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Immune Stimulatory Receptor CD40 Is Required for T-Cell Suppression and T Regulatory Cell Activation Mediated by Myeloid-Derived Suppressor Cells in Cancer

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Abstract

Immune tolerance to tumors is often associated with accumulation of myeloid-derived suppressor cells (MDSC) and an increase in the number of T-regulatory cells (Treg). In tumor-bearing mice, MDSCs can themselves facilitate the generation of tumor-specific Tregs. In this study, we demonstrate that expression of the immune stimulatory receptor CD40 on MDSCs is required to induce T-cell tolerance and Treg accumulation. In an immune reconstitution model, adoptive transfer of Gr-1$^+$CD115$^+$ monocytic MDSCs derived from CD40-deficient mice failed to recapitulate the ability of wild-type MDSCs to induce tolerance and Treg development *in vivo*. Agonistic anti-CD40 antibodies phenocopied the effect of CD40 deficiency and also improved the therapeutic efficacy of IL-12 and 4-1BB immunotherapy in the treatment of advanced tumors. Our findings suggest that CD40 is essential not only for MDSC-mediated immune suppression but also for tumor-specific Treg expansion. Blockade of CD40-CD40L interaction between MDSC and Treg may provide a new strategy to ablate tumoral immune suppression and thereby heighten responses to immunotherapy. *Cancer Res; 70(1); 99–108. ©2010 AACR.*

Introduction

A growing body of evidence suggests that host immune cells with a suppressive phenotype pose a significant hurdle to successful immune-enhancing therapy for cancer (1–4). Among these suppressor cells, T regulatory cells (Treg) and myeloid-derived suppressor cells (MDSC) have been shown to increase significantly in hosts with advanced malignancies (5, 6). Tregs play an essential role in the maintenance of self-tolerance and may impede antitumor immune responses (7, 8). MDSCs contribute to tumor-associated immune dysfunctions through a myriad of mechanisms, including production of nitric oxide (6, 9, 10), arginine depletion (3, 11–14), and production of reactive oxygen species (15, 16).

CD40, a member of the tumor necrosis factor receptor superfamily, is expressed at various levels on antigen-presenting cells, epithelial cells, hematopoietic progenitor cells (17, 18), and activated T cells (19). CD40 ligand (CD40L) is expressed predominantly on activated, but not resting, T cells (17), activated B cells (20), and activated platelets (21). The interaction between CD40 and CD40L reciprocally delivers activating signals to antigen-presenting cells and cognate T cells, respectively. This process is critically important in the development of adaptive immunity (22–24); however, the role of CD40 in MDSC suppressive function has not been investigated.

Previously, we found that Gr-1$^+$CD11b$^+$CD115$^+$ (monocytic) MDSCs but not Gr-1$^+$CD11b$^+$CD115$^-$ (granulocytic) MDSCs, induced the activation of tumor specific Foxp3$^+$ Tregs (25). The Tregs induced by MDSC have an inhibitory effect on antitumor responses in tumor-bearing mice. In the current study, we found that CD40 and MHC II expressions by MDSC were increased upon stimulation with IFN-γ. MDSC derived from CD40-deficient tumor-bearing mice failed to induce the development of Foxp3$^+$ Tregs and were unable to suppress T-cell proliferation. Blockade of CD40/CD40L interaction through the use of anti-CD40 not only suppressed the development of Tregs but also enhanced the therapeutic efficacy of an established immune modulatory therapy in an advanced tumor model. Our results show a novel role for CD40 in the tumor-associated activation of Tregs and immune suppression mediated by MDSC.

Materials and Methods

Experimental animals. Wild-type (WT) BALB/c, CD40-deficient BALB/c, ovalbumin (OVA)–specific MHC class II–restricted TCR-transgenic (OT-II) CD45.1 C57BL6, and CD40 knockout (KO) CD45.2 C57BL6 mice were purchased from National Cancer Institute and The Jackson Laboratory. Influenza hemagglutinin (HA)–specific MHC class II–restricted CD4 TCR-transgenic mice (in BALB/c background, Thy-1.2) were a gift from Dr. C. Bona (Mount Sinai School of Medicine,

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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New York, NY). Congenic Thy-1.1$^+$ BALB/c mice were a gift from Dr. R. Dutton (Trudeau Institute). MaFIA (c-fms–specific Fas-induced apoptosis) mice (26) were obtained Dr. D.A. Cohen (University of Kentucky College of Medicine, Lexington, KY). MaFIA transgenic mice express a bicistronic transcript containing c-fms (M-CSF receptor, CD115)–specific promoter-driven EGFP and the ΔLNGFR-FKBP-Fas suicide gene. CD115-specific depletion is induced by treatment of AP20187 to induce dimerization of FKBP-Fas. All animal experiments were performed in accordance with the guidelines of Mount Sinai School of Medicine.

Recombinant adenoviral vectors, mouse model of hepatic metastases, and therapeutic protocols. The MCA26 tumor line is a BALB/c-derived, chemically induced colon carcinoma with low immunogenicity (25). The MCA26 colon tumor cell line that expresses influenza HA, HA-MCA26, was established (25). The OVA expressing OVA-B16 melanoma was established (27).

The construction of Adv.mIL-12 has been described previously (28). Metastatic colon cancer was induced as described previously (6). MCA26 tumor–bearing mice were given an i.p. injection of anti-CD40 or rat immunoglobulin on days 6 and 9 after tumor implantation. Nine days later, mice with tumors measuring 8 × 8 mm^2 to 10 × 10 mm^2 were injected with Adv/mIL-12 or control vector DL312, followed by two injections of anti-4-1BB (100 μg; or control rat immunoglobulin; Accurate Chemical & Scientific Co.) and anti-CD40 (50 μg; or rat immunoglobulin) antibodies on days 1, 3 and days 6, 9, respectively, after viral injection.

Antibodies and flow cytometry. Anti-Thy1.2-FITC, anti-CD40-APC, anti-Gr-1-APC, biotinylated anti-I-A, anti-Ly-6C-PE, anti-CD115-PE, anti-CD25-APC, anti-FoxP3-PE, and isotype-matched monoclonal antibodies (mAb) were purchased from eBioscience. Flow cytometric analysis were performed using LSR-II and FACSDiVa software (BD Biosciences).

