Supplemental methods

NK cell expansion

PM-21-NK cells: 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. Cells were counted and media exchanged every 2-3 days.

FC21-NK cells: T-cell depleted PBMCs (EasySep CD3 positive selection kit; StemCell Technologies) were co-cultured with mitomycin-treated K562-mb21 cells added to culture at 1:10 ratio in RPMI media with 100 U/mL IL2 (Peprotech) and 10% FBS. On day 7, fresh K562-mb21 cells were added to the culture at a 2:1 ratio of NK cells to feeder cells. Cells were counted and media exchanged every 2-3 days.

Expanded NK cells were further T-cell depleted on day 14 using EasySep CD3+ selection kit (Stem Cell technologies) if T-cells were >1% of total cell culture.

Analysis of blood and peritoneal fluids

For submandibular bleeds, animals were injected with saline 15 minutes prior to collection to assure proper hydration and then up to 100 µL of peripheral blood was collected. Blood (50 µL) was processed using red blood cell lysis buffer and cells were stained with fluorophore conjugated antibodies against hCD45 (eBiosciences), hCD56 and hCD3 (Biolegend). Ascites were processed similarly. Peritoneal washes were obtained by flushing ~2mL of PBS in the peritoneal cavity.

Immunohistochemistry

Specimens were fixed in 10% neutral buffered formalin (Surgipath Leica), paraffin embedded prior to sectioning with a rotary microtome (5um; Leica). Sections attached to charged microscope slides were dried at 65°C for 30 minutes in a hybridization oven. Immunohistochemisty using Polymer Refine Detection reagents was performed using the Bond-Max Immunostainer (Leica). Next, tissue was dehydrated and the cover slip was placed on the slide. Antigen retrieval was optimized using sodium citrate, pH 6.0 or EDTA, pH 9.0 (Leica). Primary antibodies used for this study include CD3 (Cell Marque), CD4 (Leica), and FoxP3 (Epitomics). Images were taken using a Leica DM 2000 microscope with 40X objective.

