HMGB1 Enhances Immune Suppression by Facilitating the Differentiation and Suppressive Activity of Myeloid-Derived Suppressor Cells

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Abstract

Chronic inflammation often precedes malignant transformation and later drives tumor progression. Likewise, subversion of the immune system plays a role in tumor progression, with tumoral immune escape now well recognized as a crucial hallmark of cancer. Myeloid-derived suppressor cells (MDSC) are elevated in most individuals with cancer, where their accumulation and suppressive activity are driven by inflammation. Thus, MDSCs may define an element of the pathogenic inflammatory processes that drives immune escape. The secreted alarmin HMGB1 is a proinflammatory partner, inducer, and chaperone for many proinflammatory molecules that MDSCs develop. Therefore, in this study, we examined HMGB1 as a potential regulator of MDSCs. In murine tumor systems, HMGB1 was ubiquitous in the tumor microenvironment, activating the NF-κB signal transduction pathway in MDSCs and regulating their quantity and quality. We found that HMGB1 promotes the development of MDSCs from bone marrow progenitor cells, contributing to their ability to suppress antigen-driven activation of CD4+ and CD8+ T cells. Furthermore, HMGB1 increased MDSC-mediated production of IL-10, enhanced crosstalk between MDSCs and macrophages, and facilitated the ability of MDSCs to downregulate expression of the T-cell homing receptor L-selectin. Overall, our results revealed a pivotal role for HMGB1 in the development and cancerous contributions of MDSCs. Cancer Res; 74(20); 5723–33. ©2014 AACR.

Introduction

Antitumor immunity and immunotherapies that activate innate and/or adaptive immunity have potential for the prevention and/or treatment of primary and metastatic cancers. However, immunotherapies are frequently ineffective because patients with cancer contain immunosuppressive cells. Myeloid-derived suppressor cells (MDSC; ref. 1) are present in virtually all patients with solid tumors and are major contributors to immune suppression. They facilitate tumor progression through multiple immune mechanisms, including the inhibition of T- and NK-cell activation (2), polarization of immunity toward a tumor-promoting type II phenotype through their production of IL-10 (3), and by perturbing the trafficking of naïve T cells by downregulating L-selectin (4). MDSCs also use nonimmune mechanisms to enhance tumor growth. They produce VEGF and matrix metalloproteases that promote tumor vascularization (5) as well as invasion and metastasis (6).

Chronic inflammation has long been associated with tumor onset and progression (7). The role of chronic inflammation was originally attributed to its ability to foster genetic mutations, enhance tumor cell proliferation and survival, and promote metastases. Chronic inflammation also facilitates malignancy by inducing the accumulation and increasing the potency of MDSCs, which prevent adaptive and innate immunity from delaying tumor progression (8). Multiple redundant proinflammatory molecules drive MDSCs. Because the damage-associated molecular pattern molecule (DAMP) and alarmin high mobility group box protein I (HMGB1) is proinflammatory and is a binding partner, inducer, and/or chaperone for many of the proinflammatory molecules that drive MDSCs (9), we have studied HMGB1 as a potential regulator of MDSCs. HMGB1 was originally identified as a DNA-binding protein in the nucleus. It performs multiple functions within the nucleus, including changing the conformation of DNA to allow for the binding of regulatory proteins, facilitating the integration of transposons into DNA, and stabilizing nucleosome formation (10). Its role as a secreted protein and an immune modulator has only been recognized within the past 15 years (11).

We now report that in addition to many other cells, MDSCs release HMGB1 and that HMGB1 activates MDSCs through NF-κB and facilitates several immune suppressive mechanisms used by MDSCs to inhibit antitumor immunity. HMGB1 drives the differentiation of MDSCs from bone marrow progenitor cells, enhances crosstalk between MDSCs and macrophages by increasing MDSC production of IL-10, and reduces the expression of L-selectin on circulating naïve T cells. Collectively, these
results suggest that HMGB1 contributes to immune suppression by inducing and activating MDSCs.

Materials and Methods

Mice

BALB/c, C57BL/6, BALB/c IL-10−/−, BALB/c TLR4−/−, BALB/c DO11.10 (TCR-transgenic for the ββ-TCR specific for OVA peptide 323-339 restricted by I-Ad) and BALB/c clone 4 (OVA peptide 323-339 restricted by I-Ad) postinoculation). Tumors were measured palpable (day 7 /C2 intraperitoneally 3

Recombinant A box (300

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526 restricted by H-2Kd) mice were from The Jackson laboratory for exceptions

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> (Roswell Park Cancer Center, Buffalo, NY) approximately 5

exception of AT3, which was obtained from Dr. S. Abrams laboratory and/or bred in the UMBC animal facility. Mice <6 months of age were used for all experiments. All animal procedures were approved by the University of Maryland Baltimore County (Baltimore, MD) Institutional Animal Care and Use Committee.

Reagents and antibodies

Heparin sodium salt (grade IA) and ethyl pyruvate were from Sigma-Aldrich. Glycyrhrizin (ammonium salt) was from Calbiochem. Recombinant mouse IL-6 and GM-CSF were from BioLegend. Recombinant mouse IFNy, HMGB1, and TNFα were from R&D Systems. Recombinant LPS was from DiVco. Monoclonal antibodies (mAb) Gr1-APC-Cy7, Gr1-APC (RB6-8C5), CD45-PE (30-F11), CD8-FITC (53-67), CD4-PE, (L3T4/GK1.5), CD3-PE-Cy7 (145-2C11), CD11b-PE (M1/70), CD11c-FITC (HL3), CD54-R-B200-PE (RA3-6B2), CD62L-APC (MEL14), c-kit-PE (CD117; ACK45), iNOS, arginase, rat IgG2b isotype, and annexin V were from BD Biosciences. CD11b-PacB (M1/70), F4/80-PE (BM8), F4/80-PacB (BM8), rat IgG1a-APC (R7K2758), and CD16/32 (93) were from BioLegend. CD45-TxR (MCD4517) was from Invitrogen. Anti-mouse ADAM17 mAb was from Abcam (ab2051). Secondary for ADAM17 antibody (goat-anti-rabbit; 554020) was from BD Biosciences. Anti-CD3 was from Dako (clone F7.2.38). Recombinant A box (12, 13) and 2G7 (14) were produced as described.

