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Hyperactivation of the NLRP3 Inflammasome in Myeloid Cells Leads to Severe Organ Damage in Experimental Lupus

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Systemic lupus erythematosus (SLE) is an autoimmune syndrome associated with severe organ damage resulting from the activation of immune cells. Recently, a role for caspase-1 in murine lupus was described, indicating an involvement of inflammasomes in the development of SLE. Among multiple inflammasomes identified, the NLRP3 inflammasome was connected to diverse diseases, including autoimmune encephalomyelitis. However, the function of NLRP3 in SLE development remains elusive. In this study, we explored the role of NLRP3 in the development of SLE using the pristane-induced experimental lupus model. It was discovered that more severe lupus-like syndrome developed in *Nlrp3*$^{-R258W}$ mice carrying the gain-of-function mutation. *Nlrp3*$^{-R258W}$ mutant mice exhibited significantly higher mortality upon pristane challenge. Moreover, prominent hypercellularity and interstitial nephritis were evident in the glomeruli of *Nlrp3*$^{-R258W}$ mice. In addition, hyperactivation of the NLRP3 inflammasome in this mouse line resulted in proteinuria and mesangial destruction. Importantly, all of these phenotypes were largely attributed to the *Nlrp3*$^{-R258W}$ mutation expressed in myeloid cells, because Cre recombinase–mediated depletion of this mutant from such cells rescued mice from experimental lupus. Taken together, our study demonstrates a critical role for NLRP3 in the development of SLE and suggests that modulating the inflammasome signal may help to control the inflammatory damage in autoimmune diseases, including lupus. ***The Journal of Immunology*****, 2017, 198: 1119–1129.**

Systemic lupus erythematosus (SLE) is a serious autoimmune syndrome that predominantly affects young women, resulting in severe clinical manifestations (1). Lupus is associated with significant organ damage resulting from the activation of innate and adaptive immune signals (2). Our understanding of SLE has advanced in recent years; microRNAs, inflammation, and inflammasomes have been implicated in the development and pathogenesis of this disease (3–5).

Inflammasomes are multiprotein complexes consisting of sensor proteins, such as NLRP1, NLRP3, NLRP7, AIM2, and NAIP, adaptor proteins ASC and NLRC4, and effectors, including caspase-1 and caspase-8 (6–8). Assembly of a canonical inflammasome leads to activation of caspase-1, which triggers pyroptosis and the maturation and release of proinflammatory cytokines IL-1β and IL-18 (9). On one hand, inflammasome activity is critical for the control of invading pathogens (10); on the other hand, hyperinflammasome activation may lead to tissue damage (11).

Gain-of-function genetic mutations in some inflammasome genes were connected with multiple autoinflammatory diseases. Several recent studies showed that mutations in the *NLRC4* gene are connected with enterocolitis or macrophage activation syndrome in humans and mice (12–14). The *NLRP3* gene was also identified because of its strong connection with a group of autoinflammatory diseases, including Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and neonatal-onset multisystem inflammatory disease (15, 16). These NLRP3-associated autoinflammatory diseases are now collectively called cryopyrin-associated periodic syndromes (CAPS), and mouse lines mimicking such human diseases have been generated (17, 18). The most dominant mutation associated with CAPS in humans is R260W, whose homologous site in the mouse *Nlrp3* gene is the R258W mutation. The *Nlrp3*$^{-R258W}$ mice generated in a previous study exhibited inflammasome hyperactivation and developed autoinflammatory syndromes very similar to those in human patients (17).

As cytokines under the control of inflammasomes, IL-1β and IL-18 contribute to autoinflammation in mouse models of CAPS (19). These cytokines also were implicated in the pathogenesis of autoimmune diseases, including SLE. It was reported that IL-1β levels are increased in the plasma of patients with neuropsychiatric lupus (20). IL-18 levels are also elevated in the

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Abbreviations used in this article: BUN, blood urea nitrogen; CAPS, cryopyrin-associated periodic syndromes; PAMS-HE, periodic acid methenamine silver–H&E; PAS, periodic acid–Schiff; SLE, systemic lupus erythematosus; WT, wild-type.

serum and glomeruli of patients with lupus nephritis, which is associated with localization of plasmacytoid dendritic cells to glomeruli, triggering resident T cells and promoting renal damage (21, 22). An essential role for caspase-1 in pristane-induced murine lupus was described recently, indicating an involvement of certain inflammasomes in SLE (23). It was also found that the NLRP3 inflammasome is activated in macrophages from SLE patients (24, 25). In addition, lupus nephritis was attenuated through inhibition of the NLRP3 inflammasome in two models of murine SLE: NZB×NZW F1 mice and MRL/*Fas*^{lpr} mice (26, 27). Moreover, the kidney is the organ most often involved in SLE; NLRP3 inflammasome activation was demonstrated in several murine models of renal injury using NZB×NZW F1 lupus-prone mice (26) and in a chronic kidney disease model using C57BL/6 mice (28). Thus, according to these studies, the NLRP3 inflammasome promoted the pathogenesis of SLE. Paradoxically, however, a recent study showed that NLRP3 and ASC were suppressive factors for lupus development in lpr/lpr mice (29), and autoimmune NZB mice were defective for NLRP3 and AIM2 inflammasome function (30). Therefore, the role of NLRP3 in SLE is controversial and needs further investigation.

In the current study, we analyzed the autoimmune disease phenotypes in mice carrying the *Nlrp3*$^{-R258W}$ mutation upon challenge with pristane, which is recognized as a robust model for SLE (31). *Nlrp3*$^{-R258W}$ mutant mice developed much more severe lupus-like syndrome than did wild-type (WT) controls, which was largely due to a hyperactive inflammasome in myeloid cells. Thus, this study demonstrates a critical role for NLRP3 in the development of SLE and suggests that inhibiting the inflammasome signal should help to control the inflammatory damage in lupus.

Materials and Methods

Mice and pristane induction of SLE

The *Nlrp3*$^{-R258W}$ mouse line was provided by Dr. Warren Strober (National Institutes of Health) (17). The myeloid cell–specific cre-expressing mice (LysM cre, B60129P2-Lyz2tmlCcreHfolJ) were from the Model Animal Research Center of Nanjing University. Heterozygous *Nlrp3*$^{-R258W}$ mice were crossed with LysM cre mice for the conditional deletion of the R258W mutation. Females were used in experiments at 8–10 wk of age. Mice were maintained at the specific pathogen–free facility at the Institut Pasteur of Shanghai. Animal care, use, and experimental procedures complied with national guidelines and were approved by the Animal Care and Use Committee at Institut Pasteur of Shanghai.

For SLE induction, *Nlrp3*$^{-R258W}$ and littermate control WT mice received one i.p. injection of 500 μl of saline (as a control) or pristane (Sigma-Aldrich, St. Louis, MO), and survival was monitored twice a week for 6 mo. For ex vivo analysis, the experimental mice were sacrificed 6 mo after pristane injection.

Quantification of anti-dsDNA and total IgG via ELISA

Serum anti-DNA levels were determined by ELISA, as described (32). Briefly, 96-well plates were coated with 5 μg/ml calf thymus dsDNA (Sigma-Aldrich). A mouse anti-dsDNA mAb (Chemicon International) was used to prepare a reference standard curve. Absorbance was measured at 450 nm. Anti-dsDNA concentrations were quantified according to the standard curve. Serum was collected for the measurement of total IgG using commercial ELISA kits (eBioscience), according to the manufacturer's instructions, with normal mouse IgG as negative control.

