

Toll-like receptor 9–dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE

Jane Tian¹, Ana Maria Avalos², Su-Yau Mao¹, Bo Chen¹, Kannaki Senthil¹, Herren Wu¹, Peggy Parroche³, Stacey Drabic¹, Douglas Golenbock³, Cherilyn Sirois³, Jing Hua⁴, Ling Ling An¹, Laurent Audoly¹, Greg La Rosa⁵, Angelika Bierhaus⁶, Peter Naworth⁶, Ann Marshak-Rothstein², Mary K Crow⁴, Katherine A Fitzgerald³, Eicke Latz³, Peter A Kiener¹ & Anthony J Coyle¹

Increased concentrations of DNA-containing immune complexes in the serum are associated with systemic autoimmune diseases such as lupus. Stimulation of Toll-like receptor 9 (TLR9) by DNA is important in the activation of plasmacytoid dendritic cells and B cells. Here we show that HMGB1, a nuclear DNA-binding protein released from necrotic cells, was an essential component of DNA-containing immune complexes that stimulated cytokine production through a TLR9–MyD88 pathway involving the multivalent receptor RAGE. Moreover, binding of HMGB1 to class A CpG oligodeoxynucleotides considerably augmented cytokine production by means of TLR9 and RAGE. Our data demonstrate a mechanism by which HMGB1 and RAGE activate plasmacytoid dendritic cells and B cells in response to DNA and contribute to autoimmune pathogenesis.

Systemic lupus erythematosus (SLE), the second most common human autoimmune disease, is characterized by autoantibodies to nuclear ‘autoantigens’, including DNA, RNA-associated proteins, core histones and chromatin¹. Although the defects in normal immune homeostasis underlying this distinct autoantibody profile remain unclear, impaired clearance of apoptotic cells by macrophages, which leads to secondary necrosis, represents at least one mechanism for the immune dysregulation of SLE^{2,3}. The production of autoantigens circulating in the form of immune complexes (containing autoantigen, associated protein and even autoantigen-specific antibodies) are pathogenic because components of the complexes, such as the DNA and/or RNA, have potent immunostimulatory properties^{4,5}. Indeed, DNA fragments in immune complexes isolated from the blood of people with SLE are of a size similar to those of cleaved chromatin fragments that are released from apoptotic bodies, are unusually rich in CpG content⁶ and are particularly stimulatory through Toll-like receptor 9 (TLR9).

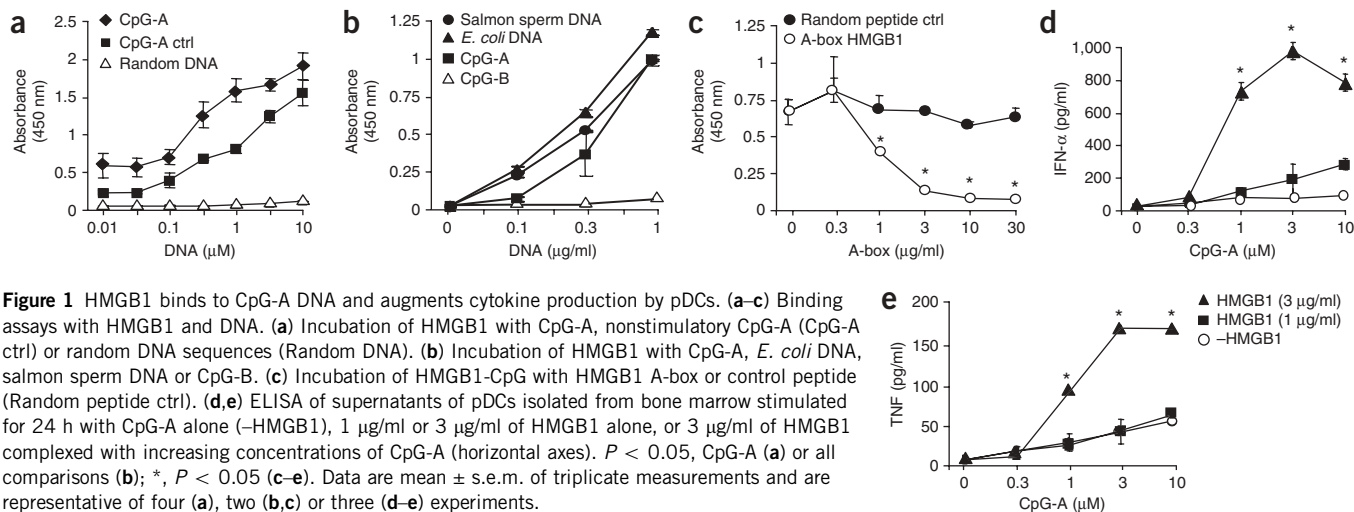
CpG DNA binds to the cell surface, enters the cell by means of clathrin-dependent pathways and is recognized by TLR9 located in the endoplasmic reticulum and endosomal compartments^{7,8}. TLR9 responds to different types of oligodeoxynucleotides (ODNs), which is in part dependent on sequence motifs and secondary structures.

Class A CpG ODNs (CpG-A), which form higher-order structures, ‘preferentially’ activate plasmacytoid dendritic cells (pDCs), whereas class B CpG ODNs (CpG-B), which in general maintain linear, single-stranded forms, activate B cells^{9,10}. Indeed, the ability of class A ODNs to form multivalent higher-order structures is believed to be critical for their activation of TLR9 (ref. 11), potentially by TLR9–TLR9 crosslinking. Other work has suggested that the endosomal localization of CpG-A is also a requisite determinant^{12,13}, although the mechanisms by which ODNs are delivered to subcellular structures are unknown.

TLR9 was originally believed to uniquely recognize bacterial or viral DNA, although it is now appreciated that TLR9 also recognizes unmethylated CpG-containing DNA sequences, including those of mammalian origin^{9,14}. However, whereas bacterial and virus-derived DNA can gain access to the endosome through phagocytosis or other cell entry strategies, the mechanisms by which mammal-derived self DNA-containing immune complexes activate TLR9 and induce cell activation remain poorly understood. TLR9 is expressed by many different cell types, most notably pDCs, which, although they represent only a very small fraction of cells in the peripheral blood, account for the most interferon- α (IFN- α) produced after TLR9 activation^{15,16}. Type I interferons are believed to be central to the pathogenesis

¹Inflammation and Autoimmune Group, Research Department, MedImmune, Gaithersburg, Maryland 20878, USA. ²Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118, USA. ³Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA. ⁴Rheumatology Research and Autoimmunity and Inflammation Program, Hospital for Special Surgery, New York, New York 10021, USA. ⁵Critical Therapeutics, Lexington Massachusetts 02421, USA. ⁶Department of Medicine, University of Heidelberg, Heidelberg D-69115, Germany. Correspondence should be addressed to A.J.C. (coylea@medimmune.com).

Received 31 July 2006; accepted 8 March 2007; published online 8 April 2007; doi:10.1038/ni1457



of many autoimmune disorders, including SLE, by promoting the maturation of immature DCs and the activation and differentiation of B cells^{17,18}. Indeed, the presence of a type I interferon gene ‘signature’ in the peripheral blood of people with SLE and Sjögren disease further link type I interferons to the pathogenesis of autoimmune disorders^{19–21}.

