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Mammalian DNA Is an Endogenous Danger Signal That Stimulates Local Synthesis and Release of Complement Factor B

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Complement factor B plays a critical role in ischemic tissue injury and autoimmunity. Factor B is dynamically synthesized and released by cells outside of the liver, but the molecules that trigger local factor B synthesis and release during endogenous tissue injury have not been identified. We determined that factor B is upregulated early after cold ischemia–reperfusion in mice, using a heterotopic heart transplant model. These data suggested upregulation of factor B by damage-associated molecular patterns (DAMPs), but multiple common DAMPs did not induce factor B in RAW264.7 mouse macrophages. However, exogenous DNA induced factor B mRNA and protein expression in RAW cells *in vitro*, as well as in peritoneal and alveolar macrophages *in vivo*. To determine the cellular mechanisms involved in DNA-induced factor B upregulation we then investigated the role of multiple known DNA receptors or binding partners. We stimulated peritoneal macrophages from wild-type (WT), toll-like receptor 9 (TLR9)-deficient, receptor for advanced glycation end products (RAGE)$^{-/-}$ and myeloid differentiation factor 88 (MyD88)$^{-/-}$ mice, or mouse macrophages deficient in high-mobility group box proteins (HMGBs), DNA-dependent activator of interferon-regulatory factors (DAI) or absent in melanoma 2 (AIM2), with DNA in the presence or absence of lipofection reagent. Reverse transcription–polymerase chain reaction, Western blotting and immunocytochemical analysis were employed for analysis. Synthesis of factor B was independent of TLR9, RAGE, DAI and AIM2, but was dependent on HMGBs, MyD88, p38 and NF-κB. Our data therefore show that mammalian DNA is an endogenous molecule that stimulates factor B synthesis and release from macrophages via HMGBs, MyD88, p38 and NF-κB signaling. This activation of the immune system likely contributes to damage following sterile injury such as hemorrhagic shock and ischemia-reperfusion.

INTRODUCTION

Ischemia/reperfusion (I/R) injury occurs when an organ is deprived of its blood supply with subsequent restoration of flow, resulting in an inflammatory response and organ injury. The complement system, a tightly regulated intricate network of over 30 serum and membrane-bound proteins, plays a critical role in I/R injury (1,2). Although the liver is the predominant source of serum complement proteins, previous studies have demonstrated that cell types outside the liver are capable of producing complement (3–11), and that complement components can be upregulated in the setting of I/R (10,11). Interestingly, it has been demonstrated that production of some complement products locally within an ischemic organ, rather than serum complement, is responsible for tissue injury in the setting of I/R injury. Utilizing a murine isograft model, Farrar and coinvestigators observed that significant tissue damage occurred when ischemic kidneys that produced C3 normally were transplanted into mice that were deficient in C3 or mice that produced C3 normally (12). Remarkably, ischemic donor kidneys deficient in C3 were functionally and structurally protected upon reperfusion when transplanted into recipients that produced C3 normally, demonstrating that local complement synthesis and activation within the graft itself, rather than serum-derived complement, was responsible for tissue injury (12).

Complement factor B (factor B) is a central component of the alternative pathway of complement (13). It has been

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previously demonstrated that factor B is an important mediator of tissue damage in the setting of a number of autoimmune disorders, because mice deficient in factor B are protected from inflammation and tissue destruction in several animal models of autoimmunity (14–19). Previous work has also shown that factor B plays an important role in the pathogenesis of I/R injury (20,21). Factor B–deficient mice developed less C3 deposition, neutrophil infiltration and functional impairment in comparison to wild-type (WT) counterparts in a renal I/R model (20). Furthermore, treatment with a monoclonal antibody against factor B protected against tissue injury in a renal I/R model (21). More recently, it has been shown that local production of factor B is markedly upregulated in a murine myocardial infarction model. In the same study, factor B$^{-/-}$ mice demonstrated less myocardial complement deposition and less adverse remodeling after ischemic myocardial injury (22). In aggregate, these observations highlight the importance of factor B in local tissue damage caused by autoimmunity and demonstrate that local production of factor B is important to the pathogenesis of ischemic tissue injury as well. Although a central role for locally produced factor B in ischemic tissue injury is evident, the endogenous molecules that trigger factor B production in response to tissue injury have not been identified.

Macrophages serve as an important source of extrahepatic complement and can dynamically modulate factor B synthesis (23–25). We have recently demonstrated that microbial double-stranded RNA can serve as a stimulus for factor B production in the macrophage (26). It has also been shown that DNA released from dying cells can act as damage-associated molecular patterns (DAMPs) and activate cells of the immune system (27). On the basis of the observation that a microbial nucleic acid can stimulate factor B synthesis, and given that much endogenous DNA can be spilled into the extracellular environment in the setting of cell death during ischemia, we hypothesized

that mammalian DNA may serve as a stimulus for factor B production. Accordingly, we found that DNA is a potent stimulus for factor B synthesis and release. Furthermore, our results demonstrate that factor B upregulation is independent of the DNA-sensing molecules DNA-dependent activator of interferon (IFN)-regulatory factors (DAI), absent in melanoma 2 (AIM2), toll-like receptor 9 (TLR9) and receptor for advanced glycation end products (RAGE) but requires myeloid differentiation factor 88 (MyD88) and high-mobility group box proteins (HMGBs), which have recently been shown to play important roles in nucleic acid–induced innate immune responses (28).

