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Lineage-Specific Analysis of Syk Function in Autoantibody-Induced Arthritis

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Autoantibody production and autoantibody-mediated inflammation are hallmarks of a number of autoimmune diseases. The K/BxN serum-transfer arthritis is one of the most widely used models of the effector phase of autoantibody-induced pathology. Several hematopoietic lineages including neutrophils, platelets, and mast cells have been proposed to contribute to inflammation and tissue damage in this model. We have previously shown that the Syk tyrosine kinase is critically involved in the development in K/BxN serum-transfer arthritis and bone marrow chimeric experiments indicated that Syk is likely involved in one or more hematopoietic lineages during the disease course. The aim of the present study was to further define the lineage(s) in which Syk expression is required for autoantibody-induced arthritis. To this end, K/BxN serum-transfer arthritis was tested in conditional mutant mice in which Syk was deleted in a lineage-specific manner from neutrophils, platelets, or mast cells. Combination of the MRP8-Cre, PF4-Cre, or Mcpt5-Cre transgene with floxed Syk alleles allowed efficient and selective deletion of Syk from neutrophils, platelets, or mast cells, respectively. This has also been confirmed by defective Syk-dependent *in vitro* functional responses of the respective cell types. *In vivo* studies revealed nearly complete defect of the development of K/BxN serum-transfer arthritis upon neutrophil-specific deletion of Syk. By contrast, Syk deletion from platelets or mast cells did not affect the development of K/BxN serum-transfer arthritis. Our results indicate that autoantibody-induced arthritis requires Syk expression in neutrophils, whereas, contrary to prior assumptions, Syk expression in platelets or mast cells is dispensable for disease development in this model.

Keywords: Syk, arthritis, neutrophils, platelets, mast cells

INTRODUCTION

A number of autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, small vessel vasculitis, or pemphigoid diseases, are characterized by production of autoantibodies against various autoantigens of the mammalian body (1). Those autoantibodies are thought to contribute to the autoimmune disease pathogenesis, either directly by engagement of their target autoantigens (activating or function-blocking autoantibodies), or by triggering an inflammatory reaction and concomitant tissue damage caused by the infiltrating inflammatory cells.

The K/BxN serum-transfer arthritis is one of the most widely used mouse model of autoantibody-induced tissue damage. This model is initiated by systemic injection of serum from so-called K/BxN mice in which the expression of a specific T-cell-receptor transgene on an autoimmunity-prone genetic background leads to the generation of high titers of autoantibodies against the ubiquitously expressed glucose 6-phosphate isomerase enzyme (2–5). Transferring those autoantibodies with the K/BxN serum to naive animals triggers robust inflammation of the distal joints and of other tissues. K/BxN serum-transfer arthritis is triggered by immune complex (IC) deposition and concomitant activation of Fcγ-receptors (5). A number of hematopoietic lineages are thought to be involved in the development of K/BxN serum-transfer arthritis. The role of neutrophils is indicated by the fact that antibody-mediated depletion (6) or genetic deletion (7, 8) of neutrophils prevents arthritis development in this model. Arthritis development was also reduced in mast cell-deficient $Kit^{W/W\text{-}v}$ mice (9) suggesting an important role of mast cells. In addition, platelets were proposed to be required for the development of K/BxN serum-transfer arthritis by releasing platelet-derived microparticles upon collagen-induced activation in the synovial tissue (10).

Syk is a nonreceptor tyrosine kinase primarily expressed in cells of the hematopoietic lineage (11). It mediates signaling by a number of cell surface receptors including B-cell-receptors (12, 13), Fcγ- and Fcε-receptors (14–18), β_2 and β_3 integrins (19–21), C-type lectins (11, 22), and other receptors coupled to immunoreceptor tyrosine-based activation motifs (ITAMs) (11, 23). Given its role in various hematopoietic lineages and signaling downstream of diverse cell surface receptors, Syk is indispensable for a number of *in vivo* processes including B-cell development (12, 13), various inflammatory disease processes (17, 24, 25), antifungal immunity (26), or lymph vessel development (27). Based on its central role in the immune system, Syk has been proposed as a therapeutic target in various autoimmune and inflammatory diseases (11, 28).

We have previously shown that Syk is critically involved in arthritis development in the autoantibody-induced K/BxN serum transfer model (25). Our additional studies indicated that Syk is involved in a pathway downstream of Fc-receptors and Src-family kinases (29) and activates further downstream processes through PLCγ2 (30) and CARD9 (31). However, it is at present incompletely understood in which lineage(s) Syk needs to be expressed for arthritis development in this model. Bone marrow chimeric experiments suggested the role for Syk in one or more hematopoietic lineages (25). Several lines of evidence suggest an important role for Syk in neutrophils (19, 31, 32). An important role for GpVI, an ITAM-coupled collagen receptor on platelets, for the development of K/BxN serum-transfer arthritis (10) suggested a role for Syk in platelets for disease development in this model (33). Finally, the proposed role of mast cells (9, 34) and the critical role for Syk in mast cell activation (14, 18) raised the possibility that Syk expression in mast cells contributes to development of K/BxN serum-transfer arthritis.

The above studies prompted us to perform lineage-specific deletion of Syk from neutrophils, platelets, and mast cells, and to test the effect of those mutations on the development of autoantibody-induced arthritis in the K/BxN serum-transfer model. Our results indicate an important role for Syk expression in neutrophils whereas, contrary to our expectations, Syk expression in platelets or mast cells appears to be dispensable for arthritis development in this model.

