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Src-family and Syk Tyrosine Kinases are Required for Neutrophil Effector Responses to Infection and Inflammation

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Src-Family and Syk Tyrosine Kinases are Required for Neutrophil Effector Responses to Infection and Inflammation

by

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DISSERTATION

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Dedication and Acknowledgments

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The analysis of the *lyn* conditional knock out mouse, and additional work described in Chapter Five, stems from a fruitful collaboration with friend and colleague Dr. Patrizia Scapini.

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You will have to experiment and try things out for yourself and you will not be sure of what you are doing. That's all right, you are feeling your way into the thing. – Emily Carr, Canadian artist

Src-family and Syk Tyrosine Kinases are Required for Neutrophil Effector Responses to Infection and Inflammation

Jessica Ann Grant Van Ziffle

Abstract

Leukocyte specific CD18-integrins are critical in mediating cell recruitment and activation during host defense responses to bacterial infection. The signaling pathways downstream of CD18-integrins are dependent on Src-family kinases, including Hck, Fgr and Lyn, as well as the spleen tyrosine kinase, Syk. In a model of pneumococcal meningitis, deficiency of Hck, Fgr and Lyn results in increased susceptibility, due in part to the reduced ability of deficient neutrophils to phagocytose the bacteria, and undergo respiratory burst. To further investigate the role integrin signaling plays in host defense, we examined the responses of Syk-deficient neutrophils to bacterial challenge with serum-opsonized *Staphylococcus aureus* and *Escherichia coli*. Syk-conditional knockout mice lacking this kinase specifically in myeloid cells or just neutrophils were also used to investigate host responses in vivo. Syk-deficient neutrophils manifested impaired exocytosis of secondary and tertiary granules, reduced cytokine release and very poor activation of the NADPH oxidase in response to serum-opsonized *S. aureus* and *E. coli*. These functional defects correlated with impaired activation of c-Cbl, Pyk2, Erk1/2 and p38 kinases. Bacterial phagocytosis, NET formation and killing were also reduced in Syk-deficient cells, with a more profound effect following *S. aureus* challenge. In vivo, loss of Syk in myeloid cells or specifically in neutrophils resulted in reduced clearance of *S. aureus* following subcutaneous or intra-

peritoneal infection, despite normal recruitment of inflammatory cells. These results indicate that loss of Syk kinase-mediated integrin signaling impairs leukocyte activation, leading to reduced host defense responses.

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Chapter One

Introduction

1.1 Neutrophil development

Neutrophils, like the other granulocytes eosinophils and basophils, are constitutively produced in the bone marrow under the direction of cytokines, the most important of which is granulocyte colony stimulating factor (G-CSF). G-CSF induces a transcriptional program that governs the full spectrum of proliferation, differentiation, functional activation, and apoptosis of hematopoietic progenitors directed along the myeloid lineage. Following regulated release, neutrophils circulate in the blood for only a few hours before migrating out of the vasculature into the connective tissues or other specific sites, where they survive for only a few days. The regulation of neutrophil number, the induction of normal maturation, and the continued balance of cell production with apoptosis are crucial to the normal homeostasis of the innate immune response, as neutrophils must be rapidly released in response to infection or inflammation.

Neutrophils develop through a series of precursors identified by the level of nuclear condensation and the types of granules present in their cytoplasm, which is in part orchestrated by the sequential production of granule components (Borregaard and Cowland, 1997). The first granules to arise during the early myeloblast and promyelocyte stages of neutrophil development are the azurophil (1°) granules, which are characterized by the abundance of myeloperoxidase, and named by their affinity for the basic dye azure A (Spicer

and Hardin, 1969). Cell division continues to take place during the first three stages of neutrophil development, which causes dilution of the 1° granules, and results in a relatively lower number of 1° granules to 2° granules, which begin to form during the third stage, the neutrophilic myelocyte, a time at which myeloperoxidase has also ceased to be produced. 2° granules, or specific granules, are characterized by the presence of lactoferrin, and continue to be formed through the neutrophilic metamyelocyte stage. Following this transition, gelatinase (3°) granules and secretory vesicles begin to form during the neutrophilic band stage, and in the mature neutrophil. The heterogeneity of neutrophil granules is best explained by the "targeting-by-timing" hypothesis, which states that granules are formed during all stages of neutrophil development in the bone marrow, from the early promyelocyte until the segmented neutrophil (Borregaard et al., 2007). Indeed, this hypothesis was recently tested by gene expression profiling of blood neutrophils and neutrophil precursors isolated from normal human bone marrow separated into three major stages of maturation: myeloblasts and promyelocytes; myelocytes and metamyelocytes; and bands and segmented neutrophils as the third (Theilgaard-Monch et al., 2005). This study confirmed that the profile of expressed genes changes dramatically as a neutrophil matures, and even more dramatically as a neutrophil enters a tissue. It has become apparent that not only do neutrophils arrive at sites of inflammation with pre-synthesized anti-bacterial proteins, but neutrophils also initiate the synthesis and secretion of inflammatory chemokines and cytokines upon arrival at such sites. This cascade allows the neutrophil to participate in the recruitment of activated monocytes, other neutrophils and T cells, and also to support wound healing (Borregaard et al., 2007).

1.2 Neutrophil Effector Functions

Engagement of activating receptors on the surface of neutrophils, including Fc receptors, integrins and chemokine receptors, leads to the activation of a variety of effector functions. These include secretion of granule components, release of cytokines, and generation of reactive oxygen intermediates (Berton and Lowell, 1999). During neutrophil activation, a number of pre-formed granule mediators are sequentially released into extracellular and (or) intracellular compartments. Secretory vesicles are the first to fuse with the membrane, increasing the surface expression of a number of neutrophil activating receptors, including the β2 integrin Mac-1 (CD11b; Complement Receptor 3), (Sengelov et al., 1993) which is re-distributed on the plasma membrane following degranulation (Borregaard et al., 1994). In addition, other receptors including CR1, fMLF receptor, and FcγRIII are stored in secretory vesicles and re-distributed to the plasma membrane (Faurschou and Borregaard, 2003). L-selectin (CD62L) is shed from the surface coincident with vesicle degranulation (Tellier et al., 2006); this event is thought to be due to membrane redistribution and co-clustering of TNF-α converting enzyme (TACE; ADAM17) with L-selectin (Schaff et al., 2008). Subsequent to secretory vesicle degranulation, neutrophils release gelatinase granules into the extracellular space. Gelatinase granules contain enzymes, such as gelatinase, leukolysin, and other matrix metalloproteinases required for the degradation of the extracellular matrix. In addition, gelatinase granules deliver additional membrane receptors that are required for neutrophil diapedesis and extravasation, including the β2 integrins (Faurschou and Borregaard, 2003). Specific granules are rich in antibiotic substances, including lactoferrin, an iron-binding protein that sequesters iron and impairs bacterial growth; and lysozyme, which cleaves the bacterial cell wall component

peptidoglycan (Faurschou and Borregaard, 2003). Finally, azurophil granules, which have limited exocytosis, are principally targeted to the phagolysosome, where they deliver a variety of anti-microbial proteins for the destruction of phagocytosed micro-organisms. The defining protein of azurophil granules is myeloperoxidase, which generates hypochlorous acid, a compound with potent antimicrobial oxidizing abilities (Goedken et al., 2007). In addition, azurophil granules contain α-defensins, which are cationic anti-microbial cytotoxic peptides; azurocidin, which is a multifunctional heparin-binding microbicide that also functions as a monocyte chemoattractant; and bacterial permeability increasing protein (BPI), a highly cationic protein that binds negative residues in LPS and causes rearrangement of the bacterial outer membrane lipids leading to bactericidal damage to the inner membrane (Faurschou and Borregaard, 2003). These pre-formed mediators stored within the heterogenous neutrophil granules are principally dedicated to bactericidal activity and clearance of microbial pathogens.

In addition to degranulation of pre-formed mediators, neutrophils synthesize a wide variety of cytokines and chemokines, including proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β; potent chemokines of both the CC and CXC families, including macrophage inflammatory protein 1α (MIP1α; CCL3), MIP-1β (CCL4), IL-8 (CXCL8), GROα (CXCL1), IFNγ-inducible protein 10 (IP-10; CXCL10), and monokine induced by IFNγ (MOG; CXCL9); as well as angiogenic factors, including vascular endothelial growth factor (VEGF) (Kasama et al., 2005; Scapini et al., 2000). The capacity of neutrophils to produce several chemokines, as well as major pro-inflammatory cytokines, indicates that these cells are important in influencing early cell trafficking and activation during pathophysiological processes. For example, the production of distinct cytokines by neutrophils has been demonstrated to influence both the differentiation of

macrophages and host responses to antibiotic resistant strains of *Staphylococcus aureus* (Tsuda et al., 2004).

1.3 Microbicidal Mechanisms of Neutrophils

Neutrophils have a variety of potent anti-microbial activities, including anti-microbial peptides (e.g., α-defensins) and broadly acting proteases (e.g., elastase); however, the critical microbial defensive mechanism provided by neutrophils is the generation of reactive oxygen intermediates (ROIs) (Dale et al., 2008). The importance of generating ROIs by the NADPH oxidase is demonstrated by the recurrent fungal and bacterial infections observed in patients with chronic granulomatous disease (CGD), a genetically heterogeneous immunodeficiency disorder characterized by the inability of neutrophils to kill ingested microbes. CGD arises by defects in one of the four main components of the NADPH oxidase enzyme complex, which is required for the generation of the neutrophil oxidative burst (Heyworth et al., 2003; Meischl and Roos, 1998). The most common mutation found in humans is in CYBB encoding gp91phox (60% of cases), and results in an X-linked inheritance pattern. The autosomal recessive forms of CGD are due to mutations in the genes encoding $p47^{phox}$ (NCF1; 30% of cases), $p22^{phox}$ (CYBA; 5% of cases), or $p67^{phox}$ (NCF2; 5% of cases) (Casimir et al., 1991; Clark et al., 1989; Dinauer et al., 1990; Roos, 1994). In addition to killing by direct products of the NADPH oxidase, indirect products, such as hypochlorous acid, which is generated by myeloperoxidase (MPO) acting on hydrogen peroxide in the presence of a halide, typically Chloride (Dale et al., 2008). While the generation of hypochlorous acid is presumed to be of some importance for microbicidal activity, human deficiency of MPO is a relatively common genetic event (estimated

frequency 1:2000) which does not result in an increase in infection incidence (Stendahl et al., 1984), although there may be a slight increase in *Candida* infections (Lehrer and Cline, 1969), a phenotype also observed in *mpo*$^{-/-}$ mice (Aratani et al., 1999). The loss or reduction in a neutrophils ability to produce ROIs clearly leads to a severe impairment of neutrophil function, resulting in immunodeficiency. Indeed, neutrophil dysfunction is associated with a number of other human immunodeficiencies.

The leukocyte-specific CD18 (β2) integrins are important receptors for neutrophil recruitment and activation. Genetic mutations in the gene encoding the CD18-integrin subunit (ITGB2) results in leukocyte adhesion deficiency (LAD) type I (OMIM 116920) (Etzioni et al., 1999). The CD18 integrin chain is common to three membrane adhesion molecules, namely, LFA-1 (ITGAL), Mac-1 (ITGAM) and CD11c (ITGAX). Thus, reduction or complete loss of CD18 expression due to genetic mutation results in poor or absent surface expression of all three adhesion molecules. The loss of these receptors results in impaired recruitment of phagocytes to areas of infection, defective phagocyte function, and ultimately leads to increased susceptibility to bacterial infections. This phenotype is recapitulated in CD18-deficient mice (Bunting et al., 2002; Rosenzweig and Holland, 2004; Scharffetter-Kochanek et al., 1998). Congenital disorder of glycosylation type IIc (CDG IIc, CDG2C), also known as LADII (OMIM 266265), is an autosomal recessive disorder caused by a mutation if SLC35C1, which encodes a GDP-fucose transporter, and is characterized by moderate to severe psychomotor retardation, mild dysmorphism, and impaired neutrophil motility (Lubke et al., 2001; Luhn et al., 2001). CDG2C is caused by a lack of fucosylated glycoconjugates, including Sialyl-Lewis X, the carbohydrate structure of selectin ligands. Sialyl-Lewis X is required for initial rolling of neutrophils, mediated by E- and P-selectin expressed on endothelial cells, and L-selectin expressed on neutrophils; the loss of Sialyl-

Lewis X results in impaired neutrophil recruitment to sites of inflammation, and thus increased susceptibility to bacterial infections, although much milder than the susceptibility seen with LAD I patients. A third, rare LAD has been described, LAD III, which emphasizes the importance of the integrin activation phase in the adhesion cascade. Although the primary defect is somewhat controversial, it is clear that all hematopoietic integrin activation processes are defective, leading to severe susceptibility to bacterial infections, as observed in LAD I, and to an increased tendency for bleeding problems (Etzioni, 2009). Initially, a mutation in CALDAGGEF1 (RASGRP2) was identified in two Turkish patients (Pasvolsky et al., 2007); however, more recently, this mutation was discovered to be a benign founder-effect in the Turkish families, with the pathogenic mutation lying within a linked gene, KINDLIN3 (Malinin et al., 2009; Svensson et al., 2009). Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells (Moser et al., 2009).

The loss or reduction in neutrophil numbers themselves also results in human immunodeficiencies. Severe congenital neutropenia is a heterogeneous disorder of hematopoiesis characterized by a maturation arrest of granulopoiesis (Skokowa et al., 2007), typically caused by an autosomal dominant mutation in the gene encoding neutrophil elastase, ELA2. Dominant mutations in ELA2 are also responsible for cyclic neutropenia, where peripheral blood neutrophil counts oscillate between zero and normal levels with an approximate frequency of 21 days (Horwitz et al., 1999). Cyclical neutropenia places affected individuals at risk for opportunistic infections during neutropenic intervals. Interestingly, neutropenia has not been reported in $NE^{-/-}$ mice; however, these mice have an increased susceptibility to Gram-negative bacteria (e.g., *E. coli*) while maintaining bactericidal activity to Gram-positive bacteria (e.g., *S. aureus*) (Belaaouaj et al., 1998). A related form of

autosomal recessive severe congenital neutropenia, known as Kostmann disease (Kostmann, 1956), was recently determined to be due to a mutation in HAX1, which encodes a mitochondrial protein involved in signal transduction and cytoskeletal control, and plays a critical role in protecting myeloid cells from apoptosis (Klein et al., 2007). Chediak-Hiagashi syndrome is caused by mutation in the lysosomal trafficking regulator gene, LYST (Nagle et al., 1996), which prevents the normal formation of the phagolysosomes and melanosomes. Chediak-Hiagashi syndrome is characterized by a spectrum of disorders including neutropenia, albinism and enlarged lysosomes. It is clear that while the pathological insult might vary between these neutrophil dysfunction disorders, the major impact is abnormal susceptibility to infections. Patients with congenital neutropenia benefit greatly from treatment with G-CSF, which boosts peripheral neutrophil numbers, reduces infection rates and thus reduces reliance on anti-bacterial and anti-fungal treatments.

For many patients with neutrophil dysfunction syndromes, including CGD and LAD, current treatment options are limited to long-term antibiotic and antifungal prophylaxis or successful acceptance in a bone marrow transplantation program. Furthering our understanding of neutrophil effector functions will contribute to the way neutrophil-mediated diseases are treated in the clinic. Boosting neutrophil function will be of obvious benefit for patients with neutropenia or neutrophil dysfunction. Conversely, understanding how best to inhibit neutrophil effector functions will be of benefit for the treatment of neutrophil-dependent inflammatory disorders, such as rheumatoid arthritis.

Chapter Two

Materials and Methods

2.1 Mice

Syk$^{f/f}$ mice were originally described by Saijo *et al.* (Saijo et al., 2003). *Syk*$^{f/f}$ mice, backcrossed to C57BL/6 for at least 8 generations, were crossed to animals expressing Cre recombinase under control of the endogenous lysozyme M promoter (*LysMcre*$^{Tg/Tg}$) (Clausen et al., 1999), or under control of the transgenic human Mrp8 promoter (*Mrp8cre*^{Tg}) (Passegue et al., 2004). Experimental mice were generated by crossing *syk*$^{f/f}$*LysMcre*$^{Tg/Tg}$ or *syk*$^{f/f}$*Mrp8cre*^{Tg} mice to *syk*$^{+/-}$ mice to generate littermate pairs of s*yk*$^{f/+}$*LysMcre*$^{Tg/+}$ and *syk*$^{f/-}$*LysMcre*$^{Tg/+}$ or *syk*$^{f/-}$ *Mrp8cre*^{Tg} mice. The genotypes of additional control mice used were: *syk*$^{f/f}$ and *syk*$^{f/-}$ to control for heterozygosity of the *syk* gene. For complete deletion of *syk* in the hematopoietic lineage, *syk*$^{f/f}$ mice were crossed to *Vav1cre*^{Tg} mice (de Boer et al., 2003). *Syk*$^{-/-}$ mice have been described (Cheng et al., 1995; Turner et al., 1995); *syk*$^{-/-}$ and WT control cells were obtained from bone marrow chimeras generated by using fetal liver cells to reconstitute lethally irradiated mice, as described (Mocsai et al., 2002). *Hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice have been described (Meng and Lowell, 1998), and then backcrossed for 11 generations into a C57BL/6 background. *CD18*$^{-/-}$ and *CD11b*$^{-/-}$ mice were purchased from the Jackson Laboratory. The generation of *lyn* conditional knock-out mice *(lyn*$^{f/f}$*)* is described in text to follow, and have been backcrossed for 9 generations into a C57BL/6 background. Animals were kept in a specific pathogen-free facility at UCSF and used according to protocols

approved by the UCSF Committee on Animal Research. All experiments were conducted with animals aged 8 to 12 weeks.

2.2 Antibodies and flow cytometry

Bone marrow was separated into single cell suspensions by flushing with a 25 gauge needle, followed by re-suspension through a 19 gauge needle. 1–3 x 10^6 cells were used for each stain. Monoclonal antibodies against Gr-1 (RB6; eBiosciences), Ly6G (1A8; BD Pharmingen), CD11b (M1/70; BD Pharmingen), 7/4 (abcam), and CD62L (MEL-14; eBiosciences) were used to delineate neutrophils. A monoclonal antibody against mouse Syk clone 5F5 conjugated to Alexa Fluor 488 or Alexa Fluor 647 as described (Palacios and Weiss, 2007) was a kind gift of Dr. Art Weiss (University of California, San Francisco). A polyclonal antibody against mouse Lyn was conjugated to Fluorescein isothiocyanate (FITC; Invitrogen), according to the manufacturers protocol. Cells were surfaced stained, followed by fixation and permeabilization (eBiosciences) and intracellular staining for Syk or Lyn. For pErk and pp38 staining, neutrophils were stimulated with *rag1*$^{-/-}$ serum opsonized-*S. aureus* or -*E. coli*, and fixed at the indicated time points with 1% para-formaldehyde, followed by permeabilization in 90% methanol, and stained in the presence of 2.4G2 with 7/4 FITC, Gr1 APC and a PE-labeled antibody to pErk1/2 (BD Pharmingen), or stained with Ly6G PE, CD11b APC and an Alexa Fluor 488-labeled antibody to pp38 (BD Pharmingen). Data were collected on a modified FACScan (BD Biosciences; Cytek Development) and analyzed using FlowJo software (TreeStar, Inc.).

2.3 Neutrophil Functional Assays and Detection of Granule Markers

Bone marrow neutrophils from WT, *syk*$^{-/-}$, *CD11b*$^{-/-}$, and *CD18*$^{-/-}$ mice were isolated with a one-step 62% percoll density gradient medium, as previously described (Pereira and Lowell, 2003). All bacterial stimulations were conducted by opsonizing either *S. aureus* or *E. coli* with 10% *rag1*$^{-/-}$ serum for 15 min at 37°C with end-over-end rotation. Induction of neutrophil oxidative burst was performed as described (Lowell et al., 1996) with the following modifications: ImmulonIV plates were blocked with 0.1% milk, and neutrophils were stimulated with *S. aureus* (MOI=5) or *E. coli* (MOI=10). Oxidative burst was assessed by reduction of cytochrome C over the indicated time course. Superoxide production was also monitored by flow cytometry following the stimulation of $1x10^6$ neutrophils loaded with 5μM of 3'-(p-aminophenyl) fluorescein (APF; Invitrogen). Secretory vesicle degranulation was performed similarly, and analyzed by flow cytometry following surface staining for CD11b and CD62L.

Gelatinase zymography was performed as in (Mocsai et al., 2006). Briefly, non-reducing sample buffer was added to cell supernatants, which were separated by zymogram gel electrophoresis (Invitrogen), were renatured in 2.5% Triton X-100 and were developed overnight at 37°C in zymography buffer followed by Coomassie blue staining. An Alpha Innotech Alphaimager was used for quantification.

Activation of elastase was detected by loading $1x10^6$ neutrophils with 10 μM ElastoLux® (OncoImmunin, Inc.) for 30 min at 37°C, followed by stimulation with *S. aureus* or *E. coli* (MOI = 5) for the indicated time points. Samples were collected by flow cytometry and analyzed for the percentage of neutrophils positive for ElastoLux® fluorescence.

Degranulation of the secondary granule marker lactoferrin was determined by ELISA as described previously (Mocsai et al., 1999). Supernatants were collected from bone marrow neutrophils stimulated for 60 min with *S. aureus* or *E. coli* (MOI = 5), (anti-human Lactoferrin antibody; Immunology Consultants Laboratory, Inc.).

Supernatants were collected from neutrophils stimulated for 60 min with *S. aureus* or *E. coli* (MOI = 5). Degranulation of the secretory vesicle marker albumin was determined by ELISA (Immunology Consultants Laboratory, Inc.). The azurophil granule marker myeloperoxidase was detected by colorimetric assay by incubating 10 μL of supernatant with 200 μL of *o*-Dianisidine solution (0.165 mg/mL) and 10 μL of 0.1% H_2O_2. Absorbance was read at 405 nm after 5 to 30 minutes. The azurophil granule marker β-D-glucuronidase was detected by fluorescence assay. 25 μL of supernatant was incubated with 50 μL of freshly prepared substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide in 0.1 M sodium acetate, pH 4.0, containing 0.1% Triton X-100) for 16hr at 37°C, followed by addition of 200 μL of stop solution (0.05 M glycine, 5 mM EDTA; pH 10.4) to each well prior to measurement with excitation wavelength of 355 nm and emission wavelength of 460 nm.

Inhibitors of MAPKs (PD98059, SB203580) were from EMD Biosciences (San Diego, CA). Bone marrow cells or purified bone marrow neutrophils were incubated with 20 μM PD09059, 3 μM SB203580, or DMSO 30 minutes prior to stimulation with *S. aureus* or *E. coli*.

2.4 Cytokine analysis

Culture supernatants harvested from neutrophils after 16 hours culture in DMEM containing 10% FCS plus penicillin and streptomycin, plus *E. coli* (MOI=4) or *S. aureus*

(MOI=2). Supernatants were analyzed in triplicate for IL-1β, IL-6 and TNF-α by ELISA using commercially available monospecific kits (BioSource), or for IL-1β, IL-6, TNF-α, MIP1α, MIP2, KC, IP-10 and MCP1 by multiplex bead array assay (Millipore) and acquired on a Bio-Rad Bio-Plex instrument.

2.5 RNA extractions and Taqman Real-Time PCR Analysis

RNA extractions were performed using an RNeasy kit (Qiagen) according to manufacturer's instructions. Reverse-transcription was performed using iScript cDNA Synthesis Kit (BIO-RAD Lab) according to manufacturer's instructions. Quantitative RT-PCR was performed on an ABI7700 sequence detection instrument (Taqman; PE Applied Biosystems). Primer pairs and probes, including their specificity, orientation (forward, F; reverse, R), and sequence were as follows: HPRT (F-AGGTTGCAAGCTTGCTGGT, R-TGAAGTACTCATTATAGTCAAGGGCA, probe-TGTTGGATACAGGCCAGACTTTGTTGGAT), TNFα, (F-CTGTCTACTGAACTTCGGGGTGAT, R-GGTCTGGGCCATAGAACTGATG, probe-ATGAGAAGTTCCCAAATGGCCTCCCTC). Values of TNFα mRNA were normalized to values of HPRT mRNA in each sample.

