Augmentation of Immune Checkpoint Cancer Immunotherapy with IL-18

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**Translational Relevance**

Although immune checkpoint inhibitors are effective in some cancer patients, the efficacy may be further improved in combination with other therapeutic modalities. In
In the present study, we analyzed the effect of IL-18 on the anti-tumor effect of immune checkpoint blockade. IL-18 is a cytokine of IL-1 family members and is formed by the action of activated inflammasomes, but its physiological roles are not completely clarified. Recently, it was demonstrated that IL-18 promoted the expansion and survival of effector cells including NK, γδ T, and CD8+ T cells. However, clinical efficacy of IL-18 cancer monotherapy has been shown to be negligible. Here, we show that IL-18 efficiently augmented the expansion of effector cells in the presence of immune checkpoint antibodies, and reduced the proportion of regulatory T cells. In addition, immune checkpoint inhibitors and IL-18 synergistically inhibited the growth of tumor cells without significant adverse events in animal models. The results strongly suggest that a combination of IL-18 and immune checkpoint inhibitors could be beneficial in treatment of cancer patients.
Abstract

Purpose: Recent clinical trials and animal models demonstrated that immune checkpoint blockade enhanced effector cell responses and tumor rejection, however, further development and improvement of cancer immunotherapy is necessary for more favorable objective responses. In this study, we examined the effect of IL-18 on the anti-tumor effect of immune checkpoint inhibitors.

Experimental design: We examined the effect of IL-18 on the peritoneal dissemination of CT-26 cells or tail vein injection metastasis of B16/F10 cells using anti-programmed death-1 ligand-1 (αPD-L1) and/or anti-cytotoxic T lymphocyte-associated antigen-4 (αCTLA-4) monoclonal antibodies.

Result: Massive ascites developed after i.p. inoculation of CT-26, resulting in animal death within 30 days. Treatment of mice with αPD-L1 and/or αCTLA-4 significantly prolonged their survival, and a combination of the antibodies and IL-18 provided a much greater therapeutic benefit. The combination modality led to the accumulation of precursor of mature natural killer (pre-mNK) cells in the peritoneal cavity together with increased CD8^+ T and decreased CD4^+CD25^+Foxp3^+ T cells. Depletion of the pre-mNK cells abrogated the therapeutic effects and increased the number of CD4^+CD25^+Foxp3^+ T cells. The combination treatment also suppressed tail vein injection metastasis of
B16/F10 cells.

**Conclusion:** The results demonstrated that IL-18 enhanced therapeutic effects of immune checkpoint blockade against peritoneal dissemination of carcinoma or tail vein injection metastasis of melanoma through accumulation of pre-mNK cells, memory-type CD8$^+$ T cells, and suppression of CD4$^+$CD25$^+$Foxp3$^+$ T cells. A combination of immune checkpoint inhibitors with IL-18 may give a suggestion to the development of next-generation cancer immunotherapy.
Introduction

T cell responses are controlled by T cell receptors (TCRs) and non-clonotypic immune co-receptors. Whereas co-stimulatory receptors such as CD28 and inducible T cell co-stimulator (ICOS) amplify the transcriptional effects of TCR signaling, co-inhibitory receptors including cytotoxic T lymphocyte (CTL)-associated protein-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene 3 (LAG3), and transforming growth factor-β (TIM3) reduce effector functions. Tumor growth can thus be restricted by co-stimulatory signaling and facilitated by co-inhibitory pathways. Tumor cells often usurp the co-inhibitory system, also termed the immune checkpoint, which controls the anti-tumor effects of immune effector cells (1-4).

Recently, research has focused on the development of cancer immunotherapy that targets the immune checkpoint pathways using monoclonal antibodies (mAb), anti-CTLA4 (αCTLA-4), anti-PD-1 (αPD-1), and/or anti-PD-1 ligand-1 (αPD-L1) (5-7). In clinical trials, αCTLA-4 was shown to improve the overall survival rate in patients with advanced melanoma, refractory to conventional therapies (8-10). Because CTLA-4 signaling is involved in the down-regulation of T cells in the priming phase, the blockade of CTLA-4 signaling promotes cytotoxic T cells, however, αCTLA-4 induces severe adverse reactions (1-4). By contrast, the PD-1 pathway plays an essential role in
the regulation of immune cells, including αβ T cells, γδ T cells, and NK cells, in the effector phase. In a phase I clinical trial of a fully human αPD-1/αPD-L1, objective responses were observed in patients with melanoma, non-small-cell-lung cancer, renal cell cancer, bladder cancer (11, 12), non-Hodgkin's lymphoma, Hodgkin's lymphoma (13), esophageal cancer (14) and hepatocellular cancer (15). The non-redundant roles and distinct adverse reactions of αCTLA-4 and PD-1 pathways suggest the potential of dual immune checkpoint blockade using a low-dose of αCTLA-4 and a high-dose of αPD-1 or αPD-L1 (16, 17).

Effective anti-tumor responses to treatments require not only a blockade of co-inhibitory pathways, but also an increase in the number of immune effector cells. It has been demonstrated that the development, cytokine production, and cytotoxicity of effector cells can be modulated by various cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21. Among them, IL-18 is a unique cytokine that is maturated by inflammasome complexes (18, 19), and modulates anti-tumor responses (20-22). It is demonstrated that IL-18 markedly enhances the expansion of NK cells, CD8+ CTLs, and γδ T cells which express IL-18 receptors α/β chains and receptors containing immunoreceptor tyrosine-based activation motif (ITAM), and produce IFN-γ (23−27). In humans, IL-18 promotes the expansion of CD56brightCD11c+HLA-DR+ helper NK cells, which may be
a human counterpart of mouse precursors of mature natural killer (pre-mNK) cells, previously referred to as IFN-producing killer dendritic cells (28, 29). IL-18-induced NK-lineage cells exhibit potent anti-tumor activity, although NK cells expressing inhibitory receptors, such as CTLA-4 and PD-1, may have regulatory functions. It is thus worthwhile to examine the effect of IL-18 on cancer immunotherapy using blocking mAbs specific to co-inhibitory pathways.

