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The Syk tyrosine kinase is required for skin inflammation in an in vivo mouse model of epidermolysis bullosa acquisita

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ABSTRACT

The inflammatory form of epidermolysis bullosa acquisita is caused by autoantibodies against type VII collagen (C7), a component of the dermal-epidermal junction. We have previously shown that myeloid Src-family kinases mediate skin inflammation triggered by anti-C7 antibodies. Here we identify the Syk tyrosine kinase as a critical component of autoantibody-induced skin inflammation downstream of Src-family kinases. Immobilized C7–anti-C7 immune complexes triggered neutrophil activation and Syk phosphorylation in a Src-family kinase-dependent manner. Bone marrow chimeric mice lacking Syk in their hematopoietic compartment were completely protected from skin inflammation triggered by anti-C7 antibodies despite normal circulating anti-C7 levels. Syk deficiency abrogated the accumulation of CXCL2, IL-1β and LTB_4 at the site of inflammation and resulted in defective in vivo neutrophil recruitment. *Syk*$^{-/-}$ neutrophils had a normal intrinsic migratory capacity but failed to release CXCL2 or LTB_4 upon activation by immobilized C7–anti-C7 immune complexes, indicating a role for Syk in the amplification of the inflammation process. These results identify Syk as a critical component of skin inflammation in a mouse model of epidermolysis bullosa acquisita and as a potential therapeutic target in epidermolysis bullosa acquisita and other mechanistically related inflammatory skin diseases such as bullous pemphigoid.

INTRODUCTION

Typical examples of subepidermal blistering skin diseases are bullous pemphigoid and epidermolysis bullosa acquisita, the latter disease having both an inflammatory and a non-inflammatory (mechanobullous) form (Ludwig and Zillikens, 2011; Kim and Kim, 2013). Both bullous pemphigoid and epidermolysis bullosa acquisita are caused by autoimmune targeting of components of the dermal-epidermal junction and are triggered by autoantibodies against the hemidesmosomal proteins BP230/BP180 or the dermal anchoring fibril protein type VII collagen, respectively (Schmidt and Zillikens, 2013; Baum et al., 2014; Turcan and Jonkman, 2015). In case of bullous pemphigoid and the inflammatory form of epidermolysis bullosa acquisita, this leads to local inflammation and disruption of the dermal-epidermal junction, mediated by complement activation and leukocyte (mainly eosinophil and neutrophil) infiltration (Schmidt and Zillikens, 2013). In contrast, autoantibodies appear to directly decrease the adhesive function of the basement membrane without initiating significant inflammation in the non-inflammatory form of epidermolysis bullosa acquisita (Kim and Kim, 2013). Unfortunately, further details of the pathomechanism of bullous pemphigoid and epidermolysis bullosa are poorly understood and, therefore, the therapeutic options are rather limited (Kasperkiewicz and Schmidt, 2009).

A number of in vivo models mimic bullous pemphigoid or the inflammatory form of epidermolysis bullosa acquisita by utilizing active or passive immunization against components of the dermal-epidermal junction in experimental animals (Iwata et al., 2015). The most widely used model relies on the passive immunization of mice with IgG antibodies against the immunodominant NC1 domain of type VII collagen (Sitaru et al., 2005; Sitaru, 2007; Ludwig, 2012). Since this intervention triggers substantial inflammation and inflammatory pathway components are critical to disease pathogenesis (see below), this model is believed to mimic the inflammatory but not the non-inflammatory form of epidermolysis bullosa acquisita.

Animal models of bullous pemphigoid and epidermolysis bullosa acquisita have revealed striking similarities between the pathomechanism of the two disease models. Critical components include activating Fc-receptors (Sitaru et al., 2005; Zhao et al., 2006; Kasperkiewicz et al., 2012; Schulze et al., 2014), the alternative pathway of complement activation (Liu et al., 1995; Sitaru et al., 2005; Mihai et al., 2007) as well as myeloid cells such as neutrophils (Liu et al., 1997; Chiriac et al., 2007). Our recent signaling studies revealed complete protection of mice lacking three Src-family kinases expressed in myeloid cells (Hck, Fgr and Lyn) from skin inflammation triggered by antibodies against type VII collagen (Kovács et al., 2014), while mice lacking PI3Kβ (Kulkarni et al., 2011) or the CARD9 adapter protein (Németh et al., 2016) were

partially protected. Additional experiments pointed to a critical role for those molecules within the neutrophil compartment in all three cases.

Syk is a nonreceptor tyrosine kinase primarily expressed in cells of hematopoietic origin (Mócsai et al., 2010). Syk is involved in signal transduction of a number of tyrosine kinase-coupled receptors including β_2-integrins and various Fc-receptors (Mócsai et al., 2002; Mócsai et al., 2003; Mócsai et al., 2010; Németh et al., 2016). Src-family kinases are involved in Syk activation in most of those cases (Mócsai et al., 2002; Mócsai et al., 2006; Mócsai et al., 2010; Kovács et al., 2014). Hematopoietic or neutrophil-specific deletion of Syk protects mice from arthritis development in the K/B×N serum-transfer model (Jakus et al., 2010; Elliott et al., 2011) and Syk has been proposed to be a potential target in rheumatoid arthritis and other autoimmune inflammatory diseases (Mócsai et al., 2010; Weinblatt et al., 2010; Geahlen, 2014). However, the role of Syk in autoimmune inflammatory skin diseases is still entirely unclear.

The above findings and the lack of information on the role of Syk in autoimmune dermatitis prompted us to test the role of Syk in an in vivo model of the inflammatory form of epidermolysis bullosa acquisita in experimental mice. Our results indicate that Syk is critically involved in skin inflammation triggered by autoantibodies against type VII collagen, identifying Syk as a potential therapeutic target in epidermolysis bullosa acquisita and, possibly, other inflammatory skin diseases of similar pathomechanism.

RESULTS

Neutrophil activation by type VII collagen-containing immune complexes

We have previously shown that immobilized human serum albumin (HSA)–anti-HSA immune complexes trigger robust activation of neutrophils (Jakus et al., 2008). To determine whether immune complexes containing dermo-epidermal junctional proteins can also activate neutrophils, we generated immobilized immune complexes using a His-tagged fragment of the immunodominant NC1 domain of murine type VII collagen (His-C7) and rabbit polyclonal IgG (anti-C7) against the GST-fusion protein of the same fragment (Sitaru et al., 2002; Csorba et al., 2010). Plating wild-type mouse neutrophils on such immobilized immune complexes triggered robust activation of the cells as measured by the release of superoxide anions (Fig 1a; $p = 4 \times 10^{-6}$). Neutrophil activation required both the antigen and the antibody components (Fig 1a). His-C7–anti-C7 immune complexes also triggered spreading of the cells over the immune complex-coated surface (Fig 1b).

