

Induced Syk deletion leads to suppressed allergic responses but has no effect on neutrophil or monocyte migration in vivo

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The spleen tyrosine kinase (Syk) is a key mediator of immunoreceptor signaling in immune cells. Thus, interfering with the function of Syk by genetic deletion or pharmacological inhibition might influence a variety of allergic and autoimmune processes. Since conventional Syk knockout mice are not viable, studies addressing the effect of Syk deletion in adult animals have been limited. To further explore functions of Syk in animal models of allergy and to shed light on the role of Syk in the *in vivo* migration of neutrophils and monocytes, we generated inducible Syk knockout mice. These mice harbor a floxed Syk gene and a tamoxifen-inducible Cre recombinase under the control of the ubiquitously active *Rosa26*-promoter. Thus, treatment of mice with tamoxifen leads to the deletion of Syk in all organs. Syk-deleted mice were analyzed in mast cell-dependent models and in models focusing on neutrophil and monocyte migration. We show that Syk deletion in adult mice reduces inflammatory responses in mast cell-driven animal models of allergy and asthma but has no effect on the migration of neutrophils and monocytes. Therefore, the inducible Syk knockout mice presented here provide a valuable tool to further explore the role of Syk in disease-related animal models.

Key words: Inducible knockout mice • Mast cells • Monocytes • Neutrophils • Syk



Supporting Information available online

Introduction

The spleen tyrosine kinase (Syk), a non-receptor tyrosine kinase, is engaged in various biological functions including FcεRI signaling in mast cells [1] and basophils [2], FcγR signaling in eosinophils [3], neutrophils [4] and macrophages [5], osteoclast

function [6], B-cell receptor signaling [7], embryonic vascular development [8, 9] and innate immune recognition [10–12]. Hence, Syk is suggested to play a key role in IgE-mediated responses in allergic asthma and rhinitis [13, 14] and is also discussed as a suitable target for the treatment of inflammatory diseases involving neutrophils or monocytic cells [15]. Since selective Syk inhibitors with oral bioavailability are not available yet [16] and due to the perinatal lethality of Syk-deficient mice [8, 9], considerable difficulty has been encountered in analyzing the role of Syk in animal models of inflammation. The generation

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of Syk-deficient BM chimeras carrying a Syk-deficient hematopoietic system partially circumvented this limitation [4, 17, 18].

For this study we generated inducible Syk knockout mice, in which Syk can be ubiquitously deleted by a tamoxifen-inducible Cre recombinase. We tested those animals in models of allergy and asthma, as well as in models of neutrophil and monocyte migration. The ability to knockout Syk in adult mice circumvented difficulties previously encountered using conventional Syk knockout animals and as such provided an appropriate tool to examine the role of Syk in various disease-related animal models.

Results

Generation of inducible Syk knockout mice

To obtain an insight into the role of Syk in animal models of allergy and inflammation, we generated an inducible Syk knockout mouse strain ($Syk^{flox/flox}$) by crossing $Rosa26-CreER^{T2}$ mice [19] with mice carrying exon 2 of the *Syk* gene flanked by *loxP*-sites (Fig. 1A) [20]. The knockout of Syk was induced in adult $Syk^{flox/flox}$ mice (8 wk or older) by the oral administration of 20 mg/kg tamoxifen on 5 consecutive days, resulting in the deletion of exon 2 containing the start codon. To assess the

efficacy and the tissue distribution of the knockout, the deletion of Syk was analyzed 10 days after the last tamoxifen administration by genomic PCR (Fig. 1B) and western blotting (Fig. 1C). PCR analysis of genomic DNA from different organs showed the successful deletion of exon 2 in all organs analyzed (Fig. 1B). Only in the brain a partial deletion was detected (Supporting Information Fig. 1). Furthermore, deletion of exon 2 led to undetectable levels of Syk protein in spleen, lymph nodes, blood, BM, lung, thymus and colon as determined by western blot analysis (Fig. 1C). Taken together, these data suggest that the $Syk^{flox/flox}$ mouse strain represents a suitable tool to address the function of Syk in various disease models and to mimic the systemic effects of pharmacological Syk inhibition.

Short-term Syk deletion does not negatively impact health status of animals

Conventional $Syk^{-/-}$ mice suffer from severe hemorrhaging and die 1–5 days after birth, indicating an essential function of Syk during embryogenesis [8, 9]. Therefore, we assessed in a first set of analyses several physiological parameters reflecting the health status of adult (14-wk old) mice after Syk deletion. There were no significant differences in body weight, spleen weight, white

