THE ROLE OF INNATE IMMUNITY IN ORCHESTRATING TH17 CELL PATHOGENESIS IN SEVERE SCHISTOSOMIASIS

A Dissertation

Submitted by

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ABSTRACT

In murine *Schistosoma mansoni* infection, the magnitude of immunopathology and proinflammatory cytokine production in response to live schistosome eggs is strain dependent. Whereas infected CBA mice develop severe disease characterized by hepatic egg-induced granulomatous inflammation that is associated with prominent Th17 and Th1 cytokine responses, milder lesions develop in the Th2-polarized cytokine environment of infected BL/6 mice. The initiation of pathogenic Th17 cell differentiation in CBA mice is largely dependent on dendritic cell (DC) production of IL-1β and IL-23 following stimulation with schistosome eggs; by contrast, low-pathology BL/6 mice fail to mount this proinflammatory cytokine pathway. While the preconditions necessary for the generation of Th17 cells induced by CBA DCs have been elucidated, the reasons for strain-dependent differences in antigen (Ag) presenting cell (APC) reactivity to live eggs are not well understood. Genome-wide genetic profiling disclosed dramatic differences in the expression of C-type lectin receptors (CLRs) by CBA and BL/6 bone marrow derived DCs (BMDCs). CLRs are a family of pattern recognition receptors (PRRs) that specifically bind carbohydrate Ags such as the fucose-containing glycans that are abundantly secreted by live schistosome eggs. Marked elevation of CD209a, a murine homologue of the well characterized human CLR DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), was detected in infected CBA tissues including liver, spleen, and granuloma cells by means of quantitative real-time PCR (qRT-PCR), flow cytometric analysis, and immunohistochemistry. Functional assessment of CD209a-expressing APC subsets determined that CBA DCs, but not macrophages, B cells, or neutrophils, elicit Th17 cell differentiation in response to eggs. Additionally, inhibition of Th17 cell induction by CBA DCs pre-treated with mannose prior to egg stimulation suggested lectin-dependency. Further gene silencing in CBA DCs and over-expression in BL/6 DCs, demonstrated that CD209a is essential for IL-1β and IL-23 production as well as subsequent differentiation of Rorγt^{+} Th17 cells. Signaling analysis revealed that DC CD209a expression was associated with SRC, RAF-1, and ERK1/2 activation; importantly, CD209a expression was necessary for ERK1/2-dependent IL-23 and IL-1β production in response to eggs. These findings reveal a novel role for CD209a in mediating pathogenic proinflammatory Th17 cytokine responses in helminthic disease.

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ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
BL/6	C57BL/6
BMDC	Bone marrow-derived dendritic cell
CBA	CBA/J
CBD	Carbohydrate binding domain
CLR	C-type lectin receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICMA3-grabbing non-integrin
EAE	Experimental Autoimmune Encephalomyelitis
ERK	Extracellular signal related kinase
FACS	Fluorescence-Activated Cell Sorting
GalNAc	*N*-Acetylgalactosamine
GC	Granuloma Cell
GlcNAc	*N*-Acetylglucosamine
GO	Gene ontology
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KO	Knockout
LDN	GalNAcβ1–4GlcNAc (LacdiNAc)
LDN-F	Fucosylated GalNAcβ1–4GlcNAc

Le^X	Lewis X
LNFPIII	Lacto-N-fucopentose III
LPS	Lipopolysaccaride
mAb	Monoclonal antibody
MLNC	Mesenteric lymph node cell
NTD	Neglected Tropical Disease
NLR	NOD-like receptor
NTD	Neglected Tropical Disease
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PZQ	Praziquantel
RBC	Red blood cell
RLR	RIG-like receptor
SEA	Soluble egg antigen
SFK	SRC family kinases
TCR	T cell receptor
Tg	Transgenic
Th	T helper
TLR	Toll-like receptor
Treg	T regulatory
WT	Wild type

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SECTION I: INTRODUCTION

CHAPTER 1

Schistosome Biology and Infection

Schistosomiasis is the second most socioeconomically devastating parasitic disease in the world, for which the causative pathogen is one of various blood-dwelling helminths of the genus Schistosoma. Severe liver, intestinal, or bladder immunopathology occurs as a result of highly immunogenic parasite eggs that become lodged in host tissues. Varying magnitudes of granulomatous pathology materialize among humans and mice with diverse genetic backgrounds. Such heterogeneity in disease has been linked to differential T cell responses. Chapter 1 will discuss the biology of schistosome parasites and the immune-mediated disease that develops in human and murine schistosome infection.

1.1 Schistosomiasis and The Life Cycle of *Schistosoma mansoni*

Schistosomiasis

Schistosomiasis is one of the seventeen infectious diseases categorized by the World Health Organization (WHO) as a Neglected Tropical Disease (NTD). NTDs are tropical infections of bacterial, parasitic, protozoan, or viral origin that predominate in developing countries of the Americas, Africa, and Asia where sufficient healthcare is unavailable and disease control efforts focus on prominent diseases such as Human Immunodeficiency Virus (HIV-1) infection, tuberculosis, or malaria (Bhopal et al., 2013; Freeman et al., 2013). Despite preventative measures to control NTDs, they are a major source of illness and devastation, often causing severe complications during co-infection with pathogens such as HIV-1 (Chenine et al., 2008; Da'dara & Harn, 2010; Hotez et al., 2010; Mbabazi et al., 2011; Andreani et al., 2012). Like numerous NTDs, schistosomiasis is a parasitic infection caused by helminths. Disease materializes following infection with one of various species of tremadote helminths, also known as blood flukes or schistosomes, that are classified in the genus *Schistosoma* for which the disease is appropriately named (Bica et al., 2000; Fallon et al., 2000; Pearce & MacDonald, 2002; Larkin et al., 2012). Presently, schistosomiasis is the second most socioeconomically devastating parasitic disease in the world, severely disrupting developing countries to a degree only surpassed by malaria. 74 countries in tropic regions of the globe are endemic for schistosome infection. The CDC estimates that a total of 600,000,000 people are at risk, while 200,000,000 people are currently affected. This amounts to an estimated 20,000,000 incidents of severe schistosomiasis and

200,000 deaths per year (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002; Larkin et al., 2012).

Schistosomiasis was first described in the mid 1800s in Cairo, Egypt by a German pathologist named Theodor Maximilian Bilharz who discovered a previously unidentified worm now recognized as *Schistosoma haematobium.* Dr. Bilharz originally described a new genus for such worms of blood-dwelling nature, which he termed *Bilharzia* (Hagan, 2009). The genus was later re-named *Schistosoma*; nonetheless, the term bilharzia is still used to refer to parasitic disease caused by infection with schistosomes including, but not limited to, *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma intercalatum,* and *Schistosoma mekongi.* Depending on the geographical region, different schistosome species prevail. For this reason, distinct forms of pathology commonly arise in particular regions of the world (Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012)(Fig.1). The vast majority of schistosomiasis cases are caused by three schistosome species: *S. mansoni and S. japonicun,* which cause hepatosplenic-intestinal disease and *S. haematobium*, which causes urinary disease. While both forms of schistosomiasis are endemic in Africa, hepatosplenic-intestinal disease is prevalent in Asia and the Americas (Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012).

Life Cycle of S. mansoni

Parasites are by definition organisms that require a specific host for nutrients and survival. As such, schistosomes evolved to progress through an intricate life cycle in order to mature, reproduce, and propagate. As digenetic organisms, schistosomes need

two separate hosts for survival. While the human generally serves as the definitive host, the intermediate host is an aquatic snail that is unique to each schistosome species. For *S. mansoni,* the species of interest in the current study, the intermediate host is one of numerous snails in the genus *Biomphalaria.* The fiery-colored *Biomphalaria glabrata* is one such snail species that infests freshwater ponds and rivers in tropic regions around the world, serving as the primary parasite vector (Bica et al., 2000; Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012) (Fig. 2). The infectious form of *S. mansoni*, the cercaria, is shed from the snail upon sensing humans in water by means of largely undescribed chemical and thermal sensory mechanisms. Cercariae are equipped with a bifurcated tail enabling them to migrate towards a human and penetrate directly into the epidermis through a wound or hair follicle. Upon entering human skin, the parasite loses its tail and transforms into an immature worm, termed schistosomulum (Fallon, 2000; Pearce & MacDonald, 2002; Wilson et al., 2007). Subsequently, the schistosomula enter circulation and undergo extensive migration through the human body, pausing for brief periods in the lungs and hepatic portal vein to mature into adult worms. Adult male and female worms are dioecious, that is, morphologically distinct and separate organisms, that will reside in the mesenteric venous plexus as pairs where they survive for years rousing little immune response. However, the female worm is capable of laying nearly 300 eggs per day, which are swept throughout the blood stream (Bica et al., 2000; B. M. Larkin et al., 2012).

Depending on the final destination of the eggs, different outcomes arise for parasite and host. Most eggs exit the mesenteric venous plexus where they elicit granulomatous inflammation that aids their passage through the intestine, from where

they are finally released into the environment (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002). Upon excretion, eggs hatch upon contacting freshwater, yielding miracidium, a larval form of the parasite. In continuation of the life cycle, miracidia infect freshwater snails, inside which many generations of sporocysts develop asexually, giving rise to infectious cercariae. As such, the life cycle of *S. mansoni* carries on (Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012)(Fig. 3). Alternatively, eggs that fail to reach the intestine are destined to a devastating fate, for both parasite and host. As the female worm continues to lay hundreds of eggs per day, many are swept up into the liver via the portal circulation. Once embolized in liver tissue, eggs stimulate robust granulomatous inflammation, which eventually leads to scar formation (fibrosis) (Bica et al., 2000; Fallon, 2000; Wilson et al., 2007). In severe cases of schistosomiasis, eggs may also deposit in the spleen, brain, or kidneys. Overall, deposition of eggs in the liver is detrimental to both the host, who develops clinical disease, as well as to the parasite for whom it represents a dead end (Pearce & MacDonald, 2002; Wilson et al., 2007).

The nature of S. mansoni eggs

The *S. mansoni* species was originally named for Patrick Manson, a British parasitologist known as the “Father of Tropical Medicine” who was renowned for his revolutionary discoveries in filarial disease (Manson-Bahr, 1962). Manson was the first to describe the unique laterally-spined eggs of *S. mansoni*, which are visually distinct from other schistosome species’ eggs that contain a terminal spine or lack a spine

entirely. After Manson's identification of this distinct species, the schistosome was suitably named, "*mansoni,"* for his discovery (Manson-Bahr, 1962; Hagan, 2009). Presently, the location of the spine is a diagnostic factor that continues to be useful for distinguishing the causative schistosome species during infection. It is believed that the spine may aid the egg's passage through the intestine (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002).

Schistosome eggs are large oval structures spanning ≈140μm in length. Living eggs that become lodged in liver tissue are highly immunogenic in nature (Wilson et al., 2007; Larkin et al., 2012). The egg surface is profusely glycosylated and leaks a vast secretome of unique glyco-proteins, which is well characterized (Cummings & Nyame, 1999; Cass et al., 2007). 188 secreted schistosome egg proteins are documented (Cass et al., 2007) including omega-1 ribonuclease (Everts et al., 2009; Steinfelder et al., 2009), IPSE/alpha 1 (SmEP25) (Schramm et al., 2003; Williams et al., 2005), the major *S. mansoni* egg Ag Sm-p40 (Nene et al., 1986; Cao et al., 1993; Cai et al., 1996) secretory glycoprotein kappa5 (k5) (Schramm et al., 2009), thioredoxin (Williams et al., 2001), and phosphoenol-pyruvate carboxykinase (Asahi et al., 2000). While the functions of these proteins are diverse, they all retain schistosome-specific glycosylation patterns (Cummings & Nyame, 1999; Hokke & Yazdanbakhsh, 2005; Prasanphanich et al., 2013).

Natural schistosome glycans are both O- and N- linked short branching saccharides that commonly contain terminal α–linked fucose, β–linked galactose, or β–linked N-acetylgalactosamine (GalNAc) residues (Hokke & Yazdanbakhsh, 2005; Prasanphanich et al., 2013). Such schistosome-specific motifs are structurally distinct

from the glycosylation patterns that usually adorn human proteins. In particular, schistosomes fail to synthesize Sialic acid, a common terminal component of human glycans (Cummings & Nyame, 1999; Hokke & Yazdanbakhsh, 2005). Abundantly produced schistosome glycans include Lewis X (Le^X), poly-Le^X, pseudo Lewis Y, GalNAcβ1–4GlcNAc (LacdiNAc (LDN)), fucosylated LDN (LDN-F), CAA, F-LDN, F-LDN-F, and HexNAc-DF (Cummings & Nyame, 1999; Hokke & Yazdanbakhsh, 2005; Prasanphanich et al., 2013)(Fig. 4). Similarly, it was demonstrated that schistosome glycolipids are distinct from host-expressed lipo-conjugates and contain the characteristic core "schisto-motif," GalNAcβ1-4Glcβ-Cer (Makaaru et al., 1992). Of note is the high fucose content of schistosome glycans. Unlike many pathogens that express a bounty of mannose-based oligosaccharides, schistosomes are one of few genera of pathogens that evolved to synthesize fucosylated antigens. To this date, the immuno-stimulatory vs. -modulatory purpose of such molecules remains a topic of debate (Cummings & Nyame, 1999; Hokke & Yazdanbakhsh, 2005; Prasanphanich et al., 2013). Ultimately, while specific Ags from each stage of the *S. mansoni* life cycle have been identified, the egg Ags are of highest interest for their inflammatory properties, which contribute to disease (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002; Shainheit et al., 2011; Larkin et al., 2012).