MATERIALS AND METHODS

Animals

Mice carrying a deleted *Syk* allele (Syk^{tm1Tyb}, referred to as Syk^{-}) (12) were kept in heterozygous form and used to obtain $Syk^{-/-}$ and control fetuses for fetal liver transplantation (19, 25). Lineage-specific deletion of *Syk* was achieved by crossing MRP8-Cre (35), PF4-Cre (36), or Mcpt5-Cre transgenic mice (37) with animals carrying a floxed Syk allele ($Syk^{tm1.2Tara}$, referred to as Syk^{flox}) (38) to obtain MRP8-Cre$^{+}Syk^{flox/flox}$, PF4-Cre$^{+}Syk^{flox/flox}$, and Mcpt5-Cre$^{+}Syk^{flox/flox}$ mice, referred to as $Syk^{\Delta PMN}$, $Syk^{\Delta PLT}$, and $Syk^{\Delta MC}$ animals, respectively. Mice carrying the KRN T-cell-receptor transgene (2) were maintained in heterozygous form by mating with C57BL/6 mice. All transgenic mice were backcrossed to the C57BL/6 genetic background for at least six generations. Genotyping was performed by allele-specific PCR.

Wild type (WT) control C57BL/6 mice were purchased from Charles River or the Hungarian National Institute of Oncology (Budapest, Hungary). NOD mice, as well as a congenic strain carrying the CD45.1 allele on the C57BL/6 genetic background (B6.SJL-$Ptprc^{a}$) were purchased from the Jackson Laboratory.

Mice were kept in individually sterile ventilated cages (Tecniplast) in a conventional facility. All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

Bone marrow chimeras were generated by intravenous injection of unfractionated bone marrow or fetal liver cells into recipients carrying the CD45.1 allele on the C57BL/6 genetic background, which were lethally irradiated before by 11 Gy from a ^{137}Cs source using a Gamma-Service Medical (Leipzig, Germany) D1 irradiator. 4 weeks after transplantation, peripheral blood samples were stained for Ly6G and CD45.2 (Clones 1A8 and 104, respectively; both from BD Biosciences) and analyzed by a BD Biosciences FACSCalibur flow cytometer as previously described (see Figure S1A in Supplementary Material) (29).

K/BxN Serum-Transfer Arthritis

Mice carrying the KRN T-cell receptor transgene on the C57BL/6 genetic background were mated with NOD mice to obtain transgene-positive (arthritic) K/BxN and transgene-negative (non-arthritic) BxN mice (2, 30). The presence of the transgene was determined by allele-specific PCR and confirmed by phenotypic assessment. Blood was taken by retroorbital bleeding and sera from arthritic and control mice were pooled separately.

Arthritis was induced by a single intraperitoneal injection of 300 µl K/BxN (arthritic) or BxN (control) serum into intact mice or bone marrow chimeras, followed by daily assessment of arthritis severity for 2 weeks as described (30, 31, 39). Visible clinical signs were scored on a 0–10 scale by two investigators

blinded for the origin and treatment of the mice. Ankle thickness was measured by a spring-loaded caliper (Kroeplin).

Isolation and Activation of Neutrophils, Platelets, and Mast Cells

Mouse neutrophils were isolated from the bone marrow of the femurs and tibias of intact mice or chimeras by hypotonic lysis followed by Percoll (GE Healthcare) gradient centrifugation using sterile and endotoxin-free reagents as described (18, 31, 39). Cells were kept at room temperature in Ca^{2+}- and Mg^{2+}-free medium until use and prewarmed to 37°C prior to activation. Neutrophil assays were performed at 37°C in HBSS supplemented with 20 mM HEPES, pH 7.4. Adhesion-dependent superoxide release by neutrophils was followed by a cytochrome *c* reduction test from 100 µl aliquots of 4×10^6/ml cells plated on fibrinogen (Calbiochem) coated surfaces in the presence of 50 ng/ml murine TNF-α (PeproTech) as described (39).

Platelets were isolated from peripheral blood by mild centrifugation in the presence of heparin. For an *in vitro* aggregation assay (40), platelets were divided into two groups, one labeled with an anti-CD9-PE (Clone EM-04; Abcam) and the other one with an anti-CD9-APC (Clone eBioKMC8; eBioscience) antibody. The two differently labeled groups were mixed in equal volumes and were activated by 50 ng/ml Convulxin (Enzo Life Sciences) at 37°C while shaking at 700 rpm for 5 min. The reaction was stopped by BD FACS Lysing Solution (BD Biosciences). The samples were analyzed by flow cytometry, where platelets were identified according to their forward and side scatter characteristics. Aggregation was determined as the percentage of CD9-PE/CD9-APC double positive events (40).

Mast cells were cultured from the bone marrow in the presence of 5 ng/ml murine IL-3 and 20 ng/ml stem cell factor (both from PeproTech). The purity of the cultures was tested by an anti-FcεR antibody (Clone MAR-1; eBioscience) by flow cytometry. For *in vitro* activation, mast cells were first incubated with an anti-dinitrophenyl (DNP) IgE antibody (Clone SPE-7) at a final concentration of 0.5 µg/ml overnight at 37°C on fetal bovine serum (FBS)-coated plates, followed by the crosslinking of Fcε receptors by the addition of 100 ng/ml DNP-human serum albumin to the cell suspensions (both reagents from Sigma-Aldrich). After 30 min, the cells were washed and mast cells were kept in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) overnight at 37°C on FBS-coated plates. The release of the inflammatory mediator MIP-1α was tested from the cell-free supernatants by a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions. The absence of Syk did not have a major effect on neutrophil, platelet, or mast cell development and numbers (data not shown).

