Short communication

HLA-DM mediates peptide exchange by interacting transiently and repeatedly with HLA-DR1

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ABSTRACT

The peptide editor HLA-DM (DM) catalyzes the exchange of peptides bound to MHC class II molecules within antigen presenting cells by generating a “peptide-receptive” MHC class II conformation (MHCreceptive) to which peptides readily bind and rapidly unbind. While recent work has uncovered the determinants of DM recognition and effector functions, the nature of MHCreceptive and its interaction with DM remains unclear. Here, we show that DM induces but does not stabilize MHCreceptive in the absence of peptides. We demonstrate that DM is out-competed by certain superantigens, and increasing solvent viscosity inhibits DM-induced peptide association. We suggest that DM mediates peptide exchange by interacting transiently and repeatedly with MHC class II molecules, continually generating MHCreceptive. The simultaneous presence of peptide and DM in the milieu is thus crucial for the efficient generation of specific peptide–MHC class II complexes over time.

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1. Introduction

The antigen-specific activation of the CD4+ “T helper” arm of the adaptive immune response is intractably linked to the processing of antigens and the presentation of the peptides generated on MHC class II molecules on the surface of antigen presenting cells (APCs). MHC class II αβ heterodimers are synthesized with an invariant chain (Ii) derived peptide CLIP occupying the peptide binding groove. As this complex traffics to the endosomal compartments of the APC, the majority of Ii is cleaved, leaving CLIP bound to MHC class II (Blum and Cresswell, 1988; Cresswell, 1994; Peters et al., 1991). HLA-DM, a nonclassical HLA molecule, has been shown to play a critical role in mediating the exchange of CLIP for antigenic peptides in the low pH environment of the endosome (Denzin and Cresswell, 1995; Fung-Leung et al., 1996). DM interacts with various conformations of the molecule (Chou and Sadegh-Nasser, 2000; Kropshofer et al., 1996, 1997; Sadegh-Nasser et al., 2008;

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Ullrich et al., 1997; Weber et al., 1996) and is thought to stabilize empty MHC class II against denaturation at physiological temperatures (Kropshofer et al., 1997). Primarily however, DM is considered as a catalyst of peptide association and dissociation, and kinetic parameters for DM-mediated peptide binding have been described (Vogt et al., 1996). This characterization has prompted the speculation that soluble forms of DM could be added exogenously to catalyze speedy association and/or dissociation of specific peptides of interest in vitro assays or even in vivo settings. Recently, we have shown that structural changes, primarily around the P1 pocket of the MHC class II allele HLA-DR1 (DR1), could provide the basis for DM sensitivity (Chou and Sadegh-Nasser, 2000), and conserved hydrogen bonds in the same region may be targeted by DM to effect peptide dissociation (Narayan et al., 2007). One of the emergent hypotheses from these and other studies (Rabinowitz et al., 1998; Zarutskie et al., 2001) was that DM could mediate both peptide association and dissociation by generating a “peptide-receptive” conformation of DR1 (DR1receptive), to which peptides could readily bind and rapidly unbind (Natarajan et al., 1999a; Sadegh-Nasser and McConnell, 1989; Vogt et al., 1996). Here, using soluble, recombinant DM and wild type and mutant DR1 molecules, along with variants of the Influenza Hemagglutinin derived peptide HA306–318.
we probe the interaction between DM and DR1. Several groups have reported the qualitative interaction between full-length DM and DR molecules in the context of cells, primarily by conventional biochemical assays (Denzin et al., 1996; Kropshofer et al., 1997; Weber et al., 2001; Zwart et al., 2005). We have targeted the soluble forms of these two molecules because of the possibility of use of soluble DM as an exogenously added catalyst of peptide exchange, but also because the usage of soluble isoforms has been used by us and other groups (Pashine et al., 2003) to accurately measure biophysical parameters of DM–DR interactions in solution. Here we characterize the central transitional conformation in the binding reaction, DR1receptive, and in light of the results, we propose a “hit and run” model describing transient and repeated interactions between DM and DR1 in order to generate DR1receptive and mediate rapid binding of peptides.

