Supplementary Materials for

PD-L1-targeting high-affinity NK cells (PD-L1 t-haNK) induce direct antitumor effects and target suppressive MDSC populations

Kellsye P. Fabian, Michelle R. Padget, Renee N. Donahue, Kristen Solocinski, Yvette Robbins, Clint T. Allen, John H. Lee[4], Shahrooz Rabizadeh, Patrick Soon-Shiong, Jeffrey Schlom, James W. Hodge

Document includes:

Table S1. Identification of 135 immune subsets

1. **CD4**: Helper T lymphocytes (33 subsets)
2. **CD8**: Cytotoxic T lymphocytes (29 subsets)
   - **Maturation status of T lymphocytes (in CD4 and CD8)**:
     - **Naïve**: CD45RA^{+} CCR7^{+}
     - **Central Memory**: CD45RA^{-} CCR7^{+}
     - **Effector Memory**: CD45RA^{-} CCR7^{-}
     - **Terminal (EMRA)**: CD45RA^{+} CCR7^{-}
   - **T lymphocyte markers (in total and memory CD4 and CD8)**:
     - **CTLA-4:** inhibition
     - **PD-1:** activation/inhibition
     - **PD-L1:** activation/cross-inhibition
     - **TIM-3:** inhibition
     - **41BB:** co-stimulation
     - **ICOS**: activation (only on CD4)
     - **Ki67:** proliferation
3. **Tregs**: Regulatory T lymphocytes (CD4^{+} CD25^{+} FoxP3^{+} CD127^{-}) (10 subsets)
   - **CD45RA**: Tregs highly expandable *in vitro*
   - **CTLA-4**: Treg suppression
   - **CD49d:** "contaminating" effector lymphocytes (non-Tregs)
   - **ICOS**: Treg suppression
   - **PD-1:** activation/inhibition
   - **PD-L1:** cross-inhibition
   - **CD38:** Treg suppression
   - **HLA-DR:** Treg suppression
   - **Ki67:** proliferation
4. **B lymphocytes**: CD19^{+} (3 subsets)
   - **PD-1:** activation/inhibition
   - **PD-L1:** cross-inhibition
5. **NK**: Natural killer cells (CD56^{+} CD3^{-}) (32 subsets)
   - **CD16^{+} CD56^{dim}** : Mature NK, lytic
   - **CD16^{+} CD56^{br}**: Functional intermediate, lytic, cytokine production
   - **CD16^{-} CD56^{br}:** Immature, cytokine production, abundant in placenta
   - **CD16^{-} CD56^{dim}**: non-lytic, non-cytokine production
   - **TIM-3:** activation
   - **PD-1:** activation/inhibition
   - **PD-L1:** cross-inhibition
   - **DNAM1:** adhesion/activation
   - **NKG2d:** activation
   - **NKp30:** activation
   - **NKp46:** activation
   - **HLADR:** proliferative activity, cytotoxic activity, cytokine production
   - **Ki67:** proliferation
6. **NK-T**: CD56^{+} CD3^{+} (4 subsets)
   - **TIM-3:** activation
   - **PD-1:** activation/inhibition
   - **PD-L1:** cross-inhibition
7. **cDCs (Conventional DCs):** CD3^{-}CD56^{-} HLA-DR^{+}CD1c^{+}CD303^{-} (4 subsets)
8. **pDCs (plasmacytoid DCs):** CD3^{-}CD56^{-} HLA-DR^{+}CD1c^{-}CD303^{+} (4 subsets)
   - **Markers of DC activation**
     - **TIM-3:** inhibition
     - **PD-L1:** cross-inhibition
     - **PD1:** activation/inhibition
9. **MDSCs**: Myeloid-derived suppressor cells (CD11b^{+} HLA-DR$^{low/-}$ CD33^{+}) (16 subsets)
   - **CD14**: Common Myeloid Marker (high in monocytes, dim in granulocytes)
   - **CD15**: Granulocyte marker
   - **CD16**: most immature monocytic MDSCs
   - **PD-1:** activation/inhibition
   - **PD-L1:** cross-inhibition