The high-mobility group box (HMGB) proteins are highly abundant chromatin-binding proteins located mainly in the cell nucleus, where they bind to the minor groove of DNA²². Their chief functions are to bend or distort the double helix and to regulate many transcriptional events by increasing the affinity of transcription factor–DNA interactions. The HMGB proteins consist of three related highly conserved family members, HMGB1, HMGB2 and HMGB3, that consist of an A-box and B-box protein with a carboxy-terminal acidic tail. The proteins are highly homologous and are believed to show little sequence specificity in their ability to bind to DNA. HMGB1 is released from cells by two distinct mechanisms: it is liberated from cells undergoing necrosis but not apoptosis²³, or is actively secreted from cells after inflammatory cytokine stimulation²⁴. It has also been suggested that HMGB1 is important in inflammatory disorders such as sepsis and rheumatoid arthritis^{24–28}. The putative cellular receptors that bind HMGB1 are at present controversial and may include TLR2 and TLR4 as well as the immunoglobulin ‘superfamily’ member RAGE (receptor for advanced glycation end-products)^{29–31}.

HMGB1 is secreted by both pDCs and myeloid DCs after stimulation with CpG ODN and regulates the production of IFN- α in an autocrine way. This raises the possibility that HMGB1 may also contribute to autoimmune disorders characterized by type I interferons^{32,33}. Notably, HMGB1 is reported to be expressed in lesions from people with cutaneous lupus³⁴.

Here we demonstrate that class A CpG-containing ODNs and HMGB1 functionally interacted and that the resultant complex activated pDCs and augmented IFN- α production through a mechanism dependent on adaptor protein MyD88–TLR9 and RAGE. HMGB1–DNA complexes resulted in the association of RAGE with TLR9. Moreover, HMGB1 was present in DNA-containing immune complexes and was a key factor in immune complex–triggered activation of autoreactive B cells and the induction of genes encoding type I interferon after stimulation with DNA-containing immune complexes dependent on RAGE engagement. Our data collectively provide a previously unknown mechanism by which DNA-containing

immune complexes activate pDCs and B cells and contribute to immune dysregulation in disorders such as SLE.

RESULTS

HMGB1–DNA complex augments cytokines release

The nuclear functions of HMGB1 are to bind to and bend DNA and to serve as an ‘architectural’ protein²². As it is now appreciated that HMGB1 can be released from cells, we hypothesized that in a way analogous to its binding of nuclear DNA, HMGB1 could bind to extracellular DNA and, by forming HMGB1–DNA complexes, modify the immune-regulatory effects of specific DNA sequences. In our initial experiments, we compared the binding of thymus-derived mammalian HMGB1 to various types of ODNs. HMGB1 bound to immunostimulatory CpG-A as well as to nonstimulatory (CG-inversion) sequences, but not to random DNA sequences (Fig. 1a). Measurement of the perturbation of intrinsic tryptophan fluorescence³⁵ showed that HMGB1 bound to CpG-A ODN with an apparent dissociation constant of 70 nM. HMGB1 also bound to DNA of both bacterial and mammalian origin, but in contrast to CpG-A ODNs, CpG-B ODNs failed to bind to HMGB1 (Fig. 1b). A-box and B-box HMGB1 peptides also bound to CpG-A ODNs with affinity to similar to that of full-length protein (data not shown). Moreover, HMGB1 A-box, which has been shown to function as a HMGB1 antagonist²⁷, prevented the binding of HMGB1 to CpG-A (Fig. 1c).

We next determined whether the formation of HMGB1–CpG complexes modified the immunostimulatory properties of CpG-A. We isolated pDCs from total bone marrow and stimulated them with immunostimulatory CpG-A alone, with 1 $\mu\text{g/ml}$ or 3 $\mu\text{g/ml}$ of HMGB1 alone, or with preformed HMGB1–CpG complexes. CpG-A induced the production of IFN- α and tumor necrosis factor, whereas HMGB1 alone was ineffective. However, stimulation with HMGB1–CpG complexes elicited an increase in the production of IFN- α and tumor necrosis factor (Fig. 1d,e).

To further investigate the mechanisms underlying the synergistic effect of the HMGB1–CpG-A complex, we stimulated cells with mammalian HMGB1 in complex with either immunostimulatory or nonstimulatory ODN sequences. Unlike the stimulatory sequences, the nonstimulatory CG-inversion DNA sequence was ineffective in inducing cytokine secretion whether it was in a ‘precomplex’ with HMGB1 or not (Fig. 2a). To determine which domain of HMGB1 was required, we stimulated cells with the A-box or B-box of HMGB1 alone or stimulated them with complexes of HMGB1 A-box–CpG-A

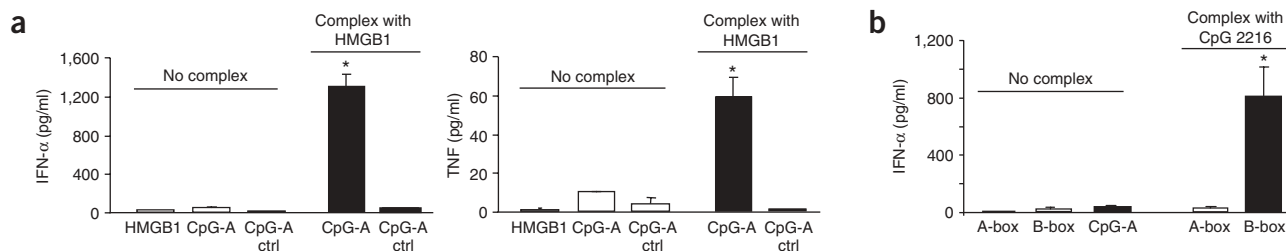


Figure 2 HMGB1 B-box augments IFN- α production induced by immunostimulatory CpG-A. **(a)** ELISA of IFN- α and tumor necrosis factor (TNF) in supernatants of pDCs stimulated with HMGB1 alone or with immunostimulatory CpG-A or nonstimulatory sequences (CpG-A ctrl) alone (No complex) or in complex with HMGB1. **(b)** ELISA of IFN- α in cells stimulated with CpG-A alone or with HMGB1 A-box or HMGB1 B-box alone or in complex with CpG-A (CpG 2216). *, $P < 0.05$. Data are mean \pm s.e.m. of triplicate measurements and are representative of three independent experiments.

or HMGB1 B-box–CpG-A. HMGB1 B-box–CpG-A but not HMGB1 A-box–CpG-A augmented the production of IFN- α relative to CpG-A alone (**Fig. 2b**). These data suggested that the B-box domain, which has been reported to contain the RAGE-binding region of HMGB1, confers immunostimulatory activity to HMGB1.

CpG-A ODN augments the binding of HMGB1 to RAGE

HMGB1 has been reported to bind to and signal through the multivalent immunoglobulin receptor RAGE as well as TLR2 and TLR4. To further assess the binding of HMGB1 to these receptors, we did *in vitro* experiments to assess the binding of plate-bound fusion proteins of RAGE, TLR2 and TLR4 and the Fc portion of immunoglobulin (RAGE-Fc, TLR2-Fc and TLR4-Fc, respectively) to HMGB1 derived from mammalian thymus. In contrast to other published reports, mammalian HMGB1 bound only to RAGE and not to either of the TLRs (**Fig. 3a**).

We next hypothesized that HMGB1 in complex with DNA might modify the ability of HMGB1 to bind its receptor. To investigate this hypothesis, we compared the binding of HMGB1 to RAGE in the presence or absence of CpG-A or CpG-B. HMGB1 bound to RAGE-

Fc, but the HMGB1–CpG-A complex bound more effectively than HMGB1 alone (**Fig. 3b**). In contrast to the HMGB1–CpG-A complex, HMGB1–CpG-B failed to augment binding to RAGE above that obtained with HMGB1 alone (**Fig. 3c**). Thus, HMGB1 ‘preferentially’ binds to class A ODNs, which augments the binding of HMGB1 to RAGE. To address whether the binding of the complex to RAGE resulted in greater internalization of DNA, we assessed the uptake of labeled HMGB1–CpG-A in DCs from wild-type and RAGE-deficient cells. The uptake of HMGB1–DNA was similar in wild-type and RAGE-deficient cells (**Fig. 3d**), suggesting that although this complex binds to RAGE, RAGE is not essential for regulation of the internalization of DNA or the HMGB1–DNA complex.

