CD27-SIGNALING INCREASES THE FREQUENCY OF REGULATORY T CELLS AND PROMOTES TUMOR GROWTH

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Precis: Findings reveal how a TNF receptor superfamily member mediates tumor promoting effects of the adaptive immune system, with implications for new immunotherapeutic strategies to target this receptor.


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Abstract

Signaling of the TNF-receptor superfamily member CD27 activates costimulatory pathways to elicit T and B cell responses. CD27-signaling is regulated by the expression of its ligand CD70 on subsets of dendritic cells and lymphocytes. Here, we analyzed the role of the CD27-CD70-interaction in the immunological control of solid tumors in CD27-deficient mice. In tumor-bearing wild-type mice, the CD27-CD70-interaction increased the frequency of regulatory T cells (Tregs), reduced tumor-specific T cell responses, increased angiogenesis and promoted tumor growth. CD27-signaling reduced apoptosis of Tregs in-vivo and induced CD4+ effector T cells (Teffs) to produce interleukin-2, a key survival factor for Tregs. Consequently, the frequency of Tregs and growth of solid tumors were reduced in CD27-deficient mice or in wild-type mice treated with monoclonal antibody to block CD27-signaling. Our findings therefore provide a novel mechanism by which the adaptive immune system enhances tumor growth and may offer an attractive strategy to treat solid tumors.
**Introduction**

Clinical observations and experimental data support the hypothesis that the immune system contributes to cancer surveillance (1). However, different “escape” mechanisms of cancer cells explain the failure of an efficient immune control in progressing cancer. These include immunological ignorance of tumors developing outside secondary lymphoid organs (2), the expression of inhibitory molecules on cancer cells such as fas-ligand (3), the generation of MHC- and/or antigen-loss variants (4) and the induction of Tregs (5). CD4⁺CD25⁺FoxP3⁺ Tregs have been shown to hinder tumor immunity in different murine models and depletion of Tregs promotes tumor-specific immune responses (6). Furthermore, the frequency of Tregs is increased in peripheral blood and tumor-infiltrating lymphocytes (TILs) of cancer patients and accumulation of Tregs in the tumor predicts poor prognosis and survival (7).

In healthy individuals, Tregs constitute 5-10% of peripheral CD4⁺ T cells. These natural (n)Tregs arise in the thymus and depend on a strong T cell receptor (TCR) activation (8). In addition, Tregs can be induced outside the thymus from CD4⁺CD25⁻FoxP3⁻ naïve T cells. This conversion of naïve T cells to induced (i)Tregs depends on TCR stimulation, IL-2 and TGF-β (9). Whether the increased frequency of Tregs in tumor tissue and draining lymphatic tissue is a result of an expansion and/or accumulation of nTregs, or if they are locally induced, is controversial (10).

CD27, a TNF-receptor-superfamily member, acts in a costimulatory pathway to elicit T and B cell responses. CD27-signaling activates NF-κB, promotes cell survival, enhances antigen-receptor-mediated proliferative signals, and increases effector function (11). In addition, CD27-signaling increases the production of the T cell growth/survival factor IL-2 (12, 13). CD27-signaling either leads to improved T cell function or to T cell dysfunction, probably depending on the amount, duration, and timing of the expression of the CD27 ligand (CD70) (14-16). As a result, CD70 expression is tightly regulated, and it is only transiently expressed on activated T and B cells, as well as on subsets of professional antigen-presenting dendritic...
cells (DCs) and NK cells (11). In contrast to the very limited expression of CD70 in a normal healthy individual, constitutive expression of CD70 has been documented in cancer (17-19) and chronic viral diseases (14).

Current evidence supports the view that the CD27-CD70-interaction improves anti-tumor immunity (20-22). Injection of CD27-activating antibody improved tumor rejection (23, 24) and Cd70 transgenic mice were protected against poorly immunogenic tumor cells (25, 26). Certain human tumors such as brain tumors (19), renal cell carcinomas (27) and some lymphomas (17, 18) have been shown to express CD70. Somewhat controversial to the proposed improved T cell response after CD27-signaling, expression of CD70 on tumors often correlates with increased aggressiveness due to increased metastasis (28), Treg formation (17, 18) or death of effector cells (19). Similar contradictory observations on tumor formation also exist for other TNF-receptor-superfamily members, e.g. improved tumor control by systemic administration of stimulating anti-CD137 (4-1BB) antibody (29, 30) versus improved tumor control in CD137-deficient mice (31).

Although stimulating CD27 by over-expressing CD70 or by monoclonal antibody clearly improves tumor control (23-25), the physiological role of the CD27-CD70-interaction in tumor formation has not been analyzed so far. Using CD27-deficient mice, we found that CD27-signaling increased tumor-specific T cell responses after immunization with tumor cells as single-cell suspension. In contrast, tumor formation of transplanted tumors and chemically induced tumors was reduced in CD27+/− mice. CD27-signaling increased the frequency of Tregs in-vivo by reducing apoptosis and thereby increased tumor growth.
Materials and Methods

Mice

C57BL/6 (BL/6), H8 (32), UBC-GFP mice (33) and RAG-1−/− mice were from the Institute of Laboratory Animal Science (Zurich, Switzerland). CD27−/− mice (34) were from the Netherlands Cancer Institute (Amsterdam, Netherlands). All experiments were approved by the local Experimental-Animal-Committee.

