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Release of High Mobility Group Box 1 by Dendritic Cells Controls T Cell Activation via the Receptor for Advanced Glycation End Products¹

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High mobility group box 1 (HMGB1) is an abundant and conserved nuclear protein that is released by necrotic cells and acts in the extracellular environment as a primary proinflammatory signal. In this study we show that human dendritic cells, which are specialized in Ag presentation to T cells, actively release their own HMGB1 into the extracellular milieu upon activation. This secreted HMGB1 is necessary for the up-regulation of CD80, CD83, and CD86 surface markers of human dendritic cells and for IL-12 production. The HMGB1 secreted by dendritic cells is also required for the clonal expansion, survival, and functional polarization of naive T cells. Using neutralizing Abs and receptor for advanced glycation end product-deficient (RAGE^{-/-}) cells, we demonstrate that RAGE is required for the effect of HMGB1 on dendritic cells. HMGB1/RAGE interaction results in downstream activation of MAPKs and NF- κ B. The use of an ancient signal of necrosis, HMGB1, by dendritic cells to sustain their own maturation and for activation of T lymphocytes represents a profitable evolutionary mechanism. *The Journal of Immunology*, 2005, 174: 7506–7515.

Dendritic cells are the most important APCs and are crucial to activate immune responses (1). Dendritic cells challenged with primary proinflammatory signals undergo a well-characterized differentiation program, referred to as maturation, that endows them with the ability to migrate to secondary lymphoid organs and to activate naive T cells (2, 3).

The high mobility group box 1 (HMGB1)³ protein is expressed in the nuclei of vertebrate cells at a level that varies in vivo over 2 log scales and appears to correlate with the cellular differentiation state (4). HMGB1 is also a cytokine that mediates endotoxin lethality, inflammation, and macrophage activation (5, 6). It has been argued that the presence of a nuclear protein outside of the cell signals unprogrammed cell death and tissue damage (7). Indeed, HMGB1 is passively released from necrotic cells, but is avidly retained within the remnants of apoptotic cells (8). However, some cell types, including monocytes and macrophages, can secrete HMGB1 actively without dying. Active secretion involves extensive acetylation of HMGB1 and transfer to a secretory en-

dolysosomal compartment (9–11). Extracellular HMGB1 activates membrane receptors, including the receptor for advanced glycation end products (RAGE), a member of the Ig superfamily (12, 13), and possibly TLR (14).

Exogenous HMGB1 is one of many signals that promote dendritic cell maturation (15, 16). In vivo, exogenous HMGB1 effectively controls dendritic cell function, with substantial adjuvant effects on the immunogenicity of soluble or corpusculate Ags (15). Under certain conditions, necrotic cells provide HMGB1 for dendritic cell activation (15); we have suggested that this reflects the need for increased immune surveillance after traumatic tissue death (17).

In this study we show that the role of HMGB1 and its receptor, RAGE, is much more important than previously suspected. Human maturing dendritic cells actively secrete HMGB1 in response to inflammatory stimuli, and secreted HMGB1 is necessary for proliferation, survival, and polarization of naive CD4⁺ T cells. The blockage of either HMGB1 or RAGE restrains T cell responses. Thus, the presence of an NF in the extracellular environment represents a bottleneck for the initiation of adaptive immune responses in humans. When HMGB1 is not passively leaked in the microenvironment of dendritic cells by necrotic cells, the dendritic cells themselves provide the required extracellular HMGB1 by an active secretion mechanism.

Materials and Methods

Media and reagents

The medium used throughout was RPMI 1640 (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 1.5 mM L-glutamine, and 10% heat-inactivated FCS. Rabbit polyclonal anti-HMGB1 Ab raised against peptide 166–181 was purchased from BD Pharmingen. Goat polyclonal Ab against human RAGE was purchased from Chemicon International. Expression and purification of recombinant HMGB1, box A, and GST protein (used as control) were performed as previously described (18). Endotoxin was removed by passage through Detoxo-Gel columns (Pierce).

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³ Abbreviations used in this paper: HMGB1, high mobility group box 1 protein; RAGE, receptor for advanced glycation end product.

Culture of dendritic cells

Human PBMCs were isolated from healthy donor blood by Ficoll density gradient centrifugation. The method for the generation of dendritic cells from PBMCs has been previously described (19). Briefly, monocytes isolated by adherence to plastic were incubated with GM-CSF (800 U/ml) and IL-4 (800 U/ml; R&D Systems) for 6 days. The purity and maturation of dendritic cells were routinely assessed by staining with Abs to CD1a, CD14, CD83, CD86, HLA-DR, and CD40 (BD Pharmingen). Immature dendritic cells were CD14⁻, CD1a⁺, CD83⁻, CD80⁻, CD86⁺, and HLA-DR⁺. Murine dendritic cells were derived from RAGE^{-/-} mice or age- and sex-matched wild-type animals. For this purpose, precursors isolated from bone marrows were propagated for 7 days in complete medium containing 1000 U/ml murine rGM-CSF and 5 ng/ml murine rIL-4 (R&D Systems). Endotoxin (LPS; 1 μg/ml; Sigma Aldrich), rabbit anti-HMGB1 (1/200 dilution), goat anti-RAGE (1/200 dilution), or box A (10 μg/ml) were added as indicated to induce dendritic cell maturation. For detection of cytokines, supernatants were collected and either used immediately or stored frozen until analysis. When indicated, the up-regulation of surface markers was expressed as the average increase, calculated as: ((mean fluorescence intensity of endotoxin treated cells – mean fluorescence intensity of untreated cells)/mean fluorescence intensity of untreated cells) × 100.

Isolation of naive CD4 T cells

Human CD4⁺ naive T cells were purified by two-step immunomagnetic selection. Briefly, CD4⁺ T cells were first negatively selected for CD4⁺

using the CD4⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Then memory/effector T cells were depleted by incubation with anti-CD45RO-conjugated magnetic beads (Miltenyi Biotec). The purity of naive CD4⁺ T cells was typically >95%.

Immunoassays for HMGB1

The levels of HMGB1 in culture medium were assessed by Western blotting as previously described (8). Cellular localization of HMGB1 was assessed by immunostaining of dendritic cells using affinity-purified rabbit anti-HMGB1 polyclonal Abs. Dendritic cells were transferred onto adhesion slides and incubated at 37°C for 1 h to allow adherence. The adherent cells were fixed with phosphate-buffered formaldehyde (4%; pH 7.4; for 15 min) and permeabilized with saponin. After blocking the slides with 10% BSA, cells were sequentially incubated with anti-HMGB1 Abs and FITC-conjugated anti-rabbit IgG. Nuclei were counterstained with Hoechst 33342 (Molecular Probes).

