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A cyclic peptide accelerates the loading of peptide antigens in major histocompatibility complex class II molecules

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ABSTRACT

Major histocompatibility complex (MHC)-loading enhancers (MLE) have recently attracted attention because of their ability to enhance the efficacy of peptide immunotherapeutics. As small molecular weight compounds, they influence the loading of peptides in MHC molecules by converting them from a non-receptive to a receptive state. Herein, we report a 14-mer cyclic peptide **1** (CP-1) as a new class of MLE-peptide. This peptide was used to investigate its loading on human leukocyte antigen (HLA)-DR molecules. It was found that CP-1 strongly accelerates peptide-loading on both soluble and cell surface HLA-DR molecules in a dose-dependent manner. The effect was evident for all subsets of HLA-DR tested, including HLA-DRB1*1501, indicating that it acts independently of P1-pocket size, which is the canonical MLE-binding site. Importantly, increased peptide-loading by CP-1 was correlated with improved CD4+ T cell responses in vitro, while propidium iodide staining indicated low peptide-induced cytotoxicity. Thus, this study revealed a new class of peptide-based enhancers that catalyze peptide-loading by allosteric interactions with MHC molecules. Because of its low cellular cytotoxicity and high MLE activity, it may be useful in stimulating antigen-specific T cell responses for therapeutic purposes.

1. Introduction

Major histocompatibility complex (MHC) class II (MHC-II) molecules are heterodimeric cell surface glycoprotein that play critical roles in cell-mediated immunity. When complexed with a peptide, they are expressed on the surface of antigen-presenting cells (APCs) to present peptides to CD4+ T cells [1,2]. Most peptides displayed by MHC molecules are derived from self-proteins, but can also originate from pathogens and pathogen-derived products, which can enter cells via endocytosis. These proteins are processed into short peptides by the exogenous antigen-processing pathway in the endosomal compartment. MHC-II proteins, in association with the invariant chain, are transported to this compartment for peptide loading [3,4]. Loaded MHC molecules are then expressed on the cell surface to interact with CD4+ T cells. Upon recognition, CD4+ T cells become activated, resulting in focused immune responses [1,2].

There is a growing interest in therapeutics designed to manipulate immune responses. One of these approaches targets the peptide-loading of MHC molecules on the cell surface. Conceptually, the effect of directly enhancing peptide loading translates to the priming of antigen-specific CD4+ T cells that, in turn, exert strong influences on tolerance and immunity [5]. Although several peptide vaccines have been developed to modulate immune responses in clinical trials, they often show only weak immunogenicity [6]. One of the strategies to used improve immunogenicity is to enhance the loading of peptides into MHC molecules for presentation on the cell surface [7]. Peptide loading on the cell surface can be hindered by the occupation of peptide binding site by endogenous ligands or, in the case of empty molecules, by the acquisition of a non-receptive state [7–9]. Consequently, only a small fraction of MHC molecules can be directly loaded on the cell surface. However, the frequency of loaded MHC molecules can be increased by the actions of MHC-loading enhancer (MLE) molecules. Upon addition of an antigen mixture, MLEs can accelerate antigen loading by converting the non-receptive conformation of the MHC molecule into a receptive state. To date, only a few organic molecules, inorganic metal complexes, and dipeptides have been reported to be MLEs [9–15]. The mechanism of action of these molecules is not yet fully elucidated, but some studies have suggested that these small molecules act either by disrupting the hydrogen bond

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network of the peptide-binding groove [5,10] or by stabilizing the receptive conformation by occupying the "P1-pocket" in the MHC molecule binding groove [12,13]. As a result of a dimorphism at the bottom of the pocket, an MLE compound can only activate a subset of MHC-II molecules [13].

The MLEs that have been discovered to date lack structural diversity. Most of these molecules only exhibit peptide-loading capacity at higher concentrations [10,12] and are often associated with cytotoxic activities [10,13]. This report describes the identification and characterization of a 14-residue cyclic peptide **1** (CP-1) as an MLE for MHC-II molecules (HLA-DRB1*0101, DRB1*1501, and DRB1*1502, which are all allelic variants of human MHC-II molecules), which is derived from the oxpholipin-11D peptide [16]. Mechanistic studies of the catalytic activities of CP-1 on peptide loading of soluble and cell surface MHC-II molecules suggest that its binding site is distinct from the actual peptide-binding groove of the protein. Enhanced T cell proliferation resulting from increased IL-2 release was observed at a lower concentration of CP-1 in an in vitro antigen-specific $CD4^+$ T cell assay, which further emphasizes its role as an effective catalyst. Moreover, the low cytotoxic effects of CP-1 that were observed at higher concentrations against human leukocyte antigen (HLA)-DR-expressing cells and mouse fibroblasts, which are negligible at lower concentrations, indicates that it might be a safe candidate for therapeutic applications. Although additional studies on the catalytic activity of CP-1 in the context of DP, DQ, and other DR subsets of MHC molecules remain to be carried out, our current findings establish it as a suitable candidate for amplifying immune responses for therapeutic purposes.

