

# Sublytic C5b-9 Complexes Induce Apoptosis of Glomerular Mesangial Cells in Rats with Thy-1 Nephritis through Role of Interferon Regulatory Factor-1-dependent Caspase 8 Activation<sup>\*[5]</sup>

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**Background:** The mechanism of sublytic C5b-9-mediated glomerular mesangial cell (GMC) apoptosis in rat Thy-1 nephritis remains unclear.

**Results:** GMC apoptosis was induced by sublytic C5b-9 via the interferon regulatory factor-1 (IRF-1)/caspase 8-dependent pathway, in which IRF-1 up-regulated caspase 8 activity.

**Conclusion:** Sublytic C5b-9 could promote GMC apoptosis in Thy-1 nephritis through IRF-1-activation of caspase 8.

**Significance:** IRF-1 might provide a potential target for human mesangioproliferative glomerulonephritis treatment.

The apoptosis of glomerular mesangial cells (GMC) in rat Thy-1 nephritis (Thy-1N), a model of human mesangioproliferative glomerulonephritis, is accompanied by sublytic C5b-9 deposition, but the mechanism of sublytic C5b-9-mediated GMC apoptosis has not been elucidated. In the present study, the gene expression profiles both in the GMC stimulated by sublytic C5b-9 and the rat renal tissue of Thy-1N were detected using microarrays. Among the co-up-regulated genes, the up-regulation of interferon regulatory factor-1 (*IRF-1*) was further confirmed. Increased caspase 8 and caspase 3 expression and caspase 8 promoter activity in the GMC were also identified. Meanwhile, overexpression or knockdown of *IRF-1* not only enhanced or inhibited GMC apoptosis and caspase 8 and 3 induction but also increased or decreased caspase 8 promoter activity, respectively. The element of *IRF-1* binding to the caspase 8 promoter was first revealed. Furthermore, silencing *IRF-1* or repressing the activation of caspases 8 and 3 significantly reduced GMC apoptosis, including other pathologic changes of Thy-1N. These novel findings indicate that GMC apoptosis of Thy-1N is associated with the *IRF-1*-activated caspase 8 pathway.

Human mesangioproliferative glomerulonephritis (MsPGN)<sup>3</sup> is a disease characterized by apoptosis and proliferation of glo-

merular mesangial cells (GMC). Several studies have revealed that complement, especially C5b-9 complexes, could deposit in the glomeruli of patients with MsPGN (1, 2). However, the mechanism by which C5b-9 causes GMC injuries remains to be further defined (3). Rat Thy-1 nephritis (Thy-1N) is an established animal model for studying MsPGN (4–6). It has been clear that Thy-1 antibody injected into rats can bind to Thy-1 antigen on GMC and then activate complement (7, 8). The consequences of complement activation result in C3a, C5a, and C5b-9 generation. C3a and C5a are regarded as mediators involved in inflammatory cells, and C5b-9 is involved in causing cell injury and proteinuria (9).

During the process of Thy-1N induction, GMC undergo apoptosis or proliferation (4, 10). The GMC apoptosis in the early phase is considered to be a contributor to the initiation of nephritis (4, 11). Previous studies have confirmed that GMC damage in Thy-1N is complement-dependent but neutrophil-independent (9). The effects of C5b-9 on nucleated cells appear to be lytic or sublytic (12, 13), and sublytic C5b-9 can induce cell apoptosis and other injuries (14–16). Nevertheless, the mechanism of GMC apoptosis mediated by sublytic C5b-9 is not understood.

As we know, cell apoptosis is associated with the expression of apoptosis-related genes (11, 17–21). In the present study, we first employed microarray analysis to identify the gene expression profiles in cultured GMC incubated with sublytic C5b-9 (*in vitro*). These profiles were then compared with the gene expression profiles in the renal tissue of Thy-1N rats (*in vivo*). Among the characterized genes, we further focused on exploring the role of interferon regulatory factor-1 (*IRF-1*), including its effects on the caspase pathway because *IRF-1* was a cell apoptosis-related gene in other reports (22–24).

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[5] This article contains supplemental Table 1 and Figs. 1–4.

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<sup>3</sup> The abbreviations used are: MsPGN, mesangioproliferative glomerulonephritis; Ab, antibody; C6DS, C6-deficient serum; GMC, glomerular mesangial cell(s); HIS, heat-inactivated serum; IRF, interferon regulatory factor; MEM, modified Eagle's medium; NS, normal serum; pIRF-1,

pcDNA3.1/IRF-1; Thy-1N, Thy-1 nephritis; Z, benzyloxycarbonyl; FMK, fluoromethyl ketone; nt, nucleotides; SD, Sprague Dawley; FAM, carboxyfluorescein; TRMRA, tetramethylrhodamine.

## EXPERIMENTAL PROCEDURES

**Reagents and Animals**—A monoclonal antibody against IRF-1 was purchased from BD Biosciences. Polyclonal antibodies against procaspase/cleaved caspase 9, procaspase/cleaved caspase 8, procaspase/cleaved caspase 3, and a Western blot detection system were purchased from Cell Signaling Technology (Beverly, MA). The chromatin immunoprecipitation (ChIP) assay kit was from Millipore (Bedford, MA). The specific inhibitors of caspase 8 (Z-IETD-FMK) and caspase 3 (Z-DEVD-FMK) were purchased from R&D Systems (Minneapolis, MN). pRL-SV40 luciferase reporter was provided by Promega (Madison, WI). pGL3-basic vector and a 1.7-kb rat IRF-1 promoter were provided by Dr. Fangming Tang (University of Chicago) and Dr. Li-yuan Yu-Lee (Baylor College of Medicine, Houston, TX). SD rats were from B&K Universal Ltd. (Shanghai, China). Institutional approval for the animal study protocol was obtained. The rat GMC strain was provided by the China Center for Type Culture Collection (Wuhan, China). Normal serum (NS) from 10 healthy adult donors, and heat-inactivated serum (HIS) was obtained by incubating NS at 56 °C for 30 min. Human complement C6-deficient serum (C6DS) was obtained from Sigma. Rabbit polyclonal antibody against Thy-1 antigen of rat thymocyte (anti-Thy-1 Ab) was prepared according to previously published procedures (6, 7).

**IRF-1 Plasmid Construction**—The expression plasmid of pcDNA3.1/IRF-1 (pIRF-1) was constructed by inserting the complete ORF of rat *IRF-1* cDNA into the expression vector (pcDNA3.1). In brief, the first strand cDNA was synthesized from total RNA of cultured GMC using M-MLV-RT (14). The *IRF-1* gene was amplified by PCR. Specific primer sequences were as follows: forward primer, 5'-GCGCGGATCCATGCCTATCACTCGGATGCGAATG-3'; reverse primer, 5'-GCGCAAGCTTTGGTGCACAAGGAATAGCCTGA-3'.

**IRF-1 shRNA Vector Generation**—Three classes of different shRNA sequences were designed to be homologous to the *Rattus norvegicus IRF-1*, complete CDS (GenBank<sup>TM</sup> accession number NM\_012591.1). The complementary oligonucleotides encoded a hairpin structure with a 21-mer stem derived from the target site. The vectors of shIRF-1-expressing plasmid were constructed using pGCSi as the vector backbone, whereas a scrambled gene shRNA (control shRNA) was used as a negative control (14, 15).

**Caspase 8 Promoter Luciferase Reporter Construction**—The luciferase reporter was constructed by inserting the 1.24-kb caspase 8 promoter (nt -1136 to +101) into pGL3-basic vector. This construct contains nucleotides 57, 417, 453–57, 418, and 689 from the reference genomic sequence NC\_005108.2 and corresponds to nucleotides -1136/+101 of the rat caspase 8 gene. The 1237-bp promoter of the rat caspase 8 gene was amplified by PCR: forward primer, 5'-GCGCGGTACCCCTTGGCTTGTGTCA-3'; reverse primer, 5'-GCGCAAGCTTTCTTCTGCGGGATGT-3' (the restriction enzyme cutting site is underlined). To determine the minimal caspase 8 promoter sequence required for constitutive and inducible activity, we constructed the following promoter deletion fragments by PCR and cloned them into the same reporter vector: nt -936 to +101, -736 to +101, -336 to +101, and -136 to +101. Spe-

cific primers for different promoter deletion fragments were as follows: 1) nt -936 to +101 of rat caspase 8 gene (forward primer, 5'-GCGCGGTACCAGTGGACAGGTCTGTG-3'; reverse primer, 5'-GCGCAAGCTTTTCTTCTGCGGGATGT-3'); 2) nt -736 to +101 of rat caspase 8 gene (forward primer, 5'-GCGCGGTACCGACTCTATAAGCTATA-3'; reverse primer, 5'-GCGCAAGCTTTTCTTCTGCGGGATGT-3'); 3) nt -336 to +101 of caspase 8 gene (forward primer, 5'-GCGCGGTACCCACTTTTCTTAAAGA-3'; reverse primer, 5'-GCGCAAGCTTTTCTTCTGCGGGATGT-3'); 4) nt -136 to +101 of the rat caspase 8 gene (forward primer, 5'-GCGCGGTACCTCTAAAGTGTCTGGAA-3'; reverse primer, 5'-GCGCAAGCTTTTCTTCTGCGGGATGT-3').

**GMC Culture and Sublytic C5b-9 Determination**—To ensure that C5b-9 attack was insufficient to lead to cell lysis, lactate dehydrogenase was detected in the supernatants of cultured GMC using a lactate dehydrogenase assay kit, and less than 5% lactate dehydrogenase release from cells was regarded as a sublytic effect (14, 15). Based on a checkerboard titration test, 5% Thy-1 Ab and 4% NS were used to form sublytic C5b-9 complexes (14). To ascertain that the effects on GMC were due to formation and attack of sublytic C5b-9, GMC were also treated with anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM as control groups (14, 15).