Western blot analysis of cellular protein kinases

Cell lysates (30–40 μg) were electrophoresed on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% (w/v) nonfat dry milk/1% (v/v) Tween 20 in PBS for 1 h at room temperature and incubated overnight with primary Ab at 4°C. Abs to phospho- and total p38, phospho- and total ERK, and phospho- and total IκBα were all purchased from Cell Signaling Technology. Abs to β-actin were obtained from Sigma-Aldrich. Detection was

FIGURE 1. Maturing dendritic cells secrete HMGB1. *A*, HMGB1 staining (white) is present in the nuclei of immature dendritic cells. *B*, Upon maturation of dendritic cells with endotoxin, HMGB1 staining is seen predominantly in the cytoplasm. The arrows point to central, nuclear regions of representative cells. *C*, HMGB1 (green) colocalizes with Hoechst 33342 (blue) in the nuclei of immature dendritic cells. *D*, Upon maturation with endotoxin, HMGB1 (green) is observed predominantly in the cytoplasm in structures resembling vesicles. *E*, Dendritic cells were stimulated with endotoxin for various time periods, and cell-conditioned culture medium (supernatants) was assayed for levels of HMGB1 protein by Western blotting analysis. The extracellular levels of HMGB1 increase with time, whereas the intracellular HMGB1 remains unchanged (pellets). Cells dying by necrosis leak lactate dehydrogenase (LDH). Accumulation of HMGB1 in the culture supernatants does not correlate with the release of LDH. Results are from representative routine experiments.

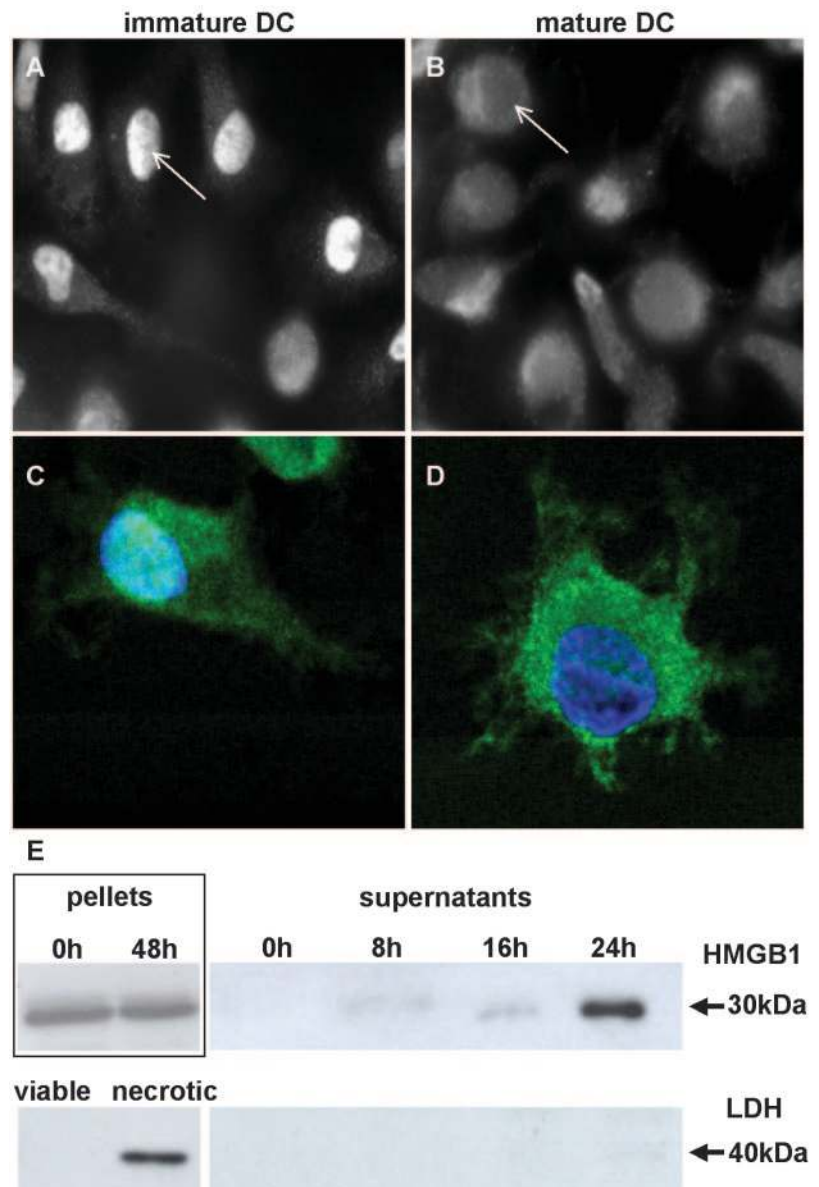


FIGURE 2. HMGB1 secreted from maturing dendritic cells is necessary for CD4⁺ T cell proliferation, survival, and Th1 differentiation. Immature dendritic cells were incubated for 8 h with endotoxin (maturing dendritic cells (DC)) alone or in the presence of anti-HMGB1 Abs (anti-HMG), box A (boxA), control Ig (ctrl. Ig), or a control GST protein (ctrl. GST). Dendritic cells were then incubated with 2×10^6 allogeneic naive CD4⁺ T cells (input) for 8 days. HMGB1 blockade abrogates T cell expansion (anti-HMGB1 Ab, $p < 0.005$; box A, $p < 0.01$; A) by reducing T cell proliferation of ($p < 0.005$, both agents; B) and increasing the number of apoptotic T cells (D). C. The number of IFN- γ ⁺ (Th1) T cells is reduced by HMGB1-blocking reagents ($p < 0.005$). A representative example of five (anti-HMGB1) and three (box A) independent experiments is shown as the mean \pm SD of duplicate cultures. *, Statistically significant values.