2. Materials and methods

2.1. Materials

Adamantane ethanol (AdEtOH), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) was obtained from SERVA (GmbH, Germany). Cells were obtained from different sources as follows: *Spodoptera frugiperda* (Sf21) insect cells from GIBCO (Invitrogen, CA, USA); immortalized human B lymphocytes expressing HLA-DRB1*0101 (15310-LN), HLA-DRB1*1501 (HO104), and HLA-DRB1*1502 (TOKUNAGA) from ECACC (Salisbury, England); and mouse cytotoxic T lymphocytes (CTLL-2) and mouse fibroblasts (NIH-3T3) from ATCC (Manassas, USA). HLA-DRB1*0101-restricted peptide, HA306-318 (Biotin-PKYVKQNTLKLAT), HLA-DRB1*1501/*1502-restricted peptide, and MBP86-100 (Biotin-NPVVHFFKNIVTPRT) were purchased from BioBasic Inc. (Markham, Ontario, Canada). Streptavidin-PE was obtained from Caltag Medsystems (Silverstone, UK) and Eu^{3+}-labeled streptavidin was purchased from Perkin Elmer (Massachusetts, USA). Kits for IL-2 detection and cell proliferation were obtained from R&D Systems (Minneapolis, USA) and Calbiochem (San Diego, CA, USA), respectively.

2.2. Peptide synthesis and characterization

Solid phase peptide synthesis of the CP-1 was accomplished using stepwise 9-flourenylmethoxycarbonyl (Fmoc) chemistry [17]; all reagents were from Chem-Impex (Wood Dale, IL) and Novabiochem (California, USA). Rink amide resin (loading level; 0.51 mmol/g) was used as solid support. Fmoc–Val–OH was coupled to resin using oxymapure and diisopropylcarbodiimide (DIC) in dichloromethane (DCM)/dimethylformamide (DMF). After loading the first amino acid (AA), the resin was treated with acetic anhydride (1.5 mL) and *N*-methylmorpholine (NMM) (3 equiv.) in DCM for 45 min to cap un-reacted amino groups. Linear peptide resin **4** (Supplementary Scheme S1) was synthesized using Fmoc AAs and oxymapure/DIC coupling reagents. After completing the linear peptide sequence, the allyl group of the side chain of the glutamic acid was removed using palladium catalyst in an inert environment in mixtures of DCM/acetic acid/NMM of 37:2:1. After washing, the terminal AA was deprotected by treatment with 4-methylpiperidene in DMF. The macrocyclization was performed as previously reported (Supplementary Scheme S1) [18]. A cocktail of trifluoroacetic acid/phenol/water/triisopropylsilane of 8.8:0.5:0.5:0.2 v/v was used to cleave the peptide from resin. Filtrates containing crude peptide were concentrated and precipitated using cold diethyl ether. After purification of CP-1, purity was confirmed by analytical HPLC (Supplementary Fig. S1).

CP-1: overall yield = 17.25%; m.p. 301 °C; matrix-assisted laser desorption/ionization-time-of-flight, *m/z* 1804 $[M+H]^+$; ^1H NMR (DMSO-d_6, 600 MHz) (Supplementary Fig. S2).

2.3. Stock solutions and cell culture

The 30 mM stock solutions of CP-1 and AdEtOH were prepared in DMSO. Cells were cultured in a CO_2 incubator at 37 °C, except for Sf21 cells that were grown in an orbital shaking incubator at 27 °C without CO_2 circulation or humidification. Cell lines were cultured as follows: HLA-DR-expressing cells in RPMI 1640 (Invitrogen, CA, USA) supplemented with 2 mM L-glutamine, 20% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin; HLA-DR1-restricted, HA306-318-specific T cell hybridoma (EvHA/X5) [13] in RPMI 1640 containing 1 mM sodium pyruvate, 2 mM HEPES, 10% FBS, 1% L-glutamine, and 1% non-essential amino acids (NEA); CTLL-2 cells in ATCC-formulated RPMI 1640, supplemented with 1 mM sodium pyruvate, 2 mM HEPES, 10% FBS, 10% T-STIM and Con A, 1% L-glutamine, and 1% NEA; and NIH-3T3 cells in DMEM, supplemented with 10% FBS, 1% L-glutamine, 1% NEA, and 50 μg/mL penicillin–streptomycin.

