Induced PD-L1 Expression Mediates Acquired Resistance to Agonistic Anti-CD40 Treatment

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Abstract

CD40 stimulation on antigen-presenting cells (APC) allows direct activation of CD8\textsuperscript{+} cytotoxic T cells, independent of CD4\textsuperscript{+} T-cell help. Agonistic anti-CD40 antibodies have been demonstrated to induce beneficial antitumor T-cell responses in mouse models of cancer and early clinical trials. We report here that anti-CD40 treatment induces programmed death ligand-1 (PD-L1) upregulation on tumor-infiltrating monocytes and macrophages, which was strictly dependent on T cells and IFN\gamma. PD-L1 expression could be counteracted by coadministration of antibodies blocking the PD-1 (programmed death-1)/PD-L1 axis as shown for T cells from tumor models and human donors. The combined treatment was highly synergistic and induced complete tumor rejection in about 50\% of mice bearing MC-38 colon and EMT-6 breast tumors. Mechanistically, this was reflected by a strong increase of IFN\gamma and granzyme-B production in intratumoral CD8\textsuperscript{+} T cells. Concomitant CTLA-4 blockade further improved rejection of established tumors in mice. This study uncovers a novel mechanism of acquired resistance upon agonistic CD40 stimulation and proposes that the concomitant blockade of the PD-1/PD-L1 axis is a viable therapeutic strategy to optimize clinical outcomes. Cancer Immunol Res; 3(3); 1–9. ©2015 AACR.

Introduction

CD40, a tumor necrosis factor (TNF) receptor superfamily member, is primarily expressed on antigen-presenting cells (APC), such as dendritic cells (DC), monocytes, and B cells (1). Ligation with CD40L (CD154) results in direct activation with upregulation of costimulatory molecules and other critical immune mediators (2). Agonistic anti-CD40 antibodies are able to fully substitute for T-cell help in vivo during the activation of CD8\textsuperscript{+} T cells and have therefore been explored for their potential to promote antitumor immunity in murine tumor models (3–5). Beatty and colleagues (6) have demonstrated that tumor regression upon systemic CD40 activation can also be mediated by innate immunity in a T cell–independent fashion. However, it remains unclear whether these findings apply only to specific cancers or cancer models, such as pancreatic ductal adenocarcinoma, in which anti-CD40 therapy is insufficient for invoking productive antitumor T-cell immunity (7). Promising early clinical trials have been conducted with different agonistic anti-CD40 antibodies leading to objective and durable tumor responses in patients suffering from melanoma, pancreatic carcinoma, and non–Hodgkin lymphoma (8–10).

Expression of PD-1 and its cognate ligand PD-L1 within the tumor microenvironment is a major resistance mechanism to escape immune surveillance (11). Inhibitors of the PD-1/PD-L1 pathway exert potent antitumor activity in both murine tumor models and clinical trials (12–15). Although PD-L1 is not detectable in most normal tissues, high levels of expression can be induced by proinflammatory cytokines, of which IFN\gamma is the most potent (16–18). Accordingly, upregulation of PD-L1 in melanoma lesions from patients is mediated by immune-cell infiltration and is mainly driven by CD8\textsuperscript{+} T cells (18, 19). Although infiltrating immune cells such as monocytes (20) or myeloid-derived suppressor cells (21) can contribute to PD-L1 expression within the tumor microenvironment, their relative contribution as compared with tumor cells, some of which can also express PD-L1, is largely unknown.

Here, we report that agonistic CD40 stimulation leads to an improved antitumor T-cell response, which is accompanied by IFN\gamma-mediated PD-L1 upregulation on tumor-infiltrating monocytes and macrophages. We show that this resistance mechanism can be successfully circumvented by coadministration of PD-1/PD-L1 blocking antibodies. Concomitant CTLA-4 blockade further improved the therapeutic efficacy of this combination. Our study clearly highlights therapeutic strategies to augment anti-CD40–induced, tumor-infiltrating T cells by targeting coinhibitory pathways.