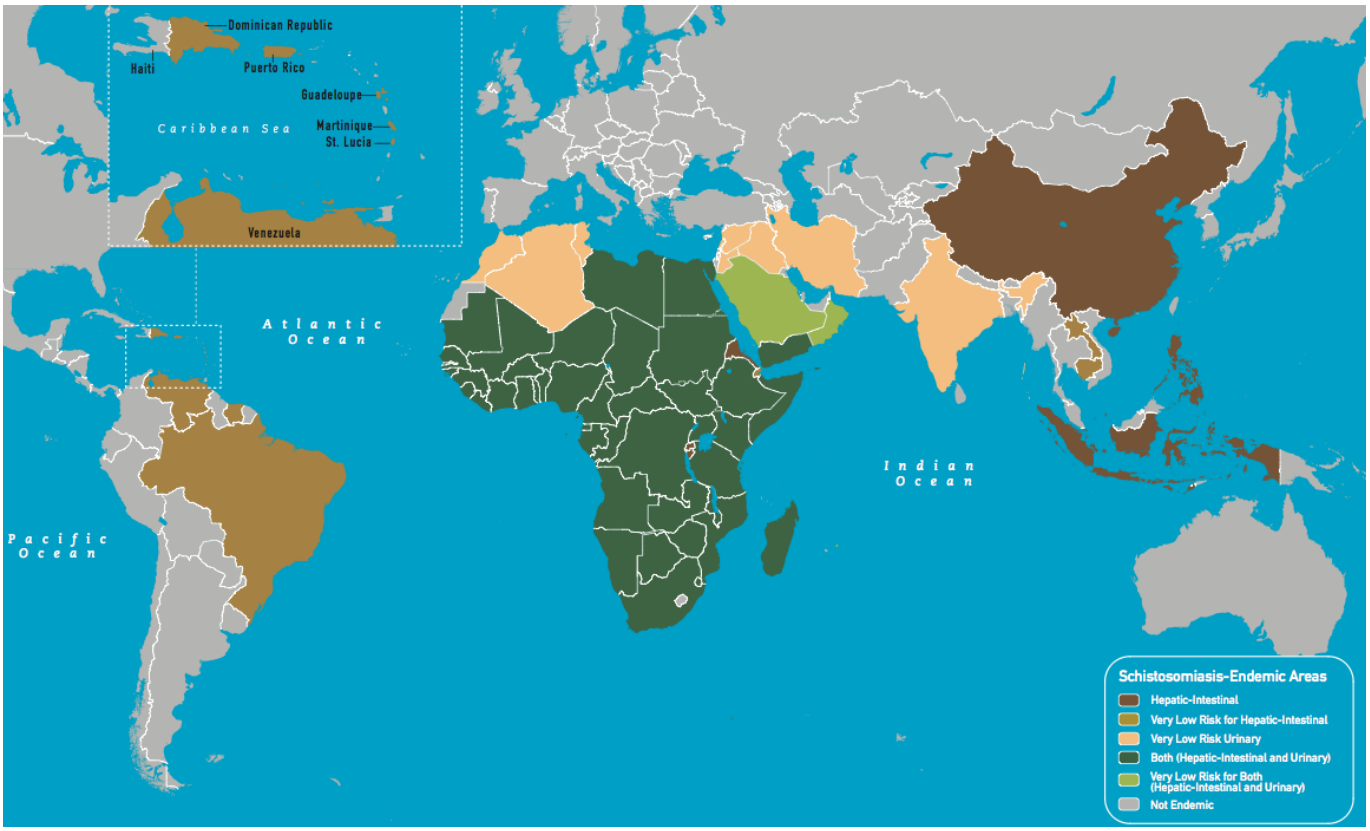

Figure 1. World map of schistosome infection. Schistosomiasis is serious parasitic disease caused by numerous blood-dwelling flukes, termed schistosomes. Schistosome infection is endemic in 74 countries around the world causing widespread morbid disease in developing countries. Distinct forms of pathology materialize depending on the causative schistosome, which varies in different regions of the globe. In Africa, both urinary and hepatosplenic pathologies are prevalent, while other regions of the world are more commonly linked to one form of disease. The CDC estimates that 600,000,000 people are risk of transmitting schistosome infection, while 200,000,000 people are affected, primarily in tropic regions (Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012). Map from CDC.gov (“Schistosomiasis," CDC, 2014).

Figure 2. Transmission of disease in schistosomiasis. Schistosomiasis is transmitted in underdeveloped countries where people bathe or wash clothing in parasite-infested freshwater ponds and rivers (*upper left, bottom right*). Such bodies of water harbor the intermediate host of schistosomes, freshwater snails from the genera *Bulinus, Biamphalaria,* or *Oncomelania* (*left to right)* for *S. haematobium*, *S. mansoni*, or *S. japonicum*, respectively. Such snails shed infectious cercariae (*lower left, S. mansoni*), which can burrow directly into human skin (Bica et al., 2000; Fallon, 2000; Wilson et al., 2007).

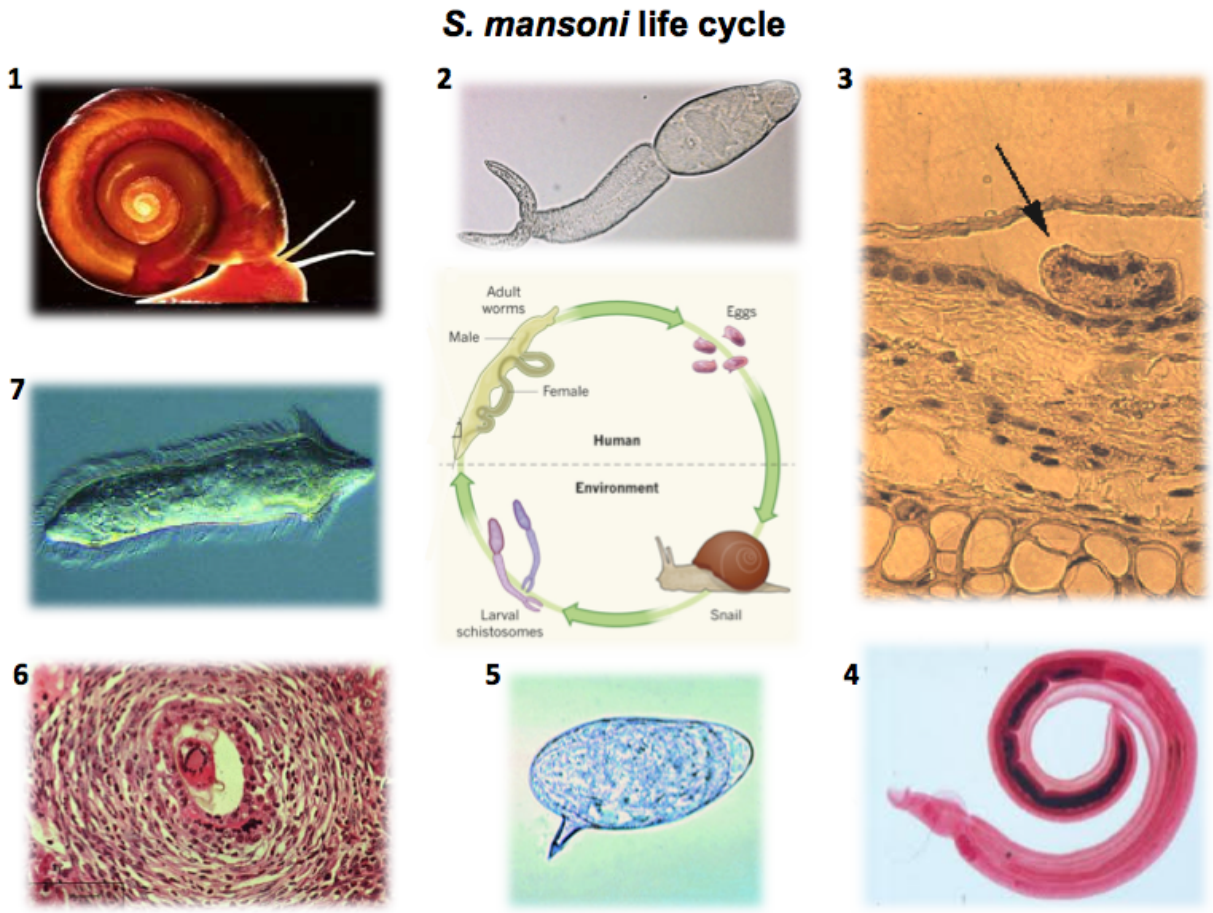

Figure 3. Life cycle of *S. mansoni.* Schistosomes are digenetic parasites that progress through an intricate life cycle including 8 main stages. The freshwater snail, *B. glabrata,* (1) is the intermediate host of *S. mansoni* and sheds infectious cercariae (2). Cercariae sense humans in freshwater and penetrate directly into skin through a wound or hair follicle where they shed their tail and transform into schistosomula (3). This immature worm enters the circulation, migrating through the lungs and hepatic portal system where it ultimately matures into an adult worm (4). Adult male and female worms pair and reside in the mesenteric venous plexus. Females produce 300 eggs (5) per day, which are swept through the vasculature and ensnare in tissue of the intestinal wall. Granulomatus inflammation (6) facilitates passage into the intestine for excretion into the environment. Upon contact with freshwater, miracidia (7) hatch from eggs and infect freshwater snails, thus continuing the life cycle. Center diagram adapted from Pearce & MacDonald, 2002 (Pearce & MacDonald, 2002).

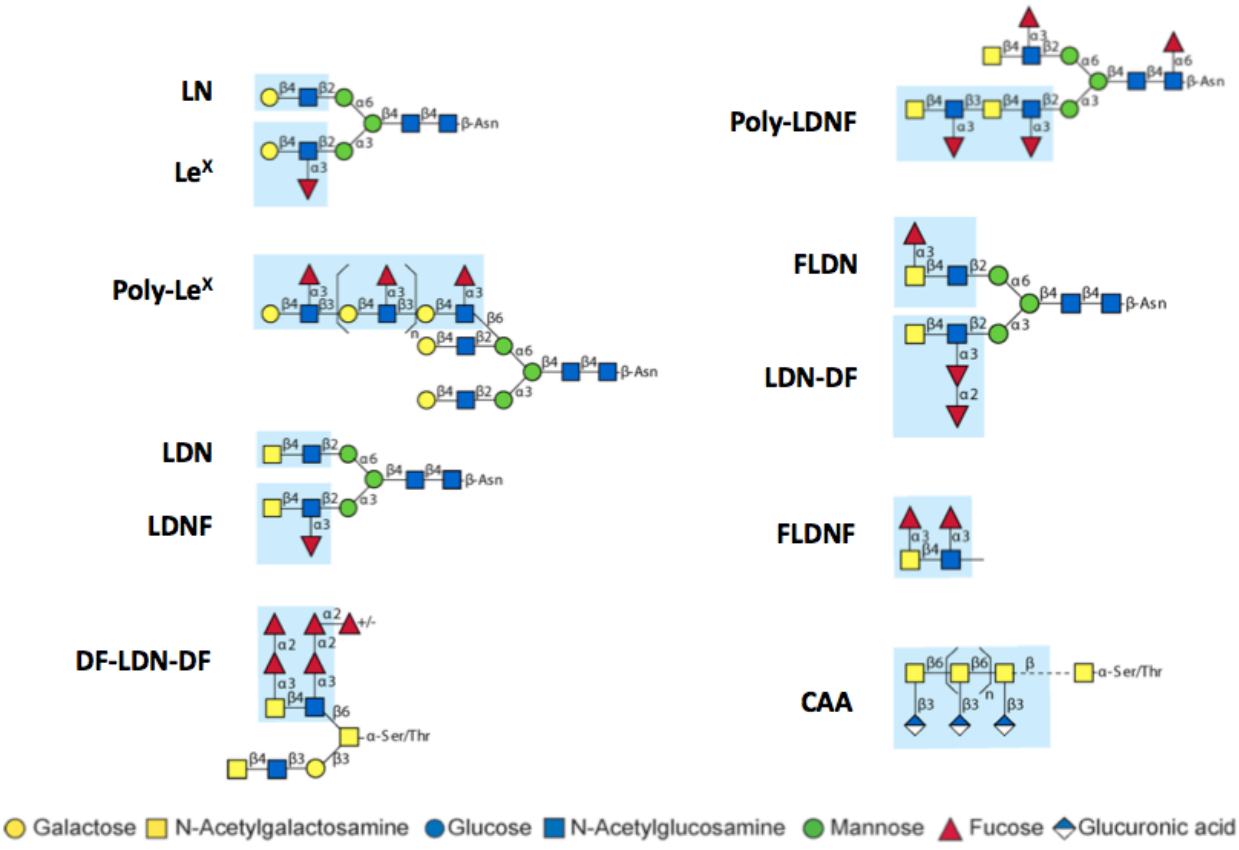

Figure 4. The schistosome glycans. Schistosome glycans are small carbohydrate chains characterized by terminal fucose, galactose or GalNAc residues. Such glycans are abundantly expressed on the surface of schistosome eggs as well as secreted in the form of glycoconjugated proteins or lipids. Glycans depicted were originally described in *S. mansoni* eggs, but are also present in other schistosome species. Schistosome glycosylation patterns are distinct from most human glycosylations, likely contributing to their immunogenic nature. For each glycan structure, shapes are representative of the indicated monosaccharides. α− and β− linkages are noted. LN, LacNAc; Le^X, Lewis X; F, fucose; CAA, Circulating Anodic Antigen (Prasanphanich et al., 2013). Diagram adapted from Prasanphanich et al., 2013.

1.2 Immunobiology Human and Murine *Schistosoma mansoni* Infection

Immunopathology

Schistosomiasis is an infectious disease characterized by detrimental immune responses that fail to eliminate the causative pathogen. Morbidity and mortality are the result of granulomatous inflammation that ensues following the entrapment of parasite eggs in vital organs (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012). In *S. mansoni* infection, eggs deposit in the liver and intestines resulting in hepato-intestinal disease. In such organs, copious egg Ag release triggers the infiltration of several innate and adaptive immune cells including T cells, B cells, macrophages, neutrophils, eosinophils, and dendritic cells (DCs). The result is the development of dense perioval granulomatous inflammation, which eventually results in fibrosis. Notably, human schistosomiasis develops in two forms. A majority of infected individuals develop mild intestinal disease characterized by moderate gastrointestinal symptoms; in contrast, 10% of infected individuals develop life-threatening hepatosplenic disease (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012). In such severe cases, chronic fibrosis of the liver causes elevated blood pressure in the portal venous system, termed portal hypertension. As a consequence, complications develop including splenomegaly, ascites, portal-systemic shunting, gastrointestinal hemorrhage, and death. To this date, the causes of heterogeneous disease in humans are not fully understood (Bica et al., 2000; Fallon, 2000; Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012).

Similar to humans, different forms of pathology have also been described in mice. The CBA/J (CBA) and C57BL/6 (BL/6) mouse strains develop contrasting forms of pathology that are analogous to severe and mild human disease, respectively. *S. mansoni*-infected CBA mice develop severe hepato-intestinal pathology characterized by large poorly circumscribed liver granulomas and pronounced splenomegaly (Fig.5, Table I). Parenchymal inflammation is evident in CBA liver histology (Fanning et al., 1981; Cheever et al., 1987; Hernandez et al., 1997). In contrast, under identical infection conditions, BL/6 mice develop mild disease characterized by significantly smaller liver granulomas and healthier parenchymal tissue (Pearce et al., 1991)(Fig. 5, Table I). Despite differences in immunopathology, CBA and BL/6 mice retain identical parasite burdens throughout the 7-week infection with equal numbers of eggs present in CBA and BL/6 liver (Larkin et al., 2012). CBA and BL/6 mice provide a faithful murine model to study the immunological mechanisms underlying the development of severe vs. mild disease in *S. mansoni* infection; as such, the following sections will focus on the details of murine schistosomiasis previously learned from studies in this experimental model.

