Anti-CD137 Suppresses Tumor Growth by Blocking Reverse Signaling by CD137 Ligand

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Abstract

CD137 (4-1BB) is a T-cell costimulatory molecule, and agonistic CD137 antibodies are currently being evaluated in the clinic as cancer immunotherapy. Recently, it was found that CD137+ mice or mice injected with agonistic anti-CD137 antibodies exhibit heightened antitumor responses, contrary to expectations based on other knowledge of CD137 function. Here, we report findings related to reverse signaling by CD137 ligand (CD137L) in antigen-presenting dendritic cells (DC) in tumors that address these paradoxical results. Specifically, CD137L suppressed intratumoral differentiation of IL12-producing CD103+ DC and type 1 tumor-associated macrophages (TAM). Differentiation of these cell types is important because they are required to generate IFNγ-producing CD8+ cytotoxic T lymphocytes (Tc1). Notably, CD137L blockade increased levels of IL12 and IFNγ, which promoted intratumoral differentiation of IFNγ-producing Tc1, IL12-producing CD103+ DC, and type 1 TAM within tumors. Our results offer an explanation for the paradoxical effects of CD137 blockade, based on differential immunomodulatory effects of CD137 signaling and reverse signaling in T cells and DC, respectively. Further, they show how CD137L blockade can send a forward-feedback loop for activation of CD103+ DC/type 1 TAM and Tc1 that can create a self-perpetuating cycle of highly effective immunosurveillance. Cancer Res; 77(21): 5989–6000. © 2017 AACR.

Introduction

The generation of effective T-cell responses to tumor antigens involves the operation of the cancer-immunity cycle, consisting of release of cancer cell antigens, cancer antigen capture, and processing by antigen-presenting cells (APC). APC migration to and T priming in the draining lymph node, T-cell trafficking to tumors, and cancer cell killing (1). However, tumors create a tumor microenvironment that suppresses one or more steps in the cancer-immunity cycle (1–3). To establish an immunosuppressive environment, the tumor stroma is progressively occupied by myeloid cells that can incubate immunosuppressive networking in cooperation with tumor cells (4). Consequently, tumor cells and their associated stromal cells upregulate pairs of T-cell inhibitory receptors (immune checkpoint receptors) and ligands and secrete a number of soluble factors that limit T-cell effector functions (2). Recent studies have demonstrated that tumors infiltrated with T cells (so-called T-cell–inflamed tumor microenvironments) are more responsive to cancer immunotherapy (3). Recruitment of CD103+ dendritic cells (DC) within tumors is a rate-limiting step for effective cancer immunotherapy (5–10), as these DCs are a major subset that cross-presents tumor antigens to cytotoxic CD8+ T lymphocytes (CTL). IL12 produced by CD103+ DCs are critical for the successful generation of CTLs, whereas type 1 interferon (IFN) seems to increase CTL activities against tumor cells through accumulation of CD103+ DCs (6, 10–12). However, it remains unclear how CD103+ DCs are activated within tumors. CTLs producing IFNγ-centered cytokine patterns (called Tc1s) may be responsible for tumor immunosurveillance, because IFNγ can promote CD103+ DC differentiation (13, 14).

CD137 (also known as 4-1BB and TNFRSF9) is a potent costimulator of CD8+ T cells (15), and agonistic anti-CD137 monoclonal antibody (mAb) has been shown to be highly effective in eradicating established tumors via activation of NK and CD8+ T cells (16). Not surprisingly, BATF3-dependent CD103+ DCs are crucial for the efficacy of anti-CD137 cancer therapy (9), suggesting that the baseline cross-priming function of CD103+ DCs is required for the responsiveness of CTLs to anti-CD137 cancer immunotherapy. The therapeutic successes against various types of cancer in preclinical animal models have led to clinical trials with humanized mAbs directed against CD137 (17).

The puzzling observation that CD137+ mice exhibit enhanced antitumor activity (18) indicates that the mere stimulation of NK and CD8+ T cells by anti-CD137 mAb treatment cannot fully explain its antitumor effect. Recently, accumulating evidence has demonstrated that CD137 ligand (CD137L) reverse signaling plays a critical role in the regulation of myelopoiesis, macrophage differentiation, and tissue inflammation (19–21). As

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both CD137<sup>-/-</sup> and anti-CD137-treated mice lack CD137 signaling, we hypothesized that blocking of the CD137 signaling pathway contributes to poor tumor growth. Indeed, our results in this study demonstrate that CD137L signaling inhibits the differentiation of CD103<sup>+</sup> DCs and classically activated macrophages (M1 macrophages) within tumors, suggesting that CD137L signaling functions as a negative regulator for antitumor immunity. Accordingly, blockade of the CD137L signaling pathway may be used to promote therapy-induced immunosurveillance through mutual activation of IL12-producing CD103<sup>+</sup> DCs/M1 macrophages and tumor cell killing. IFNγ-producing CTLs.

