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IL-12 and Type I IFN differentially program CD8 T cells for PD-1 re-expression levels and tumor control

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Abstract

Naïve CD8 T cells proliferate in response to TCR and CD28 signals, but require IL-12 or Type I IFN to survive and develop optimal effector functions. Although murine CTL generated in vitro in response to IL-12 or IFN- α had comparable effector functions, IL-12-stimulated cells were significantly more effective in controlling tumor in an adoptive immunotherapy model. They maintained high numbers and function, while IFN- α -stimulated cells declined in number and became exhausted. Consistent with this, IFN- α -stimulated cells in the tumor expressed higher levels of PD-1 inhibitory receptor than did IL-12-stimulated cells. When blocking Ab specific for the PD-L1 ligand of PD-1 was administered, the efficacy of IFN- α -stimulated CTL became comparable to that of IL-12-stimulated cells. Thus, IL-12 and IFN- α differentially program CD8 T cells to re-express distinct levels of PD-1 upon re-encountering Ag, resulting in IL-12-stimulated cells being less susceptible to exhaustion in the face of sustained tumor Ag.

INTRODUCTION

Activated CD8 T cells express inhibitory receptors that can limit responses to viruses or tumors, the best characterized being Programmed Death 1 (PD-1; CD279). PD-1 belongs to the CD28/CTLA-4 family of receptors and binds PD-L1 and PD-L2 ligands that are B7 family members (1–3). Blocking interactions of PD-1 with its ligands can increase CD8 T cell responses to chronic virus infections and tumors (3–5), and PD-1 and PD-L1 antibody therapy has demonstrated significant tumor regression in human trials (6, 7). PD-1 expression is upregulated by TCR binding to Ag and depends on NFATc1 (8), but the factors that regulate PD-1 expression levels and determine whether it mediates tolerance are poorly understood. As we show here, the extent to which effector CD8 T cells upregulate PD-1 expression when they re-encounter Ag differs depending upon whether they have differentiated in response to IL-12 or Type I IFN signals.

Naive CD8 T cells responding to Ag and costimulation develop only weak effector functions unless an inflammatory cytokine(s) is available to signal for differentiation to strong effector status (9). IL-12 and/or Type I IFN (IFN- α/β) are the major cytokines that provide this third signal for responses to transplanted tissues, viral and bacterial pathogens, and tumors (9–11). IL-12 and IFN- α stimulate development of comparable effector activities (12), consistent with both cytokines stimulating a common program of regulation of about 350 genes, many of which encode proteins known to be critical for effector functions, including grzB, T-bet,

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Eomes, FasL, etc. (12). However, each cytokine also uniquely regulates expression of 200 to 300 genes, raising the possibility that in vivo properties of effector cells may differ depending on which signal 3 cytokine drove their differentiation. To begin to examine this, we compared effector cells generated in the presence of IL-12 or IFN- α for their ability to control tumor growth in an adoptive tumor immunotherapy model. This provides an excellent model to examine the differential effects of the cytokines since it allows generation of CTL under well-defined in vitro conditions followed by assessment of the in vivo migration and function of the effector cells. The results described here demonstrate that in comparison to IFN- α , programming differentiation with IL-12 results in decreased levels of PD-1 on CD8 T cells in the tumor and increased efficacy of the cells in controlling tumor growth, strongly suggesting that provision of IL-12 during the in vitro generation of cells for adoptive immunotherapy will improve outcomes.

MATERIALS AND METHODS

Mice, tumor cell line and reagents

OT-I.PL mice were generated by crossing OT-I mice with Thy1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (The Jackson Laboratory) and C57BL/6NCr and CD45.1-congenic B6 (B6.Ly5.2) mice were from the National Cancer Institute. Experiments were conducted under specific pathogen-free conditions and performed in compliance with relevant laws and guidelines and with approval of the Institutional Animal Care and Use Committee at the U. of Minnesota. B16.OVA (OVA transfected B16.F10) cells were maintained in complete RPMI 1640 medium with 800 μ g/ml G418. Abs were purchased from Biolegend, BD Biosciences, eBioscience, or Invitrogen. Anti-PD-L1 mAb for in vivo use (clone M1H6) was generated as previously described (13). Mice received 100 μ g (i.p.) of anti-PD-L1 or isotype control IgG1 (Jackson ImmunoResearch) on the same day as OT-I-CTL cells transferred, and then every 3 days for a total of 4 injections.

