Primary Tumors Limit Metastasis Formation through Induction of IL15-Mediated Cross-Talk between Patrolling Monocytes and NK Cells

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Abstract

Metastases are responsible for the vast majority of cancer-related deaths. Although tumor cells can become invasive early during cancer progression, metastases formation typically occurs as a late event. How the immune response to primary tumors may dictate this outcome remains poorly understood, which hampers our capacity to manipulate it therapeutically. Here, we used a two-step experimental model, based on the highly aggressive B16F10 melanoma, that temporally segregates the establishment of primary tumors (subcutaneously) and the formation of lung metastases (from intravenous injection). This allowed us to identify a protective innate immune response induced by primary tumors that inhibits experimental metastasis. We found that in the presence of primary tumors, increased numbers of natural killer (NK) cells with enhanced IFNγ, granzyme B, and perforin production were recruited to the lung upon metastasis induction. These changes were mirrored by a local accumulation of patrolling monocytes and macrophages with high expression of MHC class II and NOS2. Critically, the protective effect on metastasis was lost upon patrolling monocyte or NK cell depletion, IL15 neutralization, or IFNγ ablation. The combined analysis of these approaches allowed us to establish a hierarchy in which patrolling monocytes, making IL15 in response to primary tumors, activate NK cells and IFNγ production that then inhibit lung metastasis formation. This work identifies an innate cell network and the molecular determinants responsible for “metastasis immunosurveillance,” providing support for using the key molecular mediator, IL15, to improve immunotherapeutic outcomes.

Introduction

Metastases cause more than 90% of deaths linked to solid tumors, and the lung is one of the most common metastatic sites for various tumor types, including melanoma, breast, liver, and stomach cancers. Metastasis is a complex process where cells detach from the primary tumor, cross the extracellular matrix and intravasate, enter the blood circulation, extravasate at distant sites, and colonize niches to start new processes of expansion. In order to thrive, tumor cells must escape immune surveillance, “proteasome subunits, TAPI/2, or MHC class I (1)—or NK cell recognition via downregulation of the NKG2D ligands MICA and MICB (2). In addition to NK cells and T cells, myeloid cells polarized to an inflammatory classical (CCR2/high Ly6C−) monocytes and patrolling nonclassical (CX3CR1+ Ly6C+ monocytes (7). In breast cancer models, classical monocytes are recruited to metastatic sites and promote extravasation and subsequent growth of cancer cells (8). In contrast, it was reported that patrolling monocytes prevent melanoma metastasis to the lung by recruiting NK cells (9).

The prevailing view is that metastasis occurs as a late event in cancer progression (10). However, a few studies have found tumor cells already disseminated from undetectable primary tumors (11, 12). Considering this possibility, it is likely that the immune system, primed by the primary tumor, may operate to prevent metastatic tumor cells to establish in distant locations. Thus, we hypothesized that primary tumors trigger “metastasis immunosurveillance.” To test this hypothesis, we developed a two-step model where primary B16F10 melanoma was established subcutaneously and experimental lung metastasis was then induced by intravenous injection. We showed that B16F10 lung metastasis was inhibited in primary tumor-bearing mice compared with primary tumor-free mice. By using depleting antibodies and genetic ablation, we found that NK cells and IFNγ play a critical role in suppressing metastasis establishment in the presence of primary tumors. NK cell activation and IFNγ production were abolished upon IL15 neutralization, which also abrogated...
the inhibition of metastasis formation by the primary tumor. We identified patrolling monocytes as the critical source of IL15 upstream of NK cell activation and metastasis inhibition. In summary, our work identifies an IL15-dependent patrolling monocyte/NK cell cross-talk that is induced by primary tumors and strongly inhibits experimental metastasis formation in the lung.

