

IL-18 Induces PD-1–Dependent Immunosuppression in Cancer

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Abstract

Immunosuppressive cytokines subvert innate and adaptive immune responses during cancer progression. The inflammatory cytokine interleukin-18 (IL-18) is known to accumulate in cancer patients, but its pathophysiological role remains unclear. In this study, we show that low levels of circulating IL-18, either exogenous or tumor derived, act to suppress the NK cell arm of tumor immunosurveillance. IL-18 produced by tumor cells promotes the development of NK-controlled metastases in a PD-1–dependent manner. Accordingly, PD-1 is expressed by activated mature NK cells in lymphoid organs of tumor bearers and is upregulated by IL-18. RNAi-mediated knockdown of IL-18 in tumors, or its systemic depletion by IL-18–binding protein, are sufficient to stimulate NK cell-dependent immunosurveillance in various tumor models. Together, these results define IL-18 as an immunosuppressive cytokine in cancer. Our findings suggest novel clinical implementations of anti-PD-1 antibodies in human malignancies that produce IL-18. *Cancer Res*; 71(16); 5393–9. ©2011 AACR.

Introduction

Cancer progression constitutes one of the best-characterized pathological conditions in which tolerance is actively induced. Tumors employ several mechanisms to avoid or actively suppress anticancer immune responses (1, 2). Tumor progression subverts the adaptive arm of antitumor immune responses, either directly by compromising T-cell functions or indirectly by inhibiting antigen-presenting cells (3). The secretion by tumor cells of soluble factors (such as IL-6, transforming growth factor- β , vascular endothelium growth factor, macrophage-colony stimulating factor, and indoleamine 2, 3-dioxygenase) can directly block T-cell proliferation, promote

T-cell apoptosis, or render tumor cells resistant against T-cell attack (4). The tumor microenvironment can also release IL-23 which is a key NK cell immunosuppressant promoting metastases dissemination of otherwise NK cell-controlled cancers (5).

Driven by clinical observations linking elevated serum levels of IL-18 with defective NK cell functions (6, 7) and the potential role of tumor derived-IL-18 in cell autonomous tumor progression (8, 9), we investigated the potential immunosuppressive effects of IL-18 in NK cell-controlled tumors (10, 11). We found that IL-18 could upregulate PD-1 expression on NK cells and facilitated metastases dissemination of NK cell-dependent tumors in a PD-1–dependent manner. Depletion or neutralization of IL-18 produced by tumor cells markedly stimulated NK cell-mediated immunosurveillance against cancer.

Materials and Methods

Mice and tumors

Female C57Bl/6 or BALB/c mice were obtained from C. River Laboratories. C57Bl/6 Nude mice were purchased from Taconic. MyD88^{-/-} and IL-18R α ^{-/-} mice backcrossed on a C57Bl/6 background were kindly provided by B. Ryffel. RET^{+/-} mice were obtained from A. Prévost-Blondel (Institut Cochin, Paris, France) with the permission of M. Kato (Chubu University, Japan). All animals were maintained in IGR animal facilities according to the Animal Experimental Ethics Committee Guidelines. The tumors used were B16F10 melanomas and CT26 colon carcinomas (obtained from ATCC). To establish pulmonary metastases, 3×10^5 B16F10 or CT26 were inoculated i.v. into C57Bl/6 or BALB/c mice, respectively. Mice

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were sacrificed between day 9 and 12 and pulmonary metastases were enumerated by binocular microscopy.

Cytokines and antibodies

rmIL-18 (endotoxin-and BSA-free) was purchased from R&D Systems. Neutralizing anti-PD-1 antibody (RPM1-14) was kindly provided by Dr. Hideo Yagita (12). Monoclonal antibodies (purchased from Becton Dickinson Pharmingen, eBioscience, and R&D Systems) to the following mouse antigens were conjugated to FITC, PE, PerCP, APC, Pacific Blue or biotin: NK1.1 (PK136), NKp46 (29A1.4), CD3 (145-2C11), CD117 (ACK2), PD-1 (J43). Cells were pretreated with anti-CD16/CD32 Ab before staining and were analyzed with LSRII cytofluorometer using FACS Diva Software (Becton Dickinson), and Flow-JO Software (TreeStar, Ashland, OR).

IL-18 dosage

Serum concentrations were evaluated by mouse IL-18 ELISA Kit.

