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ORIGINAL RESEARCH

PD-L1 blockade enhances anti-tumor efficacy of NK cells

Jeremiah L. Oyer [a], Sarah B. Gitto [a], Deborah A. Altomare[a], and Alicja J. Copik [a]

[a]Burnett School of Biomedical Sciences, University of Central Florida, College of Medicine, Orlando, FL, USA

ABSTRACT

Anti-PD-1/anti-PD-L1 therapies have shown success in cancer treatment but responses are limited to ~ 15% of patients with lymphocyte infiltrated, PD-L1 positive tumors. Hence, strategies that increase PD-L1 expression and tumor infiltration should make more patients eligible for PD-1/PD-L1 blockade therapy, thus improving overall outcomes. PD-L1 expression on tumors is induced by IFNγ, a cytokine secreted by NK cells. Therefore, we tested if PM21-particle expanded NK cells (PM21-NK cells) induced expression of PD-L1 on tumors and if anti-PD-L1 treatment enhanced NK cell anti-tumor efficacy in an ovarian cancer model. Studies here showed that PM21-NK cells secrete high amounts of IFNγ and that adoptively transferred PM21-NK cells induce PD-L1 expression on SKOV-3 cells *in vivo*. The induction of PD-L1 expression on SKOV-3 cells coincided with the presence of regulatory T cells (Tregs) in the abdominal cavity and within tumors. In *in vitro* experiments, anti-PD-L1 treatment had no direct effect on cytotoxicity or cytokine secretion by predominantly PD-1 negative PM21-NK cells in response to PD-L1^{+} targets. However, significant improvement of NK cell anti-tumor efficacy was observed *in vivo* when combined with anti-PD-L1. PD-L1 blockade also resulted in increased *in vivo* NK cell persistence and retention of their cytotoxic phenotype. These results support the use of anti-PD-L1 in combination with NK cell therapy regardless of initial tumor PD-L1 status and indicate that NK cell therapy would likely augment the applicability of anti-PD-L1 treatment.

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Introduction

Cancer therapies utilizing antibodies to disrupt PD-1/PD-L1 interaction have been among the most exciting developments leading to long lasting remission in patients with various malignancies and even with very advanced diseases.[1] However, PD-1/PD-L1 blockade therapy is only applicable in a small minority of patients having PD-L1 positive tumors that are infiltrated with cytotoxic lymphocytes, referred to as "hot" or "inflamed" tumors, which are present in only 10–20% of patients across tumor types.[2-4] To augment the overall applicability of PD-1/PD-L1 blockade therapy to benefit a greater number of patients, there is a need for innovative treatment strategies that would increase PD-L1 expression and work in concert with immune cells by promoting better tumor infiltration. This may not be intuitive because PD-L1 expression is generally thought to suppress activity of immune cells, but the success of PD-1/PD-L1 checkpoint blockade as measured by the improvement in overall survival, strongly correlates with PD-L1 expression on tumors.[4] Previous studies have established that expression of PD-L1 on most tumors is initially induced in response to IFNγ secreted by CD8 T cells recruited to the tumor site as part of adaptive tumor resistance.[5,6] Thus, treatment with adoptive transfer of IFNγ producing cell populations, such as activated NK cells, should lead to an increase in PD-L1 expression on tumor targets. NK cells not only produce IFNγ and other cytokines, which are secreted upon recognition of the tumor, but also recruit and orchestrate responses by other immune cells such as T- and dendritic cells in the tumor bed.[7,8] (reviewed in[9,10]) Furthermore, their direct killing of tumor cells leads to release of tumor antigens along with cytokines which likely primes the adaptive immune response for better tumor control.[11,12]

NK cells are a small subpopulation of lymphocytes that have long been recognized to hold high potential for cancer treatment. Trials with autologous IL-2 activated NK cells yielded unfulfilling results.[13] Treatment with haploidentical NK cells was more encouraging by leading to complete remission in 30% of patients with relapsed/refractory acute myelogenous leukemia (AML) and thus demonstrating the therapeutic potential of NK cells.[14] Recently, increasing evidence from preclinical and clinical studies supports the potential efficacy of NK cell therapeutics in multiple cancer types.[15-19] Clinical studies also showed a positive correlation between outcomes of the therapy and the dose of NK cells administered, prompting the development of effective methods to grow NK cell *in vitro*.[19,20]

To fulfill the promise of NK cell therapy, advances have been made to enable robust NK cell expansion with the use of feeder cells in a co-culture system.[21-23] D.A. Lee and co-workers have

CONTACT Alicja J. Copik alicja.copik@ucf.edu Burnett School of Biomedical Sciences, University of Central Florida, College of Medicine, 6900 Lake Nona Blvd., Orlando, FL 32827, USA

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developed a method for large-scale production of highly potent NK cells using K562 cells engineered to express 4-1BB ligand and membrane bound IL21 (mb21).[22] NK cells expanded by the K562-mb21 feeder cell method are currently used in multiple clinical trials and preliminary results are showing highly encouraging clinical efficacy for leukemia relapse prevention after stem cell transplant.[15] Recently, the K562-mb21 cell based method was modified to a cell-free, PM21-particle based method for both *ex vivo* and *in vivo* specific expansion of NK cells which can eliminate some logistical and safety concerns while also retaining the benefits of the feeder-cell based expansion.[24,25] These significant breakthroughs made in regards to generating large doses of NK cells allow for their potential use as a viable and attractive therapeutic option for cancer treatment.

As described above, NK cells directly lyse tumor cells and secrete IFNγ as part of their response. The secreted IFNγ can then induce PD-L1 expression on tumor cells which initiates a cascade of events including the proliferation of Tregs that creates an immunosuppressive environment.[26] Engagement of PD-1 on T cells by PD-L1 on the tumor cells also directly blocks the function of cytotoxic T cells and leads to their anergy and apoptosis. (reviewed in[27]) These changes then aid tumor progression and metastasis. Since NK cells mostly lack the PD-1 receptor on their surface, not much attention has been focused on how NK cells may be suppressed through PD-L1 on tumor surface. Thus, antibodies targeting PD-1 and PD-L1 were largely considered to only benefit T cell driven responses. However, blockade of the PD-1/PD-L1 axis may also improve NK cell treatment through indirect but important mechanisms. The effect of PD-1 blockade on NK cell function has been so far only studied in settings of multiple myeloma where NK cells collected from patients were shown to be positive for PD-1 expression.[28] We have hypothesized that adoptively transferred PM21-NK cells will secrete IFNγ and prime the tumor to induce expression of PD-L1. Since induction of PD-L1 leads to a cascade of events resulting in an immunosuppressive environment, we further postulated that inclusion of PD-L1 blockade will prevent the induction of immunosuppression and improve NK cell efficacy to increase survival of tumor-bearing animals. This study probes the combinatorial use of PM21-NK cells with PD-L1 blockade to potentially enhance outcomes of cancer immunotherapy regardless of PD-1 expression on NK cells or the initial PD-L1 status of patient's tumors.

