Immune complexes induce TNF-α and BAFF production from U937 cells by HMGB1 and RAGE

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Abstract. – OBJECTIVE: This study investigated the effects of immune complexes (ICs) on tumor necrosis factor α (TNF-α) and B cell-activating factor (BAFF) production from U937 cells and further explored the mechanism.

MATERIALS AND METHODS: U937 cells were incubated with necrosis supernatant or systemic lupus erythematosus (SLE) sera alone, or their combination. The expression of TNF-α and BAFF was determined by Real-time polymerase chain reaction and enzyme-linked immunosorbent assay. High mobility group box 1 (HMGB1) A-box was produced by gene recombination. HMGB1 A-box and anti-receptor for advanced glycation end products (RAGE) antibody were adopted in the blocking experiments. The importance of DNA for cytokine induction was investigated by DNase treatment.

RESULTS: The combination of necrosis supernatant and SLE sera induced the expression of TNF-α and BAFF significantly increased compared to necrosis supernatant or SLE sera alone. Recombinant HMGB1 A-box protein 1 (HMGB1) A-box was produced by gene recombination. HMGB1 A-box and anti-receptor for advanced glycation end products (RAGE) antibody were adopted in the blocking experiments. The importance of DNA for cytokine induction was investigated by DNase treatment.

CONCLUSIONS: ICs containing DNA can promote TNF-α and BAFF production in U937 cells, and this process can be mediated by HMGB1 and RAGE. One possible mechanism of increasing BAFF production in SLE is proposed in this study whereby B cell activation, antibody production and ICs stimulated monocytes may create a vicious cycle that leads to B cell hyperactivity, which can be of importance for SLE etiopathogenesis.

Key Words: Immune complexes, U937 cells, High mobility group box protein 1, Receptor for advanced glycation end products, Systemic lupus erythematosus.

Introduction

Systemic lupus erythematosus (SLE) is a multi-systemic involvement and chronic progressive autoimmune disorder characterized by production of autoantibodies against nucleic components such as DNA and nucleosomes and formation of immune complexes (ICs) due to polyclonal B cell activation. Being inactive in its free form, however, mammalian DNA in the form of ICs has potent immunostimulatory properties. The necessity for complex formation suggests that endogenous DNA must exist in association with another moiety or structure to enable access to the corresponding receptor. High mobility group box protein 1 (HMGB1) is known as a non-histone nuclear protein that binds DNA in a non-sequence-specific manner, modifying chromosomal architecture and facilitating gene transcription. HMGB1 has been highlighted as a pro-inflammatory mediator when it is released from cells. Binding of HMGB1 to the receptor for advanced glycation end products (RAGE), Toll-like receptor-2 (TLR-2) or TLR-4 can lead to the release of pro-inflammatory cytokines. However, highly purified HMGB1 has a weak or no pro-inflammatory activity; for full activity, it may need to form a complex with another component to activate inflammation by enhancing effects of other pro-inflammatory-
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Materials and Methods

Culture of U937 Cells and Induction of Cell Necrosis

The human monocyte line U937 (ATCC 1593) was cultured in Modified Roswell Park Memorial Institute (RPMI)-1640 medium (TransGen Biotech, Beijing, China) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA), L-glutamine (2 mM, MP Biomedicals, Shanghai, China), HEPES (20 mM, MP Biomedicals, Shanghai, China), penicillin (60 μg/ml, Solarbio, Beijing, China), and streptomycin (100 μg/ml, Solarbio, Beijing, China), at 37°C in 5% CO₂. The induction of U937 cell necrosis was performed by repeat freeze thawing as previously described14. Cell debris was removed by centrifugation (400 g for 10 min, GTR22-1, Beijing Era Beili Centrifuge Co., Ltd, Beijing, China) and necrotic cell supernatant was isolated and stored at -80°C for subsequent preparation of ICs.

