ANTIGEN PRESENTATION FUNCTION OF TYPE-I DIABETES ASSOCIATED HLA-DQ MOLECULES

by

Zemin Zhou

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

in

Microbiology and Immunology

Department of Pathology

The University of Utah

May 2016

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of **Zemin Zhou**

Dates at right indicate the members' approval of the dissertation.

Peter E. Jensen, Chair	**4/12/2016** Date Approved
Gerald J. Spangrude, Member	**1/29/2016** Date Approved
Matthew A. Williams, Member	**4/12/2016** Date Approved
Ryan M. O'Connell, Member	**4/12/2016** Date Approved
Julio Delgado, Member	**1/29/2016** Date Approved
Attila Kumánovics, Member	**1/29/2016** Date Approved
Xiao He, Member	**4/12/2016** Date Approved

The dissertation has also been approved by **Peter E. Jensen**, Chair of the Department/School/College of **Pathology**, and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Autoreactive CD4+ T cells initiate the autoimmune disease Type-1 diabetes (T1D). Expression of DQ2, DQ8, and DQ2/8 trans-dimers are highly prevalent in T1D. However, the underlying molecular mechanisms are poorly understood. HLA-DM is essential for editing peptides bound to Major Histocompatibility Complex class II (MHCII) on antigen presenting cells, thus influencing the repertoire of peptides mediating selection and activation of CD4+ T cells. Here we explore the structural characteristics of DQ and the role of DM function, as well as the potential molecular mechanism of DM editing, as they may contribute to an understanding of autoreactive CD4+ T cell development in T1D.

Cells coexpressing DM with these DQ molecules were observed to express elevated levels of class II-associated invariant chain peptides (CLIP), consistent with HPLC-MS/MS analysis of eluted peptides. Assays with purified recombinant soluble proteins further confirmed that T1D-associated DQ2 and DQ8 are resistant to DM editing. DM sensitivity was enhanced in mutant DQ8 with disruption of hydrogen bonds (H-bonds) that stabilize DQ8 near the DM-binding region.

Compared to T1D nonassociated DQ1 and DQ6, the percentages of shared peptides among T1D-associated DQ molecules were significantly higher. Predicted peptide binding motifs of T1D-associated DQ molecules shared charged anchor residues,

while hydrophobic anchors were present in DQ6 peptides. Competition binding assays and peptide dissociation rate measurements indicated that peptide with high affinity is necessary but not sufficient for DM editing resistance. DM editing dominates the stability of MHCII/peptide complexes in the cellular environment.

DM catalyzes peptide exchange in MHCII through a mechanism that has been proposed to involve the disruption of specific conserved H-bond in MHCII/peptide complexes. HLA-DR1 molecules with alanine substitutions at each of the six conserved H-bonding positions were expressed in cells, and susceptibility to DM editing was evaluated by measuring the release of CLIP. Our results support the conclusion that no individual component of the conserved H-bond network plays an essential role in the DM catalytic mechanism.

Taken together, our data support that the relative DM resistance in T1D-associated DQ molecules affects the peptide repertoires and preferential presentation of T1D-associated autoantigenic peptides may contribute to the pathogenesis of T1D.

To Qin, Angela and Andy

and

In memory of my father and father-in-law

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

ACKNOWLEDGEMENTS

This work would not have been accomplished without the support and contribution from so many individuals. First of all, I would like to thank Dr. Peter E. Jensen for providing me the opportunity to work with him in different roles in the past ten years. I thank him for providing a stimulating multidisciplinary environment and introducing me the field of immunology, which was new to me. I am also very grateful to him for the continuous support and guidance in my research and his confidence in me throughout the years, as well as his invaluable help and advice in my career.

I would like to thank the members of my large and strong thesis advisory committee, including Drs. Peter E. Jensen, Gerald J. Spangrude, Julio C. Delgado, Matthew A. Williams, Ryan M. O'Connell, Attila Kumánovics, and Xiao He, for their continuous guidance, invaluable suggestions, and enthusiastic encouragement during my graduate career. In particular, I would like to thank Dr. Delgado for his tremendous help on my research project and career development. I am grateful to Dr. Kumánovics for his help and recognition in my career development. I am also grateful to Dr. Williams for his guidance of research and critical reading of the manuscript. Especially, I would like to thank Dr. Xiao He for his constant input in supporting my research and proofreading of the manuscript. He is also a close friend who provided massive help in my life.

I want to thank Dr. Alan L. Rockwood for his help on the analysis of peptidomes of T1D associated DQ molecules. I would like to thank Dr. Kuan Y. Chang for the prediction of peptide binding motifs of T1D associated DQ molecules. I am also grateful to Dr. Adam P. Barker for his massive resources, and continuous technical support and advice. In addition, I would like to give my thanks to Dr. Mathew F. Cusick for his help on providing human B cell lines and especially for peripheral blood samples.

I am very grateful to all the former and current members of the Jensen lab. I have considerable gratitude to Dr. Lisa M. Reed-Loisel for her kind help and technical training in the beginning of my research; thanks to Dr. Kari A. Callaway for her contribution to the DM mechanism project and to the building of Fluorescence Polarization platform; thanks to Hernando Escobar for his help in identification of the eluted peptides by HPLC-MS/MS; thanks to Dr. Eugene V. Ravkov for his help on the techniques of MHC tetramer and lentiviral transduction; thanks to Hu Dai and Xiaomin Wang for their valuable help with my experiments. Especially, I would like to thank Eduardo Reyes-Vargas for his help in the elution of peptides from T1D-associated DQ molecules. He is also a perfect cofighter against the difficulties in our research on DM mechanism and a continuous source for invaluable academic discussions and language training.

Furthermore, I would like to thank Allison Boyer, Kimberly Antry, Shannon Ritzman, and all administrative staff, especially the path-IT team. Their help made my study in the Department of Pathology much easier. I am grateful to the Microbiology and Immunology Ph.D. Program for financial support during my graduate career.

Last but not least, I would like to thank my family. Thanks to my two kids, Angela and Andy, for their love, with tears and smiles, and silliness, naughtiness,

smartness, happiness, plus their Swahili slogan "*HAKUNA MATATA*". Especially, I would like to thank my beloved wife, Qin, for her rock solid support, endless love, and harmonic accompaniment in our colorful (including blue), wonderful and meaningful life.

CHAPTER 1

INTRODUCTION: STRUCTURAL CHARACTERISTICS OF HLA-DQ THAT MAY IMPACT DM EDITING AND SUSCEPTIBILITY TO TYPE-1 DIABETES

Originally published in *Frontiers in Immunology*. Zemin Zhou and Peter E. Jensen. 2013. Structural characteristics of HLA-DQ that may impact DM editing and susceptibility to type-1 diabetes. Front. Immunol. 4:262. doi: 10.3389/fimmu.2013.00262.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 29 August 2013
doi: 10.3389/fimmu.2013.00262

Structural characteristics of HLA-DQ that may impact DM editing and susceptibility to type-1 diabetes

Zemin Zhou and Peter E. Jensen*

ARUP Laboratories, Department of Pathology, University of Utah, Salt Lake City, UT, USA

Edited by:
Lawrence J. Stern, University of Massachusetts Medical School, USA

Reviewed by:
Masaaki Murakami, Osaka University, Japan
Justine Mintern, Walter and Eliza Hall Institute, Australia

***Correspondence:**
Peter E. Jensen, ARUP Laboratories, Department of Pathology, University of Utah, 15 North Medical Drive East Suite 1100, Salt Lake City, UT 84112-5650, USA
e-mail: peter.jensen@path.utah.edu

Autoreactive CD4+ T cells initiate the chronic autoimmune disease Type-1 diabetes (T1D), in which multiple environmental and genetic factors are involved. The association of HLA, especially the DR-DQ loci, with risk for T1D is well documented. However, the molecular mechanisms are poorly understood. In this review, we explore the structural characteristics of HLA-DQ and the role of HLA-DM function as they may contribute to an understanding of autoreactive T cell development in T1D.

Keywords: type-1 diabetes, HLA-DQ, HLA-DM, invariant chain, autoreactive T cells, negative selection

INTRODUCTION

Multiple factors contribute to the chronic autoimmune disease type-1 diabetes (T1D) characterized by selective destruction of pancreatic β cells. To complement β cell deficiency, life-long insulin replacement is required to maintain glucose metabolism. There is evidence that both genetic and environmental factors contribute to the etiology of T1D. Genome wide association analysis data indicate that the highly polymorphic major histocompatibility complex (MHC), including both MHC class I and class II (MHCI and MHCII), contributes approximately 50% of genetic susceptibility to T1D (1). Individuals with MHCII DR3-DQ2 and DR4-DQ8 haplotypes have a significantly higher risk of T1D and DQ6 (DQA1*0102/DQB1*0602) is dominantly protective in Caucasians, Mexicans, and other Latin American populations (1–3). A number of studies have demonstrated the peptide-binding specificity of DQ8 as well as T cells from T1D that recognize pancreatic autoantigens presented by DQ8 (4–8). Compared with the DQ2 and DQ8 homozygous individuals, DR3-DQ2/DR4-DQ8 heterozygotes (DRB1*0301-DQA1*0501-DQB1*0201/DRB1*04-DQA1*0301-DQB1*0302) have the highest risk in whites of European and Northern African decent (9). Haplotype sharing analysis in siblings also shows that the risk for T1D is dramatically increased in DR3/4-DQ2/8 siblings (10). Another study of 607 Caucasian families and 38 Asian families further confirmed the association of DQ2 and DQ8, especially the trans-dimer DQ2-8, with the highest risk of T1D (11). These striking observations raise several open questions: (a) what structural features distinguish DQ molecules associated with risk for T1D; (b) why do heterozygotes have even greater risk for T1D than individuals homozygous for DQ2 or DQ8; (c) how do the autoreactive CD4+ T cells that mediate β cell destruction develop and escape negative selection in the thymus. In this review, we will focus on the function of MHCII molecules and their role in selection of autoreactive CD4+ T cells.

MHCII FUNCTION IN ANTIGEN PRESENTATION

In the adaptive immune system, MHCI and MHCII molecules play critical roles by presenting peptides on the surface of antigen presentation cells (APC) to select or activate CD8+ and CD4+ T cells, respectively (12). MHCI and MHCII share very similar structure in the peptide-binding groove and both can load with endogenous or exogenous peptides through two sets of non-covalent interactions: sequence dependent anchor-pocket interactions and conserved hydrogen-bond networks formed between the peptide and non-polymorphic amino acids in MHC. However, the peptide-binding groove of MHCII is open in both sides, compared with the closed binding site in MHCI; therefore, MHCII can present relatively longer peptides. Extra residues in the *N*-terminus of the bound peptide, such as P-1 and P-2, are important for the stability of MHCII/peptide complexes (13). MHCII molecules initially assemble with invariant chain (Ii) in the endoplasmic reticulum (ER) and the peptide-binding groove is occupied by a disordered region of Ii to prevent the loading of other ligands in the ER. After translocation into late endosomal compartments, Ii is processed by endosomal proteases and a segment of Ii, CLIP (class II-associated Ii peptide), occupies the peptide-binding groove. The dissociation of CLIP from the peptide-binding groove is necessary for the loading of other peptides, which is accelerated by a non-classical MHC class II molecule, HLA-DM (DM) (14). DM can catalyze multiple subsequent rounds of peptide exchange, editing the repertoire of presented peptides, and favoring the most stable peptide complexes.

MOLECULAR MECHANISM OF DM-MEDIATED PEPTIDE EDITING AND ITS POTENTIAL ROLE IN T1D

The general function of DM is well defined but many questions have remained about its precise mechanism of action (14). The possibility that DM selectively disrupts conserved hydrogen bonds between peptide and MHCII had been proposed as a potential

mechanism (15, 16); however, subsequent analysis of substituted MHCII molecules with disrupted H-bonds ruled out this mechanism in its simplest form (17, 18). It has been suggested that the interaction of DM with MHCII activates the empty or inactive form of MHCII to be active for peptide loading (19, 20). MHCII molecules with an empty P1 pocket can associate with DM while the filled form has been reported to interact poorly with DM (21). Molecular dynamics simulation studies indicated that the peptide-binding groove in the bound, partially filled, or empty states are significantly different (22–24), indicating that the interaction of DM and MHCII might induce a conformational rearrangement of peptide-binding groove, especially the α53–65 region around P1 pocket of MHCII (19). Recent advances with the co-crystallization of DM and DR (25), and the co-crystallization of DM and DO (26), another non-classical MHCII that inhibits DM function (14), provide a significant advance in our understanding of the interaction of DM with MHCII, confirming that DM binding is associated with a major structural rearrangement of the MHCII α53–65 region (**Figure 1A**) that precludes occupancy of the region of the peptide-binding groove that normally accommodates the peptide *N*-terminus, including the P1 anchor residue.

Genetic studies of the limited polymorphisms of DMα and DMβ in different populations indicate that specific DM alleles are associated with T1D (27–29). Interestingly, patients with T1D show relatively high levels of CLIP on the surface of lymphocytes (30), and T1D-like NOD mice also display high CLIP levels (31), indicating that DM is inefficient in removing CLIP from specific MHCII molecules expressed in individuals with T1D and NOD mice. A natural deletion of arginine in α53 of DQ2 has

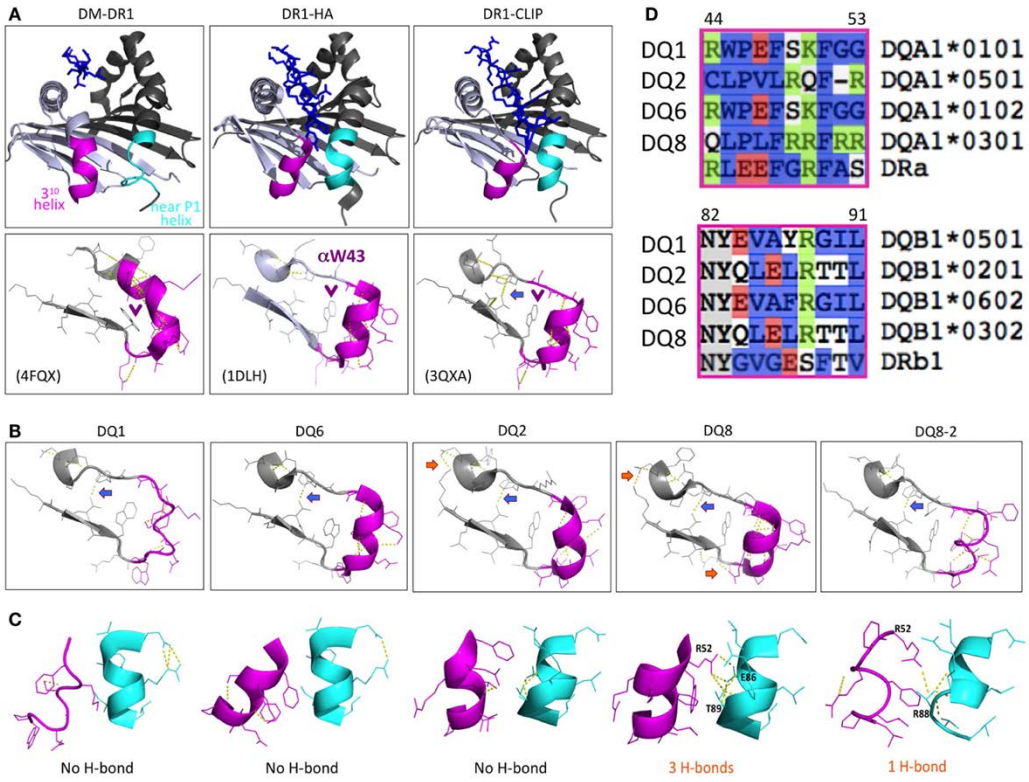


FIGURE 1 | Structure characteristics of DR1 and T1D sensitive, neutral, and protective DQ molecules. (A) The structure of DR1 showing with P1 pocket empty (left of upper panel, in co-structure of DM-DR1), bound with high affinity HA peptide (middle), and bound with low affinity CLIP (right). The purple and cyan colors show the conformational difference of the two helices near the P1 pocket of the DR1 peptide-binding groove in the crystal structures. The lower panel shows the H-bond between 3^{10} helix and β-sheet, and the αW43 position (purple arrow "→"). The unique H-bond in DR1-CLIP is showed by blue arrow "→" **(B)** Conformational difference of the 3^{10} helix, β-sheet, and inter-helix H-bond(s) in different DQ molecules. There is a conserved H-bond formed in all of the DQ molecules and DR1 bound with CLIP peptide (blue arrow "→"), indicating a similar status among these molecules. Also, extra H-bond(s) are found in T1D-associated DQ2 and DQ8 (orange arrow "→"), suggesting a stabilized conformation in this region, compared with DQ1 or DQ6. **(C)** Conformational differences in the α chain 3^{10} helix, the β chain near the P1 helix, and the H-bond(s) interactions between the two helices. DQ8 have 3 H-bonds formed between the two helices, and DQ8-2 has 1 H-bond, compared with DQ1, DQ2, and DQ6, with no H-bonds. **(D)** Sequence comparison of different DQ molecules and DR1 in the helix regions.

been demonstrated to reduce affinity for DM, explaining inefficient DM-mediated peptide exchange in T1D-associated DQ2 molecules (32, 33), further supporting the idea that inefficient DM editing may play a critical role in T1D-associated autoreactive CD4+ T cell development (32, 34). The coincidence of high CLIP expression might be a general indicator of poor DM editing function with T1D-associated DQ molecules, and it is also plausible that high levels of CLIP select CD4 T cells are cross-reactive and autoreactive. Interestingly, Ii deficient NOD mice are protected from T1D (35), providing further evidence for the potential role of CLIP in autoreactive T cell development; however, there is no direct evidence currently supporting this hypothesis.

STRUCTURAL CHARACTERISTICS OF T1D-ASSOCIATED DQ MOLECULES

The structure of the T1D sensitive, neutral, and protective DQ molecules, including DQ2 (PDB ID: 1S9V) (36), DQ8 (1JK8, 2NNA, and 4GG6) (37–39), DQ8-2 (4D8P) (40), DQ1 (3PL6) (41), and DQ6 (1UVQ) (42), have been recently solved. These DQ molecules share the general structural characteristics of MHCII with an open peptide-binding groove interacting with variable length peptides through a nine-residue binding "core". In the core, preferred amino acids anchor the peptide at positions 1, 4, 6, 7, and 9 (32). However, the conformations of the 3^{10}-helix region (43), which is in the DM-MHCII contact surface (25) and affects the sensitivity of DM-MHCII interaction (43), are apparently variable among the different DQ structures (**Figures 1A,B**). Interestingly, there are 3 H-bonds formed between the two helices in the α and β chains of DQ8 and 1 H-bond in DQ8-2, but no H-bond in the low T1D risk DQ1 or DQ6 molecules (**Figure 1C**). The conformation of the two helices and the number of inter-helix H-bonds in DQ8 are not dependent on the sequence specificity of bound peptide (37–39). Sequence comparison of the helical regions of the α and β chains among these DQ molecules shows that, in T1D-associated DQ2 and DQ8, the 3^{10} helix of the α chain includes several positively charged residues and the helix of the β chain has some negatively charged or uncharged hydrophilic residues with the potential to form H-bond(s); while in DQ1 and DQ6, those residues are hydrophobic (**Figure 1D**). The structure differences between DQ8 and other DQ molecules indicates that H-bond(s) might play a role in regulation of the sensitivity to DM editing by further stabilizing the DM contact region, providing an energetic barrier to formation of the DM-bound conformation. The structural differences between DQ8 and DQ2 suggest that different mechanisms might be responsible for the relative inefficiency of DM-mediated peptide editing in these molecules (33). The sensitivity of the T1D-associated DQ8 and DQ8-2 molecules to DM editing, and the potential inter-helix H-bond(s) or other structural features that might impact DM catalytic potency warrant further investigation.

It is still unclear why heterozygosity for DQ2/8 confers exceptionally high risk for T1D. APC in individuals with this haplotype co-express four distinct DQ molecules, including the trans-encoded DQ2-8 and DQ8-2 mixed haplotype molecules and the parental DQ2 and DQ8 proteins. Peptides eluted from the 293T cells expressing different DQ molecules show that the peptide-binding motifs of these DQ molecules are unique (8), supporting the hypothesis that the trans-dimers in heterozygotes might confer risk through independent presentation of specific self-peptides (44, 45). However, it is also possible that the higher risk of DQ2/8 heterozygous is due to an expanded repertoire of presented self-peptides by the combination of four DQ molecules. A study comparing gluten-specific T cells from Celiac disease patients demonstrated the potential for T cells to cross-react with DQ8 and the DQ2-8 trans-dimer (46), raising the possibility that T cell cross-reactivity might somehow contribute to the etiology of autoimmunity associated with DQ2/8 heterozygosity. Further studies are needed to explore these various possibilities.

THE DEVELOPMENT OF T1D-ASSOCIATED AUTOREACTIVE T CELLS

A big challenge in this field is to understand how autoreactive T cells develop, survive negative selection, and become activated to mediate tissue damage. In the thymus, the autoimmune regulator (Aire) regulates the ectopic expression of "tissue-restricted" antigens in medullary thymic epithelial cells (mTECs). The fate of thymocytes is determined by the affinity of expressed T cell receptor (TCR) for self-peptide-MHC complexes (47). Theoretically, the T cell precursors that bind strongly to self-peptide-MHC complex on thymic dendritic cells (DCs) and mTECs will be deleted, and all remaining mature T cells are self-tolerant. However, the identification of autoreactive T cells in T1D patients, and even in healthy subjects, indicates that negative selection in the thymus is incomplete (48). Several mechanisms have been proposed for inefficient deletion of autoreactive T cells in the thymus, including differences in autoantigen expression in the thymus and periphery, autoantigen posttranslational and posttranscriptional modification, autoantigen polymorphisms (49), and mechanisms through which key self-peptides can be presented on the cell surface through alternative pathways (34), or as a result of poor DM editing function (32). In addition, T cell cross-reactivity between microbial and self-antigens may also play an important role in the development of autoimmunity (50).

Based on current findings, we postulate that the T1D-associated DQ molecules (DQ2, DQ8, and the DQ2/8 trans-dimers) share a common feature, a relative resistance to DM-mediated peptide exchange, and editing. This impacts antigen presentation in two ways (**Figure 2**). A substantially increased fraction of MHCII molecules escape even one round of peptide exchange, resulting in high levels of CLIP presentation in the periphery and presumably also in the thymus. Secondly, a reduction in the efficiency of further peptide editing may lead to presentation of an array of relatively unstable peptide complexes. High levels of CLIP in the thymus might result in positive selection of T cells that cross-reactive with autoantigens in the periphery, or a reduction in the negative selection of self-reactive T cells, as is seen in the extreme case in mice with targeted deletion of DM (51). Increased presentation of unstable self-peptide complexes might also lead to inefficient negative selection and survival of T cells with a capacity to be activated in the periphery under conditions where the concentration of pancreatic β cell antigens is high. Alternatively, unstable complexes may be more susceptible to DM-independent peptide exchange in the periphery, promoting the activation of "type B" T cells that recognize β cell peptides bound to MHCII through an alternative register or conformation generated through alternative presentation pathways (34). These potential mechanisms may contribute

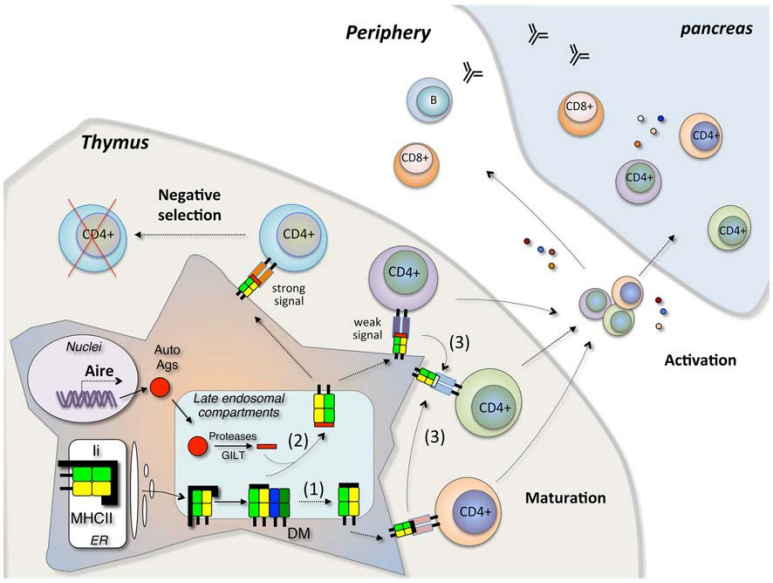


FIGURE 2 | Model of autoreactive CD4+ T cell development in T1D. In the thymus, Aire regulates tissue-specific autoantigen expression. Autoantigen peptides are processed in the late endosomal compartment and loaded in the peptide-binding groove of MHCII by DM editing. In case of inefficient DM editing, the pre-bound CLIP peptide may escape peptide exchange, resulting high levels of CLIP presentation (1). Secondly, the inefficient DM editing may lead to presentation of both low affinity and high affinity peptides on the cell surface (2). The stable MHCII-peptide complex will deliver strong signal through the T cell receptor (TCR) and induce the deletion of CD4+ T cells by negative selection, while the unstable MHCII-peptide complex will deliver weak signal and this signal may induce the positive selection of CD4+ T cells. Alternatively, the unstable complexes presented on the cell surface may be more susceptible to DM-independent peptide exchange (3). Those escaped CD4+ T cells will migrate into the periphery and initiate the β cell destruction in pancreas under certain conditions.

to the pathogenesis of T1D but further elements are needed to explain the specificity for β cells as opposed to other tissues. This is presumably related to the capacity of the T1D-associated DQ molecules to bind and present key β cell self-peptides.

CONCLUSION

Type-1 diabetes is a chronic autoimmune disease affected by both environmental and genetic factors. The mechanism(s) responsible for the high genetic risk associated with HLA genotype, and especially DQ2, DQ8, and DQ2/8 heterozygosity, remains poorly understood despite the obvious role of these molecules in antigen presentation. Reduced DM editing of T1D-associated DQ-peptide complexes combined with T cell cross-reactivity may contribute. Further analysis of structural and functional characteristics that distinguish disease-associated DQ molecules from neutral or protective alleles is likely to provide insights into the fundamental question of why HLA haplotype is such an important factor in determining risk for T1D.

REFERENCES

1. Gorodezky C, Alaez C, Murguia A, Rodriguez A, Balladares S, Vazquez M, et al. HLA and autoimmune diseases: type 1 diabetes (T1D) as an example. *Autoimmun Rev* (2006) **5**:187–94. doi:10.1016/j.autrev.2005.06.002
2. Cifuentes RA, Rojas-Villarraga A, Anaya JM. Human leukocyte antigen class II and type 1 diabetes in Latin America: a combined meta-analysis of association and family-based studies. *Hum Immunol* (2011) **72**:581–6. doi:10.1016/j.humimm.2011.03.012
3. Rojas-Villarraga A, Botello-Corzo D, Anaya JM. HLA-Class II in Latin American patients with type 1 diabetes. *Autoimmun Rev* (2010) **9**:666–73. doi:10.1016/j.autrev.2010.05.016
4. Ge X, Piganelli JD, Tse HM, Bertera S, Mathews CE, Trucco M, et al. Modulatory role of DR4- to DQ8-restricted CD4 T-cell responses and type 1 diabetes susceptibility. *Diabetes* (2006) **55**:3455–62. doi:10.2337/db06-0680
5. Geenen V, Louis C, Martens H. An insulin-like growth factor 2-derived self-antigen inducing a regulatory cytokine profile after presentation to peripheral blood mononuclear cells from DQ8+ type 1 diabetic adolescents: preliminary design of a thymus-based tolerogenic self-vaccination. *Ann N Y Acad Sci* (2004) **1037**:59–64. doi:10.1196/annals.1337.008
6. Liu J, Purdy LE, Rabinovitch S, Jevnikar AM, Elliott JF. Major DQ8-restricted T-cell epitopes for human GAD65 mapped using human CD4, DQA1*0301, DQB1*0302 transgenic IA(null) NOD mice. *Diabetes* (1999) **48**:469–77. doi:10.2337/diabetes.48.3.469
7. Mallone R, Brezar V, Boitard C. T cell recognition of autoantigens in human type 1 diabetes: clinical perspectives. *Clin Dev Immunol* (2011) **2011**:513210. doi:10.1155/2011/513210
8. van Lummel M, van Veelen PA, Zaldumbide A, de Ru A, Janssen GM, Moustakas AK, et al. Type 1 diabetes-associated HLA-DQ8 transdimer accommodates a unique peptide repertoire. *J Biol Chem* (2012) **287**:9514–24. doi:10.1074/jbc.M111.313940
9. van Autreve JE, Weets I, Gulbis B, Vertongen F, Gorus FK, van der Auwera BJ, et al. The rare HLA-DQA1*03-DQB1*02 haplotype confers susceptibility to type 1 diabetes in whites and is preferentially associated with early clinical disease onset in male subjects. *Hum Immunol* (2004) **65**:729–36. doi:10.1016/j.humimm.2004.04.004

10. Aly TA, Ide A, Jahromi MM, Barker JM, Fernando MS, Babu SR, et al. Extreme genetic risk for type 1A diabetes. *Proc Natl Acad Sci U S A* (2006) **103**:14074–9. doi:10.1073/pnas.0606349103
11. Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P, et al. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* (2008) **57**:1084–92. doi:10.2337/db07-1331
12. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* (2011) **11**:823–36. doi:10.1038/nri3084
13. O'Brien C, Flower DR, Feighery C. Peptide length significantly influences in vitro affinity for MHC class II molecules. *Immunome Res* (2008) **4**:6. doi:10.1186/1745-7580-4-6
14. Jensen PE. Recent advances in antigen processing and presentation. *Nat Immunol* (2007) **8**:1041–8. doi:10.1038/ni1516
15. Narayan K, Chou CL, Kim A, Hartman IZ, Dalai S, Khoruzhenko S, et al. HLA-DM targets the hydrogen bond between the histidine at position beta81 and peptide to dissociate HLA-DR-peptide complexes. *Nat Immunol* (2007) **8**:92–100. doi:10.1038/ni1414
16. Weber DA, Evavold BD, Jensen PE. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* (1996) **274**:618–20. doi:10.1126/science.274.5287.618
17. Ferrante A, Gorski J. Cutting edge: HLA-DM-mediated peptide exchange functions normally on MHC class II-peptide complexes that have been weakened by elimination of a conserved hydrogen bond. *J Immunol* (2010) **184**:1153–8. doi:10.4049/jimmunol.0902878
18. Zhou Z, Callaway KA, Weber DA, Jensen PE. Cutting edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. *J Immunol* (2009) **183**:4187–91. doi:10.4049/jimmunol.0901663
19. Denzin LK, Hammond C, Cresswell P. HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J Exp Med* (1996) **184**:2153–65. doi:10.1084/jem.184.6.2153
20. Grotenbreg GM, Nicholson MJ, Fowler KD, Wilbuer K, Octavio L, Yang M, et al. Empty class II major histocompatibility complex created by peptide photolysis establishes the role of DM in peptide association. *J Biol Chem* (2007) **282**:21425–36. doi:10.1074/jbc.M702844200
21. Anders AK, Call MJ, Schulze MS, Fowler KD, Schubert DA, Seth NP, et al. HLA-DM captures partially empty HLA-DR molecules for catalyzed removal of peptide. *Nat Immunol* (2011) **12**:54–61. doi:10.1038/ni.1967
22. Painter CA, Cruz A, Lopez GE, Stern LJ, Zavala-Ruiz Z. Model for the peptide-free conformation of class II MHC proteins. *PLoS ONE* (2008) **3**:e2403. doi:10.1371/journal.pone.0002403
23. Rupp B, Gunther S, Makhmoor T, Schlundt A, Dickhaut K, Gupta S, et al. Characterization of structural features controlling the receptiveness of empty class II MHC molecules. *PLoS ONE* (2011) **6**:e18662. doi:10.1371/journal.pone.0018662
24. Yaneva R, Springer S, Zacharias M. Flexibility of the MHC class II peptide binding cleft in the bound, partially filled, and empty states: a molecular dynamics simulation study. *Biopolymers* (2009) **91**:14–27. doi:10.1002/bip.21078
25. Pos W, Sethi DK, Call MJ, Schulze MS, Anders AK, Pyrdol J, et al. Crystal structure of the HLA-DM-HLA-DR1 complex defines mechanisms for rapid peptide selection. *Cell* (2012) **151**:1557–68. doi:10.1016/j.cell.2012.11.025
26. Guce AI, Mortimer SE, Yoon T, Painter CA, Jiang W, Mellins ED, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. *Nat Struct Mol Biol* (2013) **20**:90–8. doi:10.1038/nsmb.2460
27. Cucchi-Mouillot P, Lai S, Carcassi C, Sorba P, Stuart-Simoni M, Amoros JP, et al. Implication of HLA-DMA alleles in corsican IDDM. *Dis Markers* (1998) **14**:135–41. doi:10.1155/1998/705857
28. Sang YM, Yan C, Zhu C, Ni GC, Hu YM. [Association of human leukocyte antigen non-classical genes with type 1 diabetes]. *Zhonghua Er Ke Za Zhi* (2003) **41**:260–3.
29. Siegmund T, Donner H, Braun J, Usadel KH, Badenhoop K. HLA-DMA and HLA-DMB alleles in German patients with type 1 diabetes mellitus. *Tissue Antigens* (1999) **54**:291–4. doi:10.1034/j.1399-0039.1999.540313.x
30. Silva DG, Socha L, Correcha M, Petrovsky N. Elevated lymphocyte expression of CLIP is associated with type 1 diabetes and may be a useful marker of autoimmune susceptibility. *Ann N Y Acad Sci* (2004) **1037**:65–8. doi:10.1196/annals.1337.009
31. Bhatnagar A, Milburn PJ, Lobigs M, Blanden RV, Gautam AM. Nonobese diabetic mice display elevated levels of class II-associated invariant chain peptide associated with I-Ag7 on the cell surface. *J Immunol* (2001) **166**:4490–7.
32. Busch R, De Riva A, Hadjinicolaou AV, Jiang W, Hou T, Mellins ED. On the perils of poor editing: regulation of peptide loading by HLA-DQ and H2-A molecules associated with celiac disease and type 1 diabetes. *Expert Rev Mol Med* (2012) **14**:e15. doi:10.1017/erm.2012.9
33. Hou T, Macmillan H, Chen Z, Keech CL, Jin X, Sidney J, et al. An insertion mutant in DQA1*0501 restores susceptibility to HLA-DM: implications for disease associations. *J Immunol* (2011) **187**:2442–52. doi:10.4049/jimmunol.1100255
34. Mohan JF, Unanue ER. Unconventional recognition of peptides by T cells and the implications for autoimmunity. *Nat Rev Immunol* (2012) **12**:721–8. doi:10.1038/nri3294
35. Mellanby RJ, Koonce CH, Monti A, Phillips JM, Cooke A, Bikoff EK. Loss of invariant chain protects nonobese diabetic mice against type 1 diabetes. *J Immunol* (2006) **177**:7588–98.
36. Kim CY, Quarsten H, Bergseng E, Khosla C, Sollid LM. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proc Natl Acad Sci U S A* (2004) **101**:4175–9. doi:10.1073/pnas.0306885101
37. Broughton SE, Petersen J, Theodossis A, Scally SW, Loh KL, Thompson A, et al. Biased T cell receptor usage directed against human leukocyte antigen DQ8-restricted gliadin peptides is associated with celiac disease. *Immunity* (2012) **37**:611–21. doi:10.1016/j.immuni.2012.07.013
38. Henderson KN, Tye-Din JA, Reid HH, Chen Z, Borg NA, Beissbarth T, et al. A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity* (2007) **27**:23–34. doi:10.1016/j.immuni.2007.05.015
39. Lee KH, Wucherpfennig KW, Wiley DC. Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type 1 diabetes. *Nat Immunol* (2001) **2**:501–7. doi:10.1038/88694
40. Tollefsen S, Hotta K, Chen X, Simonsen B, Swaminathan K, Mathews II, et al. Structural and functional studies of trans-encoded HLA-DQ2.3 (DQA1*03:01/DQB1*02:01) protein molecule. *J Biol Chem* (2012) **287**:13611–9. doi:10.1074/jbc.M111.320374
41. Sethi DK, Schubert DA, Anders AK, Heroux A, Bonsor DA, Thomas CP, et al. A highly tilted binding mode by a self-reactive T cell receptor results in altered engagement of peptide and MHC. *J Exp Med* (2011) **208**:91–102. doi:10.1084/jem.20100725
42. Siebold C, Hansen BE, Wyer JR, Harlos K, Esnouf RE, Svejgaard A, et al. Crystal structure of HLA-DQ0602 that protects against type 1 diabetes and confers strong susceptibility to narcolepsy. *Proc Natl Acad Sci U S A* (2004) **101**:1999–2004. doi:10.1073/pnas.0308458100
43. Painter CA, Negroni MP, Kellersberger KA, Zavala-Ruiz Z, Evans JE, Stern LJ. Conformational lability in the class II MHC 310 helix and adjacent extended strand dictate HLA-DM susceptibility and peptide exchange. *Proc Natl Acad Sci U S A* (2011) **108**:19329–34. doi:10.1073/pnas.1108074108
44. Koeleman BP, Lie BA, Undlien DE, Dudbridge F, Thorsby E, de Vries RR, et al. Genotype effects and epistasis in type 1 diabetes and HLA-DQ trans dimer associations with disease. *Genes Immun* (2004) **5**:381–8. doi:10.1038/sj.gene.6364106
45. Thorsby E. Invited anniversary review: HLA associated diseases. *Hum Immunol* (1997) **53**:1–11. doi:10.1016/S0198-8859(97)00024-4
46. Kooy-Winkelaar Y, van Lummel M, Moustakas AK, Schweizer J, Mearin ML, Mulder CJ, et al. Gluten-specific T cells cross-react between HLA-DQ8 and the HLA-DQ2alpha/DQ8beta transdimer. *J Immunol* (2011) **187**:5123–9. doi:10.4049/jimmunol.1101179
47. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* (2005) **5**:772–82. doi:10.1038/nri1707
48. Mathis D, Benoist C. Aire. *Annu Rev Immunol* (2009) **27**:287–312. doi:10.1146/annurev.immunol.25.022106.141532
49. Roep BO, Peakman M. Antigen targets of type 1 diabetes autoimmunity. *Cold Spring Harb Perspect Med* (2012) **2**:a007781. doi:10.1101/cshperspect.a007781

50. Sewell AK. Why must T cells be cross-reactive? *Nat Rev Immunol* (2012) **12**:669–77. doi:10.1038/nri3279
51. Fung-Leung WP, Surh CD, Liljedahl M, Pang J, Leturcq D, Peterson PA, et al. Antigen presentation and T cell development in H2-M-deficient mice. *Science* (1996) **271**: 1278–81. doi:10.1126/science.271.5253.1278

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 July 2013; paper pending published: 02 August 2013; accepted: 18 August 2013; published online: 29 August 2013.

