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

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Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; GM-CSF/CSF2, colony stimulating factor 2; HD, healthy donor; Her2/EGFR2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; HNMT, head-and-neck malignant tumor ; IFNγ, interferon-γ; IL, interleukin; LAMP1a, lysosomal-associated membrane protein 1a; LP, long peptide; LY6K, lymphocyte antigen 6 complex locus K; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; SP, short peptide; Th, CD4+ helper T; Th1, T helper type-1

Identification of peptides that activate both tumor-specific helper T (Th) cells and cytotoxic T lymphocytes (CTLs) are important for the induction of effective antitumor immune responses. We focused on a long peptide (LP) derived from lymphocyte antigen 6 complex locus K (LY6K) encompassing both candidate Th epitopes and a known CTL epitope. Using IFNγ ELISPOT assays as a marker of activated T cells, we studied the immunogenicity and cross-priming potential of LY6K-LP, assaying human immune cell responses in vitro and immunologic activities in HLA-A24 transgenic mice in vivo. We identified $LY6K_{172–191}$-LP as an effective immunogen spanning naturally processed epitopes recognized by T helper type 1 (Th1) cells and CTLs. LY6K-specific CTLs were induced through cross-presentation of $LY6K_{172–191}$-LP in vitro and in vivo. In addition, $LY6K_{172–191}$-LP enhanced induction of LY6K-specific CTLs among the peripheral blood mononuclear cells (PBMCs) of head-and-neck malignant tumor (HNMT) patients. $LY6K_{172–191}$-LP-specific Th1 immunologic response following 1 week in vitro stimulation of PBMCs with $LY6K_{172–191}$-LP were detected in 16 of 21 HNMT patients (76%) vaccinated with CTL-epitope peptides and 1 of 11 HNMT patients (9%) prior to vaccination, but not in 9 healthy donors. Our results are the first to demonstrate the presence of LY6K-specific Th1 cell responses in HNMT patients and underscore the possible utility of $LY6K_{172–191}$-LP for the induction and propagation of both LY6K-specific Th1 cells and CTLs.

Introduction

We have recently identified a novel cancer-testis antigen, lymphocyte antigen 6 complex locus K (LY6K, synonymous with URLC10), frequently overexpressed in lung cancer, head and neck malignant tumor (HNMT), esophageal carcinomas, bladder cancer, gastric cancer, and various other malignancies.[1] Previous studies have reported that $LY6K_{177–186}$-A24 short peptide (SP) stimulated cytotoxic lymphocytes (CTLs) killed esophageal cancer cells expressing endogenous LY6K antigen.[2] Furthermore,

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$LY6K_{177–186}$-A24 SP-specific CTLs have been detected among tumor-infiltrating lymphocytes, regional lymph node-associated lymphocytes, and PBMCs obtained from HLA-A24 (*A*24:02*)-positive patients with esophageal cancer[3]. Phase I/II clinical trials of $LY6K_{177–186}$-A24 SP immunotherapy for treatment of lung, gastric, and esophageal cancers has yielded promising results and Phase I/II clinical trials assessing $LY6K_{177–186}$-A24 SP vaccines for patients with HNMTs are currently underway.[4-8] In these trials, we observed that vaccination with $LY6K_{177–186}$-A24 SP induced antigen-specific CTL responses and improved progression-free survival among patients with advanced esophageal cancer.[6] Thus, further investigation is warranted to test the premise that LY6K is an attractive target molecule for cancer immunotherapy.

Th cells are necessary elements of cellular immunity, priming tumor-specific CTLs and influencing the differentiation and expansion of tumor antigen specific CTLs essential for generating and maintaining long-lasting CTL responses.[9,10] T helper type 1 (Th1) cells have a direct role in tumor elimination and interferon-γ (IFNγ) secreted by Th1 cells mediates anti-angiogenic effects. Furthermore, Th1 cells pave the way for entry of CTLs at the tumor site.[11] Tumor-specifc Th1 cells also control tumor development and progression via the activation of dendritic cells (DCs), natural killer (NK) cells, and type 1 macrophages.[9] Vaccination-induced tumor-specific Th cells with tumor homing capabilities can deliver inflammatory cytokines such as IFNγ to the local tumor microenvironment. Finally, antigen-specific Th cells can enhance and sustain tumor-specific immunity over time. Therefore, the identification of peptides that can activate both tumor-specific Th1 cells as well as CTLs is important for induction of effective antitumor immunity in tumor-bearing hosts.

DCs are capable of processing exogenous antigens and activating tumor-specific CTL responses via cross-presentation. Melief et al. reported a synthetic long peptide (LP) vaccine encompassing a CTL epitope and possessing immunotherapeutic potential. Specifically, LP-vaccine treated patients' DCs take up the peptide, process it, and present all possible CTL epitopes and Th cell epitopes in the context of various HLA class I and class II molecules.[12,13] Disis et al. reported that vaccination with a CTL-epitope LP derived from human epidermal growth factor receptor 2 (EGFR2, better known as HER2) generated robust and persistent tumor-specific T-cell immunity in patients with metastatic breast cancer.[14] Recent clinical studies using a telomerase-derived LP encompassing CTL-epitopes (GV1001) increased the survival of cancer patients in combination with radio- and chemotherapy.[15] Thus, we proposed that an ideal peptide vaccine for cancer immunotherapy may be optimally composed of a single polypeptide spanning epitopes for both Th cells and CTLs.[12,16-18]

In this study, we sought to identify a LY6K-derived LP with the potential to induce immunologic activity in both Th1 cells and CTLs to further develop peptide-based vaccine immunotherapy. We found that $LY6K_{172–191}$-LP successfully triggered Th1-cell responses in individuals expressing several common HLA allelic variants, including *HLA-DR*, *-DP* or *-DQ* alleles, and that efficient cross-presentation of the $LY6K_{172–191}$-LP induced $LY6K_{177–186}$-A24 SP-specific CTLs. In addition, we also observed $LY6K_{172–191}$-LP enhanced induction of $LY6K_{177–186}$-A24 SP-specific CTLs in PBMCs from healthy donors and HNMT patients.

Results

Identification of immunogenic LY6K-derived LPs encompassing helper T cell epitopes

To identify LPs comprising candidate HLA-class II binding Th cell epitopes of LY6K, we first examined the amino acid sequence of LY6K using a recently developed immune epitope database (IEDB) computer algorithm previously described.[19,20] We focused on the protein regions with multiple Th cell epitope prospects (**Fig. S1A; Table S1**). An $LY6K_{172–191}$-LP predicted by IEDB to be a potent HLA class II-binding peptide, was identified proximal to a known 10-mer CTL-epitope, $LY6K_{177–186}$-A24 SP that is recognized by HLA-A24-restricted CTLs (**Fig. S1B**). Another peptide, $LY6K_{119–142}$-LP, was also predicted to be a potent HLA class II-binding peptide, although it did not include a known CTL-epitope sequence. Therefore, 2 candidate LPs, $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP, both of which were predicted to have a strong binding affinity for HLA-class II molecules (e.g., HLA-DP5, -DR8, -DR9, or -DR15), were synthesized for subsequent analyses.

Purified $CD4^+$ T cells were obtained from the PBMCs of 4 healthy donors by positive selection with magnetic microbeads. These $CD4^+$ T cells were stimulated at weekly intervals with autologous DCs or PBMCs pulsed with either $LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP, as described in Materials and Methods. After at least 3 rounds of stimulation, the LY6K-LP-specific immunologic responses of the corresponding cultured $CD4^+$ T cells were examined by IFNγ ELISPOT assays. The Th cells generated from $HLA\text{-}DP5^+$ HD1 produced a significant amount of IFNγ in response to $LY6K_{119–142}$-LP-pulsed PBMCs in an HLA-DP-dependent manner (**Fig. 1**, left panel). The bulk Th cells also specifically recognized HLA-DP5-expressing L cells (L-DP5) pulsed with $LY6K_{119–142}$-LP in an HLA-DP-dependent manner, but not irrelevant peptide (EBNA)-pulsed L-DP5 cells or $LY6K_{119–142}$-LP-pulsed HLA-DR4-expressing L cells (L-DR4; **Fig. 1A**, right panel). These results indicated that $LY6K_{119–142}$-LP was specifically presented by HLA-DP5. To investigate whether $LY6K_{119–142}$-LP induces responses in Th cells restricted by other HLA class II molecules, $CD4^+$ T-cells from $DR8^+$ and $DR15^+$ healthy donors (HD2 and HD3) were also tested. We confirmed that $LY6K_{119–142}$-LP also stimulates HLA-DR8-restricted Th cells and HLA-DR15-restricted Th cells (**Fig. 1B and C**; **Fig. S1C**). Thus, $LY6K_{119–142}$-LP binds to HLA-DP5, HLA-DR8, and HLA-DR15, suggesting that $LY6K_{119–142}$-LP encompasses several Th cell epitopes presented by high frequency HLA class II molecules.[21,22]

Next, we assessed whether $LY6K_{172–191}$-LP, a peptide spanning a known CTL-epitope, could also generate active Th cells. The Th cells originating from 2 $HLA\text{-}DR15^+$ donors (HD2 and HD3) produced a significant amount of IFNγ in response