Cell lines

The murine tumor cell lines fibrosarcoma MC57 and MC57-GP, melanoma B16F10 and B16F10-GP, colon adenocarcinoma MC38 and MC38-GP, and lymphoma RMA-S have been described before (35, 36). MC57-CD70 cells were transfected with the mCD70/pcDNA3.1(+) plasmid (provided by H. Yagita, Tokyo, Japan) and cultured in the presence of 1 mg/ml G418 (Invitrogen).

Tumor-induction

Tumors were induced as described in (35).

DC-generation

DCs were generated as described before (37).

Immunohistochemistry

Sections (2μm) of formalin-fixed, paraffin-embedded tumors were stained for Ki67, CD31 and CD3 on a NexES automated immunostainer (Ventana Instruments) using an IVIEW DAB Detection Kit (Ventana). Cleaved lamin A was stained as described before (38). Image acquisition was performed on an Olympus SZX12 microscope equipped with a JVC digital camera (KY-F70; 3CCD).
TaqMan® array

RNA was isolated from tumors using the RNeasy mini kit (Qiagen). 200ng of RNA were reversely transcribed and run on the TaqMan® mouse immune low density array using an ABI 7900 system (Applied Biosystems).

TIL-isolation

Tumors were digested at 37°C in PBS supplemented with 1mg/ml Collagenase-IA, 100μg/ml Hyaluronidase-V (Sigma), 40U/ml DNase-I (Roche), 5mM CaCl₂ and 5mM MgCl₂, washed and filtered. TILs were isolated using positive magnetic cell sorting (MACS; Miltenyi Biotec).

Antibodies

Anti-mouse/human-Helios was from BioLegend, anti-5-bromo-2'-deoxyuridine(BrdU) was from Pharmingen, Annexin-V from ImmunoTools, anti-mouse-IgG-(H+L) from Caltag and anti-mouse-CD70 (FR70), anti-mouse-CD4 (YTS131.1), anti-mouse-CD8 (YTS169.4), anti-mouse-CD25 (PC-61.5.3) from BioXCell, rat-IgG from Sigma and anti-asialo-GM1 from Wako. All other antibodies were from eBioscience. Intracellular stainings were performed using the FoxP3-staining-kit (eBioscience). Intracellular IFN-γ- and TNF-α-stainings were performed as described (12). Samples were acquired on an LSRII (BD Biosciences) and analyzed using FlowJo™ software (Tree Star).

Proliferation

Animals were administered BrdU (Sigma; 0.8mg/ml in drinking water and 1mg i.p./day) on 2 consecutive days and BrdU staining was performed as described (39). Cell cycle in FACS-sorted TILs was analyzed as described (40).
Treg-induction *in-vitro*

7x10^5 MACS-purified splenic CD4^+ T cells or 3x10^5 FACs-purified splenic CD4^+CD25^{hi} T cells were cultured in RPMI (10% FCS, 5ng/ml rh-TGF-β1 (R&D Systems) in the presence or absence of 5U/ml rm-IL-2 in anti-CD3ε coated (10µg/ml) 48-well or 96-well tissue culture plates. For CD27-stimulation, 5x10^4 irradiated (120Gy) MC57 or MC57-CD70 tumor cells were added to the culture. CD4^+ T cells were labeled with CFSE (Invitrogen). IL-2 protein levels were measured using a multiplexed particle-based flow cytometry cytokine assay (R&D Systems).

**Statistics**

Data were analyzed using GraphPad Prism® 5.0 and unpaired, two-tailed Student's t-test, Mann-Whitney-U test, one-way-ANOVA (Tukey's multiple-comparison test) or two-way-ANOVA (Bonferroni's post-test). Data are displayed as mean±SEM. p<0.05 was considered significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Results

Improved control of solid tumors in CD27-deficient mice

The transplantation of solid tumor fragments allows studying tumor development in the context of an already established tumor stroma. When transplanted as solid fragments, even very immunogenic tumors grow in immunocompetent hosts (2). To investigate the role of CD27-signaling in tumor growth, we transplanted solid fragments (~1mm³) of MC57 fibrosarcoma, MC38 colon adenocarcinoma or B16F10 melanoma s.c. into the flanks of BL/6 and CD27-deficient mice. In all experiments, tumor progression was faster in BL/6 (or CD27+/- littermates, data not shown) than in CD27-/- mice (Figures 1A-C). Similarly, MC38 and B16F10 tumors expressing the LCMV-GP as a tumor-model antigen developed faster in BL/6 mice (Figures 1E-F). As shown before (41), GP-expressing MC57 tumors were rejected in BL/6 mice due to a very efficient induction of anti-tumoral CD8+ T cells. Similarly, MC57-GP did not lead to tumor formation in CD27-/- mice (Figure 1D). In addition, MC57 single-cell suspensions failed to grow in both groups of mice (Figure 1G) due to an efficient immune response (2, 42).

