Microenvironment-Derived IL-1 and IL-17 Interact in the Control of Lung Metastasis

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Inflammatory cytokines modulate immune responses in the tumor microenvironment during progression/metastasis. In this study, we have assessed the role of IL-1 and IL-17 in the control of antitumor immunity versus progression in a model of experimental lung metastasis, using 3LL and B16 epithelial tumor cells. The absence of IL-1 signaling or its excess in the lung microenvironment (in IL-1β and IL-1R antagonist knockout [KO] mice, respectively) resulted in a poor prognosis and reduced T cell activity, compared with WT mice. In IL-1β KO mice, enhanced T regulatory cell development/function, due to a favorable in situ cytokine network and impairment in APC maturation, resulted in suppressed antitumor immunity, whereas in IL-1R antagonist KO mice, enhanced accumulation and activity of myeloid-derived suppressor cells were found. Reduced tumor progression along with improved T cell function was found in IL-17 KO mice, compared with WT mice. In the microenvironment of lung tumors, IL-1 induces IL-17 through recruitment of γδ T cells and their activation for IL-17 production, with no involvement of Th17 cells. These interactions were specific to the microenvironment of lung tumors, as in intrafootpad tumors in IL-1/IL-17 KO mice, different patterns of invasiveness were observed and no IL-17 could be locally detected. The results highlight the critical and unique role of IL-1, and cytokines induced by it such as IL-17, in determining the balance between inflammation and antitumor immunity in specific tumor microenvironments. Also, we suggest that intervention in IL-1/IL-17 production could be therapeutically used to tilt this balance toward enhanced antitumor immunity. The Journal of Immunology, 2011, 186: 3462–3471.
angiogenesis (24–26), whereas its effects on antitumor immunity encompass recruitment of NK cells, dendritic cells (DCs), and T cells to tumor sites and activation of tumor-specific CD8+ T cells (27–30). Thus, in the tumor microenvironment, pleiotropic cytokines, like IL-1 and IL-17, have multiple target cells.

Interactions between IL-1 and IL-17 were shown in inflammatory responses related to autoimmune diseases; IL-1 promotes the differentiation of pathogenic Th17 cells and induces IL-17 secretion by these cells (31–33). Nevertheless, interactions between IL-1 and IL-17 in the tumor microenvironment have not yet been investigated. This study attempts to clarify the role of host-derived IL-1 and IL-17 in the control of in situ development of antitumor cell immunity. Our findings highlight the complex role of inflammation during lung metastasis and suggest that manipulation of IL-1/IL-17 may affect local inflammatory responses at tumor sites, which may result in an improved antitumor immune response and a more favorable prognosis.

Materials and Methods

Mice

IL-18, IL-1Ra, and IL-17 knockout (KO) mice were generated by Y. Ikawaka, as described (34, 35). Wild-type (WT) C57BL/6 mice were purchased from Harlan (Jerusalem, Israel). Animal studies were approved by the Animal Care Committee of Ben-Gurion University. Male, 8- to 12-wk-old mice were used in all experiments.

Cell lines

Lewis lung carcinoma (3LL) and B16-F10 melanoma cell lines were a gift of Prof. Y. Keisari (Tel Aviv University) and cultured in RPMI 1640 or DMEM (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beit Haemek, Israel) under standard conditions. Cell lines were routinely tested for mycoplasma and for IL-1 and IL-17 secretion by these cells (31–33). Nevertheless, interactions between IL-1 and IL-17 in the control of in situ development of antitumor cell immunity. Our findings highlight the complex role of inflammation during lung metastasis and suggest that manipulation of IL-1/IL-17 may affect local inflammatory responses at tumor sites, which may result in an improved antitumor immune response and a more favorable prognosis.

In vivo depletion assays

The 3LL tumor cells were injected i.v. into WT mice (1 × 105/mouse). Five days following tumor cell inoculation, 1 μg Abs (anti–IL-18 Ab [R&D Systems; AF-401-NA] or anti–IL-17 Ab [R&D Systems; AF-421-NA]) or 1 μg IL-1β (PeproTech, Rocky Hill, NJ) were injected into the tail vein once per week, or every 3 d, respectively. Treatment started on day 5, to enable appropriate tumor cell seed and initial invasiveness as well as accumulation of sufficient amounts of tumor-associated Ags for potential induction of antitumor cell immunity. On day 21, mice were sacrificed and patterns of tumor invasiveness were evaluated by gross morphology and further processed to obtain single-cell suspensions for FACS analyses.

Histology

Lung samples were fixed in 4% paraformaldehyde, dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Four-micron sections were cleared in xylene, and embedded in paraffin. Four-micron sections were stained with H&E or subjected to immunohistochemistry, as described (36).

Processing of cells from lungs

To obtain single-cell suspensions, lungs were dissected and incubated in 3 ml HBSS (Invitrogen) containing a mixture of enzymes (1 g/100 ml collagenase type IV, 20,000 U/100 ml; DNAse type IV, 1 mg/ml; hyaluronidase type V (Sigma-Aldrich, St. Louis, MO), as described (37)). For isolation of specific cells, we used MACS separation columns that consist of magnetic beads coated with Abs (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Preparation of lung extracts and cytokine measurements

Extracts of dissected lung samples were mixed with a protease inhibitor mixture (Sigma-Aldrich), centrifuged for 20 min at 12,000 rpm, and diluted according to weight of the lungs before use in cytokine measurements or in vitro experiments. Levels of cytokines were measured by ELISA kits (BD Pharmingen, San Diego, CA).

