



## TUMORIGENESIS AND NEOPLASTIC PROGRESSION

# Generation of a Dual-Functioning Antitumor Immune Response in the Peritoneal Cavity

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Tumor cell metastasis to the peritoneal cavity is observed in patients with tumors of peritoneal organs, particularly colon and ovarian tumors. Following release into the peritoneal cavity, tumor cells rapidly attach to the omentum, a tissue consisting of immune aggregates embedded in adipose tissue. Despite their proximity to potential immune effector cells, tumor cells grow aggressively on these immune aggregates. We hypothesized that activation of the immune aggregates would generate a productive antitumor immune response in the peritoneal cavity. We immunized mice i.p. with lethally irradiated cells of the colon adenocarcinoma line Colon38. Immunization resulted in temporary enlargement of immune aggregates, and after challenge with viable Colon38 cells, we did not detect tumor growth on the omentum. When Colon38-immunized mice were challenged with cells from the unrelated breast adenocarcinoma line E0771 or the melanoma line B16, these tumors also did not grow. The nonspecific response was long-lived and not present systemically, highlighting the uniqueness of the peritoneal cavity. Cellular depletions of immune subsets revealed that NK1.1<sup>+</sup> cells were essential in preventing growth of unrelated tumors, whereas NK1.1<sup>+</sup> cells and T cells were essential in preventing Colon38 tumor growth. Collectively, these data demonstrate that the peritoneal cavity has a unique environment capable of eliciting potent specific and nonspecific antitumor immune responses. (*Am J Pathol* 2013, 183: 1318–1328; <http://dx.doi.org/10.1016/j.ajpath.2013.06.030>)

The peritoneal cavity is a unique immunologic environment that includes immune aggregates present in the peritoneal wall, mesentery, and omentum as well as free cells present in the peritoneal fluid.<sup>1,2</sup> This fluid, which mechanically acts to lubricate organ movement, also distributes a variety of immune subsets throughout the peritoneal cavity. The immune cells present in the peritoneal fluid are primarily macrophages and B cells but also include other lymphocyte and dendritic cell populations.<sup>3</sup> These free-floating immune cells have a dynamic relationship with the organized immune aggregates also present in the peritoneal cavity.<sup>4,5</sup> These structures contain immune cell subsets similar to those in the peritoneal fluid but in a highly organized manner, similar to many other tertiary immune structures.<sup>3,6,7</sup> One site of these immune aggregates, the omentum, is of particular interest because of the high density of aggregates found there.

The omentum is a thin adipose tissue located in the peritoneal cavity that is appreciated as a guardian of the peritoneal cavity, especially for its immunologic role in controlling

infections. For example, peritoneal dialysis, which can introduce bacteria into the cavity, leads to increases in the number and size of omental immune aggregates, which further increase on complications of peritonitis.<sup>8,9</sup> In addition, omental immune aggregates are the primary site of leukocyte extravasation in models of peritonitis.<sup>10,11</sup> Furthermore, bacteria are rapidly sequestered in the omentum shortly after introduction to the peritoneal cavity,<sup>12</sup> a process that slows bacterial dissemination throughout the peritoneal cavity.<sup>8</sup> Collectively, these data suggest that omental immune aggregates are capable of responding against foreign pathogens.

Similar to bacterial localization to the omentum, following tumor cell metastasis to the peritoneal cavity, the initial and most common site of tumor formation is the

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omentum.<sup>7</sup> Tumor cell metastasis to the peritoneal cavity is generally a poor prognostic indicator, and limited effective therapies are available to diagnosed individuals.<sup>13,14</sup> Omental metastasis is a common occurrence in individuals diagnosed as having cancers of the ovary and colon as well as other peritoneal organs.<sup>15,16</sup> It is specifically immune aggregates to which metastasizing cells originally bind and subsequently divide.<sup>7</sup> Tumor growth on the omentum is suggested to be a result of preferential binding to this site and the presence of factors that promote tumor growth.<sup>7,17,18</sup> After tumor formation on the omentum, tumor cells often further disseminate to other sites in the peritoneal cavity, as well as systemically, further propagating disease.

Despite data demonstrating the immune capabilities of the omentum,<sup>4,6</sup> the omental immune response to tumor metastasis is relatively understudied. Limited work shows that after cells adhere to the omentum, the vasculature of omental immune aggregates is well-suited to supporting rapid tumor growth. Under normal conditions, the vasculature of omental immune aggregates exhibits a phenotype that may be capable of rapid expansion after an immunologic stimulus, which is exploited by metastasizing tumor cells.<sup>7</sup> Despite the abundance of immune cells present at the site of tumor growth, a productive immune response does not occur naturally, and tumors grow progressively.<sup>3,19</sup>

In an attempt to determine whether the omental immune microenvironment is capable of promoting antitumor responses, we immunized mice with lethally irradiated tumor cells. Because the omentum is the initial site of tumor cell binding, *i.p.* immunization with these lethally irradiated tumor cells allows us to target the omentum to potentially generate an antitumor immune response. Herein, we found that *i.p.* immunization with lethally irradiated tumor cells led to the production of an antitumor immune response that was effective in controlling the growth of both specific and unrelated tumors after a secondary challenge with viable tumor cells. The nonspecific antitumor response was unique to the peritoneal cavity and was sustained for  $\geq 60$  days after immunization. In addition, depletion of NK1.1<sup>+</sup> cells reversed the protective effects elicited by immunization only when challenged with an unrelated tumor challenge. In contrast, depletion of NK1.1<sup>+</sup> cells and conventional T-cell populations was required to reverse the protective effects against specific tumor challenge. Thus, activation of peritoneal NK1.1<sup>+</sup> cells in addition to conventional T-cell populations may have potent antitumor capabilities that could be exploited to benefit patients therapeutically.

## Materials and Methods

### Mice and Cell Lines

C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129S2-Igh-6 tm1Cgn mice ( $\mu$ MT B cell KO) were a gift from Dr. Frances Lund (University of Rochester). All the mice were treated

following the guidelines for the humane treatment of animals as approved by the University of Rochester Committee on Animal Resources. Colon38, a murine colon adenocarcinoma, and E0771, a murine mammary adenocarcinoma, were gifts from Dr. Edward Brown (University of Rochester). The B16-F0 cell line was purchased from the ATCC (Manassas, VA). The Line1 cell line was a gift from Dr. John Yuhas (Oak Ridge National Laboratory) and the EMT6 line from Dr. Robert Sutherland (Ontario Institute for Cancer Research). Colon38/GFP, EMT6/GFP, and L1/GFP were generated by Lipofectamine reagent (Invitrogen, Grand Island, NY) transfection of parental lines with the pEGFP plasmid as previously described.<sup>7</sup> All the cell lines were tested using PCR-based testing (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO) and were found to be negative for a panel of mouse pathogens, including mycoplasma. The lines were maintained in MAT/P media (US patent No. 4.816.401) supplemented with 100 U/mL<sup>-1</sup> penicillin, 100 mg/mL<sup>-1</sup> streptomycin (Sigma-Aldrich, St. Louis, MO), and no fetal calf serum to avoid cross-reactive immune responses against serum proteins.

