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Toll-like Receptors 2 and 9 Are Important for Innate Immune Cell Activation and Recruitment in Hypersensitivity Pneumonitis

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Toll-like Receptors 2 and 9 Are Important for Innate Immune Cell Activation and Recruitment in Hypersensitivity Pneumonitis

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Kelly J. Andrews
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DEDICATION

I would like to dedicate this work to my parents, Thomas and Patricia Andrews,
and my sisters, Leslie Slusher and Ashley Andrews,
for encouraging me and supporting me while I followed my dreams.

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ABSTRACT

Hypersensitivity pneumonitis (HP) is an interstitial lung disease caused by repeated inhalation of environmental antigens. The disease is characterized by alveolitis and granuloma formation; however, some patients develop chronic HP (CHP), a restrictive lung disease characterized by fibrosis. Previous studies revealed that neutrophils are recruited into the lung via a MyD88-dependent pathway and regulate disease severity through cytokine production. Toll-like receptors (TLRs) 2 and 9 recognize conserved molecular patterns present in bacteria, and signal through the adaptor molecule MyD88. The goal of my project is to investigate the role of TLRs 2 and 9 in the pathogenesis of HP during the acute phase, granulomatous phase, and chronic phase of the disease. Using the *S. rectivirgula* (SR) animal model of HP, our studies indicated that individually, TLRs 2 and 9 contributed to neutrophil recruitment. We generated TLR2/9 double knockout (TLR2/9$^{-/-}$) mice to determine the extent to which TLRs 2 and 9 cooperate in neutrophil recruitment during HP.

During the acute response, TLR2/9$^{-/-}$ mice exposed to SR demonstrated a significant reduction in neutrophil recruitment after a single exposure to SR compared to C57BL/6 mice (WT). The reduction in neutrophils was associated with a reduction in the expression of the neutrophil chemokine CXCL2 and inflammatory cytokines TNFα and IL-6 in the BALF. Disease severity in HP is associated with granuloma formation and a Th17 response. After 3 weeks exposure to SR, our results demonstrate a decrease in IL-17 and IL-22 mRNA expression and a corresponding decrease in the percentage of Th17 cells in TLR2/9$^{-/-}$ mice compared to WT and single knockout (SKO) mice. The decrease in Th17 cells in TLR2/9$^{-/-}$ mice was not associated with a significant increase in T regulatory (Treg) cells or a switch to a Th1 response. In addition, TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice have significantly fewer activated CD4^{+} Th cells. Interstitial macrophages from TLR2/9$^{-/-}$ mice have decreased costimulatory molecule and MHCII expression suggesting they are deficient in T cell activation. However, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ still form granulomas in the lung.

To determine the extent to which TLRs 2 and 9 contributed to the development of CHP, WT and TLR2/9$^{-/-}$ mice were intranasally exposed to SR three times/week for 15 weeks. WT and TLR2/9$^{-/-}$ mice developed a neutrophilic and lymphocytic alveolitis, but TLR2/9$^{-/-}$ mice had a significant increase in eosinophils in the bronchoalveolar lavage (BAL) fluid compared to WT exposed mice. Th17 cells, but not Th2 cells were detected in the lungs of mice exposed to SR for 14 weeks, suggesting CHP is not associated with a switch to a Th2 type immune response. WT and TLR2/9$^{-/-}$ mice had significantly reduced static compliance compared to WT unexposed mice. To determine whether the restrictive lung defect was associated with fibrosis, we visualized collagen in the lung by histology using Masson's trichrome stain. Measuring collagen staining by positive pixel analysis suggested TLR2/9$^{-/-}$ mice were partially protected from lung fibrosis compared to WT mice. Altogether, the results suggest TLRs 2 and 9 cooperate in neutrophil recruitment and subsequently disease severity and play an integral role in the outcome of HP.

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LIST OF ABBREVIATIONS

βTcR   β T cell receptor

A   Total lung capacity

AM   Alveolar macrophage

ANOVA   Analysis of variance

APC(s)   Antigen presenting cell(s)

BAL   Bronchoalveolar lavage

BALF   Bronchoalveolar lavage fluid

BMDC   Bone marrow derived dendritic cell

BMDM   Bone marrow derived macrophage

C   Compliance

CHP   Chronic hypersensitivity pneumonitis

Cst   Static compliance

DC   Dendritic cell

E   Elastance

Est   Static elastance

HP   Hypersensitivity pneumonitis

IM   Interstitial macrophage

KO   Knock-out

MHCII   Major histocompatibility complex class II

MyD88$^{-/-}$ MyD88 knock-out

PRRs   Pattern recognition receptors

R   Resistance

RPKM	Reads per kilobase of exon model per million mapped reads
SKO	Single knock-out
SR	*Saccharopolyspora rectivirgula*
Th	T helper cell
TLR2$^{-/-}$	TLR2 knock-out
TLR2/9$^{-/-}$	TLR2 & TLR9 double knock-out
TLR9$^{-/-}$	TLR9 knock-out
TLR(s)	Toll-like receptor(s)
Treg	T regulatory cell
WT	Wild type

CHAPTER 1.   HYPERSENSITIVITY PNEUMONITIS

Hypersensitivity Pneumonitis (HP), or extrinsic allergic alveolitis, develops following repeated exposure to a wide variety of inhaled environmental antigens (reviewed in Kurup et al., 2006). The environmental antigens that induce HP include organic dusts, animal proteins, fungi, and bacteria. The different types of HP are frequently named after the occupation or activity that results in exposure to the inciting agent (reviewed in Selman et al., 2012). Farmer's Lung disease is one of the most common types of HP and is caused by repeated inhalation of the thermophilic gram positive bacteria *Saccharopolyspora rectivirgula* (SR), which grows in moldy hay.

Hypersensitivity pneumonitis can be classified into three subtypes: acute, subacute, and chronic. Acute HP is characterized by flu-like symptoms such as fever, sweating, myalgia, aching joints, headaches, accompanied by cough, breathlessness, dyspnea, wheeze, and chest tightness a few hours after exposure, and the disease can be intermittent or progressive (reviewed in Ismail et al., 2006; Kurup et al., 2006; Selman et al., 2012). The subacute stage often occurs after prolonged low dose exposure over weeks or months as patients develop cough, dyspnea, and hypoxemia after exertion with mild or absent acute symptoms (reviewed in Ismail et al., 2006; Kurup et al., 2006; Selman et al., 2012). In the lung, patients develop a neutrophilic and lymphocytic alveolitis, interstitial bronchiolocentric pneumonitis, and small loosely formed noncaseating granulomas (Selman et al., 2010). In some patients, the disease can progress to chronic HP (CHP), however some patients may develop CHP without ever experiencing acute symptoms. Chronic symptoms are similar to subacute HP, but in addition CHP is associated with emphysema and a restrictive lung disease characterized by fibrosis and reduced lung compliance (Costabel et al., 2012; Erkinjuntti-Pekkanen et al., 1998; Selman et al., 2012). CHP is progressive and irreversible and is associated with high morbidity and mortality (Hanak et al., 2008; Vourlekis et al., 2004).

The best treatment option for HP is antigen avoidance if the antigen can be identified. If exposure can be stopped, patients with acute and subacute HP will recover. In chronic HP, antigen identification and avoidance will not completely resolve symptoms but is associated with reduced mortality (Fernandez Perez et al., 2013). Corticosteroids are also often prescribed, but have little effect on disease outcomes (Kokkarinen et al., 1992). Deaths where HP is the underlying or contributing cause have steadily increased from 1979 to 2007 (NIOSH., 2012). Therefore, more research into how HP progresses to CHP is needed to develop new treatment options.

SR Mouse Model of HP

The SR mouse model of Farmer's Lung disease has identified many of the pathogenic mechanisms that lead to disease. Mice intranasally inoculated with SR 3 days/week for 3 weeks develop a neutrophilic alveolitis that becomes more lymphocytic as exposures continue (Denis, 1992; Denis et al., 1992). The influx of neutrophils is

accompanied by a cytokine response consisting of TNFα, IL-6, IL-17, IFNγ, and CXCL2/MIP-2, macrophage and dendritic cell activation, and lymphocyte recruitment. Previous studies have revealed HP is a T cell mediated disease. Nude mice, which lack a thymus and do not develop mature T cells, do not develop HP (Ohta et al., 1993), and TcRβ$^{-/-}$ mice, which lack CD4$^+$ and CD8$^+$ T cells, do not form granulomas or develop severe fibrosis in response to SR (Simonian et al., 2009). In addition, the adoptive transfer of CD4$^+$ T cells or ex vivo expanded Th1 cells from exposed mice into naïve mice confers disease upon antigen exposure (Schuyler et al., 1997; Schuyler et al., 1993). Granuloma formation in HP is dependent on T cells as well as the cytokine IFNγ, produced either from T cells or innate immune cells (Gudmundsson and Hunninghake, 1997; Nance et al., 2005). However, recent studies have shown IL-17 and the Th17 response is associated with increased disease severity and fibrosis in HP (Joshi et al., 2009; Simonian et al., 2009).

Chronic HP Studies

Unfortunately, little is known about the transition between HP and CHP. One hypothesis suggests the development of progressive fibrosis in CHP is associated with a switch to a Th2 response. A study which looked at T cells responses in patients with pigeon breeder's disease, another common type of HP, found an increase in CD4$^+$ and CD8$^+$ T cells expressing IL-4 and a decrease in T cells expressing IFNγ in patients with CHP compared to subacute HP (Barrera et al., 2008). This is in agreement with a mouse model of chronic pigeon breeder's disease; the authors found that IL-4 / IL-13 expression and a Th2-biased immune response correlated with increased lung fibrosis (Mitaka et al., 2011). Fibrotic diseases often develop in the presence of Th2-associated cytokines IL-4 and IL-13 (Gharaee-Kermani et al., 2001; Jakubzick et al., 2003; Lee et al., 2001); however, previous studies have shown IL-4 and Th2-biased immune responses are protective in SR induced HP (Butler et al., 2002; Gudmundsson et al., 1998; Jimenez-Alvarez et al., 2010; Matsuno et al., 2007).

Neutrophils have also been associated with lung fibrosis in CHP. There is a correlation between the level of neutrophils in the lung tissue and severity of fibrosis in patients with CHP (Pardo et al., 2000). The neutrophils expressed high levels of gelatinase B and collagenase 2, which contribute to lung damage. IL-17 expression is associated with the induction of neutrophil chemokines and neutrophil recruitment in the lung (Hellings et al., 2003; Laan et al., 1999; Miyamoto et al., 2003; Witowski et al., 2000). In addition, recent studies of HP in mice have shown that neutrophils and macrophages were the main cell type that expressed IL-17A in HP, and that neutrophil depletion decreased the amount of lung collagen (Hasan et al., 2013).

Toll-Like Receptors in HP

Because neutrophils are one of the first cell types recruited to the lung after antigen exposure, and have been implicated in the pathogenesis of CHP, our lab became

interested in the pattern recognition receptors (PRRs) responsible for SR recognition and neutrophil recruitment. PRRs recognize conserved molecular patterns present on bacteria, viruses, and other pathogens and are important in innate immune cell activation and phagocytosis. Toll-like receptors (TLRs) are a family of PRRs expressed either on the cell surface or in endosomes, and most signal through the adaptor molecule myeloid differentiation primary response gene 88 (MyD88). Previous studies in our lab demonstrated that MyD88-dependent receptors are necessary for the initial production of pro-inflammatory cytokines, neutrophil chemokines, and subsequently neutrophil recruitment following acute SR exposure (Nance et al., 2008). One of the MyD88-dependent TLRs involved in recognition of gram positive bacteria is TLR2, and our previous studies revealed that SR interacts with TLR2 and can activate the TLR2 signaling pathway that leads to NF-κB activation using a luciferase reporter assay in vitro. TLR2 forms heterodimers with either TLR1 or TLR6, and previous studies have shown TLR6 is involved in SR induced immune response in HP (Fong et al., 2010). Mice deficient in TLR2 have a significant reduction in the neutrophil chemokine CXCL2/MIP-2 but not neutrophil recruitment. Therefore, additional MyD88-dependent receptors must be involved in SR recognition and neutrophil recruitment.

Previous studies have shown the importance of TLR9 signaling in other gram positive organisms such as *Mycobacterium tuberculosis*, and *Mycobacterium avium*, (Bafica et al., 2005; Carvalho et al., 2011; Daito et al., 2011), fungi such as *Aspergillus fumigates* and *Cryptococcus neoformans* and *Stachybotrys chartarum* (Bhan et al., 2011; Ramaprakash et al., 2009; Zhang et al., 2010), parasites such as *Trypanosoma cruzi* (Bafica et al., 2006), and viruses such as Herpes simplex virus (Sorensen et al., 2008). In addition, TLR2 can cooperate with TLR9 to induce protective immunity as mice deficient in both TLRs 2 and 9 resulted in increased pathogen burden, and reduced cytokine production, particularly IL-6, TNFα, and IL-12p40 (Bafica et al., 2006; Bafica et al., 2005; Sorensen et al., 2008). SR is a gram positive bacteria, and exposure induces IL-6, TNFα, and IL-12p40 (Schuyler et al., 2000), and therefore TLR9, along with TLR2, may contribute to the immune response to SR.

The goal of my project is to investigate the role of TLRs 2 and 9 in the pathogenesis of HP during the acute phase, the granulomatous phase, and the chronic phase. We hypothesize mice doubly deficient in TLRs 2 and 9 will have reduced production of neutrophil chemokines and inflammatory cytokines TNFα, IL-6, and IL-12p40 resulting in reduced neutrophil recruitment during the acute innate immune response. During the granulomatous phase, we hypothesize the loss of TLRs 2 and 9 in SR expose mice will also result in reduced neutrophil recruitment as well as reduced antigen presenting cell activation and upregulation of costimulatory molecules and MHCII necessary for the activation of naïve T cells; and therefore CD4$^+$ Th cell activation during the adaptive immune response will be reduced. The reduced CD4$^+$ Th cell activation, combined with the reduction in inflammatory cytokines IL-6 and IL-12p40 (the common chain for IL-12 and IL-23) will result in reduced polarization of Th1 and Th17 cells in TLR2/9$^{-/-}$ mice. Granuloma formation depends on physical signals as well as cytokine signals, and the reduced interstitial macrophage and CD4$^+$ Th cell activation and inflammatory cytokine production in TLR2/9$^{-/-}$ mice will result in reduced

granuloma formation. Finally, the reduced neutrophil recruitment and Th17 cell response in TLR2/9$^{-/-}$ mice will result in reduced fibrosis during CHP and protect against the development of restrictive lung disease.

To test these hypotheses, we will use an animal model of HP where WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice will be exposed to SR once for experiments during the acute phase, 3 times/week for 3 weeks for experiments during the granulomatous phase, and 3 times/week for 14 or 15 weeks for experiments during the chronic phase. Changes in neutrophils and other immune cell populations recruited to the airways and lung tissue will measured by alveolitis and flow cytometry, and changes in Th17 cell and other Th cell populations will be evaluated by intracellular flow cytometry between WT and KO mice. At each time point, SR induced cytokine and chemokine production will be analyzed for reductions in expression and protein levels in KO mice compared to WT SR exposed mice. The development of granulomas during the granulomatous phase, and fibrosis, and reduced lung function during the chronic phase will be assessed in WT and KO mice as a measure of disease severity. Data will be analyzed by statistical software to compare differences between KO and WT SR exposed mice to assess statistical significance.

CHAPTER 2.   METHODOLOGY

Animals and SR Exposure Protocol

Care and Housing

C57BL/6 mice (WT) were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. MyD88$^{-/-}$, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice on a C57BL/6 background were bred at UTHSC and were in at least the 5th generation backcross. MyD88$^{-/-}$ and TLR9$^{-/-}$ were provided by Dr. S. Akira (Osaka University, Japan), TLR2$^{-/-}$ mice were purchased from Jackson Lab, and TLR2/9$^{-/-}$ mice were generated by breeding TLR2$^{-/-}$ and TLR9$^{-/-}$ mice. All animals were housed in sterile micro-isolator cages with sterile food and water *ad libitum* and were maintained by the Division of Comparative Medicine at the University of Tennessee Health Science Center according to the guidelines of the Animal Welfare Act. All animal care procedures were performed according to protocols approved by the UTHSC animal care and use committee.

Genotyping Knock-Out Mice

The genotype of knock-out mice was confirmed by PCR. A tail snip was collected from mouse pups and digested in digestion buffer (500mM KCl, 100mM Tris, 1% NP-40, 1% Tween, 200μg/mL proteinase K) overnight at 55°C with shaking. The enzyme was heat inactivated at 100°C for 5 minutes then centrifuged at 8500rpm (Centrifuge 5451D, Eppindorf, Hamburg, Germany) for 5 minutes. The supernatant containing DNA was transferred to a clean tube. The DNA concentration of supernatant was determined using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Piscataway, NJ). A portion of the supernatant (2μL) was diluted 1:30 in nuclease free water and the absorbance at 260nm and 280nm was measured. The concentration of DNA (μg/mL) is equal to the absorbance at 260nm multiplied by the dilution factor and 50μg/mL. The supernatant containing DNA was diluted to a concentration of 200μg/mL for use in PCR.

DNA (200ng) was added to a PCR master mix (Go Taq PCR core system I, Promega, Fitchburg, WI) containing Taq polymerase, nucleotides, gene specific forward and reverse primers (**Table 2-1**), and MgCl$_2$ in a reaction buffer. The PCR reaction was carried out in a PCR thermocycler (Mastercycler epgradient S, Eppindorf, Hamburg, Germany). Each reaction began with a denaturation step of 94°C followed by an annealing step at a primer specific temperature and an elongation step at 72°C repeated for 35 or 36 cycles (**Table 2-2**).

Table 2-1.   Genotyping primer sequences.

Gene	Forward primer	Reverse primer	Neomycin primer	Product
TLR2	CTT CCT GAA TTT GTC CAG TAC A	ACG AGC AAG ATC AAC AGG AGA	GGG CCA GCT CAT TCC TCC CAC	WT: 498bp KO: 334bp
TLR9	GCT CAT TTA GCC CTG GCT CTC ACA G	AGG TTC TGG GCT CAA TGG TCA TGT	CGT CGT GAC CCA TGG CGA TG	WT: 377bp KO: 662bp
MyD88	TGG CAT GCC TCC ATC ATA GTT AAC C	GTC AGA AAC AAC CAC CAC CAT GC	ATC GCC TTC TAT CGC CTT CTT GAC G	WT: 563bp KO: 625bp

Table 2-2.   Genotyping PCR conditions.

Gene	Denaturation		Annealing		Elongation		Cycles
	Temp	Time	Temp	Time	Temp	Time	
TLR2	94°C	45s	63°C	45s	72°C	60s	36
TLR9	94°C	45s	60°C	60s	72°C	60s	36
MyD88	94°C	45s	65°C	60s	72°C	60s	35

SR Exposure

The *S. rectivirgula* (SR) (strain designation A1313 – ATCC) was grown at 55°C in trypticase soy broth. The bacterial preparation was washed in endotoxin free distilled water 3 times followed by lyophilization. The lyophilized preparation was reconstituted with endotoxin free saline. Mice were anesthetized with isoflurane and mice inoculated intranasally with 50μg SR in 40μL sterile saline. For chronic exposure experiments, the SR dose was increased to 150μg SR in 50μL sterile saline. In acute experiments, mice were exposed once. For experiments during the granulomatous phase, mice were exposed three times/week for 3 weeks. For experiments during the chronic phase, mice were exposed three times/week for 13 weeks and once on week 14 or three times/week for 14 weeks and once on week 15.

Lung Physiology Measurements

Mice were weighed then anesthetized with intraperitoneal ketamine/xylazine (1:1) (0.15mL/10g). A tracheotomy was performed using an 18-Ga needle. Adequate anesthesia was maintained with inhalation of 1% isoflurane. Temperature was maintained with a heat lamp. On the flexiVent™ mechanical ventilator (SCIREQ Scientific Respiratory Equipment Inc, Montreal, QC), ventilation was 10mL/kg, and respiratory rate was kept at 150 breaths/min. Positive end-expiratory pressure (PEEP) was 2cmH$_2$O. Mice were inflated to total lung capacity (TLC) to open airways before taking measurements. A snapshot 150 perturbation inflated lungs in a sinusoidal forced oscillation waveform to measure dynamic lung compliance (C), elastance (E), and airway resistance (R). A pressure volume curve was generated by a step-wise inflation of the lung to total lung capacity and deflation back to functional residual capacity. The flexivent software preformed a curve fit of the data to the Salazar-Knowles equation to determine static compliance (Cst), static elastance (Est) and total lung capacity (A).

BAL and Lung Cell Isolation

Alveolitis

Bronchoalveolar lavage (BAL) was performed by intratracheal injection of 1.0-1.5mL PBS or 2mM PBS-EDTA into the lungs with immediate vacuum aspiration. The amount of fluid recovered was routinely around 70%. Cells were recovered from the BAL fluid by centrifugation (Sorvall Legend RT, Thermo Fisher Scientific, Waltham, MA) at 1500rpm for 5 minutes and live cells counted using trypan blue dye exclusion on a hemocytometer. The BAL fluid was aliquoted and frozen at -80°C until used in ELISA assays for cytokine and chemokine measurement.

Alveolar Macrophage Isolation

To isolate alveolar macrophages, the lungs were exhaustively flushed with 14-15mL 2mM PBS-EDTA injected intratracheally. Cells were then recovered by centrifugation (Sorvall Legend RT, Thermo Fisher Scientific, Waltham, MA) at 1500rpm for 5 minutes for use in flow cytometry.

Differential Cell Counting

In some experiments, an aliquot of BAL cells were centrifuged onto a slide for differential cell counting. Slides were heat fixed and stained with a modified Wright Giemsa stain (HEMA 3 stat pack, Fisher Scientific) according to manufacturer's instructions. Slides were digitized (Scanscope XT, Aperio, Vista, CA) and analyzed using Spectrum Imagescope software (Aperio). Cells in a minimum of 4 fields at 20x were analyzed until a minimum of 200 cells were counted and identified by nuclear shape and cytoplasmic staining. Multinucleated macrophages were identified as macrophages that contained two or more nuclei.

Lung Cell Isolation

Lungs were perfused with phosphate-buffered saline (PBS) to remove blood and lobes removed and placed in culture media (**Table 2-3**). Lung lymphocytes were isolated from the lung in a method similar to Abraham et al. (Abraham et al., 1990). Lung tissue was digested with collagenase (20U/mL) and deoxyribonuclease I (40μg/mL) for 45 minutes at 37°C. Cells were freed by disruption in a Stomacher 80 tissue processor (Seward, West Sussex, UK) and then isolated by centrifugation on a discontinuous percoll (Sigma, St. Louis, MO) gradient. Cells were underlaid with a 40% percoll solution, followed by an 80% percoll solution and centrifuged at 2000rpm for 20 minutes and stopped with a light brake. Mononuclear cells were isolated at the 40/80% interface and used in flow cytometry and *ex vivo* cell stimulations.

Flow Cytometry

Cell Surface Staining

Flow cytometry was performed on isolated BAL and lung cells. Cells were suspended in FACS buffer (2-3% heat inactivated FCS in PBS, in some experiments also containing 2mM EDTA). The Fc-receptor was blocked with anti-CD16/32 antibody (BD Biosciences, San Jose, CA). Unbound antibody was washed with FACS buffer and cells were stained with fluorochrome-conjugated antibodies to CD11b, CD11c, Gr-1, F4/80, CD45, CD4, CD8, βTcR, γδTcR, NK1.1, CD19, CD40, CD80, CD86, MHCII, and

Table 2-3.   Culture media.

Reagent	Manufacturer	Location
RPMI	Cellgro, Mediatech Inc.	Manassas, VA
5% heat inactivated FCS	Hyclone, Thermo Scientific	Logan, UT
2mM glutamine	Cellgro, Mediatech Inc.	Manassas, VA
100IU/mL penicillin	Cellgro, Mediatech Inc.	Manassas, VA
100μg/mL streptomycin	Cellgro, Mediatech Inc.	Manassas, VA
1μL/L β-mercaptoethanol	Fisher Scientific	Waltham, MA

CD206 (BD Biosciences, San Jose, CA; eBiosciences, San Diego, CA; Biolegend, San Diego, CA; or Biosource, Invitrogen, Carlsbad, CA) in a volume of approximately 200μL. After a 30 minute incubation in the dark, the cells were washed with FACS buffer twice then resuspended for analysis on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). The flow cytometer was compensated with fluorochrome-conjugated beads (BD Biosciences, San Jose, CA). Autofluorescence was compensated with autofluorescence$^-$ spleen cells and autofluorescence$^+$ unstained lung cells. For most experiments between 30,000 and 50,000 events were collected. Data was analyzed with FACSDiva software (BD Biosciences, San Jose, CA). Cells were identified by expression of surface markers (**Table 2-4**) and analyzed for the expression of classical or alternative activation makers.

Intracellular Cytokine Staining

For intracellular cytokine staining, 5×10^5 lung cells were incubated with SR lysate (4μg) or plate bound α-CD3 for 3 hours with an additional 3 hours in the presence of brefeldin A (Golgi Plug, BD Biosciences, San Jose, CA) or monensin (Golgi Stop, BD Biosceinces, San Jose, CA). The SR lysate was generated using the Y-PER reagent (Pierce) following manufacturer's instructions. In some experiments, cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. The cells were incubated with antibodies to CD45, CD4, and βTcR to identify CD4$^+$ T cells and then fixed (BD Cytofix, BD Biosciences, San Jose, CA) and permeabilized with 1% saponin followed by incubation with IL-17A, IFNγ, TNF, IL-10, or isotype control antibodies. Alternatively, cells were fixed with FoxP3 fixation/permeabilization solution (eBiosciences, San Diego, CA) and stained with FoxP3 and IL-17F or isotype control antibodies. A minimum of 50,000 events/sample was collected on a BD Biosciences LSRII cytometer (BD Biosciences, San Jose, CA). Expression of cell surface markers and intracellular cytokines was analyzed using FACSDiva software (BD Biosciences, San Jose, CA).

Spleen Cell Isolation, Splenic Adherent Cell Enrichment, and Stimulation

Spleen Cell Isolation

The spleen was harvested from WT, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ mice. Spleen cells were freed by disruption in a Stomacher 80 tissue processor (Seward, West Sussex, UK). RBCs were lysed with RBC lysis buffer, then washed with media and pelleted at 1500rpm for 5 minutes.

Table 2-4.   Cell surface flow cytometry.

Cell type	Identifying surface markers	Activation markers	Alternative activation markers
Neutrophil	$CD45^+$ / $CD11b^+$ / $Gr-1^+$		
Interstitial macrophage	$CD45^+$ / $CD11b^+$ / $F4/80^+$ / autofluorescence$^-$	$CD80^+$ / $CD86^+$ / $CD40^+$ / $MHCII^{high}$	
Alveolar macrophage	$CD45^+$ / $CD11b^{high/low}$ / $CD11c^+$ / $F4/80^+$ / $CD205^+$ / autofluorescence$^+$	$CD80^+$ / $CD86^+$ / $CD40^+$ / $MHCII^{high}$	$CD206^+$
Dendritic cell	$CD45^+$ / $CD11b^{+/-}$ / $CD11c^+$ / $F4/80^-$ / autofluorescence$^-$	$CD80^+$ / $CD86^+$ / $CD40^+$ / $MHCII^{high}$	
B cell	$CD45^+$ / $CD19^+$ / SSc^{low}	$CD69^+$	
$CD4^+$ Th cell	$CD45^+$ / βTcR^+ / $CD4^+$ / SSc^{low}	$CD69^+$	
$CD8^+$ T cell	$CD45^+$ / βTcR^+ / $CD8^+$ / SSc^{low}	$CD69^+$	
$\gamma\delta$ T cell	$CD45^+$ / $\gamma\delta TcR^+$ / SSc^{low}	$CD69^+$	
NK cell	$CD45^+$ / $NK1.1^+$ / SSc^{low}	$CD69^+$	

Splenic Adherent Cell Enrichment

Spleen cells were divided into two non-tissue culture petri plate and incubated at 37ºC for two hours. Non-adherent cells were removed and cells incubated in cold PBS at 4ºC for 15 minutes. Adherent cells were removed with a cell scraper, collected and pelleted at 1500rpm for 5 minutes then resuspended in media for cell stimulation assays.

Spleen and Splenic Adherent Cell Stimulation

Spleen cells (5×10^5 cells/well) were plated in a 96 well plate and stimulated with SR (0.025mg/mL) in the presence or absence of bafilomycin (0.1µM). In other experiments, spleen cells were stimulated with SR RNA complexed to DOTAP, a liposomal transfection agent at a concentration of 0.01, 0.1, 1, 10µg/mL. Imiquimod (0.25µg/mL) was used as a positive control. In all experiments, spleen cells were stimulated for 24 hours then cell culture supernatants were harvested and stored at -80°C for analysis by ELISA. Enriched splenic adherent cells (5×10^5 cells/well) were plated in a 96 well plate then stimulated with SR (0.05mg/mL) for 24 hours and cell culture supernatants were harvested and stored at −80°C for analysis by Luminex. Cytokine and chemokine concentrations were extrapolated from a standard curve and compared to cells cultured in media alone.

Proliferation Assay

SR and bafilomycin toxicity was measured using the CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Spleen cells (2.5×10^5 cells/well) were plated and stimulated with SR (0.025mg/mL) in the presence or absence of bafilomycin (1µM). During the last 4 hours of culture, MTS-PMS solution was added to the well then absorbance read at 490nm with a reference reading at 630nm. The absorbance was normalized to the average absorbance of WT spleen cells in media alone. A treatment was considered toxic if the absorbance was significantly reduced compared to cells without treatment.

Bone Marrow Derived Macrophage (BMDM) Generation and Stimulation

Bone Marrow Harvest and Storage

Bone marrow cells were harvested from the femur and tibia of WT and TLR2/9$^{-/-}$ mice. Mice were sacrificed and fur completely wetted down with 70% ethanol. The fur, skin, and large muscle tissue was removed from the hind legs. Legs were removed at the hip and the paw removed at the joint to prevent cutting the femur and tibia. The bones were sprayed with ethanol and placed in media on ice until all mice were processed. In a sterile hood, any remaining flesh was removed from the bones. The epiphysis was cut and

bone marrow flushed with sterile PBS into a 50mL tube using a 26 gauge 1/2 inch needle and syringe. The bone marrow was centrifuged at 400g and supernatant decanted. The bone marrow cells were pipetted up and down to break up clumps and washed with sterile PBS and pelleted at 400g. The supernatant was discarded and RBCs lysed with RBC lysis buffer. Bone marrow cells were passed through a 70μm cell strainer (BD Biosciences, San Jose, CA) to obtain a single cell suspension. Cells were pelleted at 400g and resuspended in media. Cells were counted by trypan dye exclusion on a hemocytometer and brought to a concentration of $6x10^6$ cells/mL. Bone marrow cells were either plated immediately or reconstituted in freezing media (90% heat inactivated FCS, 10% DMSO) and frozen at -80°C and transferred to liquid nitrogen for long term storage (Marim et al., 2010). To thaw bone marrow progenitor cells for BMDM differentiation, bone marrow progenitor cells were rapidly thawed in a 37°C water bath and were washed with media to removed DMSO.

BMDM Differentiation

To generate macrophages, bone marrow progenitor cells from one mouse (approximately $30x10^6$ cells) were cultured in a non-tissue culture sterile petri plate in 15mL BMDM differentiation media containing 30% L-cell conditioned medium (LCCM) as a source of M-CSF (**Table 2-5**). The following day, the non-adherent cells were harvested, cells counted, and plated at $5x10^6$ cells/plate in media containing 30% LCCM bone marrow differentiation media. On the fourth day, 5mL of the media was removed and 10mL of warm bone marrow differentiation media containing 60% LCCM was added so that the final concentration of the dish is 30% LCCM. On day 6-7 the adherent cells were harvested and the purity of the macrophage ($CD11b^+$ / $F4/80^+$) preparation was determined by flow cytometry and was routinely between 80-85%.

BMDM Stimulation

The BMDMs ($5x10^5$ cells/well) were plated in BMDM cultivation media containing 5% LCCM (**Table 2-5**). The BMDMs were allowed to adhere overnight then stimulated with SR (0.05mg/mL) or imiquimod (3μg/mL) for 24hrs in the presence or absence of bafilomycin (0.1μM), then cell culture supernatants were harvested and stored at -80°C for analysis by ELISA. CpG DNA stimulation was used as a positive control.

ELISA

Sandwich ELISA

Cytokines present in unconcentrated BALF or culture supernatants were measured by ELISA according to manufacturer's instructions. ELISA plates (Nunc flat-

Table 2-5.   **BMDM media.**

Reagent	Differentiation media	Cultivation media	Manufacturer	Location
DMEM	X	X	Cellgro, Mediatech Inc.	Manassas, VA
15% heat inactivated FCS	X	X	Hyclone, Thermo Scientific	Logan, UT
2mM glutamine	X	X	Cellgro, Mediatech Inc.	Manassas, VA
100IU/mL penicillin	X	X	Cellgro, Mediatech Inc.	Manassas, VA
100µg/mL streptomycin	X	X	Cellgro, Mediatech Inc.	Manassas, VA
1µL/L β-mercaptoethanol	X	X	Fisher Scientific	Waltham, MA
50µg/mL gentamycin	X		GIBCO cell culture, Invitrogen	Carlsbad, CA
LCCM	30%	5%		

bottom 96 well plate, Apogent, Portsmouth, NH) were coated with capture antibody then incubated with samples or standards diluted in a 7 point 2-fold dilution series. Manufacturer and cytokine data range can be found in **Table 2-6**. After incubation, plates were washed to remove unbound proteins and cytokine specific biotinylated detection antibody added. Plates were again washed to remove unbound antibody, then cytokine bound biotinylated antibodies were incubated with avidin or streptavidin conjugated horseradish peroxidase. To visualize positive cytokine binding, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (BD OptEIA, BD Biosciences, San Jose, CA) was added to the plate, which changes color in the presence of horseradish peroxidase. The color change reaction was stopped with 2N sulfuric acid. The color change in each well of the plate was measured with a Spectra Max 340 plate reader (Molecular Devices, Sunnyvale, CA) at 450nm and background absorbance at 570nm was subtracted. For data analysis, a curve fit was applied to the standards and the sample concentrations extrapolated from the standard curve using four-parameter logistic software (SoftMax Pro, Sunnyvale, CA).

Luminex/Bead Based ELISA

For some experiments, cytokines were detected in BALF and cell culture supernatants using a Milliplex bead based ELISA assay (Millipore, Billerica, MA) according to the manufacturer's instructions. The cytokine standard mixture was reconstituted to 10,000pg/mL and diluted in a 6 point 5-fold dilution series (10,000 – 3.2pg/mL). Cytokine standards and samples were incubated with capture antibody conjugated fluorescent microbeads in a specially designed 96-well filter bottomed plate (Millipore). All incubations were done on a plate shaker to keep beads in solution. Vacuum suction was used to remove unbound proteins and wash buffer and beads incubated with cytokine specific biotinylated detection antibody. Plates were again washed to remove unbound antibody and cytokine bound biotinylated antibodies incubated with streptavidin-phycoerythrin (PE). Plates were washed and the mean fluorescence intensity of PE labeled cytokine bound beads was detected by flow cytometry with a Bio-Plex 200 system (Bio-Plex, R&D, Minneapolis, MN). For data analysis, a curve fit was applied to the standards and the sample concentrations extrapolated from the standard curve using five-parameter logistic software (Bio-Plex Manager, R&D, Minneapolis, MN).

RNA Isolation

Lung Tissue

Total RNA was extracted from the upper right lung lobe from individual mice using the Trizol method (Rio et al., 2010). Lung tissue was harvested, snap-frozen on dry ice, and stored at -80°C until isolation. Trizol (Invitrogen, Carlsbad, CA) was added to

Table 2-6.   **Enzyme linked immunosorbent assay (ELISA).**

Cytokine	Manufacturer	Catalog number	High standard	Low standard
CXCL2/MIP-2	R&D Systems	DuoSet DY452	1000 pg/mL	15.60 pg/mL
G-CSF	PeproTech	900-K103	2000 pg/mL	31.25 pg/mL
IL-6	BD Biosciences	OptEIA 555240	1000 pg/mL	15.60 pg/mL
IL-10	eBiosciences	Ready-Set-Go 88-7104	4000 pg/mL	31.25 pg/mL
IgM	Bethyl Laboratories Inc	E90-101	1000 ng/mL	15.60 ng/mL
TNFα	BD Biosciences	OptEIA 558534	1000 pg/mL	15.60 pg/mL

the frozen tissue and immediately homogenized using an Omni TH tissue homogenizer (OMNI International, Kennesaw, GA). The tissue homogenizer was washed with 75% ethanol and water between samples. The aqueous layer was separated using chloroform. RNA was precipitated with isopropanol, and RNA pellet washed with 75% ethanol twice. The RNA pellet was then dissolved in water and heated at 55°C for 10 minutes. Contaminating genomic DNA was removed by treatment with DNA-free (Ambion, Austin, TX) according to manufacturers' directions.

Cultured Cells

Culture media was removed completely and Trizol (Invitrogen) was added to the plate and allowed to incubate for 5 minutes. Trizol was then transferred to a microcentrifuge tube for RNA isolation. The aqueous layer was separated using chloroform. Glycogen was added to the aqueous RNA then both were precipitated with isopropanol. The RNA/glycogen pellet was washed with 75% ethanol twice. The RNA/glycogen pellet was then dissolved in water and heated at 55°C for 10 minutes. The amount of RNA recovered from cultured cells was small, and therefore the RNA was not DNAse treated.

RNA Quantification and Quality

Spectrophotometer

RNA concentration was determined using Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Piscataway, NJ). RNA was diluted 1:25 in nuclease free water and the absorbance at 260nm and 280nm was measured. The concentration of RNA (μg/mL) is equal to the absorbance at 260nm multiplied by the dilution factor (25) and 40μg/mL.

Electrophoresis

RNA quality was determined by running 2-3μg RNA in a 1% agarose gel. RNA was visualized with ethidium bromide under UV light (Alpha Image 2200 Multi-image light cabinet and alphaEase FC software, Alpha Innotech Corporation, San Leandro, CA). The presence of intact 18S and 28S ribosomal RNA bands was used as an indicator of high quality RNA.

RT-PCR

RNA (2μg) was reverse transcribed into cDNA with Promega Reverse Transcription System (Promega, Madison, WI). RNA was added to a master mix containing $MgCl_2$, buffer, nucleotides, random primers, ribonuclease inhibitor, and avian

myeloblastosis virus reverse transcriptase. To initiate reverse transcription, each RNA sample was incubated at room temperature for 15 minutes followed by 42°C for 30 minutes.

The cDNA (1-3μL) was added to a PCR master mix (Go Taq PCR core system I, Promega, Madison, WI) containing Taq polymerase, nucleotides, 15μM forward and reverse gene specific primers (**Table 2-7**), $MgCl_2$, in a reaction buffer. The PCR reaction was carried out in a PCR thermocycler (Mastercycler epgradient S, Eppindorf, Hamburg, Germany). Each reaction began with a denaturation step of 94°C followed by an annealing step at primer specific temperature (**Table 2-7**) and an elongation step at 72°C repeated for 35 cycles.

The difference in gene expression was gauged by running 15mL of the amplified RT-PCR product in a 1.8% agarose gel. RT-PCR product bands were visualized with ethidium bromide under UV light (Alpha Image 2200 Multi-image light cabinet and alphaEase FC software, Alpha Innotech Corporation, San Leandro, CA).

Real Time qRT-PCR

RNA (1μg) was reverse transcribed into cDNA with the Roche Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN) or Promega Reverse Transcription System (Promega, Madison, WI). RNA was added to a master mix containing buffer, nucleotides, random primers, ribonuclease inhibitor, and transcriptor reverse transcriptase. To initiate reverse transcription, each RNA sample was incubated at room temperature followed by 55ºC or 42ºC for 30 minutes then inactivated at 85°C for 5 minutes.

The cDNA (2-3μL) was added to an LC480 master mix (Roche Applied Science, Indianapolis, IN) or Kapa Probe Fast Universal (Kapa Biosystems, Woburn, MA) containing 5μM forward and reverse gene specific primers, and 10μM product specific probe. All primers and probes were chosen using the online software Universal Probe Library (**Table 2-8**). After each sample was loaded into a 96 well plate, the plate was spun down with a short spin and placed into a LightCycler 480 real time PCR thermal cycler (Roche Applied Science, Indianapolis, IN). Each real time reaction began with a denaturation step of 95°C, followed by an annealing step at 60°C, and an elongation step at 72°C repeated for 50 cycles. Data are normalized for HPRT and plotted as fold induction over unexposed control mice.

Histology

The left lobe of lung was removed and fixed with neutral buffered formalin and embedded in paraffin. Eight micron sections were cut and stained with hematoxylin and eosin (H&E) to analyze granuloma formation or Masson's trichrome to visualize fibrosis

Table 2-7.   Primers used in RT-PCR.

Primer name	Forward primer	Reverse primer	Product length	Annealing temp
Actin	GTG GGC CGC TCT AGG CAC CA	CGG TTG GCC TTA GGG TTC AGG GGG G	245nt	60ºC
CCL11/eotaxin	TTC TAT TCC TGC TGC TCA CGG	AGG GTG CAT CTG TTG TTG GTG	157nt	57ºC
IL-4	TCG GCA TTT TGA ACG AGG TC	GAA AAG CCC GAA AGA GTC TC	261nt	60ºC
TLR3	CTC ACC TCC ACA TCT TGA AT	CTG TTC AGG TTT TGA AAA GG	232nt	55ºC
TLR4	ACC TCT GCC TTC ACT ACA GA	AGG GAC TTC TCA ACC TTC TC	223nt	55ºC
TLR5	GAT CTT TCT GAA AAT GGC TG	CTG GTC AGG ATC TCT GAT GT	144nt	55ºC
TLR7	GTT CAA AAA CAA AGA GCC AC	AGG AAT GAA AGA TGC TGA AA	132nt	55ºC

Table 2-8.   Primers used in real time qRT-PCR.

Primer name	Forward primer	Reverse primer	Product length	Probe number
HPRT	TCC TCC TCA GAC CGC TTT T	CCT GGT TCA TCA TCG CTA ATC	90nt	95
CCL2/MCP-1	CAT CCA CGT GTT GGC TCA	GAT CAT CTT GCT GGT GAA TGA GT	76nt	62
CCL4/MIP-1β	GCC CTC TCT CTC CTC TTG CT	GAG GGT CAG AGC CCA TTG	73nt	1
CXCL1/KC	AGA CTC CAG CCA CAC TCC AA	TGA CAG CGC AGC TCA TTG	130nt	83
CXCL2/MIP-2	CCT GGT TCA GAA AAT CAT CCA	CTT CCG TTG AGG GAC AGC	93nt	63
IFNγ	GGA GGA ACT GGC AAA AGG AT	TTC AAG ACT TCA AAG AGT CTG AGG	85nt	21
IL-6	ATG GAT GCT ACC AAA CTG GAT	CCA GGT AGC TAT GGT ACT CCA GA	85nt	6
IL-10	CAG AGC CAC ATG CTC CTA GA	GTC CAG CTG GTC CTT TGT TT	78nt	41
IL-12p40	GAT TCA GAC TCC AGG GGA CA	TGG TTA GCT TCT GAG GAC ACA TC	75nt	27
IL-17A	TGT GAA GGT CAA CCT CAA AGT CT	GAG GGA TAT CTA TCA GGG TCT TCA T	132nt	50
IL-22	TTT CCT GAC CAA ACT CAG CA	CTG GAT GTT CTG GTC GTC AC	67nt	17
IL-23p19	TCC CTA CTA GGA CTC AGC AAC	TGG GCA TCT GTT GGG TCT	67nt	19
TNFα	TCT TCT CAT TCC TGC TTG TGG	GGT CTG GGC CAT AGA ACT GA	128nt	49

in the lung. Slides were digitized to 40x resolution (Scanscope XT, Aperio, Vista, CA) and analyzed using Spectrum Imagescope software (Aperio, v11.2).

