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Therapeutic Targeting of Syk in Autoimmune Diabetes

Lucrezia Colonna,*† Geoffrey Catalano,*† Claude Chew,*† Vivette D’Agati,‡ James W. Thomas,§ F. Susan Wong,‡ Jochen Schmitz,∥ Esteban S. Masuda,∥ Boris Reizis,*† Alexander Tarakhovsky,# and Raphael Clynes*†

In APCs, the protein tyrosine kinase Syk is required for signaling of several immunoreceptors, including the BCR and FcR. We show that conditional ablation of the syk gene in dendritic cells (DCs) abrogates FcγR-mediated cross priming of diabetogenic T cells in RIP-mOVA mice, a situation phenocopied in wild-type RIP-mOVA mice treated with the selective Syk inhibitor R788. In addition to blocking FcγR-mediated events, R788 also blocked BCR-mediated Ag presentation, thus broadly interrupting the humoral contributions to T cell-driven autoimmunity. Indeed, oral administration of R788 significantly delayed spontaneous diabetes onset in NOD mice and successfully delayed progression of early-established diabetes even when treatment was initiated after the development of glucose intolerance. At the DC level, R788 treatment was associated with reduced insulin-specific CD8 priming and decreased DC numbers. At the B cell level, R788 reduced total B cell numbers and total Ig concentrations. Interestingly, R788 increased the number of IL-10–producing B cells, thus inducing a tolerogenic B cell population with immunomodulatory activity. Taken together, we show by genetic and pharmacologic approaches that Syk in APCs is an attractive target in T cell-mediated autoimmune diseases such as type 1 diabetes. The Journal of Immunology, 2010, 185: 000–000.

Autoimmunity is kept in check by central and peripheral tolerance mechanisms involving the elimination or regulation of self-reactive lymphocytes encountering self-Ags displayed by professional APCs. In particular, self-Ags internalized by the BCR on B cells or as immune complexes (ICs) by the activating FcγRs on myeloid cells empower these APCs with immunostimulatory capacities by facilitating antigenic uptake and processing and by inducing cellular activation through ITAM signaling pathways. Therefore, interfering with the ITAM-triggered humoral pathways would be predicted to reinforce T cell tolerance.

Self-reactive B cells may contribute to the autoimmune process by functioning as APCs (1–3). Indeed, recognition and uptake of soluble self-Ag by B cells bearing an autoreactive BCR can lead to B cell activation and MHC class II (MHC-II)–restricted Ag presentation, empowering these cells as potent APCs for CD4+ T cells. However, this model cannot explain autoreactive CD8 responses, because B cells are incapable of phagocytosis or cross priming of CD8 cells, a property unique to dendritic cells (DCs) (4).

Autoantibodies can participate in autoimmune pathogenesis indirectly by enhancing self-Ag uptake by activating FcγRs on DCs (5). Following the binding of self-Ag–containing ICs by activating FcγRs, the ICs are internalized and processed for presentation via both the exogenous and the cross-presentation pathways, with loading of self-peptides onto both MHC-II and -I molecules (6, 7). Additionally, uptake of self-Ag–containing ICs by activating FcγRs leads to the upregulation of costimulatory molecules (7, 8) and the production of immunostimulatory cytokines (9, 10).

BCR and FcγR ITAM signaling share a requirement for the protein tyrosine kinase (PTK) activity of the spleen tyrosine kinase, or Syk. Conversely, normal T cells do not use Syk to transduce the TCR signals, but rather the Syk family members ZAP70. The ITAM signaling cascade is triggered by immunoreceptor cross-linking, initiated by recruitment of Src family kinases. Src family PTK activity phosphorylates tyrosine residues within the ITAM sequences of the cytosolic domains of immunoreceptor adaptor signaling subunits, namely CD79α/CD79β in B cells and the FcγR γ-chain in myeloid cells. These phosphorylated ITAMs provide docking sites for the Src homology 2 domains of Syk, which itself is phosphorylated. Activated Syk kinase, in turn, phosphorylates a number of important substrates, including phospholipase Cγ, BTK, and PI3K. Thus, many divergent signaling pathways are induced directly by Syk activation, ultimately driving multiple BCR and FcγR-mediated biological responses, including induction of cellular activation and upregulation of the Ag presentation machinery in both B cells (11) and DCs (6, 12). Therefore, interruption of immunoreceptor signaling at the level of Syk would be predicted to attenuate both BCR and FcγR signaling, potentially uncoupling self-reactive B cells and their secreted Ig products from their pathogenic consequences in driving loss of T cell tolerance. In an effort to demonstrate Syk-mediated dependence of T cell-driven autoimmunity, we have examined the effect of Syk deletion/inhibition on an Ab-triggered CD8–mediated insulitis RIP-mOVA mouse model system (5) as well as in spontaneous type 1 diabetes (T1D) development in NOD mice.

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Abbreviations used in this paper: BMDC, bone marrow–derived dendritic cell; cernLNs, cervical lymph nodes; DC, dendritic cell; DN, double-negative; DP, double-positive; GAD, glutamic acid decarboxylase; GC, germinal center; HEL, hen egg lysozyme; IC, immune complex; INS, insulin; IPGT, i.p. glucose tolerance testing; MHC-II, MHC class II; MZ, marginal zone; panLN, pancreatic lymph node; PC, peritoneal cell; PTK, protein tyrosine kinase; RO, C57Bl/6 RIP-mOVA; SP, single-positive; T1D, type 1 diabetes; Treg, regulatory T cell; WT, wild-type.

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Materials and Methods

**Mice**

NOD/LtJ, NOD/SCID, and C57BL.6 wild-type (WT), and MD4 (13) congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and C57BL.6 FcγRy<sup>−/−</sup>, OT-I congenic mice (14) were purchased from Taconic Farms (Germantown, NY). C57BL.6 RIP-mOVA (RO) mice (15) were obtained from T. Ratliff (University of Iowa, Iowa City, IA). To conditionally ablate syk specifically in the DC compartment, CD11c-Cre RO<sup>−</sup> Syk<sup>fl Orange</sup> mice were generated by interbreeding congenic Syk<sup>fl Orange</sup> mice (16) and CD11c-Cre transgenic mice, which express Cre in CD11c<sup>+</sup> cells, mainly DCs (17). All animal experiments were performed in compliance with institutional guidelines and approved by Columbia University’s Institutional Animal Care and Use Committee (New York, NY).

