Original Report: Laboratory Investigation

American Journal of Nephrology

Am J Nephrol 2011;34:519–528
DOI: 10.1159/000333004

Received: July 12, 2011
Accepted: September 7, 2011
Published online: November 7, 2011

Natural Recovery from Antiglomerular Basement Membrane Glomerulonephritis Is Associated with Glomeruli-Infiltrating CD8α^+CD11c$^+$MHC Class II$^+$ Cells

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Key Words
Glomerulonephritis · Immunosuppression · Animal models · Apoptosis

Abstract
Background/Aims: In an antiglomerular basement membrane glomerulonephritis (GN) model, GN-resistant Lewis (LEW) rats naturally recover from early glomerular inflammation (days 21–23). We have previously identified a glomeruli-infiltrating CD8α^+CD11^{high}MHC II$^+$ cell (GIL CD8α^+ cell) in GN-prone Wistar Kyoto (WKY) rats, which terminates glomerular inflammation through inducing T cell apoptosis prior to glomerular fibrosis at days 35–40. We investigated if GIL CD8α^+ cells were also associated with the recovery in LEW rats. ***Methods:*** GIL CD8α^+ cells in LEW rats were characterized; their infiltration was observed in connection with T cell apoptosis in glomeruli. ***Results:*** An influx of GIL CD8α^+ cells into inflamed glomeruli was confirmed in the immunized LEW rats at days 17–22, which was much earlier than days 28–35 in WKY rats. Notably, LEW rats had a GIL CD8α^+CD11^{high} subpopulation after day 17, while WKY rats lacked this population until after day 30. Analyses further revealed a large number of clustered apoptotic CD4$^+$ or CD3$^+$ T cells in the glomeruli during recovery (day 23) in LEW rats, as compared to day 35 (transition to fibrosis) in WKY rats. Thus, infiltration of GIL CD8α^+ cells coincided with decline of glomerular inflammation and T cell apoptosis during recovery in LEW rats. Isolated GIL CD8α^+ cells were able to infiltrate glomeruli in both WKY and LEW rats at day 20. ***Conclusion:*** Our data revealed a strong association between GIL CD8a+ cells and recovery from early glomerular inflammation. It raises a possibility of involvement of GIL CD8a+ cells in the recovery.

Introduction

Spontaneous recovery from autoimmune diseases has been reported in both human patients and animal models [1–4]. By mimicking those natural recovery mechanisms, we may develop immunotherapeutic strategies for effective treatment of autoimmune diseases. A full understanding of the mechanism behind the recovery is not only the first step leading to development of such treatment, but may also reveal novel immune tolerance mechanisms. Recently, many immune cells, including regulatory T cells and several types of macrophages or dendrit-

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ic cells (DC), have been shown to be involved in immune tolerance [5–9]. Those cells usually reside in lymphoid organs and prohibit activation of autoreactive T cells into effecters. Thus, generation of pathogenic autoreactive T cells is prevented de novo. However, naïve autoreactive T cells may be activated and further differentiate into pathogenic effector cells through, for example, molecular mimicry or bystander activation during an infection [10, 11]. Thus, it will be equally important to ask if any mechanisms in target tissues are able to control autoimmune diseases after pathogenic autoreactive T cells have initiated tissue damage.

Antiglomerular basement membrane (GBM) glomerulonephritis (GN) is among the earliest recognized human autoimmune diseases. Mechanisms of GN pathogenesis have been well investigated at different levels [12–15]. We have developed a rat model for this disease, which is induced by the well-defined T cell epitope pCol(28–40) of autoantigen collagen 4α3 chain. GN-prone Wistar Kyoto (WKY) rats develop severe glomerular inflammation, which eventually is terminated and replaced by progressive fibrosis [16–18]. In fact, it is fibrosis rather than inflammation that leads to end-stage renal disease. In spite of sharing identical MHC molecules and mounting a similar T cell response to WKY rats, the Lewis (LEW) strain is GN resistant [19]. The GN resistance in LEW is due to a spontaneous termination of T cell-mediated glomerular inflammation at an early stage [19]. Thus, an unknown mechanism arrests autoimmune GN after pathogenic T cells have initiated tissue inflammation. As mimicking this naturally occurring recovery mechanism may lead to an antigen-specific immunotherapeutic strategy for treating autoimmune diseases, we explored this recovery mechanism. Although glomerular CD8+ T cells have been described, we have previously identified a novel CD8αα$^+$CD11c$^+$MHC II$^+$ DC-like myeloid cell population among glomeruli-infiltrating leukocytes (GIL) at transient stage from inflammation to fibrosis in WKY rats [20, 21]. This cell, designated as GIL CD8α$^+$ cells, is able to induce antigen-dependent T cell apoptosis in vitro. More importantly, infiltration of this DC-like population into glomeruli is coincident with a peak of apoptotic CD4$^+$ T cells during termination of glomerular inflammation prior to fibrosis, suggesting a direct involvement of this cell in T cell apoptosis in vivo [20]. In the present study, we further investigated whether infiltration of GIL CD8α$^+$ cells was also associated with T cell apoptosis and termination of T cell-mediated inflammation in the target tissue in LEW rats during their recovery stage.

Methods

Antibodies

Antibodies, including biotin-labeled anti-rat CD3 (G.4.18) and CD11b/c, PE-labeled anti-rat CD4 (OX35), CD8α, CD11c, TcRγδ (V65) and RT-1B (OX6), and FITC-labeled anti-rat CD8α, RT-1B (OX6), and various mouse IgG isotype controls, were from Pharmingen (BD, San Diego, Calif., USA). Biotin-labeled rabbit anti-rat caspase 3 mAb (D175) was from Cell Signaling Technologies, and goat antibody to rat CD94 (an NK cell marker) and anti-CD68 (ED1) mAb were from Santa Cruz. SR-13, which is an mAb to rat GBM, was kindly provided by Dr. Y. Sado (Okayama University, Japan). Anti-rat CD32 monoclonal antibody (D34-485, Pharmingen) was used for Fc block for immunostaining. Anti-rat IgG, PE, FITC or HRP-labeled anti-rat IgG, and purified rat IgG were from Southern Biotechnology (Birmingham, Ala., USA).

