The Mechanism of Anti–PD-L1 Antibody Efficacy against PD-L1–Negative Tumors Identifies NK Cells Expressing PD-L1 as a Cytolytic Effector

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ABSTRACT Blockade of PD-L1 expression on tumor cells via anti–PD-L1 monoclonal antibody (mAb) has shown great promise for successful cancer treatment by overcoming T-cell exhaustion; however, the function of PD-L1 on natural killer (NK) cells and the effects of anti–PD-L1 mAb on PD-L1+ NK cells remain unknown. Moreover, patients with PD-L1− tumors can respond favorably to anti–PD-L1 mAb therapy for unclear reasons. Here, we show that some tumors can induce PD-L1 on NK cells via AKT signaling, resulting in enhanced NK-cell function and preventing cell exhaustion. Anti–PD-L1 mAb directly acts on PD-L1+ NK cells against PD-L1− tumors via a p38 pathway. Combination therapy with anti–PD-L1 mAb and NK cell–activating cytokines significantly improves the therapeutic efficacy of human NK cells against PD-L1− human leukemia when compared with monotherapy. Our discovery of a PD-1–independent mechanism of antitumor efficacy via the activation of PD-L1+ NK cells with anti–PD-L1 mAb offers new insights into NK-cell activation and provides a potential explanation as to why some patients lacking PD-L1 expression on tumor cells still respond to anti–PD-L1 mAb therapy.

SIGNIFICANCE: Targeting PD-L1 expressed on PD-L1+ tumors with anti–PD-L1 mAb successfully overcomes T-cell exhaustion to control cancer, yet patients with PD-L1− tumors can respond to anti–PD-L1 mAb. Here, we show that anti–PD-L1 mAb activates PD-L1+ NK cells to control growth of PD-L1− tumors in vivo, and does so independent of PD-1.
INTRODUCTION

Inhibition of the PD-1/PD-L1 pathway has become a very powerful therapeutic strategy for patients with cancer, and has shown unprecedented clinical responses in advanced liquid and solid tumors (1). At present, two PD-1 monoclonal antibodies (mAb), pembrolizumab (Keytruda) and nivolumab (Opdivo), are FDA-approved to treat melanoma, kidney cancer, head and neck cancers, and Hodgkin lymphoma (2–4). Three PD-L1 mAbs, atezolizumab (Tecentriq), avelumab (Bavencio), and durvalumab (Imfinzi), are FDA-approved to treat non–small cell lung cancer (NSCLC), bladder cancer, and Merkel cell carcinoma of the skin (5–7). However, the overall response rate to anti–PD-L1 mAb therapy is still very low in patients with melanoma (26%), NSCLC (21%), and renal cell carcinoma (13%; ref. 8). In addition, anti–PD-L1 mAb therapy can also show an unexplained clinical response in the absence of PD-L1 expression on tumor cells (8, 9). Therefore, an improved understanding of the mechanisms for anti–PD-L1 (or anti–PD-1) mAb therapy will help direct future efforts in developing more precise cancer immunotherapeutics.

Tumor cells in the tumor microenvironment (TME) can upregulate PD-L1 after encountering activated T cells via their secretion of IFNγ (10). Upon binding to PD-1, PD-L1 delivers a suppressive signal to T cells and an antiapoptotic signal to tumor cells, leading to T-cell dysfunction and tumor survival (10). Therefore, anti–PD-1/PD-L1 therapy aims to remove this immune suppression and activate the T-cell response against cancer. It has been reported that PD-L1 is not only expressed on tumor cells but also on immune cells, including T cells, natural killer (NK) cells, and macrophages within the TME (11–14). However, the function and the mechanism of action of PD-L1 on NK cells remain unexplored. It is also unknown whether and how anti–PD-L1 mAbs can modulate the function of NK cells expressing PD-L1. Unraveling these mechanisms will likely play an important role in understanding the clinical effectiveness of anti–PD-1/PD-L1 mAb therapy.

NK cells comprise a group of innate cytolytic effector cells that participate in immune surveillance against cancer.
and viral infection. NK cells become cytolytic without prior activation, especially when they encounter cells lacking self-MHC class I molecules (15). Downregulation of MHC class I can occur in the setting of cancer (16), allowing NK cells to recognize and lyse malignant cells. Activated NK cells exert strong cytotoxic effects via multiple mechanisms involving perforin, granzyme B, TRAIL, or FASL (17). NK cells also produce IFNγ, which not only directly affects target cells, but also activates macrophages and T cells to kill tumor cells or enhance the antitumor activity of other immune cells (18). However, to our knowledge, the function of PD-L1 on NK cells and the underlying mechanisms in the normal or disease setting, as well as the involvement of PD-L1+ NK cells in anti-PD-L1 mAb therapy, has not been explored.

In the present study, we found that some myeloid leukemic cell lines and acute myeloid leukemia (AML) blasts from patients can upregulate PD-L1 on NK cells. PD-L1+ NK cells are activated effectors exerting enhanced cytotoxic activity against target cells in vitro compared with PD-L1− NK cells. NK cells from a majority of patients with AML expressed moderate to high levels of PD-L1, and the change in its level of expression following chemotherapy correlated with clinical response. Further, in vivo, anti-PD-L1 mAb treatment in combination with NK cell–activating cytokines significantly enhanced NK-cell antitumor activity against myeloid leukemia lacking PD-L1 expression, suggesting that anti-PD-L1 mAb therapy has a unique therapeutic role in treating PD-L1− cancer, acting through NK cells. This novel mechanism of direct innate immune cell activation with anti-PD-L1 mAb therapy that is PD-1− independent may explain the efficacy of the anti-PD-L1 checkpoint inhibitor in some PD-L1− tumors.

