Article

Characteristics of a Novel Target Antigen against Myeloma Cells for Immunotherapy

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Abstract: Despite the availability of therapeutic treatments, multiple myeloma is an incurable haematological disorder. In this study, we aimed to clarify the role of CXorf48 as a therapeutic target in multiple myeloma. Based on a previously identified HLA-A*24:02-restiricted epitope from this novel cancer/testis antigen, we characterized the activities of cytotoxic T lymphocytes (CTLs) specific to this antigen against myeloma cells and evaluated the effects of demethylating agents in increasing antigen expression and enhancing the cytotoxic activity of CTLs. *CXorf48* expression was examined by RT-PCR using nine myeloma cell lines. Cell lines with low *CXorf48* expression were treated by demethylating agents (DMAs), 5-azacytidine (5-aza), and 5-aza-2'-deoxycytidine (DAC) to evaluate gene expression using quantitative RT-PCR. Furthermore, CXorf48-specific CTLs were induced from peripheral blood mononuclear cells of HLA-A*24:02-positive healthy donors to evaluate antigen recognition using ELISpot and ^{51}Cr cytotoxicity assays. *CXorf48* was widely expressed in myeloma cells and gene expression was significantly increased by DMAs. Furthermore, CXorf48-specific CTLs recognized DMA-treated myeloma cells. These findings suggest that CXorf48 is a useful target for immunotherapy, such as vaccination, in combination with demethylating agents for the treatment of patients with myeloma.

Keywords: myeloma; antigen; demethylating agents

1. Introduction

Multiple myeloma is a plasma cell neoplasm accompanied by various symptoms, including anaemia, renal dysfunction, lytic bone lesion, and hypercalcemia [1,2]. Despite the development of various therapeutic drugs, such as immune modulatory drugs (IMiDs) and proteasome inhibitors, and treatment strategies, including high-dose chemotherapy and stem cell transplantation, which have prolonged the median survival of multiple myeloma patients, it remains difficult to cure myeloma patients [3]. In recent years, antibodies, antibody-drug conjugate (ADC), bispecific T-cell engager (BiTE), or chimeric antigen receptor (CAR)-T cells have also been found to be effective treatment options, suggesting that myeloma cells are highly sensitive to immunotherapies [4,5].

Recent technological developments, including multiparameter flow cytometry or next-generation sequencing techniques, have enabled the detection of minimal residual disease (MRD) in myeloma patients [6]. The meta-analysis of several clinical studies has shown that MRD-negativity is associated with a better clinical prognosis [7]. Therefore, eradicating MRD is essential to cure myeloma patients. However, most myeloma patients are elderly, and prolonged therapy, with its severe adverse effects, is often not suitable.

Vaccination with tumor-specific antigens has been reported to reduce MRD in cancer patients, without inducing severe toxicities [8]. For myeloma patients, vaccinations using myeloma-specific peptide antigens or dendritic cells pulsed with antigens or fused with myeloma cells have been investigated, and have been found to enhance anti-myeloma immunity in preclinical settings [9,10]. However, a high therapeutic efficacy is needed to obtain tangible results in clinical settings.

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In this study, we aimed to clarify the role of CXorf48 as a novel therapeutic target in multiple myeloma. In a previous study, we found this antigen to be expressed in chronic myelogenous leukaemia cells, and not expressed in normal tissues, except the testes. We also previously identified a human leukocyte antigen (HLA)-A*24:02-restiricted epitope from this novel cancer/testis antigen (CTA) [11]. In the present study, the expression of this antigen in myeloma cells was detected, and cytotoxic T lymphocyte (CTL) activity against myeloma cells was evaluated. In addition, the effects of demethylating agents, including 5-azacytidine (5-aza) or 5-aza-2'-deoxycytidine (DAC), were examined for their role in increasing antigen expression and enhancing the cytotoxic activity of myeloma cell lines with low levels of CXorf48 expression. Our findings suggest that CXorf48 is a useful target for immunotherapy in combination with demethylating agents for the treatment of myeloma patients.

2. Materials and Methods

2.1. Cell lines

The myeloma cell lines KMM1, KMS11, KMS20, KMS21, KMS26, KMS27, KMS28, and KMS34 were kindly gifted by Dr. Ohtsuki of Kawasaki Medical School (Kurashiki, Japan). CIR-A24 cells were kindly gifted by Dr. Yutaka Kawakami of Keio University, school of medicine (Tokyo, Japan). All of these cell lines were established from Japanese myeloma patients. U266 was obtained from American Type Culture Collection. The cancer cell line K562 was obtained from the Health Science Research Resources Bank (National Institute of Biomedical Innovation, Osaka, Japan). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium (Sigma-Aldrich, Saint Louis, MO) and 10% foetal bovine serum (FBS) (Invitrogen™, Life Technologies, Grand Island, NY, U.S.A.).

2.2. Reagents

Phytohemagglutinin (Sigma-Aldrich) was used to generate PHA blasts in CTL induction. 5-azacytidine and 5-aza-2′-deoxycytidine (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO). CXorf48-derived (DYGMIDESI) and HIV-derived peptides (RYLRDQQLL) were synthesized and purified to 98% by high-performance liquid chromatography (HPLC) (Sigma-Aldrich, Tokyo, Japan). Purified peptides were dissolved in DMSO and stored in aliquots at −80°C.