Adoptive transfer experiments. In the HA-MCA26/BALB/c tumor model, 9 × 10^4 HA-MCA26 cells (or *neo* transfected parental MCA26 cells as a control) were inoculated intrahepatically into Thy1.1$^+$ BALB/c mice. Seven to eight days later, mice with tumors of 7 × 7 mm^2 to 9 × 9 mm^2 were irradiated (850 rad) to eradicate endogenous MDSCs and T cells, as confirmed by flow cytometric analysis of Gr-1$^+$CD115$^+$ cells and T cells in bone marrow and spleen of irradiated mice (<0.5% of T cells and MDSC were present in the irradiated recipient mice). Thy1.2 congenic CD4 HA-specific TCR-transgenic T cells were enriched by T-cell enrichment columns per manufacturer's instructions (R&D Systems). After removal of macrophages by adherence, Gr-1$^+$CD115$^+$ monocytic MDSCs were sorted from bone marrow and spleen cells derived from large tumor-bearing WT or CD40 KO BALB/c mice. The sorted MDSC (2.5 × 10^6/mouse) and T cells (5 × 10^6/mouse) were coadoptively transferred by injection via the tail vein the day after irradiation. Mice were given three doses of anti-CD40 (50 μg/mouse) or rat immunoglobulin control, starting on the day before adoptive transfer. Mice were sacrificed at day 10 after adoptive transfer, and Thy1.2$^+$ T cells were recovered from spleen and lymph nodes of the recipient mice by cell sorting for Thy-1.2$^+$ cells.

In the OVA-B16/C57BL/6 MaFIA tumor model, MaFIA mice were implanted intrahepatically with OVA-B16 or control B16 tumor cells. When tumors reached the size of 7 × 7 mm^2 to 9 × 9 mm^2, CD115$^+$ cells were depleted by the injection of AP20187 (10 mg/kg body weight; Ariad Pharmaceuticals). On the same day of AP20187 injection, sorted WT or CD40 KO MDSCs (5 × 10^6 per mouse) were i.v. injected. Two days after MDSC transfer, purified OT-II T cells (5 × 10^6 per mouse) were injected via tail vein followed by a second dose of MDSCs 2 d later. Five days after the last injection of MDSC, mice were sacrificed. The tumor weight was measured. The presence of tumor-specific (OT-II) Tregs in the tumor was assessed by flow cytometry. The proliferative response of purified splenic tumor-specific (OT-II) CD45.1 T cells in the presence of OVA peptides and irradiated naïve splenocytes was assessed.

Proliferation assay. T cells (1 × 10^4) were cocultured with irradiated (2500 rad) naïve splenocytes (4 × 10^3; as antigen-presenting cells) in the presence or absence of HA, OVA peptide (5 μg/mL), or anti-CD3 (1 μg/mL) plus anti-CD28 (1 μg/mL) in 96-well microplates. [^{3}H]Thymidine was added during the last 8 h of a 72-h culture.

MDSC suppression assay. CD4 HA peptide (^{110}SFERFEIFPKE^{120}) and OT-II OVA peptide ^{323}ISQAVHAAHAEINEAGR^{339} were purchased from Washington Biotechnology, Inc. The suppressive activity of MDSC was assessed in a peptide-mediated proliferation assay of TCR transgenic T cells as described previously (25).

In some experiments, purified CD4$^+$CD25$^+$ Treg or CD4$^+$CD25$^-$ T cells from naïve OT-II transgenic mice were labeled with CFSE and cocultured with MDSC isolated from bone marrow or spleen of WT or CD40 KO tumor-bearing mice at a ratio of 4:1 (T cell/MDSC) in the presence of recombinant murine interleukin 2 (IL-2; 100 units/mL, R&D Systems). Irradiated (3,000 rad) OVA-EL4 cells (a kind gift from Dr. Julie M. Blander, Mount Sinai School of Medicine, New York) were used as stimulator. After a 4-d stimulation, cells were harvested and stained with anti-CD4-PerCP-Cy5.5 and anti-Foxp3-PE, or isotype control (eBioscience). In transwell culture, MDSC was added in the upper chamber whereas T cell in the lower chamber.

Cytokine detection by ELISA. IL-10 and transforming growth factor-β (TGF-β) concentrations in culture supernatants were determined by specific mouse ELISA kits (R&D Systems) as per the manufacturer's instructions.

Reverse transcription–PCR and quantitative real-time PCR. Target cells were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted per manufacturer's instructions. Reverse transcription–PCR (RT-PCR) and quantitative real-time PCR were used to determine relative quantities of mRNA as previously described (25).

Statistical analysis. Statistical analysis of survival rates was performed using the log-rank test. Student's *t* test was used in all other analyses.

Results

IFN-γ upregulates expression of CD40 and MHC class II (I-A) on MDSC. In a previous report, we found that the development of Tregs mediated by MDSCs required

antigen-associated activation of tumor-specific T cells and depended on IFN-γ signaling by MDSCs (25). We hypothesize that IFN-γ can upregulate the expression of costimulatory molecules and MHC II on MDSCs. As shown in Fig. 1, upon IFN-γ stimulation, expression of CD40 and MHC II by Gr-1$^+$CD115$^+$ MDSC was significantly increased (70.3 ± 12.8%) when compared with MDSCs cultured in the absence of IFN-γ (12.6 ± 4.2%, $P < 0.001$) (Fig. 1*B*). Among other costimulatory molecules, CD80 and CD86 were constitutively expressed by MDSCs (Fig. 1*C*). Upon IFN-γ stimulation, however, only a slight increase in the expression of CD80 and CD86 was observed.

***CD40 is required for MDSC mediated T-cell suppression and Treg expansion* in vitro.** To determine whether CD40 plays a role in the suppressive functions of MDSCs, the suppression of T-cell proliferation and Treg induction by MDSCs derived from WT or CD40 KO tumor-bearing mice were assessed. Purified naïve T cells were cocultured with MDSCs at a 4:1 ratio in the presence of anti-CD3/anti-CD28 for 5 days followed by flow cytometry to assess the presence of CD4$^+$CD25$^+$Foxp3$^+$ Tregs. As shown in Fig. 2*A*, coculture with WT MDSCs resulted in 20.7% (21.8 ± 2.6%, average from four separate experiments) Tregs, a significantly higher percentage than that from T cells alone (6.5 ± 2.9%). Interestingly, a significantly lower percentage of Tregs (9.6 ± 2.1%) was observed in coculture with CD40 KO MDSCs when compared with coculture with WT MDSCs ($P < 0.001$). To further investigate the role of the CD40 molecule in Treg induction by MDSCs, CFSE-labeled purified CD4$^+$CD25$^-$ T cells or CD4$^+$CD25$^+$ Tregs from naïve mice were cocultured with WT or CD40 KO MDSCs in the presence of anti-CD3/anti-CD28. WT MDSCs significantly inhibited the proliferation of naïve CD4$^+$CD25$^-$ T cells but efficiently expanded CD4$^+$CD25$^+$ Treg when compared with the culture without MDSCs (Fig. 2*B, middle*). In contrast, CD40 KO MDSCs not only lost the ability to suppress proliferation of naïve CD4$^+$CD25$^-$ T cells but also failed to support the expansion of Treg. Similar results were observed when CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$