RESULTS
PD-L1 Expression on NK Cells after Encountering Tumor Cells

Expression of PD-L1 has been extensively reported on tumor cells, and its binding to PD-1 on T cells suppresses the function of PD-1+ T cells (19). The expression of PD-L1 on immune cells has also been reported on macrophages, T cells, and NK cells (11–14). However, the mechanism of induction and function of PD-L1 on NK cells remains unknown. Here, we enriched fresh human NK cells from healthy donors and cocultured them with PD-L1high target tumor cells, the K562 myeloid leukemia cell line. We found that anywhere from 14.2% to 74.4% of NK cells expressed PD-L1 after encountering K562 cells (Fig. 1A; Supplementary Fig. S1A). The RNA and protein levels of PD-L1 were both markedly increased (Fig. 1B and C). To confirm the expression of PD-L1 on NK cells, we stained both PD-L1+ and PD-L1− NK cells with the human NK cell–surface marker CD56. Immunofluorescence images showed that PD-L1 (green) localized with CD56 (red) on PD-L1+ NK cells (Fig. 1D). In addition to its expression on the NK-cell surface, PD-L1 can also be secreted by NK cells (Fig. 1E). To further understand the mechanism of K562-induced NK-cell expression of PD-L1, we FACS-purified NK cells to repeat the experiments with highly enriched NK cells. We observed that PD-L1 was induced by specific interactions between K562 cells and purified NK cells (Fig. 1F). We also tested whether direct cell contact was required for PD-L1 induction. For this purpose, NK cells were cultured in the supernatants from K562 cells alone or in the supernatants from K562 cells incubated with NK cells. The conditioned media marginally induced PD-L1, significantly less so when compared with NK cells directly incubated with K562 cells (Supplementary Fig. S1B). K562 cells incubated in transwells did not induce PD-L1 on NK cells (Fig. 1G). Of note, PD-L1 expression could also be more modestly induced on CD8+ T cells and B cells when cocultured with K562 cells, but not in NK-T cells or CD4+ T cells (Supplementary Fig. S1C–S1G). Collectively, these results show that direct interaction between NK cells and K562 myeloid leukemia cells alone is sufficient to induce PD-L1 expression on NK cells.

PD-L1 Expression Marks NK-Cell Activation and Positively Correlates with Clinical Outcome of Patients with AML

We next investigated the function of PD-L1 expression on NK cells. NK-cell expression of CD107a and IFNγ production are commonly used as functional markers for NK-cell degranulation and cytokine production, respectively, following NK-cell activation (17, 20). Although degranulation and IFNγ production occurred within 2 hours of NK cells encountering K562 cells (Supplementary Fig. S2A, top and middle), PD-L1 upregulation on NK cells increased significantly only after 16 hours (Supplementary Fig. S2A, bottom). These data suggest that PD-L1 upregulation on NK cells is likely not the driver of NK-cell activation but rather the result of NK-cell activation. We compared the functional phenotype of PD-L1+ and PD-L1− NK cells following coculture with K562 cells. We found that the expression of CD107a and that of IFNγ were significantly increased in PD-L1+ NK cells compared with PD-L1− NK cells (Fig. 2A). The 51Cr release assay confirmed that the cytotoxicity of PD-L1+ NK cells was dramatically increased compared with PD-L1− NK cells (Fig. 2B). These results further suggest that PD-L1+ NK cells are highly activated immune effector cells. Giemsa staining showed that PD-L1+ NK cells were larger in size and had a thicker cytoplasm (Fig. 2C, top). The observation of the PD-L1+ NK cells appearing larger and functionally more activated than the PD-L1− NK cells was confirmed by transmission electron microscopy (Fig. 2C, bottom). The cytoplasm of freshly isolated PD-L1+ NK cells contains more mitochondria and liposomes than the PD-L1− NK cells, which may account for their larger size (Fig. 2C, bottom). We also examined the survival and proliferative capacity of PD-L1+ NK cells. Compared with PD-L1− NK cells, PD-L1+ NK cells are more apoptotic (Fig. 2D–F). There was no difference in proliferation between PD-L1+ and PD-L1− NK cells (Fig. 2G). Using flow cytometry, we measured the expression of surface markers present on these two NK-cell subsets. We observed that the expression of the two activation antigens, CD69 and CD25, was significantly increased on PD-L1+ NK cells compared with PD-L1− NK cells, whereas the receptors CD94, KLRG1, NKP44, NKG2D, and TGFβRII did not show a significant difference in expression between PD-L1+ and PD-L1− NK cells (Supplementary Fig. S2B). CXCR4 expression was decreased on PD-L1+ NK cells compared with PD-L1− NK cells, which could promote their egress from the bone marrow niche (ref. 21; Supplementary Fig. S2B). These data demonstrate that
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For this purpose, we examined samples from 79 patients related to clinical outcomes following standard chemotherapy. The percentage of PD-L1 existed in the majority of patients with AML but not in the healthy donors (Fig. 2H). The percentage of PD-L1+ NK cells (Fig. 2H). We also confirmed the induction of PD-L1 mRNA expression on NK cells incubated without or with K562 cells (n = 3) and representative example showing total PD-L1 protein in NK cells incubated without or with K562 cells. Total PD-L1 protein was measured by immunoblot (right) and the relative expression rate was calculated using ImageJ (left). D, Immunofluorescence of unstimulated (left) and K562 cell–stimulated (right) enriched human NK cells stained with PD-L1 (green), CD56 (red), or DAPI nuclear stain (blue) and then merged. Images are shown at 20× magnification (top panels). Panels at the bottom are zoomed-in areas of dashed boxes in the top panels. Scale bars, 5 μm. E, NK cells were incubated without or with K562, followed by measuring secreted PD-L1 protein levels by ELISA (right) and the relative expression rate was calculated using ImageJ (left). F, Representative flow cytometry plots and summary data (n = 5) of FACs-purified NK cells (purity > 96%) incubated with or without K562 cells. PD-L1 expression was measured by flow cytometry. G, Representative flow cytometry plots and summary data (n = 4) showing the percentages of PD-L1+ NK cells from enriched NK cells incubated with or without K562 cells in transwell plates, or incubated directly with or without K562 cells. Paired t test (A–F) was used for two-group comparisons and one-way ANOVA with repeated measures for donor-matched 3 groups (G). P values were adjusted by the Holm–Sidak method. *, P < 0.05; ***, P < 0.001; ****, P < 0.0001; NS, not significant.

