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Original Article

Attenuated Macrophage Infiltration in Glomeruli of Aged Mice Resulting in Ameliorated Kidney Injury in Nephrotoxic Serum Nephritis

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Abstract

Senescent cells have deleterious effects on the tissue microenvironment through proinflammatory senescence-associated secretory phenotypes; meanwhile, the onset of glomerulonephritis is predominant in younger adults. To clarify the influence of aging on the onset and development of glomerulonephritis, we used a murine model of antibody-mediated nephritis. Sheep nephrotoxic serum was administered in C57BL/6J mice at 12 weeks (adult) or 18 months old (aged) after pre-immunization with sheep IgG. Depositions of sheep IgG and autologous mouse IgG along the glomerular basement membrane and the serum titer of anti-sheep IgG-specific mouse IgG were similar between adult and aged mice. However, kidney injury was depressed in aged mice, accompanied by reduced macrophage infiltration in the glomeruli. The mRNA expression of most chemokines involved in monocyte/macrophage chemotaxis was not different between adult and aged mice, but the cell surface expression of C-C chemokine receptor (CCR) 1 and CCR2 was down-regulated in the monocyte/macrophage lineage cells infiltrating the kidneys of aged nephritic mice. Furthermore, expression of all four isotypes of the Fcγ receptor (FcγR) was reduced in these cells. Both CCR and FcγR expression were down-regulated in monocyte/macrophage lineage cells, resulting in attenuated glomerular infiltration of these cells and impaired glomerular injury in aged mice.

Keywords: Animal model, Immune system, Immunosenescence, Kidney/renal

Introduction

Biological aging is characterized by a chronic low-grade inflammation termed as inflammaging. The most common theories of inflammaging include redox stress, mitochondrial dysfunction, glycation, deregulation of the immune system, hormonal changes, epigenetic modifications, and telomere dysfunction, and the inflammaging is profoundly associated with age-related disease such as type II diabetes, Alzheimer's disease, cardiovascular disease, frailty, sarcopenia, osteoporosis, and cancer (1,2). Cellular senescence is one of the most important causes of inflammaging, and is deeply associated with age-related dysfunction and chronic sterile inflammation. It is a potent anticancer mechanism that permanently arrests cells at risk of malignant transformation after a potentially oncogenic insult (3,4). However, senescent cells also have deleterious effects on the tissue microenvironment, such as the acquisition of a senescence-associated secretory phenotype (SASP). SASP is linked to DNA damage responses, and alters the tissue microenvironment, attracts immune cells, and induces malignant phenotypes due to inflammatory, growth-promoting, and remodeling factors produced by senescent cells (3,5). Proinflammatory proteins such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) -α, chemokines including IL-8 and monocyte chemoattractant protein (MCP)-1, insulin-like growth factor-binding proteins, matrix metalloproteinases, serine proteases and their inhibitors, extracellular

insoluble molecules, and colony-stimulating factors are all reported to be associated with SASP (3,4).

During chronic kidney disease, considered as a type of renal aging, a variety of intrinsic and infiltrating cells suffer from cellular senescence and secrete large amounts of factors associated with SASP. When the kidneys suffer injury, the repair mechanisms for the impaired kidney tissue are started mainly by residual tubular epithelial cells, mesenchymal stem cells and hematopoietic stem cells producing vascular endothelial growth factor and/or fibroblast growth factor 2 to promote tissue repair. At the same time IL-6, TNF-α, and MCP-1 are also secreted from renal tubular epithelial cells, endothelial cells, mesangial cells, macrophages, and lymphocytes to recruit a variety of immune cells to remove harmful factors, maintain homeostasis, and secret more SASP. Once tissue repair fails, the accumulated SASP plays various roles in age-related pathological damage (6). However, several glomerular diseases, such as immunoglobulin A nephropathy and membranoproliferative glomerulonephritis, have their clinical onset in young adulthood (7,8). Anti-glomerular basement membrane (GBM) disease has two periods of peak incidence. The first peak is in the second and third decades of life and the second peak is in the sixth and seventh decades. Younger patients show high frequency of pulmonary hemorrhage, and the latter peak is considered to be partly due to the wide application of immunoassays and immunohistology, resulting in an increased awareness of the disease (9–11). To clarify the influence of the aging-related immunological microenvironment on the onset of glomerulonephritis, we adopted a murine nephrotoxic serum (NTS) nephritis (NTN) model in adult and aged mice. This model resembles human anti-GBM disease (12) by its dependence on the targeting effect of anti-GBM antibodies to direct immune complex deposition to the GBM and initiate an inflammatory response (13). Anti-GBM disease is an immune disorder with serum autoantibodies directed against α3 chain of type IV collagen (9), which composes GBM as a heterotrimer with α4 and α5 chain (14), whereas NTS is generated in the sheep or rabbit serum by immunization with mouse or rat GBM (13) and its antigen could be a variety of the main components of GBM including type IV collagen, laminin, heparin sulfate proteoglycan agrin, and nidogen (14). Passive serum transfer of NTS induces deposition of immunoglobulin along GBM in the same way as anti-GBM disease, but pre-immunization of the donner IgG is required to evoke autologous mouse anti-donner IgG antibody deposition for accelerated glomerular injury in C57BL/6 mice (13). It is also an Fcγ receptor (FcγR)-dependent disease model, in which monocyte/macrophage lineages have been shown to play important roles in the development of NTN through the interaction of FcγR and deposited mouse IgG (13,15). In the current study, we clarified the influence of aging on the development of glomerulonephritis using NTN mouse model and revealed the functional deterioration of macrophages.

Method

Experimental Animals

Male C57BL/6J mice were purchased from Charles River Japan (Yokohama, Japan) at 8 weeks of age and maintained in our animal facility. Pentobarbital (100 μg/g of body weight) was injected intraperitoneally to anesthetize mice before sacrifice. All animal experiments were performed following protocols approved by the Institutional Animal Care and Ethics Committee at Niigata University (Approved number 28-208-1).