To verify our findings in a different experimental model, we injected 3-methylcholanthrene. All BL/6 mice developed clinically detectable subcutaneous tumors within 2-3 months (101±10 days). In contrast, tumor formation in CD27-deficient mice was delayed up to 10 months (219±23 days) (Figure 1H).

Reduced tumor cell proliferation, reduced tumor angiogenesis and increased T cell infiltration of tumors in CD27-/- mice.

The complete rejection of MC57 tumors by the majority of CD27-/- mice prevented further analysis of the underlying mechanisms in the tumor. Therefore, larger fragments (8 mm³) were transplanted in all subsequent experiments, resulting in sufficient initial tumor growth to
perform histological sections and to analyze the function of TILs in both BL/6 and CD27\(^{-/-}\) mice (Supplementary Figure 1A). Tumor cell proliferation was increased in the presence of an intact CD27-CD70-interaction (Figures 2A,B). In contrast, no differences in tumor cell apoptosis were observed (Figures 2A,C). Furthermore, tumors were significantly more vascularized in BL/6 mice than in CD27\(^{-/-}\) mice (Figures 2A,D). Finally, CD3\(^{+}\) T cells infiltrated significantly less into tumors of BL/6 than CD27\(^{-/-}\) mice (Figures 2A,E). These data suggest that the enhanced tumor progression in BL/6 mice correlates with increased tumor cell proliferation, improved tumor vascularization and poor T cell infiltration.

CD8\(^{+}\) T cells are the main effector mechanisms that control tumor growth in the tumor models used so far (35). Depletion of NK cells in RAG-1\(^{-/-}\) mice carrying the primary tumor using anti-asialo-GM1-antibody before transplantation did not influence tumor growth, formally excluding that NK cells transplanted within the tumor fragments were responsible for the observed different tumor growth in BL/6 versus CD27\(^{-/-}\) mice (Figure 2F). In addition, the NK cell-controlled RMA-S tumor fragments (36) grew equally in BL/6 and CD27\(^{-/-}\) mice (Figure 2G).

**Anti-tumoral immune responses are increased in CD27\(^{-/-}\) mice.**

We compared anti-tumoral CTL responses after s.c. injection of irradiated MC57-GP single-cell suspensions. In BL/6 mice, frequencies of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing gp33-specific CD8\(^{+}\) T cells were significantly higher than in CD27\(^{-/-}\) mice (Figure 3A and Supplementary Figure 1B). Similar results were obtained with non-irradiated tumor cells or i.p.-injections (data not shown). These results confirm that after injection of tumor cells as single-cell suspensions, CD27-costimulation improves specific anti-tumoral T cell responses.

In a next step, anti-tumoral immune responses were analyzed 20-30 days after tumor transplantation. Frequencies of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing CD4\(^{+}\) and CD8\(^{+}\) T cells in spleens did not significantly differ. However, in tumor-draining inguinal LN and TILs of
CD27−/− mice, frequencies IFN-γ- and TNF-α-producing CD4+ and CD8+ T cells were significantly increased (Figure 3B and Supplementary Figure 1C).

In addition, an immune gene profile in total tumor RNA 14 days after transplantation revealed that 11 effector/activation markers were significantly increased in tumors isolated from CD27-deficient mice (Supplementary Figure 1D).

To analyze if the induction of a CTL response is suppressed in the presence of growing solid tumors, gp33-expressing DCs were injected into tumor-bearing or naïve mice. Gp33-specific IFN-γ and TNF-α CTL responses were suppressed approximately 20-40% in spleens and LN of tumor-bearing vs. naïve BL/6 mice (Figure 3C and data not shown). In contrast, gp33-specific CTL responses were comparable in tumor-bearing and naïve CD27−/− controls.

Our results indicate that the CD27-CD70-interaction increases the tumor-specific CD8+ T cell response against tumor cells injected as single-cell suspension, but suppresses CD8+ T cell responses in the presence of growing solid tumors.

The CD27-CD70-interaction induces Treg accumulation.

Tregs reduce anti-tumoral immune responses and enhance tumor progression (6, 7, 45). As reported before (15), the frequency of Tregs in naïve CD27−/− and BL/6 mice was comparable. This held also true for CD4+CD25−FoxP3+ Tregs (CD25− Tregs). In contrast, after tumor transplantation, BL/6 mice had significantly higher frequencies of CD25+ and CD25− Tregs than CD27−/− mice in the spleen and in tumor-draining inguinal LN. Importantly, the frequency of intratumoral CD25+ Tregs was increased in BL/6 compared to CD27−/− mice, while there was no difference for CD25− Tregs (Figure 4A).

To test if CD4+CD25+ Tregs are responsible for the CD27-CD70-mediated tumor progression, MC57 tumor growth was analyzed in CD4+ or CD25+ T cell-depleted mice (Figures 4B-C). CD4+ T cell-depletion induced complete tumor rejection in CD27−/− and BL/6 mice (Figure 4B). Anti-CD25-antibody was administered four days before tumor transplantation,
allowing efficient depletion of CD25+ Tregs up to 20 days after transplantation without impairing the generation of CD25+ anti-tumor effector cells (46). Initially, CD25+ T cell-depleted BL/6 and CD27−/− mice similarly controlled tumor growth (Figure 4C). However, four weeks after CD25 depletion, the frequency of Tregs increased and reached significantly higher levels in BL/6 mice (Figure 4D). As a consequence, 5 out of 10 tumors in BL/6 mice grew, whereas only one tumor grew in CD27−/− mice (Figure 4C).