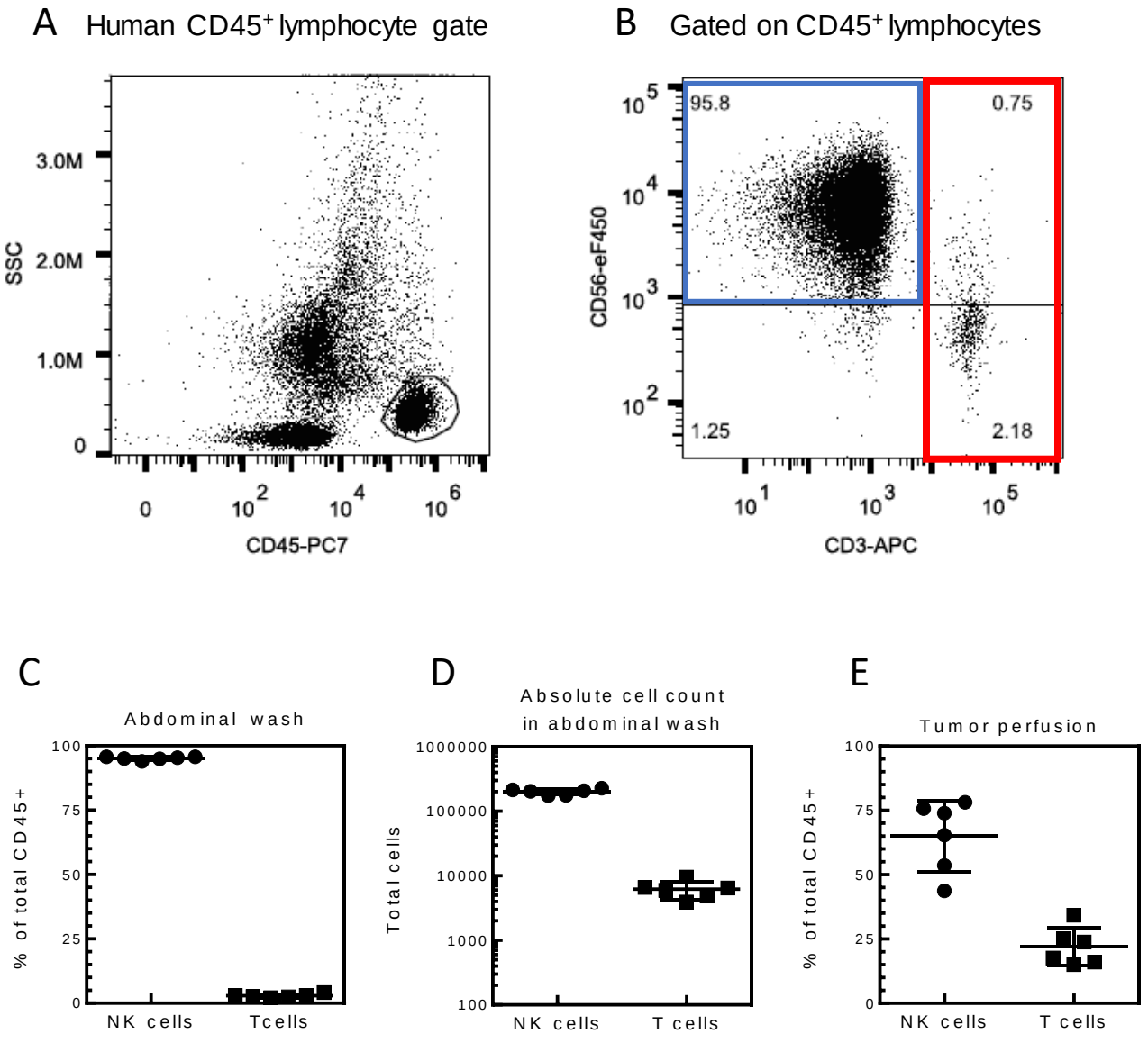
Treg induction with anti-PD-L1 beads.

Protein A/G magnetic beads (Pierce Thermo Fisher, 25 µL at 10 mg/mL) were labeled with 50 µg of human PD-L1-Fc (Biolegend) and/or IgG1 control. PM21 NK cells were seeded at 0.2 x 10^6 NK cells/mL and stimulated with 10^7 of PD-L1/IgG1 beads or ctrl. IgG1 beads. Cells were incubated at 37°C in 5% CO_2 atmosphere for 4 days. Cells were analyzed for NK cell and Treg content.

Figure	Donor	Depleted upfront	Day depleted after seeding	T-cell % pre depletion after seeding	Tcell% at day 14 (or injection)
1a &1b	L50 PM	Y	13	12.58%	0.10%
1a&1b	L50 IL2	Y	N/A	N/A	1.55%
1c & 1d	L34	N	N/A	N/A	3.10%
1c & 1d	L35	N	N/A	N/A	1.85
1c & 1d	L36	Y	N/A	N/A	0.52%
1c & 1d	L37	Y	N/A	N/A	0.61%
1e	PL5	Y	N/A	N/A	0.56%
1e	L50	Y	N/A	N/A	4.78%
1e	L36	Y	N/A	N/A	0.43%
2	L34 (1st inj)	N	N/A	N/A	2.38%
2	L34 (2nd inj)	N	N/A	N/A	2.11%
3b, c, & d	41	Y	N/A	N/A	1.87%
3b, c, & d	43	Y	N/A	N/A	1.89%
3b, c, & d	44	Y	N/A	N/A	1.05%
4	41 (1st inj)	Y	N/A	N/A	1.50%
4	41 (2nd inj)	Y	N/A	N/A	1.10%
5	L36 (1st inj)	Y	13	0.52%	0.00%
5	L36 (2nd inj)	Y	16	0.40%	0.18%
5	L36 (3rd inj)	Y	18	0.22%	0.01%
5	L36 (4th inj)	Y	18	0.22%	0.12%
5	L36 (5th inj)	Y	18	0.22%	0.16%
Supplemental 3-5	L27 FC	N	14	2.83%	0.05%
Supplemental 3-5	L27 PM	N	14	7.99%	0.02%
Supplemental 3-5	L27 FC	N	14	1.21%	0.00%
Supplemental 3-5	L27 PM	N	14	3.70%	0.03%
Supplemental 7	PL4	N	N/A	N/A	10.72% (day 9)
Supplemental 7	L50	N	N/A	N/A	21.37% (day 9)

