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A novel method to measure HLA-DM-susceptibility of peptides bound to MHC class II molecules based on peptide binding competition assay and differential IC_{50} determination

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Abstract

HLA-DM (DM) functions as a peptide editor that mediates the exchange of peptides loaded onto MHCII molecules by accelerating peptide dissociation and association kinetics. The relative DM-susceptibility of peptides bound to MHCII molecules correlates with antigen presentation and immunodominance hierarchy, and measurement of DM-susceptibility has been a key effort in this field. Current assays of DM-susceptibility, based on differential peptide dissociation rates measured for individually labeled peptides over a long time base, are difficult and cumbersome. Here, we present a novel method to measure DM-susceptibility based on peptide binding competition assays performed in the presence and absence of DM, reported as a delta-IC_{50} (change in 50% inhibition concentration) value. We simulated binding competition reactions of peptides with various intrinsic and DM-catalyzed kinetic parameters and found that under a wide range of conditions the delta-IC_{50} value is highly correlated with DM-susceptibility as measured in off-rate assay. We confirmed experimentally that DM-susceptibility measured by delta-IC_{50} is comparable to that measured by traditional off-rate assay for peptides with known DM-susceptibility hierarchy. The major advantage of this method is that it allows simple, fast and high throughput measurement of DM-susceptibility for a large set of unlabeled peptides in studies of the mechanism of DM action and for identification of CD4+ T cell epitopes.

Keywords

MHCII-peptide complex; DM-susceptibility; IC_{50}; fluorescence polarization; binding competition; off-rate

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INTRODUCTION

HLA-DM (DM)[4] is a nonclassical MHCII molecule that serves as a peptide editor by mediating the exchange of peptides loading onto MHCII during antigen presentation. DM-mediated peptide exchange has been shown to play a key role in CD4+ T cell epitope selection (Hartman et al., 2010; Lazarski et al., 2006; Lich et al., 2003; Lovitch et al., 2003; Sant et al., 2005; Yin et al., 2012). Measurements of differential DM-susceptibility of various sets of peptides have been crucial in understanding the molecular mechanism of DM-mediated peptide exchange, in identifying CD4+ T cell epitopes, and in improving the efficiency of CD4+ T cell epitope prediction algorithms.

Previously, we and others have measured DM-susceptibility by determining dissociation kinetics of a labeled peptide in the presence of different concentrations of DM, and calculated DM-susceptibility as the slope of the off-rate versus DM concentration curve (Belmares et al., 2002; Hou et al., 2011; Painter et al., 2011; Weber et al., 1996; Yin et al., 2012). This method allows for a direct measurement of peptide off-rate and DM-susceptibility. However, the assay is cumbersome in that each test peptide has to be individually labeled for detection, and difficult in that multiple time points have to be collected for reliable off-rate determination, over a time base that can extend to >10 days for stable peptide complexes as often observed for epitope peptides. These factors limit the application of this assay for measuring DM-susceptibility of a large set of peptides. Moreover, DM has been shown to catalyze peptide association to MHCII molecules, and different peptides might be differentially susceptible to DM-accelerated peptide association, even though the detailed mechanism is still in debate (Grotenbreg et al., 2007; Guce et al., 2013; Pashine et al., 2003; Zarutskie et al., 2001). Nevertheless, the traditional measurement of DM-susceptibility by off-rate has not taken into account of impact of DM on peptide association.

In this study, we developed a novel method to measure DM-susceptibility by assessing the difference of IC_{50} in the absence and presence of DM in conventional peptide competition binding assays. The resultant IC_{50} value correlates with conventional off-rate based measures of DM-susceptibility but also takes into account the effect of DM on both peptide association and dissociation reactions. The method described here allows for a reliable and high throughput measurement of DM-susceptibility for a large set of peptides.

MATERIALS AND METHODS

Peptide synthesis and labeling

Influenza hemagglutinin peptide $HA_{306-318}$ (PKYVKQNTLKLAT), class II-associated invariant chain peptide CLIP (VSKMRMATPLLMQ), human MHCI $A2_{104-117}$ (GSDWRFLRGYHQYA) and its P1 pocket residue substituted mutants (W1I and W1T) were synthesized for IC_{50} assay (21st Century Biochemicals, Marlboro, MA). N-terminally

[4]Abbreviations: DM, HLA-DM; MHCII, major histocompatibility complex class II molecules; IC_{50}, concentration required for 50% inhibition; $IC_{50,DM}$, IC_{50} measured in the presence of DM; $k_{off,DM}$ and k_{off}, DM-catalyzed and intrinsic off-rate respectively; $k_{ass,DM}$ and k_{ass}, DM-catalyzed and intrinsic association rate constant respectively; $k_{dis,DM}$ and k_{dis}, DM-catalyzed and intrinsic dissociation rate constant respectively; FP, fluorescence polarization

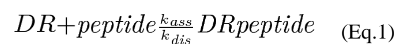
acetylated $HA_{306-318}$ analog peptide (Ac-PRFVKQNTLRLAT) was labeled with Alexa488 tetrafluorophenyl ester (Invitrogen, Eugene, OR) through primary amine of K_5 to be used as the probe peptide in the IC_{50} assay. N-terminal biotin-labeled MHCI $A2_{104-117}$ and its P1 pocket residue substituted mutants, Alexa488-labeled $HA_{306-318}$ and Alexa488-labeled CLIP were used in the dissociation kinetics assay. Labeled peptides were purified by Jupiter C18 reverse-phase chromatography (Phenomenex, Torrance, CA).

DR1 and DM expression and purification

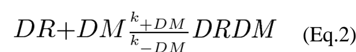
Soluble recombinant MHCII molecules DR1 (HLA-DRA*01:01;DRB1*01:01) and DM were expressed in Drosophila S2 cells and purified by immunoaffinity chromatography followed by Superdex200 (GE Healthcare) size exclusion chromatography as described previously (Busch et al., 1998; Frayser et al., 1999; Stern and Wiley, 1992).

KinTek simulation of peptide association, dissociation, and binding competition reactions

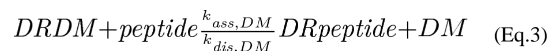
Peptide dissociation, association and binding competition reactions in the absence or presence of DM were simulated with KinTek Explorer (Johnson, 2009; Johnson et al., 2009). Peptides with various intrinsic and DM-catalyzed kinetic parameters were set as the test peptides. The model used to simulate the reactions was described as:



$$DR+peptide \underset{k_{dis}}{\overset{k_{ass}}{\rightleftharpoons}} DRpeptide \quad \text{(Eq.1)}$$



$$DR+DM \underset{k_{-DM}}{\overset{k_{+DM}}{\rightleftharpoons}} DRDM \quad \text{(Eq.2)}$$



$$DRDM+peptide \underset{k_{dis,DM}}{\overset{k_{ass,DM}}{\rightleftharpoons}} DRpeptide+DM \quad \text{(Eq.3)}$$

For intrinsic peptide association and dissociation reactions, Eq. 1 was used. For peptide binding competition reactions, two versions of Eq. 1 were used, with different parameters for each peptide. For DM-catalyzed reactions, Eqs. 2 and 3 also were included. Values used in the simulation for each kinetic parameter for various peptides were: k_{ass} = 0.048–0.114 $\mu M^{-1}min^{-1}$, $k_{ass,DM}$ = 0.114–0.912 $\mu M^{-1}min^{-1}$, k_{dis} = 0.00017–0.0108 min^{-1}, $k_{dis,DM}$ = 0.00043–0.052 min^{-1}, k_{+DM} = 0.0216 $\mu M^{-1}min^{-1}$ and k_{-DM} = 0.216 min^{-1} (detailed values for test peptides shown in Table I). For calculation of IC_{50}, the binding competition simulations using a series of test peptide concentrations were exported and IC_{50} was fitted from the concentration-dependent inhibition curve (using the equation described in the following IC_{50} assay) for each peptide. For calculation of intrinsic (k_{off}) and DM-catalyzed off-rate ($k_{off,DM}$), dissociation simulations of 0.1 μM DR-peptide complex were exported and off-rate was fitted from the dissociation curve using one-phase exponential decay for each peptide.

