Establishment of Tumor-Associated Immunity Requires Interaction of Heat Shock Proteins with CD91

Yu Jerry Zhou, Michelle Nicole Messmer, and Robert Julian Binder

Abstract

Host antitumor adaptive immune responses are generated as a result of the body's immunosurveillance mechanisms. How the antitumor immune response is initially primed remains unclear, given that soluble tumor antigens generally are quantitatively insufficient for cross-priming and tumors generally lack the classical pathogen-associated molecular patterns to activate costimulation and initiate cross-priming. We explored the interaction of the tumor-derived heat shock proteins (HSP) with their common receptor (CD91) on antigen-presenting cells (APC) as a mechanism for host-priming of T-cell–mediated antitumor immunity. Using targeted genetic disruption of the interaction between HSPs and CD91, we demonstrated that specific ablation of CD91 in APCs prevented the establishment of antitumor immunity. The antitumor immunity was also inhibited when the transfer of tumor-derived HSPs to APCs was prevented using an endogenous inhibitor of CD91. Inhibition was manifested in a reduction of cross-presentation of tumor-derived antigenic peptides in the lymph nodes, providing a molecular basis for the observed immunity associated with tumor development. Our findings demonstrate that early in tumor development, the HSP-CD91 pathway is critical for the establishment of antitumor immunity. Cancer Immunol Res; 2(3); 217–28. ©2013 AACR.

Introduction

In a majority of patients with cancer and in murine experimental tumor model systems, recognition of tumor by the host's immune system occurs, resulting in the priming of concomitant immunity and immunoediting (1–3). Conventional mechanisms of priming immune responses to pathogens do not apply to tumors because tumors generally lack sufficient antigens as native proteins for cross-priming (4, 5). In addition, being of self-origin, tumors in a sterile environment often lack the classical pathogen-associated molecular patterns (PAMP) for activating innate signals and costimulation necessary for priming T cells. Given the ability of endogenous, purified heat shock proteins (HSP) to prime specific immune responses under conditions of limiting antigen (5, 6), we tested the role of tumor-derived HSPs in situ and their receptor CD91 on antigen-presenting cells (APC) in the initiation of immune responses to tumors.

We have explored the HSP-CD91 axis as a mechanism for host-priming of antitumor immunity for two reasons: (i) antigens in the form of peptides are chaperoned by HSPs and are efficiently cross-presented by APCs (7–11). The increase in efficiency of cross-presentation of HSP-chaperoned peptides versus peptides alone is several thousand-fold and is made possible through the cell surface receptor CD91 on APCs (5, 12–16), and (ii) we have recently shown that HSPs signal through CD91 and activate APCs for costimulatory capacity based on the secretion of proinflammatory cytokines, including interleukin (IL)-1β, TNF-α, IL-6, and the improved expression of CD40, MHC II, and CD86 molecules (17–20). These observations explain the ability of six intracellular HSPs, gp96, hsp90, hsp70, calreticulin, hsp110, and grp170, to prime immune responses, specific for the peptides they chaperone in cells, once they have been purified from various antigen-bearing cells including tumors, pathogen-infected cells, allogeneic cells, and model antigen-expressing cells (6, 9, 11–14, 21–25).

The immunologic properties of HSPs make them prime candidates for the initiation of immune responses to tumors. However, HSPs are necessary for the survival of cells, so testing their requirement for priming tumor-specific immune responses in vivo through simultaneous or sequential deletion is not possible. Instead, we test their requirement by targeting and selectively deleting the HSP receptor CD91 in mice. This approach is possible because while structurally unrelated, four of the abundant and immunogenic HSPs, gp96, hsp90, hsp70, and calreticulin, utilize the common receptor CD91 to elicit their immune responses (12–14, 17). We show that, unlike wild-type mice, mice lacking CD91 expression on dendritic cells fail to elicit tumor-associated immunity. Antitumor immune responses can also be abrogated by the receptor-associated protein (RAP), an endogenous inhibitor of the HSP-CD91 pathway, which prevents exposed HSPs from binding to CD91. We show that endogenously expressed RAP inhibits the...
localization of HSPs in the draining lymph nodes, the uptake of HSPs by CD91 and the cross-presentation of HSP-chaperoned peptides. Our study demonstrates that the HSP-CD91 pathway is critical for the establishment of tumor-associated immunity.