*Citation: Zhou Z and Jensen PE (2013) Structural characteristics of HLA-DQ that may impact DM editing and susceptibility to type-1 diabetes. Front. Immunol. **4**:262. doi: 10.3389/fimmu.2013.00262*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

CHAPTER 2

TYPE 1 DIABETES ASSOCIATED HLA-DQ2 AND DQ8 RELATIVELY MOLECULES ARE RESISTANT TO HLA-DM MEDIATED RELEASE OF INVARIANT CHAIN-DERIVED CLIP PEPTIDES

Originally published in *European Journal of Immunology*. Zemin Zhou, Eduardo Reyes-Vargas, Hernando Escobar, Brant Rudd, Alan L. Rockwood, Julio C. Delgado, Xiao He, Peter E. Jensen. 2015. Type 1 diabetes associated HLA-DQ2 and DQ8 molecules are relatively resistant to HLA-DM mediated release of invariant chain-derived CLIP peptides. *Eur J Immunol,* doi: 10.1002/eji.201545942.

Summary

HLA-DM is essential for editing peptides bound to Major Histocompatibility Complex class II (MHCII), thus influencing the repertoire of peptides mediating selection and activation of CD4+ T cells. Individuals expressing HLA-DQ2 or DQ8, and DQ2/8 trans-dimerss, have elevated risk for type 1 diabetes (T1D). Cells coexpressing DM with these DQ molecules were observed to express elevated levels of CLIP (Class II associated invariant chain peptide). Relative resistance to DM-mediated editing of CLIP was further confirmed by HPLC-MS/MS analysis of eluted peptides, which also demonstrated peptides from known T1D-associated autoantigens, including a shared epitope from ZnT8 that is presented by all four major T1D-susceptible DQ molecules. Assays with purified recombinant soluble proteins confirmed that DQ2-CLIP complexes are highly resistant to DM editing, whereas DQ8-CLIP is partially sensitive to DM, but with an apparent reduction in catalytic potency. DM sensitivity was enhanced in mutant DQ8 molecules with disruption of hydrogen bonds that stabilize DQ8 near the DM-binding region. Our findings show that T1D-susceptible DQ2 and DQ8 share significant resistance to DM editing, compared with control DQ molecules. The relative resistance of the T1D-susceptible DQ molecules to DM editing and preferential presentation of T1D-associated autoantigenic peptides may contribute to the pathogenesis of T1D.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by selective destruction of pancreatic β cells. Multiple genetic and environmental factors have been implicated in the etiology of T1D. Among these factors, certain MHC haplotypes are

associated with a high risk for development of T1D, with a potential role of MHC molecules in the selection or activation of autoreactive T cells involved in T1D [1]. Genetic studies have implicated DQ2 (DQA1*0501/DQB*0201) and DQ8 (DQA1*0301/DQB1*0302) as important risk factors. DQ2/8 heterozygotes, expressing two potential DQ trans-dimerss as well as DQ2 and DQ8, have a further elevation in risk. By contrast, DQ6 (DQA1*0102/DQB*0602) has a dominant protective effect, while DQ1 (DQA1*0101/DQB1*0501) is neutral to T1D [2-8]. Beyond speculation, the mechanisms responsible for the disease risk associated with expression of specific MHC class II (MHCII) alleles remain to be established.

MHCII molecules present endogenous or exogenous peptides on the surface of APC to select or activate CD4+ T cells in the thymus or periphery [9]. Classical MHCII are initially assembled with the class II invariant chain (Ii) chaperone protein. Ii is partially released through a series of successive proteolytic events in endosomal compartments, leaving a fragment, CLIP, occupying the peptide-binding groove. Antigen presentation is critically dependent on the release of CLIP and exchange with antigenic peptides sampled in endosomal compartments, which is catalyzed by a nonclassical MHC protein HLA-DM. DM functions optimally at acidic pH to catalyze CLIP dissociation and peptide exchange. It can catalyze multiple rounds of peptide exchange, and preferentially edit unstable peptide complexes, favoring the presentation of highly stable peptide complexes under physiological conditions. Thus, DM plays a critical role in editing the repertoire of peptides available for selection and recognition by CD4+ T cells [10, 11]. It has been noted that CD4+ T cells with specificity for self-peptides with low affinity for MHC can escape negative selection in the thymus [12-16]. The identification

of autoreactive CD4+ T cells recognizing low-affinity peptides in both nonobese diabetic (NOD) mouse [17-19] and human [20] autoimmune diabetes suggests the possibility that incomplete DM editing may contribute to presentation of a unique repertoire of peptides important to the pathogenesis of T1D.

The recently published cocrystal structures of DM bound to the MHCII molecule DR1 demonstrate a major structural rearrangement in the region of the DR1 peptide-binding groove that accommodates the N-terminal segment of bound peptide proximal to the DM contact surface [21]. This conformation change precludes full occupancy of the peptide-binding groove. Polymorphisms in DM contact residues have the potential to impact DM binding affinity and catalytic potency [22]. In addition, polymorphisms in MHCII that might impact the conformational stability in the region that undergoes a major structural transition might impact DM editing function [23]. The potential effects of MHCII polymorphism on susceptibility to DM editing have been explored to a limited extent. Notably, it has been reported that DQ2 is a poor substrate for DM, due to a natural deletion of arginine (α53) in the α chain of DQ2 [22, 24]. This allele is associated with celiac disease [25] as well as T1D. However, that naturally deleted residue of DQ2 is intact in DQ8, and the susceptibility of DQ8 to DM editing is unknown. Different mechanism(s) might be responsible for the association of DQ8 with increased risk for T1D. In addition to the obvious impact on peptide binding specificity, MHCII polymorphisms might contribute to the pathogenesis of CD4+ T cell autoimmunity by influencing a variety of steps in the peptide loading, editing, and presentation process, possibly enabling alternative mechanisms for presentation of key autoantigens or selection of autoreactive T cells [22, 26, 27].

Given the potential importance of DM in modulating the repertoire of peptides that mediate thymic selection and/or peripheral activation of autoreactive T cells in T1D [17, 18, 20], we investigated the sensitivity of the major T1D-susceptible DQ molecules to DM editing. We coexpressed DQ, Ii, and DM molecules in 293T cells to compare DM mediated release of Ii derived CLIP peptides. We found that DQ2, DQ8, or DQ2/8 trans-dimerss were more resistant to DM editing than T1D-protective DQ6 or the T1D-neutral DQ1 molecules, as demonstrated by retention of CLIP. HPLC-MS/MS analysis confirmed the presence of DM-resistant CLIP, as well as known T1D-associated autoantigenic peptides specifically binding to T1D-susceptible DQ molecules. Fluorescence polarization (FP) assays with purified recombinant soluble DQ proteins demonstrated that DQ8-CLIP is partially sensitive to DM, but with an apparent reduction in catalytic potency. DM sensitivity was enhanced in mutant DQ8 molecules with disruption of hydrogen bonds that stabilize the DQ8 in the DM-binding region. We hypothesize that a relative DM editing resistance of T1D-associated DQ2, DQ8, and DQ2/8 trans-dimer molecules may contribute to the pathogenesis of T1D, by affecting the selection and activation of autoreactive CD4+ T cells.

Results

Relative resistance to DM editing of T1D-susceptible cis- and trans-DQ molecule

To investigate the DM editing sensitivity of the T1D-susceptible DQ2, DQ8, and DQ2/8 molecules, we expressed each of the four DQ molecules or the controls, T1D-protective DQ6 or T1D-neutral DQ1 molecules, in the 293T cells, which do not express

endogenous Ii or MHCII. All six DQ molecules were efficiently expressed at the cell surface in the absence or presence of Ii, confirming that the two trans-dimerss potentially expressed in DQ2/8 heterozygotes, DQ2-8 and DQ8-2, are efficiently assembled and transported to the cell surface [28, 29] (Figure 2.1). Cell surface CLIP levels, measured by flow cytometry with CerCLIP.1, a mAb specifically recognizing CLIP1 peptides with N-terminal extensions (Figure 2.S1), were high for each of the T1D-susceptible DQ molecules and the DQ6 control on cells coexpressing Ii in the absence of DM, demonstrating functional association with Ii, proteolytic processing to generate CLIP, and inefficient release of CLIP in the absence of DM (Figure 2.1). Coexpression of DM resulted in a complete loss of cell surface CLIP on cells expressing the control molecule DQ6. By contrast, DQ2-CLIP complexes were relatively resistant to DM editing, confirming previous reports demonstrating that DQ2 is a poor substrate for DM [22, 24]. Strikingly, the three other T1D-susceptible DQ molecules (DQ8, DQ2-8, and DQ8-2) were also observed to retain high levels of cell surface CLIP in the presence of DM (Figure 2.1, Figure 2.S2E). The cell lines used in these experiments expressed similar levels of DQ, Ii, and DM (Figure 2.1, Figure 2.S2D). Interestingly, there is no detectable CLIP peptide presented by DQ1 even in the absence of DM (Figure 2.1A). A hierarchy in DM sensitivity was observed for these molecules, with DQ8 being the least sensitive, followed by DQ2-8, DQ2, and DQ8-2. However, all of the four T1D-susceptible DQ molecules were relatively resistant to DM editing of CLIP as compared with DQ6. Although 293T cells have been used in MHC class II antigen presentation studies [30], they are not professional antigen presenting cells (APC). To explore the generality of the results with 293T cells, we performed additional experiments with T2 lymphoblastoid

cells. The T2 cell line, a hybrid human B cell line with endogenous Ii expression but with the deletion of entire MHC class II region in the genome [31, 32], has been used extensively as a model professional APC. We expressed the six different DQ alleles in T2 cells in the absence or presence of HLA-DM and determined the effectiveness of DM in editing CLIP by flow cytometry. The results were consistent with those obtained with 293T cells (Figure 2.S3).

Direct analysis of DQ-bound CLIP and T1D-associated autoantigen-derived peptides

We next sought to confirm DM-resistant retention of CLIP by the four T1D-associated DQ molecules by direct identification of peptides eluted from DQ molecules. Peptides were eluted from affinity-purified DQ molecules isolated from 293T cells coexpressing Ii with or without DM, and samples were analyzed by HPLC-MS/MS. Consistent with the findings from flow cytometry, CLIP peptides were prominently presented by all DQ alleles in cells expressing Ii but not DM (Figure 2.2, left). A large number of nested peptides ranging from 9 to 24 amino acids in length were identified that contained the classical CLIP1 core sequence (boxed) [33] as well as the alternative CLIP2 register (gray highlight). Consistent with the flow cytometry results, CLIP was not detected in peptide eluates from cells expressing DQ6+Ii+DM, indicating efficient DM editing on DQ6-CLIP complexes (Figure 2.2, right). By contrast, CLIP1 was detected in peptide samples from cells coexpressing DM and each of the T1D-susceptible DQ molecules, supporting the conclusion that CLIP1 bound to these alleles is resistant to DM editing. Strikingly, Ii-derived CLIP peptides, including both CLIP1 and CLIP2 registers,

were also detected in peptide samples coexpressing DQ1 and Ii, but not in samples coexpressing DM, indicating that DQ1 molecules do present CLIP peptides and DM efficiently mediates the editing of DQ1-CLIP complexes (Figure 2.2). However, most of the CLIP1 peptides presented by DQ1 molecules lack the N-terminal extensions, the epitope recognized by the CerCLIP.1 mAb [34], which is consistent with the flow cytometry observations and CerCLIP.1 mAb specificity (Figure 2.1A, Figure 2.S1). CLIP2 peptides were highly represented in peptide samples eluted from DQ2 [35-37]. It is not known if DQ2-CLIP2 is generated by an alternative association of Ii with DQ2 in the endoplasmic reticulum (ER), or from rebinding of Ii fragments in this register in endosomal compartments [24]. Our results demonstrate that CLIP2 associates with DQ molecules other than DQ2, including the T1D-protective DQ6 and T1D-neutral DQ1 molecules. It is also interesting to note that CLIP2 peptides were also present in samples from cells expressing DQ2+Ii+DM, consistent with prior findings indicating the CLIP2 register has a relatively high affinity for DQ2 [35-37].

Previous reports (*http://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-24733/*) indicate that 293T cells express variable levels of some T1D-associated autoantigens, including heat shock protein 60 (HSP60), zinc transporter 8 (ZnT8), and insulinoma antigen-2 (IA-2) [38]. Interestingly, we identified peptides from these autoantigens in samples eluted from T1D-susceptible DQ molecules but not from T1D-neutral DQ1 or T1D-protective DQ6 (Supporting Information Table 2S.1). Strikingly, a peptide from ZnT8 was identified in samples from all four T1D-susceptible DQ molecules. Peptides with overlapping sequence from HSP60, were detected in DQ8, DQ2-8, and DQ8-2 samples. These peptide sequences, as well as an additional HSP60

peptide eluted from DQ2-8 and three IA-2 peptide sequences eluted from DQ8, DQ2-8, and DQ8-2 samples have been previously reported as positive stimulators in autoreactive CD4+ T cell activation assays (Table 2.1) [39-52]. We also confirmed that the ZnT8 peptide binds T1D-susceptible DQ molecules with relatively higher binding affinity than the pan-DQ binding peptide, CLIP1 (Figure 2.3). These results confirm that functionally relevant autoantigenic peptides can be identified by HPLC-MS/MS analysis of peptide isolated from cells expressing autoantigen proteins, although the physiologic processing mechanisms for presentation of these epitopes in professional APC might be different, and they raise the possibility that key self-peptide epitopes can be presented by multiple different T1D-susceptible DQ molecules.

DM catalytic potency in CLIP dissociation from soluble DQ6, DQ2, and DQ8

To further characterize the biochemical properties of these DQ molecules, soluble DQ (sDQ) proteins, sDQ6, sDQ2, and sDQ8 were generated. The sDQ-peptide complexes were preloaded with labeled CLIP peptide (^{488}CLIP), and peptide dissociation rates were measured as a function of DM concentration (Figure 2.4). DM efficiently catalyzed the dissociation of CLIP from the control DQ6 protein. Peptide dissociation rates increased with increasing DM concentration. Note that rates appeared to saturate with increasing DM concentration. This might reflect a relatively high affinity in DM binding to DQ6-CLIP complexes. By contrast, DQ2-CLIP complexes were resistant to DM activity, showing little enhancement in dissociation with high DM concentrations. An intermediate level of DM potency was observed with DQ8-CLIP complexes. A clear

catalytic effect is evident with DQ8, but the rate enhancement was less than that observed with DQ6 as judged by the initial slope of rate versus DM concentration. Furthermore, less rate saturation was observed, consistent with the idea that DM may have a lower affinity for DQ8-CLIP as compared with DQ6-CLIP. This is illustrated in normalized plots of fold-enhancement in rates versus DM concentration (Figure 2.4B, right). Thus, experiments with purified recombinant proteins demonstrate a striking resistance of DQ2 to DM-mediated CLIP editing and an intermediate DM-resistance phenotype for DQ8.

Contribution of unique hydrogen bonds in DQ8 to its DM-editing resistance

It has previously been demonstrated that a natural α53 deletion in the DQ2 α chain contributes to the resistance of this protein to DM catalytic activity. Introduction of Gly at position 53 in DQ2 restored DM sensitivity [22]. The natural deletion in DQ2α is positioned in the DM contact interface [21] and thus it may reduce the affinity of the DM interaction with DQ2 or alter the structure of the interface such that catalytic potency is reduced. This would also provide a structural explanation for the DM resistant phenotype of the DQ2-8 trans-dimer, which shares the α chain with DQ2. However, the DQ8 α chain does not have this natural deletion and the structural basis of DQ8 and DQ8-2 trans-dimer for a relative resistance to DM editing remained to be explored.

Comparison of the crystal structures of different DQ molecules, as well as DR1 bound to different peptides and the DM-DR1 cocrystal structure, demonstrates conformational differences in the helix-β-sheet region near the P1 pocket of the peptide binding groove, the DM interaction site [23]. Compared with the other DQ molecules,

there are multiple positively charged arginine residues in the 3^{10}-helix region of DQ8 α chain (Figure 2.5A). Notably, three extra hydrogen bonds (H-bonds) are present between the two helixes near the P1 pocket of DQ8 peptide binding groove (Figure 2.5A, B), as well as one H-bond in DQ8-2, but none in DQ1, DQ6, DQ2, or DQ2-8 [23]. To test whether these H-bonds contribute to the relative resistance of DQ8 to DM editing, we replaced the residues in the DQ8 β chain that participate in these H-bonds with the amino acids present in DQ6, with the goal of disrupting the extra H-bond(s) formed with the 3^{10}-helix of DQ8α. The mutant DQ8 proteins were expressed with Ii in the absence or presence of DM in 293T cells (Figure 2.5C). The βT89G mutation, intended to disrupt one H-bond between αR52 and βT89, resulted in little change in DM sensitivity, such that CLIP levels remained high in the presence of DM. However, disruption of two H-bonds through the βE86A substitution resulted in restoration of a substantial increase in sensitivity to DM editing, as determined by comparing CLIP levels with and without coexpression of DM (Figure 2.5C, D). These findings support the idea that the additional H-bonds between the α and β chain helices in DQ8 may contribute to a phenotype of relative resistance to DM editing, possibly by stabilizing the region of MHCII that undergoes a marked conformation transition when bound to DM.

Discussion

Collectively, our findings indicate that the four T1D-susceptible DQ molecules, DQ2, DQ8, and the trans-dimerss expressed in DQ2/8 heterozygotes, share a distinct property of being relatively resistant to DM-mediated editing of CLIP, compared with the T1D-protective DQ6 or T1D-neutral DQ1 molecules that have the classical DM-sensitive

phenotype. The most direct evidence for this conclusion was obtained by analyzing surface CLIP levels on cells with or without coexpression of DM. We observed little or no reduction in CLIP levels on 293T cells coexpressing DM with DQ8 or DQ2-8, indicating a significant DM editing resistance of CLIP peptides. The phenotype was less striking for the DQ2 and DQ8-2 trans-dimer (Figure 2.1). Similar results were obtained with T2 lymphoblastoid cells; however, DQ2 and DQ2-8 were less sensitive to DM than DQ8 in these cells.

The α chain of DQ2 (DQA1*0501) contains a deletion of residue 53. Insertion of Gly at this position was shown to restore sensitivity to DM as judged by a marked increase in DM-mediated rate enhancement in the dissociation of a DQ2 binding peptide [22]. Given the location of α53 in the DM contact interface [21], it is tempting to consider the possibility that the natural α53 deletion in DQ2 (and the DQ2-8 trans-dimer) confers a general resistance to DM-mediated peptide exchange, reducing the impact of DM editing on the peptide repertoire presented by these molecules. However, additional structural elements may also contribute to the DM editing sensitivity. The DQA1*0501/DQB1*0301 DQ7 allele shares DQA1*0501 with DQ2, yet it is not associated with T1D. Other DQA alleles represented in human populations have a natural deletion at α53, including DQA1*0201 and DQA1*04. Further work will be needed to determine the DM sensitivity of the proteins generated from these alleles and the structural features that affect DM catalytic efficiency. There is an inverse relationship between the intrinsic stability of peptide complexes and sensitivity to DM-catalyzed peptide dissociation, such that less stable complexes are selectively edited, and more stable complexes survive for recognition by $CD4^+$ T cells [10, 53, 54]. Reduction in DM

editing might broaden the array of self-peptide complexes available to activate autoreactive T cells, and unstable complexes may be more susceptible to alternative peptide loading pathways for exogenous autoantigenic peptides [55].

In experiments with sDQ molecules, high concentrations of DM were observed to have little effect on the rate of dissociation of CLIP from sDQ2, whereas DM was clearly able to enhance CLIP dissociation from DQ8 in a dose-dependent manner. DM catalytic potency was intermediate between DQ2 and the DM-sensitive DQ6 control. The stability of sDQ8-CLIP complexes was observed to be considerably greater than that of sDQ6-CLIP, with intrinsic half-lives of 58 h and 7 h, respectively. The increased stability of sDQ8-CLIP might explain the relative resistance to DM. While DQ8 does not have the α53 deletion found in DQ2, it does have three H-bonds, not present in DQ2, DQ2-8, DQ1, or DQ6 molecules. The H-bonds connect the helices of the α and β chains near the DM contact site [23]. Disruption of two H-bonds through a βE86A substitution was observed to partially restore sensitivity to DM in cells expressing the substituted DQ protein. It is possible that the mutation reduces the intrinsic stability of DQ8 molecule as well as the complex of DQ8-CLIP, as a potential mechanism for enhancing DM sensitivity. Alternatively, the additional H-bonds in DQ8 might serve to stabilize the DM-binding region of DQ8, making it more difficult for the molecule to undergo the conformational rearrangement required for interaction with DM [21]. It is interesting to note that the DQ8-2 trans-dimer has one additional H-bond [23] that could potentially stabilize the conformation of the peptide-binding groove and contribute to the partial DM-resistant phenotype observed in cells expressing this mixed haplotype DQ molecule.

HPLC-MS/MS analysis confirmed that Ii-derived peptides lacking the core CLIP1

sequence and containing the alternative CLIP2 binding register were highly represented in samples from cells expressing DQ2+Ii. The CLIP2 loading mechanism is unknown, reflecting either an alternative mode of assembly of Ii with DQ2 or an exchange mechanism in which CLIP1 is replaced by CLIP2 [24]. CLIP2 peptides were also prominently represented in samples from cells expressing DQ1, DQ6, DQ8, or the DQ8/2 trans-dimers in the absence of DM expression. While the biological significance of CLIP2 is unknown, it is clear that CLIP2 is not uniquely associated with DQ2 or T1D-associated DQ molecules. The detection of CLIP peptides in DQ1 peptide samples by HPLC-MS/MS, but not by CerCLIP.1 mAb surface staining, reflects the specificity of the CerCLIP.1 mAb, for only long CLIP1 peptides with N-terminal sequence extensions. The peptide elution data confirm that DQ1-CLIP is sensitive to DM editing. However, the mechanism responsible for selective loading of DQ1 with short CLIP peptides remains to be investigated, possibly reflecting an increased sensitivity to proteolytic trimming.

A number of peptides from well known T1D-associated autoantigens were identified in the HPLC-MS/MS experiments with samples from T1D-susceptible DQ molecules. The 293T human embryonic kidney cell line fortuitously expresses some of the proteins relevant in T1D pathogenesis. Further work will be needed to determine whether specific peptides are dependent on DM for presentation, or sensitive to DM editing such that DM-independent presentation mechanisms would be required for T cell recognition. It was interesting to observe that several peptides were identified with multiple different T1D-susceptible DQ molecules, but not with DQ1 or DQ6. Strikingly, a ZnT8 peptide was eluted from cells expressing each of the T1D-susceptible DQ alleles. This raises the question of whether presentation of selected peptides by the multiple

different DQ molecules expressed on the cells in DQ8/2 heterozygotes might play a role in inducing cross-reacting autoreactive T cell responses in these individuals [56]. It was also interesting that most of the peptide sequences identified from T1D autoantigens had previously been defined as epitopes for autoreactive CD4+ T cell responses in T1D. This provides some level of validation that mass spectrometric analysis of MHCII-associated peptides may be useful in identifying candidate autoantigenic epitopes.

Overall, we conclude that the four T1D-susceptible DQ alleles have in common a shared resistance to DM-mediated editing of CLIP. Different structural elements appear to be responsible for this phenotype in DQ2, DQ8, and the DQ2/8 trans-dimers. Cell surface CLIP levels have been reported to be elevated in the peripheral blood of people with T1D [57]. It is possible that high levels of CLIP in the thymus might result in less efficient negative selection of a population of self-reactive T cells with the potential to participate in initiating or propagating immune responses to islet antigens. Alternatively, reduced susceptibility to DM editing function could potentially facilitate peripheral presentation of self-peptides loaded under inflammatory conditions through DM-independent mechanisms. Unanue and colleagues have demonstrated that DM-independent loading of free peptides can result in the formation of peptide-MHCII complexes with alterative binding registers [58]. These alternatively-generated peptide complexes can activate a population of unconventional “type B” T cells that are not eliminated through conventional mechanisms that maintain self tolerance [55]. While there is little doubt that the impact of MHCII polymorphism on CD4+ T cell repertoire selection and epitope specificity must play a major role in determining genetic susceptibility to specific autoimmune diseases, allelic differences that impact the peptide

loading pathway may also be important.

Materials and Methods

Expression of DQ, Ii and DM molecules and purification of full length and soluble DQ in 293T cells

Constructs encoding the full length α and β chains of DQ1, DQ2, DQ6, or DQ8 were fused with the T2A sequence [59] and cloned into pWPI vector (Addgene). Human Ii cDNA was cloned into another pWPI vector. The DM construct was cloned from MigR1-DM [60] and inserted behind the IRES site within lentiviral HIV\CS\Ub-ChIT (ChIT) vector provided by Dr. Vicente Planelles (University of Utah), which contains an mCherry gene driven by a human ubiquitin promoter (Figure 2.S2A, B). High-titer lentiviral supernatants were generated by transfection of 293T cells (ATCC), a human embryonic kidney cell line having no endogenous expression of Ii, DM, or DQ, with pWPI-DQ, pWPI-Ii, or ChIT-DM construct, as previously described [61]. The 293T transductants stably expressing DQ (DQ only) or DQ and Ii (DQ+Ii) were sorted for GFP and DQ expression with a FACSAria^{TM} cell sorter (BD Biosciences). DQ+Ii cells were further transduced with ChIT-DM lentivirus and sorted for GFP, DQ, and mCherry expression to obtain DQ+Ii+DM cells (Figure 2.S2C). Full length DQ (fDQ) was purified from DQ+Ii 293T cells by SPVL3 affinity column [53]. Full length DM (fDM) was purified as previously described [53]. Soluble DQ proteins were generated from pWPI-sDQ lentiviral transduced 293T cells using the construct as previously described [62]. The culture media were precleared and incubated with His-Tag Purification Resin (Roche), and the eluates were further purified by SPVL3 affinity column. Purified sDQ

were overnight cleaved with immobilized thrombin (EMD Millipore) in PBS at room temperature to facilitate the release of endogenous CLIP peptide. Soluble DM was purified as previously described [60].

Abs and flow cytometry

The following mAbs, DM-PE (clone Map.DM1; BD Pharmingen), CLIP-PerCP-Cy5.5 (clone CerCLIP.1; Santa Cruz Biotechnology) and isotype-matched negative controls, were used in cell staining. To staining DQ, purified mAb to DQ (clone SPVL3, ATCC) was labeled with Biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma) then accordingly stained by streptavidin-APC or -PE-Cy7 (Invitrogen). Purified mAb to Ii (clone PIN-1), provided by Dr. Peter Cresswell (Yale), was conjugated with Alexa Fluor® 647 (Invitrogen). Intracellular staining was performed with the Cytofix/Cytoperm kit (BD Biosciences). Stained cells were analyzed by LSRFortessa^{TM} flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (Tree Star). Based on GFP, mCherry and surface DQ expression, cells were sorted with FACSAria^{TM} cell sorter (BD Biosciences) at the core facility of University of Utah. The sDQ 293T transductants were sorted for high GFP expression, and sDQ secretion in culture media was measured by immunoassay [63], using SPVL3 and biotin-IVA12 mAbs.

Peptide elution and HPLC-MS/MS sequence analysis

To elute peptides bound to each DQ molecules, ~$1x10^9$ of each DQ+Ii or DQ+Ii+DM transductant 293T cells were lysed and the DQ molecules were captured by anti-DQ mAb (SPVL3) immobilized with protein A beads. Bound peptides were eluted

and further separated by HPLC for HPLC-MS/MS analysis as previously described [64].

Peptide competition and fluorescence polarization assay

N-acetylated CLIP (80-103, KLPKPPKPVSKMRMATPLLMQALC), in which the last residue was substituted by cysteine for labeling, was commercially synthesized (CelTek Pepitdes) and labeled with BMCC-biotin (Pierce), designated as bCLIP. The N-acetylated peptide CLIP (VSKMRMATPLLMQ) was labeled on lysine (underlined) with Alexa Fluor 488 (Molecular Probes) as previously described, designated as ^{488}CLIP [60]. The peptides, CLIP1 (LPKPPKPVSKMRMATPLLMQA), CLIPc (PSSGLGVTKQDLGPVPM) and ZnT8(126) (KPPSKRLTFGWHRAEILGAL), were used as competitor peptides in competition assays. Briefly, 100 nM fDQ, 1 μM bCLIP, 200 nM fDM, and the competitor peptide (0, 2, 20 or 200 μM) were incubated at 37^{o}C for 3 h in 100 mM citrate-phosphate buffer (pH 5.0) with 0.2% NP-40. Sample was loaded into 96-well plate coated with SPVL3 mAb, and detected by immunoassay [63]. The relative loading of bCLIP to specific DQ molecules was normalized to the reaction without competitor peptides. Thrombin-cleaved sDQ was preloaded with extra ^{488}CLIP (1:5 molar ratio) in FP buffer (10 mM citrate-phosphate buffer, pH 5.0, 150 mM NaCl, 0.05% Tween 20), at 37^{o}C for 3 h (for sDQ6) or overnight (for sDQ2) with 100 nM sDM, or at 37^{o}C overnight without sDM (for sDQ8), due to the different DM sensitivity. Unbound ^{488}CLIP peptide was removed by buffer exchange with 30-kD cut-off spin column (Millipore). In FP assay, 50 nM sDQ-^{488}CLIP complex, sDM (0 to 2000 nM), and 100 μM competitor peptide were incubated in FP buffer and measured at 37^{o}C with a Tecan Infinite F200 plate reader. FP value was normalized by the background FP signal

of free ^{488}CLIP peptides, and the data were processed with Prism 5.0 (GraphPad Software), as previously described [60].

