Published in final edited form as:

Clin Immunol. 2018 July ; 192: 50–57. doi:10.1016/j.clim.2018.04.007.

The Role of Syk in Peripheral T Cells

Jeoung-Eun Park[†], Sirshendu Majumdar[†], David D. Brand^{†,‡,§}, Edward F. Rosloniec^{†,§}, Ae-Kyung Yi[‡], John M. Stuart^{†,§}, Andrew H. Kang^{†,§}, and Linda K. Myers^{*}

^{*}Department of Pediatrics, University of Tennessee Health Science Center, Memphis TN 38163

[†]Department of Medicine and Microbiology-Immunology, University of Tennessee Health Science Center, Memphis TN 38163

[‡]Department of Biochemistry, University of Tennessee Health Science Center, Memphis TN 38163

[§]Research Service, Veterans Affairs Medical Center, Memphis TN 38104

Abstract

The aim of this study was to understand how Syk affects peripheral T cell function. T cells from Syk^{-/-} chimeric mice and DR1 Syk^{fl/fl} CD4^{cre} conditional mice gave strong CD3-induced Th1, Th2, and Th17 cytokine responses. However, an altered peptide ligand (APL) of human CII (256 276) with two substitutions (F263N, E266D), also called A12, elicited only Th2 cytokine responses from Syk^{fl/fl} T cells but not Syk^{fl/fl}-CD4^{cre} T cells. Western blots revealed a marked increase in the phosphorylation of Syk, JNK and p38 upon A12/DR1 activation in WT or Syk^{fl/fl} T cells but not in Syk^{fl/fl}CD4^{cre} cells. We demonstrate that Syk is required for the APL-induction of suppressive cytokines. Chemical Syk inhibitors blocked activation of GATA-3 by peptide A12/DR1. In conclusion, this study provides novel insights into the role that Syk plays in directing T cell activity, and may shape therapeutic approaches for autoimmune diseases.

Introduction

Spleen tyrosine kinase (Syk) is a cytoplasmic tyrosine kinase involved in signaling by many of the cells that drive immune inflammation (1). The development of new therapies for autoimmune diseases based on inhibition of Syk suggest that Syk plays an important role in the expression of autoimmunity(1). Although multiple cells are likely involved in autoimmunity and inflammation, we focused on the CD4⁺ T cell, because CD4⁺ T cells, especially Th1 and Th17 cells play a prominent role in the initiation of systemic immune responses in rheumatoid arthritis (RA) and are dysregulated in experimental animal models autoimmune arthritis (2–5).

Corresponding author: Linda K. Myers, Division of Rheumatology, 956 Court Avenue, Room G326, Memphis TN 38163. Phone: (901) 448-5774, Fax: (901) 448-7265. lmyers@uthsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The aim of the present study was to understand the role of Syk in immune reactions to type II collagen by peripheral T cells. We utilized a synthetic altered peptide ligand (APL) of human CII comprised of amino acids 256-276 with two substitutions (F263N, E266D), also called A12. This peptide is an analog of the immunodominant epitope of CII for humanized DR1 transgenic mice. The peptide will suppress arthritis when administered to CII-immunized DR1 mice (6, 7). A12 appears to exert its suppressive effect by redirecting T cells to shift their cytokine response from Th1 towards a Th2-type profile. It is also known that it can induce suppressive cytokines in human T cells in the context of both HLADR4 and HLADR1, molecules known to be associated with RA. The exact mechanism for A12 immunosuppression has not been definitively established but there is substantial circumstantial evidence that it exerts its effects through activation of an alternative signaling pathway. We have previously shown that an altered peptide ligand (A9) which is restricted by the murine I-A^q-can activate T cells to utilize an FCR γ -dependent alternative signaling pathway (8–10). In the present experiments we address two important questions. Can this pathway be activated by A12 in the context of a human HLA molecule and is the relevant cell a CD4 T cell.

To pursue these questions, we used two different mouse models. The first model was a chimeric mouse with a Syk-deficient hematopoietic compartment. Mice which are conventional homozygous Syk deficient are not viable. We also used HLA-DR1 transgenic mice as recipients of a conditional knockout in which the Syk gene was deleted in peripheral CD4 cells. Syk deficient T cells were tested for cytokine responses induced by stimulation with anti-CD3 and for antigen responses to collagen peptides, both a peptide representing the immunodominant peptide of CII (A2), and the A12 APL. Finally, we studied the biochemical signaling pathways activated following TCR stimulation in the presence or absence of Syk in peripheral T cells.

We believe that understanding the role of Syk-dependent altered signaling through the T cell receptor (TCR) should provide insight into autoimmunity and a better understanding of the development of inhibitory T cells that are immunosuppressive. New therapies that inhibit Syk kinase are currently under development in preparation for clinical trials. It is our belief that a definitive understanding of the role of biochemical pathways involving Syk kinase in immune cells, including peripheral T cells, will facilitate the development of treatments for RA.

