TAS0314, a novel multi-epitope long peptide vaccine, showed synergistic antitumor immunity with PD-1/PD-L1 blockade in HLA-A*2402 mice

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Cancer peptide vaccines are a promising cancer immunotherapy that can induce cancer-specific cytotoxic T lymphocytes (CTLs) in tumors. However, recent clinical trials of cancer vaccines have revealed that the efficacy of the vaccines is limited. Targeting single antigens and vaccination with short peptides are partly the cause of the poor clinical outcomes. We synthesized a novel multi-epitope long peptide, TAS0314, which induced multiple epitope-specific CTLs in HLA-knock-in mice. It also showed superior epitope-specific CTL induction and antitumor activity. We also established a combination treatment model of vaccination with PD-1/PD-L1 blockade in HLA-A*2402 knock-in mice, and it showed a synergistic antitumor effect with TAS0314. Thus, our data indicated that TAS0314 treatment, especially in combination with PD-1/PD-L1 blockade, is a promising therapeutic candidate for cancer immunotherapy.

Cancer vaccines that can induce a tumor-specific immune response without serious adverse events are attractive as a therapy for cancer. Numerous cancer antigens have been discovered and developed as peptide-, DNA-, or RNA-based cancer vaccines. Peptide vaccines have been developed since the 1990s, but only limited therapeutic effects have been shown in the clinical setting. EGFR, Lck, MRP3, SART2, SART3, PTHrP, TMEM189 (also referred as UBE2V), and WHSC were identified as common cancer antigens among various cancers, and epitope peptides from these antigens efficiently induced HLA-restricted cytotoxic T lymphocyte (CTL) responses in clinical trials. However, peptide vaccines using these antigens did not prolong the overall survival of patients in phase III clinical trials. Many studies on how to overcome these limited therapeutic effects of peptide vaccines have been carried out, and several problems regarding cancer peptide vaccines have been revealed.

First, peptide vaccine-induced specific CTLs fail to efficiently accumulate in tumors. Incomplete Freund's adjuvant is widely used as an adjuvant for cancer peptide vaccines. However, a recent study revealed that epitope peptides emulsified with incomplete Freund's adjuvant induced the accumulation of epitope-specific CTLs at the injection site, and failed to induce efficient antitumor effects. However, a long peptide did not induce accumulation at the injection site, and could induce an efficient antitumor effect.

Second, immune checkpoint molecules on CTLs induce exhaustion. PD-1 is a molecule that is expressed on exhausted T cells, and it interacts with PD-L1 in the tumor microenvironment. PD-1/PD-L1 interaction suppresses cytokine production and proliferation, and leads to T cell dysfunction. To enhance T cell immunity, PD-1/PD-L1 blockades have been developed, and they showed strong clinical efficacy in various types of cancers. It has been reported that vaccine-induced CTLs also express PD-1, and the combination of a peptide vaccine with anti-PD-1 antibody synergistically induced antitumor effects in a mouse tumor model. Therefore, PD-1/PD-L1 blockade is expected to augment the efficacy of cancer vaccines in clinical trials.

Third, cancers can evade vaccine-induced CTLs via tumor antigen loss. The CTLs induced by peptide vaccines mediate cytotoxicity only against antigen-expressing tumors. In some tumors, the expression of tumor antigens...
is altered by epigenetic regulation or mutation, enabling the tumors to evade the immune system. For example, in the phase II clinical trial of the glypican-3 peptide vaccine, tumors that relapsed after vaccination showed the loss of glypican-3 antigen. Immunization with multiple epitopes is one of the ways to overcome the immune evasion of tumors, and would enhance the antitumor effect. Therefore, it is possible to enhance the efficacy of anti-cancer peptide vaccines to overcome these three problems.

To overcome the issues of existing antitumor peptide vaccines, we designed and developed a novel multi-epitope long peptide vaccine, TAS0314, which contains four epitopes from SART2 and SART3. The aim of this study was to demonstrate the immunological characteristics of TAS0314 in vitro and in vivo as an antitumor peptide vaccine. We also evaluated the benefit of combination treatment with PD-1/PD-L1 blockade using recently established HLA-A*2402 mice.

Results

TAS0314 induced multiple epitope-specific CTLs. To evaluate the efficacy of a multi-epitope long peptide vaccine, we synthesized a novel peptide, TAS0314, which contains four epitopes from SART2 and SART3 linked with an arginine dimer.

First, we evaluated the CTL induction ability of TAS0314 by using HLA knock-in (KI) mice that were established previously. The CTL induction ability of HLA-A*2402-restricted epitopes SART293–101 and SART3109–118 was determined by using HLA-A*2402 KI mice (Fig. 1a). Lymph node cells from immunized mice produced interferon (IFN)-γ after stimulation from the respective epitope peptides, indicating that TAS0314 can induce HLA-A*2402-restricted epitope peptide-specific CTLs.

We also confirmed the CTL induction ability of HLA-A*0201-restricted epitope SART3302-310 as well as HLA-A3 superfamily-restricted epitope SART3734–742 using HLA-A*0201 KI mice or HLA-A*3101 KI mice (Fig. 1b,c).

