IL-18 induces PD-1-dependent immunosuppression in cancer

Magali Terme\textsuperscript{1,2,3,*}, Evelyn Ullrich\textsuperscript{1,2,4,*}, Laetitia Aymeric\textsuperscript{1,2,*}, Kathrin Meinhardt\textsuperscript{4}, Mélanie Desbois\textsuperscript{1,2}, Nicolas Delahaye\textsuperscript{1,2}, Sophie Viaud\textsuperscript{1,2}, Bernard Ryffel\textsuperscript{5}, Hideo Yagita\textsuperscript{6}, Gilles Kaplaniski\textsuperscript{7}, Armelle Prévost-Blondel\textsuperscript{8,9}, Masashi Kato\textsuperscript{10}, Joachim L. Schultze\textsuperscript{11}, Eric Tartour\textsuperscript{3}, Guido Kroemer\textsuperscript{1,12,13}, Nathalie Chaput\textsuperscript{1,14}, Laurence Zitvogel\textsuperscript{1,2,12,14}.

\textsuperscript{1} Institut Gustave Roussy, Villejuif, France
\textsuperscript{2} INSERM, U1015, Villejuif, France
\textsuperscript{3} INSERM U970 PARCC ; Université Paris Descartes. 56 Rue Leblanc. 75015. Paris. France
\textsuperscript{4} Department of Internal Medicine 5, Hematology and Oncology, University of Erlangen-Nürnberg, Erlangen, Germany
\textsuperscript{5} CNRS, IEM 2815, Institut Transgénose, Orléans, France
\textsuperscript{6} Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan
\textsuperscript{7} Service de Médecine Interne, Hôpital de la Conception, Marseille, France
\textsuperscript{8} Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France.
\textsuperscript{9} Inserm, U567, Paris, France.
\textsuperscript{10} Unit of Environmental Health Sciences, Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai-shi, Aichi 487-8501, Japan
\textsuperscript{11} Laboratory for Genomics and Immunoregulation, LIMES, University of Bonn, Bonn, Germany
\textsuperscript{12} Faculté Paris Sud-Université Paris XI, France
\textsuperscript{13} INSERM, U848, Villejuif, France
\textsuperscript{14} Center of Clinical Investigations CBT507, Biotherapy, Villejuif, France

*MT, EU and LA contributed equally to this work.

Correspondence to: Pr Laurence ZITVOGEL
U1015 INSERM
Institut Gustave Roussy
39, rue Camille Desmoulins
F-94805 Villejuif
France
Tel. 33-1-42 11 50 41
Fax 33-1-42 11 60 94
e-mail: zitvogel@igr.fr

Running title: IL-18-induced cancer immunosuppression
Edited Precis: IL-18 suppresses the crucial role of natural killer cells (NK cells) in supporting tumor immunosurveillance, with implications for clinical strategies to reverse this mechanism of tumoral immune escape.
Key words: NK cells, IL-18, PD-1, tumor immunology, tolerance
Abbreviations:

**IL-18BP**: IL-18 binding protein, **IL-18R**: IL-18 receptor, **MyD88**: myeloid differentiation primary response protein 88.

Acknowledgments:

This work was supported by INCa, ANR, Ligue contre le cancer (équipes labellisées de LZ and GK) and INFLACARE EU grant. MT, EU, KM, LA were supported by Cancéropole IDF and the Ligue Nationale Contre le Cancer, the German Research Foundation (DFG), the Fondation pour la Recherche Medicale (FRM), the German Cancer Aid, the Deutscher Akademischer Austauschdienst (DAAD) and Institut National du Cancer (INCa), respectively. We thank Pr. C. Dinarello (Denver, USA) for providing IL-18BP, and Pr. A. Mackensen (Erlangen, Germany) for critically reading the manuscript.
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 demonstrate 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.
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 anti-cancer 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.
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 (12). 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 (Supplemental Fig. 1A) 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 (2x) (Fig. 1a) while daily administration (5x) of rIL-18 reduced tumorigenesis consistent with published results (13) (Fig. 1A). Accordingly, the (2x) rIL-18-induced expansion of lung metastases was inversely correlated with intratumoral mature NK cells, defined as NK1.1+CD3-Kit (CD117) NK cells (14) (Fig. 1B). Circulating serum levels of IL-18 accumulating by day 7 were significantly higher with the 5x versus 2x regimen (Fig. 1C). These circulating levels of rIL-18 were associated with distinct NK cell phenotypes (Supplemental Fig. 1B, 1C) and cytokine patterns in spleens. The quantitative Real-time Polymerase Chain Reaction (qRT-PCR) analyses of 96 immune gene products using a microfluidic card designed for qRT-PCR on splenocytes harvested from mice treated with once (1x) versus twice a week (2x) versus 5 times (5x) daily administrations of rIL-18 revealed different gene signatures (Fig. 1D,E). We observed a significant upregulation of the IL-12p40 and inflammatory gene products (TNF, CCL5, IL-1β, IL-15, CCR4) with 5 daily injections of rIL-18 while one (1x) or two shots (2x)
of rIL-18 promoted an immunosuppressive microenvironment (significant downregulation of CCR7, Fas, upregulation of IL-10, trend towards a reduction in CXCR3, CD3ε, CD4, CD8, Pfr, CD28, CD40, STAT1) (Fig. 1D,E). These data underscore the capacity of low doses of rIL-18 to favour 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 (18kDa) bioactive IL-18 was detected in B16F10 and various tumor cell lysates (CT26, P815, RMA and RMAS) by immunoblotting (Fig. 2A, upper panel) and was released into the supernatant, as determined by an IL-18 bioassay (15) that measures IFNγ induction in splenocytes (Fig. 2A, lower panel). Transfection of B16F10 tumor cells with two distinct IL-18 small interfering RNAs (siRNAs) reduced the capacity of tumor cells to produce bioactive IL-18 (Fig. 2B, upper panel) and compromised B16F10 tumorigenesis (Fig. 2B, lower panel). 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 signalling 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) (16), the serum levels of IL-18 were markedly increased by 10 days (pre-mortem) and at 12 months respectively (Suppl. Fig. 1D, E), in the same ranges as those obtained with 2x rIL-18 scheduling (Fig. 1C). Next, we used saturating amounts of IL-18 binding protein (IL-18BP), a naturally occurring IL-18 antagonist (17), that successfully compromised tumor progression in two NK cell controlled- lung metastases models (11).
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 anti-tumor host defense.

**IL-18 upregulates PD-1 expression on mature NK cells while the pro-tumorigenic 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 (18). Therefore, we examined PD-1 expression on T and NK cells in metastases and lymph nodes (LN) of tumor bearers. Tumor sites contained significant levels of tumor infiltrating PD-1+ T lymphocytes but no PD-1+ NK cells (Fig. 3A, upper panel). Interestingly, NK cells (but not T cells) exhibited a significant upregulation of PD-1 expression in LN (Fig. 3A, lower panel). As determined by intracellular staining, PD-1 molecules were present in freshly isolated NK cells (Suppl. Fig. 2A), which however lacked surface expression of PD-1. Stimulation of NK cells with rIL-2 or other NK cell stimulatory compounds (such as IFNγ, CpG ODN, PolyI:C) induced surface exposure of PD-1 (Suppl. Fig. 2B), 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, 3C respectively). The pro-metastatic effects of rIL-18 in Nude mice (lacking 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 IFNγ and are usually obtained with high doses of IL-18 often combined with IL-2 or IL-12 (8, 19-20). 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 (rev. in (21-22). 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 (23), in part through cell autonomous effects on cancer and endothelial cells (23-24). In addition, as demonstrated 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 two-weekly schedule of IL-18 administration 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 while the pro-metastatic 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 (25) 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.

Material and Methods

Mice and tumors. Female C57Bl/6 or BALB/c mice were obtained from C.River Laboratories (L’Arbresle, France). C57Bl/6 Nude mice were purchased from Taconic (Danemark). MyD88^-/- and IL-18Rα^-/- mice backcrossed on a C57Bl/6 background were kindly provided by B. Ryffel (CDTA Orléans, France). RET^-/- mice were obtained from A. Prévost-Blondel (Institut Cochin, Paris, France) with the permission of Pr. 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, $3 \times 10^5$ B16F10 or CT26 were inoculated iv into C57Bl/6 or BALB/c mice, respectively. Mice 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 (Mipneapolis, US). Neutralizing anti-PD-1 antibody (RPM1-14) was kindly provided by Dr. Hideo Yagita (Tokyo, Japan) (26). 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 Mountain View, CA), and Flow-JO Software (TreeStar, Ashland, OR).

