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T Cell CD3 ζ Deficiency Enables Multiorgan Tissue Inflammation

Guo-Min Deng,^{*,†} Jessica Beltran,^{*} Chen Chen,[†] Cox Terhorst,[‡] and George C. Tsokos^{*}

Although a population of T cells with CD3 ζ chain deficiency has been found in patients with systemic lupus erythematosus, rheumatoid arthritis, cancer, and infectious disease, the role of CD3 ζ chain in the disease pathogenesis remains unknown. To understand the contribution of CD3 ζ deficiency to the expression of organ injury, we have performed the following studies. We used CD3 ζ -deficient mice to investigate the role of CD3 ζ in the pathogenesis of organ tissue inflammation. We found that the CD3 $\zeta^{-/-}$ mice can spontaneously develop significant organ inflammation that can be accelerated following the administration of polyinosinic:polycytidylic acid or allogeneic cells (graft versus host). T cells from CD3 $\zeta^{-/-}$ mice display increased expression of the adhesion molecules CD44 and CCR2 and produce increased amounts of IFN- γ blockade, which mitigates tissue inflammation. Our results demonstrate that CD3 ζ deficiency bestows T cells with the ability to infiltrate various tissues and instigate inflammation. Decreased CD3 ζ expression noted in T cells from various diseases contributes independently to tissue inflammation and organ damage. Approaches to restore CD3 ζ expression of the surface of T cells should be expected to mitigate tissue inflammation. *The Journal of Immunology*, 2013, 191: 3563–3567.

A population of T cells that lack the CD3 ζ chain exists in systemic lupus erythematosus (SLE), rheumatoid arthritis, cancer, and infectious diseases (1–4). SLE is a chronic autoimmune disease characterized by multiorgan tissue inflammation and high production of autoantibodies, and T cells appear to have a crucial role in the pathogenesis of SLE (5). The TCR is a multisubunit complex composed of the α/β heterodimer chain, which is responsible for Ag recognition, and the CD3 complex (δ , γ , ϵ , and ζ chain), which couples Ag recognition to intracellular signaling pathways (6). CD3 ζ chain is a 16-kDa transmembrane protein expressed by T cells and NK cells. It contains a short extracellular domain and a long intracellular domain that includes three ITAMs. Of all the TCR subunit chains, only CD3 ζ chain expression is specifically downregulated in activated T cells, and decreased levels of CD3 ζ chain have been reported in peripheral blood T cells from patients with SLE (1, 7) and HIV, T cells infiltrating the synovium in patients with rheumatoid arthritis, and tumor infiltrating T cells (2–4). Whatever the causes of decreased expression of the CD3 ζ chain, it is unclear whether T cells that lack or express decreased amounts of CD3 ζ on the surface contribute further to the disease-associated organ damage. We present evidence that T cells from mice lacking CD3 ζ display increased amounts of the adhesion molecule CD44 and

populate inappropriately tissues. The process is accelerated with polyinosinic:polycytidylic acid [poly:(IC)] or allogeneic stimulation.

Materials and Methods

Mice and reagents

Female C57BL/6, Bm12, and CD3 ζ chain-deficient mice generated by Love et al. (8) (stock no. 002704) were purchased from JAX Labs (Cold Harbor). CD3 ζ chain-deficient mice were backcrossed to the C57BL/6 strain for at least nine generations, and the whole genome was checked by Mice Genotyping Diversity Array (JAX Labs). All mice were housed in the animal facility of Beth Israel Deaconess Medical Center. The animal protocol was approved by the IACUC of Beth Israel Deaconess Medical Center. Poly:(IC), and chorea toxin-B-FITC were purchased from Sigma-Aldrich (St. Louis, MO). Anti-IFN- γ Ab (XMG 1.2) and control IgG (HRPN) were purchased from BioXCell (West Lebanon, NH).

