

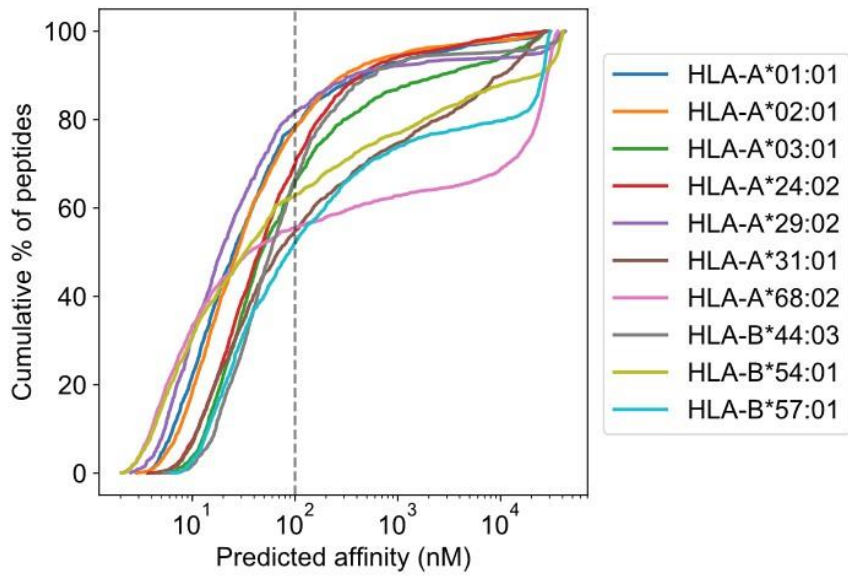
In the format provided by the authors and unedited.

# Antibody-mediated delivery of viral epitopes to tumors harnesses CMV-specific T cells for cancer therapy

David G. Millar<sup>1</sup>, Rakesh R. Ramjiawan<sup>2</sup>, Kosuke Kawaguchi<sup>2</sup>, Nisha Gupta<sup>2</sup>, Jiang Chen<sup>2</sup>, Songfa Zhang<sup>1</sup>, Takashi Nojiri<sup>2</sup>, William W. Ho<sup>2</sup>, Shuichi Aoki<sup>2</sup>, Keehoon Jung<sup>2</sup>, Ivy Chen<sup>2</sup>, Feng Shi<sup>1</sup>, James M. Heather<sup>1</sup>, Kohei Shigeta<sup>2</sup>, Laura T. Morton<sup>3</sup>, Sean Sepulveda<sup>1</sup>, Li Wan<sup>1</sup>, Ricky Joseph<sup>3</sup>, Eleanor Minogue<sup>1</sup>, Ashok Khatri<sup>1</sup>, Aditya Bardia<sup>4</sup>, Leif W. Ellisen<sup>1</sup>, Ryan B. Corcoran<sup>1</sup>, Aaron N. Hata<sup>1</sup>, Sara I. Pai<sup>5</sup>, Rakesh K. Jain<sup>2</sup>, Dai Fukumura<sup>2</sup>, Dan G. Duda<sup>2</sup> and Mark Cobbold<sup>1\*</sup>

---

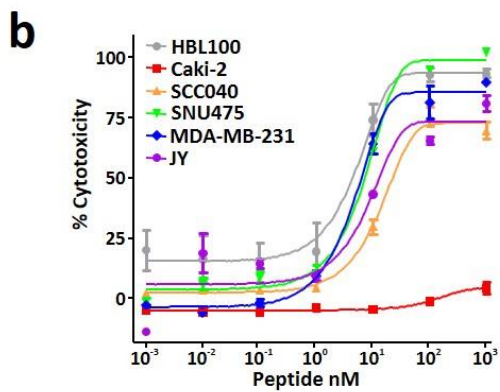
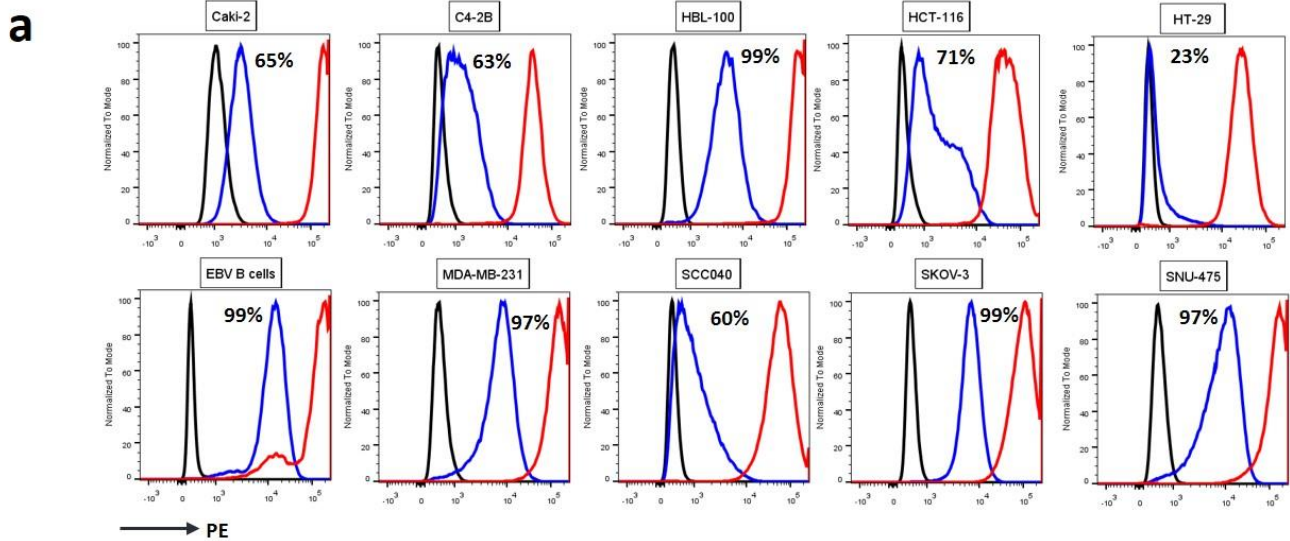
<sup>1</sup>Massachusetts General Hospital Cancer Center and Department of Medicine, Harvard Medical School, Boston, MA, USA. <sup>2</sup>Steele Laboratories, Department of Radiation Oncology, Harvard Medical School, Boston, MA, USA. <sup>3</sup>Medical Research Council Centre for Immune Regulation and Clinical Immunology Service, School of Immunity and Infection, College of Medicine and Dental Sciences, University of Birmingham, Birmingham, UK. <sup>4</sup>Massachusetts General Hospital, Boston, MA, USA. <sup>5</sup>Division of Surgical Oncology, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA. \*e-mail: [mcobbold@mgh.harvard.edu](mailto:mcobbold@mgh.harvard.edu)



**Supplementary Figure 1**

Predicted affinities of HLA-bound peptides.

