Melanoma Induces, and Adenosine Suppresses, CXCR3-Cognate Chemokine Production and T-cell Infiltration of Lungs Bearing Metastatic-like Disease

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Abstract

Despite immunogenicity, melanoma-specific vaccines have demonstrated minimal clinical efficacy in patients with established disease but enhanced survival when administered in the adjuvant setting. Therefore, we hypothesized that organs bearing metastatic-like melanoma may differentially produce T-cell chemotactic proteins over the course of tumor development. Using an established model of metastatic-like melanoma in lungs, we assessed the production of specific cytokines and chemokines over a time course of tumor growth, and we correlated chemokine production with chemokine receptor–specific T-cell infiltration. We observed that the interferon (IFN)-inducible CXCR3-cognate chemokines (CXCL9 and CXCL10) were significantly increased in lungs bearing minimal metastatic lesions, but chemokine production was at or below basal levels in lungs with substantial disease. Chemokine production was correlated with infiltration of the organ compartment by adoptively transferred CD8⁺ tumor antigen-specific T cells in a CXCR3- and host IFNγ-dependent manner. Adenosine signaling in the tumor microenvironment (TME) suppressed chemokine production and T-cell infiltration in the advanced metastatic lesions, and this suppression could be partially reversed by administration of the adenosine receptor antagonist aminophylline. Collectively, our data demonstrate that CXCR3-cognate ligand expression is required for efficient T-cell access of tumor-infiltrated lungs, and these ligands are expressed in a temporally restricted pattern that is governed, in part, by adenosine. Therefore, pharmacologic modulation of adenosine activity in the TME could impart therapeutic efficacy to immunogenic but clinically ineffective vaccine platforms.

Introduction

Active vaccination has proven immunogenic (1), increasing the number of circulating tumor antigen-specific CD8⁺ effector or memory T cells. However, therapeutic vaccination imparts minimal clinical efficacy against active disease (2), even though ex vivo analyses demonstrate that vaccine-induced cytotoxic T lymphocytes lyse tumor targets in vitro (3). The failure of vaccines in the context of active disease may be ascribed to tumor-induced local immune suppression, myeloid-derived suppressor cells (MDSC) or regulatory T cell (Treg) activity, or tumor immune editing to evade T-cell recognition (4). However, T-cell access of tumors is associated with improved prognosis in primary (5) and metastatic (6) melanomas, yet multiple studies have demonstrated that effector T-cell infiltration of primary or metastatic melanomas is variable and often absent. Thus, the efficacy of vaccination likely relies upon efficient infiltration of tumors by treatment-induced antigen-specific T cells.

Circulating effector T cells access peripheral tissues, including tumors and tumor-infiltrated organs, through the engagement of integrins with their ligands on the vascular endothelium; firm adhesion and extravasation of T cells require high-affinity integrin binding, which is activated by chemokine receptor (CCR) in response to specific ligands (chemokines). Recent studies have highlighted the clinical importance of chemokine expression for T-cell infiltration into the tumor microenvironment (TME; ref. 7). Likewise, expression of CXCR3-cognate chemokines (CXCL9, CXCL10, and CXCL11) is correlated with T-cell infiltration status in metastatic melanoma (8). Previously, we demonstrated that melanoma cells from lymph node metastases are capable of producing CXCR3-cognate chemokines following stimulation with interferon (IFN)-α or IFNγ (9). Furthermore, the presence of tumor-specific CD8 cells expressing CXCR3 was correlated with the duration of survival (10). Collectively, these observations suggest an important role for the CXCR3 chemotactic axis for the temporal and spatial targeting of circulating T effector cells to compartments with melanoma metastases.

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Although vaccination has shown minimal efficacy for treatment of advanced disease, adjuvant-setting vaccination has prolonged disease-free survival (11), suggesting that newly forming metastatic lesions may be receptive to T-cell infiltration, and by extension, replete with chemokines; in contrast, more advanced and established tumors may lack T-cell infiltration as a consequence of suppressed chemokine production. To date, no study has evaluated the possibility of differential chemokine production and T-cell infiltration over the course of tumor growth.

