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Anti-VEGF antibody enhances the antitumor effect of CD40

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As its central immunomodulatory effects, CD40 induces interleukin (IL)-12-dependent antitumor immune responses; as its local protumor effects, CD40 induces the expression of vascular endothelial growth factor (VEGF) that promotes tumor angiogenesis and growth. Therefore, using a previously established tumor model in mouse, we examined if the antitumor functions of CD40 are self-limited by VEGF induction. We observed that as the tumor mass grew during day 6 to day 18, VEGF expression in the tumor peaked with concomitant decrease in expressions of CD40 and IL-12 but not of IL-10. Among the angiogenic factors, VEGF-B, VEGFR-1, VEGFR-2, angiopoietin-1 and Tie2 expressions decreased, whereas the expressions of angiopoietin-2 and angiopoietin-3 increased with tumor growth. As significant changes in the expressions of these factors were observed on day 6, we treated the tumor-bearing mice with the agonistic anti-CD40 antibody or neutralizing anti-VEGF antibody—alone or in combination—from the fifth day after the injection of tumor cells. The anti-VEGF antibody significantly enhanced the antitumor effects of the anti-CD40 antibody, as observed through increased survival of the mice, accompanied by reduced angiogenesis and angiopoietin-2 expression but higher T-cell proliferation in response to tumor antigens, increased interferon-γ production and tumor cell cytotoxicity and higher levels of tumor antigen-specific serum IgM, IgG1 and IgG2a, indicating B-cell activation. Thus, our data show for the first time that the combined treatment with an agonistic anti-CD40 antibody and a neutralizing anti-VEGF antibody, which increases antitumor immune response or reduces local angiogenesis, respectively, is a novel antitumor strategy.

The immune system constantly eliminates potentially tumorigenic cells by recognizing unusually expressed proteins, but tumor cells exploit different strategies to evade immune responses and form tumor mass.[1,2] Mounting an effective antitumor immune response requires CD40–CD40L interaction[3,4] that activates cytotoxic T lymphocytes (CTLs), which recognize and kill tumor cells.[5,6] CD40 signaling in endothelial cells (ECs) upregulates the expression of VCAM-1, ICAM-1 and E-selectin, facilitating attachment, rolling and transmigration of tumor-reactive lymphocytes and other immune cells.[7] CD40–CD40L interaction also induces the expression of various proangiogenic factors such as vascular endothelial growth factor (VEGF) in endothelial cells resulting in neovascularization that promotes countercurrent tumor cell metastasis and immune cell infiltration into tumor.[8] CD40–CD40L interaction is thus proposed to play a dual role in tumorigenesis by effecting the T-cell-mediated antitumor immune response and formation of new blood vessels that supply nutrients to the proliferating tumor cells leading to the survival and growth of tumor.[9,10]

VEGF is the most potent angiogenic mediator in the tumor microenvironment. VEGF binds to its receptors (VEGFR-1 and VEGFR-2) on endothelial cells. It facilitates angiogenesis by extracellular matrix degradation, endothelial cell proliferation and migration and promotes growth of cancer cells. A neutralizing anti-VEGF antibody decreases angiogenesis and inhibits tumor growth.[11] Therefore, we proposed that the CD40-facilitated central antitumor immune response was self-limited by the local protumor effects of CD40-induced VEGF, such that VEGF neutralization would enhance the efficacy of CD40-induced antitumor immune responses. In our study, we report that anti-VEGF antibody efficiently enhances CD40-induced tumor antigen-specific antitumor immune response.

Material and Methods

Tumor model and antibody treatment

RM-1 prostate cancer cell line was maintained in Ham's F12K complete medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL).[12] To generate tumor in mice, 2×10^5 RM-1 cells were injected subcutaneously into the right flank of C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) in 50 μL sterile phosphate-buffered saline (PBS). The mice were injected intraperitoneally with 50 μg endotoxin-free anti-CD40 mAb (Clone-3/23; BD Biosciences, San Diego, CA) and/or 125 μg endotoxin-free anti-VEGF Ab (B20-4.1.1, a kind gift from Genentech, San Francisco, CA) in 500 μL

