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ORIGINAL ARTICLE

Interruption of MDM2 signaling augments MDM2-targeted T cell-based antitumor immunotherapy through antigen-presenting machinery

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Abstract

Identification of immunogenic tumor antigens, their corresponding T cell epitopes and the selection of effective adjuvants are prerequisites for developing effective cancer immunotherapies such as therapeutic vaccines. Murine double minute 2 (MDM2) is an E3 ubiquitin-protein ligase that negatively regulates tumor suppressor p53. Because MDM2 overexpression serves as a poor prognosis factor in various types of tumors, it would be beneficial to develop MDM2-targeted cancer vaccines. In this report, we identified an MDM2-derived peptide epitope ($MDM2_{32\text{-}46}$) that elicited antigen-specific and tumor-reactive $CD4^+$ T cell responses. These $CD4^+$ T cells directly killed tumor cells via granzyme B. MDM2 is expressed in head and neck cancer patients with poor prognosis, and the T cells that recognize this MDM2 peptide were present in these patients. Notably, Nutlin-3 (MDM2-p53 blocker), inhibited tumor cell proliferation, was shown to augment antitumor T cell responses by increasing MDM2 expression, HLA-class I and HLA-DR through class II transactivator (CIITA). These results suggest that the use of this MDM2 peptide as a therapeutic vaccine combined with MDM2 inhibitors could represent an effective immunologic strategy to treat cancer.

Keywords MDM2 · MDM2 inhibitor · Immunotherapy · Peptide vaccine · Tumor-associated antigen · Head and neck squamous cell carcinoma

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Abbreviations

APC	Antigen-presenting cell
CIITA	Class II transactivator
CTL	$CD8^+$ cytotoxic T lymphocyte
DC	Dendritic cell
E	T: Effector: Target
FFPE	Formalin fixed, paraffin embedded
HNSCC	Head and neck squamous cell carcinoma
HTL	$CD4^+$ helper T lymphocyte
ICI	Immune checkpoint inhibitor
mAb	Monoclonal antibody
MDM2	Murine double minute 2
MFI	Mean fluorescence intensity
PBMC	Peripheral blood mononuclear cell
TAA	Tumor-associated antigen

Introduction

Success of cancer immunotherapy such as immune checkpoint inhibitors (ICIs) has shown that immune cells can play a leading role in cancer treatment. Although the antitumor role of immune cells is evident, the clinical response to ICIs is far from optimal, since many patients fail to respond. The antitumor effects of ICIs rely on existing immune cells which in most instances are few in numbers or become exhausted. Accordingly, the de novo generation of tumor-reactive T cells by active immunization can be a viable strategy to optimize tumor immunotherapy. Cancer vaccines using synthetic peptides derived from tumor antigens constitute a practical approach to elicit and expand tumor-reactive T cells [1]. Tumor-specific antigens such as mutation-derived neoantigens have been suggested as the most promising tumor vaccine candidates [2]. However, requiring the identification of mutations for each patient, this approach is complex and cost-ineffective to apply to the general patient population. Moreover, the mutational status fluctuates during tumor progression [3] suggesting that the use of mutation-derived antigens is not a straightforward strategy for developing peptide vaccines and that perhaps the use of shared tumor-associated antigens (TAAs) should be reconsidered.

Ideal characteristics of a TAA to develop a peptide vaccine would be its immunogenicity (lack of complete tolerance) and relatively high levels of expression in various tumor types as compared to normal tissues [4]. In addition, the selection of effective immune adjuvants will be necessary to optimize any type of peptide vaccine [5] [6]. Our group found that peptide vaccines administered with adjuvants that mimic viral infections significantly elicited and expanded antigen-specific, tumor-reactive T cells [7] [8]. Thus, the identification of potent peptide epitopes from TAAs and the use of effective immune adjuvants will be critical for developing tumor immunotherapy.

Apoptosis-related proteins overly expressed by tumor cells could constitute unique universally expressed antigens. We reported that a posttranslational modified epitope from p53 which is aberrantly expressed and loses its anti-apoptotic function in tumors could generate tumor-reactive T cells [9] [10]. Murine double minute 2 (MDM2) is an E3 ubiquitin-protein ligase that forms a complex with p53 followed by p53 degradation [11]. Because MDM2 transcription is activated by p53, the protein is highly expressed in various cancer types with poor prognosis [12] [13]. This prompt us to investigate the potential use of MDM2 as a TAA.

MDM2 inhibitors have been shown to be effective for MDM2 expressing tumors with few adverse events [14]. Since MDM2 inhibitors block the interaction and ubiquitination of MDM2 and p53, MDM2 expression is upregulated suggesting that MDM2 inhibitors could complement an MDM2-targeted vaccine. Interestingly, Wang et al. showed that the p53 amplification by MDM2 inhibitor increased tumor HLA class I expression via endoplasmic reticulum aminopeptidase 1 [15]. However, the effect of MDM2 inhibitors with HLA class II expression remains unknown.

Here, we report the identification of an MDM2-derived peptide capable of inducing in vitro antigen-specific, tumor-reactive $CD4^+$ T cell responses with blood cells from healthy donors and patients with head and neck squamous cell carcinoma (HNSCC). Furthermore, the MDM2 inhibitor Nutlin-3 augmented antitumor responses of MDM2- -specific T cells subsequent to upregulating HLA class II expression on tumor cells. These findings suggest that MDM2 could be a promising antitumor target as vaccine, and the combination therapy with MDM2 inhibitors constitutes a novel strategy for cancer immunotherapy.

Materials and methods

Patients and Immunohistochemistry

Tissue samples were acquired from pretreatment biopsy tissues of 49 HNSCC patients treated in Asahikawa Medical University. Clinical characteristics of the patients are shown in Supplementary Table S1. TNM staging was based on 8th edition of the International Union Against Cancer. MDM2 expression was analyzed on formalin-fixed, paraffin-embedded (FFPE) tissues from the HNSCC patients. The mouse monoclonal antibody (mAb) to MDM2 (IF2, 1:50 dilution, Merck, Darmstadt, Germany) served as a primary antibody. FFPE tissues were stained in a VENTANA Benchmark GX (Roche Diagnostics, Rotkreuz, Switzerland) using Cell Conditioning 1 buffer (Rache Diagnostics) as antigen retrieval solution and a VENTANA ultraView Universal DAB Detection Kit

(Roche Diagnostics). Informed consent was obtained by the opt-out method on the Asahikawa Medical University Web site. Approval for clinical data collection and analysis was obtained from the Asahikawa Medical University Institutional Review Board (#16,217).

Cell lines

HNSCC cell lines, HSC2 (oral SCC, HLA-DR13), HSC3 (tongue SCC, HLA-DR15), HSC4 (tongue SCC, HLA-DR1/4, 53), Sa-3 (gingival SCC, HLA-DR9/10, 53) were supplied by RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). HNSCC cell line HPC-92Y (hypopharyngeal SCC, HLA-DR4/9,53) and L cells (mouse fibroblasts cell lines) expressing individual human HLA-DR molecules (HLA-DR4,8,9, and 53) were kindly provided from Dr. Syunsuke Yanoma (Yokohama Tsurugamine Hospital, Yokohama, Japan), Dr. R. Karr (Karr Pharma, St. Louis, MO), and Dr. Sasazuki (Kyushu University, Fukuoka, Japan), respectively. All cell lines were maintained in tissue culture as recommended by the supplier.