Materials and Methods

Mice

Female BALB/c, C57BL/6, C.129S7(B6)-Rag1<sup>tm1Mom/J</sup> (rag<sup>−/−</sup> BALB/c), and B6.129(Cg)-Rag2<sup>tm1Wcol/J</sup> (rag<sup>2−/−</sup> C57BL/6), C57BL/6-Tg1CrlTcrb<sup>110Mjb/J</sup> (OT-1), B6.129P-Lp<sup>+/−</sup> Pec<sup>−/−</sup>BoyJ (CD45.1) mice were purchased from the Jackson Laboratory and housed in the animal facility at the University of Pittsburgh (Pittsburgh, PA). All experimental mice were 6- to 8-week-old. B6.129S7-Lrp<sup>fl/+</sup>/J (CD91<sup>fl/+</sup>) mice (Jackson Laboratory) were mated to homozygosity. CD91 conditional knockout mice were generated by crossing B6.Cg-Tg(Itgaxcre)-1-IRE2i/J (CD11c-Cre) with CD91<sup>△<sub>calc</sub>/fl</sup>/n mouse. Specific depletion of CD91 in CD11c<sup>−</sup> cells was confirmed by using immunoblot and flow cytometry. All experiments with mice were approved by the Institutional Animal Care and Use Committee and performed in compliance with its guidelines.

Cells and reagents

Tumor cell lines, lung carcinoma D122, fibrosarcoma CMS5, and Simian Virus 40 (SV40)-induced SVB6, were obtained from American Type Culture Collection (ATCC) and cultured in complete Dulbecco’s Modified Eagle Medium (DMEM), which includes 1% sodium pyruvate, 1% l-glutamine, 1% nonessential amino acids, 1% penicillin and streptomycin, 0.1% 2-mercaptoethanol, and 10% FBS (GIBCO). RAP or control vector-transfected tumor cells were cultured in complete DMEM media plus blasticidin (3 μg/mL, Invitrogen). Double-transfected tumor cells (gp96EGFP+RAP or gp96EGFP+control vector) were cultured in complete DMEM media plus blasticidin (3 μg/mL, Invitrogen) and geneticin (0.7 g/L, GIBCO). All tumor cell lines were free of specific pathogen, tested by IMPACT I PCR Profile (Research Animal Diagnostic Laboratory, RADIL). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Ovalbumin, complete Freund’s adjuvant, incomplete Freund’s adjuvant, lipopolysaccharide (LPS), and mitomycin-C (Research Animal Diagnostic Laboratory) were mated to homozygosity. BALB/c (or C57BL/6) mice were challenged with 1 × 10<sup>6</sup> RAP- or control vector-transfected CMS5 cells. Two weeks later, mice were challenged with 1 × 10<sup>6</sup> untransfected CMS5 cells and tumor growth was measured. In the EG7 tumor system, gp96 was purified from cultured cells as described (6). Mice were immunized intradermally with 1 μg gp96 twice a week apart and challenged with 5 × 10<sup>6</sup> EG7 tumor cells in PBS 1 week later. Tumor growth was measured on two axes and expressed as average tumor diameter.

T-cell proliferation assay

Endotoxin-free OVA was introduced into CMS5 cells expressing either RAP or control protein by electroporation at 200 V for 30 ms (Bio-Rad). The OVA-loaded cells were then rendered replication-incompetent by treatment with mitomycin-C. CD45.2<sup>+</sup> OT-1 cells were harvested from spleens, enriched for CD8<sup>+</sup> T cells (Miltenyi Biotec), and labeled with CFSE (Invitrogen). To test antigen-transfer from RAP-expressing tumor cells to APCs, BALB/c mice were immunized intradermally with titrated dose of mitomycin-C-treated, RAP+, or control vector-transfected CMS5 cells. Two weeks later, mice were challenged with 1 × 10<sup>6</sup> untransfected CMS5 cells and tumor growth was measured. In the E.G7 tumor system, gp96 was purified from cultured cells as described (6). Mice were immunized intradermally with 1 μg gp96 twice a week apart and challenged with 5 × 10<sup>6</sup> EG7 tumor cells in PBS 1 week later. Tumor growth was measured on two axes and expressed as average tumor diameter.
CD8+ T cells (Miltenyi Biotec), and labeled with CFSE (Invitrogen). CFSE-labeled OT-1 cells (2.5 × 10^6 cells/mouse) were transferred into CD91+/− or CD91+/+ mice via the retro-orbital route. One day later, recipient mice were immunized intradermally with 20 μg OVA 8 peptide emulsified in Complete Freund’s adjuvant. After 3 days, cells from the draining lymph nodes were harvested and stained with anti-CD8 antibody. CFSE dilution of the previously transferred OT-1 cells was examined.