IC_{50} assay and calculation of DM-susceptibility by IC_{50}

A fluorescence polarization (FP) assay was used to measure the IC_{50} of each peptide, using Alexa488-$HA_{306-318}$ as probe peptide as previously described(Yin et al., 2012). 100 nM

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DR1 was incubated with 25 nM probe peptide Alexa488-$HA_{306–318}$, together with a series dilution of test peptides, starting with 20 μM with a diluting factor of 5. DR1 concentration is set by titrating DR1 against fixed labeled peptide concentration (25 nM), with the selected DR1 concentration which allows for ~50–75% of the maximum binding as judged by FP assay. The starting concentration and dilution factor for serial dilution of target peptides can be changed depending on specific assay configuration. Competition of each test peptide with binding of probe Alexa488-$HA_{306–318}$ to DR1 was measured by FP. FP values were converted to % bound as [(FP_sample-FP_free) / (FP_no_comp – FP_free)] ×100, where FP_sample is the FP values for sample well; FP_free is the FP values for free Alexa488-$HA_{306–318}$; FP_no_comp is the FP values for wells without competitor peptides. Typically FP_free was 70 mP, and FP_no_comp varied with experimental conditions with a maximum of 350 mP for fully bound peptide. We plotted % bound versus concentration of test peptide, and fit the curve to equation $y=1/(1+[pep]/IC_{50})$, where [pep] is the concentration of test peptide, y is the % of probe peptide bound at that concentration of test peptide and IC_{50} is the 50% inhibition concentration of the test peptide. To measure DM-susceptibility, $IC_{50,DM}$ was obtained by including DM in the binding competition assay and IC_{50} was calculated ($IC_{50,DM}$-IC_{50}). DM-susceptibility measured by IC_{50} was calculated as IC_{50}/[DM], where [DM] is the concentration of DM.

Peptide dissociation assay and calculation of DM-susceptibility by off-rate

Peptide dissociation kinetics were measured by europium time-resolved fluorescence after addition of excess unlabeled peptide to purified DR-biotinylated test peptide complexes using an antibody capture assay with streptavidin-Europium detection as previously described (Tompkins et al., 1993; Yin et al., 2012), or measured by FP if the test peptide was labeled with Alexa488 ($HA_{306–318}$ and CLIP in this study) as previously described (Anders et al., 2011; Ferrante et al., 2008; Guce et al., 2013; Painter et al., 2011). DM-susceptibility measured by off-rate was calculated as the slope of off-rate versus DM concentration curve, or as ($k_{off,DM}$-k_{off})/[DM] when only one DM concentration was included, where $k_{off,DM}$ and k_{off} are the off-rates in the presence or absence of DM respectively; and [DM] is the concentration of DM.

RESULTS

Influence of DM on MHCII-peptide binding reactions

DM is required for efficient MHCII-peptide loading in antigen presenting cells (Albert et al., 1998; Morris et al., 1994). *In vitro* DM catalyzes peptide association, dissociation, and exchange reactions (Kropshofer et al., 1996; Morris et al., 1994; Sloan et al., 1995; Weber et al., 1996). Different peptides are differentially susceptible to the action of DM (Belmares et al., 2002; Kropshofer et al., 1996; Weber et al., 1996). The DM susceptibility of a MHCII-peptide complex, usually is measured in a DM-dependent dissociation assay, and characterized as the slope of the linear portion of the off-rate versus DM concentration curve (Yin et al., 2012). DM-dependent peptide dissociation plots and off-rate vs. DM concentration plots are shown in Fig. 1A–C for DR1 complexes of two peptides with different DM-susceptibilities: influenza hemagglutinin derived $HA_{306–318}$ ($HA_{306–318}$) and class II-associated invariant chain $Ii_{105–117}$ peptide (CLIP). $HA_{306–318}$ is a well-

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characterized immunodominant epitope with high affinity to DR1 (Roche and Cresswell, 1990a). The DR1-$HA_{306-318}$ complex has extremely low DM-susceptibility (Ferrante et al., 2008; Ferrante and Gorski, 2010; Joshi et al., 2000; Narayan et al., 2007; Roche and Cresswell, 1990a; Stern et al., 1994; Yin et al., 2012; Zhou et al., 2009). CLIP is the naturally processed remnant of the class II-associated invariant chain chaperone that stabilizes nascent MHCII molecules, with CLIP exchanged for antigenic peptides during epitope selection in antigen presenting cells (Denzin and Cresswell, 1995; Kropshofer et al., 1996; Roche and Cresswell, 1990b; Xu et al., 1995). Although CLIP exhibits similar binding affinity as $HA_{306-318,}$ it has a much higher DM-susceptibility (Anders et al., 2011; Bakke and Dobberstein, 1990; Painter et al., 2011; Roche and Cresswell, 1990b). Consistent with previous studies, $HA_{306-318}$ displayed slower dissociation kinetics compared with CLIP (k_{off} of 0.00026 vs 0.20 hr^{-1}, Fig. 1A and 1B) and lower DM-susceptibility (0.0013 vs 1.43 $hr^{-1} \mu M^{-1}$, Fig. 1C). In general faster dissociating peptides are more susceptible to DM. In early studies it appeared that the ratio between the slope of the DM-susceptibility curve and intrinsic dissociation rate would be constant, however the relationship is now believed to hold only approximately with many outliers (Belmares et al., 2002; Painter et al., 2011; Stratikos et al., 2004; Weber et al., 1996)

In the experiments shown in Fig. 1A–C, peptides were labeled with the fluorophore Alexa488 and dissociation of MHCII-peptide complexes was measured by fluorescence polarization. In previous studies of the dissociation kinetics of these peptides, a variety of fluorophore, biotin, or radioactive labels were used, with dissociation tracked *in situ* by fluorescence polarization or fluorescence resonance energy transfer (FRET) assay, or after separation of bound and free peptide with fluorescence, gamma radiation, scintillation counting, or enzyme-linked assays (De Wall et al., 2006; Kim et al., 2013; Nicholson et al., 2006; Rothbard and Busch, 2001; Sidney et al., 2013; Tompkins et al., 1993; Vollers and Stern, 2008). Peptide association kinetics have been measured using similar techniques (Call et al., 2009; Ferrante et al., 2008; Guce et al., 2013; Joshi et al., 2000; Kropshofer et al., 1996; Nicholson et al., 2006; Painter et al., 2011). In every case, the test peptides need to be individually labeled in order to detect the MHCII-peptide complex, and samples at multiple time points have to be collected to plot the dissociation kinetics curve.

In contrast to studies of MHCII-peptide association and dissociation kinetics, studies of MHCII-peptide affinity often have employed competition assays. In these assays unlabeled test peptides compete for MHCII binding with a labeled probe peptide. FP-based competition assays have been used widely to measure peptide binding affinities to HLA-DR molecules (De Wall et al., 2006; Ferrante and Gorski, 2010; Ferrante and Gorski, 2012; Guce et al., 2013; Nastke et al., 2012; Nicholson et al., 2006; Pos et al., 2012; Yin et al., 2012; Zhou et al., 2009). The major advantage of this method is that only the probe peptide has to be labeled, which makes the measurement for a large set of unlabeled target peptides possible. The relative binding affinities of peptides as measured in competition assays usually are reported as IC_{50} values, i.e. the concentration of test peptide needed to inhibit 50% of the binding of probe peptide. Under certain conditions IC_{50} values can be related to equilibrium binding affinities (K_D) using a simple relationship $K_{D,test} = IC_{50,test}/(1+[probe]/K_{D,probe}$ (Cheng and Prusoff, 1973), but under the conditions typically used for FP

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assays and constraints of MHCII-peptide association/dissociation kinetics the relationship is considerably more complicated (McFarland and Beeson, 2002; Munson and Rodbard, 1988; Nikolovska-Coleska et al., 2004).