HMGB1–CpG-A induces RAGE-dependent secretion of cytokines

To further determine the function of RAGE and HMGB1–DNA complexes in IFN- α secretion, we measured IFN- α production by cells stimulated with HMGB1–CpG-A in the presence of RAGE-Fc or the HMGB1 antagonist A-box protein. In freshly isolated mouse pDCs, RAGE-Fc and the HMGB1 antagonist inhibited HMGB1–CpG-A–mediated IFN- α production by more than 95%

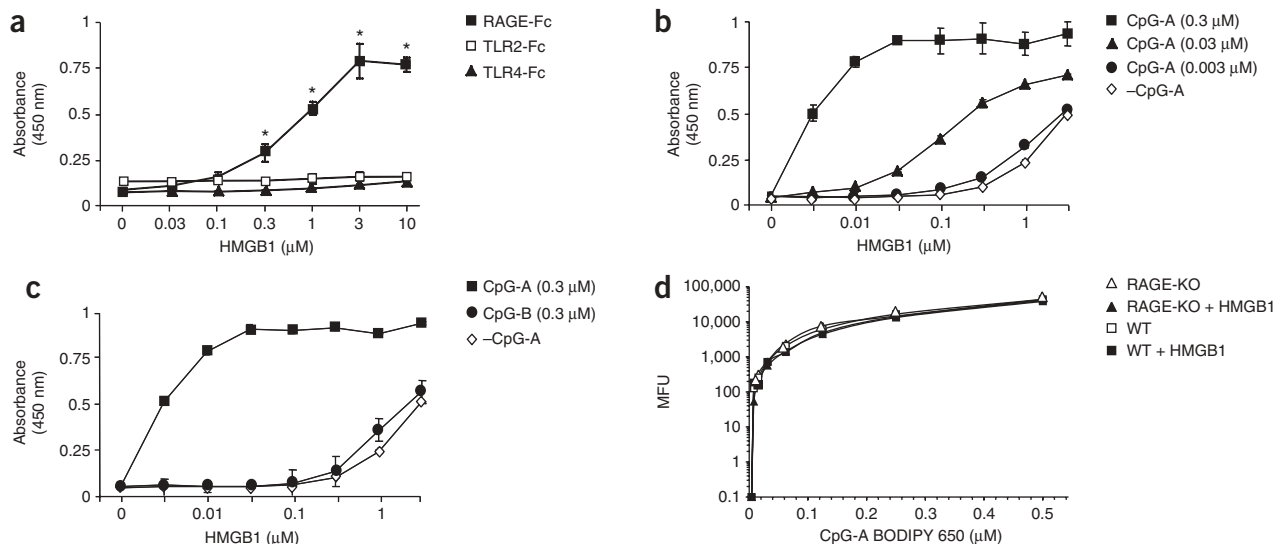


Figure 3 CpG-A but not CpG-B augments the binding of HMGB1 to RAGE. **(a)** Binding of HMGB1 to RAGE-Fc, TLR2-Fc or TLR4-Fc (10 μ g/ml). **(b)** Binding of HMGB1 to RAGE-Fc in the absence of CpG-A (–CpG-A) or in the presence of 0.003–0.3 μ M CpG-A. **(c)** Binding of HMGB1 to RAGE in the absence of CpG-A or in the presence of 0.3 μ M CpG-A or 0.3 μ M CpG-B. **(d)** Internalization of labeled CpG-A alone or labeled HMGB1–CpG-A (+ HMGB1) in wild-type (WT) or RAGE-deficient (RAGE-KO) DCs, assessed by flow cytometry and presented as mean fluorescence units (MFU). *, $P < 0.05$. Data are mean \pm s.e.m. of triplicate measurements and are representative of three **(a–c)** or two **(d)** independent experiments.