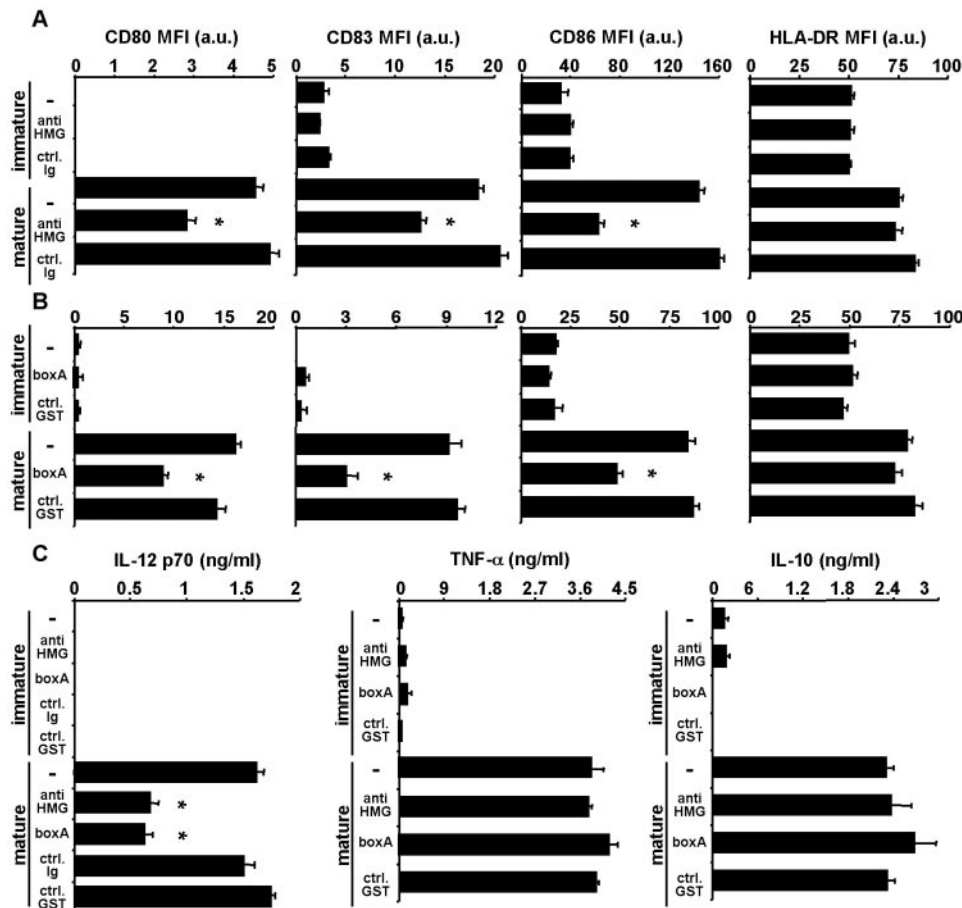
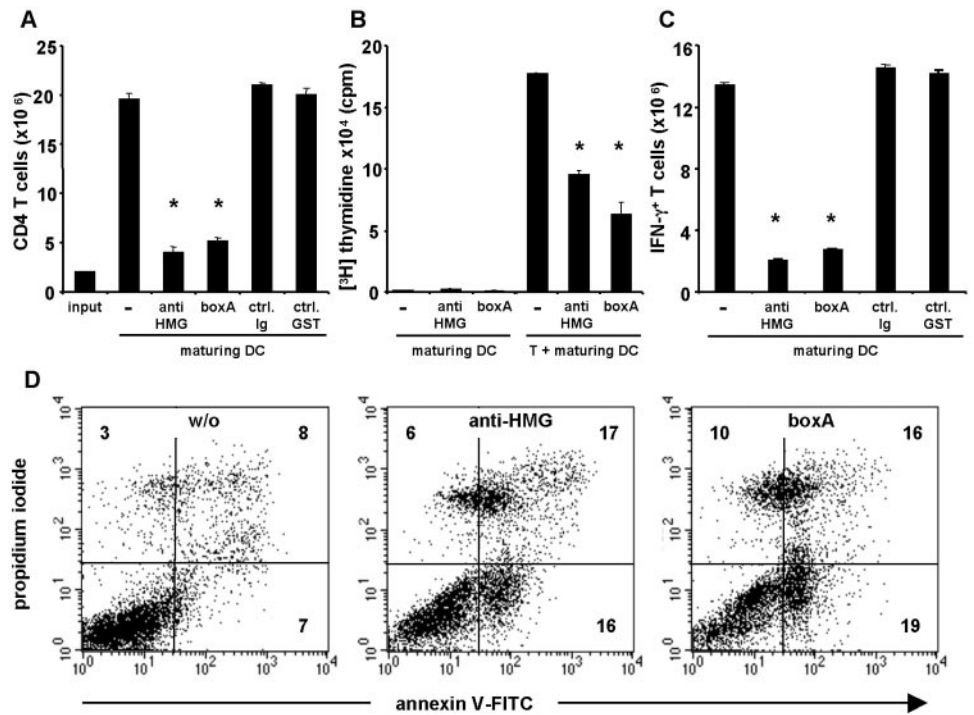


FIGURE 3. Secreted HMGB1 is necessary for the maturation of dendritic cells. Dendritic cells were cultured alone (immature) or in the presence of endotoxin (mature) for 48 h. Anti-HMGB1 Abs (anti-HMG), box A (boxA), control Ig (ctrl. Ig), or a control GST protein (ctrl. GST) were added to the culture medium as indicated. Samples were analyzed in duplicate. A and B, Endotoxin up-regulates the expression of CD80, CD83, CD86, and HLA-DR surface markers. HMGB1 blockade reduces the up-regulation of CD80, CD83, and CD86 ($p < 0.05$), but not that of HLA-DR. Results represent the mean fluorescence intensity (MFI) \pm SD, as assessed by flow cytometry of three independent experiments using dendritic cells from different donors. C, Endotoxin induces production of IL-12 p70, TNF- α , and IL-10. HMGB1 blockade significantly decreases the secretion of IL-12 p70 ($p < 0.005$), but not that of TNF- α or IL-10. Secreted cytokine levels were assessed by ELISA. Representative examples of three independent experiments are shown as the mean \pm SD of duplicate cultures. *, Statistically significant values.

performed by ECL (Amersham Biosciences). Relevant bands were quantified by densitometry using a computerized image analysis program (Amersham Biosciences).

Detection of cytokines

The concentrations of IL-12 p70, TNF- α , and IL-10 in culture medium were determined using a two-site sandwich ELISA (R&D Systems), with reference to standard curves of purified recombinant cytokines. Samples were analyzed in serial 2-fold dilutions in duplicate.

Culture of naive CD4⁺ T lymphocytes with dendritic cells

Dendritic cells were incubated with endotoxin alone or in the presence of anti-HMGB1 Abs or box A for 8 h, then extensively washed before incubation with naive CD4⁺ T cells. T cells were mixed with allogeneic dendritic cells at a ratio of 10:1 and were plated at 1.5×10^6 cells/ml. On day 3, the cultures were supplemented with 100 U/ml IL-2 (Roche). In some experiments, naive CD4⁺ T cells were stimulated with 1 μ g/ml plate-bound anti-CD3 and 10 μ g/ml soluble anti-CD28 mAbs as previously described (20). When indicated, 1 μ g/ml rHMGB1 or supernatant of stimulated dendritic cells was filtered (0.2 μ m), supplemented with 1 μ g/ml anti-IL-12 (BD Pharmingen) blocking mAb, and added to naive T cells triggered by CD3/CD28 ligation. On day 8, cells were recovered, and Th1 and Th2 polarization was assessed as previously described (21). To assess T cell proliferation, cells were plated at 10^5 cells/well in a round-bottom, 96-well plate at a T cell to dendritic cell ratio of 10:1 for 5 days. Microcultures were pulsed with [³H]thymidine (1 μ Ci/well) for 16 h. Cell cultures were harvested with an automated multiple sample harvester, and incorporated isotope was determined by liquid scintillation β emission.

Flow cytometry

Surface Ag staining was performed using mouse mAbs against CD1a, CD14, CD40, CD80, CD83, CD86, and HLA-DR (BD Pharmingen). The intracellular cytokine content was determined after stimulation of cells with 50 ng/ml PMA and 1 μ g/ml ionomycin (21) (both from Sigma-Aldrich). GolgiPlug (BD Pharmingen) was added during the last 2 h of culture to prevent cytokine secretion. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen), then stained with FITC-conjugated anti-IFN- γ and PE-conjugated anti-IL-4 mAbs (BD Pharmingen). The samples were analyzed on a FACScan (BD Biosciences).

Apoptosis detection

Apoptosis was detected by staining with annexin V (Bender MedSystems) and propidium iodide (Sigma-Aldrich) as previously described (22).

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t* test for unpaired samples with unequal variance. A value of $p < 0.05$ was considered statistically significant.

Results

Human maturing dendritic cells translocate nuclear HMGB1 into the cytosol and secrete it in the extracellular milieu

Human immature dendritic cells derived in vitro from circulating precursors and cultured in the presence of rGM-CSF and IL-4 express high levels of HMGB1 in their nuclei, as shown by immunocytochemistry (Fig. 1, A and C). TLR4 engagement elicits the maturation of dendritic cells (2, 23) and changes HMGB1 localization; HMGB1 leaves the nucleus and reaches cytoplasmic vesicles (Fig. 1, B and D). Other stimuli known to elicit the maturation of dendritic cells, such as TNF- α , induced the translocation of HMGB1 to cytoplasmic vesicles as well (not shown). Similar changes occur in human monocytes and murine macrophages after endotoxin challenge (10, 24). These changes possibly represent a common response of cells belonging to the innate immune system after recognition of pathogen-associated molecular patterns (25).