MATERIALS AND METHODS

Reagents

All tissue culture plates and flasks were purchased from Corning (Corning, NY, USA). Ultrapure lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4, TLR4 ligand) was purchased from List Biological Laboratories, Inc. (Vandell Way, CA, USA). Calf thymus DNA and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Mouse genomic DNA was purchased from Promega (Madison, WI, USA). Poly(dA-dT):poly(dT-dA) was from Invivogen (San Diego, CA, USA) and was used at a final concentration of 10 μg/mL. Heparan sulfate was purchased from Sigma (St. Louis, MO, USA). Fibrinogen was purchased from American Diagnostica (Hauppauge, NY, USA) and recombinant high-mobility group box-1 (HMGB1) was purchased from R&D Systems (Minneapolis, MN, USA). Inhibitory peptide sets for MyD88, which interfere with MyD88 homodimer formation, together with the control peptide were purchased from Imgenex (SanDiego, CA, USA) and were used at a final concentration of 100 mmol/L 24 h prior to experimentation. Inhibitors of p38 (SB 203580), c-Jun NH$_2$-terminal kinase (JNK) (JNK inhibitor II in solution) and ERK (extracellular signal-regulated kinase) (98059) were pur-

chased from Calbiochem (San Diego, CA, USA). Nuclear factor κB (NF-κB) activation inhibitor was purchased from Sigma (St. Louis, MO, USA). Concentrations of ligands and inhibitors are indicated in the corresponding figure legends.

Cells and Cell Culture

RAW264.7 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine and 100 U/mL penicillin and streptomycin. (Lonza, Basel, Switzerland). Cultures were maintained at 37ºC in a humidified atmosphere of 5% CO$_2$ and 95% air.

Thioglycollate-elicited peritoneal macrophages were collected 6 d after intraperitoneal injection of 1 mL of 3% Brewer's thioglycollate (Sigma) by peritoneal washout with 5 mL phosphate buffered saline (PBS). Cells were subsequently pelleted, resuspended and plated in RPMI1640 (Invitrogen) containing 10% FBS, 2 mmol/L L-glutamine and 100 U/mL of penicillin and streptomycin. All cell culture experiments were performed in triplicate.

AIM2$^{-/-}$ mouse bone-marrow–derived macrophage cell lines and WT control cells were obtained from Kate Fitzgerald (University of Massachusetts). Cultures were maintained in DMEM containing 10% FBS, 2 mmol/L L-glutamine and 100 U/mL of penicillin and streptomycin. AIM2 deficiency was confirmed by lack of interleukin (IL)-18 or IL-1β production in response to calf thymus DNA.

Stable Transfection Knockdown of HMGBs

RAW264.7 cells were transfected with either pSuper.retro.puroU6HMGBsi (6465 bp), a kind gift from Todatsugu Taniguchi, University of Tokyo, or a control plasmid, both of which contained a puromycin resistance gene. Plasmids were amplified with pSuper from Qiagen (Valencia, CA, USA). Transfection was performed with GeneJammer (Agilent Technologies, Santa Clara, CA, USA) ac-

cording to manufacturer instructions. After 24 h cells were split and plated with DMEM media containing 3 μg/mL puromycin. Single colonies of cells were harvested and plated out separately to grow. Expression of HMGB1in cells from each colony was then determined by Western blot.

Knockdown of DAI

DAI was knocked down in RAW264.7 macrophages by use of commercially available small-interfering RNA (siRNA) (Dharmacon, Chicago, IL, USA) transiently transfected with GeneJammer (Agilent) transfection agent. Knockdown of DAI in cells was confirmed by Western blot and produced knockdown of over 75%.

Western Blot Analysis

Samples were separated by sodium dodecyl sulfate (SDS) 10% polyacrylamide gel electrophoresis (PAGE), and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in 5% milk in Tris-buffered saline with 0.1% Tween (TBS-T), followed by immunostaining with optimized dilutions of primary antibody in 1% milk in TBS-T overnight at 4°C. Factor B antibody (1:5000) was obtained from Quidel Corporation (San Diego, CA, USA). Monoclonal anti-β-actin antibody was obtained from Novus Biologicals (Littleton, CO, USA). Membranes were washed three times for 10 min in TBS-T, and antibody binding was detected with horseradish peroxidase–conjugated secondary antibodies in a standard enhanced chemiluminescence reaction according to manufacturer's instructions (Pierce, Rockford, IL, USA) and exposed to Kodak X-Omat film (Kodak, Rochester, NY, USA).

Comparative Reverse Transcription–Polymerase Chain Reaction

RNA from cultured cells was isolated with a silica gel–based method (RNEasy Miniprep kits, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) re-

actions were performed with 1 μg of RNA and Omniscript reverse transcriptase (Qiagen). Prevalidated primers for factor B and β-actin were obtained from Qiagen. Comparative polymerase chain reaction (PCR) was performed with Brilliant SYBR Green QPCR Master Mix kits obtained from Stratagene (La Jolla, CA, USA). All samples were assayed in duplicate. An Mx3000P instrument and software were used for analysis with results normalized to actin levels and compared with baseline untreated cells.

Immunocytochemistry

RAW264.7 cells on coverslips were fixed with 4% paraformaldehyde (Canemco & Marivac, Quebec, Canada) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 2% bovine serum albumin (BSA) for 1h. Cells were then washed five times with 0.2% BSA and then incubated with antibodies against factor B (1:250, Quidel) and counterstained with a high-affinity probe for F-actin rhodamine phalloidin from Invitrogen (1:250). After washing, cells were then incubated with Alexa488-conjugated secondary antibodies (1:500) from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Mice

Male WT (WT: C57/BL6) mice (8–12 wks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice on a C57BL/6 background–deficient in TLR9 (CpG1) were provided by B Beutler (Scripps Research Institute, La Jolla, CA, USA) and bred in our facility. RAGE$^{-/-}$ mice were provided by Angelika Bierhaus (University of Heidelberg, Heidelberg, Germany) and bred in our facility. MyD88$^{-/-}$ (MyD88KO) mice were provided by Ruslan Medzhitov (HHMI, Yale University). TLR4$^{-/-}$ mice on a C57BL/6 background were generated and bred in our facility. All animals used in experiments were maintained in laminar flow cages in a specific pathogen-free atmosphere at the University of Pittsburgh (Pittsburgh, PA, USA). A standard diet and water were provided *ad libitum.*

MyD88$^{-/-}$ mice and WT matched controls were provided water supplemented with trimethoprim (4 mg/mL) and sulfamethoxazole (40 mg/mL) until 8 wks of age. Antibiotics were stopped for at least 2 wks prior to mice being used in experiments. Experimental protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh and all experiments were performed in adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (29).