Western blotting

HNSCC cell lines protein were extracted using a Total Protein Extraction Kit for Animal Cultured Cells and Tissues (Invent Biotechnologies, Inc., Plymouth, MN). The protein extracts were subjected to electrophoresis in a 4–12% NuPAGE Bis–Tris SDS-PAGE gel (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA) and transferred to an Immobilon-P membrane (Merck Millipore, Burlington, MA). The membrane was blocked in PBS containing 0.01% Tween 20 and 5% non-fat dry milk for 1 hr at room temperature and incubated with monoclonal mouse anti-human MDM2 Ab (sc965, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal mouse anti-human β-actin Ab (C4, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) for 12 hr at 4℃. After washing, the membrane was incubated with horseradish peroxidase-labeled sheep anti-mouse IgG. Amersham ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences, Logan, UT) and Invitrogen iBright Imaging Systems 1500 (Invitrogen, Thermo Fisher Scientific) were used to detect chemiluminescent. The effect of MDM2-inhibitor was evaluated by treating MDM2-expressing HNSCC cell lines with 10 μM MDM2-inhibitor (Nutlin-3, Selleck Biotech, Tokyo, Japan) for 48 hr. The expression of class II transactivator (CIITA) was assessed using monoclonal mouse anti-CIITA Ab (7-1H, sc-13556, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA).

MTS assay

The anti-tumor effect of Nutlin-3 was investigated by MTS assay (The CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI). HSC4 and HPC-92Y were seeded in a 96-well culture plate and treated with Nutlin-3 (0-100 μM) for 48 hr. After adding MTS solution, we put it in the incubator for 1 hr and measured the absorption at 490 nm by GloMax Discover Microplate Reader (Promega, Madison, WI).

Synthetic peptides

HLA-DR-binding amino acid sequences of MDM2 were predicted using the computer-based algorithms SYFPEITHI (http://www.syfpeithi.de/) [16] and Immune Epitope Database Analysis Resource [IEDB, RRID:SCR_013182, https://www.iedb.org/) [17]. The peptide which has high bind score with multiple common HLA-DR molecules was selected as a candidate epitope. We selected $MDM2_{32\text{-}46}$ (PLLLKLLKSVGAQKD) that potentially bind with DRB1*0101, DRB1*0401, DRB1*0901, DRB1*1101 and DRB1*1501. The $MDM2_{32\text{-}46}$ peptide epitope was synthesized and purified by Hokkaido System Science (Sapporo, Japan). PADRE peptide (aK-Cha- VAAWTLKAAa, where "a" is D-alanine, "Cha" is L-cyclohexylalanine) that could bind to multiple HLA class II molecules was used as a positive control (Pan HLA-DR-binding epitope).

In vitro induction of MDM2-reactive $CD4^+$ T cells

The method utilized for the induction of peptide-reactive $CD4^+$ T cell (HTL) lines from peripheral blood mononuclear cells (PBMCs) of healthy donors has been described in detail [18]. Briefly, HTLs purified by EasySep™ Human $CD4^+$ T Cell Isolation Kit (STEMCELL technology, Vancouver, Canada) were stimulated with peptide-pulsed autologous dendritic cells (DCs). DCs were derived from $CD14^+$ cells purified by using EasySep™ Human CD14 Positive selection Kit (STEMCELL) following GM-CSF (50 ng/ml, PeproTech, Rocky Hill, NJ) and IL-4 (1000 IU/ml, PeproTech, Rocky Hill, NJ) stimulation. After 2 cycles of stimulations with γ-irradiated autologous PBMCs with peptides, T cells were tested for IFN-γ production with $MDM2_{32\text{–}46}$ peptide stimulation by ELISA kits (BD Pharmingen, SanDiego, CA) according to the manufacturer's instructions. Positive microcultures

showing an significant increase in IFN-γ production after peptide stimulation compared to unstimulated control were subsequently expanded. $MDM2_{32–46}$ -specific HTL lines were isolated by the limiting dilution.

MDM2-specific responses with established $CD4^+$ T cell lines

The method to assess $CD4^+$ T cells responses to target peptide was described previously [19]. HTL lines were co-cultured with antigen-presenting cells (APCs). Autologous PBMCs (1×10^5), L-cells (3×10^4), and MDM2-expressing HNSCC cell lines (3×10^4) were used as APCs. Tumor cell lines were exposed to IFN-γ (500U/ml, PeproTech, Rocky Hill, NJ) for 48 hr to enhance HLA-DR expression before the assay. Anti-HLA-DR mAb L243 (IgG2a made from hybridoma HB-55 obtained from ATCC) and anti-HLA-class I mAb W6/32 (IgG2a, ATCC) were used to determine HLA restriction. Production level of INF-γ in each co-cultured supernatant was measured by ELISA kits (BD Pharmingen).

Cytotoxicity assay

We evaluated the cytotoxicity of HTLs by Granzyme B ELISA and flow cytometry. After coculturing target cells with the HTL lines, supernatants were collected and evaluated for Granzyme B by ELISA (MABTECH, Stockholm, Sweden) according to the manufacturer's instructions. In killing assay, tumor cell lines were labeled by the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen; Thermo Fisher Scientific, Inc.) 6 hr before coculturing with several effector/target cell (E: T) ratios of T cells. Dead cells were assessed in by flow cytometry (BD Accuri C6 flow cytometer, BD Biosciences) using 7-AAD viability staining solution (BioLegend).

Flow cytometry

HLA-DR, MHC-class I, PD-L1 and EGFR expression on HNSCC cell lines after pretreatment with 10 μM Nutlin-3 and 50U/ml IFN-γ for 48 hr were evaluated by flow cytometry using anti-HLA- DR mAB (TU36) conjugated with phycoerythrin (BD Pharmingen), anti-MHC-class I mAB (G46-2) conjugated with fluorescein isothiocyanate (BD Pharmingen), anti-PD-L1 mAb (MIH) conjugated with phycoerythrin (eBioscience, Thermo Fisher Scientific) and anti-EGFR mAb (EGFR.1) conjugated with phycoerythrin (BD Pharmingen). IgG2a antibody (MOPC-173, BioLegend) and IgG1 antibody (MOPC-21, BioLegend) were used as isotype controls. Samples were analyzed using the BD Accuri C6 flow cytometer and software (BD Biosciences).

Effects of combination with MDM2 inhibitor and peptide-specific T cells

HNSCC cell lines were pretreated with 10 μM Nutlin-3 and 50U/ml IFN-γ for 48 hr. Tumor cell lines pretreated with Nutlin-3 were co-cultured with $MDM2_{32-46}$-specific HTL lines or $EGFR_{875-889}$ -specific HTLs (T8 and H22) [20]. Production level of IFN-γ and cytotoxic assay were carried out as mentioned above.