References

1 **Pociot, F., Akolkar, B., Concannon, P., Erlich, H. A., Julier, C., Morahan, G., Nierras, C. R. et al.,** Genetics of type 1 diabetes: what's next? *Diabetes* 2010. **59**: 1561-1571.

2 **Gorodezky, C., Alaez, C., Murguia, A., Rodriguez, A., Balladares, S., Vazquez, M., Flores, H. and Robles, C.,** HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example. *Autoimmun. Rev.* 2006. **5**: 187-194.

3 **Cifuentes, R. A., Rojas-Villarraga, A. and Anaya, J. M.,** Human leukocyte antigen class II and type 1 diabetes in Latin America: a combined meta-analysis of association and family-based studies. *Hum. Immunol.* 2011. **72**: 581-586.

4 **Rojas-Villarraga, A., Botello-Corzo, D. and Anaya, J. M.,** HLA-Class II in Latin American patients with type 1 diabetes. *Autoimmun. Rev.* 2010. **9**: 666-673.

5 **van Autreve, J. E., Weets, I., Gulbis, B., Vertongen, F., Gorus, F. K., van der Auwera, B. J. and Belgian Diabetes, R.,** The rare HLA-DQA1*03-DQB1*02 haplotype confers susceptibility to type 1 diabetes in whites and is preferentially associated with early clinical disease onset in male subjects. *Hum. Immunol.* 2004. **65**: 729-736.

6 **Aly, T. A., Ide, A., Jahromi, M. M., Barker, J. M., Fernando, M. S., Babu, S. R., Yu, L. et al.,** Extreme genetic risk for type 1A diabetes. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 14074-14079.

7 **Erlich, H., Valdes, A. M., Noble, J., Carlson, J. A., Varney, M., Concannon, P., Mychaleckyj, J. C. et al.,** HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* 2008. **57**: 1084-1092.

8 **Pociot, F. and McDermott, M. F.,** Genetics of type 1 diabetes mellitus. *Genes. Immun.* 2002. **3**: 235-249.

9 **Neefjes, J., Jongsma, M. L., Paul, P. and Bakke, O.,** Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 2011. **11**: 823-836.

10 **Jensen, P. E.,** Recent advances in antigen processing and presentation. *Nat. Immunol.* 2007. **8**: 1041-1048.

11 **Sant, A. J., Chaves, F. A., Leddon, S. A. and Tung, J.,** The control of the specificity of CD4 T cell responses: thresholds, breakpoints, and ceilings. *Front Immunol.* 2013. **4**: 340.

12 **Fairchild, P. J., Wildgoose, R., Atherton, E., Webb, S. and Wraith, D. C.,** An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int. Immunol.* 1993. **5**: 1151-1158.

13 **Geluk, A., van Meijgaarden, K. E., Schloot, N. C., Drijfhout, J. W., Ottenhoff, T. H. and Roep, B. O.,** HLA-DR binding analysis of peptides from islet antigens in IDDM. *Diabetes* 1998. **47**: 1594-1601.

14 **Gross, D. A., Graff-Dubois, S., Opolon, P., Cornet, S., Alves, P., Bennaceur-Griscelli, A., Faure, O. et al.,** High vaccination efficiency of low-affinity epitopes in antitumor immunotherapy. *J. Clin. Invest* 2004. **113**: 425-433.

15 **Liu, G. Y., Fairchild, P. J., Smith, R. M., Prowle, J. R., Kioussis, D. and Wraith, D. C.,** Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* 1995. **3**: 407-415.

16 **McNeil, L. K. and Evavold, B. D.,** Dissociation of peripheral T cell responses from thymocyte negative selection by weak agonists supports a spare receptor model of T cell activation. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 4520-4525.

17 **Stadinski, B. D., Zhang, L., Crawford, F., Marrack, P., Eisenbarth, G. S. and Kappler, J. W.,** Diabetogenic T cells recognize insulin bound to IAg7 in an unexpected, weakly binding register. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 10978-10983.

18 **Crawford, F., Stadinski, B., Jin, N., Michels, A., Nakayama, M., Pratt, P., Marrack, P. et al.,** Specificity and detection of insulin-reactive CD4+ T cells in type 1 diabetes in the nonobese diabetic (NOD) mouse. *Proc. Natl. Acad. Sci. USA* 2011. **108**: 16729-16734.

19 **Levisetti, M. G., Lewis, D. M., Suri, A. and Unanue, E. R.,** Weak proinsulin peptide-major histocompatibility complexes are targeted in autoimmune diabetes in mice. *Diabetes* 2008. **57**: 1852-1860.

20 **Yang, J., Chow, I. T., Sosinowski, T., Torres-Chinn, N., Greenbaum, C. J., James, E. A., Kappler, J. W. et al.,** Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 2014. **111**: 14840-14845.

21 **Pos, W., Sethi, D. K., Call, M. J., Schulze, M. S., Anders, A. K., Pyrdol, J. and Wucherpfennig, K. W.,** Crystal structure of the HLA-DM-HLA-DR1 complex defines mechanisms for rapid peptide selection. *Cell* 2012. **151**: 1557-1568.

22 **Hou, T., Macmillan, H., Chen, Z., Keech, C. L., Jin, X., Sidney, J., Strohman, M.**

et al., An insertion mutant in DQA1*0501 restores susceptibility to HLA-DM: implications for disease associations. *J. Immunol.* 2011. **187**: 2442-2452.

23 **Zhou, Z. and Jensen, P. E.,** Structural Characteristics of HLA-DQ that May Impact DM Editing and Susceptibility to Type-1 Diabetes. *Front Immunol.* 2013. **4**: 262.

24 **Fallang, L. E., Roh, S., Holm, A., Bergseng, E., Yoon, T., Fleckenstein, B., Bandyopadhyay, A. et al.,** Complexes of two cohorts of CLIP peptides and HLA-DQ2 of the autoimmune DR3-DQ2 haplotype are poor substrates for HLA-DM. *J. Immunol.* 2008. **181**: 5451-5461.

25 **Lundin, K. E., Gjertsen, H. A., Scott, H., Sollid, L. M. and Thorsby, E.,** Function of DQ2 and DQ8 as HLA susceptibility molecules in celiac disease. *Hum. Immunol.* 1994. **41**: 24-27.

26 **Busch, R., De Riva, A., Hadjinicolaou, A. V., Jiang, W., Hou, T. and Mellins, E. D.,** On the perils of poor editing: regulation of peptide loading by HLA-DQ and H2-A molecules associated with celiac disease and type 1 diabetes. *Expert. Rev. Mol. Med.* 2012. **14**: e15.

27 **Morgan, M. A., Muller, P. S., Mould, A., Newland, S. A., Nichols, J., Robertson, E. J., Cooke, A. and Bikoff, E. K.,** The nonconventional MHC class II molecule DM governs diabetes susceptibility in NOD mice. *PLoS One* 2013. **8**: e56738.

28 **van Lummel, M., van Veelen, P. A., Zaldumbide, A., de Ru, A., Janssen, G. M., Moustakas, A. K., Papadopoulos, G. K. et al.,** Type 1 diabetes-associated HLA-DQ8 transdimer accommodates a unique peptide repertoire. *J. Biol. Chem.* 2012. **287**: 9514-9524.

29 **Tollefsen, S., Hotta, K., Chen, X., Simonsen, B., Swaminathan, K., Mathews, II, Sollid, L. M. and Kim, C. Y.,** Structural and functional studies of trans-encoded HLA-DQ2.3 (DQA1*03:01/DQB1*02:01) protein molecule. *J. Biol. Chem.* 2012. **287**: 13611-13619.

30 **Testa, J. S., Apcher, G. S., Comber, J. D. and Eisenlohr, L. C.,** Exosome-driven antigen transfer for MHC class II presentation facilitated by the receptor binding activity of influenza hemagglutinin. *J. Immunol.* 2010. **185**: 6608-6616.

31 **Kovats, S., Whiteley, P. E., Concannon, P., Rudensky, A. Y. and Blum, J. S.,** Presentation of abundant endogenous class II DR-restricted antigens by DM-negative B cell lines. *Eur. J. Immunol.* 1997. **27**: 1014-1021.

32 **Hosken, N. A. and Bevan, M. J.,** Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science* 1990. **248**: 367-370.

33 **Ghosh, P., Amaya, M., Mellins, E. and Wiley, D. C.,** The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* 1995. **378**: 457-462.

34 **Denzin, L. K., Robbins, N. F., Carboy-Newcomb, C. and Cresswell, P.,** Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1994. **1**: 595-606.

35 **Wiesner, M., Stepniak, D., de Ru, A. H., Moustakis, A. K., Drijfhout, J. W., Papadopoulos, G. K., van Veelen, P. A. and Koning, F.,** Dominance of an alternative CLIP sequence in the celiac disease associated HLA-DQ2 molecule. *Immunogenetics* 2008. **60**: 551-555.

36 **van de Wal, Y., Kooy, Y. M., Drijfhout, J. W., Amons, R. and Koning, F.,** Peptide binding characteristics of the coeliac disease-associated DQ(alpha1*0501, beta1*0201) molecule. *Immunogenetics* 1996. **44**: 246-253.

37 **Vartdal, F., Johansen, B. H., Friede, T., Thorpe, C. J., Stevanovic, S., Eriksen, J. E., Sletten, K. et al.,** The peptide binding motif of the disease associated HLA-DQ (alpha 1* 0501, beta 1* 0201) molecule. *Eur. J. Immunol.* 1996. **26**: 2764-2772.

38 **Roep, B. O. and Peakman, M.,** Antigen targets of type 1 diabetes autoimmunity. *Cold Spring Harb. Perspect. Med.* 2012. **2**: a007781.

39 **Abulafia-Lapid, R., Elias, D., Raz, I., Keren-Zur, Y., Atlan, H. and Cohen, I. R.,** T cell proliferative responses of type 1 diabetes patients and healthy individuals to human hsp60 and its peptides. *J. Autoimmun.* 1999. **12**: 121-129.

40 **Chujo, D., Foucat, E., Nguyen, T. S., Chaussabel, D., Banchereau, J. and Ueno, H.,** ZnT8-Specific CD4+ T cells display distinct cytokine expression profiles between type 1 diabetes patients and healthy adults. *PLoS One* 2013. **8**: e55595.

41 **Dang, M., Rockell, J., Wagner, R., Wenzlau, J. M., Yu, L., Hutton, J. C., Gottlieb, P. A. and Davidson, H. W.,** Human type 1 diabetes is associated with T cell autoimmunity to zinc transporter 8. *J. Immunol.* 2011. **186**: 6056-6063.

42 **Dosch, H., Cheung, R. K., Karges, W., Pietropaolo, M. and Becker, D. J.,** Persistent T cell anergy in human type 1 diabetes. *J. Immunol.* 1999. **163**: 6933-6940.

43 **Honeyman, M. C., Stone, N. L. and Harrison, L. C.,** T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents. *Mol. Med.* 1998. **4**: 231-239.

44 **Huurman, V. A., van der Meide, P. E., Duinkerken, G., Willemen, S., Cohen, I. R., Elias, D. and Roep, B. O.,** Immunological efficacy of heat shock protein 60 peptide DiaPep277 therapy in clinical type I diabetes. *Clin. Exp. Immunol.* 2008. **152**: 488-497.

45 **Jonson, C. O., Lernmark, A., Ludvigsson, J., Rutledge, E. A., Hinkkanen, A. and Faresjo, M.,** The importance of CTLA-4 polymorphism and human leukocyte antigen genotype for the induction of diabetes-associated cytokine response in healthy school children. *Pediatr. Diabetes* 2007. **8**: 185-192.

46 **Kudva, Y. C., Deng, Y. J., Govindarajan, R., Abraham, R. S., Marietta, E. V., Notkins, A. L. and David, C. S.,** HLA-DQ8 transgenic and NOD mice recognize different epitopes within the cytoplasmic region of the tyrosine phosphatase-like molecule, IA-2. *Hum. Immunol.* 2001. **62**: 1099-1105.

47 **Peakman, M., Stevens, E. J., Lohmann, T., Narendran, P., Dromey, J., Alexander, A., Tomlinson, A. J. et al.,** Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. *J. Clin. Invest* 1999. **104**: 1449-1457.

48 **Petrich de Marquesini, L. G., Fu, J., Connor, K. J., Bishop, A. J., McLintock, N. E., Pope, C., Wong, F. S. and Dayan, C. M.,** IFN-gamma and IL-10 islet-antigen-specific T cell responses in autoantibody-negative first-degree relatives of patients with type 1 diabetes. *Diabetologia* 2010. **53**: 1451-1460.

49 **Schulz, R. M., Hawa, M., Leslie, R. D., Sinigaglia, F., Passini, N., Rogge, L., Picard, J. K. and Londei, M.,** Proliferative responses to selected peptides of IA-2 in identical twins discordant for Type 1 diabetes. *Diabetes Metab. Res. Rev.* 2000. **16**: 150-156.

50 **Shpigel, E., Elias, D., Cohen, I. R. and Shoseyov, O.,** Production and purification of a recombinant human hsp60 epitope using the cellulose-binding domain in Escherichia coli. *Protein Expr. Purif.* 1998. **14**: 185-191.

51 **Szebeni, A., Schloot, N., Kecskemeti, V., Hosszufalusi, N., Panczel, P., Prohaszka, Z., Fust, G. et al.,** Th1 and Th2 cell responses of type 1 diabetes patients and healthy controls to human heat-shock protein 60 peptides AA437-460 and AA394-408. *Inflamm Res.* 2005. **54**: 415-419.

52 **Vaughan, K., Peters, B., Mallone, R., von Herrath, M., Roep, B. O. and Sette, A.,** Navigating diabetes-related immune epitope data: resources and tools provided by the Immune Epitope Database (IEDB). *Immunome. Res.* 2013. **9**, doi: 10.4172/1745-7580.1000063.

53 **Weber, D. A., Evavold, B. D. and Jensen, P. E.,** Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 1996. **274**: 618-620.

54 **Belmares, M. P., Busch, R., Wucherpfennig, K. W., McConnell, H. M. and Mellins, E. D.,** Structural factors contributing to DM susceptibility of MHC class II/peptide complexes. *J. Immunol.* 2002. **169**: 5109-5117.

55 **Mohan, J. F. and Unanue, E. R.,** Unconventional recognition of peptides by T cells and the implications for autoimmunity. *Nat. Rev. Immunol.* 2012. **12**: 721-728.

56 **Kooy-Winkelaar, Y., van Lummel, M., Moustakas, A. K., Schweizer, J., Mearin, M. L., Mulder, C. J., Roep, B. O. et al.,** Gluten-specific T cells cross-react between HLA-DQ8 and the HLA-DQ2alpha/DQ8beta transdimer. *J. Immunol.* 2011. **187**: 5123-5129.

57 **Silva, D. G., Socha, L., Correcha, M. and Petrovsky, N.,** Elevated lymphocyte expression of CLIP is associated with type 1 diabetes and may be a useful marker of autoimmune susceptibility. *Ann. NY Acad. Sci.* 2004. **1037**: 65-68.

58 **Pu, Z., Lovitch, S. B., Bikoff, E. K. and Unanue, E. R.,** T cells distinguish MHC-peptide complexes formed in separate vesicles and edited by H2-DM. *Immunity* 2004. **20**: 467-476.

59 **Szymczak, A. L., Workman, C. J., Wang, Y., Vignali, K. M., Dilioglou, S., Vanin, E. F. and Vignali, D. A.,** Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat. Biotechnol.* 2004. **22**: 589-594.

60 **Zhou, Z., Callaway, K. A., Weber, D. A. and Jensen, P. E.,** Cutting edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. *J. Immunol.* 2009. **183**: 4187-4191.

61 **Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D.,** Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 8392-8396.

62 **Gauthier, L., Smith, K. J., Pyrdol, J., Kalandadze, A., Strominger, J. L., Wiley, D. C. and Wucherpfennig, K. W.,** Expression and crystallization of the complex of HLA-DR2 (DRA, DRB1*1501) and an immunodominant peptide of human myelin basic protein. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 11828-11833.

63 **Tompkins, S. M., Rota, P. A., Moore, J. C. and Jensen, P. E.,** A europium fluoroimmunoassay for measuring binding of antigen to class II MHC glycoproteins. *J. Immunol. Methods* 1993. **163**: 209-216.

64 **Escobar, H., Reyes-Vargas, E., Jensen, P. E., Delgado, J. C. and Crockett, D. K.,** Utility of characteristic QTOF MS/MS fragmentation for MHC class I peptides. *J. Proteome Res.* 2011. **10**: 2494-2507.

Figure 2.1. DM editing sensitivity of Ii-derived CLIP peptides in T1D-neutral DQ1, -protective DQ6 or -susceptible DQ2, DQ8 and DQ2/8 trans-dimers. (A) CLIP and DQ molecules on 293T cells transduced with DQ only (top), DQ+Ii (middle) or DQ+Ii+DM (bottom) were cell surface stained with fluorescence conjugated CerCLIP.1 and SPVL3 antibodies as described in Materials and Methods. The mean fluorescence intensity (MFI) is stated in the panels, and indicates cell surface expression level of CLIP bound to DQ. (B) Relative DM editing effects indicated by comparison of the MFI ratio of cell surface CLIP and DQ levels in the absence or presence of DM expression. Cells were surface stained for CLIP and DQ and gated on the same level of GFP and/or mCherry expression. Data are representative of 3 independent experiments and shown as mean + SD in panel B.

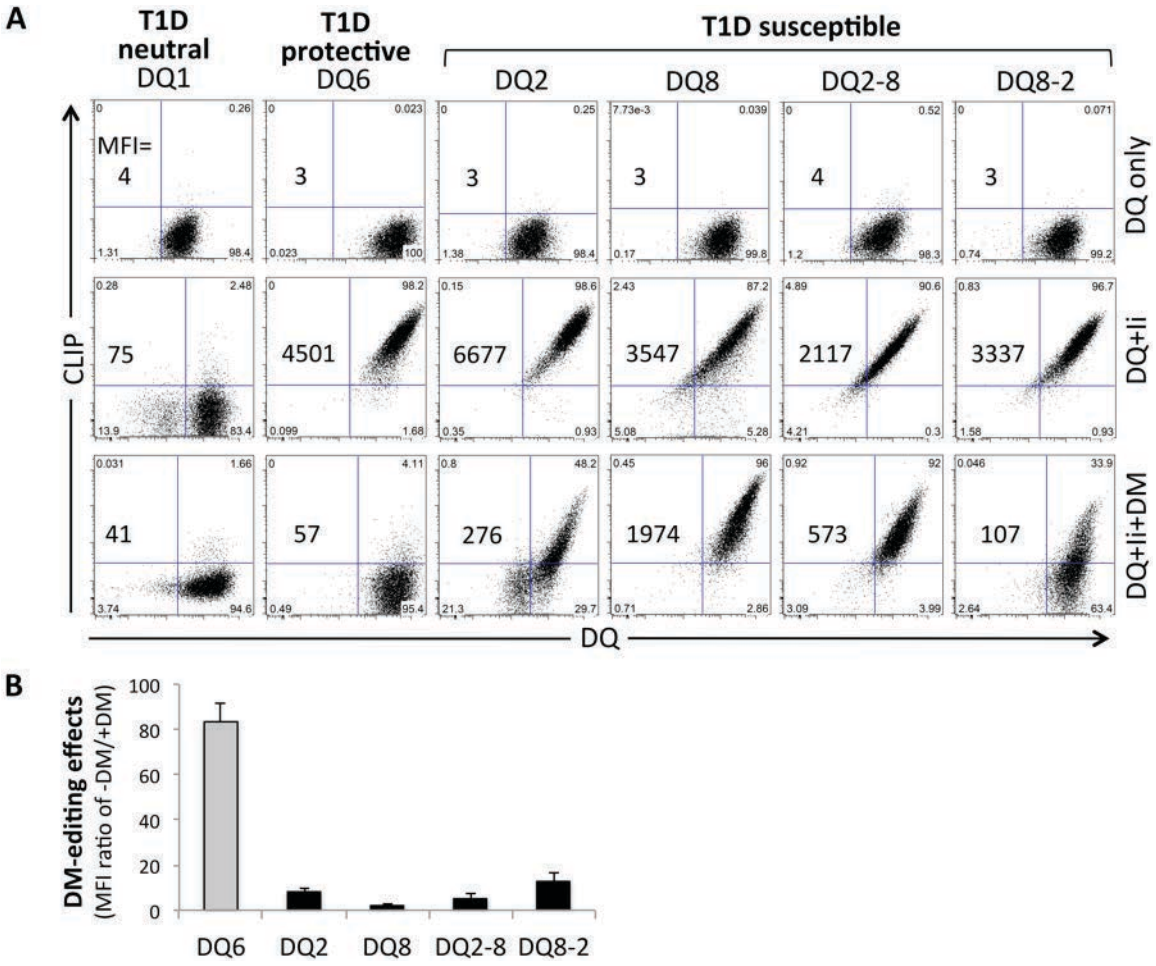

A
T1D neutral
DQ1
T1D protective
DQ6
T1D susceptible
DQ2
DQ8
DQ2-8
DQ8-2
DQ only
DQ+li
DQ+li+DM
CLIP
DQ
MFI=
4
3
3
3
4
3
75
4501
6677
3547
2117
3337
41
57
276
1974
573
107
B
DM-editing effects
(MFI ratio of -DM/+DM)
100
80
60
40
20
0
DQ6
DQ2
DQ8
DQ2-8
DQ8-2

Figure 2.2. Pattern of eluted unique CLIP peptides. 293T cells expressing different DQ molecules in the absence (left, DQ+Ii) or presence (right, DQ+Ii+DM) of DM were used to elute peptides bound to each DQ molecules captured by anti-DQ mAb (SPVL3) immobilized with protein A beads. Bound peptides were eluted and further separated by HPLC and analyzed by HPLC-MS/MS. The two well-defined registers of Ii-derived peptides are highlighted as CLIP1 (boxed) and CLIP2 (gray). Data shown are representative of 2 independent experiments.

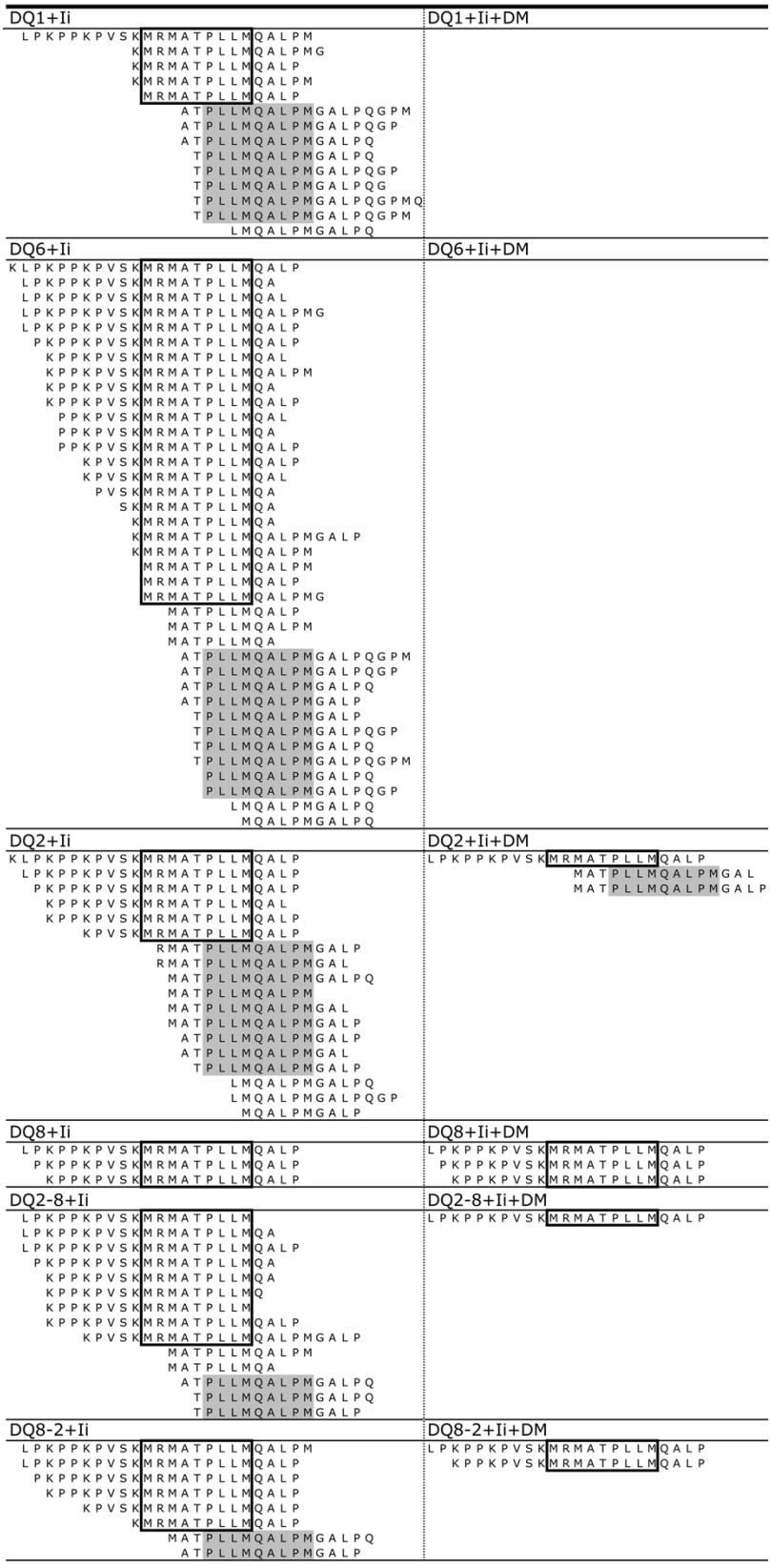

DQ1+Ii
DQ1+Ii+DM
DQ6+Ii
DQ6+Ii+DM
DQ2+Ii
DQ2+Ii+DM
DQ8+Ii
DQ8+Ii+DM
DQ2-8+Ii
DQ2-8+Ii+DM
DQ8-2+Ii
DQ8-2+Ii+DM

Figure 2.3. Binding analysis of the ZnT8-derived ZnT8(126) peptide to purified fDQ molecules. Peptide competition assays were performed with purified fDQ proteins and bCLIP, using unlabeled peptides CLIP1, CLIPc or ZnT8(126) peptides as competitor. CLIPc is a control peptide derived from the C-terminus of Ii that was detected by HPLC-MS/MS from all of the samples (DQ2, DQ2-8 and DQ8-2), except DQ8. Data shown are representative of 3 independent experiments.

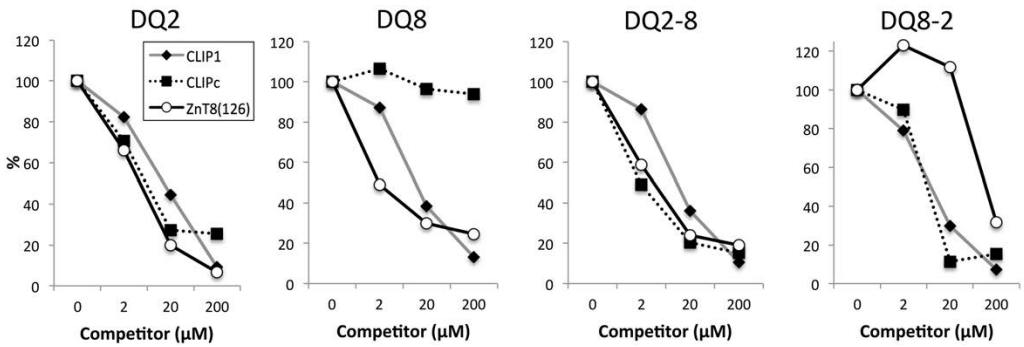

DQ2
DQ8
DQ2-8
DQ8-2
CLIP1
CLIPc
ZnT8(126)
%
120
100
80
60
40
20
0
0
2
20
200
Competitor (μM)

Figure 2.4. CLIP dissociation rate and DM catalytic potency measured with purified DQ molecules. (A) The FP measurement of CLIP dissociation and DM editing effects. Thrombin cleaved sDQ proteins were pre-loaded with ^{488}CLIP peptides. Peptide dissociation rates were measured in the presence of different concentration of sDM and 100 μM of unlabeled CLIP1 as competitor peptides. The control represents the sample without adding extra sDM or competitor peptides. (B) Peptide dissociation rates of sDQ6, sDQ2 and sDQ8 measured by FP assay in the presence of different concentration of DM (left) and the DM-catalyzed rate enhancements normalized relative to dissociation rates measured in the absence of DM (right). Data are representative of 3 independent experiments and shown as mean ± SD in panel B.

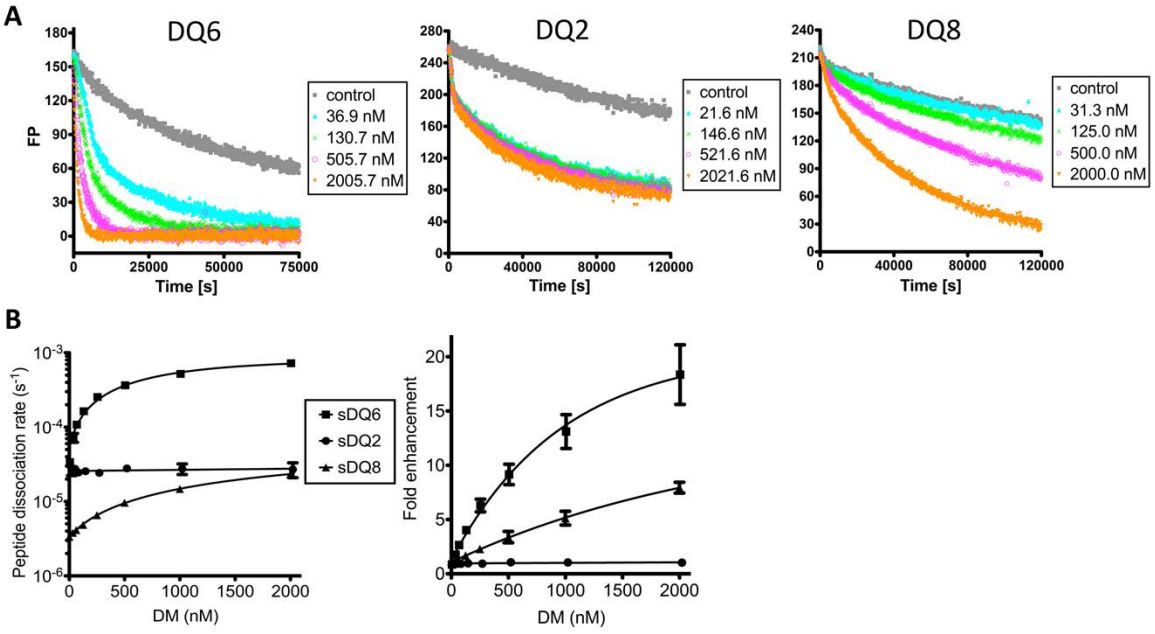

A
DQ6
DQ2
DQ8
FP
Time [s]
control
36.9 nM
130.7 nM
505.7 nM
2005.7 nM
21.6 nM
146.6 nM
521.6 nM
2021.6 nM
31.3 nM
125.0 nM
500.0 nM
2000.0 nM
B
Peptide dissociation rate (s-1)
DM (nM)
sDQ6
sDQ2
sDQ8
Fold enhancement

Figure 2.5. Impact of hydrogen bonds between the two helical regions of α and β chains near the P1 pocket of DQ8 molecule. (A) Top view of the two helical regions of DQ8 α and β chains near the P1 pocket of the peptide binding groove with bound peptide (PDB: 1JK8). The R52 residue in the 3^{10}-helix (purple) of the α chain forms three H-bonds with E86 and T89 in the helix (cyan) near the P1 pocket of the β chain. DQ8 specific residues are labeled orange. (B) A close-up view of the three H-bonds formed between the two helixes in DQ8. (C) Comparison of CLIP level on 293T cells transduced with Ii and DQ6, DQ8, or DQ8 mutants, in the absence or presence of DM expression. The DQ8 mutants were designed to disrupt one (βT89G) or two (βE86A) H-bonds, respectively. The MFI of cell surface CLIP is shown. (D) The relative DM editing effects indicated by comparison of the ratio of CLIP/DQ in the absence or presence of DM expression. Cells were surface stained for CLIP and DQ and gated on the same level of GFP and/or mCherry expression. Data are representative of 3 independent experiments and shown as mean + SD in panel D. ***, $P<0.001$; two tail t-test.

