Amlexanox enhances the antitumor effect of anti-PD-1 antibody

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ABSTRACT

Cancer immunotherapy, especially treatment with monoclonal antibodies (mAbs) that block programmed cell death-1 (PD-1)/programmed cell death-ligand 1 (PD-L1) signaling, has attracted attention as a new therapeutic option for cancer. However, only a limited number of patients have responded to this treatment approach. In this study, we searched for compounds that enhance the efficacy of anti-PD-1 mAb using mixed lymphocyte reaction (MLR), which is a mixed culture system of the two key cells (dendritic and T cells) involved in tumor immunity. We found that amlexanox enhanced production of interferon (IFN)-γ, an indicator of T cell activation, by anti-PD-1 mAb. Amlexanox also induced PD-L1 expression in dendritic cells in MLR, whereas it did not stimulate interleukin-2 production by Jurkat T cells. These results suggest that amlexanox acts on dendritic cells, not T cells, in MLR. Furthermore, it enhanced the antitumor effect of the anti-PD-1 mAb *in vivo* in a mouse tumor-bearing model. The combination of amlexanox and anti-PD-1 mAb increased the expression of *Ifng* encoding IFN-γ, IFN-γ-related genes, *Cd274* encoding PD-L1, and cytotoxic T cell-related genes in tumors. In conclusion, amlexanox stimulates the antitumor effect of anti-PD-1 mAb by acting on dendritic cells, which in turn activates cytotoxic T cells in tumors.

Keywords: Cancer; Cancer immunotherapy; Cytotoxic T cell; Immunity; Interferon-γ.

1. Introduction

Cancer immunotherapy, including blockade of the programmed cell death-1 (PD-1)/ programmed cell death-ligand 1 (PD-L1) pathway, has a therapeutic effect and can extend survival in some patients who have become resistant to other therapies. PD-1 is a type-1 membrane protein belonging to the CD28 family that is expressed on the surface of activated T lymphocytes [1]. PD-1 binds to the PD-1 ligands (PD-L1 and PD-L2) expressed on antigen-presenting cells and negatively regulates activated lymphocytes [2-4]. PD-1 ligands are also expressed in various human tumor tissues, and their expression is negatively correlated with prognosis in several cancer types [5]. Inhibition of the cytotoxic activity of $CD8^+$ T cells by PD-L1 on cancer cells can be reversed by blocking the binding of PD-1 on T cells to PD-L1 by anti-PD-L1 or -PD-1 monoclonal antibodies (mAbs) [6,7]. It has also been reported that inhibition of the PD-1/PD-1 ligand pathway between dendritic cells (DCs) and T cells contributes to antitumor effect of anti-PD-1 mAbs, and the expression of PD-L1 on DCs is important for the efficacy of anti-PD-1 mAbs [8]. Although anti-PD-1/-PD-L1 mAbs are clinically approved for the treatment of several types of cancer (including melanoma, non-small-cell lung cancer, and renal cell carcinoma), their response rates against these cancers are only 20–40% [9]. Therefore, combination therapy with other drugs has been studied with the goal of enhancing the efficacy of anti-PD-1/-PD-L1 mAbs [9]. Mixed lymphocyte reaction (MLR), in which primary human DCs are co-cultured with T cells, is a useful system for assessing the DC-mediated activation of T cells. In MLR, it has been reported that anti-PD-1/-PD-L1 mAbs enhance interferon (IFN)-γ production, which is an indicator of T cell activation [10,11]. The effect was further enhanced by cotreatment with anti-cytotoxic T-lymphocyte (CTL)-associated protein 4 (CTLA-4) mAb or anti-lymphocyte-activation gene 3 (LAG-3) mAb [12].

In this study, we screened for compounds that could enhance the production of IFN-γ by

anti-PD-1 mAb in MLR and identified amlexanox. Combination therapy using amlexanox and anti-PD-1 mAb also produced a more potent antitumor effect than anti-PD-1 mAb monotherapy *in vivo* in a tumor-bearing mouse model. Our findings thus suggest that amlexanox can be used to stimulate the antitumor effect of anti-PD-1 mAb in clinical settings.