### Major Histocompatibility Complex Class I Analysis

B16, Colon38, and E0771 cells were plated at a concentration of  $2.5 \times 10^6$  cells/mL in 2 mL of MAT/P media. Cells were incubated for 72 hours in the presence of 20 ng/mL of mouse interferon gamma (IFN- $\gamma$ ) (PeproTech, Rocky Hill, NJ) or vehicle control. After IFN- $\gamma$  treatment, cells were trypsinized, stained with anti-H-2K<sup>b</sup> (clone AF6-88.5) for 1 hour, and analyzed by flow cytometry using a BD FACSCanto cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software version 7.6 (Tree Star Inc., Ashland, OR).

### Immunization and Tumor Challenges

C57BL/6 mice were immunized by injecting  $1 \times 10^6$  Colon38 cells treated with 100 Gy of ionizing radiation *i.p.* Mice were challenged 14 or 60 days after immunization with  $1 \times 10^5$  Colon38 cells,  $1 \times 10^5$  B16-F0 cells, or  $1 \times 10^6$  E0771 cells either *i.p.* or *i.m.* in the left thigh. Mice that had been challenged *i.p.* were sacrificed after 3 or 7 days, and omenta were processed for analysis by whole-mount histology, flow cytometry, and/or colony-forming assay as described below. Tumor growth in mice that had been challenged *i.m.* was monitored over time by measuring the mean thigh diameter as previously described.<sup>20</sup>

BALB/c mice were treated in a similar manner using  $5 \times 10^6$  irradiated EMT6 cells to immunize and  $1 \times 10^5$  EMT6/GFP or Line1/GFP cells to challenge.

### Whole-Mount Histologic Analysis and Image Processing and Analysis

Omenta were removed from mice after sacrifice and were stained with fluorescently conjugated antibodies as previously

described<sup>3,7</sup> or were left unlabeled. Briefly, whole omenta were mounted on glass slides and imaged using a fluorescence microscope equipped with a monochrome CCD digital camera.

To calculate tumor burden in mice that had been challenged with GFP-expressing tumor cells, images covering the entire omentum were captured and assembled to create a montage in bright field and GFP fluorescence. Image-Pro software version 5.0 (Media Cybernetics Inc., Rockville, MD) was used to determine percentage of tumor burden as follows: bright field compositions were used to designate the area of the omentum as the area of interest. Generated areas of interest were applied to GFP fluorescence compositions to calculate a percentage of area that is GFP positive.

The antibodies used were obtained from BD Biosciences or eBioscience Inc. (San Diego, CA) and included anti-CD4 (clone RM4-5), anti-CD8 $\alpha$  (clone 53-6.7), anti-CD19 (clone 1D3), anti-CD31 (clone MEC13.3), and anti-F4/80 (clone BM8).

### Flow Cytometric Analysis

Omenta were processed into single-cell suspensions by incubation of whole omenta in collagenase D (Sigma-Aldrich) for 40 minutes at 37°C with rotation. Samples were washed with HBSS (Sigma-Aldrich), counted, and blocked using BD Fc Block (BD Biosciences). Samples were stained for 1 hour. If intracellular staining was performed, cells were fixed with BD Cytotfix/Cytoperm (BD Biosciences) for 20 minutes, and then intracellular staining was performed for 1 hour. Samples were then analyzed using a BD FACSCanto cytometer (BD Biosciences) and FlowJo software version 7.6 (Tree Star Inc.).

The antibodies used were obtained from BD Biosciences or eBioscience Inc. and were anti-CD3e (clone 145-2C11), anti-CD4 (clones GK1.5 and RM4-4), anti-CD8 $\alpha$  (clone 53-6.7), anti-CD8 $\beta$  (clone H35-17.2), anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD19 (clone 1D3), anti-CD107a (clone 1D4B), anti-B220 (clone RA3-6B2), anti-F4/80 (clone BM8), anti-granzyme B (clone NGZB), anti-IFN- $\gamma$  (clone XMG1.2), anti-NK1.1 (clone PK136), anti-NKG2D (clone CX5), anti-NKp46 (clone 29A1.4), anti-perforin (clone eBioOMAK-D), and anti-tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (clone MP6-XT22).

### Colony-Forming Assays

Colony-forming assays were performed as previously described.<sup>21,22</sup> Briefly, omenta were removed from mice and single-cell suspensions were obtained by collagenase dissociation as described previously in *Materials and Methods*. Single-cell suspensions were then serially diluted in MAT/P media supplemented with 5% fetal calf serum (Hyclone Laboratories, Lakewood, NJ) and plated in triplicate in 60-mm tissue culture-treated plates. Plates were then incubated at 37°C for 7 to 10 days to allow for the formation of colonies and were enumerated using crystal violet. Samples in which no colonies were present were given a value of 1 for graphing purposes.

### In Vivo Cell Depletions

Antibody depletion using  $\alpha$ CD4 (clone GK1.5),  $\alpha$ CD8 (clone 53-6.7), or  $\alpha$ Thy1 (clone T24) was performed by i.v. injection of 200  $\mu$ g of antibody per mouse every 4 days starting 5 days before live tumor challenge. Antibody depletion using  $\alpha$ NK1.1 (clone PK136) was performed by i.v. injection of 500  $\mu$ g of antibody per mouse 5 days before live tumor challenge and 200  $\mu$ g of antibody per mouse 1 day before and 3 days after live tumor challenge. Depletion of macrophages was performed by i.p. injection of 200  $\mu$ L of 1 $\times$  clodronate liposomes<sup>23</sup> 4 and 2 days before live tumor challenge.