Materials and Methods

Mice

C57BL/6 and Balb/c mice were obtained from Janvier Laboratories and bred in the animal facility of the Department of Biomedicine, University of Basel (Basel, Switzerland). All animals were housed under specific pathogen-free conditions and in accordance with Swiss federal regulations. Tumor cells (see below) were injected s.c. Perpendicular tumor diameters were measured using calipers and the tumor volume calculated according to the formula: \( D/2 \times d/2 \), where \( D \) and \( d \) are the longest and shortest diameter of the tumor in millimeters, respectively.
In vivo tumor models and therapeutic treatments

Eight- to 10-week-old mice were injected s.c. with $5 \times 10^5$ MC38 colon carcinoma cells (C57BL/6) in 100 µL DMEM without phenol red into the right flank or $2.5 \times 10^5$ EMT6 breast cancer cells (Balb/c) in 40 µL DMEM without phenol red into the mammary gland. On days 16, 18, 21, and 24 after injection, tumors were analyzed for growth and treatment efficacy.

Figure 1.
Agonist anti-CD40 induced antitumor responses and PD-L1 upregulation. A, expression of CD40 in MC38 tumor cells from tissue culture and whole MC38 tumors as analyzed by RT-PCR. B, gating strategy: CD45^+ CD11b^+ cells from MC38 tumors were stained for Ly6C and Ly6G (left) as well as F4/80 (Ly6C low cells, right). C, CD40 expression on monocytes and TAMs gated according to B. An isotype control antibody and tumors from CD40-deficient mice were used as control. D, treatment response of MC38 tumors upon administration of agonist anti-CD40 antibodies. Mice were sacrificed when tumors reached the size of 1500 mm^3. E, upregulation of PD-L1 on monocytes and TAMs following agonist anti-CD40 treatment. Representative histograms are shown. Independent experiments were performed at least two times. Tumor survival curves are representative of three independent, pooled experiments ($n = 15-16$ per group). All experiments were performed using the MC38 model. TAM, tumor-associated macrophage.
PD-L1 Blockade Overcomes Resistance to Anti-CD40 Therapy

A

Percent survival vs. time (days)

B

CD4/8 T-cell depletion

Monocytes

TAMs

C

IFNγR-deficient mice

Monocytes

TAMs

D

IFNγ neutralization

Monocytes

TAMs
tumor challenge, mice were treated with 5 mg/kg anti-CD40 (1C10; milg1; produced at Evitria) and/or 12.5 mg/kg anti–PD-L1 (10F.9G2; rat IgG2b; BioXCell) and/or 12.5 mg/kg anti–PD-1 (RMP1-14; rat IgG2a; BioXCell) and/or 12.5 mg/kg anti–CTLA-4 (9D9; mouse IgG2b; BioXCell) as indicated in the figures. Tumor volume was measured three times/week as described above. For MC38 rechallenge, mice were injected with 1 × 10^8 MC38 tumor cells in 100 μL DMEM without phenol red into the left flank. Naïve mice were used as controls.

Cell lines

The murine tumor cell lines MC38 and EMT6 were obtained from Mark Smyth (Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia) and ATCC, respectively. Cells were cultured in DMEM (Sigma) supplemented with 10% heat-inactivated FBS, sodium pyruvate, penicillin/streptomycin, 1-glutamine, MEM nonessential amino acids, Ciproxin (Bayer), and 0.05 mmol/L 2-mercaptoethanol. All cell lines were tested for mycoplasma free; no genomic authentication was performed.

RT-PCR

RT-PCR was performed using the Go Taq qPCR Master Mix (Promega) on a ViiA 7 Real-Time PCR System. HPRT was used as housekeeping gene for control. The following primers were used to amplify and detect CD40 cDNA: gagctgactaagtctgcgggt and accgcggaaatttgat.