Prognosis of peritoneal dissemination of cancer is poor, even though a combination of cytoreduction with hyperthermic intraperitoneal chemotherapy has become a standard therapy (30). By contrast, there have been several studies examining immunotherapy for the treatment of peritoneal disseminated cancer (31-33). In this study, we examined the effect of IL-18 on cancer immunotherapy using αPD-L1 and/or αCTLA-4 in animal models of peritoneal dissemination of CT-26 colon carcinoma, and analyzed the mechanism underlying IL-18-mediated enhancement of anti-tumor activity.
Materials and Methods

Animals and tumor cells

Six- to eight-week old male BALB/c and C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Mice were housed in a pathogen-free condition at 25°C under controlled lighting (12 h light/12 h dark), with free access to water and food pellets. The CT-26 (CRL-2638), CT-26.CL25 (CRL-2639) mouse colon carcinoma cell lines, 4T1 (CRL-2539) mouse breast carcinoma cell line and B16/F10 (CRL-6475) mouse melanoma cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The CT-26, CT-26.CL25 and 4T1 cells were maintained in RPMI1640 medium (Nacalai Tesque, Inc, Nakagyo-ku, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaill, France), 100 units/mL penicillin, 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY), B16/F10 cells were maintained in DMEM (Nacalai Tesque, Inc, Nakagyo-ku, Kyoto, Japan) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Reagents

Recombinant mouse IL-18 was provided by GlaxoSmithKline PLC (Research Triangle...
Park, NC), whereas mouse IL-2 was purchased from R&D Systems Inc. (Minneapolis, MN). Anti-mouse CD152 (CTLA-4) antibody (αCTLA-4, clone UC10-4F10-11), anti-mouse CD274 (PD-L1) antibody (αPD-L1, clone 10F.9G2), and control isotype-matched hamster IgG were purchased from BioXcell (BE0091, West Lebanon, NH). Rabbit anti-asialo GM1 (αGM1) was purchased from Wako Pure Chemical Industries Ltd (014-09801, Chuo-ku, Osaka, Japan). Rabbit IgG was purchased from Medical and Biological Laboratories Co., Ltd (PM035, Nagoya, Japan). Anti-mouse CD8 mAb (αCD8) was purchased from Santa Cruz Biotechnology, Inc. (clone 53-6.7, Dallas, TX).

**Tumor cell implantation and immune checkpoint blocking**

Sub-confluent CT-26, CT-26.CL25, 4T1 and B16/F10 cells were harvested by detaching with 0.05% trypsin and 0.53 mM EDTA (trypsin-EDTA) (Nacalai) or 0.25% trypsin and 0.53 mM EDTA (trypsin-EDTA) (Nacalai) in Ca²⁺, Mg²⁺ free Dulbecco’s PBS, pH 7.4 (Nacalai) and washed with PBS. The viable cells were counted by Trypan Blue dye exclusion and suspended in PBS. CT-26 and 4T1 cells suspensions (5 × 10⁶ cells in 250 μL of PBS) were intraperitoneally (i.p.) injected into BALB/c mice, CT-26.CL25 cells suspensions (1 × 10⁵ cells in 100 μL of PBS) were subcutaneously (s.c.) injected into
BALB/c mice, and B16/F10 cells suspensions (2 × 10^5 cells in 200 µL of PBS) were intravenously (i.v.) injected into C57BL/6J mice. After 3, 7, or 10 days post inoculation, various doses of αCTLA-4, αPD-L1, and/or IL-18 were i.p. injected 4 times every 4 days. For depletion of NK cells or CTL, αGM1 or αCD8 was administered to the mice. The abdominal perimeter was measured to estimate the amount of ascites, and the survival rate was monitored until 90 days post-inoculation of tumor cells.

**Cell preparation and cytokine assay**

Peritoneal exudate cells (PECs) were harvested from the abdominal cavity and washed in 5mL PBS three times. Red blood cells were eliminated by ACK lysis buffer (500 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA at pH 7.2–7.4), and the resulting cells were washed three times with PBS. Cells viability was assessed by Trypan Blue dye exclusion. CT-26 cells were lysed by repeated freezing and thawing, and the resultant cell debris was washed and re-suspended in PBS. PECs were stimulated with the CT-26 cell preparation and cultured in the absence or presence of IL-12 (1 ng/mL) and/or IL-18 (100 ng/mL) in RPMI1640 medium supplemented with 10 % fetal bovine serum, L-glutamine (Gibco BRL, Grand Island, NY), 100 units/mL penicillin, 100 µg/mL streptomycin and 10⁻⁵ M 2-mercaptoethanol (2ME) at 37°C in a humidified
atmosphere with 5% CO₂. After incubation, cytokines in the culture supernatants were measured using their corresponding ELISA kits according to the manufacturer’s instructions (PeproTech, Rocky Hill, NJ).

**In Vitro Cytotoxicity Assay:**

Cytotoxicity was assessed in vitro using the DELFIA EuTDA Cytotoxicity Assay System (PerkinElmer Life Sciences, Wallac Oy, Turku, Finland). Briefly, CT-26 cells were labeled with a chelate prodrug for 10 min, washed, and resuspended in RPMI1640 medium. The PECs from mice which had been treated with mAbs and/or IL-18 were suspended in RPMI1640 medium. The resulting cells were mixed at the indicated effector/target ratios and incubated for 3 hr at 37°C. The supernatants were mixed with europium solution and the time-resolved fluorescence was measured using a multiplate reader. The percentage of specific lysis was calculated as follows: 
\[
\text{percentage of specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100\%
\]