**Microscopy**

Transfected tumor cells were cultured overnight in 35 mm glass bottom dishes (MatTek Corp.) at 37°C, washed with PBS, fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked with 2% bovine serum albumin (BSA). For RAP-transfected cells, mouse anti-V5 antibody and Cy3-conjugated goat anti-rat IgG antibody were used to stain HSP90; 4', 6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. For gp96-EGFP–transfected cells, rhodamine-conjugated Phalloidin (Invitrogen/Life Technologies) was used to stain the cytoskeleton; DAPI was used to stain nuclei, in accordance with a protocol developed by the Center for Biologic Imaging (University of Pittsburgh). Tumor tissues were incubated with 2% paraformaldehyde for 1 hour, then with 30% sucrose at 4°C overnight. After freezing, tumor sections were cut into 8-μm sections using a cryo-microtome (HM505E Microm). The sections were permeabilized with 0.1% TritonX100 and blocked with 2% BSA. Mouse anti-V5 antibody and Cy3-conjugated goat anti-mouse IgG antibody were used to stain RAP; rat anti-HSP90 antibody and DyLight488-conjugated goat anti-rat IgM antibody were used to stain HSP90. DAPI was used to stain the cytoskeleton; the DAPI was used to stain nuclei. All images were captured using an Olympus FV1000 inverted confocal microscope with ×100 objective (Figs. 3C and D and 6B) or 60× objective (Fig. 3K) and Fluoview v. 2.1 acquisition software. Imaris v. 7.2.1 (Bitplane) and Photoshop v. 7.0 (Adobe) were used for analysis and to prepare the images for publication.

**HSP transfer experiments in vivo**

A total of 5 × 10^6 CMS5 cells cotransfected either with RAP and gp96-EGFP or with control vector and gp96-EGFP, were injected into one footpad of wild-type BALB/c or CD91+/− mice. Two days later, the draining (dLN) and contralateral (nondraining; ndLN) popliteal lymph nodes were harvested. EGFP signal in CD45+ cells in the lymph nodes were compared. Because of minor variations in autofluorescence observed in each mouse and in the fluorescence of the gp96-EGFP–expressing cultured cells over time, the signal was normalized to these two parameters by accounting for background signal in the ndLN and the fluorescence of gp96-EGFP–expressing cells on the day of the experiment, respectively (Supplementary Fig. S3D–S3G). The following formula was used:

\[ \text{MFI (dLN for CMS5 – EGFP)} - \text{MFI (ndLN for CMS5 – EGFP)} \times 100\% \]

**Statistical analysis**

An unpaired two-tailed Student t test or a two-way ANOVA test was used for statistical analyses, and a P value of less than 0.05 was considered statistically significant. Error bars were calculated as SEM.