### Statistical Analysis

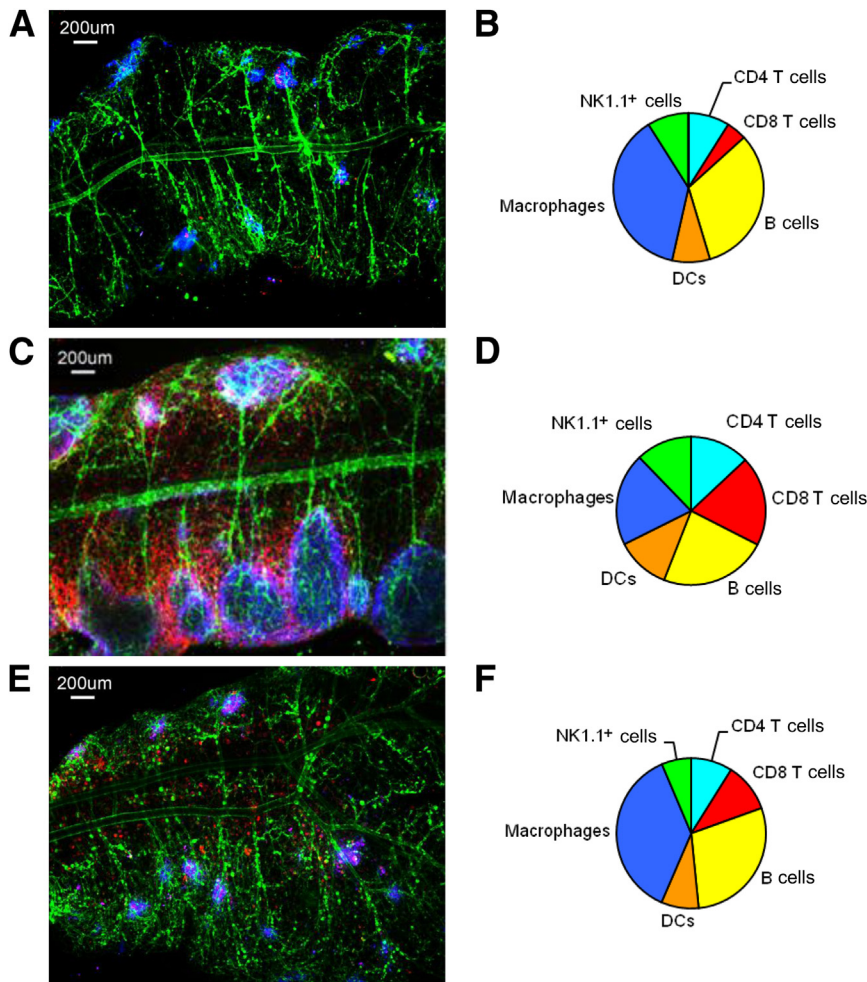
Data were plotted and statistical analysis was run using GraphPad Prism software version 4 (GraphPad Software Inc., San Diego, CA). Student's *t*-test or analysis of variance followed by a Bonferoni post-test was used where appropriate. *P* < 0.05 was considered significant.

## Results

### Intraperitoneal Immunization Results in Temporary Expansion of Omental Immune Aggregates

We hypothesized that immunization using lethally irradiated tumor cells would result in the induction of an antitumor immune response on the omentum. To assess the omental immune response to such an immunization, mice were injected i.p. with Colon38 cells that had been lethally irradiated with 100 Gy (10,000 rad) *in vitro*. At various time points after immunization, omenta were harvested for whole-mount histologic analysis. Naive immune aggregates were visible as clusters of lymphocytes and macrophages supported by dense vascular networks (Figure 1A). Enlarged immune aggregates were evident 7 days after immunization (Figure 1C), followed by a return to approximately naive size by day 14 (Figure 1E). In accordance with the visual increase in size, the number of total immune (CD45<sup>+</sup>) cells recovered per omentum was also increased at day 7 and subsequently reduced at day 14 (Table 1). Seven days after immunization, the time point when the density of omental cells was the highest, the weight of the omentum was also significantly elevated over that of naive omenta and omenta 14 days after immunization (Table 1).

Omenta were also analyzed by flow cytometry to better classify the immune cell types on the omentum that were responding to immunization. Representative flow cytometry panels are shown in Supplemental Figure S1. Naive immune aggregates are composed primarily of macrophages and B cells, with smaller populations of T cells, NK1.1<sup>+</sup> cells, and dendritic cells (Figure 1B). All immune cell subsets expand 7 days after immunization; however, at this time point, CD8<sup>+</sup> T cells, followed by CD4<sup>+</sup> T cells, constitute a proportionally larger fraction of immune cells compared with naive omenta



**Figure 1** Omental immune aggregates showed evidence of an active immune response after i.p. immunization with lethally irradiated Colon38. Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells and were sacrificed 7 or 14 days after immunization. Omenta were harvested from naive mice (**A**) or from mice 7 days (**C**) or 14 days (**E**) after immunization and were stained with  $\alpha$ CD31 (green),  $\alpha$ F4/80 (red), and  $\alpha$ CD4/ $\alpha$ CD8/ $\alpha$ CD19 (blue) and were analyzed by whole-mount microscopy. Single-cell suspensions were then stained for flow cytometry to distinguish major immune cell subsets present on the omenta of naive mice (**B**) or mice 7 days (**D**) or 14 days (**F**) after immunization. DC, dendritic cell. Data are representative of two experiments ( $n = 4$  to 5 mice per group).

(Figure 1D). Similar to immune aggregate size and cellularity, by day 14, the immune cell composition of immunized omenta had returned to approximately naive levels, except for CD8<sup>+</sup> T cells, which remained significantly increased (Figure 1F). Thus, immunization with lethally irradiated tumor cells results in temporary enlargement of omental immune aggregates, which contain increased proportions of CD8<sup>+</sup> T cells.

### Immunization Prevents the Growth of Both Specific and Unrelated Tumors on the Omentum

To determine whether the immune response generated by immunization is capable of mediating an effect on tumor

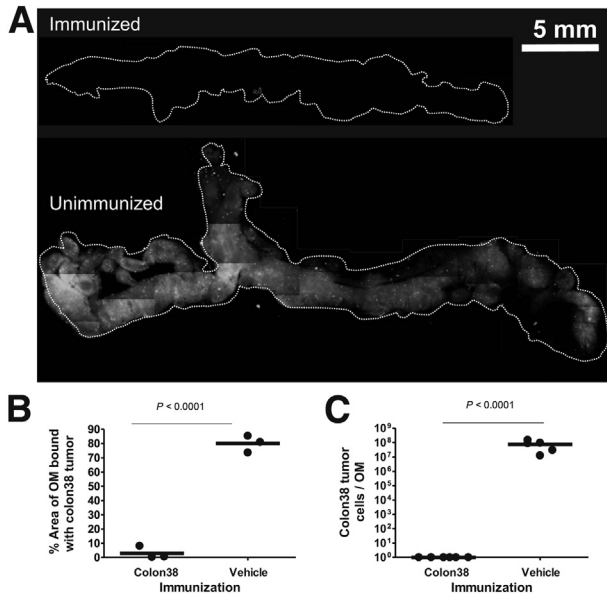
**Table 1** Immune Aggregates Are Temporarily Enlarged after Immunization

Days after immunization	CD45 <sup>+</sup> cells per omentum	Omental weight (mg)
0 (naive)	$3.5 \pm 1.7 \times 10^5$	$11.6 \pm 3.2$
7	$26.0 \pm 6.3 \times 10^5$ *	$29.6 \pm 7.5$ *
14	$7.1 \pm 3.6 \times 10^5$	$11.9 \pm 4.7$

Data are given as means  $\pm$  SD.

