Molecular Basis for T Cell Response Induced by Altered Peptide Ligand of Type II Collagen*

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Background: Alterations in peptide-MHC interactions with the T cell receptor (TCR) can lead to dramatically different T cell outcomes. We have developed an altered peptide ligand of type II collagen, referred to as A9, which differentially regulates TCR signaling in murine T cells leading to suppression of arthritis in the experimental model of collagen-induced arthritis. This study delineates the T cell signaling pathway used by T cells stimulated by the A9-I-Aq complex. We have found that T cells activated by A9 bypass the requirement for Zap-70 and CD3-ζ and signal via FcRγ and Syk. Using collagen-specific T cell hybridomas engineered to overexpress either Syk, Zap-70, TCR-FcRγ, or CD3-ζ, we demonstrate that A9-I-Aq preferentially activates FcRγ/Syk but not CD3-ζ/Zap-70. Moreover, a genetic absence of Syk or FcRγ significantly reduces the altered peptide ligand induction of the nuclear factor GATA3. By dissecting the molecular mechanism of A9-induced T cell signaling, we have defined a new alternate pathway.

Results: We have found that T cells activated by A9 signal via FcRγ and Syk.

Conclusion: By dissecting the molecular mechanism of A9-induced T cell signaling, we have defined a new alternate pathway.

Significance: Understanding this pathway may be critical for the proper application of Syk inhibitors to RA therapies.

Mounting evidence from animal models has demonstrated that alterations in peptide-MHC interactions with the T cell receptor (TCR) can lead to dramatically different T cell outcomes. We have developed an altered peptide ligand of type II collagen, referred to as A9, which differentially regulates TCR signaling in murine T cells leading to suppression of arthritis in the experimental model of collagen-induced arthritis. This study delineates the T cell signaling pathway used by T cells stimulated by the A9-I-Aq complex. We have found that T cells activated by A9 bypass the requirement for Zap-70 and CD3-ζ and signal via FcRγ and Syk. Using collagen-specific T cell hybridomas engineered to overexpress either Syk, Zap-70, TCR-FcRγ, or CD3-ζ, we demonstrate that A9-I-Aq preferentially activates FcRγ/Syk but not CD3-ζ/Zap-70. Moreover, a genetic absence of Syk or FcRγ significantly reduces the altered peptide ligand induction of the nuclear factor GATA3. By dissecting the molecular mechanism of A9-induced T cell signaling, we have defined a new alternate pathway that is dependent upon FcRγ and Syk to secrete immunoregulatory cytokines. Given the interest in using Syk inhibitors to treat patients with rheumatoid arthritis, understanding this pathway may be critical for the proper application of this therapy.

Although the canonical T cell receptor (TCR) signaling pathway has been intensively studied (1), less is known about TCR signaling that occurs in more complex biological contexts, such as when T cells are triggered by an altered peptide ligand. Although it is believed that peptides which interact strongly with the TCRs of naive CD4 T cells favor Th1 and Th17 cell differentiation and low affinity peptide-MHC/TCR interactions favor Th2 differentiation, it remains unclear precisely how weak TCR interactions affect TCR signaling (2). In altered peptide ligand (APL)-stimulated cells, a lack of ζ chain-associated protein kinase (Zap) 70 activation is believed to be critical for the generation of Th2 cytokines (2)). Moreover, it is believed that the strength of the TCR signal influences downstream signaling events, which in turn dictate transcription factor and cytokine gene transcription. Yet the precise pathway by which these events occur remains to be unraveled.

