Identification of Promiscuous KIF20A Long Peptides Bearing Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell Epitopes: KIF20A-Specific CD4<sup>+</sup> T-cell Immunity in Patients with Malignant Tumor

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Abstract

**Purpose:** To identify long peptides (LP) derived from a novel tumor-associated antigen (TAA), kinesin family member 20A (KIF20A), which induce tumor-specific T-helper type 1 (T<sub>H1</sub>) 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 T<sub>H1</sub>-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 T<sub>H1</sub>-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<sup>+</sup> 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 T<sub>H1</sub> 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 showing the presence of KIF20A-specific T<sub>H1</sub> cell responses in HNMT patients and underline the possible utility of KIF20A-LPs for propagation of T<sub>H1</sub> cells and CTLs. Clin Cancer Res; 19(16); 4508–20. ©2013 AACR.

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 (SP) derived from KIF20A, KIF20A-A2<sub>809–917</sub>, and KIF20A-A24<sub>466–75</sub> stimulated CTLs, which kill cancer cells endogenously expressing KIF20A antigen and that KIF20A-specific CTLs were present in peripheral blood mononuclear cells (PBMC) obtained from patients with pancreatic cancer (1, 4). Phase I/II clinical trials of cancer immunotherapy for lung cancer, pancreatic cancer, and cholesterolcellular 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 LP that induce both antigen-specific CD4\(^+\) helper T (Th) cells and CTLs 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). IFN-\(\gamma\) produced by Th1 cells is critical for induction and maintenance of long-lived CTL responses through multiple interactions (6, 7). IFN-\(\gamma\) secreted by Th1 cells also mediates direct antitumor or antiangiogenic effects (8). Furthermore, Th1 cells pave the way for entry of CTLs at the tumor site (9). Therefore, identification of Th1-cell epitopes that can activate tumor-specific Th1 cells is important for induction of effective tumor immunity in tumor-bearing hosts.

Melief and colleagues recently reported a synthetic LP naturally bearing a CTL epitope as an attractive vaccine compound. Following injection of LP, a patient’s dendritic cells take up the LP, process it, and present all possible CTL epitopes and Th1-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 HLA-A24–restricted CTLs were used to select candidate promiscuous Th1-cell epitopes containing the CTL epitopes. Our results show that the predicted LPs triggered Th1 responses in individuals expressing several common HLA-DR or HLA-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 Th1 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 SPs (clinical-grade 9-10-amino acid-SPs) derived from 3 cancer-testis antigens, LY6K (LY6K-A24177–186), IMP-3 (IMP-3-A24508–516), and CDCA1 (CDCA1-A24506–64) were reviewed and approved by the Institutional Review Board of Kumamoto University (Kumamoto, Japan; ref. 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 00008379 (CTR-8379). The patients with radical resection were enrolled in the trial under UMIN-CTR number 00008380 (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 (APC). The HLA-A24-positive C1R-A2402 cells were a gift from Dr. M. Takiguchi (Kumamoto University; ref. 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; refs. 18 and 19). The program analyzed 15-amino acid-long sequences offset to encompass the entire protein. The 24- and 25-amino acid LPs 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, KIF20A949–454-LP (DSMEKVKVLVRRPLLPSIVERQED), KIF20A-A499–517-LP (TLHVAKFSAIASQLVHAPPMQLGF), and KIF20A-A809–833-LP (CIAEQYHTVKLQGQVSAKKRLGTN) were selected (Supplementary Fig. S1 and Table S1).

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**Translational Relevance**

We recently identified a novel tumor-associated antigen, 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 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.
Synthetic peptides and recombinant proteins

Human KIF20A-derived SPs presented by HLA-A2 (KIF20A-A2809–817) or HLA-A24 (KIF20A-A2466–75), and 3 LPs (KIF20A60–84-LP, KIF20A494–517-LP, and KIF20A809–833-LP) were synthesized (MBL, Nagoya, Japan; purity >95%; Supplementary Fig. S1B). 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+ T cells from healthy donors

The research protocol for collecting and using PBMCs from healthy donors (HD1–HD11; Supplementary Table S2) 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 conducted at the HLA Laboratory (Kyoto, Japan; Supplementary Table S2). With some modifications, induction of antigen-specific CD4+ T cells was conducted as described previously (22). Detailed methods are provided in Supplementary 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 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 × 10^6 bulk CD4+ T cells in response to stimulation with peptide-pulsed PBMCs (3 × 10^6 cells/well), or 1 × 10^6 bulk CD4+ T cells in response to stimulation with peptide-pulsed L-cells expressing HLA-DR (5 × 10^5 cells/well) was analyzed. The frequency of KIF20A-LP–specific T-cell clone (T11 clone) producing IFN-γ by 2 × 10^5 T11 clone in response to stimulation with protein-loaded dendritic cells (5 × 10^5 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; refs. 24–26). All mAbs were used at a final concentration of 5 μg/mL. All assessments of IFN-γ ELISPOT assays were conducted 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) was conducted as described previously (1, 2, 27).