MDM2 peptide-specific responses in HNSCC patients

PBMCs of HNSCC patients were cultured with $MDM2_{32-46}$ peptides or PADRE peptides in 96-well plates as described previously [21]. PADRE peptide that could bind to multiple HLA class II molecules was used as a positive control. Since only a small amount of PBMCs was obtained from patients, short-term culture was performed. We stimulated PBMCs for 2 cycles every 7 days and evaluated the production level of IFN-γ in the supernatants by ELISA. Anti-DR Ab was used as a negative control. All experiments were approved by the institutional ethics committee on the Asahikawa Medical University (#16,217), and written informed consent was appropriately obtained.

Statistical analysis

The *P* values were examined with log-rank test. The other data were analyzed by the Student's t test or Fisher's exact test. *P* values < 0.05 indicates statistical significance.

Results

MDM2 expression in HNSCC patients and cell lines

Several reports have suggested that MDM2 is aberrantly expressed in aggressive HNSCC [22]. To validate this, immunohistochemical analyses were performed to assess correlation between MDM2 expression and prognosis in 49 HNSCC patients. MDM2 was expressed in 25/49 cases (51%) of HNSCC, which is consistent with previous reports (Fig. 1a and Supplementary Table S1). No correlation was observed between MDM2 expression and clinical characteristics including age, sex, tumor cite, or tumor stage. Similarly, the expression pattern of MDM2 in the Human Protein

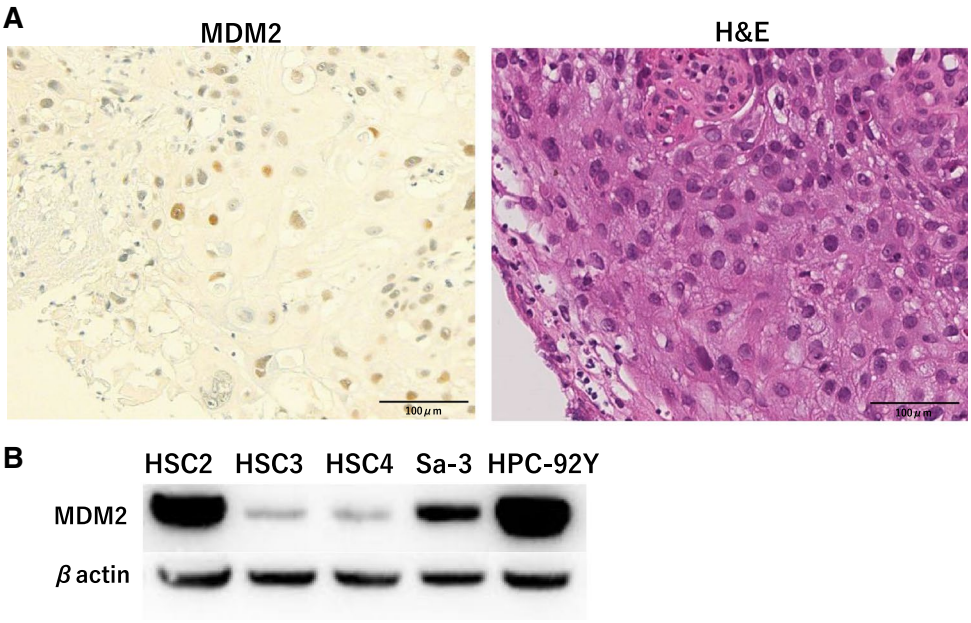


Fig. 1 MDM2 expression in HNSCC patients and cell lines **a** A representative clinical image of MDM2 expression in immunohistochemistry. MDM2 was positively stained in the nucleus of HNSCC. H&E staining was shown in the right. **b** MDM2 expression in HNSCC cell lines was examined by Western blotting

Atlas database (https://www.proteinatlas.org) [23] showed that 367/499 (73.5%) HNSCC patients have high MDM2 expression. Next, we examined the expression of MDM2 in HNSCC cell lines (HSC2, HSC3, HSC4, Sa-3 and HPC-92Y). As shown in Fig. 1b, all the cell lines tested expressed MDM2. The proliferation of HNSCC cells was decreased in a dose-dependent manner with MDM2 inhibitor Nulin-3 treatment (Supplementary Figure S1). These results suggest that MDM2 is expressed in more than half of patients with aggressive HNSCC, and MDM2 could be a feasible target to treat HNSCC.

Induction of $MDM2_{32\text{-}46}$-specific $CD4^+$ T cells responses

To develop MDM2-targeted T cell immunotherapy, using a computer-based algorithm, we identified and selected peptide $MDM2_{32\text{-}46}$ (PLLLKLLKSVGAQKD) as a potential candidate for inducing $CD4^+$ T cell responses. Purified $CD4^+$ T cells from healthy donors were repeatedly stimulated with $MDM2_{32\text{-}46}$ peptide as described in Materials and Methods. Three $MDM2_{32\text{-}46}$ peptide-reactive HTL lines, K2, R9, and H4 were induced. These HTLs responded to $MDM2_{32\text{-}46}$ peptide and released IFN-γ in a dose-dependent manner (Fig. 2a), and this response as expected was restricted by MHC class II and not MHC class I (Fig. 2b). To determine which HLA-DR allele was responsible for the T cell interaction, L-cells expressing single HLA-DR molecules were used as APCs. As a result, HTL lines K2 and R9 responded to L-cells expressing HLA-DR53, while HTL H4 responded to L-cells expressing HLA-DR4 indicating that $MDM2_{32\text{-}46}$ peptide can be presented by at least 2 different HLA-DR molecules (Fig. 2c).

Direct recognition and killing of tumor cells by $MDM2_{32\text{-}46}$-specific $CD4^+$ T cells

To evaluate whether induced HTL lines could recognize naturally processed MDM2-derived peptide, direct recognition of MDM2 expressing HNSCC cell lines by $MDM2_{32\text{-}46}$-specific HTL lines was examined. As shown in Fig. 3a, $MDM2_{32\text{-}46}$-specific HTL lines coculturing with HLA-DR-matched tumor cells produced IFN-γ while HLA-DR-unmatched tumor cells were ignored by the HTL. Recent evidence suggests that $CD4^+$ T cells can perform direct cytotoxicity against tumor cells via granzyme B [24]. In addition to IFN-γ, $MDM2_{32\text{-}46}$-specific HTL lines produced granzyme B in response to HLA-DR-matched tumor cells (Fig. 3b). More importantly, these T cells showed a direct killing activity to HLA-DR-matched tumor cells (Fig. 3c). These results suggest that $MDM2_{32\text{-}46}$ peptide could induce cytotoxic $CD4^+$ T cells, and could be a potent tumor vaccine against HNSCC.

