The Alarmin HMGN1 Contributes to Antitumor Immunity and Is a Potent Immunoadjuvant
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Abstract
Alarmins are endogenous mediators that are elicited rapidly in response to danger signals, enhancing innate and adaptive immune responses by promoting the recruitment and maturation of antigen-presenting cells (APC). The nucleosome-binding protein HMGN1 is a potent alarmin that binds TLR4 and induces antigen-specific Th1 immune responses, but its contributions to antitumor immunity have not been explored. We found that ovalbumin (OVA)-expressing EG7 mouse thymoma cells grew much faster in Hmgn1-deficient mice than littermate-matched controls. Tumor-bearing Hmgn1+/− mice generated fewer OVA-specific CD8 cells in the spleen than EG7-bearing Hmgn1+/+ mice, suggesting that HMGN1 supported T cell–mediated antitumor immunity. In addition, EG7 tumors expressing HMGN1 grew more slowly than control EG7 tumors, suggesting greater resistance to HMGN1-expressing tumors. This resistance relied on T cell–mediated immunity because it was abolished by in vivo depletion of CD4+ and CD8+ T cells. Moreover, mice vaccinated with a DNA vector expressing an HMGN1–gp100 fusion protein manifested gp100-specific, Th1-polarized immune responses, acquiring resistance to challenge with mouse B16F1 melanoma. Overall, our findings show that HMGN1 contributes to antitumor immunity and it may offer an effective adjuvant to heighten responses to cancer vaccines. Cancer Res; 74(21); 5989–98. ©2014 AACR.

Introduction
In the past two decades, several antitumor immunotherapies, including adoptive cell therapy, tumor-specific antibodies, and checkpoint inhibition have yielded considerable clinical success (1–3). Although humanpapilloma virus (HPV)–based vaccines have been successful in cancer prevention, other vaccine approaches have so far failed to mediate regression of solid tumors (3, 4). There are many hurdles in therapeutic tumor vaccine approaches, predominantly difficulties in inducing sufficient number of antitumor CD8 effector cells and the immunosuppressive tumor microenvironment that inhibits effector mechanisms (5). Although the immunosuppressive components can be overcome by countering IL10, TGFβ, VEGF, regulatory T cells, and/or myeloid-derived suppressor cells (3, 5), identifying potent adjuvant(s) capable of generating potent protective antitumor immunity is key to the development of therapeutic antitumor vaccines.

Alarmins are structurally distinct endogenous mediators that can activate the immune system by inducing recruitment and activation of immune cells, particularly antigen-presenting dendritic cells (DC; 6–8). Alarmins identified so far include defensins, cathelicidins, eosinophil-associated ribonucleases, high-mobility group (HMG) proteins, heat shock proteins (HSP), saposin-like granulysin, ion-binding proteins (e.g., S100 proteins and lactoferrin), and nucleotides/metabolites (e.g., uric acid; 6–9). Alarmins promote host defenses by inducing inflammation, immune response, and wound healing (10–23).

Various alarmins appear to have distinct effects on the types of antigen-specific immune responses. Earlier studies show that neutrophil-derived α-defensins can induce both Th1 and Th2 immune responses upon administration together with an antigen via a mucosal route (9, 10), whereas β-defensins stimulate predominantly Th1 responses upon gene gun delivery to mouse epidermis as a defensin–antigen fusion product (9, 12). Cathelicidin also promotes the generation of both Th1 and Th2 immune responses when administered intraperitoneally (13). HMGB1 and HSPs preferentially promote Th1 immune responses involved in protective antitumor immunity (9, 15, 24). In contrast, eosinophil-derived neutrotoxin, a member of the eosinophil-associated ribonuclease, and uric acid selectively enhance the development of Th2 immune responses (14, 21). Therefore, it has gradually become apparent that various alarmins differentially promote distinct types of antigen-specific immune responses.

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We have recently identified HMG nucleosome-binding protein 1 (HMGN1) as a potent Th1-polarizing alarmin (25). Recombinant HMGN1 induced the accumulation and activation of DCs at the site of injection as indicated by upregulation of costimulatory and MHC molecules as well as extracellular secretion of proinflammatory cytokines such as TNFα, IL-12 p70, and IL-1β (25). In addition, immunization of mice with an antigen mixed with HMGN1 augmented antigen-specific immune responses with greatly elevated production of IFNγ, but not IL4 (25). The critical role of HMGN1 in the induction of Th1-polarized immune response was clearly demonstrated by the lack of such responses in HMGN1 knockout mice (25). Because generation of tumor-associated antigen (TAA)–specific Th1 immune response is vital for antitumor immunity, we investigated: (i) whether HMGN1 would contribute to the induction of antitumor immune responses, and (ii) whether HMGN1 was an effective anticancer adjuvant.