Treatment of CD3 $\zeta^{-/-}$ mice with poly:(IC) or anti-IFN- γ Ab

Female CD3 $\zeta^{-/-}$ mice received i.p. injection of poly:(IC) (50 μ g; n = 8) and PBS (100 μ l; n = 6) twice per week. Treatment started at 6 wk old and lasted for 6 wk. Two mice with poly:(IC) treatment and one mouse with PBS treatment were sacrificed at 16 wk old. The rest of the mice were sacrificed at 32 wk old. For anti-IFN- γ treatment, female CD3 ζ chain-deficient mice received i.p. injection of XMG1.2 (1 mg/mouse; n = 4) and control HRPN (1 mg/mouse; n = 4) once per week for 2 wk. Treatment started at age of 13 wk. Multiorgan tissues were collected from experimental mice at 32 wk old for histopathologic examination.

Graft-versus-host experiments

We transferred splenocytes (1×10^8) isolated from female Bm12 mice into female CD3 $\zeta^{-/-}$ mice and wild C57BL/6 mice at 10 wk old by i.p. injection. In the reverse experiment, we transferred the same number of splenocytes (5×10^6) from CD3 $\zeta^{-/-}$ mice or wild C57BL/6 mice into Bm12 mice by i.p. injection. After 18 d, all host mice were sacrificed, and several organ tissues were collected for histopathologic examination.

Histopathologic and immunohistochemistry examination

After routine fixation and paraffin embedding of the tissue, tissue sections were cut and stained with H&E. Severity of tissue inflammation was scored 0–4: grade 0, normal; grade 1–4, different amounts of infiltrating inflammatory cells in the tissue. Incidence of tissue inflammation was evaluated based on histologic data. For immunohistochemistry after deparaffinization and Ag retrieval, samples were stained with primary Abs and followed by incubation with biotinylated secondary Abs, avidin-biotin-peroxidase complexes, and 3-amino-9-ethyl-carbazole containing H₂O₂. All sections were counterstained with Mayer hematoxylin.

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The online version of this article contains supplemental material.

Abbreviations used in this article: GVH, graft-versus-host; Poly:(IC), polyinosinic:polycytidylic acid; SLE, systemic lupus erythematosus.

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FIGURE 1. CD3 ζ chain-deficient mice spontaneously develop multiorgan tissue inflammation. **(A)** Expression of CD44 and IFN- γ on T cells with CD3 ζ chain deficiency or wild type T cells. T cells were from CD3 ζ chain-deficient mice or C57BL/6 mice at 12 wk old. **(B)** Stimulation of activated T cells ($\zeta^{-/-}$, $\zeta^{+/+}$) was achieved with a dose of 1 or 5 μ g anti-CD3 ϵ mAb (145.2C11) for 12 h. The percent cell loss was calculated by PI exclusion using flow cytometry. **(C)** and **(D)**. Incidence, severity and pictures of spleen (C) and tissue inflammation (D) in female CD3 ζ chain-deficient mice ($n = 4$) and wild type C57BL/6 mice ($n = 4$) at 36 wk old. Stained with H&E, original magnification $\times 20$. $*p < 0.01$.

Flow cytometry

Mononuclear cells were isolated from the spleen of CD3 ζ chain-deficient mice or C57BL/6 mice using gradient centrifugation. For surface markers such as CD44, cells were stained with Abs conjugated with FITC or PE for 45 min and then measured by flow cytometry. For the detection of intracellular molecules such as IFN- γ , cells were stained with Abs conjugated with FITC, PE, or others for 45 min after they were fixed and permeabilized. An LSR II instrument was used to detect labeled cells.

Apoptosis induction assay

T cells isolated from CD3 ζ chain-deficient mice and wild type mice using mouse T cell enrichment columns (R&D) were activated in 24-well plates using PMA plus ionomycin or anti-CD3 ϵ Ab for 3 d and with IL-2 (9) culture for an additional 2 d. Next, apoptosis assays were performed in duplicates in wells coated with anti-CD3 ϵ Ab. Samples were analyzed on an LSR II (Becton Dickinson). Apoptotic cells were measured by staining with annexin V or propidium iodide (Becton Dickinson).

ELISA

Serum IgG and ANA and anti-dsDNA autoantibodies were detected with ELISA kits of IgG, ANA (cat. no. A2298-12J; U.S. Biological) and dsDNA (cat. no. 5120; Alpha Diagnostics).