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What's new?
Both anti-tumor and pro-angiogenic responses are produced from the interaction of CD40 and CD40L, making for an intriguing duality in tumor biology. However, the possibility that pro-angiogenic responses generated through the induction of vascular endothelial growth factor (VEGF) expression self-limit anti-tumor responses also poses unique therapeutic challenges. Here, in an established mouse tumor model, simultaneous augmentation of the CD40-induced anti-tumor immune response and neutralization of CD40-induced VEGF production was found to enhance the anti-tumor function of CD40. The combined use of an agonistic anti-CD40 antibody and a neutralizing anti-VEGF antibody represents a possible anti-tumor strategy for the clinic.

sterile PBS on days 5, 7, 9 and 11 after tumor inoculation. One group was injected with rat IgG isotype control. The tumor size was measured using Vernier calipers, and the volume was calculated using the formula (length $\times$ width $\times$ width)/2. Mice were sacrificed 21 days after tumor inoculation and tumors were weighed. Photographs of tumors were taken using stereo microscope (Stemi DV4, Carl Zeiss, Hamburg, Germany) at 12$\times$ magnification and the extent of vessel growth with different treatments was quantified using Angioquant software (Angioquant Freeware; www.cs.tut.fi/sgn/csb/angioquant). All experiments were performed according to the animal use protocols approved by the Institutional Animal Care and Use Committee.

Tumor antigen-specific T-cell proliferation and interferon-γ production

Splenic T cells were purified as described earlier.[12] Isolated T cells were cultured in RPMI-1640 (GIBCO BRL) with 10% FBS for 72 hr in 96-well plates for 3 days at 2×10^5 cells/per well with RM-1 Ag-pulsed, irradiated splenocytes as antigen-presenting cells. In the last 16 hr, the cells were pulsed with 1 μCi of [methyl-^{3}H]-thymidine (BRIT, Mumbai, India). The incorporation of [methyl-^{3}H]-thymidine was measured by liquid scintillation (Packard Instruments, Meriden, CT).[12] Assays were performed in quadruplicates. Supernatants from parallel cultures were harvested and assayed for interferon (IFN)-γ by using ELISA kits (BD Bioscience), following the manufacturer's instructions.

Cytotoxic T-cell assay

Splenocytes were plated at 1.5×10^7 cells per well in a six-well culture plate containing irradiated RM-1 cells. Five days after the co-culture, viable CD8$^+$ T cells were isolated by using enrichment cocktail (BD Bioscience) and were plated at different CD8$^+$ T cell to RM-1 cell ratios. The target RM-1 cells were pulsed with [methyl-^{3}H]-thymidine (5 μCi/mL). The cytolytic activity was tested in a standard 4-hr JAM test.[12]

ELISA

Cytokines and VEGF in culture supernatants were detected by a standard sandwich ELISA following the manufacturer's instructions (BD Bioscience; R&D Systems, Minneapolis, MN). The VEGF capture antibody had specificity to mouse $VEGF_{164}$ and $VEGF_{120}$ isoforms.

Real-time PCR assay

Total RNA was isolated using TRI-Reagent (Sigma Aldrich, St. Louis, MO) and was used for first-strand cDNA synthesis using the thermoscript real-time polymerase chain reaction (RT-PCR) system (Invitrogen Life Technologies, Carlsbad, CA). Quantitative RT-PCR was performed in duplicates following the previously described protocol.[13] Relative quantitation was done using the comparative threshold ($\Delta\Delta C_t$) method. mRNA expression levels of the target genes were normalized against those of GADPH levels and expressed as relative fold change compared with untreated controls. The sequences of the primers used are given in Supporting Information Table 1.

Western blotting

Tumor tissue samples collected were washed with chilled PBS, minced and lysed in cell lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor cocktail (Pierce, Rockford, IL)]. Protein was quantified by BCA kit (Pierce) and equal amount of protein was run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were blotted to polyvinylidene fluoride (PVDF) (Millipore, Bedford, MA) followed by blocking with 5% nonfat dried milk in TBST [25 mM Tris (pH 7.6), 137 mM NaCl and 0.1% Tween-20]. Membranes were incubated with primary antibody at 4°C overnight, washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive bands were visualized using the Luminol reagent purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analyses

Statistical analyses were performed using Student's *t*-test. Survival data were analyzed using the log-rank test. A *p*-value of <0.05 was considered to indicate a statistically significant difference.