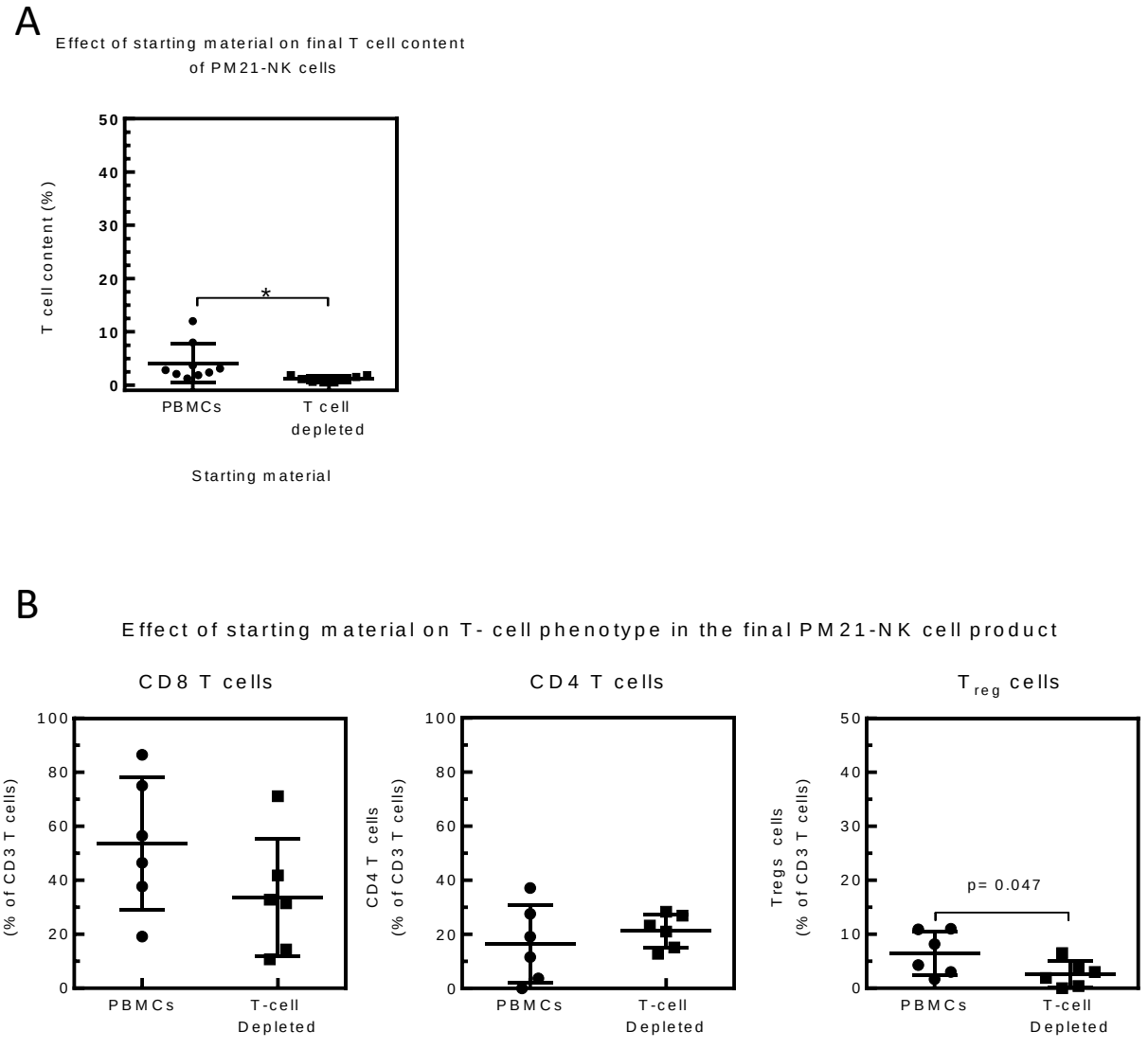
Supplemental Table 1. Donor, starting material and final T cell content of the PM21-NK cell product used in experiments 1-5 and supplemental figures

