. The control recipients were injected with TCD-SPL cells alone. We found that, compared with TCD-SPL cells, the donor- and host-reactive CD4+ T cells induced weight loss in all (12/12), proteinuria in 50% (6/12), higher serum levels of anti-dsDNA IgG in all, and hair loss in most of the recipients (p < 0.01; Fig. 5B–D). Furthermore, histopathology showed that, as compared with TCD-SPL cells, both donor- and host-reactive donor-type CD4+ T cells induced severe tissue damage in skin, jejunum, liver, lung, salivary gland, and kidney (p < 0.01; Fig. 5E), and there was no significant difference in the severity of tissue damage between the two groups (Fig. 5E). These results indicate that both donor- and host-reactive donor-type CD4+ T cells derived from the mature T cells in transplants could mediate autoimmune-like manifestations in allogeneic cGVHD recipients.

Similarly, we found that both donor- and host-reactive CD4+ T cells induced autoimmune manifestations in the syngeneic DBA/2 recipients (Fig. 5F–I). We should point out that, consistent with the in vitro proliferation result, the donor- and host-reactive CD4+ T cells induced more severe disease in host-type BALB/c recipients than in donor-type DBA/2 recipients (Fig. 5). Taken together, both donor- and host-reactive CD4+ T cells derived from mature T cells in transplants could proliferate in response to stimulation by donor-type as well as host-type APCs, and they mediate autoimmune-like manifestations in both syngeneic and allogeneic recipients. These results indicate that the donor- and host-reactive CD4+ T cells may recognize Ags presented by both donor and host APCs.

The donor- and host-reactive CD4+ T cells in transplants belong to the same population, and their expansion was triggered by an alloimmune response

TCR spectratyping has been used to measure tissue-specific and Ag-specific alloreactive T cell clonal expansion in GVHD recipients (36–41). Next, we used this technique to find out whether the alloreactive and autoreactive T cells in donor transplants express differential TCRs by comparing the H chain (Vβ) TCR spectra of donor-type CD4+ T cells before and after HCT in syngeneic and allogeneic primary and secondary recipients, using TCR spectratyping methods described by Friedman et al. (31). We found that DBA/2 donor-type CD4+ T cells showed no skewed TCR-CDR3 lengths in primary syngeneic recipients (1˚ Syn), as compared with before HCT, but they showed skewed TCR-CDR3 lengths in primary allogeneic recipients (1˚ Allo) (0/22 versus 13/22, p < 0.01; Fig. 6A, 6B). This result indicates that clonal expansion of donor-type CD4+ T cells in transplants is initiated by alloimmune response after allogeneic HCT.

Furthermore, transfer of the donor-type CD4+ T cells from the primary syngeneic recipients (1˚ Syn) into the secondary syngeneic recipients (1˚ Syn → Syn) still did not cause the skewing of TCR-CDR3 lengths (data not shown), and transfer of donor-type CD4+ T cells from primary thymectomized allogeneic recipients (1˚ Allo) into the secondary thymectomized allogeneic recipients (1˚ Allo → Allo) or syngeneic recipients (1˚ Allo → Syn) did not lead to a further increase in the frequencies of skewed CDR3 lengths, either. In addition, the CD4+ T cells with skewed TCR-CDR3 lengths in the secondary allogeneic (1˚ Allo → Allo) or secondary syngeneic (1˚ Allo → Syn) recipients were all originated from the primary allogeneic recipients (1˚ Allo) (Fig. 6A, 6B). Interestingly, the expanded donor- or host-reactive CD4+ T cells both had similar TCR spectra to the donor-type CD4+ T cells in the primary allogeneic BALB/c recipients (Fig. 6B). These results indicate that the donor- and host-reactive CD4+ T cells derived from the mature T cells in transplants may have similar Ag specificity.

Although the frequencies of the TCRs with skewed CDR3 lengths were not further increased by further in vivo or in vitro stimulation, as compared with that in the primary allogeneic recipients, the percentage of some VB subsets was increased not only in the secondary allogeneic recipients (1˚ Allo → Allo) but also in the secondary syngeneic recipients (1˚ Allo → Syn) (p < 0.01; Fig. 6C). Additional in vitro stimulation resulted in further expansion of some VB subsets. For example, after the serial in vivo and in vitro stimulation, the Vβ8.1+ subset became the dominant subset in both donor- and host-reactive CD4+ T cells, although the percentage of the Vβ8.1+ subset was higher among donor-reactive than that among host-reactive (35 versus 12%) (Fig. 6D). These results indicate that, although the donor- and host-reactive donor-type CD4+ T cells have similar TCR spectra and recognize Ags presented by both donor- and host-type APCs, different clones may differ in their expansion in response to donor- or host-type APC stimulation. Therefore, it remains unknown whether the donor and host reactivity can be mediated by a single TCR.