Results

Tumor microenvironment and VEGF regulate the growth of tumor

Because sustained nutrient supply is critical for tumor growth, we studied the kinetics of tumor growth and the

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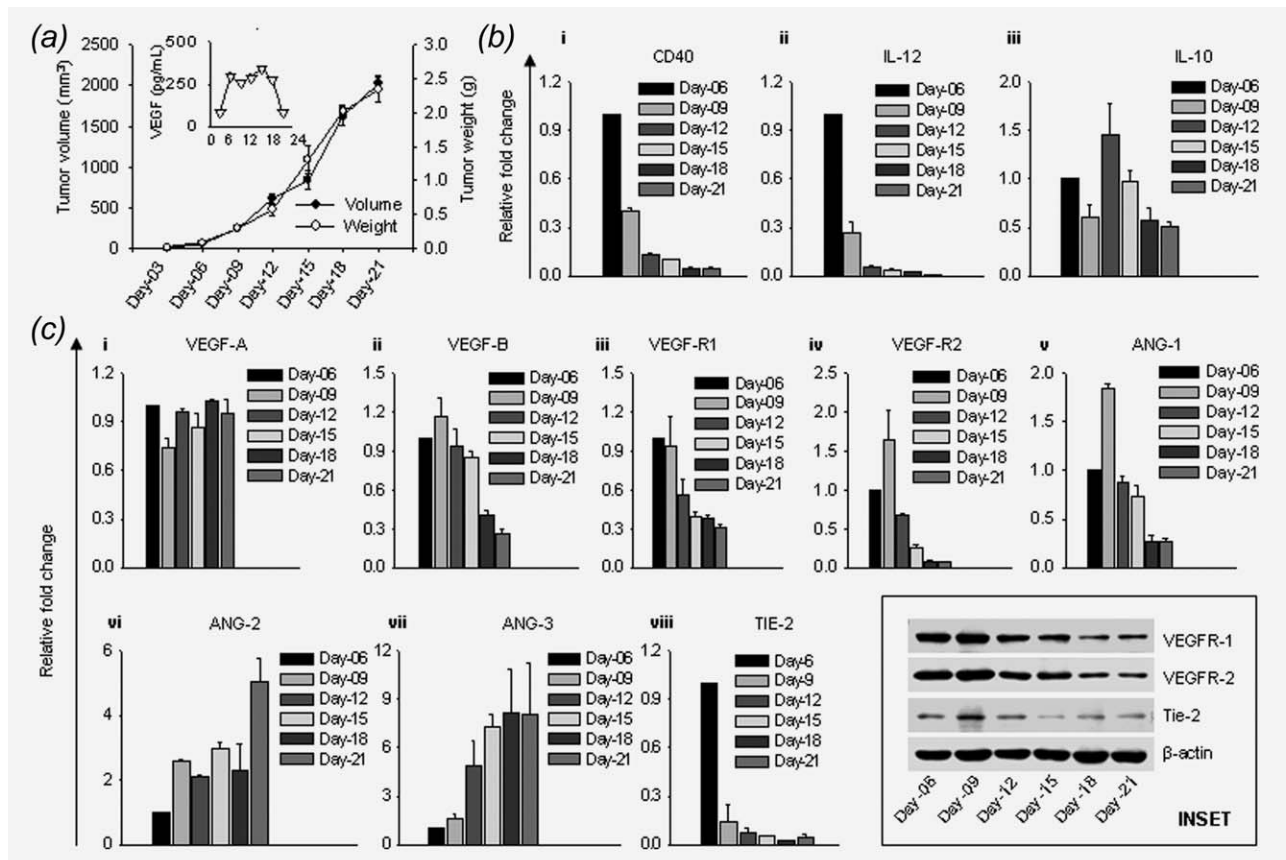