2.4. Production of HLA-DR in insect cells

Recombinant soluble HLA-DR1 (DRA*0101, DRB1*0101) and HLA-DR2 (DRA*0101, DRB1*1501 and DRB1*1502) molecules were produced in Sf21 cells, as described previously [13].

2.5. Peptide loading of soluble HLA-DR molecules

Peptide loading of HLA-DR molecules was carried out as described previously [15]. Briefly, 96-well plates were coated with anti-L243 antibody (in-house production; 100 μL/well; 10 μg/mL in sodium bicarbonate buffer). Peptide-loading reactions containing test peptides (500 μM), DR-restricted peptide (80 μg/mL), and soluble protein (100 nM) were carried out in a total volume of 150 μL in phosphate buffer saline (PBS) by incubating for 1 h at 37 °C. Loading was terminated by 1% dialyzed bovine serum albumin in PBS; 100 μL/well from the loading reaction was transferred to antibody-coated plates. After incubation for 1.5 h at 4 °C, plates were washed with wash buffer (0.05% Tween in PBS) and Eu^{3+}-labeled streptavidin (100 μL/well; 1:500 dilution) was added. Plates were incubated again for 0.5 h at 37 °C; after washing with buffer, 200 μL enhancer solution [19] was added to each well. Fluorescence was measured as counts per minute on a Multilabel Reader (Chameleon II, Hidex, Finland) using the time-resolved fluorescence mode at 340/614 nm (excitation/emission) wavelengths. Control reactions were run as spontaneous loading (SL), without any catalyst, loading with AdEtOH, or with DMSO. CP-1 and AdEtOH were also subjected to dose–response curve determination at various concentrations.

2.6. Peptide loading of cell surface HLA-DR proteins

Cell surface loading was carried out as described previously [13]. Briefly, HLA-DR-expressing cells were seeded at a density of 7×10^4 cells/well (in 100 μL) in round-bottom 96-well plates. The experimental setup included SL of biotinylated HA306-318 (100 μL; 5 μg/mL) onto the HLA-DR protein as a control, along with test reactions in the presence of 500 μM peptide and AdEtOH. Loading with DMSO was used as a vehicle control. Reactions were incubated for 4 h, after which cells were centrifuged (1300 rpm/500*g*; 5 min), rinsed with wash buffer (PBS with 5% FBS), and stained with streptavidin-PE (30 μL/well; 1:100 dilution in flow cytometry (FC) buffer. Live cells (PI-negative) were analyzed to detect the PE signal using CellQuest Pro software with a FACSCalibur (BD Biosciences, San Jose, CA, USA). The fluorophore signal intensities corresponding to the amount of peptide/MHC complex on surface were expressed as geometric mean values. CP-1 and AdEtOH were also subjected to dose–response curve determination at various concentrations.

2.7. T cell proliferation assay

T cell assays were carried out as described previously [15]. Briefly, various concentrations of CP-1 and AdEtOH were incubated with DRB1*0101-expressing cells for 4 h using the same procedure described in Section 2.6. After incubation, cells were washed; EvHA/X5 T cells were added at a density of 5×10^4 cells/well (in 200 μL) and incubated for 24 h. The culture supernatant (100 μL) was collected to estimate IL-2 release using an IL-2 detection kit. The T cell proliferation induced by the supernatant was determined by measuring cell proliferation using a BrdU cell proliferation kit in a secondary assay that was carried out with CTLL-2 cells, as described previously [12]. T cell proliferation was expressed as absorbance values.

2.8. Cytotoxicity assay

CP-1 and AdEtOH were administered to determine their cytotoxicity profile against NIH-3T3 cells in flat-bottom 96-well plates using the MTT assay, as described previously [20]. Cytotoxicity, expressed as growth inhibition (%) and cell viability (%), was calculated using the following formulae: Inhibition (%) = [1 − (OD of treated/OD of control)] × 100; Viability (%) = 100 − % inhibition. The IC_{50} values (the inhibitory concentration at which the response is reduced to half) were calculated using the EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc., Amherst, NH, USA). Cytotoxicity against immortalized human B cells expressing HLA-DRB1*0101 (15310-LN) was estimated using flow cytometry-based PI staining. Briefly, cells were seeded at 7×10^4 cells/well (in 200 μL) in the presence of titrated amounts of test samples. Cells were incubated for 48 h in an incubator at 37 °C and 5% CO_2, then were centrifuged (1300 rpm/500*g*; 5 min), rinsed with wash buffer (PBS with 5% FBS), and stained with PI (5 μL; 0.5 mg/mL) in a total volume of 300 μL FC buffer. The frequency of live (PI-negative) and dead (PI-positive) cells was determined using a FACSCalibur. Cytotoxicity was expressed as the percentage of dead cells based on a histogram of PI-stained cells.

