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Supplementary Materials for

NK cells with tissue-resident traits shape response to immunotherapy by inducing adaptive antitumor immunity

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The PDF file includes:

Figs. S1 to S9
Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data file S1
MDAR Reproducibility Checklist

Supplementary Materials

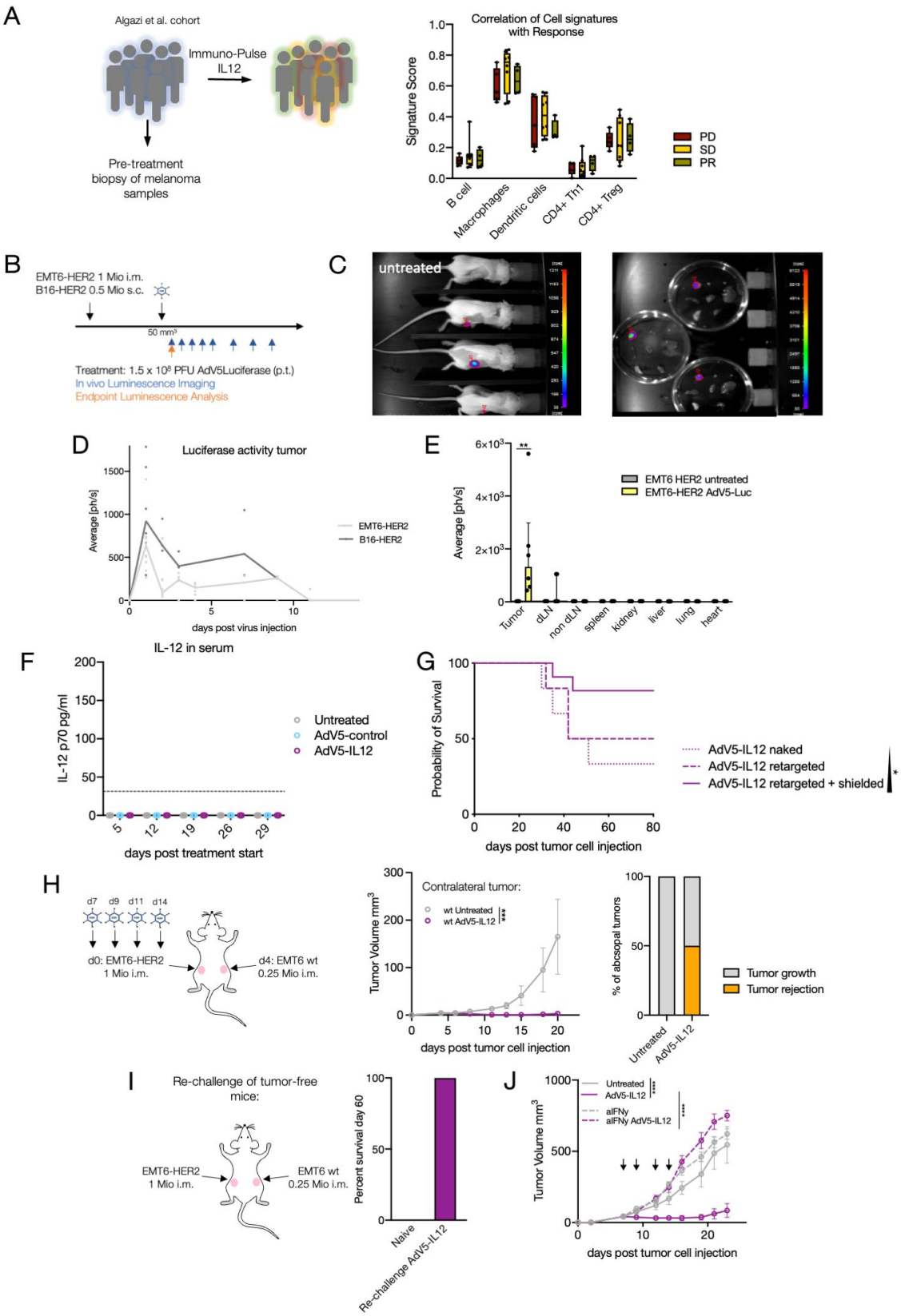

Fig. S1: Features of local IL-12 therapy.

(A) Cell signature scores measured by Nanostring in skin tumor biopsies from 19 patients with melanoma before intra-tumoral treatment with ImmunoPulse IL-12 were correlated with clinical response (PD: progressive disease, SD: stable disease, PR: partial response) (**B)** Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 (i.m.) or 0.5 x10^8B16-HER2 (s.c.). Mice were p.t. treated with AdV5-Luciferase on day 7 or day 11 (tumor size 30–70 mm^3), respectively. The luciferase signal was live imaged at the indicated time points (blue arrows; day 1, 2, 3, 4, 5, 7, 9, 11 and 13 post virus injection). *n*= 6 mice per condition. (**C**) Representative luciferase signal in three treated and one untreated animal one day post AdV5-Luciferase injection are shown. After isolation of tumor, draining lymph node, non-draining lymph node, spleen, kidney, liver, heart and lung, luciferase signal was measured again. (**D)** Quantification of in vivo luciferase signal in EMT6-HER2 (light grey) and B16-HER2 (dark grey) tumors. (**E**) Quantification of luciferase signal one day post AdV5-Luciferase treatment in isolated indicated organs of EMT6-HER2 bearing mice. (**F)** Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 intramammarily (i.m.). From day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (peritumorally) encoding for IL-12 or empty control cassette (AdV5-control) on day 7, 9, 11 and 14. On the indicated days, serum was collected and IL-12 concentration was determined by ELISA. Dotted line denotes detection limit of the ELISA. (**G)** Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 intramammarily (i.m.). From day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of naked, HER2-targeted or HER2-targeted and shielded adenoviral vectors (peritumorally, p.t.) encoding for IL-12 or an empty control cassette (AdV5-control) on days 7, 9, 11 and 14 p.t. Kaplan-Meier survival curves are shown. Black arrows denote days of treatment. n = 6. Log rank test for trend was performed to determine significant changes. (**H)** Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 (i.m.) and with 4 days delay with 0.25 x10^8EMT6 wt cells on the contralateral flank. EMT6-HER2 tumors were peritumorally treated with AdV5-IL12. Tumor growth of contralateral tumor (EMT6 wt) was measured. Tumor growth curve and percentage of rejected contralateral tumors are shown. n = 6 mice per group. (**I)** Mice which rejected tumors after AdV5-IL12 treatment were rechallenged (60d after tumor rejection) with 1 x10^8EMT6-HER2 i.m. and 0.25 x10^8EMT6 wt cells (no HER2 transgene) on each flank. Percentage of survival 60d post rechallenge is shown. n = 12, naive mice served as a control. (**J)** Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 intramammarily (i.m.). From day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (peritumorally) encoding for IL-12 on day 7, 9, 11 and 14. IFNγ neutralization was performed using anti-IFNγ antibody injected every 2-3 days starting one day before virus inoculation. Tumor growth curves are shown. n = 5-6 mice. *P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Error bar values represent SD or SEM (tumor growth curves). For comparisons between three or more groups, one-way ANOVA with multiple comparisons was used. For survival analysis, P values were computed using the Log Rank test. Two-way ANOVA was used to compare tumor growth curves.