2.3. Detection of CXorf48 expression by RT–PCR

CXorf48 expression was detected using the standard reverse transcription polymerase chain reaction (RT–PCR) or quantitative PCR, as previously described [11]. Total RNA was extracted from cancer cell lines and peripheral blood mononuclear cells (PBMNC) from healthy donor (HD) using Isogen (Nippon Gene Co. Ltd, Tokyo, Japan). Complementary DNA was synthesized from 1 μg of the total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), followed by PCR with *CXorf48* primers (forward, 5′-gttgtgcctcgccatctttatg-3′ and reverse, 5′-tgcactggggtgatagaaatcg-3′) or *GAPDH* primers (forward, 5′-tgaacgggaagctcactgg-3′ and reverse, 5′-tccaccaccctgttgctgta-3′) with Taq polymerase (Takara Bio, Shiga, Japan). Quantitative PCR was performed using SsoFast probes supermix (Bio-Rad, Hercules, CA, U.S.A.) with *CXorf48* primers and probe (TaqMan Gene Expression Assays; Applied Biosystems, CA, USA) or *GAPDH* primers (forward; 5′-gacctgacctgccgtctagaaa-3′, reverse; 5′-cctgcttcaccaccttcttga-3′) and probe (5′-(6-FAM)-acctgccaaatatgatgac-(BHQ-2)-3′). Complementary DNA from the K562 cell line was used to make standard curves. The relative gene expression level was calculated as follows: *CXorf48* expression level (sample)/*GAPDH* expression level (sample). In use of blood samples from heathy donors, written informed consents were obtained. This study was approved by the Ethics Committee of Keio University Faculty of Pharmacy (190613-10).

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2.4. Detection of cancer/testis antigen (CTA) gene expression by RT-PCR

The expression of the CTA genes *NY-ESO-1*, *MAGE-A1*, *MAGE-A2*, *MEGE-A3*, *MAGE-C1*, *LAGE-1*, and *PRAME* was detected by RT-PCR using gene-specific primers (*NY-ESO-1*: forward, 5′-CCCCACCGCTTCCCGTG-3′ and reverse, 5′-CTGGCCACTCGTGCTGGGA-3′; *MAGE-A1*: forward, 5′-CGGCCGAAGGAACCTGACCCAG-3′ and reverse, 5′-GCTGGAACCCTCACTGGGTTGCC-3′; *MAGE-A2*: forward, 5′-AAGTAGGACCCGAGGCACTG-3′ and reverse, 5′-GAAGAGGAAGAAGCGGTCTG-3′; *MEGE-A3*: forward, 5′-TGGAGGACCAGAGGCCCCC-3′ and reverse, 5′-GGACGATTATCAGGAGGCCTGCt-3′; *MAGE-C1*: forward, 5′-GACGAGGATCGTCTCAGGTCAGC-3′ and reverse, 5′-ACATCCTCACCCTCAGGAGGG-3′; *LAGE-1*: forward, 5′-CTGCGCAGGATGGAAGGTGCCCC-3′ and reverse, 5′-GCGCCTCTGCCCTGAGGGAGC-3′; *PRAME*: forward, 5′-CTGTACTCATTTCCAGAGCCAGA-3′ and reverse, 5′-TATTGAGAGGGTTTCCAAGGGGTT-3′).

2.5. Immunocytochemical staining

Immunocytochemical staining was performed as follows: cells were attached to slides using a Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA), fixed with 2% paraformaldehyde, permeabilized with Triton X-100, and blocked with 1% bovine serum albumin in PBS. The samples were then stained with mouse anti-CXorf48 antibody (Sigma-Aldrich), followed by Alexa Fluor488 goat-anti-mouse IgG (Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA).

2.6. Treatment with demethylating agent

KMS11, KMS34, and peripheral blood mononuclear cells (PBMNCs) from HD were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) in 6-well culture plates. To this, 200 nM of 5-azacytidine or 5-aza-2′-deoxycytidine (Sigma-Aldrich) was added every 24 hours for 72 hours.

2.7. Generation of CXorf48 peptide-specific CTLs from human PBMCs

CTLs were generated by *in vitro* stimulation with peptide-pulsed autologous dendritic cells and phytohemagglutinin (PHA) blasts, as previously described [12]. Briefly, PBMNCs were isolated from the whole blood of HLA-A*24:02-positive HD by Ficoll density gradient centrifugation. Dendritic cells (DC) were generated by isolating $CD14^+$ cells using a MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD14^+$ cells were cultured in AIM-V medium supplemented with 10% human serum, 100 ng/mL of IL-4 (R&D Systems) and 100 ng/mL of GM-CSF (R&D Systems) for 5 days. Then, 20 ng/mL of TNF-α (R&D Systems) was added to generate DC. PHA blasts were derived from $CD14^-$ cells by culturing in AIM-V medium supplemented with 10% human serum, 100 units of IL-2, and 1 μg/mL of PHA for 2 days. The DC or PHA blasts were pulsed with 50 μg/L of peptide at room temperature for 3 hours before irradiation (mediXtec, Chiba, Japan). On day 0, the $CD14^-$ cells were stimulated with irradiated peptide-pulsed DC in AIM-V medium with 10% human serum supplemented with 10 ng/mL of IL-7 (R&D Systems). To this, 50 ng/mL of IL-2 was added every 2 to 3 days. The cells were stimulated weekly with irradiated peptide-pulsed autologous PHA blasts. The resulting $CD8^+$ cells were purified using immunomagnetic beads (Miltenyi Biotec). In use of blood samples from heathy donors, written informed consents were obtained. This study was approved by the Ethics Committee of Keio University Faculty of Pharmacy (190613-10).