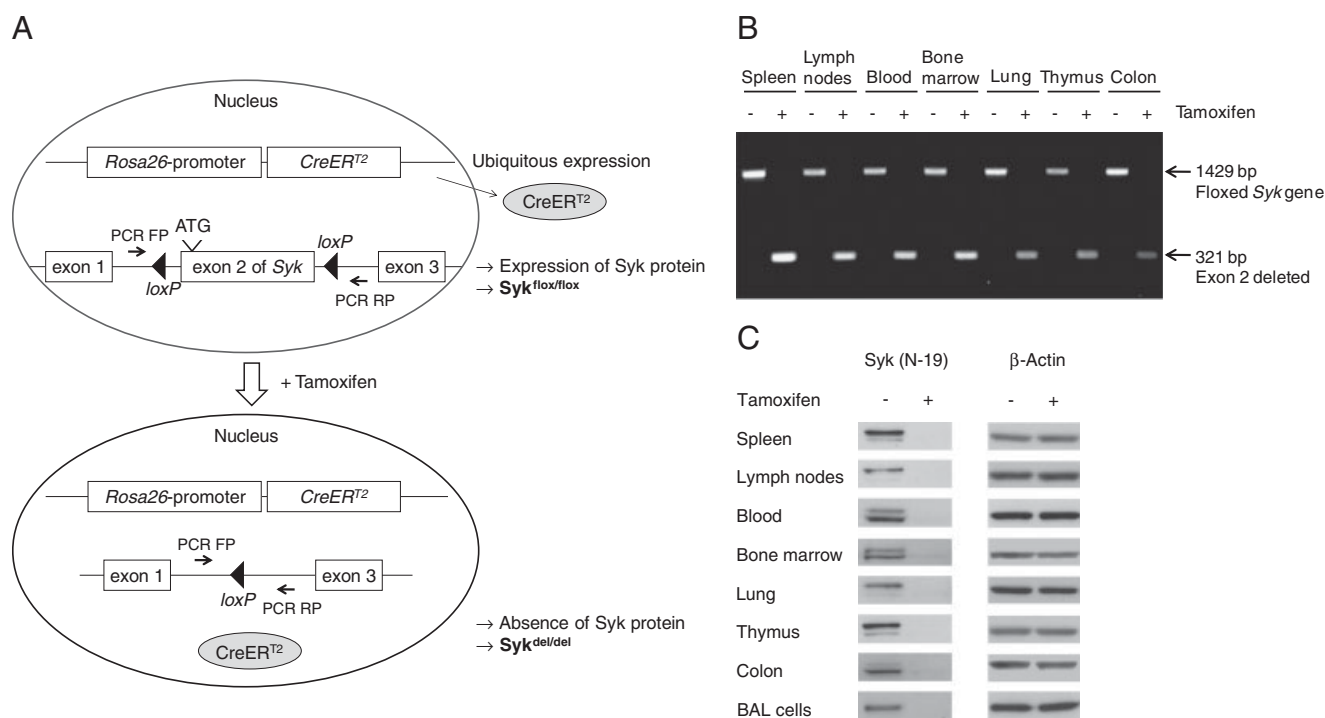


Figure 1. Inducible Syk knockout mice: Principle and proof of concept. (A) Schematic diagram of the knockout strategy. A fusion protein (CreER^{T2}) consisting of the Cre recombinase and a mutated form of the ligand binding domain of the estrogen receptor is constitutively expressed under control of the Rosa26-promoter. Nuclear translocation of the fusion protein and subsequent deletion of exon 2 of the *Syk* gene is induced by tamoxifen. Primer locations for PCR analysis are indicated. (B) PCR analysis of genomic DNA isolated from different organs of inducible Syk knockout mice after vehicle or tamoxifen treatment using primer pair as indicated (A). Floxed Syk gene: 1429 bp band. Gene locus after deletion of exon 2: 321 bp band. (C) Protein extracts from various organs of inducible Syk knockout mice after vehicle or tamoxifen treatment were analyzed by western blot for Syk and β-Actin expression. Each panel represents three to five independent experiments with similar results.

blood cell count or B lymphocyte count in blood between the vehicle-treated Syk^{flox/flox} and the tamoxifen-treated Syk^{del/del} groups 10 days after tamoxifen administration (Supporting Information Table 1). This suggests that the short-term deletion of Syk does not lead to general health impairment.

Syk-deleted BM cells differentiate into mature BMMCs in vitro but do not respond to FcεRI cross-linking

BM cells were isolated from the long bones of Syk^{flox/flox} and Syk^{del/del} mice 10 days after the last vehicle or tamoxifen application. After 4-wk culture with IL-3, surface expression levels of FcεRI and c-Kit were measured by flow cytometry (Fig. 2A) and the expression of Syk was determined by western blotting (Fig. 2B). Mouse BM-derived mast cells (mBMMCs) cultured from Syk^{del/del} mice were devoid of Syk protein but displayed equivalent FcεRI and c-Kit expression levels (93%) as cells isolated from Syk^{flox/flox} mice (90%), indicating that Syk is not necessary for the maturation of mBMMCs in vitro. To determine the responsiveness of mBMMCs, we analyzed their calcium flux and histamine release following FcεRI activation. As shown in Fig. 2C, the intracellular calcium concentration of Syk^{del/del} mBMMCs did not increase after antigen-induced cross-linking of the high affinity IgE receptor. Consistent with this observation, Syk^{del/del} mBMMCs did not release histamine in response to FcεRI cross-linking, but underwent normal histamine degranulation in response to a calcium ionophore, confirming that FcεRI-mediated degranulation absolutely relies on signaling through Syk. Total intracellular histamine levels, measured following lysis with Triton X-100, were comparable in Syk^{flox/flox} and Syk^{del/del} mBMMCs (Fig. 2D).

Pharmacological inhibition of Syk abrogates signal transduction in mBMMCs

Pharmacological inhibition of Syk kinase activity using the Syk inhibitors BAY61-3606 [21] and R406 [22] showed a concentration-dependent inhibition of calcium influx (IC₅₀ of BAY61-3606 = 43 nM; IC₅₀ of R406 = 75 nM) and histamine release (IC₅₀ of BAY61-3606 = 111 nM; IC₅₀ of R406 = 152 nM) after antigen-induced degranulation (Fig. 3A and B), suggesting that the catalytic function of Syk is required for this event. To visualize the blockade of downstream signaling events Syk^{flox/flox} or Syk^{del/del} mBMMCs were stimulated by receptor cross-linking in the absence or presence of BAY61-3606 and R406 and analyzed by western blot analysis. Both pharmacological inhibition and deletion of Syk completely inhibited the FcεRI-induced phosphorylation of Syk-binding partners PLCγ1 and SLP-76 and downstream proteins LAT, Erk, Akt and p38 MAPK (Fig. 3C).

Deletion of Syk reduces the IgE-induced passive cutaneous anaphylaxis reaction in mice

Mast cells releasing histamine and other mediators play a critical role in DNP-BSA (2,4-dinitrophenyl bovine serum albumin) -induced passive cutaneous anaphylaxis (PCA) in mice. Intravenous injection of DNP-BSA in combination with Evans blue dye elicited an edema in anti-DNP IgE-sensitized ears in Syk^{flox/flox} and WT mice. Evans blue dye leakage as a surrogate parameter for mast cell degranulation, subsequent histamine release and edema formation was reduced by 33% in Syk^{del/del} animals (Fig. 4A). Tamoxifen alone had no significant effect on the PCA reaction in WT animals