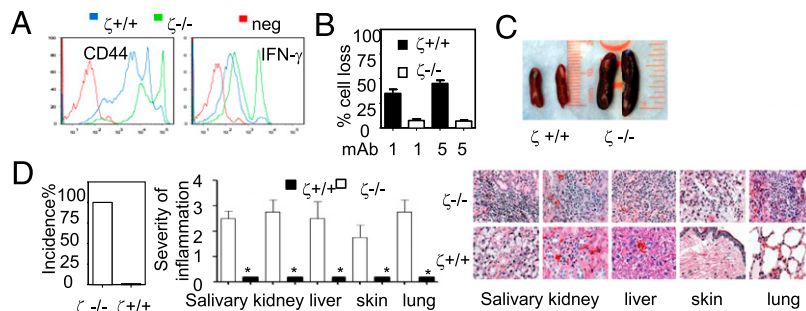
Statistics

Statistical evaluations of tissue inflammation pathology, serum IgG, ANA, and anti-dsDNA Ab were performed using the Student t test; $p \leq 0.05$ was considered statistically significant.

Results

CD3 ζ chain-deficient mice develop multiorgan tissue inflammation spontaneously

To determine whether CD3 ζ chain deficiency contributes to the pathogenesis of human disease, we studied clinical and immunologic features of CD3 ζ -deficient mice. First, we found that spleen T cells from 12-wk-old CD3 ζ -deficient mice express high levels of CD44, as it has been previously noted for SLE T cells (10), on the surface membrane and produce increased amounts of IFN- γ compared with control C57BL/6 mice (Fig. 1A). We found that the proportion of CD44^{high} cells versus CD44^{low} was 3.21:1 in wild type mice and 32:1 in CD3 ζ -deficient mice. There was



higher percentage of CD44⁺ and a lower percentage of CD62L⁺ in CD4⁺ T cells from the spleens of CD3 ζ chain-deficient mice compared with CD4⁺ T cells from wild type mice (Supplemental Fig. 1). Because T cells from patients with SLE display decreased activation-induced cell death (11), we subjected spleen T cells from CD3 $\zeta^{-/-}$ mice to stimulation with an anti-CD3 Ab, and we noted it to be decreased (Fig. 1B). Because CD44 and IFN- γ have been linked to inflammation (12, 13), we observed the mice for signs of organ inflammation for 9 mo. CD3 $\zeta^{-/-}$ mice spontaneously developed splenomegaly (Fig. 1C). In addition, we analyzed the number of CD4 and CD8 T cells, B cells, monocytes/macrophages, dendritic cells, and neutrophils in the spleens of CD3 $\zeta^{-/-}$ mice. We found that the number of T cells was decreased, but the number of B cells, monocytes/macrophages, and dendritic cells was increased in CD3-deficient mice compared with wild type mice. The number of neutrophils did not change significantly in CD3 ζ -deficient mice (Supplemental Figs. 2, 4). We also found that lymph nodes in CD3 $\zeta^{-/-}$ mice were also enlarged in size compared with lymph nodes in wild type mice (Supplemental Fig. 3). Histopathologic examination revealed inflammation in many organs, including the skin, salivary glands, liver, kidney, and lung in all CD3 $\zeta^{-/-}$ mice at the age of 36 wk (Fig. 1D). Therefore, T cells from CD3 $\zeta^{-/-}$ mice display features that enable them to infiltrate tissues.