We expected that an IC_{50} assay might be useful in measurements of DM-susceptibility, with DM influencing the IC_{50} value more for DM-susceptible peptides than for DM-resistant peptides. DM previously has been shown to influence IC_{50} of peptides based their sensitivities to DM, in studies using size-exclusion chromatography (Kropshofer et al., 1996). We measured IC_{50} for unlabeled $HA_{306–318}$ and CLIP in the absence or presence of DM after 24 hours of reaction using Alexa488-labeled $HA_{306–318}$ as the probe in a FP assay (Fig. 1D–E). DM had little impact on the IC_{50} of $HA_{306–318}$ (0.074 and 0.079 μM without or with DM respectively, Fig. 1D). In the absence of DM the IC_{50} value for CLIP was 0.046 μM, similar to that of $HA_{306–318}$. However, in the presence of DM the IC_{50} value for CLIP was substantially increased to 0.15 μM, for a IC_{50} of 0.1 μM (Fig. 1E). Considering the long half-life of $HA_{306–318}$ bound to DR1, we also performed the binding competition assay after 72 hours incubation. Consistently, we observed that DM had little effect on the IC_{50} of $HA_{306–318}$ (Fig. 1F), but a IC_{50} of 0.54 μM was observed for CLIP (Fig. 1G). DM catalyzes peptide dissociation in a dose-dependent manner (Fig. 1A–1C). We evaluated whether DM also influences IC_{50} in a dose-dependent manner by including a series of concentrations of DM in the binding competition assay (Fig. 1H–1I). We found that similar to the effect of DM on peptide dissociation, DM increased IC_{50} in a dose-dependent manner, with CLIP having a much greater slope (1.57) in the IC_{50} versus DM concentration curve compared with $HA_{306–318}$ (0.06) (Fig. 1J). Thus, our data suggested that DM influences IC_{50} and IC_{50} could be used to measure DM-susceptibility. DM-susceptibility measured by off-rate is characterized as the slope of the linear portion of the off-rate versus DM concentration curve. In this linear portion (usually DM concentration less than 1 μM, i.e. Fig. 1C), this slope can be simplified as $(k_{off,DM}-k_{off})/[DM]$ when only one DM concentration was included, where $k_{off,DM}$ and k_{off} are the off-rates in the presence or absence of DM respectively; and [DM] is the concentration of DM. This simplification is used in previous studies of DM kinetics and epitope selection (Belmares et al., 2002; Ferrante and Gorski, 2010; Hall et al., 2002; Kropshofer et al., 1996; Narayan et al., 2007; Schafer et al., 1996; Sloan et al., 1995; Yin et al., 2012; Zarutskie et al., 2001). We will also use this simplification in this study for Fig. 3–6. For DM-susceptibility measured by IC_{50} assay, as we demonstrated in Fig. 1J, IC_{50} versus DM concentration curve was also linear in the range of DM concentrations tested. Therefore, like using $(k_{off,DM}-k_{off})/[DM]$, $IC_{50}/[DM]$ is a reasonable simplification for the slope of IC_{50} versus DM concentration curve when only one specific DM concentration is performed as a measure for DM-susceptibility.

Numerical simulation of binding reactions

We used a computational approach to help understand the relationship between DM susceptibility and IC_{50} values, We simulated intrinsic and DM-catalyzed peptide dissociation and association reactions in the KinTek modeling program (Johnson, 2009; Johnson et al., 2009) using a simplified reaction scheme (see *Materials and Methods*, Fig. 2A–D) and values for intrinsic association (k_{ass}=0.114 $\mu M^{-1} min^{-1}$) and dissociation (k_{dis}=0.0027 min^{-1}) rate constants. We simulated a binding competition reaction (Fig. 2E–

F) as two concurrent binding reactions, with k_{ass} of 0.048 and 0.114 $\mu M^{-1}min^{-1}$, and k_{dis} of 0.00017 and 0.0027 min^{-1} for probe and test peptide, respectively. Previous studies of peptide binding and dissociation kinetics have provided values in this range. For peptide dissociation reactions, half-times of ~5 hours for CLIP and ~200 hours for $HA_{306-318}$ have been reported (Belmares et al., 2002; Ferrante et al., 2008; Ferrante and Gorski, 2010; Joshi et al., 2000; Kropshofer et al., 1996; Narayan et al., 2007; Pashine et al., 2003; Sloan et al., 1995; Stratikos et al., 2004; Weber et al., 1996; Zarutskie et al., 2001; Zhou et al., 2009) corresponding to k_{dis} values of 0.002 min^{-1} and 0.00006 min^{-1}, respectively. For peptide association reactions, the situation is more complicated because multiple intermediates are involved, including peptide-receptive and peptide-averse MHCII species (Natarajan et al., 1999; Rabinowitz et al., 1998) and open and closed states of the MHCII-peptide complex (Joshi et al., 2000). By combining rate constants for individual steps and estimates of the peptide-receptive fraction we can estimate k_{ass} values for $HA_{306-318}$ and CLIP as ~0.05 and ~0.1 $\mu M^{-1}min^{-1}$ respectively (Joshi et al., 2000; Zarutskie et al., 2001). Another study determined k_{ass} as 0.1 $\mu M^{-1}min^{-1}$ for a fluorescence-labeled myelin basic protein-derived peptide by computationally fitting experimental data into a kinetic model for peptide exchange (Grotenbreg et al., 2007).

Fig. 2 shows calculated concentrations of peptide-free DR, DRpeptide, DRprobe and DRtest for simulated dissociation (Fig. 2A), association (Fig. 2C), and competition binding (Fig. 2E) reactions. We modeled the effect of DM by including additional reaction intermediates: a DM-bound form of the DR-peptide complex with increased peptide dissociation ($k_{dis,DM}$), and a DM-bound form of DR with increased peptide association ($k_{ass,DM}$) (Fig. 2B and 2D) (Pashine et al., 2003; Zarutskie et al., 2001). In the peptide binding competition simulation, we used values for $k_{ass,DM}$ of 0.192 and 0.228, $k_{dis,DM}$ of 0.00043 and 0.013 for probe and test peptide, respectively. In the simulations, DM increased the rates of peptide binding, release, and exchange (Fig. 2B, 2D and 2F). Notably, DM changed the competition profile in terms of how much DRprobe relative to DRtest was formed (Fig. 2F). Similar curve shapes were observed when intrinsic and DM-dependent kinetic values were varied in a reasonable range (data not shown).

Relationship of IC_{50} and DM susceptibility

To obtain IC_{50} values from the simulations, multiple concentrations of test peptide were included in competition binding reactions, and DRprobe was plotted against concentration of test peptide with IC_{50} value determined by curve fitting (Fig. 3). To test our hypothesis that IC_{50} could be used to measure DM-susceptibility, we simulated binding competition reaction for a DM-susceptible test peptide 1 (k_{ass}=0.114 $\mu M^{-1}min^{-1}$, $k_{ass,DM}$=0.228 $\mu M^{-1}min^{-1}$, k_{dis}=0.00027 min^{-1}, $k_{dis,DM}$=0.013 min^{-1}, and calculated $k_{off,DM}$-k_{off} equals 0.0032 min^{-1}, Table I) and a DM-resistant peptide 2 (k_{ass}=0.048 $\mu M^{-1}min^{-1}$, $k_{ass,DM}$=0.192 $\mu M^{-1}min^{-1}$, k_{dis}=0.00017 min^{-1}, $k_{dis,DM}$=0.00043 min^{-1}, and calculated $k_{off,DM}$-k_{off} equals 0.0001 min^{-1}, Table I), with peptides 1 and 2 having similar K_D (k_{dis}/k_{ass} ~ 3 nM). We simulated the binding competition reactions with or without DM and calculated IC_{50} values. In the absence of DM, peptide 1 competed binding with probe peptide in a concentration-dependent manner and remained bound due its slow k_{off} (Fig. 3A and 3C). In the presence of DM, the competition capacity of peptide 1 was weakened due to its high DM-

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susceptibility, and a large fraction of peptide 1 dissociated from DR1 resulting in increased binding of probe peptide and higher IC_{50} (Fig. 3B and 3C). The influence of DM on IC_{50} for peptide 1 was observed at different time points (Fig. 3D). In sharp contrast, for the DM-resistant peptide 2 no difference for IC_{50} was observed with or without DM (Fig. 3E–H). These data indicated that IC_{50} could be used to measure DM-susceptibility.