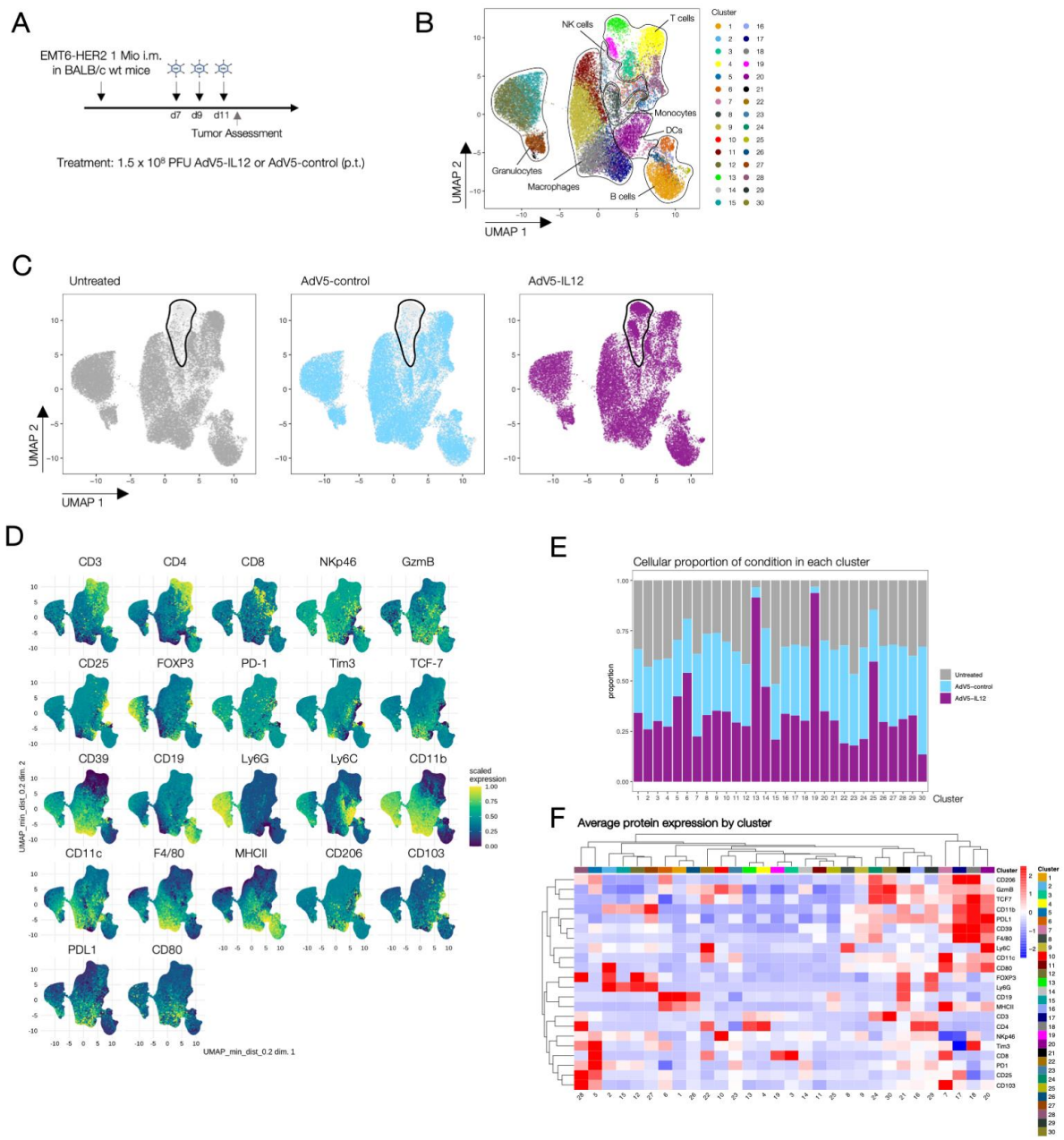

Fig. S2: Cellular composition after AdV5-IL12 therapy.

(A) Wildtype (WT) mice were engrafted with 1 x10^8EMT6-HER2 intramammarily (i.m.). Starting from day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (p.t.) encoding for IL-12 or empty control cassette (AdV5-control) on day 7, 9 and 11. On day 12 post inoculation, tumors were isolated and single cell suspensions were analyzed by flow cytometry. (**B)** UMAP projection is depicting the alive CD45+ tumor infiltrating lymphocytes colored by cluster. (**C)** UMAP projection is showing distribution of cells colored by treatment condition (dark grey: untreated; blue: AdV5-control; magenta: AdV5-IL12). (**D)** UMAP-projection of pooled conditions showing expression analyzed proteins supporting cell-type assignments. (**E)** Percentage of cells in each cluster by treatment. (**F)** Heatmap showing protein expression of 2 x 10^4 random cells assigned to conditions (Untreated, AdV5-control or AdV5-IL12) and cluster. *n* = 5-6 mice per group.

