Published in final edited form as:

Clin Immunol. 2015 October ; 160(2): 188–197. doi:10.1016/j.clim.2015.05.002.

Peptide Ligand Structure and I-A^q Binding Avidity Influence T Cell Signaling Pathway Utilization

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Abstract

Factors that drive T cells to signal through differing pathways remain unclear. We have shown that an altered peptide ligand (A9) activates T cells to utilize an alternate signaling pathway which is dependent upon FcR γ and Syk. However, it remains unknown whether the affinity of peptide binding to MHC drives this selection. To answer this question we developed a panel of peptides designed so that amino acids interacting with the p6 and p9 predicted MHC binding pockets were altered. Analogs were tested for binding to I-A^q using a competitive binding assay and selected analogs were administered to arthritic mice. Using the collagen-induced arthritis (CIA) model, arthritis severity was correlated with T cell cytokine production and molecular T cell signaling responses. We establish that reduced affinity of interaction with the MHC correlates with T cell signaling through the alternative pathway, leading ultimately to secretion of suppressive cytokine and attenuation of arthritis.

Keywords

arthritis; autoimmunity; T cells

Introduction

The development of acquired autoimmunity is essential to the pathogenesis of rheumatoid arthritis (RA). The critical role of the interaction of T cells and MHC is reinforced by the success of Abatacept (a molecule engineered to interfere with the costimulation involved in T cell activation) in treating RA [1]. These data make it clear that modulating T cells can be an effective therapeutic strategy. Substantial evidence indicates a correlation between T cell functional activity and TCR interaction with the peptide/MHC complex, and that alterations

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in peptide contact residues with either MHC or TCR can lead to dramatically different functional outcomes [2]. Therefore, to understand, at the most basic level, how autoimmunity might then be held in check by manipulating T cells is of utmost importance.

We have previously shown that immunization of H-2^a mice with type II collagen (CII) induces polyarthritis known as collagen-induced arthritis (CIA) mediated by B and T cell response to CII, and that the core immunodominant determinant presented to murine T-cells by I-A^q is within the CII region between amino acids 260 and 270 (IAGFKGEQGPK) [3]. Based on these data, we developed an analog peptide, designated A9, containing substitutions at positions 260 (I->A), 261 (A->hydroxyproline), and 263 (F->N), that could profoundly suppress immunity to CII and the development of arthritis in DBA/1 (I-A^q) mice. Subsequent studies have demonstrated that amino acids 260 and 263 within this peptide are the p1 and p4 binding residues for this MHC molecule [4]. Additionally, we have shown that this analog peptide binds very weakly to I-A^q and induces T cell signaling via an alternative pathway which is dependent upon the non-canonical signaling molecules FcR γ and Syk [5; 6], and induces T cells to secrete immunoregulatory cytokines [6]. Yet it remains unclear whether the affinity of peptide interaction with MHC influences this outcome.

To this end, we used a panel of altered peptide ligands (APL) of CII, based on the sequence of the A9 peptide that differ at amino acids known to be important for MHC binding. We have taken advantage of the fact that class II molecules have four major binding pockets located within the peptide binding groove, each of which is capable of interacting with the antigenic peptide. We designed peptides in which substitutions were made to affect the interaction with the putative binding pockets at the p6 and/or p9 binding positions, replaced with the homologous binding residues of hen egg lysozyme (HEL₇₄₋₈₈) peptide, since HEL also binds to I-A^q [4; 7]. Each APL was tested for its ability to bind to I-A^q using a competitive binding assay and for *in vivo* effects on collagen-induced arthritis (CIA), together with cytokine production and molecular T cell signaling responses. These studies give us insights into what drives T-cells to signal through alternate pathways ultimately leading to new therapeutic approaches to human diseases.

Methods

Preparation of Tissue Derived CII and Synthetic Peptides

Native CII was solubilized from fetal calf articular cartilage by limited pepsin-digestion and purified as described earlier [8]. The purified collagen was dissolved in cold 10 mM acetic acid at 4 mg/ml and stored frozen at -70°C until used. The synthetic peptides were supplied by Biomolecules Midwest Inc. (Waterloo IL). A peptide representing the immunodominant determinant of both human and bovine CII (CII₂₅₆₋₂₇₅), (GEBGIAGFKGEQGPKGEBGP), where B stands for 4-hydroxyproline is designated peptide A2 or wild type (WT), and a synthetic peptide representing the following sequence (GEBGABGNKGEQGPKGEBGP) is designated A9. The sequences of other synthetic peptides which represent variations of these peptides are described in Table 1.