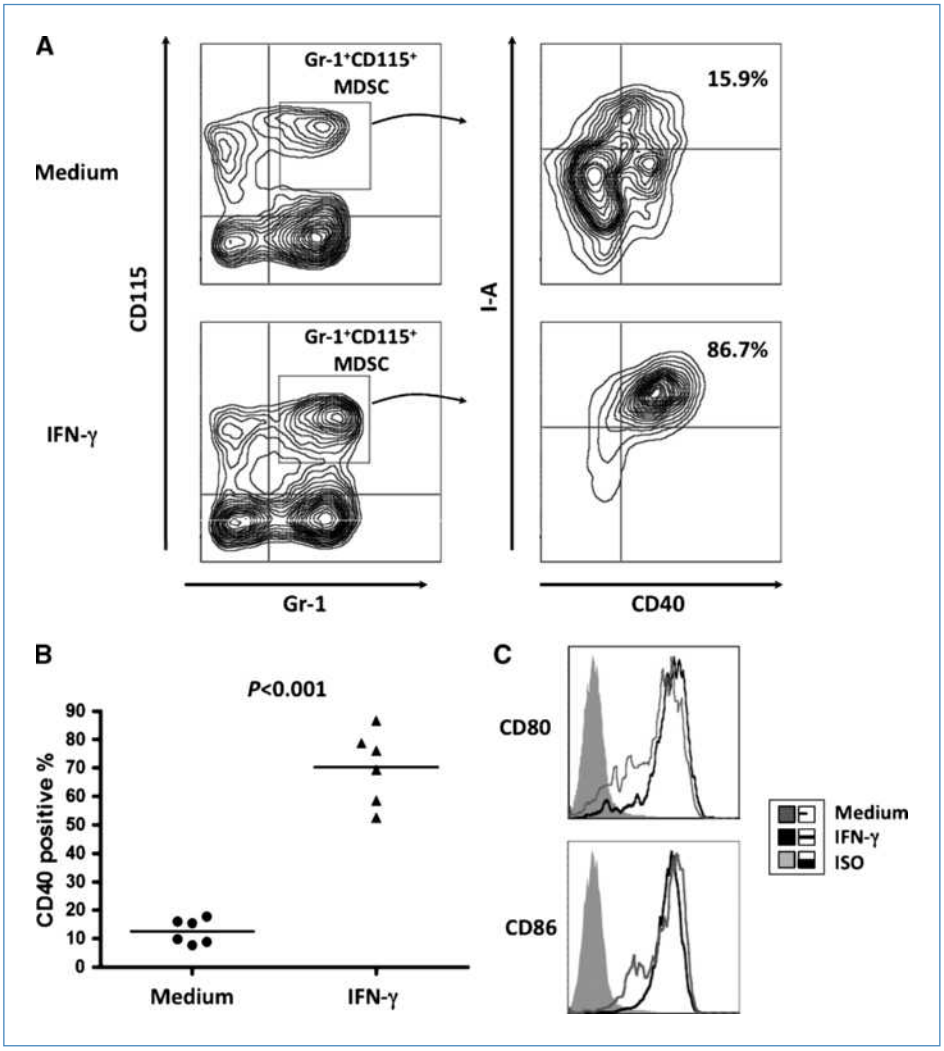

Figure 1. Expression of costimulatory molecules and MHC II by MDSC upon IFN-γ stimulation. Bone marrow Percoll fraction 2 cells, which contain MDSC, were cultured in the presence or absence of IFN-γ (100 ng/mL). Twenty-four hours later, cells were stained with fluorochrome-conjugated anti-Gr-1, anti-CD115, anti-CD40, anti-CD80, and anti-I-Ab or isotype control. *A,* induction of CD40 on MDSC by IFN-γ. Flow cytometric data obtained from one representative experiment are presented as dot plots. *B,* significant induction of CD40 by IFN-γ. The results obtained from six tumor-bearing mice are presented. *C,* constitutive expression of CD80 and CD86 on MDSC.

