Syk-dependent signaling pathways in neutrophils and macrophages are indispensable in the pathogenesis of anti-collagen antibody-induced arthritis

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Abstract

Spleen tyrosine kinase (Syk) is associated with Fcγ receptors (FcγRs) and transmits activation signals through FcγRs in myeloid cells. Thus, application of drugs to inhibit Syk activity can affect the development of immune diseases mediated by autoantibodies, while unexpected systemic effects by the inhibition may be concerned because Syk has multiple physiological functions. We used tamoxifen-inducible systemic conditional Syk knockout (KO) mice to evaluate the role of Syk in the pathogenesis of autoimmune arthritis and to investigate the systemic effects of Syk deletion.

In a collagen antibody-induced arthritis model, Syk KO mice were almost completely protected from disease induction and showed significantly attenuated accumulation of neutrophils and macrophages in the joints. Syk-deleted macrophages showed less IL-6 and MCP-1 production upon FcγR ligation and exhibited reduced FcγR-mediated phagocytosis in vitro. Syk-deleted macrophages produce more RANTES upon FcγR ligation, indicating a Syk-independent signaling through the FcγR. We further found that both wild-type and Syk-deleted macrophages induced neutrophil chemotaxis upon FcγR ligation in vitro, and air-pouch model demonstrated that Syk-deleted neutrophils have a potential to infiltrate into local tissues in response to collagen and anti-collagen antibodies. However, Syk-deleted neutrophils exhibited greatly decreased neutrophil extracellular traps formation and FcγR-mediated phagocytosis. Our results indicated that Syk deficiency rendered mice completely unresponsive to immune activation by anti-collagen antibodies with disabling one pathway of FcγR-mediated signaling that was crucial for arthritis induction.

Keywords: collagen antibody–induced arthritis (CAIA), inducible knockout mice, macrophages, neutrophils, Syk

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects close to 1% of the human population, manifesting severe chronic inflammation of the joints. Attack on the joints by the immune system leads to marked destruction and deformation, resulting in considerable disability for patients. The disease is characterized by the production of autoantibodies, synovial inflammation with formation of pannus tissue and erosion of the underlying cartilage and bone. Despite considerable efforts and significant advances of technology and research, the underlying cause of RA and its pathogenesis remain incompletely understood (1).

Tumor necrosis factor alpha (TNFα) inhibitors are currently the most potent treatment for active RA and together with methotrexate are used as a first-line therapy. However, a considerable number of patients do not respond to this therapy or lose their responsiveness over time. Side effects
Spleen tyrosine kinase (Syk), a member of nonreceptor tyrosine kinases, transmits signals from a variety of cell surface receptors including Fcγ receptors (FcγRs), B-cell receptors and integrins (3). FcγRs are expressed on most immune cells and activate these cells when they bind immune complexes composed of antibodies bound to a cognate antigen (4). Because RA is characterized by high levels of autoantibodies in the serum including rheumatoid factor, anti-citrullinated protein antibodies, anti-RA33 autoantibodies and anti-collagen antibodies (5), activation of FcγR-expressing cells by autoantibodies may be the key promoter of various cellular responses, including cytokine production that drives inflammation and tissue destruction. As evidence that FcγRs are important for initiating inflammation in the joints (6), phospho-Syk, the activated form of Syk, is detected at significantly higher levels in the synovial tissues of patients with RA than in those of patients with osteoarthritis (7). Therefore, increased signaling through Syk, via FcγR or other receptors, may be a pathway that can be targeted by drugs to hinder arthritis development or progression (8). In support of this key role for Syk, a phase II study with an orally administered Syk inhibitor, R788 (fostamatinib disodium), has shown positive effects in patients with RA who have had a suboptimal response to methotrexate (9, 10).

Although Syk inhibitors may show positive effects in the treatment of RA, possible unexpected effects associated with the inhibition of Syk must be considered in order to evaluate the long-term safety of Syk inhibitors in RA patients. The key effector cell types in RA, however, that require Syk for activation and the disease-relevant pathways downstream of Syk are largely unknown. Genetically modified gene-deleted animals are often used to explore the relevance of target molecules in disease models and can reveal unexpected effects associated with disabling the function of the target molecule. However, germ line deletion of Syk leads to perinatal death from severe hemorrhaging (11) and thus conventional Syk knockout (KO) mice have not been able to be used in in vivo models to elucidate the role of Syk in disease pathogenesis. To circumvent this limitation, in the current study, we used a conditional deletion system, in which Syk can be systemically deleted postnatally by tamoxifen administration.