Preparation of I-A^q:IgG2a constructs

In order to develop a peptide/MHC binding assay, soluble I-A^q:IgG2a Fc fusion proteins were produced, based on a design described previously by Vignali and colleagues [9] with modifications to the leucine zipper peptide and the linker peptides between I-A^q and the leucine zipper. For generating the chimeric I-A^q α chain construct, a 692 bp cDNA fragment containing the native leader sequence and extracellular domain of the I-A^q α chain, was PCR-amplified from a plasmid (pKSV) that contains full-length sequence of the I-A^q α chain. The primers used for the PCR amplification were: A α Q-sense, 5'-GATCGAATTTCGCGGCCGAGACCTCCCGGAGACCAGG-3', A α Q-antisense, 5'-AGTTTCCGTCAGCTCTGACATGGG-3'. A 105 bp cDNA containing a short linker (TTAPS) and a leucine zipper sequence was obtained by PCR using plasmid pRmHA3-A α Q-leuZ-Tet as a template. The resultant cDNA was then linked to the 3'-terminus of the A α Q by recombination PCR and the chimeric cDNA cloned into the pCR2.1-Topo vector (Invitrogen, Carlsbad, CA). For attaching the murine IgG2a Fc fragment to the A α Q-leuZ peptide and cloning the chimeric I-A^q α chain into a drosophila cell expression vector, the pCR2.1-A α Q-LeuZ DNA was digested with *EcoRI/AscI* and a 796 bp cDNA fragment was gel-purified. A construct pMT-H-2a α /E α ^k, plasmid (kindly provided by Dr. Dario Vignali from St. Jude Children's research hospital, Memphis, TN) was digested with *EcoRI/AscI* and separated on a 1 % agarose gel. A fragment that contained the vector portion and a murine IgG2a Fc fragment was gel-purified and used to ligate to the *EcoRI/AscI* fragment of the A α Q-LeuZ to create pMT-A α Q-leuZ-mG2a construct. To generate the A β q chimeric construct, a 682 bp cDNA containing the leader and extracellular domain of the I-A^q β chain was amplified using full-length I-A^q β chain DNA (pKSV-IA^q- β) as template. The primers used for the PCR amplification were: A β Q-sense, 5'-GATCGAATTCTGCATGGCTCTGCAGATCCCCAGC-3', A β Q antisense, 5'-CTTCGATCGGGCAGACTCGGACTG-3'. The second recombinant PCR was performed to link the resultant A β q with a 219 bp of chimeric cDNA that was PCR amplified from pRmHA3-A β q-leuZ-bio-flag and consisted of a leucine zipper peptide, a flexible linker (RGGASGG), a biotinylation sequence and a flag-tag sequence. The resultant chimeric A β Q:LeuZ:biotin:flag cDNA was then digested with *EcoRI/XhoI* and subcloned into the same sites of pMT-V5 vector (Invitrogen, Carlsbad, CA) to product pMT-A β Q-LeuZ-bio-flag construct. Both A α Q and A β Q constructs were verified by DNA sequencing.

Production and purification of I-A^q:IgG2a Fc fusion protein

Drosophila melanogaster S2 cells were grown in Schneider's *Drosophila* medium (Gibco/BRL, Grand Island, NY) containing 10 % heat-inactivated fetal bovine serum, 5 U/ml of penicillin/5 μ g/ml of streptomycin at 26°C to a density of 2–4 $\times 10^6$ cells/ml. The S2 cells (1×10^7) were co-transfected with pMT-A α Q-leuZ-mG2a and pMT-A α Q-LeuZ-bio-flag constructs along with a selection plasmid pCoBlast using a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA). Three days after transfection, the cells were selected in the culture medium containing 25 μ g/ml of Blasticidin (Invitrogen, Carlsbad, CA) for 2 weeks. After selection, the transfected S2 cells were expanded to a density of 0.5–1 $\times 10^7$ /ml and soluble I-A^q protein was induced by addition of 1 mM CuSO₄. Five days later the culture supernatants was collected and adjusted to 0.05% octyl glucoside (OcG). Protein production was monitored by ELISA using anti-mouse IgG2aFc (Clone R11–89, BD PharMingen, San

Diego, CA) as the capture antibody and biotin anti-mouse IgG2aFc (Clone R19-15, BD PharMingen), which recognizes a different epitope, as the developing antibody. Soluble I-A^q: IgG2aFc fusion protein was purified by passage of the supernatant over a recombinant protein A column (rProtein-A-sepharose, Amersham Pharmacia, Piscataway, NJ). The column was washed with 0.1 M phosphate buffer (PB), pH 8.0, followed by elution with 0.1 M citrate buffer (CB), pH 3.5, and the fraction were immediately neutralized with 0.2 M PB. The production was concentrated using an Amicon Stirred Cell (Amicon, Beverly, MA) and quantitated by OD 280 absorption prior to use in the MHC binding assay.