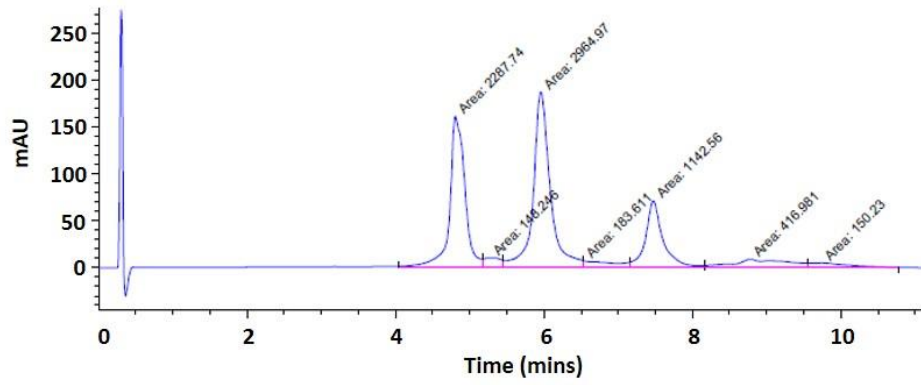
In silico prediction of peptides eluted from HLA molecules.



### Supplementary Figure 2

Presence of empty HLA molecules at the surface of tumor cell lines that can be loaded with exogenous CMV peptide with tumor cell cytotoxicity.

**(a)** Ten tumor cell lines labelled with antibodies specific for peptide-loaded HLA (red line) and empty HLA molecules (blue line) compared with control stained cells (black line). Percentages shown are percentage cells positive for empty HLA molecules (HC10) staining. All cell lines tested were 100% positive for peptide-loaded HLA (W6/32). Staining was repeated (n=3) in selected cell lines. **(b)** Five HLA-A2+ cell lines lysed by peptide-specific CMV-CTL and no lysis of HLA-A2- cell line (Caki-2) (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean.

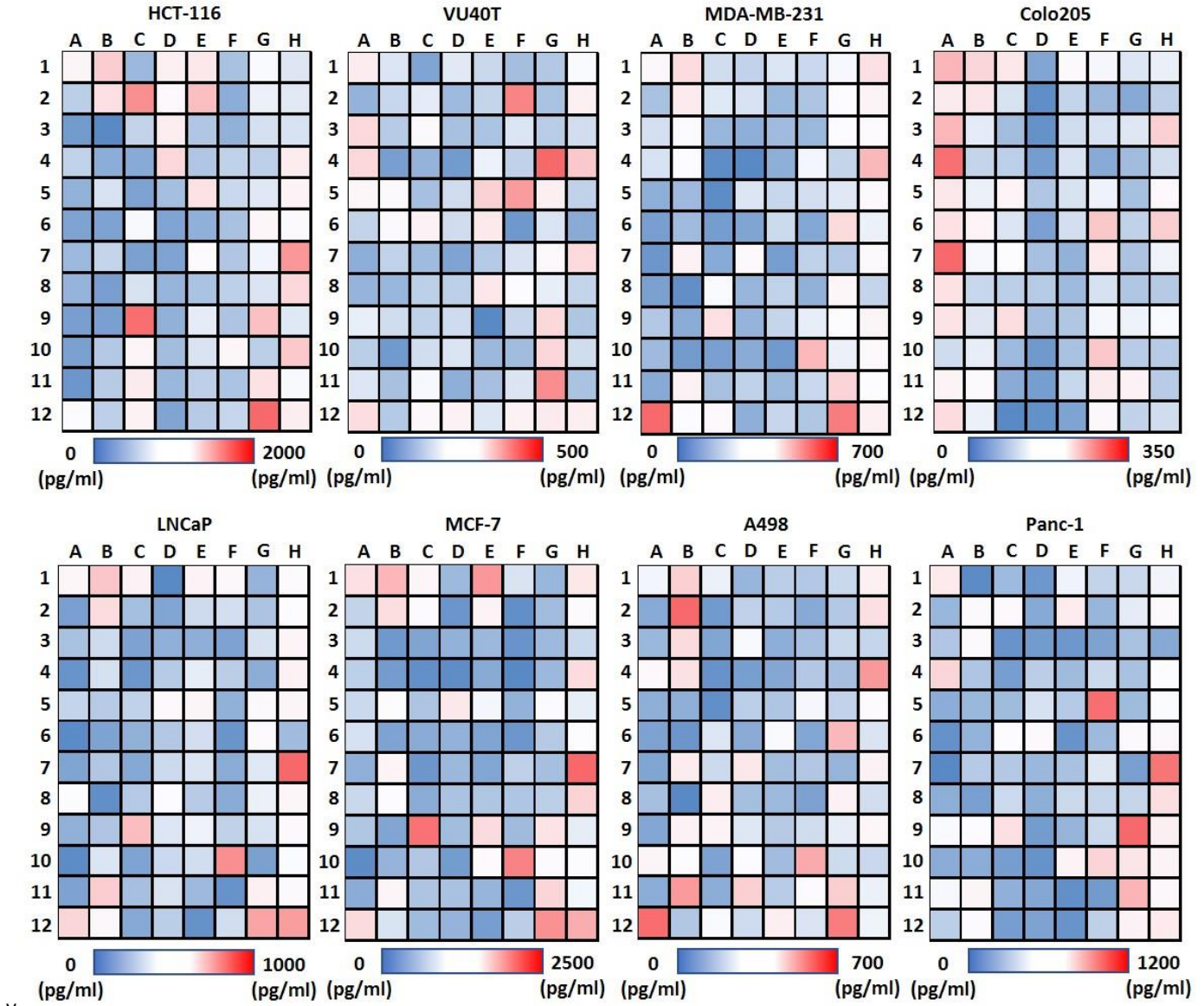


Drug antibody ratio	Area	Conjugation (%)
0	2287.74	31.36
2	2964.97	40.64
4	1142.58	15.66
6	416.98	5.71
8	150.23	2.04

### Supplementary Figure 3

Assessment of peptide antibody ratio conjugation.

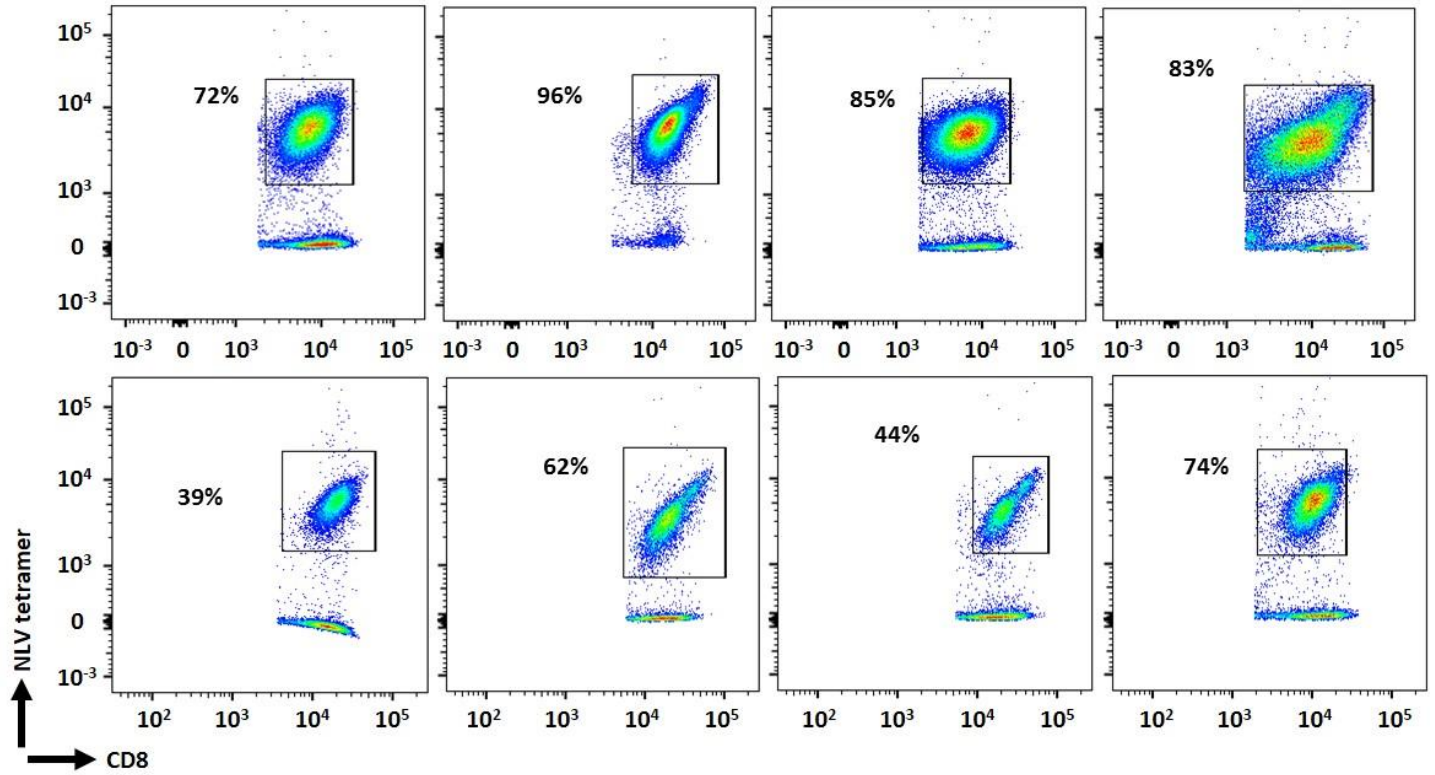
The number of peptides conjugated to each antibody was assessed by high pressure liquid chromatography (HPLC). Data from single experiment.