T cell responses to $MDM2_{32\text{-}46}$ in HNSCC patients

The presence of MDM2 peptide-reactive T cells in patients with HNSCC would encourage translation of an MDM2 peptide vaccine into clinic. To assess the existence of precursor MDM2 peptide-specific T cells in HNSCC patients, we

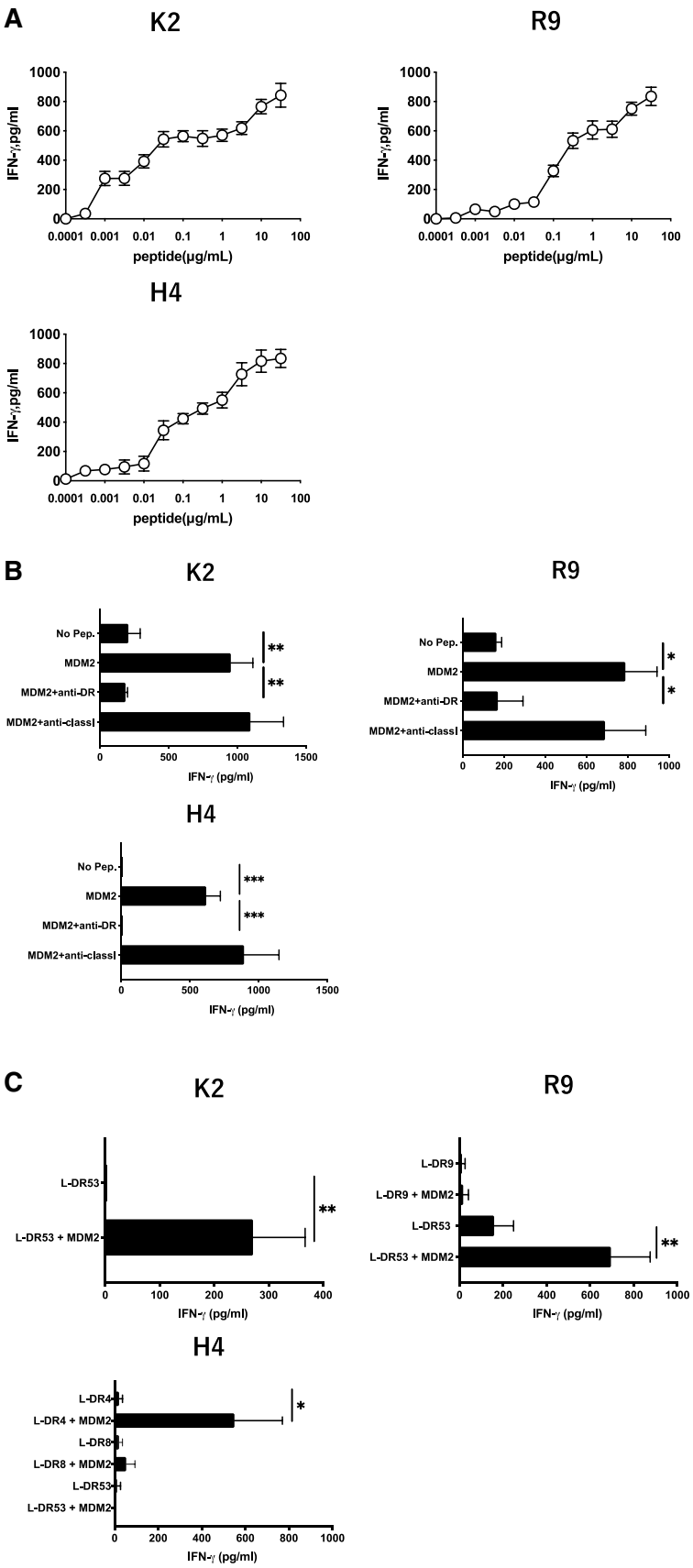


Fig. 2 Induction of $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells **a** $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells (K2, R9, and H4) were evaluated for cytokine production with various concentrations of $MDM2_{32\text{-}46}$ peptide in the context of autologous PBMCs as APCs. **b** HLA restriction of the $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells was evaluated by co-culturing anti-HLA-DR mAb or anti-HLA class I mAb with autologous PBMCs as APCs. **c** Determination of restrictive HLA-DR allele in the $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells. Peptide-reactive responses in these T cells were evaluated by co-culturing with L-cells expressing individual HLA-DR as APCs. The production of IFN-γ was examined using ELISA after coculturing with APCs for 48 hr. Bars and error bars indicate the mean and SD, respectively. Experiments were performed in triplicate (*$p < 0.05$, **$p < 0.01$, *** < 0.001, Student's *t* test)