To quantify blue stained collagen on Masson's trichrome stained slides, 8x1mm² square analysis areas were selected in Imagescope. The number of blue pixels was quantified using the positive pixel algorithm (v9.1) with a positive hue value of 0.65, a hue width of 0.2, and a color saturation threshold of 0.1. All other settings were left at default values. Positive and strong positive pixels were quantified in each analysis area. A representative image from the analysis area with the median number of positive and strong positive pixels was captured at 40x.

Statistics

Data were analyzed using one-way ANOVA with Tukey-Kramer post hoc test or two-way ANOVA with Bonferroni post hoc test using GraphPad Prism statistical software (GraphPad Software, San Diego, CA). Differences were considered significant at p values of less than 0.05. Results are expressed as mean ± S.D.

CHAPTER 3.   ACUTE PHASE[*]

Introduction

Acute hypersensitivity pneumonitis (HP) is characterized by neutrophilic alveolitis and inflammatory cytokine production. Neutrophils play an important role in the development of lung damage throughout the different stages of HP. They are the first cell population recruited into the lungs following SR exposure and they release numerous cytokines such as TNFα and IFNγ that contribute to the initiation of disease (Fournier et al., 1985; Nance et al., 2005). Our previous studies using the SR model of HP demonstrated that MyD88-dependent receptors are necessary for the initial production of pro-inflammatory cytokines, neutrophil chemokines and subsequently neutrophil recruitment following acute SR exposure (Nance et al., 2008). One of the MyD88-dependent TLRs involved in recognition of gram positive bacteria is TLR2, and our previous studies revealed that SR interacts with TLR2 and can activate the TLR2 signaling pathway that leads to NF-κB activation using a luciferase reporter assay in vitro. Mice deficient in TLR2 have a significant reduction in the neutrophil chemokine CXCL2/MIP-2 but not neutrophil recruitment. TLR9 has previously been shown to play an important role in the immune response to mycobacteria and fungus induced HP (Bhan et al., 2011; Daito et al., 2011). Therefore, we hypothesize TLRs 2 and 9 cooperate in SR induced chemokine production and neutrophil recruitment.

Toll-Like Receptors

Toll-like receptors (TLRs) are pattern recognition receptors that recognize conserved molecular patterns present in bacteria, fungi, protozoa, and viruses. TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the cell surface, whereas TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are present in endosomes. TLR2 forms heterodimers with TLR1 or TLR6 to recognize peptidoglycan and lipoproteins commonly found in gram positive bacteria. TLR4 recognizes LPS found in gram negative bacteria. TLR5 recognizes flagellin. In the endosome, TLR3 recognizes double stranded RNA found in viruses. TLR7 and TLR8 recognize viral and bacterial single stranded RNA. TLR9 recognizes the CpG motif found in bacterial and viral DNA. TLR11 and TLR12, present in mice but not humans, have been shown to recognize profilin, a protein found in the parasite T. gondii (Koblansky et al., 2013; Plattner et al., 2008; Yarovinsky et al., 2005). In addition TLR11 has also been shown to recognize bacterial flagellin (Mathur et

al., 2012). TLR13, also present in mice, has recently been shown to recognize bacterial 23S rRNA (Hidmark et al., 2012; Li and Chen, 2012). TLRs can be grouped according to whether the receptor signals through the adaptor molecule MyD88 or is MyD88-independent. MyD88-dependent receptors include TLR2/1, TLR2/6, TLR5, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13, whereas TLR3 is MyD88-independent. TLR4 can signal in a MyD88-dependent and MyD88-independent manner. Signaling through the MyD88 adaptor protein results in activation of mitogen-activated protein kinase (MAPK), NF-κB, and interferon regulatory factors (IRF) for the induction of inflammatory cytokines and type I interferons (reviewed in Kawai and Akira, 2010).

In the lung, TLR2 and TLR9 are expressed by dendritic cells as well as bronchial and airway epithelial cells and can be upregulated in response to infection (reviewed in Rohmann et al., 2011). Alveolar macrophages express TLR2 but have low levels of TLR9 (Suzuki et al., 2005). TLR7 is expressed in plasmacytoid dendritic cells (pDCs); however, recent reports have demonstrated that TLR7 can be expressed by other cells during chronic inflammation (Cherfils-Vicini et al., 2010; Hammadi et al., 2008; Nicholas et al., 2011; Nicholas et al., 2010). It is not clear what the role of TLR7 expression in these non-native cells is in regards to inflammation and host defense. TLR7 is widely recognized to be important in viral pathogen recognition; however, there have been reports that TLR7 may also recognize bacterial RNA and play a role in host defense against bacteria (Inoue et al., 2011; Mancuso et al., 2009; Rad et al., 2009). Sakai et al demonstrated that exposure to non-typeable *Haemophilus influenzae* upregulates TLR7 expression in the human airway epithelial cells and mouse whole lung in a TLR2 dependent manner (Sakai et al., 2007). Although it is becoming clear that TLR7 may play a role in the immune response to bacteria there is very little information on that role and our studies will begin to address this important question.

Leukocyte Recruitment

Neutrophils

Neutrophil recruitment from the circulation is a multistep process (reviewed in Kolaczkowska and Kubes, 2013). First, upregulation on selectins on endothelial cells initiates leukocyte rolling. P-selectin and E-selectin are upregulated on endothelial cells in response to inflammatory stimuli, cytokines, or PRRs. P-selectin and E-selectin bind to glycoproteins on leukocytes such as PSGL-1. Second, integrins bind to cell adhesion molecules. Integrins consist of two chains, an alpha chain and a beta chain. On neutrophils, the beta integrin β2/CD18 can pair with the alpha integrin αL/CD11a and αM/CD11b. CD11a/CD18 and CD11b/CD18 plays an important role in neutrophil recruitment by binding to the intracellular adhesion molecule (ICAM)-1, ICAM-2, and ICAM-3 expressed on endothelial cells to induce firm binding. Finally, neutrophils spread and cross the endothelial barrier in a process known as diapedesis.

Neutrophils reach the site of infection by following a chemokine gradient. The chemokines form a gradient and attract leukocytes to sites of inflammation through the binding of chemokine receptors. Neutrophils express many chemokine receptors including CXCR2, which binds to the chemokines CXCL1/KC, CXCL2/MIP-2, and CXCL5/ENA-78. Previous studies revealed the importance of TLR2 in neutrophil recruitment in HP as mice deficient in TLR2 had significantly reduced CXCL2/MIP-2 production accompanied by a partial reduction in neutrophil recruitment following one SR exposure (Nance et al., 2008). However, since TLR2 deficiency only affected CXCL2 production whereas MyD88$^{-/-}$ mice had a complete absence of both proinflammatory cytokine production and neutrophil recruitment following SR exposure, we will investigate the cooperativity of TLRs 2 and 9 in neutrophil recruitment and the production of neutrophil chemokines in HP. We hypothesize TLR2/9$^{-/-}$ mice will have a significant reduction in neutrophil recruitment and neutrophil chemokine production after one exposure to SR.

Monocytes

Monocytes are also recruited to sites of inflammation in a manner similar to neutrophils; however monocytes express different chemokine receptors, and therefore are attracted by alternative chemokine gradients (reviewed in Shi and Pamer, 2011). Monocytes express the chemokine receptors CCR1, CCR2, and CCR5, which overlap in ligand specificity. CCR1 binds to CCL3/MIP-1α, CCL5/RANTES, and CCL7/MCP-3. CCR2 binds CCL2/MCP-1, CCL8/MCP-2, and CCL7/MCP-3. CCR5 binds CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL11/eotaxin (reviewed in Charo and Ransohoff, 2006). After a single dose of SR, mice have shown increases in the expression of CCL3/MIP-1α, and CCL2/MCP-1 (Nance et al., 2008; Schuyler et al., 2000), and SR stimulated alveolar macrophages produced CCL3/MIP-1α in a dose dependent manner (Schuyler et al., 2000). In the present study, we will investigate the contribution of TLRs 2 and 9 to the production of monocyte chemokines CCL2/MCP-1, CCL3/MIP-1α, and CCL4/MIP-1β in response to SR exposure.

Inflammatory Cytokines

Inflammatory cytokines TNFα, IL-6, IL-1α, and IL-12p40 (the common chain for IL-12 and IL-23) are detected in the BALF within 2 hours of SR exposure and remain elevated up to 72 hours later (Schuyler et al., 2000). Alveolar macrophages are one of the first cell types to encounter SR and are a source of inflammatory cytokines. Macrophages isolated from mice exposed to SR spontaneously release IL-1, IL-6, and TNFα, and in vitro stimulation with SR induces greater IL-1, IL-6, and TNFα production (Denis et al., 1992). Previous studies in MyD88$^{-/-}$ mice revealed IL-1β and TNFα expression was dependent on MyD88; however TLR2$^{-/-}$ mice did not have a significant reduction in TNFα in the BALF at 6hrs and 24hrs compared to WT (Nance et al., 2008). Therefore, we hypothesize that in addition to TLR2, TLR9 also contributes to inflammatory cytokine production in SR exposed mice.

In the following studies, the objective is to analyze neutrophil recruitment, inflammatory cytokine, and chemokine production after a single exposure to SR. We hypothesize the combined loss of TLRs 2 and 9 will result in reduced neutrophil recruitment due to reduced neutrophil and monocyte chemokine production, and reduced inflammatory cytokine production in vitro and in vivo.

Results

TLRs 2 and 9 Contribute to Neutrophil Chemokine Production and Recruitment into the Airways Following One Exposure to SR In Vivo

Our previous studies demonstrated that neutrophil recruitment was dependent on MyD88 signaling pathways, and TLR2 was one of the MyD88-dependent PRRs that contributed to CXCL2 production following a single SR exposure (Nance et al., 2008). To examine the combined role of TLRs 2 and 9 in neutrophil recruitment following in vivo exposure to SR, we exposed WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice to SR and measured the recruitment of neutrophils into the airways (**Figure 3-1**). Analysis of the BAL from individual mice demonstrated that following a single exposure to SR, there is an increase in alveolitis in the WT mice, which is significantly reduced in the TLR2/9$^{-/-}$ mice. Although the TLR2$^{-/-}$ mice and TLR9$^{-/-}$ mice had reduced alveolitis compared to the WT mice, the differences were not statistically significant (**Figure 3-1A**). At this time point the alveolitis is almost entirely neutrophilic. The reduction in alveolitis in the TLR2/9$^{-/-}$ mice correlated with the decrease in neutrophil recruitment that was observed compared to WT mice (**Figure 3-1B**). Natural killer (NK) cells and γδ T cells are lymphocytes that can contribute to the innate immune response. A small number of NK cells were detected in the BAL of SR exposed mice, but there was no significant difference between WT and KO groups (**Figure 3-1B**). γδ T cells were not detected at this time point. Therefore, both TLRs 2 and 9 appear to contribute to neutrophil recruitment following a single SR exposure since a significant reduction in neutrophils was only observed in the TLR2/9$^{-/-}$ mice.

To analyze the expression of neutrophil chemokines, lung tissue from WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice was harvested and the expression of neutrophil chemokines CXCL1 and CXCL2 was measured. Quantitative RT-PCR analysis revealed that there is a significant decrease in CXCL2 mRNA in the TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice; however, there was no significant decrease in CXCL1 mRNA in any of the mutant mice (**Figure 3-1C**). The decrease in CXCL2 mRNA was reflected in the BALF; TLR2$^{-/-}$, TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice had a significant decrease in CXCL2 in the BALF following SR exposure (**Figure 3-2**). Although there was no difference in expression of CXCL1, the concentration of CXCL1 in the BALF was also significantly reduced in TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice. The decrease in neutrophil recruitment was likely due to a decrease in chemokine production and not an intrinsic defect in TLR2/9$^{-/-}$ neutrophil migration since neutrophils in the TLR2/9$^{-/-}$ mice migrated into the
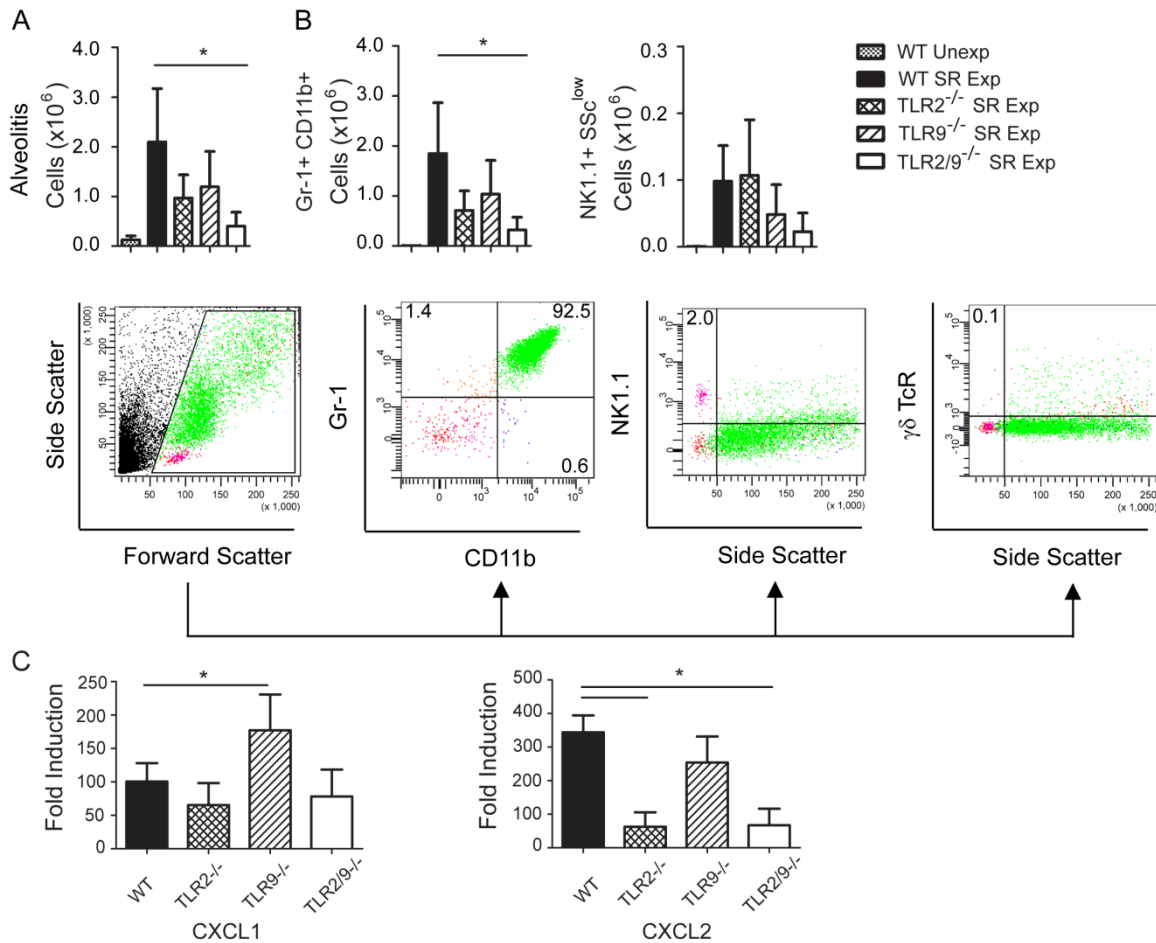
Figure 3-1. Neutrophil recruitment and chemokine expression after one exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR and analyzed 6 hours after one exposure. (A) BAL was performed and alveolitis determined using trypan blue dye exclusion. (B) Cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, γδTcR, and NK1.1 and analyzed by flow cytometry to determine the number of neutrophils (CD11b^{+} / Gr-1^{high}), and natural killer cells (NK1.1^{+} / SSc^{low}) in each group using the depicted gating strategy (Abdelsamed et al., 2011). γδ T cells were not detected. (C) Expression of mRNA for cytokines was determined by qRT-PCR on RNA isolated from individual lung lobes (n = 3 - 5 mice per group) and is expressed as fold induction over WT unexposed mice. The data represent mean ± SD of duplicate samples and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT / SR exposed mice).
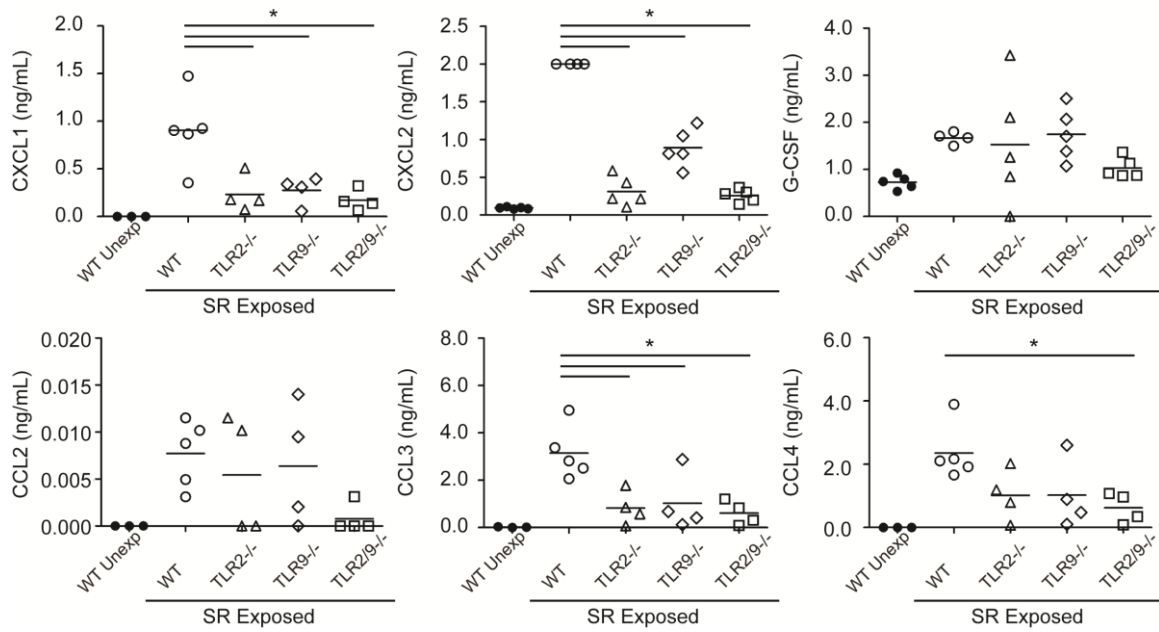
Figure 3-2.   Chemokine production after one exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR and analyzed 6 hours after one exposure. The cell-free BAL fluid was analyzed for CXCL2, G-CSF by ELISA and CXCL1, CCL3 and CCL4 by Milliplex bead based ELISA (limit of detection 0.0032ng/mL). Data represent the mean ± SD (n = 3-5 per group) significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

peritoneum in response to an IP injection of casein (**Figure 3-3**). We also observed a decrease in the chemokines CCL3 and CCL4 in the TLR2/9$^{-/-}$ mice compared to the WT mice suggesting that TLRs 2 and 9 may also be important for recruitment of monocytes and lymphocytes following a single SR exposure. We did not find a significant decrease in G-CSF in the BALF of exposed mice (**Figure 3-2**). This suggests that lack of the neutrophil growth factor was not the cause of decreased neutrophil recruitment in the mice.

To confirm the reduction in neutrophil recruitment in TLR2/9$^{-/-}$ mice was not due to increased neutrophil apoptosis, we exposed mice to SR once per day for 3 consecutive days and analyzed the percentage of neutrophils expressing the apoptosis marker annexin V by flow cytometry. Neutrophils were identified as CD11b^{+} / Gr-1^{high} expressing cells. The results show SR exposed TLR2/9$^{-/-}$ mice had a decreased percentage of neutrophils in the BAL cells compared to WT exposed mice (**Figure 3-4A**); however, we did not see an increase in Annexin V staining on neutrophils from TLR2/9$^{-/-}$ exposed mice compared to WT exposed mice (**Figure 3-4B**). The results suggest that increased apoptosis is not playing a role in the decrease in neutrophil frequency in the BALF of these mice. Altogether, these results suggest that following a single SR exposure, neutrophil chemokine production and recruitment is largely dependent on TLR2 and any role for TLR9 becomes apparent only in the absence of TLR2.

TLR2/9$^{-/-}$ Mice Have a Significant Reduction in Inflammatory Cytokine Expression In Vivo

The expression of cytokines was examined in the lung tissue of mice exposed once to SR. The expression of inflammatory cytokines TNFα, IL-6 and the anti-inflammatory cytokine IL-10 was increased in mice exposed to SR over WT unexposed mice. There was no significant difference in the expression of TNFα and IL-6 in TLR2$^{-/-}$, TLR9$^{-/-}$, or TLR2/9$^{-/-}$ mice compared to WT exposed mice. IL-10 expression was significantly decreased in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to WT exposed mice (**Figure 3-5**). The concentration of these cytokines in the BALF of mice exposed once to SR was measured. Although there was no significant difference in TNFα and IL-6 expression in the lung tissue, the BALF from TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice had a significant reduction in TNFα and TLR2/9$^{-/-}$ mice had a significant reduction in IL-6 compared to WT exposed mice (**Figure 3-6**). IL-10 was not detected in the BALF at this time point. Altogether, the results suggest TLR2 is the dominant receptor responsible for the induction of TNFα and IL-6 production, but TLR9 also contributes to the production of IL-6 after a single exposure to SR.

IL-12p40 is the common chain for heterodimeric cytokines IL-12p70 (IL-12p35 and IL-12p40) and IL-23 (IL-23p19 and IL-12p40). IL-12p70 and IL-23 are important for the polarization and proliferation of Th1 and Th17 cells respectively. IL-12p40 and IL-23p19 expression was significantly decreased in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to WT exposed mice (**Figure 3-5**), suggesting TLR2 is important for IL-12p40
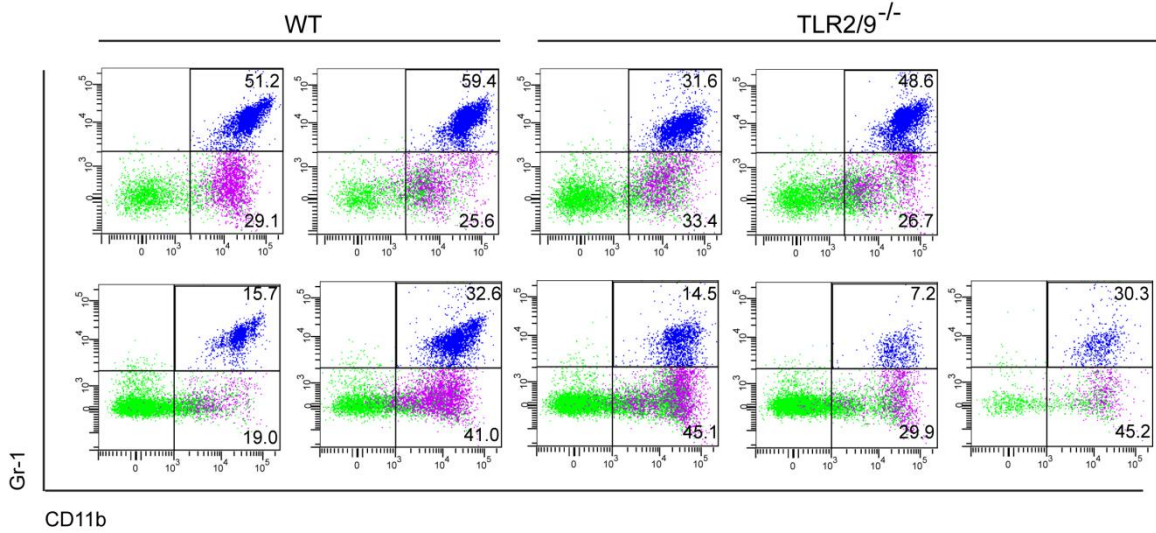
Figure 3-3.   Neutrophils are recruited to the peritoneum in response to casein injections in WT and TLR2/9$^{-/-}$ mice.

WT and TLR2/9$^{-/-}$ mice were injected IP with 1mL of a 9% casein solution, followed by a second injection 24 hours later then sacrificed 3 hours after the last injection. Peritoneal lavage was performed, and cells recovered from the peritoneal lavage were incubated with antibody to CD11b and Gr-1 and analyzed by flow cytometry to determine the percentage of neutrophils (CD11b^{+}/Gr-1^{high}) in each group. Each scatter plot represents a separate mouse (n = 4-5 per group).
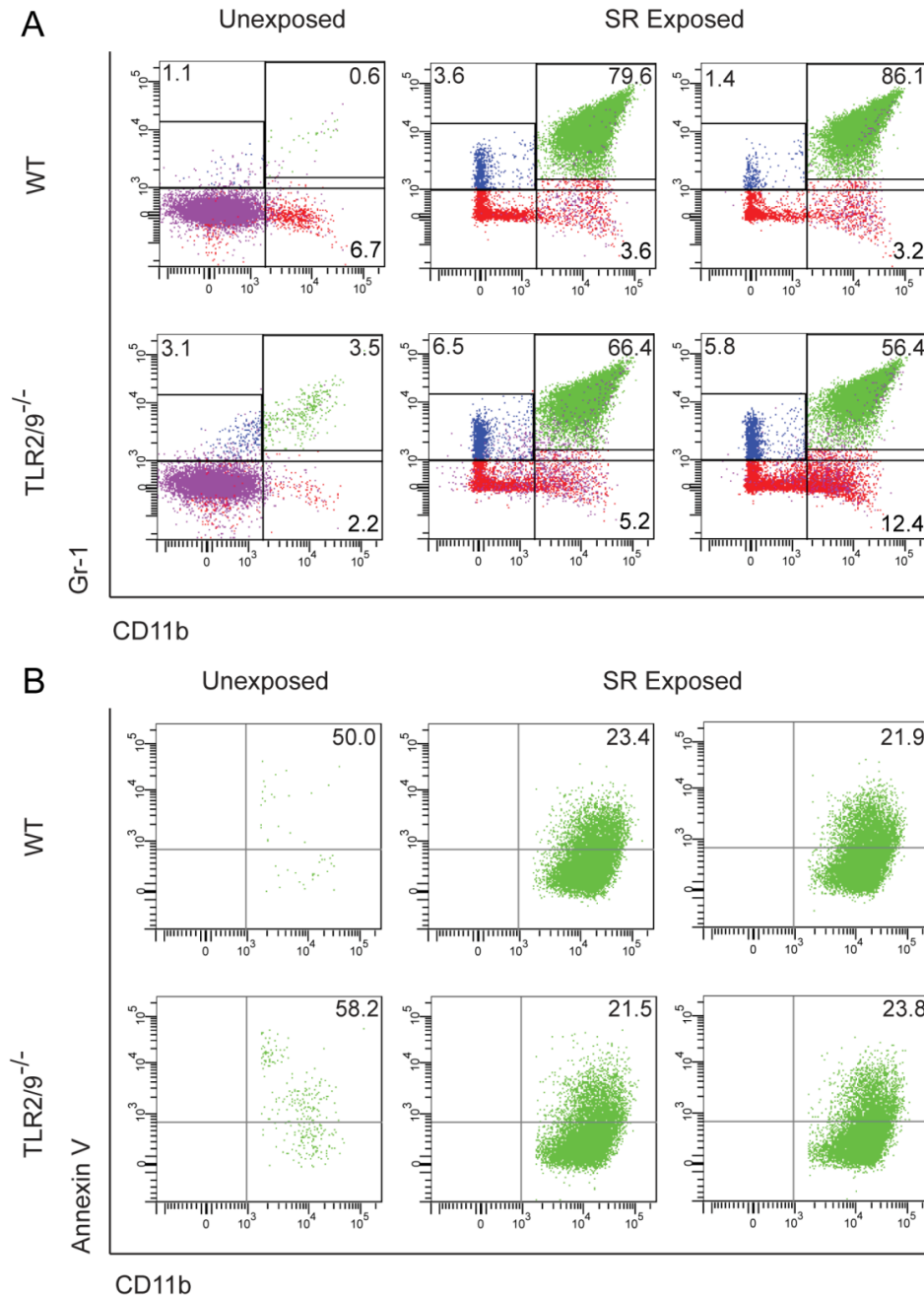
Figure 3-4.   The decrease in neutrophil recruitment in TLR2/9$^{-/-}$ mice is not due to increased neutrophil apoptosis.

WT and TLR2/9$^{-/-}$ mice were exposed to SR three times and sacrificed 12 hours after the last exposure. BAL was performed and the cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, and annexin V and analyzed by flow cytometry. (A) Cells were gated on neutrophils (CD11b^{+}/Gr-1^{high}) (B) The percentage of neutrophils (CD11b^{+} / Gr-1^{high}) positive for annexin V staining in WT and TLR2/9$^{-/-}$ mice. (n = 1-2 per group)
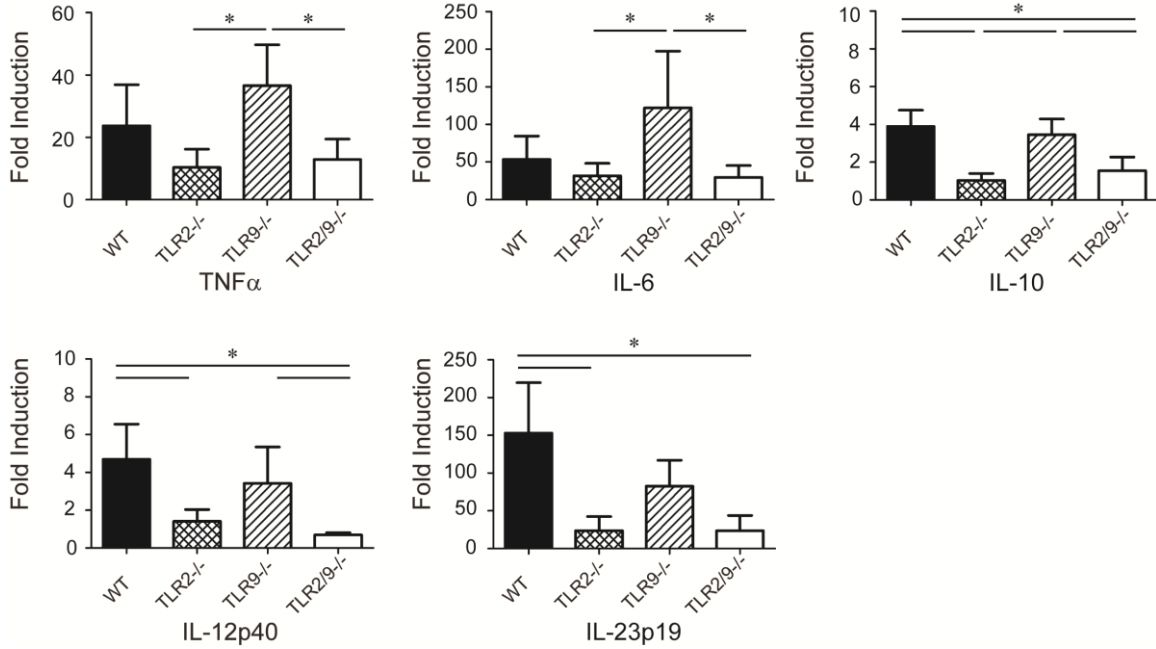
Figure 3-5.   Inflammatory cytokine expression after one exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ were exposed to SR once and sacrificed 6 hours later. RNA was isolated from lung tissue and reverse transcribed to cDNA for real time PCR. The expression of TNFα, IL-6, IL-10, IL-12p40, and IL-23p19 was normalized to HPRT. Data is expressed as fold induction over WT unexposed mice ± SD. (n = 5 mice per group) and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$).
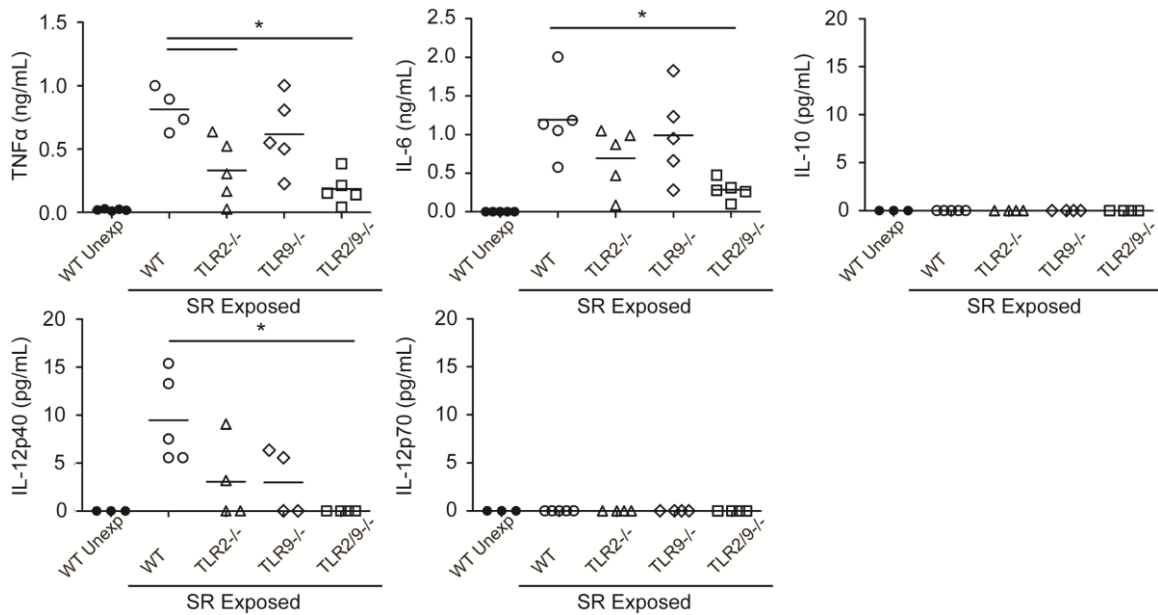
Figure 3-6. Inflammatory cytokine production after one exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR and analyzed 6 h after one exposure. The cell-free BAL fluid was analyzed for TNFα and IL-6 by ELISA and IL-10, IL-12p40, and IL-12p70 by Milliplex bead based ELISA (limit of detection 3.2pg/mL). Data represent the mean ± SD (n = 3-5 per group) significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

and IL-23 expression. The concentration of IL-12p40 was significantly reduced in the BALF of TLR2/9$^{-/-}$ mice (**Figure 3-6**); however, IL-12p70 was not detected, suggesting the significant reduction was in the production of IL-23. The results suggest TLR2 contributes to the expression of IL-12p40, and IL-23p19 after a single exposure to SR and that loss of TLR2 may result in reduced Th17 responses during the granulomatous phase.

TLR2/9-Dependent and MyD88-Dependent Chemokine and Cytokine Production in SR Stimulated Splenic Adherent Cells In Vitro

Although neutrophil recruitment and inflammatory cytokine production was significantly reduced in TLR2/9$^{-/-}$ mice compared to WT exposed mice, the reduction was not to the level previously shown in MyD88$^{-/-}$ mice (Nance et al., 2008). To investigate MyD88-dependent cytokine production in response to SR, splenic adherent cells from WT, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$ and MyD88$^{-/-}$ mice were stimulated with SR and cytokine production measured by bead based ELISA.

Neutrophil chemokines CXCL1, CXCL2, and the neutrophil growth factor G-CSF were partially dependent on TLRs 2 and 9 as the production of these proteins were significantly reduced in TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$ cells, but completely dependent on MyD88 as protein expression was reduced to WT unexposed levels (**Figure 3-7**). The results indicate an additional MyD88-dependent receptor is involved in the induction of neutrophil chemokines and growth factors. GM-CSF production was not significantly induced by SR stimulation of splenic adherent cells compared to unexposed cells. The lymphocytic chemokines CXCL9 and CXCL10 were significantly increased in TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ cells compared to WT cells, suggesting signaling through TLR9 is important in inhibiting expression of CXCL9 and CXCL10 in splenic adherent cells (**Figure 3-7**).

The induction of MyD88-dependent cytokines was also examined in splenic adherent cells. CXCL9 and CXCL10 are induced in response to IFNγ (Farber, 1997), and TLR9$^{-/-}$ and TLR2/9$^{-/-}$ cells had a significant increase in the production of IFNγ over WT SR stimulated cells. However, IFNγ was not significantly increased MyD88$^{-/-}$ cells (**Figure 3-8**), suggesting TLR9 stimulation suppresses the production of IFNγ, but an additional MyD88-dependent receptor is responsible for IFNγ induction. The production of IL-1β and IL-17 after SR stimulation was significantly increased in TLR9$^{-/-}$ cells compared to WT cells (**Figure 3-8**). TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ cells had significantly increased production of IL-1β and IL-17 over MyD88$^{-/-}$ cells suggesting a MyD88-dependent receptor is responsible for the induction of IL-1β and IL-17 production. SR stimulation significantly increased production of IL-12p70 in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice (**Figure 3-8**), which suggests TLR2 stimulation suppresses IL-12p70 production. SR stimulation of MyD88$^{-/-}$ cells did not induce IL-12p70 expression suggesting a MyD88-dependent receptor is necessary for the induction IL-12p70 protein expression.
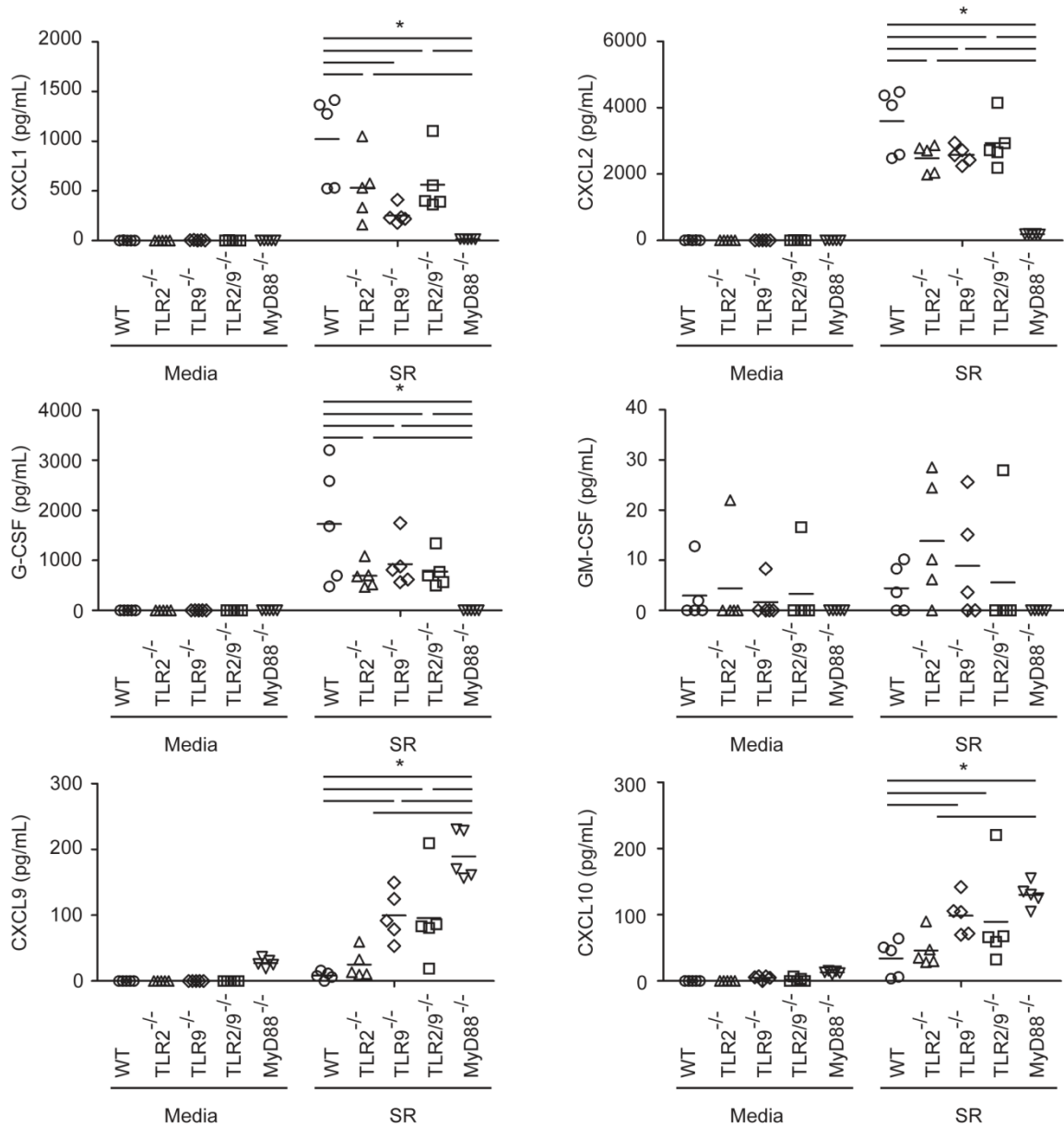
Figure 3-7.   Chemokine production in splenic adherent cells stimulated with SR in vitro.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ splenic adherent cells were plated at 5×10^{5} cells per well and stimulated with media or SR (0.05mg/mL) for 24 hours. Cell culture supernatants were collected and the expression of CXCL1, CXCL2, CXCL9, CXCL10, G-CSF, and GM-CSF was determined by Milliplex bead based ELISA (limit of detection 3.2pg/mL). Data represent the mean ± SD (n = 5 wells per group) and significance was determined using two-way ANOVA with Bonferroni post-hoc test (SR stimulation vs. genotype) (*p < 0.05 compared to WT SR exposed cells or MyD88$^{-/-}$ SR exposed cells).

