

## Short peptide sequences mimic HLA-DM functions

Chih-Ling Chou<sup>b,1</sup>, Saied Mirshahidi<sup>a,2</sup>, Katherine Wailen Su<sup>a</sup>, AeRyon Kim<sup>c</sup>, Kedar Narayan<sup>c</sup>, Stanislav Khoruzhenko<sup>a</sup>, Minzhen Xu<sup>d</sup>, Scheherazade Sadegh-Nasseri<sup>a,b,c,\*</sup>

<sup>a</sup> Department of Pathology, Johns Hopkins University, Baltimore, MD 21205, United States

<sup>b</sup> Graduate Program in Molecular and Computational Biophysics, United States

<sup>c</sup> Graduate Program in Immunology, United States

<sup>d</sup> Antigen Express, Antigen Express, Inc., Worcester, MA 01605, United States

Received 10 September 2007; received in revised form 18 October 2007; accepted 21 October 2007

Available online 3 December 2007

### Abstract

HLA-DM (DM) plays a critical role in Ag presentation to CD4 T cells by catalyzing the exchange of peptides bound to MHC class II molecules. It is known that DM interaction with MHC II involves conformational changes in the MHC II molecule leading to the disturbance of H-bonds formed between the bound peptide and the MHC II groove leading to peptide dissociation. The specific region of the DM molecule that induces this peptide dissociation is not defined. In this study, we describe three short peptides (helper peptides) that accelerate DM-catalyzed peptide exchange. Kinetic studies presented here demonstrate that these peptides act similarly to DM in; (a) enhancing peptide binding to HLA-DR1; (b) dissociation of complexes of peptide-DR1; and (c) maintaining a receptive conformation of empty DR1. We further report that helper peptides are effective in increasing peptide binding to DR1 expressed on B cells *in vitro*, and, when mixed with peptide and adjuvant, cause enhanced T cell priming in HLA-DR1 Tg mice. We suggest that helper peptides might interact with the same critical residues on MHC class II that is targeted by DM.  
© 2007 Elsevier Ltd. All rights reserved.

**Keywords:** Antigen presentation; HLA-DM; T cell activation; Vaccines; Small peptides enhancing peptide loading; Binding kinetics; HLA-DR

### 1. Introduction

Shortly after synthesis in the antigen presenting cell, MHC class II  $\alpha\beta$  heterodimers form nonameric assemblies with Invariant chain (Ii) in the endoplasmic reticulum and are then transported through the Golgi complex to the endocytic pathway (Neeffjes et al., 1990; Peters et al., 1991). During transport through the endocytic pathway the majority of Ii is removed from MHC II molecules by low pH and acid proteases (Blum and Cresswell, 1988), leaving a proteolytic fragment of Ii called CLIP (class II-associated Ii peptide) bound to MHC class II (Cresswell, 1994). CLIP acts as a place-keeper for the MHC class II groove, inhibiting conformational changes that render

the groove closed (Romagnoli and Germain, 1994; Castellino et al., 1997; Natarajan et al., 1999a; Sadegh-Nasseri, 1994; Sadegh-Nasseri and Germain, 1991, 1992; Sadegh-Nasseri et al., 1994; Sato et al., 2000) and has to be removed in order to allow binding of exogenous peptides to nascent MHC class II complexes. DM or H2-M in mice is a nonclassical HLA molecule and was discovered in B cell lines that were defective in Ag presentation by MHC class II molecules (Mellins et al., 1990) and has been shown to play a critical role in the displacement of CLIP (Denzin et al., 1994; Denzin and Cresswell, 1995; Green et al., 1995; Fung-Leung et al., 1996). In addition to displacing CLIP, DM transiently interacts with empty MHC class II to generate a peptide-receptive conformation, and plays an active role in the selection of specific peptide/MHC class II complexes during antigen processing (Weber et al., 1996; Kropshofer et al., 1996; Martin et al., 1996; Miyazaki et al., 1996; Ullrich et al., 1997; Vogt et al., 1997; Kropshofer et al., 1997; Chou and Sadegh-Nasseri, 2000; Doebele et al., 2000; Narayan et al., 2007).

DM has been shown to function in two stages: a recognition phase followed by an effector phase. Multiple studies have

\* Corresponding author at: Department of Pathology, Johns Hopkins University School of Medicine, 664C Ross Building, Baltimore, MD 21205, United States. Tel.: +1 410 614 4931; fax: +410 614 3548.

E-mail address: [ssadegh@jhmi.edu](mailto:ssadegh@jhmi.edu) (S. Sadegh-Nasseri).

<sup>1</sup> Present address: Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85715, USA.

<sup>2</sup> Present address: Dana-Farber Cancer Institute, Boston, MA 02115, USA.

shown that the recognition phase depends on structural variations among different peptide/MHC complexes (Belmares et al., 2003; Chou and Sadegh-Nasseri, 2000; Pashine et al., 2003; Pu et al., 2004; Stratikos et al., 2004). It was shown that structurally flexible complexes were susceptible to DM-induced dissociation, whereas “rigid” complexes were resistant to DM (Chou and Sadegh-Nasseri, 2000). The effector phase involves destabilization of H-bonds between the peptide main chain and the conserved residues lining the groove of MHC class II protein. Specifically, the H-bond formed between His 81 on the beta chain and peptide was shown to be most critical (Narayan et al., 2007). Thus, it was proposed that DM affects peptide/MHC class II complex dissociation by a “hit-and-run” mechanism, where a transient interaction between DM and DR1 causes a conformational change in DR1 that leads to the perturbation of the  $\beta$ 81His H-bond, resulting in destabilization of bound peptide. This characteristic of DM therefore, allows for screening of different peptides until one comes along that forms a DM-insensitive complex with MHC II and thus remains bound and is then perhaps exported to the cell surface. It has also been suggested that the conformation of peptide/MHC class II complexes formed in the presence of DM might be somewhat different from those formed in its absence (Pu et al., 2004). Thus, DM is highly significant for efficient peptide binding to MHC class II and selection of peptides that remain bound to MHC II and their recognition by T cells (Lazarski et al., 2005).

Although these findings have provided a mechanistic understanding of the DM interaction with MHC class II molecules, the specific interaction site of DM and MHC class II remains unknown. Interestingly, some earlier studies have shown that the amino-terminal segment of long CLIP (residues 81–89) that falls outside the peptide binding groove of class II molecules might facilitate the release of the groove-binding sequence of CLIP through an allosteric release mechanism (Kropshofer et al., 1995a). In designing strategies to improve peptide vaccination, Adams et al. (1997) constructed a series of peptide variants derived from the N-terminal sequence of CLIP. Originally called Ii-key peptides, but renamed as “helper peptides” in the present study, they were shown to enhance peptide presentation when mixed with the antigenic peptide and in conjugated form with peptide (Kallinteris et al., 2003, 2005a,b). A core peptide sequence, LRLKLPK, was reported to be the shortest fully active sequence of this subset of peptides. It has been hypothesized that the position of the helper peptide regulatory site lies at the end of the antigenic peptide binding groove around the pocket 1 region (Kropshofer et al., 1995b; Vogt et al., 1995). Based on such similarities, we proposed that helper peptides might mimic DM and these helper peptides may bind to the same regulatory site around pocket 1 on class II molecules and function in a similar way.