Using an established murine model of metastatic-like melanoma in the lungs, we demonstrate differential regulation of CXCR3-cognate chemokine and infiltration by CXCR3+/CD8+ T cells in early- versus advanced-stage tumors. Importantly, T-cell infiltration of advanced tumors can be partially restored through blockade of adenosine receptor signaling. Thus, we demonstrate a deficit in immune therapy of cancer and a potential means to enhance the antitumor efficacy of vaccine or adoptive T-cell-mediated therapy of established metastatic disease.

Materials and Methods

Tumor models

Metastatic-like tumors were established in C57BL/6 mice (The Jackson Laboratory strain 00664), Rag2–/– (The Jackson Laboratory strain 002216), or IFNγ–/– (The Jackson Laboratory strain 002287), as indicated, by i.v. injection of 3 × 10^6 B16-F10 (B16) melanoma cells. B16 cells were obtained from the ATCC (CRL-6475), and tumors were established from cryopreserved stocks that had been passaged less than two times. Tumor development was observed in 100% of injected mice.

Inducible melanomas were established by intradermal injection of 4-hydroxy-tamoxifen (10 μL of a 20-μmol/L solution in DMSO) in Tyr::CreER+/+; BrafFlox/+; Pten/Flox mice (12) that had been backcrossed onto a B6 background, as described previously (13).

For all studies, animals were maintained in pathogen-free facilities at the Geisel School of Medicine (Lebanon, NH), and all procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee.

Cytokine and chemokine production analyses

Protein from perfused lungs was isolated using Total Protein Extraction Reagent (Thermo Scientific) and cComplete protease inhibitor (Roche cComplete). Mouse CXCL9, CXCL10, and IFNγ ELISA (DuoSets) were obtained from R&D Systems and performed according to the manufacturer's protocol.

Gene expression analyses

Total RNA from cultured B16 melanoma cells, perfused lungs, or Braf/Pten tumors was isolated (Qiagen RNeasy kit) and then translated into cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems). Specific gene expression was quantified by qPCR using master mix and prevalidated gene-specific primers (Life Technologies) and a StepOne Plus instrument (Applied Biosystems).

Lung sections and immunohistochemistry

Mice were perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich) prior to lung dissection. Lungs were sequentially incubated for 1 hour at 4°C in (i) 4% PFA; (ii) 30% sucrose in PBS, and (iii) 15% sucrose, 50% optimum cutting temperature (OCT; Tissue Tek, Sakura) in PBS followed by embedding in OCT. Ten-micrometer sections were performed with a cryostat, fixed in cold acetone, rehydrated in PBS, and blocked in 5% BSA in PBS. Sections were stained overnight with primary antibodies against CXCL9, CXCL10 (rabbit, Bioss), and TA99 (ATCC clone HB-8704), followed by Alexa Fluor 594- and Alexa Fluor 647-conjugated secondary antibodies against mouse and rabbit IgG in 1% normal goat serum (Jackson ImmunoResearch and Life Technologies), for 1 hour at room temperature. Microscopy was performed on a Zeiss AX10 fluorescence microscope fitted with a CoolSnap HQ2 mono14-bit camera, using a ×20 Apo objective. Images were taken with ZEN Pro 2012 (Zeiss) software and processed with Fiji image processing software.

Reagents

Antibodies specific for murine CD183 (CXCR3, clone CXCR3-173), CXCL9 (clone MIG 2F5.5), IFNγ (clone XMGl.2), CD4 (clone RM4-5), CD8α (clone 5H10-1), CD45 (clone 30-F11), CD11c (clone N418), Ly6G (clone 1A8), CD335 ([NKp46], clone 29A1.4), CD90.1 (Thy1.1, clone OX-7), CD31 (clone 390), and CD16/32 (clone 93) were obtained from BioLegend. Anti-adenosine A2a receptor (ADORA2A; clone 7F6-G5-A2) was obtained from Novus Biologicals.