2.6 Western blotting

Phosphorylation of $p40^{phox}$ was determined by western blotting using a polyclonal antibody that recognizes phospho-Thr 154 (Cell Signaling Technology, 4311). Neutrophils were pre-stimulated with TNFα (50 ng/mL) for 30 min at 37°C, then incubated with *S. aureus* or *E. coli*

at an MOI of 5 for 30 min at 37°C, or with 10 nM PMA. Cell lysates were prepared for western blotting as described (Mocsai et al., 2002). Phosphorylation of c-Cbl and Pyk2 were determined by western blotting of neutrophils, stimulated with *S. aureus* or *E. coli* at an MOI of 5 for 10 min at 37°C, and lysed directly in 4X sample buffer. The following antibodies were used: phospho-c-Cbl Tyr774 (Cell Signaling Technology, 3555), total c-Cbl (Santa Cruz, 170), phospho-Pyk2 Tyr579 (Biosource 44632), total Pyk2 (Santa Cruz, 1514), actin C-2 (Santa Cruz, 8432).

2.7 Bactericidal Assay and Phagocytosis

Neutrophils were allowed to bind opsonized E. coli or S. aureus (MOI=10) for 10 minutes prior to the addition of 50 μg/mL gentamicin. At the indicated time points, neutrophils were washed, and lysed in Luria Broth + 0.1% Triton X-100 on ice for 20 min. Suitable dilutions were plated to determine the number of recovered viable bacteria.
Phagocytosis (MOI = 10) was assessed by flow cytometry using FITC-labeled heat-shock-killed bacteria as previously described (Voyich and DeLeo, 2002). In brief, heat-shock killed *S. aureus* or *E. coli* were labeled with FITC (Pierce), opsonized with 10% *rag1*$^{-/-}$ serum and incubated with neutrophils for the indicated time points. Prior to FACS analysis, an equal volume of 0.4% Trypan blue (Sigma) was added to quench the extracellular FITC-labeled bacteria.

2.8 Actin polymerization

Measurement of actin polymerization after bacterial stimulation was done by a flow cytometry–based phalloidin-binding assay. Bone marrow cells were purified and stimulated with *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E. coli* for the indicated time points. Following stimulation, bone marrow cells were fixed in 2% para-formaldehyde (methanol free) for 15 minutes at 37°C. Cells were then stained in permeabilization buffer (BD biosciences) for neutrophil markers, CD11b and Ly6G, and phalloidin-AlexaFluor546 (Invitrogen), and analyzed by flow cytometry.

2.9 Microscopy

Neutrophils were incubated for 60 minutes in the presence of 50 ng/mL TNFα with *S. aureus* (MOI=5) or *E. coli* (MOI=10). Cytospins were prepared from $5x10^4$ neutrophils, and were fixed in 4% para-formaldehyde. Slides were blocked with 4% goat serum and 1% BSA, followed by staining with anti-pan Histone (Chemicon, 3422), phalloidin-Alexa 546 (Molecular Probes), and 2 μg/mL DAPI. Image acquisition was achieved with an inverted Axiovert 200 M microscope and a ^{X}63/1.4 lens (Carl Zeiss, NJ), equipped with a 175 W xenon highspeed DG4 wavelength selector and a single emission filter wheel (Sutter Instruments, Novato, CA), a PI piezoelectric z-drive (Physik Instrument, Germany), and a cooled-CCD Coolsnap camera (Roper Instruments, NJ). Data were acquired with MetaMorph software (Molecular Devices) and images were colored and overlaid using the MetaMorph "Color Combine" command.

2.10 Measurement of Peripheral Blood Cell Count and Differential

A sample of blood was placed in an EDTA-coated vial, and a multispecies hematology instrument (Hemavet 950FS; Drew Scientific) was used to generate a complete blood count with cellular differential.

2.11 Nonlethal Skin Abscess Infection Model

$2x10^7$ early exponential phase *S. aureus* (ATCC strain SA113) or $6x10^6$ *E. coli* serotype K1, originally isolated from the blood of a patient with biliary sepsis (Matute-Bello et al., 2001), were injected into a 7 day air pouch created by the subcutaneous injection of 5 cc of sterile air on day 1 and re-inflation with 2.5 cc on day 4. After 24hr, the pouch abscess was lavaged with 3 mL of cold HBSS + 0.315% sodium citrate. Neutrophils from the pouch lavage were enumerated, and an aliquot was lysed in LB/0.1% Triton X-100. Suitable dilutions were plated on LB-agar to determine the number of recovered viable bacteria.

2.12 Intra-peritoneal infection model

$2x10^7$ early exponential phase *S. aureus* was injected i.p. After 4hr, the peritoneum was lavaged with 10mL of cold HBSS + 0.315% sodium citrate. Neutrophil numbers from the lavage were enumerated, and an aliquot was lysed in LB/0.1% Triton X-100. Suitable dilutions were plated on LB-agar to determine the number of recovered viable bacteria.

Chapter Three

Functions of Spleen Tyrosine Kinase (Syk)

3.1 Introduction

Syk is a protein-tyrosine kinase that is widely expressed in hematopoietic cells, with some reports suggesting a broader level of expression in epithelial cells, including human breast tissue (Coopman et al., 2000) and human umbilical vein endothelial cells (Inatome et al., 2001). The main function of Syk is in coupling activated immunoreceptors or integrins to downstream signaling events. These signalling events and the diverse cellular responses that they mediate will be discussed in detail below.

3.1.1 The Unexpected Role for Syk in Vascular-Lymphatic Separation

There is extensive interest in the role that the protein tyrosine kinase Syk plays in signaling downstream of immune receptors. To investigate this, Turner *et al.* and Cheng *et al.* systemically deleted *syk* by homologous recombination in mice. Surprisingly, in addition to abrogating immune receptor signaling, Syk deficiency resulted in embryonic hemorrhage and perinatal lethality (Cheng et al., 1995; Turner et al., 1995). To be able to study the consequences of Syk deficiency in immune cells, fetal liver hematopoietic stem cells from *syk*$^{-/-}$ mice were transferred into lethally irradiated adult recipients. These radiation chimeras develop fatal abdominal hemorrhages by 3 months post-fetal liver adoptive transfer (Kiefer

et al., 1998), phenocopying the vascular phenotype in *syk*$^{-/-}$ mice. Interestingly, a hemorrhagic phenotype has also been observed in SLP-76-deficient (Clements et al., 1999; Pivniouk et al., 1998) and PLCγ2-deficient (Wang et al., 2000) mice, two molecules that participate in some of the same signaling pathways as Syk. The phenotype of both SLP-76 and Syk deficient embryos was determined to be a defect in the regulation of separation of blood and lymphatic vascular systems (Abtahian et al., 2003). SLP-76 deficient bone marrow radiation chimeras further revealed a requirement for SLP-76 signaling in bone marrow-derived cells for normal vascular development (Abtahian et al., 2003), similar to that previously described for Syk. It is still unclear which cell types require signaling through Syk and SLP-76 to regulate normal lymphatic and blood vascular development, and through what receptors this regulation is functioning.

3.1.2 Syk Kinase is Required for Immune Receptor Signaling

In contrast to the role of Syk in the regulation of vascular development, the role of Syk in immune receptor signaling is well characterized. Engagement of immunoreceptors, including the T- and B-lymphocyte antigen receptors, Fcγ and Fcε receptors, NK activating receptors and the platelet receptor GPVI, results in the phosphorylation of immuno-receptor tyrosine-based activation motifs (ITAMs). ITAMs are either located in the intracellular domains of immune receptors themselves, or more commonly, they are present in the intracellular domains of the receptor adaptor molecules. The ITAM dual tyrosines are phosphorylated by Src-family kinase members, leading to the recruitment of phosphorylated and activated Syk-family kinases (Greenberg et al., 1994). Syk is recruited to the phosphorylated ITAM through its SH2 domains, forming a signaling complex at the plasma

membrane. Subsequently, Syk triggers a signaling cascade through the phosphorylation of a number of downstream adaptor molecules, including LAT, BLNK and SLP-76. These adapter proteins bind a number of other signaling molecules, including Grb2, which leads to activation of the Ras pathway, Vav, which activates Rho-GTPase signaling and PI-3 kinase, which activates lipid second messengers. The triggering of these signaling events leads to cellular changes to morphology through actin cytoskeletal rearrangement, and to gene expression through alteration of transcriptional activity. These changes have functional consequences, which differ depending on cell type, but include proliferation, cytokine secretion and antibody secretion in lymphocytes or phagocytosis and degranulation in myeloid cells (Lowell, 2004).

Antibody, or immunoglobulin, opsonization allows for the efficient elimination of foreign antigens through recognition of the Fc portion of immunoglobulins by the Fcγ immunoreceptors (FcγRs). In mice, monocytes and macrophages express all activating and inhibitory FcγRs (FcγRI–FcγRIV), neutrophils mainly express the inhibitory FcγRIIB and the activating FcγRIII and FcγRIV (Nimmerjahn and Ravetch, 2008). Furthermore, in monocytes, macrophages and neutrophils the cross-linking of activating FcγRs causes a range of cellular functions, including phagocytosis of IgG-coated micro-organisms, phagocytosis of IgG-coated red blood cells or other cells; ingestion of non-cellular immune complexes; antigen presentation; release of inflammatory mediators, including ROIs; and altered gene expression (McKenzie and Schreiber, 1998). With the notable exception of human FcγRIIA and FcγRIIC, activating FcγRs require an ITAM-bearing adaptor molecule to transduce signaling events. The requirement for Syk in mediating FcγR signaling events in myeloid cells is demonstrated by the inability of *syk*$^{-/-}$ bone marrow-derived macrophages to phagocytose IgG-coated sheep red blood cells (Crowley et al., 1997), and the decreased

production of hydrogen peroxide by *syk*$^{-/-}$ neutrophils in response to IgG-coated zymosan particles (Kiefer et al., 1998).

3.1.3 Syk Kinase Is Required for Integrin Receptor Signaling in Neutrophils

Granulocytes and macrophages express members of the β1, β2 and β3 integrins (Berton et al., 2005). Engagement of neutrophil integrins activates a variety of effector functions, including secretion of granule components, release of cytokines, and generation of reactive oxygen species (Berton and Lowell, 1999). During neutrophil activation, a number of preformed granule mediators are sequentially released in a hierarchy into extracellular and (or) intracellular compartments (Borregaard and Cowland, 1997). In addition, neutrophils synthesize a wide variety of cytokines (Scapini et al., 2000), which are stored in an uncharacterized intracellular location. The production of distinct cytokines by neutrophils has been demonstrated to influence both the differentiation of macrophages and responses to antibiotic resistant strains of *Staphylococcus aureus* (Tsuda et al., 2004). Finally, the importance of generating reactive oxygen intermediates (ROIs) by the NADPH oxidase is demonstrated by the recurrent fungal and bacterial infections observed in patients with chronic granulomatous disease (CGD), a genetic disorder arising from mutations in components of the NADPH oxidase (Heyworth et al., 2003; Meischl and Roos, 1998).

Integrin signaling in phagocytic cells is mediated by Src-family and Syk tyrosine kinases, and has been demonstrated to require ITAM-bearing adaptor molecules, such as Dap12 or FcRγ (Mocsai et al., 2006). Several downstream substrates of Src-family and Syk kinases have been identified and implicated in effector functions of integrin ligation, including Pyk2, c-Cbl, SLP-76 (Newbrough et al., 2003), Vav1/3 (Gakidis et al., 2004),

PLCγ2 (Graham et al., 2007) and Erk (Abram and Lowell, 2007; Berton et al., 2005). Signaling events triggered by the engagement of integrins are thought to be important for cellular migration; however, studies investigating the role of Syk in CD18 (β2) integrin-mediated migration demonstrated that *syk*$^{-/-}$ neutrophils migrate normally in transwell assays, thioglycollate induced sterile peritonitis (Mocsai et al., 2002), as well as during the development of Mac-1 (CD11b)-mediated thrombohemorrhagic vasculitis (Hirahashi et al., 2006). While these observations may suggest that CD18 integrin-dependent migration is independent of signaling through Syk**,** more recent studies indicate that the integrin - ITAM pathway regulates the velocity of neutrophil migration in tissues, rather than overall directionality (Graham et al., 2009). In contrast, it is clear that activation of adhesion-dependent neutrophil effector functions, such as release of specific granules or production of ROIs following adhesion to integrin ligands, are dependent on Syk signaling (Mocsai et al., 2002). Loss of this signaling pathway completely blocks tissue injury in the thrombohemorrhagic vasculitis model, despite normal recruitment of leukocytes into the inflamed tissue, indicating the importance of integrin signaling pathways in inflammatory disease. However, the physiologic effect of alterations in integrin signaling in host defense to pathogen infection *in vivo* remains unknown.

To study the role of Syk signaling in host defense mediated by myeloid cells, mice containing the loxP flanked-allele of *syk* (*syk*$^{f/f}$) were cross to mice containing Cre under the control of the neutrophil and macrophage specific promoter, Lysozyme M (Clausen et al., 1999) or the neutrophil specific promoter, Mrp8 (Passegue et al., 2004). These mice provide a system with which to assess the importance of Syk in neutrophils during acute bacterial infections in a non-lethal subcutaneous skin abscess model. Syk-deficient neutrophils fail to secrete granule components, release induced cytokines, and generate ROIs, while retaining

limited elastase activity in response to both *Staphylococcus aureus* and *Escherichia coli*. Following bacterial infection, neutrophil migration is normal in the absence of Syk. However, as a consequence of the defects in neutrophil function, killing of *S. aureus*, and to a lesser extent *E. coli*, is impaired both *in vitro* and *in vivo*. These data support a critical role for Syk signaling in the induction of neutrophil effector functions during innate immune responses.

3.2 Results

3.2.1 Neutrophil degranulation

Following neutrophil activation, secretory vesicles fuse with the plasma membrane. CD11b is stored in secretory vesicles (Sengelov et al., 1993) and is re-distributed on the plasma membrane following degranulation (Borregaard et al., 1994). In addition, CD62L (L-selectin) is shed from the surface coincident with vesicle degranulation (Tellier et al., 2006). To determine the requirement for Syk in bacterial-induced secretory vesicle degranulation, the up-regulation of CD11b and the shedding of CD62L were assessed by flow cytometry (Figure 3.1 A). In these experiments, bacteria were opsonized with fresh serum isolated from *rag1*$^{-/-}$ mice, to ensure that integrins were the primary leukocyte receptors engaged while avoiding Ig opsonization that could trigger Fc receptors. Exposure of WT neutrophils to *rag1*$^{-/-}$ serum-opsonized *S. aureus* or *E. coli* resulted in a significant increase in CD11b expression on the cell surface. The median fluorescence intensity (MFI) of CD11b increased 4 fold at 30 to 60 min following stimulation with either bacteria (Figure 3.1 B and C). Concomitant with increased CD11b expression was loss of CD62L from the surface of ~80% of WT neutrophils by 60 min (Figure 3.1 D and E). *Syk*$^{-/-}$ neutrophils failed to both up-regulate CD11b, and shed CD62L following stimulation with either type of bacteria (Figure 3.1 B and E), with a greater effect observed following *S. aureus* incubation. Albumin secretion is an additional marker of vesicle degranulation, and this was also decreased in the absence of Syk (Figure 3.2 A).

The ability to shed CD62L in response to *S. aureus* was partially dependent on CD18 integrins, as neutrophils from *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ mice showed an intermediate ability to

shed CD62L (Figure 3.1 F). CD62L shedding in response to *E. coli* depended more highly on CD18 expression than CD11b expression (Figure 3.1 G), and is likely due to the contribution of CD11c (Complement Receptor 4). Secretory vesicle degranulation was also monitored in response to bacteria opsonized with heat-inactivated (HI) *rag1*$^{-/-}$ serum, to eliminate the presence of the complement ligand. However, up-regulation of CD11b and shedding of CD62L were still compromised in the absence of Syk, and CD62L shedding was compromised in the absence of CD11b and CD18 (Figure 3.1 H,I). This suggests that there are other integrin ligands on the surface of *S. aureus* and *E. coli*, in addition to iC3b. It has been demonstrated that LPS and intact *E. coli* can bind directly to CD18-integrins (Wright and Jong, 1986). In addition, *S. aureus* expresses gene products that bind to fibronectin (GeneID: 3920926) or fibrinogen (GeneID: 3920717), allowing indirect associations with CD18-integrins, as well as β1 integrins (Schwarz-Linek et al., 2004).

Release of the three major granule types was analyzed to determine the requirement for Syk in their exocytosis. Release of gelatinase granules (Figure 3.1 J,K) and the secondary granule marker lactoferrin (Figure 3.1 L) were impaired in *syk*$^{-/-}$, *CD11b*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils in response to bacteria opsonized with *rag1*$^{-/-}$, and HI *rag1*$^{-/-}$ serum. There was no appreciable defect in the release of the azurophilic granule markers myeloperoxidase and β-glucoronidase (Figure 3.2 B and C).

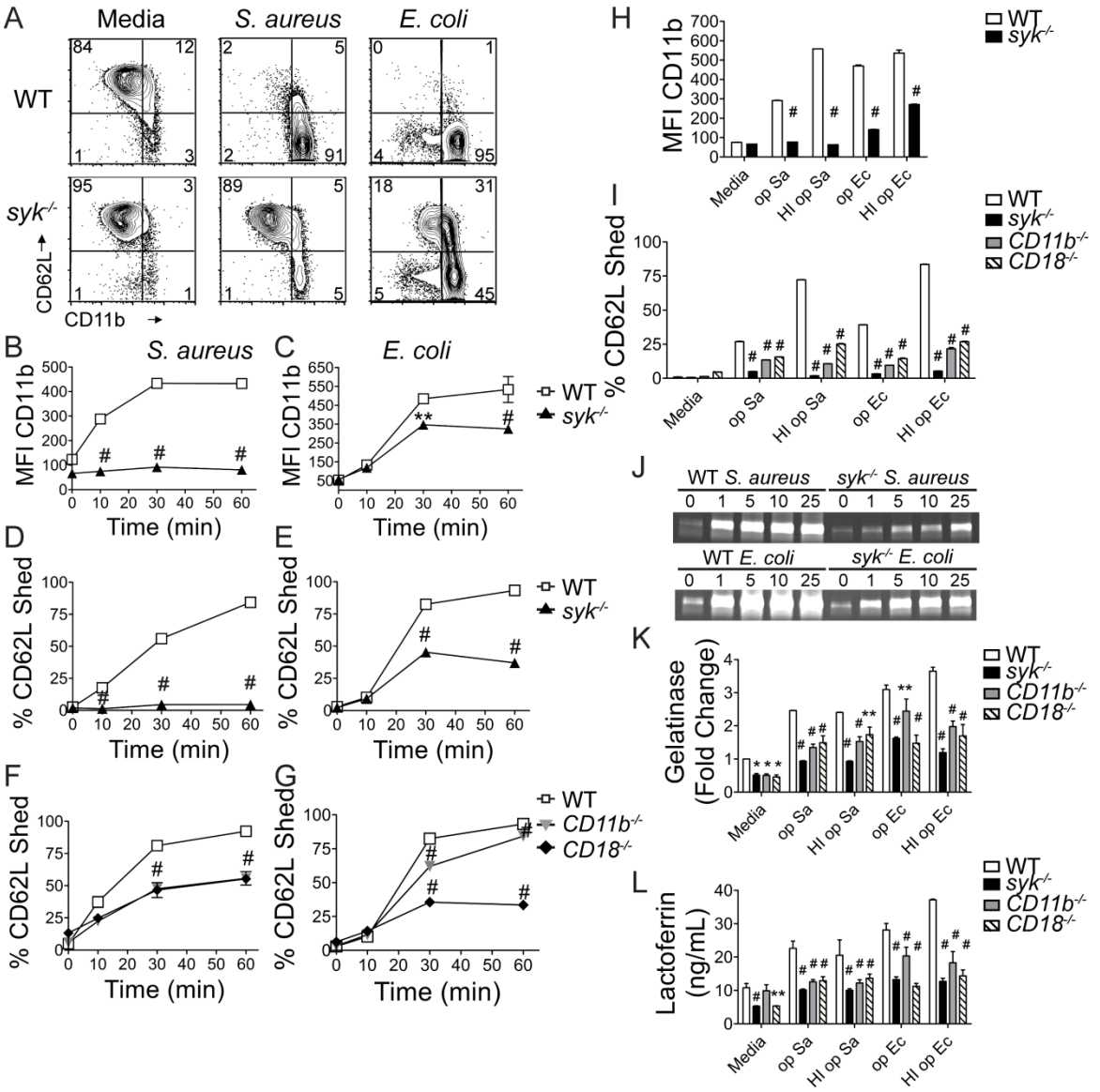

Figure 3.1 Impaired degranulation in *syk*$^{-/-}$ neutrophils in response to bacteria. (A) Flow cytometric analysis of CD11b and CD62L levels on WT or *syk*$^{-/-}$ neutrophils stimulated for 60 minutes with *rag1*$^{-/-}$ serum opsonized (op) *S. aureus* (MOI = 5) or *E. coli* (MOI = 10). Neutrophils of the indicated genotype were quantified for CD11b (median fluorescence intensity, MFI) following stimulation with (B) *S. aureus* or (C) *E. coli*; % CD62L negative (shed) following stimulation with (D,F) *S. aureus*, (E,G) *E. coli*, or (H,I) bacteria opsonized with heat-inactivated (HI op) serum. (J) Release of gelatinase granules by WT or *syk*$^{-/-}$ neutrophils stimulated in suspension with *S. aureus* or *E. coli* for 60 min and analyzed by gelatinase zymogram. (K) Quantitation of gelatinase zymogram (MOI = 1). (L) Lactoferrin release (MOI=1). Error bars are ± SD. *$p<0.05$, **$p<0.01$, $^{\#}p<0.001$ compared to WT by two-way ANOVA with Bonferroni post-tests. All data are representative of three or more independent experiments.

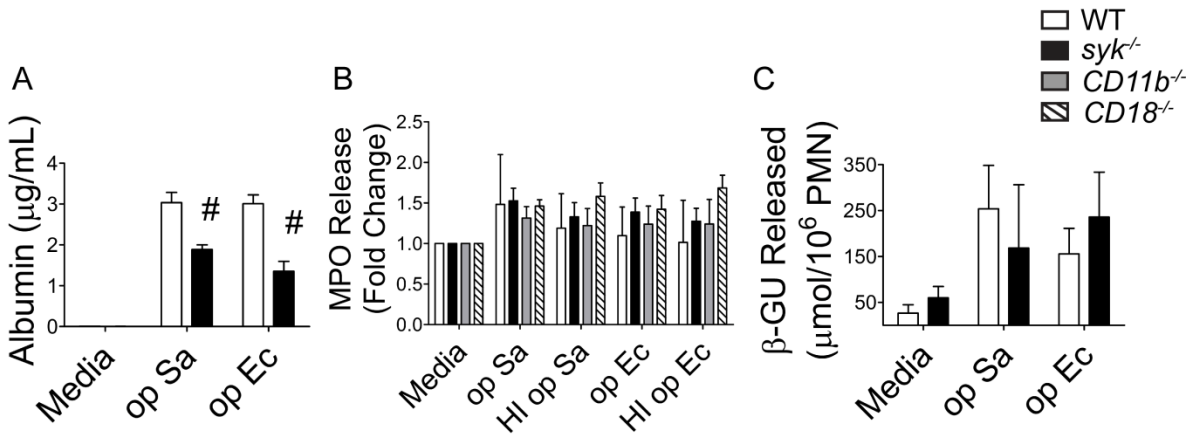

Figure 3.2 Degranulation in response to opsonized bacteria. WT or *syk*$^{-/-}$ neutrophils were stimulated in suspension with *rag1*$^{-/-}$ serum opsonized *S. aureus* (op Sa) or *E. coli* (op Ec), or with bacteria opsonized with heat inactivated serum (HI op) for 60 min, then analyzed for the release of granule components. (A) Release of albumin from secretory vesicles by WT or *syk*$^{-/-}$ neutrophils was detected by ELISA. (B) Release of myeloperoxidase (MPO) from azurophil granules was measured by colorimetric assay. (C) Release of β-glucoronidase (β-GU) from azurophil granules was assessed by fluorimetric assay. Data are presented as mean ± SD. #$p<0.001$ compared to WT by ANOVA with Bonferroni post-tests.

