Identification of promiscuous KIF20A long peptides bearing both CD4+ and CD8+ T-cell epitopes: KIF20A-specific CD4+ T-cell immunity in patients with malignant tumor

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Conflict of interests

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Translational Relevance

We recently identified a novel tumor-associated antigen (TAA), kinesin family member 20A (KIF20A), overexpressed in various malignancies. Phase I/II clinical trials of immunotherapy for several cancers using a KIF20A-derived CTL-epitope are underway. We identified promiscuous KIF20A long peptides (LPs) bearing naturally processed epitopes recognized by CD4+ T-cells and CTLs. KIF20A-LPs stimulated KIF20A-specific CTLs in vitro and in vivo. In addition, KIF20A-LP and a T-helper type 1 (Th1) cell clone enhanced induction of KIF20A-specific CTLs in vitro. Thus, KIF20A-LPs provide a useful tool for propagation of both Th1 cells and CTLs. This report also describes the first immunohistochemical detection of KIF20A expression in cases of head-and-neck malignant tumor (HNMT), and the first detection of KIF20A-LPs specific Th1 cell responses in patients with HNMT. These findings will support clinical trials of KIF20A peptide-based immunotherapy for various cancers.
Abstract

Purpose: To identify long peptides (LPs) derived from a novel tumor-associated antigen (TAA), kinesin family member 20A (KIF20A), which induce tumor-specific T-helper type 1 (Th1) cells and CTLs.

Experimental Design: We combined information from a recently developed computer algorithm predicting HLA class II-binding peptides with KIF20A-derived CTL-epitope sequences presented by HLA-A2 (A*02:01) or HLA-A24 (A*24:02) to select candidate promiscuous Th1 cell epitopes containing CTL-epitopes. Peripheral blood mononuclear cells (PBMC) derived from healthy donors or patients with head-and-neck malignant tumor (HNMT) were used to study the immunogenicity of KIF20A-LPs, and the in vitro cross-priming potential of KIF20A-LPs bearing CTL-epitopes. We used HLA-A24 transgenic mice to address whether vaccination with KIF20A-LP induces efficient cross-priming of CTLs in vivo. The Th1 cell response to KIF20A-LPs in HNMT patients receiving immunotherapy with TAA-derived CTL-epitope peptides was analyzed using IFN-γ enzyme-linked immunospot assays.

Results: We identified promiscuous KIF20A-LPs bearing naturally processed epitopes recognized by CD4+ T-cells and CTLs. KIF20A-specific CTLs were induced by vaccination with a KIF20A-LP in vivo. KIF20A expression was detected in 55% of HNMT by immunohistochemistry, and significant frequencies of KIF20A-specific Th1 cell responses were detected after short-term in vitro stimulation of PBMCs with...
KIF20A-LPs in 50% of HNMT patients, but not in healthy donors. Furthermore, these responses were associated with KIF20A expression in HNMT tissues.

**Conclusions:** These are the first results demonstrating the presence of KIF20A-specific Th1 cell responses in HNMT patients and underline the possible utility of KIF20A-LPs for propagation of Th1 cells and CTLs.
Introduction

We recently used genome-wide cDNA microarray analysis to identify a novel tumor-associated antigen (TAA), kinesin family member 20A (KIF20A), which is frequently overexpressed in lung cancer, pancreatic cancer, gastric cancer, bladder cancer, breast cancer, melanoma, and various other malignancies (1-3). KIF20A has been reported to be a promising immunotherapeutic target for cancers. Studies have reported that 2 short peptides (SPs) derived from KIF20A, KIF20A-A2809–917 and KIF20A-A2466–75, stimulated CTLs, which kill cancer cells endogenously expressing KIF20A antigen and that KIF20A-specific CTLs were present in peripheral blood mononuclear cells (PBMCs) obtained from patients with pancreatic cancer (1, 4). Phase I/II clinical trials of cancer immunotherapy for lung cancer, pancreatic cancer, and cholangiocellular carcinoma using KIF20A-derived SP are underway. In these trials, we observed that vaccination with a KIF20A-derived SP induced a KIF20A-specific CTL response and yielded promising results in patients with advanced cancer (manuscript in preparation). Therefore, we propose that KIF20A is an attractive target molecule for cancer immunotherapy. We have attempted to identify long peptides (LPs) that induce both antigen-specific CD4+ helper T (Th) cells and CTLs in order to further develop peptide vaccine immunotherapy.

Tumor-specific Th cells, particularly T-helper type 1 (Th1) cells, play a critical role in efficient induction of CTL-mediated antitumor immunity (5). Interferon-γ
(IFN-γ) produced by Th1 cells is critical for induction and maintenance of long-lived CTL responses through multiple interactions (6, 7). IFN-γ secreted by Th1 cells also mediates direct antitumor or antiangiogenic effects (8). Furthermore, Th cells pave the way for entry of CTLs at the tumor site (9). Therefore, identification of Th-cell epitopes that can activate tumor-specific Th1 cells is important for induction of effective tumor immunity in tumor-bearing hosts.

Melief et al. recently reported a synthetic long peptide (LP) naturally bearing a CTL-epitope as an attractive vaccine compound. Following injection of LP, a patient’s dendritic cells (DC) take up the LP, process it, and present all possible CTL epitopes and Th cell epitopes in the context of various HLA class I and class II molecules (10). In addition, recent clinical studies using a promiscuous telomerase-derived helper-epitope vaccine called GV1001 bearing CTL-epitopes, increased survival of cancer patients when combined with radiotherapy and chemotherapy (11, 12). Thus, we proposed that an ideal peptide vaccine for cancer immunotherapy may be a single polypeptide containing epitopes for both Th1 cells and CTLs, which are naturally proximal to each other and can be induced simultaneously (10, 13).

In this study, a recently developed computer algorithm predicting HLA class II-binding peptides and known CTL-epitope sequences recognized by HLA-A2 or -A24-restricted CTLs were used to select candidate promiscuous Th cell epitopes containing the CTL epitopes. Our results show that the predicted LPs triggered Th1
responses in individuals expressing several common HLA-DR or -DP alleles, and that the KIF20A-LPs bearing CTL-epitopes efficiently stimulated KIF20A-specific CTLs.
Materials and Methods

Patients

Blood samples were collected from 16 patients with a head and neck malignant tumor (HNMT). The immune responses of Th cells reactive to KIF20A-LPs were investigated. The patients were receiving immunotherapy with TAA-derived CTL-epitope peptides, and were enrolled in 2 peptide vaccine trials. These phase I/II clinical trials of cancer immunotherapy using 3 HLA-A24-binding short peptides (SPs), (clinical-grade 9–10-amino acid-long peptides) derived from 3 cancer-testis antigens, LY6K (LY6K-A24177–186), IMP-3 (IMP-3-A24508–516), and CDCA1 (CDCA1-A2456–64) were reviewed and approved by the Institutional Review Board of Kumamoto University, Japan (14-16). This vaccine cocktail did not include KIF20A-derived SPs. All patients with HNMT were selected on the basis of HLA-A24 presence after providing written informed consent. The patients suffered from inoperable advanced HNMT with recurrent or metastatic tumors and were resistant to standard therapy; they were enrolled in the trial under University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) number 000008379 (CTR-8379). The patients with radical resection were enrolled in the trial under UMIN-CTR number 000008380 (CTR-8380). In the latter trial, patients were treated with postoperative peptide vaccine combined with S-1, ifosfamide, or doxorubicin. These clinical trials and analyses are ongoing.
**Cell lines**

Mouse fibroblast cell lines (L-cells), genetically engineered to express DR4 (*DRB1*04:05), L-DR4; DR8 (*DRB1*08:03), L-DR8; DR15 (*DRB1*15:02), L-DR15; or DR53 (*DRB4*01:03), L-DR53 were used as antigen-presenting cells (APCs). The HLA-A24-positive C1R-A2402 cells were a gift from Dr. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan) (17).