Adoptive transfer model

Tumors were allowed to establish for 3, 11, or 18 days before adoptive transfer of T cells. T cells for adoptive transfer were collected from spleens of T-cell receptor (TCR) transgenic donor mice. To facilitate tracking of transferred cells, specific TCR mice were crossed onto a Thy1.1 x Rag2–/– background (The Jackson Laboratory strains 000406 and 002216, respectively). To ensure tumor-antigen specificity, T cells specific for an endogenous melanocyte/melanoma antigen (gp100) were obtained from Pmel × Thy1.1 x Rag2–/– mice (crossed from Pmel TCR mice; The Jackson Laboratory strain 005023; henceforth termed Pmel mice); these mice were then crossed onto a CXCR3-deficient background and henceforth termed Pmel/CXCR3–/– mice.

Before transfer, TCR transgenic CD8 T cells were enriched using a MACS negative selection magnetic kit (Miltenyi Biotec), then activated by culture (37°C/5%CO2 for 24 hours) with anti-CD3 and anti-CD28 Dynabeads (Life Technologies) at a 1:1 ratio. For adoptive transfer studies, 1 × 10^6 in vitro-activated CD8+ Pmel or Pmel/CXCR3–/– cells were i.v. injected into mice at various stages of tumor growth, as indicated, then recovered and enumerated 1 hour later (14).

To assess T-cell infiltration of tumor-bearing lungs, the lungs were perfused with 1 mL 1× PBS and homogenized to single-cell suspensions and enriched for lymphocytes using a gentleMACS tissue dissociator and lung dissociation kit (Miltenyi Biotec). Magnitude of T-cell infiltration was assessed by multi-color flow cytometry (MACSQuant, Miltenyi Biotec). Monoclonal antibodies against CD8, CD45, CXCR3, and Thy1.1 (BioLegend) were used to track adoptively transferred T cells, and viability was confirmed with violet viability dye (Life Technologies).

Flow cytometric staining and analyses

For surface staining, cells were suspended in FACS buffer (1% BSA in PBS) and incubated with purified antibodies for 30 minutes at 4°C, then washed three times in FACS buffer and fixed in 0.5% PFA. For intracellular staining, cells were stained for
In vitro migration studies

To assess the migration potential of CD8+ T cells from Pmel and Pmel/CXCR3+/− mice, magnetically enriched CD8 T cells (Miltenyi Biotec) were activated, as described above, and plated at 5 × 10^6 cells into Transwell assay plates with 5 μmol/L membranes (Costar). Chemokines (CXCL10 or CCL5; R&D Systems) were added to the bottom wells, and CCR-specific blocking antibody (anti-CXCR3, BioLegend CXCR3-173) added to the top wells, as indicated. After 3-hour incubation at 37°C, migrated cells were enumerated using an automated cell counter (Bio-Rad TC10).

Aminophylline treatment

To assess the role of adenosine-mediated signaling on cytokine and chemokine expression and production in the TME, tumor-bearing mice were treated with the nonspecific adenosine receptor antagonist aminophylline (theophylline ethylenediamine; Sigma-Aldrich) by i.p. injection of 50 μg/mL of drug every 3 days for 18 days, as described previously (15).

In vitro tumor studies

B16 melanomas were plated at 1 × 10^5 cells per well in 24-well tissue culture plates (Corning) at 37°C/5% CO2. After 24 hours, the supernatant was replaced and amended with IFNγ (0, 500, 2,500, or 5,000 pg; BioLegend), adenosine (ADO; 0, 500, or 1,000 μmol/L; Tocris), or the adenosine A2 receptor–specific inhibitor ZM241,385 (ZM; 0 μmol/L, 10 μmol/L; R&D Systems). After 24 hours at 37°C/5% CO2, the supernatant was collected and CXCL10 protein production measured by ELISA.

Results

Differential CXCR3-cognate chemokine production over the course of tumor growth

We hypothesized that tumor engraftment might transiently induce, and then suppress, CXCR3-cognate chemokine production in the lungs. To explore this possibility, we used a well-characterized model of metastatic-like melanoma and evaluated CXCL9 and CXCL10 production at various time points following injection and establishment of tumors.