**Syk inhibitor**

R788 (fostamatinib disodium; Rigel Pharmaceuticals, South San Francisco, CA) is an oral prodrug and, after conversion in the stomach, is absorbed into the circulation as R406, the physiologically relevant and biologically active component of R788 (18). In vitro studies were performed with R406 (Figs. 3, 4, 7). For in vivo studies, R788 was administered to mice either dissolved in the drinking water (Figs. 2, 5) or formulated into the food chow (Figs. 6–9). Serum levels of R406 varied dramatically with R788 provided in the drinking water (mean 1.4 SD 1.2 mg/ml range 5–9180 ng/ml; n = 7 mice), likely the pharmacokinetic consequence of intermittent drinking and rapid systemic clearance (t<sub>1/2</sub> = 1–2 h in the mouse versus 12–24 h in the human). To overcome this uneven biologic exposure, in all subsequent experiments (Figs. 6–9), R788 was provided in the food chow (2 g/kg) to slow gastrointestinal absorption, which resulted in steadier levels of drug in the blood (mean ± SD 1463 ± 937 ng/ml range 197–3280 ng/ml; n = 10 mice) that more closely approximated the drug exposures/levels seen in treated patients (18–20).

**RO<sup>+</sup> cross priming model**

Five million positively selected CD8<sup>+</sup> T cells bead-isolated (MACS technology, Miltenyi Biotec, Auburn, CA) from spleens and peripheral lymph nodes from OT-I mice were transferred i.v. together with i.p. injection of 1 mg rabbit anti-OVA IgG as described (5). Mice were treated with R788 3 mg/ml in the drinking water beginning 24 h pretransfer.

**NOD diabetes**

In prevention studies in NOD female mice, treatment began at 6 wk of age with R788 (provided by Rigel Pharmaceuticals and dissolved at 0, 1, 2, or 3 mg/ml in the drinking water). Diabetes development was monitored daily and scored positive if glucose >250 mg/dl in the urine in two successive measurements performed daily (RIP-mOVA) or biweekly (NOD). Histopathological assessment of insulitis from H&E sections of formalin-fixed paraffin-embedded sections was performed by Dr. Vivette D’Agati (Columbia University, New York, NY) in a blinded fashion by means of the following classification system: stage 0, no invasion; stage 1, perisinusitis; stage 2, >25% invasion; stage 3, >75% invasion; and stage 4, remnant islets.

**R788 treatment of i.p. glucose-intolerant mice**

Mice were considered glucose intolerant and started on R788 (2 g/kg in chow) or not if and when serum glucose readings >180 mg/dl 1 h after an i.p.

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**FIGURE 1.** Syk expression in DCs is required for Ab-mediated cross-priming and autoimmune. A. Characterization of CD11c-Cre<sup>+</sup> Syk<sup>fl Orange</sup> mice: DCs but not granulocytes show defective FcyR-mediated cellular activation. Total splenocytes from WT, FcγR y<sup>−/−</sup>, and CD11c-Cre<sup>+</sup> Syk<sup>fl Orange</sup> mice were analyzed following incubation for 1 h with anti-BSA IgG/BSA H<sub>2</sub>DCF at 37°C in vitro. Oxidized green fluorescent DCF (Oxyburst) measures FcR exposure, in all subsequent experiments (Figs. 6–9), R788 was provided in the food chow (2 g/kg) to slow gastrointestinal absorption, which resulted in steadier levels of drug in the blood (mean ± SD 1463 ± 937 ng/ml range 197–3280 ng/ml; n = 10 mice) that more closely approximated the drug exposures/levels seen in treated patients (18–20).

B. Density plots are gated for (Gr1<sup>hi</sup>, B220<sup>−</sup>) CD11c-positive cells, mainly DCs (17). All animal experiments were performed in compliance with institutional guidelines and approved by Columbia University’s Institutional Animal Care and Use Committee (New York, NY).

C. Flow cytometric analysis of OT-I proliferation in the pancreatic lymph nodes of RIP-mOVA mice 5 d posttransfer. CFSE dilution plots are gated for CD8<sup>+</sup> Vα2<sup>+</sup> VB<sup>+</sup> transgenic OT-I cells. OT-I cells represented 3.6 ± 0.5% of total CD8<sup>+</sup> T cells in the pancreatic lymph node (n = 3 mice) in control RO<sup>−</sup>CD11c-Cre<sup>+</sup> Syk<sup>fl Orange</sup> mice, whereas in RO<sup>−</sup>CD11c-Cre<sup>+</sup> Syk<sup>fl Orange</sup> mice, OT-I numbers were significantly reduced (p = 0.002) averaging 1.4 ± 0.31% (n = 3).
injection of 1.5 mg glucose/kg body weight injected after a 4-h fasting period (i.e., glucose intolerance testing, IPGTT).

NOD/SCID adoptive transfers

A total of 10 × 10⁶ splenocytes obtained from either diabetic or non diabetic NOD/LtJ mice were transferred alone or together with equal numbers of CD43⁺ or CD43⁻ splenocytes as indicated. Recipient mice were treated or not with 2 g/kg R788 in the chow.

In vitro Ag presentation assays

B cells. Enriched CD43⁻/⁻ splenic B cells (Miltenyi Biotec) isolated from BCR transgenic MD4 mice were plated at a density of 2 × 10⁵ cells/well in a 96-well, round-bottom, tissue-culture plate in RPMI 1640 supplemented with 10% FBS. B cells were preincubated for 1 h with R406 (Regel Pharmaceuticals) before the addition of 20 μg/ml hen egg lysozyme (HEL) and 5 × 10⁴ B04 HEL-specific T hybrid cells [recognizing Aβ:HEL-74-88 (21)]. In parallel, BO4 T hybrid cells were incubated alone in wells coated with 2 μg/ml anti-CD3 Ab (NA/LE, clone 145-2C11; BD Pharmingen, San Diego, CA) in the presence or absence of R406. After 72 h in culture, IL-2 production by the responding T cells was measured by sandwich ELISA assay in triplicate.