Biochemical Studies

For analysis of protein contents, neutrophils, platelets, and mast cells were lysed in 100 mM NaCl, 30 mM Na-HEPES (pH 7.4), 20 mM NaF, 1 mM Na-EGTA, 1% Triton X-100, 1 mM benzamidine, freshly supplemented with 0.1 U/ml Aprotinin, 1:100 Mammalian Protease Inhibitor Cocktail, 1:100 Phosphatase Inhibitor Cocktail 2, 1 mM PMSF, and 1 mM Na_3VO_4 (all from Sigma-Aldrich). After removal of insoluble material, lysates were boiled in sample buffer. Whole cell lysates were run on SDS-PAGE and immunoblotted using antibodies against Syk (Clone N19; Santa Cruz) or β-actin (Clone AC-74; Sigma-Aldrich) followed by peroxidase-labeled secondary antibodies (GE Healthcare). The signal was then developed using the ECL system (GE Healthcare) and exposed to X-ray film.

Presentation of the Data and Statistical Analysis

Experiments were performed the indicated number of times. Quantitative graphs and kinetic curves show mean and SEM from all independent *in vitro* experiments or from all individual mice from the indicated number of experiments. Statistical analyses were carried out by the STATISTICA software using two-way (factorial) ANOVA, with treatment and genotype being the two independent variables. In case of kinetic assays, area under the curve was used for statistical analysis. P values below 0.05 were considered statistically significant.

RESULTS

K/BxN Serum-Transfer Arthritis in $Syk^{-/-}$ Bone Marrow Chimeras

To test the role of Syk within hematopoietic lineage cells, we generated bone marrow chimeric mice by transplanting $Syk^{-/-}$ or WT control fetal liver cells into lethally irradiated recipients. As shown in **Figure 1A**, injection of arthritogenic K/BxN serum into WT control chimeras triggered robust inflammation of the ankle joints whereas no such response could be observed in $Syk^{-/-}$ bone marrow chimeras which carry Syk-deficient hematopoietic tissues. Quantitative kinetic analysis of the clinical scoring of arthritis (**Figure 1B**) or the ankle thickness (**Figure 1C**) revealed that $Syk^{-/-}$ bone marrow chimeras were completely protected from arthritis development in this model ($p = 3 \times 10^{-6}$ and $p = 1.3 \times 10^{-3}$, respectively). Those results confirmed our prior studies showing critical role for Syk in the hematopoietic compartment in K/BxN serum-transfer arthritis (25).

Syk Is Expressed in Neutrophils, Platelets, and Mast Cells

Prior studies suggested a role for neutrophils, platelets, and mast cells in K/BxN serum-transfer arthritis (6–10, 34), as well as the functional role for Syk in those cells (14, 16, 18–20). To confirm the presence of Syk in those lineages and its deletion from $Syk^{-/-}$ cells, we tested the expression of Syk in primary neutrophils and platelets, or bone marrow-derived mast cells, from WT and $Syk^{-/-}$ bone marrow chimeras. As shown in **Figure 1D**, Syk was present in all three cell types derived from WT but not $Syk^{-/-}$ bone marrow chimeras (see the entire blots in Figures S1B–D in Supplementary Material).

Efficacy and Specificity of Syk Deletion From Neutrophils, Platelets, and Mast Cells

To test the role of Syk in a linage-specific manner, we turned to Cre-lox-mediated lineage-specific conditional deletion of Syk. To this

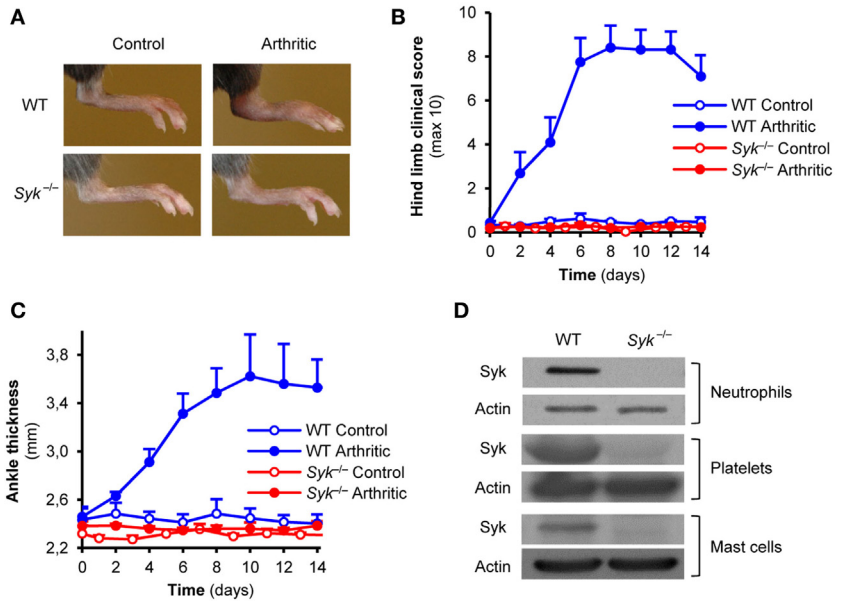

FIGURE 1 | Autoantibody-induced arthritis in Syk-deficient bone marrow chimeras. Wild type (WT) and $Syk^{-/-}$ bone marrow chimeras were injected with BxN (Control) or K/BxN (Arthritic) serum intraperitonally on day 0. Arthritis development was followed by photographing on day 7 **(A)**, clinical scoring of the hind limbs **(B)**, and ankle thickness measurement **(C)**. Panel **(D)** shows the absence of the Syk tyrosine kinase from whole cell lysates of $Syk^{-/-}$ neutrophils, platelets, and mast cells. Photos are representative of, and quantitative data show mean and SEM from, four control and four to five arthritic serum-treated individual mice per group from two independent experiments. Western blot images are representative of two to three independent experiments. See the text for actual *p* values.