TAS0314 efficiently induced memory CTLs. We compared the frequencies of epitope-specific CTLs induced by TAS0314 and SART293–101 peptide, which makes up TAS0314, at an equivalent molar mass. Interestingly, TAS0314 induced a higher frequency of SART293–101-specific CTLs than SART293–101 peptide (Fig. 2a). In order to clarify the difference in CTL function due to immunization with TAS0314, we focused on the cytokine production capacity of the CTLs in blood. TAS0314 markedly increased the population of IFN-γ+/tumor necrosis factor (TNF)-α + double-positive CTLs and IFN-γ+/TNF-α+/interleukin (IL)-2 + triple-positive multifunctional CTLs in blood when compared to SART293–101 peptide vaccination (Fig. 2b).

To evaluate whether the CTLs induced by TAS0314 can exert long-term surveillance, we examined the recall response against SART293–101 peptide. Mice were immunized with TAS0314 or SART293–101 peptide and were boosted with SART293–101 148 days after the last immunization (Fig. 2c). Mice immunized with TAS0314 had significantly increased numbers of specific CTLs after boosting when compared to those immunized with SART293–101.

To evaluate the mechanism of antigen presentation for TAS0314, we examined the antigen presentation of TAS0314 by professional antigen-presenting cells (DCs) and non-professional antigen-presenting cells (T cells). As shown in Fig. 2d, TAS0314-pulsed CD11c+ DCs efficiently activated CTLs while TAS0314-pulsed T cells exhibited a significantly lower IFN-γ response. In addition, the CTL activation by TAS0314-pulsed DCs was significantly decreased by the proteasome inhibitor epoxomicin.

CTLs induced by TAS0314 killed antigen-expressing cells in vitro. To confirm whether the TAS0314-induced CTLs had cytotoxicity against tumor cells, epitope-specific cytotoxicity was evaluated using
A 51-chromium (\(^{51}\)Cr) cytotoxicity assay. SART293–101 peptide-specific CTLs from TAS0314-immunized mice were expanded for a week, then co-cultured with B16F10 cells expressing HLA-A*2402 Ki (B16F10.A24) and epitope peptide. Strong cytotoxicity was observed against B16F10.A24 cells only when SART293–101 peptide was pulsed (Fig. 3a).

SART3302-310 epitope-specific cytotoxicity was also evaluated using T2, an HLA-A2-positive human cell line. The cultured SART3302-310 peptide-specific CTLs showed cytotoxicity against the peptide-pulsed T2 cells (Fig. 3b).

We measured the cytotoxicity against a tumor cell line, B16F10.A24/SART293-101 cells, that overexpressed both HLA-A*2402 and SART293-101 peptide. As expected, the CTLs induced by TAS0314 displayed strong cytotoxicity against B16F10.A24/\(\)SART293-101 cells (Fig. 3a). The addition of HLA-neutralizing antibody resulted in a 40% reduction of cytotoxicity (Fig. 3c). These data revealed that the CTLs induced by TAS0314 can recognize epitopes in an HLA-dependent manner.

**Antitumor effect of TAS0314 against B16F10.A24/SART293-101 tumor cells.** We evaluated the antitumor activity against subcutaneous B16F10.A24/SART293-101 tumors in a syngeneic mouse model. To compare the efficacy, mice were immunized with SART293-101 peptide or TAS0314 at a molar-equivalent dose (Fig. 4a). At 18 days after tumor implantation, TAS0314 significantly reduced the tumor volume by 70.25% when compared to the control, SART293-101 peptide only reduced the tumor volume by 47.7%, and did not show any significant difference from the control group (Fig. 4b). These data suggested that TAS0314 induced better antitumor activity than SART293-101.

We also evaluated antitumor activity with a syngeneic lung metastasis model of B16F10.A24/SART293-101 cells (Fig. 4c). The number of metastatic lung nodules was significantly decreased in the TAS0314-treated group when compared to the control group (Fig. 4d). TAS0314 showed antitumor activity not only in the subcutaneous model, but also in the metastasis model.
TAS0314 and PD-1/PD-L1 blockade synergistically suppressed tumor growth. We evaluated the synergistic antitumor activity of TAS0314 and anti-PD-1 antibody against B16F10.A24/SART293–101 tumors (Fig. 5a). Anti-PD1 antibody treatment partially suppressed tumor growth, while TAS0314 treatment significantly suppressed tumor growth (Fig. 5b). Furthermore, the combined therapy of TAS0314 and anti-PD-1 antibody dramatically suppressed tumor growth when compared to monotherapy.

We also evaluated the survival for 57 days. Anti-PD-1 antibody did not prolong the survival when compared to the control group (Fig. 5c). Combined treatment with TAS0314 and anti-PD-1 antibody significantly suppressed tumor growth (Fig. 5b). Furthermore, the combined therapy of TAS0314 and anti-PD-1 antibody dramatically suppressed tumor growth when compared to monotherapy.

