Supplementary materials and methods

Generation of antigen-specific $CD4^+$ T-cell lines and clones

$CD4^+$ T-cells were purified from peripheral blood mononuclear cells (PBMCs) by positive selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) (1). Monocyte-derived dendritic cells (DCs) were generated from $CD14^+$ cells by *in vitro* culture, as described previously (2), and used as antigen-presenting cells (APCs) to induce antigen-specific $CD4^+$ T-cells. Dendritic cells (1×10^4/well) were pulsed with 10 μg/mL long peptide (LP) for 3 h and irradiated (45 Gy), and subsequently mixed with $CD4^+$ T-cells (3×10^4/well) in 200 μL AIM-V supplemented with 5% human decomplemented plasma in each well of a 96-well, flat-bottomed culture plate. After 7 days, half of the medium was removed from each culture, and fresh medium (100 μL/well) containing irradiated (50 Gy) autologous PBMCs (1×10^5) pulsed with peptide (10 μg/mL) and 5 ng/mL recombinant human interleukin 7 (rhIL-7) was added. Two days after the second stimulation with peptide, rhIL-2 was added to each well (10 IU/mL). A week later, the stimulated $CD4^+$ T-cells in each well were analyzed for specificity in IFN-γ ELISPOT assays. The T-cells showing a specific response to the cognate peptide were transferred to 24-well plates and re-stimulated at weekly intervals

with irradiated autologous PBMCs (1 × 10^6/well) pulsed with the peptide in medium supplemented with rhIL-2 (20 IU/mL) and rhIL-7 (5 ng/mL).

CD107a mobilization assay

To identify degranulating $CD4^+$ and $CD8^+$ T lymphocytes stimulated by the peptides, CD107a on the cell surface was analyzed by flow cytometry (3, 4). In brief, a CD107a mobilization assay was performed as described previously (5). The cognate-LP, SP, or control LP (1 μg/mL) were added as stimulants, and FITC-labeled anti-human CD107a mAb or FITC-labeled isotype control mouse IgG1 and monensin were added to each well. Cells were cultured for 5 h at 37°C. After culture, the peptide-stimulated Th cells CTLs were stained with PE-conjugated anti-human CD4 antibody (eBioscience, San Diego, CA), or PerCP-labeled anti-human CD8 mAb (BioLegend) and PE-labeled tetramer of the HLA-A*24:02/KIF20A-$A24_{66–74}$ complex (MBL, Nagoya, Japan). Cells were analyzed on a FACScan (BD Bioscience, Bedford, MA) flow cytometer.

In vitro **induction of KIF20A-$A24_{66–75}$ SP-specific CTLs by stimulation of PBMCs with KIF20$A_{60–84}$-LP**

To assess induction of KIF20A-$A24_{66–75}$ SP-specific CTLs from an $HLA\text{-}A24^+$ donors (HD4 and HD8) by simulation with KIF20$A_{60–78}$-LP *in vitro*, PBMCs (2 ×

Formatted: Font color: Auto

10^6/well of 24-well plates) were incubated with KIF20A$_{60–84}$-LP (30 μg/mL) for 2 weeks. On day 0 and day 7, KIF20A$_{60–84}$-LP (30 μg/mL) was added, and then rhIL-2 (20 U/mL) was added on day 9 and day 11. On day 14 of *in vitro* stimulation with KIF20A$_{60–84}$-LP, the cells were harvested and the number of IFN-γ producing T-cells (1 × 10^5/well) in response to stimulation with KIF20A-A24$_{66–75}$ SP-pulsed C1R-A2402 cells (2 × 10^4/well) was counted by ELISPOT assay.

CD107a expression of KIF20A-specific CD8$^+$ T-cells expanded by activated KIF20A-specific Th cells

CD107a expression of KIF20A-A24$_{66–75}$ SP-specific CTLs cultured in the presence of activated KIF20A$_{809–833}$-LP-specific Th cells for 1 week was examined. KIF20A$_{809–833}$-LP-specific bulk CD4$^+$ T-cells (1 × 10^5 cells/well, 48-well plates) and KIF20A-A24$_{66–75}$ SP-specific bulk CD8$^+$ T-cells (1 × 10^5 cells/well) derived from HLA-A24$^+$/DR15$^+$ HD4 were cultured with autologous DCs (2 × 10^4 cells/well) in the presence of KIF20A-A24$_{66–75}$ SP (10 μg/mL; SP alone), KIF20A-A24$_{66–75}$ SP + Control LP (10 μg/mL; Control LP + SP), or KIF20A-A24$_{66–75}$ SP + KIF20A$_{809–833}$-LP (10 μg/mL; KIF20A$_{809–833}$-LP + SP) without addition of any cytokine. Induction of KIF20A-A24$_{66–75}$ SP-reactive bulk CTLs from an HLA-A24$^+$/DR15$^+$ donor (HD4) by stimulation with KIF20A-A24$_{66–75}$ SP was performed as described previously (5, 6).

