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TNF Receptor-Associated Factor 6 Deficiency during Hemopoiesis Induces Th2-Polarized Inflammatory Disease

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Toll-like receptors (TLR) initiate rapid innate immune responses by recognizing microbial products. These events in turn lead to the development of an efficient adaptive immune response through the up-regulation of a number of costimulatory molecules, including members of the TNF/TNFReceptor superfamily, on the surface of an APC. TNFR-associated factor 6 (TRAF6) is a common signaling adapter used by members of both the TNF and the TLR/IL-1R superfamilies, and as such plays a critical role in the development of immune responses. As TRAF6-deficient mice die prematurely, we generated chimeras reconstituted with TRAF6-deficient fetal liver cells to analyze functions of TRAF6 in vivo in the hemopoietic compartment. We found that TRAF6-deficient chimeras develop a progressive lethal inflammatory disease associated with massive organ infiltration and activation of CD4+ T cells in a Th2-polarized phenotype, and a defect in IL-18 responsiveness. When recombination-activating gene 2−/− blastocysts were complemented with TRAF6-deficient embryonic stem cells, a marked elevation of activated CD4+ T cells and progressive inflammatory disease were also observed. Moreover, T cell activation and lethal inflammation were not reversed in mixed chimeric mice generated from normal and TRAF6-deficient fetal liver cells. These results suggest that deletion of TRAF6 induces a dominant Th2-type polarized autoimmune response. Therefore, in addition to playing a critical role in innate and adaptive immunity, TRAF6 is likely to play a previously unrecognized role in the maintenance of self-tolerance. The Journal of Immunology, 2003, 171: 5751–5759.

Host defenses against microorganisms rely on a coordinated interplay between the innate and adaptive immune responses. The innate immune system initiates immediate host responses to microbial Ags and further acts as an effector by stimulating adaptive immune responses. Toll-like receptors (TLRs),1 expressed on APCs such as dendritic cells (DC), act as pattern recognition receptors, recognizing microbial products and initiating signaling events that culminate in cell maturation and cytokine production (1). In particular, through induction of MHC molecules and costimulatory ligands, the innate immune response mediated by TLRs also helps initiate T cell-mediated adaptive immunity (2). Another group of cell surface receptors found on APCs, those of the TNF and TNFR families, also play an important role in initiating and perpetuating an effective adaptive immune response. In particular, molecules such as CD40, CD27, CD30, 4-1BB (CD137), or OX-40 (CD134) regulate T cell expansion, effector function, migration, survival, and memory development (3).

TNF-associated factor 6 (TRAF6), a member of the TRAF family of proteins that were initially identified as signal transducers of the TNFR superfamily, is a key molecule that links signals from both members of the TNFR superfamily (e.g., receptor activator of NF-κB or CD40) and the IL-1R/TLR family (IL-1R, IL-18R, and IL-17R) to the nuclear factor of activated T cells (NF-κB), p38 mitogen-activated protein kinase, and Jun N-terminal kinase. DR4, which is required for osteoclast formation, has also been shown to be induced by TRAF6. In addition to this role in innate immunity, TRAF6 is also important in adaptive immunity. TRAF6-deficient mice lack normal bone marrow cavities, and succumb to premature death between 2 and 3 wk of life (11–13). Thus, although studies to date have revealed several important physiological roles of TRAF6, in-depth in vivo analysis of how the immune system is regulated by TRAF6 has been lacking.

To study the role of TRAF6 in the hemopoietic compartment, we generated bone marrow chimeric mice by reconstituting adult lethally irradiated mice with TRAF6-deficient fetal liver cells (FLC). We observed that TRAF6-deficient chimeras develop a spontaneous wasting disease characterized by progressive weight loss, cachexia, and ultimately death. They exhibit hemopoietic abnormalities with defects in the lymphoid compartment. Histologic analysis of tissues from these mice reveal T cell infiltration, inflammation, and necrosis, which are widespread through multiple visceral organs. Detailed immunohistochemical studies show that infiltrating T cells are producing Th2-type cytokines. Similar T cell defects are also observed when recombination-activating gene

1 Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; FLC, fetal liver cell; TRAF, TNFR-associated factor; RAG, recombination-activating gene.
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3 This work was supported by National Institutes of Health Grant AI-43626.
harvested and analyzed for CFSE by FACS, and for cytokine production by ELISA, according to the manufacturer instructions (PharMingen).