Heterotopic Syngeneic Heart Transplantation

Heterotopic syngeneic heart transplantation was performed as previously described (30). To summarize, hearts were procured with cold University of Wisconsin (UW) solution (Viaspan, DuPont, Wilmington, DE, USA) and placed in cold UW solution for 2 h at 4°C for preservation. Heart transplantation was performed on mice in a heterotopic position by anastomosing the graft aorta and pulmonary artery to the recipient abdominal aorta and inferior vena cava, respectively. Each anastomosis was performed in an end-to-side fashion. At 3 h after reperfusion, grafts were harvested for analysis.

In Vivo DNA Injections

Intratracheal injection of DNA. Mice were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine administered via intraperitoneal injection. A tracheotomy was performed with a 20-gauge catheter, and DNA or carrier alone was administered intratracheally. Alveolar macrophages were harvested by bronchoalveolar lavage as described previously (31). An immunomagnetic separation system (BD Biosciences Pharmingen, San Diego, CA, USA) was used to isolate alveolar macrophages from BAL fluid. Magnetic nanoparticle-conjugated antibodies (anti-mouse Gr-1, anti-CD4, anti-CD8, and anti-CD45R/B220 antibodies; BD Biosciences) were chosen to label and remove PMN and lymphocytes. The resulting cells consisted of > 98%

macrophages, and cell viability was >95%.

Intraperitoneal injection of DNA.
One milliliter of 3% Brewer's thioglycollate (Sigma) was injected into the peritoneal cavity of each mouse. After 6 d, either DNA complexed with Lipofectamine 2000 or carrier containing Lipofectamine 2000 only was injected into the peritoneal cavity. After 6 h, peritoneal washout was performed using 5 mL of ice cold PBS to collect cells. The cell populations obtained contained >96% macrophages. Cells were subsequently pelleted and RNA was harvested for RT-PCR as described above.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Experimental results were analyzed for their significance by Student *t* test or analysis of variance (ANOVA) using SigmaStat (SPSS, Chicago, IL, USA). Significance was established at the 95% confidence level ($p < 0.05$).

All supplementary materials are available online at www. molmed. org.

RESULTS

Factor B is Upregulated Early after Myocardial I/R

Factor B plays an important role in ischemic tissue injury (20,22,32). It has been previously demonstrated that factor B is upregulated 3 wks after myocardial infarction in a murine model (22). We have observed that factor B is upregulated in hearts that underwent cold I/R compared with control hearts in a syngeneic, heterotopic cardiac transplant model (Supplementary Figure S1). Together, these data suggest that endogenous danger signals, or DAMPs, may be involved in factor B upregulation after tissue damage and hypoxia.

DNA Stimulates Factor B Synthesis and Release by Macrophages

Early upregulation of factor B occurs after ischemic tissue injury. However, the endogenous molecules that stimulate factor B production in the setting of ischemic tissue injury have not been identified. We have previously shown that microbial ligands, including LPS and polyinosine–polycytidylic acid (poly I:C) stimulate dynamic synthesis and release of factor B by macrophages (26). We next sought to test the hypothesis that one of several known endogenous mediators of inflammation known to activate pattern-recognition receptors would stimulate factor B synthesis. Because we previously found that activation of TLR4 signaling by LPS resulted in synthesis of factor B in macrophages, we therefore studied the effect of several endogenous molecules that have been identified as activators of TLR4 signaling (33–35). As previously observed, stimulation of RAW264.7 macrophages with LPS resulted in upregulation of factor B mRNA (9.9-fold, $p < 0. 001$). However, no significant upregulation of factor B was observed after stimulation with fibrinogen, heparan sulfate or HMGB1 at known stimulatory concentrations (Figure 1).

Because our previous data indicated that a microbial nucleic acid could trigger factor B synthesis and significant amounts of DNA could potentially be spilled into the extracellular compartment as a result of cell death in the setting of ischemic tissue injury, we next tested whether mammalian DNA could stimulate factor B synthesis and release. RAW264.7 macrophages were treated with Lipofectamine 2000 alone, calf thymus DNA alone or calf thymus DNA complexed with Lipofectamine 2000 for 6 h. Treatment of cells with DNA alone (2.8-fold) or DNA complexed with Lipofectamine 2000 (30.1-fold) resulted in significant ($p < 0. 001$) upregulation of factor B mRNA compared with untreated controls (Figure 2A). Time-course experiments revealed that factor B mRNA levels begin to rapidly increase after 3 h, reaching a peak at 18 h and remaining elevated for greater than 24 h (data not shown). To confirm that factor B protein was also upregulated after stimulation of RAW264.7 cells by DNA, we performed immunohistochemistry with antibodies
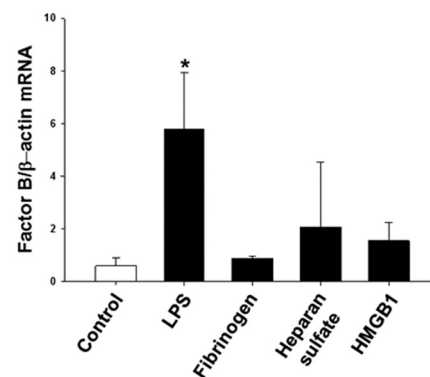

Figure 1. Factor B is not upregulated in macrophages by common known TLR4-mediated DAMPs. Expression of factor B mRNA in RAW264.7 macrophages treated with LPS (positive control), or DAMPs (fibrinogen, heparan sulfate, HMGB1) or PBS (control) for 6 h. *$p < 0.05$ versus control. Experiment performed in triplicate and repeated at least three times.

specific for factor B. As shown in Figure 2B, factor B protein was detected in the cells after stimulation with DNA-complexed Lipofectamine 2000. To confirm that factor B protein was also synthesized and released into the extracellular environment after stimulation of RAW264.7 cells by DNA complexed with lipofection reagent, we collected media from cells treated with calf thymus DNA/Lipofectamine 2000 or Lipofectamine 2000 alone and performed Western blotting with antibodies specific for factor B. Factor B protein was detected in the media after stimulation with calf thymus DNA/Lipofectamine 2000, as shown in Figure 2C. Release of factor B protein into the extracellular environment occurs in a concentration-dependent manner, as demonstrated in Figure 2D.