To test the antigen specificity of neutrophil activation, ELISA plates were coated with His-C7 or HSA, blocked and then incubated with anti-C7 or anti-HSA antibodies.

As shown in Fig 1c, neutrophils mounted a robust respiratory burst in the presence of matching antigen-antibody pairs (His-C7 with anti-C7 or HSA with anti-HSA; $p = 8 \times 10^{-6}$ and $p = 1.9 \times 10^{-3}$, respectively) but not when non-matching antigen-antibody pairs were used.

Taken together, type VII collagen-containing immune complexes are able to specifically activate neutrophil functions.

Src-family kinases mediate neutrophil respiratory burst and Syk activation by type VII collagen-containing immune complexes

We have recently shown that the Src-family kinases Hck, Fgr and Lyn are critically involved in neutrophil activation triggered by HSA–anti-HSA immune complexes (Kovács et al., 2014). To test whether this also applies to type VII collagen-containing immune complexes, we tested the responses of $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ (3× SFK KO) neutrophils. As shown in Fig 1d, 3× SFK KO neutrophils failed to release superoxide under such conditions.

Syk is a non-receptor tyrosine kinase activated downstream of Src-family kinases in several immunoreceptor-induced signaling pathways (Mócsai et al., 2010; Futosi et al., 2013). We next tested whether this is also true for neutrophil activation by type VII collagen-containing immune complexes. Wild-type neutrophils plated on immobilized His-C7–anti-C7 immune complexes showed Syk phosphorylation (a measure of Syk activation) whereas no such response could be observed in 3× SFK KO neutrophils (Fig 1e; see also the entire blots in Fig S1).

Taken together, neutrophil activation by type VII collagen-containing immune complexes triggers Syk activation downstream of the Src-family kinases Hck, Fgr and Lyn.

Generation and characterization of $Syk^{-/-}$ bone marrow chimeras

The above findings raised the possibility that Syk may also be involved in anti-C7 antibody-induced skin inflammation. Unfortunately, the perinatal lethality of genetically Syk-deficient (*$Syk^{-/-}$*) animals (Turner et al., 1995; Abtahian et al., 2003; Mócsai et al., 2010) did not allow us to test this in intact animals. To overcome that problem, we generated bone marrow chimeras lacking Syk only in their hematopoietic compartment (termed *$Syk^{-/-}$* bone marrow chimeras) by transplanting fetal liver cells of *$Syk^{-/-}$* fetuses into lethally irradiated wild-type recipients (Mócsai et al., 2002). Control chimeras (termed wild-type bone marrow chimeras) were generated by parallel transplantation of *$Syk^{+/+}$* or *$Syk^{+/-}$* fetal liver cells. Differential expression of CD45 alleles

in the donor (CD45.2) and the recipient (CD45.1) cells allowed identification of donor- and recipient-derived cells. As shown in Fig 2a, practically all peripheral blood neutrophils of wild-type or *Syk*$^{-/-}$ bone marrow chimeras expressed the donor-derived CD45.2 allele, indicating complete repopulation of the hematopoietic compartment by donor-derived cells. In addition, Syk was present in lysates of freshly isolated neutrophils and platelets, as well as of bone marrow-derived macrophages and mast cells, from wild-type but not *Syk*$^{-/-}$ bone marrow chimeras (Fig 2b). Therefore, we were able to replace the wild-type hematopoietic compartment of the recipient mice with donor-derived (e. g. *Syk*$^{-/-}$) cells.

We did not observe any difference in circulating neutrophils numbers between wild-type and *Syk*$^{-/-}$ bone marrow chimeras (Fig 2c; $p = 0.69$), and similar numbers of bone marrow neutrophils could be isolated from chimeras of the two genotypes (Fig 2d; $p = 0.37$). In addition, *Syk*$^{-/-}$ neutrophils expressed normal levels of the neutrophil maturation marker Ly6G, the β_2 integrin component CD18, the major neutrophil chemokine receptor CXCR2, as well as the Fc-receptors FcγRII/III and FcγRIV (Fig 2e). Therefore, the *Syk*$^{-/-}$ mutation did not interfere with the generation or maturation of neutrophils, or the expression of critical cell surface receptors.

Syk$^{-/-}$ *bone marrow chimeras are completely protected from skin inflammation triggered by anti-C7 antibodies*

We next subjected wild-type and *Syk*$^{-/-}$ bone marrow chimeras to repeated subcutaneous injection of anti-C7 or normal rabbit IgG/PBS, as a model of inflammatory autoimmune blistering skin diseases (Sitaru et al., 2005). Our previous studies have indicated a critical role for the Fc portion of the pathogenic antibodies in this model (Sitaru et al., 2005). As shown in Fig 3a, wild-type chimeras treated with anti-C7 antibodies developed significant signs of skin inflammation at several body areas, ranging from loss of the superficial layer of the skin (most prominent in the buccal/periorbital area) to severe inflammation and scarring at the most severely affected places (most prominent on the ears). Importantly, *Syk*$^{-/-}$ bone marrow chimeras were completely protected from development of all those skin inflammatory changes (Fig 3a).

Quantification of the overall skin changes revealed that the percentage of the affected body surface area gradually increased over the assay period in wild-type chimeras, whereas no signs of the disease could be observed in *Syk*$^{-/-}$ chimeras (Fig 3b; $p = 0.022$). Similarly, the overall clinical score also gradually increased during the assay period in wild-type chimeras without any signs of the disease in *Syk*$^{-/-}$ chimeras (Fig 3c; $p = 6.0 \times 10^{-3}$).

A possible explanation for the above findings could be that the *Syk*$^{-/-}$ mutation affects the metabolism of the injected pathogenic antibodies. However, no differences in circulating anti-C7 antibody titers could be observed between the wild-type and *Syk*$^{-/-}$ genotypes (Fig 3d; $p = 0.69$).

We next tested skin inflammation by histological analysis of the ear tissue. As shown in Fig 3e, anti-C7 antibody treatment triggered substantial thickening of the ear of wild-type chimeras with prominent leukocyte infiltration. However, no such inflammatory changes could be observed in *Syk*$^{-/-}$ bone marrow chimeras.