Induction of Nephrotoxic Serum Nephritis

Sheep NTS was prepared as described previously (13,16). NTS was heat inactivated, and then absorbed with an excess of murine red blood cells. Adult (12-week-old) or aged (18-month-old) mice were pre-immunized by intraperitoneal injection of 200 μg of sheep IgG (Bio-Rad, Hercules, CA) in a 50:50 mix with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO), followed by intravenous injection of sheep NTS (2.0 μL of serum per gram of body weight) 4 days later. Mice were sacrificed under anesthesia 7 days after NTS administration to collect blood and kidney samples. Blood urea nitrogen level in sera was measured using DetectX Urea Nitrogen Colorimetric Detection Kit (Arbor Assays, Ann Arbor, MI). Serum cystatin C concentration was measured using Mouse/Rat Cystatin C Immunoassay (R &D Systems, Minneapolis, MN). For evaluation of creatinine clearance normalized by mouse body weight and urine albumin/creatinine ratio, 24 h urine samples were collected from pre-immunized mice using metabolic cages between day 5 and 6 and blood samples were obtained on day 6 after NTS or phosphate-buffered saline (PBS) injection. Serum and urine creatinine levels were measured by enzymatic method and urine albumin concentration was determined by turbidimetric immunoassay (Oriental Yeast, Shiga, Japan). Serum levels of cytokines such as TNF-α, IL-6, IL-1β, IL-4, IL-10, IL-12, IL-13, IL-17, interferon-γ, and transforming growth factor-β were measured using each DuoSet ELISA development system (R & D Systems).

Histological Analysis

Kidneys were removed from mice, fixed in 10% buffered formalin, and embedded in paraffin. Four-micrometer paraffin sections were stained with periodic acid-Schiff (PAS) and assessed in 20 glomeruli per mouse by light microscopy in a blinded manner. The pathologic scores of glomerular injury were graded into five grades: grade 0 (no PAS-positive material), grade 1 (0–25% of glomerular cross-section PAS-positive), grade 2 (25–50%), grade 3 (50–75%), and grade 4 (75–100%) (13,16).

Immunohistochemical and Immunofluorescence Staining

For immunohistochemical analysis, 4 μm paraffin sections were subjected to heat-mediated antigen retrieval and stained with biotinylated rat monoclonal antibody specific to F4/80 (clone A3-1, Bio-Rad, Hercules, CA), rabbit monoclonal antibody specific to Thr202/Tyr204-phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (clone D13.14.4E, Cell Signaling Technology, Danvers, MA), rabbit polyclonal antibody specific to Ser32/Ser36-phosphorylated nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα) (GeneTex, Irvine, CA), or rabbit polyclonal antibody specific to C–C chemokine receptor (CCR)-2 (Abbiotech, San Diego, CA). Biotinylated rat IgG2b was used as a control for the F4/80-specific antibody. These antibodies were reacted with horseradish peroxidase (HRP)-conjugated goat anti-biotin antibody (Sigma Aldrich) or anti-rabbit IgG antibody (Sigma Aldrich). Immune complexes were detected using 3,3′-diaminobenzidine (Nichirei Biosciences, Tokyo, Japan). The number of phosphorylated ERK$^-$, phosphorylated IκBα$^-$, and F4/80-positive cells was assessed in a minimum of 20 glomeruli randomly selected high-power fields per animal in a blinded manner. For immunofluorescence staining, 4-μm thick frozen sections were fixed in acetone and stained with fluorescein (FITC)-conjugated anti-sheep IgG (Sigma-Aldrich), tetramethylrhodamine-conjugated anti-mouse IgG (Sigma-Aldrich), FITC-conjugated anti-mouse complement C3c (LSBio, Seattle, WA),

FITC-conjugated anti-mouse IgG1 (Bio-Rad), or FITC-conjugated anti-mouse IgG2b (Bio-Rad). For quantification of immunofluorescence, blinded sections were examined at 100-fold magnification and the mean intensity of 20 glomeruli for each sample was measured using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Measurement of Sheep IgG-Specific Circulating IgG Levels

Ninety-six-well ELISA plates were coated overnight at 4°C with 5 µg/mL of sheep IgG. After blocking for 1 h with 5% BSA in PBS, samples were incubated with a 1:500 dilution of test sera for 1 h. After washing with PBS containing 0.05% Tween 20, the plates were incubated with HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, or IgG3 antibodies (Bethyl Laboratories, Montgomery, TX) and color was developed with 3,3′,5,5′-tetramethylbenzidine.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from kidneys using the RNeasy Mini Kit (Qiagen, Valencia, CA). qRT-PCR analysis was performed using the Thermal Cycler Dice Real Time System II with the One Step SYBR PrimeScript Plus RT-PCR Kit (Takara Bio, Shiga, Japan) following the manufacturer's protocol. Target amplicons and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reverse transcribed and quantified using the same template RNA for relative quantification analysis. The primer sequences are shown in Table 1.

Kidney Single-Cell Preparation

Kidneys were finely minced and digested with 0.4 ng/mL collagenase D (Roche, Mannheim, Germany) and 0.01 ng/mL DNase I in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum for 45 min at 37°C. Cell suspensions were sequentially filtered through 70-µm nylon mesh, resuspended in a 33% Percoll solution, and centrifuged at 2000 rpm for 20 min at room temperature (16). After red blood cell lysis, kidney-infiltrating cells, as well as peripheral blood cells, were incubated with rat anti-mouse CD16/CD32 (clone 2.4G2) antibody to block nonspecific binding through FcγRs, and were subjected to flow cytometric analysis using the specific monoclonal antibodies described below. FITC-conjugated anti-mouse CCR1 (clone 643854), CCR2 (clone 475301), and CCR5 (clone CTC5) were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated anti-mouse FcγRI (clone AT152-9), FcγRIIB (clone AT130-5), FcγRIII (clone AT154-2), FcγRIV (clone AT137), and allophycocyanin-conjugated anti-mouse F4/80 (clone A3-1) antibodies were purchased from Bio-Rad. Phycoerythrin-conjugated anti-mouse Ly-6G (clone 1A8) antibody was purchased from BD Biosciences (San Jose, CA).