2. Materials and methods

2.1. Cell lines and culture

The murine colon cancer cell line MC38 (BioVector NTCC) and the human T cell leukemia cell line Jurkat (American Type Culture Collection) were cultured at 37 °C under 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM; Thermo Fisher Scientific) and RPMI 1640 Medium (Thermo Fisher Scientific), respectively, both supplemented with 10% fetal bovine serum (HyClone Laboratories), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone Laboratories).

2.2. MLR assay

$CD14^+$ cells were isolated from human peripheral blood mononuclear cells (HemaCare) using Human CD14 MicroBeads (Miltenyi Biotec). The isolated cells were differentiated into DCs in RPMI 1640 Medium (Thermo Fisher Scientific) containing 250 U/mL of granulocyte-macrophage colony-stimulating factor (R&D Systems), 500 U/mL of interleukin (IL)-4 (R&D Systems), 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Six days after differentiation, the DCs were activated for 24 h with 1 μg/mL of lipopolysaccharide (Merck). $CD4^+$ T cells were isolated from peripheral blood mononuclear cells derived from another donor using a Human $CD4^+$ T cell isolation kit (STEMCELL Technologies). The activated DCs were cocultured with the $CD4^+$ T cells in the presence of one of the 142 compounds listed in Supplementary Table S1 (all supplied by Selleck Biotech) and 3 μg/mL of anti-human PD-1 mAb (NDC-0003-3772-11; Bristol Myers Squibb). The cells were collected on day 2 for analysis of PD-L1 expression via flow cytometry (described below). On day 5, the culture supernatant was collected to measure the production of IFN-γ using a Human IFN-γ ELISA Kit (Dakewe) according to the manufacturer's protocol.

2.3. Measurement of IL-2 levels by ELISA

Jurkat T cells were treated with amlexanox or lenalidomide (both from Selleck Biotech) for 1 h and then stimulated by transferring them to dishes precoated with 5 μg/mL of anti-CD3 (clone OKT3; BioLegend) and incubating them in 10 μg/mL of soluble anti-CD28 mAb (clone CD28.2; BioLegend). Twenty hours after stimulation, supernatants were collected, and IL-2 levels were measured via ELISA (R&D Systems) according to the manufacturer's protocol.

2.4. Mice

Female C57BL/6 or BALB/c mice (6–8 weeks of age; Shanghai Laboratory Animal Center) were bred under specific pathogen-free conditions. Animal experiments were performed in accordance with the guidelines for animal experiments established by Ono Pharmaceutical. The animals were fed *ad libitum* with chow diet (Jiangsu Xietong Biology) and allowed free access to water.

2.5. Mouse tumor-bearing model

MC38 (1×10^6) cells suspended in 0.1 mL of phosphate-buffered saline were inoculated subcutaneously into the right flank of C57BL/6 mice. The mice were randomly assigned to the experimental groups when their tumor volume reached approximately 100 mm^3. Amlexanox (Selleck Biotech) and anti-mouse PD-1 mAb (clone RPM1-14; BioXcell) were administered orally and intraperitoneally, respectively. The doses were set at 25 or 30 mg/kg for amlexanox and 10 mg/kg for anti-PD-1 mAb, based on prior studies [13,14]. Every two or three days, tumor volume was evaluated by measuring the long diameter (l, in mm) and short diameter (s, in mm) with calipers, and was calculated as: $(l) \times (s)^2 \times 0.5$. The mice were euthanized in the middle of the experiment at a humane endpoint defined as tumor volume

exceeding 2,000 mm^3 or ulceration of the tumor mass. Tumor growth inhibition was defined as the ratio of the mean tumor volume in the experimental group to that in the vehicle group.

2.6. Analysis of mRNA expression

Tumor tissues were isolated, homogenized in RNAlater Stabilization Solution (Thermo Fisher Scientific), and stored at 4 °C until RNA extraction. RNA extraction was performed using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA expression was determined using an nCounter Mouse PanCancer Immune Profiling Panel (NanoString Technologies) according to the manufacturer's protocol. Raw data quality checking, data normalization, data analysis, and data output were performed using the software nSolver 3.0 Analysis (NanoString Technologies). Normalization was performed using the mean of the values of the following 15 housekeeping genes, which exhibit low variability in expression levels among samples: *Abcf1*, *Ercc3*, *Dnajc14*, *G6pdx*, *Polr2a*, *Psmc4*, *Pum1*, *Tfrc*, *Tbp*, *Tbc1d10b*, *Oaz1*, *Tmub2*, *Sf3a1*, *Mrpl19*, and *Nrde2*.