RAW264.7 Is a Mouse Macrophage Cell Line

To confirm that mouse genomic DNA also stimulates factor B synthesis, and that increased factor B expression is not species dependent, RAW264.7 macrophages were treated with mouse genomic DNA complexed with Lipofectamine 2000. We observed that factor B

mRNA was significantly ($p < 0.001$) up-regulated after treatment of cells with mouse genomic DNA/Lipofectamine 2000 (18.3-fold) compared with untreated cells (Figure 2E).

It is possible that other nucleic acids, nuclear proteins or other contaminants rather than DNA might result in upregulation of factor B. To evaluate this possibility we tested whether a synthetic oligonucleotide, which should be free of any potentially contaminating molecules found in the nucleus of mammalian cells, would also result in upregulation of factor B when used to stimulate cells. We hypothesized that macrophages stimulated with a synthetic poly(dA-dT)·poly(dT-dA), which may assume a B-form conformation in solution, should also result upregulation of factor B mRNA. Accordingly, after stimulation of RAW264.7 macrophages with poly(dA-dT)·poly(dT-dA) complexed with a lipofection reagent, increased factor B mRNA levels (10.5-fold increase, $p < 0.001$) compared with controls (Figure 2F). To exclude the possibility that LPS contamination of DNA preparations used in these experiments accounts for increased factor B expression, peritoneal macrophages were harvested from both WT and TLR4-deficient mice. Cells were placed in culture and stimulated with carrier, DNA/Lipofectamine or LPS. Significantly increased factor B mRNA levels ($p \leq 0.001$) were observed in both WT and TLR4-deficient mice after stimulation with DNA/Lipofectamine, whereas TLR4-deficient cells exhibited impaired synthesis of factor B mRNA after stimulation with LPS. This result demonstrates that synthesis of factor B after stimulation with DNA/Lipofectamine is unlikely to be due to LPS contamination (Supplementary Figure S2).

DNA Stimulates Factor B Upregulation *In Vivo*

We next hypothesized that DNA would similarly stimulate upregulation of factor B in macrophages *in vivo*. To test this hypothesis, we performed intratracheal injections of DNA complexed with
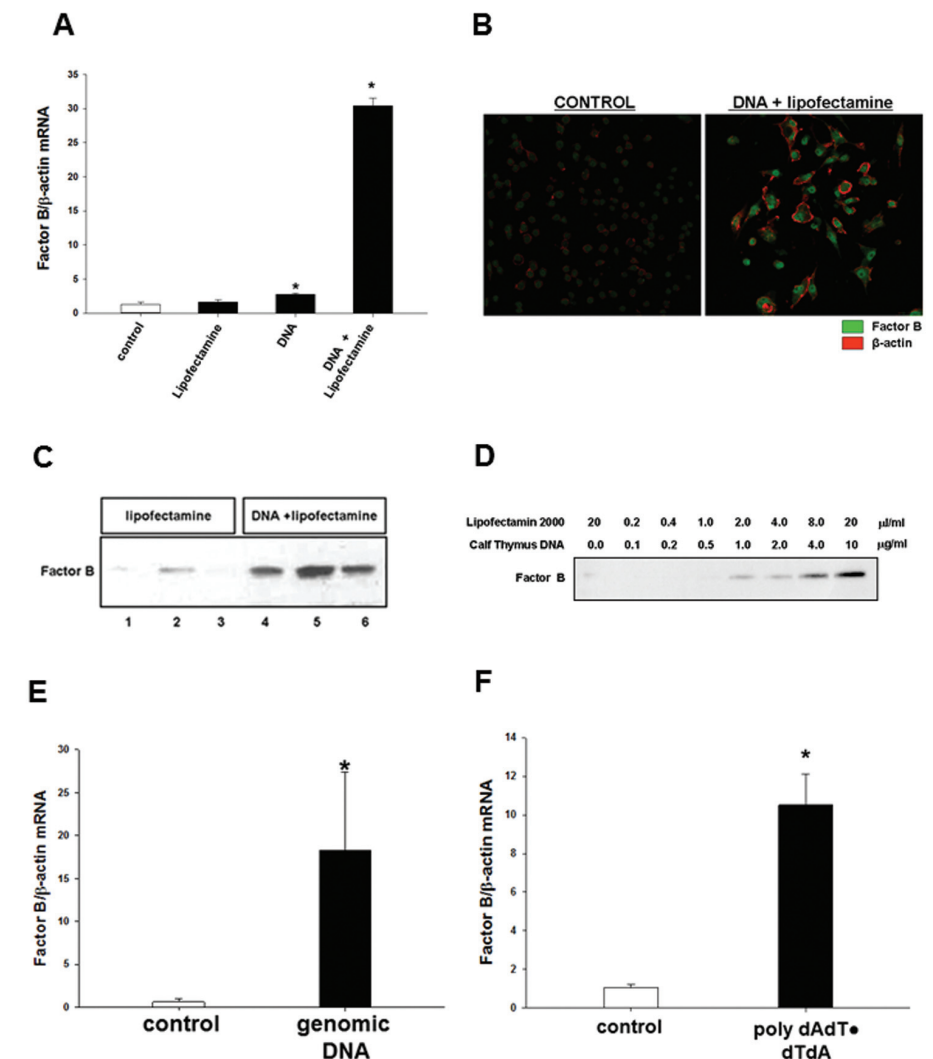

