IFN-γ–Producing Effector CD8 T Lymphocytes Cause Immune Glomerular Injury by Recognizing Antigen Presented as Immune Complex on Target Tissue

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IFN-γ–Producing Effector CD8 T Lymphocytes Cause Immune Glomerular Injury by Recognizing Antigen Presented as Immune Complex on Target Tissue

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We investigated the role of effector CD8 T cells in the pathogenesis of immune glomerular injury. BALB/c mice are not prone to autoimmune disease, but after 12 immunizations with OVA they developed a variety of autoantibodies and glomerulonephritis accompanied by immune complex (IC) deposition. In these mice, IFN-γ–producing effector CD8 T cells were significantly increased concomitantly with glomerulonephritis. In contrast, after 12 immunizations with keyhole limpet hemocyanin, although autoantibodies appeared, IFN-γ–producing effector CD8 T cells did not develop, and glomerular injury was not induced. In β2-microglobulin–deficient mice lacking CD8 T cells, glomerular injury was not induced after 12 immunizations with OVA, despite massive deposition of IC in the glomeruli. In mice containing a targeted disruption of the exon encoding the membrane-spanning region of the Ig μ-chain (μMT mice), 12 immunizations with OVA induced IFN-γ–producing effector CD8 T cells but not IC deposition or glomerular injury. When CD8 T cells from mice immunized 12 times with OVA were transferred into naive recipients, glomerular injury could be induced, but only when a single injection of OVA was also given simultaneously. Importantly, injection of OVA could be replaced by one injection of the sera from mice that had been fully immunized with OVA. This indicates that deposition of IC is required for effector CD8 T cells to cause immune tissue injury. Thus, in a mouse model of systemic lupus erythematosus, glomerular injury is caused by effector CD8 T cells that recognize Ag presented as IC on the target renal tissue. The Journal of Immunology, 2013, 191: 000–000.

Glomerular injury is a major clinical feature of systemic lupus erythematosus (SLE) and is found in up to 50% of SLE patients. This injury has been attributed to the induction of an immunopathology resulting from immune complex (IC) deposition (1–7). However, it is also clear that IC by itself is not sufficient for the development of glomerular injury (8–11) and that CD4 T cells also contribute to the glomerular injury seen in SLE. Wofsy et al. (12–14) and Jabs et al. (15) showed that anti-CD4 T cell Ab therapy could significantly reduce the frequency and the extent of glomerulonephritis in both NZB/W F1 and MRL/lpr mouse models of lupus. Jevnikar et al. (16) showed that deficiency of MHC class II results in the amelioration of autoimmune renal disease in MRL/lpr mice. In contrast, Chesnutt et al. (17) showed that nephritis is not abolished in CD4-deficient MRL/lpr mice, whereas Christianson et al. (18) and Chan et al. (19) showed that glomerular injury is prevented in MHC class I-deficient MRL/lpr mice, which lack CD8 T cells. D’Agati et al. (20) showed that CD8, rather than CD4, T cells predominate in most of the biopsied kidney samples from patients with SLE. Couzi et al. (21) showed that CD8 T cells predominantly infiltrate to the periglomerular region of lupus kidney and are significantly associated with the prognosis of lupus nephritis. Together, these findings reinforce the importance of CD8 T cells in the pathogenesis of lupus nephritis. Nevertheless, discrepancies still exist in assessing the contribution of CD4 versus CD8 T cells to glomerular injury and IC deposition (22–25). Thus, we wished to better clarify the role of CD8 T cells in this pathology.

We previously succeeded in inducing glomerular injury with characteristics almost identical to human SLE in mice normally not prone to autoimmune disease (26). In that study, we showed that both CD4 T cell help and Ag cross-presentation are fundamental for activating CD8 T cells to become fully matured CTLs and, subsequently, to induce lupus kidney disease. If this finding is correct, then discrepancies in the literature describing differing contributions of CD4 or CD8 T cells to lupus nephritis (12–21) might be reconciled. Therefore, in the current study, we used β2-microglobulin (β2m)-deficient mice, which lack functional effector CTLs, and mice harboring a targeted disruption of the exon encoding the membrane-spanning region of the Ig μ-chain (μMT), which lack B cells, to examine the relationships among IC deposition, effector CD8 T cells, and the nephritis induced in mice by repeated immunization with OVA.