We have developed an analog of type II collagen (CII) referred to as A9, that differentially regulates inducible TCR signaling in murine T cells (3). A9 is an APL of the immunodominant epitope contained in CII (CII245–270) with substitutions made at amino acid residues 260 (alanine for isoleucine), 261 (hydroxyproline for alanine), and 263 (asparagine for phenylalanine). We have previously shown that A9 profoundly suppresses immunity to CII in the murine arthritis model of collagen-induced arthritis (4) by inducing suppressive cytokines and that it binds very weakly to murine I-Aq (4). Using T cells from mice that have a genetic deletion of FcRγ or Syk, we have studied the role of A9-induced T cell signaling. We found that A9 induces TCR activation that is mediated by FcRγ and Syk to produce Th2-type cytokines. We have determined the Syk and Zap-70 are not redundant but display clear functional differences in T cells. Given that T cells can express Syk (5) and that there is a significant interest in using Syk inhibitors to treat human patients who have rheumatoid arthritis (RA) (6), this pathway should be taken into account when applying such findings to biological phenomenon.
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**EXPERIMENTAL PROCEDURES**

*Preparation of Tissue-derived CII—*Native CII was solubilized from fetal calf articular cartilage by limited pepsin-digestion and purified as described earlier (7). The purified collagen was dissolved in cold 20 mM acetic acid at 4 mg/ml and stored frozen at −70 °C until used. The synthetic peptides were purchased from Biomolecules Midwest Inc. (Waterloo, IL).

*Animals—*DBA/1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice transgenic for a CII-specific TCR-V$\alpha$11.1/V$\beta$8.3 were established and bred in the animal core facility of the Rheumatic Diseases Research Core Center, University of Tennessee as described previously (8). All mice were maintained in groups of six in polycarbonate cages and fed standard rodent chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum. The mouse facility had routine testing for mouse hepatitis and Sendai viruses.

Another strain of mice genetically deficient in the FcRγ chain has been bred onto the DBA/1 background for 12 generations (9–11). In some experiments these mice were intercrossed with DBA/1$^{CII\text{24}}$ to produce a new strain expressing the TCR transgene but deficient in the production of FcRγ. Heterozygous mice carrying a deleted Syk allele (Syktm1Tyb, which is referred to as Syk-) were obtained from Victor Tybulewicz (National Institute for Medical Research, London, UK). The mutation was backcrossed onto the DBA/1$^{CII\text{24}}$ tg strain having the DBA/1 genetic background as well as the collagen-specific TCR transgene and maintained in heterozygous (Syk$^{+/−}$) form. The TCR transgene-positive mice were identified by flow cytometric analysis of the expression of the Vb8 TCR.

*Bone Marrow Transplantation—*Bone marrow chimeras with the Syk$^{−/−}$ hematopoietic system were generated by fetal liver transplantation using fetuses from days 15.5–18.5 of embryogenesis (E15.5–18.5) that were obtained from timed matings of Syk$^{−/−}/$DBA/1qCII24 carriers. The 8–16-week-old DBA/1 recipient mice were lethally irradiated as described and then injected intravenously with unfractionated fetal liver cell suspensions. On average, fetal liver cells from a single donor were injected into 5–8 recipients. Syk$^{−/−}$/fetuses were lethally irradiated as described, and their genotype was confirmed by allele-specific PCR analysis. Fetal livers from DBA/1$^{CII\text{24}}$ Syk$^{+/−}$ mice were used to generate wild type control chimeras.

*Assessment of Chimerism—*Peripheral blood samples were taken 4–6 weeks after transplantation and stained with phycoerythrin-labeled antibodies against Vb8. For the analysis of Syk protein levels, total splenocytes were immunoblotted using anti-Syk (N-19; Santa Cruz Biotechnology) or anti β-actin (clone AC-74; Sigma) antibodies.

*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 μg/ml streptomycin, 2.5 μM β-mercaptoethanol, and 2 mM l-glutamine). CD4+ naïve 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-CD45 mAb and were >95% pure. Cells were cultured for varying lengths of time (30 s to 60 min or overnight) with APCs (I-A$^d$-positive splenocytes) that had been prepped with A2, A9, or other analog peptide(s). In some experiments, after a short culture period at 37 °C, the cells were collected and lysed in lysis buffer, and insoluble materials were removed by centrifugation at 10,000 × g at 4 °C for 15 min.