Figure 1. CD40, cytokines and angiogenic factors that contribute to tumor microenvironment change during tumor growth *in vivo*. (*a*) The size and volume of each tumor were measured ($n = 10$). After sacrificing the mice, tumors were excised ($n = 5$) and weighed. The tumor cell suspension was cultured for 24 hr and the VEGF in the supernatant was measured by ELISA (Inset). The values shown are mean ± SEM. (*b*) Total RNA from tumor tissue was isolated and the relative expression of IL-10, IL-12 and CD40 during the progression of tumor was assessed by real-time PCR. mRNA levels of the genes were normalized against GAPDH. The values shown are mean ± SEM. (*c*) Similarly, the relative expressions of the indicated angiogenic factors during the progression of tumor were assessed by real time-PCR or Western blots for VEGFR-1, VEGFR-2 and Tie-2 (inset). The values shown are mean ± SEM. The experiments were repeated four times. The cultures were set in triplicates.

factors associated with it using the previously described tumor model.[3,4] We observed a steep increase in tumor volume and weight of tumor coinciding with VEGF-A secretion by tumor cells (Fig. 1*a*). Because tumor growth is differentially associated with the expression of CD40, which regulates the secretion of proinflammatory and anti-inflammatory cytokines IL-12 and IL10, respectively,[12,14] we assessed the expressions of CD40, IL-10 and IL-12 in tumor (Fig. 1*b*, i–iii): expressions of CD40 and IL-12 decreased with tumor growth, whereas IL-10 expression remained high. We also observed that VEGF-A was expressed at high levels but VEGF-B, VEGFR-1 and VEGFR-2 decreased steadily during the progression of tumor (Fig. 1*c*, i–iv, inset). We observed that expressions of angiopoietin (Ang)-1 and Tie-2 were decreased, whereas expressions of Ang-2 and Ang-3 were found to be increased (Fig. 1*c*, v–viii, inset). The expressions of the angiogenic factors were not detectable in the controls (normal subcutaneous tissues from naïve mouse). These data indicate that the tumor microenvironment regulates tumor growth by selective modulation of various angiogenic factors.

Co-treatment with anti-CD40 and anti-VEGF effectively decreases tumor growth *in vivo*

To assess if simultaneous CD40 activation and VEGF neutralization would affect tumor growth, we treated the tumor-bearing C57BL/6 mice with anti-CD40 or anti-VEGF antibodies alone or in combination. We observed that the mice co-treated with anti-CD40 and anti-VEGF antibodies showed reduced tumor volume and weight than those treated with either antibody alone (Figs. 2*a* and 2*b*) and that the anti-VEGF antibody was more efficient in inhibiting tumor growth than the anti-CD40 antibody. Most importantly, the mice treated with anti-CD40 plus anti-VEGF antibodies showed 100% survival, whereas the control groups showed less than 50% survival; the anti-VEGF-treated mice survived longer than the anti-CD40-treated mice (Fig. 2*c*). The observed effects were accompanied by significantly reduced relative vessel abundance in anti-CD40 and anti-VEGF antibodies-co-treated mice than treatments alone and the controls (Fig. 2*d*, inset). The expression of VEGF-A, VEGF-B and VEGFR-1 did not significantly change upon treatment, suggesting that the treatment does not