Methods

Animals

DBA/1 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) and B6 mice expressing the chimeric (human/mouse) DRB1*0101 construct were obtained from Taconic Biosciences, (Hudson, NY). The chimeric DRB1*0101 construct has been previously described, as has the production of Tg mice expressing this construct (11). Mice transgenic for a CII-specific TCR-V α 11.1/V β 8.3 having a DBA/1 background, referred to as DBA^{qCII24} (12) and mice transgenic for a CII-specific TCR in the context of DR1(13) were developed and bred in the animal core facility of the Rheumatic Diseases Research Core Center, University of Tennessee as described previously (13). *Syk knockout chimeric mice:*

Syk^{+/-} mice were obtained from Victor Tybulewicz (National Institute for Medical Research, London, UK) (14). The mutation was backcrossed onto the DBA/1 qCII24 strain for 12 backcrosses so that the resulting mice had the DBA/1 genetic background as well as the collagen-specific TCR transgene and were 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 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), which were obtained from timed matings of Syk^{+/-} DBA/1qCII24 carriers. Syk^{-/-} fetuses were identified according to their characteristic petechiated appearance and their genotype was confirmed by allele specific PCR analysis. The 8 16-week-old DBA/1 recipient mice were lethally irradiated as described (41) 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. Fetal livers from DBA/1qCII24 Syk^{+/+} mice were used to generate wild type control chimeras. Samples of peripheral blood were taken 4 weeks after transplantation and stained with phycoerythrin (PE) labeled antibodies against Vb8. For the analysis of Syk protein levels, total splenocytes were immunoblotted using anti-Syk using anti-Syk (Cell Signaling) or anti β -actin (Cell Signaling) antibodies. Chimeras were used 4 8 weeks after the bone marrow transplantation.

Syk conditional T cell knock-out mice

The Cre-loxP technology was used to generate mice with a conditional inactivation of Syk in the CD4 cell population. Heterozygous mice of the conditional *Sykb* mutant strain were obtained from Alexander Tarakhovsky, The Rockefeller University (JAX stock #017309) (15). In this conditional *Sykb* mutant strain, exon 1 has been flanked by loxP sites. Homozygous floxed mice are fully viable and fertile. The conditional *Sykb* mutant strain were crossed with CD4-Cre. CD4-Cre transgenic mice, which contain a *CD4* enhancer, promoter and silencer sequences driving the expression of a Cre recombinase gene, were obtained from Christopher B. Wilson, University of Washington (JAX stock# 017336). Hemizygotes are viable and fertile. Since Cre recombinase expression is observed in CD4-expressing T cells during sequential stages of T cell development in lymphoid tissues and commences at the very late double-negative stage this generally results in >99% deletion of *loxP* flanked genes by the double-positive stage of T-cell development. Consequently both mature CD4 and CD8 cells are affected as well as some CD4⁺ dendritic cells. The *Sykb* mice (which were C57Bl6 background were backcrossed to the DR1 mice (also C57Bl6 background) for three generations so that the resulting mice had both the *Sykb* and the DR1 genes. These mice were crossed with the CD4-cre mice and screened by polymerase chain reaction to select mice that were homozygous for the *Sykb* (*Syk*^{fl/fl}) gene and heterozygous for both the DR1 gene and the CD4-cre gene. The deletion of Syk in CD4 T cells was confirmed by western blot using anti-Syk (Cell Signaling) or anti β -actin (Cell Signaling) antibodies.

All mice were fed standard rodent chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum. Sentinel mice were routinely tested for a panel of mouse pathogens. All animals were kept until the age of 7–10 weeks before being used for experiments. Animal care and housing requirements set forth by the National Institutes of Health Committee on Care and

Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the University of Tennessee Animal Care and Use Committee.

Preparation of Tissue Derived CII and Synthetic Peptides—The following nomenclature is used to define the antigens used in this study: CII = Type II collagen, A2 = a peptide containing the immunodominant determinant sequence of both bovine and human CII (GIAGFKGEQGPKGEB) (B stands for 4-hydroxyproline), A12 = an altered peptide ligand of both bovine and human CII (GIAGNKGDQGPKGEB), $\alpha 1(\text{II})$ = the constituent protein chains of bovine CII isolated by carboxymethyl-cellulose chromatography. Native CII was solubilized from fetal calf articular cartilage or murine articular cartilage by limited pepsin-digestion and purified as described earlier (16). The purified collagen was dissolved in cold 10mM acetic acid at 4 mg/ml and stored frozen at -70°C until used. Synthetic peptides representing collagenous sequences were supplied by New England Peptide Inc., (Gardner, Massachusetts) and were greater than 95% pure.

CD4+ T cell isolation and activation

Spleens were collected and a single-cell suspension prepared by mechanical disruption in DMEM medium. Cultures were performed in DMEM supplemented with 10 % FCS, 100 IU/ml of penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2.5 μM β -mercaptoethanol and 2 mM L-glutamine. CD4+ T cells were isolated using a CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, Ca) using a negative selection protocol (which includes monoclonal antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC Class II, Ter-119, and TCR γ/δ .) 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, or overnight) with APC (splenocytes) which had been prepulsed with A2, A12, or other peptide(s). In some experiments, after a short culture period at 37°C , the cells were collected, lysed in lysis buffer, and insoluble materials were removed by centrifugation at 10,000 x g at 4°C for 15 minutes prior to tissue culture.

To test for cytokines cultures were set up using cells from three mice run in triplicates and supernatants were analyzed for the presence of IL-10, IL-4, IL-2, IFN- γ , TGF- β and IL-17A 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 taken from three separate experiments.

Analysis of protein phosphorylation

Whole cell lysates were separated using SDS-PAGE gels and electrotransferred onto nitrocellulose membranes. After transfer, the membrane was blocked in tris buffered saline (TBS)-Tween 20 containing 5% BSA for 1 hour and incubated 2 hours with phospho specific antibodies. The membrane was then incubated with a secondary antibody (Bio-Rad) for 1 hour and subjected to Enhanced Chemiluminescence detection (ECL Western Blot Substrate, Pierce) according to the manufacturer's protocol. To detect protein levels, the membranes were re-stripped and reblotted with non-phospho specific antibodies.

