SUPPLEMENTAL DIGITAL CONTENT 1

Enhancing Efficacy of TCR-engineered CD4+ T Cells Via Coexpression of CD8α

Victoria E. Anderson, Sara S. Brilha, Anika M. Weber, Annette Pachnio, Guy E. Wiedermann, Sumaya Dauleh, Tina Ahmed, George R. Pope, Laura L. Quinn, Roslin Y. Docta, Adriano Quattrini, Siobhan Masters, Neil Cartwright, Preetha Viswanathan, Luca Melchiori, Louise V. Rice, Alexandra Sevko, Claire Gueguen, Manoj Saini, Barbara Tavano, Rachel J.M. Abbott, Jonathan D. Silk, Bruno Laugel, Joseph P. Sanderson, Andrew B. Gerry

SUPPLEMENTAL TABLE 1. Additional Cell Lines Used in Figure 5b

Designation	Cell Type
HSAEpC1	Small airway epithelial cell
HSAEpC11	Small airway epithelial cell
HSAEpC14	Small airway epithelial cell
HSAEpC23	Small airway epithelial cell
N15	Melanocyte
N16	Melanocyte
N17	Melanocyte
N20	Melanocyte
Nalm6	ALL
J82	Bladder carcinoma (transitional cell)
Mel526	Malignant melanoma
PANC-1	Pancreatic epithelioid carcinoma
SW480	Colorectal adenocarcinoma
TCC-SUP	Bladder carcinoma (transitional cell)
HSAEpC1	Small airway epithelial cell
HSAEpC11	Small airway epithelial cell
HSAEpC14	Small airway epithelial cell
HSAEpC23	Small airway epithelial cell
N15	Melanocyte
N16	Melanocyte
N17	Melanocyte
N20	Melanocyte
Nalm6	ALL
J82	Bladder carcinoma (transitional cell)
Mel526	Malignant melanoma
PANC-1	Pancreatic epithelioid carcinoma
SW480	Colorectal adenocarcinoma
TCC-SUP	Bladder carcinoma (transitional cell)

ALL cell lines are HLA-A*02^{+} and MAGE-A4^{-}.
ALL, acute lymphoblastic leukemia; HLA, human leukocyte antigen; MAGE-A4, melanoma-associated antigen A4.

SUPPLEMENTAL TABLE 2. HLA Class I Allele Expression of EBV-transformed B-LCLs and T-cell Donors Used in Figure 5c

Cell Type	Cell Lines	Tissue Type					
		HLA-A		HLA-B		HLA-C	
EBV-transformed B-LCL	1331-8234	A*02:01		**B*13:02**	B*51:01	C*06:02	**C*15:03**
	1333-8276	A*01:01	A*24:02	**B*39:06**	**B*58:01**	C*07:01	C*07:02
	1349-8396	A*01:01	A*02:01	**B*27:03**	B*57:01	C*02:02	C*06:02
	1416-1191	A*02:01	**A*23:01**	B*15:01	**B*41:01**	C*03:03	**C*17:01**
	230699	A*02:06	A*24:02	B*07:02	**B*51:03**	C*07:02	C*14:02
	AMALA	**A*02:17**		B*15:01		C*03:03	
	BPOT	A*02:01		**B*07:03**	**B*15:11**	C*03:03	C*03:04
	BRIP	A*24:02		**B*15:17**	B*51:01	C*07:01	**C*15:04**
	CGP04	A*02:01	A*29:02	B*35:01	B*44:03	C*04:01	C*16:01
	DUG150	**A*68:01**	A*02:01	**B*45:01**	**B*58:02**	C*06:02	C*16:01
	FB466 LCL	A*02:01		B*44:02	**B*51:08**	**C*05:01**	**C*16:02**
	FB469 LCL	A*02:01	A*24:02	**B*14:01**	B*35:01	C*04:01	C*08:02
	FB572 LCL	A*24:02	A*30:01	**B*15:10**	B*18:01	C*03:04	C*07:01
	FB578 LCL	A*03:01	A*11:01	**B*39:01**	**B*44:05**	C*02:02	C*12:03
	FH10	A*02:06	A*24:02	**B*40:02**	**B*55:02**	C*01:02	C*15:02

FH18	**A*36:01**	A*74:01	B*53:01	**B*57:03**	C*04:01	C*07:01
FH21	**A*02:30**	A*03:01	B*35:01	**B*38:01**	C*04:01	C*12:03
FH23	A*03:01	A*31:01	B*07:02	**B*27:07**	C*07:02	C*15:02
FH24	A*24:02	A*29:02	**B*39:09**	B*44:03	C*07:02	C*16:01
FH25†	A*02:05	A*26:01	B*07:02	**B*39:03**	C*07:02	
FH28	A*01:01	A*11:01	B*08:01	**B*27:04**	C*07:01	C*08:01
FH36	A*34:02	A*74:01	B*08:01	**B*15:03**	C*02:02	C*07:01
FH39	A*26:01	**A*34:01**	**B*07:06**	B*40:01	C*03:04	C*07:02
FH41†	A*02:05	A*31:01	**B*35:04**	B*49:01	C*04:01	C*07:01
FH42	A*02:01	A*24:02	B*35:01	B*51:01	C*01:02	**C*04:04**
FH43	A*30:01	**A*33:01**	B*53:01	**B*81:01**		
FH46	A*11:01	A*24:33	**B*27:22**	B*51:06		
FH53	A*24:33	**A*24:07**	**B*35:05**	B*51:06	C*04:01	**C*12:04**
FH58	A*02:01	**A*02:20**	B*40:01	B*44:02		
FH6	A*24:02	**A*29:01**	**B*07:05**	**B*27:02**	C*02:02	**C*15:05**
FH67	A*02:01	A*03:01	**B*15:71**	B*40:01	C*03:03	C*03:04
FH75	A*02:01	A*03:01	B*07:02		C*01:02	**C*07:10**
FH77	A*02:01	A*24:02	**B*15:07**	B*48:01	C*01:02	C*08:03