To more directly define the contribution of CD27-signaling on Tregs, we adoptively transferred FACS-sorted CD4+ or CD4+CD25hi T cells from CD27−/− or BL/6 mice into tumor-bearing CD27−/− mice. The transfer of BL/6 CD4+ or CD4+CD25hi T cells to CD27−/− recipient mice completely reconstituted tumor growth (Figure 4E). As a control, the transfer of CD27−/− CD4+ or CD4+CD25hi T cells did not affect tumor growth and all tumors were rejected. The adoptively transferred CD4+ or CD4+CD25hi T cells from BL/6 mice reached higher frequencies in TILs than the endogenous CD27−/− CD4+ T cells (Figure 4F). These data indicate that direct CD27-signaling on CD25+ Tregs contributes to the observed differences in tumor growth between CD27−/− and BL/6 mice.

**Anti-CD70-treatment reduces tumor progression in BL/6 mice.**

To analyze if blocking the CD27-CD70-interaction by monoclonal antibody (FR70) is a possibility to treat solid tumors, two treatment protocols were performed. First, early FR70-treatment starting one day before MC57 tumor transplantation significantly reduced tumor growth (Figure 4G). In addition, the frequency of CD25+ Tregs of rat-IgG-treated BL/6 mice significantly increased 10 days after transplantation compared to FR70-treated mice (Figure 4H). Secondly, late FR70 treatment starting 18 days after tumor transplantation (when tumors were clinically detectable) significantly delayed tumor growth (Figure 4I) and prevented intratumoral accumulation of CD25+ Tregs (Figure 4J) without affecting other T cell subsets (Supplementary Figure 2A). However, early treatment was more effective. These data
indicate that treatment with blocking anti-CD70 antibody significantly inhibits Treg accumulation and reduces tumor progression.

**Preferred expression of CD70 on CD4⁺ TILs**

Analysis of CD70-expression on tumor-infiltrating immune cells in BL/6 and CD27⁻/⁻ mice revealed highest levels of CD70-expression on CD4⁺ TILs in CD27⁻/⁻ mice (Figures 5A-B). This is in line with previous findings indicating that CD70 is shed from the cellular surface after ligating CD27 in BL/6 mice (15, 47). Analysis of CD4⁺ subsets in TILs of CD27⁻/⁻ mice revealed that CD70 was expressed on Tregs and Teffs as well as on CD4⁺CD25⁺FoxP3⁻ cells (Figures 5C,D). In contrast, CD70 expression in both CD27⁻/⁻ and BL/6 mice was low on non-tumor-infiltrating immune cells (Figure 5B and data not shown).

**CD27 is preferentially expressed and ligated on intratumoral CD25⁺ Tregs.**

In tumor-bearing BL/6 mice, the expression of the CD27 receptor was strongly down-regulated on CD4⁺ and CD8⁺ TILs as compared to CD4⁺ and CD8⁺ T cells from tumor-draining inguinal LN and spleens (Figure 5E). This down-regulation of CD27 may be due to shedding after ligation with CD70 (48). In accordance with this hypothesis, blocking CD70 in tumor-bearing BL/6 mice by FR70 resulted in an up-regulation of CD27-expression on intratumoral CD25⁺ Tregs, whereas the expression of CD27 on Tregs in the spleen remained unchanged (Figure 5F-G and data not shown). Analysis of CD27 mean-fluorescence intensity (MFI) in TILs and spleens of FR70- vs. rat-IgG-treated tumor-bearing BL/6 mice revealed that CD27 is preferentially ligated on CD25⁺ Tregs in the tumor (Figures 5F-G) but also on Teffs in the spleen. However, the frequency of CD4⁺CD25⁺FoxP3⁻ Teffs in spleen was very low (Figure 5F). These experiments indicate that CD70 is locally expressed on TILs and leads to CD27 ligation on intratumoral Tregs.
CD27-signaling favors Treg accumulation in-vivo by reducing Treg apoptosis.

To analyze the mechanism of the CD27-mediated increase in Treg numbers, purified CD4⁺ T cells from GFP-expressing BL/6 or CD27⁻/⁻ mice were transferred to recipient mice one day before transplantation. Twenty days after tumor transplantation, the frequencies and absolute numbers of GFP⁺ and GFP⁻ CD25⁺ Tregs were determined in the spleen (Figure 6A). Transferring CD27-competent CD4⁺ T cells resulted in significantly higher frequencies and numbers of GFP⁺CD25⁺ Tregs than transferring CD27-deficient CD4⁺ T cells. This held true for BL/6 and CD27⁻/⁻ recipient mice (Figure 6B). In addition, blocking the CD27-CD70-interaction of CD4⁺ T cells from BL/6 mice transferred to CD27⁻/⁻ recipient mice by FR70-treatment reduced the frequency and numbers of GFP⁺CD25⁺ Tregs (Figure 6B). Due to shedding of CD70 after ligation with CD27, CD70 expression is higher in CD27⁻/⁻ mice than in BL/6 mice (Figure 5A and data not shown). This might explain the higher frequencies of BL/6 CD25⁺ Tregs after transfer into CD27⁻/⁻ recipient mice when compared to BL/6 recipient mice.