Taken together the *Syk*$^{-/-}$ mutation blocks the capability of anti-C7 antibodies to trigger skin inflammation both at the macroscopic and microscopic levels, without affecting their removal from the circulation.

The Syk$^{-/-}$ *mutation blocks neutrophil recruitment without affecting the intrinsic migratory capacity of neutrophils*

The inflammatory form of epidermolysis bullosa acquisita is characterized by a significant influx of granulocytes (eosinophils and neutrophils) which are likely involved in the inflammation process (Schmidt and Zillikens, 2013). As shown in Fig 4a, anti-C7 antibodies triggered dramatic influx of neutrophils to the ear of wild-type bone marrow chimeras, which appears to be a peculiarity of this mouse model (as compared to the dominant infiltration by eosinophils in the inflammatory form of human epidermolysis bullosa acquisita). Importantly, and in agreement with the histological pictures (Fig 3e), no such accumulation of neutrophils could be observed in *Syk*$^{-/-}$ chimeras (Fig 4a; $p = 7.7 \times 10^{-3}$).

The simplest explanation for the above findings would be an intrinsic migration defect of *Syk*$^{-/-}$ neutrophils. However, we previously reported normal migration of *Syk*$^{-/-}$ neutrophils towards various proinflammatory agonists both under in vitro and in vivo conditions (Mócsai et al., 2002; Mócsai et al., 2003). To test this question in anti-C7-induced skin inflammation, we generated mixed bone marrow chimeras carrying leukocytes (including neutrophils) from both CD45.1-expressing wild type and CD45.2-expressing wild type or *Syk*$^{-/-}$ bone marrow cells. Such chimeras developed signs of inflammation due to the presence of wild type cells. Comparative flow cytometric analysis of CD45.1/2 expression in the inflammatory infiltrate and the peripheral blood then allowed us to compare the migration of wild type and *Syk*$^{-/-}$ neutrophils within the same animal. The percentage values for the peripheral blood and ear samples are presented in Fig 4b, whereas the relative migratory capacity of the CD45.2 (wild type or *Syk*$^{-/-}$) cells relative to the CD45.1 (wild type) reference cells (calculated based on the data in Fig 4b) is shown in Fig 4c. As expected, the percentage of wild type CD45.2

cells was comparable in the peripheral blood and the inflamed ear in the chimeras transplanted with cells from two wild type donors (WT:WT chimeras; Figs 4b-c). Importantly, the percentage of *Syk*$^{-/-}$ CD45.2 cells in the inflamed ear tissue was also similar to that in the peripheral blood in WT:*Syk*$^{-/-}$ chimeras (Fig 4b), and the calculated relative migration of *Syk*$^{-/-}$ cells was similar to, or even higher than that of wild type cells (Fig 4c; $p = 4.4 \times 10^{-6}$). Those results indicate that *Syk*$^{-/-}$ neutrophils are intrinsically capable of migrating to the site of inflammation, arguing against an intrinsic migration defect in *Syk*$^{-/-}$ neutrophils.

Taken together, although neutrophils failed to accumulate in the ear of anti-C7 antibody-treated *Syk*$^{-/-}$ bone marrow chimeras, this was not due to an intrinsic migration defect of *Syk*$^{-/-}$ neutrophils since those cells were able to accumulate at the site of inflammation when wild type cells were also present in mixed bone marrow chimeras.

The Syk$^{-/-}$ *mutation blocks the accumulation of proinflammatory mediators in vivo*

An alternative explanation for the abrogation of neutrophil accumulation in *Syk*$^{-/-}$ chimeras (Fig 4a) is that Syk deficiency may interfere with the development of a proper inflammatory (chemoattractant) microenvironment. As shown in Fig 4d, systemic administration of anti-C7 antibodies dramatically increased tissue levels of CXCL2 (MIP-2) in the ear of wild-type but not *Syk*$^{-/-}$ bone marrow chimeras ($p < 10^{-17}$). Similarly, hematopoietic deficiency of Syk abrogated the accumulation of the proinflammatory cytokine IL-1β (Fig 4e; $p = 1.6 \times 10^{-7}$). Moreover, anti-C7 antibodies also moderately increased the level of the lipid mediator LTB_4 (a major neutrophil chemoattractant in various inflammatory conditions), in wild-type but not *Syk*$^{-/-}$ bone marrow chimeras (Fig 4f; $p = 0.038$).

Taken together, hematopoietic deficiency of Syk somehow prevented the accumulation of various proinflammatory agonists at the site of inflammation, likely leading to the defective accumulation of *Syk*$^{-/-}$ neutrophils and subsequent protection from clinical disease.

Syk$^{-/-}$ *neutrophils fail to respond to type VII collagen-containing immune complexes in vitro*

Since neutrophils are capable of releasing various chemokines, cytokines and lipid mediators (Kovács et al., 2014; Tecchio et al., 2014; Németh et al., 2016), we hypothesized that the above in vivo findings may be due to defective responses of *Syk*$^{-/-}$

$^{/-}$ neutrophils to type VII collagen-containing immune complexes. Indeed, *Syk*$^{-/-}$ neutrophils failed to release superoxide (Fig 5a; $p = 8.6 \times 10^{-4}$) or spread over the adhesive surface (Fig 5b) when plated on immobilized His-C7–anti-C7 immune complexes. In addition, wild-type neutrophils released large amounts of both CXCL2 and LTB_4, whereas no such responses could be observed in *Syk*$^{-/-}$ samples (Figs 5c-d; $p = 3.8 \times 10^{-10}$ and 1.1×10^{-16}, respectively). Those results suggest that Syk is indispensable for neutrophil activation by type VII collagen-containing immune complexes, explaining the defective development of an in vivo inflammatory environment in *Syk*$^{-/-}$ bone marrow chimeras.

DISCUSSION

Together with an accompanying paper (Samavedam et al., submitted), here we report for the first time that the Syk tyrosine kinase is indispensable for an in vivo model of the inflammatory form of epidermolysis bullosa acquisita in experimental mice. These studies raise the possibility that Syk may be a suitable therapeutic target in that disease and, possibly, in other diseases with a similar pathogenesis.