We also evaluated the antitumor activity of TAS0314 in combination with anti-PD-L1 antibody (Fig. 5d). The combination of TAS0314 and anti-PD-L1 antibody also synergistically inhibited tumor growth when compared to monotherapy (Fig. 5e). Combination therapy with anti-PD-L1 antibody dramatically prolonged the survival (Fig. 5f). Eighty-five days after tumor implantation, 66.67% of the mice treated with the combination of TAS0314 and anti-PD-L1 antibody were tumor-free. In contrast, no mice were alive in the other groups.

These data strongly suggested that the combination treatment of TAS0314 and PD-1/PD-L1 blockade has synergistic antitumor effects.

Combination of TAS0314 and anti-PD-1 antibody increased the number of epitope-specific CTLs in tumors. To analyze the mechanism of the synergistic effect, we measured SART293–101-specific CTLs in tumors using the SART293–101-epitope-specific T cells in tumors (Fig. 6). A number of tetramer-positive T cells was detected after immunization with TAS0314, whereas no SART2-specific CTL infiltration was detected in the vehicle-immunized groups. Surprisingly, the number increased approximately three-fold in the group treated with the combination treatment of TAS0314 and anti-PD-1 antibody. Increases in the number of epitope-specific CTLs in tumors would contribute to the synergistic antitumor activity of TAS0314 and anti-PD-1 antibody.

Discussion

Numerous clinical trials have been conducted, but the efficacy of cancer vaccines has been limited\(^30\),\(^31\). To develop useful cancer peptide vaccines, their efficacy must be improved.
Figure 4. Antitumor activity of TAS0314 against syngeneic tumors. (a,b) Antitumor activity of prophylactic treatment with TAS0314. HLA-A*2402 KI mice (n = 15/group) were vaccinated with TAS0314 (100 µg) or SART293–101 peptide (21 µg). One week after the last immunization, B16F10.A24/SART293–101 cells were inoculated into the right flank of the mice. (a) Experimental scheme. (b) Plot of the tumor growth. Results are expressed as the mean ± standard error. (c,d) Antitumor activity of TAS0314 against lung metastasis. HLA-A*2402 KI mice (n = 10/group) were injected with B16F10.A24/SART293–101 cells, then immunized with TAS0314 (300 µg). Twenty-eight days after implantation, the number of lung metastases was counted. (c) Experimental scheme. (d) Evaluation of lung metastasis. Results are expressed as the mean ± standard error. *p < 0.05 using a two-tailed Student’s t test.

Figure 5. The synergistic antitumor effect of TAS0314 and PD-1/PD-L1 blockade. (a–c) Combination therapy of TAS0314 and anti-PD-1 mAb. HLA-A*2402 KI mice (n = 15/group) were treated with TAS0314 (300 µg/each) and/or anti-PD-1 mAb (100 µg), then implanted with B16F10.A24/SART293–101 tumors. (a) Experimental scheme. (b) Tumor growth curves. (c) Survival curves. (d–f) Combination therapy of TAS0314 and anti-PD-L1 mAb. HLA-A*2402 KI mice (n = 15/group) were treated with TAS0314 (300 µg/each) and/or anti-PD-L1 mAb (200 µg), then implanted with B16F10.A24/SART293–101 tumors. (d) Experimental scheme. (e) Tumor growth curves. (f) Survival curves. Results are expressed as the mean ± standard error. Mice were monitored until the tumor volume had reached 2000 mm<sup>3</sup> or until death. Tumor volume was assessed by a two-tailed Student’s t test, and survival was evaluated by a log-rank test. *p < 0.05; **p < 0.001.
We developed a novel multivalent long peptide vaccine, TAS0314, and evaluated its CTL induction ability, antitumor effect in monotherapy, and its antitumor effect in combination with anti-PD-1 antibody. We previously reported that the induction of epitope-specific CTLs is affected by the position of the epitope in a long peptide\(^\text{32}\). We evaluated and screened numerous peptides to obtain suitable long peptides with efficient CTL induction ability by changing the linkage order of four epitopes. Finally, we designed TAS0314, which could efficiently induce epitope-specific CTL induction. Indeed, TAS0314 showed superior CTL induction and antitumor effects when compared to short epitope peptide vaccination.

Synthetic long peptide vaccines that were efficiently processed by DCs and induced robust CD8\(^+\) T cell responses have been reported\(^\text{33}\). In our study, we confirmed that TAS0314 epitopes were presented by DCs in a proteasome-dependent pathway. In contrast, non-professional antigen-presenting cells, such as T cells, showed little antigen presentation of the epitopes in TAS0314. Antigen presentation by non-professional antigen-presenting cells induces the exhaustion of CTLs, and exhausted T cells show loss of multifunctional cytokine production\(^\text{34}\). Indeed, we obtained similar data showing that T cells induced by short epitope immunization had a smaller population of multifunctional (IFN-γ/+/TNF-α+/IL-2+, IFN-γ/+/TNF-α+) CTLs when compared to those induced by TAS0314. We considered that TAS0314 antigen presentation via DCs may contribute to the enhanced CTL induction and antitumor effects of TAS0314.