Figure 2. DNA stimulates factor B synthesis and release by macrophages *in vitro*. (A) Factor B mRNA expression in RAW264.7 macrophages treated for 6 h with Lipofectamine 2000 alone, calf thymus DNA (10 μg/mL) alone or calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000. *$p < 0.001$ versus control. (B) Immunohistochemistry for factor B and β-actin in RAW264.7 macrophages treated with DNA (10 μg/mL) complexed with Lipofectamine 2000, or Lipofectamine 2000 alone (control) for 6 h. (C) Factor B release into supernatants from RAW264.7 macrophages treated with DNA (10 μg/mL) complexed with Lipofectamine 2000 or Lipofectamine 2000 alone for 24 h. (D) Factor B protein expression in cell culture supernatants from RAW264.7 macrophages treated with DNA complexed with Lipofectamine 2000, or Lipofectamine 2000 alone for 24 h at various concentrations as indicated. (E) Expression of factor B mRNA in RAW264.7 macrophages treated for 6 h with mouse genomic DNA (10 μg/mL) complexed with Lipofectamine 2000. *$p < 0.001$ versus control. (F) Factor B mRNA expression in RAW264.7 macrophages treated for 6 h with poly (dAdT)·poly(dTdA) (10 μg/mL) complexed with lipofection reagent. *$p < 0.001$ versus control. All experiments performed in triplicate, and repeated at least three times.

Lipofectamine 2000, or carrier containing Lipofectamine 2000, harvested alveolar macrophages, and measured levels of factor B mRNA by comparative RT-PCR.

Factor B mRNA was significantly (3.9-fold, $p < 0.005$) upregulated in alveolar macrophages after DNA/Lipofectamine 2000 injection (Figure 3A).
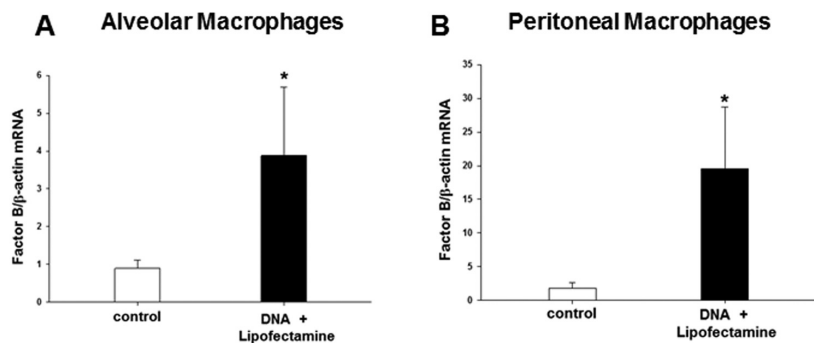
Figure 3. DNA stimulates factor B upregulation *in vivo.* (A) Factor B mRNA expression in alveolar macrophages harvested 6 h after intratracheal injection of either Lipofectamine 2000 in carrier or calf thymus DNA complexed with Lipofectamine 2000. *$p < 0.005$ versus control. (B) Factor B mRNA expression in peritoneal macrophages harvested at 6 h after intraperitoneal injections of either Lipofectamine 2000 in carrier, or calf thymus DNA complexed with Lipofectamine 2000. *$p < 0.001$ versus control; n = 6 per experimental group and experiments were repeated at least twice.

In a separate set of experiments, macrophages were elicited into the peritoneal cavities of mice using intraperitoneal thioglycollate injections. DNA complexed with Lipofectamine 2000 or carrier containing Lipofectamine 2000 was then injected into the peritoneal cavity. Peritoneal macrophages were harvested and comparative RT-PCR was performed. Increased synthesis of factor B mRNA (19.6-fold increase, $p < 0.001$) was also observed in peritoneal macrophages treated with DNA/Lipofectamine 2000 compared with controls in these experiments (Figure 3B).

Factor B Synthesis and Release after Stimulation with DNA Requires HMGBs but Not RAGE

It has been demonstrated that HMGB proteins serve as sentinels for nucleic acid–induced immune responses (28) and mouse cells lacking HMGB1 or HMGB2 demonstrate impaired responses to either RNA or DNA. Further, cells in which the expression of all three HMGBs are suppressed demonstrate markedly impaired activation in response to nucleic acids (28).

To test whether HMGBs mediate DNA-induced synthesis of factor B, we first generated RAW264.7 cells in which the expression of HMGB proteins was sup-

pressed utilizing a siRNA vector that targets HMGBs. RAW264.7 cells were stably transfected with a siRNA vector targeting HMGBs or a control siRNA vector. After stable transfection of RAW264.7 cells with the siRNA vector targeting HMGBs, we identified a clonal cell line (siHMGB-12) with impaired production of HMGB1 protein by Western blot (Figure 4A). Robust upregulation of factor B mRNA was observed after cells transfected with the siRNA control vector were stimulated with DNA complexed with Lipofectamine 2000 (160-fold, $p < 0.001$). However, cells transfected with the siRNA vector targeting HMGBs demonstrated significantly less upregulation (10.1-fold) of factor B mRNA in response to DNA complexed with Lipofectamine 2000 (Figure 4B), demonstrating that knockdown of HMGBs results in impaired synthesis of factor B after stimulation with DNA. HMGBs and HMGB1 in particular have been shown to bind many ligands, such as nucleic acids, microbial ligands and cytokines. We were therefore not surprised by our observation that similar stimulation of siHMGB-12 cells with LPS also resulted in decreased factor B levels compared with controls (data not shown).