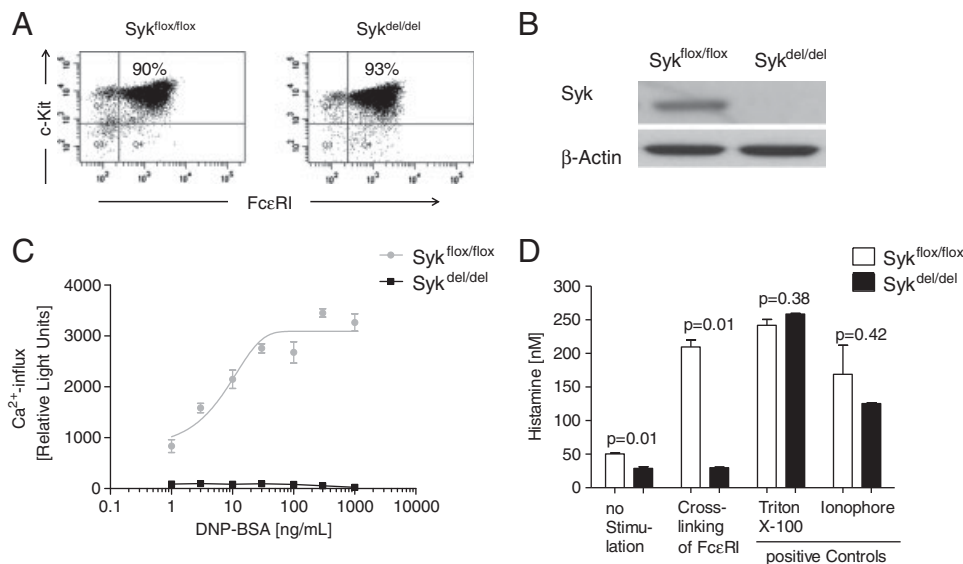


Figure 2. Effect of Syk deletion on the development and function of mBMMCs. (A) Expression levels of FcεRI and c-Kit receptors on Syk^{flox/flox} and Syk^{del/del} mBMMCs after 4 wk of culture with IL-3 were determined by FACS analysis. (B) Syk^{flox/flox} and Syk^{del/del} mBMMCs were assayed for Syk and β-Actin expression by western blot. (C) Calcium flux into Syk^{flox/flox} and Syk^{del/del} mBMMCs after stimulation with different concentrations of 2,4-dinitrophenyl bovine serum albumin (DNP-BSA) (*n* = 3). (D) Histamine release from mBMMCs after stimulation with either DNP-BSA, Triton X-100 or Calcium Ionophore A23187 (*n* = 3). In (C and D) data are presented as mean ± SEM. For calculation of *p*-values one-factorial ANOVA was performed.

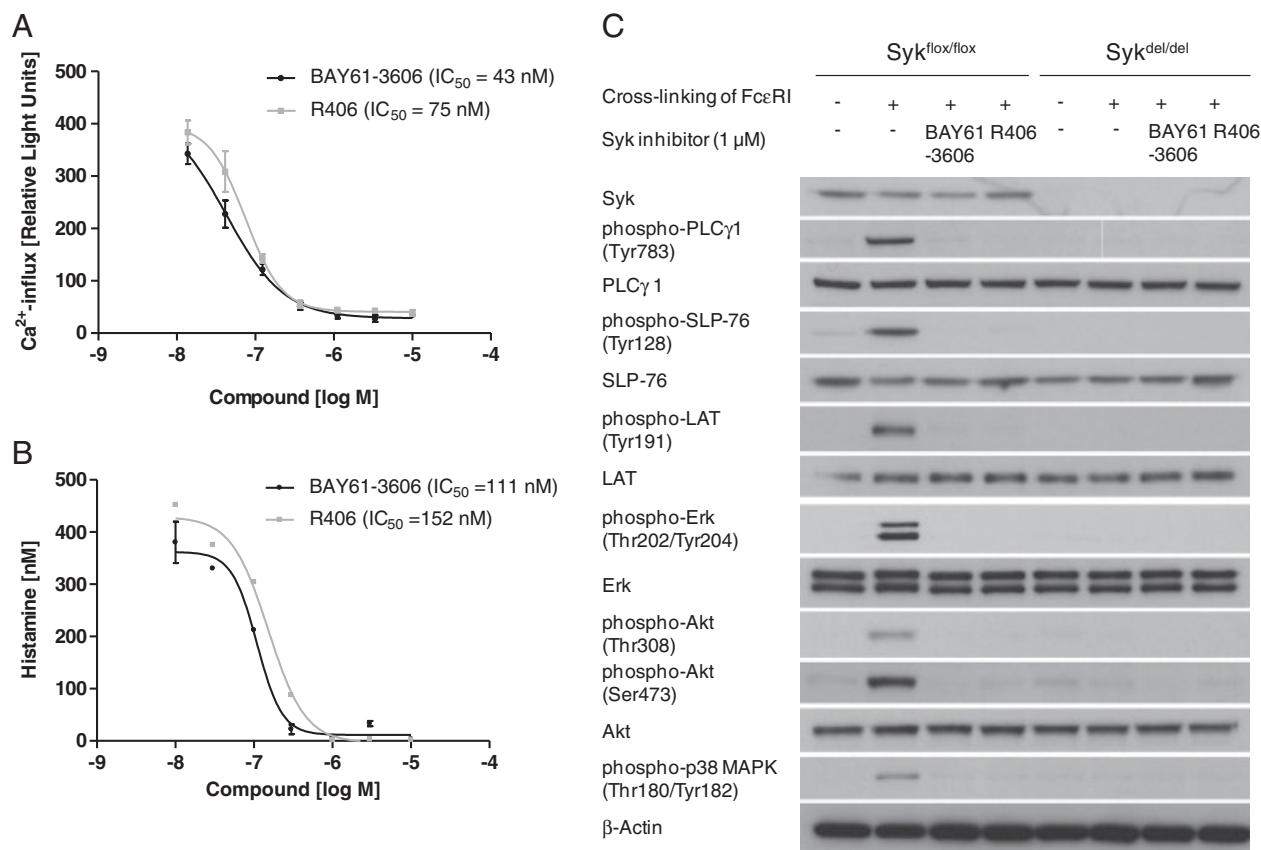


Figure 3. Effect of Syk inhibitors (BAY61-3606 and R406) on signaling events in mBMMCs. (A) Calcium flux into Syk^{flox/flox} and Syk^{del/del} mBMMCs after pre-incubation of cells with different inhibitor concentrations and cross-linking of high-affinity IgE receptor (FcεRI) with 30 ng/mL DNP-BSA (n = 3). (B) Histamine release from Syk^{flox/flox} and Syk^{del/del} mBMMCs after pre-incubation of cells with different inhibitor concentrations and stimulation with 30 ng/mL DNP-BSA (n = 2). (C) Anti-DNP IgE-sensitized Syk^{flox/flox} and Syk^{del/del} mBMMCs were pre-incubated with 1 μM BAY61-3606 or R406 and stimulated with 100 ng/mL DNP-BSA. Cell lysates were subjected to western blot analysis to determine downstream signaling events. In (A, B) data are presented as mean ± SEM.

(Fig. 4B). The results confirm the involvement of Syk in FcεRI-mediated mast cell degranulation in vivo.

Deletion of Syk has no effect on mast cell migration in vitro and in vivo

To clarify whether the reduced Evans blue dye leakage observed in Syk^{del/del} mice was a consequence of the reduced capability of mast cells to degranulate in response to receptor cross-linking or a consequence of reduced numbers of tissue resident mast cells we performed in vitro chemotaxis assays using mast cells generated from the BM of Syk^{flox/flox} and Syk^{del/del} mice. Syk^{flox/flox} and Syk^{del/del} mast cells showed the same chemotactic responsiveness to different concentrations of recombinant stem cell factor (Supporting Information Fig. 2). To further validate these data, the number of mast cells in toluidine blue-stained ear tissue sections from Syk^{flox/flox} and Syk^{del/del} mice after intradermal injection of anti-DNP IgE was determined 30 min after the intravenous injection of DNP-BSA. No significant difference in mast cell numbers was observed (Supporting Information Fig. 3),

adding evidence to the fact that the deletion of Syk has no influence on the migratory capacity of mast cells.