	FH8	A*11:01	A*34:02	**B*27:05**	**B*82:01**	C*01:02	**C*03:02**
	ISH3	A*24:02		**B*15:26**		C*04:01	
	ISH4	**A*02:18**	A*11:01	B*15:01	B*46:01	C*01:02	C*04:01
	ISH5	A*24:02		B*48:01	**B*54:01**	C*01:02	C*08:03
	J0528239	A*01:01		**B*35:02**		C*04:01	
	KT14	A*24:02	**A*26:02**	**B*40:06**	B*51:01	C*08:01	C*14:02
	MYE 2001	**A*01:04**	A*02:01	B*15:01	B*49:01	C*03:03	
	MYE 2002	A*03:01	**A*68:11**	B*07:02	B*44:02	C*07:02	
	MYE 2004	A*29:02	**A*68:18**	**B*14:02**	B*57:01	C*06:02	C*08:02
	OLGA	A*31:01		B*15:01	**B*15:20**	C*01:02	C*03:04
	RML	**A*02:04**		B*51:01		C*15:02	
	SCL-116A	A*03:01	**A*30:02**	B*18:01	**B*56:01**	C*01:02	
	T7527	A*02:01	**A*02:07**	B*46:01		C*01:02	
	TISI	A*24:02		**B*35:08**		C*04:01	
T cell	L215	A*23:01	A*24:02	B*07:02	B*49:01	C*07:01	C*07:02
	L216	A*01:01	A*02:01	B*57:01	B*57:01	C*06:02	C*06:02
	L217	A*02:01	A*24:02	B*35:03	B*50:01	C*06:02	C*12:02

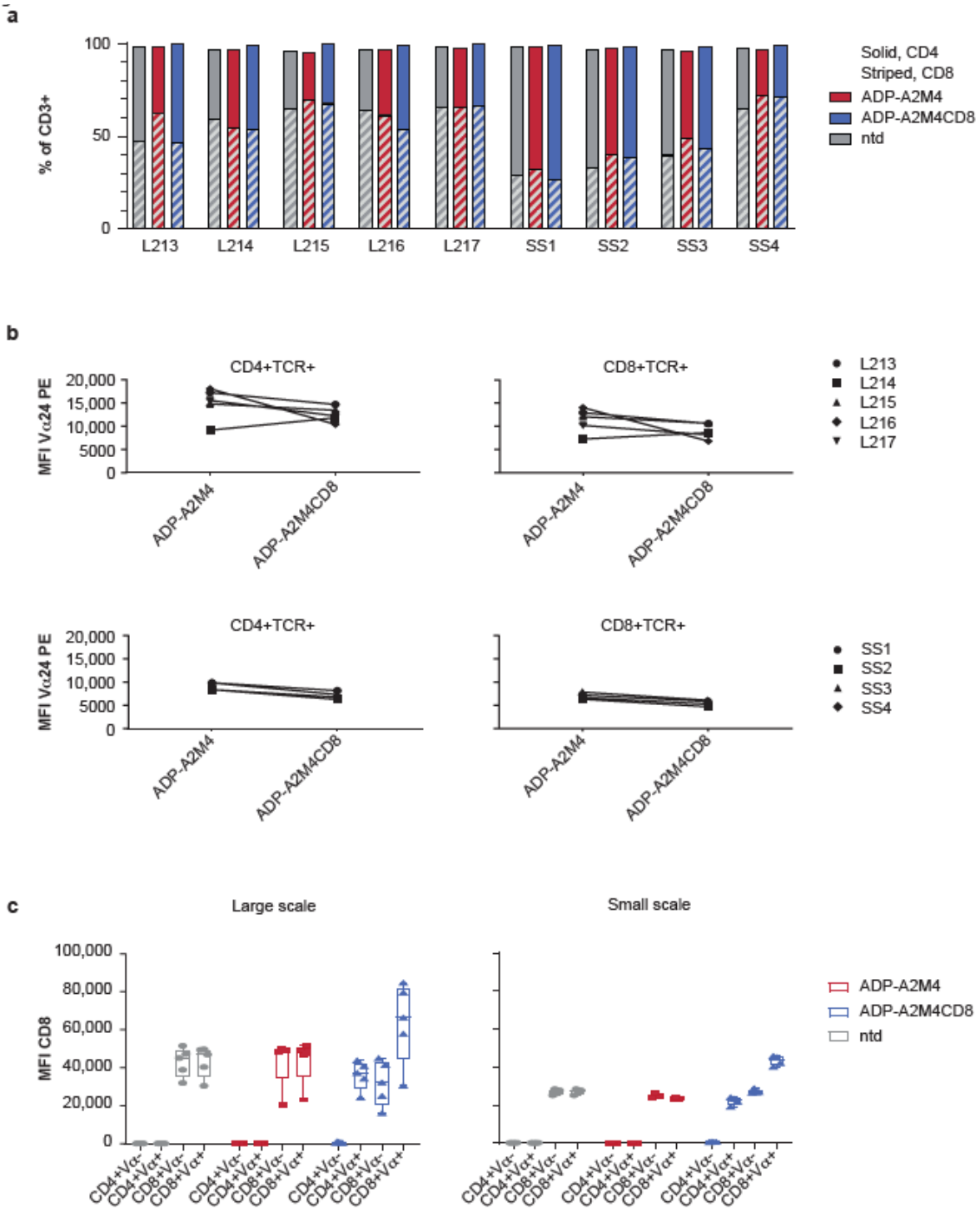
HLA alleles unique to each cell line are in bold font. †Signifies lines recognized by T cells.
B-LCL, B-lymphoblastoid cell line; EBV, Epstein-Barr virus; HLA, human leukocyte antigen.