Serum Preparation

SLE sera were prepared from the blood samples of SLE patients in our hospital who fulfilled the American College of Rheumatology (ACR) 1997 revised criteria for the classification of SLE23. Venous blood was collected from SLE patients during a flare or with active disease based on clinical classification criteria into tubes without anticoagulants. After isolated, sera were screened by anti-dsDNA antibody ELISA test (Euroimmun, Lübeck, Germany). Only sera with positive anti-dsDNA titer greater than 1:100, Shanghai KaLang Biological Technology (Co., Ltd, Shanghai, China) were selected and mixed. The mixed sera were passed through a 0.45 μm filter and stored at -80°C. Also, sera from healthy blood donors were prepared and used in some control experiments. All the samples were collected after informed written consent was obtained from all participants, and this study was approved by Ethics Committee of Shandong University.

Preparation of Immune Cells (ICs)

Necrotic U937 cell supernatant and mixed SLE sera obtained above were used for preparation...
of ICs. ICs were prepared according to previous reports. In this study, ICs were preformed by combining 0.5% (vol/vol) SLE sera and 12.5% (vol/vol) necrosis supernatant in the complete medium according to our preliminary experiments. The control groups were designed as follows: 12.5% necrosis supernatant or 0.5% SLE sera alone, the combination of 12.5% necrosis supernatant and 0.5% normal sera, or 0.5% normal sera alone. We found that the combination of 12.5% necrosis supernatant and 0.5% normal sera did not modify the effect of necrosis supernatant alone, and 0.5% normal sera alone showed no stimulatory activity (data not shown).

**Expression and Purification of Human HMGB1 A-box**

Sequence of human HMGB1 A-box was optimized with OptimumGeneTM for expression in *E. coli* and synthesized (GenScript, Piscataway, NJ, USA). Target sequence was ligated into a bacterial expression vector (pQE-T7-2; Qiagen, Hilden, Germany). After transformed by the identified recombinant vector pQE-A-box, *E. coli* BL21 was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Boston Biomedical, Inc., Boston, MA, USA) (1 mmol/L) for 4 h at 37°C. The recombinant protein in bacterium lysates was analyzed by SDS-PAGE and identified by western blot using rabbit anti-human HMGB1 polyclonal antibodies (Abcam, Cambridge, MA, USA) (Table I). The housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) EarthOx (San Francisco, CA, USA), was used as an internal control. The PCR was performed for 30 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 40 s. The relative gene expression of TNF-α and BAFF was analyzed by the 2–ΔΔCt method and was depicted as the extent of change with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values.

**Cytokine Induction**

U937 cells were incubated at a density of 1×10⁶ cells/ml in complete medium containing preformed ICs, necrosis supernatant or SLE sera alone in 24-well flat-bottomed plates (Corning, New York, NY, USA) at 37°C and 5% CO₂. The cells were collected by centrifuging at 200 g for 5 min at 4°C into 1.5 ml Eppendorf centrifuge tubes (Beijing Biocen Biotechnology Co., Ltd, Beijing, China). Also, DNase or RNase-treated ICs were used in some experiments. DNase I Roche Diagnostics product, Co., Ltd. (Shanghai, China) or RNase A (Sigma-Aldrich, St. Louis, MO, USA) of different concentrations was added to the medium containing preformed ICs. After 1 h incubation at 37°C, the enzyme-treated ICs were used as described above.

**TNF-α and BAFF mRNA Assays**

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 1 μg of total RNA from each sample was used as a template in the reverse transcription reaction to synthesize cDNA with random hexamer primers according to Fermentas RT kit (Fermentas, Glen Burnie, MD, USA). All samples were reverse-transcribed under the same condition (42°C for 60 min) and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. The cDNA (2 μl) was amplified by Real-time PCR using a QuantiTect SYBR Green kit (TaKaRa, Otsu, Shiga, Japan) in ABI 7500 PRISM system (ABI, Foster City, CA, USA). The primers were synthesized and validated by Sangon Bio (Co. Ltd., Shanghai, China) (Table I). The housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) EarthOx (San Francisco, CA, USA), was used as an internal control. The PCR was performed for 30 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 40 s. The relative gene expression of TNF-α and BAFF was analyzed by the 2–ΔΔCt method and was depicted as the extent of change with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values.

**TNF-α and BAFF Protein Assay**

Supernatants from U937 cultures were harvested after 24 h stimulation, and TNF-α and BAFF protein levels were measured by sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The sensitivity for TNF-α and BAFF determination is 1.6 and 2.43 pg/ml, respectively.