Supplemental Figure 1



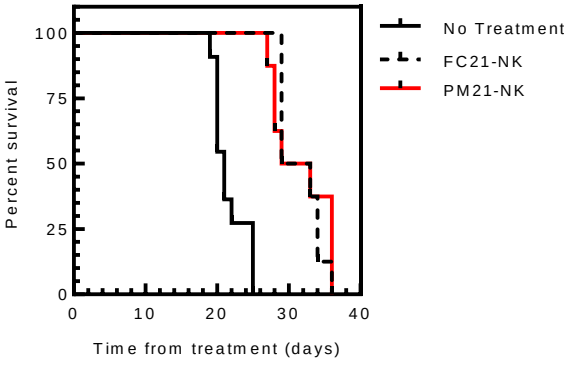
Supplemental Figure 1. Gating strategy and abdominal and tumor lymphocyte content for Figure 2. Cells collected from the peritoneal cavity are first gated based on human CD45 to distinguish human lymphocytes (A). NK cells (blue box) and T cells (red box) in human lymphocyte populations are discriminated based on CD56 and CD3 staining (B). NK cells consisted majority of human lymphocytes in the abdominal cavity (C) and an average of (0.20±0.02) x 10^6 NK cells were recovered in the abdominal wash (D) from animals euthanized 13 days from the initiation of treatment. NK cells also consisted the majority population (65± 14%) of human lymphocytes recovered from perfused tumors although the percentage was lower as compared to abdominal wash (E).

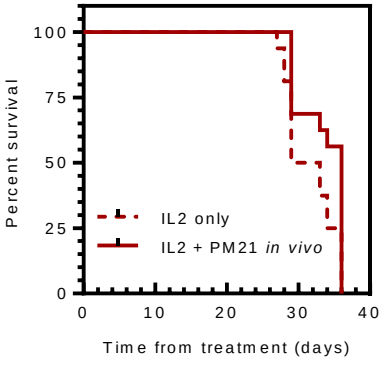
Supplemental Figure 2



Supplemental Figure 2. Characterization of T cell populations in final PM21-NK cell product with respect to starting material.
Products used in experiments included in the manuscript were analyzed for the effect of starting material on the final T cell content (A). PM21-NK cells contained significantly less T cells when expanded from T-cell depleted material as compared to starting with PBMCs (1% vs. 4%, p=0.04). To determine typical T cell phenotype of PM21-NK cell product and assess if starting material has an effect on T cell phenotype, PM21-NK cell were expanded from 6 donors using unselected or T cell depleted PBMCs (B). The final product contained similar proportions of CD8 T cells which were the predominant T cell population in most of the products, followed by CD4 T cells and T regs. Tregs accounted for minority of T cell populations in the final product with products started from unselected PBMCs containing significantly more Tregs as compared to those obtained from T cell depleted material (6% vs. 3%, p=0.047). Analysis was performed using unpaired two-tailed t-test for upper panel and paired t-test for the lower panel.