Results

PM21-particle expanded NK cells are highly cytotoxic against SKOV-3 cells and secrete IFNγ in response to stimulation

The initial experiments were designed to test the ability of NK cells expanded for 14 days with PM21-particles (denoted as PM21-NK cells) to kill SKOV-3 cells and compare their response to NK cells activated for 5 days with 2000 U of IL2 (IL2-NK cells). In comparison to IL2-NK cells, PM21-NK cells were > 10 times more efficacious at killing SKOV-3 cells, where 10–20 times fewer of PM21-NK cells were required to kill the same number of target cells (Figure 1A). PM21-NK cells were also more potent than IL2-NK cells at killing SKOV-3 cells, resulting in 3.4 times more cytotoxicity at 1:1 E:T ratio ($p < 0.0001$) . Similar results were obtained for other cancer cells tested including leukemia, lung and colon cancer cell lines with PM21-NK cells killing 2.5–28 times more targets as compared to IL2-NK cells at 1:1 ratio (Figure 1B). To further probe

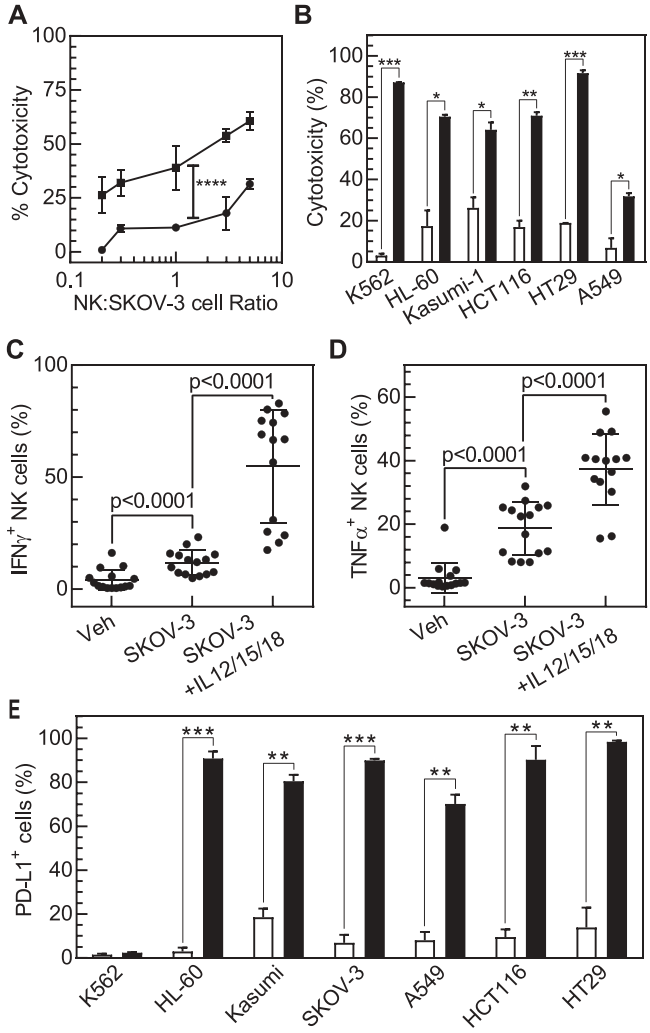

Figure 1. Particle-expanded NK cells are cytotoxic against SKOV-3 cells and secrete IFNγ in response to stimulation. NK cells were expanded with PM21-particles for 14 days or were enriched by negative selection, and activated for 5 days with 2000 U of IL2. NK cells were added to GFP^+ SKOV-3 cells at indicated ratios (A) or to various TFL4-labeled target cells at 1:1 ratio (B) and co-incubated for 60 min. Cells were stained and analyzed by flow cytometry to determine cytotoxicity. PM21-NK cells (■) were at least 10 times more efficacious at killing SKOV-3 cells as compared to IL2- NK cells (●), where at least 10 times more IL2-NK cells would be required to kill 20% of SKOV-3 cells ($p < 0.0001$). PM21-NK cells (solid black bars) were also 2.4–28 times better at killing different targets at 1:1 ratio as compared to IL2-NK cells (open, white bars) (B). PM21-NK cells expanded from five different donors were stimulated with SKOV-3 cells and tested for expression of IFNγ (C) and TNFα (D) in triplicates. PM21-NK cells secreted both IFNγ and TNFα in response to SKOV-3 stimulation. Ability of NK cells to induce PD-L1 on various tumor target cells was tested in a transwell assay (E). Sufficient amount of IFNγ was secreted from PM21-NK cells upon killing to significantly increase PD-L1 expression (black bars) on each tumor target (except for K562 cells) as compared to control cells not exposed to NK cell secretions (white, open bars) in 24 hours (E). Data are presented as scatter plots with mean and 95% confidence interval or as mean with error bars representing standard error of means (SEM). Two-way ANOVA was used to compare cytotoxicity plots for SKOV-3 cells, paired two-tailed Student's t-test analyses were performed for cytokine secretion, and multiple t-test analysis were performed to compare cytotoxicity at 1:1 and PD-L1 induction using GraphPad Software to determine p-values. P values are shown as * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, **** if $p < 0.0001$.

the anti-tumor response of PM21-NK cells, secretion of IFNγ and TNFα was examined in response to engagement of tumor cells. PM21-NK cells were co-incubated with vehicle or SKOV-3 cells at a 1:1 ratio in the presence of Brefeldin A to allow for intracellular accumulation and detection of cytokines. Stimulation of PM21-NK cells with SKOV-3 cells resulted in 3-fold ($p < 0.0001$) increase in the fraction of PM21-NK cells expressing IFNγ as compared to unstimulated cells and 6-fold ($p < 0.0001$) of cells expressing TNFα (Figure 1C and D). The number of IFNγ and TNFα-producing PM21-NK cells increased even further upon inclusion of IL12, IL15 and IL18, cytokines frequently produced in tumor microenvironment. This result led to the hypothesis that the efficient IFNγ production by PM21-NK cells in response to tumor encounter causes induction of PD-L1 on tumors.

PM21-particle expanded NK cells induce PD-L1 on tumor cells *in vitro* and *in vivo* on SKOV-3 tumors with subsequent Treg expansion

In the context of response by CD8 T cells, previous studies have shown that IFNγ secretion results in upregulation of PD-L1 expression on tumors, which in turn interacts with PD-1 on T cells leading to induction and proliferation of Tregs.[26,29] Since PM21-NK cells produce IFNγ in response to SKOV-3 cells, PM21-NK cells should also have a similar effect on tumors to upregulate PD-L1 expression on their surface.