Figure 3-8.   Cytokine production in splenic adherent cells stimulated with SR in vitro.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ splenic adherent cells were stimulated with media or SR (0.05mg/mL) for 24 hours. Cell culture supernatants were collected and the expression of TNFα, IL-6, IL-1β, IL-10, IL-12p70, IFNγ, and IL-17 was determined by Milliplex bead based ELISA. Data represent the mean ± SD (n = 5 wells per group) and significance was determined using two-way ANOVA with Bonferroni post-hoc test (SR stimulation vs. genotype) (*$p < 0.05$ compared to WT SR exposed cells or MyD88$^{-/-}$ SR exposed cells).
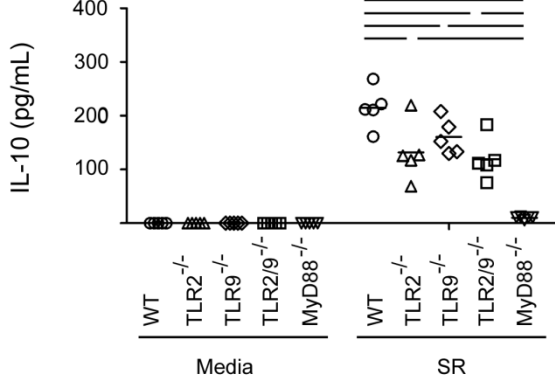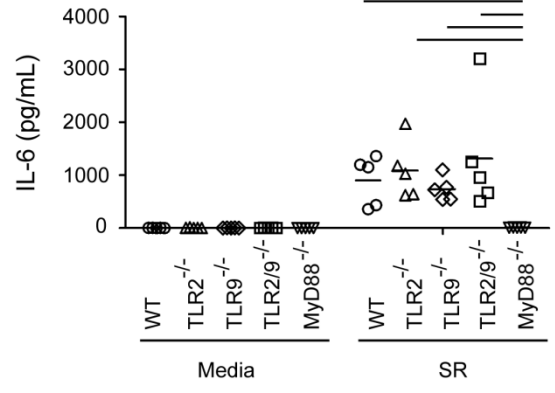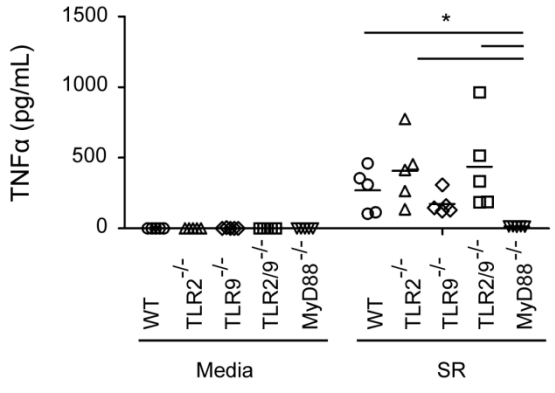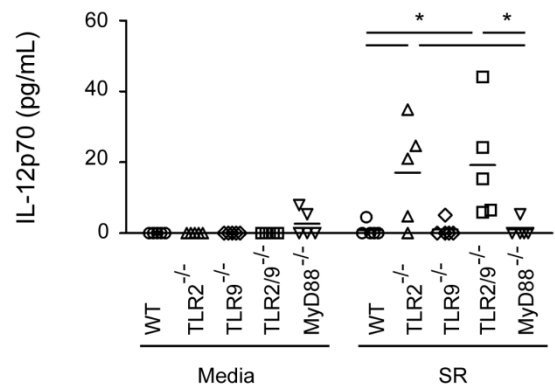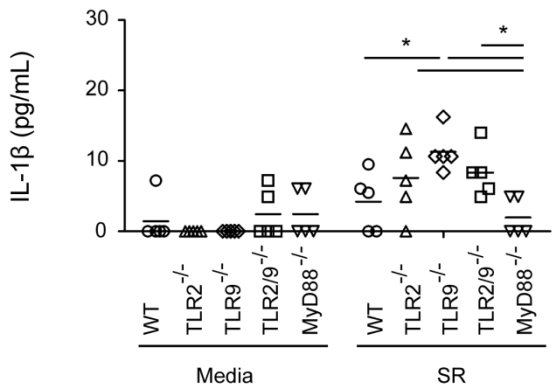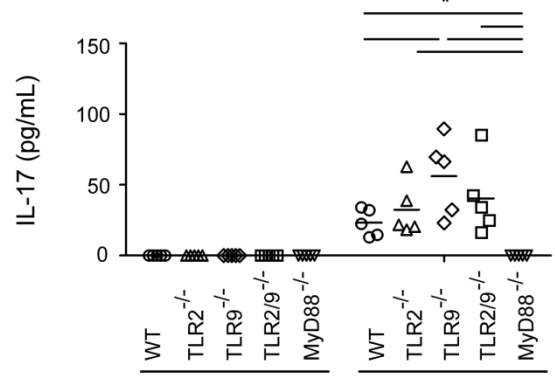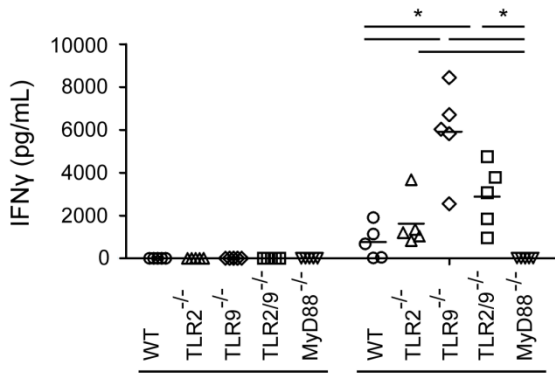
The inflammatory cytokines TNFα and IL-6 are induced in response to SR stimulation in WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ cells, but significantly reduced in MyD88$^{-/-}$ cells (**Figure 3-8**), suggesting a MyD88-dependent receptor other than TLR2 and TLR9 can induce TNFα and IL-6 expression in splenic adherent cells. The anti-inflammatory cytokine IL-10 is significantly reduced in SKO, and TLR2/9$^{-/-}$ cells (**Figure 3-8**), suggesting IL-10 expression is partially dependent on TLR2 and TLR9. However, MyD88$^{-/-}$ cells have significantly less IL-10 than WT and KO cells suggesting another MyD88-dependent receptor is responsible for IL-10 expression in response to SR. Altogether, the results suggest there is an additional MyD88-dependent receptor involved in SR recognition leading to the production of CXCL1, CXCL2, G-CSF, IFNγ, IL-17, IL-1β, IL-12p70, TNFα, IL-6, and IL-10 in WT and TLR2/9$^{-/-}$ splenic adherent cells.

TLR7 in SR Induced Cytokine Production In Vitro

MyD88-dependent receptors include the IL-1/IL-18R and TLRs. To investigate whether the additional MyD88-dependent receptor involved in the recognition of SR was another TLR, we looked at TLR expression in the lung tissue after 3 weeks exposure to SR. We found increased expression of TLR7, a MyD88-dependent receptor that recognizes single stranded RNA (**Figure 3-9**). There was no increase in the expression of TLR3, TLR4, or TLR5 in the lungs after 3 weeks SR exposure, suggesting TLR3, TLR4, and TLR5 are not required for SR recognition.

To confirm that SR RNA is able to induce cytokine production, we stimulated WT and TLR2/9$^{-/-}$ spleen cells with SR RNA complexed with DOTAP, a liposomal transfection agent (**Figure 3-10A**). SR RNA was able to stimulate TNFα production in WT and TLR2/9$^{-/-}$ spleen cells in a dose dependent manner. Non-MyD88-dependent cytoplasmic PRRs, such as RIG-I, can recognize RNA. To verify SR RNA stimulates spleen cells through a MyD88-dependent receptor, WT, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ spleen cells were stimulated with SR RNA and IL-10 production measured (**Figure 3-10B**). SR RNA stimulated IL-10 production in WT and TLR2/9$^{-/-}$ spleen cells. There was a significant reduction in IL-10 in MyD88$^{-/-}$ spleen cells stimulated with SR RNA compared to WT and TLR2/9$^{-/-}$ cells. The results suggest SR RNA is able to stimulate IL-10 production through a MyD88-dependent receptor such as TLR7.

TLR7 is present in endosomes and along with TLRs 3 and 9, and requires endosomal acidification for proteolytic cleavage of the receptor for activation (Ewald et al., 2008; Qi et al., 2012). Bafilomycin is an inhibitor of the vacuolar type H$^+$ ATPase, which inhibits endosomal acidification (Yoshimori et al., 1991); therefore, we hypothesized if an endosomal TLR was responsible for cytokine production in TLR2/9$^{-/-}$ cells, then cytokine production would be blocked by bafilomycin. We stimulated spleen cells from WT and TLR2/9$^{-/-}$ mice with SR in the presence or absence of bafilomycin (**Figure 3-11**). The production of IL-6 and TNFα was significantly increased in WT SR stimulated cells compared to unexposed cells. The presence of bafilomycin did not
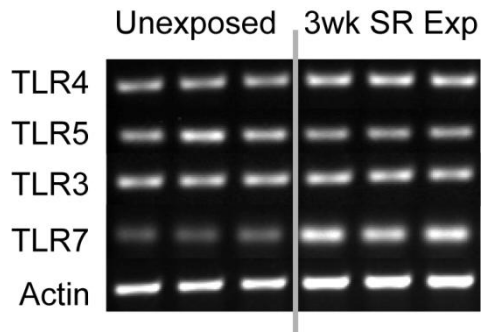
Figure 3-9.   TLR7 expression is increased in mice exposed to SR for 3 weeks.

C57Bl/6 mice were exposed to SR 3 times/week for 3 weeks and sacrificed 12 hours after the last exposure. Lung tissue was harvested and RNA isolated for reverse transcription and PCR. TLR4, TLR5, TLR3, and TLR7 expression was measured by RT-PCR. Actin expression was used as a housekeeping gene control.
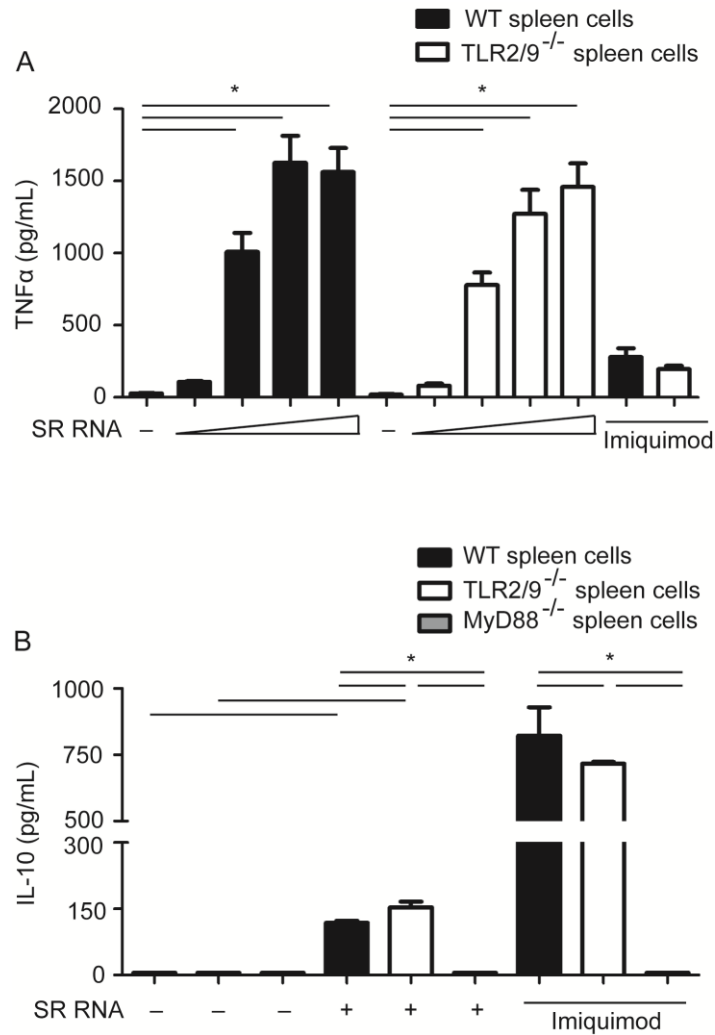
Figure 3-10. SR RNA stimulates cytokine expression in spleen cells in vitro.

(A) WT and TLR2/9$^{-/-}$ spleen cells were stimulated with SR RNA complexed with DOTAP (0.01, 0.1, 1.0, and 10µg/mL). Imiquimod (0.25µg/mL), a synthetic TLR7 agonist, was used as a positive control. Cell culture supernatants were collected and the expression of TNFα measured by ELISA. Data represent the mean ± SD (n = 4 wells per group) significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05). (B) WT, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ spleen cells were stimulated with SR RNA complexed with DOTAP (1µg/mL). Imiquimod (0.25µg/mL), a synthetic TLR7 agonist, was used as a positive control. Data represent the mean ± SD (n = 4 wells per group) and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).
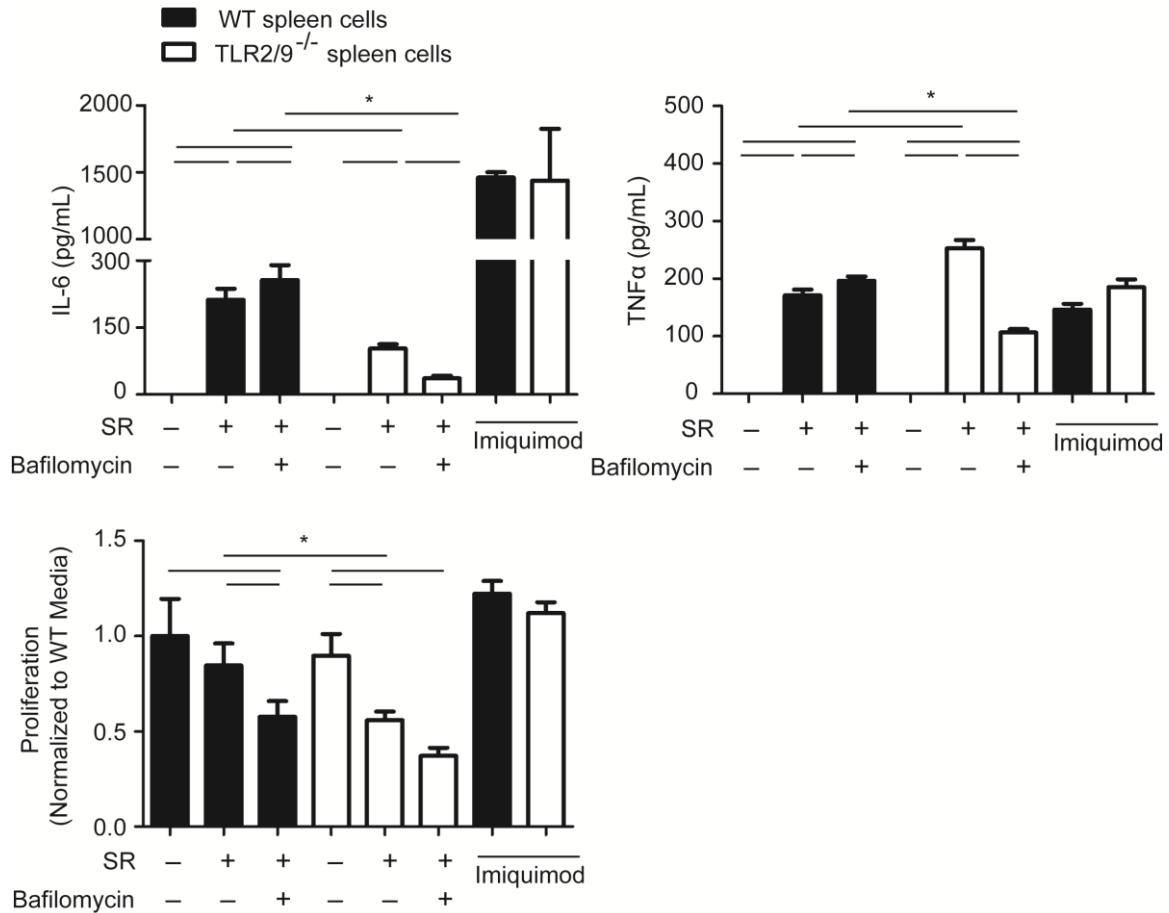
Figure 3-11. Bafilomycin inhibits IL-6 and TNFα production in TLR2/9$^{-/-}$ spleen cells stimulated with SR in vitro.

WT and TLR2/9$^{-/-}$ spleen cells were stimulated with media or SR (0.025mg/mL) in the presence of absence of bafilomycin (0.1μM), an inhibitor of endosomal acidification, for 24 hours. Imiquimod (0.25μg/mL), a synthetic TLR7 agonist, was used as a positive control. Cell culture supernatants were collected and the expression of TNFα and IL-6 measured by ELISA. Bafilomycin toxicity was assessed by Promega MTS cell proliferation assay. Data represent the mean ± SD (n = 4 wells per group) and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p <$ 0.05).

significantly decrease IL-6 or TNFα production in WT cells. The production of IL-6 was significantly decreased, and the production of TNFα was significantly increased in TLR2/9$^{-/-}$ cells stimulated with SR compared to WT SR stimulated cells. The addition of bafilomycin to SR stimulated TLR2/9$^{-/-}$ spleen cells significantly decreased the production of IL-6 and TNFα compared to SR alone. The reduction in TNFα and IL-6 production was not due to bafilomycin toxicity as there was no significant difference in proliferation between SR and SR + bafilomycin stimulated TLR2/9$^{-/-}$ spleen cells. Altogether, the results suggest an endosomal TLR contributes to IL-6 and TNFα production in SR stimulated spleen cells.

Macrophages are one of the first cell types in the lung to encounter SR upon exposure. To investigate SR stimulation of TNFα and IL-6 production in macrophages, BMDMs were differentiated from bone marrow cells for 7 days in the presence of LCCM. RNA was isolated from WT and TLR2/9$^{-/-}$ BMDMs for RT-PCR to verify BMDMs express TLR7 (**Figure 3-12A**). WT BMDMs stimulated with SR express IL-6 and TNFα, but there was no significant decrease in expression in the presence of bafilomycin (**Figure 3-12B**). TLR2/9$^{-/-}$ SR stimulated BMDMs express significantly less IL-6 and TNFα than WT SR stimulated BMDMs, but the addition of bafilomycin did not significantly decrease IL-6 and TNFα production compared to TLR2/9$^{-/-}$ SR stimulated BMDMs. Imiquimod stimulated IL-6 and TNFα production in WT and TLR2/9$^{-/-}$ BMDMs, suggesting TLR7 is functional in these cells. Bafilomycin was able to significantly reduce the production of IL-6 and TNFα in imiquimod stimulated cells, suggesting the concentration of bafilomycin used in the experiment was able to suppress TLR7 induced cytokine production. Altogether, the results suggest TNFα and IL-6 production in TLR2/9$^{-/-}$ BMDMs is not induced by an endosomal TLR, such as TLR7.

Discussion

SR is a strong inducer of neutrophil chemokines and neutrophils are rapidly recruited to the lungs after a single exposure to SR. The recruitment of neutrophils to the lungs is dependent on TLRs 2 and 9 as evidenced by the significant reduction in neutrophils recruited to the lungs of TLR2/9$^{-/-}$ mice. The reduced neutrophil recruitment can partially be explained by reduced neutrophil chemokine production in TLR2/9$^{-/-}$ mice compared to WT; however, neutrophil chemokines were also significantly reduced in TLR2$^{-/-}$, and TLR9$^{-/-}$ mice compared to WT exposed mice. Inflammatory cytokines also play a role in neutrophil recruitment through the upregulation of selectins and cell adhesion molecules on endothelial cells (Wagner and Roth, 2000). TNFα was significantly reduced in the BALF of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice, and IL-6 was significantly reduced in TLR2/9$^{-/-}$ mice compared to WT exposed mice. SR stimulation also induces the production of monocyte/lymphocyte chemokines CCL3/MIP-1α and CCL4/MIP-1β. CCL3 production was significantly reduced in all KO mice, whereas CCL4 was only significantly reduced in TLR2/9$^{-/-}$ mice suggesting TLRs 2 and 9 both contribute to the production of CCL3 and CCL4. Altogether the results suggest SR stimulation through TLRs 2 and 9 is important for the recruitment of neutrophils in HP,
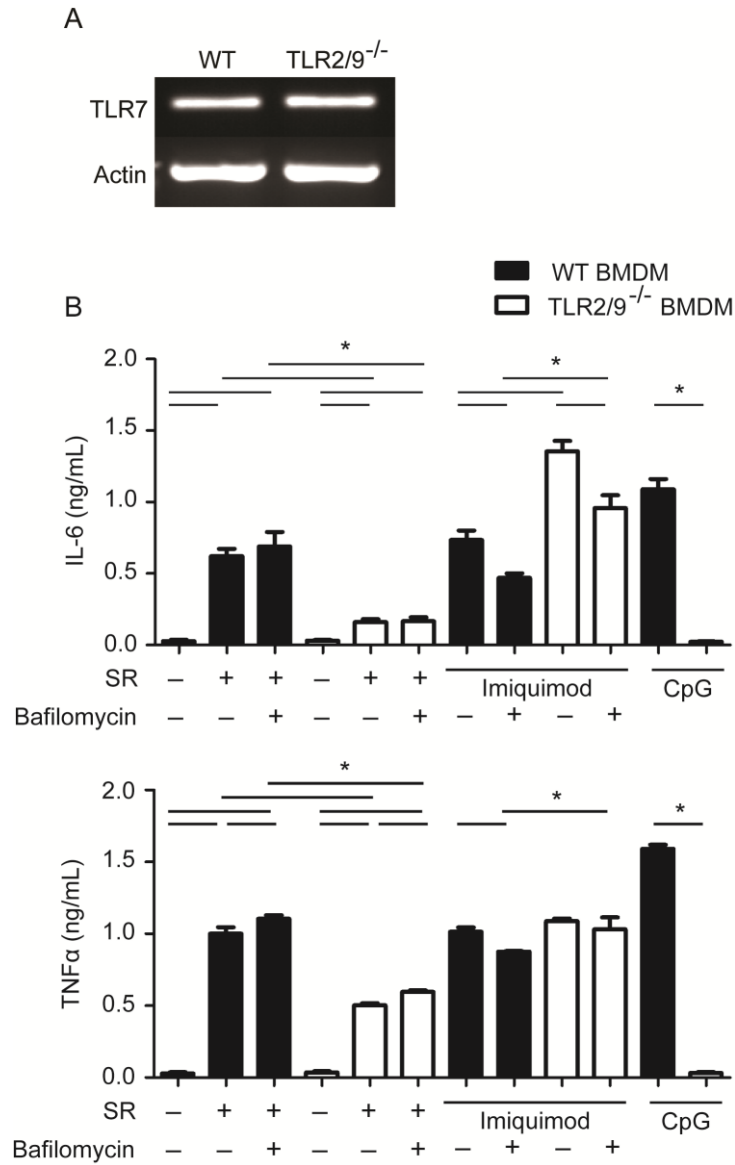
Figure 3-12. Bafilomycin does not inhibit IL-6 and TNFα in BMDM stimulated with SR in vitro.

(A) RNA was isolated from BMDMs for reverse transcription and PCR. TLR7 expression was analyzed. Actin expression was used as a housekeeping gene control. (B) WT and TLR2/9[−/−] BMDMs were stimulated with media or SR (0.05mg/mL) in the presence or absence of bafilomycin (0.1μM), an inhibitor of endosomal acidification, for 24 hours. Imiquimod (3μg/mL), a synthetic TLR7 agonist, in the presence or absence of bafilomycin was used as a positive control of TLR7 inhibition by bafilomycin. CpG, a TLR9 agonist, was used as a positive control. Cell culture supernatants were collected and the expression of TNFα and IL-6 measured by ELISA.

and may also contribute to monocyte and lymphocyte recruitment to the lung at later time points.

SR exposed mice had increased the production of IL-12p40 in the BALF. IL-12p40 is the common chain for the cytokines IL-12p70 (IL-12p35 / IL-12p40) and IL-23 (IL-23p19 / IL-12p40), two cytokines important in the polarization and proliferation of Th1 and Th17 cells. There was no IL-12p70 detected in the BALF suggesting SR induces the production of IL-23, and IL-23p19 expression was increased in SR exposed mice. SR induced expression of IL-12p40 and IL-23p19 was largely dependent on TLR2, as TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice had a significant reduction in expression compared to WT exposed mice; however, only TLR2/9$^{-/-}$ mice had a significant reduction in IL-12p40 in the BALF. IL-23 is a cytokine that is important for the proliferation and survival of Th17 cells, which have been associated with disease severity and fibrosis in HP. Therefore, TLR2/9$^{-/-}$ mice may be protected from Th17 cell associated pathogenesis at later time points. The expression of the anti-inflammatory cytokine IL-10 was also upregulated in response to SR, and the upregulation was dependent on TLR2. However, IL-10 was not detected in the BALF at this time point. Reduced anti-inflammatory cytokine production could have a negative impact on disease progression in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice at later time points.

Although TLR2/9$^{-/-}$ mice had reduced neutrophil recruitment, inflammatory cytokine and chemokine production after one exposure to SR, the levels were still greater than previous studies with MyD88$^{-/-}$ mice (Nance et al., 2008). In vitro stimulation of splenic adherent cells with SR revealed another MyD88-dependent receptor could be involved in the residual production of CXCL1, CXCL2, G-CSF, TNFα, IL-6, IL-1β, IL-10, IL-12p70, IFNγ, and IL-17 in TLR2/9$^{-/-}$ splenic adherent cells. TLR7 was a possible candidate since it was upregulated in the lungs of mice exposed to SR for 3 weeks. TLR7 recognizes single stranded RNA, and SR RNA stimulated the production of TNFα in a dose dependent manner, and the production of IL-10 through a MyD88-dependent receptor. Inhibition of endosomal TLRs with bafilomycin showed a significant reduction of TNFα and IL-6 production in TLR2/9$^{-/-}$ spleen cells, but not BMDMs. RT-PCR revealed BMDMs express TLR7, and stimulation with the TLR7 agonist imiquimod suggests the TLR7 receptor is functional and that bafilomycin can inhibit TLR7 induced TNFα and IL-6 production in BMDMs. Altogether, the results suggest an endosomal TLR such as TLR7 plays a role in SR induced TNFα and IL-6 production in the spleen, but not in BMDMs. TLR13 is an endosomal TLR, which has recently been shown to recognize bacterial 23S rRNA in mice (Oldenburg et al., 2012). TLR13 is not expressed in humans and therefore would not contribute to SR induced HP in patients. The IL-1R and IL-18R is MyD88-dependent and may play a role in SR induced inflammatory cytokine production in BMDMs.

In summary, TLR2/9$^{-/-}$ mice have significant reduction in the recruitment of neutrophils, a cell type that is important in innate immune defense against bacteria, but is associated with increased inflammation and lung pathology in HP. TLR2/9$^{-/-}$ also have significantly reduced inflammatory cytokine production after SR exposure. Importantly, TLR2/9$^{-/-}$ mice have significant reductions in IL-6 and IL-23p19, two cytokines

important for the differentiation and proliferation of Th17 cells, which have been associated with lung fibrosis in HP. In the next chapter, we will explore the role of TLRs 2 and 9 in neutrophil recruitment, macrophage and lymphocyte activation, polarized Th cell phenotypes, and granuloma formation after three weeks repeated SR exposure.

CHAPTER 4.   GRANULOMATOUS PHASE[*]

Introduction

The granulomatous phase of HP is characterized by lymphocytic alveolitis and granuloma formation. Previous studies have revealed HP is a T cell mediated disease. Nude mice, which lack a thymus and do not develop mature T cells, do not develop HP (Ohta et al., 1993), and TcRβ$^{-/-}$ mice, which lack CD4$^+$ and CD8$^+$ T cells, do not form granulomas or develop severe fibrosis in response to SR (Simonian et al., 2009). Granuloma formation depends on interactions between macrophages and T cells through cytokine stimulation and receptor interactions. In HP, the cytokine IFNγ produced from T cells, neutrophils, or other innate immune cells is required for granuloma formation (Gudmundsson and Hunninghake, 1997; Nance et al., 2005). The activation of antigen specific Th cells is also critical for granuloma formation. TLR stimulation of antigen presenting cells leads to the upregulation of costimulatory molecules CD80 and CD86, increased expression of MHCII, and induces cytokine secretion necessary for the activation and polarization of naïve Th cells into effector cells (Pasare and Medzhitov, 2003; Pasare and Medzhitov, 2004). In addition, costimulatory molecules CD80 and CD86, CD40, and MHCII have been shown to be upregulated on macrophages inside granulomas in tuberculosis, schistosomiasis, and Crohn's disease (Hara et al., 1997; Rathore et al., 1996; Soler et al., 1999). Studies during the acute phase revealed the importance of TLRs 2 and 9 in neutrophil recruitment, neutrophil chemokine production, and inflammatory cytokine production in response to a single SR exposure. During the granulomatous phase, we hypothesize TLRs 2 and 9 cooperate in neutrophil recruitment as well as T cell activation and costimulatory molecule upregulation necessary for granuloma formation and the pathogenic T cell response.

T Cell Activation

Naïve CD4$^+$ T cells require 3 signals for activation. The first signal comes from the T cells receptor, which recognizes antigen displayed on major histocompatibility complex class II (MHCII) on the cells surface of an antigen presenting cell (APC), such as a dendritic cell or macrophage. The second signal is transmitted through the binding of costimulatory molecules CD80 or CD86 to CD28 on the T cell. The third signal of activation is through cytokine stimulation released from the APC and binding to cytokine receptors on the T cell. The activated Th cell then matures and differentiates into T

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effector cells, which travel to sites of infection to augment the immune response, or the Th cell can differentiate into a memory cell, which populates lymphoid organs such as the spleen and lymph nodes. Effector and memory T cells only require 1 signal for activation, the T cell receptor interacting with antigen displayed on MHCII.

CD4$^+$ Th cells polarize into different subtypes depending on their cytokine stimulation (reviewed in Weaver et al., 2007). Th cells exposed to IL-12p70 and IFNγ upregulate the transcription factor T-bet, which leads to polarization of Th1 cells. Exposure to IL-4 and the upregulation of the transcription factor Gata3 induces Th2 cell polarization. Finally, in the mouse, TGFβ in the presence of IL-6 upregulates the transcription factor RORγ and polarizes Th17 cells, while TGFβ alone polarizes T regulatory (Treg) cells and induces the upregulation of the transcription factor FoxP3. The Th17 response in SR exposed mice is associated with increased disease severity and fibrosis (Joshi et al., 2009; Simonian et al., 2009). Previous studies have shown that TLR6$^{-/-}$ mice have significantly decreased expression of IL-6 and IL-17, cytokines associated with the Th17 response, suggesting SR stimulation through the TLR2/6 heterodimer is involved in IL-17 expression and the Th17 response (Fong et al., 2010). During the acute phase, signaling through TLR2 was revealed to be important in SR induced production of IL-6 and IL-23p19 expression, suggesting polarization of the Th17 response may be inhibited in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice.

TLR Induced Macrophage Activation

Like T cells, macrophages can exhibit polarized phenotypes depending on the chemokines and stimuli in their environment (reviewed in Mosser, 2003; Mosser and Edwards, 2008). Stimulation of TLRs on macrophages in the presence of IFNγ and TNFα leads to the classically activated phenotype characterized by upregulation of nitric oxide synthase, costimulatory molecules CD80 and CD86, and MHCII. Nitric oxide synthase generates nitric oxide, which is a potent antimicrobial agent (Bogdan et al., 2000). Classically activated macrophages release inflammatory cytokines such as TNFα, IL-12, IL-1, and IL-6. However, TLR signaling combined with immune complexes leads to a regulatory macrophage that also upregulates costimulatory molecules, MHCII, and nitric oxide production, but secretes low levels of IL-12 and high levels of IL-10 (Edwards et al., 2006), and are therefore important for dampening immune responses.

Activated effector T cells can recognize bacterial proteins displayed on the surface of macrophages on MHC molecules through the TcR. T cells are an important source of cytokines to induce macrophage activation through the secretion of cytokines such as IFNγ or IL-10. Effector T cells also express CD40 ligand, which interacts with CD40 expressed on the surface of macrophages to send contact dependent activating signals to the macrophage (Stout et al., 1996). Macrophages are well known to upregulate costimulatory molecules in response to bacterial and TLR stimulation (Bashir et al., 2010; Dong et al., 2008; Shim et al., 2009). Alveolar macrophages (AMs) have low costimulatory molecule expression in a healthy lung, however in patients with HP, costimulatory molecules are upregulated (Israel-Assayag et al., 1999).

In the following studies, the objective is to analyze neutrophil and lymphocyte recruitment, inflammatory cytokine and chemokine production, lymphocyte and macrophage activation, the Th17 response, and granuloma formation following repeated exposure to SR. We hypothesize the loss of TLRs 2 and 9 in SR exposed mice will result in reduced neutrophil recruitment due to reduced inflammatory cytokine and chemokine production during the granulomatous phase. In addition, we hypothesize TLRs 2 and 9 are required for a Th17 cell response through the induction of inflammatory cytokines IL-6 and IL-12p40 (the common chain for IL-12 and IL-23).

Results

In the following studies we measured disease severity through alveolitis, inflammatory cytokine and chemokine production, and granuloma formation. In the alveolitis section, we examined the composition of the alveolitis in WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice exposed to SR for neutrophil and lymphocyte recruitment and markers of lymphocyte activation. In the granuloma section, we explored granuloma formation through the analysis of lymphocyte populations in the lung, the upregulation of lymphocyte activation markers and polarized Th cell subtypes, the upregulation of costimulatory molecules on macrophage populations in the lung, and the formation of granulomas by histology.

Alveolitis

Neutrophil Recruitment and Chemokine Production Are Largely Dependent on TLR2 Following Repeated SR Exposures

To determine whether TLRs 2 and 9 also control neutrophil recruitment throughout 3 weeks of repeated exposures, we exposed WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice to SR 3 times/week for 3 weeks and performed BAL on day 18 (one day after the last exposure) and day 20 (3 days after the last exposure). The results demonstrate that there is no significant reduction in alveolitis in the TLR2/9$^{-/-}$ mice compared to the WT mice on day 18 and day 20 (**Figure 4-1A**). To investigate whether the cellular composition of the BAL is altered in KO mice, immune cell populations were measured by flow cytometry. **Figure 4-1B** shows the gating strategy used to analyze the percentages of Gr-1^{+} / CD11b^{+} neutrophils, Gr-1^{+} / CD11b^{-} cells, and CD11b^{+} / Gr-1^{-} monocytes present in the BAL cells. The results show the composition of the alveolitis is different during the granulomatous phase compared with an acute exposure. In WT mice, neutrophils were still the dominant cell type on day 18 but were decreased by day 20. However, the TLR2/9$^{-/-}$ mice still had a significant reduction of neutrophil frequency compared to the WT group on day 18 and day 20 (**Figure 4-1C**). The decrease in neutrophil recruitment to the airways appeared to be compensated for by a significant increase in CD11b^{+} / Gr-1^{-} cells.

Figure 4-1. TLR2 is the dominant receptor in neutrophil recruitment to the airways of mice exposed to SR for 3 weeks.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 20 (3 days after the last exposure). (A) BAL was performed and alveolitis determined using trypan blue dye exclusion. Cells recovered from the BAL fluid were incubated with antibody to CD11b and Gr-1 and analyzed by flow cytometry. (B) The gating strategy to discriminate neutrophil (CD11b$^+$ / Gr-1^{high}), Gr-1$^+$ cell (Gr-1$^+$ / CD11b$^-$), and monocyte (Gr-1$^-$ / CD11b$^+$) populations present in the alveolitis. (C) The number of neutrophils, Gr-1$^+$ cells, and monocytes in the BAL (n = 3 - 4 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

A Alveolitis

Legend:
- WT Unexp
- WT 3wk SR exp
- TLR2$^{-/-}$ 3wk SR exp
- TLR9$^{-/-}$ 3wk SR exp
- TLR2/9$^{-/-}$ 3wk SR exp

Day 18 — Day 20

B

Side Scatter / Forward Scatter → Gr-1 / CD11b

3.0   57.1
       6.1

C Gr-1+ CD11b+   Gr-1- CD11b+

Day 18   Day 20   Day 18   Day 20

Gr-1+ CD11b-

Day 18   Day 20

49
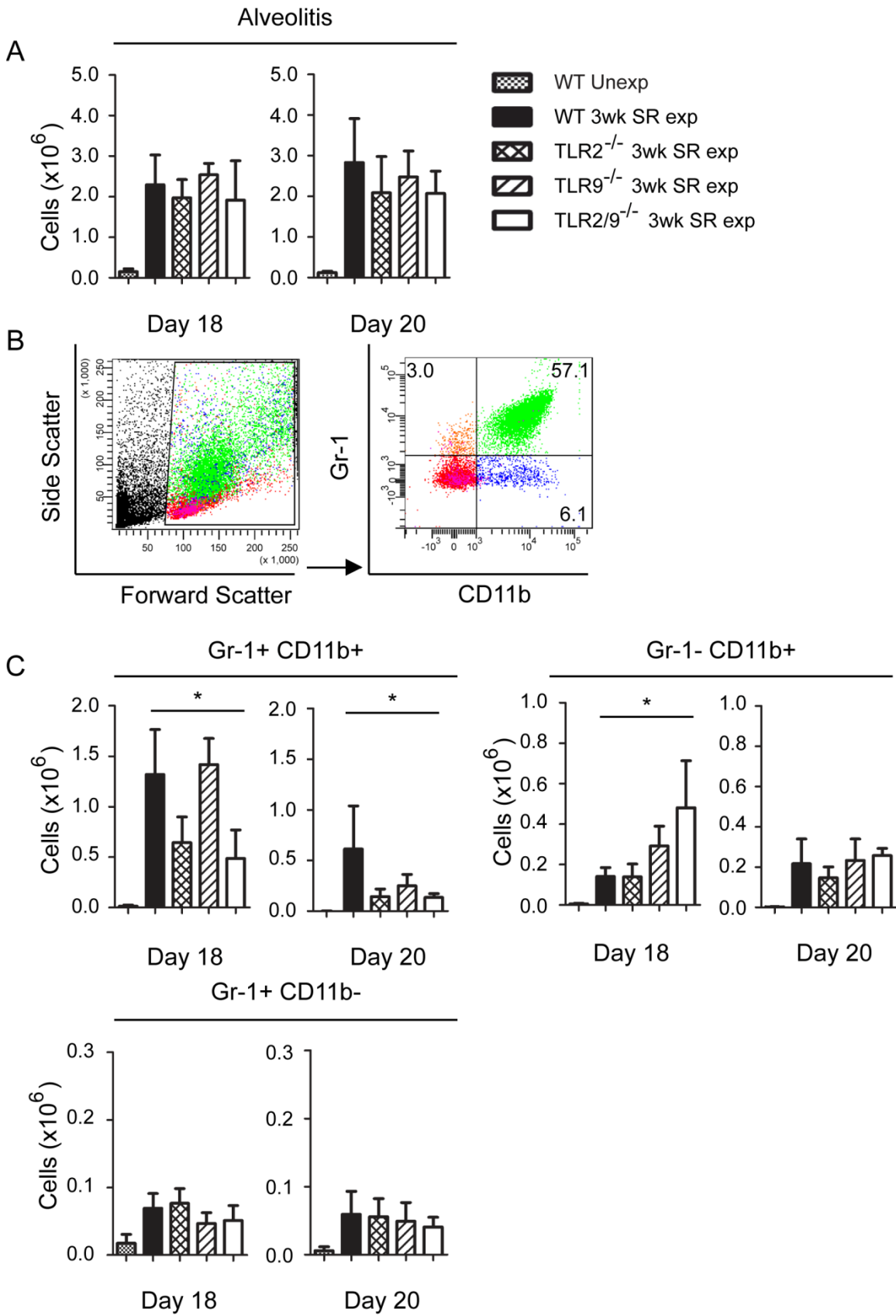
To determine if the reduced neutrophil recruitment in TLR2/9$^{-/-}$ mice was due to impaired chemokine expression, RNA was isolated from the lungs of mice exposed to SR for 3 weeks for reverse transcription and RT-PCR. The expression of CXCL2 was significantly decreased in both the TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to the WT mice (**Figure 4-2**). However, CXCL1 mRNA was not significantly reduced between the WT and mutant mice suggesting that TLRs 2 and 9 are not responsible for its production. The expression of macrophage and lymphocyte chemokines CCL2 and CCL4 was also upregulated after 3 weeks SR exposure, but the difference between WT and KO mice was not significant. Altogether, these results demonstrated that neutrophil recruitment and CXCL2 production following repeated SR exposures is largely dependent on TLR2.

Lymphocytes Are Recruited to the Airways after 3 Weeks Exposure to SR

After three weeks exposure to SR, lymphocytes were also present in greater numbers. The gating strategy for the detection of lymphocytes in the BAL is depicted in **Figure 4-3A**. Of the lymphocytes, T cells and B cells were more numerous than NK cells and γδ T cells, which is reflective of the adaptive immune response generated against SR (**Figure 4-3B**). On day 20, additional Th and B cells were recruited to the lung compared to day 18. However, there was no significant difference in the composition of lymphocytes in the BAL in single and double knockout mice.

To determine if the T cells recruited to the lung after 3 weeks exposure to SR were CD4^{+} T helper (Th) cells or CD8^{+} T cells, the percentage of T cells expressing CD4^{+} or CD8^{+} was analyzed by flow cytometry. Unexposed mice were excluded from the analysis as T cells are not present in the BAL of unexposed mice. The gating strategy to discriminate CD4^{+} and CD8^{+} T cell populations is depicted in **Figure 4-4A**. A greater percentage of T cells were CD4^{+} Th cells than CD8^{+} T cells in WT and KO mice (**Figure 4-4B**). TLR9$^{-/-}$ mice had a significant decrease in the percentage of CD8^{+} T cells on day 18. On day 20, there was a greater percentage of CD4^{+} T cells than day 18, which resulted in an increased CD4:CD8 ratio (**Figure 4-4C**) and suggests a greater number of CD4^{+} Th cells rather than CD8^{+} T cells are recruited to the airways at this time point.