2.9. Statistical analysis

The data were analyzed using a one-way ANOVA with SigmaPlot 11 software (Systat Software, Inc., San Jose, CA, USA). A *P*-value less than 0.05 was considered to denote a statistically significant difference.

3. Results and discussion

To search for effective and non-cytotoxic MLEs, this study investigated the catalytic effects of a randomly selected cyclic peptide derived from oxpholipin-11D peptide to enhance the peptide loading of MHC-II molecules. Peptide was synthesized using solid phase peptide methodology.

All MLEs that have been discovered to date belong to the category of low molecular weight compounds, including organic molecules, inorganic metal complexes, and dipeptides [9–15]. Some MLEs have been reported to interact reversibly with MHC molecules via the peptide-binding groove [12,13]. Mechanistic studies on adamantane derivatives and dipeptides revealed that they act by targeting conserved P1 anchor pocket located in the peptide-binding groove of MHC-II molecules [13]. While the overlap between the catalytic and actual binding site clearly limits the effectiveness of these compounds, this route is not yet fully established as the only site that is targeted by all MLEs. To identify an active compound that influences the conformational state through allosteric interactions, this study investigated the effects of the cyclic 14-mer peptide CP-1 (Fig. 1A; top right) on human HLA-DR molecule peptide-loading. The following allelic variants of human MHC molecules were studied: DRB1*0101, DRB1*1501, and DRB1*1502. This allowed the study of the effects of CP-1 on MHC molecules with differences in the dimorphic residue in the P1-pocket (HLA-DRB1*0101 and DRB1*1502: β86G; HLA-DRB1*1501: β86V), which is the canonical binding site for MLEs.

First, the catalytic capacity of the cyclic peptide CP-1 was determined in a loading assay by incubating it with soluble DR1*0101 molecules and the high-affinity ligand biotinylated-HA306-308 [15]. The peptide strongly accelerated peptide loading at 500 μM (Fig. 1A). Compared to AdEtOH, an MLE compound known to affect HLA-DRB1*0101 [13], even higher amounts of peptide ligand were transferred onto soluble MHC molecules. The influence on peptide loading was clearly dose-dependent (Fig. 1B). Moreover, in contrast to the effect of the known MLE compound, which quickly reached saturation, CP-1 displayed dose-dependent effects over a wider range, suggesting a different kinetic mechanism between the two molecules.

Previously, AdEtOH showed allele susceptibility with strict correlation to the presence of glycine at the dimorphic position β86 of HLA-DR molecules [13]. The latter phenomenon is based on steric interactions within the P1-pocket, whose depth is restricted by this dimorphism. To determine whether CP-1 targets the same site, experiments were extended to allelic variants of DRB1*1501 ($Val^{\beta86}$) and DRB1*1502 ($Gly^{\beta86}$). In accordance with a previous report [13], both molecules are identical except for the dimorphism at position β86. AdEtOH effectively catalyzed the loading of DRB1*1502, but not DRB1*1501 (Fig. 1C). Interestingly, CP-1 showed activity on both alleles. As previously observed for DRB1*0101, DRB1*1501, and DRB1*1502, the peptide catalyzed a five- to sixfold increase compared to SL. Moreover, similar dose–response curves of CP-1-mediated catalysis were observed for all three allelic variants of MHC-II, indicating that CP-1 acts with similar efficiency across these different allelic variants (Fig. 1B and D). Furthermore, the absence of any influence of residue β86 suggested that CP-1 interacts with sites other than the P1-pocket.

To validate the results obtained using soluble HLA-DR molecules and cell-surface MHC molecules, the loading experiments were conducted using immortalized human B lymphocytes, 15310-LN, HO104, and TOKUNAGA. These three cell lines expressed allelic variants of DRB1*0101, DRB1*1501 (β86V), and DRB1*1502 (β86G), respectively. For these experiments, cells were incubated in the absence or presence of MLE-peptide with respective biotinylated DR1-restricted HA306-318 and DR2-restricted