IC_{50} reports the DM-susceptibility for peptides with a wide range of kinetic parameters

To further test the hypothesis that IC_{50} values could be used to monitor DM susceptibility, we simulated the reactions for a set of peptides (peptides 3, 4, 5 and 6), which had equal k_{ass}, $k_{ass,DM}$ and k_{dis}, but various $k_{dis,DM}$ (Table I). Intrinsic (k_{off}) and DM-catalyzed off-rates ($k_{off,DM}$) were calculated by simulating dissociation reactions of 0.1 μM DR-peptide complex using the input k_{ass}, $k_{ass,DM}$, k_{dis} and $k_{dis,DM}$ rate constants for each peptide in the absence (k_{off}) or presence of 0.25 μM DM ($k_{off,DM}$) and fitting the dissociation curves with one-phase exponential decay (Fig. 4A). Again, we found that IC_{50} correlated with $k_{off,DM}$-k_{off} (Fig. 4B). For a set of peptides (peptides 7, 8, 9 and 10) with same k_{ass} and $k_{ass,DM}$, but different k_{dis} and $k_{dis,DM}$ (Fig. 4C, Table I), the correlation between IC_{50} and $k_{off,DM}$-k_{off} still held (Fig. 4D). Taken together, these simulations demonstrate that IC_{50} could be a reliable measure of DM-susceptibility for peptides with various kinetic parameters.

Although most studies of DM have focused on DM-mediated peptide dissociation, several studies have clearly demonstrated a role of DM on catalyzing peptide loading onto MHCII molecules during antigen presentation and epitope selection (Ferrante et al., 2008; Grotenbreg et al., 2007; Guce et al., 2013; Nicholson et al., 2006; Zarutskie et al., 2001). Therefore, we examined whether IC_{50} could capture the effect of DM on peptide association. We first simulated the binding competition reactions with or without DM for a set of peptides (peptides 11, 12, 13 and 14) with same k_{ass}, k_{dis} and $k_{dis,DM}$, but different $k_{ass,DM}$ (Table I). As shown, IC_{50} was negatively correlated with $k_{ass,DM}$ (Fig. 4E). To test the effect of DM on both peptide dissociation and association, we simulated the binding competition reactions for two additional sets of peptides. Set 1 (peptides 3, 4, 5 and 6) had the same $k_{ass,DM}$ of 0.228 $\mu M^{-1}min^{-1}$, but various $k_{off,DM}$-k_{off} (same in Fig. 4A–B). Set 2 (peptides 15, 16, 17 and 18) had the same $k_{ass,DM}$ of 0.114 $\mu M^{-1}min^{-1}$, but various $k_{off,DM}$-k_{off} (Table I). Interestingly, we found that IC_{50} correlates with $k_{off,DM}$-k_{off} for both sets of peptides, but IC_{50} for set 2 is systemically higher than that of set 1 due to its lower $k_{ass,DM}$ (Fig. 4F). These simulation data demonstrate that IC_{50} represents a measurement of DM-susceptibility of peptides that takes into account the catalytic effect of DM on both peptide association and dissociation reactions.

DM-susceptibility measured by IC_{50} assay correlates with that measured by dissociation assay

To experimentally test the hypothesis that IC_{50} is a reliable measure of DM-susceptibility, we measured DM-susceptibility by IC_{50} for a set of peptides based on the immunodominant alloantigen $A2_{104–117}$ (derived from HLA-A2) and compared these values to DM-susceptibility measured conventionally by off-rate analysis. To generate peptides with increased DM-susceptibility, we substituted the P1 pocket residue of $A2_{104–117}$ from tryptophan to isoleucine (W1I) or to threonine (W1T), because the P1 pocket residue is one

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of the major anchor residues (Murthy and Stern, 1997; Stern et al., 1994) and has been implicated in determining DM-susceptibility (Anders et al., 2011; Narayan et al., 2009; Pos et al., 2012; Schulze and Wucherpfennig, 2012; Yin and Stern, 2013). As expected, $A2_{104-117}$, W1I and W1T all showed a concentration-dependent inhibition on the binding of probe peptide Alexa488-$HA_{306-318}$ to DR1, with $A2_{104-117}$ having the highest affinity and W1T the lowest (Fig. 5A–C). Notably, DM exerted an influence on the IC_{50} of each peptide, and DM-susceptibility calculated by IC_{50} of $A2_{104-117}$, W1I and W1T exhibited the expected hierarchy (Fig. 5D). To confirm the accuracy of this IC_{50}-based method, we also measured the dissociation kinetics for each peptide bound to DR1 (Fig. 5E–G), and calculated the DM-susceptibility by off-rate, which showed consistent hierarchy as that measured by IC_{50} (Fig. 5H). The correlation coefficient of DM-susceptibility measured by IC_{50} and off-rate was 0.99 (data not shown).

Hierarchy of DM-susceptibility measured by IC_{50} is independent of time of detection over a wide range of assay times

To be noted, IC_{50} measurement both in the absence or presence of DM is dependent on the time of detection, especially for peptides with higher DM-susceptibility and early detection times (Fig. 3D and 3H). This is due to the fact that probe peptide and test peptide have different association and dissociation kinetics. To test the influence of time of detection on DM-susceptibility measured by IC_{50}, we read the FP at different time points and calculated IC_{50} and DM-susceptibility (Fig. 6). As shown, although IC_{50} in the absence or presence of DM changed during the time course of over 300 hours, the hierarchy of DM-susceptibility measured by IC_{50} of these tested peptides remained the same.

DISCUSSION

DM-mediated peptide exchange plays a key role in MHCII antigen presentation (Amria et al., 2008; Lazarski et al., 2006) and CD4+ T cell epitope selection (Hartman et al., 2010; Kremer et al., 2012; Lazarski et al., 2005; Sant et al., 2005; Yin et al., 2012). In this study, we developed a novel IC_{50}-based method to measure DM-susceptibility. The underlying principle for this measurement is that the DM-susceptibility of a test peptide would be reflected by its differential ability to inhibit binding of probe peptide to MHCII in the presence or absence of DM. In the FP-based binding competition assay, labeled probe peptide and unlabeled test peptide compete for binding to MHCII. If test peptide bound to MHCII is more susceptible to DM, it will dissociate more easily and allow for more labeled probe peptide to bind. Instead, if test peptide bound to MHCII is resistant to DM, it will stay bound and prevents more labeled probe peptide to bind. This difference is reflected in IC_{50} in the absence or presence of DM. Using both numerical simulation and experimental data, we showed that DM-susceptibility measured by IC_{50} was comparable with that measured by traditional kinetic off-rate analysis.

In this study we used a simplified reaction scheme to simulate peptide association, dissociation and binding competition reactions (Fig. 2–4). Some previous studies have suggested that these reactions may be more complex than modeled. For instance, it has been demonstrated that MHCII molecules undergo a reversible isomerization between peptide-

receptive and peptide-averse states, and DM may catalyze peptide association by accelerating the transition of peptide-averse to peptide-receptive conformation, or stabilizing the peptide-receptive form (Grotenbreg et al., 2007; Natarajan et al., 1999; Rabinowitz et al., 1998). Moreover, multiple intermediates formed between peptide, MHCII and DM during peptide association, dissociation and exchange have been proposed although the detailed mechanisms are still in debate (Anders et al., 2011; Ferrante et al., 2008; Grotenbreg et al., 2007; Narayan et al., 2009; Pashine et al., 2003; Pos et al., 2012; Zarutskie et al., 2001). It is possible that reaction steps not included in our simulation reaction scheme might put some constraints on the interpretation on the IC_{50} data. Nevertheless, simulations using the reasonably simplified reactions demonstrated that DM-susceptibility could be reliably measured by IC_{50}, which is confirmed by our experimental evaluations.