**In vitro CD4⁺ T cell differentiation**

Splenic CD4⁺ T cells (1 × 10⁶/ml) were cultured with wild-type irradiated T-depleted splenocytes (1 × 10⁶/ml) in the presence of anti-CD3 (5 μg/ml) and IL-2 (20 U/ml). Th1 polarization was achieved with the addition of IL-12 (5 ng/ml) and anti-IL-4 (4 μg/ml), whereas IL-4 (10 ng/ml) and anti-IFN-γ (4 μg/ml) were added for Th2 polarization. After 4 days of culture, cells were collected, washed extensively, and rested for 24 h before being stimulated at 1 × 10⁶/ml with plate-bound anti-CD3 (10 μg/ml). In some instances, Th1 cells were restimulated with or without plate-bound anti-CD3 (10 μg/ml) in the presence of IL-12 (1 ng/ml) and/or IL-18 (50 ng/ml) for 24 h, or unfractionated splenocytes were stimulated with IL-12 (5 ng/ml), IL-18 (20 ng/ml), or both for 24 h. Supernatants were collected after 24 h and assayed for IFN-γ and IL-4 levels by ELISA.

**Statistical analyses**

Statistical analyses were performed using Student’s t test, which was considered significant at p ≤ 0.05.

**Histology and immunohistochemistry**

Organs were preserved in 10% neutral buffered Formalin (Fisher, Swedesboro, NJ), cut into 5-μm sections, and stained with H&E. For immunohistochemical analysis, organs were snap frozen, embedded in Tissue-Tek (OCT compound; Sakura Finetek, Torrance, CA), cut into 5-μm sections, and fixed in acetone for 10 min at room temperature. Tissue sections were labeled using an indirect immunoperoxidase technique with biotinylated anti-CD4, anti-IFN-γ, anti-IL-4, anti-IL-10, and anti-TGF-β. Tissue sections were then incubated with an Envision-peroxidase kit (DAKO, Carpenteria, CA).

**Results**

Weight loss, death, multiorgan inflammation, and chronic CD4⁺ T cell activation in TRAF6-deficient chimeras

To study the function of TRAF6 in the hematopoietic compartment, we generated bone marrow chimeras in which lethally irradiated (1200 rad) adult C57BL/6 (CD45.1) mice were reconstituted with 1 × 10⁶ TRAF6-deficient or control FLC (CD45.2). As early as 4 wk after reconstitution, TRAF6-deficient chimeras began to exhibit progressive cachexia, including hunched posture, and in ~70% of the mice, focal loss of hair at the base of the head with thickened skin, scaling, and itching (Fig. 1A). By 9 wk after reconstitution, TRAF6-deficient chimeras lost up to 20% of their initial weight compared with control chimeras (Fig. 1B; n = 15, p < 0.02). In addition, the mortality of TRAF6-deficient chimeras was 100% by 18 wk after reconstitution, while no deaths were observed in control chimeras (n = 10, p < 0.02) (Fig. 1C). Progression of the illness was variable, and some mice died prematurely as young as 7 wk after reconstitution. Histologic analysis of multiple organs of the TRAF6-deficient chimeras revealed severe inflammatory infiltrates characterized by an influx of mononuclear cells and overt tissue destruction (Fig. 2A). Although virtually all organs were affected, the lesions were most prominent in the lung and the liver, and the least so in the colon, heart, and kidney. Moreover, immunohistochemical analysis of affected organs from TRAF6-deficient chimeras revealed the presence of CD4⁺ infiltrating cells that colocalized with abundant staining for IL-4, IL-10, and TGF-β, but not for IFN-γ (Fig. 2B, and data not shown). The observed multiorgan inflammatory process was accompanied by a large increase in the percentage of splenic CD4⁺ T cells that exhibited an effector phenotype. Indeed, 13.1 ± 4.9% and 22.7 ± 5.8% of the CD4⁺ T cells in TRAF6-deficient chimeras expressed CD25⁺ and CD69⁺, respectively, compared with 5.8 ± 1.6% (n = 10, p < 0.018) and 11.3 ± 2.3% (n = 10, p = 0.0064) in the control chimeras (Table I). Similarly, there was an increase in memory phenotype CD4⁺ T cells: 42.5 ± 6.6% and 39.2 ± 11% of the CD4⁺ T cells in TRAF6-deficient chimeras were

