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Modeling alternative binding registers of a minimal immunogenic peptide on two class II major histocompatibility complex (MHC II) molecules predicts polarized T-cell receptor (TCR) contact positions

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Abstract: Several major histocompatibility complex class II (MHC II) complexes with known minimal immunogenic peptides have now been solved by X-ray crystallography. Specificity pockets within the MHC II binding groove provide distinct peptide contacts that influence peptide conformation and define the binding register within different allelic MHC II molecules. Altering peptide ligands with respect to the residues that contact the T-cell receptor (TCR) can drastically change the nature of the ensuing immune response. Here, we provide an example of how MHC II (I-A) molecules may indirectly effect TCR contacts with a peptide and drive functionally distinct immune responses. We modeled the same immunogenic 12-amino acid peptide into the binding grooves of two allelic MHC II molecules linked to distinct cytokine responses against the peptide. Surprisingly, the favored conformation of the peptide in each molecule was distinct with respect to the exposure of the N- or C-terminus of the peptide above the MHC II binding groove. T-cell clones derived from each allelic MHC II genotype were found to be allele-restricted with respect to the recognition of these N- vs. C-terminal residues on the bound peptide. Taken together, these data suggest that MHC II alleles may influence T-cell functions by restricting TCR access to specific residues of the I-A-bound peptide. Thus, these data are of significance to diseases that display genetic linkage to specific MHC II alleles, e.g. type 1 diabetes and rheumatoid arthritis.

Abbreviations: APC, antigen presenting cell; CFA, complete Freund's adjuvant; MCF, median channel fluorescence; MHC, major histocompatibility complex; TCR, T-cell receptor.

T-cell recognition of antigens requires that peptides are generated within the antigen presenting cell (APC) and bound into the groove of molecules encoded by genes of the major histocompatibility complex (MHC) (1–3). Antigens derived from intracellular sources (e.g. viral infections) are primarily presented by class I MHC molecules to CD8 T cells that have critical regulatory and effector functions (e.g. cytotoxicity) in the ensuing immune response. By contrast, class II MHC molecules present immunogenic peptides derived from extracellular sources (e.g. bacterial toxins) to CD4 T cells that provide important functions, such as suppression and induction of humoral immunity and inflammation (4–6). Importantly, class I and class II MHC genes are extremely polymorphic (e.g. there are ≈ 122 recognized HLA-DRB alleles), such that distinct alleles differ in a region of the MHC molecule called the peptide binding groove (7–9). It has been recognized since the early 1970s that MHC alleles can determine whether T-cell immunity is elicited against a given antigen (1,2). However, it remains to be determined how specific types of immune responses are dictated by relatively subtle differences in the amino acids that line the peptide binding groove of allelic MHC II molecules.

To investigate this question, we adopted a system wherein distinct types of immunity were genetically linked to two MHC II (designated I-A) alleles in the mouse (10–14). Early work showed that the inflammatory cytokine, interferon gamma (IFN- γ) was elicited from T cells responding to a minimal 12-mer peptide from human collagen IV in mice of I-A^S genotype, whereas the same peptide elicited interleukin-4 (IL-4)-producing T cells in mice which differed only in the I-A allele, i.e. I-A^b mice (12). Unbalanced production of these cytokines are prototypical of T-helper (Th)₁-type (IFN- γ) and Th₂-type (IL-4) cytokine responses that have now been associated with several human disease states (14–16). In some cases, Th₁ vs. Th₂ immunity in a human disease is genetically linked to specific MHC II alleles (17–20). It is known that I-A^S molecules differ from I-A^b molecules in the peptide residues that they prefer to bind within the binding pockets P₁ → P₉ of the MHC II groove (Fig. 4) (8,9). In an attempt to explain why functionally distinct cytokines are elicited by the same peptide in mice which differ in the binding grooves of their I-A molecules, we modeled the two MHC II alleles binding the collagen 12-mer peptide. Our data suggest that the N- and C-termini are oppositely available for TCR binding in the two allelic I-A/peptide complexes. These data therefore indicate that MHC II molecules may contribute to functional immunity

via a mechanism involving the alteration of peptide conformation.