PD-L1 can be induced in NK cells when encountering tumor cells, and, compared with PD-L1− NK cells, PD-L1+ NK cells appear to possess higher levels of effector functions against tumor cells. We next addressed whether PD-L1+ NK cells exist in patients with cancer and whether this NK-cell subset is correlated to clinical outcomes following standard chemotherapy. For this purpose, we examined samples from 79 patients with AML and found that the PD-L1+ NK-cell population existed in the majority of patients with AML but not in the healthy donors (Fig. 2H). The percentage of PD-L1+ NK-cell population in the patients with AML was as high as 40%, with 77% (61/79) of the patients with AML having PD-L1+ NK cells (Fig. 2H). We also confirmed the induction of PD-L1 on NK cells from healthy donors during ex vivo incubation with primary patient AML blasts (Fig. 2I; Supplementary Fig. S2C). When comparing the percentage of PD-L1+ NK cells in patients with AML at the time of evaluation for response to two cycles of standard induction chemotherapy, we observed that patients with AML who achieved complete remission (CR; n = 31 of 47) had a significantly higher percentage of PD-L1+ NK cells at CR compared with the percentage of PD-L1+ NK cells at the time of diagnosis (Fig. 2J). In contrast, patients with AML who did not achieve CR (NCR; n = 16 of 47) showed no significant difference in the percentage of PD-L1+ NK cells between the time of diagnosis and the time of assessment for CR (Fig. 2K). Further, patients with AML who achieved CR had a significantly higher percentage...
of PD-L1+ NK cells compared with patients with AML who did not achieve CR (Fig. 2L). When the data were reanalyzed and presented as the percent change of PD-L1+ NK cells from the time of diagnosis to the time of assessment for CR, a significant difference was also observed between the patients with CR and those without CR (Fig. 2M). However, these differences were not observed in the percentage of total NK cells at diagnosis when compared with the percentage of total NK cells at the time of CR evaluation, regardless of whether or not the patients with AML achieved CR (Supplementary Fig. S2D–G). These data suggest that the percentage of PD-L1+ NK cells at the time of CR evaluation is correlated with attainment of CR, rather than the percentage of total NK cells. Taken together, the data presented thus far suggest that the activated NK cells as identified by their expression of PD-L1 may possess antileukemic activity in vivo.

Targeting PD-L1 with the Humanized Anti–PD-L1 mAb Atezolizumab Enhances NK-Cell Function

We have found that PD-L1 expression or lack thereof could divide NK cells into two morphologically and functionally distinct populations with a higher level of cytotoxicity and IFNγ production in the PD-L1+ subset compared with the PD-L1− subset. To further evaluate the function of PD-L1+ NK cells, we used atezolizumab (AZ), one of the humanized mAbs against PD-L1 that has been approved by the FDA for the treatment of NSCLC (22). K562 myeloid leukemia cells express a low level of PD-L1 (Supplementary Fig. S3A), consistent with a previous report (23). To ensure no contribution from PD-L1 expression on K562 cells, we generated PD-L1− knockout (KO) K562 cells using the CRISPR/Cas9 system (Supplementary Fig. S3A). Lack of PD-L1 expression on K562 cells did not affect their ability to induce the expression of PD-L1 on NK cells (Supplementary Fig. S3B).

AZ is an IgG1 mAb engineered with a modification in the Fc domain that eliminates mAb-dependent cellular cytotoxicity (ADCC; ref. 9). To ensure complete blockage of the potential ADCC effect in AZ-treated NK cells, we used a mAb that blocks the Fc receptor. We found that, compared with AZ-treated PD-L1− NK cells, IgG-treated PD-L1+ NK cells, and IgG-treated PD-L1− NK cells, treatment of PD-L1+ NK cells with AZ significantly increased the expression of CD107a and IFNγ in PD-L1− NK cells (Fig. 3D), and this difference was further enhanced following treatment of the PD-L1− transduced NK cells with AZ (Fig. 3D). We also found that the expression of CD107a was significantly decreased in PD-L1 knockdown (KD) NK cells compared with the empty vector control group after encountering K562 cells in the presence of AZ (Supplementary Fig. S3C).

In addition, we found that PD-L1 expression by NK cells pretreated with K562 was further elevated at both the mRNA and protein levels in a time-dependent manner after treatment with AZ (Fig. 3E and F), suggesting that PD-L1 signaling by AZ induces continuous upregulation of PD-L1, which then becomes available for additional activation by AZ.

Mouse PD-L1+ NK Cells Show Enhanced Antitumor Activity In Vivo

We next sought to test whether NK cells could be induced to express PD-L1 in the presence of tumor in an animal model, and whether PD-L1+ murine NK cells display similar functional activity as seen thus far with human NK cells ex vivo. We found that mouse NK cells constitutively express PD-L1, which is consistent with a previous report (24); however, we also found that its expression could be significantly increased in mice bearing the lymphoid tumor YAC-1 (Fig. 4A). For further in vivo functional study, we generated PD-L1− knockout YAC-1 cells (PD-L1 KO YAC-1) using the CRISPR/Cas9 system (Supplementary Fig. S4A). PD-L1+ NK cells in mice bearing PD-L1 KO YAC-1 tumors showed enhanced degranulation compared with PD-L1− NK cells (Fig. 4B). To further study the function of PD-L1 on mouse NK cells, we used PD-L1−/− mice and found that CD107a expression was significantly decreased on splenic NK cells in PD-L1−/− mice and showed a similar trend in lungs compared...
Figure 3. Analysis of PD-L1 signaling and functions in NK cells upon anti–PD-L1 mAb AZ treatment. A, NK cells were incubated with K562 cells for 24 hours and then treated with or without 20 μg/mL AZ in the presence or absence of an Fc blocker for 4 hours. PD-L1+ NK cells were gated to assess the expression of CD107a (n = 5). AZ-treated cells were stained with anti-AZ antibody and non–AZ-treated cells were stained with anti–PD-L1 antibody. B, NK cells were incubated with K562 cells for 24 hours and then treated with 20 μg/mL AZ or IgG control in the presence of an Fc blocker for 4 hours. PD-L1+ NK cells were gated to assess CD107a expression (%) and IFN-γ expression (%). Cytotoxicity was used to assess NK cell function. C, Representative flow cytometry plots and summary data (n = 3) of NK cells transduced with empty vector (EV) or PD-L1 overexpression vector with or without AZ treatment prior to measuring the expression of IFN-γ by intracellular flow cytometry (n = 3). E, Enriched NK cells were incubated with PD-L1 KO K562 cells for 20 hours and then treated with 20 μg/mL AZ for the time indicated on the x-axis. NK cells were then sorted to >96% purity, and the relative levels of PD-L1 mRNA expression were measured by qRT-PCR (n = 6). F, Representative flow cytometry plots and summary data (n = 5) showing that the expression of PD-L1 on NK cells increases in a time-dependent manner after treatment with AZ. One-way ANOVA with repeated measures [D–F] or linear mixed model [A and C] was used to compare donor-matched 3 or more groups. P values were adjusted by the Holm–Sidak method. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; NS, not significant.
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Figure 4. PD-L1 KO mice and NK-cell depletion show impaired antitumor activity in a PD-L1 KO YAC-1 tumor model treated with or without anti–PD-L1 mAb. A and B, Representative flow cytometry plots and summary data (n = 5) of (A) PD-L1 expression of spleen NK cells and (B) CD107a expression of spleen and lung NK cells of BALB/c mice after being challenged with PD-L1 KO YAC-1 cells. C and D, Representative flow cytometry plots and summary data (n = 5) of NK-cell CD107a expression in the spleen and lung of WT and PD-L1 KO BALB/c mice challenged with PD-L1 KO YAC-1 cells treated with or without anti–PD-L1 mAb. E, The number of PD-L1 KO YAC-1 cells in spleens of WT, NK cell–depleted, or PD-L1−/− BALB/c mice treated with anti–PD-L1 mAb or IgG control. Paired t test (B and C) was used for two-group comparisons and one-way ANOVA with repeated measures for 3 or more groups (A and E). P values were adjusted by the Holm–Sidak method. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. NS, not significant.