In vitro stimulation and adoptive transfer of naïve OT-I T cells

Naïve OT-I or OT-I.PL T cells were purified from LN by negative selection (Supplemental Fig. 1A). Cells were stimulated in flat-bottom microtiter wells having DimerX H-2K^b:Ig fusion protein (BD Biosciences) loaded with OVA₂₅₇₋₂₆₄ peptide (New England Peptide) and rB7-1/Fc chimeric protein (R&D Systems) immobilized on the surface (14). $1-5 \times 10^5$ cells in 0.2 ml of complete RPMI 1640 medium were placed in wells, and 2.5 U/ml IL-2 (R&D Systems) added. Where indicated, 2 U/ml murine rIL-12 (R&D Systems), 1000 U/ml murine IFN- α (PBL Biomedical Laboratories) or both were added. These cytokine concentrations stimulate optimal development of effector functions (Fig. S1). Cells were harvested on day 3 and adoptively transferred (tail vein).

Tumor growth and flow cytometric analysis of T cells

Mice were injected s.c. (flank) with 2×10^5 B16.OVA cells, and growth monitored by determining area as the product of two right angle measurements with calipers. Tumor-bearing mice received activated OT-I T cells by adoptive transfer on the day indicated (day 6 – 10 of tumor growth). Mice were sacrificed at the indicated times, spleens and tumors homogenized, and lymphocytes in tumor homogenates enriched on Percoll (GE Healthcare) gradients. OT-I and OT-I.PL cells were identified by staining for CD8 α and congenic markers Thy1.1 and/or CD45.2, and cell counts obtained using PKH26 reference microbeads (Sigma-Aldrich). Samples were stained for surface expression of PD-1, and separate samples were incubated for 4 hr with OVA₂₅₇₋₂₆₄ peptide and then permeabilized and stained for IFN- γ and IL-2. Statistical significance was determined using two-tailed Student's t tests.

RESULTS AND DISCUSSION

Effector CTL generated in response to IL-12 control tumor more effectively than CTL generated in response to IFN- α

When naïve CD8 T cells are stimulated in vitro with Ag and B7-1, either IL-12 or IFN- α stimulates development of strong effector functions by day 3 (12) and programs the cells to develop a protective memory population if they are then transferred into normal mice (15). To assess the effects of the cytokines on the ability of effector cells to control tumors, purified naïve (CD44^{low}) OT-I T cells (Supplemental Fig.1A) were stimulated for 72hr in microtiter wells having immobilized H-2K^b/OVA₂₅₇₋₂₆₄ and B7-1/Fc in the presence of either IL-12 (IL-12-CTL) or IFN- α (IFN- α -CTL). The cells were then adoptively transferred (tail vein) into mice having progressing B16.OVA tumors (B16 melanoma expressing full-length OVA) growing subcutaneously. In the experiment shown in Fig. 1, mice received tumor on day 0 and activated OT-I cells were transferred 6 days later. Despite comparable cytolytic activity and IFN- γ production at 72 hours (Supplemental Fig. S1B), IL-12-CTL were much more effective in controlling tumor growth over the next 1 to 2 weeks than were IFN- α -CTL (Fig. 1A). This differential control of tumor was reproducibly seen in numerous experiments (see below), and comparing growth following transfer of differing numbers of CTL indicated that the IL-12-CTL are greater than 5-fold more effective than the IFN- α -CTL (Fig. 1A and not shown).

IFN- α -CTL, but not IL-12-CTL, become 'exhausted' during tumor growth

OT-I effector cells increased in number in the tumor over the first 3 days post transfer, and numbers were similar for IL-12 and IFN- α effectors up to about 7 days post-transfer (day 13, Fig. 1B). Thus, differing efficacy does not result from differential ability of the effector cells to traffick to the tumor. Beyond day 13, the number of IFN- α -CTL in the tumor declined, while the number of IL-12-CTL remained high. In fact, when expressed as total OT-I per tumor volume (i.e. density of OT-I in the tumor mass), the IL-12-CTL remained constant for at least 14 days post transfer, while IFN- α -CTL declined over 100-fold as the total number of CTL declined and the tumors continued to grow (Fig. 1C).

