Expression of High Mobility Group Box Chromosomal Protein 1 and Its Modulating Effects on Downstream Cytokines in Systemic Lupus Erythematosus

JIE LI, HONGFU XIE, TING WEN, HONGBO LIU, WU ZHU, and XIANG CHEN

ABSTRACT. ***Objective.*** To compare the expression of high mobility group box chromosomal protein 1 (HMGB1) and the modulating effects on its downstream cytokines in patients with systemic lupus erythematosus (SLE) and healthy controls.

Methods. HMGB1 concentrations in serum from SLE patients and controls were measured by immunoblot analysis. HMGB1 messenger RNA (mRNA) expression in peripheral blood mononuclear cells (PBMC) was detected by real-time reverse transcription–polymerase chain reaction. Immunofluorescence assay was employed to examine the translocation of HMGB1 in monocytes after endotoxin stimulation. Release of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) by PBMC after rHMGB1 stimulation was also measured.

Results. Serum HMGB1 levels and HMGB1 mRNA expressions in PBMC were elevated in SLE patients compared with controls. A positive correlation was demonstrated between HMGB1 concentrations and SLE Disease Activity Index. There was an inverse correlation between HMGB1 levels and C4 and C3 concentrations in SLE patients. HMGB1 concentrations were higher in patients with vasculitis and myositis. Lipopolysaccharide stimulated a temporarily elevated release of HMGB1 in SLE patients compared with controls. The pattern and localization of HMGB1 staining in monocytes were similar in both groups. After stimulation with rHMGB1, TNF-α level decreased but IL-6 level increased in SLE patients compared with controls.

Conclusion. Our findings suggest that increased serum levels of HMGB1 in SLE may be associated with lupus disease activity. The altered production of TNF-α and IL-6 in response to rHMGB1 stimulation may participate in the disruption of cytokine homeostasis in SLE. (J Rheumatol First Release Feb 1 2010; doi:10.3899/jrheum.090663)

Key Indexing Terms:
DISEASE ACTIVITY HIGH MOBILITY GROUP BOX CHROMOSOMAL PROTEIN 1
INTERLEUKIN 6 SYSTEMIC LUPUS ERYTHEMATOSUS TUMOR NECROSIS FACTOR-α

Systemic lupus erythematosus (SLE) is an autoimmune disease with a complex immunopathogenesis that is characterized by variable involvement of different organ systems. Deficiencies in the recognition and phagocytosis of dead and dying cells have been shown to be one of the main alterations in patients with SLE. Multiple immunoregulatory defects have also been identified in SLE, including functional deficiencies of immunocompetent cells and altered cytokine profiles[1,2].

High mobility group box 1 (HMGB1) is a ubiquitously expressed nuclear DNA-binding protein that has a dual function. In the extracellular milieu, HMGB1 is very proinflammatory[3-6] and has been shown to play a role in the pathogenesis of rheumatic disease[7-11]. In patients with rheumatoid arthritis (RA), HMGB1 can be detected in synovial fluid, but minimally in serum[11]. In cutaneous lupus erythematosus, increased expression of HMGB1 is found in affected skin[10]. Sera of patients with SLE and other rheumatic diseases have increased amounts of HMGB1 and its antibodies[12-15]. In *in vitro* studies, Urbonaviciute, *et al*[16] reported that HMGB1-nucleosome complexes can induce secretion of cytokines as well as expression of costimulatory molecules on macrophages and dendritic cells. Iwata, *et al*[17] reported that dendritic cells secrete HMGB1 via p38 MAPK activation to participate in autoimmunity in MRL-Fas (lpr) mice. While the mechanism is uncertain, these findings suggest that extracellular HMGB1 may pro-

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Supported in part by grants from the Central South University Graduate School Research Foundation and the Science and Technology Project of Hunan Province (06FJ3150).

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Accepted for publication October 28, 2009.

mote systemic and local inflammation in lupus. However, few studies published about HMGB1 and SLE have addressed the relevance of HMGB1 and lupus disease activity as well as the clinical features.

Extracellular HMGB1 has the capacity to stimulate macrophages/monocytes to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6)[4,5,11]. TNF-α and IL-6 have been implicated in association with disease activity or involvement of some organs in SLE[1,2,18-20]. Also, TNF-α is a very important early-response cytokine for innate immunity[21,22], and HMGB1 acts as an alarmin in innate immunity[7,23,24]. Therefore, the TNF-α-inducing ability of HMGB1 may in part reflect the duration and extent of innate immune response. Studies have suggested that HMGB1 contributes to the pathogenesis of SLE by forming complexes with nucleosomes, or as an adjuvant[16,17,25-28]. However, different biological effects of HMGB1 may be related to whether it is complexed or free, and research regarding a direct immunoregulatory role of this protein in SLE continues[7]. The stimulating effects of HMGB1 on the production of TNF-α and IL-6 in SLE are not yet known. Since the polarization of immune response depends on the cytokine environment during stimulation[23], determining how the immune system orchestrates this alarmin and its downstream cytokines is important in SLE.

Inflammatory stimuli [e.g., lipopolysaccharide (LPS)] stimulate the release of early proinflammatory cytokines such as TNF-α, IL-1, IL-6, and late proinflammatory mediators (e.g., HMGB1) from human peripheral blood mononuclear cells (PBMC)[4,6]. However, the HMGB1-releasing ability of SLE PBMC in response to stimulation with inflammatory stimuli may differ from those in other diseases and in healthy individuals.

One purpose of our study was to measure HMGB1 concentrations in the serum of patients with SLE and to correlate these findings with their disease activity scores. Another objective was to determine the potential role of HMGB1 in stimulating proinflammatory cytokine release in SLE microenvironments. We investigated HMGB1 expression at the protein and messenger RNA (mRNA) levels in quiescent and activated PBMC obtained from SLE patients and healthy controls. Further, we compared LPS-stimulated HMGB1 translocation from nucleus to cytosol in monocytes of SLE patients and controls. In addition, we investigated the release of TNF-α and IL-6 in response to rHMGB1 stimulation.