Materials and Methods

Mice and tumor cell line
C57BL/6j (B6) wild-type mice were purchased from Charles River Laboratories. B6.1Fny<sup>−/−</sup> mice were purchased from The Jackson Laboratory. All animals were females, 7–13 weeks of age, which were aged matched within 3 weeks. Mice were maintained in specific pathogen-free facilities of Instituto de Medicina Molecular (iMM). All experimental procedures were performed in compliance with the relevant laws and institutional guidelines and have been approved by the local ethics committees. The B16F10 and B16F0 melanoma cell lines were purchased and authenticated from ATCC (Manassas); cultured for 1 week; and passaged 8 and 10 times, respectively, before use. Cells were maintained in Dulbecco’s modified Eagles’ medium (DMEM) with 10% (vol/vol) FCS (Gibco; Life Technologies) and 1% (vol/vol) penicillin/streptomycin, 1% (vol/vol) nonessential amino acids, 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, and 1% (vol/vol) pyruvate and gentamycin (10 μg/mL). Monoclonal antibodies (mAb) for the following markers were purchased from eBioscience: CD3ε (clone 145-2C11), CD4 (clone RM4-5), CD11b (clone M1/70), F4/80 (clone BM8), IFNγ (clone XMG1.2), and MHC II (clone M5/114.15.2); from BD Bioscience: SiglecF (clone ES0-2440); and from Biolegend: CD8α (clone 53-6.7), CD11c (clone N418), CD19 (clone 6D5), CD45 (clone 30-F11), CX3CR1 (clone SA011F11), TCРβ3 (clone GL3), Ly6C (clone NK.1.4), Ly6G (clone 1A8), NKp46 (clone 29A1.4), and NK1.1 (clone PK136). Cells were analyzed on a FACS Fortessa (BD Bioscience). For NK cell and T-cell intracellular cytokine staining, cells from lung or spleen were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma), ionomycin (1 μg/mL; Sigma), brefeldin-A (10 μg/mL; Sigma), and 2 μmol/L monensin (eBioscience) for 4 hours at 37°C. Cells were treated with Zombie Violet (BioLegend) or LIVE/DEAD Fixable Near-IR stain to exclude dead cells and then stained for surface markers, fixed, and permeabilized using the Fixation Permeabilization Solution (eBioscience) following the manufacturer’s instructions, and then incubated for 30 minutes at room temperature with the following antibodies: anti-IFNγ (clone XMG1.2) and anti-IL1β (clone eBioMAK-D); and from Biolegend: anti-granzyme B (clone GB11) and anti-TNFα (clone MP6-XT22). Data were acquired on a FACS Fortessa (BD Bioscience) and analyzed using FACSDiva or FlowJo software (TreeStar).

Ex vivo intracellular TNFα and IL1β staining for myeloid cells was performed on isolated cells 6 hours following an i.v. injection of brefeldin A (10 mg/g of body weight). Lung digestion was performed in the presence of brefeldin A (10 μg/mL). Cells were then stained for surface markers, fixed, permeabilized as described above, and then stained with anti-mouse anti-IL1β (clone N17E3; eBioscience), anti-NOX2 (clone C4N57; eBioscience), and anti-mouse anti-TNFα (clone MP6-XT22; Biolegend).

ELISA
Lungs were perfused with PBS and homogenized using a tissue homogenizer in 0.5 mL PBS containing 0.05% Tween-20 and complete, EDTA-free Protease Inhibitor Cocktail (Roche). The
Statistical analysis

Statistical testing was performed using GraphPad Prism (GraphPad Software Inc.). All data were analyzed by a two-tailed unpaired t test with 95% confidence intervals or one-way ANOVA followed by Bonferroni multiple comparison test, with data representing the mean; *, P < 0.05; **, P < 0.01; ***, P < 0.001 were considered to be statistically significant.