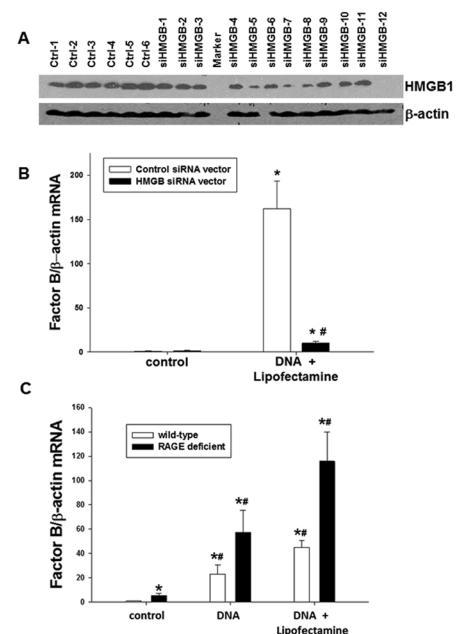One pathway through which complexes containing DNA and HMGB1 acti-

Figure 4. Factor B synthesis and release after stimulation with DNA requires HMGBs but not RAGE A. Expression of HMGB1 in RAW264.7 cell clones stably transfected with siRNA against HMGBs (siHMGB), or control siRNA (Ctrl). (A) Clonal cell line (siHMGB-12) expressing relatively low levels of HMGB1 was selected for future experiments. (B.) Factor B mRNA expression in control or siHMGB-12 cells 6 h after stimulation with calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000. *$p < 0.001$ versus unstimulated cells containing control siRNA vector, #$p < 0.001$ versus cells containing control siRNA vector stimulated with DNA/lipofectamine. (C) Factor B mRNA expression in peritoneal macrophages from WT and RAGE-deficient mice after stimulation with calf thymus DNA (10 μg/mL) alone, or calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000 for 6 h. *$p < 0.01$ versus WT control, #$p < 0.01$ versus unstimulated RAGE-deficient cells. Experiments were performed in triplicate and repeated at least three times.

vate the immune system uses the receptor RAGE (36). To test whether RAGE is involved in the synthesis of factor B after stimulation of macrophages with DNA, peritoneal macrophages were harvested from both WT and RAGE-deficient mice and placed in culture. Macrophages were

then stimulated with either DNA alone or DNA/Lipofectamine 2000. As seen in Figure 4C, after stimulation with DNA, a 24.2-fold increase in factor B mRNA was observed in WT macrophages compared with untreated control WT macrophages ($p < 0.01$). After treatment with DNA alone, significant ($p < 0.01$) upregulation (60.4-fold) of factor B mRNA was observed in RAGE-deficient macrophages. Stimulation of either WT macrophages or RAGE-deficient macrophages with DNA/Lipofectamine 2000 led to significant ($p < 0.01$) upregulation of factor B mRNA (47.4-fold and 122-fold respectively). These observations demonstrate that RAGE is not critical to synthesis of factor B by macrophages after stimulation with DNA.

Factor B Upregulation in Macrophages after Stimulation with DNA is Independent of AIM2, DAI and TLR9 but Dependent on MyD88

AIM2 is a cytosolic DNA sensor (37–40). To determine whether AIM2 mediates synthesis of factor B by macrophages in response to mammalian DNA, we used a mouse macrophage cell line deficient in AIM2 and compared responses to WT controls. AIM2 deficiency was confirmed by lack of IL-18 production in response to DNA (not shown). Macrophages were then stimulated with either DNA alone or DNA/Lipofectamine 2000. As demonstrated in Figure 5A, a 1.8-fold increase in factor B mRNA was observed in WT macrophages compared with untreated controls ($p < 0.05$) after stimulation with DNA. A 2.9-fold increase in factor B mRNA was observed in AIM2-deficient cells after stimulation with DNA ($p < 0.01$) compared with untreated AIM2-deficient macrophages. After stimulation of cells with DNA complexed with a lipofection reagent, robust upregulation of factor B mRNA was observed in both WT (20-fold, $p < 0.05$) and AIM2-deficient cells (87-fold, $p < 0.01$) compared with untreated cells, indicating that AIM2 is not required for upregulation of factor B.
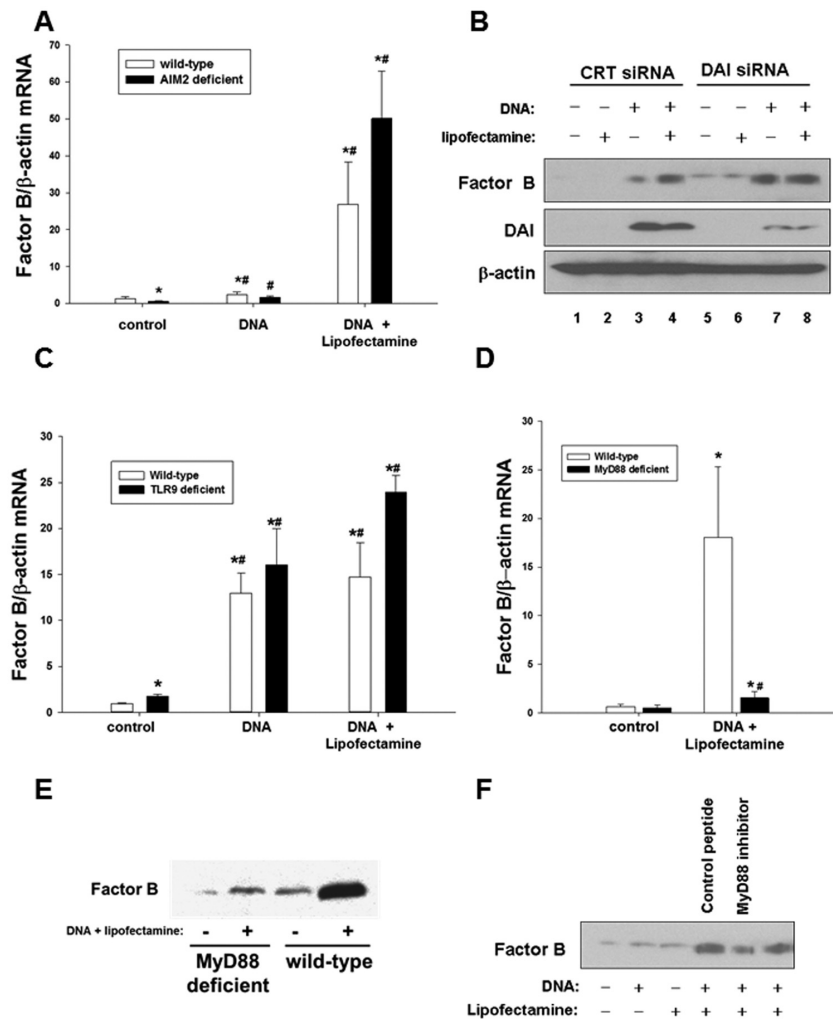