TLR2 Is Important for CD4^{+} Th Cell Activation, While TLR9 Is Important for CD8^{+} T Cell Activation in the Airways

To determine if TLRs 2 and 9 are necessary for lymphocyte activation, the percentage of lymphocytes expressing CD69, a marker of lymphocyte activation, was analyzed on T cells, B cells, NK cells, and γδT cells. Unexposed mice were excluded from the analysis as T cells, B cells, NK cells, and γδT cells are not present in the BAL of unexposed mice. In WT SR exposed mice, more than half of the CD4^{+} and CD8^{+} T cells express CD69 on day 18. TLR2$^{-/-}$ mice had a reduced percentage of CD69^{+} CD4^{+} Th cells on day 18, and TLR2$^{-/-}$ and TLR2/9$^{-/-}$ had significantly reduced percentage of CD69^{+} CD4^{+} Th cells on day 20 (**Figure 4-5**), suggesting signaling through TLR2 is
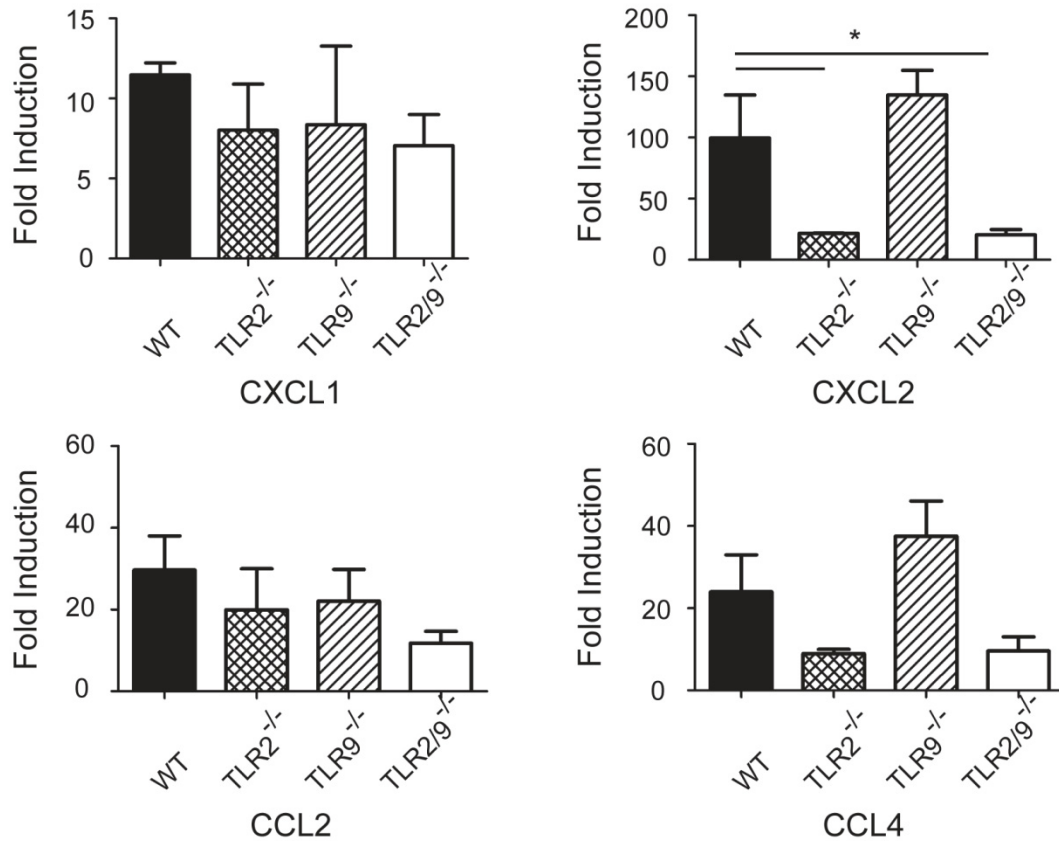
Figure 4-2. **Neutrophil chemokine expression is dependent on TLR2 following 3 weeks SR exposure.**

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure). The expression of chemokines was determined by qRT-PCR on RNA isolated from individual lung lobes (*n* = 3 mice per group) and is expressed as fold induction over WT unexposed mice. The data represent mean ± SD of duplicate samples. Data represent the mean ± SD (n=3 mice / group). Significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

Figure 4-3. Mice exposed to SR for 3 weeks have increased leukocyte populations in the BAL.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 20 (3 days after the last exposure). BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD19, βTcR, NK1.1, and γδTcR and analyzed by flow cytometry. (A) The gating strategy to discriminate B cell (CD19^{+} / SSc^{low}), Th cell (βTcR^{+} / SSc^{low}), NK cell (NK1.1^{+} / SSc^{low}), and γδ T cell (γδTcR^{+} / SSc^{low}) populations present in the alveolitis. (B) The number of, T cells, B cells, NK cells, and γδ T cells present in the BAL. (n = 3 - 4 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).
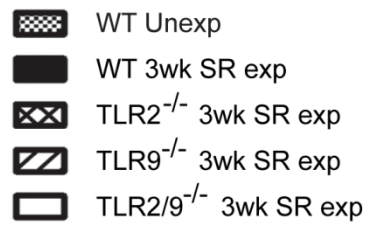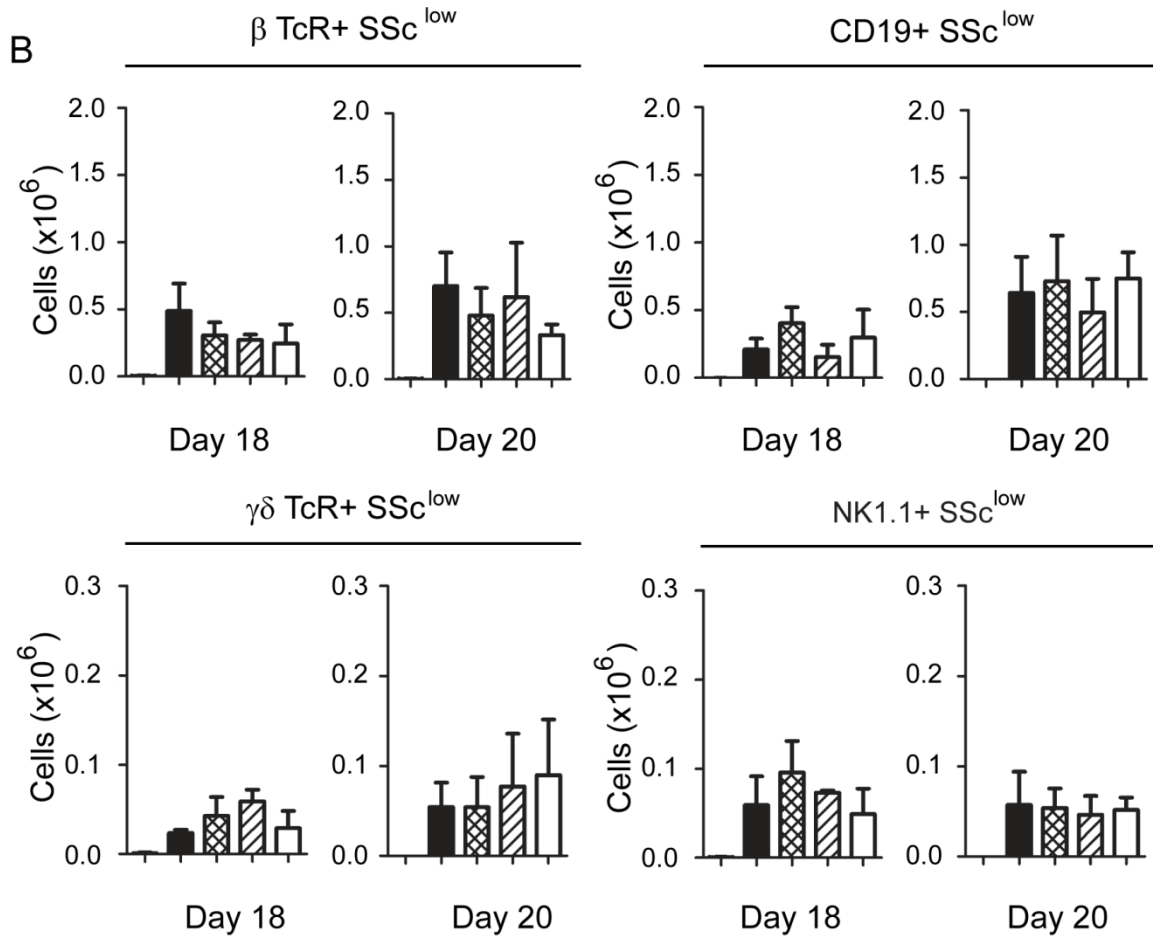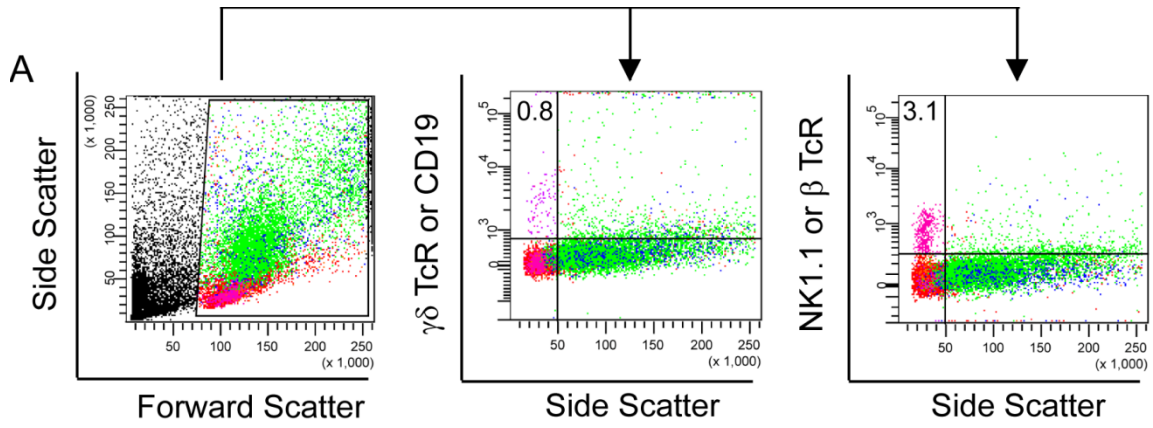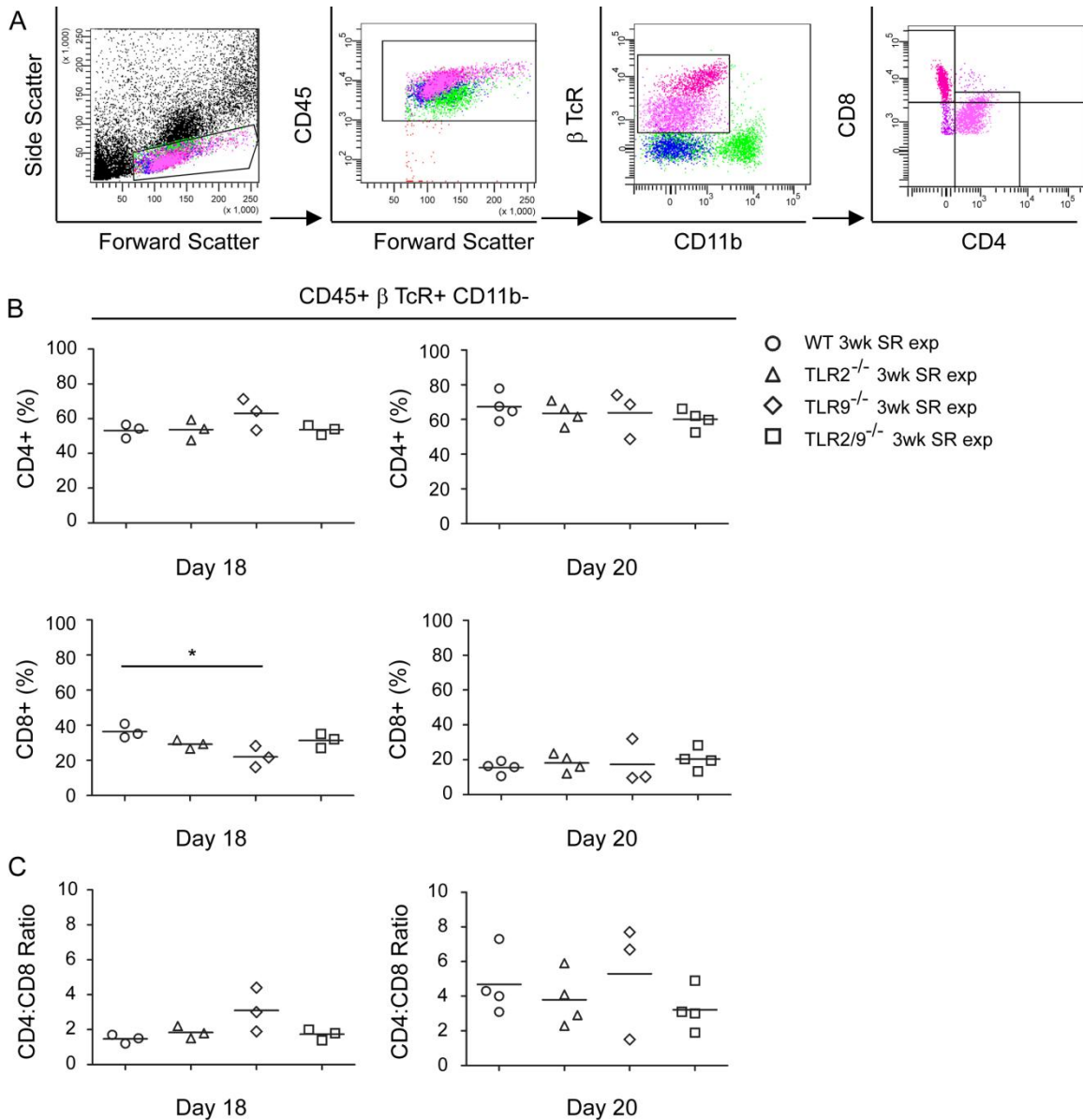
Figure 4-4.   CD4$^+$ and CD8$^+$ Th cells are recruited to the airways of mice exposed to SR for 3 weeks.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 20 (3 days after the last exposure). BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD45, CD11b, βTcR, CD4, and CD8 and analyzed by flow cytometry. (A) The gating strategy to discriminate CD4$^+$ Th cells (CD45$^+$ / CD11b$^-$ / βTcR$^+$ / CD4$^+$) and CD8$^+$ T cells (CD45$^+$ / CD11b$^-$ / βTcR$^+$ / CD8$^+$). (B) The percentage of CD4$^+$ Th cells and CD8$^+$ T cells present in the BAL. (C) The CD4:CD8 ratio was calculated. (n = 3 - 4 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

Figure 4-5.   Activated CD4$^+$ and CD8$^+$ T cells in the BAL of mice exposed to SR for 3 weeks is reduced in TLR2/9$^{-/-}$ mice.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 20 (3 days after the last exposure). BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD45, CD11b, βTcR, CD4, CD8, and CD69 and analyzed by flow cytometry. The cells were gated to discriminate activated CD4$^+$ Th cells (CD45$^+$ / CD11b$^-$ / βTcR$^+$ / CD4$^+$ / CD69$^+$) and CD8$^+$ T cells (CD45$^+$ / CD11b$^-$ / βTcR$^+$ / CD8$^+$ / CD69$^+$). The percentage of activated CD4$^+$ Th cells and CD8$^+$ T cells present in the BAL. (n = 3 - 4 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

important for CD4$^+$ Th cell activation. In contrast, TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice had a significant reduction in the percentage of CD69$^+$ CD8$^+$ T cells on day 18 (**Figure 4-5**). This suggests signaling through TLR9 may be important for CD8$^+$ T cell activation. The percentage of B cells and NK cells expressing CD69 was low, and there was no significant difference between WT and KO groups (**Figure 4-6**). Although γδ T cells were not a dominant cell type in the airways on day 18 and day 20, more than half express CD69 suggesting they are activated in response to SR and contribute to the immune response (**Figure 4-6**).

Granuloma Formation

TLR2/9$^{-/-}$ Mice Have a Significant Reduction in the Percentage of Activated T and B Cells in the Lung

With repeated SR exposures an adaptive immune response develops, which is associated with T cell dependent granuloma formation in the lung. To investigate the role of TLRs 2 or 9 in lymphocyte populations in the lung, we exposed WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice to SR 3 times/week for 3 weeks and sacrificed the mice on day 20 (3 days after the last exposure) and analyzed immune cell populations within the interstitial lung tissue by flow cytometry. CD4$^+$ Th cells were identified as CD45$^+$ / βTcR$^+$ / CD4$^+$. There was a significant reduction in the percentage of CD4$^+$ Th cells in the lung in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice on day 20 compared to WT exposed mice (**Figure 4-7A**). The reduced percentage of Th cells in the lungs of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice was also associated with a significant reduction in the percentage of Th cells that express the activation marker CD69 (**Figure 4-7B**), suggesting signaling through TLR2 is important for CD4$^+$ Th cell recruitment and activation in response to SR.

CD25 is the high affinity IL-2 receptor, and is transiently upregulated on activated proliferating T cells. Although not expressed on effector T cells at the site of infection, CD25 expression is retained on Treg cells. There was no significant difference in the percentage of Th cells that express CD25 in the lungs of WT or KO mice exposed to SR (**Figure 4-7B**). Therefore, the reduction in the percentage of activated CD4$^+$ Th cells in the lungs of TLR2/9$^{-/-}$ mice was not associated with an increased percentage of CD25$^+$ CD4$^+$ Treg cells.

Upon antigen stimulation, effector CD4$^+$ Th cells and other lymphocytes must be recruited to sites of infection. Like neutrophils, and monocytes, lymphocytes express chemokine receptors to traffic to site of infection. Previous studies have shown that CXCL9, CXCL10, and CXCL11 are induced by SR exposure, and recruit T cells that express CXCR3 (Nance et al., 2004). The expression of CCR6, which binds to CCL20, has been associated with the recruitment of Th17 cells as well as Treg cells (Yamazaki et al., 2008). The CD4$^+$ Th cells recruited to the lung of SR exposed mice expressed the
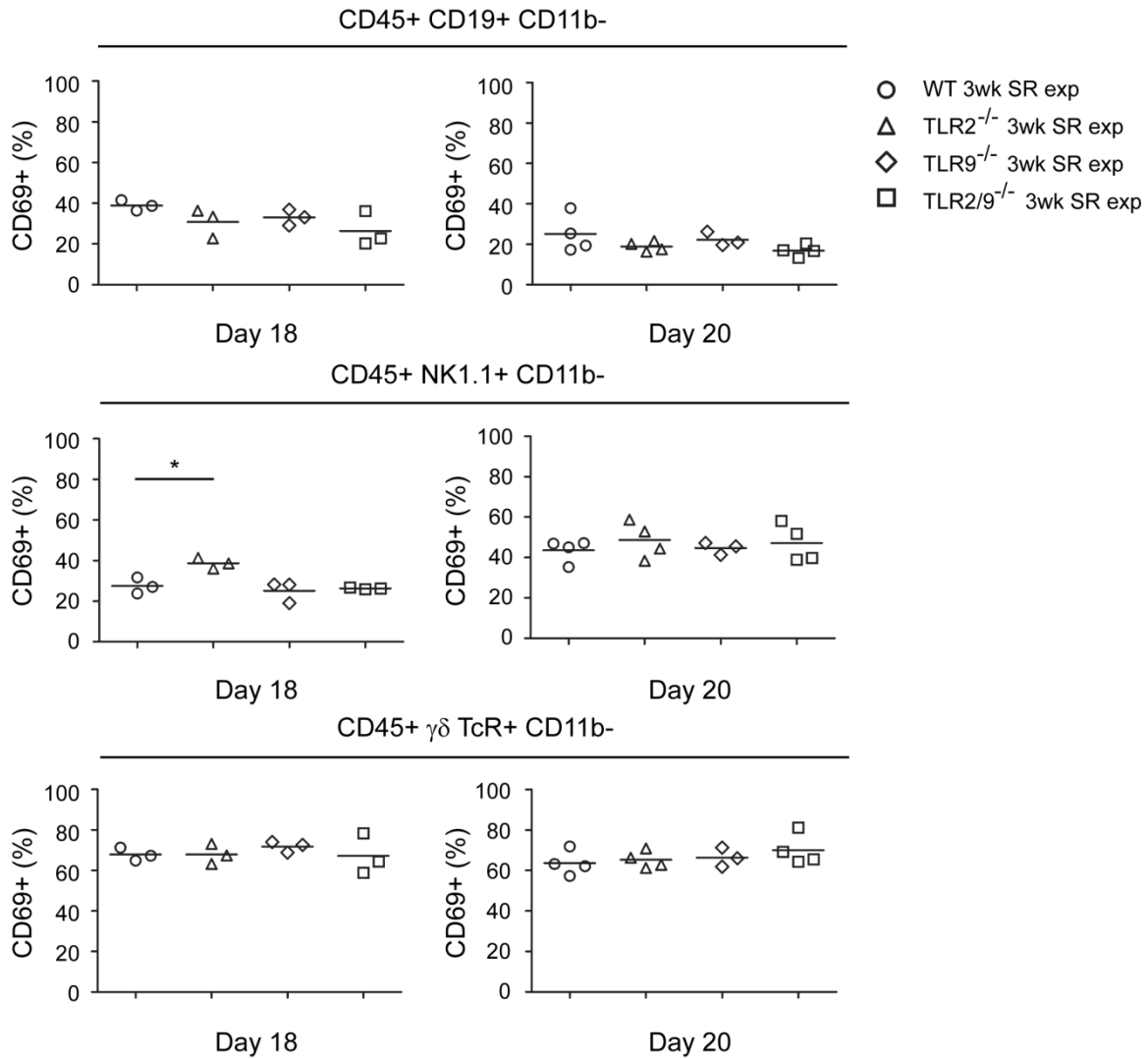
Figure 4-6. **TLRs 2 and 9 are not required for activation of B cells, NK cells, or γδ T cells in the airways of mice exposed to SR for 3 weeks.**

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 20 (3 days after the last exposure). BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD45, CD11b, CD19, NK1.1, γδTcR, and CD69 and analyzed by flow cytometry. The cells were gated to discriminate activated B cells (CD45^{+} / CD11b^{-} / CD19^{+} / CD69^{+}), NK cells (CD45^{+} / CD11b^{-} / NK1.1^{+} / CD69^{+}), and γδ T cells (CD45^{+} / CD11b^{-} / γδTcR^{+} / CD4^{+} / CD69^{+}). The percentage of activated B cells, NK cells, and γδ T cells present in the BAL ($n = 3 - 4$ mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

Figure 4-7. Reduced activation of CD4$^+$ Th cells in the lungs of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice exposed to SR for 3 weeks.
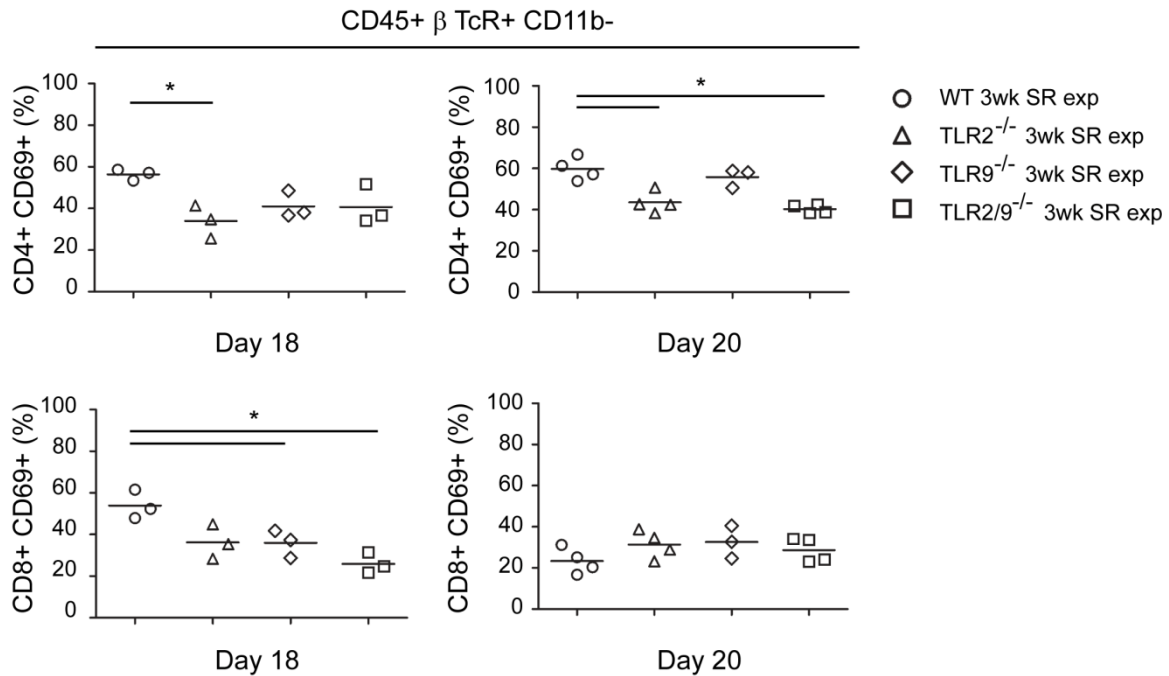
WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 20 (3 days after the last exposure). Lung cells were digested and incubated with antibodies to CD45, βTcR, CD4, CD69, CD25, and the chemokine receptors CXCR3, and CCR6 and analyzed by flow cytometry. (A) The percentage of CD4$^+$ Th cells (CD45$^+$ / CD4$^+$ / βTcR$^+$) in the lymphocyte population were identified. (B) The percentage of CD4$^+$ Th cells that express CD69, CD25, CXCR3, or CCR6 were analyzed. (n = 3 - 4 mice per group. The lung cells from 3 or 4 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).
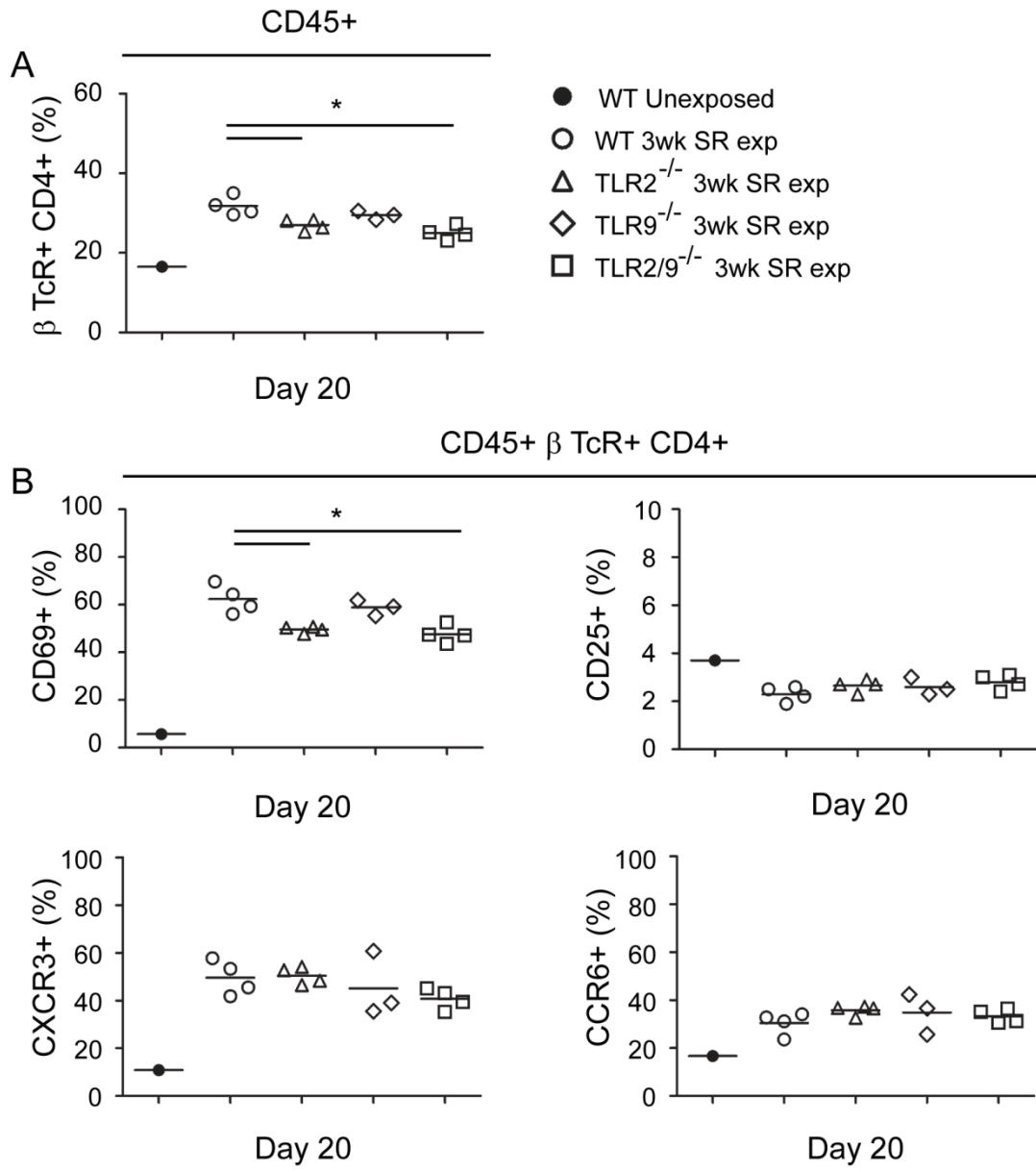
chemokine receptors CXCR3 and CCR6, but there was no difference in the percentage among WT and KO mice (**Figure 4-7B).**

TLR2 Is the Dominant Receptor for Cytokine Production Following Repeated SR Exposures

The reduced cellular recruitment to the lungs and reduced activation of lymphocytes could be due to a reduced inflammatory environment in the lungs of TLR2/9$^{-/-}$ mice. Studies during the acute phase showed TLRs 2 and 9 were involved in the expression of TNFα, IL-6, IL-12p40, and IL-23p19. To determine if inflammation is reduced in TLR2/9$^{-/-}$ mice following repeated SR exposures, the expression of TNFα, IL-6, IL-10, IL-12p40, and IL-23p19 in the lung tissue of mice exposed to SR for three weeks was measured by qRT-PCR (**Figure 4-8**). TNFα mRNA was significantly decreased in the lungs of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to the WT mice following 3 weeks of exposure. The expression of IL-6 and IL-23p19 was increased over unexposed mice, but not significantly reduced in KO mice compared to WT SR exposed mice. We did not detect an increase of IL-10 mRNA in the lungs of TLR2/9$^{-/-}$ mice exposed for 3 weeks, indicating that a decrease in T and B cell activation is not related to expression of IL-10.

The Th17 response is associated with increased disease severity and fibrosis in HP (Joshi et al., 2009; Simonian et al., 2009). Th17 cells are polarized in response to IL-6 and TGFβ and proliferate in response to IL-23, and the expression of IL-6 and IL-23p19 was increased in SR exposed lungs. IFNγ, a Th1 associated cytokine, is also critical for granuloma formation in HP. To investigate to role of TLRs 2 and 9 in the expression of Th17 associated cytokines IL-17 and IL-22 and Th1 associated cytokine IFNγ after 3 weeks exposure to SR, the expression of IL-17, IL-22, and IFNγ in the lung tissue was measured by qRT-PCR (**Figure 4-9**). Expression of IL-17 and IL-22 was significantly reduced in the TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to the WT. Expression of IFNγ was significantly reduced in TLR9$^{-/-}$ mice in comparison to WT mice following SR exposure suggesting that TLR9 is important in IFNγ expression. Altogether, these results suggest that TNFα, IL-17, and IL-22 production is largely mediated by TLR2 stimulation following repeated exposure with SR.

TLR2/9$^{-/-}$ Mice Have a Reduced Percentage of Th17 Cells in the Lung

Because TLR2/9$^{-/-}$ mice had a reduced percentage of activated CD4^{+} Th cells and Th17 associated cytokine expression, we next wanted to know if TLR2/9$^{-/-}$ mice were protected from developing a Th17 response. Mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 18 (1 day after the last exposure) and the percentage of Th17 and Th1 cells in the lungs was measured in ex vivo SR lysate stimulated lung cells by intracellular flow cytometry. The gating strategy for the detection of CD4^{+} Th cells is depicted in **Figure 4-10A**. The results show the percentage of CD4^{+} Th cells that
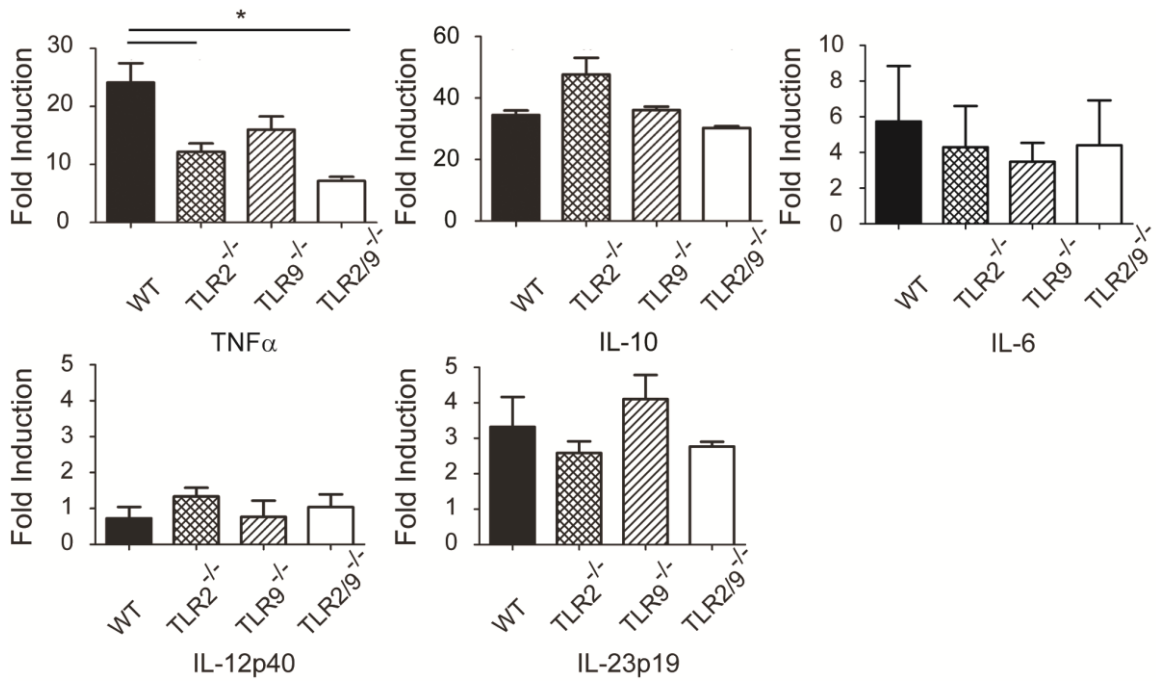
Figure 4-8.   TLRs 2 and 9 contribute to TNFα expression following repeated SR exposures.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure). RNA was isolated from lung tissue and reverse transcribed to cDNA for real time qRT-PCR to measure the expression of TNFα, IL-10, IL-6, IL-12p40, and IL-23p19 using specific primers. The results were normalized to the housekeeping gene HPRT and expressed as mean ± SD fold induction over unexposed mice. (n = 3 mice / group) Significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).
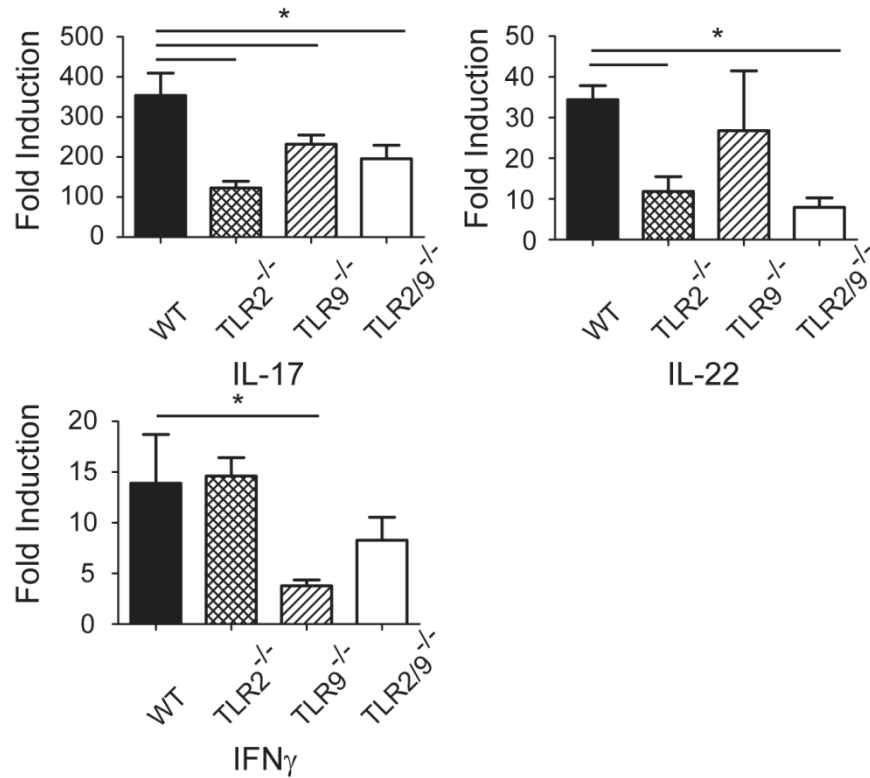
Figure 4-9.   TLRs 2 and 9 contribute to Th1- and Th17-associated cytokine production following repeated SR exposures.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure). RNA was isolated from lung tissue and reverse transcribed to cDNA for real time qRT-PCR to measure the expression of IFNγ, IL-17, and IL-22 using specific primers. The results were normalized to the housekeeping gene HPRT and expressed as mean $\pm$ SD fold induction over unexposed mice. (n = 3 mice / group) Significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

Figure 4-10. TLRs 2 and 9 are necessary for the generation of the Th17 response following 3 weeks repeated SR exposure.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure). Lung cells were digested and stimulated with SR lysate or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, IL-17A, and IFNγ for intracellular cytokine staining. (A) The gating strategy to discriminate CD4$^+$ Th cells (CD45$^+$ / CD4$^+$ / βTcR$^+$). (B) Representative dot plots of IL-17A and IFNγ expressing CD4$^+$ Th cells. (C) The percentage of CD4$^+$ Th cells expressing IL-17A and/or IFNγ. (n = 3 mice / group. The lung cells from 3 unexposed mice were combined.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*p < 0.05 compared to WT SR exposed mice).
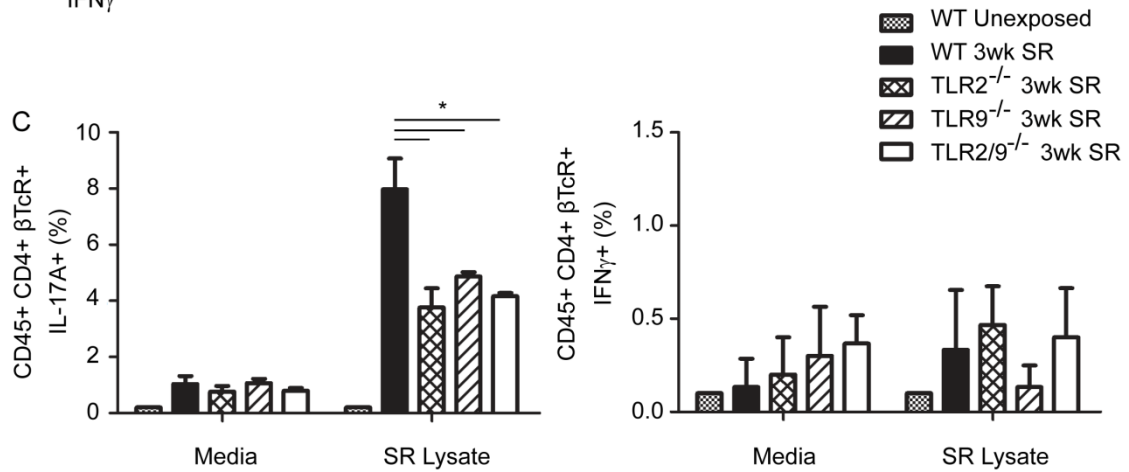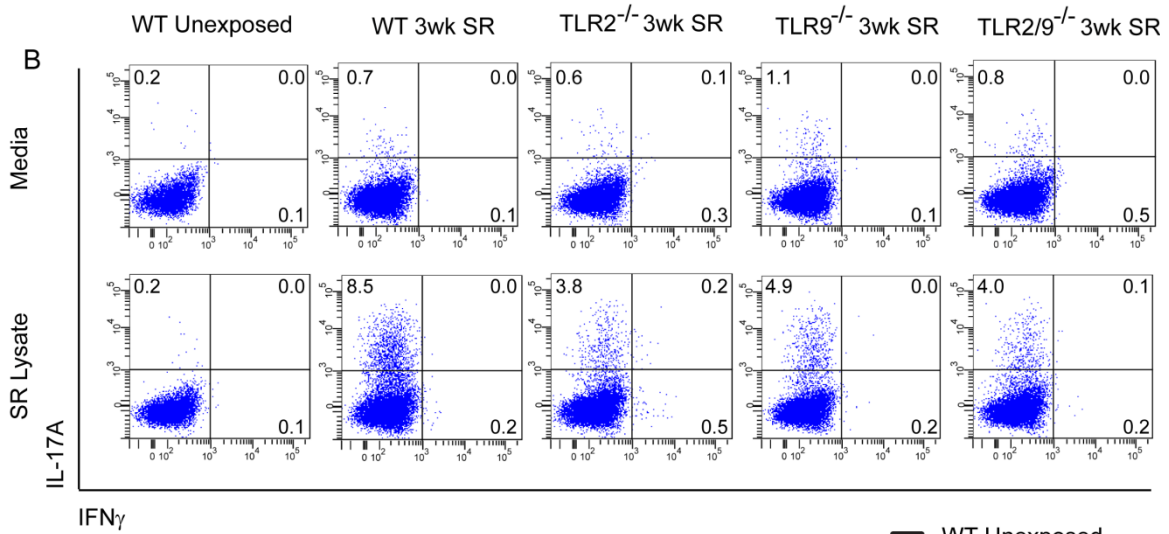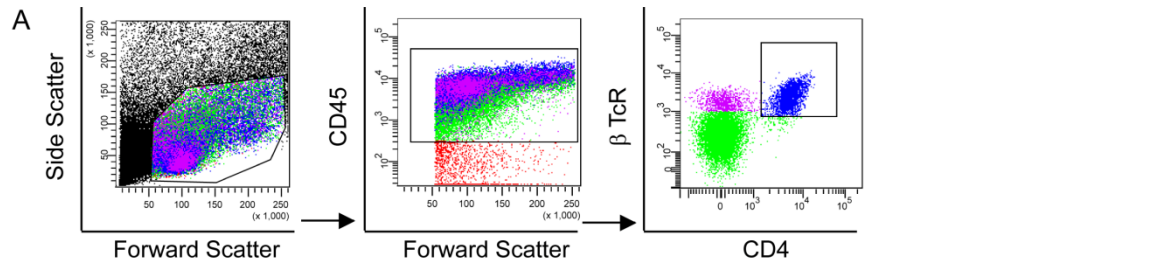
expressed IL-17A and IFNγ (**Figure 4-10B)**. In WT mice, 8% of CD4$^+$ Th cell stimulated with SR lysate respond with IL-17A production (**Figure 4-10C**). TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice had a significant decrease in the percentage of IL-17A$^+$ CD4$^+$ Th cells compared to WT exposed mice, indicating TLRs 2 and 9 both contribute to the Th17 response. Less than 1% of Th cells stimulated with SR lysate responded with IFNγ production, suggesting Th17 cells play a greater role in the Th cell response at this time point.

To determine if the Th17 response is maintained 4 days after the last exposure, the percentage of Th17 cells in the lung was measured on day 21. In addition, CD4$^+$ Th cells were stained for the expression of CD25, which is not expressed on effector Th17 cells. The results show the percentage of CD4$^+$ Th cells that expressed CD25 / IL-17A (**Figure 4-11A**) and CD25 / IL-17F, an alternative IL-17 isoform secreted by Th17 cells (**Figure 4-11B**). IL-17A expressing CD25$^-$ CD4$^+$ Th cells were still detected in WT, TLR2$^{-/-}$, TLR9$^{-/-}$ mice, but the percentage of Th17 cells in TLR2/9$^{-/-}$ mice was significantly reduced compared to WT exposed mice (**Figure 4-11C**). IL-17F expressing CD4$^+$ Th cells were not detected at this time point (**Figure 4-11C**). Altogether, these results suggest that signaling through TLRs 2 and 9 are necessary for sustaining a Th17 response in the lung after 3 weeks exposure to SR.