In this study, we show that a group of helper peptides can facilitate the binding of high affinity peptides. We show that AE206, with the core sequence LRLKLPK, can catalyze peptide exchange. The binding experiments showed that AE206 is able to convert the empty DR1 into receptive conformation, although it was less effective than DM. Moreover, just like DM, it can act as a molecular chaperone protecting empty

DR1 molecules from functional inactivation, and can distinguish conformational differences between the wild-type DR1 and mutant DR1 $\beta$ G86Y, where pocket 1 is shallow because of being filled by tyrosine. Thus, these small peptides behave like DM with the advantage that they are perfectly functional at neutral pH, making them effective additions to peptide vaccine preparations.

## 2. Materials and methods

### 2.1. Production of recombinant soluble DR1 proteins

Soluble wild-type (wt) and mutant DR1 (DR1 $\beta$ G86Y) proteins were expressed and purified as originally described (Natarajan et al., 1999a). Baculovirus DNA (BaculoGold; PharMingen, San Diego, CA) and transfer vectors carrying the wild-type or mutant genes were cotransfected into Sf9 insect cells to produce recombinant viruses. Hy5 insect cells infected with these recombinant viruses for protein production and DR1 proteins were purified from the culture supernatant by using anti-DR1 mAb (L243) immunoaffinity chromatography columns. Purified wild-type and mutant DR1 proteins migrated similarly at the expected sizes for  $\alpha$  and  $\beta$  subunits in a SDS-PAGE assay, where samples were not boiled.

### 2.2. Peptide synthesis and labeling

Influenza hemagglutinin (HA) peptides, HA<sub>306–318</sub> (CPKYVKQNTLKLAT), and several variants, HA<sub>Y308A</sub> (CPKAVKQNTLKLAT), Cys-HA<sub>Anchorless</sub> (CPKAVKAN-GAKAAT), and AE206 (LRLKLPK) were purified to apparent homogeneity of >95% by reverse-phase preparative HPLC and their identities were confirmed by mass spectrometry. Then, 0.15  $\mu$ M HA<sub>305–318</sub> or HA<sub>Anchorless</sub>, containing a single cysteine, was incubated in 10 ml PBS with 25  $\mu$ l of 75  $\mu$ M fluorescein-5-maleimide (Molecular Probes) in *N,N*-dimethylformamide (DMF) for 1 h at room temperature. The excess free fluorescence label was removed by passing through a Sephadex G-10 column (Amersham Pharmacia Biotech). The concentration was determined by the extinction coefficient of fluorescein-5-maleimide (83  $\text{mM}^{-1} \text{cm}^{-1}$ ).

### 2.3. Cells and mice

Clone 1 (CL-1) is a CD4<sup>+</sup> Th1 clone specific for the influenza hemagglutinin-derived peptide HA<sub>306–318</sub> bound to HLA-DR1 (De Magistris et al., 1992). Epstein-Barr virus (EBV) 1.24 transformed, a human HLA-DR1 (DRB1\*0101)-positive B cell line was used as the antigen presenting cell (APC) for CL-1 stimulation. T cell clones and B cells were grown as described (Korb et al., 1999). HLA-DR1 (DRB1\*0101) transgenic mice were used at 8–10 weeks of age. The chimeric HLA-DR1 molecule comprised a peptide-binding groove derived from the human DR1 sequence and a CD4-binding domain from I-A<sup>f</sup> mice (Rosloniec et al., 1997, 2002).

#### 2.4. Peptide association and dissociation assays

Purified wtDR1 or DR1 $_{\beta G86Y}$  (2.4  $\mu\text{M}$ ) was incubated in the absence or presence of 1  $\mu\text{M}$  DM or 150  $\mu\text{M}$  AE206 with 100  $\mu\text{M}$  fluorescence-labeled HA $_{\text{Anchorless}}$  peptide (FITC-HA $_{\text{Anchorless}}$ ) for various times in 0.15 M citric phosphate buffer, pH 6.0, or in PBS, at 37 °C. After removal of free peptides by a Sephadex G-50 spin column equilibrated with PBS, fluorescence emission of the DR-FITC-HA $_{\text{Anchorless}}$  complex was measured at 514 nm with an excitation at 490 nm on an LS-50B spectrofluorimeter (PerkinElmer) at room temperature.

#### 2.5. In vitro stabilization assay

In experiments to study the lifetime of the receptive conformation, unlabeled DR1/HA $_{Y308A}$  complexes were separated by HPLC as described earlier. DR1/HA $_{Y308A}$  (1.5  $\mu\text{M}$ ) was allowed to dissociate for various times at 37 °C in PBS (pH 7.4) and in 0.1 M citrate/phosphate (pH 5.5) buffer in the presence or absence of DM or AE206 and then followed by binding with 50  $\mu\text{M}$  FITC-HA $_{306-318}$  for 1.5 h in the respective buffers. The unbound peptides in both reactions were removed using Sephadex G-50 spin columns equilibrated with PBS, as the fluorescein had significantly reduced fluorescence at pH 5.5.

#### 2.6. Data analysis

All the raw association data in Figs. 1–3 were fitted into either single or double exponential association equations as previously described (Chou and Sadegh-Nasseri, 2000).

#### 2.7. Cell surface peptide loading assay

EBV 1.24, B cell line was used for detection of AE206 effects on peptide binding to HLA-DR1. One million EBV 1.24 cells were pulsed with the short-lived peptide, HA $_{\text{Anchorless}}$ , overnight. The excess peptide was washed off and then the EBV-B cells were incubated with 1  $\mu\text{M}$  fluorescence labeled HA $_{306-318}$  peptide in the presence or absence of 150  $\mu\text{M}$  AE206 at 37 °C for various times (0, 30, 60, 120 min). Cells were then washed twice and analyzed by flow cytometry with a FACScalibur instrument (Becton Dickinson, Mountain View, CA).

#### 2.8. T cell proliferation assay

Clone 1 is a human CD4 $^{+}$  Th1 clone specific for HA $_{306-318}$  peptide presented on HLA-DR1. EBV-B cells were pulsed with either 0.1  $\mu\text{M}$  or 1  $\mu\text{M}$  HA $_{306-318}$  peptide in the presence and absence of 150  $\mu\text{M}$  AE206 at 37 °C for 0, 30, 60 or 120 min. Clone 1 ( $4 \times 10^4$ ) T cells were then incubated with equal numbers of irradiated (10,000 rad) pulsed EBV-B cells at 37 °C in 5% CO $_2$ . Seventy-two hours after the addition of the peptide-pulsed APCs in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% FCS (Life Technologies), 5% pooled human serum, 2 mM L-glutamine (Life Technologies), and 10 mM HEPES (Life Technologies), each well was pulsed with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Amersham,