**Prediction of HLA class II-binding peptides**

To predict possible promiscuous HLA-class II binding human KIF20A-derived peptides, the amino acid sequence of the human KIF20A protein was analyzed by a recently developed computer algorithm (IEDB analysis resource, consensus method, [http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html](http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html)) (18, 19). The program analyzed 15-aminoacid-long sequences offset to encompass the entire protein. The 24- and 25-aminoacid-long peptides with overlapping high consensus percentile ranks for multiple HLA-class II molecules encoded by *DRB1*04:05, *DRB1*15:02, or *DRB4*01:03 alleles, KIF20A<sub>60-84</sub>-LP (DSMEKVKYLYLRPLPSELERQED), KIF20A<sub>494-517</sub>-LP (TLHVAKFSAIASQLVHAPPMQLGF), and KIF20A<sub>809-833</sub>-LP (CIAEQYHTVNLQLGQVSAKRLGTN), were selected (Supplementary figure 1 and Supplementary table 1).
Synthetic peptides and recombinant proteins

Human KIF20A-derived SPs presented by HLA-A2 (KIF20A-A2<sub>809-817</sub>) or A24 (KIF20A-A24<sub>66-75</sub>), and 3 LPs (KIF20A<sub>60-84</sub>-LP, KIF20A<sub>494-517</sub>-LP, and KIF20A<sub>809-833</sub>-LP) were synthesized (MBL, Nagoya, Japan; purity >95%; Supplementary figure 1B). A human immunodeficiency virus (HIV) peptide that binds to HLA-A24 (HIV-A24) was used as a negative control SP (17). A WT1-derived LP presented by HLA-DR4 (WT1-peptide) and a promiscuous HIV-derived LP were used as negative control LPs (20, 21). Peptides were dissolved in dimethylsulfoxide at 10 μg/μL. The recombinant whole KIF20A and CDCA1 protein were expressed by *Escherichia coli* BL21 with a pET28a vector (Novagen). The CDCA1 protein was used as a control. Each recombinant protein was purified and assessed by SDS-PAGE.

Generation of antigen-specific CD4<sup>+</sup> T cells from healthy donors

The research protocol for collecting and using PBMCs from healthy donors (HD1–HD11; Supplementary table 2) 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*, and *DPB1* was performed at the HLA Laboratory (Kyoto, Japan; Supplementary table 2). With some modifications, induction of antigen-specific CD4<sup>+</sup> T-cells was performed as described previously (22). Detailed
methods are provided in Supplemental Materials and Methods. In some instances, T-cells were cloned by limiting dilution for further studies as described previously (23).

**Assessment of T-cell responses to peptides and proteins**

The immune response of Th cells to antigen-presenting cells (APCs) pulsed with peptides (10 μg/mL) or proteins (50 μg/mL) was assessed by IFN-γ enzyme-linked immunospot (ELISPOT) assays (BD Biosciences) as described previously (17). In brief, the frequency of peptide-specific CD4+ T-cells producing IFN-γ per 3 × 10^4 bulk CD4+ T-cells in response to stimulation with peptide-pulsed PBMCs (3 × 10^4 cells/well), or 1 × 10^4 bulk CD4+ T-cells in response to stimulation with peptide-pulsed L-cells expressing HLA-DR (5 × 10^4 cells/well) was analyzed. The frequency of KIF20A-LP-specific Th cell clone (Th-clone) producing IFN-γ per 2 × 10^4 Th-clone in response to stimulation with protein-loaded DCs (5 × 10^3 cells/well) was analyzed. To determine the HLA molecules involved in antigen presentation, antigen-induced IFN-γ production was blocked by adding anti-HLA-DR monoclonal antibody (mAb; L243, BioLegend), anti-HLA-DP mAb, (B7/21, Abcam), anti-human HLA-DQ mAb (SPV-L3, Abcam), or anti-HLA class I mAb (W6/32, Abcam) (24-26). All mAbs were used at a final concentration of 5 μg/mL. All assessments of IFN-γ ELISPOT assays were performed in triplicate or duplicate, and results are presented as means ± SD.
Immunohistochemical examination

Immunohistochemical staining of KIF20A using a rabbit polyclonal antibody against KIF20A (A300-879A, Bethyl Laboratories, Montgomery, TX, USA) was performed as described previously (1, 2, 27).

Cytokine assays and CD107a mobilization assay

HLA-DP2-restricted KIF20A_{60–84}-LP-specific bulk Th cells \((3 \times 10^4 \text{ cells/well})\) were cultured with autologous PBMCs \((3 \times 10^4 \text{ cells/well})\) in the presence of cognate peptide in 96-well culture plates. HLA-DR53-restricted KIF20A_{809–833}-LP-specific bulk Th cells were cultured with L-DR53 \((5 \times 10^4 \text{ cells/well})\) in the presence of cognate peptide. After 24 h, culture supernatants were collected and cytokine (TNF-\(\alpha\), IFN-\(\gamma\), GM-CSF, MIP1\(\beta\), IL-2, IL-4, and IL-7) levels were measured using the Bio-Plex system (Bio-Rad) according to manufacturer’s instructions.

Peptide-stimulated degranulation of CD4\(^+\) T lymphocytes was determined by flow cytometric analysis of CD107a exposed on the cell surface as described previously, with some modifications (28). Detailed methods are provided in Supplemental Materials and Methods.

The synergistic effect of KIF20A-LPs on induction of KIF20A-specific CTLs

PBMCs obtained from an HLA-A2\(^+\)/DR53\(^+\)/DP2\(^+\) donor (HD2), from whom
the KIF20A_{60–84}-LP or KIF20A_{809–833}-LP-specific Th-clones were generated, were
plated in 24-well plates (3 × 10^6 cells/well). After culture for 7 days the following
additions were made: recombinant human (rh) IL-2 (20 U/mL) and SP alone
(KIF20A-A_{2809–817} SP, 20 μg/mL), or SP + LP (KIF20A_{60–84}-LP or KIF20A_{809–833}-LP,
20 μg/mL), or SP + LP + Th-clone (5 × 10^5 cells/well) in a final volume of 2 mL.
Recombinant human IL-15 (5 ng/mL) was added on day 9. On day 11, cells were
stained with PE-labeled tetramer of the HLA-A*02:01/ KIF20A-A_{2809–817}-complex and
an FITC-labeled anti-human CD8 mAb. Data acquisition was performed on a
FACSCalibur (BD Biosciences), and data files were analyzed with FlowJo software
(Tree Star, Ashland, OR).