The reduction in Th17 cells on day 21 in TLR2/9$^{-/-}$ mice could be due to an increase in the percentage of IFNγ or TNF producing Th cells. Plate bound anti-CD3 antibody, a polyclonal stimulator of T cells, was used on day 21 in case the low percentage of IFNγ producing CD4$^+$ Th cells observed on day 18 was due to poor stimulation of IFNγ by SR lysate. Mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 21 (4 days after the last exposure) and the percentage of IFNγ^+ and TNFα^+ cells in stimulated dispersed lung cells was detected by intracellular flow cytometry. The results show the percentage of CD4$^+$ Th cells expressing CD25 / IFNγ (**Figure 4-12A)** and CD25 / TNFα (**Figure 4-12B**). An average of 2.8% of CD25$^-$ CD4$^+$ Th cells expressed IFNγ and 7.5% expressed TNFα. The percentage IFNγ^+ Th1 cells was significantly reduced in TLR2$^{-/-}$ mice, and the percentage of TNFα^+ CD4$^+$ Th cells was significantly reduced in TLR2/9$^{-/-}$ mice compared to WT SR exposed mice (**Figure 4-12C**). The results suggest TLR2 may be important for Th cell expression of IFNγ, and TLRs 2 and 9 are important for Th cell expression of TNFα. Importantly, it also shows the reduction in the Th17 response in TLR2/9$^{-/-}$ mice is not due to an increase in IFNγ or TNFα producing CD4$^+$ Th cells.

We next wanted to know if the reduced percentage of Th17 cells in the lungs of TLR2/9$^{-/-}$ mice was due to an increase in Treg cells, identified by the expression of CD25 and the transcription factor FoxP3. Mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 21 (4 day after the last exposure) and the percentage of CD25$^+$ and FoxP3$^+$ cells in SR lysate stimulated dispersed lung cells was detected by intracellular flow cytometry. The results show the percentage of CD4$^+$ Th cells expressing CD25 and FoxP3 (**Figure 4-13A)**. No more than half a percent of CD4$^+$ Th cells were CD25$^+$ FoxP3$^+$ in SR exposed mice indicating few Treg cells are recruited to

Figure 4-11.   TLRs 2 and 9 cooperate in sustaining the Th17 response following 3 weeks repeated SR exposure.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 21 (4 days after the last exposure). Lung cells were digested and stimulated with SR lysate or media alone in the presence of monensin then incubated with antibodies for CD45, βTcR, CD4, CD25, IL-17A, and IL-17F for intracellular cytokine staining. (A) Representative dot plots for CD4^{+} Th cells expressing CD25 or IL-17A. (B) Representative dot plots for CD4^{+} Th cells expressing CD25 or IL-17F. (C) The percentage of CD25^{-} CD4^{+} Th cells expressing IL-17A or IL-17F. (n = 3 mice / group. The lung cells from 3 unexposed mice were combined.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*p < 0.05 compared to WT SR exposed mice).
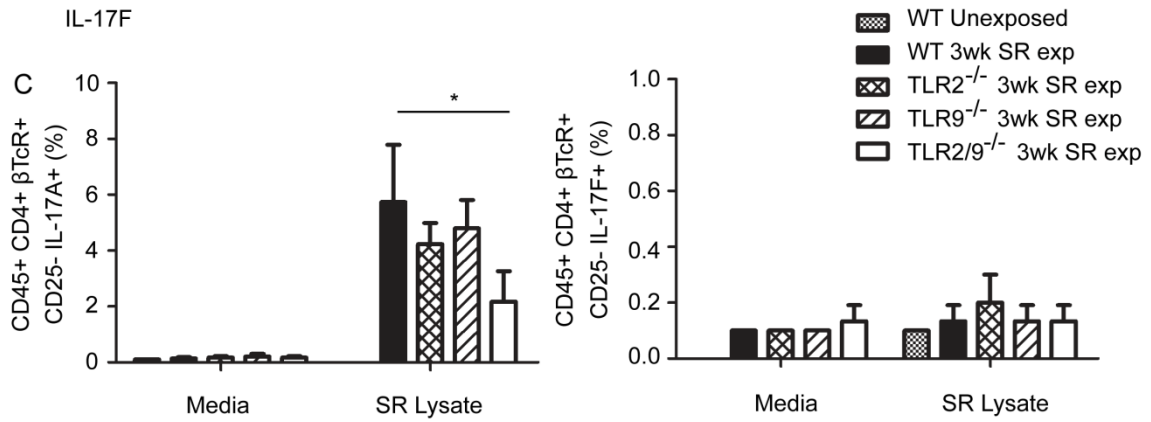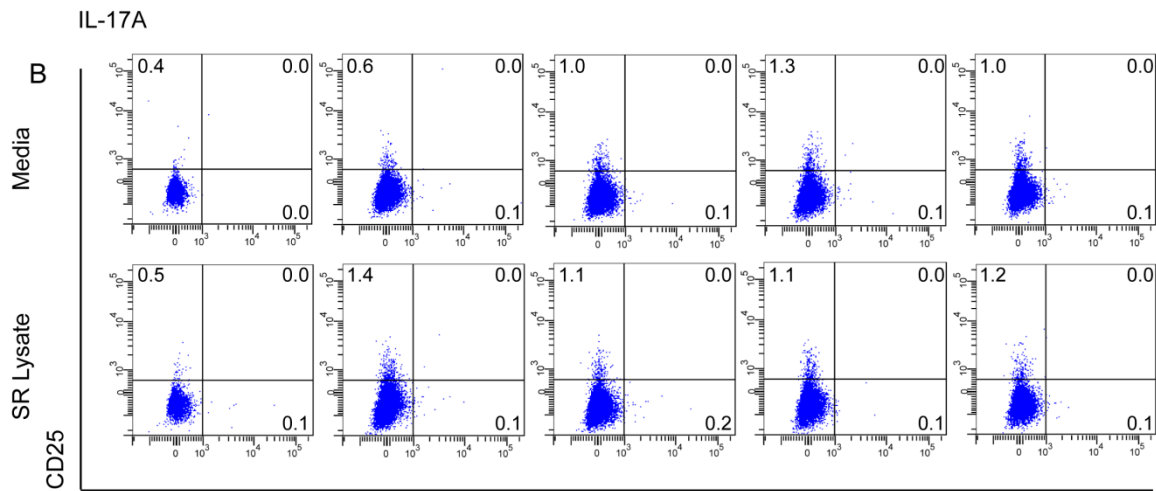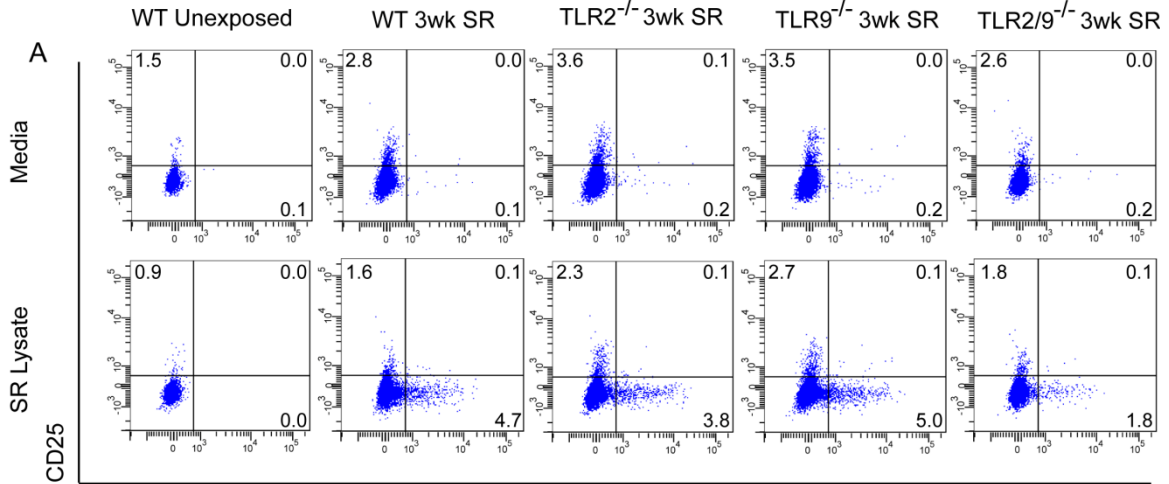
Figure 4-12. The reduction in Th17 cells in TLR2/9$^{-/-}$ mice is not due to an increase in IFNγ$^+$ or TNFα$^+$ Th1 cells.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 21 (4 days after the last exposure). Lung cells were digested and stimulated with plate bound α-CD3 (A), SR lysate (B), or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, CD25, IFNγ, and TNFα for intracellular cytokine staining. (A) Representative dot plots for CD4$^+$ Th cells expressing CD25 or IFNγ. (B) Representative dot plots for CD4$^+$ Th cells expressing CD25 or TNFα. (C) The percentage of CD25$^-$ CD4$^+$ Th cells expressing IFNγ or TNFα. (n = 3 mice / group. The lung cells from 3 unexposed mice were combined.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p <$ 0.05 compared to WT SR exposed mice).
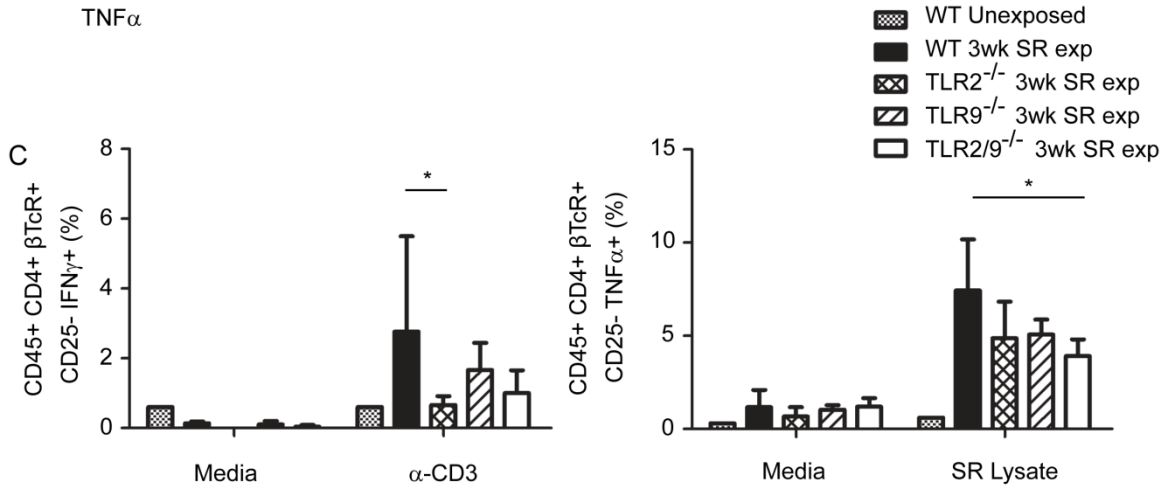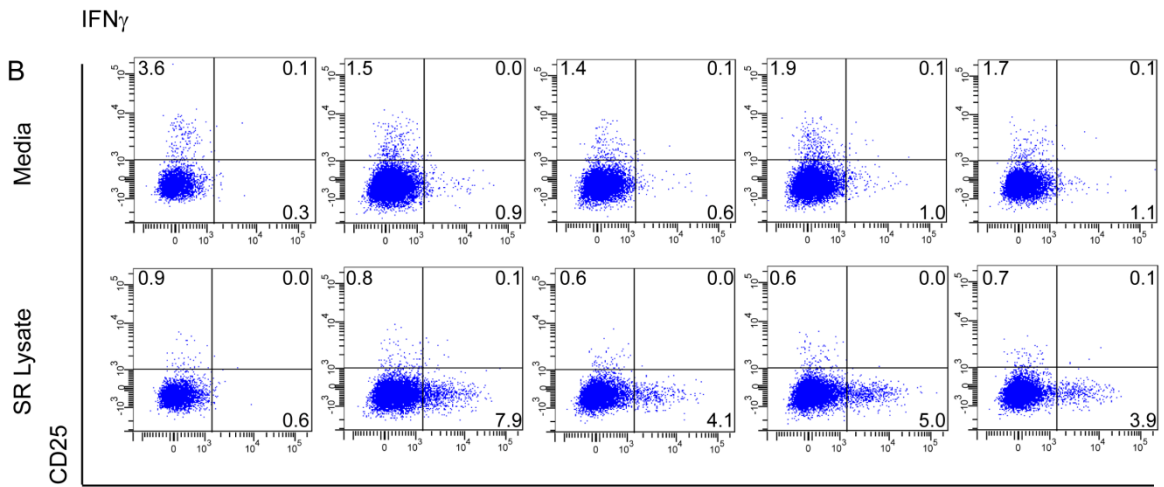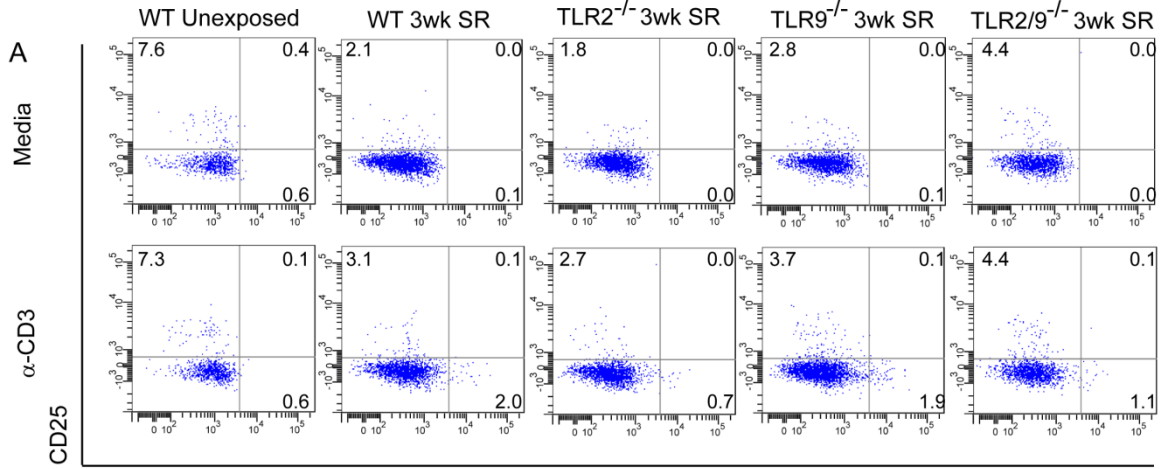
Figure 4-13. The reduction in Th17 cells in TLR2/9$^{-/-}$ mice is not due to an increase in FoxP3^{+} T regulatory cells.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 21 (4 days after the last exposure). Lung cells were digested and stimulated with SR lysate or media alone in the presence of monensin then incubated with antibodies for CD45, βTcR, CD4, CD25, FoxP3 for intracellular cytokine staining. (A) Representative dot plots for CD4^{+} Th cells expressing CD25 or FoxP3. (B) The percentage of CD25^{+} CD4^{+} Th cells or CD25^{-} CD4^{+} Th cells expressing FoxP3. (n = 3 mice / group. The lung cells from 3 unexposed mice were combined.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

the lung after SR exposure at this time point (**Figure 4-13B**). However, exposure to TGFβ at the site of infection can induce $CD25^-$ $CD4^+$ Th cells to become a Treg cells and upregulate FoxP3 (Fantini et al., 2004; Fu et al., 2004), and 14% of the $CD25^-$ $CD4^+$ Th cells express FoxP3 in WT SR exposed mice. There was no significant difference in the percentage of $CD25^-$ $CD4^+$ Th cells that express FoxP3 in KO mice compared to WT SR exposed mice. Altogether, these results suggest the reduced Th17 response in TLR2/9$^{-/-}$ mice is not due to increased polarization of Treg cells.

The production of IL-10 by Treg cells can inhibit immune responses. Although the percentage of Treg cells was not different between WT and KO mice, we next wanted to determine if the reduced percentage of Th17 cells in the TLR2/9$^{-/-}$ mice was due to increased production of IL-10 by Treg cells. Mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 21 (4 day after the last exposure) and the percentage of $CD25^+$ and IL-10$^+$ Th cells in SR lysate stimulated lung cells was detected by intracellular flow cytometry. The results show the percentage of $CD4^+$ Th cells expressing CD25 and IL-10 (**Figure 4-14A).** In WT SR exposed mice, only 1.3% of Th cells expressed IL-10. The IL-10 expressing $CD4^+$ Th cells were $CD25^-$ indicating the source of the IL-10 was not $CD25^+$ Treg cells (**Figure 4-14B**). In addition, TLR2$^{-/-}$ and TLR2/9$^{-/-}$ had a significantly reduced percentage of IL-10$^+$ $CD25^-$ $CD4^+$ Th cells, suggesting loss of TLR2 leads to decreased IL-10 production by $CD4^+$ Th cells, and the reduced Th17 response in TLR2/9$^{-/-}$ mice is not due to increased IL-10-producing Th cells.

Signaling Through TLR2 Is Important in B Cell Activation

The significant reduction in $CD4^+$ Th cells in the lungs of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice on day 20 and 21 was associated with a significant increase in the percentage of B cells (**Figure 4-15A**). However, the expression of the activation marker CD69 was significantly reduced on the B cells of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice on day 20 compared to WT SR exposed mice (**Figure 4-15A**), suggesting signaling through TLR2 is important for B cell activation. There was no significant difference in the percentage of $CD8^+$ cells or NK cells in the lungs, nor was the percentage of $CD8^+$ cells or NK cells that express of the activation maker CD69 significantly different compared to WT SR exposed mice (**Figure 4-15B** and **C**). Altogether the results suggest SR induced signaling through TLR2 is important for the activation of $CD4^+$ Th cells and B cells.

TLRs 2 and 9 Cooperate in Costimulatory Molecule Upregulation on Interstitial Macrophages

Antigen specific effector $CD4^+$ T cells are stimulated at the site of infection by antigen presenting cells, like macrophages, through the presentation of antigen on MHCII molecules. Stimulation of macrophages through pattern recognition receptors like TLR2 and TLR9 leads to the upregulation of costimulatory molecules CD80, CD86, and
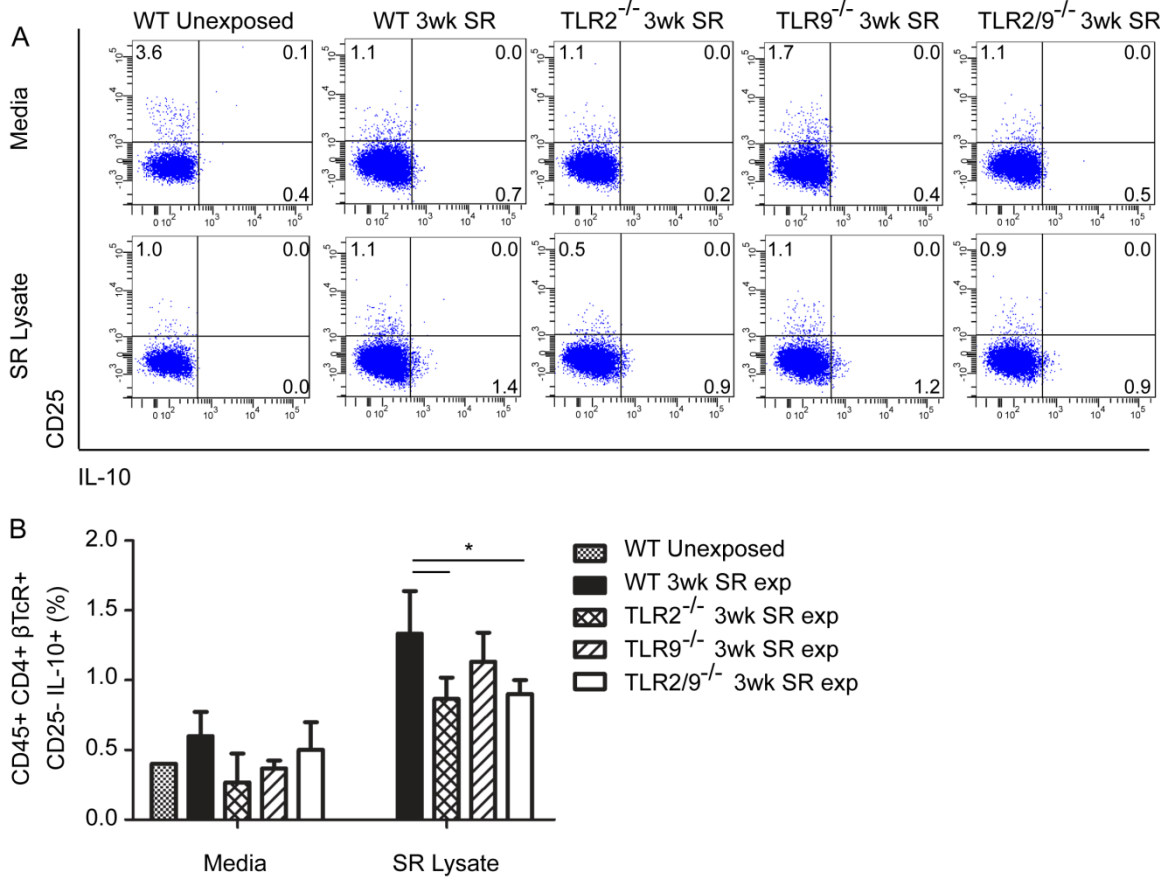
Figure 4-14. TLR2 is important for the generation of IL-10 expressing CD4$^+$ T cells.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 21 (4 days after the last exposure). Lung cells were digested and immune cells enriched on a 40/80% percoll gradient were stimulated with SR lysate or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, CD25, and IL-10 for intracellular cytokine staining. (A) Representative dot plots for CD4$^+$ Th cells expressing CD25 or IL-10 (B) The percentage of CD25$^-$ CD4$^+$ Th cells expressing IL-10. (n = 3 mice / group. The lung cells from 3 unexposed mice were combined.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

Figure 4-15. Reduced activation of B cells in the lungs of TLR2/9$^{-/-}$ mice.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 20 (3 days after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD45, βTcR, CD8, CD19, CD69, and NK1.1 and analyzed by flow cytometry. (A) The percentage of B cells (CD45$^+$ / CD19$^+$ / CD8$^-$) and B cells that express CD69. (B) The percentage of CD8$^+$ lymphocytes (CD45$^+$ / CD8$^+$ / CD19$^-$) and CD8$^+$ lymphocytes that express CD69. (C) The percentage of NK cells (CD45$^+$ / NK1.1$^+$ / CD19$^-$) and NK cells that express CD69. (n = 3 - 4 mice per group. The lung cells from 3 or 4 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).
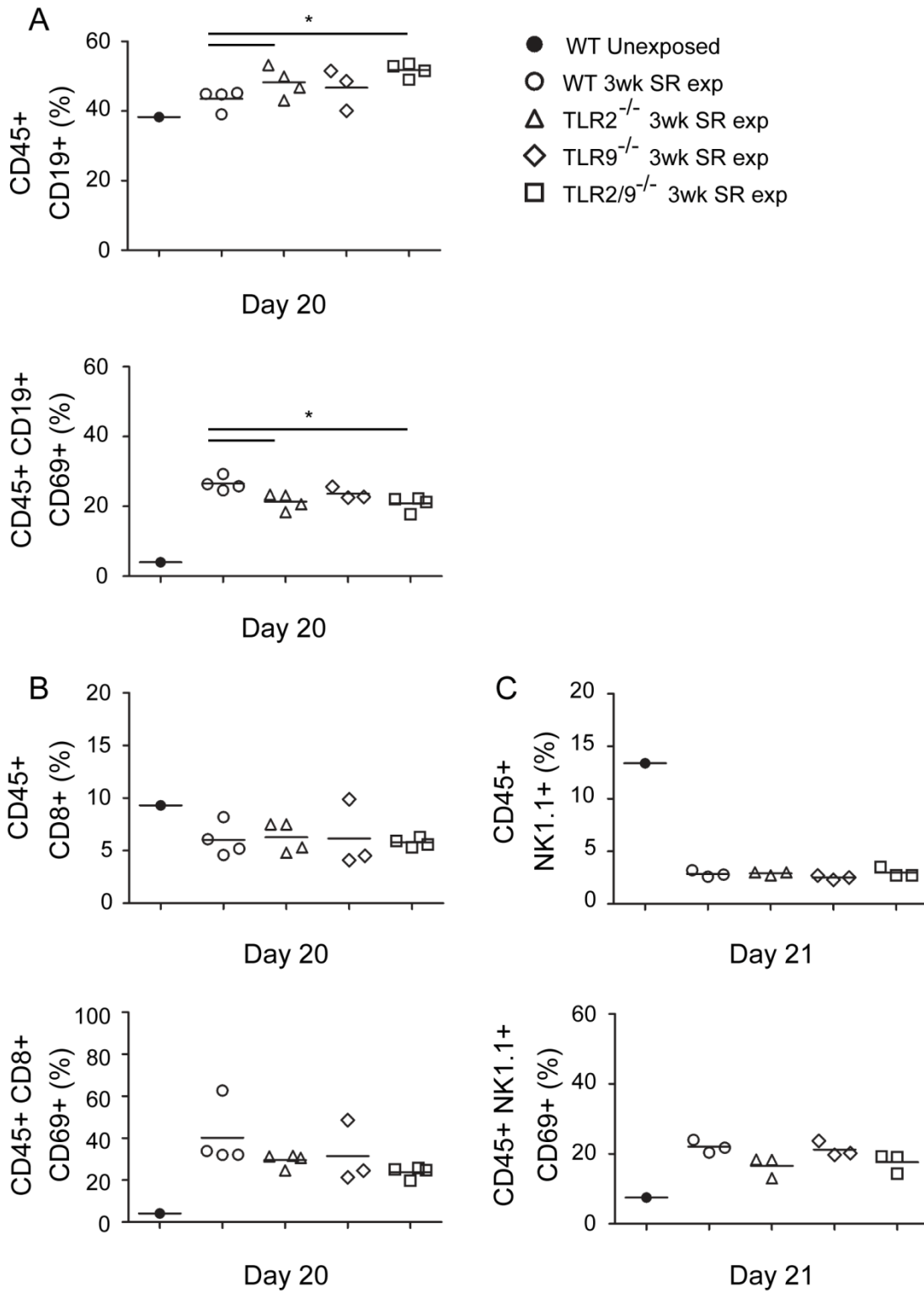
MHCII. In addition, T cells can induce macrophage activation through stimulation of CD40 through CD40 ligand interaction. To determine whether macrophage activation was deficient in the SKO or TLR2/9$^{-/-}$ mice, mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 18 (1 day after the last exposure) or day 21 (4 day after the last exposure) and co-stimulatory molecule and MHCII expression on macrophages isolated from whole lung tissue was measured by flow cytometry. Dispersed lung cells were incubated with antibodies to CD11b, F4/80, CD11c, Gr-1, CD80, CD86, MHCII, and CD40 and measured by flow cytometry. The gating strategy for the detection of interstitial macrophages (CD11b^{+} / F4/80^{+} / CD11c^{-} / Gr-1^{-}) is shown in **Figure 4-16A**.

We first examined costimulatory molecule CD80 and CD86 expression after 3 weeks exposure to SR. Interstitial macrophages had increased expression of CD80 on day 18 and day 21 after repeated SR exposure. TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice had a significant decrease in the percentage of interstitial macrophages that expressed CD80. The geometric mean fluorescence intensity (MFI) was calculated, and showed the average intensity of CD80 staining was also significantly decreased in TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ cells on day 18 and day 21 (**Figure 4-17A**). The percentage of interstitial macrophages that expressed CD86 was also decreased in TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice on day 18 (**Figure 4-17B**). The results suggest TLRs 2 and 9 cooperate in the upregulation of costimulatory molecules CD80 and CD86 on interstitial macrophages.

MHCII was also upregulated on WT interstitial macrophages after 3 weeks exposure to SR. On average, 72% of the interstitial macrophages were MHCII^{high} on day 18 and 21. TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice had a significant decrease in the percentage of MHCII^{high} cells compared to WT SR exposed mice on day 18 (**Figure 4-18A**). The percentage of MHCII^{high} cells was also significantly decreased on day 21 in TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice. In addition, the percent of interstitial macrophages that expressed CD40 was also significantly decreased in TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice compared to WT SR exposed mice (**Figure 4-18B**). The results suggest TLR9 is important for MHCII and CD40 upregulation on interstitial macrophages. Taken together, TLRs 2 and 9 appear to cooperate in the upregulation of CD80, CD86, MHCII, and CD40 on interstitial macrophages.

Signaling Through TLR2 Inhibits Costimulatory Molecule Upregulation on Alveolar Macrophages (AMs)

The lung also contains a macrophage population present in the alveoli of the lung responsible for immune surveillance and phagocytosis of microorganisms in the airways. To investigate the AM population after repeated SR exposure, mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 18 (1 day after the last exposure) or day 21 (4 day after the last exposure). To identify AMs, dispersed lung cells were incubated with antibodies to CD11c, CD205, F4/80, Gr-1, and CD11b and measured by flow cytometry. The gating strategy for the detection of AMs (CD11c^{+} / CD205^{+} / F4/80^{+} /
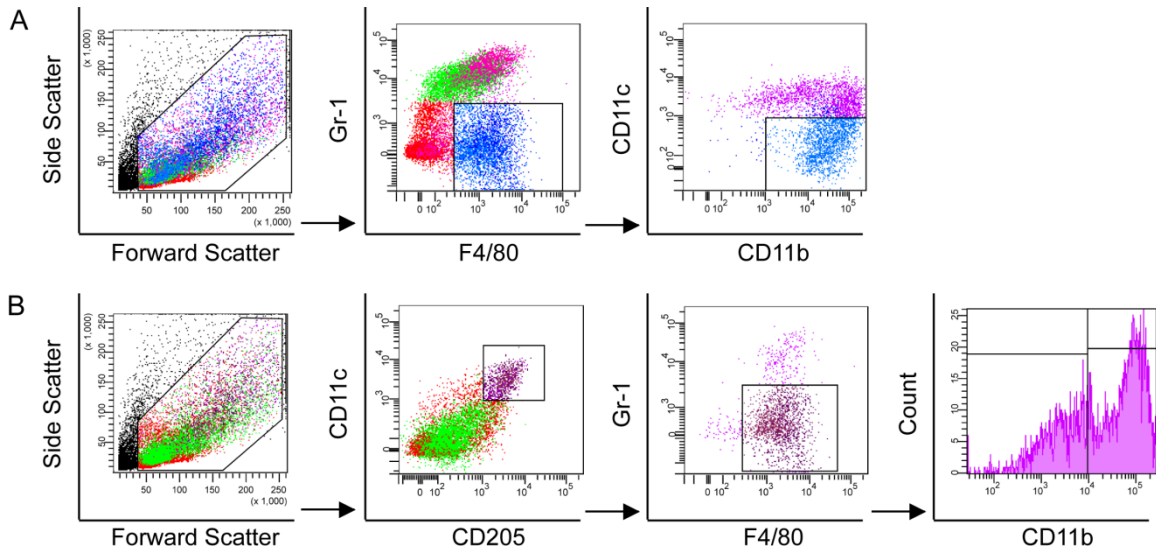
Figure 4-16. Gating strategy for the identification of interstitial and alveolar macrophages in a lung cell suspension.
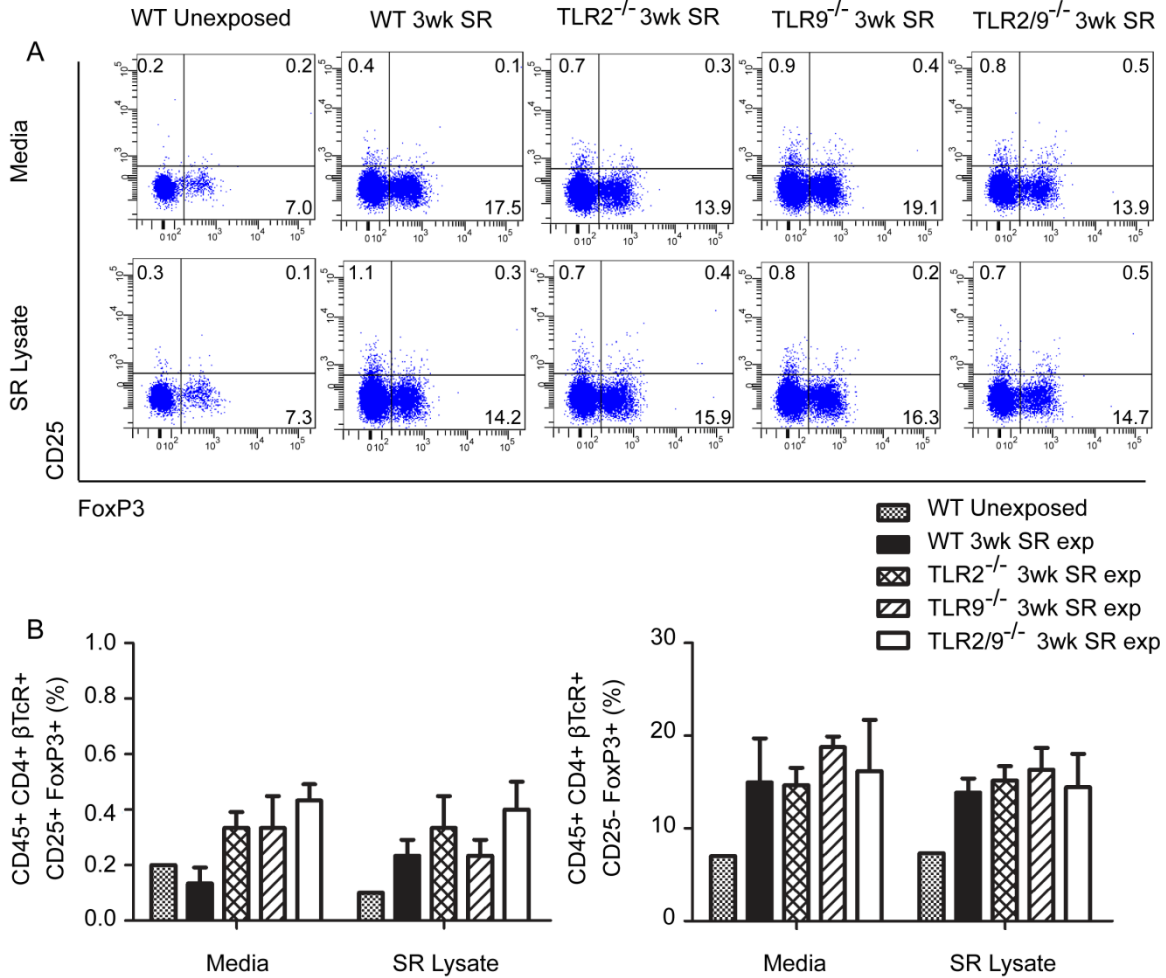
WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, and CD205 and analyzed by flow cytometry. (A) Interstitial macrophages were identified as F4/80$^+$ / CD11b$^+$ / CD11c$^-$ / Gr-1$^-$. (B) Alveolar macrophages (AMs) were identified as CD11c$^+$ / CD205$^+$ / F4/80$^+$ / Gr-1$^-$ / CD11b^{low}. In SR exposed mice, an additional AM population was identified as CD11c$^+$ / CD205$^+$ / F4/80$^+$ / Gr-1$^-$ / CD11b^{high}.
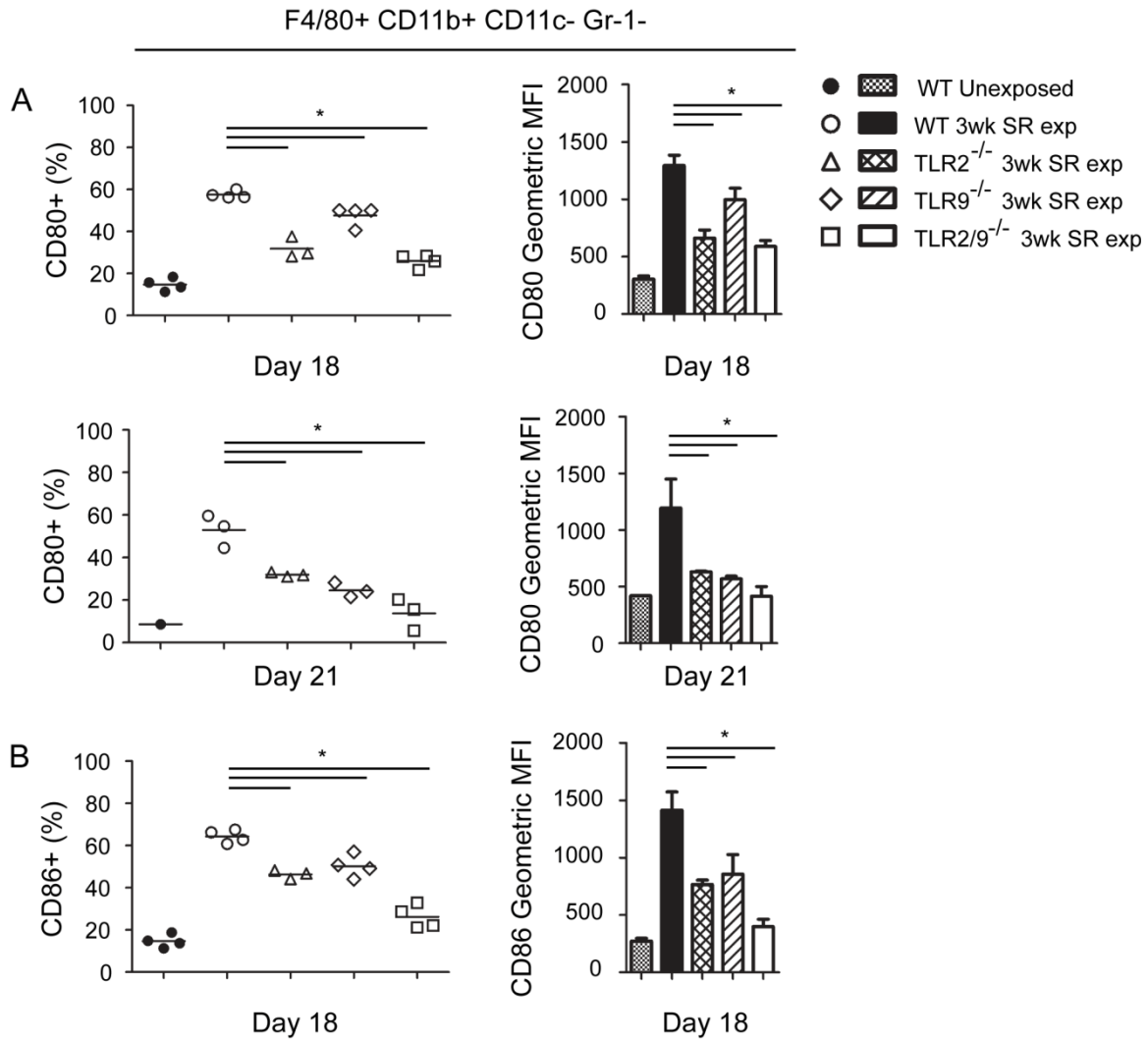
Figure 4-17. TLRs 2 and 9 cooperate in CD80 and CD86 upregulation on interstitial macrophages after 3 weeks exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, CD80, and CD86 and analyzed by flow cytometry. Interstitial macrophages were identified as (F4/80^{+} / CD11b^{+} / CD11c^{-} / Gr-1^{-}). The percentage of interstitial macrophages that express (A) CD80 and (B) CD86 and the geometric mean fluorescence intensity (MFI) was measured (n = 3 - 4 mice per group. On day 21, the lung cells from 3 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).
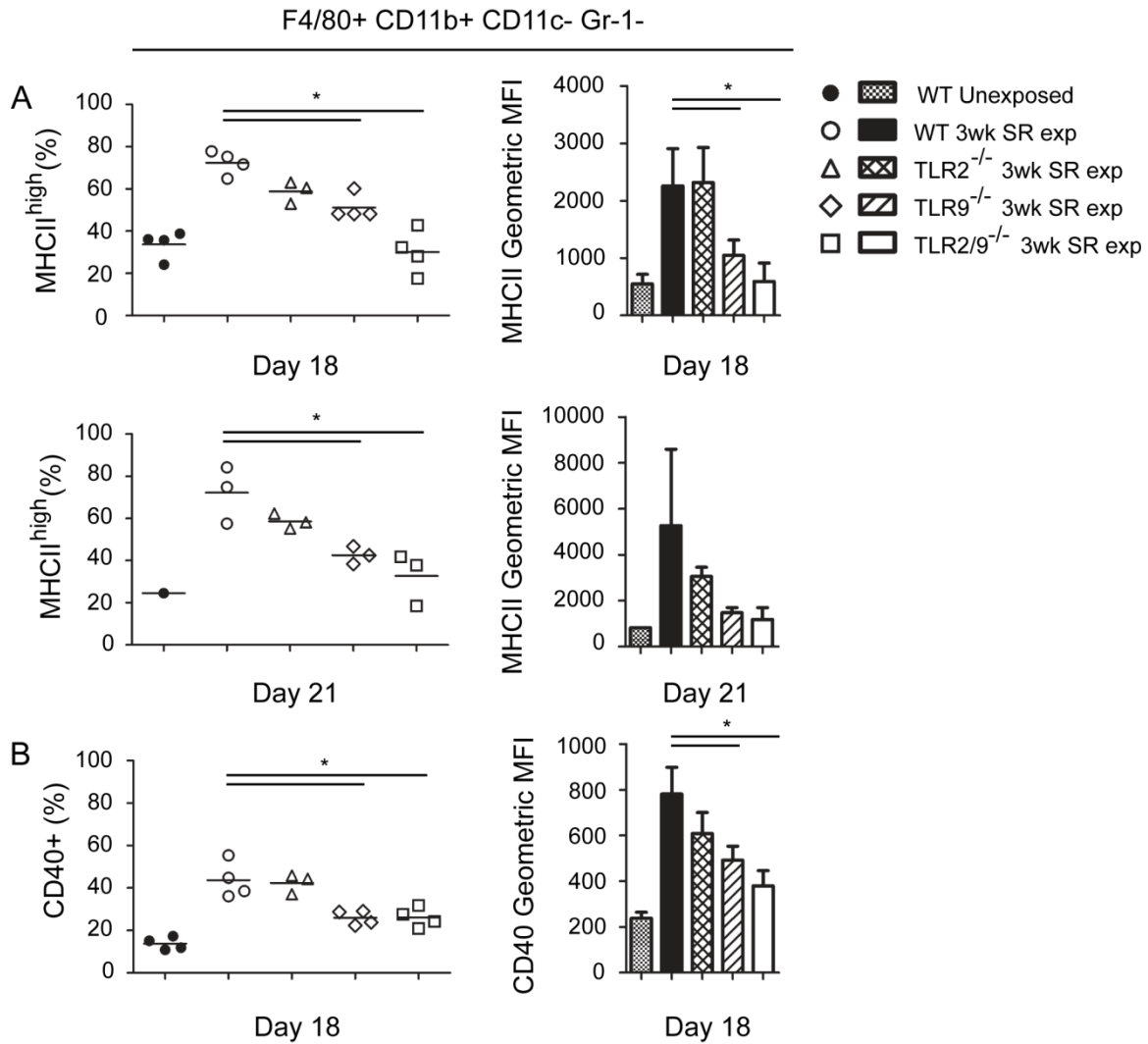
Figure 4-18. TLR9 is required for MHCII and CD40 upregulation on interstitial macrophages after 3 weeks exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, MHCII, and CD40 and analyzed by flow cytometry. Interstitial macrophages were identified as (F4/80$^+$ / CD11b$^+$ / CD11c$^-$ / Gr-1$^-$). The percentage of interstitial macrophages that express (A) MHCII and (B) CD40 and the geometric mean fluorescence intensity (MFI) was measured (n = 3 - 4 mice per group. On day 21, the lung cells from 3 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

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Gr-1$^-$ / CD11b^{low}) is shown in **Figure 4-16B**. In SR exposed mice, an additional CD11b^{high} AM population was detected, which was CD11c$^+$ / CD205$^+$ / F4/80$^+$ / Gr-1$^-$ / CD11b^{high}. WT SR exposed mice had a greater percentage of CD11b^{high} AMs than CD11b^{low} AMs on day 18 and 21 (**Figure 4-19**). However, TLR2/9$^{-/-}$ mice had a significantly greater percentage of CD11b^{low} AMs in the lung compared to WT SR exposed mice on day 18. On day 21, there was also a significantly greater percentage of CD11b^{low} AMs in TLR2$^{-/-}$ mice as well as TLR2/9$^{-/-}$ mice compared to WT SR exposed mice. Therefore, TLR2 appears to play an important role in shifting the balance between CD11b^{low} AM and CD11b^{high} AM populations in the lung after repeated SR exposure.