2.7. Flow cytometry

Cells were collected after treatment with trypsin-EDTA (Thermo Fisher Scientific), washed twice with staining buffer (BD Biosciences), and incubated at 4 °C for 20 min with an Fc receptor blocking solution (Human Trustain FcX block; BioLegend). The cells were incubated with Fixable Viability Stain 510 (BD Biosciences) to stain dead cells and with anti-human PD-L1 mAb (clone MIH1; BD Biosciences) at 4 °C for 30 min, using the concentrations specified by the respective manufacturers. Subsequently, the cells were washed twice with staining buffer and suspended in 100 μL of staining buffer. Flow cytometry was performed using a FACS LSRFortessa Cell Analyzer (Becton Dickinson). Raw data were analyzed using FlowJo (Becton Dickinson) to calculate the mean fluorescence

intensity.

2.8. Statistical analyses

Tests for the significance of differences were performed using GraphPad Prism Ver. 5.04 (GraphPad Software). For *in vitro* experiments, Dunnett's test was used for multiple-group comparisons. For *in vivo* experiments, the Wilcoxon rank-sum test was used for intergroup comparisons of tumor volume, and Welch's *t*-test was used for intergroup comparisons of gene expression in tumors. In all tests, $P < 0.05$ was assumed to indicate statistical significance.

3. Results

3.1. Identification of amlexanox by compound screening using MLR

To obtain compounds that can enhance the antitumor effect of anti-PD-1 mAb, we performed MLR-based screening using 142 compounds, measuring IFN-γ in the culture supernatant as an indicator of T cell activation. Most of the compounds used were drugs that have been clinically applied in Japan or the United States, and others are their related compounds (Figure S1). Four compounds increased IFN-γ production more than 1.5-fold when used in combination with anti-PD-1 mAb relative to anti-PD-1 mAb alone (Fig. 1A and Table S1). Of these, only the effect of amlexanox was reproducible, so we used amlexanox for further analyses. Amlexanox is an anti-asthmatic drug, although its mechanism of action is not fully understood [15]. The dose dependency of amlexanox (1.2, 6, and 30 μmol/L) was assessed in the presence of anti-PD-1 mAb in MLR. IFN-γ production was significantly higher than that in the anti-PD-1 mAb alone group only in the 6 μmol/L amlexanox group (Fig. 1B). Amlexanox alone did not increase IFN-γ production (Fig. 1C). These results indicate that amlexanox increases IFN-γ production only in combination with anti-PD-1 mAb, and not as a monotherapy, in MLR.

3.2 Enhanced antitumor effect of anti-PD-1 mAb by amlexanox in tumor-bearing mouse model

To verify the effect of anti-PD-1 mAb combined with amlexanox *in vivo*, we next used a tumor-bearing mouse model (implanted with MC38 murine colorectal cancer cells) in which the antitumor effect of anti-PD-1 mAb has been observed [16]. In the vehicle group, all six mice reached the humane endpoint (a tumor volume of 2,000 mm^3) on day 15 after treatment (Fig. 2A). Administration of anti-PD-1 mAb alone had biphasic effects — both strong and weak antitumor effects in each of the three mice — although the reason for this is unknown.

The combination of anti-PD-1 mAb and amlexanox had antitumor effects in all mice examined. The median tumor volume was 1327.8 mm^3 on day 20 after treatment in the anti-PD-1 mAb alone group, whereas it was 397.7 mm^3 in the combination group (Fig. 2B). On day 13 after the start of treatment, tumor growth inhibition was 67.5% and 87.6% and in the anti-PD-1 mAb alone and combination groups, respectively, and tumor size in the combination group was significantly smaller than that in the vehicle group (Fig. 2C). Amlexanox alone had no antitumor effect (Fig. 2D).