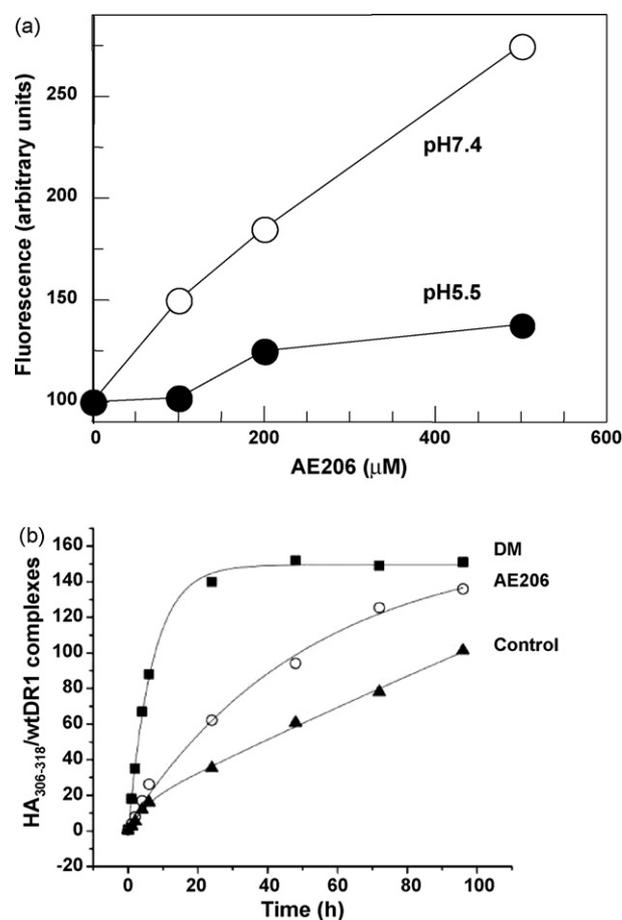


Fig. 1. Effects of AE206 and DM on peptide association with empty wtDR1. (a) The effect of AE206 on the binding of peptide to wtDR1 at pH 5.5 (●) and 7.4 (○): 2.4  $\mu\text{M}$  wtDR1 was incubated with 10  $\mu\text{M}$  FITC-HA $_{306-318}$  peptide for 30 min with various concentrations of AE206 for various times in either 0.15 M citrate phosphate buffer (pH 5.5) or PBS (pH 7.4) at 37 °C. (b) Association of FITC-HA $_{306-318}$  peptide with wtDR1 as a function of time in the absence (▲) or presence of 150  $\mu\text{M}$  AE206 (○), or 0.5  $\mu\text{M}$  of HLA-DM (■) at pH 5.5. Half-time ( $t_{1/2}$ ) for each data set was calculated by fitting the data into equations describing double exponential rise for the control, and single exponential rise for the HLA-DM and AE206 curves. The  $t_{1/2}$  for the control was calculated as 3.46 h and 533 h; for DM, 4.8 h; and for AE206, 36.4 h. Representative of three independent experiments.

Arlington Heights, IL). Cells were harvested and counted 14 h later by using a beta counter (Packard Instruments, Meriden, CT). Each assay was done in triplicate.

#### 2.9. Immunization of transgenic mice

HLA-DR1 Tg mice were immunized at the base of the tail subcutaneously (s.c.) with various doses of HA $_{306-318}$  peptide in the presence (100 nmol) or absence of AE206 peptide emulsified at a 1:1 (v/v) in Complete Freund's Adjuvant (CFA) (Sigma, St. Louis, MO). Eight days later mice were euthanized, inguinal lymph nodes were removed, and cells were cultured either with no peptide or various concentrations (0, 0.1, and 1  $\mu\text{M}$ ) of HA $_{306-318}$  at 37 °C in 5% CO $_2$  for 72 h. Proliferation assay was performed as described above.