Figure 5. Factor B upregulation in macrophages after stimulation with DNA is independent of AIM2, DAI and TLR9, but dependent on MyD88. (A) Factor B mRNA expression in WT and AIM2-deficient mouse peritoneal macrophages 6 h after stimulation with calf thymus DNA (10 μg/mL) alone or calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000. *$p < 0.001$ versus WT control, #$p < 0.001$ versus unstimulated AIM2-deficient cells. (B) Expression of factor B and DAI in cell lysates of RAW264.7 macrophages pretreated with either control (CRT) or DAI-targeting siRNA and then stimulated for 24 h with DNA alone (10 μg/mL), Lipofectamine 2000 alone or DNA (10 μg/mL) complexed with Lipofectatmine 2000 as indicated. (C) Expression of factor B mRNA in peritoneal macrophages from WT and TLR9-deficient mice stimulated with calf thymus DNA (10 μg/mL) alone or calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000 for 6 h. *$p < 0.001$ versus WT control, #$p < 0.001$ versus unstimulated TLR9-deficient cells. (D) Factor B mRNA expression in peritoneal macrophages from WT and MyD88-deficient mice 6 h after stimulation with calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000. *$p < 0.001$ versus WT control, #$p < 0.001$ versus stimulated WT cells. (E) Factor B release into cell culture supernatants of peritoneal macrophages from WT and MyD88-deficient mice stimulated with calf thymus DNA (10 μg/mL) complexed with Lipofectamine 2000 for 6 h. (F) Factor B mRNA expression in cell culture supernatants from RAW264.7 macrophages pretreated with MyD88-inhibitor peptide or control peptide, then stimulated with DNA alone (10 μg/mL), Lipofectamine 2000 alone or with DNA (10 μg/mL) complexed with Lipofectatmine 2000 for 6 h as indicated. Experiments were performed in triplicate and repeated at least three times.
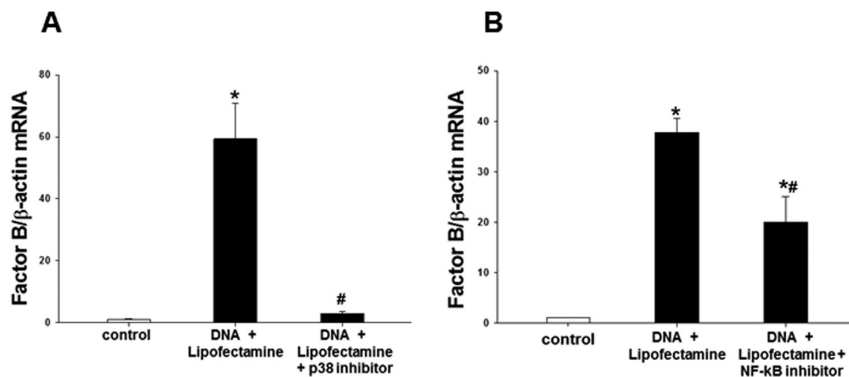
A

B

Figure 6. p38 and NF-κB mediate factor B production in macrophages. (A) Expression of factor B mRNA in RAW264.7 macrophages pretreated for 30 min with p38 inhibitor (SB 203580, 40 μmol/L) then treated with DNA (10 μg/mL) complexed with Lipofectamine 2000 for 6h. *$p < 0.001$ versus control, #$p < 0.001$ versus DNA + Lipofectamine 2000. (B) Factor B mRNA expression in RAW264.7 macrophages pretreated with NF-κB activation inhibitor (10 μmol/L) overnight, then treated with DNA (10 μg/mL) complexed with Lipofectamine 2000 for 6 h. *$p < 0.001$ versus control, #$p < 0.001$ versus DNA + Lipofectamine 2000. Experiments were performed in triplicate and repeated at least three times.

DAI is another cytosolic sensor of DNA (41,42). To evaluate whether DAI mediates factor B synthesis after stimulation with DNA, RAW264.7 were treated with siRNA directed against DAI, or a negative control siRNA. After treatment with a control siRNA, stimulation of cells with either DNA alone, or DNA complexed with a lipofection reagent, resulted in a clear increase in DAI protein levels (Figure 5B). After stimulation with either DNA or DNA/Lipofectamine 2000, substantially less DAI protein was observed in cells treated with DAI siRNA compared with those treated with the control siRNA. However, synthesis of factor B protein was not impaired in cells treated with the DAI siRNA, suggesting that DAI is not critical for mediating synthesis of factor B after stimulation with DNA.

TLR9 serves as a molecular sensor for bacterial CpG DNA and it has also been implicated in detecting mammalian DNA (36). To determine whether TLR9 mediates synthesis of factor B by macrophages in response to mammalian DNA, peritoneal macrophages from both WT and TLR9-deficient mice were then stimulated with either DNA alone or DNA/Lipofectamine 2000 *in vivo*. As seen in Figure 5C, after stimulation with DNA, a

13.0-fold increase in factor B mRNA was observed in WT macrophages compared with untreated control WT macrophages ($p < 0.001$). Similarly, after treatment with DNA alone, significant ($p < 0.001$) upregulation (16.0-fold) of factor B mRNA was observed in TLR9-deficient macrophages. Stimulation of either WT macrophages or TLR9-deficient macrophages with DNA/Lipofectamine 2000 led to significant ($p < 0.001$) upregulation of factor B mRNA (14.7-fold and 23.7-fold respectively). Because TLR9-deficient macrophages significantly upregulate factor B in response to DNA alone and DNA complexed to a lipofection reagent, TLR9 is not required for synthesis of factor B by macrophages after stimulation with DNA. This finding is consistent with our previous observation that stimulation of macrophages with CpG DNA, a microbial ligand of TLR9, does not result in synthesis of factor B (26).