Induction and Evaluation of GN

All procedures involving animals in this study were approved by the institutional Animal Welfare Committee. Female WKY or LEW rats (4–6 weeks of age) were purchased from Harlan (Indianapolis, Ind., USA). Rats were allowed to acclimate for at least 3 days in the animal facility at the University of Texas Houston Health Science Center. Rats were immunized with peptide pCol(28–40) (0.15 μmol), emulsified in CFA, in one hind footpad and at the base of tail. Rats immunized with CFA alone served as controls. GN was evaluated by albuminuria and renal histopathology. Urine albumin concentration was semiquantitated by SDS-PAGE. Renal tissues fixed in Bouin's fixative were used for HE staining, or fixed in 4% of paraformaldehyde (PFA) for immunochemical staining. Severity of GN was expressed as glo-meruli injury score (GIS). At least 100 glomeruli were classified into five categories from normal to most severe: 0: normal, 1: leukocyte infiltration, 2: mild inflammation, 3: inflammation and 4: fibrosis. Subscores for each category were calculated by multiplying the category number with glomeruli number in this category. GIS = sum of sub-scores ÷ total glomeruli number. GIS ranged from 0 (normal) to 4 (fibrosis in all glomeruli). Renal tissues were used for immunofluorescent staining following our published method [20]. DAPI was used for nuclear staining and FITC-labeled SR-13 for GBM to reveal glomerular structure.

Isolation of Glomeruli and Glomeruli-Infiltrating Cells

The renal cortex was sliced, placed in a cold DMEM medium containing 10% FCS, ground against a #100 mesh, and repeatedly rinsed. The passing-through materials included intact glomeruli, fragmented tubules and single cells. The single cells were first removed by cell strainer (80 μm). The glomeruli were isolated to a purity of approximately 95% through repeated low-speed centri-fugation [19]. The isolated glomeruli were used for isolation of glomeruli-infiltrating cells. For isolation of cells, glomeruli were incubated with collagenase IV and Accutase™ at 37°C for 30 min with periodic stirring. Digested glomerular fragments were eliminated by centrifugation (300 rpm, 5 min), and the single cells were collected by centrifugation (1,000 rpm, 10 min). Those cells were designated as glomeruli-infiltrating leukocyte (GIL). The cells were further used for flow cytometry or for other purposes.

Detection of Apoptotic T Cells in Glomeruli

The immunoperoxidase method was used for detection of apoptotic cells in vivo using an anti-caspase 3 Ab-based kit (Cell Signaling Technology, Beverly, Mass., USA). Frozen sections were cut from PFA-fixed kidney tissues and stained following the manufacturer's instruction. DAB-based substrate was used to develop a brown color for positive cells, followed by counterstaining with methylene blue. Caspase 3-positive cells were counted and statistically analyzed. Immunofluorescence was applied to colocalize caspase 3 with $CD3^+$ cells in glomeruli. Briefly, frozen sections of kidney were incubated with biotin-labeled anti-caspase 3 antibody after preincubation with various blocking reagents. The sections were further incubated with streptavidin-FITC, and finally with PE-labeled anti-rat CD3 antibody. Two-color flow cytometry was used for identifying apoptotic $CD4^+$ T cells. To maintain the originality, freshly isolated glomerular cells without further treatment were immediately incubated with PE-labeled anti-CD4 antibody, followed by incubation with FITC-annexin V from a kit (Apoptosis detection kit, BioVision, Mountain View, Calif., USA). To rule out that isolation or digestion process might cause T cell apoptosis, the cells were isolated from draining lymph nodes using an identical isolation/digestion method to that for glomerular cells, and stained by PE-labeled anti-CD4 antibody and FITC-labeled annexin V. This control showed that T cells were less affected than other cells.

Purification Labeling and Transfer of Glomerular $CD8\alpha\alpha^+CD11^+CD3^-$ Cells

The isolated bulk glomeruli-infiltrating cells were first centrifuged on a Ficoll cushion to remove dead cells or debris, and adjusted to 10^7 cells/ml. The cells were first incubated with micromagnetic bead-labeled OX52 pan T cell marker to remove T cells on an automatic cell sorter (AutoMACS, Miltenyi Biotec). The negatively selected cells were further incubated with micromagnetic bead-labeled anti-CD8α antibody (10^7 cells/3 μl beads, Miltenyi Biotec, Germany). Flow cytometery showed the positively selected $CD8\alpha^+$ cells as a $CD8\alpha^+CD3^-$ cell population with purity over 90%. For labeling experiments, freshly isolated $CD8\alpha^+CD3^-$ cells were washed with HBSS, adjusted to 10^7 cells/ml, and labeled by CFSE (Molecular Probes, Eugene, Oreg., USA) following a published method [20]. The labeled cells (10^6 cells/rat) were injected into untreated or immunized rats. The cell recipients were sacrificed 24 h after the cell transfer. Different organs including lung, liver, pancreas, spleen, heart, small intestine, and kidney were removed and either fixed in 3% PFA or snap-frozen. Serial frozen sections (3 μm) which cut through 10 glomeruli, were counterstained with SR-13 to reveal GBM. The labeled cells were counted under a fluorescent microscope (Eclipse 80i, Nikon, Japan), and expressed as cells/glomerular section.

Results

Identification of Glomeruli-Infiltrating $CD8\alpha^+$ Cells in GN-Resistant LEW Rats

We have previously shown that after immunization with nephritogenic T cell epitope pCol(28–40), GN-resistant LEW rats develop a transient, histologically recognizable glomerular inflammation at days 20–23, which is characterized by infiltration of T cells and $ED1^+$ macrophages (fig. 1a) [19]. Detailed analyses by immunofluorescence and flow cytometry showed that T cells invaded glomeruli as early as day 15 (fig. 1b) [19, 20], followed by an influx of both $ED1^+$ macrophage and $CD4^+$ T cells at days 17–20 (fig. 1b). Glomerular inflammation peaked at day 21, and rapidly declined thereafter (fig. 1a). Histology or immunofluorescence failed to detect any signs of inflammation or T cells in glomeruli after day 25, except that the affected glomeruli were slightly enlarged in size (fig. 1a, b). Thus, a natural recovery from autoimmune GN occurred during days 21–25 in LEW rats.