Antibodies and reagents

The following human mAbs were used for flow cytometry: CD4-PE, CD8-FITC, CD25-APC, HLA-DR-eFluor450 (all eBioscience), PD-1-PE-Cy7, PD-L1-PE (both from BD), and CD14-BV605 (Biolegend). Antibodies were from BD, Biolegend, and eBioscience. The following murine mAbs were used for flow cytometry: CD3-PE, CD4-PE-Cy7, CD8-BV605, CD11b-FITC, CD11c-FITC, CD11c-APC, CD19-PE, CD40-PE, CD45-PerCP, CTLA-4-PE, Ly-6C-BV605, Ly-6G-PE-Cy7, NK1.1-BV421, PD-L1-PE (all BD), F4/80-BV421, Granhyz B-Alexa647 PD-L1-BV421, PD-L1-APC, TCR-γδ-APC (all Biolegend), FoxP3-Alexa647, IFNγ-APC, and TCR-γδ-PE (all eBioscience). The live/dead fixable near-infrared dye (Invitrogen) was used to exclude dead cells. Either carboxyfluorescein succinimidyl ester (CFSE) or Cell Proliferation Dye eFluor670 (eBioscience) was used for the determination of T-cell proliferation. The therapeutic antibodies used in studies reported in this article are described in the Methods sections of the corresponding studies.

Analysis of tumor-infiltrating lymphocytes

Eight- to 10-week-old C57BL/6 mice were injected s.c. with 5 × 10^3 MC38 tumor cells in 100 μL DMEM without phenol red into the right flank. On days 16, 18, 20, and 22 after tumor challenge, mice were treated with 5 mg/kg anti-CD40 (1C10; milg1; produced at Evitria) and/or 12.5 mg/kg anti–PD-L1 (10F.9G2; rat IgG2b; BioXCell). On day 24, mice were euthanized, and tumors were mechanically dissociated and digested using accutase (PAA), collagenase IV (Worthington), hyaluronidase (Sigma), and DNase type IV (Sigma). Single-cell suspensions were prepared and stained against the indicated markers for flow cytometric analysis. For detection of IFNγ-producing cells, single-cell preparations were cultured overnight in the presence of anti-CD3/CD28 antibodies and monensin (Biolegend).

**Polyclonal human T-cell activation**

CD4+ and CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from healthy blood donors (Blood Transfusion Center, University Hospital, Basel) by CD4 or CD8 microbeads (Miltenyi Biotech). Anti-CD3e (Clone OKT3; Biolegend) was coated onto 96-well plates by overnight incubation with a 10-μg/mL solution. For polyclonal stimulation, 1 × 10^5 CD4+ or CD8+ T cells were cultured in anti-CD3e-coated wells with the addition of soluble anti-CD28 (2 μg/mL; Clone 28.2; eBioscience). For IFNγ neutralization, anti-IFNγ was added to the culture (20 μg/mL; Clone NIB42; eBioscience). After 24 hours, CD14+ monocytes isolated by CD14 microbeads (Miltenyi Biotech) from PBMCs were added. After a further 24 hours of cultivation, PD-L1 expression on monocytes, identified as CD14+ cells, was determined by flow cytometry.

**Mixed lymphocyte reaction**

Monocytes purified from PBMCs provided by healthy blood donors were isolated by plastic adherence and cultured for 6 days in the presence of 50 ng/mL recombinant human GM-CSF and 250 μL recombinant human IL4 (rhIL4, both from Peprotech). Day-6 human monocyte-derived DCs (mDC) were plated in 96-well round-bottomed plates (1 × 10^3 cells/well) and stimulated with anti-CD40 (10 μg/mL; RO7009789; previously known as CP-870,893) for 48 hours. Anti–PD-L1 (10 μg/mL; Clone 29E.2A3; Biolegend) was added as indicated. PD-1–positive and PD-1–negative CD4+ T cells were isolated by FACS sorting, labeled with the cell proliferation dye eFluor670 (eBioscience), and added to the culture (1 × 10^5 cells/well) as indicated. After 5 days of dilution of the proliferation dye in the gated CD4+, the T-cell population was measured by flow cytometry.