**Results**

**Selective loss of CD91 in CD11c+ cells renders mice unresponsive to gp96**

Using multiple approaches in vitro, we and others have demonstrated that CD91 serves as a receptor for immunogenic HSPs including gp96 (12–17). Conventional CD91 knockout mice are embryonically lethal (27); thus, we created mice lacking CD91 expression in CD11c+ cells, to test the role of CD91 in HSP-mediated immunogenicity in vivo. Mice with homozygous floxed CD91/LRP1 (CD91fl/fl) were crossed with mice expressing Cre-recombinase under the CD11c promoter. Lack of CD91 expression was confirmed in the bone marrow–derived dendritic cells (BMDC) of these mice, generated by culturing bone marrow cells in GM-CSF for 6 days and purified by CD11c expression to 96%. Cells were analyzed by SDS-PAGE and immunoblotting was done for CD91 protein expression (Fig. 1A). In addition, CD11c+ cells in the lymph nodes of CD91fl/fl/C2 mice were also able to respond to LPS by secreting IL-1α, in contrast to BMDCs from CD91+/+ mice. We next tested the ability of BMDCs from CD91+/− to mature was tested by pulsing these cells with LPS for 24 hours and monitoring the expression of several maturation markers. CD86, CD40, and MHC II were all upregulated in response to LPS, and the upregulation was to similar levels as those in BMDCs from CD91+/− littermates (Fig. 1D). BMDCs from CD91+/− mice were also able to respond to LPS by secreting IL-1β, measured by ELISA 24 hours later (Fig. 1E). Importantly, however, CD91+/− BMDCs failed to secrete IL-1β in response to gp96. Control BMDCs from CD91+/+ mice secreted IL-1β after stimulation with LPS or gp96, consistent with our previous observations (17, 18). We tested the ability of BMDCs to mature in response to gp96 as previously shown (28). BMDCs from CD91+/− mice failed to upregulate maturation markers CD86 and CD40 in response to incubation with gp96 (Fig. 1F), in contrast to BMDCs from CD91+/+ mice. We next tested the response of CD91+/− mice to gp96 immunization in a prototypical tumor-rejection assay (6, 14). CD91−/− or CD91+/+ mice were immunized with EG7-derived gp96. Mice were then
challenged with E.G7 and tumor growth was monitored. While tumors were rejected in gp96-immunized CD91⁺/⁺ mice (as observed routinely; refs. 6, 14), there was no tumor rejection in gp96-immunized CD91⁻/⁰/⁻ mice (Fig. 1G).

**Loss of CD91 expression in antigen-presenting cells abrogates tumor-associated immunity**

As a measure of tumor-associated immunity, we tested the rate of growth of tumors in CD91⁻/⁻ mice using the...
moderately immunogenic Lewis lung carcinoma D122. When mice were inoculated with $8 \times 10^3$ D122 cells, the tumor growth rate was significantly faster in CD91$^{-/-}$ recipient mice than in CD91$^{+/+}$ recipient mice (Fig. 2A). The significant difference in tumor growth rate was observed at early time points, up to day 11, with differences then dissipating. To test the role of adaptive immune response on tumor growth, we depleted CD8$^{+}$ T cells from CD91$^{-/-}$ or CD91$^{+/+}$ mice. In the absence of CD8$^{+}$ cells, D122 tumors grew with identical kinetics in mice from both groups (Fig. 2B). The rate of tumor growth in both groups was identical to CD91$^{-/-}$ mice in Fig. 2A. Therefore, CD8$^{+}$ T-cell priming was impaired in CD91$^{-/-}$ mice. To test the generality of these observations, similar experiments were performed with the highly immunogenic SV40-transformed regressor tumor SVB6. While SVB6 cells ultimately were rejected in both CD91$^{-/-}$ and CD91$^{+/+}$ mice, the tumors grew significantly larger and were rejected more slowly in CD91$^{-/-}$ mice (Fig. 2C). Because CD91$^{-/-}$ mice were deficient in their ability to mount antitumor immune responses, we tested their general competence in mounting immune responses with a regimen that bypasses a requirement for antigen uptake and intracellular processing. CD91$^{-/-}$ or CD91$^{+/+}$ mice were immunized with OVA8 peptide emulsified in CFA (week 1) or IFA (week 2). Supernatants from spleen cells cultured ex vivo with OVA8 peptide for 5 days were analyzed for IL-2. E, IL-2 ELISA was performed on supernatant of cultures. F, CFSE-labeled OT-1 cells were transferred to mice followed by immunization with OVA8 peptide in CFA. Lymph nodes were harvested on day 3. G, Percentage of dividing OT-1 cells in lymph nodes was measured by flow cytometry. n.s., not statistically significant; *, $P < 0.05$; **, $P < 0.01$. Experiments were independently performed twice with 3–5 (A, C, E, G) or 2 (B) mice per group.
plus adjuvant (Fig. 2F). Three days later, lymph nodes were harvested and the percentage of dividing OT-1 cells was quantified. OT-1 cells were observed to divide to similar levels following immunization of CD91−/− and CD91+/+ mice (Fig. 2G). These results are consistent with a normal immune phenotype, as depicted in Fig. 1C–E, and show that CD91−/− mice are capable of priming T-cell responses when immunized with peptide + adjuvant, a regimen that does not require CD91 for uptake.