Syk has diverse biological functions (Mócsai et al., 2010), primarily by mediating signaling from immunoreceptors such as Fc-receptors. Fc-receptors play a critical role in mouse models of bullous pemphigoid and the inflammatory form of epidermolysis bullosa acquisita (Sitaru et al., 2005; Zhao et al., 2006; Kasperkiewicz et al., 2012; Schulze et al., 2014). Together with our studies on the role of Src-family kinases (Kovács et al., 2014) and CARD9 (Németh et al., 2016), our experiments suggest that anti-C7-induced skin inflammation is mediated by Syk linking Fc receptor-mediated activation of Src-family kinases to downstream signaling through the CARD9 adapter protein. This is reminiscent of signal transduction in K/B×N serum-transfer arthritis and recognition of fungal pathogens (Gross et al., 2009).

In agreement with other studies on neutrophil function (Németh and Mócsai, 2012; Mócsai, 2013), our results suggest an important role for neutrophils in certain inflammatory autoimmune subepidermal blistering skin disease models. Our results of defective in vivo recruitment of *Syk*$^{-/-}$ neutrophils despite apparently normal intrinsic migratory capacity (Figs 4a-c) and the defective accumulation of neutrophil-attracting proinflammatory mediators (CXCL2 and LTB_4) in vivo (Figs 4d,f) and in in vitro neutrophil assays (Figs 5c-d) suggest that the principal role of Syk is to promote feedback amplification loops through proinflammatory mediator release by neutrophils (Németh and Mócsai, 2016). Though we cannot exclude the role of Syk expression in another cell type, neutrophil-specific deletion of Syk completely protected mice from

anti-C7-induced skin pathology (T. N. and A. M., unpublished observation), indicating the central role for neutrophil-derived Syk in the disease process.

Besides identifying Syk as a critical player in autoantibody-induced skin inflammation, our results also raise a number of novel questions. What is the role of Syk in the initial, immunization phase of autoimmune bullous skin diseases, or the very first steps of anti-C7-induced skin inflammation before the initiation of the neutrophil-mediated feedback amplification process? Would it be possible to document differential chemokine gene expression of wild type and *Syk*$^{-/-}$ neutrophils in mixed bone marrow chimeras? Do our conclusions also apply to other autoimmune subepidermal blistering skin diseases where the autoantigen is other than type VII collagen or where the inflammatory component is less pronounced? How much can we extrapolate our findings on mouse models to human autoimmune and other inflammatory skin diseases? Those and other questions need to be addressed by future follow-up studies.

Parallel to our own study initiated by the relationship between Src-family kinases and Syk (Berton et al., 2005; Kovács et al., 2014; Futosi and Mócsai, 2016), another study described in an accompanying paper (Samavedam et al., submitted) also identified Syk as a core signaling hub in the inflammatory form of epidermolysis bullosa acquisita. While both papers conclude that Syk is critical for disease development, they also complement each other by addressing different additional aspects, such as additional signaling and mechanistic experiments in our study and bioinformatics and pharmacological aspects in the accompanying paper.

The pharmacological therapy of bullous pemphigoid and epidermolysis bullosa acquisita still relies on rather nonspecific immunosuppressive and anti-inflammatory agents. We have identified Syk as a critical component of autoantibody-induced skin inflammation in experimental mice. Though there are clear differences between our mouse model and the corresponding human disease(s) (passive vs. active immunization; neutrophils vs. eosinophils dominating the inflammatory infiltrate; respectively), we believe that our mouse studies relatively closely mimic important aspects of human pathology. Syk has emerged as a potential therapeutic target in other autoimmune inflammatory diseases such as rheumatoid arthritis, as well as in Syk-dependent hematologic malignancies (Geahlen, 2014). Though fostamatinib, the first Syk inhibitor reaching clinical development raised safety concerns likely related to its poor specificity (Anastassiadis et al., 2011; Genovese et al., 2014; Weinblatt et al., 2014; Rolf et al., 2015), there is a large effort from the pharmaceutical industry to develop novel and much more specific Syk inhibitors (Norman, 2014; Walker and Croasdell, 2016). Our results, together with those described in the accompanying

paper (Samavedam et al., submitted) suggest that such Syk-specific inhibitors may be suitable for the pharmacological control of certain inflammatory autoimmune subepidermal blistering skin diseases such as the inflammatory form of epidermolysis bullosa acquisita or, possibly, the mechanistically related bullous pemphigoid.

MATERIALS AND METHODS

Animals

Mice carrying a deleted *Syk* allele (*Syk*^{tm1Tyb}, referred to as *Syk*$^{-}$) were from Victor Tybulewicz (Turner et al., 1995) and kept in heterozygous form. Recipients carrying the CD45.1 allele on the C57BL/6 genetic background (B6.SJL-*Ptprc*^{a} mice from the Jackson Laboratory) were lethally irradiated by 11 Gy from a ^{137}Cs source using a Gamma-Service Medical D1 irradiator and then injected intravenously with fetal liver cells from *Syk*$^{-/-}$ and control fetuses obtained from timed mating of *Syk*$^{+/-}$ carriers (Mócsai et al., 2002; Jakus et al., 2010). *Hck*$^{tm1Hev/tm1Hev}$*Fgr*$^{tm1Hev/tm1Hev}$*Lyn*$^{tm1Sor/tm1Sor}$ triple knockout mice (referred to as *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ or 3× SFK KO mice) were described previously (Meng and Lowell, 1998; Kovács et al., 2014). Mice were kept in individually sterile ventilated cages (Tecniplast) in a conventional facility. All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

Autoantibody-induced skin blistering model

The murine model of the inflammatory form of human epidermolysis bullosa acquisita was triggered by systemic administration of antibodies against collagen type VII which triggers skin inflammation through the antibody Fc portions (Sitaru et al., 2005). Rabbits were immunized with a GST-fusion protein containing a fragment of the immunodominant NC1 domain of murine type VII collagen (GST-C7), followed by total IgG preparation (anti-C7) (Sitaru et al., 2005; Csorba et al., 2010). The reactivity of the antibody preparation was tested with a His-tagged murine type VII collagen fragment (His-C7) (Csorba et al., 2010). Normal rabbit IgG (Sigma) or PBS (Thermo Fisher) was used as control.

