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Anti-IL-6 receptor mAb eliminates myeloid-derived suppressor cells and inhibits tumor growth by enhancing T-cell responses

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$CD11b^+Gr\text{-}1^+$ immature myeloid cells (ImCs), which are abnormally increased in tumor-bearing mice, were classified into three different subsets according to their phenotypic and morphological characteristics: $Gr\text{-}1^{low}$ $F4/80^+$ macrophages (MΦ-ImCs), $Gr\text{-}1^{mid}$ stab neutrophils ($Neut_{stab}$-ImCs), and $Gr\text{-}1^{high}$ segmented neutrophils ($Neut_{seg}$-ImCs). In the spleen, only MΦ-ImCs but not $Neut_{stab}$-ImCs and $Neut_{seg}$-ImCs exhibited a significant immunosuppressive activity in MLR. In contrast, tumor-infiltrating leukocytes (TILs) contained only two ImC subsets, MΦ-ImCs and $Neut_{seg}$-ImC, both of which exhibited stronger inhibitory activity against T cells compared with spleen-MΦ-ImCs. Thus, we concluded that tumor-infiltrating MΦ-ImCs and $Neut_{seg}$-ImCs were fully differentiated myeloid-derived suppressor cells (MDSCs) with stronger T-cell inhibitory activity. Indeed, spleen MΦ-ImCs were converted into stronger MΦ-MDSCs by tumor-derived factor (TDF). Moreover, both spleen $Neut_{stab}$-ImCs and $Neut_{seg}$-ImCs differentiated into $Neut_{seg}$-MDSCs with suppressive activity after culture with TDF. We first demonstrated that administration of anti-IL-6R mAb could downregulate the accumulation of MΦ-MDSCs and $Neut_{seg}$-MDSCs in tumor-bearing mice. The elimination of those MDSCs caused subsequent enhancement of antitumor T-cell responses, including IFN-γ-production. The therapeutic effect of anti-IL-6R mAb was further enhanced by combination with gemcitabine (GEM). Thus, we propose that anti-IL-6R mAb could become a novel tool for the downmodulation of MDSCs to enhance antitumor T-cell responses in tumor-bearing hosts.

Keywords: anti-IL-6R mAb · CTLs · Immunosuppression · MDSCs · Tumor immunotherapy

Supporting Information available online

Introduction

It has been accepted that the introduction of Th1-dependent immunity in the tumor-bearing host is critical for the induction of tumor-specific CTLs and memory T cells, which are essential for the destruction of tumor cells and the prevention of tumor recurrence, respectively [1–4]. However, it has been also demonstrated that the induction of tumor-specific T-cell responses in tumor-bearing hosts is difficult, due to strong immunosuppressive tumor

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escape mechanisms. Namely, immune responses are markedly inhibited by multiple mechanisms, which make it difficult to induce fully-activated tumor-specific CD4$^+$ and CD8$^+$ T cells essential for the inhibition of tumor growth in vivo. It has been well known that immunosuppressive soluble factors, such as IL-10 and TGF-β, impair the functions of T cells and DCs in the tumor microenvironment [5–7]. In addition, regulatory T (Treg) cells are major T-cell subset responsible for immunosuppression in tumor-bearing hosts. Indeed, several strategies attenuating Treg cell-mediated immune inhibition have been developed to enhance cytotoxic T cells (CTL)-mediated antitumor activity in tumor-bearing mice [8–11].

Recently, myeloid-derived suppressor cells (MDSCs) are also highlighted as a potent immunosuppressive population in tumor-bearing hosts. MDSCs, which accumulate in lymphoid organs and tumor tissue, contributed to tumor progression through inhibiting antitumor immune responses and/or promoting angiogenesis and metastasis [12–16]. Similar MDSCs population was also detected in human cancer patients [17, 18]. The increased numbers of MDSCs in the tumor-bearing hosts were mediated by various inflammatory factors, including VEGF, GM-CSF, IL-1β, IL-6, PGE_2, and S100A8/A9 [12, 19–24]. MDSCs negatively regulated both NK cell and CD4$^+$, CD8$^+$ T-cell responses in vitro and in vivo through their high levels of production of suppressive factors such as arginase-1, NO, and ROS [14, 23]. Interestingly, it has been reported that MDSCs also facilitate the induction and/or accumulation of Treg cells [25]. Therefore, it has been considered that the downmodulation of MDSCs would be a rational strategy for developing an efficient tumor immunotherapy.

To develop a novel strategy to downmodulate MDSC function, it is of great importance to understand the precise function of MDSC subsets and their suppressive mechanisms during tumor progression. However, the definition of MDSCs is still controversial. So far, almost all CD11b$^+$Gr-1$^+$ cell subsets, which consisted of different cell subsets, are defined as MDSC population regardless of their immunosuppressive activity against T cells [12, 13]. However, we previously revealed that spleen CD11b$^+$Gr-1$^+$ cells, which are usually poorly immunosuppressive cells, differentiated into F4/80$^+$ macrophages by stimulation with tumor-derived factors (TDF) and acquired a strong suppressive activity to inhibit T-cell responses [26]. This result encouraged us to evaluate in detail the mechanisms by which CD11b$^+$Gr-1$^+$ subsets exhibit a strong immunosuppressive activity against T cells and act as MDSCs in tumor-bearing mice. Indeed, recent reports indicated that CD11b$^+$Gr-1$^+$ cells from tumor-bearing mice were a heterogeneous population [15]. In addition, it has been also reported that monocytic and granulocytic MDSCs suppressed immune responses in altered mechanisms respectively [14, 27]. However, there are no clear explanations concerning the characteristics of morphological and functional heterogeneity and responsible factors regulating MDSC subpopulations.

In the present paper, we expanded on previous work [12, 28, 29] and found that spleen CD11b$^+$Gr-1$^+$ immature myeloid cells (ImCs) can be classified into three different subsets: Gr-1^{high} segmented neutrophil (Neut$_{seg}$-ImCs), Gr-1^{mid} stab neutrophil (Neut$_{stab}$-ImCs) and Gr-1^{low} macrophage (MΦ-ImCs). Among them, Neut$_{seg}$-ImCs and Neut$_{stab}$-ImCs did not suppress T-cell responses though MΦ-ImCs exhibited a weak suppressive activity without activation. In contrast to the spleen, tumor-infiltrating CD11b$^+$Gr-1$^+$ cells consisted of two ImCs subsets, Gr-1^{high} Neut$_{seg}$-ImCs and Gr-1^{low} MΦ-ImCs, which revealed stronger immunosuppressive activity than spleen MΦ-ImCs. It has been reported that in both mouse and human systems, hosts bearing various types of cancers showed high levels of inflammatory cytokines, such as IL-1β, GM-CSF, TGF-β, TNF-α, and IL-6 [30–33]. In particular, IL-6 has been recently demonstrated to be a candidate for Stat-3-dependent negative modulator for antitumor immunity via its capability of inducing MDSCs [34–37]. Therefore, neutralization of IL-6 activity may become a novel strategy to downmodulate MDSC function in tumor-bearing hosts to enhance antitumor immunity. Kishimoto and colleagues [38–40] have developed an anti-IL-6 receptor mAb (anti-IL-6R mAb), which revealed a great impact on the therapy of rheumatoid arthritis, Castleman's disease, and multiple myeloma. However, the impact of a humanized anti-IL-6R mAb on the treatment of patients with solid tumor remains unclear. Some studies have shown that humanized anti-IL-6R mAb (tocilizumab) exhibits antitumor effects in xenograft tumor models or in vitro studies using human tumor cell lines, such as oral squamous cell carcinoma and glioma [41, 42]. However, these reports did not demonstrate the effects of anti-IL-6R mAb on immune responses in the tumor-bearing host. Therefore, here, we initially examined whether administration of anti-IL-6R mAb resulted in an antitumor effect in mice with established solid tumors via downmodulation of MDSCs. As a result, we demonstrate, for the first time, that anti-IL-6R mAb treatment decreases the numbers of MDSCs, which subsequently causes the inhibition of tumor growth via the augmentation of CD4$^+$ T cell- and CD8$^+$ T cell-mediated antitumor immunity in tumor-bearing mice. We also propose that combination therapy with anti-IL-6R mAb and gemcitabine could be a good protocol for combination tumor immunotherapy.