Dendritic cells. At day 7 of culture, 10⁶ GM-CSF bone marrow-derived DCs (BMDCs) were plated in 96-well plates in complete GM-CSF-containing medium, either untreated or with R406 for 1 h prior to the addition of OVA ICs (OVA plus polyclonal rabbit anti-OVA IgGs) or OVA peptides (immunodominant class I-restricted OVA257–264 and class II-restricted OVA323–339, New England Peptide, Gardner, MA) (22). A total of 2 × 10⁵ responder CD8 T cells were added to each well, and proliferation was measured in triplicate 48 h later by pulsing the cells with 1 μCi/well of [³¹]H]methylenethymidine for 16–18 h.

IC binding and internalization

Day 8 GM-CSF-expanded BMDCs were incubated on ice (binding assay) or at 37°C (internalization/accumulation assay) with fluorescent ICs [10 μg/ml Alexa Fluor 488 OVA (Invitrogen, Carlsbad, CA); 50 μg/ml polyclonal rabbit anti-OVA IgG] and then washed and stained for flow cytometric analysis on gated CD11c⁺ conventional DC populations. The C4H3 Ab (23) was kindly provided by Dr. Lisa Denzin (Memorial Sloan-Kettering Cancer Center, New York, NY) to detect HEL peptide loading on MHC-II La⁺ postincubation with free HEL or rabbit anti-HEL–HEL ICs (1.3:1.0 molar ratio).

In vivo Ag presentation assays

CD8-positive T cells [insulin (INS)-CD8] from 6-9 wk-old transgenic mice expressing the cloned TCR of the G9C8 insulin-reactive CD8 T cell clone (24) or from OT-I transgenic mice were isolated from spleen suspensions by magnetic bead separation (Miltenyi Biotec) and stained with 20 μM CFSE (Molecular Probes, Eugene, OR). A total of 10 × 10⁶ INS-CD8 T cells or 5 × 10⁴ OT-I CD8 T cells were transferred i.v. into NOD or RO⁺ female mice that were either left untreated or were treated with R788 (2 g/kg in chow) for 1 wk prior to T cell transfer. Proliferation of the transgenic CD8 T cells was monitored by CFSE dilution in the pancreatic lymph nodes, spleen, and cervical lymph nodes of the recipient mice at day 5 posttransfer. For the detection of OT-I and INS-CD8 T cells in vivo, cells were stained with CD8 (clone 53-6.7) and appropriate TCR family-specific Abs [e.g., Vα2 (clone B20.1) and Vβ6 (clone RR-7) or Vβ5 (clone MR9-4)].

Flow cytometric immunophenotyping

The Abs (clones) with the following specificities were used [purchased from ebBioscience (San Diego, CA) or BD Pharmingen]: CD3 (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), CD21/CD35 (clone 7G6), CD23 (clone B3B4), CD25 (PC61), CD44 (IM7), CD42L (MEL-14), CD69 (H1.2F3), CD86 (GL-7), CD138 (clone 281-2), GL7 (clone GL7), Gr-1 (clone RB6-8C5), and 120G8 (a generous gift from Giorgio Trinchieri, National Cancer Institute, Frederick, MD). DC subsets were stained with the following Abs: CD1c (clone NC21), CD24 (clone M1/69), Sirp-α (P84), class II (clone M5/114), B220 (clone RA3-6B2), and CD11b (clone M1/70).

IL-10-producing B cells were identified poststimulation with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (500 ng/ml; Sigma-Aldrich), and LPS (10 ng/ml) in the presence of monensin (GolgiStop solution, BD Biosciences, San Jose, CA) for 5 h at 37°C prior to surface staining with PE-Cy7-B220 (clone RA3-6B2), PE CD23 (clone B3B4), and FITC CD21/CD35 (clone 7G6). Cell fixation and permeabilization...
were done using the Cytofix/Cytoperm Plus kit (BD Biosciences) before staining with APC anti-mouse IL-10 Abs (clone JES5-16E5). Regulatory T cells (Tregs) were identified by intracellular staining with the Foxp3 Ab (clone FJK-16s; eBioscience).

Measurement of oxidative burst induced by FcR-mediated internalization

Total splenocytes from WT, FcγR−/−, and CD11c-Cre+Syklox/lox mice were analyzed following incubation for 1 h in glucose Krebs’ Ringer’s PBS with 90 μg/ml rabbit anti-Bxa:10 μg/ml BSA–dichlorodihydrofluorescein (H2DCF, Molecular Probes) at 37˚C in vitro.

ELISA assays

Total IgG and IgM. ELISA Maxisorp plates (Thermo Fisher Scientific, Rochester, NY) were coated overnight at 4˚C with diluted NOD sera at 1:100,000 and 1:1,000,000 in PBS, washed, and blocked before addition of biotinylated goat anti-mouse-IgG or IgM Abs (1:500) (Southern Biotech, Birmingham, AL) for 2 h at room temperature. Glutamic acid decarboxylase autoantibody ELISA. ELISA plates were coated with 30 μg/ml glutamic acid decarboxylase (GAD) peptide 524–543 (New England Peptide) in carbonate buffer (PBS with the addition of 0.1 M NaHCO3) for 2 h at 37˚C, then blocked with PBS 1% BSA at room temperature for 2 h. NOD sera diluted 1:100 in washing/blocking buffer (PBS, 0.05% Tween 20, 0.05% BSA) were added to the plates overnight at 4˚C. Autoantibodies were detected with biotinylated goat anti-mouse-α (1:200) and goat anti-mouse-λ (1:100) Abs (Southern Biotech) followed by streptavidin-conjugated alkaline phosphatase. Insulin autoantibody ELISA. ELISA plates were coated overnight at 4˚C with 1 μg/ml insulin in PBS (pH 8) and washed (PBS 0.2% Tween, 1% BSA) before addition of diluted mouse sera. For competitive inhibition with insulin, diluted sera were preincubated with insulin at 0.05–100 μg/ml for 2 h pretransfer to insulin-coated plates (25, 26). Insulin binding in the presence or absence of soluble inhibitor was measured using goat anti-mouse IgG conjugated to alkaline phosphatase at room temperature in washing buffer. Results are presented as the percent inhibition of plate-bound insulin binding in the presence of soluble insulin.