**Results**

**Agonistic anti-CD40 induced antitumor responses and PD-L1 upregulation on tumor-infiltrating monocytes and macrophages**

Tumors were obtained by s.c. inoculation of MC38 colon adenocarcinoma cells, which do not express CD40; of note, this excludes direct or ADCC-mediated killing of MC38 tumor cells by CD40-targeting antibody therapy. Expression of CD40 was detected in MC38 tumors but not in MC38 tumor cells from
PD-L1 Blockade Overcomes Resistance to Anti-CD40 Therapy

A

Percent survival

Time (days)

B

Percent survival

Time (days)

C

Myeloid: Ly-6C high
Myeloid: Ly-6G high
Myeloid: Ly-6G low
CD4 (conventional)
CD4 (Treg)
CD8
NK
NKT
TCRβ/δ
B cell

Control

Anti-CD40

Anti-PD-L1

Anti-CD40 + Anti-PD-L1

D

CD8+ T cells

E

CD8+ T cells

F

Percent survival

Time (days)

G

Percent survival

Time (days)
tissue culture using RT-PCR normalized to two different house-keeping genes (Fig. 1A and Supplementary Fig. S1A). Importantly, CD40 was not detected on CD45<sup>+</sup> cells in MC38 tumors (Supplementary Fig. S1B). Indeed, as CD40 is primarily expressed on APCs (1), expression of CD40 could be readily observed on monocytes (defined as CD11b<sup>+</sup>Ly6C<sup>high</sup>Ly6C<sup>low</sup>) and tumor-associated macrophages (TAM; defined as CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>high</sup>/F4/80<sup>+</sup>) by flow cytometry (Fig. 1B and C). CD40<sup>+</sup> cells were negative as revealed by treatment of MC38 tumor-bearing mice with the agonistic anti-CD40 antibody 1C10 (22) statistically significantly (P < 0.0001; log-rank test), though modestly, prolonged survival compared with the control group of mock-treated mice (Fig. 1D). These data suggest that tumor-infiltrating monocytes and macrophages may play an important role during anti-CD40 therapy. Importantly, administration of 1C10 led to a strong increase in PD-L1 expression both on tumor-infiltrating monocytes and TAMs (Fig. 1E), but not on the CD45<sup>+</sup> cell fraction containing MC38 tumor cells (Supplementary Fig. S1C). Furthermore, PD-1 was upregulated upon anti-CD40 treatment both on CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Supplementary Fig. S1D and S1E). The increase of both PD-L1 and PD-1 expression may impart the capacity of 1C10 to promote robust antitumor T-cell responses and provide an explanation for the limited efficacy of 1C10 monotherapy in the MC38 model.

**Upregulation of PD-L1 is T cell- and IFNγ-dependent**

Depletion of T cells in anti-CD40-treated MC38 tumor-bearing mice completely eliminated the therapy-induced survival benefit (Fig. 2A), providing evidence for a T cell–dependent immune mechanism in this tumor model. Intriguingly, the upregulation of PD-L1 on tumor-infiltrating monocytes and macrophages following anti-CD40 treatment was completely abrogated when T cells were depleted (Fig. 2B). Similar results were obtained using IFNγR−/− mice (Fig. 2C) or IFNγ-neutralizing antibodies (Fig. 2D). Of note, untreated IFNγR−/− mice—and, to a lesser extent, CD40<sup>−/−</sup> mice (Supplementary Fig. S2A and S2B)—exhibited a lower basal expression of PD-L1 on tumor-infiltrating monocytes and macrophages compared with wild-type mice. This finding in IFNγR−/− mice indeed suggests that intratumoral IFNγ-secreting T cells also contribute to PD-L1 expression during tumor growth and development; PD-L1 did not increase during anti-CD40 agonist treatment (Fig. 2C).