3.3. Induction of PD-L1 expression on DCs by amlexanox in combination with anti-PD-1 mAb

To clarify whether DCs or T cells are the target of amlexanox in MLR, we first examined expression levels of PD-L1, which is expressed on DCs and has been found to be induced by IFN-γ [17]. Although treatment with anti-PD-1 mAb alone or amlexanox alone did not affect the expression levels of PD-L1, the combination increased them (Fig. 3A). We then examined whether amlexanox can act on T cells by examining the IL-2 levels in the activated Jurkat T cells. Amlexanox had no effect on IL-2 production, while the positive control lenalidomide increased it (Fig. 3B). These results suggest that amlexanox acts on DCs but not on T cells.

3.4. Increased expression of IFN-γ- and CTL-related genes in response to amlexanox in combination with anti-PD-1 mAb

To reveal the mechanism by which amlexanox enhances the antitumor effect of anti-PD-1 mAb *in vivo*, we conducted gene-expression analyses on tumoral tissues isolated from the tumor-bearing mice 23 days after the implantation of MC38 cells. We found that expression of 75 and 65 genes was increased at least 1.5-fold ($P < 0.05$) relative to the vehicle group in the combination and anti-PD-1 mAb alone groups, respectively (Fig. 4A). Of

these genes, 19 were increased in both groups. Several genes that are reported to be closely associated with the efficacy of anti-PD-1 mAb in clinical studies were increased in one or both groups. These genes included IFN-γ related genes (*Ifng*/IFN-γ, *Ido1*, *Cxcl10*, *Cxcl9*, *H2-dm*, and *Stat1*) and CTL-related genes (*Cd8a*, *Cd8b1*, *Gzma*, *Gzmb*, and *Prf1*), as well as *Cd274*/PD-L1 [18-20] (Fig. 4B). The expression levels of *Ifng*/IFN-γ, *Cd274*/PD-L1, *Cd8a*, and *Prf*1/Perforin were significantly higher in the combination group than in the vehicle group (Fig. 4C). We speculate that the antitumor effect of amlexanox in combination with anti-PD-1 mAb is due to the increased production of IFN-γ, which has antitumor effects, and the chemokines CXCL9 and CXCL10, which recruit and activate CTLs in tumors.

4. Discussion

Our findings from the MLR-based compound screening reveal that amlexanox enhanced the production of IFN-γ caused by anti-PD-1 mAb (Fig. 1). Amlexanox also induced PD-L1 expression on DCs when administered in combination with anti-PD-1 mAb (Fig. 3A). In addition, it enhanced the antitumor effect of anti-PD-1 mAb in the MC38 tumor-bearing mouse model (Fig. 2). Gene-expression analysis of the tumor tissues revealed that the combination of amlexanox and anti-PD-1 mAb increased the expression levels of several IFN-γ- and CTL-related genes (Fig. 4).

Amlexanox is an anti-asthmatic drug that inhibits the extracellular release of histamine and leukotrienes in mast cells, although its mechanism of action is still not fully understood [15]. Recently, it was shown to ameliorate obesity and insulin resistance in a mouse model by exerting anti-inflammatory effects through inhibition of the protein kinase TANK-binding kinase 1 (TBK-1) in macrophages in adipose tissue [14]. In that study, the *in vitro* IC_{50} of amlexanox for TBK-1 was 1 to 2 μmol/L, and a therapeutic effect was observed at 25 mg/kg in an *in vivo* animal model. These concentrations and doses were similar to the effective concentration or dose of amlexanox in the MLR and tumor-bearing mouse model in our study. Therefore, it is likely that the antitumor effect of the combination of amlexanox and anti-PD-1 mAb occurs through TBK-1 inhibition. Another study also showed that a compound with inhibitory activity against TBK-1 and IκB kinase-ε (referred to as "Compound 1" in that study) enhanced the production of IL-2 and IFN-γ in human T cells that had been stimulated by anti-CD3 or anti-CD28 mAbs [21]. Furthermore, Compound 1 showed a stronger antitumor effect in combination with anti-PD-L1 mAb in a tumor-bearing mouse model than did the mAbs alone. DC-specific deletion of *Tbk1* has been reported to increase the number of IFN-γ-producing $CD4^+$ T cells in tumors via induction of the expression of type 1 interferon and its downstream genes, which resulted in a stronger

antitumor effect in a tumor-bearing mouse model [22]. Based on these observations, together with the report that type 1 interferon induces PD-L1 expression [13], we speculate that in our study, amlexanox enhanced the expression of PD-L1 on DCs by inhibiting TBK-1 (Fig. 3A).