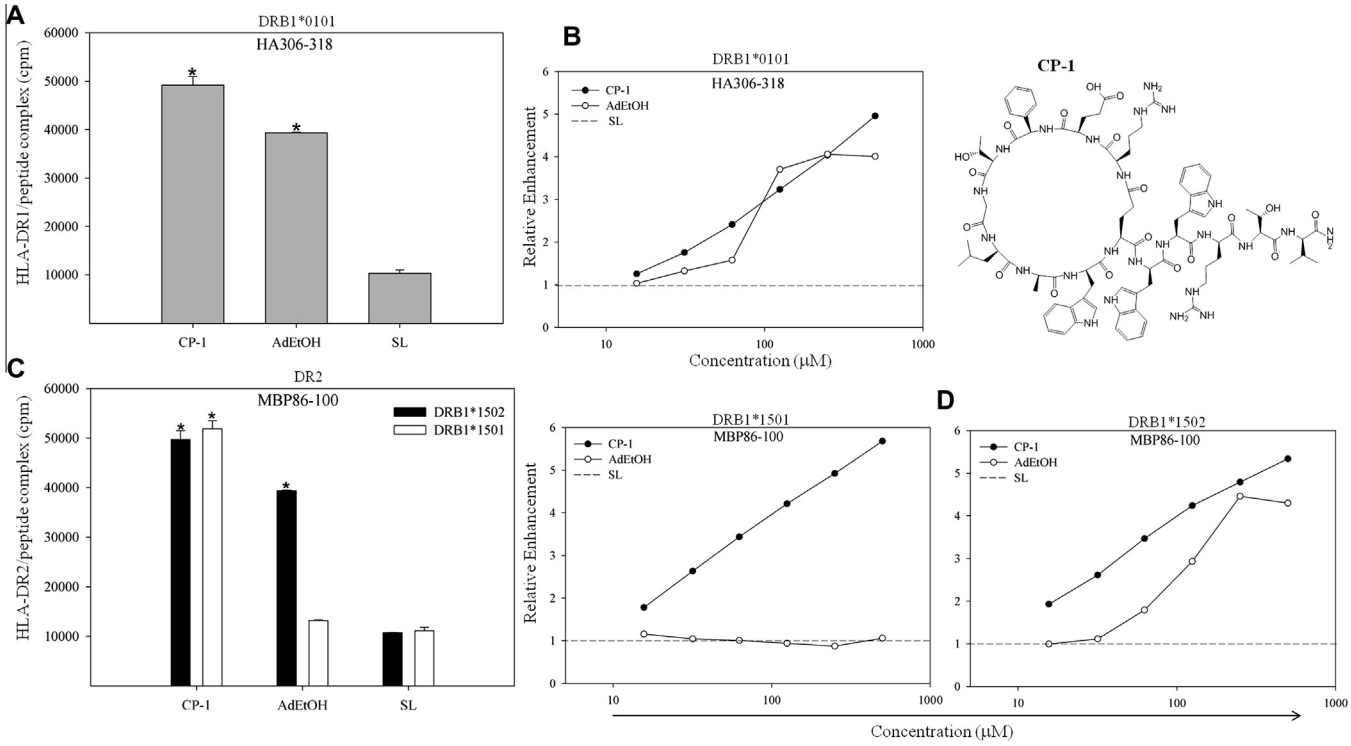

Fig. 1. The effect of CP-1 on peptide loading of soluble MHC-II molecules. (A) and (C) Soluble HLA-DR1/DR2 molecules (100 nM) were incubated with peptide antigens (80 μg/mL) in the absence or presence of test samples (500 μM). (B) and (D) Dose–response curves of CP-1 and AdEtOH for which the peptide/MHC complex is expressed as relative enhancement. AdEtOH was used as a positive control. Data represent the means of three replicates. $^{*}P < 0.05$, spontaneous loading versus test samples.

MBP86-100 peptide antigens. After incubation, the amount of peptide loaded onto the cell surface was determined by flow cytometry after staining with streptavidin-PE.

In line with the loading of soluble MHC, flow cytometric analysis showed a striking increase in the amount of peptide bound to the cell surface in the presence of CP-1 (Fig. 2A). Also, it was found that the effect was evident with all three allelic variants of DRB1. Compared to AdEtOH, the effect was even more striking. Presumably, because of lower bioavailability in serum-supplemented culture medium, only subtle increases were observed for HLA-DRB1*0101- and HLA-DRB1*1502-expressing cells in the presence of AdEtOH. By contrast, CP-1 exhibited potency across the entire concentration range (Fig. 2B). No influence of CP-1 on fibroblast cells that do not express any MHC-II molecules demonstrates the HLA-DR specificity of the peptide.