Twelve mg pathogenic or control IgG was injected subcutaneously under isoflurane anaesthesia on Days 0, 2, 4, 6 and 8 (60 mg total IgG/mouse). Disease onset and progression was followed by clinical assessment, while quantitative scoring was based on the specific dermatological abnormalities and the size of the affected skin area (Sitaru et al., 2005; Kovács et al., 2014; Németh et al., 2016). Serum anti-C7 levels were determined by ELISA using His-C7. For histological analysis, mice were sacrificed on Day 8 (when no open wounding had occurred), their ears were fixed in

paraformaldehyde, dehydrated, embedded in paraffin, sectioned at 9 µm thickness and stained with hematoxylin and eosin.

In vivo analysis of neutrophil accumulation

Wild type and Syk-deficient bone marrow chimeras were injected with control or pathogenic IgG. On Day 8-10, mice were sacrificed and their ears were removed. The samples were digested with Liberase II (Roche) (Weber et al., 2015). Single cell suspensions were obtained and neutrophil numbers were determined by flow cytometry on the basis of their forward and side scatter characteristics and Ly6G-positivity (Clone 1A8, BD Biosciences).

In vivo neutrophil migration

A competitive migration assay in mixed bone marrow chimeras was used to assess in vivo migration of neutrophils (Mócsai et al., 2002; Jakus et al., 2009; Kovács et al., 2014). Wild type (C57BL/6) or *Syk*$^{-/-}$ bone marrow cells (carrying the CD45.2 allele) were mixed at varying ratios with bone marrow cells from congenic mice expressing CD45.1 on the C57BL/6 genetic background. The cell suspension was injected intravenously into lethally irradiated CD45.1-expressing recipient mice. After bone marrow repopulation, the chimeras were subjected to experimental epidermolysis bullosa acquisita and their ears were digested (before any open wounds occurred). The percentage of CD45.2-expressing neutrophils was analyzed by flow cytometry with staining for Ly6G and CD45.2 (Clone 104, BD Biosciences).

Relative migration of the CD45.2-positive neutrophils (relative to the CD45.1-expressing wild type cells) was calculated as described (Jakus et al., 2009).

Isolation and activation of neutrophils

Mouse neutrophils were isolated from the bone marrow by Percoll (GE Healthcare) gradient centrifugation (Mócsai et al., 2003). Neutrophil assays were performed at 37 °C in HBSS (GE Healthcare) supplemented with 20 mM HEPES, pH 7.4 or in Dulbecco's Modified Eagle's Medium (DMEM, Sigma). Cell surface molecule expression was detected by anti-Ly6G-PE (Clone 1A8), anti-CD18-Biotin (Clone C71/16), anti-CXCR2-PE (Clone #242216, R&D Systems), anti-FcγRII/III (Clone 2.4G2) and anti-FcγRIV-PE (Clone 9E9) antibodies. Where required, secondary staining with Streptavidin-PE or mouse anti-rat-FITC (Clone MRK-1) was performed. If not otherwise stated, the antibodies were from BD Biosciences.

Immobilized immune complex-coated surfaces were obtained by binding His-C7 at 20 µg/ml to Nunc MaxiSorp F96 plates (Thermo Fisher) or tissue culture dishes,

blocked and then treated with anti-C7 at a 1:10 dilution, otherwise as described (Jakus et al., 2008). Human serum albumin (HSA) and anti-HSA immune complexes were generated as described (Jakus et al., 2008).

For the analysis of mediator release, neutrophils were stimulated for 1 (LTB_4) or 6 (cytokines) hours, and supernatants were collected for further analysis (Kovács et al., 2014; Németh et al., 2016). Superoxide release was followed by a cytochrome c reduction test (Németh et al., 2010).

Isolation of other cell types

Platelets were isolated from peripheral blood by mild centrifugation in the presence of heparin. Mast cells were cultured from the bone marrow by murine IL-3 and stem cell factor (both from Peprotech). Macrophages were obtained by culturing bone marrow cells in the presence of recombinant murine M-CSF from a conditioned medium (Kertész et al., 2012).

Analysis of inflammatory mediators

The removed ears were either crushed under liquid nitrogen and lysed by a Triton-based buffer or were cut into small pieces and were digested. The release of mediators was tested from the ear lysates, the cell-free supernatants of digested samples or of in vitro stimulated neutrophils. The levels of CXCL2, IL-1β and LTB_4 were tested by commercial ELISA kits (R&D Systems).

Biochemical studies

For analysis of Syk phosphorylation, neutrophils were plated on immune complex-covered 6-cm tissue culture dishes, incubated for 10 min at 37 °C, and lysed on ice (adherent and nonadherent cells combined) (Kovács et al., 2014). Immunoprecipitation was performed by an anti-Syk antibody (5F5, BioLegend) followed by capturing with Protein G-Agarose (Invitrogen) (Mócsai et al., 2000; Mócsai et al., 2004; Mócsai et al., 2006; Németh et al., 2016). Whole cell lysates from the same experiments were used as controls.

Samples were run on SDS-PAGE and immunoblotted using antibodies against phosphotyrosine (Clone 4G10, Merck Millipore), Syk (N19, Santa Cruz Biotechnology) or β-actin (Clone AC-74, Sigma) followed by incubation with peroxidase-labeled secondary antibodies (GE Healthcare). The signal was developed using the ECL system (GE Healthcare) and exposed to X-ray films.

Presentation of the data and statistical analysis

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

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AUTHOR CONTRIBUTIONS

T. N. and A. M. conceived the study, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. T. N. performed the majority of the experiments. O. V. and C. S. purified the anti-C7 antibodies, performed some of the experiments, and provided further experimental tools and scientific advice. A. M. supervised the project.

CONFLICT OF INTEREST DISCLOSURE

The authors have no conflicting financial interests.

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FIGURE LEGENDS

Figure 1

Type VII collagen-containing immune complexes trigger neutrophil activation and Syk phosphorylation through Src-family kinases

(a) Respiratory burst of wild-type (WT) mouse neutrophils plated on surfaces treated with His-C7 and/or anti-C7. (b) His-C7–anti-C7 immune complexes trigger spreading of wild-type neutrophils. (c) Specificity of the His-C7–anti-C7- and HSA–anti-HSA-induced responses. (d-e) Src-family kinases are essential for His-C7–anti-C7 immune complex-triggered superoxide release and Syk-phosphorylation. Kinetic curves and graphs in a, c and d show mean and SEM of 2-3 independent experiments. In Panels a and d, control data points were subtracted. Panels b and e are representative of 2-3 independent experiments. See the text for actual p values. C7, type VII collagen fragment; HSA, human serum albumin; 3× SFK KO, $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$.