end, we generated mice carrying the $Syk^{flox/flox}$ mutation along with a neutrophil-specific MRP8-Cre ($Syk^{\Delta PMN}$), the platelet-specific PF4-Cre ($Syk^{\Delta PLT}$), or the mast cell-specific Mcpt5-Cre ($Syk^{\Delta MC}$) transgenes. We then isolated neutrophils or platelets, and cultured bone marrow-derived mast cells, from those animals. As shown in **Figure 2A**, Syk expression was strongly reduced in $Syk^{\Delta PMN}$, but was not affected in $Syk^{\Delta PLT}$ or $Syk^{\Delta MC}$ neutrophils. Similarly, Syk was absent from $Syk^{\Delta PLT}$ but not from $Syk^{\Delta PMN}$ or $Syk^{\Delta MC}$ platelets (**Figure 2B**). Finally, Syk expression was abrogated in $Syk^{\Delta MC}$ but not in $Syk^{\Delta PMN}$ or $Syk^{\Delta PLT}$ mast cells (**Figure 2C**; see the entire blots in Figure S2 in Supplementary Material). Those results confirm both the efficacy and the specificity of the $Syk^{\Delta PMN}$, $Syk^{\Delta PLT}$, and $Syk^{\Delta MC}$ mutations.

Lineage-Specific Deletion of Syk Abrogates Functional Responses of Neutrophils, Platelets, and Mast Cells

To test the functional efficacy of lineage-specific Syk deletion, we also tested supposedly Syk-dependent functional responses of neutrophils, platelets, and mast cells. Superoxide release of neutrophils plated on a fibrinogen surface in the presence of a soluble TNF stimulus is mediated by β_2 integrins in a supposedly Syk-dependent manner (19). As shown in **Figure 3A**, this response was nearly completely blocked in neutrophils from $Syk^{\Delta PMN}$ animals ($p = 0.02$). Convulxin is a snake venom toxin activating the Fc-receptor-related collagen receptor GpVI on platelets in a Syk-dependent manner (40, 41). As shown in **Figure 3B**, convulxin induced aggregation of WT but not $Syk^{\Delta PLT}$ platelets ($p = 0.03$). Crosslinking of IgE bound to the surface of mast cells

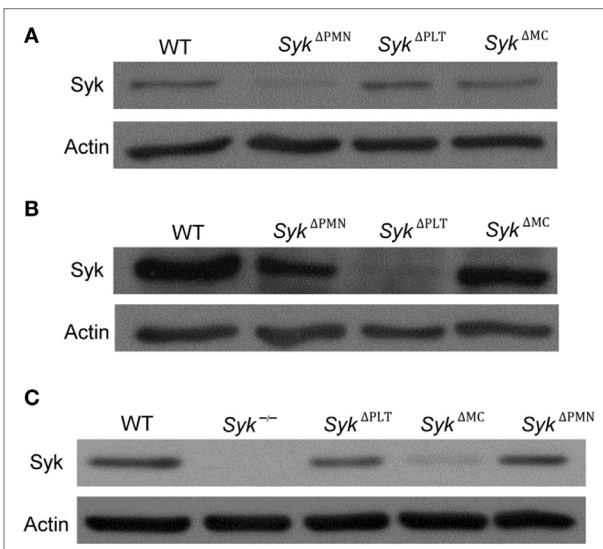

FIGURE 2 | The efficacy and specificity of lineage-specific Syk deletion from different cell types. The efficacy and specificity of lineage-specific deletion was tested by immunoblotting of whole cell lysates of neutrophils **(A)**, platelets **(B)**, and mast cells **(C)** derived from wild type (WT), $Syk^{\Delta PMN}$, $Syk^{\Delta PLT}$, or $Syk^{\Delta MC}$ mice or from $Syk^{-/-}$ bone marrow chimeras. Blots are representative of three independent experiments.

triggers release of various proinflammatory mediators through Fcε-receptors in a Syk-dependent manner (14). As shown in **Figure 3C**, MIP-1α release induced by IgE crosslinking of mast cells was abrogated by the $Syk^{\Delta MC}$ mutation ($p = 1.3 \times 10^{-4}$). Those