MHC binding assay

A competitive inhibition of binding assay using solubilized MHC:IgG2aFc fusion proteins was performed as we have previously described [9; 10]. Briefly, soluble I-A^q: IgG2aFc fusion proteins were produced and incubated with the appropriate biotinylated HEL_{74–88} peptide in the presence of varying concentrations of competitor peptides at pH 6.5. Plates were probed for bound biotinylated peptide with streptavidin-europium followed by a chelating enhancement solution. Fluorescence was quantitated using a Bio-Rad Fluormark Microplate Fluorometer utilizing a TR-EX filter with an excitation range of 260–350 and an emission filter of 615. The concentration of analog peptides inhibiting 50% of the biotinylated HEL_{74–88} peptide (IC₅₀) was calculated from the linear portion of the curve. IC₅₀ values represent the average of two determinations per peptide. These studies allowed us to categorize the relative affinity of the peptides as either high, intermediate, or weak binders to I-A^q based on IC₅₀ values.

Animals

DBA/1 mice were obtained from the Jackson Laboratories and raised in our animal facility. Mice transgenic for a CII-specific TCR-V α 11.1/V β 8.3 were established and bred in the animal core facility of the Rheumatic Diseases Research Core Center, University of Tennessee as described previously [3]. These animals are referred to as DBA^{qCII24}. Another strain of mice, genetically deficient in the FcR γ -chain has been bred onto the DBA/1 background for 12 generations [11; 12; 13]. In some experiments these mice were intercrossed with qCII24 to produce a new strain expressing the TCR transgene, but deficient in the production of the FcR γ -chain.

All mice were fed standard rodent chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum. The environment was specific pathogen-free for and sentinel mice were routinely tested for mouse for a panel of mouse pathogens. All animals were kept until the age of 7–10 weeks before being used for experiments, which were conducted in accordance with approved IACUC protocols.

CD4⁺ T cell isolation and activation

Spleens were collected from TCR transgenic mice and the single-cell suspension was prepared by mechanical disruption in complete DMEM medium (DMEM supplemented with 10 % FCS, 100 IU/ml of penicillin, 100 μ g/ml streptomycin, 2.5 μ M β -mercaptoethanol and 2 mM L-glutamine). CD4⁺ naive T cells were isolated using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, Ca) using a negative selection protocol. The purity of the

recovered CD4⁺ T cells was determined by flow cytometry after staining with anti-CD4⁺ mAb and were >95% pure. Cells were cultured for varying lengths of time (30 seconds to 60 minutes) with APC (I-A^q-positive splenocytes) which had been prepulsed with A2, A9, or other analog peptide(s). In some experiments, after a short culture period at 37°C, the cells were collected, lysed, and insoluble materials were removed by centrifugation at 10,000 x g at 4°C for 15 minutes.

Flow cytometric detection of intracellular phospho-proteins in qCII24 T cells

The magnetic purified CD4⁺ T cells were stimulated with peptide-prepulsed antigen presenting cells for five minutes. Cells were fixed with 1% formaldehyde and permeabilized with methanol. The fluorescent-conjugated phospho-specific antibodies as well as antibodies to T cell surface markers (CD3, CD4 and TCR-β) were added to the cell preparations and incubated at room temperature for 1 hour. The intracellular phosphorylation-state was analyzed on a FACS Calibur flow cytometer (Becton Dickinson), gating on CD4⁺ T cells, using CellQuest and FlowJo software.

Analysis of protein phosphorylation

Lysates of whole cells were separated using SDS-PAGE gels and electrotransferred onto nitrocellulose membranes. After transfer, the membrane was blocked in tris buffered saline (TBS) containing 3% No-fat dry milk for 2 hours and incubated overnight with phospho specific antibodies in TBS-Tween 20 /3% milk. The membrane was then incubated with a secondary antibody (Amersham) for 1 hour and subjected to Enhanced Chemiluminescence (ECL) detection (ECL Western Blot kit, Amersham) according to the manufacturer's protocol. To detect protein levels, the membranes were re-stripped and blocked with 3% no-fat milk, incubated with anti-pan antibodies and then analyzed by ECL.