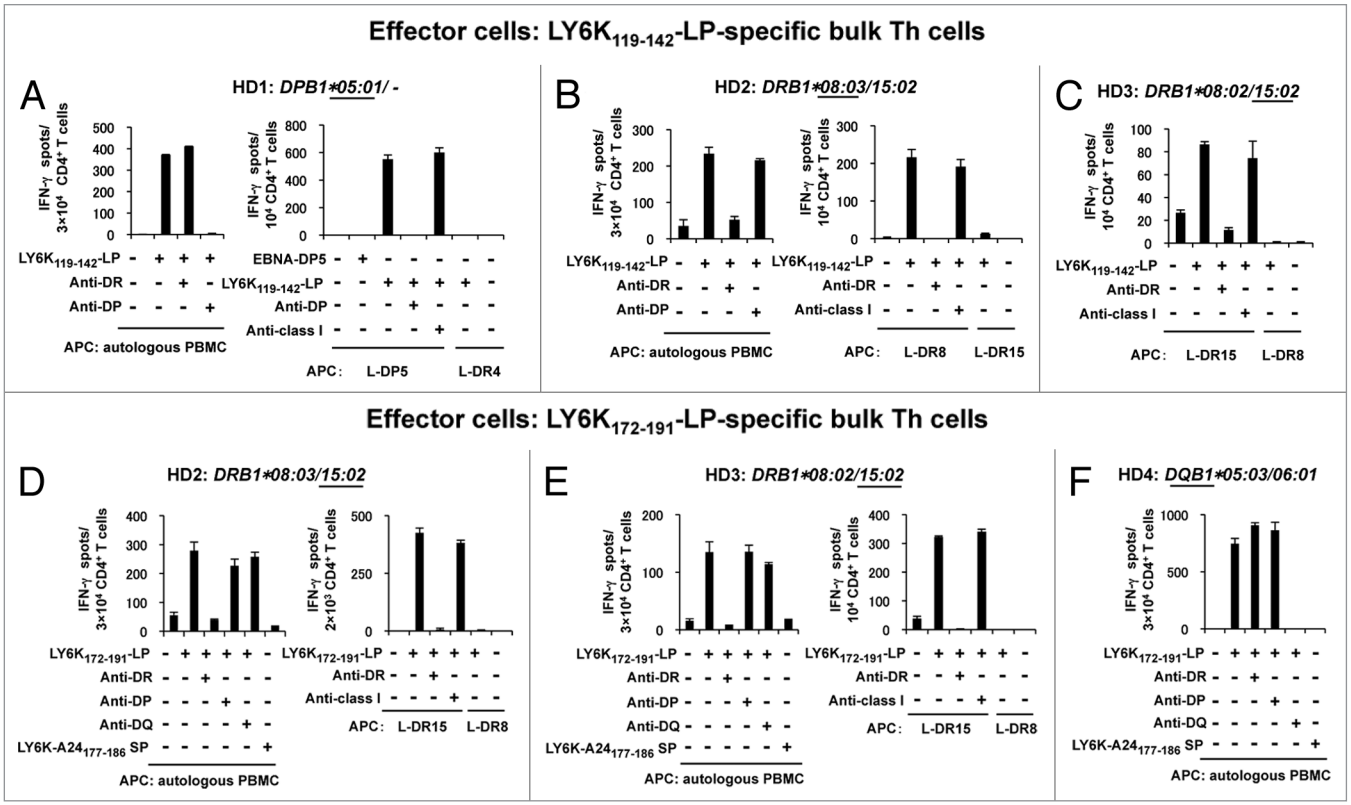

Figure 1. Induction of LY6K-specific helper T cells from healthy donors. (**A–F**) LY6K-specific helper T (Th) cells were generated from healthy donors (HDs) by stimulating purified CD4+ cells with $LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP, as indicated. The number of IFNγ-producing Th cells was analyzed by ELISPOT assay. Data are presented as the mean ± SD of triplicate assays. The HLA class-II genotype is indicated above the panels. The underlined HLA-Class II allele encodes the element presenting the peptides to Th cells. (**A**) Induction of HLA-DP5-restricted $LY6K_{119–142}$-LP-specific Th cells in donor HD1. Data are from at least 3 independent experiments with representative results shown. The Th cells were restimulated with autologous peripheral blood mononuclear cells (PBMCs; left panel) or L-cells (engineered to express the indicated HLA molecule) pulsed with $LY6K_{119–142}$-LP antigen presenting cells (APCs; right panel). An Epstein–Barr virus-derived nuclear antigen (EBNA-DP5 LP) was used as a control LP. B-F. Induction of HLA-restricted $LY6K_{119–142}$-LP **(B and C)** or $LY6K_{172–191}$-LP (**D–F**) specific Th cells, similar to (**A**) (above). (**B**) Induction of HLA-DR8-restricted $LY6K_{119–142}$-LP-specific Th cells in donor HD2. (**C**) Induction of HLA-DR15-restricted $LY6K_{119–142}$-LP-specific Th cells in donor HD3. (**D and E**) HLA-DR15-restricted $LY6K_{172–191}$-LP-specific Th cells were generated from HD2 and HD3. (**F**). HLA-DQ-restricted $LY6K_{172–191}$-LP-specific Th cells were generated from a DR15-negative HD4. LP, long peptide.

to $LY6K_{172–191}$-LP-pulsed PBMCs in an HLA-DR-dependent manner. The bulk Th cells specifically recognized HLA-DR15 expressing L cells (L-DR15) pulsed with $LY6K_{172–191}$-LP in an HLA-DR-dependent manner (**Fig. 1D and E**). $CD4^+$ T-cells from an HLA-DR15-negative donor (HD4) were tested to investigate whether $LY6K_{172–191}$-LP can induce Th cells restricted by other HLA class II molecules. $LY6K_{172–191}$-LP could stimulate HLA-DQ-restricted bulk Th cells originating from this donor (**Fig. 1F**). Indeed, $LY6K_{172–191}$-LP robustly generated Th cells expressing IFNγ, suggesting that $LY6K_{172–191}$-LP also encompasses several Th cell epitopes. In these healthy donors, $LY6K_{177–186}$-A24 SP control failed to stimulate $LY6K_{172–191}$-LP-specific Th cells (**Fig. 1D–F**). Taken together, LY6K-LPs stimulation induced IFNγ-producing Th cells derived from 4 healthy donors.

LY6K-LPs encompass naturally processed helper T cell epitopes

We proceeded to assess whether DCs take up and process the LY6K protein to stimulate LY6K-LP specific Th cells. Autologous DCs loaded with recombinant LY6K protein were prepared and used as antigen-presenting cells (APCs) in IFNγ ELISPOT assays.[23,24] HLA-DP5-restricted $LY6K_{119–142}$-LP-reactive bulk Th cells efficiently recognized DCs loaded with LY6K protein in an HLA-DP-dependent manner, but did not recognize control protein-loaded DCs, indicating that $LY6K_{119–142}$-LP encompasses an epitope naturally processed and presented by HLA-DP5 molecules (**Fig. 2A**). HLA-DR15- and HLA-DQ-restricted $LY6K_{172–191}$-LP-specific Th-clones also recognized autologous DC loaded with LY6K protein, indicating that $LY6K_{172–191}$-LP similarly constitute epitopes naturally processed and presented by HLA-DR15 and HLA-DQ molecules (**Fig. 2B**).

LY6K-LPs stimulate Th1-type $CD4^+$ T cells

In order to further characterize LY6K-LP-reactive Th cells, we next applied the Bio-Plex system to measure the levels of several cytokines released from epitope-specific Th cells in response to stimulation by the cognate peptide. $LY6K_{119–142}$-LP-specific bulk Th cells from HD1 and HD3 stimulated with cognate peptide specifically produced IFNγ, tumor-necrosis factor-α (TNF-α), interleukin-2 (IL-2), colony stimulating factor 2 (CSF2, commonly known as GM-CSF), and chemokine ligand 4 (CCL4, better known as MIP-1β), indicating Th1 polarized secretory

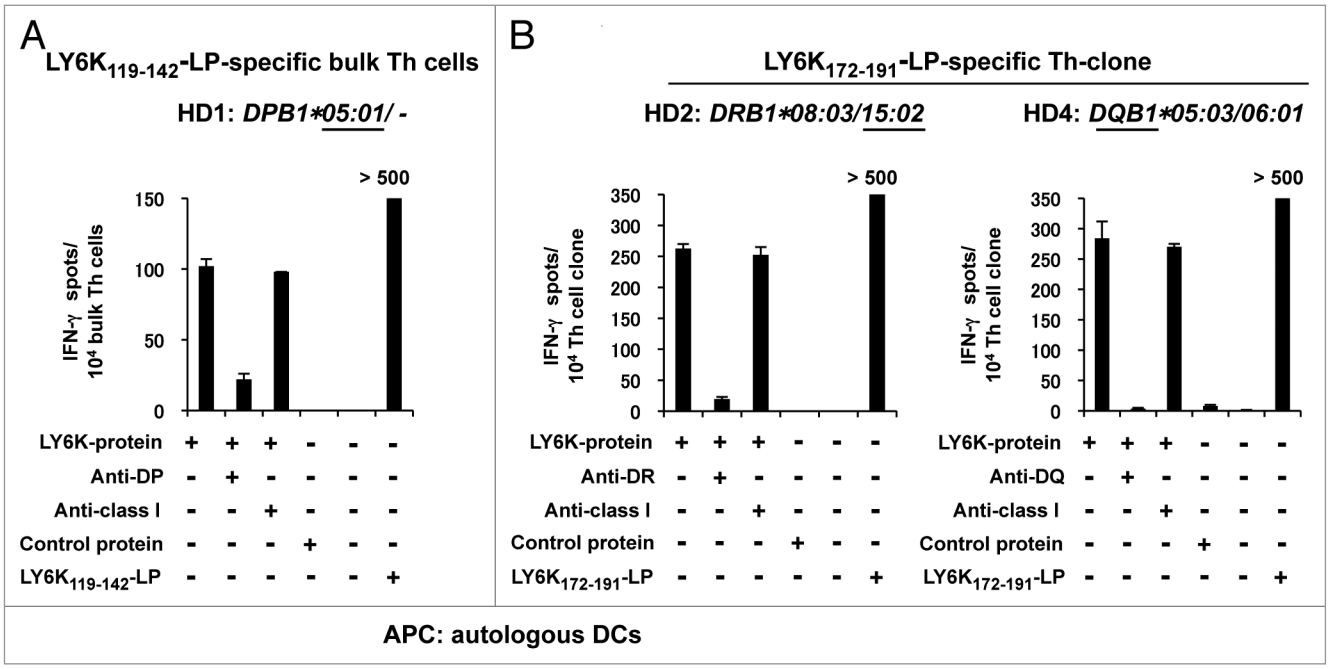

Figure 2. LY6K-LPs encompass naturally processed Th-cell epitopes. (**A and B**). The number of IFNγ-producing healthy donor (HD) derived helper T (Th) cells stimulated by antigen-presenting dendritic cells (DCs) loaded with recombinant full-length lymphocyte antigen 6 complex locus K (LY6K) protein was analyzed by ELISPOT assay. (**A**) HLA-DP5-restricted $LY6K_{119–142}$-LP-specific bulk helper T (Th) cells established from HD1 specifically recognized autologous DCs loaded with recombinant LY6K protein (left panel). Representative data are shown from 3 independent experiments with similar results achieved. (**B**) HLA-DR15 (left) or HLA-DQ (right)-restricted $LY6K_{172–191}$-LP-specific Th-clone (from HD2 and HD4, respectively) recognized autologous DCs loaded with a recombinant LY6K protein. Representative data are shown from 5 independent experiments with similar results achieved. The cognate LY6K-LPs were used as positive controls in the IFNγ ELISPOT assays. APC, antigen-presenting cells; LP, long peptide.