Results

Characterization of three distinct subsets of CD11b$^+$ Gr-1$^+$ ImCs in the spleen

When the methylcholanthrene-induced skin squamous cell carcinoma, CMC-1, was i.d. inoculated into BALB/c mice, CD11b$^+$ Gr-1$^+$ myeloid cells including immunosuppressive macrophages dramatically increased in the spleen in parallel with tumor growth [26]. To determine the precise role of CD11b$^+$Gr-1$^+$ ImCs in tumor-bearing hosts, we expanded previous results [13–25] and classified spleen ImCs in detail. As a result, three different subsets of CD11b$^+$Gr-1$^+$ ImCs were identified in spleen based on their distinct expression levels of Gr-1 molecules; Gr-1^{high} (20 ± 1%), Gr-1^{mid} (11.4 ± 2.2%), and Gr-1^{low} (7.3 ± 1.2%) (Fig. 1A). Gr-1^{high}, Gr-1^{mid}, and Gr-1^{low}

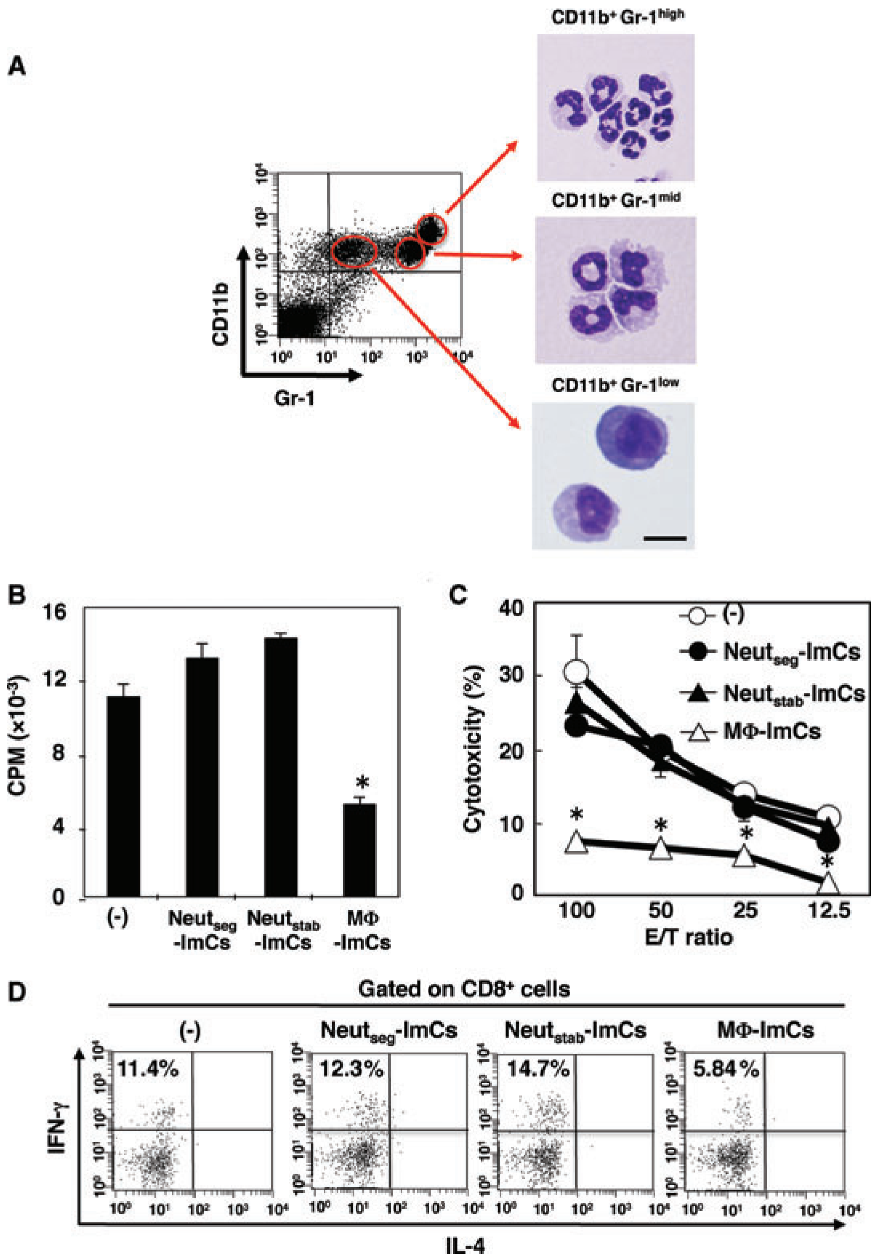

Figure 1. Characterization of three distinct subsets of CD11b$^+$ Gr-1$^+$ ImCs in the spleen. CMC-1 cells (2×10^6) were intradermally (i.d.) inoculated into BALB/c mice and the characteristics of spleen CD11b$^+$ Gr-1$^+$ ImCs cells were analyzed 30 days after tumor inoculation. (A) Cells were stained for CD11b and Gr-1 and a flow cytometry profile representative of three independent experiments is shown (left). The three populations indicated by red circles were sorted and defined their morphological features by staining with Giemsa stain (right). Bar = 5 μm. (B, C, D) The indicated ImCs populations isolated from spleen cells were added into MLR to determine their immunosuppressive activity 4 days after the culture. (B) T-cell proliferation, (C) cytotoxicity against MBL-2 lymphoma (H-2^b) and (D) IFN-γ production from CD8$^+$ T cells were determined by ^{3}H-thymidine-incorporation assay, ^{51}Cr-release assay or intracellular cytokine staining assay, respectively. (B, C) Data are shown as mean ± SD of n = 3 samples. (D) Flow cytometry profiles show the percentages of IFN-γ-producing cells gated in CD8$^+$ cells. (A–D) Data shown are representative of three independent experiments. $^*p < 0.05$: difference from control (–), two-sided Student's t-test.

ImCs were isolated by FACS to define their morphological and functional characteristics. Their morphologic characteristics were defined using cytospin preparations stained with May-Giemsa. As shown in Figure 1, the three ImCs subsets were defined as Gr-1^{low} monocyte-like cell (MΦ-ImCs), Gr-1^{mid} stab neutrophil (Neut$_{stab}$-ImCs), and Gr-1^{high} segmented neutrophil (Neut$_{seg}$-ImCs), respectively (Fig. 1A).

To evaluate immunosuppressive function, which is the most critical point for the definition of MDSCs, Neut$_{seg}$-ImCs, Neut$_{stab}$-ImCs, and MΦ-ImCs were isolated from spleens of tumor-bearing mice and examined their inhibitory activity against T-cell responses. As a result, only MΦ-ImCs, but neither Neut$_{seg}$-ImCs or Neut$_{stab}$-ImCs suppressed the proliferation, IFN-γ-production and CTL generation in MLR (Fig. 1B and C). Therefore, in the spleen of the tumor-bearing host, MΦ-ImCs but neither Neut$_{seg}$-ImCs nor Neut$_{stab}$-ImCs population should be defined as MDSCs, judging from their immunosuppressive activity.

Identification of stronger CD11b$^+$ Gr-1$^+$ ImCs in tumor-infiltrating leukocytes (TILs)

Consistent with previous reports [13–25], we confirmed the infiltration of large amounts of CD11b$^+$Gr-1$^+$ cells in the tumor tissue. Here, we first demonstrated that TILs included only two subsets of ImCs; CD11b$^+$G-1^{high} (54.5 ± 9.8%) and CD11b$^+$Gr-1^{low} (6.6 ± 0.5%) cells (Fig. 2A). Morphological analysis revealed that CD11b$^+$Gr-1^{high} cells and CD11b$^+$Gr-1^{low} cells in tumor tissues were histologically identified as segmented neutrophil (Neut$_{seg}$-ImCs) and macrophage (MΦ-ImCs) respectively (Fig. 2A). In