**Flow cytometry**

The PECs were examined for cell surface markers using a FACS flow cytometer. The cells were stained with FITC-conjugated anti-CD4 mAbs (GK1.5), APC-conjugated
anti-CD4 mAbs (RM4-5), biotin-conjugated anti-CD8 mAb (53-6.7), APC-conjugated anti-CD314/NKG2D mAb (CX5), FITC and APC-conjugated anti-β TCR mAb (H57-597), PE-conjugated Foxp3 mAb (FJK-16s), APC-conjugated anti-streptavidin (catalogue 17-4317-82), which were purchased from eBioscience Inc. (San Diego, CA). APC-conjugated anti-CD8 mAb (53-6.7), PE and APC-conjugated anti-CD45R/B220 mAbs (RA3-6B2) were purchased from Biolegend Inc. (San Diego, CA). FITC-conjugated anti-CD11b mAb (M1/70), biotin-conjugated anti-CD11c mAb (HL3), PE-conjugated anti-CD25 mAb (PC-61), FITC and PE-conjugated anti-CD49b mAbs (DX5), PE-conjugated anti-CD122 mAb (TM-β1), PE-conjugated anti-NK1.1 mAb (PK136), and FITC-conjugated anti-Streptavidin Ab (catalogue 554460) were purchased from BD Bioscience (San Jose, CA). The cells were incubated with Abs for 20 min at 4°C, and then analyzed using a FACS Calibur flow cytometer. Anti-mouse CD16/32 mAb (clone: 93, eBioscience) was used as a Fc blocker. The data were processed with CellQuest software (BD Biosciences).

**Measurement by ImageJ**

To determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined using an ImageJ software 1.48 (National Institutes of Health, USA). Tumor volume was calculated by the modified ellipsoidal
formula: Tumor volume = \( \frac{1}{2}(\text{length} \times \text{width}^2) \).

**Statistical analysis**

Data were statistically analyzed by SPSS 20.0.0 software (IBM, New York, USA) and expressed as means ± SD. Two groups measurements were analyzed with a two-tailed Student’s \( t \) test, and the other groups were analyzed using one-way ANOVA with Tukey’s multiple comparison test. A \( p \) value < 0.05 was considered significant, NS = non-significant. Survival was estimated using Kaplan-Meier followed by a log-rank test.

**Study approval**

All mice were bred and maintained in our animal facility in accordance with the guidelines for the care and use of experimental animals in Hyogo College of Medicine, Japan. All experiments were conducted with the approval of the Animal Care Committee of Hyogo College of Medicine.

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Results

IL-18 enhances anti-tumor activity elicited by αPD-L1 and/or αCTLA-4

The blockade of immune checkpoints such as PD-1 and CTLA-4 signaling pathways...
induces clinical benefits in limited population of patients with various cancers (11-15). Therefore, it is important that αPD-1/PD-L1- and/or αCTLA-4-based therapies are analyzed in a wider range of patients. Because effective anti-tumor responses require immune activation as well as immune checkpoint blockade, we examined the synergistic effect of IL-18, which was demonstrated to enhance effector functions of immune cells (25, 26, 38, 39) on the survival rate of pre-implanted mice receiving αPD-L1 and/or αCTLA-4. To develop an in-vivo model of peritoneal dissemination, mice were i.p. inoculated with the CT-26 colon carcinoma cells. When pre-implanted mice were administered with control IgG, all mice died of peritoneal dissemination of tumor cells accompanying pool of ascites and abdominal adhesions within 30 days (Fig. 1A-D, Supplementary Figs. S1A-D). Treatment of 3-day pre-implanted mice with either IL-18, αCTLA-4, or αPD-L1 alone demonstrated modest therapeutic effect. It is of note that combination of IL-18 and CTLA-4 or PD-L1 blockade promoted tumor rejection with definite significance by at least day 90 (Fig. 1A, Supplementary Figs. S1A and B). When 7-day pre-implanted mice were treated with the combination therapy of IL-18 and immune checkpoint blockade, 40% and 80% of mice survived more than 90 days for αPD-L1 and αCTLA-4, respectively (Fig. 1B). Although dual blockade of PD-L1 and CTLA-4 pathways also had modest effects on the survival rate of 7-day...
pre-implanted mice, the addition of IL-18 enhanced tumor rejection in all mice by day 90 (Fig. 1C). Although dual blockade of PD-L1 and CTLA-4 signaling elicited therapeutic effects on the survival rate of 10-day pre-implanted mice, inclusion of IL-18 further promoted tumor rejection in 20% of mice by at least 90 days (Fig. 1D).

To further analyze the mechanism underlying the anti-tumor activity elicited by IL-18 and immune checkpoint blockade, the optimal doses of the recombinant proteins were determined in the mouse model. When CT-26 pre-implanted mice were treated with IL-18 and αCTLA-4, all mice receiving 2 μg of IL-18 and 50 or 100 μg of αCTLA-4 survived until day 90, while lower doses of IL-18 and αCTLA-4 had moderate therapeutic effects. Similarly, all mice treated with 2 μg of IL-18 and 200 μg of αPD-L1 survived until day 90 and reduced effects were observed with a lower αPD-L1 dose (Supplementary Figs. S1E and F).

Moreover, combination of IL-18 and dual blockade of PD-L1 and CTLA-4 was effective also in the treatment of mice subcutaneously (s.c.) injected with CT-26.CL25 cells (Supplementary Figs. S1G and H), but the combination failed to prevent the abdominal growth of 4T1 cells (Fig. 1E). IL-18 also enhanced the therapeutic effect of immune checkpoint blockade by αPD-L1 and/or αCTLA-4 in the tail vein injection metastasis model using CT26 cells (data not shown) and in C57BL/6 mice inoculated
with B16 mouse melanoma cells (Fig. 1F, Supplementary Fig. S1I).