**Materials and Methods**

**Mice and tumor model**

Balb/c mice were purchased from Orientbio. CD137<sup>-/-</sup> Balb/c mice were bred at the animal facility of the University of Ulsan. All animal experiments were performed in accordance with the approved protocols and guidelines of the Animal Care and Use Committee. Mice were injected subcutaneously with 2 × 10<sup>5</sup> CT26 cells on the flank. Tumor-challenged mice were injected intraperitoneally with 200 μg rat IgG or anti-CD137 mAb (clone 17A2) on day 5 or 6 after tumor cell inoculation. 3E1 antibody can stimulate CD137 (15) but block CD137L (21). For depletion of CD8<sup>+</sup> T cells, 500 μg anti-CD8 mAb (clone 2.43) was injection on days 0, 5, and 10. For neutralization of IL12 or IFNγ, R4-6A.2 mAbs were purified from eBioscience: anti-CD3 (17A2), FITC-conjugated anti-CD4 (GK1.5), APC-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD45, PerCPCy5-conjugated anti-CD45, anti-CD25 (PC61.5), PE-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD8 (53-6.7), APC-conjugated anti-CD8, APCCy7-conjugated anti-CD8 and anti-CD107a antibodies.

**Reagents and antibodies**

Anti-CD137 (3E1), anti-CD8 (2.43), anti-CD137L (TKS-1), 2.4G2, and anti-IFNγ (R4-6A.2) mAbs were purified from ascites. Functional grade anti-CD3 (17A2) was purchased from eBioscience, anti-IL12p75 mAb from BioXcell, anti-Asialo GM1 from Wako and rat IgG and human IgG1 from Sigma Aldrich. The following conjugated antibodies were purchased from eBioscience: anti-CD3 (17A2), FITC-conjugated anti-CD4 (GK1.5), APC-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD8 (53-6.7), APC-conjugated anti-CD8, APC-PE-conjugated anti-CD11b (M1/70), PE-Cy7-conjugated anti-CD11c (N418), PE-conjugated anti-CD11c (N418), PE-conjugated anti-CD25 (PG61.5), PE-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD45 (30-F11), PerCP-Cy5.5-conjugated anti-CD45, PerCP-Cy5-conjugated anti-CD62L (MEL-14), APC-conjugated anti-CD103 (M290), PE-conjugated anti-CD107a (eBio1D4B), PE-conjugated anti-CD137L (17B5), PE-conjugated anti-CD137L (TKS-1), FITC-conjugated anti-F4/80 (BM8), APC-conjugated anti-F4/80 (BM8), FITC-conjugated anti-Foxp3 (FJK-16S), PE-conjugated anti-IL12/IL23 (C17.8), PE-conjugated anti-IFNγ (XM12.1), PE-conjugated anti-Flt3 (A2F10), PerCP-Cy5.5-conjugated anti-ly-6C (HK1.4), PE-Cy7-Conjugate Siglet H (eBio404c), FITC-conjugated anti-ly-6G (1A8), PE-conjugated anti-MHCII (M5/114.15.2), and APC-PE-conjugated anti-MHCII. The following conjugated antibodies were purchased from BD Pharmingen: FITC-conjugated Sirp-α (P84), PerCP-Cy5.5-conjugated anti-Gr-1(R6/8C5). The following conjugated antibodies were purchased from Biolegend: PE-conjugated anti-CD206 (C068C2). Recombinant fusion CD137-Fc protein was made in the laboratory (21). Flt3 ligand (Flt3L), GM-CSF, and IFNγ were purchased from PeproTech, Cpg ODN from Invivogen, and gp70 423-431 peptide was synthesized by Peptron. Streptavidin microbeads, anti-CD4 microbeads, and MDSC isolation kit were purchased from Miltenyi Biotec.

**Flow cytometry**

Cells were incubated with the Fc blocker 2.4G2 (10 μg/mL) at 4°C for 10 minutes, stained with specific antibodies against surface markers in FACS buffer (PBS containing 0.2% BSA) at 4°C for 15 minutes, and washed with FACS buffer. Data acquisition was performed using Canto II (BD Biosciences) and analyzed using BD FACSDiva (BD Biosciences) and FlowJo (Tree Star).

**Analysis of tumor-infiltrating leukocytes**

For analysis of tumor-infiltrating leukocytes, tumors were dissected and digested with 2 mg/mL collagenase IV and 30 mg/mL DNase I (Sigma) at 37°C for 45 minutes. After RBC lysis, dead cells were removed by centrifugation in Lympholyte M solution (Cedarlane) at 2,000 rpm at room temperature for 30 minutes. For IL12p40 intracellular staining, cells were cultured in 48-well plates containing complete RPMI 1640 medium supplemented with 10 μg/mL Brefeldin A for 4 hours. Cells were harvested, stained with PE-Cy7-conjugated anti-CD11c, FITC-conjugated anti-F4/80, and PerCP-conjugated anti-CD45 antibodies, and fixed in 4% parafomaldehyde for 15 minutes. Cells were then washed and stained with PE-conjugated anti-IL12p40 in 1 × BD Perm/Wash buffer for 30 minutes. For intracellular IFNγ staining, cells were stimulated with 50 ng/mL PMA (Sigma) and 100 μg/mL ionomycin (Sigma) in the presence of 10 mg/mL Brefeldin A for 4 hours. Cells were stained with FITC-conjugated anti-CD3, PerCP-conjugated anti-CD8, and anti-IFNγ antibodies, as described above. CD107α mobilization assay was performed using a 1:2 effector:target ratio. Purified CD8<sup>+</sup> T cells from tumors or the spleen were incubated with gp70<sub>23,431</sub> (SPSYHVHQF)-pulsed CT26 cells at 37°C for 5 hours. Cells were stained with anti-CD8 and anti-CD107α antibodies.