**Supplementary Figure 4**

Screening 96 APECs in multiple tumor cell lines.

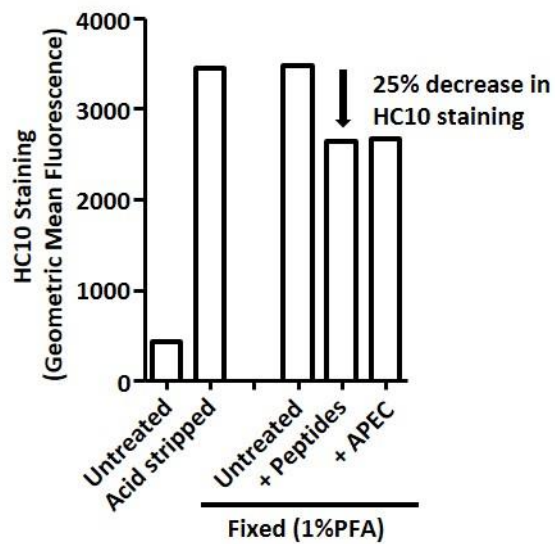
Eight tumor cell lines are labelled with cAPECs and cytokine release by peptide-specific T cells assayed.



**Supplementary Figure 5**

Peptide specificity of *ex vivo* cultured T cell lines.

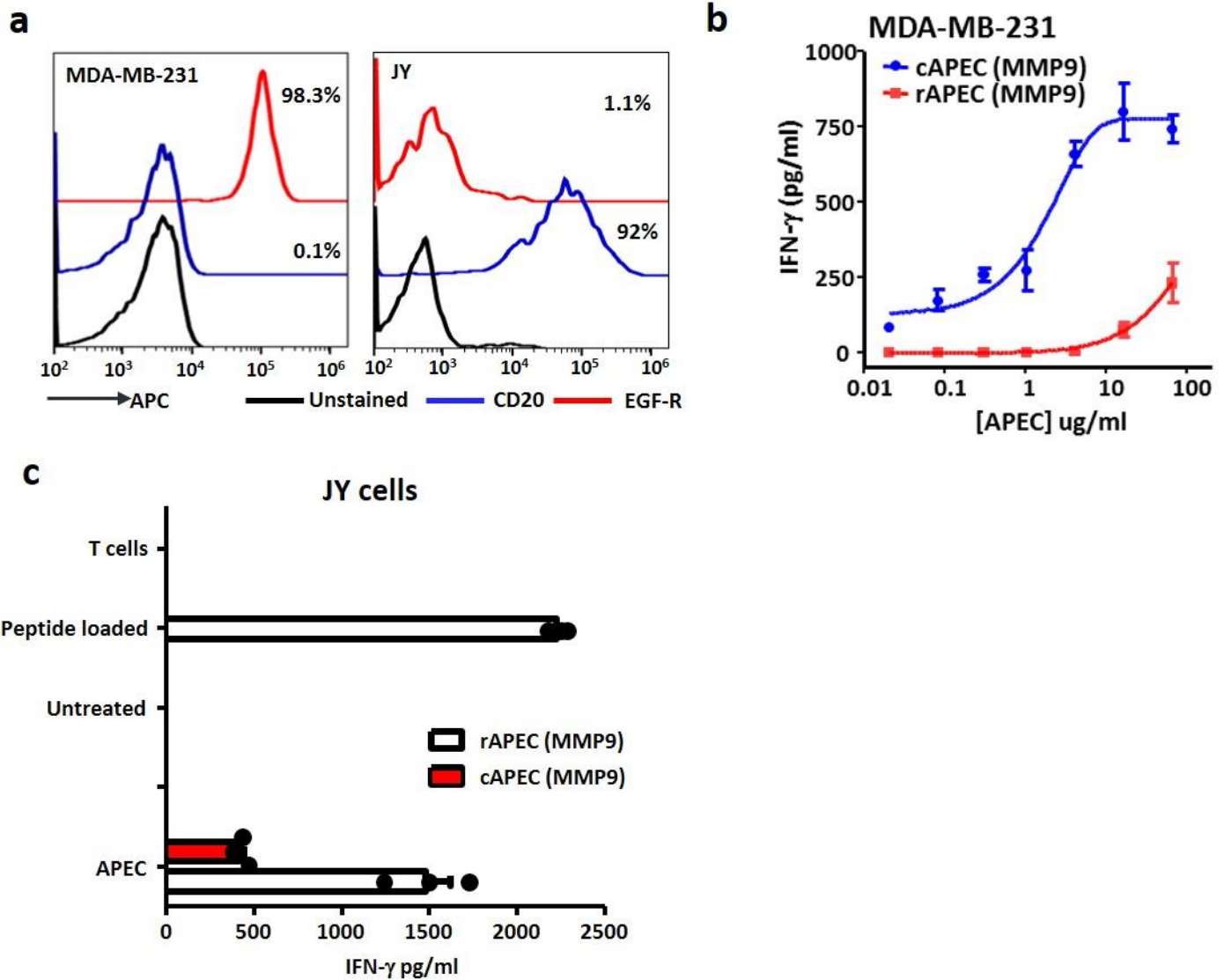
Flow cytometric analysis of *ex vivo* cultured T cell lines used for *in vitro* assays using HLA-peptide tetramers. Cell lines were cultured at various times and each cell line was analyzed for tetramer positive T cells once.



### Supplementary Figure 6

Analysis of empty MHC molecules using HC10 antibody staining.

Colo205 Tumor cells were assayed for the presence and decrease in the amount of empty MHC molecules at the cell surface by HC10 staining. Cells were acid-stripped to remove peptides from surface MHC molecules, lightly fixed or left untreated and incubated with either peptide or APEC before HC10 staining.