Cytokine assays and CD107a mobilization assay

HLA-DP2–restricted KIF20A60–84–LP–specific bulk T cell (3 × 10^4 cells/well) were cultured with autologous PBMCs (3 × 10^6 cells/well) in the presence of cognate peptide in 96-well culture plates. HLA-DR53–restricted KIF20A809–833–LP–specific bulk T cell (3 × 10^4 cells/well) were used in the presence of cognate peptide. After 24 hours, culture supernatants were collected and cytokine [IFN-α, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)1β, interleukin (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 Supplementary Materials and Methods.

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

PBMCs obtained from an HLA-A2+/DR53+ donor (HD2), from whom the KIF20A60–84–LP or KIF20A809–833–LP–specific T11 clones were generated, were plated in 24-well plates (3 × 10^6 cells/well) followed by addition of SP alone (KIF20A-A2809–817 SP, 20 μg/mL), or SP + LP (KIF20A60–84–LP or KIF20A809–833–LP, 20 μg/mL), or SP + LP + Th-clone (5 × 10^5 cells/well) in a final volume of 2 mL. After culture for 7 days, these peptides and IL-2 (20 U/mL) were added, then IL-15 (5 ng/mL) was added on day 9. On day 11, cells were stained with phycoerythrin-labeled tetramer of the HLA-A*02:01/KIF20A-A2809–817–complex and a fluorescein isothiocyanate–labeled anti-human CD8 mAb. Data acquisition was conducted on a FACS caliber (BD Biosciences), and data files were analyzed with FlowJo software (Tree Star).

In vitro stimulation of KIF20A-A2809–817 SP-specific CTLs with KIF20A809–833–LP

Induction of KIF20A-A2809–817 SP–reactive bulk CTLs from an HLA-A2+ and DR53+ donor (HD5) by stimulation with KIF20A-A2809–817 SP was conducted as described (28, 29). In brief, purified human CD8+ T cells were stimulated with KIF20A-A2809–817 SP–pulsed dendritic cells. After 3 rounds of stimulation, we confirmed that CTLs specifically produced IFN-γ in response to restimulation with T2 cells pulsed with KIF20A-A2809–817 SP; thus, the KIF20A-A2809–817 SP–reactive CTLs were used as effector cells.

To prove the stimulation of KIF20A-A2809–817 SP–reactive CTLs with KIF20A809–833–LP–loaded dendritic cells, the number of IFN-γ producing KIF20A-A2809–817 SP–specific bulk CTLs in response to stimulation with the
KIF20A<sub>60–84</sub>-LP–loaded autologous dendritic cells were counted by an ELISPOT assay. The LP-loaded mature dendritic cells 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>60–84</sub>-LP (50 μg/mL) and OK432 were added on day 5. The LP-loaded mature dendritic cells 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>494–517</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 recombinant human (rh) IL-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>494–517</sub>-LP, or control LP for 18 hours. The number of KIF20A-LP–specific T<sub>H</sub> 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 twofold 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 conducted using investigative protocols. We acknowledge the recommendations of the Minimal Information About T-cell Assays reporting framework for human T-cell assays (30).

Statistical analysis

Data were compared by the two-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 T<sub>H</sub> cell epitopes of KIF20A, we first examined the amino acid sequence of KIF20A using a recently developed computer algorithm (Supplementary Fig. S1A; refs. 18 and 19). Two LPs, KIF20A<sub>60–84</sub>-LP and KIF20A<sub>494–517</sub>-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 Fig. S1B and Table S1). Another peptide, KIF20A<sub>494–517</sub>-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, KIF20A<sub>60–84</sub>-LP, KIF20A<sub>494–517</sub>-LP, and KIF20A<sub>809–833</sub>-LP, predicted to have strong binding affinity to HLA class II molecules HLA-DR4, HLA-DR15, or HLA-DR53 were synthesized for subsequent analyses.