**Differentiation of DCs and CD8<sup>+</sup> T-cell activation**

Pre-DCs were enriched from bone marrow cells by negative selection of lineage-positive cells using anti-CD3, CD11b, and B220 biotin-conjugated Abs and streptavidin microbeads (Miltenyi Biotec). Pre-CD103<sup>+</sup> DCs [Lin<sup>-</sup>CD3<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>-</sup>Flt3<sup>-</sup>Sirp-α<sup>-</sup>Siglet H<sup>+</sup>Ly6C<sup>+</sup>] and pre-CD11b<sup>+</sup> DCs [Lin<sup>-</sup>CD3<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup>MHCII<sup>-</sup>Flt3<sup>-</sup>Sirp-α<sup>-</sup>Siglet H<sup>+</sup>Ly6C<sup>+</sup>] then were sorted from the enriched bone marrow cells using FACSaria (BD Biosciences) and cultured in Flt3 ligand (Flt3L: 160 ng/mL)-containing complete IMDM medium in the absence or presence of CD137-Fc for 3 to 6 days. Differentiated DCs were analyzed by staining with anti-CD11c, anti-MHCII, anti-F4/80, anti-CD103, and anti-CD11b. To obtain bone marrow–derived DCs (BMDCs), bone marrow cells were harvested from the femur and tibia. After RBC lysis, cells (2 × 10<sup>6</sup> cells/mL) were cultured in Flt3L-containing complete IMDM medium for 9 days. DCs were induced to polarize toward CD103<sup>+</sup> subsets in the presence of IFNγ (0.1 ng/mL) and GM-CSF (10 ng/mL) for additional 2 days. CD103<sup>+</sup> DCs then were isolated using anti-CD103–biotin and streptavidin microbeads. DCs (1 × 10<sup>6</sup> cells/mL) were stimulated with 5 μg/mL CpG alone or CpG plus 10 μg/mL CD137-Fc for 6 hours. Some of the activated DCs...
were used for intracellular staining for IL12p40 and the remaining DCs were cocultured with purified CD8+ T cells in the presence of gp70 423–431 peptide plus/minus anti-CD3 mAb (1 μg/mL) at DC:T cell = 1:5 for 3 days. CD8+ T-cell activation were identified by staining with anti-CD8, anti-CD62L, and anti-CD44 antibodies.

**Suppression assay of Tregs**

CD25+CD4+ Tregs were sorted from WT or CD137−/− spleenocytes using FACSAria. Sorted Tregs were cocultured with irradiated CT26 tumor cells at Treg:CT26 = 2:1 for 48 hours. Tregs were repurified with anti-CD4 microbeads. Naive WT splenic T cells were cocultured with tumor cell–primed Tregs at various ratios of responder-to-Treg in the presence of anti-CD3 mAb (1 μg/mL) for 3 days. 3H thymidine (1 μCi; Amersham) was added for the last 3 hours of culture. The proliferation of T cells was calculated by 3H thymidine incorporation.

**Isolation of myeloid-derived suppressor cells and differentiation of macrophages**

For splenic myeloid-derived suppressor cell (MDSC) isolation, single-cell suspensions were prepared from the spleens of tumor-bearing mice, and red blood cells were lysed using red blood cell (RBC) lysis buffer (Sigma). Splenic MDSCs were isolated using an MDSC isolation kit (Miltenyi Biotech) according to the supplier's protocol. To differentiate into macrophages, MDSCs were cultured in complete RPMI 1640 medium containing GM-CSF (10 ng/mL) in plates coated with CD137-Fc or control human IgG1 (10 μg/mL) for 3 to 5 days.

**Cytokine measurement**

For quantification of intratumoral levels of cytokines, weight-to-volume-normalized tumor lysates were analyzed using the Cytokine Bead Array (BD Biosciences) according to the manufacturer's recommendations.

**qRT-PCR**

Total RNA was extracted from cells using TRizol (Invitrogen), and reverse transcription was performed using a GoScript Reverse Transcription System (Promega). Real-time quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The expression levels of mRNAs were normalized to that of β-actin as an internal control. Specific primers were as follows: iNOS, 5′-CACCTCCTGTACAGGTGATGCG-3′; CCL5, 5′-CCCTGACCATCATTCCATGG-3′; Cox-2, 5′-AGAACAGACTTCTGCGTGG-3′; Cytokine Bead Array (BD Biosciences) according to the manufacturer's recommendations.