2.8. Enzyme-linked immunospot (ELISpot) assay

The IFN-γ secretion of CTLs in response to target cells was detected using ELISpot assay. Briefly, effector cells were incubated with target cells in plates coated with anti-IFN-γ antibody (1-D1K; Mabtech Inc., Cincinnati, OH, U.S.A.). After incubation for 20 hours, a biotinylated antibody specific for IFN γ (7-B6-1; Mabtech) was added for 2 hours at room temperature, followed by the addition of

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streptavidin-alkaline phosphatase (Mabtech) for 1 hour. To develop spots, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BioRad) were added. Colour development was stopped by rinsing with distilled water. The resulting spots were counted using a CTL-ImmunoSpot1 analyser (Cellular Technology Ltd., Shaker Heights, OH)

2.9. Cytotoxicity assay

The cytotoxic activity of the CTLs was measured using a standard ^{51}Cr release assay. The target cells were labelled with 50 μCi of ^{51}Cr (PerkinElmer Inc., Waltham, MA, U.S.A.) for 60 min at 37°C. The labelled target cells were mixed with effector cells. After 4 hours, the resulting supernatants were transferred to LumaPlates (PerkinElmer) and allowed to air dry in a hood overnight. The plates were sealed and counted using a Plate CHAMELON V (Hidex, Turku, Finland). The percentage of lysis specific to ^{51}Cr release was calculated as follows: [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release – spontaneous 51Cr release)] × 100.

2.10. Dextramer staining

PBMCs stimulated with the CXorf48-derived peptides were washed with PBS (Sigma), stained with antibodies against CD3 (33-2A3; Immunostep, Salamanca, Spain), CD8 (BW135/80; Miltenyi Biotec). and dextramer specific for HIV-derived peptide or CXorf48-derived peptide (Immudex, Copenhagen, Denmark). The resulting cells were analysed using flow cytometry (LSR II; BD Biosciences).

2.11. Statistical analysis

The results are presented as the mean ± standard error of the mean (s.e.m.). Groups were compared using Student's t-test. Differences were considered significant at $p<0.05$.

3. Results

3.1. CXorf48 is expressed in myeloma cells

We first evaluated the levels of *CXorf48* gene expression in myeloma cell lines, including KMM1, KMS11, KMS20, KMS21, KMS26, KMS27, KMS28, KMS34, and MUM24 using RT-PCR (Figure 1a). Eight out of the nine cell lines were found to express this gene. Among these cell lines, KMM1, KMS26, KMS28, KMS34, and MUM24 harboured high-risk chromosomal abnormalities, such as t(4;14) or del17. We also detected the expression of other CTA genes, namely *NY-ESO-1*, *MAGE-A1*, *MAGE-A2*, *MAGE-C1*, *LAGE-1*, and *PRAME*. *MAGE-A1* and *MAGE-A3* were found to be commonly expressed in five myeloma cell lines (KMM1, KMS20, KMS21, KMS26, and KMS27), while *NY-ESO-1* was detected in six cell lines (KMM1, KMS20, KMS21, KMS26, KMS27, and MUM24), and *MAGE-A2* and *MAGE-C1* were detected in seven cell lines (KMM1, KMS21, KMS26, KMS27, KMS20, and MUM24). *LAGE-1* and *PRAME* were expressed in all of the cell lines.

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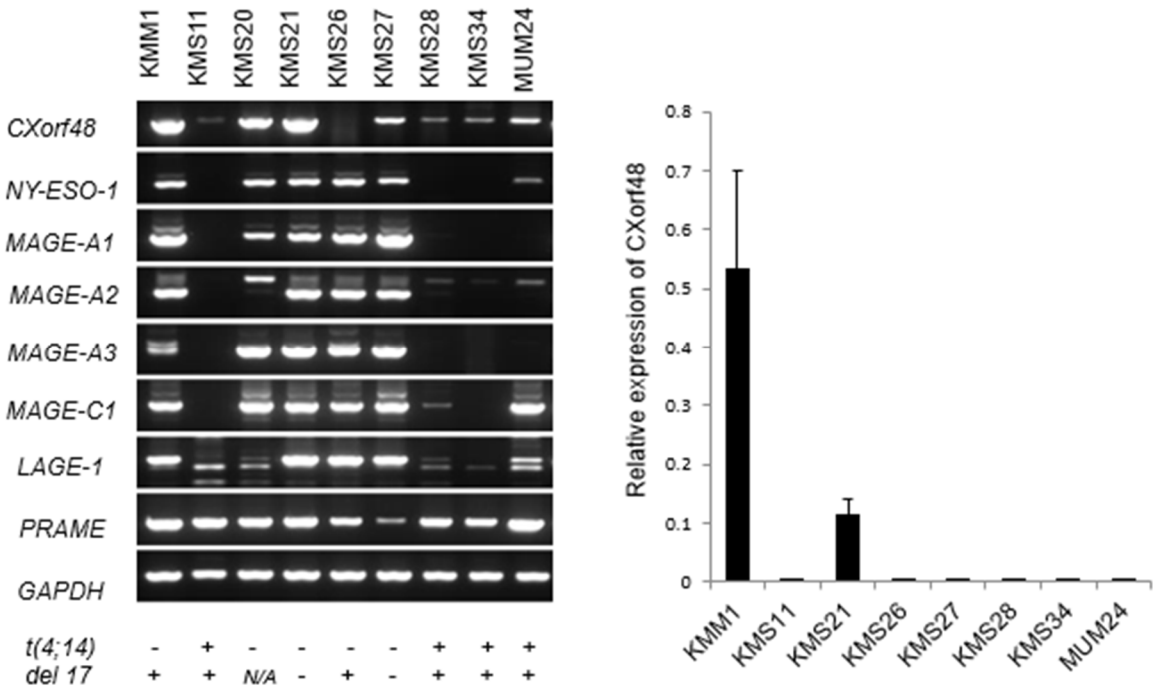