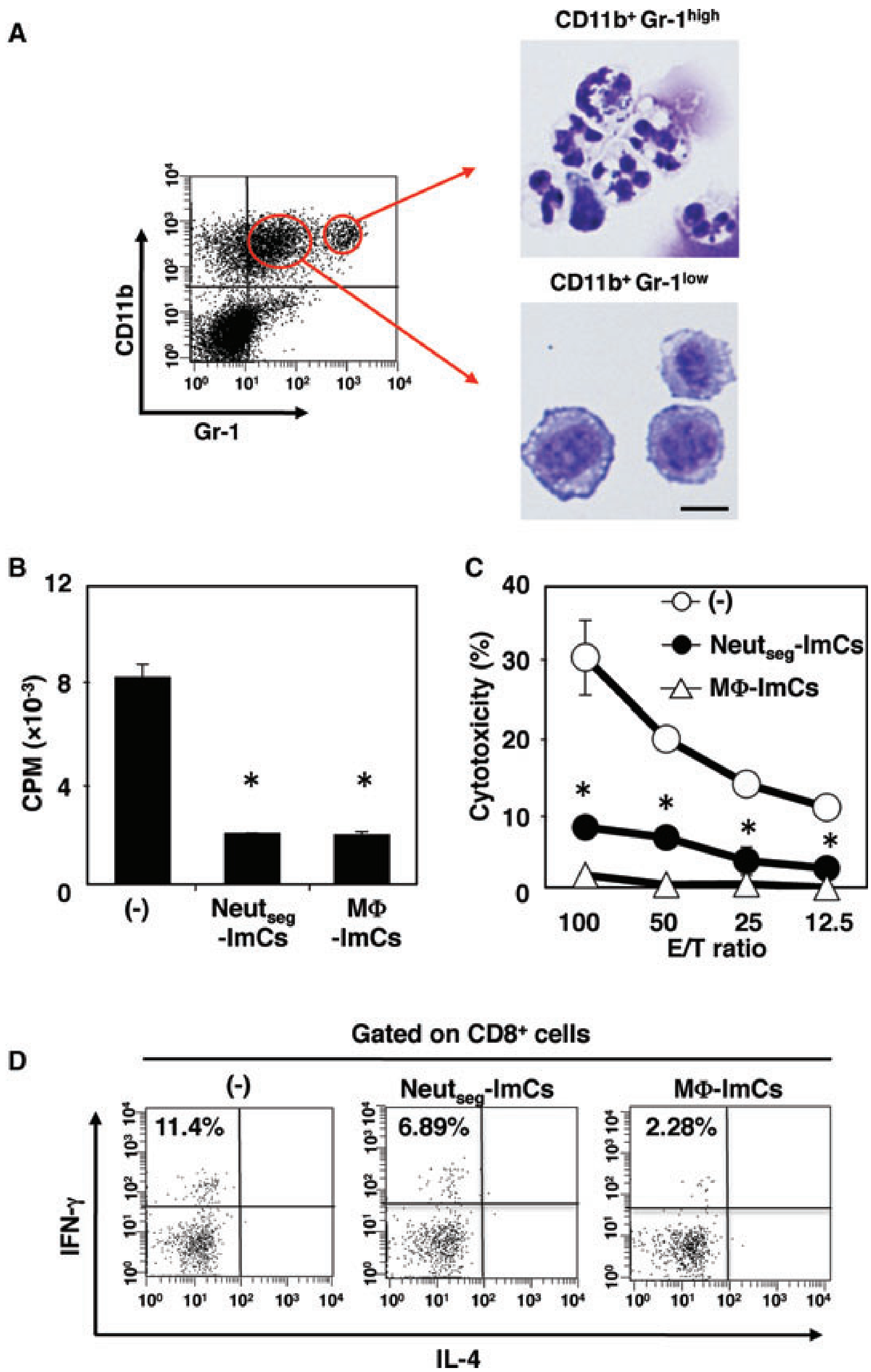

Figure 2. Identification of stronger CD11b$^+$ Gr-1$^+$ ImCs in tumor infiltrating leukocytes. CMC-1 cells (2×10^6) were i.d. inoculated into BALB/c mice and the characteristics of TILs-CD11b$^+$ Gr-1$^+$ ImCs cells were analyzed 30 days after tumor inoculation. (A) Cells were stained for CD11b and Gr-1 and a flow cytometry profile representative of three independent experiments is shown (left). The two populations indicated by red circles were sorted and defined their morphological features by staining with Giemsa stain (right). Bar = 5 μm. (B, C, D) The indicated ImC populations isolated from TILs were added into MLR to determine their immunosuppressive activity 4 days after the culture. The same inhibition assays (B, proliferation; C, CTLs generation; D, IFN-γ production) shown in Fig. 1 were carried out. (B, C) Data are shown as mean ± SD of $n = 3$ samples. (D) Flow cytometry profiles show the percentages of IFN-γ-producing cells gated in CD8$^+$ cells. (A–D) Data shown are representative of three independent experiments.$^*p < 0.05$: difference from control (–), two-sided Student's *t*-test.

contrast to spleen, CD11b$^+$Gr-1^{mid} (Neut$_{stab}$-ImCs) cells were not detected in the tumor tissues. Notably, tumor-infiltrating Neut$_{seg}$-ImCs and MΦ-ImCs showed hypersegmented nuclear and foamy morphology, which were obviously different morphology from spleen Neut$_{seg}$-ImCs and MΦ-ImCs, respectively. We next examined the immunosuppressive activity of tumor-infiltrating ImC subsets and demonstrated that both Neut$_{seg}$-ImCs and MΦ-ImCs markedly inhibited T-cell responses in MLR (Fig. 2B–D). Similar inhibitory activity of spleen and tumor ImCs were obtained from other two different tumor models (Supporting Information Fig. 1).

Consistent with other reports, we confirmed that TIL ImC subsets showed significantly higher inhibitory activity against CTL generation in MLR compared with spleen MΦ-ImCs, which was the only immunosuppressive subset in tumor-bearing mouse spleen (Fig. 3A). In parallel with the immunosuppressive function (Fig. 2B–D), TILs Neut$_{seg}$-ImCs and MΦ-ImCs exhibited higher expression levels of immunosuppressive molecules compared with the corresponding subsets of spleen ImCs (Fig. 3B). We also performed immunoblot analysis to evaluate the protein levels of those immunosuppressive molecules. As a result, higher levels of Cox-2,