\* $P < 0.001$  compared with naive as determined by one-way analysis of variance followed by a Bonferroni posttest.  $n = 4$  to 8 mice per group.

growth, we challenged immunized mice i.p. with live Colon38 cells. Mice were challenged 14 days after immunization, a time when the omentum is no longer at the peak of the initial immune response. This time point was chosen to avoid analyzing the response generated by the temporary inflammation caused by immunization with lethally irradiated cells. Mice were sacrificed 7 days after challenge, and tumor growth was assessed by two different methods. First, mice were challenged with GFP-expressing Colon38 (C38/GFP), and tumor burden was assessed by fluorescence microscopy as described in *Materials and Methods*. Omenta harvested from immunized mice did not exhibit the large tumor burden that was present on omenta from mice receiving a vehicle immunization (unimmunized) and exhibited very little GFP fluorescence, indicating little to no tumor burden (Figure 2, A and B). Thus, immunization prevented the growth of Colon38. To complement the whole-mount method, we also examined tumor burden by colony-forming assay. Similar to the fluorescence data, we detected viable tumor cells on the omentum 7 days after challenge, whereas unimmunized omenta contained abundant tumor cells (Figure 2C). These data indicate that i.p. immunization with lethally irradiated tumor cells results in an effective antitumor response capable of preventing tumor growth on the omentum.



**Figure 2** Immunization with lethally irradiated Colon38 prevented the growth of Colon38 on the omentum (OM) after tumor challenge. Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells or were treated with vehicle and challenged i.p. 14 days later with  $1 \times 10^5$  Colon38/GFP (**A** and **B**) or Colon38 (**C**). OM were harvested 7 days after challenge. Whole OM were imaged by whole-mount fluorescence microscopy (**A**), and the percentage area of the OM that was GFP<sup>+</sup> was determined (**B**). **C**: OM were also collagenase digested into single-cell suspensions, and tumor burden was determined by colony-forming assay. Each dot represents an individual mouse, and lines indicate the mean (**B** and **C**). Statistical significance was determined by Student's *t*-test. Data are representative of four experiments ( $n = 3$  mice per group).

To analyze the specificity of the antitumor response after immunization with lethally irradiated tumor cells, mice that had been immunized with Colon38 were challenged with an alternative syngeneic tumor cell line: either E0771 (a spontaneously arising mammary carcinoma) or B16.F0 (a spontaneously arising melanoma) (**Figure 3A**). Regardless of which tumor was used to challenge, mice that had been immunized with Colon38 were capable of rejecting E0771 and B16 (**Figure 3, B and C**). To determine whether this lack of specificity was unique to C57BL/6 mice immunized with Colon38, we repeated the immunization and challenge protocol in BALB/c mice using EMT6 (a mammary carcinoma) to immunize and EMT6/GFP or Line1/GFP (a spontaneous lung carcinoma) to challenge mice (**Figure 3D**). Similar to immunization with Colon38, mice that were i.p. immunized with lethally irradiated EMT6 could prevent the growth of specific (EMT6) and unrelated (Line1) tumor on the omentum (**Figure 3, E and F**). Thus, we could demonstrate nonspecific tumor protection in two different strains of mice against tumors of varying origin.

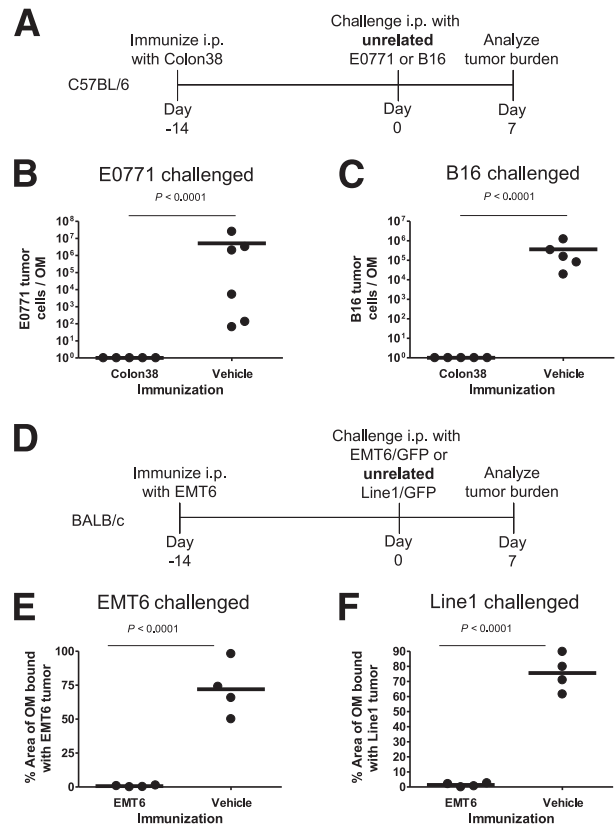
### Specific and Unrelated Tumor Control Still Exists at Least 60 Days after Immunization

We next examined the longevity of the antitumor response after immunization. If the specific and/or nonspecific antitumor responses resulted in the development of immunologic

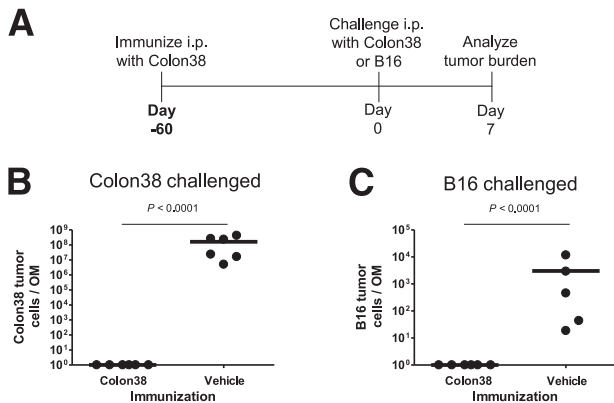
memory, we would expect that mice challenged at a later time point would still fail to develop tumor growth on the omentum. Thus, mice were immunized and rested for 60 days instead of 14 days and then were challenged with either Colon38 (specific) or B16 (unrelated) tumor cells (**Figure 4A**). As expected, mice that were challenged with Colon38 60 days after immunization could still prevent tumor growth on the omentum (**Figure 4B**). Even after 60 days, mice that were challenged with an unrelated tumor challenge (B16) also could prevent tumor growth on the omentum (**Figure 4C**). Antitumor responses were also observed when mice were challenged with E0771 60 days after immunization (data not shown). These data demonstrate that the nonspecific antitumor response is not transient and is, indeed, long-lived.