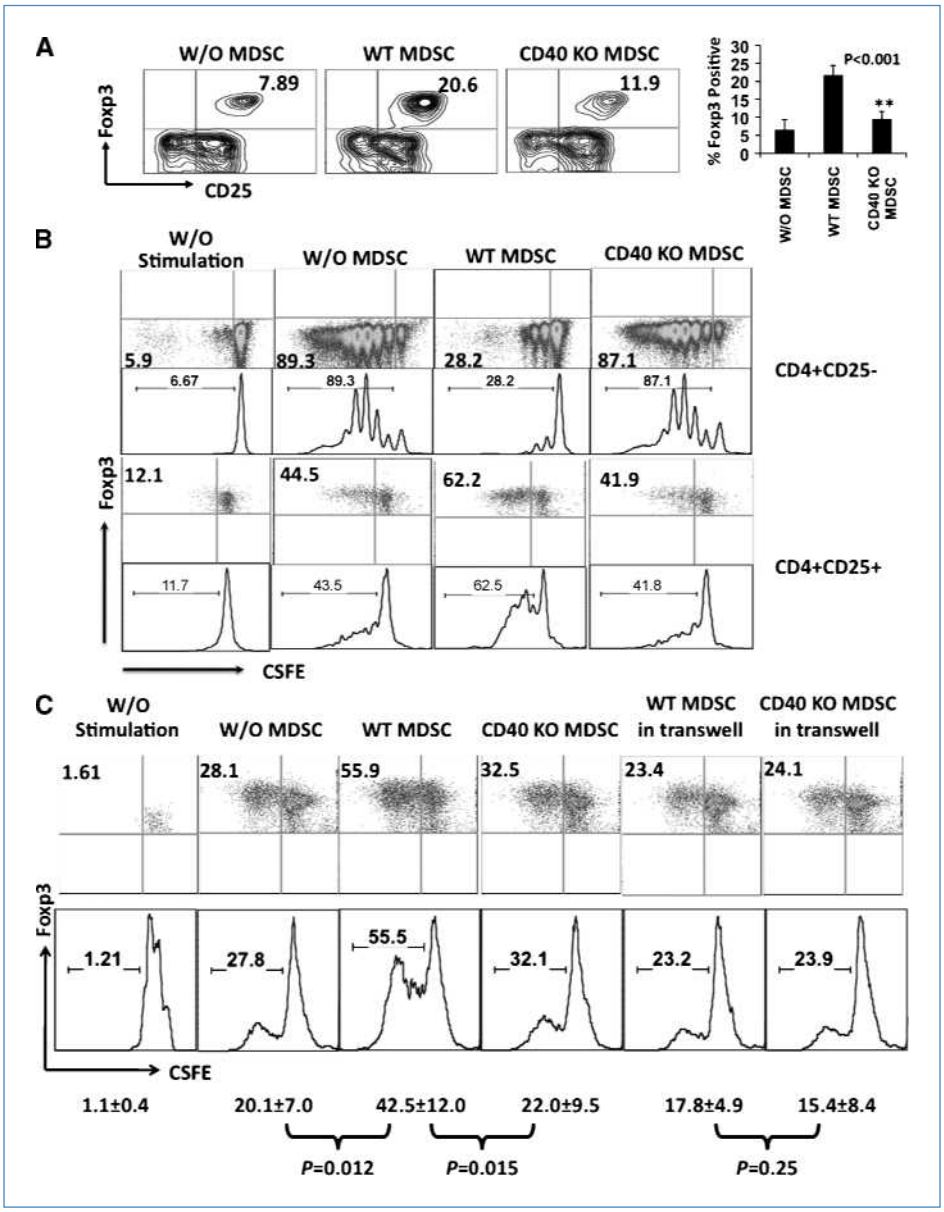

Figure 2. Requirement of CD40 for MDSC-mediated T-cell suppression and Treg expansion *in vitro*. *A,* Treg induction by WT versus CD40 KO MDSCs. Purified CD4+ T cells were cocultured with WT or CD40 KO MDSCs at 4:1 ratio for 5 d followed by flow cytometry to assess the presence of $CD4^+CD25^+Foxp3^+$ Tregs. Data are gated on $CD4^+$ populations ($n = 6$). *B,* expansion of $CD4^+CD25^+Foxp3^+$ Tregs by WT, but not CD40 KO, MDSCs. CFSE-labeled, purified $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs from naïve mice were cocultured with WT or CD40 KO MDSCs at 4:1 ratio in the presence of anti-CD3/anti-CD28 for 3 d. Proliferation was assessed by flow cytometry. Histograms of $CD4^+CD25^-$ population were gated on $CD4^+$ cells, whereas those of $CD4^+CD25^+$ population were gated on $Foxp3^+$ cells. Data from one representative of three reproducible experiments. *C,* CFSE-labeled, purified $CD4^+CD25^+$ Tregs from naïve OT-II mice were cocultured with WT or CD40 KO MDSCs at 4:1 (T cell to MDSC) ratio in the presence of irradiated OVA-EL4 cell (3,000 rad) at 10:1 ratio in the presence of IL-2 for 4 d. In the transwell experiment, MDSCs and Treg were added in the upper and lower chambers, respectively. Proliferation (CFSE dilution) was assessed by flow cytometry. Data from one representative of three reproducible experiments. Mean ± SD of each group has presented in results ($n = 6$).

Tregs from naïve OT-II mice were cocultured with WT or CD40 KO MDSCs in the presence of OVA peptides and irradiated naïve splenocytes (as antigen-presenting cells; Supplementary Fig. S1).

To address the requirement of specific antigen and cell contact between MDSC and Treg in the Treg expansion, we purified $CD4^+CD25^+$ T cells from naïve OT-II (CD4 OVA-specific transgenic) mice, followed by CFSE labeling and coculture with purified MDSCs and irradiated OVA-EL4 cells (as the source of antigen). As shown in Fig. 2*C*, purified OT-II Treg did not proliferate well without stimulation (i.e., without IL-2; 1.1 ± 0.4%). In the presence of IL-2 and irradiated OVA-EL4, but in the absence of MDSC, substantial proliferation was observed (20.1 ± 7%). In the presence of IL-2 and irradiated OVA-EL4, WT MDSCs further expanded purified OT-II Treg in the absence of other antigen-presenting cells whereas Treg expansion by CD40 KO MDSCs in the presence of irradiated OVA-EL4 was decreased significantly (42.5 ± 12% versus 22 ± 9.5%; $P = 0.015$, paired t test, $n = 6$), comparable with that in the absence of MDSC (22 ± 9.5% versus 20.1 ± 7%). When MDSC and Treg were separated in the transwell culture, neither WT nor CD40 KO MDSC mediated a significant level of Treg expansion.

Taken together, these results indicate that CD40 is required for the suppressive functions, e.g., suppression of T-cell proliferation and Treg induction, of MDSCs and that direct contact between MDSCs and T cells through ligation of CD40 on MDSC and CD40L on T cells, as well

as TCR-peptide/MHC class II engagement, is required for this process.

Treatment with anti-CD40 reverses T-cell tolerance and prevents tumor-associated expansion of Tregs. To facilitate future clinical application and to verify the potential role of CD40 in MDSC-mediated suppression, we investigated the ability of anti-CD40 to prevent T-cell tolerance and Treg development in mice with large tumor burdens. HA-MCA26 tumor-bearing mice (Thy-1.1) were injected i.p. with anti-CD40 or control rat immunoglobulin the day before adoptive transfer of $CD4^+$ tumor (HA)-specific TCR transgenic T cells (Thy-1.2). Nine days later, at which time the tumor size had reached ~12 × 12 mm^2, Thy-1.2 tumor-specific T cells were recovered from the spleens of recipient mice by sorting and various immune parameters pertinent to T-cell tolerance and Treg were measured. The tumor-specific T cells recovered from anti-CD40–treated mice proliferated upon HA stimulation, whereas those recovered from rat immunoglobulin–treated mice exhibited a hypoproliferative response when compared with naïve HA TCR transgenic T cells (Fig. 3*A*). Furthermore, tumor-specific T cells from anti-CD40–treated mice secreted significantly lower amounts of IL-10 and TGF-β than those from mice treated with rat immunoglobulin (Fig. 3*B*). A significant level of Foxp3 expression was detected in sorted, tumor-specific T cells derived from mice treated with rat immunoglobulin; however, Foxp3 gene expression was barely detectable in tumor-specific T cells recovered from anti-CD40–treated mice (Fig. 3*C*). The expression of Foxp3 protein was confirmed using intracellular staining followed by flow cytometric analysis (Fig. 3*D*). These results indicate that agonist anti-CD40 antibody can prevent tumor-specific Treg development and T-cell tolerance.