with NK cells in wild-type (WT) mice engrafted with the PD-L1 KO YAC-1 tumor cells (Fig. 4C and D). In vivo anti–PD-L1 mAb treatment of mice bearing PD-L1 KO YAC-1 tumors increased CD107a expression on NK cells in WT mice but not on NK cells in PD-L1+ mice (Fig. 4C and D). In WT mice with PD-L1+ NK cells implanted with PD-L1 KO YAC-1 tumor cells, the tumor burden was significantly decreased when the anti–PD-L1 mAb was used compared with IgG control–treated mice (Fig. 4E; Supplementary Fig. S4B). This suggests that host cells’ PD-L1 may play a positive role in controlling tumor development. However, for similar experiments with PD-L1−/− mice (i.e., lack of PD-L1 expression on NK cells), we did not observe significant antitumor activity of anti–PD-L1 mAb versus IgG control (Fig. 4E; Supplementary Fig. S4B). NK-cell percentages did not change in tumor-bearing WT and PD-L1−/− mice with or without PD-L1 mAb treatment (Supplementary Fig. S4C). We also observed that tumor burden was lower in WT mice compared with PD-L1−/− mice, which led us to investigate whether the effect of anti–PD-L1 mAb was mediated by NK cells. We depleted NK cells in WT mice implanted with PD-L1 KO YAC-1 tumor cells and the mice were treated with an anti–PD-L1 mAb or IgG control (Supplementary Fig. S4D). We observed that when NK cells were absent, there were no significant antitumor effects of the anti–PD-L1 mAb (Fig. 4E; Supplementary Fig. S4B), suggesting that NK cells play a role in mediating the effects of anti–PD-L1 mAb in our animal model. Together, these results suggest that PD-L1+ NK cells are essential for antitumor activity of the PD-L1 mAb in mice bearing PD-L1+ tumors, and that the antitumor effect of the mAb is acting directly on NK cells. Our in vivo mouse studies suggest that the use of anti–PD-L1 mAb to target PD-L1+ NK cells should be considered as a cancer immunotherapy for certain PD-L1+ tumors.

Anti–PD-L1 mAb Augments Human PD-L1+ NK Cell Antitumor Activity In Vivo

Having hypothesized from data from our patients with AML that PD-L1+ NK cells could have antitumor activity in vivo (Fig. 2L and M), and having shown an improved antitumor effect of mouse NK cells by delivering an anti–PD-L1 mAb against a malignant mouse tumor lacking PD-L1 in vivo, we next attempted to reproduce this finding in an orthotopic mouse model using human NK cells and PD-L1 KO K562 myeloid leukemia followed by in vivo delivery of AZ versus placebo (placebo was PBS but not isotype IgG, to avoid NK-cell ADCC that is not active with AZ). For this purpose, we transplanted human primary NK cells into NSG mice, without or with PD-L1 KO K562 myeloid leukemia cells. We first showed that the presence of the PD-L1 KO K562 cells resulted in a very significant increase in the expression of PD-L1 on human NK cells in vivo (Fig. 5A). Treatment of these mice with either a placebo or the AZ resulted in the latter group showing a significant increase in NK-cell expression of granzyme B, IFNγ, and CD107a when compared with NK cells in the placebo-treated group (Fig. 5B). Further, we showed that the mice treated with AZ had a significantly lower tumor burden compared with the placebo-treated mice (Fig. 5C).