MATERIALS AND METHODS

Participants included 39 consecutive patients with SLE and 24 healthy volunteers from the same geographical area matched for race, sex, and age. All patients fulfilled the criteria of the American College of Rheumatology for SLE[29]. They were 34 women (87.2%) and 5 men (12.8%); ages ranged from 17 to 52 years (mean 30.97 ± SD 8.71 yrs). Disease activity at the time of blood sampling was assessed using the SLE Disease Activity Index (SLEDAI)[30]: the mean SLEDAI score was 16.97 ± 13.15, and disease duration was 69.20 ± 98.06 months. The control group comprised 24 healthy volunteers, 21 women (87.5%) and 3 men (12.5%) aged 18 to 51 years (mean 31.38 ± 8.98 yrs). Information was obtained on patients' clinical manifestations and disease activity markers (age, disease duration, rash, arthritis, myositis, vasculitis, serositis, and renal, neurological, ocular and hematological disorder). On the day of sampling, laboratory investigations collected data on anti-dsDNA and anti-Sm antibodies, blood cell counts, hemoglobin (Hb), C3, C4, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), creatine kinase, and complete urinalysis. The SLEDAI variable was grouped into scores for active lupus (score > 2) and inactive lupus (≤ 2). Patients with active disease (n = 29) had not received corticosteroids, antimalarials, or immunosuppressors for the previous 3 months before sampling. The dose of prednisone taken by patients with inactive disease (n = 10) was not more than 15 mg/day during the previous 3 months. Patients with infections or other inflammatory conditions were excluded.

The protocol for this research conformed to the provisions of the World Medical Association Declaration of Helsinki. The study was approved by the local ethics committee at Xiangya Hospital, Changsha, and informed consent was obtained from all participants prior to the study.

Western blotting. Levels of HMGB1 in serum samples from controls and patients were assayed by Western blotting analysis. Each serum sample was centrifuged at 4°C, 12,000 g, for 10 min and then filtered through a 0.22 μm Millex-GP filter (Millipore, Natick, MA, USA), and filtered fluid was collected. For Western blotting of HMGB1, 2 ml filtered fluid was concentrated using a 10-kDa filter (Amicon Ultra-4 NMWL; Millipore) and centrifuged at 4000 g for 30–45 min to a final volume of 50 μl. Samples were concentrated 40-fold with this filter. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 10%–25% beta-mercaptoethanol) was then added to the filtered fluid in equal volume, and samples were boiled for 5 min. Then proteins were resolved on 10% SDS-PAGE gel (Invitrogen) and transferred to polyvinylidene fluoride membrane (Invitrogen) blocked with 5% dry milk in 0.1% Triton X-100/PBS (PBST) at room temperature for 1 h. The membrane was then incubated with an anti-HMGB1 rabbit polyclone antibody (1:1000; Abcam, Cambridge, UK) overnight. After washing with PBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h, then processed with an ECL kit (Pierce, Rockford, IL, USA). Membranes were then exposed to x-ray film (Kodak). Levels of HMGB1 were calculated with reference to standard curves generated with the rHMGB1.

Cell preparation and culture. PBMC were isolated from heparinized venous blood from SLE patients and controls by endotoxin-free Ficoll-Paque density centrifugation. The cells were cultured at concentration 10^6 cells/ml in RPMI-1640/10% heat-inactivated human serum/2 mM L-glutamine overnight. Then the culture medium was replaced with serum-free OPTI-MEM I medium (Gibco BRL). After preincubation for 2 h, cells were cultured alone or in the presence of purified rHMGB1 (1 μg/ml, expressed by *Escherichia coli*; a kind gift of Dr. H. Wang, Department of Emergency Medicine, North Shore University Hospital, New York University School of Medicine, NY, USA) or LPS (100 ng/ml or 200 ng/ml *E. coli* 0111:B4; Sigma-Aldrich). Endotoxin was routinely removed by passage through Detoxy-Gel columns (Pierce). The endotoxin content of HMGB1 preparations was determined by the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD, USA). Concentrations of the 2 stimuli used in the study were determined as described[4]. Cultures were supplemented with polymyxin B (10 μg/ml, 70 U/ml; Sigma) to inhibit contamination with endotoxin. The polymyxin B dose was neither toxic nor stimulating for cytokine production according to our preliminary experiments and literature reports[4].

Cell viability assay. Cell viability was assessed by trypan blue exclusion analysis after treatment at various timepoints[4], and was determined to be

95%–100% at all timepoints in all cultures. The cytotoxicity and proliferation of stimulated cells were examined further by MTT reduction assays[31]. Cell viability was unaffected by LPS and rHMGB1 at concentrations that induced effective proinflammatory cytokine release when assessed by trypan blue and MTT reduction assays.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). For analysis of HMGB1 mRNA expression, we used real-time RT-PCR. PBMC were left untreated or treated with LPS (200 ng/ml) for different timepoints, and total RNA was isolated with TRIzol reagent (Gibco BRL). Before elution of RNA, residual genomic DNA was digested using RNase-Free DNase I (Promega, Madison, WI, USA). Total RNA was converted to cDNA using the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas/MBi, Glen Burnie, MD, USA).

Quantitative real-time RT-PCR was performed using the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). We used SYBR premix Ex-Taq™ (TaKaRa Biotechnology, Dalian, China) as suggested by the manufacturer. The cycling parameters were 30 s at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The amplification efficiency of the target gene (HMGB1) and the internal control (GAPDH) was comparable. Relative gene expression data were calculated using the $2^{-\Delta\Delta C(T)}$ method[32]. Amplification of HMGB1 gene was normalized to GAPDH (unaffected by any treatments used) and expressed relative to either control or untreated sample in every group (SLE or controls). Amplified products were checked for their correct size by means of agarose gel electrophoresis.

Primers used in amplification were designed and obtained from TaKaRa Biotechnology (Dalian, China). The sequences were as follows: 5'-TCA CAG CCA TTG CAG TAC ATT GAG-3' (sense) and 5'-GGA TCT CCT TTG CCC ATG TTT AGT T-3' (antisense) for HMGB1 (National Center for Biotechnology Information/GenBank accession no. NM_002128), and 5'-GCA CCG TCA AGG CTG AGA AC-3' (sense) and 3'-TGG TGA AGA CGC CAG TGG A-5' (antisense) for GAPDH (NCBI/GenBank accession no. NM_002046).

