Supplemental Material to:

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Identification of LY6K long peptide immunogenic variants encompassing both CD4+ and CD8+ T-cell epitopes and eliciting CD4+ T-cell immunity in patients with malignant disease

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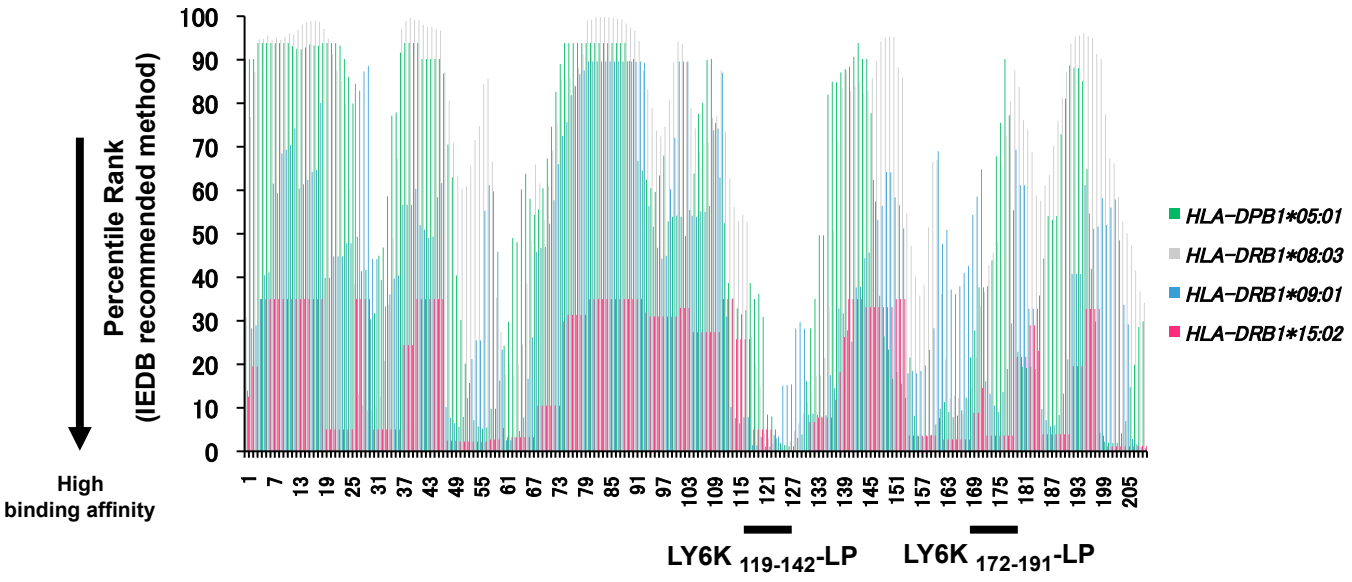
Supplementary figure 1.