We next assessed the effects of NK cell–activating cytokines on NK-cell PD-L1 expression in the absence or presence of K562 myeloid leukemia cells. Alone, IL2 had essentially no
Figure 5. Effects of the anti–PD-L1 mAb AZ and/or NK-activating cytokines on antitumor efficacy in vivo. A, Human NK cells were injected intravenously into NSG mice without or with PD-L1 KO K562 cells followed by intraperitoneal injection of 1 µg IL2 and 1 µg IL15 per mouse every other day. After 6 days, mice were sacrificed, and NK cells were isolated and assessed for PD-L1 expression by flow cytometry. Representative flow-cytometric analyses and summary data are shown (n = 5). B and C, Human NK cells and PD-L1 KO K562 cells were injected intravenously into NSG mice, followed by treatment with intraperitoneal injection of AZ or PBS every other day. PBS was used instead of IgG1 as placebo because AZ lacks antibody-dependent cellular cytotoxicity activity. After 6 days (three treatments), mice were sacrificed and human NK cells were examined for (B) their expression of granzyme B, CD107a, and IFNγ or (C) the number of PD-L1 KO K562 cells measured by flow cytometry. Representative analyses and summary data (n = 5) are shown. D, Survival curve of NSG mice injected intravenously with primary human NK cells and PD-L1 KO K562 myeloid leukemia, we treated mice with various combinations of NK-activating cytokines in the absence or presence of AZ. As predicted from our earlier in vitro work showing that IL2 alone did not increase PD-L1 expression on NK cells (Supplementary Fig. SSA), the in vivo administration of IL2 alone or its combination with AZ had minimal effect on survival, and in both cases no mice survived longer than 16 days (Fig. 5D). The administration of combinations of IL12 and IL15 or IL12 and IL18 had substantial effects on increasing PD-L1 expression on NK cells in vitro (Supplementary Fig. SSA), but the combination of these three cytokines in the absence of AZ only modestly improved survival over IL2 alone, with no survival beyond day 21 (Fig. 5D). In contrast, mice treated with IL12, IL15, and IL18 in combination with AZ showed a significant improvement in survival, with 50% of mice alive at day 40 (Fig. 5D). These data, together with Fig. 4E, suggest that AZ directly acts on PD-L1+ human NK cells in the presence of three NK-activating cytokines to significantly prolong survival in mice engrafted with a lethal dose of human myeloid leukemia.

The PI3K/AKT Signaling Pathway Regulates PD-L1 Expression on NK Cells

To investigate mechanisms by which PD-L1 is induced in NK cells by myeloid leukemia cells, we performed an RNA microarray to profile gene expression in PD-L1+ NK cells versus PD-L1− NK cells.
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NK cells, both of which were FACS-purified from bulk NK cells after being cocultured with K562 cells. The results showed that the PD-L1+ NK-cell subset had higher expression levels of TBX21 and EOMES, which encode the two signature transcriptional factors required for NK cells to gain functional maturity (27, 28). The gene encoding CD226 (also known as DNAM1), an activation marker for NK cells (29), had higher expression in PD-L1+ NK cells, whereas the gene encoding the negative regulatory transcriptional factor SMAD3 had lower expression levels in PD-L1+ NK cells (ref. 30; Fig. 6A). These gene-expression patterns indicate that PD-L1+ NK cells exhibit unique gene profiling compared with their PD-L1− counterparts and are consistent with our above characterization showing that PD-L1+ NK cells are an activated NK-cell subset. In addition, the microarray data implied that protein kinase B (AKT) signaling may be involved in regulating PD-L1 expression on NK cells (Fig. 6A). The AKT family contains three members: AKT1, AKT2, and AKT3 (31). To test whether AKT signaling regulates PD-L1 expression on NK cells, we incubated them with K562 cells in the presence of a global AKT inhibitor against AKT1/2/3 (afuresertib), followed by measuring PD-L1 expression on NK cells. Treatment with afuresertib significantly reduced PD-L1 expression (Fig. 6B, top, and C), suggesting that global AKT inhibition is capable of blocking PD-L1 expression. Upstream of the AKT cascade is PI3K. Treatment with the PI3K inhibitor wortmannin also significantly reduced PD-L1 expression on NK cells when incubated with K562 cells (Fig. 6B, middle, and C). We next sought to identify which transcription factors downstream of the PI3K/AKT signaling pathway regulate PD-L1 expression in NK cells. We cloned a PD-L1 promoter 2.1 kb upstream of the transcription start site, cotransfected it with genes for specific transcription factors into 293T cells, and measured the activity of the PD-L1 promoter by luciferase assay. We found that most transcriptional factors of the PI3K/AKT cascade, including XBP1, FOXO1, NFAT2, and NFAT4, did not activate the PD-L1 promoter (32–34); however, the PI3K/AKT downstream transcription factor p65 enhanced PD-L1 promoter activity 5-fold compared with empty vector control (Fig. 6D). The p65 subunit comprises part of the nuclear factor kappa B (NFκB) transcription complex, which plays a crucial role in inflammatory and immune responses (35). We then confirmed the regulatory role of p65 in PD-L1 expression using a specific p65 inhibitor, TPCK (Fig. 6B, bottom, and C). To validate the role of the PI3K/AKT/p65 pathway in regulating PD-L1 expression, we examined the binding of p65 with the PD-L1 promoter. For this purpose, we cotransfected the PD-L1 promoter with AKT or p65 in 293T cells and observed that the introduced AKT or p65 led to enhanced association of p65 with the PD-L1 promoter compared with IgG control (Fig. 6E and F). These results collectively show that signaling through PI3K/AKT/NFκB plays a critical role in regulating not only NK-cell activation, but also PD-L1 expression.

Because NK-cell activation is usually triggered by recognizing the absence of MHC class I molecules (15), we next wondered if tumor susceptibility to NK-cell lysis is associated with NK-cell induction of PD-L1 expression. For this purpose, we examined the expression of HLA-A, HLA-B, and HLA-C on target cell lines and found that K562 and AML3 cells have significantly lower expression of HLA-A, HLA-B, and HLA-C, and are highly susceptible to NK-cell lysis and induce PD-L1 expression on NK cells (Supplementary Fig. S6A–S6C). In contrast, RPMI 8226, MOLM-13, and MV-4–11 cells have high levels of HLA-A, HLA-B, and HLA-C, are not susceptible to NK-cell lysis, and could not efficiently activate NK cells or induce NK-cell expression of PD-L1 (Supplementary Fig. S6A–S6C), even following an extended incubation time (Supplementary Fig. S6D). These data suggest that target susceptibility to NK-cell cytotoxicity may be associated with the capacity of PD-L1 induction by tumor cells.

