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Knockdown of HMGB1 in Tumor Cells Attenuates Their Ability To Induce Regulatory T Cells and Uncovers Naturally Acquired CD8 T Cell-Dependent Antitumor Immunity

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Although high mobility group box 1 (HMGB1) in tumor cells is involved in many aspects of tumor progression, its role in tumor immune suppression remains elusive. Host-derived IL-10 suppressed a naturally acquired CD8 T cell-dependent antitumor response. The suppressive activity of tumor-associated Foxp3+CD4+CD25+ regulatory T cells (Treg) was IL-10 dependent. Neutralizing HMGB1 impaired tumor cell-promoted IL-10 production by Treg. Short hairpin RNA-mediated knockdown of HMGB1 (HMGB1 KD) in tumor cells did not affect tumor cell growth but uncovered naturally acquired long-lasting tumor-specific IFN-γ– or TNF-α–producing CD8 T cell responses and attenuated their ability to induce Treg, leading to naturally acquired CD8 T cell- and IFN-γ–dependent tumor rejection. The data suggest that tumor cell-derived HMGB1 may suppress naturally acquired CD8 T cell-dependent antitumor immunity via enhancing Treg to produce IL-10, which is necessary for Treg-mediated immune suppression. The Journal of Immunology, 2011, 187: 118–125.

High mobility group box 1 (HMGB1), a small protein of 215 aa resides with extensive various posttranslational modifications, is a highly conserved protein in the nucleus, cytoplasm, or extracellular environment (e.g., released from cells via necrosis and autophagy, secreted from inflammatory or cancer cells) with multiple distinguished functions (e.g., binding/bending DNA to facilitate transcription factor assembly on site-specific DNA targets, promoting autophagosome, inducing cell death, acting as a signaling molecule to alert innate immunity) (18–28). Advanced glycation end products (RAGE), TLR2, TLR4, TLR9, and CD24 have been suggested to be the receptors of HMGB1 (26–28).

HMGB1 is highly expressed in tumor cells, and increased levels of HMGB1 in tumor cells are usually associated with a greater tumor angiogenesis, growth, invasion, and metastasis (23–28). HMGB1 released by tumor cells is involved with either antitumor or protumor effects under certain circumstances or models, or both (23–31). As an endogenous adjuvant, HMGB1, released from dying tumor cells after chemotherapy, virotherapy, or radiation therapy, promotes DC maturation and Treg Ag presentation via acting on TLR2 or TLR4, or activates innate immunity, thereby resulting in antitumor activity (29–31). As a tumor-promoting factor, tumor cell-derived HMGB1 enhances tumor angiogenesis, growth, invasion, and metastasis (23–29).

Although HMGB1 produced by tumor cells exhibits the inhibitory effect on DC in both mouse and human studies (32), it is largely unknown whether and how tumor cell-derived HMGB1 mediates tumor immune suppression. In this study, we examined the impact of tumor cell-derived HMGB1 on Treg and naturally acquired antitumor immunity.

Materials and Methods

Mice and tumor cell lines

BALB/c, BALB/c–IL-10−/−, (C.129P2(B6)–IL-10tm1Cgn/J), BALB/c– C.129S7(B6)–Il10tm1Tch/J, BALB/c–Foxp3-eGFP mice (C.3H–Foxp3tm1Rch/J), and C57BL/6 (B6) mice (female, 6–8 wk) were purchased from JAX and Taconic, and housed and bred in specific pathogen-free conditions in the University of Pittsburgh animal facility. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. Murine breast tumor 4T1.2-Neu (33), lung cancer 3LL (American Type Culture
IL-10 production by Treg