Evaluation of renal histopathological features and immune complex deposition

Paraffin-embedded sections of renal tissues were stained with H&E, periodic acid–Schiff (PAS), or periodic acid methenamine silver–H&E (PAMS-HE). Histopathological features of glomerular lesions were graded semiquantitatively for severity, in a double-blinded manner. The histological signs of lupus nephritis were assessed by pathologists, as described elsewhere (33). In brief, the extent of the pathological lesions was scored on a semiquantitative scale ranging from 0 to 4: 0, normal; 1, a small increase in cells in the glomerular mesangium; 2, a larger number of cells in the mesangium; 3, glomerular lobular formation and thickened basement membrane; and 4, glomerular crescent formation, sclerosis, tubular atrophy, and casts. The score for each animal was calculated by dividing the total score for the number of glomeruli observed. An activity index was generated by compiling these scores.

To detect immune complex deposition, frozen kidney sections were stained for mouse Igs and C3 with FITC-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology) and FITC-conjugated goat IgG against mouse C3 (Cedarlane Laboratories), respectively (34, 35). The fluorescence intensity was confirmed by flow cytometry.

Biochemistry

Urine samples were collected prior to euthanasia of mice and were assessed for total protein using a BCA kit (Beyotime Biotechnology). Blood urea nitrogen (BUN) levels were analyzed using a commercial ELISA kit (Shanghai Jianglai Biocompany) at the end of the experiment. The samples were also assessed for creatinine levels using a Quantitative Colorimetric Creatinine Determination Kit (BioAssay Systems).

Real-time PCR

Total RNA was extracted from kidney using TRIzol Reagent (Invitrogen). Reverse transcription of mRNA and synthesis of cDNA were performed using TaqMan Reverse Transcription Reagents (Promega). Real-time PCR was performed using SYBR qPCR Mix (Toyobo) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative quantification of genes was achieved via normalization against GAPDH. The primers used are listed in Table I.

Flow cytometry

Isolation and characterization of mononuclear phagocytes from the kidneys of mice were performed as previously described (36). Briefly, freshly isolated kidneys were placed in ice-cold DMEM mixed with Hams F12 (1:1 ratio; Life Technologies, Grand Island, NY) on a petri dish. Kidney capsule was removed by peeling with forceps, and the kidney was sliced coronally and homogenized by mincing into 1–2 mm^3 pieces. The homogenized kidney tissue pieces were resuspended and mixed in 10 ml of collagenase type I for 30 min to obtain single-cell suspensions. After digestion, the cell suspension was filtered through 70-μm cell strainers. The filtered cell suspensions were

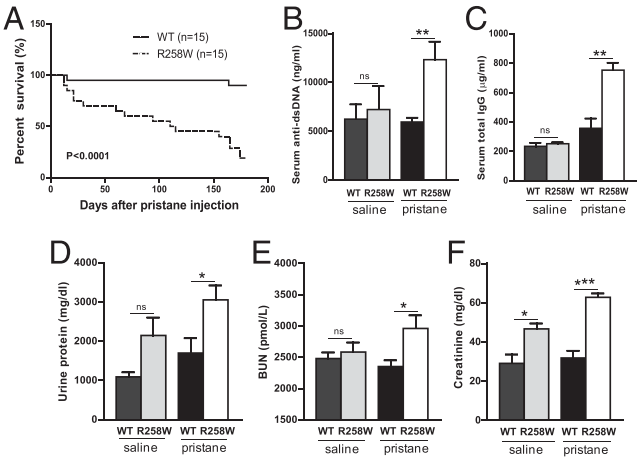

FIGURE 1. The R258W mutation in NLRP3 protein exacerbated the autoimmune responses of mice upon pristane challenge. Mice were injected with 500 μl of saline or pristine, as described in *Materials and Methods*. (**A**) Survival was analyzed. Mice were sacrificed 6 mo after pristane treatment. Data were analyzed using the Gehan–Breslow–Wilcoxon test, data are mean ± 95% confidence interval. Serological levels of anti-dsDNA (**B**) and total IgG (**C**). Urine protein excretion (**D**), BUN (**E**), and urine creatinine (**F**) were measured at the end of the experiment. Data in (B)–(F) are mean ± SEM from five to eight mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant,

centrifuged at 300 × *g* for 2 min at 4°C. The pellet was incubated with ACK Lysing Buffer (Beyotime Biotechnology) for 2 min at room temperature to remove RBCs and centrifuged at 1000 × *g* for 5 min at 4°C. The pellet was then washed with PBS containing 1% BSA and 0.1% sodium azide (Sigma-Aldrich) and passed through a 40-μm cell strainer. Cells were incubated with anti-mouse CD16/32 (eBioscience) to block nonspecific Fc binding and incubated with F4/80 (eBioscience), CD11b (BD Pharmingen), Ly6G (eBioscience), Ly6C (eBioscience), CD3 (BD Pharmingen), CD4 (BD Pharmingen), CD8 (BD Pharmingen), CD11c (BD Pharmingen), FITC-conjugated goat IgG fraction to mouse IgG (Santa Cruz Biotechnology), or and C3 (Cedarlane Laboratories). Data were acquired using a BD FACSCanto and analyzed using FlowJo software.

Statistical analysis

Statistical analyses were performed with Prism 5 software (GraphPad, San Diego, CA) using the paired *t* test, unless noted otherwise. Data are presented as mean ± SEM for 3–10 animals in each group, as indicated. The *p* values < 0.05 versus respective controls were taken as a significant difference.

Results

Nlrp3^{-R258W} *mutant mice exhibited compromised survival and enhanced autoimmune response in pristane-induced lupus*

To test whether the *Nlrp3*$^{-R258W}$ mice that are hyperactive for inflammasome activity carry any abnormalities in autoimmune lupus, mutant and littermate control (WT) mice were injected with saline or pristane and observed for 6 mo. The mortality of *Nlrp3*$^{-R258W}$ mice was significantly higher compared with that of WT mice following pristane injection (Fig. 1A). In addition, it is well known that the pathogenic hallmark of SLE is the autoimmune response against self-antigens, including nuclear dsDNA (37, 38). The IgG autoantibody, especially IgG2a, also plays important pathogenic roles in lupus nephritis (37, 38). Therefore, we examined whether autoantibody and total IgG production was altered in *Nlrp3*$^{-R258W}$ mice after pristane challenge. Of note, we found that anti-dsDNA (Fig. 1B) and total IgG (Fig. 1C) were increased significantly in the serum of *Nlrp3*$^{-R258W}$ mice compared with WT mice. To assess the effects of the R258W mutation in NLRP3 on renal function, urine protein excretion, BUN, and urine creatinine were measured 6 mo after pristane injection. As expected, all of these parameters were increased significantly in *Nlrp3*$^{-R258W}$ mutant mice compared with WT mice (Fig. 1D–F). In mock-treated groups of mice, the autoantibody, total IgG, urine protein, and BUN were comparable, whereas creatinine was slightly increased in *Nlrp3*$^{-R258W}$ mice compared with WT controls (Fig. 1B–F). All of these data indicate that *Nlrp3*$^{-R258W}$ mutant mice exhibited enhanced autoimmune responses after pristane treatment.

