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Deletion of Syk in Neutrophils Prevents Immune Complex Arthritis

Emily R. Elliott,*† Jessica A. Van Ziffle,*† Patrizia Scapini,*† Brandon M. Sullivan,‡ Richard M. Locksley,* and Clifford A. Lowell*†

The K/BxN serum transfer model of arthritis is critically dependent on FcγR signaling events mediated by spleen tyrosine kinase (Syk). However, the specific cell types in which this signaling is required are not known. We report that deletion of Syk in neutrophils, achieved using sykf/f MRP8-cre+ mice, blocks disease development in serum transfer arthritis. The sykf/f MRP8-cre+ mice display absent joint disease and reduced deposition of pathogenic anti-glucose-6-phosphate isomerase Abs in the joint (with a reciprocal accumulation of these Abs in the peripheral circulation). Additionally, sykf/f MRP8-cre+ mice manifest poor edema formation within 3 h after formation of cutaneous immune complexes (Arthus reaction). Together, this suggests that neutrophil-dependent recognition of immune complexes contributes significantly to changes in vascular permeability during the early phases of immune complex disease. Using mixed chimeric mice, containing both wild-type and sykf/f MRP8-cre+ neutrophils, we find no impairment in recruitment of Syk-deficient neutrophils to the inflamed joint, but they fail to become primed, demonstrating lower cytokine production after removal from the joint. They also display an increased apoptotic rate compared with wild-type cells in the same joint. Mast cell-deficient c-kitsh/sh mice developed robust arthritis after serum transfer whereas c-kit+/+/sh mice did not, suggesting that previous conclusions concerning the central role of mast cells in this model may need to be revised. Basophil-deficient mice also responded normally to K/BxN serum transfer. These results demonstrate that Syk-dependent signaling in neutrophils alone is critically required for arthritis development in the serum transfer model. The Journal of Immunology, 2011, 187: 4319–4330.

Neutrophils are the most numerous leukocytes in the peripheral blood, comprising up to 50% of the compartment. They are among the first cells recruited at the initiation of inflammatory responses, and they make up the majority of cells found in sites of infection and tissue injury. Neutrophils are able to respond quickly to a large variety of stimuli, including immune complexes, complement, and pathogen-associated molecular patterns. After activation, neutrophils initiate phagocytosis, generate reactive oxygen species and cytokines, and release preformed granules containing inflammatory mediators. Different types of granules are released depending on the strength and type of signal, allowing neutrophils to modulate their responses (1, 2). Neutrophils are therefore able to shape the immune response by affecting the early inflammatory milieu. Indeed, neutrophils are known to be critical for the effective response to multiple infectious organisms (3). Recent discoveries have greatly broadened our knowledge about the functional role of this cell type in pathologic processes besides infection (4). It is noteworthy that neutrophils are responsible for much of the damage to host tissues in some types of autoimmune disorders, such as rheumatoid arthritis (5–7).

The K/BxN serum transfer mouse model of inflammatory arthritis reproduces many of the pathological hallmarks of human rheumatoid arthritis. In this model, transient arthritis is induced in naïve mice by transfer of serum from K/BxN transgenic mice containing Abs against glucose-6-phosphate isomerase (GPI) (8, 9). Clinical swelling and immune cell infiltrates are associated with IgG and C3 deposition along the cartilaginous surface leading to joint damage, including pannus formation and erosion of cartilage and bone (10). Although inflammation peaks between days 7 and 9 after transfer, disease may persist up to a month with no evidence of extra-articular inflammation (8). Inflammation in the K/BxN serum transfer model requires a variety of cellular mediators, including macrophages (11) and neutrophils (12), but does not require the adaptive immune system, making it a useful model for studying the role of the innate immune system in autoinflammation (8). In addition to IgG, FcγRs, and complement, inflammation depends on the production of leukotriene B4 (LTB4) (13), TNF-α, and IL-1β (14). There is some evidence that tissue-resident mast cells are primarily responsible for recognizing immune complexes in the joint, then initiating disease by inducing neutrophil and monocyte extravasation through release of chemokines (15–17).

Neutrophils are the predominant infiltrating cell in the arthritic joint and are required for K/BxN serum-induced disease (18). The LTB4–BLT1 (the cellular receptor for LTB4) pathway plays a crucial role in neutrophil recruitment in this model (13, 19). In addition to FcγRs and BLT1, neutrophils express most of the other

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Abbreviations used in this article: Cre, Cre-recombinase; GPI, glucose-6-phosphate isomerase; LTB4, leukotriene B4; PI, propidium iodide; poly-IC, polyinosinic-polycytidylic acid; Syk, spleen tyrosine kinase; UCSF, University of California, San Francisco.

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receptors known to be required for inflammation, including C5aR (20), and thus are able to recognize disease-causing immune complexes as well as other inflammatory mediators in the peripheral blood and the joint. These data suggest that neutrophils are key mediators of joint inflammation in the K/BxN serum transfer model. However, it is unknown if neutrophils are required for the recognition of immune complexes. Mice expressing the human FcγRIIA receptor only in neutrophils develop both arthritis (after KxB/N serum transfer) and nephritis (in response to injection of nephrototoxic serum) (7, 20), demonstrating the ability of neutrophils to initiate Ab-mediated inflammation. However, these mice lack FcRs in all other cell types, perhaps exaggerating the role of neutrophil FcRs. Previous data in the K/BxN model suggest that neutrophils initially respond to the inflammatory signals from other Fcγ-expressing cells such as mast cells (15) and macrophages (11). It is currently unknown how neutrophil Fcγ signaling participates in the initiation and progression of inflammation within the articular space.