Poly:(IC) promotes the development of spontaneous tissue inflammation in CD3 $\zeta^{-/-}$ mice

Because TLR3 ligands are available in chronically inflamed tissues and the activation of the TLR3 pathways contributes to inflammation in autoimmune diseases (14), we asked whether spontaneous tissue inflammation in CD3 ζ -deficient mice could be modulated by poly:(IC), a TLR3 ligand (15). Accordingly, CD3 $\zeta^{-/-}$ mice were treated with 50 μ g poly:(IC) or PBS for 6 wk. At the age of 16 wk, we found that multiorgan tissue inflammation developed in CD3 ζ -deficient mice treated with poly:(IC), but not with PBS (Fig. 2A). At the age of 32 wk, similar severity of inflammation developed in both poly:(IC) and PBS-treated CD3 ζ -deficient mice (Fig. 2B). In addition, we did not

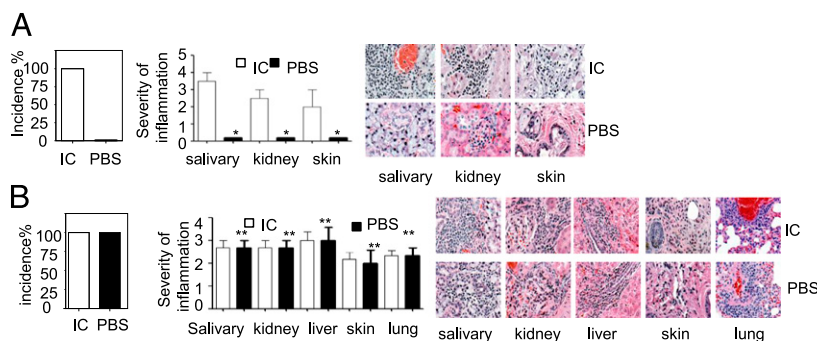
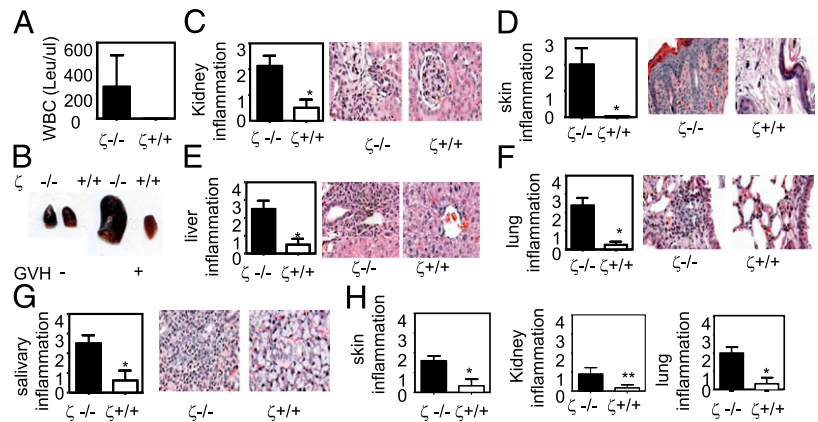


FIGURE 2. Poly:(IC) treatment promoted multiorgan tissue inflammation in CD3 ζ chain-deficient mice. **(A)** Incidence, severity, and pictures of tissue inflammation from female CD3 ζ chain-deficient mice treated with poly:(IC) ($n = 2$) or PBS ($n = 3$) at 16 wk old ($*p < 0.01$). **(B)** Incidence, severity, and pictures of tissue inflammation from female CD3 ζ chain-deficient mice treated with poly:(IC) ($n = 6$) or PBS ($n = 3$) at 32 wk old. Stained with H&E, original magnification $\times 20$. $**p > 0.05$.

FIGURE 3. Multiorgan inflammation following allogeneic cell transfer (GVH). Transfer of splenocytes from Bm12 mice into female $CD3\zeta^{-/-}$ mice or wild type C57BL/6 mice at 12 wk old. (**A** and **B**) Urine leukocytes and spleen size of $CD3\zeta^{-/-}$ mice and wild C57BL/6 mice with GVH. (**C–G**) Severity of inflammation and pictures in kidney, skin, lung, salivary gland, and liver from $CD3\zeta^{-/-}$ mice ($n = 8$) and C57BL/6 mice ($n = 8$) 18 d after transplant of splenocytes of Bm12 mice. (**H**) Severity of tissue inflammation in skin, kidney, and lung from Bm12 mice at 10 wk old ($n = 5$ per group) 18 d after transplant of splenocytes from $CD3\zeta^{-/-}$ mice or wild C57BL/6 mice. Stained with H&E, original magnification $\times 20$. * $p < 0.01$, ** $p > 0.05$.



observe the development of multiorgan tissue inflammation in C57BL/6 mice treated with the same or a higher dose of poly:(IC). These data indicate that poly:(IC) treatment can accelerate tissue inflammation in $CD3\zeta$ -deficient mice.