To test the hypothesis that PM21-NK cells induce PD-L1 on tumor cells, an *in vitro* transwell experiment was performed. The cell lines tested included K562, HL-60 and Kasumi leukemia cell lines, HCT116 and HT29 colon carcinoma cells, A549 lung cancer cells and SKOV-3 ovarian cancer cell line. The upper chamber of the transwell contained PM21-NK cells together with tumor target cells to stimulate IFNγ secretion while the lower chamber contained only the respective target cells. Cells were co-incubated for 24 hours and the PD-L1 expression was analyzed on the cells from the lower chamber. PM21-NK cells induced expression of PD-L1 on all target cells tested except for K562 cells (Figure 1E). To test if PM21-NK cells are able to induce PD-L1 *in vivo*, an end-point experiment was performed in which animals seeded with SKOV-3 cells, were treated or not treated with PM21-NK cells and then euthanized 13 days after initiation of treatment to examine PD-L1 expression on extracted tumors. Tumors from untreated and PM21-NK cell-treated groups were removed, dissociated and analyzed for PD-L1 expression by flow cytometry. As predicted, treatment with PM21-NK cells of SKOV-3 implanted mice resulted in upregulation of PD-L1 expression on tumor cells (Figure 2A-histogram overlay). This resulted in a greater percentage of PD-L1 expressing cells on tumors from animals treated with PM21-NK cells as compared to those from the untreated group (30% *vs.* 14%, $p < 0.0001$) (Figure 2B). Human $CD45^+$ lymphocytes recovered from the peritoneal cavity of treated animals contained mostly NK cells (95 ± 1%), that lacked PD-1 expression, and some T cells (3 ± 1%), that were mostly $PD\text{-}1^+$ (54 ± 8%) (Supplemental Figures S1 and Figures 2C-D). Further characterization of the T cell populations revealed almost a complete lack of cytotoxic CD8 T cells and presence of high levels

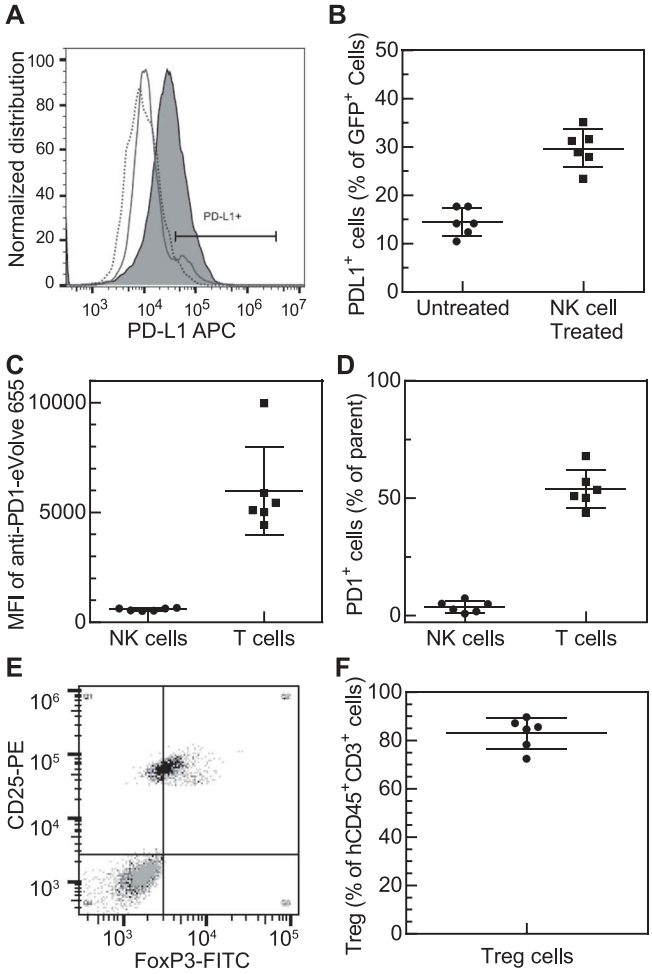

Figure 2. Adoptively transferred PM21-NK cells induce PD-L1 on SKOV-3 cells and drive Treg expansion *in vivo*. NSG mice were implanted with 1×10^6 SKOV-3 cells i.p. Mice were treated with 10×10^6 of PM21 NK cells (n = 6) or with vehicle control (n = 6) on day 8 and 13 followed by IL2 injections (25,000 U, 3x/week). Mice were sacrificed on day 13 after initiation of treatment with the first NK cell injection and tumors and peritoneal wash were collected for analysis. Tumors were perfused to obtain single cell suspension and were stained with anti-PD-L1 (A, B). Histogram overlays are shown with the dotted line representing SKOV-3 cells cultured *in vitro*, solid black line represents tumor cells from untreated animals and solid filled histogram represents tumor cells recovered from NK cell-treated group. Peritoneal wash cells were analyzed for presence of PD-1 expression on NK cells and T cells (C, D) as well as for presence of $CD3^+CD4^+CD25^{Bright}FoxP3^+$ T cells (E, F). Isotype control shown in gray on the overlay. Tregs represented 83 ± 6% of total human T cells recovered from the abdominal cavity. Data are presented as scatter plots with mean and 95% confidence interval. Unpaired two-tailed Student's t-test analyses were performed using GraphPad Software to determine p-values.

of Tregs, where over 80% (mean 83%; range 73 – 90%) of the $CD3^+$ T cells recovered were of $CD3^+CD4^+CD25^{high}FoxP3^+$ phenotype (Figure 2E and F). This is in contrast to the typical T cell composition observed in the final PM21-NK cell product, which consist of majority CD8 T cells with Tregs accounting on average for 3–6% of T cells in the culture dependent if peripheral blood mononuclear cells (PBMCs) or T cell depleted starting material is used (Figure S2). This strongly supports the mechanism that NK cells induce the expression of PD-L1 on tumors through the secretion of IFNγ, which then leads to Treg expansion.

Interestingly, increased IFNγ secretion and CD8 T cell suppression was recently reported in clinical settings, in a

trial of K562-mb21 feeder cell-expanded NK cells (FC21-NK cells) for treatment of blood cancers in combination with stem cell transplantation (SCT).[18] In this study, FC21-NK cells were given before and after stem cell transplant and resulted in decreased relapse rate as well as lower occurrence of post-transplant viral reactivation and graft-*versus*-host disease. Peripheral blood taken from treated patients on day 30 post-SCT (21 days after 2 NK cell infusions) was analyzed for NK and T cell content, phenotype and function. Although there was no difference between the absolute NK cell counts, the NK cells from treated patients had much greater IFNγ secretion as compared to control group ($p = 0.003$). Furthermore, the composition of T cell populations in the blood of NK cell-treated patients was much different as compared to control group resulting in almost complete lack of CD8 T cells in the NK cell-treated group ($p = 0.00015$). To determine if FC21-NK cells and PM21-NK cells are similar in terms of their anti-tumor response and modulation of tumor microenvironment, we have performed long term survival experiments that comparatively tested anti-tumor efficacy of PM21-NK cells and FC21-NK cells with or without application of PM21-particles *in vivo* in SKOV-3 tumor bearing mice (Supplemental Figures 3–5). In both PM21-NK ($N = 8$) and FC21-NK cell ($N = 8$) treated animals, significant decrease in tumor growth was observed which resulted in longer animal survival (mean of 31 days) as compared to untreated group (mean of 21 days, $p < 0.0001$) (Figure S3). Although human $CD45^+$ cells recovered from ascites and abdominal wash of treated animals at the time when moribund (days 27–33 post treatment) contained predominantly NK cells (mean 75%, range 23–92%), high amounts of T cells were also present (mean 23%, range 4–49%) of which the majority were $CD4^+$ $CD25^{high}$. The $CD4^+$ $CD25^{high}$ cells were associated with the presence of ascites, which were observed in more than 70% of animals (Figure S4). Histological analysis of tumors extracted from treated animals also showed infiltration with Tregs. This was observed despite the use of a highly pure population of NK cells being initially injected into animals (NK cells > 99.9%; T cells < 0.05% – Figure S5) and NK cells monitored in blood of treated animals remaining at ≥97% of total hCD45 cells (Figure S5). This indicates that the tumor microenvironment drives the changes in lymphocyte composition and the extent of this effect is time dependent.