We addressed the effect of Syk deletion at the age of 7 weeks on arthritic disease susceptibility using a collagen antibody–induced arthritis (CAIA) model. We also addressed the impact of Syk deletion in the function of immune cell types in vitro. This allowed us to evaluate the role of Syk in disease pathogenesis and the systemic effects of Syk deletion and to specifically investigate the molecular function of Syk in cells of both hematopoietic origin and in cells of hematopoietic origin, especially macrophages, that are difficult to target with a conditional gene KO due to the lack of specific gene promoters for running Cre-loxP system. Our study revealed that the postnatal deletion of Syk clearly protected mice from CAIA, suggesting that Syk plays an important role in the pathogenesis of arthritis. In addition, we confirmed that postnatal Syk deletion per se did not lead to any apparent adverse effects. Therefore, Syk is expected to be a good target for arthritis therapy.

### Methods

#### Mice

To obtain inducible Syk KO (Syklox/lox) mice, mice homozygous for the Syk gene flanked by loxP sites (obtained from Dr. A. Tarakhovsky, Rockefeller University) were crossed with homozygous Rosa26-Cre ER T2 mice at the Department of Respiratory Disease Research, Boehringer Ingelheim Pharma GmbH & Co. KG (12), and maintained under specific pathogen-free conditions at Kobe Pharma Research Institute, Nippon Boehringer Ingelheim Co., Ltd. All experiments were approved by the Animal Care and Use Committee at Nippon Boehringer Ingelheim Co., Ltd. Syk complete null mice (Sykdel/del mice) were prepared by tamoxifen treatment of Syklox/lox mice (12). B cell–deficient mMT mice (13) were kept at the division of Molecular Biology, Research Institute for Biological Sciences, Tokyo University of Science. C57BL/6J mice as a control were purchased from Japan SLC, Inc.

#### Syk deletion, PCR analysis of genomic DNA and western blotting

Syk deletion in Syklox/lox mice to produce Sykdel/del mice was induced by gavage of 20 mg/kg tamoxifen (Sigma, MO, USA) in 9% (v/v) ethanol in sunflower oil (Sigma) for 5 consecutive days (12). For Syk deletion in vitro, cultured cells derived from Syklox/lox mice were incubated with 0.6 μM of the active metabolite of tamoxifen, 4-hydroxytamoxifen (Sigma), for 2 days (14). Syk deletion was confirmed by PCR amplification of genomic DNA using the Extract-N-Amp Tissue PCR kit (Sigma) and the primers (Sigma Genosys, Hokkaido, Japan) derived from exon 2 of Syk, FP—5’-GCCCGTCTGCCTACTGG-3’ and RP—5’-GCTGGTCTGTCTCCTGG-3’, and western blotting using anti-Syk(N-19) and anti-β-actin (Santa Cruz Biotechnology, CA, USA), as described previously (12). When Sykdel/del mice or cells were used for any experiments, Syklox/lox mice or cells were used as controls.

#### Induction of CAIA model in mice

To induce CAIA, 5 mg of an anti-collagen II mAbs (CII-Ab, Arthritis-CIA Arthritogenic Monoclonal Antibody, # 53010; Chondrex, Inc., WA, USA) was injected on day 0 followed by 50 μg of LPS on day 3, both by intraperitoneal (i.p.) injection. To evaluate arthritis severity, the following scoring system was employed: 0, no evidence of erythema or swelling; 1, erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot and digits. The total combined score of all limbs was recorded each day (maximum score 16) (15).

#### Evaluation of immune cell populations

Immune cell populations of PBMCs, spleen and bone marrow (BM) cells were detected by flow cytometry (FACS Caliber or FACS Aria; BD Biosciences, NJ, USA). Antibodies against cell surface markers (BD Biosciences; Biolegend, CA, USA; R&D systems, MN, USA) used were as follows: CD3ε, CD4, CD8, TCRβ and TCRδ mAbs for T cell populations, B220 and...
IgD mAb for mature B cells in spleen, CD3e and NK1.1 mAbs for NK and NKT cells, CD11b and CD11c mAbs for macrophages and dendritic cells (DCs).

**Preparation and culture of BM-derived macrophages**

BM-derived macrophages (BMMFs) were prepared by culturing BM cells from 8-week-old to 12-week-old mice with 20 ng/ml M-CSF (eBioscience, CA, USA) for 4 days.

**Measurement of cytokines and chemokines**

For *in vitro* studies, BMMFs were plated at a density of 5 x 10⁴ cells/well in a 96-well plate and cultured with or without stimulation. BMMFs were stimulated with plate-bound IgG [anti-mouse CD16/CD32 (BD Biosciences)] or CII-Ab, with 100 μg/ml CII-Ab and 5 μg/ml bovine type II collagen (CII; Collagen Gijutsu Kensyukai, Tokyo, Japan) with or without LY294002 (Cellbiochem, CA, USA), PP1, Wortmannin or Y-27632 (Enzo Life Science, NY, USA), and supernatants were collected after the indicated times. For *in vivo* studies, the air-pouch exudates were collected by washing. Measurements of secreted cytokines and chemokines in both *in vitro* and *in vivo* samples were performed on a suspension array system (Luminex 200; Bio-Rad Laboratories, CA, USA) or ELISA (eBioscience; R&D systems) according to the manufacturer’s instructions.