2. Materials and methods

2.1. Production of proteins and peptide synthesis

Soluble recombinant DR1 and DM proteins were expressed and purified as originally described (Narayan et al., 2007). Baculovirus DNA (BaculoGold; PharMingen) and transfer vectors carrying the wild type or mutant genes were cotransfected into Sf9 insect cells to produce recombinant viruses and infect Hy5 cells; DR1 and DM proteins were purified from the culture supernatant using an anti-DR1 mAb (L243) or M2 (αFLAG) mAb sepharose resin (Sigma), respectively. Proteins were eluted at low pH or with 0.1 M FLAG peptide, and purified by gel filtration chromatography (Superdex 200 HR 10/30 column, Amersham Pharmacia). The peptides HA306–318 (PKYVKQNTLKLAT), HAY308A (PKAYVQNTLKLAT) and HAAnchorless (PKAVKANGAKAAT) were synthesized and purified to >90% by reverse-phase preparative HPLC (Elim BioPharmaceuticals). The irrelevant control peptide ETEC (IIQYQDEKKK) is a peptide corresponding to amino acids 111–123 of Cs6, a subunit of enterotoxigenic Escherichia coli pili protein (Natarajan et al., 1999b). Fluoresceinated peptides were similarly pre-synthesized, with the N-terminal addition of FAM and Glycine linker.

2.2. Peptide association, dissociation and receptive conformation assays

Purified DR1wt (1 μM) was incubated in the absence or presence of 0.2 μM DM with 50 μM fluoresceinated peptides for various times in solutions of varying amounts of sucrose or glycerol in 0.15 M citrate phosphate buffer, pH 5.5 (CP) at 37 °C. After removal of free peptides by a Sephadex G-50 spin column equilibrated with PBS pH 7.4, fluorescence emission of the DR–FITC–peptide complexes was measured at 514 nm with an excitation at 492 nm on an LS-50B spectrofluorimeter (PerkinElmer) at room temperature. In case of the “pre-incubation” experiments, DR1wt (1 μM) was pre-incubated with DM (0.2 μM) for 5 min at 37 °C, and then the association experiment was carried out as before. When required, excess αFLAG MAB resin was added to all groups for 20 min and quickly centrifuged to spin out any DM in the solution. Equal volumes of the supernatant were then used for association experiments. The dissociation experiments in the presence of SAg was essentially performed as described previously (Chou and Sadegh-Nasser, 2000); here, 7 μM SAg (SEA, SEB or SEH, Toxin Technologies) was added at the start of the dissociation reaction. The mutant SEA molecule was kindly donated by Dr. Robert G. Ulrich, US Army Medical Research Institute of Infectious Diseases at Ft. Detrick. For DR1receptive lifetime assays, DR1 variants were incubated with non-fluorescent HA306–318 in CP at 37 °C to yield maximal loading. Excess unbound peptides were removed by a spin, and samples were incubated at 37 °C with no competitor peptide in the presence or absence of DM (DR:DM = 5:1), for required lengths of time. At the end of the incubations, the pH of the solution was changed to 7.5 by the addition of appropriate molar concentrations of phosphate buffer pH 8.0 to inactivate DM, and the samples were pulsed with an excess (50 μM) of fluoresceinated peptide for 15 min (DR1β81N or DR1G86Y) or 60 min (DR1wt). After one more spin through a Sephadex G-50 spin column to remove excess fluorescein-labeled peptide, fluorescence was measured as above. All the raw dissociation data were fitted into single exponential dissociation equations as follows:

\[ Y = Y_0 + A_1 e^{-\lambda t} \]

All curves were normalized, with the arbitrary value of 1.0 assigned to the fluorescence obtained at the start of measured dissociation curves.

2.3. Preparation of co-solvent buffers at various viscosities

Solutions of varying concentrations of sucrose (Fisher) or glycerol (Sigma) in CP pH 5.5 were run through an Ubbelode viscometer to determine viscosity of each sample. From the %w/v vs. viscosity standard curves, co-solvent buffers of specified viscosities were produced and used.