We have reported a special GIL $CD8\alpha^+$ population, which resembles DC, in glomeruli at the late inflamma-

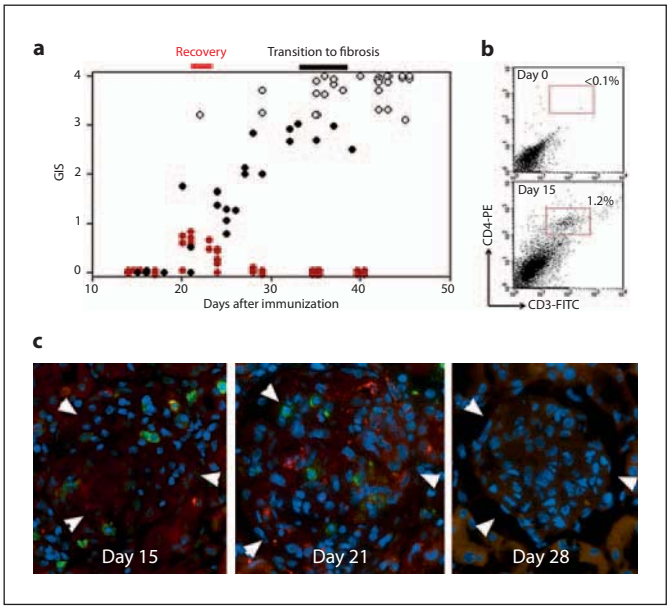

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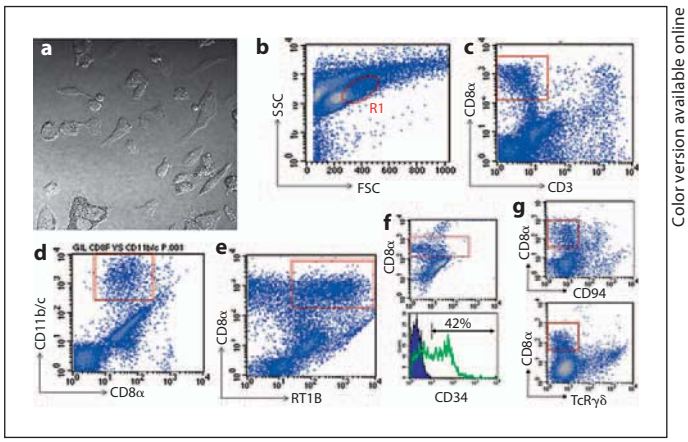
Fig. 1. Development of transient T cell-mediated anti-GBM GN in GN-resistant LEW rats in comparison to GN-prone WKY rats. **a** Time course of GN development after immunization with nephritogenic T cell epitope pCol(28–40). Each dot represents one individual. Severity of GN is expressed as glomerular injury score, from 0 (normal) to 4 (100% of glomeruli with fibrous crescentic lesions). Black dots = LEW; red dots = WKY; empty dots = WKY, fibrotic. Time frames for recovery in LEW (red letters) and transition to fibrosis in WKY rats (black letters) are indicated at the top. **b** Flow cytometry identification of glomeruli infiltrating $CD4^+$ T cells. **c** Immunofluorescent micrographs show infiltrating $CD4^+$ T cells (green) and $ED\text{-}1^+$ macrophages (red) at days 15, 21, and 28 after immunization in GN-resistant LEW rats. Note that no $CD4^+$ T cells and $ED\text{-}1^+$ macrophages are present at day 28. Nuclei were counterstained by DAPI, and glomeruli outlined by arrowheads. ×300. For colors, see online version.



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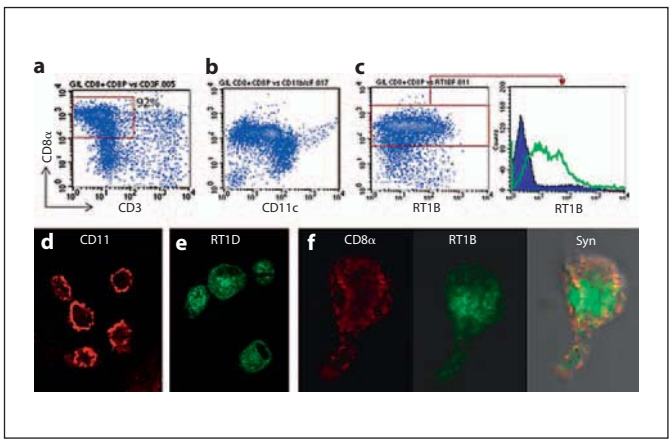

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Fig. 2. Identification of glomeruli-infiltrating CD8α$^+$ cells (GIL CD8α$^+$ cells) in GN-resistant LEW rats at day 21 after immunization with pCol(28–40). **a** Phase contrast micrograph shows various cells released from the glomeruli of LEW rats after digestion. **b** Flow cytometry reveals several morphologically different cell populations isolated from the glomeruli. Cells in gate R1 contain a GIL CD8α$^+$ population. **c–e** Flow cytometry analyses based on R1 show a GIL CD8α$^+$ population (red gate), which was CD8α$^+$CD3$^-$ (**c**), CD11b/c$^+$ (red gate in **d**) and RT1B$^+$ (red gate in **e**). **f** Flow cytometry shows 42% of GIL CD8α$^+$ cells were CD34$^+$. **g** GIL CD8α$^+$ cells did not express CD94 (upper panel) or TcRγδ$^-$ (lower panel). For colors, see online version.