SUPPLEMENTAL TABLE 3. Peptides Used in Figure 5

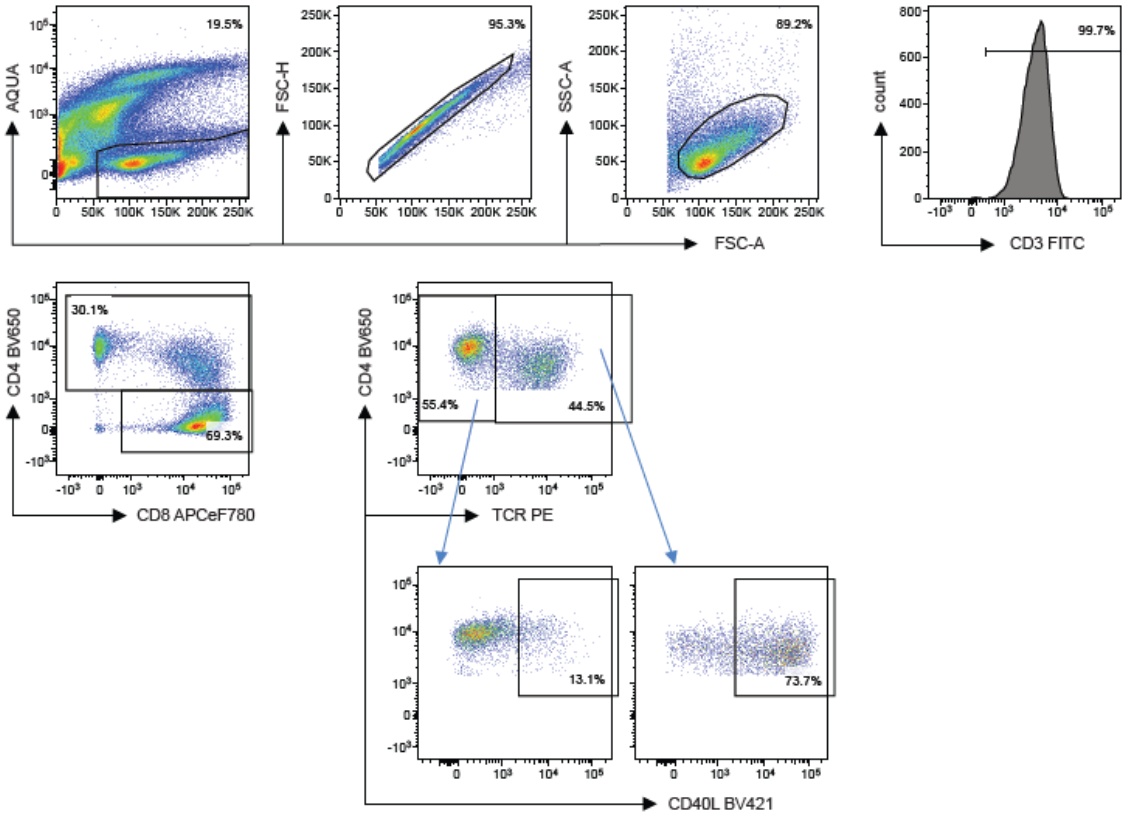
Protein Name	Peptide
ATHL1	GVYNGAGGDT
DHX36	HLYNGLRASL
FBX10	GIYDNRGHGI
FMO3	GVFNGKRVLV
FMOD	GVFDNATGLL
MORN4	GLFNGFGVLT
MOT10	GLFDGCFISI
MPCP	GIFNGFSVTL
MPRD	HIFNGSNWIM
NCOA1	GVYNNMSITV
NOD1	GLYNNQITDV
NRK2	GLFDGHVWPM
PAPP2	GVFDNCSHTV
PDP1	GVFDGHAGCA
PDP2	GIFDGHGGHA
PP2D1	GLFDGHHGAS
TLR7	GVFDGMPPNL
UL18	GIFDGQHFFT
ZN721	GVYNGINKCL
MAGEA1	EVYDGREHSA
MAGEA2	EVFEGREDSV
MAGEA3/A6	EVFEGREDSI
MAGEA4	GVYDGREHTV
MAGEA8	GLYDGREHSV
MAGEA9	GVYVGKEHMF
MAGEA10	GLYDGMEHLI
MAGEB1	GAYDGEEHLI
MAGEB2*	GVYDGEEHSV
MAGEB3	RIYDGKKHFI
MAGEB4	GIYDGKRHLI

MAGEB5	QIYDGKKYYI
MAGEB6	GIYDGILHSI
MAGEB16	GVYSGKKHFI
MAGEC1	GVRAGREHFA
MAGEC2	GVYAGREHFV
MAGED1	GLRPGVRHPL
MAGED4	GLRPGVRHPF
MAGEE1	GVQRERRLSI
Trophinin	GLRPGVRHSL

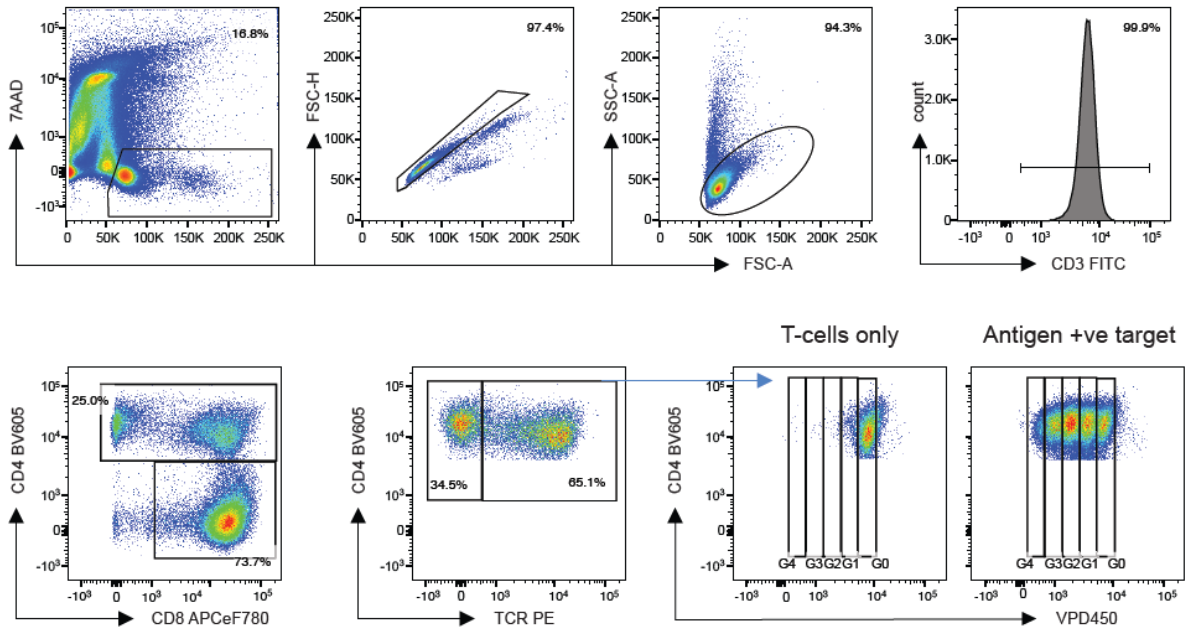
SUPPLEMENTAL FIGURE 1. Analysis of CD4, CD8, and T-cell receptor (TCR) surface expression on manufactured T-cell products. (a) Summarizes the frequency of $CD4^+$ and $CD8^+$ T cells within large-scale (L) and small-scale (SS) T-cell products; cells were gated on live, single lymphocytes and $CD3^+$ events as shown in Supplemental Figure 2. (b) Levels of TCR surface expression median fluorescence intensity (MFI) on $CD4^+$ and $CD8^+$ T cells for each product transduced with ADP-A2M4 or ADP-A2M4CD8. The top two panels summarize data for large-scale T-cell products, and the bottom two panels for small-scale T-cell products. (c) Surface levels of CD8α on $CD4^+$ or $CD8^+$ T-cell subsets were analyzed by determining the CD8 MFI on both $V\alpha24^+$ and $V\alpha24^-$ subsets within both subsets for large-scale cells on the left and small-scale cells on the right. In (b) and (c), large-scale and small-scale products were run in separate assays, so MFI is not directly comparable between graphs. ntd, non-transduced.