**GMC Transfection and Identification**—Transient transfection of pcDNA3.1/IRF-1 (pIRF-1) or IRF-1 shRNA expression plasmid into cultured GMC was conducted with GenEscort<sup>TM</sup> III. The IRF-1 protein expression was assessed by Western blot (supplemental Fig. 1A), and the efficiency of shRNA transfection was examined by green fluorescence protein (GFP) expression in supplemental Fig. 1B. As for IRF-1 shRNA, the three classes of shRNA to *IRF-1*, different target sites were designed and transfected into GMC to repress the target gene. The shRNA-2 (caGCTCTACTCTGCCTGATGATTCAAGAGATCATCAGGCAGAGTAGAGctg) that could effectively silence IRF-1 expression (supplemental Fig. 1C) was chosen.

**Thy-1N Model Reproduction and Experimental Design**—Normal male SD rats (160–200 g) were randomly divided into two groups ( $n = 8$ ): 1) Thy-1N group (rats were given anti-Thy-1 Ab (0.75 ml/100 g body weight) by a single intravenous injection; 2) NS group (rats were injected intravenously with normal rabbit serum (0.75 ml/100 g body weight)). Samples of renal cortexes were obtained at 0 min, 20 min, 40 min, 80 min, 3 h, 6 h, and 12 h after the administration of serum by biopsies or sacrifice (6). Some samples obtained at fixed times were first examined using microarray, real-time PCR, and Western blot for the expressions of IRF-1 and caspases 9, 8, and 3.

In order to find the most optimal time of IRF-1 shRNA transfer, the plasmids were transferred into rat kidneys via renal artery perfusion for 24, 48, 72, or 96 h, followed by anti-Thy-1 Ab injection. The efficiency of transferring the plasmids into glomeruli was determined by observing GFP expression (supplemental Fig. 1D). Besides, the IRF-1 expression in the renal tissue was examined using Western blot, and the result showed that IRF-1 shRNA pretreatment for 72 h before giving anti-Thy-1 Ab could markedly silence IRF-1 expression induced by anti-Thy-1 Ab injection for 3 h (supplemental Fig. 1E).

## IRF-1 Role in GMC Apoptosis Mediated by Sublytic C5b-9

To examine the roles of IRF-1 or caspases in mediating GMC apoptosis of Thy-1N rats, other normal male SD rats (160–200 g) were divided into four groups ( $n = 5$ ), namely 1) NS, 2) Thy-1N, 3) control shRNA + Thy-1N, and 4) IRF-1 shRNA + Thy-1N, and the rats in the NS and Thy-1N groups were injected with the corresponding sera as mentioned above, but the rats allocated to control shRNA + Thy-1N and IRF-1 shRNA + Thy-1N were treated by the same method described previously (14). Furthermore, other normal SD rats (160–200 g) were again divided into six groups ( $n = 5$ ): 1) control shRNA + Thy-1N; 2) IRF-1 shRNA + Thy-1N; 3) Z-IETD-FMK (caspase 8 inhibitor) + Thy-1N; 4) Z-DEVD-FMK (caspase 3 inhibitor) + Thy-1N; 5) DMSO + Thy-1N; and 6) NS. The rats allocated to Z-IETD-FMK + Thy-1N and Z-DEVD-FMK + Thy-1N were given the caspase inhibitors at a final concentration of 1  $\mu\text{M}$  blood volume by intravenous injection and then treated with anti-Thy Ab (0.75 ml/100 g body weight). The renal cortices of rats were collected at 3 h and day 7 by biopsies or sacrifice, the samples were embedded in paraffin or Epon 812, and the GMC changes were determined by TUNEL, electron microscopy, and light microscopy.

**Microarray Analysis**—Total RNA was extracted from GMC (6 wells) incubated with sublytic C5b-9 or renal cortices of Thy-1N rats (6 rats) for 40 min and 3 h using an RNeasy minicolumn (11). Biotinlabeled cRNA prepared from template cDNA was fragmented and hybridized to Affymetrix RAE230A arrays (done in triplicate). The arrays were biotin-labeled prior to scanning with a confocal scanner. Only those genes with a  $p$  value of  $\leq 0.01$  were included in the subsequent analysis. The expression data for each time point were compared with control, and a signal log ratio of 1 or greater was taken to identify genes with significantly different regulation.

**Real-time PCR**—The total RNA from cultured GMC or renal tissues was extracted, and IRF-1 mRNA was measured using real-time PCR. 1) To detect IRF-1, a fluorescence-labeled probe was used (forward primer, 5'-GTACAACTTGCAGGTGTCGC-3'; reverse primer, 5'-GCTGCCACTCAGACTGTTCA-3') as well as a FAM/TAMRA-labeled probe, 5'-CCTCCACCTCTGAAGCTGCAACA-3'). 2)  $\beta$ -Actin was quantified using forward primer (5'-TCACCCACACTGTGCCCATCTATGA-3'), reverse primer (5'-CATCGGAACCGCTCATTGCCGATAG-3'), and FAM/TAMRA-labeled probe (5'-ACGCGCTC-CCCCATGCCATCCTGCGT-3'). -Fold change was calculated with the relative  $C_T$  method as 2 raised to (normalized  $C_T$  in control sample - normalized  $C_T$  in stimulated sample).

**Western Blot**—The lysates of cultured GMC or extracts of renal cortices were prepared. Equal amounts of protein were subjected to electrophoresis on 12% SDS-PAGE, and the electroblotted membranes were then incubated with the primary antibodies to IRF-1, procaspase/cleaved caspase 9, procaspase/cleaved caspase 8, and procaspase/cleaved caspase 3, followed by incubation with horseradish peroxidase-conjugated secondary antibodies.

**Renal Histological Examination**—Paraffin sections were stained with H&E. Numbers of glomerular cells were quantified from counts of positive-stained nuclei and performed in a double-blinded manner by two independent observers counting under light microscopy. Ultrastructural changes were examined by transmission electron microscopy (14).

**TUNEL Analysis**—Tissue sections were treated with proteinase K and incubated with a 50- $\mu\text{l}$  reaction mixture of terminal deoxynucleotidyltransferase for 60 min. The number of TUNEL-positive nuclei in 100 glomerular cross-sections was counted in a double-blinded manner under fluorescence microscopy (14).

**Flow Cytometry**— $5 \times 10^5$  GMC were resuspended in binding buffer containing Annexin V-APC and propidium iodide. The samples were analyzed on a FACScan flow cytometer. The percentage of apoptotic cells in a 10,000-cell cohort was determined by flow cytometry.

**Luciferase Reporter Assay**—To analyze IRF-1 and caspase 8 promoter activity,  $2 \times 10^5$  GMC were transfected with the rat 1.7-kb IRF-1 promoter or the rat 1.24-kb caspase 8 promoter (nt -1136 to +101) accompanied by pRL-SV40 as a transfection efficiency control. After 48 h, the cells were treated with sublytic C5b-9, anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM for 40 min and 3 h. The luciferase activity was assayed in a TD20/20 luminometer. Moreover, part of the cells transfected with full-length caspase 8 promoter (nt -1136 to +101) or different promoter deletion fragments (nt -936 to +101, -736 to +101, -336 to +101, or -136 to +101) for 48 h was attacked by sublytic C5b-9 for another 3 h, and then the luciferase activity was detected.

In order to further assess the effects of IRF-1 overexpression or knockdown on caspase 8 promoter activity, GMC were first co-transfected with IRF-1 expression plasmids (pIRF-1) or IRF-1 shRNA and caspase 8 promoter plasmids for 48 h. The cells were subsequently divided into six groups, namely sublytic C5b-9, IRF-1 shRNA + sublytic C5b-9, control shRNA + sublytic C5b-9, MEM, pIRF-1, and pcDNA3.1, and the luciferase activity of each group was measured. Additionally, to further identify which caspase 8 promoter deletion fragments can be activated by IRF-1 overexpression, GMC were co-transfected with pIRF-1 and caspase 8 promoter plasmids of full-length or deletion fragments for 48 h, and luciferase activity was also examined.

**Chromatin immunoprecipitation (ChIP)**—DNA-binding proteins were cross-linked to DNA and lysed in SDS lysis buffer. DNA was sheared to 200–500-bp fragments by sonication for 30 s. The chromatin solution was precleared with salmon DNA/protein A-agarose 50% slurry for 30 min, and the supernatant was incubated with anti-IRF-1 antibody overnight. A proximal region in the caspase 8 promoter (nt -336 to -136) was amplified from the immunoprecipitated chromatin by PCR using four pairs of primers: primer 1 (sense, 5'-CTGTGACC-AGTGGCTTTC-3'; antisense, 5'-CCAGACACTTTAGAG-GGA-3') (nt -235 to -122); primer 2 (sense, 5'-CCAGGAG-GTGACTCTGTG-3'; antisense, 5'-GAGGGAAGAAATGG-AGAA-3') (nt -248 to -134); primer 3 (sense, 5'-CAGCGT-CACCAACAATCC-3'; antisense, 5'-GGTCACAGAGTCAC-CTCC-3') (nt -411 to -228); primer 4 (sense, 5'-CACCAAC-AATCCGTTCTA-3'; antisense, 5'-GGTCACAGAGTCACC-TCC-3') (nt -405 to -228).

**Urine Protein Detection**—The urinary samples in rats were collected at 24 h and day 7. The total protein contents in urine were measured by the total protein UC FS (DiaSys Diagnostic Systems, Holzheim, Germany).

**Statistical Analysis**—All data are given as mean  $\pm$  S.D. The statistical significance (defined as  $p < 0.05$ ) of the groups was evaluated by one-way analysis of variance with simultaneous multiple comparisons between groups by the Bonferroni method. All measurements of each sample were performed in triplicate.

## RESULTS

**Comparison of Gene Expression Profiles both in GMC following Sublytic C5b-9 Stimulation and in Renal Tissues of Rats with Thy-1N**—Among 15,923 gene sequences expressed on the Affymetrix RAE230A oligonucleotide microarray, 928 genes and 979 genes were disturbed in the cultured GMC following sublytic C5b-9 stimulation (*in vitro*) at 40 min and 3 h, respectively, in which 536 and 486 genes were up-regulated at 40 min and 3 h, respectively. Moreover, 681 genes and 749 genes of them were perturbed at 40 min or 3 h after Thy-1N induction, and 667 genes as well as 173 genes were up-regulated at 40 min or 3 h from the renal tissue of Thy-1N rats (*in vivo*). Supplemental Table 1, A–D, highlights in part the genes whose mRNA levels were increased (*i.e.* related to signal transduction, apoptosis, extracellular matrix, early response, inflammation, etc.).