$CD4^+$ T cells in schistosomiasis

Schistosomiasis is mainly an immune-mediated disease; thus, the innate and adaptive immune cell responses that occur at sites of granulomatous liver pathology are of prime interest. The development of pathology in murine schistosomiasis is dependent on MHC Class II-restricted $CD4^+$ T cells. As a result, in the absence of $CD4^+$ T cells, liver granuloma size is significantly reduced (Hernandez et al., 1997; Phillips et

al.,1977). More specifically, $CD4^+$ T cells expressing rearranged αβ T cell receptors (TCR), but not γδ TCRs, are necessary for the formation of egg-induced liver granulomas (Iacomini et al., 1995; Hernandez et al., 1997). Similarly, in human schistosomiasis, the requirement of $CD4^+$ T cell responses for the development of immunopathology is nicely demonstrated in patients co-infected with HIV-1. Such individuals fail to have eggs present in their stool due to an absence of intestinal granulomatous inflammation (Karanja et al., 1997). Therefore, $CD4^+$ T cell responses are necessary for the development of pathology in schistosomiasis as well as for continuation of the parasite life cycle.

In light of the critical role of $CD4^+$ T cells in *S. mansoni* infection, the TCR repertoire of schistosome egg Ag-specific T cells was previously described in infected CBA and C3H mouse strains, which both develop severe disease. Analysis of T cells specific for the major schistosome egg Ag, Sm-p40, demonstrated that a majority of Sm-p40-specific $CD4^+$ T cells from infected CBA and C3H mice, both having the $H\text{-}2^K$ haplotype, express an expanded αβ TCR repertoire specific for a single immunodominant epitope spanning amino acids 234-246 of Sm-p40 (Hernandez & Stadecker, 1999; Finger et al., 2005). The discovery of a principal Sm-p40 epitope, together with a specific TCR documented as Vα11.3Vβ8, gave rise to a transgenic CBA mouse that expresses the Vβ8 TCR on >95% of T cells (Shainheit et al., 2011). Such a mouse proved to be a valuable tool for studying schistosome egg-specific $CD4^+$ T cell responses. Notably, no immunodominant TCR epitope was identified in BL/6 mice that develop mild pathology.

Cytokine responses in severe and mild disease

As mentioned, CBA and BL/6 mice provide a faithful murine model for exploring the immunological processes in severe vs. mild schistosomiasis (Fig. 6). Previous studies demonstrated that infected CBA and BL/6 mice mount divergent cytokine responses in *S. mansoni* infection. Severe pathology in CBA mice is mediated by $CD4^+$ T cell IL-17 production (Rutitzky et al., 2005; Rutitzky & Stadecker, 2006). IL-17 is largely the product of T helper (Th) 17 (Th17) cells, a highly proinflammatory subset of $CD4^+$ effector T cells that also produce IL-22, colony stimulating factors (CSFs), CXCL1, CXCL2, and TNF-α (Dong, 2006; Bettelli et al., 2007; Stockinger & Veldhoen, 2007; Iwakura et al., 2008). The development of such Th17 cells requires IL-1β and IL-23 production by professional antigen presenting cells (APCs) (Laura I Rutitzky et al., 2008; Shainheit et al., 2008). Notably, CBA mice also develop simultaneous Th1 and Th2 cell responses during infection (Stadecker et al., 2004). The result is a strong proinflammatory environment consisting of Th17, Th1, and Th2 cells (Stadecker et al., 2004; Rutitzky & Stadecker, 2006; Larkin et al., 2012). In contrast, infected BL/6 mice elicit a Th2-polarized $CD4^+$ T cell response, accompanied by the development of T regulatory (Treg) cells (Pearce et al., 1991). This relatively protective immune response is responsible for the occurrence of mild pathology.

Schistosome egg-stimulated cytokine responses occurring in CBA and BL/6 mice were nicely described by *in vivo* and *in vitro* methods. The role of IL-17 in the development of severe schistosomiasis was demonstrated with mice deficient for IL-17 as well as Th17-associated innate cytokine subunits (Rutitzky et al., 2005; Rutitzky et

al., 2008; Rutitzky & Stadecker, 2011). As cytokine-deficient mice are readily available on a BL/6 background, BL/6 mice immunized with schistosome soluble egg Ag (SEA) in combination with Complete Freund's Adjuvant (CFA) were used as a surrogate model to study cytokine requirements in the development of severe Th17-mediated pathology (Rutitzky et al., 2001). *S. mansoni*-infected SEA/CFA-immunized BL/6 mice develop severe pathology characterized by T cell IL-17 and IFN-γ production; however, in infected IL-17-deficient SEA/CFA-immunized BL/6 mice, pathology was significantly decreased (Rutitzky & Stadecker, 2011). Additionally, SEA/CFA-immunized BL/6 mice deficient for the p19 subunit of IL-23 developed greatly reduced pathology concurrent with a significant drop in IL-17 (Rutitzky et al., 2008). In contrast, in IL-12p35 deficient mice, that lack IL-12 but not IL-23, IL-17 production and severe pathology was unchanged from SEA/CFA-immunized WT BL/6 mice during infection (Rutitzky et al., 2005). Such *in vivo* studies confirmed that Th17, but not Th1 or Th2, cell responses are required for the development of marked granulomatous inflammation in murine schistosomiasis (Larkin et al., 2012). The necessity of IL-17 for severe pathology was also verified utilizing an anti-IL-17 neutralizing antibody treatment (Rutitzky et al., 2005). Finally, such *in vivo* studies demonstrated that the Th17-associated cytokine IL-23 was required for pathogenic Th17 cell development (Rutitzky et al., 2008). IL-1β, yet another innate Th17-associated cytokine, was also proven critical for Th17 cell development in infected CBA mice in studies utilizing an IL-1 receptor (IL-1R) antagonist (Shainheit et al., 2011).

In vitro co-culture systems were extremely useful tools for further elucidating the intricate innate and adaptive cytokine responses occurring during schistosome infection.

Several studies demonstrated that dendritic cells (DCs) are potent APCs that facilitate pathogen-specific Th cell responses. Thus, an *in vitro* system of DC and T cell stimulation was designed utilizing bone marrow-derived DCs (BMDCs), syngeneic naïve splenic $CD4^+$ T cells and schistosome eggs to assess egg-specific cytokine responses (Fig. 7). In this system, anti-CD3/CD28-coated beads were included to amplify cytokine responses, which are dependent on DC – T cell contact (Shainheit et al., 2008). Cytokine production by wholly-CBA or -BL/6 co-cultures reflects the cytokine responses that occur in infected CBA and BL/6 mice. Specifically, egg-stimulated CBA DCs sequentially produce IL-23 and IL-1β promoting T cell IL-17 production in response to eggs (Shainheit et al., 2008; Shainheit et al., 2011). CBA DCs also produce IL-6, IL-12, and TGF-β, leading to simultaneous Th1 and Th2 cell development indicative of T cell IFN-γ, IL-4, IL-5, and IL-13 production (Shainheit et al., 2008). In contrast, BL/6 DC – T cell co-cultures produce fewer cytokines in response to eggs, including DC production of TGF-β and IL-10, which leads to Th2 cell development. Such BL/6 Th2 cells mainly produce IL-4, IL-5, and IL-13 (Shainheit et al., 2008)(Fig. 6).

To this date, few studies have explored inflammatory responses in human schistosomiasis and the mechanisms underlying the variation in egg-induced immunopathology are largely unknown. Nonetheless, it is noteworthy that a recent study of *S. haematobium* infection in humans similarly linked the development of pathology to an increase in Th17 cells in the peripheral blood. In contrast, Treg cells were reduced. Likewise, infected CBA mice had significantly increased numbers of Th17 cells in peripheral blood in comparison to BL/6 mice (Mbow et al., 2013). This observation

suggests that Th17 cell responses are associated with human pathology and validates the experimental murine model.

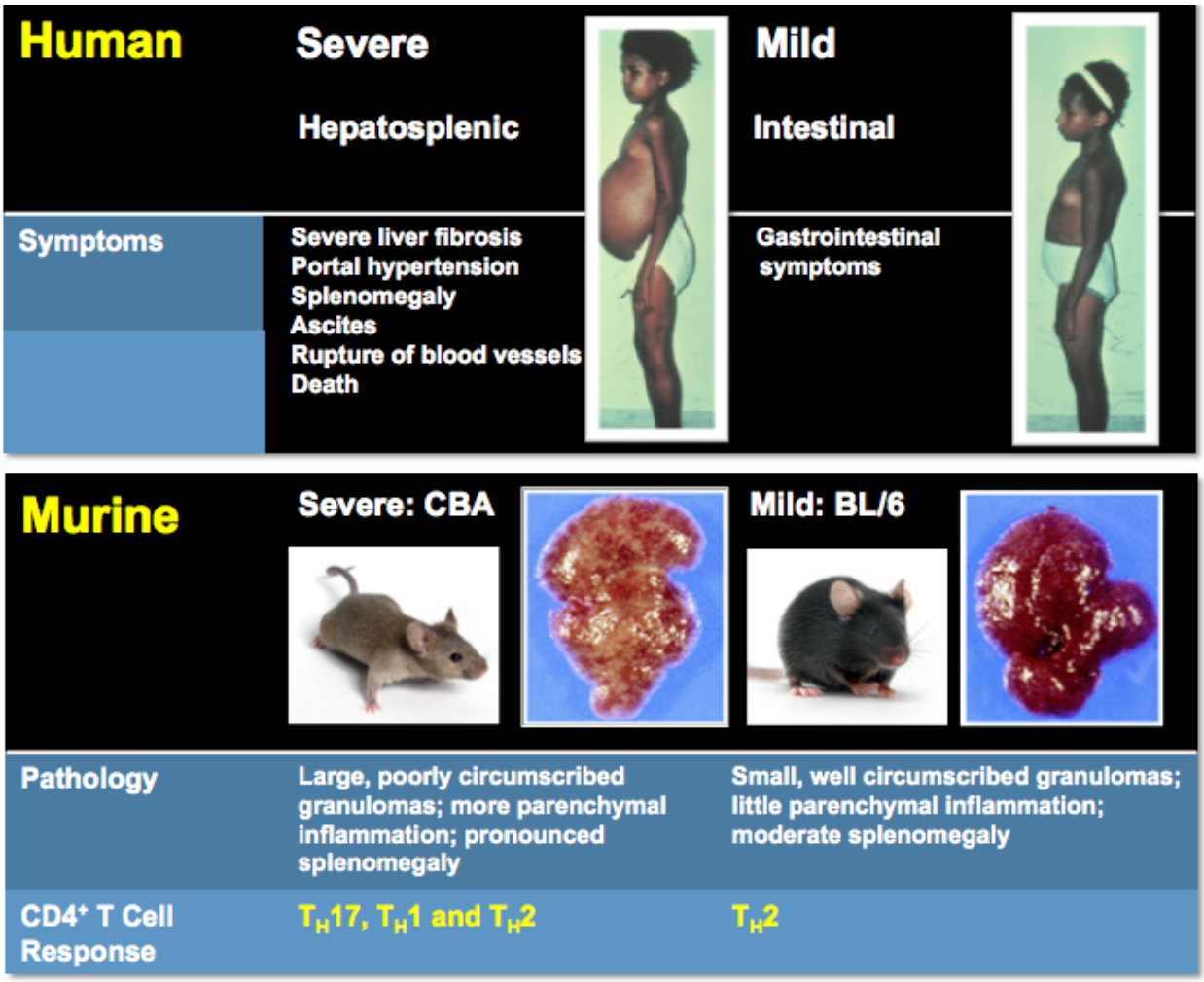

Table I. Heterogeneity of disease in humans and mice. Highly contrasting forms of pathology occur in human and murine schistosomiasis. In humans, 10% of people develop severe hepatosplenic schistosomiasis, a life-threatening condition consisting of a granulomatous and fibrosing inflammatory response against parasite eggs lodged in liver and intestinal tissues. Severe liver fibrosis, ascites, and splenomegaly are hallmarks of disease. In contrast, 90% of people develop mild intestinal pathology characterized by moderate abdominal and gastrointestinal discomfort (Fallon, 2000; Pearce and MacDonald, 2002; Wilson et al., 2007). Severe and mild pathologies are exemplified by an experimental murine model including CBA (severe pathology) and BL/6 (mild pathology) mice. Under identical infection conditions, CBA mice develop large hepatic granulomas associated with Th17, Th1, and Th2 responses, while BL/6 mice develop small hepatic granulomas and Th2-polarized responses to schistosome

Ag (Pearce et al., 1991; Stadecker, 2004; Rutitzky & Stadecker, 2006; Rutitzky et al., 2005; Rutitzky et al., 2008; Shainheit et al., 2008; Shainheit et el. 2011; Larkin et al., 2012).

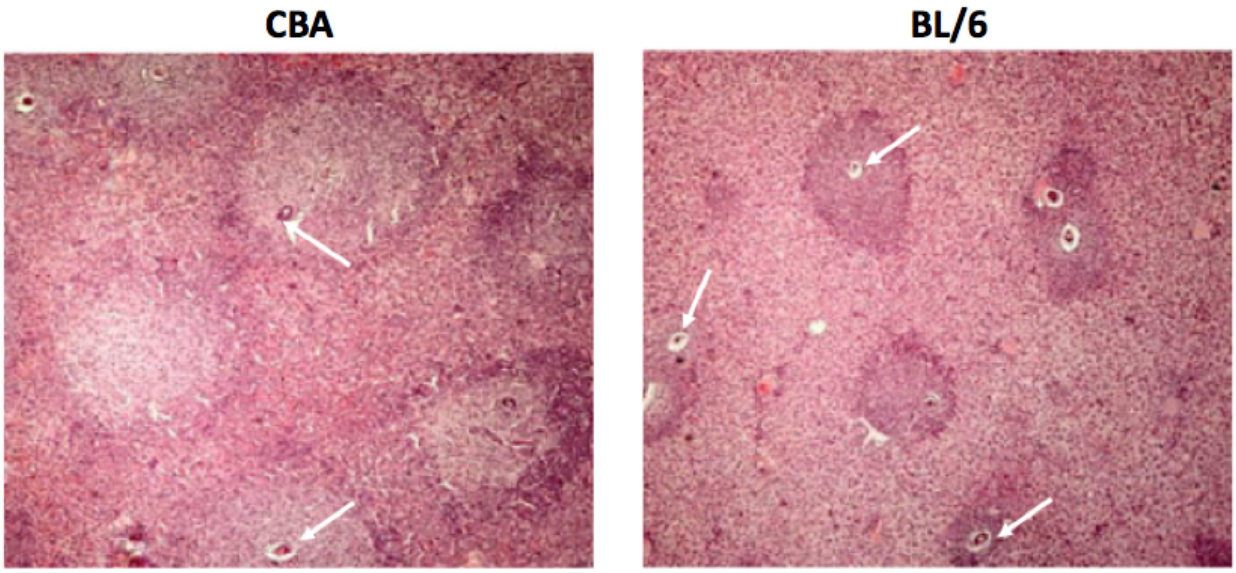

Figure 5. Hepatic granuloma formation in murine schistosomiasis. Liver histology from infected CBA and BL/6 mice demonstrates highly contrasting forms of pathology in murine *S. mansoni* infection. Severe pathology in infected CBA mice is characterized by large, poorly circumscribed liver granulomas. Parenchymal inflammation is evident. In contrast, small, well circumscribed granulomas form during mild pathology in BL/6 mice. Arrows indicate schistosome eggs (Larkin et al., 2012). Figure adapted from Larkin et al. 2012.