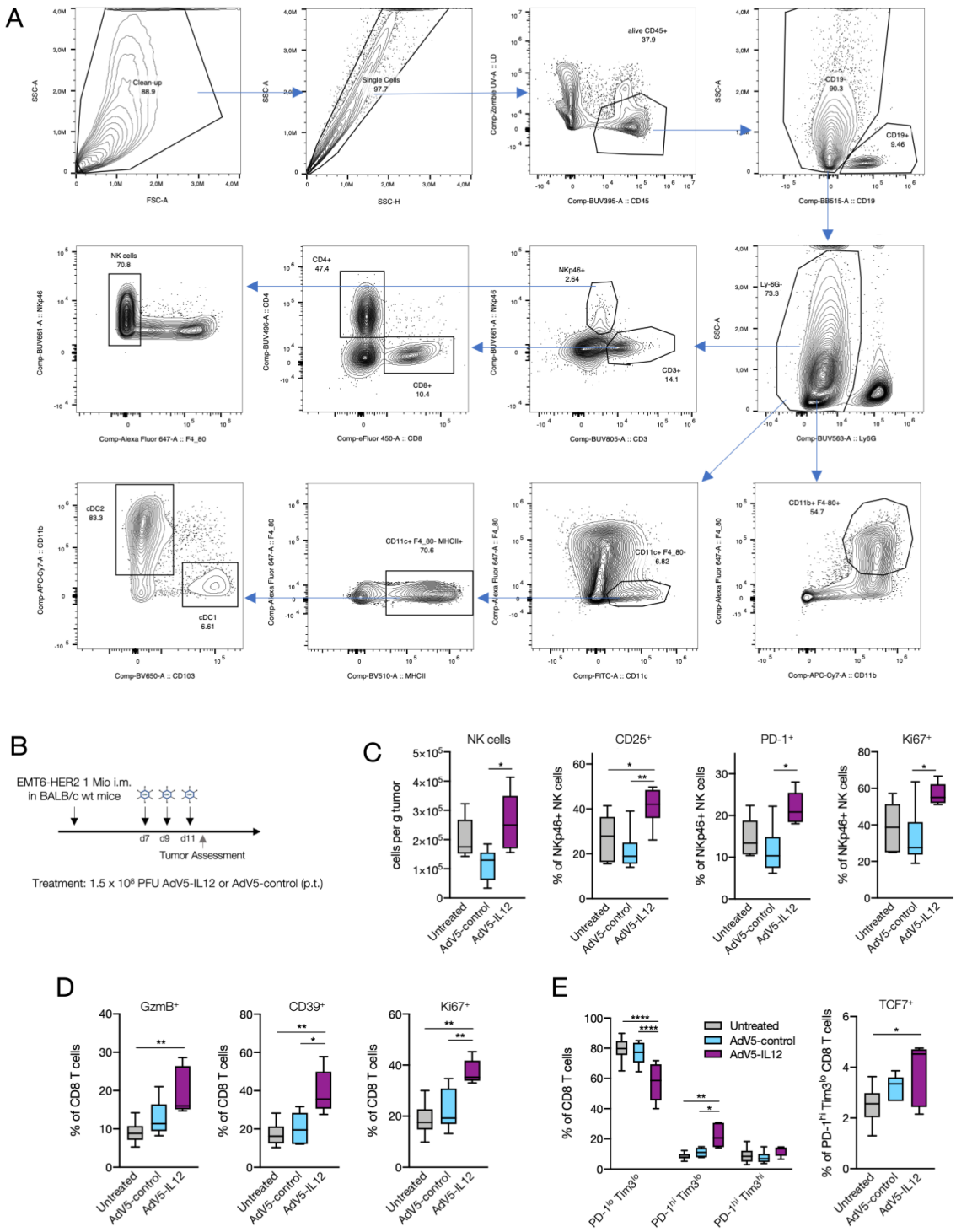

Fig. S3: Gating strategy and cellular changes after AdV5-IL12 therapy.

(A) To gate on singlets, SSC-A and SSC-H were used. After gating on alive CD45+ cells, CD19+ B cells and Ly-6G+ Granulocytes were excluded. To define T cells (CD3+), NK cells were excluded (NKp46+ F4/80-) and further distinguished between CD8 T cells and CD4 T cells. Macrophages were defined using F4/80+ and CD11b+. DCs were defined as CD11c+ F4/80- cells. MHCII was used to gate on cDCs. Subsequently to distinguish cDC1s from cDC2s, CD11b and CD103 was used. **(B)** Wildtype (WT) mice were engrafted with 1 x10^8 EMT6-HER2 intramammarily (i.m.). Starting from day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (p.t.) encoding for IL-12 or empty control cassette

(AdV5-control) on day 7, 9 and 11. On day 12 post inoculation, tumors were isolated and single cell suspensions were analyzed by flow cytometry. (**C**) Quantification of NK cells (NKp46+, CD3-, Ly6G-, CD19-, F4/80-) per gram tumor and proportion of CD25+, PD-1+ or Ki67+ of NK cells between the different treatment conditions. (**D**) Proportion of granzyme B+ (GzmB+), CD39+ or Ki67+ of CD8 T cells (CD3+, CD4-, NKp46-, CD19-) between the different treatment conditions. (**E**) Proportion of PD-1^{lo}TIM3^{lo}, PD-1^{hi}TIM3^{lo} or PD-1^{hi}TIM3^{hi} intra-tumoral CD8 T cells in each treatment group. n = 5-6 mice per group. *P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Error bar values represent SD. For comparisons between three or more groups, one-way ANOVA with multiple comparisons was used.