Spleen tyrosine kinase (Syk) is required for signaling through FcγRs, integrins, and other scavenger receptors that use ITAMs to initiate intracellular signaling (21–23). Mice deficient in Syk die late in gestation due to dysregulated macrophage inflammation leading to vascular and lymphatic malformations (24, 25). The role of Syk in immune cells has been studied by transfer of syk−/− fetal liver cells into lethally irradiated recipient mice to generate chimeric mice lacking Syk in all hematopoietic lineages. Syk-deficient fetal liver chimeras are completely resistant to disease in the K/BxN serum transfer model, reflecting the critical role of FcγR signaling in inflammatory cells (26). However, the relative importance of Syk-mediated signaling in different cell types for the initiation or progression of disease cannot be determined using chimeric mice. To address this question, we have generated strains of Syk conditional mutant mice, which lack Syk in specific myeloid cell lineages. Using these mice, we have recently demonstrated the importance of Syk-based signaling in neutrophils alone as being critical for appropriate host defense to Staphylococcus aureus infection in vivo (3). We report for the first time, to our knowledge, that specific deletion of Syk in neutrophils is sufficient to block the initiation of arthritis in the K/BxN serum transfer model. These results suggest that neutrophils alone are essential for establishment of immune complex-mediated arthritis in this model. Indeed, using mast cell- and basophil-deficient animals, we find no requirement for these cells in the K/BxN model. These observations suggest that models for the pathophysiologic processes in immune complex arthritis may need to be revised.

Materials and Methods

**Mice**

Syk+/- mice (27) were back-crossed to C57BL/6 (Charles River) for eight generations, then crossed to MRP8-cre-/- (28), Ms-1-cre-/- (29), LynM-cre+/- (30), or CD11c-cre+/- (31) strains for conditional syk deletion. Importantly, the MRP8-cre gene also contains an ires-GFP marker, allowing us to track cells expressing the Cre-recombinase (Cre) by flow cytometry. Induction of Mx-1 expression by injection of polyinosinic-polycytidylic acid (poly-IC) was performed as described (29). Control mice for all experiments included either syk+/- or syk-/- with the relevant Cre or syk+/- or syk-/- without Cre to control for both Syk and Cre expression in the various cell lineages. NOD/ShiLtJ, C57BL/6-JtkI-wt-Sh/SamJ (c-kir+/-) (32), C57BL/6-JtkI-wt/v (c-kir+/-), and WR/Rej KitW/v (c-kir+/-) mice were purchased from the Jackson Laboratory. The c-kir+/- and c-kir+/- were intercrossed to obtain c-kir+/- mice (16). NOD/ShiLtJ mice were crossed to C57BL/6 using the KRN TCR transgene to obtain KxB/N mice (8). B6.SJL mice carrying the Ly5.1 allele, used for generation of mixed chimeric mice, were purchased from Taconic Farms. Basophil-deficient mice were generated by interbreeding Basophil8 mice (containing a YFP-ires-Cre gene inserted into the mast cell protease 8 gene: a basophil-specific marker) with Rosa26-tdTomato mice (containing a loxP-flanked diphtheria toxin a-chain gene inserted into the Rosa26 locus) as described (33). Mice were transferred with lethally irradiated B6 recipients with bone marrow cells, as described (34). Briefly, male 8- to 12-wk-old B6.SJL.CD45.1+ mice were lethally irradiated and injected i.v. with 5 × 10^6 mixed bone marrow cells. Chimeric animals were used for experiments after 8–10 wk. The percentage of chimerism was determined by flow cytometry on peripheral blood or inflammatory joint neutrophils using CD45.1 versus CD45.2 mAbs as described (34). Syk−/− chimeras were obtained by reconstituting lethally irradiated wild-type mice with Syk−/− fetal liver, as described (26). All animals were kept in a specific-pathogen-free facility at the University of California, San Francisco (UCSF) and used according to protocols approved by the UCSF Committee on Animal Research.

**K/BxN serum transfer and clinical scoring**

Arthritogenic serum was collected in Serum Gel Z1.1 tubes (Seadist) and pooled from 8-wk-old arthritic K/BxN mice. Disease was induced by injecting 200 µl serum i.p. into 6- to 8-wk-old recipient mice on days 0 and 2. Clinical scores were assessed on a 0–12 scale (0–3 per paw) as follows: 0, no edema: 1, localized edema or erythema; 2, localized edema and erythema on three or more toes and at talocural joint or over one entire surface of paw; 3, marked edema and erythema over entire paw surface. Mice were scored daily (19).

**Histology**

Paws were removed from euthanized mice above the tibiotalar and radiocarpal joint and flash frozen in OCT (Tissue-Tek) prior to sectioning. Frozen sections were obtained using the CryoJane Tape-Transfer System (Instrumedics). Sections were fixed in cold acetone and stained with fluorescently labeled goat anti-mouse IgG F(ab’2) (Jackson ImmunoResearch Laboratories) (19).