Results

Primary tumors suppress experimental metastasis

We set up a two-step experimental model to determine the effect of primary tumors on the establishment of lung metastasis. Mice in the test group received 5 × 10^5 B16F10 melanoma cells subcutaneously (s.c.) in the flank, and formed black colonies easily distinguishable from normal tissue (Fig. 1A). We found 5-fold more metastatic nodules in the lungs of primary tumor-free mice compared with primary tumor-bearing mice (Fig. 1A and B). Colonies established in primary tumor-bearing mice were 2-fold smaller than those in primary tumor-free mice (Fig. 1C). The protective effect of s.c. primary tumor establishment was also observed on abdominal lesions derived from the i.p. injection of secondary B16F0 cells (Supplementary Fig. S1A–S1B). Our results also showed that B16 primary tumors from both F10 and F0 sublines inhibited lung metastasis (i.v.) of E0771 breast cancer cells, which was also suppressed by primary (s.c.) E0771 cell inoculation (Supplementary Fig. S1C). These data collectively suggest a broad activity spectrum for the underlying protective mechanism.

We next aimed to assess whether competition for essential nutrients (the athrepsia hypothesis; refs. 13, 14), or increase in circulating angiogenesis inhibitors, such as angiostatin and endostatin (15–17), accounted for the inhibition of lung metastases by the subcutaneous primary tumor. To do so, we surgically removed primary tumors before, but on the same day of, i.v. B16F10 metastasis induction. We confirmed that no relapse occurred during the time frame of the metastasis formation and analysis. Even when resected, the previous presence of primary tumors (on day 7 or 14) was sufficient to inhibit lung metastases when compared with sham-operated mice (Fig. 1D; Supplementary Fig. S1D). These results suggest that competition for nutrients and/ or survival factors do not account for metastasis inhibition by the primary tumor. Instead, we hypothesized that the host immune response to primary tumors would protect from metastases.

NK cells inhibit experimental metastasis formation in primary tumor-bearing mice

Several lines of evidence point toward NK cells in regulating metastasis formation in B16F10 experimental lung metastasis model (18, 19). Thus, we next assessed the number of NK cells and their activation status within the lung after metastasis induction in primary tumor-bearing versus primary tumor-free mice (gating strategies in Supplementary Fig. S2A–S2B). NK cell numbers and activation peaked on day 2 after metastasis challenge in primary tumor-bearing mice compared with controls (Fig. 2A). Lung NK cells in primary tumor-bearing mice displayed higher expression of CD11b (maturation marker of NK cells), granzyme B, perforin, and IFNγ (Fig. 2A; Supplementary Fig. S2C). These data collectively suggest a broad activity spectrum for the underlying protective mechanism.

Antimetastatic Innate Response to Primary Tumors

Figure 1. Primary B16F10 tumor inhibits experimental metastasis formation in the lung. A, Representative pictures of pulmonary metastatic nodules produced on day 14 after intravenous injection of 5 × 10^5 B16F10 cells. Data derive from three independent experiments (Expt). B, C57BL/6 mice were injected with PBS (n = 42) or B16F10 melanoma cells (n = 34) subcutaneously in the flank for establishment of primary tumor. On day 7 after tumor establishment, mice were injected with B16F10 cells (1 × 10^5) intravenously via tail vein. Lungs were resected on day 14 after the i.v. injection and metastatic nodules were quantified. C, The size of each superficial nodule was measured by ImageJ software. The five lobes of the lungs from 5 mice without and 10 mice with primary tumor from 3 independent experiments were analyzed (numbers of colonies; n = 514, 127, respectively). D, Tumor resection was performed (gray circles) on the same day as metastasis induction in one of three experimental groups (n = 5–7 for each group). Each circle represents one animal. ***, P < 0.001 (B and C: unpaired two-tailed t test; D: one-way ANOVA followed by Bonferroni multiple comparison test).

homogenates were centrifuged at 6,000 × g for 10 minutes at 4°C. The supernatant was collected and incubated with recombinant mouse IL15Rα-Fc (500 pg/mL; R&D Systems) for 30 minutes. The concentration of IL15 in the supernatant was evaluated using the mouse IL15R/IL15 Complex ELISA Ready-SET-Go (eBioscience) according to the manufacturer’s recommendations.
Figure 2.  
Experimental metastasis inhibition by primary tumors is mediated by NK cells and IFN-γ. A, Total cell numbers of NK cells and NK cell activation status in the lungs on days 0, 1, 2, and 3 after metastasis induction in primary tumor-free (open symbols) or tumor-bearing (closed symbols) mice (n = 4 for each group). Data are representative of two independent experiments. B, Number of B16F10 lung metastatic nodules in primary tumor-free or primary tumor-bearing mice (as in Fig. 1B) upon NK cell depletion using anti-NK1.1 mAb (n = 4 for each group). C, B16F10 lung metastatic nodules in IFN-γ−/− mice in the presence or absence of primary tumor (n = 3–4 for each group). Each symbol represents one animal. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired two-tailed t test).