Summarizing the results of the above experiments, adoptive application of PM21-NK cells leads to a strong initial anti-tumor response with production of IFNγ, which in turn leads to induction of PD-L1 expression on tumor cells and *in situ* proliferation of immune suppressive Tregs in the tumor microenvironment. PD-L1 induction is unlikely to directly inhibit NK cell function (as it does in the case of CD8 T cells) since NK cells isolated from the intraperitoneal cavity were mostly negative for PD-1 expression. To confirm this, the effect of PD-L1 blockade on the cytotoxicity of PM21-NK cells expanded from PBMCs obtained from three different donors was tested against SKOV-3 cells that were untreated or pretreated with exogenous IFNγ to induce surface PD-L1 expression (Figure 3A). To study the sole effect of PD-L1 blockade, a non-CD16 engaging antibody (clone 6E11, Genentech) was selected for this experiment, which will not induce antibody-dependent cell cytotoxicity (ADCC) by NK cells. As expected, blocking PD-L1 on SKOV-3 cells did not affect the cytotoxicity (Figure 3B) nor cytokine production (Figure 3C–F) by PM21-NK cells irrespective of PD-L1 expression or presence of anti-PD-L1.

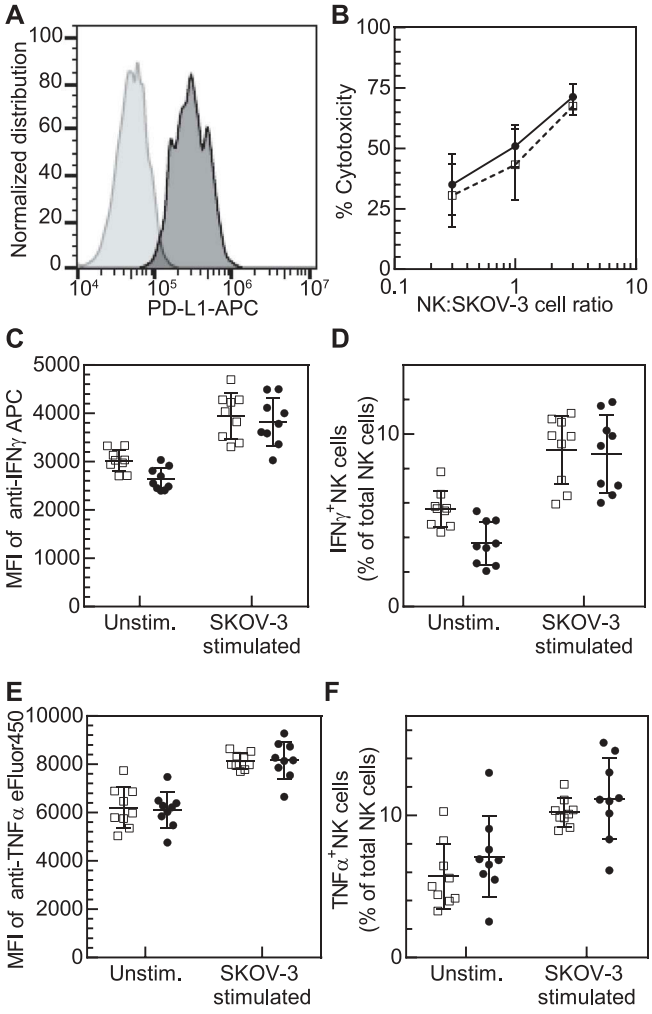

Figure 3. PD-L1 blockade does not affect NK cell direct cytotoxicity against PD-L1$^+$ SKOV-3 cells. A) SKOV-3 cells were pretreated with 20 ng/mL of IFNγ for 48 hours to induce PD-L1 expression. Expression was confirmed by flow cytometry (dark gray- IFNγ-treated, light gray-untreated). B) PM21-NK cells derived from 3 different donors were added at indicated ratios into IFNγ-treated SKOV-3 cells that were preincubated for 30 minutes with vehicle or anti-PD-L1 (clone 6E11, Genentech). PM21-NK cell cytotoxicity was found to be similar in the presence (●) and absence (□) of PD-L1 blockade using anti-PD-L1 (clone 611E) which does not mediate ADCC. PD-L1 blockade did not affect secretion of IFNγ (C, D) and TNFα (E, F) in response to PD-L1$^+$ SKOV-3 stimulation. Responses were tested for 3 different donors and performed in triplicates. Data are presented as scatter plots with mean and 95% confidence interval.

PD-L1 blockade improves survival and phenotype of NK cells

Differentiation and proliferation of suppressive Tregs is induced upon stimulation by PD-L1 expressed on tumor cell surface. Tregs are known to suppress the anti-tumor response and cytotoxic function of NK cells by several mechanisms including surface presentation of TGFβ. TGFβ has been shown to downregulate CD16 expression on NK cells and lead to their conversion to a more immunoregulatory-like