Among all differentially expressed genes, *IRF-1*, *ATF3* (activating transcription factor 3), *egr-1* (early growth response 1), *C/EBP $\beta$*  (CCAAT/enhancer-binding protein  $\beta$ ), *gadd45* (growth arrest and DNA-damage-inducible 45), *CTGF* (connective tissue growth factor), *IL-6* (interleukin 6), *IL-1 $\beta$*  (interleukin 1 $\beta$ ), *HO-1* (heme oxygenase 1), *schlafen 3*, and *cyclin L1* were found to be co-up-regulated both *in vitro* and *in vivo* (supplemental Table 1, A–D), indicating that these genes might be critically involved in Thy-1N pathogenesis.

**Up-regulation of IRF-1 Expression both in GMC Induced by Sublytic C5b-9 Attack and in Renal Tissues of Rats with Thy-1N**—Of the co-up-regulated genes, *IRF-1* was selected to further study its function because several experiments have reported that IRF-1 is associated with tumor cell apoptosis (20, 22). Thus, IRF-1 expression levels both in the GMC attacked by sublytic C5b-9 and in the renal tissues of Thy-1N rats were determined. As expected, the data showed that IRF-1 mRNA and protein both *in vitro* and *in vivo* were increased at 20 min, peaked at 3 h, and then reduced at 6 and 12 h (supplemental Fig. 2, A–D). In order to avoid the effects of MEM, Thy-1 Ab, or human serum, and to determine that C6 is necessary for C5b-9 assembly on GMC membrane, cultured GMC were also treated with sublytic C5b-9, Thy-1 Ab + C6DS, Thy-1 Ab + HIS, Thy-1 Ab, and MEM for 3 h. The results demonstrated that IRF-1 expression was up-regulated markedly after sublytic C5b-9 exposure (Fig. 1, A and B). Furthermore, IRF-1 expression increased at 3 h in the renal tissues of Thy-1N rats (Fig. 1, C and D). These findings confirmed that the GMC attacked by sublytic C5b-9 and renal tissue of Thy-1N rats displayed increased IRF-1 expression.

**Activation of Caspase 9, Caspase 8, and Caspase 3 both in GMC Induced by Sublytic C5b-9 Attack and in Renal Tissues of Rats with Thy-1N**—To ascertain whether sublytic C5b-9 attack can activate caspase, the expression of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3 both in the GMC upon sublytic C5b-9 attack and in the renal tissues of Thy-1N rats was deter-

mined. The results suggested that procaspase 8 and cleaved caspases 8 and 3 increased significantly at 40 or 80 min (cleaved caspase 9 emerged at 3 h) and peaked at 3 h *in vitro*. Procaspase 8 and cleaved caspases 9, 8, and 3 began to increase at 40 min, 80 min, or 3 h, peaking at 3 h *in vivo* (supplemental Fig. 2, E and F). In addition, the results from different groups showed that the protein levels of cleaved caspases 9, 8, and 3 were up-regulated significantly in the GMC at 3 h after sublytic C5b-9 incubation (Fig. 2A) and in the renal tissues of Thy-1N rats (Fig. 2B), indicating that sublytic C5b-9 induced caspase pathway activation in the GMC in Thy-1N rats.

**Effects of IRF-1 Overexpression or Knockdown on GMC Apoptosis Induced by Sublytic C5b-9 Stimulation**—To confirm the effect of IRF-1 on the GMC apoptosis in response to sublytic C5b-9, the rate of GMC apoptosis mediated by sublytic C5b-9 was detected, and the result demonstrated that sublytic C5b-9 could increase GMC apoptosis (supplemental Fig. 3A). Afterward, the plasmids of pcDNA3.1 expressing IRF-1 (pIRF-1) or pcDNA3.1 (vector) and IRF-1 shRNA were transfected into GMC for 48 h, respectively, and then with or without sublytic C5b-9 stimulation for 3 h. In fact, the cultured GMC were divided into the following treatments: 1) MEM, pcDNA3.1, sublytic C5b-9, pcDNA3.1 + sublytic C5b-9, pIRF-1, and pIRF-1 + sublytic C5b-9 and 2) MEM, sublytic C5b-9, control shRNA + sublytic C5b-9, and IRF-1 shRNA + sublytic C5b-9. The results revealed that IRF-1 expression in the sublytic C5b-9, pcDNA3.1 + sublytic C5b-9, pIRF-1 and pIRF-1 + sublytic C5b-9 groups was greatly enhanced, especially much more in the pIRF-1 + sublytic C5b-9 group. In contrast, IRF-1 expression decreased in the IRF-1 shRNA + sublytic C5b-9 group (supplemental Fig. 3, B and C). In addition, GMC apoptosis in the pIRF-1 + sublytic C5b-9 group or in the IRF-1 shRNA + sublytic C5b-9 group exhibited a significant increase or decrease, respectively (Fig. 3A), providing evidence that IRF-1 plays a role in the GMC apoptosis triggered by sublytic C5b-9 attack.

**Changes of Caspase 9, 8, and 3 Activation by IRF-1 Gene Knockdown in GMC Stimulated by Sublytic C5b-9**—To determine whether GMC apoptosis induced by sublytic C5b-9 can be caused by IRF-1 activation of caspases, the cultured GMC were again divided into MEM, sublytic C5b-9, control shRNA + sublytic C5b-9, and IRF-1 shRNA + sublytic C5b-9 groups. Then activation of caspases 9, 8, and 3 in the GMC transfected with IRF-1 shRNA for 48 h followed by sublytic C5b-9 treatment for another 3 h was examined by measuring the corresponding cleavages. As presented in Fig. 3B, cleaved caspases 8 and 3 in the IRF-1 shRNA + sublytic C5b-9 group were significantly lower than that in the sublytic C5b-9 and control shRNA + sublytic C5b-9 groups, but the cleaved caspase 9 did not decrease in the same group, implying that IRF-1 gene knockdown had an inhibiting effect on the activation of caspases 8 and 3 induced by sublytic C5b-9. These data implicated that IRF-1-promoted GMC apoptosis was related to caspase 8 activation.

**Effects of Inhibiting Caspase 8 and 3 Activation on GMC Apoptosis Mediated by Sublytic C5b-9 or Induced by IRF-1 Overexpression**—To further demonstrate the roles of caspase 8 and 3 activation in GMC apoptosis mediated by sublytic C5b-9

