**Original Article**

**A20 overexpression alleviates pristine-induced lupus nephritis by inhibiting the NF-κB and NLRP3 inflammasome activation in macrophages of mice**

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**Abstract:** Background: Lupus nephritis is an autoimmune inflammatory disease and urgently needs effective anti-inflammation therapies. A20, tumor necrosis factor alpha induced protein 3 (TNFAIP3), is a key negative regulator of inflammation, however whether A20 can regulate lupus nephritis has not been clarified. This study aimed at investigating the potential therapeutic effect of A20 on renal inflammation in mouse pristine model of lupus. Methodology/Principal Findings: Female BALB/c mice were intraperitoneally injected with pristine to establish lupus renal injury. The levels of serum IL-1β, IL-6 and autoantibodies and the degrees of renal injury and CCL2 and F4/80 levels were measured. The levels of the NF-κB and NLRP3 inflammasome activation in peritoneal macrophages were determined. We found that injection with pristine increased the levels of serum IL-1β, IL-6, autoantibodies and CCL20 and F4/80 expression in the kidney and induced renal injury, accompanied by enhancing the NF-κB and NLRP3 inflammasome activation in macrophages of mice. In contrast, treatment with Ad-A20, but not with Ad-control, significantly mitigated pristine-induced inflammatory responses and renal injury, and reduced the NF-κB and NLRP3 inflammasome activation in macrophages in mice. Conclusion/Significance: Our data indicated that induction of A20 overexpression inhibited pristane induced lupus inflammation and renal injury in mice and may be a new therapeutic strategy for treatment of lupus nephritis.

**Keywords:** A20, pristine, cytokine, lupus nephritis, adenovirus, macrophages, NF-κB, NLRP3 inflammasome, mice

**Introduction**

Systemic lupus erythematosus (SLE) is an autoimmune disease predominantly affecting females. During the process of SLE, loss of tolerance to nucleic acids and their interacting proteins results in the production of pathogenic autoantibodies that cause inflammation and tissue damage [1]. Although advances in the medical management of infections and renal failure have improved the survival of patients with lupus, the efficacy of available therapeutic strategies for lupus nephritis and SLE is limited. The current broad-spectrum immunosuppressive agents are not always adequate to control clinical symptoms or prevent disease flares. Chronic inflammation in SLE patients can induce aberrant activation of signaling cascades that confer resistance to immunosuppressive drugs and deteriorate tissue damage [2, 3]. Therefore, development of new therapies for control of systemic inflammation is of significance in management of patients with SLE.

Macrophages act as professional antigen presenting cells and are crucial for the development of SLE and lupus nephritis [4]. Macrophages can infiltrate in the kidney and secrete pro-inflammatory cytokines, such as IL-1β and IL-6, and chemokines, such as CCL2, which are important for the development and progression of nephritis [5, 6]. Furthermore, inflammatory stimuli can activate the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) expression, which interacts with apoptosis-associated speck-like protein containing a CARD (ASC) to recruit caspase-1, leading to inflammasome activation [7]. In addition, the enhanced NF-κB signaling and subsequent activation of the NLRP3 promote inflammatory...
cytokine production in macrophages [8]. Hence, down-regulation of NLRP3 inflammasome activation and the NF-κB signaling may be critical for control of macrophage-related inflammatory.

A20, also known as tumor necrosis factor alpha induced protein 3 (TNFAIP3) is an anti-inflammatory factor induced by TNF [9]. A20 can inhibit TNFα-induced cell apoptosis and the NF-κB and IRF activation [10]. Mice deficient for A20 expression spontaneously develop severe inflammation and cachexia, are hypersensitive to both lipopolysaccharide and TNF, and die prematurely [11]. Previous studies have shown that A20 inhibits the pathogenic process of inflammatory diseases, such as myocarditis [12], hepatitis [13], and arthritis [14]. However, the effect of A20 on inflammation during the process of lupus nephritis has not been clarified.