BALB/c or BALB/c-Foxp3−/−eGFP mice were s.c. inoculated with 4T1.2-Neu (1 × 10^5) in 20 μl endotoxin-free 1× PBS (Sigma) at the fourth mammary fat pad (33). B6 mice were s.c. inoculated with 3LL (1 × 10^5) at the left flank. After 3–4 wk, Treg were purified from splenocytes of tumor-bearing mice using mouse CD4^+CD25^+ regulatory T cell isolation kit according to the vendor’s instruction (Miltenyi Biotec) (34). In some experiments, Treg (eGFP^+) were sorted from splenocytes or single-cell suspensions of tumors using a BD FACSAria High Speed Cell Sorter (BD Biosciences). Purity of Foxp3 Treg was confirmed by flow cytometry and consistently resulted in >95%. Tumor cells (1 × 10^7/150 μl) were cultured 24 h and culture media were centrifuged at room temperature, 2000 rpm for 5 min, to obtain tumor cell supernatants. Treg (2 × 10^5) were cultured alone or with tumor cells (1 × 10^6) in 200 μl RPMI 1640 10% FBS or tumor cell culture supernatants, at 37°C, 5% CO_2 for 2 d. In some experiments, functional anti-HMGB1 Ab (anti-HMGB166-181 kindly provided by Dr. Michael T. Lotze, University of Pittsburgh; ab18256 purchased from Abcam) or rabbit IgG (10–20 μg/ml; eBioscience) was added. The concentration of IL-10 in the culture supernatant was determined by ELISA (BD Biosciences, eBioscience).

HMGB1 knockdown in tumor cells

HMGB1 small interfering RNA (siRNA; GCUGAAGAGCAGAAGAAATTTTCTTGCTCTTTTCAGCTTTTTGGAAG-3') was synthesized (IDT DNA). The annealed oligonucleotides including HMGB1 siRNA sequence (sense: 5'-GATCGGCTGTAAGAGCAGAAGAAATTTTCTTGCTCTTTTCAGCTTTTTGGAAG-3'; antisense: 5'-AGC-TCTCCTTAAAAAGCTTGGAAAAAGAAAATCTTTCATTTCTTCTCCTTCTACTGGA-3') were synthesized (IDT DNA). The annealed oligonucleotides were cloned into retroviral vector pTReoSuper (pRS, a generous gift from Dr. Joan Massague at Memorial Sloan-Kettering Cancer Center; resultant pRS-HMGB1 short hairpin RNA [shRNA]). Inserted shRNA was confirmed by DNA sequencing. DNA was purified using EndoFree Plasmid kits (Qiagen). 4T1.2-Neu or 3LL wild type (WT) were transfected with pRS-HMGB1 shRNA or pRS (vector control) using Lipofectamine 2000 (Invitrogen) and selected with puromycin (InvivoGen). Selected 4T1.2-Neu shRNA HMGB1 (4T1.2-Neu HMGB1 knockdown [KD]), 4T1.2-Neu vector control (4T1.2-Neu KD control), 3LL shRNA HMGB1 (3LL HMGB1 KD), or 3LL vector control (3LL KD control) were used in experiments. The equivalent amounts of proteins from cell lysate (20 μg) or tumor cell culture supernatants (50 μg) were loaded to confirm HMGB1 KD in tumor cell lysates or culture supernatants using Western blotting (WB) with rabbit anti-HMGB1 Ab (ab18256; primary Ab), goat anti-rabbit poly-HRP (secondary Ab; Cell Signaling Technology, Pierce), and ECL WB Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent Substrate (Pierce).

Tumor-specific CD8 T cell responses

BALB/c or BALB/c-Foxp3−/−eGFP mice were s.c. inoculated with 4T1.2-Neu WT, HMGB1 KD, or KD control (1 × 10^5). Day 21 or 60 after tumor inoculation, CD8 T cells were isolated from splenocytes of those mice (naive mice as non–tumor-bearing control) using anti-mouse CD8 microbeads according to the vendor’s instruction (Miltenyi Biotec). Purified CD8 T cells (4 × 10^5) were transduced with retroviral vector pTReoSuper (pRS, a generous gift from Dr. Joan Massague at Memorial Sloan-Kettering Cancer Center; resultant pRS-HMGB1 short hairpin RNA [shRNA]). Inserted shRNA was confirmed by DNA sequencing. DNA was purified using EndoFree Plasmid kits (Qiagen). 4T1.2-Neu or 3LL WT-bearing BALB/c-IL-10−/− mice, were adoptively cotransferred i.v. into naive BALB/c mice (3/group) on day −1. Those mice were inoculated s.c. with 4T1.2-Neu (1 × 10^5) on day 0. On day 5, CD4^+CD127− tumor-draining lymph node (TDLN) cells (4 × 10^5) were restimulated with mitomycin C (Sigma)-treated 4T1.2-Neu or CT26 (tumor-specific control; 4 × 10^5) in the presence of purified naive syngeneic splenic DC (8 × 10^5) in 200 μl RPMI 1640 10% FBS at 37°C, 5% CO_2 for 5 d. The concentration of IFN-γ in the culture supernatants was determined by ELISA (34).