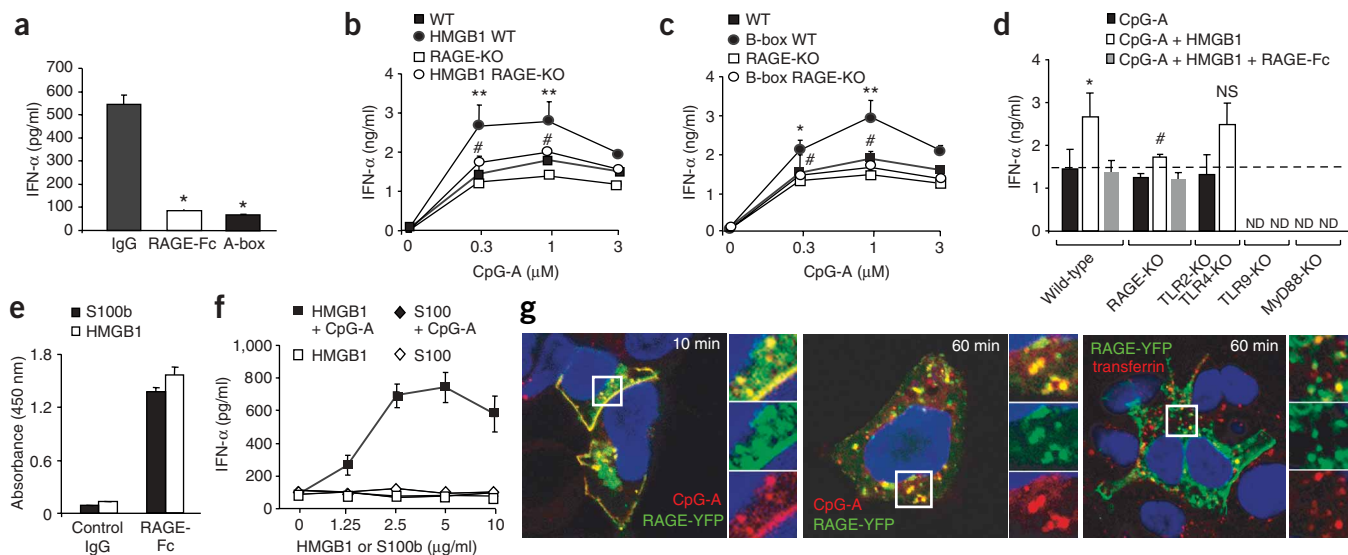


Figure 4 RAGE-TLR9-dependent IFN- α production induced by HMGB1-CpG-A. **(a-d)** ELISA of IFN- α in supernatants of freshly isolated cells. **(a)** Bone marrow pDCs stimulated with HMGB1-CpG-A (immunostimulatory ODN 2216) in the presence of control immunoglobulin (IgG), RAGE-Fc (10 μ g/ml) or A-box (10 μ g/ml). **(b)** Total wild-type or RAGE-deficient bone marrow cells stimulated with CpG-A alone (ODN 2336) or the HMGB1-CpG-A complex (HMGB1). **(c)** Wild-type or RAGE-deficient bone marrow cells stimulated with CpG-A alone (ODN 2336) or the CpG-A and B-box complex (B-box). **(d)** Bone marrow cells from wild-type mice or mice deficient in RAGE, TLR2 and TLR4 (TLR2-KO TLR4-KO), TLR9 (TLR9-KO) or MyD88 (MyD88-KO), stimulated with CpG-A alone (ODN 2336), the CpG-A-HMGB1 complex or CpG-A-HMGB1 plus RAGE-Fc (10 μ g/ml). NS, not significant; ND, not detected. **(e)** Binding of S100b and HMGB1 to RAGE or control immunoglobulin (Control IgG). **(f)** ELISA of IFN- α in supernatants of freshly isolated bone marrow pDCs stimulated with HMGB1 or S100b alone or with HMGB1-CpG-A or S100-CpG-A. **(g)** Confocal microscopy of HEK293 cells expressing yellow fluorescent protein-tagged RAGE (RAGE-YFP; green) incubated with BODIPY 630/650-conjugated CpG-A (red) and HMGB1 (left and middle) or with Alexa Fluor 647-conjugated human transferrin (red; right). RAGE plus CpG-A or RAGE plus transferrin, yellow. White boxes indicate areas magnified in insets. * or #, $P < 0.05$; **, $P < 0.01$. Data are mean + s.e.m. **(a-c,e)** or the mean + s.e.m. of quadruplicate **(d)** or triplicate **(f)** measurements and are representative of three **(a)**, two **(b,d)**, two **(c)**, four **(f)** or two **(g)** experiments.

(Fig. 4a). To extend those observations, we stimulated total bone marrow cells from wild-type mice or RAGE-deficient mice with CpG-A alone or with HMGB1-CpG-A. Although the response to CpG-A alone was similar in wild-type and RAGE-deficient mice, the response to HMGB1-CpG-A was reduced by 70% in bone marrow cells from RAGE-deficient mice (Fig. 4b). The synergistic response to HMGB1-CpG-A was absent in RAGE-deficient bone marrow cells (Fig. 4c). Treatment of wild-type cells with RAGE-Fc inhibited the response to the complex, whereas cytokine production by RAGE-deficient cells treated with RAGE-Fc remained unchanged (Fig. 4d). In contrast to the response of RAGE-deficient cells, the response to CpG-A and HMGB1-CpG-A was abolished in bone marrow cells from mice deficient in MyD88 and TLR9 (Fig. 4d). These results were also supported by studies of pDCs differentiated with the receptor tyrosine kinase Flt3 (data not shown).

To further rule out the possibility of involvement of TLR2- and/or TLR4-mediated signaling in response to the HMGB1-DNA complex, we obtained bone marrow cells from mice doubly deficient in both TLR2 and TLR4 and stimulated the cells with HMGB1 alone or HMGB1-CpG complex. In contrast to RAGE-deficient cells, the cells doubly deficient in both TLR2 and TLR4 responded to both CpG-A and HMGB1-CpG complex similarly to cells obtained from wild-type mice (Fig. 4d). Thus, our data support the hypothesis that RAGE is the main receptor involved in mediating the effect of the HMGB1-DNA complex.

RAGE is a multivalent receptor that binds many ligands, including advanced glycation end-products and proinflammatory cytokine-like mediators such as members of the calgranulin-S100 family. To determine whether other RAGE ligands would act in synergy

with CpG-A, we assessed the binding of S100b to soluble RAGE and then assessed whether S100b alone or in combination with CpG-A would induce IFN- α secretion. S100b and HMGB1 bound to soluble RAGE; however, despite that binding, neither S100b or S100b-CpG-A augmented IFN- α secretion (Fig. 4e,f). These data suggested that although engagement of RAGE by HMGB1-CpG was required for cytokine secretion, ligand engagement of RAGE itself was insufficient to augment CpG-A-mediated secretion of cytokines from pDCs.

To further elucidate the interaction between the HMGB1-CpG-A complex and RAGE, we did a series of confocal microscopy experiments to visualize the interaction of RAGE with the complex using HEK293 cells stably transfected with RAGE. At 10 min after stimulation of RAGE-transfected cells with the HMGB1-CpG-A complex, RAGE and CpG-A were both detectable on the cell surface as well as in the cytoplasm. Indeed, some RAGE-positive cells were also CpG-A positive (Fig. 4g). By 60 min, RAGE was no longer detectable on the cell surface; it was present in the cytoplasm and was associated with DNA (Fig. 4g, middle inset). To further determine the subcellular localization of RAGE, we stained cells with transferrin as a marker of early endosomes. Overlay analysis of transferrin and RAGE showed that some of the RAGE-positive cells were localized together with transferrin-positive structures, suggesting that RAGE can be internalized, some of which is sequestered in early endosome-like structures in the cytoplasm (Fig. 4g).

HMGB1 in the activation of autoreactive B cells

Next we sought to determine whether HMGB1 is involved in cellular responses to more physiologically relevant immune complexes. To

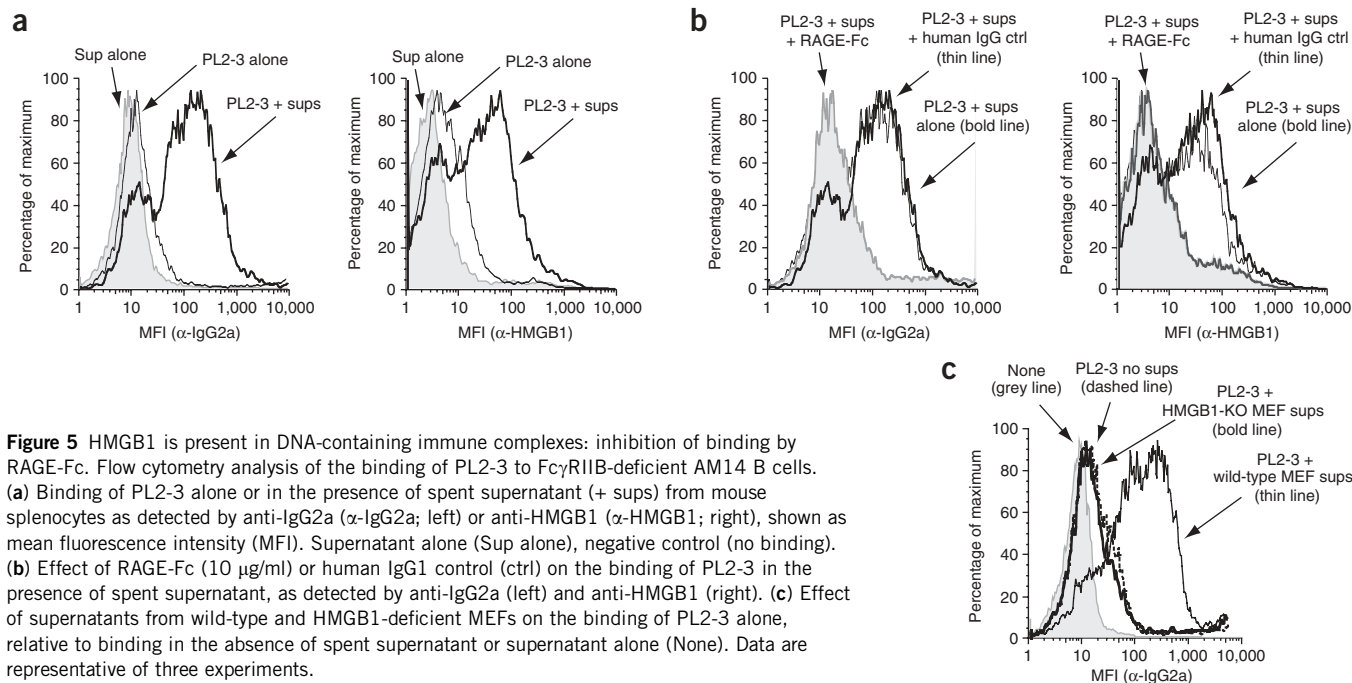


Figure 5 HMGB1 is present in DNA-containing immune complexes: inhibition of binding by RAGE-Fc. Flow cytometry analysis of the binding of PL2-3 to Fc γ RIIB-deficient AM14 B cells. (a) Binding of PL2-3 alone or in the presence of spent supernatant (+ sups) from mouse splenocytes as detected by anti-IgG2a (α -IgG2a; left) or anti-HMGB1 (α -HMGB1; right), shown as mean fluorescence intensity (MFI). Supernatant alone (Sup alone), negative control (no binding). (b) Effect of RAGE-Fc (10 μ g/ml) or human IgG1 control (ctrl) on the binding of PL2-3 in the presence of spent supernatant, as detected by anti-IgG2a (left) and anti-HMGB1 (right). (c) Effect of supernatants from wild-type and HMGB1-deficient MEFs on the binding of PL2-3 alone, relative to binding in the absence of spent supernatant or supernatant alone (None). Data are representative of three experiments.