2.4. Real-time binding experiments

2.4.1. DM is out-competed by DR1

2.4.2. Real-time binding experiments

2.4.3. Preparation of co-solvent buffers at various viscosities

2.5. Results and discussion

3.1. DM is out-competed by DR1β81His binding superantigens

Using various soluble DR1 mutants, we have demonstrated the importance of the P1 pocket of DR1 for DM recognition and effector functions (Narayan et al., 2007). To characterize the interaction between DM and DR1 without genetic manipulation, we measured DM-mediated peptide exchange in the presence of certain “superantigens” (SAg), bacterial toxins (Proft and Fraser, 2003) that bind DR1β81His, a conserved residue near the P1 pocket that forms a short strong hydrogen bond with peptide (McFarland et al., 2001; Stern et al., 1994). We tested the ability of one such toxin, Staphylococcal Enterotoxin A (SEA) to out-compete DM. SEA binds DR1 via a high (nM) affinity site, coordinating a zinc with β81His, as well as a weaker (μM affinity) zinc independent site on the α chain of DR1 (Thibodeau et al., 1997).

In a real-time binding experiment utilizing surface plasmon resonance (SPR) measured in a BiAcore machine, we injected a solution of DR1 and DM on a chip decorated with HA306–318 or its synthetic variant HAAnchorless (see Section 2). As expected, DR1 aided by DM bound both peptides immobilized on the chip (Fig. 1a, blue). The pre-incubation of DR1 with SEA abrogated peptide binding (black), suggesting the binding of β81His by SEA either physically blocked the peptide binding groove of DR1, or prevented the...
Fig. 1. DM is out-competed by superantigens that bind DR1β81His. (a) DM-mediated peptide binding is blocked by SEA. 2.4 μM DR1–HA306–318 complexes were produced, isolated, and incubated with or without 1 μM DM or 7 μM SEA for 20 min, and injected over either HAAnchorless or HA306–318 peptide bound CM5 chip surface in a BIAcore experiment. Samples in citrate phosphate (CP) pH 6.0 were injected at 4 μL/min followed by washout (CP pH 6.0 + 0.01% Tween). Raw real-time binding data of DR1 to the peptide chip is shown; in the presence of DM, blue; in the presence of SEA, red; in the presence of DM + SEA, black; in the presence of DM + SEA + EDTA, green. (b) Same as (a), except that SEB or TSST were used instead of SEA. Real-time binding data of DR1 to peptide in the presence of DM, red; SEB + DM, blue; or TSST + DM, black. (c) DM-mediated peptide dissociation is blocked by SEA and SEH. 2.4 μM DR1–FITC–HAAnchorless complexes were allowed to dissociate for 30 min in CP pH 5.5, in the presence or absence of 1 μM DM, either without SAg (black), or in the presence of 7 μM SEA (grey) or 7 μM SEH (white). The fluorescence of the complexes is expressed as a fraction of complexes dissociated at 0 min, which was assigned an arbitrary value of 1.0. (d) Same as (c), except that the experiment was performed over 20 min, and the SAg used were SEA (grey), SEB (stripes), or a mutant SEA that does not bind β81His (dotted). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

conformational changes required by DR1 to bind peptide. On incubating DR1 with both DM and SEA, there was minimal binding of DR1 to the peptide surface (Fig. 1a, red). There is considerable evidence that the acidic face of DM may interact with the region of the peptide–DR1 complex that contains the N-terminus of peptide and the P1 pocket of DR1 (Chou and Sadegh-Nasseri, 2000; Doebele et al., 2000; Pashine et al., 2003). Given that both SEA and DM have significant “footprints” of interaction with the DR1 molecule, it is possible that SEA physically out-competes DM by binding DR1 with higher affinity, or alternatively, it could be that SEA inhibits DM-induced conformational changes in DR1 by binding β81His. That this binding site is critical in the blocking DM was underscored when these molecules were incubated in the presence of EDTA. Chelating the zinc presumably disrupted the zinc-dependent binding of SEA to DR1, and DM was now able to generate DR1receptive and mediate peptide binding (Fig. 1a, green). Importantly, SEA could still bind DR1 through the zinc independent binding site on the DR1α chain, resulting in a larger complex binding the peptide surface, and therefore a greater RU response.