Single donor-type Vβ8.1+CD4+ T clones possessed both donor and host reactivity

Because we observed that the Vβ8.1+ subset became dominant among the donor- and host-reactive donor-type CD4+ T cells after several rounds of in vitro expansion by syngeneic DBA/2 or allogeneic BALB/c DCs, we used the Vβ8.1+CD4+ T subset as an example for comparing their donor and host reactivity. First, we tested whether the reactivity of the Vβ8.1+CD4+ T cells was similar to that of the whole population. Accordingly, Vβ8.1+CD4+ T cells from the expanded donor-reactive donor-type CD4+ T cells were sorted and stimulated with DCs from syngeneic donor

\[ n = 6 \]. F and G. Proliferation of CD4+ T cells. Sorted donor-type CD4+ T cells (0.2 × 10⁶ per well) from the primary allogeneic (1˚ Allo) recipients or the primary syngeneic (1˚ Syn) recipients were stimulated with DCs (0.1 × 10⁶ per well) from wild-type (WT) or MHC II−/− donor DBA/2 or host BALB/c mice for 5 d, and [3H]TdR incorporation was measured. Mean ± SE of the stimulating index (SI); the stimulating index of six replicated experiments is shown.

Downloaded from http://www.jimmunol.org/ by guest on April 17, 2017
DBA/2 or allogeneic host BALB/c mice. We found that Vβ8.1+ CD4+ T cells proliferated vigorously to either type of DC stimulation, and there was no difference between the two groups (Fig. 7A). This is consistent with the donor and host reactivity of whole CD4+ T population (Fig. 5A).

Second, we tested whether the donor and the host reactivity was mediated by T cells with dual TCRs, because it was reported that alloreactive CD4+ T cells could have dual TCR α-chains (42). We checked the Vα expression by a panel of available anti-Vα Abs. We found that, although the Vβ8.1+ cells expressed different Vα subsets (including Vα2, 8, and 11), no Vβ8.1+ cells expressed dual Vα (Fig. 7B, 7C). In addition, we used the panel of anti-Vβ Abs used in Fig. 6C to check whether Vβ8.1+CD4+ T cells expressed a second Vβ, and we found no expression of a second...
FIGURE 6. Expansion of donor-reactive CD4+ T cells in transplants was initiated by alloimmune response. Day 15 after HCT, donor-type CD4+ T cells from the primary syngeneic DBA/2 recipients (1˚ Syn-Rec) or allogeneic recipients (1˚ Allo-Rec) given CD25-DBA/2 donor spleen cells were sorted for TCR spectratyping analysis. Naive donor DBA/2 CD4+ T cells were used as a control. At the same time, CD4+-SPL cells from the primary allogeneic BALB/c recipients (1˚ Allo-Rec) were enriched and transferred into sublethally irradiated secondary syngeneic DBA/2 recipients (1˚ Allo→Syn) or allogeneic recipients (1˚ Allo→Allo). Fifteen days after the cell transfer, donor-type CD4+ T cells from the 1˚ Allo→Syn or 1˚ Allo→Allo recipients were sorted for TCR spectratyping analysis again. In addition, the TCR spectra of donor- and host-reactive donor-type CD4+ T cells used in Fig. 5 were also measured. A, Representative TCR spectra: Skewed TCR-CDR3 length was shaded. A representative of at least three repetitions is shown. B, Summary of TCR spectratyping: A check mark indicates that tested samples contained at least one peak exhibiting significant skewing, whereas a dash indicates a lack of skewing. A representative of at least three replicate experiments is shown. C, The left panel shows the percentage of Vβ subsets among total donor-type CD4+ T cells from spleens of the DBA/2 donor, 1˚ Allo→Syn recipient, or 1˚ Allo→Allo recipient, as measured by flow cytometry. The right panel shows...
Vb by the Vβ8.1+ T cells from primary allogeneic recipients or T hybridoma cells (Supplemental Fig. 3). Thus, the Vβ8.1+CD4+ T cells that possess both donor and host reactivity are unlikely to express dual TCRs.

Third, we tested whether a single Vβ8.1+CD4+ T cell clone possessed both donor and host reactivity, using Vβ8.1+CD4+ hybridoma T cell clones. Accordingly, the donor-reactive CD4+ T cells were fused with the TIB-48 thymoma cell line as described previously (32). After selective culture for 14 d, Vβ8.1+CD4+ hybridomas were cloned with single-cell flow cytometry sorting, and two plates of 96 clones each were set up (Fig. 7D). After clonal expansion, 10 clones with different Vβs from each plate were stimulated with syngeneic donor-type DBA/2 or allogeneic host-type BALB/c DCs, and their production of IL-2 was measured. We found that, although the hybridoma T clones produced little IL-2 without DC stimulation (data not shown), they produced large amounts of IL-2 in response to syngeneic donor-type or allogeneic host-type DC stimulation, and there was no significant difference in the TCR spectratyping (Fig. 7E). These results indicate that a single Vβ8.1+CD4+ T cell clone can possess both donor and host reactivity.