Flow cytometry

Single suspensions or single-cell suspensions obtained from lungs of tumor-bearing mice were analyzed using flow cytometry (FACSQuant®; BD Biosciences). Datasets were analyzed using FlowJo software (Tree Star). mAbs conjugated to FITC, PE, PE-Cy7, PE-Cy5.5, allophyocyanin, PE-Cy7, allophyocyanin-Cy7, or pacific blue specific for the following Abs were used (eBioscience, San Diego CA): CD3e (17A2), CD4 (GK1.5), CD8α (53-67), γ/δ TCR (eBioGL3), CD11c (N418), CD11b (M1/70), Gr-1 (RB6-8C5), F4/80 (clone BM8), I-A (A6-120.1), CD40 (2,23), and B7.1 (16-10A1). Dead cells were excluded by using the Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen). Intracellular cytokine stainings in permeabilized cells were performed, as described (26), using the following anti-mouse Abs (eBioscience): IL-17A (eBio1787), IFN-γ (XM1G1.2), and TNF-α (MP6-XCT2).

RT-PCR

Total mRNA was extracted from enriched CD4+ cells recovered from lungs of tumor-bearing mice (RNeasy; Qiagen, Valencia, CA), and cDNA was prepared according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). RT-PCR was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems). The following primers were used: β-actin (Mm00465992_m1), IL-17A (Mm00439619_mL), Foxp3 (Mm00751655_m1), T-bet (Mm01299453_m1), GATA-3 (Mm01337569_m1), and RORγ (Mm01326122_m1). PCR results were analyzed with SDS 2.02 software (Applied Biosystems).

Suppression activity of enriched CD11c+ cells

To analyze cytokine secretion, enriched CD11c+ cells from the lungs of tumor-bearing mice were cocultured with enriched CD8+ splenocytes from naive WT mice (2 × 10^5/well/200 μl) stimulated with anti-CD3 Abs (0.5 μg/ml; BioLegend, San Diego, CA) at a ratio of 1:4 in round-bottom 96-well plates. After 72 h, intracellular production of IFN-γ and TNF-α in CD8+ cells was assessed by FACS.

To measure proliferation, enriched CD8+ cells labeled with 10 μM CFSE (Invitrogen) were cocultured with enriched CD11c+ cells obtained from the lungs of tumor-bearing mice under the same conditions as above. The pattern of progressive reduction in CFSE intensity after cell division was analyzed to determine the percentage of proliferating CD8+ T cells.

T regulatory cell differentiation

Enriched CD4+ cells from spleens of naive WT mice were cultured in medium containing extracts of dissected lung samples (20% v/v) and anti-CD3 Abs. After 3 d, levels of CD25/Foxp3+ cells were assessed by FACS.

Cytotoxicity assay

The 3LL tumor cells were labeled with 1 μCi [35S]methionine (Amersham Biosciences) and mixed with enriched CD8+ cells from tumor-bearing mice (2 × 10^5/well/200 μl) at different E:T cell ratios, as described (38). Assays were terminated after 4 h.

Statistical analysis

Each experiment was performed five times. In vivo tumor assays, each experimental group consisted of five mice. Significance of the results was determined using the two-sided Student t test. Results of micrographs and FACS analyses are of a representative single experiment. The bar graphs present mean values (±SEM) of five experiments.

Results

Microenvironment-specific effects of IL-1 and IL-17 on tumor invasiveness

Initially, 3LL and B16 cells were injected i.v. into WT, IL-1β, IL-1Ra, and IL-17 KO mice, to assess the role of IL-1β and IL-17 on invasiveness patterns in the lung tumor microenvironment. After...
21 d, metastasis development was assessed, concomitantly to evaluating immune/inflammatory cells and cytokines in the lungs. Remarkably, although IL-1β and IL-1Ra KO mice express opposite patterns of IL-1β production, they both manifested poor pathology compared with WT mice, as indicated by gross morphology of the lungs (Fig. 1A, upper panel), number of metastatic nodules (Fig. 1A, lower panel), lung morphology (occupancy of the tumor and residual lung structure on days 14 and 22) (Fig. 1B, upper panel), lung weight (Fig. 1B, lower panel), and survival rates of 3LL tumor-bearing mice (Fig. 1C). In contrast, the number of metastases, lung pathology, and especially survival rates were improved in IL-17 KO mice compared with WT mice (Fig. 1A–C). To further corroborate these results, we have shown that injection of rIL-1β or its neutralization by Abs in WT mice enhanced invasiveness of 3LL cells in the lungs, whereas neutralization of IL-17 significantly decreased invasiveness patterns (Fig. 1D, p < 0.005). It was surprising to observe that in IL-1β KO mice, which manifest attenuated inflammatory responses, lung tumors were more invasive than in WT mice and resembled invasiveness patterns in IL-1Ra KO mice, where much of the IL-1 activity was reduced.