We also tested the ability of two other SAgs, Staphylococcal Enterotoxin B (SEB) and Toxic Shock Syndrome Toxin-1 (TSST-1) to block DM-mediated generation of DR1receptive. Both SEB and TSST-1 bind in a similar fashion to the α chain of DR1 (Papageorgiou and Acharya, 2000), on the opposite side of the molecule purported to interact with DM. We observed that neither the addition of SEB (Fig. 1b, blue) nor TSST-1 (black) affected the ability of DM to generate DR1receptive and mediate peptide binding; again, the increase in the size of the ligand (toxin-DR1 complexes) resulted in greater bulk binding than DR1 alone (red). It is important to note that we are not following the kinetics of peptide binding in this experiment; the assay was designed to follow the total change in bulk binding of the various molecules. Thus while the slow injection precluded the accurate measurements of on/off rates, it allowed binding of all available analytes to the peptide ligand, which was central to the evaluation of SEA effect on DM-mediated DR1-peptide complex formation. The presence of EDTA by itself does not increase the “stickiness” of the chip, as seen in a control experiment where soluble DR1/HAY308A complexes in the presence or absence of DM were incubated in EDTA and then injected on to the peptide-decorated chip. The EDTA group showed a far greater RU change during the injection (approximately 2200 RU as compared to 300 RU for the –EDTA group, a difference of 1900 RU), which was expected given the change in refractive indices between flow buffer and injection buffer, but there was no stable non-specific binding after at the end of the injection. On flowing wash buffer at the end of the injection, the presence of EDTA in the injection buffer was seen to cause a change of only 16.9 RU in the DM-mediated DR1 binding to the HAAnchorless chip (Supplementary Figure S1). As an additional control, we also tested the contribution of the peptide-decorated chip towards non-specific binding, if any. We immobilized an irrelevant peptide, ETEC and performed a DR1 injection as above. We observed minimal binding of ≤10 RU even in the presence of DM (Supplementary Figure S2) suggesting that non-specific binding of soluble DR1 or DM to the chip
Fig. 2. DM does not maintain the peptide-receptive conformation of DR1. (a) DR1 molecules complexed to short-lived peptides were allowed to dissociate for various times in the absence or presence DM at pH 5.5 and then pulsed for 1 h with FITC–HA306–318 at pH 8 (●, +DM; ▲, −DM). Alternatively they were pulsed at pH 5.5 for 1 h (○) or 2 h (▲). (b) DR1G86Y–HAanchorless (●) or DR1H81N–HAanchorless (▲) complexes were allowed to dissociate for various times and pulsed with FITC–HA306–318 or FITC–HAanchorless respectively, for 15 min in the absence (○) or presence (▲) of DM. Excess peptide was separated and relative fluorescence from bound FITC–peptide was plotted against the time of dissociation of initial peptide–DR1 complexes. In all cases, the fluorescence intensity at time $t_0$ was arbitrarily assigned a value of 1.0 and the data at later time points plotted relative to the initial intensity. One of at least two experiments.

3.2. DM does not maintain the peptide-receptive conformation of DR1

We and others (Chou and Sadegh-Nasseri, 2000; Narayan et al., 2007; Rabinowitz et al., 1998; Zarutskie et al., 2001) have previously shown that DM generates a short-lived conformation DR1<sub>receptive</sub> that allows rapid peptide exchange. Given that our results above suggest a weak and perhaps transient interaction between DM and DR1, we wanted to test the effect of DM on DR1<sub>receptive</sub> over time, since it is well documented that DM can mediate rapid peptide binding for long periods (Chou and Sadegh-Nasseri, 2000; Vogt et al., 1996). Specifically, we wished to test this interaction in the absence of excess peptides, since it is known that DR1<sub>receptive</sub> rapidly binds offered peptides with kinetics similar to "DM-assisted" peptide association, and DM has no added effect (Chou and Sadegh-Nasseri, 2000). We allowed soluble recombiant complexes of HA<sub>anchorless</sub>–DR1 to dissociate at pH 5.5 in the presence or absence of DM. As peptide dissociates, DR1<sub>receptive</sub> also decays with time. To measure the fraction of DR1 that was present as DR1<sub>receptive</sub>, we exposed the complexes to a quick pulse (1 h) of a molar excess of fluorescent HA306–318 peptide at various points during the dissociation. Based on previous observations (Chou and Sadegh-Nasseri, 2000; Narayan et al., 2007), most of HA306–318 available within this short time window would be bound by the conformation of DR1 that most readily binds peptide, i.e. DR1<sub>receptive</sub>. We ensured that little or no fresh DR1<sub>receptive</sub> was generated during the association reaction by limiting the duration of the pulse (thus limiting the natural conversion of DR1 to DR1<sub>receptive</sub> with time), and also inactivated DM when present by changing pH to 7.5. Thus, in the groups with DM, DM was active only during the time at which DR1<sub>receptive</sub> was decaying without excess available peptide. When the experiment was conducted in the absence of DM, DR1<sub>receptive</sub> decayed with a half-life of ~25 min (Fig. 2a, ▲). However, even in the presence of DM (DR:DM = 5:1), the half-life of DR1<sub>receptive</sub> was unchanged (●). When we repeated the experiment without altering the pH of the solution, allowing for DM to be active during peptide loading (○), and doubled the length of peptide pulse to 2 h, we observed that (i) there was a flattening of the curve, and (ii) the 1/2 of DR1<sub>receptive</sub> still was ~25 min (Fig. 2a, □). The observation that the two curves are superimposable at the earlier timepoints suggests that DM does not extend the lifetime of the receptive conformation of DR1; however, the increase in binding during peptide pulse in the presence of DM, reflected in the flattening of the +DM curve, emphasizes the ability of DM to convert DR1 to DR1<sub>receptive</sub>. Thus a longer incubation with active DM allows accelerated peptide association (compare plateaus of curves marked by ○ and □) when DR and excess peptides are present simultaneously in the milieu, as seen previously (Chou and Sadegh-Nasseri, 2000).