**In vitro stimulation of KIF20A-A_{2809–817} SP-specific CTLs with KIF20A_{809–833}-LP**

Induction of KIF20A-A_{2809–817} SP-reactive bulk CTLs from an HLA-A2^+ and
DR53^+ donor (HD5) by stimulation with KIF20A-A_{2809–817} SP was performed as
described (28, 29). In brief, purified human CD8^+ T-cells were stimulated with
KIF20A-A_{2809–817} SP-pulsed DCs. After 3 rounds of stimulation, we confirmed that
CTLs specifically produced IFN-γ in response to restimulation with T2-cells pulsed
with KIF20A-A_{2809–817} SP; thus, the KIF20A-A_{2809–817} SP-reactive CTLs were used as
effector cells.

To assess the stimulation of KIF20A-A_{2809–817} SP-reactive CTLs with
KIF20A<sub>809–833</sub>-LP-loaded DCs, the number of IFN-γ producing-KIF20A-A2<sub>809–817</sub> SP-specific bulk CTLs in response to stimulation with the KIF20A<sub>809–833</sub>-LP loaded autologous DCs was counted by an ELISPOT assay. The LP-loaded mature DCs were prepared from positively isolated CD14<sup>+</sup> cells (day 0). CD14<sup>+</sup> cells were cultured in the presence of IL-4 (10 ng/ml) and GM-CSF (100 ng/ml). KIF20A<sub>809–833</sub>-LP (50 μg/ml) and OK432 were added on day 5. The LP-loaded mature DCs were harvested on day 7, washed, and used as APCs in an ELISPOT assay.

Assessment of KIF20A-LPs-specific CD4<sup>+</sup> T-cell responses in patients with HNMT.

Fresh PBMCs from patients with HNMT or healthy donors were cultured with a mixture of KIF20A<sub>60–84</sub>-LP and KIF20A<sub>809–833</sub>-LP (10 μg/mL each) in a final volume of 2 ml AIM-V supplemented with 5% human decomplemented plasma at 37°C (2 × 10<sup>6</sup> cells/well, 24-well plates); both rhIL-2 and rhIL-7 were added on day 0 and 2. After 1 week of cell culture, the PBMCs were collected, washed, and cultured in ELISPOT plates (1 × 10<sup>5</sup> cells/well) with KIF20A<sub>60–84</sub>-LP, KIF20A<sub>809–833</sub>-LP, or control LP for 18 h. The number of KIF20A-LP-specific Th cells expressed as spot-forming cells/10<sup>5</sup> cells was calculated after subtracting control values (background). Responses were scored as positive when the mean number of IFN-γ spots was more than 15 and more than 2-fold over background. The ELISPOT assays on patients’ cells were conducted in single, duplicate, or triplicate wells because of the limited number of available cells.
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 Assays (MIATA) reporting framework for human T-cell assays (30).

**Statistical analysis**

Data were compared by the 2-tailed Student *t*-test (bar graphs), or by the nonparametric Mann–Whitney *U* test (scatter-dot graph). Differences with a *P* value < 0.05 were considered statistically significant for all tests.
Results

Prediction and selection of possible promiscuous HLA class II-binding KIF20A-LPs

To identify possible promiscuous HLA-class II binding Th cell epitopes of KIF20A we first examined the amino acid sequence of KIF20A using a recently developed computer algorithm (Supplementary figure 1A) (18, 19). Two LPs, KIF20A60–84-LP and KIF20A809–833-LP, predicted by the computer algorithm to be potent promiscuous HLA class II-binding peptides, were identified proximal to known 9- or 10-mer CTL-epitopes recognized by HLA-A2- or A24-restricted CTLs (Supplementary figure 1B and Supplementary table 1). Another peptide, KIF20A494–517-LP, was also predicted to be a potent promiscuous HLA class II-binding peptide, although it did not include a known CTL-epitope sequence. Therefore, 3 candidate LPs, KIF20A60–84-LP, KIF20A494–517-LP, and KIF20A809–833-LP, predicted to have strong binding affinity to HLA-class II molecules HLA-DR4, -DR15, or -DR53, were synthesized for subsequent analyses.

Identification of promiscuous KIF20A-derived Th cell epitopes

CD4+ T-cells isolated from PBMCs of healthy donors were stimulated at weekly intervals with autologous DCs or PBMCs pulsed with KIF20A60–84-LP as described in the Materials and Methods section. After at least 3 rounds of stimulation,
KIF20A\textsubscript{60–84}-LP-specific responses of CD\textsuperscript{4}+ T-cells were examined by IFN-\(\gamma\) ELISPOT assays. In an HLA-DR\textsubscript{15}\textsuperscript{+} HD1, the generated Th cells produced a significant amount of IFN-\(\gamma\) in response to KIF20A\textsubscript{60–84}-LP-pulsed PBMCs in an HLA-DR-dependent manner. The bulk Th cells specifically recognized L-DR\textsubscript{15} cells pulsed with KIF20A\textsubscript{60–84}-LP in an HLA-DR-dependent manner, but not KIF20A\textsubscript{60–84}-LP-pulsed L-DR\textsubscript{8} cells (Figure 1A). These results indicated that KIF20A\textsubscript{60–84}-LP was presented by HLA-DR\textsubscript{15}.

To investigate whether KIF20A\textsubscript{60–84}-LP induces responses in Th cells restricted by other HLA class II molecules, CD\textsuperscript{4}+ T-cells from HLA-DR\textsubscript{15}-negative healthy donors were tested. The Th cells generated from HLA-DP\textsubscript{2}\textsuperscript{+} HD2 produced a significant amount of IFN-\(\gamma\) in response to KIF20A\textsubscript{60–84}-LP-pulsed PBMCs in an HLA-DP-dependent manner. L-cells transduced with \textit{HLA-DP2} were unavailable; therefore, we established a KIF20A\textsubscript{60–84}-LP-reactive Th cell clone (Th-clone) and used allogeneic PBMCs from 4 different donors as APCs to determine restriction by shared HLA-DP molecules. We confirmed that KIF20A\textsubscript{60–84}-LP generates HLA-DP\textsubscript{2}-restricted Th cells (Figure 1B). Thus, KIF20A\textsubscript{60–84}-LP binds to HLA-DR\textsubscript{15} and HLA-DP\textsubscript{2}, which suggests that KIF20A\textsubscript{60–84}-LP is a promiscuous Th cell epitope presented by frequent HLA class II molecules (31, 32). In addition, KIF20A\textsubscript{60–84}-LP could induce antigen-specific and HLA-DR-restricted bulk Th cells from HD3, who was HLA-DR\textsubscript{4}\textsuperscript{+} and DR\textsubscript{53}\textsuperscript{+}, but DR\textsubscript{15}- and DP\textsubscript{2}-negative (Supplementary figure 2A). Although we
were unable to define the HLA-class II molecule presenting the LP to the Th cells, this result supports the promiscuous nature of KIF20A_{60-84}-LP.