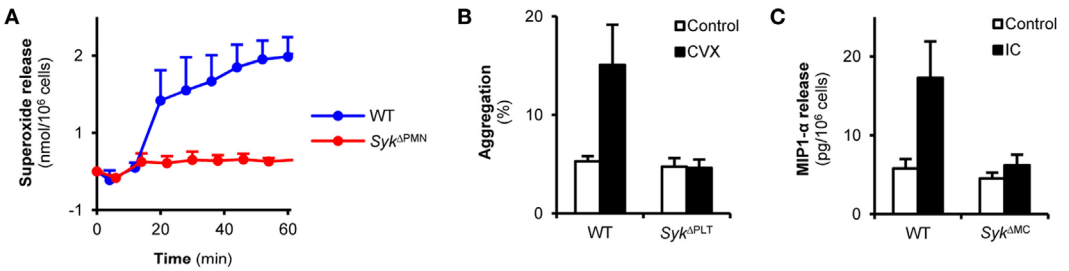

FIGURE 3 | Syk is indispensable for immunoreceptor tyrosine-based activation motif-mediated *in vitro* cellular responses of neutrophils, platelets, and mast cells. **(A)** Wild type (WT) or $Syk^{\Delta PMN}$ neutrophils were plated on fibrinogen-coated surfaces in the presence of TNF-α and their superoxide release was measured by a cytochrome *c* reduction test. Control data points were subtracted. **(B)** WT or $Syk^{\Delta PLT}$ platelets were isolated from peripheral blood, labeled by two different fluorochrome-conjugated CD9 antibodies and were activated by convulxin (CVX) for 5 min. Aggregation was measured as the percentage of CD9-PE/CD9-APC double positive events. **(C)** WT or $Syk^{\Delta MC}$ bone marrow-derived mast cells were incubated with anti-DNP IgE antibodies followed by an Fcε receptor crosslinking step with DNP-HSA. MIP-1α levels were determined from the cell-free supernatant by an ELISA assay. Kinetic curves and graphs represent mean and SEM from three **(A,C)** or six **(B)** samples from three **(A)** or two **(B,C)** independent experiments. See the text for actual *p* values. DNP, dinitrophenyl; HSA, human serum albumin, IC, immune complex.

results indicate that lineage-specific deletion of Syk from neutrophils, platelets, or mast cells leads to the expected functional consequences in those cells.

Neutrophil-Specific Deletion of Syk Abrogates Autoantibody-Induced Arthritis

We next tested the consequence of neutrophil-specific deletion of Syk on the development of K/BxN serum-transfer arthritis. As shown in **Figure 4A**, arthritogenic K/BxN serum triggered visible arthritis development in WT animals. However, no signs of arthritis could be observed in $Syk^{\Delta PMN}$ animals (**Figure 4A**). Quantitative kinetic analysis revealed that $Syk^{\Delta PMN}$ mice were nearly completely protected from development of clinical signs of arthritis (**Figure 4B**; $p = 1.5 \times 10^{-5}$) and arthritis-induced ankle swelling (**Figure 4C**; $p = 1.7 \times 10^{-3}$). Similar results could be observed when testing a larger cohort of bone marrow chimeras generated by transplanting WT or $Syk^{\Delta PMN}$ bone marrow cells into lethally irradiated WT recipients (**Figures 4D,E**; $p = 6.2 \times 10^{-7}$ and $p = 3.2 \times 10^{-6}$, respectively). Those results indicate a critical role for Syk expression within neutrophils for the development of autoantibody-induced arthritis *in vivo*.

Normal Arthritis Development Upon Platelet-Specific Deletion of Syk

Boilard et al. previously showed that genetic deletion of the Syk-coupled GpVI collagen receptor of platelets strongly reduced arthritis development in the K/BxN serum-transfer model (10), suggesting an important role for Syk expression in platelets in this model (33). To test this hypothesis experimentally, we tested K/BxN serum-transfer arthritis in $Syk^{\Delta PLT}$ mice in which Syk was deleted in a platelet-specific manner. Contrary to our expectations, platelet-specific Syk deletion did not affect the development of visual signs of arthritis in our model (**Figure 5A**). Quantitative kinetic analysis did not reveal any effect of the $Syk^{\Delta PLT}$ mutation on the clinical appearance (**Figure 5B**; $p = 0.51$) or on the ankle thickness increase (**Figure 5C**; $p = 0.76$) either. Similar results were obtained when using bone marrow chimeras generated by transplanting WT or $Syk^{\Delta PLT}$ bone marrow cells into lethally irradiated WT recipients (**Figures 5D,E**; $p = 0.49$ and $p = 0.9$, respectively). Those results, together with the lack of Syk (**Figure 2B**) and the defective Syk-dependent functional activation (**Figure 3B**) of $Syk^{\Delta PLT}$ platelets indicate that Syk expression in platelets is not required for the development of K/BxN serum-transfer arthritis.

Mast Cell-Specific Syk Deletion Does Not Affect Autoantibody-Induced Arthritis

Mast cells are one of the major targets of Syk function (14, 18) and they have also been proposed to participate in the development of K/BxN serum-transfer arthritis (9, 34). Therefore, we hypothesized that Syk expression in mast cells may be required for arthritis development in this model. To this end, we tested the development of K/BxN serum-transfer arthritis in $Syk^{\Delta MC}$ mice. As shown in **Figure 6A**, the $Syk^{\Delta MC}$ mutation did not affect the development of visible signs of arthritis in our model. Quantitative kinetic analysis did not reveal any inhibition of arthritis development either when scoring clinical signs of arthritis (**Figure 6B**; $p = 0.38$) or when measuring arthritis-induced increase of ankle thickness (**Figure 6C**; $p = 0.37$). By contrast, there was even a tendency of earlier arthritis development in the $Syk^{\Delta MC}$ animals (**Figures 6B,C**), raising the possibility of a negative role of Syk expressed in mast cells. Because of the radioresistance of mast cells, no bone marrow chimeras have been generated using $Syk^{\Delta MC}$ mice. The lack of inhibition of arthritis in $Syk^{\Delta MC}$ animals, together with the dramatic reduction of Syk expression (**Figure 2C**) and Syk-mediated functional responses (**Figure 3C**) in $Syk^{\Delta MC}$ mast cells indicates that Syk expression within mast cells is dispensable for arthritis development in the K/BxN serum-transfer model.