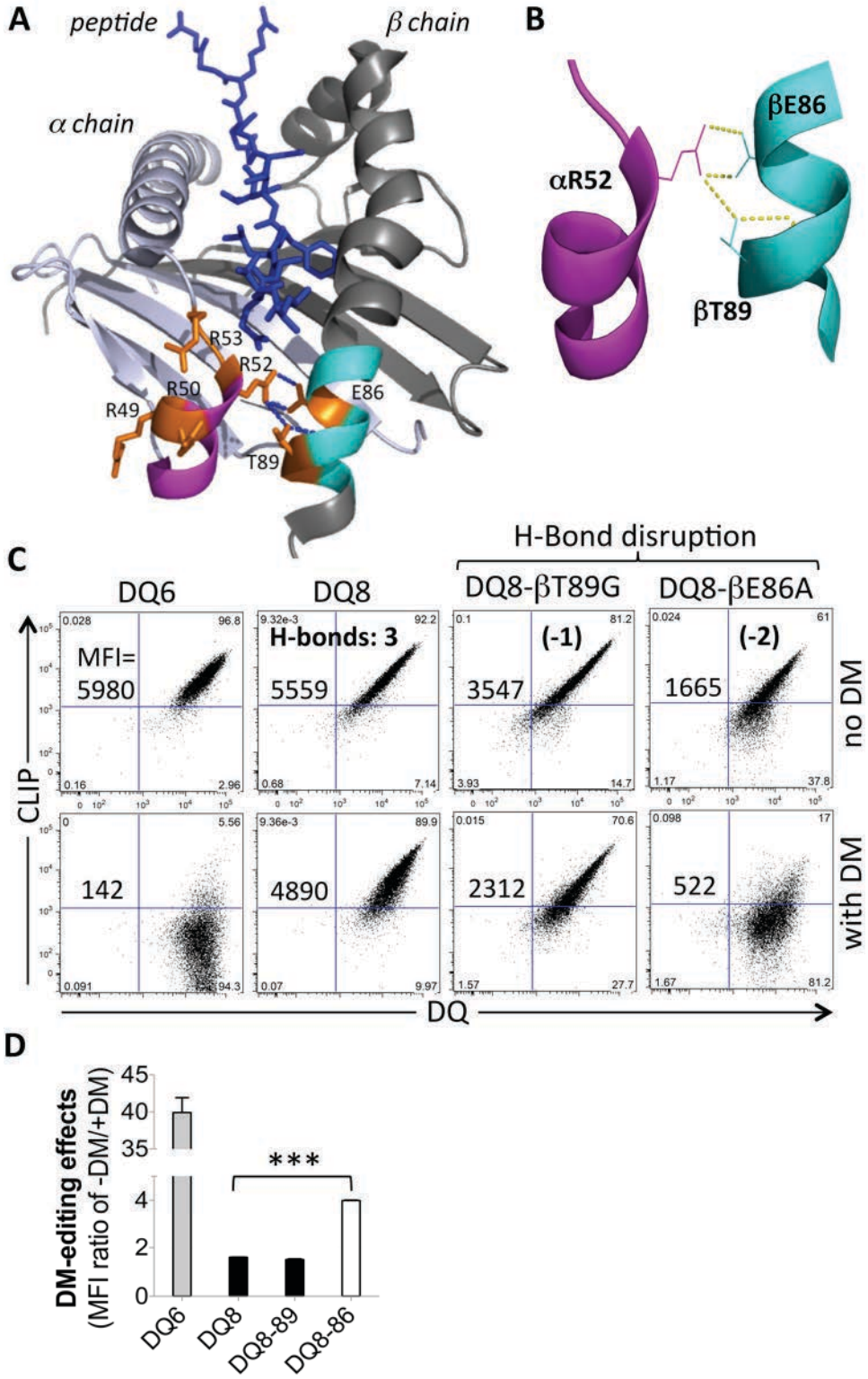

A
peptide
β chain
α chain
R53
R52
R50
R49
E86
T89
B
βE86
αR52
βT89
C
H-Bond disruption
DQ6
DQ8
DQ8-βT89G
DQ8-βE86A
MFI= 5980
H-bonds: 3
5559
(-1)
3547
(-2)
1665
no DM
142
4890
2312
522
with DM
CLIP
DQ
D
DM-editing effects
(MFI ratio of -DM/+DM)
45
40
35
4
2
0
***
DQ6
DQ8
DQ8-89
DQ8-86

Table 2.1. Eluted T1D-associated autoantigen-derived peptides with previously reported positive function in autoreactive CD4+ T cell activation assays.

AutoAg	Methods (ref.)	Start AA	Sequence
ZnT8	**DQ2, 8, 8-2 LC-MS**	**126**[a]	**K P P S K R L T F G W H R A E I L G A L**
(Q8IWU4)	**DQ8, 2-8, 8-2 LC-MS**	**126**	**K P P S K R L T F G W H R A E I L G A L L S**
	ELISPOT[c] [41]	120-146	S L W L S S K P P S K R L T F G W H R A E I L G A L L
	ELISPOT [41]	134-174[b]	***F G W H R A E I L G A L L*** S I L C I W V V T G V L V Y L A C E R L L Y P D Y Q I Q
	Cytokine [41]	124-138	S S ***K P P S K R L T F G W H R***
	ELISPOT [40]	106-132	H L L I D L T S F L L S L F S L W L S S ***K P P S K R L***
IA-2	**DQ8 LC-MS**	**876**	**P A E G T P A S T R P L L D F R R K V N K C Y R**
(Q16849)	3H thymidine [43]	889-904	D F R R K V N K C Y R G R S C P
	DQ2-8 LC-MS	**622**	**G D T T F E Y Q D L C R Q**
	3H thymidine [46]	616-633	G P E G A H G D T T F E Y Q D L C R
	DQ8-2 LC-MS	**956**	**K D Q F E F A L T A V A E E V N A I L K A L P**
	3H thymidine [46]	961-979	F A L T A V A E E V N A I L K A L P Q
	3H thymidine [49]	957-969	D Q F E F A L T A V A E E
	3H thymidine [43]	959-974	F E F A L T A V A E E V N A I L
	3H thymidine [47]	955-975	S K D Q F E F A L T A V A E E V N A I L K
	ELISPOT [48]	955-976	S K D Q F E F A L T A V A E E V N A I L K A
HSP60	**DQ8, DQ2-8 LC-MS**	**448**	**I P A L D S L T P A N E D Q K**
(P10809)	3H thymidine [39, 42, 44, 50]	437-460	V L G G G V A L L R V I P A L D S L T P A N E D
	ELISPOT [51]	437-460	V L G G G V A L L R V I P A L D S L T P A N E D
	Cytokine [45]	437-460	V L G G G V A L L R V I P A L D S L T P A N E D
	DQ2-8 LC-MS	**523**	**K V V R T A L L D**
	3H thymidine [39]	511-530	V N M V E K G I I D P T K V V R T A L L

[a] The sequence identified in different samples of this study by LC-MS is bold. [b] The bold and italic sequence shows the overlapped portion of peptide compared with the peptide identified in this study. [c] The listed method that was used to identify the function of each peptide in each of the references. The Immune Epitope Database (www. iedb.org) was applied for the search of T cell response to specific epitope as described [52].

Figure 2.S1. N-terminus sequence of CLIP peptides and the specificity of CerCLIP.1 mAb. (A) Sequence alignment of Ii-derived CLIP1, CLIP1* and CLIP2 peptides for the identification of CerCLIP.1 mAb specificity. (B) The CerCLIP.1 mAb specificity. ELISA plate was coated with 100 μM or 10 μM of CLIP peptides (in 50 μL) listed in (A); then detected with 2 μg/ml of purified CerCLIP.1 mAb (Santa Cruz Biotech), followed by 2 μg/ml of biotinylated goat-anti-mouse IgG mAb (SouthernBiotech), and developed with Eu-ELISA, n = 2.

A

CLIP1: LPKPPKPVSKMRMATPLLMQA
CLIP1*: KMRMATPLLMQA
CLIP2: MATPLLMQALPM

B

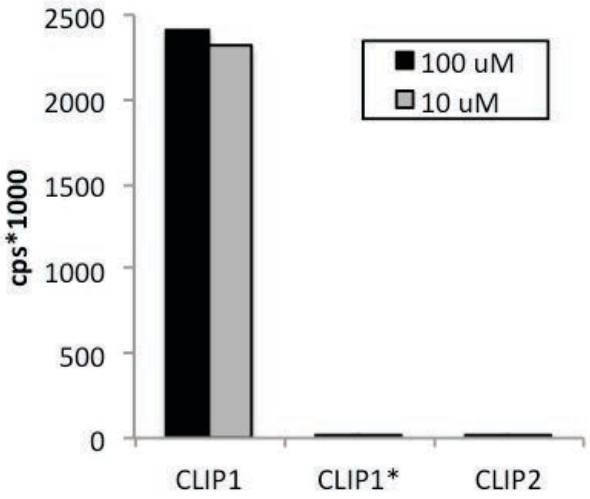
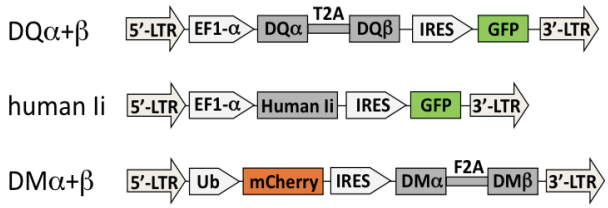


Figure 2.S2. The expression of Ii, DQ and DM in 293T cells. (A) The α and β chain gene allele information of T1D-neutral, -protective and -susceptible DQ molecules. (B) Diagrams of the constructs and the markers for the expression of Ii, DQ or DM in 293T cells. (C) Strategies of lentiviral mediated gene transduction and the markers used for the cell sorting. 293T cells were firstly transduced by high titer of fresh lentivirus generated in producer cells transfected with DQ or Ii construct. Then, the transductants were sorted by GFP and DQ (SPVL3) expression. To generate the DQ+Ii+DM cells, DQ+Ii cells were further transduced by lentivirus with DM, and sorted by GFP, mCherry and cell surface DQ expression. (D) Comparison of the relative expression level of GFP and mCherry (top panel), intracellular Ii (PIN-1, middle panel) and intracellular DM (Map.DM1, bottom panel) in DQ+Ii+DM cells with the negative control. The MFI showed in the top panel with (x, y) represents the MFI of GFP and mCherry expression level, respectively. The MFI showed in the middle or the bottom panel represents the MFI of Ii or DM expression level, respectively. (E) The MFI ratio of cell surface CLIP and DQ levels in the absence or presence of DM expression. Cells were surface stained for CLIP (CerCLIP.1) and DQ (SPVL3) and gated on the same level of GFP and/or mCherry expression, n = 3.

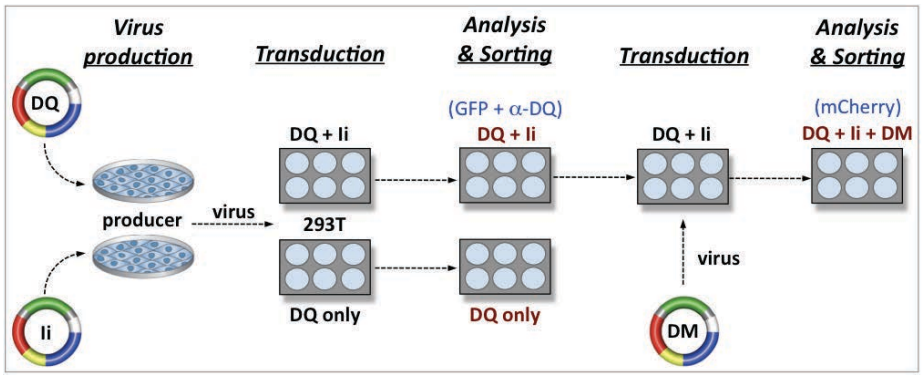
A

MHC	α chain	β chain	T1D association
DQ1	DQA1*0101	DQB1*0501	Neutral
DQ6	DQA1*0102	DQB1*0602	Protective
DQ2	DQA1*0501	DQB1*0201	Susceptible
DQ8	DQA1*0301	DQB1*0302	
DQ2-8	DQA1*0501	DQB1*0302	
DQ8-2	DQA1*0301	DQB1*0201	

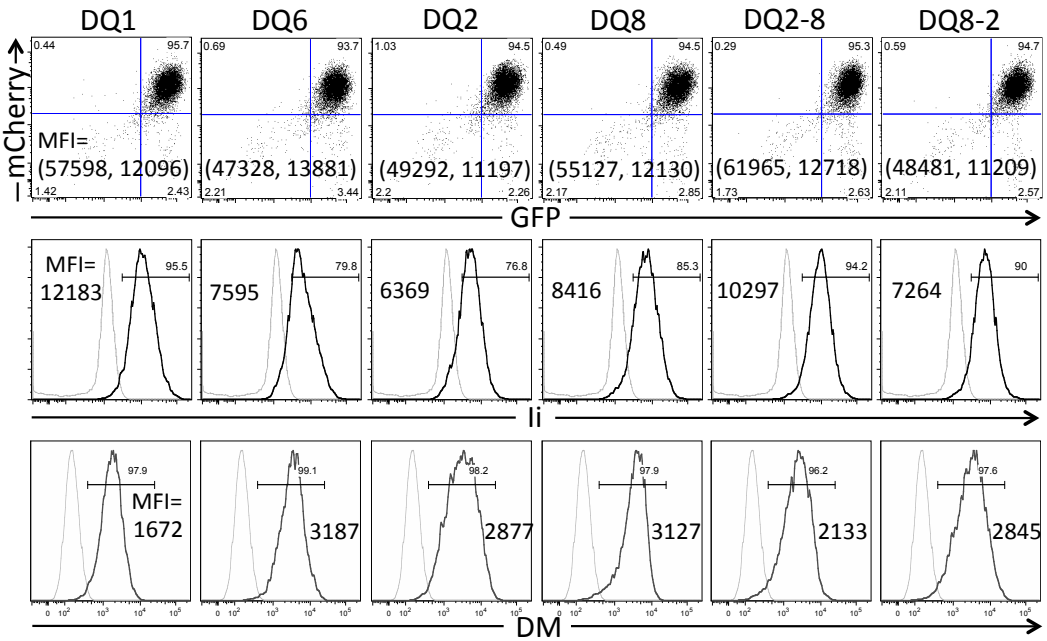
B



C



D



E

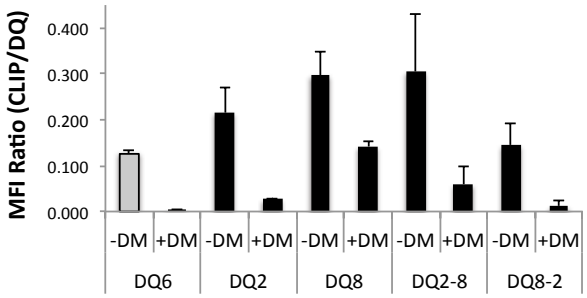
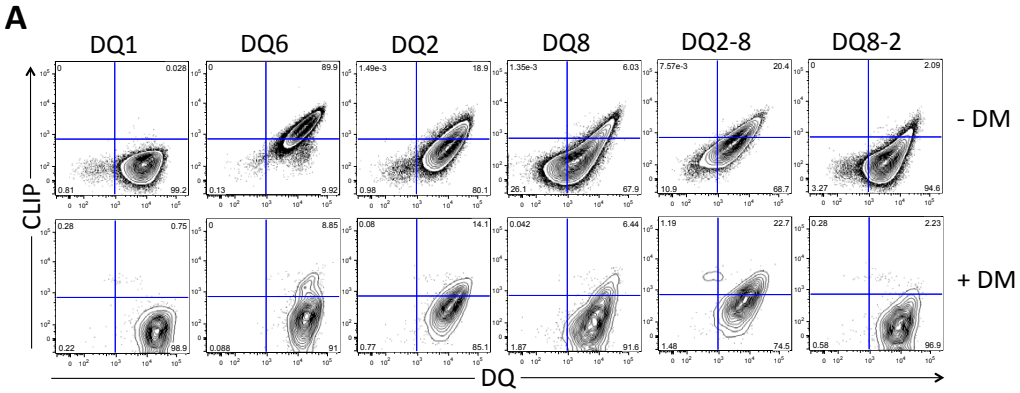


Figure 2.S3. DM editing effects of T1D-associated DQ molecules on Ii-derived CLIP peptides in T2 transductants. (A) Cell surface presentation of Ii-derived CLIP peptides and DQ molecules in T2 transductants in the absence or presence of DM expression. T2 cells were transduced with DQ and DM, using GFP and mCherry as the marker of DQ and DM expression, respectively. Cells were gated on GFP+mCherry- (top panel) or GFP+mCherry+ (bottom panel). (B) Difference of DM editing effects between T1D-associated and nonassociated DQ molecules. The DM editing effects are indicated by the ratio of surface CLIP/DQ MFI (mean fluorescence index) between the cells in the absence or presence of DM expression, as shown in (A), n = 3.


A
DQ1
DQ6
DQ2
DQ8
DQ2-8
DQ8-2
- DM
+ DM
CLIP
DQ

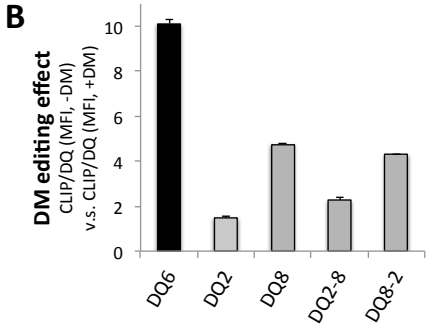

B
DM editing effect
CLIP/DQ (MFI, -DM)
v.s. CLIP/DQ (MFI, +DM)
10
8
6
4
2
0
DQ6
DQ2
DQ8
DQ2-8
DQ8-2

Table 2.S1. T1D-associated auto-antigen derived peptides eluted from 293T transductants with or without DM expression [a].

DQ Molecules	Start AA	Sequence	AutoAg (-DM)	AutoAg (+DM)	Accession #
DQ2	126	KPPSKRLTFGWHRAEILGAL	ZnT8		Q8IWU4
	321	GGAVFGEEGLTLNLEDV	HSP60		P10809
	323	AVFGEEGLTLNLEDV	HSP60		P10809
DQ8	876	PAEGTPASTRPLLDFRRKVNKCYR	IA-2		Q16849
	989	VQTKEQFEFALTAVAEEVNAILKALP	IA-2beta		Q92932
	126	KPPSKRLTFGWHRAEILGALLS	ZnT8	ZnT8	Q8IWU4
	126	KPPSKRLTFGWHRAEILGAL	ZnT8	ZnT8	Q8IWU4
	448	IPALDSLTPANEDQK		HSP60	P10809
DQ2-8	424	VPSPVSSEPPKAARPPVTPVLLE	IA-2		Q16849
	622	GDTTFEYQDLCRQ		IA-2	Q16849
	330	DGMAELMAGLMQGVDHG		IA-2beta	Q92932
	126	KPPSKRLTFGWHRAEILGALLS		ZnT8	Q8IWU4
	448	IPALDSLTPANEDQK		HSP60	P10809
	523	KVVRTALLD		HSP60	P10809
DQ8-2	541	LEAQTGLQILQTGVGQREEAAAVLP		IA-2	Q16849
	956	KDQFEFALTAVAEEVNAILKALP	IA-2		Q16849
	126	KPPSKRLTFGWHRAEILGALLS	ZnT8	ZnT8	Q8IWU4
	126	KPPSKRLTFGWHRAEILGAL		ZnT8	Q8IWU4
	455	TPANEDQKIGIEIIK		HSP60	P10809

[a] Nested peptides derived from ZnT8 and identified from different DQ molecules is highlighted in light gray. Nested peptides derived from HSP60 and identified from different DQ molecules is highlighted in dark gray. The "-DM" represents autoantigen-derived peptides detected in DQ+Ii samples, while the "+DM" represents autoantigen-derived peptides detected in DQ+Ii+DM samples.

CHAPTER 3

PEPTIDOMIC ANALYSIS OF TYPE 1 DIABETES ASSOCIATED HLA-DQ MOLECULES AND THE IMPACT OF HLA-DM ON PEPTIDE REPERTOIRE EDITING

Abstract

HLA-DM is critical in editing peptide repertoires presented by MHCII on antigen presenting cells (APC), thus influencing the selection/activation of $CD4^+$ T cells. We previously reported that type 1 diabetes (T1D) associated HLA-DQ2, DQ8, and DQ2/8 molecules are relatively resistant to DM editing of the invariant chain (Ii) derived CLIP peptides. In this study, we further studied DM sensitivity of T1D-associated DQ molecules by peptidomic analysis. Compared to T1D nonassociated DQ1 and DQ6, the percentages of shared peptides among T1D-associated DQ molecules were significantly higher. There were greater numbers of nested peptide sets and long peptides (>20-mer) eluted from T1D-associated DQ molecules. Predicted peptide binding motifs of DQ2, DQ8, and DQ2/8 shared charged anchor residues, while hydrophobic anchors were present in DQ6 peptides. Competition binding assays comparing the eluted peptides from DM sensitive, dependent, or resistant groups showed that the binding affinities of the DM resistant and dependent peptides were consistently high; however, the DM sensitive peptides were complicated, in which some of the DM sensitive peptides were with relatively higher affinity, indicating that high affinity is necessary but not sufficient for DM editing resistance, and other mechanisms are also involved. Measurements of peptide dissociation rates and DM editing effects further confirmed that DM editing dominates the biological stability of MHCII-peptide complexes. The increased complex stability that formed with DM resistant or dependent peptides, combined with the poor DM editing effects on T1D-associated DQ molecules, illustrates the critical impact of DM editing on peptide repertoire and highlights the potential role of DM resistance in T1D-associated DQ molecules in T1D etiology. Taken together, our new data further support

the hypothesis that DM resistance in T1D-associated DQ molecules affects the peptide repertoires, thus contributing to the pathogenesis of T1D.

Introduction

Human major histocompatibility complex class II (MHCII), especially the HLA-DQ alleles, DQ2 (DQA1*0501/DQB1*0201) and DQ8 (DQA1*0301/DQB1*0302), are highly associated with type 1 diabetes (T1D), an autoimmune disease with the loss of insulin production due to self-destruction of pancreatic β cells [1-5]. However, the involved molecular mechanisms are still uncertain. The characteristics of the peptide-binding groove of MHCII molecules, including both the anchor-pocket interaction and the conserved hydrogen-bond network between peptide and MHCII, contribute to the peptide binding and presentation on the antigen-presenting cell (APC) surface [6]. Meanwhile, the repertoire of presented peptides by MHCII determines the selection or activation of $CD4^+$ T cells [7]. Therefore, it is believed that the unique properties of T1D-associated DQ molecules may contribute to the etiology of T1D. These properties, such as the unique peptide binding specificity and the different sensitivity to HLA-DM (DM) editing, will affect the presentation of key autoantigen epitopes associated with T1D and potentially differentiate the selection of the $CD4^+$ T cell repertoire [8-11].

DM plays a critical role to accelerate peptide exchange in the peptide-binding groove of MHCII [12]. Various findings indicate that DM selectively releases the lower affinity peptides and helps the loading of higher affinity peptides (“DM editing”), therefore potentially favoring the presentation of stable MHCII-peptide complexes on the surface of APC [12-14]. However, less study has focused specifically on the T1D-

associated DQ molecules; there is no direct evidence exploring the impact of DM editing in T1D-associated DQ molecules. Previously, van Lummel et al. expressed T1D-associated cis-dimers DQ2 or DQ8, or the trans-dimer DQ2-8 (DQA1*0501/ DQB1*0302) or DQ8-2 (DQA1*0301//DQB1*0201) molecules in 293T cells and firstly revealed the peptide repertoire difference of these T1D-associated DQ molecules [15]. However, there is no endogenous DM or human invariant chain (Ii) expressed in 293T cells. Ii plays multiple roles in antigen presentation process. First, it is required for the translocation of MHCII molecules to late endosomal compartments, in which the Ii is enzymatically processed and other peptides are loaded with the help of DM [6, 7]. Second, the occupancy of the peptide-binding groove of MHCII molecules by the Ii-derived CLIP peptides inhibits the early loading of peptides in the endoplasmic reticulum (ER) and/or the MHCII trafficking compartments [16, 17]. Therefore, the deficiency of Ii and DM expression in their system might skew the identified peptide repertoire of T1D-associated DQ molecules, and the impact of DM editing on DQ peptide repertoire was also not considered.

We previously expressed in 293T cells each of the T1D-associated DQ2, DQ8, or the trans-dimer, DQ2-8 or DQ8-2 molecules, in the absence or presence of Ii and/or DM. We concluded that these T1D-associated DQ molecules are relatively resistant to DM editing for the release of Ii-derived CLIP peptides; therefore potentially affecting the efficient loading of other peptides for the selection or activation of $CD4^+$ T cells involved in T1D etiology [11]. To further investigate the peptide repertoires of each T1D-associated DQ molecules and the impact of DM editing on peptide repertoires, we analyzed the peptidome of each DQ molecules identifying eluted peptides by the HPLC-

MS/MS methods [18, 19]. We found that T1D-associated DQ molecules share several distinct characteristics that are not observed with T1D nonassociated DQ molecules. In addition, we determined that high affinity is necessary but not sufficient to confer resistance to DM editing in stable MHCII-peptide complexes. Our findings further confirmed that T1D-associated DQ molecules are with distinct characteristics that may contribute to the pathogenesis of T1D.

Results

The impact of Ii and DM on the DQ bound peptide repertoire

To investigate the impacts of Ii and DM editing on T1D-associated DQ molecules peptide repertoires, we eluted DQ bound peptides from each group of 293T cells (18 cell lines) expressing each of the DQ (DQ-only), DQ+Ii (-DM), or DQ+Ii+DM (+DM) molecules that we previously generated [11]. Interestingly, with the identified variable numbers of unique peptides in each cell lines, there was relatively little overlap of peptides between DQ-only and DQ+Ii, or DQ and DQ+Ii+DM samples (Figure 3.1A). It was also found that the sources of eluted peptides are apparently different when we compared the cells expressing DQ (Figure 3.S1, upper panel) with the cells expressing DQ+Ii (Figure 3.S1, middle panel), or with the cells expressing DQ+Ii+DM (Figure 3.S1, bottom panel); however, there is no visible source difference between the DQ+Ii and DQ+Ii+DM samples. These results suggested that expression of Ii has a major impact on the DQ bound peptide repertoire. Similarly, the small fraction of unique peptides that overlapped between the DQ+Ii and DQ+Ii+DM samples indicated an apparent difference of DQ bound peptide repertoire that results from DM editing (Figure 3.1A). Moreover,

for the length of each DQ presented peptides, we can rarely identify long (>20-mer) peptides from the DQ cell samples, but we can identify many longer peptides from cells expressing DQ+Ii or DQ+Ii+DM (Figure 3.1B and Figure 3.S2). In addition, the predicted peptide binding motifs from DQ-only eluted peptides (Figure 3.S4) are consistent with the motifs previously reported from experiments without Ii coexpression [15], but are different with the predicted motifs from cells expressing DQ+Ii or DQ+Ii+DM (Figure 3.1E and Figure 3.S4). Taken together, we conclude that the dual roles of Ii and the function of DM editing have a major effect on the bound peptide repertoires of each DQ molecule.

Peptidomic analysis of T1D-associated DQ molecules that are less sensitive to DM editing

As previously reported, when we coexpress DQ and Ii in 293T cells and compare the impact of DM editing among those DQ molecules, it was found that the T1D-associated DQ molecules, including DQ2, DQ8, DQ2-8, and DQ8-2, are all relatively resistant to DM editing of Ii-derived CLIP peptides, with high surface CLIP levels on DM expressing cells; however, the T1D nonassociated DQ1 and DQ6 molecules are both highly sensitive to DM editing, with nondetectable surface CLIP on DM expressing cells [11]. These findings indicated that T1D-associated DQ molecules may have distinct DM editing sensitivity and the weakened DM editing sensitivity may change the T1D-associated DQ bound peptide repertoires, and potentially affect the selection or activation of autoreactive $CD4^+$ T cells. To further confirm this observation, we analyzed the peptidome of each T1D-associated DQ molecules and T1D nonassociated DQ1 and DQ6

molecules (Figure 3.1).

We firstly compared the numbers of nested cores (two or more unique peptides sharing at least 8-mer of the same sequence) edited by DM, as defined by the nested cores identified in peptides from DQ+Ii but not from the DQ+Ii+DM samples. Interestingly, for DQ1 and DQ6, 82.1% and 88.9% of nested cores were edited by DM expression; while for the T1D-associated DQ2, DQ8, DQ2-8, and DQ8-2, there are only 65.6%, 70.4%, 78.2%, and 62.7%, respectively, of nested cores edited by DM, showing a significant difference between T1D-associated and nonassociated DQ molecules (Figure 3.1C).

MHCII molecules can present both short and long peptides. The peptide-binding grooves of MHCII are opened at both sides, a unique property of MHCII distinguished from MHCI molecules [20, 21]. We analyzed the length of unique peptides eluted from each group of cells expressing Ii and T1D-associated or nonassociated DQ molecules in the absence or presence of DM expression. As expected, each of the DQ molecules can present both short (median length of ~15-mer) and long (~23-mer) peptides, and the majority of presented peptides by T1D nonassociated DQ1 and DQ6 molecules are short peptides in DQ+Ii samples; however, even greater percentages of long peptides were presented by T1D-associated DQ molecules in DQ+Ii samples. Surprisingly, when DM is expressed in these cell lines, the long peptides from T1D nonassociated DQ1 and DQ6 molecules are edited by DM, as shown by a significant decrease in long peptides, while there are still a substantial number of long peptides retained with T1D-associated DQ molecules (Figure 3.1B). The different length distribution of the eluted peptides from DQ+Ii or DQ+Ii+DM cells between T1D-associated and nonassociated DQ molecules

further indicated that their DM editing sensitivity is different.

Kooy-Winkelaar et al. previously reported gluten-specific T cells that are cross-reactive with DQ8 and DQ2-8 molecules presenting the same peptides [22], indicating that the peptide-binding groove of DQ8 and DQ2-8 might share some similar characteristics. Van Lummel et al. also showed that some peptides can bind with multiple DQ molecules [15]. We calculated the number of same peptides eluted from cells expressing Ii and different DQ molecules (shared peptides) in the absence or presence of DM expression. Interestingly, in the absence of DM editing, there are 5.4% to 10.2% of DQ6 presented peptides shared in all of the other five DQ molecules and this is higher than the peptides shared among the other five DQ molecules (Figure 3.S3); however, in the presence of DM editing, the frequencies of shared peptides within T1D-associated DQ molecules are increased, between 4.8% and 10.1%, compared with the frequencies in DQ6 with a range between 2.0% to 3.6%, or in DQ1 with a range between 0.1% and 0.4% (Figure 3.1D, highlighted). These data support the conclusion that T1D-associated DQ molecules share some similar properties in peptide binding. Moreover, the increased frequencies within the T1D-associated DQ molecules in the presence versus the absence of DM editing, while the decreased frequencies within the T1D nonassociated DQ molecules in the presence versus the absence of DM editing, indicates the enrichment of shared peptides that are resistant to DM editing (Figure 3.1D). This also supports our previous observation that some autoantigen-derived peptides can be presented by multiple different T1D-associated DQ molecules [11]. The results further indicate that DM editing is relatively less efficient in T1D-associated DQ molecules. They are also consistent with previous findings that these T1D-associated DQ molecules share some

similar peptide binding properties [15, 22], which may contribute to the T1D-associated different DQ molecules share with similar mechanism(s) in T1D etiology.

To further analyze the differences between T1D-associated and nonassociated DQ molecules, we predicted the peptide binding motifs of each DQ molecule and compared the difference of motifs in the absence or presence of DM expression. We firstly compared the DQ8 peptide binding motif from the peptide dataset with the DQ8+Ii+DM samples with a previously published DQ8 motif, in which the peptide dataset were from naturally processed peptides using a B cell line generated from humanized NOD.DQ8 mice [23, 24], to validate both the dataset we generated from 293T cells and the applied HPLC-MS/MS method [19]. As expected, the DQ8 peptide binding motif predicted with the DQ8+Ii+DM peptide dataset was very similar to the previously published DQ8 motif [24], with a dominant preference for acidic residues at P9, a preference for acidic residues at P1, and preference for smaller residues at P4 and P6, as well as proline and valine at P6 (Figure 3.1E, panel 4). We then predicted the peptide binding motifs for each of the DQ molecules. As expected, within all of the six DQ molecules, there is clear evidence of DM editing effects between the motifs from DQ+Ii and DQ+Ii+DM samples, such as the enrichment of major anchor residues on P1 and P9 (Figure 3.1E and Figure 3.S4). All of the four T1D-associated DQ molecules share similar predicted peptide binding motifs, with the charged residues at the major anchor residues P1 and P9. With the DQ6 peptide binding motifs, these residues are not charged. Interestingly, the peptide binding motifs of T1D neutral DQ1 molecule showed similar characters as T1D-associated DQ molecules, but it is sensitive to DM editing [11]. It is possible that other mechanism(s) might be involved in its sensitivity to DM editing, as we previously proposed [8].

High peptide binding affinity is necessary but not sufficient for resistance to DM editing

To study the relationship of peptide binding affinity and DM editing sensitivity, we firstly synthesized three categories of peptides that were eluted from DQ6 samples representing the DM resistant, DM dependent, or DM sensitive peptide group (Table 3.1 and Figure 3.S5). To increase the grouping accuracy, we confirmed the recurrence of these peptides by the total hits of each peptide sharing the same nested core sequence within the raw dataset. As shown, for the group of DM sensitive peptides, they are only detectable in the DQ6+Ii sample in the absence of DM expression; for the DM dependent peptides, they are only detectable in the presence of DM expression, whereas for the DM resistant peptides, they are detectable in both situations (Table 3.1).

We first measured the IC50 of each peptide, which is indicated by the concentration of peptides required for 50% inhibition of the loading of HA^{488} peptide to DQ6 in FP competition binding experiments [25]. As expected, all of the tested peptides from the DM resistant group, including ABP230, ABP634, CBC114, GRP198, and SCM57 can efficiently inhibit the loading of the indicator HA^{488} peptide with the measured IC50 value from 0.06 μM to 0.53 μM, compared with the unlabeled HA peptide with the measured IC50 at 1.63 μM (Figure 3.2A and 2E). Consistently, all of the peptides from DM dependent group, PRP348, TPP425, and NEP494, efficiently inhibited the loading of indicator HA^{488} peptide as well, with low IC50 values at 0.11 μM, 0.16 μM and 0.07 μM, respectively (Figure 3.2B and 2E). While for most of the DM sensitive peptides, the IC50 values, from 2.67 μM to 18.03 μM, are significantly higher than those of the DM resistant and DM dependent groups (Figure 3.2C and 2E). These data

confirmed that DM selectively favors the presentation of high affinity peptides by DQ6 and the peptides with low binding affinity are sensitive to DM editing.