Materials and Methods

Cell lines and mice

All cell lines used in this study were originally obtained from the American Type Culture Collection. Human embryonic kidney 293 (HEK293) cells were maintained in DMEM [DMEM (Meditech) supplemented with 10% FBS (Hyclone), 2 mmol/L L-glutamine, 25 mM L-HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol]. Mouse melanoma cell line B16F1 was cultured in DMEM medium additionally supplemented with 1× vitamin solution (Life Technologies), and 1× nonessential amino acids solution (Life Technologies). EG7, a cell line derived from EL4 thymoma and constitutively expressing ovalbumin (OVA) as a surrogate TAA, was maintained in RPMI-1640 medium [RPMI-1640 (Meditech) supplemented with 10% FBS, 2 mmol/L L-glutamine, 25 mM L-HEPES, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol] containing 0.4 mg/mL G418 (Life Technologies). The insect cell lines Sf9 and High-Five were purchased from Invitrogen and cultured at 27°C in SF-900 III serum-free medium (SFM; Invitrogen) or Express Five SFM (Invitrogen), respectively.

C57BL/6 mice were obtained from Charles River. Hmgn1<sup>+/−</sup> and littermate-matched Hmgn1<sup>−/−</sup> mice were generated as reported previously (25). All mice were kept under specific pathogen-free conditions with water and food given ad libitum. All experiments with mice were performed in compliance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals and were approved by the National Cancer Institute at Frederick Animal Care and Use Committee.

Generation and treatment of mouse DCs

Mouse DCs were generated from C57BL/6 bone marrow progenitors as previously reported (14). DCs were incubated with recombinant human HMGN1 at 1 to 5 μg/mL, for 24 to 48 hours before analysis of DCs. Cytokines produced by DCs in the culture supernatants were quantitated by SearchLight (Aushon). Recombinant human HMGN1 used in this study was expressed in insect cells using a baculovirus expressing system and purified as previously reported (25).

Plasmid construction, purification, transfection, and cell line establishment

The recombinant plasmids encoding HMGN1 (Clone Id LFISEEQ128711) and gp100 (OriGene SC122763) were purchased from the Open Biosystems and OriGene Technologies, Inc., respectively. The cDNAs encoding the mature form of the target gene (HMGN1 or gp100) were amplified by PCR, with the introduction of the insulin signal peptide at the N-terminus to facilitate secretion of the targets. The fusion gene HMGN1–gp100 was constructed by overlapping PCR, with the insertion of a flexible linker (Gly<sub>4</sub>Ser), between HMGN1 and gp100. After sequence confirmation, the three target genes were subcloned to eukaryotic expressing plasmid pcDNA3.1-hygromycin (Invitrogen) or pcDNA3.1/myc-His B (Invitrogen) to generate pcDNA3.1-HMGN1, pcDNA3.1-gp100, or pcDNA3.1-HMGN1–gp100, respectively. The plasmid pcDNA3.1/myc-His B also provided a c-myc and a poly-His motifs in-frame after the inserted genes so that the target proteins could be detected using anti-myc or anti-poly-His antibodies. Plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen).

Transfection of HEK293, EG7, or EL4 cells was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s recommendations. For determining the production of targets, proteins in supernatants collected 24 hours after transfection were precipitated by trichloroacetic acid and subsequently analyzed by Western blot analysis. Some transfected cells were selected with hygromycin B (Invitrogen) at 700 μg/mL and cloned by limiting dilution.