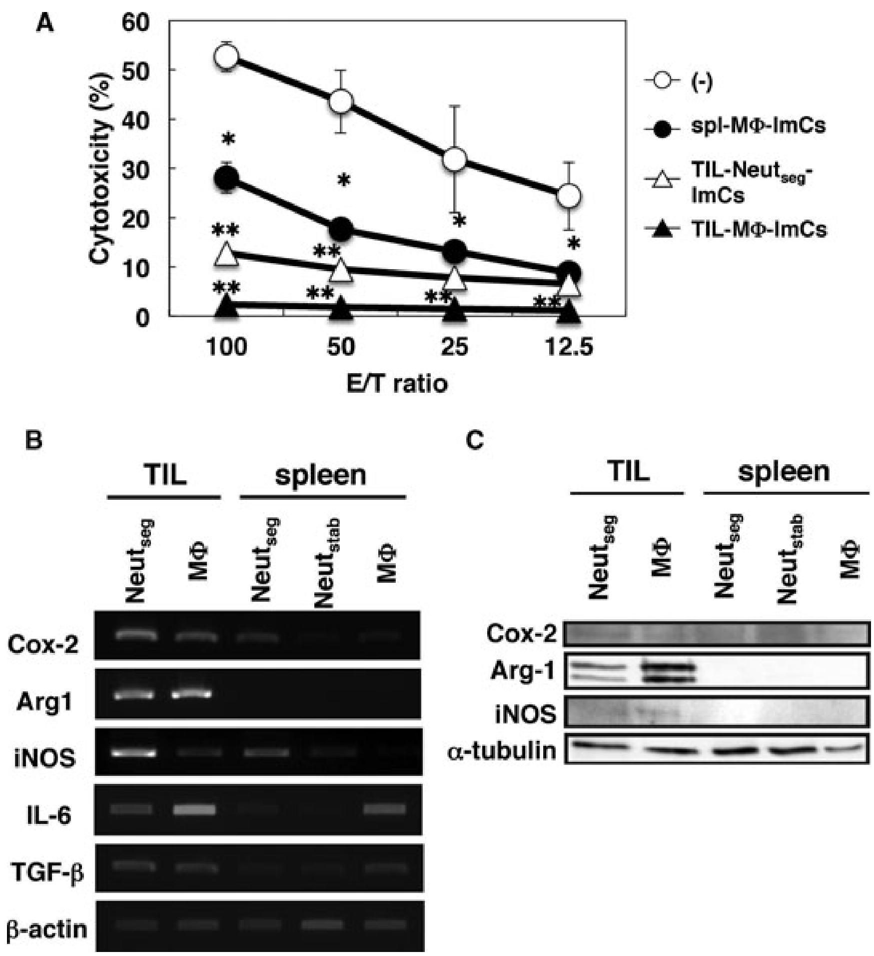

Figure 3. TILs-Neut$_{seg}$-ImCs and TILs-MΦ-ImCs exhibit stronger inhibitory activities against T cells compared with spleen-MΦ-ImCs. The characteristics of ImC subsets prepared from spleen and TILs of CMC-1-bearing mice were analyzed 30 days after tumor inoculation. (A) The indicated ImCs populations isolated from spleen cells and TILs were added into MLR. Four days after the incubation, MBL-2 (H-2^b)-specific cytotoxicity was determined by ^{51}Cr-release assay. Data are shown as mean ± SD of $n = 3$ samples. $^*p < 0.05$: difference from control, $^{**}p < 0.05$: difference from spl-Mφ-ImCs, two-sided Student's *t*-test. (B) mRNA or (C) protein expression in the indicated ImCs populations was determined by RT-PCR and immunobloting, respectively. β-actin and α-tubulin were used as control. (A–C) Data shown are representative of three independent experiments.

Arginase-1, and iNOS were detected in TILs-Neut$_{seg}$- and Mφ-ImCs than those in spleen ImCs populations (Fig. 3C). Although we evaluated the protein levels of IL-6 and TGF-β by ELISA, we could not detect these cytokines in the present experiments. This might be because that low levels of these cytokines functioned in tumor microenvironment.

Spleen CD11b$^+$Gr-1$^+$ cells differentiate into stronger immunosuppressive cells by tumor-derived factors

From the above data (Figs. 1, 2, and 3), it was speculated that non or low-suppressive spleen-ImCs (spl-Neut$_{stab}$-ImCs, spl-Neut$_{seg}$-ImCs, and spl-MΦ-ImCs) might possess the potential to differentiate into stronger suppressive MDSCs (TILs-Neut$_{seg}$-ImCs and TILs-MΦ-ImCs) when they were exposed to the factors in tumor microenvironment.

To evaluate this working hypothesis, we isolated the three subsets (spl-Neut$_{stab}$-ImCs, spl-Neut$_{seg}$-ImCs, and spl-MΦ-ImCs) of ImCs from spleen cells of CMC-1 tumor-bearing mice and cultured each ImC population with the conditioned medium containing TDFs of CMC-1 cells. Morphological analysis showed that spl-Neut$_{seg}$-ImCs and spl-Neut$_{stab}$-ImCs become hypersegmented cells like the TILs-Neut$_{seg}$-ImCs after incubation with TDF for 2.5 days. In addition, spleen MΦ-ImCs were differentiated into foamy macrophages, which were morphologically similar to TILs-MΦ-ImCs (Fig. 4A). In parallel with the morphological changes, Neut$_{seg}$-ImCs and Neut$_{stab}$-ImCs exhibited a strong suppressive activity after culturing with TDF, although freshly isolated these cells did not inhibit T-cell responses in MLR (Figs. 1 and 4). Moreover, after culture with TDF, spleen MΦ-ImCs were also converted into stronger suppressive MDSCs, which exhibited markedly inhibitory activity against cell proliferation, CTLs generation, and IFN-γ production in MLR (Fig. 4B–D). In parallel with the inhibitory activity, we demonstrated that the expression levels of Arg-1, iNOS markedly and Cox-2, TGF-β, and IL-6 were slightly upregulated in spleen ImCs subpopulations in response to TDF, indicating that these molecules might be involved in the suppressive activity of the cultured spleen ImCs (Supporting Information Fig. 2). As shown in Fig. 4E, culture of spleen ImCs subsets with IL-6 and TGF-β caused the induction of cells with a strong immunosuppressive activity. Thus, these data clearly indicated that TDF influenced on the differentiation and activation of MDSCs in tumor microenvironment.

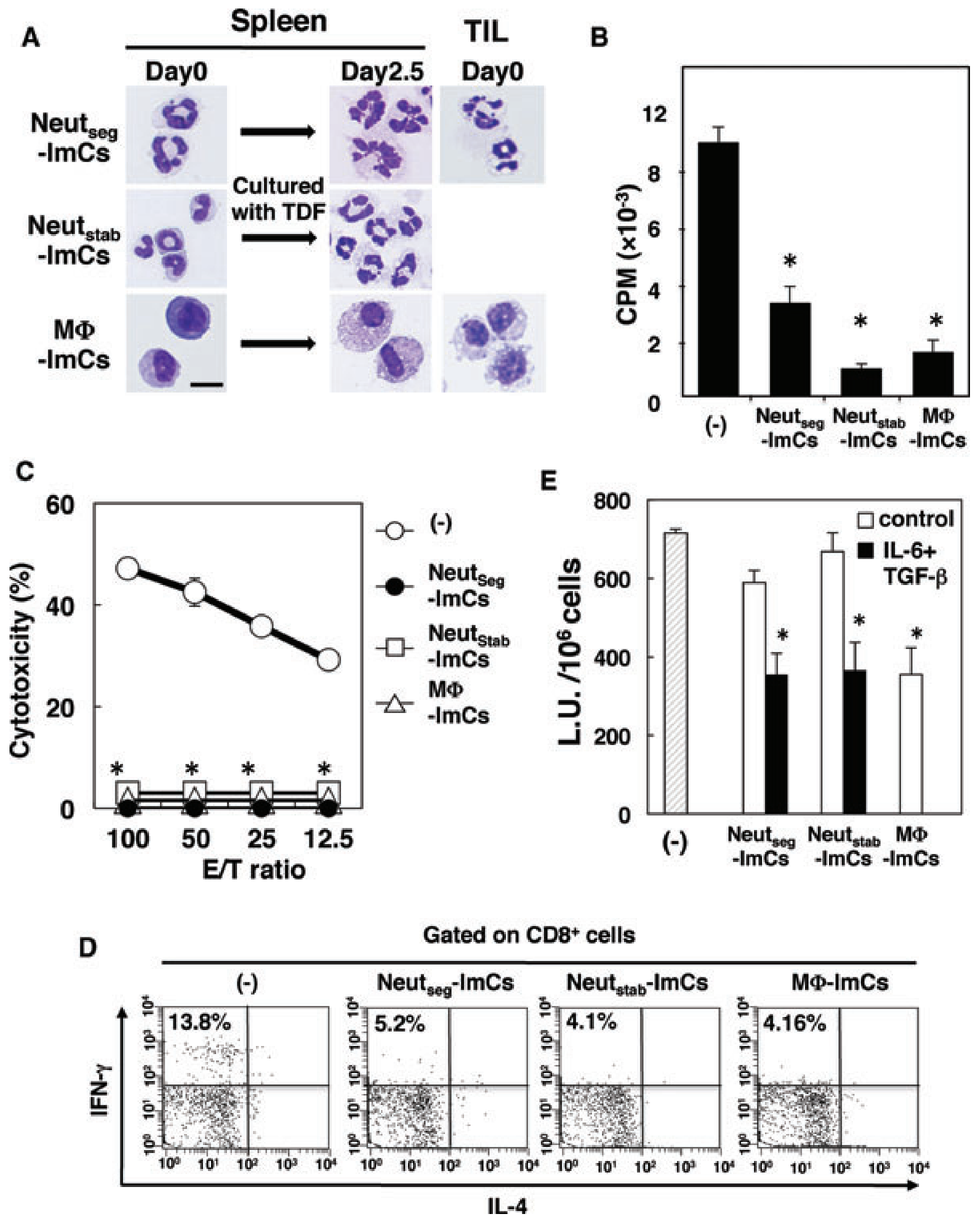

Figure 4. Spleen CD11b$^+$Gr-1$^+$ cells differentiate into stronger immunosuppressive cells by TDFs. Morphological and functional characteristics of ImC subsets prepared from spleen and TILs of CMC-1-bearing mice were analyzed 30 days after tumor inoculation. (A) The isolated spleen ImC subsets were incubated with culture supernatants of CMC-1 cells (tumor-derived factor; TDF) for 2.5 days. Freshly isolated spleen ImC subsets, TDF-cultured spleen ImCs and freshly isolated TILs-ImCs subsets were stained with Giemsa stain. Representative morphology data of three independent experiments is shown. Bar = 5 μm. (B, C, D) The inhibitory activity of the induced-ImCs subsets from corresponding spleen ImC subsets by TDF was determined by adding them into MLR 4 days after the culture. The same inhibition assays (B, proliferation; C, CTLs generation; IFN-γ production) shown in the legend to Fig. 1 were carried out. (B, C) Data are shown as mean ± SD of $n = 3$ samples. (D) Flow cytometry profiles show CD8$^+$ cells. (E) The isolated spleen ImC subsets were incubated with GM-CSF (open bar) or GM-CSF+IL-6+TGF-β (filled bar) for 2.5 days. The inhibitory activity of the cultured cells was determined by adding them into MLR. Cytotoxicity was measured by ^{51}Cr-release assay and indicated as Lytic unit (LU)/10^6 effector cells. (A–E) Data shown are representative of three independent experiments. $^*p < 0.05$: difference from control (–), two-sided Student's *t*-test.