Table S2. Antibody panels

Panel 1: T cells			Panel 2: Tregs			Panel 3: NK/DC			Panel 4: MDSC /B cells		
Antibody	**Company**	**Catalog #**	**Antibody**	**Company**	**Catalog #**	**Antibody**	**Company**	**Catalog #**	**Antibody**	**Company**	**Catalog #**
CTLA4	LS Bio	ls-c62860	CTLA4	LS Bio	ls-c62860	CD3	Biolegend	300306	CD15	Biolegend	301904
PD-1	Biolegend	329906	PD-1	Biolegend	329906	PD-1	Biolegend	329906	PD-1	Biolegend	329906
4-1BB	Biolegend	309814	ICOS	Biolegend	313518	CD303	Biolegend	354210	CD19	Biolegend	302230
PD-L1	BD Bioscience	558017	PD-L1	BD Bioscience	558017	PD-L1	BD Bioscience	558017	PD-L1	BD Bioscience	558017
Tim3	Biolegend	345008	FoxP3**	Biolegend	320116	Tim3	Biolegend	345008	CD14	Biolegend	301830
CCR7	Biolegend	353232	CD49d	Biolegend	304318	NKp30	BD Bioscience	743170	CD16	Biolegend	302048
CD4	Biolegend	317438	CD4	Biolegend	317438	CD56	Biolegend	318334	HLA-DR	Biolegend	307640
Ki67**	Biolegend	350516	Ki67**	Biolegend	350516	NKp46	Biolegend	331927	CD11b	Biolegend	101243
CD8	Biolegend	301046	CD38	Biolegend	303530	Ki67**	Biolegend	350516	live/dead	Invitrogen	L23105
live/dead	Invitrogen	L23105	HLA-DR	BD Bioscience	564040	NKG2D	BD Bioscience	743560	CD33	Biolegend	303408
CD45RA	Biolegend	304120	live/dead	Invitrogen	L23105	CD226	BD Bioscience	742498			
			CD25	Biolegend	356110	live/dead	Invitrogen	L23105			
			CD45RA	Biolegend	304120	HLA-DR	Biolegend	307610			
			CD127	Ebioscience	47-1278-42	CD16	Biolegend	302026			
						CD1c	Biolegend	331520			

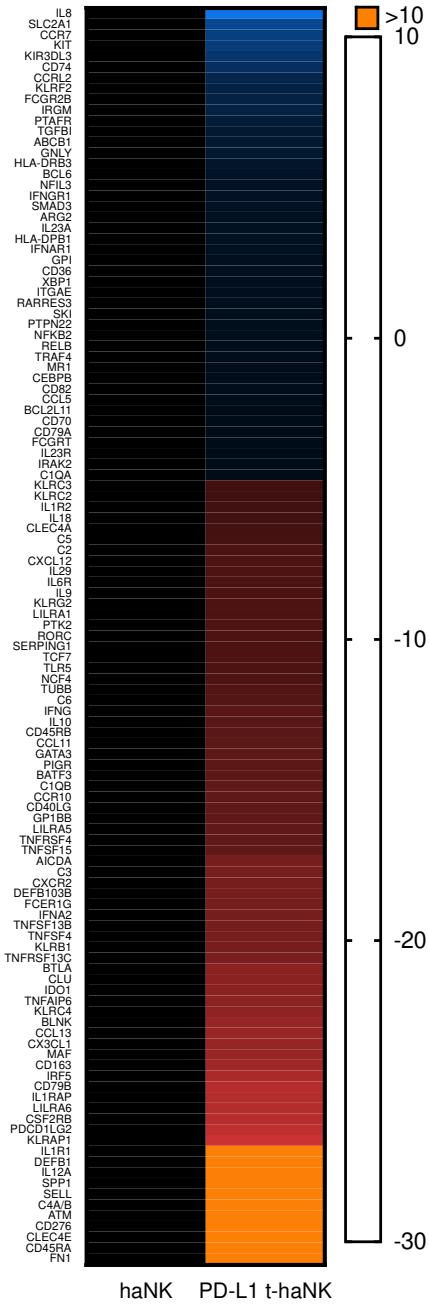

Figure S1. Transcript changes in PD-L1 t-haNK vs haNK cells. nCounter was run to compare PD-L1 t-haNK and haNK gene expression. Reported are transcripts changed by greater than 3 fold.

Table S3. Ingenuity pathway analysis (IPA) of the transcripts deregulated in PD-L1 t-haNK vs haNK. Transcripts deregulated by >3 fold in PD-L1 t-haNK vs haNK were selected and used for analysis by IPA 4.0 (Ingenuity Systems Inc., www.ingenuity.com). Here are reported the top classes in each category.