**Arthus reaction**

The cutaneous Arthus reaction was performed as previously described (35). Briefly, 30 µl 1 mg/ml rabbit anti-chicken OVA IgG or control rabbit IgG was injected intradermally on the backs of anesthetized mice, followed by the administration of 0.5% Evans blue and 2.5 mg/ml chicken OVA (no. 5503; Sigma) in PBS i.v. (200 µl per mouse). Skin from the intradermal injection site was collected 3 h later and placed in N,N-dimethylformamide for 48 h to extract the Evans blue. Evans blue concentration was determined by absorbance at 650 nm, and edema was evaluated as the ratio of Evans blue concentration in the tissue to that in the peripheral blood.

**Leukocyte isolation**

To isolate bone marrow neutrophils, femora, tibiae, and humeri were collected from euthanized 8- to 10-wk-old mice and flushed with saline. Collected marrow was homogenized with a 19-gauge needle and filtered through a 70-µm filter, followed by hypotonic RBC lysis. Neutrophils were purified by isolating cells from the Percoll layer of a 62% Percoll gradient, as previously described (34). The resulting neutrophils were at least 85% pure. Splenocytes were isolated by mechanical homogenization of whole spleens through a 70-µm filter, followed by hypertonc RBC lysis. Cells sorted by F4/80 and Ly6G surface markers were cytospun and stained by Wright–Giemsa. To obtain peritoneal cells, the peritoneum of euthanized mice were lavaged with 5 ml PBS/2 mM EDTA to isolate resident cells. Peripheral blood leukocytes were isolated by collecting 100 µl blood into heparinized saline, followed by hypertonc RBC lysis (3). Synovial fluid leukocytes were isolated by ankle joint aspiration using a 26-gauge needle into PBS/2 mM EDTA for staining, flow cytometry, and stimulation. Anesthetized mice were exsanguinated to reduce peripheral blood contamination of joint aspirates.
Leukocyte stimulation

Synovial leukocytes or bone marrow neutrophils were isolated as above, suspended in RPMI 1640 plus 10% FCS, then stimulated for 6 h at 37°C under CO2 with 1 μg/ml of brefeldin A (eBioscience) at 4 x 10^5 cells/100 μl. After stimulation, cells were placed on ice, surface stained for Ly6G, and stained intracellularly for TNF-α and IL-6 using the eBioscience IC Fixation Kit as described by the manufacturer. Reagents for stimulation were as follows: 10 ng/ml LPS or rabbit IgG immune complexes at 420 μg/ml for cytokine stimulation and 10 μg/ml for superoxide production. Immune complexes were generated by incubating rabbit anti-chicken OVA (no. 555419; BD Biosciences) with OVA in saline in a 10:1 ratio for 2-h incubation at 37°C, forming a visible insoluble immune complex precipitate (36).

Flow cytometry

For flow cytometry, isolated leukocytes were treated with 1 μg/ml Fc block (eBioscience), then surface stained with the following anti-mouse FcTC-, allophycocyanin-, allophycocyanin-Cy7-, PE-Cy7-, biotin-, PE-, PerCP-Cy5.5-, or Alexa Fluor 647-conjugated specific Abs (3): CD11b (M1/70), CD11c (HL3), CD62L (MLE-14), CD45.1 (A20), CD45.2 (104), Ly6G (1A8), Ly6C (AL-21), SiglecF (29A1.4), NKp46, DX5 (J0R50), CD131, FceR1a (MAR1), c-KIt (2B8), all from eBiosciences or BD Pharmingen; F4/80 (CI:A3-1; Serotec); 7/4 (Caltag); followed by streptavidin–Pacific orange. After final wash, cells were resuspended in staining/wash buffer containing 1 μg/ml propidium iodide (PI; Sigma-Aldrich) and/or annexin V (no. 555419; BD Biosciences) for viability staining according to the manufacturer’s instructions. For intracellular Syk and cytokine staining, cells were fixed using the eBioscience kit as described (3) and stained for TNF-α or IL-6 (MP6-XT22) or IL-6 (MP5-20F3) from eBioscience, and/or Alexa Fluor 488-conjugated mouse anti-Syk (5F5). Eight-color flow cytometry was performed on a Becton Dickinson LSR Fortessa and data analyzed with FlowJo software (Tree Star). Peripheral blood and synovial neutrophils and monocytes/macrophages were defined as CD11b^+ Ly6G^- and CD11b^- Ly6G^+, respectively (3). Macrophages were gated as CD11b^- and F4/80^- cells (37). Peritoneal mast cells were defined as c-KIt FceR1 double-positive cells.

Cytokine assays

Serum cytokine concentrations were determined using Cytokine Multiplex Kits for the Luminex technology according to the manufacturer’s protocol (Invitrogen).

ELISA for serum anti-GP1

Anti-GP1 Abs were identified by sandwich ELISA. Briefly, serum was incubated on plates coated with rabbit GPI (Sigma) and detected with an HRP-conjugated anti-mouse IgG-Fc Ab and TMB reagent (KPL 50-76-00). The reaction was stopped with acid and read at 450 nm on a SpectraMax M5 (Molecular Devices). Results are displayed as arbitrary units and standardized between experiments by normalizing to K/BxN serum (12).

Analysis and statistics

Graphs and statistical test were performed using Prism software. Statistical significance was determined by one-way ANOVA unless otherwise indicated, and error bars represent SEM (*p < 0.05, **p < 0.01, and ***p < 0.0001).