Statistical Analysis

Values were expressed as means ± SD. Clinical scores and phosphorylate ERK$^-$, phosphorylated IκBα$^-$, and F4/80-positive cells per glomerulus were evaluated with Mann–Whitney *U* test or Kruskal–Wallis test followed by Mann–Whitney *U* test with Bonferroni correction as a post-hoc test, respectively. Otherwise, the Student's *t*-test was used for comparison between two groups. For comparison among four groups a one-way analysis of variance was used, followed by Scheffe's *F* test as a post-hoc test. $p < .05$ was considered significant.

Results

Attenuated Kidney Injury of Aged Mice in Nephrotoxic Serum Nephritis

To investigate the influence of aging in NTN, we administered NTS in adult and aged mice pre-immunized with sheep IgG 4 days beforehand. Seven days after injection of NTS, the blood urea nitrogen level of adult mice, but not of aged mice, was significantly elevated (adult, 241.8 ± 54.7 mg/dL; aged, 44.0 ± 15.9 mg/dL; $p < .01$; Figure 1A). The serum cystatin C level was also significantly elevated in adult mice (adult, 1.54 ± 0.34 mg/L; aged, 0.72 ± 0.15 mg/L; $p < .01$; Figure 1A). Twenty-four-hour creatinine clearance between days 5 and 6 was significantly decreased in NTN adult mice (adult, 0.90 ± 0.90 µL/min/g body weight; aged, 3.33 ± 0.64 µL/min/g body weight; $p < .01$; Figure 1A) and urine albumin/creatinine ratio was also elevated in NTN adult mice (adult, 3.14 ± 0.50 g/g creatinine; aged, 0.48 ± 0.50 g/g creatinine; $p < .01$; Figure 1A). Kidney sections from NTN adult mice stained with PAS on day 7 demonstrated severe tissue damage with intracapillary cellular proliferation and glomerular thrombosis. Adult NTN mice also showed a dilated tubule containing a proteinaceous cast. In contrast, kidney injury in aged mice, even though treated with NTS, was markedly attenuated (Figure 1B). The pathologic scores for glomerular injury were also remarkably decreased in aged mice compared with adult mice (Figure 1C).

Systemic and Local Inflammatory Response in the Glomeruli of Adult and Aged NTN Mice

We measured serum levels of several cytokines to evaluate systemic inflammatory response prior and after NTS stimulation in adult and

Table 1. Sequences of primers used in the study

Gene	Sense (5′–3′)	Antisense (3′–5′)
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
IL-12p35	AAATGAAGCTCTGCATCCTGC	TCACCCTGTTGATGGTCACG
CCL2	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
CCL3	ACCTGCTCAACATCATGAAGG	AGATGGAGCTATGCAGGTGG
CCL4	ACGTTCAGATTTCCTGCCCC	GCAGAGAAACAGCAATGGTGG
CCL5	ATATGGCTCGGACACCACTC	ACTTGGCGGTTCCTTCGAG
CCL7	CGCTGCTTTCAGCATCCAAG	CTTCCCAGGGACACCGACTA
CCL8	GCCAGATAAGGCTCCAGTCA	TCCATGGGGCACTGGATATTG
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

Note: CCL, C-C motif chemokine ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin.

aged mice. Serum level of TNF-α was significantly elevated in aged mice without any treatment and after pre-immunization. Serum IL-6 was also augmented in aged mice, but not statistically significant (Figure 2A). Other cytokines such as IL-1β, IL-4, IL-10, IL-12, IL-13, IL-17, interferon-γ, and transforming growth factor-β were under detectable level in the serum of each mouse. On the contrary, phosphorylated ERK was detected in the Bowman's cells and glomerular cells, and phosphorylated IκBα was also positive in the glomeruli of NTN adult mice (Figure 2B). Nuclear factor κB (NF-κB) is released from its inhibitor IκB protein in the cytosol by phosphorylation of IκB and activates gene transcription after nuclear translocation (17). Phosphorylation of ERK and IκBα indicated that inflammation pathways were activated locally in the kidney of adult NTN mice. The number of phosphorylated ERK- and phosphorylated IκBα-positive cells in the glomeruli was remarkably increased in adult NTN mice compared with aged NTN mice (Figure 2C).