Statistics

Kaplan-Meier survival curves were constructed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). Survival curve p values were calculated using a one-tailed log-rank test. Differences between groups were evaluated using a two-tailed unpaired Student t test.

Results

Syk is required for Ab-mediated cross priming in vivo in RIP-mOVA mice

We previously showed in RIP-mOVA mice that autoantibodies can participate pathogenically by enhancing self-Ag uptake and driving autoreactive T cell responses in an activating FcγR-dependent manner (5). In this study, the role of cross priming DCs was implicated through cellular transfer and depletion approaches. To genetically investigate the role of FcγR signaling in DCs, we generated RIP-mOVA mice lacking syk expression selectively in CD11c-positive DCs. In these CD11cCre+RIP-mOVA Syklox/lox mice, targeted disruption of syk in DCs was confirmed functionally by showing that FcγR-mediated delivery of ICs to lysosomes ex vivo was abrogated in the DC compartment but was maintained in the granulocyte compartment (Fig. 1A). Deletion of syk reduced Ab-mediated cross priming posttransfer of anti-OVA IgG and OVA-specific OT-I CD8 cells and protected RIP-mOVA from diabetes induction (Fig. 1B, 1C).

FIGURE 3. R406 inhibits BCR- and FcR-mediated cellular activation and Ag presentation. A, Primary HEL-specific BCR transgenic MD4 splenic B cells were incubated with graded doses of the cognate Ag HEL in the presence of the indicated [R406]. R406 is the physiologically relevant and biologically active component of the oral prodrug R788 and significantly reduced CD86 expression (two-way ANOVA, p < 0.01). Representative results of three experiments are provided as fold-increase in CD86 mean fluorescence intensity of B220 gated cells. B, Primary MD4 B cells were preincubated with R406 and stimulated with cognate Ag HEL and HEL-specific T cell hybrids (B04). Ag presentation was monitored by measuring T cell activation via an IL-2-specific ELISA immunoassay. R406 inhibits Ag presentation to T cell hybrids in a dose-dependent manner (p = 0.042, 0.006, 0.002, and 0.002 for 30 nM, 100 nM, 300 nM, and 1 μM R406, respectively). In parallel, tissue-culture plates were coated with anti-CD3 Abs to stimulate the TCR complex in a B cell-independent manner. R406 has no effect on direct T cell activation through the triggering of the TCR complex. C, BMDCs were preincubated with R406 and different concentrations of OVA-ICs. DC maturation was assessed by flow cytometric analysis for CD86 expression selectively in CD11c-positive DCs. R406 significantly inhibited DC maturation (two-way ANOVA, p = 0.01). D, BMDCs were preincubated with or without R406 and subsequently challenged with graded concentrations of OVA-ICs (left panel) or SIINFEKL peptide (right panel) and Ag presentation to OT-1 assessed by [3H]thymidine incorporation 48 h later. The mean ± SD of triplicate wells are shown. R406 significantly inhibited IC-triggered Ag presentation (p = 0.01, 0.008, and 0.001 for 3:0.3, 10:1, and 30:3, respectively).
To address the potential to block this pathway in syk-sufficient mice, WT RIP-mOVA mice were treated with R788 (fostamatinib disodium; Rigel Pharmaceuticals), an orally available small molecule that acts as a competitive antagonist for ATP binding to the Syk catalytic domain (18). R788 treatment phenocopied syk deficiency and protected RIP-mOVA mice from insulitis and diabetes development (Fig. 2A, 2B) and abrogated diabetogenic T cell priming in the draining pancreatic lymph node (Fig. 2C, 2D).

**R788 blocks BCR- and FcγR-mediated cellular activation and Ag presentation**

To directly evaluate the activity of R788 on APC function, the effect of R788 on B cells and DCs was examined in vitro using R406, the physiologically relevant and biologically active component of the oral prodrug R788. BCR-triggered immunophenotypic activation was inhibited by R406 at an IC_{50} in the 300 nM range, as exhibited by reduced CD86 costimulatory molecule induction postincubation of HEL-specific primary splenic transgenic MD4 B cells with graded doses of the cognate Ag HEL (Fig. 3A). To examine BCR-mediated Ag presentation, MD4 splenic B cells were incubated with HEL Ag and syngeneic HEL-specific B04 CD4 T cell hybrids. R406 blocked BCR-mediated Ag presentation (Fig. 3B; IC_{50} 100–300 nM). The effect was specific for B cells because R406 had no effect on direct TCR-mediated activation by plate-bound anti-CD3. Next, we examined the inhibitory effects of R406 on FcγR function on DCs. R406 specifically decreased activation of bone marrow-derived GM-CSF–cultured DCs poststimulation with OVA-containing ICs (Fig. 3C; IC_{50} <300 nM) and inhibited OVA IC-mediated cross-presentation to OT-I CD8 T cells, without altering presentation of OVA peptide (SIINFEKL) Ag (Fig. 3D). Thus, R406 interferes with ITAM-mediated cellular activation and presentation by both the BCR and FcγR.

The mechanistic block in FcγR-mediated Ag presentation was examined further. R406 did not interfere with either the binding or internalization of ICs (Fig. 4A, 4B). To directly examine MHC loading, the C4H3 Ab was employed, which recognizes a HEL peptide in the context of MHC-II Ia^{k} molecules (23). R406 did not impair loading of MHC–peptide complexes postincubation with free HEL, but specifically impaired peptide loading onto MHC molecules poststimulation with HEL ICs (Fig. 4C, 4D), implicating a postendocytic block in antigenic processing.

