IL2/Anti-IL2 Complex Combined with CTLA-4, But Not PD-1, Blockade Rescues Antitumor NK Cell Function by Regulatory T-cell Modulation

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Abstract

High-dose IL2 immunotherapy can induce long-lasting cancer regression but is toxic and insufficiently efficacious. Improvements are obtained with IL2/anti-IL2 complexes (IL2Cx), which redirect IL2 action to CD8+ T and natural killer (NK) cells. Here, we evaluated the efficacy of combining IL2Cx with blockade of inhibitory immune pathways. In an autochthonous lung adenocarcinoma model, we show that the IL2Cx/anti-PD-1 combination increases CD8+ T-cell infiltration of the lung and controls tumor growth. In the B16-OVA model, which is resistant to checkpoint inhibition, combination of IL2Cx with PD-1 or CTLA-4 pathway blockade reverses resistance. Both combinations work by reinvigorating exhausted intratumoral CD8+ T cells and by increasing the breadth of tumor-specific T-cell responses. However, only the IL2Cx/anti–CTLA-4 combination is able to rescue NK cell antitumor function by modulating intratumoral regulatory T cells. Overall, association of IL2Cx with PD-1 or CTLA-4 pathway blockade acts by different cellular mechanisms, paving the way for the rational design of combinatorial antitumor therapies.

Introduction

Administration of high-dose IL2 immunotherapy, which can induce durable cancer regression, is approved by the FDA for treatment of metastatic melanoma and renal carcinoma (1). IL2 therapy can, however, be toxic and has low efficacy (5%–20% of responders) due to its effect on regulatory T cells (Tregs), which express the high-affinity IL2 receptor (composed of subunits IL2R-α, IL2Rβ, and IL2Rγc) and can block the antitumor immune response (2). Antitumor activity requires activation of CD8+ T and natural killer (NK) cells, which respond to IL2 through the intermediate affinity IL2 receptor (composed of IL2Rβ and IL2Rγc subunits; ref. 3).

One strategy to avoid unwanted activation of Tregs is to use IL2/anti-IL2 complexes (IL2Cx). IL2Cx can redirect IL2 to Treg or to IL2Rβγc-expressing cells that can mount an antitumor response (4). IL2Cx has a longer half-life than IL2, resulting in better pharmacodynamics (5). High-dose IL2 therapy can be toxic due to vascular leak syndrome. IL2-associated pulmonary edema seems to be caused by interaction of IL2 with its receptors (IL2-Rα, CD25) on lung endothelial cells (6). This side effect can be abrogated by use of IL2Rβγc-directed IL2Cs, which should not activate CD25 on the endothelial cells (5). In murine models of cancer, the IL2Rβγc-directed IL2Cx can boost the antitumor response by stimulating NK and CD8+ T cells, encouraging the clinical development of IL2Cx (7). Nevertheless, in experimental models, administration of IL2Cx as monotherapy delays tumor growth only modestly (6–8).

Given the success of antitumor therapies in cancer, combination of IL2Cx with antitumor mAbs that target CTLA-4 or PD-1/PD-L1 inhibitory pathways could improve results. Indeed, single-agent administration of anti–CTLA-4 or anti–PD-1 shows clinical efficacy in around 20% of patients with tumors (9). Combination of anti–CTLA-4 and anti–PD-1 increases both response rate and toxicities (10), although the majority of cancer patients still do not respond. Acquired resistance to treatment has also been observed (11, 12). Efficient combination therapies are needed to increase the number of responding patients.

In this study, we evaluated whether combining IL2Cx administration with the blockade of inhibitory immune pathways represents an effective antitumor therapy. We tested two experimental models: B16-OVA, which is resistant to checkpoint inhibition (13), and the KrasLSL-G12D/p53fl/+ genetically engineered mouse lung adenocarcinoma model (14). In both models, we observed that combination of IL2Cx with antitumor mAbs was more effective. We found that IL2Cx works in a cell-intrinsic manner, boosting tumoral NK, reinvigorating exhausted CD8+ T cells, and increasing the breadth of tumor-specific T-cell responses. We also found that combination of IL2Cx with blockade of the PD-1 or CTLA-4 pathways increases tumor infiltration by CD8+ T cells. Anti–CTLA-4 contributes to efficacy by a CD8+ T and NK cell-extrinsic mechanism.
releasing effector CD8+ T and NK cells from Treg suppression. Consequently, the combined treatment of IL2Cx with CTLA-4 blockade relies on the action of NK cells. These two combinations, which act by different cellular mechanisms, suggest avenues for the design of combinatorial immunotherapies adapted to the individual tumor microenvironment of each patient. Our preclinical results show the validity of combining IL2Cx with anti-checkpoint mAbs to fight cancer.

**Materials and Methods**

**Mice**

C57BL/6 female mice (5–6 weeks old) were purchased from The Charles River Laboratories. KrasLSL-G12D/+/p53fl/fl mice (referred to as KP mice) were kindly given by T. Jacks (ref. 14; NIH) and backcrossed on the C57BL/6 background for more than 10 generations at CNRS Central Animal Facility TAAM (Orléans, France). Experiments with KP mice were performed with females of 8 to 12 weeks old. Experimental animal procedures were approved by the ethics committee of the Institut Curie CEEA-IC #118 (DAP n° 05302.03), in compliance with international guidelines.