(a) **(b)**

Figure 1. CXorf48 gene expression in MM cell lines. (a) Expression of *CXorf48* gene and other cancer-testis antigen (CTA) genes in 9 myeloma cell lines were detected using conventional RT-PCR. Chromosomal abnormalities were indicated below. N/A; not analyzed. (b) Expression of *CXorf48* gene genes in myeloma cell lines were evaluated by quantitative PCR. Relative expression was calculated by dividing expression level of *CXorf48* by that of *GAPDH*. Data are mean with standard deviations.

Quantitative RT-PCR revealed that KMM1 and KMS21 expressed high levels of CXorf48 (Figure 1b). By contrast, *CXorf48* gene expression was not detected in KMS11 by quantitative PCR, although a small band was found to express CXorf48, detected using standard PCR, suggesting that KMS11 expresses extremely low levels of this gene.

We then conducted an immunocytochemistry assay to confirm protein expression. The cytoplasm of KMS21 and KMM1 was strongly stained with anti-CXorf48 antibody (Figure 2). We detected low levels of CXorf48 protein expression in KMS11, which was consistent with the RT-qPCR results. Our previous study showed that the *CXorf48* gene is not expressed in blood mononuclear cells or bone marrow mononuclear cells [11]. Therefore, *CXorf48* can be a myeloma-specific antigen.

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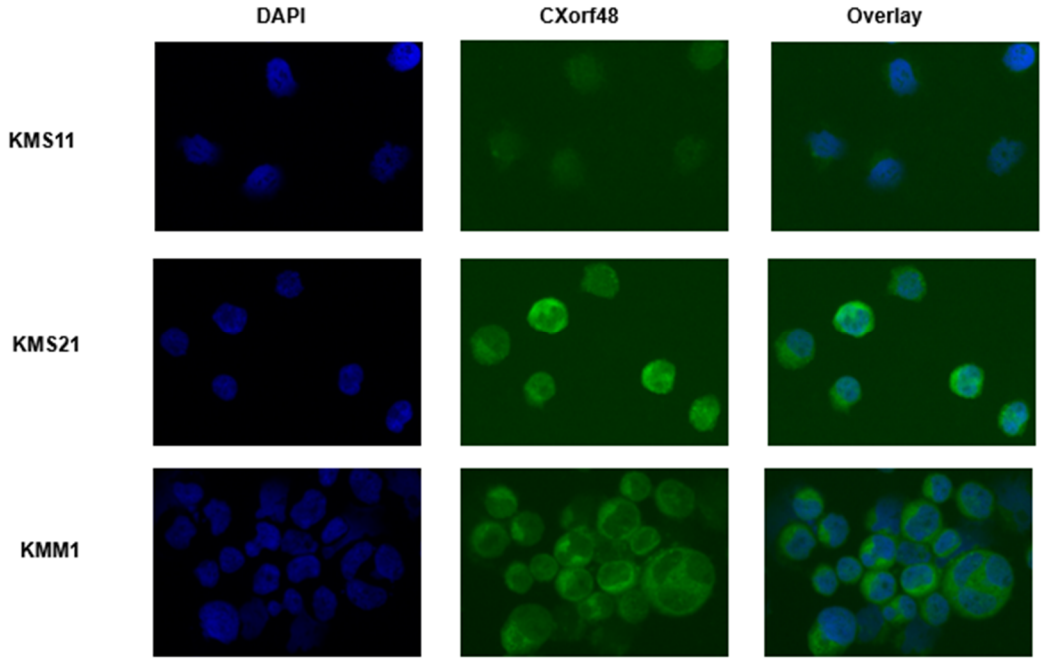

Figure 2. CXorf48 protein expression in MM cell lines. Expression of protein was detected immunocytochemistry in KMS11, KMS21, and KMM1. The myeloma cell lines were stained with DAPI and mouse anti-CXorf48 antibody followed by staining with Alexa488-labeled goat anti-mouse IgG.

