Augmentation of Immune Checkpoint Cancer Immunotherapy with IL18

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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 IL18 on the antitumor effect of immune checkpoint inhibitors.

Experimental Design: We examined the effect of IL18 on the peritoneal dissemination of CT-26 cells or tail vein injection metastasis of B16/F10 cells using antiprogrammed death-1 ligand-1 (aPD-L1) and/or anti-CTLA-4 (aCTLA-4) mAbs.

Result: Massive ascites developed after intraperitoneal inoculation of CT-26, resulting in animal death within 30 days. Treatment of mice with aPD-L1 and/or aCTLA-4 significantly prolonged their survival, and a combination of the antibodies and IL18 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.

Conclusions: The results demonstrated that IL18 enhanced the 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 IL18 may give a suggestion to the development of next-generation cancer immunotherapy.

Introduction

T-cell responses are controlled by T-cell receptors (TCR) and noncostimulatory immune coreceptors. Whereas costimulatory receptors such as CD28 and inducible T-cell costimulator (ICOS) amplify the transcriptional effects of TCR signaling, coinhibitory receptors including CTL-associated protein-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene 3 (LAG3), and T cell immunoglobulin and mucin domain 3 (TIM-3) reduce effector functions. Tumor growth can thus be restricted by costimulatory signaling and facilitated by coinhibitory pathways. Tumor cells often usurp the coinhibitory system, also termed the immune checkpoint, which controls the antitumor effects of immune effector cells (1–4).

Recently, research has focused on the development of cancer immunotherapy that targets the immune checkpoint pathways using mAbs, anti-CTLA4 (aCTLA-4), anti-PD-1 (aPD-1), and/or anti-PD-1 ligand-1 (aPD-L1; refs. 5–7). In clinical trials, aCTLA-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 downregulation of T cells in the priming phase, the blockade of CTLA-4 signaling promotes cytotoxic T cells; however, aCTLA-4 induces severe adverse reactions (1–4). In contrast, the PD-1 pathway plays an essential role in the regulation of immune cells, including regulatory T cells, γδ T cells, and NK cells, in the effector phase. In a phase I clinical trial of a fully human aPD-1/aPD-L1, objective responses were observed in patients with melanoma, non–small cell lung cancer, renal cell cancer, bladder cancer (11, 12), non-Hodgkin lymphoma, Hodgkin lymphoma (13), esophageal cancer (14), and hepatocellular cancer (15). The nonredundant roles and distinct adverse reactions of CTLA-4 and PD-1 pathways suggest the potential of dual immune checkpoint blockade using a low dose of CTLA-4 and a high dose of aPD-1 or aPD-L1 (16, 17).

Effective antitumor responses to treatments require not only a blockade of coinhibitory 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 IL2, IL12, II15, IL18, and IL21. Among them, IL18 is a unique cytokine that is maturated by inflammasome complexes (18, 19), and modulates antitumor responses (20–22). It is demonstrated that IL18 markedly enhances the expansion of NK cells, CD8+ CTLs, and γδ T-cells.
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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 the current study, we analyzed the effect of IL18 on the antitumor effect of immune checkpoint blockade. IL18 is a cytokine of IL1 family members and is formed by the action of activated inflammasomes, but its physiologic roles are not completely clarified. Recently, it was demonstrated that IL18 promoted the expansion and survival of effector cells including NK, γδ T, and CD8+ T cells. However, clinical efficacy of IL18 cancer monotherapy has been shown to be negligible. Here, we show that IL18 efficiently augmented the expansion of effector T cells in the presence of immune checkpoint antibodies, and reduced the proportion of regulatory T cells. In addition, immune checkpoint inhibitors and IL18 synergistically inhibited the growth of tumor cells without significant adverse events in animal models. The results strongly suggest that a combination of IL18 and immune checkpoint inhibitors could be beneficial in treatment of cancer patients.