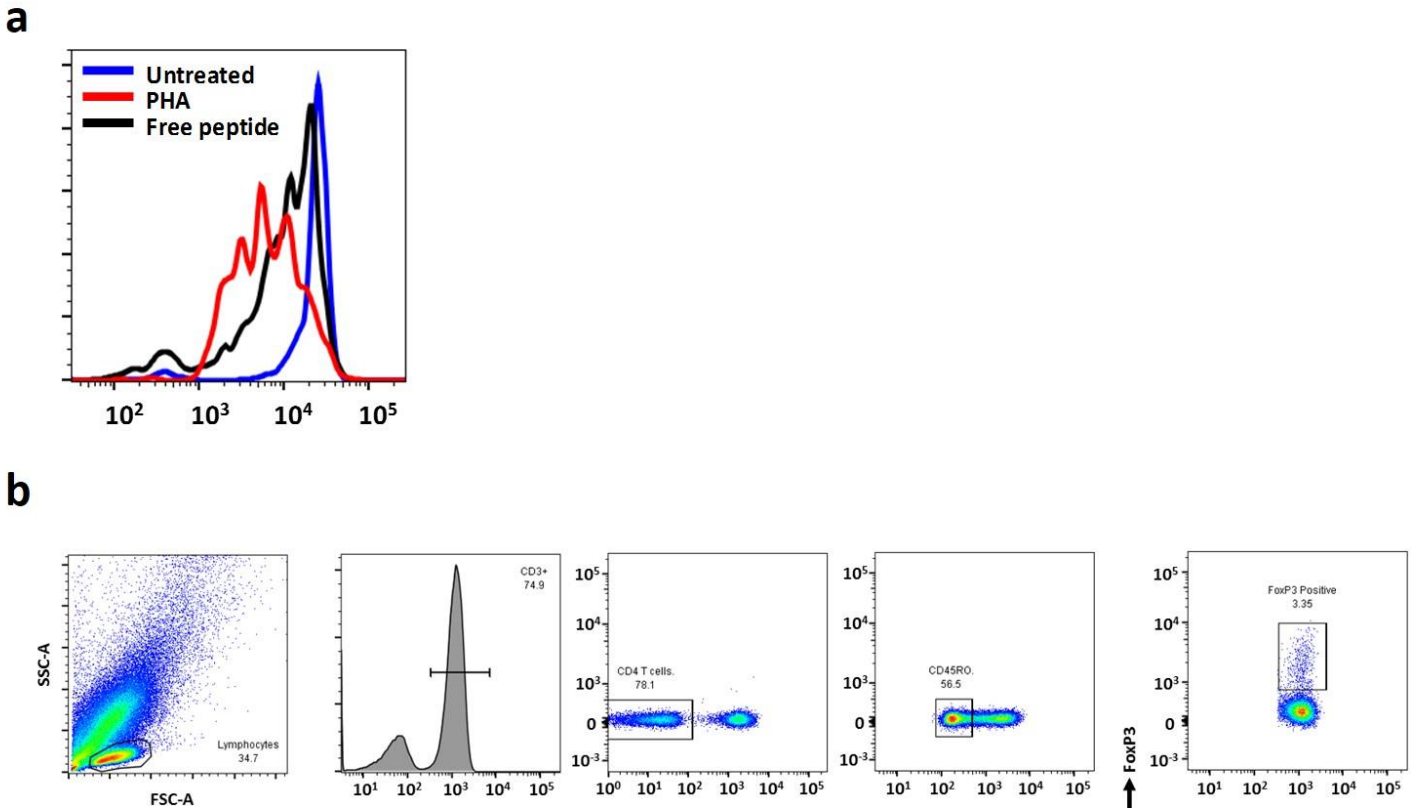


### Supplementary Figure 7

APEC binding to tumor cell surface essential for T cell recognition.

**(a)** Flow cytometric analysis of EGF-R and CD20 expression on MDA-MB-231 and JY tumor cell lines. Staining was repeated  $n=3$ . **(b)** Surface binding of APEC is required for antigenic reprogramming and can be inhibited by the pre-treatment with unconjugated antibody ( $n=3$  independent samples). Data represented as mean and error bars represent standard error of the mean. **(c)** CD20+ tumor cells labelled with cAPEC or rAPEC (350nM) and demonstrating T cell activation only when bound by the anti-CD20 rAPEC. Peptide loaded ( $1\mu\text{M}$ ) target cells were used to determine efficacy of T cells ( $n=3$  independent samples). Data represented as mean and error bars represent standard error of the mean.

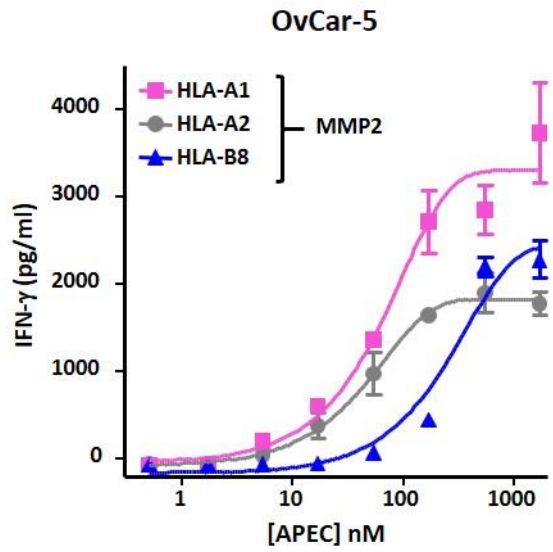




**Supplementary Figure 8**

Flow cytometric analysis of T cell proliferation and regulatory T cells.

**(a)** Flow cytometric staining to allow analysis of T cell proliferation after treatment with free peptide (black), phytohaemagglutinin (PHA, red) or untreated T cells (blue) (data from single experiment). **(b)** Flow cytometric staining to allow analysis of CD4+ CD45RO FoxP3+ regulatory T cells (data from single experiment).