Measuring IC_{50} by a FP-based method has been a standard protocol in determining MHCII-peptide interactions (De Wall et al., 2006; Ferrante and Gorski, 2012; Guce et al., 2013; Pos et al., 2012; Yin et al., 2012; Zhou et al., 2009). By incorporating DM into the binding competition reaction, we developed a novel IC_{50}-based method to measure DM-susceptibility. Compared with the previous used kinetic method, the major advantage of the method described in this study is only a single probe peptide needs to be labeled and only a single final read is needed, allowing for measurement of DM-susceptibility of many peptides at the same time. The protocol we described here uses 96-well plate, but should be easily converted to 384-well or 1536-well format for screening of peptides with higher or lower DM-susceptibility, because the basic FP measurement is concentration- and volume-independent. Another advantage of this method to measure DM-susceptibility is that it considers the catalyzing effect of DM on both peptide association and dissociation, which is important in antigen presentation and epitope selection. In this protocol, we used a well-characterized and widely-used $HA_{306-318}$ derived peptide as the probe peptide, which has high affinity, high kinetic stability, and low DM-susceptibility ($k_{off,DM}$-k_{off} =0.0001 min^{-1}). However, when we changed the $k_{off,DM}$-k_{off} of probe peptide to 0.1 min^{-1}, the correlation between IC_{50} and $k_{off,DM}$-k_{off} of test peptides still held, which indicated that having a probe peptide with low DM-susceptibility is not necessary for using IC_{50} as a measure of DM-susceptibility. This might be important for applying this method to other MHCII alleles, for which a probe peptide with low DM-susceptibility may not be established. One potential disadvantage of this protocol is that it only measures relative DM-susceptibility from binding competition with the probe peptide, instead of absolute DM-susceptibility calculated from dissociation kinetics. However, in most cases, we are interested in screening out peptides with lowest DM-susceptibility to identify epitopes, and measurement of the relative DM-susceptibility is sufficient for this goal.

Epitope prediction algorithms have been widely used to help identify CD4 T cell epitopes from various pathogens (Borras-Cuesta et al., 2000; Calvo-Calle et al., 2007; Doolan et al., 2003; Wang et al., 2010). Most current CD4+ T cell epitope prediction algorithms are based explicitly on measurements of peptide binding affinity to MHCII (Hammer et al., 1992; Peters et al., 2005), with no consideration of the effect of DM. We have previously demonstrated that DM susceptibility is a strong and independent factor governing peptide immunogenicity and epitope selection (Yin et al., 2012). Therefore, the method developed here capable of measuring DM-susceptibility for a large set of peptides might be useful in

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training the prediction algorithms to account for DM effects, and should be directly useful in predicting epitopes based on differential DM-susceptibilities of tested peptides.

In summary, in this study we describes a novel IC_{50}-based protocol for reliable, fast, simple and high throughput measurement of DM-susceptibility of MHCII-peptide complexes, which will facilitate our understanding of the mechanism of DM-mediated peptide exchange and improve our ability to screen and predict CD4+ T cell epitopes.

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Highlights

- IC_{50} is a fast, reliable and high-throughput measure of MHCII DM-susceptibility
- IC_{50} correlates with DM-susceptibility measured conventionally by off-rate
- IC_{50} accounts for the effect of DM in both peptide association and dissociation

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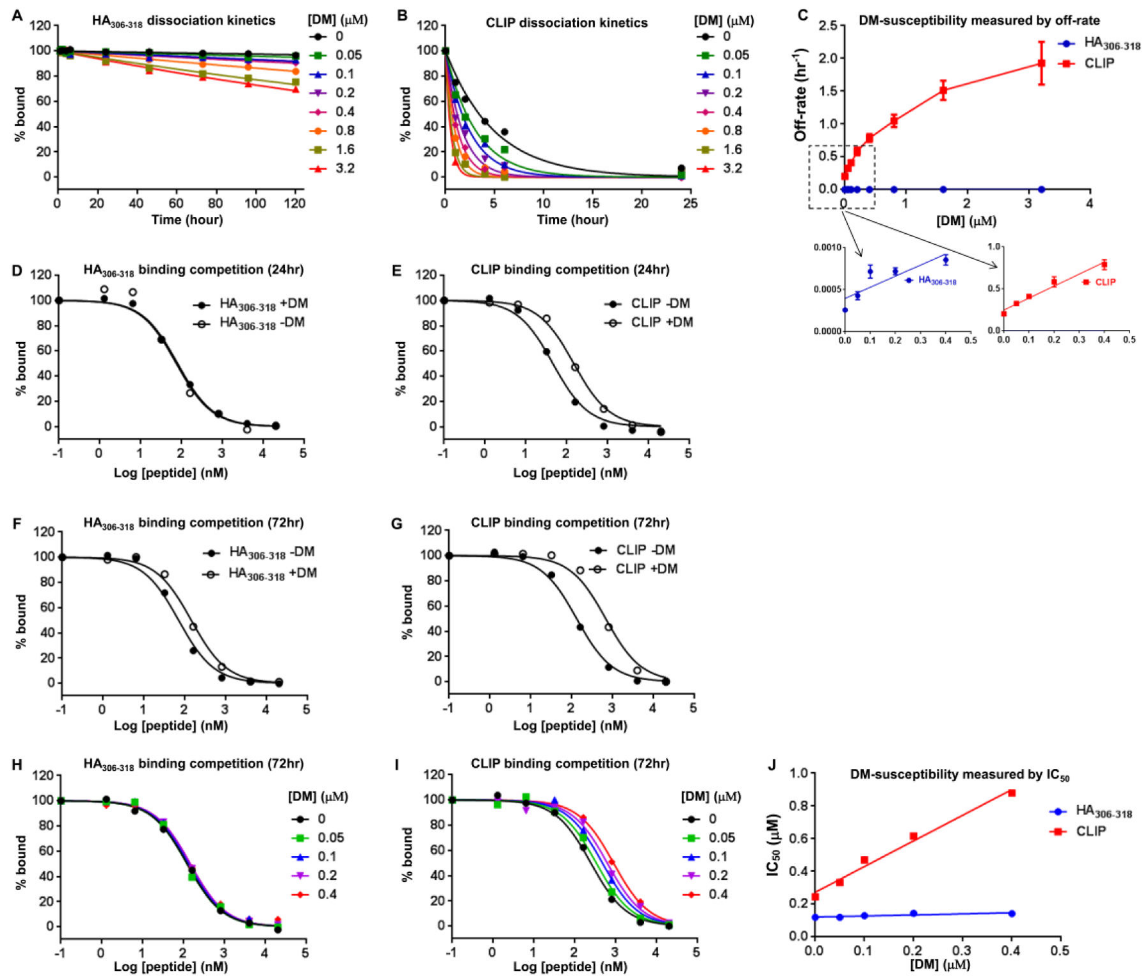

FIGURE 1. DM-susceptibility measured by off-rate and influence of DM on IC_{50}

(**A, B**) Dissociation kinetics of 0.1 μM (**A**) DR1-$HA_{306–318}$ or (**B**) DR1-CLIP were measured in the absence or presence of various concentrations of DM. (**C**) The off-rate versus DM concentration of $HA_{306–318}$ and CLIP bound to DR1 were plotted. The linear range of this plot was amplified and DM-susceptibility measured by off-rate was calculated as the slope. (**D–G**) IC_{50} values in the absence or presence of 0.25 μM DM were measured for (**D**) $HA_{306–318}$, and (**E**) CLIP after incubation at 37 °C for 24 hours, and (**F**) $HA_{306–318}$, and (**G**) CLIP after incubation at 37 °C for 72 hours. Alexa488-$HA_{306–318}$ was used as the probe peptide. These data represent at least three independent experiments with two replicates each. (**H, I**) Binding competition assay in the presence of a series concentration of DM for (**H**) $HA_{306–318}$, and (**I**) CLIP after incubation at 37 °C for 72 hours. (**J**) IC_{50} versus DM concentration was plotted for $HA_{306–318}$ and CLIP.