3.2.2 Neutrophil cytokine secretion

Neutrophils can be induced to synthesize and secrete a variety of cytokines upon encountering inflammatory stimuli (Scapini et al., 2000). To determine if Syk is required for neutrophil cytokine synthesis or secretion, a multiplex bead array was used to assess cytokine levels in supernatants and cell lysates of neutrophils stimulated with *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E.coli* (Figure 3.3). Syk-deficiency resulted in the decreased secretion of tumor necrosis factor α (TNFα), macrophage inflammatory protein 1α (MIP1α), macrophage inflammatory protein 2 (MIP2) and chemokine (C-X-C motif) ligand 1 (KC) in response to bacterial stimuli, but relatively normal secretion of interleukin 1β (IL1β), interleukin 6 (IL6),

10 kDa interferon-gamma-induced protein (IP-10) and monocyte chemotactic protein-1 (MCP1). Analysis of the cytokine profiles in the cell pellets indicates that those dependent on Syk for secretion (TNFα, MIP1α, MIP2, and KC) were induced and stored normally upon stimulation. This is in contrast to cytokines that were not dependent on Syk for secretion (IL1β, IL6, IP-10 and MCP1), which were either pre-formed and stored in granules prior to stimulation and release (IL1β, IP-10), or directly secreted from the neutrophil without significant storage (IL6, MCP-1). The ability of neutrophils to secrete TNFα in response to *S. aureus* is dependent on CD18 integrins, as demonstrated by reduced TNFα secretion in supernatants from stimulated *CD18*$^{-/-}$ neutrophils. However, this response is not through CD11b, as TNFα secretion from *CD11b*$^{-/-}$ neutrophils is at least equivalent to WT (Figure 3.3 B). The decrease in TNFα secretion is not due to a reduction in mRNA transcription, as *syk*$^{-/-}$ neutrophils induced TNFα mRNA equivalently to WT neutrophils, as assessed by real-time RT-PCR (Figure 3.4). These data indicate that Syk signaling through CD18 integrins is required for the release of a subset of inflammatory neutrophil cytokines.

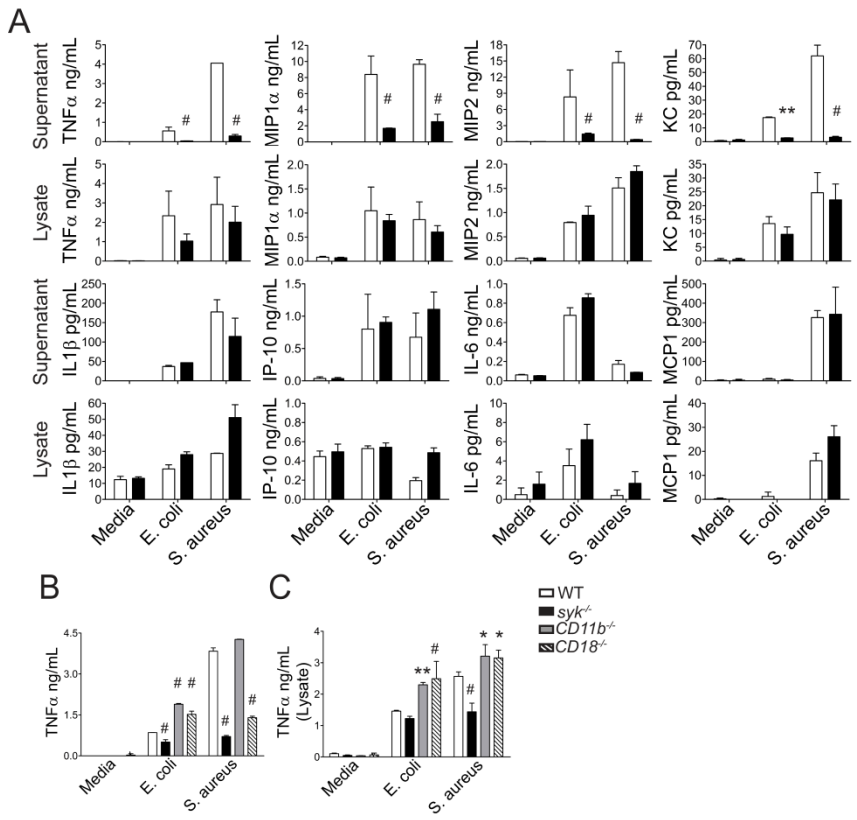

Figure 3.3 Syk-deficient neutrophils have altered cytokine profiles following stimulation with bacteria. (A) WT (white bars) or *syk*$^{-/-}$ (black bars) neutrophils were stimulated with *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E. coli* (MOI=5) for 16 hours. Culture supernatants or cell pellets lysed in low detergent lysis buffer were analyzed for TNFα, MIP1α, MIP2, KC, IL1β, IP-10 or MCP1 by multiplex bead array (Milliplex). Data are the mean of two independent experiments, each with an N=2 or 3. (B) WT, *syk*$^{-/-}$, *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ neutrophils were stimulated with *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E. coli* (MOI= 2) for 16 hours and assessed for (B) TNFα secretion into the supernatant or (C) TNFα synthesis and storage in cell lysates by plate based ELISA, and represent at least 3 independent experiments. Data are presented as mean ± SD. *$p<0.05$, **$p<0.01$, # $p<0.001$ compared to WT by two-way ANOVA with Bonferroni post-tests.

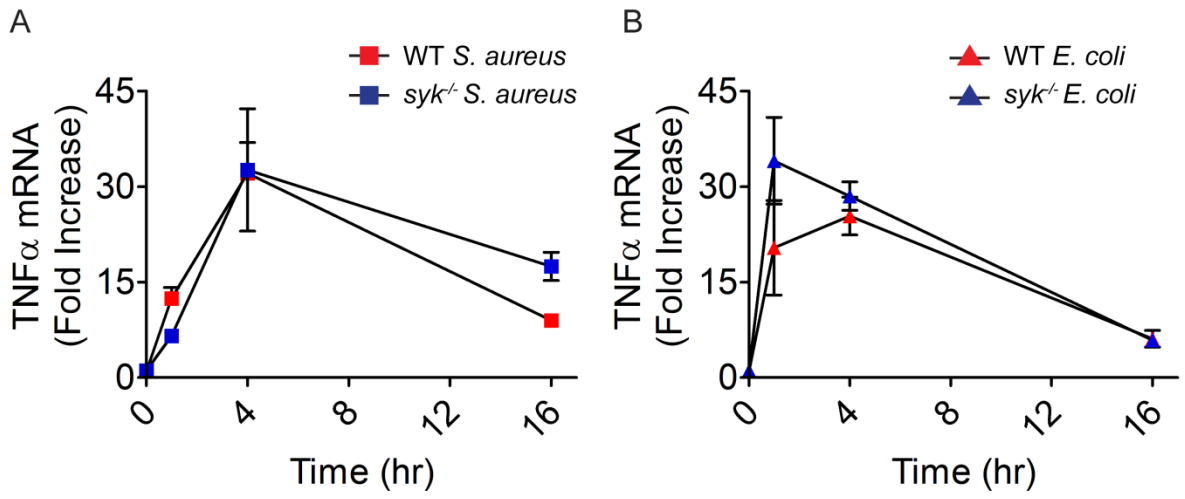

Figure 3.4 Normal Transcription of TNFα mRNA in response to opsonized bacteria. Induction of TNFα transcription was assessed by quantitative RT-PCR of WT or *syk*$^{-/-}$ neutrophils following stimulation with *rag1*$^{-/-}$ serum opsonized (A) *S. aureus* (MOI=2) or (B) *E. coli* (MOI=4) for 1, 4 or 16hrs. The origin is set to the level of TNFα mRNA in unstimulated neutrophils of the respective genotype. Data is represented as mean ± SD, n=3.

3.2.3 Respiratory burst and elastase activation

The requirement for neutrophil superoxide production in the host defense against *S. aureus* has been demonstrated in both human patients with CGD (Liese et al., 2000) and mouse mutants lacking either the gp91^{phox} or p40^{phox} subunits of the NADPH oxidase (Ellson et al., 2006; Pollock et al., 1995). To determine whether Syk is required for oxidative burst in response to bacteria, WT or *syk*$^{-/-}$ neutrophils were stimulated with *rag1*$^{-/-}$ serum opsonized-*S. aureus* and assessed for superoxide production using a non-adherent flow cytometric assay. WT and *syk*$^{-/-}$ neutrophils bound similar levels of APC-labeled *S. aureus*, but a reduced percentage of *syk*$^{-/-}$ neutrophils produced superoxide, as detected by the fluorescent conversion of 3'-(p-aminophenyl) fluorescein (APF) (Figure 3.5 A). To

independently measure the amount of superoxide produced, a plate-based cytochrome c reduction assay was also used. Syk deficient neutrophils produced less superoxide following treatment with opsonized *S. aureus* (Figure 3.5 B). Neutrophils lacking either CD11b or CD18 were also defective in superoxide production in response to opsonized *S. aureus*. Impaired superoxide production was also observed following challenge of CD11b-, CD18- or Syk-deficient neutrophils with *rag1*$^{-/-}$ serum-opsonized *E. coli* (Figure 3.5 C). To determine if the failure to produce superoxide was due to a defect in activation of the NADPH-oxidase complex, the phosphorylation of the p40^{phox} subunit on Thr-154, a phosphorylation event that occurs during NADPH oxidase activation (Bouin et al., 1998), was assessed. Compared to WT neutrophils, phosphorylation of p40^{phox} Thr-154 was reduced 2 – 6 fold in *syk*$^{-/-}$ neutrophils stimulated with *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E. coli*, with the more profound effect seen with *S. aureus* (Figure 3.5 D and E). A similar level of impairment of p40^{phox} phosphorylation was observed with CD18-deficient cells, again confirming that the majority of this signaling response was proceeding through the CD18/Syk pathway.

The requirement for neutrophil elastase has been demonstrated to have a specific role in host defense against Gram negative bacteria, including *E. coli* (Belaaouaj et al., 1998). To determine whether the CD18/Syk pathway is involved in elastase activation following stimulation with bacteria, WT, *syk*$^{-/-}$, *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ neutrophils were stimulated with either *rag1*$^{-/-}$ serum opsonized *S. aureus* or *E. coli*, and assessed by flow cytometry for intracellular elastase activity (Figure 3.5 E and F). Elastase was activated in WT neutrophils in response to both bacteria and this activation decreased slightly in the absence of Syk, especially following *E. coli* stimulation. However, elastase activation in response to both bacteria was dependent on both CD11b and CD18, indicating that at least one mechanism

of bacterial killing, namely the activation of elastase, remained intact in Syk-deficient neutrophils.

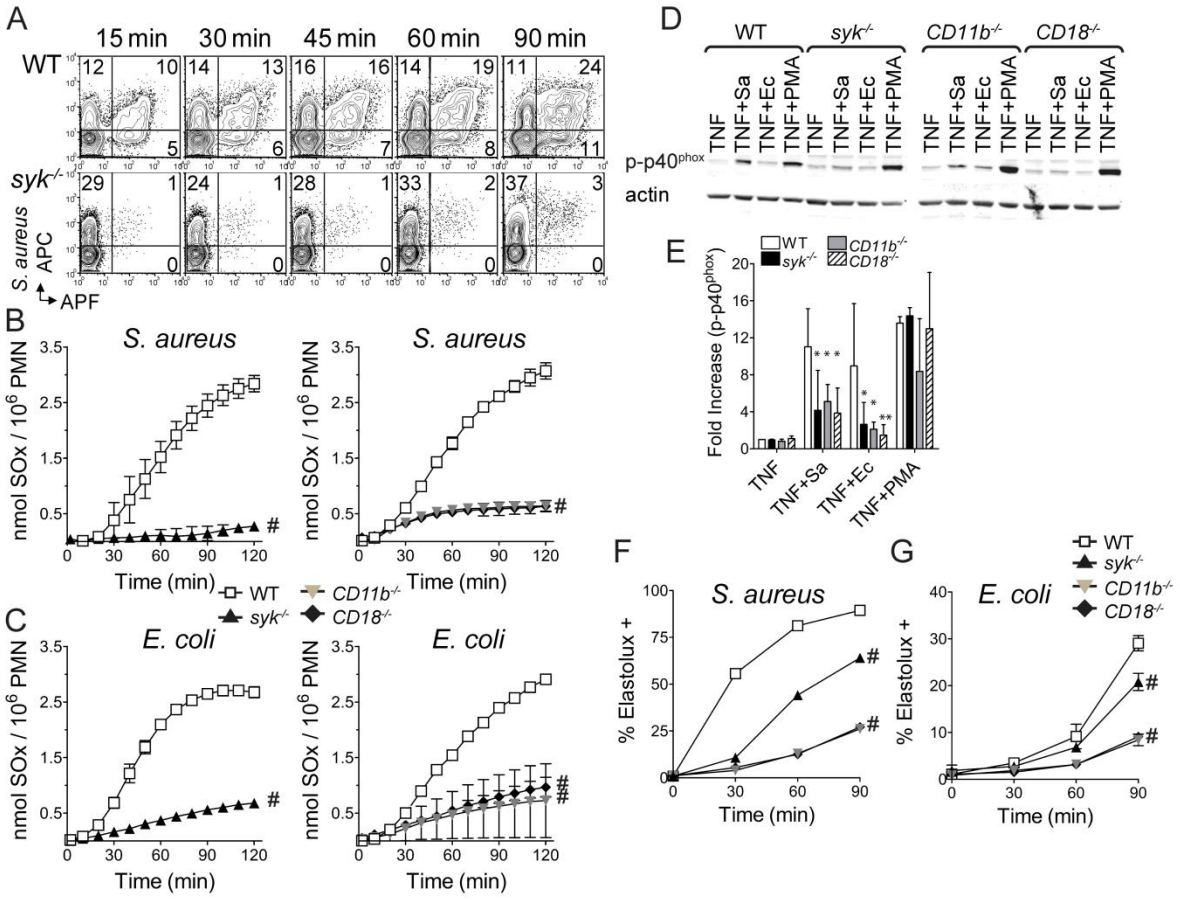

Figure 3.5 Syk-deficient neutrophils display reduced integrin-mediated superoxide production in response to bacteria. (A) Flow cytometric detection of superoxide by fluorescence conversion of APF in WT or *syk*$^{-/-}$ neutrophils following stimulation with APC-labeled *S. aureus* (MOI = 5). (B) WT, *syk*$^{-/-}$, *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ neutrophils were plated in microtiter wells containing *S. aureus* (MOI=5) or (C) *E. coli* (MOI=10), in cytochrome c media. Production of superoxides (SOx) by respiratory burst was measured as reduction of cytochrome c. Data are representative of at least three independent experiments, and are mean ± SD. (D) Western blot analysis of p40^{phox} phosphorylation (Thr 154) following priming of neutrophils for 30 min with 50ng/mL TNFα, and where indicated, 30 min with *S. aureus* (Sa) MOI=20, *E. coli* (Ec) MOI=40 or 10nM PMA. (E) Quantitation of phospho-p40^{phox} normalized to actin. (F,G) Intracellular activation of elastase was detected by flow cytometry following cleavage of ElastoLux®. Neutrophils of the indicated genotype were stimulated with (F) *S. aureus* or (G) *E. coli* (MOI = 5) for the indicated time points. Data are representative of three independent experiments, and are mean ± SD. *p<0.05, **p<0.01, $^{\#}$p<0.001 compared to WT by ANOVA.

3.2.4 MAP Kinase signaling

An assessment of the signaling events following stimulation with opsonized bacteria indicated that the phosphorylation of c-Cbl (Figure 3.6 A) and Pyk2 (Figure 3.6 B) were reduced in *syk*$^{-/-}$ neutrophils compared to WT. Given the reduced phosphorylation of the up-stream signaling molecules c-Cbl and Pyk2, it was of interest to determine the phosphorylation status of Erk1/2 and p38. The magnitude of Erk1/2 phosphorylation was decreased in *syk*$^{-/-}$ neutrophils stimulated with *rag1*$^{-/-}$ serum opsonized-*S. aureus* or -*E. coli* (Figure 3.6 C). The kinetics and magnitude of Erk1/2 phosphorylation differed depending on the bacteria, with maximal pErk1/2 signal detectable at 10 min in response to *S. aureus*, and a lesser degree of pErk1/2 detectable at 15 min in response to *E. coli* (Figure 3.6 C). The phosphorylation of p38 was marginally decreased in *syk*$^{-/-}$ neutrophils (Figure 3.6 D). *E. coli* stimulation induced a more robust level of p38 phosphorylation than *S. aureus*, peaking at 20 minutes. CD18-deficient neutrophils also showed poor phosphorylation of Erk1/2 and p38, at levels equivalent to *syk*$^{-/-}$ cells, indicating that these pathways are activated, in part, through engagement of integrins by the serum opsonized bacteria. It is likely that much of the p38 phosphorylation caused by *E. coli* particles reflects signaling through neutrophil TLR4 and hence is independent of the CD18/Syk pathway.

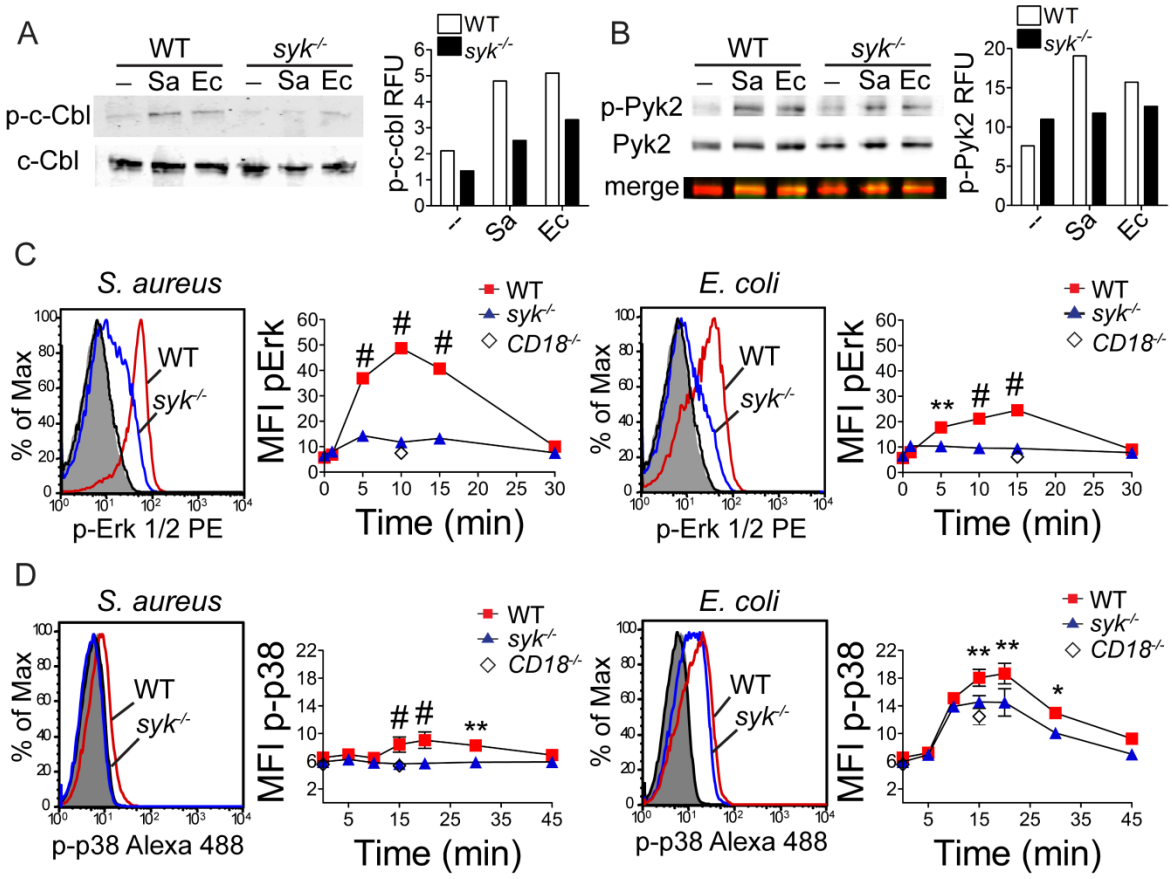

Figure 3.6 Syk is required for normal phosphorylation events following bacterial stimulation. Western blot analysis of (A) c-Cbl or (B) Pyk2 phosphorylation following stimulation of WT or *syk*$^{-/-}$ neutrophils with *S. aureus* (Sa) or *E. coli* (Ec) for 10 min. For quantitation, c-Cbl and p-c-Cbl were detected on separate gels, and were each normalized to actin; p-Pyk2 was normalized to total Pyk2 detected on the same gel. (C,D) Flow cytometric histograms of intracellular (C) p-Erk1/2 at 10 min in response to *S. aureus* or at 15 min in response to *E. coli* or (D) p-p38 at 20 min in response to *S. aureus* or *E. coli*. Histograms of unstimulated WT (black dash) or *syk*$^{-/-}$ (filled grey) and stimulated WT (black) or *syk*$^{-/-}$ (grey) neutrophils are shown. Time course quantitation is of the Median Fluorescence Intensity (MFI). Error bars are ± SD. *p<0.05, **p<0.01, #p<0.001 by two-way ANOVA with Bonferroni post-tests. All data are representative of three or more independent experiments.

Given the strong phosphorylation of Erk1/2 and p38 in response to bacterial stimulation, it was important to determine if decreased signaling through the MEK/Erk and p38 affected neutrophil degranulation responses to *rag1*$^{-/-}$ serum opsonized bacteria. To investigate these pathways, WT neutrophils were treated with either the MEK-1 inhibitor

PD98059 or the p38 inhibitor SB203580 prior to bacterial challenge. Treatment with SB203580 impaired secretory vesicle degranulation, as assessed by CD11b up-regulation and CD62L shedding, whereas treatment with PD98059 only weakly impaired CD62L shedding (Figure 3.7 A to C). Interestingly, inhibition of p38 had a greater effect on both *S. aureus* and *E. coli* treated neutrophils, even through *S. aureus* induced more robust Erk1/2 responses and less p38 activation. Both the MEK/Erk and p38 pathways are required for secretory vesicle degranulation, as treatment of WT neutrophils with both PD98059 and SB203580 synergized to inhibit up-regulation of CD11b and shedding of CD62L.