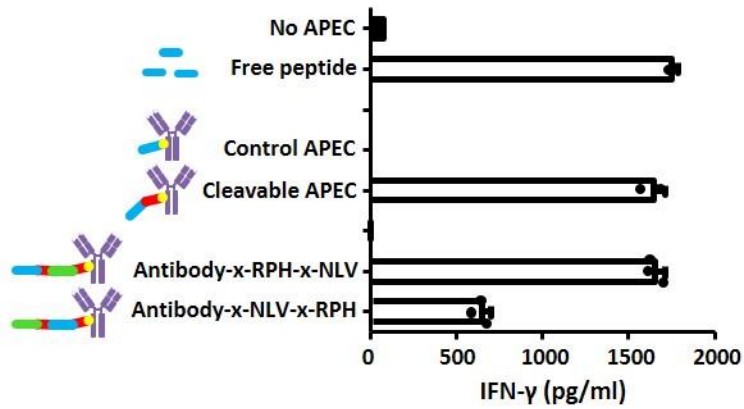


**Supplementary Figure 9**

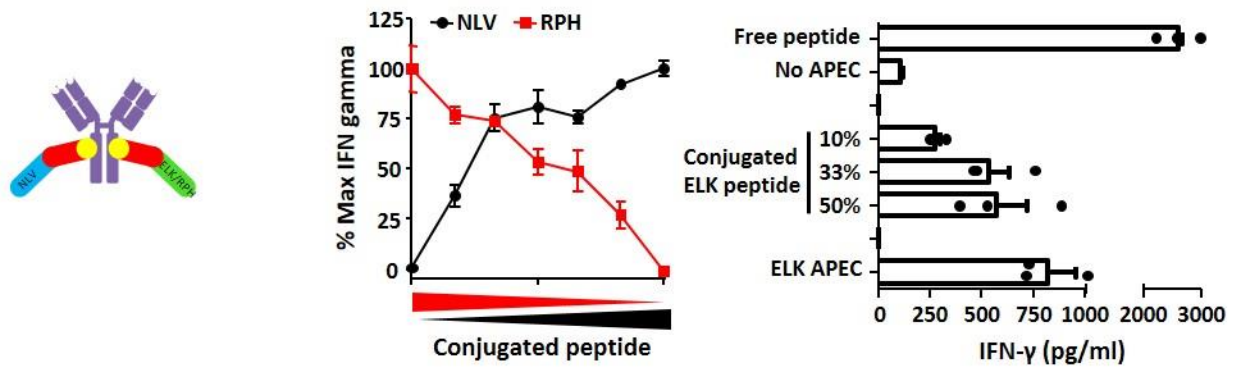
Expanding APEC to include peptides that bind to other HLA alleles.

APECs conjugated with CMV epitopes covering multiple HLA alleles are able to activate and trigger cytokine release of peptide-specific CMV-CTL (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean.

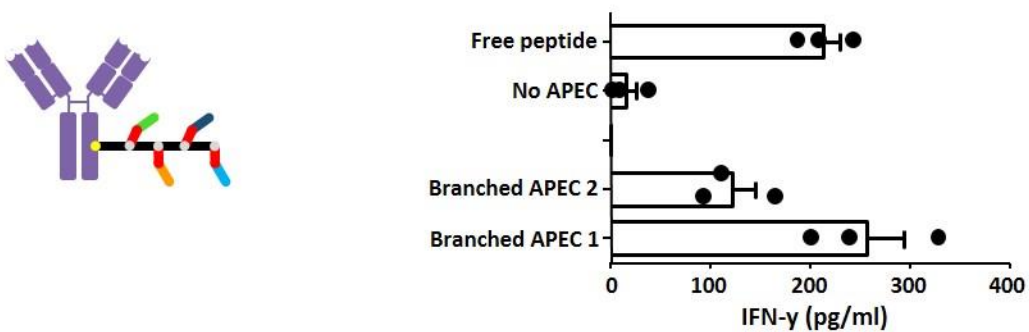
**a** Concatemer peptide APEC



**b** Multiple peptide APEC



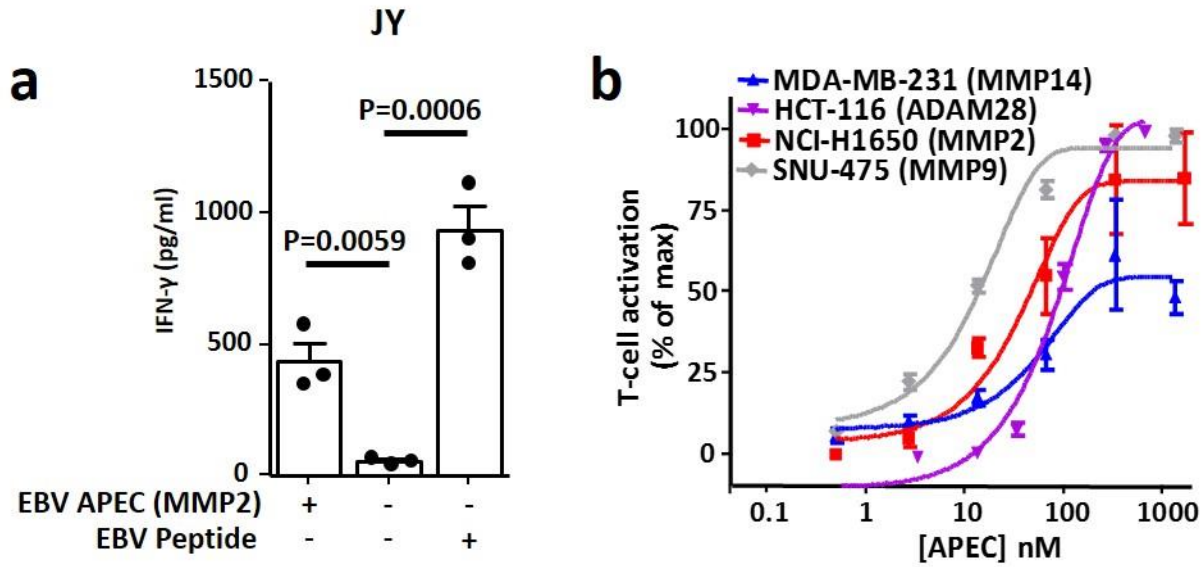
**c** Branched APEC



Supplementary Figure 10

Construction of APECs containing multiple T-cell epitopes (polytopes).

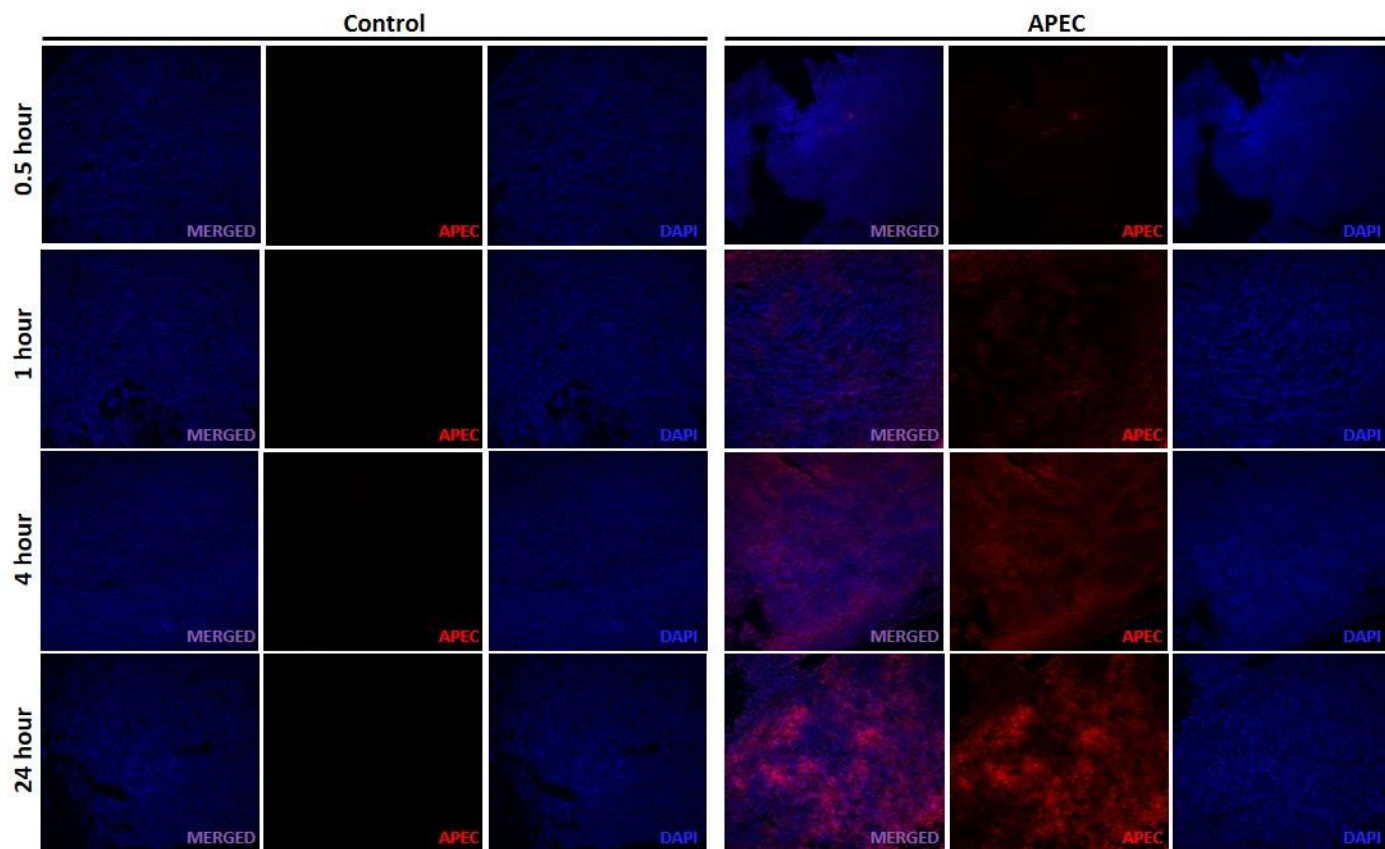
We tested whether it was possible to generate APECs that contained multiple T-cell epitope peptide payloads. **(a)** The initial concatemer design utilized linear peptides with tandem T-cell epitopes (NLV and RPH) juxtaposed by proteolytic cleavage sequences (-x-). Using T-cells against each epitope, both epitopes elicited T-cell responses (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean. A second method **(b)** involved the mixed conjugation of peptides to antibodies. In this case the peptide payloads were the same as original APEC design, but two different peptides (with 7-different ratios) were conjugated onto a single APEC. These mixed APECs were able to activate the two different T-cell populations (NLV or RPH) but with varying potency. In a separate experiment three different ratios were tested against two different epitopes (NLV and ELK) which gave concordant results (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean.. Lastly, we created a single APEC species that was conjugated to a branched peptide that contained multiple different cleavable peptides **(c)**. These branched peptides were able to activate T-cell populations (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean.