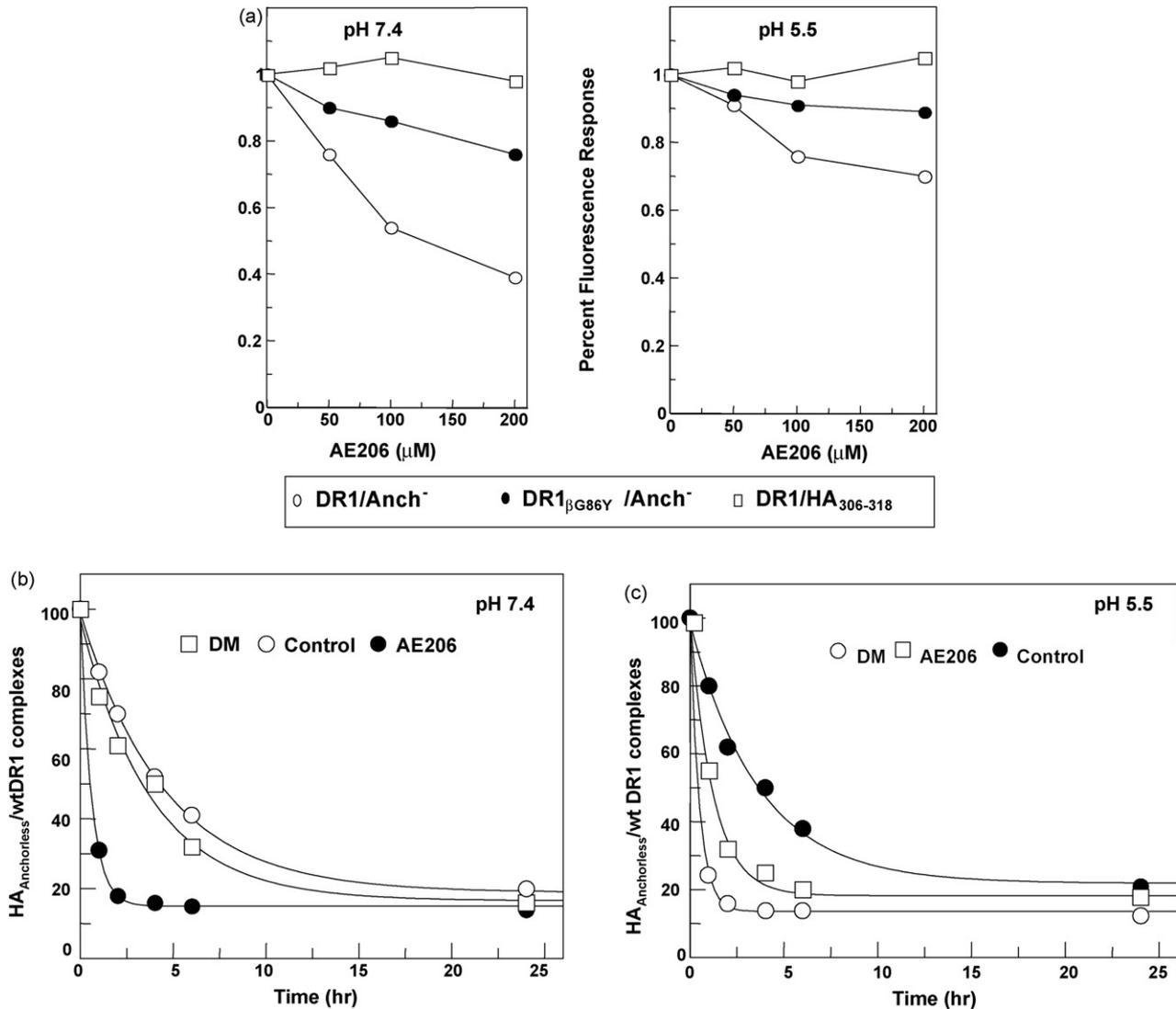


Fig. 2. Effects of AE206 and DM on peptide/DR1 complex dissociation. (a) Effects of concentration of AE206 on dissociation of peptide/DR1 at pH 5.5 and pH 7.4: 2.4 μM wtDR1 in complex with FITC-HA<sub>Anchorless</sub> (○) or HA<sub>306-318</sub> (□), and 2.4 μM DR1<sub>βG86Y</sub> in complex with FITC-HA<sub>Anchorless</sub> (●). Titrated amounts of AE206 were incubated with the shown complexes at 37 °C for 30 min, and dissociation of the complexes was measured. (b and c) Effects of AE206 on rate of dissociation of peptide/DR1 at pH 5.5 and pH 7.4: 150 μM AE 206 or 0.5 μM DM was incubated with wtDR1 in complex with FITC-HA<sub>Anchorless</sub> at pH 7.4 (b), and pH 5.5 (c). The labeled complexes were dissociated in the presence of 100 times molar excess of unlabeled HA<sub>306-318</sub> peptides at 37 °C for the indicated times in the absence (●) or presence of 150 μM AE206 (□), or 0.5 μM of HLA-DM (○). The fluorescence of the labeled complex before dissociation ( $f_0$ ) was arbitrarily assigned a value of 1.0. The fluorescence of the labeled complex after dissociation for various times is expressed as a percentage of  $f_0$ . The dissociation data are fitted to a single exponential curve that yields  $t_{1/2}$  of 2.6 h for DM, 0.4 h for AE206, and 3.15 h for control at pH 7.4, and  $t_{1/2}$  of 0.34 h for DM, 0.86 h for AE206 and 2.5 h for control at pH 5.5. The y-axis represents arbitrary fluorescence units. Representative of four to five independent experiments.

### 3. Results

#### 3.1. Influence of AE206 on peptide association with wtDR1

To test the influence on peptide loading, we first examined the molar ratio of AE206 required to facilitate association of wtDR1 in complex with FITC-HA. Soluble HLA-DR1 was incubated with a fluorescein-labeled high affinity peptide, HA<sub>306-318</sub>, in the presence of various concentration of AE206 at 37 °C for 18 h in 0.1 M citrate phosphate buffer (pH 5.5) or PBS (pH 7.4). In both buffers, the addition of AE206 to the binding assay enhanced the amount of peptide bound to wtDR1, although AE206 was more effective at pH 7.4 (Fig. 1a). A similar enhancing effect

was induced by AE107 upon peptide binding to sDR1 (data not shown). To examine the influence of AE206 on peptide loading to DR1 in more detail, the association rate of the binding was measured in the presence and absence of either AE206, or DM at pH 5.5 (Fig. 1b). In control samples, where no catalyst was present, empty sDR1 showed the typical biphasic binding kinetics and maximal binding was not obtained even after 96 h of incubation in the presence of 10 μM peptide. In the presence of DM, the formation of peptide complex significantly increased, as shown previously. DM converted the biphasic peptide-binding pattern of wtDR1 to the monophasic-binding pattern of receptive wtDR1 (Chou and Sadeh-Nasser, 2000; Narayan et al., 2007). Interestingly, the same effect was also evident when AE

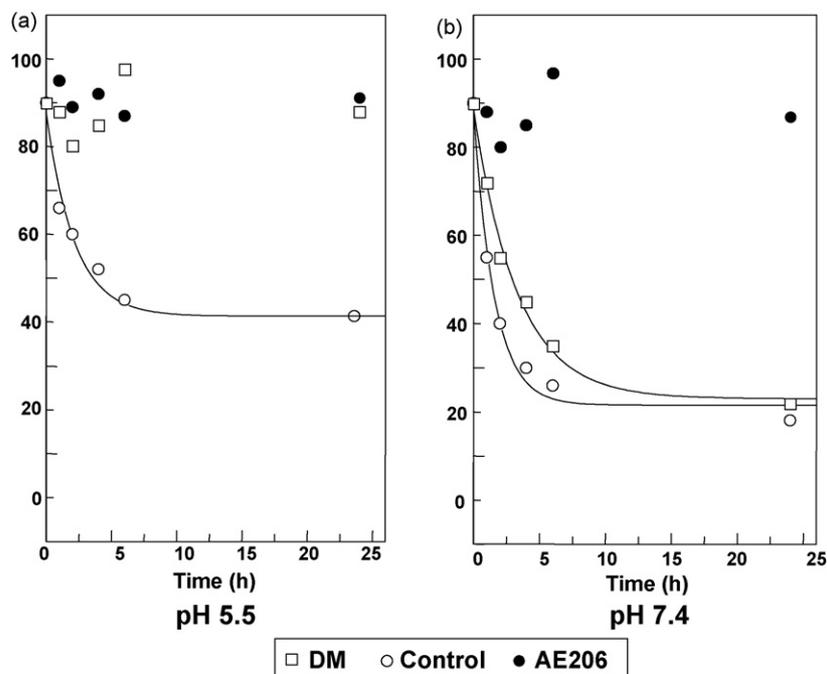


Fig. 3. Stabilization of empty class II molecules by AE206 at pH 5.5 and pH 7.4. FPLC-isolated DR1/Y308A complexes (2.4  $\mu$ M) were incubated in the absence of any free peptide for the indicated times at 37  $^{\circ}$ C in (a) PBS (pH 7.4) or in (b) 0.1 M citrate/phosphate buffer (pH 5.5) in the absence ( $\circ$ ) or presence of 150  $\mu$ M AE206 ( $\bullet$ ), or 0.5  $\mu$ M HLA-DM ( $\square$ ) followed by binding to 50  $\mu$ M FI-HA for 1.5 h in the same buffer. The samples were then passed over a Sephadex G-50 column equilibrated with PBS, to remove the excess free peptide and to exchange the buffer. The amount of HA binding to DR1 immediately after dissociation from DR1/Y308A complexes was arbitrarily assigned a value of 100%. The data were fit to a single exponential decay function. The  $t_{1/2}$  for inactivation of the receptive molecule is 1.1 h at pH 7.4 and 1.5 h at pH 5.5 for control. Representative of three to four independent experiments.

206 was used but at neutral pH, although the effect was less prominent than the DM sample at pH 5.5.

### 3.2. Effect of AE206 on peptide release from Wt and DR1 $\beta$ G86Y

Because both AE206 and AE107 displayed same HLA-DM-like catalytic activity in the loading experiment, the effect of the AE206 was further investigated. We next tested the influence of AE206 on peptide release by the dissociation of FITC-labeled peptide from DR1. WtDR1 and DR1 $\beta$ G86Y in complex with HA<sub>306–318</sub> and HA<sub>Anchorless</sub> were incubated in the presence of increasing concentrations of AE206 for 30 min at 37  $^{\circ}$ C at pH 5.5 and at pH 7.4 (Fig. 2a). Consistent with effects on peptide binding, AE206 enhanced wtDR1/HA<sub>Anchorless</sub> complex dissociation more effectively at pH 7.4 compared to pH 5.5. Importantly, AE206 was not effective in dissociation of wtDR1/HA<sub>306–318</sub>, or DR1 $\beta$ G86Y/HA<sub>Anchorless</sub> consistent with the previous data showing that DR1 $\beta$ G86Y is less sensitive to the catalytic function of DM (Chou and Sadegh-Nasseri, 2000). Thus, AE206 can mediate the release of peptides according to their affinity and the conformation of the complexes.