phenotype.[30,31] Tregs also sequester IL-2 which results in a decrease of NK cell survival.[31,32] Tregs and/or PD-1 engagement of T cells may also inhibit beneficial T cell/NK cell crosstalk that can potentially provide NK cells with IL2 or other stimulatory signals. Thus, blocking PD-L1 to mitigate induction of Treg expansion and the associated immunosuppression should lead to improved NK cell function and persistence. To test this, SKOV-3 bearing animals were treated with PM21-NK cells either alone or in combination with PD-L1 antibody. Animals were sacrificed on day 26 after initiation of NK cell treatment, and the immune cells were recovered from the intraperitoneal cavity. The recovered immune cells were enumerated and examined for CD16 expression and other phenotypic markers. Anti-PD-L1 treated animals were found to contain a significantly higher number of NK cells in the intraperitoneal cavity as compared to no antibody control (2.7×10^5 *vs.* 1.1×10^5, p = 0.02, Figure 4A). NK cells recovered from the anti-PD-L1 treated animals were almost uniformly positive for CD16 expression with a mean of 97% (range 94–98%) of NK cells staining positive for anti-CD16. In contrast, NK cells recovered from animals treated in the absence of anti-PD-L1 revealed significant downregulation of CD16 (mean 78%) with a disparate spread of CD16 expression ranging from 30 to 98% (Figure 4B). Loss of CD16 expression has been reported on NK cells recovered from ovarian patient ascites and was connected to loss of cytotoxic function.[33,34] Interestingly, NK cells from animals treated with anti-PD-L1 also had greater frequencies of $CD57^+$ NK cells (30 *vs.* 47%, p = 0.0014; Figure 4C) which have been correlated with memory-like phenotype and are associated in several studies with better outcomes and improved long-term survival across many tumor types. (reviewed in[35]) Anti-PD-L1 treated animals had very few PD-1 positive NK cells (median 2.9%, range 2.5–4.5%) and also significantly lower frequencies of PD-1 positive T cells (11% *vs.* 5%, p = 0.0006) (Figure 4D and E). There were also virtually no Tregs in the *ip* cavity of mice treated with anti-PD-L1 although Tregs were also low in the NK only treated group for this donor and the difference did not reach statistical significance (Figure 4F). In conclusion, as predicted PD-L1 blockade improved phenotype and survival of NK cell *in vivo* which should enhance NK cell efficacy and increase overall survival in a treatment context.

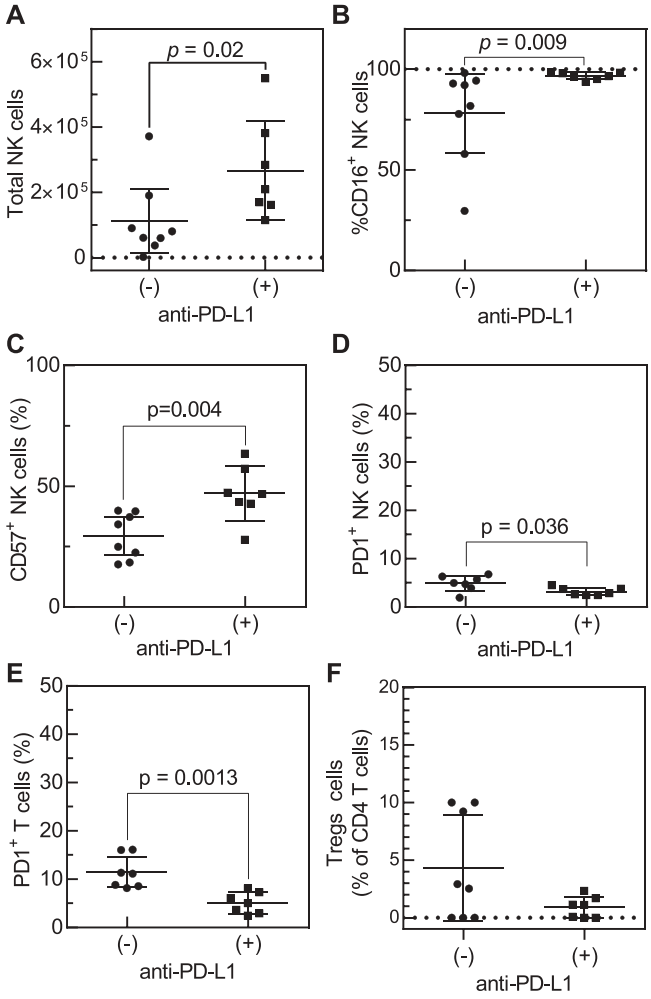

Figure 4. PD-L1 blockade improves NK cell persistence and preserves cytotoxic phenotype. Combination of anti-PD-L1 and PM21-NK cells was tested in NSG mice implanted with SKOV-3 cells. Mice were implanted with 1×10^6 SKOV-3 cells i.p. on day −4 and then given 2 doses of 10×10^6 T cell-depleted PM21-NK cells (n = 16) i.p. starting on day 0. Mice were subdivided and half of the animals received vehicle control while the remaining animals were given 10 mg/kg of anti PD-L1 (clone 6E11, Genentech) on day 0 following with 5 mg/kg twice weekly. All animals were given 25,000 U IL2 (3x/week). Mice were monitored for luminescence signal from SKOV-3. Animals were euthanized on day 26 and peritoneal wash was collected and analyzed for presence and activation state of immune cells. Treatment with PD-L1 antibody resulted in greater numbers of NK cells still residing in the peritoneal cavity at day 26 (A). Close to 100% of NK cells recovered from anti-PD-L1 treated animals had expression of CD16 while expression was significantly diminished in control animals (B). Treated animals had greater percentage of $CD57^+$ NK cells (C) and lower content of $PD\text{-}1^+$ NK (D) and T cells (E). There was also a trend toward lower levels of Tregs in PD-L1 treated animals (F). Data are presented as scatter plots with mean and 95% confidence interval. Unpaired two-tailed Mann-Whitney analyses were performed using GraphPad Software to determine p-values.

PD-L1 blockade improves anti-SKOV-3 efficacy of PM21-NK cell treatment

To test the hypothesis that PD-L1 blockade improves the anti-tumor efficacy of PM21-NK cells, animals were implanted with SKOV-3 cells as previously described and were 1) untreated as control group, 2) treated with anti-PD-L1 alone, 3) treated with PM21-NK cells alone, or 4) treated with a combination of PM21-NK cells with anti-PD-L1 (Figure 5A). Similarly to prior experiments shown on Figure 3, the non-CD16 engaging antibody (clone 6E11, Genentech) was selected for this experiment, to study the sole effect of PD-L1 blockade. As would be expected in immunocompromised NSG mice lacking endogenous immune cells, anti-PD-L1 alone had no effect on tumor progression or animal survival. Treatment with PM21-NK cells slowed down tumor growth as determined by tracking of luminescence (Figure 5B and C) and significantly improved animal survival over the untreated group (median survival of 40 *vs.* 24, p = 0.0006, Figure 5D). Combining anti-PD-L1 and PM21-NK cells resulted in doubling of animal survival as compared to untreated and anti-PD-L1 treated groups (48 *vs.* 24 days, *p* = 0.0001). There was also further significant improvement of survival of this group over PM21-NK cells alone group (48 *vs.* 40 days, p = 0.042). Furthermore, there were two animals (25%) remaining in good health at day 58 when the experiment was terminated. One of the two animals was completely free of any peritoneal tumors while the other had only one very small tumor