The effects of CP-1 and AdEtOH were correlated with the effects observed previously with soluble molecules (see Fig. 1). The dose–response curves of CP-1- and AdEtOH-mediated catalysis were determined by incubating cells with respective biotinylated peptide antigens in the presence of a titrated amount of MLEs. Quantification of peptide loading by flow cytometry showed a nearly similar pattern of curves for CP-1 not only with all alleles (Fig. 2B), but also with the curves determined previously with soluble molecules (see Fig. 1). It is evident from the curves that CP-1 is susceptible to all HLA-DR alleles tested without showing any correlation with position β86. Importantly, an enhanced effect was observed with cell surface molecules at a low dose (i.e., 15 μM) of CP-1, showing more than a onefold increase in loading.

The findings presented here along with previous reports indicate that CP-1 exhibits catalytic activity without showing any correlation with the dimorphism at position β86. Indeed, a comparison of the catalytic activity with AdEtOH suggests a different route for CP-1 to interact with the DR molecule. Our data show that CP-1 acts on all of the DR alleles tested in the same manner. The large size of CP-1 (Fig. 1; top right) is one factor that might restrict it to interact via the P1-pocket of the peptide-binding groove of HLA-DR molecules. Based on these results, it can be inferred that CP-1 likely binds to a conserved surface feature of DRα- or DRβ-chains and, thus, acts allosterically to induce conformational changes in MHC-II molecules, resulting in the widening of the peptide-binding groove.

As the major function of MHC-II molecules is to display peptide antigens to $CD4^+$ T cells, the effects of CP-1-mediated increased MHC-II loading on $CD4^+$ T cell responses were studied using an in vitro antigen-specific T cell assay. In these experiments, the antigen-specific mouse $CD4^+$ T cell hybridoma EvHA/X5, which recognizes HA306-318 antigen in the context of DRB1*0101, were stimulated with DRB1*0101-expressing cells that had been previously loaded in the presence of CP-1 and AdEtOH. The effect of increased loading resulted in drastic enhancement in the sensitivity of T cell responses at a suboptimal antigen dosage (Fig. 3A and B). The dose–response curves revealed increased IL-2 release in the presence of CP-1 (Fig. 3A). Cell culture supernatants collected after stimulation of EvHA/X5 cells also triggered enhanced proliferation of CTLL-2 cells (Fig. 3B). These data demonstrated that the effect of CP-1 on peptide antigen loading directly translates into improved $CD4^+$ T cell responses.

Lastly, the cytotoxic effects of CP-1 were assessed using mouse fibroblasts (NIH-3T3) and EBV-transformed B cells (15310-LN) using the MTT assay and a flow cytometry-based PI staining method (Fig. 4). Cells were incubated with CP-1 and AdEtOH at various concentrations to determine the % growth inhibition and the number of dead cells. It was found that CP-1 exhibited low cytotoxicity on B cells, as only 24.9% of cells were dead at the highest concentration, 500 μM (Fig. 4B). However, increased cytotoxic effects were observed on NIH-3T3 cells, with 50% growth inhibition