address this issue, we investigated the function of HMGB1 in the activation of autoreactive B cells. B cells expressing a transgene-encoded AM14 receptor (AM14 B cells) recognize immunoglobulin G2a (IgG2a) and proliferate in response to IgG2a monoclonal antibodies (mAbs) reactive with chromatin or DNA in a DNase-sensitive, TLR9- and MyD88-dependent way^{36,37}. PL2-3 is an IgG2a mAb specific for H2A-H2B histones and is thought to bind chromatin released from the damaged and/or dying cells present in 'spent' cell cultures. Preincubation of PL2-3 with supernatants from 'spent' cell cultures increases binding avidity of PL2-3 for AM14 B cells.

To determine whether HMGB1 was present in these chromatin immune complexes, we stained Fc γ receptor IIB (Fc γ RIIB)-deficient AM14 B cells with preformed PL2-3 immune complexes. HMGB1 was present in the immune complexes bound to the surface of Fc γ RIIB-deficient AM14 B cells (Fig. 5a). Furthermore, the addition of RAGE-Fc inhibited the binding of HMGB1-containing DNA-containing immune complexes to the surface of B cell cells, as detected by mAb to IgG2a or mAb to HMGB1 (Fig. 5b). Although supernatants from wild-type cells augmented PL2-3 binding, supernatants obtained from HMGB1-deficient cells did not augment PL2-3 binding (Fig. 5c). These results indicated that HMGB1 was bound to DNA-containing immune complexes and that HMGB1 was required for binding of the chromatin immune complex to AM14 B cells. Notably, both the binding of the PL2-3 complex and inhibition of this binding by RAGE-Fc were Fc γ R independent, as these assays used Fc γ RII-deficient AM14 B cells.

To investigate the function of HMGB1 in activation of autoreactive B cells, we stimulated AM14 B cells with either DNA-containing immune complexes, the TLR2 ligand Pam₃CysK₄, the TLR4 ligand LPS or antibody to IgM (anti-IgM), in the presence of RAGE-Fc or the HMGB1 antagonist A-box. RAGE-Fc considerably inhibited the PL2-3-dependent proliferation of AM14 B cells relative to control immunoglobulin (Fig. 6a) but had no effect on proliferation induced by TLR2 or TLR4 ligand or on B cell receptor-mediated B cell activation (Fig. 6 and data not shown). Similarly, the antagonist A-box inhibited DNA-containing immune complexes but not

Pam₃CysK₄ (Fig. 6b). These data suggested that HMGB1 is crucial in the activation of DNA or chromatin-reactive B cells.

TLR9 and RAGE interaction after HMGB1-CpG-A stimulation

To further delineate the preceding mechanisms, we immunoprecipitated TLR9 from HEK293 cells transfected with hemagglutinin-tagged TLR9. Stimulation with HMGB1-CpG-A resulted in considerable association between TLR9 and RAGE, which did not occur after stimulation with either HMGB1 or CpG-A alone (Fig. 7a). CpG-A alone induced the association of MyD88 with TLR9, which was

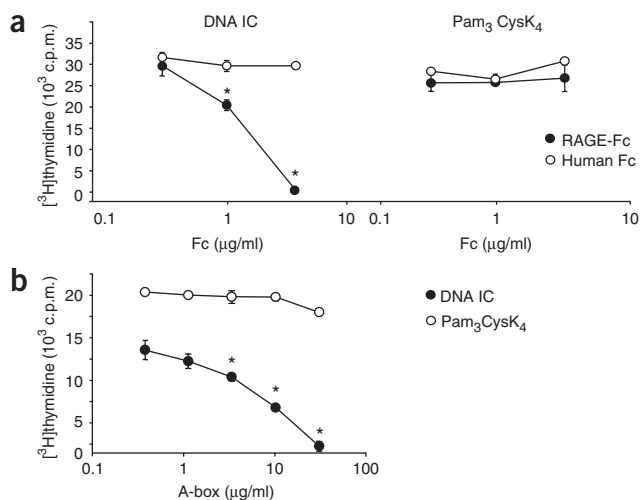


Figure 6 Inhibition of AM-14 B cell proliferation induced by DNA-containing immune complexes by RAGE-Fc and A box antagonist. Proliferation assay of AM14 B cells stimulated with DNA-containing immune complexes (DNA IC; 0.1 μ g/ml) or Pam₃CysK₄ (1 μ g/ml) and treated with RAGE-Fc or human IgG1 (Human Fc; a) or stimulated with PL2-3 (DNA IC) or Pam₃CysK₄ in the presence of A-box antagonist (b). *, $P < 0.05$. Data are mean \pm s.e.m. of triplicate measurements and are representative of three to four experiments.

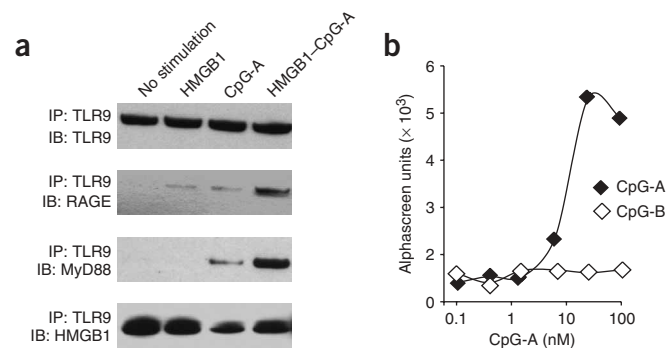


Figure 7 The HMGB1-CpG-A complex recruits RAGE, MyD88 and TLR9. **(a)** Immunoblot (IB) analysis of HEK293 cells expressing hemagglutinin-tagged TLR9 and transfected with human RAGE cDNA, to detect the association of RAGE, MyD88 and HMGB1 with immunoprecipitated (IP) TLR9. **(b)** AlphaScreen analysis of RAGE-Fc and TLR9-Fc coupled to acceptor and donor beads, with CpG-A or CpG-B. Data are representative of four independent experiments.

enhanced when cells were stimulated with the complex. These data suggested that RAGE, MyD88 and TLR9 physically interact after stimulation with HMGB1-CpG-A.

HMGB1 and TLR9 were also physically associated, although in contrast to RAGE, HMGB1 was constitutively associated with TLR9 and was not regulated after stimulation. To further investigate this interaction, we covalently coupled RAGE-Fc and TLR9-Fc to AlphaScreen acceptor and donor beads, respectively. In the absence of CpG DNA, RAGE and TLR9 interacted poorly; however, CpG-A but not CpG-B substantially augmented the RAGE and TLR9 interactions (Fig. 7b).