When the functional capacities of the OT-Is in the tumor were assessed by their ability to produce IFN- γ upon ex vivo restimulation, IL-12-CTL and IFN- α -CTL were comparable on days 8 and 10 (days 2 and 4 post transfer). However, by day 13 most of the IFN- α -CTL no longer produced IFN- γ while a large fraction of the IL-12-CTL retained activity (Fig. 1D). Few if any of the IL-12-CTL or IFN- α -CTL produced detectable IL-2 over the first few days post transfer (Fig. 1E), but by day 13 the IL-12-CTL were regaining the ability to produce IL-2 while the IFN- α -CTL were not. Thus, the IFN- α -CTL not only decline in number in comparison to the IL-12-CTL, they undergo increased functional impairment over time in the tumor environment.

Intratumoral IL-12-CTL and IFN- α -CTL differentially express PD-1

The declining numbers and loss of functional capacities of the IFN- α -CTL resembled the exhaustion that CD8 effector CTL undergo in the face of chronic virus infections and tumors. In virus and tumor models considerable evidence has accumulated to implicate the inhibitory receptor PD-1 (CD279) in development of exhaustion (1, 3). Furthermore, the B16 melanoma tumor expresses PD-L1 (B7-H1; CD274), a ligand for PD-1 (16). We therefore examined the expression levels of PD-1 on IL-12-CTL and IFN- α -CTL.

Naïve CD8 T cells do not express PD-1, but it is upregulated within 72 hours of in vitro stimulation with Ag/B7 alone, and levels do not differ when either IL-12 or IFN- α are present (Fig. 2A). This is consistent with oligonucleotide microarray analysis that showed

mRNA levels for PD-1 to be strongly up-regulated by just Ag and B7-1 signals, and not changed when the cytokines are present (12). Thus, PD-1 expression is comparably high on IL-12-CTL and IFN- α -CTL at the time of transfer into tumor bearing mice. Within 4 days of transfer, the OT-I CTL in the spleens of the mice have down-regulated PD-1 expression (Fig. 2B and D), as expected for cells no longer exposed to Ag. In contrast, PD-1 expression is high on intratumoral OT-I cells, and is at a significantly higher level on IFN- α -CTL than on IL-12-CTL (Fig. 2C and D). By 14 days post transfer (day 20) the number of IFN- α -CTL had declined substantially in comparison to the IL-12-CTL (Fig. 2E). In additional experiments, we found that PD-1 expression was also higher on IFN- α -CTL when five-fold higher concentrations of the cytokines were used for the in vitro stimulation (Fig. S2).

Differential expression of PD-1 might result from the cytokines mediating differential programming for receptor upregulation upon Ag re-encounter. Alternatively, other functional differences between the IL-12 and IFN- α stimulated cells might account for differing control of tumor growth, with PD-1 expression being secondarily affected. To address this, IL-12-CTL and IFN- α -CTL having differing congenic markers were transferred in equal numbers into B16.OVA-bearing mice. Six days later the tumors were smaller in mice that received 5×10^6 IL-12-CTL than in mice that received 5×10^6 IFN- α -CTL, and tumors were of intermediate size when 2.5×10^6 of each type of CTL (Mix(IFN- α -CTL+IL-12-CTL) was transferred into the same mouse (Fig. 3A). As expected, intratumoral OT-I cells in mice that received just IL-12-CTL had lower PD-1 expression than those in mice that received just IFN- α -CTL (Fig. 3B). For mice that received both types of CTL, congenic markers were used to distinguish IL-12-CTL (Mix IL-12 CTL) from IFN- α -CTL (Mix IFN- α CTL) in the tumors, and the IL-12-CTL still exhibited significantly lower PD-1 expression than did IFN- α -CTL (Fig. 3B). Thus, OT-I effector cells in the same tumor express lower or higher levels of PD-1 depending upon whether they initially responded to IL-12 or IFN- α .