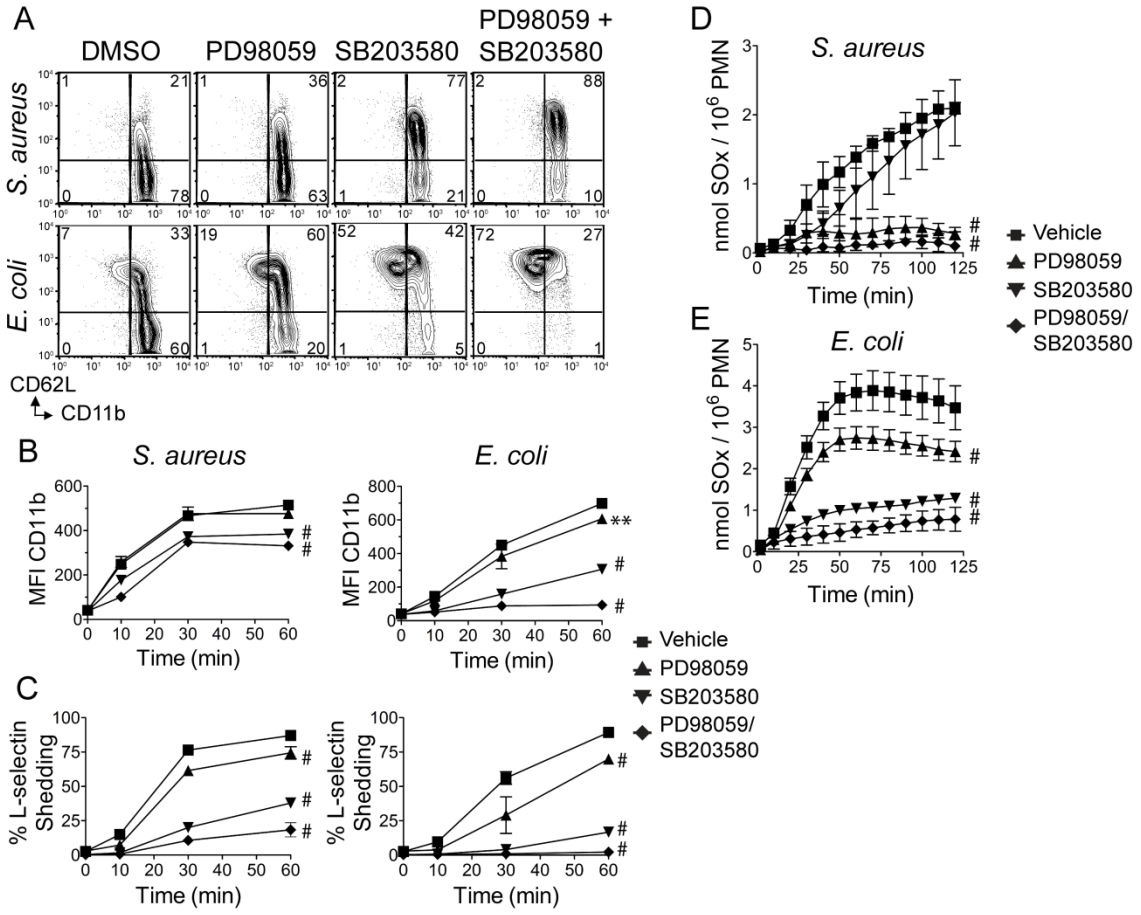

Figure 3.7 Signaling through Erk and p38 is required for neutrophil degranulation. (A) WT neutrophils pre-treated with DMSO vehicle, 20μM PD98059, 3μM SB203580 or both compounds were stimulated for 60 minutes with *S. aureus* (MOI = 5) or *E. coli* (MOI = 10), then analyzed by flow cytometry for CD11b and CD62L. (B) Quantitation of CD11b Median Fluorescence Intensity (MFI) and % CD62L negative. Data are ± SD, and are representative of three or more independent experiments. (D) WT neutrophils pre-treated as in (A) were plated in microtiter wells containing *S. aureus* (MOI=5) or *E. coli* (MOI=10), in cytochrome c media, and assessed for superoxide (SOx) production as in Figure 3. Data are representative of at least three independent experiments. Error bars are ± SD. **p<0.01, #p<0.001 by two-way ANOVA with Bonferroni post-tests.

The relative requirement for signaling through the MEK/Erk versus p38 pathways for neutrophil oxidative burst in response to *rag1*$^{-/-}$ serum opsonized bacteria was compared. As expected from the increased Erk phosphorylation in response to *S. aureus*, treatment of WT neutrophils with PD98059 completely prevented oxidative burst in response to *rag1*$^{-/-}$

serum opsonized *S. aureus*, whereas treatment with SB203580 had a minimal effect (Figure 3.7 D). Treatment of WT neutrophils with both PD98059 and SB203580 had a similar, but slightly more dramatic effect than treatment with PD98059 alone. This supports the requirement for Erk signaling downstream of the CD18/Syk pathway in response to serum-opsonized *S. aureus*. Consistent with the increased phosphorylation of p38 in response to *E. coli*, treatment of WT neutrophils with SB203580 significantly decreased the oxidative burst in response to *rag1*$^{-/-}$ serum opsonized *E. coli*, whereas treatment with PD98059 resulted in a modest decrease in superoxide production (Figure 3.7 E). Treatment of WT neutrophils with both PD98059 and SB203580 had a cumulative effect, causing significant inhibition of superoxide production. This supports the predominant requirement for p38 signaling in response to serum-opsonized *E. coli*, in a CD18/Syk dependent manner.

3.2.5 Phagocytosis and killing

To determine if Syk is required for the phagocytosis of serum opsonized bacteria, a flow cytometric assay was used. Heat shock killed *S. aureus* or *E. coli* was labeled with FITC, then opsonized with *rag1*$^{-/-}$ serum, and incubated with TNFα stimulated neutrophils. Prior to acquisition by flow cytometry, trypan blue was used to quench FITC fluorescence of cell-surface bound bacteria, allowing examination of only internalized organisms (Bjerknes and Bassoe, 1984). *Syk*$^{-/-}$ neutrophils displayed a reduced ability to phagocytose *S. aureus*, but a more modest defect in uptake of *E. coli* (Figure 3.8 A). Phagocytosis of both of these bacteria is dependent on CD11b and CD18, as neutrophils deficient in either of these integrin components fail to take up bacteria. Following stimulation with either bacteria, there is a rapid burst in actin polymerization, which is maintained in the absence of Syk

(Figure 3.9), and is thus not the defect leading to reduced phagocytosis. To assess whether *syk*$^{-/-}$ neutrophils kill internalized bacteria, an *in vitro* phagocytosis and killing assay was utilized. In this assay, *syk*$^{-/-}$ neutrophils exhibited a reduced ability to kill internalized *S. aureus* and to a lesser extent, a reduced ability to kill internalized *E. coli* (Figure 3.8 B). Concomitant with uptake of the *rag1*$^{-/-}$ serum-opsonized *S. aureus* or *E. coli*, WT neutrophils formed large cellular aggregates, containing cells with nuclear disruption and extracellular DNA with trapped bacteria, suggestive of neutrophil extracellular trap (NET) formation (Figure 3.8 C). The percentage of neutrophils that had formed NETs was quantified as those that had lost both the ring-shaped nuclear structure and excluded the actin cytoskeleton (Figure 3.8 D). The aggregation of WT neutrophils and the presence of NETS were more apparent in responses to *S. aureus* than *E. coli*. In contrast, *syk*$^{-/-}$ neutrophils did not tend to aggregate or show signs of NET formation following challenge with *rag1*$^{-/-}$ serum-opsonized bacteria (Figure 3.8 C and D).

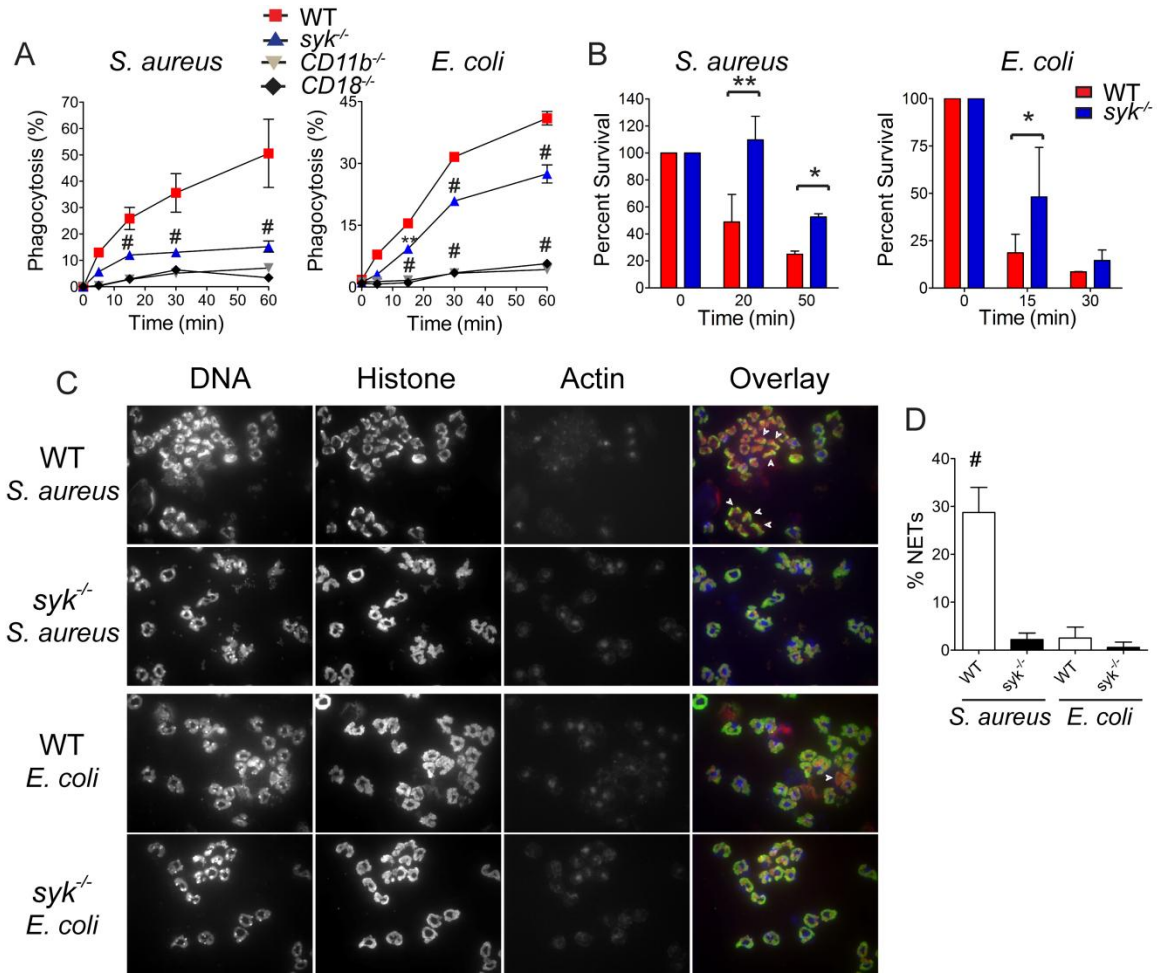

Figure 3.8 Syk is required for optimal phagocytosis and killing of *rag1*$^{-/-}$ serum opsonized bacteria. (A) Phagocytosis was determined by flow cytometric analysis of FITC-labeled *S. aureus* or *E. coli* by WT, *syk*$^{-/-}$, *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ neutrophils. (B) Intracellular bacterial killing capacity assessed by viable *S. aureus* or *E. coli* CFU. Data are the mean (n=3 replicates) of the percent remaining CFU relative to the addition of gentamicin (set as Time = 0 min). (C) Cytospins of TNFα primed neutrophils stimulated for 2hrs with *S. aureus* or *E. coli*, and stained with DAPI (DNA/Red), anti-Histone antibody (Green), and phalloidin (Actin/Blue). White arrowheads indicate cells classified as Neutrophil Extracellular Traps (NETs). (D) NETs quantified by both loss of nuclear structure and exclusion of the actin cytoskeleton, expressed as a percent of total neutrophils scored. Error bars are ± SD. Data are representative of 3 independent experiments. *p<0.05, **p<0.01, #p<0.001, by ANOVA.

Overall, these *in vitro* experiments demonstrate that Syk-deficient neutrophils manifest reduced phagocytosis and cellular activation (degranulation, release of cytokines, and ROI production) following exposure to serum opsonized *S. aureus* and *E. coli.* The impaired degranulation responses and reduced superoxide production correlate with poor activation of Erk1/2 and p38 pathways, and additionally, the reduced superoxide production results, in part, from reduced phosphorylation of NADPH oxidase subunits. These signaling defects occur primarily through the CD18 integrins, given that CD18 deficient cells manifest a similar phenotype, and are more apparent in *S. aureus* versus *E. coli* stimulated neutrophils.

3.2.6 Lineage specific deletion of syk

To determine the role of Syk in neutrophils, mice with *loxP* sites flanking exon 1 of *syk* (Saijo et al., 2003) were crossed to mice expressing the Cre recombinase (Cre) under the control of the endogenous Lysozyme M promoter (*LysMcre*$^{Tg/Tg}$), restricting Cre activity to myeloid cells (Clausen et al., 1999), or under the control of the human Mrp8 promoter (*Mrp8cre*^{Tg}), restricting Cre activity to neutrophils (Passegue et al., 2004). For complete deletion of *syk* in the hematopoietic system, the IFNα/β inducible cre, *Mx1-cre*^{Tg} (Kuhn et al., 1995), and the *Vav-1cre*^{Tg} (de Boer et al., 2003) lines were used. *Syk*$^{fl/-}$*LysMcre*$^{Tg/+}$ and *syk*$^{f/-}$ *Mrp8cre*^{Tg} mice were born at the expected Mendelian ratio, show no signs of abnormal blood-lymphatic endothelial connections or abnormal B cell development (data not shown), and have normal differential blood leukocyte counts (Figure 3.10 and Table 3.1).

Table 3.1 Complete blood leukocyte counts in conditionally deleted *syk* mice.

	Neutrophils		Monocytes		Lymphocytes		
	%	Number	%	Number	%	Number	n
syk$^{f/-}$LysMcre	20.8±4.0	1.9±0.4	3.0±0.6	0.3±0.1	76.2±3.7	6.8±0.4	5
syk$^{f/-}$Mrp8cre	20.2±4.7	1.9±0.4	4.0±2.8	0.3±0.2	75.9±7.0	7.8±2.8	5
syk$^{f/f}$ Vavcre	51.3±5.2*	3.5±1.3	3.1±0.3	0.2±0.04	44.5±5.7*	2.9±0.5	3
syk$^{-/-}$ chimera	46.0±0.9*	1.6±0.1	5.4±1.0	0.2±0.1	46.0±1.8*	1.6±0.1*	2
syk$^{f/f}$	23.1 ±9.0	1.6±0.7	3.4±1.1	0.3±0.1	73.1±9.0	5.8±3.5	3
syk$^{f/-}$	22.6 ±3.9	2.2±0.7	3.1±0.7	0.3±0.1	73.6±3.1	7.2±1.8	5
syk$^{f/+}$ LysMcre	22.0±2.7	2.0±0.2	2.6±0.3	0.2±0.1	75.1±3.5	6.8±1.3	3

The data represent the mean ± SD of the indicated measurement of complete blood count with cellular differential, assessed by a Hemavet 950FS (Drew Scientific) multispecies hematology instrument. *$p<0.05$ by one-way ANOVA with Bonferroni post-tests.

Syk$^{f/-}$Mx1-cre^{Tg} mice do not exhibit any gross signs of vascular abnormalities in the steady state. In contrast, *syk$^{f/f}$Vav-1cre^{Tg}* mice are born at reduced numbers than expected (25% observed versus 37.5% expected), display leukocyte infiltration into organs, including the liver and lung and show signs of blood and lymph vessel fusion, as indicated by blood and chylous lymph fluid in the peritoneum (Figure 3.10 A), and exhibit splenomegaly due to accumulation of myeloid cells in the spleen (Figure 3.10 B). In addition, Vav-1 driven expression of Cre in the hematopoietic system results in a loss of B cells in the bone marrow and the spleen (Figure 3.10 C), presumably due to the B cell developmental block first described in the conventional *syk* knock-out (Cheng et al., 1995; Turner et al., 1995). This is also observed as lymphopenia similar to that observed in the peripheral blood of *syk$^{-/-}$* bone marrow chimeras (Table 3.1). The early and complete expression of Vav-1cre in the

hematopoietic system results in complete loss of Syk protein from peripheral blood Ly6G$^+$CD11b$^+$ neutrophils (Figure 3.10 D).

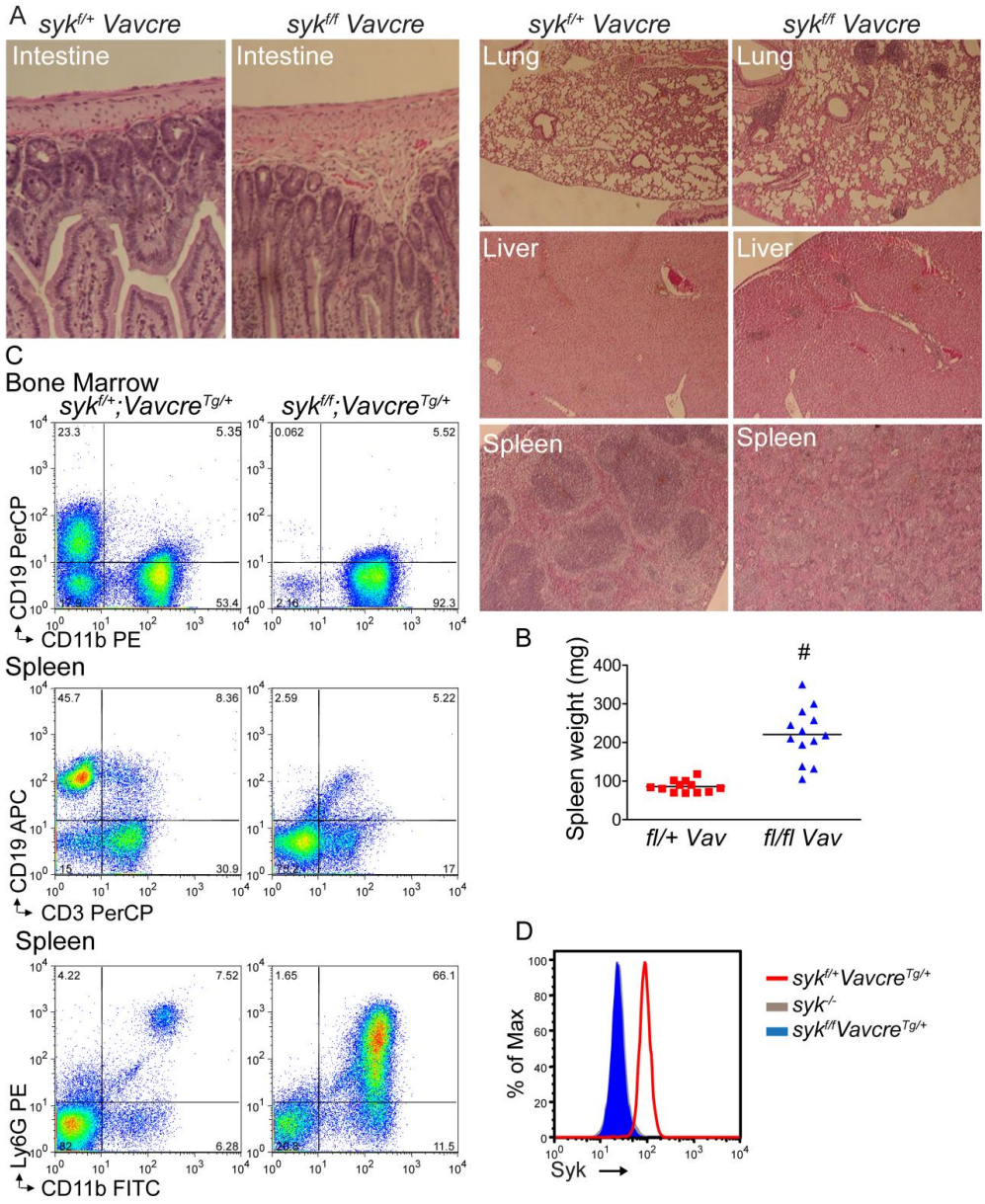

Figure 3.10 Syk is required for normal organ maintenance and B cell development. (A) Hematoxylin and eosin staining of histological sections from the intestine, lung, liver and spleen of *syk*$^{fl/+}$*Vav-1cre*$^{Tg/+}$or *syk*$^{fl/fl}$*Vav-1cre*$^{Tg/+}$ mice, at an original magnification of X50. (B) Spleen weights from *syk*$^{fl/+}$*Vav-1cre*$^{Tg/+}$or *syk*$^{fl/fl}$*Vav-1cre*$^{Tg/+}$ mice. #p<0.001, by t-test. (C) Analysis of leukocytes from bone marrow or spleen, analyzed for the presence of B cells (CD19$^+$), T cells (CD3$^+$) and myeloid cells (CD11b$^+$). (D) Analysis of *syk* deletion by flow cytometry of Syk protein in neutrophils (Ly6G$^+$CD11b$^+$) from the peripheral blood. Cells from a *syk*$^{-/-}$ chimera are negative controls (grey histogram) relative to *syk*$^{fl/fl}$*Vav-1cre*$^{Tg/+}$ (blue line) or *syk*$^{fl/+}$*Vav-1cre*$^{Tg/+}$ (red line) mice. Histograms are representative of at least 4 animals.

Intracellular staining for Syk was used to investigate the loss of Syk protein from myeloid cells (Figure 3.11 A and B, Table 3.2). Analysis of bone marrow from *syk*$^{fl/-}$ *LysMcre*$^{Tg/+}$ revealed that ~40% of neutrophils (Ly6G^{+}CD11b^{+}) and ~60% of monocytes (Ly6G^{-}CD11b^{+}) were positive for Syk protein, whereas ~11% of neutrophils and ~60% of monocytes were positive for Syk in *syk*$^{fl/-}$*Mrp8cre*^{Tg} mice. Analysis of peripheral blood from *syk*$^{fl/-}$*LysMcre*$^{Tg/+}$ mice indicates that ~20% of neutrophils and 40-50% of monocytes retained Syk protein, at a level lower than that of wild type expression. In contrast, peripheral blood from *syk*$^{fl/-}$*Mrp8cre*^{Tg} mice indicates that ~12% of neutrophils and ~75% of monocytes retained Syk protein. Analysis of Syk expression in cells migrating into an inflammatory airpouch indicates that ~10% of neutrophils were positive for Syk protein in mice from either Cre strain. Loss of Syk protein was incomplete in the bone marrow where neutrophils develop; however, as mature neutrophils enter the peripheral blood and sites of inflammation, protein loss increased up to 90%. Furthermore, LysMcre-mediated deletion of *syk* occurred in neutrophils and monocytes, whereas Mrp8cre-mediated deletion occurred predominantly in neutrophils. Female *syk*$^{f/-}$*Mx1-cre*^{Tg} mice, upon cre-induction following injection with polyI:polyC, show near complete loss of Syk protein from both peripheral blood and bone marrow neutrophils.

Table 3.2 Syk protein level in conditionally deleted mice.

	Bone Marrow			Peripheral Blood			Air Pouch		
	$Ly6G^+$ $CD11b^+$	$Ly6G^-$ $CD11b^+$	n	$Ly6G^+$ $CD11b^+$	$Ly6G^-$ $CD11b^+$	n	$Ly6G^+$ $CD11b^+$	$Ly6G^-$ $CD11b^+$	n
syk$^{f/-}$ *LysMcre*	39 ± 10	57 ± 7	6	20 ± 11	45 ± 12	29	9 ± 8	29 ± 19	11
syk$^{f/-}$ *Mrp8cre*	11 ± 2	58 ± 10	9	12 ± 4	75 ± 5	17	9 ± 6	46 ± 22	9
syk$^{f/-}$ *Mx1cre*	32 ± 5	27 ± 3	3	5 ± 2	15 ± 6	8	9 ± 4	30 ± 16	3
syk$^{f/f}$ *Vavcre*	n.d.	n.d.		2 ± 2	2 ± 2	10	1 ± 0	5 ± 3	7
syk$^{-/-}$ *chimera*	1 ± 0	8 ± 2	3	1 ± 0	11 ± 10	9	n.d.	n.d.	
syk$^{f/f}$	95 ± 5	93 ± 0	5	85 ± 3	99 ± 1	16	84 ± 19	68 ± 8	7
syk$^{f/-}$	90 ± 2	74 ± 14	4	92 ± 6	62 ± 12	8	91 ± 3	71 ± 11	4
syk$^{f/+}$ *LysMcre*	98 ± 1	78 ± 7	5	96 ± 3	89 ± 3	18	68 ± 9	76 ± 4	5

The data represent the mean ± SD of the percent Syk positive cells as assessed by intracellular flow cytometry. For each analysis the respective cell type from *syk*$^{f/f}$*Vavcre*^{Tg} or *syk*$^{-/-}$ mice were used as a negative control for Syk protein level, and used to set the lower limit gate.