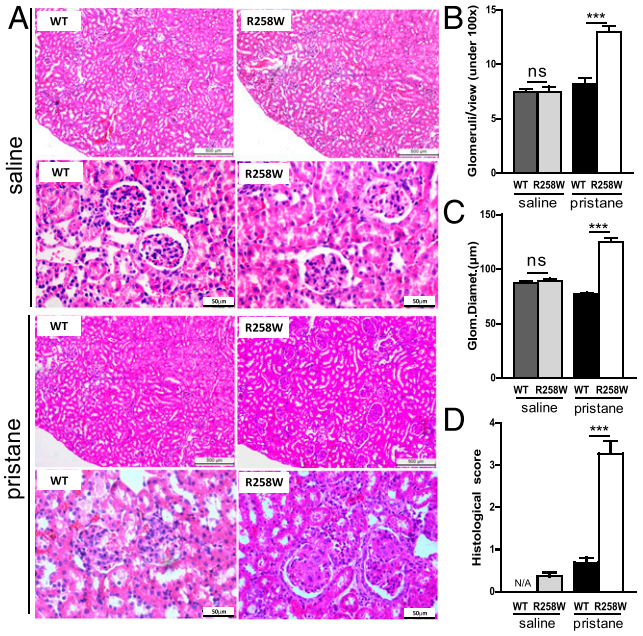

FIGURE 2. *Nlrp3*$^{-R258W}$ mutant mice exhibited more severe renal damage than did WT controls after pristane challenge. Renal histological parameters were analyzed 6 mo after saline or pristane injection. (**A**) Kidney sections were stained with H&E. Scale bars, 500 μm (first and third rows) and 50 μm (second and fourth rows). (**B**) Glomeruli were counted on H&E-stained sections, with the operator blinded to mice genotypes. (**C**) Glomerular diameter was measured digitally; the average glomerular width and height from individual glomerulus are shown. (**D**) Histologic damage index of kidneys from *Nlrp3*$^{-R258W}$ mice (mean ± SEM). Representative images of three to five mice are shown for each genotype. ***$p < 0.001$. ns, not significant.

Table I. Primers used for real-time PCR in this study

Genes	Primers (5′–3′)
GAPDH	AGGTCGGTGTGAACGGATTTG
	TGTAGACCATGTAGTTGAGGTCA
IL-1β	CACGATGCACCTGTACGATCA
	GTTGCTCCATATCCTGTCCCT
IL-17A	TTTAACTCCCTTGGCGCAAAA
	TTTCCCTCCGCATTGACAC
IL-6	TAGTCCTTCCTACCCCAATTTCC
	TTGGTCCTTAGCCACTCCTTC
IL-21	CAGATCGCCTCCTGATTAGA
	GCTTCTGTTTCTTTCCTCCC
IL-18	GACTCTTGCGTCAACTTCAAGG
	CAGGCTGTCTTTTGTCAACGA
IL-22	GACCAAACTCAGCAATCAGCTC
	CTCAGACGCAAGCATTTCTCAG
IFN-γ	GCCACGGCACAGTCATTGA
	TGCTGATGGCCTGATTGTCTT
IFN-α	GCTAGGCTCTGTGCTTTCCT
	GGCTCTCTTGTTCCTGAGGT
IFN-β	AGCTCCAAGAAAGGACGAACA
	GCCCTGTAGGTGAGGTTGAT
IL-12p40	TGGTTTGCCATCGTTTTGCTG
	ACAGGTGAGGTTCACTGTTTCT
IL-12p35	AGACATCACACGGGACCAAAC
	CCAGGCAACTCTCGTTCTTGT
IL-23p19	AGCGGGACATATGAATCTACTAAGAGA
	GTCCTAGTAGGGAGGTGTGAAGTTG
CCL2	TTAAAAACCTGGATCGGAACCAA
	GCATTAGCTTCAGATTTACGGGT
CCL3	CAAGTCTTCTCAGCGCCATA
	GGAATCTTCCGGCTGTAGG
CCL5	GCTGCTTTGCCTACCTCTCC
	TCGAGTGACAAACACGACTGC
CCL20	GGTACTGCTGGCTCACCTCT
	TGTACGAGAGGCAACAGTCG
CXCR3	GCTCTATGCCTTTGTGGGAG
	GCGTGAACAACATCCACATT
BAFF	AATATGCCCAAAACACTGCC
	CATCTCCTTCTTCCAGCCTC
APRIL	CCGTGCCTACAATAGCTGC
	CCGTGGAATTTTGACAGTGAT
NLRP3	AAGGGCCATGGACTATTTCC
	GACTCCACCCGATGACAGTT
TNF-α	GGAACACGTCGTGGGATAATG
	GGCAGACTTTGGATGCTTCTT
TGF-β	AAGTTGGCATGGTAGCCCTT
	GCCCTGGATACCAACTATTGC
Vimentin	AGAGAGAGGAAGCCGAAAGC
	TCCACTTTCCGTTCAAGGTC
Fibronectin	GGAGTGGCACTGTCAACCTC
	ACTGGATGGGGTGGGAAT

R258W mutation in NLRP3 led to severe renal damage after pristane challenge

The progression from acute kidney injury to chronic renal disease is influenced by many intrarenal factors, including glomerular hypertension, endothelial injury, vascular dropout, interstitial inflammation, and fibrosis (39). *Nlrp3*$^{-R258W}$ and WT mice were sacrificed 6 mo after saline or pristane treatment, and several renal histological parameters were observed. H&E staining of kidney sections revealed an increase in the number of glomeruli and cortical thickness in *Nlrp3*$^{-R258W}$ mutant mice (Fig. 2A). When glomeruli were counted in H&E-stained sections, 59% more nephrons were observed in *Nlrp3*$^{-R258W}$ mice kidney compared with WT mice (Fig. 2B). Glomerular diameter was measured digitally under higher magnification. In terms of width or height, for each individual glomerulus, glomeruli were clearly bigger in the *Nlrp3*$^{-R258W}$ kidney (Fig. 2C). The histological damage index also showed that kidney damage in *Nlrp3*$^{-R258W}$ mice was significantly more severe than in WT mice (Fig. 2D). As expected, although the kidney pathology score was slightly higher in *Nlrp3*$^{-R258W}$ mice, no difference in terms of the number and diameter of glomeruli was observed compared with WT mice after saline treatment (Fig. 2). Taken together, these data suggested that the R258W mutation in NLRP3 promoted the development of SLE manifestations.

Hypercellular and interstitial nephritis developed in the glomeruli of Nlrp3^{-R258W} *mice upon pristane challenge*

With additional analysis through PAS and PAMS-HE staining, we demonstrated that the R258W mutation in NLRP3 led to severe renal damage in glomeruli that was characterized by hypercellularity, mesangial expansion, crescent formation, and interstitial mononuclear cell infiltration; these changes were not apparent in the glomeruli of WT mice (Fig. 3A). Moreover, PAMS-HE staining showed clearly thicker glomerular basement membrane, prominent crescent formation, cellular proliferation in the Bowman's space, collapse of the capillary loops, and tuft narrowing in the glomeruli of pristane-treated *Nlrp3*$^{-R258W}$ mice but not control mice (Fig. 3B). In addition, we noticed that some glomeruli were necrotizing, with occasional microcysts and rare large cysts (~10 per section), in *Nlrp3*$^{-R258W}$ mice (Fig. 3A, 3B). Vimentin is a major component of the intermediate filament protein produced by cells of mesenchymal origin. Abs to vimentin were found in a variety of inflammatory and autoimmune conditions, including rheumatoid arthritis and experimental murine lupus (40). In addition, soluble fibronectin is a known inducer of proinflammatory cytokines in renal epithelial cells, and enhanced fibronectin accumulation is observed in several glomerulopathies (41). In our experiments, real-time PCR showed that mRNAs of fibronectin and vimentin were increased in pristane-treated *Nlrp3*$^{-R258W}$ mice compared with WT mice, indicating that more damage and fibrosis were induced in the kidney of *Nlrp3*$^{-R258W}$ mice (Table I, Fig. 3C, 3D). Mock-treated mice did not exhibit any clear signal for pathogenesis in either mouse line (Fig. 3).