We also examined effector cells generated in the presence of both IL-12 and IFN- α (IFN- α /IL-12-CTL). Upon adoptive transfer these cells controlled tumor growth comparably to cells stimulated with Ag/B7 and IL-12, and more effectively than cells stimulated with Ag/B7 and IFN- α (Fig. 3A). Expression of PD-1 on IFN- α /IL-12-CTL in the tumors was low, comparable to levels on IL-12-CTL and lower than on IFN- α -CTL (Fig. 3B). Thus, the IL-12-dependent PD-1^{low} phenotype predominates when both IL-12 and IFN- α are present to drive differentiation in response to Ag/B7.

Peptide Ag stimulates differential levels of PD-1 expression on IL-12- versus IFN- α -CTL in the absence of tumor

To confirm that IL-12 and IFN- α differentially program cells for PD-1 re-expression, and further rule out the potential of other effects in the tumor microenvironment, we examined a simpler model that avoids the complexity of an anti-tumor response. Normal mice received either IL-12-CTL or IFN- α -CTL by adoptive transfer, and 4 days later groups were challenged by i.v. injection of 10 ug of OVA₂₅₇₋₂₆₄ peptide Ag in PBS. OT-I cells were then examined 24 hours later for PD-1 expression in comparison to levels on cells from mice that did not receive Ag. PD-1 expression is high at the time of cell transfer, but declines to almost naïve levels after 4 days in vivo in the absence of Ag (Fig. 3C). PD-1 is re-expressed in response to Ag challenge, and the level is substantially higher on IFN- α -CTL than on IL-12-CTL. Here too, cells stimulated with both IL-12 and IFN- α have the same low PD-1 level as cells stimulated with just IL-12 (not shown). Thus, IL-12 and IFN- α differentially program cells during the first 3 days of differentiation to re-express PD-1 to differing levels when the cells subsequently re-encounter Ag, and the PD-1^{low} IL-12 phenotype dominates when both cytokines are present.

Blocking PD-L1 enhances tumor control by IFN- α -CTL

The above results suggested that the markedly reduced efficacy of IFN- α -CTL in controlling tumor might be a consequence of the higher PD-1 re-expression on these cells. To examine this, we determined the effects of blocking PD-1 interaction with its ligand PD-L1 that is expressed by B16 melanoma (16). Anti-PD-L1 mAb treatment of tumor-bearing mice that did not receive OT-I cells by adoptive transfer did not significantly affect tumor growth (Fig. 4), i.e. it was not sufficient to allow an effective response by endogenous cells. Similarly, anti-PD-L1 treatment did not significantly reduce the moderate tumor control achieved by a low number (1×10^5 /mouse) of IL-12-CTL, suggesting that the relatively low level of PD-1 expressed by the IL-12-CTL does not significantly decrease their efficacy. In contrast, 1×10^6 IFN- α -CTL alone did not control tumor, but controlled growth very effectively in combination with anti-PD-L1 mAb treatment; as effectively as the same number of IL-12-CTL. These results support the conclusion that the reduced efficacy of CTL that have differentiated in response to IFN- α in comparison to IL-12-CTL is due primarily to the increased level at which PD-1 is re-expressed when the cells encounter Ag in the tumor.

PD-1 is upregulated when T cell activation results in translocation of NF-ATc1 (NFAT2) to the nucleus where it interacts with the *pdcd1* promoter (8). Upon transition of naïve to effector cells the *Pdcd1* locus is demethylated to facilitate transcription, and is remethylated as Ag is cleared, but remains demethylated in exhausted CD8 T cells in chronic virus infections (17). Thus, a potential mechanism for differential regulation of PD-1 re-expression might be differences in the level of demethylation/remethylation of the *pdcd1* locus by IL-12 versus IFN- α . In our experiments, IFN- α does not increase expression of PD-1 on primary effector cells, but does program the cells to subsequently re-express higher levels of PD-1 upon re-encountering Ag. Terawaki et.al. (18) also reported that IFN- α does not increase PD-1 levels during initial priming of T cells, but showed that when it is provided beyond the peak of the primary response it has the effect of maintaining PD-1 expression at a higher level. A post-programming effect of IL-12 on PD-1 expression has also been observed. Schurich et.al. (19) showed that treatment of exhausted human HBV-specific T cells with IL-12 results in down-regulation of PD-1 expression and recovery of effector functions, suggesting that PD-1 re-expression levels programmed during priming can be altered by an IL-12 signal at later times in the response. Whether these late effects of signal 3 cytokines involve similar mechanisms to those of the differential programming that occurs during priming, as shown here, remains to be determined.