In vivo IL-18 neutralization

IL-18BP was kindly provided by C. Dinarello (13). 3×10^5 B16F10 or CT26 tumor cells were injected i.v. into C57Bl/6 or BALB/c mice, respectively. Mice received 20 μ g of IL-18BP (or saline buffer) i.p. daily from day 0 to day 8 with or without depleting anti-NK1.1 Ab (300 μ g injected i.p. on day 0, 3, 6, 9). Anti-PD-1 antibody was injected i.p. (250 μ g/mice) at day 0, 1, 3, 6, 9.

siRNA transfections of tumor cells

B16F10 tumor cells were transfected with mouse IL-18 siRNA [IL-18 Stealth Select RNAi (MSS205424, MSS205426)] and control siRNA (Stealth RNAi Negative Control Med GC; Invitrogen) using HiPerfect reagent.

Western blot analyses

Protein lysates of tumor cells transfected or not with control or IL-18 siRNA were examined by immunoblotting. Aliquots of 20 μ g of total protein extracts were solubilized in Laemmli loading buffer, separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad Laboratories). After blocking for 1 h, blots were incubated overnight at 4°C with antibodies against IL-18 (rabbit anti-mouse IL-18, 2 μ g/mL; BioVision, Inc.) and GAPDH (mouse anti-glyceraldehyde-3-phosphate dehydrogenase, 1/10,000; Chemicon International). Protein detection was carried out with HRP-conjugated secondary antibodies (Southern Biotech) and SuperSignal West Pico Chemiluminescent Substrate.

Results and Discussion

Low dosing of rIL-18 promotes metastases dissemination

The cell-autonomous protumorigenic effects of endogenous IL-18 have been described in B16F10 melanoma where IL-18 induced adhesiveness of melanoma cells to sinusoidal

endothelium, thereby facilitating metastatic dissemination (14). Since B16F10 metastatic spreading is controlled by NK cells (10), we investigated the mechanisms by which rIL-18 could facilitate tumor progression. We compared different schedules of recombinant mouse endotoxin-free IL-18 (rIL-18) administration (Supplementary Fig. S1A) for their ability to modulate the establishment of B16F10 lung metastases. rIL-18 enhanced the development of B16F10 metastases when it was injected twice a week (2 \times ; Fig. 1A), whereas daily administration (5 \times) of rIL-18 reduced tumorigenesis consistent with published results (15; Fig. 1A). Accordingly, the (2 \times) rIL-18-induced expansion of lung metastases was inversely correlated with intratumoral mature NK cells, defined as NK1.1⁺CD3⁺Kit (CD117)⁻ NK cells (16; Fig. 1B). Circulating serum levels of IL-18 accumulating by day 7 were significantly higher with the 5 \times versus 2 \times regimen (Fig. 1C). These circulating levels of rIL-18 were associated with distinct NK cell phenotypes (Supplementary Fig. S1B and C) and cytokine patterns in spleens. The quantitative real-time PCR (qRT-PCR) analyses of 96 immune gene products using a microfluidic card designed for qRT-PCR on splenocytes harvested from mice treated with once (1 \times) versus twice a week (2 \times) versus 5 times (5 \times) daily administrations of rIL-18 revealed different gene signatures (Fig. 1D and E). We observed a significant upregulation of the IL-12p40 and inflammatory gene products (TNF, CCL5, IL-1 β , IL-15, and CCR4) with 5 daily injections of rIL-18, whereas 1 (1 \times) or 2 shots (2 \times) of rIL-18 promoted an immunosuppressive microenvironment (significant downregulation of CCR7, Fas, upregulation of IL-10, trend toward a reduction in CXCR3, CD3 ϵ , CD4, CD8, Pfr, CD28, CD40, STAT1; Fig. 1D and E). These data underscore the capacity of low doses of rIL-18 to favor tumor progression of B16F10 metastases.