Experimental Procedures

I-A^S constructs and L-cell transfection

The full-length cDNA for A α^S and A β^S , each in pBluescript SK+ (the gift of Dr H. O. McDevitt, Stanford University) (21,22) were each subcloned into the *EcoRI* site of the expression vector, pcDNA3 (Invitrogen, San Diego, CA, USA). Predicted fragment sizes for correct 5'→3' orientation were 4975 and 1357 bp (pcDNA3-A α^S /*StuI* cut) and 5089 and 1149 bp (pcDNA3-A β^S /*EcoNI* + *StuI* cut), which were observed. Fragment sizes for incorrect 3'→5' orientation were not observed. Miniprep DNA was made from clones represented by lanes 2 and 4 (Fig. 1), linearized at the unique *BglII* site in each plasmid, and 5 μ g of each used to transfect DAP.3 (class II negative) fibroblasts (gift of Dr R.N. Germain, NIAID) (23) by electroporation in a CellPoratorTM (Gibco/BRL). For the selection of stable transfectants, the

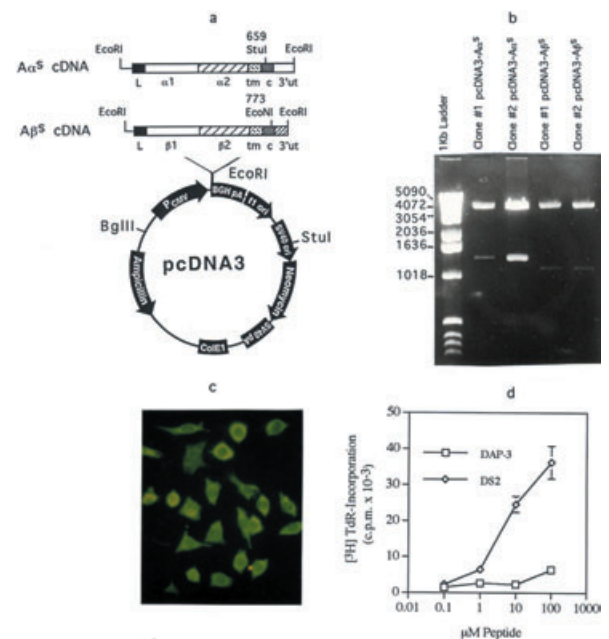


Figure 1. Generation of I-A^S L cells. Construction of the pcDNA3-A α^S and pcDNA3-A β^S expression vectors, showing restriction enzyme sites used to map 5' → 3' orientation for expression by the CMV promoter (A). Restriction fragments of the recombinant plasmids in 1.2% agarose, ethidium bromide stained minigel (B). Cell surface expression of I-A^S on the transfected DAP.3 L-cell clone, DS2 (C). Presentation of the 12-mer peptide to CD4⁺ T-cell clone (S7) by the DS2 transfectant and the parent L-cell line, DAP.3 (D).

media was replaced with fresh media containing 200 µg/mL G418 (GeneticinTM, Gibco/BRL) every 4 days for 2 weeks. Screening the transfectants for cell surface I-A^s was with biotinylated monoclonal antibodies (mAbs) 10–3.6.2 (Aβ^s epitope) and Y3P (Aα^s/Aβ^s epitope), followed by avidin–FITC and fluorescence microscopy or flow cytometry (12).

T-Cell proliferation assay

L-Cell transfectants were cloned by limiting dilution then tested for efficient antigen presentation of the 12-mer collagen IV peptide to the I-A^s-derived CD4⁺ T-cell clone, S7. Briefly, transfected L-cell clones, or the parent line, DAP.3, were plated at 2×10⁴ per well overnight, irradiated on the plate (3000R), and washed before the addition of the T-cell clone (1×10⁵) in Clicks/EHAA/5% fetal calf serum (FCS) containing increasing concentrations of the 12-mer peptide. Cultures were incubated for 48 h, followed by a 16-h pulse with 1 µCi/well of tritiated thymidine, followed by liquid scintillation counting (12,13).