*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 h and incubated overnight with phospho-specific antibodies in TBS-Tween 20, 3% milk. The membrane was then incubated with a secondary antibody (Amersham Biosciences) for 1 h and subjected to Enhanced Chemiluminescence detection (ECL Western blot kit, Amersham Biosciences) according to the manufacturer’s protocol. To detect protein levels, the membranes were re-striped and blocked with 3% no-fat milk, incubated with anti-pan antibodies, and then analyzed by ECL.

The detection of intracellular phosphoproteins in T cells was carried out as follows. CD4+ T cells were purified by magnetic separation then stimulated with peptide-prepulsed antigen-presenting cells for differing time periods (1–60 min). Cells were fixed with 1% formaldehyde and permeabilized with methanol. Both fluorescent-conjugated phospho-specific antibodies and antibodies specific for T cell surface markers (CD3, CD4, and TCR-β) were added to the cells and incubated at room temperature for 1 h. Evaluation of the status of the intracellular phosphorylation was determined using CellQuest and FlowJo software after analysis with a Calibur flow cytometer (BD Biosciences).

*Reagents—*The peptide representing the immunodominant determinant of CII, ATGGLPKGQTGEBGIAGFKGEQPK (CII246–270), where B stands for 4-hydroxyproline, is designated peptide A2 (12). The APL containing three specific amino acid substitutions at positions 260, 261, and 263, CII245–270 (A260, B261, and N263), were chemically synthesized by a solid-phase procedure and purified by high performance liquid chromatography (13). Antibodies including anti-phospho-specific antibodies recognizing Erk, p38, JNK/SAPK, Zap-70, and Syk were developed as previously described (14). Briefly, the coding region of each was amplified by PCR using murine spleen cDNA as a template and then inserted into a pIRES-hrGFPII (Stratagene, La Jolla, CA) vector. T hybridoma cells expressing a collagen-reactive TCR were transfected with the construct vectors using Lipofectamine Plus transfection reagents. Stable transfectants were selected, and the resulting T cell lines were cultured with APCs. As a source of APCs, we transfected RAW264.7 cells with retroviral constructs coding for both the α and β chains of I-A$^d$. APCs were pulsed with saturating concentrations of A9, wild type peptide A2, or an irrelevant peptide overnight. To confirm functionality, CII-reactive T cell hy-
briodoma cells transfected with an empty control retroviral construct were cultured with peptide pulsed APCs (I-A<sup>+</sup>-expressing RAW 264.7 cells), and supernatants were analyzed for cytokine expression. As expected, culture with A2-pulsed APCs induced both Th1 cytokines (IL-2) and Th2 cytokines (IL-4), whereas culture with A9 peptide induced only Th2 cytokines (IL-4). An irrelevant peptide was unable to induce cytokines.

**Statistics**—Statistical analyses were performed using Student's t test.

**Measurement of Cytokines**—To measure cytokines, inguinal lymph node cells were cultured (5 × 10<sup>5</sup> CD4<sup>+</sup> T cells/ml) with wild type APCs (I-A<sup>+</sup>-positive splenocytes) (1:2 ratio) that had been prepulsed with 100 μg/ml concentrations of the various peptides (A2 or A9) (each cell population was confirmed by flow cytometry to have >95% purity). Supernatants were collected at 72 h 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) according to the manufacturer's protocol. Values are expressed as pg/ml and represent the mean values for each group.

**RESULTS**

**T Cell Stimulation by A9 Requires FcRγ to Activate Intracellular Substrates**—We have previously established that mice immunized with CII and treated with A9 use an alternative to the canonical T cell signaling pathway. T cells from these mice use an activation pathway in which Syk replaces Zap-70 to initiate T cell functions (3). Utilization of this pathway, instigated by A9-I-A<sup>+</sup>, results in the secretion of immunoregulatory cytokines that function to down-regulate CIA. It is known that, FcRγ shares structural similarities with CD3-ζ and under some circumstances can substitute for it (15). Therefore, we wanted to determine whether FcRγ activation was required for Syk to function in T cells. To execute these studies rapidly and efficiently, we used the DBA/1<sup>95C<sup>CL24</sup></sup> mouse (FcRγ<sup>++/−</sup>) or FcRγ-deficient mice (FcRγ<sup>−/−</sup>) that had been prepulsed with 200 μg/ml of A2 or A9 or no peptide for the indicated time period. The cells were harvested, and total RNA was purified using RNeasy Mini kit (Qiagen). cDNA was synthesized from 0.1 mg of total RNA in a 20-ml reaction volume at 37°C using reverse transcriptase (Invitrogen). T-bet or actin fragment was amplified using RT-PCR (2 ml cDNA in 20 ml reaction volume) using Tag polymerase (PCR conditions: Step 1, 95°C for 5 min; Step 2, 95°C for 30 s, 58°C for 3 s, 72°C for 30 s; Step 3, 72°C for 10 min; 4°C 1 h (T-bet: 35 cycle; actin, 20 cycle).