Supplemental Figure 3





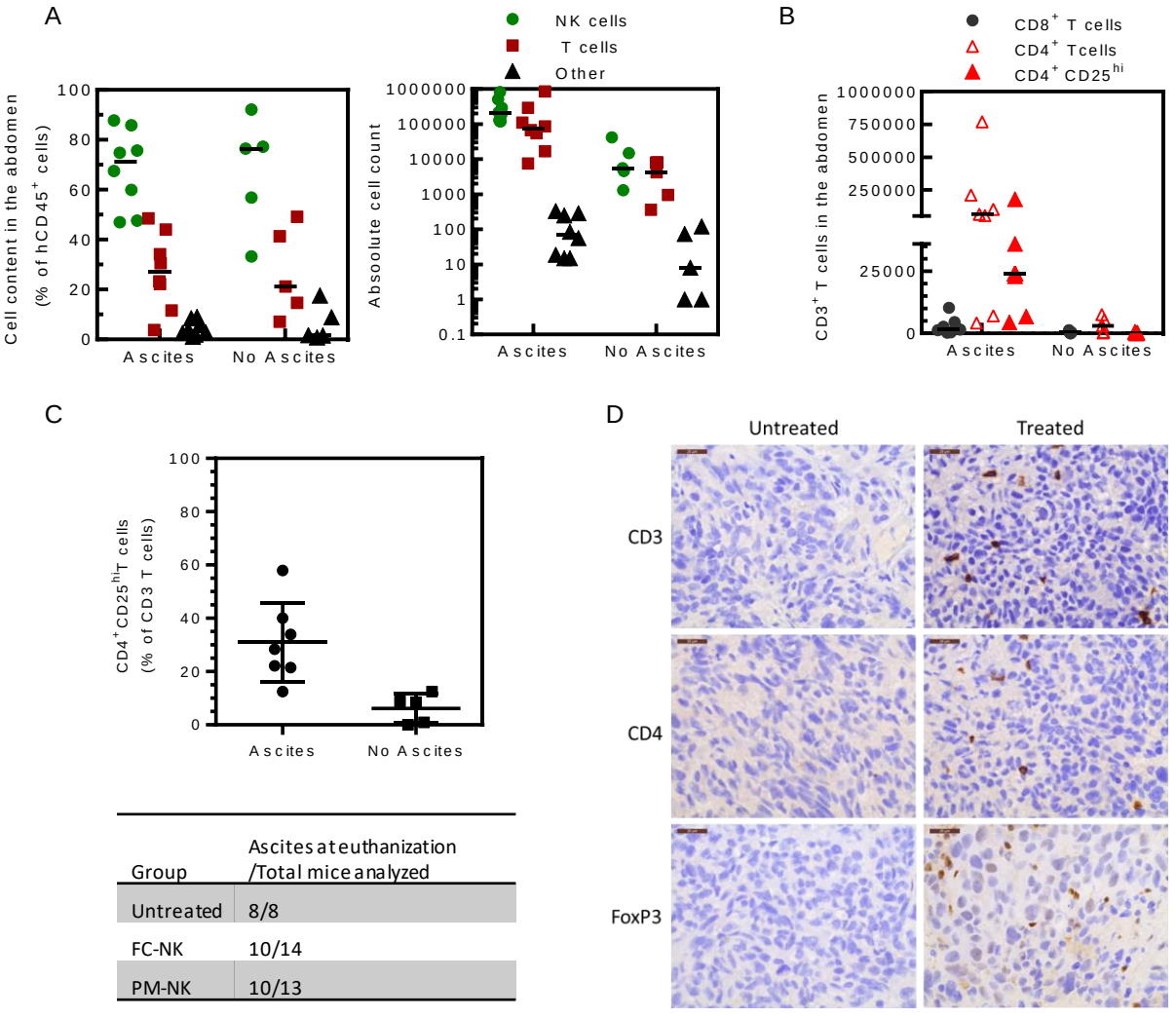
	Ctrl.	FC21-NK	PM21-NK
Median survival	21.0	31	31