According to the current study, immunization with TAS0314 and boosting 148 days after the last immunization significantly induced a large number of CTLs, suggesting that TAS0314 vaccination resulted in memory CTL induction. Among peripheral blood mononuclear cells, central memory T cells produce IL-2\(^\text{35}\), and autocrine IL-2 production is required for efficient expansion after a second challenge\(^\text{36}\). Therefore, multifunctional CTLs that are induced by TAS0314 might differentiate into memory T cells, and contribute to long-term surveillance. It has been reported that epitope-specific memory T cells remained for a long time in long-surviving patients treated with cancer vaccines\(^\text{37}\). Thus, TAS0314 might also contribute to the long-term control of cancer progression by inducing memory CTLs.

Cancer antigen-specific T cells upregulate immunosuppressive molecules (e.g., PD-1) after activation, and cause dysfunction by interacting with their ligands (e.g., PD-L1) in tumors\(^\text{17}\). Indeed, it has been reported that epitope-specific CTLs induced by peptide vaccines highly expressed PD-1 in cancer patients\(^\text{38}\). It is believed that the combination therapy of a cancer vaccine and PD-1/PD-L1 blockade synergistically enhances the antitumor activity. However, few HLA-A2-restricted epitope peptides from human cancer antigens have been evaluated for their efficacy in combination with anti-PD-1 antibody due to the HLA restriction in mouse models\(^\text{39}\). To evaluate the combination of other HLA allele-restricted epitopes with anti-PD-1 antibody, mice expressing the HLA-A*2402 allele are required. The synergistic antitumor effect of HLA-A24-restricted epitopes and anti-PD-1 antibody was evaluated using our HLA-A*2402 KI mice.

Combination treatment with TAS0314 and PD-1/PD-L1 blockade also showed synergistic antitumor activity. The combination therapy dramatically inhibited tumor growth, and prolonged survival. The number of
epitope-specific CTLs in tumors were increased by approximately three-fold, and this increase was considered to be a mechanism of the synergistic antitumor effect of both PD-1 and PD-L1 antibody treatment with TAS0314.

Although a short peptide vaccine containing the SART293–101 epitope did not induce a significant clinical response in a recent Phase III clinical trial, our data suggest that the combination of PD-1/PD-L1 blockade might improve its clinical efficacy.

It has been reported that anti-PD-1 antibody enhanced chemokine expression in tumors and T cell infiltration. Others have also reported that anti-PD-1 antibody inhibited T cell apoptosis, and enhanced the proliferation of T cells. According to these reports and our current data, we believe that anti-PD-1 antibody treatment induces the infiltration and accumulation of TAS0314-specific CTLs in the tumor microenvironment, which contributes to the synergistic antitumor effects. However, the detailed mechanism should be elucidated in future studies.

There are some limitations in this study. First, we evaluated the antitumor activity only against tumor cells expressing SART293–101 as a representative epitope peptide. Other epitopes are also expected to show superior CTL induction and antitumor activity, because they likely behave similarly.

We also developed different multivalent long peptides, TAS0315 and TAS0316, which contain 8 epitopes from EGFR, Lck, MR3P, PTHR1, TMEM189, and WHSC2. Similar to TAS0314, these two long peptides also showed CTL induction ability in HLA-KI mice (Supplementary Fig. 1). According to our results, TAS0314, TAS0315, and TAS0316 are expected to have an antitumor effect and synergistic effect with anti-PD-1 antibody; however, further investigations are needed with the establishment of new HLA-A2- or A24-dependent syngeneic anti-tumor models.

Second, the antitumor effect of TAS0314 was examined only by an artificial model using HLA-A*2402 and SART293–101 stably overexpressing cells. Although we confirmed that TAS0314-induced antigen-specific CTLs recognized and killed HLA-A24 human cancer cells in vitro (Supplementary Fig. 2), the anti-tumor effect of TAS0314 against human cancers remains to be clarified through clinical trials.

In summary, a novel multi-epitope long peptide vaccine, TAS0314, induced HLA-A2, HLA-A24, and HLA-A3 superfamily-restricted multiple epitope-specific CTLs. TAS0314 showed superior antitumor activity when compared to short epitope vaccines. Furthermore, we developed a combination model of vaccine and PD-1/PD-L1 blockade in HLA-A*2402 KI mice; combination therapy with anti-PD-1 antibody or anti-PD-L1 antibody increased epitope-specific CTLs in tumors and synergistically enhanced the antitumor activity. We have been developing a peptide mixture, TAS0313, that contains TAS0314, TAS0315, and TAS0316 for clinical use. Our preliminary data obtained during this development suggest that TAS0313 treatment, especially the combination therapy of TAS0313 and PD-1/PD-L1 blockade, represents a novel treatment option for patients suffering from various cancers. Moreover, the induced multiple antigen-specific CTLs that are expected to protect from immune escape due to antigen loss would contribute to long-term antitumor activity.

**Methods**

**Mice.** Heterozygous HLA-A*2402, heterozygous HLA-A*0201, and heterozygous HLA-A*3101 KI mice were generated as previously described. All animal procedures were performed in compliance with the National Institutes of Health Guidelines, and were approved by the Taiho Institutional Animal Care and Use Committee.