### Protection against Unrelated Tumor Challenge Is Not Systemic

To determine whether the nonspecific antitumor response is localized to the peritoneal cavity or whether it is a systemic



**Figure 3** Immunization prevented the growth of unrelated tumors on the omentum (OM) of C57BL/6 and BALB/c mice. C57BL/6 mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells (**A**) or were treated with vehicle and challenged i.p. 14 days later with  $1 \times 10^5$  E0771 cells (**B**) or  $1 \times 10^5$  B16 cells (**C**). OM were harvested 7 days after tumor challenge, and tumor burden was assessed by colony-forming assay. BALB/c mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated EMT6 cells (**D**) or were treated with vehicle and challenged i.p. 14 days later with  $1 \times 10^5$  EMT6/GFP cells (**E**) or  $1 \times 10^5$  Line1/GFP cells (**F**). Each dot represents an individual mouse, and lines indicate the mean (**B, C, E, and F**). Statistical significance was determined by Student's *t*-test. Data are combined from two experiments ( $n = 2$  to 3 mice per group).



**Figure 4** Specific and nonspecific tumor immunity was long-lived. **A:** Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells or were treated with vehicle and challenged i.p. 60 days later with  $1 \times 10^5$  Colon38 cells (**B**) or  $1 \times 10^5$  B16 cells (**C**). Omenta (OM) were harvested 7 days after tumor challenge, and tumor burden was assessed by colony-forming assay. Each dot represents an individual mouse, and lines indicate the mean (**B** and **C**). Statistical significance was determined by Student's *t*-test. Data are combined from two experiments ( $n = 2$  to 3 mice per group).

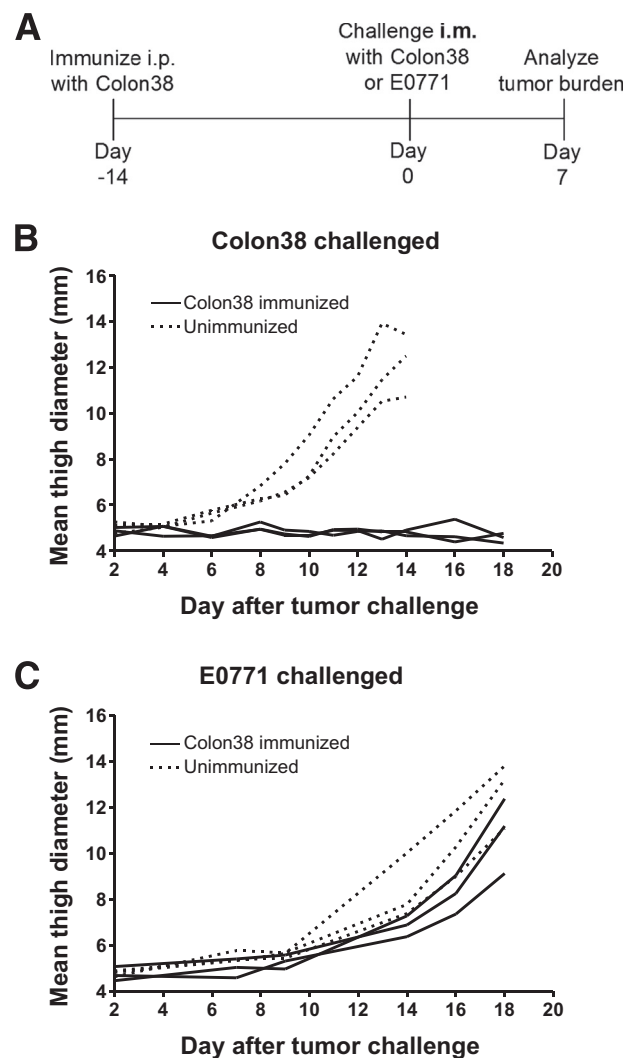
effect, mice were immunized i.p. with irradiated Colon38 as before and were challenged i.m. in the hind flank with either Colon38 or E0771 (Figure 5A). Tumor growth was then monitored over time by measuring mean thigh diameter. As expected, mice that had been immunized and challenged with Colon38 had no measureable tumor growth in the thigh (Figure 5B), indicating a systemic immune response. Unlike the results in the peritoneal cavity, mice that were challenged with unrelated E0771 i.m. developed large tumors regardless of whether they were immunized (Figure 5C). These data indicate that whereas the specific antitumor response after immunization is systemic, the nonspecific antitumor response is not.