**Quantitative real-time PCR**

Total RNA was extracted with RNeasy mini kits (Qiagen, Hilden, Germany) and was reverse transcribed using ReverTra Ace qPCR RT Master Mix (FQS-201; Toyobo, Osaka, Japan). Quantitative PCR was performed using a CFX384 real-time system (Bio-Rad Laboratories) following the manufacturer’s protocols. Oligonucleotide primers for amplification were purchased from Perfect Real Time Support system (TaKaRa Bio, Shiga, Japan). The calculated expression level of a tested gene was normalized relative to levels of β-actin in the same sample.

**Preparation of neutrophils**

Mouse peritoneal neutrophils were prepared by i.p. injection of 1 ml of 5% thioglycollate solution per mouse. Peritoneal exudates were collected after 18 h and checked for the percentage of neutrophils by flow cytometry.

**IgG-mediated phagocytosis**

BMMFs were seeded at a density of 2.5 x 10⁴ cells/well in a 96-well plate and then cultured overnight. After incubation with latex beads coated with fluorescein isothiocyanate (FITC)-labeled rabbit-IgG (Phagocytosis Assay Kit, #S50290; Cayman, MI, USA) for 2 h at 37°C, BMMFs were washed with PBS three times and then observed using fluorescence microscopy. For neutrophils, 1 x 10⁶ cells were incubated with latex beads for 2 h at 37°C and were washed with PBS three times. Cells were observed using fluorescence microscopy and analyzed by flow cytometry.

**Chemotaxis assay**

An EZ-TAXIScan six-channel chamber (BD Bioscience; Effector Cell Institute, Tokyo, Japan) was assembled with a 260-μm-wide x 4-μm-thick silicon chip on a 2-mm untreated glass base and filled with RPMI-HEPES medium containing 0.1% BSA. Neutrophils (1 μl, 2 x 10⁶ cells/ml) were added to the lower reservoir of the six channels each and allowed to line up at the upper–lower reservoir interface by removing buffer from the upper reservoir. RPMI-HEPES medium containing 0.1% BSA was then added to fill both reservoirs to the brim. One microliter of cultured supernatant from BMMFs with CII plus CII-Ab was then added to the upper reservoir and time-lapse images of neutrophil migration in each of the channels was captured sequentially every 30 s for 120 min.

**Mouse air-pouch model**

A dorsal air pouch was created by injecting mice (8–10 weeks old) with 5 ml of air subcutaneously on the back at day 0. On day 3, the pouches were reinflated with 3 ml of air. On day 6, inflammatory cell recruitment was induced by injecting 1 ml of PBS containing CII (10 μg) only or CII plus CII-Ab (100 μg) into the pouches. After 5 or 24 h, mice were anesthetized and sacrificed by cervical dislocation. The air pouches were lavaged with 2 ml of cold PBS and exudates were collected. Total migrated cells were counted, and neutrophil or macrophage contents were assessed with anti-Ly-6G or anti-CD11b and -MHC class II staining by flow cytometry.

**NETosis**

Neutrophils of 1 x 10⁵ cells were incubated with 5 μg/ml CII only or CII plus 100 μg/ml CII-Ab for 30 min at 37°C. CII-Ab was pretreated for 10 min before adding CII. Cells were centrifuged by CytoSpin (Thermo Fisher Scientific, MA, USA) and their DNA stained (SlowFade Gold Antifade Reagent with DAPI (4’,6-diamidino-2-phenylindole), #S36938; Molecular Probes, OR, USA), then were checked for neutrophil extracellular traps (NETs) formation by fluorescence microscopy. Percentages of cells producing NETs were determined by counting cells in eight fields each.

**Histological analysis**

Fore and hind limbs were fixed in 10% formalin. After fixation, samples were decalcified with Decalcifying Solution B (Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 10 days and then embedded in paraffin. After embedding, 2- to 4-μm-thick sections were obtained and stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections (2.4-μm) were prepared from formalin-fixed, paraffin-embedded tissue specimens and deparaffinized. Antigen retrieval was done in 10mM citrate buffer solution (pH 6). After quenching endogenous peroxidase with 3% H₂O₂ in methanol for 10 min and treatment with 1% BSA for 10 min at room temperature, the sections were incubated with a rat monoclonal anti-CD68 antibody (DA:11; Abcam, Cambridge, UK) at 1:200 dilution at 4°C overnight, incubated with second antibody (Histofine simple stain; Nichirei, Tokyo, Japan) for 30 min at room temperature and subsequently incubated with a DAB solution for staining.