**Statistical analysis**

GraphPad Prism 5 (GraphPad Software) was used to analyze and present data. Differences between groups were analyzed using a two-tailed t-test or one- or two-way ANOVA with post hoc analysis, as appropriate. Values are expressed as the means ± SEM. P < 0.05 was considered statistically significant.

**Results**

**Induction of systemic antitumor immunity in CD137−/− and anti-CD137-injected mice**

Consistent with previous data (18), tumor growth was severely impaired in CD137−/− mice subcutaneously challenged with CT26 colorectal carcinoma cells (Supplementary Fig. S1A). To address the mechanism responsible for the suppression of tumor growth in CD137−/− mice, we first analyzed the systemic antitumor immune response in CT26 tumor-bearing mice at days 14 to 16 after inoculation with tumor cells, when tumor growth progressed vigorously in WT versus CD137−/− mice (Supplementary Fig. S1A). Tumor-bearing CD137−/− mice had a higher percentage of activated CD4+ and CD8+ T cells (CD62LhiCD44hi) in the spleen (Supplementary Fig. S1B). Consistently, splenic CD8+ T cells of CD137−/− mice exhibited significantly increased CTL activities, as determined by the cell surface mobilization of CD107a after tumor cell challenge evidenced in evident suppression of tumor growth (Supplementary Fig. S1D) and marked expansion of CD8+ T cells with an activated phenotype in the spleen (Supplementary Fig. S1E). CTL activity against CT26 tumor cells was also markedly increased (Supplementary Fig. S1F). Importantly, tumor growth was completely restored in both CD137−/− and anti-CD137-injected mice when CD8+ T cells were depleted (Supplementary Fig. S1G). These results indicate that the elevated antitumor immunities of CD137−/− and anti-CD137-injected mice both involve increased CTL activity. We confirmed that CD137−/− and anti-CD137-injected mice commonly showed poor tumor growth in two other subcutaneously challenged tumor models (Supplementary Fig. S2A–S2C).

**IL12 is required for the intratumoral accumulation of Tc1s in CD137−/− and anti-CD137-injected mice**

Systemic activation of tumor-specific CTLs indicated that there might be active APCs capturing tumor antigens within the tumors of CD137−/− mice. CD103+ DCs not only transfer tumor antigens to draining lymph nodes for T-cell priming (22) but also activate intratumoral CD8+ T cells (7, 8). Indeed, we observed a higher percentage of CD45F4/80−MHCII−CD11c−CD103+ DCs and IL12p40-producing DCs within the tumors of CD137−/− mice compared with that in WT mice on days 9 and 10 after tumor cell inoculation (Fig. 1A and B). A marked decrease in tumor-associated macrophages (TAM; CD45+CD11b+F4/80+) expressing CD206, a marker of M2 macrophages, was also prominent within tumors of CD137−/− mice (Fig. 1C). Gene expression analysis showed that TAMs of CD137−/− mice were M1-like macrophages: they had a lower expression of Arg1, CD206, and IL10 transcripts compared with that in WT TAMs, while their expression of iNOS and IL12p35 mRNAs showed the opposite pattern (Fig. 1D). We noticed that increased percentages of CD103+ DCs were detected in 9- or 10-day tumors but not in 14-day tumors of CD137−/− mice, while the dominance of M1-like TAMs was sustained (data not shown). Administration of
Figure 1.
Increases in intratumoral CD103⁺ DCs and M1-like TAMs in CD137⁻/⁻ and anti-CD137-injected mice. Tumors were analyzed on day 9, 10, or 16. A and E, FACS dot plots for intratumoral CD103⁺ DCs and CD11b⁺ DCs isolated on day 9 or 10 (left), and the ratio of CD103⁺ DCs to CD11b⁺ DCs (right). Tumors were pooled from 4 mice/group. CD103⁺ DCs and CD11b⁺ DCs were gated from CD45⁺ F4/80⁻ CD11c⁺ cells. B and F, FACS dot plots for intracellular staining of IL12p40 in CD45⁺ F4/80⁻ CD11c⁺ DCs on day 9 or 10. Data are representative of two independent experiments (n = 4/group). C and G, FACS analysis of TAMs isolated on day 16. Left, CD206⁺/macrophages were gated from CD45⁺ Gr-1⁻ F4/80⁺ cells. Right, the ratio of CD206⁻ TAMs to CD206⁺ TAMs. ***, P < 0.001; **, P < 0.01; *, P < 0.05. D and H, qRT-PCR was performed using total RNA extracted from purified F4/80⁺ TAMs (n = 4/group) on day 16. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are representative of two independent experiments.
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anti-CD137 mAb induced almost the same pattern of changes in the intratumoral composition of CD103+ DCs and M1-like TAMs that was seen in CD137−/− mice (Fig. 1E–H).