Western blot analysis

Samples or SeeBlue Plus2 Pre-Stained Standard (Invitrogen) were loaded onto a NuPAGE Novex 4% to 12% Bis–Tris gel (Invitrogen) and separated using either MOPS or MES buffer. After transfer of separated proteins onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore), the membranes were rinsed with tris-buffered saline containing 0.05% Tween 20 (TBST), blocked with 5% nonfat dry milk at room temperature for 1 hour, and incubated overnight at 4°C with rabbit anti-HMGN1 (ProteinTech; 11695-1-AP), rabbit anti-poly-His (Cell Signaling Technology; #2365), or goat anti-gp100 (Santa Cruz Biotechnology; sc-15010). After washing with TBST, the membranes were reacted with horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (Cell Signaling Technology; #70741) or HRP-conjugated rabbit anti-goat IgG (Calbiochem; 401515), washed, and developed in the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher). The images were collected using the G BOX Chemi systems (Syngene).

Preparation of gene gun bullets and DNA vaccination

Gene gun bullets were prepared as previously described with optimization (26). Briefly, 100 μL of 0.05 mol/L spermidine was added into 45 mg of 1-μm gold beads (Bio-Rad) and mixed by brief sonication. Forty-five μL of plasmid (2 mg/mL) was immediately added into the gold/spermidine mixture and the
plasmids were coprecipitated onto the gold beads by adding 100 μL of 1 mol/L CaCl2 drop-wise while vortexing. After washing with 100% ethanol, the plasmid-coated gold beads were resuspended in 6 mL of 100% ethanol in a 15-mL tube and loaded onto predripped Teflon tubing (Bio-Rad). The Teflon tubing was rotated at 20 rpm in a Tube turner (Bio-Rad) for 1 minute to allow the gold beads smear evenly on the inner surface of the tubing, and finally was dried by flowing dry nitrogen gas through the rotating tubing at a rate of 0.35 L/min for about 4 minutes. The dried tubing was subsequently cut into 0.5-inch sections (bullets) and stored at 4°C until use.

For DNA vaccination, C57BL/6 mice were anesthetized and their abdominal skin was shaved. Subsequently, plasmid DNA was delivered into the epidermal layer of the shaved abdominal skin via a Helios Genegun (Bio-Rad) with a discharge pressure of 400 psi (four bullets/mouse/immunization). Mice were vaccinated either once a week for 3 weeks, twice a week for 3 weeks, or only twice in 3 weeks as specified. Each mouse received 4 μg of plasmid DNA at each vaccination.

**Mouse tumor models**

Mice (C57BL/6, Hmgn1-/-, or Hmgn1+/+, female, 7–10-week old, n = 5–10), untreated or DNA-vaccinated, were implanted subcutaneously with 0.2 ml sterile PBS containing indicated number (2 × 105–5 × 106/injection) of tumor cells (EG7, control/ HMGN1-expressing EG7, or B16F1) into the right flank. The appearance and growth of tumors were monitored twice every week. The greatest longitudinal diameter (length) and the greatest transverse diameter (width) of a palpable tumor were measured to the nearest 0.1 mm using a caliper. Tumor volume (mm3) was calculated by the ellipsoidal formula: Tumor volume = (length × width2) × 1/6. For depleting CD4 or CD8 cells, mice were injected intraperitoneally with 0.2-mL PBS containing 150 μg of either control rat IgG (clone 2A3; BioXcell), anti-mouse CD4 (clone GK1.5; BioXcell), or anti-mouse CD8 (clone 53–6.72) for 3 consecutive days before tumor inoculation and once a week after tumor inoculation for 4 weeks.

**Immunostaining and flow cytometry**

Mouse DCs or splenocytes suspended in FACS buffer (PBS containing 0.5% BSA and 0.05% NaN3) were blocked with 2% normal mouse serum on ice for 20 minutes and stained with various combinations of fluorochrome-conjugated anti-mouse antibodies on ice for 30 minutes. The antibodies used to stain mouse DCs were FITC-anti-mouse CD80 (clone 16-10A1; BD/Pharmingen), PE-anti-mouse CD86 (clone GL1; BD), FITC-anti-mouse I-A/E (clone 2G9; BD), and PE-anti-mouse CD11c (clone IHL3; BD). Stained DCs were analyzed on a flow FACSscan cytometer (BD). For determining the frequency of OVA-specific CD8 cells, splenocytes of EG7-bearing Hmgn1-/- and Hmgn1+/+ mice were stained with FITC-anti-mouse CD3 (clone I45–2C11; BD), PE-OVA–specific tetramer (Class I ITAg MHC Tetramer; Beckman Coulter), PerCP-Cy5.5-anti-mouse CD8 (clone 53–6.7; eBioscience), and APC-conjugated anti-mouse CD4 (clone RM4–5; BD), and analyzed on a FACS Calibur flow cytometer (BD).