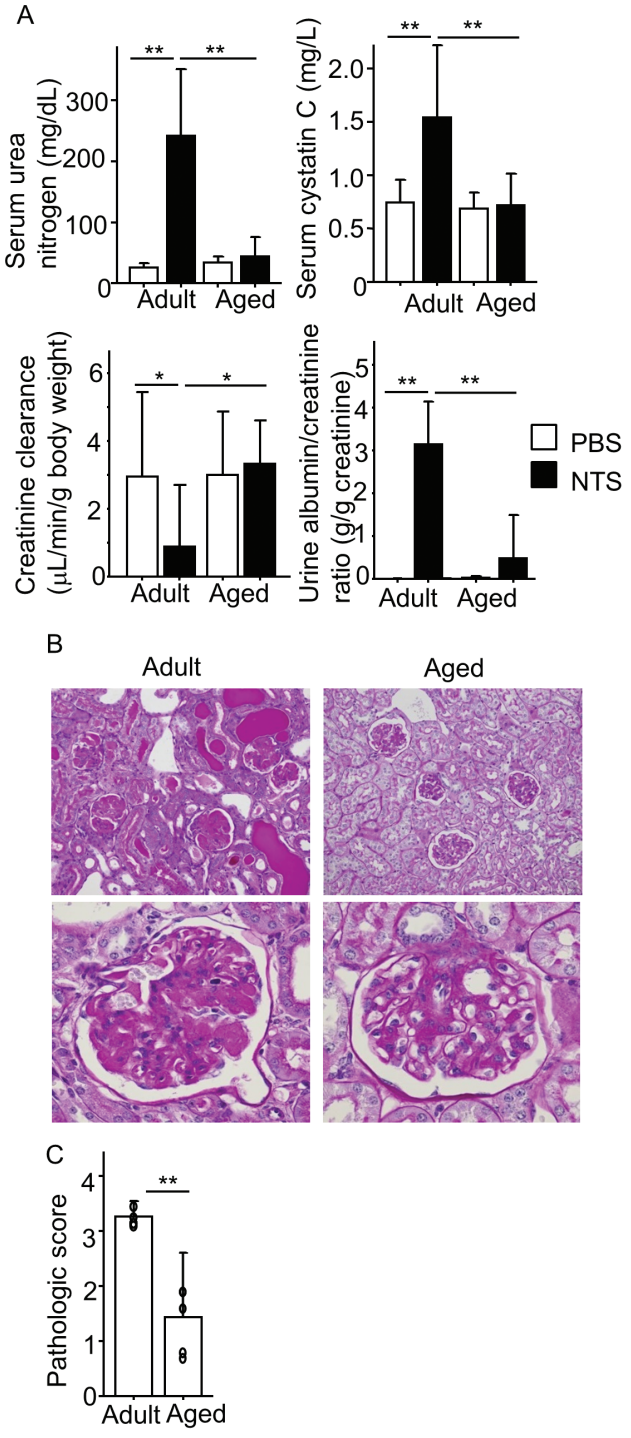

Figure 1. Attenuated kidney injury caused by nephrotoxic serum (NTS) nephritis in aged mice. (**A**) Serum levels of blood urea nitrogen and cystatin C, 24 h creatinine clearance and urine albumin/creatinine ratio from pre-immunized adult and aged mice measured 7 days after NTS or phosphate-buffered saline (PBS) injection ($n = 5$). (**B**) Representative periodic acid-Schiff-stained kidney sections of adult and aged mice (upper, ×100; lower, ×400). Kidneys were removed and examined on day 7. (**C**) Quantitative evaluation of glomerular injury in kidney sections of adult and aged mice 7 days after NTS injection ($n = 5$). Values are mean ± SD. *$p < .05$, **$p < .01$.

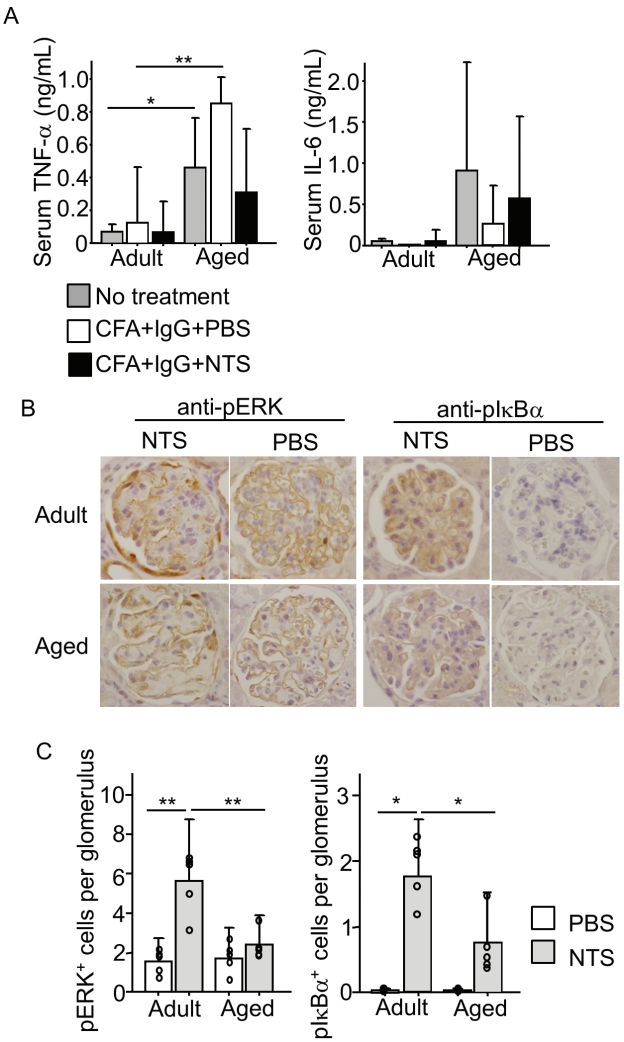

Figure 2. Systemic and local immune response in glomeruli of adult and aged nephrotoxic serum nephritis (NTS) mice. (**A**) Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL) -6 from no-treated, or pre-immunized adult and aged mice measured 7 days after NTS or phosphate-buffered saline (PBS) injection ($n = 5$). (**B**) Phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα) was examined in the glomeruli of adult (upper) and aged (lower) mice pre-immunized with sheep IgG and treated with NTS or PBS 7 days previously (×400). (**C**) Quantitative evaluation of phosphorylated ERK (pERK)- or phosphorylated IκBα (plκBα)-positive cells in glomerulus of pre-immunized adult and aged mice 7 days after NTS or PBS injection ($n = 5$). Values are mean ± SD. *$p < .05$, **$p < .01$.