**Blocking test**

HMGB1 A-box was generated by gene recombination as described herein. Rabbit anti-human RAGE antibody was purchased from Abcam (Cambridge, MA, USA). HMGB1 A-box and anti-RAGE antibody at different doses were respectively added to the U937 cultures before the addition of preformed ICs.

**Statistical Analysis**

All analyses were based on three or more separate experiments. Data were presented as mean ± standard deviation. One-way ANOVA followed by the Bonferroni test for multiple groups or Student’s *t*-test for two groups was used (*p<0.05* was considered to be significant). Statistical analyses were performed by the SPSS16.0 (SPSS Inc., Chicago, IL, USA) statistical package.
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Results

ICs augment TNF-α and BAFF Expression in U937 Cells

Necrotic cells release significant quantities of nuclear antigens, which bind specific autoantibodies in SLE sera to form ICs. We investigated whether the combination of necrosis supernatant and SLE sera modified the immunostimulatory activity of either necrosis supernatant or SLE sera alone. To determine the kinetics of cytokine expression, U937 cells were stimulated for 1, 4, 8, 16, or 24 h with necrosis supernatant combined with SLE sera, necrosis supernatant or SLE sera alone. We detected maximal TNF-α mRNA at about 4 h of stimulation, while BAFF mRNA expression peaked around 8 h (Figure 1a). The remaining experiments in this study were based on

![Graphs showing the expression of TNF-α and BAFF](image)

**Figure 1.** Necrosis supernatant combined with SLE sera activates TNF-α and BAFF production in U937 cells. (a) U937 cells were stimulated with 12.5% necrosis supernatant, 0.5% SLE sera or their combination. Cells were harvested at the indicated time points for RNA. The relative expression of TNF-α and BAFF was determined by Real-time PCR and was depicted as the extent of change on GAPDH values. (b) Expression of TNF-α and BAFF mRNA at the 8 h time point after stimulation. (c) ELISA performed on the supernatants of the stimulated cells to detect TNF-α and BAFF production at the 24 h time point.
this data. The mRNA expression of both TNF-α and BAFF in the group of necrosis supernatant combined with SLE sera was significantly higher than that in untreated, necrosis supernatant or SLE sera alone group (p<0.05 for TNF-α and p<0.01 for BAFF) (Figure 1b). Higher TNF-α mRNA expression was observed in necrosis supernatant group compared to the untreated group, but no statistical difference was reached. Also, no significant difference in BAFF mRNA expression was found between necrosis supernatant and untreated groups. SLE sera alone showed no significantly stimulating effect in both TNF-α and BAFF mRNA expression. To demonstrate that the increases in TNF-α and BAFF mRNA levels were accompanied by protein production, ELISA was performed on the supernatants of U937 cells stimulated with necrosis supernatant combined with SLE sera, necrosis supernatant or SLE sera alone for 24 h. The results were consistent with the mRNA data (Figure 1c). These data suggested that the combination of necrosis supernatant and SLE sera promoted a robust TNF-α or BAFF production from U937 cells.

Identification and Purification of Recombinant Human HMGB1 A-box Protein

We established the high-efficiency expression system E. coli BL21-pQE-A-box by gene recombination technique and induced the expression of target protein. sodium Sodium dodecyl sulpha-

te-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the relative molecular mass (Mr) of the recombinant protein was about 14 kD, which was consistent with the expected (Figure 2a). We identified the recombinant protein as HMGB1 A-box by Western blot (Figure 2b). Then, by Ni²⁺-NTA affinity chromatography and dialysis, we obtained purified recombinant human HMGB1 A-box protein (Figure 2c).