**CD91–HSP interaction is required for priming tumor immunity**

CD91 is both an endocytic (12–15) and signaling receptor (17) for immunogenic HSPs. We next tested whether CD91 was required for its role in binding extracellular ligands by using the universal CD91-binding antagonist, RAP (29). RAP interacts with CD91 with high affinity and inhibits the binding of immunogenic HSPs (14). To determine whether abrogation of ligand-binding to CD91 was responsible for the decreased immune responses to tumors shown in Fig. 2, we expressed and quantified RAP in the moderately immunogenic fibrosarcoma CMS5 or SVB6 (Fig. 3A–D and Supplementary Fig. S1A–S1E). Tumors were determined to express approximately 10 fg of RAP per cell for both tumors. Tumors expressing RAP or a control (non-CD91 binding) protein were then implanted into BALB/c mice and tumor growth was monitored. In the tumor growth assay, RAP-expressing CMS5 grew with significantly faster kinetics when compared with control CMS5 tumor cells (Fig. 3E). However, when the same tumor cells were implanted into immunocompromised rag1−/− mice, there was no difference in growth rate between the RAP and non-RAP–expressing tumor cells (Fig. 3F). Since tumor growth rate is determined largely by the balance between tumor cell proliferation and lysis (mediated by immune effectors), we tested whether RAP transfection had any effect on cellular proliferation. There was no difference in growth rate between RAP and non-RAP–expressing cells either expressed RAP or a control protein (Fig. 3G). This number of cells was estimated to be loaded into BALB/c mice, however, the growth of tumors was comparable irrespective of RAP expression (Fig. 3H). In immunocompromised rag2−/− mice, however, the growth of tumors was comparable irrespective of RAP expression (Fig. 3I). Similar to the CMS5 model, SVB6 tumors proliferated comparably whether they expressed RAP or not (Fig. 3J).

The reduction of immunogenicity of RAP-transfected cells depends on the continual expression of RAP. Therefore, RAP expression in tumors was monitored throughout the duration of the experiment and was found to be preserved 15 days after implantation (Fig. 3K). These results highlight the role of CD91 as a receptor for ligand binding in mounting tumor-associated immunity.

**RAP abrogates antigen transfer in vivo and inhibits T cell priming**

To examine the transfer of antigen from tumor cells to APCs in the presence or absence of RAP in vivo, we rendered the transfected tumor cells replication incompetent and used them as a source of antigen. Mice were immunized with titrated doses of replication-incompetent CMS5 tumor cells expressing RAP or control protein (Fig. 4A). Two weeks later, mice were challenged with 1 × 106 wild-type CMS5 cells and tumor growth was monitored. There was no protection in either group at the 1,000 immunizing dose (Fig. 4B). However, when mice were immunized with 10,000 cells, RAP-expressing cells failed to protect mice from a subsequent tumor challenge whereas the control tumor cells were able to elicit protection (Fig. 4C). These results are consistent with RAP-expressing cells failing to transfer their antigen to APCs leading to inefficient cross-priming of T cells. At more than 1 × 105 immunizing cells, tumor cells expressing either protein were able to protect mice, suggesting that at higher antigen doses, CD91-independent mechanisms for antigen transfer to cross-prime APCs may come into play (Fig. 4D and E).

**Inhibition of antigen cross-presentation by RAP reduces T-cell proliferation in vivo**

We tested the ability of RAP to prevent antigen transfer by measuring levels of antigen presented to T cells in the draining lymph nodes. CFSE-labeled CD45.2+ OT-1 cells were adoptively transferred into naïve mice (CD45.1+) which were immunized a day later with 1 × 106 ovalbumin-loaded CMS5 tumor cells (Fig. 5A). This number of cells was estimated to be loaded with a total of approximately 1 ng of ovalbumin (Supplementary Fig. S2A and S2B). The ovalbumin-loaded CMS5 tumor cells either expressed RAP or a control protein. The CMS5 tumor is of the H-2b haplotype and cannot directly present the OVA8 peptide to OT-1 cells. Therefore stimulation of the OT-1 cells will be an indication of cross-presentation by resident APCs of the (H-2b, CD45.1+) C57BL/6 mouse. Lymph node cells were harvested after 3 days, and the percentage of dividing CD8+CD45.2+ cells was determined by flow cytometry. We show CFSE dilution in OT-1 cells from representative mice.
in Fig. 5B and C. In multiple mice (Fig. 5D), we show that the presence of RAP in the tumor cells accounted for a decrease in proliferation of OT-1 cells when compared with cells expressing a control protein. This is despite the fact that both cell types were loaded with exactly the same amount of OVA. In parallel experiments, CFSE-labeled OT-1 cells were transferred into CD91−/− mice 1 day before immunization with replication-incompetent, ovalbumin-loaded CMS5 cells (Fig. 5E) expressing RAP or an irrelevant protein. Lymph node cells were harvested after 3 days, and the percentage of all dividing CD8+ cells was determined by flow cytometry. Regardless of RAP expression, no OT-1 proliferation was observed in these CD91−/− mice. When mice were immunized with 10 times more OVA-loaded CMS5 cells (1×10^6, 10 ng), no difference was observed in dividing OT-1 cells when cells expressed RAP or a control protein (Supplementary Fig. S2C). This latter result was consistent with results in Fig. 4D and E.