**Cell lines**

B16F10 melanoma cell line expressing OVA (B16-OVA) was kindly given by K. Rock in 1999 and was cultured in RPMI (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Biosera), 2 mM L-glutamine (Life Technologies), β-mercaptoethanol, and 1% penicillin/streptomycin (Life Technologies). For all experiments, cells were thawed from the same stock generated in 2016. Cells were cultured during maximum 2 weeks before injection into mice. Cells were not authenticated in the past year. Cells were checked for the absence of Mycoplasma by PCR reaction (GAT-Biotech).

HEK293-LTV and 3TZ cells were obtained from ATCC in 2013 and were used upon a second passage. Cells were cultured in Dubeco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Biosera). Cells were not authenticated in the past year. Cells were checked for the absence of mycoplasma by PCR reaction (GAT-Biotech).

**Lentiviral particle production and titration**

A second generation of lentivirus (LV) was produced as previously described (15). Briefly, LV particles were produced by transfection of the HEK-293LTV cell line (Cell Biolabs, Inc.) with pAX2 (packaging plasmid; Addgene), pCMV-VSV-G (envelope plasmid; Addgene), and luciferase and recombinase Cre-expression plasmid. The latter was modified to express SIIFNFKL and DBY epitopes fused to the C-terminal end of luciferase protein. Moreover, this luciferase–SIIFNFKL–DBY sequence was modified to restrict SIIFNFKL and DBY expression to nonhematopoietic cell lineage as previously described (15). Functional particles were quantified using a Cre activity readout system based on the reporter cell line 3TZ expressing β-galactosidase upon Cre-mediated recombination.

**Tumor experiments**

For the transplantable tumor model, C57BL/6 females (5–6 weeks old) were injected subcutaneously in the flank with 0.5 × 10^6 B16-OVA cells in 200 μL of PBS. Tumor growth was measured using a metric caliper 2 to 3 times a week. Lung adenocarcinoma was induced in KP mice (8–12 weeks old) by intratracheal injection of 2 × 10^4 lentiviral particles expressing the fusion protein luciferase–SIIFNFKL–DBY and Cre-recombinase as previously described (15). In vivo tumor growth was measured by bioluminescence. Briefly, lentiviral-injected mice were shaved and anesthetized with isoflurane (Sigma-Aldrich). D-luciferin substrate (150 mg/kg, Promega) dissolved in PBS was injected intraperitoneally (i.p.) 15 minutes before imaging in IVIS Spectrum (PerkinElmer). Photon fluxes were transformed into pseudocolour images using the Living Image software (PerkinElmer).

**In vivo treatments**

Recombinant human IL2 (rhIL2, Proleukine, Novartis), anti-CTLA4 (clone 9H10; Bio X Cell), anti–PD-1 (clone RMP1-14; Bio X Cell), anti–PD-L1 (clone 10F9G2; Bio X Cell), anti-human IL2 (clone MAB602; Bio-Techne), and anti-human IL2 (clone 5344.111; BD Biosciences) were purchased. IL2Cx were prepared mixing 15,000 UI of rhIL2 with 4.5 μg of Ab (molar ratio 2:1) and incubated for 30 minutes at 37°C. Treatment of B16-OVA engrafted mice began when tumors became measurable (at approximately day 10). Treatment of KrasLSL-G12D/+/p53fl/fl mice was started when luciferase signal achieved 1 × 10^5 photons/second (between 12 and 14 weeks after viral inoculation). Where indicated, tumor-bearing mice received i.p. injections of IL2Cx for 5 consecutive days. Anticheckpoint mAbs were injected i.p. at 200 μg/dose 3 times a week, for 7 total doses with the B16-OVA tumor or 8 weekly cycles for KrasLSL-G12D/+/p53fl/fl mice. For depletion experiments, CD8+ cell depletion was performed using 200 μg anti-CD8 (clone 53.6.72; Bio X Cell) and NK cells depletion using 200 μg anti-NK (clone PK136; Bio X Cell). Depleting mAbs were administered i.p. 1 or 2 days prior to therapeutic treatment and 1 or 2 days after. Depletion was maintained by twice-a-week injection of depleting Abs until the end of the experiment. CD8+ T-cell and NK cell depletion was monitored in blood after 4 and 6 doses of depleting mAbs.

**Preparation of cell suspensions**

Subcutaneous tumors were collected in CO2 independent medium (Gibco). Then, tumors were cut into small pieces and placed into 2.5 mL of CO2 independent medium containing 0.1 mg/mL DNase I (Roche) and 0.1 mg/mL Liberase TL (Roche) in C tubes (Miltenyi Biotec). After mechanical dissociation with gentleMACS Dissociator, samples were incubated with shaking at 37°C for 30 minutes and processed again with gentleMACS. The cell suspension was then filtered with a 100-μm cell strainer for direct tumor cell analysis and further separated on a Percoll gradient (GE Healthcare Life Sciences) from 40% to 75% interface to recover mononuclear cells for immune cell infiltrate analysis.

Inguinal tumor-draining lymph nodes (dLN) were collected in CO2 independent medium. Single-cell suspensions were obtained by mechanical disruption over a 40-μm cell strainer. Lungs were perfused with 20 mL of cold PBS to remove circulating blood cells prior to preparation of single-cell suspension. Mononuclear cells from lungs were obtained as described above for subcutaneous tumors.
Detection of Ag-specific T cells by ELISPOT

IFNγ-producing antigen-specific CD4+ or CD8+ T cells were measured by ELISPOT. Briefly, microplates (MAIPS4510; Millipore) were coated with anti-murine IFNγ (Diaclone). PBMCs (0.3 × 106) were cultured overnight in the presence of either control medium or class I–restricted OVA-I peptide (257–264, SIINFEKL, 10 mmol/L) or the class II–restricted DBY peptide (NAGFNSRANSRSS, 40 mmol/L), or B16-OVA melanoma neoepitopes Pool 1 (10 mmol/L) or melanoma neoepitopes Pool 2 (10 mmol/L). Cells and peptides were resuspended in complete medium RPMI (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Biosera), 2 mmol/L L-glutamine (Life Technologies), and penicillin/streptomycin (Life Technologies), 1% penicillin/streptomycin (ThermoFisher) intracellular staining was performed using intracellular Fixa
tomycin (Life Technologies), and avidin–alkaline phosphatase (Mabtech) and revealed using the appropriate substrate (Bio-Rad). Spots were counted using an ELISPOT Reader System ELR02 (AID). Results were expressed as number of cytokine-producing cells (SFC) per 1 × 106 of total cells.