Immune complexes in SLE serum stimulate interferon production

Stimulation of peripheral blood mononuclear cells (PBMCs) with plasma or serum containing anti-DNA-DNA-containing immune complexes, obtained from people with SLE, has been reported to induce secretion of type I interferon, as assessed by the induction of *IFIT1* mRNA, encoded by a gene that can be induced by type I interferon³⁸, or as assessed by IFN- α secretion³⁹. To evaluate the function of HMGB1-RAGE in the regulation of genes encoding type I interferon mediated by DNA-immune complex, we did two series of experiments. We stimulated PBMCs with a solution containing 20% plasma from people with lupus (called 'lupus plasma' here) and measured *IFIT1* mRNA by quantitative PCR or, conversely, stimulated PBMCs with 1% lupus serum and 25% necrotic cell supernatant and measured IFN- α , as described^{38,39}. Both mAb to human HMGB1 and RAGE-Fc inhibited, in a dose-dependent way, the induction of *IFIT1* mRNA by lupus serum by approximately 75% (Fig. 8a). Those observations were further supported by data showing that IFN- α secretion induced by 1% lupus serum and necrotic cell supernatants was inhibited by mAb to HMGB1, RAGE-Fc and mAb to human RAGE (Fig. 8b). Moreover, unlike supernatants from necrotic wild-type cells, supernatants from necrotic HMGB1-deficient cells failed to induce IFN- α production. These data used plasma from one patient, whose serum contained anti-double-stranded DNA, anti-Sm and anti-cardiolipin. We noted similar inhibition with mAb to HMGB1 and RAGE-Fc in plasma from two other patients with lupus that contained anti-double-stranded DNA but undetectable anti-ribonucleoprotein (data not shown). These data suggested that HMGB1 is important in the induction of target genes encoding type I interferon after stimulation of cells with DNA-containing immune

complexes that are present in people with SLE, and that this is dependent on RAGE.

DISCUSSION

Recognition of bacterial or viral DNA by TLR9 is crucial in innate immune responses by providing protective immune responses against invading viral and bacterial pathogens^{9,40,41}. However, activation of TLR9 by mammalian self DNA in the form of immune complexes is thought to contribute to systemic autoimmune disease through the production of type I interferons^{41,42}. The mechanism by which immune complexes containing double-stranded DNA activate TLR9-dependent responses remains unclear. Here we have provided evidence that HMGB1 can mediate the activation of TLR9 by DNA-containing immune complexes through a mechanism involving the immunoglobulin superfamily member RAGE.

HMGB1 has been recognized for many years as an abundant nuclear chromatin protein that binds to and distorts DNA; in doing so, it regulates many transcriptional events^{22,43}. With the realization that HMGB1 is liberated from both necrotic cells²³ and cytokine-stimulated cells²⁴, we hypothesized that extracellular HMGB1 would bind to extracellular DNA and modify the immunostimulatory properties of DNA. We have shown here that HMGB1 'preferentially' bound to CpG-A ODNs but not to CpG-B ODNs or to random DNA sequences. These observations were somewhat unexpected, because although some HMGB proteins bind in a sequence-specific way²²,

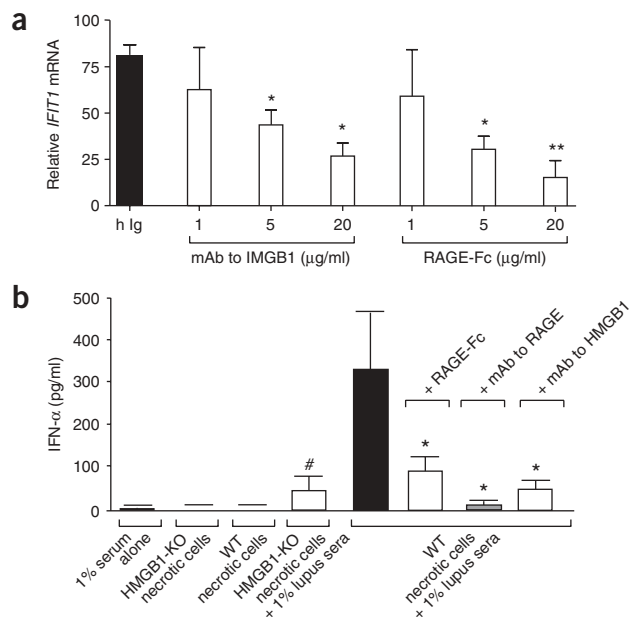


Figure 8 HMGB1 and RAGE mediate the induction of genes encoding type I interferons mediated by DNA-containing immune complexes present in sera from people with lupus. **(a)** Analysis of *IFIT1* mRNA in PBMCs stimulated with 20% lupus sera treated with human immunoglobulin (h Ig) or treated with mAb to HMGB1 or RAGE-Fc. *, $P < 0.05$. Data are mean \pm s.e.m. of two experiments. **(b)** ELISA of supernatants of PBMCs stimulated with 1% lupus serum alone, supernatants from necrotic cells alone or 1% lupus serum plus supernatants in the presence of control immunoglobulin (filled bars), RAGE-Fc (10 μ g/ml), mAb to RAGE (10 μ g/ml) or mAb to HMGB1 (4 μ g/ml). Supernatants from necrotic cells derived from HMGB1 deficient cells were also used in place of supernatants from wild-type MEFs. * or #, $P < 0.01$. Data are mean \pm s.e.m. of four different serum samples evaluated on PBMCs from four different donors.

HMGB1 itself has been thought to bind to DNA mainly in a sequence-independent way.

The structures of CpG-A and CpG-B ODNs are very different. CpG-A contains poly(G) motifs that form stable, higher-ordered structures and a central phosphodiester region containing one or more CpG motifs in self-complementary palindromes forming double-stranded DNA structures. In contrast, CpG-B has a phosphorothioate backbone and, in general, exists as linear single-stranded structures. Data suggest that the capacity of CpG-A to augment secretion of IFN- α from pDCs is dependent on its ability to form multivalent higher-order structures, which maybe necessary for TLR9 crosslinking¹¹. The interaction of HMGB1 with DNA has been determined by nuclear magnetic resonance spectroscopy, which showed that the A-box and B-box of HMGB1 bind to the minor groove of DNA⁴⁴. However, whether HMGB1 allows CpG-A ODNs to assemble into larger, multivalent complexes remains to be determined.

There is emerging evidence that DNA-containing immune complexes are involved in the pathogenesis of autoimmune disorders; here, the HMGB1-CpG-A complex considerably increased the production of IFN- α from mouse bone marrow-derived pDCs relative to CpG-A alone. Neither pretreatment with CpG-A before HMGB1 treatment nor HMGB1 treatment before CpG-A augmented cytokine secretion (data not shown). These observations suggest that the synergy noted after HMGB1-CpG-A treatment is different from simply augmented cytokine production induced by TLR ligands⁴⁵ and that it results instead from direct activation of cells by the complex. There was synergy with the B-box and not the A-box of HMGB1, consistent with published work showing that the proinflammatory sequence of HMGB1 is in the B-box^{24,27}.

Our data contrast with other published reports^{29,32,46,47} in that in our experiments, HMGB1 alone was inactive on mouse pDCs (and bone marrow-derived macrophages; data not shown). In all our experiments described here, we used HMGB1 protein purified from calf thymus, containing minimal contaminating DNA or RNA. In contrast, recombinant HMGB1 from *Escherichia coli* is a potent activator of pDCs and macrophages, and *E. coli*-derived HMGB1 activity is reduced by treatment with DNase (data not shown). These data suggest that at least part of the activity of HMGB1 demonstrated with recombinant protein is mediated by HMGB1 associated with DNA.