In healthy individuals AMs are usually in a quiescent state; however, AMs in patients with HP have increased costimulatory molecule expression (Israel-Assayag et al., 1999). Previous studies in mice have shown an increased percentage of CD11c$^+$ cells that expressed CD86 and MHCII in the lung after exposure to SR and sendai virus, but only low levels when exposed to SR alone (Girard et al., 2009). To determine if loss of TLRs 2 and 9 alters costimulatory molecule and MHCII expression after repeated SR exposure, AMs were additionally stained with CD80 and MHCII, and analyzed by flow cytometry. In WT SR exposed mice, the percentage of AMs that expressed CD80 was similar to unexposed mice on day 18 (**Figure 4-20A**). However, TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice had a significant increase in the percentage of AMs that expressed CD80. On day 21, the percentage of AMs that expressed CD80 was lower than day 18, however TLR2/9$^{-/-}$ still had a significant increase in the percentage of cells that expressed CD80. The results suggest signaling through TLR2 is important in inhibiting CD80 expression on AMs after SR exposure.

The expression of MHCII was also measured on AMs. On day 18, TLR2$^{-/-}$ mice had a significant increase in the percentage of AMs that expressed MHCII^{high} compared to WT SR exposed mice (**Figure 4-20B**). In contrast, TLR9$^{-/-}$ mice had a significant decrease in the percentage of AMs that expressed MHCII^{high} and the geometric MFI. However, there was no significant difference in TLR2/9$^{-/-}$ mice compared to WT exposed mice. On day 21, there is no significant difference in the percentage of AMs that express MHCII^{high} in KO mice compared to WT exposed mice, suggesting the increase in MHCII expression was transitory. Altogether, the results suggest signaling through TLR2 is important for inhibiting MHCII expression on AMs. In addition, signaling through TLR9 may be important for MHCII upregulation as was seen in interstitial macrophages.

The expression of CD80 and MHCII was also examined on the CD11b^{high} AMs in SR exposed mice on day 18 and 21. In contrast to the CD11b^{low} AMs, TLR2/9$^{-/-}$ cells had a significant decrease in the percentage of cells that expressed CD80 on day 18, and the geometric MFI of CD80 was significantly decreased in TLR2$^{-/-}$ as well as TLR2/9$^{-/-}$ mice compared to WT SR exposed mice (**Figure 4-21A**). By day 21, there was no significant difference in CD80 expression between WT and KO SR exposed mice, suggesting the upregulation of CD80 was delayed in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice. The results suggest signaling through TLR2 is important for CD80 expression on CD11b^{high} AMs after repeated SR exposure. Although CD80 expression was decreased, MHCII

Figure 4-19. TLR2/9$^{-/-}$ mice have a significant increase in the percentage of CD11b^{low} AMs after 3 weeks exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, CD205 and analyzed by flow cytometry. AMs were identified as (CD11c^{+} / F4/80^{+} / CD205^{+} / Gr-1^{-} / CD11b^{low}). In SR exposed mice, a CD11c^{+} / CD205^{+} / F4/80^{+} / Gr-1^{-} / CD11b^{high} AM population was also detected. The percentage of CD11b^{low} AMs and CD11b^{high} AMs in SR exposed mice (n = 3 - 4 mice per group. On day 21, the lung cells from 3 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

Figure 4-20. Loss of TLR2 leads to upregulation of CD80 and MHCII on AMs after 3 weeks exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested and incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, CD205, and CD80 and analyzed by flow cytometry. (A) The percentage of AMs (CD11c^{+} / F4/80^{+} / CD205^{+} / Gr-1^{-} / CD11b^{low}) that express CD80 and the geometric MFI was measured. (B) The percentage of AMs that express MHCII^{high} and the geometric MFI was measured. (n = 3 - 4 mice per group. On day 21, the lung cells from 3 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

CD11c+ CD205+ F4/80+ Gr-1- CD11b^{low}

A

Day 18

Day 18

Legend:
- ● / crosshatch — WT Unexposed
- ○ / black — WT 3wk SR exp
- △ / crosshatch — TLR2$^{-/-}$ 3wk SR exp
- ◇ / diagonal — TLR9$^{-/-}$ 3wk SR exp
- □ / white — TLR2/9$^{-/-}$ 3wk SR exp

Day 21

Day 21

B

Day 18
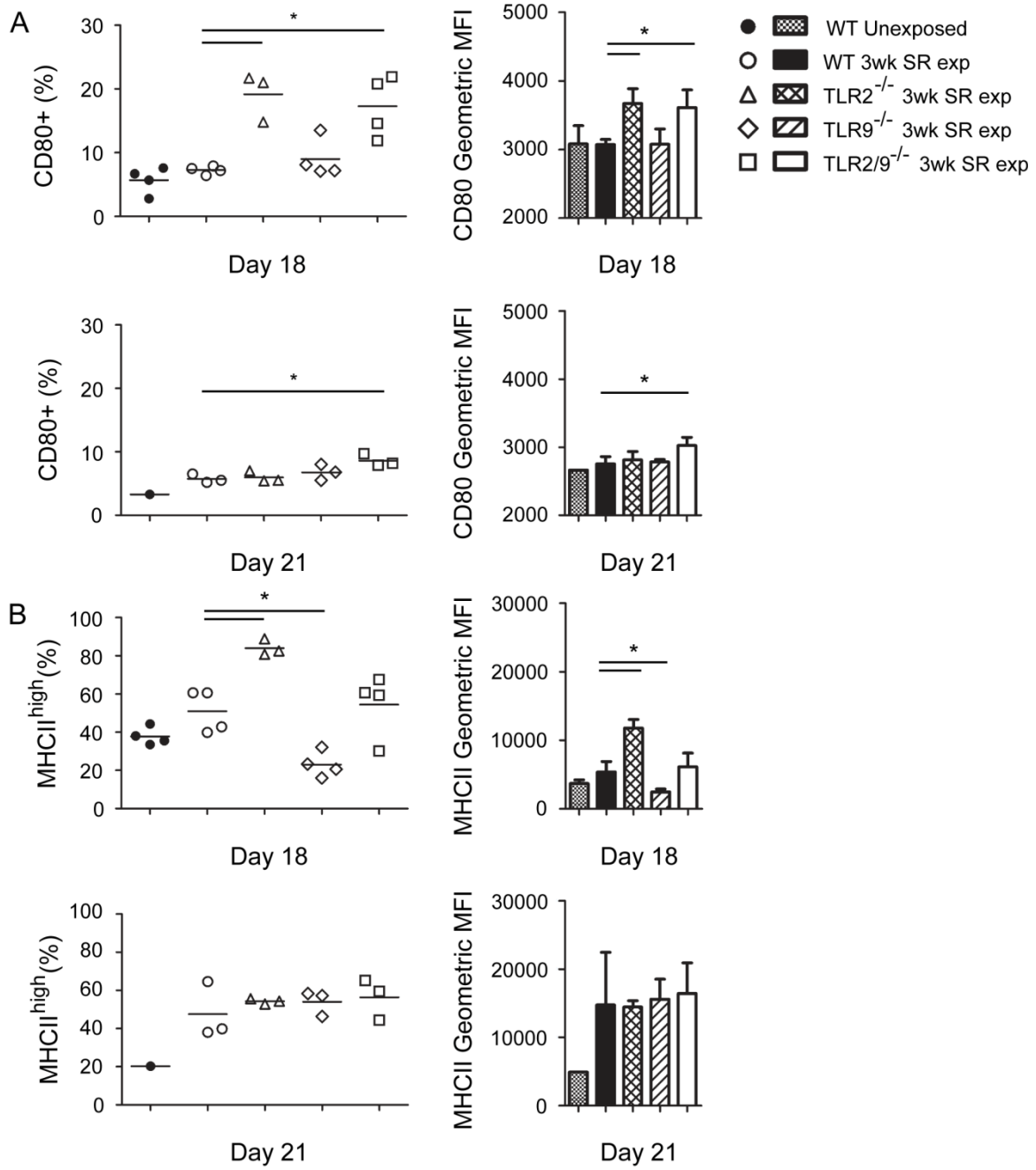
Day 18

Day 21

Day 21

Figure 4-21. Loss of TLR2 leads to downregulation of CD80 on CD11b^{high} AMs after 3 weeks exposure to SR.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed on day 18 (1 day after the last exposure) or day 21 (4 days after the last exposure). Lung cells were digested incubated with antibodies to CD11b, F4/80, Gr-1, CD11c, CD205, and MHCII and the cells analyzed by flow cytometry. (A) In SR exposed mice, the percentage of CD11b^{high} AMs (CD11c$^+$ / F4/80$^+$ / CD205$^+$ / Gr-1$^-$ / CD11b^{high}) that express CD80 and the geometric mean fluorescence intensity (MFI) was measured. (B) In SR exposed mice, the percentage of CD11b^{high} AMs that express MHCII^{high} and the geometric mean fluorescence intensity (MFI) was measured (n = 3-4 mice per group. On day 21, the lung cells from 3 unexposed mice were combined). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05 compared to WT SR exposed mice).

CD11c+ CD205+ F4/80+ Gr-1- CD11b^high

A

B

Legend:
- ● WT Unexposed
- ○ WT 3wk SR exp
- △ TLR2^-/- 3wk SR exp
- ◇ TLR9^-/- 3wk SR exp
- □ TLR2/9^-/- 3wk SR exp

expression was significantly increased in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ CD11b^{high} AMs compared to WT SR exposed mice similarly to CD11b^{low} AMs on day 18 (**Figure 4-21B**). However on day 21, there was no significant difference in MHCII expression in WT and KO SR exposed groups suggesting the upregulation of MHCII was accelerated in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice. Altogether, the results suggest signals through TLR2 are also important for inhibiting MHCII expression on CD11b^{high} AMs.

TLRs 2 and 9 Are Not Required for Granuloma Formation

Cellular interactions through receptor binding and cytokine stimulation between antigen presenting cells and T cells are crucial for granuloma formation. TLR2/9$^{-/-}$ mice have significant reductions in inflammatory cytokine expression, T cell activation, the Th17 response, and MHCII and costimulatory molecule expression on interstitial macrophages. To determine if loss of TLRs 2 and 9 also results in reduced granuloma formation, mice were exposed to SR 3 times a week for 3 weeks and sacrificed on day 20 (3 days after the last exposure). The left lung lobe was harvested, fixed in formalin, and paraffin embedded sections were stained with H&E (**Figure 4-22A-E**). Wild type mice exposed to SR displayed peribronchiolar areas of granuloma formation while unexposed mice exhibited normal lung architecture (**Figure 4-22A** and **B**). The TLR2$^{-/-}$, TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice exposed to SR all demonstrated granuloma formation (**Figure 4-22C-E**). The results suggest loss of TLRs 2 and 9 is not sufficient to inhibit granuloma formation in SR exposed mice. This suggests that additional PRRs must be involved in granuloma formation in HP.

Discussion

Initial studies during the acute phase of HP showed the alveolitis after a single SR exposure was predominantly neutrophilic, and neutrophil recruitment was dependent on TLRs 2 and 9. After 3 weeks repeated SR exposure, the alveolitis is comprised of neutrophils, monocytes, and lymphocytes. At this time point, TLR2/9$^{-/-}$ mice still have significant reductions in neutrophil recruitment, as well as neutrophil chemokine production, with TLR2 being the dominant receptor for CXCL2 expression. The reduction in neutrophil recruitment in TLR2/9$^{-/-}$ mice was offset by a significant increase in CD11b$^+$ Gr-1$^-$ monocytes. Lymphocytes also make up a large percentage of the alveolitis. T cells and B cells were more numerous than γδ T cells or NK cells; however there was also no significant difference in the number of lymphocytes in WT or KO mice at this time point. Altogether the results suggest TLR2 is the dominant receptor for neutrophil recruitment with minor contributions from TLR9, but in the absence of TLRs 2 and 9, additional pattern recognition receptors can also contribute to monocyte and lymphocyte recruitment in response to repeated SR exposure.

Although the number of lymphocytes recruited to the airways was not significantly different between WT and KO SR exposed mice, there was a significant
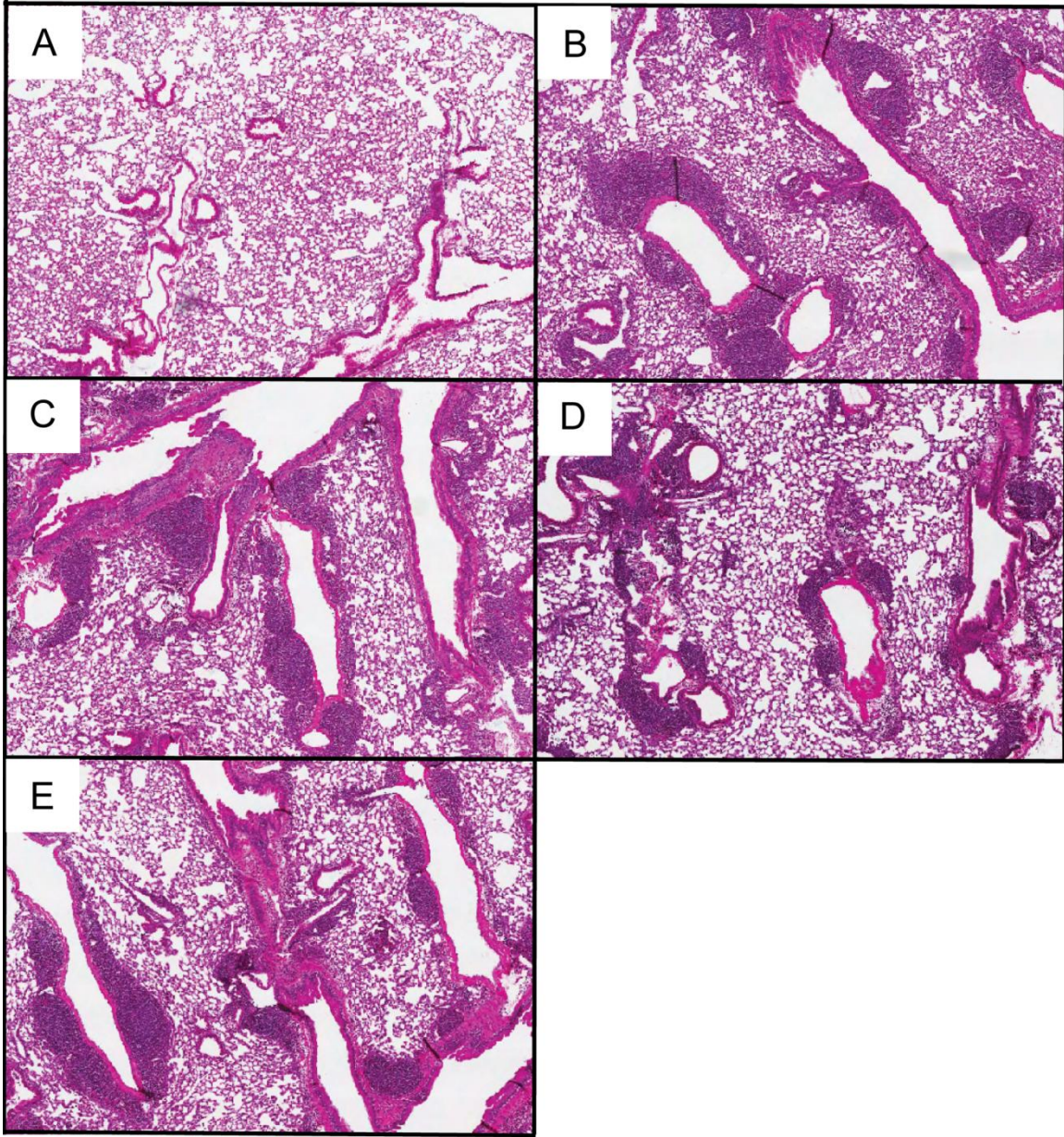
Figure 4-22. Granuloma development is not dependent on TLRs 2 and 9.

WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 3 weeks and analyzed 3 days after the last exposure. Representative H&E stained lung sections from WT unexposed mice (A), WT SR exposed mice (B), TLR2$^{-/-}$ SR exposed mice (C), TLR9$^{-/-}$ SR exposed mice (D), and TLR2/9$^{-/-}$ SR exposed mice (E). (Original magnification x4).

reduction in the percentage of CD4$^+$ T cells in the lung of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ on day 20. There was no difference in the percentage of CD4$^+$ Th cells that expressed CXCR3 or CCR6 between WT and KO mice, although we cannot rule out a reduction in percentage of Th cells that express other chemokine receptors. The expression of chemokines CCL2 and CCL4, which bind to CCR2 and CCR5 respectively, was reduced in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice, but not significantly different compared to WT SR exposed mice. However, the reduction in chemokine expression could contribute to the reduced percentage of CD4$^+$ T cells in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice.

The percentage of CD4$^+$ T cells that expressed the activation marker CD69 was significantly reduced in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice the BAL and in the lung on day 20. This suggests signaling through TLR2 is important for T cell activation. Activation of naïve CD4$^+$ T cells requires stimulation of the T cell receptor via antigen presented on MHCII, as well as CD28 stimulation through binding of costimulatory molecules CD80 and CD86. Effector memory T cells also require MHCII stimulation for activation. The expression of CD80 and CD86 on interstitial macrophages was significantly reduced in KO mice compared to WT after repeated SR exposure. MHCII expression was also significantly reduced in TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice. Therefore, the reduced percentage of CD4$^+$ Th in the lung after repeated SR exposures, as well as the reduced percentage of CD4$^+$ Th cells expressing the activation marker CD69 could be due to the reduced capacity of antigen presenting cells to activate naïve T cells in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice.

However, naïve T cells require a third signal for activation through cytokine stimulation from antigen presenting cells. Previous studies rev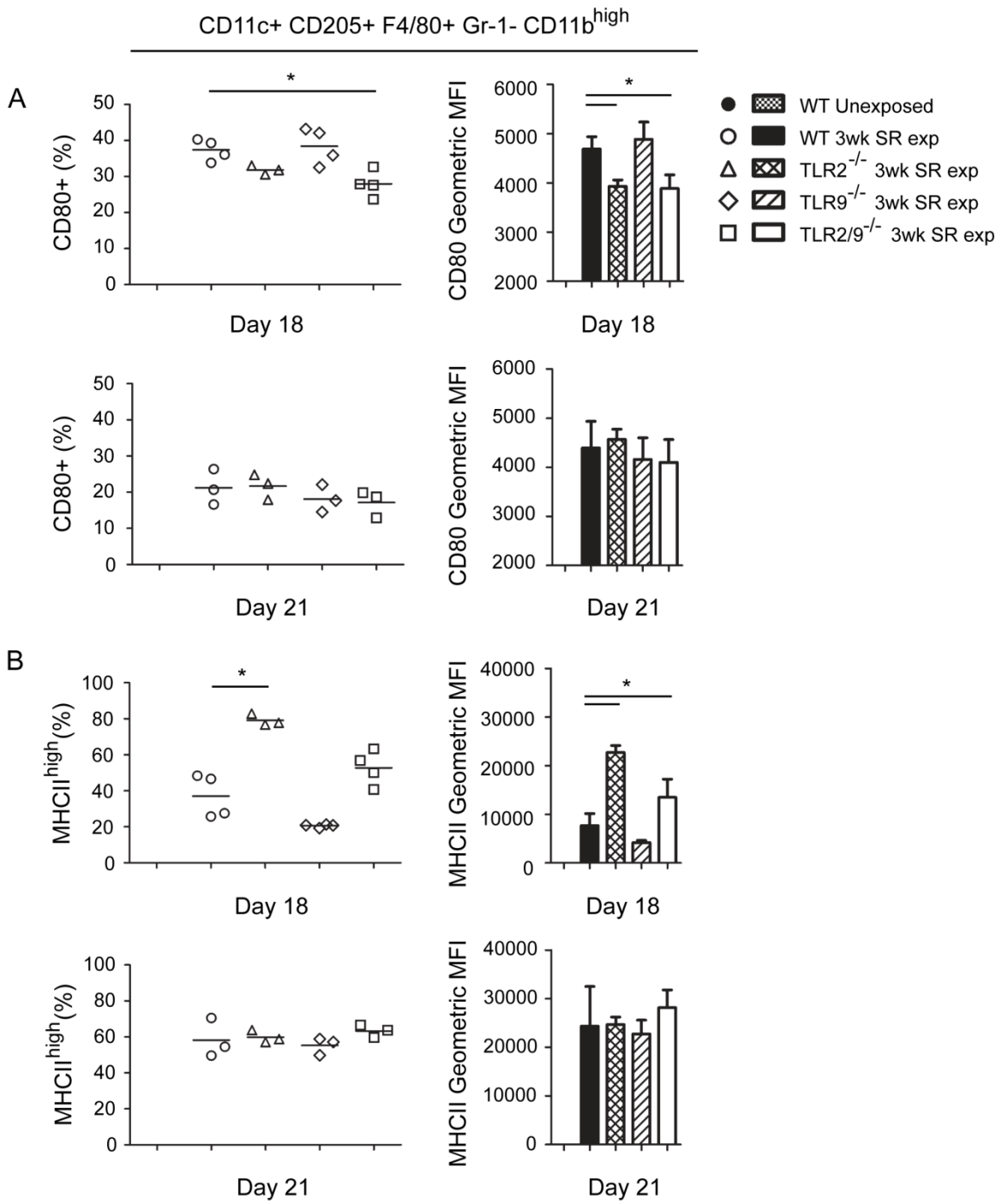ealed LPS induced MyD88-independent stimulation was sufficient for maturation of dendritic cell including upregulation of costimulatory molecules and migration to the draining lymph node, but that MyD88-dependent receptor induced upregulation of cytokine secretion was necessary to overcome Treg suppression of T cell activation (Pasare and Medzhitov, 2004). This suggests SR induced signaling through TLR2 may be important for the induction of cytokines in antigen presenting cells necessary for naïve T cell activation. IL-6 was shown to be the critical cytokine stimulation required to overcome Treg suppression of naïve T cell activation (Pasare and Medzhitov, 2003). The expression of TNFα was significantly reduced in the lung in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice, but not IL-6, which may explain why CD4$^+$ T cell activation was reduced, but not absent.

IL-6 in combination with TGFβ leads to the polarization of Th17 cells in the mouse, while IL-23 induces proliferation and expansion of pathogenic Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; McGeachy et al., 2007; Zhou et al., 2007). The Th17 response is generated after repeated SR exposure and is associated with increased lung severity and fibrosis (Joshi et al., 2009; Simonian et al., 2009), and previous studies have shown that the expression of Th17 cytokines was reduced in the absence of TLR6 (Fong et al., 2010). In this study, we found the expression of IL-17 was significantly reduced in TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice. IL-22, another Th17 associated cytokine was also significantly reduced in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice.

The reduced IL-17 expression after 3 weeks exposure to SR correlated with a reduced percentage of Th17 cells in the lung in all KO mice compared to WT SR exposed mice on day 18, suggesting TLRs 2 and 9 contribute to the Th17 response. However, only TLR2/9$^{-/-}$ mice had a significant reduction in the percentage of Th17 cells in the lung on day 21, suggesting TLR2/9$^{-/-}$ are additionally deficient in maintaining the Th17 response. The Th17 cells from SR exposed mice secreted IL-17A but not IL-17F as was shown previously (Simonian et al., 2009). Studies during the acute phase showed TLR2/9$^{-/-}$ mice were deficient in IL-23p19 expression, which could contribute to reduced Th17 proliferation in the lung; however there was no significant difference in the expression of IL-23p19 in WT and KO mice on day 18. The reduction in the Th17 response in TLR2/9$^{-/-}$ mice on day 21 was also not associated with a switch to a Th1 response.

CD25^{+} FoxP3^{+} Treg cells develop in the thymus and are important in controlling inflammatory responses, and previous studies have shown that CD25^{+} CD4^{+} regulatory cells are important for controlling IFNγ producing T cells in HP (Park et al., 2009). A small percentage of CD25^{+} FoxP3^{+} CD4^{+} T cells were detected in the lung on day 21, whereas the vast majority of FoxP3^{+} cells were CD25^{-}. Previous studies have shown CD25^{-} CD4^{+} T cells can be induced to upregulate FoxP3 and adopt a regulatory phenotype in the presence of TGFβ (Fantini et al., 2004; Fu et al., 2004). The increase in CD25^{-} FoxP3^{+} CD4^{+} Treg cells suggests that induced Treg cells may play a more important role in inhibiting the immune response in the lung after SR exposure.

However, the presence of TNFα has been shown to inhibit the suppressive activity of Tregs by inducing dephosphorylation of FoxP3 (Nie et al., 2013; Valencia et al., 2006). TLR2/9$^{-/-}$ mice have a significant decrease in TNFα expression in the lung, as well as decreased percentage of CD25^{-} CD4^{+} Th cells that express TNFα in response to SR lysate stimulation. Therefore, the reduction in the percentage of Th17 cells in TLR2/9$^{-/-}$ mice may not be due to an increase in the percentage of natural or induced Treg cells, but instead the Treg cells may have an increased ability to suppress T cells responses in TLR2/9$^{-/-}$ mice due to the significant decrease in TNFα in the lungs compared to WT SR exposed mice. In support of this hypothesis, previous studies have shown that Tregs isolated from patients with active HP and high concentrations of IL-17 and TNFα in the BALF are deficient in suppressing T cell proliferation ex vivo (Girard et al., 2011). Further studies are needed to test this hypothesis.

The reduction in CD4^{+} Th cells in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice was associated with an increase in the percentage of B cells in the lung on day 20, and TLR2/9$^{-/-}$ mice on day 21. Although not statistically significant, an increased number of B cells was also seen in the BAL on day 18 and day 20. However, the percentage of B cells that expressed the activation maker CD69 was significantly reduced in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice, suggesting signaling through TLR2 is important for activation of B cells. Further experiments are necessary to establish if the reduced activation of B cells is due to loss of TLR2 on the B cell, or a result of the reduced activation of Th cells. B cells bind antigen through their B cell receptor and display antigen on MHCII. When CD4^{+} Th cells recognize the antigen displayed on MHCII, they stimulate B cells through CD40-CD40L interactions to enter germinal centers and undergo affinity maturation and class

switching. Therefore, the reduction in the percentage of activated B cells in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice on day 20 could be due to the reduced percentage of activated CD4$^+$ Th cells also seen on day 20.

Alveolar macrophages are normally in a quiescent state in a healthy lung; however, in patients with HP, the expression costimulatory molecules increased (Israel-Assayag et al., 1999). WT mice did not significantly upregulate CD80 on AMs compared to unexposed mice; however, TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice had increased expression of CD80 on AMs on day 18, and CD80 expression remained elevated on TLR2/9$^{-/-}$ mice on day 21. This suggests signaling through TLR2 is required to inhibit costimulatory molecule expression on AMs. SR exposed mice had an increase in the percentage of CD11b^{high} AMs, and this macrophage population was significantly reduced in TLR2/9$^{-/-}$ mice. CD11b^{high} AMs have been reported in patients with HP, as well as sarcoidosis, and idiopathic pulmonary fibrosis (Krombach et al., 1996). Previous studies have suggested CD11b is important for the recruitment of AMs into the lung after *Streptococcus pneumoniae* challenge (Kirby et al., 2006). In contrast to the CD11b^{low} AMs, the expression of costimulatory molecule CD80 was significantly reduced on the CD11b^{high} AMs in TLR2/9$^{-/-}$ mice suggesting TLRs 2 and 9 are required for CD80 upregulation in this cell type. MHCII was upregulated in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice on day 18 on AMs, as well as CD11b^{high} AMs, but there was no difference between groups on day 21, suggesting loss of TLR2 accelerates MHCII upregulation. Altogether, the results suggest repeated SR exposure leads increased MHCII expression on AMs, and costimulatory molecule upregulation on CD11b^{high} AMs, as has been observed in patients; but loss of signaling through TLR2 leads to increased CD11b^{low} AM activation. Previous studies have shown increased expression of CD86 on CD11c$^+$ cells in the lung after sendai virus infection and exposure to SR, which supports the authors hypothesis that prior viral infection leads to increased immune responses to HP antigens (Girard et al., 2009; Israel-Assayag et al., 1999). TLR-induced immune responses have been shown to be inhibited following viral infections (Didierlaurent et al., 2008; Oliver et al., 2008), which is associated with increased risk of secondary bacterial infection. Further research into the relationship between viral infection and subsequent TLR induced SR sensitization is needed.

Three weeks exposure to SR resulted in increased interstitial macrophage activation as evidenced by an increased percentage of interstitial macrophages that expressed CD80, CD86, CD40, and high levels of MHCII. Mice deficient in TLR2 or TLR9 had a reduced percentage of interstitial macrophages that expressed CD80 or CD86, which was further reduced in TLR2/9$^{-/-}$ mice, suggesting TLRs 2 and 9 cooperate in the upregulation of CD80 and CD86. In addition, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice had a significant reduction in the percentage of MHCII^{high} and CD40 expressing cells. Altogether, the results suggest signaling through TLRs 2 and 9 cooperate in interstitial macrophage activation in response to SR exposure. Classically activated macrophages as well as regulatory macrophages require TLR stimulation for activation (Edwards et al., 2006). Further experiments would be necessary to determine if repeated SR exposure leads to classically activated macrophages typified by inflammatory cytokine secretion and the upregulation of nitric oxide synthase, regulatory macrophages identified by IL-10

secretion, or if in the absence of TLR 2 and 9 signaling, SR exposure induces an alternatively activated macrophage phenotype.

Granuloma formation depends on the interaction between T cells and macrophages through physical contact as well as cytokine signals. TLR2/9$^{-/-}$ mice had reduced T cell activation, cytokine expression, as well as interstitial macrophage costimulatory molecule expression and MHCII upregulation, which suggested TLR2/9$^{-/-}$ may also be deficient in granuloma formation. However, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice formed granulomas similarly to WT SR exposed mice. Further experiments would be necessary to determine if granulomas resolve faster, or if the cellular composition of the granuloma is altered in TLR2$^{-/-}$, TLR9$^{-/-}$, or TLR2/9$^{-/-}$ mice.

In summary, mice deficient in TLRs 2 and 9 are partially protected from SR induced HP. TLR2/9$^{-/-}$ mice have a significant reduction in neutrophils at this time point, as well as a significant reduction in activated CD4$^+$ Th cells, two cell types that have been associated with increased lung pathology. In addition, TLR2/9$^{-/-}$ have a significant reduction in the percentage of Th17 cells after three weeks SR exposure, a cell type that has been associated with lung fibrosis in HP. In the next chapter, we will explore whether TLR2/9$^{-/-}$ mice are protected from developing chronic HP.

CHAPTER 5.   CHRONIC PHASE

Introduction

Chronic HP (CHP) is associated with emphysema and a restrictive lung disease characterized by fibrosis and reduced lung compliance (Costabel et al., 2012; Erkinjuntti-Pekkanen et al., 1998). CHP is progressive and irreversible and is associated with high morbidity and mortality (Hanak et al., 2008; Vourlekis et al., 2004). At this stage, antigen avoidance will not resolve symptoms but can prolong survival (Fernandez Perez et al., 2013). Deaths where HP is the underlying or contributing cause have steadily increased from 1979 to 2007 (NIOSH., 2012). However little is known about the transition between HP and CHP. One hypothesis suggests the transition to progressive fibrosis in CHP is associated with a switch to a Th2 response. A study which looked at T cell responses in patients with subacute and chronic pigeon breeder's disease, another common type of HP, found an increase in $CD4^+$ and $CD8^+$ T cells expressing IL-4 and a decrease in T cells expressing IFNγ in patients with CHP compared to subacute HP (Barrera et al., 2008). This is in agreement with a mouse model of chronic pigeon breeder's disease in which the authors found that IL-4 / IL-13 expression and a Th2-biased immune response correlated with increased lung fibrosis (Mitaka et al., 2011). Fibrotic diseases are often associated with the expression of Th2-associated cytokines IL-4 and IL-13 (Gharaee-Kermani et al., 2001; Huaux et al., 2003; Jakubzick et al., 2003; Lee et al., 2001). Wound healing alternatively activated macrophages, which are polarized in the presence of IL-4, have been shown to be both pathogenic and protective in fibrosis development (Gibbons et al., 2011; Pesce et al., 2009). However, previous studies have shown IL-4 and Th2-biased immune responses are associated with reduced disease severity in SR induced HP (Butler et al., 2002; Gudmundsson et al., 1998; Jimenez-Alvarez et al., 2010; Matsuno et al., 2007). Therefore, further examination of Th2 immune responses during SR induced CHP is necessary.

Neutrophils have also been associated with lung fibrosis in CHP. There is a correlation between the level of neutrophils in the lung tissue and severity of fibrosis in patients with CHP (Pardo et al., 2000). The neutrophils expressed high levels of gelatinase B and collagenase 2, which contribute to lung damage. The IL-17 response is associated with the induction of neutrophil chemokines, neutrophil recruitment in the lung (Hellings et al., 2003; Laan et al., 1999; Miyamoto et al., 2003; Witowski et al., 2000). Other models of lung fibrosis such as bleomycin and IL-1-mediated lung fibrosis have also found IL-17 production by Th17 and γδ T cells is associated with increased lung fibrosis (Wilson et al., 2010). In addition, recent studies in mice have shown that neutrophils and macrophages were the main cell type that expressed IL-17A in HP and that neutrophil depletion decreased the amount of collagen in the lungs after SR exposure (Hasan et al., 2013). However, the role of IL-17 in fibrosis is controversial. A mouse model of experimental silicosis found that IL-17 produced by $CD4^+$ T cells and γδT cells was important for early inflammation and neutrophil recruitment, but IL-17 and signaling through the IL-17R was dispensable for TGFβ production and fibrosis development at later time points (Lo Re et al., 2010).

Studies during the acute and granulomatous phase show TLR2/9$^{-/-}$ mice have significant reductions in neutrophil recruitment, IL-17 expression, and the Th17 response. We hypothesize the reduced neutrophil recruitment and Th17 response in TLR2/9$^{-/-}$ mice will protect against the development of fibrosis during CHP. In this chapter, we will examine the immune response that develops in a mouse model of SR induced CHP to determine if mice switch from a Th17 to a Th2 response. In addition, we will measure lung compliance and fibrosis to determine if loss of TLRs 2 and 9 protect against the development of interstitial lung disease after long term exposure to SR.

Results

Mice with Long Term Exposure to SR Develop a Neutrophilic and Lymphocytic Alveolitis

To measure the cellular composition of the alveolitis in mice with CHP, mice were intranasally exposed to SR 3 times/week for 15 weeks, and BAL was performed 3 days after the last exposure. BAL cells were spun onto slides and stained for differential counting (**Figure 5-1**). WT and TLR2/9$^{-/-}$ mice exposed to SR for 15 weeks had a significant increase in the percentage of neutrophils compared to unexposed mice. Interestingly, TLR2/9$^{-/-}$ mice also had a significant increase in eosinophils compared to WT unexposed mice and SR exposed mice. However, neutrophils were still the dominant cell type in the alveolitis, as the percentage of eosinophils accounted for no more than 6% of the total BAL cells in TLR2/9$^{-/-}$ mice and less than 1% in WT SR exposed mice. The increase in the percentage of neutrophils was associated with a significant increase in the expression of the neutrophil chemokine CXCL2 mRNA in the lung tissue of 15 week SR exposed mice compared to unexposed mice (**Figure 5-2A**). The increase in eosinophils in TLR2/9$^{-/-}$ mice was not associated with an increase in the expression of CCL11/eotaxin, a chemokine associated with eosinophil recruitment (**Figure 5-2B**). The percentage of lymphocytes was also significantly increased compared to unexposed mice (**Figure 5-1**). There was a decrease in the percentage of macrophages in the BAL cells of mice exposed to SR for 15 weeks compared to unexposed mice due to the influx of immune cells into the lungs. Monocytes and large multinucleated macrophages were also observed in SR exposed mice. The results suggest mice with long term exposure to SR develop a neutrophilic and lymphocytic alveolitis.

We next wanted to know if the composition of the alveolitis differs in mice exposed long term to SR versus short term. Mice were intranasally exposed to SR for 4 or 14 weeks and BAL performed 2 days after the last exposure. BAL cells were stained with antibody to CD11b, CD11c, Gr-1, F4/80, and βTcR and immune cell populations were determined by flow cytometry. The gating strategy to discriminate neutrophils, AMs/macrophages, CD11c^{+} DCs, and T cells is depicted in **Figure 5-3A**. There was no significant difference in the percentage of neutrophils or T cells in the BAL cells, however there was a significant increase in the percentage of AMs and a significant
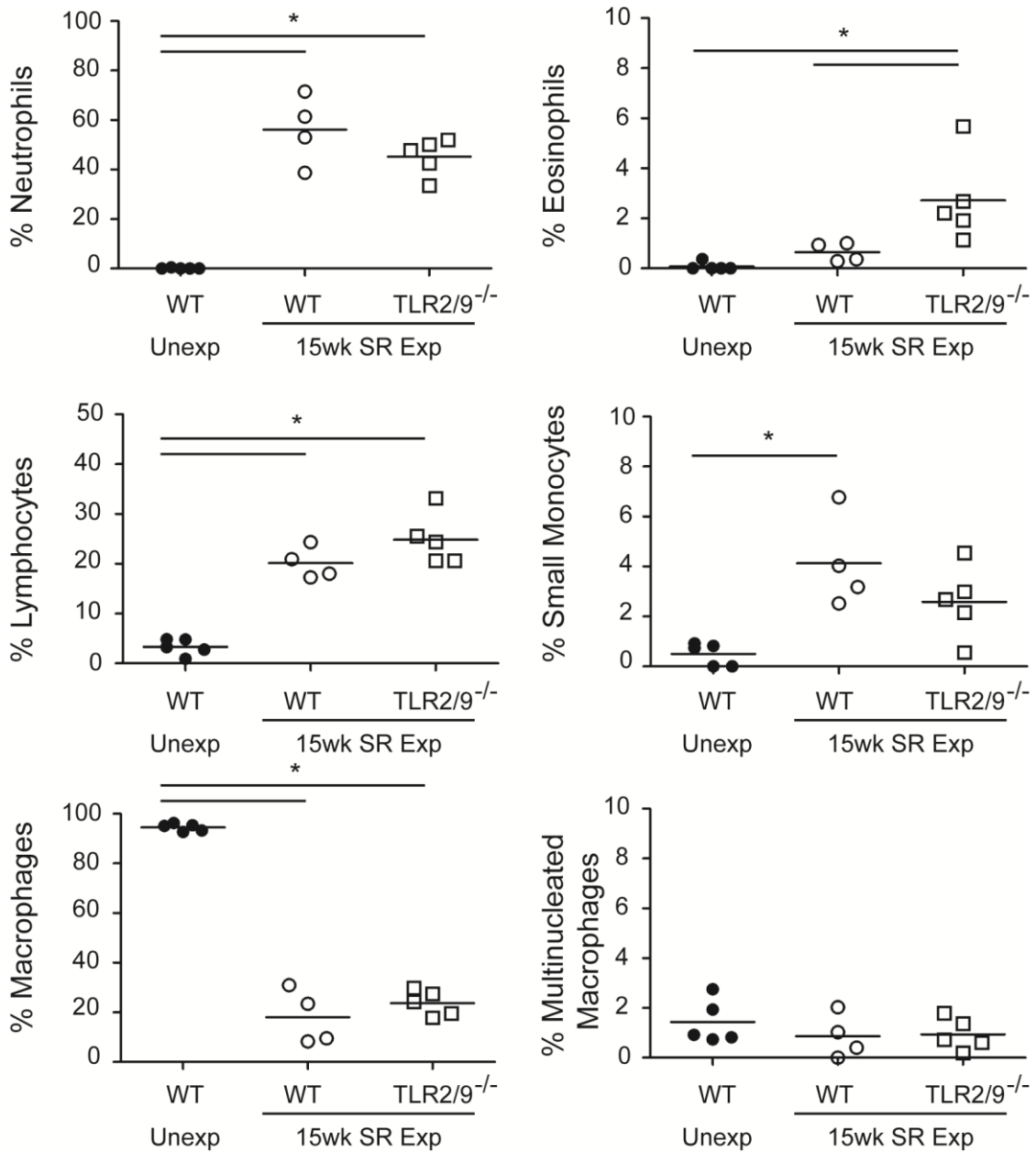
Figure 5-1.   Composition of alveolitis in mice exposed to SR for 15 weeks.

WT and TLR2/9$^{-/-}$ mice were exposed to SR for 15 weeks and analyzed 3 days after the last exposure. BAL was performed and cells recovered from the BAL fluid spun onto slides and stained with a modified Wright Giemsa stain (Diff quick) for differential counting (n = 4-5 mice per group). A minimum of 4 areas at 20x were examined until a minimum of 200 cells were counted. The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).
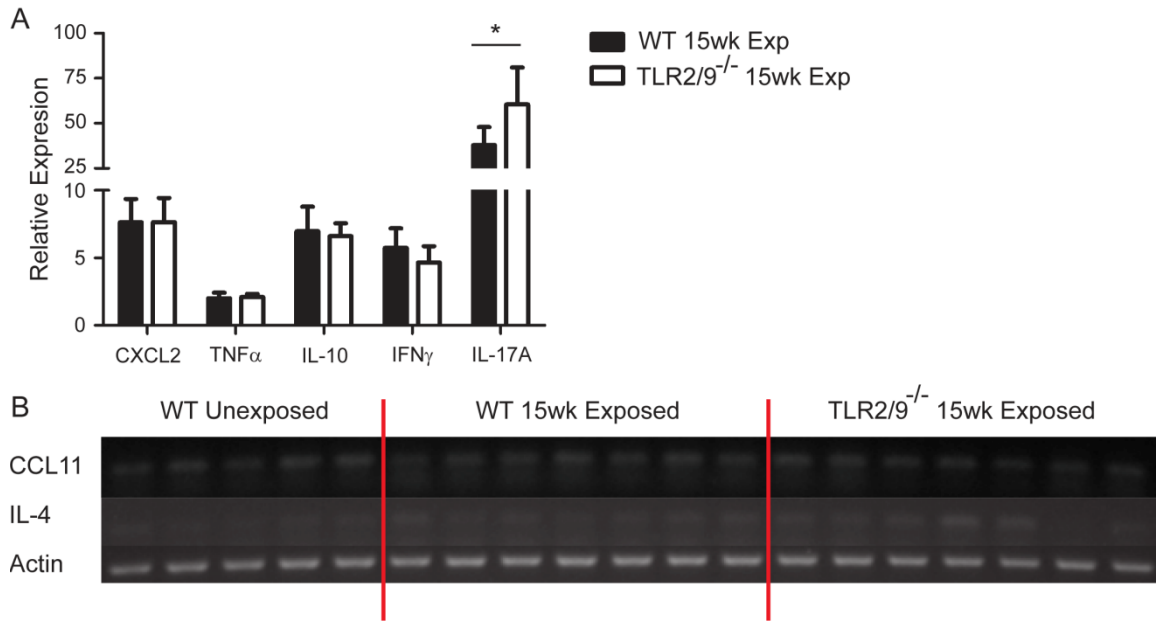
Figure 5-2.   Th17-associated cytokine expression detected in the lungs of mice exposed to SR for 15 weeks.