The above experiments reveal that tumor cells induce PD-L1 expression on NK cells via the PI3K/AKT/NFκB pathway (Fig. 6B and C). We also showed that the binding of AZ to tumor-induced PD-L1 on NK cells led to further NK-cell activation (Fig. 3A and B), suggesting that the induced PD-L1 on NK cells may signal following treatment with AZ. We thus continued to explore the molecular mechanism(s) downstream of the PD-L1–AZ interaction in NK cells. For this purpose, we screened four kinases that have been reported to be involved in regulating PD-L1 signaling (36–38). Intracellular flow cytometry analysis in Supplementary Fig. S7 showed that the activity of p38, a kinase involved in regulating NK-cell function and antitumor activity (39), was increased by AZ treatment, whereas the activities of pERK, pAKT, or p-mTOR were not increased. These data were confirmed by immunoblotting of p38 and its downstream NFκB signaling family members, including p65 (RelA), pRelB, and pRelC (Fig. 6G and H). Interestingly, it was previously reported that PD-L1 signaling in PD-L1+ tumor cells occurs via the PI3K/AKT pathway (38); however, our data showed that PD-L1 signaling in PD-L1+ NK cells did not occur via the PI3K/AKT pathway in the presence of AZ (Supplementary Fig. S7A). The p38 inhibitors SB203580 and SB202190 were found to reduce AZ-induced PD-L1 expression in the presence of PD-L1 KO K562 tumor cells, suggesting a positive regulatory effect of p38 signaling in this setting (Fig. 6I). Consistent with this, chromatin immunoprecipitation (ChIP) assays also showed that p38 signaling induced the binding of p65 to the PD-L1 promoter (Fig. 6J). Functional assays demonstrated that the two p38 inhibitors SB203580 and SB202190 also inhibited AZ-induced CD107a and IFN-γ cytokine secretion features (Supplementary Fig. S8).

Taken together, our data suggest a model involving PD-L1 upregulation on activated NK cells via the PI3K/AKT signaling pathway after NK cells and tumor cells encounter each other (Supplementary Fig. S8); the model also involves subsequent anti–PD-L1 mAb binding to the upregulated PD-L1 on NK cells by tumor cells and further activation of NK cells via p38 signaling, both events leading to NFκB activation, resulting in a positive feedback loop in the presence of excess AZ to continuously induce PD-L1 expression and to further activate NK cells. In the loop, the engagement of the anti–PD-L1 mAb with PD-L1 leads to an increase in PD-L1 expression on the NK-cell surface, providing more binding sites for anti–PD-L1 mAb, which leads to continuous activation of p38, which further transduces strong activation signaling to NK cells to maintain their cytotoxic and cytokine secretion features (Supplementary Fig. S8).

**DISCUSSION**

It is well known that PD-L1 is typically expressed on tumor cells, allowing them to suppress PD-1+ T-cell function, thereby
"PD-L1" represents PD-L1.

Figure 6. Signaling pathways involved in the anti-PD-L1 antibody-induced NK-cell activation in the presence of tumor cells. A, Gene-expression profile using RNA microarray of PD-L1− and PD-L1+ NK cells sorted from K562 and IL2-stimulated enriched NK cells of three healthy donors (D1, D2, and D3). D1+ represents PD-L1+ NK cells from donor 1, whereas D1− represents PD-L1− NK cells from donor 1, each purified by FACS sorting. Similar definitions are applied to "D2−", "D3−", and "D3−". Expression of Cd274 (PD-L1), Cd226 (DNAM1), Tbx21, Eomes, Smad3, and Akt1 is highlighted by transcription factor names. B, NK cells were incubated without or with K562 cells in the absence or presence of the pan-AKT inhibitor afuresertib, the PI3K-specific inhibitor wortmannin, or the p65-specific inhibitor TPCK at a concentration of 1 or 10 μmol/L. The percentages of PD-L1 expression of D1+ NK cells were measured by flow cytometry. C, Inhibition rate of PD-L1+ NK cells was measured as the relative proportion of PD-L1+ cells in each treatment condition compared with untreated control (no inhibition; n = 5). D, 293T cells were transfected with the PD-L1 promoter and the indicated expression plasmids. Relative promoter activity was measured by luciferase assay after 48 hours. E and F, The ChIP assay was used to assess the binding of p65 to the PD-L1 promoter when AKT or p65 was overexpressed. The experiments were repeated three times. (continued on following page)
Figure 6. (Continued) G and H, Representative and summary data (n = 3) showing the activity of p38 and NFκB family members, including p65 (RelA), RelB, and RelC in NK cells incubated with IL2 plus PD-L1 KO K562 cells for 20 hours and then with the anti–PD-L1 mAb AZ or IgG for various periods as indicated. Fc blocker was added 15 minutes prior to adding IgG or AZ antibodies to minimize ADCC effect. Total PD-L1 protein was measured by immunoblotting. Numbers beneath each lane represent relative density quantified by ImageJ software, normalized for equivalent loading as determined by β-actin. Increased phosphorylation of some members by AZ at 0 minutes was likely due to the activation of the cells by AZ during sample harvesting. A linear mixed model was used to assess the treatment × time interaction and group difference between IgG and AZ at each time point by accounting for the repeated measures from the same donor. 

I, Flow cytometry to measure PD-L1 expression on NK cells incubated with PD-L1 KO K562 cells for 20 hours and then with the anti–PD-L1 mAb AZ in the absence or presence of 1 μM p38 inhibitor SB202190 or SB203580 for 4 hours (n = 4). J, The ChIP assay was used to assess binding of p65 to the PD-L1 promoter in 293T cells transduced with p38 for 24 hours using a p65 or IgG antibody. The experiments were repeated three times. K, Representative example and summary data (n = 5) quantifying the expression of CD107a and IFNγ in NK cells following their incubation with K562 cells for 24 hours and then with the anti–PD-L1 mAb AZ in the absence or presence of 1 μM p38 inhibitor SB202190 or SB203580. Two paired groups were compared by paired t test. Paired t test (E, F, and J) was used for two-group comparisons and one-way ANOVA with repeated measures was used for donor-matched 3 or more groups ([C, D, I, and K]). P values were adjusted by the Holm–Sidak method.* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; NS, not significant.
enhancing the tumor’s ability to evade the immune system (40). However, the functional consequences of PD-L1 expression on NK cells and the role of PD-L1+ NK cells in regulation of the immune response have not been characterized. In this study, we studied PD-L1+ and PD-L1− NK cells in both humans and mice in the setting of PD-L1+ tumors. PD-L1+ NK cells were found to have significantly enhanced cytotoxicity and IFNγ production compared with PD-L1− NK cells. We show that NK cells, upon encountering and being activated by NK-susceptible tumor cells, not only secrete cytokines and cytolytic granules, but also upregulate PD-L1 on their surfaces via the PI3K/AKT/NFκB pathway. Upon engagement with the anti-PD-L1 mAb AZ, PD-L1 is able to further upregulate NK-cell function through the p38 signaling pathway, thus serving as a functional activation antigen for NK cells. Using these in vitro observations, we used a mouse model and an orthotopic mouse model of human NK cells and PD-L1+ KS62 myeloid leukemia to show that in both models, anti–PD-L1 mAb acts directly on PD-L1+ NK cells to improve the antitumor activity of NK cellsensitive yet lethal tumors in vivo. Interestingly, in an attempt to find a correlation with human disease, we performed an analysis that provides preliminary evidence for a correlation between an increase in the percentage of PD-L1+ NK cells from the time of diagnosis to the completion of induction chemotherapy, and the attainment of CR. Collectively, the extensive experimental data presented here may provide an explanation for why some patients lacking PD-L1 expression on tumor cells still respond to anti-PD-L1 mAb therapy (8, 9). Further, when combined with our analysis of NK-cell PD-L1 expression in 79 patients with AML, one could speculate that the presence of PD-L1+ NK cells may identify certain patients whose tumors are more susceptible to NK-cell lysis and may therefore benefit from a trial of anti–PD-L1 mAb without or with NK cell–activating cytokines, exploiting a novel pathway that is independent of T cells and PD-1.