Intrathymic injections and measuring Th1/Th2 frequencies by flow cytometry

Six to eight-week-old female MHC class II-deficient (C2D) mice (24) (Taconic Farms) were age matched for these studies. Three to five mice per group received the I-A^s-bearing transfectant (clone DS2) prepared as described above, and injected intrathymically (i.t.) at 2×10⁶ cells/10 µL sterile (endotoxin-free) saline, as described previously (25). An identical group received the I-A^b transfectant (FT7.1) by the same i.t. injection technique. T-cell maturation was allowed to proceed for 4 weeks (26), each group was then injected subcutaneously with 3×10⁶ peptide-pulsed B cells bearing a high ligand density of the 12-mer peptide (peptide sequence in Fig. 3A), as described previously (13). Eight days later, the peripheral lymph nodes were removed and cultured for 3 days in Clicks/EHAA/5% FCS containing 100 µM 12-mer peptide. On day 4, 50 units/mL of recombinant human IL-2 was added, and on day 5 the cells were collected over lymphocyte separation medium (LSM, Gibco/BRL). Viable cells were stimulated with PMA (50 ng/mL) plus ionomycin (0.5 µg/mL; Sigma) for 6 h in Clicks-EHAA–5% FCS, wherein the last 2 h included the addition of monensin (GolgiStopTM, Pharmingen, San Diego, CA, USA). The cells were washed extensively in PBS/5% FCS and incubated with 10 µg/mL of anti-CD4 (RM4-5-cyochrome, Pharmingen), then washed and permeabilized with saponin-containing buffer (Cytotfix-CytoPerm kitTM,

Pharmingen). Anti-cytokine mAbs (anti-IL-4, clone BVD4-1D11-PE; and anti-IFN-γ, clone XMG1.2-FITC, Pharmingen) were incubated together in saponin buffer at 10 µg/mL with the anti-CD4 stained cells on ice, followed by one wash in saponin-buffer and two in PBS/5% FCS. The cells were then subjected to flow cytometry using a FACScanTM (Becton Dickinson, Mountain View, CA, USA) as described previously (12,13). For analysis, 40 000 events were acquired and dead cells excluded by forward/side-scatter gating. Statistical quadrants were set on negative and single color controls for each cytokine, and the data analyzed with CellQuestTM software (Becton Dickinson).

Analysis of TCR specificity for the 12-mer peptide

CD4⁺ T-cell clones were derived as described previously (12). Clonality was confirmed by staining with a panel of Vβ-specific mAb (Pharmingen). Peptides were synthesized using solid-phase chemistry and purified by reverse-phase HPLC (Research Genetics, Huntsville, AL, USA). Sequences were verified by mass spectroscopy (MS Laboratory, Department of Chemistry). For proliferation, 1×10⁵ cloned T cells/well were cultured in triplicate with 5×10⁵ irradiated (2000R) syngeneic (T-cell depleted) spleenocyte APC in 200 µL of Clicks/EHAA/5% FCS containing increasing concentrations of the various 12-mer peptide derivatives as shown in Fig. 3A. Subsequent steps of the assays were as described above.

Molecular modeling of the TCR ligands

To model I-A/peptide structures, docking studies were performed on a Silicon Graphics OctaneTM workstation, using INSIGHTIITM software (MSI/Biosym, version 980, San Diego, CA, USA). The 12-mer was modeled into the two I-A structures by using the coordinates of the I-A^k/HEL peptide complex (Protein Data Bank, Brookhaven National Laboratory, Accession no. 1IAK), wherein each of the polymorphic Aα and Aβ chain amino acids were substituted with the s- and b-allelic residues (9) using INSIGHTIITM. The collagen 12-mer peptide was used in place of the HEL peptide, assuming that Ile³ occupies the specificity binding pocket number 1 (P1) within the peptide-binding groove of I-A^s, whereas Gln⁴ occupies P1 within I-A^b; both according to the anchor motifs identified for these alleles (Fig. 4A) (27,28). The primary structure of the peptide was built with INSIGHTIITM and the structure was energy minimized for 100 steps using the steepest decent method. This structure was modeled onto the I-A proteins and the affinity program

was used to calculate the best-docked structure. This docked structure was subjected to energy minimization using the steepest descent and conjugate-gradient methods until the most stable structures were determined, i.e. the root mean square derivative (rmsd) was -0.8 kcal/mole \AA^{-1} (29).

Results

Construction of an I-A^s fibroblast clone (DS2)

For the I-A^s transfectant, the full-length cDNA for A α ^s and A β ^s were subcloned into the *EcoRI* site of the expression vector, pcDNA3 (Fig. 1A,B). As illustrated by Fig. 1C, we obtained a clone (DS2), that expressed I-A^s MHC class II molecules on the cell surface. This transfectant was efficient at antigen presentation of the 12-mer peptide to 12-mer-elicited Th1 CD4⁺ T-cell clones derived from I-A^s mice (Fig. 1D). Comparatively, the clone, FT7.1, was derived from the same fibroblast line (DAP.3) (18), displays I-A^b at a similar level on its cell surface, and is similarly efficient at antigen presentation of the 12-mer peptide to I-A^b-derived CD4⁺ T-cell clones (13).