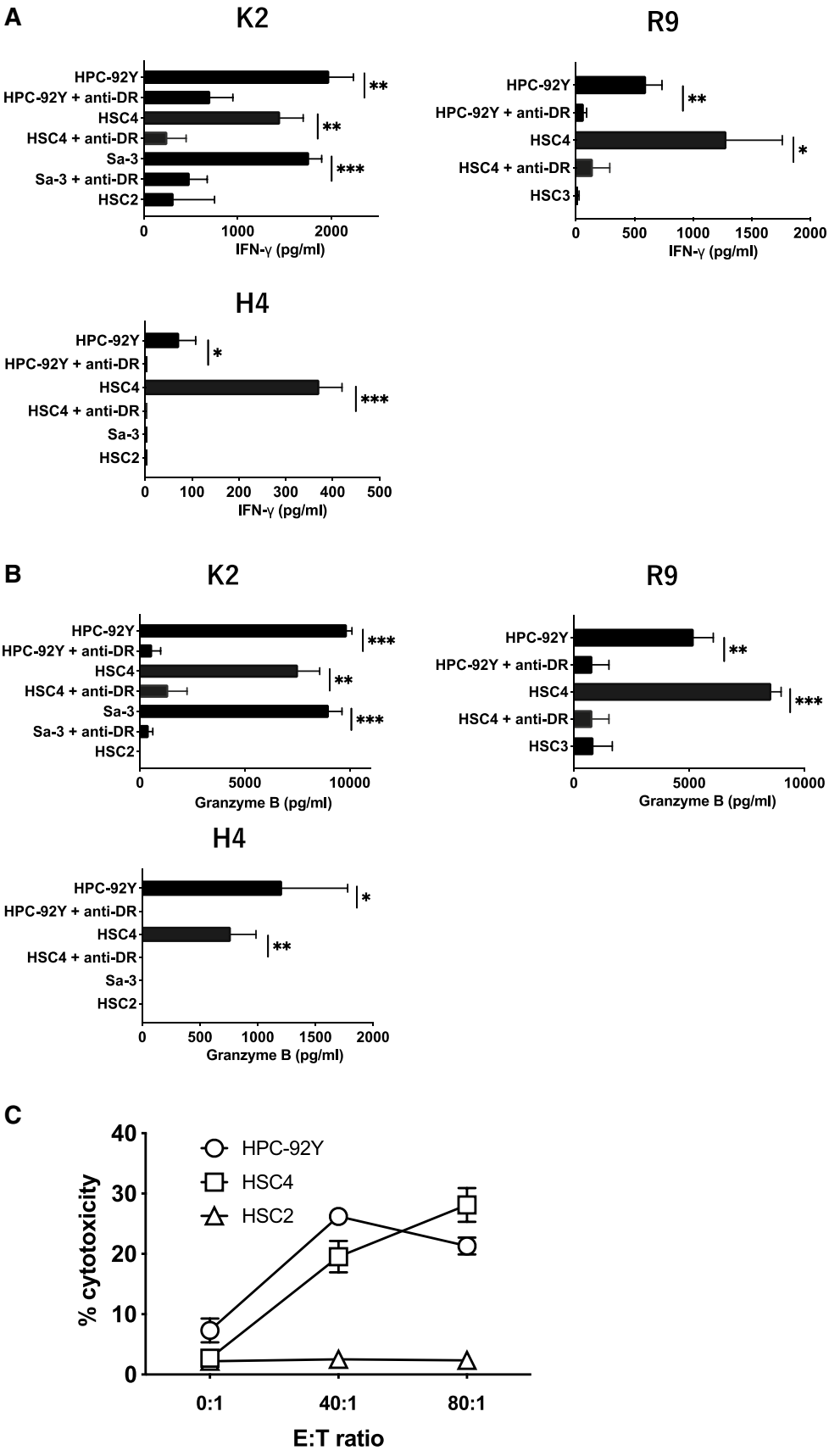


Fig. 3 Direct recognition and cytotoxicity to MDM2 expressing HNSCC cell lines by $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells **a** Direct recognition of tumor by $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells was evaluated by co-culturing HLA-DR matched or unmatched tumor cell lines and measuring IFN-γ production. K2 and R9 were restricted to HLA-DR53, whereas H4 was restricted to HLA-DR4. HSC2: HLA-DR13; HSC3: HLA-DR15; HSC4: HLA-DR1/4, 53; Sa-3: HLA-DR9/10, 53: HPC-92Y: HLA-DR4/9, 53. **b** Cytotoxicity of $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells to HLA-DR matched or unmatched tumor cell lines was examined measuring Granzyme-B production. **c** Killing ability of $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells to HLA-DR matched or unmatched tumor cell lines. HLA-DR53-restricted R9 cell lines were co-cultured with CSFE-labeled tumor cell lines (HSC2: HLA-DR13; HSC4: HLA-DR1/4, 53; HPC-92Y: HLA-DR4/9, 53) for 6 hr with several E: T (Effector: Target cells) ratio and evaluated the percentages of dead cells ($CFSE^+$ $7\text{-}AAD^+$ cells) with flow cytometry. Symbols and error bars indicate the mean and SD, respectively. Experiments were performed in triplicate. (*$p < 0.05$, **$p < 0.01$, *** < 0.001, Student's *t* test)

Table 1 Evaluation of T cell responses to $MDM2_{32-46}$ peptide in HNSCC patients

	Sex	Age (years)	Stage	Tumor site	No antigen IFN-γ.pg/ml	MDM2	MDM2 + anti-DR	PADRE
1	M	64	T4aN2bM0	Oropharynx pl6 −	<	253 ± 46	64 ± 61	187 ± 45
2	M	59	T4aN2cM0	Oralcavitity	<	121 + 23	25 + 20	202 ± 27
3	M	72	T4aN2bM0	Oropharynx pl6−	<	637 + 117	119 + 46	495 ± 89
4	M	88	T1N0M0	Hypopharynx	<	296 ± 29	<	169 ± 29
5	M	52	T4N1M0	Oropharynx pl6 +	<	669 ± 122	<	651 ± 141
6	M	67	T4N1M0	Oropharynx pl6 +	<	1694 ± 276	242 ± 48	1381 ± 278

<: less than the lower limit of detection

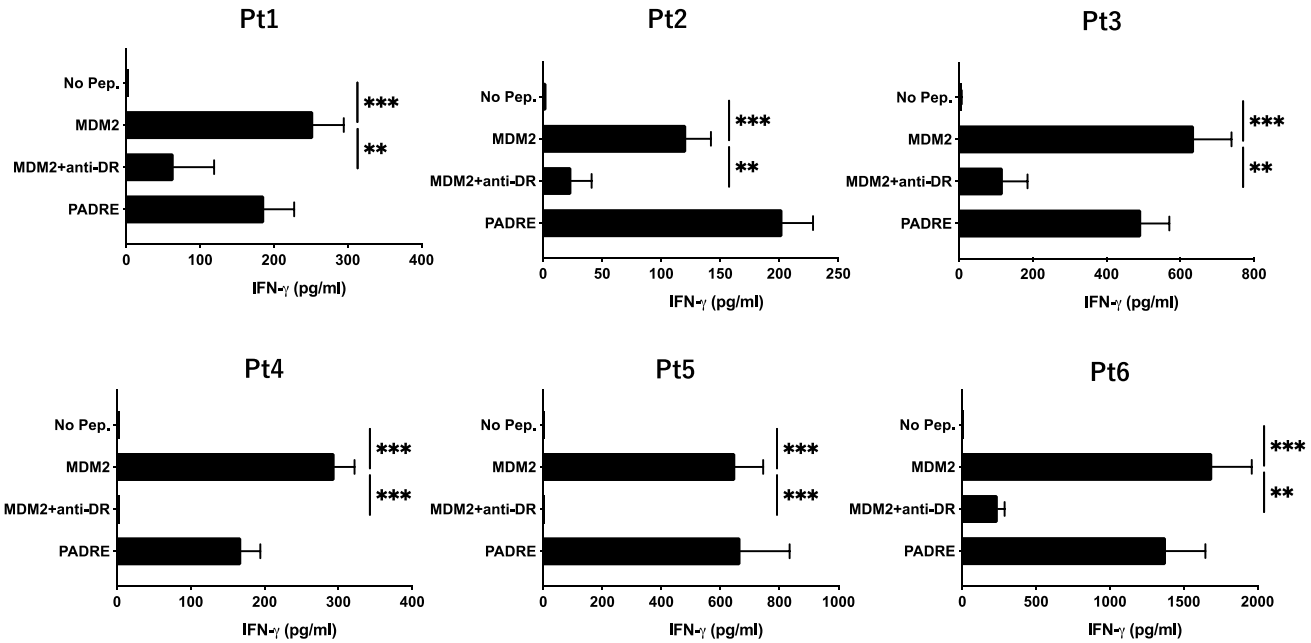


Fig. 4 Reactivity to $MDM2_{32-46}$ peptide in HNSCC patient PBMCs PBMCs from six HNSCC patients were stimulated with $MDM2_{32-46}$ peptide for 2 cycles. IFN-γ production in the supernatant was measured by ELISA. PADRE was used as positive control. Bars and error bars indicate the mean and SD, respectively. Experiments were performed in triplicate. (*$p < 0.05$, **$p < 0.01$, ***< 0.001, Student's *t* test)

performed short-term stimulation assays using PBMCs from HNSCC patients. The clinical characteristics of 6 HNSCC patients are summarized in Table 1. As shown in Table 1 and Fig. 4, T cell responses to $MDM2_{32-46}$ peptide were observed in HNSCC patients. This result indicates that the patients with HNSCC could be capable of inducing T cell responses with MDM2-targeted peptide vaccine.