T cells infiltrating the TME are heterogeneous, containing both effector and bystander CD8+ T-cell populations (41, 42). The higher the percentage of effector T cells in the TME, the better the prognosis, and vice versa (43). However, there are fewer studies of NK cells within the TME, and little is known about PD-L1+ NK cells. We found that after encountering myeloid leukemia cells, a proportion of NK cells lost most of their cytotoxic activity and became “bystander like,” with little or no expression of PD-L1, although a second fraction of NK cells emerged with strong induction of PD-L1 expression, indicating a state of activation with enhanced effector function toward tumor target cells. We found that the more sensitive the target cell was to NK cytotoxicity (by virtue of its inverse correlation with MHC class I expression), and the more direct was the cell–cell contact of the NK cells with the target cells, the higher was the expression of PD-L1 and the stronger was the activation of the NK cell. As noted above, PD-L1 expression on patient NK cells may serve as an in vivo biomarker for patient tumors that are more likely to be susceptible to NK-cell lysis.

We and others have previously shown that PD-1 expression on NK cells results in a negative regulatory event upon engagement with its ligand (44–46), as is well known in T cells (40, 47). Previous functional analysis indicates that, compared with PD-1− NK cells, PD-1+ NK cells are less activated with a lower level of degranulation and impaired cytokine production upon their interaction with tumor targets (46). Interestingly, our current study showed that PD-L1+ NK cells are more activated compared with their PD-L1− counterparts upon their interaction with tumor targets. We also showed that PD-L1 signaling via the p38/NFκB pathway is a positive regulatory event for NK cells upon PD-L1 engagement by anti–PD-L1 mAb, resulting in further expression of PD-L1 which, in the presence of excess anti–PD-L1 mAb, further increased p38 signaling. This positive feedback loop continually provides intracellular signaling that allows the NK cell to retain an activated effector state (Supplementary Fig. S8). Both effects are very likely to contribute to the potent antitumor effect seen in NK cells in both the in vitro and in vivo modeling performed in this study. Importantly, we also discovered that the induction of PD-L1 expression following the NK-cell interaction with an NK cell–susceptible tumor cell target occurs via the PI3K/AKT/NFκB signaling pathway, distinct from the p38/NFκB signaling pathway that mediates the NK-cell activation resulting from the interaction between NK-cell PD-L1 and anti–PD-L1 mAb. However, we show that the simultaneous interaction of the PD-L1+ NK cell with both its tumor cell target and anti–PD-L1 mAb does result in an additive effect on NK-cell activation, likely as a result of both pathways ultimately driving increased activation of NFκB as shown in Supplementary Fig. S8. Thus far, in immunotherapy, reversal and even enhancement of T-cell antitumor activity through checkpoint blockade has had great success in the cancer clinic (48–50). It is therefore not unreasonable to believe that NK cell–based antitumor activity in the TME could also be reversed and enhanced. Here we show that anti–PD-L1 mAb therapy enhanced PD-L1+ NK-cell function against PD-L1+ tumors in both mouse and human systems in vitro and significantly improved survival against a human PD-L1+ tumor in a PD-1–independent fashion in vivo. Further, in our in vivo mouse model, we demonstrated that depleting NK cells or using PD-L1− mice significantly decreased the antitumor effect of anti–PD-L1 mAb therapy, suggesting that the antitumor effects are mediated directly by the NK cells following PD-L1 signaling within the TME. Although our study could not exclude the possible role of other PD-L1+ immune cells, including myeloid cells, in PD-L1 antibody therapy, we documented the role of PD-L1+ NK cells in mediating the antitumor effect in this setting, revealing a new strategy for an increased and prolonged immune response by NK cells in the TME, likely applicable to some but not all tumors, and provide an explanation as to how immune therapy with anti–PD-L1 mAb can be effective in individuals whose tumors lack PD-L1 expression (8, 9).

IL12, IL15, and IL18 cytokines are known to activate and expand NK cells, and each has been investigated in clinical studies (51–54). IL12 has demonstrated antitumor effects through its regulation of both innate and adaptive immune cells (55). Recombinant human IL15 has entered phase 1/II clinical trials for various types of cancer (56). IL15 has shown promising antitumor effects alone or in combination with other treatments (57, 58). IL18 also plays an important role in expansion and priming of NK cells (59, 60). Our current study showed that the anti–PD-L1 mAb AZ had a significantly enhanced antitumor effect when administered in combination with these NK cell–activating cytokines, leading to a prolonged survival in mice engrafted with human NK cells.
and human myeloid leukemia, likely through the enhancement of NK-cell function. Together with the clinical correlative data presented on 79 patients with AML in this report, our in vitro and in vivo studies here suggest that for select patients with AML with a significant fraction of PD-L1+ NK cells at CR, an anti-PD-L1 mAb clinical trial at the time of CR could be considered in order to prevent disease relapse, ultimately in combination with an NK-activating cytokine such as IL15. In addition to measuring NK-cell function against autologous patient blasts pre- and post-anti-PD-L1 mAb administration ex vivo, one could get a preliminary indication of in vivo activity by assessing the time to relapse. This is particularly relevant when considering a highly vulnerable population such as elderly patients with AML, where the vast majority relapse within 2 years (61). It would also be interesting to determine if patients with AML treated with anti-KIR mAb to block the NK KIR interaction with MHC class I had a higher expression of PD-L1 on their NK cells (62).