## IRF-1 Role in GMC Apoptosis Mediated by Sublytic C5b-9

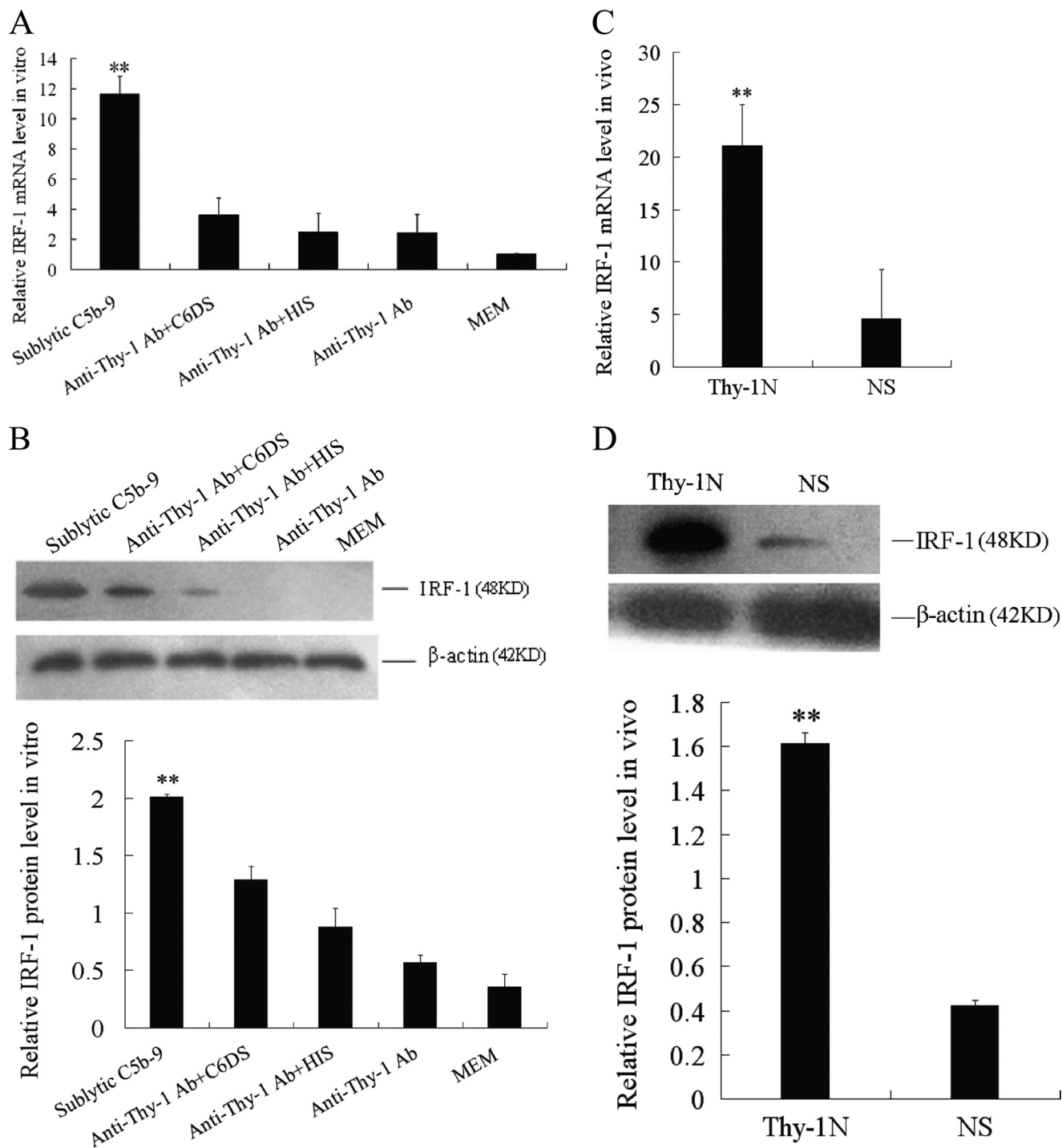
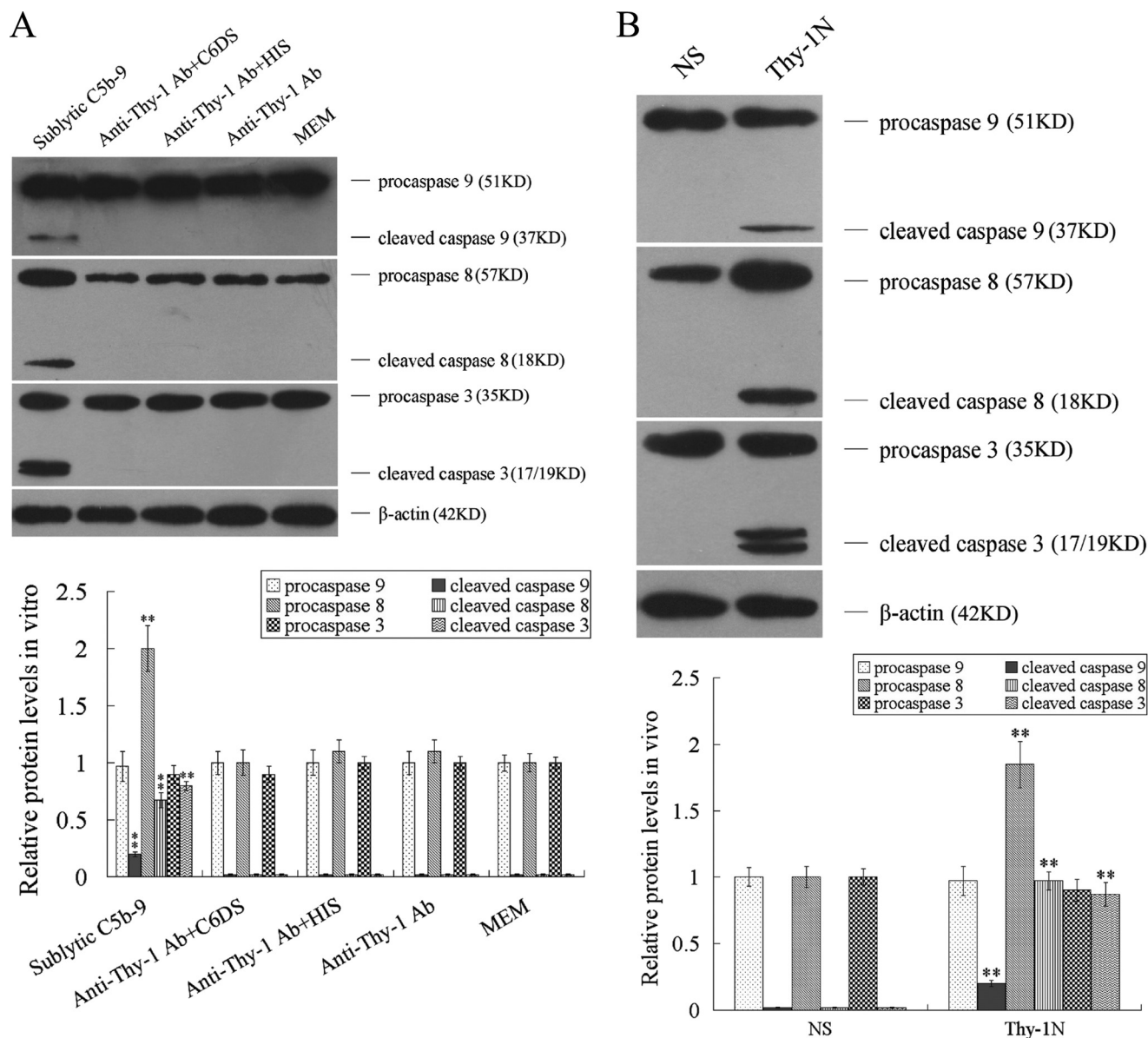


FIGURE 1. The level of IRF-1 gene expression was measured at 3 h in different groups using real-time PCR (A and C) and Western blot (B and D) both in cultured GMC triggered by sublytic C5b-9 attack (*in vitro*) and in the renal tissue of rat Thy-1N (*in vivo*). A and B, the expression of IRF-1 in the sublytic C5b-9 group at 3 h was significantly increased compared with other groups (\*\*,  $p < 0.01$  versus anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, and anti-Thy-1 Ab and MEM group, respectively). C and D, the expression of IRF-1 in the Thy-1N group at 3 h was obviously increased compared with the NS group (\*\*,  $p < 0.01$ ). Each experiment was done in triplicate. Error bars, S.D.

or induced by IRF-1 overexpression, the GMC were divided into six groups with the following treatments: DMSO + sublytic C5b-9, Z-IETD-FMK + sublytic C5b-9, Z-DEVD-FMK + sublytic C5b-9, DMSO + pIRF-1, Z-IETD-FMK + pIRF-1, and Z-DEVD-FMK + pIRF-1. GMC apoptosis was evaluated at 3 h (here the method and time of pIRF-1 transfected into GMC was the same as previously mentioned). The results indicated that

the relative amount of GMC apoptosis induced by sublytic C5b-9 or IRF-1 overexpression could be significantly reduced by using caspase 8 or 3 inhibitor, compared with the DMSO + sublytic C5b-9 or DMSO + pIRF-1 group (Fig. 3C), suggesting that inhibiting caspase 8 or 3 activation could actually decrease GMC apoptosis in response to sublytic C5b-9 stimulation or IRF-1 overexpression.



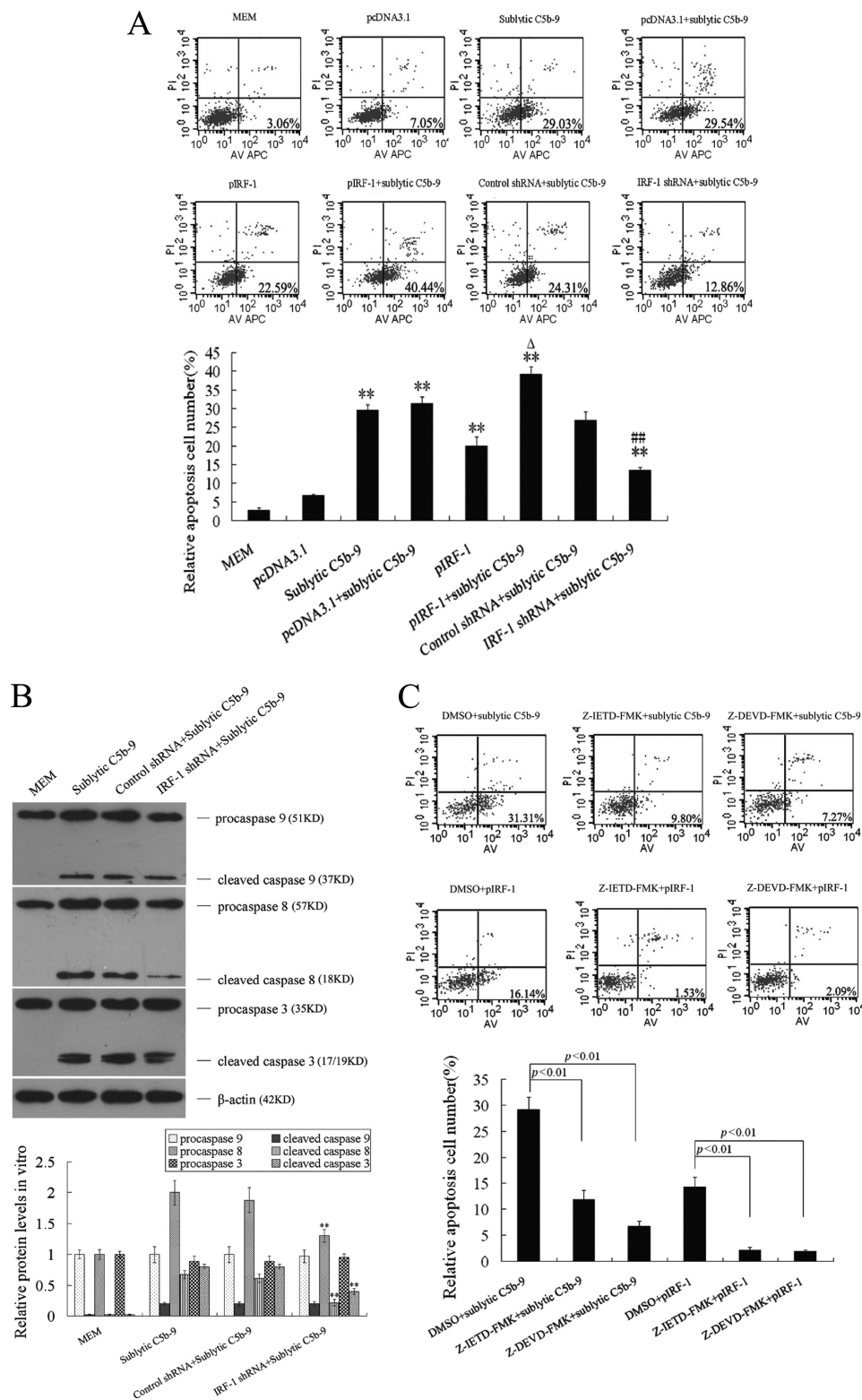
**FIGURE 2. Expression levels of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3 were detected using Western blot in different groups at 3 h in cultured GMC induced by sublytic C5b-9 stimulation (*in vitro*) and in the renal tissue of Thy-1N rat (*in vivo*).** A, protein levels of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3 in the GMC *in vitro*. The result showed that protein levels of procaspase 8 and cleaved caspases 9, 8, and 3 in cultured GMC attacked by sublytic C5b-9 (sublytic C5b-9 group) were significantly increased compared with other groups (\*\*,  $p < 0.01$  versus anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM groups). B, protein levels of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3 *in vivo*. The results show that the protein levels of procaspase 8 and cleaved caspases 9, 8, and 3 in the renal tissues of Thy-1N rats were markedly up-regulated compared with the corresponding NS group (\*\*,  $p < 0.01$ ). Error bars, S.D.