**Materials and Methods**

**Generation of chimeric mice following lethal irradiation**

TRAF6-deficient mice (mixed 129/C57BL/6 background) were generated, as previously described (13). To generate chimeras, we performed adoptive transfer (by i.v. injection) of 1 × 10⁶ FLC isolated from TRAF6-deficient (CD45.2) or wild-type littermate (CD45.2) fetuses into irradiated (1200 rad) adult C57BL/6 mice (CD45.1) (13). For mixed chimeras, 0.5 × 10⁶ FLC from both CD45.1 and control CD45.2 TRAF6-deficient fetuses were mixed and injected into lethally irradiated (1200 rad) adult C57BL/6 mice (CD45.1). Antibiotics (sulfatrim; Alpharma USPD, Baltimore, MD) were given for the first 2 wk after reconstitution, and mice were analyzed at 6–12 wk. CD45.1 or CD45.2 staining was used to evaluate the efficiency of reconstitution. Chimerism was 99% in the thymus and bone marrow, and 95% in spleen and blood from mice reconstituted with control or TRAF6-deficient CD45.2 cells (data not shown). For mixed chimeras, ~50% of the cells in the bone marrow were CD45.1 (control derived) and 50% CD45.2 (of TRAF6-deficient origin) (data not shown).

**Generation of TRAF6/RAG-deficient chimeric mice**

TRAF6-deficient embryonic stem cells (TRAF6 homozygous mutant) were generated as previously described (13), and were injected, as described, into RAG2-deficient blastocysts, yielding chimeric mice (14).

**Reagents and FACS analysis**

Cells were grown in RPMI 1640 (Mediatech Cellgro, Herndon, VA) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Life Technologies, Rockville, MD), and 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO). Purified unlabeled, biotinylated, FITC-, PE-, or APC-labeled Abs were purchased from BD Pharmingen (San Diego, CA) and include anti-CD3, anti-CD25, anti-MHC class II, anti-Gr1, anti-CD4, anti-CD8, anti-CD44, anti-CD69, anti-CD44, anti-CD62L, anti-IL-2, anti-IFN-γ, anti-IL-4, anti-IL-10, and anti-TGF-β. The anti-CD28 Ab hybridoma was a gift of J. Allison (University of California, Berkeley, CA). Murine rTGF-β was purchased from R&D Systems (Minneapolis, MN), and murine recombinant IL-10, IFN-γ, IL-2, and IL-4 were purchased from BD Pharmingen. Cells were analyzed on a BD FACScalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software.

**CD4⁺ T cell purification**

Cell suspensions from spleens were subjected to hypotonic shock with water to lyse erythrocytes. The resultant mononuclear cells were enriched for T cells by negative selection using the MACS system (Magnetic Cell Sorting; Miltenyi Biotec, Auburn, CA). Briefly, cells were incubated with purpuriated biotinylated anti-MHC class II, anti-CD220, anti-Gr1, and anti-CD8 Abs at 2 μg/ml at 20 × 10⁶ cells/ml in MACS buffer (PBS with 2% FCS, 2 mM EDTA) for 30 min at 4°C. After two washes, cells were incubated with streptavidin microbeads (100 μl/10⁶ cells) at 40 × 10⁶/ml for 20 min at 4°C in MACS buffer. After two additional washes, bead-bound cells were isolated using a separation column placed in a strong magnetic field, and the negative fraction containing the CD4⁺ T cells was harvested. CD4⁺ T cell purity was monitored by FACS analysis with a FITC anti-CD4 mAb, and purity was ~95%. For purification of naive CD4⁺ T cells, biotinylated anti-CD44 (200 ng/ml) was added to the cocktail of Abs.

**CD4⁺ T cell stimulation and analysis**

Plates were coated with anti-CD3 in PBS (1 μg/ml) for 2 h at 37°C and then washed three times with PBS. CD4⁺ T cells were labeled with CFSE and resuspended at 0.5 × 10⁶ cells/ml in complete medium, which also contained soluble anti-CD28 mAb (1 μg/ml). After 72 h, the cells were
CD44$^{\text{high}}$ and CD62L$^{\text{low}}$, respectively, compared with 13.2 ± 3% ($n = 10$, $p < 0.0000013$) and 19.5 ± 3.7% ($n = 10$, $p < 0.011$) of the CD4$^+$ T cells of control chimeras (Table I).

Because TRAF6-deficient mice ((129 × C57BL/6)$F_1$ background) have not yet been fully backcrossed onto the B6 background, we considered the possibility that the observed abnormalities might be due to graft vs host disease directed at minor histocompatibility Ags. However, the recipient mice were reconstituted with FLC containing no T cells, and none of the above abnormalities were observed in the control chimeras generated using FLC from control littermates (also (129 × C57BL/6)$F_1$ background), effectively excluding this explanation. Thus, these results indicate that deletion of TRAF6 limited to the hemopoietic compartment leads to lethal Th2-type inflammatory disease.