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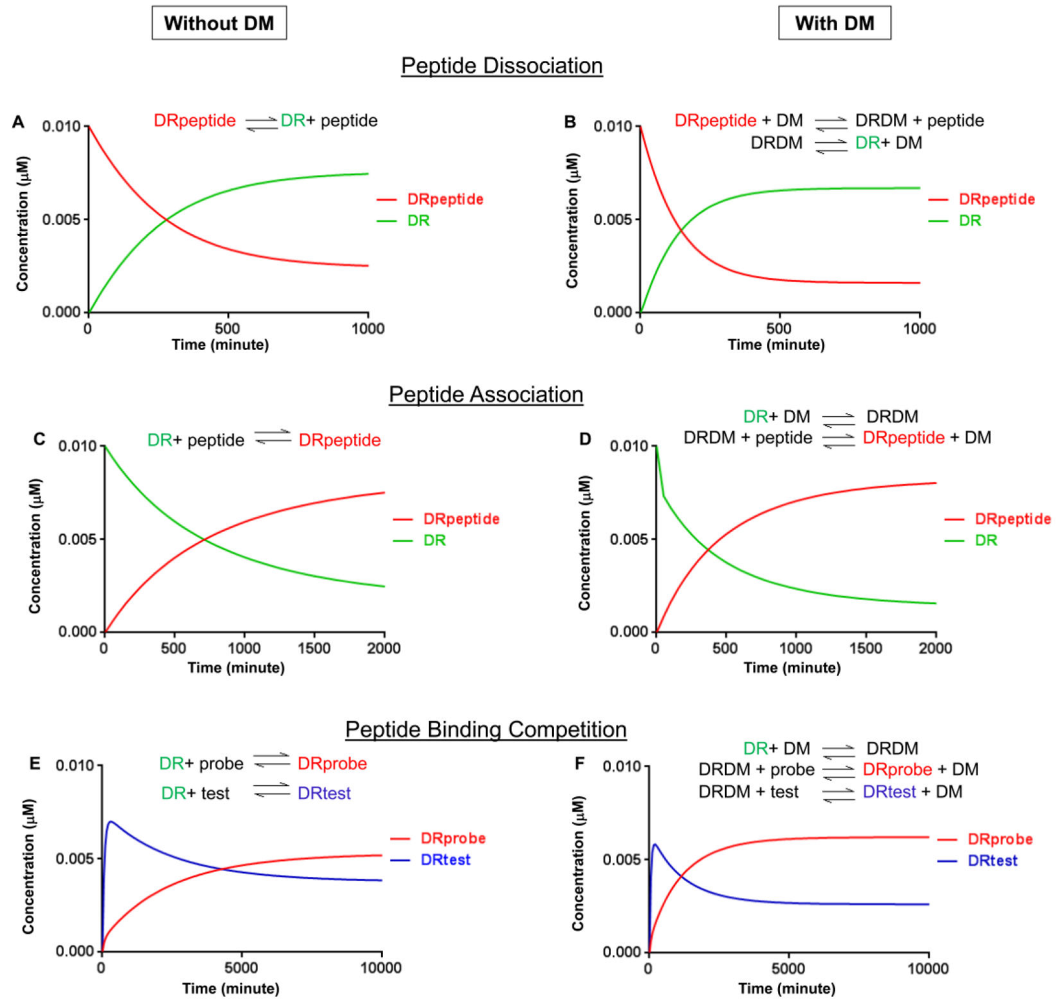

FIGURE 2. Simulation of peptide dissociation, peptide association, and peptide binding competition in the absence or presence of DM

(**A, B**) Simulation of 0.01 μM DRpeptide dissociation (**A**) without, or (**B**) with 0.25 μM DM. (**C, D**) Simulation of 0.025 μM peptide association with 0.01 μM DR (**C**) without or (**D**) with 0.25 μM DM. (**E, F**) Simulation of competition of 0.025 μM test peptide with 0.025 μM probe peptide for the binding to 0.01 μM DR (**E**) without DM or (**F**) with 0.25 μM DM. The equations for each reaction have been indicated, and the simulated values for peptide-bound and peptide-free DR species in each reaction have been plotted.

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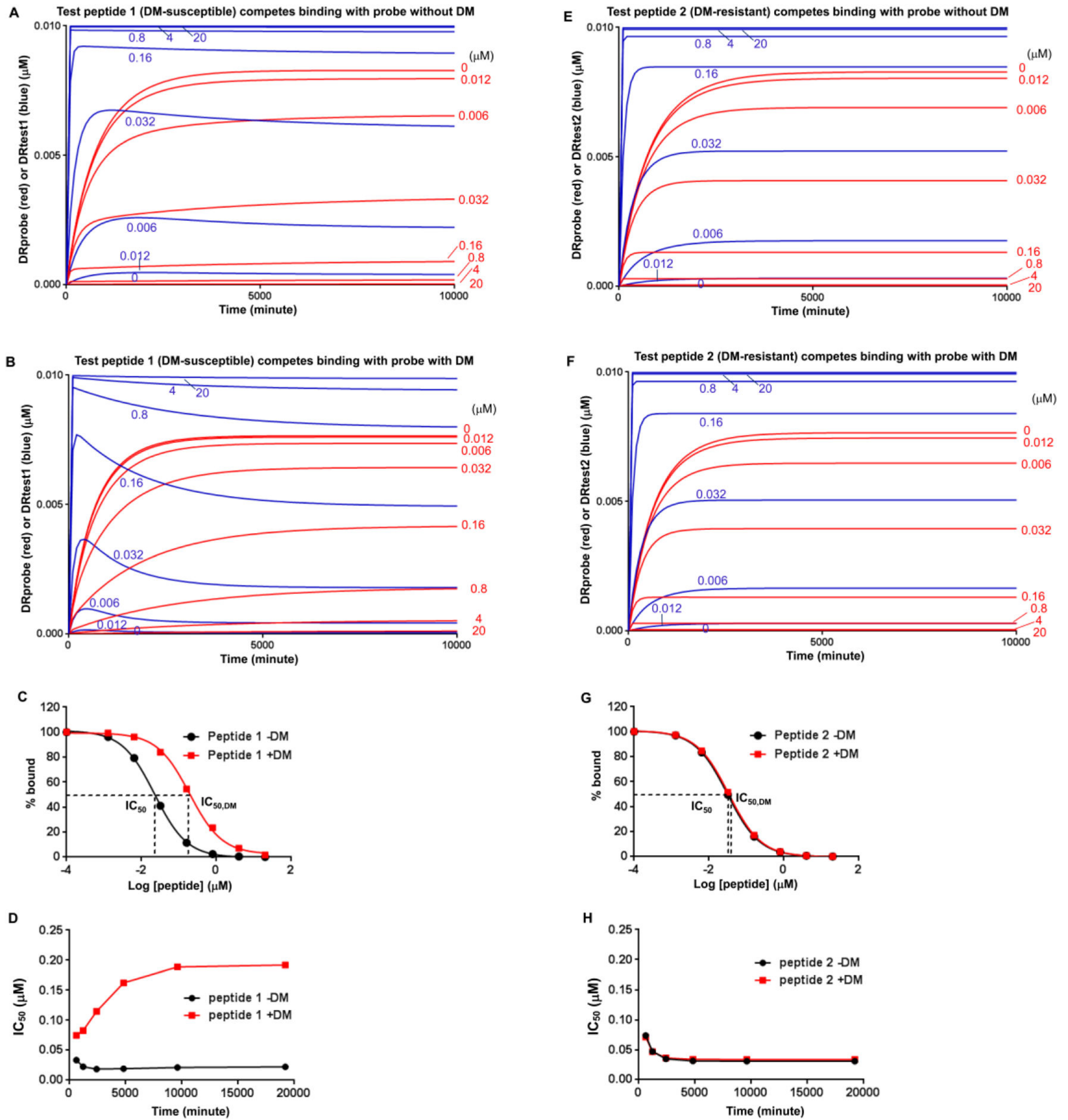

FIGURE 3. Significant difference in IC_{50} value (IC_{50}) is observed for DM-susceptible peptide but not for DM-resistant peptide

(**A, B**) Competition of test peptide 1 (DM-susceptible) with 0.025 μM probe peptide for binding of 0.01 μM DR (**A**) without DM, or (**B**) with 0.25 μM DM. Test peptide 1 was included as a 5-fold dilution series from 20 to 0.012 μM. Concentration of complex formed with probe peptide (DRprobe, red) and test peptide 1 (DRtest1, blue) at different initial concentrations of test peptide 1 are shown. (**C**) Concentration-dependent inhibition plots for test peptide 1 without or with 0.25 μM DM using 19200 minute data from panels (**A**) and (**C**). (**D**) IC_{50} without or with 0.25 μM DM shown for various time points. (**E–H**) Competition of test peptide 2 (DM-resistant).