In the unperturbed lung compartment, we detected CXCL9 and CXCL10 protein (Fig. 1A and B), consistent with a high level of cxcl9 and cxcl10 expression in CXCR3-competent C57BL/6 mice (16). Three days after injection of B16 melanoma, we detected significant increases in CXCL9 (Fig. 1A; P < 0.0002) and CXCL10 (Fig. 1B; P < 0.05) proteins, relative to lungs without tumor. At this time point, there was minimal detectable gp100 (melanoma-specific gene) in the lungs (Supplementary Fig. S1A), suggesting minimal residual tumor burden. By 11 days after tumor injection, CXCL9 and CXCL10 production was significantly (P < 0.001 for both proteins) lower than that observed at day 3. After 18 days of tumor growth, when tumor burden was substantial, chemokine levels were comparable (CXCL9; P = 0.06) or lower (CXCL10; P = 0.0003) than basal levels. From day 3 to 18, CXCL9 and CXCL10 were significantly (P < 0.0001 and R^2 = 0.7401, and P = 0.0002 and R^2 = 0.6879, respectively) inversely correlated with gp100 signal (Supplementary Fig. S1B and S1C). Cxcl9 and cxcl10 expression by qPCR corresponded with observed protein production (not shown). Thus, melanoma transiently induces the expression and production of CXCL9 and CXCL10 in lungs bearing metastatic-like tumors.

The observed production of CXCL9 and CXCL10 3 days after tumor injection is not likely the consequence of tumor cell injection-induced transient ischemia. We observed no change in CXCL9 and CXCL10 production at day 1 after injection, but significant increases in chemokine production by day 2 (Supplementary Fig. S2A and S2B). In contrast, Medoff and colleagues...
(17) reported a rapid and short-lived production of CXCL10 in an injury-induced ischemia model. These data discount the role of transient ischemia in chemokine production in our model and suggest that chemokine production in tumor-burdened lung is a consequence of tumor engraftment.

To assess whether spontaneous tumors also suppress CXCR3-cognate chemokine production as a function of growth, we assessed the expression of \textit{cxcl9} and \textit{cxcl10} in early (\(\sim 5\) mm\(^2\), day 25) and late (\(\sim 25\) mm\(^2\), day 40) Braf/Pten tumors using an inducible model (13). The timing of assessment is offset, relative to a transplantable model, as inducible tumors require approximately 2 weeks to become palpable and assessable (13).

Consistent with our observations in the transplantable model, we observed higher levels of chemokine gene expression in early tumors, as compared with those in late tumors (Supplementary Fig. S3). Likewise, we have observed a similar temporal regulation of CXCL9 and CXCL10 in subcutaneously implanted B16 melanoma (data not shown). These data suggest that tumor-induced CXCR3-cognate chemokine production, and subsequent suppression, may occur in clinically relevant cancers.

Our primary studies evaluated chemokine production in the tumor-bearing lung compartment, rather than in isolated tumors. Thus, our chemokine data from the lung studies represent the total tumor-bearing organ. To more precisely define the source of...
Adenosine Suppresses Melanoma-Induced CXCR3 Chemokines

Differential IFN production over the course of tumor growth

IFNs are major inducers of CXCL9 and CXCL10 expression and production (18). Therefore, we evaluated IFNγ (type II) and IFNα (type I) in our murine tumor model of metastatic-like melanoma in the lungs. IFNγ production in the lungs was significantly (P < 0.005) enhanced at day 3 after tumor induction, relative to mice with no tumor. Importantly, IFNγ production was significantly diminished by day 18, relative to day 3 levels (P < 0.0005) or basal levels (P < 0.01; Fig. 3A). Similarly, Ifnar expression was significantly enhanced on day 3 (P < 0.001) versus mice with no tumor, and significantly reduced from days 3 to 18 (P < 0.0001; Fig. 3B). The induction of IFN expression is not a consequence of transient tumor injection-induced ischemia, as no increase in IFN production or expression was observed at day 1 after tumor injection (data not shown). Comparable temporal regulation of IFNγ expression was also observed in the Braf/Pten inducible tumor model (data not shown). These data demonstrate that engrafting melanomas induce IFNγ and IFNα, and these cytokines likely induce CXCL9 and CXCL10 production in the tumor-bearing organ.