TRAF6/RAG2-deficient chimeras also develop lymphoproliferative disease

DC and other APCs play an important role in central and peripheral tolerance by facilitating the inactivation and/or deletion of autoreactive T cells (1, 15–18). The fetal liver chimeras studied above lack TRAF6 in their entire hemopoietic compartment. Thus, we could not use this system to discriminate whether a defect in T cell tolerance was T cell intrinsic or might be due to a defect in APCs. If the latter was the case, then the presence of normal APCs might prevent the development of disease. To address this issue, we generated TRAF6/RAG2-deficient chimeras. Similar to what we observed in the chimeras on a wild-type background, by 6 wk of age the TRAF6/RAG2-deficient chimeras mice exhibited a...
runted posture, cachexia, and hair loss on the head and on the back (data not shown). In addition, these mice displayed severe lung, liver, heart, skin, and colon infiltrates characterized by the presence of CD4+ T cells and the presence of the Th2 cytokines, IL-4, IL-10, and TGF-β (data not shown). Similar also to chimeras on a wild-type background, peripheral T cells from TRAF6/RAG2-deficient chimeras exhibited an effector/memory phenotype: 13.6 ± 3.5% and 28.2 ± 1.5% of the CD4+ T cells in the TRAF6/RAG2-deficient chimeras expressed CD25 and CD69, respectively, vs 5.7% ± 2.6% (n = 5, p < 0.04) and 13.2 ± 3.7% (n = 5, p < 0.002) in the control mice, respectively (Table II). These results demonstrate that the development of Th2-type autoinflammatory responses and lymphoproliferative disease is not due to the lack of normal APCs.

Enhanced proliferation and Th2 cytokine production by in vitro stimulated CD4+ T cells

To determine whether the increase in the effector/memory phenotype of T cells in TRAF6-deficient chimeras was associated with enhanced or skewed responses to stimulation, purified CD4+ T cells were CFSE labeled and stimulated in vitro with plate-bound anti-CD3 (1 μg/ml) ± soluble anti-CD28 (1 μg/ml) mAbs. Proliferation, measured as loss of CFSE fluorescence, was assessed by FACS analysis, and cytokine production was analyzed by ELISA. We observed (Fig. 3) a significant increase in the frequency of cells that had proliferated in response to anti-CD3 stimulation in TRAF6-deficient chimeras compared with control chimeras. This difference was most pronounced under suboptimal stimulation
Th1 polarization. To explore this issue, we stimulated purified chimeras suggested the possibility of a defect in normal Th1 differentiation, but impaired IL-18 response in TRAF6-deficient CD4+ T cells

The preferential production of Th2 cytokines by T cells from the TRAF6-deficient chimeras suggested the possibility of a defect in Th1 polarization. To explore this issue, we stimulated purified naive CD4+ T cells in vitro under Th1-polarizing conditions, and measured the production of IFN-γ. We observed that TRAF6-deficient CD4+ T cells differentiating in Th1 conditions produced comparable levels of IFN-γ, as did the control CD4+ T cells (Fig. 5A), demonstrating intact ability to differentiate into Th1-type T cells under manipulated conditions.

IL-12 plays an important role in Th1 differentiation, and this function is specifically enhanced by IL-18 (19). The IL-18R belongs to the Toll/IL-1R family and uses the Myd88/IL-1R-associated kinase/TRAF6 signaling pathway (19, 20). Therefore, we next assessed the potential of TRAF6-deficient cells to respond to IL-18. To do so, we stimulated control or TRAF6-deficient differentiated Th1 T cells or total splenocytes with IL-18 and/or IL-12, as this is known to elicit IFN-γ production from Th1 T cells or the NK population of spleen without a requirement for any other stimuli (19). We observed that TRAF6-deficient Th1 CD4+ T cells produced similar levels of IFN-γ, as did control Th1 CD4+ T cells following IL-12 stimulation alone. However, they produced markedly less IFN-γ in response to IL-18, and modestly less in response to IL-12 plus IL-18 (Fig. 5A). Identical results were seen with NK cells, which are the responding cell population contained in total splenocytes (Fig. 5B). These results demonstrate that TRAF6 is required for IFN-γ production in response to IL-18 stimulation, but not in response to TCR or IL-12 stimulation.