The contribution of $CD3\zeta^{-/-}$ T cells to multiorgan tissue inflammation in graft-versus-host disease

Allogeneic transfer of immune cells promotes autoimmunity and the development of multiorgan tissue damage (graft-versus-host [GVH] disease) (16–18). When we injected splenocytes from Bm12 mice into 12-wk-old $CD3\zeta^{-/-}$ mice, we observed (18 d later) the development of a remarkably large spleen and more severe inflammatory cell infiltration into the kidney, skin, liver, lung, and salivary glands compared with controls (Fig. 3A–3G). We noted the development of similarly enhanced tissue inflammatory response when we performed the reverse experiment—that is, when we transferred splenocytes containing $CD3\zeta^{-/-}$ T cells from $CD3\zeta^{-/-}$ into Bm12 mice (Fig. 3H). These data indicate that the presence of $CD3\zeta^{-/-}$ T cells promotes the development of multiorgan tissue inflammation in GVH disease.

$CD3\zeta^{-/-}$ mice fail to produce autoantibodies

At least in patients with SLE, multiorgan tissue inflammation has been linked to the presence of autoantibodies. Accordingly, we asked whether $CD3\zeta^{-/-}$ mice display increased amounts of autoantibodies. We noted that $CD3\zeta^{-/-}$ mice had lower levels of serum total IgG, anti-nuclear and anti-dsDNA Abs compared with wild type mice, and this fact did not change after the injection of poly:(IC) (Fig. 4A). In addition, we did not detect IgG deposited in sites of tissue inflammation in $CD3\zeta^{-/-}$ (Fig. 4B). Therefore, multiorgan tissue inflammation in $CD3\zeta^{-/-}$ mice is not associated with the presence of autoantibodies.

Features of $CD3\zeta^{-/-}$ T cells that explain tissue migration

Immunohistochemistry staining of the inflammatory infiltrates demonstrated the presence of $CD3^{+}$ and $CD4^{+}$ T cells (Fig. 4C). Fig. 1 shows evidence that T cells from $CD3\zeta^{-/-}$ mice express CD44 on the surface membrane. Using immunohistochemistry, we found that the tissue-infiltrating T cells in 32-wk-old mice express CD44, as do spleen T cells (Fig. 5A). Furthermore, T cells from the spleen and T cells infiltrating tissues express CCR2 (Fig. 5B, left), and the CCR2 ligand (MCP-1) was found abundantly present in the inflamed sites (Fig. 5B, right).

IFN- γ has been shown to play an important role in the pathogenesis of SLE, at least in certain lupus-prone mice (19). Spleen T cells from $CD3\zeta^{-/-}$ mice and T cells present in inflamed sites express high levels of IFN- γ (Fig. 5C). We considered that IFN- γ might represent a driving force in the expression of tissue inflammation in $CD3\zeta^{-/-}$ mice as it was claimed for other lupus-prone mice. Indeed, treatment of 32-wk-old $CD3\zeta^{-/-}$ mice with an anti-IFN- γ Ab for 2 wk abrogated the development of tissue inflammation (Fig. 5D), thus assigning IFN- γ an important role in organ damage.