CD40 expression by MDSC is required for MDSC-mediated Treg induction and tolerance. Since we found a significant decrease of suppressive functions in CD40 KO MDSCs *in vitro*, we sought to determine whether CD40 on MDSCs is required for the suppressive activities mediated by MDSCs in tumor-bearing mice. We used a previously established adoptive transfer model in which Thy-1.2^+ congenic tumor (HA)–specific TCR transgenic T cells and sorted Gr-1^+ MDSC (WT versus CD40 deficient) were injected i.v. into sublethally irradiated HA-MCA26 tumor-bearing recipient mice (25). A

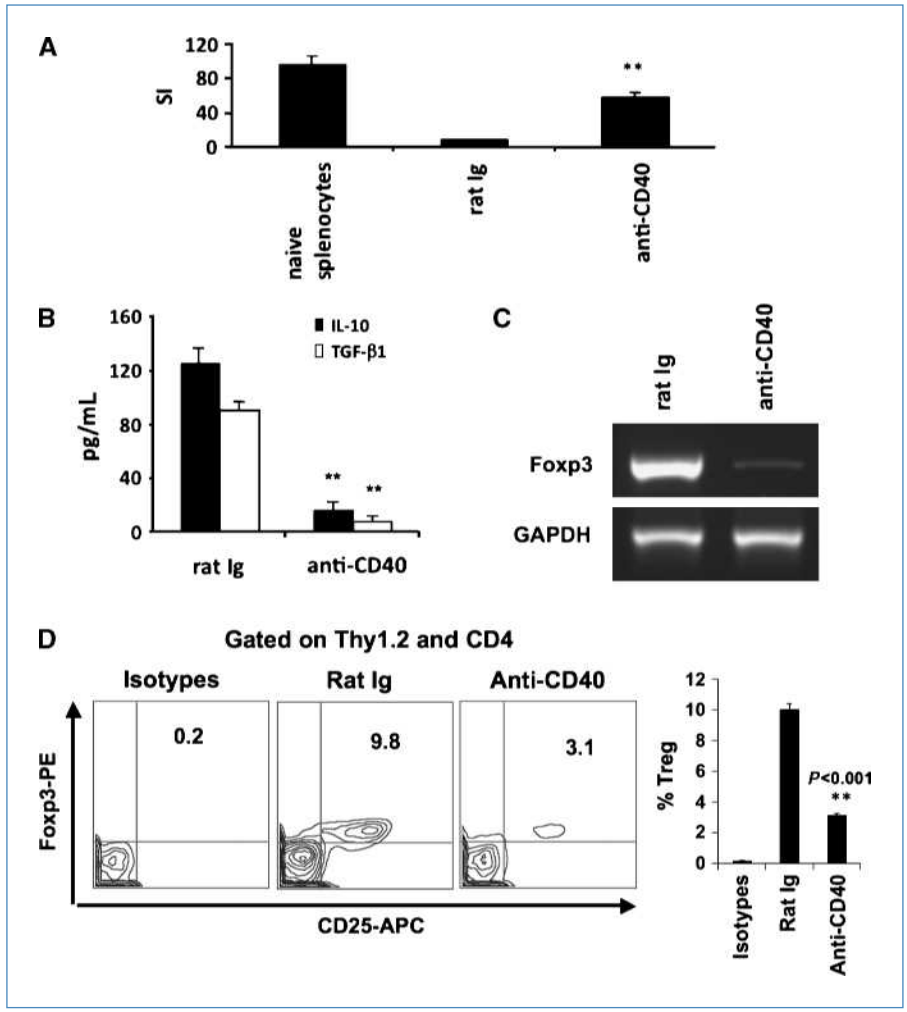

Figure 3. Reversal of MDSC mediated immune suppression by anti-CD40. HA-TCR tumor (HA)–specific Thy-1.2 T cells were sorted from recipient HA-MCA26 tumor-bearing mice. Three mice per group were used in each of three reproducible and independent experiments. *A,* proliferative response of sorted Thy-1.2 T cells. Data are expressed as stimulation index (*SI*) relative to the cpm of T-cell proliferation in the absence of peptide (rat immunoglobulin versus anti-CD40; $P < 0.001$). *B,* IL-10 and TGF-β secretion by Thy-1.2 T cells. The concentrations of IL-10 and TGF-β in the culture supernatants were measured by ELISA (**, $P < 0.001$). *C,* prevention of Treg development by anti-CD40 *in vivo*. Foxp3 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was assessed by RT-PCR of total RNA derived from sorted T cells. *D,* intracellular staining of Foxp3 in recovered tumor (HA)–specific T cells.