Melanoma is an unlikely source of IFN production, and we have not detected IFNγ or IFNα protein or mRNA or Ifnα gene expression in cultured B16 or human melanoma cell lines, even in response to stimulation with exogenous TNFα (data not shown). We assessed IFNγ production in nontumor cells by intracellular flow cytometry (9), in tandem with specific lineage markers. CD4 T cells are the predominant nontumor population of IFNγ-producing cells in tumor-bearing lungs (Fig. 3C and Supplementary Fig. S5B); as observed for chemokine, only a small number of CD45−CD31+ endothelial cells were positive for CXCL9 (Fig. 2D). Thus, tumor cells and immune cells are likely to be the major contributors to CXCR3-cognate chemokine in the lung during early metastatic tumor growth.

Differential T-cell infiltration over the course of tumor growth corresponds with production of CXCR3 ligands

We previously associated survival with the presence of circulating CXCR3+CD8+ tumor-specific T cells (10), and the presence of infiltrating CD8 T cells in human melanoma metastases has
Figure 4.
CXCR3-expressing Pmel CD8\(^+\) T cells traffic to lungs more efficiently than CXCR3-deficient Pmel T cells. To assess T-cell infiltration of tumor-bearing lungs, mice were given i.v. B16 injection on day 0, then received adoptive transfer with either CXCR3\(^+\)/CXCR3\(^+\) (Pmel) or CXCR3\(^-\)/CXCR3\(^-\) (Pmel/CXCR3\(^-\)/CXCR3\(^-\)) tumor antigen-specific CD8\(^+\) T cells. A, number of recovered Pmel CD8\(^+\) T cells from lungs of RAG\(^-\)/RAG\(^-\) hosts at various stages of tumor growth. (Continued on the following page.)
CXCR3-expressing CD8 T cells are preferentially recruited to tumor-bearing lung during early phases of tumor growth

The observed correlation between CXCL9 and CXCL10 production and T-cell infiltration in lungs suggest that CXCR3 may play a significant role in CD8 T-cell access of tumor-bearing lungs. To assess the role of T-cell–expressed CXCR3, we compared the infiltration of in vitro–activated CD8⁺ Pmel and Pmel/CXCR3⁻/⁻ T cells in Rag⁻/⁻ hosts bearing day 3 lung metastases. Tumor antigen–specific CXCR3⁺ CD8⁺ T cells infiltrated tumor-bearing lungs to a greater extent than cells lacking CXCR3 (P < 0.0005; Fig. 4C and D), even though adoptively transferred CXCR3⁺ and CXCR3⁻/⁻ cells were equivalently represented in the spleen (Supplementary Fig. S7C).

Over the course of tumor growth, infiltration by CXCR3⁺ Pmel T cells was significantly and consistently greater than that of Pmel/CXCR3⁻/⁻ cells (Fig. 4E); CXCR3⁻/⁻ T-cell infiltration coordinated with the level of observed CXCR3 ligands in the organ compartment, whereas infiltration by Pmel/CXCR3⁻/⁻ T cells did not significantly change over the course of tumor growth. Therefore, CXCR3 is critical for T-cell infiltration during early phases of tumor development, but is insufficient to facilitate T-cell access of organs with significant tumor load and a dearth of CXCR3-cognate chemokines.

Consistent with data from Thapa and colleagues (19), we observed that Pmel/CXCR3⁻/⁻ CD8 T cells were less capable of lysing target cells (Supplementary Fig. S8A), indicating reduced effector function. However, pharmacologic activation induced comparable proportions of IFNγ⁺ cells from Pmel or Pmel/CXCR3⁻/⁻ mice (Supplementary Fig. S8B), and ablation of CXCR3 did not diminish the capacity of the CD8 cells to migrate in response to CXCR3-specific chemokine (CCL5, Supplementary Fig. S8C). Thus, although loss of CXCR3 may negatively affect cell-mediated effector function, our short-term studies are designed to assess the impact of CXCR3 on T-cell infiltration (14), and the uncoupling of effector function and migratory activity (19, 20) validates our model for this purpose.