T cells which express IL18 receptors α/β chains and receptors containing immunoreceptor tyrosine-based activation motif (ITAM), and produce IFNγ (23–27). In humans, IL18 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 IFNγ-producing killer dendritic cells (28, 29). IL18-induced NK lineage cells exhibit potent antitumor 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 IL18 on cancer immunotherapy using blocking mAbs specific to coinhibitory pathways.

Prognosis of peritoneal dissemination of cancer is poor, even though a combination of cytoreduction with hyperthermic intra-peritoneal chemotherapy has become a standard therapy (30). In this study, we examined the effect of IL18 on cancer immunotherapy using blocking mAbs specific to coinhibitory pathways.

**Materials and Methods**

**Animals and tumor cells**

Six- to 8-week-old male BALB/c and C57BL/6J mice were purchased from Japan SLC (Hamamatsu). Mice were housed in a pathogen-free condition at 25°C under controlled lighting (12 hours light/12 hours 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 ATCC. The CT-26, CT-26.CL25, and 4T1 cells were maintained in RPMI1640 medium (Nacalai Tesque, Inc.) supplemented with 10% FBS (BioWest), 100 units/mL penicillin, 100 µg/mL streptomycin, 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin in a humidified atmosphere with 5% CO2.

**Reagents**

Recombinant mouse IL18 was provided by GlaxoSmithKline PLC (Research Triangle Park), whereas mouse IL2 was purchased from R&D Systems Inc. Anti-mouse CD152 (CTLA-4) antibody (aCTLA-4, clone UC10-4F10-11), anti-mouse CD274 (PD-L1) antibody (aPD-L1, clone 10F.9G2), and control isotype-matched hamster IgG were purchased from BioXcell (BE0091). Rabbit antiasialo GM1 (aGM1) was purchased from Wako Pure Chemical Industries Ltd (014-09801). Rabbit IgG was purchased from Medical and Biological Laboratories Co., Ltd (PM035). Anti-mouse CD8 mAb (oCD8) was purchased from Santa Cruz Biotechnology, Inc. (clone 53-6-7).

**Tumor cell implantation and immune checkpoint blocking**

Subconfluent CT-26, CT-26.CL25, 4T1, and B16/F10 cells were harvested by detaching with 0.05% trypsin and 0.53 mmol/L EDTA (trypsin-EDTA; Nacalai) or 0.25% trypsin and 0.53 mmol/L EDTA (trypsin-EDTA; Nacalai) in Ca2+–Mg2+–free Dulbecco PBS, pH 7.4 (Nacalai) and washed with PBS. The viable cells were counted by Trypan Blue dye exclusion test and suspended in PBS. CT-26 and 4T1 cell suspensions (5 × 10^6 cells in 250 µL of PBS) were intraperitoneally injected into BALB/c mice, CT-26.CL25 cell suspensions (1 × 10^7 cells in 100 µL of PBS) were subcutaneously injected into BALB/c mice, and B16/F10 cell suspensions (2 × 10^5 cells in 200 µL of PBS) were intravenously injected into C57BL/6J mice. After 3, 7, or 10 days postinoculation, various doses of aCTLA-4, aPD-L1, and/or IL18 were intraperitoneally injected four times every 4 days. For depletion of NK cells or CTL, aGM1 or oCD8 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 postinoculation of tumor cells.

**Cell preparation and cytokine assay**

Peritoneal exudate cells (PEC) were harvested from the abdominal cavity and washed in 5 mL PBS three times. Red blood cells were eliminated by ACK lysis buffer (500 mmol/L NH4Cl, 10 mmol/L KHCO3, and 0.1 mmol/L Na2EDTA at pH 7.2–7.4), and the resulting cells were washed three times with PBS. Cells viability was assayed by Trypan Blue dye exclusion test. CT-26 cells were lysed by repeated freezing and thawing, and the resultant cell debris was washed and resuspended in PBS. PECs were stimulated with the CT-26 cell preparation and cultured in the absence or presence of IL12 (1 ng/mL) and/or IL18 (100 ng/mL) in RPMI1640 medium supplemented with 10% FBS, α-glutamine (Gibco BRL), 100 units/mL penicillin, 100 µg/mL streptomycin, and 10−3 mol/L 2-mercaptoethanol 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).