Discussion

Peripheral blood T cells from patients with SLE (1, 7), infected with HIV (4), T cells infiltrating tumors (3), and the synovium of patients with rheumatoid arthritis (2) have decreased amounts of CD3 ζ chain, which is an important component of the CD3/TCR complex. Similarly, in an infectious animal model of gingivitis, T cells were found to have decreased CD3 ζ levels (20). Multiple causes, at least in SLE T cells, have been identified as contributing to decreased CD3 ζ expression (5). Regardless of the causes of decreased expression of CD3 ζ by T cells in various disease states, an important unanswered question has been whether T cells with decreased CD3 ζ contribute independently to the expression of tissue inflammation and organ damage. In this study, we used

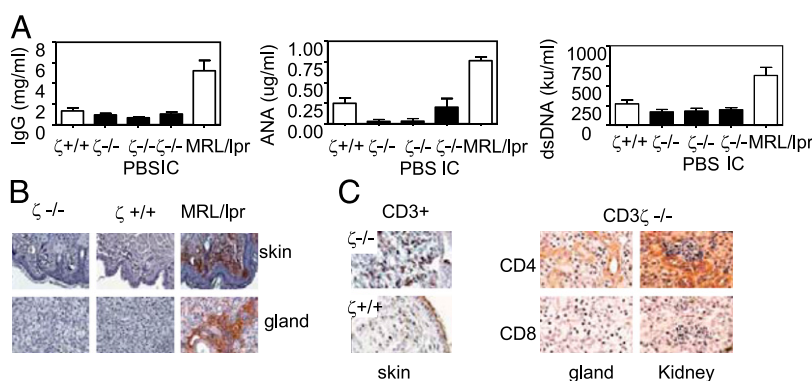
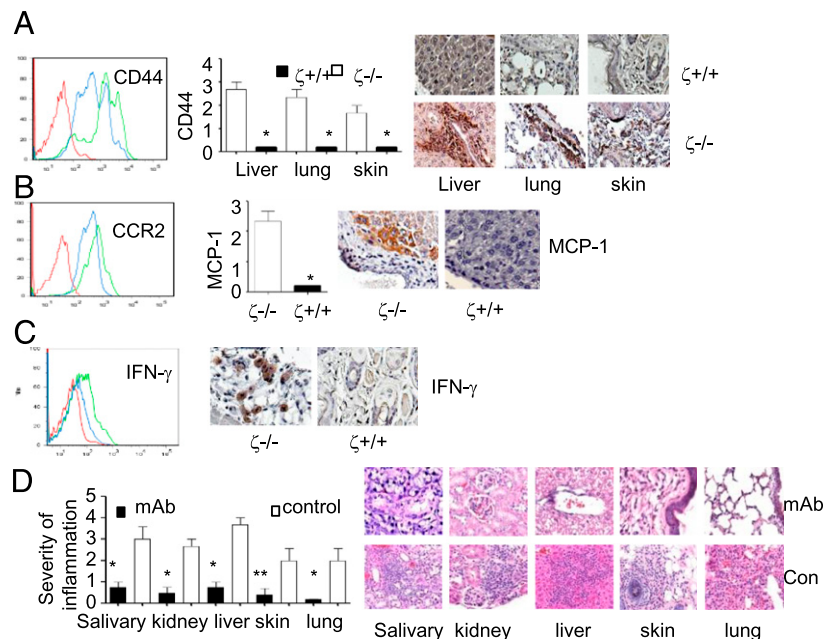


FIGURE 4. Humoral or cellular immunity in tissue inflammation in $CD3\zeta^{-/-}$ -deficient mice. (**A**) Serum levels of IgG, autoantibody of ANA, and ds-DNA in $CD3\zeta$ chain-deficient mice ($\zeta^{-/-}$) and C57BL/6 mice ($\zeta^{+/+}$) at 36 wk old, $CD3\zeta$ chain-deficient mice treated with IC [poly:(IC)] or PBS at 32 wk old, and MRL/lpr mice at 20 wk old. (**B**) IgG staining in tissue (skin, salivary gland) from $CD3\zeta$ chain-deficient mice, wild C57BL/6 mice at 32 wk old, and MRL/lpr mice at 20 wk old. (**C**) Immunohistochemistry staining of $CD3^{+}$, $CD4^{+}$, and $CD8^{+}$ T cells in tissue (salivary gland, kidney) of $CD3\zeta$ chain-deficient mice and wild type mice at 32 wk old. Stained with H&E, original magnification $\times 20$.