FIGURE 1. Neutrophil-specific deletion of Syk in syk^−/−MRP8-cre^+ mice. Leukocytes from mice of the indicated genotypes were stained for surface markers and Syk for analysis by flow cytometry, as described in Materials and Methods. Cell populations were analyzed for either Syk or GFP expression. Leukocytes from syk^−/- fetal liver chimeras served as a negative control for Syk staining. GFP positivity indicates the current expression of the MRP8-cre^+ires-GFP construct. A and B, Syk and GFP expression in peripheral blood, peritoneal, and splenic leukocytes. Histograms are representative of at least three separate experiments, with at least three mice per genotype per experiment. C, Mean fluorescence intensity of Syk staining in peripheral blood populations in syk^+/− MRP8-cre^+ mice (n = 8) compared with C57BL/6 wild-type mice and syk^+/− cells (n = 2–3). Only the significant differences by one-way ANOVA for Ly6G^+ and 7/4^+Ly6G^- cells are shown. Only staining in Ly6G^+ cells was significantly different between syk^+/− MRP8-cre^+ and wild-type controls. D, Percentage GFP^+ of peripheral blood leukocytes from syk^−/−MRP8-cre^+ and syk^+/− MRP8-cre^+ mice (n = 6). Only the significant differences by one-way ANOVA between Ly6G^+ and 7/4^+Ly6G^- cells in syk^+/− MRP8-cre^+ mice are shown. GFP positivity in Ly6G^+ cells was significantly higher than in all other cell populations analyzed. *p < 0.05, **p < 0.01, ***p < 0.001.
Results

Syk deletion in neutrophils protects against Ab-mediated arthritis

To determine the requirement for Syk in Ab-mediated arthritis, mice with LoxP flanked Syk alleles (syk<sup>fl</sup>) were crossed with cell-specific Cre-expressing strains. Inducing Cre expression under the control of the human MRP8 promoter (syk<sup>fl</sup> MRP8-Cre<sup>+</sup>) resulted in nearly complete Syk deletion in neutrophils as measured by intracellular flow cytometry (Fig. 1A–C, Supplemental Fig. 1). Intracellular Syk staining in neutrophils from syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice overlapped staining in cells from syk<sup>f/f</sup> fetal liver chimeras, confirming loss of Syk expression. In contrast, peripheral blood 7/4<sup>+</sup> Ly6G<sup>+</sup> monocytes as well as peritoneal F4/80<sup>+</sup> macrophages and splenic DX5<sup>+</sup> basophils from syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice exhibited similar Syk expression to syk<sup>fl</sup> littermate controls (Fig. 1A, 1B). The relative levels of Syk in monocyte cell types from Syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice are similar to those seen in wild-type C57BL/6 controls, in contrast to the Syk staining intensity in Syk<sup>fl</sup> MRP8-Cre<sup>+</sup> neutrophils, which is similar to Syk<sup>f/f</sup> neutrophils (Fig. 1C). Use of intracellular staining for Syk allowed us to confirm that every animal used in the following experiments demonstrated >90% reduction in Syk expression in peripheral blood neutrophils, as previously reported for syk<sup>f/f</sup> MRP8-Cre<sup>+</sup> mice (3).

The MRP8-Cre allele contains an irex-GFP construct that allows us to detect cells expressing Cre (3). While 95–98% of Ly6G<sup>+</sup> neutrophils expressed GFP, <20% of the various monocyte populations were GFP<sup>+</sup>, in general at a lower intensity, resembling a shoulder on the negative peak (Fig. 1A, 1D). Because this level of GFP expression did not correlate with significant differences in Syk expression (Fig. 1A, 1C), it is unclear if this level of potential Cre expression is physiologically relevant. Nevertheless, determining GFP levels allows us a potentially more sensitive approach to follow cellular expression of Cre in the MRP8-Cre<sup>+</sup> animals. Resident macrophages, basophils, mast cells, and some peripheral blood monocyte populations show low levels (5–20%) of GFP expression (Fig. 1B, 1D, Supplemental Fig. 1). Though we cannot rule out the effects of this low level of non-neutrophil Cre expression, no other cell population besides neutrophils shows efficient GFP expression and Syk deletion. We have also crossed the MRP8-Cre<sup>+</sup> strain to the Rosa26-YFP reporter strain (38) and have confirmed recombination predominantly in neutrophils, with <10% recombination in monocytes/macrophages (C. Abram and C.A. Lowell, unpublished observations). We also crossed syk<sup>fl</sup> mice to the Mx-1-Cre<sup>+</sup>, LysM-Cre<sup>+</sup>, and CD11c-Cre<sup>+</sup> strains to afford inducible deletion of Syk in all hematopoietic cells, in myeloid leukocytes only, or in CD11c<sup>+</sup> dendritic cells, respectively. The specificity and efficiency of Syk deletion in these strains was also monitored by intracellular flow cytometry and was previously described (3, 39).

The multiple syk<sup>fl</sup> Cre-expressing strains were treated with serum from K/BxN arthritic mice to determine the importance of Syk expression in various cell types for the initiation and progression of arthritis. Syk deletion in all hematopoietic cells using poly-IC-treated syk<sup>fl</sup> Mx1-Cre<sup>+</sup> mice (29) resulted in complete protection against Ab-mediated arthritis (Fig. 2A). These results confirm previous findings using Syk-deficient fetal liver chimeras, which lack Syk in all hematopoietic lineages and were completely resistant to serum transfer arthritis (26). Deletion of Syk in myeloid cells (syk<sup>f/f</sup> LysM-Cre<sup>+</sup>) was also protective (Fig. 2B), reflecting the previously published importance of macrophages and granulocytes in the development of disease (12). However, deletion of Syk in dendritic cells (syk<sup>f/f</sup> CD11c-Cre<sup>+</sup>) had no effect on the course of arthritis (Fig. 2C). This was expected, as dendritic cells have not been shown to contribute to inflammation during the effector phase of arthritis (9, 40).