Deposition of Mouse IgG in the Glomeruli and Serum Titer of Antigen-Specific IgG were not Affected by Aging

Heterologous sheep NTS induces the glomerular deposition of pathogenic immune complexes, and pre-sensitization of sheep IgG triggers the production of IgG anti-sheep IgG antibodies in mice, which leads to accelerated glomerular injury (13). To assess the humoral immune responses, deposition of heterologous sheep IgG, autologous mouse IgG, and complement C3c in the glomeruli were quantified via immunofluorescence of frozen kidney sections. There were no significant differences in the amount of heterologous sheep IgG, autologous mouse IgG, or complement C3c deposited in the glomeruli of adult and aged mice 7 days after NTS administration (Figure 3A and B). Serum titers of sheep IgG-specific IgG1, IgG2a, IgG2b, and IgG3 were measured by ELISA and were found to be at comparable levels in adult and aged mice (Figure 3C). IgG1 and IgG2b were the isotypes mainly introduced and consumed in the NTN model, and there were no significant differences in the amount of autologous mouse IgG1 or IgG2b deposited in the glomeruli of adult and aged mice 7 days after NTS administration (Figure 3D and E).

Reduced Glomerular Infiltration of Macrophages in Aged NTN Mice

Then we investigated the difference of macrophage infiltration in the diseased kidney by detecting F4/80 antigen-positive cells. F4/80 antigen is contained in a 160 kDa glycoprotein exclusively expressed on mouse monocyte and macrophage plasma membrane. Monocytes replenish F4/80$^+$ tissue-resident macrophages associating with endothelia and epithelia as they migrate through tissues (18,19). In the kidney large proportion of F4/80$^+$ macrophages normally exist in medullary interstitium, but also in cortical interstitium along with the outer surface of proximal and distal tubular cells and Bowman's capsule (20). In contrast to the equal humoral immune responses between the adult and the aged mice, the number of F4/80$^+$ macrophages infiltrating the glomeruli revealed that infiltration of macrophages was remarkably decreased in aged NTN mice compared with adult NTN mice (Figure 4A and B). mRNA expression of IL-6 and IL-12 p35, which are produced by inflammatory M1 macrophages as proinflammatory cytokines (21,22), was also significantly elevated only in adult NTN mice (Figure 4C), consistent with impaired infiltration of M1 macrophages in the diseased kidney of aged mice. To clarify the mechanism of diminished macrophage infiltration in aged NTN mice, we focused on the chemotaxis of monocyte/macrophage lineage cells through chemokine-chemokine receptor interactions and examined the expression levels of several chemokines associated with monocyte/macrophage chemotaxis. As shown in Figure 5A, mRNA expression of C–C motif chemokine ligand (CCL) 2 was specifically up-regulated in adult NTN mice compared with adult control mice. CCL4 expression was significantly augmented in aged NTN mice compared with adult NTN mice, but no other significant differences in chemokine expression were observed among these mice. On the contrary, cell surface expression of CCR1 and CCR2 on F4/80$^+$ Ly-6G$^-$ monocyte/macrophage lineage cells infiltrating the NTN kidney was significantly impaired in aged NTN mice compared with adult NTN mice. This down-regulated expression of CCR1 and CCR2 in aged NTN mice was specifically observed in kidney-infiltrating monocyte/macrophage lineage cells, but not in peripheral monocytes (Figure 5B, C, and D).