DISCUSSION

The Syk tyrosine kinase is critically involved in various inflammatory disease processes including the development of autoantibody-induced arthritis and dermatitis models (11, 17, 25). Given the wide expression of Syk in practically all hematopoietic lineages (11), understanding Syk function in a lineage-specific manner is of particular importance. Our results presented in this work indicate that of the three most prominent Syk-expressing lineages supposedly involved in the development of autoantibody-induced

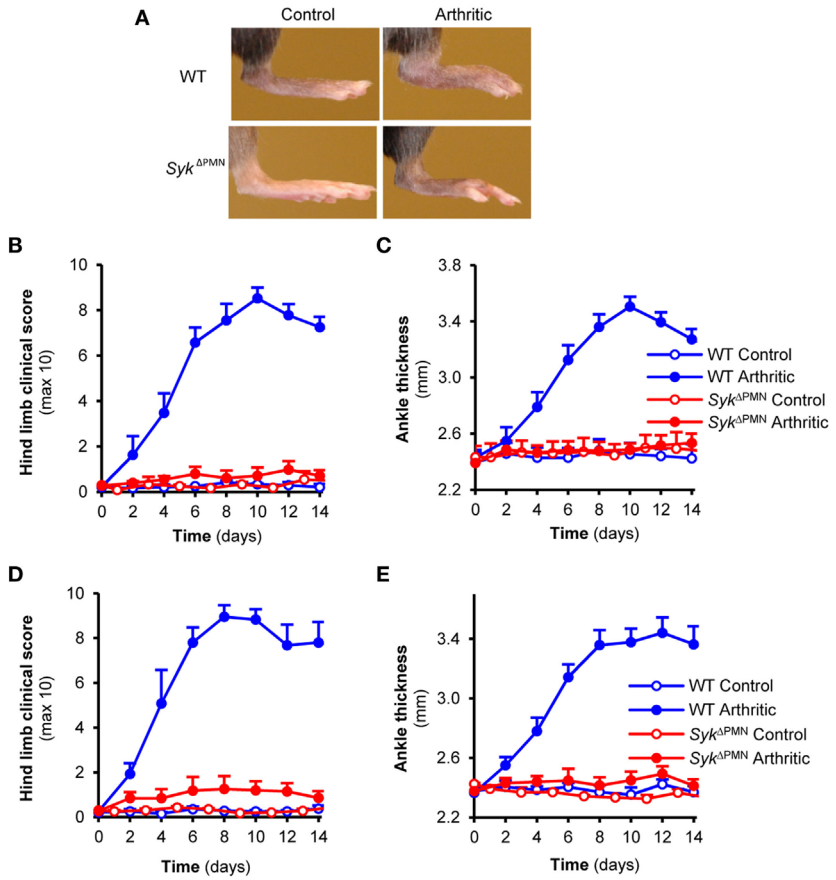

FIGURE 4 | Neutrophil-specific Syk deletion attenuates experimental arthritis. Wild type (WT) and *Syk*$^{\Delta PMN}$ intact animals **(A–C)** or bone marrow chimeras **(D,E)** were injected with BxN (Control) or K/BxN (Arthritic) serum intraperitonally on day 0. Arthritis development was followed by photographing **(A)**, clinical scoring of the hind limbs **(B,D)**, and ankle thickness measurement **(C,E)**. Quantitative data show mean and SEM from three control and five to six arthritic serum-treated individual mice per group from three independent experiments **(B,C)** or from five control and five to seven arthritic serum-treated mice per group from three independent experiments **(D,E)**. See the text for actual *p* values.

arthritis, Syk expression in neutrophils is critical, whereas that in platelets or mast cells is dispensable, for the development of K/BxN serum-transfer arthritis.

We and others have shown that Syk plays a critical role in various functional responses of neutrophils (16, 17, 19, 42, 43) without affecting neutrophil development (17, 19). Neutrophils have also been shown to be critical for the development of autoantibody-induced arthritis (6–8), likely at least in part through IgG IC-mediated activation of Fcγ-receptors expressed on the neutrophil cell surface (29), as well as by yet incompletely understood neutrophil-mediated initial vascular changes (8). Based on those studies, we hypothesized that Syk expression within neutrophils is critical for autoantibody-induced arthritis development. Our results confirmed that hypothesis, and they were also in line with prior studies from other groups (32) and our own analysis of neutrophil-specific deletion of the CARD9 adapter protein, a supposedly downstream effector of Syk (31). Though it is at present incompletely understood how Syk within neutrophils participates in autoantibody-induced arthritis development, our prior studies showing defective release of proinflammatory mediators by Syk-deficient neutrophils despite normal intrinsic migratory capacity of the cells (17, 19, 31) suggest that Syk, similar to Src-family kinases (29), participates in the amplification of neutrophil recruitment by neutrophil-derived proinflammatory mediators (44).