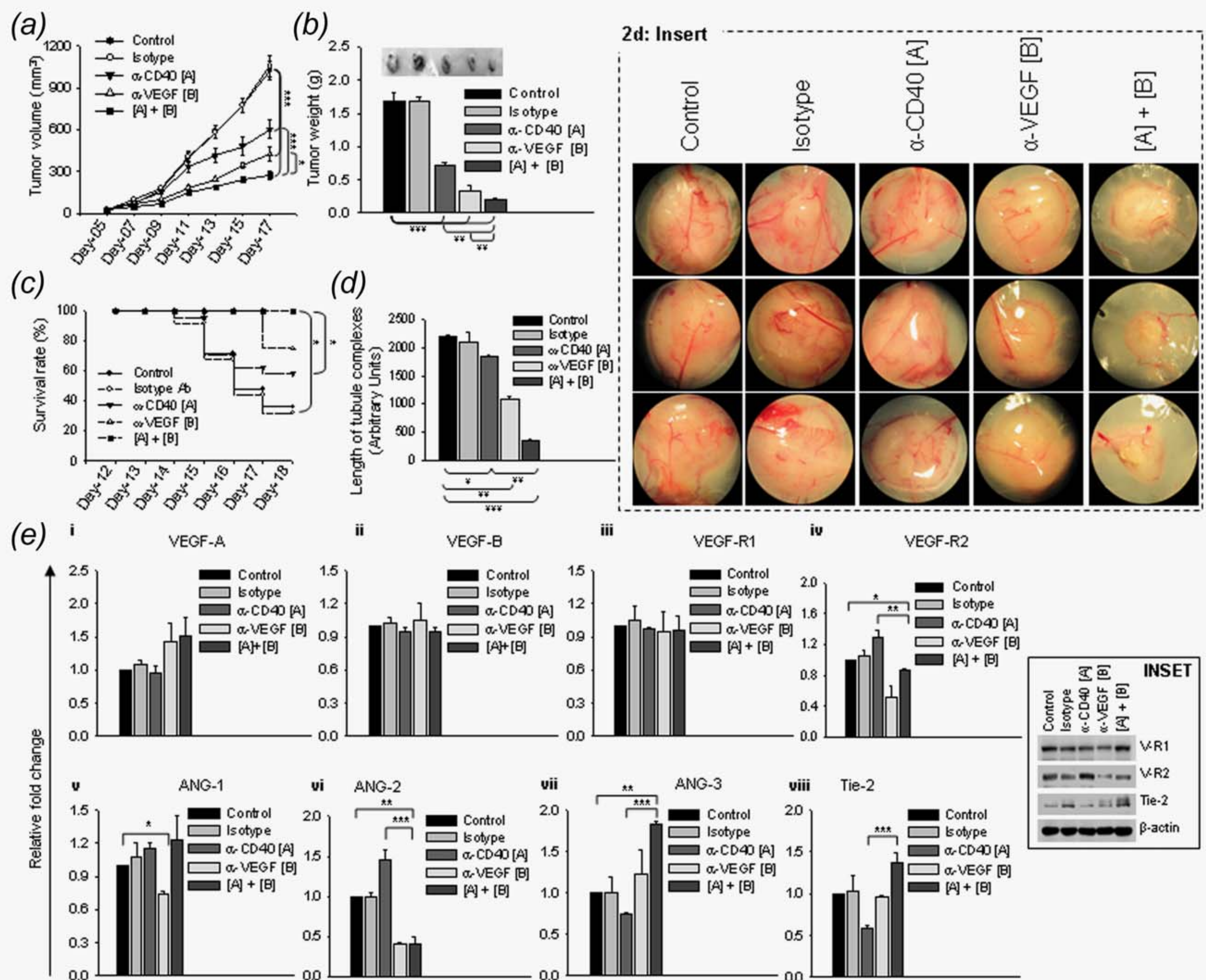

Figure 2. Co-treatment with anti-CD40 and anti-VEGF antibodies effectively decreased tumor growth *in vivo*. (*a*) The size and volume of each tumor were measured ($n = 8$). (*b*) Tumors from the tumor-bearing C57BL/6 mice were collected ($n = 5$) and weighed. The values shown are mean ± SEM. (*c*) The survival kinetics of treated and control mice ($n = 24$) are shown. (*d*) Extent of angiogenesis represented by total length of tubule complexes of treated and control mice is shown. (d, insert) The photographs were taken using stereo microscope at ×12 magnification and quantified as described in Material and Methods. (*e*) Total RNA from tumor tissue was collected and relative expressions of different angiogenic factors are shown. Western blots for VEGFR-1, VEGFR-2 and Tie-2 are shown in the inset. The data shown are mean ± SEM of individual biological replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The experiments were repeated thrice.

affect its expression (Fig. 2*e*, i–iii). The expression of VEGFR-2 was decreased significantly in co-treated group and mice treated with anti-VEGF but increased after anti-CD40 treatment (Fig. 2*e*,iv, inset). Ang-1 showed no significant change in expression after anti-CD40 treatment or co-treatment but showed significant decrease after anti-VEGF treatment, whereas Ang-2 showed increased expression after anti-CD40 treatment but anti-VEGF-treated and co-treated group showed decreased expression (Fig. 2*e*, v and vi). Ang-3 showed decreased expression after anti-CD40 treatment but increased expression upon co-treatment (Fig. 2*e*, vii). The expression of Tie-2 was decreased on anti-CD40 treatment but showed increased expression upon co-treatment (Fig. 2*e*, viii). These data indicate that co-treatment effectively decreases tumor growth and increases the rate of survival of mice.