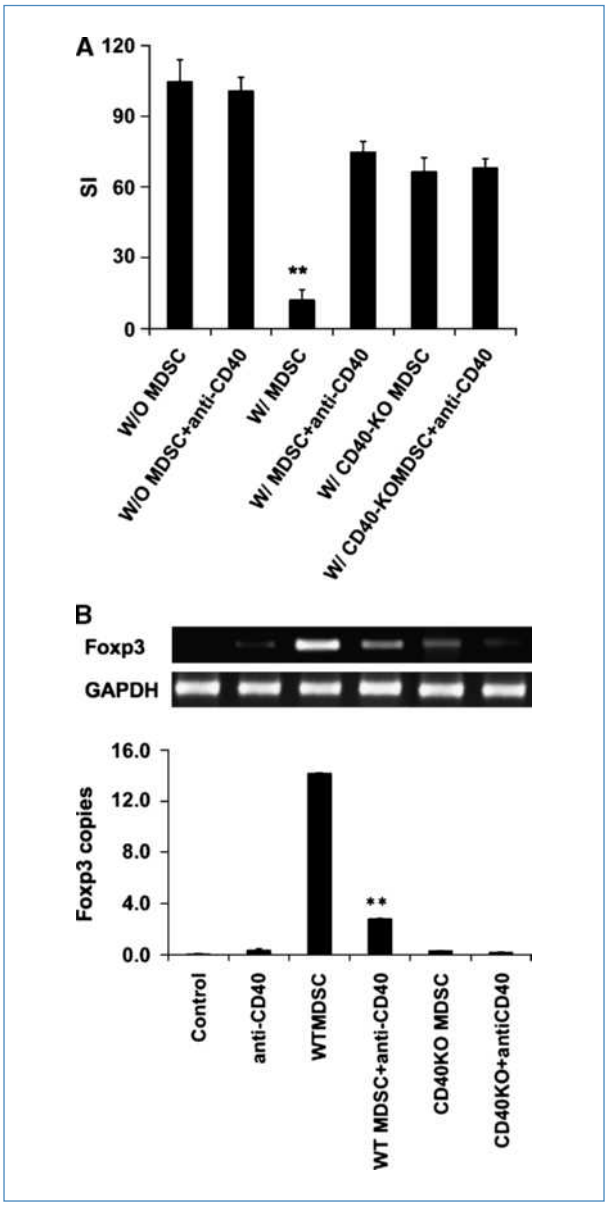

Figure 4. CD40 expression by MDSC is required for MDSC mediated tumor specific T cells immune suppression *in vivo*. After 9 d of adoptive transfer, tumor (HA)–specific CD4 Thy-1.2$^+$ T cells were sorted from recipient HA-MCA26 tumor-bearing mice (Thy1.1$^+$). One of three reproducible experiments was presented. Three mice per group were used in each of three independent experiments. *A,* proliferative response of sorted tumor-specific T cells. Data are expressed as stimulation index (*SI*) relative to the cpm of T-cell proliferation in the absence of peptide (*W/MDSC* versus *W/O MDSC*; $P < 0.001$, and no significant difference in other group). *B,* reduction in Foxp3 expression by tumor (HA)–specific T cells recovered from mice that also received CD40-deficient MDSCs. Foxp3 gene expression was assessed by RT-PCR on total RNA prepared from sorted T cells (WT MDSC versus WT MDSC + anti-CD40; $P < 0.001$).

cohort of recipient mice was treated with anti-CD40 or control rat immunoglobulin via i.p. injection.

Thy-1.2$^+$ HA-specific T cells recovered from recipient HA-MCA26 tumor-bearing mice that received WT MDSCs exhibited a hypoproliferative response upon *in vitro* stimulation with HA peptide when compared with those recovered from mice that did not receive MDSC (Fig. 4*A, third versus first column*). However, CD40 KO MDSCs failed to induce significant T-cell tolerance, as evidenced by the higher proliferative response of tumor-specific T cells recovered from mice that received CD40 KO MDSCs (Fig. 4*A, fifth column*). The lack of a significant difference in proliferative capability between tumor-specific T cells recovered from anti-CD40–treated mice and those from mice that received CD40-deficient MDSCs suggests that anti-CD40 exerts its effect directly on the MDSC (Fig. 4*A, fourth versus fifth column*). This notion is further supported by the lack of a significant additive effect upon the treatment of CD40 KO MDSCs with anti-CD40 (Fig. 4*A, sixth column*).

The effect of CD40 deficiency in MDSCs on the development of Tregs was also determined. A significantly lower level of Foxp3 gene expression was detected in tumor-specific T cells recovered from tumor-bearing mice that received CD40 KO MDSCs when compared with mice that received WT MDSC (Fig. 4*B, top*: RT-PCR, *bottom*: real-time PCR). Foxp3 gene expression in tumor-specific T cells recovered from mice that were injected with anti-CD40 was significantly reduced, albeit by a low, but substantial, level.

To directly address whether the CD40 expressed by MDSCs is involved in the suppressive functions of MDSCs, MaFIA mice, whose endogenous CD115$^+$ monocytic MDSCs can be depleted (Supplementary Fig. S2), were used to interrogate the role of CD40 in Treg expansion mediated by monocytic MDSCs in a model of adoptive transfer of tumor-specific T cells and reconstitution of MDSCs. CD45.1 OT-II T cells were adoptively transferred into MaFIA mice with pre-existing OVA-B16 tumors followed by deletion of CD115$^+$ cells and reconstitution of CD115$^+$ monocytic MDSCs derived from WT or CD40 KO tumor-bearing mice. As shown in Fig. 5*A*, 24.4% of tumor (OVA)–specific T cells in the tumor were CD4$^+$CD25$^+$Foxp3$^+$ Tregs whereas the percentage of tumor-specific Tregs was decreased to 7.28% in MaFIA mice depleted of CD115$^+$ cells. The reconstitution of CD115-depleted MaFIA mice with WT MDSCs restored the percentage of Tregs to 21.6%. Interestingly, the adoptive transfer of CD40 KO MDSCs did not restore the level of tumor-specific Tregs in the tumor (8.57%). These findings are consistent with the absolute number of tumor-specific Tregs present within the tumor (Fig. 5*B*).

We also measured the proliferative response of tumor-specific T cells reisolated from the spleen. A stronger proliferative response was observed in CD45.1 OT-II T cells isolated from CD115-depleted MaFIA mice when compared with those from CD115-repleted (without depletion) MaFIA mice (Fig. 5*C*). The reconstitution of WT MDSCs resulted in a decreased proliferative response comparable with that of MaFIA mice without CD115 depletion whereas adoptive transfer of CD40 KO MDSCs had no significant effect. More importantly, CD115 depletion resulted in a significantly decreased tumor weight ($P < 0.05$), and reconstitution of WT MDSCs, but not CD40 KO MDSCs, reestablished the tumor growth similar to that of MaFIA mice without CD115 depletion (Fig. 5*D*).

Taken together, the results indicate that the CD40 molecule on MDSCs is essential for MDSC-mediated Treg induction and tumor promotion in tumor-bearing mice.

Treatment with anti-CD40 increases the therapeutic efficacy of immune-based therapy in a large tumor setting. To determine whether inhibition of Treg expansion and intervention with MDSC-mediated immune suppression by CD40 blockade can improve the therapeutic efficacy of immune enhancing therapy in the large tumor setting, we combined anti-CD40 treatment with an existing immune enhancing therapy (IL-12 + 4-1BB activation) that is currently being used in a clinical trial at the Mount Sinai Medical Center. Mice bearing tumors measuring 8 × 8 mm^2 to 10 × 10 mm^2 were randomly assigned to one of the following groups: Adv.mIL-12 + anti-4-1BB + anti-CD40, Adv.mIL-12 + anti-4-1BB + rat immunoglobulin, Adv.mIL-12 + anti-CD40 + rat immunoglobulin, DL312 + anti-4-1BB + anti-CD40, or DL312 + rat immunoglobulin. As shown in Fig. 6, 65% of mice that were treated with Adv.mIL-12 + anti-4-1BB + anti-CD40 were alive at 100 days after tumor implantation compared with a 23% survival rate for mice treated with Adv.mIL-12 + anti-4-1BB + rat immunoglobulin ($P < 0.01$, log-rank test). Only 3 of 12 (25%) and 1 of 12 mice (8%) receiving DL312 + anti-4-1BB + anti-CD40 or adv.mIL-12 + anti-CD40 + rat