characteristics (**Fig. S2A**, upper panels and data not shown). $LY6K_{172–191}$-LP-specific Th-clone from HD4 produced a large amount of these cytokines after stimulation, suggesting $LY6K_{172–191}$-LP can potently induce multifunctional Th1 cells (**Fig. S2A**, lower panels). Similar results were obtained from $LY6K_{172–191}$-LP-specific Th cells established from HD3 (data not shown). The lysosomal-associated membrane protein 1a (LAMP1a, also known as CD107a) was detected on the $LY6K_{119–142}$-LP-specific bulk Th1 cells (HD1, HD2 and HD3) and $LY6K_{172–191}$-LP-specific bulk Th1 cells (HD2, HD3, and HD4) stimulated with cognate peptides (**Fig. S2B** and data not shown), as has been previously demonstrated for antiviral $CD4^+$ effectors and tumor-infiltrating lymphocytes.[25-28]

Presence of LY6K-specific Th1 cells in HNMT patients before and after vaccination with $LY6K_{177–186}$-A24 SP

We next assessed LY6K-LPs-specific Th1 cell responses in the peripheral blood of 23 HNMT patients enrolled in 2 CTL-epitope peptide-vaccine trials. LY6K-reactive Th1 cells were derived from patients pre- and post-$LY6K_{177–186}$-A24 SP vaccination and characterized by their production of IFNγ upon stimulation with LY6K-LP variants in vitro.[9] The donor clinical characteristics and T-cell responses are summarized in **Table 1**. After 1 wk of stimulating patient-derived PBMCs with LY6K-LPs in vitro, the frequency of individual LY6K-LP-specifc Th1 cells among blood lymphocytes was detected by IFNγ ELISPOT assay (**Fig. 3A**). PBMCs from 9 healthy donors were used as control. Responses were considered positive when the number of IFNγ-secreting cells was at least 2-fold above the negative control. LY6K-specific Th1 cell responses were observed in $LY6K_{177–186}$-A24 SP vaccinated HNMT patients. As summarized in **Table 1**, specifically, 10 of 23 (44%) of patients were reactive to $LY6K_{119–142}$-LP, and 16 of 23 (70%) of patients responded to $LY6K_{172–191}$-LP but no specific immune responses to LY6K-LPs were detected among the 9 healthy donors (**Fig. S3A**; **Table 1**). We also found that the number of specific IFNγ-spots per 10^5 CTL-epitope vaccinated patient-derived PBMCs immunoreactive against $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP were significantly larger than in PBMCs from healthy donors (**Fig. 3B**; $P < 0.001$). Although the increase of $LY6K_{119–142}$-LP-specific spots from vaccinated patient PBMCs was not significant compared with those derived from HNMT patients before vaccination, the mean number of $LY6K_{172–191}$-LP-specific IFNγ spots in vaccinated patient PBMCs was significantly larger than that of HNMT patients before vaccination (**Fig. 3B**: $P < 0.05$). The proportion of donors responding to $LY6K_{172–191}$-LP in HNMT patients after vaccination was also significantly higher than in HNMT patients before vaccination ($P < 0.05$; **Fig. 3C**; **Fig. S3B**). Interestingly, specific responses to LY6K-LPs were elicited or augmented by repeated vaccinations (**Fig. S3C**). In comparing the numbers of LY6K-LP-specific IFNγ spots in vaccinated HNMT patients with advanced cancer (CTR-8379, n = 13) and vaccinated HNMT patients receiving postoperative adjuvant immunotherapy (CTR-8380, n = 7), the number of LY6K-LP-specific IFNγ spots in those patients receiving postoperative adjuvant immunotherapy was significantly higher than in vaccinated

Table 1. Clinical characteristics of HNMT patients in LY6K-epitope immunotherapy trials

		LY6K-LP-specific T-cell responsesa				No. of vaccinations			
Patient ID	Age/Sex	LY6K119–142-LP		LY6K172–191-LP			Histologic subtype	HLA-DRB1	HLA-DPB1
CTR-8379 + CTR-8380									
Positive/ Total (%)		10/23 (44%)		16/23 (70%)					
CTR-8379		Pre-vac.	Post-vac.	Pre-vac.	Post-vac.				
Positive / Total (%)		2/7 (29%)	4/13 (31%)	1/7 (14%)	8/13 (62%)				
HNMT10	61/M	n.t.	-	n.t.	-	60	Squamous Cell Carcinoma	01:01 / 04:05	05:01 / -
HNMT20	57/F	n.t.	+	n.t.	-	32	Squamous Cell Carcinoma	01:01 / 09:01	02:01 / 05:01
HNMT26	70/M	n.t.	-	n.t.	+	24	Basaloid Squamous Cell Carcinoma	04:05 / 15:02	05:01 / 09:01
HNMT29	64/F	n.t.	-	n.t.	+	16	Squamous Cell Carcinoma	09:01 / 14:54	03:01 / 05:01
HNMT31	69/F	n.t.	+	n.t.	+	16	Adenoid Cystic Carcinoma	01:01 / 11:01	02:01 / 04:02
HNMT34	65/M	n.t.	-	n.t.	+	12	Squamous Cell Carcinoma	08:03 / 15:02	02:01 / 05:01
HNMT35	85/F	n.t.	-	n.t.	-	8	Squamous Cell Carcinoma	04:05 / 08:02	05:01 / -
HNMT39	77/M	-	-	-	+	8	Adenoid Cystic Carcinoma	04:06 / 14:54	05:01 / 19:01
HNMT40	76/M	-	-	-	-	4	Squamous Cell Carcinoma	01:01 / 09:01	04:02 / 05:01
HNMT41	51/F	+	+	+	+	4	Adenoid Cystic Carcinoma	01:01 / 04:05	04:02 / 05:01
HNMT42	36/F	+	+	-	+	4	Unknown	01:01 / 08:02	04:02 / 05:01
HNMT43	50/M	-	-	-	+	4	Squamous Cell Carcinoma	08:02 / 09:01	05:01 / -
HNMT44	55/M	-	n.t.	-	n.t.	0	Malignant Melanoma	01:01 / 13:02	04:01 / 09:01
HNMT45	82/M	-	-	-	-	8	Squamous Cell Carcinoma	04:03 / 14:03	05:01 / -
CTR-8380		Pre-vac.	Post-vac.	Pre-vac.	Post-vac.				
Positive / Total (%)		0/4 (0%)	6/8 (75%)	0/4 (0%)	8/8 (100%)				
HNMT102	80/F	n.t.	+	n.t.	+	33	Squamous Cell Carcinoma	15:02 / -	02:01 / 09:01
HNMT103	78/F	n.t.	+	n.t.	+	20	Mutinous Adenocarcinoma	04:05 / 15:01	02:01 / 05:01
HNMT105	65/M	n.t.	+	n.t.	+	20	Angiosarcoma	04:05 / 13:02	03:01 / 04:01
HNMT107	20/M	n.t.	+	n.t.	+	20	Osteosarcoma	09:01 / -	02:01 / 02:02
HNMT108	41/M	n.t.	-	n.t.	+	17	Osteosarcoma	04:05 / 09:01	05:01 / -
HNMT109	60/F	-	-	-	+	4	Squamous Cell Carcinoma	04:03 / 04:05	02:01 / 05:01
HNMT110	72/F	-	+	-	+	8	Verrucous Carcinoma	04:10 / 15:02	03:01 / 09:01
HNMT111	70/F	-	+	-	+	4	Squamous Cell Carcinoma	09:01 / 15:02	05:01 / -
HNMT112	54/M	-	n.t.	-	n.t.	0	Squamous Cell Carcinoma	08:02 / 15:01	02:01 / 04:01

Summary of the immune responses of helper T (Th) cells reactive to long-peptide (LP) derived from lymphocyte antigen 6 complex locus K (LY6K) from 23 patients enrolled in 2 CTL-epitope peptide-vaccine clinical trials. aLY6K-LP-specific T-cell responses were measured by IFNγ ELISPOT assay. In brief, patient-derived peripheral blood mononuclear cells (PMBCs) were stimulated with the indicated LY6K-LP. Responses were scored as positive when both the mean number of IFNγ spots exceeded 15 and was greater than 2-fold over background. Positive and negative responses are denoted by (+) and (–), respectively. No., Number; CTR, Clinical Trials Registry; vac., vaccination; HNMT, Head and neck malignant tumor; M/F, male/female; LP, long peptide; n.t., not tested