The source of the immunosuppressive IL-18 is the tumor cell

Next, we determined whether tumor cells may spontaneously secrete bioactive IL-18. Mature (18 kDa) bioactive IL-18 was detected in B16F10 and various tumor cell lysates (CT26, P815, RMA, and RMAS) by immunoblotting (Fig. 2A, top) and was released into the supernatant, as determined by an IL-18 bioassay (17) that measures IFN γ induction in splenocytes (Fig. 2A, bottom). Transfection of B16F10 tumor cells with 2 distinct IL-18 siRNAs reduced the capacity of tumor cells to produce bioactive IL-18 (Fig. 2B, top) and compromised B16F10 tumorigenesis (Fig. 2B, bottom). In IL-18R^{-/-} or MyD88^{-/-} mice, the dissemination of B16F10 metastases was markedly reduced, suggesting that B16F10 progression was dependent on the IL-18R/MyD88 signaling pathway of the host (Fig. 2C). It is noteworthy that upon inoculation of transplantable tumors (B16F10) and in spontaneous melanomas arising in RET transgenic mice (expressing the RET proto-oncogene driven by the metallothionein promoter; ref. 18), the serum levels of IL-18 were markedly increased by 10 days (premortem) and at 12 months, respectively (Supplementary Fig. 1D and E), in the same ranges as those obtained with 2 \times rIL-18 scheduling (Fig. 1C). Next, we used saturating amounts of IL-18 binding protein (IL-18BP),