Hyperactivation of inflammasome in Nlrp3^{-R258W} *mice resulted in renal immune complex deposition*

Deposition of immune complex in the kidney triggers production of the proinflammatory mediators, results in macrophage and lymphocyte infiltration and diffused glomerular proliferation, and ultimately leads to glomerulosclerosis (42). In the current study, kidney sections of experimental mice were stained with FITC-conjugated anti-C3 or anti-IgG Abs. No obvious immune complex was observed in saline-treated WT mice; however, moderate deposition of IgG and C3 was detected in saline-treated *Nlrp3*$^{-R258W}$ mice. Of note, in pristane-treated groups, more pronounced scattered granular deposits of total IgG and C3 were observed in the mesangium capillary wall and glomerular basement membrane of glomeruli in kidney sections from *Nlrp3*$^{-R258W}$ mice (Fig. 4A). Furthermore, total IgG and C3 expression in kidney was quantified using flow cytometry. Our results showed that the fluorescence intensity and the total number of cells positive for C3 and IgG

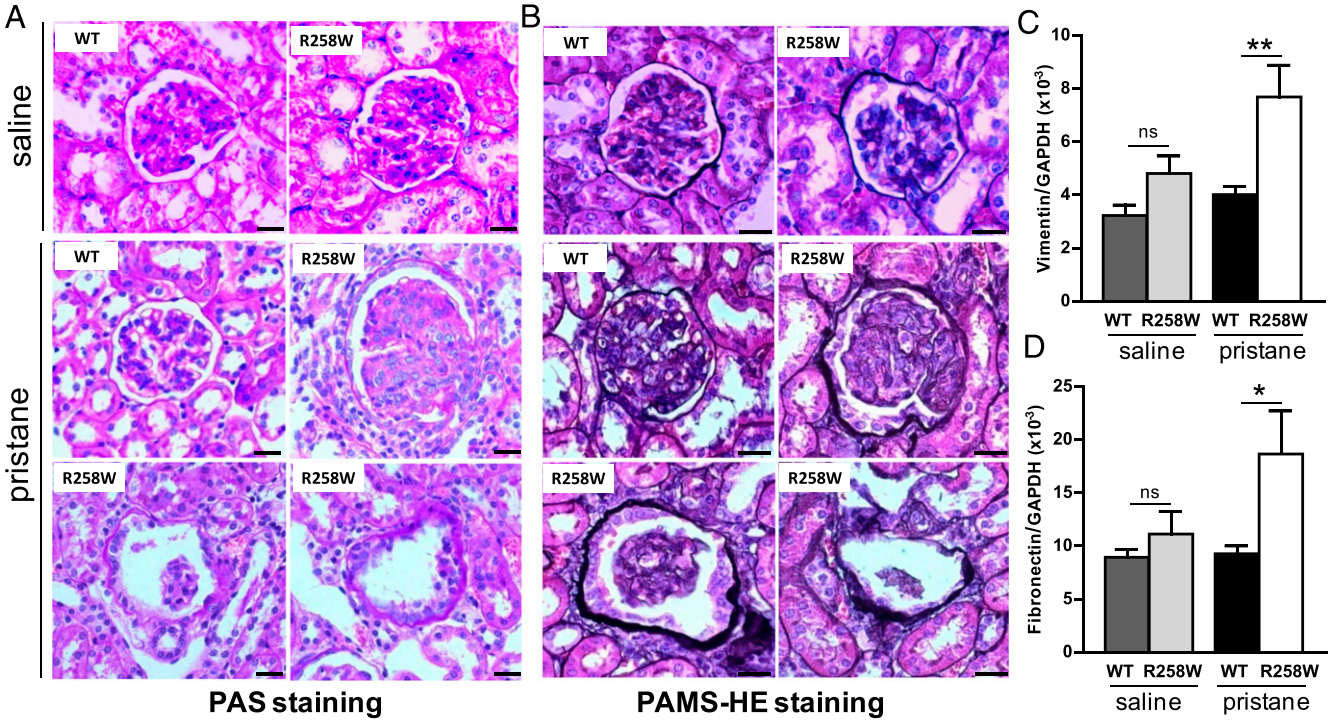

FIGURE 3. *Nlrp3*$^{-R258W}$ mutant mice exhibited accelerated glomerular lesions with a higher chronicity index than WT controls in lupus model. Kidney sections were stained with PAS (**A**) or PAMS-HE (**B**). Representative images of three to five mice are shown for each genotype. Scale bars, 10 μm. (**C** and **D**) Transcripts of vimentin and fibronectin were quantified through real-time PCR. Data are mean ± SEM from five to eight mice. *$p < 0.05$, **$p < 0.01$. ns, not significant.

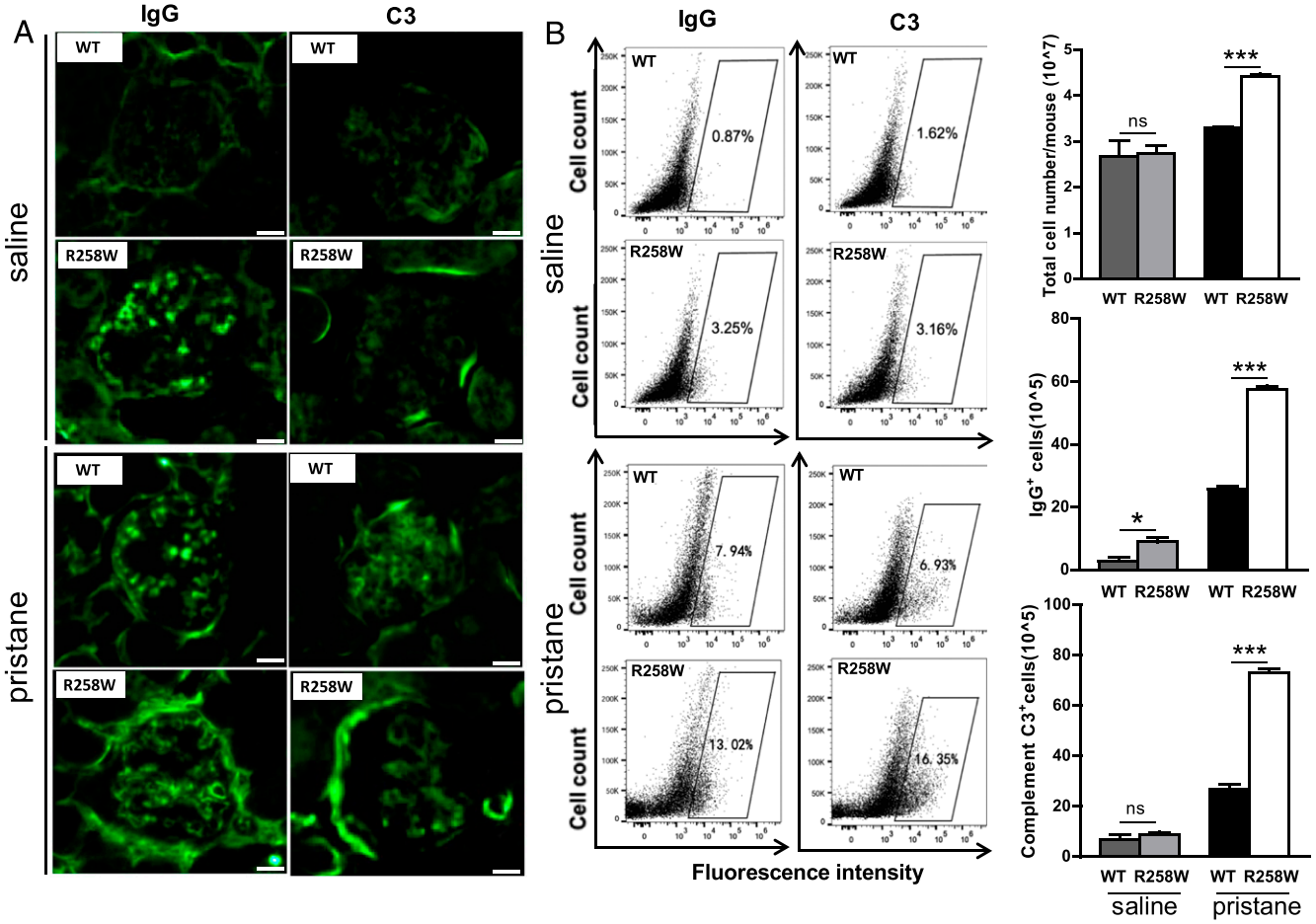

FIGURE 4. The R258W mutation in NLRP3 rendered mice susceptible to increased deposition of immune complex in the kidney. (**A**) Snap-frozen sections were stained with FITC-conjugated Abs against C3 or IgG. Representative images of five mice are shown for each group of mice. Scale bars, 10 μm. (**B**) Fluorescence intensity and the number of cells positive for IgG and C3 staining were analyzed by flow cytometry. The quantified data are shown as mean ± SEM from three mice in each group. $*p < 0.05$, $***p < 0.001$. ns, not significant.