Flow cytometry

Murine spleens or lymph node cells were collected and the phenotype was determined by multiparameter flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Cells were labeled with fluorochrome antibodies including: PE (phycoerythrin)-conjugated anti-CD4, FITC (fluorescein isothiocyanate)-conjugated anti-CD8, APC (allophycocyanin)-conjugated anti-CD19, AF(alexa fluor) 700-labelled CD3, PE-labelled PD1 (Programmed death protein 1), strep-avidin-conjugated CXCR5 with biotinylated AF405 (BD Biosciences, San Jose, CA). All were used according to the manufacturer's recommendations. A minimum of 10,000 cells was analyzed from each sample and the final analysis was performed using FlowJo software (Tree Star, Ashland, OR).

The detection of intracellular phospho-proteins in T cells was carried out as follows: CD4⁺ cells were purified by magnetic separation, then stimulated with peptide-prepulsed antigen presenting cells for differing time periods (1 to 60 minutes). Cells were fixed with 1% formaldehyde and permeabilized with methanol. Both fluorescent-conjugated phospho-specific antibodies and antibodies specific for T cell surface markers [AF(alexa fluor) 700-labelled CD3 and PE (phycoerythrin)-conjugated anti-CD4, BD Biosciences, San Jose, CA] were added to the cells and incubated at room temperature for 1 hour. All antibodies, including the anti-phospho specific antibodies that recognize Erk -p44/42 MAPK (Erk1/2) (Thr202/Tyr204)Alexa Fluor[®] 488 Conjugate) and Syk (Phospho-Syk (Tyr323) Alexa Fluor[®] 488) were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Evaluation of the status of the intracellular phosphorylation was determined using CellQuest and FlowJo software following analysis with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey).

Statistical Analysis

Cytokine levels and other analyses were compared using the Mann-Whitney test.

Results

T cell responses in Syk^{-/-} chimeric mice

To examine the importance of Syk in T cells, CD4⁺ T cells from CII-immune Syk^{-/-} bone marrow chimeric mice were collected from spleens and cultured with the immunodominant collagen peptide (A2) together with APCs from wildtype mice. The resulting supernatants were evaluated for cytokines using a multiplexed ELISA (Table I). There were vigorous T cell responses to the collagen peptide from all T subsets (Th1, Th17, and Th2) (Table I). More importantly, both Syk ^{+/+} and Syk ^{-/-} chimeric T cells secreted all three (Th1, Th2, and Th17) cytokines, although the T cell responses were not identical. These data demonstrate that Syk is not required for the induction of Th1, Th2, or Th17 cytokine responses in peripheral T cells.

Development and characterization of Syk^{fl/fl} CD4^{-cre} mice

To corroborate these results in another system, we turned to the Cre-loxP system. A DR1 Syk^{fl/fl} mouse was crossed with CD4-Cre transgenic mice, driving the expression of a Cre

recombinase gene in CD4 cells. The CD4-cre line was selected due to its high deletion efficiency. The resulting mice that are $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{-cre}}$ survived normally and the deletion of Syk in the CD4 T cells was confirmed by western blot (Figure 1). To characterize the phenotype of these conditional mice, splenocytes were collected, and the numbers of immune cells, evaluated by flow cytometry (Figure 2). The percentages of CD4⁺ cells, CD8⁺ cells, and CD19⁺ cells were equivalent among the $\text{Syk}^{\text{fl/fl}}$ mice, and the $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{cre}}$ mice (Figure 2A and 2B). When gated on the lymphocyte population (n=3), the results were as follows:

CD4/CD3: $\text{Syk}^{\text{fl/fl}} = 15.55 \pm 2\%$, $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}} = 19.2 \pm 6\%$, WT = $17.0 \pm 1\%$;

CD8/CD3: $\text{Syk}^{\text{fl/fl}} = 7.56 \pm 2\%$, $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}} = 9.2 \pm 1\%$, WT = $9.5 \pm 1\%$;

CD19: $\text{Syk}^{\text{fl/fl}} = 58 \pm 12\%$, $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}} = 54.6 \pm 4\%$, WT = $59.2 \pm 5\%$.

Another subset of the CD4 cells which is important for autoimmunity is the follicular B helper T cell subset (also known as just follicular helper T cells or T_{FH}). These are antigen-experienced CD4⁺ T cells identified by their constitutive expression of the B cell follicle homing receptor CXCR5. In order to analyze this population, both $\text{Syk}^{\text{fl/fl}}$ and $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{cre}}$ mice were immunized with CII/CFA and 10 days after immunization, cells from the spleens and lymph nodes were evaluated by flow cytometry to determine the numbers of Tfh cells (Figure 2C). Percentages of PD-1/CXCR5 positive cells (n=3) from spleens were as follows: $\text{Syk}^{\text{fl/fl}} = 37.9 \pm 14\%$, $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}} = 30.4 \pm 5\%$. Cells from draining lymph nodes (n=3) were as follows: $\text{Syk}^{\text{fl/fl}} = 7.5 \pm 1\%$, $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}} = 6.4 \pm 1\%$. Although there was a trend toward decreased Tfh cells in the splenocytes and lymph nodes cells from $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{cre}}$ mice compared to $\text{Syk}^{\text{fl/fl}}$ mice the differences did not reach statistical significance. Taken together these data demonstrate that syk does not affect the numbers of immune cells *in vivo*.

Characterization of anti-CD3-induced T cell responses in $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{-cre}}$ mice

Since CD4⁺ helper T cells control responses primarily through cytokine secretion, splenocytes were collected from the $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{cre}}$ conditional mice and controls and were stimulated in culture with anti-CD3. The resulting supernatants were collected and evaluated for T cell cytokine responses by multiplexed ELISA. As shown in figure 3, T cells from both WTCD4^{-cre} and $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{cre}}$ gave cytokine responses greater than background for all cytokines tested (Th1, Th2, and Th17), figure 3. These data corroborate the findings from the Syk chimeric mice, that Syk is not necessary for induction of Th1, Th2, and Th17 T cell cytokines in response to TCR stimulation.