Antagonizing HMGB1 or Blocking RAGE Inhibits the Stimulatory Activity of ICs

To further investigate whether the stimulatory activity of necrosis supernatant combined with SLE sera on monocytic U937 cells was initiated by HMGB1 and RAGE, we adopted the blocking reagent, anti-RAGE antibody and recombinant human HMGB1 A-box, which has been shown to function as HMGB1 antagonist. U937 cells were treated with the combination (necrosis supernatant and SLE sera) alone or in the presence of anti-RAGE or A-box. We found that both HMGB1 A-box (20 μg/ml) and anti-RAGE (0.5 μg/ml) antibody significantly reduced TNF-α mRNA expression induced by necrosis supernatant combined with SLE sera in U937 cells (p<0.05) (Figure 3a). The TNF-α mRNA level in the blocked ICs group was higher than that in the untreated group, but no statistical significance was achieved. Moreover, we observed that either anti-RAGE (0.5 μg/ml) or HMGB1 A-box (20 μg/
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BAFF-inducing activity (p<0.05) (Figure 4a and b, respectively). However, RNase treatment was unable to lessen ICs-induced TNF-α and BAFF production in U937 cells (data not shown). Thus, these results demonstrated that the DNA component of ICs was important for inducing cellular activity.

Discussion

ICs can activate the immune cells to produce cytokines13,22,26. In this study we have investigated the possibility that ICs may stimulate the production of TNF-α and BAFF in SLE. We have demonstrated that ICs can significantly promote the expression of TNF-α and BAFF from human monocytic U937 cells. Furthermore, our data have provided evidence that HMGB1 can also induce ICs to mediate U937 activation through a mechanism involving RAGE, and the DNA component of ICs was necessary for its immunostimulatory activity. Recent reports26,27 have demonstrated that lupus ICs induce IFN-α and TNF production by PBMCs or PDCs. Here we observed that material from necrotic U937 cells, when combined with SLE sera, augmented TNF-α and BAFF production from U937 cells. Although necrotic cells were considered to be pro-inflammatory13, however, necrotic supernatant alone didn’t show significantly stimulatory activity in this study, which was consistent with a previous finding that necrotic material alone failed to induce IFN-α from PBMCs12. It wasn’t surprising that SLE sera alone were inactive, because the concentration of circulating ICs in serum was low and the sera amount used in this study was much smaller compared to that in previous reports26. Given that nuclear material is released by necrotic cells after freeze-thawing due to a rapid loss of membrane integrity, and autoantibodies specific to nucleic components are present in SLE sera, a likely reason for the stimulatory activity of the combination of necrosis supernatant and SLE sera is the formation of ICs. TNF is a strong mediator of inflammatory responses and is implicated in the tissue inflammation and damage. TNF is increased in the serum of active SLE and in renal tissue with active nephritis, and the levels of TNF is correlated with renal disease activity6,28. The present work showed that the combination of necrosis supernatant and SLE sera stimulated TNF-α production from U937 cells, which suggests that ICs may be an important

| ![Image](image.png) |

**Figure 3.** HMGB1 A-box or anti-RAGE antibody inhibits TNF-α and BAFF production from U937 cells induced by preformed immune complexes (ICs). HMGB1 A-box or anti-RAGE antibody at indicated concentrations was added to the U937 cultures prior to the addition of preformed ICs. Cells were harvested at the 8 h time point for RNA. The relative expression depicted as the extent of change with respect to GAPDH values: (a) TNF-α and (b) BAFF.

ml) significantly inhibited BAFF mRNA expression triggered by the combination of necrosis supernatant and SLE sera (p<0.05) (Figure 3b). The blocked ICs group showed higher BAFF mRNA level compared to the untreated group, whereas no significant difference was found. These data suggested that the TNF-α and BAFF expression induced by the combination of necrosis supernatant and SLE sera in U937 cells were mediated by HMGB1 and RAGE.