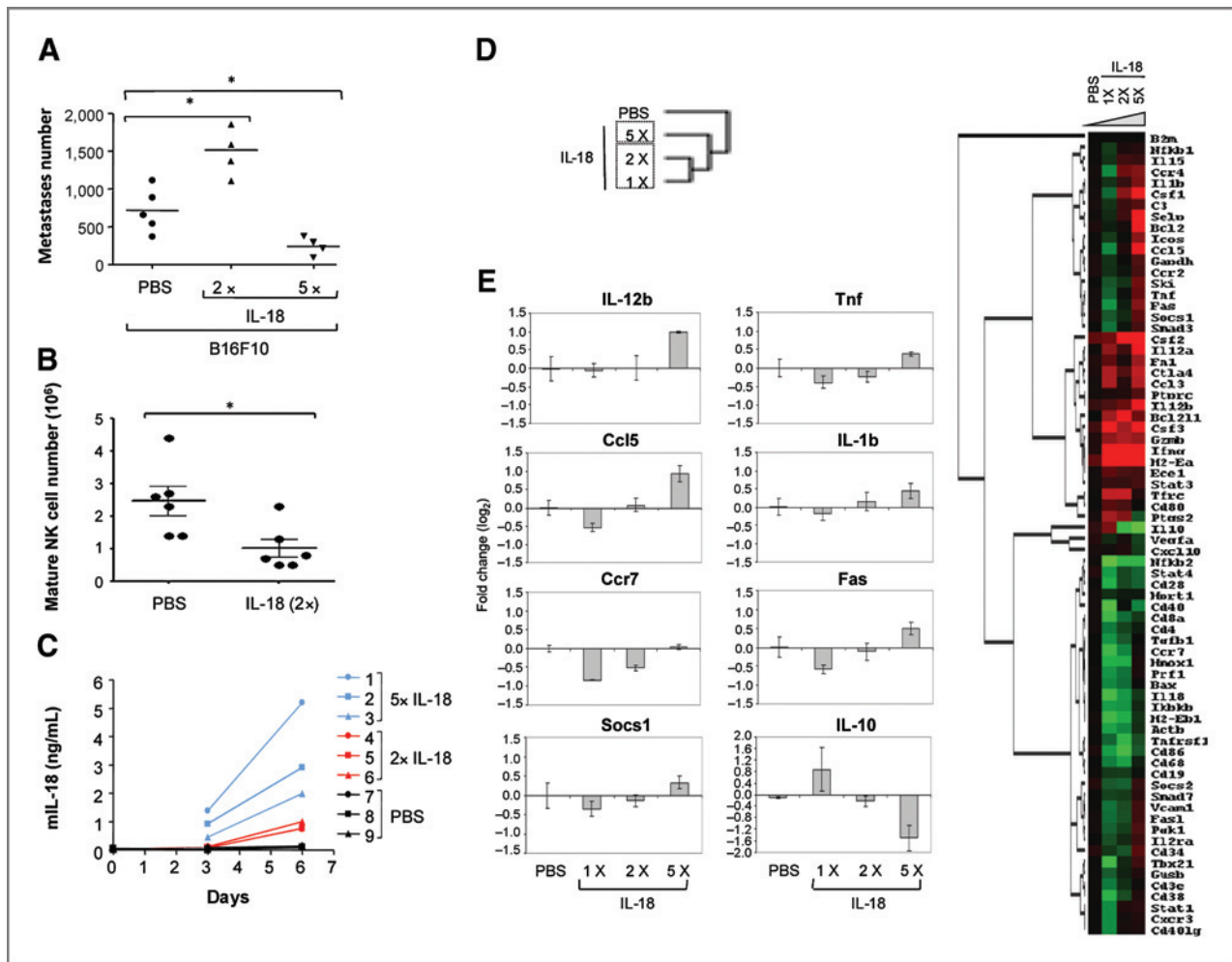


Figure 1. Administration of rIL-18 twice a week promotes metastases dissemination. **A**, dose-dependent effects of rIL-18 on B16F10 lung metastases. C57Bl/6 mice were inoculated i.v. with 3×10^5 B16F10 cells and received twice a week 1 μ g of rIL-18 ip (2 \times) or every day for 5 days (5 \times). Pulmonary metastases were enumerated at day 12. One representative experiment of 3 with 4 to 5 mice/group is shown. **B**, concomitant attrition of mature NK cells in tumor beds upon twice a week rIL-18 scheduling. Same experimental setting as in (A) but mice received only twice a week rIL-18 administrations. Mice were euthanized at day 7 when tumor burdens were comparable between the 2 groups. The absolute numbers of mature NK cells [NK1.1⁺CD3⁻Kit(CD117)⁻ cells] were assessed in lung metastases. One representative experiment is shown of 2 experiments yielding similar results. **C**, circulating levels of IL-18 in various scheduling of administration of rIL-18. Serum concentrations of mL-18 assessed by ELISA in 3 animals per group at day 3 and 6 in the daily (5 \times) versus twice a week (2 \times) scheduling. **D** and **E**, transcriptional profile of cytokines in spleens after exposure to various schedules of rIL-18. Transcriptional analyses of 96 immune gene products in splenocytes using a microfluidic card designed for qRT-PCR (TaqMan Low Density Mouse Immune Array from Applied Biosystems). **D**, a hierarchical clustering was conducted on the 72 genes for which we obtained detectable signals by qRT-PCR from the original set of 96 immune genes. Each row represents a gene and each column represents a sample (mean of 3 mice). Red and green indicate expression levels above and below the PBS-treated control value, respectively. Dendrograms of genes (to the left of matrix) and of samples (**D**) represent overall similarities in gene expression profiles. Graphs (**E**) represent log fold changes (relative to PBS-treated controls, calculated with the $\Delta\Delta C_T$ method (normalization with B2m as endogenous control) of transcripts pooled from 2 independent experiments containing 3-4 mice/group for the most significant differences between each schedule. The data were clustered using the Cluster and TreeView programs (average linkage clustering using Pearson's uncentered correlation as similarity metric). SEM of log fold changes are shown.

a naturally occurring IL-18 antagonist (13), that successfully compromised tumor progression in 2 NK cell-controlled lung metastases models (Refs. 10, 11; CT26 and B16F10, Fig. 2D, top and bottom). IL-18 neutralization by IL-18BP did not cause tumor regression in the absence of NK1.1⁺ cells, suggesting that IL-18 modulated NK cell functions (Fig. 2E). Therefore, IL-18 produced by tumor cells could subvert the NK-mediated antitumor host defense.

IL-18 upregulates PD-1 expression on mature NK cells, whereas the protumorigenic activity of IL-18 depends on PD-1 receptors

PD-1 is one of the major checkpoints downregulating T-cell functions in tumor beds. Recently, myeloma associated-human NK cells were reported to express PD-1 that may cause their exhaustion (19). Therefore, we examined PD-1 expression on T and NK cells in metastases and lymph nodes (LN) of tumor