HMGB1 has been proposed to regulate inflammatory responses by functioning as a chemoattractant factor for monocytes⁴⁸ and smooth muscle cells⁴⁹, and it induces mesoangioblast migration and proliferation⁵⁰. In addition, HMGB1 causes upregulation of adhesion molecules⁵¹ and is crucial in impairing the integrity of epithelial surfaces⁵². Many of the biological effects of HMGB1 are proposed to be mediated by RAGE^{29,32,33,53}, although studies have also suggested involvement of TLR2 and TLR4 (ref. 30). In our studies, although mammalian HMGB1 bound to RAGE, we were unable to detect binding to TLR2 or TLR4. In addition, the capacity of HMGB1-DNA complexes to induce pDC activation was inhibited by soluble RAGE fusion protein and the antagonist A-box, both of which have been described as inhibiting many RAGE-HMGB1-dependent responses *in vitro* and *in vivo*^{27,53-55}. Our data suggest that B-box protein signals exclusively through RAGE and that although RAGE is important in the response to the HMGB1-DNA complex, other surface receptors may also be involved. Although additional receptors for the HMGB1-DNA complex remain to be identified, it is unlikely that TLR2 or TLR4 is involved. Also, whereas CpG-A-induced secretion of IFN- α was dependent on TLR9 and MyD88, the response to CpG-A alone was

independent of RAGE, and RAGE contributed only to the augmented response to the HMGB1-DNA complex. One explanation for the enhanced immunostimulatory effect of the complex on pDC activation is increased binding efficiency of HMGB1-DNA due to a conformational change in HMGB1, although this remains to be formally demonstrated.

Our data contrast with other published studies of human pDCs showing that HMGB1, rather than augmenting IFN- α secretion, suppressed cytokine secretion in response to TLR9 agonists⁵⁶. Although the explanation for these differences is unclear at present, different forms of HMGB1 were used in these studies. Indeed, we have found using circular dichroism that different types of HMGB1 (mammalian and *E. coli*) interact in a different ways with DNA (data not shown). Another explanation is that RAGE expression and/or function maybe distinct in mouse pDCs and human pDCs. In contrast to results obtained with recombinant proteins⁵⁶, endogenously secreted HMGB1 has been reported to induce pDC maturation and IFN- α secretion through a RAGE-dependent mechanism³². Whether such results are related to hyperacetylated forms of HMGB1 released after cytokine stimulation⁵⁷ is unclear.

In addition to inducing the production of type I interferons by pDCs, DNA-containing immune complexes have been reported to activate autoreactive B cells^{36,37,58} through DNA- and TLR9-dependent mechanism⁵⁸. We have shown here that HMGB1 was present in the immune complexes that bound to AM14 B cells and that supernatants from HMGB1-deficient cells had the unique function of HMGB1 in regulating the binding of immune complexes coautoreactive B cells. HMGB1-dependent binding of immune complexes was inhibited by soluble RAGE, suggesting that RAGE may be a coreceptor, although this remains to be formally demonstrated by studies of B cells from RAGE-deficient AM14 cells. HMGB1 is required for the binding of immune complexes and for the activation of B cells, as an antagonist A-box and soluble RAGE inhibited proliferation induced by PL2-3 but by TLR2 or TLR4 ligands.

Activation of autoreactive B cell by DNA complexes also requires engagement of the B cell receptor followed by TLR9 activation. Studies using mono- and tri-haptenated DNA conjugates suggest that crosslinking of the B cell receptor is not required, and a model has been proposed in which B cell receptor engagement facilitates the transport of autoantigens to cytoplasmic TLR9 (ref. 36). One explanation for our findings is that binding of the HMGB1-DNA complex to RAGE results in internalization of the complex and to more-efficient delivery of DNA to TLR9. Indeed, facilitation of the delivery of DNA to the cytoplasm by various transfection protocols is sufficient to activate TLR9. That hypothesis is further supported by the demonstration of a mutant TLR9 that translocates to the cell surface and confers responsiveness to normally nonstimulatory vertebrate mammalian DNA¹⁴. In the absence of RAGE, HMGB1-ODN complexes are still efficiently taken up by cells, as indicated by binding experiments as well as by confocal analysis (data not shown), suggesting that RAGE is not the main receptor involved in internalization, which suggests uptake through other mechanisms, such as clathrin-dependent, caveolin-independent pathways^{8,59}.

We found here that stimulation of cells with the HMGB1-CpG-A complex resulted in the association of TLR9 with RAGE and recruitment of the TLR adaptor molecule MyD88. Furthermore, RAGE and TLR9 strongly interacted only in the presence of CpG-A (not CpG-B), suggesting that multimeric DNA, when in complex with HMGB1, may provide a link between a surface transmembrane receptor and an endosome-localized TLR, possibly by leading to a

greater degree of TLR9 crosslinking and/or altering the intracellular compartmentalization of CpG-A. It has been shown that the localization of ODNs to early endosomes is critical in determining whether CpG DNA results in IFN- α secretion^{12,13}. We visualized RAGE after stimulation and noted that RAGE is internalized and associates with DNA, some of which is sequestered in endosome-like structures. It is possible that through RAGE and HMGB1, CpG-A maybe retained in the early endosome, where it enhances TLR9 activation. Indeed, we found HMGB1 to be constitutively associated with TLR9. However, the precise function of non-nuclear, cytoplasmic and/or endosomal HMGB1 remains to be determined.

An additional mechanism may also involve signaling through RAGE after binding of DNA-containing immune complexes. RAGE activation by HMGB1 has been reported to induce phosphorylation of the kinases Erk and Erk2 and induction of the transcription factor NF- κ B^{55,60}. However, binding of RAGE itself is unlikely to explain our observations, as the S100b cytokine-like molecule and HMGB1 bound to RAGE in a similar way, but the S100b cytokine-like molecule failed to augment CpG-A-mediated cytokine secretion.

Immune complexes containing double-stranded DNA present in the plasma of people with lupus are potent inducers of type I interferons, most produced from pDCs^{42,61–63}. We have shown here that stimulation of normal PBMCs with either 20% lupus serum or with 1% lupus serum plus necrotic cells induced *IFIT1* mRNA or the secretion of IFN- α , respectively^{38,62}. The induction of *IFIT1* mRNA after stimulation with immune complexes was reduced substantially by mAb to HMGB1 and by soluble RAGE. Similarly, IFN- α secretion induced by 1% lupus serum plus necrotic cells was inhibited by mAb to HMGB1, by soluble RAGE and also by mAb to RAGE. The pharmacological evidence suggesting that HMGB1 is critical in the regulation of IFN- α production in the context of lupus-associated immune complexes was further supported by the demonstration that when combined with lupus serum, necrotic debris from HMGB1-deficient cells, unlike that from wild-type cells, failed to induce IFN- α secretion.

In conclusion, our data are consistent with a model in which necrosis or tissue injury causes HMGB1 to be released from cells; it then binds to DNA-containing immune complexes in serum and then the resultant complexes regulate the population expansion of autoreactive B cells and the production of IFN- α by pDCs. These effects may be important in perpetuating inflammatory ‘amplification loops’ in diseases such as lupus. RAGE is involved in the recognition of HMGB1- and DNA-containing immune complexes, and the DNA-dependent association of TLR9 and RAGE, along with the endosomal localization of RAGE, raise the possibility that RAGE determines the subcellular localization and/or retention of DNA-TLR9 complexes in the endosome. Recognition and/or activation of HMGB1- and DNA-containing immune complexes by means of RAGE may therefore be important in the loss of tolerance to self antigens.