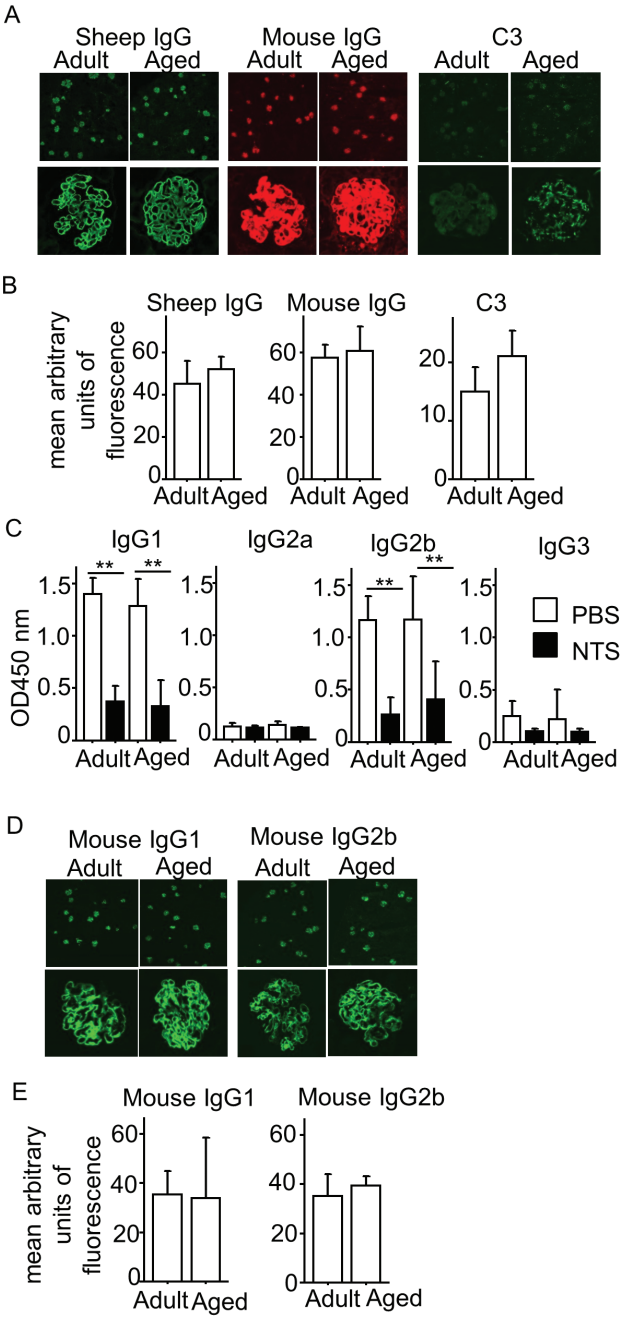

Figure 3. Secondary immune responses in adult and aged mice. (**A**) Representative immunofluorescence microscopy of kidney sections detecting sheep IgG, mouse IgG, and C3c deposited along the GBM in the kidney of adult and aged mice 7 days after nephrotoxic serum (NTS) injection. Upper row, ×40; Lower row, ×400. (**B**) Quantitative evaluation of immunofluorescence detection of sheep IgG, mouse IgG, and C3c deposited in glomeruli on day 7 ($n = 5$). (**C**) Serum levels of IgG1$^-$, IgG2a$^-$, IgG2b$^-$, and IgG3-specific antibodies against sheep IgG measured by ELISA on day 7 ($n = 5$). (**D**) Representative immunofluorescence microscopy of kidney sections detecting mouse IgG1 and IgG2b deposited along the GBM in the kidney of adult and aged mice 7 days after NTS injection. Upper row, ×40; lower row, ×400. (**E**) Quantitative evaluation of immunofluorescence detection of mouse IgG1 and IgG2b deposited in glomeruli on day 7 ($n = 5$). Values are mean ± SD. $^{**}p < .01$.

Reduced FcγR Expression in Monocyte/Macrophage Lineage Cells of Aged NTN Mice

Finally, we evaluated the functional impairment of monocyte/macrophage lineage cells from the aspect of FcγR expression. We investigated the cell surface expression of the IgG-Fc receptors, FcγRI, FcγRIIB, FcγRIII, and FcγRIV, in monocyte/macrophage lineage cells and granulocytes infiltrating the NTN kidney. All four FcγRs showed down-regulated expression, specifically in F4/80$^+$ Ly-6G$^-$ monocyte/macrophage lineage cells of aged NTN mice, but not in Ly-6G$^+$ granulocytes (Figure 6).

Discussion

In the present comparative study of young and aged normal mice, we demonstrated that cell surface expression of both CCRs and FcγRs is down-regulated in F4/80$^+$ Ly-6G$^-$ monocyte/macrophage lineage

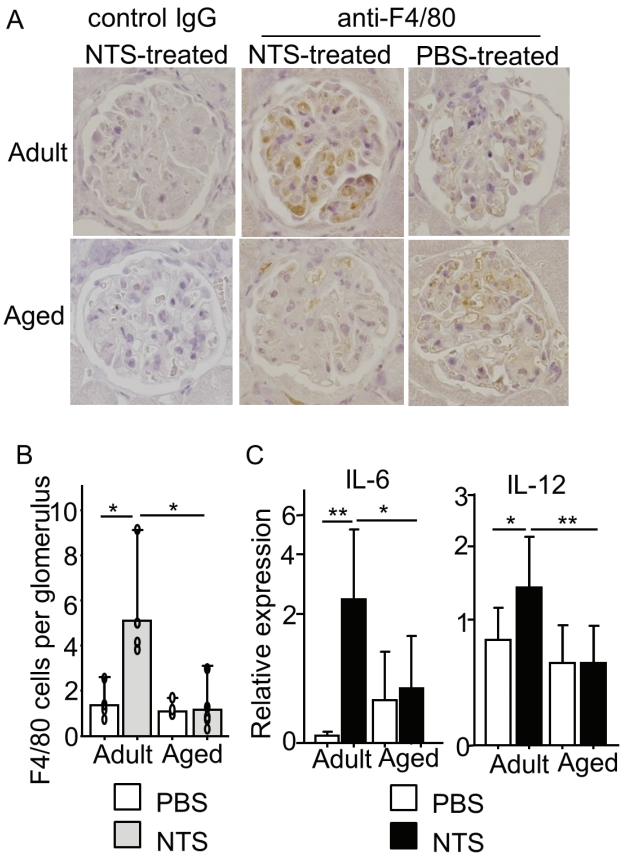

Figure 4. Impaired macrophage infiltration in glomeruli and immune responses of aged mice. (**A**) The macrophage marker, F4/80, was examined in the glomeruli of adult (upper) and aged (lower) mice pre-immunized with sheep IgG and treated with nephrotoxic serum (NTS, middle) or phosphate-buffered saline (PBS, right) 7 days previously. Rat control IgG was used as a control against anti-F4/80 (left) (×400). (**B**) Quantitative evaluation of infiltrated F4/80 positive cells per glomerulus in kidney sections of pre-immunized adult and aged mice 7 days after NTS or PBS injection (n = 5). (**C**) Total RNA was extracted from the kidney of pre-immunized adult or aged mice treated with NTS or PBS 7 days previously. mRNA expression of the M1 macrophage-related cytokines, interleukin (IL)-6 and IL-12 p35, were evaluated by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Data are shown as the fold change (n = 5). Values are mean ± SD. *p < .05, **p < .01.