IFN-γ signaling has been reported to be important for enabling immune checkpoint inhibitors such as anti-PD-1/-PD-L1 mAbs to exert their antitumor effects, both in mouse models [16] and in clinical practice [18]. In our study, amlexanox induced the expression of *Ifng*/IFN-γ and its related genes when administered in combination with anti-PD-1 mAb (Figs. 1 and 4). Therefore, combination therapy with amlexanox and anti-PD-1/-PD-L1 mAbs may be a useful therapeutic tool for patients who have become resistant to immune checkpoint inhibitors.

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Declaration of competing interest

Kazuhiko Takeda, Kaoru Yamada, and Hiroshi Yano are employees of Ono Pharmaceutical.

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Figure legends

Fig. 1. Identification of amlexanox as a candidate compound that enhances the antitumor effect of anti-PD-1 mAb. (**A**) The results of MLR-based screening of 142 compounds. The values represent the fold increase in IFN-γ production caused by each compound in combination with anti-PD-1 mAb relative to anti-PD-1 mAb alone. (**B**, **C**) Amlexanox dose dependency in the presence (**B**) or absence (**C**) of 3 μg/mL anti-PD-1 mAb in MLR. IFN-γ levels in the culture supernatant on days 5 (**B**) and 3 **(C**) were measured via ELISA. Values presented are means ± SD (**B**; n = 4, **C**; n = 3), and significant differences from the anti-PD-1 mAb alone group (**B**) or vehicle group (**C**) are indicated (* $P < 0.05$; ** $P < 0.01$; Dunnett's test).

Fig. 2. Amlexanox treatment is effective in combination with anti-PD-1 mAb *in vivo* in a mouse tumor model. (**A**–**C**) MC38 cells were implanted subcutaneously in C57/BL6 mice (n = 6). After the tumor volume reached approximately 100 mm^3, vehicle (saline) or anti-PD-1 mAb (10 mg/kg) was administered intraperitoneally once a week for three weeks, with or without daily oral administration of amlexanox (25 mg/kg). (**A** and **B**) Time course of the tumor volume for individual mice (**A**) and the average for each group (**B**). (**C**) Median and individual tumor volumes on day 13. * $P < 0.05$; Wilcoxon rank-sum test. (**D**) Time course of median tumor volume. MC38 cells were subcutaneously implanted in C57/BL6 mice (n = 10). After the tumor volume reached approximately 100 mm^3, amlexanox (30 mg/kg) was orally administered daily.

Fig. 3. Amlexanox enhances the expression levels of PD-L1 on DCs in combination with anti-PD-1 mAb. (**A**) Expression levels of PD-L1. DCs and allogeneic $CD4^+$ T cells in MLR were incubated with dimethyl sulfoxide (DMSO), amlexanox (10 μmol/L) alone, anti-PD-1

mAb (3 μg/mL) alone, or a combination of both for two days, and PD-L1 levels were examined via flow cytometry. Values represent the mean fluorescence intensities ± SD from three independent experiments (** $P < 0.01$; Dunnett's test). (**B**) Production levels of IL-2. Jurkat T cells activated by anti-CD3 mAb for 20 h were incubated with DMSO, 10 μmol/L lenalidomide (positive control), or amlexanox (0.1, 1, or 10 μmol/L) for a further 24 h. The quantity of IL-2 in the cell supernatant was measured via ELISA. Values presented are means from two independent experiments.