Characterization of antigen-induced T cell responses in $\text{Syk}^{\text{fl/fl}}\text{CD4}^{\text{-cre}}$ mice

Syk deficient T cells were next evaluated for responses to protein antigens. We selected peptides representing, both the immunodominant determinant of type II collagen, A2, and an altered peptide ligand, A12. The APL A12 was of interest, because it has been shown to suppress autoimmune arthritis (6, 17). The $\text{Syk}^{\text{fl/fl}}$ and $\text{Syk}^{\text{fl/fl}}\text{-CD4}^{\text{cre}}$ mice were immunized with CII and the draining lymph nodes collected for *in vitro* studies. As shown in Table II, the supernatants from cultures of CD4⁺ cells gave differing responses to A12. Cells from $\text{Syk}^{\text{fl/fl}}$ mice that were cultured with A12/DR1 secreted IL-4, IL-5, IL-10, and TGF- β

cytokines with levels that were comparable to those elicited by A2/DR1. On the other hand T cell cytokine responses to A12 were absent in Syk^{fl/fl}CD4^{cre} cells. The T cell responses to A2 were equivalent in both Syk^{fl/fl} and Syk^{fl/fl}-CD4^{cre} cells for Th1, Th17, and Th2 cytokines. These data corroborate the hypothesis that Syk is required for the APL-induced T cell cytokine responses to A12.

T Cell Signaling in Syk conditional mice in response to peptides A2 and A12

We have previously established that T cells restricted by murine I-A^q can use an alternative to the canonical T cell signaling pathway in which FCR γ replaces CD3- η to initiate T cell functions (9). Therefore we wanted to determine whether T cells responding to A12 in the context of a human HLA molecule can use a similar activation pathway. To examine the appearance of tyrosine phosphorylated intracellular substrates, CD4⁺ cells from DR1TCRtg mice were cultured with DR1 positive APCs pre-pulsed with either a peptide representing the immunodominant determinant of CII (A2) or A12. As shown in figure 4 (left panel), triggering by either peptide induced an increase in the phosphorylation of a number of intracellular proteins within five minutes of activation. However, there are striking differences in the patterns of phosphorylation obtained between the two peptides. A2/DR1 activates the canonical pathway, specifically Zap-70 followed by activation of the three mitogen activated protein kinases (MAPKs) Erk-1/2, JNK, and p38. On the other hand, A12/DR1 activates Syk but not Zap-70, followed by phosphorylation of only two of the MAPKs; JNK and p38 but not Erk-1/2.

To confirm that A12/DR1 stimulation induced the tyrosine phosphorylation of Syk in CD4 T cells, we compared signaling in CD4 T cells from Syk^{fl/fl} mice compared to Syk^{fl/fl}-CD4^{cre} mice. Both groups also contained the CII-specific TCR. Examination by Western blots (Figure 4, right panel), revealed a marked increase in the phosphorylation of Syk, JNK and p38 upon A12/DR1 activation in DR1TCRtg cells. In contrast, no major activation was detected in the phosphorylation of the corresponding molecules in the lysates of cells from Syk^{fl/fl}CD4^{cre} TCRtg mice. The same Syk^{fl/fl}CD4^{cre} TCRtg T cells, however, successfully activated ZAP-70 and all the MAPKs when stimulated by A2, giving results comparable to those of T cells obtained from mice sufficient in Syk. Taken together, these data show that Syk is required for a MAPK activation by A12/DR1 but plays no role in the canonical pathway triggered by A2/DR1.

To corroborate that the signaling changes were produced by CD4⁺T cells, flow cytometry was performed on wild type T cells using antibodies specific for phospho-Syk and phosphor-Erk, with gating on CD3⁺/CD4⁺ T cells (Figure 5).

Expression of GATA3 by A2 and A12

A final outcome of A12/DR1 stimulation is the secretion of cytokines associated with the Th2 T cell phenotype (18). As the zinc finger transcription factor GATA3 is known to upregulate the expression of Th2 cytokines in T cells, we examined the induction of GATA3 following A12/DR1-stimulation. Therefore, CD4⁺ T cells from Syk^{fl/fl} or Syk^{fl/fl} CD4^{cre} conditional knockout mice were activated by APCs prepulsed with A2 or A12. When GATA3 expression was examined by Western blot analysis (Figure 6), GATA3 expression

was increased following activation by either A2 or A12 in Syk^{fl/fl} mice. On the other hand, in Syk^{fl/fl} CD4^{-cre} conditional knockout mice a significant decrease in GATA3 induction was detected in CD4+ T cells stimulated by A12, while the GATA3 induction by A2 remained intact.

Similarly, in the presence of the Syk inhibitor piceatannol, a significant decrease in GATA3 induction was detected in CD4+ T cells stimulated by A12, while GATA3 was induced by A2. These data confirm that Syk is required for Th2-type cytokine activation by A12/DR1 but does not play a role in the canonical pathway triggered by A2/DR1.

Discussion

The purpose of this study was to establish a better understanding of how Syk functions in T cells. Using an altered peptide ligand (A12) that is presented to T cells in the context of human HLA-DR1 or DR4, we established that an alternate signaling pathway, dependent upon Syk is required for APL- induced suppressive Th2 cytokines. T cells from Syk chimeric mice and Syk conditional mice in which Syk was deleted from CD4 T cells, cannot respond to the APL A12, although the canonical signaling pathway induced by the immunodominant peptide A2 remains intact. Given that Syk is an attractive target for treatment of autoimmune diseases because of its role in antigen receptor signaling, and its abnormal regulation has been implicated in the pathogenesis of several autoimmune diseases, including RA and SLE (1), these findings have implications for understanding how to use new therapies targeting Syk to treat autoimmune arthritis.