Combination of immune checkpoint blockade with IL-18 allows for an accumulation of the precursors of mature NK cells

In order to elucidate the mechanism by which immune checkpoint blockade combined with IL-18 provides favorable therapeutic effects, we next examined the number of PECs. When tumor-free mice were injected with a combination of αCTLA-4, αPD-L1, and IL-18, no detectable increase in the number of PECs was observed (data not shown). Treatment of CT-26 pre-implanted mice with either IL-18, αCTLA-4, or αPD-L1 alone induced modest accumulation of PECs. By contrast, there was a large accumulation of PECs when CT-26 pre-implanted mice were treated with a combination of immune checkpoint blockade with IL-18 (Fig. 2, A-C). Although the accumulation of PECs reached a maximum at day 6 and declined thereafter, a subsequent injection of mAbs and IL-18 on day 7 promoted re-accumulation of PECs and a sustained level of PECs was observed even at day 11. The number of CT26 tumor cells contained in the PECs of CT-26 pre-implanted mice injected with control IgG was significantly greater compared with the mice treated with combination of immune checkpoint blockade and IL-18 (Fig. 2D, E).
To characterize the PECs recruited by the tumor cells, mAbs, and/or IL-18, the cells were stained with mAbs specific for DX5 (CD49b), B220 (CD45R), CD11b, CD11c and NKG2D (CD314). It was of note that 10 - 30 % of PECs comprised DX5⁺B220⁺ NK cells 4 days after mAb plus IL-18 administration to CT26 cell-implanted mice (7 days post-inoculation of CT26 cells). In addition, B220 and NKG2D were expressed on the majority of the NK cells in the PECs and the expression levels of B220, CD11b and CD11c were significantly increased by the injection of IL-18 (Fig. 3A, Table 1, and Supplementary Fig. S2A). The expression of B220 on NK cells was further enhanced 8 days after mAbs plus IL-18 administration (day 11 post-inoculation of CT 26 cells) (Fig. 3B, Table 1). The B220⁺ NK cells also expressed CD11b, CD11c and CD122 and were most likely to be pre-mNK cells (Table 2), formerly called IFN-γ-producing killer dendritic cells (IKDC) (28, 29).

Phenotypic analyses of T cells in the PECs from pre-implanted mice treated with mAbs and IL-18

Analysis of the T cell subsets in the PEC was undertaken by staining the cell surface markers with mAbs. Although IL-18 did not affect the proportion of CD4⁺ cells in the PECs of mice administrated αCTLA-4, IL-18 strongly reduced CD4⁺ cells in the PECs
stimulated by αPD-L1 or by αCTLA-4 plus αPD-L1 (Fig. 4A and Supplementary Fig. S2B). CD4⁺ T cells occupied 10.95 % ± 1.21 % of the PECs from mice injected with αPD-L1 plus IL-18 four days after CT-26 inoculation, although 20.35 % ± 2.16 % of PECs from mice treated with αPD-L1 alone were CD4⁺ T cells. This effect was pronounced when αCTLA-4 plus αPD-L1 regimen (23.18 % ± 2.76 %) was compared with the αCTLA-4 plus αPD-L1 plus IL-18 regimen (5.88 % ± 1.03 %). This suggests that a combination of IL-18 and immune checkpoint blockade potently decreases CD4⁺ T cell/effector T cell ratios in the PECs (Fig. 4A and Supplementary Fig. S2B). This was confirmed by measuring the proportion of CD25⁺Foxp3⁺ cells in the PECs. Administration of αCTLA-4 plus αPD-L1 reduced the proportion of CD25⁺Foxp3⁺ cells, while a combination of IL-18 with the mAbs further reduced CD25⁺Foxp3⁺ cells. The proportion of CD25⁺Foxp3⁺ cells in the control IgG CD4⁺CD25⁺ PECs was 65.23 % ± 3.11 %, and this was reduced to 54.88 % ± 3.07 % by αCTLA-4 plus αPD-L1, and further reduced to 47.67 % ± 3.15 % by αCTLA-4 plus αPD-L1 plus IL-18. By contrast, in the TCR-β chain⁺CD8⁺ cells, IL-18 appeared to have no effect on their proportion (Fig. 4A). The decrease in the proportion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the PECs by IL-18 was observed even when they were analyzed 8 days after administration of αCTLA-4 plus αPD-L1 with or
without IL-18 (51.03 % ± 2.75 % vs. 59.31 % ± 4.32 %, respectively), while the proportion of the control was rather high (77.02 % ± 4.12 %) (Fig. 4B). In addition, the proportion of CD8⁺ T cells was decreased by IL-18 administration, although the expression of NKG2D on the CD8⁺ T cells was slightly increased (Fig. 4B).

**Functional analyses of PECs from pre-implanted mice treated with immune checkpoint blocking mAbs and/or IL-18**

Because immune checkpoint blockade in combination with IL-18 promoted accumulation of pre-mNK cells in the PECs and shifted the peritoneal environment from dominant suppression to an inflammatory milieu ideal for immune effector cell-mediated tumor rejection, we next analyzed responses of PECs to various stimuli to determine their functions. The results show that IFN-γ was released from the PECs derived from CT-26 mice. Treatment of mice with αCTLA-4 plus αPD-L1 enhanced the production of IFN-γ, and IL-18 further promoted IFN-γ production (Supplementary Fig. S3A). By contrast, a high level of IL-10 was produced by the PECs and the IL-10 secretion was reduced when mice were treated with immune checkpoint blocking mAbs. The decrease in IL-10 production was more prominent in the presence of IL-18 (Supplementary Fig. S3B), and TGF-β production was also attenuated by mAbs.
treatment. The addition of IL-18 reduced the cytokine secretion to a background level (Supplementary Fig. S3C). A low level of IFN-γ was produced by PECs after administration of IL-18 plus mAbs 10 days after tumor inoculation (Supplementary Fig. 3D). By contrast, a high level of TGF-β was produced by PECs induced by control IgG, and the secretion of TGF-β was reduced when mice were treated with immune checkpoint blocking mAbs (Supplementary Fig. S3D).