**In vitro cytotoxicity assay**

Cytotoxicity was assessed in vitro using the DELFIA ExTDA Cytotoxicity Assay System (PerkinElmer Life Sciences, Wallac Oy). Briefly, CT-26 cells were labeled with a chelate prodrug for 10
minutes, washed, and resuspended in RPMI1640 medium. The PECs from mice which had been treated with mAbs and/or IL18 were suspended in RPMI1640 medium. The resulting cells were mixed at the indicated effector/target ratios and incubated for 3 hours at 37°C. The supernatants were mixed with europium solution and the time-resolved fluorescence was measured using a multicycle reader. The percentage of specific lysis was calculated as follows: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 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-β220 mAbs (RA3-6B2) were purchased from Biolegend Inc. (San Diego). FITC-conjugated anti-CD11b mAb (M1/70), biotin-conjugated anti-NK1.1 mAb (PK136), and FITC-conjugated anti-CD4 mAbs (GK1.5), APC-conjugated anti-CD4 mAbs (RM4-5), APC-conjugated anti-streptavidin (catalog 17-4317-82), which were purchased from BD Biosciences Inc. (San Diego). 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). FITC-conjugated anti-CD11b mAb (M1/70), biotin-conjugated anti-CD11c mAb (HL3), PE, APC and B6515-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 (catalog 554460) were purchased from BD Biosciences. The cells were incubated with antibodies for 20 minutes 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 Image

To determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined using an ImageJ software 1.48 (NIH, Bethesda, MD). Tumor volume was calculated by the modified ellipsoidal formula: Tumor volume = 1/2(length × width²).

Statistical analysis

Data were statistically analyzed by SPSS 20.0.0 software (IBM) and expressed as means ± SD. Two groups measurements were analyzed with a two-tailed Student t test, and the other groups were analyzed using one-way ANOVA with Tukey multiple comparison test. A P value < 0.05 was considered significant, NS denotes nonsignificant. 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.

Results

IL18 enhances antitumor 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 PD-1/PD-L1- and/or αCTLA-4–based therapies are analyzed in a wider range of patients. Because effective antitumor responses require immune activation as well as immune checkpoint blockade, we examined the synergistic effect of IL18, which was demonstrated to enhance effector functions of immune cells (25,26,38,39) on the survival rate of preimplanted mice receiving αPD-L1 and/or αCTLA-4. To develop an in vivo model of peritoneal dissemination, mice were intraperitoneally inoculated with the CT-26 colon carcinoma cells. When preimplanted 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 and D and Supplementary Figs. S1A–S1D). Treatment of 3-day preimplanted mice with either IL18, αCTLA-4, or αPD-L1 alone demonstrated modest therapeutic effect. It is of note that combination of IL18 and CTLA-4 or PD-L1 blockade promoted tumor rejection with definite significance by at least day 90 (Fig. 1A and Supplementary Figs. S1A and S1B). When 7-day preimplanted mice were treated with the combination therapy of IL18 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 preimplanted mice, the addition of IL18 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 preimplanted mice, inclusion of IL18 further promoted tumor rejection in 20% of mice by at least 90 days (Fig. 1D).

To further analyze the mechanism underlying the antitumor activity elicited by IL18 and immune checkpoint blockade, the optimal doses of the recombinant proteins were determined in the mouse model. When CT-26 preimplanted mice were treated with IL18 and αCTLA-4, all mice receiving 2 μg of IL18 and 50 or 100 μg of αCTLA-4 survived until day 90, whereas lower doses of IL18 and αCTLA-4 had moderate therapeutic effects. Similarly, all mice treated with 2 μg of IL18 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 S1F).

Moreover, combination of IL18 and dual blockade of PD-L1 and CTLA-4 was effective also in the treatment of mice subcutaneously injected with CT-26.CL25 cells (Supplementary Figs. S1G and S1H), but the combination failed to prevent the abdominal growth of 4T1 cells (Fig. 1E). IL18 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 and Supplementary Fig. S1I).