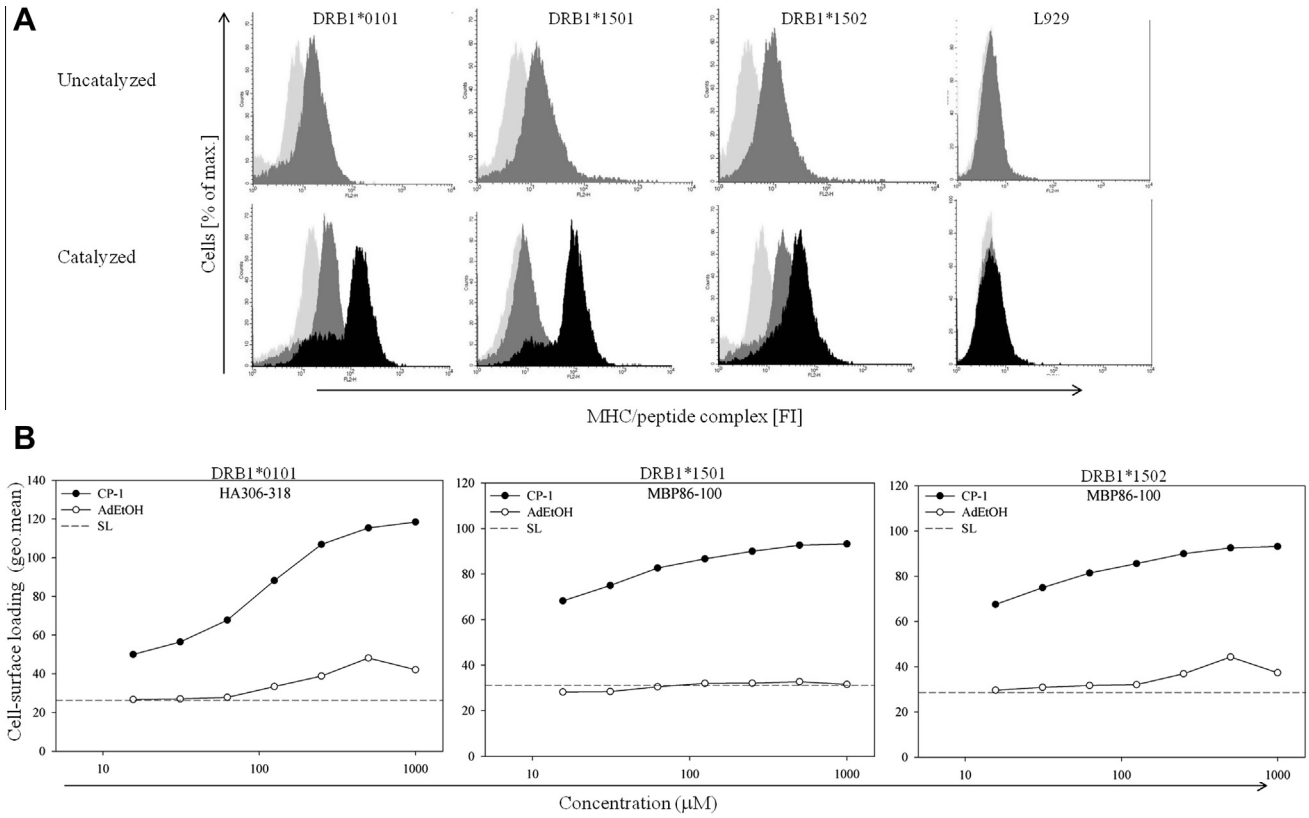

Fig. 2. The effect of CP-1 on cell surface peptide loading of MHC-II molecules. (A) HLA-DRB1-expressing cells and L929 fibroblast cells (as a negative control) were incubated with respective peptide antigens (5 μg/mL) in the absence (uncatalyzed) or presence (catalyzed) of peptide and AdEtOH (500 μM). Representative histogram plots show the fluorescence recorded in the absence (light gray) or presence of peptide antigens (dark gray; upper panel) and in the presence of peptide antigens (light gray) and catalysts (i.e., AdEtOH; dark gray) and peptide (black) molecules (lower panel). (B) Dose–response curves. AdEtOH was used as a positive control. Data represent the means of three replicates.