Measurement of LPS-stimulated HMGB1 production. At indicated timepoints after treatment with LPS (200 ng/ml), levels of HMGB1 in cell culture medium were assayed using a sandwich ELISA, as described[14]. For this ELISA, a monoclonal anti-HMGB1 antibody (Abcam) was used for capture antibody and an anti-HMGB1 antibody (R&D Systems) biotinylated as a second antibody. Streptavidin-alkaline phosphatase (Sigma-Aldrich) was used as a conjugate.

Immunofluorescence assay. Freshly isolated PBMC from patients and controls were cultured on glass coverslips for 4 h, and nonadherent cells were subsequently removed. Adherent monocyte-enriched cultures were left untreated or treated with LPS (200 ng/ml), and fixed in 4% paraformaldehyde for 15 min at room temperature prior to 1% Triton X-100, 10 min at 4°C. Coverslips were saturated with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 30 min at room temperature and processed for immunofluorescence with rabbit anti-HMGB1 polyclonal antibody (1:300; Abcam) followed by FITC-conjugated goat anti-rabbit Ig (1:100; KPL, Gaithersburg, MD, USA). Between incubation steps, cells were washed 3 times for 5 min with PBS containing 0.2% BSA. Fluorescence signals were analyzed using a fluorescence microscope (Olympus, Japan). The fluorescence intensity in the nuclear or cytoplasm region of stained cells was determined using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA).

Measurement of proinflammatory cytokines. Concentrations of TNF-α and IL-6 in the culture supernatants treated with rHMGB1 (1 μg/ml) and LPS (100 ng/ml) and medium alone were measured using commercial ELISA kits (R&D Systems) according to the supplier's instructions. All samples were measured in triplicate.

RT-PCR analysis. We determined expression of TNF-α mRNA by RT-PCR. PBMC were stimulated with rHMGB1 (1 μg/ml), and total RNA was isolated as described above. After treatment with RNase-free DNase I, reverse transcription was performed with the reverse transcription system (Promega) according to the manufacturer's protocol. Primers used in PCR were designed using the online utility Primer 3 (http://frodo.wi.mit.edu/primer3/) as follows: 5'-TCA GCC TCT TCT CCT TCC TG-3' (sense) and 5'-TGA GGT ACA GGC CCT CTG AT-3' (antisense) for TNF-α (NCBI/GenBank accession no. NM_000594); and 5'-AAG GTC ATC CCT GAG CTG AA-3' (sense) and 5'-CCC CTC TTC AAG GGG TCT AC-3' (antisense) for GAPDH as an internal control. The predicted lengths of TNF-α and GAPDH amplification products were 303 bp and 495 bp, respectively. The cDNA was amplified after determining the optimal number of cycles. The cycling conditions were 94°C for 3 min, followed by 28 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final cycle at 72°C for 10 min. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide and were visualized and photographed using ultraviolet transillumination. Relative optical density (OD) values of bands were measured and normalized to values of the housekeeping gene GAPDH.

Statistical analysis. Data were coded and summarized using SPSS version 13.0 for Windows. Quantitative variables were described using mean ± SD and categorical data by frequency and percentage. Data in which variables demonstrated nonparametric distributions were expressed as median with interquartile range (IQR), and analyzed by Mann-Whitney U test for unpaired samples and Kruskal–Wallis test for more than 2 groups. Spearman's rank correlation test was used as a measure of association of quantitative variables. Other data were analyzed with Student's t test for unpaired values to compare differences between different control cultures. One-way ANOVA (LSD and Duncan tests) was used to compare differences of more than 2 groups. A p value < 0.05 was considered statistically significant.

RESULTS

The general and disease characteristics of the 39 SLE patients were representative of our lupus population (Table 1).

We analyzed concentrations of HMGB1 in serum of SLE patients and healthy controls. HMGB1 concentrations in SLE patients were significantly higher (median 27 ng/ml, IQR 68 ng/ml) than those in controls (median 0 ng/ml, IQR 0 ng/m; $p < 0.001$) as shown in Figure 1A. Moreover, serum HMGB1 concentrations in patients with active SLE were higher than those in controls as well as in patients with inactive SLE (Kruskal–Wallis test, $p < 0.001$; Mann-Whitney U test, $p = 0.001$) as shown in Figure 1B. There was a significant difference in the HMGB1 concentrations between patients with and without vasculitis (median 70.5 ng/ml, IQR 50 ng/ml vs median 13.0 ng/ml, IQR 34 ng/ml, respectively; $p < 0.001$). Similarly, we observed a significant difference in the HMGB1 concentrations between patients with and without myositis (median 86 ng/ml, IQR 63.5 ng/ml, vs median 16 ng/ml, IQR 41.5 ng/ml; $p = 0.001$) as shown in Figure 2.

However, the HMGB1 concentrations did not differ between SLE patients with and those without renal disorder. Nor was any difference found when the levels of HMGB1 were compared between patients with and without rash, arthritis, hematological disorder, neurological disorder, serositis, or ocular disease, or who were anti-dsDNA or anti-Sm-positive ($p > 0.05$).

Because the SLE patients in the study were unselected, possible correlations between HMGB1 concentrations and clinical disease manifestations were considered. For the

Table 1. SLE patients' characteristics and disease manifestations (total n = 39).

Characteristic		r	p	Correlation
Female/male, n (%)	34/5 (87.2/12.8)			
Age, mean ± SD, yrs	30.97 ± 8.71	–0.181	0.269	None
Disease duration, mean ± SD, mo	69.20 ± 98.06	9.265	0.103	None
Rash (malar/discoid), n (%)	29 (74.4)			
Arthritis, n (%)	20 (51.3)			
Myositis, n (%)	10 (25.6)			
Vasculitis*, n (%)	16 (41.0)			
Renal disorders, n (%)	20 (51.3)			
Neurological disorder, n (%)	7 (17.9)			
Ocular disease, n (%)	4 (10.3)			
Serositis, n (%)	6 (15.4)			
Hematological disorder, n (%)	13 (33.3)			
C4, mg/l, mean ± SD	127.94 ± 70.35	–0.689	< 0.001	Negative
C3, mg/l, mean ± SD	742.74 ± 305.98	–0.633	< 0.001	Negative
ESR, mean ± SD	61.92 ± 25.13	0.001	0.995	None
CRP, mg/dl	2.72 ± 2.69	0.292	0.071	None
Creatinine kinase, U/l**	1773.6 ± 940.36			
Presence of anti-dsDNA, n (%)	21 (53.8)			
Presence of anti-Sm, n (%)	14 (35.9)			
SLEDAI scores				
Active, n/mean ± SD	29/22.62 ± 10.28	0.504	0.005	Positive
Inactive, n/mean ± SD	10/0.6 ± 0.52			
Total, n/mean ± SD	39/16.97 ± 13.15	0.632	< 0.001	Positive

* Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis[29]. ** Patients with myositis. SLEDAI: SLE Disease Activity Index.