Tumor challenge

BALB/c-IL-10−/−, –WT, or –IFN-γ−/− mice were s.c. inoculated with 1 × 10^5 4T1.2-Neu WT, HMGB1 KD, or KD control in 20 μl endotoxin-free 1× PBS at the fourth mammary fat pad on day 0. B6-WT mice (3–7/group) were s.c. inoculated with 1 × 10^5 3LL WT, HMGB1 KD, or KD control at the left flank on day 0. In some experiments, anti-mouse CD8 Ab (53-6-7; 200 μg/injection) were i.p. injected into WT mice on days −1, 1, 3, 6, and 9 to deplete CD8 T cells. CD8 T cell depletion was confirmed by flow cytometry and resulted in >95% reduction of CD8 T cells. Tumors were measured using digital slide calipers (Fisher Scientific) in the two perpendicular diameters every 2 d. Mice were dead naturally or sacrificed when tumor reached 10 mm in mean diameter (33–35).

FIGURE 1. Host cell-derived IL-10 inhibits naturally acquired CD8 T cell-dependent antitumor immunity. BALB/c-WT or –IL-10−/− mice (female, 4–5 wk; 2–5/group) were inoculated with 4T1.2-Neu. Because 13% of homozygotes BALB/c-IL-10−/− mice at 9 wk had adenocarcinomas and there was a 65% incidence rate of colorectal carcinoma at 10–31 wk, BALB/c-IL-10−/− mice bearing an inoculated 4T1.2-Neu with spontaneous adenocarcinomas or colorectal carcinoma were removed from these experiments. Nineteen BALB/c-IL-10−/− mice presented in this article were adenocarcinoma or colorectal carcinoma free, at least in the experimental period. Data are combined from four independent experiments. IL-10−/− versus WT or IL-10−/− + anti-CD8: p < 0.0001. Animal survival is presented using Kaplan–Meier survival curves.
Statistics

Data were statistically analyzed using Student t test (Graph Pad Prism, version 5). Data from animal survival experiments were statistically analyzed using log rank test (Graph Pad Prism, version 5). A p value <0.05 was considered to be statistically significant.

Results

Host cell-derived IL-10 inhibits naturally acquired CD8 T cell-dependent antitumor immunity

BABL/c mice s.c. inoculated with 4T1.2-Neu (1 × 10^5) at mammary fat pad bore a primary solid tumor at the site of injection and metastatic tumors at various distant organs, and were naturally dead or sacrificed within 3–4 wk (Fig. 1) (33–35). Although 4T1.2-Neu initially grew well in BALB/c-IL-10^-/- mice (data not shown), the primary tumors were eventually rejected in around 60% of mice (Fig. 1). Metastatic tumors were not found in those (primary) tumor-rejection mice (data not shown). Moreover, depletion of endogenous CD8 T cells abrogated the tumor rejection (Fig. 1). 4T1.2-Neu cultured in vitro did not produce detectable soluble IL-10 (Fig. 2A). The data suggest that, in this breast tumor model, host cell-derived IL-10 inhibits naturally acquired CD8 T cell-dependent antitumor immunity.