In this study, we generated recombinant adenovirus to induce A20 overexpression and examined the therapeutic effect of A20 overexpression on systemic inflammation, autoantibody responses, renal inflammation and injury in mouse pristine model of lupus nephritis. Furthermore, we explored the potential mechanisms underlying the action of A20 in regulating the NF-κB and NLRP3 inflammasome activation in peritoneal macrophages of mice.

Materials and methods

Mice

Female BALB/c mice at 4 weeks of age were purchased from the Experimental Animal Centre of the Third Military Medical University and housed in a specific pathogen-free facility at 25±2°C and 40-70% relative humidity with a 12-h day/night lighting cycle and free access to standard rodent chew and filtered water. After being acclimated for 2 weeks, the mice were used for experiment. The experimental protocol was proved by the Ethics Committee of the Third Military Medical University.

Preparation of recombinant adenoviruses

TNFAIP3 cDNA was from Oobio (Shanghai, China) and cloned into the plasmid of pADV-MCMV-3FLAG-IRES-EGFP (AddGene, US). After being sequenced, the recombinant or control plasmid was co-transfected with packaging helper plasmids (Oobio) into 293T cells to generate the recombinant Ad-A20 and control Ad-control. The generated recombinant Ad-A20 and control Ad-control were titered.

Generation and treatment of mouse model of SLE

Individual BALB/c mice at 6-8 weeks of age were injected intraperitoneally (i.p) with 0.5 ml pristine (Sigma-Aldrich) or PBS. Three months later, the pristine-injected mice were randomized and injected i.p with 1.0×10⁹ plaque forming units (PFU) of Ad-A20, Ad-control or PBS (100 µl, n=6-8 per group). The PBS-injected mice served as the healthy PBS control group. One month after treatment with Adenovirus, 24-h urinary and blood samples from individual mice were collected for measurement of urinary albumin and preparation of serum sample, respectively. The mice were sacrificed and their peritoneal macrophages were harvested by peritoneal lavage. Furthermore, their kidney tissues were immediately frozen in liquate nitrogen and stored in -80°C or fixed with 10% formalin overnight and paraffin-embedded, respectively.

Preparation of murine peritoneal macrophages

Peritoneal macrophages from healthy and pristine-injected mice were harvested by peritoneal lavage with cold DMEM medium supplemented with 1% penicillin/streptomycin. The cells were washed, and re-suspended in DMEM medium supplemented with 10% FBS (complete medium). Peritoneal macrophages were incubated in complete medium at 37°C 5% CO₂ for 2 h, and after removing non-adherent cells, the adherent cells were cultured in freshDMEM medium as macrophages.

Enzyme-linked immunosorbent assay (ELISA)

The levels of serum IL-1β and IL-6 in individual mice were measured using mouse cytokine kits (Invitrogen/BioSource International, Camarillo, USA). Similarly, the levels of serum IgG, anti-nRNP, and anti-dsDNA antibodies were also measured using specific ELISA kits, according to the manufacturer’s instructions (Alpha Diagnostic, San Antonio, TX). Briefly, serum samples (50 µL each) at 1:4 dilutions were added in triplicate into 96-well plates that had
been coated with specific antibodies and incubated at room temperature for 2 h, and after being washed, the bound antigens were detected with biotinylated antibodies at room temperature for 0.5 h. Subsequently, the bound biotinylated antibodies were detected with horse-radish peroxidase (HRP)-conjugated Streptavidin and chromogen reagent, followed by reading the absorbance at 450 nm in an ELISA reader. The levels of urinary albumin in individual 24-h urinal samples were analyzed using a mouse albumin ELISA quantification kit (Bethyl Laboratories, USA).