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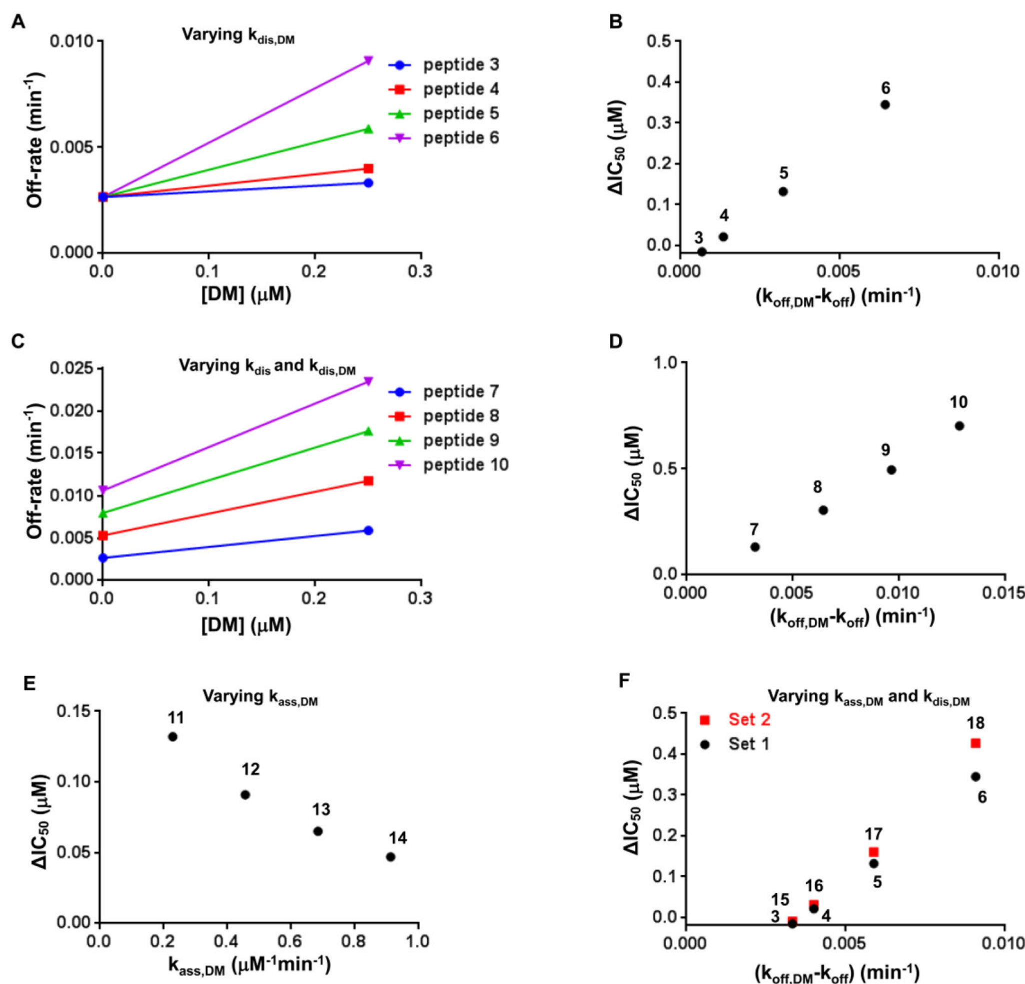

FIGURE 4. IC_{50} is a reliable measure of DM-susceptibility for peptides with various kinetic parameters

(**A**) Intrinsic (k_{off}) and DM-catalyzed off-rates ($k_{off,DM}$) were calculated by simulating dissociation reactions of 0.1 μM DR-peptide complex using the input k_{ass}, $k_{ass,DM}$, k_{dis} and $k_{dis,DM}$ rate constants for each peptide in the absence (k_{off}) or presence of 0.25 μM DM ($k_{off,DM}$) and fitting the dissociation curves with one-phase exponential decay. Simulated reactions for peptides 3, 4, 5 and 6 used the same k_{ass}, $k_{ass,DM}$, k_{dis}, but various $k_{dis,DM}$. (**B**) Calculated IC_{50} correlated with calculated $k_{off,DM}$-k_{off} of peptides 3, 4, 5 and 6. (**C**) Simulated reactions for peptides 7, 8, 9 and 10 used the same k_{ass}, $k_{ass,DM}$, various k_{dis} and $k_{dis,DM}$. (**D**) Calculated IC_{50} correlated with calculated $k_{off,DM}$-k_{off} of peptides 7, 8, 9 and 10. (**E**) Simulated reactions for peptides 11, 12, 13 and 14 used the same k_{ass}, k_{dis}, $k_{dis,DM}$, but various $k_{ass,DM}$. Calculated IC_{50} negatively correlated with $k_{ass,DM}$ for these peptides. (**F**) Set 1 (peptides 3, 4, 5 and 6, black circle) had the same k_{ass}, $k_{ass,DM}$, and k_{dis}, but various $k_{dis,DM}$. Set 2 (peptides 15, 16, 17 and 18, red square) had the same k_{ass}, $k_{ass,DM}$, and k_{dis}, but various $k_{dis,DM}$. Set 1 had a $k_{ass,DM}$ of 0.228 $\mu M^{-1}min^{-1}$, while set 2 had a $k_{ass,DM}$ of 0.114 $\mu M^{-1}min^{-1}$. Calculated IC_{50} correlated with calculated $k_{off,DM}$-k_{off} of set 1 and set 2 peptides, but IC_{50} of set 2 was systemically higher than that of set 1.

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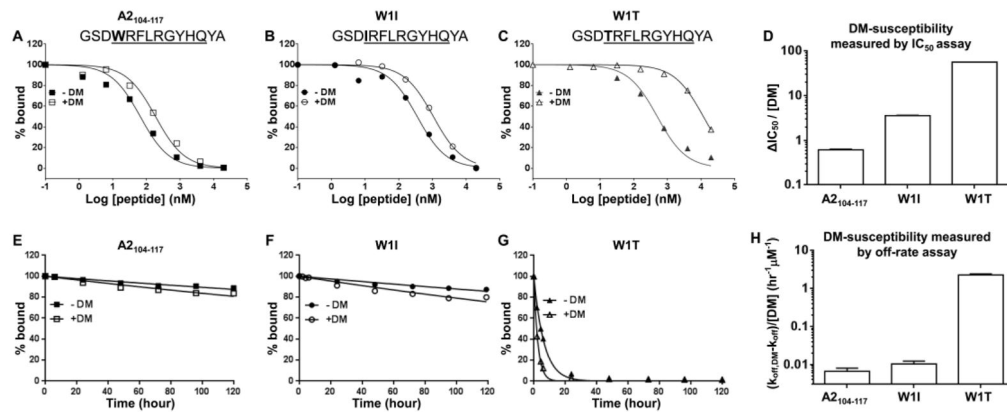

FIGURE 5. DM-susceptibility measured by IC_{50} correlates with that calculated by off-rate
(**A–C**) IC_{50} measurements for in the absence or presence of 0.2 μM DM measured for (**A**) $A2_{104–117}$, and its pocket 1 substituted variants (**B**) W1I, and (**C**) W1T. The sequence of each peptide is indicated with peptide binding motif underlined and P1 pocket residue highlighted in bold. This assay was read at 24 hours after incubation at 37 °C. Alexa488-$HA_{306–318}$ was used as the probe peptide. (**D**) DM-susceptibility by IC_{50} for each peptide was calculated as IC_{50} divided by DM concentration. (**E–G**) Dissociation kinetics measurements for 0.1 μM DR1 bound with (**E**) $A2_{104–117}$, (**F**) W1I, and (**G**) W1T measured in the absence or presence of 0.1 μM DM. (**H**) DM-susceptibility by off-rate was calculated for each peptide, using function $(k_{off,DM}-k_{off})/[DM]$. These data are representative of two independent experiments with at least two replicates each.