Next, we assessed whether KIF20A_{809-833}-LP could generate antigen-specific Th cells. The Th cells generated from HLA-DR15^+ healthy donors (HD1 and HD4) produced a significant amount of IFN-γ in response to KIF20A_{809-833}-LP-pulsed PBMCs in an HLA-DR-dependent manner (Figure 1C and Supplementary figure 2B). The bulk Th cells specifically recognized L-DR15 cells pulsed with KIF20A_{809-833}-LP in an HLA-DR-dependent manner (Figure 1C, HD1). CD4^+ T-cells from an HLA-DR15-negative healthy donor (HD2) were tested to investigate whether KIF20A_{809-833}-LP induces responses in Th cells restricted by other HLA class II molecules. KIF20A_{809-833}-LP could generate HLA-DR53-restricted Th cells in this donor (Figure 1D). These results demonstrate that KIF20A_{809-833}-LP is also a promiscuous Th cell epitope.

The immunogenicity of KIF20A_{494-517}-LP, which did not include a known CTL-epitope was evaluated. KIF20A_{494-517}-LP could generate HLA-DR4-restricted bulk Th cells in HLA-DR4^+ HD2 (Supplementary figure 2C). Although the immunogenicity of KIF20A_{494-517}-LP was assessed in other donors, it was determined to be less immunogenic. Thus, we focused on promiscuous LPs bearing known CTL-epitopes, such as KIF20A_{60-84}-LP and KIF20A_{809-833}-LP, for further studies.
**KIF20A_{60-84}-LP and KIF20A_{809-833}-LP are naturally processed and presented by DCs**

We proceeded to assess whether DCs take up and process the KIF20A protein to stimulate KIF20A-specific Th-clones. DCs loaded with recombinant KIF20A protein were prepared and used as APCs in IFN-γ ELISPOT assays (16, 28). An HLA-DP2-restricted KIF20A_{60-84}-LP-specific Th-clone efficiently recognized DCs loaded with KIF20A protein in an HLA-DP-dependent manner, but did not recognize control protein-loaded DCs, indicating that this epitope was naturally processed and presented by HLA-DP2 molecules (Figure 2A). An HLA-DR53-restricted KIF20A_{809-833}-LP-specific Th-clone efficiently recognized DCs loaded with KIF20A protein in an HLA-DR-dependent manner, but did not recognize control protein-loaded DCs, indicating that this epitope was also naturally processed and presented by HLA-DR53 molecules (Figure 2B).

**KIF20A-LPs stimulate Th1-type CD4^+ T cells**

In order to further characterize KIF20A-LP-reactive Th cells, we used the Bio-Plex system to measure the levels of several cytokines released in response to stimulation by the cognate peptide. KIF20A_{60-84}-LP- or KIF20A_{809-833}-LP-specific bulk Th cells from HD2 produced a large amount of TNF-α, IFN-γ, GM-CSF, MIP-1β, and...
IL-2, but less IL-4 and IL-17 after restimulation with cognate peptide, indicating Th1 polarized characteristics (Figure 3A). The cytotoxicity marker CD107a was detected on the KIF20A-LPs-specific bulk Th cells stimulated with cognate peptide (Figure 3B), as was previously demonstrated for antiviral CD4+ effectors and tumor-infiltrating lymphocytes (33-36). These data suggest that KIF20A-specific Th cells provide a helper function and direct cytotoxic activity, both of which are advantageous for cancer immunotherapy.

Enhanced induction of KIF20A-specific CTLs by KIF20A-LP-specific CD4+ T-cells

Next, we tested whether KIF20A-LPs could enhance induction of KIF20A-A2809–817 SP-specific CTLs as shown in Figure 4A. When PBMCs from an HLA-A2+/DP2+/DR53+ donor (HD2) were stimulated with KIF20A-A2809–817 SP alone (SP), the frequency of KIF20A-A2809–817 SP-specific tetramer+ cells was 0.24% of CD8+ T-cells (Figure 4B). Addition of KIF20A60–84-LP (LP) into the SP culture (SP + LP) induced a slight increase in the frequency of tetramer+ cells. In contrast, when the PBMCs were co-stimulated with KIF20A-A2809–817 SP, KIF20A60–84-LP, and KIF20A60–84-LP-specific Th-clone (SP + LP + Th-clone), the frequency of KIF20A-A2809–817 SP-specific CTLs increased significantly to 0.87%. As shown in Supplementary figure 3A-B), KIF20A809–833-LP alone (LP), which encompasses
KIF20A-A2\textsubscript{809-817} SP, or addition of KIF20A\textsubscript{809-833}-LP into the SP culture (SP + LP) induced a slight increase in the frequency of KIF20A-A2\textsubscript{809-817} SP-specific CTLs. On the other hand, the KIF20A\textsubscript{809-833}-LP-specific Th-clone rapidly increased in response to the KIF20A\textsubscript{809-833}-LP when we added both LP and Th-clone into the PBMCs without KIF20A-A2\textsubscript{809-817} SP (LP + Th-clone) and then we could not detect the increase of frequency of KIF20A-A2\textsubscript{809-817} SP-specific CTLs. We also observed that the stimulation of PBMCs with KIF20A-A2\textsubscript{809-817} SP, KIF20A\textsubscript{809-833}-LP, and KIF20A\textsubscript{809-833}-LP-specific Th-clone (SP + LP + Th-clone) most strongly enhanced induction of KIF20A-A2\textsubscript{809-817} SP-specific tetramer\textsuperscript{+} T-cells. These results indicate that the activated Th1 cells enhanced induction of KIF20A-specific CTLs in the presence of KIF20A-A2\textsubscript{809-817} SP.

Next, we examined that the CD107a expression of KIF20A-A2\textsubscript{466-75} SP-specific CTLs cultured in the presence of KIF20A\textsubscript{809-833}-LP-specific Th cells stimulated with the relevant LP for 1 week to assess the function of KIF20A-specific CTLs expanded by activated KIF20A-specific Th cells. KIF20A\textsubscript{809-833}-LP-specific bulk CD4\textsuperscript{+} T-cells and KIF20A-A2\textsubscript{466-75} SP-specific bulk CD8\textsuperscript{+} T-cells derived from HLA-A2\textsuperscript{4} and HLA-DR15\textsuperscript{+} HD4 were cultured with autologous DCs in the presence of KIF20A-A2\textsubscript{466-75} SP (SP alone), KIF20A-A2\textsubscript{466-75} SP + Control LP (Control LP + SP), or KIF20A-A2\textsubscript{466-75} SP + KIF20A\textsubscript{809-833}-LP (KIF20A\textsubscript{809-833}-LP + SP) without addition of any cytokine. After 1-week in vitro culture with peptides, the cultured cells
were stained with tetramer of the HLA-A*24:02/KIF20A-A2467–75 complex, anti-human CD107a mAb, and anti-human CD8 mAb as described in the Supplementary Materials and Methods section. As shown in Supplementary figure 3C, the addition of KIF20A-A2466–75 SP + KIF20A809–833-LP (KIF20A809–833-LP + SP) significantly increased the absolute number of KIF20A-A2466–75 SP-specific CD8+ T-cells compared with the addition of KIF20A-A2466–75 SP alone (SP) or KIF20A-A2466–75 SP + Control LP (Control LP + SP). The absolute number of KIF20A-A2466–75 SP-specific CTLs expressing CD107a on the cell surface after re-stimulation with KIF20A-A2466–75 SP was also significantly augmented by the addition of KIF20A-A2466–75 SP + KIF20A809–833-LP (KIF20A809–833-LP + SP; Supplementary Figure 3D). These results suggest that activated KIF20A-LP-specific Th cells enhanced induction of KIF20A-A2466–75 SP-specific CTLs expressing CD107a.