To assess whether these patterns of invasiveness are unique to the lung microenvironment, 3LL and B16 cells were injected i.f.p. into IL-1β/IL-17 KO mice. Indeed, deviations in the above-mentioned invasiveness patterns were observed (Fig. 2A). Invasiveness levels of tumor cells were lowest in IL-1β KO mice, followed by WT mice, and the highest levels were observed in IL-1Ra KO mice, corresponding to degrees of IL-1 expression in the host and consistent with previously described i.f.p. patterns of invasiveness of fibrosarcomas (16, 36, 39). In IL-17 KO mice, invasiveness patterns were similar to those in control mice. When IL-1 and IL-17 expression was assessed in extracts of i.f.p. tumors and lung tumors in IL-1 and IL-17 KO mice, IL-17 could be detected only in lung tumors, whereas IL-1β was detected in tumors from both sites (Fig. 2B). Patterns of IL-1β expression in lung tumors corresponded to the IL-1 genotype of the mice; there were high levels in tumors from IL-1Ra KO mice, intermediate levels in tumors from WT mice, and no expression in tumors from IL-1β KO mice, indicating that the IL-1β originated in the microenvironment (Fig. 2B, left). Levels of IL-1β were only modestly reduced in IL-17 KO mice compared with WT mice. To further corroborate these results, we assessed expression of IL-1β or IL-17 in extracts of tumor-bearing lungs from untreated WT mice or mice treated with anti-cytokine Abs. Neutralization of IL-1β largely reduced IL-17 levels, whereas IL-17 neutralization had only a modest effect on levels of IL-1β in the tumor microenvironment (Fig. 2C). Elevated levels of IL-17 were found in lung tumors in IL-1Ra KO mice, compared with tumors in WT mice, whereas significantly lower levels of IL-17 were detected in IL-1β KO mice, indicating that these cytokines possibly mutually interact in the lung tumor microenvironment. rIL-17 did not show direct effects on the proliferation of the tumor cells or their cytokine-producing potential (results not shown), further suggesting that effects of host-derived IL-17 are mainly on the tumor microenvironment.

**FIGURE 1.** Effects of microenvironment-derived IL-1 and IL-17 on patterns of lung metastasis. A, Gross morphology of tumor-bearing lungs of WT or IL-1/IL-17 KO mice injected i.v. with 3LL or B16-F10 cells (day 21) (naive = lungs from control WT mice). Lower panels, Number of visible nodules per lobe. B, Upper panels, Show histological sections of lungs of mice bearing 3LL tumors (days 14 and 21), H&E staining (original magnification ×200). Lower panels, Show mean values of lungs weight (±SEM). C, Survival rates of mice inoculated i.v. with 3LL tumor cells (±SEM, n = 25). D, Gross morphology of 3LL tumor-bearing lungs of WT mice, of WT mice injected with rIL-1β, or of WT mice injected with neutralizing Abs against IL-17 (αIL-17) or IL-1β (αIL-1β). Lower panels, Show the mean numbers of visible nodules per lobe (±SEM).
Further experiments to decipher interactions between IL-1β and IL-17 focused on the invasiveness of 3LL tumor cells in the lung microenvironment.

**IL-1β derived from DCs/macrophages is essential for recruitment and in situ activation of IL-17-producing γδ T cells in the lung tumor microenvironment**

We next assessed the cellular sources of IL-1β and IL-17 in the microenvironment of lung tumors. FACS analyses indicated that IL-1β is mainly expressed in F4/80⁺/Gr1⁻/CD11b⁻ cells (macrophages and DCs), rather than in Gr1⁺/CD11b⁺ immature myeloid cells (myeloid precursor cells) (Fig. 3A, left). These patterns of IL-1β secretion were further confirmed in supernatants of enriched CD11c⁺ adherent cells (macrophages/DCs) derived from the lungs of tumor-bearing mice (Fig. 3A, right).

When the cellular source of IL-17 in lung tumors was assessed, we found that this cytokine is produced by γδ T cells rather than Th17 cells (Fig. 3B, left). Thus, CD4⁺ T cells produced only minute amounts of IL-17 in tumors from all groups of mice tested (Fig. 3B, right). Furthermore, similar baseline levels of RORγt transcripts were observed in enriched CD4⁺ T cells from lung tumors from all groups of mice (Fig. 3B, middle). Macrophages from lung tumors from WT did not express significant levels of IL-17, as detected by intracellular staining of or in supernatants of tumor-derived isolated CD11b⁺ cells cultured in vitro for additional 24 h (37) (results not shown).