Spleen tyrosine kinase (Syk) is a cytosolic non-receptor protein tyrosine kinase (PTK), which was discovered in 1990 (19, 20). It is a key integrator of intracellular signals triggered by activated immunoreceptors, and is important for the development and function of lymphoid cells (21). The human Syk gene encodes a 635-aa polypeptide with an estimated molecular weight of 72 kDa. It is localized on chromosome 9q22 and is mainly expressed in hematopoietic cells. Since it belongs to the Src family of non-receptor type tyrosine kinases, it is highly homologous to ZAP-70, containing two N-terminal SH2 domains and one C terminal tyrosine kinase domain. Syk has an autophosphorylation site at Tyr-518 although it can also be phosphorylated by Src kinases, such as Lyn. Syk is activated by binding to immunoreceptor tyrosine-based activation motifs (ITAMs) via its SH2 domains. Receptor engagement initiates the signaling pathway, leading to an increase in second messenger IP3 which stimulates calcium ion mobilization and the induction of downstream effects (1).

Syk and ZAP-70 are not redundant but instead display clear functional differences. In the canonical pathway, ZAP70 requires the additional co-expression of the Src-family kinases Fyn or Lck to efficiently phosphorylate SLP-76 and ITAM signaling. In contrast, Syk can signal using a Src-kinase independent ITAM-based signaling pathway, which may be involved in calibrating the threshold for lymphocyte activation (22). T cells can be influenced to utilize Syk over ZAP70 when Src-family kinases are not activated. In a murine system, by using altered peptide ligands with varying affinities of interaction with the MHC we established that peptide/MHC affinity correlates with TCR signaling, i.e. ZAP-70/ ζ or Syk/FcR γ (23). 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 absence of activation of src kinases and the induction of the FcR γ -dependent alternate signaling pathway and suppressive cytokines (23).

One fascinating aspect of T cell biology is that a T cell can productively interact through its TCR with less-than-optimal ligands resulting in partial T-cell activation. These ligands are called 'altered peptide ligands' (APLs). Moreover there is evidence that endogenous APLs exist *in vivo* and affect T-cell development (24). It is very likely that endogenous APLs are generated *in vivo* whenever a new protein is expressed. Therefore, T cells have to possess the capacity to interact with sub-optimal ligands for an immune response to develop. One could clearly hypothesize that these types of interactions do occur for peripheral T cells under limited attainable circumstances and are an integral part of the life of a T cell (24). The finding that APLs can induce T cells to utilize Syk has led to a renewed interest in the importance of Syk in autoimmunity. In the present study, we focus on A12 peptide which is clinically relevant because it is currently in a phase one clinical trial (Postlethwaite, NCT01123655, clinicaltrials.gov). It can induce human T cells to secrete suppressive cytokines in the context of HLA-DR1 and HLA-DR4, both of which are associated with RA (6, 7, 17).

In a similar manner, human T cells from patients with SLE exhibit a rewiring of their T cell receptor (TCR) wherein the expression of the CD3- ζ chain is decreased, replaced by the homologous FcR γ chain, which recruits the downstream signaling Syk kinase rather than the CD3- ζ partner Zap70 (25). Other investigators have demonstrated that naïve CD4 T-cells treated with immune complexes and C5b-9 can phosphorylate TCR signaling proteins and spleen tyrosine kinase (Syk) (26) so that the FcR γ chain displaces the CD3- ζ chain in TCR-CD3 complex in SLE T-cells. Moreover, ZAP-70-deficient patients express and signal via Syk. Their Syk^{high} T-cells show decreased Erk, JNK, and MAPK activity, suggesting a distinct signaling (26).

There is an unmet need in autoimmune arthritis for new nonchemotherapy drugs. Rheumatoid arthritis is a systemic disease characterized by a chronic inflammation of the synovial membrane of diarthrodial joints which leads to destruction of cartilage, joint deformity and disability. Current treatment usually consists of glucocorticoids, NSAIDs, DMARDs, and biologic agents, although they are not always effective and significant side effects occur. Syk inhibitors are a new group of small molecule inhibitors targeting signaling pathways (27). Although the Syk inhibitor fostamatinib (R788) has been used in clinical trials, it displays some off-target activities (27), suggesting that, newer, more selective, Syk inhibitors may be more effective. Several new Syk inhibitors are in development and early clinical trials. Among others, the second generation compound, entospletinib, showed promising results in clinical trials against B-cell malignancies. Moreover, investigators have developed an alternative small molecule SYK inhibitor, designated RO9021, which could complement the current arsenal of tools in development for treatment of inflammation-related and autoimmune-related disorders (21).

Although multiple cells are likely involved in autoimmune arthritis, we have chosen to focus on the CD4+ T cell, because Th1 and Th17 cells, play a prominent role in the initiation of systemic immune responses in RA. Moreover, the induction of long-lasting, protective immunity largely relies on the activities of T lymphocytes as well. While uncontrolled hyperactive T cells can cause significant tissue damage which could eventually lead to autoreactive disease, there are T regs, and other inhibitory T cells which serve as a counterbalance to hold the immune system in check. T cell responses are usually tightly regulated to prevent T cell hyperactivation and their dysregulation may end up in autoimmune pathology. Our finding that the induction of an inhibitory cell utilizes a signaling pathway involving Syk suggests that therapies against Syk will be more effective if Syk is targeted toward B cells neutrophils, dendritic cells, and monocytes, while bypassing the regulatory and other inhibitory T cells. This focus should lead to more effective Syk-directed therapies with better outcomes.