Treg-derived IL-10 is necessary for mediating immune suppression in vitro and in vivo

Treg from spleens of tumor-bearing mice (spleen-derived Treg) exhibited potent function in suppressing IFN-γ production by T cells stimulated by DC in vitro (Fig. 3A) (34). Because tumor-infiltrating Treg are postulated to suppress tumor-infiltrating CD8 T cells, the phenotype and function of Treg from tumors (tumor-derived Treg) were examined. Although tumor-derived Treg expressed less CD25, more membrane-bound CTLA-4, and comparable GITR compared with spleen-derived Treg (Supplemental Fig. 1A), their suppressive activity was comparable (Supplemental Fig. 1B). Spleen is the most feasible source of Treg; thus, spleen-derived Treg were used in most of the experiments in this study. To determine the role for Treg-derived IL-10 in mediating immune suppression, IL-10 signaling was blocked using functional anti–IL-10R Ab (rat IgG1 as isotype control). Blocking IL-10 signaling in the cell culture diminished Treg-mediated immune suppression in vitro (Supplemental Fig. 2). To confirm this observation, we obtained IL-10^-/- Treg from 4T1.2-Neu–bearing BALB/c–IL-10^-/- mice. As shown in Fig. 3A, IL-10^-/- Treg lost their suppressive function in vitro. To examine whether Treg-derived IL-10 is necessary for mediating immune suppression of tumor-specific CD8 T cell activation in vivo, we coadipotively transferred tumor-primed CD4 T cells and WT-Treg or IL-10^-/-

FIGURE 2. Tumor cell-derived HMGB1 is involved in promoting IL-10 production by Treg in vitro. A, Treg from 4T1.2-Neu-bearing mice were cultured alone or cocultured with tumor cells (4T1.2-Neu). Treg versus tumor cells + Treg: p < 0.0005. B, Treg from 4T1.2-Neu-bearing mice were cocultured with 4T1.2-Neu in the presence or absence of anti-HMGB1 Ab or rabbit IgG. Tumor cells + Treg versus tumor cells + Treg + anti-HMGB1: p < 0.0005; tumor cells + Treg versus tumor cells + Treg + rabbit IgG: NS. C, Treg from 4T1.2-Neu–bearing mice were cultured in 4T1.2-Neu culture supernatants. Treg versus supernatants + Treg: p < 0.05. D, Treg from 4T1.2-Neu–bearing mice were cultured in 4T1.2-Neu culture supernatants in the presence or absence of anti-HMGB1 Ab or rabbit IgG. Supernatants + Treg versus supernatants + Treg + anti-HMGB1: p < 0.05; supernatants + Treg versus supernatants + Treg + rabbit IgG: NS. Data (A–D) are represented as mean ± SEM. Results are combined from four (A) or five (B, C) independent experiments and representative of three (D) independent experiments with at least one to two mice per group in each experiment.
compared with IL-10
Treg were much more potent to suppress adoptively transferred ex vivo. WT-Treg exhibited a certain degree of suppressive function in vivo. WT-Treg versus IL-10 mice (3/group) were adoptively cotransferred with CD4 and WT-Treg or experiment. WT-Treg versus IL-10 dependent experiments with at least one to two mice per group in each 6
Data are represented as mean SEM and combined from three independent experiments. WT-Treg on day 2, BALB/c-WT mice adopted with CD4 alone served as positive control. On day 5, CD4 CD11c+ TDLN cells were restimulated by mitomycin C-treated 4T1.2-Neu or CT26 (tumor-specific control). As previously reported (35), adoptively transferred tumor-prime CD4 T cells induced tumor-specific CD8 T cell activation (Fig. 3B). Although IL-10−/− Treg exhibited a certain degree of suppressive function in vivo, WT-Treg were much more potent to suppress adoptively transferred CD4 T cell-induced, tumor-specific CD8 T cell activation in vivo compared with IL-10−/− Treg (Fig. 3B). The data indicate that Treg-derived IL-10 is necessary for mediating immune suppression in vitro and in vivo.

