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Selection of immunodominant epitopes during antigen processing is hierarchical

Scheherazade Sadegh-Nasseri*, AeRyon Kim[1]

Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

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ABSTRACT

MHC II proteins present processed antigens to CD4 + T cells through a complex set of events and players that include chaperons and accessory molecules. Antigen processing machinery is optimized for the selection of the best fitting peptides, called '*immunodominant epitopes*', in the MHC II groove to which, specific CD4 + T cells respond and differentiate into memory T cells. However, due to the complexity of antigen processing, understanding the parameters that lead to immunodominance has proved difficult. Moreover, immunodominance of epitopes vary, depending on multiple factors that include; simultaneous processing of multiple proteins, involvement of multiple alleles of MHC II that can bind to the same antigen, or competition among several suitable epitopes on a single protein antigen. The current dogma assumes that once an antigenic determinant is selected under a specific condition, it would emerge immunodominant wherever it is placed. Here we will discuss some established parameters that contribute to immunodominance as well as some new findings, which demonstrate that slight changes to antigen structure can cause a complete shift in epitope selection during antigen processing and distort the natural immunodominant epitope.

1. Introduction

It is well established that the immune system focuses on and responds to very few *immunodominant epitopes* from pathogenic insults such as infectious agents and antigenic targets in autoimmune diseases, allergy, and cancer (Sercarz et al., 1993). However, despite its importance, obtaining a mechanistic understanding of immunodominance has proved difficult due to both the complexity of the antigen processing pathways and various contributors to antigen processing (see below).

For MHC class II presentation, antigens internalized from exogenous sources move through a series of endosomal compartments in antigen presenting cells (APCs) containing a suitable denaturing environment, the accessory molecules HLA-DM/H2-M (DM) and HLA-DO/H2-O (DO), and a set of proteolytic and denaturing enzymes (mainly cathepsins and Gamma-interferon-inducible lysosomal thiol reductase, GILT) that generate and trim antigenic determinants (Maric et al., 2001; Bryant and Ploegh, 2004; Jensen, 2007). Meanwhile, newly synthesized MHC class II molecules associate with the Class II invariant chain (Ii) in the ER, which guides the nascent MHC II through Golgi, trans Golgi, and to the compartments specialized for antigen processing and epitope selection, MHC II compartment (MIIC) (Peters et al., 1991; Neefjes et al., 1990), where it encounters the exogenous antigens. Ii is sequentially cleaved until only a short peptide, CLIP, remains in the MHC II groove (Cresswell, 1994). CLIP has been described as a surrogate peptide needed for shaping and completion of MHC class II folding (Sadegh-Nasseri and Germain, 1992; Sadegh-Nasseri, 1994; Natarajan et al., 1999; Rabinowitz et al., 1998), which must be displaced from the MHC groove and exchanged for antigenic peptides. This important task requires the accessory molecule DM (Morris et al., 1994). In the following two sections we would discuss our current understanding of the contributions of DM and DO to immunodominant epitope selection.

2. DM

DM is a non-polymorphic MHC II-like accessory molecule that does not bind peptides itself (Mosyak et al., 1998) but is necessary for the efficient displacement of the CLIP peptide from the MHC groove and its exchange for the exogenous peptides (Riberdy et al., 1992; Denzin et al., 1994; Denzin and Cresswell, 1995; Cresswell, 1996; Denzin et al., 1996; Spies et al., 1990; Mellins et al., 1991, 1994; Ghosh et al., 1995). Mechanistic insight into the role of DM in epitope selection was gained when biochemical and biophysical studies were performed by multiple laboratories including ours. The key observation was that DM

* Corresponding author.
E-mail address: ssadegh@jhmi.edu (S. Sadegh-Nasseri).
[1] Present address: Department of Oncology, Amgen Inc. South San Francisco, DA 94080, USA.

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interacted with peptide MHC class II molecules (pMHCII) differentially. DM was most effective in dissociating poor binding peptides that left the P1 pocket of HLA-DR1 (DR) molecules partially filled (Kropshofer et al., 1996). Peptide/DR1 complexes where the P1 was filled were not good substrates for DM, and were left unaffected by DM (Chou and Sadegh-Nasseri, 2000). Moreover, it was demonstrated that DM mediated peptide exchange by interacting transiently and repeatedly with MHC class II molecules, continually generating a peptide-receptive conformation of MHC II (Natarajan et al., 1999; Rabinowitz et al., 1998) that can readily scan suitable stretches of partially folded antigens by binding and dissociating quickly until an optimal peptide is found (Narayan et al., 2007, 2009).