**Histological examination of the kidney injury**

Paraffin-embedded renal tissue sections (4 μm) were stained with hematoxylin and eosin (H&E) and examined under a light microscope. The pathologic changes in the kidney tissues were evaluated for the glomerular and tubulointerstitial activity scores of 8 fields of each mouse in a blinded manner using a scale of 0-3, as reported previously [15].

**Immunofluorescence of glomerular IgG and C3**

The frozen kidney sections (5 μm) were treated with 10% FBS and stained with Alexa Fluor 555-conjugated donkey anti-mouse IgG (Beyotime, Jiangsu, China) or FITC-conjugated goat anti-mouse C3 (Cedarlane Lab, Burlington, USA). The sections were examined under a fluorescent microscope and the mean intensity of fluorescent antibody staining in individual sections was evaluated using Image J software and scored for 0-3, as described previously [16].

**Western blot**

Total proteins were extracted from individual kidney samples or peritoneal macrophages from different groups of mice using lysis buffer, according to the manufacturer’s instructions (Beyotime, Jiangsu, China). After being centrifuged and quantified for the protein concentrations, the lysate proteins (50 μg/lane) were separated by NuPAGE on 4-12% Bis-Tris Gel (Life Technology, Grand Island, USA) and transferred onto polyvinylidene fluoride membrane (PVDF, Millipore, USA). The membranes were incubated with 5% fat skimmed dry milk in TBST at room temperature for 1 h and incubated with primary anti-A20, anti-NLRP3, anti-ASC, anti-p-IκB, anti-IκB, anti-F4/80, anti-CCL2 (Abcam, Cambridge, USA), anti-Caspase-1p20, anti-p-NF-κBp65, anti-NF-κBp65, anti-GAPDH and anti-fibrillarin (Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C overnight. After being washed, the bound antibodies were detected with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h and visualized using the enhanced chemiluminescence detection system (Millipore, Minnesota, USA). The relative levels of target protein expression to the internal control were qualified using the Image J analysis software.

**Statistical analysis**

Quantitative data are expressed as mean ± SD. The difference among groups was analyzed by the Mann-Whitney u test or one-way ANOVA when the data were normally distributed using the GraphPad Prism (Version 5.0). A p value of <0.05 was considered statistically significant.

**Results**

**Adenoviral-mediated A20 overexpression in the peritoneal macrophages decreases pro-inflammatory cytokine production in pristine induced lupus mice**

A20 is a key regulator of inflammation. To determine whether A20 could inhibit pro-inflammatory cytokine production in lupus mice, BALB/c mice were injected i.p with pristine to induce lupus and three months later, the mice were treated i.p with Ad-A20 or Ad-control adenovirus. One month after treatment, their peritoneal macrophages were harvested by peritoneal lavage and the relative levels of A20 expression in macrophages were determined by Western blot (Figure 1A). The relative levels of A20 in the macrophages from the pristine-injected without Adv treatment were similar to that in the macrophages from the Ad-control treated mice and were significantly higher than that in the macrophages from the PBS-injected healthy control mice (Figure 1B). However, the levels of A20 expression in the macrophages from the Ad-control treated mice were significantly lower than that in the macrophages from the Ad-A20 treated mice. Hence, injection with pristine up-regulated the A20 expression in macrophages and injection with Ad-A20 induced A20 overexpression in peritoneal macrophages of mice.
A20 overexpression alleviates pristine-induced lupus nephritis

Pro-inflammatory cytokines play indispensable roles in the development of SLE. We tested the serum levels of IL-1β and IL-6 in the different groups of mice by ELISA. We detected similar higher levels of serum IL-1β and IL-6 in the pristine and Ad-control treated mice (Figure 1C and 1D), indicating that injection with pristine induced systemic inflammation, which was not affected by treatment with Ad-control in mice. In contrast, the levels of serum IL-1β and IL-6 in the mice treated with Ad-A20 were significantly lower than that in the Ad-control injected mice although they remained significantly higher than that in the PBS healthy controls. Therefore, induction of A20 overexpression inhibited pristine-induced systemic pro-inflammatory cytokine responses and inflammation in mice.