**Increase of IRF-1 and Caspase 8 Promoter Activity in GMC Induced by Sublytic C5b-9 Stimulation**—In order to further clarify whether the IRF-1 or caspase 8 gene was directly activated by sublytic C5b-9 attack, the activity of the IRF-1 and caspase 8 promoters was analyzed. As shown in Fig. 4 A, the luciferase activity of the 1.7-kb IRF-1 promoter increased in the GMC exposed to sublytic C5b-9 for 40 min (1.95-fold versus MEM) and 3 h (2.61-fold versus MEM). Moreover, as shown in Fig. 4B, the luciferase activity of the 1.24-kb caspase 8 promoter was elevated at 40 min (1.56-fold versus MEM) and markedly increased in the GMC at 3 h after sublytic C5b-9 stimulation (2.24-fold versus MEM). These results were in agreement with the findings that sublytic C5b-9 up-regulated IRF-1 and caspase

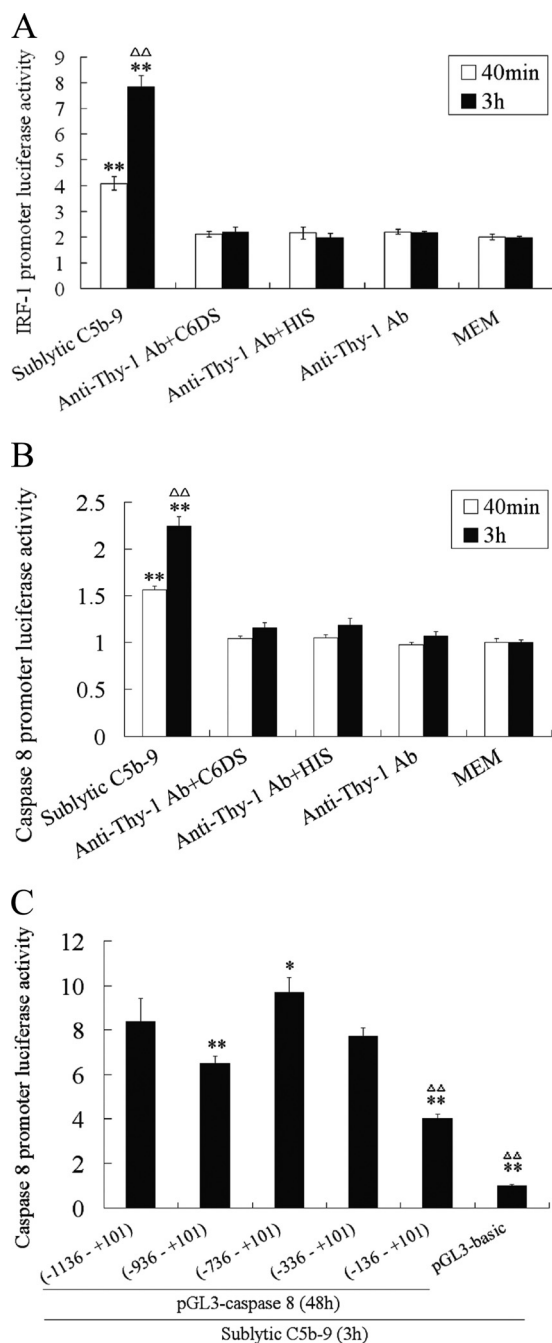
8 expressions *in vitro*, providing evidence that IRF-1 and caspase 8 promoter activity in the GMC incubated with sublytic C5b-9 can be directly up-regulated.

To further decide which element was involved in caspase 8 gene activation upon sublytic C5b-9 attack, caspase 8 promoter fragments were generated, and the promoter activities of the GMC transfected with these constructs for 48 h and attacked by sublytic C5b-9 again for 3 h were detected. The data presented in Fig. 4C suggested that only the activity of the caspase 8 promoter deletion fragment (nt -136 to +101) was notably less than that of the full-length caspase 8 promoter (nt -1136 to +101) or other deletion fragments (nt -936 to +101, -736 to +101, and -336 to +101), indicating that a key element in

## IRF-1 Role in GMC Apoptosis Mediated by Sublytic C5b-9



**FIGURE 3. Effects of IRF-1 gene overexpression or knockdown on GMC apoptosis and caspases expression induced by sublytic C5b-9.** A, the results of IRF-1 gene overexpression or knockdown on GMC apoptosis by flow cytometry. The number of apoptotic GMC manifested an obvious increase in the sublytic C5b-9, pcDNA3.1 + sublytic C5b-9, pIRF-1, and pIRF-1 + sublytic C5b-9 groups, and the number of apoptotic GMC in the pIRF-1 + sublytic C5b-9 group was also significantly higher than that in other groups. However, the number of apoptotic GMC in the IRF-1 shRNA + sublytic C5b-9 group was remarkably lower than that in the control shRNA + sublytic C5b-9 group (\*\*,  $p < 0.01$  versus MEM or pcDNA3.1;  $\Delta$ ,  $p < 0.05$  versus sublytic C5b-9; ##,  $p < 0.01$  versus control shRNA + sublytic C5b-9). B, protein expression of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3 in the four groups at 3 h using Western blot analysis. The result showed that the protein levels of procaspase 8 (not procaspase 9 or 3) and cleaved caspases 8 and 3 (not cleaved caspase 9) in the IRF-1 shRNA + sublytic C5b-9 group were significantly lower than those in the sublytic C5b-9 and control shRNA + sublytic C5b-9 groups; \*\*,  $p < 0.01$  versus other groups, respectively. C, effects of inhibiting caspase 8 and 3 activation on the GMC apoptosis triggered by sublytic C5b-9 or induced by IRF-1 overexpression. The results show that GMC apoptosis in response to sublytic C5b-9 attack or IRF-1 overexpression could be strikingly reduced by giving caspase 8 inhibitor (Z-IETD-FMK) and caspase 3 inhibitor (Z-DEVD-FMK), compared with the corresponding groups ( $p < 0.01$ ). Error bars, S.D.



**FIGURE 4. Analysis of IRF-1 and caspase 8 promoter activity in the GMC attacked by sublytic C5b-9.** A, rat GMC were transfected with 1.7-kb IRF-1 promoter luciferase reporter plasmids for 48 h, and then the cells were treated with sublytic C5b-9, anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM for 40 min or 3 h. The luciferase activity of the IRF-1 promoter increased in GMC induced by sublytic C5b-9. \*\*,  $p < 0.01$  versus sublytic C5b-9;  $\Delta\Delta$ ,  $p < 0.01$  versus sublytic C5b-9 (40 min) group. B, rat GMC were transfected with the luciferase reporter constructs containing 1.24-kb rat caspase 8 promoter (nt -1136 to +101) accompanied by pRL-SV40 transfection as the control of transfection efficiency for 48 h, and then the cells were given different treatments (sublytic C5b-9, anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM) for 40 min or 3 h. The luciferase activity of caspase 8 promoter showed an increase in the GMC exposed to sublytic C5b-9 for 40 min and became more prominent at 3 h. \*\*,  $p < 0.01$  versus sublytic C5b-9, anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM groups;  $\Delta\Delta$ ,  $p < 0.01$  versus sublytic C5b-9 (40 min) group. C, rat GMC were transfected with the luciferase reporter constructs containing the 1.24-kb rat caspase 8 promoter (nt -1136 to +101) or different promoter deletion fragments (nt -936 to +101, -736 to +101, -336 to +101, and -136 to +101)

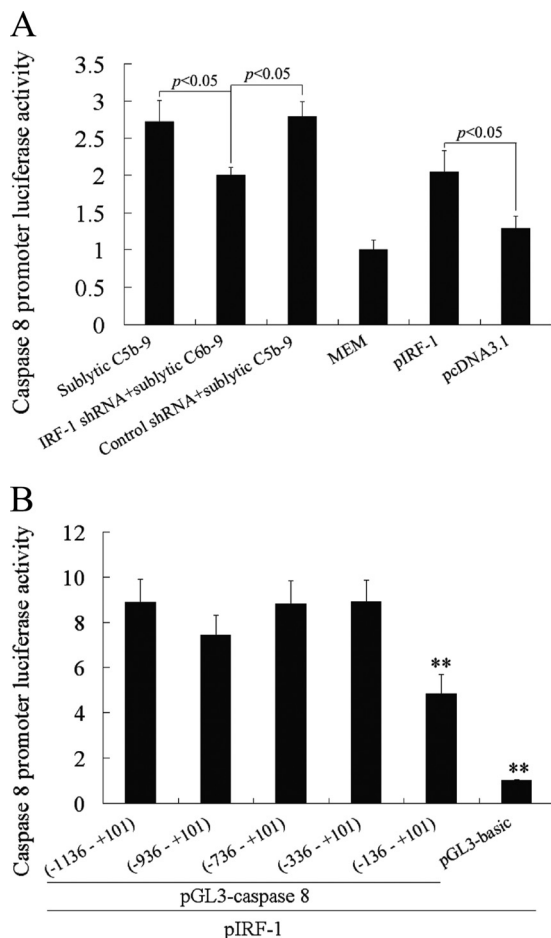
response to sublytic C5b-9 stimulation might be located within the nt -336 to -136 proximal region of the caspase 8 promoter.