Tumor inoculations, tumor measurements, 2G7, and A box treatment

C57BL/6 mice were inoculated subcutaneously in the flank with 5 × 105 MC38 colon carcinoma cells, 1 × 105 B78H1 melanoma cells, or 1 × 106 AT3 mammary carcinoma cells (15). BALB/c mice were inoculated in the abdominal mammary fat pad with 7 × 105 4T1 mammary carcinoma cells or subcutaneously with 1 × 106 CT26 colon carcinoma cells. With the exception of AT3, which was obtained from Dr. S. Abrams (Roswell Park Cancer Center, Buffalo, NY) approximately 5 years ago, all tumor cell lines have been in the authors’ laboratory for >15 years. Cell lines are routinely checked for mycoplasma and early freeze-downs are preferentially used. Recombinant A box (300 µg/100 µL/mouse), vehicle (PBS), 2G7 mAb (5 µg/200 µL/mouse), or control IgG2b antibody (MOPC 195; 5 µg/200 µL/mouse; Sigma Aldrich) were administered intraperitoneally 3x/week starting when tumors were first palpable (day 7–9 postinoculation). Tumors were measured in two perpendicular diameters every 2 to 3 days. Tumor volume = (4/3)πr², where r = (diameter 1 + diameter 2)/2. IHC for tumor-infiltrating T cells was performed by CD3 staining of O.C.T. embedded tumors.

Tumors, MDSCs, and macrophage supernatants; MEF cell lysates

The 4T1, CT26, B78H1, MC38, and AT3 tumor cells were cultured at 5 × 10⁶ cells/mL in 6-well plates in serum-free HI-1 medium at 37°C, 5% CO₂. MDSCs and thioglycollate-elicited macrophages were similarly cultured except some wells contained 100 ng/mL LPS. Supernatants were harvested after 18 hours and concentrated 10x using 10 kDa Centricon filters (Millipore). Excised tumors were minced into small pieces using scissors, and placed in 10 mL of serum-free HI-1 media containing 0.8 µg/mL DNase. Tumor chunks were then dissociated into single cell suspensions using a GentleMACS Dissociator equipped with a GentleMACS C tube and program m_tumor 01.01 (Miltenyi Biotec). Dissociated material, including medium, was then plated in 10 cm dishes and incubated at 37°C, 5% CO₂ for 18 hours, after which the supernatants were collected and concentrated to 2 mL using 10 kDa Centrificon filters. Wild-type and HMGB1-knocked out MEF cells (16) were lysed in 300 µL of M-Per buffer Mammalian Protein Extraction Reagent (Thermo Scientific) using a GentleMACS Dissociator fitted with an M tube and program protein 01.01. Lysates were centrifuged at 10°C and 650 × g for 5 minutes, and the supernatants removed and centrifuged at 10°C and 160 × g for 15 minutes. Protein concentration of the supernatants was determined at 280 absorbance.

Blood MDSCs

Mice were bled from the submandibular vein into 1 mL of PBS containing 0.008% heparin. RBCs were removed by Gey’s treatment (17). The remaining white blood cells were stained for Gr1 and CD11b and analyzed by flow cytometry. White blood cells that were >90% Gr1+CD11b+ were used in experiments.

MDSC–macrophage cocultures

Peritoneal macrophage (>80%CD11b+F4/80+ cells) and MDSC cocultures were performed as described elsewhere (3). Briefly, cells were plated at 7.5 × 10⁵ MDSCs and 7.5 × 10⁵ macrophages/well in 500 µL of DMEM with 5% FBS, 100 ng/mL LPS, and 20 U/mL IFNy in 24-well plates. Cocultures were incubated at 37°C, 5% CO₂ for 16 to 18 hours. Supernatants were stored at −80°C until analyzed by ELISA.

Cytokine detection

IL-10, IL-6, IL-12, and IL-1β were measured by ELISA according to the manufacturer’s protocol (R&D Systems). Plates were read at 450 nm using a Bio-Tek synergy microplate reader. Data are the mean ± SD of triplicate wells.

MDSC generation from bone marrow cells

MDSCs were generated (18) with the following adaptations: Bone marrow was flushed aseptically from femurs with RPMI medium using a syringe fitted with a 27 g needle. RBCs were lysed with Gey’s solution. Resulting cells were cultured at 37°C, 5% CO₂ at 4.2 × 10⁵ cells/2 mL in 6-well plates containing RPMI medium supplemented with 10% FCS, 40 ng/mL IL-6, and 40 ng/mL GM-CSF. After 4 days of culture, percent decrease in Gr1medCD11b− cells was determined (Gr1med CD11b− cells = 100% [(number of vehicle-treated cells – number of inhibitor

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treated cells)/(number of vehicle-treated cells)]. Absolute number of cells = (total number of cells) × % of a given cell type as determined by flow cytometry.

T-cell activation assays

T-cell activation assays were performed as described (17). Briefly, splenocytes and irradiated (2500 Rad) 4T1-induced MDSCs or bone marrow generated MDSCs were cocultured in 96-well plates at 10^5 cells/well of HL-1 media containing 1% penicillin, 1% streptomycin, 1% Glutamax, and 5 × 10^{-5} M β-mercaptoethanol. Of note, 14 μmol/L OVA 323-339 peptide or 28 μmol/L H3A318-326 peptide was included for D011.10 and clone 4 cells, respectively. Wells were pulsed with 1 μCi of [3H] thymidine/well on day 3, and 18 hours later, the cells were harvested. Data are expressed as cpm ± SD of triplicate cultures. Hydrogen peroxide levels were measured as described (19).