Combination of immune checkpoint blockade with IL18 allows for an accumulation of the precursors of mNK cells

To elucidate the mechanism by which immune checkpoint blockade combined with IL18 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 IL18, no detectable increase in the number of PECs was observed (data not shown). Treatment of CT-26 preimplanted mice with either IL18, αCTLA-4, or αPD-L1 alone induced modest accumulation of PECs. In contrast, there was a large accumulation of PECs when CT-26 preimplanted mice were treated with a combination

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Effect of IL18 on the survival rate of preimplanted mice receiving αCTLA-4 and/or αPD-L1. BALB/c mice were intraperitoneally (i.p.) implanted with 5 x 10^4 CT-26 cells on day 0, isotype-matched IgG 100 μg, αCTLA-4 100 μg, αPD-L1 200 μg, IL18 2 μg alone or in various combinations in 250 μL of PBS were intraperitoneally 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 μg, αCTLA-4 100 μg, αPD-L1 100 μg alone or combined with IL18 2 μg in 250 μL 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 intraperitoneally implanted with 5 x 10^4 4T1 cells on day 0, isotype-matched IgG 100 μg, αCTLA-4 100 μg, αPD-L1 200 μg, IL18 2 μg alone or in various combinations in 250 μL of PBS which were intraperitoneally 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 intravenously injected with 2 x 10^5 B16/F10 cells, isotype-matched IgG 100 μg, αCTLA-4 100 μg, αPD-L1 100 μg, and IL18 2 μg in different combinations in 250 μL of PBS which were intraperitoneally administered on days 3, 7, 11, and 15 following B16/F10 inoculation. Each group included 5 mice and tumor volume was measured on day 28. Error bars represent mean ± SD; one-way ANOVA with corresponding Tukey multiple comparison test; *, P < 0.05; ***, P < 0.005 (F). Representative results of four independent experiments are shown (A–F).
of immune checkpoint blockade with IL18 (Fig. 2A–C). Although the accumulation of PECs reached a maximum at day 6 and declined thereafter, a subsequent injection of mAbs and IL18 on day 7 promoted reaccumulation 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 preimplanted mice injected with control IgG was significantly greater compared with the mice treated with combination of immune checkpoint blockade and IL18 (Fig. 2D and E).

To characterize the PECs recruited by the tumor cells, mAbs, and/or IL18, the cells were stained with mAbs specific for DX5 (CD49b), B220 (CD45R), CD11b, CD11c, and NKG2D (CD314). It was of note that 10% to 30% of PECs comprised DX5⁺B220⁺ NK cells 4 days after mAb plus IL18 administration.
to CT26 cell-implanted mice (7 days postinoculation of CT26 cells). In addition, B220 and NK2D 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 IL18 (Fig. 3A, Table 1, and Supplementary Fig. S2A). The expression of B220 on NK cells was further enhanced 8 days after mAbs plus IL18 administration (day 11 postinoculation 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 IFNγ-producing killer dendritic cells (28, 29).

Phenotypic analyses of T cells in the PECs from preimplanted mice treated with mAbs and IL18

Analysis of the T-cell subsets in the PEC was undertaken by staining the cell surface markers with mAbs. Although IL18 did not affect the proportion of CD4⁺ cells in the PECs of mice administrated αCTLA-4, IL18 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 IL18 4 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 IL18 regimen (5.88% ± 1.03%). This suggests that a combination of IL18 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, whereas a combination of IL18 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 IL18. In contrast, in the TCR-β chain "CD8" cells, IL18 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 IL18 was observed even when they were analyzed 8 days after administration of αCTLA-4 plus αPD-L1 with or without IL18 (51.03% ± 2.75% vs. 59.31% ± 4.32%, respectively), whereas 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 IL18.
administration, although the expression of NKG2D on the CD8+ T cells was slightly increased (Fig. 4B).