Induction of A20 overexpression mitigates pristine-induced humoral responses in mice

Autoantibodies, such as anti-dsDNA and anti-nRNP, are biomarkers for diagnosis of SLE and crucial for the pathogenesis of lupus [17]. To evaluate the impact of A20 overexpression on the pathogenesis of lupus, the levels of serum anti-dsDNA and anti-nRNP in individual mice were measured by ELISA. As shown in Figure 2A, levels of serum anti-dsDNA were detected...
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Induction of A20 overexpression inhibits lupus-related renal injury in mice

To determine the therapeutic effect of A20 overexpression on lupus-related renal injury in mice, the kidney sections from individual groups of mice were stained with H&E and immunofluorescence. We observed mesangial-endocapillary proliferation and increased thickness of mesangial membranes in many glomeruli of the Ad-control treated mice (Figure 3A-C). However, there were obviously less frequent glomeruli with these pathogenic changes in the Ad-A20 treated mice. Semi-quantitative analysis revealed that the glomerular and tubulointerstitial activity scores in the Ad-A20 group of mice were only slightly higher than that in the PBS healthy controls, but were significantly lower than that in the Ad-control treated mice (Figure 3D). Quantitative measurements of 24-h urinary albumin indicated that the levels of urinary albumin in the pristine and Ad-control treated groups of mice were indistinguishable and significantly higher than that in the Ad-A20 treated and PBS healthy groups of mice (Figure 3E). Together, these data indicated induction of A20 overexpression inhibited lupus-related renal injury in mice.

Induction of A20 overexpression inhibits CCL2 expression and macrophage infiltration in the kidneys of mice

During the process of lupus nephritis, activated macrophages migrate into the glomeruli and secrete a number of inflammatory cytokines and chemokines. Chemokines, such as CCL2, are crucial for recruiting activated macrophages and have been associated with the progression of lupus nephritis [16, 17]. To determine the impact of A20 overexpression on macrophage infiltration, we characterized the relative levels of CCL2 and F4/80 expression in the kidneys of different groups of mice by Western blot (Figure 4). While several-fold increased levels of CCL2 and F4/80 expression were detected in the kidneys of the pristine and Ad-control injected mice they were significantly reduced in

Figure 2. Induction of A20 overexpression reduces serum levels of anti-dsDNA and anti-nRNP IgG in lupus mice. The levels of serum anti-dsDNA and anti-nRNP IgG in individual mice were examined by ELISA. Data are expressed as individual values and mean of each group of mice (n=6-8 per group) from three separate experiments. A: The levels of anti-dsDNA IgG. B: The levels of anti-nRNP IgG. Data were analyzed by Mann-Whitney U test. The horizontal lines show the means. NS: Not significant; *p<0.05; **p<0.01; ***p<0.001.
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The kidneys of the Ad-A20 treated mice. Therefore, induction of A20 overexpression significantly reduced lupus-related macrophage infiltration in the kidneys of mice.

Figure 3. Induction of A20 overexpression mitigates pristine-induced renal injury and improves the kidney function in mice. The kidney tissue sections were stained with H&E and four months after pristine injection, the cumulative glomerular activity score (GAS) and tubulointerstitial activity score (TIAS) in individual mice were evaluated. The degrees of immunoglobulin and complement deposition in the glomeruli of individual mice were examined by immunohistochemistry. Furthermore, the levels of 24-h urinary albumin in individual mice were measured by ELISA. Data are representative images (magnification×200) or expressed as the means ± SD of each group (n=6-8) of mice from three separate experiments. A: H&E staining of the glomeruli of mice. B, C: Immunohistochemistry analysis of immunoglobulin and complement deposition on the glomeruli of mice. D: The GAS and TIAS. E: The levels of albuminuria. *p<0.05, determined by one way ANOVA; ****p<0.0001 vs. the PBS group or the Ad-control group, determined by Mann-Whitney u test.
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A20 overexpression inhibits the NF-κB activation and reduces the NLRP3 inflammasome in peritoneal macrophages of lupus mice