Identification of promiscuous KIF20A-derived T<sub>H</sub>-cell epitopes

CD4<sup>+</sup> T cells isolated from PBMCs of healthy donors were stimulated at weekly intervals with autologous dendritic cells or PBMCs pulsed with KIF20A<sub>60–84</sub>-LP as described in the Materials and Methods section. After at least 3 rounds of stimulation, KIF20A<sub>60–84</sub>-LP–specific responses of CD4<sup>+</sup> T cells were examined by IFN-γ ELISPOT assays. In an HLA-DR15<sup>+</sup> HD1, the generated T<sub>H</sub> cells produced a significant amount of IFN-γ in response to KIF20A<sub>60–84</sub>-LP–pulsed PBMCs in an HLA-DR–dependent manner. The bulk T<sub>H</sub> cells specifically recognized L-DR15 cells pulsed with KIF20A<sub>60–84</sub>-LP in an HLA-DR–dependent manner, but not KIF20A<sub>60–84</sub>-LP–pulsed L-DR8 cells (Fig. 1A). These results indicated that KIF20A<sub>60–84</sub>-LP was presented by HLA-DR15.

To investigate whether KIF20A<sub>60–84</sub>-LP induces responses in T<sub>H</sub> cells restricted by other HLA class II molecules, CD4<sup>+</sup> T cells from HLA-DR15-negative healthy donors were tested. The T<sub>H</sub> cells generated from HLA-DP<sup>2</sup> HD2 produced a significant amount of IFN-γ in response to KIF20A<sub>60–84</sub>-LP–pulsed PBMCs in an HLA-DP–dependent manner. L cells transduced with HLA-DP<sup>2</sup> were unavailable; therefore, we established a KIF20A<sub>60–84</sub>-LP–reactive T<sub>H</sub> cell clone (T<sub>H</sub> clone) and used allogeneic PBMCs from 4 different donors as APCs to determine restriction by shared HLA-DP molecules. We confirmed that KIF20A<sub>60–84</sub>-LP generates HLA-DP<sup>2</sup>–restricted T<sub>H</sub> cells (Fig. 1B). Thus, KIF20A<sub>60–84</sub>-LP binds to HLA-DR15 and HLA-DP<sup>2</sup>, which suggests that KIF20A<sub>60–84</sub>-LP is a promiscuous T<sub>H</sub> cell epitope presented by frequent HLA class II molecules (31, 32). In addition, KIF20A<sub>60–84</sub>-LP could induce antigen-specific and HLA-DR–restricted bulk T<sub>H</sub> cells from HD3, who was HLA-DR4<sup>+</sup> and DR53<sup>+</sup>, but DR15- and DP2-negative (Supplementary Fig. S2A). Although we were unable to define the HLA class II molecule presenting the LP to the T<sub>H</sub> cells, this result supports the promiscuous nature of KIF20A<sub>60–84</sub>-LP.

Next, we assessed whether KIF20A<sub>60–84</sub>-LP could generate antigen-specific T<sub>H</sub> cells. The T<sub>H</sub> cells generated from HLA-DR15<sup>+</sup> healthy donors (HD1 and HD4) produced a significant amount of IFN-γ in response to KIF20A<sub>60–84</sub>-LP–pulsed PBMCs in an HLA-DR–dependent manner (Fig. 1C and Supplementary Fig. S2B). The bulk T<sub>H</sub> cells specifically recognized L-DR15 cells pulsed with KIF20A<sub>60–84</sub>-LP in an HLA-DR–dependent manner (Fig. 1C, HD1). CD4<sup>+</sup> T cells from an HLA-DR15-negative healthy donor (HD2) were tested to investigate whether KIF20A<sub>60–84</sub>-LP induces responses in T<sub>H</sub> cells restricted by other HLA class II molecules. KIF20A<sub>60–84</sub>-LP could generate HLA-DR53–restricted T<sub>H</sub> cells in this donor (Fig. 1D). These results show that KIF20A<sub>60–84</sub>-LP is also a promiscuous T<sub>H</sub> cell epitope.