Reagents

All antibodies, including the anti-phospho specific antibodies that recognize Erk, P38, JNK/SAPK, Zap-70 and Syk were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Measurement of Cytokines

To measure cytokines, inguinal lymph node cells or spleen cells were cultured (5 X 10⁵ CD4⁺ T cells/ml) with wild type APC's (I-A^q-positive splenocytes) (1:2 ratio) which had been prepulsed with differing concentrations of the various peptides (A2, A9, or other variants). (Each cell population was confirmed by flow cytometry to have > 95% purity). Supernatants were collected at 72 hours and analyzed for the presence of IL-4, IL-10, IL-2, INFγ, and IL-17 using a Bio-plex mouse cytokine assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Values are expressed as picograms per ml and represent the mean values for each group.

Immunization

CII was dissolved in 0.01 M acetic acid (4 mg/ml) and emulsified with complete freunds' adjuvant (CFA) containing 4 mg/ml of *M. tuberculosis* (H37 Ra strain) (Difco Microbiology

Products, Becton Dickinson, NJ, USA) [14]. Each mouse received 100 µg of CII emulsified in CFA subcutaneously at the base of the tail in order to induce arthritis.

Measurement of the Incidence and Severity of Arthritis

The severity of arthritis was determined by visually examining each limb and scoring them on a scale of 0–4 as described previously [8]. Scoring was conducted by an examiner who was unaware of the identity of the treatment groups. Each mouse was scored thrice weekly beginning three weeks post immunization and continuing for 8 weeks. The mean severity score (sum of the severity scores for the group on each day /total number of animals in the group) was recorded at each time point.

Statistical Analysis

Mean severity scores, and cytokine levels were compared using the Mann-Whitney test.

Results

Binding affinity of analog peptides for I-A^q

In order to determine how peptide interaction with MHC leads to different T cell outcomes, we designed variants of the A9 peptide, taking advantage of the fact that class II molecules have 4 major binding pockets located within the peptide binding groove. Two of the amino acids that give A9 its unique properties are involved in MHC (I-A^q) binding, residues 260 and 263, and most likely extend into the p1 and p4 I-A^q binding pockets respectively, as determined by our previous studies (Figure 1) [4] and predicted from crystal structures of other murine I-A molecules [15]. Therefore, we designed peptides in which additional substitutions were introduced into the peptide amino acid positions predicted to interact with peptide binding pockets p6 and p9 [4; 15], namely amino acids at positions 265 and 268 in the CII immunodominant epitope. These amino acids in the wild type CII peptide are both Gly and therefore unable to contribute to corresponding binding pocket interactions beyond their main chains. To enhance the affinity of an analog CII peptide, we chose amino substitutions based on the homologous amino acids of hen egg lysozyme (HEL_{74–88}), since HEL also binds to I-A^q [4; 7; 15; 16; 17]. These substitutions allowed the generation of APLs based on the sequence of CII but with varying affinities for the I-A^q (Figure 1).

To measure the relative affinity of the analog peptides for I-A^q, a soluble I-A^q: IgG2aFc fusion protein-peptide binding assay was used in which various concentrations of the analog peptides were tested for their ability to compete with biotinylated HEL_{74–88} for binding to I-A^q. As shown in Figure 1, amino acid substitutions at positions 265 (Gly → A), and 268 (Gly → Ala) resulted in peptides that had the weakest binding to I-A^q. On the other hand a Ser substitution at position 268 enhanced the binding of the analog in comparison to A9, but is still lower than the relative affinity of the wild type peptide in binding to I-A^q. Although a larger panel of peptides was initially tested, two peptides were selected for further study because they demonstrated variations in MHC binding affinities that were less than A2 but either greater than or equal to that of A9. These peptides were used to correlate peptide affinity to MHC with function.

Function of APLs *in vivo* in suppressing immunity to CII and development of arthritis

In order to determine which peptides give the most effective suppression of arthritis, analog peptides shown by the preceding experiments to have differing affinities were evaluated *in vivo* to test our hypothesis that low avidity to MHC correlates with an active immunosuppression. Groups of 10 DBA/1 mice were immunized with CII/CFA, and observed for the appearance of arthritis. After arthritis had clearly developed, each mouse was treated with analog, A9, A9AA, or A9S peptide and evaluated over time to determine the severity of arthritis.