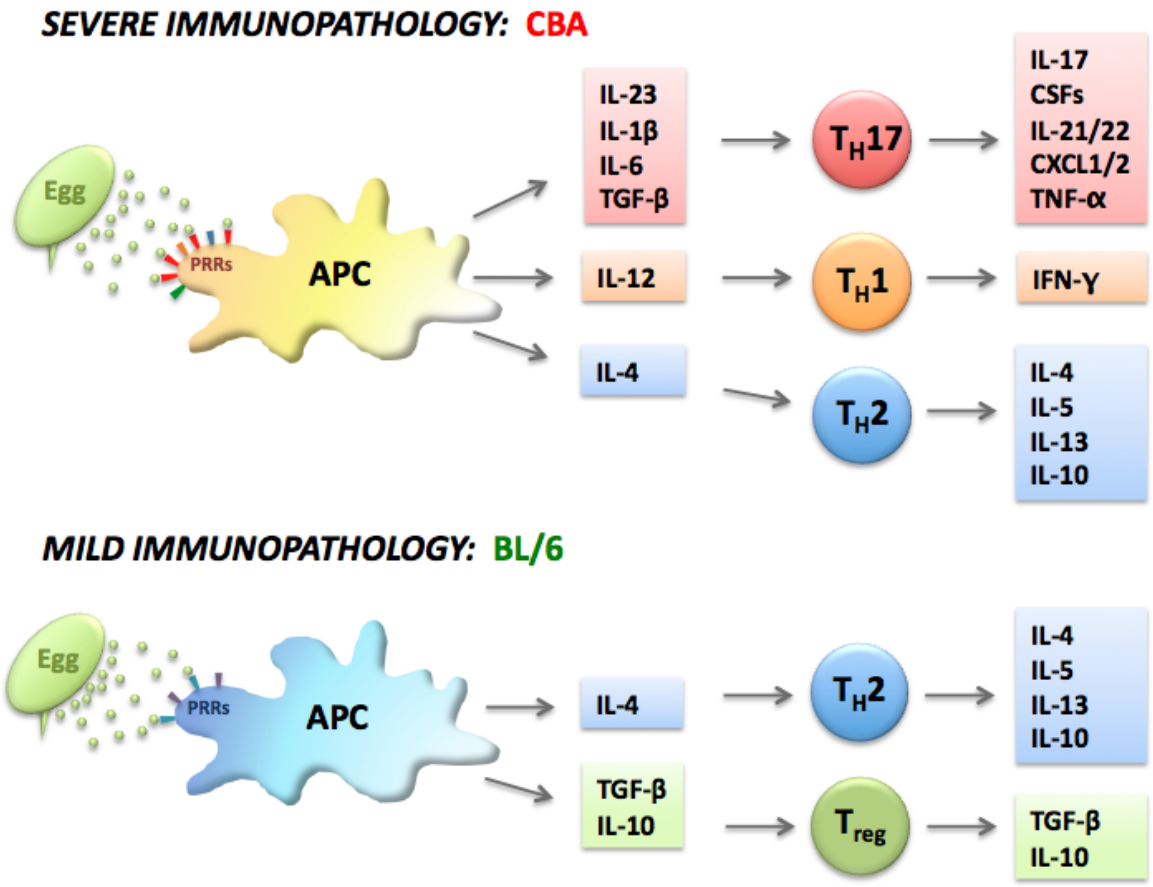

Figure 6. Role of CD4$^+$ T cell subsets in murine schistosomiasis. Cytokine responses associated with the development of severe and mild pathology in murine schistosomiasis were elucidated via cytokine analysis of infected CBA and BL/6 mice as well as *in vitro* DC-T cell-egg co-culture systems (Shainheit et al., 2008). In severe pathology (CBA), egg-stimulated APCs produce various cytokines including IL-23, IL-1β, IL-6, TGF-β, IL-12, and IL-4 leading to subsequent Th17, Th1, and Th2 development. IL-23 and IL-1β are necessary for the development of Th17-dependent severe immunopathology, which is associated with production of numerous proinflammatory cytokines and chemokines (Shainheit et al., 2008; Shainheit et al., 2011). In mild pathology (BL/6), APCs produce fewer cytokines in response to eggs including IL-4, TGF-β, and IL-10 leading to the development of Th2 and Treg cells (Shainheit et al., 2008; Larkin et al. 2012).

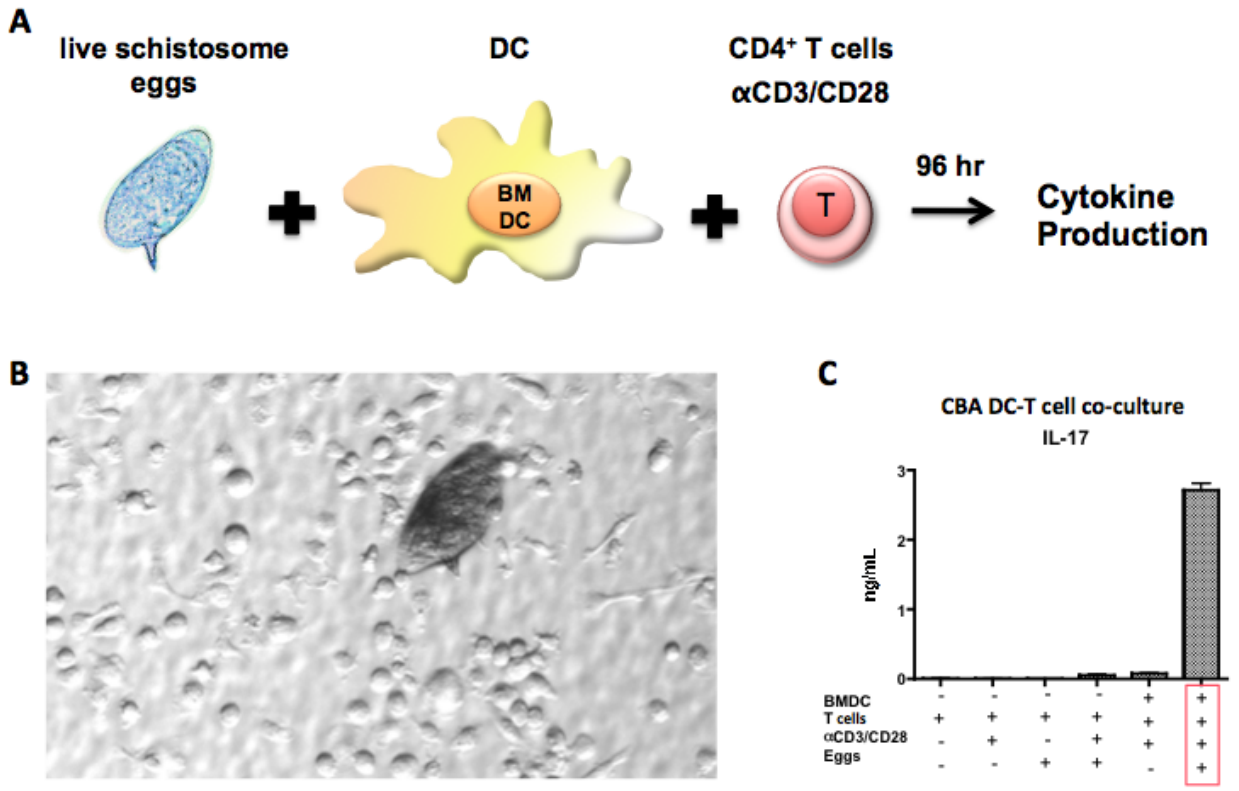

Figure 7. *In vitro* model of dendritic cell and T cell stimulation by schistosome Ag. To compare CBA and BL/6 schistosome egg-specific cytokine responses in detail, a co-culture system was created that combines BMDCs and naïve $CD4^+$ T cells together in co-culture with anti-CD3/CD28-coated beads ± freshly isolated live eggs (Shainheit et al., 2008) (A,B) Cytokine production in supernatant is assessed by ELISA after 96 hr. For controls, single cell cultures ± anti-CD3/CD28-coated beads and/or eggs are included. All components are required for Ag-specific cytokine production (C) as seen in a complete egg-stimulated CBA co-culture producing IL-17 (ELISA). BL/6 DC – T cell co-cultures fail to produce IL-17.

1.3 Treatment of Schistosomiasis

In spite of the widespread devastation caused by schistosomiasis around the globe, an anti-helminth drug is available that efficiently kills the parasite. Praziquantel (PZQ), also commonly labeled as Biltricide, Cesol, or Distocide, is a small hydrophobic molecule originally marketed for use in veterinary medicine (Doenhoff & Pica-Mattoccia, 2006; Doenhoff et al., 2009). It has been used to treat a variety of diseases caused by parasitic worms in horses, dogs, cats, and fish. Currently, PZQ is the only effective drug used for the treatment of human schistosomiasis caused by any of the schistosome species and the only drug recommended by the WHO. One dose of PZQ treatment is generally enough to kill the parasite (Doenhoff & Pica-Mattoccia, 2006; Doenhoff et al., 2009). The drug is believed to work by interfering with calcium ion channels on the surface of the worm, inducing paralysis, death, and dislodging from the walls of the mesenteric veins (Doenhoff et al., 2008). To lower transmission rates of *S. mansoni* into the environment, as well as prevent morbidity in later life, PZQ has been made readily available in high-risk regions through a donation from Merck Serono to the WHO. Annual mass-treatment of high-risk groups has aided in preventing disease transmission in some countries (Doenhoff & Pica-Mattoccia, 2006; McManus & Loukas, 2008; Doenhoff et al., 2009).

Unfortunately, there are a number of reasons why schistosomiasis is still a major problem despite the fact that PZQ, an affordable and efficient anti-helminth drug, exists. First, although one dose of PZQ eliminates the parasite, re-infection is extremely common. Second, PZQ does not readily resolve the hepato-intestinal pathology caused by schistosome eggs; thus, PZQ treatment provides minimal alleviation of advanced

disease (King et al., 2006; Doenhoff et al., 2008). Finally, despite extensive efforts, no vaccine is available at this time (Siddiqui et al., 2011).

A vaccine will ultimately be required to eradicate schistosomiasis. Justification for the development of vaccines includes high re-infection rates following treatment, high disease burden in developing countries, reliance on PZQ for treatment, and the occurrence of malnutrition and anemia in infected individuals (Hotez et al., 2010). There are also concerns that resistance to PZQ may develop due to a lack of new anti-helminth drugs; thus, efforts to develop vaccines against *S. mansoni, S. haematobium,* and *S. japonicum* are underway (McManus & Loukas, 2008; Siddiqui et al., 2011). Vaccines are generally designed to target the adult worm or infectious cercaria; as such, approaches include irradiated or recombinant proteins from the worm or cercaria (Hotez et al., 2010). One vaccine for *S. haematobium* has progressed to Phase I and Phase II clinical trials. Termed Bilhvax, this vaccine is a recombinant 28 kDa glutathione-*S*-transferase from *S. haematobium* combined with aluminum hydroxide adjuvant (McManus & Loukas, 2008; Bethony et al., 2011; Siddiqui et al., 2011). While the vaccine was documented to be immunogenic and safe in healthy individuals, no further information is currently available.

Vaccines for *S. mansoni* have not progressed to clinical trials, but several candidates exist. A vaccine designed with Sm14, a 14 kDa protein with fatty acid-binding properties, protected against *S. mansoni* infection in rabbits and mice. Sm14 also conferred protection against *Fasciola hepatica*, a parasite that mainly affects sheep and cattle, making it an attractive vaccine candidate for both human and livestock disease (Tendler & Simpson, 2008). Another vaccine under development involves Smp-

80, the *S. mansoni* protein calpain. Studies demonstrated that mice and baboons immunized with Sm-p80 had significantly reduced worm burden, egg retention, and egg excretion in stool during *S. mansoni* infection (Karmakar et al., 2014b). Recent investigations in rodents and non-human primates suggested that Sm-p80 may confer resistance to both *S. mansoni* and *S. haematobuim* (Karmakar et al., 2014a). Future trials will determine if the Sm-p80 vaccine protects against schistosome infection in humans. A vaccine that confers protection against several schistosome species would significantly decrease disease burden and deaths due to schistosomiasis around the globe.

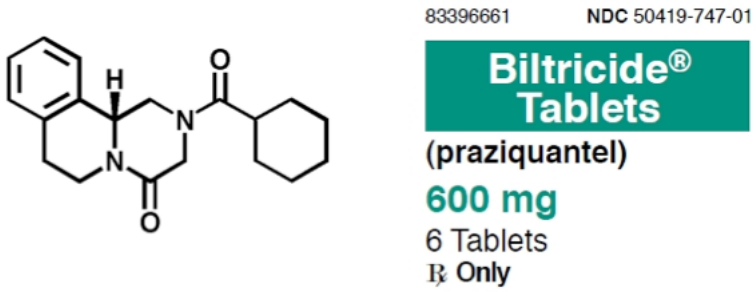

Figure 8. Treatment of schistosome infection: Praziquantel. Praziquantel is currently the only drug recommended for treatment of human schistosomiasis caused by infection with any schistosome species. The drug causes paralysis and death of worms, efficiently eliminating the parasite in one dose. While PZQ is readily available in high-risk regions, it does not prevent re-infection parasite (Doenhoff & Pica-Mattoccia, 2006; Doenhoff et al., 2009).

CHAPTER 2

Innate Immunity in Schistosome Infection

The innate immune system is evolutionarily one the oldest branches of the immune system, dating back to host defense mechanisms in primitive microorganisms nearly 500 million years ago. Innate immunity encompasses several different cell types that are engineered to sense and rapidly respond to invading pathogens of diverse types. Antigen presenting cells (APCs), such as DCs and macrophages, are essential for the detection of foreign antigen by innate sensors, termed pattern recognition receptors (PRRs). In schistosome infection, DCs are key players in the recognition of egg glycans, which are recognized by various different carbohydrate-specific PRRs, known as C-type lectin receptors (CLRs). This chapter will discuss the elements of innate immunity that are most relevant to schistosome antigen recognition and host defense.

2.1 Dendritic Cells

Discovery

DCs were first described in the late 1970's by Ralph Steinman and Zanvil Cohn at Rockefeller University. For years, the theory that DCs played a unique and important role in the immune system was controversial. Not until decades later was DC function in Ag recognition and the orchestration of adaptive immunity fully recognized. For novel revelations in the DC system, Steinman received the Nobel Prize in Medicine in 2011 (Merad et al., 2013). DCs were named for their unique dendrite-like morphology, which is characterized by long philapodia that extend into the surrounding environment to survey for signs of infection or abnormality. Presently, a vast number of DC subsets have been classified based on expression of surface markers, transcription factors, and tissue localization; however, DCs are mainly recognized for their functions in Ag recognition and the elicitation of adaptive T cell immunity (Villadangos & Schnorrer, 2007; Merad et al., 2013).

