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*J Immunol* published online 14 September 2011
http://www.jimmunol.org/content/early/2011/09/14/jimmunol.1100341

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/09/14/jimmunol.1100341.1.DC1

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Deletion of Syk in Neutrophils Prevents Immune Complex Arthritis

Emily R. Elliott,*† Jessica A. Van Zifflé,*† 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 syk<sup>f/f</sup> MRP8-cre<sup>+</sup> mice, blocks disease development in serum transfer arthritis. The syk<sup>f/f</sup> MRP8-cre<sup>+</sup> 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, syk<sup>f/f</sup> MRP8-cre<sup>+</sup> 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 syk<sup>f/f</sup> MRP8-cre<sup>+</sup> 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-kit<sup>sh/sh</sup> mice developed robust arthritis after serum transfer whereas c-kit<sup>W/W</sup> 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: 000–000.

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 pre-formed 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 naive 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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Received for publication February 1, 2011. Accepted for publication August 10, 2011.

This work was supported by the National Institutes of Health (AI65495 and AI68150 to C.A.L.) and the National Science Foundation Graduate Research Fellowship Program (to E.R.E.).

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The online version of this article contains supplemental material.

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

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100341
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 nephrotoxic 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<sup>fl</sup> mice (27) were back-crossed to C57BL/6 (Charles River) for eight generations, then crossed to Mrp8<sup>cre</sup>-<sup>−</sup> (28), Ms-1<sup>cre</sup>-<sup>−</sup> (29), Lyn-M<sup>cre</sup>-<sup>−</sup> (30), or Cd11c<sup>cre</sup>-<sup>−</sup> (31) strains for conditional syk deletion. Importantly, the Mrp8<sup>cre</sup>-<sup>−</sup> gene also contains an *ires-GFP* marker, allowing us to track cells expressing the Cre recombinase (Cre) by flow cytometry. Induction of Ms-1 expression by injection of polyinosinic-polycytidylic acid (poly-IC) was performed as described (29). Control mice for all experiments included either syk<sup>fl</sup> or syk<sup>−/−</sup> with the relevant Cre or syk<sup>fl</sup> or syk<sup>−/−</sup> without Cre to control for both Syk and Cre expression in the various cell lineages. NOD/ShiLtJ, C57BL/6-Kit-w-sh/ByJ (c-Kit<sup>max/0</sup>) (32), C57BL/6-Ki-t-W/v (c-Kit<sup>max</sup>), and WB/ReJ KI/W/v (c-Kit<sup>max</sup>) mice were purchased from The Jackson Laboratory. The c-Kit<sup>max</sup> and c-Kit<sup>max</sup> were intercrossed to obtain c-Kit<sup>max</sup> mice (16). NOD/ShiLtJ mice were crossed to C57BL/6 mice to obtain C57BL/6 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 Basoph8 mice (containing a YFP-ires-Cre gene inserted into the mast cell protease 8 gene: a basophil-specific marker) with *Ros份额* mice (containing a loxp-flanked diphteria toxin α-chain gene inserted into the Rosade2 locus) as described (33). Mixed chimeric mice were generated by injecting lethally irradiated B6 recipients with bone marrow cells, as described (34). Briefly, male 8- to 12-wk-old B6.SJL CD45.1<sup>−</sup> mice were lethally irradiated and injected i.v. with 5 × 10<sup>6</sup> 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<sup>−/−</sup> chimeras were obtained by reconstituting lethally irradiated wild-type mice with Syk<sup>−/−</sup> 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 (Seistadt) 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 talocrural 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 fluoresently labeled goat anti-mouse IgG F(ab')<sub>2</sub> (Jackson ImmunoResearch Laboratories) (19).

**Immunofluorescent staining**

Frozen ankle sections were prepared as previously described (19). Briefly, 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')<sub>2</sub> (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 cytopsin and stained by Wright–Giemsa. To obtain peritoneal cells, the peritonea of euthanized 8- to 12-wk-old mice were flushed with PBS containing 5 ml PBS/2 mM EDTA for isolation. 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 with 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 in the presence of antibodies to CD11b, 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. 55304; Cappel) 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 FITC-, 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 (JORO50), CD131, FcrR1a (MAR1), c-Kit (2B8), all from eBioscience 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 (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 FcεR1 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-GPI
Anti-GPI 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).
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 in staining in cells from syk<sup>−/−</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 monocytic 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>−/−</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>−/−</sup> MRP8-cre<sup>+</sup> mice (3).

The MRP8-cre allele contains anires-GFP construct that allows us to detect cells expressing Cre (3). While 95–98% of Ly6G<sup>+</sup> monocytes and 85–90% of 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 monocytic 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>−/−</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>−/−</sup> MRP8-cre<sup>+</sup> mice (3).

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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>+/+</sup> MRP8-cre<sup>+</sup>) (Fig. 2D). 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>−/−</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>fl</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).

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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> MR8-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> MR8-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 types ranged from 5 to 20% (Fig. 3F). This level of GFP expression was low and of unclear significance, especially as monocyte/macrophage cell types make 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> MR8-cre<sup>+</sup> mice with wild-type hematopoietic cells responded normally (Supplemental Fig. 2B). These
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 whether neutrophil-specific Syk deficiency affected anti-GPI immune complex-induced cutaneous edema. A lack of induced vascular permeability in the sykf/f 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.