M1-like myeloid cells accumulate in the lungs of primary tumor-bearing mice

We next set out to dissect the link between the primary (s.c.) tumor and the accumulation and activation of NK cells in the lung. It is well known that the number and functions of NK cells depend on the cytokine environment and on cell-to-cell interactions with myeloid cells such as monocytes, macrophages, and neutrophils (22–27). We, therefore, monitored myeloid populations in the lung in response to metastasis induction (gating strategies in Supplementary Fig. S4A). Patrolling (CX3CR1+ LY6C−) and classical (CX3CR1− LY6C+) monocytes, macrophages, and alveolar macrophages all accumulated from day 1 after metastasis challenge in primary tumor-bearing mice (Fig. 3A). This accumulation was specific to the lung as no alterations were observed in the spleen upon metastatic challenge (Supplementary Fig. S2D). To formally test whether NK cells contributed to metastasis inhibition by the primary tumor, we injected anti-NK1.1 mAb, which resulted in a significant increase in the number of metastatic foci when compared with WT controls (Fig. 2C), thus implicating IFN-γ in the loss of the protective effect of the primary tumor in IFN-γ−/− mice (Fig. 2B). This is consistent with previous reports on NK cell–mediated elimination of lung B16F10 metastases (9, 18). Thus, our data support the key role of NK cells in controlling metastasis formation and further activation of the protective NK cell response.

IFN-γ–producing NK cells have been reported to play an early protection against metastasis (20, 21). Therefore, given that we observed a rapid increase in IFN-γ–producing NK cells upon metastasis challenge (Fig. 2A), we went on to determine if IFN-γ played a role in inhibiting lung metastasis. We observed a clear loss of the protective effect of the primary tumor in IFN-γ−/− mice compared with WT controls (Fig. 2C), thus implicating IFN-γ production as a key molecular mechanism underlying metastasis suppression in primary tumor-bearing mice. Although the phenotype in IFN-γ−/− mice cannot be directly ascribed to NK cells, the contribution of IFN-γ–producing NK cells, compared with IFN-γ–producing T cells, increased in primary tumor-bearing mice (Supplementary Fig. S3A–S3D).

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We also analyzed the activation and polarization status of patrolling and classical monocytes and macrophages in the lung (Supplementary Fig. S4E). In mice, classically activated M1 macrophages, which are associated with antitumor functions, are characterized by increased microbicidal activity, including the expression of NO synthase 2 (NOS2) and high antigen-presenting function. M1 macrophages, which are associated with antitumor functions, are characterized by increased microbicidal activity, including the expression of NO synthase 2 (NOS2) and high antigen-presenting function. M1-like (antitumor) polarization of patrolling monocytes and macrophages at the metastatic site of primary tumor-bearing mice. Also, given that patrolling monocytes and macrophages in the lung shared similar polarization status, it is possible that the former give rise to the latter, as previously proposed (33).
Patrolling monocytes activate NK cells via IL15 to control metastasis formation

To assess the contribution of these myeloid cells, we used a depleting antibody against CD115, which is expressed on monocytes and macrophages (34) but significantly higher on patrolling monocytes (Supplementary Fig. S4D). We found that anti-CD115 mAb injection in primary tumor-free or primary tumor-bearing mice was efficient at depleting only patrolling monocytes from the bone marrow, blood, and lung (Supplementary Figs. S4C and S5A–S5B) and resulted in an abrogation of the NK cell response (numbers and functional properties) in the lung of primary tumor-bearing mice (Fig. 4A; Supplementary Fig. S3D).