Functional analyses of PECs from preimplanted mice treated with immune checkpoint blocking mAbs and/or IL18

Because immune checkpoint blockade in combination with IL18 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 IFNγ was released from the PECs derived from CT-26 mice. Treatment of mice with αCTLA-4 plus αPD-L1 enhanced the production of IFNγ, and IL18 further promoted IFNγ production (Supplementary Fig. S3A). In contrast, a low level of IL10 was produced by the PECs and the IL10 secretion was reduced when mice were treated with immune checkpoint blocking mAbs. The decrease in IL10 production was more prominent in the presence of IL18 (Supplementary Fig. S3B), and TGFβ production was also attenuated by mAbs treatment. The addition of IL18 reduced the cytokine secretion to a background level (Supplementary Fig. S3C). A low level of IFNγ was produced by PECs after administration of IL18 plus mAbs 10 days after tumor inoculation (Supplementary Fig. S3D). In contrast, a high level of TGFβ was produced by PECs induced by control IgG, and the secretion of TGFβ was reduced when mice were treated with immune checkpoint blocking mAbs (Supplementary Fig. S3D).

Next, the effect of immune checkpoint blocking mAbs and/or IL18 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 IL18 markedly enhanced the cytotoxicity of PECs against CT-26 cells (Supplementary Fig. S4A). Repeated injection of αCTLA-4 plus αPD-L1 and/or IL18 revealed that IL18 helped sustain a high level of antitumor activity of PECs (Supplementary Fig. S4B). On the basis of 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 IL18

We next attempted to identify cell subsets in the PECs responsible for tumor rejection and improved survival rate. Upon intraperitoneal inoculation of tumor cells and treatment by immune checkpoint blockade and IL18, 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 αCTLA-4, αPD-L1, IL18, and control IgG into CT-26 preimplanted mice, all mice survived more than 90 days. In contrast, injection of anti-asialoGM1 Ab (αGM1) 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 αGM1 (Fig. 5B). Repeated injections of αGM1 further reversed the beneficial effects of the tumor immunotherapy using immune checkpoint blockading mAbs and IL18 (Fig. 5C).

Modulation of pre-mNK cell and CD4+CD25+ T-cell accumulation by αGM1

The numbers of PECs were essentially the same between preimplanted mice which had been injected with αGM1, followed by αCTLA-4, αPD-L1, and IL18, and those with control IgG. The percentage of TCR-β+ 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 antitumor 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 preimplanted mice were treated with immune checkpoint blocking mAb plus IL18, the inclusion of αGM1 reduced the secretion of IFNγ from PECs (Supplementary Fig. S6A). In contrast, the production of IL10 and TGFβ was markedly enhanced (Supplementary Figs. S6B and S6C), demonstrating that αGM1 converted the inflammatory environment in the peritoneum into immunosuppressive milieu.

Table 1. Percentage and absolute number of DX5+ B220+ NK induced by IgG, αCTLA-4, αPD-L1, and IL18

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<tr>
<td></td>
<td>Percentage (%)</td>
<td>10.85 ± 1.74</td>
<td>28.94 ± 0.09*</td>
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<td>Absolute number (×10^5)</td>
<td>0.47 ± 0.08</td>
<td>1.68 ± 0.12*</td>
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Table 2. Surface markers of pre-mNK induced by IgG, αCTLA-4, αPD-L1, and IL18

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Note: CT-26 preimplanted mice were administered IgG, αCTLA-4 plus αPD-L1 with or without IL18. Surface markers of pre-mNK cells were checked by using a flow cytometer.
Essential role of CD8+ T cells in tumor rejection

Because αGM1 treatment decreased efficacy of immune checkpoint blockade and/or IL18 with concomitant reduction of CD8+ T cells, we analyzed the role of CD8+ T cells in tumor immunotherapy. When preimplanted mice received αCTLA-4, αPD-L1, and IL18, 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 preimplanted mice treated with αCTLA-4, αPD-L1, II.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 antitumor responses.