Syk deletion reduces ovalbumin-induced pulmonary eosinophilic inflammation

The role of Syk in eosinophilic lung inflammation was tested using Syk^{flox/flox}, Syk^{del/del} and WT mice. The allergic response was assessed by counting eosinophil numbers and measuring IL-4 and IL-5 levels in the BALF. Eosinophil numbers in the BALF of Syk^{del/del} mice ($0.54 \pm 0.16 \times 10^5$) were reduced by 83% compared with Syk^{flox/flox} mice ($3.26 \pm 1.23 \times 10^5$) (Fig. 4D). Tamoxifen alone had no influence on cell numbers in the BALF of WT mice in this experimental setup (Fig. 4E). The reduced Th2 response in Syk^{del/del} mice was also reflected by decreased IL-4 and IL-5 levels in BALF of Syk^{del/del} mice (28.8 ± 3.4 pg/mL and 78.7 ± 18.0 pg/mL respectively) compared with Syk^{flox/flox} mice (68.1 ± 23.4 pg/mL and 235.4 ± 88.6 pg/mL respectively). To confirm that the reduced eosinophil number in BALF observed in the OVA-model actually reflects a reduced Th2 response and is not a consequence of the

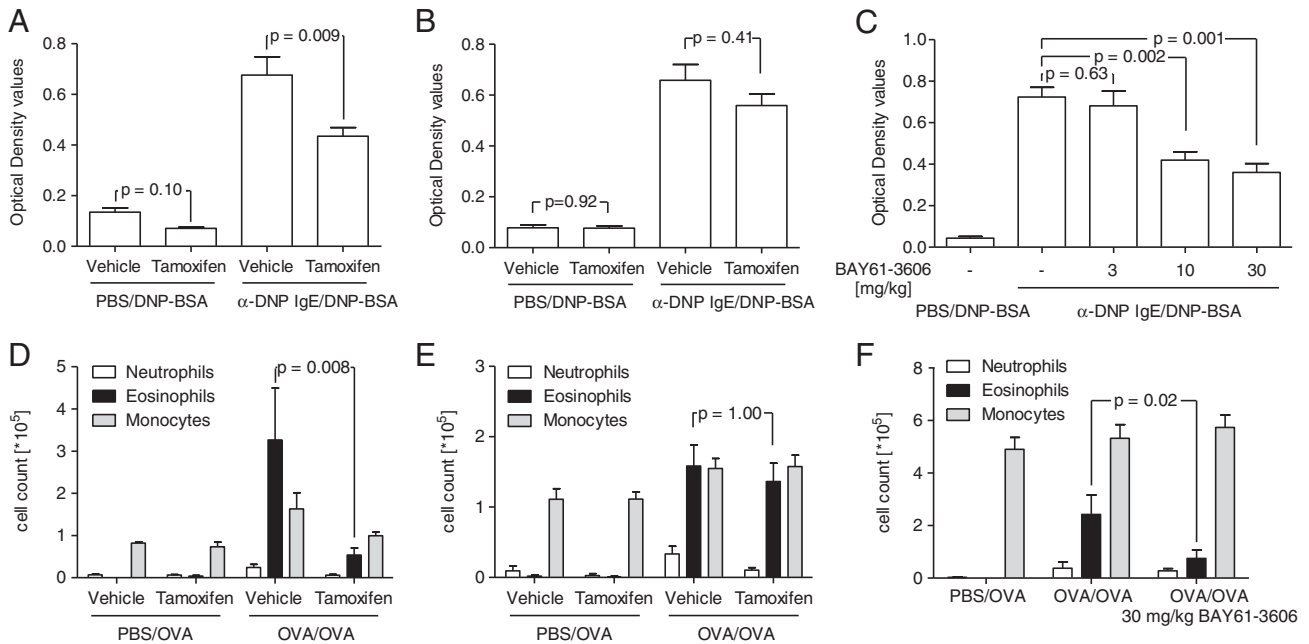


Figure 4. Effect of Syk deletion or pharmacological inhibition of Syk on mast cell-driven models of allergy and asthma. (A, B) Amount of dye leakage into ear tissue 30 minutes after DNP-BSA challenge measured colorimetrically at 620 nm in (A) inducible Syk knockout mice and (B) WT animals in a model of PCA. The graphs represent combined results of two independent experiments ($n = 11$ – 17). (C) Effect of BAY61-3606 on Evans blue dye leakage into ear tissue of BALB/c mice in a model of PCA ($n = 5$). (D, E) Neutrophil, eosinophil and monocytic cell count in the BALF of (D) inducible Syk knockout mice and (E) WT mice in a model of ovalbumin-induced pulmonary eosinophilia ($n = 7$ – 8). (F) Effect of BAY61-3606 on neutrophil, eosinophil and monocytic cell numbers in the BALF of BALB/c mice in a model of ovalbumin-induced pulmonary eosinophilia ($n = 8$). Data are presented as mean $\pm$ SEM. For calculation of p -values one-factorial ANOVA was performed.

reduced migratory capability of Syk^{del/del} eosinophils we tested the inducible Syk knockout mice in a model of eotaxin-induced lung inflammation. Eosinophil numbers in BALF were not significantly different between Syk^{flox/flox} and Syk^{del/del} mice confirming that the deletion of Syk does not negatively impact the chemotactic capability of eosinophils to migrate towards eotaxin in vivo (Supporting Information Fig. 4).

Pharmacological inhibition of Syk reduces PCA reaction and OVA-induced pulmonary eosinophilia

To address whether the effects observed in Syk^{del/del} mice were due to the absence of Syk protein or were dependent on the catalytic activity of Syk, we tested the Syk inhibitor BAY61-3606 in the PCA- and OVA-models. As shown in Fig. 4C, BAY61-3606 dose-dependently inhibited the PCA reaction with an ID₅₀ of 20.6 mg/kg and a maximum effect of 53% at 30 mg/kg ($p.o.$). BAY61-3606 (*i.p.*) also significantly inhibited the pulmonary eosinophil accumulation by 70% at 30 mg/kg (Fig. 4F), indicating that both effects are dependent on the catalytic activity of Syk.