A



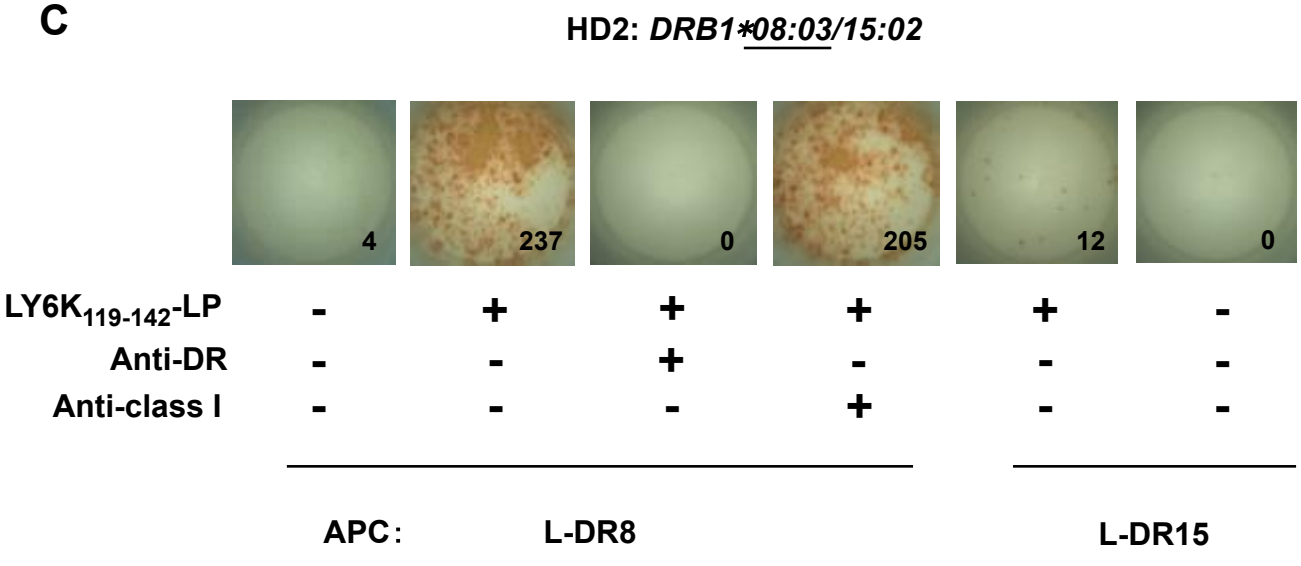
B

LY6K $_{119\text{-}142}$-LP: KWTEPYCVIAAVKIFPRFFMVAKQ

LY6K $_{172\text{-}191}$-LP: KCCKI**RYCNLEGPPI**NSSVF

LY6K$_{177\text{-}186}$-A24

C



Supplementary figure 1. LY6K-derived HLA class II-binding LPs predicted by immune epitope database (IEDB) analysis resource. **A**, The amino acid sequence of the human LY6K protein was analyzed using IEBD (IEDB recommended method), http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html. Numbers on the horizontal axis indicate amino acid positions at the N-terminus of LY6K-derived 15-mer peptides. Lower number of percentile rank indicates stronger binding affinity to HLA class II molecules. **B**, The 24-mer LP, $LY6K_{119–142}$-LP with high percentile ranks for multiple *HLA-class II* allelic (*DPB1*05:01*, *DRB1*08:03*, *DRB1*09:01*, and *DRB1*15:02*) products was synthesized (**A**, left black bar). The 20-mer LP, $LY6K_{172–191}$-LP with high percentile ranks for 2 HLA-class II allelic (*DRB1*09:01* and *DRB1*15:02*) products and encompassing a 10-mer CTL-epitope recognized by HLA-A24-restricted CTLs was synthesized (**A**, right black bar). **C**, Induction of LY6K-specific Th cells from healthy donors. Supplementary figure 1C shows representative images of wells in IFN-γ ELISPOT assay from one healthy donor HD2.