VEGF neutralization increases the efficacy of the CD40-induced antitumor responses

As CD40-mediated tumor regression is associated with T cells, we studied the effector function of T cells *in vitro*: T-cell proliferation, IFN-γ production and CTL activity. We observed that splenic T cells from the anti-CD40 and anti-VEGF antibodies-co-treated tumor-bearing mice showed significantly higher proliferation and IFN-γ production than any other groups of mice (Figs. 3*a* and 3*b*). We observed that CTLs from the anti-CD40 and anti-VEGF antibodies-co-treated mice showed higher cytolytic activity than the CTLs from other groups (Fig. 3*c*). We observed that anti-CD40 and anti-VEGF antibodies-co-treated mice showed higher IL-12 expression than mice from other groups (Fig. 3*d*). In addition, anti-CD40 and anti-VEGF antibodies-co-treated mice

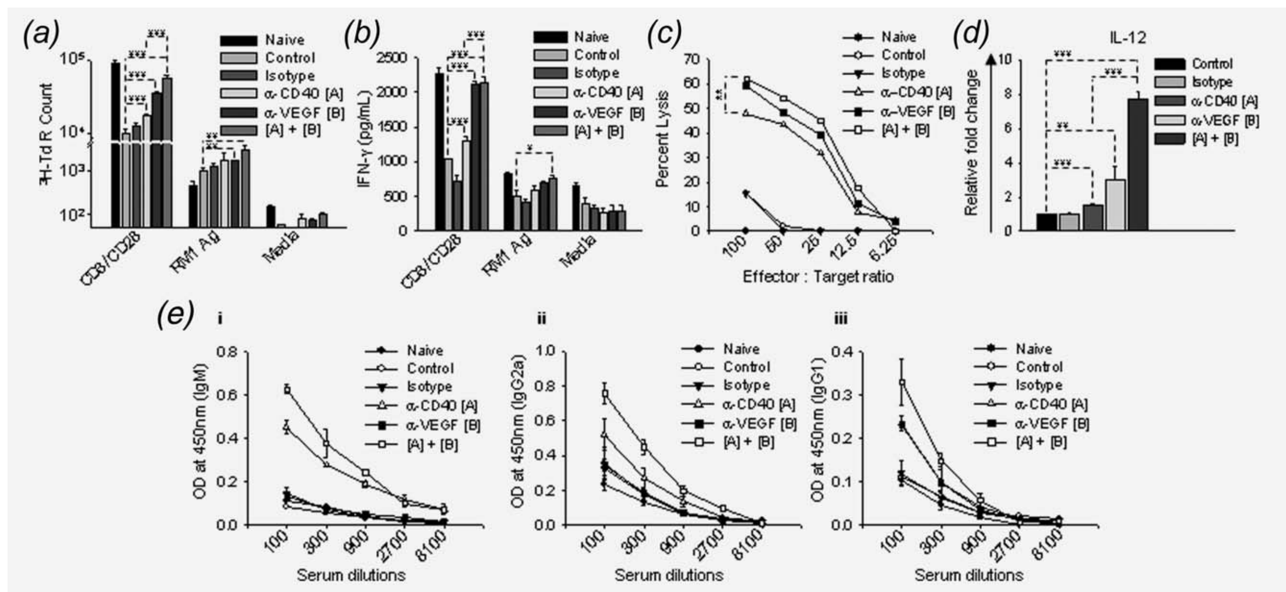

Figure 3. Enhanced antitumor response on co-treatment is attributed to high IFN-γ production and CTL activity. (*a*) Splenic T cells ($n = 4$) from different treatment and control mice were cultured in the presence or absence of RM-1 cell Ag and cells were pulsed with ^{3}H]-thymidine to determine tumor Ag-specific T-cell proliferation. Data represent the mean ± SEM of quadruplicate assays. (*b*) Splenic T cells ($n = 4$) from different treatment and control mice were cultured with RM-1 Ag in triplicates. Cell-free supernatants were harvested and assayed for IFN-γ production by ELISA. The data shown are mean ± SEM. (*c*) Splenocytes were co-cultured with irradiated RM-1 cells. After 5 days, viable CD8^{+} T cells were harvested and tested for their cytolytic activity against ^{3}H]-thymidine-incorporated target cells (RM-1) in a standard 4-hr JAM test. The E:T ratio is shown. Each data point is the mean of quadruplicate samples. (*d*) Relative expression of IL-12 has been shown. The data shown are mean ± SEM of individual biological replicates. (*e*) The sera from these mice ($n = 5$) were assayed for tumor Ag-reactive Ig isotypes (IgM, IgG1 and IgG2a) on RM-1 Ag-coated (10 μg/mL) ELISA plates. The data shown are OD of tumor Ag-reactive Ig isotypes IgM, IgG2a and IgG1, respectively. The results shown are mean ± SEM of a given group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The experiments were performed thrice.

showed higher IgM, IgG1 and IgG2a (Fig. 3*e*, i–iii). These data indicate that VEGF neutralization significantly enhanced CD40-induced tumor regression accompanied by T-cell responses.

Discussion