Syk is not required for the in vitro and in vivo migration of neutrophils

To explore the function of Syk in the migration of neutrophils, we first examined the chemotactic responsiveness of isolated

Syk^{flox/flox} and Syk^{del/del} BM neutrophils to LTB₄ in an in vitro transwell assay and found no difference (Supporting Information Fig. 5). To test whether this effect translates into the in vivo situation, we used Syk^{flox/flox} mice in models of thioglycollate-induced sterile peritonitis, LPS- and short-term cigarette smoke (CS)-induced pulmonary neutrophilia. In the thioglycollate-induced sterile peritonitis model, the total cell count and neutrophil cell count in the peritoneal lavage fluid was similar or slightly higher in the Syk^{del/del} animals compared with the Syk^{flox/flox} animals (Fig. 5A). Both LPS and short-term CS treatment induced prominent pulmonary neutrophilic inflammation. Again, deletion of Syk had no effect on neutrophil cell numbers in BALF in both experimental setups (Fig. 5B and C). The absence of Syk was verified by western blot analysis of cells isolated from the BALF of Syk^{del/del} animals (Fig. 1C). These results confirm that the deletion of Syk has no effect on the in vivo migration of neutrophils.

Syk is not required for the migration of monocytes to the lung after MCP-1 treatment or chronic CS exposure

Since studies exploring the role of Syk in the migration of macrophages have been limited to in vitro systems [23, 24] we next tested the migratory capability of Syk^{del/del} monocytes in models of MCP-1- or chronic CS-induced lung inflammation. The intratracheal application of MCP-1 led to a comparable increase in monocyte counts in the BALF of both Syk^{flox/flox} and Syk^{del/del}

mice (Fig. 5D). To confirm that this observation was not dependent on the experimental model used, the animals were additionally tested in a chronic CS model. In this experiment animals were exposed to CS over a period of 3 wk and 18 h after the last CS exposure the lungs were lavaged. The exposure of mice to CS led to a prominent accumulation of monocytic cells in the lungs of both *Syk^{flox/flox}* and *Syk^{del/del}* mice (Fig. 5E) confirming that the deletion of Syk has no impact on the in vivo migration of monocytes.

Discussion

Based on the tamoxifen-induced CreER^{T2} system [19] we established an inducible Syk knockout mouse strain enabling us for the first time to directly compare the function of Syk in cellular systems and various animal models of allergy and inflammation. Consistent with the ubiquitous activity of the *Rosa26*-promoter used to drive the expression of CreER^{T2} recombinase, we demonstrated by genomic PCR and western blotting that Syk deletion occurred in all tissues tested apart from the brain, which probably reflects a lower local concentration of 4-hydroxytamoxifen in the brain [19]. This novel omnipresent inducible Syk knockout model surpasses previous findings obtained in Syk-deficient BM chimeric mice carrying a Syk-deficient hematopoietic system [17] and in mice treated with

inhaled Syk antisense oligonucleotides, which are acting topically only [25]. Hence, the broad tamoxifen-induced deletion of Syk in the inducible Syk knockout mice described here may resemble the systemic inhibition of Syk by an oral small molecule inhibitor.

In our short-term experiments tamoxifen-treatment and Syk-deletion had no overt effects on basic body functions as assessed by body weight, spleen weight, blood cell counts and B-cell counts 10 days after the last tamoxifen administration. Animals exhibited normal behavior and were healthy.

The development of mast cells generated from the BM (mBMMCs) of *Syk^{del/del}* animals was not impaired, which is in agreement with a previous study that showed normal differentiation of mast cells from Syk-deficient fetal liver cells [26]. However, FcεRI-induced calcium release and mast cell degranulation was abrogated in mBMMCs derived from *Syk^{del/del}* mice. This was accompanied by abrogated phosphorylation of Syk binding partners and downstream proteins involved in FcεRI signaling.

Syk deletion also reduced mast cell-dependent edema formation in vivo, as measured using the PCA reaction. In addition, treatment of BALB/c mice with BAY61-3606 resulted in a comparable inhibition of the PCA reaction, suggesting that the catalytic activity of Syk is required in this processes. However, the deletion of Syk had neither an effect on the capability of mast cells to migrate towards recombinant mouse stem cell factor (rmSCF) in an in vitro chemotaxis assay nor did it affect the number of tissue resident mast cells in the ears of *Syk^{del/del}* mice