The prevalence of γδ T cells was shown to be dependent on IL-1 in the tumor microenvironment. Thus, markedly elevated levels of γδ T cells were found in lung tumors from IL-1Ra KO mice and significantly reduced levels in tumors from IL-1β KO mice, compared with WT mice (Fig. 3C, upper panel). Furthermore, the percentage of IL-17-producing γδ T cells in lung tumors was also IL-1 dependent; it was lower in tumors in IL-1β and higher in IL-1Ra KO mice, compared with WT mice (Fig. 3C, lower panel). To directly demonstrate the in vivo role of IL-1 in recruitment and activation of γδ T cells into lung tumors, manipulation of IL-1β levels was performed in WT mice injected with 3LL cells. In WT mice, 39% of γδ T cells expressed IL-17 in lung tumors, and increased up to 60% following rIL-1β injection and 15% following neutralization of IL-1β (Fig. 3D, p < 0.005). Neutralization of IL-17 did not significantly reduce IL-1β levels in lung tumors in WT mice. Thus, in the tumor microenvironment of the lungs, IL-1 and IL-17, both of innate cell origin, interact. Taken together, these results suggest that in our experimental system, IL-1 is dominant in IL-17 induction, whereas the effects of IL-17 on IL-1β induction are much less pronounced.

**Antitumor T cell-mediated immunity is impaired in IL-1-deficient mice**

A large body of evidence suggests that 3LL tumor growth is controlled by CTL activity. Indeed, depletion of CD8⁺ cells in 3LL tumor-bearing WT mice resulted in increased tumor growth and a higher mortality rate (Supplemental Fig. 1A). We next assessed CD8⁺ T cell prevalence and activity in lung tumors from WT, IL-1β, IL-1Ra, and IL-17 KO mice. As shown by FACS analysis (Fig. 4A, upper panel) and immunohistochemistry (Supplemental Fig. 1B, upper panel), the prevalence of CD8⁺ T cells was low in lung tumors from IL-1Ra KO compared with WT mice and comparable in tumors from WT, IL-1β, and IL-17 KO mice. In addition, production of IFN-γ by CD8⁺ T cells was much lower in tumors from IL-1β and IL-1Ra KO mice, compared with WT and IL-17 KO mice (Fig. 4A, lower panel, 4B). Taken together, our results indicate significant low levels of IFN-γ and TNF-α production in CD8⁺ cells from IL-1 KO mice compared with WT and IL-17 KO mice (Fig. 4B, p < 0.0005). Furthermore, reduced IFN-γ production in CD8⁺ cells was also observed in tumor-bearing WT and IL-17 KO mice treated with anti–IL-1β Abs or with rIL-1β (Supplemental Fig. 2B). In a killing assay using [³⁵S]methionine-tagged 3LL cells as targets, enriched CD8⁺ T cells from lungs of IL-1β and IL-1Ra KO mice showed reduced killing activity, compared with those from WT mice (Fig. 4C). In contrast, a higher killing activity was observed in lung-derived CD8⁺ T cells from IL-17 KO compared with WT mice. We next assessed the status of CD4⁺ T Th1 cells, which supply auxiliary cytokines for CTL proliferation and activation. Although equivalent numbers of CD4⁺ T cells were observed in all experimental groups, IFN-γ production in CD4⁺ cells was reduced in tumor-bearing IL-1β and IL-1Ra KO mice and elevated in IL-17 KO mice, compared with WT mice (Fig. 4D, p < 0.005). IL-4 production by CD4⁺ cells was similar in tumors from the different mice. These results were also confirmed at the level of T-bet transcripts in enriched CD4⁺ T cells from lung tumors (Fig. 4D, lower right). These results indicate impaired Th1 and CTL activity in lung tumors in mice with either deficient or non-attenuated IL-1β production.

A tumor microenvironment lacking IL-1β favors T regulatory cell differentiation

We next examined immunosuppressive effector cells, which might contribute to the low CTL and Th1 activity in lung tumors in IL-1β and IL-1Ra KO mice. Initially, we showed that depletion of T regulatory cells (Treg) by anti-CD25 Abs reduced invasiveness of 3LL cells in the lungs of WT mice (Supplemental Fig. 1C). Next, we assessed the role of IL-1/IL-17 in Treg development in tumor-bearing mice. As shown, the number of Treg in the lungs increased...
during tumor progression. Significantly higher levels of Treg were found in the lungs of IL-1β KO compared with WT mice, whereas levels of Treg in IL-1Ra KO mice were markedly lower (Fig. 5A, p < 0.005). The prevalence of Treg in the lungs of IL-17 KO mice was comparable to that in WT mice. These results were further confirmed by manipulation of IL-1β in tumor-bearing WT and IL-17 KO mice using neutralizing Abs or rIL-1β, which resulted in enhanced or reduced levels of Foxp3+ CD4+ T cells, respective-
ly (Fig. 5A, lower panel, and Supplemental Fig. 2B), and were further corroborated by quantification of Foxp3 transcript levels in enriched CD4+ T cells from lung tumors (Fig. 5A, upper right panel). Also, CD4+ T cells from lung tumors in IL-1β KO mice most strongly suppressed the proliferation of CD8+ T cells, whereas much less inhibition was observed with CD4+ T cells from WT, IL-17, or IL-1Ra KO mice (data not shown).