Tumor cell-derived HMGB1 is involved in promoting IL-10 production by Treg in vitro
Treg-derived IL-10 appears to be a protumor factor in 4T1.2-Neu growth (Figs. 1, 3). A lung cancer 3LL, which did not produce detectable soluble IL-10 in cell culture (Supplemental Fig. 2), grows rapidly in IL-10 transgenic mice, and T cell-derived IL-10 promotes its growth by suppressing both T cell and APC function (36, 37), suggesting that host cell-derived IL-10 is a protumor factor in 3LL progression. Both 4T1.2-Neu and 3LL did not secrete detectable IL-10 in cell culture (Fig. 2A, Supplemental Fig. 3). Treg isolated from 4T1.2-Neu- or 3LL-bearing mice secreted detectable (4T1.2-Neu) or undetectable (3LL) IL-10 in cell culture without stimulation, activation, or both (Fig. 2A, Supplemental Fig. 3). Tumor cells or tumor cell culture supernatants promoted Treg from tumor-bearing mice to produce IL-10 in vitro (Fig. 2A, 2C, Supplemental Fig. 3, data not shown). It has been reasoned that the increase of IL-10 production by Treg is caused by either tumor cell-stimulated Treg proliferation (number) or tumor cell-enhanced Treg capacity to produce IL-10 (function). Tumor cells did not stimulate Treg proliferation in vivo (data not shown). It seems that tumor cell-derived soluble factors promote tumor-associated Treg to produce IL-10. Unexpectedly, neutralization of HMGB1 signaling using functional anti-HMGB1 Ab (antiHMGB1,66–181 or ab18256; rabbit IgG as isotype control) dampened tumor cell- or tumor cell culture supernatant-promoted IL-10 production by Treg (Fig. 2B, 2D, Supplemental Fig. 3). The data suggest that tumor cell-derived HMGB1 is involved in promoting IL-10 production by Treg in vitro.

FIGURE 3. Treg-derived IL-10 is necessary for mediating immune suppression in vitro and in vivo. A, Treg obtained from 4T1.2-Neu-bearing BALB/c-WT (WT-Treg) or −IL-10−/− (IL-10−/−Treg) mice were cocultured with 4T1.2-Neu–primed CD4 T cells (CD4), 4T1.2-Neu–loaded naive syngenic splenic DC (DC), and naive syngenic CD8 T cells (CD8). Data are represented as mean ± SEM and combined from three independent experiments with at least one to two mice per group in each experiment. WT-Treg versus IL-10−/−Treg; p < 0.05. B, BALB/c-WT mice (3/group) were adoptively cotransferred with CD4 and WT-Treg or IL-10−/−Treg on day −1, and inoculated with 4T1.2-Neu on day 0. BALB/c-WT mice adoptively transferred with CD4 alone served as positive control. On day 5, CD4 CD11c+ TDLN cells were restimulated by mitomycin C-treated 4T1.2-Neu or CT26 (tumor-specific control) in the presence of purified naive syngenic splenic DC. Data are represented as mean ± SEM and combined from three independent experiments. WT-Treg versus IL-10−/−Treg; p = 0.005.

Tumor cell-derived HMGB1 does not affect tumor cell growth but uncovers naturally acquired CD8 T cell-dependent antitumor immunity
Tumor cell-derived HMGB1 promoted Treg to produce IL-10, which was important to inhibit naturally acquired CD8 T cell-dependent antitumor immunity (Figs. 1–3). Tumor cell-derived HMGB1, in both 4T1.2-Neu and 3LL models, may play a critical role in tumor progression as a protumor factor. Efficient siRNA-mediated HMGB1 KD in tumor cells did not affect the tumor cell growth in those models in vitro (Fig. 4A, Supplemental Fig. 4A–C). The growth of 4T1.2-Neu or 3LL HMGB1 KD in syngenic WT mice was comparable with 4T1.2-Neu or 3LL KD control or WT (Fig. 4B, Supplemental Fig. 4D). Although they initially grew well (Fig. 4B, Supplemental Fig. 4D), substantial 4T1.2-Neu or 3LL HMGB1 KD were eventually rejected (Fig. 4C, 4D, Supplemental Fig. 4E), suggesting that HMGB1 KD uncovers a naturally acquired antitumor activity leading to the effective tumor rejection. Importantly, endogenous CD8 T cell depletion or IFN-γ deficiency abrogated the observed tumor rejection (Fig. 4D, Supplemental Fig. 4E), implying that endogenous immune effectors mediate HMGB1 KD-associated tumor rejection in syngenic immune-component mice. The data demonstrate that HMGB1 KD does not impair tumor cell growth but uncovers naturally acquired CD8 T cell-dependent antitumor immunity.