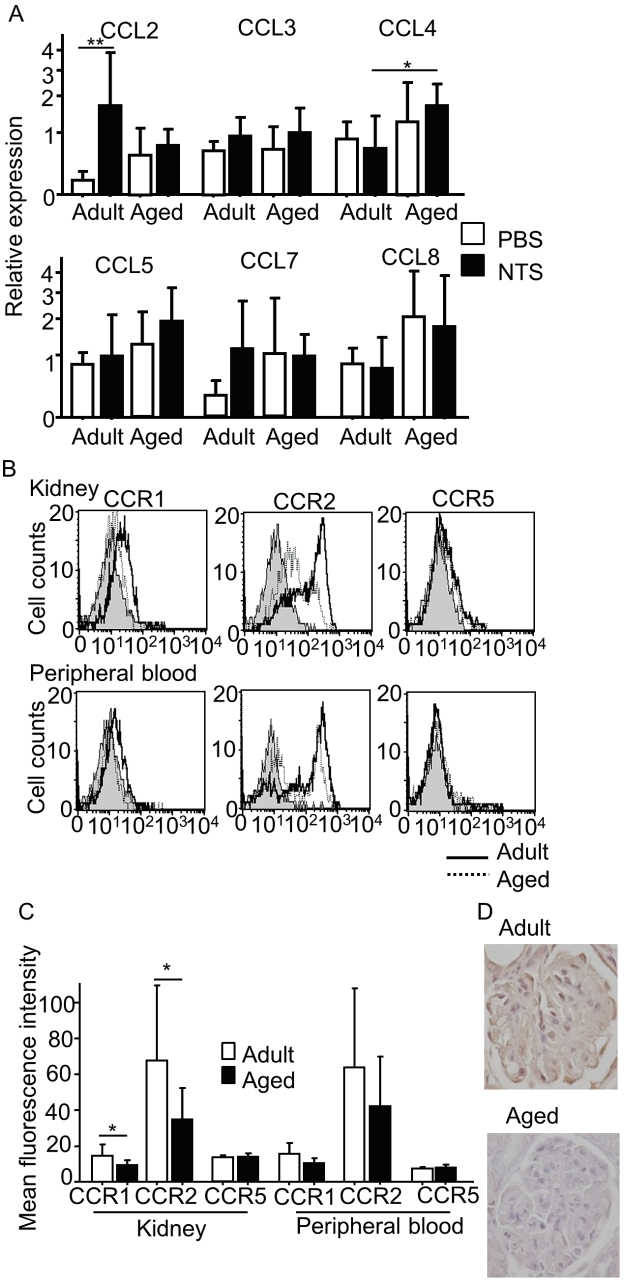

Figure 5. Attenuated chemokine production and chemokine receptor expression in aged mice. (**A**) Total RNA was extracted from kidneys of pre-immunized adult or aged mice treated with nephrotoxic serum (NTS) or phosphate-buffered saline (PBS) 7 days previously. mRNA expression of the macrophage-related chemokines, CCL2, CCL3, CCL4, CCL5, CCL7, and CCL8 were evaluated by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Data are shown as the fold change (n = 5). (**B**) Representative flow cytometry analysis of CCR1, CCR2, and CCR5 expression on F4/80$^+$ Ly-6G$^-$ monocyte/macrophages infiltrated in the NTS nephritis kidney (upper) or peripheral blood (lower) of adult and aged mice 7 days after NTS injection. Bold lines, adult mice; dotted lines, aged mice; grey shadows, isotype control. (**C**) Quantitative evaluation of CCR1, CCR2, and CCR5 expression on F4/80$^+$ Ly-6G$^-$ monocyte/macrophages. Mean fluorescence intensity was compared between adult and aged mice 7 days after NTS injection (n = 4). (**D**) Representative kidney sections of adult and aged mice 7 days after NTS injection stained with a CCR2-specific antibody (×400). Values are mean ± SD. *p < .05, **p < .01.

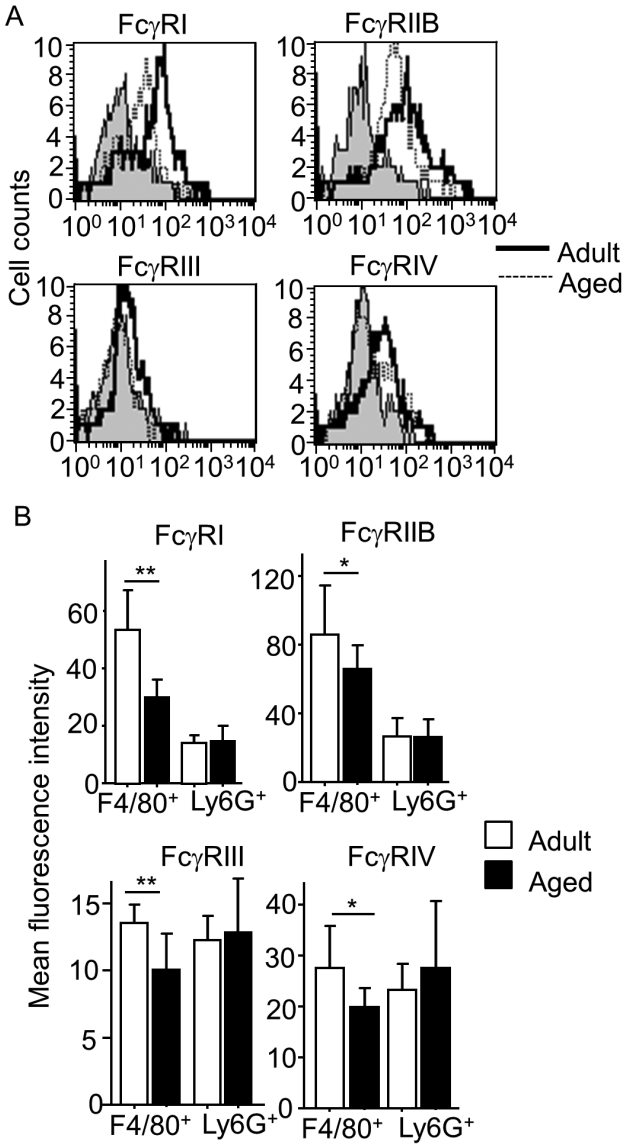

Figure 6. Reduced FcγR expression specific to monocyte/macrophages in aged mice. (**A**) Representative flow cytometry analysis of FcγRI, FcγRIIB, FcγRIII, and FcγRIV expression on F4/80$^+$ Ly-6G$^-$ monocyte/macrophages infiltrated in the NTS nephritis kidney of adult and aged mice 7 days after nephrotoxic serum (NTS) injection. Bold lines, adult mice; dotted lines, aged mice; grey shadows, isotype control. (**B**) Quantitative evaluation of FcγRI, FcγRIIB, FcγRIII, and FcγRIV expression on F4/80$^+$ Ly-6G$^-$ monocyte/macrophages and Ly6G$^+$ granulocytes. Mean fluorescence intensities were compared between adult and aged mice 7 days after NTS injection (n = 4). Values are mean ± SD. *p < .05, **p < .01.