Because of the transient nature of interactions of DM with DR molecules, co-crystallization of DM/DR complexes was a challenge until recently. Anders et al. cleverly designed a DR1/peptide complex that allowed for the DR1 groove to remain open. Through the use of a partial peptide, which acted as a barrier to the collapse of the hydrophobic P1 pocket in DR1 molecules, a conformation that DM could interact with and form stable complexes was generated. Hence, a stable DM/DR complex was achieved and was crystallized (Anders et al., 2011; Painter et al., 2011; Pos et al., 2012). Consistent with previous reports, the 3D structure of DM/DR1 revealed that during the interaction, DM causes flipping of a critical tryptophan (DRα W43), which in the DR1/HA peptide complex interacts with the P1 Tyr residue of the peptide and neighboring hydrophobic residues. In the DR1–DM complex, this tryptophan has rotated away from the P1 pocket leading to conformational changes in the P1 area of DR1, the distortion of multiple H-bonds and, destabilization of the bound peptide. The findings suggested that an overall dynamic MHC II conformation, in addition to P1 pocket occupancy, determines susceptibility to HLA-DM-mediated peptide exchange and provides a molecular mechanism for HLA-DM to efficiently target poorly fitting pMHC II complexes, editing them for more stable ones. Upon removal of CLIP, DM helps in the selection of immunodominant epitopes by skewing the pMHC II balance towards the immunodominant pMHC II that are resistant to DM-mediated dissociation (Kim and Sadegh-Nasseri, 2015; Kim et al., 2014). By mediating dissociation of those pMHCII complexes that are DM-sensitive, DM, releases those peptides from MHC II groove, making them targets for antigen processing enzymes.

3. DO

Unlike our good mechanistic understanding of how DM functions, the field has not been able to efficiently study DO. While the genes encoding the DO molecule were first identified in mid 1980s, and its unique tissue distribution was discovered in the early 90s, it took another decade or so before the first studies exploring some of its contributions to class II antigen processing were performed (Karlsson et al., 1991; Trowsdale and Kelly, 1985). Now, two decades later, we still do not have a clear understanding of its biological impacts. A major difficulty has been that DO knockout mice did not show a clear phenotype (Brocke et al., 2003; Perraudeau et al., 2000; Liljedahl et al., 1998), and production of recombinant DO for biophysical studies proved difficult. Our group, along with others, have vested interest in understanding DO and its relationship to DM and MHCII molecules.

All we know about DO so far can be distilled into two working hypotheses: (1) DO binds to DM to inhibit its activity, mainly removal of the CLIP peptide and, (2) DO differentially affects presentation of structurally diverse peptides and acts as a second chaperone together with DM in fine tuning MHC II repertoire selection. Data in support of the former hypothesis mainly comes from over-transfection of DO genes in cell lines, or dendritic cells (Denzin, 2013; Kim et al., 2017), and the recent structural studies of DM/DO interaction, which showed that DO binds to DM at the same interface with which DM contacts DR1 (Pos et al., 2012; Guce et al., 2013). Studies supporting the latter hypothesis came from biochemical (Kropshofer et al., 1998) and kinetic studies demonstrating that DO only affected *association* of certain peptides to DR, but had no effect on the *dissociation* of any tested peptide/DR1 complexes (Poluektov et al., 2013a, b). The effect of DO on association directly correlated with peptide sensitivity to *DM-mediated dissociation*. DO reduced binding of peptides that formed *DM-sensitive* complexes with DR, and enhanced the binding of peptides that formed *DM-resistant* complexes. In a nutshell, it was clearly shown that; i) DO works directly on DR1, and not by regulating the effect of DM, ii) DO forms complexes with peptide-receptive MHC Class II, and iii) A *peptide-receptive* conformation is generated by DM. Hence, it was proposed that DM and DO cooperate for a more effective epitope selection. Because of the significant role that DM plays in determinant selection and immunodominance (Kim et al., 2014; Hartman et al., 2010), it makes sense that two accessory molecules are evolved to ensure optimal selection of immunodominant epitopes from autoantigens in the thymic medulla and in B cells where DO is mainly expressed.