**Overcoming agonistic anti-CD40–induced resistance by immune checkpoint blockade**

PD-L1 upregulation upon treatment with agonistic anti-CD40 antibodies might be a major limiting factor for the efficacy of the induced antitumor T-cell response and a potential mechanism of acquired resistance. To this end, we concomitantly treated MC38 tumor-bearing mice with the agonistic anti-CD40 antibody 1C10 and a PD-L1–blocking antibody, which resulted in complete tumor rejection in >50% of the mice, whereas anti-CD40 treatment alone led to tumor rejection only in <5% of the mice (Fig. 3A; Supplementary Fig. S2C). Administration of anti–PD-L1 as monotherapy led to prolonged survival but did not induce rejection of tumors (Supplementary Fig. S2C). These data demonstrate that PD-L1 blockade strongly enhances anti–CD40-induced antitumor immunity by overcoming resistance mediated by PD-L1 upregulation. The combination of anti-CD40- and anti–PD-L1–induced immunologic memory formation as tumor-free survivors were protected upon rechallenge with MC-38 tumor cells, while all naïve control mice developed tumors (Fig. 3B). Compared with controls and mice receiving anti-CD40 monotherapy, flow cytometry–based analysis of tumor-infiltrating immune cells revealed a relative and predominant increase of CD8<sup>+</sup> T cells in mice receiving the combination therapy, whereas the proportion of regulatory T cells (Treg, defined as CD4<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>−</sup>Ter119<sup>−</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>) declined and therefore the CD8/Treg ratio increased (Fig. 3C; Supplementary Fig. S2D). When analyzing the functional characteristics of CD8<sup>+</sup> T cells, we found a strong increase in IFNγ and granulocyte B production (Fig. 3D and E).

We independently confirmed the synergistic interaction of agonistic anti-CD40 with blocking anti–PD-L1 using a second tumor model. To this end, Balb/c mice were orthotopically injected with the breast cancer cell line EMT-6 and treated as described for Fig. 3A–F.

Owing to their distinct immunologic mechanisms of action, concomitant blockade of PD-1 and CTLA-4 has shown superior therapeutic activity in patients with melanoma as compared with either of the antibodies alone (23). Intriguingly, we have noticed an increase in the frequency of CTLA-4–expressing CD8<sup>+</sup> T cells (Supplementary Fig. S1F and S1G) upon agonistic anti-CD40 treatment. We have, therefore, in a last experimental effort, assessed rejection of MC38 tumors by applying therapeutic combinations of agonistic anti-CD40 with CTLA-4/PD-L1 as well as CTLA-4/PD-1 blocking antibodies (Fig. 3G). When combining both anti–PD-L1 and anti–CTLA-4 with anti-CD40 treatment, more than 90% of tumors were rejected (Fig. 3G). Importantly, we have noted no toxicity in any treatment group. Replacement of anti–PD-L1 with anti–PD-1 yielded similar results (Fig. 3G), indicating that blocking either of the components of the PD-1/PD-L1 axis, at least in this model, is an equivalent therapeutic option when combined with anti-CD40.

**CD40 stimulation and concomitant PD-L1 blockade recovers T-cell function in human T cells**

To investigate whether T-cell activation and the subsequent cytokine secretion lead to PD-L1 upregulation on human monocytes, we performed a polyclonal stimulation of CD8<sup>+</sup> and CD4<sup>+</sup>
Figure 4.
T-cell and IFNγ dependence of PD-L1 upregulation in human monocytes. A, polyclonal in vitro stimulation of CD4+ and CD8+ human T cells isolated from PBMCs induced PD-L1 upregulation on CD14+ monocytes, which could be largely annihilated by the addition of neutralizing anti-IFNγ antibody. B, a mixed lymphocyte reaction with monocyte-derived DCs and sorted PD-1+ or PD-1− CFSE-labeled CD4+ T cells was performed. Agonistic anti-CD40 and anti-PD-L1 antibodies were added as indicated. Dilution of CFSE as a marker of proliferation was determined by flow cytometry. One representative out of three independent experiments with similar results is shown. C, supernatants from B were analyzed for IFNγ using ELISA. Statistically significant differences (one-way ANOVA with Bonferroni post-test) are indicated: *, P < 0.05; **, P < 0.005; ***, P < 0.001. n.s., not statistically significant.
T cells isolated from PBMCs of healthy human donors and cocultured them with CD14+ monocytes. T-cell activation and the subsequent IFNγ production led to a strong upregulation of PD-L1 on human monocytes, which could be largely diminished by IFNγ neutralization (Fig. 4A). These data confirm that, comparable with the mouse tumor model, PD-L1 upregulation following human T-cell activation also largely depends on IFNγ. Subsequently, to analyze whether a blocking antibody against human PD-L1 is able to overcome secondary resistance induced by an agonistic antibody against CD40 (8), we tested both antibodies in a human mixed lymphocyte reaction using moDCs and PD-1–expressing or PD-1− CD4+ T cells sorted from whole PBMCs by flow cytometry. Coadministration of anti–PD-L1 significantly increased the anti–CD40-induced proliferation and IFNγ secretion of PD-1+ but not of PD-1− T cells (Fig. 4B and C). Utilizing human PBMCs, these results clearly confirm a potential synergistic activity of agonistic anti-CD40 and blocking anti–PD-L1 treatment.