HMGBl1 Western blot analyses and ELISA

Of note, 50 μL of equivalent quantities of concentrated supernatants of cultured tumor cells, in vivo grown tumors, MDSCs, macrophages, or 60 μg of MEF cell lysates were mixed with 10 μL or the appropriate amount of 6× sample buffer and electrophoresed on 12% SDS-PAGE gels in SDS running buffer (Bio-Rad) at 150 volts for 1 hour, and trans-ferred overnight in transfer buffer (Bio-Rad) at 30 volts to PVDF membranes (GE Healthcare). Membranes were blocked with 5% milk in TBST. HMGBl1 was detected with anti-HMGBl antibody (Epitomics; 5 ng/mL in 10 mL of 2.5% milk/TBST) followed by goat-anti-rabbit-HRP (Millipore; 40 ng/mL in 10 mL of 2.5% milk/TBST). Protein was visualized using an HRP detection kit (Denville Scientific, Inc). HMGBl1 levels were measured by ELISA according to the manufacturer’s directions (IBL International).

Flow cytometry

Cells were labeled and analyzed by flow cytometry for cell surface molecules as described (17). For bone marrow experiments, cells were first stained using the LIVE/DEAD fixable yellow dead cell stain kit (Invitrogen) as per the manufacturer’s protocol, followed by staining for cell surface markers with antibodies diluted in PBS/2% FCS (HyClone). For NF-κB staining of MDSCs, 3 × 10^6 to 5 × 10^6 leukocytes/mL RPMI were incubated with/without 50 ng/mL HMGBl1 for 15 minutes at 37° C, fixed and permeabilized, and then stained with rabbit mAb phospho-NF-κB p65 (Ser536; clone 93H1) and goat-anti-rabbit (Fab')2-AlexaFluor 647 (Cell Signaling Technology, Inc.) according to the manufacturer’s protocol, followed by staining for Gr1 and CD11b. Peritoneal macrophages (5 × 10^6/5 mL DMEM) were similarly stained, except they were rested for 2 hours at 37° C before stimulation with 20 mg/mL TNFα or 100 μg/mL LPS, and subsequently incubated with Fc block (CD16/32) for 15 minutes, followed by staining with NF-κB, CD11b, and F4/80 mAbs. For ADAM17 staining, 3 × 10^6 to 5 × 10^6 leukocytes were incubated with or without HMGBl1 (50 ng/mL) or ethyl pyruvate (10 mmol/L) for 0, 2, and 4 hours and stained with mAb to ADAM17. For tumor-infiltrating MDSCs, solid tumors were prepared as they were for tumor supernatants, except collagenase (300 U/mL) was included in the dissociation medium, and the resulting cells were centrifuged through ficoll to remove dead cells. Samples were run on a CyaN ADP flow cytometer and analyzed using Summit Software (Beckman Coulter).

Statistical analysis

Statistical analysis of tumor growth rate was conducted utilizing the compare Growth Curves function of the Statmod software package (http://bioinf.mvh.edu.au/software/compareCurves). A Student t test was used to determine statistical significance between two sets of data. Single-factor ANOVA was used to determine statistical significance between groups of data.

Results

HMGBl1 is ubiquitously present in the tumor microenvironment and activates MDSCs via the NF-κB pathway

If HMGBl1 is associated with the induction of MDSCs, then HMGBl1 will be present in the tumor microenvironment. To test this hypothesis, BALB/c-derived 4T1 mammary carcinoma and CT26 colon carcinoma cells, and C57BL/6-derived B78H1 melanoma, MC38 colon carcinoma, and PyMT-MMTV-derived AT3 mammary carcinoma cells were cultured in serum free-media, and the supernatants assessed by Western blot analysis for HMGBl1. Whole-cell lysates of wild-type MEF cells and their HMGBl1-knocked out counterparts served as positive and negative controls, respectively. All tumors constitutively secreted HMGBl1 (Fig. 1A). Secretion of HMGBl1 was confirmed and quantified by ELISA (Supplementary Table S1).

Because MDSCs are driven by inflammation and themselves produce proinflammatory mediators (8, 20), we tested MDSCs for secretion of HMGBl1. MDSCs generated in 4T1 tumor-bearing BALB/c mice were harvested from the blood, stained for Gr1 and CD11b, and assessed for their ability to suppress T-cell activation (Fig. 1B). More than 90% of the blood leukocytes were CD11b⁺Gr1⁺ and they were suppressive. We then tested MDSCs for their ability to secrete HMGBl1 by culturing them overnight and assaying the supernatant for HMGBl1 by Western blot analysis and ELISA (Fig. 1C and Supplementary Table S1). Macrophages are established producers of HMGBl1 (11), and LPS is reported to increase their secretion of HMGBl1 (21). To determine whether LPS similarly affects MDSCs, MDSCs were cultured with and without LPS. Both LPS-treated and untreated MDSCs produced more HMGBl1 than equivalent numbers of LPS-treated macrophages, demonstrating that MDSCs constitutively secrete HMGBl1.