4. Current status

4.1. A reductionist antigen processing system helps in teasing out contributing factors to immunodominance

A better understanding of immunodominant epitope selection during antigen processing became feasible by the development of a reductionist antigen processing system (Hartman et al., 2010), which provided evidence that antigen processing and immunodominant epitope selection could be achieved by a minimal number of purified proteins in a tube. Using this system, we identified immunodominant epitopes from several proteins implicated in autoimmunity as well as protein antigens from influenza, malaria, and HIV-1. We showed that pathogen-derived epitopes were highly sensitive to proteolysis unless protected by the groove of MHC II, and that for successful processing and presentation of the immunodominant epitopes, antigenic determinants must first be captured by MHC II, and then proteolyzed by endo- and exoproteases (Kim and Sadegh-Nasseri, 2015; Kim et al., 2014). These observations contradicted the prevailing dogma that immunodominant peptides are first subjected to proteolysis by antigen processing enzymes, cathepsins, and then bound to the MHC II groove with help of DM (Janeway, 2008). As such, these observations also placed focus on structural factors such as the location of the epitopes, and their accessibility to being captured by the groove of MHC II molecules (Dai et al., 2001, 2002).

The phenomenon of *'Epitope hierarchy'*, originally described by Sercarz and colleagues, proposed that while many epitopes within a protein antigen can bind to the groove of MHC II, some gain dominance over the others, hence the terms subdominant or cryptic epitopes were introduced to the immunology world (Sercarz et al., 1993). Cryptic epitopes were defined as epitopes that would only elicit a T cells response if the immunodominant epitopes had been removed or altered. One molecular mechanisms envisioned in explaining the phenomenon was that MHC II binding to antigenic determinants could occur prior to peptide generation in the endosomal compartments (Gammon et al., 1987; Moudgil and Sercarz, 2005), a concept that was directly supported by the use of our reductionist system. We were able to clearly demonstrate that immunodominant epitopes bind to MHC II as either: (i) part of the full antigen or, (ii) in a large fragment of the parent antigens, and DM helps with the selection of the right determinants (Kim and Sadegh-Nasseri, 2015; Kim et al., 2014).

4.2. Artificial spacer sequences designed to facilitate recombinant protein purification can emerge as immunodominant epitopes

The competition to gain dominance among potential epitopes of an antigen as previously described by Sercarz and colleagues considered only the natural antigen sequences. However, when a recombinant malaria vaccine candidate, LSA-NRC, was tested to determine its

immunodominant epitope(s), LSA(434–453) located at the C-terminus of the protein (residues 434–443) emerged as dominant. Interestingly, this epitope contained part of a spacer sequence artificially added to the protein for purification purposes (residues 444–453) (Hillier et al., 2005). While the LSA sequence ends at Leucine 443, the immunodominant epitope includes the spacer sequence GGSGSP and four histidine residues. Importantly, the artificial epitope was the selected determinant *in vivo*, as verified by its ability to induce T cell responses in DR1 transgenic mice immunized with LSA-NRC, and in human volunteers immunized with LSA-NRC vaccine preparations (Hartman et al., 2010). The peptide containing the spacer sequence, LSA(436–449) was the only peptide to recall strong dose-dependent T cell responses. In this regard, LSA-NRC protein that had been used as a vaccine candidate in humans, did not protect vaccinated individuals against malaria challenge (Spring et al., 2009), likely because immunized individuals selectively recognized the spacer containing epitope as the dominant epitope (Hartman et al., 2010).

Another example of a new immunodominant determinant generated by adding a spacer sequence to a recombinant protein came from studying a recombinant peptidylargine deimnase 4 (PAD4) protein (Kim et al., 2017). To determine the PAD4 dominant epitopes, we once again used our cell free reductionist antigen processing system. Recombinant PAD4 protein was incubated with DR1 and DM followed by the addition of CatB, CatH, and CatS allowing for processing and determinant selection. DR1 bound peptides (8 PAD4-derived peptide sequences) were eluted and analyzed by mass spectrometry and their immunogenicity were verified. Again, the only peptide which induced a recall response contained a portion of the N-terminal spacer sequence (YKKAGFT) and the adjacent N-terminal PAD4 residues *i.e.*, MAQGTLRVTPEQPTHA. Notably, the spacer added to this PAD4 protein (to be called PAD4-A) was different in sequence, and its placement from the spacer added to LSA-NRC. Surprisingly, yet another recombinant PAD4 protein (PAD4-B) identical in its native PAD4 sequence except for a different spacer sequence, GSAEGSS, and manufactured by a different company, also demonstrated a shift in its immunodominant epitope to the spacer region, and was confirmed by recall response. These results further substantiated that with the addition of the YKKAGFT spacer sequence, the immunogenicity of the PAD4-A protein was shifted from it natural sequences to an artificial sequence. Hence, when it comes to design of recombinant proteins as vaccine candidates, even smallest changes in protein sequence and/or structure can completely disturb the natural processing and immunodominant epitope selection.