As we anticipated, CII-induced arthritis was significantly suppressed when DBA/1 mice were treated with the A9 analog peptides when compared to mice treated with a control antigen (Ova, Figure 2). Similarly, mice treated with the peptide A9AA, which had a low binding affinity similar to A9, had suppression of arthritis. On the other hand, peptide A9S, which had an intermediate relative binding affinity to I-A^q, had only a partial effect in attenuating the severity of collagen-induced arthritis (Figure 2). These data demonstrate that the two peptides that have the lowest affinity for the arthritis susceptibility allele I-A^q were the most effective, while the peptide with a stronger binding avidity to I-A^q was less effective.

Activation of intracellular substrates in T cells following stimulation by analog peptides

We have previously established that T cells from mice immunized with CII and treated with A9 use an alternative to the canonical TCR signaling pathway. T cells from these mice use an intracellular activation pathway in which Syk functions to initiate T cell functions via the TCR instead of Zap-70 [5; 6]. Utilization of this pathway, results in the secretion of immunoregulatory cytokines that function to downregulate CIA. Therefore we wanted to determine whether T cell activation by peptides A9AA and A9S induced the canonical or the alternate T cell pathway. In these studies we used the DBA/1^{qCII24} mouse which is transgenic for a TCR specific for the immunodominant epitope of CII and is expressed on the majority of naïve T cells [18]. These T cells can be activated *in vitro* without prior *in vivo* priming and can be isolated in large numbers. To examine the activation patterns of tyrosine phosphorylated intracellular substrates, the CD4⁺ cells from the transgenic mice were cultured with I-A^q positive splenocytes pre-pulsed with either a peptide representing the immunodominant determinant of CII (A2), A9, A9AA or A9S. As shown in Figure 3, I-A^q presentation of the peptides induced striking differences in the patterns of protein phosphorylation among the peptides. Presentation of the A2 peptide to these T cells activates the canonical pathway, specifically Zap-70 followed by activation of the mitogen activated protein kinases (MAPKs) Erk-1/2. On the other hand, I-A^q presentation of A9 activates Syk but not Zap-70, and there is no phosphorylation of Erk-1/2. We also found an increase in the phosphorylation of Syk, upon stimulation with A9AA and A9S (Figure 3). However, stimulation of the CII T cells with A9S also partially activated the canonical pathway as evidenced by the presence of pZap-70 and pErk1/2. Taken together, these data show that the affinity of peptide binding to I-A^q directly correlates with triggering of either the canonical or the alternate pathway following activation of the T cell receptor.

Cytokine Responses induced by Analog Peptides

We have previously used IL-4 and IL-10 knockout mice to determine that both IL-4 and IL-10 play critical roles in the APL-induced suppression of arthritis [19; 20]. To determine how cytokine production is affected by each of our new analog peptides, we measured cytokines using T-cells from CII-immune mice. As shown in Figure 4, T cells that were stimulated with A2 secreted a full spectrum of Th1, Th2, and Th-17 cytokines (IFN- γ , IL-2, IL-10, and IL-17, respectively). On the other hand, T cells stimulated with the low affinity A9AA or A9 peptides produce a predominantly Th2 cytokines, IL-4 and IL-10, and no Th1 or Th17 cytokines. Conversely, A9S, which binds with a higher affinity than A9 or A9AA, stimulated a mixture of Th2 and Th17 cytokines. This peptide induced both of the production of IL-4 and IL-10 as well as an intermediate IL-17 and IL-2 response, but did not induce the Th1 cytokine IFN- γ (Figure 4). These data are consistent with our prior observation that A9S induced only an intermediate level of suppression of arthritis compared to A9 and A9AA.

T cell Activation of Syk requires FcR γ

A9-immune T cells require FcR γ to suppress collagen-induced arthritis[21]. To determine whether activation with A9AA or A9S required FcR γ , we used FcR $\gamma^{-/-}$ CD4 $^{+}$ T cells that were stimulated for various times and compared the results to those obtained using FcR $\gamma^{+/+}$ cells. Western blot analysis of signaling molecules (Figure 5) revealed a marked increase in the phosphorylation of Syk, upon A9AA/I-A q and A9S activation of T cells from DBA/1 qCII24 FcR $\gamma^{+/+}$ cells. In contrast, no significant activation was detected in the phosphorylation of the corresponding molecules in the lysates of T cells from DBA/1 qCII24 FcR $\gamma^{-/-}$ mice. The same FcR $\gamma^{-/-}$ T cells, however, successfully activated Zap-70 when stimulated by A2 or A9S, results comparable to those of T cells obtained from mice sufficient in FcR $\gamma^{+/+}$. Taken together, these data show that FcR γ is required for Syk activation by A9, A9AA, or A9S when presented by I-A q but it plays no role in the canonical pathway triggered by A2/I-A q .