Discussion

We have demonstrated that, in a cGVHD model of DBA/2 donor to MHC-matched but minor Ag-mismatched BALB/c recipient, autoimmune-like manifestations in cGVHD recipients can be mediated by a population of donor-type CD4+ T cells derived from mature T cells in transplants that possess both donor and host reactivity.

First, we observed that donor-type CD4+ T cells from thymectomized cGVHD recipients proliferated in response to stimulation by donor-type as well as host-type DCs, and they induced autoimmune-like manifestations in both syngeneic as well as allogeneic secondary recipients. Second, donor- and host-reactive donor-type CD4+ T cells established by serial in vivo transfer and in vitro culture from cGVHD recipients still proliferated in response to stimulation by donor- and host-type DCs as well as induced autoimmune-like manifestations in donor-type syngeneic and host-type allogeneic recipients. Third, TCR spectratyping analysis revealed that TCR-CDR3 skewing of donor CD4+ T cells took place in primary allogeneic recipients, and the TCR spectra of the donor- and host-reactive CD4+ T cells from the cGVHD recipients were similar. Finally, hybridoma T cell clones derived from the donor-reactive CD4+ T cells also proliferated in response to stimulation by syngeneic donor-type and allogeneic host-type DCs. Taken together, donor CD4+ T cells that possess both donor and host reactivity in transplants can mediate autoimmune-like manifestations in allogeneic recipients.

We have also observed that the activation and expansion of the donor CD4+ T cells with both donor and host reactivity are initiated by the alloimmune response. However, the mechanisms are not yet clear. Because we have reported that the depletion of donor B cells can prevent the induction of autoimmune-like manifestations in allogeneic recipients (12) and this is associated with reduction of autoreactive CD4+ T cells in the recipients (D. Zhao and D. Zeng, unpublished observations), we hypothesize that the donor CD4+ T cells in transplants include both alloreactive and autoreactive T cells, and the latter recognize non-polymorphic Ags presented by both donor and host APCs. After alloimmune HCT, alloreactive donor T cells are activated by interaction with host APCs presenting alloantigens, then the activated autoreactive T cells interact with donor B cells that present both allo- and autoantigens, including non-polymorphic Ags from both donor and host. Thereafter, the activated donor B cells subsequently activate and expand the autoreactive donor CD4+ T cells in transplants that contribute to the development of autoimmune-like manifestations in the recipients. We should also point out that the donor CD4+ T cells with both donor and host reactivity have high percentages of Vβ subsets after expansion with either donor- or host-type APCs. This is consistent with a previous report that the majority of the autoreactive CD4+ T cell clones from experimental autoimmune encephalomyelitis mice were Vβ8.1 (43). Therefore, the donor CD4+ T cells with both donor and host reactivity are most likely to be autoreactive T cells that recognize non-polymorphic Ags presented by MHC-matched donor and host APCs, as recently proposed by Shlomchik et al. (44). The role of donor B cells in the expansion of the donor-type autoreactive CD4+ T cells in cGVHD recipients is under investigation.

However, we cannot exclude the possibility that some of those donor CD4+ T cells have both allo- and autoreactivity, and their expansion was initiated by their autoreactivity and continued by their autoreactivity, because it was reported by Felix et al. (45) that an alloreactive CD4+ T cell could express a polyspecific TCR that recognizes multiple distinct ligands, each in a highly specific way. Thus, some donor CD4+ T cells may recognize both allo- and autoantigens. In addition, it was reported that many autoreactive T cells possess dual TCRs (42), but we did not find dual TCRs on the donor CD4+ T cells with donor and host reactivity.

To our knowledge, this is the first demonstration that autoimmune-like manifestations in cGVHD recipients can be mediated by donor CD4+ T cells in transplants that possess both donor and host reactivity. Our current studies have also made several new novel observations in contrast to a recent publication by Tivol et al. (11): 1) Our study is based on a MHC-matched cGVHD model in which the primary recipients showed signs of autoimmunity, including high levels of serum autoantibodies and tissue damage in small intestine and salivary glands that represent characteristic futures of human cGVHD (1, 7). However, the observation of Tivol et al. was based on a MHC-mismatched aGVHD model in which the primary recipients did not show clear signs of cGVHD or autoimmunity (11). 2) The autoreactive CD4+ T cells in our studies induced a similar autoimmune syndrome in both allogeneic and syngeneic secondary recipients with a variety of organ tissue damage; however, in the studies of Tivol et al., the autoreactive CD4+ T cells induced only autoimmune tissue damage in colon but not in other GVHD target tissues in the secondary syngeneic recipients, and they did not induce tissue damage in the secondary allogeneic recipients, either. Colitis is the typical sign of aGVHD but not for cGVHD in humans (1, 7).