Materials and Methods

Animal studies

Animal studies were performed with the approval of the Institutional Animal Care And Use Committee and according to animal experimental regulations at Kobe University and Kyushu University. Eight-week-old...
female BALB/c mice (Japan SLC, Hamamatsu, Japan), β-m-deficient mice (BALB/c background) (27), and μMT mice (BALB/c background) (28) were immunized with 500 μg OVA (grade V; Sigma, St. Louis, MO), 100 μg keyhole limpet hemocyanin (KLH; Sigma), or PBS by i.p. injection every 5 d. Nine days after the final immunization, proteinuria was measured semiquantitatively using urine dipsticks (Albtest; Siemens Healthcare Diagnostics, Tarrytown, NY), and B, T, CD4 T, and CD8 T cells were isolated from spleen to care Diagnostics, Tarrytown, NY), and B, T, CD4 T, and CD8 T cells were isolated from mice immunized 12 times with OVA with ≥90% purity using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells were adoptively transferred i.v. into naïve BALB/c mice (2.5 × 10^7/mouse), and an additional booster i.p. injection of 500 μg OVA or 500 μl sera from mice immunized 12 times with OVA was given at 24 h after transfer. Sera, urine, and organs were collected 2 wk later. CD4 and CD8 T cells in the kidney were analyzed by flow cytometry 9 d after the final immunization with allophycocyanin-conjugated anti-CD8 Abs (RM4-5; BioLegend, San Diego, CA) and PerCP-conjugated anti-CD8 Abs (53-6.7; BD Pharmingen, San Diego, CA). Glomerular injury in mice was evaluated by studying 30 glomeruli/mouse.

**Immunofluorescent staining**

Frozen kidney sections were stained for C3 and IgG using goat anti-C3 Abs (Bethyl Laboratories, Montgomery, TX), Alexa Fluor 488–conjugated anti-goat IgG Abs, or Alexa Fluor 594–conjugated anti-mouse IgG Abs (both from Molecular Probes, Eugene, OR).

**Intracellular IFN-γ staining**

Spleen cells (1 × 10^7/ml) were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of brefeldin A (10 μg/ml) for 4 h and stained with PerCP-conjugated anti-CD8 Ab, followed by fixation in 2% formaldehyde, permeabilization with 0.5% saponin (Sigma), and staining with PE-conjugated anti–IFN-γ Ab (XMGl2; BD Pharmingen).

**ELISA**

Sera were assayed for rheumatoid factor (RF) by ELISA (Shibayagi, Gunma, Japan), for anti-Sm Ab using plates coated with Sm Ag (InnuoVision, Springfield, AR), and for anti-dsDNA Ab using plates coated with dsDNA (Worthington Biochemical, Lakewood, NJ) that had been digested using S1 nuclease (Promega, Madison, WI). Serum IgG, IgG1, and IgG2a were measured by ELISA (Bethyl Laboratories). Serum IC was measured using anti-C3 Ab (Bethyl Laboratories) and HRP-conjugated anti-mouse IgG Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD), followed by reaction with o-phenylenediamine (Sigma). An arbitrary unit (AU) of 1.0 is the equivalent of the titer found in sera of 25-wk-old MRL/lpr mice. Anti-OVA Ab in sera was quantified as a reference using mouse anti-OVA mAb (OVA-14; Sigma).

**Statistical analysis**

Statistical analyses were performed using the Student t test, and the data are expressed as the mean ± SD.

**Results**

**Requirement of activated CD8 T cells for glomerular injury**

Wild-type (WT) BALB/c mice, which are normally not prone to autoimmune disease, were repeatedly immunized with OVA every 5 d. After 12 immunizations, we observed an increase in autoantibodies, including RF, anti-Sm, and anti-dsDNA Abs, and an increase in serum IC and glomerular injury (Fig. 1A, 1B). Glomerular injury was assessed by proteinuria and glomerulonephritis, according to the International Society of Nephrology/Renal Pathology Society classification. Glomerular injury consisted of the following: class II mesangial proliferative glomerulonephritis in 26.29 ± 7.53% of the specimens (n = 9, mean ± SD) (Fig. 1Ca), class III focal glomerulonephritis in 13.70 ± 7.53% of the specimens (Fig. 1Cb), class IV diffuse glomerulonephritis in 38.14 ± 7.28% of the specimens (Fig. 1Cc), class V membranous glomerulonephritis in 3.57 ± 3.51% of the specimens (Fig. 1Cd), and class VI advanced sclerosing glomerulonephritis in 10.37 ± 3.09% of the glomerular specimens (n = 9, mean ± SD) (Fig. 1Ce). We also immunized mice 12 times with KLH and observed a similar induction of RF and anti-Sm Ab; however, there was no anti-dsDNA Ab induction, proteinuria, or glomerular injury (Fig. 1A, 1B). We also observed an increase in serum IC and the massive deposition of IC in the glomeruli of mice. Serum IgG1 and IgG2a, which were reported to increase concomitantly with autoimmune renal diseases (29, 30), were also increased after immunization with either OVA or KLH (Fig. 1D). Importantly, however, we noted that IFN-γ–producing activated CD8 T cells were increased in OVA-immunized, but not KLH-immunized, mice (Fig. 1E). These IFN-γ–producing producing CD8 T cells infiltrated into the sites of OVA deposition in the glomeruli of the mice immunized 12 times with OVA (26). Compared with KLH-immunized and control mice, there were increased numbers of CD8 T cells, but not CD4 T cells, in the kidneys of OVA-immunized mice (Fig. 1F). This indicates that mice immunized 12 times with KLH do not induce effector CD8 T cells, which suggests that, despite the massive deposition of IC in the kidneys, the lack of glomerular injury in these mice is due to the fact that KLH was not cross-presented to T cells.