a
% of CD3+
100
50
0
L213
L214
L215
L216
L217
SS1
SS2
SS3
SS4
Solid, CD4
Striped, CD8
ADP-A2M4
ADP-A2M4CD8
ntd
b
CD4+TCR+
CD8+TCR+
MFI Vα24 PE
20,000
15,000
10,000
5000
0
ADP-A2M4
ADP-A2M4CD8
L213
L214
L215
L216
L217
SS1
SS2
SS3
SS4
c
Large scale
Small scale
MFI CD8
100,000
80,000
60,000
40,000
20,000
0
CD4+Vα-
CD4+Vα+
CD8+Vα-
CD8+Vα+
ADP-A2M4
ADP-A2M4CD8
ntd

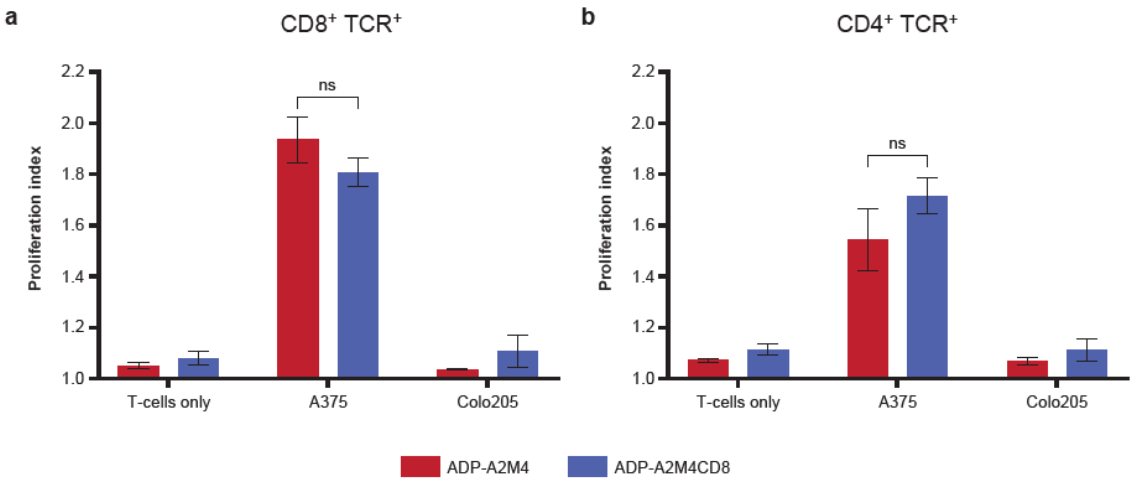
SUPPLEMENTAL FIGURE 2. Gating strategy to assess CD40L expression. Live cells were gated by lack of Aqua staining, followed by single cells, lymphocytes, and $CD3^+$ T cells. Within the $CD3^+$ subset, we identified $CD4^+$ T cells (CD4 single-positive cells and those that coexpress CD4 and CD8α) and $CD8^+$ T cells (single-positive). Within the $CD4^+$ T-cell population, T-cell receptor (TCR)-negative and TCR-positive subsets were gated on and CD40L expression assessed on these cell populations. FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.



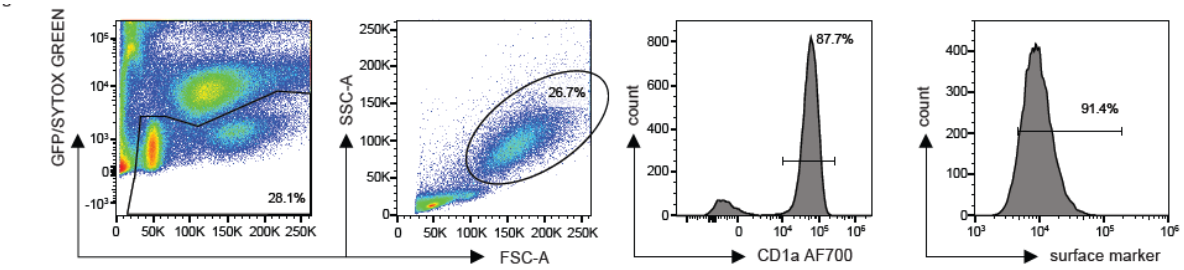
SUPPLEMENTAL FIGURE 3. Gating strategy to assess T-cell proliferation. Live cells were gated by lack of 7-AAD staining, followed by single cells, lymphocytes, and $CD3^+$ T cells. Within the $CD3^+$ subset, we identified $CD4^+$ T cells (CD4 single-positive cells and those that coexpress CD4 and CD8α) and $CD8^+$ T cells (single-positive). Within the $CD4^+$ T-cell subset, T-cell receptor (TCR)-positive cells were identified and cytoplasmic dye dilution of VPD450 in this population was evaluated to determine T-cell proliferation. FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter; VPD, Violet Proliferation Dye.



SUPPLEMENTAL FIGURE 4. Assessment of antigen-driven proliferation of transduced $CD8^+$ and $CD4^+$ T cells. ADP-A2M4 or ADP-A2M4CD8 transduced T cells were cocultured in the presence of melanoma-associated antigen A4 (MAGE-A4)$^+$ (A375) or MAGE-A4$^-$ (Colo205) tumor cell lines, or alone for 3 days, to assess proliferation in response to antigen. The mean proliferation index (the number of divisions of proliferating cells) was calculated for (a) $CD8^+$ T-cell receptor (TCR)$^+$ and (b) $CD4^+$ TCR^+ cells (n = 4). Statistical significance was assessed by two-way analysis of variance followed by a Sidak post hoc multiple comparison test. ns, not significant.