HMGB1 KD uncovers a naturally acquired, long-lasting, tumor-specific, IFN-γ– or TNF-α–producing CD8 T cell response
To examine tumor-specific CD8 T cell responses, we inoculated BALB/c mice with 4T1.2-Neu WT, HMGB1 KD, or KD control. Day 21 or 60 (only 4T1.2-Neu HMGB1 KD-rejection mice survived at this time point) posttumor inoculation, CD8 T cells isolated from splenocytes of tumor-bearing mice (naive mice as non–tumor-bearing control) were restimulated with irradiated 4T1.2-Neu or CT26 (tumor-specific control)-pulsed naive syngenic CD8+ splenocytes. As shown in Fig. 5, HMGB1 KD uncovered a naturally acquired, long-lasting, tumor-specific, IFN-γ– or TNF-α–producing CD8 T cell response.
HMGB1 KD attenuates the ability of tumor cells to induce Treg

To determine the impact of HMGB1 KD in tumor cells on tumor-associated Treg frequencies and numbers in tumor-bearing mice, we s.c. inoculated BALB/c-WT mice (3–4/group) with 4T1.2-Neu WT, HMGB1 KD, or KD control. Two weeks posttumor inoculation, the frequencies and absolute numbers of Treg in spleen and TDLN were examined. HMGB1 KD reduced the ability of tumor cells to increase absolute numbers (not frequencies) of Treg in both TDLN and spleen (Fig. 6A, data not shown). Three weeks after tumor inoculation, the ability of those splenic Treg in suppressing splenic DC maturation was measured. Treg from 4T1.2-Neu HMGB1 KD-inoculating mice showed a reduced activity of suppressing the expression of CD80 or CD86 on DC and the production of IL-12 by DC compared with Treg from 4T1.2-Neu WT, HMGB1 KD, or KD control on day 0. Data are combined from six independent experiments. HMGB1 KD versus WT or KD control: p < 0.0001; WT versus KD control: NS. C, BALB/c-WT mice (2–5/group) were inoculated with 4T1.2-Neu WT, HMGB1 KD, or KD control on day 0. Data are combined from six independent experiments. HMGB1 KD versus WT or KD control: p = 0.0001; WT versus KD control: NS. D, BALB/c-WT or –IFN-γ−/− mice (3–5/group) were inoculated 4T1.2-Neu HMGB1 KD on day 0. Anti-mouse CD8 Ab were injected into some WT mice on days −1, 1, 3, 6, and 9. Data are combined from three independent experiments. WT versus CD8 depletion or IFN-γ−/−: p < 0.0001. Animal survival is presented using Kaplan–Meier survival curves.

Discussion

Overproduction of HMGB1, which occurs in various tumor cells, is associated with the hallmarks of cancer (e.g., tumor angiogenesis, growth, inflammation, invasion, and metastasis) (27, 38). HMGB1 plays multiple roles in either antitumor or protumor effects (23–31). Recombinant human HMGB1 induces a distinct form of cell death in cancer cells that may provide therapeutic benefits for cancer patients (39). HMGB1 released from dying tumor cells after chemotherapy, virotherapy, or radiation therapy, as a “danger” molecule, stimulates DC maturation and tumor Ag presentation via TLR2/4 or activates innate immunity, leading to an antitumor immune response (29–31). However, in the physiological condition (tumor progression), the role of HMGB1 in tumor immune suppression is largely unknown even though it has been reported that HMGB1 produced by tumor cells exhibits the inhibitory effect on DC in both mice and humans (32).