4.3. An immune hierarchy is established when two different epitopes compete for the same HLA-DR1 molecules

The above observations indicated that immune hierarchy exists between epitopes of one antigen. What about the simultaneous processing of two different protein antigens? To investigate if hierarchy is established among immunodominant epitopes of more than one protein, we used a mixture of LSA-NRC and influenza HA1-H5N1 protein at a 1:1 ratio for simultaneous processing in our cell free system. Mass spectrometric results indicated only a trace signal for LSA(436–449), but prominent H5N1-rHA1-derived peaks containing HA(259–274). Interestingly, the results were validated *in vivo* when DR1 transgenic mice were immunized with a 1:1 ratio mixture of LSA-NRC and HA1-H5N1 proteins. The dominance of HA1-H5N1 epitope was not reduced even when the two proteins were mixed at ratios of, 2:1, or 4:1 in favor of LSA Ag. A recall T cell proliferation assay showed that T cells responded to the HA1 and its dominant epitope HA(259–274) only, and no recall responses were observed to LSA-NRC and LSA(436–449). Because LSA-NRC protein is immunogenic when injected alone, the observed absence of T cell responses to LSA-NRC epitope (Hartman et al., 2010) cannot be explained by a 'hole in CD4 T cell repertoire' hypothesis (Ogasawara et al., 1987).

4.4. Epitope hierarchy can occur among epitopes of the same protein, or immunodominant epitopes of different proteins- but what determines immunodominance?

4.4.1. pMHC II complex stability and/or DM resistance?

Multiple reasons might contribute to immunodominance including the possibility of differences in the stability and/or DM resistance of HA(259–274)/DR1 *versus* LSA(436–449)/DR1 complexes. We tested both HA(259–274) or LSA(436–449) peptides for DR1 binding and sensitivity to DM, and demonstrated that both peptides formed stable complexes with DR1 and were DM-insensitive (Kim et al., 2017), validating that lack of presentation of the LSA dominant epitopes was not due to poor DR1 binding, or lack of resistance to DM-mediated dissociation.

4.4.2. Differential sensitivity to cathepsins induced degradation?

In light of our previous findings that epitopes from various source might have differential sensitivity to cathepsins, we compared LSA-NRC and H5N1-rHA1 protein for differential sensitivity to our cathepsin mixture, CatB, CatH and CatS, by incubating them individually for different lengths of time (3 h, 1 h, or 15 min), before adding, or concurrently with DR1 and DM for epitope binding and selection. The outcome, as analyzed by MS, indicated that the LSA-derived dominant epitope was highly sensitive to the cathepsins, whereas a slight MS peak corresponding to the H5N1-rHA1 dominant epitope, H5N1-HA1(259–274), remained detectable even after three hours of pre-incubation with cathepsins in the absence of DR1. This difference was not due to a lack of susceptibility to cathepsin digestion (Kim et al., 2014), as both synthetic epitopes were readily digested when incubated with just the cathepsin mixture. However, as full-length proteins, the location of the two epitopes; C-terminus of the protein (LSA-NRC epitope), or part of the tightly folded HA1 head (H5N1-HA1(259–274) epitope), made cathepsin accessibility more difficult. Hence, providing evidence for the location the epitope and the associated structural constraints as contributors to the selection of dominant epitopes. In this context, it is to be reminded of the role of γ-interferon inducible lysosomal thiol reductase (GILT) in reducing disulfide bonds in antigens and exposing new epitopes (Maric et al., 2001; West et al., 2013; West and Cresswell, 2013).

4.4.3. GILT reduction of disulfide bonds reveals additional epitopes

Originally discovered by Cresswell and colleagues, GILT is a thioredoxin-related oxidoreductase that is constitutively expressed in APCs, but is Interferon-γ inducible in other cell types (Maric et al., 2001; West et al., 2013; West and Cresswell, 2013). It localizes to MHC-II containing compartments and is active at low pH. GILT has been shown to be necessary for presentation of peptides from antigens that contain multiple disulfide bonds such as hen egg lysozyme (HEL), influenza hemagglutinin, and house dust mite allergen Der p 1 (West and Cresswell, 2013). Using our reductionist cell free antigen processing system to directly examine the influence of GILT in the diversity of HA1 epitopes of influenza H5N1 and H1N1 presented by DR1 and DR4, we found that indeed, recombinant murine GILT leads to presentation of new epitopes that locate near disulfide bonds (Ishizuka, Kim, Barrantes Gomez, West, Cresswell, Sadegh-Nasseri, research in progress). Overall, these findings show that epitope hierarchy is likely established during competition for binding to the MHC II groove, and as such, any factor that can cause an increase in longevity of an epitope, or its rapid capture might contribute to its emergence as dominant.