Interestingly, the peptides LEP147 and CKA362 can also efficiently inhibit the binding of indicator peptides, with IC50 values at 0.32 μM and 0.12 μM, respectively (Figure 3.2D and 2E), even though these were grouped as DM editing sensitive due to the multiple hits in the DQ6+Ii but not in the DQ6+Ii+DM samples. The inconsistency of observed DM editing sensitivity and peptide binding affinity by IC50 measurement indicated that peptide affinity is not sufficient to predict resistance to DM editing in cells, and other factor(s) might be involved in the determination of MHCII-peptide sensitivity to DM editing. To support this hypothesis, we further measured the MHCII-peptide complex stability and the effect of DM editing using labeled DQ6-LEP147^{488} and DQ6-CKA362^{488} complexes. Both of these complexes have significantly higher dissociation rates at different concentration of DM as compared with the DQ6-CBC114^{488} complex, which is resistant to DM editing (Figure 3.2 and Figure 3.3). This result is consistent with the observed peptide elution results showing that neither LEP147 nor CKA362 was detectable in the samples from cells with DM expression (Table 3.1). It also confirmed our hypothesis that high affinity is necessary but not sufficient for the resistance of DM editing.

DM editing favors the formation of stable MHC II/peptide complex

To further study the role of DM editing in the formation of stable MHCII/peptide complex, we labeled more of the synthesized peptides (Table 3.1) and preloaded each of them onto sDQ6 and measured the peptide dissociation rates. As expected, all of the DM

sensitive peptides, CLIP, ABP625, LEP147, HA, and CKA362 showed significantly higher peptide dissociation rates in the presence of DM, while the DM resistant or DM dependent peptides, and the T1D-associated DQ2 and DQ8 that are relatively resistant to DM editing of CLIP peptide [11], consistently had significantly higher half-life, indicating higher complex stability, and lower peptide dissociation rates in the presence of DM (Figure 3.4A, Table 3.2, and Table 3.3). It is also consistent with data that was normalized relative to the interpolated intrinsic rates (Figure 3.4B and Figure 3.S6). These data confirmed that MHCII-peptide complexes formed with DM sensitive peptides are less stable in the presence of DM, and therefore unlikely to be presented by APC, while the MHCII-peptide complexes formed with DM dependent or resistant peptides are more stable, and likely to be immunologically dominant and involved in the selection or activation of $CD4^+$ T cells.

Interestingly, the intrinsic dissociation rates of DM sensitive peptides are variable and no significant difference was found between DM sensitive and DM insensitive (including DM resistant and DM dependent) groups; however, in the presence of DM, the complex stability and peptide dissociation rates are significantly different between DM sensitive and DM insensitive groups (Figure 3.4C, Table 3.2, and Table 3.3). These data further support the conclusion that peptide affinity is necessary but not sufficient for the formation of stable MHCII-peptide complexes in cells expressing DM. Taken together, these data confirmed the critical role of DM editing as a final checkpoint for the stability of MHCII-peptide complexes.

Discussion

The association of DQ2 and DQ8 alleles with T1D raises several fundamental questions in this field regarding why these DQ alleles are associated with T1D and what unique properties of these T1D-associated DQ molecules, including the two trans-dimers DQ2-8 and DQ8-2, contribute to disease risk. In this study, we evaluated the properties of the T1D-associated DQ molecules by peptidomic analysis. We found further evidence, such as less editing of nested core numbers, significant DM resistance of longer peptides, combined with a relative resistance to DM editing of Ii-derived CLIP peptides that we previously reported [11]. It supports the conclusion that the four T1D-associated DQ molecules share distinct properties with relatively broad resistance to DM-mediated peptide editing, compared to the control molecules, T1D-protective DQ6 and T1D-neutral DQ1, which are both sensitive to DM editing. We also systematically investigated the impact of DM editing effects on the selection of peptides that can form stable complex with MHCII and found that high affinity peptide binding is a necessary but not sufficient for the formation of DM-resistant MHCII-peptide complex. Collectively, our findings further highlight the critical role of DM editing in autoantigen presentation for the selection and/or activation of $CD4^+$ T cells and its potential impact in the pathogenesis of T1D.

Different mechanisms are involved in the relative DM editing resistance of T1D-associated DQ2 and DQ8 molecules, as well as the trans-dimers DQ2-8 and DQ8-2 [8-11]. The same DQ α chain (DQA1*0501) is shared between DQ2 and DQ2-8 molecules, in which there is a natural deletion of residue 53, and the insertion of Gly at this position restored its DM editing sensitivity [9]. In DQ8 and DQ8-2 molecules, extra hydrogen

bonds (H-bonds), with three in DQ8 and one in DQ8-2, were identified between the two helices of α and β chain and near to the DM contact site [11, 26]. Substitution on βE86A to disrupt two of the three H-bonds in DQ8 partially restored DM sensitivity, accompanied with decreased stability of the DQ8 complex [11]. Interestingly, for the two distinct mechanisms in DQ2 and DQ8, the same final output is shared: DM editing resistance. As described, we found more evidence for shared properties among the four T1D-associated DQ molecules in the current study. In particular, we found that there are more long peptides in all of the four T1D-associated DQ molecules that are resistant to DM editing. The biological impact of the DM resistant long peptides is unknown. It is also possible that these peptides result from incomplete antigen processing in 293T cells, but not in professional APC cells. However, the underlying mechanism(s) that is specifically linked to T1D-associated DQ molecules but not to T1D nonassociated DQ1 or DQ6 molecules needs to be further investigated. It appears that long peptides may be more likely to be strong autoantigen agonists in T1D [27]. Potentially, the extra sequences in the long peptides might be beneficial for the competition of prebound CLIP peptides, as well as further disfavoring the interaction of DM and T1D-associated DQ molecules, based on the study of the spatial structure of DM and MHCII interaction [26].

The measurement of peptide dissociation rates confirmed our peptide elution results, showing that all of the DM resistant or dependent peptides have significantly lower dissociation rates, indicating that these peptides can form biologically stable MHCII-peptide complexes. Consistently, these peptides show high binding affinity as illustrated by lower IC50 values. However, in the DM sensitive peptide group, some of the peptides that had significantly lower IC50 values, indicating high binging affinity,

were observed to be sensitive to DM editing. Intrinsic peptide dissociation rates were not found to be a reliable predictor of DM sensitivity (Table 3.2 and Table 3.3). These data indicated that peptide affinity is a necessary but not a sufficient determinant of biological MHCII-peptide complex stability in cells expressing DM. This is consistent with previous findings that in the MHCI pathway, MHCI-peptide complex stability is a better predictor than peptide affinity for CTL immunogenicity [28, 29], as well as in the MHCII pathway, where the stability of MHCII-peptide determines the clonal selection of $CD4^+$ T cells [30, 31].

Multiple factors might be involved in the determination of MHCII-peptide complex stability, such as the peptide affinity, peptide length (with positive or negative effects on peptide binding, and/or the interaction of DM and MHCII), the structure of peptide that favors or disfavors interaction of DM and the direct interaction of DM and MHCII affected by polymorphism of MHCII or DM, etc. As a final checkpoint, DM editing dissociates those peptides that form unstable MHCII-peptide complexes and favors the formation of stable MHCII-peptide complex. However, in the T1D-associated DQ molecules, the DM editing effects are less efficient due to the two distinct mechanisms [8, 9, 11]. In this situation, the peptide repertoire of T1D-associated DQ molecules could be skewed, and autoreactive $CD4^+$ T cells might escape negative selection in thymus and be activated in peripheral as previously proposed [8, 11].

In summary, in this study, we further confirmed that T1D-associated DQ molecules are relatively resistant to DM editing, a property that might affect the peptide repertoires involved in the selection and/or activation of autoreactive $CD4^+$ T cells in T1D etiology. These findings will facilitate our understanding of the mechanism of

autoreactive $CD4^+$ T cell development and improve our ability to design better strategy in early screening and treatment of T1D.

Materials and Methods

Cell lines used for peptide elution or the purification of soluble DQ and DM molecules

Three types of 293T cell lines that were previously generated and maintained in our lab were used for peptide elution [11]. The 293T-DQ cell lines (DQ only) express the full length α and β chains of DQ1 (DQA1*0101/DQB1*0501), DQ2 (DQA1*0501/DQB1*0201), DQ6 (DQA1*0102/DQB1*0602), DQ8 (DQA1*0301/DQB1*0302), or the trans-dimer DQ2-8 (DQA1*0501/DQB1*0302) or DQ8-2 (DQA1*0301/DQB1*0201), the 293T-DQ+Ii cell lines (DQ+Ii) express each DQ and the human Ii, and the 293T-DQ+Ii+DM (DQ+Ii+DM) cell lines express each DQ, Ii and the full length α and β chains of DM expression (DQ+Ii+DM). The transduced 293T cell lines expressing secreted soluble form of DQ molecules (sDQ), such as sDQ2, sDQ6, or sDQ8, were generated and maintained in the complete DMEM media, and the soluble proteins were purified as previously described [11]. The soluble DM (sDM) was purified from S2-sDM insect cells as previously described [32].

Peptide elution and HPLC-MS/MS sequence analysis

About $1x10^9$ of each DQ only, DQ+Ii, DQ+Ii+DM transductants or the control cells were lysed and the DQ molecules were captured by anti-DQ antibody (SPVL3) immobilized with protein A beads. Bound peptides were eluted and further separated by

HPLC for HPLC-MS/MS analysis. The detail procedure and data process followed the previously described methods [18, 19].

DQ-eluted peptide alignment

The sequences were aligned using a specialized expectation-maximization (EM) algorithm [33]. This EM algorithm focuses on the five major anchor residues with MHCII, P1, P4, P6, P7, and P9. Because each position has up to 20 different amino acids, there are 100 parameters in the alignment model. Two general steps, expectation (E) step and maximization (M) step, were undergone iteratively to build the alignment. The formulas for the E and M steps are listed as follows:

E step: Calculate the Q function. $Q(\theta|\theta^t) = \sum_{Z^*} P(Z^*|Z, \theta^t) \log P(Z^*|Z, \theta^t)$

M step: Maximize $Q(\theta|\theta^t)$ with respect to θ. $\theta^{t+1} = \arg\max_\theta Q(\theta|\theta^t)$

where Z refers to the residues of the binding sites in the sequences, Z* refers to the unobserved binding positions, and θ is the alignment model. More details of this model can be found as previously described [33].

Peptide synthesis and labeling

The N-acetylated peptide CLIP (VSKMRMATPLLMQ) and HA (PRFVKQNTLRLAT) were previously labeled on lysine (underlined) with Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Molecular Probes) as described [32], and designated as $CLIP^{488}$ and HA^{488}, respectively. The peptides, with the sequences listed in Table 3.1, were commercially synthesized (PepMic). Some of the peptides without lysine residue in the sequence, such as ABP625, CKA362, LEP147, CBC114,

PRP348, TPP425, ABP625, were further labeled on the N-terminus free amine with the same Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Molecular Probes), following the manual and the purification method as previously described [32].

Peptide competition experiment and IC50 calculation

Peptide competition experiment, measured by fluorescence polarization (FP) assay, was performed as previously described [34], using HA^{488} as probe peptide. Briefly, 150 nM of sDQ6, pretreated with thrombin beads as previously described [11], was incubated in FP buffer (100 mM citrate-phosphate buffer, 0.05% Tween-20, pH 5.0) with 25 nM of HA^{488} probe peptide, 500 nM of sDM, and various concentration (0 to 20 μM, with a dilution factor of 5) of testing peptides (Table 3.1) as competitor peptides. The samples were incubated at 37°C for 18 h, then the FP signal were detected, as previously described [32]. The 50% inhibition concentration (IC50) of the testing peptides was calculated by fitting the curve of relative binding versus concentration of unlabeled testing peptide with the equation $y = 1/[1+(pep)/IC50]$, in which the (pep) is the concentration of testing peptide, as previously described [34].

Fluorescence polarization assay and peptide dissociation rate calculation

Thrombin-cleaved sDQ molecules were preloaded with each of the Alexa Fluor 488 labeled peptides. Briefly, the sDQ and extra amount of each labeled peptides (1:5 molar ratio) were incubated in FP buffer (pH5.0) at 37°C for 6 h (for sDQ6) or overnight (for sDQ2) with 50 nM or sDM, or at 37°C overnight without sDM (for sDQ8). Then, the

unbound peptides were removed by buffer exchange with 30-kD cut-off spin column (Millipore). In FP assay, 50 nM sDQ-^{488}peptide complex, sDM (0 to 2000 nM) and 100 μM competitor peptides were incubated in FP buffer and measured at 37^{o}C with a Tecan Infinite F200 plate reader. FP value was normalized with the background (free ^{488}peptide) FP signal. The data were processed with Prism 5.0 (GraphPad Software) and the DM catalytic peptide dissociation rate was calculated as previously described [32]. The peptide intrinsic dissociation rate was interpolated using a linear fit from the DM catalytic peptide dissociation rates with DM concentrations below 125 nM.

References

1 **Pociot, F., Akolkar, B., Concannon, P., Erlich, H. A., Julier, C., Morahan, G., Nierras, C. R., Todd, J. A. et al.,** Genetics of type 1 diabetes: what's next? *Diabetes* 2010. **59**: 1561-1571.

2 **Gorodezky, C., Alaez, C., Murguia, A., Rodriguez, A., Balladares, S., Vazquez, M., Flores, H. et al.,** HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example. *Autoimmun. Rev.* 2006. **5**: 187-194.

3 **Cifuentes, R. A., Rojas-Villarraga, A. and Anaya, J. M.,** Human leukocyte antigen class II and type 1 diabetes in Latin America: a combined meta-analysis of association and family-based studies. *Hum. Immunol.* 2011. **72**: 581-586.

4 **Aly, T. A., Ide, A., Jahromi, M. M., Barker, J. M., Fernando, M. S., Babu, S. R., Yu, L. et al.,** Extreme genetic risk for type 1A diabetes. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 14074-14079.

5 **Erlich, H., Valdes, A. M., Noble, J., Carlson, J. A., Varney, M., Concannon, P., Mychaleckyj, J. C. et al.,** HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* 2008. **57**: 1084-1092.

6 **Jensen, P. E.,** Recent advances in antigen processing and presentation. *Nat. Immunol.* 2007. **8**: 1041-1048.

7 **Neefjes, J., Jongsma, M. L., Paul, P. and Bakke, O.,** Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 2011. **11**: 823-836.

8 **Zhou, Z. and Jensen, P. E.,** Structural Characteristics of HLA-DQ that May Impact DM Editing and Susceptibility to Type-1 Diabetes. *Front Immunol.* 2013. **4**: 262.

9 **Hou, T., Macmillan, H., Chen, Z., Keech, C. L., Jin, X., Sidney, J., Strohman, M. et al.,** An insertion mutant in DQA1*0501 restores susceptibility to HLA-DM: implications for disease associations. *J. Immunol.* 2011. **187**: 2442-2452.

10 **Busch, R., De Riva, A., Hadjinicolaou, A. V., Jiang, W., Hou, T. and Mellins, E. D.,** On the perils of poor editing: regulation of peptide loading by HLA-DQ and H2-A molecules associated with celiac disease and type 1 diabetes. *Expert. Rev. Mol. Med*. 2012. **14**: e15.

11 **Zhou, Z., Reyes-Vargas, E., Escobar, H., Rudd, B., Rockwood, A. L., Delgado, J. C., He, X. et al.,** Type 1 diabetes associated HLA-DQ2 and DQ8 molecules are relatively resistant to HLA-DM mediated release of invariant chain-derived CLIP peptides. *Eur. J. Immunol.* 2015. In press. DOI: 10.1002/eji.201545942.

12 **Weber, D. A., Evavold, B. D. and Jensen, P. E.,** Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 1996. **274**: 618-620.

13 **Kropshofer, H., Vogt, A. B., Moldenhauer, G., Hammer, J., Blum, J. S. and Hammerling, G. J.,** Editing of the HLA-DR-peptide repertoire by HLA-DM. *EMBO J.* 1996. **15**: 6144-6154.

14 **Nanda, N. K. and Bikoff, E. K.,** DM peptide-editing function leads to immunodominance in CD4 T cell responses in vivo. *J. Immunol.* 2005. **175**: 6473-6480.

15 **van Lummel, M., van Veelen, P. A., Zaldumbide, A., de Ru, A., Janssen, G. M., Moustakas, A. K., Papadopoulos, G. K. et al.,** Type 1 diabetes-associated HLA-DQ8 transdimer accommodates a unique peptide repertoire. *J. Biol. Chem.* 2012. **287**: 9514-9524.

16 **Stumptner-Cuvelette, P. and Benaroch, P.,** Multiple roles of the invariant chain in MHC class II function. *Biochim. Biophys. Acta.* 2002. **1542**: 1-13.

17 **Thayer, W. P., Ignatowicz, L., Weber, D. A. and Jensen, P. E.,** Class II-associated invariant chain peptide-independent binding of invariant chain to class II MHC molecules. *J. Immunol.* 1999. **162**: 1502-1509.

18 **Escobar, H., Crockett, D. K., Reyes-Vargas, E., Baena, A., Rockwood, A. L., Jensen, P. E. and Delgado, J. C.,** Large scale mass spectrometric profiling of peptides eluted from HLA molecules reveals N-terminal-extended peptide motifs. *J. Immunol.* 2008. **181**: 4874-4882.

19 **Escobar, H., Reyes-Vargas, E., Jensen, P. E., Delgado, J. C. and Crockett, D. K.,** Utility of characteristic QTOF MS/MS fragmentation for MHC class I peptides. *J. Proteome. Res.* 2011. **10**: 2494-2507.

20 **Fremont, D. H., Hendrickson, W. A., Marrack, P. and Kappler, J.,** Structures of an MHC class II molecule with covalently bound single peptides. *Science* 1996. **272**: 1001-1004.

21 **Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. and Wilson, I. A.,** Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science* 1992. **257**: 919-927.

22 **Kooy-Winkelaar, Y., van Lummel, M., Moustakas, A. K., Schweizer, J., Mearin, M. L., Mulder, C. J., Roep, B. O. et al.,** Gluten-specific T cells cross-react between HLA-DQ8 and the HLA-DQ2alpha/DQ8beta transdimer. *J. Immunol.* 2011. **187**: 5123-5129.

23 **Suri, A., Walters, J. J., Gross, M. L. and Unanue, E. R.,** Natural peptides selected by diabetogenic DQ8 and murine I-A(g7) molecules show common sequence specificity. *J. Clin. Invest* 2005. **115**: 2268-2276.

24 **Chang, K. Y. and Unanue, E. R.,** Prediction of HLA-DQ8beta cell peptidome using a computational program and its relationship to autoreactive T cells. *Int. Immunol.* 2009. **21**: 705-713.

25 **Yin, L. and Stern, L. J.,** 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. *J. Immunol. Methods* 2014. **406**: 21-33.

26 **Pos, W., Sethi, D. K., Call, M. J., Schulze, M. S., Anders, A. K., Pyrdol, J. and Wucherpfennig, K. W.,** Crystal structure of the HLA-DM-HLA-DR1 complex defines mechanisms for rapid peptide selection. *Cell* 2012. **151**: 1557-1568.

27 **Jin, N., Wang, Y., Crawford, F., White, J., Marrack, P., Dai, S. and Kappler, J. W.,** N-terminal additions to the WE14 peptide of chromogranin A create strong autoantigen agonists in type 1 diabetes. *Proc. Natl. Acad. Sci. USA* 2015. **112**: 13318-13323.

28 **Harndahl, M., Rasmussen, M., Roder, G., Dalgaard Pedersen, I., Sorensen, M., Nielsen, M. and Buus, S.,** Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. *Eur. J. Immunol.* 2012. **42**: 1405-1416.

29 **Geironson, L., Roder, G. and Paulsson, K.,** Stability of peptide-HLA-I complexes and tapasin folding facilitation--tools to define immunogenic peptides. *FEBS Lett.* 2012. **586**: 1336-1343.

30 **Baumgartner, C. K., Ferrante, A., Nagaoka, M., Gorski, J. and Malherbe, L. P.,** Peptide-MHC class II complex stability governs CD4 T cell clonal selection. *J. Immunol.* 2010. **184**: 573-581.

31 **Siklodi, B., Vogt, A. B., Kropshofer, H., Falcioni, F., Molina, M., Bolin, D. R., Campbell, R. et al.,** Binding affinity independent contribution of peptide length to

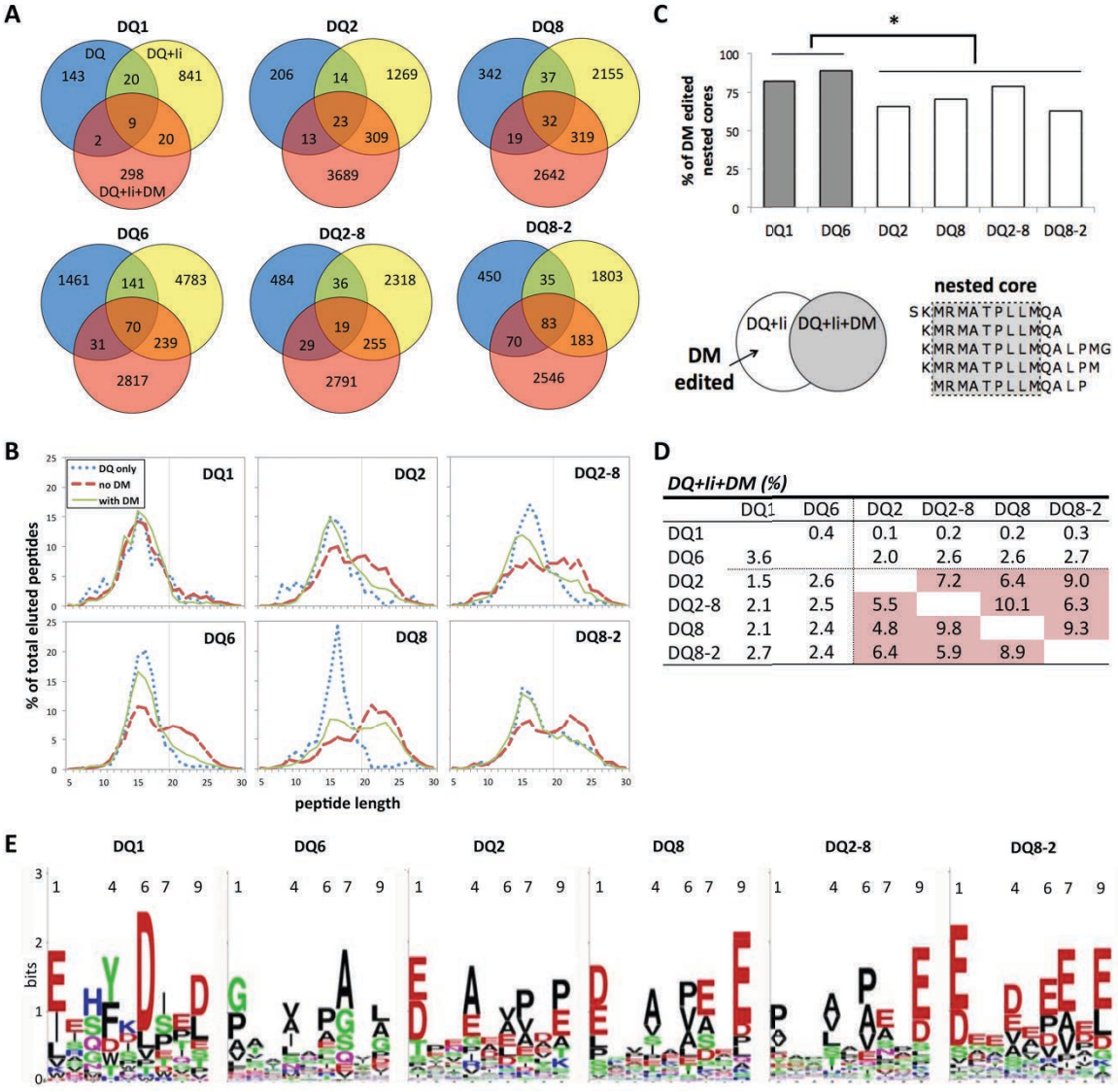
the stability of peptide-HLA-DR complexes in live antigen presenting cells. *Hum. Immunol.* 1998. **59**: 463-471.

32 **Zhou, Z., Callaway, K. A., Weber, D. A. and Jensen, P. E.,** Cutting edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. *J. Immunol.* 2009. **183**: 4187-4191.

33 **Chang, K. Y., Suri, A. and Unanue, E. R.,** Predicting peptides bound to I-Ag7 class II histocompatibility molecules using a novel expectation-maximization alignment algorithm. *Proteomics* 2007. **7**: 367-377.

34 **Yin, L. and Stern, L. J.,** Measurement of Peptide Binding to MHC Class II Molecules by Fluorescence Polarization. *Curr. Protoc. Immunol.* 2014. **106**: 5 10 11-15 10 12.

Figure 3.1. Peptidomic analysis of T1D-associated and nonassociated DQ molecules with the peptides eluted from 293T transductants. (A) Overlapping of the identified unique peptides eluted from DQ only (blue), DQ+Ii (yellow) and DQ+Ii+DM (orange) samples. (B) The distribution of peptide length in the absence or presence of Ii or DM editing. The dotted gray line separates the bimodal distributions with median lengths of ~15 and ~23 amino acids. (C) The percentage of nested core numbers that were edited by DM. The significance between T1D-associated and nonassociated DQ molecules are shown with *, P=0.043, two-tail T-test, unpaired. The bottom left illustrated the population of peptides that was edited in the presence of DM expression. The bottom right showed an example of a nested core, in which multiple unique peptides share the same 9 amino acids core sequence as marked. (D) The frequency of unique peptides that were identified in common between samples from different DQ molecules in the presence of DM editing. The highlighted frequency indicated the increase of shared peptides within T1D-associated DQ molecules. (E) The predicted peptide binding motifs of each DQ molecules in the presence of DM editing. The numbers on the top of each motif showed the major anchor residues on position 1, 4, 6, 7, and 9.



DQ+Ii+DM (%)	DQ1	DQ6	DQ2	DQ2-8	DQ8	DQ8-2
DQ1		0.4	0.1	0.2	0.2	0.3
DQ6	3.6		2.0	2.6	2.6	2.7
DQ2	1.5	2.6		7.2	6.4	9.0
DQ2-8	2.1	2.5	5.5		10.1	6.3
DQ8	2.1	2.4	4.8	9.8		9.3
DQ8-2	2.7	2.4	6.4	5.9	8.9	

Figure 3.2. Comparison of the peptide binding affinity among DM resistant, dependent and sensitive peptides by the measurement of IC50 in peptide competition experiment. (A) The percentage of indicator peptides HA^{488} bound to DQ6 molecules with DM resistant peptides as competitors. Peptide competition experiment was performed in the presence of variant concentration competitor peptides, from 0 nM to 20000 nM, with 5 times dilution. After treated at 37^{o}C for 18 h, five FP signal reads were averaged and normalized with the signal of bound indicators without competitor peptides. (B) The percentage of indicator peptides HA^{488} bound to DQ6 molecules with DM dependent peptides as competitors. (C) The percentage of indicator peptides HA^{488} bound to DQ6 molecules with DM sensitive peptides as competitors. (D) The percentage of indicator peptides HA^{488} bound to DQ6 molecules with DM sensitive peptides that showing high affinity in competition assays as competitors. (E) Comparison of IC50 among DM resistant, dependent and sensitive peptides. The asterisks mark the two peptides LEP147 and CKA362 that are not detectable in peptide elution experiments in the presence of DM expression but with high affinity in peptide competition experiments. Data shown are representative of three independent experiments.

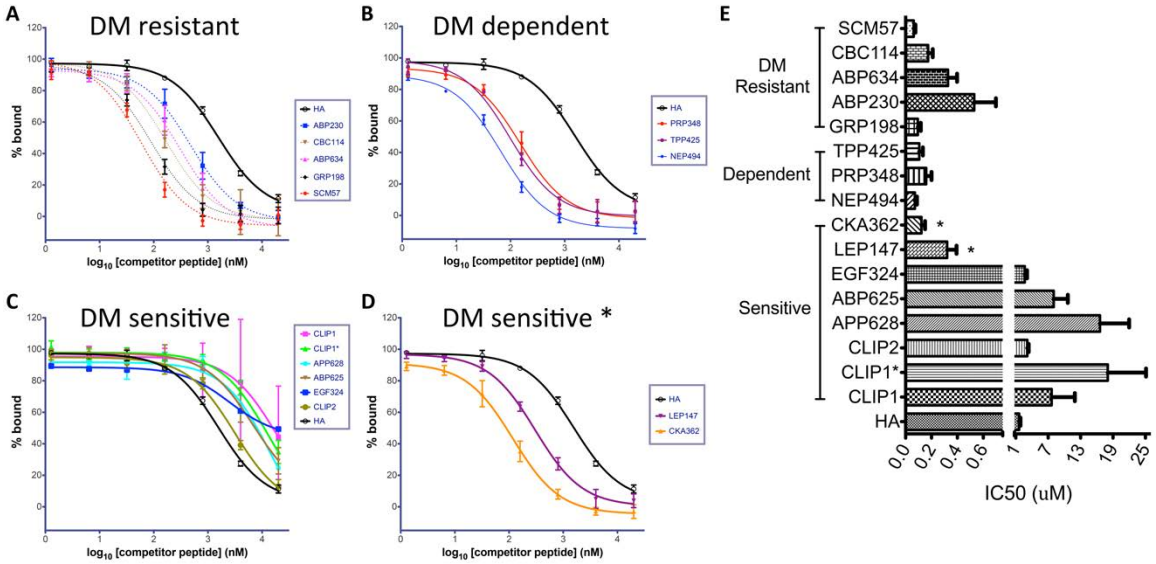

A
DM resistant
% bound
log10 [competitor peptide] (nM)
HA
ABP230
CBC114
ABP634
GRP198
SCM57
B
DM dependent
% bound
log10 [competitor peptide] (nM)
HA
PRP348
TPP425
NEP494
C
DM sensitive
% bound
log10 [competitor peptide] (nM)
CLIP1
CLIP1*
APP628
ABP625
EGF324
CLIP2
HA
D
DM sensitive *
% bound
log10 [competitor peptide] (nM)
HA
LEP147
CKA362
E
DM Resistant
Dependent
Sensitive
SCM57
CBC114
ABP634
ABP230
GRP198
TPP425
PRP348
NEP494
CKA362
LEP147
EGF324
ABP625
APP628
CLIP2
CLIP1*
CLIP1
HA
*
*
0.0
0.2
0.4
0.6
1
7
13
19
25
IC50 (uM)

Figure 3.3. Peptide dissociation rates and DM editing effects on LEP147 and CKA362 peptides. (A). The peptide dissociation rates of the four different peptides measured by FP experiments. The labeled DQ6/peptide complex was incubated with 20 μM excess amount of unlabeled peptide and various concentration of DM (pH 5.0) and measured at 37^{o}C by FP. (B) DM-catalyzed rate enhancements normalized for differences in interpolated intrinsic peptide dissociation rates. Data shown are representative of three independent experiments.

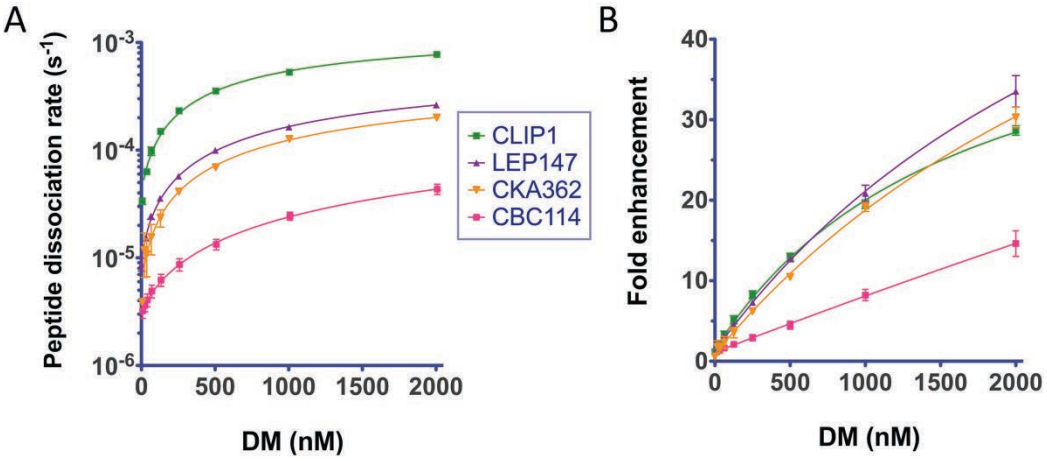

A
Peptide dissociation rate (s-1)
10-3
10-4
10-5
10-6
0
500
1000
1500
2000
DM (nM)
CLIP1
LEP147
CKA362
CBC114
B
Fold enhancement
40
30
20
10
0
0
500
1000
1500
2000
DM (nM)

Figure 3.4. Effects of DM editing in different DQ-peptide complex stability. (A) Peptide dissociation rates of DM sensitive, resistant, and dependent peptides. The peptide dissociation rates of DQ8/CLIP, DQ2/CLIP and DQ6/CLIP were previously measured [11]. (B) DM-catalyzed rate enhancements normalized for differences in interpolated peptide intrinsic dissociation rates. (C) Distribution of IC50 that are measured in the presence of 500 nM of sDM versus the interpolated peptides intrinsic dissociation rates. (D) Distribution of IC50 versus DM-catalyzed peptide dissociation rates in the presence of 500 nM of sDM. Data shown are representative of three independent experiments.