Fig. 3. Characterization of GIL CD8α$^+$ cells isolated from immunized LEW rats. **a**, **b** Flow cytometries show purified GIL CD8α$^+$ cells to be CD3$^-$CD11c$^+$. **b** Note two CD11c$^+$ subpopulations: a majority of CD11^{high} and a minority of CD11^{low}. **c** The expression of various levels of MHC class II molecule (RT1B) in purified CD8α$^+$ cells. The histogram on the right is based on the red gate in the left panel. Green line, antibody to RT1B (OX6), blue line, IgG control. **d**, **e** Confocal micrographs show surface CD11b/c (red), and surface/intracellular MHC II molecule RT1D (green) in CD8α$^+$ cells. ×400. **f** Confocal micrograph demonstrates colocalization of RT1B (green) and CD8α in a GIL CD8α$^+$ cell. ×600. Cells were cultured for one day before being processed for staining. For colors, see online version.

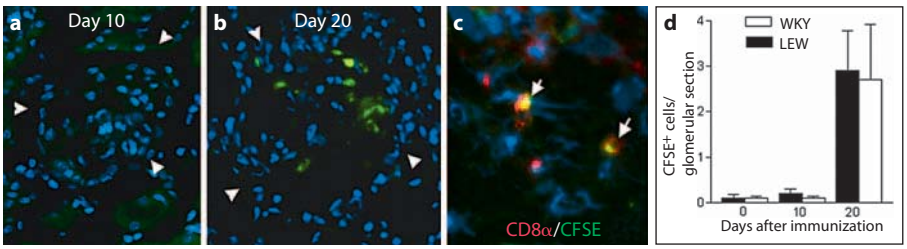

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Fig. 4. GIL CD8α$^+$ cells of LEW rats are able to infiltrate inflamed glomeruli of both LEW and WKY rats at an early stage. **a** Fluorescence shows no CFSE$^+$ cells in a glomerulus of a normal LEW rat. **b** Fluorescent micrographs show transferred CFSE$^+$ GIL CD8α$^+$ cells (green) in a glomerulus of immunized LEW rats. Glomeruli are outlined by arrowheads. **c** Immunofluorescence shows two transferred CFSE$^+$CD8α$^+$ cells (arrows) among other endogenous CD8α$^+$ cells in an inflamed glomerulus of a LEW rat at day 20. ×300. **d** Summary of transferred CFSE$^+$ cells in glomeruli in different groups. For colors, see online version.

tory stage prior to fibrosis in GN-prone WKY rats (for this transition stage, refer to fig. 1a). We first asked whether a similar GIL CD8α$^+$ population was also present in the inflamed glomeruli during the recovery stage in LEW rats. Glomerular cells were isolated from glomeruli at days 22–23 after immunization (fig. 2a). The isolated glomerular cells formed at least 6 morphologically distinct populations (fig. 2b). Flow cytometry analyses showed a population which was in gate R1 to be CD8α$^+$CD3$^-$ (red gate in fig. 2c). This population was also CD11b/c$^+$ (red gate in fig. 2d). Importantly, the cells expressed MHC class II molecules RT1B and RT1D (red gate in fig. 2e). A portion of these cells also expressed low levels of CD34, suggesting their myeloid origin (fig. 2f). However, the cells did not express TcRγδ or ED1 (fig. 2g). Thus, this cell population was similar to GIL CD8α$^+$ cells in WKY

rats. There was an additional $CD8^+CD94^+$ population. However, R1 gating on FSC-SSC showed that the $CD8^+CD94^+$ population was not $CD8^+CD3^-$ population.

Next, we isolated this population for further characterization through positive selection using CD8α antibody-labeled magnetic beads after removal of pan T cells (fig. 3a). Flow cytometry showed that the purified $CD8\alpha^+CD3^-$ cells (92%) expressed CD11c and various levels of RT1B (fig. 3b, c). Similar to the GIL $CD8\alpha^+$ cells isolated from WKY rats at day 35 [16], there were two subpopulations based on CD11 expression levels: a majority of $CD11^{high}$ population with a minority of $CD11^{low}$ population (fig. 3b). Finally, expression of CD11 and RT1D were also confirmed by immunofluorescence (fig. 3d, e). The cells expressed both intracellular and surface RT1D (fig. 3e). Coexpression of CD8α and RT1B in the $CD8\alpha^+CD3^-$ cells was further confirmed by confocal immunofluorescence (fig. 3f). In summary, this glomeruli-infiltrating cell population isolated from LEW rats had a phenotype (i.e. $CD8\alpha^+CD3^-CD11^+RT1B^+$) identical to GIL $CD8\alpha^+$ cells from GN-prone WKY rats. Thus, this population was the counterpart of WKY rats' GIL $CD8\alpha^+$ cells in LEW rats.

GIL $CD8\alpha^+$ Cells of LEW Rats Are Capable of Infiltrating Inflamed Glomeruli at an Early Stage in Both LEW and WKY Rats

We asked whether GIL $CD8\alpha^+$ cells isolated from immunized LEW rats were capable of infiltrating glomeruli just like those in WKY rats. LEW and WKY rats were immunized with pCol(28–40) and used for cell transfer at day 10 or day 20 (3 for LEW rats/group, and 2 for WKY rats/group); these rat groups were designated as 10d or 20d group, respectively. Normal WKY or LEW rats were used as a control and designated as 0d group. CFSE-labeled GIL $CD8\alpha^+$ cells from LEW rats (10^6 cells/rat) were transferred into each experimental or control rats (fig. 4). After 24 h, fluorescence microscope revealed no $CFSE^+$ cells, if any, in both LEW and WKY rats of the 0d and the 10d group (fig. 4a, d). However, a significant number of $CFSE^+$ cells were observed in the glomeruli of LEW rats of the 20d group (fig. 4b, d); immunofluorescence showed that the transferred $CFSE^+CD8\alpha^+$ cells were among endogenous GIL $CD8\alpha^+$ cells (fig. 4c) in immunized LEW rats. We have reported GIL $CD8\alpha^+$ cells of WKY rats only invade glomeruli at day 35, but not day 20 [16]. Interestingly, labeled cells were also found in the glomeruli of WKY rats of the 20d group (fig. 4d). Except for only a few in the lung, no $CFSE^+$ cells were observed in other organs. Thus, GIL $CD8\alpha^+$ cells were able to migrate into inflamed glomeruli of both LEW and WKY rats at an early stage of inflammation. Since there was a small $CD8^{low}$ population (7%) in the purified GIL $CD8\alpha^+$ cells, we estimated if the infiltrating cells were those $CD8^{low}$ cells. Average $CFSE^+$ cells in each glomerulus were 4.3 (by a serial sections which cut through 15 glomeruli). Thus, total $CFSE^+$ cells in glomeruli were approximately 2.8×10^5, which accounted for nearly 28% of transferred $CFSE^+$ cells. Thus, majority of $CFSE^+$ cells were $CD8\alpha^{high}$ cells. In a separate experiment, we also demonstrated that these GIL $CD8\alpha^+$ cells induced T cell apoptosis in a similar way to those in WKY [unpubl. data].