**Statistical analysis**

Data were presented as the mean ± SEM. Student’s *t*-test was used to determine the significance of differences versus respective control.
Results

Postnatal Syk deletion rendered mice resistant to CAIA induction

To examine the effect of Syk on the inflammation process in a mouse CAIA model, we deleted the Syk locus with the tamoxifen treatment (Syk\(^{floxflox}\) mice), and after 4 weeks deletion efficacy was examined by genomic PCR and western blotting (12). Deletion of Syk locus occurred in a tamoxifen dose-dependent manner and diminished expression of Syk protein was confirmed (Fig. 1A and B). In the current study, thereafter, high dose as described in the Materials and Methods (20 mg/kg) was used for complete deletion of Syk locus. The complete deletion was sustained during whole experimental period we conducted. When Syk\(^{floxflox}\) mice were treated with CII-Ab for induction of CAIA, no arthritis developed, as indicated by the absence of obvious paw inflammation. In contrast, Syk\(^{floxflox}\) mice showed severe inflammation of the paws and an elevation in arthritis score by day 7 after administration of CII-Ab (Fig. 1C and D). As shown in Fig. 1E and F, histological analysis at 14 days after CII-Ab injection indicated that infiltration of leukocytes mainly including macrophages into the synovium was found around the joints, and bones were severely eroded in the Syk\(^{floxflox}\) mice. In contrast, the Syk\(^{floxflox}\) mice showed no signs of arthritis, such as increased infiltration of neutrophils and macrophages, necrosis of synovium or bone erosion. These data reiterated that Syk played a crucial role in the pathogenesis of CAIA.

Profiling of immune cells in Syk-deleted mice

Because Syk is known to express in various types of immune cells, we investigated whether postnatal deletion of Syk affected hematolymphopoiesis. Using tamoxifen to induce deletion, we found that Syk\(^{floxflox}\) mice had no effect on survival and renewal of red blood cells, granulocytes, platelets, NK/NKT cells, macrophages and DCs in peripheral blood, spleen and BM (Fig. 2A and Supplementary Tables I and II, available at International Immunology Online). We analyzed four Syk\(^{floxflox}\) mice to compare with four Syk\(^{floxflox}\) mice and we confirmed that T cell profile in Syk-deleted mice was comparable to that in wild-type (WT) mice (data not shown). However, B cell population in Syk\(^{floxflox}\) mice was relatively lower than in control Syk\(^{floxflox}\) mice (Fig. 2B). To check the effect of Syk in B cell development in Syk\(^{floxflox}\) mice, tamoxifen treatment was started on day 4 after birth, just before B cells develop from precursor cells, and the B cell component was analyzed by flow cytometry. We found that the pre-B cell population was reduced and both immature and mature B cells almost completely disappeared in Syk\(^{floxflox}\) mice. Impact of Syk deletion on detailed B-cell development was also shown (Supplementary Figure 1A, 1B and 1C are available at International Immunology Online). Syk deletion greatly impaired the development and survival of B cells during many stages of their development.

Therefore, we examined whether the reduced numbers in B cells could explain the attenuation of arthritis development. For this aim, we examined the arthritis induction in B cell-deficient muMT mice (13). Counterintuitively, muMT mice developed arthritis by CII-Ab treatment and were more susceptible to the arthritis induction than control mice (Fig. 2C). This result indicated that the reduced inflammatory responses in Syk\(^{floxflox}\) mice induced by CII-Ab in vivo were independent of the presence of B cells.

IL-6 production and FcγR-mediated phagocytosis were impaired in Syk-deleted BMMFs

Mice with CAIA often show bone erosion, influx of neutrophils, deposition of IgG and complement (C3) in articular cartilage (16). Likewise, in human RA, the joint is infiltrated by multiple inflammatory cells including macrophages and neutrophils (17). In our CAIA model, a single treatment with CII-Ab similarly induced joint inflammation (manufacturer's description on the product and data not shown). Therefore, we assumed that neutrophils and macrophages would be key players for inflammatory tissue damage in CAIA model. Macrophages in the inflamed synovium of RA can potentially produce inflammatory mediators that facilitate pathogenesis of arthritis, and this cell type is resident in joint cavities even in a normal state (18). We, thus, studied whether production of any proinflammatory cytokines was also impaired in Syk-deleted BMMFs upon stimulation with CII-Ab in vitro. We first confirmed deletion of Syk locus in BMMFs treated by tamoxifen in vitro and used these cells for our study (Fig. 3A). As shown in Fig. 3B, Syk\(^{floxflox}\) macrophages had significantly lower IL-6 production, a key proinflammatory cytokine regulating RA development, than that in Syk\(^{floxflox}\) cells when the cells were stimulated with plate-coated CII-Ab in vitro. However, we found IL-6 production was comparable between Syk\(^{floxflox}\) and Syk\(^{floxflox}\) BMMFs stimulated with CII plus CII-Ab, probably because CII plus CII-Ab initiated much stronger signal toward IL-6 production Syk independently (data not shown). Production of TNFα, another key cytokine for development of arthritis, could not be detected after stimulation with CII-Ab even in Syk\(^{floxflox}\) cells. We further investigated phagocytosis by macrophages of Ig-coated particles, which is one of the effector functions of macrophages. Phagocytosis of Ig-coated beads was drastically decreased in Syk\(^{floxflox}\) macrophages compared with Syk\(^{floxflox}\) macrophages (Fig. 3C), indicating that FcγR-mediated phagocytosis by macrophages was also a Syk-dependent event. These data suggested that deletion of Syk caused a distinct impairment of macrophage activation and interfered with the production of proinflammatory cytokines and phagocytic activities initialized by FcγR engagement.