The translocation of nuclear HMGB1 to cytoplasmic vesicles precedes its release in the medium (Fig. 1E). Lactate dehydrogenase is not released in the medium (Fig. 1E), ruling out the possibility that cells dying in culture passively leak their contents. The level of intracellular HMGB1 does not change over the 48-h in-

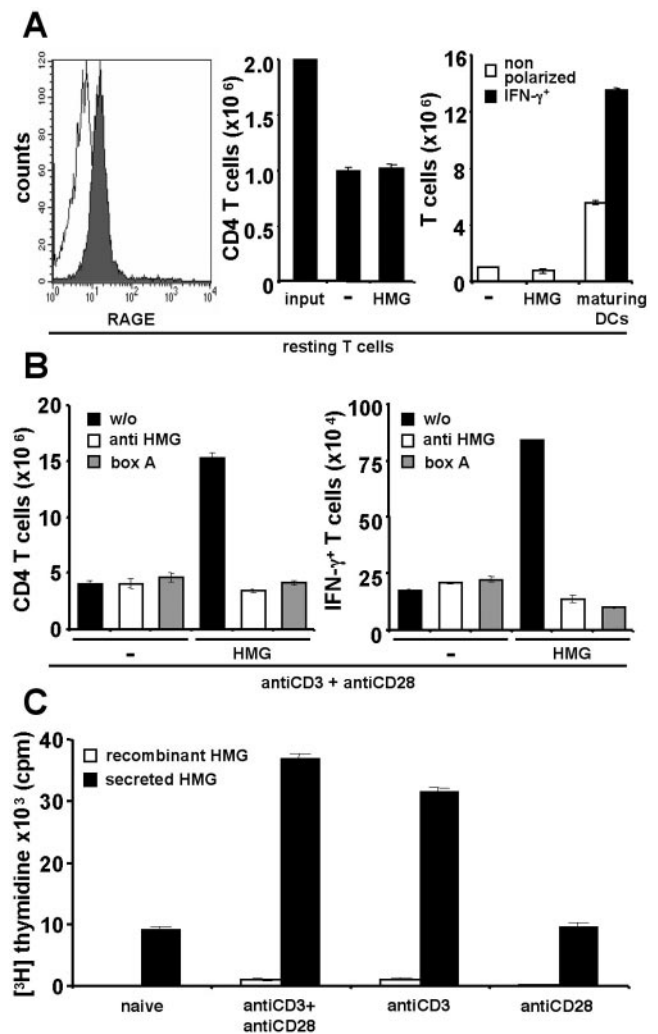


FIGURE 4. HMGB1 secreted by dendritic cells acts on activated T cells, but not on naive T cells. **A**, Freshly isolated naive CD4⁺ T cells (resting T cells) express RAGE (gray). Staining with control, isotype-matched Abs is shown in white (left panel). Naive CD4⁺ T cells (input; 2×10^6) were cultured alone (-) or with secreted HMGB1 (HMG; middle and right panels). Secreted HMGB1 was prepared from supernatants of endotoxin-stimulated dendritic cells that were filtered (0.2 μ m pore size) and supplemented with 1 μ g/ml mAb to IL-12. Allogeneic dendritic cells were triggered with endotoxin and cultured with T cells as described in Fig. 2 (maturing DCs; right panel). Secreted HMGB1 does not induce expansion of naive T cells (middle panel) or Th1 polarization (right panel). **B**, Naive CD4⁺ T cells (1.5×10^6) were stimulated with anti-CD3 and anti-CD28 Abs alone (-) or in the presence of secreted HMGB1 (HMG). Where indicated, anti-HMGB1 Abs (anti-HMG) or box A (boxA) were added to the cultures. Secreted HMGB1 induces an increase in the number of recovered T cells (left panel) and their polarization toward Th1 (right panel). This effect is abrogated by HMGB1 blockade. HMGB1 blockade does not change the number of recovered CD4⁺ T cells (left panel) or Th1 cells (IFN- γ ⁺ cells; right panel) if T cells are activated by cross-linking CD3 and CD28. **C**, Naive CD4⁺ T cells (1.5×10^5) were incubated alone (naive) or were stimulated with anti-CD3 and/or anti-CD28 Abs in the presence of recombinant (\square) or secreted (\blacksquare) HMGB1. Preactivated T cells proliferate more in response to secreted HMGB1 than to the recombinant protein. Representative results of one of three independent experiments are expressed as the mean \pm SD of duplicate cultures.

incubation period (Fig. 1E, inset), indicating that sustained production of the molecule supports active secretion during dendritic cell maturation.

Secretion of nuclear HMGB1 by dendritic cells is required for CD4⁺ T cell proliferation and survival

We then analyzed whether the active secretion of nuclear HMGB1 from maturing dendritic cells influences the cross-talk with naive CD4⁺ T cells. This event controls T cell activation, proliferation, and polarization (2, 3). We committed dendritic cells to maturation and verified their action on naive allogeneic CD4⁺ T cells in the absence or the presence of reagents that antagonize secreted HMGB1. We used anti-HMGB1 polyclonal Abs and an N-terminal fragment of HMGB1 (box A) that behaves as an HMGB1 antagonist (26). The Abs we used recognize peptide 166–181 of HMGB1, which is part of the domain that interacts with RAGE (27); they inhibit the release of TNF- α by activated murine macrophages *in vitro* and reduce sepsis-induced mortality *in vivo* after cecal ligation and puncture (26). Box A attenuates HMGB1-induced release of proinflammatory cytokines and reduces mortality after cecal ligation and puncture or endotoxin injection (26).

Maturing dendritic cells induce the expansion of CD4⁺ T cells (from 2×10^6 to $19.6 \times 10^6 \pm 0.6$; mean \pm SE; Fig. 2A). HMGB1 blockade by either Abs or box A abrogated the T cell expansion ($p < 0.005$ and $p < 0.01$, respectively; Fig. 2A). Irrelevant control Igs or a GST control protein were ineffective (Fig. 2A).

The T cell expansion reflects a combination of at least two events, i.e., the proliferation of T cells and the acquired resistance to activation-induced cell death (28). Fig. 2B shows that T cell proliferation abates in the presence of HMGB1 inhibitors, indicating that T cell proliferation requires the paracrine release of endogenous HMGB1. Moreover, HMGB1 blockade causes a substantial increase in T cell death; 18% of T cells primed with maturing dendritic cells alone died, whereas 39 and 46% of T cells died in the presence of anti-HMGB1 Abs or box A, respectively (Fig. 2D).