In contrast to our neutrophil-specific deletion studies, our platelet-specific deletion experiments did not support our hypothesis based on literature data. Though Syk is not required for platelet development (20), it plays a critical role in various platelet functions (11) including $\alpha_{IIb}\beta_3$ integrin-dependent platelet spreading (20), responses mediated by the hemITAM-coupled C-type lectin CLEC-2 (45), as well as signaling downstream of GpVI, an ITAM-coupled collagen-receptor of platelets (40, 41). GpVI is closely related to Fcα-receptors and it is directly associated with, and supposedly signals through, the ITAM-containing Fc-receptor γ-chain (FcRγ) (40, 41, 46–49). Platelets and, specifically, GpVI has been shown to play a critical role in the development of K/BxN serum-transfer arthritis (10), suggesting that Syk expression downstream of platelet GpVI is critically involved in arthritis development in this model (33). Our results of normal arthritis development upon platelet-specific deletion of Syk (**Figure 5**) despite practically complete lack of Syk from platelets

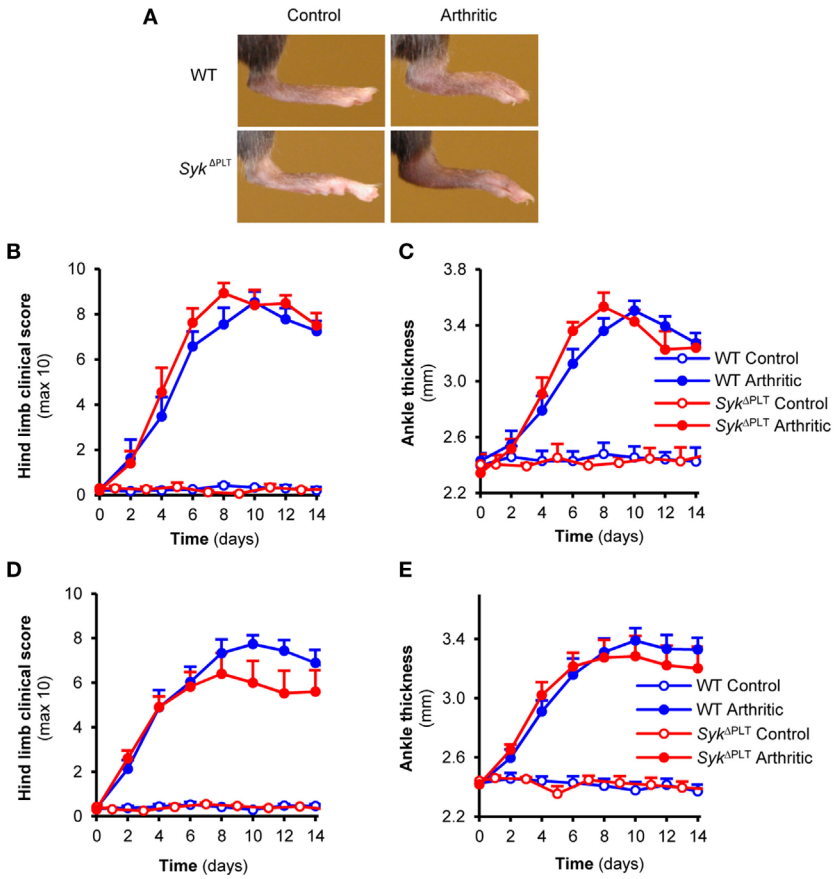

FIGURE 5 | Platelet-specific Syk deletion has no effect on autoantibody-induced arthritis. Wild type (WT) and *Syk*$^{\Delta PLT}$ intact animals **(A–C)** or bone marrow chimeras **(D,E)** were injected with BxN (Control) or K/BxN (Arthritic) serum intraperitonally on day 0. Arthritis development was followed by photographing **(A)**, clinical scoring of the hind limbs **(B,D)**, and ankle thickness measurement **(C,E)**. Quantitative data show mean and SEM from three control and five arthritic serum-treated individual mice per group from three independent experiments **(B,C)** or from six control and eight to nine arthritic serum-treated mice per group from three independent experiments **(D,E)**. See the text for actual *p* values.

(**Figure 2**) and completely defective GpVI-mediated *in vitro* platelet function (**Figure 3**) argues against that hypothesis. There are several possible explanations for those findings. Though GpVI is associated with the ITAM-containing FcRγ adapter, it may be able to bypass the ITAM-Syk pathway under certain conditions, using FcRγ as a chaperone required for cell surface expression but not as an ITAM-mediated signaling adaptor. Platelets have also been proposed to interact with fibroblast-like synoviocytes in a COX-1-dependent manner which is independent of platelet GpVI or FcRγ expression (50). This pathway may be able to compensate for the defective GpVI–FcRγ–Syk pathway upon platelet-specific Syk deletion. We also cannot exclude the possibility that GpVI needs to be expressed in a non-platelet lineage to support autoantibody-induced arthritis in mice. Finally, technical details such as a role for the small remaining Syk expression after Cre-mediated *Syk* deletion, or different experimental conditions may also account for the different conclusions drawn from our study and from those proposing a critical role for the platelet GpVI–FcRγ–Syk pathway in autoantibody-induced arthritis (10, 33). It should also be mentioned that our study focused on visible signs of arthritis and therefore we cannot exclude the possibility that Syk expression in platelets modulates the inflammation process by a mechanism not clearly visible by macroscopic inspection.