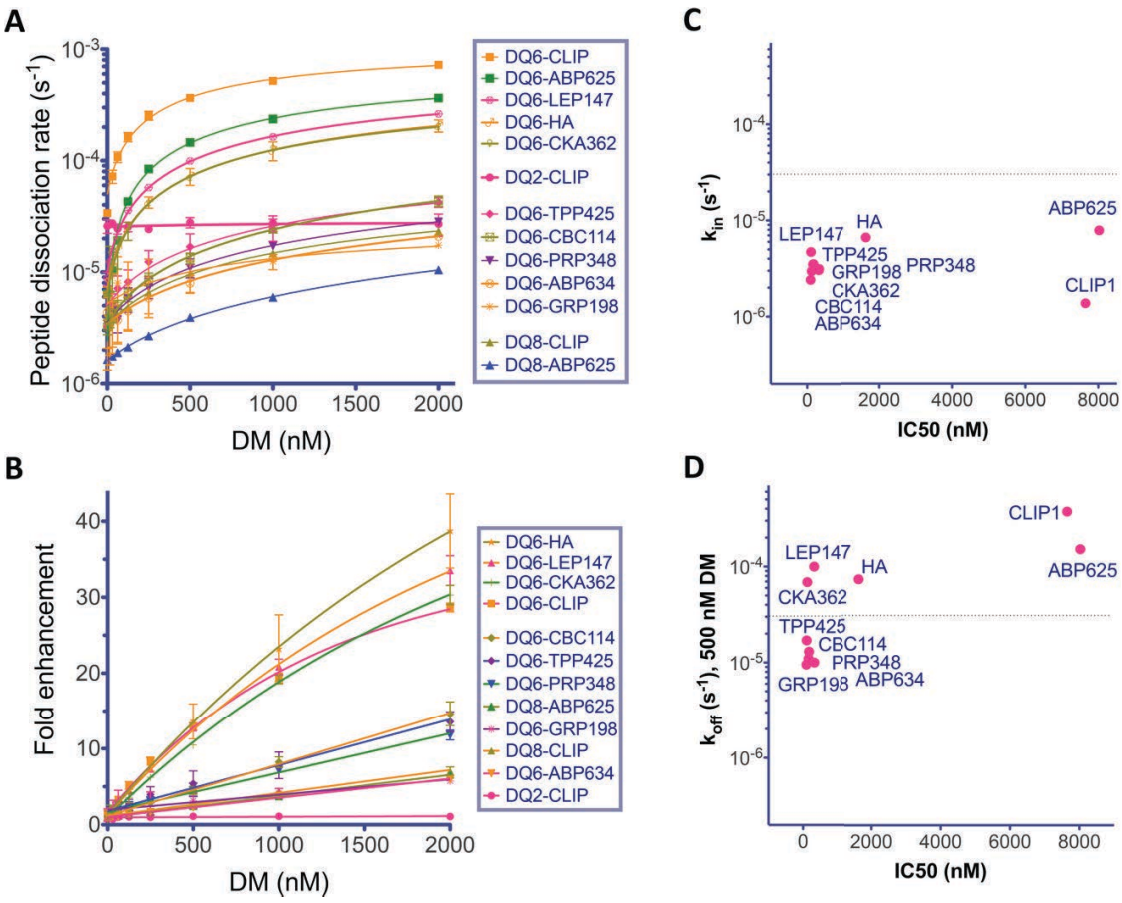

A
Peptide dissociation rate (s^{-1})
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
500
1000
1500
2000
DM (nM)
DQ6-CLIP
DQ6-ABP625
DQ6-LEP147
DQ6-HA
DQ6-CKA362
DQ2-CLIP
DQ6-TPP425
DQ6-CBC114
DQ6-PRP348
DQ6-ABP634
DQ6-GRP198
DQ8-CLIP
DQ8-ABP625
B
Fold enhancement
40
30
20
10
0
500
1000
1500
2000
DM (nM)
DQ6-HA
DQ6-LEP147
DQ6-CKA362
DQ6-CLIP
DQ6-CBC114
DQ6-TPP425
DQ6-PRP348
DQ8-ABP625
DQ6-GRP198
DQ8-CLIP
DQ6-ABP634
DQ2-CLIP
C
k_{in} (s^{-1})
10^{-4}
10^{-5}
10^{-6}
LEP147
HA
TPP425
GRP198
PRP348
CKA362
CBC114
ABP634
ABP625
CLIP1
0
2000
4000
6000
8000
IC50 (nM)
D
k_{off} (s^{-1}), 500 nM DM
10^{-4}
10^{-5}
10^{-6}
CLIP1
LEP147
HA
ABP625
CKA362
TPP425
CBC114
PRP348
GRP198
ABP634
0
2000
4000
6000
8000
IC50 (nM)

Table 3.1. Peptides with different DM sensitivity that were eluted from DQ6+Ii or DQ6+Ii+DM 293T cells.

DM sensitivity	Name	Peptide sequence	Accession Number	Hits (-DM) [a]	Hits (+DM) [b]	Entry name
Resistant	ABP230	EEEEVAEVEEEEADDD	P05067	8 of 11	4 of 17	Amyloid beta A4 protein precursor
	ABP634	ENEVEPVDARPAADR	P05067	1 of 8	1 of 9	Amyloid beta A4 protein precursor
	CBC114	GVVRGASIPFQFRP	Q13137	6 of 11	2 of 9	Calcium-binding and coiled-coil domain-containing protein 2
	GRP198	IINEPTAAAIAYG	P11021	16 of 75	2 of 13	78 kDa glucose-regulated protein precursor
	SCM57	SSQPAVLQPSVE	O15127	0 of 8	3 of 22	Secretory carrier-associated membrane protein 2
Dependent	PRP348	APSIGFVSVRQGALS	O14917	--	9 of 9	Protocadherin-17 precursor
	TPP425	EVQQTAARIAGALG	Q9NX61	--	4 of 16	Transmembrane protein 161A precursor
	NEP494	TPKTVKGVIIQGARGGD	O60462	--	7 of 19	Neuropilin-2 precursor
Sensitive	CLIP1	LPKPPKPVSKMRMATPLLMQA	P04233	1 of 250	--	HLA class II histocompatibility antigen gamma chain
	CLIP1*	KMRMATPLLMQA	P04233	4 of 250	--	HLA class II histocompatibility antigen gamma chain
	CLIP2	MATPLLMQALPM	P04233	1 of 127	--	HLA class II histocompatibility antigen gamma chain
	ABP625	GADSVPANTENEVEPV	P05067	4 of 17	--	Amyloid beta A4 protein precursor
	APP628	VGEQDGGLIGAEEKVIN	Q06481	2 of 12	--	Amyloid-like protein 2 precursor
	EGF324	LARCPEAGLAAQVISPLLTPKA	Q8TE67	13 of 21	--	Epidermal growth factor receptor kinase substrate 8-like protein 3
	LEP147	DHGSTGILVFPNEDL	Q99538	17 of 25	--	Legumain precursor
	CKA362	SPLGPLAGSPVIAAANPL	P68400	9 of 15	--	Casein kinase II subunit alpha

[a] The hits (n of m) represent the number of times (n) that the sequence was identified in the raw dataset from a total number of peptides (m) that share a common core sequence; [b] the (-DM) or (+DM) represents the source of the peptides that are eluted from the DQ+Ii or DQ+Ii+DM samples, respectively.

Table 3.2. Half-life (t1/2) of DQ/peptide complexes in the absence or presence of DM and the peptide IC50 values.

DM sensitivity	Complex	$t_{1/2}$ (hour, no DM)	IC50 (nM)[a]	$t_{1/2}$ (500 nM DM)	$t_{1/2}$ (2 mM DM)
Sensitive	DQ6/CLIP1	7.1	7649.7	0.5	0.3
	DQ6/ABP625	137.5	8026.7	1.3	0.5
	DQ6/LEP147	64.2	321.0	1.9	0.7
	DQ6/CKA362	29.2	122.8	2.8	1.0
Resistant	DQ6/ABP634	55.0	327.2	24.2	9.2
	DQ6/CBC114	64.2	174.0	14.4	4.4
	DQ6/GRP198	64.2	94.7	20.2	11.0
Dependent	DQ6/PRP348	80.2	158.7	17.5	6.8
	DQ6/TPP425	62.1	105.9	11.4	4.5
	DQ2/CLIP1	7.7		6.9	7.1
	DQ8/CLIP1	58.3		20.0	8.2
	DQ8/ABP625	120.3		49.2	18.4

[a] The IC50 values were measured in the presence of 500 nM of DM.

Table 3.3. The intrinsic and DM mediated catalytic peptide dissociation rates (s^{-1})

Complex	0 [a]	DM (nM)							
		31.3	62.5	125	250	500	1000	2000	n
DQ6/CLIP1	2.7E-05	7.2E-05	1.1E-04	1.6E-04	2.5E-04	3.7E-04	5.2E-04	7.2E-04	4
DQ6/HA	5.3E-06	1.1E-05	1.6E-05	2.5E-05	4.2E-05	7.3E-05	1.2E-04	2.1E-04	3
DQ6/ABP625	1.4E-06	1.0E-05	1.9E-05	4.3E-05	8.4E-05	1.5E-04	2.4E-04	3.6E-04	3
DQ6/CKA362	6.6E-06	1.1E-05	1.6E-05	2.4E-05	4.1E-05	6.9E-05	1.3E-04	2.0E-04	3
DQ6/LEP147	7.8E-06	1.5E-05	2.4E-05	3.6E-05	5.7E-05	1.0E-04	1.6E-04	2.6E-04	3
DQ6/CBC114	3.0E-06	4.1E-06	4.9E-06	6.2E-06	8.7E-06	1.3E-05	2.4E-05	4.3E-05	3
DQ6/GRP198	3.0E-06	5.8E-06	8.0E-06	7.7E-06	8.5E-06	9.5E-06	1.2E-05	1.7E-05	2
DQ6/ABP634	3.5E-06	6.5E-06	6.0E-06	6.9E-06	8.6E-06	1.0E-05	1.4E-05	2.1E-05	4
DQ6/PRP348	2.4E-06	4.5E-06	3.8E-06	5.8E-06	7.3E-06	1.1E-05	1.7E-05	2.8E-05	3
DQ6/TPP425	3.1E-06	5.3E-06	7.1E-06	8.2E-06	1.2E-05	1.7E-05	2.4E-05	4.2E-05	3
DQ8/ABP625	1.6E-06	1.8E-06	1.9E-06	2.1E-06	2.7E-06	3.9E-06	5.9E-06	1.0E-05	2
DQ8/CLIP1	3.3E-06	3.8E-06	4.2E-06	4.9E-06	6.6E-06	9.6E-06	1.5E-05	2.3E-05	4
DQ2/CLIP1	2.5E-05	2.7E-05	2.4E-05	2.6E-05	2.4E-05	2.8E-05	2.8E-05	2.7E-05	3

[a] The interpolated intrinsic peptide dissociation rates.

Figure 3.S1. Distribution of the subcellular localization of proteins identified by the eluted unique peptides. The accession number of each protein that was identified by each of the eluted unique peptides was used to query the protein subcellular localization information from the UniProt human protein database (www.uniprot.org). The proteins with multiple localizations will be classified in combined groups. ER, endoplasmic reticulum; ER/G/L/En, the combined group of ER, Golgi, Lysosome, and Endosome; Multiple, the combined group that proteins are with multiple localizations other than the classified; Other, the group of proteins with unknown subcellular localization information; -DM, peptides eluted from DQ+Ii samples; +DM, peptides eluted from DQ+Ii+DM samples.

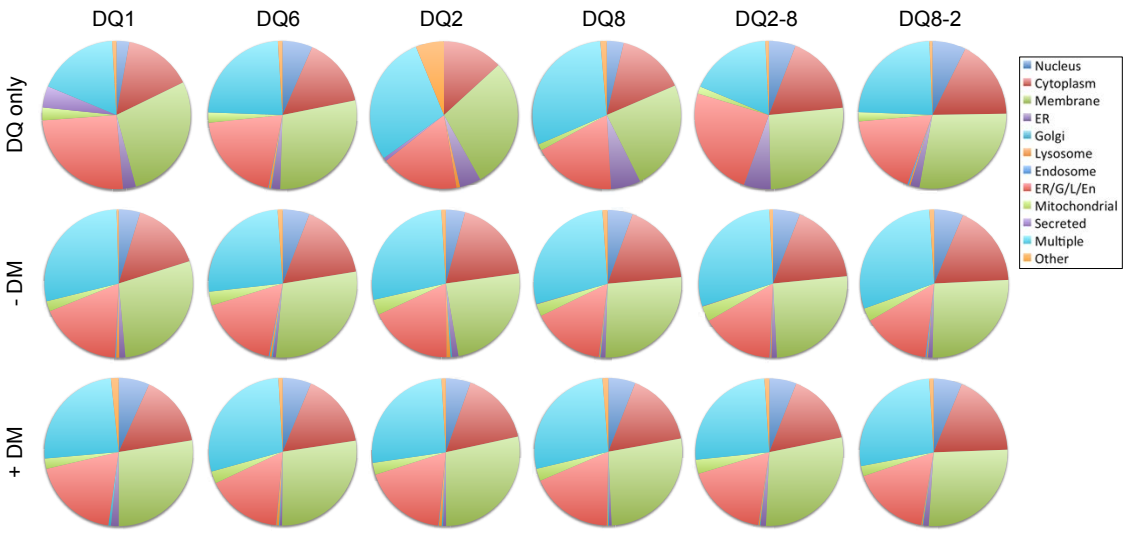

DQ1
DQ6
DQ2
DQ8
DQ2-8
DQ8-2
DQ only
- DM
+ DM
Nucleus
Cytoplasm
Membrane
ER
Golgi
Lysosome
Endosome
ER/G/L/En
Mitochondrial
Secreted
Multiple
Other

Figure 3.S2. Distribution of the length of unique peptides that were detected from each DQ samples. The unique peptides were eluted from DQ-only, DQ+Ii or DQ+Ii+DM samples and illustrated as the total number of peptides versus peptide length, the counts of amino acids.

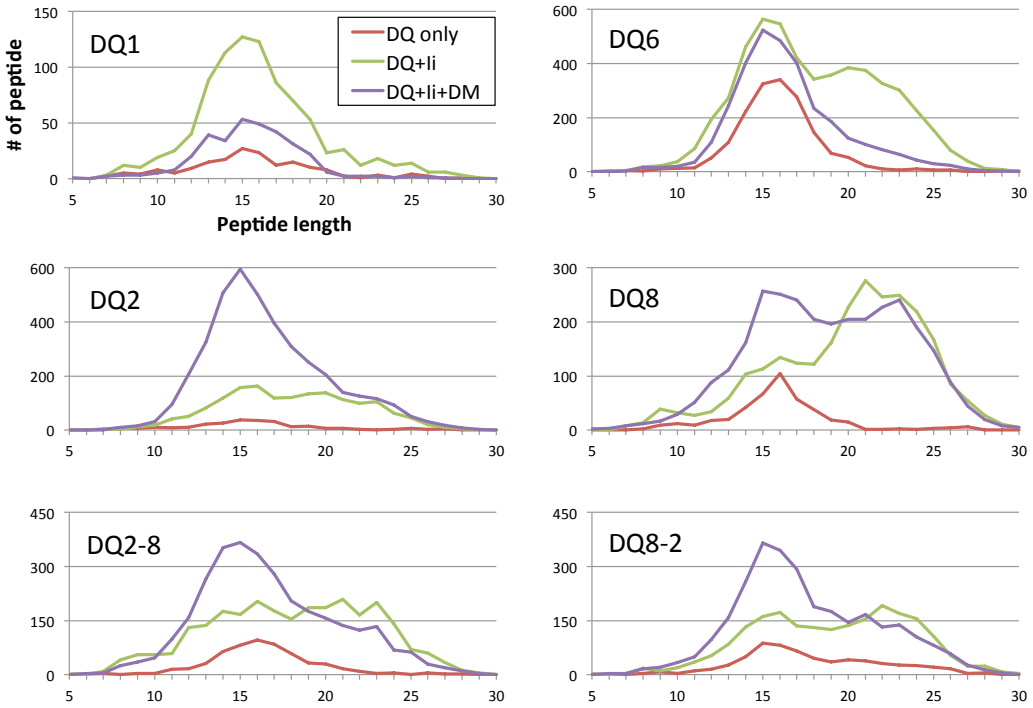

DQ1
DQ only
DQ+Ii
DQ+Ii+DM
# of peptide
Peptide length
DQ6
DQ2
DQ8
DQ2-8
DQ8-2

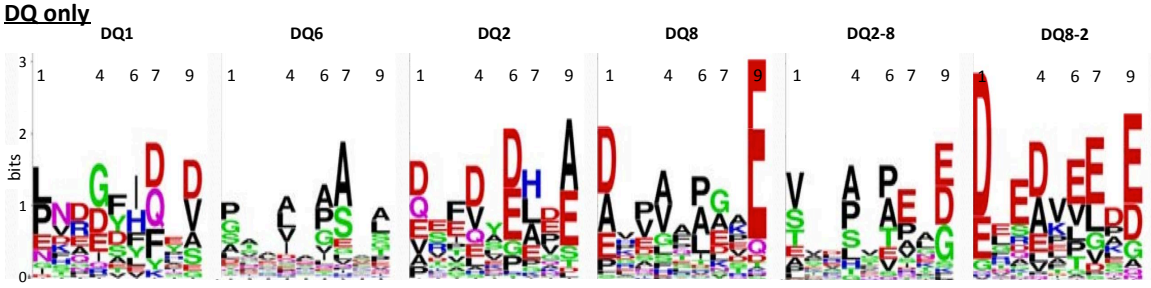
Figure 3.S3. The frequency of unique peptides that were identified in common between samples from different DQ molecules in the absence of DM editing. This is supportive data for Figure 3.1D, showing no frequency difference of shared peptides between T1D-associated and nonassociated DQ molecules.

DQ+Ii (%)

DQ1		10.2	1.6	3.5	1.7	1.7
DQ6	1.8		2.1	2.7	2.7	2.0
DQ2	1.1	7.9		2.9	3.6	5.0
DQ2-8	1.3	5.7	1.6		3.6	2.1
DQ8	0.7	6.0	2.1	3.8		3.6
DQ8-2	0.8	5.4	3.5	2.7	4.3	

Figure 3.S4. Predicted peptide binding motifs of each DQ molecules. Each of the DQ molecules peptide binding motifs was predicted using the eluted peptides from the transduced 293T-DQ samples in the absence (top panel) or presence (bottom panel) of human invariant chain expression. The numbers on the top of each motif showed the major anchor residues on position 1, 4, 6, 7, and 9. The predicted motifs were displayed with WebLogo.

DQ only



DQ + Ii

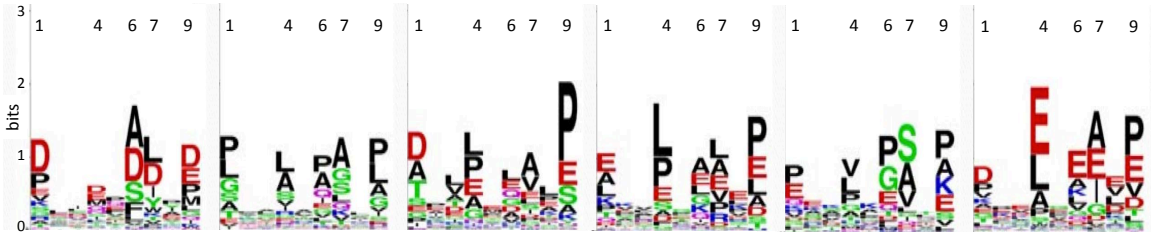


Figure 3.S5. The classification of eluted peptides for their sensitivity to DM editing. The unique peptides eluted from DQ+Ii samples were not DM edited, while the peptides eluted from DQ+Ii+DM samples were DM edited. Based on the overlapping of two types of peptides, DM sensitive peptides represent the group of peptides that are in DQ+Ii samples but not in DQ+Ii+DM samples (yellow); DM dependent peptides represent the group of peptides that are in DQ+Ii+DM samples but not in DQ+Ii samples (red); DM resistant peptides represent the group of peptides that are in both samples (orange), indicating that those peptides are resistant to DM editing.

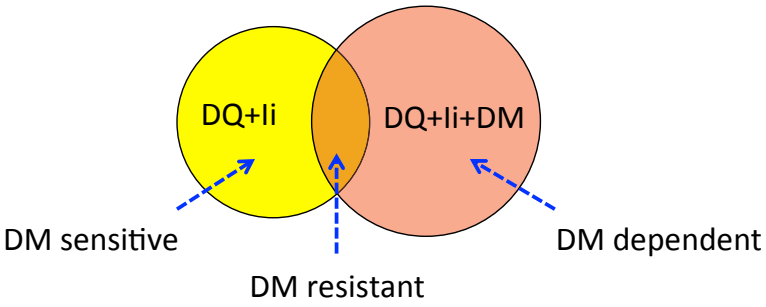
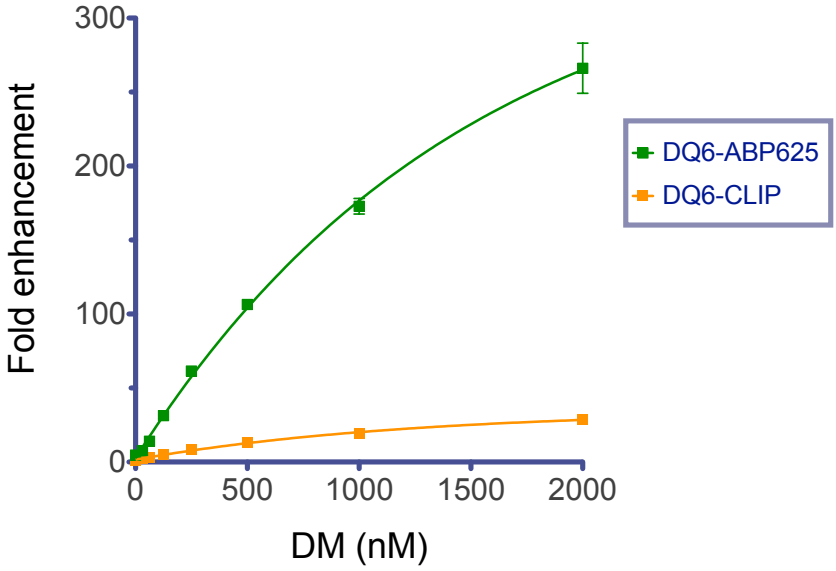

DQ+Ii
DQ+Ii+DM
DM sensitive
DM resistant
DM dependent

Figure 3.S6. DM-catalyzed rate enhancements in DQ6/ABP625 complex. The DM editing effect is represented with the DM-catalyzed peptide dissociation rate normalized for fold enhancement with the interpolated intrinsic peptide dissociation rates. Data shown are representative of three independent experiments.


300
200
100
0
Fold enhancement
0
500
1000
1500
2000
DM (nM)
DQ6-ABP625
DQ6-CLIP

CHAPTER 4

HLA-DM FUNCTIONS THROUGH A MECHANISM THAT DOES NOT REQUIRE SPECIFIC CONSERVED HYDROGEN BONDS IN CLASS II MHC-PEPTIDE COMPLEXES

Originally published in *The Journal of Immunology*. Zemin Zhou, Kari A. Callaway, Dominique A. Weber, Peter E. Jensen. 2009. Cutting- edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. J. Immunol. 183: 4187-4191.

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: HLA-DM Functions through a Mechanism That Does Not Require Specific Conserved Hydrogen Bonds in Class II MHC-Peptide Complexes[1]

Zemin Zhou,[2*§] Kari A. Callaway,[2*] Dominique A. Weber,[†] and Peter E. Jensen[3*‡]

HLA-DM catalyzes peptide dissociation and exchange in class II MHC molecules through a mechanism that has been proposed to involve the disruption of specific components of the conserved hydrogen bond network in MHC-peptide complexes. HLA-DR1 molecules with alanine substitutions at each of the six conserved H-bonding positions were expressed in cells, and susceptibility to DM catalytic activity was evaluated by measuring the release of CLIP. The mutants αN62A, αN69A, αR76A, and βH81A DR1 were fully susceptible to DM-mediated CLIP release, and βN82A resulted in spontaneous release of CLIP. Using recombinant soluble DR1 molecules, the amino acid βN82 was observed to contribute disproportionately in stabilizing peptide complexes. Remarkably, the catalytic potency of DM with each β-chain mutant was equal to or greater than that observed with wild-type DR1. Our results support the conclusion that no individual component of the conserved hydrogen bond network plays an essential role in the DM catalytic mechanism. *The Journal of Immunology,* **2009, 183: 4187–4191.**

Class II MHC molecules initially assemble with the chaperone invariant chain (Ii)[4] followed by transport to endosomal compartments and proteolytic cleavage of Ii, leaving a fragment, CLIP, largely buried in the peptide-binding groove (1). HLA-DM catalyzes CLIP dissociation and peptide exchange reactions in class II molecules, accelerating the loading process for peptide Ags (2–4) and editing the repertoire of peptides presented to $CD4^+$ T cells. DM is a nonpolymorphic MHC class II protein that is structurally similar to other class II molecules (5). However, DM does not have the capacity to bind peptide Ags, and it functions as a chaperone-catalyst, stabilizing peptide-free ("empty") class II molecules (6) and accelerating CLIP dissociation and peptide exchange through a mechanism that involves transient direct physical interaction with class II-peptide complexes. DM accelerates the rate of dissociation of all peptides (7), not just CLIP, but catalytic potency differs depending on the kinetic stability of the complex (7–10) and other less defined features of the complex (11–13). The capacity of DM to differentially "edit" peptide complexes has important biological implications through skewing of the repertoire of foreign and self-peptide complexes available for activation or tolerance induction in $CD4^+$ T cells.

The structural basis for the DM catalytic mechanism remains poorly understood. The general orientation of the physical interaction between DM and substrate MHC class II molecules (i.e., HLA-DR) has been defined by using mutational and cross-linking approaches (14–16). It is likely that DM preferentially binds to and stabilizes a relatively unpopulated conformational isomer of MHC class II-peptide complexes, characterized by a loss or weakening of noncovalent interactions that stabilize peptide binding (7, 17, 18). Two general sets of interactions are largely responsible for peptide binding: 1) peptide sequence-dependent interactions between peptide side chains (anchors) and subsites or "pockets" in the peptide-binding groove; and 2) a conserved hydrogen bond network formed by nonpolymorphic amino acids in the MHC protein and main chain atoms in bound peptide (19). The anchor-pocket interactions are primarily responsible for determining peptide-binding specificity, whereas the conserved hydrogen bond network provides a basal contribution to stability and constrains the orientation of peptide in the binding site. Destabilization of conserved hydrogen bonds has been hypothesized to be a primary component of the DM catalytic mechanism (5, 7, 20, 21). This is attractive because the hydrogen bond network is a conserved feature, consistent with the universal capacity of DM to accelerate the dissociation of peptide complexes. There is strong evidence that the network contributes greatly to stabilizing peptide complexes (22, 23). In addition, this mechanism would account for the results indicating that catalytic potency is inversely proportional to kinetic stability (7). If one or more

*Department of Pathology, University of Utah, Salt Lake City, UT 84112; †Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322; ‡ARUP Laboratories, University of Utah, Salt Lake City, UT 84108; and §College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China 430070

Received for publication May 26, 2009. Accepted for publication August 6, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Research Grants AI30554 and AI33614.

[2] Z.Z. and K.A.C. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Peter E. Jensen, Department of Pathology, University of Utah, Emma Eccles Jones Medical Research Building, 15 North Medical Drive East, Suite 1100, Salt Lake City, UT 84112-5650. E-mail address: peter.jensen@path.utah.edu

[4] Abbreviations used in this paper: Ii, invariant chain; s, soluble (prefix); WT, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901663

conserved hydrogen bonds were the primary target for disruption in the catalytic mechanism, one might predict that the energy of stabilization would be reduced by an approximately constant factor independent of the sequence of the bound peptide. Indeed, Narayan et al. recently proposed that DM specifically targets the hydrogen formed by the conserved histidine at position β81 in MHC class II molecules (21). HLA-DR1 molecules with an asparagine substitution at this position were reported to form highly unstable peptide complexes, and peptide dissociation was not further enhanced by DM, possibly because DM cannot further disrupt a hydrogen bond that does not exist in the mutant molecule.

In the present study, two approaches were used to systematically analyze the effect of conserved hydrogen bond-disrupting mutations on DM catalytic potency. We postulated that mutational disruption of specific hydrogen bonds targeted in the catalytic mechanism would result in reduced catalytic potency, consistent with the results reported by Narayan et al. (21). Instead, our results indicate that the conserved hydrogen bond formed by histidine β81 is not a primary target in the DM catalytic mechanism. Indeed, our findings support the conclusion that none of the conserved hydrogen bonds is a critical target necessary for DM-catalyzed peptide dissociation.

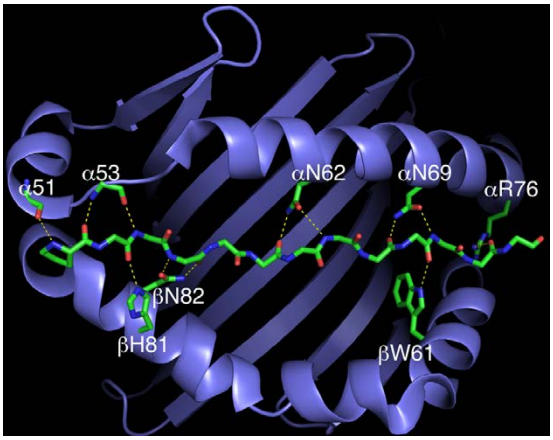


FIGURE 1. The conserved hydrogen bond network in class II MHC-peptide complexes. The binding site of DR1 is represented as a ribbon diagram and the backbone of bound peptide is shown as a stick representation. Hydrogen bonds with main chain atoms of bound peptide are shown as yellow dashed lines. Main chain atoms of amino acids at DR1 positions α51 and α53 mediate hydrogen bonds, whereas conserved side chains form hydrogen bonds at other positions in the MHC molecule. The figure was generated with PyMOL software (www.pymol.org; PyMOL molecular graphics system; DeLano Scientific) using Brookhaven Protein Data Bank coordinate file 1DLH (19).

Materials and Methods

Expression of mutant DR1 molecules in T2 cells

Full length DR1α and DR1β (DRA*0101/DRB1*0101) and mutant constructs were cloned into the retroviral vectors pLPCX or pLXSN (Clontech). Constructs encoding full-length DM α- and β-chains were fused with the FMDV.2A sequence (24) by PCR. The DMA-2A-DMB construct was cloned into the retroviral vector MigR1, which has a GFP marker driven by an internal ribosomal entry site. The T2 and Phoenix cell lines were obtained from the American Type Culture Collection. High-titer retroviral supernatants were generated by transfection of Phoenix cells with pLPCX-DRA, pLXSN-DRB or MigR1-DMA-2A-DMB (25). T2-DRα-DRβ double positive cell lines were obtained by DRα retroviral infection and puromycin selection, followed by DRβ retroviral infection and G418 selection (Invitrogen). To coexpress DM, cells expressing wild-type (WT) or mutant DR1 were infected with DMA-2A-DMB retrovirus and sorted for GFP expression with a FACSVantage cell sorter (BD Biosciences).

Abs and flow cytometry

Fluorophore-conjugated mAbs to HLA-DR (L243), CLIP (CerCLIP), and HLA-DM (MaP.DM1) and isotype-matched negative control mAbs were purchased from BD Pharmingen. Cell surface staining was performed with a combination of mAbs according to the standard procedures. Intracellular staining was performed using BD Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences). Stained cells were analyzed on a FACScan flow cytometer (BD Biosciences) and data were analyzed with FlowJo 8.4 software.

Expression and purification of soluble (s) DR1 (sDR1) and sDM

Stable S2 transfectants expressing sDR1A (residues 1–192) and sDR1B (residues 1–198) were induced for 7 days in BD BaculoGold Max-XP serum-free medium (BD Biosciences) with 1 mM $CuSO_4$. Cell culture supernatant was collected by centrifugation, sDR1 was purified with L243 mAb affinity chromatography, and aggregates were removed using a TSK-GEL G3000SW analytical gel filtration column. Soluble DM was purified from supernatants of S2 transfectants as described (4, 14).

Peptide labeling and sDR1-peptide complex formation

The peptides HA (PRFVKQNTLRLAT) and CLIP (VSKMRMATPLLMQ) were commercially synthesized with the N terminus acetylated. Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester was used to covalently attach the Alexa Fluor 488 fluorophore to the lysine residue (underlined in the sequences above) in the peptide. Labeled peptides were purified by reverse-phase chromatography and labeling was confirmed by MALDI mass spectrometry. sDR1-peptide complexes were formed by incubating sDR1 (5–10 μM) with 50 or 250 μM peptide in 10 mM citrate-phosphate buffer (pH 5.0) and 150 mM NaCl. After an overnight incubation at 37°C, complexes were purified on a TSK-GEL G3000SW analytical gel filtration column and concentrated with a micron centrifugal device.

Fluorescence polarization peptide dissociation assays

To measure peptide dissociation rates, 50 nM sDR1 complexes containing Alexa Fluor 488-labeled peptide was incubated with 20 μM unlabeled peptide in the absence or presence of sDM (0.25–2 μM). Assays were performed at 37°C in 10 mM citrate-phosphate buffer (pH 5.0), 150 mM NaCl, and 0.05% Tween 20 in a final volume of 75 μl. All reactions were conducted in Corning 384-well, low-flange, black, flat-bottom, polystyrene NBS microplates. To prevent evaporation during measurements, 10 μl of mineral oil was layered over the reactions and the plate was covered with a VIEWseal plate seal (Greiner Bio-One). Fluorescence polarization measurements were made using a Tecan Infinite F200 plate reader equipped with polarizers and two 485-nm (±20 nm) bandwidth filters for excitation and two 535-nm (±25 nm) bandwidth filters for emission. A total of 25 flashes were used for each reading and the integration time was set to 20 μs. The program i-Control was used to collect data and to calculate the fluorescence polarization, p, which is defined as $p = (I_V - GI_H)/(I_V + GI_H)$, where I_V and I_H are the intensity of the emission at polarizations both parallel and perpendicular to the excitation source, and G is a factor to correct for instrumental differences in detecting emission components. The experiments containing complexes of WT, W61A, or H81A DR1 were monitored for 1000 cycles of 432 s each. For assays containing the ^{488}HA-N82A complex, the reactions were monitored for 1000 cycles of 8 s each in the absence of sDM and for 600 cycles of 3 s each in the presence of sDM. Rate constants were obtained by fitting the data points to the single exponential decay equation $p = Ae^{-kt} + C$ using Prism 4.0 (GraphPad Software).