FIGURE 5. Molecular requirements for induction of multiorgan tissue inflammation in CD3 ζ chain-deficient mice. **(A and B)** Flow cytometry-measured expression of CD44 (A), CCR2 (B) on CD3 ζ chain-deficient T cells and immunohistochemistry staining of CD44 (A) and MCP-1 (B) on infiltrating cells in tissue of CD3 ζ chain-deficient mice at 32 wk old. $*p < 0.01$. **(C)** Expression of IFN- γ on CD3 ζ chain-deficient T cells and in the skin of CD3 ζ chain-deficient mice and wild type mice at 36 wk old. **(D)** Severity and pictures of multiorgan tissue from CD3 ζ chain-deficient mice treated with anti-IFN- γ Ab (XMG1.2) or control Ab (HRPN) at 32 wk old. $*p < 0.01$, $**p > 0.05$. Stained with H&E, original magnification $\times 20$.



CD3 $\zeta^{-/-}$ mice to demonstrate that although these animals do not mount an autoantibody response, they gradually develop inflammation of many organs, including the kidney, skin, salivary glands, and liver, when they age to 7 mo. Interestingly, the inflammatory response can be accelerated in younger mice after the injection of poly:(IC) or allogeneic cells. Therefore, CD3 ζ deficiency is sufficient to enable inappropriate homing of T cells to tissues possibly instigating organ damage.

Inflammation in multiple organs of CD3 $\zeta^{-/-}$ mice is not caused by humoral immunity. In the GVH model, multiorgan tissue inflammation developed in a short course of 18 d. In aged CD3 $\zeta^{-/-}$ mice, ANA and dsDNA Abs were also significantly lower in CD3 $\zeta^{-/-}$ mice than in wild type mice. A patient with CD3 $\zeta^{-/-}$ deficiency exhibited immunodeficiency (21), but it is not known whether an inflammatory response was smoldering in the tissues or whether additional manifestations would develop later in life. CD3 $\zeta^{-/-}$ T cells are polarized into IFN- γ -producing cells (22, 23), which inhibit Th2 cytokine production and then probably inhibit Ab production. This line of information demonstrates that humoral immunity is not necessary in the development of tissue inflammation in CD3 $\zeta^{-/-}$ mice.

T cells from even young CD3 $\zeta^{-/-}$ mice displayed a memory cell-like CD44^{high}CD62^{low} phenotype (22, 23), and these T cells migrate easily from the blood organ tissues. Specifically, CD3 $\zeta^{-/-}$ T cells, like SLE T cells (24), display increased expression of the adhesion molecular CD44, CCR2, and produce increased amounts of IFN- γ blockade, which mitigates tissue inflammation. These molecules alone or concurrently with other factors enable the exit of T cells to tissues. For example, the presence of the CCR2 ligand MCP-1 in the tissues, as noted in this study (Fig. 5), probably contributes to the homing of CD3 $\zeta^{-/-}$ cells to tissues.

T cells with CD3 ζ deficiency from mice or patients with SLE produce a large amount of IFN- γ (22, 25). IFN- γ in some animal models of autoimmune disease is important in the expression of disease (13), although its levels can be decreased, at least in peripheral blood T cells for patients with SLE (1, 7). Anti-IFN- γ Ab inhibited the development of inflammation in multiorgan tissues of CD3 ζ -deficient mice (Fig. 5).

There are limitations that are inherent to the mice used in our studies and that might qualify the interpretation of our results. The

CD3 ζ deletion was made in 129 cells, which can transfer genes facilitating the expression of autoimmunity. The mice did not define the autoimmune disease-associated SLAMF molecules, although other genes facilitating the expression of autoimmune disease may be present. It can be argued though that because the CD3 $\zeta^{-/-}$ mice did not develop signs of humoral autoimmunity, as would be expected from the contribution of 129-defined genes, the observed immunopathology should be attributed to CD3 ζ deficiency. Obviously, the absence of CD3 ζ significantly affects T cell development, and it has been claimed that both positive and negative selection are affected (3). A conditional deletion of CD3 ζ at later stages of life should represent the human disease conditions that display T cells with decreased CD3 ζ chain.

Disclosures

The authors have no financial conflicts of interest.

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