	IL2	IL2+PM21	*P*
Median survival	31	36	0.056

p-values	No treatment
PM21-NK	<0.0001
FC21-NK	<0.0001

Supplemental Figure 3. NK cells expanded either with PM21 or FC21 significantly improve survival of SKOV-3 bearing mice. NK cells were expanded by culturing CD3 depleted PBMCs with PM21 particles or by co-culture with K562.mb21 cells for 14 days as described in method section. NSG mice (8 per group) were implanted i.p. with 1 x 10^6 SKOV-3 ovarian tumor cells, allowed to seed for 8 days and then treated with vehicle or NK cells expanded with PM21 or K562.mb21 cells (two doses of 10 x 10^6, injected 6 days apart), with or without *in vivo* administration of PM21 particles (600 µg, 3x weekly), and IL2 (25 KU, 3x weekly), all delivered i.p.. Treatment of SKOV-3 engrafted NSG mice with NK cells, expanded with K562.mb21 cells (FC21-NK cells) or with PM21 particles (PM21-NK cells), allowed significant (P< 0.0001) 10 day increase in survival compared to untreated animals that succumbed on average 21 days after start of treatment. Administration of PM21 particles i.p. enhanced survival by 5 days (p = 0.056) over no *in vivo* PM21 groups. Survival analysis was performed with log rank (Mantel-Cox) test.

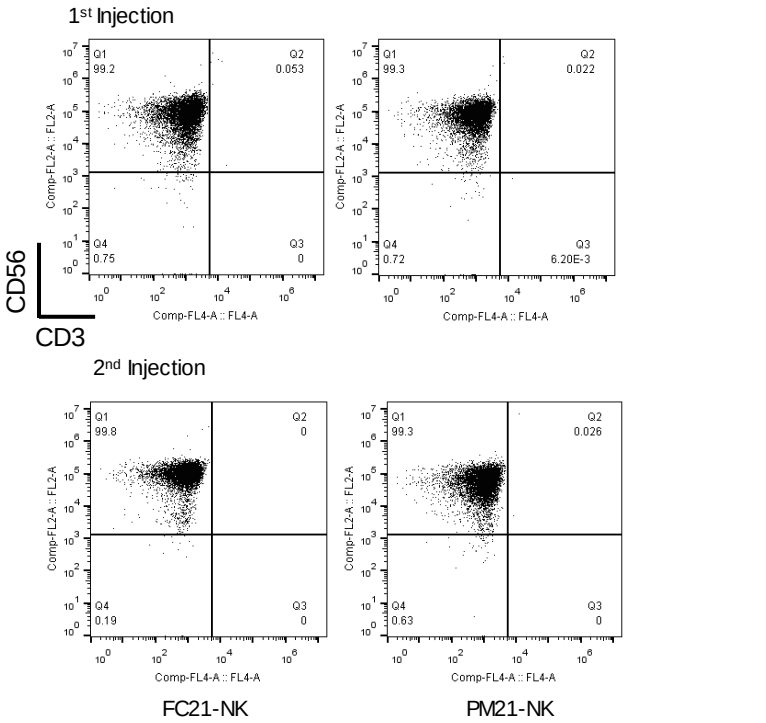
Supplemental Figure 4

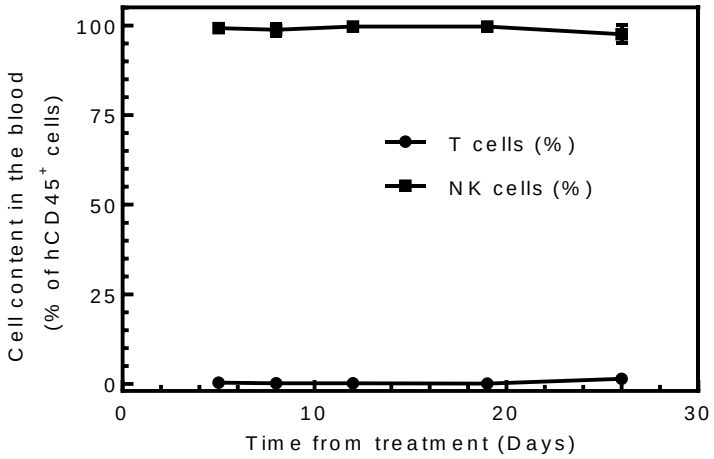


Group	Ascites at euthanization /Total mice analyzed
Untreated	8/8
FC-NK	10/14
PM-NK	10/13