KIF20A-LPs stimulate KIF20A-specific CD8+ T-cells in vitro and in vivo

We assessed whether the KIF20A-LPs bearing CTL-epitopes could stimulate KIF20A-SP-specific CTLs. The capacity of KIF20A809–833-LP to stimulate KIF20A-A2809–817 SP-specific CTLs was examined by IFN-γ ELISPOT assay as described in the Materials and Methods section. As shown in Figure 4C, KIF20A-A2809–817 SP-reactive bulk CTLs from an HLA-A2+ and -DR53+ donor (HD5) specifically produced IFN-γ in response to stimulation with KIF20A809–833-LP-loaded
DC, but not with control LP-loaded DC. The specific IFN-γ production was significantly inhibited by addition of the anti-HLA-class I mAb, but not by the anti-HLA-DR mAb, thus suggesting that KIF20A-A2\textsubscript{809–817} SP-reactive CTLs were stimulated through the cross-presentation of KIF20A\textsubscript{809–833}-LP by DCs \textit{in vitro}.

Subsequently, the capacity of KIF20A\textsubscript{60–84}-LP to induce KIF20A-A2\textsubscript{466–75} SP-specific CTLs was examined by IFN-γ ELISPOT assay. After \textit{in vitro} stimulation of PBMCs with KIF20A\textsubscript{60–84}-LP for 2 weeks, the cells were harvested and the number of IFN-γ-producing T-cells in response to stimulation with KIF20A-A2\textsubscript{466–75} SP-pulsed C1R-A2402 cells was counted by ELISPOT assay. Details are provided in Supplemental Materials and Methods. As shown Figure 4D, KIF20A-A2\textsubscript{466–75} SP-specific CTLs were induced in PBMCs cultured with KIF20A\textsubscript{60–84}-LP. In addition, KIF20A\textsubscript{809–833}-LP also induced KIF20A-A2\textsubscript{809–817} SP-specific CTLs from PBMCs derived from HLA-A2\textsuperscript{+} donors (HD2 and HD3; data not shown).

The capacity of KIF20A\textsubscript{60–84}-LP to prime KIF20A-A2\textsubscript{466–75} SP-specific CTLs \textit{in vivo} was examined by an \textit{ex vivo} IFN-γ ELISPOT assay. HLA-A24 Tgm were immunized 3 times with KIF20A\textsubscript{60–84}-LP. The CD8\textsuperscript{+} T-cells of HLA-A24 Tgm vaccinated with KIF20A\textsubscript{60–84}-LP produced IFN-γ in response to stimulation with BM-DCs pulsed with the KIF20A-A2\textsubscript{466–75} SP (Figure 4E). These results suggest that after uptake of KIF20A\textsubscript{60–84}-LP, APCs cross-prime KIF20A-A2\textsubscript{466–75} SP-specific CTLs \textit{in vitro} and \textit{in vivo}.
Presence of KIF20A-specific Th1 cells in patients with HNMT receiving immunotherapy with TAA-derived CTL-epitope peptides.

To the best of our knowledge, no studies have examined KIF20A expression in HNMT. Immunohistochemical analysis of KIF20A expression was performed on 56 cases of HNMT (39 squamous cell carcinoma, 14 adenoid cystic carcinoma, 2 osteosarcoma, and 1 angiosarcoma tissue specimens). Twenty-six of the 39 head and neck squamous cell carcinomas (67%), 4 of the 14 adenoid cystic carcinomas (29%), and 1 of the 2 osteosarcoma (50%) showed positive expression of KIF20A (Supplementary table 3). No staining was detected in the benign tumor samples.

In context of cancer immunotherapy, there is strong evidence suggesting that vaccines using restricted epitopes can result in broad CD8+ T-cell responses to antigens not present in the vaccine (37-39). Thus, we considered that KIF20A-specific Th cell responses may be induced by vaccination with TAA-derived CTL-epitope peptides, which do not include KIF20A-derived SPs. We assessed T-cell responses specific for KIF20A in peripheral blood from 16 patients who were receiving immunotherapy for treatment of HNMT. The donor characteristics are summarized in Table 1. After 1 week of in vitro stimulation of PBMCs with KIF20A-LPs, the frequency of individual KIF20A-LP-specific T-cells was detected by IFN-γ ELISPOT assay (Figure 5A). PBMCs isolated from 9 healthy volunteers were used as controls. Responses were
considered positive when the number of IFN-γ-secreting cells was at least 2-fold above the negative control. KIF20A-specific Th cell responses were observed in 8 of 16 patients (KIF20A\textsubscript{60–84}-LP, 2 of 16, 13%; KIF20A\textsubscript{809–833}-LP, 7 of 16, 44%), but no specific immune responses to KIF20A-LPs were detected in the 9 healthy donors (Table 1). We also found that the number of specific spots against KIF20A\textsubscript{60–84}-LP and KIF20A\textsubscript{809–833}-LP in patients were significantly larger than in healthy donors (Figure 5B). KIF20A\textsubscript{809–833}-LP-specific IFN-γ production by T-cells in HNMT31 and HNMT43 was significantly inhibited by addition of anti-HLA-DR mAb but not by anti-HLA-class I mAb (Figure 5C). Interestingly, specific responses to KIF20A\textsubscript{809–833}-LP were augmented in some patients (HNMT31 and 42), or induced in HNMT43 during the course of immunotherapy (Figure 5D). As shown in Figure 5E, KIF20A antigen was expressed in patients with HNMT in whom KIF20A-specific Th1 cell responses were detected (Table 1, HNMT31 and HNMT108), but was not expressed in those for whom KIF20A-specific Th1 cell responses were not detected (Table 1, HNC102 and 107). These observations suggest that APCs collected and processed a KIF20A antigen derived from tumor cells expressing KIF20A, and then activated KIF20A-specific Th1 cells \textit{in vivo}. 
Discussion

We identified 2 promiscuous KIF20A-derived Th cell epitopes bearing known HLA-A2- or 24-restricted CTL-epitopes. KIF20A-specific CTLs were induced by stimulation with KIF20A-LPs \textit{in vitro} and \textit{in vivo}. This is the first demonstration of KIF20A expression in HNMT, and the presence of KIF20A-specific Th1 cell responses in patients with HNMT. Furthermore, these responses were related to KIF20A expression in HNMT tissues. We postulate that these KIF20A-LPs-specific Th1 cell responses may occur through the KIF20A antigen in apoptotic bodies released from tumor cells. When collected and processed by APCs, the KIF20A antigen most probably activates KIF20A-specific Th1 cells \textit{in vivo}.