MCP-1 was produced by macrophages in a Syk-dependent manner upon FcγR engagement

We then studied whether production of a variety of cytokines and chemokines was impaired in FcγR-stimulated Syk-deleted macrophages. Both Syk\(^{floxflox}\) and Syk\(^{floxflox}\) BMMFs were treated with the mixture of CII and CII-Ab in vitro. First, to identify which chemokine was profoundly produced by BMMFs in the treatment with CII plus CII-Ab, we analyzed chemokine and cytokine production secreted in the culture supernatants of BMMFs with Luminex 200 and identified that significant amount of monocyte chemoattractant protein-1 (MCP-1), MIP-1α, MIP-1β, RANTES, and KC (IL-8) were produced by BMMFs upon FcγR engagement (data not shown). We then prepared Syk\(^{floxflox}\) and Syk\(^{floxflox}\) BMMFs from Syk\(^{floxflox}\) and Syk\(^{floxflox}\) mice treated with tamoxifen in vivo.
mice and stimulated with CII plus CII-Ab. We found that MCP-1 production by Syk-del/del BMMFs was dramatically impaired compared with Syk-flox/flox BMMFs, whereas production of KC, MIP-1α and MIP-1β were comparable between Syk-flox/flox and Syk-del/del cells. Interestingly, RANTES production was significantly upregulated in Syk-del/del cells by CII plus CII-Ab engagement but not by plate-coated IgG (Fig. 4B and Supplementary Figure 2, available at International Immunology Online). Therefore, while MCP-1 production was dependent on Syk activity, production of KC, MIP-1α, MIP-1β and RANTES upon FcγR engagement appeared Syk independent. When Syk-flox/flox BMMFs were treated with various inhibitors, in addition to CII plus CII-Ab, PIP3 kinase inhibitor (LY294002, also known as Akt inhibitor), Src inhibitor (PP1) and PIP3 kinase inhibitor (Wortmannin) suppressed MCP-1 expression, whereas ROCK inhibitor (Y-27632) enhanced MCP-1 expression (Fig. 4C and D; Table 1). Interestingly, RANTES expression was rather enhanced by treatment of cells with Akt/Pi3K inhibitor or Src inhibitor, whereas ROCK inhibitor suppressed the expression of RANTES. These data collectively demonstrated that expression of these chemokines was regulated by various pathways downstream of FcγR and that deletion of Syk did not completely shut down the expression of these chemokines in macrophages.

Syk was dispensable in CII-Ab–induced neutrophil migration

We showed that MCP-1 was produced by macrophages in Syk-independent manner upon FcγR engagement. In case of RA, we hypothesize that residential macrophages that initially respond to CII-CII-Ab immune complex via FcγR attract influx of neutrophils. Therefore, in order to determine the effect of CII-Ab activated macrophages on infiltration and accumulation of neutrophils, we investigated whether Syk-deleted BMMFs could control chemotaxis of WT neutrophils using EZ-TAXIScan. In the absence of supernatant from normal macrophages, chemotaxis was barely observed in neutrophils. Addition of either supernatant derived from FcγR-stimulated Syk-flox/flox BMMFs or Syk-del/del mouse at 14 days after CII-Ab injection. (E) Histological analysis of joint sections of Syk-flox/flox and Syk-del/del mouse stained with H&E (original magnification: low, ×4 and high, ×20). (F) Immunohistochemical staining of joint sections of Syk-flox/flox and Syk-del/del mouse stained for macrophages with CD68 (low and high magnification).
in Syk\textsubscript{flox/flox} or Syk\textsubscript{del/del} mice, and injection with CII alone was performed as a negative control. The numbers of neutrophils in the lavage fluids of the air pouches were counted after 5 h. As shown in Fig. 5C, neutrophil accumulation was strongly induced by CII plus CII-Ab administration, and the accumulation rate was similar between Syk\textsubscript{flox/flox} and Syk\textsubscript{del/del} mice (total migrated cell number, Syk\textsubscript{flox/flox} mice: 18.4 ± 3.14 × 10^5 cells, Syk\textsubscript{del/del} mice: 16.2 ± 3.01 × 10^5 cells). Therefore, neutrophil infiltration in toto did not require Syk-mediated secretion of attractants. We also examined macrophage infiltration into the air pouches at 24 h after CII plus CII-Ab injection, finding there was no significant differences in macrophage recruitment between two lines of mice (Table 2).