To determine whether HMGBl1 is present in vivo within the tumor microenvironment, 4T1, CT26, B78H1, MC38, and AT3 tumors of BALB/c and C57BL/6 tumor-bearing mice were measured, and then excised and weighed. Explanted tumors were then dissociated into single cell suspensions without disrupting cell integrity, and incubated in serum-free medium. The resulting supernatants were assessed by Western blot analysis and ELISA for HMGBl1 (Fig. 1C right-hand five lanes and Supplementary Table S1). All excised tumors released HMGBl1; however, the quantity of HMGBl1 released did not directly correlate with tumor burden. Because different types of tumors contain...
Figure 1. HMGB1 is ubiquitously present in the tumor microenvironment, is secreted by MDSCs, and activates the NF-κB signaling pathway in MDSCs. A, 4T1, CT26, B78H1, AT3, and MC38 tumor cells were cultured in serum-free medium and their supernatants assessed by Western blot analysis for HMGB1. B, BALB/c 4T1– and untreated macrophages and MDSCs, and excised, dissociated tumors of BALB/c (4T1, CT26) and C57BL/6 (B78H1, AT3, MC38) mice were cultured overnight in serum-free medium. Resulting dissociated tumors of BALB/c (4T1, CT26) and C57BL/6 (B78H1, AT3, MC38) mice were cultured under conditions that affect their functional activities. To assess whether HMGB1 regulates MDSCs by either controlling their accumulation and/or affect their functional activities. To assess whether HMGB1 affects MDSC differentiation, bone marrow cells from the femurs of healthy BALB/c mice were cultured with or without HMGB1, subsequently transduced to determine whether HMGB1 activates MDSCs, leukocytes from the blood of tumor-free BALB/c mice were treated with or without HMGB1, subsequently stained for phosphorylated NF-κB (pNF-κB), and the Gr1+ CD11b+ cells gated and analyzed for pNF-κB (Fig. 1D). HMGB1 treatment caused phosphorylation of NF-κB.

To confirm the specificity of the pNF-κB staining, macrophages from either TLR4+/− or TLR4−/− mice were treated with either LPS or TNFα. If the pNF-κB mAβ is specific, then TNFα will activate NF-κB in both TLR4+/− and TLR4−/− cells because it acts via the TNFα receptor. In contrast, NF-κB will only be activated by LPS in TLR4+/+ cells, because LPS activates NF-κB via TLR4. TNFα activated NF-κB in both TLR4+/+ and TLR4−/− cells, whereas LPS activated NF-κB in TLR4+/+, but not TLR4−/− cells, confirming the specificity of the pNF-κB mAβ (Supplementary Fig. S1).

These data indicate that HMGB1 is ubiquitously present in the tumor microenvironment, multiple cell populations within the tumor microenvironment produce HMGB1, MDSCs contribute to the production of HMGB1, and HMGB1 activates the NF-κB signal transduction pathway in MDSCs.

**HMGB1 drives the differentiation of MDSCs from bone marrow progenitor cells**

Because the differentiation, accumulation, and function of MDSCs are driven by inflammation (8, 20, 24, 25), HMGB1 may regulate MDSCs by either controlling their accumulation and/or affecting their functional activities. To assess whether HMGB1 affects MDSC differentiation, bone marrow cells from the femurs of healthy BALB/c mice were cultured under conditions that drive the differentiation of MDSCs (18). The HMGB1 inhibitors ethyl pyruvate and glycyrrhizinic acid were included in some cultures. Ethyl pyruvate prevents extracellular secretion of HMGB1 from activated monocytes and macrophages by blocking NF-κB signal transduction (26). Glycyrrhizinic acid prevents the binding of extracellular HMGB1 by attaching to two distinct regions of HMGB1 (27). At the end of the differentiation period, the presence of HMGB1 was confirmed by Western blot analysis (Fig. 2A) and quantified by ELISA (Supplementary Table S1), and the absolute number of Gr1med CD11b+ cells was determined by cell counting and flow cytometry (Fig. 2B). At the start of culture, 5.5×10^5 cells were Gr1med CD11b+. At the end of the culture period, the vehicle control–treated cultures contained 1.6×10^6 Gr1med CD11b+ cells, indicating that MDSCs had expanded by almost 3-fold. Both HMGB1 inhibitors significantly reduced the absolute number of MDSCs (Fig. 2C and Supplementary Table S2). The highest dose of glycyrrhizin reduced the number of Gr1med CD11b+ cells by 82%, whereas ethyl pyruvate reduced the number by 80%. Gr1− CD11b+ MDSCs induced under these conditions were just as suppressive as tumor-induced MDSCs isolated from mice with 4T1 tumors (Fig. 2D). Glycyrrhizin and ethyl pyruvate also increased the number of DC (CD11c+ cells, 43% and 67%, respectively) macrophages (F4/80+ CD11b+ cells, 66% and 68%, respectively), consistent with published reports showing that HMGB1 also drives the maturation of these cells (28). In contrast, B cells (B220+ cells) and T cells (CD3ε+ cells) were either not affected or only minimally decreased.
To determine whether inhibition of HMGB1 reduces MDSC accumulation by inhibiting the proliferation of MDSC progenitor cells or by causing apoptosis of differentiated MDSCs, bone marrow cells were vehicle or ethyl pyruvate treated, and the levels of c-kit+ (CD117) progenitor cells and Annexin V+PI+ apoptotic cells were determined by flow cytometry (Fig. 2E and Supplementary Fig. S2A). Ethyl pyruvate reduced the level of progenitor cells but did not...
induce apoptosis as compared with vehicle treatment. These data indicate that HMGB1 facilitates the expansion of myeloid cells, including MDSCs, from bone marrow progenitor cells.