Recent reports showed that a new wave of tumor-specific CTL clones become detectable in the blood after vaccination and provide convincing evidence that the phenomenon of antigen spreading is critical to the development of effective antitumor immunity (37-41). The interaction between antivaccine CTLs and the tumor facilitates stimulation of large numbers of antitumor CTLs that proceed to destroy the tumor cells. However, CD4$^+$ T-cell responses against a TAA in tumor-bearing patients vaccinated with CTL-epitope peptides have not been investigated in detail. In this study, we analyzed CD4$^+$ T-cell responses to a KIF20A antigen, which was not present in the vaccine cocktail, in the patient’s blood before and after vaccination to investigate the phenomenon of antigen spreading triggered by CTL-epitope vaccination. In patient
HNMT43 with advanced cancer, the KIF20A\textsubscript{809–833}-LP-specific Th1 response was absent before vaccination, but after vaccination this response was significantly induced (Figure 5D, right panel). We believe that this KIF20A\textsubscript{809–833}-LP-specific Th1 cell response was a phenomenon of antigen spreading triggered by vaccination with CTL-epitopes.

Aarntzen et al. have recently reported that targeting Th cells with DCs pulsed with both HLA class I and II-restricted epitopes enhances vaccine-specific immunologic responses and improves clinical responses (42). We demonstrated that KIF20A-LPs and Th1-clones enhanced induction of HLA-A2-restricted KIF20A-specific CTLs \textit{in vitro}. These findings indicate that KIF20A-LPs can augment the induction of antigen-specific CTLs in combination with immunotherapy using KIF20A CTL-epitope peptides. We speculate that immunotherapy with peptide vaccinations using KIF20A-LPs and KIF20A-SPs may improve clinical outcomes in KIF20A-expressing cancer.

HLA-class II restriction of the KIF20A\textsubscript{809–833}-LP specific CD4\textsuperscript{+} T-cell response in HNMT31 was confirmed by inhibition of IFN-\(\gamma\) secretion in the presence of HLA-DR blocking antibody (Figure 5C), although HLA-DR alleles of HNMT31 (Table 1; \textit{DRB1*01:01/11:01}) were not shared by the HLA-DR alleles which encode HLA-class II-molecules presenting KIF20A\textsubscript{809–833}-LP to Th cells in healthy donors (HLA-DR15 and DR53, Figure 1). This result supports the promiscuous nature of KIF20A\textsubscript{809–833}-LP and indicates that LP was naturally processed and presented by HLA-class II molecules.
In vivo.

In this study, significant frequencies of KIF20A-specific Th1 cell responses were detected in patients with HNMT receiving immunotherapy (8 of 16, 50%). Godet et al. reported a possible synergistic effect of the telomerase-specific CD4+ T-cell response with chemotherapy in lung cancer. They demonstrated that the existence of spontaneous telomerase-specific Th1 cells prior to first-line chemotherapy significantly increases overall survival in lung cancer that responds to chemotherapy (43). In addition, the recent introduction of immunotherapy in clinical practice emphasized the influence of immune responses on cancer prognosis and chemotherapy effectiveness (11, 12, 44). These pieces of evidence support the hypothesis that induction of KIF20A-specific Th1 cells by KIF20A-LP vaccination may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies (45-47). Weide et al. have recently reported that the presence of circulating Th1 cells responding to Melan-A or NY-ESO-1 has a strong independent prognostic impact on survival among chemotherapy-treated advanced melanoma patients (48). Thus, KIF20A-LPs-specific Th1 cell responses in patients with HNMT receiving immunotherapy may positively influence overall survival. The impact of KIF20A-specific Th1 cell responses on clinical outcome will be evaluated in a future study.

Recent studies of LPs have shown that vaccines containing natural CTL-epitopes are superior to those comprising minimal CTL-epitopes in antitumor CTL
immunity because of long-lasting cross-presentation of the LPs (10, 49). We showed that KIF20A-LPs stimulated KIF20A-specific CTLs in vitro and in vivo. However, we did not compare the capacity to induce KIF20A-specific CTLs between KIF20A-LPs and KIF20A-SPs in human cells. In vivo assay using HLA-A24 Tgm, vaccination with KIF20A_{60–84}-LP was not superior to KIF20A-A24_{66–75} SP in induction of KIF20A-specific CTLs (data not shown). Therefore, we are not able to conclude that whether we should use KIF20A-LP encompassing CTL-epitope alone or both KIF20A-SP and the KIF20A-LP to elicit stronger anti-tumor T-cell immunity at this moment. These issues will be evaluated in a future study.

In this study, we did not confirm the response of generated Th cells to the shorter, 15-mer Th epitope from KIF20A-LPs, because we considered that these 25-mer amino-acid long KIF20A-LPs encompass various both HLA-class I and class II-restricted T-cell epitopes including unconfirmed T-cell epitopes by in vitro experiments and could cover many cancer patients. Based on the findings from healthy donors, two KIF20A-derived LPs, KIF20A_{60–84}-LP and KIF20A_{809–833}-LP, cover at least 93% of Japanese individuals. We are planning to use these 25-mer KIF20A-LPs in a future clinical trial of peptide-based immunotherapy.

In conclusion, we identified promiscuous Th cell epitopes derived from KIF20A and present the first clinical evaluation of KIF20A-specific Th1 cell responses in patients with HNMT receiving immunotherapy. These Th cell epitopes provide a tool
for propagation of KIF20A-specific Th1 cells and CTLs. These findings support a clinical trial of KIF20A peptide-based immunotherapy for cancer treatment.
Acknowledgments

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References


Figure Legends

Figure 1. Induction of KIF20A-specific Th cells from healthy donors. A, KIF20A-specific Th cells were generated from a DR15\(^+\) healthy donor (HD1) by stimulation of purified CD4\(^+\) T-cells with KIF20A\(_{60-84}\)-LP. The generated Th cells were restimulated with autologous PBMCs or L-cells pulsed with KIF20A\(_{60-84}\)-LP. The number of IFN-\(\gamma\)-producing Th cells was analyzed by ELISPOT assay. Data are presented as the mean ± SD of triplicate assays. Representative data from at least 3 independent experiments with similar results obtained from HD1 are shown. The HLA class-II genotype of HD1 is indicated above the panels. The underlined HLA-class II alleles encode the HLA-class II-molecule presenting the peptides to Th cells. B, HLA-DP2-restricted KIF20A\(_{60-84}\)-LP-specific bulk Th cells (left panel) or a Th-clone (right panel) derived from HD2 were established. An HLA-DP-restricted Th-clone was cocultured with allogeneic PBMCs derived from HLA-DP2-positive or negative donors pulsed/unpulsed with KIF20A\(_{60-84}\)-LP. C, KIF20A\(_{809-833}\)-LP-specific HLA-DR15-restricted Th cells were generated from a healthy donor (HD1). D, KIF20A\(_{809-833}\)-LP-specific HLA-DR53-restricted Th cells were generated from a healthy donor (HD2).