Anti-IL-6R mAb inhibits tumor growth and downmodulates MDSC subsets

Since high amounts of IL-6, a key cytokine of immunosuppression and decreased quality of life in tumor-bearing hosts [35,36], is elevated in the serum of CMC-1-bearing mice and cancer patients, we investigated the role of IL-6 on MDSCs accumulation in vivo. To block IL-6-signaling in vivo, tumor-bearing mice were administered with anti-IL-6R mAb. Humanized anti-IL-6R mAb (tocilizumab) has been already applied to a clinical therapy of autoimmune diseases but not yet for the immunotherapy of solid cancer, except B lymphoma [38,39]. We found that injection of

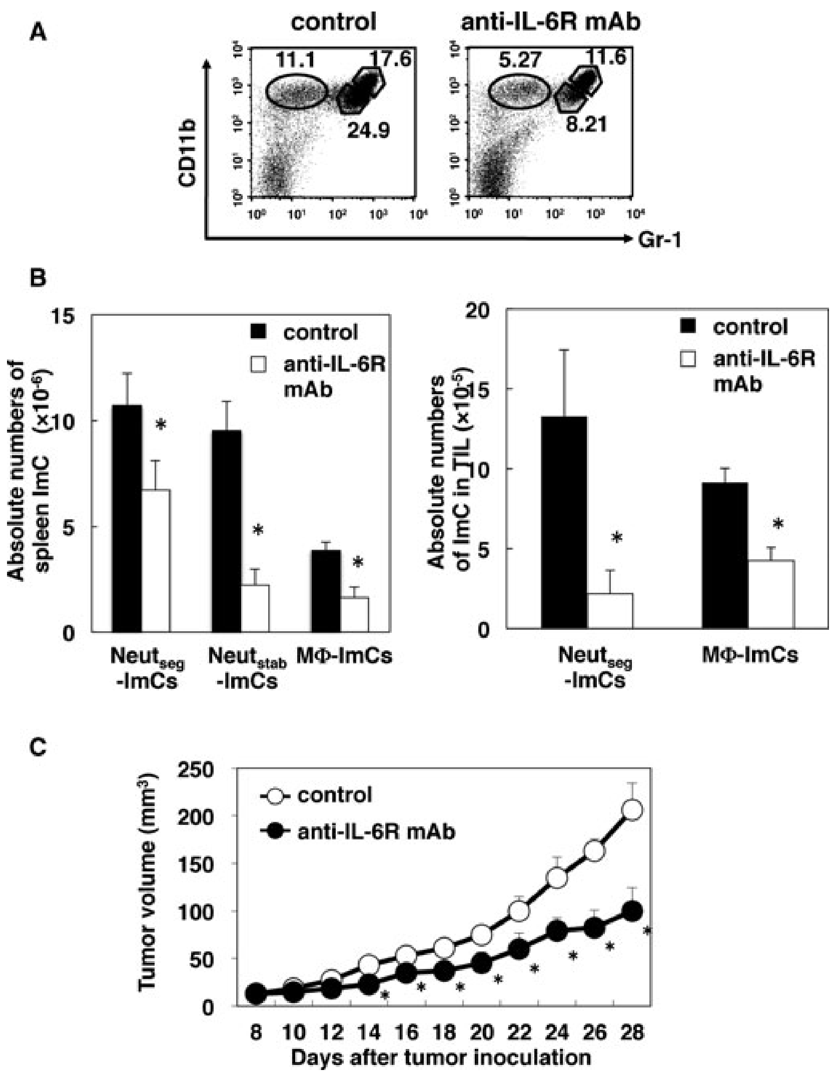

Figure 5. Anti-IL-6R mAb inhibits tumor growth and downmodulates MDSC subsets. BALB/c mice i.d. inoculated with CMC-1 cells (2×10^6) were treated i.v. with control IgG or anti-IL-6R mAb every 3 days. Then, their effect on ImC subsets in spleen cells or TILs was determined 2 days after final injection of antibodies. (A) The effect of control or anti-IL-6R mAb injection on the percentages of three distinct CD11b$^+$Gr-1$^+$ spleen-ImC subsets. A flow cytometry profile representative of three independent experiments is shown. (B) The effect of control or anti-IL-6R mAb injection on the absolute numbers of spleen-ImC subsets (left) and TILs-ImC subsets (right). Data represent mean + SD of five samples. The representative of three independent experiments is shown. (C) The effect of control IgG or anti-IL-6R mAb injection on tumor growth was determined by measuring tumor size as described in *Materials and methods*. The data are shown as mean + SD of $n = 5$ per group from one experiment representative of three independent experiments. $^*p < 0.05$: difference from control, two-sided Student's *t*-test.

anti-IL-6R mAb into tumor-bearing mice significantly decreased the numbers of cells within ImC subsets in both spleen and tumor tissue (Fig. 5A and B). Moreover, we also revealed that administration of anti-IL-6R mAb significantly suppressed the growth of tumor (Fig. 5C). These findings suggested that IL-6 might be involved in the differentiation and activation of MDSCs from ImCs and it might be a critical factor for regulating MDSC accumulation at tumor site, which caused the promotion of tumor progression in tumor-bearing mice.

Anti-IL-6R mAb augments antitumor T-cell responses in tumor-bearing mice

Based on the finding that suppressive MDSCs were depleted by anti-IL-6R mAb, we next tested whether anti-IL-6R mAb administration could rescue and augment T-cell responses in tumor-bearing mice. Spleen cells isolated from the tumor-bearing mice, which were treated with or without anti-IL-6R mAb, were stimulated with anti-CD3ε mAb to determine their IFN-γ-producing ability. As shown in Fig. 6A, administration of anti-IL-6R mAb, but not control mAb resulted in the enhancement of IFN-γ production of tumor-bearing mouse spleen cells. Intracellular cytokine staining analysis also demonstrated that IFN-γ-producing cells increased in both CD4$^+$ and CD8$^+$ T cells by anti-IL-6R mAb treatment (Fig. 6B and C). In consistent with above results (Fig. 6A, B, and C), we showed an administration of anti-IL-6R mAb caused the inhibition of tumor growth via downmodulation of MDSCs. However, pretreatment of tumor-bearing mice with anti-CD8 mAb caused the cancellation of anti-IL-6R mAb-induced tumor growth inhibition (Fig. 6D). Thus, these results indicate that depletion of MDSCs by anti-IL-6R mAb rescued CD8$^+$ T-cell activation, which might be essential for the inhibition of tumor progression.