In contrast, the transfer of CD27-competent CD4⁺ T cells to CD27⁻/⁻ recipient mice did not affect the frequency of the endogenous CD27⁻/⁻ CD25⁺ Tregs (GFP⁻) (Supplementary Figure 2B). This further confirms a direct effect of CD27-signaling on CD25⁺ Tregs.

Tumor-infiltrating Tregs consist of nTregs and iTregs (9, 10). To discriminate whether CD27-signaling leads to a preferential accumulation of nTregs or an increased generation of iTregs, we used the recently identified transcription factor Helios (49). About 60% of CD25⁺ Tregs in the spleens and inguinal LN of naïve and tumor-bearing BL/6 and CD27⁻/⁻ mice were Helios⁺ nTregs. In contrast, only about 40% of tumor-infiltrating CD25⁺ Tregs were Helios⁺ nTregs. In Figure 6C. Importantly, no differences in the frequencies of CD25⁺ nTregs in BL/6 versus CD27⁻/⁻ mice were found. These data suggest that the increased frequency of CD25⁺ Tregs in BL/6 mice is the consequence of an accumulation of both, nTregs and iTregs.
A higher frequency of Tregs in CD27-competent mice may be due to prolonged cell survival and/or due to increased cell cycling. BrdU-incorporation and DAPI experiments revealed no significant differences in proliferation of Tregs between tumor-bearing BL/6 and CD27⁻/⁻ mice (Figures 6D-E). In contrast, CD25⁺ Tregs isolated from tumor-draining LN and tumors of BL/6 mice contained a significantly lower percentage of Annexin-V⁺ cells (Figure 6F). Similarly, the frequency of Annexin-V⁺CD4⁺ Teffs in tumor-draining LN and tumors was lower in BL/6 mice (Supplementary Figure 2C). Interestingly, a reduction of T cell apoptosis was not detected in the spleen, correlating well to the low expression of CD70 in this organ (Figure 5B and Supplementary Figure 2C). Therefore, CD27-signaling reduces apoptosis of both, CD25⁺ Tregs and Teffs. However, as outlined above and shown in Figure 5F, the frequency of CD4⁺CD25⁺FoxP3⁺ Teffs within total CD4⁺ T cells is very low. Importantly, the reduced apoptosis of CD25⁺ Tregs decreases the intratumoral ratio of Teffs/Tregs in BL/6 mice (Supplementary Figure 2A).

**CD27-signaling increases survival of Tregs in-vitro by augmenting IL-2 production.**

To analyze the mechanism of the CD27-induced increase in Tregs in more detail, we generated Tregs in-vitro in the presence of anti-CD3ε and TGF-β. Splenic CD4⁺ T cells from naïve BL/6 or CD27⁻/⁻ mice were co-cultured with irradiated MC57 or MC57-CD70 cells. CD27-ligation in co-cultures with MC57-CD70 significantly increased the frequency of CD25⁺ Tregs from BL/6 mice when compared to CD25⁺ Tregs from CD27⁻/⁻ mice at days 2 (Figure 7A) and 4 (not shown). Similarly, the frequency of BL/6 CD25⁺ Tregs was higher in co-cultures with MC57 cells when compared to CD27⁻/⁻ CD25⁺ Tregs. This may be due to the expression of CD70 by activated CD4⁺ T cells (data not shown). The CD25⁺ Treg population contained nTregs and iTregs, which both expanded similarly (Figure 7B) and down-regulated the CD27-receptor (Figure 7C) upon CD70-stimulation. In contrast, we observed no differences in the frequency of CD25⁺ Tregs, regardless of CD27-ligation.
To distinguish between Treg cell division and apoptosis in-vitro, we performed a CFSE dilution assay. CD27−/− and BL/6 CD25+ Tregs underwent a similar number of cell divisions at days 2 (Figure 7D) and 4 (not shown). However, CD27-ligation led to significantly higher numbers of BL/6 Tregs before and after the first cell division.

CD27-signaling has been shown to increase IL-2 secretion and IL-2 is a crucial growth and survival factor for Tregs (50). We therefore analyzed the IL-2 concentrations and found significantly higher amounts of IL-2 when the CD27-CD70-interaction was present (Figure 7E). Intracellular IL-2 stainings revealed that CD27-signaling significantly increased IL-2 production in FoxP3-negative CD4+ T cells but not Tregs (Figure 7F). This observation is in line with previous findings indicating that Tregs do not produce IL-2 (51).