To evaluate the effect of AE206 on peptide release in more detail, the off-rates of FITC-HA<sub>Anchorless</sub>/wtDR1 complexes were tested in the presence of either 0.5  $\mu$ M soluble DM, or 150  $\mu$ M AE206 at both pH 5.5 (Fig. 2C) and pH 7.4 (Fig. 2B). At pH 5.5, the dissociation  $T_{1/2}$  of HA<sub>Anchorless</sub> was enhanced by HLA-DM from 2.5 h (in the control samples) to 0.34 h. Similar

results were observed with 150  $\mu$ M AE206. As expected, HLA-DM molecules showed little or no catalytic activity at pH 7.4 (Denzin and Cresswell, 1995). In accordance with these reports, only little enhancement of off-rate from 3.15 h to 2.6 h was observed. Compared with HLA-DM, the ligand release capacity of AE206 appeared to be more dramatic at pH 7.4, with an enhancement of almost 8-fold.

### 3.3. AE206 stabilizes empty class II molecules

Empty class II  $\alpha\beta$  dimers have been shown to aggregate and lose their ability to bind antigenic peptides (Beeson and McConnell, 1994; Natarajan et al., 1999a; Sadegh-Nasseri, 1994; Sadegh-Nasseri et al., 1994; Sato et al., 2000; Stern and Wiley, 1992). HLA-DM has been shown to prevent the functional inactivation and aggregation of class II molecules. Stabilization of empty class II molecules may be an important property of HLA-DM in facilitating antigen processing. To investigate whether AE206 also can stabilize the empty DR1 molecules, we measured the lifetime of the empty receptive DR1 molecules (Natarajan et al., 1999a). HPLC-purified wtDR1/HA<sub>Y308A</sub> was incubated at 37  $^{\circ}$ C for various times in the presence and absence of DM or AE206, and the availability of functional DR1 molecules by binding of fluorescent labeled HA peptide was measured. In the absence of DM or AE206 only 50% of peptide-receptive DR1 molecules were present after 24 h incubation at pH 5.5, whereas almost all of the class II molecules retained their peptide binding function when DM or AE206 was present (Fig. 3). At physiological pH, however, only AE206

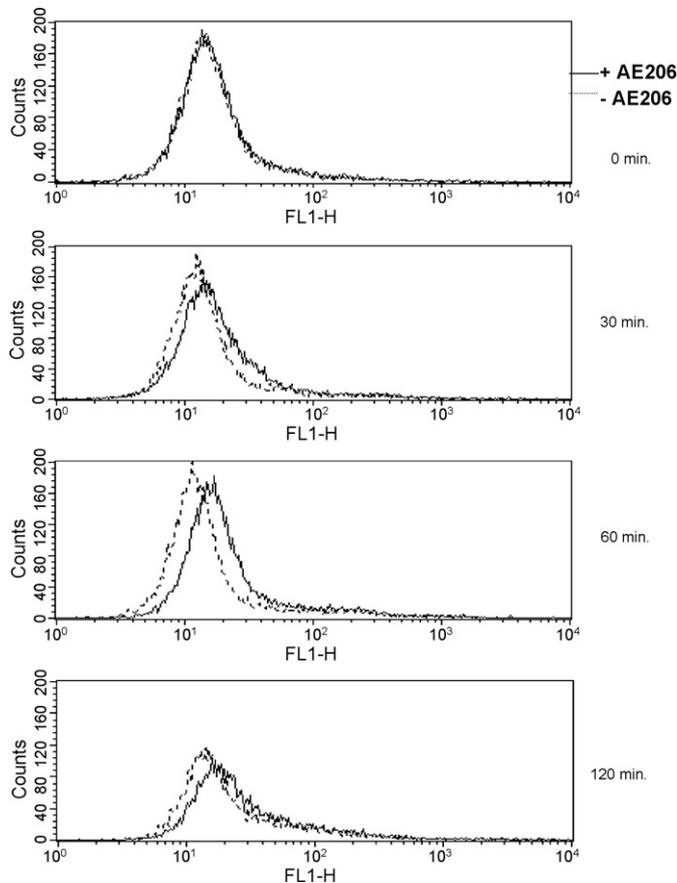


Fig. 4. Enhanced antigen loading efficiency in the presence of AE206 on HLA-DR1 expressing B cell line. HLA-DR1 EBV B cells were pulsed with HA<sub>Anchorless</sub> overnight and then washed. FITC-HA<sub>306-318</sub> peptide loading was carried out in the absence (dotted line) or presence (solid line) of 150  $\mu$ M AE206 onto EBV 1.24 B cells for the duration of time indicated to the right. The amount of peptide loading was determined by FACS analysis. Representative of at least three independent experiments.

stabilized the empty DR1 molecules in receptive form in the absence of peptide.

### 3.4. Peptide loading of antigen presenting cells in the presence of AE206

Because AE206 retains effectiveness at neutral pH, we next tested its efficacy in peptide loading onto DR1 expressed on the cell surface under physiological conditions. EBV 1.24, a human HLA-DR1 (DRB1\*0101)-positive, EBV-immortalized, activated B cell line (EBV-B, or B cells) were pulsed with the short-lived peptide, HA<sub>Anchorless</sub>, overnight. The excess peptide was washed off and then the cells were incubated with 1  $\mu$ M fluorescence-labeled HA<sub>306-318</sub> peptide in the presence or absence of AE206 for various time points. The excess labeled peptide was washed off before the analysis was carried out by flow cytometry. The mean channel fluorescence intensity, MFI, showed a 2-fold increase in the presence of AE206 after 2-h incubation with HA<sub>306-318</sub>. Thus, the presence of AE 206 resulted in a 2-fold increase in the level of peptide exchange (Fig. 4).

### 3.5. Enhancement of T cell proliferation by AE206

T cell proliferation assays were used to test whether the increase in loading efficiency also translated into improved T cell responses. EBV cells expressing HLA-DR1 were loaded with 0.1  $\mu$ M or 1  $\mu$ M of HA<sub>306-318</sub> peptide in the absence or presence of 150  $\mu$ M AE206 for various lengths of time (0, 30, 60 and 120 min) and used to stimulate clone 1 T cell reporter specific for HLA-DR1/HA<sub>306-318</sub>. In accordance with the previous peptide loading experiment, the HLA-DR1-restricted T cell response was increased by  $\sim$ 2-fold with 0.1  $\mu$ M HA peptide pulsed for 2 h. In the case of 1  $\mu$ M HA, a  $\sim$ 2-fold increase of T cell response was observed upon 1 h coincubation, and increased only 25% over the control APC that was pulsed with HA<sub>306-318</sub> peptide alone. This is not surprising because in 2 h, at 1  $\mu$ M HA<sub>306-318</sub> peptide concentration, we see a less significant enhancement in the presence of AE peptide, likely because at higher concentration of peptide, sufficient numbers of peptide/DR1 complexes sufficient for T cell activation were formed (Fig. 5). The above cell experiments clearly illustrated that the presence of AE206 amplifies the T cell responses.