Treatment with anti-IL-6R mAb and GEM augments antitumor T-cell immunity via inhibition of MDSCs

It has been reported that treatment with GEM, an anticancer drug, significantly downmodulated MDSCs in tumor-bearing mice

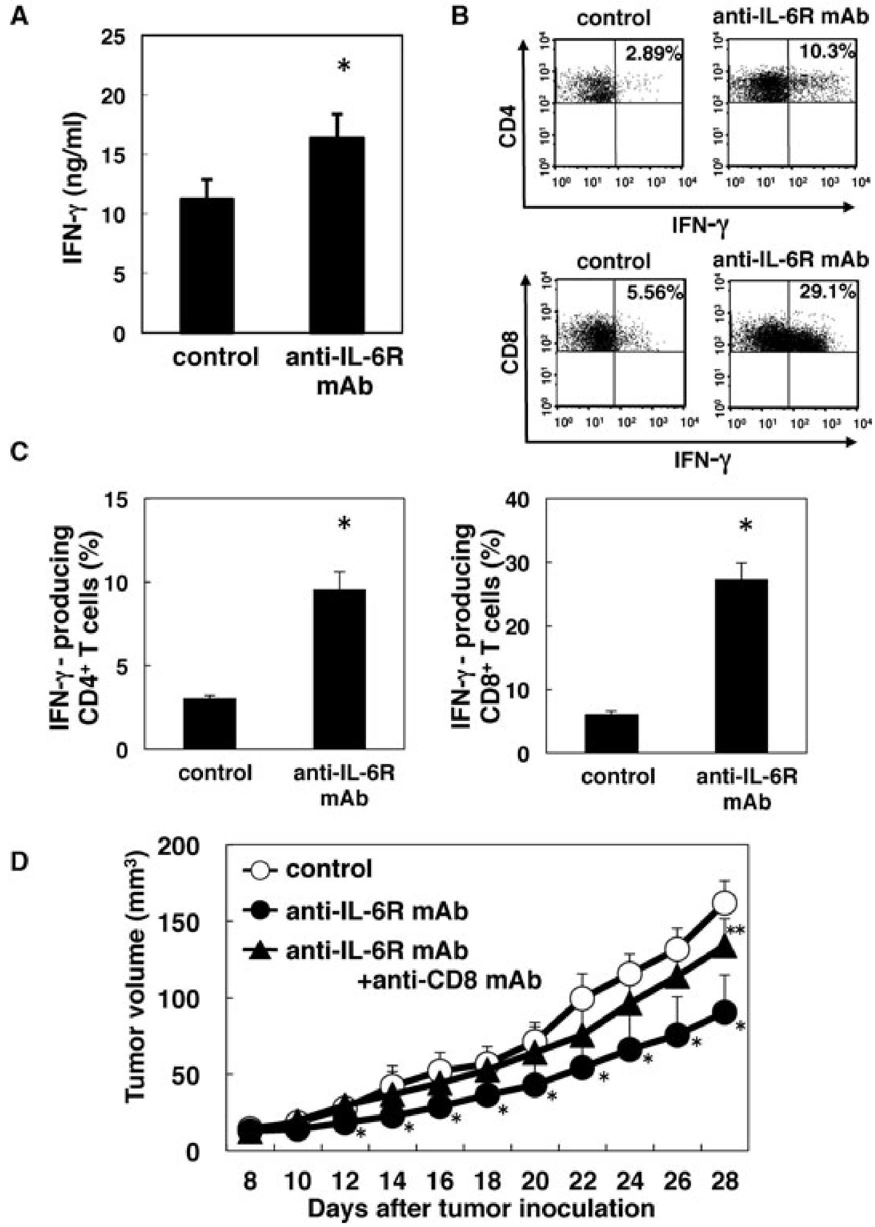

Figure 6. Anti-IL-6R mAb augments antitumor T-cell responses in tumor-bearing mice. BALB/c mice i.d. inoculated with CMC-1 cells (2×10^6) were injected i.v. with control IgG or anti-IL-6R mAbs. Two days after the final injection of antibodies, spleen cells were harvested and their effect on T-cell functions was determined. (A) IFN-γ-producibility of unfractionated spleen cells: spleen cells obtained from control or anti-IL-6R mA-treatment were stimulated with soluble anti-CD3ε mAb for 48 h to determine IFN-γ levels in the supernatants by ELISA. (B, C) IFN-γ-producibility of isolated $CD4^+$ and $CD8^+$ T cells from control or anti-IL-6R mA-treated mice. After stimulation with plate-bound anti-CD3ε mAb for 6 h, the percentages of IFN-γ-producing $CD4^+$ or $CD8^+$ T cells were determined by flow cytometry. (B) A representative flow cytometry profile and (C) mean ± SD of $n = 5$ per group representative of three separate experiments are shown. (D) CMC-1 cells (2×10^6) were inoculated i.d. into BALB/c mice, and the mice were treated with anti-IL-6R and/or anti-CD8 mAb every 3 days. The antitumor effect of mAbs was determined by measuring the tumor size. The data are shown as mean + SD of $n = 5$ per group from one experiment representative of three independent experiments. $^*p < 0.05$: difference from control, $^{**}p < 0.05$: difference from anti-IL-6R mAb, two-sided Student's *t*-test.

[43, 44]. We also confirmed that the treatment of tumor-bearing mice with GEM alone reduced the numbers of all three different spleen ImC subsets and two suppressive MDSC subsets at tumor local site (Fig. 7A and B). In addition, combined treatment of tumor-bearing mice with both anti-IL-6R mAb and GEM revealed more efficient reduction of MDSCs compared with anti-IL-6R mAb alone or GEM alone. Although GEM is an anticancer drug, it exhibited no significant toxic effect on T cells compared with other anticancer drugs such as 5-FU, cyclophosphamide (data not shown), and GEM-treated tumor-bearing mice exhibited rather increased T-cell responses via downmodulation of MDSCs, which were also observed in the case of anti-IL-6R mAb treatment (Fig. 6A, B, and C). As shown in Fig. 7C, combination therapy of tumor-bearing mice with anti-IL-6R mAb and GEM exhibited more efficient antitumor activity in comparison with a single therapy with anti-IL-6R mAb or GEM alone. Moreover, the antitumor activity of the combination therapy was significantly reduced by depletion of $CD8^+$ T cells by anti-CD8 mAb injection. Thus, $CD8^+$ T-cell activation appeared to be involved in the inhibition of tumor growth by combination therapy with anti-IL-6R mAb and GEM via reducing MDSC accumulation at tumor site.

Discussion

It has been considered that it is essential to downmodulate strongly immunosuppressive tumor escape mechanisms to induce Th1-dependent tumor-specific CTLs for establishing more efficient

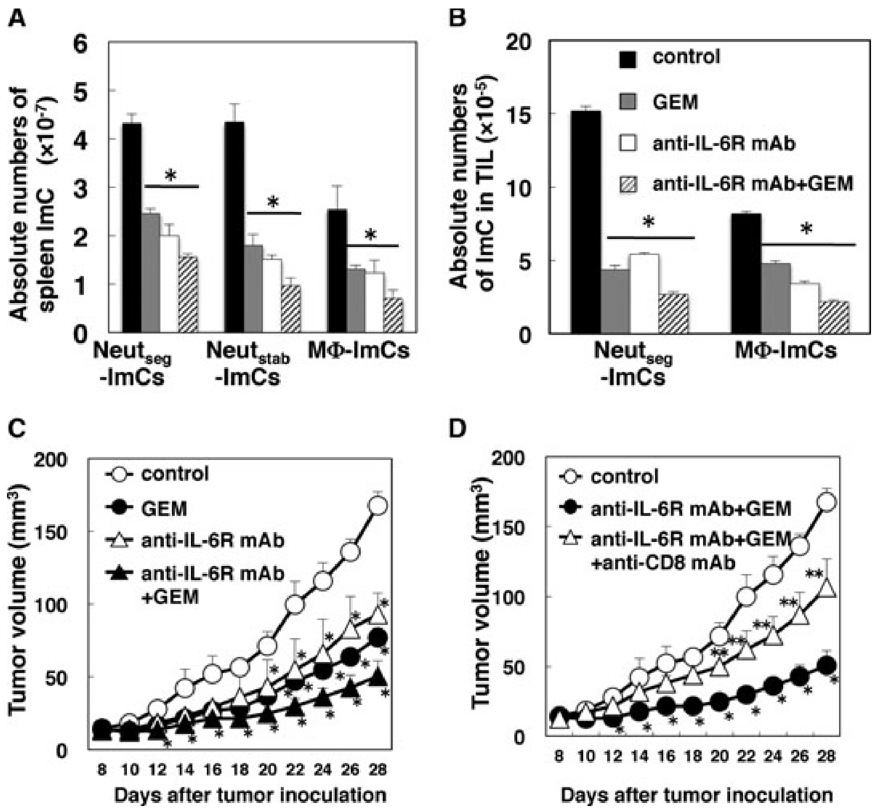

Figure 7. Treatment with anti-IL-6R mAb and GEM augments antitumor T-cell immunity via inhibition of MDSCs. BALB/c mice i.d. inoculated with CMC-1 cells (2×10^6) were treated with anti-IL-6R mAb and/or GEM and their effect on the percentages of (A) spleen ImCs subsets or (B) TILs-ImCs subsets was evaluated by calculating absolute cell numbers of each ImC subsets. The data are shown as mean + SD of $n = 5$ per group. (C, D) CMC-1-bearing mice were treated with anti-IL-6R mAb and/or GEM and thereafter the tumor size was monitored to determine their antitumor effects. As a control (open circle), mice were treated with anti-rat IgG mAb. The data are shown as mean + SD of $n = 5$ per group. (A–D) Data shown are representative from one out of three independent experiments. $^{*}p < 0.05$: difference from control, $^{**}p < 0.05$: difference from anti-IL-6R mAb+GEM, two-sided Student's t-test.

tumor immunotherapy. In the present paper, we initially demonstrate that an administration of anti-IL-6R mAb is a novel strategy to downmodulate MDSCs to enhance antitumor T-cell immunity in tumor-bearing mice.