Altered hemopoiesis, but normal T cell development in TRAF6-deficient chimeras

To assess potential mechanisms responsible for autoimmune-like diseases observed in TRAF6-deficient chimeric mice, we next examined T cell development. We observed severe atrophy of the thymus in TRAF6-deficient chimeras (8–12 wk after reconstitution) with 10.8 × 106 ± 6.3 total cells vs 80.2 × 106 ± 19.4 cells in control chimeras (n = 10, p = 1.8 × 10−7) (Table III). However, the proportions of CD4+CD8− subpopulations (Table III), as well as the proportions of the CD5+, CD69+, CD44+, and CD25+ cells within different subpopulations (data not shown) were not significantly altered in TRAF6-deficient chimeras, suggesting no obvious skewing in T cell maturation.

As with the thymic compartment, the total cellularity of the spleen was reduced in TRAF6-deficient chimeras (11.3 × 106 ± 6.7) compared with the control chimeras (34.4 × 106 ± 11.1; n = 10, p = 6.1 × 10−6; Table III). Consistent with observed thymic atrophy, we also found a reduction in the percentage of T cells in the spleen from TRAF6-deficient chimeras with 26.4% ± 12.4 vs 38.8% ± 9.9 in the control chimeras (n = 10, p = 5 × 10−7). As was also noted in the thymus, the proportions of CD4+CD8− subpopulations were not altered (Table III). We also found a dramatic expansion of cells expressing CD11b and Gr1, previously reported as immature myeloid cells derived from the monocyte-macrophage lineage (21), in the spleen of TRAF6-deficient chimeras; 42 ± 22.9% vs 4.8 ± 1.8%, n = 10, p < 4 × 10−4. As well, the mesenteric, inguinal, cervical, mandibular, axillary, and para-aortic lymph nodes from the TRAF6-deficient chimeras were extremely small and contained very few T cells (0.5 ± 0.2 × 105 total cells from TRAF6-deficient chimeras vs 12 ± 2.3 × 105 in the control chimeras). This is consistent with findings in TRAF6-deficient mice, indicating that lymph node development requires TRAF6 expression (11–13), and extends this finding to show that this expression is required in a cell of hemopoietic origin.

We observed a decrease in the number of B cells in the bone marrow and spleen of TRAF6-deficient chimeras (26 ± 13.8% in TRAF6-deficient chimeras vs 50.6 ± 9.9% in controls; n = 10, p > 4.1 × 10−7; Table III). Although the absolute number of bone

Table I. Phenotypic markers on splenic T cells

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Control Chimeras (n = 10)</th>
<th>TRAF6-Deficient Chimeras (n = 10)</th>
<th>Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25+ (% in CD3+CD4+)</td>
<td>5.8 ± 1.6</td>
<td>13.1 ± 4.9</td>
<td>p &lt; 1.8E-02</td>
</tr>
<tr>
<td>CD60+ (% in CD3+CD4+)</td>
<td>11.3 ± 2.3</td>
<td>22.7 ± 5.8</td>
<td>p &lt; 6.4E-03</td>
</tr>
<tr>
<td>CD44high (% in CD3+CD4+)</td>
<td>13.2 ± 3.0</td>
<td>42.5 ± 6.6</td>
<td>p &lt; 1.3E-06</td>
</tr>
<tr>
<td>CD62Llow (% in CD3+CD4+)</td>
<td>19.5 ± 3.7</td>
<td>39.2 ± 11.0</td>
<td>p &lt; 1.1E-02</td>
</tr>
</tbody>
</table>

Table II. Activation markers on T cells from TRAF6/RAG2-deficient chimeras

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Control Chimeras (n = 5)</th>
<th>TRAF6/RAG2-Deficient Chimeras (n = 5)</th>
<th>Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenocytes</td>
<td>CD25+ (% in CD3+CD4+)</td>
<td>5.7 ± 2.6</td>
<td>13.6 ± 3.5</td>
</tr>
<tr>
<td>CD60+ (% in CD3+CD4+)</td>
<td>13.2 ± 3.7</td>
<td>28.2 ± 1.5</td>
<td>p &lt; 2E-03</td>
</tr>
<tr>
<td>CD44high (% in CD3+CD4+)</td>
<td>16.1 ± 2.8</td>
<td>55.4 ± 18.4</td>
<td>p &lt; 7E-03</td>
</tr>
<tr>
<td>CD62Llow (% in CD3+CD4+)</td>
<td>19.7 ± 6.9</td>
<td>55.1 ± 18.2</td>
<td>p &lt; 3E-02</td>
</tr>
</tbody>
</table>
For example, CD4, the function of these cells could be impaired in the absence of TRAF6. However, these cells could represent activated cells that recirculate for self-tolerance (22, 23). We observed normal proportions of CD4 T cells in the spleen and thymus of TRAF6-deficient mice. CD4 T cells from control or TRAF6-deficient chimeras were labeled with CFSE and cultured in the presence of 1 μg/ml of plate-bound anti-CD3 mAb alone or with 1 μg/ml of soluble anti-CD28 mAb for 72 h, after which the cells were harvested and analyzed by flow cytometry. Histograms show the CFSE fluorescence profile of the live cells. These results are representative of four different experiments.