### 3.6. Enhancement of specific T cell response by AE206 *in vivo*

Because of an enhancement in peptide loading on B cells, we sought to determine whether this effect was also reproducible *in vivo*. HLA-DR1 (DR B1\*0101) transgenic mice (Rosloniec et al., 1997) were immunized with 0.1, 1, or 10 nmol HA<sub>306-318</sub> peptide alone, or together with 100 nmol AE206 subcutaneously as described under Section 2. Cells from the draining nodes were assayed for T cell proliferation *in vitro* as previously described (Mirshahidi et al., 2001, 2004). The results, which are shown in Fig. 6, are impressive, indicating that Ii helper peptides when injected together with different quantities of HA<sub>306-318</sub> peptide generated significant increases in immunizing specific T cell response up to 4-fold over groups injected with HA<sub>306-318</sub> peptide alone. Overall, these results clearly demonstrate that small peptides such as AE206 can function similarly to DM and can be used to enhance the efficacy of immunization.

## 4. Discussion

Identification of small molecules and/or peptides to mimic DM functions is of great value for understanding the mechanisms of peptide binding and DM functions, for mapping of DM/MHC-class II interaction sites, and for the enhancement of peptide vaccine efficiency. Several recent studies have focused on the identification and characterization of such molecules (Hopner et al., 2006; Marin-Esteban et al., 2004; Nicholson et al., 2006). In this study, we have provided evidence that several short peptides, derived from the N-terminal sequence of long CLIP, and bearing the core sequence of LRLKLPK, share common characteristics with HLA-DM in regulation of peptide loading to HLA-DR1. We also demonstrate that these short peptides, similar to HLA-DM can preserve the peptide-binding groove

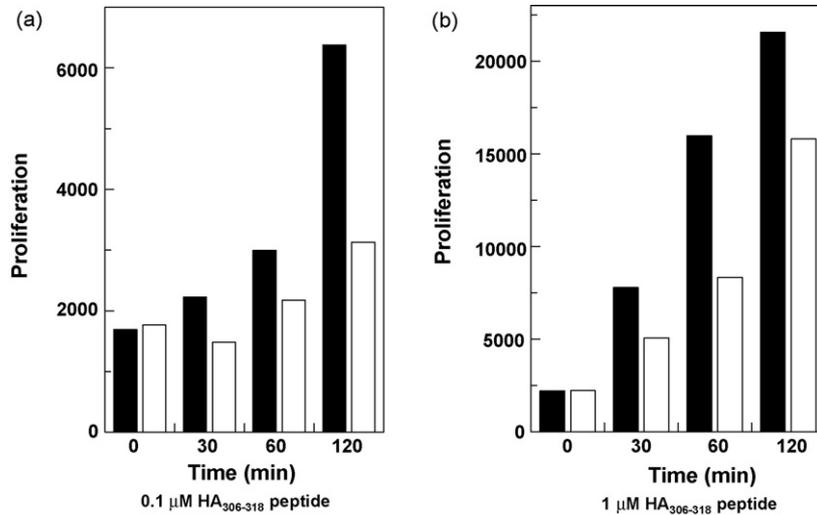


Fig. 5. Increased T cell sensitivity in the presence of AE206. HLA-DR1<sup>+</sup> EBV-B cells were pulsed with either 0.1 μM (a) or 1 μM (b) HA<sub>306-318</sub> peptide in the absence (open bars) or presence (filled bar) of 150 μM AE206 at indicated time points. The specific T cell response was determined by using clone 1 T cell line. EBV-B cells were then washed and used to challenge CL-1 cells. The response was measured by [<sup>3</sup>H]thymidine incorporation. Representative of two independent experiments.

of class II from inactivation at physiological temperatures. As an added bonus, these peptides function at neutral pH, making them excellent candidates for inclusion in peptide vaccine preparations.

HLA-DM has been shown to be in command of several major functions related to MHC class II peptide binding: (1) acceleration of MHC class II/Peptide complex dissociation; (2) generation of peptide-receptive conformation; (3) prevention of denaturation of the peptide-binding groove at physiological temperature; and (4) enhancement of formation of stable peptide/MHC complexes. We have shown that DM can perform these functions by recognizing different conformations

of class II. Class II molecules acquire different conformations based on whether they are empty or are bound to peptides of different sequences. If the conformation of a complex is floppy, i.e., flexible, DM dissociates the complex. However, if the peptide/MHC shapes into a rigid complex, such as HLA-DR1/HA<sub>306-318</sub>, DM can no longer be effective in dissociating them. We have demonstrated this by using a DR1<sub>βG86Y</sub> mutant DR1 that has a filled pocket 1 and has a relatively rigid conformation, as detected by resistance to denaturation by heat and SDS (Natarajan et al., 1999b; Sato et al., 2000). DM does neither enhance binding of peptides to DR1<sub>βG86Y</sub>, nor does it accelerate dissociation of peptide/DR1<sub>βG86Y</sub> complexes. Most available data demonstrate that the interaction of DM with MHC class II is transient (Grotenbreg et al., 2007; Narayan et al., 2007; Zwart et al., 2005). In agreement with those data, by using two different assays, we observed that while AE206 bound minimally to the empty DR1, there was no detectable binding with HA<sub>306-318</sub>/DR1 complexes, or DR1<sub>βG86Y</sub> (data not shown). Thus, all of these characteristics of DM are exactly mimicked by the helper peptides studied here, suggesting that the helper peptides may also function through conformational recognition of MHC class II.

Previously, we have shown that DM converts the biphasic peptide-binding pattern of wtDR1 to the monophasic-binding pattern in peptide binding assays (Chou and Sadegh-Nasseri, 2000; Narayan et al., 2007). In the AE206 study, we found that AE206 also converts the biphasic binding pattern toward a monophasic pattern, although less effectively than DM. Thus, AE206 only facilitates peptide binding to DR1 molecules that have non-receptive conformation. Another main function for DM is to stabilize empty class II molecules. AE206 clearly can stabilize the empty class II molecules and prevent them from functional inactivation. This strongly suggests that DM and the helper peptides may function through the same mechanism.