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As A9-I-A<sup>+</sup> stimulation induced the tyrosine phosphorylation of Syk rather than Zap-70, we asked whether this activation required FcRγ. To answer this question we used FcRγ<sup>−/−</sup>-CD4<sup>+</sup> T cells that were stimulated at varying times and compared the results to those obtained using FcRγ<sup>++/−</sup> cells. Examination by Western blots (Fig. 1) revealed a marked increase in the phosphorylation of Syk, JNK, and p38 upon A9-I-A<sup>+</sup> activation in DBA/1<sup>95C<sup>CL24</sup></sup> FcRγ<sup>++/−</sup> cells. In contrast, no major activation was detected in the phosphorylation of the corresponding molecules in the lysates of cells from DBA/1<sup>95C<sup>CL24</sup></sup> FcRγ<sup>−/−</sup> mice. The same FcRγ<sup>−/−</sup> T cells, however, successfully activated Zap-70, and all the MAPKs when stimulated by A2, giving results comparable with those of T cells obtained from mice sufficient in FcRγ<sup>++/−</sup>. Taken together these data show that FcRγ is required for Syk and MAPK activation by A9-I-A<sup>+</sup> but plays no role in the canonical pathway triggered by A2-I-A<sup>+</sup>.

**Expression of GATA3 and T-bet by A2 and A9**—A9-I-A<sup>+</sup> stimulation is the secretion of cytokines associated with the Th2 T cell phenotype (4). As the zinc finger transcription factor GATA3 is known to up-regulate the
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expression of Th2 cytokines in T cells, we examine the induction of GATA3 after A9-I-A^q stimulation. CD4^+ T cells from DBA/1^{qcII24} mice either sufficient or deficient in FcR\gamma were activated by APCs prepulsed with A2 or A9 for 1 h. When GATA3 expression was examined by Western blot analysis (Fig. 2), no GATA3 induction was detected in FcR\gamma^{-/-} CD4^+ T cells stimulated by A9, whereas the GATA3 induction by A2 remained intact. The FcR\gamma sufficient T cells showed GATA3 induction after stimulation with either peptide (Fig. 2A). These data confirm that FcR\gamma is required for GATA3 up-regulation by A9-I-A^q but plays no role in the GATA3 activation triggered by A2-I-A^q. In contrast, the absence of FcR\gamma had no effect on the induction of the transcription factor T-bet (the transcription factor that controls the expression of Th1 cytokines) when the T cells were stimulated by A2-I-A^q (Fig. 2B), whereas A9 did not induce T-bet regardless of whether T cells were FcR\gamma-sufficient or -deficient.

Activation of MAPKs by A2 and A9 in T Cells from Syk-Knock-out ("^/-") Chimeric Mice—Because we have demonstrated that A9-induced T cell activation is dependent upon FcR\gamma, we resolved to definitively establish the requirement for Syk in this alternative signaling pathway. Because a homozygous genetic deletion of syk is embryonic lethal, we developed bone marrow chimeras, generated by fetal liver transplantation, using fetal livers from DBA/1 mice containing both the qCl24 transgene and the Syk^{-/-} phenotype as a source of CD4^+ T cells. The Syk^{-/-} CD4^+ T cells were collected from chimeric spleens, and activated by APCs prepulsed with A2 or A9 for various time periods, and the cell lysates were collected and analyzed for activation of three MAPKs: JNK, p38, and Erk. As shown in Fig. 3, there was a marked increase in the phosphorylation of JNK and p38 upon A9-I-A^q activation in cells from DBA/1^{qcII24} Syk^{+/+} mice. By contrast, no major phosphorylation of the corresponding molecules in the lysates of cells from Syk^{-/-} mice was detected. On the other hand, the activation of MAPKs by A2 in CD4^+ T cells from Syk-deficient mice was comparable with that of cells from Syk-sufficient mice.