Flow-cytometric analysis

For flow-cytometry analysis, mAbs specific for mouse CD152 (UC10-F410-11), CD274 (M1H5), CD4 (RM4-5), CD8 (53-6-7), GITR (DTA-1), Ki67 (B56), KLRG1(2F1), and TCRβ (H57-597) were purchased from BD Biosciences. mAbs against mouse CD19 (6D5), CD25 (3C7), CD27 (LG.3A10), CD4 (GK1.5), CD44 (IM7), CD45.2 (104), Foxp3 (MF-14), GzmB (GB11), ICOS (C398.4A), IFNγ (XMG1.2), NK 1.1 (PK136), PD-1 (29F.1A12), Tbet (4B10), and TCRβ (H57-597) were purchased from BioLegend. mAb against mouse EOMES (Dan11mag) was purchased from ebioscience/Thermo Fisher, unconjugated gp100 (EP4863) was purchased from Abcam, and anti-rabbit IgG (H + L)-A488 secondary Ab was purchased from Thermo Fisher.

Surface staining was performed at 4°C with antibodies resuspended in PBS with 2% PBS and 2 mmol/L EDTA. For intracellular staining of cytokines, cells were restimulated with phorbol 12-myristate 13-acetate (PMA, 20 ng/mL) and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 hours at 37°C in the presence of GolgiStop and GolgiPlug (BD Biosciences). All intracellular staining was performed using intracellular Fixation/Permeabilization buffer set (ebioscience/Thermo Fisher) according to the manufacturer’s instructions. Live-cell detection was performed using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies).

FACS data were acquired using an LSRSforrtesa flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 10x, Tree Star).

Statistical analyses

Statistical analyses were performed in GraphPad Prism v7. P values were calculated with one-way ANOVA and Kruskal–Wallis posttest.

Results

IL2Cx with anti–PD-1 mAb controls established tumors in a lung adenocarcinoma model

In patients with lung cancer, inhibition of the PD-1/PD-L1 pathway has proven to be efficient and safe, leading to the approval of these treatments (16, 17). Nevertheless, not all patients respond, and combination with IL2Cx, which activates NK and CD8+ T cells, could improve treatment efficacy. Thus, we evaluated the therapeutic effect of IL2Cx (made of IL2/a-IL2 mABMAB602) alone or combined with an anti–PD-1 blocking mAB in an autochthonous lung tumor model. With this aim, we used the spontaneous tumor model described by DuPage and colleagues (14), in which autochthonous lung tumors are induced by lentiviral infection of a limited number of somatic cells in which oncogenesis is stimulated by recombination of LoxP sites, leading to the activation of one oncogene (Kras) and deletion of one suppressor gene (p53; KP mice). This model not only recapitulates the most frequent gene mutations found in human lung tumors, but also mimics features of the human situation, such as vascularature, tumor architecture, and speed of growth. We modified the lentivirus to induce tumor expression of luciferase and one class I (SIINFEKL)– and one class II (DBY)–restricted nominal antigen. To reduce expression of the nominal antigens after virus inoculation and unwanted priming, we used a lentivirus harboring four tandem target sequences of the hematopoietic-specific mir142-3p after the luciferase-Ag cassette, to induce its degradation in hematopoietic cells (ref. 15; see Materials and Methods).

KP mice infected with the lentivirus were monitored for tumor development by whole-body bioluminescence imaging. The first tumors were detected at 13 to 26 weeks after virus inoculation. Once mice reached a bioluminescence signal of 1 × 106 photons/second, they were randomized to receive one of the following treatments: PBS, IL2Cx (made of 15,000 IU of IL2 and anti-IL2; MAB602, 2.1 molar ratio), anti–PD-1 (200 μg twice per week), or IL2Cx plus anti–PD-1, for at least 7 weeks, and tumor growth followed (Fig. 1A and B). Bioluminescence detection usually correlated with tumor burden when evaluated by hematoyxin and eosin staining, which also revealed that IL2Cx-treated mice did not develop lung edema, previously associated with high-dose IL2 therapy (ref. 6; Fig. 1B). Single-agent therapies showed little antitumor effect, whereas the combination of IL2Cx with anti–PD-1 controlled tumors (Fig. 1C). No tumor regression was observed in the PBS group, whereas 33% of the mice showed tumor control or regression in the IL2Cx group, 27% in the anti–PD-1 group, and 42% in the combination group (Fig. 1C). Treatment efficacy was associated with more circulating antitumor CD8+ T cells (specific for the MHC class I–restricted peptide, SIINFEKL) and CD4+ T cells (specific for the MHC class II–restricted DBY epitope; Supplementary Fig. S1A) in treated groups than in the PBS group. At the end of the experiment, no changes were observed on the proportions of CD4+ T effector (Teff) or NK cells in lung tissue (Supplementary Fig. S1B and S1C). IL2Cx administration increased the frequency and the cycling of CD8+ CD44High T cells (Fig. 1D), an effect reinforced by combination with anti–PD-1. The frequency of Tregs also increased in mice receiving combination therapy (Fig. 1E), but the CD8+CD44High/Treg ratio was unaltered (Fig. 1F). Thus, the combination of IL2Cx...
with anti–PD-1 increases CD8⁺ T-cell infiltration of the lung and controls cancer growth, rendering autochthonous lung tumors more sensitive to immune attack.