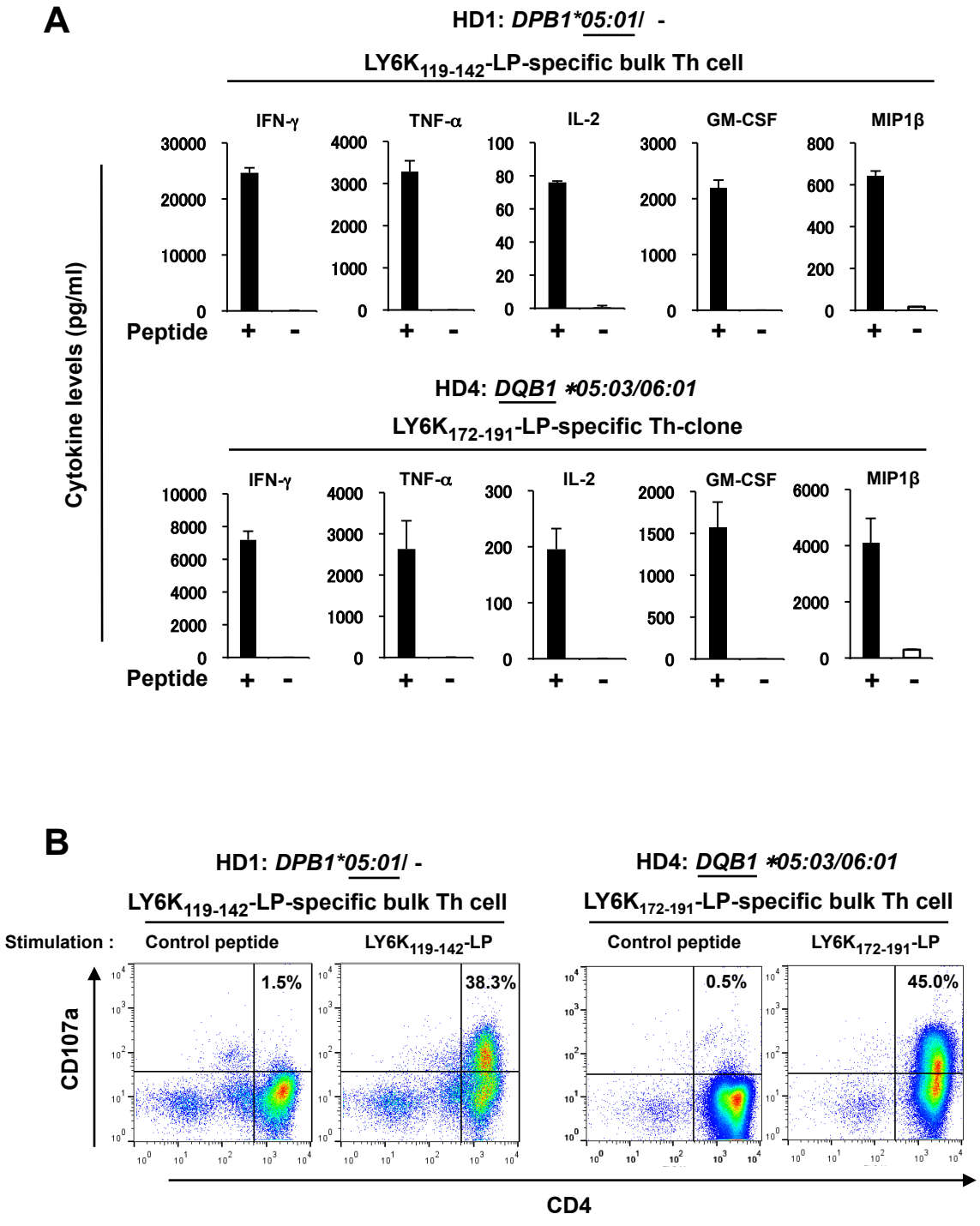
Supplementary table 1

Binding algorithm scores of LY6K-derived peptides for HLA-class II molecules

	Percentile Rank			
Amino acid residues position	HLA-DP5 (*DPB1*05:01*)	HLA-DR8 (*DRB1*08:03*)	HLA-DR9 (*DRB1*09:01*)	HLA-DR15 (*DRB1*15:02*)
$LY6K_{119-142}LP$				
119-133	35.0	20.1	1.4	5.0
120-134	36.2	14.8	3.3	5.0
121-135	30.9	4.1	1.1	5.0
122-136	8.4	3.0	1.1	5.0
123-137	8.0	1.8	3.7	5.0
124-138	3.0	1.0	3.4	0.02
125-139	1.9	1.2	15.1	0.02
126-140	1.5	1.4	15.2	0.02
127-141	1.2	2.3	15.4	0.02
128-142	1.2	4.7	28.2	0.02
$LY6K_{172-191}LP$				
172-186	37.7	36.6	16.1	3.6
173-187	37.9	42.7	13.2	3.6
174-188	43.9	45.7	10.5	3.6
175-189	67.8	48.2	9.1	3.6
176-190	75.5	65.6	13.7	3.6
177-191	90.2	72.6	18.9	3.6