We subsequently addressed possible mechanisms whereby reduced levels of IL-1, as manifested in IL-1β KO mice, induce activation of Treg in lung tumors. It was previously suggested that

FIGURE 5. Lack of IL-1 induces Treg differentiation. A, Upper left, Treg prevalence in the lungs of tumor-bearing mice along progression, assessed by counting CD25+Foxp3+ T cells by FACS analysis. Upper right, Relative quantification (RQ) of Foxp3 transcripts in enriched CD4+ T cells. Lower panel, Shows Treg prevalence in WT mice and in WT mice injected with rIL-1β, or with neutralizing Abs against IL-1β (αIL-1β). B, Levels of TGF-β (left) and IL-6 (right) in extracts of lung tumors. C, Enriched CD4+ splenic T cells from naive mice (2 × 10^7/well) were cocultured for 3 d with lung extracts of tumor-bearing lungs (20% v/v) in the presence of anti-CD3 Abs (0.5 µg/ml), and percentages of Foxp3+ cells from CD4+-gated T cells were determined. D, Mean numbers of visible nodules per lobe in mice treated with neutralizing Abs against CD25 (open bars; αCD25), or treated with isotype control Abs (solid bars; CT). In FACS analyses, shown is a representative analysis of one experiment, and the bar graphs show mean levels of five experiments performed (±SEM, n = 25). *p < 0.01, **p < 0.005.

FIGURE 6. Nonattenuated levels of IL-1 promote recruitment and enhanced activity of myeloid cells in lung tumors. A, Prevalence of MDSCs in lungs of tumor-bearing mice (CD11c-gated). B, Prevalence of MDSCs in WT mice and in WT mice injected with rIL-1β, or with neutralizing Abs for IL-1β (αIL-1β) (upper panel). Lower panel, Shows the prevalence of IFN-γ–producing CD8+ cells in mice tested as the above. C, Upper panel, Intracellular production of IFN-γ in enriched CD8+ splenic T cells from naive mice stimulated with anti-CD3 Abs upon coculture with enriched CD11c+ cells from tumor-bearing mice. Lower panel, CFSE-tagged enriched CD8+ T cells from naive mice were cocultured, as described in B. Shown are the percentages of nonproliferating CD8+ T cells. In FACS analyses, shown is a representative analysis of one experiment, and the bar graphs show mean levels of five experiments performed (±SEM, n = 25). *p < 0.01, **p < 0.005.
TGF-β, in the absence of IL-1β and/or IL-6, induces Treg differentiation (40). Indeed, levels of TGF-β were higher in IL-1β KO and lower in IL-1Ra KO mice compared with WT and IL-17 KO mice (Fig. 5B, left). IL-6 was significantly reduced in IL-1β KO mice and was comparable among WT, IL-1Ra, and IL-17 KO mice (Fig. 5B, p < 0.005). To test the physiological significance of this cytokine milieu, lung extracts from tumor-bearing mice were incubated with enriched CD4+ T cells from naive WT mice, which were stimulated with anti-CD3 Abs, and after 3 d, the frequency of Foxp3+ T cells was measured. A marked increase in the frequency of Treg was found when CD4+ cells were incubated with lung extracts from IL-1β KO mice (p < 0.005), whereas extracts from IL-17, IL-1Ra KO, or WT mice were comparable and were less potent (Fig. 5C). Using depletion of CD25+ T cells, we next tested the effects of Treg on metastasis formation in vivo. Thus, 3LL cells were injected i.v. into WT, IL-17, IL-1β, and IL-1Ra KO mice treated with anti-CD25 Abs, as described in Materials and Methods, and patterns of invasiveness were examined. As seen in Fig. 5D and Supplemental Fig. 1D, the largest reduction in lung metastases was observed in IL-1β KO mice, which express the highest levels of Treg. Remarkably, the lungs of tumor-bearing IL-17 and IL-1Ra KO mice were not affected upon treatment with anti-CD25 Abs, whereas a relatively minor reduction in the number of lung metastases was observed in WT mice. These results point to a dominant role of Treg in the control of invasiveness in a microenvironment lacking the major proinflammatory cytokine, IL-1β. Importantly, neutralization of IL-1β in IL-17 KO mice resulted in a dramatic increase in the levels of metastasis (Supplemental Fig. 2A) due to an increase in Treg cells (Supplementary Fig. 2B).

Nonattenuated levels of IL-1β in the tumor microenvironment promote accumulation and activation of CD11b+CD11c+ MDSCs in lung tumors

Although the number of Treg found in the lungs of IL-1Ra KO tumor-bearing mice was remarkably low (Fig. 5A), CTL activity was impaired in tumors from these mice (Fig. 4C). We next assessed the prevalence/activity of MDSCs from lung tumors as a possible cause of suppression of T cell activity in these mice. The prevalence of MDSCs in the lungs of IL-1Ra KO mice was significantly higher than that in WT mice (p < 0.005), whereas equivalent or lower numbers of MDSCs were found in the lungs of IL-17 and IL-1β KO mice (Fig. 6A, Supplemental Fig. 1B, lower panel, Supplemental Fig. 2A, 2B). The necessity of microenvironment-derived IL-1β to enhance accumulation of MDSCs in lung tumors was further confirmed in WT tumor-bearing mice treated with anti–IL-1β Abs or with rIL-1β (Fig. 6B). As shown, the prevalence of MDSCs was significantly elevated upon treatment with rIL-1β and reduced following treatment with anti–IL-1β (p < 0.005), which was followed by significantly decreased levels of IFN-γ expression in CD8+ T cells in lung tumors. The decrease in IFN-γ expression following neutralization with anti–IL-1β Abs possibly results from the activation of the Treg circuit, as earlier shown in Fig. 5A. We next showed that CD11b+Gr1+ MDSCs from IL-1Ra KO mice are more suppressive on a per-cell basis compared with MDSCs from lungs of tumor-bearing mice of the other strains. This was demonstrated by IFN-γ expression and proliferation of CD8+ T cells from spleens of WT mice, activated with anti-CD3 Abs in the presence of enriched CD11c+ cells from lungs of IL-1Ra KO mice, although comparable and significantly less suppressive activity of CD11c+ cells from the lungs of WT, IL-17, and IL-1β KO mice was detected. This indicates that a tumor microenvironment enriched in IL-1 favors MDSC accumulation and activation, resulting in suppression of T cell function.