Next, the effect of immune checkpoint blocking mAbs and/or IL-18 on the cytotoxicity of PECs was determined. A combination of αCTLA-4 and αPD-L1 slightly increased the tumoricidal activity of the PECs and the addition of IL-18 markedly enhanced the cytotoxicity of PECs against CT-26 cells (Supplementary Fig. S4A). Repeated injection of αCTLA-4 plus αPD-L1 and/or IL-18 revealed that IL-18 helped sustain a high level of anti-tumor activity of PECs (Supplementary Fig. S4B). Based on the results, it was most likely that enhanced secretion of inflammatory cytokines and tumoricidal activity of the PECs were responsible for the protection of mice from aggressive tumor cells.

**Negation by pre-mNK cell deletion of the therapeutic effect of immune checkpoint blockade and IL-18**
We next attempted to identify cell subsets in the PECs responsible for tumor rejection and improved survival rate. Upon i.p. inoculation of tumor cells and treatment by immune checkpoint blockade and IL-18, the pre-mNK cells were accumulated in the peritoneum as shown in Fig. 3. The result prompted us to examine the effect of NK cell depletion on the survival rate and cytotoxic activity of effector cells in the PECs. After administration of $\alpha$CTLA-4, $\alpha$PD-L1, IL-18, and control IgG into CT-26 pre-implanted mice, all mice survived more than 90 days. By contrast, injection of anti-asialoGM1 Ab ($\alpha$GM1) instead of control IgG almost completely negated the therapeutic effect of the combination therapy, indicating that pre-mNK cells play a pivotal role in tumor rejection (Fig. 5A). In addition, the cytotoxic activity of the PECs against CT-26 was significantly reduced by the addition of $\alpha$GM1 (Fig. 5B). Repeated injections of $\alpha$GM1 further reversed the beneficial effects of the tumor immunotherapy using immune checkpoint blocking mAbs and IL-18 (Fig. 5C).

Modulation of pre-mNK cell and CD4$^+$CD25$^+$ T cell accumulation by $\alpha$GM1

The numbers of PECs were essentially the same between pre-implanted mice which had been injected with $\alpha$GM1, followed by $\alpha$CTLA-4, $\alpha$PD-L1, and IL-18, and those with control IgG. The percentage of TCR-\(\beta^\)DX5$^+$B220$^+$ pre-mNK cells was, however,
significantly reduced by the treatment with αGM1 (Supplementary Fig. S5A). It is noteworthy that αGM1 markedly increased the accumulation of CD4⁺CD25⁺ T cells, and that the administration of αGM1 significantly reduced the number of CD8⁺ T cells (Supplementary Fig. S5B). The results clearly demonstrated that αGM1 treatment led to an increase in the ratio of CD4⁺CD25⁺ regulatory T cells to TCR-β⁺DX5⁺B220⁺ pre-mNK effector cells, shifting anti-tumor cellular responses to immune suppression.

**Immunosuppressive milieu induced by αGM1**

To examine the tumor environment in the peritoneum, cytokine production was determined after administration of αGM1. When pre-implanted mice were treated with immune checkpoint blocking mAb plus IL-18, the inclusion of αGM1 reduced the secretion of IFN-γ from PECs (Supplementary Fig. S6A). By contrast, the production of IL-10 and TGF-β was markedly enhanced (Supplementary Figs. S6B, C), demonstrating that αGM1 converted the inflammatory environment in the peritoneum into immunosuppressive milieu.

**Essential role of CD8⁺ T cells in tumor rejection**

Because αGM1 treatment decreased efficacy of immune checkpoint blockade and IL-18
with concomitant reduction of CD8⁺ T cells, we analyzed the role of CD8⁺ T cells in tumor immunotherapy. When pre-implanted mice received αCTLA-4, αPD-L1, and IL-18, these mice survived more than 90 days. The addition of anti-CD8 mAb (αCD8), however, significantly reduced the survival rate and all mice died within 32 days (Fig. 6A). In functional analyses, cytotoxic activity of the PECs derived from pre-implanted mice treated with αCTLA-4, αPD-L1, IL-18, and αCD8 was significantly impaired, when compared with treatment without αCD8 (Fig. 6B). The immune impairment in the PECs was observed even 9 days after treatment (Fig. 6C), which strongly suggests that CD8⁺ T cells played a critical role in anti-tumor responses.

**Phenotypic analyses of PECs after treatment with αCD8**

Administration of αCD8 to mice receiving CT-26, αCTLA-4, αPD-L1, and IL-18 greatly reduced the CD8⁺ population in the PECs, whereas the proportion of CD4⁺ T cells was not significantly altered (Supplementary Fig. S7A). The proportion of TCR-β⁺B22₀⁺DX₅⁺ pre-mNK cells was not markedly influenced by the addition of αCD8 (Supplementary Fig. S7B), demonstrating that the accumulation of CD4⁺ T cells and pre-mNK cells in the peritoneum was regulated by a mechanism that is independent of CD8⁺ T cells, and that the anti-tumor activity of the CD8⁺ T cells was essential for
tumor rejection.
Discussion

Immunotherapy targeting immune checkpoint pathways is effective in some cancer patients such as metastatic melanoma, lung cancer, and kidney cancer (1, 11-15, 31). The blockade of immune checkpoints like PD-1/PD-L1 and CTLA-4/CD80 pathways reinvigorates immune effector cells and leads to tumor rejection (5-7). Even though dual blockade of the PD-1 and CTLA-4 pathways results in synergistic effects in anti-tumor responses (16, 17), the therapeutic intervention fails to completely eliminate malignant tumors. It is thus necessary to further develop and improve immune checkpoint blocking therapy. In the present study, we demonstrated that IL-18 markedly improved the survival rate of pre-implanted mice treated with αCTLA-4 and αPD-L1. In a tail vein injection metastasis model using B16 melanoma cells, IL-18 exerted a similar effect on treatment by immune checkpoint blockade. In contrast IL-18 failed to enhance the effect of immune checkpoint blockade against intraperitoneal growth of 4T1 breast cancer cells. This indicated that the effect of IL-18 as well as immune checkpoint blockade is not effective on all the tumor cells type as is the fact that the therapy is not effective on all of the cancer patients. Although the reasons for different responses among tumor cell types, it seems that the efficacy of the therapy is dependent on whether the cancer is able to induce inflammatory responses or not. Inflammagogenic tumors may be susceptible to the therapy, while others may not respond to it effectively.
The strength of antigenicity may be dependent on the frequency of antigenic mutation of the tumor cells, or on the intensity of PD-L1 or CTLA4 in tumor cells, or on the expression of stress-induced molecules such as MICA/MICB (34). These must be further explored.