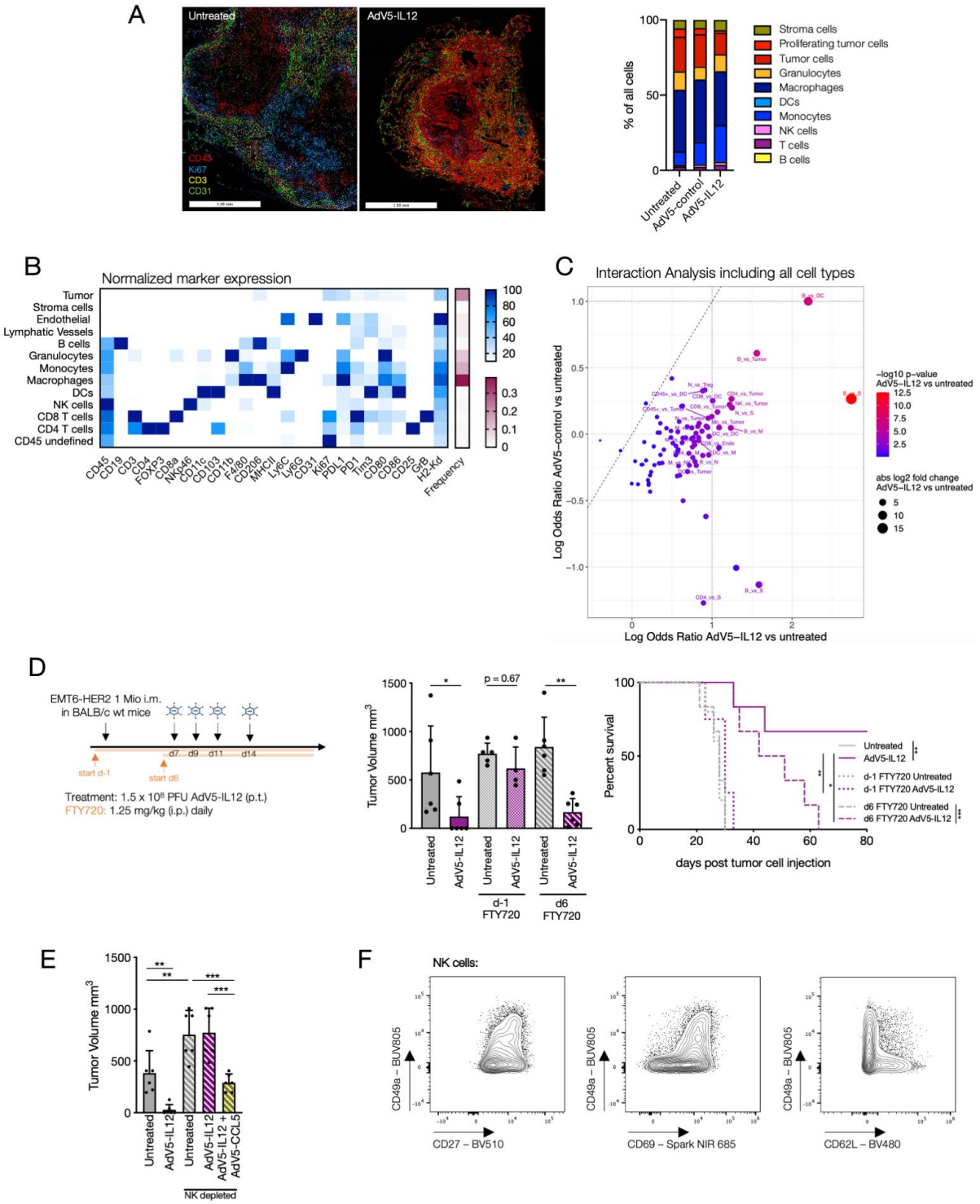

Fig. S4: Interaction analysis and functions of NK cells in efficacy of AdV5-IL12 treatment.

(A–B) Wildtype (WT) mice were engrafted with 1 x10^8 EMT6-HER2 intramammarily (i.m.). Starting from day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (peritumorally) encoding for IL-12 or empty control cassette (AdV5-control) on day 7, 9 and 11. On day 12 post inoculation, tumors were isolated, embedded in OCT and analyzed by multiparameter immuno-fluorescence microscopy. *n*= 3 mice per condition. (**A)** Representative IF pictures are showing untreated and AdV5-IL12 treated tumors (CD45: red, Ki67: blue, CD3: yellow, CD31: green) including quantification of main clusters

between conditions. (**B)** Heatmap showing normalized marker expression and frequency of identified main populations. (**C)** Visualization of log odds ratios and *P* values for changes in all cell-cell type interactions between experimental conditions. (**D)** 1 x10^8 EMT6-HER2 cells were injected in WT mice (i.m.). Starting from day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (peritumorally) encoding for IL-12 on day 7, 9, 11 and 14. Lymphocyte trafficking was inhibited using FTY720 as indicated (orange arrow and line). Tumor volume on day 23 post tumor inoculation and Kaplan-Meier survival curves are shown. (**E)** EMT6-HER2-engrafted mice were treated with AdV5-IL12 and AdV5-CCL5. Starting one day prior adenoviral therapy, NK cells were depleted using anti-AsialoGM1 antibody. Tumor volume on day 23 post tumor inoculation is shown. (**F)** Protein expression on intratumoral NK cells analyzed by flow cytometry of AdV5-IL12 treated EMT6-HER2-bearing mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Error bar values represent SEM. For survival analysis, *P* values were computed using the Log Rank test. For comparisons between three or more groups, one-way ANOVA with multiple comparisons was used.