As a consequence of the inability to produce IL-2, FACS-purified CD4+CD25hi T cells from BL/6 or CD27−/− mice could not be expanded in the absence of exogenous IL-2 (Figure 7G). The addition of IL-2 to the culture media resulted in comparable frequencies of CD25+ and CD25− Tregs in BL/6 and CD27−/− CD4+ T cell cultures (Figure 7H). The addition of IL-2 resulted in a similar survival and proliferation of FACS-purified CD4+CD25hi T cells from BL/6 and CD27−/− mice (Figure 7I). Blocking IL-2 by neutralizing antibody completely blocked Treg formation (data not shown). In summary, these in-vitro experiments confirm a role of CD27-signaling on CD25+ Treg survival but not proliferation and underline the importance of IL-2 in Treg development and maintenance.

**CD27−/− and BL/6 Tregs are similarly capable to inhibit CD8+ T cell expansion.**

CD27 expression has been reported to correlate with the inhibitory capacity of Tregs (52). Therefore, we tested the capacity of titrated numbers of CD27−/− or BL/6 Tregs to inhibit the proliferation of CD8+ T cells in a 3H-Thymidin incorporation assay FACS-sorted CD4+CD25hi T cells of CD27−/− and BL/6 mice similarly inhibited the expansion of CD8+
responder T cells (Supplementary Figure 3A,B). These experiments suggest that CD27-signaling mainly increases Treg numbers but not their T cell suppressive capacity.
Discussion

CD27-signaling increases T cell expansion and function and is of importance in the maintenance of T cell memory (12, 43). This improves the control of acute viral infections and of tumors in certain model situations (15, 20, 22). However, it is well documented that the tumor microenvironment favors a chronic, smoldering inflammation that differs substantially from inflammatory processes in secondary lymphoid organs during an acute infection. This chronic inflammatory condition may promote tumor progression by generating an immunosuppressive environment (53). Especially Tregs account for substantial inhibition of anti-tumoral immunity by reducing effector cell expansion and function as well as the function of professional APCs (5).

In our model, the CD27-CD70-interaction promoted tumor growth. It is of importance to state that in mice transplanted with solid tumor fragments, significant expression of CD70 was only detected on TILs. Therefore, the CD27-CD70 interaction occurred mainly in the immunosuppressive tumor microenvironment. This is in contrast to previous studies (23-25) that analyzed the potential to provide maximal CD27 stimulation by the administration of stimulating anti-CD27 monoclonal antibodies or by injecting tumors into CD70 transgenic mice to improve tumor control. In these situations, CD27 ligation is not only provided in the immunosuppressive tumor microenvironment but in the entire lymphoid tissue, especially in the proinflammatory microenvironment of LN and spleen.

Similarly divergent observations have been made with other TNF-receptor superfamily members, like CD137 (4-1BB). Agonistic anti-CD137 antibodies improved CD8+ T cell responses and tumor control (29, 30). In contrast, tumor growth was reduced in CD137−/− mice (31). Therefore, as reported before (15), co-stimulatory molecules of the TNF-receptor-superfamily have different effects on the immune response depending on the signaling conditions.
We could demonstrate that CD25⁺ Treg frequencies were increased in TILs and tumor-draining lymph nodes of BL/6 vs. CD27⁻/⁻ mice. In addition to classical CD4⁺CD25⁺FoxP3⁺ Tregs, CD4⁺CD25⁻FoxP3⁺ Tregs exist in the tumor environment and may contribute to immunosuppression. However, CD27-signaling only increased the frequency of CD25⁺ Tregs in tumors and tumor-draining LN of BL/6 mice, suggesting that in the tumor microenvironment, CD25⁻ Tregs are less dependent on CD27-signaling. Our depletion and adoptive transfer experiments clearly indicate that CD25⁺ Tregs are responsible for the increased tumor growth.

The adoptive transfer of purified CD27-competent CD25⁺ Tregs to CD27⁻/⁻ recipients suggests a Treg intrinsic effect of CD27-signaling on cell survival but not proliferation. In addition, CD25⁺ Tregs constitutively express high levels of the IL-2Rα subunit (CD25) and the IL-2 receptor pathway is crucial for Treg survival (50). Our *in-vitro* experiments demonstrate a CD27-dependent accumulation of CD25⁺ Tregs due to an increase in IL-2 produced by non-Treg CD4⁺ T cells. Therefore, the accumulation of CD25⁺ Tregs in tumors of CD27⁻/⁻ mice is most likely a consequence of both, direct CD27-singaling on Tregs but also on CD4⁺ Teffs leading to an increase in IL-2 production. The effect was similar on Helios⁺ nTregs as well as of Helios⁻ iTregs.

CD27-signaling increases cell survival of both, Teffs and Tregs. The effect on tumor growth depends on the relative contribution of Teffs vs. Tregs. In our model, the ratio of Teffs/Tregs was decreased in solid tumors of BL/6 compared to CD27⁻/⁻ mice. In addition, T cell function after *in-vitro* restimulation and antigen-specific CTL responses *in-vivo* were suppressed in solid tumor-bearing BL/6 but not CD27⁻/⁻ mice. Therefore, at least part of the increased tumor growth in BL/6 mice may be consequent to a Treg-mediated reduced CD8⁺ T cell responses. Indeed, we demonstrated before that CD8⁺ T cells are the main effector mechanism in control of the tumors used in our study (35). Besides suppression of the adaptive anti-tumoral immune response, Tregs also favor tumor progression via modulation of innate immunity. For
example, Tregs are able to skew monocyte differentiation towards alternatively activated macrophages (M2 type) (54) that are known to support tumor angiogenesis (55). A reduction of Tregs in the absence of CD27 may lead to a shift in monocyte differentiation from protumoral M2 to anti-tumoral M1 macrophages. Indeed, IL-1α and Nos2, both markers of anti-tumoral M1 macrophages (56), were expressed at higher levels in tumors of CD27−/− than BL/6 mice.