METHODS

Reagents. Immunostimulatory CpG-A (ODN 2216), CpG-B (ODN 2006), *E. coli* DNA, salmon sperm DNA and HEK293 cells stably expressing TLR9 were from Invivogen. Immunostimulatory CpG-A (ODN 2216) molecules labeled at the 3' end with biotin or fluorescence were synthesized by Qperon. CpG-A ODN 2336 was from Coley Pharmaceuticals (**Supplementary Table 1** online). Enzyme-linked immunosorbent assay (ELISA) kits for mouse and human RAGE-Fc, TLR2-Fc, TLR4-Fc, granulocyte-macrophage colony-stimulating factor and the Flt3 ligand as well as IFN- α and tumor necrosis factor were from R&D Systems. HMGB1 A-box and B-box were synthesized by New England Peptides. HMGB1 was purified from bovine thymus at MedImmune as described⁶⁴. Serum from patients with SLE was from Bioreclamation. Bovine

brain S100b was from Calbiochem. The pDC isolation kit was from Miltenyi Biotec. Fully human mAbs to human HMGB1 were generated by phage display. TLR9-Fc protein was purified from HEK293 cells stably expressing a fusion protein containing the ectodomain of human TLR9 linked to the Fc portion of mouse IgG2a. Full-length human RAGE was cloned from a human splenic library and was subcloned into the pcDNA-3 expression vector. The Total RNA Purification Kit was from Gentra.

Mice. C57BL/6 mice (from The Jackson Laboratories) were maintained in specific pathogen-free conditions in conventional animal facilities at MedImmune; all experiments were approved by the Institutional Animal Care and Use Committee of MedImmune. TLR2- and TLR4-deficient mice were backcrossed to generate mice deficient in both TLR2 and TLR4. RAGE-deficient mice were generated as described⁶⁵. AM14 B cell receptor-transgenic mice were crossed with Fc γ RII-deficient mice to produce the Fc γ RII-deficient AM14 line and were maintained in the animal facility of Boston University Medical Center.

Protein binding and ELISA. HMGB1 was coated onto 96-well plates and binding of biotin-labeled CpG-A was detected with horseradish peroxidase-labeled streptavidin. In the inhibition assay, HMGB1 A-box was incubated with biotin-labeled CpG-A, which was then added to HMGB1-coated plates. Recombinant RAGE-Fc, TLR2-Fc or TLR4-Fc were coated onto 96-well plates, HMGB1 alone or HMGB1-CpG-A was added, and binding of HMGB1 was detected by biotin-labeled mAb to HMGB1.

In vitro pDC bone marrow stimulation. First, pDCs (over 60% purity) were isolated from bone marrow cells with the Miltenyi Biotec Plasmacytoid Dendritic Cell Isolation kit, then they were plated at a density of 2.5×10^4 cells per well in 96-well plates and were stimulated for 24 h with immunostimulatory CpG-A alone, HMGB1 alone or HMGB1 plus CpG-A. In some experiments, cells were pretreated with 10 μ g/ml of RAGE-Fc or 10 μ g/ml of A-box protein. In a separate series of experiments, either bone marrow cells were used or bone marrow-derived DCs generated by differentiating cells for 8 d in the presence of granulocyte-macrophage colony-stimulating factor and the Flt3 ligand. Cells were stimulated with CpG-A alone (ODN 2336) or with HMGB1-CpG-A. Cytokines in the supernatant were measured by ELISA.

Tryptophan emission for analysis of the binding of CpG-A to HMGB1. A SPEX Fluoromax-3 spectrofluorimeter was used for fluorescence titrations and spectra analysis of HMGB1; HMGB1-intrinsic tryptophan emission was monitored after the stepwise addition of CpG-A. Complex formation between HMGB1 and nucleic acid was assessed by the change in the initial fluorescence of the protein and binding affinity calculated from graphs of relative fluorescence intensity versus [nucleic acid base] / [protein] as described³⁵ (**Supplementary Methods** online).

Flow cytometry of DNA uptake. Bone marrow-derived DCs were prepared from wild-type or RAGE-deficient mice. Cells were incubated in the absence or presence of HMGB1 (10 μ g/ml) with increasing concentrations of immunostimulatory CpG-A labeled with BODIPY 650 (boron dipyrromethane dye; Molecular Probes) and were analyzed by flow cytometry. Median fluorescent units were calculated with FlowJo software and were plotted against the ‘dose’ of CpG-DNA added.

Confocal microscopy. HEK293 cells stably expressing a fusion protein of yellow fluorescent protein and RAGE were treated with Alexa Fluor 647-conjugated human transferrin (Molecular Probes) or a complex of HMGB1 and BODIPY 630/650-conjugated CpG-A (**Supplementary Methods**). Nuclei were stained with Hoechst 33342 dye and cells were analyzed by laser-scanning confocal microscopy.

DNA-containing immune complex and AM14 cell binding assay. DNA-containing immune complexes were performed by the mixture of ‘spent’ culture supernatants with 15 μ g/ml of PL2-3 (anti-nucleosome IgG2a) as described^{36,37}. RAGE-Fc (10 μ g/ml) or Fc control was added to the immune complexes. HMGB1 was detected with biotinylated anti-HMGB1-B and PL2-3 was detected with fluorescein isothiocyanate-conjugated anti-IgG2a. In some

experiments, supernatants from wild-type or HMGB1-deficient mouse embryonic fibroblasts (MEFs) were used.

AM14 B cell proliferation assay. Purified AM14 B cells were preincubated for 30 min at 37 °C with RAGE-Fc or control human IgG1, and were stimulated for 24 h at 37 °C with 0.1 µg/ml of PL2-3. Cells were then pulsed with [³H]thymidine for 6 h as described^{37,58}.

AlphaScreen binding assay. RAGE-Fc or TLR9-Fc was coupled on beads and CpG-A or CpG-B DNA was added. After 30 min, samples were 'read' with the Envision HT microplate reader (Perkin Elmer).

Lupus serum-induced *IFIT1* mRNA. PBMCs from healthy donors were cultured for 24 h with 20% (vol/vol) lupus plasma; total RNA was purified and *IFIT1* mRNA was measured by real-time PCR analysis.

Induction of IFN-α in human PBMCs. MEFs were grown in DMEM with 10% (vol/vol) FCS and necrosis was induced by the 'freeze-thaw' method. Supernatants from HMGB1-deficient necrotic MEFs were prepared as described above. Human PBMCs were stimulated with 10% (vol/vol) MEFs plus 1% (vol/vol) lupus serum (**Supplementary Methods**), and IFN-α was measured by ELISA.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank M. McCarthy and J. Suzich for comments on the manuscript; J.-C. Finet for tryptophan intrinsic fluorescence measurements; and L. Xu for technical assistance. TLR9- and MyD88-deficient mice were from S. Akira (Research Institute for Microbial Diseases); HMGB1-deficient MEFs were from M. Bianchi (San Raffaele University). Supported by Deutsche Forschungsgemeinschaft (DFG/SFB 405 to P.P.N.), the Alliance for Lupus Research and the National Institutes of Health (AI0677497-01).

AUTHOR CONTRIBUTIONS

J.T. and S.-Y.M. carried out binding assays; A.M.A. and A.M.R., the B cell assays; B.C., immunoblots; H.W., L.L.A., L.A., K.S. and G.L.R., antibody generation and protein purification; E.L., P.P., S.D., D.G., C.S. and K.A.F., AlphaScreen assays and confocal microscopy; P.N. and A.B., generation of RAGE mice; and M.K.C. and J.H., lupus serum experiments. A.J.C. and P.A.K. conceived the experiments and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/natureimmunology/.

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