Effects of microenvironment IL-1 and IL-17 on maturation of APCs in lung tumors

We next assessed the maturation state of DCs in lung tumors. DCs in the lungs were characterized as Gr1neg/CD11cdim/CD40high/MHC-IIhigh (41). As shown, the percentage of DCs of myeloid cells (CD11c gated) was highest in lung tumors in IL-17 and IL-1β KO mice, whereas in IL-1Ra KO and WT mice, levels were lower and comparable (Fig. 7A). In the lungs of IL-1β KO and IL-1Ra KO mice, immature DCs were most prevalent, whereas in tumors from IL-17 KO and WT mice, a more mature phenotype, demonstrated by MHC class II and CD80 high levels, was observed (Fig. 7B). Finally, we cultured adherent CD11c+ cells (DCs and macrophages) from lung tumors and measured IL-12/IL-10 secretion, as auxiliary cytokines that promote Th cell polarization. IL-12 secretion was the highest and IL-10 was the lowest in IL-17 KO mice (Fig. 7C), indicating a better Th1-promoting potential of APCs from IL-17 KO mice. In the other strains of mice, this...
tendency was less pronounced. These findings were further corroborated in enriched CD11c+ cell cultures obtained from the lungs of tumor-bearing WT mice, treated with anti-IL-17 neutralizing Abs, which displayed increased secretion of IL-12 and decreased secretion of IL-10 (results not shown). To further substantiate this issue, bone marrow (BM)-derived enriched CD11b+ cells from WT mice were incubated in vitro for 3 d with extracts of lungs from tumor bearing, and DC differentiation was assessed. In accordance with the previous results, the highest levels of mature DCs (CD11c+/CD80+) were found in cultures stimulated with extracts from IL-17 KO mice, whereas extracts of tumors from IL-1Ra KO mice dominantly induced CD11b/high/Gr1/high myeloid progenitors (MDSCs), and extracts from IL-1β KO induced immature DCs (CD11c−/CD80−) as compared with extracts of lung tumors from WT mice (Fig. 7D). Improved levels of mature DCs were also found when BM-derived CD11b+ were cultured with extracts of lung tumors obtained from WT mice treated with neutralizing Abs for IL-17 (Supplemental Fig. 2C). In Fig. 7D, CD11c+ represent the percentage of overall DCs from the recovered cells, whereas CD11c+/CD80+ represent mature DCs expressing costimulatory molecules. Thus, our results demonstrate accumulation of mature DCs in lung tumors from IL-17 KO mice, which may further support the development of antitumor T cell–mediated immunity.

Discussion

In this study, we show unique effects of IL-1 and IL-17 in the development of experimental lung metastases. Initially, we observed that in IL-1β KO and IL-1Ra KO mice, which manifest opposite patterns of expression of IL-1, experimental metastasis is much more pronounced than in WT mice. However, the same malignant cells (B16 melanoma and 3LL epithelial carcinoma) develop very poorly locally (i.f.p.) in IL-1β KO mice. These results indicate distinct effects of IL-1 in diverse microenvironments and suggest differential interaction patterns of IL-1 with local cytokines and inflammatory/immune cells in the relevant tissue. Indeed, in tumors developing i.f.p., we could not detect expression of IL-17, whereas in the lungs of tumor-bearing mice, IL-17 was abundant. We have further focused on the mutual interactions between IL-1 and IL-17 in invasiveness of lung tumors, especially focusing on the composition and function of in situ immune effector and regulatory cells.

In the lungs of tumor-bearing mice, we have found that IL-1 induces the expression of IL-17 in γδ T cells, which consist of the major IL-17–producing cells, whereas IL-1 is generated by cells of the myeloid lineage. We have shown that local IL-1 recruits γδ T cells into the lungs and activates them to produce IL-17, which was most pronounced in IL-1Ra KO, moderate in WT, and minor in IL-1β KO mice. We could not detect in situ expression of IL-17 in Th cells or macrophages in lung tumors. The role of IL-1 in the induction of IL-17 production in Th17 cells (31, 33) and γδ T cells (32, 42) was shown in experimental models of infection and autoimmunity, but not in tumors. Taken with our observations on IL-17 induction, we show a dominant role of IL-1 on IL-17 production in the microenvironment of lung tumors. This conclusion was further corroborated by IL-1 neutralization during tumor invasiveness in WT mice, which almost completely abolished γδ T cell infiltration and IL-17 expression in the lungs. The effects of IL-17 on IL-1β induction were much less pronounced, as observed in IL-17 KO mice or in neutralization experiments. Thus, in the tumor microenvironment, IL-1 and IL-17, as well as a broad cascade of cytokines induced by them, determine the net effect of the microenvironment on the direction of the malignant process. Although IL-1 and IL-17 have redundant functions in experimental lung metastasis, each cytokine displays distinct dominant effects.