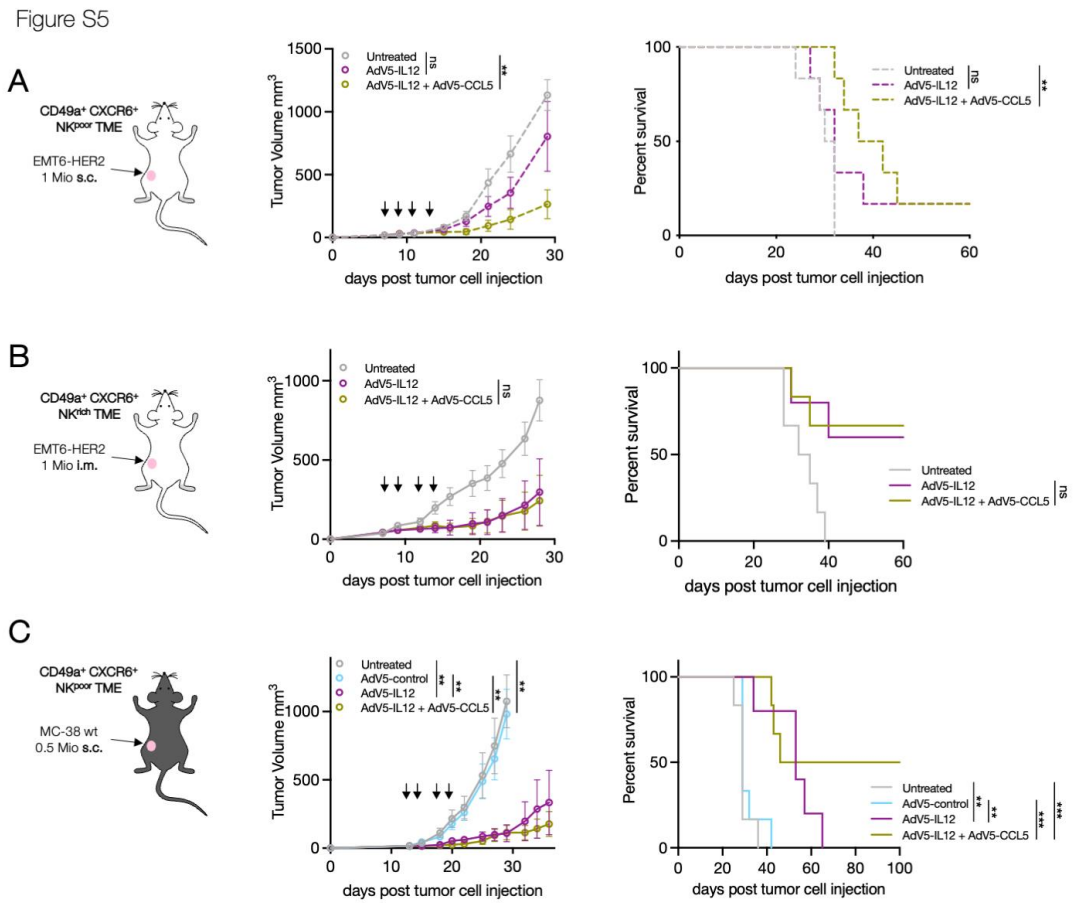

Fig. S5: AdV5-IL12 and AdV5-CCL5 efficacy in CD49a$^+$ CXCR6$^+$ **NK rich and poor tumor mouse models. (A–B)** WT mice were engrafted with 1 x10^{8}EMT6-HER2 s.c. (**A**) or i.m. (**B**). From day 7 (tumor size 30–70 mm^3), mice were treated with 1.5x10^8 PFU of HER2-targeted and shielded adenoviral vectors (peritumorally, p.t.) encoding for IL-12 alone or in combination with AdV5-CCL5 on days 7, 9, 11 and 14 p.t. Tumor growth curves and Kaplan-Meier survival curves are shown. (**C)** Mice were engrafted with MC-38 wt cell (s.c.) treated with shielded AdV5-control, AdV5-IL12 and AdV5-CCL5 on day 11, 13, 15 and 18 (tumor size 30–70 mm^3) after tumor inoculation as indicated. Tumor growth and Kaplan-Meier survival curves are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Error bar values represent SEM. For survival analysis, P values were computed using the Log Rank test. Two-way ANOVA was used to compare tumor growth curves. $n = 6$ mice per group.

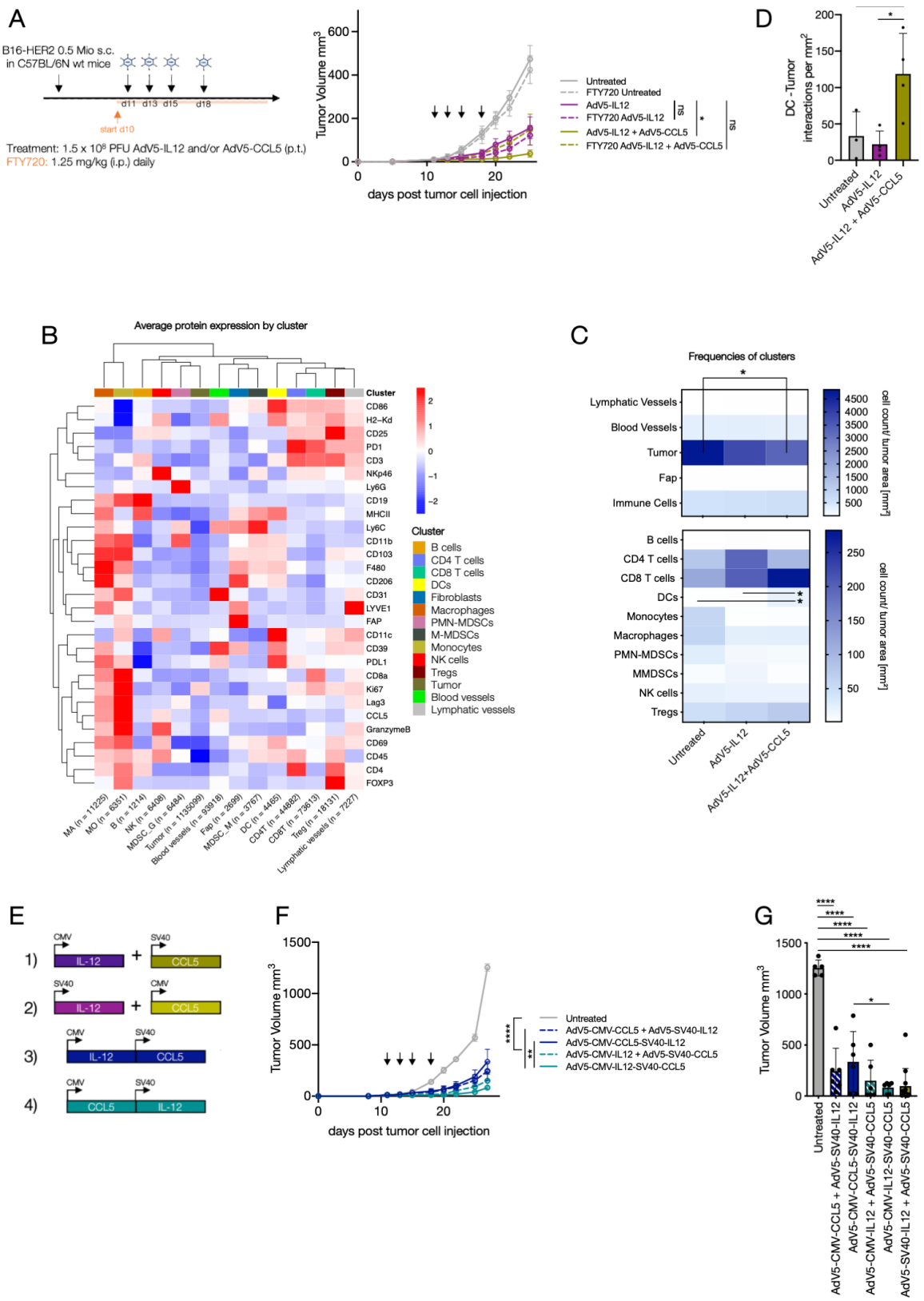

Fig. S6: Features of AdV5-IL12 and AdV5-CCL5 combinatorial therapy.