IL2Cx delays tumor growth in the B16F10-OVA melanoma mouse model

To confirm our results in a different tumor type, we switched to the transplantable syngeneic B16F10-OVA melanoma mouse model. The IL2α:IL2 mAb Mab602 complex can control B16F10 melanoma growth (4, 5). We used an Ova-expressing tumor that allows us to follow the antitumor-specific T-cell response. For this, mice were inoculated with 0.5 × 10⁶ B16-OVA melanoma tumor cells. When tumors were palpable, mice were randomized to different treatments: high-dose IL2 (200,000 IU), IL2Cx (made of 15,000 IU of IL2 and anti-IL2 Mab602 at 2:1 molar ratio), or PBS (Fig. 2A). As in ref. 6, both high-dose (HD) IL2 and the IL2Cx administered at a 13 times lower dose of IL2 (LD) delayed tumor growth (Fig. 2B). IL2 action was monitored in the blood by measuring the increase of activated and highly cycling CD44High CD8⁺ T cells (Fig. 2C). ELISPOT analysis at day 6 after the beginning of the treatment showed that administration of both HD IL2 or LD IL2 in the form of IL2Cx induced or amplified tumor-specific T cells, as indicated by the increase of IFNγ-producing T cells upon ex vivo restimulation with an OVA peptide or with peptides containing tumor neoepitopes (Fig. 2D). IL2Cx, at lower doses, thus less toxic than HD IL2, induced a delay in B16 tumor growth and an increase of tumor-specific IFNγ-producing T cells.
We considered the effect that dissociation of IL2 from the IL2Cx in vivo might have on Tregs and cancer growth. Most of the studies evaluating the role of Tregs in cancer have assessed the impact of depleting (18) but not of increasing Tregs in the cancer context (19). Thus, we studied the consequences of administering LD IL2 (15,000 IU of IL2, same amount of IL2 present in the complex), an IL2Cx that redirects IL2 action to Tregs (mAb 5344), and the IL2Cx that redirects IL2 action to CD8+ T and NK cells (mAb MAB602). In tumor-free mice, LD IL2 did not cause changes in circulating Tregs and CD8+ T cells. Both IL2Cx variants induced an increase of Tregs and enhanced their cycling. Only the IL2Cx that redirect IL2 action to CD8+ T and NK cells increased the proportion and the cycling of circulating CD8+ CD44High T cells (Supplementary Fig. S2A–S2C). In tumor-bearing mice, neither LD IL2 nor the IL2/anti-IL2 mAb MAB602 Cx accelerated tumor growth (Fig. 2E–H). Thus, an unwanted boosting effect of free IL2 on Tregs during cancer appears unlikely. These data indicate that although IL2/anti-IL2 MAB602 Cx boosts circulating Tregs (Supplementary Fig. S2C), it also delays tumor growth (Fig. 2H).

IL2Cx enhances therapeutic efficacy of immune-checkpoint-blocking mAbs

IL2Cx and checkpoint-blocking mAbs each alone have limited efficacy (Fig. 2; ref. 13). We observed a synergistic effect when both were used in combination in mice with palpable
B16-OVA tumors (Fig. 3A–C). Figure 3D and E, depicting the results of five pooled experiments, show that monotherapy with anti-CTLA-4 was inefficient; blockade of the PD-1 pathway alone and IL2Cx alone both conferred only intermediate control of tumor growth. The combination treatments were more efficient, as reflected by a significant delay in the tumor development (Fig. 3D). The maximum reduction of the mean tumor volume (measured at the end of the experiment, between days 16 and 20) was obtained with the combinatorial treatments, reaching a 6-fold diminution for the IL2Cx with anti-CTLA-4 and 4-fold with PD-1 blockade relative to the PBS group (Fig. 3E). Mice receiving either single or combined treatments showed no signs of toxicity, such as hair or weight loss. Overall, these results show the greater antitumor effect of the combination therapies over the IL2Cx or inhibitory checkpoint blockade monotherapies.

IL2Cx together with anti-CKP increases tumor infiltration by CD8⁺ T cells

Given that IL2Cx, anti-CTLA-4, or anti-PD-1/anti-PD-L1 may be acting by different mechanisms, we explored the contribution of different immune cell subsets to the efficacy of the combined treatments by flow cytometry analysis of tumor infiltrate. To verify the interpretation of our results, we assessed the relative contribution of blood circulating and tumor-resident lymphocytes among cells retrieved from tumor cell suspensions. Regardless of the treatment, more than 98% of the analyzed hematopoietic cells (total CD45⁺ cells) are tumor tissue-infiltrating cells (Supplementary Fig. S3).