**Supplementary Figure 11**

EBV-specific T cells can be re-directed to target tumor cells using APEC and CMV-CTL activation using different proteases to cleave APECs.

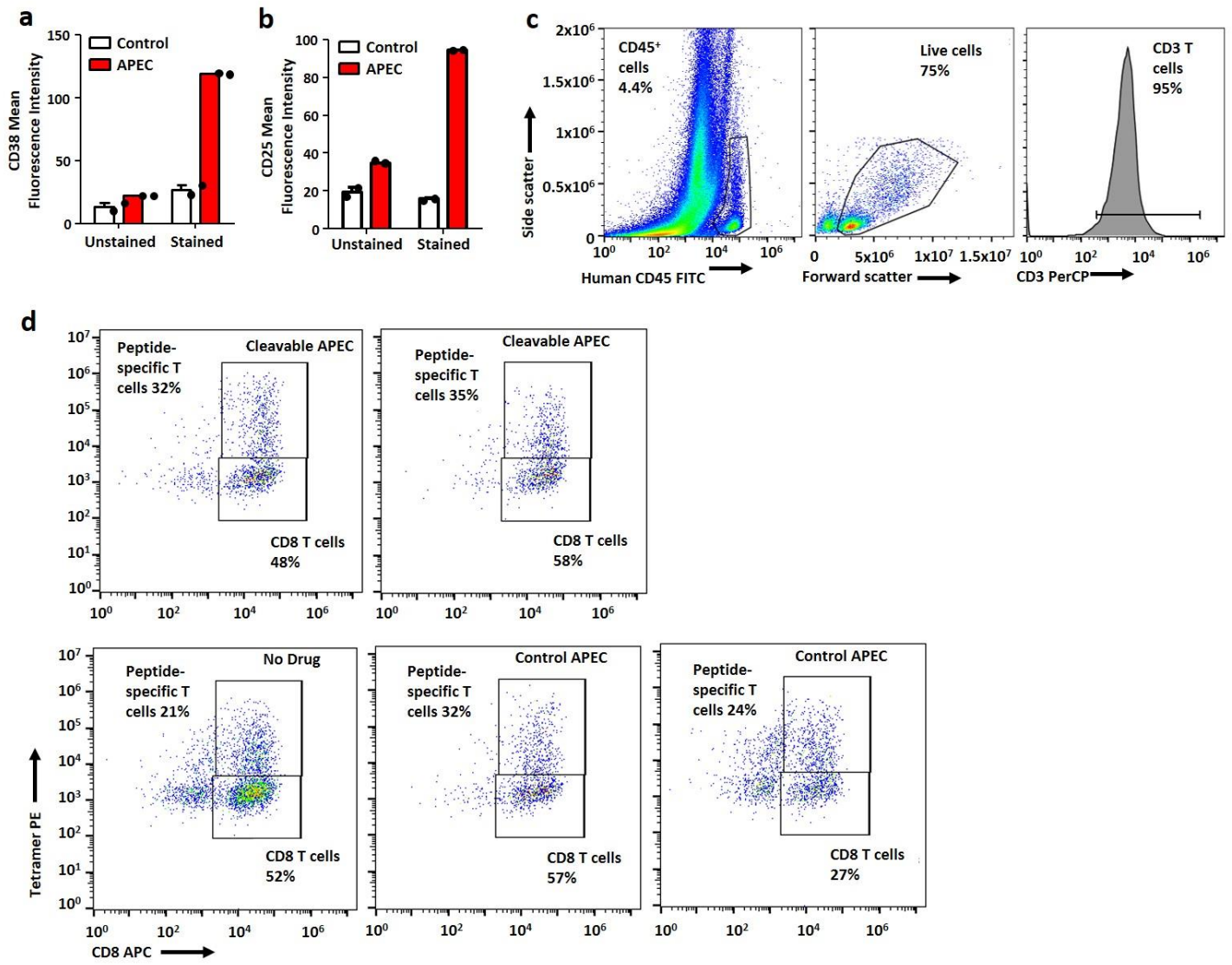
**(a)** Tumor cells treated with APEC conjugated with EBV-derived epitopes are recognised by EBV-specific T cells (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean. Significance was determined by unpaired two-tailed t-test. **(b)** Multiple tumor types can be recognized by peptide-specific T cells after treatment with cAPEC containing ADAM28, MMP2, MMP9 or MMP14 cleavable peptides (n=3 independent samples). Data represented as mean and error bars represent standard error of the mean.



**Supplementary Figure 12**

Tumor penetration of APEC in orthotopic breast cancer model.

Tumor-bearing NOD/SCID mice (n=5) were injected with either PBS or MMP14-cAPEC and tumors resected at timepoints up to 24h. Tumors were taken for immunofluorescence to stain for the presence of APEC.