**CD91 is required for uptake of immunogenic HSPs**

HSPs transfer antigen from tumor cells to APCs for cross-priming (5). To determine whether CD91 was essential for HSP-peptide cross-presentation in vivo, we developed an assay to monitor HSP transfer to APCs and the localization of the HSP in the lymph node. CMS5 cells were constructed to express gp96-EGFP (Fig. 6A and B). The amount of gp96-EGFP expressed in the CMS5 cells was equivalent to the levels of endogenous gp96 (Supplementary Fig. S3A and S3B). RAP or a control protein was simultaneously expressed in the gp96-EGFP-expressing cells. The two tumor types were implanted into individual foot pads of BALB/c mice, and the corresponding draining lymph nodes were harvested 2 days later. The popliteal lymph node cells were stained and gated on CD45 to identify hematopoietic cells and to exclude tumor cells that potentially drained directly from the foot pad, from the analysis (Supplementary Fig. S3C). The amount of EGFP fluorescence was measured by flow cytometry (Supplementary Fig. S3D and S3E). Because of minor variations in auto-fluorescence observed in each mouse and in fluorescence of the gp96-EGFP-expressing cultured cells (Fig. 6A), the signal was normalized to these two parameters by accounting for background signal in contralateral (nondraining) popliteal lymph nodes and fluorescence of gp96-EGFP-expressing cells on the day of the experiment, respectively (Supplementary Fig. S3D–S3G). Significantly less EGFP was detected in the lymph nodes when tumors expressed RAP compared with control protein (P = 0.0002; Fig. 6C). We tested the transfer of HSP to APCs in CD91−/− mice using the same system. CMS5 cells expressing gp96-EGFP with or without RAP were implanted in the foot pads of CD91−/− mice. Draining lymph node cells were harvested after 2 days and analyzed as shown in Fig. 6C. As shown in Fig. 6D, we did not detect gp96-EGFP signal in the lymph node regardless of whether the cells expressed RAP or not. These results highlight the importance of CD91 for HSP transfer and trafficking.

**Discussion**

Herein we report our finding that mice deficient in the receptor CD91 on APCs are unable to mount a substantial CD8+ T-cell-mediated immune response to tumors. In addition, tumors expressing molecules known to inhibit binding of
ligands to CD91 exhibited the same phenotype of decreased antitumor immunity. We demonstrate that the inhibition was due to the inability of APCs to cross-present antigens chaperoned by HSPs. To our knowledge, these findings are the first reports of a direct role for CD91 in antigen cross-priming in vivo. We have utilized a novel system we have developed in which the tumor and the induction of tumor-associated immunity did not require experimental manipulation in vitro.

When antigen is abundant, acquisition of antigens by APCs for cross-presentation and cross-priming can occur through several mechanisms as in most infectious diseases (30). These mechanisms are less reliant on the source of antigen or the type of APC (31). However, most tumor-associated antigens are derived from mutations of self-proteins and thus are likely to be limited in quantity at the time of the development of tumor-associated immunity (32). As previously demonstrated for transplantable tumors, concomitant immunity is established by day 3 when intradermal tumors are barely palpable (1). A careful quantification and titration of the amount of antigens in tumors available for cross-presentation is approximately six orders of magnitude less than is required if the antigen is transferred as an intact protein (5). This necessitates a special mechanism to efficiently transfer antigens from the bearing (tumor) cell to the cross-presenting cell. Here, we show that the HSP–peptide complex with its receptor CD91 provides such a mechanism.