SLEDAI score, HMGB1 concentrations in serum showed a highly significant correlation (rho = 0.632, $p < 0.001$) as shown in Figure 3A. Correlations of HMGB1 concentrations with C4 and C3 levels were significant (correlation coefficient rho = –0.689, –0.633, respectively; both $p < 0.001$; Figure 3B, 3C). But HMGB1 concentrations showed no significant correlations with ESR or CRP ($p > 0.05$).

Similar results were obtained in the 29 patients with active SLE, and analysis also indicated a positive correlation between the HMGB1 concentrations and SLEDAI in those 29 patients (rho = 0.504, $p = 0.005$). There was also an inverse correlation between HMGB1 concentrations and C4 and C3 levels in patients with active disease (rho = –0.557, –0.454 and $p = 0.002$, 0.013, respectively). HMGB1 concentrations were higher in patients with vasculitis than in those without vasculitis ($p = 0.015$). The same was true when patients with and without myositis were analyzed ($p = 0.003$).

LPS-induced extracellular release of HMGB1, cytoplasm translocation, and mRNA expression in mononuclear cells. PBMC were cultured with LPS for 0, 12, 24, and 32 h, and concentrations of HMGB1 released into conditioned supernatants were determined by sandwich ELISA. HMGB1 was not detectable in the culture medium in the absence of LPS in either SLE patients or controls. After incubation with LPS for various times, HMGB1 production increased in a time-dependent manner in both groups. Compared with controls, LPS-induced levels of HMGB1 in SLE patients were higher after 12 h or 24 h incubation (Figure 4A), especially in patients with renal disorder (Figure 4B). There were no significant differences in concentrations of HMGB1 between the groups after 32 h incubation (Figure 4A, 4B). These data indicate that HMGB1 release by SLE PBMC was temporarily elevated following LPS stimulation, compared with normal PBMC.

Immunofluorescence assay was performed to evaluate whether the HMGB1 translocation in active SLE monocytes differed from that in normal monocytes. Adherent cells were left untreated or were treated with LPS for various times. Cytoplasm staining of HMGB1 appeared as early as 3 h after LPS stimulation both in SLE and in normal monocytes (Figure 5B, 5C). The relative intensity of intracellular HMGB1 levels after stimulation for 3, 12, and 24 h was slightly higher in SLE samples, but with no significant difference between the 2 groups (data not shown).

Expression of HMGB1 mRNA in PBMC from patients with active SLE and controls was determined by real-time RT-PCR. As shown in Table 2, the HMGB1 mRNA level was significantly upregulated in unstimulated PBMC from patients with active SLE. However, HMGB1 mRNA levels were not significantly changed by LPS stimulation in the 2 groups. These data suggest that HMGB1 transcription was already activated *in vivo* in patients with active SLE.

Proinflammatory cytokine release by PBMC with stimulation by rHMGB-1. To investigate the role of rHMGB1 in stimulating TNF-α and IL-6 release in SLE, cultures of

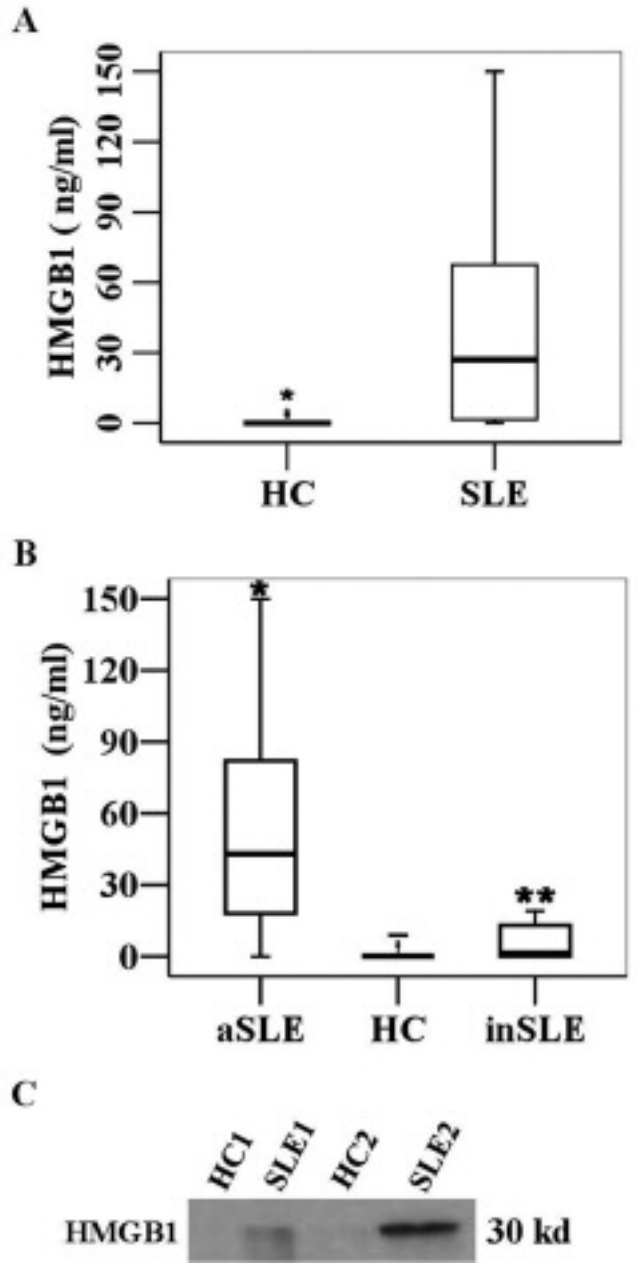

Figure 1. A. HMGB1 concentrations were assessed by Western blot in serum obtained from SLE patients and healthy controls (HC). HMGB1 concentrations in SLE patients were higher than in controls: median 27 ng/ml, IQR 68 ng/ml, versus median 0 ng/ml, IQR 0 ng/ml, respectively. *Mann-Whitney U test, $p < 0.001$, compared with SLE patients. B. HMGB1 concentrations were significantly higher in patients with active SLE (aSLE) than in healthy controls as well as patients with inactive SLE (inSLE). *Kruskal-Wallis test, $p < 0.001$ compared with inSLE and HC; **Mann-Whitney U test, $p = 0.001$ compared with aSLE. Horizontal lines, boxes, and error bars represent median, interquartile range, and maximum and minimum values in the groups, respectively. Lower panel: Representative bands from SLE patients and healthy controls are shown.