Infiltration of $CD8\alpha\alpha^+CD11c^{high}MHC\ II^+$ Cells into Glomeruli in GN-Resistant LEW Rats Is Coincident with Termination of Glomerular Inflammation during Recovery

We have previously shown that infiltration of GIL $CD8\alpha^+$ cells into glomeruli in WKY rats peaks at days 28–35 during the transient stage from inflammation to fibrosis [16]. Infiltration of GIL $CD8\alpha^+$ cells were examined with immunofluorescence in the immunized LEW rats at days 17, 21, 28, and 45. Because we have shown that glomerular $CD8\alpha^+$ cells were mostly GIL $CD8\alpha^+$cells (refer to fig. 2c), single staining with anti-CD8α antibody was performed for identification of GIL $CD8\alpha^+$ cells. GIL $CD8\alpha^+$ cells were not detectable in normal glomeruli (day 0), and were observed in a significant number at days 17 and 21 (55.20 ± 15.0 and 53.5 ± 19.2 cells/glomerulus, respectively; fig. 5a and 6a). Numbers of glomerular $CD8\alpha^+$ cells were significantly reduced at day 28 when the glomeruli had fully recovered from the transient inflammation (fig. 5a, 6a). $CD8\alpha^+$ cells were not detected in the glomeruli at day 45 (fig. 6a). Thus, infiltration of $CD8\alpha\alpha^+$ cells was coincident with decline of glomerular inflammation during recovery stage in LEW rats. As we reported previously, $CD8\alpha^+$ cell infiltration plateaus from day 28 to 35 in WKY, which is also coincident with a rapid decline in glomerular inflammation before fibrosis [20].

Glomerular cells were isolated at days 0, 15, 20 and 30 from immunized LEW and WKY rats, and analyzed with flow cytometry for GIL $CD8\alpha^+$ cells (fig. 5b). Both LEW and WKY showed a detectable population of glomerular $CD8\alpha^+CD11^+$ cells at day 15. However, significant differences in both quantity and quality of $CD8\alpha^+CD11^+$ cell population were observed between LEW and WKY rats at day 20. That is, LEW rats showed a much larger population of $CD8\alpha^+CD11^+$ cells with a majority $CD11^{high}$ subpopulation (red gate in fig. 5b) with a minor $CD11^{low}$ pop-

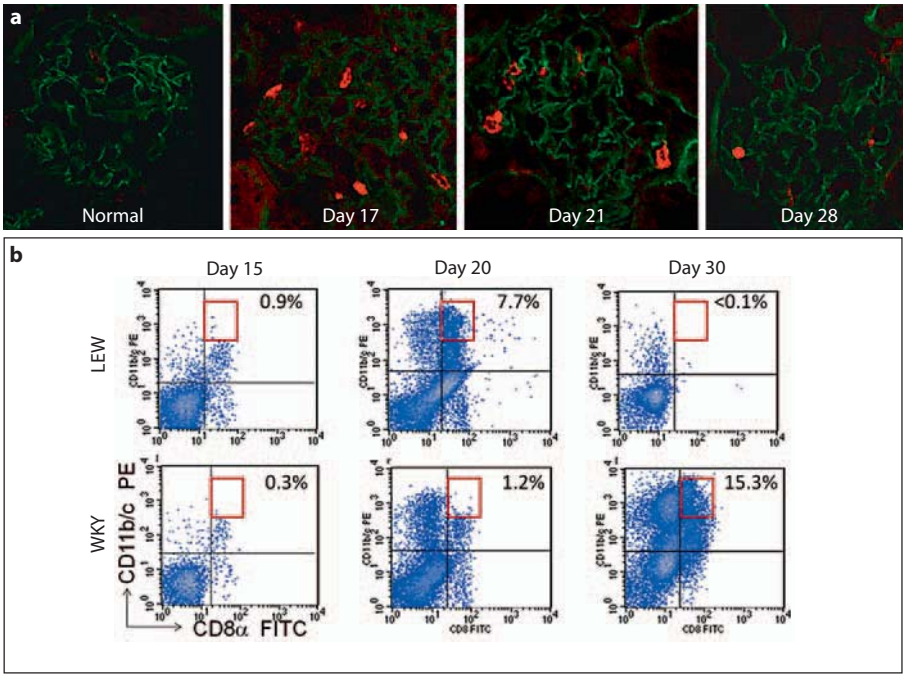

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Fig. 5. CD8α$^+$ cells infiltrate glomeruli earlier in GN-resistant LEW rats than in GN-prone WKY rats after immunization. **a** A group of confocal micrographs show GIL CD8α$^+$ cells (red) in glomeruli after pCol(28–40) immunization as indicated. GBM (green) was counterstained with SR13. Average numbers of GIL CD8α$^+$ cells per glomerulus are shown in figure 6a. Normal rats have 7.6 ± 5 cells/glomerulus. ×300. **b** Flow cytometry comparison of GIL CD8α$^+$ cells between LEW and WKY after pCol(28–40) immunization as indicated; red gates indicate the population of CD8α$^+$CD11^{high} cells. Note that LEW rats had CD8α$^+$CD11^{high} population at day 20, which WKY rats lacked. See summarized data in figure 6b. For colors, see online version.