DCs are unquestionably the most powerful APC population in the innate immune system. They are equipped with a broad array of cell surface- and intracellulary-expressed PRRs that recognize conserved pathogen associated molecular patterns (PAMPs) on bacteria, viruses, fungi, and parasites (Gordon, 2002; Takeuchi & Akira, 2010; Merad et al., 2013). High PRR expression on DCs confers the ability to recognize foreign Ag and rapidly alert the surrounding immune system of irregularities. As such, immediate innate proinflammatory responses ensue, including signaling cascades and cytokine secretion required for successful clearance of infection (Gordon, 2002; Akira et

al., 2006; Takeuchi & Akira, 2010). As professional APCs, DCs have the ability to phagocytose foreign molecules and microbes for processing and subsequent peptide presentation in the context of Major Histocompatibility Complex (MHC) Class I and/or MHC Class II molecules. Presentation of Ag peptide to naïve lymphocytes, such as $CD4^+$ or $CD8^+$ T cells, leads to a second major function of DCs: the elicitation of adaptive immune responses, particularly, effector Th cell development and memory T cell expansion (Villadangos & Schnorrer, 2007; Hivroz et al., 2012; Merad et al., 2013;).

Depending on environmental cues, DCs may stimulate Th1, Th2, Th17, or Treg cell growth during infection (Zhu et al., 2010; Hivroz et al., 2012). To orchestrate Th cell development, Ag-presenting DCs establish direct contact with naïve T cells through adhesion molecules, co-stimulatory markers, and co-receptors, leading to the formation of a tight junction, termed the immunological synapse (Hivroz et al., 2012; Kulpa et al., 2013). Within the immunological synapse, TCR/CD3 complexes cluster to form a highly organized supramolecular activation cluster (SMAC) supported by lymphocyte function-associated antigens (LFAs), intercellular adhesion molecules (ICAMs), as well as various co-stimulation and activation molecules CD28, CD40, CD80, and CD86. This highly organized DC – T cell interface initiates efficient TCR activation and signaling (Bunnell, 2010; Yokosuka & Saito, 2010). Dendritic cell ICAM-3-grabbing nonintegrin (DC-SIGN), a DC-expressed molecule that binds ICAM-3 on T cells, is also necessary for the formation of stable DC – T cell conjugates (Geijtenbeek et al., 2000). Cell interactions are further stabilized by contact of MHC Class I or Class II molecules with CD8 or CD4, respectively, amplifying TCR signaling (West et al., 1994; Yokosuka & Saito, 2010; Hivroz et al., 2012). Overall, many molecular interactions are required at

the DC – T cell interface. Subsequent DC – T cell crosstalk as well as DC cytokine production promotes the development of effector Th cell populations (Zhu et al., 2010; Hivroz et al., 2012), which will be discussed in Chapter 3. The induction of T cell development depends on a variety of factors including DC subtype, DC PRR expression, genetic influence, and environmental cues.

DC Subpopulations

Presently, an increasing number of DC subpopulations have been described, all exhibiting varying degrees of phenotypic plasticity. Two major subcategories of DCs have been documented: namely, the classical DCs (cDCs) and the plasmacytoid DCs (pDCs) (Villadangos & Schnorrer, 2007; Merad et al., 2013). pDCs are a small population of DCs that circulate through the blood and lymph nodes characterized by expression of the characteristic DC integrin, CD11c (low), as well as MHC Class II, and, by definition, “pDC antigen-1” (PDC-1). They acquire typical DC morphology after activation, and although they do not function to stimulate T cells, pDCs are known for their capacity to secrete abundant Type I interferons in response to antigenic nucleic acids (Villadangos & Schnorrer, 2007; Merad et al., 2013).

The second major subcategory of DCs, the cDCs, express CD11c (high) and include all DCs other than pDCs. cDCs are the original DCs exhibiting potent APC capacity that were first described by Steinman. While cDCs comprise a small fraction of all hematopoietic cells in the body, they are powerful Ag-sensing cells that process and present MHC-bound peptide to naïve T cells, thus initiating T cell-mediated immunity. cDCs localize to lymphoid and nonlymphoid tissues where they survey surrounding

environments for foreign Ag through PRRs. This DC subpopulation has enhanced capacity to migrate into the T cell zones of lymphoid tissue after obtaining Ag (Villadangos & Schnorrer, 2007; Merad et al., 2013).

Within cDCs, numerous murine subpopulations with distinct function have been described. Such cDC subpopulations are classified according to a vast profile of surface markers that include, but are not limited to, CD11c, CD11b, MHC Class II, CD8, CD4, B220, CD40, CD45 and CD103 (Merad et al., 2013). One such lymphoid tissue-resident cDC subpopulation is categorized by CD8 expression (Shortman & Heath, 2010). $CD8^+$ DCs, expressing CD8α but not CD8αβ expressed on CD8 T cells, generally represent 20-40% of lymph node (LN) DCs and reside in a phenotypically immature state in the LN prior to activation (Wu & Shortman, 2005; Shortman & Heath, 2010; Merad et al., 2013). This cDC subpopulation is largely CD11b-negative and expresses elevated FLT3 (CD135), an important surface molecule for hematopoietic cell development (McKenna et al., 2000; Waskow et al., 2008). Interestingly, $CD8^+$ cDCs express a distinct profile of Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) including CD205, Clec9A, and langerin, which is believed to enhance the recognition of particular families of Ag (Crowley et al., 1989; Jiang et al., 1995; Merad et al., 2013). $CD8^+$ cDCs are a DC population that strongly promotes $CD8^+$ T cell activation as well as Th1 polarization through IL-12 production (Maldonado-López et al., 1999; Dudziak et al., 2007). Upon maturation, activated cDCs, both $CD8^+$ and CD8-, up-regulate surface-expressed activation markers such as CD80 and CD86, enhancing their ability to interact with naïve T cells (Merad et al., 2013). Such markers are utilized for characterizing the activation state of diverse DC populations. In contrast to $CD8^+$ DCs, CD8- cDCs

represent a large portion of lymphoid and non-lymphoid tissue resident cDCs that are derived from monocyte precursors (Maldonado-López et al., 1999; Merad et al., 2013).

The CD11b and CD103 integrins are two additional markers utilized to classify major cDC populations. $CD11b^+$ cDCs may develop from monocyte or cDC-precursors and localize to various lymphoid and connective tissues in the body. As CD11b is co-expressed by macrophages, the function of $CD11b^+$ DCs is more clearly understood in the context of other surface markers, such as CD103 (Ginhoux et al., 2009; Schulz et al., 2009). In particular, $CD11b^+CD103^+$ cDCs have enhanced migratory capacity. These cDCs enter lymphoid tissue following encounter with Ag, where they effectively present Ag to naïve T cells (Merad et al., 2013). In contrast, CD11b-negative $CD103^+$ cDCs represent 20-30% of all cDCs and can be found in a variety of tissues including Peyers Patches of the intestine, lymph nodes, and connective tissue (Bogunovic et al., 2009). $CD103^+$ cDCs are similar to $CD8^+$ cDCs in function and origin and often overlap with the $CD8^+$ cDC population, particularly in the case of lymphoid tissue-resident DCs. $CD103^+$ DCs express lower levels of MHC Class II, high levels of the CLR langerin, and expand upon stimulation with FLT3 ligand (FLT3L) (Ginhoux et al., 2009; Del Rio et al., 2010). In sum, diverse cDC subpopulations exist, which are categorized according to surface protein expression and tissue localization. The vast majority of cDCs are potent APCs capable of T cell activation (Villadangos & Schnorrer, 2007; Merad et al., 2013).

The role of DC subpopulations in schistosome infection has not been described; however, a few reports suggest that DCs orchestrate schistosome-specific Th cell development. As described, CBA and BL/6 DCs elicit divergent T cell cytokine production in response to schistosome Ag (Shainheit et al., 2008; Shainheit et al.,

2011). Additionally, Phythian-Adams et al. demonstrated that DCs were necessary for the development of Th2 cell responses in *S. mansoni*-infected BL/6 mice (Phythian-Adams et al., 2010). Finally, several reports revealed that PRR expression on human DCs is necessary for recognition and internalization of schistosome egg Ag (van Die et al., 2003; Meyer et al., 2005; van Liempt et al., 2007). Thus, the next section will discuss the PRRs typically expressed by DCs that play a role in DC recognition of schistosome Ag.

2.2 Pattern Recognition Receptors

PRR families

As mentioned earlier, PRRs are innate sensors that detect bacterial, viral, fungal, and parasitic antigen through conserved molecular patterns (Akira et al., 2006; Takeuchi & Akira, 2010). PRR expression enables APC subsets, such as DCs and macrophages, to recognize both intracellular and extracellular foreign Ag and stimulate immediate inflammatory responses (Gordon, 2002). Well known PRR families include TLRs, CLRs, Galectins, Sialic acid binding Ig-like receptors (Siglecs), Nod-like receptors (NLRs), Retinoic Acid Inducible Gene-I (RIG-I)-like receptors, as well as AIM2 (Absent in Melanoma 2) (Figdor et al., 2002; Gordon, 2002; Akira et al., 2006; Stutz et al., 2009; Takeuchi & Akira, 2010). In general, each PRR family recognizes a specific category of antigenic molecules. The TLR family is a large family of PRRs, which has been meticulously characterized in humans and mice. While TLR4 is the innate receptor specific for lipopolysaccharide (LPS) on Gram-negative bacteria (Beutler et al., 2001), other TLRs bind to microbial PAMPs found in flagellin (TLR5) (Hayashi et al., 2001), single-stranded RNA (TLR7, TLR8) (Diebold et al., 2004; Heil et al., 2004), unmethylated CpG DNA (TLR9) (Bauer et al., 2001), or Lipoteichoic acid (TLR2) (Schwandner et al., 1999). Such TLRs are expressed both on the cell surface as well as intracellularly (Akira et al., 2006).

The NLRs are another intracellular PRR family including more than 20 members, which are subcategorized as NLRPs, NODs, NLRCs, or NAIPs depending on the

biochemical properties of the N-terminal domain. A range of viral and bacterial PAMPs stimulate NLRs leading to inflammasome assembly, activation, caspase-1 activity, and mature IL-1β production (Stutz et al., 2009). While the TLR and NLR families recognize a broad variety of microbial PAMPs, other PRR families evolved to bind Ag of specific composition or motif. Examples are lectin-based receptors including the Siglecs, an inhibitory set of PRRs that recognize Sialic acid-containing Ag (Pillai et al., 2012), or the Galectins, which bind N-acetyllactosamine-containing-glycans on pathogens and cells to mediate diverse functions in innate and adaptive immune responses (Rabinovich & Toscano, 2009). Important to the present study, the CLRs are a large family of calcium-dependent lectin receptors that recognize carbohydrate Ag on numerous pathogens including schistosomes (Figdor et al., 2002; van Die et al., 2003; Geijtenbeek & Gringhuis, 2009). The remaining sections of Chapter 2 will discuss the current understanding of CLR-carbohydrate Ag recognition.

2.3 C-type Lectin receptors and Schistosome Antigen Recognition

CLR properties

Of all PRRs characterized, the CLRs have won the greatest attention in the field of schistosome-Ag recognition. CLRs are calcium-dependent Type II transmembrane receptors that bind glycoconjugates on pathogen and cell surfaces through extracellular carbohydrate binding domains (CBDs). All CLRs also contain a calcium-binding domain in the extracellular region, thus requiring bound-calcium ions to function (Robinson, et al., 2006). Two subfamilies of CLRs exist: the mannose binding receptors (Group I CLRs) such as the Mannose Receptor (MR) and DEC205, which have multiple extracellular CBDs, and the asialoglycoprotein-binding receptors (Group II CLRs) such as DC-SIGN, the Dectins, and Mincle, which have only one extracellular CBD (Figdor et al., 2002). There is broad redundancy in the binding properties of all, Group I and Group II, CLRs; for instance, MR, DC-SIGN, Dectin-2, as well as Langerin have all been reported to bind high-mannose moieties (Fig. 9). Low-affinity carbohydrate-binding interactions enable CLRs to bind an assortment of carbohydrate-based Ags. Notably, CLRs contain diverse cytoplasmic tail regions, including immunoreceptor tyrosine-based activation motifs (ITAMs), immunoreceptor tyrosine-based inhibitory motifs (ITIMs), proline-rich domains, triacidic clusters, and di-leucine motifs that promote different pro- and anti-inflammatory signaling pathways (Figdor et al., 2002; Robinson et al., 2006) (Fig. 9). Proinflammatory CLR signaling generally occurs through ITAM-adaptor proteins and kinases associated with the cytoplasmic tail; as such, some CLRs are capable of mediating more than one proinflammatory pathway depending on the ligand (Figdor et

al., 2002; Fuller et al., 2007; Gringhuis et al., 2009). Gringhuis et al. 2009, showed that human DC-SIGN, a Group I type CLR, initiates different Th1 or Th2 cytokine responses depending on the mannose or fucose content of the ligand (Gringhuis et al., 2009).

Indeed, CLRs have diverse signaling properties and elicit distinct pro- and anti-inflammatory responses (Figdor et al., 2002; Geijtenbeek & Gringhuis, 2009). CLRs are the well known for their role in proinflammatory cytokine responses to fungal Ag. In particular, Dectin receptors expressed on human and mouse DCs, recognize β−glucans in fungal cell walls, as well as bacteria, and stimulate IL-23 production and Th17 cell development. Dectin-dependent T cell IL-17 production is critical for efficient clearance of fungal infection (Robinson et al., 2009; Lin et al., 2013; Plato et al., 2013). In contrast, a few different PRRs have anti-inflammatory properties. Macrophage galactose N-acetylgalactosamine specific lectin 1 (MGL1) was demonstrated to play an anti-inflammatory role in murine experimental colitis (Saba et al., 2009). Additionally, DC immunoreceptor (DCIR) contains a tyrosine-based inhibitory motif in its cytoplasmic region that promotes anti-inflammatory signals (Kanazawa et al., 2002; Meyer-Wentrup et al., 2009). Evidence exists that simultaneous stimulation of CLRs with other PRR families may amplify pathogen-specific cytokine responses. In particular, several reports suggest that concurrent activation of Dectins and TLRs leads to elevated proinflammatory cytokine responses, including IL-17A, to bacterial and fungal Ag (Ferwerda et al., 2008; Nakamura et al., 2008; van de Veerdonk et al., 2010; Chang et al., 2014).

Conversely, CLR signaling may also interfere with TLR-mediated proinflammatory pathways. In the presence of fucose-based glycan, DC-SIGN signaling

via RAF-1 inhibited LPS-stimulated NFκB activation downstream of TLR4 (Gringhuis et al., 2007; Gringhuis et al., 2009). By and large, the inflammatory outcome of CLR stimulation varies widely depending on the receptor, ligands, and PRR co-stimulation (Figdor et al., 2002).