We predicted that the tumor microenvironment of CD137−/− and anti-CD137-injected mice would be favorable for the generation of abundant Tc1s within tumors, one of the cardinal features of natural or therapy-induced immunosurveillance (13). Indeed, tumors of CD137−/− and anti-CD137-injected mice contained higher percentages of IFNγ-producing CD8+ T cells (Fig. 2A and B), exhibiting a more activated phenotype, compared with those in WT mice (Fig. 2C and D). As expected, intratumoral levels of soluble IFNγ were higher in CD137−/− and anti-CD137-injected mice (Fig. 2E and F). Like splenic CD8+ T cells (Supplementary Fig. S1C and F), those isolated from tumors of CD137−/− and anti-CD137-injected mice showed increased CTL activities against tumor cells (Fig. 2G and H). Taken together, our results indicate that CD137 deficiency or administration of anti-CD137 antibody results in increased intratumoral production of IL12 and IFNγ mainly by CD103+ DCs/M1-like TAMs and Tc1 cells, respectively.

CD103+ DCs efficiently cross-present antigens to CD8+ T cells, and IL12 is required for effective type 1 immune responses (23). In addition, IL12 is an indispensable, positive regulator of therapy-induced immunosurveillance (10) and IL12 synergistically enhances the antitumor activity of CD137 mAb (24, 25). As IL12 was expressed by DCs and TAMs within tumors of CD137−/− and anti-CD137-injected mice, we predicted that IL12 drives the active generation of intratumoral Tc1s in these mice. In vivo neutralization of IL12 markedly reduced intratumoral Tc1s in CD137−/− and anti-CD137-injected mice (Fig. 3A and B) and subsequently restored tumor growth to levels observed in WT mice (Fig. 3C and D). Intriguingly, CD137−/− and anti-CD137-injected mice also recovered percentages of intratumoral CD103+ DCs and M1-like TAMs after IL12 neutralization (Fig. 3E–H).

IFNγ is required for the generation of intratumoral CD103+ DCs and M1-like TAMs in CD137−/− and anti-CD137-injected mice

The data presented in Fig. 3 suggest that IL12 is involved in the differentiation of intratumoral CD103+ DCs and M1-like TAMs in CD137−/− and anti-CD137-injected mice. Considering the role of IL12 in Tc1 generation, we speculated that IFNγ acts on this differentiation process downstream of IL12. Neutralization of IFNγ in CD137−/− and anti-CD137-injected mice induced almost the same changes in the antitumor response as were observed with the neutralization of IL12: marked reduction in intratumoral Tc1s, CD103+ DCs, and M1-like TAMs (Fig. 4A–F) and suppression of tumor growth (Fig. 4G and H). These results suggest that increased intratumoral levels of IL12 and IFNγ induce secretion of each other within tumors of CD137−/− and anti-CD137-injected mice by mutually promoting differentiation of CD103+ DCs/M1-like TAMs and Tc1s, respectively.

We wanted to confirm that CD137−/− and anti-CD137-injected mice showed what we had observed in the CT26 tumor model, in response to another type of tumor cell. Like CT26 tumors, the ratio of CD103+ DCs to CD11b+ DCs was greater in Renca renal carcinomas of CD137−/− and anti-CD137-injected mice compared with those of WT mice (Supplementary Fig. S3A), even though increase in intratumoral M1-like macrophages was not evident in anti-CD137-injected mice (Supplementary Fig. S3B). We also observed a significant reduction in tumor-infiltrating CD8+ T cells in anti-CD137-injected mice (Supplementary Fig. S3C).

Figure 2.
Increase in intratumoral Tc1 cells in CD137−/− and anti-CD137-injected mice. Tumors were analyzed on day 14 or 16. A and B, Intratumoral leukocytes were isolated, stimulated in vitro with PMA and ionomycin, and stained for intracellular IFNγ. FACS dot plots for intracellular IFNγ staining of CD3+CD8+ T cells (left) and percentages of CD62L+ cells in CD8+ T cells (right; n = 4/group). **, P < 0.01; C and D, FACS dot plots for activated intratumoral CD62L+CD8+ T cells (left) and percentages of CD103+ cells in CD8+ T cells (right; n = 4/group). **, P < 0.01. E and F, Levels of IFNγ in tumor lysates (n = 4/group). *, P < 0.05; **, P < 0.001. G and H, CTL assays. Purified intratumoral CD8+ T cells were incubated with gp70 peptide–pulsed CT26 cells for 5 hours, and CD107a-expressing CD8+ T cells were counted using FACS. *, P < 0.05; **, P < 0.001 between the two groups.
Figure 3. IL12 is responsible for changes in the tumor microenvironment induced by CD137 gene deletion or anti-CD137 antibody. Mice were injected with control IgG or anti-IL12 antibody on days 0, 5, and 10. Tumors were analyzed on day 9, 10, or 16. A and B, Percentages of IFNγ+ cells among CD3+CD8+ T cells on day 16. Tumors were pooled from 4 mice (WT and control IgG-treated CD137-/- mice) or individually analyzed for anti-IL12-injected CD137-/- mice (n = 4). Data are representative of two experiments. *P < 0.05, **P < 0.01, ***P < 0.001. C and D, Tumor growth curves (n = 4–6/group). *P < 0.05, **P < 0.01. E and F, The ratio of intratumoral CD103+ DCs to CD11b+ TAMs on day 9 or 10. Data were pooled from two experiments (n = 4/group/experiment). *, P < 0.05; **, P < 0.01. G and H, The ratio of CD206+ TAMs to CD206− TAMs on day 16. **, P < 0.01; ***, P < 0.001 (n = 4/group). Data are representative of two independent experiments.