To confirm a functional consequence to conditional loss of Syk, neutrophils from *syk*$^{f/-}$*Mx1-cre*^{Tg}, s*yk*$^{fl/-}$*LysMcre*$^{Tg/+}$, or *syk*$^{fl/-}$*Mrp8cre*^{Tg} were subjected to an adhesion-dependent superoxide assay (Figure 3.11 C). The complete loss of Syk protein from *syk*$^{f/-}$*Mx1-cre*^{Tg} neutrophils abrogates TNFα-primed superoxide production in response to FCS and fibronectin; however, partial retention of Syk protein in the bone marrow neutrophil population of both s*yk*$^{fl/-}$*LysMcre*$^{Tg/+}$ and *syk*$^{fl/-}$*Mrp8cre*^{Tg} results in a partial ability to produce TNFα-primed superoxide in response to either FCS or fibronectin (Figure 3.11 C). This supports the level of *syk* gene deletion as assessed by intracellular flow cytometric staining for Syk protein.

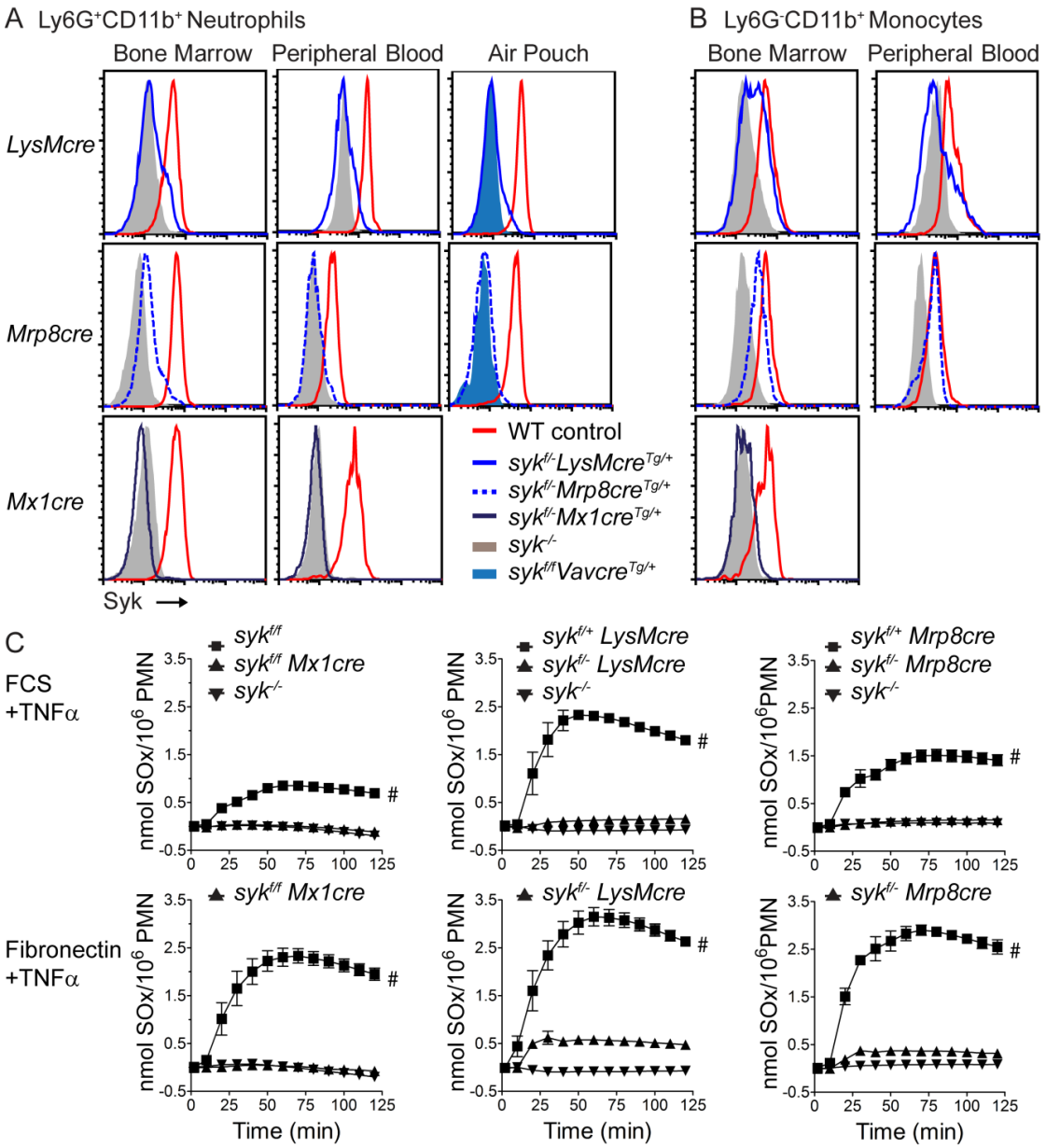

Figure 3.11 Conditional deletion of *syk* results in a reduction in adhesion-dependent superoxide production. Analysis of *syk* deletion by flow cytometry of Syk protein in (A) neutrophils ($Ly6G^+CD11b^+$) and (B) monocytes ($Ly6G^-CD11b^+$) from the bone marrow (BM), peripheral blood (PB) and airpouch lavage (AP). Syk^{KO} cells from $syk^{-/-}$ chimeras (grey filled histograms) or $syk^{f/f}Vav1cre^{Tg}$ mice (blue filled histograms) are negative controls relative to $syk^{f/-}LysMcre^{Tg/+}$, $syk^{f/-}Mrp8cre^{Tg}$ or $syk^{f/-}Mx1cre^{Tg}$ (indicated blue lines) or wild-type control (red line) mice. Histograms are representative of at least 4 animals. (C) Neutrophils of the indicated genotype were primed with 50ng/mL TNFα and plated in microtiter wells coated with FCS or fibronectin, in cytochrome c media. Production of superoxides (SOx) by respiratory burst was measured as reduction of cytochrome c. Data are representative of at least three independent experiments, and are mean ± SD. $^{\#}p<0.001$ compared to WT by ANOVA.

3.2.7 Bacterial clearance in a skin abscess model

The requirement for CD11b and CD18 was confirmed for a robust clearance of either *S. aureus* or *E. coli* following airpouch challenge, as the bacterial CFU counts were higher in either *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ mice compared to WT. In addition, CD18 appears to be required for migration of neutrophils into the airpouch following bacterial challenge (Figure 3.12).

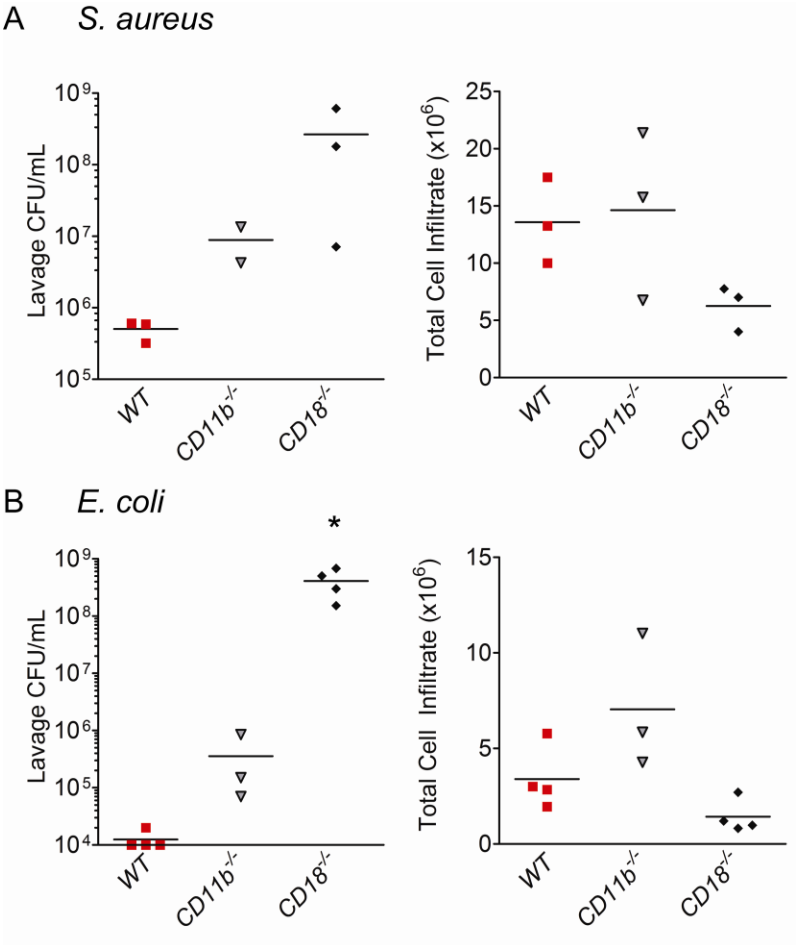

Figure 3.12 CD11b and CD18 integrins are required for an effective host response to *S. aureus* or *E. coli*. (A) CFU of *S. aureus* and neutrophil infiltrate and (B) CFU of *E. coli* and neutrophil infiltrate in the airpouch lavage of WT, *CD11b*$^{-/-}$ and *CD18*$^{-/-}$ mice 24 hr after the initiation of infection. Data represent at least 2 independent experiments, with at least 3 animals per group. *$p < 0.05$, Mann Whitney U-test.

The skin abscess model was then used to determine whether the defects in CD18 integrin-dependent neutrophil activation observed in the absence of Syk resulted in an increased bacterial load following challenge. At 24 hours following bacterial infection, 80-90% of the cells recruited into the skin abscess were neutrophils, as defined by expression of the cell surface markers Ly6G and CD11b (Figure 3.13, Table 3.3).

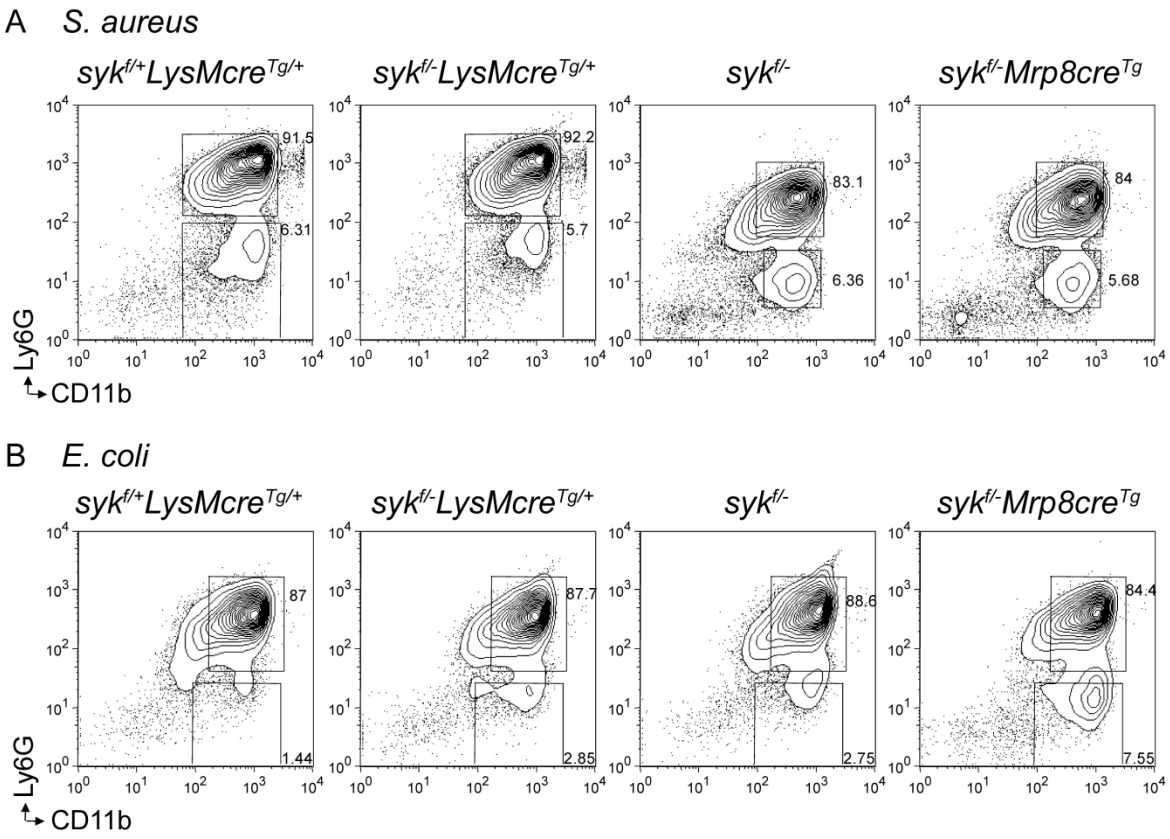

Figure 3.13 Flow cytometric analysis of airpouch infiltrate 24 hours post-infection. Airpouch lavage cells were analyzed for surface expression of Ly6G and CD11b, and the relative percentages of neutrophils ($Ly6G^+CD11b^+$) and monocytes/macrophages ($Ly6G^-CD11b^+$) were determined following (A) *S. aureus* or (B) *E. coli* infection, for the indicated genotypes.

Table 3.3 Airpouch infiltrate 24 hours post-infection.

	S. aureus			*E. coli*		
	% $CD11b^+Ly6G^+$	% $CD11b^+$ $Ly6G^-$	(n)	% $CD11b^+Ly6G^+$	% $CD11b^+$ $Ly6G^-$	(n)
syk$^{f/-}$LysMcre	89.1±2.6	7.3±1.8	5	86.5±1.4	1.9±0.8	4
syk$^{f/-}$Mrp8cre	78.2±6.6	6.9±2.4	9	83.4±4.9	5.3±2.8	10
syk$^{f/f}$Vavcre	81.9±13.9	4.5±1.3	2	88.0±4.5	4.5±3.2	2
syk$^{f/f}$	81.2±8.9	5.6±3.22	3	88.9±3.2	3.2±1.8	3
syk$^{f/-}$	80.0±2.2	7.2±1.7	4	82.6±8.9	4.5±2.7	3
syk$^{f/+}$LysMcre	87.5±3.2	8.6±2.1	4	83.7±2.1	2.4±0.9	5

Quantitation of the percentages of neutrophils and monocytes migrating into an infected subcutaneous airpouch, 24 hours post-infection. Data are mean ± SD.

Mice with relatively broad Syk-deficiency in the myeloid lineage (*syk$^{f/-}$LysMcre$^{Tg/+}$*) had a 3- to 4-fold increase in the number of viable *S. aureus* recovered from a subcutaneous abscess, despite enhanced migration of neutrophils into the site of infection (Figure 3.14 A). While there was a tendency toward higher *E. coli* bacterial counts following airpouch infection of *syk$^{f/-}$LysMcre$^{Tg/+}$* mice, this result was somewhat variable, and not statistically significant (Figure 3.14 B). Utilizing the Mrp8cre model for neutrophil-restricted deletion, a similar pattern was observed. *syk$^{f/-}$Mrp8cre^{Tg}* mice had a 4-fold increase in the number of viable *S. aureus* recovered from a subcutaneous abscess, despite equivalent migration of neutrophils into the site of infection (Figure 3.14 D). Similarly, *syk$^{f/-}$Mrp8cre^{Tg}* mice, while having a tendency toward higher bacterial burden, exhibited a near normal ability to clear *E. coli*, and displayed normal neutrophil migration (Figure 3.14 E). Control mice *syk$^{f/f}$* and *syk$^{f/-}$* showed no differences, ruling out effects from heterozygosity of the *syk* gene.

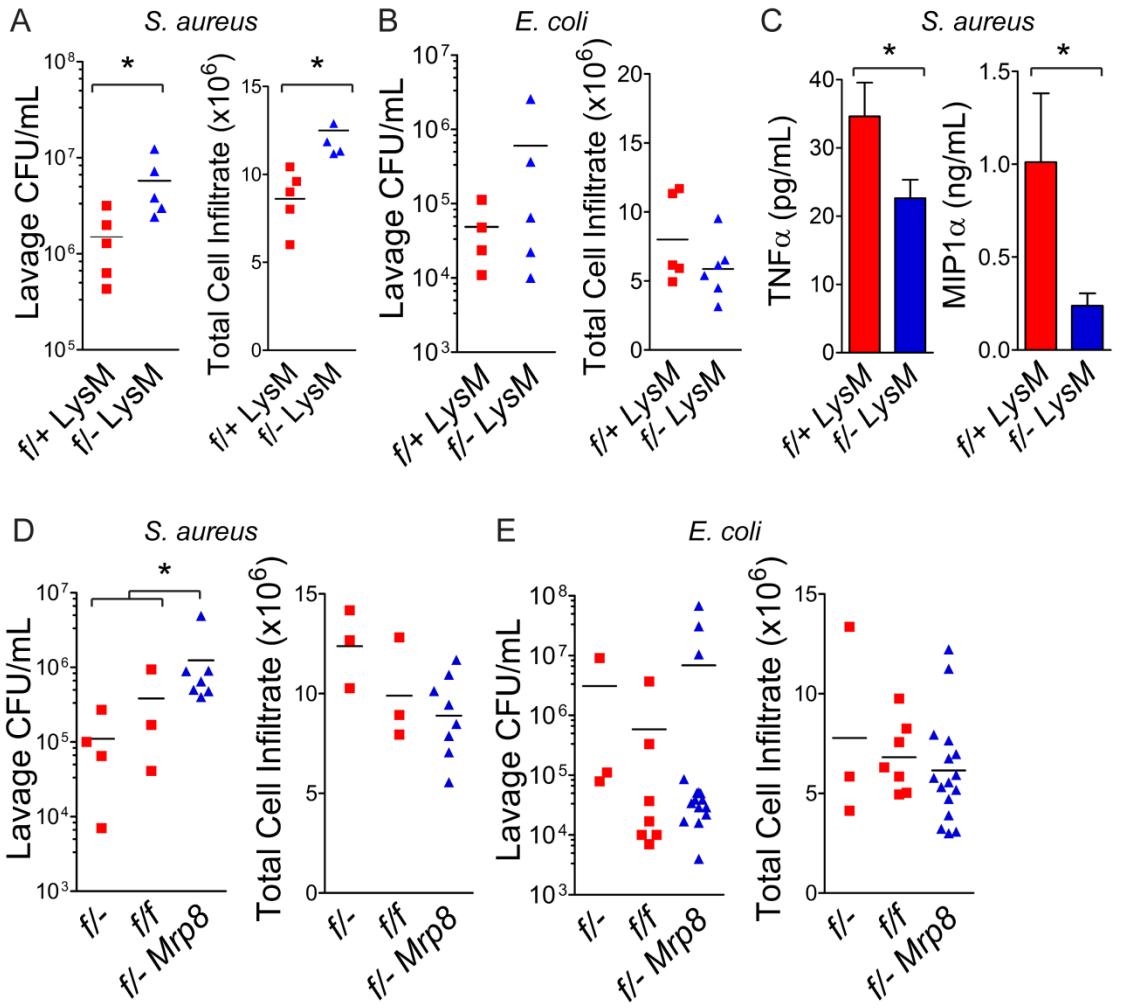

Figure 3.14 Neutrophil-specific deletion of *syk* results in a defective host response to *S. aureus*. (A) CFU of *S. aureus* and neutrophil infiltrate and (B) CFU of *E. coli* and neutrophil infiltrate in the airpouch lavage of $syk^{f/+}LysMcre^{Tg/+}$ and $syk^{f/-}LysMcre^{Tg/+}$ mice 24 hr after the initiation of infection. (C) Analysis of TNFα and MIP-1α levels in *S. aureus*-infected airpouches from $syk^{f/+}LysMcre^{Tg/+}$ and $syk^{f/-}LysMcre^{Tg/+}$ mice. *$p < 0.05$, Unpaired t-test. (D) CFU of *S. aureus* and neutrophil infiltrate and (E) CFU of *E. coli* and neutrophil infiltrate in the airpouch lavage of $syk^{f/f}$, $syk^{f/-}$ and $syk^{f/-}Mrp8cre^{Tg}$ mice 24 hr after the initiation of infection. Data represent at least 3 independent experiments, with at least 3 animals per group. The difference in *S. aureus* counts in the air pouch is statistically significant, *$p < 0.05$, Mann Whitney U-test.

Cytokine analysis following *S. aureus* challenge indicated that the levels of TNFα and MIP-1α were decreased in the airpouch lavage in $syk^{f/-}LysMcre^{Tg/+}$ animals relative to $syk^{f/+}LysMcre^{Tg/+}$ littermate controls (Figure 3.14 C), supporting the impaired *in vitro* cytokine

release by *syk*$^{-/-}$ neutrophils (Figure 3.3). Since neutrophil recruitment to the airpouch was normal in *syk*$^{f/-}$*LysMcre*$^{Tg/+}$ mice, the residual level of TNFα and MIP-1α may be sufficient to induce neutrophil recruitment to the infected site. Alternatively, other cytokines secreted by neutrophils or other cell types may act to recruit neutrophils following bacterial infection.

We also tested host defense responses in the *syk*$^{f/-}$*LysMcre*$^{Tg/+}$ mice following intraperitoneal challenge with *S. aureus* and *E. coli*. These models produced more variable results than the subcutaneous model, with a narrow window of dose response to lethal infection, in particular following *E. coli* challenge. However, following i.p. challenge with *S. aureus*, *CD11b*$^{-/-}$ mice and *syk*$^{f/-}$*LysMcre*$^{Tg/+}$ mice had an increased bacterial burden in the i.p. lavage relative to their WT controls (Figure 3.15). This supports the requirement for Syk signaling downstream of CD18 integrins for bacterial clearance at an additional tissue site following infection with *S. aureus*.

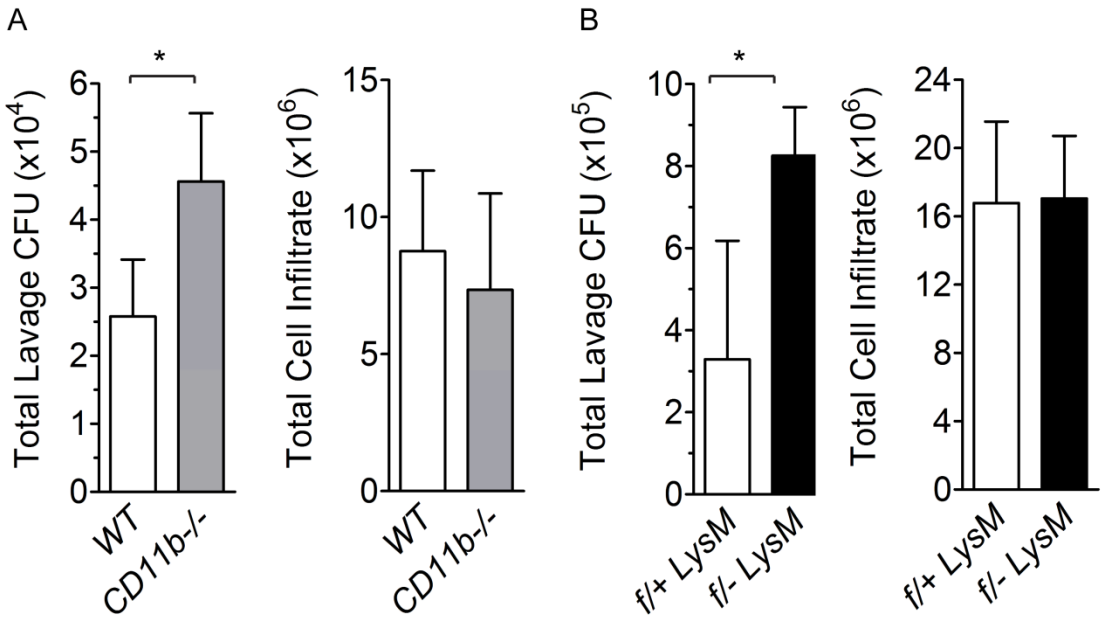

Figure 3.15 Neutrophil-specific deletion of *syk* results in a defective host response to *S. aureus* in a CD11b-dependent intra-peritoneal infection model. (A) CFU of *S. aureus* and PMN numbers in the peritoneal lavage of WT and *CD11b*$^{-/-}$ mice 4 hr after the initiation of infection. (B) CFU of *S. aureus* and PMN numbers in the peritoneal lavage of *syk*$^{f/+}$*LysMcre*$^{Tg/+}$ and *syk*$^{f/-}$*LysMcre*$^{Tg/+}$ mice 4 hr after the initiation of infection. Data are mean ± SD. The difference in bacteria counts in the peritoneum is statistically significant, *$p < 0.05$, Mann Whitney U-test.