Discussion

The purpose of this study was to establish whether affinity of peptide interaction with the MHC correlates with T cells signaling through the alternative pathway. By using altered peptide ligands with varying affinities of interaction with the MHC we established that peptide/MHC affinity does correlate with TCR signaling, i.e. ZAP-70/ ζ or Syk/FcR γ . These differences lead to two fundamentally different outcomes: high affinity peptides correlated with induction of the canonical signaling pathway and secretion of inflammatory cytokines while low affinity analog peptides correlated with the induction of the alternate signaling pathway and induction of suppressive cytokines).

Peptide binding motifs for class II MHC molecules have been reported and the contributions of specific binding pockets appear to play a critical role in the selection of epitopes [15; 22; 23]. Most, if not all class II binding motifs are P1, P4, P6, and P9 pocket-based. We have previously reported that the A9 peptide, which contains amino acid substitutions at the P1 and P4 positions has a very weak binding affinity for I-A q [6]. In this study, we used a

competitive inhibition assay using soluble recombinant class II molecule and a panel of analog peptides to design peptides in which the A9 substitutions were held constant while changes were introduced into the residues which interact with the other two binding pockets not involved in the A9 substitutions, namely P6 and P9. By altering amino acid residues 265 and 268 using homologous residues of hen egg lysozyme (HEL₇₄₋₈₈) peptide, we generated APLs with varying affinities for I-A^g. We report that alterations in the affinity of binding of the peptides clearly correlate with selection of the T cell signaling pathway.

Krishnan and coworkers previously observed that Syk and FcR γ were up-regulated in human effector CD4⁺ T cells which had been activated by antibody to CD3 in the presence of activated monocytes [24]. Moreover, these molecules coimmunoprecipitated with antibody to TCR or CD3 and could transduce signals through an alternate TCR signaling complex that contained CD3 ϵ , FcR γ and the proximal Syk kinase. The significance of this alternate signaling pathway was corroborated by the finding that in T cells from patients with Systemic Lupus Erythematosus (SLE) there is higher expression of Syk kinase compared with normal T cells and there is a greater association of Syk with key signaling molecules that shape T cell responses [25]. These findings suggested a novel role for both FcR γ and Syk in effector T cell signaling and suggested distinct signaling mechanisms that might play a role in autoimmunity, although the precise trigger for signaling through the alternate pathway remained unknown [25].

We do not fully understand the complexity of events that trigger autoimmune T-cell behaviors [26]. There is often something unusual about the interaction between an autoimmune TCR and its ligand. For example, the target peptide may bind weakly to MHC [27] or become truncated or modified [28]. It is believed these features prevent negative selection of thymocytes bearing the relevant TCR in the thymus, allowing their escape to drive autoimmunity. It behooves us, therefore, to understand, at the most basic level, how antigen recognition by T cells is achieved and how autoimmunity might be modified.

We propose that biased agonism plays a role in the differential signaling induced by A2 in comparison to A9 or the other analogs described in this study [29]. Traditional pharmacology has posited the dogma that a receptor will always induce the same second messengers and that variations are based on either full, partial, or inverse stimulation of the receptor by its ligand. More recently, other investigators have introduced the concept of biased agonism, based on the idea that there can be ligand-dependent selectivity for certain signal transduction pathways in one and the same receptor [29]. This occurs if a receptor has several possible signal transduction pathways. The determining factor as to which pathway is activated is dependent upon which ligand binds to the receptor. We believe that affinity of peptide binding to MHC can play a role in the differential signaling induced by A2 in comparison to A9 or the other analogs described in this study [29].

We recognize that changes in MHC pockets might also have a significant impact on neighboring TCR contact residues. There are several studies showing that changes in anchor residues impact TCR binding. For example Allen and coworkers showed that a critical substitution at a MHC contact residue (P6 Glu to Asp) next to a major TCR contact (P5 Asn) was able to significantly alter TCR recognition without changing MHC affinity [30]. The P6

Glu peptide was a weak agonist, requiring ~10,000-fold higher concentrations than the native Hb peptide for IL-2 production and a crystal structure confirmed that the P6 Glu was indeed located in a pocket of the MHC protein. Although our data show strong correlations between affinity of binding and TCR signaling, we cannot rule out the possibility that the changes we introduced into A9AA and A9S influenced neighboring TCR contact residues as well. Moreover, our predicted I-A^g binding pattern of P1, P4, P6, and P9 was based on our study utilizing a soluble I-A binding assay and clonally distinct CII-specific T cells to identify the residues of the immunodominant collagen peptide that bind to I-A^g [4]. However, we cannot rule out the possibility that P7 may also play a role in peptide binding to I-A^g as some investigators have suggested [31]. More work will be needed to completely understand the molecular interactions.