First, we analyzed the effect of the treatments on the tumor microenvironment. Tumors from PBS-treated mice were little infiltrated by hematopoietic cells (mean ± SD of 8.5% ± 2.7% of CD45⁺ cells among live cells, n = 10), as previously
described for this nonimmunogenic tumor (ref. 17; Supplementary Fig. S4A). Compared with the PBS group, treatment with IL2Cx alone or PD-1 pathway blockade alone did not induce a significant increase in the tumor infiltrate [mean ± SD of 12.3% ± 3.4% (n = 10), and 11.2% ± 2.8% (n = 9) of CD45+ cells among live cells, respectively]. Other treatments promoted a significant recruitment of immune cells [mean ± SEM of 13.1% ± 5.2% of CD45+ cells among live cells for anti-CTLA-4, P < 0.05 (n = 10); 17.7% ± 7.0% for IL2Cx/anti–CTLA-4, P < 0.001 (n = 10); and 18.6% ± 5.6% for IL2Cx/PD-1 blockade, P < 0.0001 (n = 10)].

Then, we evaluated the impact of the different therapies on CD8+ T cells, which mediate antitumor immunity. Compared with the PBS-treated group, all therapies except PD-1 blockade alone induced a significant increase in the percentage of CD8+ TILs among the CD45+ infiltrate, the vast majority being CD44High, in accordance with the finding that only effector cells reach the tumor (Supplementary Fig. S4A and S4B). Moreover, only mice receiving the combination therapies showed significantly higher absolute numbers of CD8+ CD44High TILs (Fig. 4A). To study CD8+ T-cell function, we analyzed T-cell division and IFNγ production. We observed that compared with mice receiving PBS, IL2Cx alone and the combination therapies induced an increase in the division of CD8+ CD44High TILs (Fig. 4B) and in circulating OVA-specific CD8+ T cells, as well as in tumor neopetitope-specific T cells (Fig. 4C).

We observed that in the B16-OVA model, a high proportion of CD8+ CD44High TILs expressed PD-1 (Supplementary Fig. S4C) and that the tumor cells expressed its ligand PD-L1, though at different amounts depending on the treatment (Supplementary Fig. S4D). During chronic viral inflammation, increased expression of PD-1 has been associated with a state of T-cell exhaustion, characterized by lower effector cytokine production and lower replication. In the cancer setting, exhausted CD8+ T cells coexpress the transcription factor eomesodermin (Eomes) along with PD-1 (20). Using these markers, we observed that the percentage of TILs with an exhausted phenotype (Eomes+ PD-1+ CD44High CD8+) was not significantly different among the various groups of treatment (Fig. 4D; Supplementary Fig. S4E). Notwithstanding, it has been reported that during treatment with anti–PD-1, exhausted cells may undergo a reinvigoration process marked by an upregulation of Ki67 and an increase in granzyme B (GzmB) production (20, 21). As shown in Fig. 4E, upon ex vivo stimulation, most of the CD8+ TILs from mice treated with IL2 alone or in combinatory treatments significantly increased Ki67 and GzmB expression, whereas CD8+ TILs from mice treated with PBS, anti–CTLA-4 alone, or PD-1 pathway blockade alone maintained a pool of cells unable to divide and produce GzmB (Fig. 4F).

Altogether, these results suggest that tumor growth control during IL2Cx administration is associated with a significant increase of the CD8+ T-cell tumor infiltrate (which is higher in combination with anticheckpoints Abs), an increase in magnitude and breadth of tumor-specific T-cell responses and the reinvigoration of exhausted CD8+ TILs.

**Therapy with IL2Cx/anti–CTLA-4 combination is associated with higher CD8+ cell/Treg ratio**

The antitumor effect of anti–CTLA-4 in mice may be in part due to Treg depletion (22). On the other hand, IL2 can boost Treg numbers and function (23). We analyzed the effect of the combinations on Tregs. As observed in Fig. 5A and B, IL2Cx administration was not associated with an increase in intratumoral Treg frequencies. Blockade of the PD-1 pathway alone or in combination with IL2Cx did not affect Treg proportions. However, administration of the anti-CTLA-4 alone induced a decrease of intratumoral CD4+Foxp3+ Treg proportions, which was more pronounced in association with the IL2Cx. The proportion of CD4+ Teff cells was not significantly affected by the different treatments (Supplementary Fig. S5A).

Therapeutic efficacy of anticancer immunotherapies is associated with increased CD8+/Treg ratios (22, 24). In our model, only administration of anti–CTLA-4 induced an increase of the tumoral CD8+/Treg ratio compared with untreated mice. This increase was greatest when anti–CTLA-4 was combined with IL2Cx (Fig. 5C).

To better understand where CTLA-4–mediated Treg depletion was occurring, we also quantified Treg proportions in the tumor-draining lymph nodes. As observed in Fig. 5A and B, Treg frequencies were only reduced in the tumor. Selective depletion of a specific T-cell population may be due to the differential expression density of the target molecule (25). To explore this hypothesis, we quantified the median fluorescence intensity (MFI) value of CTLA-4 on the T cells in the different organs (Fig. 5D). Tregs from the tumor of PBS-treated mice expressed the highest MFI values of CTLA-4, compared with CD4+ T eff and CD8+ T cells, which expressed less CTLA-4. Upon IL2Cx administration, CTLA-4 MFI was not significantly modified, and anti–CTLA-4 administration induced a reduction on its target expression on Tregs, probably due to internalization or to blockade of the target CTLA-4 molecule. T cells in the draining lymph nodes barely expressed the CTLA-4 molecule, validating our hypothesis that CTLA-4 mAb-selective depletion of tumor-infiltrating Tregs is likely due to the high expression of its target uniquely on Tregs in this location. We extended this analysis to other immune checkpoints and showed (Fig. 5D; quantified in Supplementary Fig. S5B–S5E) that Tregs in the tumor showed the highest MFI not only of CTLA-4, but also of ICOS, and GITR, compared with Treg in lymph nodes, and to CD4+ T eff and CD8+ T cells in all organs. Conversely, CD8+ T cells in the tumor expressed the most PD-1. As previously described (23, 26), CD25 expression on Tregs increased upon IL2 administration; this increase serves as a biomarker of IL2 in vivo activity.