Recently, a role for the CD27-CD70-interaction in the induction of Tregs has been suggested for non-Hodgkin B cell lymphoma and for chronic lymphocytic leukemia (17, 18). CD70+, but not CD70− lymphoma cells contributed to the activation-induced FoxP3 expression in intratumoral CD4+CD25+ T cells in-vitro. In contrast, we did not observe an effect of CD27-signaling on the frequency of iTregs in TILs or tumor-draining lymph nodes. Of note, the lymphoma cells that were used in these studies express CD70 directly, whereas in our tumor models CD70 was mainly expressed on tumor-infiltrating T cells and none of our tumor cell lines expressed CD70 (data not shown).

Previous studies have shown that CD27 expression on human CD4+CD25+ Tregs positively correlates with their suppressive activity in-vitro and with the expression of FoxP3 (52, 57). The reduced CD27 expression on intratumoral Tregs in our experiments, though, was reversible upon blocking CD70. In addition, we did not observe impaired CD8+ T cell suppression by CD27−/− Tregs in-vitro; indicating that Treg function on CD8+ T cell expansion is independent of CD27-signaling.

The role of Tregs in the suppression of anti-tumoral immune responses has been recognized 40 years ago and has been confirmed in many experimental tumor models and in cancer patients (5). Therefore, defining pathways involved in the formation, accumulation and function of Tregs in solid tumors is of importance and might lead to novel therapeutic strategies. In combination with other therapeutic regimens, blocking CD27-signaling is a
promising approach to reduce Treg formation in the tumor environment and to improve tumor control.

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Author Contributions

C.C., C.R., C.S. and M.S.M. designed experiments, performed research, analyzed and interpreted results and wrote the manuscript. T.H. performed experiments. A.F.O. directed the study, analyzed and interpreted results and wrote the manuscript.
References

Figure Legends

Figure 1

Tumor growth in CD27−/− and BL/6 mice

Indicated tumors were transplanted as (A-F) small fragments (1mm³) or injected as single-cell suspension (G) into flanks of mice. Total numbers of transplanted fragments or injections and numbers of growing tumors are indicated. (H) 3-methylcholanthrene was injected and the time interval until tumors could be clinically detected (>0.2cm³) was statistically compared.

Figure 2

Tumor cell proliferation, angiogenesis, lymphocyte infiltration and role of NK cells

8mm³ MC57 tumor fragments were transplanted into flanks of BL/6 and CD27−/− mice (n=3-4). Immunohistochemistry at day 25: (A,B) Ki67, (A,C) cleaved lamin A, (A,D) CD31 and (A,E) CD3. Scale bars, 50μm. Growth of transplanted 8mm³ tumor fragments of (F) RMA-S or (G) MC57 from NK-cell depleted RAG-1−/− mice.

Figure 3

Anti-tumoral immune responses in CD27−/− mice

(A) Irradiated MC57-GP cells were injected into naïve BL/6 and CD27−/− mice. Eight days later, IFN-γ-production of CD8+ T cells in spleens and inguinal LN was analyzed after in-vitro restimulation with gp33. (B) Twenty days after tumor fragment transplantation, IFN-γ-production of CD4+ and CD8+ T cells in spleens, inguinal LN and TILs was analyzed after in-vitro restimulation with PMA/ ionomycin. (C) DCs were injected into naïve or tumor-bearing mice 30 days after tumor fragment transplantation. Eight days later, IFN-γ- and TNF-α-production of CD8+ T cells in inguinal LN of tumor-bearing mice was analyzed after in-vitro restimulation with gp33 and normalized to the frequencies of naïve mice (dotted line).
**Figure 4**

**The frequency of Tregs is increased in tumor-bearing BL/6 mice.**

(A) Frequencies of CD25+ and CD25− Tregs within total CD4+ T cells in spleens, tumor-draining inguinal LN and TILs in naïve mice and 30 days after transplantation of MC57 tumor fragments. (B) MC57 tumor growth in untreated or CD4+ T cell-depleted mice. (C) MC57 tumor growth in mice treated with depleting anti-CD25-antibody 4 days before tumor transplantation. (D) Frequencies of Tregs in CD25-depleted mice at different time points after tumor transplantation. (E,F) On the day of tumor transplantation, BL/6 or CD27−/− CD4+ or CD4+CD25hi T cells were injected i.v. into recipient mice. (E) Analysis of tumor growth. (F) Frequencies of adoptively transferred congeneric BL/6 and endogenous CD27−/− CD4+ T cells in TILs of tumor-bearing CD27−/− mice 26 days after transplantation. (G-J) Tumor-bearing BL/6 mice were treated with FR70-antibody or rat-IgG (arrows), starting (G) 1 day before or (I) 18 days after transplantation. (H) CD25+ Treg frequency in blood before and 10 days after transplantation. (J) CD25+ Treg frequency in tumors 38 days after transplantation.