3.3 Discussion

In this study, we report that Syk-deficient neutrophils fail to secrete granule components, release induced cytokines, and generate ROIs, while retaining partial elastase activity in response to *S. aureus* and *E. coli*. This results in a reduced ability of Syk-deficient neutrophils to kill bacteria *in vitro*. We also measured the consequences of Syk-deficiency *in vivo*, and while neutrophil migration is normal in the absence of Syk, the defects in neutrophil function result in impaired killing of *S. aureus*, and to a lesser extent *E. coli*. These data indicate a requirement for Syk-signaling during innate immune responses to bacteria.

Syk was previously shown to be required for CD18 integrin-dependent adhesive responses to extracellular matrix proteins (Mocsai et al., 2002). Our results implicate Syk in host responses to bacterial infection. In particular, the release of granules containing cytokines that are induced and stored prior to secretion following bacterial stimulation are impaired in Syk-deficient neutrophils. The mechanisms and location of neutrophil cytokine production, storage and exocytosis are relatively poorly understood. It has been demonstrated that human IL-8 is produced and released from activated neutrophils *in vitro*, and is stored in an organelle that is distinct from classically defined granules and secretory vesicles (Pellme et al., 2006). The production of IL-8 by neutrophils is initiated by accumulation of mRNA transcripts, followed by post-transcriptional and post-translational regulation, varying in response to the stimulus (Scapini et al., 2000). The normal accumulation of TNFα mRNA and protein in *syk*$^{-/-}$ neutrophils suggests that the kinase is not involved in these initial steps, but has a primary function in granule release. Why *syk*$^{-/-}$ neutrophils fail to over accumulate cytokines due to impaired release is unclear. Since neutrophils themselves are responsive to TNFα, MIP1α, MIP2 and KC, the reduced

secretion of these cytokines by Syk-deficiency could result in a reduction in feedback responses, leading ultimately to reduced storage or increased cellular turnover of these cytokines.

Previous studies demonstrated that macrophages (Hamerman et al., 2005) and myeloid dendritic cells (Chu et al., 2008) require Syk for negative regulation of cytokine secretion in response to bacterial cell wall products. Our data demonstrate that neutrophils require Syk for maximal secretion of inflammatory cytokines in response to serum-opsonized bacteria. This suggests that use of Syk-dependent signaling events differs between neutrophils, macrophages and myeloid dendritic cells following stimulation with bacteria. Analysis of signaling events triggered during serum-opsonized bacterial stimulation support this finding, in that *syk*$^{-/-}$ neutrophils have decreased phosphorylation of Erk1/2 in contrast to hyper-phosphorylation of Erk1/2 in macrophages in response to LPS (Hamerman et al., 2005). Other studies ruled out a role for Syk in complement-mediated phagocytosis of *E. coli* (Crowley et al., 1997) or zymosan (Kiefer et al., 1998) in macrophages, which until now had not been examined in neutrophils. Our results show a defect in complement-mediated phagocytosis of *S. aureus*, and a less pronounced defect in complement-mediated phagocytosis of *E. coli* in Syk-deficient neutrophils. This again highlights the diverse roles that Syk plays in different cell types.

Here we show that Syk is required for CD18-dependent responses to bacteria in neutrophils, illustrating the importance of integrin signaling during innate immune responses. Our results support and extend a previous report demonstrating the requirement of CD18 for efficient phagocytosis and superoxide production in response to serum-opsonized bacteria (Anderson et al., 2008). One important difference between the receptor, CD18, and the signaling pathway, namely Syk, is that while CD18 is required for normal

intracellular elastase activation in response to both *S. aureus* and *E. coli*, Syk is only partially required for this response. One possible explanation for this phenotypic divergence is the failure of $CD18^{-/-}$ neutrophils to phagocytose complement opsonized bacteria. The requirement of CD18/Syk signaling for superoxide production, but not elastase activation has important implications for bacterial clearance. Mice deficient in components of the NADPH-oxidase complex are more susceptible to *S. aureus* challenge than *E. coli*, indicating that ROI production has less of a protective role in Gram negative infections (Ellson et al., 2006; Pollock et al., 1995). Conversely, mice deficient in neutrophil elastase are susceptible to *E. coli*, and resistant to *S. aureus* (Belaaouaj et al., 1998). Thus, the increased susceptibility of mice lacking Syk in neutrophils to *S. aureus* is likely due to their inability to produce ROIs, whereas the less pronounced susceptibility to *E. coli* could be due to retention of partial elastase activity.

CD11b and CD18 are required for a robust clearance of *S. aureus* following airpouch challenge (Chen et al., 2003, and our data presented here). The susceptibility of mice lacking Syk in myeloid cells, and specifically in neutrophils, to *S. aureus* following airpouch challenge supports an *in vivo* requirement for Syk-signaling downstream of CD18-integrins. Given that Syk has a dominant role in neutrophil-dependent host defense responses relative to macrophages, the impaired clearance of bacteria in the $syk^{f/-}LysMcre^{Tg/+}$ mice likely represents predominantly neutrophil dysfunction downstream of CD18 integrins. This is further supported by the impaired clearance of bacteria in the $syk^{fl/-}Mrp8cre^{Tg}$ mice, where *syk* deletion is restricted to neutrophils. However, in both our cre^{Tg} lines, deletion of Syk from neutrophils was not complete, as determined by intracellular flow cytometry. Hence, the reduced ability of $syk^{f/-}LysMcre^{Tg/+}$ and $syk^{fl/-}Mrp8cre^{Tg}$ mice to clear bacterial infections likely represents an underestimate of the role of Syk in host defense. More complete deletion

would likely result in more impaired bacterial clearance *in vivo*. However, the *syk*$^{-/-}$ radiation chimeras are not a suitable choice to resolve this potential issue, due to the complication of the abdominal hemorrhage and vascular phenotype (Kiefer et al., 1998). The concept that impaired signaling through neutrophil CD18 integrins can lead to impaired host defense is also evidenced by reduced clearance of *S. aureus* in Vav1/3 deficient mice, during a lung infection (Graham et al., 2007). Vav proteins are known to be required downstream of Syk in the leukocyte integrin signaling pathway (Gakidis et al., 2004).

The differences in neutrophil effector phenotypes between *syk*$^{-/-}$ and *CD18*$^{-/-}$ cells are most likely explained by the fact that *E. coli* and *S. aureus* can trigger a different variety of activating receptors on the surface of the neutrophil in addition to CD11b. These receptors may include TLRs (TLR4 versus TLR2), C-type lectins, and Ig-superfamily members such as TREM-1 (Bouchon et al., 2000) and TREM-2 (Daws et al., 2003). Additionally, CD11c/CD18 dimers may recognize bacterial ligands, for example on *S. aureus*, which would explain normal TNFα release from *CD11b*$^{-/-}$ compared to *CD18*$^{-/-}$ neutrophils. It is likely that a number of receptors, not just CD18, are engaged during neutrophil responses to bacteria, and that the CD18/Syk pathway is not the only mechanism for neutrophil activation, particularly *in vivo* where the bacterium are not directly incubated with serum opsonins. In addition, Syk is required for some of the signal transduction from receptors other than CD18, such as TREM-1 and TREM-2, which are known to signal via ITAM-bearing molecules, and some C-type lectins, including Dectin-1, which signal via an ITAM-like motif through Syk (Reid et al., 2009). Hence, it is possible that some of the host defense deficits in *Syk*-deficient neutrophils may represent defects in signaling pathways other than CD18 integrins. This may explain why some of the functional defects of *syk*$^{-/-}$ cells, such as TNFα secretion, appear to be CD18 independent. For example, Syk is involved in coupling

TLR signaling to downstream pathways through CARD9 (Ruland, 2008), which could lead to impaired TNFα release following *E. coli* challenge.

Overall, these results demonstrate that Syk is a critical signaling molecule for neutrophil effector responses to bacteria. While inhibitors of Syk are being actively pursued in clinical trials for the treatment of neutrophil-dependent inflammatory diseases, the data presented here raises the possibility that the use of such inhibitors may reduce the ability of patients to clear certain types of bacterial infections, in particular those arising from Gram positive bacteria. Recent demonstrations of the involvement of Syk in anti-fungal host-defenses, through signaling involving inflammasome activation or Dectin-1 also raise concern that anti-Syk therapeutics may put patients at risk for these types of infections (Gringhuis et al., 2009; Gross et al., 2009). A deeper understanding of Syk signaling in neutrophils during infection and inflammation may provide a rationale for differential dosing of Syk inhibitors, allowing attenuation of tissue destructive inflammation, while maintaining Syk-dependent bactericidal activity.

Chapter Four

The Role of Src-family Kinases in Integrin-mediated Responses to Microbial Infection

4.1 Introduction

The main Src-family kinases (SFKs) expressed in neutrophils are Hck, Fgr and Lyn. In addition to these, monocytes and macrophages are reported to express Fyn, Yes and Src to variable degrees (Baruzzi et al., 2008). Studies using mice deficient in the major Src-family kinases present in myeloid leukocytes, namely *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice, have demonstrated that the primary positive signaling roles for these enzymes are in the integrin pathway (Lowell, 2004). Double deficiency of Hck and Fgr renders neutrophils and macrophages non-responsive to adhesion-mediated activation after cross-linking of β1, β2, or β3 integrins. *In vitro* studies have shown that *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ macrophages have impaired FcγR-mediated phagocytosis and respiratory burst, but normal internalization of complement-opsonised zymosan particles (Fitzer-Attas et al., 2000). Studies investigating the role of Src-family kinases in β2 (CD18) integrin-mediated migration demonstrated that *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils migrate normally to CD18 integrin ligands in both transwell assays and during thioglycollate induced sterile peritonitis (Mocsai et al., 2002). In contrast to neutrophils, *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ macrophages undergo decreased migration to CD18 integrin ligands in a transwell assay, and during an *in vitro* wound healing assay (Caveggion et al., 2003; Meng and Lowell, 1998; Suen et al., 1999). Interestingly, macrophages from *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice display decreased migration to

thioglycollate-induced sterile peritonitis (Meng and Lowell, 1998). Furthermore, Hck and Fgr have been implicated in three models of inflammation. *Hck*$^{-/-}$*fgr*$^{-/-}$ mice display decreased neutrophil accumulation and enhanced survival during endotoxic shock (Lowell and Berton, 1998) as well as impaired eosinophil accumulation in an allergic model of airway inflammation (Vicentini et al., 2002). In addition, *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice are resistant to the development of Mac-1 (CD11b)-mediated thrombohemorrhagic vasculitis, while maintaining normal neutrophil migration into the inflamed tissue (Hirahashi et al., 2006).

Limited data is available assessing the role of myeloid Src kinases in mediating the inflammatory response to infections *in vivo*. It has been shown that *hck*$^{-/-}$*fgr*$^{-/-}$ mice are resistant to challenged with the protozoan *Leishmania major*, but have increase sensitivity to challenge with the intracellular bacterial pathogen *Listeria monocytogenes* (Lowell et al., 1994). This indicates a requirement of myeloid SFKs to clearance of some microbial pathogens.

Meningitis, caused by *Streptococcus pneumoniae*, is the most common bacteria-induced CNS inflammation in adults and is associated with high mortality and morbidity (Kastenbauer and Pfister, 2003). The inflammatory response to bacterial meningitis is characterized by an influx of neutrophils into the CNS, leading to cerebral inflammation, causing intracranial complications which often results in death (Kastenbauer and Pfister, 2003). It is known that mononuclear phagocytes and neutrophils mediate the innate immune response in pneumococcal infections (Casal and Tarrago, 2003), and that phagocytosis of *S. pneumoniae* is mainly mediated by CD11b through recognition of complement deposited on the bacterium (Gasque, 2004). It is of interest to determine the relevance of CD18 integrin signaling through the myeloid Src kinases during *S. pneumoniae* – induced meningitis.

4.2 Results

Leukocyte recruitment into the CSF space and bacterial clearance is hampered in mice deficient in all three myeloid SFKs (*hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$) during pneumococcal meningitis. As a result, the *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice showed an increased intracranial pressure and a worse clinical outcome as manifested by increased neurologic deficits and mortality, as well as significantly increased cerebellar and blood titers of *S. pneumoniae* compared to infected wild-type mice (Paul et al., 2008). To determine the role for CD18 integrins in the development of *S. pneumoniae* –induced meningitis, *CD11b*$^{-/-}$ mice were challenged. Interestingly, recruitment of neutrophils into the cerebellum remained intact, but the mice developed worse clinical scores and a higher mortality rate than infected WT mice after 24 hours (Paul et al., 2008).

Impaired bacterial killing was associated with a lack of phagocytosis. WT neutrophils were able to both bind bacteria to their membrane, and also internalize the bacteria (Figure 4.1 A). In contrast, neutrophils from *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice had an impaired ability to phagocytose *rag1*$^{-/-}$ serum-opsonised *S. pneumoniae* as demonstrated by the ability of bacteria to attach to the neutrophil membrane, but a lack of intracellular inclusion of pathogens (Figure 4.1 B). In addition, phagocytosis was monitored by flow cytometry of propidium iodide-labelled heat-shock killed *rag1*$^{-/-}$ serum-opsonised *S. pneumonia* (Figure 4.1 B). While a similar percentage of *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ and WT neutrophils are able to bind *S. pneumonia*, there is an inability of *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils to internalize the labelled bacteria.

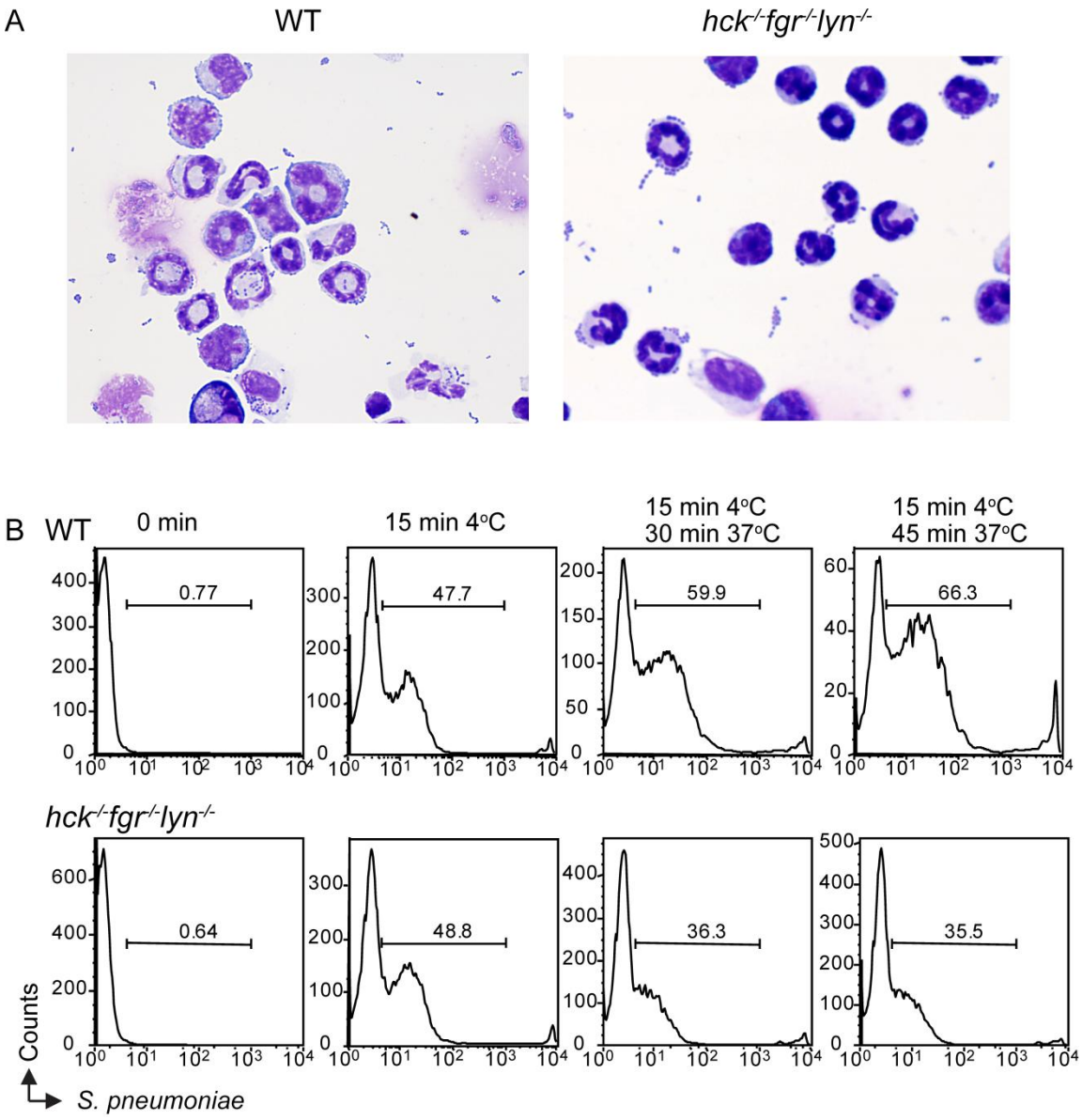

Figure 4.1 Defective bacterial phagocytosis in the absence of Hck, Fgr and Lyn. (A) Cytospins of WT or *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils stimulated with serum opsonised *S. pneumoniae* and stained with H&E to assess bacterial up-take. Many bacteria were attached to the cell surface of both WT and *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils, but intracellular inclusion of *S. pneumoniae* was absent in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils. (B) Flow cytometric analysis of PI-labelled heat-shock-killed *S. pneumoniae*. Bacterial binding is detected at the 15 minute time point, and increased percentages at following time points are indicative of bacterial phagocytosis.

In addition to an inability to phagocytose opsonized *S. pneumonia*, *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils produced less superoxide than WT neutrophils in response to *rag1*$^{-/-}$ serum-opsonized *S. pneumonia* (Figure 4.2 A). Neutrophils lacking either CD11b or CD18 were also defective in superoxide production in response to opsonized *S. pneumonia*. To determine if the failure to produce superoxide was due to a defect in activation of the NADPH-oxidase complex, the phosphorylation of the p40^{phox} subunit on Thr-154, a phosphorylation event that occurs during NADPH oxidase activation (Bouin et al., 1998), was assessed. Compared to WT neutrophils, phosphorylation of p40^{phox} Thr-154 was reduced in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils stimulated with *rag1*$^{-/-}$ serum-opsonized *S. pneumonia* (Figure 4.2 B).

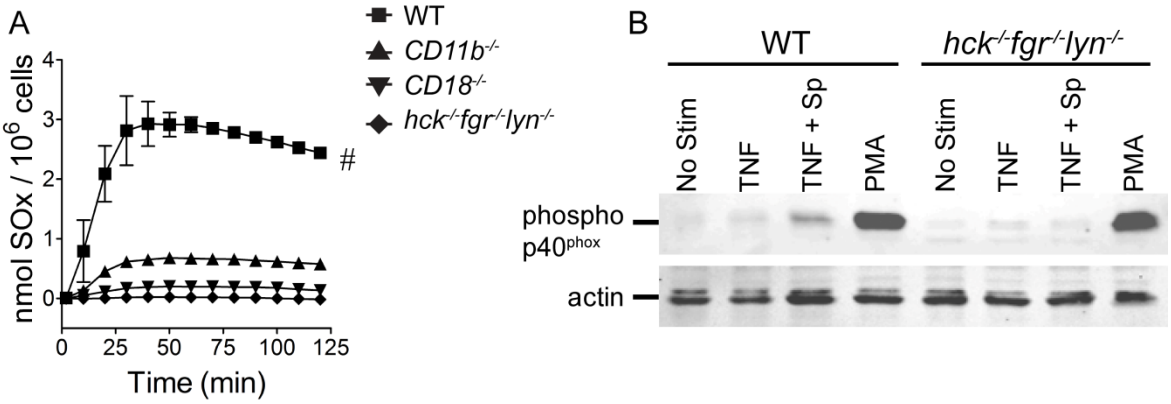

Figure 4.2 Hck, Fgr, Lyn-deficient neutrophils display reduced superoxide production in response to *S. pneumonia*. (A) WT *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$, *CD11b*$^{-/-}$ or *CD18*$^{-/-}$ neutrophils were plated in microtiter wells containing *S. pneumonia* (MOI=40), in cytochrome c media. Production of superoxides (SOx) by respiratory burst was measured as reduction of cytochrome c. Data are representative of at least three independent experiments, and are mean ± SD. (D) Western blot analysis of p40^{phox} phosphorylation (Thr 154) following priming of neutrophils for 30 min with 50 ng/mL TNFα, and where indicated, 30 min with *S. pneumonia* (Sp) MOI=40 or 50 nM PMA.

4.3 Discussion

It is now clear that the myeloid Src kinases Hck, Fgr, and Lyn play a pivotal role in the effective defence against CNS infection with *S. pneumonia*. This defective host defence is due to reduced leukocyte recruitment, impaired phagocytosis and blocked activation of the NADPH-oxidase during acute bacterial meningitis. Moreover, deficiency in CD11b, which is upstream of myeloid Src kinases, also causes impairment of phagocytosis in pneumococcal meningitis, while retaining leukocyte recruitment.

Src kinases and CD18 integrins, together with the urokinase plasminogen activator receptor (uPAR; CD87) establish a membrane-bound complex in leukoyctes. In addition, the Src kinase Hck is activated by binding uPAR to its ligand, uPA (Guiet et al., 2008). Recently, uPAR was implicated in host defense during bacterial meningitis (Paul et al., 2005), suggesting that myeloid SFKs, together with uPAR, may regulate leukocyte adhesion during inflammation *in vivo*. CD11b and Hck have been implicated in neutrophil activation and degranulation of elastase, but not in neutrophil accumulation in the subendothelial area of a model of thrombohemorrhagic vasculitis (Hirahashi et al., 2006). In addition, a *S. pneumonia*-induced pneumonia model demonstrated an important role for CD11b in effective bacterial clearance, but not in the recruitment of neutrophils into the lung (Rijneveld et al., 2005). This further confirms a role for CD11b and Src-family kinases in neutrophil activation, but not migration.

The effective clearance of *S. pneumoniae* from tissues outside of the CNS by innate immune cells at least partially mediated by naturally occurring anti-phosphocholine antibodies (Briles et al., 1981) and C-reactive protein (CRP) (Mold et al., 1981). Both require an intact complement system, but not Fcγ receptors (Mold et al., 2002). The effective

clearance of *S. pneumoniae* from CNS infections requires the complement components C1q and C3, as demonstrated by an impaired ability of mice lacking either of these components to kill bacteria during pneumococcal meningitis (Rupprecht et al., 2007). This supports a requirement for complement-mediated phagocytosis and killing of *S. pneumonia* during pneumococcal meningitis, which has now been shown to be further dependent on signalling through CD11b and the myeloid Src-family kinases. In contrast to macrophages (Fitzer-Attas et al., 2000), complement-dependent phagocytosis in neutrophils is regulated by Hck, Fgr and Lyn.

Overall, these results demonstrate that the Src-family kinases Hck, Fgr, and Lyn are critical signaling molecules for neutrophil effector responses to bacteria, including cellular recruitment and activation. While reduced superoxide production and neutrophil recruitment could be of some benefit to both infectious pneumococcal meningitis and other neutrophil-dependent inflammatory disease, such as rheumatoid arthritis, there is a significant potential for uncontrolled growth and spread of bacteria, or for a general increased susceptibility to bacterial infections.