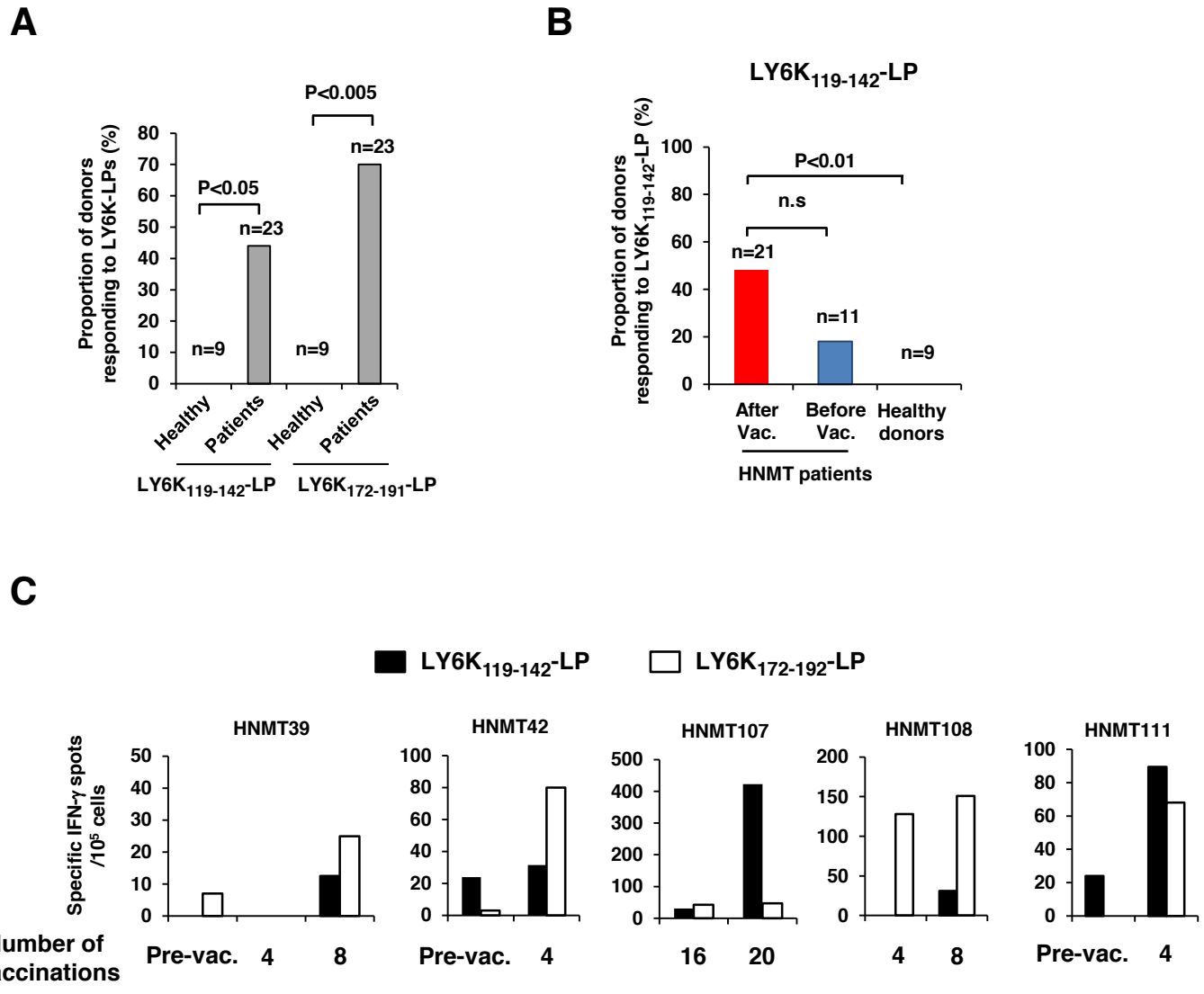
Peptide-binding algorithm scores for indicated HLA-class II molecules are shown for each15-mer LY6K-derived peptide.