While IL-18 was originally discovered as an IFN-γ-inducing factor (22, 24), precise physiological functions of this cytokine have not yet been fully elucidated. IL-18 is converted to its mature form by the action of inflammasome complexes (18, 20, 21), which are a caspase-1-activating apparatus composed of multiple proteins (19). They sense and respond to various stresses such as infection, reactive oxygen species, and tissue damage (20-22). In addition, they are linked to cellular metabolism and to events such as pyroptosis and mitochondria autophagy, regulating cell viability. IL-18 may thus play an important role in the responses to cellular stress and is involved in maintenance of energy metabolism homeostasis and in quality control of organelles, leading to cell survival and expansion of activated lymphocytes (25-27).

Beneficial effects of IL-18 in immune checkpoint blocking therapy may be attributable to multiple mechanisms including activation and expansion of cytotoxic pre-mNK cells, reduced accumulation of regulatory T cells, and suppression of soluble inhibitor secretion such as IL-10 and TGF-β. In humans, IL-18 promotes development
and expansion of \( \text{CD56}^{\text{high}}\text{CD11c}^+\text{CD25}^{\text{high}}\text{CD86}^{\text{high}}\text{HLA-DR}^{\text{high}}\text{HLA-DQ}^{\text{high}} \) NK cells (termed helper NK cells) in the presence of IL-2, which in turn enhance the expansion of effector \( \gamma\delta \) T cells (25-27). It is most likely that human helper NK cells are a counterpart of mouse pre-mNK cells (28, 29). If this is the case, mouse pre-mNK cells may induce the development and expansion of effector T cells. Because both pre-mNK cells and effector T cells exhibit cytotoxic activity against tumor cells, IL-18 unambiguously promotes induction of immune effector cells, leading to eradication of tumors.

It has been demonstrated that dual blockade of the PD-1 and CTLA-4 pathways increased the immune effector cell/regulatory T cell ratio in an animal model (16, 17). The addition of IL-18 in this system further decreases the number of regulatory T cells, although the precise mechanism remains elusive. Thus it will be of interest to examine the relationship between increased pre-mNK and decreased regulatory T cells. Because the functions of IL-18 can be defined by the environment, it is likely that IL-18 simply enhanced the effect of \( \alpha \text{CTLA-4} \) and \( \alpha \text{PD-L1} \), resulting in a decrease in the number of regulatory T cells. Similarly, effective suppression by IL-18 of soluble inhibitor secretion is also achieved by the promotion of immuno-modulatory effects of \( \alpha \text{CTLA-4} \) and \( \alpha \text{PD-L1} \).
Upon engagement with its receptor, IL-18 activates p38 MAPK, PI3K/AKT, and ERK pathways as well as the MyD88-IRAK-TRAF6 signaling pathway (35-37). IL-18 thus executes multiple signals involved in cellular viability, growth, differentiation, migration, and cytokine secretion. In addition, IL-18 activates signals related to Bcl-2 and Bcl-XL, which may account for the protection of lymphocytes from activation-induced cell death and programmed cell death (25, 26, 38, 39), resulting in a large accumulation of PECs. Because efficient cytokine production is often accompanied by large expansion of cytokine-producing cells, immune effector cells like pre-mNK cells may be expanded via the Bcl family molecule-mediated signals executed by IL-18.

It is worth noting that IL-18-deficient mice are prone to the development of spontaneous tumors, and that IL-18 has potent anti-tumor activity. The cytokine, however, fails to directly act on and eradicate tumor cells, suggesting that IL-18 activates and expand immune effector cells and indirectly exhibits anti-tumor activity. In line with the findings, depletion of pre-mNK cells or CD8\(^+\) T cells reversed anti-tumor effects of immune checkpoint blockade plus IL-18 therapy. Although pre-mNK cells accumulated in the peritoneum after the therapy, the number of CD8\(^+\) T cells was rather reduced. This is possibly because CD8\(^+\) T cells without tumor
specificity were not infiltrated into the tumor, even though a substantial number of tumor-specific T cells were mobilized into the peritoneum.

It has been demonstrated that dual blockade of the PD-1 and CTLA-4 pathways increased the immune effector cell/regulatory T cell ratio in an animal model (16, 17). In the present study, the addition of IL-18 in this system further decreases the number of regulatory T cells, although the precise mechanism remains elusive. Thus it will be of interest to examine the relationship between increased pre-mNK and decreased regulatory T cells.

On the other side, it was shown that IL-18-induced NK cells express PD-1 and are immunoablative (40, 41). Therefore, it is necessary to examine whether IL-18 induces NK cells susceptible to regulation by PD-L1-expressing cells such as tumor cells. In fact, IL-18 was shown to suppress immune responses to cancer through induction of PD-1 and immunoablative NK cells (34, 40). However, many studies indicate that IL-18 has a dual role in cancer immunology depending on the context, one is anti-cancer activity and the other is tumor-promoting activity (42). Our present results demonstrated that IL-18 can promote the anti-cancer activity of immune checkpoint inhibitors. Although the mechanism must be elucidated in detail, it was considered that the expansion of effector cells such as NK cells by IL-18 could overcome the immune suppression by
PD-1, which may be up-regulated by IL-18. The dual role of IL-18 in tumor immunology must be analyzed. This may be related with the dual roles of NK cells, induction of tolerance or activation of immunity.