I-A alleles are linked to Th1 or Th2 immunity against the minimal peptide

We observed the predicted relative frequencies of Th1 vs. Th2 associated with each I-A genotype (Fig. 2). Specifically, I-A^s constructed mice elicited an immune response

characterized by a high frequency of IFN- γ producers, with very few IL-4-producing cells against the peptide presented by I-A^s B cells (Fig. 2A). Conversely, intrathymic delivery of the I-A^b molecule into the same C2D genotype gave a response essentially devoid of IFN- γ producers, but high in IL-4-expressing cells when primed with the same density of the peptide bound to I-A^b B cells (Fig. 2B).

TCR contacts at opposite ends of the minimal peptide depend upon I-A allele

Recall-proliferation and cytokine release by a panel of CD4⁺ T-cell clones revealed two distinct peptide-specificity patterns that depend upon the I-A molecules of the mice injected with the 12-mer peptide. As illustrated in Fig. 3B (left panel), I-A^s-derived T cells (e.g. clone 3S8) respond to the wild-type 12-mer minimal peptide at relatively low concentrations, and I-A^s-derived clones recognize the A11-mer peptide, that has a truncated N-terminus (thus starts with A, Fig. 3A). I-A^s-derived T cells also effectively recognize a peptide derivative with an E \rightarrow V substitution at the N-terminus. In contrast, C-terminus truncation (the 11-mer and 10-mer peptides), or substitution of the corresponding mouse amino acid (T) at the C-terminus (the T12 peptide) severely limits T-cell recognition (Fig. 3B, left panel, data compiled in Fig. 3C). Strikingly, with respect to the portion of the peptide recognized, are the I-A^b-derived T-cell clones. They do not respond to the N-terminus derivatives (i.e. the A11-mer, or V1 peptides), but do respond equally to the C-substituted derivative (T12 peptide) and,

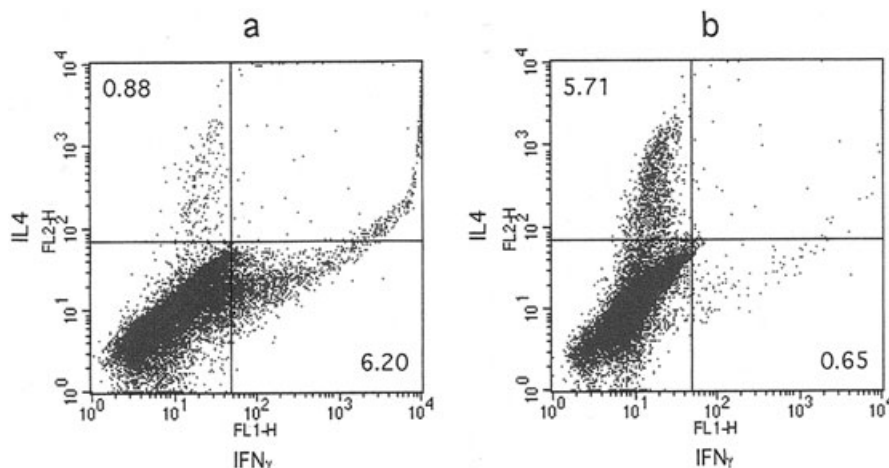


Figure 2. Intrathymic injection of I-A^s- vs. I-A^b-transfected L cells yields predicted Th1 vs. Th2 Immunity. DS2 (I-A^s) L cells by intrathymic (i.t.) injection into MHC class II knockout (C2D) mice directs Th1-immunity to the 12-mer peptide: IFN γ /IL4 expression by intracellular staining (A). FT7.1 (I-A^b) L cells injected i.t. into C2D mice reconstitutes Th2-immunity to the same peptide: IFN γ /IL4 intracellular staining (B). Values are the percentage of gated cells in each quadrant. Frequencies of CD4⁺ cells in the activated lymph node cell pools from DS2 and FT7.1 intrathymic chimeras were 36.33 and 38.36%, respectively (data not shown). These are representative results of three experiments.