(A) Mice were treated with AdV5-IL12 and AdV5-CCL5 on day 11, 13, 15 and 18 (tumor size 30–70 mm^3) after B16-HER2 inoculation. Lymphocyte trafficking was inhibited using FTY720 as indicated (orange arrow and line). Tumor growth curves are shown. n = 5-6 mice per condition. Black arrows denote days of treatment. **(B)** Wildtype (WT) mice were engrafted with 0.5 Mio B16-HER2 (s.c.). Starting from day 11 (tumor size 30–70 mm^3), mice

were treated with $1.5x10^8$ PFU of HER2-targeted and shielded adenoviral vectors (p.t.) encoding for IL-12 and CCL5 on day 11, 13 and 15. On day 16 post inoculation, tumors were isolated, embedded in OCT and analyzed by multiparameter immuno-fluorescence microscopy. Heatmap showing normalized marker expression and (**C**) frequency of identified main populations. (**D)** Interaction count per mm^2 of tumor cells in close proximity to DCs. (**E)** Design of combinatorial vectors compared to mixture of single viruses. (**F)** Mice were treated with each $1.5x10^8$ PFU AdV5-IL12 and AdV5-CCL5 or $1.5x10^8$ PFU combinatorial vectors on day 11, 13, 15 and 18 (tumor size 30–70 mm^3) after B16-HER2 inoculation as indicated (black arrows). n = 5-6 mice per condition. Tumor growth curves are shown. (**G)** Quantification of tumor volume on day 27 post tumor inoculation. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Error bar values represent SD or SEM (tumor growth curves). For comparisons between three or more groups, one-way ANOVA with multiple comparisons was used. Two-way ANOVA was used to compare tumor growth curves.

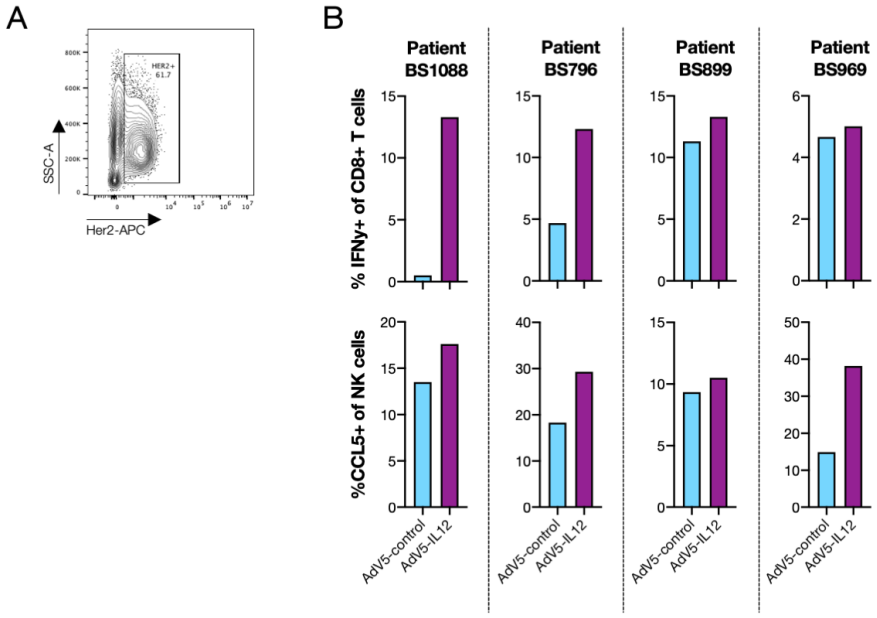

Fig. S7: CCL5 and IFNγ expression in primary human tumor fragments after AdV5-IL12 treatment. HER2+ ovarian cancer samples were dissected into tumor fragments and cultivated embedded in matrigel. Tumor fragments were treated with HER2-targeted AdV5 encoding human IL-12 for 48 h (8-12 fragments per condition). (**A)** Representative dot plot of HER2 expression analyzed by flow cytometry. (**B)** After treatment tumor fragments per condition were pooled and analyzed by flow cytometry. Quantification of IFNγ+ CD8 T cells (CD3+, CD56-) and NK cells (CD56+, CD3-) after treatment.