**Syk was indispensable in CII-Ab–induced function of neutrophils as determined by NETs formation and FcyR-mediated phagocytosis**

Histological examinations (Fig. 1E and F) and data obtained in migration assay (Fig. 5 and Table 2) were apparently in disagreement. We assumed that induction and/or augmentation of inflammation by neutrophils was impaired in the absence of Syk, as demonstrated for macrophages in Fig. 3. We, thus,
Fig. 3. Effector function of BMMFs. (A) PCR analysis of genomic DNA isolated from tail of Syk\textsuperscript{flox/flox} (a), Syk\textsuperscript{del/del} (b) mice and from Syk\textsuperscript{flox/flox} (c), Syk\textsuperscript{del/del} (d) BMMF. Expected PCR product sizes are shown; floxed Syk gene: 1428 bp, gene locus after deletion: 320 bp. (B) IL-6 production by BMMFs cultured with plate-bound CII-Ab (0.1, 1, 10 and 100 μg/ml) or IgG (anti-mouse CD16/CD32, 10 μg/ml) for 1 day. Values are mean ± SEM. n = 4. Results are representative of two independent experiments. *P < 0.001, **P < 0.01 versus respective control. (C) Phagocytosis was determined by fluorescence microscopy of BMMFs incubated with FITC-labeled rabbit-IgG for 2 h at 37°C. Representative data from two independent experiments with similar results are shown.

Fig. 4. Effect of Syk-deletion on chemokine production in BMMFs. (A) MCP-1 and KC production from BMMFs in vitro cultured with 5 μg/ml CII only or CII plus 100 μg/ml CII-Ab for 1 day. Values are mean ± SEM. n = 4. Results are representative of three independent experiments. **P < 0.01 versus respective control. (B) mRNA expression of MCP-1, MIP-1α, MIP-1β and RANTES in BMMFs in vitro cultured with 5 μg/ml CII only or CII plus 100 μg/ml CII-Ab for 1 day. Values are mean ± SEM. n = 3. Results are representative of four independent experiments. **P < 0.01 versus respective control. (C and D) Effect of signal inhibitors on chemokine expression in BMMFs. mRNA expression of MCP-1 (C) and RANTES (D) in Syk\textsuperscript{flox/flox} BMMFs in vitro cultured with 5 μg/ml CII only, CII plus 100 μg/ml CII-Ab or CII plus CII-Ab plus inhibitors [25 μM Akt/PI3K inhibitor (LY294002), 50 μM Src inhibitor (PP1), 0.1 μM PI3K inhibitor (Wortmannin) or 10 μM ROCK inhibitor (Y-27632)] for 1 day. Values are mean ± SEM. n = 3. Results are representative of four independent experiments. *P < 0.001, **P < 0.01, ***P < 0.05 versus respective control.
addressed whether some proinflammatory functions of neutrophils controlled by FcγR engagement were affected by Syk deficiency. As one of the typical neutrophil functions during inflammation, we studied NETs formation. NETs are chromatin structures released as one of the first lines of defense against pathogens, or autoantigens (20). We found that FcγR engagement induced strong NETs formation (70% of cells undergoing NETosis) in control neutrophils. In contrast, NETs formation was greatly reduced in Syk−/− neutrophils (less than 10% undergoing NETosis) (Fig. 6A). We further investigated whether phagocytosis of Ig-coated particles in neutrophils, which is also an important effector function of neutrophils, was affected by Syk deficiency. As shown in Fig. 6B, Syk−/− neutrophils showed relatively low level of phagocytosis compared with that in Syk+/+ neutrophils, indicating that FcγR-mediated phagocytosis was Syk dependent. We, therefore, concluded that Syk was essential for the neutrophil function in FcγR-mediated NETs formation and phagocytosis by neutrophils, and in the absence of Syk, these processes by activated neutrophils were substantially reduced.

Discussion

There is a high unmet medical need for RA patients who are failing currently available biologic treatment, and to meet this need, new drugs to treat RA are required. Syk is a potential target for treatment of RA because of its involvement in various intracellular signaling pathways related to RA pathogenesis. One important role for Syk is in FcR signaling, which occurs in B cells, macrophages, DCs and neutrophils in response to autoantibodies, such as anti-collagen antibodies, in RA. Indeed, a kinase inhibitor targeting Syk (fostamatinib disodium, R788) is reported to have positive effects in RA (9, 10). However, the precise molecular and cellular mechanisms by which Syk are involved in disease initiation and progression are yet to be determined.