The role of host-derived IL-1 and IL-17 on tumor invasiveness is still controversial, as they are pleiotropic cytokines and affect both inflammation and immunity (9, 24–30, 43, 44). These effects of IL-1 and IL-17, as well as of other proinflammatory cytokines induced by them, are context dependent and affected by various parameters, such as the tumor model and site of its development, the cell types that produce the cytokines, the levels of cytokines, the timing of cytokine expression, and their integration in the local network of cytokines and inflammatory cells (24–30, 43). In our experimental model, patterns of lung metastasis were lower in IL-17 KO than in WT mice, but more pronounced was the survival advantage endowed by the lack of IL-17. In contrast, i.f.p. tumors developed equally well in WT and IL-17 KO mice, indicating that the effects of IL-17 on invasiveness patterns, as for IL-1, are specific to the tumor microenvironment. The dominant effect of IL-17, and possibly also of γδ T cells that produce it, in experimental lung metastasis is proinvasive and it mainly acts to induce immune suppression, as immune parameters in IL-17 KO mice were less impaired than in lung tumors from WT mice.

CTLs represent the major adaptive immunsosurveillance cells that control invasiveness of 3LL tumors. Indeed, lower levels of CD8+ T cells and reduced activity (cytokine production and killing capacity) were observed in lung tumors in IL-1β and IL-1Ra KO mice, where tumors are the most invasive. In addition, in these mice, reduced levels of Th1 cells, which promote CTL activation, were observed, whereas no differences in the frequency of IL-4–producing CD4+ T cells were detected.

Impaired specific antitumor cell activity in lung tumors in IL-1β and IL-1Ra KO mice may stem from defects in priming antitumor immunity, in situ suppressive activity of regulatory cells, or both. We show in this study that in lung tumors, levels of Treg increased along with tumor progression; their number and per cell suppressive activity were especially elevated in IL-1β KO mice, moderate in WT and IL-17 KO mice, and lowest in tumors from IL-1Ra KO mice. This was also confirmed at the level of Foxp3 expression in enriched CD4+ T cells from tumors. In the lungs of tumor-bearing IL-1β KO mice, a local cytokine milieu that favors Treg development (high TGF-β and low IL-6) has been observed (40). As IL-1 is a major inducer of proinflammatory cytokines, including IL-6, a weak inflammatory milieu is prevalent in IL-1β KO mice, whereas in IL-1Ra KO mice, high levels of inflammatory cytokines, such as IL-1 and IL-6, inhibited Treg induction. This was substantiated by showing that extracts from tumors that had developed in IL-1β KO mice were the best inducers of Treg differentiation from enriched naive CD4+ T cells from WT mice. It is interesting that in tumor-bearing IL-1Ra KO mice, reduced TGF-β levels were observed. It has been previously shown that reduced TGF-β production/signaling in the lungs of IL-1Ra KO mice is due to excessive NF-κB activation, which also results in delayed wound healing (45).

In lung tumors in IL-1Ra KO mice, we found elevated levels of MDSCs, which are known to suppress the function of T cells, NK, and NKT cells, and other innate immune cells (46–48). This pattern of MDSC accumulation in lung tumors from IL-1Ra KO mice was also observed in i.f.p. developing tumors; a dominant role of local and systemic IL-1β in MDSC recruitment has been initially described by us (11, 12). In this study, we show that in 3LL lung tumors from IL-1Ra KO mice, MDSCs are also more suppressive on a per cell basis (inhibition of T cell proliferation and IFN-γ production), in comparison with tumors in the other strains of mice. As levels of IL-17 are high in IL-1Ra KO mice, IL-17 may also contribute to MDSC accumulation in tumors, as already
described (25). It was previously shown that MDSCs can activate Treg; however, in our experimental system, in the lung tumor microenvironment, there was no correlation between these two types of regulatory cells. It is noteworthy that unbalanced patterns of IL-1 expression in the tumor microenvironment induced potent immuno-suppressive cells (Tregs in IL-1β KO mice and MDSCs in IL-1Ra KO mice) that contribute to tumor escape and invasiveness. Mice with approximately physiological levels of IL-1 in the microenvironment, i.e., WT and IL-17 KO mice, do not show accentuated levels of these suppressor cells, as compared with the other mouse strains.

The composition and maturity of APCs in the lungs of tumor-bearing mice are detrimental for induction of tolerance versus antitumor immunity (41). Thus, in the lungs of IL-17 and IL-1β KO tumor-bearing mice, elevated DC numbers were observed compared with WT and IL-1Ra KO mice. According to analysis of MHC class II and B7.1 expression, the most mature DCs were from IL-17 KO and WT mice. The highest levels of IL-12 and lowest levels of IL-10, which promote antitumor cell-mediated immunity, were observed in cultures of enriched CD11c+ cells from lungs of tumor-bearing IL-17 KO mice. These variations in APC maturation possibly stem from unique tumor microenvironments in mice deficient in IL-1 or IL-17, but they do not necessarily reflect direct effects of the cytokines. It has yet to be established whether these in situ effects on DC number/maturation and function in IL-1/IL-17 KO mice stem from alterations in hemopoiesis in the BM, differential recruitment of cells from the BM, or alterations in local maturation of MDSCs in the tumor’s milieu.