Overall, compared with anti–PD-1 action, additional immune modulation is achieved by anti–CTLA-4, which specifically depletes intratumoral Tregs and tips the effector/regulatory balance in the tumor in favor of CD8+ T cells, an imbalance further amplified when anti–CTLA-4 is combined with IL2Cx.

**IL2Cx with anti–CTLA-4 enhances NK cell activation and increases NK cell/Treg ratio**

NK cells, which are highly cytotoxic and produce proinflammatory cytokines, are effectors of antitumor immune responses. Thus, we investigated the effect of the combinatioral therapies on tumor-infiltrating NK cells, which are known to be sensitive to IL2 (27). Mice were treated as described in Fig. 3A and sacrificed 6 days after the beginning of the treatment. As observed in Fig. 6A and B (and Supplementary Fig. S6A), the IL2Cx/anti–CTLA-4
Figure 4.
Combination of IL2Cx with anti-CKP reinvigorates tumor-infiltrating CD8⁺ T cells. Mice harboring B16F10-OVA melanoma tumors were treated as described in Fig. 3A. Tumors and blood were analyzed 6 days after the beginning of the treatment. A, Quantification of effector CD8⁺ TILs expressed as number of CD8⁺ CD44High cells per gram of tumor. B, Frequency (%) of Ki67⁺ cells among tumor-infiltrating CD8⁺ CD44High cells. C, IFNγ ELISPOT of circulating T cells against OVA-SIINFEKL peptide (top) and B16-F10 melanoma neoepitopes Pool1 (M30⁺ M23⁺ M49; middle) and Pool 2 (NB02⁺ NB08⁺ M27⁺ M47; bottom), analyzed as in Fig. 2. SFC, spot-forming cells. D, Representative dot plots showing the expression of Eomes and PD-1 among infiltrating CD8⁺ CD44High T cells (top), and of Ki67 and Granzyme B (GzmB) among Eomes⁺ PD-1⁺ cells (bottom). Numbers indicate the frequency (%) of cells in each quadrant. E, Frequency (%) of GzmB⁺ Ki67⁺ T cells among PD-1⁺ Eomes⁺ CD8⁺ CD44High T cells infiltrating the tumor. F, Frequency (%) of GzmB⁺ Ki67⁺ T cells, among PD-1⁺ Eomes⁺ CD8⁺ CD44High T cells infiltrating the tumor. Data are a pool of three (A–C) or two (D–F) independent experiments. P values were calculated using one-way ANOVA and Kruskal–Wallis test or Student t test. *P < 0.05; **P < 0.01; ***P < 0.001. See also Supplementary Figs. S3 and S4.
combination induced the largest increase in the number of NK cells and in the proportion of IFN-γ-producing tumor-infiltrating NK cells. Otherwise, IL2Cx alone and combined with the checkpoint blockers enhanced NK division and activation, as reflected by the significant increase in the proportion of Ki67+ NK cells, as well as by the increment in the expression of KLRG1 and the stimulatory receptor NKG2D by these cells (Fig. 6C and D; Supplementary Fig. S6B–S6D).

Figure 5.
IL2Cx alone or in combination with anti–CTLA-4 increases the CD8/Treg ratio in the tumor. C57BL/6 mice harboring B16F10-OVA tumors were treated as described in Fig. 3A. Flow-cytometry analysis of tumor-infiltrating T cells was done 6 days after the beginning of the treatment. A, Representative dot plots of Foxp3 and CD4+ expression on CD45+ cells from the tumor and tumor-draining lymph nodes (dLN). Numbers indicate the frequency (%) of Tregs in the indicated gates. B, Frequency (%) of Tregs among CD45+ cells in the tumor (left) and in dLN (right) from A. C, Ratio of CD8+ T cells among CD45+ cells/frequency of total Tregs among CD45+ cells. D, Quantification of CTLA4 median fluorescence intensity (MFI) in the indicated TILs (left). Heat map of the MFI of CTLA4, CD25, ICOS, GITR, and PD-1 on Treg, Teff, and CD8+ T cells in the tumor and dLNs (right). Data are from three (A–C) or one (D) independent experiments. P values were calculated using one-way ANOVA and Kruskal–Wallis test. **, P < 0.01; ****, P < 0.0001. See also Supplementary Fig. S5.
We also analyzed the impact of the treatments on NK maturation, which is modulated by IL2 (27, 28). For that, based on the expression of CD27 and CD11b, we subdivided NK cells into three subsets with different maturation status (CD27+CD11b− → DP → CD27−CD11b+) (ref. 29). None of the treatments modified the percentage of immature CD27+CD11b− NK cells, but administration of IL2Cx alone and in both combinations significantly increased the more mature CD27+CD11b− NK cells, at the expense of the more terminally differentiated/senescence CD27−CD11b+ NK cells (ref. 30; Fig. 6E). Given that both NK and Tregs can be boosted by IL2 (26), that CTLA-4 mAb can deplete Tregs (22), and that Tregs suppress NK cells (31, 32), we investigated the impact of the treatments on the NK cell/Treg ratio. The only treatment that significantly increased the NK cell/Treg ratio was the combination IL2Cx plus anti-CTLA-4 (Fig. 6F). Overall, these data indicate that intratumoral NK cells are sensitive to IL2Cx, which induces their accumulation, division, maturation, and activation, and it is the combination of IL2Cx with anti-CTLA-4 that maximizes this effect. In view of these results and on our previous observation of the anti-CTLA-4-mediated depletion on Tregs, the increase in the NK cell/Treg ratio observed with the IL2Cx/anti-CTLA-4 combination can be explained by the fact that IL2-boosting effect on NK cells is potentiated in the absence of Tregs, which both compete for IL2