The CD8 epitope of gp100, gp100(225–230) (KPVPRNQDWL), was synthesized and purified as reported previously (27). For intracellular cytokine staining, the splenocytes and lymph node cells of mice vaccinated with various plasmids were stimulated at 5 × 106/mL in RPMI-1640 medium in vitro with 1 μmol/L gp100(225–230) peptide for 6 hours at 37°C in humidified air containing 5% CO2 with the addition of GolgiPlug (eBioscience) for the last 4 hours of culture. Subsequently, the cells were stained with PE-anti-mouse CD3 (clone 17A2; BD), APC-anti-mouse CD4 (clone RM4–5; BD), and PE-Cy7-anti-mouse CD8 (clone 53–6.7; eBioscience) on ice for 30 minutes, fixed with IC Fixation Buffer (eBioscience; cat. no. 00-8222) at room temperature for 20 minutes, permeabilized with Perm/Permeabilization Buffer (BD; cat. no. 00-8333), and incubated with FITC-anti-mouse IFN-γ (clone XMG1.2; BioLegend) and eFluor450-anti-mouse IL13 (clone eBio13A; eBioscience) in Permeabilization Buffer for 20 minutes at room temperature. After three washes with Permeabilization Buffer, the samples were resuspended in FACS buffer and data were acquired on a LSRII flow cytometer (BD). All flow cytometry data were analyzed using FlowJo.

**Statistical analysis**

Unless otherwise specified, all experiments were performed at least three times, and the results of one representative experiment or the mean of multiple experiments are shown. Differences in the in vivo tumor growth were determined by repeated measures ANOVA, whereas differences between other control groups and experimentally treated groups were evaluated by one-way ANOVA after arcsine square-root transformation.

**Results**

**Endogenous HMGN1 contributed to the development of antitumor immunity**

We compared the development and growth of EG7 tumors in Hmgn1-/- and littermate-matched Hmgn1+/+ mice. The use of EG7 mouse thymoma allowed convenient measurement of tumor-specific immune response because EG7 cells express OVA as a surrogate TAA (28). Following subcutaneous implantation, EG7 tumors grew much more rapidly in Hmgn1-/- mice than in Hmgn1+/+ mice (Fig. 1A). The increased rate of EG7 tumor growth in Hmgn1-/- mice was unlikely due to the lack of intranuclear HMGN1, because EG7 tumor cells were not Hmgn1-/- (data not shown). These data suggested that knockout of HMGN1 might reduce the antitumor immune defenses of the host. Analysis of splenic lymphocytes from EG7-bearing mice by tetramer staining and flow cytometry showed that Hmgn1-/- mice generated considerably higher frequency of splenic OVA-specific CD8 cells than Hmgn1+/+ mice (Fig. IB and C). Tumor-draining lymph node also contained higher level of OVA-specific CD8 cells (data not shown). These data showed that Hmgn1-/- mice developed lower antigen-specific antitumor immune responses than littermate-matched Hmgn1+/+ mice in response to EG7 tumors, demonstrating the contribution of HMGN1 to the development of antigen-specific antitumor immune defense responses.
Exogenous HMGN1 promoted DC activation and host defense against tumor

We next determined whether recombinant human HMGN1 could activate mouse DCs. Mouse DCs were therefore incubated without (sham) or with human HMGN1 and subsequently analyzed for the expression of surface markers and production of cytokines, two hallmarks of DC activation. DCs treated with human HMGN1 at 1 or 5 μg/mL for 48 hours upregulated their expression of CD80, CD86, and I-A/E in a dose-dependent manner as shown by flow cytometry analysis (Fig. 2A and Supplementary Fig. S1). Incubation of mouse DCs with human HMGN1 at 5 μg/mL for 48 hours induced the production of multiple proinflammatory cytokines, including IL-1β, IL-6, IL-10, IL-12p70, and TNFα (Fig. 2B). Thus, human HMGN1 could act as an alarmin in mice.