Inhibitory mechanisms clearly exist *in vivo* and affect therapeutic outcomes. For example, genetically modified lymphocytes expressing antigen-specific T cell receptors that recognize tumor cells [32] can unexpectedly be less potent than lower affinity counterparts if inhibitory mechanisms are triggered *in vivo*. In order to efficiently modulate autoimmunity, it is necessary to understand how TCRs are influenced by peptide structures they might encounter *in vivo* [33; 34; 35]. Such uncertainties need to be resolved for us to fully understand and manipulate, perhaps via vaccines, T cell immune responses.

Conclusions

In conclusion, these results demonstrate that it is possible to modulate T cell function *in vivo* by manipulating the affinity of peptide binding to the MHC. We propose a therapeutic strategy in which peptides are designed to have low affinity with the MHC. We believe that carefully designed peptides which bind to the MHC with low avidity can influence autoreactive T cells to become inhibitory and counteract inflammation. We propose that the avidity of interaction of peptide with MHC plays a significant role in determining which signaling pathway the T cell takes, i.e. whether it becomes primarily inflammatory or whether it becomes inhibitory, signaling via the alternate Syk/FcR γ pathway. Although our studies answer important questions as to the role MHC/peptide/TCR avidities play in determining T cell function, further investigations into optimizing TCRs interactions with their ligands may improve therapeutic outcomes. Ultimately these studies will lead to safer peptide-based therapies for autoimmune diseases.

Acknowledgments

This work was supported, in part, by USPHS Grants AR-55661, AR064825 and program-directed funds from the Department of Veterans Affairs and the Arthritis Foundation.

List of Abbreviations

CII	Type II collagen
RA	rheumatoid arthritis
MHC	major histocompatibility complex

APC	antigen presenting cell
CIA	collagen induced arthritis
TCR	T cell receptor
APL	altered peptide ligand
B	hydroxyproline
tg	transgene

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Highlights

1. We propose a therapy in which peptides induce inhibitory T cells.
2. The avidity of interaction of peptide with MHC determines the T cell signaling pathway.
3. These studies will lead to safer peptide-based therapies for autoimmunity.

Figure 1.

Model of CII immunodominant determinant interaction with I-A^q and T cell receptor, (Upper panel). Residues involved in peptide binding to I-A^q identified using binding assays and residues interacting with the TCR identified by antigen presentation studies as previously described [4]. This model was used as the basis for the design of analog peptides in Table 1.

(Lower panel) Concentration of analog peptides of CII₂₅₆₋₂₇₅ required to prevent binding of HEL₇₄₋₈₈ to purified I-A^q. Analog peptides and biotinylated HEL₇₄₋₈₈ were incubated with affinity purified I-A^q; IgG2aFc fusion protein and the quantity of biotinylated HEL₇₄₋₈₈ bound determined as described in Materials and Methods. Data are expressed as the percentage of biotinylated HEL₇₄₋₈₈ bound in the absence of competitor and are

representative of at least three experiments. For clarity, binding patterns for only four peptides are displayed, as they were selected for in vivo testing.

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Figure 2.

Treatment of established CIA using APL. DBA/1 mice were immunized with 100 μ g of CII emulsified in CFA for the induction of arthritis (n= 10 mice per group). Treatment with the peptides was initiated on day 25 after immunization by a single intravenous injection with 0.3mg of either A9, A9AA or A9S. The severity of disease was determined by visual scoring as described in Materials and Methods. Control mice treated with ovalbumin showed progression of disease while those treated with A9S (closed circles) had slower progression, and both A9AA (closed triangles) and A9 (closed diamonds) had the slowest progression. Mean severity of both the A9AA and A9 groups was less than that of the control OVA on day 49 through 55 ($p < 0.001$). The arthritis severity in A9S treated mice differed from the Ova controls and the A9 and A9AA peptide treated mice ($p = 0.05$) on days 49 through 55. In the treatment protocol, the final incidence was 80% for mice treated with A9AA, A9, or A9S and 90% for the control mice. The data shown are representative of data obtained from four separate experiments, each with similar results. We have previously demonstrated that Ova-treated animals do not differ from untreated animals in severity and incidence scores [36].