A previous report by Jakus et al. clearly demonstrated the biological effect of Syk inhibition in a mouse arthritis model (21). In this study, BM chimeras carrying a Syk-deficient hematopoietic system generated by transplanting Syk−/− fetal liver cells into lethally irradiated WT recipients were used. When examined in a K/BxN serum-transfer study, a model of autoantibody-induced arthritis, Syk−/− BM chimeras exhibited complete resistance to disease induction. However, their experimental approach did not show which cell types were responsible for the resistance provided by transfer of fetal liver cells and also did not provide information on the role of Syk within cells of nonhematopoietic origin.

In this study, we established a conditional Syk-deleted mouse system to evaluate the role of Syk in the development of arthritis using a CAIA model. The advantage of using the

Table 1. Summary of effect of signal inhibitors on chemokine expression in BMMFs

<table>
<thead>
<tr>
<th>Expression</th>
<th>MCP-1</th>
<th>RANTES</th>
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<tbody>
<tr>
<td>Syk deletion</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Akt/PI3K inhibitor</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Src inhibitor</td>
<td>↓</td>
<td>↑</td>
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<tr>
<td>PI3K inhibitor</td>
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<td>ROCK inhibitor</td>
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Chemokine expressions were shown by arrows according to the results in Fig. 4C and 4D.

↑ = upregulated; ↓ = downregulated.

*Statistically not significant and tendency.
the inflammatory sites for augmentation of inflammation by cytokine/chemokine production and proinflammatory processes, such as NETs formation and phagocytosis, may be more important, which was impaired by Syk deficiency. In a recent report, neutrophil-specific Syk deletion ameliorated development of arthritis induced by K/BxN serum and the importance of Syk-dependent cytokine production by neutrophils in disease development was clearly shown (24). We, however, identified that the amounts of chemokines and proinflammatory cytokines produced by normal (Syk sufficient) neutrophils stimulated with CII-Ab in vitro were far lower than those produced by BMMFs stimulated in the same way (our unpublished observation). In this context, macrophages activated via FceRI may play dominant roles as shown in Fig. 3A, 4A and 4B. Although production of ROS contributes to development of inflammation, CII-Ab treatment-induced ROS production by neutrophils was quite low in vitro (Supplementary Figure 3, available at International Immunology Online). Therefore, ROS production by neutrophils may not play significant roles in this experimental model. However, other important effector functions of neutrophils such as NET formation and Ig-dependent phagocytosis were strongly induced by FceRI engagement and these were largely Syk dependent (Fig. 6A and B). High levels of inflammatory cytokines in autoimmune patients are believed to sensitize neutrophils to NETosis (25). Similarly autoantibodies may trigger a switch from apoptosis to NETosis (25). Because NETs expose self-molecules extracellularly, they may lead to autoimmunity (25). These findings also indicate that neutrophils play a certain role in development of arthritis.

In contrast to neutrophils, macrophages produced relatively large amounts of chemokines and proinflammatory cytokines upon CII-Ab stimulation (Fig. 3B and 4A). While production of IL-6 and MCP-1 was largely dependent on Syk (Fig. 3B, 4A, and 4B). Presumably, various chemokines, produced either in a Syk-dependent or -independent way, collectively induced neutrophils migration, which in toto was not impaired by Syk deficiency in the chemotaxis assay and in vivo air-pouch model (Fig. 5). Despite these findings, mice with systemic deletion of Syk showed almost no inflammatory signs including cellular infiltration after CII-Ab injection (Fig. 1C), suggesting that MCP-1 and IL-6 produced in a Syk-dependent manner by macrophages may be important players for the disease progression in this model. MCP-1 is known to be a strong chemoattractant for macrophages, and it is a well-known factor which is highly expressed in synovial fluid and in serum of RA patients (26). Production of MCP-1 by macrophages in this context was largely dependent on Syk and may result in further accumulation of pathogenic macrophages into the inflamed sites (Fig. 1F). IL-6 is involved in both the initiation and the maintenance of the inflammatory and immunologic responses in certain autoimmune diseases, and contributes to the persistent damage of bone and cartilage and chronic inflammation that are observed in RA (27). In addition, IL-6 can stimulate synoviocyte proliferation and osteoclast maturation and activation (27). Production of IL-6 by macrophages in this context was also dependent on Syk (Fig. 3A). On the
other hand, it has been reported that IL-6 KO mice were not resistant to CAIA induction (28), and TNFα is rather a key factor. Although we could not detect TNFα production in our CAIA model (data not shown), it is likely that Syk deletion caused reduction of multiple cytokine productions at barely detectable levels.