We have demonstrated that the autoreactive CD4+ T cells belong to a population of donor CD4+ T cells that express a single TCR that can interact with both allogeneic host-type and syngeneic donor-type APCs, indicating that they may recognize the non-polymorphic Ags presented by both donor- and host-type APCs. In contrast, in the study of Tivol et al., the origin of the autoreactive donor CD4+ T cells was not clear; they could be donor CD4+ T cells in transplants or those from de novo thymic de-
development. It was also not clear in their study whether the donor APC-reactive and the host APC-reactive donor CD4+ T cells belong to the same population and why the donor CD4+ T cells induced GVHD in the primary but not in the secondary allogeneic recipients. Therefore, our report is significantly different from the report of Tivol et al. in many aspects.

FIGURE 7. Donor-reactive V\textsubscript{B}8.1+CD4+ T cells derived from primary cGVHD recipients expressed different V\textsubscript{a}s, and their clones proliferated in response to both syngeneic donor and allogeneic host DC stimulation. A, In vitro-expanded donor-reactive CD4+ T cells were stained with anti-V\textsubscript{B}8.1 versus anti-CD4, and the V\textsubscript{B}8.1+CD4+ T cells were sorted for proliferation in response to restimulation by DBA/2 or BALB/c DCs for 5 d. [\textsuperscript{3}H]Tdr incorporation was measured, and mean ± SE of cpm of the triplicate culture was calculated. One representative of two replicate experiments is shown. B, The donor-reactive CD4+ T cells were stained with anti-V\textsubscript{B}8.1, CD4, and different anti-V\textsubscript{a} (V\textsubscript{a}2, V\textsubscript{a}3, V\textsubscript{a}8, or V\textsubscript{a}11). The gated V\textsubscript{B}8.1+CD4+ T cells were shown in V\textsubscript{a}2 versus V\textsubscript{a}8, V\textsubscript{a}8 versus V\textsubscript{a}11, V\textsubscript{a}8 versus V\textsubscript{a}11. One representative of three replicate experiments is shown. C, The TIB-48 thymoma cell line and hybridoma T cells were stained for V\textsubscript{B}8.1 and CD4. Hybridoma V\textsubscript{B}8.1+CD4+ T cells were cloned by single-cell sorting. E, IL-2 production of hybridoma V\textsubscript{B}8.1+ T clones in response to stimulation by syngeneic donor DBA/2 or allogeneic host BALB/c DCs. Ten clones with different V\textsubscript{a}s from each plate were randomly picked up and expanded. Each clone cells (0.1 × 10\textsuperscript{6} per well) was stimulated with DBA/2 or BALB/c DCs for 48 h. Then, IL-2 concentration in the culture supernatant was measured by ELISA.
Our observations may provide new insights into some clinical observations. First, although aGVHD damage of the thymus is proposed to be the cause of cGVHD (13, 46–50), it has been reported that some patients develop severe autoimmune-like cGVHD without obvious aGVHD that causes thymus damage (51, 52) and that old patients with little thymocyte production often develop more severe autoimmune-like cGVHD (1). Therefore, we speculate that, in those cases, the autoreactive T cells that mediate the cGVHD may be derived from donor T cells in transplants. Second, G-CSF-mobilized blood transplants cause reduction of aGVHD but augmentation of autoimmune-like cGVHD (22, 23). It is possible that the G-CSF-mobilized blood transplants contain higher numbers of the autoreactive CD4+ T precursors. Third, we have observed that, although the activation and expansion of the donor-type autoreactive CD4+ T cells in transplants are initiated by host-type APCs, those T cells can also interact with donor-type APCs and continue to expand and mediate GVHD after the elimination of host-type APCs. This observation provides a new explanation about why the majority of cGVHD in patients is after aGVHD and autoimmune-like chronic GVHD can occur in complete chimeric patients. This observation can also explain why cGVHD is often associated with better graft-versus-leukemia activity (53).

However, we should still be cautious about the relevance of this mouse model to human cGVHD. Although this animal model has important autoimmune features as well as GVHD target organs (i.e., small intestine and salivary gland) that are similar to those of human cGVHD (1, 7, 54), the high serum levels of anti-dsDNA, high frequency of glomerulonephritis, and the temporal time course in this mouse model do not mirror human cGVHD. Interestingly, recent reports showed a significant increase of glomerulonephritis in allogeneic recipients with non-myeloablative conditioning (55–58). Therefore, further study about the clinical manifestations and therapy. In

References