marrow cells was similar, the percentage of B cells in the bone marrow of TRAF6-deficient chimeras was also severely depressed (5.8 ± 2.1% vs 29.1 ± 5.2%; n = 10, p < 1.5 × 10−9; data not shown).

Taken together, these results suggest that TRAF6 may play a role in hemopoiesis. However, the lymphoproliferative diseases caused by TRAF6 deletion are not likely due to defects in T cell development.

Dominant autoimmunity in mixed chimeras

CD4+CD25+ regulatory T cells have been described to be crucial for self-tolerance (22, 23). We observed normal proportions of CD4+CD25+ T cells in the thymus of TRAF6-deficient chimeras; however, these cells could represent activated cells that recirculate from the periphery to the thymus. In addition to their development, the function of these cells could be impaired in absence of TRAF6. For example, CD4+CD25+ regulatory T cells have recently been described to express and respond to TLR4, whose signaling pathway requires TRAF6 (11, 24).

To examine whether the autoimmune seen in TRAF6-deficient chimeras might be due to a lack or intrinsic dysfunction of regulatory cells, we generated mixed chimeras by combining control FLC (CD45.1) and TRAF6-deficient FLC (CD45.2) at a ratio of 1:1. The resultant mixed chimeras behaved similarly to the TRAF6-deficient chimeras, i.e., by 6–8 wk of age, we observed runting, hair loss, and mononuclear cell infiltrations in most visceral organs. Analysis of the bone marrow at this point in time revealed that roughly 50% of the cells arose from the control donor (CD45.1) and 50% from the TRAF6-deficient donor (CD45.2) (data not shown). In the thymus and spleen, two-thirds of the cells were of control donor origin and one-third of TRAF6-deficient donor origin, suggesting a competition for hemopoietic reconstitution of thymus and spleen in favor of control cells (Table IV). The thymus was again severely reduced in total cell number, and no differences were observed in the proportion of CD3+/CD4+CD8− as a function of donor origin (Table IV, and data not shown). The proportions of regulatory phenotype CD4+CD25+ T cells in the thymus of control (4.1 ± 1.9%) or TRAF6-deficient (3.1 ± 0.8%) donor origin were also similar (Table IV). As we had observed in TRAF6-deficient chimeras, the spleen contained large percentages of CD4+ T cells with an activated phenotype. Strikingly, however, T cells of control origin were as likely to display this abnormality as were T cells of TRAF6-deficient origin (Table IV). Collectively, these results show that the presence of normal hemopoietic cells does not prevent the development of autoimmune disease, arguing that regulatory cell deficiency by itself is not the cause of the inflammatory process.

Discussion

TRAF6 is a key signaling adapter for TNFR family and IL-1R/TLR family members (4–7). In particular, TRAF6 has been implicated in the regulation of innate immune responses and the function of APCs such as macrophages and DC (12, 13). Additionally, because TRAF6 is critical for CD40-induced signals, it has been shown to be important for B cell responses (25). Despite its pleiotropic roles in the immune system, whether TRAF6 regulates T cell fates per se has not been examined to date. In this study, we find that TRAF6 deletion during hemopoiesis causes chronic activation of CD4+ T cells that leads to lethal inflammatory disease. Moreover, similar inflammatory disease associated with T cell abnormalities is also observed when RAG2-deficient mice are complemented with TRAF6-deficient lymphocytes, suggesting that TRAF6 has a previously unrecognized role in maintaining normal T cell fate and function.