**R788 prevents autoimmune diabetes development in NOD mice and delays disease progression in IPGTT-positive mice**

Because islet-specific autoantibodies and self-reactive B cells appear in the diabetes prodrome in both NOD mice and patients with T1D, the humoral response is likely to play an early pathogenic role in autoimmune diabetes development. In NOD mice, both BCR and FcγR (1, 27) pathways contribute to pathogenesis, suggesting the therapeutic utility of a selective Syk inhibitor. Thus, female adult NOD mice were treated in the prevention setting beginning at 6 wk of age with graded doses of R788 in the drinking water. Remarkably, R788 treatment delayed diabetes development and prolonged survival in a dose-dependent manner (Fig. 5A, 5B).

![FIGURE 4. R406 inhibits postendocytic FcγR-mediated antigenic processing.](http://www.jimmunol.org/) A, IC binding/internalization: flow cytometry for OVA-Alexa 488 binding to BMDC cultures incubated for 30 min on ice with OVA-Alexa 488 ICs (left panels) and for 90 min at 37°C incubation to assess internalization/accumulation (right panels). B, IC internalization over time: experiments performed as in A and harvested at indicated times. Data are representative of at least four experiments. C, MHC-II loading: flow cytometric analysis of BMDCs stained with CD11c and C4H3 Abs, the latter recognizing a HEL peptide in the context of MHC-II Ia^{k}. BMDCs were preincubated with 300 nM R406 and subsequently incubated with either unbound HEL or with HEL-containing ICs (HEL–rabbit anti–HEL IgGs) for 5 h at 37°C. D, Results are expressed as percent of C4H3^{+} CD11c^{+} cells as the mean ± SD of triplicate wells are shown.
Histopathologically, protection was associated with a delay in the appearance of invasive insulitis (Fig. 5C).

To address whether R788 could reverse established diabetes, treatment was delayed until after the development of sustained glucosuria; in this study, only one of seven R788-treated glucosuric mice demonstrated reversal and became nonglucosuric (data not shown). In contrast, R788 treatment did successfully prevent diabetes progression in late-stage prediabetes. In this study, NOD mice were screened weekly by IPGTT, and begun on R788 only upon developing glucose intolerance (Fig. 5D). In untreated mice, progression to overt diabetes was rapid, with glucosuria developing at a mean interval of 14 d after the onset of glucose intolerance. Thus, R788 significantly delayed diabetes progression, with a median interval of onset of 140 d.

**R788 does not block the diabetogenic effector phase but instead inhibits islet-reactive T cell priming**

Although T cells are the major effectors in autoimmune diabetes, Syk inhibition would be unlikely to interfere with the diabetogenic effector phase, ZAP70 being the principal PTK responsible for transducing activating signals downstream of the TCR. Indeed, R788 treatment failed to protect NOD/SCID mice that received diabetogenic splenocytes (Fig. 6A), indicating that R788 did not inhibit the effector function of transferred, already-primed diabetogenic splenocytes. To address whether R788 treatment was instead associated with impaired T cell priming, NOD mice were either left untreated or were treated between the age of 6 and 14 wk, prior to the development of overt diabetes. After 8 wk of treatment, the treated or untreated prediabetic splenocytes were harvested and transferred into untreated NOD/SCID mice to determine whether R788 protection could be extended in the untreated recipients. Protection was sustained in NOD/SCID recipients of R788-treated splenocytes (mean latency of 11 wk versus 5 wk; \( p = 0.05 \); Fig. 6B). These studies suggested that continuous R788 treatment may not be required to sustain disease protection, which was examined directly in a window study. NOD mice were treated with R788 during a therapeutic window of 12 wk, beginning at 6 wk of age and continuing until the mice were 18 wk of age. Once off treatment, the mice continued to be followed for the development of glucosuria. After 11 wk off treatment, 12 of 15 R788-treated mice continued to be disease free, whereas most untreated mice developed diabetes (11 out of 15; \( p = 0.0003 \)) (Fig. 6C).
these studies suggest that R788 inhibits diabetogenic priming in a sustained manner, potentially through the induction of dominant acting tolerogenic mechanisms.

Because syk deletion was associated with reduced cross priming in RIP-mOVA mice, and R788-mediated Syk inhibition reduced cross-presentation by BMDCs in vitro, CD8 priming was examined directly in the NOD model in vivo. To this end, CFSE-labeled, TCR-transgenic, insulin-specific CD8 T cells (INS-CD8) were transferred into R788-treated and untreated 6-wk-old NOD mice, and proliferative responses were assessed 5 d later. The proliferation of INS-CD8 was significantly impaired in the pancreatic lymph nodes of R788-treated hosts (11.9% versus 3.2% divided; \( p = 0.01 \); Fig. 6D), indicating that R788 treatment inhibits cross-presentation of the autoantigen insulin in vivo.

R788 treatment reduces total numbers of peripheral B cells and DCs

Hematopoietic development was assessed after 3 mo of R788 treatment (Table I). Total numbers of B cells in the spleen were reduced (Tables I, II), consistent with the predicted consequences of reduced BCR signaling (28–30). Within the T cell compartment, thymic development was normal (Table III), and total mature CD4 and CD8 cells in the periphery were unchanged. Percentages of CD8+ T cells were reduced by R788 treatment in both the thymus