Formatted: Font color: Auto

Formatted: Font color: Auto

Formatted: Font color: Auto

After 1-week *in vitro* culture with peptides, the cultured cells were stained with PE-labeled tetramer of the HLA-A*24:02/KIF20A-A24$_{67–75}$ complex (MBL, Nagoya, Japan) (7), FITC-labeled anti-human CD107a mAb (MBL, Nagoya, Japan), and PerCP-labeled anti-human CD8 mAb (BioLegend).

Formatted: Font color: Auto

Formatted: Font color: Auto

***In vivo* cross-priming assay**

HLA-A24 (HHH) transgenic mice (Tgm) were kindly provided by Dr. F. A. Lemonnier (8). Mice were immunized by intravenous transfer of KIF20A$_{60–84}$-LP-pulsed (50 μg/mouse, 3h) bone marrow-derived DCs (BM-DCs, 5 × 10^5 cells/mouse), and then intradermally injected at the base of the tail with KIF20A$_{60–84}$-LP solution (100 μg/mouse) emulsified in incomplete Freund adjuvant (IFA; used to stimulate the immune system), twice at 7-day intervals. Seven days after the third vaccination with KIF20A$_{60–84}$-LP, $CD8^+$ T cells were isolated from inguinal lymph nodes by positive selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). The number of IFN-γ producing $CD8^+$ T cells (2.5 × 10^5/well) in response to stimulation with KIF20A-A24$_{66–75}$ SP-pulsed BM-DCs (2 × 10^4/well) was counted by *ex vivo* ELISPOT assay (2, 9)

Formatted: Font color: Auto

References

1. Inoue M, Senju S, Hirata S, Ikuta Y, Hayashida Y, Irie A, et al. Identification of SPARC as a candidate target antigen for immunotherapy of various cancers. Int J Cancer. 2010;127:1393-403.
2. Harao M, Hirata S, Irie A, Senju S, Nakatsura T, Komori H, et al. HLA-A2-restricted CTL epitopes of a novel lung cancer-associated cancer testis antigen, cell division cycle associated 1, can induce tumor-reactive CTL. Int J Cancer. 2008;123:2616-25.
3. Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med. 2003;9:1377-82.
4. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003;281:65-78.
5. Tomita Y, Harao M, Senju S, Imai K, Hirata S, Irie A, et al. Peptides derived from human insulin-like growth factor-II mRNA binding protein 3 can induce human leukocyte antigen-A2-restricted cytotoxic T lymphocytes reactive to cancer cells. Cancer Sci. 2011;102:71-8.
6. Imai K, Hirata S, Irie A, Senju S, Ikuta Y, Yokomine K, et al. Identification of a novel tumor-associated antigen, cadherin 3/P-cadherin, as a possible target for immunotherapy

of pancreatic, gastric, and colorectal cancers. Clin Cancer Res. 2008;14:6487-95.

7. Osawa R, Tsunoda T, Yoshimura S, Watanabe T, Miyazawa M, Tani M, et al. Identification of HLA-A24-restricted novel T Cell epitope peptides derived from P-cadherin and kinesin family member 20A. J Biomed Biotechnol. 2012;2012:848042.

8. Jung KO, Khan AM, Tan BY, Hu Y, Simon GG, Nascimento EJ, et al. West Nile virus T-cell ligand sequences shared with other flaviviruses: a multitude of variant sequences as potential altered peptide ligands. J Virol. 2012;86:7616-24.

9. Inoue M, Senju S, Hirata S, Irie A, Baba H, Nishimura Y. An in vivo model of priming of antigen-specific human CTL by Mo-DC in NOD/Shi-scid IL2rgamma(null) (NOG) mice. Immunol Lett. 2009;126:67-72.

Supplementary figure legends

Supplementary figure 1. KIF20A-derived and promiscuous HLA class II-binding peptides predicted by a recently developed computer algorithm. **A**, the amino acid sequence of the human KIF20A protein was analyzed using an algorithm (IEDB analysis resource, consensus method), http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html. Numbers on the horizontal axis indicate amino acid positions at the N-terminus of KIF20A-derived 15-mer peptides. Higher consensus percentile rank indicates stronger binding affinity to HLA class II molecules. **B**, The 25-mer LPs, $KIF20A_{60–84}$-LP and $KIF20A_{809–833}$-LP with high consensus percentile ranks for 3 HLA-class II allelic (*DRB1*04:05*, *DRB1*15:02*, and *DRB4*01:03*) products and bearing 9- or 10-mer CTL-epitopes recognized by HLA-A2- or HLA-A24-restricted CTLs were synthesized (**A**, right and left black bar). The 24-mer LP $KIF20A_{494–517}$-LP with high consensus percentile ranks for multiple HLA-class II allelic products, which does not include a known CTL-epitope, was also synthesized (**A**, middle black bar).