PBMC were incubated with rHMGB1 and LPS, and medium alone were used as positive and negative control conditions, respectively. At different timepoints, cells and cell supernatants were harvested. The concentrations of TNF-α and IL-6 released into conditioned supernatants were measured by ELISA. Spontaneous IL-6 production was detected in SLE patients (79.71 ± 33.95 pg/ml), but not in healthy controls. Spontaneous TNF-α production in SLE patients was comparable to that in controls (84.93 ± 3.23 pg/ml in SLE, 111.64 ± 65.37 pg/ml in controls; $p > 0.05$). In agreement with a previous report[4], TNF-α release after rHMGB1 stimulation was biphasic and was delayed compared with LPS stimulation (Figure 6A, 6B). The most striking observation was that TNF-α level was continuously low, without a peak after rHMGB1 stimulation, in patients with active SLE, but not in controls or patients with inactive SLE. The trypan blue exclusion analysis and MTT assays confirmed that neither rHMGB1- nor LPS-induced cytokine release was due to the cell death and altered proliferation of the cultured cells (data not shown).

As shown in Figure 6C, rHMGB1-induced production of IL-6 in every group was biphasic and increased in a time-dependent manner, and rHMGB1-induced IL-6 levels by PBMC from patients with active SLE were delayed by 2 h and more compared with controls. LPS-induced IL-6 levels of both SLE- and control-derived PBMC supernatants were increased in a time-dependent manner (Figure 6D). These results indicate that rHMGB1-induced IL-6 release by PBMC from patients with active SLE was markedly higher and delayed before reaching a peak.

We then measured the expression of TNF-α mRNA using RT-PCR. PBMC from patients with active SLE and controls were cultured with rHMGB1 for 10 h. According to Andersson, *et al*[4], the TNF-α mRNA reaches peak levels after stimulation with rHMGB1 for 10 h. As shown in Figure 7, TNF-α mRNA levels in active SLE PBMC were downregulated compared with normal PBMC ($p < 0.001$).

DISCUSSION

In agreement with previous findings[14,15], HMGB1 concentrations in serum were higher in patients with SLE than in healthy controls, which confirms the association of elevated levels of circulating HMGB1 with SLE in the Chinese population. In addition, HMGB1 concentrations were correlated positively with SLEDAI scores and negatively with C4 and C3 levels, which further confirms that HMGB1 may be involved in the inflammatory process of this disease. Despite presence of renal disorder, we found no association between HMGB1 and other organ involvement in SLE. These data suggest that HMGB1 may play a more critical role in disease activity than in organ damage. Studies have shown that HMGB1 release can occur in apoptosis[33,34], and this protein may be released along with other nuclear constituents to form immune complexes[15,34]. As the removal of apoptotic debris is abnormal in patients with lupus[1], our results suggest that increased circulating HMGB1 levels may result from the decreased uptake of apoptotic cells.

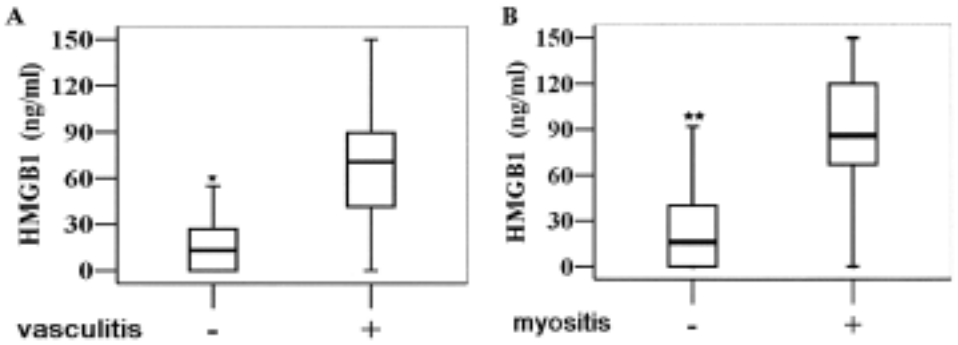

Figure 2. A. HMGB1 concentrations in SLE patients with vasculitis and without vasculitis: median 70.5 ng/ml, IQR 50 ng/ml versus median 13.0 ng/ml, IQR 34 ng/ml, respectively. *Mann-Whitney U test, $p < 0.001$. B. HMGB1 concentrations in SLE patients with myositis and without myositis: median 86 ng/ml, IQR 63.5 ng/ml, versus median 16 ng/ml, IQR 41.5 ng/ml, respectively. **Mann-Whitney U test, $p = 0.001$. HMGB1 concentrations were assessed by Western blot.