HNMT patients with advanced cancer (**Fig. S3D**). In assessments of HLA-subtype restriction, LY6K-LP-specific IFNγ production by Th1 cells derived from 7 HNMT patients was significantly inhibited by addition of the anti-HLA-Class II mAbs, but not by the anti-HLA-Class I mAb (**Fig. 3D**). *HLA-Class II* alleles of $LY6K_{119–142}$-LP-specific HLA-DR-restricted Th1 cells derived from 2 HNMT patients were negative for HLA-DR8 and -DR15 (**Fig. 3D**; $LY6K_{119–142}$-LP; HNMT31 and 107). *HLA-Class II* alleles of $LY6K_{172–191}$-LP-specific HLA-DR-restricted Th1 cells in 5 HNMT patients were negative for HLA-DR15 (**Fig. 3D**; $LY6K_{172–191}$-LP; HNMT31, 41, 42, 107, and 108). These results suggest that $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP can be recognized by Th1 cells restricted by other HLA-Class II molecules not previously identified by the in vitro experiments assaying lymphocytes from healthy donors. Taken together, these observations suggest that HNMT patients are able to mount LY6K-specific Th1 cell responses and

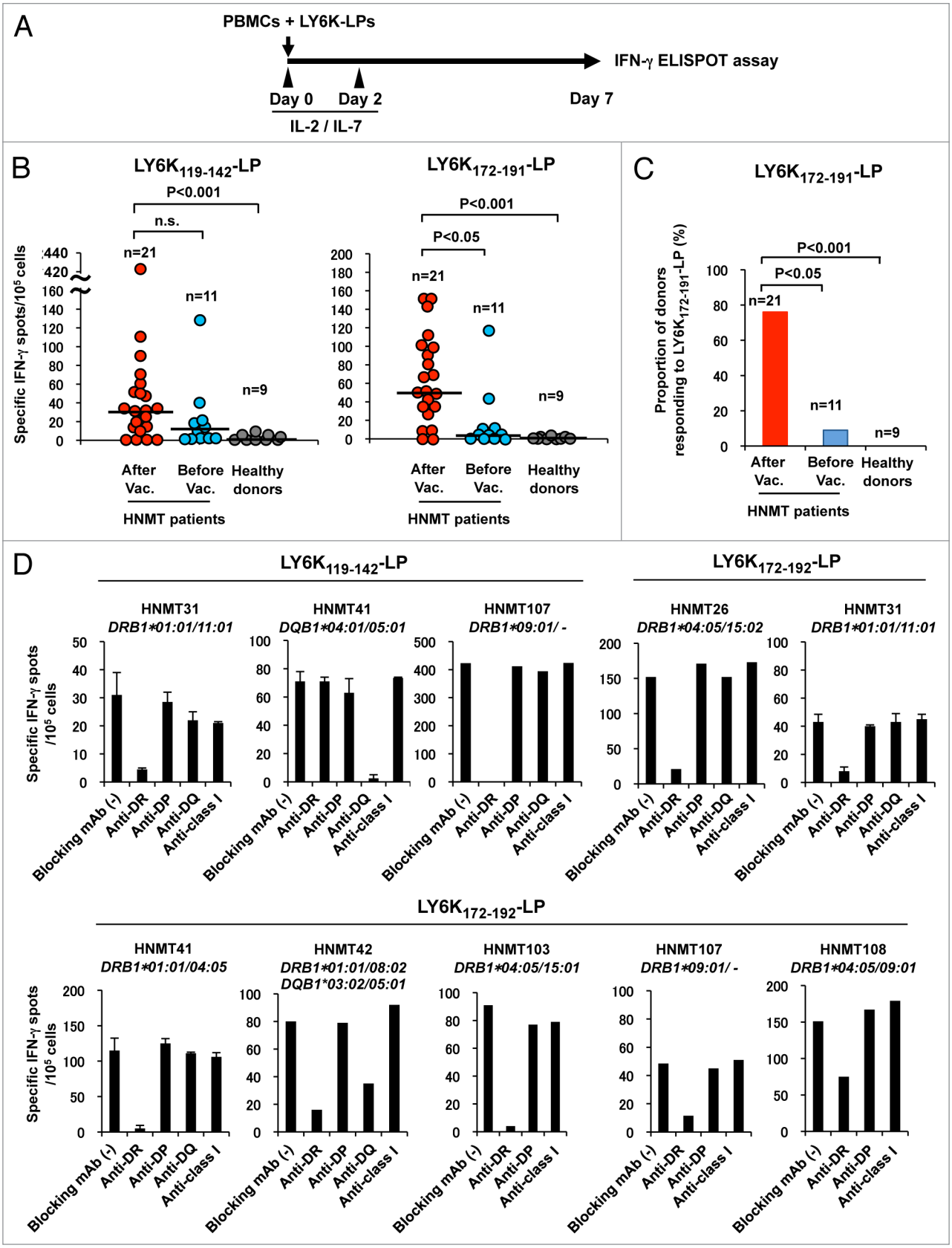

Figure 3. Presence of LY6K-LPs-specific Th1 cells in the peripheral blood of LY6K$_{177-186}$-A24 SP vaccinated HNMT patients. (**A–D**). Fresh peripheral blood mononuclear cells (PBMCs) derived from patients vaccinated with LY6K$_{177-186}$-A24 SP (refer to Table 1) were stimulated with a mixture of LY6K$_{119-142}$-LP and LY6K$_{172-191}$-LP plus IL-2 and IL-7 in vitro. After 1 wk, the frequency of LY6K-LP-specific T cells was detected by **(Continued on following page)**

Figure 3. (Continued from previous page) IFNγ ELISPOT assay. (**B and C**) LY6K-LP-specific-Th1 cell responses were assessed in 23 HNMT patients. Samples were tested from 21 HNMT patients vaccinated with $LY6K_{177–186}$-A24 SP (After Vac.), 11 HNMT patients prior to vaccination (Before Vac.), and 9 healthy donors as controls. (**B**) Th-cell responses to the indicated LY6K-LP as IFNγ spots per 10^5 cells detected 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. (**C**) Column graph showing proportion of patients and healthy donors responding to $LY6K_{172–191}$-LP (from **B**) prior to and after vaccination. *P* values were calculated using Fisher's exact probability test. (**D**) HLA class II-restriction of LY6K-LP-specific Th1 cells. Fresh PBMCs stimulated with LY6K-LPs for 1 wk were re-stimulated with each LY6K-LP ($LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP, as indicated) in the presence (or absence, first column) of monoclonal antibodies (mAbs) specific to HLA-DR, -DP, -DQ class II or HLA-Class I. SP, short peptide; LP, long peptide.

LY6K-LP based immunotherapies may be efficacious in the majority of patients.

Stimulation of $LY6K_{177–186}$-A24 SP-specific CTLs through cross-presentation of $LY6K_{172–191}$-LP in vitro

Next, we tested whether $LY6K_{172–191}$-LP-pulsed DCs could stimulate $LY6K_{177–186}$-A24 SP-specific bulk CTLs via cross-presentation of $LY6K_{172–191}$-LP.

IFNγ production by $LY6K_{177–186}$-A24 SP-specific CTLs was measured by intracellular labeling. Fixed DCs, unable to cross present but able to present $LY6K_{177–186}$-A24 SP as efficiently as live DCs (**Fig. 4A**), were used to exclude or evaluate the contribution of exogenous presentation of LP degradation products in the T-cell response. $LY6K_{172–191}$-LP induced a significant proportion of IFNγ secreting tetramer$^+$ (i.e., HLA-A24 (*A*24:02*)/$LY6K_{177–186}$) $CD8^+$ T-cells only when they when immunogens were cross presented by unfixed DCs (DC + LP). $LY6K_{172–191}$-LP-pulsed fixed DCs could not stimulate $LY6K_{177–186}$-A24 SP-specific CTLs, similar to control LP-pulsed unfixed DCs (Fixed DC + LP and DC + control LP).

We next investigated whether $LY6K_{172–191}$-LP-pulsed autologous DCs could induce expansion of $LY6K_{177–186}$-A24 SP-specific CTLs. $LY6K_{177–186}$-A24 SP-specific bulk CTLs generated from ex vivo SP-stimulated purified $CD8^+$ T cells derived from HLA-$A24^+$ HD3 were restimulated with $LY6K_{172–191}$-LP-pulsed autologous DCs. As shown in **Figure 4B**, the population of HLA-A24/$LY6K_{177–186}$-specific tetramer$^+$ $CD8^+$ cells was expanded by stimulation with $LY6K_{172–191}$-LP-pulsed DCs on day 7, but decreased when the $LY6K_{177–186}$-A24 SP-specific bulk CTLs were stimulated with control-LP-pulsed DCs. These results suggest that the expansion of $LY6K_{177–186}$-A24 SP-specific CTLs was induced by DC cross-presentation of $LY6K_{172–191}$-LP.

$LY6K_{172–191}$-LP induce efficient expansion of $LY6K_{177–186}$-A24 SP-specific CTLs derived from vaccinated HNMT patients

We next assessed whether $LY6K_{172–191}$-LP could induce in vitro expansion of $LY6K_{177–186}$-A24 SP-specific CTLs among PBMCs obtained from 5 HNMT patients vaccinated with $LY6K_{177–186}$-A24 SP. HNMT patient PBMCs were cultured with 10 μg/mL $LY6K_{172–191}$-LP to which 20 IU/mL IL-2 and 5 ng/mL IL-7 were added on days 0 and 2. On days 0 (ex vivo) and 7, PBMCs were stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer. As shown in **Figure 4C**, when PBMCs isolated from HNMT108 were stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer before in vitro culture (ex vivo), the frequency of tetramer$^+$ cells was only 0.05% of $CD8^+$ T-cells. The tetramer$^+$ cells were markedly expanded by 1-wk in vitro stimulation of PBMCs with $LY6K_{172–191}$-LP (without adding $LY6K_{177–186}$-A24 SP) with the frequency of $LY6K_{177–186}$-A24 SP-specific CTLs increasing to 0.35% of $CD8^+$ T-cells. LY6K-specific IFN-γ production by CTLs was also assayed upon stimulation with $LY6K_{177–186}$-A24 SP on day 7 (**Fig. 4C**, bar graph). The increase in the proportion of tetramer$^+$ $CD8^+$ cells was more than 2-fold that of control peptide treated cells in 4 of 5 HNMT patients (**Fig. 4D**; HNMT43, 105, 108, and 110). These results suggest that $LY6K_{177–186}$-A24 SP-specific CTLs might be augmented by DC cross-presentation of $LY6K_{172–191}$-LP without direct stimulation by $LY6K_{177–186}$-A24 SP in HNMT patients. Similar results were obtained from 9 of 11 HNMT patients vaccinated with $LY6K_{177–186}$-A24 SP when the PBMCs were cultured with a mixture of $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP (**Fig. S4A and B**). Interestingly, HLA-A24/$LY6K_{177–186}$-specific tetramer$^+$ cells detected in the peripheral blood of HNMT42 before $LY6K_{177–186}$-A24 SP vaccination were also significantly expanded by stimulation with a mixture of $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP (**Fig. S4C**).