Other investigators have used murine models that contain genetic deletions of Syk, to evaluate the severity of arthritis. In autoantibody induced experimental arthritis, the K/BxN serum transfer model, bone marrow chimeras with hematopoietic-specific Syk deletion were completely protected from arthritis development (28). Moreover, inflammation was completely abrogated by deletion of Syk either in the entire myeloid compartment or in neutrophils (29). No inflammatory defect could be observed in mice lacking Syk in platelets or mast cells, despite effective deletion of Syk from the respective lineages (29). Taken together, those studies suggested that tyrosine kinases expressed within neutrophils (and, possibly, macrophages) are critical for autoantibody-induced arthritis development (29).

Conclusion

In conclusion, this study provides novel insights into the role that Syk plays in directing T cell activity in vivo, and may shape therapeutic approaches to autoimmune disease treatment. These data also suggest that Syk inhibitors may be more effective as treatments for autoimmune arthritis, if targeted towards B cells, neutrophils, dendritic cells, or monocytes, and avoiding the regulatory and inhibitory T cells. The elucidation of various molecular pathways in T cells has revealed putative targetable molecules, such as Syk. These measures should lead to more effective Syk-directed therapies with better outcomes.

Acknowledgments

This work was supported, in part, by USPHS Grants AR064825, AR069010, AR064723 and program-directed funds from the Department of Veterans Affairs, and the Arthritis Foundation.

References

1. Deng GM V, Kyttaris C, Tsokos GC. 2016; Targeting Syk in Autoimmune Rheumatic Diseases. *Front Immunol.* 7:78. [PubMed: 27014261]
2. Cope AP, Schulze-Koops H, Aringer M. 2007; The central role of T cells in rheumatoid arthritis. *Clin Exp Rheumatol.* 25:S4. [PubMed: 17977483]
3. Choy E. 2012; Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford).* 51(Suppl 5):v3. [PubMed: 22718924]

4. Perdry H, Clerget-Darpoux F. 2011; Modeling the effect of susceptibility factors (HLA and PTPN22) in rheumatoid arthritis. *Methods Mol Biol.* 713:201. [PubMed: 21153621]
5. Iannone F, Lapadula G. 2012; The inhibitor of costimulation of T cells: abatacept. *J Rheumatol Suppl.* 89:100. [PubMed: 22751606]
6. Kimata M, Cullins DL, Brown ML, Brand DD, Rosloniec EF, Myers LK, Stuart JM, Kang AH. 2012; Characterization of inhibitory T cells induced by an analog of type II Collagen in an HLA-DR1 humanized mouse model of autoimmune arthritis. *Arthritis Res Ther.* 14:R107. [PubMed: 22569209]
7. Myers LK, Sakurai Y, Tang B, He X, Rosloniec EF, Stuart JM, Kang AH. 2002; Peptide-induced suppression of collagen-induced arthritis in HLA-DR1 transgenic mice. *Arthritis Rheum.* 46:3369. [PubMed: 12483744]
8. Park JE, Cullins D, Zalduondo L, Barnett SL, Yi AK, Kleinau S, Stuart JM, Kang AH, Myers LK. 2012; Molecular basis for T cell response induced by altered peptide ligand of type II collagen. *J Biol Chem.* 287:19765. [PubMed: 22511761]
9. Tang B, Zhou J, Park JE, Cullins D, Yi AK, Kang AH, Stuart JM, Myers LK. 2009; T cell receptor signaling induced by an analog peptide of type II collagen requires activation of Syk. *Clin Immunol.* 133:145. [PubMed: 19596610]
10. Park JE, Rotondo JA, Cullins DL, Brand DD, Yi AK, Stuart JM, Kang AH, Myers LK. 2016; Characterization of the Syk-Dependent T Cell Signaling Response to an Altered Peptide. *J Immunol.* 197:4569. [PubMed: 27837109]
11. Rosloniec EF, Brand DD, Myers LK, Whittington KB, Gumanovskaya M, Zaller DM, Woods A, Altmann DM, Stuart JM, Kang AH. 1997; An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. *J Exp Med.* 185:1113. [PubMed: 9091584]
12. Myers LK, Rosloniec EF, Cremer MA, Kang AH. 1997; Collagen-induced arthritis, an animal model of autoimmunity. *Life Sci.* 61:1861. [PubMed: 9364191]
13. Tang B, Kim S, Hammond S, Cullins DL, Brand DD, Rosloniec EF, Stuart JM, Postlethwaite AE, Kang AH, Myers LK. 2014; Characterization of T cell phenotype and function in a double transgenic (collagen-specific TCR/HLA-DR1) humanized model of arthritis. *Arthritis Res Ther.* 16:R7. [PubMed: 24405551]
14. Ackermann JA, Nys J, Schweighoffer E, McCleary S, Smithers N, Tybulewicz VL. 2015; Syk tyrosine kinase is critical for B cell antibody responses and memory B cell survival. *J Immunol.* 194:4650. [PubMed: 25862820]
15. Saijo K, Schmedt C, Su IH, Karasuyama H, Lowell CA, Reth M, Adachi T, Patke A, Santana A, Tarakhovsky A. 2003; Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. *Nat Immunol.* 4:274. [PubMed: 12563261]
16. Rosloniec EF, Cremer M, Kang AH, Myers LK, Brand DD. 2010; Collagen-induced arthritis. *Curr Protoc Immunol Chapter.* 15(Unit 15 5 1)
17. Sakurai Y, Brand DD, Tang B, Rosloniec EF, Stuart JM, Kang AH, Myers LK. 2006; Analog peptides of type II collagen can suppress arthritis in HLA-DR4 (DRB1*0401) transgenic mice. *Arthritis Res Ther.* 8:R150. [PubMed: 16982003]
18. Myers LK, Tang B, Rosloniec EF, Stuart JM, Kang AH. 2007; An altered peptide ligand of type II collagen suppresses autoimmune arthritis. *Crit Rev Immunol.* 27:345. [PubMed: 18197813]
19. Kurosaki T, Takata M, Yamanashi Y, Inazu T, Taniguchi T, Yamamoto T, Yamamura H. 1994; Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. *J Exp Med.* 179:1725. [PubMed: 7513017]
20. Kobayashi T, Nakamura S, Taniguchi T, Yamamura H. 1990; Purification and characterization of a cytosolic protein-tyrosine kinase from porcine spleen. *Eur J Biochem.* 188:535. [PubMed: 2331984]
21. Liao C, Hsu J, Kim Y, Hu DQ, Xu D, Zhang J, Pashine A, Menke J, Whittard T, Romero N, Truitt T, Slade M, Lukacs C, Hermann J, Zhou M, Lucas M, Narula S, DeMartino J, Tan SL. 2013; Selective inhibition of spleen tyrosine kinase (SYK) with a novel orally bioavailable small molecule inhibitor, RO9021, impinges on various innate and adaptive immune responses:

- implications for SYK inhibitors in autoimmune disease therapy. *Arthritis Res Ther.* 15:R146. [PubMed: 24286216]
22. Fasbender F, Claus M, Wingert S, Sandusky M, Watzl C. 2017; Differential Requirements for Src-Family Kinases in SYK or ZAP70-Mediated SLP-76 Phosphorylation in Lymphocytes. *Front Immunol.* 8:789. [PubMed: 28736554]
23. Myers LK, Cullins DL, Park JE, Yi AK, Brand DD, Rosloniec EF, Stuart JM, Kang AH. 2015; Peptide ligand structure and I-A_q binding avidity influence T cell signaling pathway utilization. *Clin Immunol.* 160:188. [PubMed: 25982319]
24. Evavold BD, Sloan-Lancaster J, Allen PM. 1993; Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunology Today.* 14:602. [PubMed: 8305133]
25. Moulton VR, Suarez-Fueyo A, Meidan E, Li H, Mizui M, Tsokos GC. 2017; Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective. *Trends Mol Med.* 23:615. [PubMed: 28623084]
26. Chauhan AK, Moore TL, Bi Y, Chen C. 2016; FcγRIIIa-Syk Cosignal Modulates CD4⁺ T-cell Response and Up-regulates Toll-like Receptor (TLR) Expression. *J Biol Chem.* 291:1368. [PubMed: 26582197]
27. Liu D, Mamorska-Dyga A. 2017; Syk inhibitors in clinical development for hematological malignancies. *J Hematol Oncol.* 10:145. [PubMed: 28754125]
28. Jakus Z, Simon E, Balazs B, Mocsai A. 2010; Genetic deficiency of Syk protects mice from autoantibody-induced arthritis. *Arthritis Rheum.* 62:1899. [PubMed: 20201079]
29. Futosi K, Mocsai A. 2016; Tyrosine kinase signaling pathways in neutrophils. *Immunol Rev.* 273:121. [PubMed: 27558332]

Figure 1. Expression of Syk in Syk conditional knockout mice

Whole cell lysates taken from purified CD4⁺ cells from DR1 (Syk^{fl/fl}) or Syk conditional knockout (Syk^{fl/fl} CD4^{-cre}) mice were collected and the proteins were separated by SDS-PAGE. The proteins were transferred onto PVDF membranes and analyzed for expression of Syk by an anti-Syk antibody. Actin was used as control.

Figure 2. Flow cytometric analysis of immune cell populations in Syk^{fl/fl} CD4-cre mice
 Splenocytes from Syk^{fl/fl} mice and Syk^{fl/fl} CD4-cre mice were collected, labeled with fluorochrome antibodies, and analyzed by flow cytometry. In 2A, scatter plots were gated on the total lymphocyte population. In 2B, scatter plots were gated on the CD3 population. Data shown are representative of three separate analyses. In 2C mice which were either Syk^{fl/fl} or Syk^{fl/fl}-CD4 cre were immunized with CII/CFA, splenocytes were collected, and the cells were labeled with fluorochrome antibodies to be analyzed by flow cytometry (2C). In the scatter plot, gates were placed on the CD4/CD45 high population. Data shown are representative of three separate analyses.

Figure 3. Anti-CD3 induced Responses of T cells from

Syk^{fl/fl}-CD4^{cre} mice. Splenocytes from mice which were either WT-CD4^{cre} or *Syk*^{fl/fl}-CD4^{cre} were collected and the cells were cultured with antibodies against CD3. The supernatants were analyzed for cytokines using a multiplexed ELISA. Data shown represent the means (pg/ml ± SD) from three different experiments. All cytokine responses to CD3 were significantly greater than background and were equivalent between the WT-CD4^{cre} and the *Syk*^{fl/fl}-CD4^{cre} mice.

Fig. 4. T cell signaling in response to A2 and A12

CD4⁺ T cells from DR1 transgenic mice (Left Panel) or CD4⁺ T cells from DR1 (Syk^{fl/fl}) or Syk conditional knockout (Syk^{fl/fl}-CD4^{cre}) mice (Right panel) were incubated with APCs prepulsed with 100 mg/ml of A2, A12 or no peptide (No Ag) for the time periods indicated. Western blot analysis of the total cell lysates was performed using specific antibodies against phospho-Zap70(pZap70), phospho-Syk(pSyk), phospho-Erk(pErk), phospho-P38(pP38), and phospho-JNK(pJNK). Data shown are representative of three separate analyses.