Supplementary figure 2. Induction of KIF20A-LPs-specific Th cells from healthy donors. **A**, Th cells generated by stimulation of purified $CD4^+$ T-cells with

KIF20A$_{60–84}$-LP were restimulated with autologous PBMCs pulsed with KIF20A$_{60–84}$-LP. The number of IFN-γ -producing Th cells was analyzed by ELISPOT assay. The HLA class-II genotype of HLA-DR15- and DP2-negative donor (HD3) is indicated at the top of the panels. This result suggests that KIF20A$_{60–84}$-LP-specific Th cells from HD3 are restricted by HLA-DR4 or DR53. Data are presented as the mean ± SD of triplicate assays. Representative data from at least 5 independent experiments with similar results are shown. **B**, The Th cells generated from HLA-DR15^{+} donor (HD4) by stimulation with KIF20A$_{809–833}$-LP were restimulated with autologous PBMCs pulsed with KIF20A$_{809–833}$-LP. The HLA class-II genotype of HD4 is indicated at the top of the panels. The Th cells were suggested to be restricted by HLA-DR. **C**, induction of HLA-DR4-restricted KIF20A$_{494–517}$-LP-specific Th cells from an HLA-DR4^{+} healthy donor (HD2). KIF20A-specific Th cells were generated from HD2 by stimulation of purified CD4^{+} T-cells with KIF20A$_{494–517}$-LP-pulsed autologous DCs or PBMCs. The generated Th cells were restimulated with autologous PBMCs or L-cells pulsed with KIF20A$_{494–517}$-LP. The number of IFN-γ-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 HD2 are shown. The HLA class-II genotype of HD2 is indicated above

the panels. The underlined HLA-class II alleles (*HLA-DRB1*04:05*) encode HLA-class II-molecule presenting the peptides to Th cells.

Supplementary figure 3. Enhanced induction of KIF20A-SP-specific CTLs by KIF20A-LP-specific $CD4^+$ T-cells **A**, PBMCs from an HLA-$A2^+$/$DR53^+$ healthy donor (HD2), from which an HLA-DR53-restricted $KIF20A_{809–833}$-LP-specific Th-clone was generated, were cultured for 11 days with KIF20A-$A2_{809–817}$ SP (SP), $KIF20A_{809–833}$-LP (LP), KIF20A-$A2_{809–817}$ SP + $KIF20A_{809–833}$-LP (SP + LP), $KIF20A_{809–833}$-LP + $KIF20A_{809–833}$-LP-specific Th clone (LP + Th-clone) or SP + LP + $KIF20A_{809–833}$-LP-specific Th-clone (SP + LP + Th-clone). On day 11, the cells were stained with KIF20A-$A2_{809–817}$ SP-specific tetramer with an anti-human CD8 mAb and were analyzed by flow cytometry. **B**, Representative KIF20A-$A2_{809–817}$ SP-specific tetramer staining (gated on $CD8^+$ T-cells) obtained from 3 independent experiments with similar results is shown. **C**, CD107a expression of KIF20A-$A24_{66–75}$ SP-specific $CD8^+$ T-cells expanded by activated $KIF20A_{809–833}$-LP-specific Th cells. $KIF20A_{809–833}$-LP-specific bulk $CD4^+$ T-cells and KIF20A-$A24_{66–75}$ SP-specific bulk $CD8^+$ T-cells derived from HLA-$A24^+$/$DR15^+$ HD4 were cultured with autologous DCs in the presence of KIF20A-$A24_{66–75}$ SP (SP alone), KIF20A-$A24_{66–75}$ SP + Control LP

Formatted: Font color: Auto

Formatted: Font color: Auto

Formatted: Font color: Auto

(Control LP + SP), or KIF20A-A24$_{66–75}$ SP + KIF20A$_{809–833}$-LP (KIF20A$_{809–833}$-LP + SP) without addition of any cytokine. After 1-week *in vitro* culture with peptides, the cultured cells were stained with PE-labeled tetramer of the HLA-A*24:02/KIF20A-A24$_{67–75}$ complex and PerCP-labeled anti-human CD8 mAb. Data are presented as the mean ± SD of triplicate assays. Representative data from 3 independent experiments with similar results are shown. **D**, After 1-week *in vitro* culture with peptides, the cultured cells were re-stimulated with KIF20A-A24$_{66–75}$ SP and stained with PE-labeled tetramer of the HLA-A*24:02/KIF20A-A24$_{67–75}$ complex, FITC-labeled anti-human CD107a mAb, and PerCP-labeled anti-human CD8 mAb. The absolute number of KIF20A-A24$_{66–75}$ SP-specific CTLs expressing CD107a on the cell surface after re-stimulatiton with KIF20A-A24$_{66–75}$ SP was shown. Data are presented as the mean ± SD of triplicate assays. Representative data from 3 independent experiments with similar results are shown.

Formatted: Font color: Auto

Supplementary figure 4. Induction of KIF20A-A24$_{66–75}$ SP-specific CTLs in mice immunized with KIF20A$_{60–84}$-LP. HLA-A24 Tgm were immunized with KIF20A$_{60–84}$-LP. After the third vaccination with KIF20A$_{60–84}$-LP, mouse CD8^{+} T-cells in the inguinal lymph nodes were stimulated with BM-DC pulsed with

KIF20A-A24$_{66–75}$ SP. The number of IFN-γ-producing murine CD8^{+} T-cells was analyzed by *ex vivo* ELISPOT assay. Representative data from 5 independent experiments with similar results are shown.