Supplementary figure 2



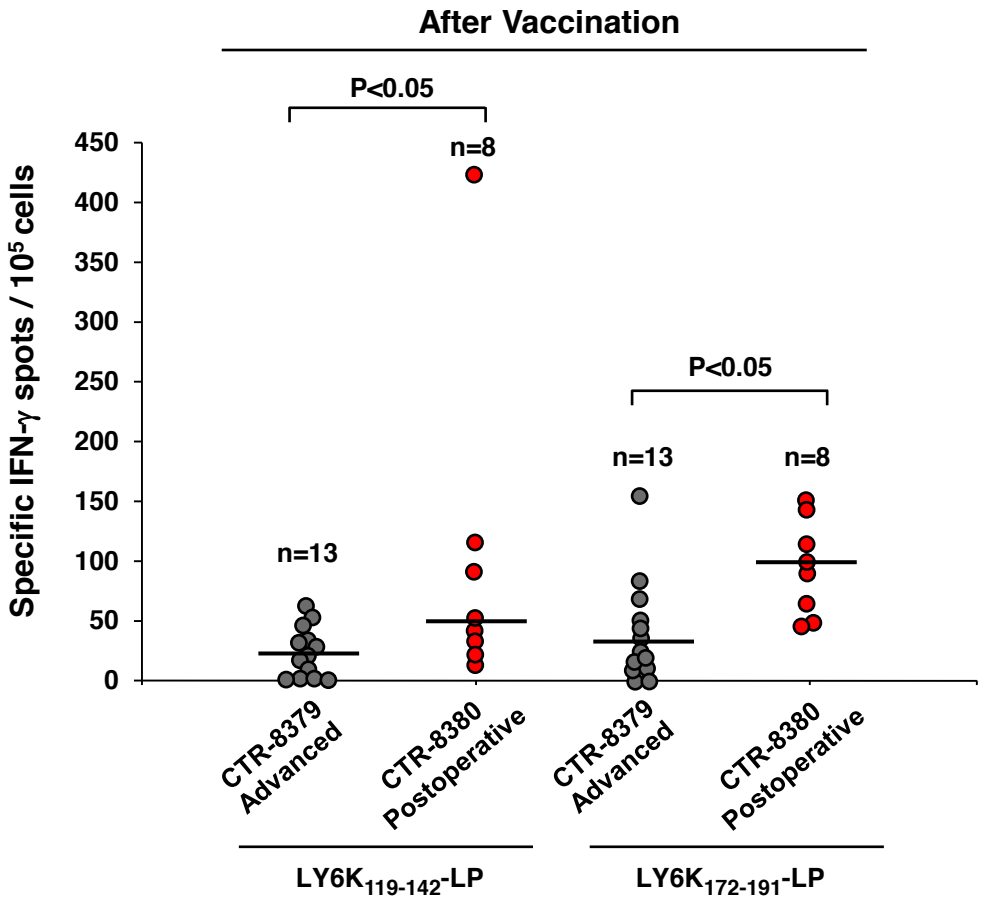
Supplementary figure 2. Cytokine profile produced by $LY6K_{119–142}$-LP-specific bulk Th cells and a $LY6K_{172–191}$-LP-specific Th-clone. **A**, After 24 h incubation of Th cells co-cultured with cognate peptides-pulsed autologous PBMCs, the culture supernatant was collected and the concentration of cytokines (IFN-γ, TNF-α, IL-2, GM-CSF, and MIP1β) was measured using the Bio-Plex assay system. Data are presented as the mean ± SD of triplicate assays. **B**, Detection of CD107a on the cell surface of bulk LY6K-LP-specific $CD4^+$ T-cells after antigenic stimulation. Cells were restimulated with LY6K-LPs or a control peptide. The numbers inside the plots indicate the percentage of the cell population with the quadrant characteristic ($CD4^+$ $CD107a^+$ T-cells).