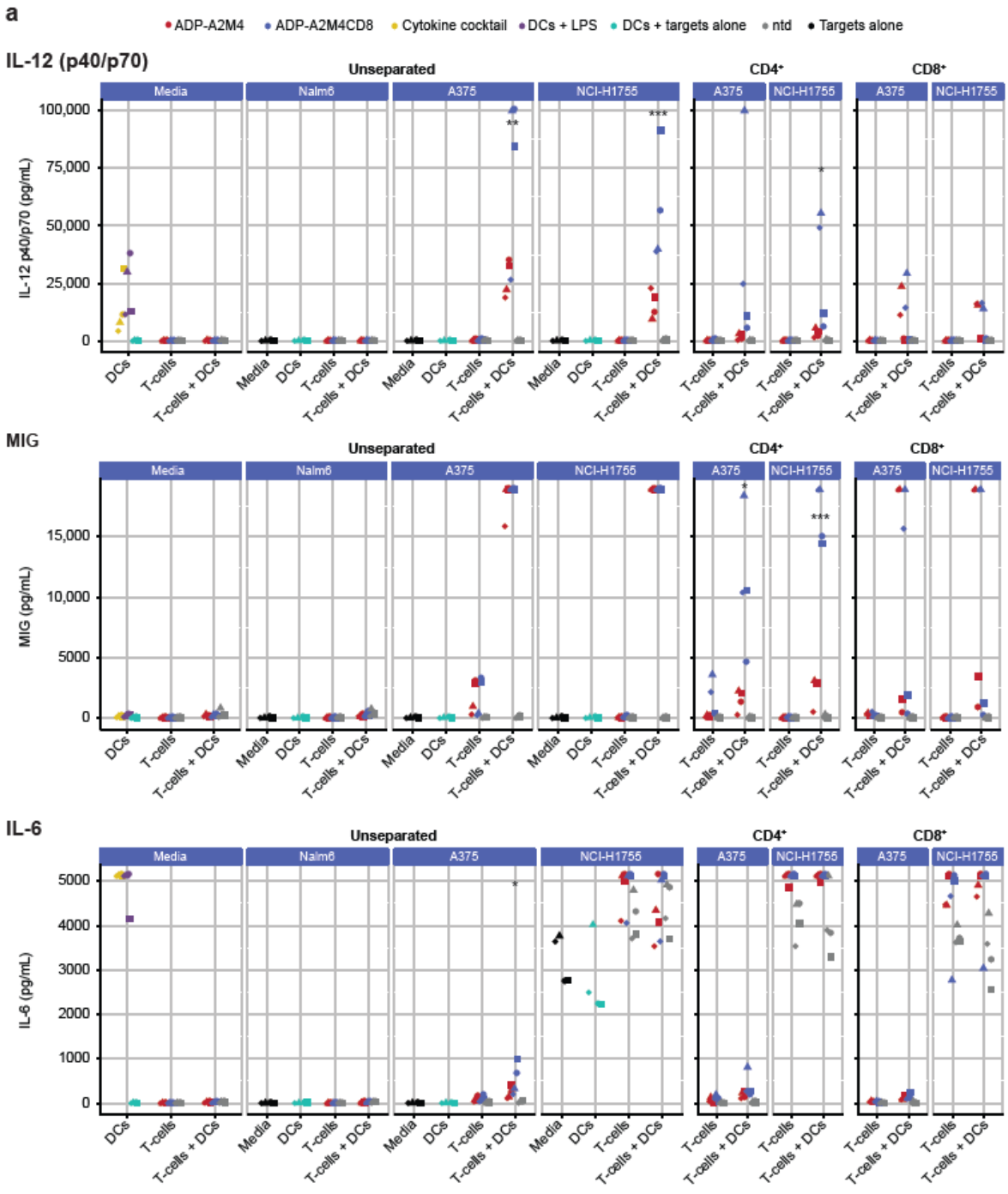


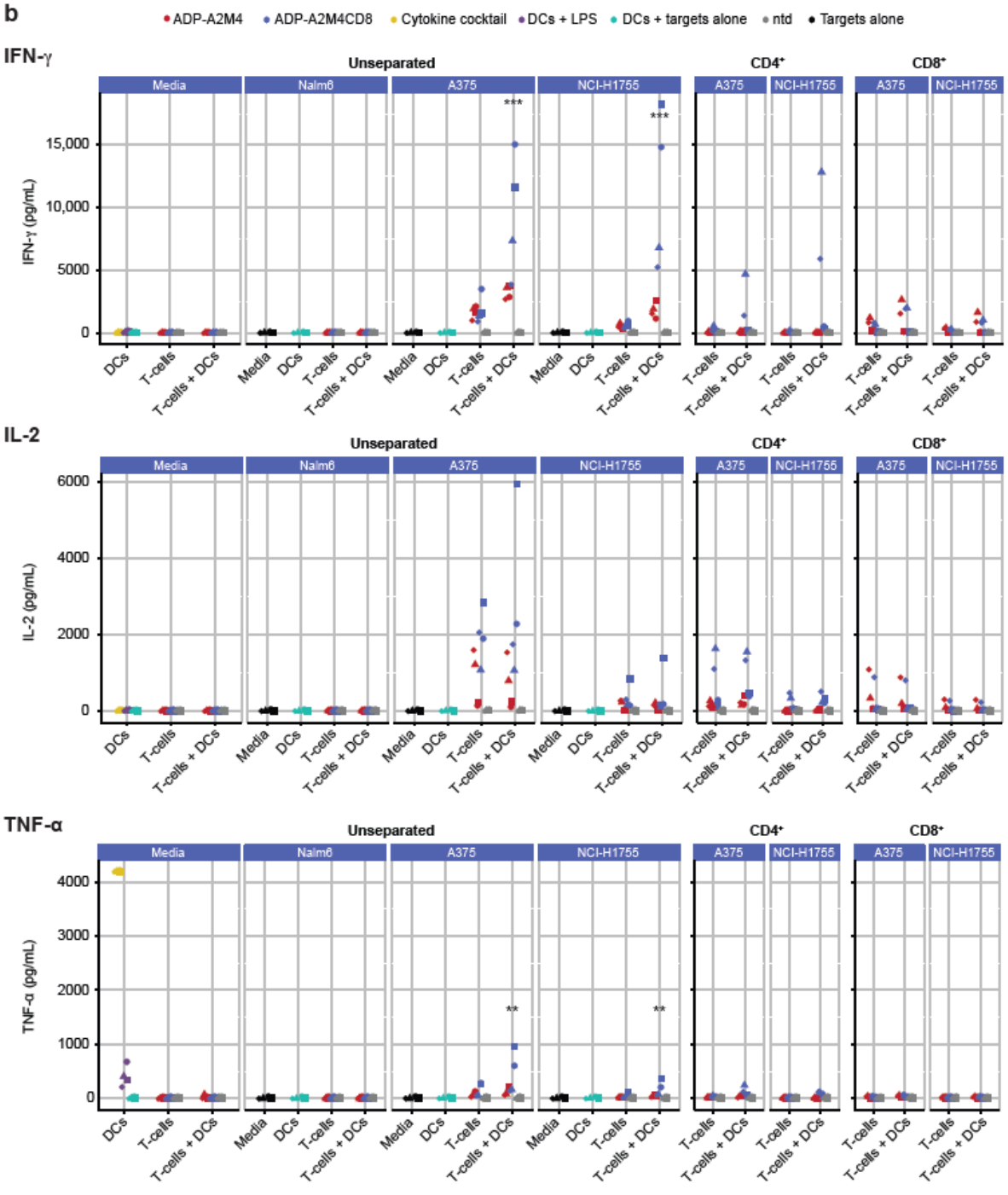
SUPPLEMENTAL FIGURE 5. Gating strategy to assess the dendritic cell (DC) phenotype after 48 cocultures. Live cells were gated on by exclusion of green fluorescent protein $(GFP)^+$ (tumor targets) and SYTOX $Green^+$ (Thermo Fisher Scientific, Waltham, MA, USA) (dead) cells; from these, the DC population was gated on in a forward scatter (FSC)/side scatter (SSC) plot and further identified as a $CD1a^+$ population.