Induction of GATA-3 in Syk^{-/-} T Cells Stimulated by A2 and A9—Studies of the induction of GATA3 in cells from Syk-deficient mice closely followed the design of the experiments used to investigate FcR\gamma-deficient cells. The CD4^+ T cells from chimeric Syk^{+/+} or Syk^{-/-} DBA/1^{qcII24} mice were activated by APCs prepulsed with 200 \mu g/ml A2 or A9 or no peptide (no) for 5 or 20 min. Cells were harvested, and activation of MAPKs was examined with Western blot analysis. The data shown are representative of two separate experiments. The activation of JNK and p38 by A9 could not be detected in cells from Syk-deficient mice. In contrast, the activation of MAPKs by A2 in was comparable in both Syk-sufficient and -deficient mice.
Cytokines in Syk\(^{-/-}\) T Cells—We have demonstrated previously that the A9 APL induces T cells to secrete suppressive cytokines, specifically IL-4 and IL-10 (17, 18). Therefore, it was of interest to quantify cytokine secretion of T cells genetically deficient in Syk when responding to A2 and A9. The Syk\(^{-/-}\) mice were immunized with CII, and the draining lymph nodes were collected for in vitro studies to determine the response to both A2 and A9. As shown in Table 1, the supernatants from cultures of CD4\(^+\) cells from Syk\(^{-/-}\) mice that were cultured with A2 contained levels of Th1, Th17, and Th2 cytokines significantly greater than background. These values are similar to those obtained when T cells were cultured with Syk\(^{+/+}\) T cells. On the other hand supernatants from cells cultured with A9 failed to elicit Th1, Th2, or Th17 cytokines, confirming that Syk is required for the APL-induced T cell cytokine responses.

Protein Interactions between CD3-ζ, FcRγ, Zap-70, and Syk When Stimulated by A2 or A9—To unravel the complex sets of interactions that occur in this alternate signaling pathway, we developed reagents to precisely define the protein-protein interactions. As a source of APCs, we transfected RAW264.7 cells with constructs coding for both the α and β chains of I-A\(^q\). These cells were then pulsed with either A2 or A9 and served as antigen-presenting cells. To determine the critical interactions involved in signaling when T cells were activated with either A9-I-A\(^q\) or A2-I-A\(^q\), we generated retroviral constructs coding for the various signaling molecules incorporating either Myc or FLAG (Fig. 5). A collagen-reactive T cell hybridoma was transfected with various combinations of retroviral constructs so that it contained one of the following four combinations: 1) MSCV/Zap-70-Myc and CD3-ζ-FLAG; 2) MSCV/Zap-70-Myc and FcRγ-FLAG; 3) MSCV/Syk-Myc and CD3-ζ-FLAG; 4) MSCV/Syk-Myc and FcRγ-FLAG. The use of these constructs allowed us to determine whether Zap-70 associated with either CD3-ζ or FcRγ and in addition whether Syk associated with either CD3-ζ or FcRγ. We used a combination of immunoprecipitation and immunoblotting to demonstrate that activation of T cells overexpressing both CD3-ζ and Zap-70 with A2 but not A9 resulted in a physical association between CD3-ζ and Zap-70 (Fig. 5A). Neither peptide induced the association of CD3-ζ with Syk (Fig. 5B). In addition we demonstrated that when T cells that overexpressed both FcRγ and Syk were activated by A9 but not A2, there was a physical association of FcRγ and Syk (Fig. 5D). Neither peptide induced the association of FcRγ with Zap-70 (Fig. 5C). We conclude that the activation of T cells through the interaction of the class II MHC-A2-TCR complex leads to the recruitment of Zap-70 to CD3-ζ, whereas activation of the MHC-A9-TCR complex leads to the alternative recruitment of Syk to FcRγ.