CD40 induces the production of both proinflammatory and anti-inflammatory mediators in the tumor microenvironment, indicating that this functional duality of CD40 self-limits its antitumor function and reduces the efficacy of CD40-targeted immunotherapy.[8] Here, we report paradoxical counter-effective functions of CD40-central induction of antitumor T cells that cause tumor regression but local angiogenesis through induction of proangiogenic factors and tumor growth. We observed that VEGF is highly expressed, whereas its receptor expressions decreased during tumor progression. Despite the reduced receptor expressions, VEGF facilitates tumor progression possibly by one or more of the following mechanisms: redundant signaling ability of VEGF through various receptors for angiogenesis,[15] reduced signaling thresholds for receptor activation and subsequent angiogenesis upon binding and enhanced angiogenic functions of VEGFR-1/VEGFR-2 heterodimer.[16,17] We observed that the expressions of Ang-1 were diminished but that of Ang-2 and Ang-3 were increased during tumor progression and the expression profile was reversed upon co-treatment with anti-CD40 and anti-VEGF antibodies. Ang-1 and Ang-2 are reported to reciprocally regulate the VEGF-induced angiogenesis and tumor growth: increased expression of Ang-1 in the absence of Ang-2 is correlated to fewer normal vessels, whereas increased Ang-2 expression corresponds to abundant abnormal vessels.[18–20] Reduced expressions of CD40 and IL-12 but high IL-10 during tumor growth corroborate with our previous reports that CD40 expression and CD40-induced IL-12 production decrease as head and neck squamous cell carcinomas progress from Stage I to Stage IV.[14] As our tumor model is CD40 negative, the counteracting regulation of IL-10 and IL-12 expression is due to decreased expression of CD40 on tumor-infiltrating cells and not the tumor cells.[12] Although CD40 stimulation is important for effective T-cell functions and T-cell-mediated antitumor immune response,[5,6] VEGF inhibits DC activation, maturation and function and affects normal hematopoiesis to paralyze immune effector functions.[2] The reduced tumor growth following CD40 stimulation along with anti-VEGF antibody treatment may have two mechanisms: lesser tumor angiogenesis with lowered immunosuppressive microenvironment caused by VEGF neutralization and CD40-enhanced IL-12-dependent antitumor immune response. Thus, this tumor model nicely reflects angiogenesis and the antitumor effects of CD40 corroborating

the previously recorded immunological effects. However, the mechanisms of the paradoxical counteractive functions of CD40 in a metastatic tumor model remain to be deciphered.

In conclusion, CD40-induced antitumor response is self-limited by induction of several protumor factors such as VEGF. Therefore, the antitumor response can be enhanced by co-treatment with a neutralizing anti-VEGF antibody. The inhibition of angiogenesis favors the CD40-promoted T-cell-mediated tumor regression, increasing the survival of the tumor-bearing mice. Thus, we establish a novel and effective combined antiangiogenic and CD40-mediated antitumor immunotherapeutic strategy.

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