4.5. Immunodominance of an epitope is not absolute

It has been an accepted dogma that once an epitope gains the title '*immunodominant*', it is always immunodominant. However, when this concept was tested directly by a series of experiments using our reductionist antigen processing system, followed by *in vivo* validation of

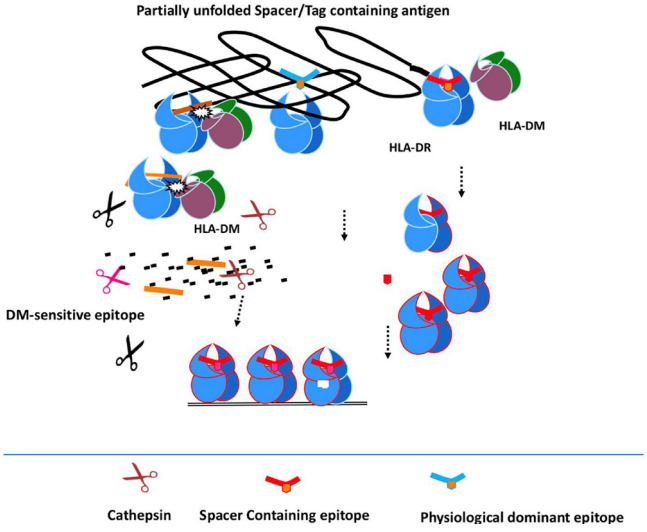

Fig. 1. Model for preferential capture of an artificial chimeric epitope *versus* a physiological one. A chimeric spacer/tag containing epitope, place at C-or N-termini of an antigen, might be preferentially captured by MHC II because of an easier access and having a suitable MHC II groove binding structure. While the physiological dominant epitope is hard to reach by MHC II, other non-dominant epitopes are sensitive to DM, and are prone to proteolysis, while the spacer containing epitope that is DM-insensitive accumulates.

the findings, we did not find support for the dogma. On the contrary, we found that small changes in antigen structure, as small as adding spacer sequences commonly used for attachment of a His-tag to the C- or N-termini of antigens, distort the processes that lead to the selection of the actual physiological epitopes. In three tested recombinant proteins, each having a different spacer, we found that the selected immunodominant epitopes were chimeric sequences composed in part from spacer as well as the natural antigen (Fig. 1).

Next, we found that the immunodominant epitope of one antigen might become subdominant or cryptic when other more robust epitopes are simultaneously present. It appears that when proteins are being simultaneously processed, a race for binding to the groove of MHC II is ongoing. A combination of factors contributes to the emergence of one epitope becoming dominant. We found that the location of an epitope at the C- or N-termini of proteins might increase the likelihood of its capture by the MHC II, as well as to proteolysis by the processing enzymes (Kim et al., 2014, 2017).

The concept of epitope accessibility also sets the rules for the emergence of epitopes that include the spacer sequences as dominant epitopes. Accessibility of an epitope combined with the right amino acid sequences to form DM resistant complexes with MHC class II, can establish dominance over the other epitopes by being presented at higher quantities to T cells (Kim et al., 2014). The above findings highlight the underlying principles for immunodominance for MHC class II by providing direct evidence for the contribution of factors intrinsic to antigenic structure or epitope accessibility. Notwithstanding, these observations suggest that determinant *hierarchy* is established in the antigen processing compartments rather than at T cell level.

Thus, these findings tell a cautionary tale to vaccine designers (Kim and Sadegh-Nasseri, 2015; Yewdell and Del Val, 2004), warning against the assumption that placement of dominant determinants in any context would not affect their selection as dominant epitopes, or mixing several vaccines together might result in effective immunity against all the immunogens in the mix.

Competing financial interests

SS-N is the author an issued patent no. 8,916,340 B2 entitled "Method for Identifying and Validation Dominant T-helper Cell Epitopes using an HLA-DM-assisted Class II Binding System" utilized in this paper. Other author has no competing financial interest.

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