Secretion of HMGB1 by dendritic cells is required for Th1 polarization

T cells differentiate after priming into effector type 1 (Th1) or type 2 (Th2) cells. The two subsets can be identified by their intracellular cytokine profiles (3). Priming with maturing dendritic cells results in polarization toward a Th1 cytokine pattern, as evidenced by the intracellular expression of IFN- γ (Fig. 2C). The number of Th1 cells was dramatically reduced when the interaction between secreted HMGB1 and RAGE was blocked with either anti-HMGB1 Abs or box A (Fig. 2C). In contrast, the percentage of Th2 cells, evaluated as IL-4⁺ T lymphocytes, was not influenced

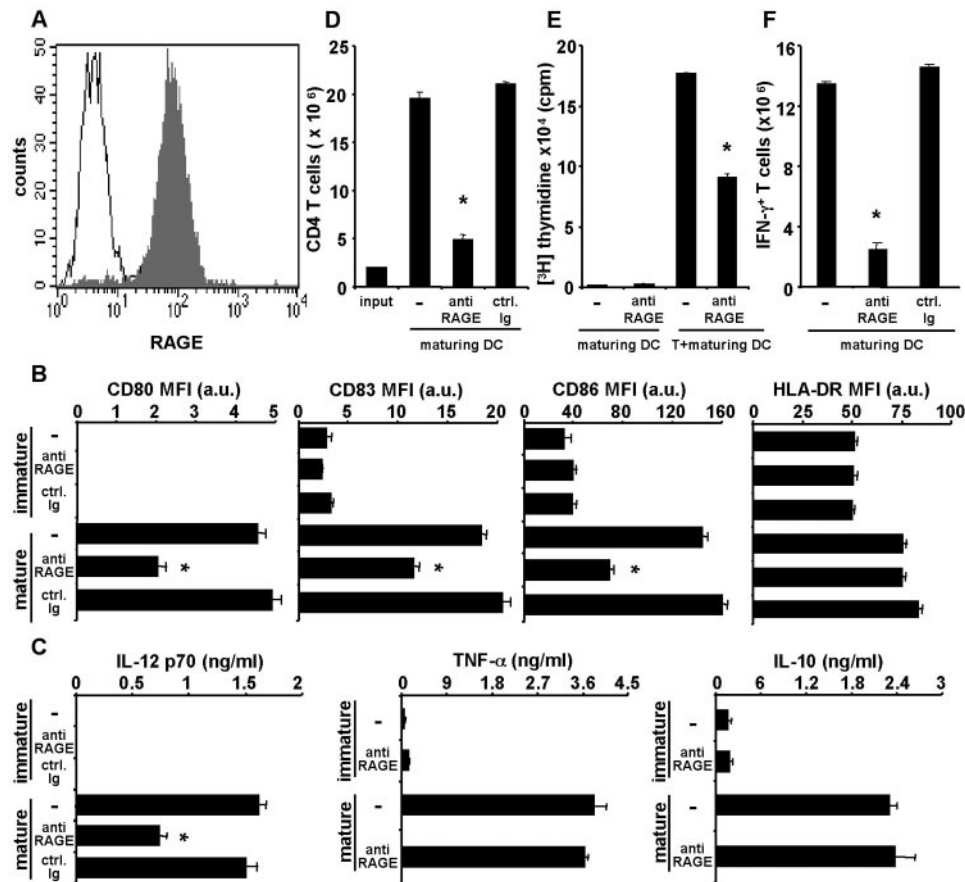


FIGURE 5. The HMGB1 receptor, RAGE, mediates the effect of secreted HMGB1 on dendritic cells. *A*, Immature dendritic cells express RAGE (■). Staining with control, isotype-matched Abs is shown (□). *B* and *C*, Dendritic cells were cultured alone (immature) or in presence of endotoxin (mature) for 48 h. Anti-RAGE Abs (anti-RAGE) or control Ig (ctrl. Ig) were added to the culture medium as indicated. The up-regulation of surface markers and the secretion of cytokines were tested. RAGE blockade reduces the up-regulation of CD80, CD83, and CD86 ($p < 0.05$) in mature dendritic cells as well as the secretion of IL-12 p70 ($p < 0.005$), but not that of TNF- α or IL-10. *D*, Immature dendritic cells were incubated with endotoxin (maturing DC) alone or in the presence of anti-RAGE Abs (anti-RAGE) or control Ig (ctrl. Ig) for 8 h. Dendritic cells were then incubated with 2×10^6 allogeneic naive CD4⁺ T cells (input) for 8 days. Anti-RAGE Abs ($p < 0.005$) abrogate T cell expansion induced by maturing dendritic cells. *E*, RAGE blockade reduces the proliferation of CD4⁺ T cells ($p < 0.005$) upon priming by dendritic cells. *F*, The number of IFN- γ ⁺ (Th1) T cells is reduced by RAGE blockade ($p < 0.05$). Representative examples of four independent experiments are shown as the mean \pm SD of duplicate cultures. *, Statistically significant values (p indicated above).