Human HMGN1 gene was cloned into a pcDNA3.1-hygro-mycin vector with insulin signal peptide directing extracellular expression of HMGN1 in mammalian cells (Fig. 3A). EG7 cells were transfected with pcDNA3.1 or pcDNA3.1-HMGN1 and subsequently selected with hygromycin to establish control and HMGN1-expressing EG7 cell lines, respectively. Western blot analysis confirmed that HMGN1 was indeed secreted by HMGN1-expressing EG7 cells (data not shown). The effect of HMGN1 expression on in vitro EG7 growth was determined by comparing ^3H-TdR incorporations of control and HMGN1-expressing EG7 cells (Fig. 3B). Both control and HMGN1-expressing EG7 cell lines grew similarly, indicating that expression of human HMGN1 in EG7 did not influence the proliferative capacity of the cells (Fig. 3B).

The control and HMGN1-expressing EG7 cells were then implanted subcutaneously into C57BL/6 mice and tumor growth was monitored. Control EG7 formed palpable tumors at about 2 weeks, and progressed during the third weeks after implantation (Fig. 3C). In contrast, implantation with HMGN1-expressing EG7 cells did not develop palpable tumors within 4 weeks (Fig. 3C). When mice were followed for longer than 4 weeks, all mice inoculated with control EG7 died or became moribund within 5 weeks, whereas 80% of the mice inoculated with HMGN1-expressing EG7 had no or small tumors and remained alive for at least 60 days (Fig. 3D). Because expression of HMGN1 did not alter the proliferation of HMGN1-expressing EG7 cells in vitro (Fig. 3B), the failure of implanted HMGN1-expressing EG7 to form tumors was unlikely due to any change in tumorigenicity of the implanted tumor cells. Presumably, human HMGN1 secreted by implanted HMGN1-expressing EG7 tumor cells promoted antitumor immune defense.

We also studied whether HMGN1 could promote antitumor defense in the absence of surrogate TAA using HMGN1-expressing EL4 cell lines. Both control and HMGN1-expressing EL4 cell lines grew identically in vitro (Supplementary Fig. S2A). When implanted into C57BL/6 mice, control EL4 formed rapidly growing tumors, whereas HMGN1-expressing EL4 formed smaller, slowly growing tumors (Supplementary Fig. S2B). Therefore, HMGN1 induced antitumor defense even in the absence of the potent surrogate TAA OVA.

To investigate whether antitumor immunity was responsible for the observed anti-EG7 resistance (Fig. 3C), mice inoculated with HMGN1-expressing EG7 cells were depleted of CD4 and/or CD8 T cells. Mice depleted of CD4 or CD8 T cells and inoculated with HMGN1-expressing EG7 cells showed faster tumor growth (closed and open triangles, respectively) than mice treated with control antibody (open circles, Fig. 3E). Depletion of both CD4 and CD8 cells (open diamonds) resulted in even faster tumor growth than in mice inoculated with control EG7 cells (closed circles, Fig. 3E), indicating that CD4 and CD8 T cells together yielded optimal anti-EG7 immune defense.
defense. Therefore, extracellular HMGN1 at the site of tumor elevated host antitumor immune defense.