Supplementary figure 3.



Supplementary figure 3.

D



Supplementary figure 3. Presence of LY6K-LPs-specific Th cells in PBMCs obtained from HNMT patients before and after vaccination. After *in vitro* stimulation of PBMCs with a mixture of $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP for 1 week, the frequency of LY6K-LP-specifc T-cells was detected by IFN-γ ELISPOT assay. **A**, HNMT patients (n=23) showed elevated LY6K-LP-specific Th cell immunity compared to normal healthy individuals (n=9). Bar graph showing proportion of patients and healthy donors (control) responded to $LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP. *p* values were calculated using Fisher's exact probability test. **B**, The frequency of $LY6K_{119–142}$-LP-specific immune response in HNMT patients after vaccination was significantly higher than in healthy donors. Colom graph showing proportion of patients and healthy donors (control) responded to $LY6K_{119–142}$-LP. *p* values were calculated using Fisher's exact probability test. **C**, The repeated CTL-epitope peptide vaccinations augmented or elicited $LY6K_{119–142}$-LP (black bars) and $LY6K_{172–191}$-LP (white bars)-specific Th cell responses. **D**, A comparison of the numbers of LY6K-LP-specific IFN-γ spots between vaccinated HNMT patients with advanced cancer (CTR-8379, Advanced, n = 13) and vaccinated HNMT patients receiving postoperative adjuvant immunotherapy (CTR-8380, Postoperative, n = 8). After 1-week *in vitro* stimulation of PBMCs with a mixture of $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP, the frequency of individual LY6K-LP-specifc Th cells was detected by IFN-γ ELISPOT assay. The results represent specific IFN-γ spots after background subtraction. Each dot represents an individual donor. Horizontal lines denote median values, and *p* values represent statistical results from a nonparametric Mann-Whitney *U* test. n.s., not significant.