**Regulation of Rat Caspase 8 Promoter Activity in GMC by IRF-1 Gene Knockdown or Overexpression in GMC Exposed to Sublytic C5b-9 Stimulation**—To test whether IRF-1 knockdown or overexpression can affect caspase 8 gene transcription triggered by sublytic C5b-9 attack, the GMC were transfected with the 1.24-kb caspase 8 promoter and IRF-1 shRNA or pIRF-1 plasmids for 48 h, and then the GMC was treated with or without sublytic C5b-9 for another 3 h. The caspase 8 promoter activity in the six groups (sublytic C5b-9, IRF-1 shRNA + sublytic C5b-9, control shRNA + sublytic C5b-9, MEM, pIRF-1, and pcDNA3.1) was measured, and data suggested that caspase 8 promoter activity was significantly decreased in the IRF-1 shRNA + sublytic C5b-9 group and markedly increased in the pIRF-1 group (Fig. 5A), indicating that IRF-1 expression was involved in the elevation of caspase 8 promoter activity in the GMC triggered by sublytic C5b-9. In addition, to identify the elements required for caspase 8 promoter activity in response to IRF-1, the GMC were transfected with the luciferase reporter constructs containing the 1.24-kb caspase 8 promoter (nt -1136 to +101) or different promoter deletion fragments (nt -936 to +101, -736 to +101, -336 to +101, and -136 to +101) and pIRF-1 (overexpression plasmid) for 48 h and then with sublytic C5b-9 attack for 3 h, and caspase 8 promoter activity was again determined. As presented in Fig. 5B, only the caspase 8 promoter activity (nt -136 to +101) + pIRF-1 group showed an effective decrease relative to the full-length caspase 8 promoter + pIRF-1 or other deletion fragments + pIRF-1 group, suggesting a critical element in the nt -336 to -136 region at the 5'-end of caspase 8 exon-1 for IRF-1.

**Identification of IRF-1 Binding to Rat Caspase 8 Promoter Element in GMC Induced by Sublytic C5b-9 Attack or by IRF-1 Overexpression**—In order to further reveal that IRF-1 can bind to the region of nt -336 to -136 of the caspase 8 promoter, GMC were first divided into the following treatment groups for 3 h: sublytic C5b-9, anti-Thy-1 Ab + C6DS, anti-Thy-1 Ab + HIS, anti-Thy-1 Ab, and MEM. Then a ChIP assay was performed using anti-IRF-1 antibody or control IgG, and immunoprecipitated DNA was amplified using four different pairs of primers for the proximal promoter regions of the caspase 8 gene. The positive result was obtained only by using primer 3 (nt -411 to -228), indicating that IRF-1 induced by sublytic C5b-9 could bind to the element (nt -411 to -228) of rat caspase 8 promoter in the GMC upon sublytic C5b-9 attack (Fig. 6A). Meanwhile, the negative PCR result was achieved by using primers 1, 2, and 4 (data not shown). Furthermore, the cultured GMC were transfected with pIRF-1 or pcDNA3.1 plasmids for 48 h, and a ChIP experiment was also performed. The immunoprecipitated DNA was again amplified by PCR

accompanied by pRL-SV40 transfection as the control of transfection efficiency for 48 h, and then the cells were given sublytic C5b-9 stimulation for 3 h. The luciferase activity of the caspase 8 promoter (nt -136 to +101) + sublytic C5b-9 group showed a notable decrease. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus total-length caspase 8 promoter (nt -1136 to +101) + sublytic C5b-9 group;  $\Delta\Delta$ ,  $p < 0.01$  versus other deletion fragments + sublytic C5b-9 group, respectively. Error bars, S.D.

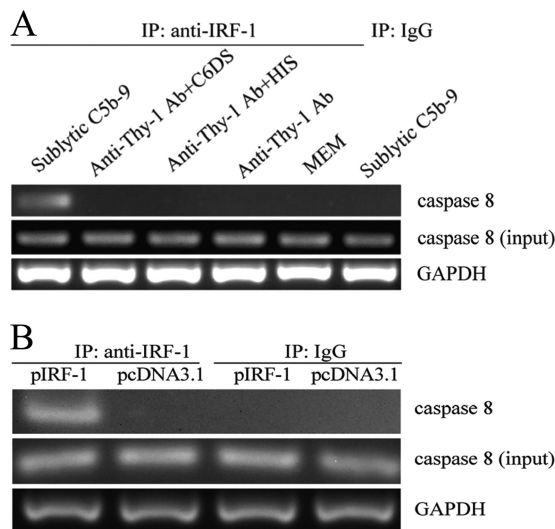




**FIGURE 5. The effect of IRF-1 expression on caspase 8 promoter activity in rat GMC attacked by sublytic C5b-9.** *A*, GMC were transfected with the luciferase reporter constructs containing 1.24-kb caspase 8 promoter (nt -1136 to +101) and pRL-SV40 accompanied by transfection of IRF-1 shRNA, control shRNA, or pIRF-1 or pcDNA3.1 plasmids for 48 h. Thereafter, the cells were subjected to (or not subjected to) sublytic C5b-9 attack (3 h). The luciferase activity of the caspase 8 promoter showed an obvious decrease in the IRF-1 shRNA + sublytic C5b-9 group compared with the control shRNA + sublytic C5b-9 and sublytic C5b-9 groups ( $p < 0.05$ ). Meanwhile, the luciferase activity of the caspase 8 promoter in the pIRF-1 group was significantly higher than that in the pcDNA3.1 group ( $p < 0.05$ ). *B*, rat GMC were transfected with the IRF-1 overexpression plasmid (pIRF-1) and luciferase reporter constructs containing 1.24-kb caspase 8 full-length promoter (nt -1136 to +101) or different promoter deletion fragments (nt -936 to +101, -736 to +101, -336 to +101, -136 to +101) accompanied by pRL-SV40 transfection as the control of transfection efficiency for 48 h. Compared with the full-length caspase 8 promoter + pIRF-1 group, the luciferase activity of the caspase 8 promoter (nt -136 to +101) + pIRF-1 group showed a marked decrease in the GMC. \*\*,  $p < 0.01$  versus full-length caspase 8 promoter + pIRF-1 group and other deletion fragments + pIRF-1 group, separately. Error bars, S.D.

using the above mentioned four pairs of primers, resulting in a positive result from primer 3 (Fig. 6B) and negative results from the other three pairs of primers (data also not shown). Collectively, the findings manifested that overexpressed IRF-1 could bind to the element (nt -411 to -228) of caspase 8 promoter (Fig. 6B), indicating that up-regulation of rat caspase 8 promoter activity in response to sublytic C5b-9 attack was due to increasing IRF-1 binding to the caspase 8 promoter element.

It is worth mentioning that the potential transcription factor-binding sites in the promoter of rat caspase 8 gene (nt -336 to -228) were predicted using the computer program TFSEARCH (version 1.3), and all of the possible elements are

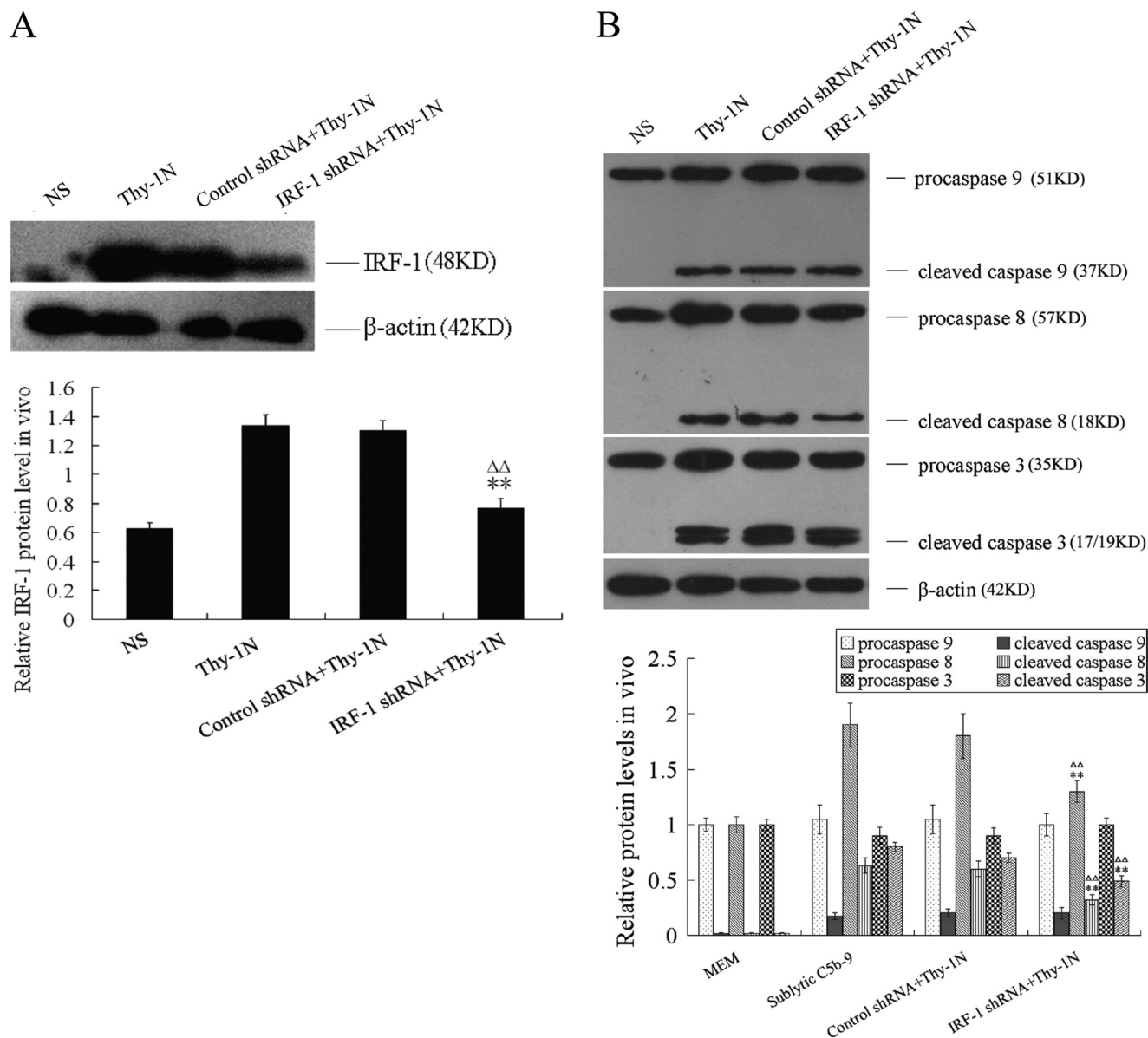


**FIGURE 6. Identification of IRF-1 binding to the element of rat caspase 8 gene promoter in the GMC attacked by sublytic C5b-9 or transfected with pIRF-1 plasmids.** *A*, the cultured GMC were stimulated with or without sublytic C5b-9 for 3 h, the ChIP assay was performed using anti-IRF-1 antibody or control IgG, and the immunoprecipitated (IP) DNA was amplified by PCR using primer 3 for the proximal promoter regions (nt -411 to -228) of the caspase 8 gene. The results showed that IRF-1 induced by sublytic C5b-9 stimulation did enhance binding to the element (nt -411 to -228) of the rat caspase 8 promoter in the GMC upon sublytic C5b-9 attack. *B*, the cultured GMC were transfected with pIRF-1 or pcDNA3.1 for 48 h, and the ChIP assay was also done using anti-IRF-1 antibody or control IgG. The immunoprecipitated DNA was amplified by PCR using primer 3 for the proximal element (nt -411 to -228) of caspase 8 promoter, and the results showed that the over-expressed IRF-1 could bind to the element of the rat caspase 8 promoter.