WT and TLR2/9$^{-/-}$ mice were exposed to 150 µg SR three times/week for 15 weeks and sacrificed 3 days after the last exposure. RNA was isolated from lung tissue and reverse transcribed to cDNA for real time PCR. (A) The expression of the chemokine CXCL2 and cytokines TNFα, IL-10, IFNγ, and IL-17A was normalized to HPRT. Data is expressed as relative expression compared to WT unexposed mice ± SD (n = 7-9 mice per group). Significance was determined using One-way ANOVA with Tukey post hoc test (*p < 0.05). (B) RNA was isolated from lung tissue and reverse transcribed to cDNA for RT-PCR. The expression of the Th2-associated chemokine CCL11/eotaxin and cytokine IL-4 was detected on an agarose gel stained with ethidium bromide. Actin expression was used as a housekeeping gene control (n=5-7 mice per group).

Figure 5-3.   Changes in BAL composition between 4 week and 14 week exposed mice.

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 4 or 14 weeks and analyzed 2 days after the last exposure. BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, CD11c, F4/80, and βTcR and analyzed by flow cytometry. (A) The gating strategy to discriminate neutrophil (CD11b^{+} / Gr-1^{high}), AM (CD11c^{+} / F4/80^{+}), macrophage (CD11c^{-} / F4/80^{+}), DC (CD11c^{+} / F4/80^{-}), and T cell (βTcR^{+} / SSc^{low}) populations present in the alveolitis. Autofluorescence^{+} AMs were detected in the βTcR^{+} / SSc^{high} gate due to spectral overlap with PE (Duan et al., 2012). (B) The percentage of neutrophils, T cells, AMs, CD11c^{+} DCs, and macrophages in the BAL (n = 4-5 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$).
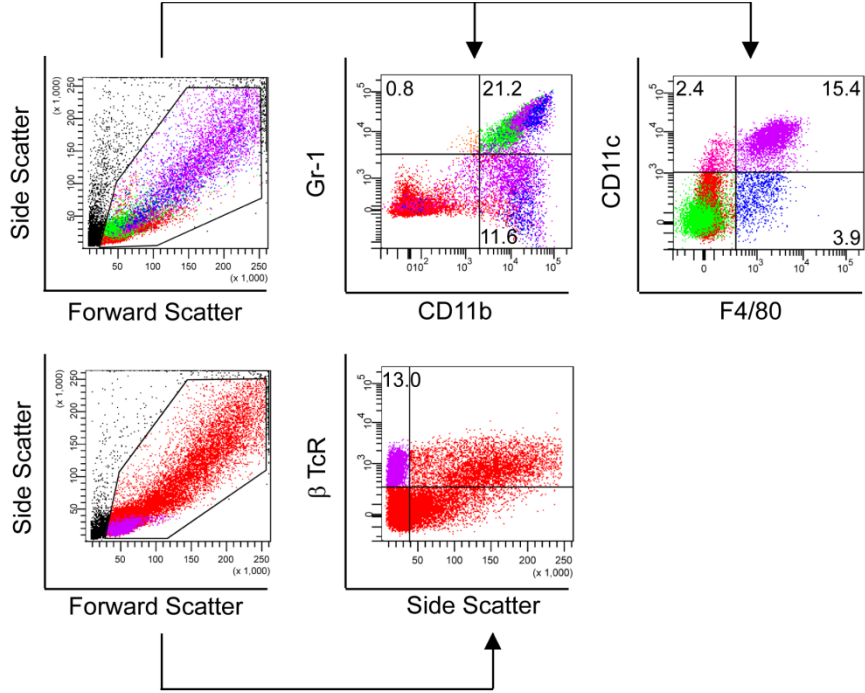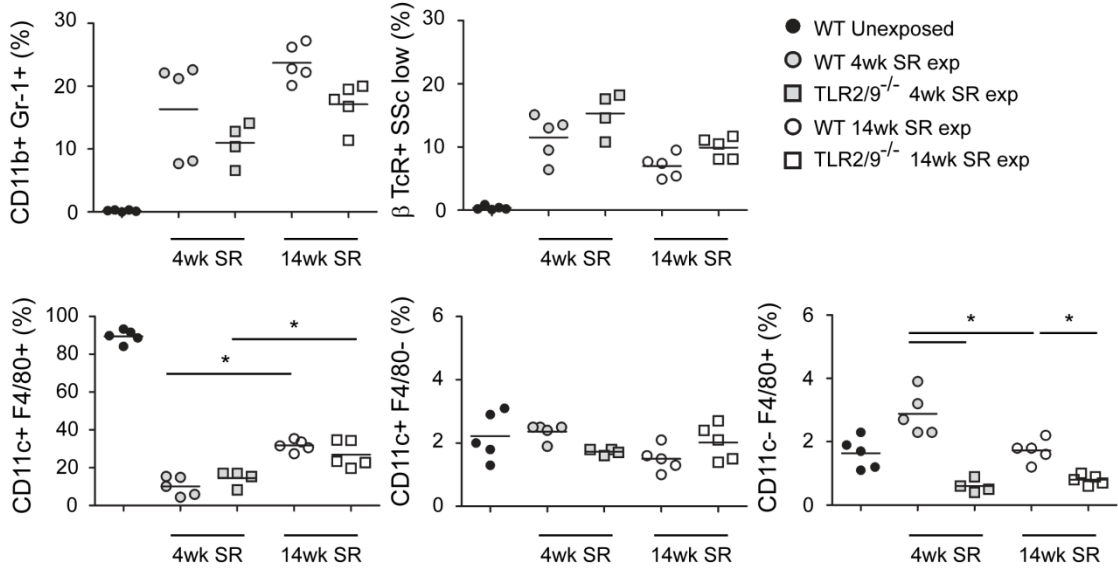
decrease in CD11c$^-$ / F4/80$^+$ macrophages between 4 and 14 week exposed mice. The percentages of CD11c$^-$ / F4/80$^+$ macrophages was significantly decreased in TLR2/9$^{-/-}$ mice exposed to SR for 4 and 14 weeks compared to WT SR exposed mice. Altogether, the results suggest that neutrophils and lymphocytes are predominant cell types in the alveolitis during the granulomatous phase as well as the chronic phase of HP, but the chronic phase is associated with increased percentages of AMs.

AMs Have Increased Expression of CD86 and CD206 after Long Term Exposure to SR

During the granulomatous phase, AMs from TLR2/9$^{-/-}$ mice had increased expression of costimulatory molecule CD80 and MHCII, markers of classically activated or type II regulatory macrophages (reviewed in Mosser, 2003; Mosser and Edwards, 2008). However, the alternative activation of macrophages is induced in the presence of IL-4, and alternatively activated macrophages are associated with the development of fibrosis (Edwards et al., 2006). The mannose receptor (CD206) is upregulated on macrophages in response to IL-4 stimulation, and is expressed on alternatively activated macrophages (Pechkovsky et al., 2010; Stein et al., 1992). Therefore, we investigated whether the development of CHP was associated with an increase in CD206 expression on AMs. In addition, we measured the expression of CD86 to determine if costimulatory molecules remain upregulated on TLR2/9$^{-/-}$ AMs after long term exposure to SR. Mice were intranasally exposed to SR for 4 or 14 weeks and BAL performed 2 days after the last exposure. BAL cells were stained with antibody to CD11b, CD11c, Gr-1, F4/80, CD206, and CD86 and AM expression of CD86 and CD206 determined by flow cytometry. The gating strategy to discriminate AMs is shown in **Figure 5-4A**. As seen previously during the granulomatous phase, TLR2/9$^{-/-}$ mice exposed to SR for 4 and 14 weeks had a significant reduction in the percentage of AM that express high levels of CD11b compared to WT SR exposed mice (**Figure 5-4B**). There was a significant increase in the geometric MFI of CD86 and CD206 on AMs from WT mice exposed to SR for 14 weeks compared to 4 weeks, but not TLR2/9$^{-/-}$ mice (**Figure 5-5**). The significant increase in CD206 geometric MFI was also seen on CD11b^{high} AMs in mice exposed to SR for 14 weeks compared to 4 weeks (**Figure 5-6**), suggesting long term exposure to SR leads to increased AM activation that is dependent on TLRs 2 or 9. However, on CD11b^{high} AMs, TLR2/9$^{-/-}$ mice had a significant increase in the percentage of cells that expressed CD86 at 4 and 14 weeks compared to WT exposed mice, suggesting TLR2 inhibits CD86 upregulation on CD11b^{high} AMs after short term and long term exposure to SR.

TLR2/9$^{-/-}$ Mice Have a Significant Decrease in Activated CD4$^+$ Th Cells after Long Term Exposure to SR

While HP has often been associated with a lower CD4:CD8 ratio than other fibrotic lung diseases such as sarcoidosis, previous studies have suggested the

Figure 5-4. TLR2/9$^{-/-}$ mice have a significant decrease in CD11b^{high} AMs after long term exposure to SR.

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 4 or 14 weeks and analyzed 2 days after the last exposure. BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, CD11c, F4/80, and analyzed by flow cytometry. (A) The gating strategy to discriminate CD11b^{high} and CD11b^{low} AM populations (CD11c$^+$ / F4/80$^+$ / autofluorescence$^+$ / Gr-1$^-$) present in the alveolitis. (B) The percentage of CD11b^{low} and CD11b^{high} AMs in the BAL (n = 4-5 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).
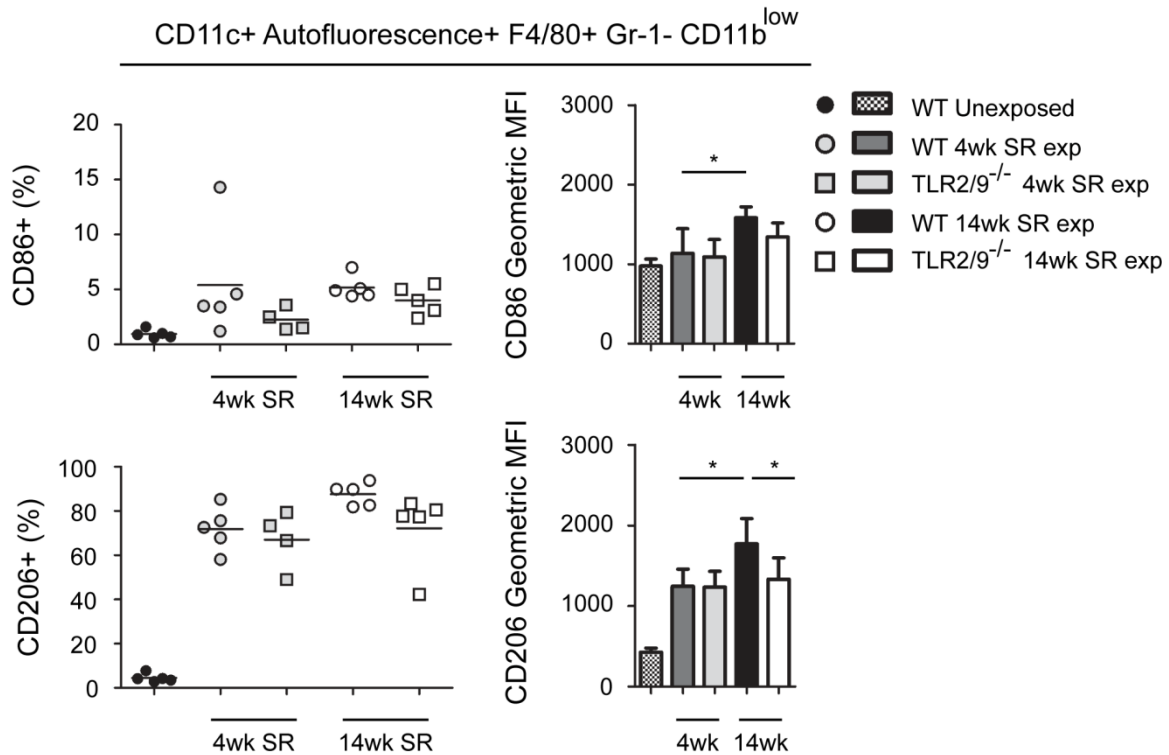
Figure 5-5. **WT AMs have a significant increase in the expression of CD86 and CD206 expression after 14 weeks SR exposure.**

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 4 or 14 weeks and analyzed 2 days after the last exposure. BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, CD11c, F4/80, CD86 and CD206 and analyzed by flow cytometry. The percentage of AMs (CD11c^{+} / autofluorescence^{+} / F4/80^{+} / Gr-1^{-} / CD11b^{low}) that express CD86 and CD206 and the geometric MFI was measured (n = 4-5 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).
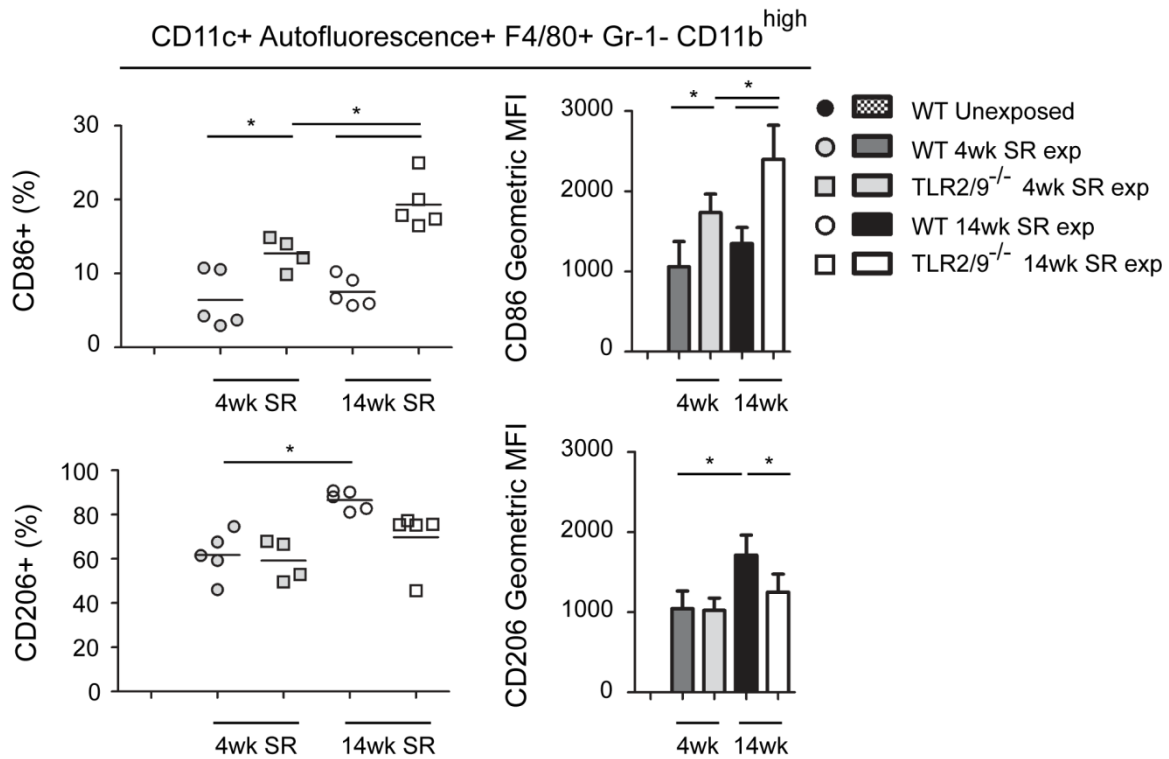
**Figure 5-6.** **TLR2/9$^{-/-}$ CD11b^{high} AMs have increased expression of CD86 and WT CD11b^{high} AMs have increase expression of CD206 after 14 weeks exposure to SR.**

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 4 or 14 weeks and analyzed 2 days after the last exposure. BAL was performed and cells recovered from the BAL fluid were incubated with antibody to CD11b, Gr-1, CD11c, F4/80, CD86 and CD206 and analyzed by flow cytometry. The percentage of CD11b^{high} AMs (CD11c$^+$ / autofluorescence$^+$ / F4/80$^+$ / Gr-1$^-$ / CD11b^{high}) that express CD86 and CD206 and the geometric MFI was measured (n = 4-5 mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).

development of CHP leads to an increase in the number of CD4$^+$ T cells in the BAL and thus an increase in the CD4:CD8 ratio (Barrera et al., 2008; Welker et al., 2004). However, a recent study has shown the CD4:CD8 ratio can be variable in all stages of HP (Caillaud et al., 2012). To investigate possible increases in the CD4:CD8 ratio in mice that have been exposed long term to SR, BAL cells were collected from mice exposed to SR for 4 and 14 weeks and incubated with antibody against βTcR, CD4, and CD8 and the percentage of CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry. Unexposed mice were excluded from the analysis as T cells were not present in the BAL of unexposed mice. The gating strategy to identify CD4$^+$ and CD8$^+$ T cells in the BAL is depicted in **Figure 5-7A**. We found no differences in the percentages of CD4$^+$ and CD8$^+$ T cells between WT and TLR2/9$^{-/-}$ mice, and no difference between mice that had been exposed to SR for 4 weeks compared to 14 weeks (**Figure 5-7B**). The CD4:CD8 ratio was also not significantly different between groups. The results suggest long term SR exposure does not lead to changes in the CD4:CD8 ratio in mice.

Studies during the granulomatous phase of HP found that SR induced signaling through TLR2 was important for CD4$^+$ Th cell activation, and that TLR2/9$^{-/-}$ mice had a significant reduction in the percentage of activated CD4$^+$ Th cells in the BAL and lungs after 3 weeks exposure to SR. To determine whether TLR2/9$^{-/-}$ mice have a significant reduction in the percentage of activated CD4$^+$ Th cells after long term SR exposure, BAL cells from WT and TLR2/9$^{-/-}$ mice exposed to SR for 4 or 14 weeks were additionally incubated with antibody to CD69, and the percentage of CD4$^+$ and CD8$^+$ T cells that express CD69 was measured by flow cytometry. TLR2/9$^{-/-}$ mice had a significant reduction in the percentage of CD4$^+$ Th cells that express CD69$^+$ compared to WT mice after 4 and 14 weeks SR exposure (**Figure 5-7C**). There was no significant difference in the percentage of CD69$^+$ CD8$^+$ T cells between WT and TLR2/9$^{-/-}$ mice. The results suggest SR induced signaling through TLR2 is also important for CD4$^+$ Th cell activation during chronic HP.

Mice with Long Term Exposure to SR Have Increased Expression of IFNγ and IL-17A in the Lung but not IL-4

During the granulomatous phase, the expression of TNFα was significantly reduced in the lungs of TLR2$^{-/-}$ and TLR2/9$^{-/-}$ mice. To investigate the expression of TNFα and as well as the anti-inflammatory cytokine IL-10 during chronic HP, mice were exposed to SR 3 times a week for 15 weeks and the lung tissue harvested for RNA isolation and qRT-PCR. The relative expression of the inflammatory cytokine TNFα and the anti-inflammatory cytokine IL-10 was increased in mice exposed to SR for 15 weeks over unexposed mice (**Figure 5-2A**); however, there was no significant difference between WT and TLR2/9$^{-/-}$ SR exposed mice. The results suggest the loss of TLR2 does not impact TNFα expression during chronic HP.

Previous studies of CHP in the pigeon breeder's disease HP model have suggested the expression of Th2-associated cytokines, IL-4 and IL-13, is associated with increased
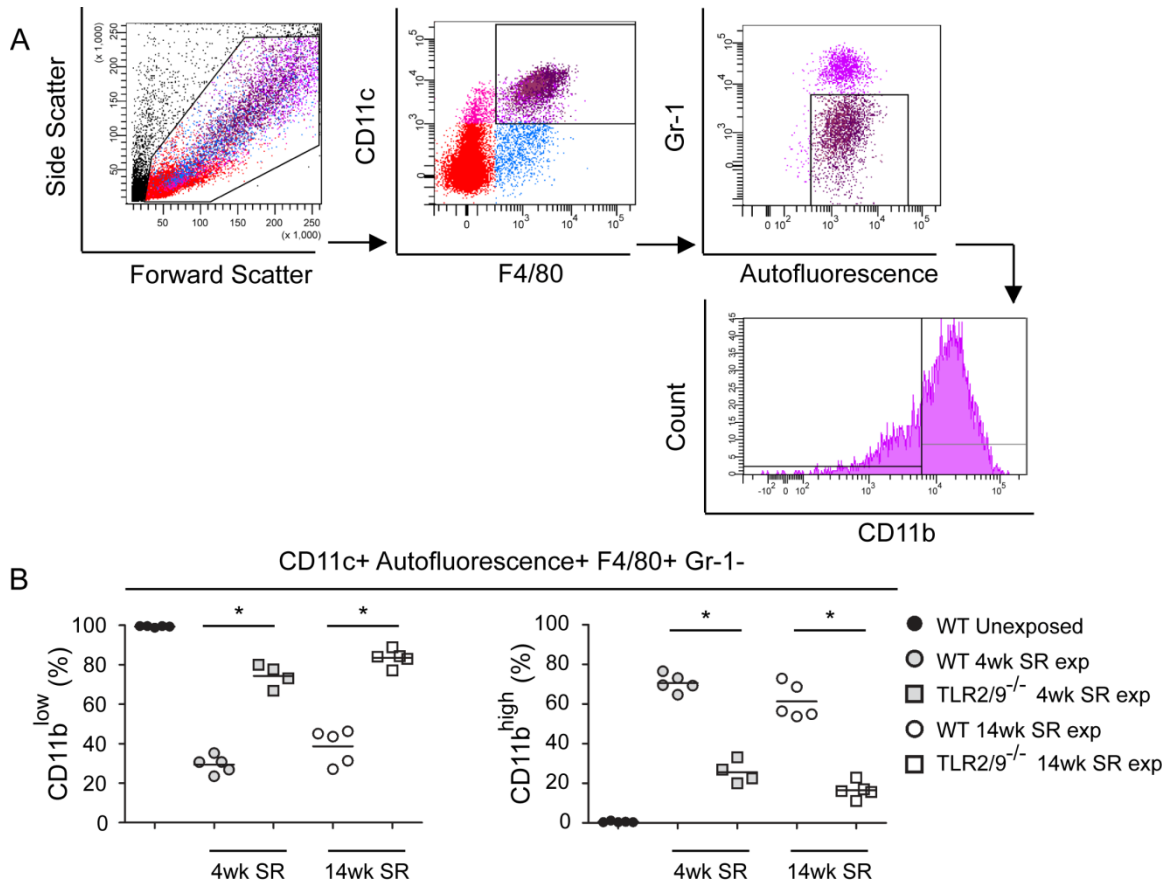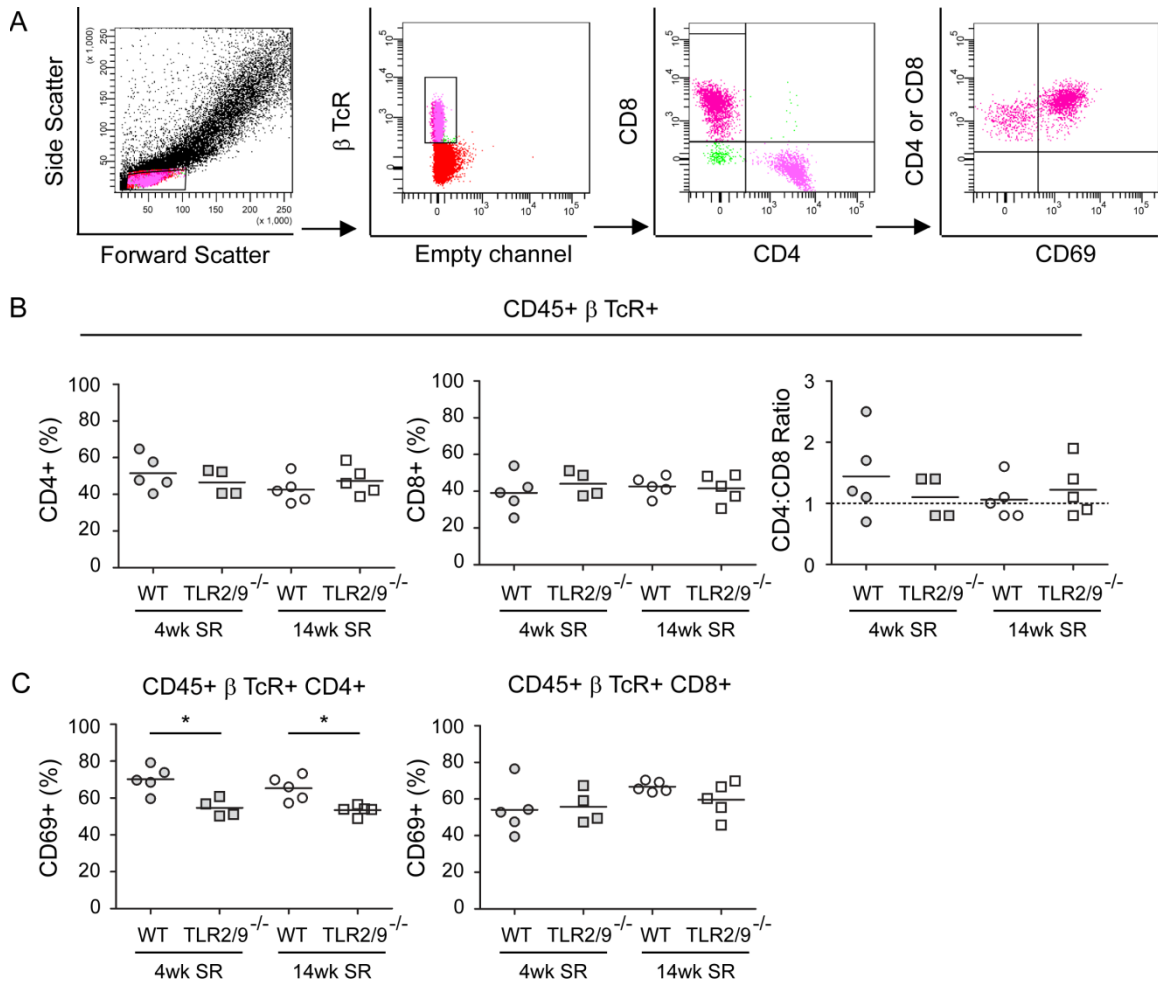
Figure 5-7. CD4^{+} and CD8^{+} T cells in mice exposed to SR 4 and 14 weeks.

WT and TLR2/9[−/−] mice were exposed to SR 3 times/week for 4 or 14 weeks and analyzed 2 days after the last exposure. BAL was performed and cells recovered from the BAL fluid were incubated with antibody to βTcR, CD4, CD8, and CD69 and analyzed by flow cytometry. (A) The gating strategy to discriminate CD4^{+} Th cells (CD45^{+} / βTcR^{+} / CD4^{+}) and CD8^{+} T cells (CD45^{+} / βTcR^{+} / CD8^{+}) that express CD69 in the lymphocyte gated alveolitis population. (B) The percentage of CD4^{+} Th cells and CD8^{+} T cells in the BAL and the CD4:CD8 ratio. (C) The percentage of CD4^{+} Th cells or CD8^{+} T cells that express CD69 ($n = 3 - 4$ mice per group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$).

lung fibrosis in the Th2-biased mouse strain A/J (Mitaka et al., 2011). However, in the SR model of HP, IL-17 and a Th17 response is associated with increased fibrosis (Joshi et al., 2009; Simonian et al., 2009). Therefore, cytokine expression was examined in mice with SR induced CHP to quantify the relative expression of cytokines associated with Th1, Th2, and Th17 responses, IFNγ, IL-4, and IL-17A respectively. RNA was isolated from whole lungs for reverse transcription and real time RT-PCR. The relative expression of IFNγ and IL-17A was significantly increased in 15 week exposed mice compared to unexposed mice (**Figure 5-2A**). During the granulomatous phase, TLR2/9$^{-/-}$ mice had a significant decrease in IL-17 expression and the Th17 response during the granulomatous phase. Surprisingly, TLR2/9$^{-/-}$ mice exposed long term to SR had a significant increase in IL-17A expression compared to WT SR exposed mice. The expression of IL-4 was not detected at this time point (**Figure 5-2B**), and IL-22, a Th17 associated cytokine that has been shown to protect against lung fibrosis in a mouse model of HP (Simonian et al., 2010), was also not detected at this time point. Therefore, the results show that mice exposed to SR express inflammatory cytokines IFNγ, IL-17A, and TNFα as well as the anti-inflammatory cytokine IL-10, but do not express IL-4 or IL-22. Altogether, the results suggest after long term exposure to SR, mice have increased expression of Th1 and Th17 associated cytokines IFNγ and IL-17, but not the Th2 associated cytokine IL-4.

CD4^{+} Th Cells from Mice with Long Term Exposure to SR Express IL-17A

To investigate whether the polarization of T cells in the lung switches to a Th2 response in mice after long term exposure to SR, WT and TLR2/9$^{-/-}$ mice were exposed to SR for 14 weeks and dispersed lung cells were stimulated ex vivo with PMA/ionomycin in the presence of brefeldin A and incubated with antibody to CD45, F4/80, βTcR, CD4, IL-17, and IFNγ and the percentage of Th17 and Th2 cells in the lung was examined by intracellular cytokine staining. The gating strategy to discriminate CD4^{+} Th cells in a population of dispersed lung cells is shown in **Figure 5-8A**. The results show the percentage of IL-17A and IL-4 expressing CD4^{+} Th cells in **Figure 5-8B**. A small percentage of IL-17A expressing CD4^{+} Th cells was detected in WT SR exposed mice, and TLR2/9$^{-/-}$ mice had a significant increase in the percentage of IL-17A expressing CD4^{+} Th cells compared to unexposed mice (**Figure 5-8C**). There was no significant increase in the percentage of Th cells that express IL-4 in WT or TLR2/9$^{-/-}$ SR exposed mice at this time point. Therefore, after long term SR exposure Th cells are polarized to a Th17 response, and do not switch to a Th2 response.

To investigate the contribution of other T cells populations in IL-17A and IL-4 expression after long term exposure to SR, dispersed lung cells were gated on CD4^{-} βTcR^{+} T cells (**Figure 5-9A**), and the percentage of IL-17 and IL-4 expressing CD4^{-} T cells is shown in (**Figure 5-9B**). There was no significant increase in the percentage of CD4^{-} Th cells expressing IL-17A or IL-4 after 14 weeks SR exposure (**Figure 5-9C**). Recent studies have suggested neutrophils and macrophages also contribute to IL-17 production and the fibrotic response in HP (Hasan et al., 2013). To determine if macrophages are a greater contributor to IL-17A or IL-4 production after long term

Figure 5-8. **CD4$^+$ Th cells in TLR2/9$^{-/-}$ mice express IL-17 but not IL-4 after long term exposure to SR.**

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 14 weeks and analyzed 2 days after the last exposure. Lung cells were digested and stimulated with PMA/Ionomycin or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, F4/80, IL-17A, and IL-4 for intracellular cytokine staining. (A) Gating strategy for the identification of CD4$^+$ Th cells (CD45$^+$ / F4/80$^-$ / βTcR$^+$ / CD4$^+$) in dispersed lung cells. (B) Representative dot plots of media or PMA/ionomycin stimulated CD4$^+$ Th cells expressing IL-17A or IL-4. (C) The percentage of CD4$^+$ Th cells expressing IL-17 or IL-4. (n = 5 mice / group.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

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Figure 5-9. CD4$^-$ T cells do not have a significant increase in IL-17 or IL-4 expression after long term exposure to SR.

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 14 weeks and analyzed 2 days after the last exposure. Lung cells were digested and stimulated with PMA/Ionomycin or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, F4/80, IL-17A, and IL-4 for intracellular cytokine staining. (A) Gating strategy for the identification of CD4$^-$ Th cells (CD45$^+$ / F4/80$^-$ / βTcR$^+$ / CD4$^-$) in dispersed lung cells. (B) Representative dot plots of media or PMA/ionomycin stimulated CD4$^-$ Th cells expressing IL-17A or IL-4. (C) The percentage of CD4$^-$ Th cells expressing IL-17 or IL-4. (n = 5 mice / group.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

exposure to SR, dispersed lung cells stimulated with PMA/ionomycin were gated on macrophages (**Figure 5-10A**), and the percentage of macrophages that express IL-17 or IL-4 are shown in **Figure 5-10B**. A small percentage of IL-17 expressing macrophages were detected in SR exposed mice, and TLR2/9$^{-/-}$ mice had a significant increase in the percentage of IL-17 expressing macrophages over unexposed mice (**Figure 5-10C**). IL-4 expression was not detected in macrophages at this time point. Altogether, the results suggest Th17 cells and IL-17A expressing macrophages are present in the lungs of mice with CHP, and long term exposure to SR does not lead to a switch to a Th2 response.

Mice with CHP Have Decreased Barrier Function and Lung Fibrosis

Lung pathology was examined by assessing barrier function and lung fibrosis in mice exposed long term to SR. Total protein and IgM was measured in the BALF in mice exposed to SR for 4 or 14 weeks as a measure of protein leak across the lung epithelium. WT and TLR2/9$^{-/-}$ mice exposed to SR for 14 weeks had a significant increase in total protein compared to unexposed mice (**Figure 5-11**). Interestingly, total IgM was significantly decreased after 14 weeks exposure to SR compared to 4 weeks exposure. To measure lung fibrosis that develops following 14 weeks of SR exposure, lungs from WT and TLR2/9$^{-/-}$ SR exposed and control mice were stained with Masson's trichrome. The slides were digitized and blue pixels quantified in the lung sections with a positive pixel algorithm. Representative images show increased collagen deposition in the alveolar walls in mice exposed to 14 weeks SR compared to normal lung architecture unexposed mice (**Figure 5-12A-C**). The positive pixel analysis showed WT mice exposed to SR for 14 weeks had a significantly greater number of positive and strong positive pixels per analysis area compared to unexposed mice and TLR2/9$^{-/-}$ mice (**Figure 5-12D**). When only strong positive pixels were considered, WT mice again had a significant increase compared to unexposed and TLR2/9$^{-/-}$ mice. This suggests TLR2/9$^{-/-}$ may be partially protected from the development of fibrosis after long term SR exposure. Altogether, the results suggest mice exposed to SR for 14 weeks have loss of barrier function and lung fibrosis compared to unexposed mice.

Mice with CHP Have Reduced Static Lung Compliance and Total Lung Capacity

Edema as a result of epithelial barrier disruption as well as lung fibrosis can contribute to reduced lung compliance. To assess whether mice exposed to SR develop reduced lung compliance and reduced total lung capacity similar to patients with CHP, mice exposed to SR for 15 weeks were placed on a flexiVent™ ventilator to assess lung function through a snap shot perturbation and a pressure volume curve. During a snap shot perturbation, the air volume delivered to the lungs is oscillated in a sinusoidal wave form (**Figure 5-13A**). Pressure and volume data is collected and fit to the single compartment model of the lung to calculate lung compliance, elastance, and resistance (reviewed in Bates and Lutchen, 2005). To generate a pressure volume loop, the air volume delivered to the mouse is increased in a step-wise fashion up to total lung

Figure 5-10. Macrophages contribute to IL-17 expression in TLR2/9$^{-/-}$ mice after long term exposure to SR.
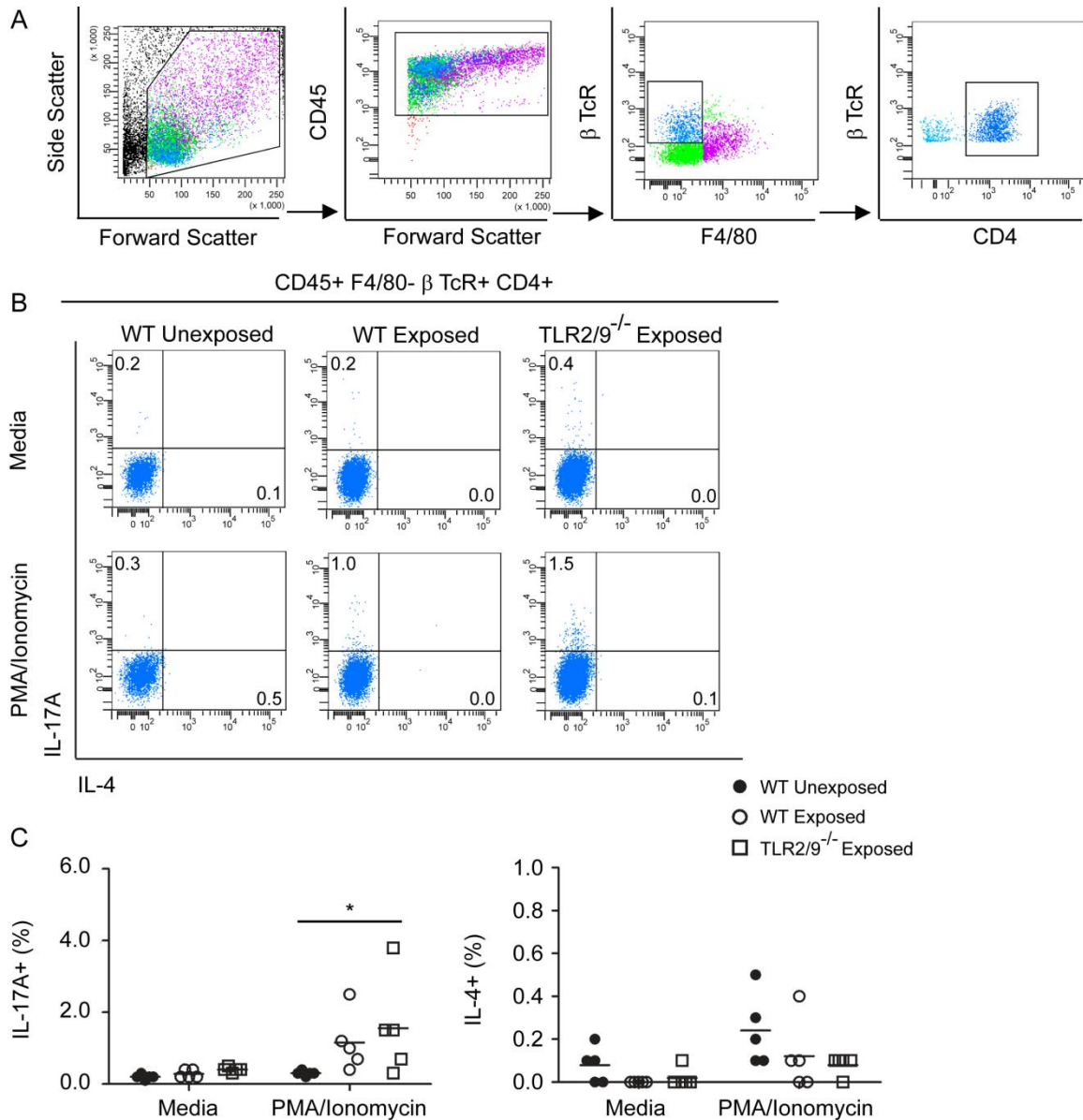
WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 14 weeks and analyzed 2 days after the last exposure. Lung cells were digested and stimulated with PMA/Ionomycin or media alone in the presence of brefeldin A then incubated with antibodies for CD45, βTcR, CD4, F4/80, IL-17A, and IL-4 for intracellular cytokine staining. (A) Gating strategy for the identification of macrophages (CD45^{+} / F4/80^{+} / βTcR^{-}) in dispersed lung cells. (B) Representative dot plots of media or PMA/ionomycin stimulated macrophages expressing IL-17A or IL-4. (C) The percentage of macrophages expressing IL-17 or IL-4. (n = 5 mice / group.) Significance was determined using two-way ANOVA with Bonferroni post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).