**Peptide synthesis.** TAS0314, TAS0315, TAS0316, and the epitope peptides in TAS0314 were synthesized and analyzed with liquid chromatography-mass spectrometry (LC–MS) by BACHEM Americas, Inc. (Torrance, CA, USA). HER2p63, WT1p126, and HIV gp41770–780 were synthesized and analyzed with liquid chromatography-mass spectrometry (LC–MS) by BACHEM Americas, Inc. (Torrance, CA, USA).

**Antibodies and reagents.** R-Phycocerythrin (PE)-conjugated anti-IFN-γ monoclonal antibody (mAb) (clone: XMG1.2), phycocerythrin–cyanin 7 (PE-Cy7)-conjugated anti-IL-2 mAb (clone: JES6-5H4), allophycocyanin (APC)-conjugated anti-TNF-α mAb (clone: MP6-XT22), Brilliant Violet (BV) 421 conjugated anti-CD8α mAb (clone: 53-6.7), BV 510-conjugated anti-CD90.2 mAb (clone: M0-12), Brilliant Violet 421-conjugated anti-CD90.2 mAb (clone: MR9-3), phycoerythrin–cyanin 7 (PE-Cy7)-conjugated anti-IL-2 mAb (clone: JES6-5H4), allophycocyanin (APC)-conjugated anti-TNF-α mAb (clone: MP6-XT22), Brilliant Violet (BV) 421 conjugated anti-CD8α mAb (clone: 53-6.7), BV 510-conjugated anti-CD90.2 mAb (clone: M0-12), purified anti-HLA-ABC mAb (clone: w6/32), GoInVivo purified anti-mouse CD274 (PD-L1) mAb (clone: 10F.9G2), GoInVivo purified rat IgG2b, κ Isotype Ctrl Antibody (clone: RTK4530), and purified mouse IgG2a, κ Isotype Ctrl (clone: MOPC-173) were purchased from BioLegend, Inc. (San Diego, CA, USA). PE-conjugated HLA-A*2402 SART293–101 tetramer.
and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (clone: KT15) were purchased from Medical and Biological Laboratories Co., Ltd. (Aichi, Japan). CD11c MicroBeads UltraPure, mouse, Pan T Cell Isolation Kit II, mouse, CD90.2 MicroBeads, and Tumor Disassociation Kit, mouse, were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Epoxomicin was purchased from Peptide Institute, Inc. (Osaka, Japan).

**Tumor cell lines.** B16F10 and T2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). B16F10.A24 cells and B16F10.A24/SART293–101 cells were established by transducing the HLA-A24 (HHD) gene or both the HLA-A24 (HHD) gene and SART293–101 mini-gene using the piggyBac vector (Transposagen Biopharmaceuticals, Inc., Lexington, KY, USA) as previously described.

**Immunization of mice.** Mice were immunized three times at 7-day intervals. A mixture of peptides emulsified in Montanide ISA-51VG (Seppic, Paris, France) was subcutaneously injected at the base of the tail (100 µL/mouse) and 148 days after the last immunization, the mice were immunized with SART293–101 as a booster treatment.

**Culture of epitope-specific CTLs.** Immunized mice were sacrificed 7 days after the last immunization, and lymphocytes from the lymph node were prepared. The lymphocytes were cultured for 8 days in culture medium supplemented with target peptide (10 µg/mL), rIL-15 (100 ng/mL) and mouse rIL-21 (100 ng/mL). Living cells were collected by density gradient centrifugation using Lymphocyte-M (Cedarlane, Hornby, ON, Canada).

**Enzyme-linked immunospot (ELISPOT) assay.** Epitope-specific IFN-γ production was detected using IFN-γ Plus ELISPOT (Mabtech, Stockholm, Sweden) as previously reported. The lymphocytes from HLA-KI mice were cultured with the target epitope peptide (10 µg/mL) or negative control peptide (WT1p126 for HLA-A*0201; Her2p63 for HLA-A*2402; HIV-1 gp41p770-780 for HLA-A*3101; all 10 µg/mL) in culture medium (RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol). For cases in which epitope-specific IFN-γ production was detected in the cultured CTLs, the CTLs were subsequently cultured with irradiated (30 Gy) splenocytes and the indicated peptide (100 µg/mL) in culture medium. Detection of IFN-γ production was performed according to the Mabtech protocol. Then, IFN-γ spots were counted with an ELISPOT analyzer Immunospot S6 (Cellular Technology Ltd., Shaker Heights, OH, USA).

**Antigen presentation assay.** CD11c+ dendritic cells (DCs) and CD3+ T cells were purified with CD11c MicroBeads UltraPure, mouse, and the Pan T Cell Isolation Kit II, mouse (Miltenyi Biotec) from the splenocytes of HLA-A*0201 KI mice. The antigen-presenting cells were incubated with peptides for 3 h (37 °C, 5% CO2) and washed three times. Epoxomicin was added 30 min before the addition of TAS0314. Cultured SART3302–310-specific CTLs from immunized HLA-A*0201 KI mice with SART3302–310 (100 µg/mice) were used as effector cells. The CTLs were co-cultivated with antigen-presenting cells on an anti-mouse IFN-γ-coated plate. Epitope-specific IFN-γ production was detected using an ELISPOT assay.