**HMGN1 acted as an effective adjuvant for induction of antimalanoma immunity**

We evaluated the immunoadjuvant activity of HMGN1 using the rapidly growing mouse B16 melanoma with gp100 as the target TAA. It has previously been shown that covalent linkage of an alarmin to a target TAA can better promote TAA-specific immune responses and immunoprotection (12, 29). Therefore, a series of mammalian expression plasmids encoding HMGN1, gp100, or fusion of HMGN1–gp100 were constructed with the insulin signal peptide fused in-frame with our target proteins (Fig. 4A). A flexible linker consisting of three tandems of Gly4 Ser (12, 29) was introduced between HMGN1 and gp100 in the pcDNA3.1-HMGN1–gp100 plasmid (Fig. 4A). To confirm that the target gene products could be expressed and secreted by mammalian cells, the supernatants of HEK293 cells transfected with pcDNA3.1-HMGN1 contained a protein band of approximately 16 kDa as probed with either anti-poly-His or anti-HMGN1 antibody (Fig. 4B, left). This band consisted of HMGN1 because it showed identical electrophoretic mobility with purified HMGN1 in the same SDS-PAGE gel (Fig. 4B, left). When probed with anti-poly-His antibody, the supernatants of HEK293 cells transfected with pcDNA3.1-gp100 and pcDNA3.1-HMGN1–gp100 yielded a detectable protein band of approximately 86 and 102 kDa, respectively, corresponding to the expected size of gp100 and HMGN1–gp100, respectively (Fig. 4B, right). The supernatant of HEK293 cells transfected with pcDNA3.1 plasmid had no positive band as expected (Fig. 4B, right). Furthermore, probing the supernatants of HEK293 cells transiently transfected with pcDNA3.1, pcDNA3.1-gp100, or pcDNA3.1-HMGN1–gp100 with anti-HMGN1 (Fig. 4C, left) or anti-gp100 (Fig. 4C, right) revealed a band of approximately 102 kDa for HMGN1–gp100 fusion protein and a band of approximately 86 kDa for gp100 protein (Fig. 4C). Therefore, the plasmids constructed were indeed capable of directing secreted expression of the target proteins in mammalian cells.
To investigate whether HMGN1 could promote an antigen-specific immune response, C57BL/6 mice vaccinated with various plasmids were analyzed for cytokine-producing T cells in the spleens and draining lymph nodes. Intracellular cytokine staining and flow cytometry analysis (analysis gating illustrated in Supplementary Fig. S3) showed that in both inguinal lymph nodes and spleen, mice vaccinated with pcDNA3.1–HMGN1–gp100 generated significantly more IFN-γ+ CD8 cells than control (vaccinated with pcDNA3.1) mice (Fig. 5). There was no significant increase in IL13+ CD8 T cells in the inguinal lymph nodes or spleens of mice vaccinated with pcDNA3.1–HMGN1–gp100 in comparison with the control group (Fig. 5). These data demonstrated that the fusion product of HMGN1 and gp100 induced a potent Th1-polarized T-cell response against gp100. Mice vaccinated with either pcDNA3.1–HMGN1 or pcDNA3.1–gp100 did not show any increase in the percentage of either IFNγ+ or IL13+ CD8 T cells, indicating that HMGN1 or gp100 alone had no effect (Fig. 5). Therefore, HMGN1 acted as an adjuvant to stimulate Th1-polarized gp100-specific immune responses.

We next determined whether DNA vaccination with pcDNA3.1–HMGN1–gp100 could induce sufficiently potent immune responses capable of protecting against a melanoma challenge. C57BL/6 mice were vaccinated with various plasmids, subcutaneously implanted with B16F1 melanoma, and the appearance and growth of tumors were monitored. Tumors grew at a similar rate in mice vaccinated with pcDNA3.1 (control group) or pcDNA3.1–gp100, suggesting that vaccination with TAA (gp100) alone had no effect (Fig. 6B). Although mice vaccinated with pcDNA3.1–HMGN1 showed a slower tumor growth than controls, the difference was not statistically significant (Fig. 6B). Tumors in mice vaccinated with a mixture of pcDNA3.1–HMGN1 and pcDNA3.1–gp100 grew significantly slower than in the controls, indicative of some immunoprotection (Fig. 6B). In contrast, none of the mice vaccinated with pcDNA3.1–HMGN1–gp100 either six times or just twice, formed any palpable tumors, indicative of the most potent immunoprotection (Fig. 6B). Interestingly, mice given the HMGN1–gp100 fusion construct also developed much greater immunoprotection to subsequent B16F1 challenge than mice