To determine whether the interaction between Syk and FcRγ was dependent upon tyrosine residues, a mutant FcRγ was developed that was FLAG-tagged and contained critical mutations in the two tyrosines of the immunoreceptor tyrosine-based activation motifs (ITAMs), i.e. Y65F and Y76F (Fig. 6). T cells were generated that overexpressed both Syk-Myc and FcRγ-FLAG, either the wild type or the mutant. As shown in Fig. 6, immunoprecipitation with either FLAG or Myc followed by blotting with the other antibody led to the detection of Syk and FcRγ only when cells contained the wild type FcRγ. These data confirm that the interaction between Syk and FcRγ is dependent upon the tyrosines contained within the ITAM of FcRγ.

Protein Interactions between TCR-β and CD3-ζ or FcRγ—Based on the results of these experiments, it seemed likely that FcRγ must associate with the TCR-CD3 complex. To evaluate this possibility, we transfected collagen-specific T cell hybridoma cells with either CD3-ζ-FLAG or FcRγ-FLAG and per-

**TABLE 1**

**Cytokine responses**

Cytokine responses in Syk\(^{-/-}\) T cells. Groups of three DBA/1 chimeric mice, either wild type (lower panel) or genetically deficient in Syk, were immunized with CII/CFA. Draining inguinal lymph node cells were harvested 14 days after the immunization, and the CD4\(^+\) cells were isolated and cultured (5 \(\times\) 10\(^5\) cells/ml) with 1 μg/ml of either the immunodominant wild type peptide A2 or the analog A9 or media alone. Supernatants were collected 72 h later and analyzed for the presence of the indicated cytokines. Before this particular set of analyses, T cell cytokine production was measured at multiple time points including 18, 24, 48, 72, and 96 h. All time points revealed essentially the same cytokine patterns. Values are expressed as pg/ml and represent the mean values for each group.

| Geno-type | Antigen | IL-2 | IFN-γ | IL-17 | IL-4 | IL-10 |
|-----------|---------|------|-------|-------|------|-------|
| Syk\(^{-/-}\) | No Ag   | 49 ± 4 | 170 ± 43 | 154 ± 55 | 5 ± 5 | 105 ± 25 |
|           | A2      | 6011 ± 10\(^a\) | 4149 ± 85\(^b\) | 3,219 ± 85\(^b\) | 67 ± 12\(^*\) | 7,458 ± 18\(^*\) |
|           | A9      | 6 ± 63 | 271 ± 55 | 234 ± 83 | 3 ± 2 | 139 ± 42 |
| Syk\(^{+/+}\) | No Ag   | 62 ± 4 | 352 ± 255 | 272 ± 27 | 9 ± 2 | 95 ± 8 |
|           | A2      | 1352 ± 19\(^a\) | 7,350 ± 382\(^b\) | 15,210 ± 207\(^*\) | 75 ± 6\(^*\) | 3,511 ± 28\(^*\) |
|           | A9      | 21 ± 2 | 659 ± 195 | 359 ± 22 | 67 ± 3\(^*\) | 2,850 ± 20\(^*\) |

\(^a\) \(p < 0.005\), \(^b\) \(p < 0.001\).
formed immunoprecipitation using antibodies specific for TCR Vβ8 or FLAG and immunoblotting with the other antibody. These experiments confirm that the TCR receptor complexes contain both CD3-ζ and FcRγ (Fig. 7).

**DISCUSSION**

In this study we characterize a TCR signaling pathway utilized when T cells are activated by the APL A9 and contrast it with the canonical CD3-ζ-associated pathway used when cells are activated by a peptide representing the immunodominant epitope of CII. A qualitative change in the pathways involved causes polarization of the T cell activation response so that there is production of GATA3 and not T-bet. This alternate pathway requires both FcRγ and Syk.