In addition to cellular immunity, cytokines appear to play an essential role in the rejection of tumors. In fact, the addition of IL-18 to immune checkpoint blockade therapy converts the tumor environment from one of suppression to one of inflammatory milieu, leading to efficient rejection of tumors. In contrast, inclusion of αGM1 or αCD8 reversed the cytokine milieu, suggesting that pre-mNK cells and CD8+ T cells play an important role in determining the tumor environment. Taken together, IL-18 is not merely a cytokine producer, but a key factor to modulate anti-tumor activity via the transcription network downstream of the IL-18 receptor during immune checkpoint blockade therapy. Although much needs to be done to elucidate the divergent roles of IL-18 at the molecular level, inclusion of IL-18 in immune checkpoint blockade therapy may extend the currently evolving treatment landscape of cancer.
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34. Desrichard A, Snyder A, Chan TA. Cancer Neoantigens and Applications for


Figure Legends

Figure 1. Effect of IL-18 on the survival rate of pre-implanted mice receiving \( \alpha \)CTLA-4 and/or \( \alpha \)PD-L1. BALB/c mice were i.p. implanted with \( 5 \times 10^4 \) CT-26 cells on day 0, isotype-matched IgG 100 \( \mu \)g, \( \alpha \)CTLA-4 100 \( \mu \)g, \( \alpha \)PD-L1 200 \( \mu \)g, IL-18 2 \( \mu \)g alone or in various combination in 250 \( \mu \)L of PBS were i.p. administered on days 3, 7, 11, and 15 (A) or days 7, 11, 15 and 19 (B) following CT-26 inoculation. Isotype-matched IgG 100 \( \mu \)g, \( \alpha \)CTLA-4 100 \( \mu \)g, \( \alpha \)PD-L1 100 \( \mu \)g alone or combined with IL-18 2 \( \mu \)g in 250 \( \mu \)L of PBS were administered on days 7, 11, 15, and 19 (C) or days 10, 14, 18, and 22 (D) following CT-26 inoculation. BALB/c mice were i.p. implanted with \( 5 \times 10^4 \) 4T1 cells on day 0, isotype-matched IgG 100 \( \mu \)g, \( \alpha \)CTLA-4 100 \( \mu \)g, \( \alpha \)PD-L1 200 \( \mu \)g, IL-18 2 \( \mu \)g alone or in various combination in 250 \( \mu \)L of PBS were i.p. administered on days 3, 7, 11, and 15 (E) following 4T1 inoculation. Each group included 5 mice and survival rate of mice was monitored by day 90, Kaplan-Meier plots followed by log-rank \( p < 0.005 \) (A-C), \( p < 0.05 \) (D), \( p = 0.088 \) (E). C57BL/6J mice were i.v. injected with \( 2 \times 10^5 \) B16/F10 cells, isotype-matched IgG 100 \( \mu \)g, \( \alpha \)CTLA-4 100 \( \mu \)g, \( \alpha \)PD-L1 100 \( \mu \)g and IL-18 2 \( \mu \)g in different combination in 250 \( \mu \)L of PBS were i.p. administered on days 3, 7, 11, and 15 following B16/F10 inoculation. Each group included 5 mice and tumor volume were measured on day 28. Error bars
represent mean ± SD, one-way ANOVA with corresponding Tukey’s multiple comparison test, *p < 0.05, **p < 0.005 (F). Representative results of four independent experiments are shown (A-F).

**Figure 2. Accumulation of PECs after immune checkpoint blockade therapy combined with IL-18.** BALB/c mice were i.p. injected with 5 × 10⁴ CT-26 cells in 250 μL of PBS on day 0, isotype-matched IgG 100 μg, αCTLA-4 100 μg, αPD-L1 100 μg, IL-18 100 μg alone or in various combinations in 250 μL of PBS were i.p. administered on days 3 and 7 following CT-26 inoculation (A-C). Each group included 5 mice and PECs were harvested and viable cells were counted on days 4, 5, 6, 7, and 11. Error bars represent mean ± SD, one-way ANOVA with corresponding Tukey’s multiple comparison test, *p < 0.05 (A-C). Representative results of four independent experiments are shown (A-C). Isotype-matched IgG or αCTLA-4 plus αPD-L1 and IL-18 were i.p. administered into the tumor pre-implanted mice 3 days following CT-26 inoculation. Each group included 5 mice and PECs were harvested and stained by Giemsa staining buffer, CT-26 cells number was counted on day 7 (D). Images are displayed at 20 × or 40 × magnification (E) and are representative of three independent experiments. Error bars represent mean ± SD, two-tailed Student’s t test, *p < 0.05 (D).
Figure 3. Phenotypic analyses of NK cells in PECs derived from CT-26 pre-implanted mice receiving immune checkpoint blocking mAbs and IL-18. BALB/c mice were i.p. injected with $5 \times 10^4$ CT-26 cells in 250 μL of PBS on day 0. Isotype-matched IgG 100 μg, or αCTLA-4 100 μg, αPD-L1 100 μg, with or without IL-18 2 μg in 250 μL of PBS were i.p. administered 3 days following CT-26 inoculation. Four days later, PECs were harvested and examined for DX5 (CD49b), B220 (CD45R) using a flow cytometer. TCR-β fractions in the PECs were gated and further examined for NKG2D (CD314) or CD11b (Mac-1) and CD11c expression (A). Similarly, PECs prepared 8 days after administration of mAbs and IL-18 were analyzed by flow cytometry (B). Similar dot plots displays are representative of three independent experiments (n=3) (A, B).