Top Canonical Pathways

Name	p-value	Overlap
T Helper Cell Differentiation	9.99E-12	23.3%
Th17 Activation Pathway	9.99E-12	23.3%
Altered T Cell and B cell Signaling in Rheumatoid Arthritis	3.28E-11	20.8%
Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.23E-11	15.5%
Th1 Pathway	2.23E-09	13.9%

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Inflammatory Response	4.16E-02 - 1.02E-05	12
Immunological Disease	1.06E-02 - 8.12E-04	3

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cellular Development	2.21E-02 - 1.48E-08	11
Cellular Growth and Proliferation	3.14E-02 - 1.48E-08	11
Cell-To-Cell Signaling and Interaction	4.16E-02 - 9.04E-08	10
Cellular Movement	4.16E-02 - 1.37E-07	7
Cellular Function and Maintenance	2.21E-02 - 1.91E-06	11

Physiological System Development and Function

Name	p-value range	# Molecules
Hematological System Development and Function	4.16E-02 - 1.48E-08	21
Lymphoid Tissue Structure and Development	2.21E-02 - 1.48E-08	17
Tissue Morphology	2.10E-02 - 7.31E-08	9
Immune Cell Trafficking	4.16E-02 - 1.37E-07	12
Cell-mediated Immune Response	4.16E-02 - 1.91E-06	9

Top Networks

Associated Network Functions	Score
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Trafficking	7
Lymphoid Tissue Structure and Development, Tissue Morphology, Cell-mediated Immune Response	4
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	1
Cell Death and Survival, Cellular Compromise, Inflammatory Response	1
Cell Death and Survival, Cell-mediated Immune Response, Cellular Development	0

Fig. S2

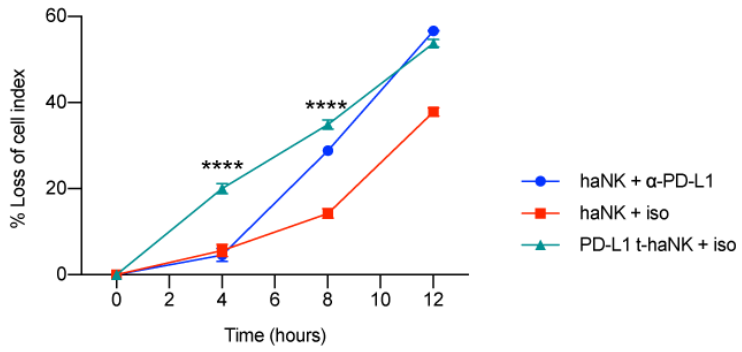

Fig. S2. PD-L1 t-haNK and haNK killing measured using real-time impedance-based cell assay. Real-time impedance-based cell assay (xCELLigence RTCA MP, ACEA) was employed to track the cell death of MDA-MB-231 mediated by irradiated haNK cells with α-PD-L1 antibody (1μg/mL) and PD-L1 t-haNK over time at 5:1 E:T ratio. MDA-MB-231 (20,000 cells/well) were allowed to grow overnight on impedance plates. When the cell index reached approximately 1.0, haNK and PD-L1 t-haNK cells (100,000 cells) were added to the wells. anti-PD-L1 antibody at a final concentration of 1μg/mL was also added to appropriate wells. The cell index was normalized to the time of NK addition and decrease in cell index was monitored. % decrease in cell lysis was calculated using the formula: [((average cell index of control - cell index of sample)/average cell index of control) x 100] for each time point. Results shown are the means with SEM of triplicate measurement. Two way ANOVA with Tukey's multiple comparisons test were used for statistical analysis. Significance shown compares PD-L1 t-haNK with haNK with anti-PD-L1 antibody. ****$p < 0.001$.

Fig. S3

A.

Cell Line	ATCC	Source	Mutation(s)	Zygosity	PDL1 expression %+ (MFI)
AGS	CRL-1739	Primary	KRAS PIK3CA CDH1 CTNNB1	*Heterozygous* *Heterozygous* *Homozygous* *Heterozygous*	99.6 (4912)
SNU-1	CRL-5971	Primary	KRAS MLH1	*Heterozygous* *Homozygous*	87.5 (2215)
SNU-16	CRL-5974	Metastatic	TP53 CDKN2A	*Homozygous* *Homozygous*	46.9 (403)
SNU-5	CRL-5973	Metastatic	TP53 CDH1 CDKN2A	*Homozygous* *Heterozygous* *Homozygous*	64.3 (1286)
KATO-III	HTB-103	Metastatic	TP53	*Homozygous*	99.2 (2767)

B.