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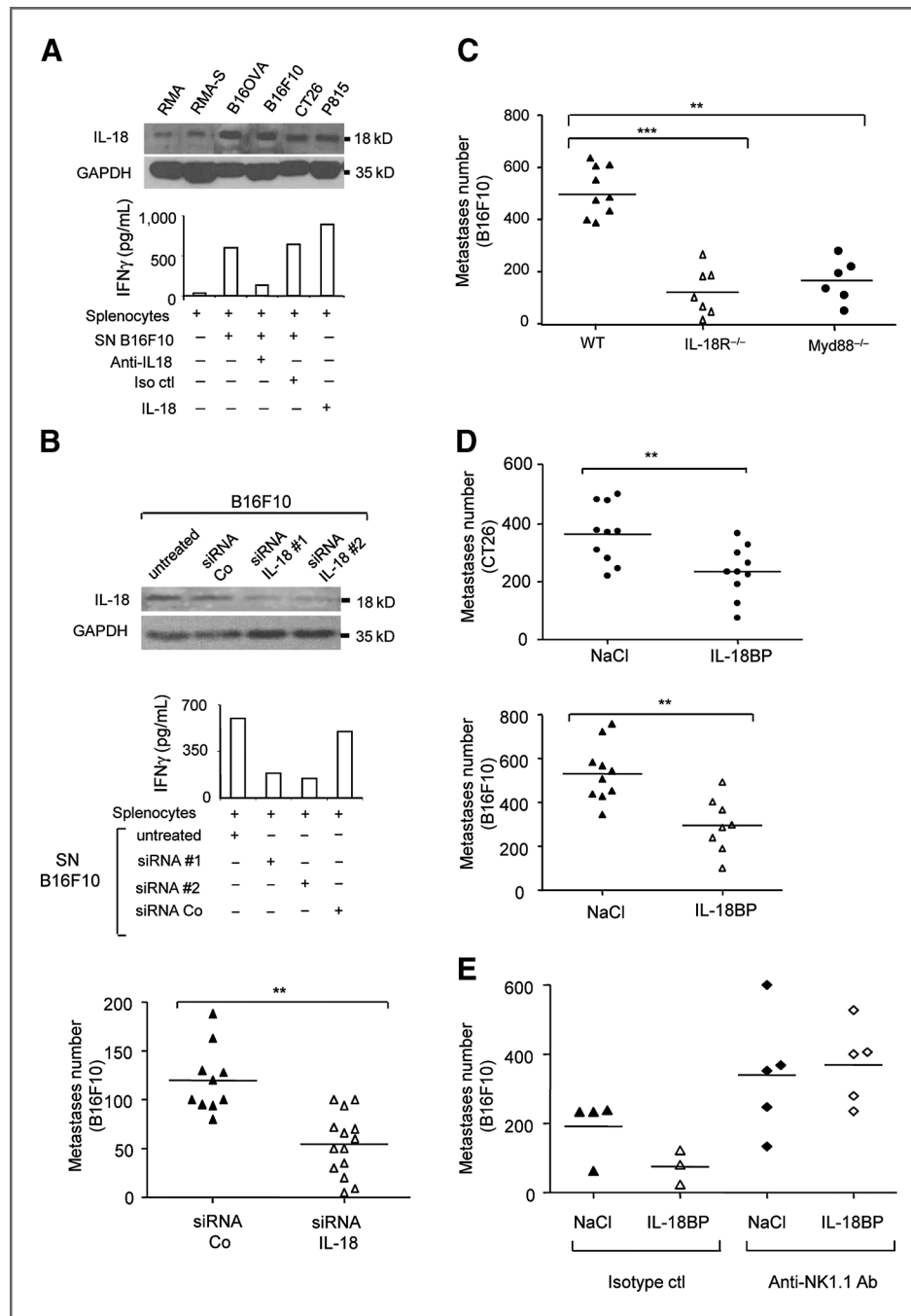
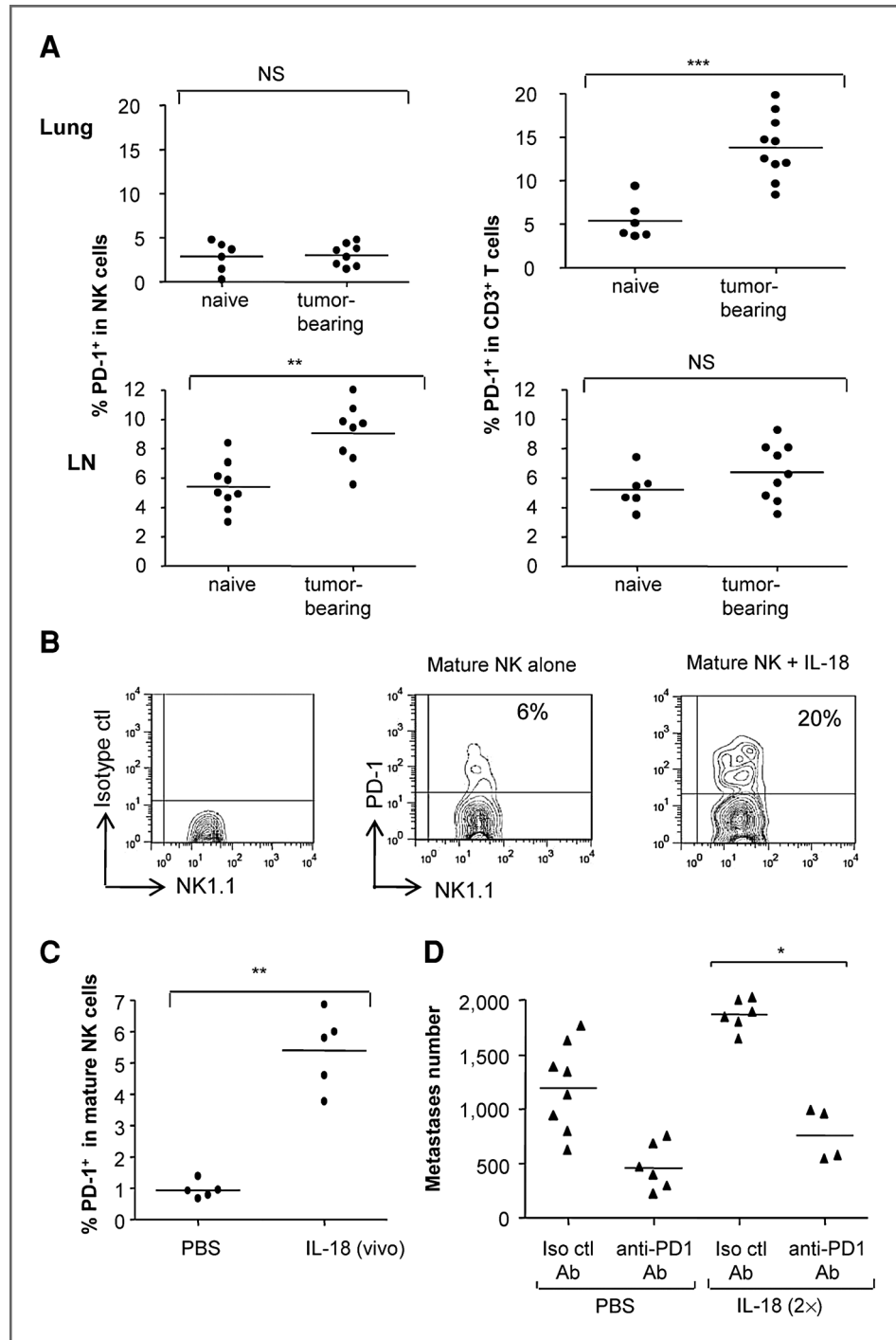