Adoptive cell therapy for treatment of cancers holds considerable promise (20), and numerous studies have evaluated methods for activating and expanding tumor-specific CD8 T cells in vitro for adoptive transfer, including the use of various cytokines. Differentiation of naïve CD8 T cells to acquire optimal effector functions and develop memory requires a signal from an inflammatory cytokine, and either IL-12 or Type I IFN provides this signal (9–11), suggesting that CTL generated using either might be equally effective in controlling tumor. The results described here demonstrate that this is not the case. CTL generated in response to IL-12 are much more effective as a result of the fact that they do not upregulate PD-1 expression to as high a level upon re-encountering Ag as do CTL generated in response to IFN- α . Thus, including IL-12 during activation of CD8 T cells for adoptive immunotherapy is likely to yield cells with greater efficacy. Furthermore, the IL-12-driven PD-1^{low} phenotype dominates when both cytokines are present, obviating any need to eliminate Type I IFN signaling during generation of the effectors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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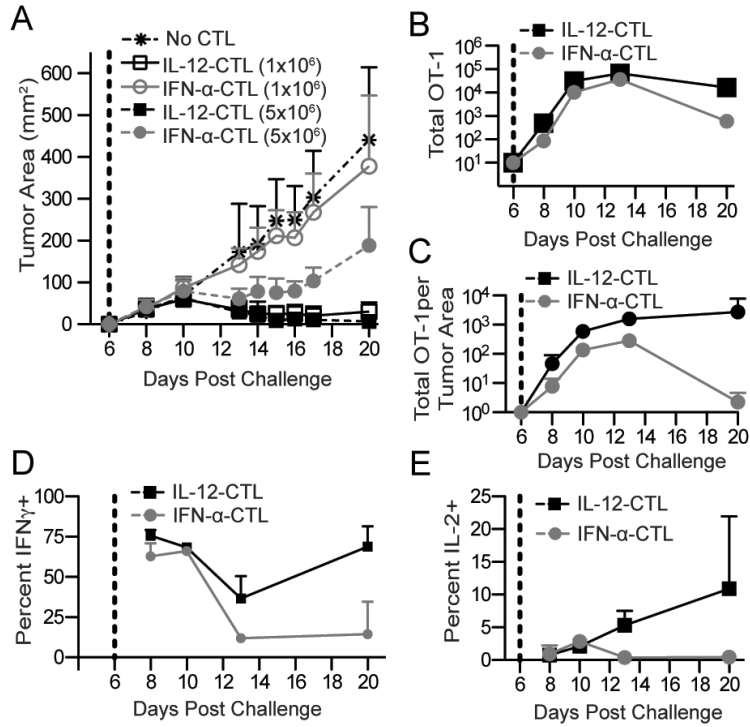


Figure 1. CD8 T cells that differentiate in response to IFN- α control tumor less well than IL-12 differentiated CTL

B16.OVA cells (2×10^5) were injected s.c. (flank) on day 0. Six days later mice received either no CTL, or CTL (i.v. tail vein) that had been stimulated for 3 days in vitro in the presence of IL-12 (IL-12-CTL) or IFN- α (IFN- α -CTL). (A) Tumor growth expressed as area. (B) Numbers of OT-I T cells in tumors. (C) Density of OT-I cells in tumors (number per mm² of tumor area). (D) IFN- γ production by OT-I cells isolated from tumors and stimulated ex vivo with Ag for 4 hr. (E) IL-2 production by OT-I cells isolated from tumors and stimulated ex vivo with Ag for 4 hr. Results in all panels are shown as average \pm SD for 3–4 mice/group.