Recently, in addition to Treg cells, MDSCs have been highlighted as crucial immunosuppressor cells accumulating into tumor tissue at the late stage of tumor growth [45]. Because all CD11b$^+$Gr-1$^+$ ImC subsets are initially defined as MDSCs regardless of their immunosuppressive function, the definition and characteristics of MDSCs are still unclear. Recently, Dolcetti et al. [15] showed that there were (i) Gr-1^{high} subset; mature polymorphonuclear cells, (ii) Gr-1^{int} subset; cells with "doughnut"-shaped nuclei, and (iii) Gr-1^{low} subset; monocytic cells of MDSCs in spleen of tumor-bearing mice. In consistent with their finding, we also demonstrate that ImCs, accumulating in spleens of tumor-bearing mice, contain three distinct ImCs subsets (Gr-1^{low}MΦ-ImCs, Gr-1^{mid}Neut$_{stab}$-ImCs, and Gr-1^{high}Neut$_{seg}$-ImCs) judging from their morphological, phenotypical, and functional difference. Although Dolcetti et al. [15] reported that both Gr-1^{int} and Gr-1^{low} subsets exhibited a strong immunosuppressive function, but Gr-1^{high} subset exerted low or no immunosuppressive activity. However, we demonstrate that two granulocytic cell subsets (Gr-1^{high}Neut$_{seg}$- and Gr-1^{mid}Neut$_{stab}$-ImCs) in tumor-bearing mouse spleen show no significant suppressive activity against T-cell responses. The discrepancy between our results and theirs may be due to the difference of tumor models used in the experiments. We, at least, revealed that among the spleen ImC subsets, Gr-1^{low}MΦ-ImCs act as MDSCs with a weak suppressive activity, while Gr-1^{mid}Neut$_{stab}$-ImCs and Gr-1^{high}Neut$_{seg}$-ImCs exhibit no significant immunosuppressive function in three distinct tumor systems (Fig. 1 and Supporting Information Fig. 1).

Many investigators have evaluated the characteristics of spleen MDSCs by morphological, phenotypical, and functional analysis [12–16]. However, the precise feature of TILs-CD11b$^+$Gr-1$^+$ ImCs is poorly understood. Here, we first demonstrate TILs-CD11b$^+$Gr-1$^+$ ImCs consist of only two distinct ImC subsets, Gr-1^{low}MΦ-ImCs and Gr-1^{high}Neut$_{seg}$-ImCs. This is totally different from spleen-ImC subsets (Fig. 1A). It is surprising that TILs-Gr-1^{high}Neut$_{seg}$-ImCs exhibit a stronger T-cell suppression activity in contrast to spleen Gr-1^{high}Neut$_{seg}$-ImCs (Fig. 1 and 2). Such data are supported by recent reports that exposure of ImCs to tumor microenvironment or inflammatory site enhanced suppressive function of MDSCs [46,47]. Notably, both of TILs-Gr-1^{high}Neut$_{seg}$-ImCs and Gr-1^{low}MΦ-ImCs exhibited different morphology and function compared with corresponding spleen CD11b$^+$Gr-1$^+$ ImCs subsets (Fig. 3 and 4); TILs-Neut$_{seg}$-ImCs have hypersegmented nucleus, TILs-MΦ-ImCs were foamy macrophages. Spleen Neut$_{stab}$-ImCs were immediately differentiated into hypersegmented neutrophils and acquired immunosuppressive activity when they were cultured in the presence of TDF. Such evidence strongly indicates that less immunosuppressive immature MDSCs in the spleen are converted into phenotypically, morphologically, and functionally maturated MDSCs with a strong immunosuppressive activity after recruiting into tumor tissues. We are now pursuing what factor(s) contribute to the induction of immunosuppressive function of CD11b$^+$Gr-1$^+$ cells in tumor microenvironment. In our and other recent observation, IL-6 and TGF-β would be possible factors to activate MDSCs as well as GM-CSF. Indeed, we demonstrate that IL-6 and TGF-β are involved in the conversion of spleen ImCs subsets into immunosuppressive TILs-Neut$_{seg}$-ImCs and TILs-MΦ-ImCs as well as TDFs (Fig. 4E).

It is a critical issue to develop a novel strategy to decrease the MDSC population to enhance antitumor T-cell responses. Sawanobori et al. [28] indicated that the precursors of MDSCs vigorously proliferated in the bone marrow but not in the peripheral tissues and subsequently migrated into spleen and tumor tissue. We initially indicate that anti-IL-6R mAb successfully reduced the numbers of MDSCs in tumor-bearing mice (Fig. 5A and B), suggesting that anti-IL-6R mAb may directly affect on proliferation, survival, and/or mobilization of MDSCs in tumor-bearing state. Indeed, flow cytometric analysis revealed that IL-6R was expressed on the MDSC subpopulations, except TILs-Neut$_{seg}$-ImCs (data not shown). These results strongly indicate that spleen ImCs and TILs-Mφ-ImCs could directly respond to IL-6. It has been well known that IL-6 is a multifunctional cytokine. In the tumor-bearing condition, IL-6, which is produced by tumor cells, macrophages, and stromal cells, is involved in immunosuppression through its negative effect on DC function [48, 49]. IL-6 directly influences on the promotion of tumor cell survival and proliferation in STAT3-dependent manner [6]. It has been also demonstrated that IL-6 is a key cytokine to induce MDSCs [12, 37].

Here, we first demonstrate that IL-6 is a key cytokine to regulate the accumulation of MDSCs in tumor-bearing mice (Fig. 5B). Thus, administration of anti-IL-6R mAb may cause the cancellation of all IL-6-mediated tumor-promoting mechanisms in tumor-bearing mice and augment antitumor T-cell responses. Moreover, we demonstrate that the combination therapy of tumor-bearing mice with anti-IL-6R mAb and GEM induces downmodulation of MDSCs and enhances T-cell responses (Fig. 7). This result is greatly supported by previous results that GEM treatment is a preferable anticancer drug to downmodulate MDSC function during cancer therapy [43].

The accumulation of MDSCs was detected in peripheral blood and tumor tissues of cancer patients as well as tumor-bearing mouse system [17, 18]. It is reported that IL-6 levels in the plasma of cancer patients were markedly correlated with numbers of CD33$^+$HLA-DR$^-$CD15$^+$ MDSCs [30]. In addition, Lechner et al. [50] reported that IL-6 plus GM-CSF condition effectively generated potent suppressive CD33$^+$ MDSCs from PBMCs of a healthy donor. These reports strongly indicated the involvement of IL-6 in the increase of MDSCs in cancer patients. Therefore, it would be possible to apply humanized anti-IL-6R mAb (tocilizumab) for human cancer therapy through the decrease of MDSC-mediated immunosuppression and the augmentation of T-cell-mediated antitumor immunity in cancer patients.

Materials and methods

Animals

Female BALB/c mice were purchased from Charles River, Kanagawa, Japan. The mice were maintained in specific pathogen-free conditions and used at 6–8 weeks of age according to the guidelines of animal department at Hokkaido University.