TRAF6-deficient chimeras exhibited progressive weight loss, cachexia, and a high mortality rate. This was associated with progressive accumulation of effector/memory phenotype CD4+ T cells in the spleen and visceral organs. By 16 wk of age (for surviving mice), all the T cells were of the effector-memory phenotype, with no remaining naive T cells (data not shown). Although we found severe thymic atrophy in TRAF6-deficient chimeras, we did not see an effect of deletion of TRAF6 on the pattern of thymocyte maturation. Moreover, in mixed chimeras, both the number of normal and of TRAF6-deficient thymocytes was reduced, suggesting that chronic activation of T cells may have induced a stress.
response that, through the action of corticosteroids, impeded thymic output. Therefore, it appears that TRAF6 is not likely to intrinsically regulate T cell maturation, and as a corollary, the autoimmune-like disease we observe in TRAF6-deficient chimeras is not likely attributable to abnormalities in T cell development.

We observed a decrease in the number of B cells in the bone marrow and spleen of TRAF6-deficient chimeras. However, given the prominent T cell infiltrates and presence of Th2 cytokines within visceral organs, we believe that a B cell developmental defect is unlikely to be the cause of the Th2 inflammatory autoimmune type disease that we observed. We also found a dramatic expansion of CD11b^+ Gr1^+ cells in TRAF6-deficient chimeras. A similar expansion of CD11b^+Gr1^+ cells has been described in several mouse models of inflammatory or infectious disease, although a precise role for these cells in the observed diseases has not been defined (26, 27). The CD11b^+Gr1^+ cell population has been reported as immature myeloid cells derived from the monocye-macrophage lineage (21). They appear to possess suppressive function through secretion of NO and IL-10, or by cytolytic activity, and may down-regulate the intensity of immune stimulation in the face of persistent Ag (28–30). Therefore, the expansion of CD11b^+Gr1^+ myeloid cells could be secondary to, rather than the cause of, chronic activation of the CD4^+ T cells.

Autoimmune disease is observed in mice deficient in regulatory cells such as CD4^+CD25^+ T cells (22) or Vα14 NK T cells (31), or in mice deficient in molecules involved in the function of regulatory cells as CTLA-4 (32, 33) or Foxp3 (34, 35). Moreover, DCs also play a crucial role in self-tolerance by depleting autoreactive T cells during negative selection in the thymus and also by eliminating or inactivating them in the periphery (1, 15–18). However, the inflammatory disease in TRAF6-deficient chimeras is not likely due to a lack of functional regulatory cells or APCs, because the presence of normal regulatory T cells in mixed chimeras, or normal APCs in mixed chimeras and RAG2/TRAF6 chimeras did not prevent disease development.

Table III. Analysis of lymphoid organs

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Control Chimeras (n = 10)</th>
<th>TRAF6-Deficient Chimeras (n = 10)</th>
<th>Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymocytes (×10^6)</td>
<td>80.2 ± 19.4</td>
<td>10.8 ± 6.3</td>
<td>p &lt; 1.8E-07</td>
</tr>
<tr>
<td>CD4^+CD8^- (%)</td>
<td>83.8 ± 2.5</td>
<td>73.1 ± 4.6</td>
<td>p &lt; 4.0E-04</td>
</tr>
<tr>
<td>CD4^-CD8^+ (%)</td>
<td>10.6 ± 1.7</td>
<td>14.9 ± 4.7</td>
<td>p &lt; 5.0E-02</td>
</tr>
<tr>
<td>CD4^-CD8^+ (%)</td>
<td>3.5 ± 2.8</td>
<td>4.7 ± 1.3</td>
<td>p &lt; 1.8E-07</td>
</tr>
<tr>
<td>CD25^- (% in CD3^-CD4^-)</td>
<td>5.1 ± 1.6</td>
<td>5.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Splenocytes (×10^6)</td>
<td>34.4 ± 11.1</td>
<td>11.3 ± 6.7</td>
<td>p &lt; 6.1E-06</td>
</tr>
<tr>
<td>B220^- (%)</td>
<td>50.6 ± 9.9</td>
<td>26.0 ± 13.8</td>
<td>p &lt; 4.1E-04</td>
</tr>
<tr>
<td>CD11b^-Gr1^- (%)</td>
<td>4.8 ± 1.8</td>
<td>42.8 ± 22.9</td>
<td>p &lt; 4.0E-04</td>
</tr>
<tr>
<td>CD3^- (%)</td>
<td>38.8 ± 9.9</td>
<td>26.4 ± 12.4</td>
<td></td>
</tr>
<tr>
<td>CD4^- (% in CD3^-)</td>
<td>71.7 ± 2.7</td>
<td>71.7 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>CD8^- (% in CD3^-)</td>
<td>23.7 ± 1.5</td>
<td>21.5 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>
At least two complementary mechanisms may account for the chronic T cell activation we observed. First, signaling via TRAF6-binding receptors may negatively regulate T cells through mechanisms such as altering thresholds for signals generated by TCR stimulation or controlling cell cycle activity. In this way, the absence of TRAF6 could lead to chronic activation by self Ags. This could account for the fact that in mixed chimeras, abnormal numbers of control T cells have an effector/memory phenotype, as the secretion of inflammatory cytokines by TRAF6-deficient T cells could lead to bystander activation of control cells (36, 37). Second, TRAF6-deficient APCs may be able to dominantly sustain prolonged and pathologic activation of T cells. Indeed, we previously demonstrated a role for TRAF6 in DC development, maturation, and cytokine production (13).

