IL17A-Mediated Endothelial Breach Promotes Metastasis Formation

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Abstract

The role of the IL23/IL17A axis in tumor-immune interactions is a matter of controversy. Although some suggest that IL17A-producing T cells (T\(_\text{H}17\)) can suppress tumor growth, others report that IL17A and IL23 accelerate tumor growth. Here, we systematically assessed the impact of IL17A-secreting lymphocytes in several murine models of tumor lung metastasis. Genetic fate mapping revealed that IL17A was secreted within lung metastases predominantly by γδ T cells, whereas T\(_\text{H}17\) cells were virtually absent. Using different tumor models, we found IL17A\(^{-/-}\) mice to consistently develop fewer pulmonary tumor colonies. IL17A specifically increased blood vessel permeability and the expression of E-selectin and VCAM-1 by lung endothelial cells in vivo. In transgenic mice, specific targeting of IL17A to the endothelium increased the number of tumor foci. Moreover, the direct impact of IL17A on lung endothelial cells resulted in impaired endothelial barrier integrity, showing that IL17A promotes the formation of lung metastases through tumor-endothelial transmigration. Cancer Immunol Res; 4(1): 26–32. ©2015 AACR.

Introduction

There is clear evidence that the immune system controls the development of malignancies, a process termed tumor immune surveillance (1). Nonetheless, cancers usually subvert antitumor immune responses, resulting in progressive tumor growth (2). Given the current limitations of conventional cancer treatment, reinforcement of antitumor immune responses remains an attractive therapeutic strategy (3). Studies on the role of the IL23/IL17A axis and T\(_\text{H}17\) cells in cancer have yielded controversial data (4, 5), because both pro- and antitumorigenic properties have been proposed for IL17A (6, 7). Nonetheless, the infiltration of IL17A-producing lymphocytes into human tumors suggests a potential function of IL17A in human malignancies (8).

The metastasis of tumor cells is the main cause of cancer-related deaths. Metastasis formation requires the invasion of cancer cells into the lymphatic system or the bloodstream. To disseminate, tumor cells need to clasp hold in the vascular or lymphatic endothod. To extravasate, and colonize target organs (9), a process involving both integrins and selectins (10, 11). The receptors required to transduce IL17A signals, the IL17RA and IL17RC complex, are expressed predominantly by epithelial and endothelial cells (12, 13). Using transplantable and spontaneous mouse models of tumor metastasis, we found IL17A to promote the formation of lung metastasis by regulating endothelial barrier function and extravasation of tumor cells from the circulation into the lung parenchyma.

Materials and Methods

Mice

C57BL/6 mice were purchased from Janvier. IL17A\(^{-/-}\) mice were provided by Y. Ikawa (University of Tokyo, Tokyo, Japan), IL17A\(^{+/+}\) and IL17F-Cre were described before (14, 15), ROSA26-EYFP mice were provided by A. Diefenbach (University of Freiburg, Freiburg, Germany), VE-cadherin-Cre mice were provided by C. Halin-Winter (University of Zurich, Zurich, Switzerland), and Tcrd\(^{-/-}\) were bred in-house from Tcrb\(^{-/-}\) and Tcrd\(^{-/-}\) mice obtained from The Jackson Laboratory. Mice were kept in house under specific pathogen-free conditions.

Cell lines

Mouse B16F10 melanoma was purchased from the ATCC (CRL-6475). B16F10 cells expressing luciferase (B16F10-luc) were purchased from Caliper LifeScience, and Lewis lung carcinoma (LLC) was kindly provided by R. Schwendener (University of Zurich, Zurich, Switzerland). All cell lines were tested to be mycoplasma free. No genomic reauthentification was performed. Tumors were established from cryopreserved stocks that have been split less than four times. B16F10 and LLC cells were cultured in DMEM medium supplemented with 10% FCS, 1% penicillin, 1% streptomycin, and 1% glutamine. B16F10-luc cells were selected with a DMEM medium supplemented with 0.2 mg/ml zeocin (Invitrogen), 10% FCS, 1% penicillin, 1% streptomycin, and 1% glutamine.
Experimental and spontaneous tumor metastasis model

Six- to 10-week-old mice were used for all the experiments. For subcutaneous tumor challenge, 5 \times 10^5 LLC cells were s.c. injected into the lateral flank of the mouse. The tumor area was calculated from two perpendicular diameters of the tumor measured with a digital caliper. Once the tumor size reached 80 to 100 mm^2, the tumor was surgically removed. Animals were kept for an additional 4 weeks before being sacrificed for tumor colony counts in the lung.

