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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 FcRγ 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 cytokines and attenuation of arthritis.
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1. 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

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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effective therapeutic strategy. Substantial evidence indicates a correlation between T cell functional activity and TCR interaction with the peptide/MHC complex, and that alterations 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^q 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 FcRγ 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 ($HEL_{74–88}$) 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.

2. Methods

2.1. 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 ($CII_{256–275}$), (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.

2.2. 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: AαQ-sense, 5′-GATCGAATTCGCGGCCGCAGAGACCTCCCGGAGACCAGG-3′, AαQ-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-AαQ-leuZ-Tet as a template. The resultant cDNA was then linked to the 3′-terminus of the AαQ 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 AαQ-leuZ peptide and cloning the chimeric I-A^q α chain into a drosophila cell expression vector, the pCR2.1-Aaq-LeuZ DNA was digested with *EcoRI*/*AscI* and a 796 bp cDNA fragment was gel-purified. A construct pMT-H-2aα/Eα^k, plasmid (kindly provided by Dr. Dario Vignali from St. Jude Childrens' 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 AαQ-LeuZ to create pMT-AαQ-leuZ-mG2a construct. To generate the Aβq 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: AβQ-sense, 5′-GATCGAATTCTGCATGGCTCTGCAGATCCCCAGC-3′, AβQ antisense, 5′-CTTCGATCGGGCAGACTCGGACTG-3′. The second recombinant PCR was performed to link the resultant Aβq with a 219 bp of chimeric cDNA that was PCR amplified from pRmHA3-Aβq-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

Table 1 Sequences of analog peptides.

	Sequence [a]
Analog peptide	256 260 263 266 269 272 275
A2	GEBGIAGFKGEQGPKGEBGP
A9	———AB–N————————
A9S	———AB–N———S————
A9AA	———AB–N–A––A————
A9A	———AB–N–A—————
A9-A	———AB–N———A————
A9-AS	———AB–N–A––S————

[a] The letter "B" denotes hydroxyproline.

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