Phenotypic analyses of PECs after treatment with αCD8

Administration of αCD8 to mice receiving CT-26, αCTLA-4, αPD-L1, and II.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-
b<sup>+</sup>B220<sup>+</sup>DX5<sup>+</sup> pre-mNK cells was not markedly influenced by the addition of αCD8 (Supplementary Fig. S7B), demonstrating that the accumulation of CD4<sup>+</sup>T cells and pre-mNK cells in the peritoneum was regulated by a mechanism that is independent of CD8<sup>+</sup>T cells, and that the antitumor activity of the CD8<sup>+</sup>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/CtLA80 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 antitumor 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 current study, we demonstrated that IL18 markedly improved the survival rate of preimplanted mice treated with αCTLA-4 and αPD-L1. In a tail vein injection metastasis model using B16 melanoma cells, IL18 exerted a similar effect on treatment by immune checkpoint blockade. In contrast, IL18 failed to enhance the effect of immune checkpoint blockade against intraperitoneal growth of 4T1 breast cancer cells. This indicated that the effect of IL18 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. Inflammatogenic tumors may be susceptible to the therapy, whereas 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 expression of IL18 plus anti-PD-L1 and anti-CTLA-4 Antibodies
stress-induced molecules such as MICA/MICB (34). These must be further explored.

Although IL18 was originally discovered as an IFN-γ-inducing factor (22, 24), precise physiologic functions of this cytokine have not yet been fully elucidated. IL18 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. IL18 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 IL18 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 IL10 and TGFβ. In humans, IL18 promotes development and expansion of CD56high CD16+CD25highCD86highHLA-DRhighHLA-DQhigh NK cells (termed helper NK cells) in the presence of IL2, which in turn enhance the expansion of effector γδ 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, IL18 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 IL18 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 IL18 can be defined by the environment, it is likely that IL18 simply enhanced the effect of cCTLA-4 and αPD-L1, resulting in a decrease in the number of regulatory T cells. Similarly, effective suppression by IL18 of soluble inhibitor secretion is also achieved by the promotion of immunomodulatory effects of cCTLA-4 and αPD-L1.

Upon engagement with its receptor, IL18 activates p38 MAPK, PI3K/AKT, and ERK pathways as well as the MyD88-IRAK-TRAF6 signaling pathway (35–37). IL18 thus executes multiple signals involved in cellular viability, growth, differentiation, migration, and cytokine secretion. In addition, IL18 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 IL18.

It is worth noting that IL18-deficient mice are prone to the development of spontaneous tumors, and that IL18 has potent antitumor activity. The cytokine, however, fails to directly act on and eradicate tumor cells, suggesting that IL18 activates and expands immune effector cells and indirectly exhibits antitumor activity. In line with the findings, depletion of pre-mNK cells or CD8⁺ T cells reversed antitumor effects of immune checkpoint blockade plus IL18 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.

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On the other side, it was shown that IL18-induced NK cells express PD-1 and are immunomodulatory (40, 41). Therefore, it is necessary to examine whether IL18 induces NK cells susceptible to regulation by PD-L1—expressing cells such as tumor cells. In fact, IL18 was shown to suppress immune responses to cancer through induction of PD-1 and immunomodulatory NK cells (34, 40). However, many studies indicate that IL18 has a dual role in cancer immunology depending on the context, one is anticancer activity and the other is tumor-promoting activity (42). Our current results demonstrated that IL18 can promote the anticancer 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 IL18 could overcome the immune suppression by PD-1, which may be upregulated by IL18. The dual role of IL18 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 IL18 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, IL18 is not merely a cytokine producer, but a key factor to modulate antitumor activity via the transcription network downstream of the IL18 receptor during immune checkpoint blockade therapy. Although much needs to be done to elucidate the divergent roles of IL18 at the molecular level, inclusion of IL18 in immune checkpoint blockade therapy may extend the currently evolving treatment landscape of cancer.

Disclosure of Potential Conflicts of Interest
H. Tahara reports receiving a commercial research grant from and has received speakers bureau honoraria from Ono Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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