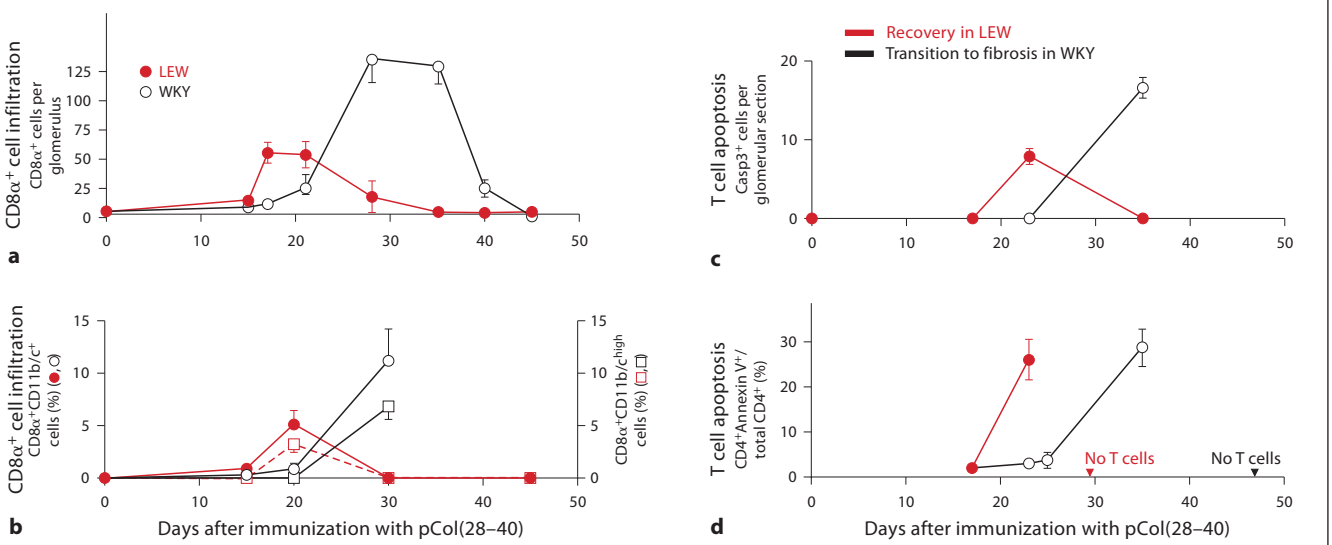

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Fig. 6. GIL CD8α$^+$ cell infiltration is coincident with T cell apoptosis during GN recovery in LEW rats or transition to fibrosis in WKY rats. Summary of time courses for infiltration of GIL CD8α$^+$ cells (**a** and **b**) and apoptosis in CD3$^+$ or CD4$^+$ cells (**c** and **d**) after immunization in GN-resistant LEW (red symbols) and GN-prone WKY rats (black symbols). Time frames for recovery stage in LEW rats and transition from inflammatory to fibrotic stage in WKY rats are indicated at the bottom. For colors, see online version.

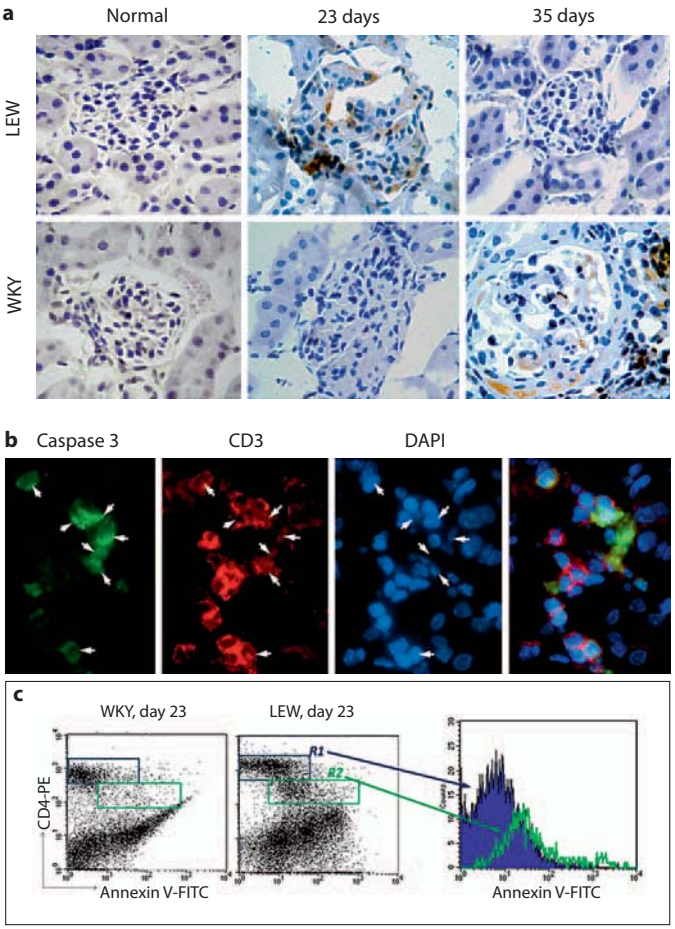

Fig. 7. Apoptosis in $CD3^+CD4^+$ cells in glomeruli of GN-resistant LEW during recovery stage. **a** Immunohistoperoxidase method detection of caspase-3-positive apoptotic cells in glomeruli of LEW and WKY as indicated. Clustered caspase 3^+ cells are present in glomeruli of LEW at day 23, but not in WKY rats despite prominent glomerular inflammation in both strains. Clustered caspase 3^+ cells are detectable in the periglomerular area of a glomerulus undergoing fibrosis in WKY at day 35. ×200. See summarized data for numbers of caspase$^+$ cells in figure 6c. **b** Immunofluorescence shows colocalization of cytoplasmic caspase 3 (green) with cell surface CD3 (red) among clustered caspase 3^+ cells in a glomerulus of a LEW rat at day 23. Arrows indicate the cells positive for both intracellular caspase 3 and surface CD3. Nuclei were counterstained with DAPI. ×400. **c** Flow cytometry analysis of isolated glomerular cells shows a significant size of apoptotic $CD4^+$ T cell population (annexin V^+ cells) in LEW but not in WKY rats at day 23. Gates R1 and R2 represent $CD4^+$annexinV^- and $CD4^+$annexinV^+ populations. Histogram on the right shows comparison of expression level of annexin V between R1 and R2 populations. Percentages of annexin V^+ cells among total $CD4^+$ cells are summarized in figure 6d. For colors, see online version.