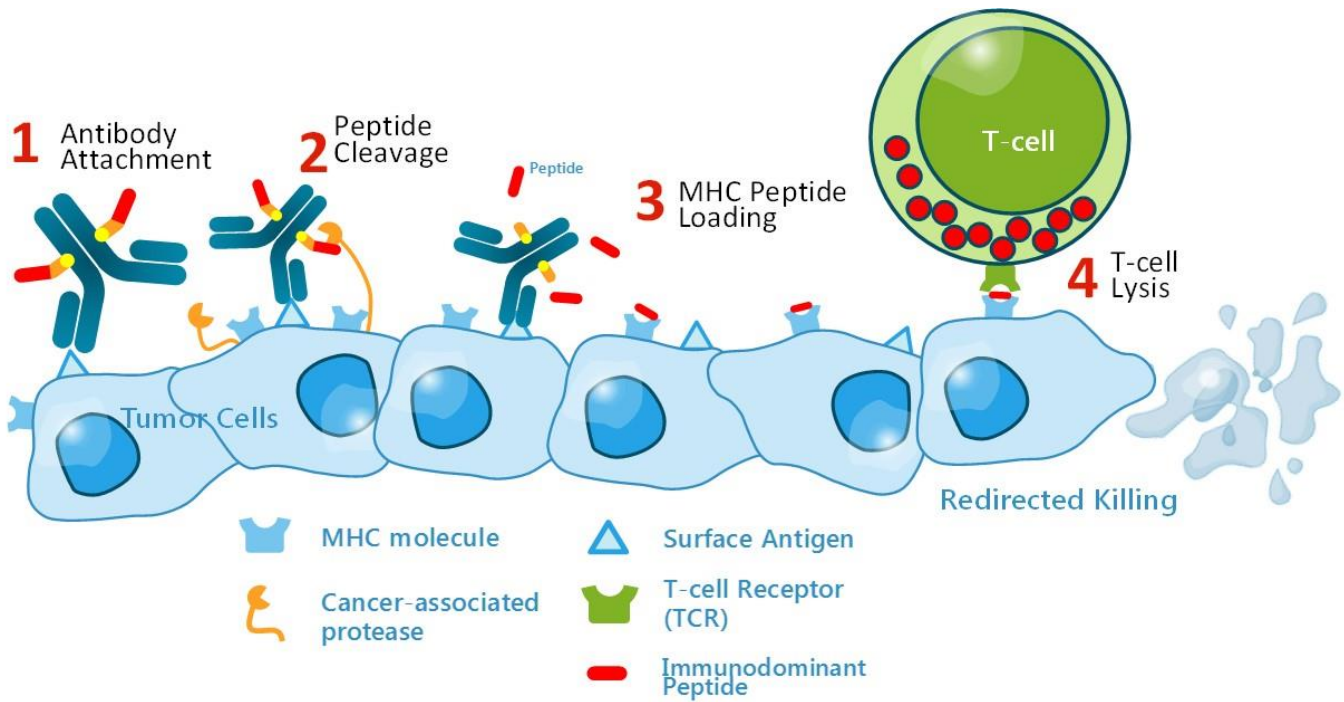
### Supplementary Figure 13

In vivo T cell activation by APEC in orthotopic breast cancer model and the presence of peptide-specific T cells within tumors.

Breast cancer tumor-bearing mice were injected intratumorally with freshly isolated peptide-specific CMV-CTL and 24h post-injection, tumors were resected and T cells isolated. Flow cytometric analysis of intratumoral CD3<sup>+</sup> T cells was undertaken for the presence of T cell activation markers (a) CD38 (n=2 independent samples) and (b) CD25 (n=2 independent samples). Data represented as mean and error bars represent standard error of the mean. (c) Gating strategy to select human T cells from the excised tumor sample. Firstly, CD45<sup>+</sup> was used to gate human lymphocytes before live cells were gated using forward and side scatter. T cells were then gated using CD3. (d) Peptide-specific T cells were labelled using HLA-peptide tetramer complexes conjugated to phycoerythrin (PE) and the cells were co-labeled with CD8 APC (data from single experiment).







### Supplementary Figure 15

APEC mechanism of action.

Proposed mechanism of action for APEC with antibody attachment to target antigen (1), release of virus-derived epitope at the cell surface (2). Released peptide loads into empty MHC class I molecule at the cell surface (3) and T cell lysis by the re-directed anti-viral immune response.

**Table S1**

Protein	Sequence	Position	HLA restriction
pp65	YSEHPTFTSQY	363–373	HLA-A1
pp50	VTEHDTLly	245–253	HLA-A1
IE1	YILEETSVM	315–323	HLA-A2
pp65	NLVPMVATV	495–503	HLA-A2
IE1	VLEETSVMl	316–324	HLA-A2
pp50	TVRSHCVSK	52–60	HLA-A3
pp65	TPRVTGGGAM	417–426	HLA-B7
pp65	RPHERNGFTVL	265–275	HLA-B7
IE-1	QJKVRVDMV	88-96	HLA-B8
IE1	ELRRKMMYM	199–207	HLA-B8
IE1	ELKRKMIYM	199–207	HLA-B8