Figure 2

Characterization of wild-type and $Syk^{-/-}$ bone marrow chimeras and their neutrophils

(a) Flow cytometric analysis of the donor-specific CD45.2 epitope in peripheral blood neutrophils tested 4 weeks after bone marrow (BM) transplantation. (b) Immunoblot analysis of Syk expression in various cell types from wild-type (WT) and $Syk^{-/-}$ bone marrow chimeras. (c) Circulating neutrophil (PMN) counts in wild-type and $Syk^{-/-}$ chimeras. (d) Average neutrophil number isolated from the bone marrow of the different chimeras. (e) Cell surface molecule expression of wild-type and $Syk^{-/-}$ bone marrow-isolated neutrophils. Panels a, b and e are representative of 3-10 independent experiments. Graphs in Panel c and d show mean and SEM from 9-11 mice. See the text for actual p values.

Figure 3

Syk is required for experimental epidermolysis bullosa acquisita

Skin disease was triggered in wild-type (WT) or $Syk^{-/-}$ bone marrow chimeras by anti-C7 antibodies and was followed by photographing the heads, the trunks and the ears (a), clinical assessment of the body surface affected (b) and the overall disease severity (c). (d) Serum anti-C7 levels were tested by ELISA. (e) Hematoxylin and eosin stained ear histological sections are presented from WT and $Syk^{-/-}$ chimeras. The right images were magnified from the pictures seen on the left (10x original magnification). Representative images (a, e) or mean and SEM (b-c) from 3 control and 12-13 anti-C7-treated mice per genotype from 3 independent experiments are shown. Panel d shows mean and SEM from 2-3 control and 9-11 anti-C7-treated mice from 2 independent experiments. See the text for actual p values.

Figure 4

Syk is essential for forming the inflammatory environment

(a) The accumulation of neutrophils in the ear upon anti-C7 injection was determined by flow cytometry. (b-c) Mixed bone marrow chimeras with CD45.1-expressing wild type (WT) and

CD45.2-expressing WT or *Syk*$^{-/-}$ hematopoietic cells were injected with anti-C7 antibodies and neutrophil accumulation was determined by flow cytometric analysis. In Panel b, each dot represents an individual ear, while Panel c shows the relative migration of WT and *Syk*$^{-/-}$ neutrophils. (d-f) Decreased in vivo CXCL2, IL-1β and LTB_4 levels in the ears of Syk-deficient chimeras. Graphs represent mean and SEM from 3-5 (a) or 2-6 mice (c), 3-4 control or 5-7 anti-C7-treated mice (d-f) per group from 2-3 independent experiments. See the text for actual p values.

Figure 5

Syk is required for neutrophil responses on immobilized His-C7–anti-C7 immune complexes

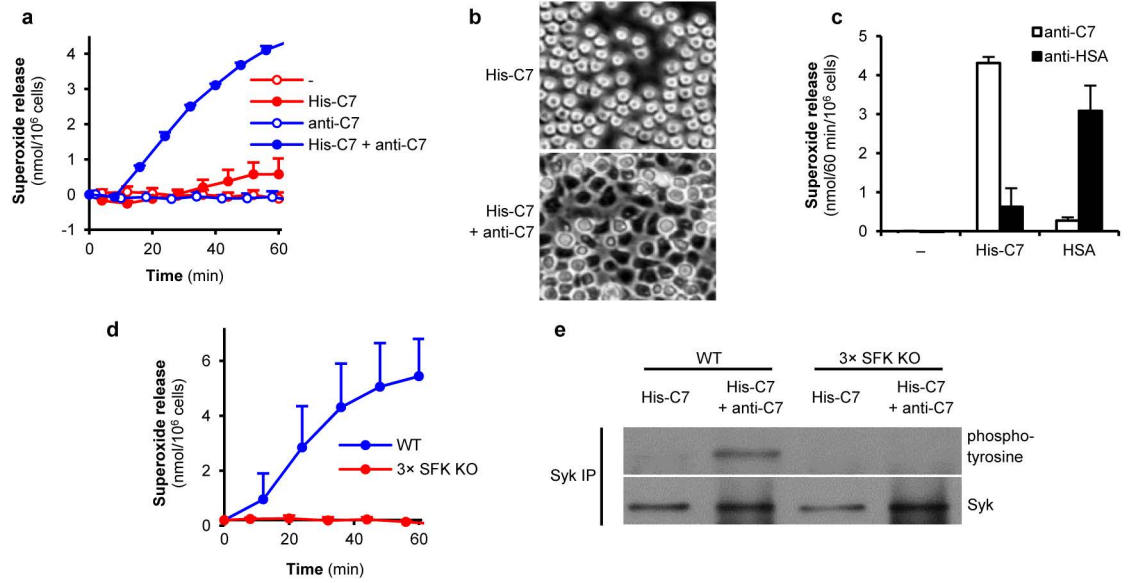
Respiratory burst (a), cell spreading (b), CXCL2 (c) and LTB_4 release (d) of wild-type (WT) and *Syk*$^{-/-}$neutrophils. Curves in Panel a show mean and SEM of 3 independent experiments; control data points were subtracted. Panel b is representative of 3 independent experiments. Graphs in (c-d) show mean and SEM from 3 independent experiments. See the text for actual p values.