The CAIA model can be used to evaluate the function of FcγR-bearing neutrophils, macrophages, B cells and other types of cells, without the induction phase of adaptive immune responses. It was previously reported that arthritis development was not observed in FcγR KO mice (29). Although muMT mice without mature B cells were not protected from CII-Ab-induced arthritis in our study, the importance of B cells in RA development has been proved (30). Indeed, a clinically available, chimeric anti-CD20 mAb, Rituximab, depletes B cells, and its efficacy in RA has been supported by several studies (31). In the current study, we confirmed that B cells were not required for development of CAIA. Interestingly, clinical score was even higher in muMT mice compared with WT mice. Reason for the greater disease severity might be that muMT mice have no ability to create endogenous antibodies, resulting in a stronger impact of exogenously added antibodies. The efficacy of Syk inhibitor shown in clinical trials might be in part due to its effect on B cells because adaptive immune responses do play a role in RA development. As such, the reduction in B-cell number in Syk−/− mice (Fig. 2B) may be beneficial when Syk inhibitor was considered for treatment. The effects of Syk inhibition on B-cell numbers/function must be followed up in both animal studies and clinical studies.

Synovial fibroblasts are nonimmune cells involved in development of RA pathogenesis, possessing the capability to produce proinflammatory cytokines (32). Syk is highly phosphorylated in the RA synovium (7) and it has also been reported that Syk is activated by TNFα in cultured fibroblasts (33). Reportedly, TNFα-induced production of certain cytokines by fibroblasts is dependent on Syk (7). The chemokine MCP-1 and the cytokine IL-6 are produced by fibroblasts (34). Advantage of our inducible Syk KO model is that we can study the function of Syk even in nonhematopoietic cells. We, therefore, prepared primary synovial fibroblasts from either Syk+/+ or Syk−/− mice and examined TNFα, IL-1β, or LPS-induced production of IL-6 and MCP-1. Both Syk+/+ and Syk−/− primary synovial fibroblasts grew at a comparable rate in vitro (data not shown). As shown in Supplementary Figure 4, available at International Immunology Online, primary synovial fibroblasts from Syk−/− mice expressed similar levels of IL-6 and MCP-1 in response to IL-1β, LPS and TNFα relative to those by cells from control Syk+/+ mice. This finding suggested that Syk in these cells was not required for the inflammation-associated function of synovial fibroblasts such as proliferation and cytokine/chemokine production. This fact is in contrast to the previous finding about the function of Syk inhibitors on human synovial fibroblasts (7). This discrepancy may arise from differences in the origin of the synovial fibroblasts used or the condition of the cells from RA patients. In addition, the effect of the Syk inhibitor on other molecules besides Syk cannot be excluded.

Osteoclast is also a key player in disease pathogenesis of arthritis. We checked serum level of RANKL among nontreated control C57BL6, Syk+/+ or Syk−/− CAIA mice, but the serum levels of RANKL were comparable among three groups (data not shown). However, bone erosion was detected only in Syk+/+ mice (Fig. 1E and F) indicating that osteoclast activity, presumably mediated by FcγR

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**Fig. 6.** Effector function of neutrophils. (A) NETs formation was induced by 5 μg/ml CII plus 100 μg/ml CII-Ab for 30 min at 37°C. Samples were fixed and stained for DNA (blue). Representative data from two independent experiments with similar results are shown. Percentages of cells producing NETs were determined by counting from eight fields each. Values are mean ± SEM. *P < 0.001 versus respective control. (B) Phagocytosis was determined by fluorescence microscopy and flow cytometric analysis of neutrophils incubated with FITC-labeled rabbit-IgG for 2 h at 37°C. Representative data from three independent experiments with similar results are shown.
engagement, might be severely affected by Syk deficiency. In line with our data, essential function of Syk in osteoclasts has been already studied by using BM chimera mice with transplanting WT and Syk−/− fetal liver cells into lethally irradiated WT recipients and it was shown that bone-resorptive capacity of Syk−/− osteoclasts was arrested even after receiving immunoreceptor tyrosine-based activation motif (ITAM)-dependent stimulation, such as FcγR-mediated one (35). This suggested that inhibiting Syk would also promise for treating arthritis in the context of inhibition of mature osteoclast's activity.

As a conclusion, we showed that Syk is indispensable for initiation and progression of disease courses of arthritis activated by CII-Ab in CAIA model. In this model of RA, we described a new role of Syk in macrophages for production of chemokines and cytokines. We also showed that in the downstream of FcγR, there are Syk-dependent and -independent pathways that drive inflammation-associated cell functions. Importantly, inhibition of Syk itself seems to induce no apparent adverse manifestations except for B-cell development, and it is possible that having an inhibitory effect on B cell development could even be of advantage in the treatment of arthritis. Taken together, a specific inhibitor of Syk appears to be an attractive target for the treatment of RA.

Supplementary data
Supplementary data are available at International Immunology Online.

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References
Syk-dependent signaling is indispensable in CAIA