For the induction of lung metastasis, 2 \times 10^5 B16F10, 3 \times 10^5 B16F10-luc, or 2 \times 10^5 LLC were injected i.v. After 6, 12, or 24 hours or at days 14 or 16 after tumor inoculation, mice were sacrificed and perfused, followed by lung extraction for tumor colony counts, flow cytometry analysis, or RNA isolation.

In vivo bioluminescence imaging

Tumor-bearing mice were injected with D-Luciferin (50 mg/kg body weight; Caliper Lifesciences). Luminescence signal was measured using the Xenogen IVIS 100 (Caliper Lifesciences) imaging system. Recorded data were analyzed using Living Image 2.5 software (Caliper Lifesciences). A region of interest was defined around the tumor site and photon flux from that area was read out.

Electric cell-substrate impedance sensing

Impact of 1,000 ng/mL of IL17A (PeproTech) on transendothelial electrical resistance was measured using electric cell-substrate impedance sensing (ECIS). Endothelial cells were seeded into gelatine-coated 8-well ECIS slides (8W1E PET; ibidi GmbH) at a concentration of 1 \times 10^5 wells/well. Impedance at a frequency of 4,000 Hz was measured every 48 seconds (ECIS-zeta system; Applied BioPhysics Inc.) while cells were continuously maintained in a humidified atmosphere at 37°C and 5% CO_2.

Evans Blue vascular permeability test

Mice were injected i.v. with 2 \times 10^5 tumor cells and 24 hours later, 2 mg of Evans Blue (Sigma-Aldrich) was i.v. applied. After 30 minutes, animals were euthanized and lungs were perfused with PBS and homogenized in 1 mL of 50% trichloroacetic acid (Sigma-Aldrich). Evans Blue fluorescence was measured at an excitation of 620 nm and an emission wavelength of 680 nm.

Study approval

Animal experiments were approved by the Swiss Cantonal Veterinary Office (16–2009 and 147/2012; Zurich, Switzerland). See the Supplementary Material and Methods for additional details.

Results and Discussion

IL17A promotes the formation of lung tumor foci

To determine the role of IL17A in lung tumor development, we injected B16F10 cells i.v. into Il17a^−/− and wild-type C57BL/6 (WT) control mice. Mice lacking IL17A showed a significant decrease in tumor burden compared with control mice (Fig. 1A). Injection of luciferase-expressing B16F10 cells (B16F10-luc) permitted in vivo monitoring of tumor growth (Fig. 1B). Tumor-free mice (control) were used to set up background detection signal of luminescence. Again, tumor cells transplanted into Il17a^−/− mice showed a significant delay in accumulation in the lungs, suggesting a tumor-promoting function of IL17A in the lung microenvironment (Fig. 1C).

These findings contrast with those from a report in which the same B16F10 cell line was shown to be more metastatic in IL17A-deficient animals (6). In that study, T117 cells promoted CD8^+ T-cell activation, which was necessary for lung tumor eradication. Although we have no obvious explanation for this discrepancy, we found that, similar to B16F10 cells, LLC tumor colonies also were reduced in Il17a^−/− mice (Fig. 1D).

γδ T cells are the main source of IL17A in tumor-bearing lungs

In previous reports, both γδ T cells and γδ T cells (4, 16) were found to produce IL17A in tumor tissues. To delineate the source of IL17A in the metastatic lung tissue, on day 16 after tumor challenge, lung-invading leukocytes were isolated, stimulated for 4 hours with phorbol myristate acetate (PMA) and ionomycin, and next analyzed by intracellular cytokine staining. Of the IL17A-producing cell types, approximately 50% could be attributed to CD3^+ T cells. Among CD3^+ IL17A^+ T cells, only small fractions of them were CD4^+ T117 cells. IL17A^+ γδ TCR^+ T cells were significantly more abundant (Fig. 2A and B). The CD3^− lymphocytes, but not natural killer cells, accounted for the remaining IL17A-expressing cell types. We found that the majority of CD3^+ IL17A^+ cells were CD5^+ positive (Fig. 2C), showing that this population consists of T cells that downregulate the CD3/TCR complex after PMA activation.