*underlined* in supplemental Fig. 4, including elements for binding of heat shock factor, caudal type homeobox A, CAP1 (adenylate cyclase-associated protein 1), EVI-1 (ecotropic viral integration site 1), MZF1 (myeloid zinc finger 1), IK-2 (I $\kappa$ B kinase-like 2), YY1 (Yin and Yang 1 protein), GATA-1 (GATA-binding factor 1), NIT2 (nitrilase 2), GCN4 (general control non-repressible 4), and AP-1 (activator protein 1) but not the IRF-1-binding site. However, the ChIP assay suggested that the IRF-1 protein could bind to this element of rat caspase 8 promoter in the GMC upon sublytic C5b-9 attack (Fig. 6A).

**Effects of IRF-1 Gene Knockdown on Caspase 8 and Caspase 3 Expression in Renal Tissue of Rats with Thy-1N**—To demonstrate the role of IRF-1 in promoting GMC apoptosis *in vivo*, normal SD rats were classified into four groups: 1) NS, 2) Thy-1N, 3) control shRNA + Thy-1N, and 4) IRF-1 shRNA + Thy-1N. The expression of renal IRF-1, caspase 8, and caspase 3 protein in the four groups at 3 h after nephritis establishment was examined, and the results showed that expression of IRF-1 (Fig. 7A) and cleaved caspases 8 and 3 (Fig. 7B) in the IRF-1 shRNA + Thy-1N group was markedly down-regulated as compared with the Thy-1N or control shRNA + Thy-1N group, although the protein levels of IRF-1 and cleaved caspases 8 and 3 in the IRF-1 shRNA + Thy-1N group were higher than in the NS group. Taken together, the data suggest that renal IRF-1 gene knockdown could inhibit renal caspase 8 and 3 activation of Thy-1N rats.

**Effects of IRF-1 Knockdown or Caspase 8 and 3 Inhibition on GMC Apoptosis and Other Pathologic Changes, Including Urinary Protein of Rats with Thy-1N**—To further evaluate the role of IRF-1 in promoting GMC apoptosis of Thy-1N rats, normal



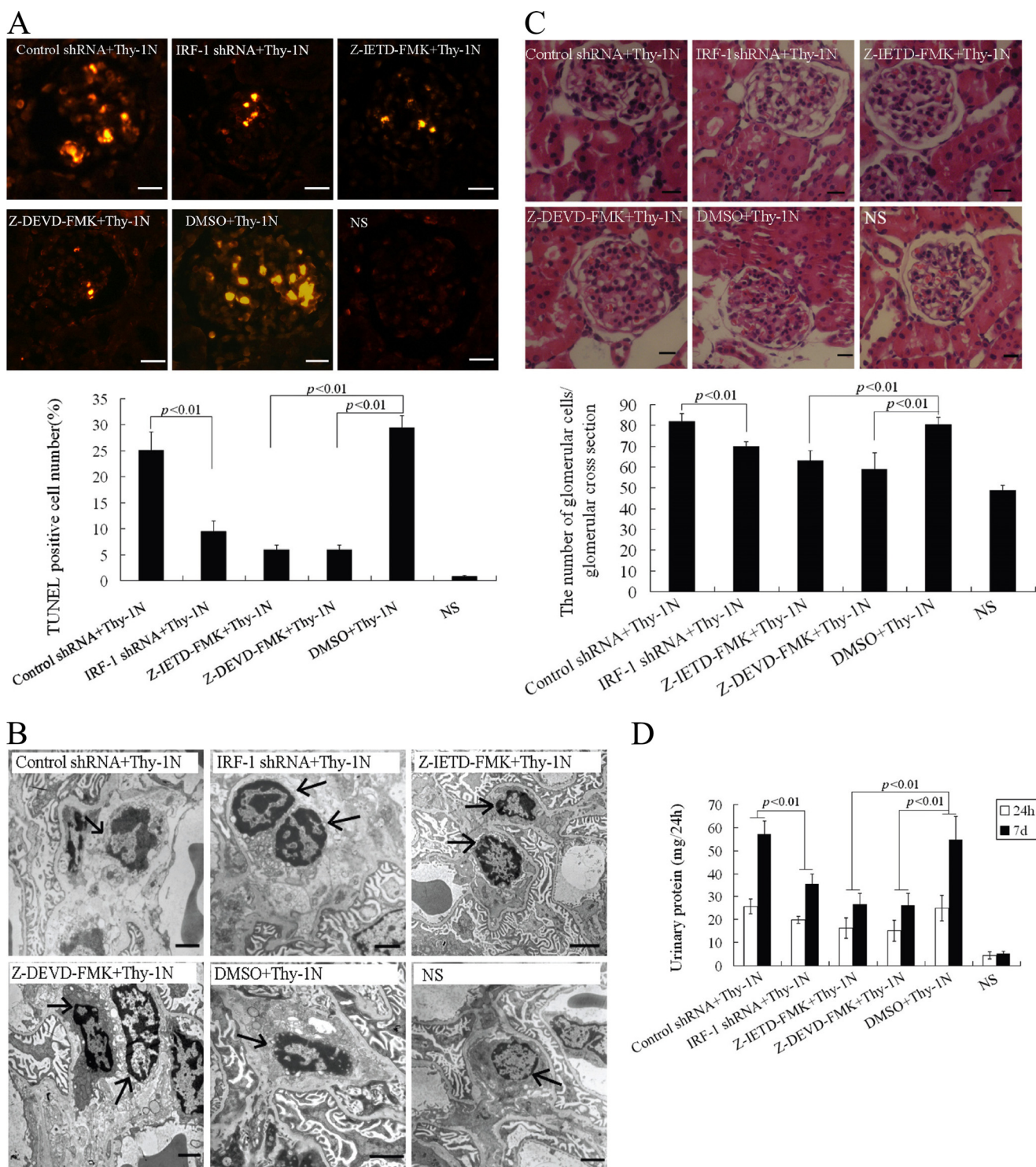
**FIGURE 7. Effects of IRF-1 gene knockdown on the protein expression of relative genes in the renal tissues of rats with Thy-1N.** A, IRF-1 protein expression. B, protein expression of procaspases 9, 8, and 3 and cleaved caspases 9, 8, and 3. The semiquantitative analysis showed that the protein levels of IRF-1, procaspase 8 (not procaspases 9 and 3), and cleaved caspases 8 and 3 (no cleaved caspase 9) in the IRF-1 shRNA + Thy-1N group were significantly lower than those in the Thy-1N group (\*\*,  $p < 0.01$ ) and the control shRNA + Thy-1N group ( $\Delta\Delta$ ,  $p < 0.01$ ), although the protein levels of cleaved caspases 8 and 3 in the IRF-1 shRNA + Thy-1N group were obviously higher than that in the NS group. Error bars, S.D.

SD rats were divided into six groups: 1) control shRNA + Thy-1N, 2) IRF-1 shRNA + Thy-1N, 3) Z-ITED-FMK + Thy-1N, 4) Z-DEVD-FMK + Thy-1N, 5) DMSO + Thy-1N, and 6) normal serum (NS). The numbers of TUNEL-positive cells and glomerular cells in renal tissues at 3 h and 7 days were observed. The results revealed that the numbers of TUNEL-positive cells were substantially decreased in IRF-1 shRNA + Thy-1N or Z-ITED-FMK + Thy-1N and Z-DEVD-FMK + Thy-1N groups (Fig. 8A), and under electron microscopy, the apoptotic changes, including irregular aggregation of chromatin at the periphery of the nucleus and condensation of the nuclear chromatin in IRF-1 shRNA + Thy-1N, Z-ITED-FMK + Thy-1N, and Z-DEVD-FMK + Thy-1N groups, were reduced (Fig. 8B). Meanwhile, the numbers of glomerular cells in the above mentioned groups on day 7 under light microscopy were also signif-

icantly less than the number in control groups, although the numbers were still higher than that in the NS group (Fig. 8C). Additionally, urinary protein content (mg/24 h) at 24 h and on day 7 was also significantly less than that in control groups (Fig. 8D), implicating that IRF-1 gene silencing or caspase 8 and 3 inhibition could alleviate GMC apoptosis, secondary proliferation, and urinary protein in the rats with Thy-1N.