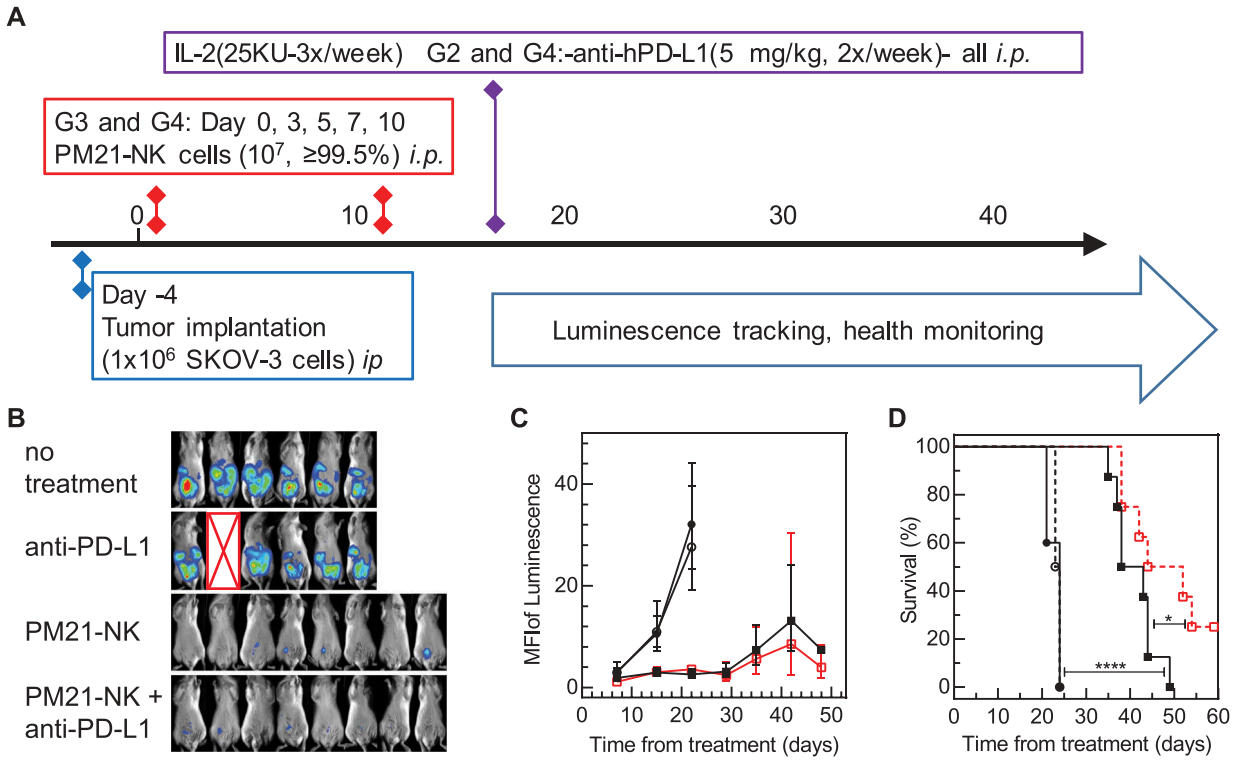

Figure 5. Adoptive transfer of PM21 NK cells with PD-L1 blockade leads to improved survival. A) Combination of anti-PD-L1 and PM21-NK cells was tested in NSG mice implanted with SKOV-3 cells. Mice were implanted with 1×10^6 SKOV-3 cells i.p. on day −4 and then given 5 doses of 10×10^6 T cell-depleted PM21-NK cells (n = 16, circles) or vehicle (n = 12, squares) i.p. starting on day 0. Groups were further subdivided and half of the animals received vehicle control (closed symbols) while the remaining animals were given 10 mg/kg of anti PD-L1 (clone 6E11, Genentech; open symbols) on day 0 following with 5 mg/kg twice weekly. All animals were given 25,000 U IL2 (3x/week). Mice were monitored for luminescence signal from SKOV-3 (day 23-B, C) and survival (D). Untreated and anti-PD-L1 alone mice had mean survival of 24 days. Treatment with PM21-NK cells (■) significantly improved survival over the untreated (p = 0.0003, ●) and PD-L1 alone (p = 0002, ○) groups resulting in mean survival of 40 days. Combination of PM21-NK cells with anti-PD-L1 (red□) resulted in further improvement of survival over the PM21-NK cells alone group (48 days, p = 0.042) with 25% of mice still remaining in good health at day 58 at the termination of experiment. Survival was determined using Kaplan-Meier method with log rank test to determine statistical significance. Pairwise comparisons were performed to determine individual p-values. Error bars represent standard error of means (SEM). P values are shown as * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, **** if $p < 0.0001$.

that was likely reseeded from an invading subcutaneous tumor, a likely artifact reported in this model.[36]

Discussion

In this paper we demonstrated that administration of PM21-NK cells leads to a strong anti-tumor response, resulting in tumor shrinkage and significant improvement of survival in treated animals. Since PM21-NK cells secrete IFNγ in response to SKOV-3 cells, we hypothesized that PM21-NK cell treatment results in upregulation of PD-L1 expression on tumors and facilitates subsequent immunosuppression (Figure 6). Indeed, tumors isolated from PM21-NK cell treated mice had a greater fraction of PD-L1 positive cells as compared to untreated mice. Furthermore, human lymphocytes isolated from the tumor microenvironment of the PM21-NK cell treated mice contained T cells of which a majority were Tregs, particularly in mice that developed ascites. Thus, Tregs developed from a miniscule subpopulation of donor T cells (<0.1% of total human lymphocytes) transferred with PM21-NK cells. Prior studies have shown that PD-L1 is critically required for the induction of Tregs from naïve CD4 T cells as well as their maintenance and immunosuppressive function[29] and the induction of its expression was thought to be caused mostly by CD8 T cells.[26] PD-L1 can also increase Treg content in PM21-NK cell product and decrease the NK/T cell ratio *in vitro* (Supplemental Figure 6). This study showed for the first time that NK cells are also capable of PD-L1 induction which likely results in Treg expansion. This finding suggests that the anti-tumor

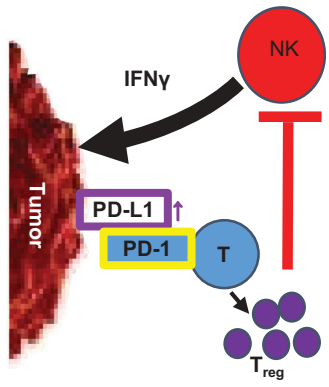

Figure 6. Adoptively transferred PM21 NK cells secrete IFNγ, which induces PD-L1 on tumor cells driving Treg expansion *in vivo*. Depicted is a schematic showing a model for cross-talk between NK cells and Tregs in the tumor microenvironment. PM21-NK cells secrete high amounts of IFNγ that induce PD-L1 expression on the tumor surface. PD-L1 on tumor cells can interact with T cells to promote expansion of Tregs which are known to inhibit NK cell function and persistence.

effect of NK cell treatment, such as with PM21-NK cells, can be further enhanced by co-administration of anti-PD-L1 to thwart immunosuppression and thus significantly improve outcomes. The present work also shows that non-responsive tumors could be turned to responsive tumors by priming with adoptive PM21-NK cells to make them amenable for PD-L1 therapy. This can potentially augment the patient population that would be eligible for PD-L1 therapy to expand therapeutic avenues for greater overall patient benefit. To note, such induction of PD-L1 on tumors have been contemplated by administration of IFNγ, but systemic IFNγ would be toxic. In contrast, an activated NK cell population, such as adoptive PM21-NK cells, would perform targeted IFNγ secretion only when encountering a tumor.