### NK1.1<sup>+</sup> Cells Are Necessary to Mediate Unrelated Tumor Protection

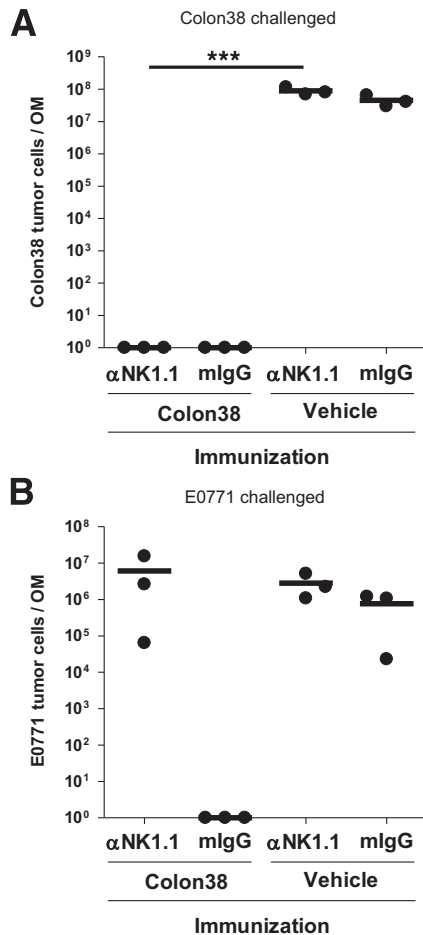
We next sought to determine which cell type(s) was responsible for preventing unrelated tumor growth on the omentum after immunization. We focused on the natural killer (NK) and NK T (NKT) cell populations owing to their reported antitumor functions.<sup>24–26</sup> Therefore, we depleted NK and NKT cells with anti-NK1.1 just before challenge with Colon38 or E0771 (during the effector phase). This procedure routinely resulted in 80% knockdown of NK1.1<sup>+</sup> cells on the omentum. In mice challenged with unrelated E0771, we observed that depletion of NK1.1<sup>+</sup> cells reversed the protective effects of immunization, and tumor growth was apparent on the omentum (Figure 6B), demonstrating that NK1.1<sup>+</sup> cells are necessary during the effector phase to prevent the growth of unrelated E0771 tumor after immunization. Flow cytometry analysis of H-2K<sup>b</sup> surface expression demonstrated that the tumor cell lines used express major histocompatibility complex class I at varying levels that can

be increased in the presence of an inflammatory environment, such as exposure to IFN- $\gamma$  (Supplemental Figure S2), suggesting that NK cell recognition is not due to lack of major histocompatibility complex class I expression. Mice that were challenged with specific Colon38 had no omental tumor burden regardless of the presence of NK1.1<sup>+</sup> cell populations (Figure 6A). This indicated that although NK1.1<sup>+</sup> cells may be playing a role in tumor prevention nonspecifically (as demonstrated in E0771-challenged mice), on their own, NK1.1<sup>+</sup> cells were not necessary to prevent the growth of specific Colon38 and that another cell population(s) was equally effective in controlling specific tumor growth.

Because specific tumor control was still apparent after depletion with anti-NK1.1, we hypothesized that i.p. immunization with lethally irradiated tumor cells was inducing a specific and nonspecific antitumor immune response.



**Figure 5** Nonspecific tumor immunity was not found peripherally. Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells (**A**) or were treated with vehicle and challenged i.m. in the hind flank 14 days later with  $1 \times 10^5$  Colon38 cells (**B**) or  $1 \times 10^6$  E0771 cells (**C**). Tumor growth was monitored over time by measuring mean thigh diameter. Data are representative of two experiments ( $n = 3$  mice per group).



**Figure 6** NK1.1<sup>+</sup> cells were necessary to prevent the growth of E0771 after immunization. Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells or were treated with vehicle. Before challenge, NK and NKT cells were depleted using  $\alpha$ NK1.1. Mice were then challenged i.p. 14 days after immunization with  $1 \times 10^5$  Colon38 cells (**A**) or  $1 \times 10^6$  E0771 cells (**B**). Omenta (OM) were harvested 7 days after tumor challenge, and tumor burden was assessed by colony-forming assay. Each dot represents an individual mouse, and lines indicate the mean. Statistical significance was determined by two-way analysis of variance followed by a Bonferroni posttest. Data are representative of two experiments ( $n = 3$  mice per group). \*\*\* $P < 0.001$ .

Depletion of macrophages (70% depletion) did not alter tumor protection against specific or unrelated tumor challenges (Supplemental Figure S3, A and B). Furthermore, immunized  $\mu$ MT B cell KO mice were capable of preventing tumor growth, to the same extent as wild-type mice (Supplemental Figure S3, C and D), although in unimmunized  $\mu$ MT B cell KO mice, tumors grew progressively. These mice exhibited a 99% reduction in the number of omental CD19<sup>+</sup> B cells (data not shown). Previous reports looking at immunization with lethally irradiated tumor cells outside the peritoneal cavity demonstrated that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are necessary for specific antitumor immunity.<sup>27</sup> Depletion of either CD4<sup>+</sup> (75% depletion) or CD8<sup>+</sup> (70% depletion) T cells alone did not alter the protective effect of immunization, and no tumor growth was detected on the omentum after specific tumor challenge (Supplemental Figure S3, E and F); simultaneous depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells using

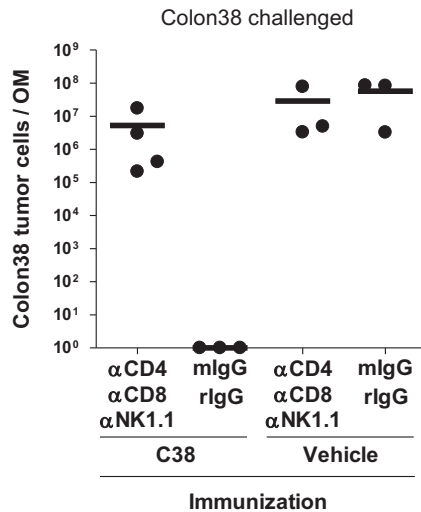
anti-Thy1 had no effect on specific tumor growth (Supplemental Figure S3G). Depletion using anti-Thy-1 resulted in 70% to 80% knockdown of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and no depletion of NK1.1<sup>+</sup> cells. In addition, depletion of either CD8<sup>+</sup> and NK1.1<sup>+</sup> cells (Supplemental Figure S3H) or CD4<sup>+</sup> and NK1.1<sup>+</sup> cells (Supplemental Figure S3I) did not result in tumor growth after immunization. It was only following depletion of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and NK1.1<sup>+</sup> cells that specific tumor growth was observed in immunized mice (Figure 7).

Because an NK1.1<sup>+</sup> cell population was capable of preventing the growth of both specific and unrelated tumor challenges, we further examined the possible role for NK cells in preventing tumor growth after immunization. We examined expression of the NK cell activating ligand NKG2D on omental NK cells after i.p. immunization with lethally irradiated Colon38 and challenge with unrelated E0771 (Supplemental Figure S4A). Mice that had been immunized, regardless of challenge or not, exhibited increased NK cell expression of NKG2D compared with NK cells from naive mice. In addition, challenge alone did not increase NK cell expression of NKG2D (Supplemental Figure S4, B and C). These data are consistent with a possible role for the activating ligand NKG2D in preventing tumor growth after immunization.