**DISCUSSION**

Reportedly, GMC injury in Thy-1N is complement-dependent but neutrophil-independent (6–9). Complement activation leads to C5b-9 insertion into the cell membrane. Our previous studies have revealed that although C5b-9 deposits were found on the GMC surface in Thy-1N rats, the morphology of most GMC deposited with C5b-9 still remained intact, indicat-



**FIGURE 8. Roles of IRF-1 and caspases 8 and 3 in the pathologic changes in the renal tissues of Thy-1N rats at fixed time.** *A*, photographs of glomerular TUNEL-positive cells at 3 h (scale bar, 10  $\mu$ m). The numbers of TUNEL-positive cells in the IRF-1 shRNA + Thy-1N group, Z-IETD-FMK + Thy-1N group, and Z-DEVD-FMK + Thy-1N group were markedly lower than those in the control shRNA + Thy-1N group and DMSO + Thy-1N groups ( $p < 0.01$ ). The photographs are representative of rat glomeruli in the six groups. *B*, ultrastructural changes detected by electron microscopy (scale bar, 2.5  $\mu$ m). The pathologic changes, including irregular aggregation of chromatin in the periphery of the nucleus and clear condensation of the nuclear chromatin (arrows) in the IRF-1 shRNA + Thy-1N group, Z-IETD-FMK + Thy-1N group, and Z-DEVD-FMK + Thy-1N group, were obviously reduced compared with the control shRNA + Thy-1N or DMSO + Thy-1N group. The photographs are representative of rat glomeruli in the six groups at 3 h. *C*, changes of glomerular cells on day 7 (7d) were determined using H&E staining under light microscopy (scale bar, 10  $\mu$ m). The numbers of the glomerular cells in the IRF-1 shRNA + Thy-1N group or Z-IETD-FMK + Thy-1N and Z-DEVD-FMK + Thy-1N groups were obviously less than those in the control shRNA + Thy-1N or DMSO + Thy-1N group ( $p < 0.01$ ). *D*, the total contents of urinary protein excretion (mg/24 h) at 24 h and day 7 in the IRF-1 shRNA + Thy-1N, Z-IETD-FMK + Thy-1N, and Z-DEVD-FMK + Thy-1N groups were also significantly lower than those in the control shRNA + Thy-1N or DMSO + Thy-1N group ( $p < 0.01$ ). Error bars, S.D.

ing that the C5b-9 is sublytic (6, 14). At a lower dose, C5b-9 can induce sublytic injury (*i.e.* cell apoptosis) (14), but some studies from mouse experimental allergic encephalomyelitis show that sublytic C5b-9 can protect oligodendrocytes from apoptosis and lead to survival (25, 26), suggesting that the different roles in these cells may be due to different biochemical reactions, including cell states, upon sublytic C5b-9 attack (3, 9, 27).

In the process of Thy-1N, GMC showed apoptosis or proliferation (11). There is growing interest in GMC apoptosis as a potential contributor to the renal lesion (14, 15, 23, 27). Shimizu *et al.* (4) demonstrated that GMC apoptosis in Thy-1N was very rapid (about 1–2 h). Our previous experiments (11, 14) have also confirmed that glomerular TUNEL-positive cells emerge at 40 min and markedly increase at 3 h, and the damaged GMC display typical morphological apoptotic features (*i.e.* nuclear chromatin aggregation at periphery of the nucleus and chromatin condensation) at 3 h. Moreover, administration of cobra venom factor for complement depletion could greatly inhibit the pathologic injury. Furthermore, rat GMC attacked by sublytic C5b-9 could result in apoptosis, which supports the findings regarding Thy-1N rats *in vivo* (11, 14, 15). Together, these data provide evidence that sublytic C5b-9 mediates GMC apoptosis, but the mechanism of sublytic C5b-9 attack in GMC apoptosis remains elusive.

The expression profiles of early responsive genes and individual up-regulated genes were determined in order to find key genes involved in the GMC apoptosis of Thy-1N. Our results displayed that, at 40 min and 3 h after Thy-1N reproduction, 667 genes and 173 genes were up-regulated among 15,923 gene spots. Likewise, at the same times following sublytic C5b-9 attack, 536 and 486 genes were elevated in cultured GMC, respectively. Notably, among all of the differentially expressed genes, *IRF-1*, *ATF3*, *egr-1*, *C/EBP $\beta$* , *gadd45*, connective tissue growth factor, *IL-6*, *IL-1 $\beta$* , *HO-1*, *schlafen 3*, and *cyclin L1* were revealed to be co-up-regulated both *in vitro* and *in vivo*. To further explore the function of up-regulated expression genes, we subsequently selected *gadd45 $\gamma$*  and *ATF3* to study their functions and revealed that up-regulation of *gadd45 $\gamma$*  and *ATF3* genes in the GMC induced by sublytic C5b-9 stimulation could cause apoptosis (14, 28). In the study, we focused on the role of *IRF-1* in GMC apoptosis.

IRF-1 is the first member of the IRF family and is expressed at very low levels in normal cells but can be rapidly induced through IFN- $\gamma$ , IL-1, and Toll receptors (29–32). Early reports have shown that IRF-1 can increase some target genes involved in inflammation and apoptosis, including interferons (IFNs), matrix metalloproteinases, *VCAM-1* (vascular cell adhesion molecule-1), and *COX-2* (cyclooxygenase-2) (33–40). Reilly *et al.* (41) found that *IRF-1* gene deletion could suppress lupus nephritis in MRL/lpr mice, and Fantuzzi *et al.* (42) also confirmed decreased production of IFN- $\gamma$  and IL-18 in *IRF-1*<sup>-/-</sup> mice exposed to inflammatory stimuli. Further evidence provided by Nakazawa (43) demonstrated a complete suppression of insulinitis and diabetes in NOD mice lacking *IRF-1*. However, little is known about whether sublytic C5b-9 enhances IRF-1 expression and its role in GMC apoptosis in Thy-1N rats.

Our results manifested that the GMC after sublytic C5b-9 treatment not only elevated IRF-1 expression but also up-reg-

ulated *IRF-1* promoter activity. On the other hand, overexpression or knockdown of the IRF-1 gene could effectively increase or decrease GMC apoptosis upon sublytic C5b-9 attack, respectively. Accordingly, renal IRF-1 gene silencing *in vivo* also greatly reduced GMC apoptosis and secondary proliferation as well as urinary protein secretion in the rats with Thy-1N. Collectively, these findings suggested that IRF-1 induction was a necessary component of sublytic C5b-9-triggered GMC apoptosis of Thy-1N, and IRF-1 promoted GMC apoptosis. Because our previous studies demonstrated that GMC apoptosis in Thy-1N rats was associated with overexpression of *Gadd45 $\gamma$*  and *ATF3* (14, 28), the results here indicated that GMC apoptosis upon sublytic C5b-9 might require synergetic activation of some apoptosis-related genes.

Cell apoptosis occurs by two pathways to activate caspases (44, 45). In one, caspase 8-induced cleavage of Bid generates a truncated form that is translocated to mitochondria and promotes release of cytochrome *c*, thus allowing activation of caspase 9 and effector caspases. In contrast, in the mitochondria-independent pathway, activated caspase 8 directly processes and activates caspase 3, indicating that caspase 8 is an indispensable mediator for cell apoptosis (44–46). Previous studies reported that human caspase 8 up-regulation by IFN- $\gamma$  required IRF-1 induction (47–50). In the present experiment, our data showed that the GMC attacked by sublytic C5b-9 could result in caspase activation and enhance the levels of cleaved caspases 9, 8, and 3; however, *IRF-1* gene knockdown in GMC had opposite effects (but did not down-regulate the cleaved caspase 9 level). Meanwhile, blockage of caspase 8 and 3 activation with the corresponding inhibitors could effectively decrease GMC apoptosis upon sublytic C5b-9 attack or IRF-1 overexpression. In contrast, silencing of the renal *IRF-1* gene significantly reduced the expression of cleaved caspases 8 and 3, and inhibition of caspase 8 or 3 activation also alleviated GMC apoptosis, including secondary damage, suggesting that IRF-1-induced GMC apoptosis of Thy-1N rats was correlated with the mitochondria-independent caspase 8 activation.

In the early stage of GMC apoptosis in our experiment, the activity of *IRF-1* and caspase 8 promoters could be clearly up-regulated after sublytic C5b-9 incubation. Given that IRF-1 is an early transcription factor, we predicted that IRF-1 might induce the transcription of the rat caspase 8 gene. To verify this hypothesis, further luciferase reporter assays confirmed that IRF-1 overexpression or knockdown could markedly increase or decrease caspase 8 promoter activity in response to sublytic C5b-9. The deletion tests showed that sublytic C5b-9 increased activity of the caspase 8 promoter located in the nt -336 to -136 region at the 5'-end of caspase 8 exon-1, and further ChIP analysis demonstrated that IRF-1 could bind to the element (nt -411 to -228) of the caspase 8 promoter, suggesting that up-regulation of rat caspase 8 promoter activity induced by sublytic C5b-9 was dependent on IRF-1 binding to the element of the caspase 8 promoter. Notably, the potential transcription factor-binding sites in the promoter of the rat caspase 8 gene located between nucleotide positions -336 and -228 were predicted using the computer program TFSEARCH, and the possible binding transcription factor consisted of heat shock factor, caudal type homeobox A, CAP1, EVI-1, MZF1, IK-2,

YY1, GATA-1, NIT2, GCN4, and AP-1 but not IRF-1. This may be explained as follows. 1) Software prediction could not achieve 100% accuracy. 2) The IRF-1 protein might be combined with other transcription factors that bound to the element of the caspase 8 gene promoter.

In summary, our studies reveal that the *IRF-1* gene is one of the co-up-regulated genes both in GMC induced by sublytic C5b-9 attack (*in vitro*) and in the renal tissue of Thy-1N (*in vivo*). Overexpression or knockdown of the *IRF-1* gene in GMC could markedly enhance or reduce GMC apoptosis mediated by sublytic C5b-9, respectively, and silencing of the renal *IRF-1* gene could effectively attenuate GMC apoptosis and secondary damage of Thy-1N rats. The mechanism of GMC apoptosis upon sublytic C5b-9 is associated with subsequent activation of caspases 8 and 3 followed by IRF-1 overexpression, and IRF-1 significantly up-regulates caspase 8 promoter activity because IRF-1 could bind to the response element (nt -411 to -228) of the caspase 8 promoter, indicating that caspase 8 promoter transcription in response to sublytic C5b-9 is dependent on IRF-1 binding. Additionally, inhibiting caspase 8 and 3 activation mitigates GMC apoptosis of Thy-1N rats. Taken together, these findings implicate that GMC apoptosis is triggered by sublytic C5b-9 in Thy-1N via an IRF-1/caspase 8-dependent mechanism, and IRF-1 induction plays a partially proapoptotic role, which might provide a potential target for treatment of human MsPGN in the future.

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