**IL2Cx plus anti-CTLA-4 therapeutic effect depends on NK and CD8+ T cells**

Our results suggested that the additive effects of the two combinations could involve different cellular effectors. Thus, to
evaluate the relative contribution of NK and CD8\(^+\) T cells on the therapeutic efficacy of the combined treatments, we performed Ab-mediated selective depletion (Fig. 7A). We verified the efficacy of depletion by periodic immunomonitoring of CD8\(^+\) T and NK cells in the blood (Supplementary Fig. S7). Figure 7B–D illustrates the effect of NK or CD8\(^+\) T-cell depletion on the efficacy of the combination treatments. In mice receiving the IL2Cx/anti–CTLA-4 combo, both depletion of CD8\(^+\) T or of NK cells abrogated treatment efficacy (Fig. 7B and C). In mice receiving the IL2Cx/anti–PD-1/anti–PD-L1 combo, depletion of CD8\(^+\) T cells completely abrogated the therapeutic effect of the treatment. However, although NK cell depletion induced a nonstatistically significant diminution in the efficacy of the combo, the treatment still delayed tumor growth (Fig. 7B–D). Overall, these results indicate that both combinations work through CD8\(^+\) T-cell activation, but only the IL2Cx/anti–CTLA-4 combination is dependent on NK cells to be effective.

**Discussion**

Successful anticancer immunotherapies aimed at inducing effective antitumor T-cell responses have delivered impressive clinical results, but only in some patients and in some tumor types (34, 35). Combinatory strategies represent
powerful therapeutic tools (36). Thus, we evaluated the efficacy of combining a T-cell growth factor, IL2, formulated as an IL2/anti-IL2 complex, with clinically approved immune-checkpoint inhibitors. In an autochthonous lung adenocarcinoma model, we show that the IL2Cx/anti–PD-1 combination durably controls tumor growth and induces lung infiltration by activated CD8\(^+\) T cells. Also, in a transplantable melanoma model refractory to monotherapy with immune-checkpoint blockers we found that IL2Cx synergizes with anti–CTLA-4 and anti–PD-1/anti–PD-L1, improving the clinical outcome of the monotherapies. In addition, we show that combination of IL2Cx with PD-1 or CTLA-4 pathway blockade acts by different cellular mechanisms, differentially targeting CD8\(^+\) T, NK, and Tregs.

High-dose IL2 was the first immunotherapy assessed for the treatment of human cancer, achieving similar objective responses in metastatic melanoma as anti-CTLA-4 treatment (15% refs. 37, 38). However, fewer than 10% of eligible patients receive this potentially curative treatment, likely due to its associated toxicity and need for hospitalization (3, 39), as well as concern about the expansion of Tregs. Consequently, different approaches have been tried to bring IL2 back to the clinics, trying to bypass Treg activation and to improve IL2 pharmacodynamics; and some of these approaches are under clinical evaluation. These strategies include, among others, a mutated IL2 superkine with increased affinity for IL2R\(\gamma\), a mutein IL2 fused to tumor-specific Abs (40), an IL2 bound to releasable polyethylene glycol chains (41); and IL2Cx (4) that have longer half-life and improved pharmacodynamics. Our results, showing that administration of either the IL2Cx that activates effector cells, low doses of IL2 (that could be released upon dissociation from anti-IL2), or even an IL2Cx designed to preferentially stimulate Tregs (42–44), did not affect tumor growth, casting doubt on a potential unwanted stimulation of Tregs. Consequently, IL2-based therapeutic approaches in general, and IL2Cx, especially in combination with immune-checkpoint blockers, may be useful in addition to the existing arsenal of antitumor immunotherapies.

Few studies have evaluated the efficacy of immunomodulatory treatments in genetic mouse models. In a model of spontaneous skin melanoma (Nra\(\delta^{Q61K}\) Ink4a\(^{+/−}\)), IL2Cx have shown some efficacy as monotherapy (7) and greater efficacy when combined with an epigenetic modulator (8). In another model of autochthonous melanoma (Braf/Pten model), antitumor effects required a multidrug therapy including a tumor antigen–targeting antibody, IL2, anti–PD-1, and a T-cell vaccine, which activated innate and adaptive immune responses (45). Finally, another study using a genetic model of lung adenocarcinoma showed that the KP-OVA lung adenocarcinoma model, which was non–T-cell infiltrated, was refractory to an anti–PD-1 plus anti–CTLA-4 combination therapy, and only combination of these two mAbs with immunogenic drugs oxaliplatin and cyclophosphamide induced antitumor CD8\(^+\) T cells and controlled tumor progression (46). In light of these results underscoring the elevated resistance of this tumor model to checkpoint blockade therapy, our data showing that IL2Cx increases the sensibility of autochthonous lung tumors to anti–PD-1 provide proof of concept of the efficacy of this combined therapy for lung tumors. The lack of observed toxicity, plus the obtained mechanistic insight on the mechanism of action of this therapeutic combination that increases tumor infiltration by CD8\(^+\) T cells and induces systemic tumor-specific CD4\(^+\) and CD8\(^+\) T-cell responses, provides a rationale for the design of combination therapies that would increase the response rate of patients to immune-checkpoint therapy.