The immunogenicity of KIF20A<sub>494–517</sub>-LP, which did not include a known CTL epitope was evaluated. KIF20A<sub>494–517</sub>-LP could generate HLA-DR4–restricted bulk T<sub>H</sub> cells in
specific TH clone efficiently recognized dendritic cells load-and process the KIF20A protein to stimulate KIF20A-specific processed and presented by dendritic cells KIF20A60–84-LP and KIF20A809–833-LP, for further studies.

on promiscuous LPs bearing known CTL epitopes, such as DR53–restricted KIF20A809–833-LP–specific TH clone effi-ciently recognized dendritic cells loaded with KIF20A protein in an HLA-DR–dependent manner, but did not recog-nize control protein–loaded dendritic cells, indicating that this epitope was naturally processed and presented by HLA-DR15–restricted TH cells were generated from a healthy donor (HD1). D, KIF20A809–833-LP–specific HLA-DR53–restricted TH cells were generated from a healthy donor (HD2).

Figure 1. Induction of KIF20A–specific Tc9 cells from healthy donors. A, KIF20A–specific Tc9 cells were generated from a DR15+ healthy donor (HD1) by stimulation of purified CD4+ T cells with KIF20A60–84-LP. The generated Tc9 cells were restimulated with autologous PBMCs or L cells pulsed with KIF20A60–84-LP. The number of IFN-γ–producing Tc9 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 Tc9 cells. B, HLA-DP2–restricted KIF20A60–84-LP–specific bulk Tc9 cells (left) or a Tc9 clone (right) derived from HD2 was established. An HLA-DP–restricted Tc9 clone was cocultured with allogeneic PBMCs derived from HLA-DP2-positive or HLA-DP2-negative donors pulsed/unpulsed with KIF20A60–84-LP. C, KIF20A809–833-LP–specific HLA-DR15–restricted Tc9 cells were generated from a healthy donor (HD1). D, KIF20A809–833-LP–specific HLA-DR53–restricted Tc9 cells were generated from a healthy donor (HD2).
tetramer+ cells was 0.24% of CD8+ T cells (Fig. 4B). Addition of KIF20A809–833-LP (LP) into the SP culture (SP + LP) induced a slight increase in the frequency of tetramer+ cells. In contrast, when the PBMCs were costimulated with KIF20A-A2809–817 SP, KIF20A60–84-LP, and KIF20A809–833-LP (LP)-specific T11 clone (LP + T11 clone), the frequency of KIF20A-A2809–817 SP-specific CTLs increased significantly to 2.87%. As shown in Supplementary Fig. S3A and S3B, KIF20A809–833-LP alone (LP), which encompasses KIF20A-A2809–817 SP, or addition of KIF20A809–833-LP (LP) into the SP culture (SP + LP) induced a slight increase in the frequency of KIF20A-A2809–817 SP-specific CTLs. However, the KIF20A809–833-LP (LP)-specific T11 clone rapidly increased in response to the KIF20A809–833-LP when we added both LP and T11 clone into the PBMCs without KIF20A-A2809–817 SP (LP + T11 clone) and then we could not detect the increase of frequency of KIF20A-A2809–817 SP-specific CTLs. We also observed that the stimulation of PBMCs with KIF20A-A2809–817 SP, KIF20A809–833-LP, and KIF20A809–833-LP-specific T11 clone (LP + T11 clone) strongly enhanced induction of KIF20A-A2809–817 SP-specific tetramer+ T cells. These results indicate that the activated T11 cells enhanced induction of KIF20A-specific CTLs in the presence of KIF20A-A2809–817 SP.

Next, we examined that the CD107a expression of KIF20A-A2466–75 SP-specific CTLs cultured in the presence of KIF20A809–833-LP-specific T11 cells stimulated with the relevant LP for 1 week to assess the function of KIF20A-specific CTLs expanded by activated KIF20A-specific T11 cells. KIF20A809–833-LP-specific bulk CD4+ T cells and KIF20A-A2466–75 SP-specific bulk CD8+ T cells derived from HLA-A24+ and HLA-DR15+ HD4 were cultured with autologous dendritic cells in the presence of KIF20A-A2466–75 SP (LP alone), KIF20A-A2466–75 SP + Control LP (Control LP + SP), or KIF20A-A2466–75 SP + KIF20A809–833-LP (KIF20A809–833-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*2402/KIF20A-A2466–75 complex, anti-human CD107a mAb, and anti-human CD8 mAb as described in the Supplementary Materials and Methods section. As shown in Supplementary Fig. S3C, 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 restimulation 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 Fig. S3D). These results suggest that activated KIF20A-LP-specific T11 cells enhance 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-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 Fig. 4C, KIF20A809–833-LP-reactive bulk CTLs from an HLA-A2+ and -DR53+ donor (HD5) specifically produced IFN-γ in response to stimulation with KIF20A809–833-LP-loaded dendritic cell, but not with control LP-loaded dendritic cell. 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-A2809–817 SP-reactive CTLs were stimulated through the cross-presentation of KIF20A809–833-LP by dendritic cells in vitro.