Fig. 4. Increased expression levels of PD-L1, IFN-γ, and CTL-related genes in tumors treated with a combination of amlexanox and anti-PD-1 mAb. (**A**–**C**) MC38 cells were implanted subcutaneously in C57/BL6 mice (n = 4). After the tumor volume had reached approximately 100 mm^3, anti-PD-1 mAb (10 mg/kg) was administered intraperitoneally every week for three weeks and amlexanox (30 mg/kg) was orally administered daily. On day 15 after the start of treatment, tumors were collected from each animal to prepare total RNA. Target transcript levels in the total RNA were determined using an nCounter. (**A**) Venn diagram representing the number of genes whose expression was 1.5-fold higher ($P < 0.05$; Welch's *t*-test) than in the vehicle group. (**B**) Colors in the heatmap indicate the mean *Z* score (the deviation from the mean of the distribution) for each group in comparison to the mean of all 16 samples (four samples per group). (**C**) Transcript levels of *Ifng*/IFN-γ, *Cd274*/PD-L1, *Cd8a*/CD8a, and *Prf1*/Perforin. * $P < 0.05$; ** $P < 0.01$; Welch's *t*-test. Aml, amlexanox; Anti-PD-1, anti-PD-1 mAb.

Figure 1

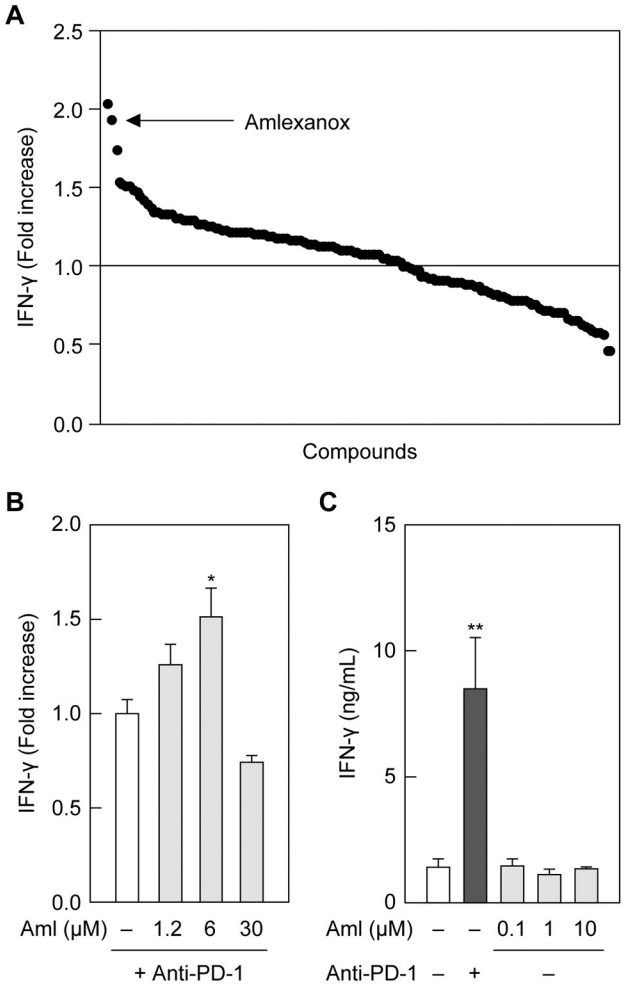
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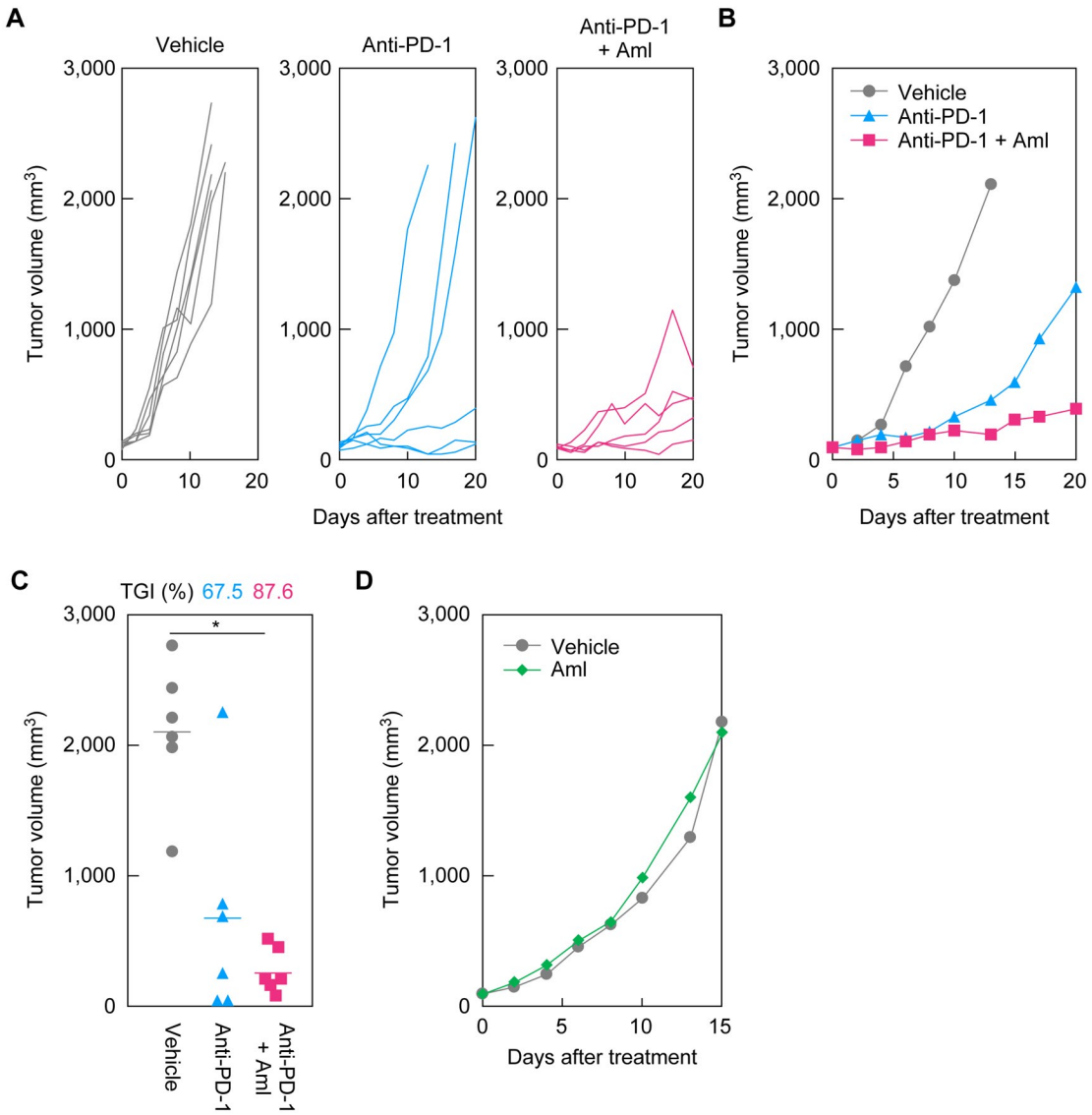