were increased in WT and *Nlrp3*$^{-R258W}$ mice after pristane challenge, and the increase in *Nlrp3*$^{-R258W}$ mice was markedly greater (Fig. 4B). $C3^+$ and IgG^+ cells were much less abundant in saline-treated groups, but both populations of cells were increased in *Nlrp3*$^{-R258W}$ mice compared with WT controls, indicating the presence of a low-level autoimmune response in the mutant animals before pristane injection (Fig. 4).

The kidney of Nlrp3^{-R258W} *mice generated more proinflammatory mediators upon pristane challenge*

Cytokines play key roles in the regulation of systemic inflammation, local tissue damage, and immune modulation. Some cytokines often play direct roles in the pathogenesis of SLE. Many inflammatory genes were upregulated in SLE patients and mouse models, including IL-1 family cytokines, TNF family cytokines, IFN family cytokines, and IL-6, IL-17, CCL2, and B cell–activating genes (43, 44). Therefore, the expression of chemokines, chemokine receptors, and proinflammatory cytokines was analyzed via RT-PCR in this study (Table I). No differences in the expression of these parameters were observed in saline-treated groups. However, 6 mo after pristane treatment, it was very evident that kidneys from *Nlrp3*$^{-R258W}$ mice exhibited significantly increased levels of IL-1β, IL-17A, IL-6, and IL-21 (Fig. 5A), although the transcription of IL-22, IL-23p19 (data not shown), IL-18, and IFN-γ remained at similar levels in *Nlrp3*$^{-R258W}$ and WT mice (Fig. 5A). Type I IFN plays a central role in the development of autoimmune disease (45); however, in our study, the expression levels of IFN-α and IFN-β were similar in *Nlrp3*$^{-R258W}$ mice and WT mice. Although IFN-γ was increased slightly in *Nlrp3*$^{-R258W}$ mice, the difference did not reach statistical significance (Fig. 5A). IL-12 is produced primarily by macrophages and dendritic cells, and it plays an important role in SLE (46, 47). Some studies reported that IL-12 levels were increased significantly in active lupus patients compared with normal subjects, whereas another study demonstrated that the production of IL-12 was impaired in SLE (48). Of note, in this study, the expression level of IL-12 was decreased significantly in *Nlrp3*$^{-R258W}$ mice compared with WT controls (Fig. 5A), which was consistent with the findings of Liu and Jones (48).

It was suggested that cytokines and chemokines derived from T and B cells play important roles in the pathogenesis of experimental lupus. The main function of chemokines is to guide inflammatory cells migrating to sites of inflammation (49). Proinflammatory cytokines, such as TNF-α and IL-1β, are the major mediators responsible for chemokine induction. In mouse macrophages, IL-17 enhances the production of CCL2, CCL3, and CCL20 through MAPK pathways (p38 and ERK1/2) to recruit T cells and monocytes. Of note, accumulating data showed that CCL2, also termed MCP-1, is a potential therapeutic target and biomarker for disease activity in lupus nephritis (50, 51). In this study, transcription of chemokines, including CCL2, CCL3, CCL5, CCL20, and CXCR3, was amplified significantly in *Nlrp3*$^{-R258W}$ mice (Fig. 5B).

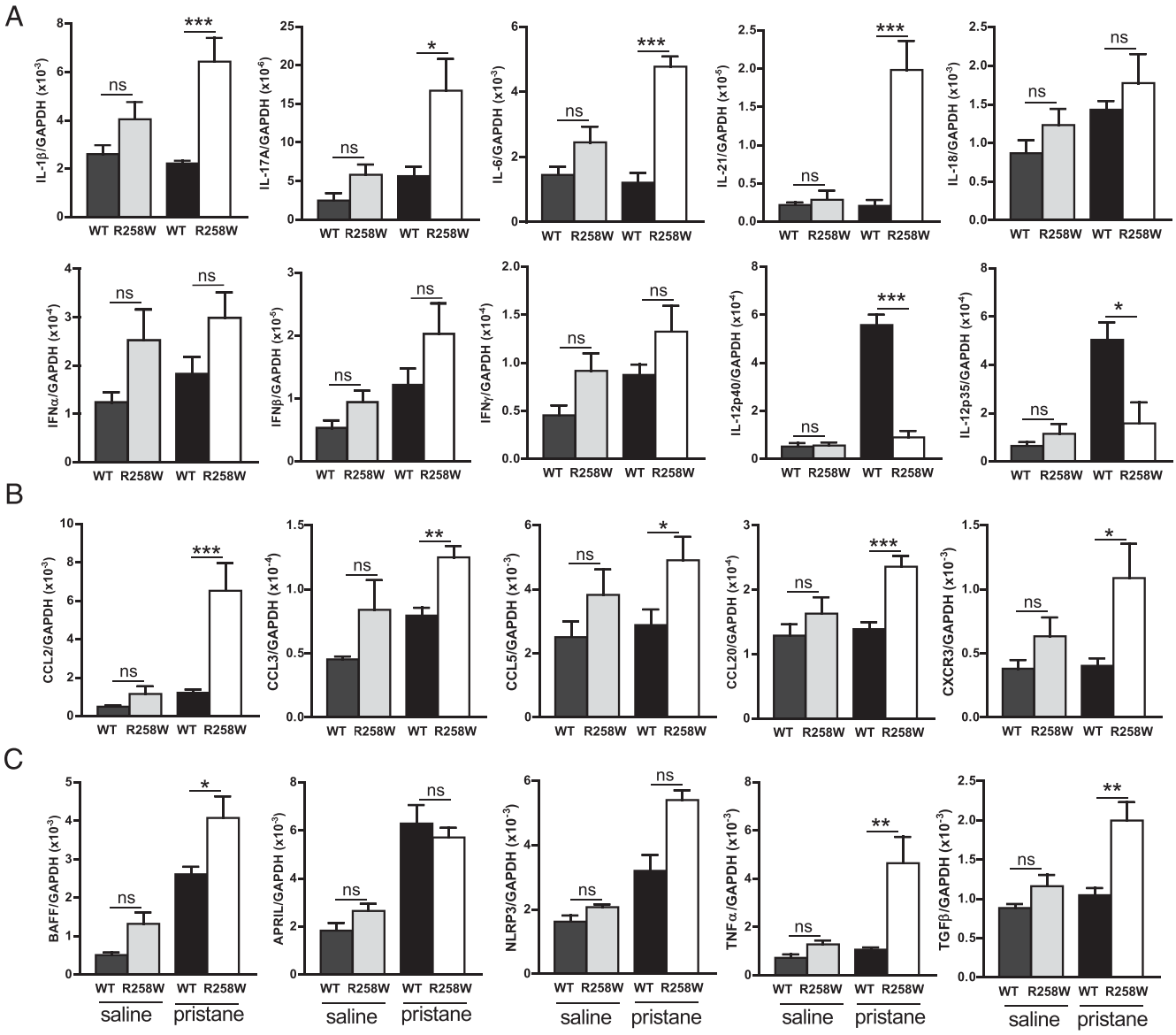

FIGURE 5. Kidney of *Nlrp3*$^{-R258W}$ mice generated more proinflammatory mediators upon pristane challenge. (**A** and **B**) Proinflammatory cytokines and chemokines were monitored via real-time PCR from kidney samples obtained after saline or pristane challenge for 6 mo. (**C**) Transcriptional expression of B cell–activating factors and NLRP3 were analyzed as in (A). Data are mean ± SEM. Five to eight mice were used in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