Fig S3B). Nonetheless, these mice had a higher portion of IFNγ-producing Tc1 cells within tumors (Supplementary Fig. S3C). Thus, the CD103+Tc1 axis seems to play a more important role in suppressing Renca tumor growth in CD137−/− and anti-CD137-injected mice than the M1-like TAM–Tc1 axis does.

CD137 expression on Treg cells is required for M2 macrophage differentiation in tumors

As CD137L signaling is blocked in both CD137−/− and anti-CD137-injected mice, we hypothesized that changes in the tumor microenvironment were due to the absence of CD137L signaling. CD137-expressing cells were found among CD11b+ non-myeloid cells within tumors (Fig. 5A), with CD4+Foxp3+ Tregs expressing the highest levels of CD137 among T cells (Fig. 5B). By contrast, CD137L expression was observed mainly in CD11b+Gr-1int/null myeloid cells (Fig. 5C). To examine whether CD137 expression on Tregs is required for the characteristic phenotypic changes seen in tumors of CD137−/− mice, we adoptively transferred WT Tregs into CD137−/− mice before inoculation of tumor cells. Adoptive transfer of WT Tregs restored not only the accumulation of intratumoral F4/80+CD206+ M2-like TAMs but also tumor growth in CD137−/− mice, both of which were comparable with that seen in WT mice (Fig. 5D and E). As in this experimental system, it was possible that the absence of CD137L signaling in Tregs was associated with inhibition of tumor growth, probably resulting from loss of their immunosuppressive activity, we investigated this question. As seen in Fig. 5F, tumor cell-primed Tregs of WT and CD137−/− mice were comparably potent in suppressing proliferation of T cells. These results suggest that the function of CD137 on Tregs may be mainly to engage CD137L on myeloid cells, stimulating their differentiation toward M2-like TAMs within tumors.

CD137L signaling inhibits differentiation of CD103+ DCs and M1-like macrophages

To test whether CD137L signaling inhibits differentiation of CD103+ DCs, we cultured bone marrow cells of WT and CD137−/− mice in the presence of Flt3L plus control human IgG, or CD137L-Fc for 9 days, and then IFNγ and GM-CSF were added to induce polarization of CD103+ subsets for additional 2 days (14, 23). As shown previously (19), CD137−/− bone marrow cells were more active during DC differentiation (Supplementary Fig. S4), and CD137−/− BMDCs were also more efficiently polarized toward CD103+ subsets (Supplementary Fig. S4). By contrast, recombinant CD137L-Fc protein markedly inhibited BMDC differentiation from bone marrow cells of WT and CD137−/− mice, and the BMDCs lost their ability to polarize to CD103+ subsets (Supplementary Fig. S4). Instead, CD137-Fc preferentially skewed differentiation toward CD11c+CD11b+ F4/80+ macrophages (Supplementary Fig. S4). Therefore, our observation indicates that CD137L signaling tends to block differentiation of CD103+ DCs.
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We identified an existence of CD11c⁺CD103⁻CD11b⁻ DC progenitors within tumors (26), which expressed detectable levels of CD137L and CD137 (Fig. 6A). While intratumoral CD103⁺ DCs expressed distinguishable levels of CD137 with undetectable levels of CD137L expression, CD11b⁺ DCs showed the opposite pattern of expression (Fig. 6A), indicating that CD137L signaling may be involved in the DC progenitor–CD11b⁺ DC differentiation axis. To test this hypothesis, we first examined the role of CD11c⁺CD103⁻CD11b⁻ DCs. CD137-Fc markedly drove the differentiation of either isolated pre-CD103⁺ or pre-CD11b⁺ DCs (26) toward CD11b⁺ DCs (Fig. 6B), confirming again that CD137L signaling is a differentiation factor for CD11b⁺ DCs. We next assessed the immunosuppressive effect of CD137L signaling on CD103⁺ DCs. CpG strongly stimulated in vitro differentiated CD103⁺ DCs to produce IL12p40 (Fig. 6C) but CD137-Fc treatment barely affected CpG-induced IL12 production (Fig. 6C). However, CD137-Fc-priming of CD103⁺ DCs mildly (approximately 10%) suppressed tumor antigen-specific activation of CD8⁺ T cells induced by CpG-activated CD103⁺ DCs (Fig. 6D). Altogether, our results suggest that although repression of CD103⁺ DC differentiation seems to be a major mechanism of tumor growth mediated by CD137L signaling, a possibility cannot be excluded that CD137L signaling can directly suppress the activity of CD103⁺ DCs.