MyD88 functions as an intracellular adaptor that mediates intracellular signaling by TLR9 and other TLRs. However, DNA-sensing pathways that use MyD88 but are independent of TLR9 have been described (43–46). Because the results of our previous experiments demonstrated that synthesis of factor B by macrophages

does not require AIM2, DAI or TLR9, we next hypothesized that synthesis and release of factor B upon stimulation by DNA is mediated by MyD88-dependent DNA-sensing pathways. Peritoneal macrophages were harvested from both WT and MyD88-deficient mice, placed in culture and then stimulated with DNA/Lipofectamine 2000. Stimulation of WT macrophages with DNA/Lipofectamine 2000 led to significant ($p < 0.05$) upregulation of factor B mRNA (28.1-fold compared with untreated WT). In comparison, upregulation of factor B mRNA in MyD88-deficient macrophages was markedly impaired (3.0-fold, $p < 0.05$; Figure 5D). Likewise, factor B protein release was reduced in MyD88-deficient macrophages following DNA/lipofectamine stimulation (Figure 5E).

We hypothesized that if MyD88 mediates upregulation of factor B in response to DNA, then a soluble inhibitor of MyD88 should also impair synthesis and release of factor B in response to DNA. To test this hypothesis, RAW264.7 macrophages were pretreated with a peptide that inhibits MyD88, a control peptide, or no pretreatment and then stimulated with DNA with or without a lipofection reagent. As can be seen in Figure 5F, the MyD88 inhibitor peptide inhibited synthesis and release of factor B, confirming that MyD88 mediates factor B synthesis and release after stimulation with DNA.

P38 and NF-κB Mediate Factor B Production in the Macrophage

In our previous work, we observed that the MAP kinases p38 and JNK, as well as the transcription factor NF-κB, mediate downstream signaling that results in upregulation of factor B after stimulation of macrophages with LPS or poly I:C (26). To determine if these pathways also mediate synthesis of factor B by macrophages after stimulation with DNA, RAW264.7 macrophages were first pretreated with a small molecule inhibitor of p38. As in previous experiments, robust upregulation of factor B mRNA was observed after stimulation of

cells with DNA (59.3 fold) (Figure 6A). Pretreatment of cells with an inhibitor of p38 prevented upregulation of factor B mRNA (2.9-fold upregulation compared with untreated control, p = not significant) Pretreatment of macrophages with a small molecule inhibitor JNK did not inhibit synthesis of factor B (data not shown). Furthermore, inhibition of NF-κB partially prevented upregulation of factor B mRNA after stimulation of cells with DNA (Figure 6B).

DISCUSSION

A critical role for complement factor B in ischemic tissue injury has been previously established (20,32). In this study we demonstrated that factor B is upregulated early within organs after ischemia/reperfusion. Previous work has demonstrated that DNA can serve as an inflammatory mediator (47). Our results indicate that mammalian DNA stimulates synthesis and release of factor B from macrophages. After knocking down HGMBs by use of an siRNA vector, substantially less upregulation of factor B was observed compared with cells containing a control vector. This observation demonstrates that HMGBs play an important role in mediating synthesis of factor B in response to DNA. Interestingly, previous work has shown that cell lysates from necrotic cells could activate immune cells, and that treating the lysates with either DNAse I or proteinase K abrogated this effect, suggesting that both self-proteins and DNA contributed to cell activation (27).

We (26) and others (48,49) have shown that ischemic insults such as hemorrhagic shock and ischemia with reperfusion can lead to DNA release into the circulation to trigger early inflammation via TLR9. However, factor B levels, or the role of complement, were not addressed in these studies. Here we show that TLR9 is not required for macrophage upregulation of factor B by DNA. In accord with this observation, we previously found that stimulation of TLR9 with microbial ligands did not result in synthesis of factor B.

Our data demonstrate that DNA alone is capable of stimulating cells to increase synthesis of factor B. However, when entry of DNA into the cytoplasm is facilitated with a lipofection reagent, markedly greater synthesis of factor B occurs. This observation suggests that one or more of the cytoplasmic sensors of DNA other than TLR9 are largely responsible for dynamically modulating upregulation of factor B in response to DNA.

Several cytoplasmic DNA sensors have been identified. DAI, which has also been called DLM-1 and ZBP-1, has been shown to sense the presence of cytosolic DNA and activate the immune system (41). On detection of cytosolic DNA through direct interaction, DAI recruits TBK1 and IRF3, which results in the expression of type I IFN and other genes (41,42).AIM2 is a cytosolic DNA sensor that binds to DNA, recruits ASC and then caspase-1, and ultimately results in IL-1β maturation (37-40). Our results demonstrate that neither DAI or AIM2 is required for synthesis and release of factor B after stimulation with DNA.

Several MyD88-dependent DNA-sensing pathways have been described (40–43). Our results indicate that factor B synthesis and release is highly dependent on MyD88, but independent of TLR9. We have previously demonstrated that myocardial injury after cold I/R is also largely dependent on MyD88 (50). RNA polymerase III has also been shown to detect DNA in the cytosol and trigger activation of the immune system through RIG-I (51,52). IFI16 has recently been identified as a pyrin-domain–containing sensor of DNA (53). These receptors provide redundancy in the ability of the cell to detect cytoplasmic DNA but do not utilize the MyD88-signaling pathway. From our data, MyD88-dependent pathways appear to be largely responsible for factor B synthesis and release in response to DNA, so the role of RNA polymerase III or IFI16 was not investigated in this study.

CONCLUSION

Previous work has demonstrated a central role for complement factor B in I/R

injury (20,32), as well as a number of autoimmune disorders including arthritis, lupus and airway hyperresponsiveness (15–19). The data presented here demonstrate that mammalian DNA is an endogenous molecule that stimulates synthesis and release of factor B through a pathway involving HMGBs, MyD88, p38 and NF-κB. A better understanding of the mechanisms underlying synthesis and release of complement in response to endogenous molecules may allow development of novel therapeutic strategies that target inflammation and tissue destruction in diverse settings including ischemic tissue injury, trauma and autoimmunity.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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