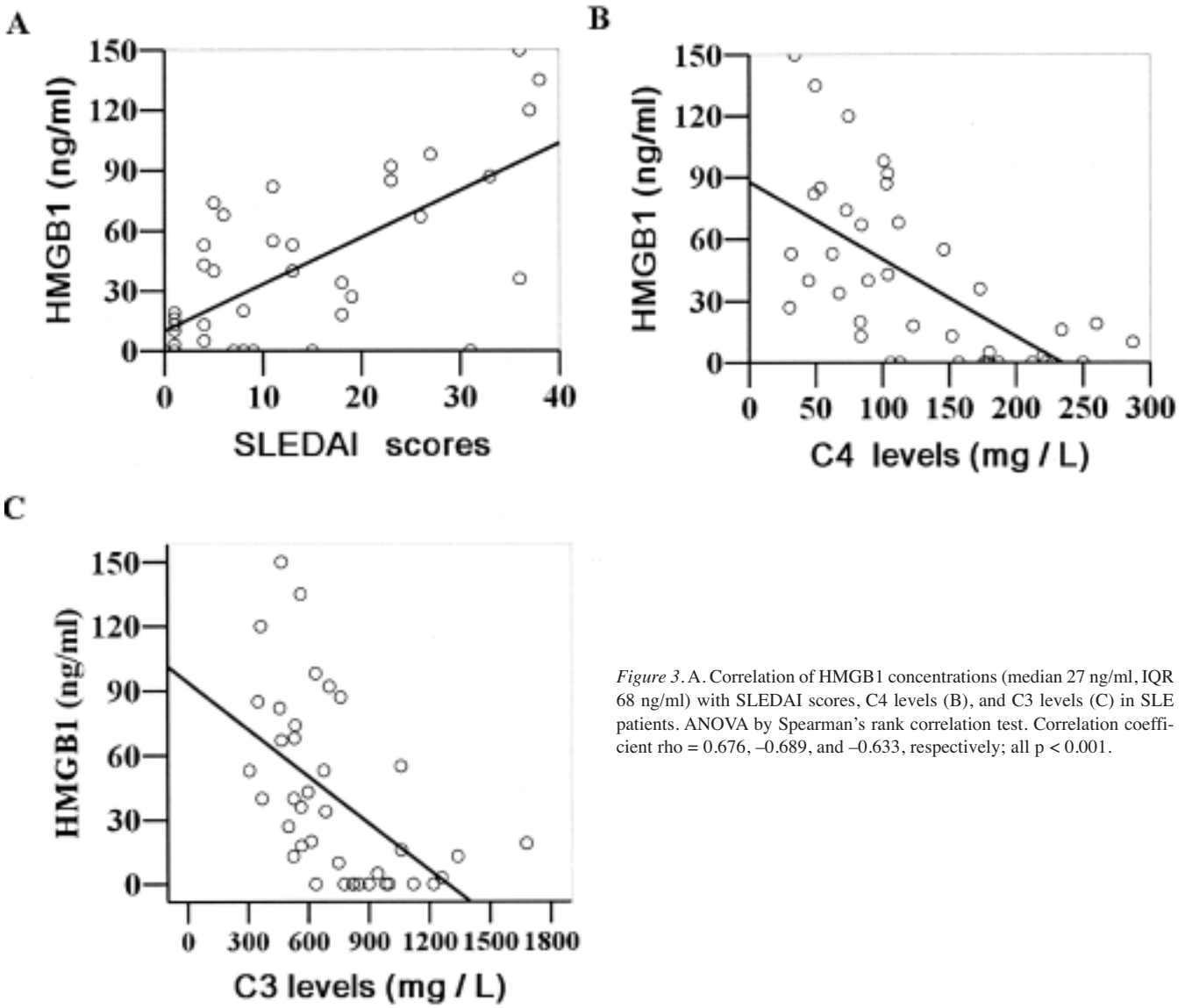

Figure 3. A. Correlation of HMGB1 concentrations (median 27 ng/ml, IQR 68 ng/ml) with SLEDAI scores, C4 levels (B), and C3 levels (C) in SLE patients. ANOVA by Spearman's rank correlation test. Correlation coefficient rho = 0.676, –0.689, and –0.633, respectively; all $p < 0.001$.