Finally, we tested the impact of A20 overexpression on the NF-κB and NLRP3 inflamma-
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Figure 6. A20 inhibits the NLRP3 inflammasome activation in macrophages from lupus mice. The relative levels of NLRP3, ASC, caspase-1p20 expression in macrophages from different groups of mice were determined by Western blot. Data are representative images or expressed as the means ± SD of each group (n=6-8) of mice from three separate experiments. A: Western blot analysis. B: Quantitative analysis. ***p<0.001, ****p<0.0001, vs. the PBS group; **p<0.01, ###p<0.001, vs. the Ad-control group.

to the healthy control (Figure 5). In contrast, the relative levels of IκB and NF-κBp65 phosphorylation in macrophages from the Ad-A20 treated mice were significantly reduced, as compared with that in the Ad-control mice. These indicated that A20 overexpression inhibited the NF-κB activation in peritoneal macrophages of lupus mice. A similar pattern of the relative levels of NLRP3, ASC, and Caspase-1p20 expression was detected in the macrophages from the different groups of mice (Figure 6), clearly demonstrating that A20 overexpression reduced the NLRP3 inflammasome activation in peritoneal macrophages of lupus mice.

Discussion

The present study was designed to investigate the impact of A20 overexpression on pristine-induced lupus in mice. First, we generated recombinant Ad-A20 and Ad-control and we found that intraperitoneal treatment significantly up-regulated A20 expression in peritoneal macrophages. We found that treatment with Ad-A20, but not Ad-control, significantly ameliorated the severity of pristine-induced renal nephritis in mice. Evidently, treatment with Ad-A20 significantly decreased the levels of serum IL-1β, IL-6 and autoantibodies in lupus mice. Furthermore, treatment with Ad-A20 significantly reduced the GAS and TIAS and immune complex deposition in the glomeruli and improved albuminuria in lupus mice. In addition, treatment with Ad-A20 significantly reduced the relative levels of F4/80 and CCL2 expression in the kidneys of lupus mice. Given that F4/80 is a reliable biomarker for macrophages the reduced levels of F4/80 in the kidney tissues suggest that induction of A20 overexpression may inhibit macrophage infiltration into the inflammatory kidneys of lupus mice. The reduced macrophage infiltration may stem from decreased levels of CCL2, which is a potent chemotactic factor for macrophage migration. To the best of our knowledge, these data may provide the first experimental evidence that A20 inhibits the development of pristine-induced lupus in mice.

Recent gene targeting studies have clearly demonstrated that A20 plays an important role in multiple cell types, including myeloid cells, dendritic cells, B cells, keratinocytes, and intestinal epithelial cells (IECs). These have provided insights into how genetic variants in the A20/TNFAIP3 gene may predispose to various inflammatory and autoimmune pathologies [10]. Genome-wide association studies have revealed that three independent SNPs in human A20 region are associated with SLE, including one coding and two noncoding polymorphisms [18]. Therefore, A20 may serve as both a predictive and prognostic biomarker and induction of A20 overexpression may be a promising strategy for intervention of SLE.
In our experiment, we found that injection with pristine induced moderate levels of A20 expression in peritoneal macrophages and promoted aberrant activation of the NF-κB signaling in lupus mice. The induced A20 expression by pristine injection may be feedback regulated by the upregulated NF-κB activity because the NF-κB can bind to the κB sequence binding element in the A20 gene promoter to induce A20 expression [19]. Because the pristine-injected mice developed severe inflammation and lupus nephritis, the moderately upregulated A20 expression may be insufficient in inhibiting inflammation and renal injury in mice. On the other hands, induction of A20 overexpression by injection with Ad-A20 significantly mitigated pristine-induced nephritis, accompanied attenuating the NF-κB signaling in mice. Therefore, therapeutic strategy using A20 should consider high levels of A20 expression in order to achieve therapeutic efficacy.