Supplemental Figure 4. Human CD4⁺ CD25^{hi} T cells are prevalent in ascites. NSG mice implanted with SKOV-3 cells were treated with PM21-NK or FC21-NK cells as described in prior figure legend. Human lymphocytes content was analyzed in abdominal fluids collected from moribund animals euthanized due to health status (days 27-33 after initiation of NK cell treatment. Although NK cells consisted majority of the human lymphocyte population recovered from the peritoneal cavity, T cells were also prevalent consisting 23 (4-49)% of hCD45⁺ cells (A). Further analysis of the T cell subpopulations revealed higher abundance of CD4⁺ T cells including CD25^{hi} T (Treg) cells in mice correlating with presence of ascites which were noted in majority of the treated animals (B, C). Resected tumors from treated animals had cells that were positive for CD3, CD4 and FoxP3 confirming presence of Tregs in the tumor microenvironment (D).

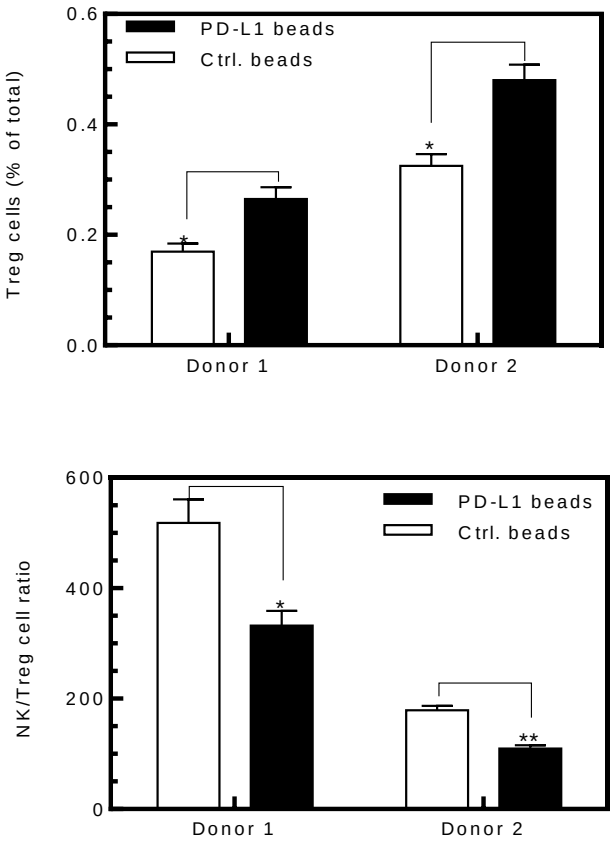
Supplemental Figure 5





Supplemental Figure 5. Initial population of NK cells injected into mice as well as peripheral blood collected from mice contained miniscule amounts of T cells. NK cells were expanded from PBMC with PM21-particles (PM21-NK) or feeder cells (FC21-NK) for 14 days. Expanded cultures on day 14 contained under 10% T cells and were further T-cell depleted prior to injection into SKOV-3 engrafted mice. Based on flow cytometry analysis injected NK cells were highly pure (>99%) and virtually devoid of T cells. The blood sampled from the animals via cheek bleeds shows that human NK cells consisted majority ≥98%) of CD45+ human lymphocytes.

Supplemental Figure 6



Supplemental Figure 6. PD_L1 treatment leads to increased Treg content and decreased NK/Treg ratios in PM21-NK cell cultures *in vitro*. NK cells were expanded from PBMC obtained from two donors with PM21-particles (PM21-NK). Cultures were stimulated for 4 days with beads coated with PD-L1/IgG or IgG and then NK and T cell content was analyzed. Multiple t-test analysis were performed to determine p values. P values are shown as * if $p<0.05$, ** if $p<0.01$, *** if $p<0.001$, **** if $p<0.0001$.