**IL-18 dosage.** Serum concentrations were evaluated by mouse IL-18 ELISA Kit (MBL, USA).

**In vivo IL-18 neutralization.** IL-18BP was kindly provided by Pr. C. Dinarello (Denver, USA) (17). At day 0, $3 \times 10^5$ B16F10 or CT26 tumor cells were injected iv into C57Bl/6 or BALC/c mice respectively. Mice received 20 μg of IL-18BP (or saline buffer) ip daily from day 0 to day 8 with or without depleting anti-NK1.1 Ab (300 μg injected ip on day 0, 3, 6, 9). Anti-PD-1 antibody was injected ip (250 μg/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 StealthTM Select RNAi (MSS205424, MSS205426) and control siRNA (Stealth RNAi Negative Control Med GC) (Invitrogen, Carlsbad CA) 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 μg of total protein extracts were solubilized in Laemmli loading buffer, separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules CA). After blocking for 1 h, blots were incubated overnight at 4°C with antibodies against IL-18 (rabbit anti-mouse IL-18, 2 μg/ml; BioVision, Inc., Mountain View CA) and GAPDH (mouse anti-glyceraldehyde-3-phosphate dehydrogenase, 1/10000; Chemicon international, Temecula, CA). Protein detection was performed with HRP-conjugated secondary antibodies (Southern Biotech, Birmingham, AL) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford IL).
References


Figure Legends

**Figure 1. Biweekly administration of rIL-18 promotes metastases dissemination.**

**A. Dose-dependent effects of rIL-18 on B16F10 lung metastases.** C57Bl/6 mice were inoculated i.v. with 3x10^5 B16F10 cells and received biweekly 1 μg of rIL-18 ip (2x) or every day for 5 days (5x). Pulmonary metastases were enumerated at day 12. One representative experiment out of 3 with 4-5 mice/group is shown. **B. Concomitant attrition of mature NK cells in tumor beds upon biweekly rIL-18 scheduling.** Same experimental setting as in (A.) but mice received only biweekly 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 out of 2 experiments yielding similar results. **C. Circulating levels of IL-18 in various scheduling of administration of rIL-18.** Serum concentrations of mIL-18 assessed by ELISA in 3 animals per group at day 3 and 6 in the daily (5x) versus biweekly (2x) scheduling. **D-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 performed 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 three mice). Red and green indicate expression levels above and below the PBS treated control value, respectively. Dendograms 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 ΔΔCT method (normalization with B2m as endogenous control) of transcripts pooled from two 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.

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 (upper panel). A bioassay measuring the IL-18-driven IFNγ production by murine splenocytes (15) was performed by incubating splenocytes with rmIL-18 or B16F10 supernatants (1:4 dilution) in the presence of anti-IL-18 neutralizing Ab or isotype control Ab. The levels of IFNγ were monitored by ELISA in the splenocyte cultures at 72 hrs (lower panel). One representative experiment out 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 two different IL-18-depleting siRNAs (siRNA #1, #2) (upper and middle panels). Enumeration of lung metastases after inoculation of B16F10 transfected with various siRNA (lower panel). 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 Materiel & Methods. Quantification of metastases numbers for CT26 in BALB/c (upper panel) and for B16F10 in C57Bl/6 mice.
(lower panel) was performed. **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,C,D) depict the pooled results of 3 independent experiments containing at least 2-4 mice/group. Panel E shows a representative experiment out of 2. The Mann-Whitney test is used for statistical analyses in panel B and D, the Kruskal Wallis multiple comparison test was used for statistical analyses in panel C, **p<0.01, ***p<0.001.

**Figure 3. IL-18 upregulates PD-1 expression on NK cells and mediate a PD-1-dependent prometastatic effect.**

**A. PD-1 expression on T and NK cells in tumors and lymph nodes.** PD-1 expression was examined by gating on CD3⁺ T cells (right panel) and CD3⁻NK1.1⁺ NK (left panel) contained in B16F10 lung metastases and lymph nodes (LN) from tumor-bearing or naive mice. The results are pooled from 2 independent experiments with 3-5 mice/group. **B-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 hrs in vitro in 25ng/ml of rmIL-18 (B) or of LN residing Kit⁻NK cells 24 hrs after ip administration of 1μg of rmIL-18 (C). A representative experiment out 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 (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 two experiments containing 3 mice/group. The Mann-Whitney test is used for statistical analyses in panel A, C, the Kruskal Wallis multiple comparison test was used for panel D. *p<0.05, **p<0.01, ***p<0.001.
Figure 1

A- Metastases number

B- Mature NK cell number (10^6)

C- miR-18 (ng/mL) vs days

D- IL-18

E- Pathway analysis

PBS

IL-18

5x

2x

1x

IL-12b

Tnf

Ccl5

IL-1b

Ccr7

Fas

Socs1

IL-10

Downloaded from cancerres.aacrjournals.org on November 10, 2017. © 2011 American Association for Cancer Research.
A -

B -

C -

D -

E -

Figure 2
Figure 3
IL-18 induces PD-1-dependent immunosuppression in cancer.

Magali Terme, Evelyn Ullrich, Laetitia Aymeric, et al.

Cancer Res  Published OnlineFirst July 1, 2011.