Figure 2. Tumor derived IL-18 is involved in metastases dissemination. **A**, production of bioactive IL-18 by a variety of tumor cells. Immunoblot analyses of several tumor lysates using anti-IL-18 mAb or anti-GAPDH Ab (top). A bioassay measuring the IL-18-driven IFN γ production by murine splenocytes (15) was conducted by incubating splenocytes with rIL-18 or B16F10 supernatants (1:4 dilution) in the presence of anti-IL-18 neutralizing Ab or isotype control Ab. The levels of IFN γ were monitored by ELISA in the splenocyte cultures at 72 h (bottom). One representative experiment of 3 is shown. **B**, knock-down of IL-18 secretion in tumor cells reduced the number of metastases. Immunoblot and bioassay as in (A) but using B16F10 transfected with control siRNA (siRNA Co) or 2 different IL-18-depleting siRNAs (siRNA #1, #2; top and bottom). Enumeration of lung metastases after inoculation of B16F10 transfected with various siRNA (bottom). **C**, IL-18R/Myd88-mediated host responsiveness is required for tumor dissemination. Enumeration of lung metastases after inoculation of B16F10 in C57Bl/6 WT, IL-18R^{-/-}, or Myd88^{-/-} mice. **D**, pharmacological neutralization of IL-18 by IL-18-BP decreases metastases. IL-18BP was administered as described in Materials and Methods. Quantification of metastases numbers for CT26 in BALB/c (top) and for B16F10 in C57Bl/6 mice (lower panel) was conducted. **E**, NK cells are involved in IL-18-induced immunosuppression. Same as in D, but mice received anti-NK1.1 antibody to deplete NK cells. The graphs (B–D) depict the pooled results of 3 independent experiments containing at least 2 to 4 mice/group. Also, E shows a representative experiment of 2. The Mann–Whitney test is used for statistical analyses in B and D, the Kruskal–Wallis multiple comparison test was used for statistical analyses in C, **, $P < 0.01$; ***, $P < 0.001$.