Chapter Five

The Role of Lyn in Leukocyte Effector Functions and Autoimmunity

5.1 Introduction

Lyn is a Src-family kinase (SFK) expressed in all hematopoietic cells, save T lymphocytes, and is the first SFK member found to have a dual role acting both as a positive and a negative signaling molecule (Scapini et al., 2009; Xu et al., 2005). *Lyn*$^{-/-}$ mice develop a progressive lupus-like autoimmunity with auto-antibody production (including production of anti-nuclear and anti-cardiolipin antibodies), lymphocyte activation and glomerulonephritis due to immune complex deposition (Chan et al., 1997; Hibbs et al., 1995; Nishizumi et al., 1995). The development of autoimmunity in *lyn*$^{-/-}$ mice has largely been attributed to alterations in B-cell signaling thresholds, leading to abnormal B-cell selection and/or tolerance, and resulting in the production of auto-antibodies (Lowell, 2004; Xu et al., 2005). Indeed, Lyn plays a crucial role in establishing signaling thresholds that negatively regulate B-cell receptor (BCR) activation, mainly by phosphorylating inhibitory receptors and molecules, such as FcγRIIb, CD22, PIR-B, CD5, SHP-1, SHP -2 and SHIP-1 (Scapini et al., 2009; Xu et al., 2005).

Similar to B cells, Lyn is a negative regulator of myeloid-cell signaling thresholds. In particular, *lyn*$^{-/-}$ mice display dramatically increased myelopoiesis due to the enhanced sensitivity of progenitor cells to Colony Stimulating Factors (CSFs), in particular, myeloid progenitors are more responsive to granulocyte (G)-CSF, granulocyte-macrophage (GM)-

CSF and macrophage (M)-CSF (Chu and Lowell, 2005; Harder et al., 2001; Pereira and Lowell, 2003). Furthermore, *lyn*$^{-/-}$ myeloid cells become hyper-responsive after engagement of surface integrins, leading to hyper-adhesion, enhanced respiratory burst and secondary granule release (Pereira and Lowell, 2003). Furthermore, serum levels of cytokines, such as BAFF/BLyS, are elevated in *lyn*$^{-/-}$ mice due to hyper-production of biologically active BAFF by *lyn*$^{-/-}$ myeloid cells both *in vitro* and *in vivo*, mainly in response to interferon gamma (IFNγ) (P. Scapini and C. Lowell, unpublished data). These results suggest that deregulated production of BAFF/BLyS by the hyper-active *lyn*$^{-/-}$ myeloid cells may be an important cause or contributor of autoimmunity in this model. Despite this experimental evidence, the contribution of myeloid cells to the development of autoimmune disorders in *lyn*$^{-/-}$ mice has not been investigated.

5.2 Results and Discussion

5.2.1 Generation of a Lyn Conditional Knock-out Allele

To generate a conditional allele of *lyn*, which could be specifically deleted by lineage-restricted expression of Cre-recombinase, mice containing a loxP flanked-allele of *lyn* (*lyn*$^{f/f}$) were generated by introducing 34 base pair LoxP sites on either side of exons 3 and 4 of the gene by homologous recombination in E14 embryonic stem cells, which were then used to generate chimeric mice in the C57BL/6 background by standard procedures (Figure 5.1).

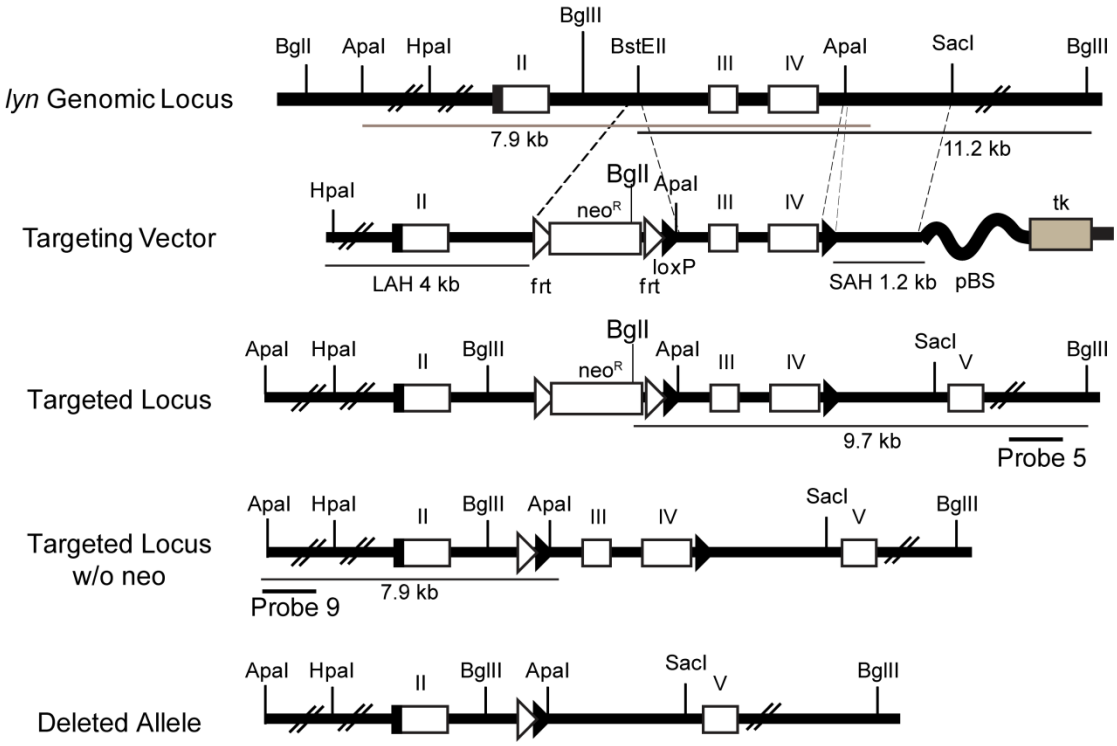

Figure 5.1 Generation of a conditional Lyn-deficient mice. Part of the *lyn* genomic locus, the *lyn* targeting vector and the targeted *lyn* locus before and after Cre-mediated recombination are shown. Exons 2, 3 and 4 are indicated by white boxes. LoxP sites are represented by black triangles, and the neomycin resistance gene (*neo*^{R}) and herpes simplex virus thymidine kinase gene (*tk*) are shown as white and grey boxes, respectively. DNA fragments used for Southern blot analysis are represented as thin lines extending between two restriction sites. Probes 5 and 9 used for Southern blot analysis are shown as thick lines.

The genomic targeting of the *lyn* locus was assessed by Southern blot, and standard screening of gene transmission is assessed by PCR (Figure 5.2 A to C). The analysis of Lyn protein levels by intracellular flow cytometry indicated that expression from the $lyn^{f/f}$ locus is unaltered by insertion of loxP sites, in that Lyn protein level is unaltered from WT levels (Figure 5.2 D).

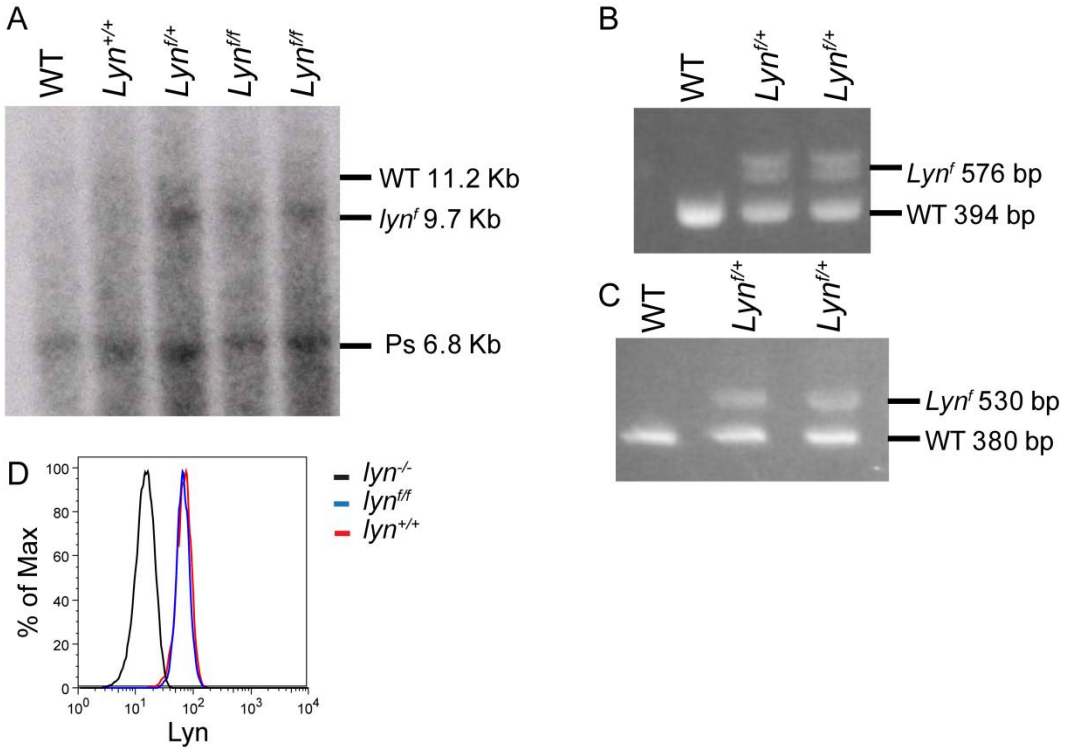

Figure 5.2 Targeting of the *lyn* genomic locus. (A) Southern blot analysis showing the WT (11.2 kb) and loxP-flanked (9.7 kb) alleles of *lyn* genomic locus, and the *lyn* pseudogene (6.8 kb) in DNA isolated from tail biopsies (BglI/BglII digestion, Probe 5). (B) PCR genotyping of the 5' loxP site (Forward primer: 5'-ccactttggccgtgttttat-3'; Reverse primer: 5'-gcacaagcacacacaggagt-3'). (C) PCR genotyping of the 3' loxP site (Forward primer: 5'-cagcaagtaggtcccagctc-3'; Reverse primer: 5'-tccataggcttggatcttgc-3'). (D) Expression from the *lyn* locus, as assessed by the level of Lyn protein and determined by intracellular flow cytometry of peripheral blood neutrophils ($Ly6G^{+}CD11b^{+}$).

5.2.2 Lineage specific conditional deletion of lyn

To assess the ability of the Cre recombinase to delete exons 3 and 4 of the l*yn* conditional allele, *lyn*$^{f/f}$ mice were crossed to the Vav1-cre transgenic mouse line, which expresses Cre very early during and throughout hematopoiesis (de Boer et al., 2003). Loss of Lyn protein was analyzed in peripheral blood cells from *lyn*$^{f/f}$*Vav1-Cre* mice. In addition to having a reduced percentage of peripheral blood B cells and an expansion of CD11b^{+} myeloid cells, as is seen in *lyn*$^{-/-}$ mice, *lyn*$^{f/f}$ *Vav1-Cre* mice show a complete loss of Lyn protein in all peripheral blood cells by intracellular flow cytometry (Figure 5.3).

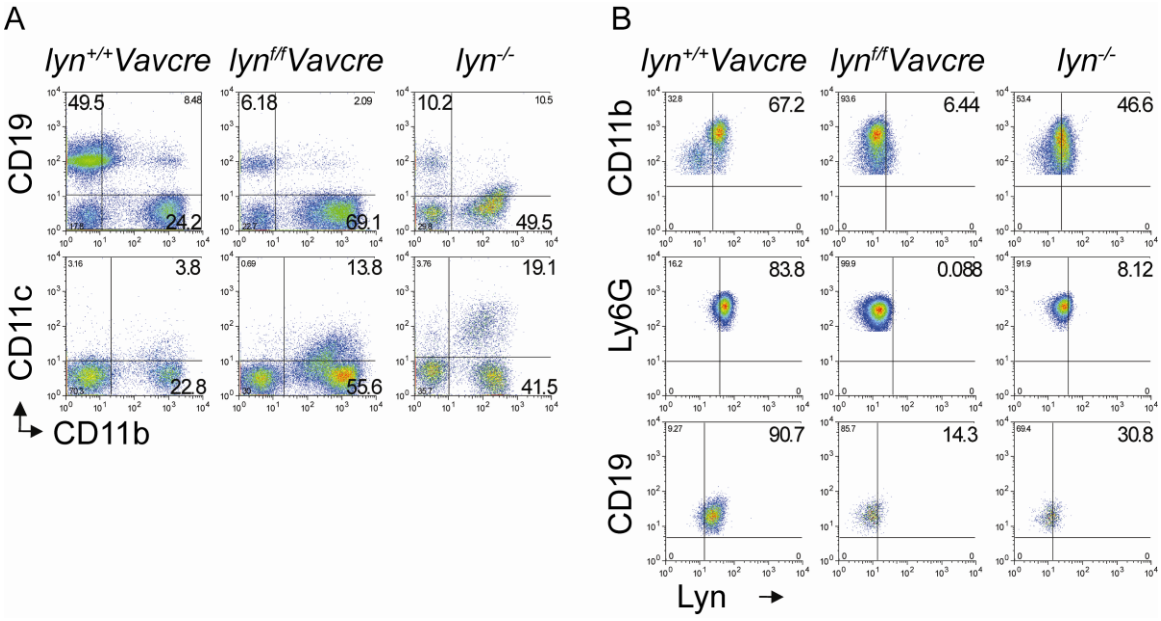

Figure 5.3 Complete loss of Lyn protein in hematopoietic cells of *lyn*$^{f/f}$ *Vav1-cre* mice. (A) Analysis of B cell percentages and CD11b^{+} myeloid cell percentages from the peripheral blood of *lyn*$^{+/+}$ *Vav1-Cre* (WT), *lyn*$^{-/-}$, and *lyn*$^{f/f}$ *Vav1-Cre* mice. (B) Lyn protein levels in peripheral blood leukocytes from *lyn*$^{+/+}$ *Vav1-Cre* (WT), *lyn*$^{-/-}$, and *lyn*$^{f/f}$ *Vav1-Cre* mice.

In order to study the role that Lyn-deficiency plays in different cell types for the initiation and progression of autoimmunity in *lyn*$^{-/-}$ mice, *lyn*$^{f/f}$ mice have been crossed to animals expressing Cre under a variety of hematopoietic lineage specific promoters. To address the role of Lyn in neutrophils, *lyn*$^{f/f}$ mice were crossed to mice expressing Cre under control of the neutrophil and macrophage specific promoter, Lysozyme M (Clausen et al., 1999). To specifically address the role of Lyn in dendritic cells and CD11c^{+} inflammatory monocytes/macrophages, we crossed *lyn*$^{f/f}$ mice to the CD11c-cre transgenic line (Caton et al., 2007). To clarify the intrinsic versus extrinsic effects of Lyn-deficiency in B cells, *lyn*$^{f/f}$ mice were crossed to *mb1-cre*^{Tg} mice (Hobeika et al., 2006), where Cre expression is controlled by the Igα promoter. B cell specific deletion of *lyn* will also enable an analysis of the developmental requirement for Lyn during B cell differentiation and activation.

Initial analysis of these mice demonstrate that *lyn*$^{f/f}$*LysMcre*$^{Tg/+}$ mice retain ~8% Lyn protein in Ly6G^{+}CD11b^{+} peripheral blood neutrophils, and ~20% Lyn protein in total CD11b^{+} peripheral blood myeloid cells. In addition, these mice show an increase in the percentage of peripheral blood CD11b^{+} cells, which could be suggestive of an increase in "inflammatory monocytes" in the blood. An expansion of total CD11b^{+} cells, as well as CD11b^{+}CD11c^{+} double positive cells (data not shown), is also seen in the peripheral blood from *lyn*$^{f/f}$*CD11c-cre*$^{Tg/+}$ mice (Figure 5.4), which retain ~37% Lyn protein in total CD11b^{+} peripheral blood cells, and retain normal protein levels of Lyn in other peripheral blood leukocytes (Table 5.1). Deletion of *lyn* specifically in B cells (*lyn*$^{f/f}$*mb1cre*$^{Tg/+}$ mice) results in retention of ~22% Lyn protein in total CD19^{+} peripheral blood B cells (Table 5.1), and leads to a decrease in the percentage of peripheral blood CD19^{+} B cells, as well as an expansion of the percentage of peripheral blood CD11b^{+} cells (Figure 5.4).

Table 5.1 Lyn protein level in conditionally deleted mice.

	Peripheral Blood			
	$CD11b^+$	$Ly6G^+CD11b^+$	$CD19^+$	(n)
$lyn^{f/f}LysMcre^{Tg/+}$	17.4±9.2	7.6±4.8	78.1±15.4	14
$lyn^{f/f}CD11c\text{-}cre^{Tg/+}$	37.6±18.6	n.d.	80.2±9.6	6
$lyn^{f/f}mb1cre^{Tg/+}$	44.5±17.8	57.1±30.0	21.9±8.1	8
$lyn^{f/f}Vav1cre^{Tg/+}$	13.6±6.3	0.25±.02	21.1±8.0	3
$lyn^{-/-}$	20.6±23.1	7.6±0.7	30.7±5.4	3
$lyn^{f/f}$	58.9±6.9	92.1±9.7	84.8±10.4	4
$lyn^{+/+}Vav1cre^{Tg/+}$	62.6±23.9	78.2±16.0	78.1±12.7	3
$lyn^{+/+}mb1cre^{Tg/+}$	48.8±11.8	76.0±17.9	77.7±8.8	7

The data represent the mean ± SD of the percent Lyn positive cells as assessed by intracellular flow cytometry. For each analysis the respective cell type from $lyn^{-/-}$ mice were used as a negative control for Lyn protein level, and used to set the lower limit gate.

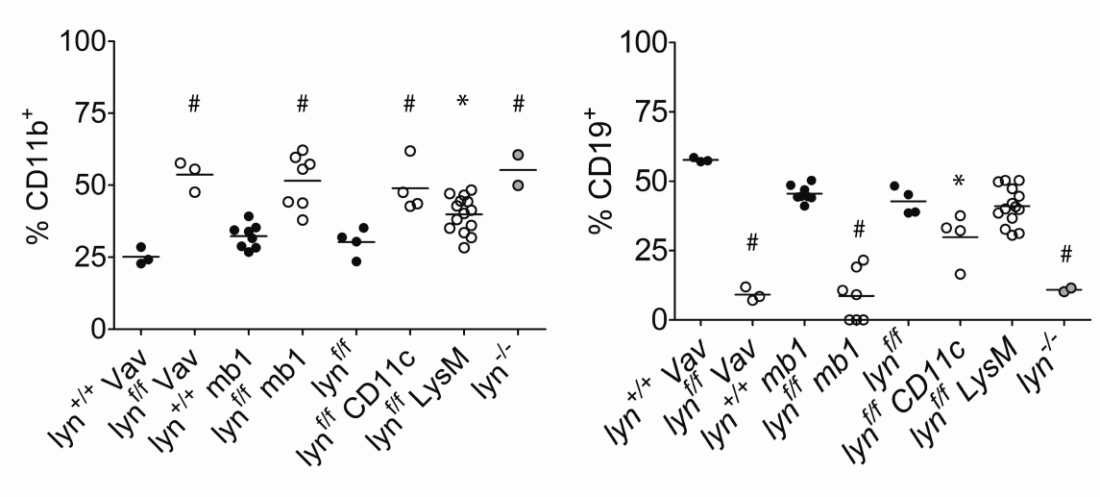

Figure 5.4 Alterations in peripheral blood leukocyte percentages following lineage specific deletion of *lyn*. Percentages of $CD11b^+$ cells or $CD19^+$ cells, as assessed by flow cytometry, of *lyn* conditional knock-out mice (*lyn*$^{f/f}$*Vav1cre*$^{Tg/+}$, *lyn*$^{f/f}$*mb1cre*$^{Tg/+}$, *lyn*$^{f/f}$*CD11c-cre*$^{Tg/+}$, *lyn*$^{f/f}$*LysMcre*$^{Tg/+}$; white circles), relative control mice (black circles), or *lyn* knock-out (*lyn*$^{-/-}$) mice (grey circles). *$p<0.05$, #$p<0.001$ compared to relative WT control by ANOVA with Bonferroni's post-test.

The ability to conditionally delete *lyn* upon lineage-restricted expression of Cre-recombinase will allow for a careful analysis of the differential role that Lyn plays during leukocyte differentiation, maturation and effector function. It will be of particular interest to determine the requirement for Lyn signalling during B cell development and B cell-dependent immune responses. It will also be interesting to further define the inhibitory role of Lyn, particularly in myeloid cells, during models of infection and inflammation. In addition, the analysis of lineage-specific loss of Lyn, and the resultant effects on the initiation and progression of spontaneous lupus-like autoimmunity in *lyn*$^{-/-}$ mice will further our understanding of signaling anomalies during autoimmune progression.

Chapter Six

Conclusions

Src-family and Syk kinases signal downstream of ITAM-bearing adaptor molecules during neutrophil activation. These signalling events are critical for neutrophil activation through the leukocyte-specific CD18 (β2) integrins, as well as other activating receptors. These receptors and signalling molecules are important for neutrophil recruitment to the sites of infection and inflammation, as well as for the cellular activation of neutrophils upon their encounter with invading pathogens or other inflammatory stimuli.

Syk-deficient neutrophils fail to secrete granule components, release induced cytokines, and generate ROIs, while retaining partial elastase activity in response to *S. aureus* and *E. coli*. This results in a reduced ability of Syk-deficient neutrophils to kill bacteria *in vitro*. We also measured the consequences of Syk-deficiency *in vivo*, and while neutrophil migration is normal in the absence of Syk, the defects in neutrophil function result in impaired killing of *S. aureus*, and to a lesser extent *E. coli*. These data indicate a requirement for Syk-signaling during innate immune responses to bacteria.

In addition to the role for Syk in host defense to *S. aureus*, it is now clear that the myeloid Src kinases Hck, Fgr, and Lyn play a pivotal role in the effective defence against CNS infection with *S. pneumonia*. This defective host defence is due to reduced leukocyte recruitment, impaired phagocytosis and blocked activation of the NADPH-oxidase during acute bacterial meningitis. Moreover, deficiency in CD11b, which is upstream of myeloid Src

kinases, also causes impairment of phagocytosis in pneumococcal meningitis, while retaining leukocyte recruitment.

Furthermore, the conditional loss of the Src-family kinase Lyn will allow for the assessment of the role of Lyn in normal leukocyte function, and provide a tool to address the cell-specific consequences of loss of Lyn during the development of spontaneous lupus-like autoimmune disease.

Some questions that the neutrophil field is left with include, how do neutrophils regulate the differential trafficking, storage and secretion of cytokines? It has become clear that the density of v-SNARES vesicle-associated membrane protein (VAMP-2) determines the propensity of granules to exocytose. This provides one mechanistic explanation for the graded release of granules following neutrophil activation(Borregaard et al., 2007). While steps have been taken to further our understanding of neutrophil exocytosis, it is still unclear how newly translated proteins are sorted into granules in mature neutrophils, and how these non-classical granules form. It is of particular interest to ascertain the role of Syk in the control of exocytic events. It is possible that tyrosine kinase signaling simply acts to bring neutrophils to a certain "threshold-level" of activation, required prior to exocytosis of granules; however, Syk may also be directly involved in the activation of the exocytic machinery.

Current interest has greatly increased our understanding of the signaling events stemming from the ligation of Toll-like receptors (TLRs). Tyrosine kinases, including Src-family kinases, Tec kinases and Syk kinases, have been implicated in the activating signals downstream of TLRs (Page et al., 2009); however, the precise role of Syk in TLR signaling is still unclear. Furthermore, it is unknown if either signaling or functional responses to TLR-ligation are normal in Syk-deficient neutrophils.

The critical role of Syk in signaling downstream of ITAM-bearing molecules places it as a central effector to a number of activating receptor-ligand interactions. It is of particular interest to investigate further the requirement for other Syk-dependent receptors in neutrophil responses to pathogens. These receptors include TREM-1 (Bouchon et al., 2000), which binds to currently unknown ligands, TREM-2, which has been reported to bind to anionic ligands on the surface of Gram-positive and Gram-negative bacteria (Daws et al., 2003), and Dectin-1, known to recognize fungal cell wall components (Reid et al., 2009). The integration of these signals through Syk, other signaling molecules, or a combination, may be critical for a robust innate immune response to microbial pathogens.

Tyrosine kinases play critical roles in cellular activation during the initiation, continuation and resolution of inflammation. They provide signals downstream of receptors responding to both bacterial and fungal cell wall components, and are thus integral to responses to these microbial pathogens. In addition, tyrosine kinases play critical roles in responses to immune-complex mediated inflammatory and autoimmune diseases, which place them as a potential targets for the treatment of neutrophil-dependent inflammatory diseases. Understanding the balance that these kinases play in neutrophils during infection and inflammation may provide a rationale for differential dosing of Src-family or Syk kinase inhibitors, allowing attenuation of tissue destructive inflammation, while maintaining neutrophil-dependent bactericidal activity.