Finally, although no tumor regressions were observed in this study, kinetics of tumor development, pathological state of tumor-bearing lungs, and survival rates correlated with the functional status of T cells in tumors. It has recently been shown that survival of cancer patients, even with metastatic disease, correlates with numbers of activated CTL and Th1 cells, as well as the presence of lymphoid follicles inside the tumor (3–5, 49, 50). Intervention in regulatory circuits in tumors may thus represent a suitable approach to improve in situ insufficient antitumor immune responses. Indeed, we have previously shown increased invasiveness of malignant cells overexpressing IL-1β, concomitantly with MDSC-mediated immune anergy, which resulted in the rapid mortality of tumor-bearing mice. However, if such large tumors were excised, the surviving mice manifested specific resistance to challenge with the malignant cells, indicating that antitumor immunity had been induced, but was subverted by MDSCs (11). Elimination of the source of IL-1β enabled the function of antitumor memory cells, which had developed in mice with primary tumors, albeit concomitant with suppression of effector T cell function by MDSCs. In this study, we have described in situ, in lung tumors, networks of immune and inflammatory cells, such as CTLs, Th1 cells, γδ T cells, Treg, MDSCs, and APCs, which are all influenced by IL-1 and IL-17 in the tumor microenvironment. Although the effects of IL-1 and IL-17 on the malignant process are complex, we could show their dominant effects on in situ antitumor cell immunity. Thus, unbalanced levels of IL-1 have been associated with activation of immuno-suppressive cells, whereas balanced and close to physiological levels of IL-1 better enable induction of antitumor immunity. In contrast, IL-17 expressed in physiological levels in the tumor microenvironment dominantly acts to inhibit antitumor immunity, mainly through inhibition of in situ accumulation of mature APCs. Further understanding the mechanisms of IL-1/IL-17–mediated inflammation, in tilting the balance between destructive invasiveness and activation of antitumor immunosurveillance, will hopefully facilitate the development of new treatments to potentiate antitumor immunity.

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Disclosures
The authors have no financial conflicts of interest.

References


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Supplemental Figure legends

Supplemental legend to Figure 1
Lung tumor growth is controlled by CTL activity. 3LL tumor cells were injected i.v. into WT mice (1x10⁶/mouse) followed by i.p. administration of anti-CD25, or anti-CD8 antibodies every 3 days. A. Mean count of visible nodules per lobe (left) and mortality rates (right) of WT mice treated with antibodies against CD8 (open triangles) or with isotype control antibodies (solid squares). B. Histological sections of lung tumors stained with anti-CD8 (upper panel) and anti-Gr1 (lower panel) antibodies in WT, IL-17, IL-1β and IL-1Ra KO mice (day 21). C. Mean count of visible nodules per lobe (left) and mortality rates (right) of WT mice treated with antibodies against CD25 (open triangles) or with isotype control antibodies (solid squares). D. Gross morphology of lungs from WT, IL-17, IL-1β and IL-1Ra KO mice treated with antibodies against CD25 (lower panel) or without these antibodies (upper panel). This figure represents 1 out of 3 experiments (n=15). Results shown as photomicrographs are from one representative experiment out of 3 performed. In all cases, P values relate to statistical significance compared to the relevant values in WT mice. Asterisks (*) denote p<0.005.

Supplemental legend to Figure 2
WT and IL-1Ra KO mice were injected i.v. with 3LL tumor cells (1x10⁶/mouse) and were treated with neutralizing anti-IL-1β antibodies (αIL-1β) or isotype antibodies (CT). On day 21, lungs were removed and invasiveness and immunity patterns were assessed. A. Gross morphology of lung tumors and the mean count of visible nodules per lobe in WT mice and IL-17 KO mice treated (solid bars), or untreated with αIL-1β antibodies (open bars). B. Left panel; Levels of IFNγ⁺ producing CD8⁺ T cells in WT and IL-17 KO mice untreated (open bars), or treated with αIL-1β antibodies (solid bars). Middle panel; Levels of Gr1⁺/CD11b⁺ immature myeloid cells in
WT and IL-17 KO mice untreated (open bars), or treated with αIL-1β antibodies (solid bars).

Right panel; Levels of FoxP3+/CD4+ T cells in WT and IL-17 KO mice untreated (open bars), or treated with αIL-1β antibodies (solid bars).

C. Levels of immature myeloid cells (Gr-1+/CD11b+) and mature DC (CD11c+/MHCII+) from CD11b+ gated cells in lung tumors from WT mice untreated (solid bars), or mice treated with αIL-17 (open bars), and IL-1Ra KO mice untreated (gray bars), or treated with αIL-17 (striped bars). This figure represents 1 out of 3 experiments performed (n=15).