3.2. CXorf48-specific CTLs recognized myeloma cells with high expression of CXorf48

To assess the immunogenic property of CXorf48, we evaluated whether CTLs against CXorf48 were able to recognize myeloma cells. CXorf48-specific CTLs were induced from the PBMNCs of HLA-A*24:02-positive healthy donors using the CXorf48$^{49-57}$ peptide. After 3 stimulations with peptide-pulsed dendritic cells or PHA blasts, CD8^{+} cells were sorted and stained with dextramer containing a complex of the HLA-A*24:02 and CXorf48$^{49-57}$ peptides. We found that 0.6% of CD3^{+} cells were positive for the antigen-specific dextramer (Figure 3a). Then, we assessed the cytotoxicity of the CTLs against myeloma cell lines. The CTLs recognized KMS21, which is HLA-A*24:02-positive with high levels of CXorf48 expression. On the other hand, the CTLs showed no cytotoxicity against KMS11, which is HLA-A*24:02-positive, but has little CXorf48 expression. The CTL also did not lyse K562 cells, which lack HLA-A*24:02, with high levels of CXorf48 expression (Figure 3b). ELISpot assay confirmed the antigen-specific IFN-γ secretion from the CTLs. The CTLs secreted significantly high level of IFN-γ when co-cultured with CIR-A24 cells pulsed with CXorf48$^{49-57}$ peptide compared to CIR-A24 cells pulsed with irrelevant peptide (Figure 3c). We then detected IFN-γ secretion from CTLs against myeloma cells with or without CXorf48 expression (Figure 3d). The number of cells secreting IFN-γ was significantly higher when CTLs were cultured with KMS21 and KMM1, which has high levels of CXorf48 expression, compared to KMS11 or KMS34, which has low CXorf48 expression.

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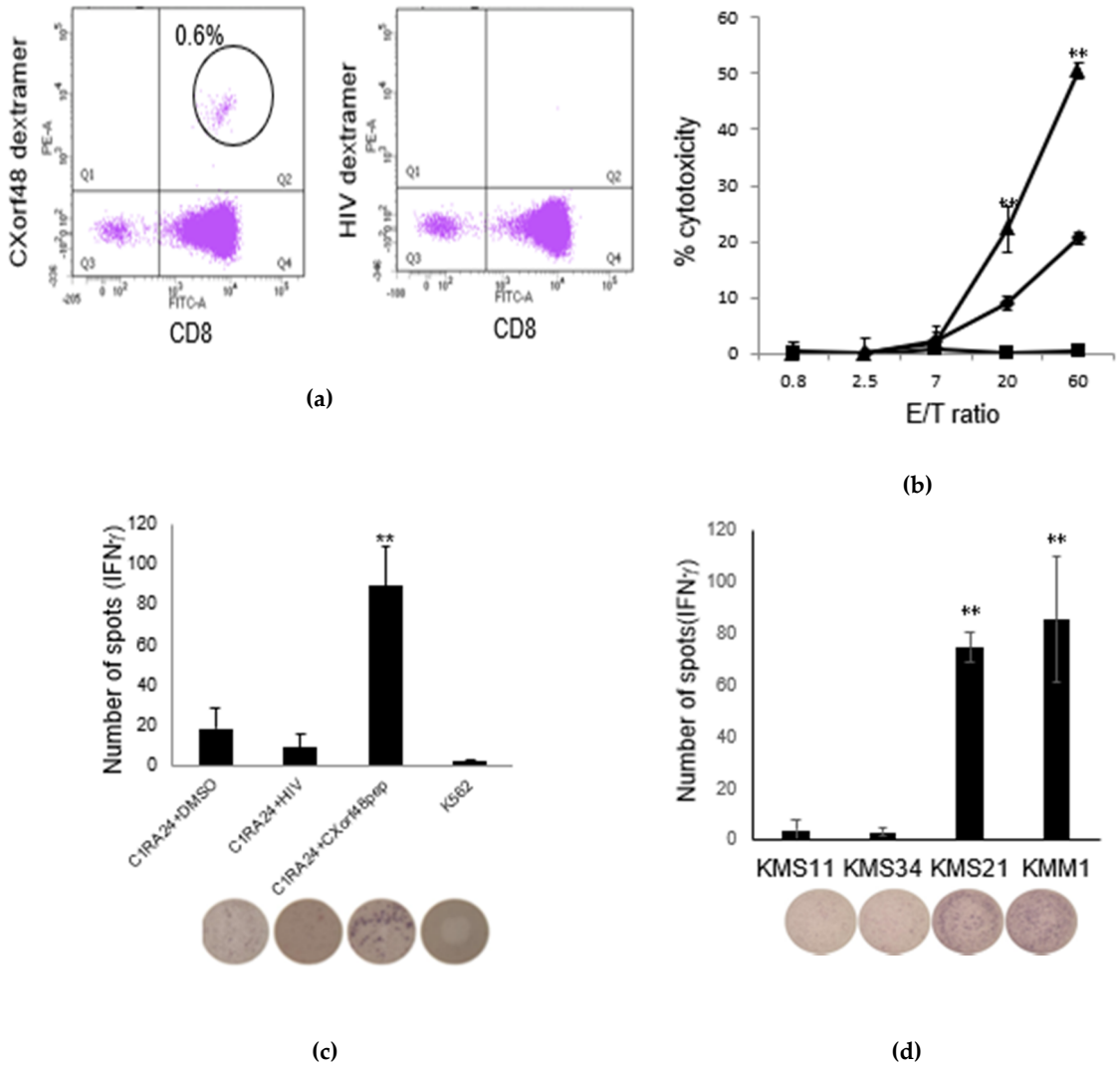