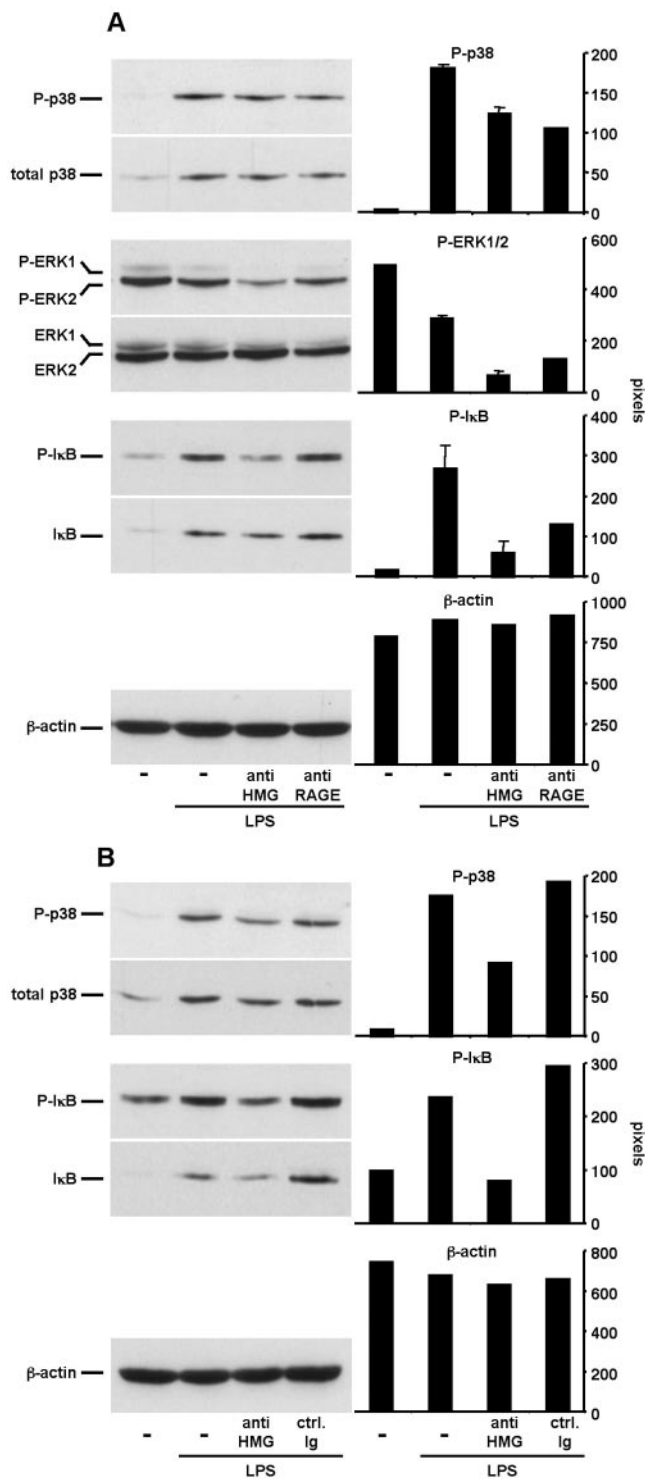


FIGURE 6. Endogenous HMGB1 acts by inducing the activation of MAPKs p38 and ERK1/2 protein and of NF- κ B. Immature dendritic cells were incubated with endotoxin (LPS) alone (–) or in the presence of anti-HMGB1 Abs (anti-HMG) or anti-RAGE Abs (anti-RAGE) or were left untreated (–). Lysates were prepared at 24 h if not indicated otherwise. Samples were analyzed by Western blot with anti-phospho-p38 (P-p38), anti-phospho-ERK1/2 (P-ERK1/2), and anti-phospho-I κ B (P-I κ B) Abs. Total amounts of proteins were evaluated after stripping the membranes and reblotting with anti-p38, -ERK1/2, -I κ B, and - β -actin Abs. We observed an increase in the phosphorylation of p38 and I κ B in dendritic cells 24 h after endotoxin stimulation. *A*, Anti-HMGB1 Abs decreased the amounts of phosphorylated p38, ERK1/2, and I κ B. RAGE blockade with anti-RAGE Abs inhibited p38, ERK1/2, and I κ B phosphorylation. *B*, Immature dendritic cells were stimulated with endotoxin (LPS) alone or in the

presence of HMGB1 inhibitors (2.02% when dendritic cells were triggered with endotoxin compared with 2.42% when dendritic cells were stimulated with endotoxin in the presence of anti-HMGB1 Abs). The ratio of IFN- γ ⁺ to IL-4⁺ T cells was 103 when dendritic cells triggered with endotoxin alone were used for priming. This ratio decreased to 28 and 51 in the presence of anti-HMGB1 and box A, respectively.

Secretion of HMGB1 modulates the maturation of dendritic cells and IL-12 production

Endotoxin-treated dendritic cells fail to sustain the proliferation, survival, and polarization of naive T cells when the HMGB1/RAGE interaction is blocked. This suggests that sustained HMGB1 secretion is necessary for the maturation of dendritic cells. We therefore evaluated by flow cytometry the membrane expression of CD80, CD83, CD86, and HLA-DR. In parallel, we measured the secretion of IL-10, IL-12, and TNF- α . Although mature dendritic cells up-regulate the expression of all markers (Fig. 3, *A* and *B*), anti-HMGB1 Abs and box A significantly hindered the up-regulation of CD80, CD83, and CD86 ($p < 0.05$) while leaving unaffected the expression of HLA-DR, the MHC molecules directly involved in Ag presentation to CD4⁺ T cells (Fig. 3, *A* and *B*). This is not surprising given the specific constraints regulating the intracellular trafficking of nascent HLA-DR to the plasma membrane (29, 30). No differences were detected in the presence of irrelevant Abs or a GST control protein (Fig. 3, *A* and *B*). HMGB1 blockade does not influence the expression of membrane molecules by immature dendritic cells (Fig. 3, *A* and *B*), which is in agreement with the observation that regulated HMGB1 secretion occurs only after induction of the maturation program (Fig. 1).

Maturing dendritic cells produce factors that sustain the maturation program, such as TNF- α (2, 3), or that contribute to limit it, such as IL-10 (31). A key factor for T cell priming is IL-12, which is produced upon dendritic cell maturation and behaves as a master regulator of the T cell polarization toward a Th1 phenotype (3). HMGB1 blockade does not influence the production of TNF- α and IL-10 (Fig. 3C). However, in agreement with the requirement of HMGB1 for Th1 polarization of CD4⁺ T cells, HMGB1 blockade decreases the production of IL-12 (Fig. 3C). Irrelevant Abs or the GST control do not influence the release of any of these cytokines (Fig. 3C).

HMGB1 secreted by dendritic cells acts on preactivated T cells but not on resting T cells

These results indicate that HMGB1 secretion by human dendritic cells enables them to prime naive T cells. Resting T cells express RAGE, the best-characterized receptor for HMGB1 (Fig. 4A, *left panel*). We found that the endogenous HMGB1 protein secreted in the microenvironment by dendritic cells is not sufficient to influence the survival or the functional polarization of naive CD4⁺ T cells (Fig. 4A, *middle and right panels*). T cell activation requires both stimulation via the T cell Ag receptor (signal 1) and additional costimulatory signals (signal 2) (32). In vitro T cell activation via signals 1 and 2 can be mimicked by CD3 and CD28 cross-linking (20). T cells activated by simultaneous triggering of CD3 and CD28 receptors become sensitive to secreted HMGB1, as revealed by proliferation and Th1 polarization (Fig. 4, *B* and *C*). The

presence of anti-HMGB1 Abs or irrelevant Abs (ctrl. Ig), and lysates were prepared at 24 h. Treatment with control Igs did not alter the phosphorylation of proteins 24 h after endotoxin stimulation. Densitometric quantifications are reported on the *right*. Results are represented as the mean of three independent experiments \pm SD.

effect of secreted HMGB1 on CD3/CD28-triggered T cells was completely inhibited by HMGB1 blockade with either anti-HMGB1 Abs or box A (Fig. 4B). As expected, T cell activation and polarization induced by the simultaneous cross-linking of CD3 and CD28 receptors in the absence of secreted HMGB1 were not influenced by HMGB1 blockade (Fig. 4B). We observed that T cells triggered by CD3/CD28 cross-linking were more sensitive to secreted HMGB1 than to the recombinant protein (Fig. 4C).

Anti-RAGE Abs prevent maturation of dendritic cells, clonal expansion, and Th1 polarization of CD4⁺ T cells

HMGB1 is a specific ligand for RAGE (12), and immature dendritic cells express RAGE (Fig. 5A) (16). To directly test whether RAGE is responsible for the effect of released nuclear HMGB1 on dendritic cells, we used anti-RAGE Abs. These Abs are directed against peptide 42–59 of human RAGE and hinder the interaction of RAGE with its ligand, HMGB1 (33). Blockade of RAGE prevents the up-regulation of CD80, CD83, and CD86 after TLR4 activation (Fig. 5B; $p < 0.05$) while leaving unaffected the expression of HLA-DR. No effects were observed in the presence of control Abs (Fig. 5B). RAGE blockade per se does not influence the expression of membrane molecules by immature dendritic cells (Fig. 5B), nor does it influence the production of TNF- α and IL-10 (Fig. 5C), whereas it significantly decreases the production of IL-12 p70 (Fig. 5C; $p < 0.005$). Irrelevant control Abs do not influence the release of IL-12 p70 (Fig. 5C).

Anti-RAGE Abs abrogate the expansion (Fig. 5D; $p < 0.005$) and proliferation (Fig. 5E; $p < 0.005$) of naive CD4⁺ T cells induced by maturing dendritic cells. The number of IFN- γ ⁺ T cells was dramatically reduced by anti-RAGE Abs (Fig. 5F; $p < 0.05$). Irrelevant control Abs did not alter the expansion or Th1 polarization of T cells (Fig. 5, D and F). Furthermore, anti-RAGE Abs caused substantial T cell death (from 18% for T cells primed with maturing dendritic cells alone to 35% in the presence of anti-RAGE Abs).