The NF-κB signaling is crucial for the development of both organ-specific and systemic autoimmunity and renal tubulointerstitial injury [20, 21]. Inhibition of the NF-κB activation by pyrrolidinedithiocarbamate (PDTC) [22] or by gene transfer of truncated IκB-α [23] can attenuate the kidney injury. Although activation of the NF-κB signaling induces A20 expression [24], the NF-κB signaling can be downregulated by A20 [25]. Indeed, A20 deficient mice spontaneously develop severe inflammation and become premature death [26]. Induction of A20 overexpression dramatically inhibits the NF-κB activation in several inflammatory diseases, including bronchial asthma, rheumatoid arthritis, atherosclerosis, myocarditis and hepatitis [27-30]. Consistently, our results showed that Ad-A20 treatment significantly inhibited the NF-κB activation in macrophages from pristine-induced lupus mice, accompanied by reduced serum levels of pro-inflammatory cytokines IL-6 and IL-1β. These data suggest that the therapeutic effect of Ad-A20 treatment may be partly due to the inhibition of the NF-κB activation in macrophages.

Previous studies have shown that the NLRP3/ASC/Caspase-1 signal pathway is critical for inflammation and immunity [31, 32]. Recent evidence suggests that immune complexes can activate the NLRP3 inflammasomes in macrophages of SLE mice and enhances pro-inflammatory production by macrophages [8]. Indeed, the NLRP3 inflammasome is hyperactivated in macrophages of both male and female SLE patients [31]. Inhibition of the NLRP3/ASC/caspase-1 activation can reduce significantly the severity of nephritis and the levels of circulating anti-dsDNA antibodies [32]. A previous study has shown that A20 is a negative regulator of the NLRP3 inflammasome activation, and protects against arthritis [33]. We found that injection with pristine significantly up-regulated the NLRP3 inflammasome activation in macrophages of lupus mice while treatment with Ad-A20 significantly mitigated pristine-induced NLRP3 inflammasome activation in macrophages as well as reduced the levels of serum IL-1β in lupus mice. These outcomes likely stem from that A20 restricts ubiquitination of pro-IL-1β protein complexes and suppresses the NLRP3 inflammasome activity [34]. The inhibition of the NLRP3 inflammasome activation can further inhibit the NF-κB activation and pro-inflammatory cytokine production, contributing to inhibition of nephritis and renal injury in mice.

In this study, we found that induction of A20 overexpression not only reduced the levels of serum IL-1β and IL-6, but also reduced the levels of serum autoantibodies and immune complex deposition in the glomeruli of lupus mice. The reduced levels of serum autoantibodies may be because induction of A20 overexpression in antigen-specific B cells inhibits B cell activation and differentiation in mice. Alternatively, A20 overexpression may also inhibit antigen presenting activity of macrophages and others, resulting in attenuated B cell activation in mice. Because immune complex deposition in the glomeruli can induce CCL2 expression, which can recruit macrophages and other inflammatory cells into the kidney, the reduced antibody production and immune complex deposition in the glomeruli by A20 overexpression may attenuate CCL2 expression and macrophage infiltration in the kidney of mice. We are interested in further investigating the molecular mechanisms underlying the therapeutic effect of A20 overexpression in pristine-related lupus mice.

In conclusion, our data indicated that A20 overexpression significantly mitigated pristine-induced systemic inflammation and renal injury in mice. Our data suggest that the therapeutic effect of A20 may be associated with inhibition
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of the NLRP3 inflammasome and NF-κB activation in macrophages. Because of dual inhibition of A20 on the NLRP3 inflammasome and NF-κB activation, A20 may be a promising new candidate for the treatment of SLE.

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Disclosure of conflict of interest

None.

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