**Evaluation of antitumor activity against SART293–101-expressing syngeneic tumors.** For the prophylactic treatment, HLA-A*2402 KI mice (n = 10) were immunized three times with TAS0314 (100 µg/mouse), SART293–101 epitope (21 µg/mouse), or vehicle control. Seven days after the last immunization, immunized mice were subcutaneously inoculated with B16F10.A24/SART293–101 tumor cells (5 × 106) into the right flank. Seven days after tumor inoculation, the tumor size was measured with a caliper. The tumor volume was calculated using the following formula:

\[ \text{Tumor volume (mm}^3\) = length (mm) \times \text{[width (mm)]}^2/2. \]

For the therapeutic treatment, HLA-A*2402 KI mice (n = 15) were intravenously injected with B16F10.A24/SART293–101 tumor cells (1 × 106). The mice were immunized with TAS0314 (300 µg/mouse) three times with intervals of 7 days starting from the day after the tumor inoculation. On day 28 after tumor inoculation, mice were euthanized, and the number of lung metastases was counted.

**Combination therapy of TAS0314 and anti-PD-1/PD-L1 antibody.** To evaluate the efficacy of the combination therapy of TAS0314 and anti-PD-1/PD-L1 antibody, HLA-A*2402 KI mice (n = 15) were immunized three times with TAS0314 vaccine (300 µg/mouse) or vehicle control. Seven days after the last immunization, anti-PD-1 mAb (100 µg/mouse), anti-PD-L1 mAb (200 µg/mouse), or isotype rat IgG2b (200 µg/mouse) was injected intraperitoneally, and then B16F10.A24/SART293–101 tumor cells were subcutaneously inoculated (5 × 106) into the right flank of the mice.

**51Cr cytotoxicity assay.** The cytotoxicity of cultured CTLs against B16F10.A24 cells as well as SART293–101 peptide-pulsed B16F10.A24 cells or B16F10.A24/SART293–101 cells was evaluated with a 6h 51Cr-release assay as reported previously. Cultured SART293–101 epitope-specific CTLs from TAS0314-immunized mice (100 µg/
immunization) were used as effector cells. \textsuperscript{51}Cr (5 MBq/\texttimes10\textsuperscript{6} cells) and peptide (100 \textmu mol/L) were added to the target cells and incubated for 1.5 h at 37 °C. After incubation, the target cells were washed with phosphate-buffered saline, and were suspended in culture medium. Five thousand \textsuperscript{51}Cr-labeled target cells were cultured with cultured CTLs in 96-well round bottom plates at the indicated effector/target ratios in triplicate assays.

The cytotoxicity of cultured CTLs against T2 cells as well as SART3\textsubscript{302-310} peptide-pulsed T2 cells was evaluated using cultured SART3\textsubscript{302-310} epitope-specific CTLs from TAS0314-immunized mice (300 \textmu g/mice) as effector cells.

Based on the measurement of radioactivity (cpm) in the supernatant, the percent specific lysis was calculated by the following formula:

\[
\% \text{ specific lysis} = \frac{[\text{test} \ \textsuperscript{51}Cr \ \text{release} \ (\text{cpm}) - \text{mean spontaneous} \ \textsuperscript{51}Cr \ \text{release} \ (\text{cpm})]}{[\text{mean maximal} \ \textsuperscript{51}Cr \ \text{release} \ (\text{cpm}) - \text{mean spontaneous} \ \textsuperscript{51}Cr \ \text{release} \ (\text{cpm})]} \times 100. \]

**Isolation of tumor-infiltrating lymphocytes.** Single-cell suspensions from tumors were prepared using the Tumor Disassociation Kit, mouse, and GentleMACS (Miltenyi Biotec) according to the manufacturer's procedures. Living lymphocytes were isolated followed by density gradient centrifugation on a 70%/40% Percoll (GE Healthcare, Chicago, IL, USA) gradient. After density gradient centrifugation, T cells were isolated with CD90.2 MicroBeads and cultured overnight in culture medium. The cultured T cells were stained with SART2\textsubscript{303-310} tetramer, anti-CD8-APC mAb (clone KT15), and anti-CD90.2-BV510 mAb. The cells were fixed and permeabilized using the Fixation/Permeabilization Solution Kit (BD Bioscience), then stained with anti-IFN-γ-PE mAb, anti-TNF-α-APC mAb, and anti-IL-2-PE-Cy7 mAb. Stained cells were analyzed by FACSVerse.

**Detection of cytokine production by CD8\textsuperscript{+} T cells.** To evaluate cytokine multi-functionality, whole blood from immunized HLA-A-A\textsubscript{2402} KI mice was lysed with BD Pharm Lyse (BD Bioscience), stimulated with 10 \mu g/mL SART2\textsubscript{303-310} peptide, and cultured for 18 h supplemented with Golgiplug (BD Bioscience). Then, stimulated peripheral blood mononuclear cells were stained with anti-CD8-BV421 mAb and anti-CD90.2 BV510 mAb. The cells were further analyzed by FACSVerse (BD Bioscience, San Jose, CA, USA).