Figure 2. Natural processing and presentation of KIF20A-LPs by DCs. A, An
HLA-DP2-restricted KIF20A<sub>60-84</sub>-LP-specific Th-clone established from HD2 recognized autologous DCs loaded with a recombinant KIF20A protein. B, An HLA-DR53-restricted KIF20A<sub>809-833</sub>-LP-specific Th-clone established from HD2 recognized autologous DCs loaded with a recombinant KIF20A protein. The relevant KIF20A-derived LP-pulsed DCs were used as positive controls. Representative data from 3 independent experiments with similar results are shown.

**Figure 3.** Cytokine profile produced by KIF20A-LPs-specific bulk Th cells. A, After 24 h incubation of Th cells cocultured with autologous PBMCs (for KIF20A<sub>60-84</sub>-LP-specific bulk Th cells) or L-DR53 (for KIF20A<sub>809-833</sub>-LP-specific bulk Th cells) in the presence of cognate peptides, the culture supernatant was collected and the concentration of cytokines (TNF-α, IFN-γ, GM-CSF, MIP1β, IL-2, IL-4, and IL-7) 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 KIF20A-specific CD4<sup>+</sup> T-cells after antigenic stimulation. Cells were restimulated with cognate LPs or a control peptide. Events shown are gated for CD4<sup>+</sup> T-cells. The numbers inside the plots indicate the percentage of the cell population with the quadrant characteristic (CD4<sup>+</sup> CD107a<sup>+</sup> T-cells).

**Figure 4.** KIF20A-LPs induce efficient expansion of KIF20A-specific CTLs *in vitro*
and in vivo. A, Enhanced induction of KIF20A-A2<sub>809–817</sub> SP-specific CTLs in response to stimulation with KIF20A<sub>60–84</sub>-LP and KIF20A<sub>60–84</sub>-LP-specific Th clones. PBMCs obtained from HLA-A2<sup>+</sup>/DP2<sup>+</sup> HD2, from which an HLA-DP2-restricted KIF20A<sub>60–84</sub>-LP-specific CD4<sup>+</sup> Th-clone was generated, were cultured for 11 days with KIF20A-A2<sub>809–817</sub> SP (SP), SP + KIF20A<sub>60–84</sub>-LP (LP), or SP + LP + KIF20A<sub>60–84</sub>-LP-specific Th clone (Th-clone). On day 11, the cells were stained with KIF20A-A2<sub>809–817</sub> SP-specific tetramer with an anti-human CD8 mAb and analyzed by flow cytometry. B, Representative KIF20A-A2<sub>809–817</sub> SP-specific tetramer staining (gated on CD8<sup>+</sup> T-cells) obtained from 3 independent experiments with similar results has been shown. C, The KIF20A<sub>809–833</sub>-LP stimulated KIF20A-A2<sub>809–817</sub> SP-specific CD8<sup>+</sup> T cells in vitro. The number of IFN-γ-producing KIF20A-A2<sub>809–817</sub> SP-specific bulk CTL in response to stimulation with the KIF20A<sub>809–833</sub>-LP-loaded or control LP-loaded autologous DCs was counted by ELISPOT assay. Statistically significant differences (p < 0.05) are indicated with asterisks. Representative data from 3 independent experiments with similar results are shown. D, PBMCs from HLA-A24<sup>+</sup> donors (HD4 and HD8) were incubated with KIF20A<sub>60–84</sub>-LP for 2 weeks. On days 0 and 7, KIF20A<sub>60–84</sub>-LP was added. On day 14, the cells were harvested and the number of IFN-γ producing T-cells (1 × 10<sup>5</sup> cells/well) in response to stimulation with KIF20A-A2<sub>466–75</sub> SP-pulsed C1R-A2402 cells (2 × 10<sup>4</sup> cells/well) was counted by ELISPOT assay. Representative data from 5 independent experiments with similar
results are shown.

**Figure 5.** Presence of KIF20A-LPs-specific Th cells in PBMCs isolated from patients with HNMT receiving immunotherapy with TAA-derived CTL-epitope peptides. **A,** After *in vitro* stimulation of PBMCs with a mixture of KIF20A<sub>60-84</sub>-LP and KIF20A<sub>809-833</sub>-LP for 1 week, the frequency of individual KIF20A-LPs-specific T-cells was detected by IFN-γ ELISPOT assay. **B,** KIF20A-LPs-specific Th1 cell responses were assessed in 16 patients with HNMT receiving immunotherapy and in 9 healthy donors. 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. **C,** HLA class II-restriction of the IFN-γ-producing KIF20A<sub>809-833</sub>-LP-specific Th cells in HNMT31 and HNMT43. Peripheral blood mononuclear cells stimulated with LPs for 1 week were restimulated with KIF20A<sub>809-833</sub>-LP in the presence of mAbs specific to HLA-DR, -DP, -DQ, or HLA-class I. **D,** KIF20A-LPs-specific Th1 cell responses in patients with HNMT were detected during the course of immunotherapy. **E,** Immunohistochemical analyses of the KIF20A protein in cancer tissues and osteosarcoma (original magnification × 400). Positive KIF20A immunohistochemical staining on tissue sections of adenoid cystic carcinoma in HNMT31 and osteosarcoma in HNMT108 are shown. Negative KIF20A immunohistochemical staining on tissue sections of
squamous cell carcinoma in HNMT102 and osteosarcoma in HNMT107 are also shown. Malignant cells positive for KIF20A showed homogeneous cytoplasmic staining. Expression of KIF20A in HNMT tissues was associated with KIF20A-LPs-specific Th1 cell responses in patients with HNMT.
**Figure 1**

**Effector cells: KIF20A<sub>60-84</sub>-LP-specific bulk Th cells**

**A**

HD1: DRB<sup>*</sup>08:03/15:02

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APC: autologous PBMC

**B**

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APC: autologous PBMC

**D**

HD2: DRB<sup>*</sup>01:04/05:09:01, DR53

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APC: autologous PBMC

**HLA-DPB1 genotypes of allo-PBMCs**

DP<sup>*</sup>02:01/09:01  DP<sup>*</sup>02:01/  DP<sup>*</sup>05:01/  DP<sup>*</sup>05:01/09:01

**APC:** autologous PBMC

**APC:** allo-PBMC

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Figure 2

A

HD2 : DPB1*02:01/04:02

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Effector cells

KIF20A_{60-84}LP specific Th-clone

B

HD2 : DRB1*04:05/09:01, DR53

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Effector cells

KIF20A_{809-833}LP specific Th-clone

APC: DC
Figure 3

A

HD2: DPB1*02:01/04:02

KIF20A_60-84-LP-specific bulk Th cells

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</table>

Cognate Peptide

HD2: DRB1*04:05/09:01, DR53

KIF20A_809-833-LP-specific bulk Th cells

Cytokine levels (pg/ml)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Peptide</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
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<td>TNF-α</td>
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<td>3000</td>
<td>0</td>
<td>8000</td>
<td>0</td>
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<td>0</td>
<td>8000</td>
<td>0</td>
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<tr>
<td>IFN-γ</td>
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<td>800</td>
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<td>0</td>
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<td>0</td>
<td>2000</td>
<td>0</td>
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<tr>
<td>GM-CSF</td>
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<td>400</td>
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<td>1000</td>
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<td>MIP1β</td>
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<td>IL-2</td>
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<tr>
<td>IL-17</td>
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</tbody>
</table>