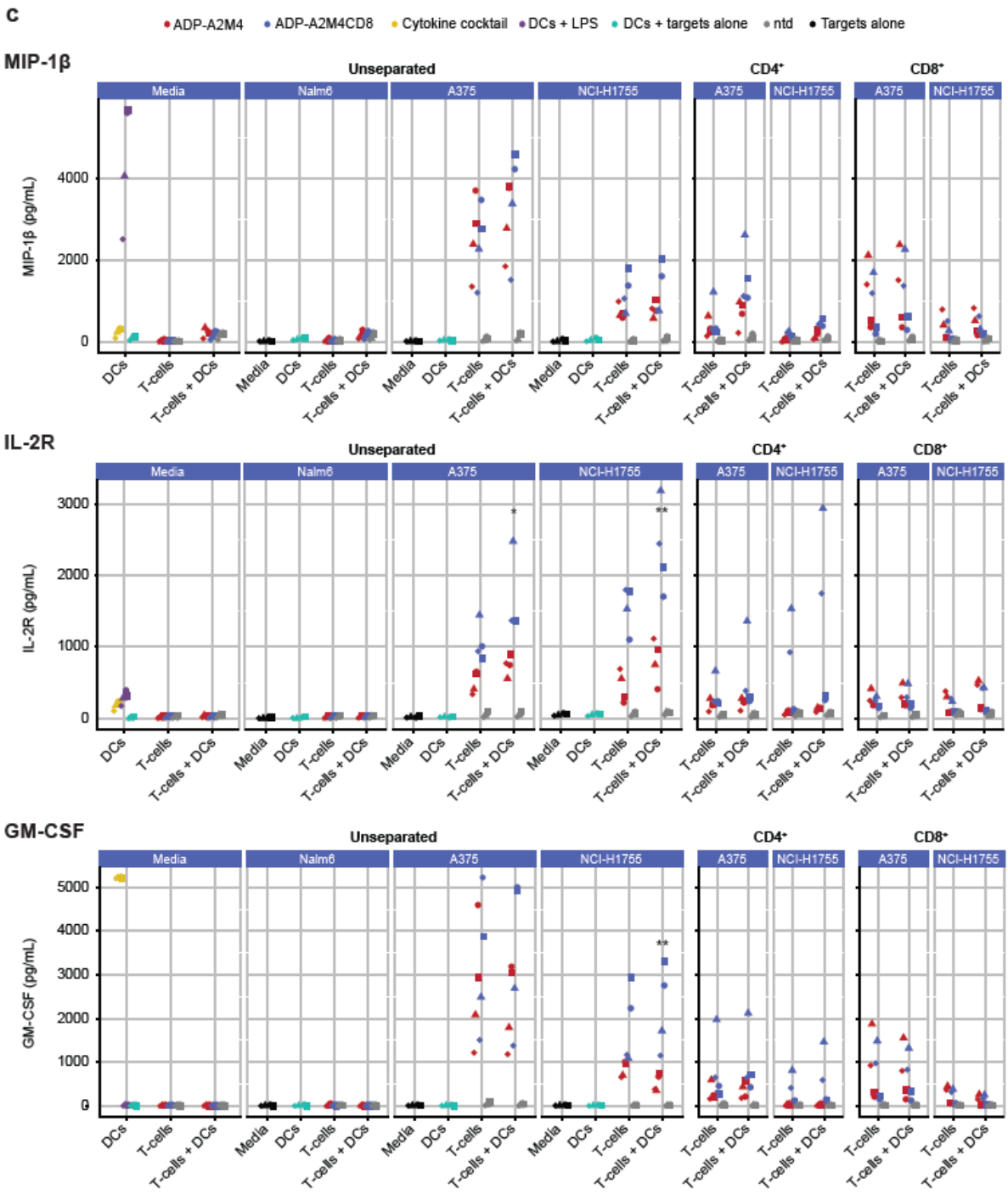


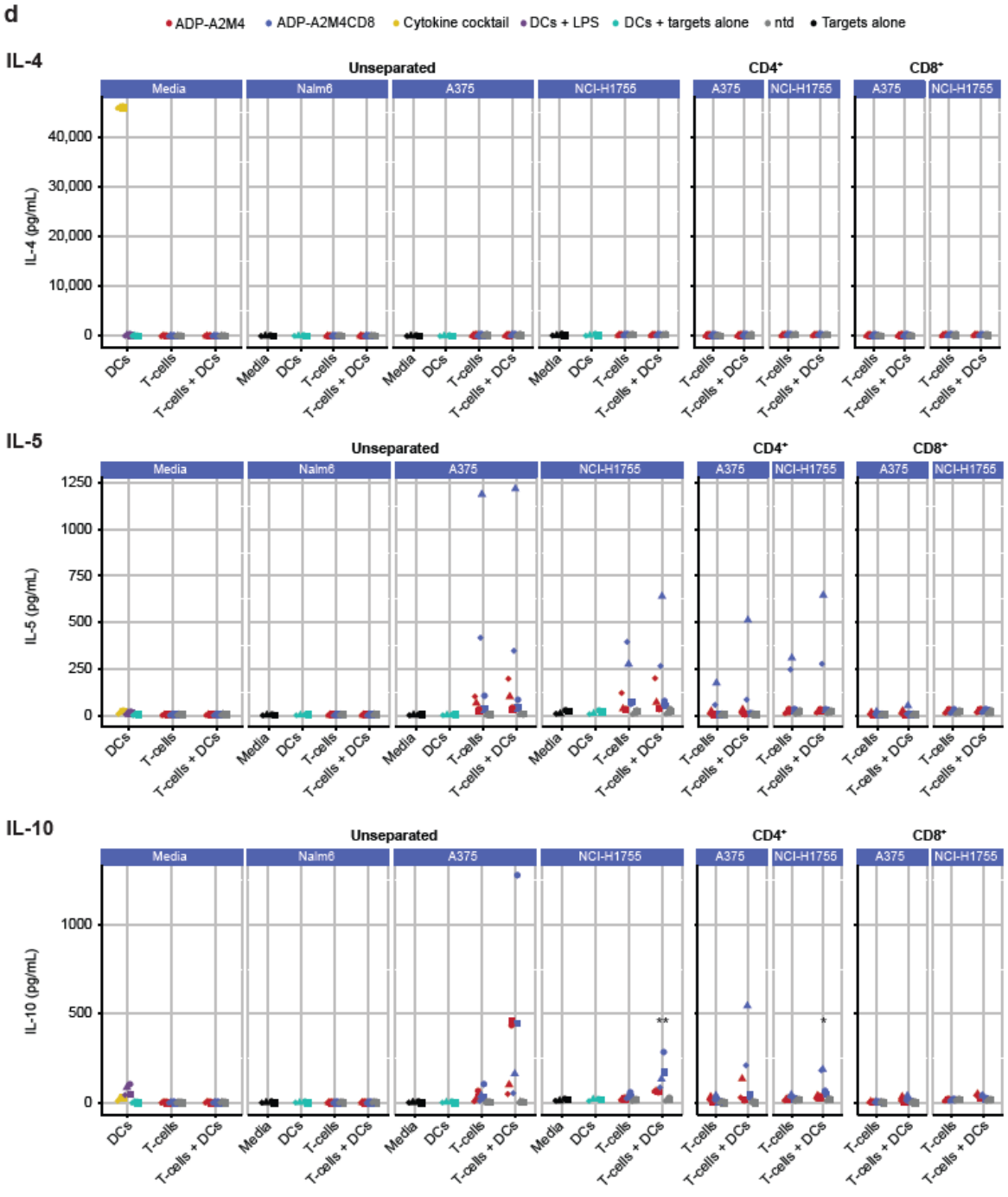
SUPPLEMENTAL FIGURE 6. Melanoma-associated antigen A4 (MAGE-A4)$^{+}$ (A375 and NCI-H1755) or MAGE-A4^{-} (Nalm6) tumor cell lines, or no target control ("Media"), were cocultured in a 48-well plate with immature dendritic cells (DCs) and non-transduced (ntd) T cells (gray), ADP-A2M4 (red), or ADP-A2M4CD8 (blue). Controls for DC activation: lipopolysaccharide (LPS; purple) and a cytokine cocktail (yellow); controls without T cells (teal) and targets alone (black). Culture supernatants were harvested after 48 hours and cytokines were analyzed by Bio-Plex MAGPIX (Bio-Rad Laboratories, Hercules, CA, USA). Each symbol represents one donor. Three-way repeated measures analysis of variance was run for each cytokine and positive-control target, with transduction, T-cell fraction, and presence or absence of DCs as within-subject factors, followed by pairwise *post hoc* tests for each combination; *P* values were adjusted using the Holm method. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. (a) Cytokines typically secreted by DCs. (b, c) Type 1 T-helper/antitumor cytokines. (d, e) Other T-cell cytokines.

GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon gamma; IL, interleukin; MIG, monokine induced by gamma interferon; MIP-1β, macrophage inflammatory protein-1 beta; TNF-α, tumor necrosis factor alpha.

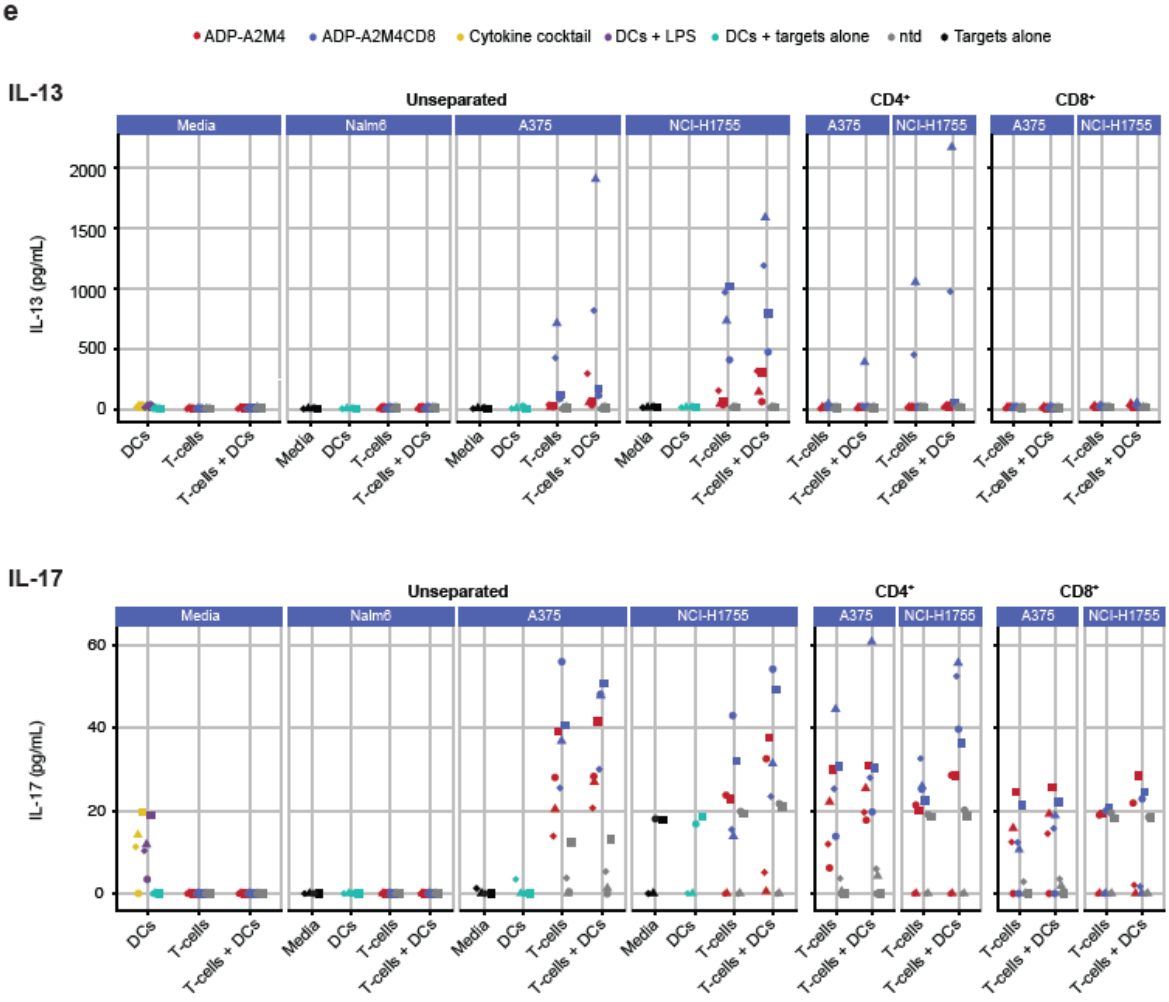

a
ADP-A2M4
ADP-A2M4CD8
Cytokine cocktail
DCs + LPS
DCs + targets alone
ntd
Targets alone
IL-12 (p40/p70)
MIG
IL-6
Unseparated
CD4+
CD8+
Media
Nalm6
A375
NCI-H1755
IL-12 p40/p70 (pg/mL)
MIG (pg/mL)
IL-6 (pg/mL)
DCs
T-cells
T-cells + DCs


b
ADP-A2M4
ADP-A2M4CD8
Cytokine cocktail
DCs + LPS
DCs + targets alone
ntd
Targets alone
IFN-γ
IL-2
TNF-α
Unseparated
CD4+
CD8+
Media
Nalm6
A375
NCI-H1755
IFN-γ (pg/mL)
IL-2 (pg/mL)
TNF-α (pg/mL)
DCs
T-cells
T-cells + DCs
***
**