Results and Discussion

Susceptibility of mutant HLA-DR molecules to DM-catalyzed CLIP dissociation in cells

We mutated the amino acids in MHC class II molecules that participate in conserved hydrogen bonds to evaluate the effect on DM catalytic activity. As an initial approach, mutant HLA-DR1 molecules were expressed in T2 lymphoid cells in the presence or absence of DM. T2 cells express endogenous Ii but not DR or DM. Retroviral expression constructs were generated encoding DR1 molecules with alanine substitutions for each of the three conserved MHC class II α-chain amino acids that form hydrogen bonds, αN62A, αN69A, and αR76A (Fig. 1), thus preventing the

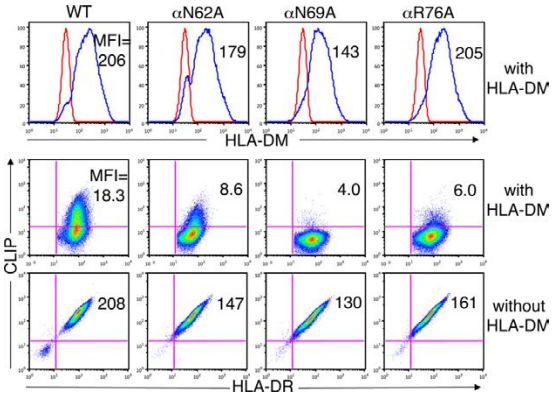


FIGURE 2. Effect of mutations in conserved positions in the DR1 α-chain on susceptibility to DM-mediated CLIP release. Expression of DR and CLIP on the surface of T2 cells expressing WT or mutant DR1 molecules in the absence (*bottom panels*) or presence (*middle panels*) of DM was measured by flow cytometry with mAbs L243 (DR) and CerCLIP (CLIP). Total cellular expression of DM was measured in permeabilized cells by flow cytometry using the mAb MaP.DM1 (*top panels*).

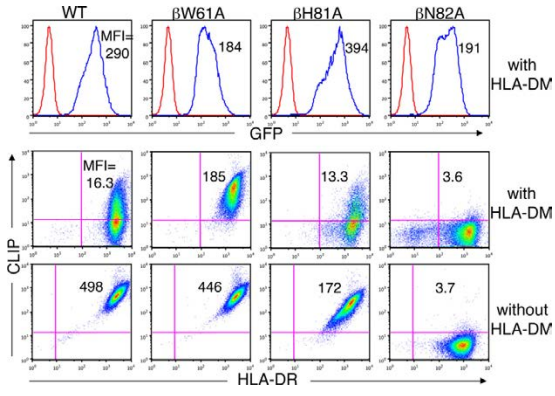


FIGURE 3. Effect of mutations in conserved positions in the DR1 β-chain on susceptibility to DM-mediated CLIP release. Expression of DR and CLIP on the surface of T2 cells expressing WT or mutant DR1 molecules in the absence (*bottom panels*) or presence (*middle panels*) of DM was measured by flow cytometry. GFP fluorescence was determined by flow cytometry as a surrogate measure of DM expression (*top panels*).

formation of specific hydrogen bonds. Cell lines were generated expressing comparable levels of total and cell surface DR1. High levels of CLIP were present on the surface of cells expressing WT DR1 in the absence of DM (Fig. 2). Spontaneous dissociation of CLIP from DR1 is inefficient, and DM is required to catalyze CLIP dissociation and replacement with other peptides. CLIP expression was markedly reduced on cells coexpressing DM with WT DR1, reflecting normal DM catalytic activity. High CLIP levels were also present on cells expressing each of the α-chain mutant DR1 molecules, αN62A, αN69A, or αR76A, and this phenotype was reversed in cells coexpressing DM (Fig. 2). Thus, none of the five conserved hydrogen bonds formed by the amino acids αN62, αN69, and αR76 is critical for the stable association of CLIP with DR1, and each mutant is fully susceptible to DM-catalyzed CLIP dissociation.

To evaluate the conserved hydrogen bonds formed by the MHC class II β-chain (Fig. 1), alanine substitutions were engineered at each of the three conserved positions to generate βW61A, βH81A, and βN82A DR1 molecules. The mutants were observed to assemble efficiently with Ii (data not shown). In the absence of DM, high levels of CLIP were present on cells expressing βW61A or βH81A DR1 (Fig. 3). By contrast, CLIP was completely absent from the surface of cells expressing βN82A DR1, suggesting that the bidentate hydrogen bonds formed by βN82 play a disproportionate role in stabilizing CLIP-DR1 binding.

CLIP levels were markedly reduced on cells coexpressing DM and βH81A DR1 (Fig. 3), demonstrating that DM can efficiently catalyze CLIP dissociation from DR1 complexes lacking the βH81 hydrogen bond, a result that was further confirmed with the asparagine substitution mutant βH81N (supplemental Fig. S1),[5] directly contradicting the conclusions of Narayan et al. (21). βH81A DR1 molecules in DM-expressing cells were stable in SDS detergent, evidence that CLIP is exchanged for high affinity peptides in these cells (data not shown). We were also interested in determining whether the histidine at β81 influences the pH-dependence of DM-catalyzed peptide binding, which is optimal at acidic endosomal pH (2–4). The βH81A and βH81N mutations had little or no effect on the pH-dependence of DM-catalyzed peptide binding (supplemental Fig. S2).

By contrast to the βH81 mutations, βW61A DR1 molecules appeared to be partially resistant to DM-mediated CLIP dissociation. This was true even when subpopulations that gated for identical expression of DR and DM were analyzed. Thus, the hydrogen bond formed by βW61 was a candidate target in the DM catalytic mechanism. The capacity of DM to catalyze CLIP dissociation from βN82A DR1 could not be evaluated with this approach because of the high rate of spontaneous dissociation, leaving βN82 as an additional candidate.

Effect of MHC class II β-chain mutations on DM catalytic potency

To further evaluate the role of conserved hydrogen bonds involving the MHC class II β-chain in the DM catalytic mechanism, we generated soluble recombinant DR1 and βW61A,

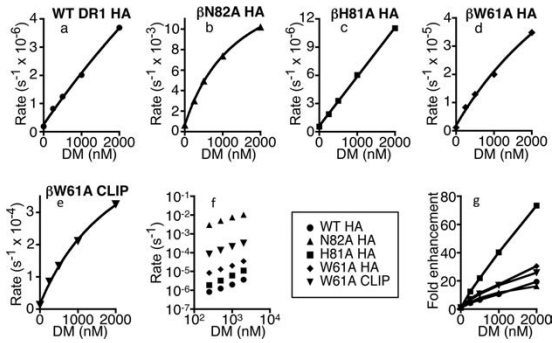


FIGURE 4. Effect of mutational disruption of DR1 β-chain hydrogen bonds on DM catalytic potency. *a–e*, Preformed complexes (50 nM) of Alexa Fluor 488-labeled HA or CLIP peptides bound to mutant sDR1 were incubated with excess unlabeled peptide (20 μM) and various concentrations of soluble DM (pH 5.0) at 37°C. Peptide dissociation rates were measured using a fluorescence polarization assay as described in *Materials and Methods*. *f*, Data for mutant sDR1 molecules are compared with WT sDR1 in a log-log plot of peptide dissociation rates vs DM concentrations. *g*, DM-catalyzed rate enhancements normalized for differences in intrinsic peptide dissociation rates.

[5] The online version of this article contains supplemental material.

Table I. *Peptide dissociation rates for mutant DR1 molecules*[a]

	DM (nM)					
	0	250	500	1000	2000	*n*
DR1 (HA)	1.9×10^{-7}	8.2×10^{-7}	1.2×10^{-6}	2.0×10^{-6}	3.7×10^{-6}	3
N82A (HA)	6.3×10^{-4}	3.0×10^{-3}	4.9×10^{-3}	7.4×10^{-3}	1.0×10^{-2}	3
H81A (HA)	1.5×10^{-7}	1.9×10^{-6}	3.3×10^{-6}	6.0×10^{-6}	1.1×10^{-5}	2
W61A (HA)	1.1×10^{-6}	8.3×10^{-6}	1.3×10^{-5}	2.0×10^{-5}	3.5×10^{-5}	2
W61A (CLIP)	1.3×10^{-5}	8.5×10^{-5}	1.3×10^{-4}	2.1×10^{-4}	3.3×10^{-4}	1

[a] Rates are expressed in units of s^{-1}.

βH81A, and βN82A mutant proteins. A fluorescence polarization assay was used to quantify the impact of DM on the kinetics of peptide dissociation. A DM concentration-dependent acceleration of the rate of peptide dissociation was observed with WT DR1 complexes containing the high affinity peptide HA (Fig. 4*a*). The βH81A mutation had essentially no effect on the intrinsic stability of the DR1-HA complex (Table I), and the mutation did not reduce the potency of DM in catalyzing peptide dissociation (Fig. 4, *c*, *f*, and *g*). Indeed, catalytic potency was somewhat greater for βH81A compared with WT DR1. Thus, the hydrogen bond formed by βH81 does not play a major role in stabilizing DR1-peptide complexes and is not an essential target in the DM catalytic mechanism.

The βW61A mutation reduced peptide complex stability with an ~5-fold increase in the intrinsic HA dissociation rate (Table I). However, DM catalytic potency was not reduced, even after normalization for the effect on the intrinsic peptide dissociation rate (Fig. 4, *d*, *f*, and *g*). These results contrasted with the finding that CLIP release from βW61A DR1 was relatively resistant to DM in cells (Fig. 3). We considered the possibility that DM might selectively catalyze the dissociation of HA but not CLIP from βW61A DR1. However, DM was observed to catalyze the dissociation of a variant CLIP peptide from βW61A DR1 (Fig. 4*e*) with potency similar to that observed for HA (Fig. 4*f*). Full-length βW61A DR1-CLIP complexes were also highly susceptible to DM (data not shown). Although we did not observe any gross alteration in endosomal colocalization of βW61A DR1 with DM (data not shown), it seems likely that this mutation affects the capacity of DR1-CLIP complexes to interact optimally with DM in T2 cells, either by impacting colocalization in membrane subdomains or through an effect on trafficking kinetics (26).

Strikingly, the βN82A mutation was observed to increased the intrinsic HA dissociation rate by >3000-fold (Table I). This is consistent with the spontaneous release of CLIP observed with this mutant expressed in cells (Fig. 3). Previous studies with mouse class II molecules support the idea that βN82 may in general have a dominant role in stabilizing MHC class II-peptide complexes (23, 27). Remarkably, despite the very rapid spontaneous dissociation of HA from βN82A DR1, DM further accelerated peptide dissociation (Fig. 4, *b* and *f*). The catalytic potency was similar to that observed with WT DR1, even with data normalized for intrinsic rates (Fig. 4*g*). Thus, the two hydrogen bonds formed by βN82 appear to be critical for stabilizing DR1-peptide complexes, yet they are not essential targets in the DM catalytic mechanism.

Concluding Remarks

In addition to the nine hydrogen bonds that we analyzed by mutational analysis in the current study, three conserved hydrogen bonds that interact with main chain atoms in the amino terminus of bound peptide are contributed by the main chain atoms of amino acids at positions α51 and α53 in DR1 (Fig. 1) (19). The potential role of these hydrogen bonds in the DM catalytic mechanism cannot be analyzed by mutation. However, Stratikos et al., used peptide truncation and amide *N*-methylation to prevent the formation of these hydrogen bonds in DR1-HA peptide complexes (20), demonstrating that DM catalytic potency was enhanced ~6- to 9-fold with peptide complexes lacking one or more of these hydrogen bonds. They observed only minor effects on catalytic potency with *N*-methylated peptide analogues designed to disrupt each of four additional conserved hydrogen bonds (20). It is possible that the disruption of hydrogen bonds mediated by α51, α53, or β81 increases DM binding affinity by increasing DR structural flexibility, resulting in small increases in DM catalytic potency.

The central conclusion of the current study is that no individual component of the conserved hydrogen bond network plays an essential role in the DM catalytic mechanism.

DM catalytic activity is preserved or even enhanced with the artificial disruption of any component of the conserved network. Given the universal capacity of DM to catalyze peptide dissociation, the conserved network has been an attractive potential target in the catalytic mechanism. If this is the case, however, individual components of the network must play a redundant role. It has become increasingly clear that MHC class II-peptide binding involves cooperative interactions throughout the peptide-binding groove, and the hydrogen bond network appears to be relatively isolated energetically from the anchor-pocket interactions (27–29). It is possible, for example, that DM might initially promote the destabilization of *N*-terminal hydrogen bonds, leading to a global change in conformation that destabilizes multiple elements of the hydrogen bond network. Removing any subset or single H-bond does not prevent DM from having its catalytic effect, but instead, it might even enhance the catalytic impact. Alternatively, DM might reduce the stability of peptide complexes by impacting other global features such as the dynamics or general structural flexibility of the peptide-binding groove, possibly by altering the interaction of the groove domain with the supporting Ig domains.

Acknowledgments

We are grateful to Dr. Elizabeth Mellins for providing critical reagents.

Disclosures

The authors have no financial conflict of interest.

References

1. Jensen, P. E. 2007. Recent advances in antigen processing and presentation. *Nat. Immunol.* 8: 1041–1048.

2. Sherman, M. A., D. A. Weber, and P. E. Jensen. 1995. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* 3: 197–205.
3. Denzin, L. K., and P. Cresswell. 1995. HLA-DM induces CLIP dissociation from MHC class II $\alpha\beta$ dimers and facilitates peptide loading. *Cell* 82: 155–165.
4. Sloan, V. S., P. Cameron, G. Porter, M. Gammon, M. Amaya, E. Mellins, and D. M. Zaller. 1995. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375: 802–806.
5. Mosyak, L., D. M. Zaller, and D. C. Wiley. 1998. The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity* 9: 377–383.
6. Denzin, L. K., C. Hammond, and P. Cresswell. 1996. HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J. Exp. Med.* 184: 2153–2165.
7. Weber, D. A., B. D. Evavold, and P. E. Jensen. 1996. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 274: 618–620.
8. Kropshofer, H., A. B. Vogt, G. Moldenhauer, J. Hammer, J. S. Blum, and G. J. Hammerling. 1996. Editing of the HLA-DR-peptide repertoire by HLA-DM. *EMBO J.* 15: 6144–6154.
9. van Ham, S. M., U. Gruneberg, G. Malcherek, I. Broker, A. Melms, and J. Trowsdale. 1996. Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J. Exp. Med.* 184: 2019–2024.
10. Lazarski, C. A., F. A. Chaves, and A. J. Sant. 2006. The impact of DM on MHC class II-restricted antigen presentation can be altered by manipulation of MHC-peptide kinetic stability. *J. Exp. Med.* 203: 1319–1328.
11. Fallang, L. E., S. Roh, A. Holm, E. Bergseng, T. Yoon, B. Fleckenstein, A. Bandyopadhyay, E. D. Mellins, and L. M. Sollid. 2008. Complexes of two cohorts of CLIP peptides and HLA-DQ2 of the autoimmune DR3-DQ2 haplotype are poor substrates for HLA-DM. *J. Immunol.* 181: 5451–5461.
12. Raddrizzani, L., E. Bono, A. B. Vogt, H. Kropshofer, F. Gallazzi, T. Sturniolo, G. J. Hammerling, F. Sinigaglia, and J. Hammer. 1999. Identification of destabilizing residues in HLA class II-selected bacteriophage display libraries edited by HLA-DM. *Eur. J. Immunol.* 29: 660–668.
13. Pu, Z., S. B. Lovitch, E. K. Bikoff, and E. R. Unanue. 2004. T cells distinguish MHC-peptide complexes formed in separate vesicles and edited by H2-DM. *Immunity* 20: 467–476.
14. Doebele, R. C., R. Busch, H. M. Scott, A. Pashine, and E. D. Mellins. 2000. Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity* 13: 517–527.
15. Pashine, A., R. Busch, M. P. Belmares, J. N. Munning, R. C. Doebele, M. Buckingham, G. P. Nolan, and E. D. Mellins. 2003. Interaction of HLA-DR with an acidic face of HLA-DM disrupts sequence-dependent interactions with peptides. *Immunity* 19: 183–192.
16. Stratikos, E., L. Mosyak, D. M. Zaller, and D. C. Wiley. 2002. Identification of the lateral interaction surfaces of human histocompatibility leukocyte antigen (HLA)-DM with HLA-DR1 by formation of tethered complexes that present enhanced HLA-DM catalysis. *J. Exp. Med.* 196: 173–183.
17. Chou, C. L., and S. Sadegh-Nasseri. 2000. HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J. Exp. Med.* 192: 1697–1706.
18. Zarutskie, J. A., R. Busch, Z. Zavala-Ruiz, M. Rushe, E. D. Mellins, and L. J. Stern. 2001. The kinetic basis of peptide exchange catalysis by HLA-DM. *Proc. Natl. Acad. Sci. USA* 98: 12450–12455.
19. Stern, L. J., J. H. Brown, T. S. Jardetzky, J. C. Gorga, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 368: 215–221.
20. Stratikos, E., D. C. Wiley, and L. J. Stern. 2004. Enhanced catalytic action of HLA-DM on the exchange of peptides lacking backbone hydrogen bonds between their N-terminal region and the MHC class II α-chain. *J. Immunol.* 172: 1109–1117.
21. Narayan, K., C. L. Chou, A. Kim, I. Z. Hartman, S. Dalai, S. Khoruzhenko, and S. Sadegh-Nasseri. 2007. HLA-DM targets the hydrogen bond between the histidine at position β81 and peptide to dissociate HLA-DR-peptide complexes. *Nat. Immunol.* 8: 92–100.
22. McFarland, B. J., C. Beeson, and A. J. Sant. 1999. Cutting edge: a single, essential hydrogen bond controls the stability of peptide-MHC class II complexes. *J. Immunol.* 163: 3567–3571.
23. McFarland, B. J., J. F. Katz, C. Beeson, and A. J. Sant. 2001. Energetic asymmetry among hydrogen bonds in MHC class II• peptide complexes. *Proc. Natl. Acad. Sci. USA* 98: 9231–9236.
24. Donnelly, M. L., G. Luke, A. Mehrotra, X. Li, L. E. Hughes, D. Gani, and M. D. Ryan. 2001. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J. Gen. Virol.* 82: 1013–1025.
25. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 90: 8392–8396.
26. Zwart, W., A. Griekspoor, C. Kuijl, M. Marsman, J. van Rheenen, H. Janssen, J. Calafat, M. van Ham, L. Janssen, M. van Lith, et al. 2005. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity* 22: 221–233.
27. McFarland, B. J., J. F. Katz, A. J. Sant, and C. Beeson. 2005. Energetics and cooperativity of the hydrogen bonding and anchor interactions that bind peptides to MHC class II protein. *J. Mol. Biol.* 350: 170–183.
28. Anderson, M. W., and J. Gorski. 2005. Cooperativity during the formation of peptide/MHC class II complexes. *Biochemistry* 44: 5617–5624.
29. Ferrante, A., and J. Gorski. 2007. Cooperativity of hydrophobic anchor interactions: evidence for epitope selection by MHC class II as a folding process. *J. Immunol.* 178: 7181–7189.

CHAPTER 5

DISCUSSION

Overview

T1D is an autoimmune disease characterized by the self-destruction of pancreatic β cells. Based on decades of studies on the etiology of T1D, it is widely accepted that autoreactive CD4+ T cells initiate this self-destruction [1, 2]. However, the underlying triggers and mechanisms that include both environmental and genetic factors are extremely complicated. Genome wide association analysis and genotyping studies indicated that individuals with MHCII of DQ2 or DQ8 haplotypes, especially DQ2/DQ8 heterozygotes, with the expression of both DQ2 and DQ8, and the assembling of trans-dimers DQ2-8 and DQ8-2 molecules, are associated with significantly higher risk of T1D [3-9]. To illustrate the molecular mechanism that is involved in the development of autoreactive CD4+ T cells, we investigated the unique properties of these T1D-associated DQ molecules, compared with the T1D nonassociated DQ6 and DQ1 molecules, and the molecular mechanism of DM editing and its potential role in T1D.

In Chapter 1 of this thesis, we summarized the role of MHCII, especially the unique structural characteristics of T1D-associated DQ molecules, and the role of DM mediated peptide editing in antigen presentation and in the selection and/or activation of autoreactive CD4+ T cells in T1D. Chapter 2 was focused on the characteristics of T1D-associated DQ2 and DQ8 molecules with demonstration that the presentation of invariant chain (Ii) derived CLIP peptides is relatively resistant to DM editing. Chapter 3 of this thesis further addresses the characteristics of these T1D-associated DQ molecules by peptidomic analysis, illustrating the critical role of DM editing in MHCII presented peptide repertoires, while the DM editing function is less efficient in T1D-associated DQ molecules. In Chapter 4, we describe experiments that disproved the prevailing

hypothesis that hydrogen bonds in the conserved hydrogen network in MHC-peptide complexes are specifically disrupted in the DM catalytic mechanism.

Role of Invariant Chain in MHC Class II Antigen Presentation Pathway

Ii is associated with MHCII molecules to help transport MHCII molecules into the late endosomal compartment. The binding of Ii with the peptide-binding region of MHCII molecules also protects the peptide-binding groove from early loading of peptide from the endoplasmic reticulum (ER). After proteolytic cleavage, the Ii-derived CLIP peptide remains in the peptide-binding groove and needs to be released for the loading of other endogenous or exogenous peptides [10, 11]. Due to the highly polymorphic nature of MHCII, especially in the peptide-binding region, the interaction of Ii chain and MHCII can be distinct and lead to variable outputs. First, due to the accessibility of proteases affected by conformational variation in the Ii-MHCII complex, the events of proteolytic cleavage of Ii might be different. As supported in Chapter 2, most of the Ii-derived CLIP1 peptides were processed more efficiently in DQ1, with short or no N-terminus extensions in eluted CLIP1 peptides; however, most of the CLIP1 peptides eluted from the other DQ molecules contained long N-terminus extensions. This finding is consistent with the structural differences in the conformation of DQ1 near to the N-terminus of CLIP1 peptide, a region that may be more flexible than the other DQ molecules, as we discussed in Chapter 1. Second, the binding affinity of Ii-derived CLIP peptides with different MHCII molecules might be distinct. In the presence of DM editing, CLIP peptides were efficiently released from DQ1 or DQ6 molecules; however, in T1D-

associated DQ molecules, large quantities of retained CLIP peptides were found on the cell surface or associated with DQ molecules in peptide elution assay in the presence of DM editing. As a result, the CLIP peptide being presented on the APC surface either directly interacts with TCR or indirectly affects the efficient presentation of other peptides. Third, the alternative binding or rebinding of Ii-derived CLIP peptides might be different, including the well-defined CLIP1 and CLIP2 registers, which might also affect the peptide repertoire of MHCII. A large quantity of CLIP2 peptides were identified bound with DQ2 molecules but not with the other DQ molecules in the presence of DM editing, although the direct biological relevance of CLIP2 peptides in T1D etiology is still unknown, as discussed in Chapter 2 and previously reported [12, 13]. Moreover, the alternative binding of Ii-derived peptides, including register shifting [14-16] and the bidirectional binding of CLIP peptides [17, 18] might also affect the peptide repertoire due to the polymorphism of peptide binding groove region.

The relevance of Ii in the development of autoreactive CD4+ T cells still need to be determined. In previous studies, high levels of Ii-derived CLIP1 and CLIP2 peptides have been detected in T1D-like NOD mice [19]. Patients with T1D also show relatively high levels of CLIP on the surface of lymphocytes [20]. Celiac disease patients with DQ2 expression are also associated with high CLIP2 levels [12, 13, 21]. It is notable that the Ii deficiency in Ii-/- NOD mice completely protects NOD mice against T1D [22]. In Chapter 2 of this thesis, we also found high levels of Ii-derived CLIP1 peptides retained on T1D-associated DQ molecules, and a large amount of CLIP2 peptides associated with DQ2 molecules. The coincidence of high level of CLIP with T1D-associated DQ molecules, as well as with T1D individuals, might be a general indicator of inefficient

DM editing with T1D-associated DQ molecules, or the CLIP peptides are high affinity binders to these T1D-associated DQ molecules. Both of these mechanisms have the potential to change the peptide repertoire of T1D-associated DQ-peptide complexes and might mediate alternative selection or activation of autoreactive CD4+ T cells involved in T1D, as we proposed in Chapter 1. Alternatively, it is also plausible that Ii does not directly contribute to the etiology of T1D, which is supported by the observation that DM knockout NOD mice are completely protected against T1D, despite high expression levels of MHCII-CLIP complexes [23]. To directly address the potential role of Ii or the Ii-derived CLIP peptides in T1D etiology, it would be valuable but challenging to directly estimate the role of CLIP peptides in thymic selection of autoreactive CD4+ T cell or the identification of CLIP cross-reactive autoreactive CD4+ T cells in T1D individuals.

Role of DQ Molecules in Autoreactive CD4+ T Cell Development and Activation

The fate of thymocytes is determined by the affinity of expressed T cell receptor (TCR) that interacts with the antigen-presenting cell (APC) surface self-peptide-MHC complexes. Those thymocytes with high affinity TCR will be deleted by negative selection; the remaining T cells are theoretically self-tolerant [24, 25]. However, it is known that a fraction of autoreactive T cells escaped negative selection through several potential mechanisms, as we summarized in Chapter 1.

In our research, we found that the DM-mediated peptide editing of T1D-associated DQ molecules is inefficient, which is affected by the unique properties of T1D-associated DQ molecules. First, the polymorphisms of DQ alleles, especially in the

peptide binding groove region of T1D-associated DQ molecules, that may determine that T1D-associated DQ molecules favor selective binding of specific autoantigen-derived peptides. As shown by the peptide binding motifs that we predicted in Chapter 3, the major anchor residues at P1, P4, P6, P7, and P9 of the bound peptides are charged in T1D-associated DQ molecules; however, those anchor residues are nonpolar amino acids in the bound peptides of T1D protective DQ6 molecules. Second, the distinct structural characteristics in T1D-associated DQ molecules may make these molecules poor substrates for DM editing. As previously reported, a natural deletion of α53 in DQ2 diminishes the sensitivity to DM editing [26]. Similarly, as we identified in Chapter 2 and Appendix B, the extra hydrogen bonds in the β chain of DQ8 contribute to the inefficiency of DM editing, and disruption of two hydrogen bonds in a DQ8-βE86A mutant protein significantly increased both the DQ8/peptide complex stability and sensitivity to DM editing.

Based on these findings, we can conclude that the unique properties of T1D-associated DQ molecules affect the DM editing efficiency and might change the peptide repertoires presented on the APC surface, which could deliver distinct strength of MHC/peptide signals to the TCR that may potentially be beneficial for the survival of the autoreactive CD4+ T cells in the thymus. Similarly, distinct signals could be delivered for the activation of autoreactive CD4+ T cells in the peripheral as well. In future studies, direct identification of autoreactive CD4+ T cells that are selected in the context of inefficient DM editing is required to further test this hypothesis. A humanized mice model with DQ2-α53R insertion transgene, or DQ8-βE86A mutation transgene to increase the DM editing sensitivity might be valuable to estimate the importance of DM

editing efficiency in the negative selection of autoreactive CD4+ T cells, especially its role in the pathogenesis of T1D or other autoimmune diseases, such as Rheumatoid Arthritis [27-30] and celiac disease [31-35].

Molecular Mechanism of DM Editing and Its Effect

Two general sets of interactions contribute in the binding of peptide with MHCII, including peptide sequence-dependent interactions between peptide side chains (anchors) and subsites (pockets) in the peptide-binding groove, and a conserved hydrogen bond network formed by nonpolymorphic amino acids in the MHCII and main chain atoms in bound peptide [36]. It has been hypothesized that the destabilization of one or more of these conserved hydrogen bonds to be the primary event in the DM catalytic mechanism [37-42]. The conserved hydrogen bond formed between βH81 of the MHCII and the peptide backbone has been reported to dominate DM susceptibility [42]. To systematically analyze the effect of the conserved hydrogen bond network and further test this hypothesis, in Chapter 4, we mutated each of the residues in DR1 protein involved in the formation of hydrogen bonds with the backbone of bound peptides, postulating that mutational disruption of specific hydrogen bonds targeted by DM would result in reduced catalytic potency as previously reported [42]. Instead, our results confirmed that disruption of the conserved hydrogen bond formed by βH81 affected neither the intrinsic stability of the DR1-HA complex nor the potency of DM in catalyzing peptide dissociation, which is further confirmed by other groups [43, 44]. The mutation of other α or β chain residues involved in the formation of conserved hydrogen bonds with bound peptides further confirmed our conclusion that none of the conserved hydrogen bonds is a

critical target necessary for DM-catalyzed peptide dissociation.

Interestingly, based on the increased intrinsic peptide dissociation rate and DM catalytic potency measured in βN82A mutation as reported in Chapter 4, even though the two hydrogen bonds formed by βN82 with the backbone of bound peptide are not the direct target in the DM catalytic mechanism, they are critical for stabilizing DR1-peptide complexes, which is consistent with previous findings [45, 46]. Molecular dynamics simulation and mutagenesis analysis also suggested that βN82 is involved in stabilizing the nonreceptive structure of empty DR1 by narrowing of the peptide-binding groove close to the P1 pocket region with the formation of a critical hydrogen bond between αQ9 and βN82 [44]. Moreover, structural analysis of DM-DR1 interaction further illustrated that the interaction of DM and DR1 mediates DR1 αE55 moving into the peptide-binding groove and forming a water-mediated hydrogen bond with βN82 [47]. Therefore, it is plausible that the two hydrogen bonds formed by βN82 are indirectly involved in hydrogen bonds exchange during the DM-MHCII interaction.

Mutagenesis and recent structural studies confirmed that the acidic concave surface of DM directly interacts with α chain of MHCII close to the P1 pocket, and barely with β chain of MHCII [47-51]. The DM-MHCII interaction involves a significant conformational rearrangement of peptide-binding groove, especially in the α53-65 region around the P1 pocket of MHCII [47-49], which is consistent with the predicted conformation of the peptide-binding groove in the bound, partially filled, or empty states in previous molecular dynamics simulation and recent thermodynamics studies [44, 52-54]. The mechanism of DM-mediated MHCII peptide exchange includes the process of dissociation of prebound peptide, interaction with the empty MHCII, and the association

of new peptide. The mechanism of DM-catalyzed peptide dissociation has been investigated in the past two decades [40-43, 47-49, 54-59]. Different models have been proposed to understand the mechanism of DM-catalyzed peptide association or the role of DM to facilitate peptide loading, based on the potentially different roles of DM in interacting and/or stabilizing empty MHCII, converting nonreceptive to receptive form MHCII, or formation of a peptide-loading DM-MHCII complex [60-64]. Recently, Stern's group reevaluated these potential roles of DM in the peptide association process and concluded that DM neither mediates the conversion from nonreceptive to receptive forms of MHCII, nor the stabilization of empty MHCII, and this study supported a previously reported model in which DM contributes to peptide association by formation of a peptide-loading DM-MHCII complex between DM and empty MHCII [64, 65].

According to the current evidence relating to DM in MHCII peptide dissociation and association processes, DM might play a role as a sensor to check the conformational flexibility of MHCII complexes. In this model, DM preferentially binds to MHCII with a flexible conformation, including conformational states of MHCII with an empty peptide-binding groove, partially occupied or bound with weak ligand, but DM is disfavored in binding MHCII with inflexible conformations. The association of DM with unstable MHCII complexes may induce secondary conformational rearrangements, especially the rearrangement of several key residues around the P1 pocket and the exchange of hydrogen bonds in the conserved hydrogen network. This rearrangement may result in a thermodynamic "open" state with a broad conformation change in the whole peptide-binding groove, favoring the release of if any prebound weak peptide and the acceptance of new peptide. The association of new peptide will further shape the conformation of

MHCII to increase the stability of the MHCII-peptide complex. The release of peptide and reloading of another peptide could reoccur if the peptide binding mediated conformational change is not enough to induce the dissociation of DM-MHCII interaction. If the peptide mediated conformational change is sufficient to stabilize the MHCII-peptide complex and that stable conformation is unfavorable for DM interaction, the dissociation of DM from MHCII-peptide complex will occur.

For T1D-associated DQ8 molecules, as studied in Chapter 2 and 3, although DM can still catalyze the peptide exchange, the catalytic activity is inefficient. The poor catalytic efficiency may relate to unique properties of DQ8 molecules, including the extra hydrogen bonds between α and β chain present in DQ8. However, a structural explanation for this inefficient DM catalysis needs to be further investigated. It is plausible that the extra hydrogen bonds might increase the stability of DQ8-peptide complex, which might be already over the range of DM-mediated conformational change, therefore the conformation of DQ8 molecules might be "frozen" in that intermediate state. On the other side, the binding of high affinity peptide might be insufficient to overcome the energy barrier of the extra hydrogen bonds to mediate further conformational rearrangement that would promote the dissociation of DM from DQ8. Therefore, the DQ8-peptide complex stability would be less dependent on the contribution of the bound peptide affinity, even though the high affinity peptide might have the advantage to compete with the low affinity peptide. Moreover, the association of DM with DQ8 could be further affected by extra factor(s), such as the extra N-terminus sequence of bound peptide that might contribute to the binding, and the environment of peptide-binding groove in DQ8 that might contribute as well. Further structural analysis

of DQ8, especially the DQ8-E86A mutation with CLIP or T1D-associated auto-antigenic peptides, might be able to distinguish these possibilities in the future.