The above experiment was repeated with DR1<sub>G86Y</sub>, a recombiant DR1 molecule with a mutation in the base of the P1 pocket that fills the pocket with a bulky tyrosine. DR1<sub>G86Y</sub> is fixed in a DR1<sub>receptive</sub>-like conformation and is refractory to DM (Chou and Sadegh-Nasseri, 2000). Here, the ability of DR1<sub>G86Y</sub> to bind peptide is purely determined by the availability of empty groove; the stable DR1<sub>receptive</sub>-like conformation is unmasked by the rate-limiting step, which is the dissociation of bound peptide. As peptide molecules dissociated with time, empty DR1<sub>G86Y</sub> became available to bind the offered fluorescent peptide, resulting in an exponential association curve (Fig. 2b-i, ○) that was unchanged even upon the addition of DM (●). The resulting curves are very similar to an ordinary association reaction of peptide with DR1<sub>G86Y</sub>, confirming that the peptide-receptive conformation generated by DM is similar to the conformation of this mutant. On the other hand, another DR1 mutant, DR1<sub>H81N</sub> displayed a very short half-life for its receptive conformation (Fig. 2b-ii, ○). This mutant fails to form a strong hydrogen bond between DR1 and peptide, hence the bound peptides rapidly dissociate even in the absence of DM (Narayan et al.,...
Fig. 3. DM-mediated peptide association is inhibited by increased solvent viscosity. (a) DR1 molecules were allowed to bind to FITC–HA306–318 peptides for 1 h in the absence (white) or presence of DM in solutions of glycerol (black) or sucrose (grey) of increasing viscosities, made in citrate phosphate buffer pH 5.5. “The “DM effect”. (b) The association of FITC–HA306–318 peptides to DR1 in the absence (white) or presence (grey) of DM was followed over time in (i) CP pH 5.5 or viscous solutions of (ii) glycerol or (iii) sucrose in CP pH 5.5, and fluorescence from bound peptide plotted against time. DR:DM = 5:1. (c) DR1 molecules pre-incubated with DM for 5 min (black) or 0 min (grey) were allowed to bind for 1 h to FITC–HA306–318 peptides. The control of 1 h association without DM (white) is also included. One of at least three experiments. (c, i) DR1 molecules were either not pre-incubated with DM (white), or were pre-incubated with DM for 5 min, followed by removal of DM by immunoprecipitation, IP (black) or no removal of DM (grey), and then incubated with fluorescent HA306–318 for 1 h. Fluorescence of DR1–HA306–318 Complexes was plotted as histograms. Error bars indicate standard deviations (SD) of at least three experiments.

2007). The results shown in Fig. 2b-ii (■) indicate that the groove of DR181His closes quickly after peptide dissociation, with a τ1/2 of just 3 min in the presence or absence of DM. The lack of an effect of DM on DR181His and the unusually rapid closure of its peptide binding groove may suggest a role played by β81His in stabilizing the groove of DR1. This property of DR181His might lead to promiscuous peptide binding, a speculative, but intriguing hypothesis to test. At any rate, the results from both mutants suggest that DM can generate, but cannot stabilize the transient conformation DR1receptive.