Cross-presentation of $LY6K_{172–191}$-LP efficiently primes LY6K-specific $CD8^+$ T cells in vitro and in vivo

Subsequently, we examined the capacity of $LY6K_{172–191}$-LP to prime naïve $LY6K_{177–186}$-A24 SP-specific human CTLs by tetramer labeling. HLA-A24-restricted $LY6K_{177–186}$-A24 SP-specific CTLs were significantly induced by culturing PBMCs from 3 HLA-$A24^+$ healthy donors (HD1, HD3, HD4) with $LY6K_{172–191}$-LP for 2 wk (**Fig. 4E** and data not shown).

Next, humanized transgenic mice expressing HLA-A24 were immunized 3 times with $LY6K_{172–191}$-LP. The capacity of $LY6K_{172–191}$-LP to prime $LY6K_{177–186}$-A24 SP-specific murine CTLs was then examined by an ex vivo IFNγ ELISPOT assay. The $CD8^+$ T-cells isolated from $LY6K_{172–191}$-LP-vaccinated HLA-A24 mice produced IFNγ in response to stimulation with bone marrow-derived DCs pulsed with the $LY6K_{177–186}$-A24 SP (**Fig. 4F**). These results demonstrate that following uptake $LY6K_{172–191}$-LP, APCs can cross-prime $LY6K_{177–186}$-A24 SP-specific CTLs in vitro and in vivo.

Enhanced induction of $LY6K_{177–186}$-A24 SP-specific CTLs by LY6K-LP-specific Th cells

We next sought to investigate whether LY6K-LP stimulated Th cells could enhance induction of $LY6K_{177–186}$-A24 SP-specific CTLs. LY6K-LP-specific bulk $CD4^+$ T cells and $LY6K_{177–186}$-A24 SP-specific bulk $CD8^+$ T cells derived from HD3 were co-cultured with autologous DCs in the presence of $LY6K_{177–186}$-A24 SP, $LY6K_{177–186}$-A24 SP + control LP, or $LY6K_{177–186}$-A24 SP + LY6K-LP. After 1-wk in vitro culture with peptides, the cells were stained with HLA-A24/$LY6K_{177–186}$-specific tetramer. As shown in **Figure 5A**, the addition of $LY6K_{177–186}$-A24 SP + $LY6K_{119–142}$-LP or $LY6K_{177–186}$-A24 SP + $LY6K_{172–191}$-LP significantly increased the absolute number of $LY6K_{177–186}$-A24 SP-specific $CD8^+$ T cells compared with the addition of $LY6K_{177–186}$-A24 SP alone or $LY6K_{177–186}$-A24 SP + control LP. We also observed that the activated

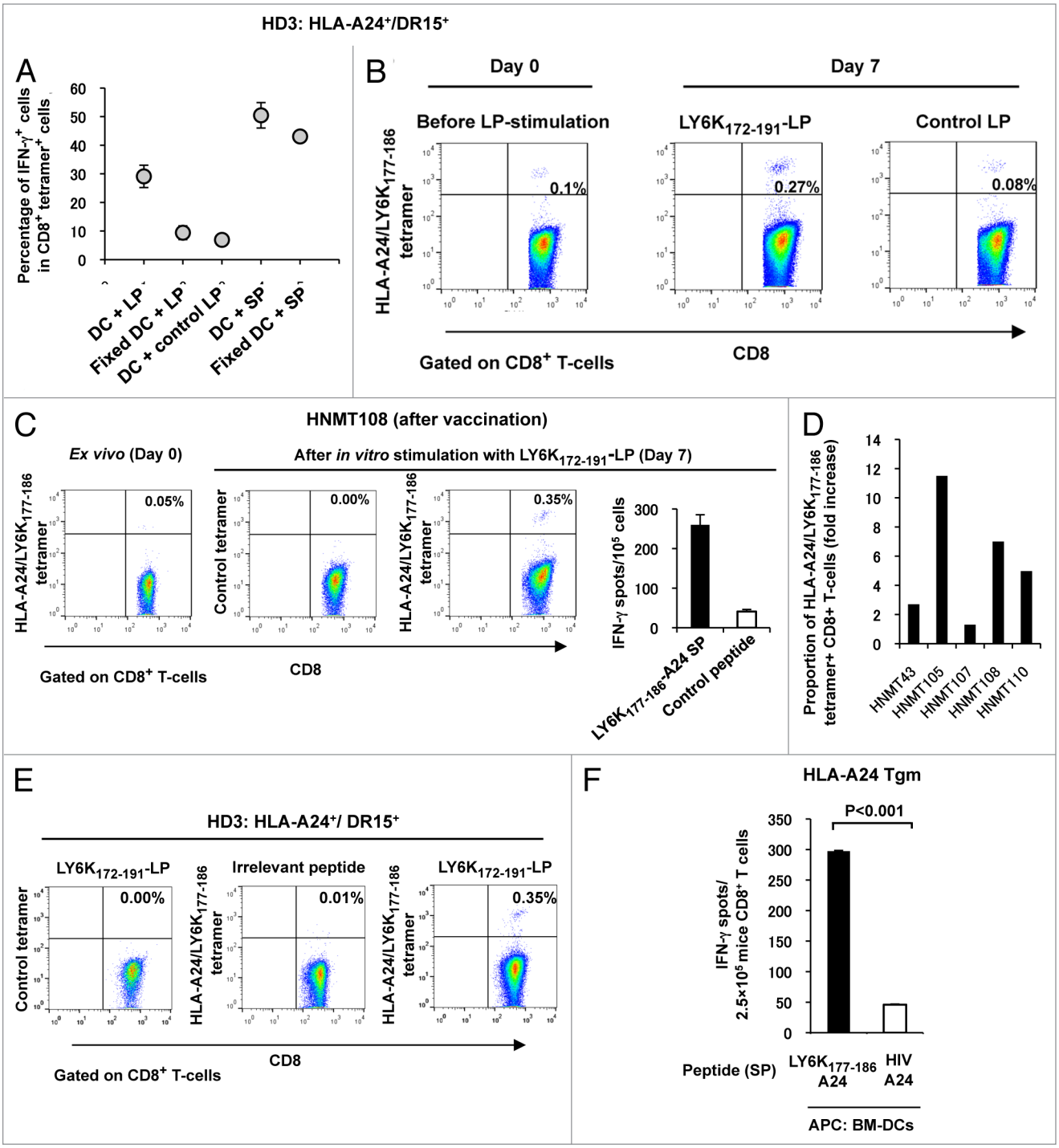

Figure 4. LY6K$_{172-191}$-LP induces efficient expansion of LY6K$_{177-186}$-A24 SP-specific CD8+ T-cells in vitro and in vivo. (**A and B**) Autologous dendritic cells (DCs) uptake and cross-present LY6K$_{172-191}$-LP to LY6K$_{177-186}$-A24 SP-specific CD8+ cytotoxic T lymphocytes (CTLs) derived from healthy donor (HD3). (**A**) Unfixed or fixed DCs were pulsed for 3 h with LY6K$_{172-191}$-LP or LY6K$_{177-186}$-A24 SP. Bulk HD3-derived LY6K$_{177-186}$-A24 SP-specific CTLs were co-cultured with the pulsed DCs for 6 h and responses were measured by intracellular IFNγ staining and flow cytometry. Representative data from 3 independent experiments with similar results are shown. (**B**) HD3-derived LY6K$_{177-186}$-A24 SP-specific bulk CTLs were stimulated with LY6K$_{172-191}$-LP (middle panel) or irrelevant LP (right panel)-pulsed autologous DCs in vitro. The peptide treated cells were stained with a HLA-A24/LY6K$_{177-186}$-specific tetramer in combination with an anti-CD8 mAb at the indicated time points. A pre-treatment (d0) control is shown in the left panel. Stained cells were analyzed by flow cytometry gating on CD8+ T cells. Representative data are from 3 independent experiments with similar results achieved. (**C and D**) LY6K$_{172-191}$-LP expands LY6K$_{177-186}$-A24 SP-specific CTLs derived from vaccinated HNMT patients. Fresh PBMCs from the HNMT patient (HNMT108) vaccinated with LY6K$_{177-186}$-A24 SP were cultured with LY6K$_{172-191}$-LP. On day 0 (ex vivo) and day 7, the PBMCs were stained with a HLA-A24/LY6K$_{177-186}$-specific tetramer or control tetramer and analyzed by flow cytometry gating on CD8+ T cells. (**C**) Dot plot (left) of tetramer+ CD8+ T cells . On day 7, the frequency of IFNγ-expressing LY6K$_{177-186}$-A24 SP-specific CTLs was also detected by ELISPOT assay (bar graph). Representative data from 4 vaccinated HNMT patients (refer to Table 1, HNMT43, 105, 108, and 110) with similar results achieved. (**D**) The increases (fold increase) in proportion of **(Continued on following page)**

Figure 4. (Continued from previous page) CD8+ tetramer+ cells (from **C**) are shown. E. $LY6K_{172–191}$-LP cross-priming of CTLs in vitro. PBMCs obtained from HD3 were incubated with $LY6K_{172–191}$-LP for 2 wk and $LY6K_{172–191}$-LP was added on d0 and d7. Cells were harvested d14 and stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer. Representative data are from 3 independent experiments (both technical and biological replicates) with similar results achieved. (**F**) Induction of $LY6K_{177–186}$-A24 SP-specific CTLs in mice immunized with $LY6K_{172–191}$-LP. HLA-A24-expressing transgenic mice were immunized with $LY6K_{172–191}$-LP. After the third vaccination with $LY6K_{172–191}$-LP, murine CD8+ T-cells in the inguinal lymph nodes were stimulated with bone marrow-derived DCs pulsed with $LY6K_{177–186}$-A24 SP. The number of IFNγ-producing murine CD8+ T-cells was analyzed by ELISPOT. Representative data are from 8 independent experiments with similar results achieved. HNMT, head and neck malignant tumor; LP, long peptide; SP, short peptide.