| Table I. Lymphoid and myeloid development/survival in R788-treated mice |
|-----------------|-----------------|-----------------|
|                 | Percent of Total Cells | Absolute Number of Cells (\( \times 10^6 \)) |
|                 | Untreated | R788 | \( p \) Value | Untreated | R788 | \( p \) Value |
| Bone marrow     |           |      |               |           |      |               |
| Total bone marrow cells | 22±5.9 | 15.7±3.14 | 0.046 | 34.5±7.4 | 31.1±4.9 | NS |
| Total B220+   | 13.5±3.9 | 12.8±4 | NS | 7.5±2.5 | 4.9±1.3 | 0.048 |
| CD11b+        | 50.9±4.3 | 54.8±4.2 | NS | 17.5±3.5 | 17±3.9 | NS |
| Spleen         |           |      |               |           |      |               |
| Total splenocytes | 40.9±4 | 39.4±5.5 | 0.17 | 64.1±24.9 | 36.6±6.2 | 0.0014 |
| B220+ B cells | 13.2±2.9 | 10.8±1.3 | 0.045 | 26.8±11.9 | 14.6±3.8 | 0.0025 |
| CD8+ T cells  | 28.7±3.2 | 25.2±2.8 | NS | 15.7±6.4 | 10.3±1.6 | NS |
| CD4+ T cells  | 6.6±1.6 | 9.2±0.6 | 0.019 | 3.5±1.4 | 3.8±0.5 | NS |
| Tot CD11b+ myeloid | 4.7±1.7 | 6±0.4 | NS | 2.4±0.7 | 2.5±0.3 | NS |
| CD11b+ Gr1+ MACS | 2.6±0.9 | 3.4±0.3 | NS | 1.3±0.5 | 1.4±0.2 | NS |
| CD11c+ DCs    | 0.81±0.2 | 0.57±0.2 | 0.002 | 0.51±0.2 | 0.24±0.1 | 0.008 |

NOD mice (five per group) were treated with 2 g/kg R788 in the chow for 3 mo beginning at 6 wk of age.
and spleen, likely an effect independent of BCR and FcR signaling but speculatively the result of R788 inhibitory effects on other Syk-dependent immunoreceptor pathways that contribute to MHC-I–restricted Ag presentation (31, 32). Immunophenotypic activation of T cells in the spleen and lymph node were also not altered by R788, because percentages of effector/memory CD44+ T cells or recently activated CD69+ T cells were similar in the spleen and pancreatic lymph node of treated and untreated mice. Surprisingly, R788 treatment lowered the numbers of DCs in the spleen, pancreatic lymph node, and tissues of treated mice (Fig. 7 and data not shown). This result was unexpected, because syk deficiency has not been reported to impact DC development (12). This was likely the result of off-target effects of R788 on FLT3 signaling because the total number of DCs was also reduced in the bone marrow, and FLT3–mediated differentiation of DCs from BM precursors in vitro was inhibited by R406 (IC50 <100 nM; Fig. 7B). Indeed, off-target inhibitory activity on FLT3 autophosphorylation has been previously reported at concentrations of R406 3–5-fold higher than those needed to inhibit Syk catalytic activity (18). The reduction in Tregs in the spleen and pancreatic lymph nodes of treated mice (Table III) occurred in parallel with reduced DC numbers, potentially the direct consequence of a reduced homeostatic capacity of DCs to support Treg maintenance (33, 34).

R788 modestly inhibits humoral autoimmunity and promotes IL-10–producing B cells

Syk is known to be critically involved in transducing BCR-mediated differentiation and survival signals. Thus, after 1–3 mo of R788 treatment, B cell numbers were reduced by 45% in the spleen (Table I). With regards to B cell subsets, marginal zone (MZ) B cell frequencies were increased by 44% at the expense of follicular B cells, whereas frequencies of activated B cells, germinal center (GC) B cells, and plasma cells were not changed (Fig. 8A, Table II, and data not shown). Total IgM and IgG levels were modestly reduced after 3 mo of treatment (Fig. 8B). This total IgG reduction was mirrored by a modest reduction in levels of anti-GAD anti-islet autoantibodies but unchanged anti-insulin autoantibody levels (Fig. 8C). Because IL-10–producing B cells with immunsuppressive

| Table II. R788 inhibits B cell survival/development with relative sparing of marginal zone B cell populations |
|-------------------------------------------------|-------|----------------|------|----------------|-------|
| Bone marrow                                      |       | Absolute Number of Cells (× 10⁶) |      |
| Percent of Total Cells                           |       | Untreated R788 p Value       |      |
| Plasma cells                                    |       |                               |      |
| Splenic                                          |       |                               |      |
| Fo/T2                                           | 69.3 ± 3.4 | 63.5 ± 6.0 | 0.007 | 18.4 ± 8.2 | 9.4 ± 3.1 | 0.002 |
| MZ                                               | 16.1 ± 3.4 | 23.2 ± 3.2 | 0.00007 | 4.3 ± 2 | 3.3 ± 0.7 | 0.07 |
| B1                                               | 10.6 ± 2  | 9.6 ± 1.9 | NS       | 2.6 ± 1.6 | 1.7 ± 0.4 | NS       |
| GC B cells                                      | 1.2 ± 0.1 | 1.4 ± 0.1 | NS       | 0.5 ± 0.3 | 0.6 ± 0.1 | NS       |
| Lymph node                                       |       |                               |      |
| Pancreatic lymph node node B220⁺                 | 27.1 ± 2.7 | 23.5 ± 1.8 | 0.05    |                   |      |
| Cervical lymph node B220⁺                        | 28.3 ± 4.5 | 22.3 ± 0.8 | 0.03    |                   |      |

B cell splenic subset percentages are indicated as percent of total B220⁺ B cells; elsewhere, percentages are of total cells in the spleen, bone marrow, and lymph node. NOD mice (five per group) were treated with 2 g/kg R788 in the chow for 3 mo beginning at 6 wk of age.