In relation to schistosome Ag recognition, reports suggest that CLR expression on human APCs is necessary for binding and internalization of schistosome egg glyco-proteins. Human CLRs including DC-SIGN, DC-SIGNR, MGL, and MR bind to the schistosome egg glycans Le^X, LDN, and LDN-F, aiding in Ag internalization (van Die et al., 2003; van Vliet et al., 2005; Meyer et al., 2005; van Liempt et al., 2004; van Liempt et al., 2007; van Stijn et al., 2010; Meevissen et al., 2012). Additionally, Ritter et al. demonstrated that components of schistosome soluble egg Ag (SEA) stimulate murine Dectin-2 leading to inflammasome activation and IL-1β production (Ritter et al., 2010). While two murine homologues of DC-SIGN, CD209b and CD209d, were shown to bind the schistosome-expressed glycan Le^X (Powlesland et al., 2006; Saunders et al., 2009), no *in vivo* function has to this date been attributed to these CLRs and their role in pathology has not been previously described in murine schistosomiasis. Take together, the studies mentioned above demonstrate that CLRs play a critical role in schistosome Ag recognition by DCs; however, no Th17-inducing murine CLR associated with severe pathology in schistosomiasis has been described.

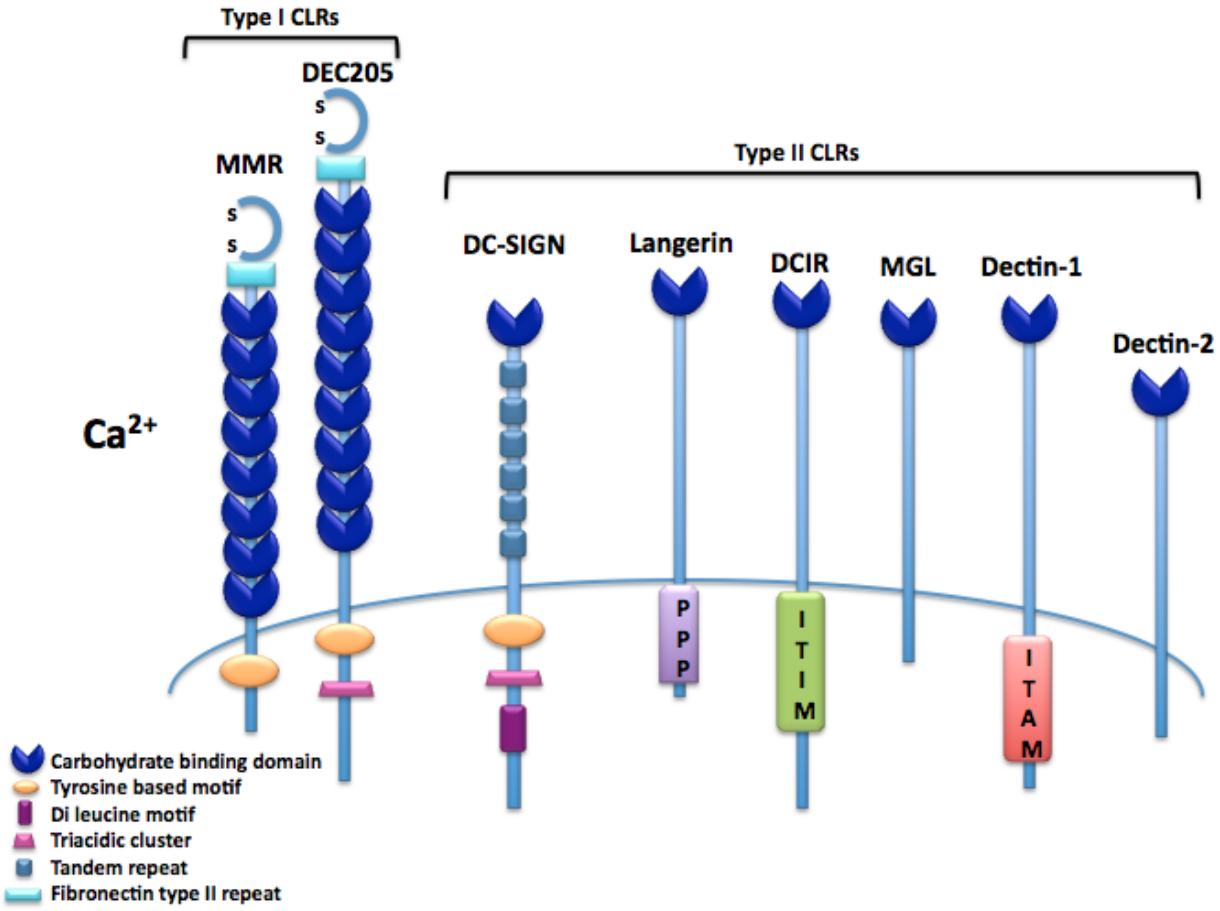

Figure 9. The C-type lectin receptor family. CLRs are type II transmembrane receptors of the innate immune system that recognize carbohydrate antigen on pathogens and cell surfaces through CBDs in a calcium-dependent manner. Additionally, Type I CLRs have several CBDs in their extracellular region as well as cysteine-rich repeats at the N terminus. In contrast, Type II CLRs have one CBD in the extracellular region. While CLRs bind various carbohydrate components with low affinity, each receptor retains distinct cytoplasmic tail motifs that elicit widely contrasting signaling cascades or interfere with other PRR responses (Figdor et al., 2002; Geijtenbeek & Gringhuis, 2009). APC cell surface is depicted.

CHAPTER 3

Th17 Cells

$CD4^+$ effector T cells, also known as T helper (Th) cells, are an essential component of the adaptive immune system. Well characterized Th cell subpopulations include Th1, Th2, and Th17 cells. These subsets play diverse roles in host defense and arise depending on environmental cues, nature of pathogen, genetic background, and PRR expression. The differentiation of Th cell populations is dependent on APC innate cytokine production, which promotes the development and maintenance of specific T cell lineages. Th17 cells are the most pro-inflammatory of all Th cell subsets and play a significant role in host defense against infection as well as the development of pathology in inflammatory and autoimmune diseases. Chapter 3 will focus on the development of $CD4^+$ effector Th cell populations with particular emphasis on the pathogenic Th17 cells responsible for severe disease in schistosome infection.

3.1 Th17 Cell Differentiation

T cell subsets and the development of Th17 cells

Naïve $CD4^+$ T cells circulate in the periphery sampling processed Ag peptide that is presented in the context of MHC Class II molecules on professional APCs. Such APCs generally include DCs and macrophages, but may occasionally be granulocytes or B cells (Abi Abdallah et al., 2011; Lankar, 2002; Villadangos & Schnorrer, 2007; West et al., 1994). Following encounter with a MHC Class II – Ag peptide complex that is specifically recognized by the TCR, T cells become activated, mature, and undergo clonal expansion. The development of $CD4^+$ T cells into diverse Th subsets depends on several stimulatory, environmental, and genetic factors (West et al., 1994; Zhu et al., 2010).

Innate cytokine production by APCs is a major determining factor in the outcome of Th cell lineage (Fig. 10). Innate cytokine responses vary depending on the APC subset, genetic background of the host, as well as the source of stimulation and PRRs involved. DC – T cell crosstalk promotes innate cytokine production by DCs at the immunological synapse during which T cells are influenced to differentiate into Th1, Th2, Th17, or Treg cells (Gordon, 2002; Zhu et al., 2010; Hivroz et al., 2012). These Th cell lineages carry out diverse functions in different inflammatory environments.

Th17 cells are the most proinflammatory and pathogenic of the T cell subsets. They play a significant role in host defense against bacterial and fungal infection, as well as the development of autoimmune disease (Dong, 2006; Iwakura et al., 2008). APCs inducing Th17 cell differentiation secrete several cytokines including IL-23, IL-1β,

IL-21, IL-6, TGF-β and IL-1β. Th17 cells are also characterized by the expression of the transcription factor Rorγt as well as the secretion of the proinflammatory cytokines IL-17A and IL-17F (Bettelli et al., 2007; Iwakura et al., 2008; Stockinger & Veldhoen, 2007). In contrast to Th17 cells, other Th cell lineages, such as Th2, have been documented to play a role in various diseases including helminthic disease and allergic allergy inflammation (Herrick & Bottomly, 2003; van Riet et al., 2007; Taylor et al., 2012). Th2 cell differentiation is induced by APCs through the production of innate cytokines including as TGF-β and IL-33. Recently it was shown that IL-33 signaling through the ST2 receptor is necessary for the maintenance of Th2 cells, which are characterized by expression of the transcription factor Gata3 and produce IL-4, IL-5, and IL-13 (Schmitz et al., 2005; Zhu et al., 2010).

Th1 effector T cells are another lineage of proinflammatory T cells well known for their role in host defense against vesicular pathogens. Th1 cells have also been implicated in various autoimmune diseases (Romagnani, 1999; Damsker et al., 2010). IL-12 is the innate cytokine produced by APCs that stimulates the development of the Th1 subset and it has been shown that IL-18 plays a role in lineage maintenance (Gee et al., 2009; Nakanishi et al., 2010). Committed Th1 cells are characterized by the expression of the transcription factor T-bet and they abundantly produce IFN-γ (Zhu et al., 2010). Finally, Treg cells, also known as suppressor T cells, are a lineage of $CD4^+$ effector T cells with an immunoregulatory function that are characterized by Foxp3 transcription factor expression. Treg cells control inflammation through IL-10 and TGF-β production. In the absence of the Treg lineage autoimmune disease readily develops (Guzmán-Flores & Portales-Pérez, 2008; Larkin et al., 2013). While other Th cell

subsets such as Th9 and Th22 cells have been reported, the function and development Th1, Th2, Th17, and Treg in host defense mechanisms are the best understood to this date (Zhu et al., 2010). This Chapter will focus on the pathogenic potential of Th17 cells and the role of IL-17 in inflammatory disease as well as schistosomiasis.

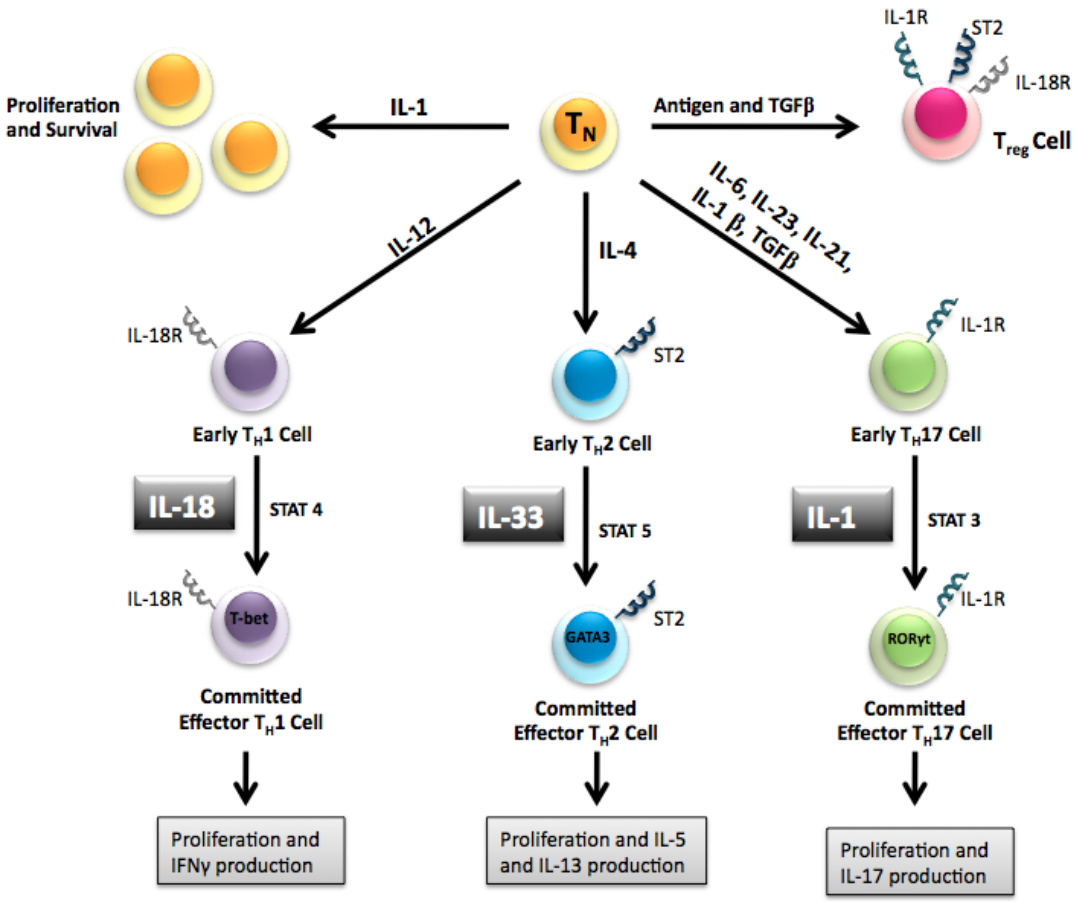

Figure 10. The differentiation of T helper cells. Several signals are required for the differentiation and maintenance of Th cell lineages. Subset-specific cytokines, generally produced by APCs, including DCs and macrophages, promote the development of the different $CD4^+$ effector cell types, Th1, Th2, Th17, and Treg cells. Naïve T cells are induced into Th1 cell development by APC secretion of IL-12. IL-18 promotes maintenance of differentiated T-bet^+ Th1 cells that produce abundant amounts of IFN-γ. IL-4 production readily elicits the differentiation of Th2 cells (Zhu et al., 2010). Th2 cells are marked by ST2 (also known as IL-1 receptor-like 1 [IL1RL1] and IL-33 receptor) expression and Gata3 transcription factor expression (Schmitz et al., 2005; Zhu et al., 2010). The Th2 lineage is sustained by IL-33 and Th2 cells produce various cytokines including IL-5 and IL-13. Th17 cells development is elicited by APC production of IL-23, IL-1β, IL-21, IL-6, and TGF-β. Th17 cells express the transcription factor Rorγt and secrete the potent pro-inflammatory cytokine IL-17 (Zhu et al., 2010).

3.2 IL-17 Production in Host Defense and Autoimmunity

IL-17 and receptors

IL-17 is largely produced by pathogenic Th17 cells, but may also secreted by cells such as neutrophils, eosinophils, Natural Killer (NK) cells, NKT cells, γδ T cells, and epithelial cells (Korn et al., 2009). IL-17, more specifically known as IL-17A, is one of various cytokines within the IL-17 cytokine family that includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (also known as IL-25), which is produced not by Th17 cells, but Th2 cells. Of the IL-17 cytokine family members, IL-17A and IL-17F are most abundantly secreted by Th17 cells (Korn et al., 2009). Of all IL-17 family members, IL-17A, henceforth referred to as IL-17, is of most relevance to the present study.