Nearly 20 years ago, Bottomly, O’Garra, Allen, and others (2) began an area of work showing that weak signaling through a TCR could lead an undifferentiated naive CD4 T cell to acquire a Th2 phenotype independent of the exogenous cytokines present at the time of priming (2, 19). Peptides that interact strongly with the TCRs of naive CD4 T cells favor Th1 and Th17 cell differentiation, whereas low affinity peptide-MHC/TCR interactions favor Th2 effector T cell differentiation. Since then, intriguing pieces of evidence have emerged to shed light on ways in which weak
TCR interactions affect TCR signaling (2). Most of this work has been done in murine models. It appears that the strength of the TCR signal influences downstream signaling events, which in turn dictate transcription factor and cytokine gene transcription (20). In altered peptide ligand-stimulated cells, a lack of \( \gamma \) chain-associated protein kinase 70 (Zap-70) activation together with a significant decrease in early canonical TCR signaling is believed to be critical for Th2 differentiation. In support of this observation, mutations in Zap-70 have resulted in aberrant but not absent cytokine production (21–23). For example, Zap-70 null mutants have normal levels of IgE, an antibody class known to be dependent upon IL-4. Two separate reports identify hypomorphic point mutations in Zap-70 that lead to increased plasma IgE (22, 23). Interestingly, in another report, a point mutation in the kinase domain of Zap-70 results in increased IgE production together with T cell deficiency (21). The observed phenotypic differences induced by differing degrees or forms of Zap-70 functional impairment have led to speculation as to whether the quantity of signal propagated by Zap-70 causes these variations or whether there might be other not as yet described qualitative differences. Our data suggest that in some circumstances qualitative differences in signaling can lead to these disparate phenotypes.

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It appears that the strength of the TCR signal influences downstream signaling events, which in turn dictate transcription factor and cytokine gene transcription (20). In altered peptide ligand-stimulated cells, a lack of \( \zeta \) chain-associated protein kinase 70 (Zap-70) activation together with a significant decrease in early canonical TCR signaling is believed to be critical for Th2 differentiation. In support of this observation, mutations in Zap-70 have resulted in aberrant but not absent cytokine production (21–23). For example, Zap-70 null mutants have normal levels of IgE, an antibody class known to be dependent upon IL-4. Two separate reports identify hypomorphic point mutations in Zap-70 that lead to increased plasma IgE (22, 23). Interestingly, in another report, a point mutation in the kinase domain of Zap-70 results in increased IgE production together with T cell deficiency (21). The observed phenotypic differences induced by differing degrees or forms of Zap-70 functional impairment have led to speculation as to whether the quantity of signal propagated by Zap-70 causes these variations or whether there might be other not as yet described qualitative differences. Our data suggest that in some circumstances qualitative differences in signaling can lead to these disparate phenotypes.

Clearly, T cell stimulation by A9-I-A\(^{\beta} \) is followed by activation of the MAPKs JNK and p38 without any phosphorylation of ERK. Other investigators have reported that Th2 responses induced by altered peptide ligands can correlate with decreased ERK phosphorylation (24). In fact, T cells treated with an inhibitor of ERK phosphorylation up-regulate GATA3 and IL-4
expression, implying that ERK activation can repress GATA3 transcription (25). Our data clearly show that ERK activation is not required for the A9-induced GATA3 up-regulation and the subsequent secretion of Th2 cytokines, although ERK may contribute to the T-bet expression induced by A2.

Current models of antigen/MHC-induced T cell activation of the canonical pathway suggest that Src kinases such as Lck or Fyn are necessary to activate Zap-70. For example, in the absence of Src kinases, Zap-70 cannot transmit TCR-mediated signaling (26). On the other hand, it is believed that Syk can activate ITAMs independently of Src kinases (27–29). Hesslein et al. (30) have demonstrated that insufficient activation of the src kinases in NK cells will favor Syk activation rather than Zap-70. Variations in stereochemical interactions likely cause the two fundamentally different outcomes that occur after culture of CII-specific T cells with either A2 (induction of the full spectrum of cytokines) or A9 (induction of only the suppressive cytokines). We hypothesize that APLs such as A9 cause conditions that favor Syk by inadequately activating the src kinases. Because TCR-α/β cells contain both CD3-ζ homodimers and CD3-ζ/FcRγ heterodimers, it is very likely that TCRs containing FcRγ are preferentially utilized when T cells are stimulation by APLs such as A9.