Figure 4. Phenotypic analyses of T cells in the PECs from CT-26 pre-implanted mice treated with immune checkpoint blockade and/or IL-18. BALB/c mice were i.p. injected with $5 \times 10^4$ CT-26 cells in 250 μL of PBS on day 0. Isotype-matched IgG 100 μg, or either αCTLA-4 100 μg, αPD-L1 100 μg, with or without IL-18 2 μg in 250 μL of PBS were i.p. administered 3 days after CT-26 inoculation. After addition 4 days,
PECs were harvested and stained for TCR-β, CD4, CD25 and Foxp3, or TCR-β, CD8, and NKG2D (A). Eight days after the treatment and examined for expression of surface molecules in the same way (B). Similar dot plots or histograms displays are representative of three independent experiments (n=3) (A, B).

**Figure 5. Critical role of pre-mNK cells in the therapeutic effect of immune checkpoint blockade and IL-18.** BALB/c mice were injected with control rabbit IgG 50 μg or 50 μl αGM1 in 250 μL of PBS on days -1, 2, 6, and 10, 5 × 10⁴ CT-26 cells in 250 μL of PBS on day 0, isotype-matched IgG 100 μg, αCTLA-4 100 μg plus αPD-L1 100 μg, or combined with IL-18 2 μg in 250 μL of PBS on day 3, 7, 11, and 15. Each group included 5 mice and survival rate of mice was monitored by day 90, Kaplan-Meier plots followed by log-rank *p < 0.005. A representative result of four independent experiments is shown (A). As in Figure 5A, CT-26 pre-implanted mice were administered with αGM1, αCTLA-4, αPD-L1 and IL-18. PECs were harvested and examined on day 7 (B) or day 12 (C) for cytotoxic activity against CT-26 cells at effector/target ratios of 3:1, 10:1, and 30:1 in decuplicate. Error bars represent mean ± SD, two-tailed Student’s *t* test, *p < 0.05. Representative results of three independent experiments are shown (B, C).
Figure 6. Critical role of CD8\(^+\) T cells in the therapeutic effect of immune checkpoint blockade and IL-18. BALB/c mice were injected with \(\alpha\text{CD8} \) 100 \(\mu\text{g}\) in 250 \(\mu\text{L}\) of PBS on days -1, 2, 6, and 10, \(5 \times 10^4\) CT-26 cells in 250 \(\mu\text{L}\) of PBS on day 0, isotype-matched IgG 100 \(\mu\text{g}\), \(\alpha\text{CTLA-4} \) 100 \(\mu\text{g}\) plus \(\alpha\text{PD-L1} \) 100 \(\mu\text{g}\) and IL-18 2 \(\mu\text{g}\) in 250 \(\mu\text{L}\) of PBS on days 3, 7, 11, and 15. Each group included 5 mice and survival rate of mice was monitored by day 90, Kaplan-Meier plots followed by log-rank \(p < 0.005\). A representative result of four independent experiments is shown (A). Similar to Figure 6A, CT-26 pre-implanted mice were administered with \(\alpha\text{CD8}\), \(\alpha\text{CTLA-4}\), \(\alpha\text{PD-L1}\) and IL-18. PECs were harvested and examined on day 7 (B) or day 12 (C) for cytotoxic activity against CT-26 cells at effector/target ratios of 3:1, 10:1, and 30:1 in decuplicate. Error bar represent mean ± SD, two-tailed Student’s \(t\) test, \(*p < 0.05\). Representative results of three independent experiments are shown (B, C).
Tables and Table legends

Table 1. Percentage and absolute number of DX5⁺B220⁺ NK induced by IgG, αCTLA-4, αPD-L1 and IL-18.

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<td></td>
<td>(%)</td>
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<td>10-days</td>
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<tr>
<td>DX5⁺B220⁺ NK</td>
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<td></td>
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<tr>
<td>Percentage (%)</td>
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<td>28.94±2.05 a</td>
<td>35.57±3.12 a, b</td>
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<td>Absolute number (×10⁶)</td>
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<td>1.68±0.12 a</td>
<td>4.05±0.36 a, b</td>
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NOTE: CT-26 pre-implanted mice were administrated IgG, αCTLA-4 plus αPD-L1 with or without IL-18, PECs were harvested (same as Figure 3), percentage of DX5⁺B220⁺ NK cells were checked by using a flow cytometer. Data given are the average values from three independent experiments in triplicate. a p < 0.05, significant when compared with IgG group, b p < 0.05, significant when compared with αCTLA-4 plus αPD-L1 group.
Table 2. Surface markers of pre-mNK induced by IgG, αCTLA-4, αPD-L1 and IL-18.

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<td>NKG2D</td>
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**NOTE:** CT-26 pre-implanted mice were administrated IgG, αCTLA-4 plus αPD-L1 with or without IL-18. Surface markers of pre-mNK cells were checked by using a flow cytometer.
Figure 8

A

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DX5 - cells gated
Figure 6

A

CT-26 i.p. 1 i.p. 2 i.p. 3 i.p. 4
Day -1 2 6 10 15
αCD8 αCD8 αCD8 αCD8

Survival (%)

0 20 40 60 80 100

Days

0 10 20 30 40 50 60 70 80 90

Control IgG
αCTLA-4+αPD-L1 +IL-18
αCTLA-4+αPD-L1 +IL-18+αCD8

B

CT-26 i.p. 1
Day 0 3 7

Harvest PECs

CT-26 Tumor cells lysis (%)

0 20 40 60 80 100

3:1 10:1 30:1

αCTLA-4+αPD-L1 +IL-18
αCTLA-4+αPD-L1 +IL-18+αCD8

C

CT-26 i.p. 1 i.p. 2 i.p. 3
Day 0 3 7 11 12

Harvest PECs

CT-26 Tumor cells lysis (%)

0 20 40 60 80 100

3:1 10:1 30:1

αCTLA-4+αPD-L1 +IL-18
αCTLA-4+αPD-L1 +IL-18+αCD8

*
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* a $p < 0.05$, significant when compared with IgG group

* b $p < 0.05$, significant when compared with αCTLA-4 plus αPD-L1 group
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Clinical Cancer Research

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