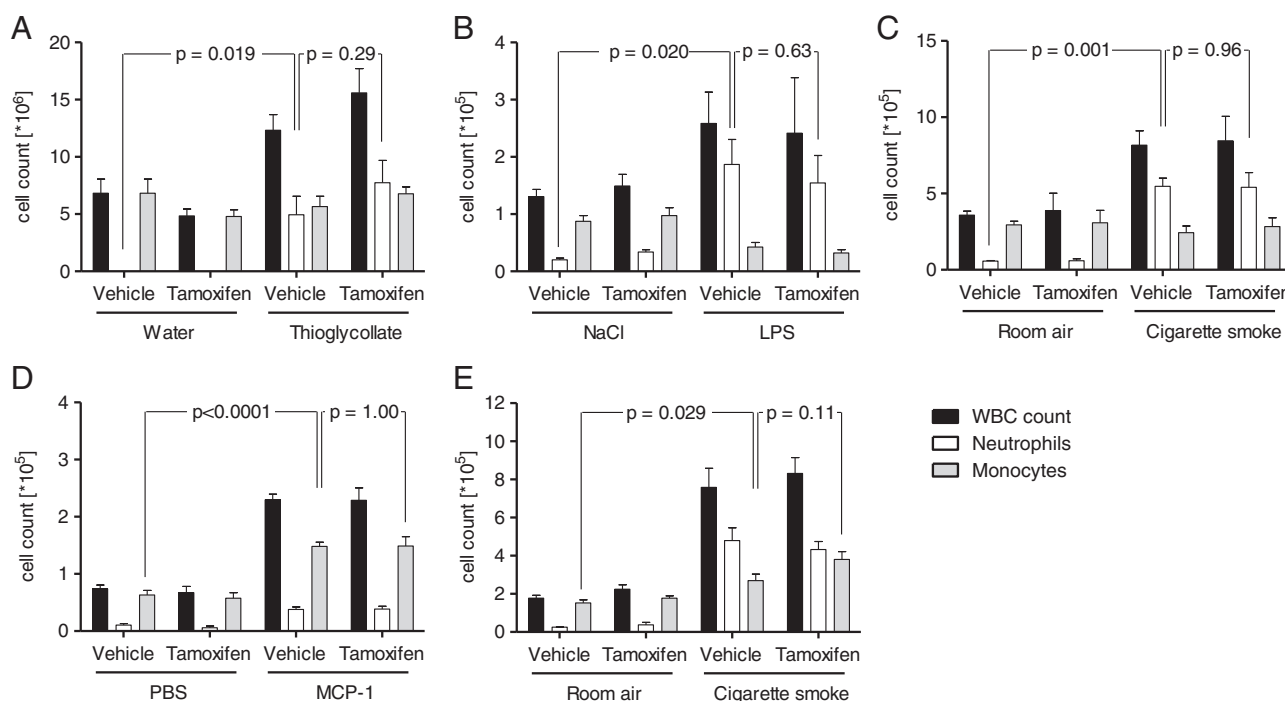


Figure 5. Effect of Syk deletion on the in vivo migration of neutrophils and monocytes. To determine the effect of Syk deletion on the in vivo migration of neutrophils, differential cell counts were measured (A) in the peritoneal lavage fluid in a model of thioglycollate-induced sterile peritonitis (n = 6) and in the BALF in models of (B) LPS-induced lung inflammation (n = 8) and (C) short-term CS-induced lung inflammation (n = 5–7). To determine the effect of Syk deletion on the in vivo migration of monocytes, differential cell counts were measured in the BALF in models of (D) MCP-1-induced lung inflammation (n = 6–8) and (E) chronic CS-induced lung inflammation (n = 6–8). Data are presented as mean $\pm$ SEM. For calculation of p-values one-factorial ANOVA was performed.

in the PCA model, suggesting that the deletion of Syk impairs mast cell degranulation but not mast cell migration. Furthermore, in a model of ovalbumin-induced pulmonary eosinophilia we were able to show that the pulmonary eosinophilic inflammation was reduced in Syk^{del/del} mice. Since in this model the Th2 cytokines IL-4 and IL-5 are strongly reduced in the BALF this effect can probably be attributed to the lack of induction of an allergic response resulting in reduced eosinophil counts. A direct role of Syk in eosinophil migration is rather unlikely since the influx of eosinophils was not significantly inhibited in Syk^{del/del} mice in a model of eotaxin-induced lung inflammation. Moreover, these data are in accordance with findings with the Syk inhibitor BAY61-3606 in an OVA-induced pulmonary inflammation model in rats [21, 25]. The inhibitor R406 has also been reported to inhibit OVA-induced pulmonary eosinophilia, airway hyperresponsiveness and goblet cell metaplasia in mice [27, 28]. However, one should keep in mind that R406 is a potent inhibitor of multiple proteins including Syk, Flt3, Ret, c-Kit, Lck, Jak 1/3 and the adenosine receptor [17], which makes it difficult to ascertain which effects observed with this compound are attributable to Syk inhibition.

Earlier reports have suggested a role of Syk in the activation, migration, and mediator release in neutrophils and monocytic cells [4, 23, 29, 30]. Using an in vitro chemotaxis assay we were not able to detect a difference between Syk^{flox/flox} and Syk^{del/del} BM neutrophils in their capability to migrate towards LTB₄ at physiologically relevant concentrations. Since the function of Syk had not been studied in animal models of pulmonary neutrophilic and monocytic inflammation and especially since studies on the involvement of Syk in the in vivo migration of neutrophils have led to conflicting results [4, 11, 18, 31], we tested the inducible Syk knockout mice in animal models broadly used to address this type of inflammation. Thioglycollate-induced neutrophilic peritonitis was unaltered in Syk^{del/del} mice compared with control animals. This finding is in agreement with experiments showing that lethally irradiated recipient mice reconstituted with Syk^{-/-} BM cells showed unaltered peritoneal neutrophil numbers in the thioglycollate model [4]. However, our findings contrast recent findings suggesting that the CD95-Src-family-Syk-axis has a pivotal function in the migration of neutrophils in the same model [32].

Normal neutrophil migration in the absence of Syk was also verified in an in vivo LPS-induced pulmonary neutrophil accumulation model. Furthermore, we demonstrated for the first time that the absence of Syk does not protect mice from CS-induced pulmonary neutrophilic inflammation. At first sight these findings are surprising and seem to be in contrast to the reduced neutrophil accumulation elicited by the reverse-passive Arthus reaction in BM chimera carrying a Syk-deficient hematopoietic system and also seem to contradict the reduced leukocyte counts found after injection of arthritogenic K/BxN serum in the joints of mice carrying a Syk-deficient hematopoietic system [17, 18]. To further elucidate the role of Syk in rheumatoid arthritis the inducible Syk knockout mice were tested in a model of collagen antibody-induced arthritis. Again, the deletion of Syk completely blocked the development of arthritis,

confirming the observations made by Jakus et al. (Ozaki et al., manuscript in preparation).

We hypothesize that a different involvement of mast cells in the various animal models might be the reason for these apparent discrepancies. Mast cells elicit an important contribution to the reverse-passive Arthus reaction [33] and the K/BxN arthritis model [34], but have not been described to be involved in the LPS- and the CS-induced pulmonary inflammation models. Thus, it is possible that the reduced neutrophil accumulation observed in Syk-deficient mice in the reverse-passive Arthus reaction is not due to a direct defect in neutrophil migration but rather a result of the inhibition of mast cell activity. Likewise, the inhibition of leukocyte infiltration observed in Syk^{-/-} BM chimera in the K/BxN arthritis model may also not reflect a neutrophil migration defect. Again, in this model the induction of disease development is dependent on mast cells, since mast cell-deficient mice are resistant to K/BxN serum transfer arthritis [34]. Therefore, in both experimental setups the reduced neutrophil accumulation is most likely a consequence of attenuated disease onset reflecting the inhibited mast cell activity as a result of Syk deletion.

Taken together, the results of our studies with inducible Syk-deficient mice confirm and complement the consensus that Syk is a suitable target for mast cell-driven allergic diseases including allergic asthma or rhinitis and further demonstrate that Syk is dispensable for the in vivo migration of neutrophils and monocytes. Furthermore, the study identifies the inducible Syk knockout mice described here as a highly valuable experimental tool, which can be used for the investigation of Syk function in different disease settings and to evaluate the systemic effects of pharmacological Syk inhibition, which is of special interest since several Syk inhibitors are currently in clinical testing.

Materials and methods

Reagents

The Syk (N-19, SYK-01), PLCγ1 and β-Actin (C4) antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Phospho-LAT (Tyr191), LAT, phospho-PLCγ1 (Tyr783), phospho-Erk (Thr202/Tyr204), Erk, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt and phospho-p38 MAPK (Thr180/Tyr182) antibodies were from Cell Signaling Technology (Danvers, MA). Phospho-SLP-76 (Tyr128), SLP-76, FITC-conjugated FcεRI and PE-conjugated c-Kit antibodies were from BD Biosciences (San Jose, CA). DNP-BSA was from Invitrogen (Eugene, Oregon). Monoclonal anti-DNP (mouse IgE isotype) antibody, tamoxifen and Evans Blue were from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IL-3 and rmSCF was purchased from R&D Systems (Minneapolis, MN). LTB₄ was from Calbiochem (La Jolla, CA). Recombinant human eotaxin-2 was purchased from Biotrend (Cologne, Germany). The Syk inhibitors BAY61-3606 and R406 were synthesized at Boehringer Ingelheim Pharma (Biberach, Germany).