$LY6K_{172–191}$-LP-specific Th cells enhanced induction of HLA-A24/$LY6K_{177–186}$-specific tetramer+ T cells derived from HLA-A24+ HD4 (data not shown).

Next, we assessed the synergistic effect of LY6K-LP on induction of $LY6K_{177–186}$-A24 SP-specific CTLs among PBMCs from HNMT patients (HNMT31, 42, and 43). PBMCs collected from HNMT43 2 mo after immunization were cultured for 7 d with $LY6K_{177–186}$-A24 SP alone, $LY6K_{177–186}$-A24 SP + control LP, $LY6K_{177–186}$-A24 SP + $LY6K_{119–142}$-LP, or $LY6K_{177–186}$-A24 SP + $LY6K_{172–191}$-LP. After 1-wk in vitro culture with peptides, the cells were stained with HLA-A24/$LY6K_{177–186}$-specific tetramer. As shown in **Figure 5B and C**, when the patient-derived PBMCs were cultured with $LY6K_{177–186}$-A24 SP + $LY6K_{172–191}$-LP, the absolute number of $LY6K_{177–186}$-A24 SP-specific CD8+ T cells was significantly increased in comparison with those cultured with $LY6K_{177–186}$-A24 SP alone or $LY6K_{177–186}$-A24 SP + control LP. On the contrary, there was no synergistic effect of $LY6K_{119–142}$-LP on induction of $LY6K_{177–186}$-A24 SP-specific CTLs obtained from HNMT43 (**Fig. 5C**). Similar results were obtained when patient CTLs were assessed at 1 mo after immunization (data not shown). This result was reproducible in that similar results were obtained using CTLs derived from HNMT31 and HNMT42 showing that either $LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP could enhance CTL responses to $LY6K_{177–186}$-A24 SP (**Fig. S5**). These results suggest that LY6K-LPs can synergistically induce $LY6K_{177–186}$-A24 SP-specific CTLs.

Discussion

We identified 2 LY6K-LPs, specifically $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP, constituting Th epitopes. A known CTL-epitope, $LY6K_{177–186}$-A24 SP was embedded within $LY6K_{172–191}$-LP. Although not shown, we did test 2 other LY6K-LPs, $LY6K_{58–72}$-LP and $LY6K_{206–223}$-LP, that were predicted to have strong binding affinity to multiple HLA-class II molecules. However, we could not induce LP-specific Th cells in peripheral blood obtained from healthy donors.

In this study, we determined that $LY6K_{177–186}$-A24 SP-specific CTLs can be induced by cross-presentation of $LY6K_{172–191}$-LP in vitro and in vivo. We also demonstrated the presence of LY6K-LPs-specific Th1 responses HNMT patients, whereas there was no response among PBMCs derived from healthy donors after 1-wk in vitro stimulation with LY6K-LPs. This is the first report of the presence of LY6K-specific Th responses in patients with HNMT.

Recent studies evaluated the CTL repertoire of melanoma patients exhibiting complete regression of cutaneous metastases after melanoma antigen A3 (MAGE-A3) vaccination with canary pox viral vector combined with peptide.[29-31] After immunization, a new wave of antigen-specific CTL clones arose in the peripheral blood, providing solid evidence that the phenomenon of epitope spreading is critical to the development of effective anticancer immunity elicited by peptide vaccines. These results further implicate functional interactions between vaccine-induced CTLs and malignant cells that facilitate the induction of large numbers of tumor-specific CTLs, cytocidal effector immune cells that subsequently proceed to destroy tumor cells. Interestingly, the frequency of $LY6K_{172–191}$-LP-specific Th cell responses and the number of IFNγ-producing $LY6K_{172–191}$-LP-specific Th1 cells in HNMT patients after CTL-epitope vaccination were significantly larger than those detected in patients prior to vaccination. LY6K-LPs-specific responses in some HNMT patients were augmented or elicited by repeated $LY6K_{177–186}$-A24 SP vaccination. We speculate that LY6K-LP-specific Th responses occurring in $LY6K_{177–186}$-A24 SP vaccinated HNMT patients may be a phenomenon of intramolecular epitope spreading triggered by CTL-epitope vaccination.

Disis et al. reported that vaccination with a herceptin-2 (HER-2/neu)-derived LP encompassing an HLA-A2-restricted CTL-epitope elicited the embedded CTL-epitope specific CD8+ T cells in cancer patients.[14] They showed that tumor-specific CTLs can be elicited in vivo via cross-presentation of HER-2/neu-derived LP. Such T-cell responses are considered to be crucial for tumor eradication and generating long-term memory.[32] With this premise in mind, we identified an immunogenic $LY6K_{172–191}$-LP that encompasses both Th epitopes and CTL-epitope and demonstrated that cross-presentation of $LY6K_{172–191}$-LP induced priming and expansion of LY6K-specific CTLs in vitro and in vivo. Nevertheless, it remains possible that $LY6K_{177–186}$-A24 SP was produced from degradation of $LY6K_{172–191}$-LP and the degraded product may subsequently stimulate LY6K-specific CTLs. Vaccination with $LY6K_{172–191}$-LP may, therefore, potentially elicit combined Th and CTL responses. A recent clinical trial showed that targeting Th cells with DCs pulsed with both HLA class I and II-restricted epitopes effectively enhanced vaccine-specific immune responses and improved clinical outcome.[33] LY6K-LPs bolstered the induction of $LY6K_{177–186}$-A24 SP-specific CTLs derived from both healthy donors and HNMT patients in vitro. Thus, LY6K-LPs administered in combination with $LY6K_{177–186}$-A24 SP immunotherapy may be able to augment the elicitation of antigen-specific CTLs. The checkpoint blockade induced by specific immunoregulatory antibodies such as those targeting cytotoxic T lymphocyte associated protein-4 (CTLA-4), programmed cell death 1 (PD-1), or its ligand PD-L1, applied in combination with therapeutic vaccines have been previously illustrated to act synergistically.[34] The checkpoint blockade may be a good candidate for future combinatorial therapy with LY6K-derived LPs vaccine.