In summary, our study identified a novel and unique subset of NK cells characterized by surface expression of PD-L1 in a fraction of patients with AML, and we reproduced this finding with both in vitro and in vivo animal and human tumor modeling. We showed that binding of anti-PD-L1 mAb to PD-L1+ NK cells induced strong antitumor activity in vitro and in vivo, and this process was independent of the PD-1/PD-L1 axis. These antitumor effects were shown to be dependent on both NK cells and their expression of PD-L1 and were effective against tumors lacking expression of PD-L1. Collectively, our experimental data suggest these PD-L1+ NK cells can be further activated in vivo for an additional antitumor effect, likely in combination with an NK-activating cytokine. PD-L1+ NK cells may identify a subset of patients with AML whose tumors are more susceptible to NK-cell lysis. We believe it may therefore be reasonable to consider anti-PD-L1 mAb therapy for the subset of patients with AML with a fraction of PD-L1+ NK cells at CR, especially in the elderly where a substantial number of patients with AML achieve CR but the vast majority relapse and die within 2 years (61). Finally, we believe that the data from this report can at least partly explain why some patients with tumors lacking PD-L1 expression can respond favorably to anti-PD-L1 mAb checkpoint inhibitor therapy. PD-L1+ NK cells may prove to be another important immune effector cell for checkpoint inhibitor–based cancer immunotherapy.

**METHODS**

**Patient Samples**

Peripheral blood samples from 48 healthy donors and 79 patients newly diagnosed with AML were recruited in this study between December 2017 and November 2018 at the First Affiliated Hospital of Soochow University, Suzhou, China. The diagnosis and classification of patients with AML were based on the revised French-American-British (FAB) classification and the 2008 World Health Organization criteria (63, 64). Then, we followed 47 newly diagnosed patients for collection of peripheral blood mononuclear cells before and after treatment. The clinical characteristics of these 47 patients are listed in Supplementary Table S1. Among these 47 patients, 31 patients achieved CR, whereas 16 patients did not respond to the treatment. We compared the PD-L1 expression on the NK cells before and after the treatment among these patients. The patients received standard induction chemotherapy followed by consolidation and autologous hematopoietic stem cell transplantation.

**Cell Lines**

K562 and MV-4-11 cells were obtained from ATCC within 6 months of this study. RPMI 8226, YAC-1, MOLM-13, and AML3 cells were obtained from the laboratory of M.A. Caligiuri. These cells were cultured with RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and were grown in 37°C with 5% CO2. No further authentication of these cell lines was performed. Cell morphology and growth characteristics were monitored during the study and compared with published reports to ensure their authenticity. All cell lines used in this study were determined to be negative for Mycoplasma prior to experiments using the MycoAlert Mycoplasma Detection Kit (Lonza; cat. #LT7-318). The latest test was on June 12, 2019. All cell lines were used within 10 passages after thawing.

**Mice**

NSG and BALB/c mice were purchased from the Jackson Laboratory and housed at the City of Hope Animal Facility. PD-L1−/− mice were kindly provided by Dr. Defu Zeng, City of Hope Beckman Research Institute, who originally received them from Dr. Lieping Chen (Yale University) and Dr. Haidong Dong (Mayo Clinic). All experiments were approved by the City of Hope Animal Care and Use Committee.

**Microarray**

High-quality total RNA isolated from FACS-sorted PD-L1+ and PD-L1− NK cells was used for microarray analysis. The integrity and quantity of the RNA were checked by Agilent Bioanalyzer and Nanodrop RNA 6000, respectively. The Clarion D Assay chip was used for hybridization following the manufacturer's protocol. Gene-expression profiles were analyzed using Transcriptome Analysis Console (TAC) 3.0 software. Data collected from three donors were used for microarray analysis.

**Cytotoxicity Assay**

Cytotoxicity assays were performed as described previously (26). K562 target cells were labeled with 51Cr for 1 hour at 37°C. Cells were washed and coincubated with effector cells (PD-L1+ and PD-L1− NK cells) in a 96-well V-bottom plate at various effector to target (E:T) ratios for 4 hours at 37°C. Following culture, supernatants were collected in a scintillation vial for analysis. The standard formula of 100 × (cpmexperimental − cpmspontaneous) / (cpmmaximal − cpmspontaneous) was used to calculate percentages of specific lysis, which were displayed as the mean of triplicate samples.

**Statistical Analysis**

Two independent or paired groups were compared by the Student t test or paired t test. Multiple groups were compared using an ANOVA or linear mixed model for repeated measures. For survival data, the Kaplan-Meier method was used to estimate survival functions and log-rank test was applied to group comparisons. P values were corrected for multiple comparisons by the Holm method or the Holm–Sidak method. A P value less than 0.05 was considered statistically significant.
significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). For microarray data, paired t-test with variance smoothing was applied to group comparisons for each gene after log base 2 transformation and noise-level gene filtering. Both fold change and mean number of false positives (e.g., 5 of 10,000 genes) were used to identify the top genes.

The following details are included in the supplementary material: cell culture; antibody staining and flow cytometry (flow antibodies used in this study were listed in Supplementary Table S2); immunostaining assay; immunoblotting assay; real-time PCR; ChIP assay; PD-L1–knockout cell line; mouse models; and data availability.

Disclosure of Potential Conflicts of Interest
M.A. Caligiuri has ownership interest (including stock, patents, etc.) in Beigene. No potential conflicts of interest were disclosed by the other authors.

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PD-1–Independent Anti–PD-L1 Therapy Involves PD-L1+ NK Cells


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The Mechanism of Anti–PD-L1 Antibody Efficacy against PD-L1–Negative Tumors Identifies NK Cells Expressing PD-L1 as a Cytolytic Effector

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