Glomerular injury induced by repeated OVA immunization could be adoptively transferred into naïve recipients via CD8+ T cell transfer (Fig. 2A). This adoptive transfer of glomerular injury was not accompanied by the generation of autoantibodies (26) or by any significant increase in IC or anti-OVA Ab in sera of the recipient mice (Fig. 2B). IC was only minimally deposited in the glomeruli of recipients, because they were boosted only once with OVA after cell transfer. Thus, these findings suggest that CD8 T cells are required for the generation of glomerular injury, whereas massive IC deposition is not required, although low amounts of IC deposition may still be necessary.

To prove that effector CD8 T cells are required for immune glomerular injury, we immunized β-m-deficient mice, which lack functional effector CTLs (27). Following 12 immunizations of these mice with OVA, there was a marked increase in serum autoantibodies, including anti-dsDNA Ab (26), IC, and anti-OVA Ab, and there was massive deposition of IC in the kidney (Fig. 3B, 3C). However, glomerular injury was minimal, as demonstrated by low proteinuria (Fig. 3A) and the absence of glomerulonephritis by histopathologic analysis (Fig. 3B). This finding indicates that functional effector CD8 T cells are required for the induction of immune glomerular injury.

**Requirement of IC**

We next tested whether the presence of Ag in the form of IC is required for immune glomerular injury. For this, we used μMT mice, which lack B cells, do not induce Ag-specific Ab responses (28), and do not generate detectable IC in sera even after 12 immunizations with OVA (data not shown). In these mice, IC deposition and renal disease were both absent (Fig. 4A, 4B). However, IFN-γ–producing CD8 T cells developed to levels comparable to those seen in WT mice (Fig. 4C). This indicates that deposition of Ag in the form of IC is required before effector CD8 T cells can cause glomerular injury. To verify this further, we performed serum-transfer experiments. When CD8 T cells from mice immunized 12 times with OVA were transferred into naïve recipients, they could reproducibly induce glomerular injury, but only when a single injection of OVA was given simultaneously (Fig. 2A). We subsequently tested whether one injection of sera from mice immunized 12 times with OVA could substitute for the single booster injection of OVA. We found that IC was indeed deposited in the recipients’ glomeruli and was accompanied by glomerular injury at 2 wk after the transfer of CD8 T cells (Fig. 5). This demonstrates that deposition of at least some amount of Ag
in the form of IC is required for effector CD8 T cells to exert their cytotoxicity and induce immune glomerular injury.

Discussion
The results in this study show that IFN-γ-producing effector CD8 T cells, which can recognize Ag presented as IC on target tissue, are required for the induction of glomerular injury in this mouse model of SLE. This finding is consistent with results showing that CD8 T cells are the dominant T cell population in renal biopsy specimens from lupus patients who presented with class III and IV glomerulonephritis (21). Heymann et al. (11) showed that glomerular Ag-specific CTLs induce renal immunopathology with the help of CD4 T cells. Such cooperation between CD8 and CD4 cells is indeed likely and may also explain several somewhat contradictory reports describing the contributions of CD8 and/or CD4 T cells to the pathogenesis of immune glomerular disease (12–21). In a previous study of experimentally induced SLE, we found that both Ag cross-presentation and CD4 T cell help were essential for generating effector CD8 CTLs, leading to glomerular injury (26). We proposed the “self-organized criticality theory,” which explains that systemic autoimmunity, or SLE, necessarily takes place when a host’s immune system is overstimulated by repeated exposure to Ag, achieving levels that surpass the immune system’s stability limit (i.e., self-organized criticality). This theory proposes that autoreactive lymphocyte clones are newly generated via de novo TCR revision from nonautoreactive clones at the periphery (26, 31). We named this novel T cell type an autoantibody-inducing CD4 T cell and postulated that these cells stimulate B cells to generate various autoantibodies, as well as to promote the final differentiation of CD8 T cells into CTLs via Ag cross-
presentation, leading to the tissue injuries found in SLE. Such a scenario is consistent with the previously demonstrated roles of CD8 and/or CD4 T cells in the pathogenesis of kidney disease (12–21) (i.e., CD8 T cells must mature into effector CD8 T cells with the help of CD4 T cells, primarily in the induction phase of glomerulonephritis).