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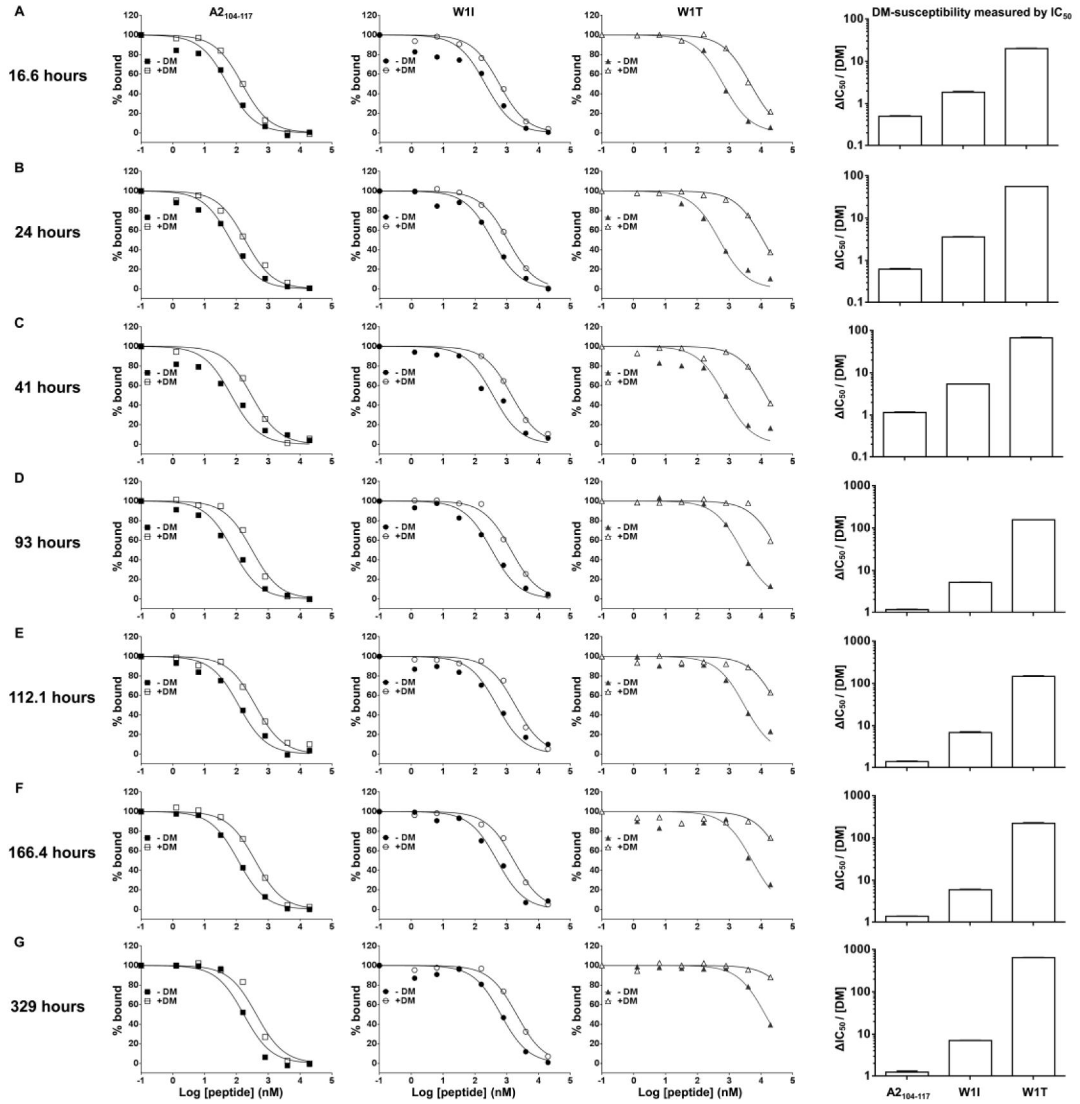

FIGURE 6. Hierarchy of DM-susceptibility measured by IC$_{50}$ is independent of time of detection over a long period

IC$_{50}$ in the absence or presence of 0.2 μM DM were measured for A2$_{104–117}$, W1I, and W1T, and DM-susceptibility by IC$_{50}$ was calculated for each peptide. The IC$_{50}$ assay was read at (**A**) 16.6 hours, (**B**) 24 hours, (**C**) 41 hours, (**D**) 93 hours, (**E**) 112.1 hours, (**F**) 166.4 hours, and (**G**) 329 hours.

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Table I

Kinetic parameters and calculated IC_{50} and off-rate for peptides used in the simulations

Peptide name	k_{ass} (μM^{-1}min^{-1})	$k_{ass,DM}$ (μM^{-1}min^{-1})	k_{dis} (min^{-1})	$k_{dis,DM}$ (min^{-1})	k_{off} [a] (min^{-1})	$k_{off,DM}$ (min^{-1})	IC_{50} [b] (μM)	$IC_{50,DM}$ (μM)
Probe peptide	0.048	0.192	0.00017	0.00043	0.0002	0.0003	0.0308	0.0336
Peptide 1	0.114	0.228	0.00027	0.013	0.0003	0.0035	0.0224	0.1919
Peptide 2	0.048	0.192	0.00017	0.00043	0.0002	0.0003	0.0308	0.0336
Peptide 3	0.114	0.228	0.0027	0.0027	0.0027	0.0033	0.1803	0.1645
Peptide 4	0.114	0.228	0.0027	0.0054	0.0027	0.0040	0.1803	0.2011
Peptide 5	0.114	0.228	0.0027	0.013	0.0027	0.0059	0.1803	0.3123
Peptide 6	0.114	0.228	0.0027	0.026	0.0027	0.0091	0.1803	0.5255
Peptide 7	0.114	0.228	0.0027	0.013	0.0027	0.0059	0.1803	0.3123
Peptide 8	0.114	0.228	0.0054	0.026	0.0053	0.0117	0.3531	0.6585
Peptide 9	0.114	0.228	0.0081	0.039	0.0080	0.0176	0.5255	1.021
Peptide 10	0.114	0.228	0.0108	0.052	0.0106	0.0234	0.6959	1.399
Peptide 11	0.114	0.228	0.0027	0.013	0.0027	0.0059	0.1803	0.3123
Peptide 12	0.114	0.456	0.0027	0.013	0.0027	0.0059	0.1803	0.2713
Peptide 13	0.114	0.684	0.0027	0.013	0.0027	0.0058	0.1803	0.2455
Peptide 14	0.114	0.912	0.0027	0.013	0.0027	0.0058	0.1803	0.2273
Peptide 15	0.114	0.114	0.0027	0.0027	0.0027	0.0033	0.1803	0.1706
Peptide 16	0.114	0.114	0.0027	0.0054	0.0027	0.0040	0.1803	0.2111
Peptide 17	0.114	0.114	0.0027	0.013	0.0027	0.0059	0.1803	0.3402
Peptide 18	0.114	0.114	0.0027	0.026	0.0027	0.0091	0.1803	0.6069

[a] Intrinsic (k_{off}, without DM) and DM-catalyzed ($k_{off,DM}$, with 0.25 μM DM) off-rates were calculated by simulating dissociation reactions of 0.1 μM DR-peptide complex for each peptide and fitting the dissociation curves with a one-phase exponential decay equation. Input values for k_{ass}, $k_{ass,DM}$, k_{dis} and $k_{dis,DM}$ rate constants for simulation of each peptide dissociation reaction are shown. Values for k_{+DM} and k_{-DM} were kept constant at 0.0216 μM^{-1}min^{-1} and 0.216 min^{-1} respectively for all the peptides.

[b] IC_{50} was calculated at equilibrium time point of 19200 minutes.