Syk deletion in neutrophils (syk<sup>fl</sup> MRP8-Cre<sup>+</sup>) completely protected mice from K/BxN serum-induced arthritis compared with control mice (either Syk<sup>fl</sup> or Syk<sup>f/f</sup> MRP8-Cre<sup>+</sup>) (Fig. 2D, Supplemental Fig. 2A). Histological evaluation of ankle sections from syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice after K/BxN serum transfer showed no evidence of cellular infiltration, pannus formation, or cartilage and bone erosion, whereas these features were readily apparent in joints from control syk<sup>fl</sup> mice (Fig. 3A, 3B). Moreover, syk<sup>fl</sup> mice developed robust synovial fluid accumulation containing neutrophils and macrophages (Fig. 3B, 3C), whereas syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice showed no joint swelling, and no synovial fluid could be collected from these animals. Syk<sup>fl</sup> MRP8-Cre<sup>+</sup> mice also

![Figure 2](http://www.jimmunol.org/Downloadedfrom)
displayed less evidence of systemic inflammation after serum transfer, as demonstrated by lower serum levels of KC, MCP-1, and TNF-α (Fig. 3D). Thus, the lack of Syk in neutrophils greatly diminished both the systemic and local inflammatory response to articular immune complexes.

Deletion of Syk in the neutrophil lineage in syk<sup>−/−</sup> MRP8-cre<sup>+</sup> mice did not alter myeloid cell development as determined by cell counts (3), expression of myeloid cell markers (Table I), or morphologic examination (Fig. 3E). Similarly, syk<sup>−/−</sup> fetal liver chimeric mice demonstrated no alteration in myeloid cell development or marker expression, but of course did have a block in B cell maturation (3). To address further the possibility that Syk deletion was occurring in monocyte/macrophage lineages, we examined GFP expression in the synovial leukocytes of syk<sup>+/−</sup> MRP8-cre<sup>+</sup> mice after K/BxN serum transfer. As in resting animals, the peripheral blood and joint Ly6G<sup>+</sup> neutrophils of arthritic mice showed 90–95% expression of GFP, whereas monocyte/macrophage cell types made up only 5% of the synovial leukocytes (Fig. 3C).

To rule out the possibility that deletion of syk in non-hematopoietic cells may be contributing to the lack of response in the K/BxN arthritis model, we generated chimeric mice by transferring wild-type bone marrow into lethally irradiated syk<sup>−/−</sup> MRP8-cre<sup>+</sup> recipients, then tested these animals for arthritis development. The syk<sup>−/−</sup> MRP8-cre<sup>+</sup> mice with wild-type hematopoietic cells responded normally (Supplemental Fig. 2B). These
results suggest that Syk signaling in neutrophils is required during the initiation and progression of Ab-mediated arthritis and that Syk-dependent signaling pathways in other cell types is not sufficient to induce arthritis. As Syk is necessary for Fc receptor signaling in response to immune complexes, we expect that the requirement for Syk in K/BxN serum-induced arthritis reflects a nonredundant role of neutrophils in responding to pathogenic immune complexes in both the joint and possibly in the serum.

Loss of Syk affects the kinetics of Ab deposition

The deposition of anti-GPI immune complexes in the joint after K/BxN serum injection is influenced both by rapid (minutes) changes in vascular permeability as well as more long-term (hours to days) changes (10, 41). Though Ab accumulation is required for disease, it can occur despite the absence of clinical arthritis. To explore whether neutrophil-specific Syk deficiency affected anti-GPI immune complex deposition, we examined both joints and serum for the presence of anti-GPI Abs. To our surprise, the amount of IgG deposited along the cartilage at day 7 was greatly reduced in syk−/− MRP8-cre+ mice (Fig. 4A). The reduced accumulation of anti-GPI Abs in the joints of syk−/− MRP8-cre+ mice was not due to a reduced serum half-life of the Abs, as the syk−/− MRP8-cre+ animals consistently had greater anti-GPI serum titers throughout the course of disease (Fig. 4B). Syk−/− MRP8-cre+ chimeric mice with wild-type bone marrow do not maintain this trend (Supplemental Fig. 2C), ruling out an effect from possible syk deletion in inflamed endothelial cells. These results suggested that loss of Syk-dependent signaling in neutrophils resulted in a failure of anti-GPI Abs to exit the vasculature and deposit in joints, potentially due to a failure to induce vascular permeability.