bearers. Tumor sites contained significant levels of tumor infiltrating PD-1⁺ T lymphocytes but no PD-1⁺ NK cells (Fig. 3A, top). Interestingly, NK cells (but not T cells) exhibited a significant upregulation of PD-1 expression in LN (Fig. 3A, bottom). As determined by intracellular staining, PD-1 molecules were present in freshly isolated NK cells (Supplementary Fig. S2A), which, however, lacked surface expression of PD-1.

Stimulation of NK cells with rIL-2 or other NK cell stimulatory compounds (such as IFN γ , CpG ODN, PolyI:C) induced surface exposure of PD-1 (Supplementary Fig. S2B), indicating that NK cell activation results in surface expression of PD-1. Importantly, rIL-18 could induce PD-1 expression on splenic NK cells, both *in vitro* and *in vivo* (Fig. 3B and C, respectively). The prometastatic effects of rIL-18 in Nude mice (lacking

Figure 3. IL-18 upregulates PD-1 expression on NK cells and mediates a PD-1-dependent prometastatic effect. **A**, PD-1 expression on T and NK cells in tumors and LN. PD-1 expression was examined by gating on CD3⁺ T cells (right) and CD3⁻ NK1.1⁺ NK (left) contained in B16F10 lung metastases and LN from tumor-bearing or naive mice. The results are pooled from 2 independent experiments with 3 to 5 mice/group. **B** and **C**, IL-18 induced PD-1 expression on mature NK cells *in vitro* and *in vivo*. PD-1 expression levels on Kit⁻ NK cells incubated 20 h *in vitro* in 25ng/mL of rIL-18 (**B**) or of LN residing Kit⁻ NK cells 24 h after i.p. administration of 1 μ g of rIL-18 (**C**). One representative experiment of 3 is shown. **D**, the protumorigenic effects of exogenous or tumor derived-IL-18 can be attributed partly to PD-1. Identical setting as in Fig. 1A but in C57Bl/6 Nude mice with concomitant administration of isotype control or blocking anti-PD-1 Abs. The graph depicts the pooled results of 2 experiments containing 3 mice/group. The Mann-Whitney test is used for statistical analyses in A and C, and the Kruskal-Wallis multiple comparison test was used for D. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.



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T lymphocytes) was mostly attributable to PD-1 triggering because neutralizing anti-PD-1 Ab administration abrogated the IL-18-mediated flare up of B16F10 metastases (Fig. 3D).

IL-18 plays a pivotal role in inflammation and immune responses. Evidence in favor of the IL-18-mediated anticancer effects involve NK, T cells, or IFN γ and are usually obtained with high doses of IL-18 often combined with IL-2 or IL-12 (8, 20, 21). Cytokine doses and schedules have been discussed in the context of pathogenesis and cancer therapy and molecules such as IL-2 or IL-10 have led to opposite biological outcomes depending on their cellular targets and/or their mode of delivery [reviewed in (22, 23)]. In patients with cancer, increased IL-18 serum levels accompany tumor progression and have a negative prognostic impact (9). In the absence of Th1-like cytokines, IL-18 alone accelerates tumor progression (24), in part through cell autonomous effects on cancer and endothelial cells (24, 25). In addition, as here and to our knowledge for the first time, low dosing of IL-18 could mediate immunosuppression on the NK cell arm of immunity (as shown using a schedule of IL-18 administration twice a week or a natural tumor outgrowth leading to low-circulating levels of IL-18). Importantly, IL-18 could drive the expression of PD-1 on mature NK cells, whereas the prometastatic effects of recombinant or tumor-derived IL-18 on NK-controlled tumors involved PD-1 receptors. However, we cannot exclude that other players of innate immunity such as DC could also

express PD-1 (26) in the context of IL-18 (data not shown) that might inhibit the DC/NK cell cross-talk. Further studies will be needed to clarify this question. These data could potentially impact the clinical development of anti-PD1 and PDL-1 antibodies in IL-18-secreting human malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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