Although it has previously been thought that MHC binding was mostly independent of MHC-peptide surface conformation, new technology using MHC-peptide tetramers reveal that changes in the residues interacting with the P1 and P4 MHC binding pockets can induce subtle but important stereochemical changes on the neighboring residues positioned to interact with the TCR (31, 32).

A2
\[ \text{TCR} \\rightarrow \text{Src} \rightarrow \text{Zap-70} \rightarrow \text{Ca}^{2+} \rightarrow \text{NFAT} \rightarrow \text{GATA3} \]

A9
\[ \text{TCR} \\rightarrow \text{Syk} \rightarrow \text{ITAMs} \rightarrow \text{GATA3} \]

The phenomenon of attenuated signals through the TCR clearly affects patients with autoimmune diseases. Lupus patients, for example, have strikingly reduced expression of CD3-ζ in effector CD4+ T cells (34–36). Moreover, SLE patients have been reported to have T cells that preferentially phosphorylate Syk rather than Zap-70 while expressing FcRγ at high levels (36–38). Although various molecules have been hypothesized to be involved in alternative T cell signaling pathways, including members of the Src family and of the Syk/Zap-70 family, the functional importance of Syk and its link to Th2 cytokine secretion has not been recognized previously.

Although studies using the DBA/1 rats transgenic mice have proven quite valuable to understanding the immuno-

![Hypothetical model of T cell signaling pathway used by A2 and A9.](image)
pathogenesis of CIA, we recognize the possibility that the TCR expressed by DBA/1\textsuperscript{RtCH} TCR Tg mice does not represent the full T cell repertoire of wild type mice. For these reasons we have been careful to confirm that T cells from TCR transgenic mice are identical to heterogenous T cells from DBA/1 non-transgenic mice in their ability to transfer suppression of arthritis and to secrete predominantly Th2 cytokines (especially IL-4) after stimulation with A9 (4). Moreover, another APL of collagen (A12) that has an entirely different MHIC restriction pattern (DR\textsuperscript{1*0101} and DRB\textsuperscript{1*0401}) behaves identically to A2 in both induction of cytokine profile and signaling pathway preferences (39).

In conclusion, we have identified FcR\textgamma and Syk as critical molecules in the TCR signaling induced by the A9 peptide and determined their physiological roles in the signal transduction pathway (Fig. 8). The wild type peptide (A2) recruits Zap-70 to the TCR-\zeta subunit and induces the activation of Zap-70 and its downstream pathway, including the MAPKs (JNK, p38, and Erk). The activation of the MAPKs leads to the activation of transcription factors including GATA3 and T-bet, inducing the production of Th1 and Th2 cytokines. In contrast, the altered peptide ligand (A9) utilized TCR complexes that contain FcR\gamma and recruits Syk to the FcR\gamma subunit, resulting in the activation of Syk, JNK, and p38 but not ERK. The differential phosphorylation induced by A9 leads to the activation of GATA3 and induces the production of Th2 cytokines but not the Th1 cytokines. Although FcR\gamma- and/or Syk-deficient CD4\textsuperscript{+} T cells cannot respond to A9, the absence of FcR\gamma or Syk did not alter the A2-mediated activation of Zap-70 and MAPKs, the induction of GATA-3, and the production of Th1 and Th2 cytokines. Taken together, our data provide evidence that T cells use an alternative pathway in response to A9 through FcR\gamma and Syk. Future studies should help us understand how altered peptide ligands can be used to develop therapies for rheumatoid arthritis and to clearly delineate the roles that Syk and FcR\gamma play in regulating autoimmunity.

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