To evaluate whether neutrophil-specific Syk deficiency affected early events of immune complex-induced vascular permeability, we used the cutaneous reverse passive Arthus reaction. Syk−/− MRP8-cre+ mice developed less severe edema at early time points, 3 h after immune complex induced inflammation, compared with wild-type animals (Fig. 4C). The decrease in edema was not as complete as that seen in FcγR-deficient mice, suggesting that Fc signaling in other cell types also contributes to increased vascular permeability. A lack of induced vascular permeability in the Syk−/− MRP8-cre+ mice may contribute to the reduced joint deposition of anti-GPI Abs, which would further protect these animals after K/BxN serum transfer.
Syk-deficient neutrophils migrate to the inflamed joint

The lack of clinical disease in syk<sup>−/−</sup> MR8-cre<sup>+</sup> mice may reflect both an inability of Syk-deficient neutrophils to respond to joint immune complexes or a block in their ability to migrate into the inflamed joint. To help distinguish these possibilities, we generated mixed chimeric mice to assess the behavior of Syk-deficient neutrophils in the presence of wild-type neutrophils that could generate inflammatory arthritis. Congenically marked mixed bone marrow chimeras were generated from various ratios of wild-type (CD45.1) and syk<sup>−/−</sup> MR8-cre<sup>+</sup> (CD45.2) bone marrow. The mixed chimeric mice were then treated with K/BxN serum. The disease course in mixed bone marrow chimeras depended on the ratio of wild-type to Syk-deficient neutrophils, as assessed by peripheral blood examination. Chimeras with a neutrophil compartment composed of 75% or greater Syk-deficient neutrophils developed less severe arthritis, with decreased maximum clinical scores (Fig. 5A). Animals with a lower percentage of Syk-deficient cells were not significantly different from wild-type (data not shown). Syk-deficient neutrophils were easily found in the synovial fluid of inflamed joints based on their congenic marker (CD45.2) and lack of Syk expression by intracellular staining (Fig. 5B). Importantly, the ratio of wild-type to Syk-deficient cells in the joint was a linear reflection of the extent of chimerism in the peripheral blood over multiple chimeric mice (Fig. 5C). These results indicate that Syk-deficient neutrophils are able to migrate normally into the inflamed joint. Hence, the lack of disease in syk<sup>−/−</sup> MR8-cre mice must reflect an impaired ability of the neutrophils to become activated rather than a migratory defect. Indeed, Syk-deficient neutrophils show a profound block in respiratory burst when stimulated with immune complexes in vitro (Supplemental Fig. 3) (21).

Syk deficiency affects specific effector functions in the inflamed joint

To determine the particular functional defects of Syk-deficient neutrophils in the K/BxN model, we induced arthritis in mixed chimeras to compare expression activation markers, apoptosis, and cytokine production by the two neutrophil types present in the same inflammatory joint environment. Syk-deficient neutrophils in the inflamed joint showed equivalent upregulation of CD11b and shedding of CD62L (Fig. 6A, 6B) compared with wild-type cells in the same joint, indicating that Syk deficiency did not reduce exocytosis of secretory granules (3, 42). Further, activation in the peripheral blood is unaffected by Syk deficiency, as wild-type and Syk-deficient peripheral blood neutrophils from K/BxN serum-treated chimeras equivalently shed CD62L compared with wild-type neutrophils from untreated mice. However, a greater proportion of Syk-deficient neutrophils in the joint were undergoing cell death than wild-type cells, as defined by being both annexin V and PI positive (Fig. 6C). No difference was seen in the proportion of neutrophils in earlier stages of apoptosis, defined as annexin V positive but PI negative (2, 43).

Though early markers of neutrophil activation were intact, there was evidence that Syk-deficient neutrophils in the inflamed joint were less primed for late-stage effector functions. The Syk-deficient neutrophils were less competent to induce TNF-α production, as determined by intracellular staining after stimulation, compared with wild-type cells (Fig. 6D). As expected, immune complex stimulation elicited TNF-α production in wild-type but not Syk-deficient cells. Similarly, bone marrow-derived Syk-deficient neutrophils failed to produce TNF-α when stimulated with immune complexes (Fig. 6E, 6F). Surprisingly, Syk-deficient neutrophils from the inflamed joint did not respond to LPS as robustly as the wild-type cells. In contrast, the percentage of naive Syk-deficient neutrophils from the bone marrow that induced TNF-α expression was equivalent to wild-type bone marrow neutrophils (Fig. 6E), and on a per cell basis, they produced more TNF-α in total, consistent with the increased TLR responses reported in Syk-deficient macrophages (Fig. 6F) (44). This indicates that the reduced TNF-α production in Syk-deficient synovial neutrophils is not due to a general defect in responsiveness, but instead suggests that these cells are poorly primed in the inflamed joint.