| Table III. T cell development/activation in R788-treated mice |
|---------------------------------------------------------------|-------|----------------|------|----------------|-------|
| Percent of Total Cells                                        |       | Absolute Number of Cells (× 10⁶) |      |
| Thymus                                                        |       |                               |      |
| CD4⁺ CD8⁻ DN                                                  | 6 ± 1.3 | 5.7 ± 0.6 | NS       | 4.1 ± 2 | 3.9 ± 1.5 | NS       |
| CD4⁺ CD8⁺ DP                                                  | 78 ± 6.4 | 82 ± 4.1 | NS       | 84 ± 27 | 62.1 ± 31 | NS       |
| CD4⁺ SP                                                       | 11.6 ± 3.7 | 8.6 ± 2.9 | NS       | 7.8 ± 4.4 | 5.5 ± 2.2 | NS       |
| CD8⁺ SP                                                       | 3.5 ± 1.6 | 2.0 ± 0.8 | 0.04    | 2.0 ± 1.6 | 1.1 ± 0.3 | NS       |
| Percent of Spleen Cells                                       |       |                               |      |
| Total CD4⁺                                                    | 28.7 ± 3 | 25.2 ± 2.8 | NS       | 48.0 ± 6.3 | 44.6 ± 6.2 | NS       |
| CD4⁺ CD62L⁺                                                   | 66.4 ± 2.9 | 67.6 ± 2.1 | NS       | 67.7 ± 3.0 | 72 ± 11.3 | NS       |
| CD4⁺ CD44⁺                                                    | 45.2 ± 5.5 | 42.4 ± 5.2 | NS       | 22.6 ± 2.0 | 21.3 ± 4.8 | NS       |
| CD4⁺ CD69⁺                                                    | 14.7 ± 4.4 | 15.8 ± 2.6 | NS       | 38.6 ± 3.8 | 47 ± 3.0 | NS       |
| CD4⁺ CD25⁺FoxP3⁺                                              | 11.3 ± 1.32 | 9.02 ± 0.7 | 0.01    | 11.1 ± 1.2 | 9.9 ± 1.4 | 0.03    |
| Total CD8⁺                                                    | 13.2 ± 2.9 | 10.8 ± 1.3 | 0.05    | 16.3 ± 0.5 | 15.7 ± 2.6 | NS       |
| CD8⁺ CD62L⁺                                                   | 89.7 ± 2.2 | 88.9 ± 2.3 | NS       | 87.3 ± 1.2 | 87.0 ± 6.3 | NS       |
| CD8⁺ CD44⁺                                                    | 44.1 ± 4.9 | 42.3 ± 5.7 | NS       | 14.9 ± 1.1 | 12.7 ± 1.0 | NS       |
| CD8⁺ CD69⁺                                                    | 8.6 ± 0.5 | 9.5 ± 1.1 | NS       | 53 ± 5.9 | 64.6 ± 6.5 | NS       |

CD4 or CD8 percentages are provided as percent of total cells for the thymus, spleen, and lymph node. CD62L⁺, CD44⁺, and CD69⁺-positive populations are expressed as percent of total CD4 or CD8 gated populations. NOD mice (five per group) were treated with 2 g/kg R788 in the chow for 3 mo beginning at 6 wk of age. DN, double-negative; DP, double-positive; SP, single-positive.
activity have been associated in some studies with an MZ-like immunophenotype, we further examined the percentages of IL-10–producing B cells in the spleen and peritoneum of treated and untreated mice. R788 treatment was consistently associated with increased percentages of IL-10–producing B cells in both the spleen and peritoneum (Fig. 9A). Absolute numbers of IL-10–producing B cells were not increased with treatment, however (2.2 versus 1.92 regulatory B cells [× 10^5 cells]/spleen, treated versus untreated; NS), because of a concomitant treatment related decline in total B cell numbers (Table I, II). In the spleen, the IL-10 producers were largely MZ-type (B220+, CD21^hi, CD23^lo), whereas the peritoneal populations were B-1 type (B220^hi, CD5^hi) (data not shown). In contrast, CD4^+ , CD25^+ , FoxP3^+ positive Treg populations were modestly decreased by percentages and absolute numbers in R788-treated mice (Fig. 9B). Treg absolute numbers were reduced in both the spleen (7.0 ± 3.4 versus 3.3 ± 1.2 untreated versus treated [× 10^6 cells/spleen]; p = 0.08) and in the pancreatic lymph node (0.27 ± 0.14 versus 0.11 ± 0.05, untreated versus treated [× 10^6 cells/spleen]; p = 0.03).

B cells obtained from the peritoneum lacked functional immunoregulatory capacity because peritoneal B cells from R788-treated mice cotransferred with untreated NOD splenocytes failed to protect NOD/SCID recipients from autoimmune diabetes (data not shown). Conversely, transferred splenic B cell populations contained dominant-acting tolerogenic cells because diabetes development in NOD/SCID recipients of untreated NOD splenocytes was suppressed by cotransferred splenic B cells (Fig. 9C). Non-B cells from R788-treated mice failed to protect consistent with the

FIGURE 8. R788 modestly inhibits humoral immunity. A, Frequencies of different splenic B cell subsets: B220^+CD21^+CD23^+ follicular/transitional-2 B cells (FO/T2), B220^+CD21^hiCD23^lo/neg MZ B cells, B220^+CD21^- CD23^- B-1 cells, and B220^+GL7^+ GC B cells. Splenic MZ B cells are significantly increased after 3 mo of treatment (2 g/kg in chow) with FO/T2 cells reciprocally decreased. *p = 0.007; **p = 0.0007. B, Total IgG and IgM ELISA immunoassays of the serum from untreated and treated NOD mice. Two months of R788 treatment modestly reduces total serum (IgG) and (IgM) in treated mice (p = 0.021 and p = 0.0505, respectively). C, ELISA for anti-insulin (left panel) and anti-GAD (right panel) autoantibodies. R788 treatment lowers the levels of anti-GAD autoantibodies in treated mice to the levels seen in nonautoimmune strains (C57BL.6 mice; p = 0.036), but did not inhibit the production of anti-insulin autoantibodies determined as the percent inhibition of plate-bound insulin binding in the presence of soluble insulin.
observation that the numbers of CD4*CD25*FoxP3* Tregs were mildly reduced in R788-treated mice. Thus, R788 treatment promotes the accumulation of IL-10–producing B cells with regulatory capacity that may contribute to sustained protection from diabetes development/progression in NOD mice.