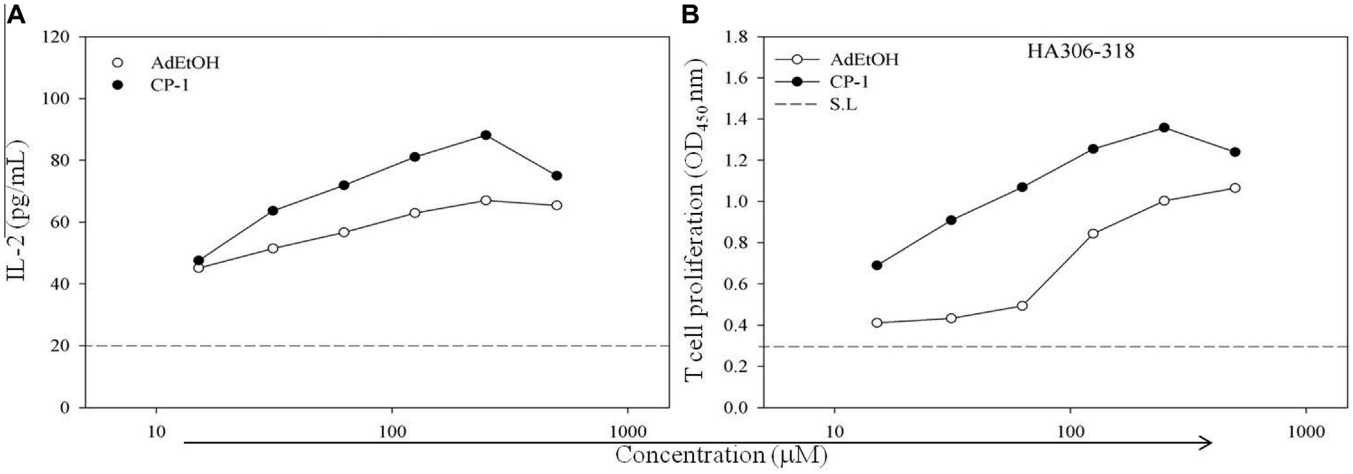

Fig. 3. The effect of CP-1 on antigen-specific CD4⁺ T cell responses. DRB1*0101-expressing cells were incubated with the peptide antigen HA306-318 (5 μg/mL) in the presence of titrated amounts of CP-1 and AdEtOH. Cells were then washed and used to stimulate the antigen-specific mouse T cell hybridoma EvHA/X5. (A) The influence of CP-1 and AdEtOH at multiple doses on antigen-specific T cell responses was determined by estimating IL-2 release in supernatants. (B) The influence of CP-1 and AdEtOH on antigen-specific T cell responses was determined by measuring CTLL-2 cell proliferation after 24 h incubation with supernatants containing EvHA/X5 T cell-secreted IL-2 using BrdU dye. Data represent the means of three replicate experiments.

at the same dose (Fig. 4A). Furthermore, a higher IC_{50} value of 497.5 ± 3.5 μM was obtained for CP-1 compared to AdEtOH, which showed an IC_{50} value of 62.5 ± 1.3 μM.

Thus, our study identified and characterized a cyclic peptide, CP-1, as a new highly active MLE-peptide that is susceptible to DRB1*0101, DRB1*1501, and DRB1*1502 without any correlation to position β86 of the P1-pocket. The finding that CP-1 acts on all DR alleles tested without interacting with the P1-pocket indicates that it could bind to nonpolymorphic DRα-chains or to the site that is conserved among polymorphic DRβ-chains. The significant

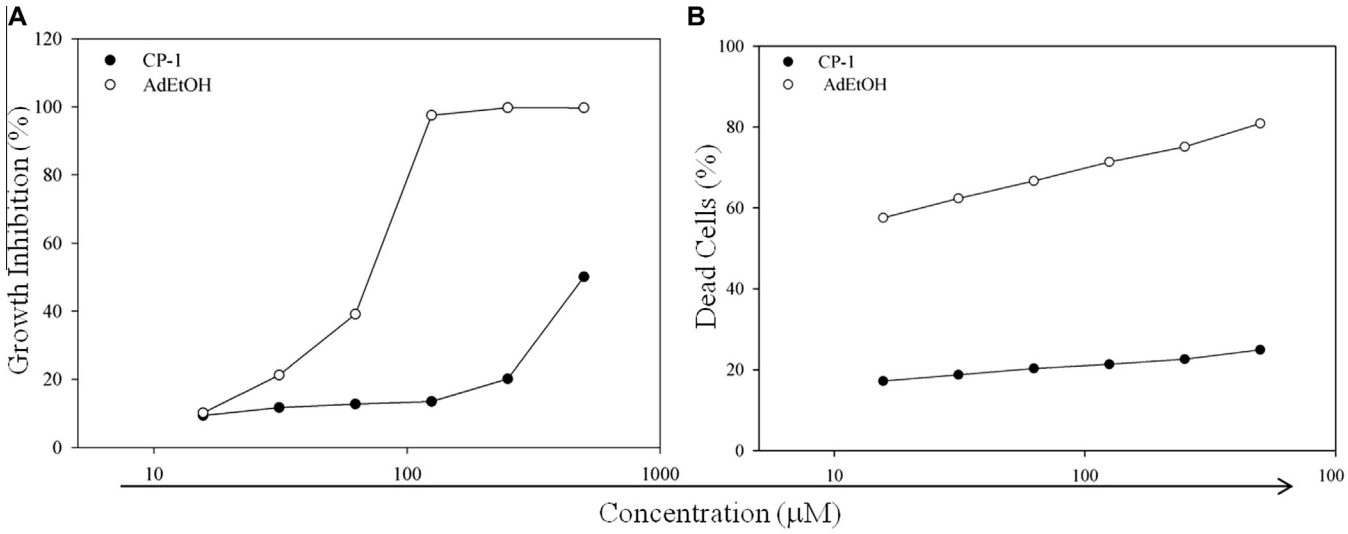

Fig. 4. The cytotoxic effects of CP-1 and AdEtOH on mouse fibroblasts (NIH-3T3) using the MTT assay, and on EBV-transformed B cells expressing DRB*0101 measured by flow cytometry. (A) Cytotoxicity against NIH-3T3 cells, shown as growth inhibition (%). (B) Cytotoxicity against B cells, shown as the percentage of dead cells (PI-positive). Data represent the means of three replicates.

enhancement ($P < 0.001$) in peptide loading by CP-1 was not correlated with any cytotoxic effects at a low concentration, indicating that it is a highly potent MLE-peptide. Therefore, we predict that the peptide could be useful for amplifying immune responses for therapeutic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.047.

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