Adenosine signaling suppresses IFN and CXCR3-cognate chemokine production in melanoma

Our data suggest that IFN and CXCR3-cognate chemokine may be suppressed in lungs bearing advanced melanomas. However, the mechanism of suppression is undefined. Because free adenosine accumulates in hypoxic TMEs (21) such as the advanced metastatic lesions in our model system, and adenosine signaling via A2 receptors (A2R) suppresses IFN-inducible CXCL9 and CXCL10 production in immune cells (22), we assessed the possibility that adenosine signaling may suppress cytokine and chemokine production in metastasis-bearing lungs. Administration of the clinically available nonselective adenosine receptor antagonist aminophylline (23) to tumor-bearing mice significantly increased CXCL9 (P < 0.001), CXCL10 (P < 0.0005), and IFNγ (P < 0.01) production in lungs bearing established day 18 melanoma metastases (Fig. 5A–C). Aminophylline treatment had no effect on CXCL9, CXCL10, or IFNγ production in tumor-free lungs (Supplementary Fig. S9 and data not shown).

As aminophylline treatment partially restored chemokine production in lungs with advanced melanoma, we assessed whether treatment with this adenosine receptor antagonist could enhance T-cell infiltration in lungs with advanced melanoma that suppresses CXCR3-cognate chemokine production. Using the adoptive transfer model described above, we assessed T-cell infiltration of lungs bearing day 18 melanoma following treatment with aminophylline or vehicle (PBS). T-cell infiltration was modestly but significantly (P < 0.05) enhanced by aminophylline treatment (Fig. 6A and B), while T-cell populations in the spleen were unaffected (Fig. 6C). Importantly, AMO treatment enhanced the antitumor efficacy of adoptively transferred pmel (tumor-specific) T cells into mice with advanced (day 15–18) tumors, as assessed by the level of trp1 gene expression in lungs, whereas adoptive transfer of pmel T cells into mice without AMO had no significant effect on tumor burden (Fig. 6D). Collectively, these data suggest that suppression of chemokine in tumor-bearing lungs is partially due to adenosine accumulation, and that chemotactic activity is
may be maintained or restored with specific adenosine receptor antagonists.

Our prior data suggest that melanoma is a source of CXCR3-cognate chemokine in the TME, so we assessed the possibility that adenosine suppresses tumor production of chemokine. We confirmed that B16 melanoma expresses the adenosine receptor A2AR (ADORA2A; Fig. 7A) as well as ADORA2A and ADORA2B (which encodes A2BR) gene products (data not shown). In vitro, exogenous adenosine suppressed B16 production of CXCL10, even after activation of chemokine production via treatment with IFNγ (Fig. 7B). Importantly, the A2R-specific inhibitor ZM-241,385 (24) significantly reversed adenosine-mediated suppression of CXCL10 (Fig. 7B). Similarly, five of seven human melanoma cell lines tested expressed surface A2AR (representative data for line VMM12; Fig. 7C), and exogenous adenosine suppressed chemokine production by VMM12 melanoma (Fig. 7D).

Although the source of adenosine in the TME remains to be determined, we observed an increase in the proportion of regulatory (Foxp3+CD4+) cells in the day 18 TME (Supplementary Fig. S10), and these cells expressed high levels of the ecto-5′-nucleotidase CD73, which converts AMP to adenosine. Thus, infiltrating regulatory cells may mediate the accumulation of bioactive adenosine in the melanoma microenvironment, and this possibility remains under study. Collectively, these data demonstrate that tumors have a temporal “window of vulnerability” of chemokine production and associated T-cell infiltration.