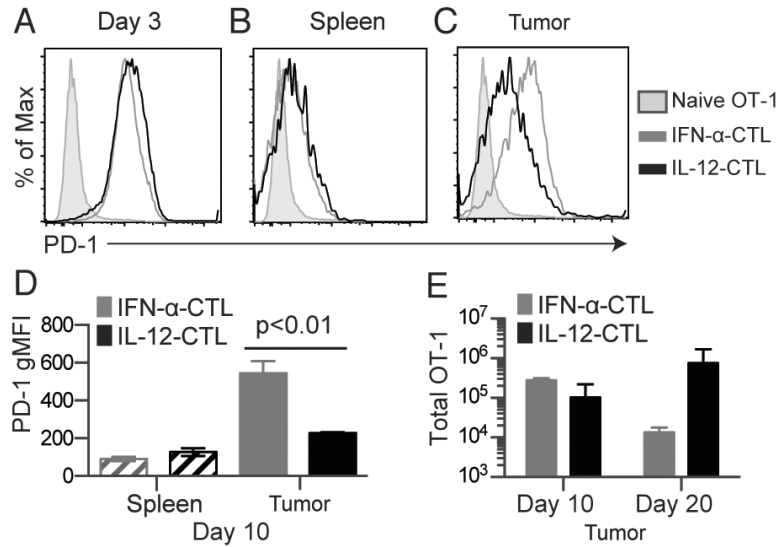


Figure 2. PD-1 is expressed at higher levels on IFN- α -differentiated CTL when they re-encounter Ag in the tumor

(A) Naïve OT-1 cells were stimulated for 3 days in vitro in the presence of IL-12 (IL-12-CTL) or IFN- α (IFN- α -CTL) and PD-1 expression determined. (B and C) Cells stimulated as in (A) were transferred into mice that had 6 day old B16.OVA tumors. 4 days later PD-1 expression on OT-I cells from spleens and tumors was determined. Representative histograms (2–3 mice/group) are shown. (D) Cells were stimulated in vitro as in (A) and transferred at 5×10^6 cells/mouse into mice that had received B16.OVA tumor 6 days earlier. PD-1 expression was determined 10 days later on OT-I cells in the spleens and tumors and results are shown as mean fluorescence intensity (MFI; geo mean) \pm SD (2–3 mice/group). Significantly higher PD-1 expression on IFN- α -CTL versus IL-12-CTL in tumors was seen in 5 independent experiments when examined at varying times post transfer. (E) Numbers of OT-I cells in tumors of mice from panel D, shown as average \pm SD (2–3 mice/group) on days 10 and 20.