**Figure 3.**
Primary tumors induce a myeloid response dominated by M1-like monocytes and macrophages. A, Total cell numbers of myeloid populations in the lungs on days 0, 1, 2, and 3 after metastasis induction in mice without (open circles) or with (closed circles) primary tumor. Data are representative of two independent experiments (n = 4 for each group). B, Activation and polarization status of monocytes (patrolling and classical) and macrophages (analyzed as in Supplemental Fig. 2) in the lungs of primary tumor-free versus primary tumor-bearing mice on day 2 after metastasis induction (n = 4 for each group). Each circle represents one animal. *, P < 0.05; **, P < 0.01 (unpaired two-tailed t test).
suggesting that patrolling monocytes are upstream of NK cells in preventing metastasis formation. Conversely, NK cell depletion did not affect the accumulation of patrolling monocytes or macrophages in the lung (Fig. 4B).

Trying to further dissect the link between patrolling monocytes and NK cells, we looked into IL15, because it is the dominant cytokine for NK cell homeostasis (35) and can be abundantly produced by monocytes and macrophages (33, 36). We measured the amount of IL15/soluble IL15Rα in the lungs of primary tumor-bearing mice in response to metastasis induction. Our results showed 2-fold higher amounts of IL15 in lungs of primary tumor-bearing mice compared with primary tumor-free mice (Fig. 4C).
Depletion of patrolling monocytes using antibody to CD115 resulted in an abrogation of the increase of IL15 production in the lungs of primary tumor-bearing mice after metastasis challenge (Fig. 4C). This shows that the IL15 response to primary tumors is mediated by patrolling monocytes. Next, to explore the role of the IL15 response in downstream NK cell activation, we blocked IL15 using an antagonizing antibody. Neutralization of IL15 led to the reduction of lung NK cell accumulation and effector functions upon metastasis formation in primary tumor-bearing mice (Fig. 4D). On the other hand, IL15 neutralization did not affect the increase of patrolling monocytes and macrophages in the lungs of primary tumor-bearing mice (Supplementary Fig. S5C), further establishing IL15 downstream of patrolling monocytes in the response to primary tumors.

Finally, to assess the contribution of IL15 and monocytes and macrophages in the prevention of metastasis to the lungs of primary tumor-bearing mice, we used anti-IL15 mAb, anti-CD115 mAb, and clodronate-loaded liposomes. Clodronate liposomes are taken up and eliminate phagocytic cells such as monocytes and macrophages (37). Treatment with clodronate liposomes effectively depleted circulating macrophages and patrolling monocytes but not classical monocytes (Supplementary Fig. S5A). Both IL15 neutralization and the depletion of patrolling monocytes and macrophages completely abolished the inhibitory effect of the primary tumors on metastasis formation (Fig. 4E). In contrast, neutrophil depletion using anti-Ly6G mAb did not affect metastasis inhibition (Fig. 4E). Although clodronate liposomes depleted patrolling monocytes, macrophages, and alveolar macrophages in the lungs, anti-CD115 mAb mainly depleted patrolling monocytes (Supplementary Fig. S5B). These data collectively suggest that patrolling monocytes are the key myeloid population to convey the inhibition of metastasis in the presence of primary tumors, and that IL15 is the molecular link to NK cells and their antimetastatic effector functions.

Discussion

Our studies investigated how the immune response to the primary tumor may affect metastasis formation during cancer progression. We demonstrated that the primary tumor sets up an innate immune cell network responsible for metastasis immunosurveillance. Patrolling monocytes respond to the primary tumor by producing IL15, which activates the antitumoral activities of NK cells, particularly IFNγ production that is essential for protection. NK cell depletion, but not monocye elimination, led to significant increase in metastatic colonies established in primary tumor-free mice. Thus, NK cells seem to play two types of antitumoral roles in primary tumor-free versus primary tumor-bearing mice. In support of this postulate, our data showed that IFNγ was required for metastasis inhibition in primary tumor-bearing mice, whereas previous reports have shown that IL15 was dispensable for the rapid NK cell–mediated elimination of lung B16F10 metastases in the absence of primary tumors (18).