Figure 3. Activity of CXorf48-specific CTL against myeloma cells. (a) CXorf48-specific CTLs induced from PBMNCs from healthy donors using CXorf48$^{49–57}$ peptide were stained with dextramer specific to CXorf48$^{49–57}$ peptide (left) or dextramer specific to HIV-derived peptide (right), and analyzed by flow cytometer. (b) Cytotoxicity of CXorf48-specific CTLs against cancer cell lines, KMS11 (HLA-A*24:02-positive, CXorf48-negative: ■), KMS21 (HLA-A*24:02-positive, CXorf48-positive :▲ , and K562 (HLA-A*24:02- negative, CXorf48- positive: ◆) was assessed by ^{51}Cr release assay. (c, d) IFN-γ secretion by CTLs responding to CIR-A24 cells pulsed with CXorf48$^{49–57}$ peptide or HIV-derived peptide, K562, and myeloma cell lines, KMS 11, KMS34, KMS21, and KMM1, was evaluated by ELISpot assay Data are mean with standard deviations. * * $p < 0.01$ (Students t-test).

3.3. Up-regulation of CXorf48 expression by demethylating agents

A threshold amount of antigen is needed for recognition by cytotoxic T cells (CTLs). Therefore, increasing antigen expression in myeloma cells with low or no antigen expression may enhance the recognition of CTLs against a wide variety of myeloma cells. It has been previously reported that the gene expression of many CTAs is controlled by the methylation of their promoter regions [13,14]. Therefore, we examined whether CXorf48 expression is also up-regulated by demethylating agents in myeloma cells. As shown in Figure 4a, *CXorf48* gene expression in KMS11 or KMS34 increased after treatment with 5-azacitidine (5-aza) or 5-aza-2'-deoxycytidine (DAC). Notably, treatment with DAC significantly up-regulated *CXorf48* gene expression ($p<0.05$) (Figure 4a,b), and 5-aza tended to increase CXorf48 gene expression (KMS11; $p=0.078$, KMS34; $p=0.058$). In contrast, gene expression was not detected in PBMCs from healthy donors, regardless of treatment with demethylating agents (Figure 4c,d).

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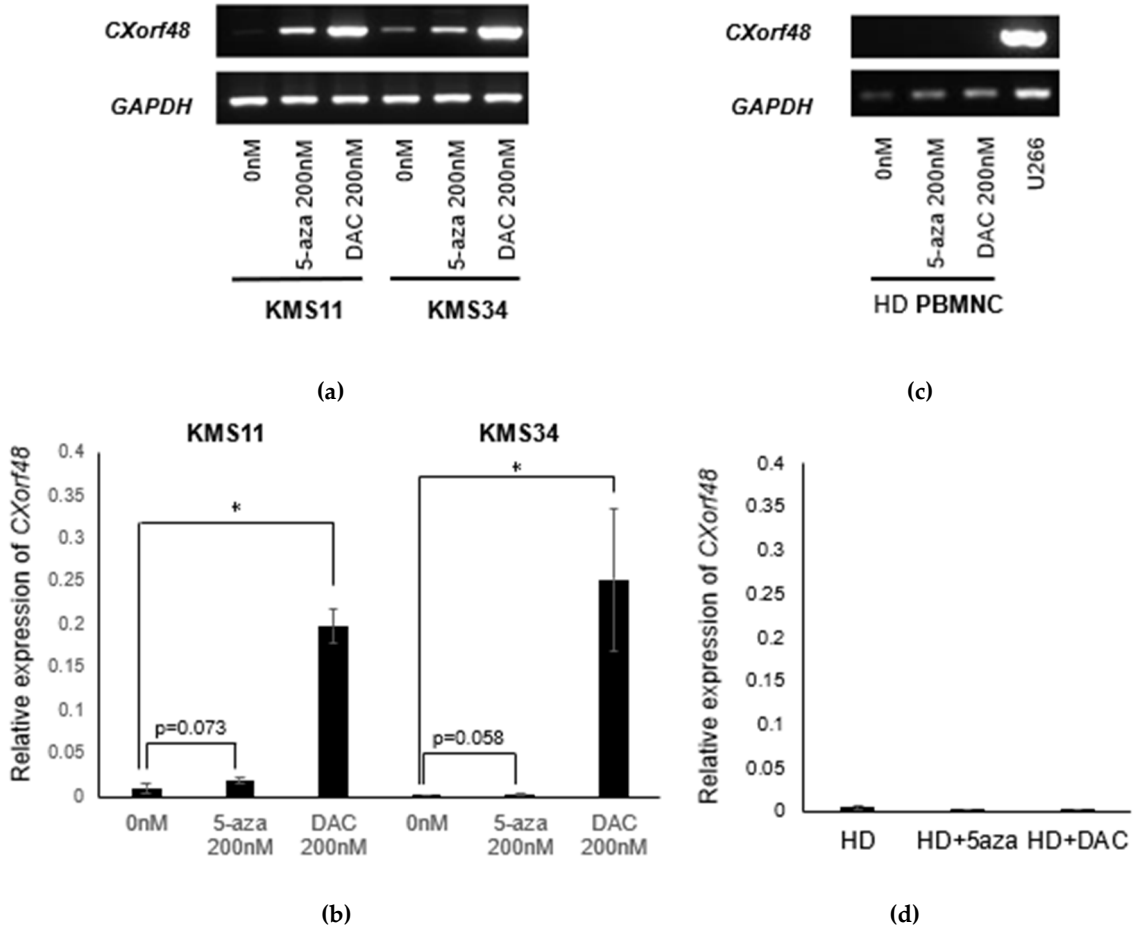