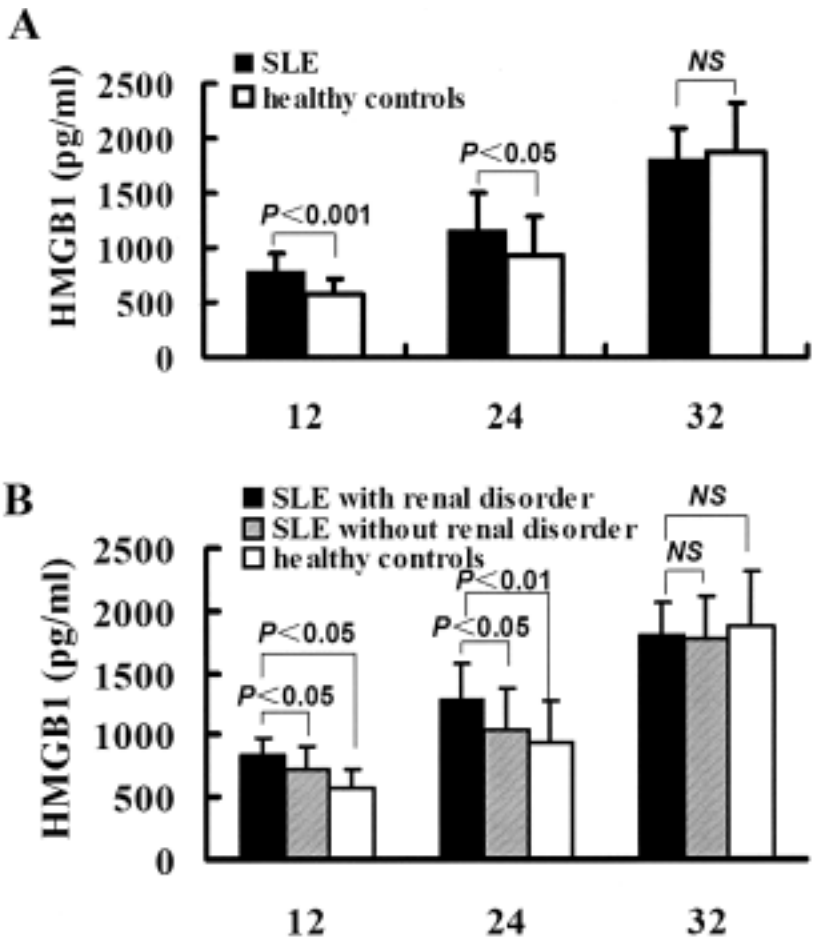

Figure 4. A. LPS-induced production of HMGB1 by PBMC from SLE patients (n = 39) and controls (n = 24) at indicated timepoints. Concentrations of HMGB1 released into conditioned supernatants were determined by ELISA. After stimulation with LPS, HMGB1 was released in a time-dependent manner in both groups. HMGB1 levels in SLE patients were higher than those in healthy controls, after 12 h or 24 h incubation (782.0 ± 167.87 pg/ml vs 564.57 ± 153.50 pg/ml, respectively, at 12 h; 1165.08 ± 328.16 pg/ml vs 933.68 ± 345.70 pg/ml at 24 h). There was no significant difference between the 2 groups after 32 h incubation ($p > 0.05$). Statistical analyses were performed using Student's t test. B. HMGB1 levels in SLE patients with renal disorder were higher than those in patients without renal disorder and in controls, after 12 h or 24 h incubation (836.41 ± 143.72 pg/ml, 724.73 ± 175.88 pg/ml, 564.57 ± 153.50 pg/ml, respectively, at 12 h; 1284.33 ± 286.36 pg/ml, 1039.55 ± 329.17 pg/ml, 933.68 ± 345.70 pg/ml, respectively, at 24 h. Analysis of variance by one-way ANOVA test (LSD and Duncan tests). NS: not significant.

HMGB1 has been implicated in the pathogenesis of lupus nephritis[25-27]. This observation (Figure 4) suggests that SLE patients with renal disorder may be prone to produce more HMGB1 than healthy subjects when triggered by inflammatory stimuli. We also observed that the levels of HMGB1 mRNA in quiescent PBMC from patients with active SLE were upregulated, while mRNA levels were not affected by stimulation with LPS in SLE patients and healthy controls (Table 2). These results indicate that the increased extracellular release of HMGB1 by PBMC from SLE patients is unlikely to be linked to increased transcription of the gene.

In patients with RA, TNF-α release by synovial fluid macrophages peaks at 6 h after stimulation with HMGB1 and tends to decrease after 12 h[11]. Our results showed significantly decreased TNF-α production by mononuclear cells from patients with active SLE when stimulated with rHMGB1 (Figure 6A). However, there is abundant literature data that, in contrast to some mouse models, TNF-α is not increased only in SLE sera, but also in lupus nephritis, suggesting that TNF-α may have a dual role in lupus[1,35]. Our findings may result from the moderate to severe disease activity of our patients. Based on these observations, we speculate that the role of TNF-α in the circulation or locally in sites of stimulation may be different in lupus. This finding is partly in contrast to one report that LPS-stimulated

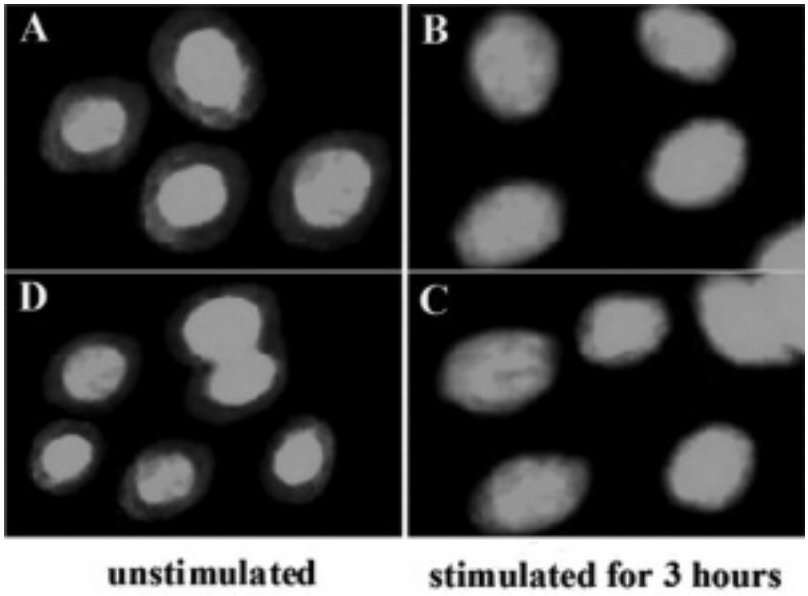

Figure 5. Comparison of LPS-stimulated HMGB1 cytoplasm translocation in monocytes from patients with active SLE (A, B) and healthy controls (C, D). Monocyte-enriched cells were left untreated or stimulated with LPS for various periods, and were fixed, permeabilized, and stained for HMGB1 using indirect immunofluorescence assay. Representative cells from untreated SLE patients (A) and healthy controls (D) are shown, in which HMGB1 (lighter grey areas) was predominantly in the nuclear region. Following stimulation with LPS, HMGB1 was translocated from the nucleus to the cytosol as early as 3 h in both SLE patients (B) and controls (C). Original magnification ×400.

Table 2. Relative quantification of HMGB1 mRNA expression in PBMC from SLE patients and healthy controls.

	Unstimulated	Stimulated for 12 h	24 h	32 h
Controls	1	1.628	1.034	0.679
SLE	10.11*	0.973	1.125	1.428

As target gene, HMGB1 expression was normalized to housekeeping gene expression and presented as n-fold of the expression in controls. * $p < 0.01$ versus unstimulated PBMC from controls. HMGB1 gene expression after stimulation with LPS was presented as n-fold of the expression in unstimulated cells from every group.

production of TNF increases in the whole-blood cell cultures of patients with SLE[36]. However, another study showed a decrease in TNF-α production by cultured SLE monocytes stimulated with LPS[37]. Further, significantly decreased TNF-α production was reported following stimulation of PBMC and peripheral blood leukocytes with phytohemagglutinin[38]. These findings, together with our own, suggest that in SLE, the immunocompetent cells may be deficient in producing TNF-α, especially upon stimulation with specific stimuli like HMGB1. TNF-α is a key cytokine mediating innate and adaptive immune responses, and a peak induction of TNF-α within 24 h is one of the characteristics of host innate immune responses[39]. The impaired level of TNF-α after rHMGB1 stimulation may indicate ineffectiveness of innate immunity in active SLE. It is also well recognized that defects in innate immune responses contribute to autoimmunity[40]. The weak production of TNF-α induced by rHMGB1 suggests that HMGB1 may participate in lupus pathogenesis by inhibiting a peak and sufficient production of TNF-α. However, chronic exposure to proinflammatory stimuli causes counterregulation. The decreased TNF-α release in SLE patients may lead to negative feedback causing an overproduction of HMGB1 through which a normal immune response is achieved in the defective production of TNF-α.