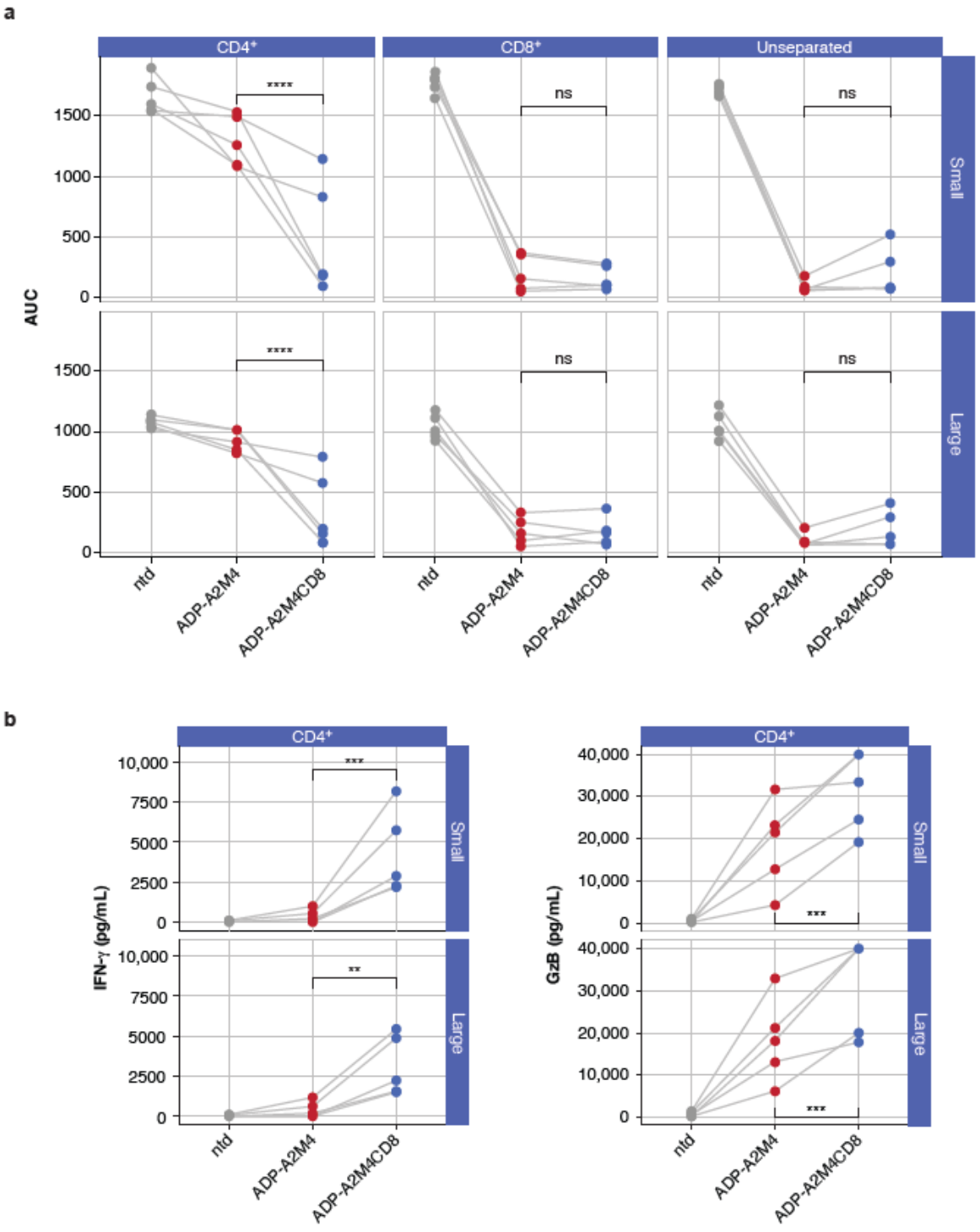

c
ADP-A2M4
ADP-A2M4CD8
Cytokine cocktail
DCs + LPS
DCs + targets alone
ntd
Targets alone
MIP-1β
IL-2R
GM-CSF
Unseparated
CD4+
CD8+
Media
Nalm6
A375
NCI-H1755
MIP-1β (pg/mL)
IL-2R (pg/mL)
GM-CSF (pg/mL)
DCs
T-cells
T-cells + DCs
*
**


d
ADP-A2M4
ADP-A2M4CD8
Cytokine cocktail
DCs + LPS
DCs + targets alone
ntd
Targets alone
IL-4
IL-5
IL-10
Unseparated
CD4+
CD8+
Media
Nalm6
A375
NCI-H1755
IL-4 (pg/mL)
IL-5 (pg/mL)
IL-10 (pg/mL)
DCs
T-cells
T-cells + DCs
**
*

e


ADP-A2M4
ADP-A2M4CD8
Cytokine cocktail
DCs + LPS
DCs + targets alone
ntd
Targets alone
IL-13
Unseparated
CD4+
CD8+
Media
Nalm6
A375
NCI-H1755
IL-13 (pg/mL)
2000
1500
1000
500
0
DCs
T-cells
T-cells + DCs
IL-17
IL-17 (pg/mL)
60
40
20
0

SUPPLEMENTAL FIGURE 7. Cytotoxic activity against melanoma-associated antigen A4 $(MAGE\text{-}A4)^+$ spheroids is enhanced with ADP-A2M4CD8 T cells. 3D spheroids (~600 μm ("small") or ~800 μm ("large") of $MAGE\text{-}A4^+$ A375 melanoma cells expressing cytoplasmic green fluorescent protein (GFP; A375.GFP) were cultured with ADP-A2M4 (red), ADP-A2M4CD8 (blue), or non-transduced (ntd) T cells (gray). (a) Scatter plots showing spheroid area under the curve (AUC) of isolated $CD4^+$ (left) or $CD8^+$ (center) T-cell fractions, or unseparated T cells (right). Each data point corresponds to the mean of six replicate wells for each donor (n = 5 donors). All data are shown normalized to the time of T-cell addition. (b) Levels of interferon gamma (IFN-γ) and granzyme B (GzB) produced by isolated $CD4^+$ ntd, ADP-A2M4, or ADP-A2M4CD8 T cells following approximately 48–50 hours of coculture with A375.GFP 3D spheroids. Each data point shows the mean of six replicate wells for each donor (n = 5 donors). Statistical analysis was performed using repeated measures analysis of variance. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. ns, not significant.


a
CD4+
CD8+
Unseparated
Small
Large
AUC
****
ns
1500
1000
500
0
ntd
ADP-A2M4
ADP-A2M4CD8
b
IFN-γ (pg/mL)
GzB (pg/mL)
***
**
10,000
7500
5000
2500
40,000
30,000
20,000