Figure 4. *CXorf48* gene expression is up-regulated by treatment of demethylating agents. KMS11, KMS34, and PBMNCs from healthy donor (HD) were incubated with 200 nM of 5-azacytidine or 5-aza-2`- deoxycytidine for 72 hours. RNA was extracted and RT-PCR (a, c) or quantitative PCR (b, d) was performed. cDNA from U266 was used as a positive control for *CXorf48* gene expression. Quantitative PCR was conducted in triplicate. Data are mean with standard deviations. *p < 0.05 (Students t-test).

3.4. CXorf48-specific CTL recognized DMA-treated myeloma cells with low CXorf48 expression

After finding that CXorf48 expression was up-regulated by demethylating agents in myeloma cells, we evaluated the activity of CXorf48-specific CTLs against CXorf48-low myeloma cells treated with demethylating agents. We induced antigen-specific CTLs from the PBMNCs of healthy donors using the CXorf48$^{49–57}$ peptide. As a result, 62.3% of the CD3^{+} cells were positive for dextramer specific to the CXorf48$^{49–57}$ peptide (Figure 5a). These cells strongly lysed KMS11 treated with 5-aza or DAC, compared to non-treated KMS11 (Figure 5b), suggesting that the induction of CXorf48 in KMS11 allowed antigen-specific CTLs to recognize KMS11.

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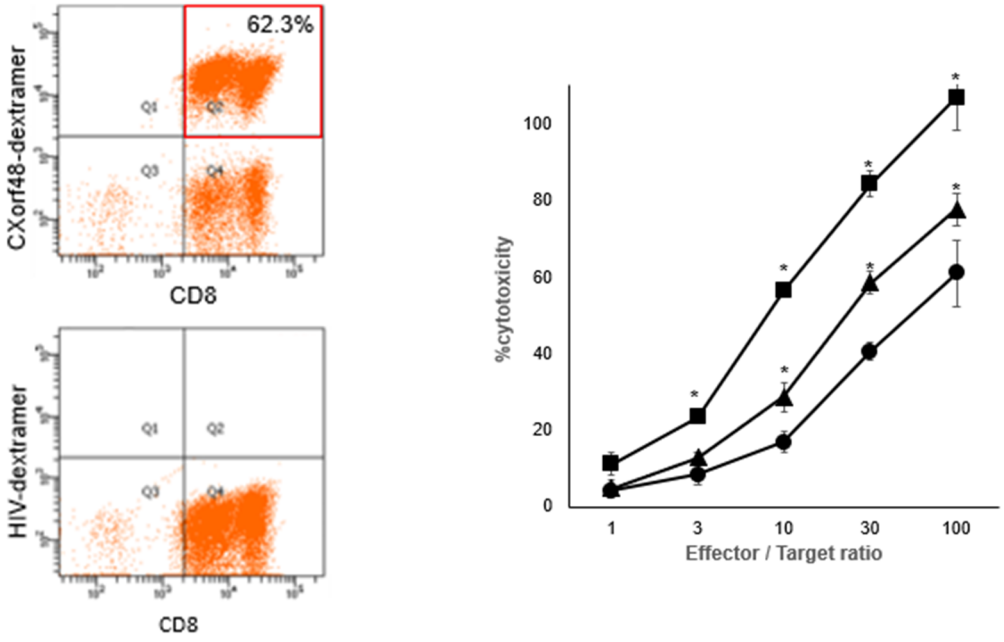

Figure 5. Activities of CXorf48-specific CTLs against demethylating agents-treated myeloma cells. (a) CXorf48-specific CTLs induced from PBMNCs of healthy donors using CXorf48$^{49–57}$ peptide were stained with dextramer specific to CXorf48$^{49–57}$ peptide (upper) or dextramer specific to HIV-derived peptide (lower), and analyzed by flow cytometer. (b) Cytotoxicity of CXorf48-specific CTLs against cancer cell lines, KMS11 (HLA-A*24:02-positive, CXorf48-negative) treated with DMSO (●), or KMS11 treated with 5-aza (▲), or KMS11 treated with DAC (■) was assessed by ^{51}Cr release assay. Data are mean with standard deviations. *p < 0.05 (Students t-test).