From our observations, the decreased production of TNF-α by PBMC from active SLE after stimulation with rHMGB1 may result, at least in part, from the downregulated expression of TNF-α mRNA (Figure 7). It has been reported that IL-6 mRNA expression cannot be induced by HMGB1 stimulation[5]. In addition, HMGB1 fails to stimulate cytokine release in lymphocytes[4]. Therefore, TNF-α and IL-6 were produced mainly by monocytes in PBMC after stimulation with rHMGB1. We speculate that the increased release of IL-6 (Figure 6C) may be due in part to the lymphocytopenia in active SLE.

Our investigation suggests that circulating HMGB1 could potentially be used in the assessment of disease activity in patients with SLE. Further studies with larger samples are needed to verify our findings and to clarify the clinical relevance of HMGB1 during disease development in SLE. Secretion of TNF-α and IL-6 by PBMC from SLE patients

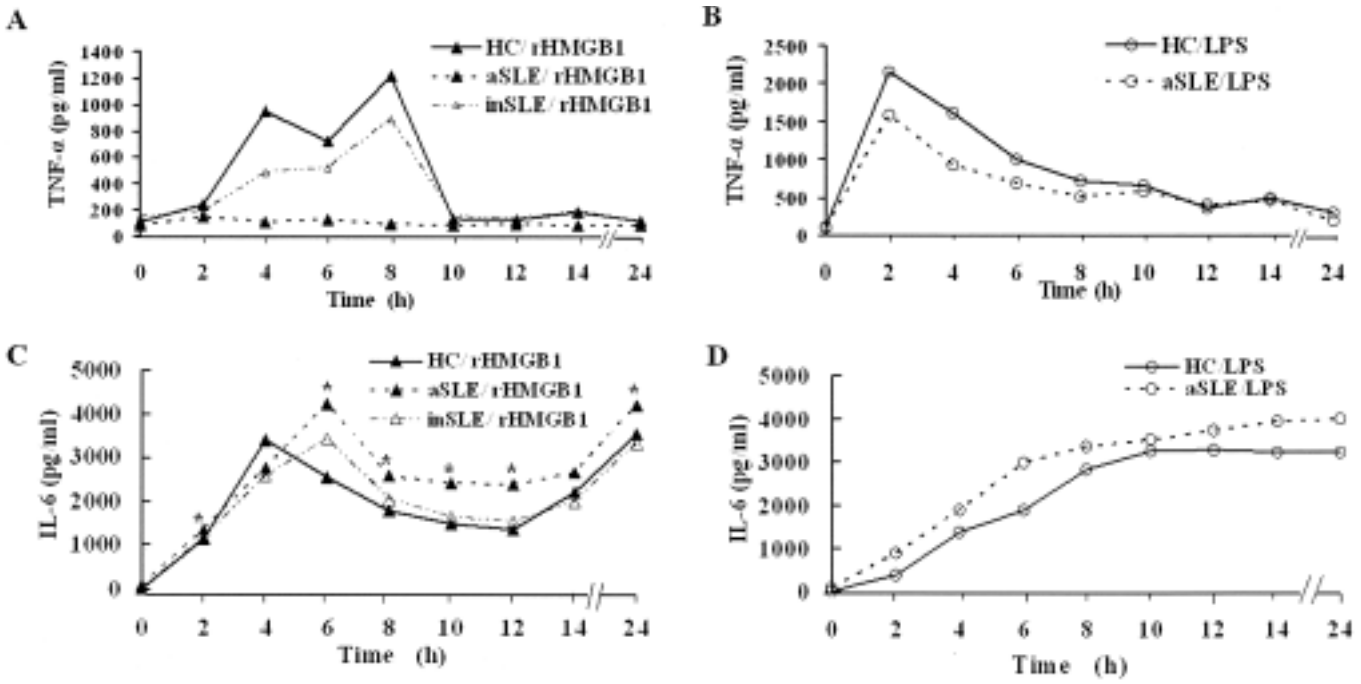

Figure 6. Production of TNF-α and IL-6 by PBMC stimulated with LPS and rHMGB1. PBMC were obtained from patients with active SLE (aSLE), inactive SLE (inSLE), and healthy controls (HC), cultured in triplicate with LPS and rHMGB1 and medium alone. Levels of TNF-α and IL-6 (pg/ml) were measured by ELISA; data are mean ± SD. A. TNF-α release after rHMGB1 stimulation was very low and with no peak in aSLE. B. LPS-induced production of TNF-α peaked early at 2 h both in aSLE and HC. C. After stimulation with rHMGB1, IL-6 release by aSLE PBMC was delayed by 2 h and was greater compared with normal PBMC. *$p < 0.05$ vs rHMGB1-stimulated normal PBMC at the same timepoints. ANOVA by Student's t test. D. LPS-induced production of IL-6 increased in a time-dependent manner in both aSLE and HC. n = 7 in every group.

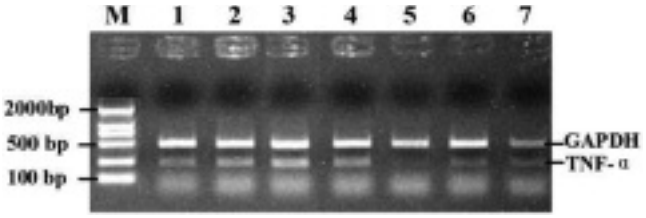

Figure 7. Expression of TNF-α mRNA in PBMC was determined by RT-PCR after stimulation with rHMGB1 for 10 h. Relative optical density (OD) values of bands were measured and normalized to values of the housekeeping gene GAPDH. Levels of TNF-α mRNA in patient with active SLE (lanes 5-7) were downregulated compared with healthy controls (lanes 1-4). Relative OD values were 0.35 ± 0.09 in patients and 0.72 ± 0.08 in controls. Student's t test, $p < 0.001$. N = 7 in every group.

was also altered following rHMGB1 stimulation; however, further studies are required to determine the mechanisms of HMGB1 and other proinflammatory cytokines in the pathogenesis of lupus.

ACKNOWLEDGMENT

We gratefully acknowledge Jianbin Tong and Bo Li for excellent technical assistance. We appreciate the provision of some samples used in this study by the Department of Rheumatology, Xiangya Hospital, Central South University, Changsha, China.

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