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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$^+$ cells were essential in preventing growth of unrelated tumors, whereas NK1.1$^+$ 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.[1,2] 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.[3] These free-floating immune cells have a dynamic relationship with the organized immune aggregates also present in the peritoneal cavity.[4,5] 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.[3,6,7] 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.[8,9] In addition, omental immune aggregates are the primary site of leukocyte extravasation in models of peritonitis.[10,11] Furthermore, bacteria are rapidly sequestered in the omentum shortly after introduction to the peritoneal cavity,[12] a process that slows bacterial dissemination throughout the peritoneal cavity.[8] 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.[7] Tumor cell metastasis to the peritoneal cavity is generally a poor prognostic indicator, and limited effective therapies are available to diagnosed individuals.[13,14] Omental metastasis is a common occurrence in individuals diagnosed as having cancers of the ovary and colon as well as other peritoneal organs.[15,16] It is specifically immune aggregates to which metastasizing cells originally bind and subsequently divide.[7] 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.[7,17,18] 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,[4,6] 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.[7] 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.[3,19]

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 $NK1.1^+$ cells reversed the protective effects elicited by immunization only when challenged with an unrelated tumor challenge. In contrast, depletion of $NK1.1^+$ cells and conventional T-cell populations was required to reverse the protective effects against specific tumor challenge. Thus, activation of peritoneal $NK1.1^+$ 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 (μMT 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.[7] 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^{-1} penicillin, 100 mg/mL^{-1} 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 2.5×10^6 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-γ) (PeproTech, Rocky Hill, NJ) or vehicle control. After IFN-γ treatment, cells were trypsinized, stained with anti−H-$2K^b$ (clone AF6-88.5) for 1 hour, and analyzed by flow cytometery 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 1×10^6 Colon38 cells treated with 100 Gy of ionizing radiation i.p. Mice were challenged 14 or 60 days after immunization with 1×10^5 Colon38 cells, 1×10^5 B16-F0 cells, or 1×10^6 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.[20]

BALB/c mice were treated in a similar manner using 5×10^6 irradiated EMT6 cells to immunize and 1×10^5 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