Macrophages are differentiated from monocytic MDSCs in tumors (27–29). We isolated MDSCs from the spleens of tumor-bearing mice and cultured them in the presence of GM-CSF plus control IgG or CD137-Fc. The purified splenic MDSCs were a mixture of Gr-1⁺CD11b⁻ granulocytic and monocytic myeloid cells but contained very few F4/80⁺ macrophages (Fig. 7A). Under these neutral culture conditions, stimulation of CD137L induced marked inhibition of neutrophil differentiation but promotion of CD206⁺ M2-like macrophage differentiation (Fig. 7B–D). Analysis of mRNA expression showed increasing levels of Arg1 and YM1 mRNA as differentiation progressed (Fig. 7E). Consistent with the in vitro data, tumors of CD137⁻⁻ and anti-CD137-injected mice contained higher percentages of neutrophils as well as a lower proportion of M2-like TAMs (Fig. 7F and G).

Discussion

Even though CD137 agonists are well known to activate CD8⁺ T cells during antigen presentation in the secondary lymphoid organ, it remains controversial whether or not their action is restricted to the priming and activation step of the cancer-immunity cycle. It is also not clear to what extent the blocking effect of CD137 agonists on CD137L signaling contributes to antitumor immune responses.
CD137 expression on Tregs is required for inhibition of intratumoral M1-like TAMs and tumor growth. **A-E**, Tumors were analyzed on day 14. **A**, FACS analysis of CD137 expression in intratumoral leukocytes. CD45<sup>+</sup> leukocytes were gated, and CD137 expression was analyzed in CD11b<sup>+</sup>Gr-1<sup>−</sup> myeloid cells and CD11b<sup>+</sup> cells. **B**, CD137 expression on T cells. CD137 expression was analyzed in CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup> T cells. **C**, CD137L expression on intratumoral leukocytes. **D** and **E**, CD4<sup>+</sup>CD25<sup>+</sup>Tregs of WT mice were isolated by FACS Aria and adoptively transferred to WT and CD137<sup>−/−</sup> mice 1 day before tumor cell inoculation. As a negative control, CD137<sup>−/−</sup> Tregs were also isolated and adoptively transferred to WT and CD137<sup>−/−</sup> mice. The ratio of CD206<sup>+</sup> TAMs to CD206<sup>−</sup> TAMs on day 16. **F**, Tumor growth curves (n = 4/group). **A–E**, P < 0.01. Data are representative of two independent experiments. **F**, Tumor cell–primed Tregs of WT and CD137<sup>−/−</sup> mice have no difference in suppressing the proliferation of splenic T cells. Tumor cell–primed Tregs were cocultured with naive WT T cells in the presence of anti-CD3 mAb.

immunity. In this study, we provided evidence supporting the idea that anti-CD137 agonists can function as inhibitors of CD137L signaling, resulting in the creation of tumor microenvironments unfavorable for tumor immune evasion. It is surprising that the blocking effect of CD137L signaling overrides that of CD137 signaling in inhibiting tumor growth. This is likely to be because CD137L delivers a negative signal for the generation of CD103<sup>+</sup> DCs and M1-like TAMs producing IL12, a key cytokine required for the differentiation of tumor-killing CD8<sup>+</sup> T cells within tumors. CD103<sup>+</sup> DCs are also one of the major subsets that migrate tumor antigens to draining lymph nodes to present them to and activate CD8<sup>+</sup> T cells, driving their differentiation toward effector cells through IL12 (22, 23).

Seemingly paradoxically, tumor escape from immune-mediated destruction also depends upon CD8<sup>+</sup> T cells, which play a role in the upregulation of IDO and PD-L1 and in the recruitment of Tregs in tumors (6). It is now becoming clear that chronic stimulation of type I and II IFNs induces adaptive resistance in tumors by increasing the expression of resistance genes, such as T cell inhibitory receptors and ligands and IFN-stimulated genes (JCS), in tumor cells and immune cells (30–34). PD-L1 and other inhibitory ligands on tumor cells and DCs/TAMs are particularly important for the development of exhausted T cells, necessitating blockade of PD-1/PD-L1 to reinvigorate exhausted T cells or allow for the development of effector T cells (31, 34, 35). However, there is an IFN-driven PD-L1–independent resistance mechanism, and thus the blocking of tumor IFN signaling effectively bypasses the need for combination checkpoint blockade (34). Interestingly, a recent study has demonstrated that PD-L1 signaling in tumor cells increases the expression of glycolytic enzymes and thus helps tumor cells to deprive T cells of glucose utilization, which mediates T-cell hyporesponsiveness within tumors (36). In this study, we also identified CD137L as an inhibitory receptor mediating the adaptive resistance of tumors. CD137L signaling integrates the immunosuppressive mechanisms of Tregs through CD137 engagement with CD137<sup>−/−</sup> on pre-DCs and monocytic MDSCs within tumors. The subsequent CD137L signaling negatively regulates the differentiation of CD103<sup>−/−</sup> DCs and M1-like TAMs.

It is noteworthy that the early tumor microenvironment is replete with large amounts of proinflammatory mediators, including differentiation factors for CD103<sup>+</sup> DCs and M1 macrophages, such as GM-CSF and IFNγ, which are co-opted for immunosuppression by tumors. Therefore, CD137L signaling is likely to be critical in switching the tumor microenvironment from immunostimulatory to immunosuppressive. This interpretation for the function of CD137L signaling seems to be generally applicable to chronic inflammatory situations (37). For example, CD137<sup>−/−</sup> mice are impaired in resolving colitis after withdrawal of the damage inducer (38). We also routinely observe that induction of tissue injury results in dysregulated chronic inflammation in
Various organs of CD137<sup>−/−</sup> or CD137L<sup>−/−</sup> mice (our unpublished data).