Generation of inducible Syk knockout mice and induction of knockout

Animals were housed in isolated ventilated cages under a 12-h light-dark cycle and received food and water ad libitum. All animal experimentation was conducted in accordance with German national guidelines and legal regulations.

C57BL/6 mice heterozygous for a *Syk* gene carrying an exon 2 flanked by two *loxP*-sites (*Syk*^{lox/+}) were obtained from Alexander Tarakhovsky (Rockefeller University) [20] and bred to homozygous *Syk*^{lox/lox} mice. These mice were crossed with homozygous *Rosa26-CreER*^{T2} mice (B6D2F1 background, TaconicArtemis Pharmaceuticals Cologne, Germany). Double homozygous F1 animals were interbred to expand the colony and F2 animals were used for the experiments (= *Syk*^{lox/lox}). In all experiments Syk knockout was induced by oral administration of 20 mg/kg tamoxifen in 9% v/v ethanol in sun flower oil (4 mL/kg) on 5 consecutive days. Animals in vehicle-treated control groups received 9% v/v ethanol in sun flower oil (4 mL/kg) on 5 consecutive days.

PCR analysis of genomic DNA

For isolation of genomic DNA we used the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). To confirm the knockout of exon 2 of *Syk*, PCR analysis (FP: 5'-GCCCCGTTCTGTGCCTACTGG-3', RP: 5'-GCTGGTTCCCTTTTCCTTCC-3') was performed using 250 ng of isolated genomic DNA and PCR Core Kit (Roche, Mannheim, Germany).

Western blot analysis of tissue samples

Tissue samples were homogenized in Tissue Extraction Reagent (Invitrogen) containing Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). After centrifugation the supernatants were solubilized in 1 × LDS Sample Buffer (Invitrogen). Samples were run on 4–12% Bis-Tris gels (Invitrogen), electroblotted onto a PVDF-membrane (Millipore, Bedford, MA) and blocked with 5% w/v non-fat milk prior to antibody incubation.

Preparation of mBMMCs

After treatment with either vehicle or tamoxifen, 8–15 wk old female *Syk*^{lox/lox} mice were sacrificed with pentobarbital (Narcoren, Merial Hallbergmoos, Germany). Long bones were removed and flushed with 2% v/v FCS in PBS. Red blood cells were lysed using ammonium chloride-potassium (ACK) lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Cells were cultured at 1 × 10⁶ cells/mL in mast cell medium (RPMI1640 medium supplemented with 10% v/v FCS, 55 μM β-mercaptoethanol, penicillin, streptomycin and 5 ng/mL recombinant mouse IL-3). Medium was replaced three times a wk. After 4 wk of culture expression levels of FcεRIα and c-Kit were

analyzed by FACS. Cell populations co-expressing both markers > 90% were used for functional assays.

Calcium assay with mBMMCs

mBMMCs sensitized with anti-DNP IgE (1 μg/mL) overnight were loaded with Fluo-4 AM (Invitrogen). Cells were pre-incubated with different concentration of the inhibitor of interest for 2 min, stimulated with 30 ng/mL DNP-BSA and calcium influx was determined using FLIPR^{TETRA} (Molecular Devices) (excitation 485 nm, emission 525 nm) with a read time of 120 s.

Histamine release assay with mBMMCs

mBMMCs were seeded at 1 × 10⁶ cells/mL in mast cell medium and sensitized with anti-DNP IgE (1 μg/mL) overnight. After washing, cells were incubated for 30 min with either BAY61-3606 or R406 followed by activation of the FcεRI with 30 ng/mL DNP-BSA for 30 min. Triton X-100 (0.2%) or calcium ionophore A23187 (2 μM) were added as positive controls. Histamine content in supernatants was measured by ELISA (Beckman Coulter Immunotech, Marseille, France).

Stimulation of mBMMCs for Western blotting

mBMMCs were incubated with anti-DNP IgE (1 μg/mL) overnight. After washing twice with mast cell medium, cells were incubated for 30 min with either BAY61-3606 or R406 (1 μM) followed by stimulation with DNP-BSA (100 ng/mL) for 10 min. Cells were lysed in 1 × LDS Sample Buffer containing Sample Reducing Agent, Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were subjected to western blot analysis as detailed above.

Mast cell migration

Migration of mBMMCs was evaluated using a modified Boyden chamber assay. Varying concentrations of rmSCF in chemotaxis buffer (RPMI 1640 supplemented with 0.5% BSA) were added to the lower wells of a 96-well chemotaxis plate (5 μm pore size, NeuroProbe, Gaithersburg, MD). A total of 200 000 mast cells in 80 μL chemotaxis buffer were loaded onto each well of the fibronectin-coated polycarbonate filter. The plates were incubated for 3 h at 37°C and 5% CO₂. Migrated cells were quantified using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Madison, WI).

Neutrophil migration

Neutrophils were isolated from single cell suspensions of bone marrow by negative selection using the EasySep[®] Mouse

Neutrophil Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada). Neutrophil migration was determined using a 96-well migration chamber (NeuroProbe, Gaithersburg, MD) with an uncoated polycarbonate membrane of 5 μ m pore size. LTB₄ was diluted in chemotaxis buffer consisting of HBSS buffer (containing calcium and magnesium) supplemented with 0.1% BSA and placed in the lower well of the 96-well chemotaxis plate. Chemotaxis buffer without LTB₄ was added to the lower well for the negative control. In total, 100 000 neutrophils in 80 μ L chemotaxis buffer were loaded on top of each well. For the positive control 100 000 neutrophils were directly applied to the lower chamber and chemotaxis buffer without cells was loaded on top of these wells. The plates were incubated for 3 h at 37°C and 5% CO₂. Migrated cells were quantified using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega).

Total and differential cell count

For the evaluation of total and differential cell counts in BALF and blood a Sysmex XT1800 iVet cell analyzer was used (Sysmex Europe Norderstedt, Germany).