The DNA Component of ICs Contributes to Cellular Activity

To test our hypothesis that the DNA component in ICs is necessary for inducing cellular activation, we stimulated U937 cells with ICs, DNase or RNase-treated ICs. We found that DNase (at a final concentration of 20 u/ml) treatment of ICs significantly neutralized their TNF-α and
factor responsible for elevated level of TNF-α in the serum and renal tissue of patients with active SLE. Also, monocytes activated by ICs may be a critical source of TNF-α. TNF-α can mediate monocyte migration and induce human endothelial cell activation and monocyte-endothelial cell adhesion, which facilitates monocyte chemotaxis and deposition to tissues. Renal pathology shows that lupus nephritis is characterized by ICs deposition and inflammatory cell infiltration. In this study, mononuclear cell infiltration of the kidney was observed during the initial murine lupus nephritis. Moreover, data from patients with lupus nephritis demonstrated that infiltrating monocytes vigorously participate in renal inflammation and injury. Thus, TNF-α over-expression of monocytes induced by ICs will promote the inflammatory course and aggravate tissue injury in SLE. Abnormal activation of B cells and production of pathogenic autoantibodies are important contributors to the development of SLE. BAFF is a prominent factor critical for B cell activation and function. BAFF deficiency results in decreased peripheral B cells and a diminished ability to mount robust humoral immune responses, whereas transgenic murine overexpressing BAFF developed a SLE-like syndrome. Moreover, circulating BAFF levels are elevated and correlate positively with anti-dsDNA antibody titers and disease activity in SLE patients. These data suggest that BAFF is closely associated with elevated production of anti-DNA antibodies and it is likely to play a critical role in the activation of antigen-driven autoimmune B cells in human SLE. Notably, the present study indicates that ICs have the potential to drive the production of BAFF from monocytes in SLE patients. Our finding provides an explanation for the ongoing BAFF production that is commonly found in SLE patients, and also we have suggested that the mechanism is to be pivotal in the population expansion of autoreactive B cells. In vivo high-level of BAFF may relax B cell selection, which leads to the increase of B cells hyper-reactive to auto-antigens. These B cells produce pathological auto-antibodies common to nucleic antigens, exacerbating SLE disease state. Thus, this established effect of ICs may contribute to the induction and development of SLE. Several investigations suggested that HMGB1 is associated with circulating DNA/nucleosomes-containing ICs from SLE patients and is required for the immunostimulatory activity of ICs. The pro-inflammatory activity of HMGB1 is mostly attributed to its ligation with RAGE, which is the first described receptor for HMGB1, and is expressed on monocytes, macrophages and other cell types. Many kinds of biological effects of HMGB1 are proposed to be mediated by RAGE. Our recent report has also shown that ICs can induce endothelial cell dysfunction via the HMGB1-RAGE axis in the pathogenesis of SLE vasculitis. Here the capacity of ICs to induce TNF-α and BAFF expression was inhibited by HMGB1 antagonist A-box, which suggests that HMGB1 plays a critical role in the expression of TNF-α and BAFF. Moreover, the capacity of ICs to trigger TNF-α and
BAFF production was also inhibited by anti-RAGE antibody, which further suggests that RAGE is involved in response to ICs. Altogether, these data demonstrate that the effects of ICs on TNF-α and BAFF expression in U937 cells are mediated via HMGB1 and RAGE. Our observations are consistent with previous findings that HMGB1 was critical in the pro-inflammatory activity of lupus-associated ICs. These findings provide evidence that ICs can activate the immune cells by HMGB1, and, thereby, may contribute to the loss of immunological tolerance against self-antigens. The sticky nature of HMGB1 enables it to synergize with and complex with other molecules such as DNA. Thus, HMGB1 has been regarded as one of the components in DNA-containing ICs. The present finding that the DNA component was required for ICs’ activity, which suggests that HMGB1 and DNA component are both involved in the immune-stimulatory effect of ICs in patients with SLE. Our data support previous reports that ICs stimulate the activation and cytokine production of immune cells via cooperative interactions among different receptors, in which TLR, Fc-receptor and RAGE are involved. Therefore, one explanation for the enhanced immune-stimulatory activity of the complex is that the components of ICs can synergize and cooperate to cellular effects through the participation of different receptors. In view of established roles for Fc-receptor, TLR-9 and RAGE in response to ICs, the exact roles of the components in ICs as well as the downstream mechanism remain to be further clarified.

Conclusions
Our data are in line with a model in which necrotic cells release HMGB1. HMGB1 binds to DNA-containing ICs in serum and then the resultant complexes stimulate TNF-α and BAFF production from monocytes by HMGB1 and RAGE. Also, the effect observed here provides a possible mechanism for increasing BAFF production in SLE, whereby B cell activation, antibody production and ICs stimulated monocytes create a vicious cycle that leads to B cell hyperactivity. Our results collectively provided evidence that ICs containing DNA are implicated in monocyte disorder, which may contribute to the immune dysregulation in SLE, and that interference with the action of HMGB1 in these ICs could be one strategy for the development of new therapies.

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Conflict of interest
The authors declare no conflicts of interest.

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