MDM2 inhibitor enhances cytotoxicity to tumor by peptide-specific $CD4^+$ T cells through upregulation of tumor HLA expression

Finally, we evaluated the effects of MDM2 inhibitor Nutlin-3 as an immune adjuvant. Interestingly, Nutlin-3 enhanced HLA-DR and HLA-class I expressions on tumor cells (Fig. 5a) in addition to MDM2 expression (Supplementary Figure S2). The upregulation of HLA-DR was mediated through CIIITA (Fig. 5b). Subsequent to increased HLA expression, preconditioning of tumor cells with Nutlin-3 augmented the $MDM2_{32-46}$-specific $CD4^+$ T cell responses to HNSCC cells (Fig. 6a). In

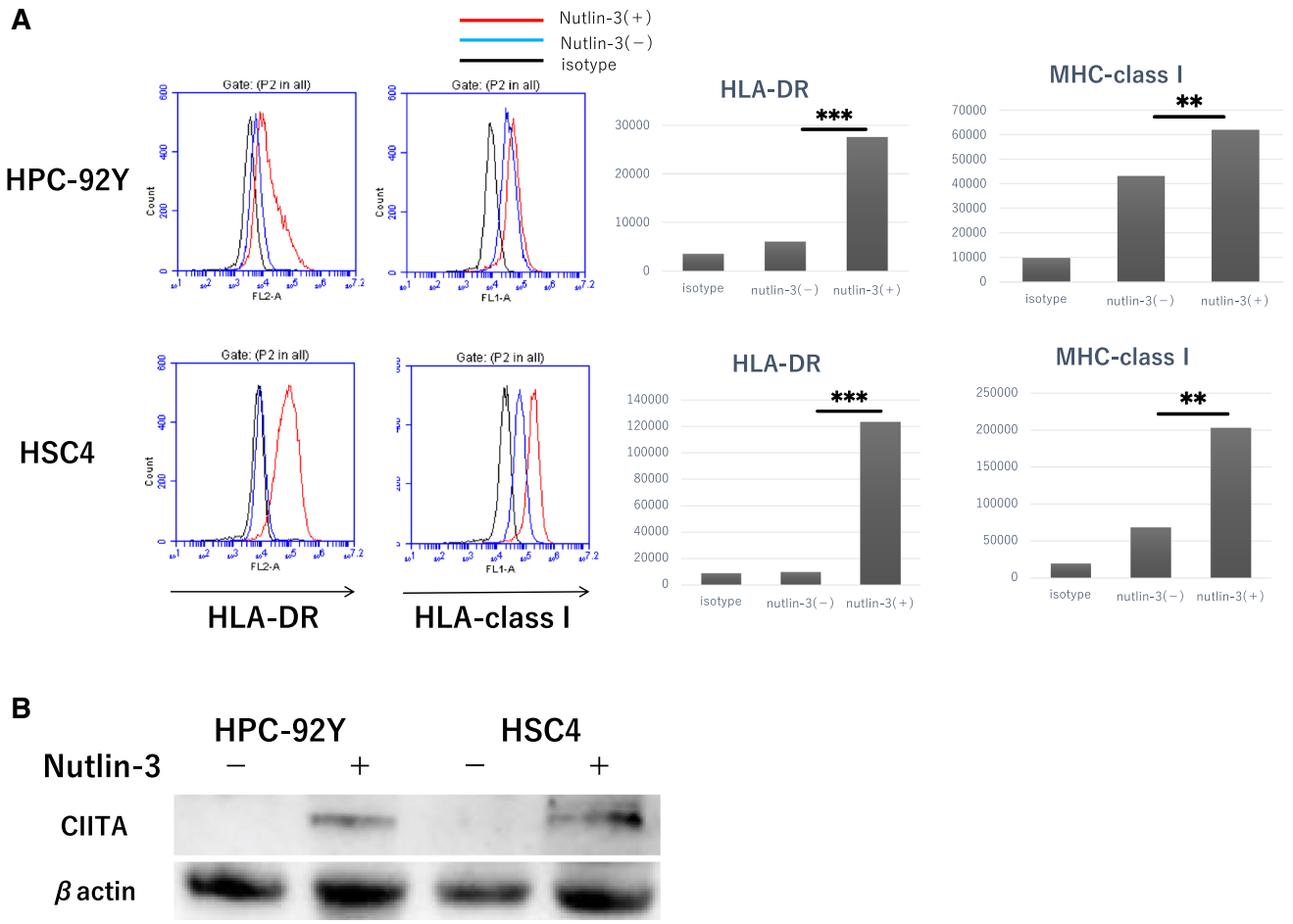
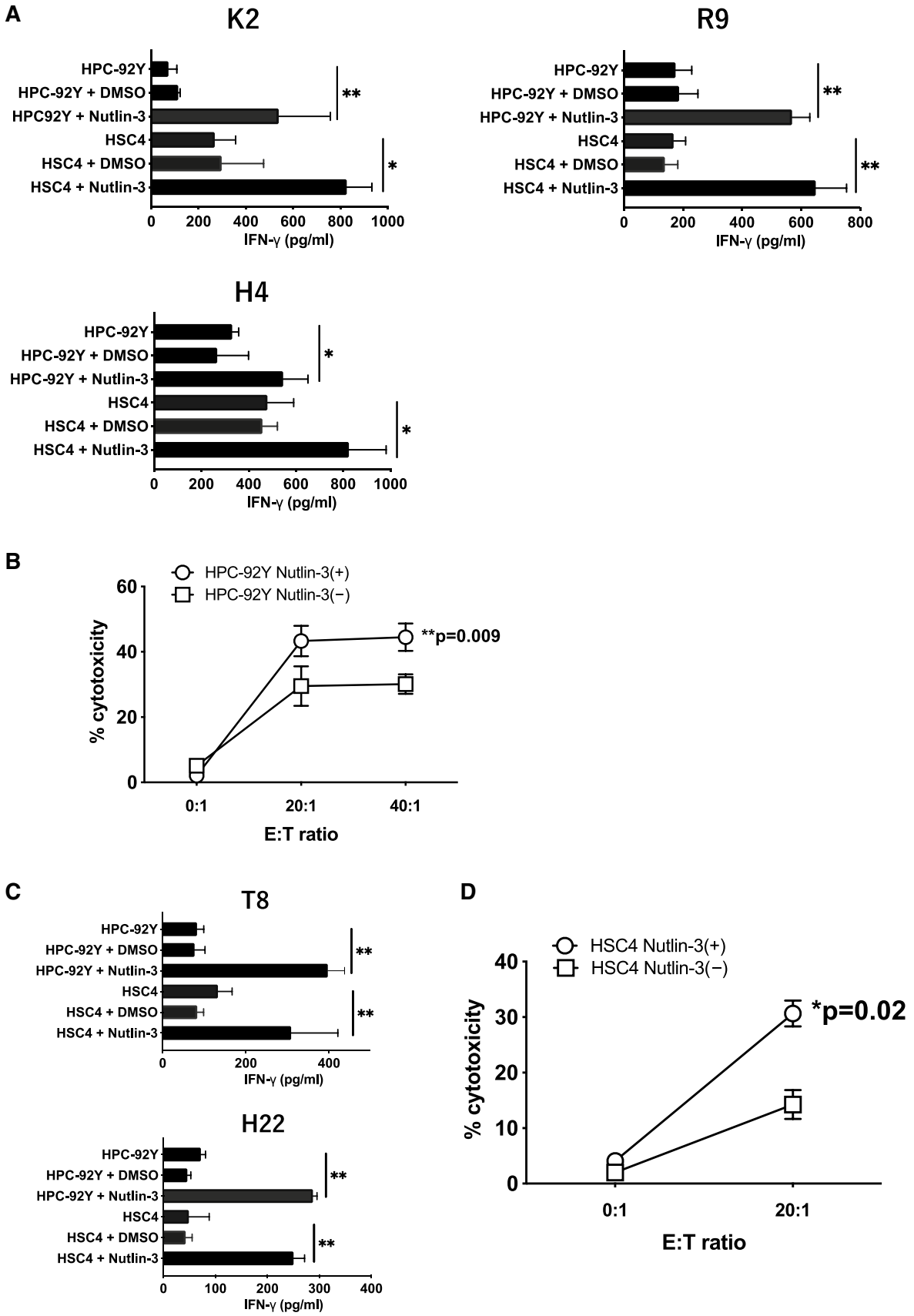