Supplementary figure 4
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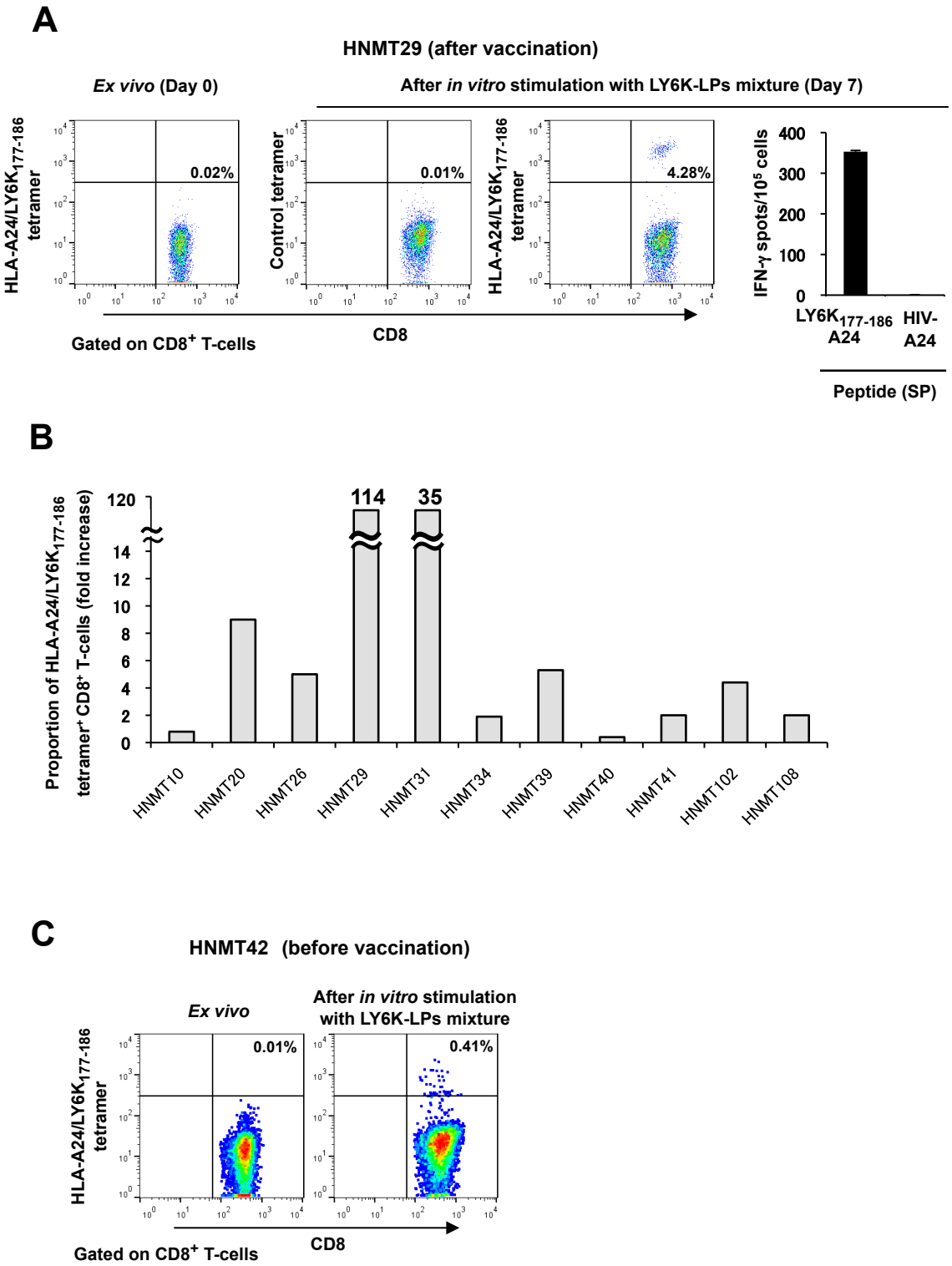
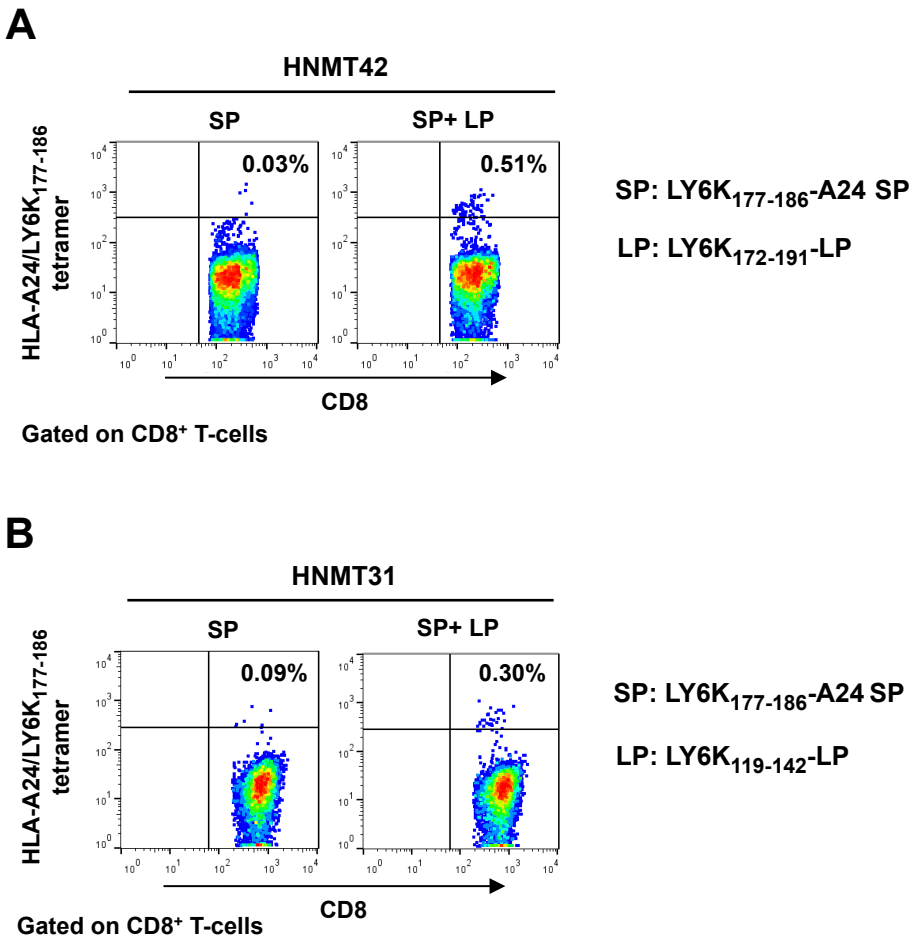