PD-1/PD-L1 blockade has been generally regarded to benefit CD8 T cell mediated response. The reason is NK cells are mostly devoid of the expression of the checkpoint receptor PD-1; however, the presence of PD-1$^+$ NK cells have been reported in settings of multiple myeloma.[28] In the referenced study, PD-1 inhibition improved NK cell mediated lysis of autologous multiple myeloma cells. Similar to previous reports, PM21-NK cells either expanded *ex vivo* or isolated from the tumor microenvironment mostly lacked PD-1 expression. Further, their cytotoxicity and cytokine production was not affected by PD-L1 blockade *in vitro*. However, PD-L1 expression on tumors likely leads to induction and expansion of Tregs in the tumor microenvironment, which then inhibit NK cell survival and function. Tregs and/or PD-1 engagement can also negatively affect function of T cell including CD4$^+$ T cells that aid NK cell by secreting IL2 or other stimulatory signals.[37] This suggests that PD-L1 blockade should have a positive impact on NK cell function *in vivo*. Indeed, the study herein showed that PD-L1 blockade improved NK cell survival and preserved their cytotoxic phenotype, leading to better overall anti-tumor efficacy. In the absence of anti-PD-L1, NK cells downregulated CD16 expression, a conversion that, based on clinical studies, also occurs when endogenous NK cells attack ovarian cancer. NK cells are highly enriched in the ascites collected from ovarian carcinoma patients but are mostly CD16$^-$ and show a lack of cytotoxic function with diminished IFNγ production.[33,34] Similar changes and dysfunction were reported in NK cells isolated from patients with esophageal squamous cell carcinoma and non-small cell lung carcinoma, particularly in pleural effusion.[38,39] The downregulation of CD16 on NK cells has been shown to be driven by TGFβ and leads to conversion from cytotoxic to a more immunoregulatory phenotype similar to decidual NK cells.[30,38] In the current study, we found that NK cells downregulated their CD16 expression upon exposure to ovarian tumor microenvironment but this conversion was mitigated by PD-L1 blockade. PD-L1 blockade also resulted in higher amounts of CD57$^+$ NK cells, a subpopulation that have been reported to have a higher cytotoxic capacity with greater responsiveness via CD16 engagement and its presence correlated with better outcomes in squamous cell lung cancer.[40,41]

Adoptive NK cell therapy offers great therapeutic potential in cancer treatment but is an emerging modality. NK cells have shown to be very effective in preventing relapse in residual disease setting, under low tumor burden.[18,42] In several trials testing efficacy of haploidentical NK cells in patients with advanced relapsed or refractory tumors, responses were observed only in a small subgroup of patients and were hampered by co-expansion of Tregs which were thought to be of patient origin.[43-46] Our results suggest that expansion of Tregs may arise in the tumor microenvironment upon PD-L1 expression, and can even occur by expansion from the very small amount of T cells transferred as part of an adoptive NK cell product. To mitigate such *in situ* Treg generation, PD-L1 blockade may prevent Tregs induction and improve overall efficacy of adoptive NK cell therapy. Association of NK cells with better outcomes have been shown in several studies. Transcriptome analysis of head and neck cancers (HNSCC) showed that independent of human papilloma virus (HPV) status, these cancers are highly infiltrated with CD8 T cells, Tregs and CD56^{dim} NK cells and infiltration with either of these populations correlated with superior survival.[47] Cetuximab has shown success in treatment of HNSCC patients but its efficacy, which is dependent on NK cell mediated ADCC, is observed only in 15–20% of patients.[48] Poor prognosis was correlated to Cetuximab induction of Tregs which were shown to suppress NK cells and decrease ADCC. PD-L1 expression in such context was not assessed as part of this study but Cetuximab stimulates IFNγ production from NK cells *in vitro* which would likely induce PD-L1, suggesting potential benefit of combination with PD-L1 blockade.

Currently, with the efficacy and approval of anti-PD-L1/anti-PD-1 therapy in several indications, there is an increasing focus to screen the tumors of patients for PD-L1 expression to examine the suitability of PD-L1 blockade therapy. Recent analysis examining the effect of PD-L1 status on outcomes of PD-1/PD-L1 checkpoint blockade in the setting of non-small-cell lung cancer showed that selection of patients based on PD-L1 expression greatly improved overall survival while lowering the cost.[4] Conventionally, PD-L1 is expressed on tumors for a minority of patients and thus only a minority of patients can benefit from PD-1 blockade therapy. Application of adoptive PM21-NK cells could broaden the patient pool that have PD-L1$^+$ tumors and co-administration of anti-PD-L1 together with PM21-NK cells could lead to even greater efficacy. Such clinical investigations would be desirable and may benefit a greater portion of cancer patients.

Induced PD-L1 could also serve as a "universal" ligand for tumor targeting with humanized ADCC competent antibodies. A recent study reported that one such anti-PD-L1 antibody engages CD16 on NK cells and can induce NK cell killing *via* ADCC.[49] Furthermore, IFNγ has been shown to induce PD-L1 on the majority of tumor cell lines tested across many tumor types including panel of ovarian and pediatric tumors.[6,49,50] In this study NK cells induced PD-L1 on most cell line tested including leukemia, lung, colon and ovarian cancer cell lines. Recently, oncolytic vaccinia virus (OV) has been shown to also lead to induction of PD-L1 largely through IFNγ produced by immune cells recruited into tumor milieu in response to viral infection and its efficacy was improved by PD-L1 blockade.[51] It is plausible that part of this response was mediated by NK cells, since an increase in cytotoxic markers-such as IFNγ, granzyme B and perforin-was observed in the dual treatment group while

depletion of either CD4 or CD8 T cells had only a partial effect on the efficacy of OV/PD-L1 combination. Clinical trial results from testing a combination of talimogene laherparepvec virus with another checkpoint inhibitor, anti-CTLA-4 also points at a potential role of NK cells in mediating the efficacy of this combination in treatment of melanoma, where enrichment of genes related to a burst of virus-specific NK cells was detected.[52]

In summary, this study demonstrated that treatment of ovarian cancer with adoptive PM21-NK cells leads to induction of PD-L1 and *in situ* expansion of Treg cells. Hence this result leads to the rationale for combination of NK cell therapeutics with PD-L1 blockade. Combinatorial application of PM21-NK cells and anti-PD-L1 improved NK cell function and significantly extended animal survival in an aggressive model of disseminated peritoneal ovarian cancer. Adoptive application of PM21-NK cells can be used to prime tumors for PD-L1 blockade to greatly extend its utility and therapeutic success. Likewise, inclusion of anti-PD-L1 treatment with adoptive NK cell therapy should improve its efficacy and unleash the full potential of NK cells. Immunotherapy combinations, such as described here with a cell therapy and a targeted monoclonal antibody are likely to play a significant role in advancing clinical innovation in the near future.