ulation. Although WKY rats showed a slightly expanded $CD8\alpha^+CD11^+$ population at day 20, the $CD8\alpha^+CD11^{high}$ population was absent (fig. 5b). At day 30, there were no more glomerular $CD8\alpha^+CD11^+$ cells in LEW rats. On the other hand, WKY rats now showed a large number of $CD8\alpha^+CD11^+$ cells in their glomeruli with a major population of $CD11^{high}$ cells (fig. 5b). Figure 6b summarizes the statistical data for flow cytometry analysis. In summary, in GN-resistant LEW rats, a large number of $CD8\alpha^+CD11^+$ cells infiltrated glomeruli at an early stage with a $CD8\alpha^+CD11^{high}$ subpopulation. In contrast, GN-susceptible WKY showed much less $CD8\alpha^+CD11^+$ cells without $CD8\alpha^+CD11^{high}$ cells at the same stage. The $CD8\alpha^+CD11^{high}$ subpopulation was present in a large number only at days 30–35 in WKY, when fibrosis was replacing glomerular inflammation [20]. We concluded that infiltration of $CD8\alpha^+$ cells with a $CD8\alpha^+CD11^{high}$ subpopulation was coincident with spontaneous recovery at an early stage in GN-resistant LEW rats.

Early Infiltration of GIL $CD8\alpha^+$ Cells in GN-Resistant LEW Rats Was Associated with Early Apoptosis of $CD4^+$ T Cells in Glomeruli

We have previously shown that the peak infiltration of GIL $CD8\alpha^+$ cells into glomeruli in WKY rats occurs at days 30–35, coincident with a peak of apoptosis in glomerular $CD3^+/CD4^+$ T cells [20]. Next, we investigated whether T cell apoptosis occurred in the glomeruli of immunized LEW rats during peak infiltration of GIL $CD8\alpha^+$ cells in comparison to WKY rats. Kidneys from immunized LEW and WKY rats were sampled at day 23 (recovering stage in LEW rats) and day 35 (transient stage from inflammation to fibrosis in WKY rats), 3 rats/group/time point. Immunohistochemistry detected a significant number of clustered caspase 3^+ apoptotic cells at day 23 in the immunized LEW rats. In contrast, no apoptotic cells were observed in the immunized WKY rats despite the presence of prominent inflammation in the glomeruli at day 23 (fig. 7a; for statistical data, see fig. 6c). At day 35, LEW rats showed no caspase 3^+ apoptotic cells in their glomeruli; glomeruli were completely free of inflammation and slightly enlarged. In contrast, clustered apoptotic cells were detected in the periglomerular area of glomeruli undergoing fibrotic changes in WKY at day 35 (fig. 6c, 7a). Double-color immunofluorescence was performed to determine the phenotype of those clustered caspase 3^+ cells in three immunized LEW at day 23. Approximately 1/3 of clustered $CD3^+$ T cells in the glomeruli expressed intracellular caspase 3 (fig. 6b). On the other hand, only a small number of non-$CD3^+$ cells expressed

caspase 3 (fig. 6b). Thus, caspase 3^+ cells were mostly $CD3^+$ cells. Next, glomerular cells were isolated from two LEW rats at days 17 and 23 and analyzed by flow cytometry. A $CD4^+$annexin V^+ population was detected, which accounted for 21% of total $CD4^+$ cells (fig. 6d, 7c). As we reported previously, a $CD4^+$annexin V^+ population was detected in a significant number in WKY rats only at day 35. Analyses on $CD4^+$annexin V^+ populations at day 30 for LEW and day 46 for WKY were not successful due to a lack of T cells (fig. 6d).

Figure 6 summarizes time courses for infiltration of GIL $CD8\alpha^+$ cells and apoptosis in T cells, in comparison to recovery stage in LEW rats and transition from inflammation to fibrosis in WKY rats. From this figure, we are able to conclude that infiltration of $CD8\alpha^+$ cells is coincident with $CD4^+$ T cell apoptosis before termination of T cell-mediated inflammation in both strains.

Discussion

Glomeruli-infiltrating $CD8^+$ cells, which were probably T cells, in anti-GBM GN models have been reported [12–14]. On the other hand, we have previously identified a $CD8\alpha\alpha^+CD11b/c^+MHC^+OX62^-$ DC-like population, which actively infiltrates the target tissue glomeruli at late inflammatory stage before fibrosis, suggesting presence of multiple CD8+ populations among glomeruli-infiltrating leukocytes [20, 21]. This $CD8\alpha^+$ DC-like population, designated as GIL $CD8\alpha^+$ cells, induces T cell apoptosis through antigen presentation, suggesting that they may terminate autoimmune inflammation in vivo before advancing to fibrosis [20]. In the present study, we further revealed a strong association between GIL $CD8\alpha^+$ cells and the recovery from early glomerular inflammation. In GN-resistant LEW rats, first we confirmed the presence of a GIL $CD8\alpha^+$ cell population in GN-resistant LEW rats with almost identical phenotype to that in WKY rats. Second, time course studies showed a time window for infiltration of GIL $CD8\alpha^+$ cells into inflamed glomeruli in the immunized LEW rats: an influx of GIL $CD8\alpha^+$ cells into target tissue plateaued at days 17–20. Thus, GIL $CD8\alpha^+$ cells infiltrated glomeruli at a much earlier stage in LEW rats than in WKY rats. Third, we observed a $CD11^{high}$ subpopulation among GIL $CD8\alpha^+$ cells. As we reported previously, this subpopulation induces T cell apoptosis [20]. Fourth, our study also revealed a close association of infiltration of $CD8\alpha^+$ cells with T cell apoptosis in the glomeruli during recovery in LEW rats. Coincidence of $CD8\alpha^+$ cell infiltration with the transition stage from glomerular inflammation to fibrosis in GN-prone WKY rats has been reported [20]. Thus, termination of glomerular inflammation is closely associated with infiltration of the $CD8\alpha^+$ cells in both strains, though at different time points (fig. 6). Our findings lead to the following hypothesis: the $CD8\alpha^+$ DC-like cells timely infiltrate inflamed target tissue and terminate autoimmune attack by selectively inducing apoptosis in autoreactive T cells through local presentation of autoantigens. It leads to a full recovery from autoimmune attack as seen in GN-resistant LEW rats. However, defects in timely infiltration of this population into inflamed target tissue may result in irreversible tissue damage, as seen in GN-prone WKY rats. Currently, we are testing this hypothesis using a cell transfer method. Since GIL $CD8\alpha^+$ cells were able to invade WKY's glomeruli at an early stage (refer to fig. 4d), it would be interesting to examine if those cells could 'turn off' glomerular inflammation in WKY rats as well.