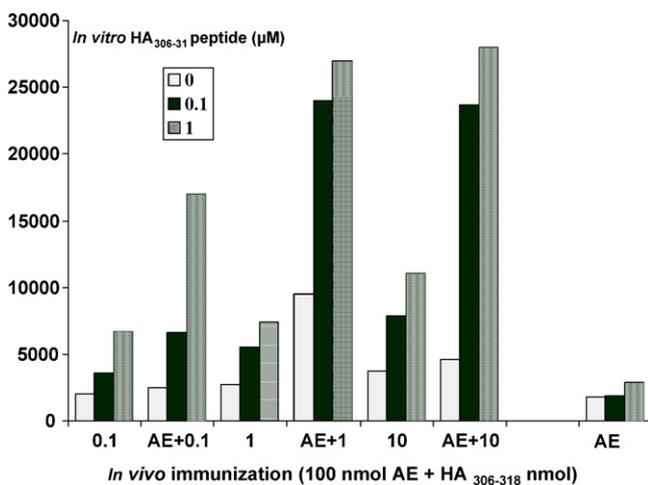


Fig. 6. AE206 peptide enhances T cell responses to HA<sub>306-318</sub> peptide immunization. HLA-DR1 Tg mice were primed with HA<sub>306-318</sub> peptide at nmol quantities shown, alone or together with 100 nmol AE206 at the base of tail. Ten days later, cells from the draining lymph nodes were challenged with 0, 0.1, or 1 μM HA<sub>306-318</sub> peptide *in vitro* and assayed for T cell proliferation by using [<sup>3</sup>H]thymidine incorporation assay. Representative of three independent experiments.

Earlier work (Kropshofer et al., 1995b) indicated an enhancing effect in dissociation of long CLIP that was due to the N-terminal CLIP extension. Although the helper peptides used in this study do not carry the same sequence as N-terminus of CLIP, they do share several common residues. One explanation might be that the N-terminus of CLIP interacts with the region outside of the P1 pocket of the peptide binding groove (Doebele et al., 2000; Pashine et al., 2003) and would interfere with the formation of an H-bond between His 81 and the peptide backbone, leading to destabilization of the peptide (Narayan et al., 2007). This hypothesis might explain peptide dissociation, but it does not offer explanations for other observed effects of helper peptides, such as their effects on the association of peptide with empty DR1, or the maintenance of the peptide-receptive conformation of empty DR molecules. Nevertheless, knowing that such small peptides can effectively enhance peptide exchange and convert MHC molecules into a peptide-receptive conformation is striking. Such functional resemblance suggests that all the different functions of DM are monitored by a single molecular mechanism.

Helper peptides studied here were quite effective at increasing the antigen loading efficiency on HLA-DR1 expressed on EBV B cell lines at neutral pH, and at increasing T cell sensitivity. More important, inclusion of helper peptides instead of DM, together with antigenic peptide led to higher T cell responses. Thus, such peptides can be of great help in any clinical settings whenever efficient peptide exchange is desired. A most outstanding example would be in peptide charging of dendritic cells for priming with cancer peptides.

## Acknowledgements

This work was supported by grants R01GM53549 and R01AI063764 to SS-N.

## References

- Adams, S., Albericio, F., Alsina, J., Smith, E.R., Humphreys, R.E., 1997. Biological activity and therapeutic potential of homologs of an Ii peptide which regulates antigenic peptide binding to cell surface MHC class II molecules. *Arzneimittelforschung* 47, 1069–1077.
- Beeson, C., McConnell, H.M., 1994. Kinetic intermediates in the reactions between peptides and proteins of major histocompatibility complex class II. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8842–8845.
- Belmares, M.P., Busch, R., Mellins, E.D., McConnell, H.M., 2003. Formation of two peptide/MHC II isomers is catalyzed differentially by HLA-DM. *Biochemistry* 42, 838–847.
- Blum, J.S., Cresswell, P., 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. U.S.A.* 85, 3975–3979.
- Castellino, F., Zhong, G., Germain, R.N., 1997. Antigen presentation by MHC class II molecules: invariant chain function, protein trafficking, and the molecular basis of diverse determinant capture. *Hum. Immunol.* 54, 159–169.
- Chou, C.L., Sadegh-Nasseri, S., 2000. HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J. Exp. Med.* 192, 1697–1706.
- Cresswell, P., 1994. Assembly, transport, and function of MHC class II molecules. *Annu. Rev. Immunol.* 12, 259–293.
- De Magistris, M.T., Alexander, J., Coggeshall, M., Altman, A., Gaeta, F.C., Grey, H.M., Sette, A., 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* 68, 625–634.
- Denzin, L.K., Cresswell, P., 1995. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82, 155–165.
- Denzin, L.K., Robbins, N.F., Carboy-Newcomb, C., Cresswell, P., 1994. Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1, 595–606.
- Doebele, R.C., Busch, R., Scott, H.M., Pashine, A., Mellins, E.D., 2000. Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity* 13, 517–527.
- Fung-Leung, W.P., Surh, C.D., Liljedahl, M., Pang, J., Leturcq, D., Peterson, P.A., Webb, S.R., Karlsson, L., 1996. Antigen presentation and T cell development in H2-M-deficient mice. *Science* 271, 1278–1281.
- Green, J.M., DeMars, R., Xu, X., Pierce, S.K., 1995. The intracellular transport of MHC class II molecules in the absence of HLA-DM. *J. Immunol.* 155, 3759–3768.
- Grotenbreg, G.M., Nicholson, M.J., Fowler, K.D., Wilbuer, K., Octavio, L., Yang, M., Chakraborty, A.K., Ploegh, H.L., Wucherpfennig, K.W., 2007. Empty class II major histocompatibility complex created by peptide photolysis establishes the role of DM in peptide association. *J. Biol. Chem.* 282, 21425–21436.
- Hopner, S., Dickhaut, K., Hofstatter, M., Kramer, H., Ruckerl, D., Soderhall, J.A., Gupta, S., Marin-Esteban, V., Kuhne, R., Freund, C., Jung, G., Falk, K., Rotzschke, O., 2006. Small organic compounds enhance antigen-loading of class II MHC proteins by targeting the polymorphic P1 pocket. *J. Biol. Chem.*
- Kallinteris, N.L., Lu, X., Wu, S., Hu, H., Li, Y., Gulfo, J.V., Humphreys, R.E., Xu, M., 2003. Ii-Key/MHC class II epitope hybrid peptide vaccines for HIV. *Vaccine* 21, 4128–4132.
- Kallinteris, N.L., Wu, S., Lu, X., Humphreys, R.E., von Hofe, E., Xu, M., 2005a. Enhanced CD4+ T-cell response in DR4-transgenic mice to a hybrid peptide linking the Ii-Key segment of the invariant chain to the melanoma gp100(48–58) MHC class II epitope. *J. Immunother.* 28 (1997), 352–358.
- Kallinteris, N.L., Wu, S., Lu, X., von Hofe, E., Humphreys, R.E., Xu, M., 2005b. Linkage of Ii-Key segment to gp100(46–58) epitope enhances the production of epitope-specific antibodies. *Vaccine* 23, 2336–2338.
- Korb, L.C., Mirshahidi, S., Ramyar, K., Sadighi Akha, A.A., Sadegh-Nasseri, S., 1999. Induction of T cell anergy by low numbers of agonist ligands. *J. Immunol.* 162, 6401–6409.
- Kropshofer, H., Vogt, A.B., Hammerling, G.J., 1995a. Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8313–8317.
- Kropshofer, H., Vogt, A.B., Stern, L.J., Hammerling, G.J., 1995b. Self-release of CLIP in peptide loading of HLA-DR molecules. *Science* 270, 1357–1359.
- Kropshofer, H., Vogt, A.B., Moldenhauer, G., Hammer, J., Blum, J.S., Hammerling, G.J., 1996. Editing of the HLA-DR-peptide repertoire by HLA-DM. *EMBO J.* 15, 6144–6154.
- Kropshofer, H., Arndt, S.O., Moldenhauer, G., Hammerling, G.J., Vogt, A.B., 1997. HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. *Immunity* 6, 293–302.
- Lazarski, C.A., Chaves, F.A., Jenks, S.A., Wu, S., Richards, K.A., Weaver, J.M., Sant, A.J., 2005. The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance. *Immunity* 23, 29–40.
- Marin-Esteban, V., Falk, K., Rotzschke, O., 2004. Chemical analogues of HLA-DM can induce a peptide-receptive state in HLA-DR molecules. *J. Biol. Chem.* 279, 50684–50690.
- Martin, W.D., Hicks, G.G., Mendiratta, S.K., Leva, H.I., Ruley, H.E., Van Kaer, L., 1996. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell* 84, 543–550.
- Mellins, E., Smith, L., Arp, B., Cotner, T., Celis, E., Pious, D., 1990. Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* 343, 71–74.