This study is supported by a large body of work in vitro demonstrating the efficient cross-presentation of HSP-chaperoned peptides by a variety of CD91-expressing APCs (7–11, 28, 33). Our present study has made clear that upward titration of tumor cell-associated antigen renders the cross-presentation less dependent on the HSP-CD91 pathway. One possibility is through the utilization of alternative undefined HSP receptors (5). Recently, CD91 was also demonstrated to be a signaling receptor for the immunogenic HSPs allowing the initiation of signaling cascades necessary for secretion of various proinflammatory cytokines (17). As a single entity, the HSP–peptide complex is able to provide the dual signals of antigen and costimulation that are necessary for priming tumor-associated immunity.
Following priming of antitumor immunity, a period called immunoediting occurs during which the tumor may develop several mechanisms allowing for coexistence with the immune response (3, 34, 35). These mechanisms include induction of immunosuppressive factors, activation of regulatory cells, loss of antigen expression, or tumor-induced impairment of antigen presentation. As a mechanism of evasion of the immune response by tumors, preceding immunoediting, we hypothesize that tumors may overexpress RAP, or other as yet identified endogenous CD91 inhibitors, to impair antigen transfer. A previous study showed a correlation between elevated RAP levels and progressive disease in patients with colon cancer (36). We expect this mechanism to occur during tumor initiation because antigen burden is very low and HSP-mediated antigen uptake prevails and allows the tumor to be firmly established. We note that as tumors grow and tumor antigen becomes more abundant, other mechanisms of antigen transfer may be evoked. This is hinted at in Fig. 4 and supported by data in Supplementary Fig. S2C. These other mechanisms of antigen transfer may become dominant in cases of overexpressed self or oncoviral tumor antigens.

We used transplantable tumors in these studies because we were thus able to carefully titrate the input of tumors into mice, measure antigen-specific immune responses, and stably introduce CD91 inhibitors such as RAP into the tumors. However, the role of CD91 in de novo induction of tumors remains to be tested, as was performed for the dependence of antitumor immunity on IFN-γ (2). Currently, we are investigating carcinogen- and UV induction of tumors in CD91+/− mice. Key steps in spontaneously developed tumors or oncogenesis elicited by carcinogens or UV can be regulated by the immune system. Tumors that are established in CD91−/− mice, and harvested less than 6 to 8 days, are expected to be more immunogenic and rejected faster when transplanted into wild-type mice, when compared with tumors established in wild-type mice and transplanted into wild-type mice. We expect this because those early tumors established in CD91−/− mice are presumed to be less edited, if edited at all, due to a failure in the establishment of antitumor immunity in these mice.

The impact of the loss of expression of CD91 in CD11c+ cells raises two issues; first, CD91 is known to bind to other ligands (29), and of these, only αvM has been shown to have any immunologic function (37, 38). Thus although not tested here, αvM bound to extracellular tumor antigens may be another mechanism for cross-priming. We also do not know the relative contribution of each HSP known to bind CD91 to the global immune response, because only gp96 was measured here. Second, given the recent observation that CD169+ macrophages are bona fide professional APCs in the setting of apoptosis-associated antigen in lymph nodes (39), we expect a more robust phenotype when CD91 expression is eliminated in both macrophages and dendritic cells. Studies are currently under way create mice with loss of CD91 in both CD11c+ and CD11b+ cells. Our present study is supported by work from the Podack laboratory showing that enforced secretion of gp96 from tumor cells enhances the generation of antitumor CD8 T-cell responses. In those reports, CD11c+ cells were identified as the necessary APCs for the immune response (40, 41).

Our findings have a bearing on immunotherapy for cancer. A prevalent approach for cancer immunotherapy involves the isolation of tumor-specific T cells from the blood of...
patients, expanding them in vitro, and reinforcing these autologous, in vitro-expanded T cells back into the patient (42). Correlations of the absence of T cells and the expression of endogenous CD91 inhibitors by tumors should be firmly established.

Disclosure of Potential Conflicts of Interest

R.J. Binder is a named inventor of intellectual property that is being evaluated for commercialization of his invention. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y.J. Zhou, R.J. Binder

Development of methodology: Y.J. Zhou, R.J. Binder

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.J. Zhou, M.N. Messmer, R.J. Binder

Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): Y.J. Zhou, R.J. Binder

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Yu Jerry Zhou, Michelle Nicole Messmer and Robert Julian Binder


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