Figure 2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Tumor volume (mm3)
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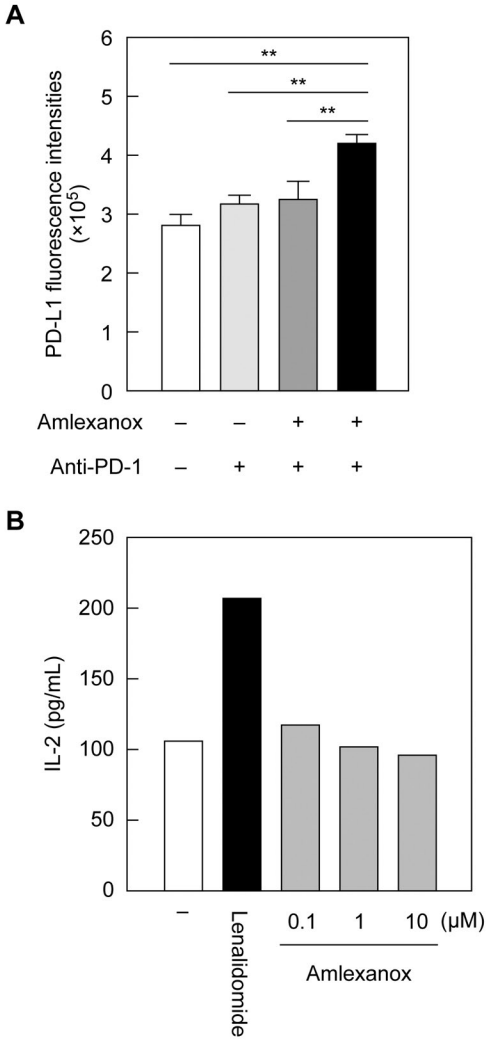
Figure 3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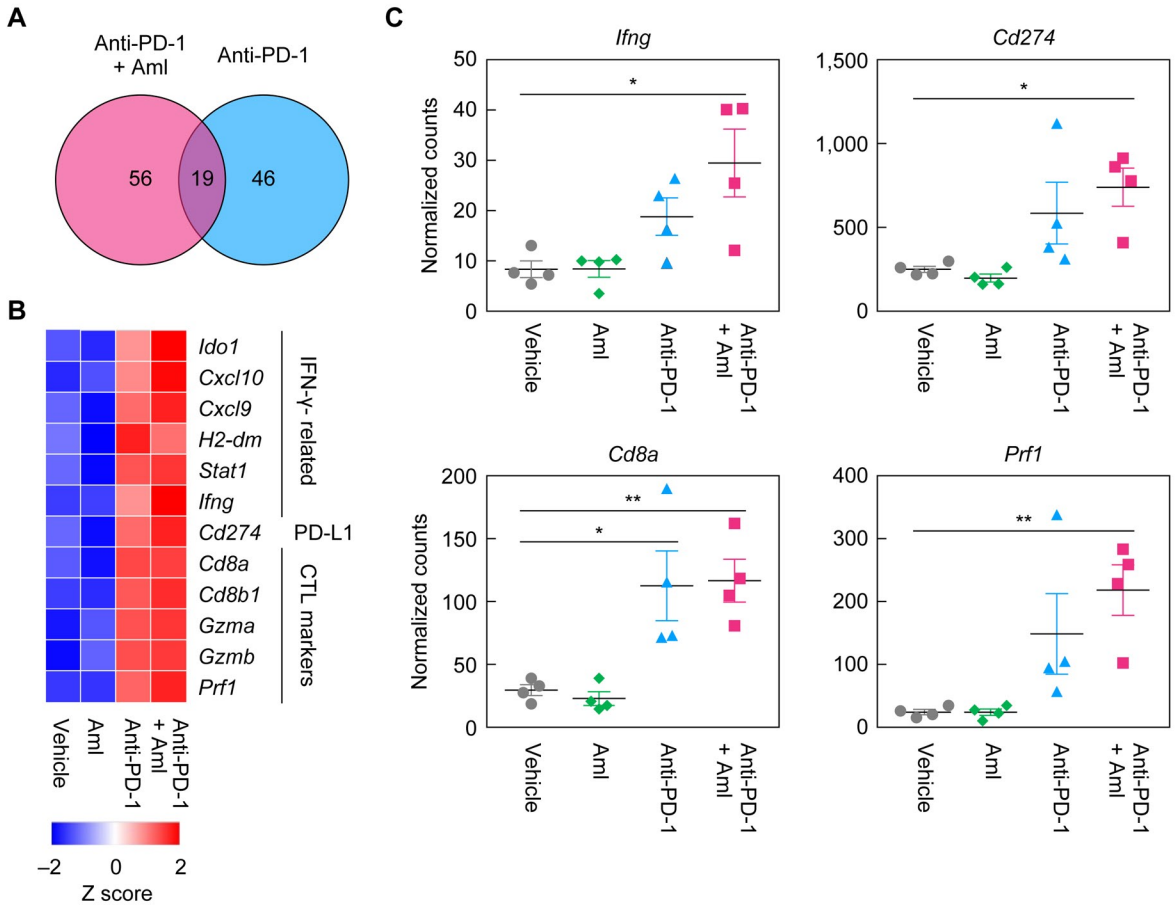
Amlexanox

Figure 4


A
Anti-PD-1 + Aml
Anti-PD-1
56
19
46
B
Ido1
Cxcl10
Cxcl9
H2-dm
Stat1
Ifng
Cd274
Cd8a
Cd8b1
Gzma
Gzmb
Prf1
IFN-γ- related
PD-L1
CTL markers
Vehicle
Aml
Anti-PD-1
Anti-PD-1 + Aml
−2
0
2
Z score
C
Ifng
Cd274
Cd8a
Prf1
Normalized counts
Vehicle
Aml
Anti-PD-1
Anti-PD-1 + Aml