Higher serum concentrations of BAFF were reported in patients with SLE, and BAFF-transgenic mice have a lupus-like phenotype that is characterized by deregulated B cell activation and maturation (52). In *Nlrp3*$^{-R258W}$ mice, the B cell–activating marker BAFF was upregulated, but APRIL was not. Meanwhile, the proinflammatory cytokine TNF-α and the inflammasome gene NLRP3 were upregulated in *Nlrp3*$^{-R258W}$ mice (Fig. 5C). Intriguingly, the expression level of TGF-β was also increased in *Nlrp3*$^{-R258W}$ mice, although it is considered an anti-inflammatory cytokine in general.

Altogether, these results suggested that the R258W mutation in NLRP3 promoted the expression of proinflammatory cytokines and chemokines after pristane challenge.

Amplified inflammatory cell infiltration in Nlrp3^{-R258W} *mice*

Lupus nephritis is characterized by immune complex deposition and inflammatory cell infiltration. Inflammatory cell infiltration plays a key role in the kidney injury and progression of SLE (53). Renal infiltration by T cells, macrophages, and dendritic cells was reported, but neutrophils play a dominant role in the progression of lupus glomerulonephritis to renal failure (53). Total body weight, as well as spleen and kidney weight, was measured at the end of our experiments. Kidneys and spleens from *Nlrp3*$^{-R258W}$ mice were 1.38- and 4-fold heavier, respectively, than those from littermate controls (Fig. 6A). Total cell number in the kidney was also significantly higher in mutant mice than in WT mice (Fig. 6A). Furthermore, renal infiltrating immune cells were increased significantly in *Nlrp3*$^{-R258W}$ mice (Fig. 6B–E). Among these infiltrated cells, myeloid cells, such as macrophages (CD11b$^+$ and F4/80$^+$) and neutrophils (Ly6G$^+$ and Ly6C$^+$), were dominant (Fig. 6B, 6C), whereas dendritic cells (CD11c$^+$) were rather rare, although a clear increase was evident in *Nlrp3*$^{-R258W}$ mice (Fig. 6D). This is consistent with early findings showing that macrophages are involved in human glomerulonephritis, including lupus nephritis (54–56). Infiltration of T cells (CD3$^+$, CD4$^+$, or CD8$^+$) in *Nlrp3*$^{-R258W}$ mice was also evident, although these cells were not as abundant as macrophages or neutrophils (Fig. 6B, 6E). The distribution of

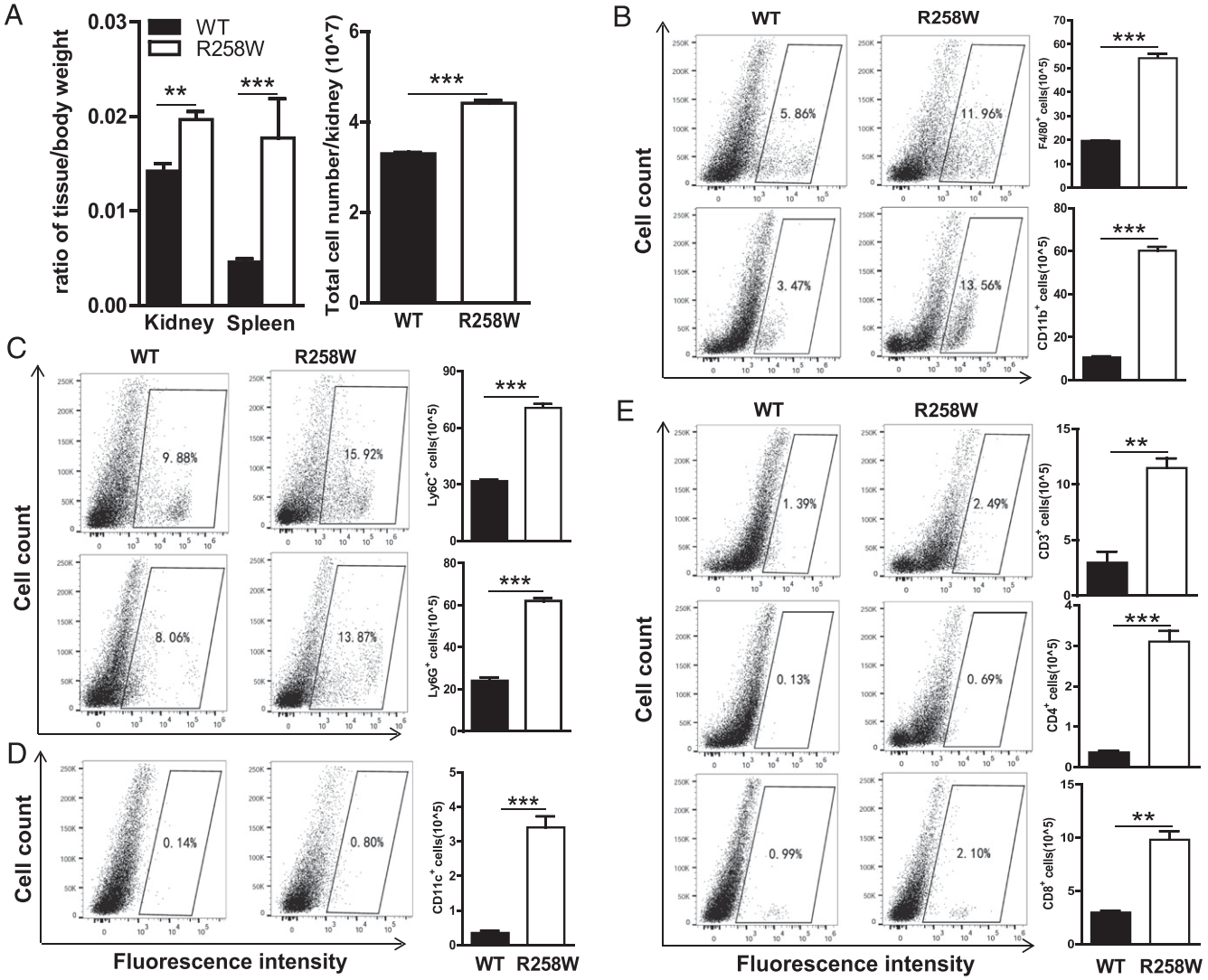

FIGURE 6. Renal infiltration of inflammatory cells was enhanced in *Nlrp3*$^{-R258W}$ mice with pristane-induced SLE. (**A**) The relative weight of spleen and kidney by body weight was monitored in pristine-challenged mice, and total cell number in the kidney was counted. (**B**–**E**) Flow cytometry analysis of kidney cells isolated from WT and *Nlrp3*$^{-R258W}$ mice after staining with the indicated Abs. The percentages of certain cell populations are depicted in the dot plots; total cell numbers are shown as bar graphs. A total of five to eight mice was used in each group. Data are mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

B cells was not clearly different in these two groups of mice (data not shown). These results suggested that changes in cytokines and chemokines influenced leukocyte infiltration into the kidney, which precedes proteinuria and renal damage in *Nlrp3*$^{-R258W}$ mice.