Our data establish the requirement for CXCR3 expression by CD8+ T cells to efficiently infiltrate melanoma-engrafted lungs. As vaccination with peptide and adjuvant can induce CXCR3-expressing tumor-specific T cells (30), these data suggest the protective aspects of adjuvant setting vaccine may arise from preexisting tumor-reactive CXCR3+CD8+ T cells that rapidly infiltrate and eradicate newly arising tumors, whereas established tumors may be recalcitrant to infiltration by the same cells. By extension, these data suggest that tumors may be conditioned for

Discussion
The failure of immunogenic vaccines to mediate regression of established tumors or metastases has generally been ascribed to an immunosuppressive TME, which arises as a consequence of tumor-derived immunomodulatory factors and the function of suppressor cells. Conversely, vaccines in the adjuvant setting have demonstrated modest clinical effects, suggesting that newly forming metastases may be more susceptible to T-cell infiltration and eradication. Previous literature has not considered the possibility that cytokine and chemokine in the metastatic TME may be differentially regulated over the course of tumor growth. In a melanoma metastasis model, we demonstrated a significant tumor-induced production of CXCR3-cognate chemotactic molecules in the microenvironment, but not in systemic circulation. In animals with substantial tumor burden, chemokine production was suppressed. Thus, our data demonstrate that tumors have a temporal “window of vulnerability” of chemokine production and associated T-cell infiltration.

In keeping with our previous observation that CXCR3-expressing T cells are associated with enhanced survival (10), we found that CXCR3 is essential for T-cell infiltration of lungs bearing early metastatic lesions. The requirement for CXCR3 expression by CD8+ T cells to efficiently infiltrate lungs bearing metastatic tumors, although not previously demonstrated, is consistent with the observed association between CXCR3 and T-cell access of lungs in situations of viral infection (25, 26), graft rejection (27), chronic inflammation (16, 28), and nonallergic asthma (29). Our data establish the requirement for CXCR3 expression by CD8+ T cells to efficiently infiltrate melanoma-engrafted lungs.

As vaccination with peptide and adjuvant can induce CXCR3-expressing tumor-specific T cells (30), these data suggest the protective aspects of adjuvant setting vaccine may arise from preexisting tumor-reactive CXCR3+CD8+ T cells that rapidly infiltrate and eradicate newly arising tumors, whereas established tumors may be recalcitrant to infiltration by the same cells. By extension, these data suggest that tumors may be conditioned for
enhanced T-cell infiltration through pharmacologic or immunologic interventions to restore chemokine production in the local microenvironment, thereby enhancing or inducing the antitumor efficacy of therapeutic-setting vaccines. For example, bacteria have been used to induce CXCL9 and CXCL10 in the TME; recent studies have demonstrated the induction of CXCR3 chemokines...
in solid melanomas following infection with *Toxoplasma gondii* (31) and in lung metastases following infection with *Trichinella spiralis* (32).

The rapid expression, then contraction, of IFNγ, CXCL9, and CXCL10 in lungs has been observed in an infection model using chronic *Rickettsia conorii* infection (33), suggesting that induction of these factors may be a common response of the reparatory microenvironment to tissue damage. Our data extend this paradigm to cancer, demonstrating that metastatic-like lesions in the lungs rapidly induce IFNγ and chemokine production. The molecular mechanisms of tumor-induced cytokine and chemokine remain under investigation, although we demonstrate that melanoma may be a major contributor of CXCL9 and CXCL10, in keeping with our *in vitro* observation in human melanoma cell lines (9). Surprisingly, a significant number of IFNγ- and chemokine-producing CD4 T cells, but not NK cells, are apparent in early melanoma-infiltrated lungs. The role of these CD4 cells, and the mechanism by which they infiltrate the lungs, remains under investigation.

The observation that melanoma suppresses IFNs, CXCL9, and CXCL10 over the course of metastatic tumor growth has not been reported, and these data have implications for T cell–mediated therapy of cancer. To explain the active suppression of IFNs and chemokines, as opposed to a failure to induce these factors, we assessed the activity of the adenosine signaling axis in this model. Present in both intra- and extracellular compartments, in the nanomolar range (34), adenosine suppresses antitumor immune responses and can enhance tumor outgrowth. Cekic and colleagues (15) demonstrated that antagonism of adenosine slows the progression of bladder or breast carcinoma growing as