**HMGB1 contributes to the ability of MDSCs to suppress antigen-driven T-cell activation**

MDSCs use multiple mechanisms to suppress antitumor immunity. Suppression of antigen-driven T-cell activation was one of the first mechanisms identified (29, 30). To determine whether HMGB1 impacts MDSC suppression of T-cell activation, MDSCs from 4T1 tumor-bearing BALB/c mice were tested for their ability to prevent the proliferation of transgenic CD4+ (DO11.10) or CD8+ (clone 4) T cells activated with cognate peptides (Fig. 3A). Increasing concentrations of the HMGB1 inhibitor ethyl pyruvate restored T-cell activation in the presence of MDSCs. Because ethyl pyruvate prevents signaling through NF-κB and T-cell activation requires NF-κB signaling (31), transgenic T cells were treated with ethyl pyruvate to ascertain that these doses were not affecting T-cell proliferation (Fig. 3B). Ethyl pyruvate did not increase T-cell activation in the absence of MDSCs, demonstrating that the increase in T-cell activation seen in Fig. 3A is an effect of ethyl pyruvate on MDSCs and not an effect on T cells.

To determine how ethyl pyruvate inhibits MDSCs, vehicle and ethyl pyruvate–treated MDSCs were assayed by flow cytometry for their content of molecules that mediate T-cell suppression (arginase, iNOS, and H2O2), and for its impact on MDSC viability. Ethyl pyruvate did not decrease arginase or iNOS levels or alter MDSC apoptosis levels (Supplementary Fig. S2B and S2C), but modestly reduced H2O2 levels (Supplementary Fig. S2D) as compared with vehicle-treated cells. In previous studies, another NF-κB inhibitor, Withaferin A, also reduced the suppressive potency of MDSCs (19). These results suggest that HMGB1 contributes to MDSC-mediated T-cell suppression by increasing their expression of H2O2.

**HMGB1 increases MDSC production of IL-10 and MDSC–macrophage crosstalk**

One of the mechanisms MDSCs use to inhibit antitumor immunity is their production of IL-10. MDSC-produced IL-10 reduces macrophage production of IL-12, thereby skewing macrophages toward a type II tumor–promoting phenotype (3). Crosstalk between MDSCs and macrophages increases MDSC production of IL-10, thereby contributing to MDSC suppression. MDSC-produced IL-10 also drives the differentiation and accumulation of T regulatory cells (32), further increasing immune suppression. To determine whether HMGB1 drives MDSC production of IL-10 or MDSC–macrophage crosstalk with respect to IL-10, MDSCs and macrophages were cocultured with or without ethyl pyruvate and glycyrrhizin and IL-10 production was measured (Fig. 4A). Both ethyl pyruvate and glycyrrhizin dose dependently reduced the production of IL-10 by MDSCs and by mixtures of MDSCs plus macrophages. To ascertain that MDSCs, rather than macrophages, are the producers of IL-10, macrophages and MDSCs from IL-10-deficient BALB/c mice were used in conjunction with MDSCs or macrophages, respectively, from wild-type BALB/c mice (Fig. 4B). Only marginal levels of IL-10 were detected in cultures containing IL-10−/− MDSCs with wild-type macrophages, demonstrating that MDSCs are the cells producing the IL-10. The reduction of IL-10 is not due to reduced MDSC viability because ethyl pyruvate–treated MDSCs cultured under the crosstalk conditions (with 5% serum) are more viable than vehicle-treated MDSCs (Supplementary Fig. S2C). These findings indicate that HMGB1 regulates MDSC production of IL-10 and macrophage-induced increases in MDSC production of IL-10.

MDSCs also promote a type II immune response by downregulating macrophage production of IL-12 (3) and IL-6 (unpublished). To determine whether HMGB1 mediates either of these effects, MDSCs and macrophages were cocultured with or without ethyl pyruvate and glycyrrhizin and IL-12 and IL-6 were quantified by ELISA (Fig. 5). Ethyl pyruvate and glycyrrhizin reduced macrophage production of IL-12 and IL-6, and did not restore production of these cytokines in MDSC–macrophage cocultures. IL-1β, a
HMGB1 Regulates MDSC Development and Suppressive Activity

Neutralization of HMGB1 delays tumor growth and reduces MDSCs in tumor-bearing mice

HMGB1 includes two functional domains: the proinflammatory B box and the anti-inflammatory A box. The B box is a RAGE agonist, whereas the A box is an HMGB1 antagonist (13). Although the A box is a competitor for the B box, the B box of HMGB1 is dominant in vivo (27). However, if administered in vivo as a recombinant protein, A box neutralizes endogenous HMGB1 (14). To determine whether A box impacts tumor progression, BALB/c and C57BL/6 mice bearing 4T1 or MC38 tumor, respectively, were treated with A box or vehicle controls starting when the tumors were first palpable (approximate day 7–9 after tumor cell inoculation; Fig. 6A). In both strains, A box delayed tumor progression, supporting the concept that HMGB1 facilitates tumor growth. 4T1 tumor cells were also knocked down by shRNA for HMGB1 (4T1/575 cells) and their tumorigenicity compared with that of 4T1 cells transfected with an irrelevant shRNA (4T1/irrelevant; Supplementary Fig. S3A). The effect of HMGB1 on spontaneous metastatic disease was assessed by treating 4T1 tumor-bearing mice with glycyrrhizin and ethyl pyruvate and assessing the number of metastatic cells by clonogenic assay (Supplementary Fig. S3B; ref. 35). 4T1/575 tumor–bearing mice survived significantly longer than mice with 4T1/irrelevant cells supporting previously published work (36). Tumor-bearing mice treated with the inhibitors trended toward fewer metastatic cells however, the values were not statistically significantly different. These results further confirm that HMGB1 enhances tumor progression.