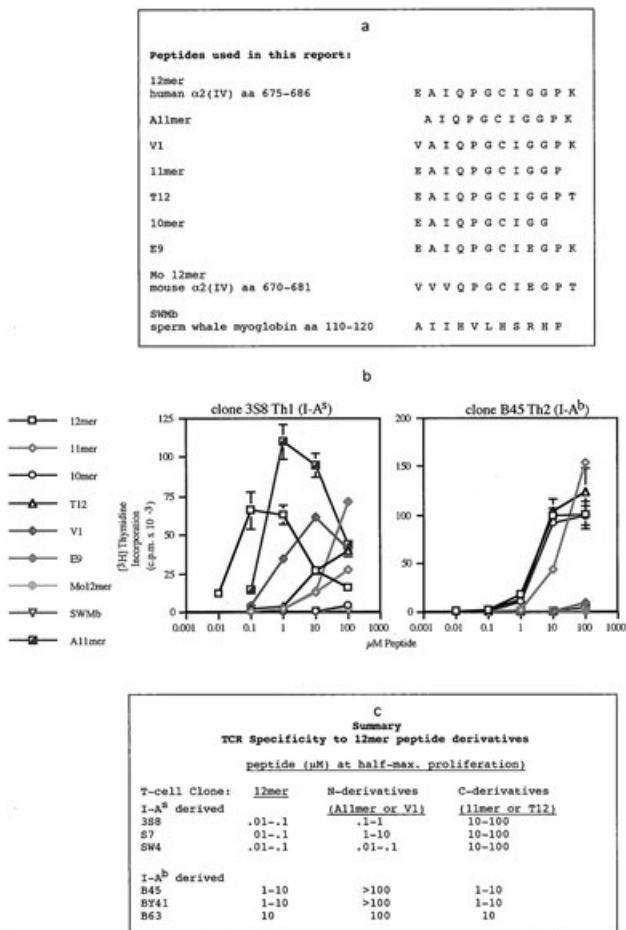


Figure 3. CD4⁺ T cells from I-A^S- vs. I-A^B genotypes recognize opposite ends of the 12-mer peptide. Sequences of peptides used in this study (A). Peptide fine-specificity of the I-A^S-derived CD4⁺ T-cell clone, 3S8 (B, left panel). Peptide fine-specificity of the I-A^B-derived CD4⁺ T-cell clone, B45 (B, right panel). Summary of I-A^S- vs. I-A^B-derived CD4⁺ T-cell clone recognition of peptide derivatives (C). Clonality was confirmed by staining with a panel of V β -specific mAb (PharMingen). Values are the mean \pm SD for each triplicate at each peptide dose. These results are representative of at least three experiments.

almost as well, to the C-truncated 11-mer and 10-mer peptides (Fig. 3B, right panel, and compiled in Fig. 3C).

Molecular modeling predicts an N- vs. C-terminus dichotomy in the preferred conformations of the minimal peptide bound to I-A^S and I-A^B.

We sought to explain this dichotomy of TCR specificity by modeling the minimal 12-mer peptide bound to each I-A molecule. The crystallographic structure of the I-A^k/HEL(50-62) peptide complex (9) was used to construct each complex (Fig. 4B-E). The HEL peptide was replaced by the collagen 12-mer into the predicted grooves of I-A^S and I-A^B [generated by substitution of the I-A polymorphic positions with s- or b-allele side chains on the

I-A^k coordinates (9) (Fig. 4)]. Shown in Fig. 4A are consensus anchor residues of peptides that bind to I-A^S and I-A^B molecules (27,28). The peptide position 3 isoleucine (I) is the P1 anchor for I-A^S, so the 12-mer was docked to the protein with the Ile³ side-chain oriented into the P1 pocket. The complex was subjected to energy minimization; and, importantly, the peptide assumed an orientation for Pro¹¹ predicted by the motif (i.e. Pro¹¹ was oriented into the P9 pocket) (Fig. 4A-C). Strikingly, in this structure, the C-terminal lysine extends out of the groove, above the A α - and A β -chain α -helices; and, the N-terminus (Glu¹) forms a hydrogen bond with the T52 side-chain and is pointing down into the P1 pocket (Fig. 4B,C). Thus, the relative accessibility of the N- and C-termini of the peptide when bound to I-A^S is remarkably consistent with the recognition pattern of the I-A^S-derived T-cell clones (Fig. 3B, left panel, and Fig. 3C). In contrast, since peptide position 4, glutamine (Q), is the predicted P1 anchor in I-A^B (Fig. 4A), docking Gln⁴ into the P1 pocket shifts the peptide to the left in the modeled I-A^B groove (Fig. 4D,E). After energy minimization, this orientation effectively rotates the N-terminus (Glu¹) and the position 3 isoleucine side-chains so that they extended up and out of the I-A^B groove (i.e. instead of down, as they are in the I-A^S model). Moreover, the C-terminus (Lys¹²) is buried into the P7 pocket (near residue W61; Fig. 4D,E). Therefore, the predicted accessibility of the N- vs. C-termini side-chains of the peptide when bound to I-A^B also exactly fits the peptide-specificity observed with I-A^B-restricted TCR (Fig. 3B, right panel and Fig. 3C). Taken together, these data suggest that TCR contact positions at opposite poles of the bound peptide are determined by the induced-fit of the peptide within each I-A molecule.