Subsequently, the capacity of KIF20A60–84-LP to induce KIF20A-A2466–75 SP-specific CTLs was examined by IFN-γ ELISPOT assay. After in vitro stimulation of PBMCs with KIF20A60–84-LP for 2 weeks, the cells were harvested and the number of IFN-γ-producing T cells in response to stimulation with KIF20A-A2466–75 SP-pulsed C1R-
A2402 cells was counted by ELISPOT assay. Details are provided in Supplementary Materials and Methods. As shown in Fig. 4D, KIF20A-A2466–75 SP–specific CTLs were induced in PBMCs cultured with KIF20A60–84-LP. In addition, KIF20A 809–833-LP also induced KIF20A-A2 809–817 SP–specific CTLs from PBMCs derived from HLA-A2+ donors (HD2 and HD3; data not shown).

The capacity of KIF20A60–84-LP to prime KIF20A-A2466–75 SP–specific CTLs in vivo was examined by an ex vivo IFN-γ ELISPOT assay. HLA-A24 Tgm were immunized three times with KIF20A60–84-LP. The CD8+ T cells of HLA-A24 Tgm vaccinated with KIF20A60–84-LP produced IFN-γ in response to stimulation with bone marrow–dendritic cells pulsed with the KIF20A-A2466–75 SP (Supplementary Fig. S4). These results suggest that after uptake of KIF20A60–84-LP, APCs cross-prime KIF20A-A2466–75 SP–specific CTLs in vitro and in vivo.

Figure 3. Cytokine profile produced by KIF20A-LPs–specific bulk TH cells. A, after 24 hours incubation of TH cells cocultured with autologous PBMCs (for KIF20A60–84-LP–specific bulk TH cells) or L-DR53 (for KIF20A809–833-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+ T cells after antigenic stimulation. Cells were restimulated with cognate LPs or a control peptide. Events shown are gated for CD4+ T cells. The numbers inside the plots indicate the percentage of the cell population with the quadrant characteristic (CD4+ CD107a+ T cells).
Presence of KIF20A-specific T<sub>H1</sub> 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 done 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%),
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<th>Table 1. Clinical characteristics of HNMT patients</th>
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Abbreviations: CTR, clinical trials registry; DR53, DRB4; IHC, immunohistochemistry; M/F, male/female; n.t., not tested.

aKIF20A-specific CD4+ 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 (Fig. 1; [1]).
and 1 of the 2 osteosarcoma (50%) showed positive expression of KIF20A. (Supplementary Table S3). No staining was detected in the benign tumor samples.

In contrast to cancer immunotherapy, there is strong evidence suggesting that vaccines using restricted epitopes can result in broad CD8+ T-cell responses to antigens not presented in the vaccine (37–39). Thus, we considered that KIF20A-specific T11 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 ELISPOT assay (Fig. 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 twofold above the negative control. KIF20A-specific T11 cell responses were observed in 8 of 16 patients (KIF20A60–84–LP, 2 of 16, 13%; KIF20A609–813–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 KIF20A60–84–LP and KIF20A609–813–LP in patients were significantly larger than in healthy donors (Fig. 5B). KIF20A609–813–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 (Fig. 5C). Interestingly, specific responses to KIF20A609–813–LP were augmented in some patients (HNMT31 and HNMT42), or induced in HNMT43 during the course of immunotherapy (Fig. 5D). As shown in Fig. 5E, KIF20A antigen was expressed in patients with HNMT in whom KIF20A-specific T11 cell responses were detected (Table 1; HNMT31 and HNMT108), but was not expressed in those for whom KIF20A-specific T11 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 T11 cell responses in vivo.

Discussion

We identified 2 promiscuous KIF20A-derived T11-cell epitopes bearing known HLA-A2– or HLA-A24–restricted CTL epitopes. KIF20A-specific CTLs were induced by stimulation with KIF20A-LPs in vitro and in vivo. This is the first demonstration of KIF20A expression in HNMT, and the presence of KIF20A-specific T11-cell responses in patients with HNMT. Furthermore, these responses were related to KIF20A expression in HNMT tissues. We postulate that these KIF20A-LPs–specific T11-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 T11 cells in vivo.