Discussion

We have evaluated the potential for targeting the PTK Syk in APCs in animal models of autoimmune diabetes using genetic and pharmacologic approaches. In addition to its well-known function in transducing FcγR- and BCR-mediated events, Syk signals downstream of a growing list of immunoreceptor pathways that modulate the innate and adaptive response (32). Because genetic disruption in the germline is embryonically lethal due to vascular abnormalities (28, 29), the consequences of deletion in APCs are not well understood, and, even in bone marrow chimeric studies, syk deletion results in a superimposed B cell deficiency. In this study, we show that syk deficiency in DCs results in impaired Ab-mediated cross priming in vivo, establishing the necessity of the activating pathways downstream of Syk in the induction of Ab-mediated T cell autoimmunity and minimizing the efficacy of FcγR-mediated effector cell activation in this model of T cell-mediated autoimmunity (5).

In T1D, anti-islet Abs can be detected prior to the onset of overt diabetes and are a harbinger of disease in both human patients and NOD mice (35–37). This suggests that autoantibodies participate early in the pathogenesis of the disease and provides a valuable tool to identify at-risk patients that might benefit from preventive intervention prior to the development of islet-reactive T cell effector responses. Notably, μMT IgM knockout NOD mice, lacking all mature B cells, were shown to be resistant to autoimmune diabetes (1). Therapeutically, preclinical B cell-depleting strategies with anti-CD20 and anti-CD22 Abs have proven successful in preventing disease in NOD mice (38–40), and a single treatment of rituximab improved β cell preservation over a 1-y period in early-onset clinical T1D (41).

The prevailing view is that B cells contribute to the autoimmune process mainly as APCs (2, 3). Support for an additional pathogenic role of secreted IgG in promoting disease came from NOD mice that were genetically engineered to produce B cells bearing a membrane-bound form of Ig (IgM H chain) that cannot be secreted. Only 5% of such mice that cannot secrete Ig but retain B cell APC functionality develop diabetes, as compared with 80% of WT NOD mice (42). Similarly, NOD mice deficient in activating FcγRs are protected from diabetes (27). Furthermore, the mere elimination of maternally transmitted autoantibodies renders the NOD progeny diabetes resistant (43). These observations suggest dual roles for B cells in diabetes pathogenesis both as APCs and as producers of autoantibodies. However, the B cell-depleting therapies currently available fail to eliminate either plasma cells or their secreted circulating autoantibodies. We show in this paper that Syk inhibition blocks both BCR- and FcγR-mediated Ag presentation, providing the rationale to more broadly target the contributions of humoral immunity to autoimmune pathogenesis, as mediated by both B cells and their secreted products.

In preclinical rodent studies, R788 has been shown to ameliorate autoimmunity in models of asthma (44, 45), arthritis (46), and lupus nephritis (47). Early clinical studies with R788 has demonstrated efficacy in immune thrombocytopenic purpura (48), a disease in which Abs are the ultimate effectors, and in rheumatoid arthritis (20), a disease similar to T1D, in which autoimmune T cells are the predominant effectors. In addition to effects of Syk inhibition on FcγR- and BCR-mediated Ag presentation, our studies revealed two unanticipated effects of R788 on B cells and DCs that plausibly contribute to its therapeutic activity. First, DC numbers are reduced in vivo, likely through the inhibition of FLT3-mediated differentiation and survival signals. The reduction in DC populations could be the basis of the limiting immunogenic presentation of self-Ag as examined in this study for GAD and insulin (49, 50). It will be interesting to see whether other multi-PTK inhibitors also noted to have preclinical activity in autoimmune models (51) also share this off-target and potentially
beneficial effect of interfering with FLI3 kinase activity in vivo. The reduction in Tregs in the spleen and pancreatic lymph nodes of treated mice occurred in parallel with reduced DC numbers, potentially the direct consequence of a reduced homeostatic capacity of DCs to support Treg maintenance (33, 34). As a result, B cell-depleted splenocytes, mainly T cells, isolated from R788-treated NOD mice were unable to suppress disease in SCID recipients, consistent with a lack of R788-induced Treg activity.

Perhaps surprisingly, given the well-known role for Syk in BCR signaling, R788 treatment was not associated with large reductions in autoantibody levels. This was also seen in lupus-prone NZB/NZW mice, which were also successfully treated with R788 (47) without showing major changes in anti-nuclear Ab titers. Perhaps a more potent Syk inhibitor would more effectively reduce B cell development and activation. Syk inhibition was associated with the induction of IL-10–producing B cells, so-called B10 cells, a potent immunoregulatory subset (52–57) for which the inductive mechanisms in vivo are largely unknown (58–61). It has been previously shown that IL-10 production by murine and human B cells can be suppressed with concomitant BCR signaling (62, 63); thus, it is tempting to speculate that Syk inhibition acts by relieving BCR-mediated tonic repression of B10 differentiation, thereby promoting the accumulation of IL-10–producing regulatory B cells that may contribute to tolerogenic protection in R788-treated mice.

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Disclosures

J.S. and E.M. are employees of Rigel Pharmaceuticals.

References


THERAPEUTIC TARGETING OF Syk IN AUTOIMMUNE DIABETES


Corrections


The original description of the statistical analysis in the *Materials and Methods* section was incomplete. The statistical analysis for all figures and tables is provided below.

**Statistical analysis**

Kaplan-Meier survival curves were constructed using GraphPad Prism 4.0 software. Survival curve *p* values were calculated using a one-tailed log-rank test (Figs. 1B, 2B, 5A, 5B, 5D, 6A, 6B, 6C, 9C). The *p* values provided for comparisons between groups were calculated using either a two-way ANOVA (Fig. 3A, 3C), or a one-tailed paired (Figs. 2D, 3B, 3D, 4D, 6D, 7A, 8B, 9A, 9B, Table I [splenic T cells and myeloid cells], Table III [spleen and pancreatic lymph node]) or unpaired (Fig. 8A, 8C, Table I [bone marrow, total splenocytes, and splenic B cells], Table II, Table III [thymus]) Student *t* test.

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