To unambiguously identify the source of IL17A in the tumor microenvironment, we used the IL17F-CreEYFP fate mapping strain, where IL17-expressing cells are reversibly labeled with enhanced yellow fluorescent protein (EYFP) after IL17F promoter activity (15). IL17A and IL17F are juxtaposed in the genome, and both genes are usually coexpressed (17–19). Thus, IL17F-CreEYFP mice can be used to faithfully track IL17A-producing lymphocytes (20). On day 16 after tumor challenge, tumor-infiltrating leukocytes were isolated and directly analyzed. A clear majority of EYFP^+ cells in the lung tissue were CD5^+ positive, among which 80% were γδ TCR^+ (Fig. 2D), confirming that γδ T cells and not γδ T cells are the major IL17A-producing tumor-infiltrating lymphocyte subset in tumor-bearing lungs.

In line with a recent study in which IL17A-secreting γδ T cells were shown to promote tumor cell dissemination in a mouse model of spontaneous breast cancer metastasis (21), we confirmed the tumor-promoting role of IL17A-producing lymphocytes, using Tcrbd^−/− mice, which lack both γδ and γδ T cells. These mice formed significantly fewer tumor lung colonies (Supplementary Fig. S1).

IL17A promotes the formation of lung metastasis

Tumor cell arrest in the lung vasculature and subsequent tissue extravasation requires interaction between cell surface receptors and their ligands on tumor cells, leukocytes, and endothelial cells (22). Selectins, which belong to the family of vascular adhesion molecules, mediate tumor cell interactions with the endothelium (23). Because epithelial and endothelial cells are the main targets of IL17A, we tested the impact of IL17A on E-selectin expression by injecting B16F10 cells i.v. into Il17a^−/− and WT mice. First, we confirmed the source of IL17A in tumor-bearing lungs 24 hours after tumor injection. Again, γδ T cells were the main producers of IL17A (Supplementary Fig. S2A and S2B). Next, after 6, 12, and 24 hours, lungs were isolated and E-selectin expression was...
quantiﬁed by qPCR. Six hours after cell transfer, we found that WT mice displayed signiﬁcantly higher levels of the E-selectin transcripts compared with lungs of Il17a−/− mice (Fig. 3A), suggesting that IL17A induces the expression of adhesion molecules by the endothelium when challenged with tumor cells. As with E-selectin, expression of integrin ligands, such as vascular cell adhesion molecule 1 (VCAM-1), was also reduced in Il17a−/− mice (Fig. 3B). When we concomitantly injected mice with recombinant IL17A and B16F10 cells, we found that IL17A alone slightly induced E-selectin expression, which was further augmented by the presence of tumor cells (Fig. 3C). This was apparently not due to upregulation of the IL17R expression in lung tissues after encountering B16 melanoma cells and/or IL17A (Fig. 3D).

Because IL17A regulated adhesion molecule expression, we determined the impact of IL17A on vessel permeability by measuring Evans Blue dye uptake. Lungs of Il17a−/− mice were signiﬁcantly less permeable compared with those from WT mice (Fig. 3E). To functionally conﬁrm the impact of IL17A on vascular integrity, we performed electrical resistance measurements of primary lung endothelial monolayers. IL17A caused decrease of the relative electrical resistance already at 1 hour after treatment (Fig. 3F). Lastly, IL17A engagement of primary lung endothelial cells increased B16F10 melanoma cell transmigration in vitro (Fig. 3G).

IL17A regulates tumor dissemination in a spontaneous lung metastasis model

To conﬁrm that IL17A promotes vessel activation and permeability and thereby contributes to tumor dissemination, we chose a different model in which lung metastases arise from a primary tumor mass located at a distant site. WT and Il17a−/− mice were injected s.c. with LLC cells, and the tumor mass was resected after reaching a size of 80 to 100 mm2. On the day of resection, no
Figure 2.
IL17A production by lung-infiltrating leukocytes. WT or IL17F-Cre^{EYFP} mice were challenged i.v. with B16F10 cells. On day 16, lungs were resected and processed for flow cytometry analysis. A, representative dot plots display IL17A secretion among CD45^{+}CD11b^{-} leukocytes in WT mice. B, percentage distribution of IL17A-producing cells in WT mice. Pooled data from two independent experiments (n = 7, average ± SEM). C, representative dot plots display IL17A secretion among CD45^{+} lineage negative (CD11b^{-}CD11c^{-}B220^{-}) leukocytes in WT mice. D, representative dot plots and percentages of IL17F/EYFP-positive cells among CD45^{+}CD11b^{-} leukocytes. Pooled data from two independent experiments (n = 3–7, average ± SEM). B and D, each data point represents an individual mouse.
A significant difference in primary tumor size was observed between the two mouse strains (Fig. 4A). Four weeks after surgery, Il17a−/− mice showed a reduced number of lung metastases compared with WT controls (Fig. 4B). Of note, primary tumor size at the time of resection did not correlate with the number of metastases (not shown).