Fig. 5 HLA expression changes on HNSCC cell lines by MDM2 inhibitor. **a** Expression levels of HLA-DR and HLA-class I in HNSCC cell lines incubated with Nultin-3 were evaluated by flow cytometry. Left panels show representative data of flow cytometry. Black: isotype control. Blue: HLA-DR or HLA-class I expression on untreated tumor cell lines. Red: HLA-DR or HLA-class I expression on tumor cell lines treated with Nutlin-3 (10 μM). Right panels show averages values of mean fluorescence Intensity (MFI). **b** Nutlin-3 (10 μM) upregulated class II transactivator (CIITA) expression in HNSCC cell lines

addition to tumor recognition, the tumoricidal activity of $MDM2_{32\text{-}46}$-specific HTL lines was also increased (Fig. 6b). To elucidate whether the upregulation of T cell responses is mediated by increase in antigen expression or antigen-presenting machinery, we took advantage of using $EGFR_{875\text{-}889}$-specific HTL lines as effector T cells [20]. As a result, both production of IFN-γ and killing activity of $EGFR_{875\text{-}889}$-specific HTL lines were enhanced by Nutlin-3 (Fig. 6c and d). Nutlin-3 did not enhanced EGFR expression (Supplementary Figure S3). Collectively, these results suggest that MDM2 blockade is a unique immune adjuvant to combine with MDM2-targeted peptide vaccine as well as with other TAAs-targeted peptide vaccine (Fig. 7).

Discussion

Earlier studies have shown that MDM2-derived peptides could induce $CD8^+$ cytotoxic T lymphocytes (CTLs) [25] [26]. However, there are no reports that show the antigenic capacity of MDM2 to activate $CD4^+$ helper T lymphocytes (HTLs). Here, we have identified a novel T cell epitope peptide from MDM2, which generates antitumor HTL responses against MDM2 expressing tumors. Although the development of antitumor vaccine has mainly focused on CTLs, 'helpless' CTLs alone are insufficient to generate T cell memory that is required for the sustained antitumor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K2
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HPC-92Y + DMSO
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% cytotoxicity
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C
T8
HPC-92Y
HPC-92Y + DMSO
HPC-92Y + Nutlin-3
HSC4
HSC4 + DMSO
HSC4 + Nutlin-3
**
**
0 200 400
IFN-γ (pg/ml)
H22
HPC-92Y
HPC-92Y + DMSO
HPC-92Y + Nutlin-3
HSC4
HSC4 + DMSO
HSC4 + Nutlin-3
**
**
0 100 200 300 400
IFN-γ (pg/ml)
D
HSC4 Nutlin-3(+)
HSC4 Nutlin-3(−)
*p=0.02
% cytotoxicity
0 10 20 30 40
0:1 20:1
E:T ratio

◂**Fig. 6** Antitumor effects of combination with peptide-specific T cells and MDM2 inhibitor. **a** Responses to tumor cell lines pretreated with Nutlin-3 by $MDM2_{32\text{-}46}$-reactive $CD4^+$ T cells were evaluated by measuring IFN-γ production. DMSO was used as negative control. **b** Killing ability of T cells to tumor cell lines pretreated with Nutlin-3. $MDM2_{32\text{-}46}$-reactive R9 cell lines were co-cultured with CSFE-labeled Nutlin-3 pretreated tumor cell lines for 6 hr. The percentages of dead cells with several E: T (Effector: Target cells) ratio were evaluated using 7-AAD staining with flow cytometry. Symbols and error bars indicate the mean and SD, respectively. **c** Cytokine production of HLA-DR53-restricted $EGFR_{875\text{-}889}$-reactive $CD4^+$ T cells (T8 and H22) in response to $EGFR^+$ $HLA\text{-}DR53^+$ tumor cell lines pretreated with Nutlin-3. IFN-γ production was measured as output. DMSO was used as negative control. **d** Killing activity to tumor cell lines pretreated with Nutlin-3 by $EGFR_{875\text{-}889}$-reactive $CD4^+$ T cells (T8). T8 cell lines was co-cultured with CSFE-labeled Nutlin-3 pretreated tumor cell lines for 6 hr. The percentages of dead cells with several E: T (Effector: Target cells) ratio were evaluated using 7-AAD staining with flow cytometry. Symbols and error bars indicate the mean and SD, respectively. Experiments were performed in triplicate. (**$p < 0.01$, *** < 0.001, Student's *t* test)

effects with vaccine [1, 27]. Alspach et al. have reported that the expression of MHC class II-restricted antigens in tumors, which might be captured by APCs following HTL activation, was necessary to achieve antitumor effects with immunotherapy [28]. Because HTLs have crucial effects for the activation of macrophages in addition to CTLs and NK cells [29], HTL activation should take place in the tumor microenvironment to stimulate tumor-resident APCs. Accordingly, the induction of tumor-specific HTLs via TAAs-derived peptide vaccine could be valuable to provide cytokines and costimulatory molecule stimulation such as CD40 activation in tumor-resident APCs and provide help to $CD8^+$ CTLs. In addition to their helper function, some $CD4^+$ HTLs also have the ability to kill tumor cells [30]. We showed that the MDM2-specific HTL lines had cytotoxic activity against HNSCC cells in addition to releasing IFN-γ in response to MDM2 expressing tumors. These results suggest that the novel MDM2-derived peptide epitope is a potent candidate as tumor vaccine.

We showed that the $MDM2_{32\text{-}46}$ peptide bound to at least 2 HLA-DR alleles (DR53 and DR4). In addition, in silico predictions suggest that this peptide could also bind to HLA-DR1, DR11 and DR15 raising the possibility that this peptide would be applied to a broad population of patients. The presence of antigen-reactive T cell precursors in cancer patients is indispensable to expand these T cells with peptide vaccine. In addition to healthy donors, the precursor of MDM2-specific T cells existed in PBMCs from HNSCC patients. Notably, these T cells were capable of producing Th1 cytokine indicating that MDM2-specific T cells are not exhausted in HNSCC patients. In addition, the novel peptide epitope $MDM2_{32\text{-}46}$ contains a potential HLA-A0201-binding amino acid sequence ($MDM2_{33\text{-}41}$ LLLKLLKSV). Although the peptide-reactive activation of CTLs is outside the scope of this study, it would be of interest to evaluate the possibility of $MDM2_{32\text{-}46}$ peptide as a vaccine to generate both CTLs and HTLs.