A

B

CD45+ F4/80+ β TcR-

WT Unexposed | WT Exposed | TLR2/9⁻/⁻ Exposed

Media

PMA/Ionomycin

IL-17A

IL-4

- WT Unexposed
- WT Exposed
- TLR2/9⁻/⁻ Exposed

C
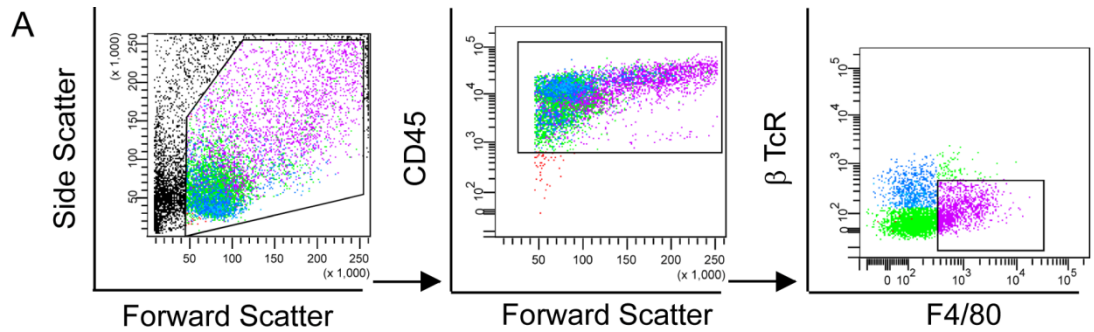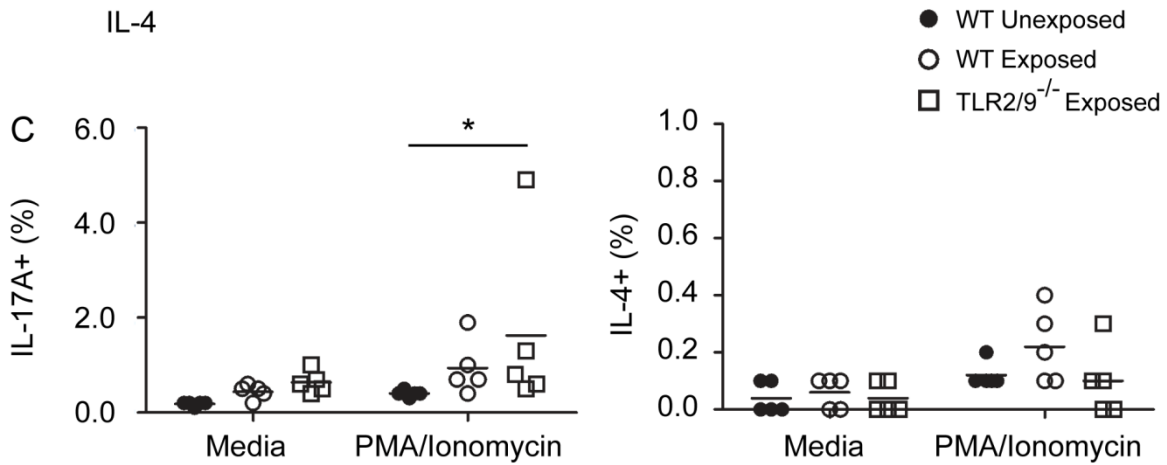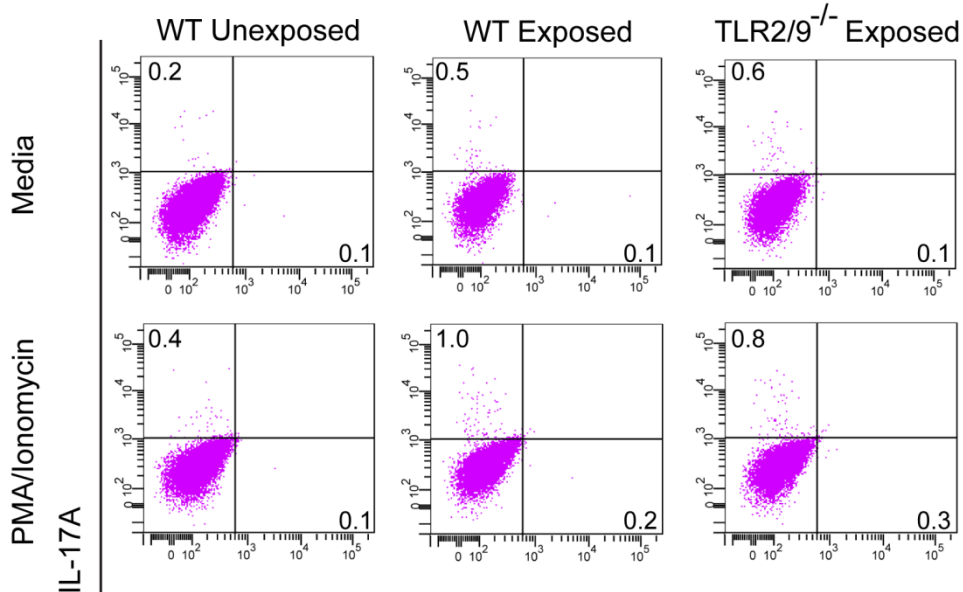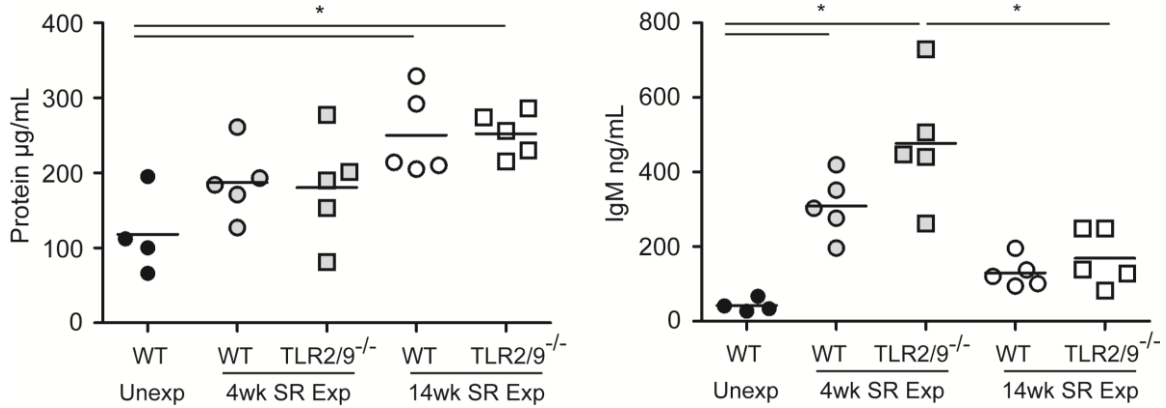
Figure 5-11. Evidence of plasma protein leakage across the lung epithelium in the BALF of mice exposed to SR for 4 and 14 weeks.

WT and TLR2/9$^{-/-}$ mice were exposed to SR 3 times/week for 4 or 14 weeks and sacrificed 2 days after the last exposure. The cell-free BAL fluid was analyzed for total protein by Bio-rad protein assay and IgM by ELISA to detect protein leakage across the epithelium as an indicator of disrupted barrier disruption. Data represent the mean ± SD (n = 4-5 per group). Significance was calculated using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$ compared to WT SR exposed mice).
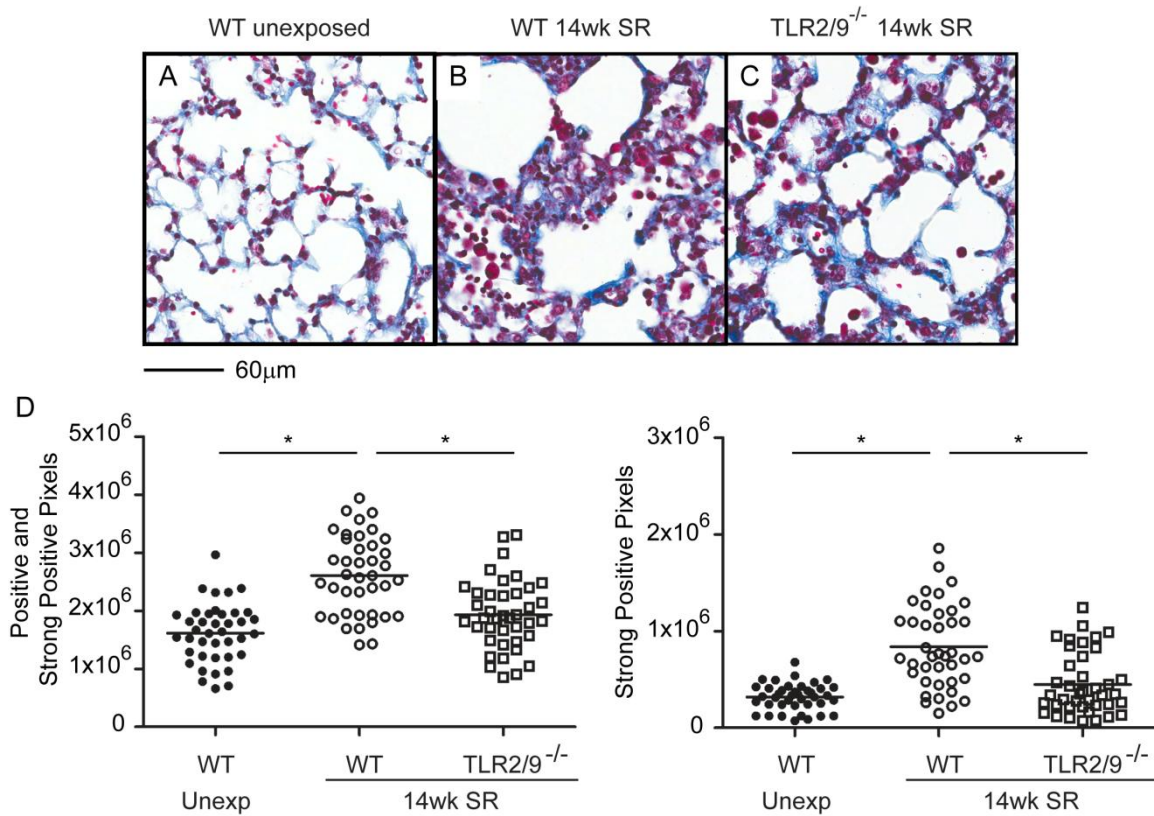
Figure 5-12. WT mice have a significant increase in lung fibrosis.

WT and TLR2/9$^{-/-}$ mice were exposed to SR for 14 weeks and lung tissue was harvested for histological analysis. Lung slides were stained with Masson's trichrome then digitized for image analysis. Representative images from (A) WT unexposed, (B) WT 14 week SR exposed, and (C) TLR2/9$^{-/-}$ 14 week SR exposed mice were captured at 40x. (D) The Aperio positive pixel algorithm was used to quantify blue collagen pixels in 8 x 1mm^2 areas / section. The total number of positive and strong positive pixels was counted. (n = 5 mice / group). The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*$p < 0.05$).

A Snap Shot

Volume

Time

Single compartment model of the lung

Pressure = Elastance * Volume + Resitance * Flow + P0

Elastance = Δ Pressure / Δ Volume
Compliance = 1 / Elastance = Δ Volume / Δ Pressure
Resistance = Δ Pressure / Flow

B Pressure Volume Curve

Volume

Time

Pressure Volume Curve

Exhalation

Inhalation

A

Volume

Slope

Pressure

Total Volume = A - Be$^{-\kappa \text{Plateau Pressure}}$

Static Compliance = Slope of curve at given volume
Static Elastance = 1 / Static Compliance
A = Upper bounds estimate of the difference
between total lung capacity and zero volume

Figure 5-13.   FlexiVent™ snap shot and pressure volume curve perturbations.

The flexiVent™ mechanical ventilator is a computer controlled piston pump capable of manipulating volume and pressure during ventilation to collect lung function data. (A) In a snap shot perturbation, the air volume delivered to the lungs is oscillated in a sinusoidal waveform. Pressure and volume data are collected during the maneuver and fit to the single compartment model of the lung consisting of a rigid tube connected to a flexible balloon to calculate elastance, compliance, and resistance of the lung. (B) To generate a pressure volume curve, the volume of air delivered to the lungs is increased in stepwise increments up to the total lung capacity and decreased to functional residual lung capacity. Pressure and volume data is collected when the air is not flowing (static) and fit to the Salazar-Knowles equation to calculate static compliance, static elastance, and total lung capacity.

capacity, and returned to functional residual capacity (**Figure 5-13B**). Pressure and volume data is collected when air is not moving or "static", then the Salazar-Knowles equation is fit to the data (Salazar and Knowles, 1964). The static compliance is the slope, and the total lung capacity is the upper bounds limit of the Salazar-Knowles equation.

During the snap shot perturbation, WT and TLR2/9$^{-/-}$ mice exposed long term to SR had a significant reduction in lung compliance compared to unexposed mice (**Figure 5-14A**). Elastance is the inverse of compliance, and therefore, WT and TLR2/9$^{-/-}$ mice had a significant increase in lung elastance compared to unexposed mice. Lung resistance was also significantly increased in WT and TLR2/9$^{-/-}$ mice compared to unexposed mice. The pressure volume curve also revealed WT and TLR2/9$^{-/-}$ mice exposed to SR for 15 weeks had a significant reduction in static compliance, and a significant increase in static elastance compared to unexposed mice (**Figure 5-14B**). The total lung capacity was significantly decreased in WT SR exposed mice compared to unexposed mice, but not TLR2/9$^{-/-}$ mice. These results suggest WT and TLR2/9$^{-/-}$ mice exposed to SR for 15 weeks have reduced lung compliance consistent with the development of restrictive lung disease.

Discussion

The results of our studies suggest mice exposed long term to SR do not switch to a Th2 immune response. Th2 cells were not detected by intracellular cytokine staining in the lungs of mice exposed to SR for 14 weeks. Recruitment of eosinophils to sites of inflammation is associated with Th2 immune responses. Analysis of the BAL cells reveals only a small percentage of eosinophils are recruited to the lungs of WT and TLR2/9$^{-/-}$ mice after 15 weeks SR exposure, and little to no IL-4 expression, one of the signature cytokines of a Th2 response, is detected in the lungs by RT-PCR at this time point. The majority of CD11b^{low} and CD11b^{high} AMs in 4 and 14 week exposed mice expressed CD206, and CD206 upregulation on macrophages is associated with alternative activation and exposure to IL-4. However, previous studies have shown TGFβ and GM-CSF also upregulate CD206 on macrophages in vitro (Porcheray et al., 2005), suggesting the upregulation of CD206 on AMs is not dependent on IL-4 in SR exposed mice.

IL-17 expression and a Th17 immune response after 3-4 week exposure to SR is associated with increased disease severity and fibrosis in HP (Hasan et al., 2013; Joshi et al., 2009; Simonian et al., 2009). The results of this study show long term exposure to SR is also associated with a Th17 response. Th17 cells are detected in the lungs of WT and TLR2/9$^{-/-}$ mice exposed to SR for 14 weeks by intracellular flow cytometry. Results from real time RT-PCR show lung tissue from mice exposed to SR for 15 weeks had a strong induction of IL-17 expression over unexposed mice, and was significantly increased in TLR2/9$^{-/-}$ mice over WT exposed mice. IL-17A is expressed by Th17 cells, but can also be expressed by other cell types such as γδT cells and NK cells. However,
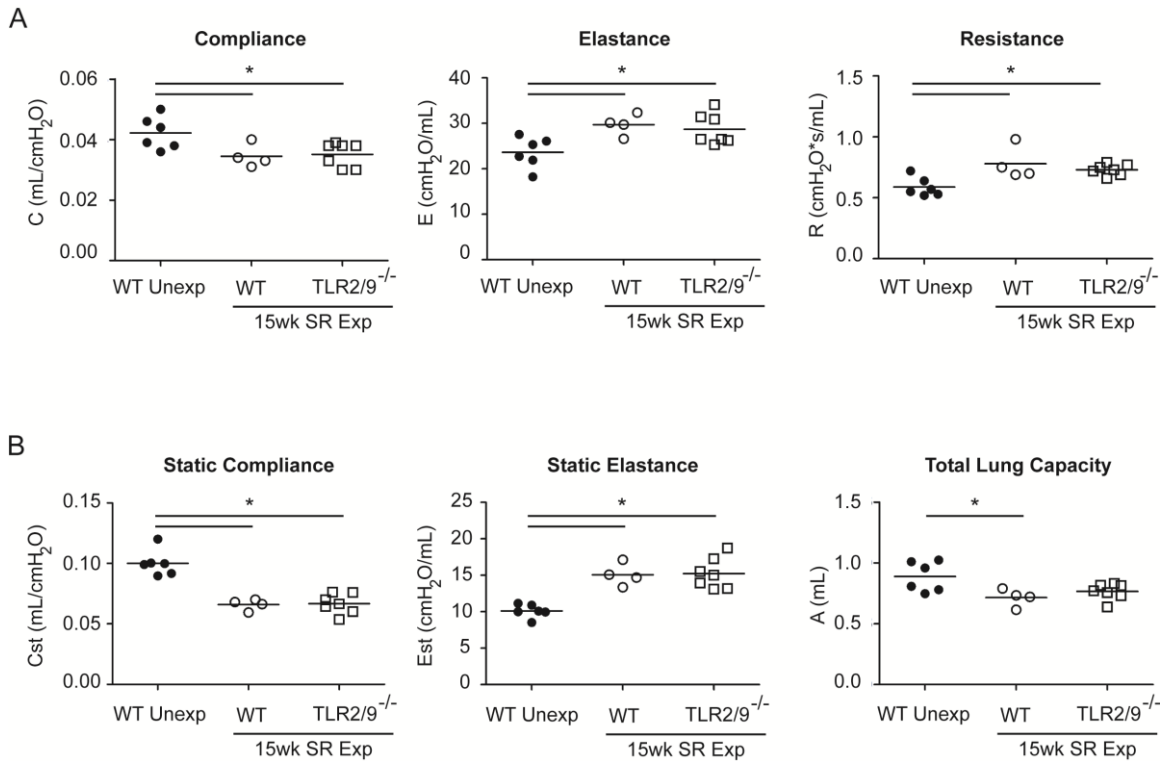
Figure 5-14. WT and TLR2/9$^{-/-}$ mice have a significant decrease in lung compliance.

WT and TLR2/9$^{-/-}$ mice were exposed to SR for 15 weeks and analyzed 3 days after the last exposure. Mice were placed on a flexiVent™ mechanical ventilator for lung function analysis (n = 4-7 mice per group). (A) A snap shot perturbation was performed to measure compliance, elastance, and resistance. (B) A pressure volume curve was performed to measure static compliance, static elastance, and total lung capacity. Each point represents the average value of at least 2 measurements for each mouse. The data represent mean ± SD and significance was determined using one-way ANOVA with Tukey post-hoc test (*p < 0.05).

recent studies in mice exposed to SR for 3 weeks showed that neutrophils and macrophages were the main cell type that expressed IL-17A and that neutrophil depletion decreased lung collagen (Hasan et al., 2013). This is in agreement with studies that have shown a positive correlation between the percentage of neutrophils in the lung tissue and the percentage of lung fibrosis in patients with HP (Pardo et al., 2000). Intercellular cytokine staining revealed a small percentage of macrophages expressed IL-17A in WT and TLR2/9$^{-/-}$ mice exposed to SR for 15 weeks. Differential counting of BAL cells and analysis of BAL cell populations by flow cytometry revealed an increase in the percentage of neutrophils and lymphocytes suggesting both cell types contribute to lung pathology at this time point.

However, the relationship between inflammation and fibrosis is not so clear cut. Inflammation mediated by the innate and adaptive immune system precedes fibrosis not only in the lung, but in multiple organ systems (reviewed in Wick et al., 2013), but the mechanisms that tip the balance between fibrosis and normal wound repair are still the subject of ongoing research. One hypothesis put forward by Dr. Robert Streiter suggests lung fibrosis develops when the integrity of the basement membrane is lost, which prevents the reestablishment of normal lung architecture, and progresses in the presence of chronic inflammation and persistent antigen (Strieter, 2008). In HP, neutrophils may damage basement membrane integrity through the secretion of collagenase-2 and gelatinase B (Pardo et al., 2000).

Granuloma formation is dependent on CD4^{+} T cells and the cytokines TNFα and IFNγ (Denis et al., 1991; Gudmundsson and Hunninghake, 1997). Mice exposed to SR for 15 weeks have increased expression of TNFα and IFNγ compared to unexposed mice and peri-vascular and peri-bronchial granulomas can be observed in lung sections. Activated CD4^{+} T cells are also significantly increased in the BALF of mice exposed to 14 weeks SR but significantly reduced in TLR2/9$^{-/-}$ mice as seen previously during the granulomatous phase. Additional experiments are necessary to determine if T cells or non-T cells are the source of IFNγ and TNFα expression at this time point.

Increased lung fibrosis leads to reduced lung compliance and restrictive lung disease. Other mouse models of fibrotic lung diseases have shown reduced lung compliance (Vanoirbeek et al., 2010; Wang et al., 2011), however, this is the first study to measure lung function in a mouse model of HP. WT and TLR2/9$^{-/-}$ mice exposed to SR for 15 weeks have a significant decrease in static lung compliance compared to unexposed mice. Lung barrier disruption leading to edema may contribute to reduced lung compliance as WT and TLR2/9$^{-/-}$ mice had a significant increase in total protein in the BALF compared to unexposed mice. The decrease in lung compliance was associated with reduced total lung capacity and increased fibrosis in WT mice exposed to SR for 14 weeks suggesting WT mice developed restrictive lung disease. Fibrosis was present in TLR2/9$^{-/-}$ mice, but positive pixel analysis revealed a significant reduction collagen deposition in the lungs of TLR2/9$^{-/-}$ mice compared to WT SR exposed mice. In addition, the total lung capacity in TLR2/9$^{-/-}$ mice was not significantly reduced compared to unexposed mice. Together, the results suggest TLR2/9$^{-/-}$ mice may be

partially protected from the development of lung fibrosis, but are not protected from the development of CHP.

Overall, the results show mice with long term exposure to SR develop lung fibrosis and reduced lung compliance. We find no evidence of a switch to a Th2 immune response, but rather detect Th17 cells and increased expression of IL-17A. In addition, TLR2/9$^{-/-}$ mice have a greater increase in the percentage of Th17 cells in the lung and expression of IL-17. TLR2/9$^{-/-}$ mice may have a partial reduction in the development of lung fibrosis compared to WT SR exposed mice, but are not protected from the development of interstitial lung disease as they have similar reductions in lung compliance as WT mice.

CHAPTER 6.   FUTURE DIRECTIONS

Treatment options for HP are limited, and include antigen avoidance and corticosteroids. The results of my study suggest TLRs 2 and 9 play an important role in the pathogenesis of HP. Mice deficient in TLRs 2 and 9 have significant reductions in neutrophil recruitment, inflammatory cytokine production, macrophage and lymphocyte activation, and the Th17 response. In addition, TLR2/9$^{-/-}$ mice were partially protected from the development of lung fibrosis. This suggests TLRs 2 and 9 are attractive targets for pharmacological inhibition of HP. TLR antagonists are currently in development to modulate immune responses in inflammatory disorders and autoimmune diseases. Drugs currently in development block TLR binding or disrupt TLR signaling, and many are entering clinical trials.

Among the TLR antagonist currently being developed, OPN-305 is a humanized version of the mouse-anti human TLR2-blocking antibody OPN-301, which has shown promise in the prevention of cardiac and kidney reperfusion injury, and inhibition of cytokine release by ex vivo stimulated synovial cells in rheumatoid arthritis (Arslan et al., 2012; Arslan et al., 2010; Farrar et al., 2012; Ultaigh et al., 2011). OPN-305 has recently finished phase I clinical trial and shows promise for further development. AN3485 is another compound that has been shown to antagonize TLR2, TLR3, TLR4, and TLR5 signaling; and mice treated with the compound had reduced TNFα, IL-1β, and IL-6 production in mouse models of sepsis, contact-dermatitis, and collagen-induced arthritis (Dong et al., 2013). Synthetic oligonucleotides are also being developed to inhibit TLR9 and have been shown to be effective in inhibiting immune responses in mouse models of rheumatoid arthritis, lupus, multiple sclerosis, and psoriasis (Barrat et al., 2007; Ho et al., 2003; Jiang et al., 2013; Zeuner et al., 2002). Further experiments to investigate the therapeutic potential of TLR2 and TLR9 antagonists in SR exposed mice are warranted.

Perhaps more importantly, during the course of my study, our lab has developed a mouse model of SR induced CHP. By establishing a time point where mice exposed to SR develop lung fibrosis and decreased lung compliance, we can study other immune modulatory compounds that may inhibit the progression to CHP. The CD200R is an inhibitory receptor expressed on myeloid cells and lymphocytes, which binds CD200 expressed on immune and non-immune cells (Rijkers et al., 2008). CD200 induced signaling through CD200R has been shown to be important for controlling inflammation as mice deficient in CD200 exhibit exacerbated immune responses during viral infection or autoimmune disease (Hoek et al., 2000; Snelgrove et al., 2008). Treatment with a CD200 fusion protein (CD200-Fc) has shown promise as an inhibitor of innate immune inflammation in mouse models of collagen-induced arthritis and multiple sclerosis (Liu et al., 2010; Simelyte et al., 2008). Experiments to test the efficacy of CD200-Fc treatment in SR exposed mice are planned. For further discussion on future directions see Appendix.

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APPENDIX. TRANSCRIPTOME ANALYSIS

Introduction

The advancement in RNA sequencing (RNAseq) technology has made this technique faster and more affordable than ever. Analysis of gene transcription by RT-PCR is limited to preselected genes, whereas RNAseq measures the expression of all transcripts in the sample. Previous studies in our lab identified MyD88 as a critical signaling adaptor molecule for the induction of inflammatory cytokine and chemokine production in response to SR (Nance et al., 2008). Studies during the acute phase found many cytokines and chemokines induced by SR were partially dependent on TLRs 2 and 9. In addition, TLR7 may play a role in SR induced cytokine production in spleen cells. However, other non-MyD88-dependent pattern recognition receptors may also contribute to the immune response to SR.

NOD-like receptors (NLRs) are a family of cytosolic pattern recognition receptors. Like TLRs, NLRs recognize pathogen associated molecular patterns (PAMPs) as well as danger associated molecular patterns (DAMPs) such as molecules associated with tissue damage such as uric acid (Gasse et al., 2009). NOD1 and NOD2 are NLRs that recognize the breakdown products of the bacterial cell wall, *meso*-diaminopimelic acid (*meso*-DAP) and muramyl dipeptide (MDP) respectively. Bacterial cell walls typically contain MDP, but gram negative and some gram positive bacterial cell walls also contain *meso*-DAP. SR is a gram positive actinomycete, which has a type IV cell wall structure that contains *meso*-DAP, arabinose and galactose, but not mycolic acids (Embley et al., 1988), and can therefore potentially stimulate NOD1 and NOD2.

To explore changes in global gene transcription after SR stimulation, bone marrow derived dendritic cells (BMDCs) from WT, TLR2/9$^{-/-}$, MyD88$^{-/-}$, TLR7$^{-/-}$ and NOD2$^{-/-}$ mice were stimulated with SR in vitro. We hypothesize loss of MyD88, TLRs 2 and 9, TLR7, and NOD2 will lead to a decrease in global gene transcription. Comparing levels of transcription between WT and KO cells will identify genes that are potentially dependent TLRs 2 and 9, TLR7, NOD2, and signaling through MyD88.

Methods

Bone Marrow Harvest and Storage

Bone marrow cells were harvested from the femur and tibia of WT, TLR7$^{-/-}$, TLR2/9$^{-/-}$, MyD88$^{-/-}$, and NOD2$^{-/-}$ mice. Mice were sacrificed and fur completely wetted down with 70% ethanol. The fur, skin, and large muscle tissue was removed from the hind legs. Legs were removed at the hip and the paw removed at the joint to prevent cutting the femur and tibia. The bones were sprayed with ethanol and placed in media on ice until all mice were processed. In a sterile hood, any remaining flesh was removed

from the bones. The epiphysis was cut and bone marrow flushed with sterile PBS into a 50mL tube using a 26 gauge 1/2 inch needle and syringe. The bone marrow was centrifuged at 400g and supernatant decanted. The bone marrow cells were pipetted up and down to break up clumps and washed with sterile PBS and pelleted at 400g. Supernatant was discarded and RBCs lysed with RBC lysis buffer. Bone marrow cells were passed through a 70μm cell strainer (BD Biosciences, San Jose, CA) to obtain a single cell suspension. Cells were pelleted at 400g and resuspended in media. Cells were counted by trypan dye exclusion on a hemocytometer and brought to a concentration of $6x10^6$ cells/mL. Bone marrow cells were either plated immediately or reconstituted in freezing media (90% heat inactivated FCS, 10% DMSO) and frozen at -80°C and transferred to liquid nitrogen for long term storage (Marim et al., 2010). To thaw bone marrow progenitor cells for BMDC differentiation, bone marrow progenitor cells were rapidly thawed in a 37°C water bath and were washed with media to removed DMSO.

Bone Marrow Derived Dendritic Cell (BMDC) Differentiation

To generate dendritic cells, $5x10^6$ bone marrow progenitor cells from WT, MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, and NOD2$^{-/-}$ mice were plated in 15mL BMDC differentiation media containing 35% F10 media (**Table A-1**) in a 15cm cell culture dish. The cells were cultured 8 days. On day 3, an additional 15mL of BMDC differentiation media containing 35% F10 media was added. On day 6, 15mL media was removed from the plate and spun down at 400g for 4 minutes. The supernatant was decanted, and 15mL fresh BMDC differentiation media containing 35% F10 media was added to the pellet and returned to the plate. On day 8, the tissue culture plates were gently washed with media 2 times to collect loosely adherent cells. The harvested cells were pelleted at 400g for 4 minutes, and resuspended in fresh BMDC cultivation media containing 17.5% F10 media. An aliquot of cells was used to determine the percentage of conventional DC (CD11b$^+$ CD11c$^+$ Gr-1$^-$), monocyte derived inflammatory DC (CD11b$^+$ CD11c$^+$ Gr-1$^-$), inflammatory monocytes (CD11b$^+$ CD11c$^-$ Gr-1$^+$), and monocyte/macrophage (CD11b$^+$ CD11c$^-$ Gr-1$^-$) was determined by flow cytometry based upon the gating strategy of Bourgeois et al. (Bourgeois et al., 2011). The BMDC preparation was 89-95% conventional DCs, monocyte derived inflammatory DCs, and inflammatory monocytes.

BMDC Stimulation

BMDCs ($2.5x10^6$ cells/well) were plated in a 6 well plate. Cells were stimulated with media or 0.05mg/mL SR for 12 hours. The next day cells from all wells were harvested by scraping, and washed with PBS. Cells were lysed with TRIzol for poly-A RNA isolation and transcriptome analysis.

Table A-1.   BMDC media.

Reagent	Differentiation media	Cultivation media	Manufacturer	Location
RPMI	X	X	Cellgro, Mediatech Inc.	Manassas, VA
10% heat inactivated FCS	X	X	Hyclone, Thermo Scientific	Logan, UT
2mM glutamine	X	X	Cellgro, Mediatech Inc.	Manassas, VA
100IU/mL penicillin	X	X	Cellgro, Mediatech Inc.	Manassas, VA
100μg/mL streptomycin	X	X	Cellgro, Mediatech Inc.	Manassas, VA
1μL β-mercaptoethanol	X	X	Fisher Scientific	Waltham, MA
F10 media	35%	17.5%		

RNA Isolation

Culture media was removed completely and Trizol (Invitrogen) was added to the plate and allowed to incubate for 5 minutes. Trizol was then transferred to a microcentrifuge tube for RNA isolation. The aqueous layer was separated using chloroform. Glycogen was added to the aqueous RNA then both were precipitated with isopropanol. The RNA/glycogen pellet was washed with 75% ethanol twice. The RNA/glycogen pellet was then dissolved in water and heated at 55°C for 10 minutes.

Poly-A RNA Enrichment

RNA isolated from BMDCs was precipitated a second time using ammonium acetate, glycogen, and 100% ethanol to remove additional salt. The pellet was washed with 70% ethanol, and resuspended in nuclease free water. The RNA was then incubated with Oligo(dT) resin (Poly-A purist kit, Ambion, Naugatuck, CT). The resin was washed to remove unbound ribosomal RNA and other sources of non-messenger RNA. The poly-A mRNA was eluted with THE RNA storage solution and re-precipitated with ammonium acetate, glycogen, and 100% ethanol. The pellet was washed with 70% ethanol, and resuspended in nuclease free water for a second round of Poly-A purification.

An aliquot of total RNA isolated for transcriptome analysis was run on a eukaryote nano chip in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) by personnel in the Molecular Resource Center of UTHSC. The RNA integrity number (RIN) for all samples was above 9.0. To check for ribosomal RNA contamination, an aliquot of purified poly-A RNA samples were again run on the Agilent Bioanalyzer to verify the 18S and 28S ribosomal bands were absent. One sample, after two rounds of poly-A RNA isolation, still had a small ribosomal RNA peak and required a third round of poly-A RNA isolation.

BMDC Transcriptome Analysis

Poly-A RNA Fragmentation and Reverse Transcription

Poly-A RNA samples were prepared for RNA sequencing (RNA-seq) by personnel in the Molecular Resource Center of UTHSC (Mortazavi et al., 2008; Nagalakshmi et al., 2010). Using the SOLiD Total RNA-seq Kit (Applied Biosystems, Carlsbad, CA), the purified poly-A RNA was fragmented and cleaned using a spin column. The fragmented RNA was then run on a pico chip on the Agilent Bioanylizer to assess the fragmentation size range. The RNA fragments were hybridized to adaptors, then reverse transcribed to cDNA.

The cDNA was washed and underwent two rounds of size selection to select for cDNA products greater than 150bp. The cDNA was then amplified using different barcoded primers for each sample and purified to remove unincorporated primers and run on a DNA 1000 chip in the Agilent Bioanalyzer for a smear analysis. (A smear analysis determines the percentage of cDNA products that fall between 25-200bp. Less than 50% is considered optimal.)

SOLiD System Sequencing

The cDNA from WT, MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, and NOD2$^{-/-}$ SR stimulated BMDCs was sequenced on a SOLiD 5500xl (Applied Biosystems, Carlsbad, CA) by personnel in the Molecular Resource Center of UTHSC. Sequences were aligned to mouse mm9 annotated reference genome using Lifescope genomic analysis software. The read count for each gene was normalized for transcript length in nucleotides and the total number of reads in the sample by calculating the reads per kilobase of exon model per million mapped reads (RPKM).

$$RPKM \ = \ (Read \ Count_{gene} \ x \ 10^9) \ / \ (Length_{Gene \ transcript}) \ (Read \ Count_{Total})$$

The fold induction was calculated by dividing the RPKM of SR stimulated WT and KO BMDCs by WT media. A cut off value of 5x or greater fold induction was used to identify genes upregulated by SR stimulation. Out of the total number of genes upregulated in WT SR stimulated BMDCs, a fold reduction was determined by dividing the RPKM of WT SR stimulated BMDCs by the RPKM of KO SR stimulated BMDCs. A cut off value of 5x or greater was used to identify which genes were dependent on MyD88, TLRs 2 and 9, TLR7, and NOD2. The list of upregulated and downregulated genes was entered into the MGI Gene GO_Slim Chart Tool (http://www.informatics.jax.org/gotools/MGI_GO_Slim_Chart.html) for biological process and function analysis to identify commonalities among genes upregulated by SR.

Results

To assess the global changes in cell transcription in response to SR, total RNA was harvested from media or SR stimulated BMDCs. BMDCs were polarized from bone marrow harvested from WT, MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, and NOD2$^{-/-}$ mice and cultured in the presence of F10 media. The cells were polarized for 8 days and stimulated with SR for 12 hours. Poly-A RNA was isolated then sequenced for transcriptome analysis. The read counts were normalized to transcript size by calculating the reads per kilobase of exon model per million mapped reads (RPKM). The number of genes upregulated by SR stimulation was calculated by dividing the RPKM of SR stimulated BMDCs by the RPKM of WT media BMDCs (**Figure A-1**). SR stimulation upregulated 648 genes ≥5x in WT BMDCs compared to media. The number of genes upregulated in TLR7$^{-/-}$ and NOD2$^{-/-}$ SR stimulated BMDCs were similar to WT SR stimulated BMDCs
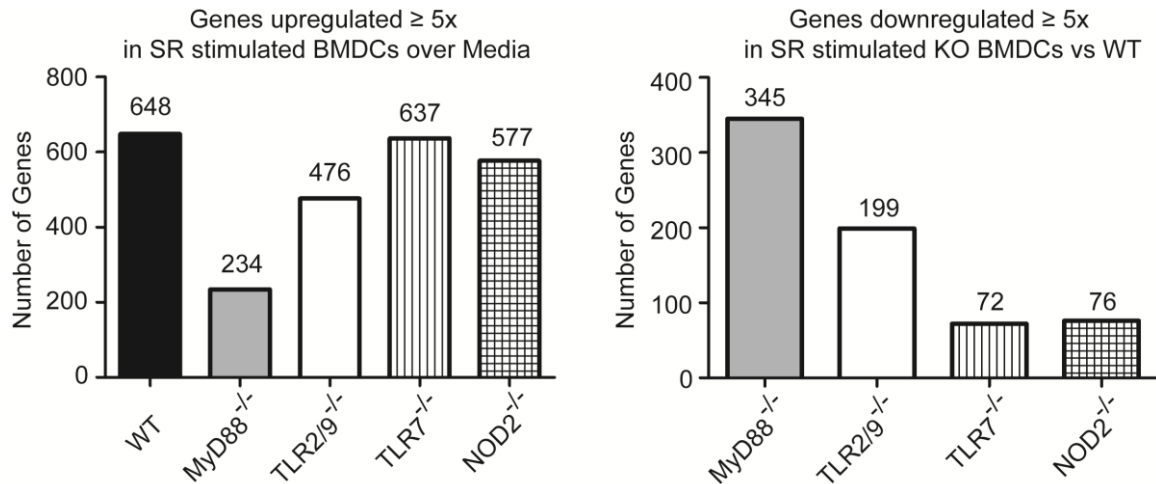
Figure A-1. **Changes in gene expression in WT, MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, NOD2$^{-/-}$ BMDCs stimulated with SR in vitro.**

WT, MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, and NOD2$^{-/-}$ BMDCs were stimulated with SR (0.05mg/mL) for 12 hours. RNA was harvested and enriched for poly-A RNA for RNA sequencing. Sequences were aligned to the mouse genome. Transcripts were mapped to a total of 24226 genes. The read count for the genes expressed in each sample was normalized for transcript length by calculating the reads per kilobase of exon model per million mapped reads (RPKM). The number of genes with RPKM values ≥5x higher in SR stimulated BMDMs compared to media stimulated BMDMs was calculated. Out of the genes upregulated ≥5x in WT SR stimulated BMDMs over media, the number of genes that had RPKM values ≥5x lower in MyD88$^{-/-}$, TLR2/9$^{-/-}$, TLR7$^{-/-}$, and NOD2$^{-/-}$ SR stimulated BMDMs was calculated.

(637 and 577 genes respectively). TLR2/9$^{-/-}$ BMDCs stimulated with SR upregulated fewer genes than WT SR stimulated BMDCs (476 vs 648 genes). However, MyD88$^{-/-}$ SR stimulated BMDCs upregulated about 1/3 the number of genes compared to WT SR (234 genes vs 648 genes). Out of the 648 genes upregulated ≥5x in WT BMDCs stimulated with SR compared to WT media, approximately half of those genes (345 genes) were downregulated ≥5x in MyD88$^{-/-}$ BMDCs. In contrast, approximately one third of those genes (199 genes) were downregulated ≥5x in TLR2/9$^{-/-}$ BMDCs. However, in TLR7$^{-/-}$ and NOD2$^{-/-}$ SR stimulated BMDCs, only 72 and 76 genes respectively were downregulated >5x compared to WT SR.

To investigate the biological process of genes upregulated in response to SR, a list of genes that were upregulated ≥5x in SR stimulated BMDCs over WT media was entered into the MGI Gene Ontology GO_Slim chart tool, an online analysis program which groups genes based on shared biological process or function. Biological processes were grouped into 13 categories, and the percentage of genes in each category was not dramatically different in WT, TLR7$^{-/-}$, and NOD2$^{-/-}$ SR stimulated BMDCs (**Table A-2**). The percentage of genes involved in cell adhesion, cell-cell signaling, cell cycle and proliferation, and death was slightly reduced in TLR2/9$^{-/-}$ BMDCs. The percentage was further reduced in MyD88$^{-/-}$ BMDCs in cell adhesion, cell-cell signaling, cell cycle and proliferation, death, cell organization and biogenesis, protein metabolism transport, and developmental processes. Out of the upregulated genes, many shared a biological function related to the induction of an immune response, such as cytokines, chemokines, cytokine and chemokine receptors, and integrins (**Table A-3**). In addition, growth factors and growth factor receptors were upregulated, as well as genes potentially involved in the fibrotic response such as extracellular matrix constituents and metalloproteinases. Altogether, the results suggest a majority of genes require a signal through a MyD88-dependent receptor to upregulate gene transcription after SR stimulation, and some genes are dependent on TLRs 2 or 9. The loss of TLR7 or NOD2 alone was not enough to dramatically alter cellular gene transcription.

Discussion

The transcriptome analysis revealed MyD88-dependent receptors to be crucial for SR induced upregulation of transcription. MyD88$^{-/-}$ BMDCs upregulated fewer genes in response to SR and had the greatest number of genes downregulated >5x compared to WT SR exposed BMDCs. TLRs 2 and 9 were revealed to be partially responsible for the MyD88-dependent induction of transcription; however, in agreement with studies during the acute phase, other MyD88-dependent receptors contribute to the response to SR. The loss of TLR7 or NOD2 alone was not enough to dramatically alter gene transcription in BMDCs, suggesting they play only a minor role in the innate immune response to SR.

Although the transcriptome analysis was limited in sample size, the data can be used to identify candidate genes for further analysis. One of the genes highly upregulated in BMDCs in response to SR stimulation was integrin β8 (WT SR RPKM 15.66 vs WT

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Table A-2. **Percentage of genes upregulated ≥5x over WT media that share a biological process.**

Biological process	WT SR	MyD88$^{-/-}$ SR	TLR2/9$^{-/-}$ SR	TLR7$^{-/-}$ SR	NOD2$^{-/-}$ SR
Cell adhesion	6.64%	3.42%	4.83%	5.49%	6.41%
Cell-cell signaling	6.17%	4.27%	4.62%	5.97%	6.76%
Cell cycle and proliferation	14.04%	7.69%	9.87%	12.24%	13.69%
Death	9.72%	5.13%	7.56%	10.68%	11.27%
Cell organization and biogenesis	15.12%	12.39%	14.08%	14.76%	16.98%
Protein metabolism	17.13%	12.82%	15.76%	15.07%	15.42%
DNA metabolism	1.85%	2.14%	2.10%	2.20%	2.25%
RNA metabolism	10.80%	9.40%	11.13%	11.15%	11.61%
Other metabolic processes	16.67%	15.81%	15.55%	15.86%	15.94%
Stress response	15.59%	14.10%	17.86%	16.95%	16.46%
Transport	16.20%	12.39%	14.71%	15.07%	15.77%
Developmental processes	23.61%	19.23%	20.59%	21.51%	24.96%
Signal transduction	20.37%	21.79%	18.07%	19.31%	19.06%
Total genes	648	234	476	637	577

Table A-3. Genes upregulated ≥5x over WT media BMDCs that share a biological function related to an immune response or fibrosis.

Biological function	Number of genes
Cytokines	20
Chemokines	12
Cytokine and chemokine receptors	9
Growth factors	13
Growth factor receptors and binding proteins	9
Extracellular matrix constituents	16
Metalloproteinases	6
Integrins and integrin binding proteins	8

media RPKM 0.05, upregulation 313x), and the upregulation was dependent on MyD88 (RPKM 0.56 vs WT SR RPKM 15.66, downregulation 28x) and TLRs 2 and 9 (RPKM 1.94 vs WT SR RPKM 15.66, downregulation 8x). Integrins are a family of adhesion molecules that bind to the extracellular matrix and are critical for cellular trafficking of immune cells. They consist of two chains, an alpha chain and a beta chain, and the pairing determines substrate specificity. Integrin αv pairs with β3 to form the vitronectin receptor, which binds vitronectin and other components of the extracellular matrix (Horton, 1997). Integrin αv can also pair with β8 to activate TGFβ. TGFβ is secreted in an inactive form bound to the latency associated peptide (LAP). TGFβ activating integrins such as αvβ8 bind LAP through an RGD-motif to release active TGFβ (reviewed in Worthington et al., 2012). Previous studies have shown that αv and specifically αvβ8 are required for TGFβ activation and polarization of Tregs and Th17 cells in a mouse model of EAE and colitis (Acharya et al., 2010; Melton et al., 2010; Paidassi et al., 2011; Travis et al., 2007). The upregulation of αvβ8 on fibroblasts has also been associated cytokine and chemokine secretion and the development of lung fibrosis in a mouse model of asthma and COPD (Kitamura et al., 2011). Importantly, treatment of mice with a cyclic RGD peptide protected mice from the development of EAE, and blocked the activation of TGFβ and the polarization of Th17 cells in vitro (Acharya et al., 2010). Further studies to confirm the upregulation of αvβ8 in SR exposed mice are necessary, but may reveal a critical mediator of the Th17 response and lung fibrosis in HP.

VITA

Kelly Andrews was born in 1981 in Lansing, Michigan. She graduated from Western Michigan University in 2004 with a major in Biomedical Sciences, and a minor in Chemistry and Environmental Sciences. She continued to take math and physics classes at Lansing Community College in 2005 and 2006. In 2007, she took classes in biochemistry and immunology at Michigan State University and volunteered in the lab of Dr. Animesh Sinha. In 2008, she entered the Integrated Program in Biomedical Sciences (IPBS) at the University of Tennessee Health Science Center. She joined the laboratory of Dr. Elizabeth Fitzpatrick where she investigated the role of toll-like receptors 2 and 9 in the pathogenesis of hypersensitivity pneumonitis. She graduated in December of 2013 with a PhD in immunology.