Secreted HMGB1 acts via activation of MAPKs p38 and ERK1/2 and NF- κ B

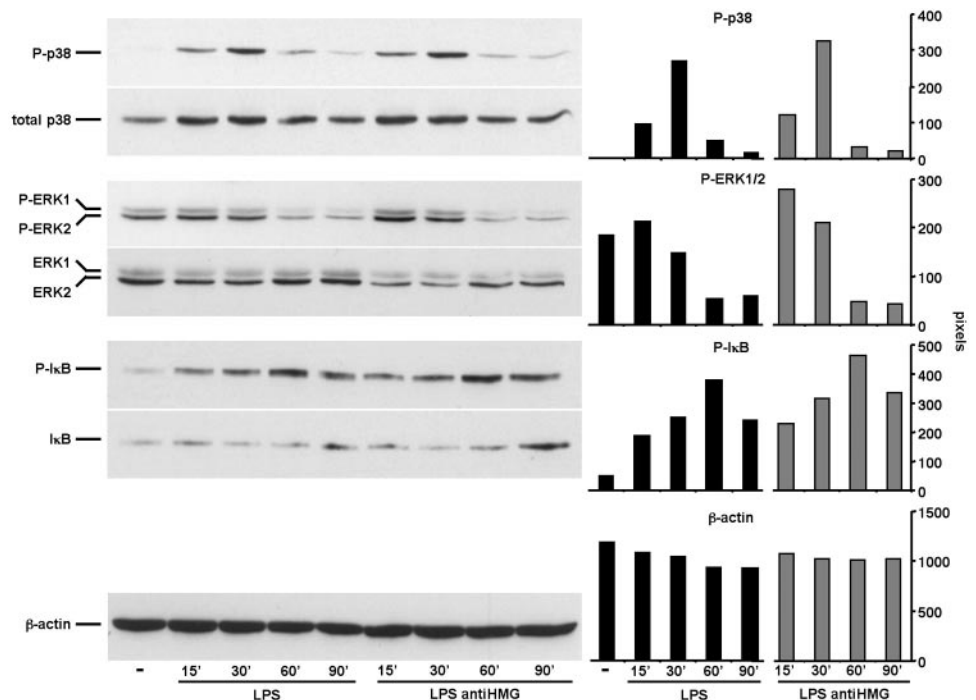
ERK1/2 and p38 stress-activated protein kinase (p38) together with the transcription factor NF- κ B are involved in the maturation of dendritic cells (34) and IL-12 secretion (35). The activation of NF- κ B can be assessed by analyzing the NF- κ B binding inhibitory protein, I κ B. Phosphorylation of I κ B leads to its degradation; this allows the nuclear translocation of NF- κ B and the subsequent activation of transcription. HMGB1 binding to RAGE in neuroblastoma cells or rat macrophages activates MAPK pathways and culminates in NF- κ B activation and nuclear translocation (12, 13, 36).

We assessed the phosphorylation of p38, ERK1/2, and I κ B in dendritic cells after TLR4 activation in the absence or the presence of HMGB1/RAGE blockade. Twenty-four hours after TLR4 activation, we observed HMGB1 secretion (as shown previously), which correlated with a significant increase in the phosphorylation of p38 and I κ B (Fig. 6). Anti-HMGB1 Abs induced a significant decrease in the phosphorylation of p38, ERK1/2, and I κ B (Fig. 6) at 24 h after TLR triggering; control Abs had no effect (Fig. 6B). Anti-RAGE Abs had the same inhibitory effect as anti-HMGB1 Abs on p38, ERK1/2, and I κ B phosphorylation (Fig. 6A).

TLR4 activation per se induces an early and transient phosphorylation of p38, ERK, and I κ B (37). Indeed, the activation of p38, ERK1/2, and I κ B after TLR4 triggering in dendritic cells follows a fast kinetic and abates after 1 h (Fig. 7). Moreover, this early phosphorylation of p38, ERK1/2, and I κ B is not influenced by anti-HMGB1 Abs (Fig. 7), an expected result because HMGB1 is not secreted until 8 h after TLR4 triggering.

Taken together, these data suggest that interaction of secreted HMGB1 with RAGE leads to late activation of p38, ERK1/2, and NF- κ B in maturing dendritic cells. This late activation is necessary for dendritic cell maturation. The early and transient activation of the same MAPKs and NF- κ B initiated by TLR4 triggering is not influenced by HMGB1 blockade.

FIGURE 7. The early and transient activation of MAPKs p38 and ERK1/2 and of NF- κ B is not influenced by blockade of HMGB1. Immature dendritic cells were incubated with endotoxin (LPS) alone or in the presence of anti-HMGB1 Abs (anti-HMG) or were left untreated (-). Lysates were prepared at the indicated time points (minutes). Endotoxin induced an early and transient activation of MAPKs p38 and ERK1/2 and of I κ B. Anti-HMGB1 Abs did not affect the early activation of p38, ERK1/2, and I κ B induced by endotoxin. Densitometric quantifications are reported on the right. Results are representative of three independent experiments.