IgE-induced PCA

Male Syk^{flox/flox} or WT mice were treated with either vehicle or tamoxifen as above. Twelve days later animals were anesthetized with isoflurane (3–4%). Anti-DNP IgE (20 ng in PBS) was injected intradermally into the right ear and PBS into the left ear of each animal. After 24 h 1 mg DNP-BSA diluted in 0.9% NaCl with 1% Evans blue was injected into the tail vein. Thirty minutes after the challenge animals were sacrificed by cervical dislocation and ear tissue (8 mm diameter) was taken from both ears. Evans blue was extracted by incubation in 0.35 μ L of formamide at 65°C overnight. The absorbance was measured in the supernatant at 620 nm using a spectrophotometer. To test BAY61-3606 male BALB/c mice were treated with inhibitor (p.o.) 30 min prior to DNP-BSA application.

Histology and quantitation of tissue mast cells

Mice were treated with either vehicle or tamoxifen as above. Twelve days later mice were sensitized by intradermal injection of anti-DNP IgE in the right ear and challenged 24 h later by i.v. injection with DNP-BSA as described in *IgE-induced PCA*. Animals were sacrificed by cervical dislocation 30 min after the injection of antigen. Ears were fixed in 4% paraformaldehyde for 24 h and processed for 3- μ m paraffin-embedded, toluidine blue-stained sections. The sections were examined by light microscopy at 400 $\times$, and the numbers of mast cells present were recorded. The same tissue sections were then scanned with a Zeiss Mirax Midi slide scanner. Images were generated from virtual slides by an export using level 4 (1:16). Ear tissue was segmented

from background by combining saturation and texture information. Calculated tissue area (in pixel) was converted to mm².

Ovalbumin-induced pulmonary eosinophilia

On day 0, 14 and 21 mice were immunized by injection of 200 μ L ovalbumin (i.p. 0.1 mg/mL) (Serva, Heidelberg, Germany) absorbed to Imject Alum (Thermo Scientific) in PBS. Control animals received Imject Alum in PBS. For knockout and control experiments female Syk^{flox/flox} and WT mice were treated with vehicle or tamoxifen from day 28 to 32 as above. Mice were challenged with nebulized OVA (1% in PBS) for 20 min on days 42 and 43. To test the Syk inhibitor, BAY61-3606, female BALB/c mice were immunized on day 1, 15 and 22 and challenged with OVA on days 27 and 28. BAY61-3606 (i.p. 30 mg/kg) was injected 1 h before, 3 and 6 h after OVA-challenge. Animals were euthanized 24 hours after the last OVA-challenge by pentobarbital injection (i.p. 400 mg/kg Narcoren) and the lungs were lavaged two times with 0.8 mL Hanks solution containing EDTA (0.6 mM).

Eotaxin-induced lung inflammation

Inducible Syk knockout and WT animals were treated with vehicle or tamoxifen on days 0–4 as above. On day 14, 18 and 22 mice were immunized by injection of 200 μ L ovalbumin (i.p. 0.1 mg/mL) (Serva) absorbed to Imject Alum (Thermo Scientific) in PBS. On day 26 mice were anesthetized with isoflurane (3–4%), fixed in supine position and 50 μ L of eotaxin solution (recombinant human eotaxin-2, 60 μ g/mL in PBS containing 0.1% BSA) was administered intratracheally. And, 4 h after eotaxin application animals were euthanized by pentobarbital injection and the lungs were lavaged as described in *Ovalbumin-induced pulmonary eosinophilia*.

Thioglycollate-induced sterile peritonitis

Male Syk^{flox/flox} mice were treated with either vehicle or tamoxifen. Twelve days later mice were treated with 1 mL sterile thioglycollate (i.p. 4% w/v). Peritoneal exudates were collected 4 h after thioglycollate application.

Lipopolysaccharid-induced pulmonary inflammation

Twenty-four days after treatment with either vehicle or tamoxifen inducible Syk knockout mice were anesthetized with isoflurane (3–4%), fixed in supine position and 50 μ L of LPS solution (*E. coli* serotype O111:B4; 100 μ g/mL in 0.9% NaCl) was administered intratracheally. 4 h after LPS application animals were euthanized by pentobarbital injection and the lungs were lavaged as described in *Ovalbumin-induced pulmonary eosinophilia*.

CS-induced pulmonary inflammation

Inducible Syk knockout mice were treated with tamoxifen or vehicle. Ten days after the last vehicle or tamoxifen application mice were exposed to CS or room air in a whole body exposure box as described previously [35]. In the short-term model mice were exposed to the mainstream smoke of six cigarettes (Roth-Händle without filters, Badische Tabakmanufaktur Roth-Händle®, Lahr, Germany) on day 1 and 2, of eight cigarettes on day 3 and of ten cigarettes on day 4. Control animals were exposed to room air. In the chronic model animals were exposed to smoke for a total of 3 wk, again starting 10 days after the last vehicle or tamoxifen application. In wk 1 and 2 animals were exposed to the mainstream smoke of five cigarettes on 5 consecutive days followed by a 2-day recovery period. In wk 3 animals were exposed to the smoke of five cigarettes on 4 consecutive days. In both setups the animals were euthanized by pentobarbital injection (i.p. 400 mg/kg Narcoren) 18 h after the final CS exposure and the lungs were lavaged as described in *Ovalbumin-induced pulmonary eosinophilia*.

MCP-1-induced lung inflammation

WT and Syk^{flox/flox} mice were treated with tamoxifen or vehicle as above. Ten days after the last vehicle or tamoxifen application animals were short-term anesthetized by inhalation of isoflurane (3–4%) as described before, were fixed in supine position and received 10 µg human MCP-1 (PeproTech, Rocky Hill, NJ) in PBS containing 0.1% w/v BSA and 10 ng LPS (*E. coli* serotype O111:B4) in a total volume of 50 µL by intratracheal application. Control animals received 50 µL PBS containing 0.1% w/v BSA. Animals were euthanized 48 h after intratracheal application by pentobarbital injection and the lungs were lavaged.

Statistical analyses

All graphs were generated using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA). All data are presented as means ± SEM of *n* animals. One-factorial analyses of variance (ANOVA) with heteroscedastic variances were applied for all experiments except Figs. 4F and 5A where homogeneous variances were assumed. The error terms of the ANOVA were taken as estimates for the variability in the following selected pairwise comparisons of the different experimental groups. To adjust for multiple testing the method of Bonferroni-Holm was used. The statistical evaluation was prepared using the software package SAS Version 9.2 (SAS Institute, Cary, NC).

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Abbreviations: CS: cigarette smoke · DNP-BSA: 2,4-dinitrophenyl bovine serum albumin · mBMMC: mouse BM-derived mast cell · PCA: passive cutaneous anaphylaxis · p.o.: per os · rmSCF: recombinant mouse stem cell factor · Syk: spleen tyrosine kinase · Syk^{del/del}: tamoxifen-treated inducible Syk knockout mice · Syk^{flox/flox}: vehicle-treated inducible Syk knockout mice

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