Cognate Peptide

B

HD2: DPB1*02:01/04:02

KIF20A_60-84-LP-specific bulk Th cells

Stimulation: Irrelevant peptide

Gated on CD4+ T-cells

HD2: DRB1*04:05/09:01, DR53

KIF20A_809-833-LP-specific bulk Th cells

Stimulation: Irrelevant peptide

Gated on CD4+ T-cells

CD107α

1.5% 38.3%

KIF20A_60-84-LP

0.5% 45.0%

KIF20A_809-833-LP

CD4
Figure 4

A

HD2 : HLA-A2⁺, DP2⁺, DR53⁺

SP : KIF-A₂₈₀₉-₈₁₇
LP : KIF2₀₆₅₆₄⁻LP

Th-clone : KIF2₀₆₅₆₄⁻LP specific Th cell clone

PBMCs +

Peptides (SP ± LP) ± Th-clone

Day 0

Day 7

Day 9

Day 11

Flow cytometry

B

 SP

SP+LP

SP + LP + Th-clone

CD8

Gated on CD8⁺ T-cells

C

HD5 : HLA-A2⁺, DR53⁺

Effector cell:
KIF2₀₆₅₆₄⁻SP specific bulk CTLs

D

HD8 : HLA-A₂₄⁺, DP2⁺

In vitro stimulation with KIF2₀₆₅₆₄⁻LP

APC: C₁R-A₂₄₀₂ cell

APC: DC
Figure 5

A PBMCs + KIF20A-LPs → IFN-γ ELISPOT assay

Day 0  Day 2  Day 7
IL-2 / IL-7

B KIF20A_{60-84}-LP

KIF20A_{809-833}-LP

Specific IFN-γ spots / 10^5 cells

HNMT patients Healthy donors

P<0.01
n=16
n=9

P<0.001
n=16
n=9

C

KIF20A_{809-833}-LP

Specific IFN-γ spots / 10^5 cells

HNMT31

HNMT43

Blocking mAb (○)
Anti-DR
Anti-DQ
Anti-class I

D

KIF_{60-84}-LP  KIF20A_{809-833}-LP

Specific IFN-γ spots / 10^5 cells

HNMT31

HNMT42

HNMT43

Number of vaccinations

16  20  Pre-vac. 4  Pre-vac. 4

E

IHC staining : Positive

IHC staining : Negative

HNMT31

Adenoid cystic carcinoma

Positive

HNMT108

Osteosarcoma

Positive

HNMT107

Osteosarcoma

Negative

HNMT102

Squamous cell carcinoma

Negative

KIF20A-specific CD4^+ T-cell response
Table 1. Clinical characteristics of HNMT patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age/Sex</th>
<th>KIF20A-specific CD4&lt;sup&gt;+&lt;/sup&gt; T-cell responses&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Histologic subtype</th>
<th>IHC analysis of KIF20A</th>
<th>HLA-DRB1</th>
<th>HLA-DRB4</th>
<th>HLA-DPB1</th>
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<tbody>
<tr>
<td>CTR-8379</td>
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<td>Positive / Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2/16 (13%) 7/16 (44%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HNMT10</td>
<td>61/M</td>
<td>-</td>
<td>Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>01:01 / 04:05</td>
<td>DR53</td>
<td>05:01 / -</td>
</tr>
<tr>
<td>HNMT20</td>
<td>57/F</td>
<td>-</td>
<td>Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>01:01 / 09:01</td>
<td>DR53</td>
<td>02:01 / 05:01</td>
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<tr>
<td>HNMT26</td>
<td>70/M</td>
<td>-</td>
<td>Basaloid Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>04:05 / 15:02</td>
<td>DR53</td>
<td>05:01 / 09:01</td>
</tr>
<tr>
<td>HNMT29</td>
<td>64/F</td>
<td>-</td>
<td>Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>09:01 / 14:54</td>
<td>DR53</td>
<td>03:01 / 05:01</td>
</tr>
<tr>
<td>HNMT31</td>
<td>69/F</td>
<td>-</td>
<td>Adenoid Cystic Carcinoma</td>
<td>Positive</td>
<td>01:01 / 11:01</td>
<td>-</td>
<td>02:01 / 04:02</td>
</tr>
<tr>
<td>HNMT34</td>
<td>65/M</td>
<td>-</td>
<td>Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>08:03 / 15:02</td>
<td>-</td>
<td>02:01 / 05:01</td>
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<tr>
<td>HNMT39</td>
<td>77/M</td>
<td>-</td>
<td>Adenoid Cystic Carcinoma</td>
<td>n.t.</td>
<td>04:06 / 14:54</td>
<td>DR53</td>
<td>05:01 / 19:01</td>
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<tr>
<td>HNMT40</td>
<td>76/M</td>
<td>-</td>
<td>Squamous Cell Carcinoma</td>
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<td>DR53</td>
<td>04:02 / 05:01</td>
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<tr>
<td>HNMT41</td>
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<td>01:01 / 04:05</td>
<td>DR53</td>
<td>04:02 / 05:01</td>
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<td>36/F</td>
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<td>Unknown</td>
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<td>04:02 / 05:01</td>
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<td>HNMT43</td>
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<td>Squamous Cell Carcinoma</td>
<td>n.t.</td>
<td>08:02 / 09:01</td>
<td>DR53</td>
<td>05:01 / -</td>
</tr>
</tbody>
</table>

| CTR-8380   |         | Positive / Total                                  |                 |                      |         |         |         |
|            |         | 2/16 (13%) 7/16 (44%)                             |                 |                      |         |         |         |
| HNMT102    | 80/F    | -                                                 | Squamous Cell Carcinoma | Negative | 15:02 / - | - | 02:01 / 09:01 |
| HNMT105    | 65/M    | -                                                 | Angiosarcoma | Negative | 04:05 / 13:02 | DR53 | 03:01 / 04:01 |
| HNMT107    | 20/M    | -                                                 | Osteosarcoma | Negative | 09:01 / - | DR53 | 02:01 / 02:02 |
| HNMT108    | 41/M    | +                                                 | Osteosarcoma | Positive | 04:05 / 09:01 | DR53 | 05:01 / - |

<sup>a</sup>KIF20A-specific T-cell responses measured by IFN-γ ELISPOT assay as detailed in the Materials and Methods section. Positive and negative responses are denoted by (+) and (-), respectively. The underlined HLA-class II alleles encode HLA-class II-molecules presenting KIF20A-LP to Th cells in healthy donors (Figure 1; HLA-DRB1*15:02, DR53, and DPB1*02:01). IHC, Immunohistochemistry; CTR, Clinical Trials Registry; HNMT, Head-and-neck malignant tumor; M/F, male/female; LP, long peptide; n.t., not tested; DR53, DRB4*01:03.
Identification of promiscuous KIF20A long peptides bearing both CD4+ and CD8+ T-cell epitopes: KIF20A-specific CD4+ T-cell immunity in patients with malignant tumor

Yusuke Tomita, Yuno Akira, Hirotake Tsukamoto, et al.

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