RAGE is required for maturation of dendritic cells

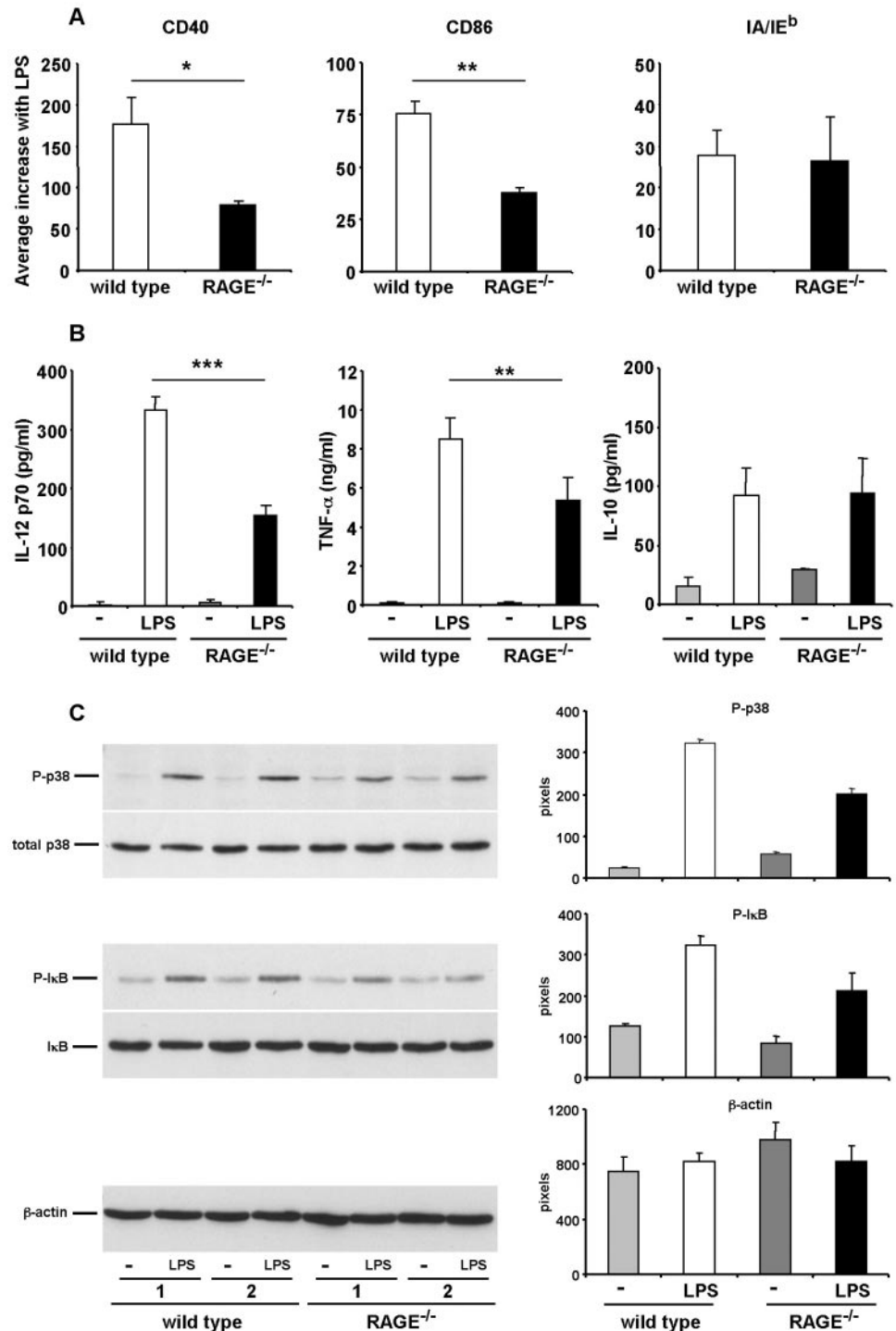
We derived dendritic cells from the bone marrow of RAGE^{-/-} mice and wild-type counterparts. We stimulated these cells with endotoxin and assessed their maturation. Up-regulation of surface markers such as CD40 and CD86 was significantly decreased in dendritic cells derived from RAGE^{-/-} mice compared with wild-type animals, whereas no change was observed for MHC class II (Fig. 8A). The production of IL-12 p70 and TNF- α upon endotoxin treatment was significantly decreased as well in RAGE^{-/-} dendritic cells (Fig. 8B). TLR4 triggering led to decreased phosphorylation of p38 and I κ B kinases in RAGE^{-/-} dendritic cells compared with wild-type counterparts (Fig. 8C). These results support

a role for secreted HMGB1 in the activation of dendritic cells by acting on its receptor, RAGE.

Discussion

HMGB1, a nuclear protein, has been recently described as an important extracellular mediator of inflammation (5, 17, 38). Its relocation outside the cell occurs as a consequence of either passive release after necrotic cell death (8) or active secretion by myeloid cells, involving extensive acetylation and transfer to secretory lysosomes (9–11). Exogenous HMGB1 is already known to play a role in dendritic cell activation (15, 16) and to behave as a potent

FIGURE 8. RAGE is required for dendritic cell maturation. Dendritic cells derived from bone marrow precursors of wild-type and RAGE^{-/-} mice were cultured alone (–) or with endotoxin (LPS) for 48 h. *A*, Up-regulation of surface markers after treatment is expressed as the average increase (y-axis; see *Materials and Methods*). Up-regulation of CD40 and CD86 abates in dendritic cells derived from RAGE^{-/-} mice. *B*, After treatment with endotoxin, dendritic cells from RAGE-deficient mice produced reduced amounts of IL-12 p70 and TNF- α , whereas the IL-10 levels were similar to those in their wild-type counterparts. *C*, Lysates of dendritic cells incubated alone (–) or with endotoxin (LPS) were prepared at 48 h. Samples were analyzed by Western blot with anti-phospho-p38 (P-p38) and anti-phospho-I κ B (P-I κ B) Abs. After stripping the membranes, the amount of total proteins was evaluated by reblotting with anti-p38, -I κ B, and - β -actin Abs. We observed a decreased phosphorylation of p38 and I κ B after endotoxin stimulation of RAGE-deficient dendritic cells compared with wild-type cells. Densitometric quantifications are reported on the right. Results are presented as the mean \pm SD of three independent experiments. Statistically significant values are marked with an asterisk(s) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



adjuvant for immunization against soluble and cell-associated Ags in murine models (15).

In this study we have found that dendritic cells actively release their own HMGB1 into the microenvironment upon activation. This event is necessary for the maturation of dendritic cells themselves and for the cross-talk with naive T cells, because it is required for T cell survival, proliferation, and functional Th1 polarization. In particular, the active release of nuclear HMGB1 by dendritic cells is required for their ability to produce IL-12 and to up-regulate the expression of membrane molecules involved in T cell costimulation. We show that secreted, but not recombinant, HMGB1 stimulates proliferation and Th1 polarization of preactivated T cells. This highlights the importance of post-translational modifications for the biological activity of HMGB1 (39).

Furthermore, our data suggest a role for RAGE in the effects of HMGB1 on dendritic cell maturation via activation of MAPKs (p38 and ERK1/2) and NF- κ B. Interestingly, the HMGB1/RAGE interaction determines the late activation of the MAPK and NF- κ B pathways, contrary to the engagement of TLRs, which apparently only induces an early transient activation of the same pathways (Fig. 7). It may be that the late activation of the MAPK/NF- κ B pathways, as opposed to an early transient activation, is key to the maturation of dendritic cells; alternatively, RAGE may activate an as yet unknown signaling pathway whose contribution is most relevant.

RAGE has been reported to be involved in innate immunity responses by recruiting inflammatory cells and contributing to the perpetuation of inflammation (40, 41). Our results suggest that RAGE plays a key role in regulating the responses of both human and murine dendritic cells to TLR triggering by microbial components. The human gene coding for RAGE, *AGER*, is located on chromosome 6 in the MHC class III region near the junction with class II (42). We note, however, that no null mutations in either the *HMGB1* or *AGER* gene are known in humans; *AGER* variant alleles have been actively sought, because one missense mutation (G82S) and several silent point substitutions affect the predisposition to diabetic complications (43). In general, the rarity of loss-of-function alleles is correlated with a strongly selected phenotype, as immunodeficiency is sure to be. Additional receptors for HMGB1 are likely to be involved, because blockage of the HMGB1/RAGE pathway does not abrogate the maturation of dendritic cells upon TLR4 triggering.

In conclusion, our data indicate that HMGB1 secreted by maturing dendritic cells orchestrates the priming, activation, and Th1 polarization of T cells. This event may influence the initiation/maintenance of autoimmune diseases by regulating the outcome of Ag presentation under conditions involving tissue damage and infection. HMGB1 is normally a nuclear protein that is passively released in the microenvironment by necrotic cells, signaling cell damage. Cell necrosis is an event that reflects trauma or the presence of cytopathic pathogens, such as high virus loads (44), and can lead to inflammation. The ability of dendritic cells to recreate the environment associated with necrosis via the regulated secretion of HMGB1 represents a successful evolutionary strategy and places HMGB1 at the crossroads between innate and adaptive immunity.

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Disclosures

M. E. Bianchi and A. A. Manfredi are named as investors in a patent application based partially on the data reported in this paper, with the assignee as Fondazione San Raffaele.

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