**Statistical analysis.** The statistical significance of CTL induction, tumor volume, and lung metastasis was evaluated with a two-tailed Student's \textit{t} test. The statistical significance of survival was evaluated with a log-rank test. In all analyses, \( P < 0.05 \) was considered to indicate statistical significance.

**Data availability**

The datasets generated and/or analyzed in the current study are available from the corresponding author on reasonable request.

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**References**

1. Hu, Z., Ott, P. A. & Wu, C. J. Towards personalized, tumour-specific, therapeutic vaccines for cancer. \textit{Nat. Rev. Immunol.} \textbf{18}, 168–182. https://doi.org/10.1038/nri.2017.131 (2018).
2. Shomura, H. et al. Identification of epidermal growth factor receptor-derived peptides recognised by both cellular and humoral immune responses in HLA-A24+ non-small cell lung cancer patients. \textit{Eur. J. Cancer} \textbf{40}, 1776–1786. https://doi.org/10.1016/j.ejca.2004.04.003 (2004).
3. Imai, N. et al. Identification of Lck-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with distant metastases. \textit{Int. J. Cancer} \textbf{94}, 237–242. https://doi.org/10.1002/ijc.1461 (2001).
4. Naito, M. et al. Identification of Lck-derived peptides applicable to anti-cancer vaccine for patients with human leukocyte antigen-A3 supertype alleles. \textit{Br. J. Cancer} \textbf{97}, 1648–1654. https://doi.org/10.1038/sj.bjc.6600471 (2007).
5. Harashima, N. et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. \textit{Eur. J. Immunol.} \textbf{31}, 323–332. https://doi.org/10.1002/1521-4141(200102)31:2<323::aid-immu323>3.0.co;2-0 (2001).
6. Yamada, A., Kawano, K., Koga, M., Matsumoto, T. & Itoh, K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes. \textit{Cancer Res.} \textbf{61}, 6459–6466 (2001).
7. Nakao, M. et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. \textit{J. Immunol.} \textbf{164}, 2565–2574. https://doi.org/10.4049/jimmunol.164.5.2565 (2000).
8. Yang, D. et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A2-restricted cytotoxic T lymphocytes in cancer patients. \textit{Cancer Res.} \textbf{59}, 4056–4063 (1999).
9. Ito, M. et al. Identification of SART3-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with different HLA-A2 subtypes. \textit{Int. J. Cancer} \textbf{98}, 633–639. https://doi.org/10.1002/1097-0215(20001115)98:4<633::aid-ijc18>3.0.co;2-n (2000).
10. Minami, T. et al. Identification of SART3-derived peptides having the potential to induce cancer-reactive cytotoxic T lymphocytes from prostate cancer patients with HLA-A3 supertype alleles. \textit{Cancer Immunol. Immunother.} \textbf{56}, 689–698. https://doi.org/10.1007/s00262-006-0216-9 (2007).
11. Yao, A. et al. Identification of parathyroid hormone-related protein-derived peptides immunogenic in human histocompatibility leukocyte antigen-A24+ prostate cancer patients. \textit{Br. J. Cancer} \textbf{91}, 287–296. https://doi.org/10.1038/sj.bjc.6601960 (2004).
12. Ito, M. et al. Molecular basis of T cell mediated recognition of pancreatic cancer cells. \textit{Cancer Res.} \textbf{61}, 2038–2046 (2001).
13. Noguchi, M. et al. Phase I trial of a cancer vaccine consisting of 20 mixed peptides in patients with castration-resistant prostate cancer: dose-related immune boosting and suppression. \textit{Cancer Immunol. Immunother.} \textbf{64}, 493–505. https://doi.org/10.1007/s00262-015-1660-1 (2015).
14. Noguchi, M. et al. Immunological evaluation of neoadjuvant peptide vaccination before radical prostatectomy for patients with localized prostate cancer. *Prostate* **67**, 933–942. https://doi.org/10.1002/pros.20572 (2007).
15. Narita, Y. et al. A randomized, double-blind, phase III trial of personalized peptide vaccination for recurrent glioblastoma. *Neuro Oncol.* **21**, 348–359. https://doi.org/10.1093/neuonc/noy200 (2019).
16. Hailmichael, Y. et al. Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and depletion. *Nat. Med.* **19**, 465–472. https://doi.org/10.1038/nm.3105 (2013).
17. Iwai, Y. et al. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12293–12297. https://doi.org/10.1073/pnas.192461099 (2002).
18. Freeman, G. J. et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* **192**, 1027–1034. https://doi.org/10.1084/jem.192.7.1027 (2000).
19. Robert, C. et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N. Engl. J. Med.* **372**, 320–330. https://doi.org/10.1056/NEJMoA1512082 (2015).
20. Gandhi, L. et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N. Engl. J. Med.* **378**, 2078–2092. https://doi.org/10.1056/NEJMoA1801005 (2018).
21. Motzer, R. J. et al. Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N. Engl. J. Med.* **378**, 1277–1290. https://doi.org/10.1056/NEJMoA1712126 (2018).