In the third part of our study, we tested the role of Syk in mast cells during autoantibody-induced arthritis. Syk has been shown to play a critical role in mast cell function without affecting mast cell survival (14, 18) and mast cells were proposed to be important players in autoantibody-induced arthritis development (9). Therefore, we hypothesized that Syk expression in mast cells may play a role in the development of K/BxN serum-transfer arthritis. Our results showing normal arthritis development in that model upon mast cell-specific Syk deletion (**Figure 6**) despite strongly reduced Syk expression (**Figure 2**) and defective Syk-mediated functional responses (**Figure 3**) in mast cells argue against that possibility. There are several possible explanations for those findings. Since the mechanism of how mast cells contribute to IgG autoantibody-induced disease pathogenesis is incompletely understood, it is possible that mast cells use a Syk-independent signal transduction pathway during K/BxN serum-transfer arthritis (e.g., when mast cells are not directly activated by the autoantibody-containing ICs, but rather indirectly through Syk-independent chemokine, cytokine, or PRR pathways). It

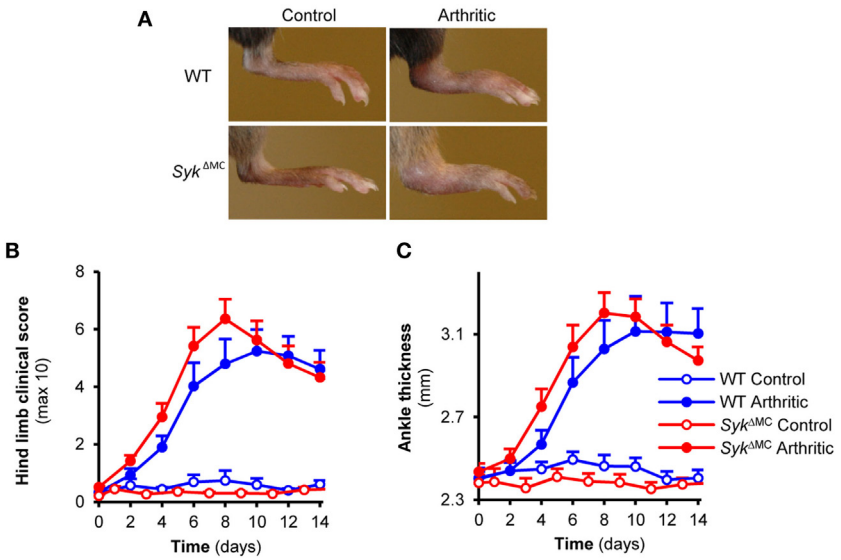

FIGURE 6 | Mast cell-specific Syk deletion does not affect the effector phase of experimental arthritis. Wild type (WT) and *Syk*$^{\Delta MC}$ animals were injected with BxN (Control) or K/BxN (Arthritic) serum intraperitonally on day 0. Arthritis development was followed by photographing **(A)**, clinical scoring of the hind limbs **(B)**, and ankle thickness measurement **(C)**. Quantitative data show mean and SEM from seven to nine control and twelve to thirteen arthritic serum-treated individual mice per group from four independent experiments. See the text for actual *p* values.

should also be mentioned that follow-up studies have questioned the critical role of mast cells in autoantibody-induced arthritis development (51, 52), pointing to difficulties of the interpretation of data obtained with different mast cell-deficient mouse strains. Indeed, our limited preliminary studies also suggested that the role of mast cells is highly dependent on the experimental conditions used for triggering autoantibody-induced arthritis in mice (Z. Jakus and A. M., unpublished observations). Finally, given that our experiments focused on visible signs of inflammation, we cannot exclude the possibility that Syk expression in mast cells may modulate arthritis development or the overall inflammation process in a manner not clearly visible by macroscopic assessment.

Besides neutrophils, platelets, and mast cells, Syk is also expressed in other lineages possibly involved in arthritis development. B-cells are one of the most prominent lineages requiring Syk function (12, 13). However, it is unlikely that Syk expressed in B-cells contributes to K/BxN serum-transfer arthritis since that model mimics the post-immunization effector phase of autoimmune arthritis and it develops normally even in the absence of B-cells in μMT-deficient or Rag-deficient mice (3). Macrophages have been proposed to be important players in the development of K/BxN serum-transfer arthritis (53). Unfortunately, currently available techniques do not allow the proper analysis of the *in vivo* relevance of Syk expression within macrophages because of the limited spectrum/specificity of the available macrophage-specific Cre-expressing mouse strains (54). We have previously shown that Syk is critically involved in osteoclast development and function (23). Though understanding the role of Syk in arthritis-induced bone erosions would be of clear importance, this question is beyond the scope of the present study focusing on the inflammatory aspect of autoantibody-induced disease processes.

Taken together, our results provide understanding of the role of Syk in autoantibody-induced arthritis at the cellular lineage level. Our findings indicate a critical role for Syk expression in neutrophils, but refute prior assumptions for the role of Syk in platelets and argue against a role for Syk expression in mast cells. Those results will strongly contribute to the understanding of the pathomechanism of autoantibody-mediated disease processes at the cellular and molecular level.

ETHICS STATEMENT

All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

AUTHOR CONTRIBUTIONS

TN and AM conceived the study, designed the experiments, and wrote the manuscript. TN, KF, KS, OV, and LK-P performed the experiments. TN, KF, and AM analyzed and interpreted the data. AM supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00555/full#supplementary-material.

FIGURE S1 | Flow cytometric analysis of bone marrow chimeras and Western blot images with more details. **(A)** Flow cytometric analysis of CD45.2-positive donor-derived neutrophils in the peripheral blood of wild type (WT) and *Syk*$^{\Delta PMN}$ bone marrow chimeras 4 weeks after bone marrow transplantation. **(B–D)** Detailed Western blot images showing the expression of Syk in neutrophil **(B)**, platelet **(C)**, or mast cell **(D)** lysates from **Figure 1D**.

FIGURE S2 | Detailed Western blot images showing the efficacy and specificity of lineage-specific Syk deletion in whole cell lysates of neutrophils **(A)**, platelets **(B)**, and mast cells **(C)** from **Figure 2**.

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