4. Discussion

Although the development of immune checkpoint inhibitors and advances in next-generation sequencing technology have revealed the existence of neoantigens derived from somatic mutations in cancer cells, several types of cancers, including leukaemia, have only a small number of neoantigens, due to rare somatic mutations [15,16]. Multiple myeloma is known to be resistant to ICIs [17], suggesting that there are few neoantigens in this haematological malignancy. Therefore, shared cancer antigens highly expressed in myeloma cells are promising targets in immunotherapies for this disease, including vaccination using cancer-specific peptides [18,19]. However, vaccination using single antigen is not enough to elicit clinical benefits in cancer patients in some cases, and the use of multiple antigens is known to strengthen the effects of vaccination during the treatment of cancer or infectious diseases [**20**]. Therefore, clarification of other antigens than previously reported antigens is needed. In this study, we evaluated the role of CXorf48 as a novel immunological target in multiple myeloma.

CXorf48 is a CTA that is expressed in chronic myelogenous leukaemia cells and not in normal blood cells [11]. This antigen is also detected in some of solid cancers including head and neck carcinoma [21]. However, its expression in myeloma cells has not yet been reported. Therefore, we evaluated the expression of *CXorf48* in nine myeloma cells and found that most cells expressed this antigen. However, the expression levels varied among cell lines. While the KMM1, KMS20, KMS21, and KMS27 cell lines were found to highly express this gene, KMS11, KMS28, KMS34, and MUM24 showed low levels of expression. All the cells expressing low levels of CXorf48 (CXorf48-low cells) were found to have chromosomal abnormalities, such as t(4;14) and del17. However, the relationship

between these abnormalities and the *CXorf48* gene, which is locate on the X chromosome, is still unknown. Translocation (4;14) is observed in 20% of myeloma patients, and causes the activation of the multiple myeloma SET domain-containing protein (MMSET), which in turn increases the methylation of lysine 36 in Histone H3, leading to the translation of other genes [22,23]. If t(4;14) is related to levels of *CXorf48* expression, there is a possibility that this translocation promotes the expression of the suppressor gene of *Cxorf48*, although little is known about control of *Cxorf48* gene expression.

Immunocytochemical staining confirmed that CXorf48 protein is highly expressed in cytoplasm of KMM1 or KMS21. CTAs located inside the cells are processed by the proteasome, modified by the endoplasmic reticulum and Golgi apparatus, moved to cell surface, and finally presented by the HLA molecule. We previously identified an epitope, CXorf48 $^{49-57}$, which binds to HLA- A*24;02, the most popular HLA class I haplotype in the Japanese population [11]. *PRAME*, another CTA located in the 22nd chromosome [24], was also strongly expressed in all myeloma cell lines, which is consistent with reports that gene expression levels are lower in X chromosomal genes compared to autosomal genes [25]. However, an epitope presented by HLA-A*24;02 has not been reported in *PRAME*, although the HLA-A*02;01-restricted epitope has been investigated and previously used in clinical trials [26]. This suggests that CXorf48 is an important immunological target in Japanese patients.

In order to clarify the immunogenicity of CXorf48 against myeloma cells, antigen-specific CTLs were induced from healthy donors, confirmed by antigen-specific dextramer staining. These CTLs were able to recognize myeloma cells, such as KMS21 and KMM1, with high levels of CXorf48 expression, but not those with low levels of antigen expression, suggesting that this antigen could be a therapeutic target, at least in myeloma patients expressing high levels of CXorf48.

To overcome this limitation, we tried to enhance antigen expression using DMAs, 5-aza, and DAC, since DMAs are known to increase the expression of several CTAs by lowering the methylation of their promotor region [13,14]. Both 5-aza and DAC were found to enhance CXorf48 expression in a myeloma-specific manner. Moreover, we evaluated the effect of DMAs on the recognition of myeloma cells by CTLs. Anti-CXorf48 CTLs showed a stronger cytotoxic activity against KMS11 treated with DMAs, which may be caused by up-regulated antigen expression in myeloma cells.

These results suggest that vaccination in combination with DMAs may be useful as effective immunotherapy for wide variety of myeloma patients. 5-aza and DAC have been approved for the treatment of myelodysplastic syndrome without severe side effects [27]. However, few reports exist on their use in myeloma patients [28]. In particular, DAC showed a more potent response compared to 5-aza in inducing both antigen expression and CTL recognition. DAC is known to have stronger effect of demethylation compared to 5-aza and does not affect the functions of T cells at low concentrations [14]. Recently, Zhou et al. reported that DAC could deplete myeloid-derived suppressor cells *in vivo* [29], suggesting that DAC may not only enhance CTL recognition of myeloma cells but also overcome immunosuppressive microenvironments. Furthermore, Wajnberg et al. reported that DAC treatment suppresses myeloma cell growth by suppressing the MYC oncogene [30]. DAC was found to increase the expression of TAZ, a transcriptional co-activator in Hippo-signaling pathway, via demethylating its promotor region, leading to decreased MYC expression. These data indicate that immunotherapy against CXorf48 with DAC could contribute to strong tumor regression in myeloma patients.

5. Conclusions

The findings of the present study suggest that CXorf48 has potential as a novel target antigen in the immunotherapy of multiple myeloma. Thus, a vaccination using CXorf48-derived epitope peptide or CXorf48 protein in combination with DMAs represents an attractive strategy for the treatment of patients with myeloma.

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