We observed a Th2-type infiltrate in organs and accumulation of effector and Th2-type memory CD4+ T cells in the spleens of TRAF6-deficient chimeras. However, naive TRAF6-deficient CD4+ T cells were able to differentiate in vitro into Th1-type cells, demonstrating no intrinsic defect of Th1 polarization in absence of TRAF6. We previously found that TRAF6-deficient DC have impaired IL-12 production in response to TLR or CD40 stimulation (13). Although IL-12 plays an important role in Th1 differentiation, Th1 effector differentiation in response to pathogens can still occur in IL-12-deficient mice (38). Therefore, a defect in IL-12 production by APCs is by itself unlikely to explain Th2 polarization in TRAF6-deficient chimeras. However, we also noted a defect in IL-18 responsiveness, indicating that TRAF6 is functionally involved in the IL-18R signaling pathway. Furthermore, one effect of IL-12 is to enhance IL-18R expression, and signals through the IL-18R synergize with IL-12 to promote IFN-γ production by both Th1 and NK cells (19, 39). Thus, diminished IL-18R expression due to attenuated IL-12 production by TRAF6-deficient APCs could further exacerbate IL-18 hyporesponsiveness, thereby preferentially inducing the development of a Th2 response.

The downstream signaling pathways through which TRAF6 maintains normal T cell homeostasis are not yet defined, although we may speculate on some likely candidates. Various members of the TNFR superfamily interact with TRAF proteins that, through activation of NF-κB, Src family kinases, and mitogen-activated protein kinases, regulate diverse immunological processes, including cell proliferation, survival, and effector function (40). For example, TRAF1 and TRAF2 have been shown to play a role in T cell proliferation and survival (41–43). Little is known about the role of TRAF6 in signaling of TNFR family members expressed by T cells. However, TRAF family members can hetero-oligomerize with each other. For example, TRAF1 positively regulates survival signals mediated by TRAF2 by controlling its subcellular localization (44). In contrast, TRAF3 has been described to negatively regulate B cell-activating factor-R-mediated NF-κB activation in B cells (45). By binding with other TRAF family members such as TRAF2, TRAF6 may positively or negatively regulate their downstream activity. Indeed, a number of lines of evidence suggest that the T cell phenotype we observe in TRAF6-deficient chimeras may be linked to abnormalities in NF-κB pathways. For example, CD4+ T cells lacking the NF-κB subunit p50, c-rel (46, 47) or RelA (48), have defects in proliferation and IL-2 or IL-2R expression, both of which are needed for the maintenance of self-tolerance. Moreover, Th2-type organ inflammation has also been described in mice deficient in the NF-κB subunit RelB, which can be activated by TRAF6 (49). Intriguingly, CD4+ T cells lacking Rip2 kinase, which contains a putative TRAF6 binding site (10) and is involved in a signaling pathway leading to NF-κB activation, have defective proliferation, IL-2 expression, and Th1 differentiation associated with impairment of IL-12R and IL-18R signaling, a phenotype very similar to what we have observed.

In summary, our data demonstrate a critical role for TRAF6 in the regulation of T cell homeostasis. In the absence of TRAF6 in the hematopoietic compartment, mice develop a severe wasting disease, associated with chronic T cell activation and Th2 infiltration and inflammation in multiple visceral organs. The continued occurrence of the phenotype even in mixed chimeras strongly suggests an underlying intrinsic T cell defect. Thus, in addition to its known roles in innate and adaptive immune responses, we find that TRAF6 is also involved in the maintenance of self-tolerance.

References

development but synergizes with IL-12 for interferon-γ production and activates IRAK and NFKB. Immunity 7:571.
38. Jankovic, D., M. C. Kullberg, S. Hieny, P. Caspar, C. M. Colliazo, and A. Sher. 2002. In the absence of IL-12, CD4+ T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10+/- setting. Immunity 16:429.