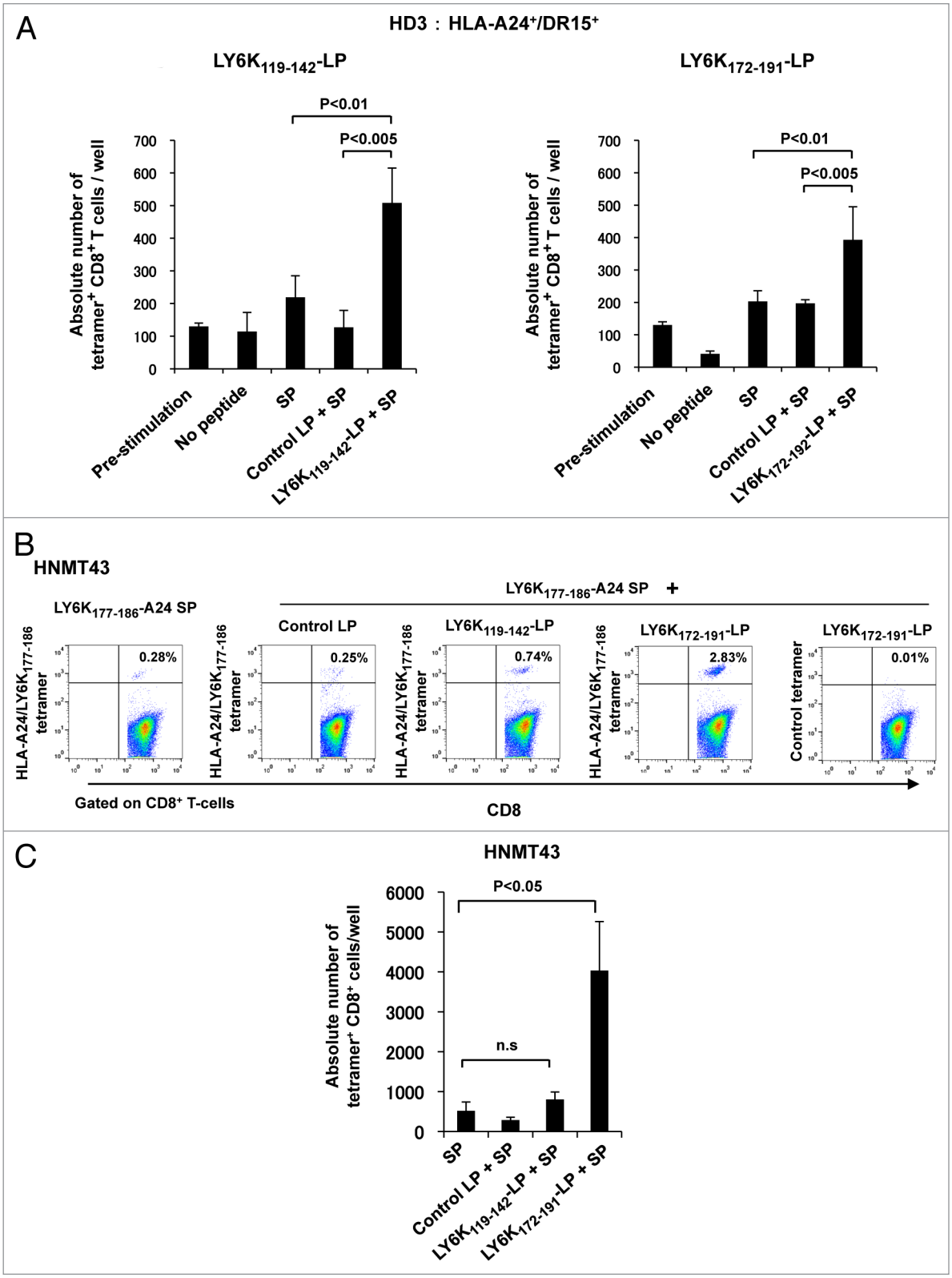

Figure 5. The synergistic effect of LY6K-LPs on induction of $LY6K_{177-186}$-A24 SP-specific CTLs. (**A**) $LY6K_{119-142}$-LP-specific or $LY6K_{172-191}$-LP-specific bulk $CD4^+$ T cells and $LY6K_{177-186}$-A24 SP-specific bulk $CD8^+$ T cells derived from HLA-$A24^+$/$DR15^+$ HD3 were cultured with autologous DCs in the presence of $LY6K_{177-186}$-A24 SP (SP) alone, $LY6K_{177-186}$-A24 SP + Control LP (Control LP + SP), or $LY6K_{177-186}$-A24 SP + LY6K-LP (LY6K-LP + SP) without cytokine for 1-wk. The cultured cells were then stained with a PE-labeled HLA-A24/$LY6K_{177-186}$-specific tetramer and a FITC-labeled anti-CD8 antibody and analyzed by flow cytometry. "Pre-stimulation" indicates the absolute number of tetramer$^+$ $CD8^+$ T cells/well of $LY6K_{177-186}$-A24 SP-specific bulk $CD8^+$ T cells in the healthy donor line. Data are the mean ± SD of triplicate assays. Representative data shown are from 3 independent experiments with similar results achieved. (**B**) Fresh PBMCs obtained from HNMT43 vaccinated with $LY6K_{177-186}$-A24 SP were plated in a 96-well, round-bottomed **(Continued on following page)**

Figure 5. (Continued from previous page) culture plate (1×10^5 cells/well) in 10 μg/mL $LY6K_{177–186}$-A24 SP alone, $LY6K_{177–186}$-A24 SP + control LP, $LY6K_{177–186}$-A24 SP + $LY6K_{119–142}$-LP or $LY6K_{177–186}$-A24 SP + $LY6K_{172–191}$-LP without addition of cytokines. On day 7, cells were stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer and anti-CD8 antibody and analyzed by flow cytometry. Representative HLA-A24/$LY6K_{177–186}$-specific tetramer staining is shown (dot plots). (**C**) Absolute number of tetramer$^+$ CD8$^+$ cells/well. Data are the mean ± SD of triplicate assays. Representative data are from 3 independent experiments with similar results achieved.

Based on the HLA-subtypes found to be capable of antigen presentation from studies using healthy donors, $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP are predicted to be useful in approximately 82% of the total population.[21,22] We showed that $LY6K_{119–142}$-LP induced HLA-DP5, HLA-DR8, or HLA-DR15-restricted Th cells in healthy donors and also induced HLA-DR or HLA-DQ-restricted Th cells in 3 HNMT patients (HNMT31, 41, and 107). However, these HNMT patients were negative for *HLA-DP5*, *-DR8*, or *-DR15* alleles. We also showed that $LY6K_{172–191}$-LP induced HLA-DR15 or HLA-DQ-restricted Th cells in healthy donors. Although we observed that the presence of HLA-DR-restricted $LY6K_{172–191}$-LP-specific Th cells in 7 HNMT patients, *HLA-Class II* alleles of 5 HNMT patients (HNMT31, 41, 42, 107, and 108) were negative for *HLA-DR15*. These results suggest that LY6K-LPs may encompass Th cell epitopes not previously identified in experiments in vitro involving cells derived from healthy donors and LY6K-LPs may be broadly useful in the majority of patients.

In this study, 44% and 70% of HNMT patients demonstrated immunity to $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP respectively, and these are significantly higher than the proportion of healthy donors responding to LY6K-LPs. Weide et al. reported that the presence of circulating Th cells responding to melanoma antigens Melan-A or NY-ESO-1 has a strong independent prognostic impact on survival among chemotherapy-treated advanced melanoma patients.[35] Another study has shown a possible synergy between the telomerase-specific Th response with chemotherapy in lung cancer.[36] The introduction of immunotherapy in clinical practice also emphasized the influence of immune responses on cancer prognosis and chemotherapy effectiveness.[15,37,38] These pieces of evidence support the hypothesis that induction or augmentation of LY6K-specific Th1 cells by vaccination with LY6K-LPs may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies.[39,40] LY6K-LPs-specific Th responses in HNMT patients may, therefore, be predicted to positively influence overall survival. The impact of LY6K-specific Th responses on clinical outcome will be evaluated in future studies.

In conclusion, LY6K-LPs provide a useful tool for propagation of both LY6K-specific Th1 cells and CTLs, and may synergize with CTL-epitopes to effect cancer cell killing. These findings provide rationale for a clinical trial of LY6K-LP-based immunotherapy against a broad spectrum of cancer types.

Materials and Methods

LY6K-epitope immunotherapy clinical trial HNMT patients

Blood samples were collected from 23 patients with HNMTs enrolled in 2 CTL-epitope vaccine trials. The immune responses of Th cells reactive to LY6K-LPs were investigated. These Phase I/II clinical trials of cancer immunotherapy using 3 HLA-A24-binding short peptides (SPs), (clinical grade peptides 9–10 amino acids in length) derived from 3 cancer-testis antigens, LY6K ($LY6K_{177–186}$-A24), IMP-3 ($IMP\text{-}3_{508–516}$-A24), and CDCA1 ($CDCA1_{56–64}$-A24) were reviewed and approved by the Institutional Review Board of Kumamoto University, Japan.[2,18] Peptides were emulsified in 500 μL Montanide ISA51 and injected subcutaneously (s.c.) on days 0, 7, 14, 28, 42, 56, 63, and 70, then monthly until tumor progression or toxicity were observed. The immunization protocol using LY6K, IMP-3, and CDCA1-derived SPs (1 mg each) was determined on the basis of other peptide-vaccine clinical trials using these SPs.[4,6] Toxicities caused by the vaccination therapy were assessed according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3. All patients with HNMTs were selected on the basis of HLA-A24 presence after providing written informed consent and according to the Helsinki Accord. The patients suffered from inoperable advanced HNMT with recurrent or metastatic tumors that were resistant to standard therapy. Patients were enrolled in the trial under the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) number 000008379 (CTR-8379). HNMT patients with radical resection were enrolled in the trial under UMIN-CTR number 000008380 (CTR-8380). In the latter trial, HNMT patients were treated with postoperative peptide vaccine combined with S-1, ifosfamide, or doxorubicin. As of the date of manuscript submission, these clinical trials and analyses remained ongoing.

Genetically engineered antigen-presenting cell lines

Mouse fibroblast cell lines (L-cells), genetically engineered to express the HLA class II heterodimeric pairs DR4 (*DRB1*04:05*) and L-DR4, DR8 (*DRB1*08:03*) and L-DR8, DR15 (*DRB1*15:02*) and L-DR15, or DP5 (*DPB1*05:01*) and L-DP5, were used as antigen-presenting cells (APCs).

Prediction of HLA Class II-binding peptides

To predict possible promiscuous HLA class II-binding human LY6K-derived peptides, the amino acid sequence of the human LY6K protein was analyzed by the immune epitope database (IEBD) recommended method, http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html.[19,20] The program analyzed 15 amino-acid-long sequences offset to encompass the entire protein. We defined the score of percentile rank less than 10 as indication of stronger binding affinity to HLA class II molecules and selected the regions predicted to have high binding affinity to at least 2 frequently observed HLA class II molecules. The 24-mer LP, $LY6K_{119–142}$-LP (KWTEPYCVIAAVKIFPRFFMVAKQ) was predicted to have a strong binding affinity to multiple HLA class II molecules encoded by *DPB1*05:01*, *DRB1*08:03*, *DRB1*09:01*, and *DRB1*15:02* genes. The 20-mer LP, $LY6K_{172–191}$-LP (KCCKIRYCNLEGPPINSSVF) was predicted to have a strong binding affinity to HLA class II molecules encoded by *DRB1*09:01* and *DRB1*15:02* genes and identified as a LP that

spanned a known 10-mer CTL-epitope ($LY6K_{177–186}$-A24 SP) recognized by HLA-A24-restricted CTLs (**Fig. S1B; Table S1**). Given the predicted epitope affinities for HLA class II molecules, in this study we elected to focus on these 2 LY6K-LPs.

Synthetic peptides and recombinant proteins

The human LY6K-derived SP to be presented by HLA-A24 ($LY6K_{177–186}$-A24 SP) and the 2 candidate LPs ($LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP) were biochemically synthesized (MBL, Nagoya, Japan; purity > 95%). A human immunodeficiency virus (HIV) peptide capable of binding to HLA-A24 (HIV-A24) was used as a SP negative control.[41] An Epstein–Barr virus nuclear antigen (EBNA)-derived LP presented by HLA-DP5 (EBNA-DP5) and a promiscuous HIV-derived LP were synthesized and used as LP negative controls.[42,43] Peptides were dissolved in dimethylsulfoxide at 10 μg/μL. The recombinant whole LY6K and CDCA1 protein were expressed by *Escherichia coli* BL21 via a pET28a expression vector (Novagen). The CDCA1 protein was used as a control. Each His-tagged recombinant protein was affinity-purified and assessed by sodium-dodecyl sulfate PAGE (SDS-PAGE).