cells in the NTN kidney, resulting in attenuated glomerular infiltration of these cells and impaired glomerular injury in aged mice. It should be highlighted that influence of aging on immune reaction was not determined by secretory phenotype alone, but was also regulated by cellular receptor expression of effector cells. However, the present study was limited to the age-related dysfunction of monocyte/macrophage lineage. Other immune cells such as granulocytes, T lymphocytes and B lymphocytes are also indispensable for the induction of NTN (16,23), and influence of aging on the entire immune system network should be further investigated. Nevertheless, macrophage accumulation is a prominent feature in most types of human glomerulonephritis (24) and in animal models of experimental glomerulonephritis (15,24) and tubulointerstitial injury (25). Chemokines and their specific receptors are increased in the NTN model and play a major role in leukocyte infiltration in the diseased kidney. CCL2/MCP-1 is most prominently increased in nephritic kidneys, but CCR1, CCR2, and CCR5 are also up-regulated (26). CCL2/MCP-1 plays a major role in the pathogenesis of kidney disease in both humans and animal models, including the NTN model (27,28), and many cells contribute to its production, e.g. tubular epithelial cells, mesangial cells, endothelial cells, and infiltrating mononuclear cells (29). In contrast to the pro-inflammatory character of SASP, CCL2/MCP-1 expression was diminished in aged NTN mice. CCL2/MCP-1 mRNA increases in concordance with macrophage infiltration in the NTN model (26). Therefore, we surmise that accumulated macrophages are the main source of enhanced CCL2/MCP-1 in the diseased kidney and that up-regulation of CCL2/MCP-1 expression, as well as IL-6 and IL-12, reflects accelerated macrophage infiltration. CCR2 is the main receptor for MCP-1 (30). CCR2$^+$ monocytes are known as "inflammatory" monocytes and are most crucial during acute inflammation, whereas CCR2$^-$ monocytes are known as "patrolling" monocytes (31). We speculate that impaired up-regulation of CCR2$^+$ monocyte/macrophages and CCL2/MCP-1 expression explains the decreased monocyte/macrophage infiltration and ameliorated kidney injury in aged mice.

In the present study, we also observed decreased expression of FcγRI, FcγRIIB, FcγRIII, and FcγRIV in monocyte/macrophage lineage cells in the kidneys of aged NTN mice. These FcγRs are categorized as type I Fc receptors and belong to the immunoglobulin receptor superfamily represented by the canonical Fcγ receptors (32). In mice and humans, FcγRIIB is a single-chain receptor which carries an immunoreceptor tyrosine-based inhibitory motif and acts as an inhibitory receptor binding IgG1, IgG2a, and IgG2b with low affinity. On the other hand, FcγRI, FcγRIII, and FcγRIV are associated with the accessory common γ chain, which carries an immunoreceptor tyrosine-based activation motif and activates FcγRs in mice. FcγRI displays high affinity for and exclusive binding to IgG2a, whereas FcγRIII and FcγRIV have low affinity for the Fc region of IgG. FcγRIII binds IgG1, IgG2a, and IgG2b, while FcγRIV binds IgG2a and IgG2b with higher affinity than FcγRIII (33). NTN develops in both FcγRI- and FcγRIII-deficient mice as severely as wild type mice, whereas a blocking antibody against FcγRIV inhibits disease progression (13). Therefore, FcγRIV was determined to be a dominant activating FcγR and IgG2b, a pathological autologous mouse IgG isotype in the development of NTN in this mouse model (13). Cell surface expression of both activating and inhibitory FcγRs was depressed in monocyte/macrophage lineage cells infiltrating the kidneys of aged NTN mice. However, we surmise that the reduction of FcγRIV would be more influential than the reduction of inhibitory FcγRIIB in regulating the function of monocyte/macrophage lineage cells, because the affinity of FcγRIV for the IgG2b Fc portion is about 10-fold higher than that of FcγRIIB (34,35). In the present study mean fluorescence intensity of FcγRIII was especially lower than other three FcγRs. Even though the specificity of the antibodies against each FcγR was verified by ELISA (36), we could not exclude the possibility that rat anti-mouse CD16/CD32 (clone 2.4G2) antibody used to block nonspecific binding through Fc-FcγR interaction would have interfered reaction of each FcγR-specific antibody. $F(ab')_2$ antibody specific to each FcγR would be a solution to eliminate the necessity and possible interference of 2.4G2 antibody.

The present study highlights the novel influence of aging on the kidney and immune system using a nephritis model. This knowledge

is important in identifying the molecular mechanisms responsible for the younger age of onset of glomerulonephritis in humans. Even though elderly patients with IgA nephropathy, the most common form of primary glomerulonephritis, have a higher incidence of end-stage renal disease (37,38), our results are not contradictory because non-immunological factors, such as the presence of hypertension or arteriolosclerosis are influential in the progression of kidney dysfunction (37–39). Anti-GBM disease shows a bimodal age distribution. In spite of general decline in immunocompetence, propensity for autoreactivity increases with age (40) due to the cessation of thymic T cell generation and homeostatic T cell proliferation for T cell replenishment, which can lead to the selection of T cells with increased affinity to self-antigens and following autoantibody production (41). We conjecture that increased susceptibility of autoantibody production with aging would be one of the etiologies of the second peak of human anti-GBM disease. Decreased effector function of monocyte/macrophage lineage cells, including proinflammatory cytokine production, cytotoxicity, intracellular killing, and antigen presentation has been reported and determined to be the cause of the diminished effectiveness of the immune system (42–44). Down-regulated expression of both CCRs and FcγRs in the inflammatory response may be one of the causes of the younger onset of some glomerulonephritis, and be related to the molecular mechanisms of the immunosenescence.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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