### Omental NK Cells Are Unique

We hypothesized that because the NK1.1<sup>+</sup> cell-dependent, nonspecific antitumor response seemed to be localized to the peritoneal cavity and omentum that omental NK cells may have a uniquely activated phenotype. Thus, we examined the basal expression of various activation and effector proteins on splenic and omental NK cells from naive mice. Omental NK cells expressed higher levels of the natural cytotoxicity receptor Nkp46 (Supplemental Figure S5A). In addition, omental NK cells had higher surface expression of the endosomal marker CD107a (Supplemental Figure S5B). Surface expression of CD107a indicates cellular secretion, suggesting that more omental NK cells may secrete higher levels of effector proteins basally. Thus, we also examined the basal intracellular expression of cytokine effector proteins and cytolytic effector proteins. A higher percentage of omental NK cells expressed TNF- $\alpha$  (Supplemental Figure S5C) and IFN- $\gamma$  (data not shown) compared with splenic NK cells. In contrast, omental and splenic NK cells expressed similar levels of the cytolytic proteins granzyme B (Supplemental Figure S5D) and perforin (data not shown). These data suggest that omental NK cells may be more prone to cytokine secretion basally. Consistent with this hypothesis, the omentum contains a substantial population of CD11c<sup>+</sup>B220<sup>+</sup> NK cells (Supplemental Figure S5E), which have been identified to be high cytokine producers in systems of viral infection.

Collectively, this indicates that in the peritoneal cavity, immunization induces a unique NK1.1<sup>+</sup> cell-mediated response that is capable of broadly preventing the growth of both specific and unrelated tumors potentially by increased



**Figure 7** T cells and NK1.1<sup>+</sup> cells were necessary to prevent the growth of specific Colon38 after immunization. Mice were immunized i.p. with  $5 \times 10^6$  lethally irradiated Colon38 cells or were treated with vehicle. Before challenge, mice were treated with a combination of  $\alpha$ CD4,  $\alpha$ CD8, and  $\alpha$ NK1.1. Mice were then challenged i.p. 14 days after immunization with  $1 \times 10^5$  Colon38 cells. Omenta (OM) were harvested 7 days after tumor challenge, and tumor burden was assessed by colony-forming assay. Each dot represents an individual mouse, and lines indicate the mean. Statistical significance was determined by two-way analysis of variance followed by a Bonferroni posttest.

signaling through the activating ligand NKG2D. In addition, immunization also induces a CD4<sup>+</sup>/CD8<sup>+</sup> T-cell-mediated response that is capable of controlling the growth of specific Colon38 but not unrelated E0771.

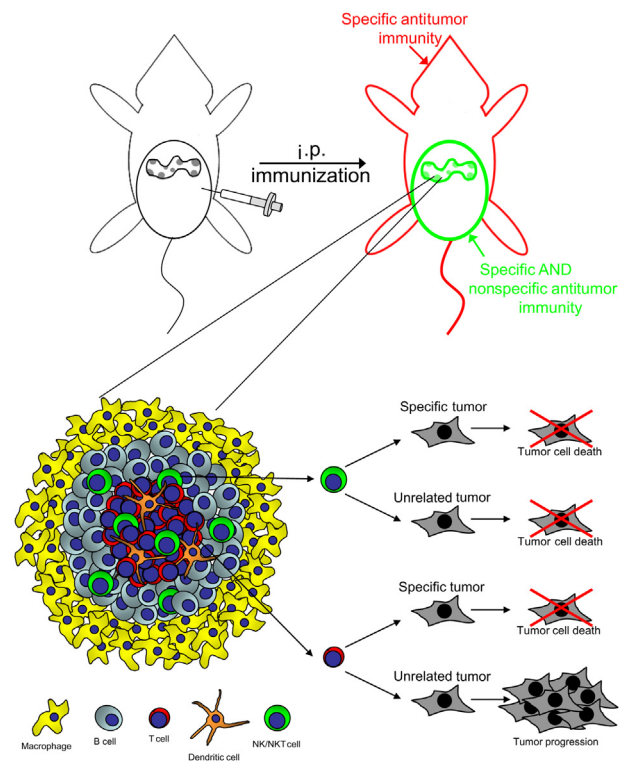
## Discussion

Herein, we generated an antitumor immune response that involves a peritoneal, nonspecific, NK1.1<sup>+</sup> cell-mediated response and a systemic, specific, T-cell-mediated response (Figure 8). The nonspecific immune response seems to be localized to the peritoneal cavity and is not effective against tumor challenge at a distal site. In contrast, the specific immune response generated by immunization in the peritoneal cavity is capable of preventing tumor growth locally on the omentum and systemically, as observed by i.m. challenge. In addition, different cell populations mediate the specific and nonspecific immune responses to these tumor challenges. Depletion of NK1.1<sup>+</sup> cells alone results in only the growth of the unrelated tumor challenge, demonstrating that another cell type(s) is capable of controlling the growth of the specific tumor challenge. To observe specific tumor growth after immunization, it is necessary to deplete the effector cells of both the specific and nonspecific responses. Thus, it is only following depletion of CD4<sup>+</sup>, CD8<sup>+</sup>, and NK1.1<sup>+</sup> cells that Colon38 grows in immunized mice.

Although we demonstrated that NK1.1<sup>+</sup> cells are required to mediate this nonspecific antitumor immune response in the peritoneal cavity, it is unclear whether NK1.1<sup>+</sup> cells

themselves in the peritoneal cavity are distinctive or whether another cell type exclusive to the peritoneal cavity is capable of inducing this nonspecific response in NK1.1<sup>+</sup> cells. The peritoneal cavity is enriched for many unique cell populations that may be involved in or influencing activation of these NK1.1<sup>+</sup> cells. This is of particular interest when considering that the studies looking at various cellular depletions focused on depletion only during the challenge phase and not when the initial immune response to immunization was greatest. For example, a larger portion of T cells in the peritoneal cavity express markers indicative of activation, such as CD44 and CXCR3, compared with other lymphoid organs.<sup>28,29</sup> These activated T cells produce increased levels of cytokines, including IFN- $\gamma$ , following T cell receptor stimulation, which, in this model, could account for the activation of NK1.1<sup>+</sup> cells to elicit antitumor responses. This hypothesis is consistent with published data by Tietze et al,<sup>30</sup> who demonstrated that immunotherapy ( $\alpha$ CD40/IL-2) results in T-cell activation and a reduction in tumor growth even when T cells cannot recognize tumor cells.<sup>30</sup>

Previous reports have found that the antitumor response induced by lethally irradiated tumor cells outside the peritoneal cavity depends on T cells.<sup>27</sup> These same studies,