**Figure 5**

**CD70 is expressed on tumor-infiltrating CD4+ T cells**

(A) CD70-expression on immune cells in MC57 tumors. (B) Frequencies of CD70+ immune cells in tumor-bearing CD27−/− mice (n=3). (C-D) CD70 expression on CD4+ subsets in tumor-bearing CD27−/− mice (n=4). (E) CD27-expression on immune cells of tumor-bearing BL/6 mice. (F-G) Tumor-bearing BL/6 mice were treated once with FR70-antibody or rat-IgG on the day before analysis. (F) Representative histograms of CD27-expression on CD25+ Tregs and (G) MFIs of CD27-expression on CD4+ T cell subsets in spleens and TILs.
Figure 6

CD27-CD70-interaction in the tumor environment favors the survival of Tregs

(A-B) GFP^+ BL/6 or CD27^/- CD4^+ T cells were injected i.v. into irradiated recipients. Recovery of Tregs was identical in irradiated BL/6 and CD27^/- mice (data not shown). One day later, tumor fragments were transplanted. Twenty days later, the frequencies and total numbers of CD25^+ Tregs in the GFP^+CD4^+ T cell populations in the spleens were analyzed (n=2-5). (C) Frequencies of Helios^+ nTregs in tumor-bearing and naïve mice. (D) BrdU-incorporation in FoxP3^+ and FoxP3^- CD4^+ T cells in tumor-bearing and naïve mice. (E) DAPI staining of CD4^+CD25^hi TILs. (F) Annexin-V staining of CD4^+CD25^hi T cells in tumor-bearing and naïve mice.

Figure 7

CD27-signaling improves survival but not function of Tregs

(A-F) 7x10^5 MACS-purified CD4^+ T cells from spleens of naïve mice were cultured in 48-well tissue culture plates coated with 10µg/ml anti-CD3ε antibody in the presence of 5ng/ml rh-TGF-β and 5x10^4 irradiated (120 Gy) MC57 or MC57-CD70 tumor cells. (A) Frequencies of Tregs after two days of culture. (B) Frequencies, total numbers and (C) CD27-expression of CD25^+ Tregs, Helios^+ nTregs and Helios^- iTregs. (D) Division steps of CFSE-labeled CD4^+ cells (E) IL-2-concentrations in cell supernatants. (F) Intracellular IL-2 staining. (G) IL-2 concentrations in cell supernatants of 3x10^5 FACS-purified CD4^+CD25^hi T cells pooled from spleens of naïve mouse (n=15) and cultured in quadruplicates as described in (A-F). (H) Frequencies of Tregs in CD4^+ T cells cultured as described in (A-F) in the presence of 5U/ml rm-IL-2. (I) 7x10^4 FACS-purified CD4^+CD25^hi T cells from spleens of naïve mice were labeled with CFSE and cultured in quadruplicates as described in (A-F) in the presence of 5U/ml rm-IL-2. After two days, the frequencies and numbers of CD25^+ Tregs in each division step were assessed.
Figure 1

Tumor fragments (1 mm³)

A. MC57, 10/10
   3/14

B. MC38, 8/8
   6/6

C. B16F10, 6/6

D. MC57-GP

E. MC38-GP, 5/6
   8/8

F. B16F10-GP, 7/8
   6/8

G. MC57 (2 x 10⁶ cells)
   0/6
   0/6

H. 3-methylcholanthrene (200 μg s.c.)
   BL/6 (n=10)
   CD27⁻/⁻ (n=10)
   ****

Single-cell suspension

3-methylcholanthrene (200 μg s.c.)

Figure 2

(A) Images showing immunohistochemical staining for various markers: Ki67, anti-cleaved lamin A, anti-CD31, and anti-CD3. The images are labeled as BL/6 and CD27−/−.

(B) Bar graph showing Ki67+ cell percentages for BL/6 and CD27−/−, with a significant difference indicated by an asterisk (*).

(C) Bar graph showing cleaved lamin A cell percentages for BL/6 and CD27−/−, with no significant difference (n.s.).

(D) Bar graph showing CD31+ cell counts per mm² for BL/6 and CD27−/−, with a significant difference indicated by an asterisk (*).

(E) Bar graph showing CD3+ cell percentages for BL/6 and CD27−/−, with a highly significant difference indicated by three asterisks (***).

(F) Graph showing tumor size over time for NK-depleted MC57 and CD27−/−, with a highly significant difference indicated by four asterisks (****).

(G) Graph showing tumor size over time for RMA-S and BL/6 and CD27−/−, with no significant difference (8/8).
Figure 3
Figure 4

A

B

C

D

E

F

G

H

I

J
CD27-SIGNALING INCREASES THE FREQUENCY OF REGULATORY T CELLS AND PROMOTES TUMOR GROWTH

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