Supplementary figure 4. LY6K-LPs induces *in vitro* expansion of $LY6K_{177–186}$-A24 SP-specific CTLs in HNMT patients. **A**, Fresh PBMCs from the HNMT patient (HNMT29) vaccinated with $LY6K_{177–186}$-A24 SP were cultured with a mixture of $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP. On day 0 (*ex vivo*) and day 7 (after *in vitro* stimulation with LY6K-LPs), the PBMCs were stained with HLA-A*24:02/$LY6K_{177–186}$-specific tetramer (gated on $CD8^+$ T-cells). On day 7, the frequency of $LY6K_{177–186}$-A24-specific CTLs was also detected by IFN-γ ELISPOT assay (bar graph). Representative data from 9 vaccinated HNMT patients (HNMT20, 26, 29, 31, 34, 39, 41, 102, and 108) with similar results are shown. **B**, The increases (fold increase) in proportion of $CD8^+$ $tetramer^+$ cells are shown. **C**, Fresh PBMCs from an HNMT patient before vaccination (HNMT42) were stimulated with a mixture of LY6K-LPs.

Supplementary figure 5



Supplementary figure 5. The synergistic effect of LY6K-LPs on induction of $LY6K_{177–186}$-A24 SP-specific CTLs. Fresh PBMCs obtained from a vaccinated HNMT patients (A, HMNT42; B, HNMT31) were cultured for 7 days with $LY6K_{177–186}$-A24 SP (SP) or SP + LY6K-LP (SP + LP). Representative HLA-A24/$LY6K_{177–186}$-specific tetramer staining (gated on $CD8^+$ T-cells) obtained from duplicate wells with similar results is shown.

Supplementary table 2

***HLA-A*, *-DR*, *and -DP* genotypes of healthy donors**

Donor ID	*HLA-A* genotype	*HLA-DRB1* genotype	*HLA-DPB1* genotype
HD1	*A*02:01 / 24:02*	*DRB1*04:05 / -*	*DPB1*05:01 / -*
HD2	*A*11:01 / 31:01*	*DRB1*08:03 / 15:02*	*DPB1*02:01 / 09:01*
HD3	*A*24:02 / -*	*DRB1*08:02 / 15:02*	*DPB1*05:01 / 09:01*
HD4	*A*24:02 / 31:01*	*DRB1*08:03 / 14:05*	*DPB1*02:02 / 05:01*
HD5	*A*02:01 / 02:06*	*DRB1*04:05 / 09:01*	*DPB1*02:01 / 04:02*
HD6	n.t.	*DRB1*04:06 / 08:03*	*DPB1*02:01 / 04:02*
HD7	*A*26:01 / 33:03*	*DRB1*04:05 / 13:02*	*DPB1*04:01 / 09:01*
HD8	*A*26:01 / -*	*DRB1*04:10 / 08:02*	*DPB1*02:01 / 05:01*
HD9	*A*31:01 / 33:03*	*DRB1*09:01 / 13:02*	*DPB1*03:01 / 04:01*

PBMCs derived from healthy donors (HD1-HD9) were used as controls in Figure 5B.
HLA, human histocompatibility leukocyte antigen; n.t., not tested