**Figure 8** Intraperitoneal immunization resulted in local antitumor immunity that can prevent the growth of specific and unrelated tumor challenges (green) as well as systemic antitumor immunity that is capable of preventing the growth of specific tumor challenge (red). In the peritoneal cavity, the omentum contains populations of immune cells localized in immune aggregates that are necessary for both specific and nonspecific antitumor immunity. After immunization, NK1.1<sup>+</sup> cells were capable of preventing the growth of specific and unrelated tumors, whereas T cells were capable of preventing the growth of a specific tumor challenge only.



however, did not demonstrate a critical role for NK cells. In the present model of immunization, T-cell populations are similarly induced to elicit antitumor immune responses; however, the NK1.1<sup>+</sup> cell response is highly unique because it can control both specific and unrelated tumor challenges. Typically, NK cells are triggered to lyse target cells following an imbalance in triggering of activating and inhibitory receptors. Thus, NK cells can be activated by either loss of ligands that trigger inhibitory receptors or up-regulation of ligands that trigger activating receptors. Both of these processes have been reported to occur in tumor cells. Many ligands for activating NK cell receptors are up-regulated in response to cellular stress, such as MULT-1, RAE1, and H60, which are recognized by NKG2D.<sup>31–33</sup> Thus, the observed increase in NKG2D expression after immunization may contribute to tumor cell death following either specific or unrelated tumor challenge. In addition to the gain of activating ligands, many tumor cells express low levels of major histocompatibility complex class I molecules, which, when present, trigger inhibitory receptors.<sup>34,35</sup> The clearance of E0771 is NK1.1<sup>+</sup> cell dependent. This cell line expresses abundant surface major histocompatibility complex class I molecules and, thus, is not likely recognized by NK cells owing to their absence. It is more probable that immunization increases the expression of activating ligands on peritoneal NK cells or increases NK cell susceptibility to activating cell ligand triggering.

In addition to activating and inhibitory receptors, NK cells can be activated by exposure to cytokines. Although stimulation under either Th1 or Th2 conditions activates NK cells against tumors,<sup>36</sup> exposure to Th1 cytokines, such as IL-12, along with IL-15 and IL-18, increases NK cell survival *in vivo* compared with unactivated cells.<sup>37</sup> As such, they have been identified as a population of memory NK cells. In the present model of peritoneal immunization, nonspecific tumor immune responses are observed even 60 days after immunization, suggesting a memory NK or NKT cell response. However, this response may differ from what has been described by memory NK cells in other models. For example, memory NK cells have been defined in viral- and hapten-specific immunity and under these circumstances are antigen specific.<sup>38,39</sup> The memory NK cells that have been described after cytokine activation, however, are not generated against a specific target and are not antigen specific.<sup>37</sup> It is likely that the memory-like NK1.1<sup>+</sup> cell population generated after immunization with lethally irradiated Colon38 more closely resembles this type of nonspecific memory NK cell.

Many of the possible modes of NK1.1<sup>+</sup> cell activation involve the presence of cytokines. Introduction of particulate antigen alone into the peritoneal cavity can induce an immune response in the peritoneal cavity,<sup>3</sup> but immunization with lethally irradiated tumor cells provides more than just a source of antigen. Administration of a lethal dose of radiation results in the generation of DNA damage that prevents further cell divisions, which is why it is termed lethal. Despite the terminal fate of lethally irradiated cells,

they are not immediately destroyed and are still capable of producing various metabolic products. In fact, irradiation has been shown to induce the production of various factors in tumor cells and other cell types, which can result in radiation-mediated effects outside the cells directly receiving radiation, termed *bystander responses*. These factors include products that are directly inflammatory, such as TNF- $\alpha$  and IL-6, and products that can induce inflammatory responses in other cells, such as reactive oxygen species and danger-associated molecular patterns.<sup>40–43</sup> These danger-associated molecular patterns subsequently alert the immune system by signaling immune cells through receptors such as the Toll-like receptors, resulting in the formation of a proinflammatory response. Although activation of the omentum by other mechanisms has been demonstrated to result in an anti-inflammatory response,<sup>44</sup> we believe that it is the production of these factors after irradiation that promotes the formation of a proinflammatory immune response, resulting in tumor control/rejection.

In addition, macrophages, which are numerous in the peritoneal cavity, have been suggested to be a critical cell type in propagating bystander responses *in vivo*.<sup>43,45</sup> These bystander responses may be acting as an adjuvant that is critical in promoting the nonspecific antitumor response, and, thus, introduction of irradiated tumor cells into the peritoneal cavity could be expected to have pleiotropic effects. First, the inflammatory mediators released directly by the tumor cells could aid in activating an immune response and/or relieving the suppressive factors present in the peritoneal cavity and on the omentum. Second, phagocytosis of the tumor cells would be expected to activate the macrophage population and result in the production of cytokines and additional inflammatory mediators that can further act on other cell populations in the peritoneal cavity to modify the immune response. Thus, immunization with lethally irradiated Colon38 results in a compounding inflammatory response, which would be consistent with the marked changes we observed in the structure and cell composition of the omentum after immunization and could result in induction of nonspecific NK cell memory.

The current standard practice for diagnosis of peritoneal metastasis is omentectomy<sup>13,14</sup>; however, the potential for generating a productive antitumor immune response in the peritoneal cavity has largely been ignored. These data involve immunization with syngeneic tumors before tumor challenge, which is generally not feasible in the clinical setting. However, such treatment could be delivered at the same time as primary tumor treatment as a means of preventing or limiting the formation or further growth of metastatic foci. Because the antitumor response we could generate was nonspecific, it would be theoretically possible to immunize patients who have a high probability of peritoneal tumor metastasis to prevent tumor growth in the peritoneal cavity. Important to either this direct translation or immunotherapy derived from these data would be a greater understanding of the mechanism by which NK1.1<sup>+</sup> cells are activated to induce tumor cell death and the mechanism by which this killing occurs. The present immunization has a very complex composition, and

an understanding of which of these components are essential to generate this nonspecific antitumor response would be critical in the generation of a therapeutic treatment. In addition, if these components were better understood, it may be possible to activate patient NK1.1<sup>+</sup> cells *ex vivo* similar to IL-2–stimulated lymphokine-activated killer cells, which have been used in multiple clinical trials.<sup>46</sup> It is possible that only NK1.1<sup>+</sup> cells from the peritoneal cavity can elicit this type of nonspecific antitumor response. There are several reports that demonstrate unique populations of NK cells located specifically in the liver that have either memory or antitumor functions.<sup>47,48</sup> The NK cells in the peritoneal cavity may represent a similar scenario in which cells capable of this type of nonspecific tumor response are located in this specific micro-environment. Our current studies seek to address these issues and further characterize this unusual antitumor response.

## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.06.030>.

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