Supplementary Figure 1

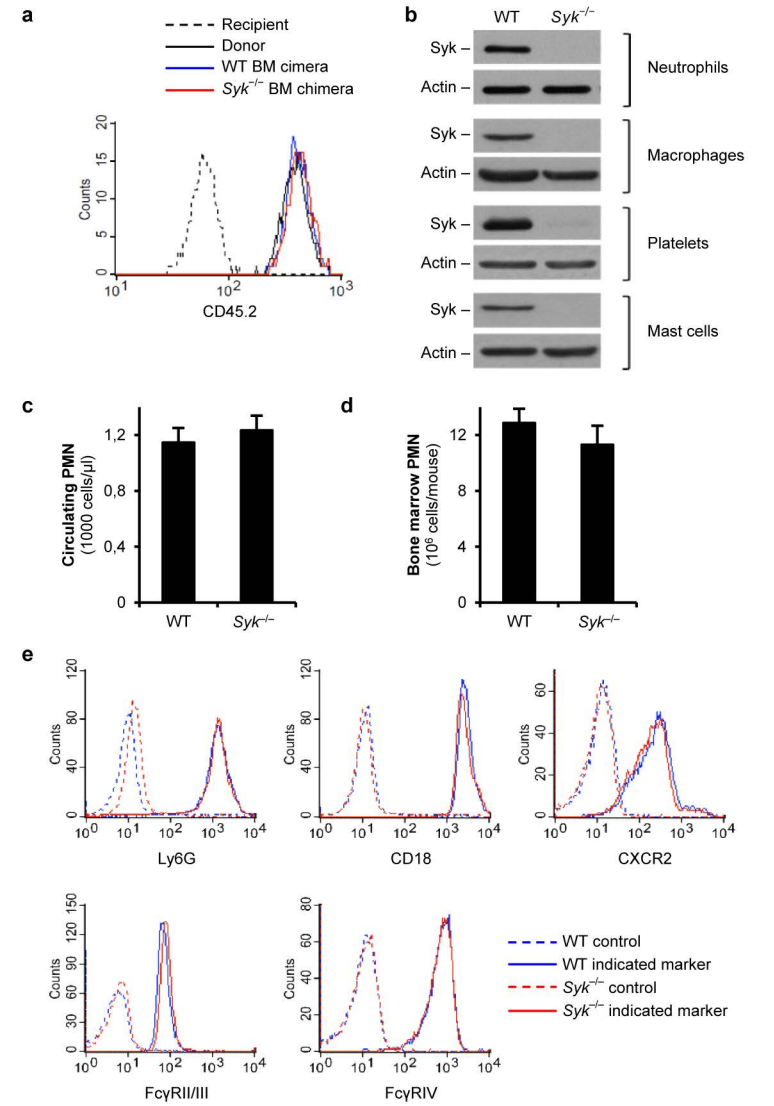
Western blot images with more details

Western blot images showing Syk phosphorylation and Syk expression in Syk-immunoprecipitated samples and whole cell lysates of neutrophils from Fig 1e. IP, immunoprecipitation; 3× SFK KO, *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$; WB, Western blot; WCL, whole cell lysate; WT, wild-type

Németh et al. - Figure 1



Németh et al. - Figure 2



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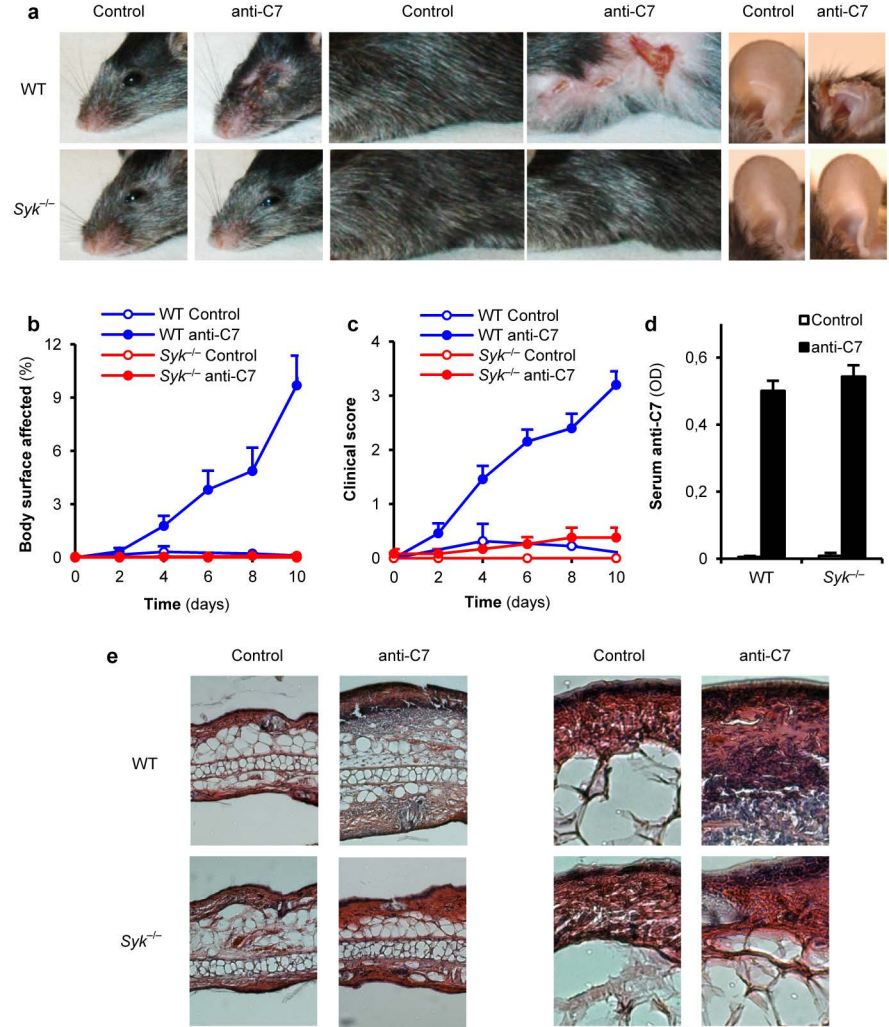