**Discussion**

Although initial phase I studies of agonistic anti-CD40 mono-therapy have demonstrated objective tumor responses in solid tumors (8), possibly the greatest potential for these drugs lies in the combination with other therapeutic agents. The combination of anti-CD40 agonists with chemotherapy (e.g., tumor-cell killing results in the release of tumor antigens) may lead to therapeutic synergies, as indicated by recent phase I clinical trials (24, 25). We propose here that antitumor T-cell responses induced and/or strengthened by CD40 stimulation can be greatly improved by targeting the PD-1/PD-L1 axis.

We found that CD40 engagement with agonistic antibodies leads to a T-cell- and IFNγ-dependent upregulation of PD-L1 on tumor-infiltrating monocytes and macrophages, thereby feeding into a negative feedback loop, which hampers anti–CD40-induced T-cell responses. A high level of PD-L1 expression on monocytes and macrophages in peritumoral stroma has been associated with high mortality and reduced survival in patients with cancer, as these monocytes effectively suppressed tumor-specific T-cell immunity (20), supporting the clinical relevance of the MC38 tumor model. Our data are in accordance with recent data from murine melanoma models showing an IFNγ-dependent upregulation of PD-L1 expression following CD8+ T-cell infiltration (18) or immune-cell infiltration in general (19). Using antibodies targeting the PD-1/PD-L1 axis in anti–CD40-treated mice, we were able to overcome PD-L1–mediated secondary resistance and improve T-cell function. In our tumor model, PD-L1 expression was largely restricted to immune cells, though in other models and, importantly, patients with cancer, PD-L1 may be induced on tumor cells. Clearly, translational studies need to be performed in future clinical trials to assess PD-L1 expression upon treatment with CD40 agonistic antibodies in patients. Importantly, using *in vitro* stimulation of human T cells, we demonstrated that a profound improvement of T-cell function could be achieved using concomitant CD40 activation and PD-L1 blockade. This suggests that a similar benefit may be achieved in patients with cancer. By integrating CTLA-4–blocking antibodies into the anti–CD40/PD-L1 and anti–CD40/PD-1 treatment regimen, we were able to further enhance durable tumor rejection.

Although a clinical trial evaluating the agonistic anti-CD40 antibody RO7009789 in combination with the CTLA-4–blocking antibody tremelimumab is currently under way at the University of Pennsylvania, to our knowledge, a combination with PD-1/PD-L1–blocking antibodies has not yet been attempted in patients with cancer. Our data clearly provide a strong rationale for the combination of these antibodies, which could be rapidly tested in a clinical setting.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: A. Zippelius, P. Müller

Development of methodology: A. Zippelius, J. Scheirner, P. Müller

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Zippelius, J. Scheirner, P. Herzig, P. Müller

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Zippelius, J. Scheirner, P. Müller

Writing, review, and/or revision of the manuscript: A. Zippelius, J. Scheirner, P. Müller

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Zippelius, P. Herzig

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