Figure 3. Human HMGN1-expressing EG7 tumors grew slower than wild-type EG7 tumors in vivo. A, schematic illustration of pcDNA3.1-hHMGN1-hygromycin. EG7 cells were transfected with pcDNA3.1-hygromycin or pcDNA3.1-hHMGN1-hygromycin and selected in the presence of hygromycin for generating control and human HMGN1-expressing EG7 clones. B, in vitro proliferation of control and HMGN1-expressing EG7 tumor cells. Control and HMGN1-expressing EG7 cells were plated in triplicate in a 96-well plate at the indicated number and incubated overnight. Subsequently, the culture was pulsed with 0.5 μCi/well of H-Tdr for 4 hours, harvested, and measured for the incorporation of H-Tdr. Shown is the average incorporation of H-Tdr (mean ± SD) of triplicate wells. C, C57BL/6 mice (8-week-old, female) were divided into two groups (n = 10) and were subcutaneously injected with 5 × 10^5 control or HMGN1–expressing EG7 into the right flank. Tumor growth was monitored twice a week for up to 4 weeks. Shown are the results of one experiment representative of three. *, P < 0.05 by repeated measures ANOVA. D, two groups of C57BL/6 mice (8-week-old, female, n = 10) were subcutaneously injected with 5 × 10^5 control or HMGN1–expressing EG7 into the right flank. Mouse morbidity was monitored for 60 days and compared after graphing with Prism software. **, P < 0.05 by the Mantel–Cox test. E, five groups of C57BL/6 mice (8-week-old, female, n = 10) were subcutaneously injected with 5 × 10^5 control (PBS group) or HMGN1–expressing EG7 (groups with Ab treatment) into the right flank on day 1. For depleting CD4 or CD8 T cells, mice were intraperitoneally injected with anti-CD4, anti-CD8, or a combination of anti-CD4 and anti-CD8 antibodies on day −4, −3, −2, 3, 10, 17, and 24 at 150 μg per mouse. Tumor volume was monitored twice a week. Shown are the results of one experiment representative of two. ***, P < 0.05 by repeated measures ANOVA.
given a mixture of HMGN1- and gp100-expressing plasmids (Fig. 6B). Consequently, HMGN1 augments protective antitumor immunity and may provide a potent adjuvant for the development of effective antitumor vaccines.

Discussion

HMGN1 is a nuclear protein that binds specifically to nucleosomes and affects chromatin structure and function (30). We have recently reported that extracellular HMGN1 acts as an alarmin and plays a critical role in the induction of antigen-specific immune responses (25). In this study, we demonstrated the importance of HMGN1 in promoting antitumor immunity in two ways. EG7 tumors grew more rapidly in Hmgn1−/− mice than in littermate-matched Hmgn1+/+ mice, which was accompanied by the generation of lower levels of splenic EG7-specific (OVA-specific) CD8+ cells (Fig. 1). Furthermore, when implanted into C57BL/6 mice, HMGN1-expressing EG7 tumor cells failed to form palpable tumors, whereas control EG7 tumor cells grew progressively into large solid tumors (Fig. 3C). Depletion of CD4 and CD8 T cells in mice inoculated with HMGN1-expressing EG7 cells nullified the antitumor defense and allowed the mice to grow even larger tumors than mice inoculated with control EG7 cells (Fig. 3E). Thus, both loss-of-function and gain-of-function approaches demonstrate that HMGN1 is important for the development of antitumor immune defenses.

Knockout of HMGN1 has been shown to affect DNA repair (31) and to cause increased susceptibility to radiation-induced DNA damage as well as tumorigenicity (30, 32). Therefore, the failure of HMGN1-expressing EG7 to grow into solid tumors might have been due to the adverse growth potential resulting from expression of human HMGN1 in EG7 cells (Fig. 3C). However, this was considered unlikely for two reasons. One is that pcDNA3.1-HMGN1-hygromycin used for transfecting EG7 cells was constructed to achieve secreted expression of HMGN1 (Fig. 3A). Consequently, human HMGN1 expressed by
transfected EG7 cells would not reach the nucleus. Second, because control and HMGN1-expressing EG7 cell lines proliferated equally in vitro (Fig. 3B), the expression of human HMGN1 in EG7 cells did not adversely influence their growth potential. Therefore, implantation of HMGN1-expressing EG7 cells probably enabled the host immune system to develop greater resistance to the tumor cells. This conclusion is supported by data showing that human HMGN1 activated mouse DCs (Fig. 2), and the induced antitumor defense was dependent on cell-mediated immunity (Fig. 3E).

In this study, human instead of mouse HMGN1 cDNA was used in making various expression constructs. This raises the possibility that adaptive immune responses against xenogenic human HMGN1 instead of antitumor immunity was responsible for the inhibition of HMGN1-expressing EG7 tumors (Fig. 3C–E). This was considered unlikely because (i) human and mouse HMGN1 are highly homologous; (ii) anti-human HMGN1 antibody was not detected in the serum of mice inoculated with HMGN1-expressing EG7 cells (data not shown); and (iii) comparison of the predicted H-2-Kb epitopes of human and mouse HMGN1 shows that the nine likely H-2-Kb epitopes of human HMGN1 are all covered by H-2-Kb epitopes of mouse HMGN1 (Supplementary Table S1), suggesting that it is unlikely for C57BL/6 mice to mount a CD8 response against human HMGN1.