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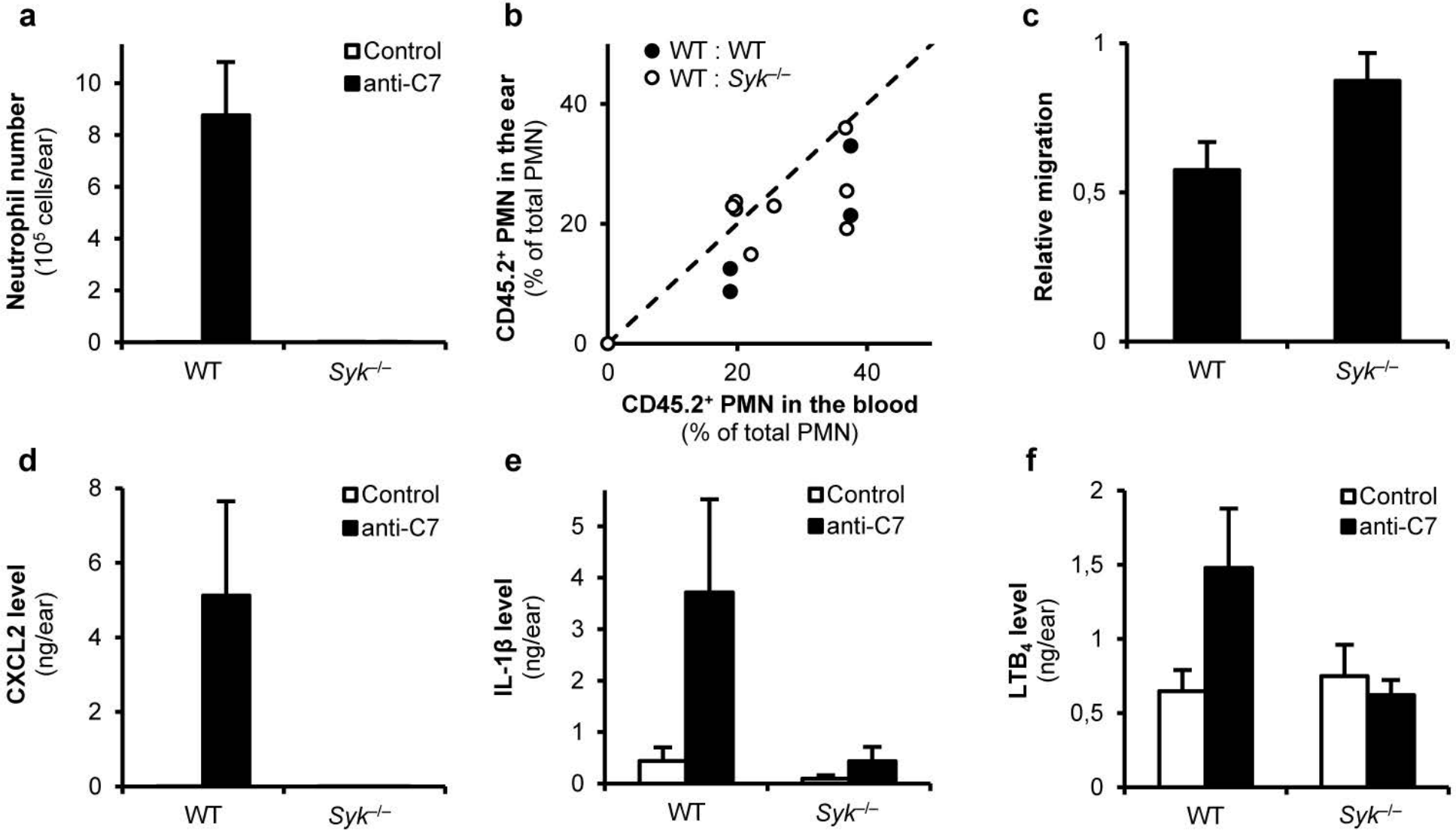

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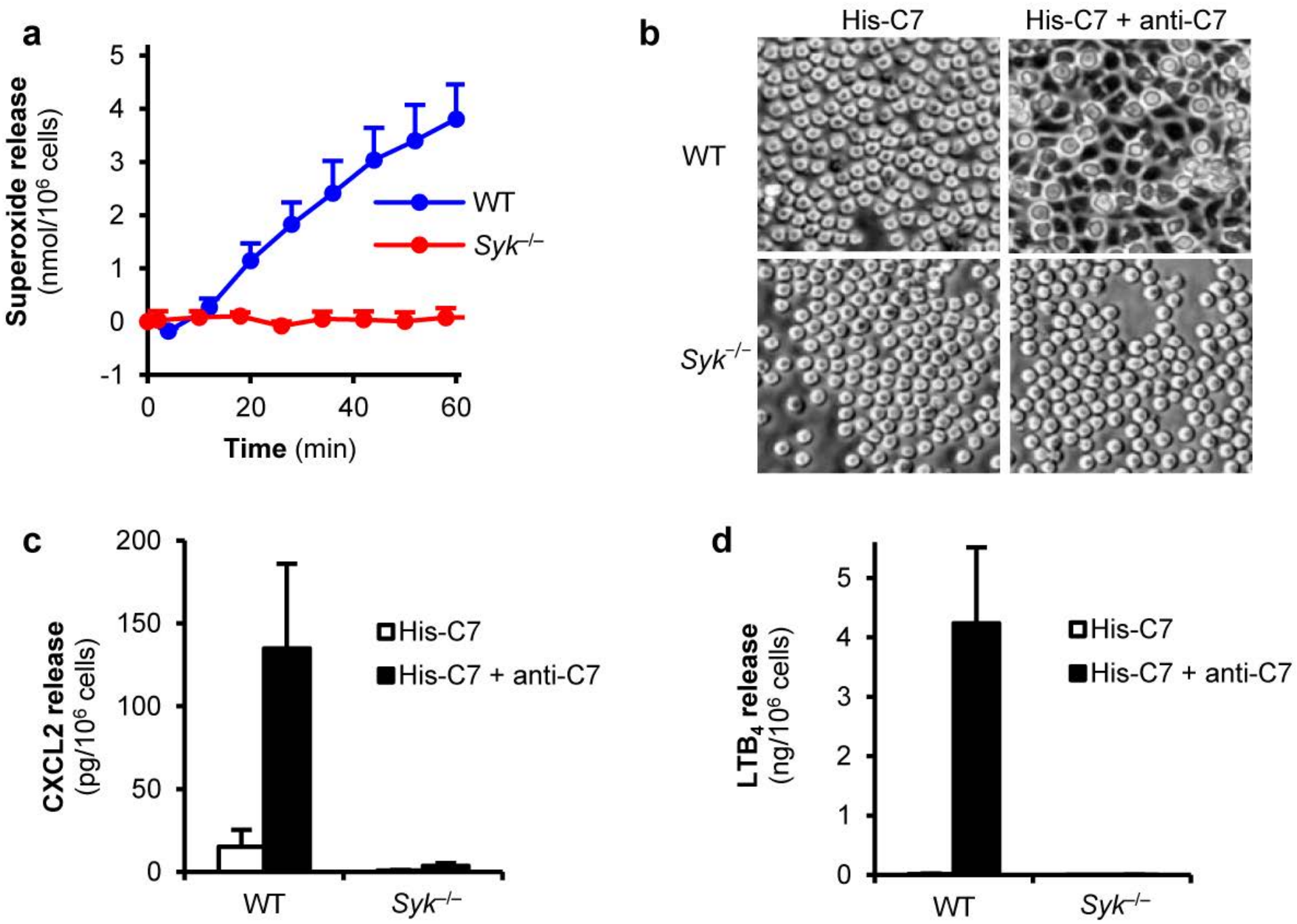

Németh et al. - Suppl. Figure 1