A key finding of our study is the role played by primary tumors in setting up the innate immune network of patrolling monocytes and NK cells. This was possible due to our two-step experimental model that allowed us to analyze the immune response to primary tumors first and then assess its impact on metastases formation. Although our conclusions based on this experimental model cannot be directly extrapolated to spontaneous metastasis formation, they are strengthened by the consistency of three distinct settings in which B16 primary tumors inhibited the development of secondary B16 tumors in the lung and in the peritoneum (derived from i.v. versus i.p. secondary injections) and suppressed the experimental metastases of i.v. E0771 breast cancer cells to the lung. Our data provide evidence that innate immune cross-talk is a potent component of metastasis immunosurveillance.

A study by Kirstein and colleagues showed reduced experimental metastases in mice bearing a primary B16F10 tumor (compared with controls without a primary tumor), but this was attributed to a reduction of circulating platelets and reduced formation of metastatic tumor cell–associated thrombs (38). Mice in that study developed splenomegaly (correlated with primary tumor size), which we did not observe in our experiments. Concomitant antimetastatic effects of primary tumors may consist of nonimmunological and/or immune-mediated mechanisms (39). Our results support the latter, with the dissected cellular and molecular mechanisms building on the study by Hanna and colleagues that identified an antimetastatic role for nonclassical patrolling monocytes through the recruitment of NK cells (9). Our work adds to this by showing that primary tumors trigger an IL15 response from patrolling monocytes which activate NK cells to secrete IFNγ that inhibit metastasis. Although we cannot discount NK cell recruitment via the chemokines CCL3, CCL4, and CCL5 as previously suggested (9), we propose here a dominating role for IL15 in the cross-talk between patrolling monocytes (whose activation mechanisms are still to be dissected) and NK cells. Monoocyte–derived IL15 has long been known to be critical for IFNγ production by NK cells (36), and IFNγ is a crucial cytokine in cancer immunomodulating (40, 41). Our data suggest that NK cells increase rapidly in primary tumor-bearing mice due to the help of patrolling monocytes, which results in the elimination of metastasizing B16F10 cells at the earlier stages of attachment to the lung endothelium and establishment of the initial colonies.

At a time when the manipulation of adaptive immunity is already producing therapeutic benefit in cancer immunotherapy, this study focuses on innate immunity and consolidates the patrolling monocyte–NK cell axis as the key determinant of metastasis immunosurveillance. As such, our data show that the manipulation of this innate cellular network, via its key molecular mediator, IL15, has potential for future cancer immunotherapeutic approaches.

IL15 is a key determinant of NK cell homeostasis, activation, and effector functions (35, 42). Although NK cell–based immunotherapy has thus far met with limited clinical success (43), Huntington and colleagues identified a critical negative regulator of IL15 signaling in NK cells (44) that may underlie the difficulty in harnessing the tumor-killing abilities of NK cells. Cytokine-inducible SH2-containing protein (CIS) interacted with the tyrosine kinase JAK1, inhibiting its enzymatic activity and targeting it for proteosomal degradation, thus blocking IL15 signaling. Genetic ablation of CIS rendered NK cells hyperresponsive to IL15, as evidenced by enhanced proliferation, survival, IFNγ production, and cytotoxicity toward tumors and resulted in resistance to melanoma, prostate, and breast cancer metastasis in vivo (44). Thus, CIS–mediated regulation of IL15 signaling seems to be a potent intracellular checkpoint in NK cell–mediated tumor immunity. Given the impact of T-cell checkpoint inhibitors on cancer treatment, overcoming NK cell inhibition (e.g., via CIS blockade) may hold the key to translate the enormous antitumor
(and antimetastasis) potential of NK cells into clinical benefit for cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Kubo, B. Silva-Santos
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Kubo, N. Gonçalves-Sousa
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Mensurado
Study supervision: B. Silva-Santos

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