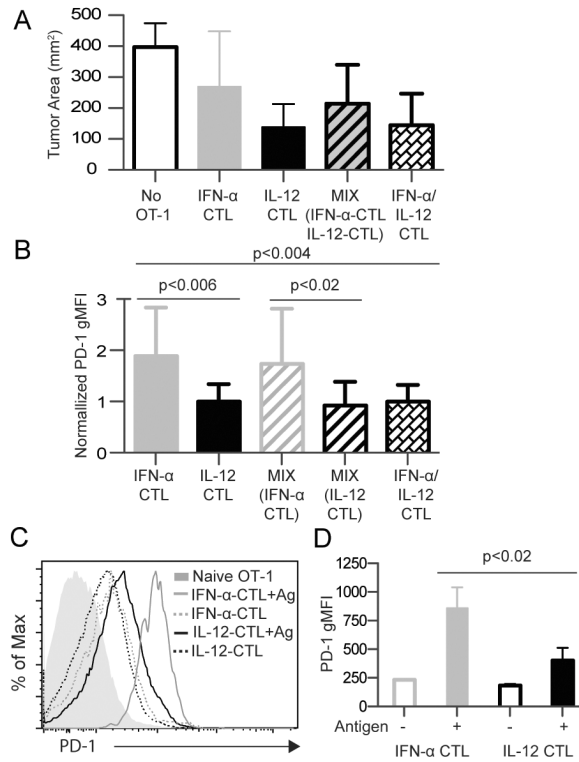


Figure 3. IL-12 and IFN- α differentially program naive CD8 T cells for PD-1 re-expression
 OT-I cells were stimulated for 3 days *in vitro* with IL-12 alone (IL-12-CTL), IFN- α alone (IFN- α -CTL), or IFN- α and IL-12 together (IFN- α /IL-12-CTL), and were transferred into mice having progressing B16.OVA tumors. Groups received either no cells, 5×10^6 IL-12-CTL (CD45.2⁺Thy1.1⁻), 5×10^6 IFN- α -CTL (CD45.2⁺Thy 1.1⁺), or 5×10^6 IFN- α /IL-12-CTL. In addition, one group of mice received 2.5×10^6 /mouse of IL-12-CTL and 2.5×10^6 /mouse of IFN- α -CTL (Mix(IFN- α -CTL IL-12-CTL)). (A) Tumor area was determined 6 days after transfer of cells into mice having 12 day old tumors. Results are average \pm SD (3–5 mice/group) of tumor area. (B) PD-I expression of transferred OT-I cells in tumors. Results are pooled for 3 independent experiments by normalizing to the PD-1 MFI on IL-12 CTL in each experiment, and are expressed as average normalized MFI \pm SD. For groups that received both IL-12- and IFN- α -CTL (Mix), the OT-I cells were distinguished based on Thy 1 expression, and PD-1 expression is shown separately for the IL-12-CTL and the IFN- α -CTL. (C) IL-12-CTL or IFN- α -CTL were transferred into normal mice (5×10^6 cells/mouse). 4 days later groups of mice were challenged by *i.v.* injection of 10 μ g of OVA_{257–264} peptide Ag in PBS. One-day later PD-1 expression on OT-I cells in the spleens was determined and compared to controls that did not receive Ag. (D) PD-1 expression is shown as average MFI \pm SD for groups in (C). Similar results were obtained in 6 independent experiments.

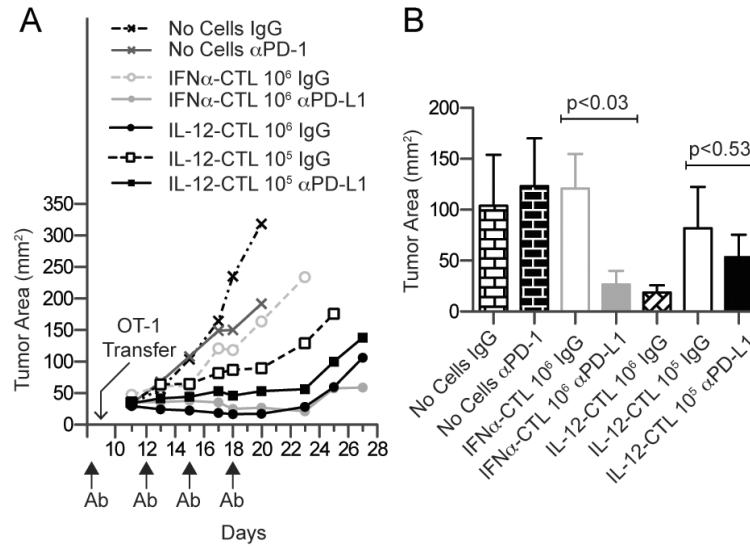


Figure 4. PD-1 blockade increases the efficacy of IFN- α -differentiated CTL in controlling tumor growth

(A) Mice were challenged with B16.OVA (day 0) and in vitro activated OT-I cells transferred into mice on day 9 (3–5 mice/group). Groups received either control IgG or anti-PD-L1 mAb (100 μ g/mouse i.p.) on the days indicated by arrows. Tumor area was determined, and analysis of a group was stopped when one or more mice developed a tumor larger than 300 mm². (B) Tumor size (average \pm SD) on day 17 for experiment in panel (A), with p values determined by Student's t test. Differences between IFN- α -CTL treated with IgG versus anti-PD-L1 mAb remained significant through day 23, while the differences between IL-12-CTL with IgG versus anti-PD-L1 were not significant at any time. Combined analysis of two independent experiments (n=7 to 9 mice/group) showed that anti-PD-L1 treatment controlled tumors better than control IgG treatment in mice that received IFN- α -CTL (p values were 0.013, 0.028 and 0.028 on days 19, 23 and 27 respectively). In contrast, for mice that received IL-12-CTL, anti-PD-L1 treatment was not significantly different from control IgG treatment at any time point. In addition, tumor control by IL-12-CTL in IgG treated mice was not significantly different from that of IFN- α -CTL in mice treated with anti-PD-L1 mAb.