### Table: Gene Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>Il17a−/−</th>
<th>B16F10 IL17A</th>
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<td>Vcam1</td>
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### Figure 3.

IL17A impact on lung endothelial activity and permeability. A and B, WT and Il17a−/− mice were challenged i.v. with B16F10 cells. After 6, 12, and 24 hours, lungs were resected and quantitative PCR was performed using whole lung tissue. Depicted data are from one experiment (n = 3 per group, average ± SEM). C and D, WT mice were challenged i.v. with B16F10 cells, 500 ng IL17A, or in combination. Six hours later, lungs were resected and quantitative PCR was performed on the whole lung tissue. Pooled data from three independent experiments (n = 9 per group, average ± SEM). E, spectrophotometric quantification of EB extracted from whole lungs of WT and Il17a−/− mice 24 hours after B16F10 challenge. Pooled data from two independent experiments (n = 7 per naive group, n = 9–10 per B16F10 group, average ± SEM). F, representative transendothelial electrical resistance. Primary lung endothelial cell monolayers were stimulated with 1,000 ng/mL of IL17A at 0 hour. The loss of electrical resistance was quantified 1 hour later. Experiment performed independently three times. G, transendothelial migration assay. Primary lung endothelial cells grown to confluence on 8-μm pore sized transwell inserts were stimulated with 100 ng/mL of IL17A for 10 hours. Next, media were changed and cells were incubated with red fluorescent-labeled B16F10 tumor cells for the next 16 hours. Results show the number of transmigrated tumor cells per field view. Pooled data from two independent experiments (n = 6 per medium group, n = 9 per IL17A group, average ± SEM). A–E, each data point represents individual mouse; G, each data point represents individual insert; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (one-way ANOVA with Bonferroni posttest for A–D or unpaired two tailed t-test for E–G). n.s., not statistically significant.
Role of IL17A in Tumor Control

To validate the protumorigenic function of IL17A, we took advantage of a mouse strain in which IL17A expression can be conditionally regulated (IL17Aind; ref. 14). Crossing the IL17Aind mouse with VE-cadherin-Cre–expressing mice led to expression of IL17A (and EGFP) by endothelial cells (Fig. 4C), allowing us to directly target IL17A to the endothelium, in which IL17A acts in an autocrine/paracrine manner. Serum from naïve VE-cadherin-CreIL17Aind/+ mice had elevated levels of IL17A (Fig. 4D). VE-cadherin-CreIL17Aind/+ mice and littermate controls were challenged with LLC cells as described above. Whereas transgenic expression of IL17A again did not have a significant impact on the primary tumor size (Fig. 4E), a significantly higher number of metastases were found in the lungs of VE-cadherin-CreIL17Aind/+ mice compared with littermate controls (Fig. 4F).

Whereas we present here one tangible mechanism by which IL17A promotes the formation of lung metastasis, namely through IL17A-induced survival or angiogenic genes (24), several lines of evidence suggest that IL17A may play a protective role in tumor surveillance in the context of T-cell responses (25). In vitro–generated IL17A–secreting T effector cells for instance were able to eradicate established tumors in an IFNγ-dependent manner upon transfer into tumor-bearing mice (5). Conversely, a growing body of data support the protumorigenic function of IL17A.
which is associated with its impact on the nonhematopoietic compartment (16, 24, 26). Here, we found γδ T cells to be the main source of IL17A in the context of lung metastasis using several preclinical models. IL17A modulates endothelial integrity, leading to enhanced tumor cell extravasation into the lung parenchyma. By combining an array of IL17-transgenic mice with several models of tumor metastasis, we propose a function of IL17A in promoting tumor cell–endothelial interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: P. Kulig, J. Mikita-Geoffroy, A.L. Croxford, B. Becher
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Kulig, S. Burkhard, J. Mikita-Geoffroy, N. Hövelmeyer, C. Gorzelanny, L. Borsig
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Kulig, S. Burkhard, J. Mikita-Geoffroy, C. Gorzelanny

References


Writing, review, and/or revision of the manuscript: P. Kulig, A.L. Croxford, N. Hövelmeyer, B. Becher
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Burkhard
Study supervision: B. Becher

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