The pathogenetic mechanism for GN has been well investigated in rodent models especially through comparison between GN-susceptible and -resistant strains [12]. It seems clear that multiple cell populations or mechanisms govern susceptibility to GN. For example, copy number of FcγR has been linked to GN susceptibility [12]. While $CD8^+$ T cell-deficient mice still developed anti-GBM GN [13], anti-CD8 antibody-mediated in vivo cell depletion prevented anti-GBM GN in WKY rats. These findings are especially interesting because the same WKY rats were used for disease induction. Together with findings from other groups, these findings suggested a positive role of $CD8^+$ or $CD8^+$ T cells in promotion of pathogenesis of GN. On the other hand, our study suggested a negative role of $CD8\alpha\alpha^+$ cells in GN. Those results do not necessarily contrast each other, because there are multiple $CD8^+$ cell populations. Depletion of $CD8^+$ cells may have not only eliminated $CD8^+$ T cells, but also GIL $CD8\alpha^+$ DC-like cells and potential $CD8^+CD94^+$ cells (see fig. 1). It is conceivable that some of them promote GN, while others inhibit GN. More studies are needed to determine whether GIL $CD8\alpha^+$ play a positive or negative role in GN.

Recently, amounting evidence has suggested special roles of myeloid cells such as DCs and macrophages in self-tolerance [6–8]. Our discovery may represent a unique tolerance mechanism due to cell type and location of its function. First, immune regulation of autoimmune disease can act at two locations, either at the secondary lymphoid organs or in the target organ itself. Most regulations of autoimmunity by APCs occur in peripheral lymphoid organs such as the spleen [22]. In contrast, this DC-like cell actively infiltrates inflamed target tissue

glomeruli to terminate autoimmune inflammation. Thus, this new cell type acts as 'damage control' in the target tissue after an autoimmune attack. Second, inhibition of autoimmune inflammation in target tissue has been reported. However, they are different in the mechanism from what is described in the present study. For example, Treg may contribute to local inhibition of autoimmune inflammation in the experimental allergic encephalomyelitis (EAE) model [3]. A subset of macrophages may have anti-inflammatory functions in target tissue. However, their anti-inflammatory functions in autoimmune tissue damage are largely mediated by their expression of anti-inflammatory cytokines such as IL-10 rather than their APC functions [23, 24]. In contrast, elimination of T cells by GIL CD8α$^+$ cell in the target tissue is through its APC function, and thus, is antigen specific [20]. Finally, various types of DCs have been studied in animal models for autoimmune GNs or human GNs [21, 25–28]. For example, depletion of conventional DCs attenuated nephrotoxin nephritis in a Tg murine model [25]. Interestingly, the same model showed a critical role of conventional DCs in promotion of various GNs [26]. A type of OX62$^+$ED1$^-$ DC was observed in glomeruli in a rat GN model with a function to be determined [27]. Obviously, all those studies showed glomeruli-infiltrating DCs, which differ from the GIL CD8α$^+$ cells either in their phenotypes or functions. Thus, the GIL CD8α$^+$ cells described in the present study are a novel type of cell.

Target-tissue-infiltrating CD8α$^+$ DC-like cells may not be limited to our model. Results from several previous studies on various autoimmune or inflammatory disease models suggest presence of similar CD8α$^+$ cells in other animal models for autoimmune or inflammatory diseases. Depletion of CD8$^+$ cells leads to a severe, late airway response, a model for asthma [29]. Although the CD8$^+$ population has not been well characterized in this study, the result suggests that the CD8$^+$ may abrogate T cell-mediated inflammation. A tumor-infiltrating CD8$^+$ cell is believed to be involved in tolerance to tumor antigens [7]. Infiltration of CD8$^+$ phagocytes into the central nervous system has been described in chronic EAE in a rat model [30]. CD8$^+$ phagocytes have been linked to variants of rat models for EAE [31]. Infiltration of CD8$^+$ phagocytes is only seen in tissue damage (demyelination). EAE in LEW rats are self-limited after a transient CNS inflammation induced by myelin basic protein [32]; modulation of PBMC alters disease severity [33]. Interestingly, in vivo depletion of CD8$^+$ cells aggravated the disease in this strain [34]. A report described infiltration of CD8$^+$ macrophages into injured brain tissue with unknown functions [35]. All the above studies suggest a critical role of CD8$^+$ phagocytes or DCs. It will be interesting to characterize those CD8$^+$ phagocytes in those models for comparison. Since all those observations were made in animal models, it will also be critical to ask whether a similar type of CD8$^+$ cell is present in human autoimmune GN [21, 28].

Acknowledgements

This study was supported by NIH R01-DK60029 and R01-DK077857 (to Y.-H. Lou) and partially by R01-HD049613 (Y.-H. Lou). Julie Robertson was supported by NIH T32-DE015355. We thank Dr. Y. Sado, Okayama University, Japan, for providing antibody to rat glomerular basement membrane. Histology support was provided by the Pathology Laboratory, University of Texas Dental Branch. Confocal fluorescent images were taken in Core Facilities, Department of Integrated Biology of Pharmacology, University of Texas Medical School at Houston.

Disclosure Statement

The authors declare no conflict of interest.

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