To determine whether HMGB1 drives MDSC accumulation in vivo, tumor-bearing mice were treated with a neutralizing HMGB1 mAb (2G7), and tumor-infiltrating MDSCs and MDSCs from the blood and spleen were compared with MDSCs in vehicle-treated tumor-bearing mice. C57BL/6 mice were inoculated with the MC38 tumor on day 1 and 2G7 treatment was started on days 10 to 13. Mice were sacrificed at a late stage of disease when their primary tumors were approximately the same diameter, and total MDSCs, monocytic MDSCs, and granulocytic MDSC levels in the blood, spleen, and infiltrating the tumors were determined by flow cytometry (Fig. 6B). Total, monocytic, and granulocytic MDSCs were reduced in the spleens, blood, and tumors of 2G7-treated mice with the exception of tumor-infiltrating granulocytic MDSCs. These decreases were not a secondary effect of reduced tumor size since, at the time of analysis, the 2G7-treated and control-treated mice had similar-sized primary tumors. MDSCs were similarly reduced in the blood of A box-treated tumor-bearing mice. These results indicate that in vivo, neutralization of HMGB1 reduces the accumulation of MDSCs in tumor-bearing mice.

Tumors from the HMGB1 mAb–treated (2G7) and isotype control–treated mice of Fig. 6 were assessed by IHC for the presence of CD3+ T cells (Supplementary Fig. S4). Both types of tumors contained few T cells; however, there was a trend toward more CD3+ cells in the tumors of 2G7-treated mice.

HMGB1 downregulates T-cell expression of L-selectin

MDSCs also impair T-cell immunity by perturbing the homing of naïve T cells to lymph nodes where they could become activated. To enter lymph nodes, naïve T cells must first be tethered via proinflammatory cytokine that is produced by MDSCs and also drives the suppressive potency of MDSCs (33, 34), was also assessed. Ethyl pyruvate and glycyrrhizin decreased MDSC production of IL-10; however, HMGB1 inhibition restored IL-10 levels in cocultures of MDSCs and macrophages. These results indicate that HMGB1 regulates MDSC production of IL-10 during MDSC–macrophage crosstalk; however, it is not involved in MDSC-mediated downregulation of macrophage-produced IL-12 or IL-6.

Figure 4. HMGB1 increases MDSC production of IL-10 and MDSC–macrophage crosstalk. A, cocultures of 4T1-induced BALB/c MDSCs and macrophages from tumor-free mice were incubated with or without ethyl pyruvate or glycyrrhizin, and the supernatants were assayed by ELISA for IL-10. B, MDSCs from 4T1-tumor-bearing BALB/c and C57BL/6 mice bearing 4T1 or MC38 tumor, respectively, were treated with A box or vehicle control starting when the tumors were first palpable (approximate day 7–9 after tumor cell inoculation; Fig. 6A). In both strains, A box delayed tumor progression, supporting the concept that HMGB1 facilitates tumor growth. 4T1 tumor cells were also knocked down by shRNA for HMGB1 (4T1/575 cells) and their tumorigenicity compared with that of 4T1 cells transfected with an irrelevant shRNA (4T1/irrelevant; Supplementary Fig. S3A). The effect of HMGB1 on spontaneous metastatic disease was assessed by treating 4T1 tumor-bearing mice with glycyrrhizin and ethyl pyruvate and assessing the number of metastatic cells by clonogenic assay (Supplementary Fig. S3B; ref. 35). 4T1/575 tumor–bearing mice survived significantly longer than mice with 4T1/irrelevant cells supporting previously published work (36). Tumor-bearing mice treated with the inhibitors trended toward fewer metastatic cells however, the values were not statistically significantly different. These results further confirm that HMGB1 enhances tumor progression.

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HMGB1 downregulates T-cell expression of L-selectin

MDSCs also impair T-cell immunity by perturbing the homing of naïve T cells to lymph nodes where they could become activated. To enter lymph nodes, naïve T cells must first be tethered via proinflammatory cytokine that is produced by MDSCs and also drives the suppressive potency of MDSCs (33, 34), was also assessed. Ethyl pyruvate and glycyrrhizin decreased MDSC production of IL-10; however, HMGB1 inhibition restored IL-10 levels in cocultures of MDSCs and macrophages. These results indicate that HMGB1 regulates MDSC production of IL-10 during MDSC–macrophage crosstalk; however, it is not involved in MDSC-mediated downregulation of macrophage-produced IL-12 or IL-6.

Figure 4. HMGB1 increases MDSC production of IL-10 and MDSC–macrophage crosstalk. A, cocultures of 4T1-induced BALB/c MDSCs and macrophages from tumor-free mice were incubated with or without ethyl pyruvate or glycyrrhizin, and the supernatants were assayed by ELISA for IL-10. B, MDSCs from 4T1-tumor-bearing BALB/c and C57BL/6 mice bearing 4T1 or MC38 tumor, respectively, were treated with A box or vehicle control starting when the tumors were first palpable (approximate day 7–9 after tumor cell inoculation; Fig. 6A). In both strains, A box delayed tumor progression, supporting the concept that HMGB1 facilitates tumor growth. 4T1 tumor cells were also knocked down by shRNA for HMGB1 (4T1/575 cells) and their tumorigenicity compared with that of 4T1 cells transfected with an irrelevant shRNA (4T1/irrelevant; Supplementary Fig. S3A). The effect of HMGB1 on spontaneous metastatic disease was assessed by treating 4T1 tumor-bearing mice with glycyrrhizin and ethyl pyruvate and assessing the number of metastatic cells by clonogenic assay (Supplementary Fig. S3B; ref. 35). 4T1/575 tumor–bearing mice survived significantly longer than mice with 4T1/irrelevant cells supporting previously published work (36). Tumor-bearing mice treated with the inhibitors trended toward fewer metastatic cells however, the values were not statistically significantly different. These results further confirm that HMGB1 enhances tumor progression.