The observations in this work suggest that tumor cell-derived HMGB1 may suppress a naturally acquired immune effector cell (CD8)- or cytokine (IFN-γ)-dependent antitumor response, probably by enhancing tumor-associated Treg to produce IL-10, which is necessary for immune suppression in vitro and in vivo. Treg-derived IL-10 may dampen DC, CD4, or CD8 T cell function to diminish the priming of tumor-specific CD8 T cells. DC have been suggested to be the most relevant targets of Treg in vivo.
decommissioning of DC probably is a realistic mechanism underlying Treg-mediated immune suppression (40). The mechanisms underlying Treg-derived, IL-10–mediated suppression of the priming of antitumor CD8 T cells via a DC effect will be precisely dissected, such as using the tetramer analysis of antitumor CD8 T cell frequencies in future work.

IL-10–producing Treg have been shown to be highly suppressive (14). The in vitro data indicate that tumor cell-derived HMGB1 may act, as an extracellular signal, on tumor-associated Treg to promote IL-10 production for an enhanced suppressive functionality. Whether HMGB1-KD tumor immunity in vivo involves interference with IL-10–producing Treg needs to be investigated in future studies. In a burn injury model, massive HMGB1 released from burn injury has been suggested to activate Treg via RAGE to produce (detectable) IL-10 in vitro (41). It is possible that tumor cell-derived HMGB1 may interact with RAGE (or other putative HMGB1 receptors) on Treg to activate p38 MAPK, ERK1/2, and JNK, leading to the activation of transcriptional factors (AP-1 and NF-κB) for IL-10 production in Treg (19, 28). The exact intrinsic molecular pathway triggered by tumor cell-derived HMGB1 and whether anti-HMGB1 treatment alters the downstream signaling pathway need to be elucidated in future studies.

HMGB1 KD in tumor cells resulted in a CD8 T cell- or IFN-γ–dependent tumor rejection, clearly suggesting that HMGB1 KD-mediated antitumor activity in vivo is due to naturally acquired antitumor immunity but not modulation on tumor cells for death suggested in the in vitro prostate tumor cell study (42). HMGB1 KD may render tumor cell susceptibility to CTL-mediated killing via altering tumor phenotype. Because Treg depletion provoked antitumor immunity and tumor increased and activated Treg, which suppressed antitumor immunity (34, 35), attenuation of the ability of tumor cells to expand and activate Treg in vivo by HMGB1 KD may allow for CD8 T cells to operate in an unopposed manner leading to enhanced CD8 T cell responses. How HMGB1 KD reduce tumor cell capacity to induce Treg in vivo is still mysterious. It is possible that HMGB1 KD may modulate tumor cells to produce immune stimulatory molecules and/or to reduce immune suppressive factors.

The conflicting data shown in the literature and in this article on HMGB1 in tumor immune responses may be explained by the different sources of HMGB1 under different tumor cell conditions. HMGB1 released from dying tumor cells after chemotherapy, virotherapy, or radiation therapy may complex with soluble moieties in tumors (e.g., nucleic acids, microbial products, and cytokines) to exert its inflammatory properties (29–31). HMGB1 released (secreted) from tumor cells in the physiological condition (tumor progression) may not be able to do so or may be specifically modified posttranslationally (e.g., oxidation) to exhibit its ability to promote tumor invasion, metastasis, or immune tolerance (23–28, 43). Indeed, the state of oxidation of HMGB1 is critical in determining immune response versus nonresponsiveness (43).

RAGE-HMGB1 blockade by administration of soluble RAGE, anti-RAGE, and/or anti-HMGB1 Ab inhibits tumor growth and metastases (44). Because HMGB1 KD in tumor cells uncovered naturally acquired CD8 T cell-dependent antitumor immunity, we are actively investigating whether anti-HMGB1 treatment (e.g., intratumorally blocking HMGB1 signaling using: i) anti-HMGB1...
mAb (neutralizing HMGB1 signaling), ii) siRNA HMGB1 (HMGB1 KD), or iii) glycyrrhizin (a specific inhibitor of extracellular HMGB1) (inhibiting HMGB1) can be used therapeutically to rescue an antitumor CD8 T cell response to established tumors.

In summary, the data suggest a new function for tumor cell-derived HMGB1 in suppressing naturally acquired CD8 T cell-dependent antitumor immunity probably via promoting tumor-associated IL-10-producing Treg.

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Disclosures
The authors have no financial conflicts of interest.