Abolishing R258W expression in myeloid cells rescued pristane-induced lupus in Nlrp3^{-R258W} *mutant mice*

Because myeloid cells, such as macrophages and neutrophils, are the major cell types wherein NLRP3 expresses and functions, we tested whether the lupus phenotype mentioned above was due to hyperinflammasome activation in *Nlrp3*$^{-R258W}$ mice in such cell populations. Taking advantage of our gene-targeted mice carrying the *Nlrp3*$^{-R258W}$ mutation, in which the R258W mutant can be deleted through breeding with cre recombinase–expressing mice (17, 57), we removed the *Nlrp3*$^{-R258W}$ mutation from myeloid cells via crossing with lysozyme cre mice (58). Because *Nlrp3*$^{-R258W}$ mice are heterozygous, removal of R258W from myeloid cells in these mice led to a *Nlrp3*$^{-/+}$ genotype in macrophages and neutrophils, but the *Nlrp3*$^{-R258W}$ mutation was retained in other cells. Of note, the *Nlrp3*$^{-R258W}$ mutant crossed with LysM cre mice were all protected from lupus-induced lethality or tissue damage, although serum anti-dsDNA and total IgG levels did not change significantly (Fig. 7A, 7B). H&E and PAS staining showed that the size and morphology of the glomeruli in *Nlrp3*$^{-R258W}$-LysM cre mice were similar to those in WT mice, with no clear hypercellularity, mesangial expansion, crescent formation, or interstitial mononuclear cell infiltration, as seen in *Nlrp3*$^{-R258W}$ mice (Figs. 2C, 3A, 7C, 7D).

Removal of R258W expression from myeloid cells attenuated the generation of proinflammatory mediators in Nlrp3^{-R258W} *mice upon pristane challenge*

In addition, we assayed the expression levels of proinflammatory cytokines and chemokines in *Nlrp3*$^{-R258W}$ mutant or *Nlrp3*$^{-R258W}$-LysM cre mice. As expected, most of these proinflammatory mediators, including IL-1β, IL-17A, and IL-6, were decreased in the kidney of *Nlrp3*$^{-R258W}$-LysM cre mice compared with *Nlrp3*$^{-R258W}$ mice. However, the level of IFN-γ was increased, whereas IL-12 was decreased. Other cytokines, such as IL-22, IL-23p19 (data not shown), IL-18, IL-21, IFN-α, and IFN-β, remained unchanged (Fig. 8A). Consistent with the change in proinflammatory mediators, the expression of some chemokines, such as CCL2, CCL3, CCL5,

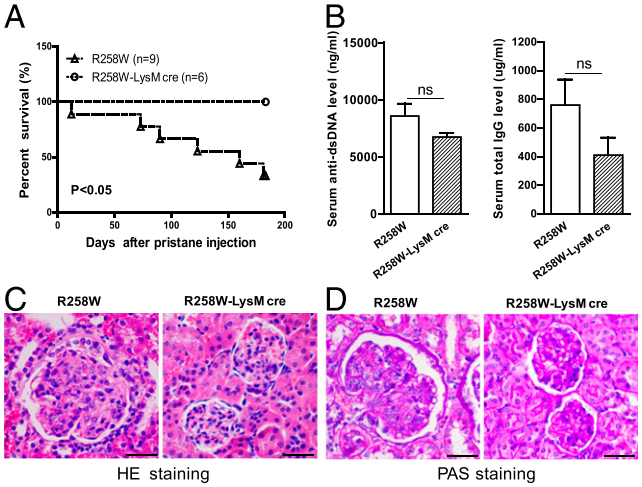

FIGURE 7. Removal of the *Nlrp3*$^{-R258W}$ mutation from myeloid cells rescued mice from pristane-induced SLE development. R258W-LysM cre mice and littermate R258W mice were analyzed after pristane challenge. (**A**) Survival was recorded, and the data were analyzed using the Gehan–Breslow–Wilcoxon test. Data are mean ± 95% confidence interval. (**B**) Disease parameters, including anti-dsDNA Ab and total IgG levels, were analyzed from serum samples of indicated mice. Three to eight mice were used in each group. Data are mean ± SEM. Kidney damage was analyzed by H&E (**C**) and PAS (**D**) staining. Representative images of three mice are shown for each genotype. Scale bars, 10 μm. ns, not significant.

CCL20, and CXCR3, was markedly downregulated in the kidney of *Nlrp3*$^{-R258W}$-LysM cre mice compared with *Nlrp3*$^{-R258W}$ mice (Fig. 8B). The B cell–activating marker BAFF was also downregulated, but APRIL was not (Fig. 8C). Meanwhile, the proinflammatory cytokine TNF-α and the inflammasome gene NLRP3 were also downregulated in the kidney of *Nlrp3*$^{-R258W}$-LysM cre mice, whereas no significant difference in the expression of TGF-β was found (Fig. 8C). Taken together, these data suggested that the *Nlrp3*$^{-R258W}$ mutation in myeloid cells played a critical role in the pathogenesis of SLE.

Discussion

In this study, we found that *Nlrp3*$^{-R258W}$ mice developed a much more severe lupus-like syndrome in the pristane-induced SLE model. Pristane (Tetramethylpentadecane) is a naturally occurring hydrocarbon oil that can induce chronic inflammation when injected into the peritoneal cavity of mouse (59). The pristane-induced lupus mouse model replicates many phenotypic and functional abnormalities of human SLE and proved to be very useful in identifying putative pathogenic mechanisms and environmental triggers of this disease (31). Using the same pristane-induced model, Kahlenberg et al. (23) recently found that caspase-1, the executive protein for inflammasome activation, was essential for the development of autoantibody production and nephritis. Mice deficient for caspase-1 are resistant to lupus-associated vascular damage (23). Although our *Nlrp3*$^{-R258W}$ mice are on the C57BL/6 genetic background, and the caspase-1–deficient mice used by Kahlenberg et al. (23) were on the BALB/c genetic background, both studies implied that the NLRP3 inflammasome has a detrimental role in experimental SLE.

The inflammasome is a cytoplasmic platform responsible for the proteolytic processing and maturation of inflammatory cytokines IL-1β and IL-18 (60). A number of recent studies proposed that the inflammasome machinery is related to the development of lupus (61–65). Increased expression of inflammasome components, including NLRP3 and caspase-1, was reported in lupus nephritis biopsies, suggesting that the kidney may have been primed for inflammasome activation (61). Neutrophil extracellular traps and LL-37–mediated activation of the inflammasome are enhanced in macrophages from lupus patients, resulting in a feed-forward loop that may lead to organ damage (62). Immune complexes formed secondary to Ab recognition of DNA also were shown to stimulate inflammasome activation through upregulation of TLR-dependent activation of NF-κB and subsequent activation of the NLRP3 inflammasome (63, 64). Moreover, AIM2 was also found to be essential for apoptotic DNA-induced SLE (65). The *Nlrp3*$^{-R258W}$ mice used in this study exhibit hyperactivation of the NLRP3 inflammasome, which is associated with autoinflammatory disorders characterized by continuous activation of caspase-1 and excessive production of IL-1β and IL-18 (17). Although there has been no report of SLE-like diseases from NLRP3-related autoinflammation in humans, this possibility cannot be excluded. Chronic inflammation may result in kidney damage when those patients carrying NLRP3 mutations grow older.