IL-17 is a proinflammatory cytokine that greatly enhances the production of other inflammatory cytokines and chemokines including TNF, IL-6, GM-CSF, G-CSF, IL-1β, CXCL1, CXCL8, and CXCL10 (Bettelli et al., 2007; Korn et al., 2009). The production of IL-17 also elevates proinflammatory processes during host defense by serving as a potent chemoattractant for neutrophils and inducing their migration to the site of infection or inflammation. IL-17 is also an activator of granulopoiesis (Ye et al., 2001). In the case of autoimmune and inflammatory disorders, such neutrophil infiltration can contribute to host tissue damage (Bettelli et al., 2007; Damsker et al., 2010). Interestingly, IL-17 production by Th17 cells was also demonstrated to play a role the establishment of germinal centers within lymph follicles. There, IL-17 aids in the retention of B cells within the germinal center and also promotes somatic hypermutation (Hsu et al., 2008).

There are a number of IL-17 family member receptors expressed by immune cells including IL-17 receptors (IL-17R) A-E. IL-17 signals through both IL-17RA and IL-17RC (Korn et al., 2009). Stimulation of such receptors, which often exist in dimerized form on the surface of hematopoietic cells as well as endothelial and epithelial cells, induces pro-inflammatory processes including granulopoiesis and neutrophil migration (Ye et al., 2001; Korn et al., 2009). Such inflammatory mechanisms are necessary for efficient clearance of pathogens and mice that are IL-17RA-deficient are susceptible to many bacterial and fungal infections including *Klebsiella* and *Candida* (Ye et al., 2001; Huang et al., 2004). Thus, it is not surprising that infection with various pathogens, particularly fungi, induces Th17 cell responses through innate receptors such as Dectin-1 and Dectin-2 (Robinson et al., 2009; Gringhuis et al., 2012).

Implications in disease

Over the past decade, the importance of Th17 cells, IL-17 production, and Th17-assocaited cytokines in the development of inflammatory diseases has been explored in depth. Th17 cells were first demonstrated to play a pathogenic role in the development of murine experimental autoimmune encephalomyelitis (EAE) where the Th17-inducing innate cytokine IL-23 was required for immunopathology. Following the discovery that IL-17 contributed to inflammatory disease, Th17 cells were shown to play a role in the development of various inflammatory conditions including myocarditis, inflammatory bowel disease (IBD), psoriasis, and collagen-induced arthritis (CIA), where there is evidence of elevated IL-23 and IL-17 production (Matusevicius et al., 1999; Kirkham et al., 2006; Krueger et al., 2007; Kobayashi et al., 2008). Elevated numbers of Th17 cells

have been identified in skin lesions from human psoriatic patients (Pène et al., 2008). Similarly, the expression of IL-17 has been demonstrated to be predictive for joint tissue damage in rheumatoid arthritis (RA), where Th17 cells were demonstrated to play a major role in the induction of osteoclastogenesis and subsequent cartilage destruction (Koenders et al., 2005). In relation to the original Th17 studies conducted in the EAE model, research has also linked the production of IL-17 to multiple sclerosis (MS) in humans (Lock et al., 2002). Some studies have suggested that, during autoimmune disease, Th17 cells may be capable of crossing the blood brain barrier and migrating into the central nervous system (Kebir et al., 2007). Additionally, it was demonstrated that elevated cerebrospinal fluid IL-17 levels correlate with higher instance of spinal lesions (Matusevicius et al., 1999). Thus, it is no surprise that IL-17 has become a major target for the treatment of autoimmune diseases.

Interestingly, it was not until after the discovery of Th17 cell involvement in autoimmune disease that IL-17 was demonstrated to play a role in host defense during the elimination of bacterial and fungal infections (Bettelli et al., 2006; Korn et al., 2009; Plato et al., 2013). Without question, despite the pathogenic potential of Th17 cells, they are an essential cell lineage in immunity to infectious disease. Stimulation of human and murine Dectin family receptors on APCs by fungal and bacterial Ags stimulates a robust Th17-inducing inflammatory response that is required for pathogen clearance (Robinson et al., 2009; Marakalala et al., 2011; Lin et al., 2013; Plato et al., 2013). In humans, polymorphisms in Dectin-1 have very clearly been linked to susceptibility to fungal infection during invasive aspergillosis (Sainz et al., 2012). As such, it is likely that the

Th17 cell lineage evolved to promote survival against such fungal and bacterial infections (Korn et al., 2009).

3.3 Th17 Cells in Human and Murine Schistosomiasis

As described in Chapter 1, Th17 cells play are critical to the development of pathology in murine schistosomiasis, a parasitic disease characterized by excessive immune-mediated inflammation that is deleterious to the host (Rutitzky et al., 2006; Rutitzky et al., 2008). Immunopathology occurring in murine schistosomiasis is largely $CD4^+$ T cell-mediated and our previous findings have demonstrated that Th17 cells promote severe granulomatous liver inflammation that can lead to death. Specifically, IL-17 production promotes robust infiltration of neutrophils to the site of tissue-lodged parasite eggs (Phillips et al., 1997; Rutitzky et al. 2006; Rutitzky et al., 2008). Th17 cells, but not Th1 or Th2 cells, are required for large hepatic liver granulomas, which was elegantly demonstrated in mice deficient for IL-17 and Th17-associated innate cytokine subunits. Mice that are predisposed to mild pathology fail to produce IL-17 and mount predominantly Th2-polarized responses during *S. mansoni* infection (Rutitzky et al., 2008; Rutitzky & Stadecker, 2011; Larkin et al., 2012). Importantly, the significant role of IL-17 in schistosomiasis was recently confirmed in human disease where $Ror\gamma t^+$ Th17 cells were linked to pathology in *S. haematobium* infection (Mbow et al., 2013).

While Th17 cells are known mediators of disease in murine schistosomiasis, the mechanism of schistosome-stimulated Th17 cell development is not clear; however, various studies report the role of innate immunity in the development of Th cell responses (Shainheit et al., 2008; Phythian-Adams et al., 2010; Zhu et al., 2010; Hivroz et al., 2012). To further understand the development of severe schistosomiasis, the present study investigates schistosome-stimulated innate pathways that lead to Th17 cell differentiation. The following sections will present major developments on the role of

innate immunity in the induction of schistosome-Ag stimulated pathogenic Th17 cell differentiation.

SECTION II: PRESENTATION OF RESULTS

CHAPTER 4

Characterization of C-type Lectin Receptor Expression in Murine Schistosomiasis

4.1 Rationale

DCs are potent APCs that elicit pro-inflammatory cytokine production and initiate adaptive immune responses during infection (van Vliet et al., 2008; Takeuchi & Akira, 2010; Merad et al., 2013). In response to schistosome Ag, DC cytokine production induces $CD4^+$ Th cell differentiation, which leads to the development of hepatic granulomatous pathology in murine schistosomiasis (Rutitzky et al., 2008; Shainheit et al., 2008; Phythian-Adams et al., 2010; Larkin et al., 2012). To this date, several studies have demonstrated that different mouse strains develop varying degrees of pathology, which is largely due to the development of divergent Th cell responses (Cheever et al., 1987; Hernandez et al., 1997; Fallon, 2000; Larkin et al., 2012). In particular, CBA DCs produce IL-1β and IL-23 in response to schistosome Ag, which results in the differentiation of pathogenic Th17 cells accountable for severe pathology. In comparison, BL/6 DCs produce TGF-β and IL-10, which ultimately lead to an expansion of Th2 and Treg cells that are responsible for low pathology in the BL/6 strain (Rutitzky et al., 2005, 2008; Shainheit et al., 2008, 2011). Moreover, in the absence of $CD11c^+$ cells, largely representative of DCs, BL/6 mice are incapable of mounting

protective schistosome-specific Th2 responses during infection (Phythian-Adams et al., 2010). Taken together, these observations demonstrate the supreme significance of DCs as APCs.

To effectively screen the surrounding environment for signs of infection, DCs are equipped with a vast assortment of PRRs that mediate pathogen recognition and rapid proinflammatory defense mechanisms (Akira et al., 2006; Takeuchi & Akira, 2010; Merad et al., 2013). Due to the diverse properties of PRR families, signaling pathways and pro-inflammatory cytokine production following PRR stimulation are widely variant (Robinson et al., 2006; Mosser & Edwards, 2008; van Vliet et al., 2008; Takeuchi & Akira, 2010). For example, while Dectin-family expressing DCs elicit robust anti-fungal Th17 cell responses, DCs expressing DC-SIGN may induce Th1 or Th2 cell differentiation in response to mannose- or fucose-based Ag (LeibundGut-Landmann et al., 2007; Gringhuis et al., 2009; Gringhuis et al., 2012). In contrast, expression of DCIR, a CLR that contains a tyrosine-based inhibitory motif, confers immunoregulatory properties to APCs (Kanazawa et al., 2002). Thus, differential PRR expression by APCs greatly influences APC phenotype and the development of Th cell responses during infection (Mosser & Edwards, 2008; van Vliet et al., 2008; Murray & Wynn, 2011; Merad et al., 2013; Lundberg et al. 2014).

In light of PRR function in the shaping of $CD4^+$ Th1, Th2, and Th17 cell responses during infection with bacteria, viruses, fungi, and parasites (Gordon, 2002; Takeuchi & Akira, 2010; Broz & Monack, 2013), we chose to explore the immune gene profile of DCs from CBA vs. BL/6 mouse strains. We hypothesized that differential schistosome-specific cytokine production by CBA and BL/6 DCs may be the result of intrinsic strain-

dependent differences in gene expression, such as genes representing the DC PRR repertoire.

4.2 Materials and Methods

Mice, parasites, and infection

5- to 6-week old female CBA and BL/6 mice were obtained from The Jackson Laboratory. Swiss Webster mice were obtained from Charles River Laboratories. A CBA mouse expressing a Tg TCR specific for the Sm-p40 schistosome egg Ag was made in house as previously described (Shainheit et al., 2011). All mice were maintained at the Tufts University School of Medicine Animal Facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. For some experiments, CBA and BL/6 mice were infected with 85 *S. mansoni* cercariae (Puerto Rico strain) by intraperitoneal injection. Cercariae were shed from infected *Biomphalaria glabrata* snails provided to us by BEI Resources, Manassas, VA. All Swiss Webster mice were infected in an identical fashion for the purpose of isolating schistosome eggs. Eggs were isolated from livers of 7- to 8-week infected mice under sterile conditions by a series of blending and straining techniques, as described in Shainheit et al., 2008.

Gene expression profiling

CBA and BL/6 BMDCs prepared from individual mice were plated in replicate at $1x10^6$ cells/ml in 48-well tissue culture plates (BD Falcon). Replicates were pooled after 4hr

and total RNA was obtained by Trizol® (Invitrogen) extraction according to the manufacturer's instructions. Amplified and labeled cRNA was assessed with Affymetrix Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA) following manufacturer's instructions. Affymetrix Expression Console 1.1 software was used to generate annotated NetAffx CSV files for analysis. Microarray data were deposited in the NCBI GEO database under accession number GSE55307 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55307). Data from a single chip is representative of gene expression of unstimulated DCs from an individual mouse and two chips were run per mouse strain.

Gene ontology analysis

Genes with ≥2 fold difference in expression between CBA and BL/6 DCs and known biological function based on Ensembl release 54 gene archive (http://may2009.archive.ensembl.org/index.html) were defined as differentially expressed. Differentially expressed genes characterized to have putative immunological functions were further selected for GO and pathway analysis using the public web server, g:Profiler with default settings (http://biit.cs.ut.ee/gprofiler/; Institute of Computer Science, University of Tartu, Estonia) (Reimand et al., 2011).

CD209a analysis

Flow cytometry and cell sorting. Spleen cells from individual normal and infected CBA and BL/6 spleens were isolated and RBCs were lysed. Cells were blocked with rat IgG and stained with fluorescently labeled antibodies specific for CD11c (BD

Pharmingen 553802), CD19 (BD Pharmingen 553786), Gr-1 (BD Pharmingen 553127), or F4/80 (AbD Serotec MCA497APC) in combination with biotin-conjugated anti-CD209a (BD Pharmingen 558073). Subsequently, cells were stained with Alexa Fluor® 647-conjugated streptavidin (Invitrogen S-21374). For DC subset analysis, cells were co-stained with antibodies specific for MHC Class II, CD8, CD80, CD11b, and CD103. Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with Summit for FlowJo Software. Spleen and granuloma cells were gated for viably based on forward scatter and side scatter parameters as well as propidium iodide exclusion. Normal CBA splenocytes were sorted at the Tufts Flow Cytometry Core Facility using the Abs listed above.

qRT-PCR. Normal and 7-week infected CBA and BL/6 spleen or liver tissue was homogenized in Trizol® and cDNA was synthesized as described above. A TaqMan probe for *Cd209a* (Applied Biosystems Mm00460067) was used in combination with TaqMan® Gene Expression Master Mix.

Immunohistochemistry

10µm OCT-embedded liver and spleen cryostat sections were fixed in acetone. Liver sections were stained with anti-CD209a Ab (BMD10) for 18 hr at 4°C, followed by mouse-anti-rat IgG2a-HRP for 30 minutes and Tyramide-signal amplification (Invitrogen) or with anti-CD209a plus anti-CD11c (N418) followed by streptavidin-HRP and Tyramide-signal amplification. Spleen sections were additionally stained with anti-B220-Alexa 647. Fluorescent staining was observed using a LSM710 confocal

microscope (Zeiss).

Cytokine analysis

Supernatants from 96 hour co-cultures were assessed for IL-17A using R&D Systems® ELISA kits.

Statistical analysis

ANOVA and student's t-tests were used to statistically analyze differences between groups. p-values of < 0.05 were considered significant, ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

4.3 Results

Gene profiling reveals elevated C-type lectin expression by CBA vs. BL/6 DCs

CBA and BL/6 DCs were previously demonstrated to induce widely divergent schistosome egg-specific cytokine profiles *in vitro* (Rutitzky et al., 2008, 2005; Shainheit et al., 2008, 2011). To identify genetic differences intrinsic to CBA and BL/6 DCs that may influence the cytokine response to schistosome Ags, Affymetrix microarray analysis was used to comprehensively evaluate the relative baseline expression of over 22,000 genes in resting CBA and BL/6 bone marrow-derived DCs (BMDCs) (Fig. 11A). Genes with a defined biological function that displayed at least 2-fold or greater difference in expression between CBA and BL/6 BMDCs were further categorized according to function by gene ontology (GO) analysis using the public web server g:Profiler (Reimand et al., 2011) (Fig. 11B, Table II, Shainheit Thesis). 35 out of 180 biologically characterized genes that were elevated at least 2-fold in CBA compared to BL/6 BMDCs had well-known immunological functions (Table III, Shainheit Thesis). The GO analysis revealed a higher expression of genes with predicted roles in cell membrane-intrinsic immune defense response to external stimuli, carbohydrate binding, and molecular transducer activity (Fig. 11B, Table II, Table III, Shainheit Thesis). 17 out of 157 genes that were elevated at least 2-fold in BL/6 compared to CBA BMDCs had known immunological function (Table IV). The GO analysis highlighted that BL/6 BMDCs exhibited a markedly different expression profile punctuated by genes involved in the regulation of immune effector processes and various metabolic processes (Fig. 11B, Table II, Shainheit Thesis).