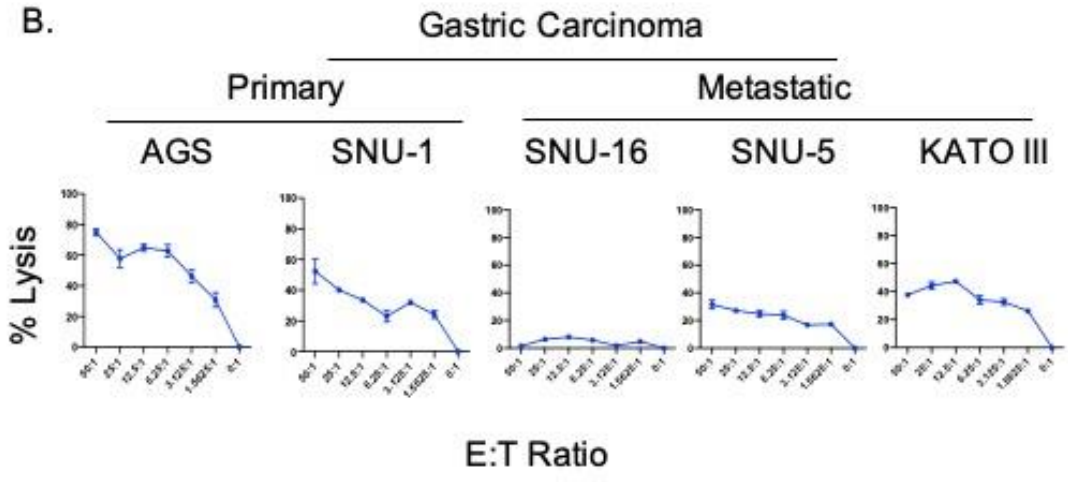

Fig. S3. PD-L1 t-haNK induced the lysis of human gastric cancer cell lines. A. Description of the mutations, zygosity, and PD-L1 expression (measured via flow cytometry) of the gastric cell lines AGS, SNU-1, SNU16, SNU-5, and KATO III. **B.** Cell lysis of AGS, SNU-1, SNU16, SNU-5, and KATO III mediated by PD-L1 t-haNK at different E:T ratios evaluated in ^{111}In-release assay. Results shown are the means with SEM of triplicate measurement and representative of at least 2 independent experiments for each cell line.

Fig. S4

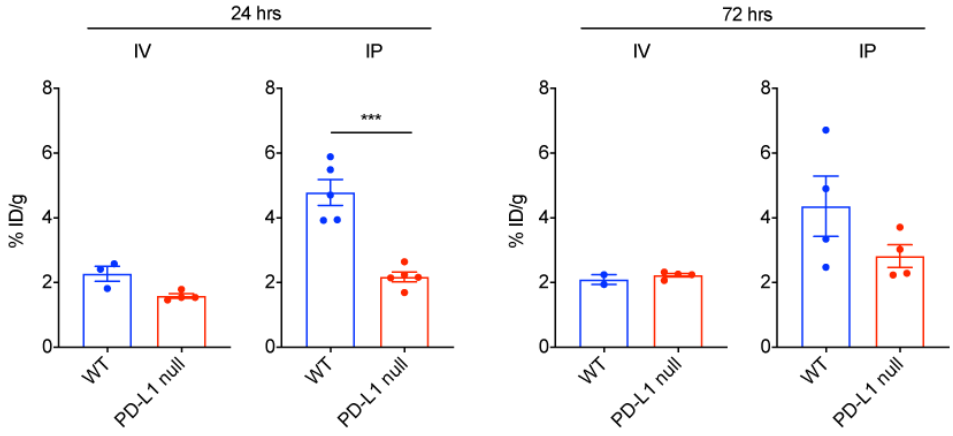

Fig. S4. PD-L1 t-haNK tracking *in vivo*. NSG mice (n = 3/group/time point) with established WT MDA-MB-231 and PD-L1 null MDA-MB-231 were injected with ^{111}In-labeled irradiated PD-L1 CAR haNK (i.p. or i.v.) when the tumors reached ~100mm^3. 24 hours and 72 hours post-treatment, the tumors were collected and assessed for ^{111}In signal. Student's *t*-test was used for statistical analysis. ***p < 0.005.