Blockade of the inhibitory PD-1/PD-L1 pathway has been reported to reinvigorate chronically stimulated exhausted CD8\(^+\) T cells during lymphocytic choriomeningitis virus (LCMV) infection (47, 48). Similar results were later observed during cancer immunotherapy in mice models and in patients treated with mAbs to PD-1 (49, 50). The benefits of combining IL2 with PD-1/PD-L1 blockade were explored in the LCMV model, in which IL2 synergized with anti–PD-L1 treatment, improving CD8\(^+\) T-cell viral-specific responses and decreasing viral load (21). Our results show that IL2 (in the form of IL2Cx) can synergize with immune-checkpoint blockade to rescue CD8\(^+\) T cells function in cancer. Indeed, IL2Cx increased the breadth of CD8\(^+\) T-cell responses, including reactivities against tumor neoepitopes, boosted CD8\(^+\) T-cell division, and reinvigorated exhausted CD8\(^+\) TILs. This action was reinforced by an increase in tumor infiltration by CD45\(^+\) cells and CD8\(^+\) activated T cells when IL2Cx was combined with blockade of immune checkpoints.

CTLA-4 blockade during cancer is associated with increased CD8\(^+\) T cell/Treg ratios and tumor rejection. Vaccination of B16-bearing mice with a GM-CSF–transduced tumor cell vaccine (GVAX) induced T-cell activation and infiltration, an effect potentiated by CTLA-4 blockade (24). Additional blockade of the PD-1/PD-L1 pathway allowed further tumor-specific T-cell activation (51). The antitumoral activity of anti–CTLA-4 required binding of the Ab to Fcγ receptors, which mediate the reduction of Tregs at the tumor site (22, 52). In the preclinical cancer setting, one study reported CTLA-4 blockade combined with IL2 administration (53). Although this work used IL2 (and not IL2Cx) and different doses and treatment timing (beginning of treatment at day 3 after tumor implantation) than our study, they observed, as we did, a synergistic effect of the combination on tumor growth delay and on the increase in tumor-infiltrating CD8\(^+\) T and NK cells. However, they did not observe Treg depletion when an mAb to a–CTLA-4 was combined with IL2, indicating that CTLA-4 mAb–mediated Treg depletion can be differently modulated when administered with plain IL2 or with IL2 in the form of IL2Cx. Given our results suggesting that CTLA-4 mAb–mediated depletion correlates with the high expression of CTLA-4 on Tregs, it could be interesting to compare CTLA-4 kinetics of expression on intratumoral Tregs during these different therapeutic interventions.

The combination of radiation with CTLA-4 and PD-L1 blockade in both mice and human works by an increase in the CD8\(^+\) T cell/Treg ratio induced by CTLA-4 blockade, and by reversion of T-cell exhaustion by PD-L1 blockade; radiation contributed to enhanced diversity of the tumor T-cell repertoire (20). Here, we observed that (i) the IL2Cx/anti–PD-1/ PD-L1 combination worked through increasing tumor infiltration by reinvigorated CD8\(^+\) T cells; and (ii) that the IL2Cx/ anti–CTLA-4 therapy was the only combination dependent upon CD8\(^+\) T and NK cells, as depletion of either of these cells disabled the antitumoral effect of this combination. Indeed, we report that only the IL2Cx/anti–CTLA-4 combination induced an increased NK cell/Treg ratio in the tumor, which was due to the diminution of tumor-infiltrating Tregs (likely
meditated by anti–CTLA-4), and the accumulation of NK cells (likely mediated by the exogenously administrated IL2Cx and the increased availability of endogenous IL2 secondary to decreased consumption by Tregs). During homeostasis, depletion of Tregs using an anti-CD25 can boost NK cell proliferation and cytotoxicity in vivo (31). Gasteiger and colleagues (27) proposed that Tregs can suppress mature NK cell function via TGFβ and restrain NK cell maturation by IL2 consumption. These two works support our hypothesis that CTLA-4 mAB–mediated Treg depletion in the tumor facilitates NK cell accumulation and maturation.

B16F10 melanoma tumors recruit CD27+CD11b+ NK cells with low proliferative capacity, reduced KLRG1 and NKGD2 expression, and low IFNγ and GM-CSF production (54), suggesting reduced effector functions. Our results showed that IL2Cx alone or combined with immune-checkpoint blockers also increased the frequency of CD27+CD11b+ NK cells, which represented the predominant NK subset in the tumor. However, the phenotype of NK cells was modified by the treatments, as KLRG1 and NKGD2 expression was increased, and proliferation and IFNγ production was enhanced, likely overcoming the inhibitory phenotype induced by the tumor described by these authors. Overall, these results suggest that depletion of Tregs with concomitant IL2-mediated activation of NK cells could complement immune-checkpoint blockade for cancer therapy.

In conclusion, our data suggest that IL2-Cx acts on T and NK cells by boosting their expansion and effector function. Blockade of the PD-1/PD-L1 pathway reinforces IL2Cx-induced reinvigoration of exhausted CD8+ T cells. Anti–CTLA-4 also acts by depleting Tregs, which in turn affects CD8+ T and NK cells, increasing the CD8+ T cell/Treg and NK cell/Treg ratios and unleashing the effector cells from Treg suppression. Although activation of CD8+ T cells accounts for most of the efficacy of the IL2Cx/PD-1 pathway blockade combination treatment, CD8+ T-cell reinvigoration by the IL2Cx-anti–CTLA-4 combination is not sufficient to control tumor progression, as the latter combination was the only one dependent on NK cell activation. Thus, our results clarify the cellular and molecular consequences that follow from the combination of IL2Cx with CTLA-4 or PD-1/PD-L1 blockade, and should help refine combinatorial cancer immunotherapies.

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

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