- Mirshahidi, S., Huang, C.T., Sadegh-Nasseri, S., 2001. Anergy in peripheral memory CD4(+) T cells induced by low avidity engagement of T cell receptor. *J. Exp. Med.* 194, 719–731.
- Mirshahidi, S., Ferris, L.C., Sadegh-Nasseri, S., 2004. The magnitude of TCR engagement is a critical predictor of T cell anergy or activation. *J. Immunol.* 172, 5346–5355.
- Miyazaki, T., Wolf, P., Tourne, S., Waltzinger, C., Dierich, A., Barois, N., Ploegh, H., Benoist, C., Mathis, D., 1996. Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell* 84, 531–541.
- Narayan, K., Chou, C.L., Kim, A., Hartman, I.Z., Dalai, S., Khoruzhenko, S., Sadegh-Nasseri, S., 2007. HLA-DM targets the hydrogen bond between the histidine at position beta81 and peptide to dissociate HLA-DR-peptide complexes. *Nat. Immunol.* 8, 92–100.
- Natarajan, S.K., Assadi, M., Sadegh-Nasseri, S., 1999a. Stable peptide binding to MHC class II molecule is rapid and is determined by a receptive conformation shaped by prior association with low affinity peptides. *J. Immunol.* 162, 4030–4036.
- Natarajan, S.K., Stern, L.J., Sadegh-Nasseri, S., 1999b. Sodium dodecyl sulfate stability of HLA-DR1 complexes correlates with burial of hydrophobic residues in pocket I. *J. Immunol.* 162, 3463–3470.
- Neeffjes, J.J., Stollorz, V., Peters, P.J., Geuze, H.J., Ploegh, H.L., 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* 61, 171–183.
- Nicholson, M.J., Moradi, B., Seth, N.P., Xing, X., Cuny, G.D., Stein, R.L., Wucherpfennig, K.W., 2006. Small molecules that enhance the catalytic efficiency of HLA-DM. *J. Immunol.* 176, 4208–4220.
- Pashine, A., Busch, R., Belmares, M.P., Munning, J.N., Doebele, R.C., Buckingham, M., Nolan, G.P., Mellins, E.D., 2003. Interaction of HLA-DR with an acidic face of HLA-DM disrupts sequence-dependent interactions with peptides. *Immunity* 19, 183–192.
- Peters, P.J., Neeffjes, J.J., Oorschot, V., Ploegh, H.L., Geuze, H.J., 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349, 669–676.
- Pu, Z., Lovitch, S.B., Bikoff, E.K., Unanue, E.R., 2004. T cells distinguish MHC-peptide complexes formed in separate vesicles and edited by H2-DM. *Immunity* 20, 467–476.
- Romagnoli, P., Germain, R.N., 1994. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J. Exp. Med.* 180, 1107–1113.
- Rosloniec, E.F., Brand, D.D., Myers, L.K., Whittington, K.B., Gumanovskaya, M., Zaller, D.M., Woods, A., Altmann, D.M., Stuart, J.M., Kang, A.H., 1997. An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. *J. Exp. Med.* 185, 1113–1122.
- Rosloniec, E.F., Whittington, K.B., Zaller, D.M., Kang, A.H., 2002. HLA-DR1 (DRB1\*0101) and DR4 (DRB1\*0401) use the same anchor residues for binding an immunodominant peptide derived from human type II collagen. *J. Immunol.* 168, 253–259.
- Sadegh-Nasseri, S., 1994. Peptide, invariant chain, or molecular aggregation preserves class II from functional inactivation. In: Humphreys, R.E., Pierce, S.K. (Eds.), *Antigen Processing and Presentation*, vol. 1. Academic Press, San Diego, pp. 170–187.
- Sadegh-Nasseri, S., Germain, R.N., 1991. A role for peptide in determining MHC class II structure. *Nature* 353, 167–170.
- Sadegh-Nasseri, S., Germain, R.N., 1992. How MHC class II molecules work: peptide-dependent completion of protein folding. *Immunol. Today* 13, 43–46.
- Sadegh-Nasseri, S., Stern, L.J., Wiley, D.C., Germain, R.N., 1994. MHC class II function preserved by low-affinity peptide interactions preceding stable binding. *Nature* 370, 647–650.
- Sato, A.K., Zarutskie, J.A., Rushe, M.M., Lomakin, A., Natarajan, S.K., Sadegh-Nasseri, S., Benedek, G.B., Stern, L.J., 2000. Determinants of the peptide-induced conformational change in the human class II major histocompatibility complex protein HLA-DR1. *J. Biol. Chem.* 275, 2165–2173.
- Stern, L.J., Wiley, D.C., 1992. The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell* 68, 465–477.
- Stratikos, E., Wiley, D.C., Stern, L.J., 2004. Enhanced catalytic action of HLA-DM on the exchange of peptides lacking backbone hydrogen bonds between their N-terminal region and the MHC class II alpha-chain. *J. Immunol.* 172, 1109–1117.
- Ullrich, H.J., Doring, K., Gruneberg, U., Jahmig, F., Trowsdale, J., van Ham, S.M., 1997. Interaction between HLA-DM and HLA-DR involves regions that undergo conformational change at lysosomal pH. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13163–13168.
- Vogt, A.B., Stern, L.J., Amshoff, C., Dobberstein, B., Hammerling, G.J., Kropshofer, H., 1995. Interference of distinct invariant chain regions with superantigen contact area and antigenic peptide binding groove of HLA-DR. *J. Immunol.* 155, 4757–4765.
- Vogt, A.B., Moldenhauer, G., Hammerling, G.J., Kropshofer, H., 1997. HLA-DM stabilizes empty HLA-DR molecules in a chaperone-like fashion. *Immunol. Lett.* 57, 209–211.
- Weber, D.A., Evavold, B.D., Jensen, P.E., 1996. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 274, 618–620.
- Zwart, W., Griekspoor, A., Kuijl, C., Marsman, M., van Rheenen, J., Janssen, H., Calafat, J., van Ham, M., Janssen, L., van Lith, M., Jalink, K., Neeffjes, J., 2005. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity* 22, 221–233.