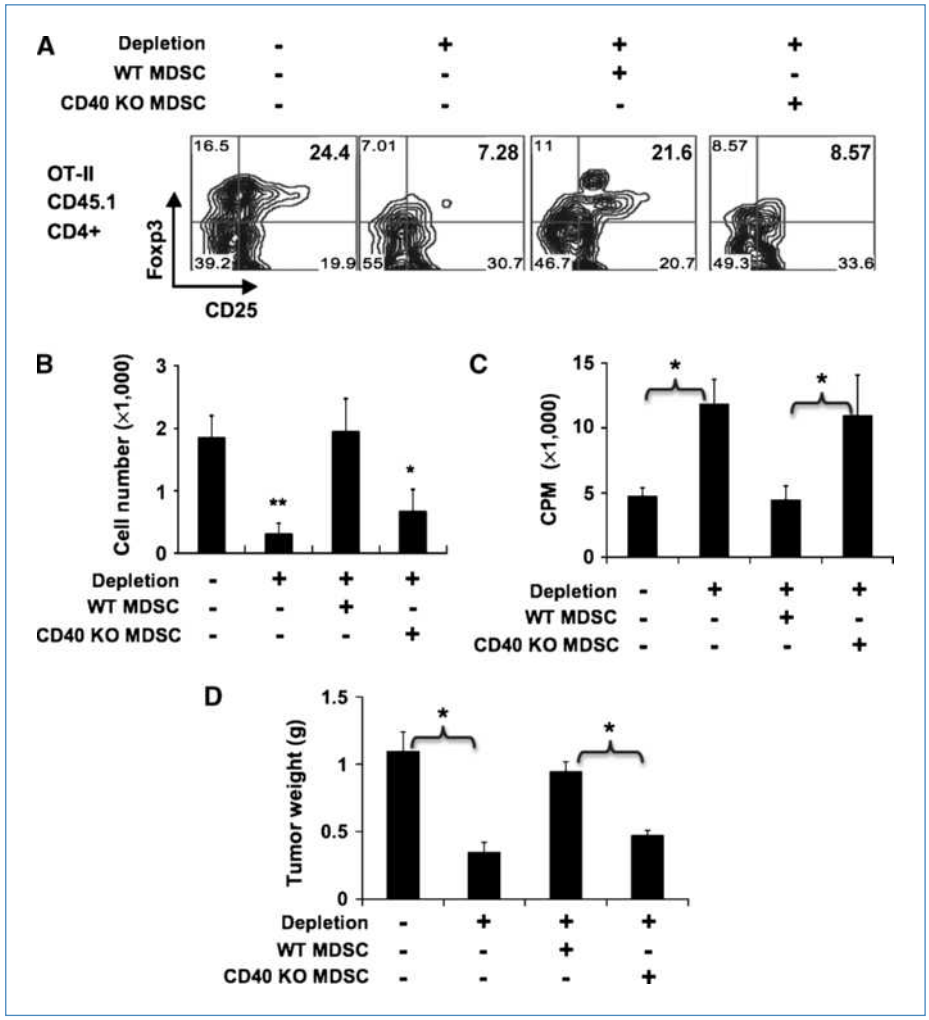

Figure 5. CD40 is essential for Treg expansion, immune suppression, and tumor promotion mediated by MDSCs. OVA-B16–bearing MaFIA mice (CD45.2) were left untreated or depleted of $CD115^+$ cells followed by adoptive transfer of CD45.1 OT-II T cells and reconstitution of WT or CD40 KO MDSC. *A,* tumor (OVA)–specific $CD4^+CD25^+Foxp3^+$ Tregs in the tumor. Tumor infiltrated leukocytes were isolated and stained with antibodies against CD45.1, CD4, CD25, and Foxp3 or isotype controls followed by flow cytometry. Contour plots gated on $CD45.1^+CD4^+$ population are presented. One of three reproducible experiments (n = 3–4 per group) was presented. *B,* the number of tumor (OVA)–specific Tregs in the tumor. The numbers of $CD45.1^+$ OT-II Tregs in the tumor were calculated (second versus first column, $P < 0.001$; fourth versus third column, $P = 0.004$). *C,* proliferative response of tumor (OT-II)–specific T cells reisolated from the recipient MaFIA mice. $CD45.1^+$ OT-II T cells were reisolated from the spleen of recipient mice and stimulated with OVA peptide (1 μg/mL) in the presence of irradiated naïve splenocytes for 3 d. [^{3}H]Thymidine was added in the last 8 h of culture (second versus first column, $P = 0.002$; fourth versus third column, $P = 0.009$). *D,* tumor weight in various treatment groups. Tumors were resected from tumor-bearing mice and weighed (second versus first column, $P = 0.011$; fourth versus third column, $P = 0.006$). Data were combined from three reproducible experiments (n = 9).

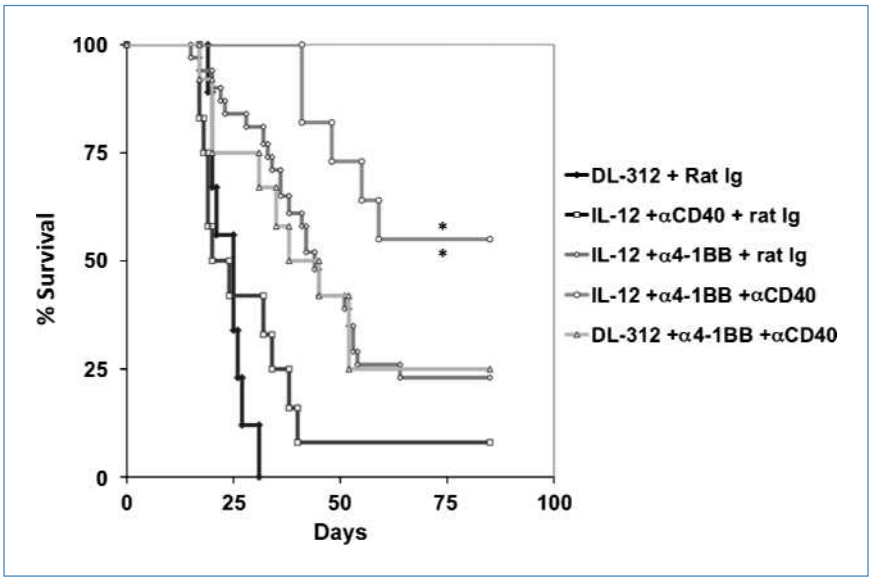

Figure 6. Effect of the combination of anti-CD40 and Adv/mIL-12 plus anti-4-1BB mAb on antitumor immunity. Mice bearing large MCA26 tumors were randomly assigned to four treatment groups. The long-term survival rate of mice treated with Adv/mIL-12 + anti-4-1BB + anti-CD40 (n = 12) is significantly higher than that of mice treated with Adv/mIL-12 + anti-4-1BB + rat immunoglobulin (n = 20; **, P < 0.01, log-rank test) and that of mice treated with DL312 + anti-4-1BB + anti CD40 (n = 12) or Adv/mIL-12 + anti-CD40 + rat immunoglobulin, respectively (n = 12; **, P < 0.01, log-rank test). All of the mice in the DL312 + rat immunoglobulin treatment group (n = 10) died on or before day 30 after tumor implantation. The combined results from two separate and reproducible experiments are presented.

immunoglobulin, respectively, were alive at 100 days (P < 0.01, log-rank test). All of the mice in the control DL312 + rat immunoglobulin treatment group died on or before day 30 after tumor implantation.