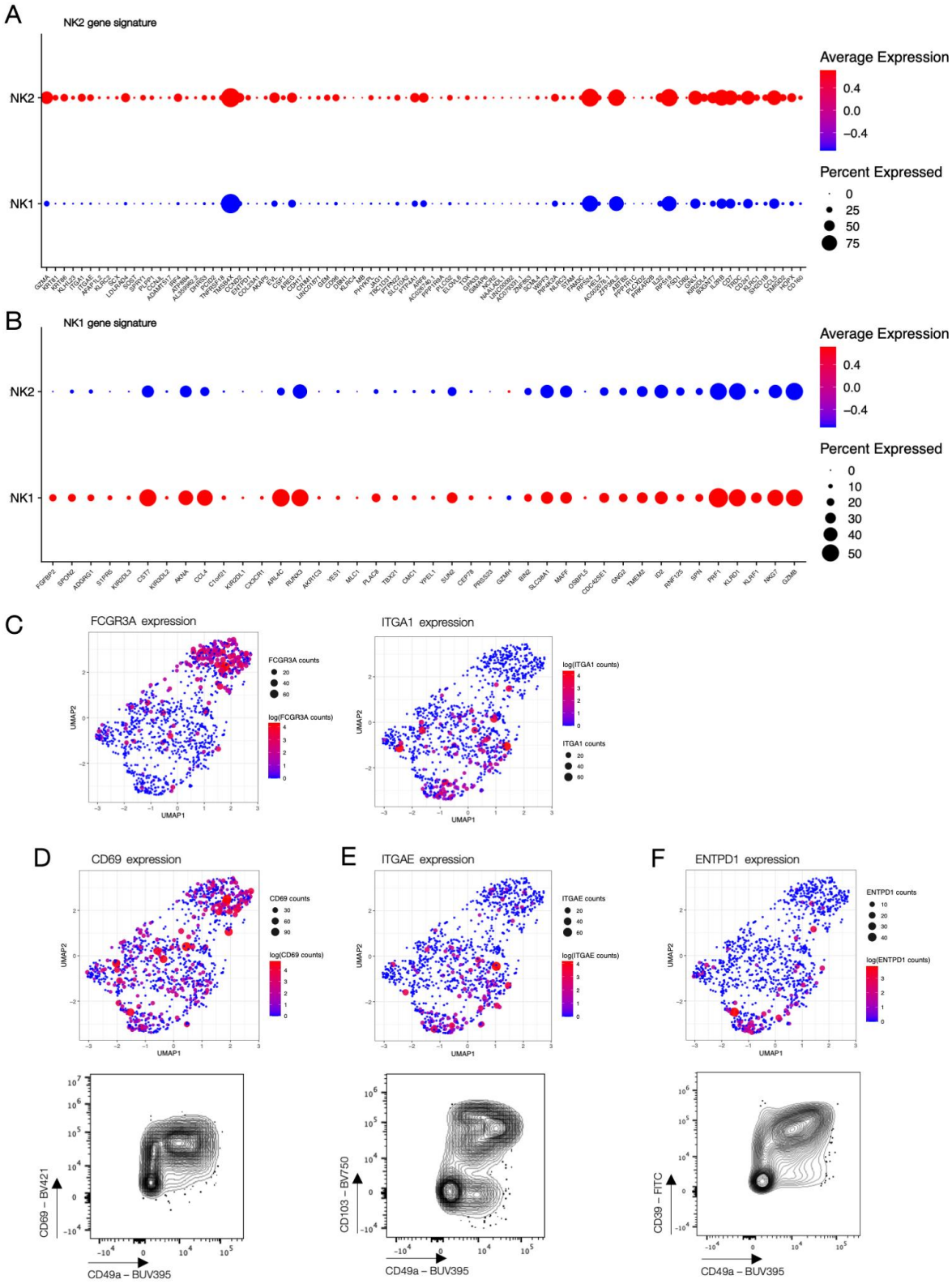

Fig. S8: Gene and protein expression of intratumoral NK cells.

(A–B) Dotplot to visualize expression of genes in tumor-infiltrating NK cells of patients with NSCLC defining generated (**A**) NK2 and (**B**)NK1 signature is shown. **(C)** *FCGR3A* and *ITGA1* expression is visualized on UMAP projection of tumor-infiltrating NK cells of patients with NSCLC. **(D–F)** Comparison of gene expression of

scRNASeq data set of tumor-infiltrating NK cells to protein expression analyzed by flow cytometry of patients with NSCLC (**D**) CD69, (**E**) *IGTAE*/CD103, (**F**)*ENTPD1*/CD39).

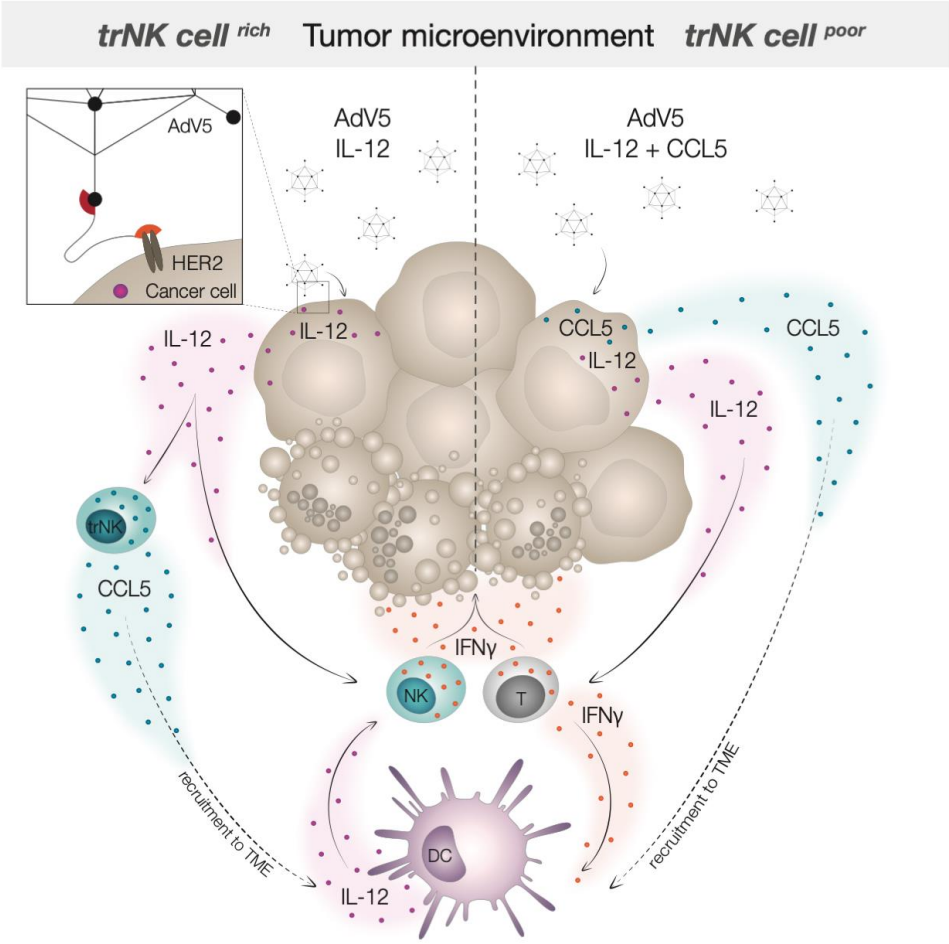

Fig. S9: Graphical abstract.