Figure 3.

Activation of Zap-70, Syk and MAPKs by wild type A2 or analog peptides. Panel A: CD4 T cells from DBA/1^{qCII24} mice were activated by APCs prepulsed with 100 μ g/ml of A2, A9 or no peptide (none) for 5 min. Cell lysates were collected and subjected to gel electrophoresis followed by immunoblotting using specific antibodies as indicated in Materials and Methods. The data indicated are representative of data taken from three separate experiments. These data confirm that activation of T cells by antigen presenting cells expressing I-A^q/A9 results in activation of Syk while activation by I-A^q/A2 results in activation of ZAP-70 and Erk1/2. Activation by I-A^q/A9AA resulted in activation of Syk only while activation by I-A^q/A9S resulted in activation of SYK as well as ZAP-70 and Erk1/2. As a control, stimulation of T cells with native CII induced signaling through the canonical pathway, identical to that induced by peptide A2 (results not shown). Panel B. Flow cytometry, using antibodies specific for phospho-syk, was performed with gating on CD4⁺ T cells as described in Material and Methods to confirm that the signaling changes were produced by CD4⁺ T cells.

Figure 4.

Spleen cells from DBA/1 mice immunized with CII respond differently to each of the analog peptides. Spleen cells from DBA/1 mice previously immunized with CII/CFA were harvested on day 10 after immunization and cultured with A2, A9, A9AA, or A9S as described in Materials and Methods. The concentration of each cytokine in the supernatant is expressed as μM and represents the mean ± SEM of 3 separate experiments. The Th2-type responses do not differ among the four peptides. However, there are marked differences in IFN-γ compared to IL-17 and IL-2 production. IFN-γ responses to A2 are robust while very little IFN-γ is produced by A9, A9AA or A9S stimulation of the T cells. On the other hand both the IL-17 and IL-2 responses to A2 were robust but only a minimal response to A9 and A9AA stimulation was detected. The IL-17 response differed significantly between A9 and A9S at 30μM ($p < 0.001$). Similarly, the IL-2 response differed significantly between A9 and A9S at 30μM ($p < 0.005$). The cytokine profile induced by native CII was similar to that induced by peptide A2 (data not shown)

Figure 5.

Culture of CD4⁺ T cells from DBA/1^{qcII24} mice with analog peptides results in differential activation of ZAP-70, Syk or Erk1/2 in mice deficient for FcRγ. CD4⁺ T cells were isolated from spleens of DBA/1^{qcII24} transgenic mice, either FcRγ^{+/+} or FcRγ^{-/-}, and cultured with wild type (FcRγ^{+/+}) APCs that had been pulsed with 200 μg/ml of A9, A9-AA, A9-S or no peptide (none). After 5 minutes cells were collected by centrifugation and whole cell lysates were removed and the proteins were separated by SDS-PAGE. The proteins were transferred onto PVDF membranes and analyzed for activated ZAP-70, Syk or Erk1/2 using anti-phospho ZAP-70 (Tyr493), anti-phospho Syk (Tyr525/526) or anti-phospho Erk1/2 antibodies. The membrane was stripped and re-blotted with non-phospho-specific antibody. CD4⁺ T cells from FcRγ^{+/+} mice responded to A9 with phosphorylation of Syk and p38. In contrast, T cells from FcRγ^{-/-} mice failed to phosphorylate either of these proteins. Cells from both FcRγ^{+/+} and FcRγ^{-/-} mice stimulated with A2 showed activation of, and Erk1/2, however, there was no phosphorylation of Syk. The data indicated are representative of data taken from three individual experiments. These data confirm that activation of T cells by antigen presenting cells expressing I-A^q/A9 results in activation of Syk. This activation is dependent upon the availability of FcRγ.

Table 1

Sequences of analog peptides.

Sequence *									
Analog Peptide	256	260	263	266	269	272	275		
A2	G	E	B	G	I	A	G	F	K
A9	---	---	A	B	---	N	---	---	---
A9S	---	---	A	B	---	N	---	S	---
A9AA	---	---	A	B	---	N	---	A	---
A9A	---	---	A	B	---	N	---	A	---
A9-A	---	---	A	B	---	N	---	A	---
A9-AS	---	---	A	B	---	N	---	A	---

* The letter "B" denotes hydroxyproline.