We chose MDM2 as a TAAs for this study. As with previous findings regarding the relationship between MDM2 overexpression and poor prognosis in various types of cancer [12] [22], HNSCC patients with high MDM2 expression tended to have a poor prognosis in this study (Supplementary Figure S4). Moreover, the expression pattern of MDM2 in the Human Protein Atlas database [23] also confirmed that HNSCC patients with MDM2 expression had relatively poor survival compared to MDM2 negative patients ($p = 0.069$). Because MDM2 inhibits tumor apoptosis by p53 ubiquitination, it is rational that the tumor with MDM2 expression has an aggressive behavior, and the establishment of MDM2-targeted therapy could be of value in the treatment of patients with aggressive tumors. MDM2 binds to 18–26 residue on p53. If there is a mutation at this site, MDM2 inhibitor will not work. However, the mutation at this site is rare in HNSCC. Mutations at other sites that are frequently harbored in HNSCC are known to weaken p53 inhibition by MDM2 [31] [32]. Thus, the anti-tumor effect of MDM2 inhibitor on tumor with p53 mutation may be attenuated.

Autoimmunity could a potential adverse effect of targeting any TAA such as MDM2. However, the presence of MDM2-reactive T cells in human PBMCs indicates that these T cells pass the negative selection in thymus and the affinity of T cell receptor is low enough to ignore normal tissues, which MDM2 expression is markedly lower than in tumors [33]. It is noteworthy that these T cells directly recognized and killed HNSCC cells, which aberrantly overexpressed MDM2. Thus, MDM2-targeted peptide vaccine may activate moderate affinity T cells that react to high MDM2 tumor cells while sparing low MDM2 expressing normal tissues. Moreover, the clinical trials have shown that MDM2 inhibitors are tolerable with manageable toxicities suggesting the safety of targeting MDM2 [34] [35]. High clinical tolerability of p53-specific peptide vaccine in HNSCC [36] also supports the safety evidence of targeting apoptosis-related proteins as a TAA.

In addition to its direct antitumor effects, we described an immune adjuvant effect of MDM2 inhibitors. The upregulated expression of MDM2 in tumors by inhibiting MDM2 degradation, which is consistent to previous reports [37], is favorable for MDM2-targeted immunotherapy through the increase in antigen. Moreover, we demonstrated that the MDM2 inhibitor augmented the antitumor effects of MDM2-irrelevant T cells subsequent to the increased expression of HLA-DR and HLA-class I on tumor cells. Therefore, MDM2 inhibitor could serve as an immune adjuvant for any tumor immunotherapy. Since the effect of MDM2 inhibitor to increase in MHC class II on mouse dendritic cells has been reported [38], this is the

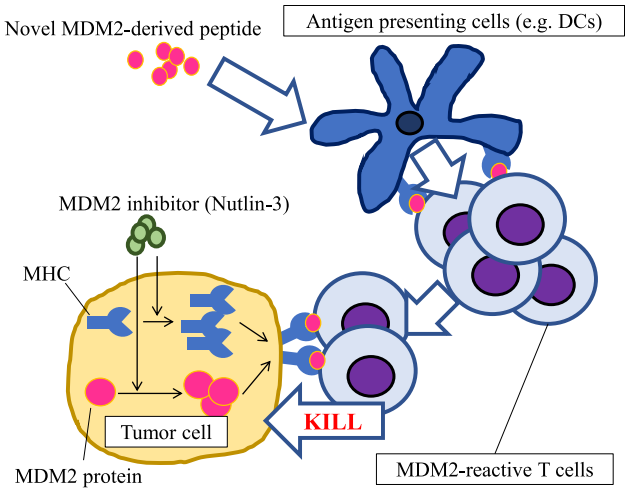

Fig. 7 Graphical abstract. Interruption of MDM2 signaling augments MDM2-targeted T cell-based antitumor immunotherapy through antigen-presenting machinery

first study to show that MDM2 blockade increases HLA-DR and HLA-class I expression on tumor cells. As with EGFR blockade [20, 39], MDM2 blockade induced tumor HLA-DR expression through CIITA. Since Wang et al. have shown that p53 amplifies HLA-class I expression via regulating endoplasmic reticulum aminopeptidase 1[15], the expression of HLA-class I might be upregulated by a similar mechanism following MDM2 blockade and p53 stimulation in the current study.

Luo et al. have reported that the expression of a negative checkpoint PD-L1 increases in accordance with MHC on tumor [40]. We also revealed that PD-L1 expression was elevated with MDM2 blockade in addition to HLA-class I and HLA-DR (Supplementary Figure S5). Interestingly, recent studies suggested that MDM2 amplification predicts poor response to ICIs in a variety of cancers [41] [42]. It is rational that ICIs resistance might be mediated by MDM2 amplification, which inhibits the HLA expression on tumor followed by immune ignorance. Accordingly, it is feasible that ICIs augment the response of MDM2 inhibitor, and vice versa. Recent reports have proved this concept by showing that MDM2 inhibitor augmented T-cell killing ability against tumor cells in combination with anti-PD-1 antibody treatment [43]. Fang et al.have shown that MDM2 inhibitor decreased M2 macrophage population through c-Myc and c-Maf, and combination therapy with anti-PD-1 enhanced antitumor activity in syngeneic tumor models [44]. Furthermore, NK cells are also activated via MDM2 inhibitors [45]. Thus, a broad range of immunotherapy including peptide vaccine and ICIs would be applied with MDM2 inhibitor as an immune adjuvant.

Conclusions

Our results suggest that an MDM2 peptide can induce effective $CD4^+$ T cell responses including direct recognition and cytotoxic activity against MDM2 expressing tumor cells. The peptide epitope, which immunogenicity was confirmed in this study, could be a feasible candidate for peptide vaccine. Moreover, Nutlin-3, which inhibits the MDM2-p53 interaction, enhanced the expression of HLA-DR and HLA-class I in addition to MDM2. As well as MDM2-specific T cells, the antitumor effects of EGFR-specific T cells were also augmented with Nultin-3 suggesting that the MDM2 inhibitor has a synergistic effect in a wide range of T cell-based immunotherapy. Peptide vaccine targeting MDM2 combined with the MDM2 inhibitor could be a novel immunologic approach to treat cancer.

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Authors' contributions MK, TK, RH, HY, HK, RW, TN and KO took part in acquisition, analysis and interpretation of data. KK, MT and AKa involved in statistical analysis of data. TO, AKo, HK participated in material support. TK, TH and YH involved in development of methodology. TK took part in conception, design and supervision of the study. MK and TK involved in writing of the paper. TK, YH and EC participated in review of the paper.

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Declarations

Conflict of interest The authors declare no potential conflicts of interest

Availability of data and material All data relevant to the study are included in the article or uploaded as supplementary information

Ethics approval, consent to participate and consent for publication All experiments were approved by the institutional ethics committee on the Asahikawa Medical University (#16217). The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The patients have given their written informed consent to participate and publish their case.

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