The role of NLRP3 in the development of SLE is likely due to caspase-1 activation and excessive production of proinflammatory cytokines, including, but possibly not limited to, IL-1β and IL-18. Indeed, we noticed a strong increase in inflammatory cytokines and chemokines that may have been induced by IL-1β or IL-18 indirectly (Fig. 5), although the protein secretion of IL-1β and IL-18 was hard to detect in serum and kidney tissues (data not shown). Because SLE is a chronic disease, these strong proinflammatory cytokines might have been kept at a low level and, thus, could not be detected by ELISA.

We observed that C3 and IgG were increased significantly in kidney sections from *Nlrp3*$^{-R258W}$ mice 6 mo after pristane challenge, suggesting that the *Nlrp3*$^{-R258W}$ mutation led to more renal deposition of immune complex compared with control mice. Immune complex deposition and complement consumption in organs affected by SLE may have further promoted activation of the inflammasome and, thus, contributed to organ damage (25).

Notably, Sester et al. (30) found that autoimmune NZB mice carry aberrant splicing for the *Nlrp3* gene and highly express p202 (AIM2 antagonist), so that the function of NLRP3 and AIM2 inflammasome is defective. However, it should be kept in mind that NZB mice may carry several more defects, in addition to their deficiencies for NLRP3 and AIM2 inflammasome function. A causal relationship between these abnormalities and SLE in these mice needs further clarification. If a genetic correction of the *Nlrp3* gene and/or suppressing the expression of p202 can effectively change the lupus phenotype of NZB mice, it will clarify the function of inflammasomes in this animal model of SLE. With the rapid developments in genetic engineering based on CRISPR/Cas9 technology, the above-mentioned genetic modification of NZB mice is quite likely to be achieved in the future.

In addition, another study showed that NLRP3 and ASC are suppressive for lupus development in lpr/lpr mice (29). It should be noted that the lpr/lpr mouse used in that study is a SLE mouse model in which *Il1r1*$^{-/-}$ or *Il18*$^{-/-}$ mice did not show any phenotype, whereas *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice exhibited more severe disease (29). This implied that a canonical function of inflammasome was not the mechanism behind the phenotype of *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice; rather, an inflammasome-independent function from NLRP3 or ASC likely accounts for the more severe disease. Indeed, NLRP3 and ASC were reported to function in inflammasome-independent manners in

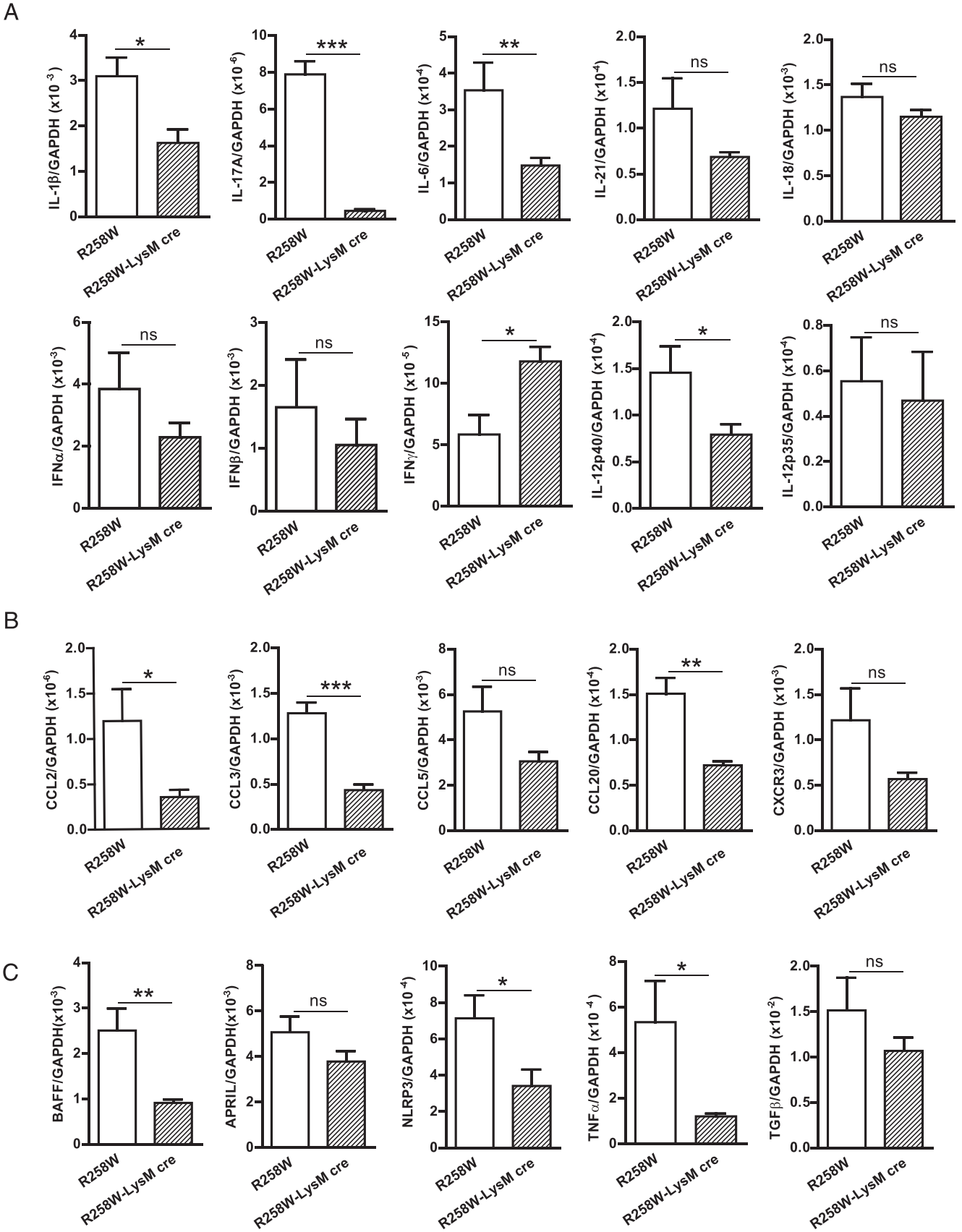

FIGURE 8. Deficiency of the *Nlrp3*^{R258W} mutation in myeloid cells decreased the production of proinflammatory mediators after pristane challenge. (**A** and **B**) Proinflammatory cytokines and chemokines were monitored via real-time PCR from kidney samples obtained after pristane challenge. (**C**) Transcriptional expression of B cell–activating factors and NLRP3 were analyzed as in (A). Data are mean ± SEM from three mice in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

diverse diseases (66–68). Therefore, in a context in which inflammasome is activated, such as in the case of pristane injection, the NLRP3–ASC–caspase-1 signaling cascade plays a detrimental role in SLE. Conversely, in models in which inflammasome is not apparently activated, NLRP3 or ASC may be suppressive for deleterious signals in stromal cells. Thus, a cell-type–specific role for NLRP3 and/or ASC in lupus awaits further investigation.

Importantly, we demonstrated that myeloid cell expression of the R258W mutation was critical for the lupus phenotype of *Nlrp3*$^{-R258W}$ mice. Although expressed in multiple lineages of cells, a cell-type–specific role for NLRP3 is still lacking. Our study demonstrates that the cells in which NLRP3 functions to drive renal inflammation during lupus are primarily myeloid cells, including macrophages, neutrophils, and some dendritic cells. This can be confirmed in the future using conditional NLRP3-knockout mice.

Taken together, our study implies a critical role for NLRP3 inflammasome activity in myeloid cells for the development of SLE. Therefore, regulating the inflammasome signal in such cells should be helpful for the control of inflammatory damage in autoimmune diseases, such as lupus.

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Disclosures

The authors have no financial conflicts of interest.

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