**FIGURE 5.** Syk-deficient neutrophils migrate equivalently to the inflamed joint. A, Mixed bone marrow chimeras were generated from syk<sup>−/−</sup> MR8-cre<sup>+</sup> (CD45.2<sup>+</sup>) and congenically marked wild-type mice (CD45.1<sup>+</sup>; CD45.1<sup>+</sup>) in ratios varying from 75% syk<sup>−/−</sup> MR8-cre<sup>+</sup> with 25% wild type to 25% syk<sup>−/−</sup> MR8-cre<sup>+</sup> control chimeras were also made with 100% wild-type or syk<sup>−/−</sup> MR8-cre<sup>+</sup> bone marrow. Eight weeks after bone marrow transfer, chimeras were injected with K/BxN serum, and clinical score was recorded as described in Materials and Methods. Data shown are for control chimeras and the 75% syk<sup>−/−</sup> MR8-cre<sup>+</sup> mix only (n = 5 mice per group). B, Synovial fluid neutrophils were isolated at day 7 after serum transfer from a mixed chimeric mouse (containing roughly 50% syk<sup>−/−</sup> MR8-cre<sup>+</sup> and 50% wild-type cells) then stained for CD45.1, CD45.2 (top panel), and Syk protein (bottom panel). Peripheral blood neutrophils from a syk<sup>−/−</sup> chimeric mouse were used to define Syk<sup>−</sup> cells (data not shown). C, Peripheral blood and synovial neutrophils from mixed chimeric mice were stained for Ly5.1 versus Ly5.2 to determine the percentage of syk<sup>−/−</sup> MR8-cre<sup>+</sup> versus wild-type cells in each compartment as shown in B. The percentage of syk<sup>−/−</sup> MR8-cre<sup>+</sup> (designated % Syk<sup>−</sup>) in peripheral blood versus synovial fluid is shown for each individual mouse (R<sup>2</sup> = 0.96).
FIGURE 6. Altered cytokine production, but not activation marker expression, by Syk-deficient neutrophils in the arthritic joint. A and B, Ly6G+ neutrophils were isolated from the peripheral blood and synovial fluid of arthritic mixed bone marrow chimeras on day 7 after K/BxN serum transfer and stained for CD11b and CD62L (L-selectin). Mean fluorescence intensity of CD11b and CD62L for Syk-deficient (CD45.2+) or wild-type (CD45.1+) gray bars) neutrophils are shown compared with peripheral blood neutrophils from a control B6 mouse not treated with serum (black bars). C, Day 7 synovial fluid neutrophils from mixed chimeric mice were stained with Ly6G, annexin V, and PI, and the percentage of positive cells for each marker is shown. A–C, n = 7. D, Day 7 synovial fluid neutrophils were pooled from eight mixed chimeric mice and stained for intracellular TNF-α, CD11b, and CD62L (L-selectin). Mean fluorescence intensity of CD11b and CD62L for Syk-deficient neutrophils, to examine the role of these cells in the innate immune-effector stage of inflammatory arthritis. Because Syk is required for FcγR-mediated effector function (45), which would be a major inducer of cell migration, these data suggest that neutrophils are responding directly to immune complexes to mediate disease in this arthritis model.

Mast cells and basophils are not required for Ab-mediated arthritis

Previous research suggested that mast cells play a central role in the K/BxN disease process, as the c-kit+/– mast cell-deficient strain of mice is protected from arthritis development (46). A general model for the progression of arthritis in K/BxN serum transfer has been recognized of joint immune complexes by resident mast cells (47). Given that we observed disease protection in mice with a neutrophil-specific mutation, we sought to reevaluate the role of mast cells. Indeed, we found that the c-kit+/– strain of mast cell-deficient mice responded normally to K/BxN serum challenge (Fig. 7A). In contrast, the mast cell-deficient c-kit+/– strain showed only minimal response to K/BxN serum challenge (Fig. 7B) as previously reported (46). We confirmed that both strains of mice lacked mast cells in the peritoneum (Fig. 7C) and joint (Fig. 7D). Mast cells were also readily detectable in the joints of syk+/+ MRP8-cre+ mice after K/BxN serum transfer (Fig. 7E), showing that this population was grossly unaffected by MRP8-cre–induced Syk deletion. We conclude that the lack of arthritis in c-kit+/– mice reflects the relative neutropenia and poor neutrophil recruitment found in these animals (48).

Basophils have been recently shown to play a large role in immune complex-mediated inflammation (49). As some MRP8-cre expression, marked by GFP, was seen in splenic basophils (Supplemental Fig. 1), we sought to determine the effect of basophils on K/BxN serum-induced arthritis. For this purpose, we induced arthritis in basophil-deficient mice, generated using a basophil-specific Cre under the Mcpt8 promoter (Basophil8) crossed to mice containing the Rosa-floxDTa allele (Dox+) (33). Basophil-deficient mice develop a course of arthritis identical to control littermates (Fig. 8A). Expression of Dto in DX5+ basophils, which can be tracked by theires-YFP construct within the Basophil8 allele, leads to apoptosis and depletion (Fig. 8B) (33). Combined with the results from the syk+/ MRP8-cre+ mice, we conclude that basophils are not required for the development of K/BxN serum-induced arthritis.

Discussion

We have used syk+/ MRP8-cre+ mice, which lack Syk in neutrophils, to examine the role of these cells in the innate immune-mediated effector stage of inflammatory arthritis. Because Syk is required for FcγR-induced signaling, the neutrophils in these mice fail to respond to immune complexes. Loss of Syk signaling in neutrophils is sufficient to protect mice from K/BxN serum-induced arthritis. Clinical swelling is greatly reduced or absent
in the syk/−/− MRP8-cre+ mice; the synovium of these animals is undisturbed, with no evidence of bone and cartilage damage. Syk/−/− MRP8-cre+ mice also demonstrate decreased Ab accumulation along the cartilage and lower serum cytokine levels. Further, Syk signaling pathways in neutrophils are required at several stages of immune response, including early induction of vascular permeability. However, using mixed chimeras, it is clear that Syk is not required for neutrophil migration into the joint if inflammation is already established. Neither mast cells nor basophils are required for arthritis development after K/BxN serum challenge. These observations help redefine the pathogenesis of inflammatory arthritis in this model and emphasize the neutrophil dependence of this disease.

These conclusions are based on the neutrophil specificity of the MRP8-cre gene. Although monocytes/macrophages may express MRP8 (50), we found no significant deletion of Syk or upregulation of Cre expression in these cell types. Therefore, although we cannot completely rule out a contribution from syk deletion in monocytes/macrophages, it would be minor compared with neutrophil deletion.