Recent reports showed that a new wave of tumor-specific CTL clones became 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 KIF20A609–813–LP–specific T11 response was absent before vaccination, but after vaccination this response was significantly induced (Fig. 5D, right panel). We believe that this KIF20A609–813–LP–specific T11-cell response is a phenomenon of antigen spreading triggered by vaccination with CTL epitopes.

Aarnitzen and colleagues have recently reported that targeting T11 cells with dendritic cells pulsed with both HLA class I- and II-restricted epitopes enhances vaccine-specific immunologic responses and improves clinical responses (42). We showed that KIF20A-LPs and T11 clones enhanced induction of HLA-A2–restricted KIF20A-specific CTLs 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 KIF20A609–813–LP–specific CD4+ T-cell response in HNMT31 was confirmed by inhibition of IFN-γ secretion in the presence of HLA-DR–blocking antibody (Fig. 5C), although HLA-DR alleles of HNMT31 (Table 1; DRB1*01:01/11:01) were not shared by the HLA-DR alleles, which encode HLA class II molecules presenting KIF20A609–813–LP to T11 cells in healthy donors (HLA-DR15 and DR53; Fig. 1). This result supports the promiscuous nature of KIF20A609–813–LP and indicates that LP is naturally processed and presented by HLA class II molecules in vivo.

In this study, significant frequencies of KIF20A-specific T11 cell responses were detected in patients with HNMT receiving immunotherapy (8 of 16, 50%). Godet and colleagues reported a possible synergistic effect of the telomerase-specific CD4+ T-cell response with chemotherapy in lung cancer. They showed that the existence of spontaneous telomerase-specific T11 cells before first-line chemotherapy significantly increased overall survival in lung cancer that responded 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 T11 cells by KIF20A-LP vaccination may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies.
Figure 5. Presence of KIF20A-LPs–specific T<sub>H</sub> 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 T<sub>H</sub>1 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 T<sub>H</sub> cells in HNMT31 and HNMT43. PBMCs stimulated with LPs for 1 week were restimulated with KIF20A<sub>809–833</sub>-LP in the presence of mAbs specific to HLA-DR, HLA-DP, HLA-DQ, or HLA class I. D, KIF20A-LPs–specific T<sub>H</sub>1 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 T<sub>H</sub>1-cell responses in patients with HNMT.
(45–47). Weide and colleagues have recently reported that the presence of circulating T<sub>11</sub> cells responding to Melan-A or NY-ESO-1 had a strong prognostic impact on survival among chemotherapy-treated advanced melanoma patients (48). Thus, KIF20A-LPs—specific T<sub>11</sub>-cell responses in patients with HNMT receiving immunotherapy may positively influence overall survival. The impact of KIF20A-specific T<sub>11</sub>-cell responses on clinical outcome will be evaluated in a future study.

Recent studies of LPs have shown that vaccines containing natural CTL epitopes were 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<sub>50-84</sub>-LP was not superior to KIF20A<sub>2A46-72</sub>-SP in induction of KIF20A-specific CTLs (data not shown). Therefore, we are not able to conclude that we should use KIF20A-LP encompassing CTL epitope alone or both KIF20A-SP and the KIF20A-LP to elicit stronger antitumor T<sub>11</sub>-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 T<sub>11</sub> cells to the shorter, 15-mer T<sub>11</sub> epitope from KIF20A-LPs, because we considered that these 25-mer amino acid long KIF20A-LPs encompass both HLA class I and 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, 2 KIF20A-derived LPs, KIF20A<sub>50-84</sub>-LP and KIF20A<sub>50-533</sub>-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 T<sub>11</sub>-cell epitopes derived from KIF20A and present the first clinical evaluation of KIF20A-specific T<sub>11</sub>-cell responses in patients with HNMT receiving immunotherapy. These T<sub>11</sub>-cell epitopes provide a tool for propagation of KIF20A-specific T<sub>11</sub> cells and CTLs. These findings support a clinical trial of KIF20A peptide–based immunotherapy for cancer treatment.

Disclosure of Potential Conflicts of Interest
K. Yoshida and T. Tsunoda are employed (other than primary affiliation; e.g., consulting) by OncoTherapy Science, Inc. Y. Nakamura is a stockholder and scientific advisor for OncoTherapy Science, Inc. Y. Nishimura has a commercial research grant from OncoTherapy Science, Inc. No potential conflicts of interest were disclosed by the other authors.

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