Reduced serum half-life of the Abs, as the syk0/0 MRP8-cre+ animals consistently had greater anti-GPI serum titers throughout the course of disease (Fig. 4B). Syk0/0 MRP8-cre+ chimeric mice with wild-type bone marrow did 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. Syk0/0 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 Fcγ signaling in other cell types also contributes to increased vascular permeability. A lack of induced vascular permeability in the Syk0/0 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.

FIGURE 4. Neutrophil-specific deletion of Syk results in decreased joint Ab deposition after K/BxN serum transfer. A, Cryosections from the forepaws of syk+/+ MRP8-cre+ and syk0/0 MRP8-cre+ mice 7 d after K/BxN serum transfer were stained for the Fc portion of IgG. Deposition of IgG lining the cartilage surface is shown by the arrows (original magnification ×50). B, Serum anti-GPI titers on days 2, 4, and 6 after K/BxN serum transfer in syk0/0 and syk0/0 MRP8-cre+ mice were determined by ELISA as described in Materials and Methods and analyzed by two-way ANOVA. C, Immune complex-induced cutaneous edema was evaluated in syk+/+ MRP8-cre+, wild-type, and FcγR−/− mice by Evans blue extraction 3 h after i.v. injection of Ag, as described in Materials and Methods. Results are shown as the ratio of Evans blue concentration in the tissue to concentration in the peripheral blood. Statistics shown compared with C56BL/6 mice by one-way ANOVA. **p < 0.01, ***p < 0.001.
Syk-deficient neutrophils migrate to the inflamed joint

The lack of clinical disease in syk<sup>−/−</sup> MRP8-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> MRP8-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> MRP8-cre<sup>+</sup> mice may 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.
joint. Because Syk is required for FcγR and integrin but not G protein-coupled receptor signaling (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<sup>W/Wv</sup> 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 recognition of joint immune complexes by resident mast cells followed by production of chemokines that lead to neutrophil recruitment and disease (8, 9, 16, 47). Given that we observed disease protection in mice containing the FcγR- and integrin but not G protein-coupled receptor signaling, we sought to reevaluate the role of mast cells in this arthritis model.

![Image](http://www.jimmunol.org/)

**FIGURE 6.** Altered cytokine production, but not activation marker expression, by Syk-deficient neutrophils in the arthritic joint. A and B, Ly6G<sup>+</sup> 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<sup>+</sup>, white bars) or wild-type (CD45.1<sup>+</sup>, gray bars) neutrophils were 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 as described in Materials and Methods after 6 h of incubation with media or media plus immune complexes (indicated as IC) or media plus 10 ng/ml LPS. Wild-type versus syk<sup>−/−</sup> MRP8-cre<sup>+</sup> cells were distinguished by CD45.1 versus CD45.2 staining. E and F, Naïve bone marrow neutrophils were isolated from either wild-type or syk<sup>−/−</sup> fetal liver chimeric mice and stimulated in vitro with the indicated agonists for 6 h, then (E) stained for intracellular TNF-α or (F) culture supernatant was collected for TNF-α ELISA as described in Materials and Methods and assessed by two-way ANOVA. *p < 0.05, **p < 0.001. IC, insoluble immune complex.

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 (Basoph8<sup>−/−</sup>) crossed to mice containing the Rosa-fbxDT<sub>α</sub> allele (Fig. 8A). Expression of Dto in DX<sup>+</sup> basophils, which can be tracked by the inx-YFP construct within the Basoph8<sup>−/−</sup> allele, leads to apoptosis and depletion (Fig. 8B) (33). Combined with the results from the syk<sup>−/−</sup> MRP8-cre<sup>+</sup> mice, we conclude that basophils are not required for the development of K/BxN serum-induced arthritis.

**Discussion**

We have used syk<sup>−/−</sup> MRP8-cre<sup>+</sup> 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...
In contrast, FcR types would have a compensatory effect on disease development. It is not known whether forcing Fc receptor expression in other cell models, FcRg, FcRg neutrophil FcRg signaling is not required for the disease process as previously suspected (48). Together, these results show that mast cell Fc/Syk signaling in neutrophils during inflammation. Combined with data from direct to compare the behavior of Syk-deficient and wild-type plexes is the major driver of K/BxN serum-induced 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 syk<sup>-/-</sup> MRP8-cre<sup>+</sup> 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 LTB4 receptor, in which reconstitution with wild-type neutrophils induced arthritis and the recruitment of BLT1<sup>-/-</sup> 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 LTB4, 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 syk<sup>-/-</sup> MRP8-cre<sup>+</sup> 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 syk<sup>-/-</sup> MRP8-cre<sup>+</sup> 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, RiIB, and RIIIB 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
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cells. This would have direct implications in the development of
cell-targeted therapeutics for treatment of immune complex dis-
ease.

Acknowledgments
We thank Clare Abram and Lynn Kamen for suggestions and comments.
Additionally, we thank the A. Luster laboratory (Harvard) for the IgG stain-
ning protocol, as well as the S. Rosen laboratory (UCSF) for help with the
cryostat tissue sectioning. We also thank the A. Weiss laboratory (UCSF)
for use of the 5F5.2 anti-mouse Syk hybridoma.

Disclosures
The authors have no financial conflicts of interest.

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