Although Tregs mediate immunosuppressive mechanisms through cell contact–dependent pathways and secretion of soluble factors (e.g., IL10 and TGF<sub>B</sub>), it is not clear how the tumor microenvironment supports their excessive immunosuppressive activity. It is possible that Tregs mediate the adaptive resistance of tumors by engaging cell surface proteins on DCS and macrophages. For example, B7 and GITR ligand (GITRL) reverse signaling is shown to induce IDO expression in DCS, resulting in tryptophan catabolism (39, 40). In these cases, action by reverse signaling is mediated through type I or II IFN depending upon DC subsets (39, 40). Considering that Tregs express numerous costimulatory/coinhibitory receptors and that DCS and macrophages express their ligands, it will be intriguing to investigate whether reverse signaling through ligands for costimulatory/coinhibitory receptors in DCS and TAMs contributes to adaptive resistance of tumors. If this is demonstrated to be true, it does not preclude the possibility that the potent antitumor effect of immune checkpoint blockade is caused at least partially by a ligand blockade effect.

Traditionally, the therapeutic benefit of anti-CD137 antibody is thought to be achieved by directly increasing T-cell activation. Unexpectedly, our study demonstrated that anti-CD137 antibody can indirectly induce T-cell activation. As strong T-cell activation is unavoidably followed by increased T-cell inhibition, the interruption of T-cell–inhibitory mechanisms is likely to bring about greater therapeutic success (37). Our results also predict that CD137L blockade combined with various other cancer therapies may provide a greater clinical benefit. The use of CD137L blockade to promote intratumoral differentiation of CD103<sup>+</sup> DCS and M1-like macrophages may provide benefits in combination with other cancer therapies including immune checkpoint blockade, DC therapy, T cell therapy, and immunogenic chemo- and radiotherapy (reviewed in refs. 41 and 42). As anti-CD137 agonists exhibit liver toxicities, we propose that anti-CD137 or anti-CD137L antagonists should be considered as CD137L blockers for cancer therapy. Another potential advantage of CD137L blockers is that they protect the kidney from drug-induced injury (21, 43, 44).

In our hands, CD137L<sup>−/−</sup> or neutralizing anti-CD137L (TKS1)-injected mice were shown to be less potent in antitumor immunity than CD137<sup>−/−</sup> or agonistic anti-CD137-injected mice (Supplementary Fig. SSA and SSB). This observation may be explained if assuming the existence of another form of CD137L, which has a similar function with the originally identified one. Nonetheless, the data presented in this study showed that signaling via CD137L was responsible for differentiation of DCS and macrophages, which were co-opted to support tumor growth. In this respect, a soluble form of CD137L in tumor-bearing mice may function as a CD137L agonist that can contribute to tumor growth (45), whereas a cleaved form of CD137L, which is released into the sera of cancer patients (46), may actively increase antitumor immunity through either T cell costimulation or blockade of CD137L signaling, as seen in agonistic anti-CD137 mAb.

CD137L stimulation enhances killing of tumor cells through antibody-dependent cell-mediated cytotoxicity (ADCC) by target antibodies in which NK cells and macrophages play a pivotal role (47–49). In addition, the synergistic effect of anti-CD137 mAb and IL12 is mediated by NK cells and/or CD8<sup>+</sup> T cells (25, 50).

In our CT26 tumor model, however, depletion of NK cells did not affect tumor growth, intratumoral macrophage differentiation, and generation of Tc1 cells within tumors (Supplementary
Fig. S6A–S6C), suggesting that NK cell–mediated ADCC might not play a major role in anti–CD137-injected and CD137−/− mice. In addition, considering basal levels of CD137 expression on macrophages, they were not likely to be involved in ADCC (Supplementary Fig. S7A). Also, there was a low possibility that anti-CD137 mAb directly mediated ADCC against tumor cells, as CT26 tumor cells did not express CD137 (Supplementary Fig. S7B). Finally, NK cells in CD137−/− mice play a greater role in killing tumor cells than anti–CD137-injected mice, because CD137−/− mice have developmental advantages for NK cells (18). In our CT26 tumor model, depletion of NK cells did not affect tumor growth in CD137−/− mice, indicating that CD8+ T cells participated in killing tumor cells more actively than NK cells.

In conclusion, the novel mechanism underlying anti-CD137 cancer therapy may provide a good basis for therapeutic combination of anti-CD137 antibody with other cancer therapies. In particular, priority should be given to activators of CD103+ DCs, such as type I/II IFN, and targets of T-cell inhibition, such as CTLA-4, IDO, PD-1, and IL10. As specific subsets of DCs or macrophages responsible for immunosuppression mediated by CD137L reverse signaling were not defined in this study, relating CD137L signatures to the immunosuppressive pathway of those subsets would be an important future direction.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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