**Table S1.** CMV peptides used for tetramer staining and their HLA restriction.

**Table S2**

Peptide	Sequence	Protease	Peptide	Sequence	Protease	Peptide	Sequence	Protease
A1	YLGRSYKVNLPVMVATV	C1s	C9	HPVGLLARNLPVMVATV	MMP2	F5	RNLVPMVATV	Trypsin
A2	FNKLNLPVMVATV	Cathepsin B	C10	KGPLGVRGNLPVMVATV	MMP2	F6	RNLVPMVATV	Trypsin
A3	GGGGFNLPVMVATV	Cathepsin B	C11	PLGLAGNLPVMVATV	MMP2	F7	GGSGRSANANLPVMVATV	uPA
A4	PRSFRLGKKNLPVMVATV	Cathepsin D	C12	PLGLWANLPVMVATV	MMP2	F8	SGRSANAKNLPVMVATV	uPA
A5	EVLWSAVNLPVMVATV	Cathepsin G	D1	PLGVRGNLPVMVATV	MMP2	F9	SGRSANLPVMVATV	uPA
A6	PVSLYRCNLPVMVATV	Cathepsin G	D2	AIPVSLRNLPVMVATV	MMP2	F10	SRRRVNSLNLPVMVATV	uPA
A7	AAPVNLPVMVATV	Elastase 2	D3	PQGIAMGNLPVMVATV	MMP2	F11	CPGRVVGGNLPVMVATV	uPa / tPA
A8	TSQVNGLNLPVMVATV	Elastase 2	D4	CGLDDNLPVMVATV	MMP2/9	F12	PRGMASNLPVMVATV	MMP9
A9	TPEHVVPYNLPVMVATV	Endothelin-converting enzyme 1	D5	GPLGIAGQNLVPVMVATV	MMP2/9	G1	PLGVRGNLPVMVATV	MMP2/9
A10	PQGRIVGGNLPVMVATV	Hepsin	D6	GPLGMLSQNLVPVMVATV	MMP2/9	G2	PLGLAGNLPVMVATV	MMP2/9
A11	PRFKIIGNLPVMVATV	Hepsin	D7	PLGLNLPVMVATV	MMP2/9	G3	PQGLAGNLPVMVATV	MMP9
A12	GKAFFRRLNLPVMVATV	Hk2	D8	PVGLIGNLPVMVATV	MMP2/9	G4	GPQGARGQNLVPVMVATV	MMP9
B1	AANLNLPVMVATV	Legumain	D9	GPQGIWQNLVPVMVATV	MMP2/9, MT1-MMP	G5	PLGLYLNLPVMVATV	MMP2/9
B2	SLGRKIQLNLPVMVATV	MASP2	D10	QPVGINTSNLPVMVATV	MMP3 (stromelysin-1)	G6	PLGLYALNLPVMVATV	MMP2/9
B3	APPPVLLNLPVMVATV	Matrilysin (MMP7)	D11	STAVIVSANLPVMVATV	MMP3 (stromelysin-1)	G7	AAALGNVAPNLPVMVATV	MMP9
B4	IPENFFGNLPVMVATV	Matrilysin (MMP7)	D12	VASSSTAVNLPVMVATV	MMP3 (stromelysin-1)	G8	GTQFFNLPVMVATV	Cathepsin D
B5	LRELHLDNLPVMVATV	Matrilysin (MMP7)	E1	GPLGLARKNLPVMVATV	MMP7	G9	GSTFFNLPVMVATV	Cathepsin D
B6	MLEDEASGNLPVMVATV	Matrilysin (MMP7)	E2	RPLALWRSNLPVMVATV	MMP7	G10	QVVAGNLPVMVATV	Cathepsin B
B7	KQSRKFVNPVPMVATV	Matriptase(ST14)	E3	NKSRRLGNLPVMVATV	MMP7/ Cathpsin B	G11	TYRSRYLNLPVMVATV	uPA
B8	RQARVVGGNLPVMVATV	Matriptase2 /Hepsin	E4	GPQGIAGQRNLPVMVATV	MMP9	G12	NSGRAVTYNLPVMVATV	uPA
B9	GPLGLWAQNLVPVMVATV	MMP (esp 2/9)	E5	KPVLSYRNLPVMVATV	MMP9	H1	PSSRRRVNLPVMVATV	uPA
B10	PVSLRNLPVMVATV	MMP1	E6	PLGMTSNLPVMVATV	MMP9	H2	PMKRLTLGNLPVMVATV	Cathepsin B
B11	AAATSIAMNLPVMVATV	MMP11 (stromelysin-3)	E7	PRALMNLPVMVATV	MMP9	H3	DDDKIVGGNLPVMVATV	Cathepsin B
B12	AAGAMFLENLPVMVATV	MMP11 (stromelysin-3)	E8	GPLPLRNLPVMVATV	MT1-MMP	H4	HLVEALYNLPVMVATV	Cathepsin B
C1	EAAAATSNLPVMVATV	MMP11 (stromelysin-3)	E9	KQLRVVNGNLPVMVATV	MT-SP1 / ST14/ uPA / Hepsin	H5	EVDLLIGSNLPVMVATV	Cathepsin B
C2	PRHLRNLPVMVATV	MMP14	E10	PLGLYANLPVMVATV	Pan-MMP	H6	PRFKIIGGNLPVMVATV	Cathepsin B
C3	PRGLRKNLPVMVATV	MMP14	E11	AFKNLPVMVATV	Plasmin	H7	AVRWLLTANLPVMVATV	MMP9
C4	PRGLRPNLPVMVATV	MMP15/16/24/25	E12	GGRNLPVMVATV	Plasmin / TMPRSS2	H8	RPLALWRSNLPVMVATV	MMP7
C5	PRHLRNNLPVMVATV	MMP15/16/24/25	F1	HSSKLQNLVPVMVATV	PSA	H9	PVGLIGNLPVMVATV	MMP2/9
C6	PRWLRSNLPVMVATV	MMP15/16/24/25	F2	SSKYQNLVPVMVATV	PSA	H10	GGGRRNLPVMVATV	uPA
C7	GPLGLWAGGNLPVMVATV	MMP2	F3	LVPRGSNLPVMVATV	Thrombin	H11	GGGGGNLPVMVATV	Control
C8	GPLGVRGNLPVMVATV	MMP2	F4	RNLVPMVATV	Trypsin	H12	V{Cit}GSV{Cit}NLPVMVATV	Cathepsin B

**Table S2.** Peptide sequences used for 96 APEC screening and the protease previously published to cleave the peptide.

**Table S3**

Peptide	Sequence	Protease	Reference from Table S2
FRET1	PRSFRLGK	Cathepsin D	A4
FRET2	KPVLSYR	MMP9	E5
FRET3	AANL	Legumain	B1
FRET4	YLGRSYKV	C1s	A1
FRET5	PRHLR	MMP14	C2
FRET6	GPLGVRGK	MMP2	C8
FRET7	GPLGLWAQ	MMP (esp2/9)	B9
FRET8	PLGLYL	MMP2/9	G5
FRET9	PRGLRK	MMP15/16/24/25	C3
FRET10	GPQGIAGQR	MMP9	E4
FRET11	GPLGIAGQ	MMP2/9	D5
FRET12	GGFRGG	Cathepsin B	-
FRET13	FRFRFR	Cathepsin B	-
FRET14	GGGGGG	Uncleavable	H11
FRET15	AIPVSLR	MMP2	D2
FRET16	KPAKFFRL	ADAM28	-
FRET17	PRSAKELR	MMP14	-

**Table S3.** Peptide sequences used for the FRET assay and the protease suggested to cleave the peptide.

**Table S4**

Peptide	Sequence	Protease	Peptide	Sequence	Protease
#14	CIPENFFGVSIINFEKL	Matrilysin (MMP7)	#42	CPVGLIGSIINFEKL	MMP2/9
#15	CLRELHLDNSIINFEKL	Matrilysin (MMP7)	#43	CGPQGIWQSIINFEKL	MMP2/9, MT1-MMP
#19	CGPLGLWAQSIINFEKL	MMP (esp 2/9)	#44	CQPVGINTSSIINFEKL	MMP3 (stromelysin-1)
#20	CPLGLLGSIIINFEKL	MMP1	#45	CSTAVIVSASIINFEKL	MMP3 (stromelysin-1)
#21	CAAATSIAMSIINFEKL	MMP11 (stromelysin-3)	#46	CVASSSTAVSIINFEKL	MMP3 (stromelysin-1)
#22	CAAGAMFLESIIINFEKL	MMP11 (stromelysin-3)	#47	CGPLGLARKSIINFEKL	MMP7
#26	CPRGLRPSIIINFEKL	MMP15/16/24/25	#48	CRPLALWRSSIINFEKL	MMP7
#27	CPRHLRNSIIINFEKL	MMP15/16/24/25	#50	CGPQGIAGQRSIIINFEKL	MMP9
#30	CGPLGVGRKSIINFEKL	MMP2	#51	CKPVSLYSRSIIINFEKL	MMP9
#31	CHPVGLLARSIIINFEKL	MMP2	#52	CPLGMTSSIINFEKL	MMP9
#32	CKGPLGVGRSIINFEKL	MMP2	#53	CPRALMSIIINFEKL	MMP9
#33	CPLGLAGSIINFEKL	MMP2	#54	CGPLPLRSIIINFEKL	MT1-MMP
#34	CPLGLWASIINFEKL	MMP2	#59	CGPQGARGQSIINFEKL	MMP9
#35	CPLGVRSIIINFEKL	MMP2	#60	CPLGLYSIIINFEKL	MMP2/9
#36	CAIPVSLRSIIINFEKL	MMP2	#61	CPLGLYALSIIINFEKL	MMP2/9
#38	CSGLDDSIINFEKL	MMP2/9	#67	CGGGGSIINFEKL	Control
#40	CGPLGMLSQSIINFEKL	MMP2/9	#68	CV{Cit}GSV{Cit}SIINFEKL	Cathepsin B
#41	CPLGLSIINFEKL	MMP2/9	MMP14	CPRSAKELRSIIINFEKL	MMP14

**Table S4.** Peptide sequences incorporating the ovalbumin-derived SIINFEKL peptide used for APEC screening of the murine colorectal cell line SL-4.