The original model of disease progression in K/BxN arthritis postulates that tissue-resident mast cells are required for the initial recognition of anti-GPI immune complexes, promoting vascular permeability and Ab deposition and elaborating proinflammatory mediators leading to neutrophil recruitment, activation, and tissue injury (8, 9, 16, 47). Indeed, mast cell-deficient c-ki+/− mice are

**FIGURE 7.** Mast cell deficiency does not affect the development or persistence of Ab-mediated arthritis. A and B, K/BxN serum was administered to c-ki+/− versus c-ki+/− mice or wild-type versus c-ki+/− mice, and disease was followed as described in Materials and Methods. C, Flow cytometric staining of resting peritoneal cells, stained for the mast cell markers c-Kit and FcrR1 from c-ki+/−, c-ki+/−, and control c-ki+/− mice. D and E, Toluidine blue staining (original magnification ×50) as described in Materials and Methods on (D) forepaws from serum-untreated c-ki+/−, c-ki+/−, and control mice and (E) hindpaws from syk/−/− and syk/−/− MR8-cre+ mice 7 d after serum transfer. Magnified views of the regions highlighted by the squares are shown to the lower right of each panel. Dotted lines approximate the epidermis, arrows indicate mast cells, white arrowheads indicate cartilage, and black arrowheads indicate the epiphysial plate. Data shown are averaged from three to five mice per cohort.
Systemic immune complexes induce both acute (5–10 min after administration) and long-term (hours to days) changes to the vascular endothelium that increase permeability and extravasation (19, 41). Arthritis can occur in the absence of acute vascular leak in the joint, though disease is slightly attenuated (41, 51). Notably, Fc receptor expression by neutrophils can rescue arthritis on an FcRγ−/− background but does not rescue the vascular leak defect observably at 45 min after serum injection (20). This observation appears to be at odds with our finding that deletion of Syk in neutrophils causes a decrease in Ab deposition in the joint. However, our results in the cutaneous Arthus reaction show that edema in response to immune complexes is reduced in sykf/f MRP8-cre+ mice after several hours. We suggest that very early acute vascular changes occur through a separate mechanism from that of the more long-term process of edema, where the latter at least partially depends on IgG recognition by neutrophils.

Syk is also responsible for signaling through other neutrophil receptors that use ITAM-dependent signaling pathways, such as integrins, some C-type lectin receptors, and cytokine receptors (52). Reduced integrin signaling may contribute to impaired neutrophil migration, as Syk-deficient neutrophils do show reduced recruitment in the cremaster muscle model after superfusion with chemoattractant peptides (53). However, we have found no defect in Syk-deficient neutrophil recruitment in thioglycolate-induced sterile peritonitis (54), hemorrhagic vasculitis in the skin (55), or as shown in the current study in the K/BxN model of arthritis. We conclude that the loss of integrin signaling in Syk-deficient neutrophils has variable effects on cellular migration dependent on the inflammation model. Whether Syk deficiency would alter cellular recruitment in other tissue sites remains to be tested.

Because sykf/MRP8-cre+ mice are protected from arthritis despite normal migration in Syk-deficient neutrophils, the block in inflammation likely occurs upstream of substantial neutrophil recruitment. Similar findings were reported with mice lacking BLT1, the LTβ4 receptor, in which reconstitution with wild-type neutrophils induced arthritis and the recruitment of BLT1+/− neutrophils to the joint (19). We propose that Syk-dependent signaling in neutrophils is required for the elaboration of chemokines and cytokines, such as TNF-α and LTβ4, which induce further recruitment of additional monocytes and neutrophils, leading to more cytokine production and tissue damage, in the fashion of a self-amplifying loop. Activation of tissue-resident cells alone is not sufficient to induce significant neutrophil recruitment in sykf/MRP8-cre+ mice, even though Syk-deficient neutrophils could otherwise migrate into the joint if a sufficient inflammatory signal was present. Undoubtedly, the majority of neutrophil activation occurs in response to tissue-deposited immune complexes. However, the decreased Ab deposition in joints of sykf/MRP8-cre+ mice suggests that at least part of the neutrophil activation occurs in the peripheral blood.

The role of Fc receptors in human autoimmune disease is complex, as these molecules mediate both activating and inhibitory signaling. Hypomorphic alleles of the human FcγRIIA, RIIA, and RIIB are associated with increased disease severity and nephritis in patients with systemic lupus erythematosus (56, 57). These hypoactive FcγRs could result in decreased immune complex clearance, paradoxically leading to accumulation of IgGs in tissues that would mediate chronic immune cell activation (58). Syk is therefore an attractive therapeutic target, as it is required for signaling through all FcγRs. Indeed, Syk inhibitors are efficacious in multiple animal models of autoimmune arthritis and systemic lupus erythematosus; phase II clinical trials with rheumatoid arthritis patients show promise (59). Our data suggest that part of the
efficacy of Syk inhibitors could stem from the inhibition of im-
mune complex-induced activating signals in innate immune cells
(7, 20, 26).

Overall, these results suggest that signaling through Syk in neutrophils is the major mediator of arthritis in the K/BxN model, whereas immune complex recognition by other cells, in particular mast cells and basophils, plays a less important role in disease development. In combination with similar findings in immune complex nephritis (7), this suggests that neutrophils are the dominant pathogenic cell in most immune complex-mediated diseases. Obviously, this hypothesis will require further testing, but it does significantly alter the pathogenic models of immune complex involvement, particularly diminishing the role of mast cells. This would have direct implications in the development of cell-targeted therapeutics for treatment of immune complex dis-
cease.

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Disclosures

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