22. Kang, Y. K. et al. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* **390**, 2461–2471. https://doi.org/10.1016/S0140-6736(17)31827-5 (2017).
23. Rittmeyer, A. et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OA2): a phase 5, open-label, multicentre randomised controlled trial. *Lancet* **389**, 255–265. https://doi.org/10.1016/S0140-6736(16)32517-X (2017).
24. Sawada, Y. et al. Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes. *Int. J. Oncol.* **46**, 28–36. https://doi.org/10.3892/ijo.2014.2737 (2015).
25. Campoli, M. & Ferrone, S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* **27**, 3869–3885. https://doi.org/10.1038/onc.2008.273 (2008).
26. Sawada, Y. et al. Phase II study of the GP33-derived peptide vaccine as an adjuvant therapy for hepatocellular carcinoma patients. *Oncovaccine* **5**, e1124983. https://doi.org/10.1016/j.oncv.2015.1124983 (2016).
27. Vermeire, R. et al. Potential target antigens for a universal vaccine in epithelial ovarian cancer. *Clin. Dev. Immunol.* https://doi.org/10.1155/2010/891505 (2010).
28. Harada, N. et al. Generation of a novel HLA class I transgenic mouse model carrying a knock-in mutation at the beta2-microglobulin locus. *J. Immunol.* **198**, 516–527. https://doi.org/10.4049/jimmunol.1502367 (2017).
29. Schwarz, K. et al. The selective proteasome inhibitors lactacystin and epoxomicin can be used to either up- or down-regulate antigen presentation at nontoxic doses. *J. Immunol.* **164**, 6147–6157. https://doi.org/10.4049/jimmunol.164.12.6147 (2000).
30. Vansteenkiste, J. F. et al. Efficacy of the MAGE-A3 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small-cell lung cancer (MAGRIT): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* **17**, 822–835. https://doi.org/10.1016/S1470-2045(16)30099-1 (2016).
31. Yamaura, H. et al. Randomized phase II/III clinical trial of elapomotide for patients with advanced pancreatic cancer: PEGASUS-PC Study. *Cancer Sci.* **106**, 883–890. https://doi.org/10.1111/cas.12674 (2015).
32. Wada, H. et al. Development of a novel immunoproteasome digestion assay for synthetic long peptide vaccine design. *PLoS ONE* **13**, e0199249. https://doi.org/10.1371/journal.pone.0199249 (2018).
33. Rosalia, R. A. et al. Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *Eur. J. Immunol.* **43**, 2554–2565. https://doi.org/10.1002/eji.201343324 (2013).
34. Reiser, J. & Banerjee, A. Effector, memory, and dysfunctional CD8(+) T cell fates in the antitumor immune response. *J. Immunol.* **2016**, 8941260. https://doi.org/10.1155/2016/8941260 (2016).
35. Wherry, E. J. et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* **4**, 225–234. https://doi.org/10.1038/ni889 (2003).
36. Feau, S., Arens, R., Togher, S. & Schenberger, S. P. Autocrine ICI-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat. Immunol.* **12**, 908–913. https://doi.org/10.1038/ni.2079 (2011).
37. Weden, S. et al. Long-term follow-up of patients with resected pancreatic cancer following vaccination against mutant K-ras. *Int. J. Cancer* **128**, 1120–1128. https://doi.org/10.1002/ijc.25449 (2011).
38. Fourcade, J. et al. PD-1 and Tim-3 regulate the expansion of tumor antigen-specific CD8(+) T cells induced by melanoma vaccines. *Cancer Res.* **74**, 1045–1055. https://doi.org/10.1158/0008-5472.CAN-13-2868 (2014).
39. Xue, W. et al. SCIB2, an antibody DNA vaccine encoding NY-ESO-1 epitopes, induces potent antitumor immunity which is further enhanced by checkpoint blockade. *Oncomunology* **5**, e1169335. https://doi.org/10.1080/2162402X.2016.1169335 (2016).
40. Peng, W. et al. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. *Cancer Res.* **72**, 5209–5218. https://doi.org/10.1158/0008-5472.CAN-12-1187 (2012).
41. Chin, Y. M. et al. PD-1 and PD-L1 up-regulation promotes T-cell apoptosis in gastric adenocarcinoma. *Anticancer Res.* **38**, 2069–2078. https://doi.org/10.21873/anticancres.12446 (2018).
42. Miyagi, Y. et al. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. *Clin. Cancer Res.* **7**, 3950–3962 (2001).

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**Author contributions**

Y.T. designed the study, performed most of the experiments, analyzed data, and drafted the manuscript. T.O. managed the project, co-designed experiments, and made substantial contributions to the revision of the manuscript. S.F., A.S., and M.O. made substantial contributions to the design of the peptide. H.W., R.G., and K.Y. performed the experiments, and analyzed the data. K.M., E.S., and K.I. revised the manuscript and co-managed the project. T.U. contributed to the conception of this work, and gave final approval of the manuscript. All authors critically reviewed and approved the final manuscript.

**Competing interests**

Y.T., H.W., R.G., T.O., K.Y., A.S., S.F., M.O., K.M., E.S., and K.I. are employees of Taiho Pharmaceutical Co. Ltd. T.U. is a board member of Taiho Pharmaceutical Co. Ltd.