To determine whether HMGB1 drives MDSC accumulation in vivo, tumor-bearing mice were treated with a neutralizing HMGB1 mAb (2G7), and tumor-infiltrating MDSCs and MDSCs from the blood and spleen were compared with MDSCs in vehicle-treated tumor-bearing mice. C57BL/6 mice were inoculated with the MC38 tumor on day 1 and 2G7 treatment was started on days 10 to 13. Mice were sacrificed at a late stage of disease when their primary tumors were approximately the same diameter, and total MDSCs, monocytic MDSCs, and granulocytic MDSC levels in the blood, spleen, and infiltrating the tumors were determined by flow cytometry (Fig. 6B). Total, monocytic, and granulocytic MDSCs were reduced in the spleens, blood, and tumors of 2G7-treated mice with the exception of tumor-infiltrating granulocytic MDSCs. These decreases were not a secondary effect of reduced tumor size since, at the time of analysis, the 2G7-treated and control-treated mice had similar-sized primary tumors. MDSCs were similarly reduced in the blood of A box-treated tumor-bearing mice. These results indicate that in vivo, neutralization of HMGB1 reduces the accumulation of MDSCs in tumor-bearing mice.

Tumors from the HMGB1 mAb–treated (2G7) and isotype control–treated mice of Fig. 6 were assessed by IHC for the presence of CD3+ T cells (Supplementary Fig. S4). Both types of tumors contained few T cells; however, there was a trend toward more CD3+ cells in the tumors of 2G7-treated mice.

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MDSCs also impair T-cell immunity by perturbing the homing of naïve T cells to lymph nodes where they could become activated. To enter lymph nodes, naïve T cells must first be tethered via proinflammatory cytokine that is produced by MDSCs and also drives the suppressive potency of MDSCs (33, 34), was also assessed. Ethyl pyruvate and glycyrrhizin decreased MDSC production of IL-10; however, HMGB1 inhibition restored IL-10 levels in cocultures of MDSCs and macrophages. These results indicate that HMGB1 regulates MDSC production of IL-10 during MDSC–macrophage crosstalk; however, it is not involved in MDSC-mediated downregulation of macrophage-produced IL-12 or IL-6.
L-selectin (CD62L) to the walls of high endothelial venules (HEV) so they can extravasate from the bloodstream. Our previous in vitro studies showed that MDSCs reduce T cell levels of L-selectin through their constitutive expression of ADAM17 (a disintegrin and metalloproteinase domain 17), an enzyme that cleaves the ectodomain of L-selectin (4). Subsequent in vivo imaging studies showed that T cells with reduced expression of L-selectin do not enter HEVs (J. Muhich, S. Ostrand-Rosenberg, S. Abrams, and S. Evans; unpublished data). To determine whether HMGB1 impacts MDSC-mediated downregulation of T cell-expressed L-selectin, a box and control-treated mice were sacrificed 29 days after tumor inoculation and circulating CD4+ CD3+ CD44+ and CD45+ CD3+ CD68+ T cells were analyzed for L-selectin by flow cytometry (Fig. 7A). Circulating CD4+ and CD8+ T cells from tumor-free mice were controls for normal L-selectin expression. L-selectin was reduced in CD4+ and CD8+ T cells of tumor-bearing vehicle-treated mice, whereas a box treatment partially restored L-selectin expression (Fig. 7B). To confirm that HMGB1 acts on MDSCs to reduce L-selectin, Gr1+CD11b+ cells from tumor-free and tumor-bearing mice were treated for 0.2, or 4 hours with HMGB1 or ethyl pyruvate, respectively. The cells were then stained with mAbs to Gr1, CD11b, and ADAM17, and the gated Gr1+CD11b+ cells were analyzed for plasma membrane expression of ADAM17 (Fig. 7C). HMGB1-treated Gr1+CD11b+ cells from tumor-free mice expressed more ADAM17, whereas ethyl pyruvate–treated MDSCs from tumor-bearing mice had less ADAM17, as compared with vehicle-treated cells. These observations indicate that plasma membrane ADAM17 turns over on MDSCs and that HMGB1 contributes to the downregulation of L-selectin on T cells by sustaining MDSC expression of ADAM17.

Discussion

The DAMP and alarmin HMGB1 is released by many tumor cells, is elevated in the serum of many patients with cancer (37), and is recognized as an enhancer of tumor progression by its direct action on tumor cells (9, 10, 28). The studies reported here identify MDSCs, along with tumor cells and macrophages, as producers of HMGB1. The observed decrease in MDSCs of tumor-bearing mice following treatment with HMGB1 inhibitors, combined with the in vitro mechanistic studies demonstrate that HMGB1 (i) promotes the differentiation of MDSCs from bone marrow progenitor cells; (ii) increases MDSC–macrophage crosstalk and MDSC production of IL-10; and (iii) increases MDSC-mediated downregulation of L-selectin on naïve T cells. These findings support the conclusion that HMGB1 contributes to the elevation and suppressive potency of MDSCs in tumor-bearing mice, and identify a new proinflammatory mediator that regulates MDSCs.