Chapter Seven

References

Abram, C.L., and Lowell, C.A. (2007). Convergence of immunoreceptor and integrin signaling. Immunol Rev *218*, 29-44.

Abtahian, F., Guerriero, A., Sebzda, E., Lu, M.M., Zhou, R., Mocsai, A., Myers, E.E., Huang, B., Jackson, D.G., Ferrari, V.A.*, et al.* (2003). Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science *299*, 247-251.

Anderson, K.E., Boyle, K., Davidson, K., Chessa, T.A., Kulkarni, S., Jarvis, G.E., Sindrilaru, A., Scharffetter-Kochanek, K., Rausch, O., Stephens, L.R., and Hawkins, P.T. (2008). CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of *E.coli* or *S.aureus* is regulated by Class III but not Class I or II PI3Ks. Blood.

Aratani, Y., Koyama, H., Nyui, S., Suzuki, K., Kura, F., and Maeda, N. (1999). Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. Infect Immun *67*, 1828-1836.

Baruzzi, A., Caveggion, E., and Berton, G. (2008). Regulation of phagocyte migration and recruitment by Src-family kinases. Cell Mol Life Sci *65*, 2175-2190.

Belaaouaj, A., McCarthy, R., Baumann, M., Gao, Z., Ley, T.J., Abraham, S.N., and Shapiro, S.D. (1998). Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. Nat Med *4*, 615-618.

Berton, G., and Lowell, C.A. (1999). Integrin signalling in neutrophils and macrophages. Cell Signal *11*, 621-635.

Berton, G., Mocsai, A., and Lowell, C.A. (2005). Src and Syk kinases: key regulators of phagocytic cell activation. Trends Immunol *26*, 208-214.

Bjerknes, R., and Bassoe, C.F. (1984). Phagocyte C3-mediated attachment and internalization: flow cytometric studies using a fluorescence quenching technique. Blut *49*, 315-323.

Borregaard, N., and Cowland, J.B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. Blood *89*, 3503-3521.

Borregaard, N., Kjeldsen, L., Sengelov, H., Diamond, M.S., Springer, T.A., Anderson, H.C., Kishimoto, T.K., and Bainton, D.F. (1994). Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol *56*, 80-87.

Borregaard, N., Sorensen, O.E., and Theilgaard-Monch, K. (2007). Neutrophil granules: a library of innate immunity proteins. Trends Immunol *28*, 340-345.

Bouchon, A., Dietrich, J., and Colonna, M. (2000). Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol *164*, 4991-4995.

Bouin, A.P., Grandvaux, N., Vignais, P.V., and Fuchs, A. (1998). p40(phox) is phosphorylated on threonine 154 and serine 315 during activation of the phagocyte NADPH oxidase. Implication of a protein kinase c-type kinase in the phosphorylation process. J Biol Chem *273*, 30097-30103.

Briles, D.E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J., and Barletta, R. (1981). Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J Exp Med *153*, 694-705.

Bunting, M., Harris, E.S., McIntyre, T.M., Prescott, S.M., and Zimmerman, G.A. (2002). Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving β2 integrins and selectin ligands. Curr Opin Hematol *9*, 30-35.

Casal, J., and Tarrago, D. (2003). Immunity to *Streptococcus pneumoniae*: Factors affecting production and efficacy. Curr Opin Infect Dis *16*, 219-224.

Casimir, C.M., Bu-Ghanim, H.N., Rodaway, A.R., Bentley, D.L., Rowe, P., and Segal, A.W. (1991). Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. Proc Natl Acad Sci U S A *88*, 2753-2757.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J Exp Med *204*, 1653-1664.

Caveggion, E., Continolo, S., Pixley, F.J., Stanley, E.R., Bowtell, D.D., Lowell, C.A., and Berton, G. (2003). Expression and tyrosine phosphorylation of Cbl regulates macrophage chemokinetic and chemotactic movement. J Cell Physiol *195*, 276-289.

Chan, V.W., Meng, F., Soriano, P., DeFranco, A.L., and Lowell, C.A. (1997). Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity *7*, 69-81.

Chen, H., Mocsai, A., Zhang, H., Ding, R.X., Morisaki, J.H., White, M., Rothfork, J.M., Heiser, P., Colucci-Guyon, E., Lowell, C.A.*, et al.* (2003). Role for plastin in host defense distinguishes integrin signaling from cell adhesion and spreading. Immunity *19*, 95-104.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303-306.

Chu, C.L., and Lowell, C.A. (2005). The Lyn tyrosine kinase differentially regulates dendritic cell generation and maturation. J Immunol *175*, 2880-2889.

Chu, C.L., Yu, Y.L., Shen, K.Y., Lowell, C.A., Lanier, L.L., and Hamerman, J.A. (2008). Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRγ. Eur J Immunol *38*, 166-173.

Clark, R.A., Malech, H.L., Gallin, J.I., Nunoi, H., Volpp, B.D., Pearson, D.W., Nauseef, W.M., and Curnutte, J.T. (1989). Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. N Engl J Med *321*, 647-652.

Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res *8*, 265-277.

Clements, J.L., Lee, J.R., Gross, B., Yang, B., Olson, J.D., Sandra, A., Watson, S.P., Lentz, S.R., and Koretzky, G.A. (1999). Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. J Clin Invest *103*, 19-25.

Coopman, P.J., Do, M.T., Barth, M., Bowden, E.T., Hayes, A.J., Basyuk, E., Blancato, J.K., Vezza, P.R., McLeskey, S.W., Mangeat, P.H., and Mueller, S.C. (2000). The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature *406*, 742-747.

Crowley, M.T., Costello, P.S., Fitzer-Attas, C.J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V.L., and DeFranco, A.L. (1997). A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. J Exp Med *186*, 1027-1039.

Dale, D.C., Boxer, L., and Liles, W.C. (2008). The phagocytes: neutrophils and monocytes. Blood *112*, 935-945.

Daws, M.R., Sullam, P.M., Niemi, E.C., Chen, T.T., Tchao, N.K., and Seaman, W.E. (2003). Pattern recognition by TREM-2: binding of anionic ligands. J Immunol *171*, 594-599.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J., and Kioussis, D. (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol *33*, 314-325.

Dinauer, M.C., Pierce, E.A., Bruns, G.A., Curnutte, J.T., and Orkin, S.H. (1990). Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J Clin Invest *86*, 1729-1737.

Ellson, C.D., Davidson, K., Ferguson, G.J., O'Connor, R., Stephens, L.R., and Hawkins, P.T. (2006). Neutrophils from *p40phox*$^{-/-}$ mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. J Exp Med *203*, 1927-1937.

Etzioni, A. (2009). Defects in the Leukocyte Adhesion Cascade. Clin Rev Allergy Immunol.

Etzioni, A., Doerschuk, C.M., and Harlan, J.M. (1999). Of man and mouse: leukocyte and endothelial adhesion molecule deficiencies. Blood *94*, 3281-3288.

Faurschou, M., and Borregaard, N. (2003). Neutrophil granules and secretory vesicles in inflammation. Microbes Infect *5*, 1317-1327.

Fitzer-Attas, C.J., Lowry, M., Crowley, M.T., Finn, A.J., Meng, F., DeFranco, A.L., and Lowell, C.A. (2000). Fcγ receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J Exp Med *191*, 669-682.

Gakidis, M.A., Cullere, X., Olson, T., Wilsbacher, J.L., Zhang, B., Moores, S.L., Ley, K., Swat, W., Mayadas, T., and Brugge, J.S. (2004). Vav GEFs are required for β2 integrin-dependent functions of neutrophils. J Cell Biol *166*, 273-282.

Gasque, P. (2004). Complement: a unique innate immune sensor for danger signals. Mol Immunol *41*, 1089-1098.

Goedken, M., McCormick, S., Leidal, K.G., Suzuki, K., Kameoka, Y., Astern, J.M., Huang, M., Cherkasov, A., and Nauseef, W.M. (2007). Impact of two novel mutations on the structure and function of human myeloperoxidase. J Biol Chem *282*, 27994-28003.

Graham, D.B., Robertson, C.M., Bautista, J., Mascarenhas, F., Diacovo, M.J., Montgrain, V., Lam, S.K., Cremasco, V., Dunne, W.M., Faccio, R.*, et al.* (2007). Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice. J Clin Invest *117*, 3445-3452.

Graham, D.B., Zinselmeyer, B.H., Mascarenhas, F., Delgado, R., Miller, M.J., and Swat, W. (2009). ITAM signaling by Vav family Rho guanine nucleotide exchange factors regulates interstitial transit rates of neutrophils in vivo. PLoS ONE *4*, e4652.

Greenberg, S., Chang, P., and Silverstein, S.C. (1994). Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. J Biol Chem *269*, 3897-3902.

Gringhuis, S.I., den Dunnen, J., Litjens, M., van der Vlist, M., Wevers, B., Bruijns, S.C., and Geijtenbeek, T.B. (2009). Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-KB activation through Raf-1 and Syk. Nat Immunol *10*, 203-213.

Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschlager, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V.*, et al.* (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature *459*, 433-436.

Guiet, R., Poincloux, R., Castandet, J., Marois, L., Labrousse, A., Le Cabec, V., and Maridonneau-Parini, I. (2008). Hematopoietic cell kinase (Hck) isoforms and phagocyte duties - from signaling and actin reorganization to migration and phagocytosis. Eur J Cell Biol *87*, 527-542.

Hamerman, J.A., Tchao, N.K., Lowell, C.A., and Lanier, L.L. (2005). Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. Nat Immunol *6*, 579-586.

Harder, K.W., Parsons, L.M., Armes, J., Evans, N., Kountouri, N., Clark, R., Quilici, C., Grail, D., Hodgson, G.S., Dunn, A.R., and Hibbs, M.L. (2001). Gain- and loss-of-function Lyn mutant mice define a critical inhibitory role for Lyn in the myeloid lineage. Immunity *15*, 603-615.

Heyworth, P.G., Cross, A.R., and Curnutte, J.T. (2003). Chronic granulomatous disease. Curr Opin Immunol *15*, 578-584.

Hibbs, M.L., Tarlinton, D.M., Armes, J., Grail, D., Hodgson, G., Maglitto, R., Stacker, S.A., and Dunn, A.R. (1995). Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell *83*, 301-311.

Hirahashi, J., Mekala, D., Van Ziffle, J., Xiao, L., Saffaripour, S., Wagner, D.D., Shapiro, S.D., Lowell, C., and Mayadas, T.N. (2006). Mac-1 signaling via Src-family and Syk kinases results in elastase-dependent thrombohemorrhagic vasculopathy. Immunity *25*, 271-283.

Hobeika, E., Thiemann, S., Storch, B., Jumaa, H., Nielsen, P.J., Pelanda, R., and Reth, M. (2006). Testing gene function early in the B cell lineage in mb1-cre mice. Proc Natl Acad Sci U S A *103*, 13789-13794.

Horwitz, M., Benson, K.F., Person, R.E., Aprikyan, A.G., and Dale, D.C. (1999). Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. Nat Genet *23*, 433-436.

Inatome, R., Yanagi, S., Takano, T., and Yamamura, H. (2001). A critical role for Syk in endothelial cell proliferation and migration. Biochem Biophys Res Commun *286*, 195-199.

Kasama, T., Miwa, Y., Isozaki, T., Odai, T., Adachi, M., and Kunkel, S.L. (2005). Neutrophil-derived cytokines: potential therapeutic targets in inflammation. Curr Drug Targets Inflamm Allergy *4*, 273-279.

Kastenbauer, S., and Pfister, H.W. (2003). Pneumococcal meningitis in adults: spectrum of complications and prognostic factors in a series of 87 cases. Brain *126*, 1015-1025.

Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. Mol Cell Biol *18*, 4209-4220.

Klein, C., Grudzien, M., Appaswamy, G., Germeshausen, M., Sandrock, I., Schaffer, A.A., Rathinam, C., Boztug, K., Schwinzer, B., Rezaei, N.*, et al.* (2007). HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). Nat Genet *39*, 86-92.

Kostmann, R. (1956). Infantile genetic agranulocytosis; agranulocytosis infantilis hereditaria. Acta Paediatr Suppl *45*, 1-78.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Lehrer, R.I., and Cline, M.J. (1969). Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. J Clin Invest *48*, 1478-1488.

Liese, J., Kloos, S., Jendrossek, V., Petropoulou, T., Wintergerst, U., Notheis, G., Gahr, M., and Belohradsky, B.H. (2000). Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. J Pediatr *137*, 687-693.

Lowell, C.A. (2004). Src-family kinases: rheostats of immune cell signaling. Mol Immunol *41*, 631-643.

Lowell, C.A., and Berton, G. (1998). Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. Proc Natl Acad Sci U S A *95*, 7580-7584.

Lowell, C.A., Fumagalli, L., and Berton, G. (1996). Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J Cell Biol *133*, 895-910.

Lowell, C.A., Soriano, P., and Varmus, H.E. (1994). Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev *8*, 387-398.

Lubke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Korner, C. (2001). Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency. Nat Genet *28*, 73-76.

Luhn, K., Wild, M.K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001). The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. Nat Genet *28*, 69-72.

Malinin, N.L., Zhang, L., Choi, J., Ciocea, A., Razorenova, O., Ma, Y.Q., Podrez, E.A., Tosi, M., Lennon, D.P., Caplan, A.I.*, et al.* (2009). A point mutation in *KINDLIN3* ablates activation of three integrin subfamilies in humans. Nat Med *15*, 313-318.

Matute-Bello, G., Frevert, C.W., Kajikawa, O., Skerrett, S.J., Goodman, R.B., Park, D.R., and Martin, T.R. (2001). Septic shock and acute lung injury in rabbits with peritonitis: failure of the neutrophil response to localized infection. Am J Respir Crit Care Med *163*, 234-243.

McKenzie, S.E., and Schreiber, A.D. (1998). Fc γ receptors in phagocytes. Curr Opin Hematol *5*, 16-21.

Meischl, C., and Roos, D. (1998). The molecular basis of chronic granulomatous disease. Springer Semin Immunopathol *19*, 417-434.

Meng, F., and Lowell, C.A. (1998). A β1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J *17*, 4391-4403.

Mocsai, A., Abram, C.L., Jakus, Z., Hu, Y., Lanier, L.L., and Lowell, C.A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. Nat Immunol *7*, 1326-1333.

Mocsai, A., Ligeti, E., Lowell, C.A., and Berton, G. (1999). Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. J Immunol *162*, 1120-1126.

Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V.L., and Lowell, C.A. (2002). Syk is required for integrin signaling in neutrophils. Immunity *16*, 547-558.

Mold, C., Nakayama, S., Holzer, T.J., Gewurz, H., and Du Clos, T.W. (1981). C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. J Exp Med *154*, 1703-1708.

Mold, C., Rodic-Polic, B., and Du Clos, T.W. (2002). Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc γ receptors. J Immunol *168*, 6375-6381.

Moser, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H.V., Sperandio, M., and Fassler, R. (2009). Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells. Nat Med *15*, 300-305.

Nagle, D.L., Karim, M.A., Woolf, E.A., Holmgren, L., Bork, P., Misumi, D.J., McGrail, S.H., Dussault, B.J., Jr., Perou, C.M., Boissy, R.E*., et al.* (1996). Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. Nat Genet *14*, 307-311.

Newbrough, S.A., Mocsai, A., Clemens, R.A., Wu, J.N., Silverman, M.A., Singer, A.L., Lowell, C.A., and Koretzky, G.A. (2003). SLP-76 regulates Fcγ receptor and integrin signaling in neutrophils. Immunity *19*, 761-769.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcγ receptors as regulators of immune responses. Nat Rev Immunol *8*, 34-47.

Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., and Yamamoto, T. (1995). Impaired proliferation of peripheral B cells and indication of autoimmune disease in Lyn-deficient mice. Immunity *3*, 549-560.

Page, T.H., Smolinska, M., Gillespie, J., Urbaniak, A.M., and Foxwell, B.M. (2009). Tyrosine kinases and inflammatory signalling. Curr Mol Med *9*, 69-85.

Palacios, E.H., and Weiss, A. (2007). Distinct roles for Syk and ZAP-70 during early thymocyte development. J Exp Med *204*, 1703-1715.

Passegue, E., Wagner, E.F., and Weissman, I.L. (2004). JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell *119*, 431-443.

Pasvolsky, R., Feigelson, S.W., Kilic, S.S., Simon, A.J., Tal-Lapidot, G., Grabovsky, V., Crittenden, J.R., Amariglio, N., Safran, M., Graybiel, A.M.*, et al.* (2007). A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets. J Exp Med *204*, 1571-1582.

Paul, R., Obermaier, B., Van Ziffle, J., Angele, B., Pfister, H.W., Lowell, C.A., and Koedel, U. (2008). Myeloid Src kinases regulate phagocytosis and oxidative burst in pneumococcal meningitis by activating NADPH oxidase. J Leukoc Biol *84*, 1141-1150.

Paul, R., Winkler, F., Bayerlein, I., Popp, B., Pfister, H.W., and Koedel, U. (2005). Urokinase-type plasminogen activator receptor regulates leukocyte recruitment during experimental pneumococcal meningitis. J Infect Dis *191*, 776-782.

Pellme, S., Morgelin, M., Tapper, H., Mellqvist, U.H., Dahlgren, C., and Karlsson, A. (2006). Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. J Leukoc Biol *79*, 564-573.

Pereira, S., and Lowell, C. (2003). The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling. J Immunol *171*, 1319-1327.

Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F.W., and Geha, R.S. (1998). Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. Cell *94*, 229-238.

Pollock, J.D., Williams, D.A., Gifford, M.A., Li, L.L., Du, X., Fisherman, J., Orkin, S.H., Doerschuk, C.M., and Dinauer, M.C. (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nat Genet *9*, 202-209.

Reid, D.M., Gow, N.A., and Brown, G.D. (2009). Pattern recognition: recent insights from Dectin-1. Curr Opin Immunol.

Rijneveld, A.W., de Vos, A.F., Florquin, S., Verbeek, J.S., and van der Poll, T. (2005). CD11b limits bacterial outgrowth and dissemination during murine pneumococcal pneumonia. J Infect Dis *191*, 1755-1760.

Roos, D. (1994). The genetic basis of chronic granulomatous disease. Immunol Rev *138*, 121-157.

Rosenzweig, S.D., and Holland, S.M. (2004). Phagocyte immunodeficiencies and their infections. J Allergy Clin Immunol *113*, 620-626.

Ruland, J. (2008). CARD9 signaling in the innate immune response. Ann N Y Acad Sci *1143*, 35-44.

Rupprecht, T.A., Angele, B., Klein, M., Heesemann, J., Pfister, H.W., Botto, M., and Koedel, U. (2007). Complement C1q and C3 are critical for the innate immune response to *Streptococcus pneumoniae* in the central nervous system. J Immunol *178*, 1861-1869.

Saijo, K., Schmedt, C., Su, I.H., Karasuyama, H., Lowell, C.A., Reth, M., Adachi, T., Patke, A., Santana, A., and Tarakhovsky, A. (2003). Essential role of Src-family protein tyrosine kinases in NF-kB activation during B cell development. Nat Immunol *4*, 274-279.

Scapini, P., Lapinet-Vera, J.A., Gasperini, S., Calzetti, F., Bazzoni, F., and Cassatella, M.A. (2000). The neutrophil as a cellular source of chemokines. Immunol Rev *177*, 195-203.

Scapini, P., Pereira, S., Zhang, H., and Lowell, C.A. (2009). Multiple roles of Lyn kinase in myeloid cell signaling and function. Immunol Rev *228*, 23-40.

Schaff, U., Mattila, P.E., Simon, S.I., and Walcheck, B. (2008). Neutrophil adhesion to E-selectin under shear promotes the redistribution and co-clustering of ADAM17 and its proteolytic substrate L-selectin. J Leukoc Biol *83*, 99-105.

Scharffetter-Kochanek, K., Lu, H., Norman, K., van Nood, N., Munoz, F., Grabbe, S., McArthur, M., Lorenzo, I., Kaplan, S., Ley, K.*, et al.* (1998). Spontaneous skin ulceration and defective T cell function in CD18 null mice. J Exp Med *188*, 119-131.

Schwarz-Linek, U., Hook, M., and Potts, J.R. (2004). The molecular basis of fibronectin-mediated bacterial adherence to host cells. Mol Microbiol *52*, 631-641.

Sengelov, H., Kjeldsen, L., Diamond, M.S., Springer, T.A., and Borregaard, N. (1993). Subcellular localization and dynamics of Mac-1 (αm β2) in human neutrophils. J Clin Invest *92*, 1467-1476.

Skokowa, J., Germeshausen, M., Zeidler, C., and Welte, K. (2007). Severe congenital neutropenia: inheritance and pathophysiology. Curr Opin Hematol *14*, 22-28.

Spicer, S.S., and Hardin, J.H. (1969). Ultrastructure, cytochemistry, and function of neutrophil leukocyte granules. A review. Lab Invest *20*, 488-497.

Stendahl, O., Coble, B.I., Dahlgren, C., Hed, J., and Molin, L. (1984). Myeloperoxidase modulates the phagocytic activity of polymorphonuclear neutrophil leukocytes. Studies with cells from a myeloperoxidase-deficient patient. J Clin Invest *73*, 366-373.

Suen, P.W., Ilic, D., Caveggion, E., Berton, G., Damsky, C.H., and Lowell, C.A. (1999). Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. J Cell Sci *112 (Pt 22)*, 4067-4078.

Svensson, L., Howarth, K., McDowall, A., Patzak, I., Evans, R., Ussar, S., Moser, M., Metin, A., Fried, M., Tomlinson, I., and Hogg, N. (2009). Leukocyte adhesion deficiency-III is caused by mutations in *KINDLIN3* affecting integrin activation. Nat Med *15*, 306-312.

Tellier, E., Canault, M., Rebsomen, L., Bonardo, B., Juhan-Vague, I., Nalbone, G., and Peiretti, F. (2006). The shedding activity of ADAM17 is sequestered in lipid rafts. Exp Cell Res *312*, 3969-3980.

Theilgaard-Monch, K., Jacobsen, L.C., Borup, R., Rasmussen, T., Bjerregaard, M.D., Nielsen, F.C., Cowland, J.B., and Borregaard, N. (2005). The transcriptional program of terminal granulocytic differentiation. Blood *105*, 1785-1796.

Tsuda, Y., Takahashi, H., Kobayashi, M., Hanafusa, T., Herndon, D.N., and Suzuki, F. (2004). Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. Immunity *21*, 215-226.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298-302.

Vicentini, L., Mazzi, P., Caveggion, E., Continolo, S., Fumagalli, L., Lapinet-Vera, J.A., Lowell, C.A., and Berton, G. (2002). Fgr deficiency results in defective eosinophil recruitment to the lung during allergic airway inflammation. J Immunol *168*, 6446-6454.

Voyich, J.M., and DeLeo, F.R. (2002). Host-pathogen interactions: leukocyte phagocytosis and associated sequelae. Methods Cell Sci *24*, 79-90.

Wang, D., Feng, J., Wen, R., Marine, J.C., Sangster, M.Y., Parganas, E., Hoffmeyer, A., Jackson, C.W., Cleveland, J.L., Murray, P.J., and Ihle, J.N. (2000). Phospholipase Cγ2 is essential in the functions of B cell and several Fc receptors. Immunity *13*, 25-35.

Wright, S.D., and Jong, M.T. (1986). Adhesion-promoting receptors on human macrophages recognize *Escherichia coli* by binding to lipopolysaccharide. J Exp Med *164*, 1876-1888.

Xu, Y., Harder, K.W., Huntington, N.D., Hibbs, M.L., and Tarlinton, D.M. (2005). Lyn tyrosine kinase: accentuating the positive and the negative. Immunity *22*, 9-18.

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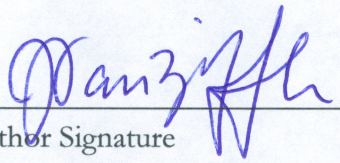

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