In TME with high amount of NK cells with tissue-resident traits (left panel; trNK cell rich), AdV5-IL12 imposes expression of the DC-attractant CCL5, inducing T cell immunity. Failure to respond to IL-12 in tumor models with low CD49a+ CXCR6+ NK cell infiltration (right panel; trNK cell poor) could be overcome by intra-tumoral delivery of CCL5.

Table S1: Flow cytometry antibodies.

Fluorophore	**Antigen**	**Clone**	**Supplier**	**Cat#**
PE	CCL5	VL1	BioLegend	515504
PE	CCL5	2E9/CCL5	BioLegend	149104
PE-Cy7	CCL5	2E9/CCL5	BioLegend	149105
BV570	CCR5	C34-3448	BD Biosciences	746501
BV650	CD103	2E7	BD Biosciences	748256
BV750	CD103	BER-ACT8	BD Biosciences	747099
APC-Cy7	CD11b	M1/70	BioLegend	101226
FITC	CD11c	N418	BioLegend	117306
Pac Blue	CD14	M5E2	BioLegend	301828
BUV496	CD16	3GA	BD Bioscience	612945
BB515	CD19	1D3	BD Biosciences	564509
FITC	CD19	1D3	BD Biosciences	553785
BV711	CD206	C068C2	BioLegend	141727
PE-Cy5.5	CD25	PC61.5	Thermo Fisher Scientific	35-0251-82
BV510	CD27	LG.3A10	BioLegend	124229
APC-H7	CD3	SK7	BD Biosciences	560275
BUV805	CD3	145-2C11	BD Biosciences	741895
BUV805	CD3	UCHT-1	BD Bioscience	612896
BV605	CD3	SK7	BioLegend	344836
PE-Dazzle594	CD3	17A2	BioLegend	100246
FITC	CD39	A1	BioLegend	328206
PE	CD39	24DMS1	Thermo Fisher Scientific	eBio 12-0391-82
BUV496	CD4	GK1.5	BD Biosciences	612952
AF532	CD45	HI30	Thermo Fisher Scientific	58-0459-41
BUV395	CD45	30-F11	BD Biosciences	564279
PerCP-Cy5.5	CD45	2D1	Thermo Fisher Scientific	9045-9459-120
BUV395	CD49a	SR84	BD Bioscience	742363
BUV805	CD49a	Ha31/8	BD Biosciences	741976
BUV661	CD56	NCAM16	BD Biosciences	750478
BV785	CD56	5.1H11	BioLegend	362550
BV480	CD62L	MEL-14	BD Biosciences	746726
BV421	CD69	FN50	BioLegend	310930
SparkNIR685	CD69	H1.2F3	BioLegend	104557
BUV805	CD8	RPA-T8	BD Biosciences	749366
eFluor 450	CD8	53-6.7	Thermo Fisher Scientific	eBio 48-0081-82
PE-Cy7	CD8	SK1	Thermo Fisher Scientific	9025-0087-120
BV605	CD80	16-10A1	BioLegend	104792
BUV737	CXCR3	CXCR3-173	BD Biosciences	741895

PerCp-Cy5.5	CXCR6	SA051D1	BioLegend	115111
AF647	F4/80	BM8	BioLegend	123122
FITC	F4/80	BM8	BioLegend	123108
APC	FoxP3	FJK-16s	Thermo Fisher Scientific	17-5773-82
PE-eFluor610	GzmB	NGZB	Thermo Fisher Scientific	61-8898-82
APC	IFNy	XMG1.2	BioLegend	505810
BB700	IFNy	B27	BD Biosciences	566395
FITC	IFNy	C4.B3	Thermo Fisher Scientific	11-7319-82
AF532	Ki67	SolA15	Thermo Fisher Scientific	58-5698-82
PerCP	Ly-6C	HK1.4	BioLegend	128028
BUV563	Ly-6G	1A8	BD Biosciences	612921
FITC	Ly6G	1A8	BD Biosciences	551460
BV510	MHCII I-A/I-E	M5/114.15.2	BioLegend	107636
FITC	MHCII I-A/I-E	M5/114.15.2	BioLegend	107606
BUV661	NKp46	29A14	BD Biosciences	741678
BV421	NKp46	29A1.4	BD Biosciences	562850
BV785	PD-1	29F.1A12	BioLegend	135225
BV421	PD-L1	10F.9G2	BioLegend	124315
AF700	TCF-7	# 812145	R&D Systems	FAB8224N
BB700	Tim-3	5D12/TIM-3	BD Biosciences	747619

Table S2: Microscopy antibodies.

Antigen	**Clone**	**Supplier**	**Cat#**
Granzyme B	GB11	invitrogen	MA1-80734
CXCL9	MIG-2F5.5	BioLegend	515602
CD40	1C10	BioLegend	102802
PD1	29F.1A12	BioLegend	135202
FOXP3	FJK-16s	eBioscience	14-5773-82
CD80	16-10A1	BioLegend	104702
IFNy	H22	BioLegend	513202
H-2Kd	34-1-2S	ThermoFisher	MA5-18008
Tim-3	RMT2-23	BioLegend	119702
NKp46	29A1.4	BioLegend	137602
CD25	PC61	BioLegend	102002
F4/80	BM8	BioLegend	123102
Ly-6C	HK1.4	BioLegend	128002
IL12	C15.6	BioLegend	505202
CD103	2.00E+07	BioLegend	121402
PD-L1	10F.9G2	BioLegend	124302
CD107a	1D4B	BioLegend	121602
CD206	C068C2	BioLegend	141702

CD11c	N418	Akoya	4350013
CD3	17A2	Akoya	4350014
Ly6G	1A8	Akoya	4350015
CD45	30-F11	Akoya	4150002
CD11b	M1/70	Akoya	4150015
CD31	MEC13.3	Akoya	4250001
CD44	IM7	Akoya	4250002
MHCII	M5/114.15.2	Akoya	4250003
CD19	6D5	Akoya	4250014
CD4	RM4-5	Akoya	4250016
CD8a	53-6.7	Akoya	4250017
Ki67	B56	Akoya	4250019