Generation of antigen-specific $CD4^+$ T cells from healthy donors

The research protocol for collecting and using PBMCs from healthy donors was approved by the Institutional Review Board of Kumamoto University. We obtained PBMCs from 11 healthy donors with written informed consent. Genotyping of *HLA-A, DRB1*, *DPB1*, and *DQB1* was performed at the HLA Laboratory (**Table S2**). Induction of antigen-specific $CD4^+$ T cells was performed as previously described.[20] In some instances, T cells were cloned by limiting-dilution assay and expansion in vitro, as previously described.[44]

Assessment of antigen-specific T-cell responses

The immune response of Th cells to APCs pulsed with 10 μg/mL peptides or 50 μg/mL recombinant proteins was assessed by IFNγ ELISPOT assays (BD Biosciences) according to manufacturer's instructions and as described previously.[41,45] In brief, the frequency of peptide-specific $CD4^+$ T-cells producing IFNγ was analyzed per 3×10^4 bulk $CD4^+$ T-cells stimulated with equal numbers of peptide-pulsed autologous PBMCs, or alternatively, 1×10^4 bulk $CD4^+$ T-cells stimulated with 5×10^4 peptide-pulsed L-cells expressing HLA-DR or -DP molecules To determine the HLA molecules involved in antigen presentation, antigen-induced IFNγ production was blocked by adding anti-HLA-DR monoclonal antibody (mAb; L243, IgG2a, BioLegend), anti-HLA-DP mAb (B7/21, IgG3, Abcam), anti-HLA-DQ mAb (SPV-L3, IgG2a, Abcam), or the broad spectrum anti-HLA class I mAb, (W6/32, IgG2a, Abcam). All mAbs were used at a final concentration of 5 μg/mL. Cells cultured with HIV-derived peptides were used as negative control. Cells cultured with 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) and 500 ng/mL ionomycin (Sigma–Aldrich) were used as positive controls. All IFNγ ELISPOT assays were performed in triplicate or duplicate, and results are presented as means ± SD.

T cell receptor tetramer staining

Expression of $LY6K_{177–186}$-A24 SP specific T cell receptor (TCR) was examined via cytofluorometric analysis on a FACSCalibur (BD Biosciences) flow cytometer using a phycoerythrin (PE)-labeled tetramer of the HLA-A24 (*A*24:02*)/$LY6K_{177–186}$ complex (MBL), according to the manufacturer's instructions. A PE-labeled tetramer of the HLA-A24/HIV-A24 (RYLRDQQLL) complex was used as a negative control.

In vitro cross-presentation assay

Induction of $LY6K_{177–186}$-A24 SP-reactive CTLs from an $HLA\text{-}A24^+$ and $HLA\text{-}DR15^+$ human donor (HD3) was performed by stimulating donor-derived lymphocytes with $LY6K_{177–186}$-A24 SP, as previously described.[24,46] Monocyte-derived DCs were generated from $CD14^+$ cells by in vitro culture, as described previously.[23] Autologous immature DCs were maintained alive or fixed for 3 min in 0.1% glutaraldehyde (Sigma-Aldrich) for use as controls. DCs were pulsed with 16 μM each $LY6K_{177–186}$-A24 SP, $LY6K_{172–191}$-LP, or control LP for 3 h, and washed 3 times. 0.1 KE/mL OK432 was added during and after the peptide pulse to induce the maturation of DCs. $LY6K_{119–142}$-LP, which does not include a known CTL-epitope, was used as a control LP. $LY6K_{177–186}$-A24 SP-reactive bulk CTLs were added at a 2:1 ratio for 6 h in medium containing 10 μg/mL brefeldin A (Sigma–Aldrich). IFNγ production by $LY6K_{177–186}$-A24 SP-specific CTLs was measured by intracellular labeling. The cells were stained with a fluorescein isothiocyanate (FITC)-labeled anti-IFNγ mAb (BioLegend) in combination with a PerCP-labeled anti-$CD8^+$ mAb (BioLegend) and a PE-labeled HLA-A24/$LY6K_{177–186}$-specific tetramer.

Propagation of $LY6K_{177–186}$-A24 SP specific CTLs by stimulation with $LY6K_{172–191}$-LP in HNMT patients

Fresh PBMCs from 5 HNMT patients vaccinated with $LY6K_{177–186}$-A24 SP were cultured at 1×10^5/well with 10 μg/mL $LY6K_{172–191}$-LP in a 96-well plate. Twenty IU/mL recombinant human (rh)IL-2 and 5 ng/mL rhIL-7 were added on day 0 and day 2. On day 0 (ex vivo) and day 7, the PBMCs were stained with a PE-labeled HLA-A24/$LY6K_{177–186}$-specific tetramer and a FITC-labeled anti-$CD8^+$ mAb.

In vitro induction of $LY6K\text{-}A24_{177–186}$ SP-specific CTLs by stimulating PBMCs with $LY6K_{172–191}$-LP

To assess induction of $LY6K_{177–186}$-A24 SP-specific CTLs from $HLA\text{-}A24^+$ donors (HD1, HD3, and HD4) by stimulation with $LY6K_{172–191}$-LP in vitro, 2×10^6 cells/well PBMCs were incubated with 7 μM $LY6K_{172–191}$-LP in 24-well plates for 2 wk without addition of cytokines. Specifically, on day 7 post-plating, 7 μM $LY6K_{172–191}$-LP was added to each well. On day 14 of in vitro stimulation with $LY6K_{172–191}$-LP, the cells were harvested and stained with a PE-labeled HLA-A24/$LY6K_{177–186}$-specific tetramer and a FITC-labeled anti-$CD8^+$ mAb.

In vivo cross-priming assay

HLA-A24 (HHH) transgenic mice were kindly provided by Dr FA Lemonnier.[47] Mice were intradermally injected at the base of the tail with 100 μg $LY6K_{172–191}$-LP solution emulsified in incomplete Freund's adjuvant (IFA), 3 times at 7-d intervals. Seven days after the third vaccination with $LY6K_{172–191}$-LP, $CD8^+$ T cells were isolated from inguinal lymph nodes by positive selection with magnetic microbeads (Miltenyi Biotec). The number of IFNγ producing $CD8^+$ T cells (of 1×10^5 cells/well plated)

in response to stimulation with $LY6K_{177–186}$-A24 SP-pulsed bone-marrow derived DCs (2×10^4 cells/well) was counted by ex vivo ELISPOT assay.[20]

The synergistic effect of LY6K-LPs on induction of LY6K-specific CTLs

To test whether LY6K-LPs could enhance induction of $LY6K_{177–186}$-A24 SP-specific CTLs, 1×10^5 $LY6K_{119–142}$-LP-specific or $LY6K_{172–191}$-LP-specific bulk $CD4^+$ T cells and 1×10^5 $LY6K_{177–186}$-A24-specific bulk $CD8^+$ T-cells derived from donor HD3 were cultured in 48-well plates with 2×10^4 autologous DCs in the presence of 10 μg/mL $LY6K_{177–186}$-A24 SP alone, $LY6K_{177–186}$-A24 SP + 10 μg/mL control LP, or $LY6K_{177–186}$-A24 SP + 10 μg/mL LY6K-LP derivatives ($LY6K_{119–142}$-LP or $LY6K_{172–191}$-LP). After 1-wk in vitro culture with peptides, the cultured cells were stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer.

In another experiment, fresh PBMCs obtained from HNMT patients vaccinated with $LY6K_{177–186}$-A24 SP were plated in a 96-well, round-bottomed culture plate (1×10^5 cells/well), followed by addition of 10 μg/mL $LY6K_{177–186}$-A24 SP alone or $LY6K_{177–186}$-A24 SP applied together with either control LP, $LY6K_{119–142}$-LP, or $LY6K_{172–191}$-LP (10 μg/mL) in a final volume of 200 μL AIM-V supplemented with 5% human decomplemented plasma without addition of cytokines. On day 7 of culture, cells were stained with a HLA-A24/$LY6K_{177–186}$-specific tetramer.

Assessment of LY6K-LP-specific Th cell responses in HNMT patients

Fresh PBMCs obtained from 23 HNMT patients before and after CTL-epitope peptide-vaccination were cultured at 2×10^6 cells/well in 24-well plates with a mixture of 10 μg/mL each $LY6K_{119–142}$-LP and $LY6K_{172–191}$-LP in a final volume of 2 mL AIM-V supplemented with 5% human decomplemented plasma at 37 °C. IL-2 and IL-7 were added on days 0 and 2. After 1 wk of cell culture, the PBMCs were collected, washed, and cultured in ELISPOT plates at 1×10^5 cells/well with 10 μg/mL each $LY6K_{119–142}$-LP, $LY6K_{172–191}$-LP, or control LPs for 18 h. The number of LY6K-LPs-specific Th cells expressed as spot-forming cells/10^5 initially-plated cells was calculated after subtracting control values (background). Responses were scored as positive when both the mean number of IFNγ spots was more than 15 and the number exceeded 2-fold over background. The ELISPOT assays on patients' cells were conducted in single, duplicate, or triplicate wells with replicates constrained due to the limited number of available cells. Cells cultured with PMA and ionomycin were used as positive controls. This study was conducted in a laboratory that operates under exploratory research principles, and was performed using investigative protocols. We acknowledge the recommendations of the Minimal Information About T-cell Assay (MIATA) reporting framework for human T cell assays.[48]

Statistical analysis

Statistical analyses were performed by 2-tailed Student's *t*-test, Fisher's exact probability test, or nonparametric Mann–Whitney *U* test. Differences with a P value < 0.05 were considered statistically significant for all tests.

Disclosure of Potential Conflicts of Interest

K.Y. and T.K. are current employees of OncoTherapy Science, Inc. Y.N. is a stockholder and a scientific advisor for OncoTherapy Science, Inc. Y.N. is supported by a funding from OncoTherapy Science, Inc.

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Supplemental Material

Supplemental materials may be found here:

www.landesbioscience.com/journals/oncoimmunology/53/article/28100

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