Although DM interacts with MHCII near the P1 pocket that interacts with the N-terminus of peptide, the contribution of the interaction between the peptide-binding groove and the C-terminus of the peptide sequence in MHCII-peptide complex stability and DM editing sensitivity needs to be emphasized as well. As supported by previous reports, the salt bridge formed between the polymorphic residue aspartic acid β57 in DR1 and DQ isotypes with the C-terminus of the bound peptide have been found linked to be protective in T1D [66-71]; moreover, extra interactions between peptides and residues outside of peptide-binding groove also contribute, as has been confirmed with two peptides differing only at P10 and showing distinct binding affinities in DR1 [72, 73]. These factors might cooperatively determine the DM interaction with MHCII and the DM editing sensitivity. As we found in Chapter 3, the peptides CKA362 and LEP147 both have relatively high binding affinities for DQ6 yet, in the presence of DM, they have a high peptide dissociation rate and they are sensitive to DM editing, results that are consistent with previous reports [74-77]. It is plausible that these peptides might have undefined properties that can efficiently compete with the indicator peptides to bind to DQ6, while not efficiently mediating the conformational change that is resistant to DM editing. Further structural, molecular dynamic simulation, and biochemical measurements of these peptide complexes with anchor residue replacement might provide more information to address this possibility.

The link of inefficient DM editing with T1D-associated DQ molecules indicates a critical role for DM in editing the MHCII presented peptide repertoire that determines the

selection and/or activation of autoreactive CD4+ T cells involved in T1D etiology [2, 78, 79]. It is interesting to note that genetic studies of the limited polymorphisms of DMα and DMβ in different populations has implied that specific DM alleles are associated with T1D [80-82]. These various findings highlight the possibility that clinical targeting in the early stage treatment of T1D to enhance DM function, with short peptides [83] or small molecules [84, 85] that mimic DM function, may be worthy of investigation.

In summary, we distinguished some unique properties of T1D-associated DQ2 and DQ8 molecules, including extra hydrogen bonds in DQ8 that contribute to inefficient DM editing and might affect the peptide repertoire presented by T1D-associated DQ molecules. We also ruled out the possibility of DM editing by disruption of specific hydrogen bond of the conserved hydrogen network formed between bound peptide and MHCII, the previously proposed prevailing hypothesis for the DM catalytic mechanism. Our findings provided molecular insights into the potential mechanisms through which DQ2 and DQ8 confer high genetic risk for T1D, further supported the reliability of genetic screening as a powerful tool in the prediction of T1D and highlighting the regulation of DM editing as a potential target for the early stage control of T1D.

References

1 **Gorodezky, C., Alaez, C., Murguia, A., Rodriguez, A., Balladares, S., Vazquez, M., Flores, H. et al.,** HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example. *Autoimmun. Rev.* 2006. **5**: 187-194.

2 **Waldron-Lynch, F. and Herold, K. C.,** Immunomodulatory therapy to preserve pancreatic beta-cell function in type 1 diabetes. *Nat. Rev. Drug. Discov.* 2011. **10**: 439-452.

3 **Cifuentes, R. A., Rojas-Villarraga, A. and Anaya, J. M.,** Human leukocyte antigen class II and type 1 diabetes in Latin America: a combined meta-analysis of association and family-based studies. *Hum. Immunol.* 2011. **72**: 581-586.

4 **Rojas-Villarraga, A., Botello-Corzo, D. and Anaya, J. M.,** HLA-Class II in Latin American patients with type 1 diabetes. *Autoimmun. Rev.* 2010. **9**: 666-673.

5 **Ge, X., Piganelli, J. D., Tse, H. M., Bertera, S., Mathews, C. E., Trucco, M., Wen, L. et al.,** Modulatory role of DR4- to DQ8-restricted CD4 T-cell responses and type 1 diabetes susceptibility. *Diabetes* 2006. **55**: 3455-3462.

6 **Erlich, H., Valdes, A. M., Noble, J., Carlson, J. A., Varney, M., Concannon, P., Mychaleckyj, J. C. et al.,** HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* 2008. **57**: 1084-1092.

7 **Aly, T. A., Ide, A., Jahromi, M. M., Barker, J. M., Fernando, M. S., Babu, S. R., Yu, L. et al.,** Extreme genetic risk for type 1A diabetes. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 14074-14079.

8 **Noble, J. A., Johnson, J., Lane, J. A. and Valdes, A. M.,** HLA class II genotyping of African American type 1 diabetic patients reveals associations unique to African haplotypes. *Diabetes* 2013. **62**: 3292-3299.

9 **van Autreve, J. E., Weets, I., Gulbis, B., Vertongen, F., Gorus, F. K., van der Auwera, B. J. and Belgian Diabetes, R.,** The rare HLA-DQA1*03-DQB1*02 haplotype confers susceptibility to type 1 diabetes in whites and is preferentially associated with early clinical disease onset in male subjects. *Hum. Immunol.* 2004. **65**: 729-736.

10 **Neefjes, J., Jongsma, M. L., Paul, P. and Bakke, O.,** Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 2011. **11**: 823-836.

11 **Jensen, P. E.,** Recent advances in antigen processing and presentation. *Nat. Immunol.* 2007. **8**: 1041-1048.

12 **Fallang, L. E., Roh, S., Holm, A., Bergseng, E., Yoon, T., Fleckenstein, B., Bandyopadhyay, A. et al.,** Complexes of two cohorts of CLIP peptides and HLA-DQ2 of the autoimmune DR3-DQ2 haplotype are poor substrates for HLA-DM. *J. Immunol.* 2008. **181**: 5451-5461.

13 **Wiesner, M., Stepniak, D., de Ru, A. H., Moustakis, A. K., Drijfhout, J. W., Papadopoulos, G. K., van Veelen, P. A. et al.,** Dominance of an alternative CLIP sequence in the celiac disease associated HLA-DQ2 molecule. *Immunogenetics* 2008. **60**: 551-555.

14 **He, X. L., Radu, C., Sidney, J., Sette, A., Ward, E. S. and Garcia, K. C.,** Structural snapshot of aberrant antigen presentation linked to autoimmunity: the immunodominant epitope of MBP complexed with I-Au. *Immunity* 2002. **17**: 83-94.

15 **Bankovich, A. J., Girvin, A. T., Moesta, A. K. and Garcia, K. C.,** Peptide register

shifting within the MHC groove: theory becomes reality. *Mol. Immunol.* 2004. **40**: 1033-1039.

16 **Mohan, J. F., Petzold, S. J. and Unanue, E. R.,** Register shifting of an insulin peptide-MHC complex allows diabetogenic T cells to escape thymic deletion. *J. Exp. Med.* 2011. **208**: 2375-2383.

17 **Gunther, S., Schlundt, A., Sticht, J., Roske, Y., Heinemann, U., Wiesmuller, K. H., Jung, G. et al.,** Bidirectional binding of invariant chain peptides to an MHC class II molecule. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 22219-22224.

18 **Schlundt, A., Gunther, S., Sticht, J., Wieczorek, M., Roske, Y., Heinemann, U. and Freund, C.,** Peptide linkage to the alpha-subunit of MHCII creates a stably inverted antigen presentation complex. *J. Mol. Biol.* 2012. **423**: 294-302.

19 **Bhatnagar, A., Milburn, P. J., Lobigs, M., Blanden, R. V. and Gautam, A. M.,** Nonobese diabetic mice display elevated levels of class II-associated invariant chain peptide associated with I-Ag7 on the cell surface. *J. Immunol.* 2001. **166**: 4490-4497.

20 **Silva, D. G., Socha, L., Correcha, M. and Petrovsky, N.,** Elevated lymphocyte expression of CLIP is associated with type 1 diabetes and may be a useful marker of autoimmune susceptibility. *Ann. NY Acad. Sci.* 2004. **1037**: 65-68.

21 **Stepniak, D., Wiesner, M., de Ru, A. H., Moustakas, A. K., Drijfhout, J. W., Papadopoulos, G. K., van Veelen, P. A. et al.,** Large-scale characterization of natural ligands explains the unique gluten-binding properties of HLA-DQ2. *J. Immunol.* 2008. **180**: 3268-3278.

22 **Mellanby, R. J., Koonce, C. H., Monti, A., Phillips, J. M., Cooke, A. and Bikoff, E. K.,** Loss of invariant chain protects nonobese diabetic mice against type 1 diabetes. *J. Immunol.* 2006. **177**: 7588-7598.

23 **Morgan, M. A., Muller, P. S., Mould, A., Newland, S. A., Nichols, J., Robertson, E. J., Cooke, A. et al.,** The nonconventional MHC class II molecule DM governs diabetes susceptibility in NOD mice. *PLoS One* 2013. **8**: e56738.

24 **Hogquist, K. A., Baldwin, T. A. and Jameson, S. C.,** Central tolerance: learning self-control in the thymus. *Nat. Rev. Immunol.* 2005. **5**: 772-782.

25 **Klein, L., Kyewski, B., Allen, P. M. and Hogquist, K. A.,** Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 2014. **14**: 377-391.

26 **Hou, T., Macmillan, H., Chen, Z., Keech, C. L., Jin, X., Sidney, J., Strohman, M., Yoon, T. et al.,** An insertion mutant in DQA1*0501 restores susceptibility to HLA-DM: implications for disease associations. *J. Immunol.* 2011. **187**: 2442-2452.

27 **Nabozny, G. H., Baisch, J. M., Cheng, S., Cosgrove, D., Griffiths, M. M., Luthra,**

H. S. and David, C. S., HLA-DQ8 transgenic mice are highly susceptible to collagen-induced arthritis: a novel model for human polyarthritis. *J. Exp. Med.* 1996. **183**: 27-37.

28 **Krco, C. J., Watanabe, S., Harders, J., Griffths, M. M., Luthra, H. and David, C. S.,** Identification of T cell determinants on human type II collagen recognized by HLA-DQ8 and HLA-DQ6 transgenic mice. *J. Immunol.* 1999. **163**: 1661-1665.

29 **Snijders, A., Elferink, D. G., Geluk, A., van Der Zanden, A. L., Vos, K., Schreuder, G. M., Breedveld, F. C. et al.,** An HLA-DRB1-derived peptide associated with protection against rheumatoid arthritis is naturally processed by human APCs. *J. Immunol.* 2001. **166**: 4987-4993.

30 **Zanelli, E., Krco, C. J., Baisch, J. M., Cheng, S. and David, C. S.,** Immune response of HLA-DQ8 transgenic mice to peptides from the third hypervariable region of HLA-DRB1 correlates with predisposition to rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA* 1996. **93**: 1814-1819.

31 **de Kauwe, A. L., Chen, Z., Anderson, R. P., Keech, C. L., Price, J. D., Wijburg, O., Jackson, D. C. et al.,** Resistance to celiac disease in humanized HLA-DR3-DQ2-transgenic mice expressing specific anti-gliadin CD4+ T cells. *J. Immunol.* 2009. **182**: 7440-7450.

32 **Vader, W., Stepniak, D., Kooy, Y., Mearin, L., Thompson, A., van Rood, J. J., Spaenij, L. et al.,** The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 12390-12395.

33 **Bao, F., Yu, L., Babu, S., Wang, T., Hoffenberg, E. J., Rewers, M. and Eisenbarth, G. S.,** One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. *J. Autoimmun.* 1999. **13**: 143-148.

34 **Tollefsen, S., Arentz-Hansen, H., Fleckenstein, B., Molberg, O., Raki, M., Kwok, W. W., Jung, G. et al.,** HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. *J. Clin. Invest* 2006. **116**: 2226-2236.

35 **Venhoff, N., Emmerich, F., Neagu, M., Salzer, U., Koehn, C., Driever, S., Kreisel, W. et al.,** The role of HLA DQ2 and DQ8 in dissecting celiac-like disease in common variable immunodeficiency. *J. Clin. Immunol.* 2013. **33**: 909-916.

36 **Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L. and Wiley, D. C.,** Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 1994. **368**: 215-221.

37 **Sant, A. J., Beeson, C., McFarland, B., Cao, J., Ceman, S., Bryant, P. W. and Wu, S.,** Individual hydrogen bonds play a critical role in MHC class II: peptide

interactions: implications for the dynamic aspects of class II trafficking and DM-mediated peptide exchange. *Immunol. Rev.* 1999. **172**: 239-253.

38 **Bandyopadhyay, A., Arneson, L., Beeson, C. and Sant, A. J.,** The relative energetic contributions of dominant P1 pocket versus hydrogen bonding interactions to peptide:class II stability: implications for the mechanism of DM function. *Mol. Immunol.* 2008. **45**: 1248-1257.

39 **Mosyak, L., Zaller, D. M. and Wiley, D. C.,** The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity* 1998. **9**: 377-383.

40 **Weber, D. A., Evavold, B. D. and Jensen, P. E.,** Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 1996. **274**: 618-620.

41 **Stratikos, E., Wiley, D. C. and Stern, L. J.,** Enhanced catalytic action of HLA-DM on the exchange of peptides lacking backbone hydrogen bonds between their N-terminal region and the MHC class II alpha-chain. *J. Immunol.* 2004. **172**: 1109-1117.

42 **Narayan, K., Chou, C. L., Kim, A., Hartman, I. Z., Dalai, S., Khoruzhenko, S. and Sadegh-Nasseri, S.,** HLA-DM targets the hydrogen bond between the histidine at position beta81 and peptide to dissociate HLA-DR-peptide complexes. *Nat. Immunol.* 2007. **8**: 92-100.

43 **Ferrante, A. and Gorski, J.,** Cutting edge: HLA-DM-mediated peptide exchange functions normally on MHC class II-peptide complexes that have been weakened by elimination of a conserved hydrogen bond. *J. Immunol.* 2010. **184**: 1153-1158.

44 **Rupp, B., Gunther, S., Makhmoor, T., Schlundt, A., Dickhaut, K., Gupta, S., Choudhary, I. et al.,** Characterization of structural features controlling the receptiveness of empty class II MHC molecules. *PLoS One* 2011. **6**: e18662.

45 **McFarland, B. J., Katz, J. F., Beeson, C. and Sant, A. J.,** Energetic asymmetry among hydrogen bonds in MHC class II*peptide complexes. *Proc. Natl. Acad. Sci. USA* 2001. **98**: 9231-9236.

46 **McFarland, B. J., Katz, J. F., Sant, A. J. and Beeson, C.,** Energetics and cooperativity of the hydrogen bonding and anchor interactions that bind peptides to MHC class II protein. *J. Mol. Biol.* 2005. **350**: 170-183.

47 **Pos, W., Sethi, D. K., Call, M. J., Schulze, M. S., Anders, A. K., Pyrdol, J. and Wucherpfennig, K. W.,** Crystal structure of the HLA-DM-HLA-DR1 complex defines mechanisms for rapid peptide selection. *Cell* 2012. **151**: 1557-1568.

48 **Guce, A. I., Mortimer, S. E., Yoon, T., Painter, C. A., Jiang, W., Mellins, E. D. and Stern, L. J.,** HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. *Nat. Struct. Mol. Biol.* 2013. **20**: 90-98.

49 **Painter, C. A., Negroni, M. P., Kellersberger, K. A., Zavala-Ruiz, Z., Evans, J. E. and Stern, L. J.,** Conformational lability in the class II MHC 310 helix and adjacent extended strand dictate HLA-DM susceptibility and peptide exchange. *Proc. Natl. Acad. Sci. USA* 2011. **108**: 19329-19334.

50 **Doebele, R. C., Busch, R., Scott, H. M., Pashine, A. and Mellins, E. D.,** Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity* 2000. **13**: 517-527.

51 **Busch, R., Pashine, A., Garcia, K. C. and Mellins, E. D.,** Stabilization of soluble, low-affinity HLA-DM/HLA-DR1 complexes by leucine zippers. *J. Immunol. Methods* 2002. **263**: 111-121.

52 **Painter, C. A., Cruz, A., Lopez, G. E., Stern, L. J. and Zavala-Ruiz, Z.,** Model for the peptide-free conformation of class II MHC proteins. *PLoS One* 2008. **3**: e2403.

53 **Yaneva, R., Springer, S. and Zacharias, M.,** Flexibility of the MHC class II peptide binding cleft in the bound, partially filled, and empty states: a molecular dynamics simulation study. *Biopolymers* 2009. **91**: 14-27.

54 **Ferrante, A., Templeton, M., Hoffman, M. and Castellini, M. J.,** The Thermodynamic Mechanism of Peptide-MHC Class II Complex Formation Is a Determinant of Susceptibility to HLA-DM. *J. Immunol.* 2015. **195**: 1251-1261.

55 **Pos, W., Sethi, D. K. and Wucherpfennig, K. W.,** Mechanisms of peptide repertoire selection by HLA-DM. *Trends Immunol.* 2013. **34**: 495-501.

56 **Mellins, E. D. and Stern, L. J.,** HLA-DM and HLA-DO, key regulators of MHC-II processing and presentation. *Curr. Opin. Immunol.* 2014. **26**: 115-122.

57 **Zhou, Z., Callaway, K. A., Weber, D. A. and Jensen, P. E.,** Cutting edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. *J. Immunol.* 2009. **183**: 4187-4191.

58 **Anders, A. K., Call, M. J., Schulze, M. S., Fowler, K. D., Schubert, D. A., Seth, N. P., Sundberg, E. J. et al.,** HLA-DM captures partially empty HLA-DR molecules for catalyzed removal of peptide. *Nat. Immunol.* 2011. **12**: 54-61.

59 **Ferrante, A., Anderson, M. W., Klug, C. S. and Gorski, J.,** HLA-DM mediates epitope selection by a "compare-exchange" mechanism when a potential peptide pool is available. *PLoS One* 2008. **3**: e3722.

60 **Chou, C. L. and Sadegh-Nasseri, S.,** HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J. Exp. Med.* 2000. **192**: 1697-1706.

61 **Denzin, L. K., Hammond, C. and Cresswell, P.,** HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty

HLA-DR molecules. *J. Exp. Med.* 1996. **184**: 2153-2165.

62 **Kropshofer, H., Arndt, S. O., Moldenhauer, G., Hammerling, G. J. and Vogt, A. B.,** HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. *Immunity* 1997. **6**: 293-302.

63 **Zarutskie, J. A., Busch, R., Zavala-Ruiz, Z., Rushe, M., Mellins, E. D. and Stern, L. J.,** The kinetic basis of peptide exchange catalysis by HLA-DM. *Proc. Natl. Acad. Sci. USA* 2001. **98**: 12450-12455.

64 **Grotenbreg, G. M., Nicholson, M. J., Fowler, K. D., Wilbuer, K., Octavio, L., Yang, M., Chakraborty, A. K. et al.,** Empty class II major histocompatibility complex created by peptide photolysis establishes the role of DM in peptide association. *J. Biol. Chem.* 2007. **282**: 21425-21436.

65 **Yin, L., Maben, Z. J., Becerra, A. and Stern, L. J.,** Evaluating the Role of HLA-DM in MHC Class II-Peptide Association Reactions. *J. Immunol.* 2015. **195**: 706-716.

66 **Todd, J. A., Acha-Orbea, H., Bell, J. I., Chao, N., Fronek, Z., Jacob, C. O., McDermott, M. et al.,** A molecular basis for MHC class II--associated autoimmunity. *Science* 1988. **240**: 1003-1009.

67 **Horn, G. T., Bugawan, T. L., Long, C. M. and Erlich, H. A.,** Allelic sequence variation of the HLA-DQ loci: relationship to serology and to insulin-dependent diabetes susceptibility. *Proc. Natl. Acad. Sci. USA* 1988. **85**: 6012-6016.

68 **Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C.,** Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 1993. **364**: 33-39.

69 **Kwok, W. W., Domeier, M. E., Johnson, M. L., Nepom, G. T. and Koelle, D. M.,** HLA-DQB1 codon 57 is critical for peptide binding and recognition. *J. Exp. Med.* 1996. **183**: 1253-1258.

70 **Erlich, H. A., Zeidler, A., Chang, J., Shaw, S., Raffel, L. J., Klitz, W., Beshkov, Y. et al.,** HLA class II alleles and susceptibility and resistance to insulin dependent diabetes mellitus in Mexican-American families. *Nat. Genet.* 1993. **3**: 358-364.

71 **Lee, K. H., Wucherpfennig, K. W. and Wiley, D. C.,** Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type 1 diabetes. *Nat. Immunol.* 2001. **2**: 501-507.

72 **Zavala-Ruiz, Z., Strug, I., Anderson, M. W., Gorski, J. and Stern, L. J.,** A polymorphic pocket at the P10 position contributes to peptide binding specificity in class II MHC proteins. *Chem. Biol.* 2004. **11**: 1395-1402.

73 **Yassai, M., Afsari, A., Garlie, J. and Gorski, J.,** C-terminal anchoring of a peptide

to class II MHC via the P10 residue is compatible with a peptide bulge. *J. Immunol.* 2002. **168**: 1281-1285.

74 **Baumgartner, C. K., Ferrante, A., Nagaoka, M., Gorski, J. and Malherbe, L. P.,** Peptide-MHC class II complex stability governs CD4 T cell clonal selection. *J. Immunol.* 2010. **184**: 573-581.

75 **Siklodi, B., Vogt, A. B., Kropshofer, H., Falcioni, F., Molina, M., Bolin, D. R., Campbell, R. et al.,** Binding affinity independent contribution of peptide length to the stability of peptide-HLA-DR complexes in live antigen presenting cells. *Hum. Immunol.* 1998. **59**: 463-471.

76 **Harndahl, M., Rasmussen, M., Roder, G., Dalgaard Pedersen, I., Sorensen, M., Nielsen, M. and Buus, S.,** Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. *Eur. J. Immunol.* 2012. **42**: 1405-1416.

77 **Geironson, L., Roder, G. and Paulsson, K.,** Stability of peptide-HLA-I complexes and tapasin folding facilitation--tools to define immunogenic peptides. *FEBS Lett.* 2012. **586**: 1336-1343.

78 **Unanue, E. R.,** Antigen presentation in the autoimmune diabetes of the NOD mouse. *Annu Rev Immunol* 2014. **32**: 579-608.

79 **Mohan, J. F., Levisetti, M. G., Calderon, B., Herzog, J. W., Petzold, S. J. and Unanue, E. R.,** Unique autoreactive T cells recognize insulin peptides generated within the islets of Langerhans in autoimmune diabetes. *Nat. Immunol.* 2010. **11**: 350-354.

80 **Cucchi-Mouillot, P., Lai, S., Carcassi, C., Sorba, P., Stuart-Simoni, M., Amoros, J. P., Genetet, B. et al.,** Implication of HLA-DMA alleles in corsican IDDM. *Dis. Markers* 1998. **14**: 135-141.

81 **Sang, Y. M., Yan, C., Zhu, C., Ni, G. C. and Hu, Y. M.,** [Association of human leukocyte antigen non-classical genes with type 1 diabetes]. *Zhonghua Er Ke Za Zhi* 2003. **41**: 260-263.

82 **Siegmund, T., Donner, H., Braun, J., Usadel, K. H. and Badenhoop, K.,** HLA-DMA and HLA-DMB alleles in German patients with type 1 diabetes mellitus. *Tissue Antigens* 1999. **54**: 291-294.

83 **Chou, C. L., Mirshahidi, S., Su, K. W., Kim, A., Narayan, K., Khoruzhenko, S., Xu, M. et al.,** Short peptide sequences mimic HLA-DM functions. *Mol. Immunol.* 2008. **45**: 1935-1943.

84 **Nicholson, M. J., Moradi, B., Seth, N. P., Xing, X., Cuny, G. D., Stein, R. L. and Wucherpfennig, K. W.,** Small molecules that enhance the catalytic efficiency of HLA-DM. *J. Immunol.* 2006. **176**: 4208-4220.

85 **Call, M. J., Xing, X., Cuny, G. D., Seth, N. P., Altmann, D. M., Fugger, L., Krogsgaard, M. et al.,** In vivo enhancement of peptide display by MHC class II molecules with small molecule catalysts of peptide exchange. *J. Immunol.* 2009. **182**: 6342-6352.

APPENDIX A

SUPPLEMENTAL DATA FOR CHAPTER 4

Originally published in *The Journal of Immunology*. Zemin Zhou, Kari A. Callaway, Dominique A. Weber, Peter E. Jensen. 2009. Cutting- edge: HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes. J. Immunol. 183: 4187-4191.

Figure A.1. Asparagine substitution at position 81 in the DR1 beta chain does not affect susceptibility to DM-mediated CLIP release. Expression of DR and CLIP on the surface of T2 cells expressing βH81N DR1 molecules in the absence (middle panel) or presence (right panel) of DM was measured by flow cytometry with mAbs L243 (DR) and CerCLIP (CLIP). A retroviral vector encoding full-length DRβ-H81N was generated using pLXSN-DRβ-H81A as a template with primers (DRβ-H81N-F: 5'-GGTGGACACCTACTGCAGAaaCAACTACGGGGTTGG -3') and DRβ-H81N-R: 5'-CCAACCCCGTAGTTGttTCTGCAGTAGGTGTCCACC -3'), and the product was confirmed by DNA sequencing. T2 cells stably expressing the mutant DR1 heterodimer in the absence or presence of DM were generated as described in Materials and Methods.

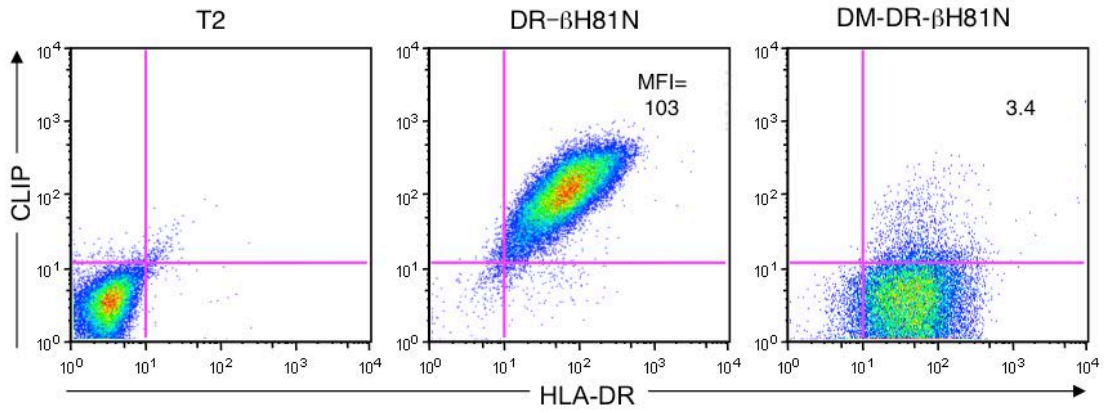
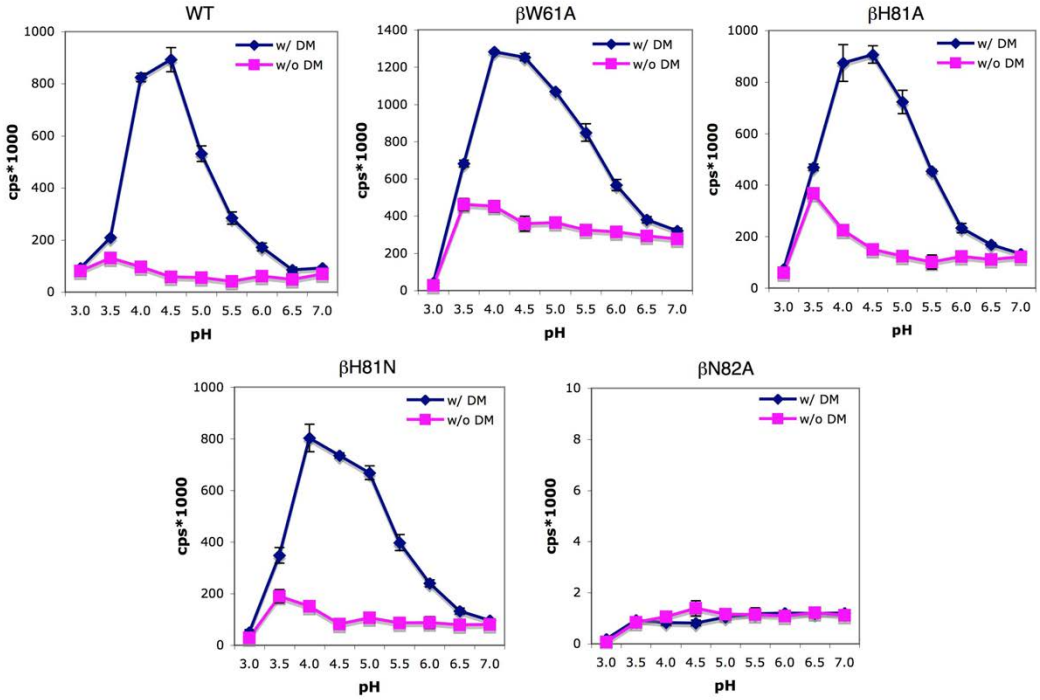

T2
DR−βH81N
DM-DR-βH81N
MFI=
103
3.4
CLIP
HLA-DR

Figure A.2. Replacement of the histidine at position 81 in the DR1 beta chain does not change the pH dependence of DM-catalyzed peptide binding. Wild type or mutant purified soluble recombinant DR1 molecules (50 nM) were incubated with 1 μM biotinylated MAT peptide in the presence or absence of 1μM purified soluble DM in a buffer containing 0.2% NP-40 and 40 mM citrate/Na_2HPO_4 (pH 3.0-7.0), in a total volume of 30 μl, for 3 hours at 37^{o}C. Following the incubation, samples were pH neutralized and DR-bound peptide was measured using a europium fluoroimmunoassay as previously described [1, 2]. No peptide binding was detected under these experimental conditions with βN82A DR1, which forms very unstable peptide complexes.

References

1 **Tompkins, S. M., Rota, P. A., Moore, J. C. and Jensen, P. E.,** A europium fluoroimmunoassay for measuring binding of antigen to class II MHC glycoproteins. *J. Immunol. Methods* 1993. 163: 209-216.

2 **Sherman, M. A., Runnels, H. A., Moore, J. C., Stern, L. J. and Jensen, P. E.,** Membrane interactions influence the peptide binding behavior of DR1. *J. Exp. Med.* 1994. 179: 229-234.


WT
βW61A
βH81A
βH81N
βN82A
w/ DM
w/o DM
cps*1000
pH

APPENDIX B

EFFECTS OF EXTRA HYDROGEN BONDS ON THE DQ8/PEPTIDE COMPLEX STABILITY AND DM EDITING SENSITIVITY

Figure B.1. Effects of extra hydrogen bonds on DQ8/peptide complex stability and DM editing sensitivity. (A) The FP measurement of peptide dissociation rates and DM editing effects between DQ8 wild type and E86A mutation bound with $CLIP^{488}$ or $ABP625^{488}$ peptides. Data shown are representative of four independent experiments. (B) The DM-catalyzed rate enhancements normalized with the interpolated intrinsic peptide dissociation rates. Data shown are representative of four independent experiments.

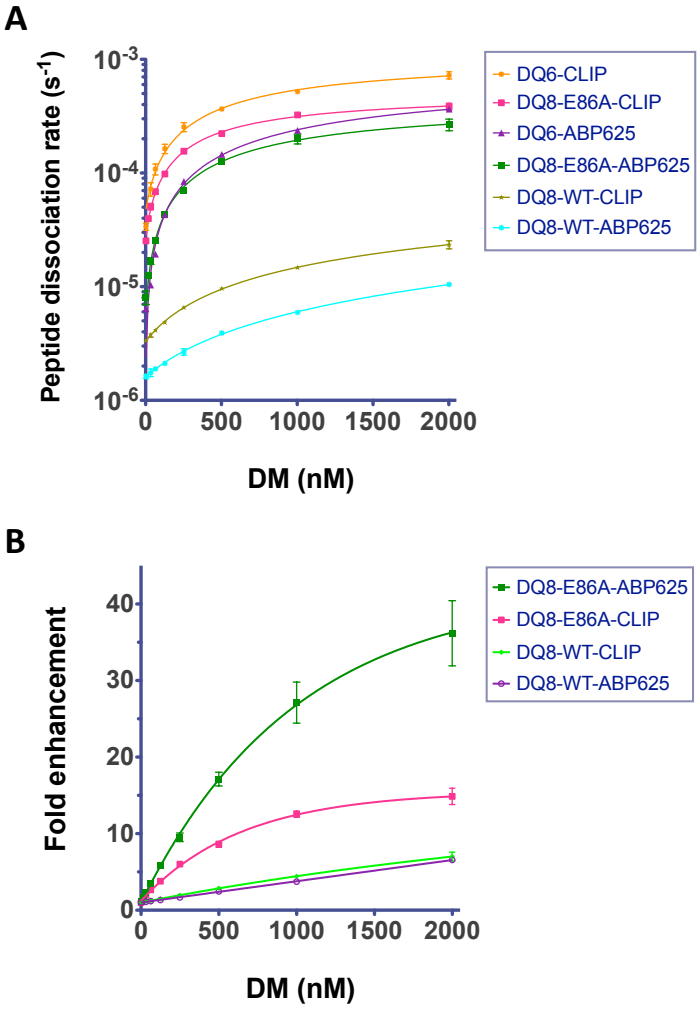

A
Peptide dissociation rate (s^{-1})
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
500
1000
1500
2000
DM (nM)
DQ6-CLIP
DQ8-E86A-CLIP
DQ6-ABP625
DQ8-E86A-ABP625
DQ8-WT-CLIP
DQ8-WT-ABP625
B
Fold enhancement
40
30
20
10
0
0
500
1000
1500
2000
DM (nM)
DQ8-E86A-ABP625
DQ8-E86A-CLIP
DQ8-WT-CLIP
DQ8-WT-ABP625

Table B.1. Comparison of the interpolated peptide intrinsic dissociation rates (K_{in}) between DQ8 wild type and E86A mutation that disrupted two of the three extra hydrogen bonds.

MHC complex	K_{in}	Ratio of K_{in} (E86A/WT)
DQ8-WT-ABP625	1.6×10^{-6}	
DQ8-E86A-ABP625	7.4×10^{-6}	4.6
DQ8-WT-CLIP	3.3×10^{-6}	
DQ8-E86A-CLIP	2.6×10^{-5}	7.7