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**Figure 7.** Adenosine suppresses, and adenosine receptor 2 antagonist restores, CXCL10 production by melanoma cells *in vitro*. A, flow cytometric staining of B16 melanoma for adenosine receptor A2A (ADORA2A). Shaded histogram, unstained; open histogram, A2AR-specific staining. B, adenosine regulates CXCL10 production by B16 melanoma. Supernatant from B16 melanoma, cultured in the presence or absence of exogenous IFNγ (0 pg, 2,500 pg), ADO (0 μmol/L, 500 μmol/L, 1,000 μmol/L), and ZM241,385 (ZM; 0 μmol/L, 10 μmol/L), was assessed for CXCL10 production by specific ELISA. C, flow cytometric staining of human VMM12 melanoma for adenosine receptor A2A (ADORA2A). Shaded histogram, unstained; open histogram, A2AR-specific staining. D, adenosine regulates CXCL10 production by human VMM12 melanoma. Supernatant from VMM12 melanoma, cultured in the presence or absence of exogenous IFNγ (0 pg, 2,500 pg) and adenosine (0 μmol/L, 500 μmol/L) was assessed for CXCL10 production by specific ELISA. Differences were assessed by a *t* test: *, *P* < 0.05; ***, *P* < 0.01; ****, *P* < 0.001.
solid tumors in a CXCR3-dependent manner; therefore, we evaluated the possibility that adenosine signaling blockade, in the context of metastatic melanoma in the lung, may influence the CXCR3 chemotactic axis. We demonstrate that interfering with adenosine signaling restores IFN-γ, CXCL9, and CXCL10 levels and partially reconstitutes T-cell infiltration in situ. CD4 cells are significant sources of IFN-γ in our model, and adenosine has been shown to suppress IFN-γ production in CD4 cells (35). Furthermore, we found that adenosine restores IFN-γ-induced production of chemokines by melanoma. Therefore, we conjecture that hypoxia in the microenvironment of advanced metastatic disease leads to an accumulation of adenosine, which suppresses IFN-γ and CXCR3-cognate chemokine production, and we are currently validating these mechanisms. Interestingly, the capacity of adenosine to directly suppress CXCL10 production in IFN-γ-stimulated melanoma cells indicates a specific blockade of gene expression, suggesting that adenosine-mediated suppression of chemokine in the TME may occur even in the presence of exogenous IFNs. Our data also demonstrate that adenosine receptor–mediated suppression of CXCR3-cognate chemokine extends to human melanoma cells, consistent with the single prior report of A2BR in a single human melanoma cell line (36). Our data demonstrate that blocking adenosine A2 receptor function is sufficient to restore chemotactic gradients, and our ongoing studies will elaborate the relative contributions of A2AR and A2BR.

A recent study has demonstrated that cutaneous melanomas decrease CXCL9 production because of immune editing and selection of CXCL9-deficient clones (37). We have not excluded the possibility of immune editing in the metastatic-like model; however, the maintenance of IFN-γ, CXCL9, and CXCL10 expression in late-stage metastases following aminophylline treatment supports an argument against the selection and predominance of loss-of-function clones in our model.

The paucity of responses to immunogenic vaccines and adoptive transfer protocols suggests the need for adjuvant therapy to induce or restore T-cell infiltration into tumor compartments. Although chemokine expression is not sufficient to induce or restore antitumor function, chemokines are essential for effective T-cell chemoattraction and therefore an important aspect of cancer immunotherapy. Collectively, our data demonstrate that CXCR3-cognate ligand expression is required for efficient T-cell access of tumor-infiltrated lungs, but these ligands are expressed in a temporally restricted pattern. We further demonstrate a means to partially overcome tumor-induced suppression of lymphocyte chemotaxis, suggesting that metastases may be conditioned for enhanced or restored chemokine expression and T-cell infiltration, thereby potentially enhancing the therapeutic efficacy of immunogenic but clinically ineffective vaccine platforms.

Disclosure of Potential Conflicts of Interest

D.W. Mullins is Chief Scientific Officer at Qu Biologics and reports receiving a commercial research grant from the same. No potential conflicts of interest were disclosed by the other authors.

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# Cancer Immunology Research

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Eleanor Clancy-Thompson, Thomas J. Pereksis, Walburga Croteau, et al.

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