Figure 5. Flow cytometry analysis of phospho-proteins induced by A2 and A12

Flow cytometry was performed using purified CD4⁺ T cells from spleens of DR1 TCR Tg mice. The T cells were stimulated by APCs pre-pulsed with A2, A12 or no peptide (Control) for 5 minutes. The cells were then fixed, permeabilized, and stained with an antibody specific for phospho-Syk (Tyr 323) (left panel) or phospho- ERK 1/2 (right panel) and analyzed by flow cytometry. Both plots are gated on CD3⁺/CD4⁺ T cells. Data shown are representative of three separate analyses.

Fig. 6. Induction of GATA-3

Panel A: The CD4⁺ T cells from Syk^{fl/fl} or Syk^{fl/fl} CD4^{cre} conditional knockout mice were activated by APCs prepulsed with 100 µg/ml of A2, A12 or no peptide (No Ag) for 1 h. Whole cell lysates were collected and the proteins were separated by SDS-PAGE. The proteins were transferred onto PVDF membranes and analyzed for induction of GATA3. Actin was used as a control Panel B: CD4⁺ T cells from DR1 TCR transgenic mice were cultured in the presence of a Syk inhibitor piceatannol (2mM) or DMSO as control. Then the T cells were activated by APCs prepulsed with 100 mg/ml of A2, A12 or no peptide (N/A) for 1 hr. After culture, the cells were collected and the extracts were subjected to immunoblot analysis using an antibody against GATA-3. Actin was used as a control. Data shown are representative of three separate analyses.

Table 1

T cell responses to a collagen peptide using Syk^{-/-} chimeric mice

Pg/ml	IL-2	IFN- γ	IL-17	IL-10	IL-4	IL-5
Syk^{+/+}						
No Ag	8 $\pm$ 5	302 $\pm$ 12	92 $\pm$ 20	36 $\pm$ 7	5 $\pm$ 1	20 $\pm$ 10
A2	52 $\pm$ 15	47770 $\pm$ 110	12850 $\pm$ 155	260 $\pm$ 25	24 $\pm$ 12	750 $\pm$ 58
Syk^{-/-}						
No Ag	7 $\pm$ 6	304 $\pm$ 15	272 $\pm$ 25	18 $\pm$ 5	4 $\pm$ 2	18 $\pm$ 9
A2	36 $\pm$ 9	71631 $\pm$ 125	15430 $\pm$ 128	443 $\pm$ 72	76 $\pm$ 10	99 $\pm$ 22

CD4⁺ T Cells were collected from spleens of chimeric mice bearing either Syk^{-/-} or Syk^{+/+} lymphocytes. They were cultured with the immunodominant peptide of CII (A2) and APCs from wildtype mice and supernatants were analyzed for cytokines using a multiplexed ELISA. Data shown represent the means from three different experiments and all responses to the A2 peptide were significantly greater than background. Both Syk^{+/+} and Syk^{-/-} cells gave Th1, Th2, and Th17 responses, but IFN- γ , IL-17, IL-10, and IL-4 responses were greater and the IL-5 responses were lower in the Syk^{-/-} cells when compared to the Syk^{+/+} responses (p 0.05).

Table II

T Cell Responses to collagen peptides using T cells from Syk^{fl/fl}-CD4^{cre} mice

Pg/ml	IL-2	IFN- γ	IL-17	IL-10	IL-4	IL-5	TGF- β
Syk^{fl/fl}							
No Ag	8 $\pm$ 1	110 $\pm$ 18	988 $\pm$ 45	42 $\pm$ 17	7 $\pm$ 3	40 $\pm$ 12	24 $\pm$ 9
A2	44 $\pm$ 8	55960 $\pm$ 123	28000 $\pm$ 258	187 $\pm$ 25	55 $\pm$ 14	750 $\pm$ 58	142 $\pm$ 22
A12	9 $\pm$ 3	160 $\pm$ 14	1001 $\pm$ 98	262 $\pm$ 32	97 $\pm$ 33	854 $\pm$ 62	123 $\pm$ 18
Syk^{fl/fl}-CD4^{cre}							
No Ag	7 $\pm$ 8	179 $\pm$ 35	122 $\pm$ 26	22 $\pm$ 8	8 $\pm$ 5	120 $\pm$ 17	29 $\pm$ 11
A2	38 $\pm$ 9	65000 $\pm$ 155	29150 $\pm$ 128	486 $\pm$ 85	50 $\pm$ 9	1513 $\pm$ 42	101 $\pm$ 20
A12	6 $\pm$ 3	85 $\pm$ 15	0	20 $\pm$ 39	6 $\pm$ 2	44 $\pm$ 21	15 $\pm$ 8

Mice which were either Syk^{fl/fl} or Syk^{fl/fl}-CD4^{cre} were immunized with CII/CFA and CD4⁺ splenocytes and lymph node cells were collected and the cells were cultured with wild type APCs that had been prepulsed with either peptide A2 or A12 and supernatants were analyzed for cytokines using a multiplexed ELISA. Data shown represent the means from three different experiments. All responses to the A2 peptide were significantly greater than background and were equivalent between the Syk^{fl/fl} and the Syk^{fl/fl}-CD4^{cre} mice with the exception of an increased IL-10 response in the Syk^{fl/fl}-CD4^{cre} mice. There were significant differences noted in cytokine responses from the two mice, in the response to A12. In the Syk^{fl/fl}-CD4^{cre} mice cells responded to A12 with a significant IL-10, IL-5, IL-4 and a TGF β response which were all greater than background, while the cells from the Syk^{fl/fl}-CD4^{cre} mice did not give responses greater than background.