HMGB1 is likely to activate and drive MDSCs because it induces, chaperones, and/or enhances the activity of several proinflammatory molecules that regulate MDSCs. For example, IL-1β drives MDSC accumulation and T-cell suppressive activity (33, 38) and is induced by HMGB1 (14). Complexes of HMGB1 and IL-1β have increased proinflammatory activity relative to either molecule alone (39). HMGB1 also enhances the proinflammatory activity of IL-6 (40), TNFα (14), and prostaglandin E2 (41), three other proinflammatory mediators that drive MDSCs (24, 34, 42, 43). Although neutralization of HMGB1 significantly downregulates MDSC-suppressive activity, it does not globally neutralize MDSCs, most likely because the multiple proinflammatory mediators that drive MDSCs are redundant and can also be regulated by molecules other than HMGB1. HMGB1 is known to facilitate tumor progression by coopting other immune cells and by directly affecting tumor cell growth (9, 10, 28). It increases the accumulation of T regulatory cells and diverts type I T helper cells to a protumor type II phenotype (36, 44). HMGB1 also acts directly on tumor cells to enhance tumor progression by binding to tumor cell–expressed RAGE. Many tumor cells express RAGE (45), and the binding of...
Figure 6. Tumor-bearing mice treated with mAbs to HMGB1 or with A box have reduced levels of MDSCs. A, C57BL/6 and BALB/c mice were inoculated subcutaneously with $5 \times 10^5$ MC38 colon carcinoma cells or in the mammary fat pad with $7 \times 10^3$ 4T1 mammary carcinoma cells, respectively. Mice were given recombinant A box (300 mg/mouse) or vehicle (PBS) three times per week starting when tumors were first palpable (day 7–9 postinoculation). P values were obtained by the log-rank test. B, C57BL/6 mice were inoculated as in A. Treatment with 2G7 (5 mg/200 mL/mouse, 3×/week), irrelevant IgG, or A box was started on days 10 to 13 when tumors were first palpable. Treatment was terminated on day 45 and blood leukocytes were analyzed by flow cytometry for total (Gr1+CD11b+), monocytic (MO; CD11b+Ly6C0Ly6G+), and granulocytic (PMN; CD11b+Ly6GLy6C-) MDSCs. Mice were sacrificed on day 50 when their tumors were approximately the same size, and spleen and tumor-infiltrating leukocytes (CD45+ cells) were analyzed by flow cytometry. n = 7 (blood, control treated for 2G7), 4 (A box, PBS treated), 6 (tumor-infiltrating and spleen, control-treated; blood, 2G7-treated), 4 (tumor-infiltrating and spleen, 2G7-treated), and 4 (A box-treated) mice/group. Data for 2G7 and their control-treated mice are from two independent experiments; data for A box and their control-treated mice are from a single experiment.

Figure 7. HMGB1 downregulates T-cell expression of L-selectin. A, twenty-nine days after tumor inoculation, the MC38 tumor-bearing mice from Fig. 6A were sacrificed and blood leukocytes were analyzed by flow cytometry for L-selectin expression and compared with blood leukocytes from tumor-free C57BL/6 mice. Representative histograms showing L-selectin expression from gated CD45+CD3+CD4+ and CD45+CD3+CD8+ T cells expressing L-selectin. n = 5 mice/group (PBS-treated and tumor-free groups); n = 3 mice/group (A box-treated group). P values were obtained by the Student t test. Data are from one of two independent experiments. B, average percent ± SD of CD45+CD3+CD4+ or CD45+CD3+CD8+ T cells expressing L-selectin. n = 5 mice/group (PBS-treated and tumor-free groups); n = 3 mice/group (A box-treated group). P values were obtained by the Student t test. Data are from one of two independent experiments.
HMGB1 to RAGE promotes tumor cell autophagy, inhibits tumor cell apoptosis, and increases tumor cell invasiveness (46, 47). Collectively, these effects produce an immune suppressive and protumor environment. MDSCs contribute to tumor growth through their immune suppressive mechanisms. However, their elimination may not be sufficient for tumor rejection, and active immunization of T cells and/or repolarization of macrophages to a M1-like phenotype may also be required (17). The studies reported here demonstrate that HMGB1 affects MDSC development and function. Because HMGB1 affects tumor progression through multiple mechanisms that act on both tumor cells and immune cells, its effects on MDSCs represent only one of its modes of action.

Paradoxically, under some conditions, HMGB1 facilitates the activation of tumor-reactive T cells. HMGB1 facilitates dendritic cell maturation (48) and enhances DC-mediated antigen presentation during chemotherapy and radiotherapy (49). In contrast with the protumor effects of HMGB1, which are thought to be transmitted through RAGE, the enhancement of DC function requires the release of HMGB1 by dead tumor cells and is mediated through DC-expressed TLR4. Whether the in vivo protumor or antitumor effects of HMGB1 balance each other, or whether one dominates is unclear. However, the potential for HMGB1 to both inhibit and promote antitumor immunity makes it difficult to evaluate whether neutralization of HMGB1 will be beneficial or harmful.

The quantity of HMGB1 within different solid tumors differs significantly (see Supplementary Table S1). MDSCs, macrophages, tumor-infiltrating cells, and tumor cells themselves all contribute to the amount of HMGB1 in the tumor microenvironment. Live tumor cells secrete HMGB1, whereas necrotic tumor cells induced by suboptimal vascularization and hypoxia release nuclear HMGB1. Because the quantity of tumor-infiltrating cells and the extent of vascularization and hypoxia differ in different types of tumors, it is not unexpected that the quantity of HMGB1 within solid tumors does not correlate with tumor mass.

HMGB1 binds to both TLR4 and RAGE, and MDSCs express both receptors (20, 50). TLR4 and RAGE signaling converges at NF-κB (9, 10, 28, 51), so that activation through either receptor may produce similar effects. Previous studies demonstrated that MDSC production of IL-10 is regulated by TLR4 (50). In the current report, A box partially restores T-cell expression of L-selectin, suggesting that this effect of MDSCs may be regulated through RAGE. The HMGB1 inhibitors ethyl pyruvate and glycyrrhizin reduced MDSC production of IL-10 during MDSC–macrophage crosstalk and the differentiation of MDSCs from bone marrow progenitor cells, and ethyl pyruvate restored T-cell activation in the presence of MDSCs. These reagents either bind exogenous HMGB1 (glycyrrhizin) or inhibit NF-κB signaling (ethyl pyruvate) and therefore do not distinguish whether HMGB1 is acting through TLR4 or RAGE. Regardless of which receptor is utilized, HMGB1 is a potent inducer of MDSCs and immune suppression, and both its protumor and antitumor activities must be considered when designing cancer immunotherapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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