Tumor immunotherapy

The carcinoma cells, CMC-1 were generated by intradermal injection of methylcholanthrene (MCA) into BALB/c mice [51]. BALB/c mice were i.d. inoculated with 2×10^6 CMC-1 cells, and the tumor size was measured by micrometer calipers. The size of the tumor was calculated as following formula; Volume (mm^3) = 0.2 × (length (mm) × width (mm)) × (high (mm))^2. In the antibody treatment experiments, CMC-1 cells were inoculated i.d. into BALB/c mice, and when the tumor mass reached palpable (7–8 mm), anti-IL-6R mAb (MR16–1) (200 μg) and/or anti-CD8 mAb (53.6.7) (200 μg), control rat IgG was intravenously (i.v.) injected every 3 days. In the chemotherapy experiments, when the tumor mass reached 7–8 mm, CMC-1-bearing mice received ip treatment with gemcitabine (GEM) (120 mg/kg) weekly, and i.v. treatment with anti-IL-6R mAb and/or anti-CD8 mAb every 3 days. Anti-IL-6R mAb (MR16–1) and control rat IgG was a kind gift from Chugai Pharmaceutical Co. Ltd., Shizuoka, Japan gemcitabine (Gemzar®) was purchased from Eli Lilly, Indianapolis, IN, USA.

Lymphocytes preparation

CMC-1 cells were i.d. inoculated into BALB/c mice, and 30 days after the injection, spleen cells were prepared. The tumor tissues were dissected from the mice and minced. Single-cell suspensions were obtained by digestion with 1 mg/mL collagenase (Sigma, Tokyo, Japan) for 30 min at 37°C.

For isolation of CD11b$^+$Gr-1^{high}, CD11b$^+$Gr-1^{mid}, and CD11b$^+$Gr-1^{low} cells, the cells prepared from spleens and tumor tissues were stained with PE-conjugated anti-CD11b mAb (M1/70, BD BioScience, San Jose, CA, USA) and FITC-conjugated anti-Gr-1 mAb (RB6–8C5, BD BioScience) after treatment with purified anti-CD16/CD32 mAb (2.4G2, BD BioScience). The percentages of CD11b$^+$Gr-1$^+$ cells were determined using FACSCant II (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR, USA). Each subpopulation of CD11b$^+$Gr-1$^+$ cells was isolated by FACSAria (BD BioSciences). The purity of the isolated cells was consistently more than 95%.

In vitro differentiation of spleen ImCs

CMC-1 cells (5×10^4) were incubated for 72 h with 6-well plate (NUNC, Roskilde, Denmark). The supernatants were collected and used as conditioned medium containing TDF. Indicated spleen $CD11b^+Gr\text{-}1^+$ subpopulations were isolated from CMC-1-bearing mice and cultured in the presence of conditioned medium (50%), GM-CSF (0.3 ng/ml, Wako, Osaka, Japan) or GM-CSF+IL-6 (50 ng/ml, Wako)+TGF-β (2 ng/ml, Wako) for 2.5 days.

Morphological analysis

Indicated $CD11b^+Gr\text{-}1^+$ subpopulations were isolated from spleen and tumor tissues of CMC-1-bearing mice. Cytospins of the isolated cells were prepared with Shandon Cytospin 3 (Thermo Electron, Waltham, MA, USA) and stained with May-Giemsa stain. Morphological differences were observed using a light microscopy (BX50, OLYMPUS OPTICAL Co, Ltd., Tokyo, Japan).

Generation of CTLs in MLR

Spleen cells (5×10^6) from BALB/c mice were cocultured with C57BL/6 spleen cells (1×10^6) which were pretreated with mitomycin C (60 μg/mL; Kyowa Hakko, Tokyo, Japan). Isolated $CD11b^+Gr\text{-}1^{high}$, $CD11b^+Gr\text{-}1^{mid}$, or $CD11b^+Gr\text{-}1^{low}$ cells (1×10^5) were added into the culture and these cells were incubated for 4 days in the 5 mL round-bottom tubes (BD Falcon, San Jose, CA, USA). After culture, cells were collected and used for analysis.

^{3}H-thymidine incorporation assay

Cell proliferation was evaluated as previously described [1]. Briefly, ^{3}H-thymidine was added to culture medium for 6 h and the radioactivity of the incorporated cell was detected and indicated as proliferation T cells.

Cytotoxicity assay

Cytotoxicity of cells generated by MLR was measured by 4 h ^{51}Cr-release assays as described previously [52]. Antigen-specific cytotoxicity was determined using MBL-2 T-lymphoma cells (H-2^b) as target cells. As a negative control, P815 mastocytoma cells (H-2^d) were used in the same experiments. The percentage of cytotoxicity was calculated and indicated in the figures as described previously [52]. In Fig. 4E, cytotoxicity was indicated as lytic units (L.U.)/10^6 effector cells. An L.U. was defined as the numbers of effector cells required for causing 25% lysis of 5×10^3 target cells.

Intracellular cytokine staining

For detection of cytoplasmic cytokine expression of T cells, spleen cells prepared from CMC-1-bearing mice were stimulated with inactivated allogeneic spleen cells for 4 days or 2 μg/mL soluble anti-CD3ε mAb (145–2C11, BD Bioscience) for 4 h. After the stimulation, cells were restimulated with plate-bound anti-CD3ε mAb in 96-well flat-bottomed plates for 6 h. Brefeldin A was added at 4 h. Then, the cells were harvested and stained with anti-CD4, and anti-CD8 mAbs (BD Bioscience), and fixed with 4% paraformaldehyde. After permeabilization, the fixed cells were stained with anti-IFN-γ and anti-IL-4 mAbs (BD bioscience). Data were acquired by FACSCant ll and analyzed with FlowJo software.

PCR analysis

Total RNA was extracted from sorted spleen or tumor-infiltrating $CD11b^+Gr\text{-}1^{high}$, $CD11b^+Gr\text{-}1^{mid}$, and $CD11b^+Gr\text{-}1^{low}$ cells using Isogen RNA extraction kit (Nippongene, Toyama, Japan) according to manufacturer's instructions. cDNA was prepared using Superscript lll RT (Invitrogen, Carlsbad, CA, USA). The indicated gene cDNAs were specifically amplified using a GeneAmp 9700 thermalcycler (PE Applied Biosystems) and corresponding primer pairs. The sequences were as follows: mouse β-actin, (sense) 5′-GTGATG GTGGGAATG GGTCAG-3′, (antisense) 5′-TTTGAT GTCACGCACG ATTTCC-3′, mouse COX-2, (sense) 5′-ACACACT CTATCACTG GCACC-3′, (antisense) 5′-TTCAGGGAGAAG CGTTTGC-3′, mouse Arg-1, (sense) 5′-GCA GAGGTCCAGA AGAATGGAA-3′, (antisense), 5′-CTTTT CTTCCTTCC CAGCAGGT-3′, mouse iNOS, (sense) 5′-GGGCTGTCACGGAGATCA-3′, (antisense) 5′-CCATGA TGGTCA-CAT TCTGC -3′, mouse IL-6, (sense) 5′-GAGGAT ACCACTCCCA ACAGACC-3′, (antisense) 5′-AAGTGCA TCATCGTTG TTCATACA-3′, mouse TGF-β, (sense) 5′-TACGTCAG ACATTCGGGA AGCA-3′, (antisense) 5′-GAAGTTG GCATGGT AGCCCTTG-3′

Immunoblotting

Antibodies were purchased from the following sources: Monoclonal Anti-Arginase I antibody and Monoclonal Anti-iNOS/NOS Type II antibody from BD Transduction Laboratories, and polyclonal Anti-COX-2 antibody from Cayman, and monoclonal anti-α-tubulin (DM1A) from SIGMA. Cells were lysed in a buffer consisting of 20 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 1% Nonidet P-40, 2 mM dithiothreitol, 1 mM Vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin and 10% glycerol. Cell lysates were subjected to immunoblotting with the indicated antibodies.

ELISA

Whole spleen cells of CMC-1-bearing mice treated with control rat IgG or anti-IL-6R mAb were stimulated with soluble anti-CD3ε mAb (1 μg/mL). After 48 h, IFN-γ levels in the culture supernatants were determined by OptEIA™ mouse IFN-γ (BD Bioscience).

Statistical analyses

Significant differences in the results were determined by using the two-sided Student's *t*-test. The $p<0.05$ was considered significant in the present experiments.

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Abbreviations: **GEM**: gemcitabine · **ImC**: immature myeloid cell · **MDSC**: myeloid-derived suppressor cell · **TDF**: tumor-derived factor · **TIL**: tumor-infiltrating leukocyte

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