Methods

Mice

NSG (NOD-*scid* IL-2Rγ^{null}) mice were purchased from Jackson Laboratory and then bred in house. Female 7- to 12-week-old were used for all *in vivo* experiments. Mice were housed and handled in accordance with protocols approved by the University of Central Florida Institutional Animal Care and Use Committee, an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility.

Cell culture

Discarded buffy coats (Leukocyte Source) from de-identified, healthy donors were purchased from local blood bank (One Blood) and were used as source of NK cells for experiments. Peripheral blood mononuclear cells were separated by density using Ficoll-Paque Plus solution (GE Healthcare). PBMCs were aliquoted and cryopreserved prior to use in experiments or expansion. NK cells were expanded using PM21-particles as previously described.[24,25] Briefly, whole PBMCs or T-cell depleted PBMCs (EasySep CD3 positive selection kit; StemCell Technologies) were cultured for 14 days with 100 U/mL IL2 (Peprotech) and 200 μg/mL PM21 particles in SCGM media (CellGenix) supplemented with 10% FBS, 2 mM Glutamax. For mouse experiments, expanded NK cells were further T-cell depleted on day 14 if T cell content was above 3% and used fresh or frozen for future use (see Supplemental Table 1 for exact information about T cell content and procedure used). SKOV-3-GFP-luc (referred to as SKOV-3) cells purchased from CellBiolabs were passaged once through NSG mice to increase their tumorigenicity and sorted based on GFP expression. CSTX-002 cells (K562 cell line expressing 41BBL and membrane bound IL21) used for preparation of PM21 particles were provided by CytoSen Therapeutics. SKOV-3 and CSTX-002 cells were maintained in RPMI + 10% FBS. K562, HL-60, Kasumi-1, A-549, HTC116 and HT29 were from ATCC and were maintained according to recommendations specific for each cell line.

Flow cytometry

The following pre-conjugated antibodies were used for flow cytometry: CD56-PE (clone 5.1H11), CD56-PC7 (clone 5.1H11), CD56-PacBlue (clone 5.1H11), CD3-FITC (clone OKT3), CD16-BV786 (clone 3G8), CD57-PE (clone HCD57), CD3-APC (clone OKT3), CD3-PacBlue (clone UCHT1), CD25-PE (clone PC61.5), PD-L1-APC (clone 29E.2A3), CD8-PC7(clone RPA-T8), IFNγ-Alexa647(clone 4S.B3), TNFα-PacBlue(clone Mab11) (BioLegend); CD45-APC(clone 2D1), CD45-eFluor450(clone 2D1), PD-1-eVolve655 (clone J105), FoxP3-FITC (clone PCH101), TNFα-eF450 (clone Mab11) (eBioscience); and CD4-APC-H7 (clone RPA-T4) (BD Biosciences). All samples were acquired on a CytoFlex flow cytometer and analyzed using CyteExpert or FlowJo software (v10.0.7).

Functional assays

Cytotoxicity assays of NK cells utilized measurement of Annexin V. NK cells were isolated and preactivated with 2000 U IL2 for 5 days or were expanded with PM21-particles as above. Target cells (SKOV-3-GFP or TFL4 labeled leukemia, lung and colon cancer cell lines) were culture alone (control wells) or co-cultured at 0.5×10^6 cells/mL with NK cells at indicated effector-to-target (E:T) ratios for 60 minutes in 37°C, 5% CO_2 atmosphere. The cells were then centrifuged and resuspended in Annexin V labelling buffer containing Annexin V-PacBlue antibody and incubated for 15 minutes at 4°C prior to analysis by flow cytometry. The cytotoxicity was determined based on the absolute amount of Viable Target Cells (GFP+/Annexin V-) remaining in each well with effectors ($VTC^{E:T}$) and referenced to average VTC in "target alone" control wells ($VTC^{T\ ctrl.}$).

$$\text{Cytotoxicity}^{E:T}(\%) = \left(VTC^{E:T} / \text{Average}VTC^{Tctrl.}\right) * 100$$

IFNγ and TNFα production

NK cells were stimulated with vehicle, SKOV-3 cells, or SKOV-3 + cytokines (10 ng/mL IL12, 50 ng/mL IL18, 100 ng/mL IL15) in presence of 3 μg/mL Brefeldin A (eBiosciences) for 4 hours. For IFNγ/TNFα experiments with anti-PD-L1, SKOV-3 cells were pretreated with 20 ng/mL of IFNγ for 48 h to induce PD-L1 expression which was confirmed by antibody staining followed by flow cytometry. IFNγ pretreated SKOV-3 cells were incubated with 100 ng/mL anti-PD-L1 (clone 6E11, Genentech) at 37°C for 30 minutes and then co-incubated with NK cells at 1:1 ratio in 37°C, 5% CO_2 atmosphere for 4 hours. Samples were then stained with CD56 and CD3 antibodies, fixed and permeabilized (eBioscience IC Fixation and Permeabilization buffers), and probed with dye-conjugated antibodies for IFNγ and TNFα followed by analysis using flow cytometry.

PD-L1 induction transwell assay

Target cells (25,000) were added to the upper and lower chamber of each well of a 24-well transwell plate (Corning, CAT# 3460). Next, 250,000 of PM21-NK cells or vehicle were added to the upper chamber and co-incubated at 37°C, 5% CO_2 atmosphere for 24 hours. Next, cells from the lower chamber were lifted with 0.25% Trypsin (if attached) and/or transferred to a tube, washed, stained with anti-PD-L1-APC and analyzed by flow cytometry.

Mouse model

For *in vivo* experiments, 1×10^6 SKOV-3-GPF-luc cells were injected to the intraperitoneal (i.p.) cavity of NSG (NOD-*scid* IL-2Rγ^{null}) and allowed to seed for 4–8 days. Mice were then treated as specified in the figure legends with 2–5 doses of 10^7 NK cells injected i.p. along with IL2 (25,000 U, 3x/week) and anti-PDL1 (10 mg/kg on day 0 followed by 5 mg/kg, 2x/week). Mice were imaged once a week using In vivo Xtreme II imager (Bruker), and checked periodically for health status.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0. Pairwise comparisons were performed to determine individual p-values. Two-way ANOVA was used to compare cytotoxicity curves and determine p-value. Paired, two-tailed Student's t test was used to analyze cytokine secretion, while for all others, unpaired two-tailed Mann-Whitney or Student's t-tests were used as indicated in each figure legend. Survival analysis was performed using Kaplan-Meier method with log rank test to determine statistical significance. Values less than 0.05 were considered significant. P values are shown as * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, **** if $p < 0.0001$.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

ORCID

Jeremiah L. Oyer http://orcid.org/0000-0002-9368-9323
Sarah B. Gitto http://orcid.org/0000-0003-4109-5539
Alicja J. Copik http://orcid.org/0000-0003-4109-5539

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