3.3. DM-mediated peptide loading in solution is dependent on solvent viscosity

DM and MHC class II molecules have long been thought of having a low-affinity interaction in solution, although the actual affinity of this interaction is unclear (Vogt et al., 1996). Given that we found that DM did not stabilize DR1receptive, we posited that DM may interact with DR1 repeatedly and transiently in order to sustain rapid peptide binding to DR over time. Thus, the ability of DM to generate DR1receptive should be dependent on the rate of diffusion of these molecules in solution. We generated sucrose or glycerol solutions of varying viscosities and tested the ability of DM to mediate the association of fluorescent HA306–318 to DR1. While the overall rate of peptide binding decreased slightly (Fig. 3a, white bars), there was a substantial decrease in the “DM effect” on increasing the viscosity of the solution, independent of the co-solvent used (black bars, glycerol; grey bars, sucrose). In a kinetic association experiment, the presence of DM resulted in a ∼4-fold increase in total peptide bound in 1 h when the reaction was carried out in citrate phosphate pH 5.5 (Fig. 3b-i). This “DM effect” was reduced to a 2.83-fold, or 2.74-fold increase in total peptide bound when the reaction was carried out in higher viscosity solutions of glycerol or sucrose in the same buffer, respectively (Fig. 3b-ii, iii). Such increase in viscosity only affected peptide binding marginally in the absence of DM. Thus, the ability of DM to generate DR1receptive and thereby mediate peptide binding was dependant on its diffusion in solution, affirming that these two soluble molecules interact with low affinity.

We also tested whether pre-conditioning DR1 molecules to DM made these molecules more readily accessible to peptide binding. We allowed fluorescent HA306–318 to bind to soluble DR1 that were either pre-treated with DM for 5 min (Fig. 3c, white bars) or not (black bars), in solvents of varying viscosities. Peptide was also allowed to bind DR1 in the absence of DM (grey bars). We observed that the ability of DR1 to bind peptide was unchanged, irrespective of whether or not it had been pre-incubated with DM. In a variant of this experiment, DR1 was pre-incubated with DM, separated by removing DM by immunoprecipitation, and then allowed to bind fluorescent HA306–318 for 1 h. The amount of peptide bound (Fig. 3c, black bars) was the same as peptide bound to DR1 alone (white bars), whereas if DM was present with DR1 during incubation with peptide, it accelerated peptide association (grey bars). These data further reinforce the idea that DR1receptive is a transient conformation, and that DM cannot stabilize this conformation for any significant period of time. Instead, DM interacts transiently and repeatedly with DR1, in a “hit-and-run” mechanism similar to that reported for enzyme catalyzed reactions (McNally et al., 2000; Rigaud et al., 1991) to generate the short-lived DR1receptive to which peptides may bind. A recent paper (Grotenbreg et al., 2007) concludes that the DM-mediated association reaction proceeds even faster than peptide association to the groove of DR that has just been vacated by a peptide. This would suggest that there may be two variants of DR1receptive, one which is formed in the absence of DM, and one that may be stabilized by DM. While we do not rule out the possibility of DM independent and DM-induced DR1receptive we posit that the data may be explained by just one central conformation,
DR<sub>1</sub><sup>receptive</sup>-It is conceivable that in the presence of DM, this fleeting conformation is generated constantly due to multiple and transient interactions with DM, resulting in very rapid peptide binding, even faster than if DR<sub>1</sub><sup>receptive</sup> was generated by peptide dissociation alone. However, our data unequivocally show that there is indeed a temporality to the “DM effect” that results in the rapid decay of DR<sub>1</sub><sup>receptive</sup> unless active DM is continuously present in the milieu. We feel that this observation lends strong support to our model of repeated transient DM–DR<sub>1</sub> interactions.

In the antigen-presenting cell, the processes of degradation of antigens into peptides, the activation of DM, and the formation of MHC class II<sub>receptive</sub> converge spatially and temporally in order for the central event in antigen processing, the formation of the peptide–MHC class II complex, to occur.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.07.001.

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