Because most TAAs are weakly immunogenic, it was relevant to determine whether HMGN1 could be used to protect against tumors with weaker TAAs. We therefore investigated whether cutaneous vaccination with a plasmid encoding the expression of a fusion protein consisting of HMGN1 and gp100, a melanoma TAA, could induce gp100-specific CD8 response as well as antimelanoma protection. Vaccination of C57BL/6 mice with pcDNA3.1-HMGN1–gp100 increased gp100-specific CD8 response as well as antimelanoma protection. Vaccination of C57BL/6 mice with pcDNA3.1-HMGN1–gp100 increased gp100-specific IFNγ-producing CD8 cells (Fig. 5). Furthermore, vaccination with pcDNA3.1-HMGN1–gp100 induced full prophylactic protection to a subsequent challenge with mouse B16F1 melanoma (Fig. 6). It is noteworthy that vaccination with pcDNA3.1-HMGN1–gp100 provided more effective protection than vaccination with a mixture of pcDNA3.1-HMGN1 and pcDNA3.1-gp100 (Fig. 6). This was presumably based on observations that antigens linked to mediators capable of interacting with receptors on antigen-presenting cells (APC) are more efficiently taken up and processed (9, 33).

Data obtained in this study demonstrate the importance of HMGN1 in the generation of antitumor immune responses. However, EG7 tumor cells do not release HMGN1 at least
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Figure 6. DNA vaccination with pcDNA3.1-HMGN1–gp100 plasmid protected mice from B16F1 inoculation. C57BL/6 mice (7-week-old, female, 5 mice/group) were DNA vaccinated with indicated plasmids via gene gun twice a week for three consecutive weeks with the exception of group 6, in which mice received only two (the first and the last) vaccinations. Six days after the last vaccination, mice were subcutaneously implanted with B16F1 melanoma (2 × 10^5/0.2 mL/mouse). Tumor growth was monitored twice a week for up to 4 weeks, starting on day 6 after B16 implantation. A, schematic illustration of the timeline of various treatments. B, tumor growth curve. Shown are the results of one experiment representative of two. *, P < 0.05 by repeated measures ANOVA in comparison with pcDNA3.1 control group.

vitro (data not shown). Bone marrow chimera experiments reveal that HMGN1 responsible for promotion of antigen-specific immune response does not seem to come from cells of hematopoietic origin (25). Therefore, it remains to be investigated which type of cells is the major source of extra-cellular HMGN1 that promotes antitumor immunity.

Several alarmins have been reported to have the capacity to enhance antitumor immunity. Mouse β-defensin 2 can promote the generation of antitumor immunity against leukemia and melanoma through activation of DCs. T cell, and NK cells (12, 29, 34). HSPs are also reported to be capable of promoting antitumor CD8 responses (35, 36). HMGB1 is shown to be an important alarmin for the generation of antitumor immunity in both mouse and human species (15, 37). Our data showing the enhancement of antitumor immunity against mouse thymoma and melanoma by HMGN1 suggest that HMGN1 can be an adjuvant of choice for the design of antitumor vaccines. HMGN1 offers some advantages over other adjuvants. First of all, endogenous HMGN1 may exhibit fewer adverse side effects than exogenous pathogen-associated molecular patterns such as bacterial lipopolysaccharides (LPS). Second, HMGN1 consistently promotes Th1 type immune responses when used intraperitoneally (25) or delivered intradermally as a DNA vaccine (Fig. 5). This preferential Th1-polarizing capability of HMGN1 makes it highly favorable for inclusion in tumor vaccines because only TAA-specific Th1 type immune responses are protective against tumors. Third, HMGN1 is more potent and stable compared with other alarmins capable of promoting antitumor immune responses. HMGN1 stimulates DC activation at 0.2 to 1.0 μg/mL (approximately 10–60 nmol/L), whereas mouse β-defensin 2, HSPs, and HMGB1 need 5- to 10-fold higher concentrations to activate DCs (12, 25, 38–41). In addition, the presence of intramolecular disulfide bonds in defensins and HMGB1 enables their activities to be affected by their redox status (42, 43), whereas HMGN1 is consistently active due to the lack of disulfide bond. Although it remains to be determined whether HMGN1 can be used to treat established solid tumors, our findings suggest that HMGN1 may potentially be a promising tumor vaccine adjuvant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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