Discussion

It has been shown that treatment with activating anti-CD40 antibodies preserves the responsiveness of $CD4^+$ T cells to vaccination and prevented peptide-induced $CD4^+$ T-cell anergy (29). Furthermore, *in vivo* ligation of CD40 with agonistic anti-CD40 antibodies prevented the induction of tolerance in tumor (HA)–specific T cells in mice with established pulmonary metastases of a renal cell carcinoma expressing HA. Although antigen-presenting cells were presumed to play an important role in the CD40-mediated conversion of T-cell tolerance to T-cell activation, the identity of the antigen-presenting cells involved and the underlying mechanism of reversion of T-cell tolerance in anti-CD40-treated mice were not determined. Our findings indicate that CD40 is required for MDSC-mediated immune suppression. Importantly, the treatment with agonistic anti-CD40 antibody prevented MDSC-mediated expansion of Tregs in mice with large tumor burdens, leading to augmented anti-tumor responses.

CD40/CD40L interaction plays a role in the development and homeostasis of naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs (30, 31). The $CD4^+CD25^+CD45RB^{low}$ subpopulation, which regulates autoreactivity, has been shown to be markedly reduced in CD40 KO mice. *In vitro*, CD40-deficient, but not WT, dendritic cells produced diminished amounts of IL-2 upon Treg encounter and showed impaired ability to expand Tregs (30). The identity of the $CD40^+$ cells *in vivo* that can mediate Treg expansion is not clear, however. Under pathologic conditions Treg homeostasis can be disrupted efficiently. An increased number of Tregs has been observed in tumor-bearing mice and in patients with various carcinomas (5). Tumor-associated expansion of Tregs is the result of conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Tregs (32). Interestingly, this process occurs independent of both the thymus and proliferation; however, the mechanisms underlying the activation and expansion of tumor-specific $CD4^+CD25^+Foxp3^+$ Tregs in the tumor-bearing host remains to be elucidated.

In this report, we show that the expression of CD40 on MDSCs is critically important for MDSC-mediated Treg expansion. Interference with CD40/CD40L interactions, through the use of CD40-deficient MDSC or anti-CD40, significantly reduces Tregs and prevents the suppression of tumor-specific T-cell responses in tumor-bearing mice. Previously, we showed that upon IFN-γ stimulation, the expressions of IL-10 and TGF-β by MDSCs were significantly enhanced and that the activation of T cells was required for Treg induction (25). Our current working model is that activated T cells secrete IFN-γ, which enhances the expression of CD40, IL-10, and TGF-β. The engagement of CD40/CD40L between MDSC and Treg in the presence of TGF-β and IL-10 leads to activation and expansion of Treg.

We used two complementary models to show that CD40 expression was required for Treg expansion and tumor promotion mediated by $Gr\text{-}1^+CD115^+$ monocytic MDSCs. In the HA-MCA26 tumor model, endogenous MDSCs were depleted by sublethal irradiation. Reconstitution of CD40 KO MDSCs in combination with treatment of agonistic anti-CD40 antibodies did not yield additive or synergistic effects on the restoration of *ex vivo* proliferation and the decrease of Foxp3 expression by tumor-specific T cells that were reisolated from recipient mice. The result suggests that agonistic anti-CD40 antibodies disrupted CD40/CD40L ligation between MDSCs and Tregs, leading to a decrease in Treg expansion.

In the OVA-B16 tumor model, CD115-specific depletion was induced in MaFIA tumor-bearing mice by the treatment of AP20187. Although other CD115-expressing cells were also

depleted, reconstitution of WT, but not CD40-deficient, MDSCs alone was sufficient to reestablish a high number of OT-II Tregs in the tumor, the *ex vivo* hypoproliferative response of OT-II T cells, and tumor growth to the level similar to those of MaFIA mice without CD115 depletion. Taken together, the data obtained from these studies using two complementary approaches strongly suggest that CD40 expression on MDSCs plays an important role in the establishment of immune tolerance in tumor-bearing mice through the expansion of Tregs.

At first glance, our findings seem to be contradictory to the well-established paradigm that CD40 triggering induces and enhances adaptive immunity. However, the outcome of CD40 ligation may vary, depending on the cell type that expresses CD40. Ligation on professional antigen-presenting cells, such as dendritic cells, induces immune activation, whereas on MDSC, it may promote the expansion of Tregs and is required for the suppression of the tumor-specific T-cell immune response. Furthermore, engagement of CD40L on T cells in an environment where the local cytokine milieu is rich in suppressive cytokines, such as IL-10 and TGF-β (25), may favor Treg expansion. The use of agonistic anti-CD40 in this study may exert dual functions: blockade of the CD40/CD40L interaction between MDSCs and T cells, thereby preventing the activation and expansion of Tregs, and induction of maturation of dendritic cells or other antigen-presenting cells, thereby preventing the establishment of tolerance. Furthermore, we have observed an additional benefit of anti-CD40 treatment, the differentiation of MDSCs into dendritic cells or macrophages.[3]

Immune-based therapy remains one of the most promising options for the treatment of advanced metastatic cancer. However, therapeutic efficacy is severely compromised by the immune tolerance and suppression associated with advanced malignancy. In this report, we show that CD40 expression by MDSCs is required for MDSC suppressive activity and Treg expansion in tumor-bearing mice. Our findings reveal a novel role for CD40 in tumor-bearing hosts. The use of agonistic anti-CD40 in conjunction with immune-based therapy may represent a novel approach to not only to block MDSC-mediated Treg activation and expansion but to also intervene in MDSC-mediated immune suppression in hosts with advanced malignancies.

[3] S-H. Chen, unpublished observation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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