Discussion

For a model peptide (from moth cytochrome *c*), there are three positions of this nine amino acid peptide that must be occupied by a restricted set of amino acids in order for the peptide to bind significantly to the MHC II molecule, I-E^k (30). Such positions along a peptide antigen are often referred to as 'anchor' positions, and in some cases, X-ray crystallographic data are available to show that these side-chains actually extend into discreet pockets of the MHC II peptide binding groove (3,9,31,32). Thus, peptide side-chains at these positions form favorable (i.e. polar/hydrophobic/steric) interactions with I-A side-chains in these *pockets* (viz., P1 \rightarrow P9) lining the binding groove (Fig. 4) (9,32). The

There are several possibilities for how MHC II could effect Th₁/Th₂ polarization, including mechanisms based on ligand density, APC type/costimulatory ligands, or the TCR repertoire (14). We have presented evidence that the collagen IV 12-mer peptide binds to the Th₁-associated I-A^s molecule with the N-terminus buried in the groove and the C-terminus exposed above the binding groove (Fig. 4B,C). Strikingly in contrast, the same peptide docked with the C-terminus buried and the N-terminus exposed in the Th₂-associated I-A^b/peptide model (Fig. 4D,E). Importantly, the recognition patterns of T cells derived from the two MHC II genotypes confirmed these predictions, suggesting that the TCR actually contact the differentially exposed termini of the peptide. Although it is possible that truncation or substitution of an anchor position would abrogate overall binding and halt T-cell activation; this however, was not observed with the single changes we made in the 12-mer peptide (Fig. 3). Most likely, this reflects on the large number of H-bonds between the peptide and MHC II protein (31–33) (Fig. 4). Indeed, previous measurements of the binding of V₁ and T₁₂ peptides demonstrated that these peptides bind to I-A as well as the wild-type 12-mer (37). Thus, another mechanism of Th₁/Th₂ selection in this system may be determined by I-A-linked distinctions in the conformation of the bound collagen peptide. In this study, we examined only a limited number of T-cell clones from each I-A genotype. As is generally the case, we maintained these cells by repeated restimulation with the peptide in long-term culture – a method known to select for high avidity TCR-bearing cells (12). We cannot rule out that lower avidity clonotypes of the intact immune system would display TCR contacts distinct from those of the clones we have examined. Here, we have used these recognition patterns primarily to support the modeling data. Nevertheless, the striking correlation between the modeled termini of the peptide and recognition by the three-member panels may be biologically significant. Indeed, it

has been demonstrated that changing peptide reactivity of just high-affinity TCR bearing clones can shift immunity in an intact (polyclonal) immune system (38,39).

How could the conformation of the bound peptide determine the frequency of Th₁- vs. Th₂-type cells elicited by the peptide immunogen? Because the altered conformations determined which residues of the peptide contact the TCR, these results are similar to the known effects on Th₁/Th₂ immunity that have been described for so-called altered peptide ligands (APL) (40,41). APL are minimal immunogenic peptides that have been substituted at positions that are thought to contact the TCR and not the MHC. APL have been shown to shift Th₁/Th₂ frequency *in vitro*, an effect that may be linked to altered calcium mobilization, and tyrosine phosphorylations of TCR-zeta and ZAP-70 molecules during intracellular signaling of T-cell differentiation (40). Increasing the dose of some APL, but not others, causes a Th₂ to Th₁ deviation in the responding *in vitro* population, and this appeared to reflect the effects of IFN- γ on Th₁/Th₂ polarization (41). As indicated above, the *in vivo* effects of an APL may alternatively involve indirect polarization of uncommitted Th cells via changes in the reactivity of only a few ‘regulatory’ clonotypes (38,39). Our data suggest that MHC II molecules can indirectly effect the TCR contacts of a specific peptide immunogen. As the particular alleles of the system examined here determine Th₁/Th₂ immunity, it follows that bound peptide conformation may be a critical factor in the mechanism by which MHC II molecules control immunity. The challenge for the future is to understand how such a mechanism is integrated into the hierarchy of MHC control.

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