In the current study, with regard to the role of effector CD8 T cells in the effector phase of glomerulonephritis, we focused on whether effector CD8 cells were directly responsible for the induction of glomerular injury. First, we found that effector CD8 T cells recognized Ag presented as IC on target renal tissue and consequently exerted immune glomerular injury. Glomerulonephritis is transferrable via fully matured effector CD8 T cells. Splenocytes of OVA-immunized BALB/c mice were adoptively transferred to naïve recipients, and the recipients were injected with 500 μg of OVA 24 h after transfer. (A) Proteinuria, histopathology (H&E; scale bar, 50 μm; original magnification ×400) and the deposition of IC, IgG, and C3 in the glomeruli of recipient mice (scale bar, 50 μm; original magnification ×200) 2 wk after cell transfer. (B) Serum IC and anti-OVA Ab in recipients as measured by ELISA 2 wk after cell transfer (mean ± SD). Thin or bold dotted lines represent the averaged value in the donor mice immunized 12 times with PBS or OVA, respectively. AU refers to the value obtained with sera of MRL/lpr mice. Each experiment was performed twice independently.

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In the current study, with regard to the role of effector CD8 T cells in the effector phase of glomerulonephritis, we focused on whether effector CD8 cells were directly responsible for the induction of glomerular injury. First, we found that effector CD8 T cells recognized Ag presented as IC on target renal tissue and consequently exerted immune glomerular injury. Glomerulonephritis is transferrable via fully matured effector CD8 T cells. Splenocytes of OVA-immunized BALB/c mice were adoptively transferred to naïve recipients, and the recipients were injected with 500 μg of OVA 24 h after transfer. (A) Proteinuria, histopathology (H&E; scale bar, 50 μm; original magnification ×400) and the deposition of IC, IgG, and C3 in the glomeruli of recipient mice (scale bar, 50 μm; original magnification ×200) 2 wk after cell transfer. (B) Serum IC and anti-OVA Ab in recipients as measured by ELISA 2 wk after cell transfer (mean ± SD). Thin or bold dotted lines represent the averaged value in the donor mice immunized 12 times with PBS or OVA, respectively. AU refers to the value obtained with sera of MRL/lpr mice. Each experiment was performed twice independently.
Phritis was not observed in the absence of effector CD8 T cells. Second, there must be at least some minimal amount of IC deposited on the target renal tissues for effector CD8 T cells to cause immune injury.

Previous studies showed that β2m is required for the surface expression of MHC class I, as well as CD1d and Qa-1. Although lack of CD1d expression can lead to NKT cell deficiency (32), we found that repeated OVA immunization of CD1d knockout mice led to both the production of various autoantibodies and the development of proteinuria to the same degree observed in WT mice (K. Tsumiyama and S. Shiozawa, manuscript in preparation). Qa-1 plays important roles in the suppression of CD4 T cells and CD8 regulatory T cell (Treg) functions (33, 34). Deficiency of Qa-1 causes exaggerated secondary CD4 responses against virus or self-peptide and impairs CD8 Treg function, ultimately leading to autoimmunity (35, 36). However, we found that the levels of autoantibodies generated after 12 repeated immunizations with OVA were similar between β2m-deficient mice and WT mice (Supplemental Fig. 3 in Ref. 26). In addition, tissue injuries, including glomerulonephritis, were clearly not induced in the β2m-deficient mice (Fig. 3), even in mice that had received CD8 T cells transferred from OV A-stimulated WT mice (figure 1C of in Ref. 26). In previous studies, the phenotype of inhibitory CD8 Tregs was shown to fluctuate (37–40). Inhibitory CD8 Treg function was reported to be defective in human SLE patients and in animal models of SLE (41–43). However, it was also observed that lupus nephritis can be suppressed by anti-CD8 Ab treatment or by MHC class I deficiency (18, 19, 44). Further, recent studies show that Tregs are actually required for the final differentiation of CD8 T cells (45). Thus, CD1d, Qa-1, NKT cells, or CD8 Tregs do not appear to play a causative role in immune-mediated glomerular disease.

In summary, immune tissue injury requires, first, that CD8 T cells mature into effector cells with the help of CD4 T cells, primarily in the induction phase. Second, in the effector phase, effector CD8 T cells recognize Ag presented as IC on target tissue, and this recognition is required for their cytotoxic actions.

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Disclosures
The authors have no financial conflicts of interest.