Among all genes assessed by microarray analysis, the most apparent difference between CBA and BL/6 BMDCs was the expression of PRRs. In CBA DCs, there were many more overexpressed PRR genes, the majority of which belonged to the CLR family (Figure 12A). A striking 18-fold increase was noted in the expression of the CLR *Cd209a*, also known as mouse DC-SIGN and SIGNR5. *Cd209a* is one of eight murine homologues of human DC-SIGN, one of few CLRs know to bind to schistosome egg glycans (Park et al., 2001; van Die et al., 2003; Powlesland et al., 2006)(Fig. 55). Significantly elevated expression of *CD209a* by CBA DCs was confirmed in BMDCs from individual CBA and BL/6 mice by quantitative RT-PCR (qRT-PCR) (Fig. 12B). These results indicate a considerable difference in baseline CLR gene expression between CBA and BL/6 BMDCs.

Of note, various chitinases were also elevated in CBA and BL/6 DCs. Chitinases are enzymes that break down chitin, a common component of fungal cell walls and the exoskeletons of crustaceans (Lee et al., 2011). In CBA DCs, *Chi3l1* (chitinase-3-like-1) was elevated 24 fold. Elevated *Chi3l1* expression was previously associated with severe disease and poor prognosis in various cancers and autoimmune disorders (Kamba et al., 2013; Coffman, 2008). In BL/6 DCs, the chitinases *Chi3l3* (alternative activation marker Ym1) and *Chi3l4* were significantly elevated. DC-expressed chitinases may play a role in the processing of chitin Ag from pathogens (Lee et al., 2011). Reese et al. demonstrated that chitin induces the infiltration of innate immune cells during allergic and helminth-stimulated inflammation (Reese et al., 2007). Further investigation of chitinase expression and function in CBA and BL/6 DCs may clarify their role in schistosome immunity.

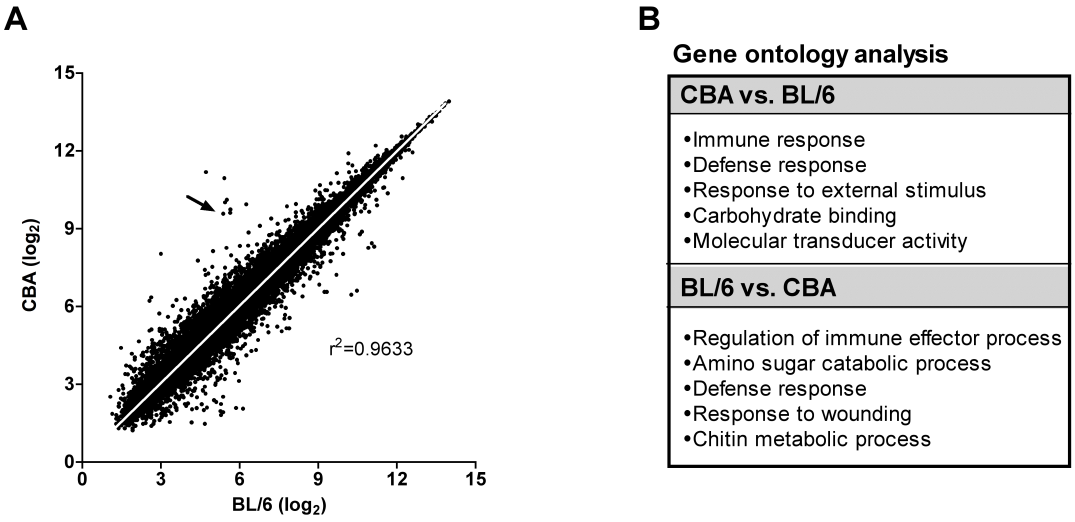

Figure 11. Microarray analysis of resting CBA vs. BL/6 BMDCs. BL/6 and CBA BMDCs were differentiated in medium containing GM-CSF over 7 days. RNA was purified and gene expression was assessed by Affymetrix Mouse 1.0 ST Gene Array technology. (A) Scatter plot shows log2-transformed gene expression. Arrow points to *CD209a*. (B) Genes with two fold or greater difference in expression between mouse strains with characterized biological function were selected for gene ontology (GO) analysis. A functional profile for differentially expressed genes was obtained using the web-server g:Profiler (Reimand et al., 2011).

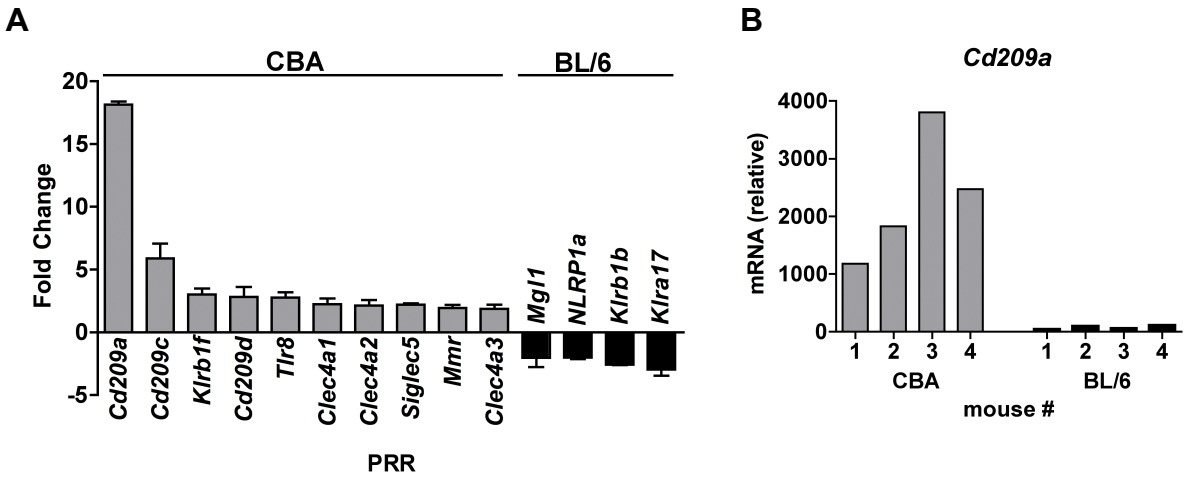

Figure 12. **C-type lectin receptor expression is elevated in CBA vs. BL/6 DCs.** BL/6 and CBA BMDCs were differentiated in medium containing GM-CSF over 7 days. RNA was purified and gene expression was assessed by Affymetrix Mouse 1.0 ST Gene Array technology. (A) Pattern recognition receptors (PRR) with two fold or greater difference in expression by CBA DC vs. BL/6 DC are shown. Bars represent means ± S.D. of two independent microarray chips for each strain. (B) *CD209a* expression by BMDC from four individual CBA and BL/6 mice was determined by qRT-PCR (mRNA relative to GAPDH).

Table II. Gene ontology (GO) analysis of genes preferentially expressed by CBA or BL/6 DCs*

	CBA vs. BL/6	BL/6 vs. CBA

Biological Processes	• **Immune Response**
	• **Defense response**
	  ○ *Innate immune response*
	• **Response to external stimulus**
		• **Regulation of immune effector process**
		  ○ *Regulation of leukocyte mediated immunity*
		  ▪ *Regulation of lymphocyte mediated immunity*
		• **Amino sugar catabolic process**
		  ○ *Glucosamine catabolic process*
		  ▪ *N-acetylglucosamine catabolic process*
		• **Chitin metabolic process**
		  ○ Chitin catabolic process
		• **Defense response**
		• **Response to wounding**
		  ○ *Inflammatory response*

Cellular Component	• **Intrinsic to membrane** • **Cell surface** • **External side of plasma membrane**	• Extracellular space

Molecular Function	• **Molecular transducer activity**
	  ○ *Signal transducer activity*
	  ▪ *Receptor activity*
	• **Carbohydrate binding**
	  ○ *Sugar binding*
	  ▪ *Monosaccharide binding*
	• **C-C chemokine binding**
	• **C-C chemokine receptor activity**
	• **Cytokine binding**
		• **Chitinase activity**
		• **G-protein-coupled receptor binding**
		  ○ *Chemokine receptor binding*
		• **Cytokine activity**
		  ○ *Chemokine activity*

***** *Gene ontology analysis of immunological genes with two fold or greater difference between CBA vs. BL/6 DCs from Tables II and III. (Shainheit Thesis; Ponichtera et al., 2014)*

Table III. Genes preferentially expressed by unstimulated CBA DCs*

Gene Symbol	mRNA Access Number	Fold Diff	mRNA Description
H2-Ea	NM 010381	88.23	Histocompatibility 2, class II antigen E alpha
Ifi202b	NM 008327	32.73	Interferon activated gene 202B
Chi3l1	NM 007695	24.14	Chitinase-3-like 1
Ctse	NM 007799	23.64	Cathepsin E
Cd209a (SIGNR5)	NM 133238	18.32	CD209a antigen
Colec12 (CL-P1)	NM130449	16.95	Collectin sub-family member 12
Retnla (FIZZ1)	NM 020509	10.22	resistin like alpha
Cd209c (SIGNR2)	NM 130903	6.73	CD209c antigen
Ly6g (Gr1)	ENSMUST00000023246	5.92	Lymphocyte antigen 6G precursor
Ngp	NM 008694	4.53	Neutrophilic granule protein
Ccr2	NM 009915	4.13	Chemokine (C-C motif) receptor 2
Rapgef5	NM 175930	3.43	Rap guanine nucleotide exchange factor (GEF) 5
Cd209d (SIGNR3)	NM 130904	3.39	CD209d antigen
Fn1	NM 010233	3.34	Fibronectin 1
Tlr8	NM 133212	3.07	Toll-like receptor 8
Cd200r3	NM 029018	2.90	CD200 receptor 3
Cfh	NM 009888	2.77	Complement component factor h
Ifi44	NM 133871	2.77	Interferon-induced protein 44
Cd2	NM 013486	2.71	CD2 antigen
Klrb1f (Nkrp1f)	NM 153094	2.70	killer cell lectin-like receptor subfamily B member 1F
C3ar1	NM 009779	2.71	Complement component 3a receptor 1
Clec4a1	NM 199311	2.57	C-type lectin domain family 4, member a1
Ccr6	NM 009835	2.54	Chemokine (C-C motif) receptor 6
Cxcl14	NM 019568	2.52	Chemokine (C-X-C motif) ligand 14
Ncf4	NM 008677	2.52	Neutrophil cytosolic factor 4
Clec4a2 (DCIR)	NM 011999	2.46	C-type lectin domain family 4, member a2
Cxcl5	NM 009141	2.31	Chemokine (C-X-C motif) ligand 5
Il18rap	NM 010553	2.22	Interleukin 18 receptor accessory protein
Il18r1	NM 008365	2.16	Interleukin 18 receptor 1
Siglec5 (Siglecf)	NM 145581	2.15	Sialic acid binding Ig-like lectin 5
Mrc1	NM 008625	2.13	Mannose receptor, C type 1
Clec4a3	NM 153197	2.12	C-type lectin domain family 4, member a3
Il10ra	NM 008348	2.08	Interleukin 10 receptor, alpha
Cysltrl	NM 021476	2.04	Cysteinyl leukotriene receptor 1
Cmklr1	NM 008153	2.04	Chemokine-like receptor 1

***** *Immunological genes with a two fold or greater baseline expression in CBA compared to BL/6 DCs. Data are one representative microarray analysis of two with similar findings. (Shainheit Thesis; Ponichtera et al., 2014)*

Table IV. Genes preferentially expressed by unstimulated BL/6 DCs*

Gene Symbol	mRNA Access Number	Fold Diff	mRNA Description
Chi3l3 (Ym1)	NM 099892	8.00	Chitinase 3-like 3
Ccl5 (RANTES)	NM 013653	6.34	Chemokine (C-C motif) ligand 5
Tnfsf4 (OX40L)	NM 009452	4.59	Tumor necrosis factor (ligand) superfamily, member 4
Tnfrsf9 (CD137)	NM 011612	4.41	Tumor necrosis factor receptor superfamily member 9
Slamf7	NM 144539	4.35	SLAM family member 7
Ly6a	NM 010738	4.34	Lymphocyte antigen 6 complex, locus A
Klra17 (Ly-49Q)	NM 133203	3.59	Killer cell lectin-like receptor, subfamily A, member 17
Ly6c1	NM 010741	3.25	Lymphocyte antigen 6 complex, locus C1
Chi3l4 (Ym2)	NM 145126	2.89	Chitinase 3-like 4
Klrblb	NM 008526	2.83	Killer cell lectin-like receptor subfamily B member 1B
Ly6i	NM 020498	2.70	Lymphocyte antigen 6 complex, locus I
Mgl1	NM 010796	2.69	Macrophage galactose N-acetyl-galactosamine lectin 1
CD59a	NM 001111060	2.42	CD59a antigen
H2-K1	NM 001001892	2.29	Histocompatibility 2, Kl, K region
Il10rb	NM 008349	2.22	Interleukin 10 receptor, beta
Fyn	NM 001122893	2.06	Fyn proto-oncogene
Cd28	NM 007642	2.05	CD28 antigen

** Immunological genes with a two fold or greater baseline expression in BL/6 compared to CBA DCs. Data are one representative microarray analysis of two with similar findings. (Shainheit Thesis; Ponichtera et al., 2014)*

CD209a expression is elevated in the spleen of normal and schistosome-infected CBA mice.

In murine *S. mansoni* infection, the spleen is proximal to the major site of hepatic immunopathology. Pronounced splenomegaly develops in severe cases of disease, mainly due to an expansion of T and B lymphocyte clones in response to Ag released by highly immunogenic schistosome eggs. Eggs may additionally become trapped in the spleen leading to splenic granuloma formation characterized by dense infiltration of DCs, macrophages, and granulocytes (Pearce & MacDonald, 2002; Wilson et al., 2007; Larkin et al., 2012).

To investigate the expression of *Cd209a* in CBA and BL/6 spleen, whole spleen tissue was collected from normal and 7 week-infected CBA and BL/6 mice and *Cd209a* expression was assessed by qRT-PCR. *Cd209a* expression is significantly higher in the spleens of both normal and infected CBA vs. BL/6 mice (Fig. 13A). The overall relative expression of *Cd209a* decreases in infected spleen due to the development of pronounced splenomegaly after 7-weeks of infection, primarily caused by clonal T and B cell expansion; however, elevation of *Cd209a* in CBA compared to BL/6 spleen remains intact (Fig. 13A).

To investigate CD209a cell surface expression on bulk splenocytes, whole spleen cells were obtained from individual normal and 7 week-infected CBA and BL/6 mice and CD209a expression was assessed by flow cytometric analysis. No difference in CD209a expression by splenocytes from uninfected CBA and BL/6 mice was visible. After infection, the overall percentage of $CD209a^{+}$ splenocytes increased in CBA compared to BL/6 mice in which the percentage of CD209a-expressing cells remained

largely unchanged (Fig. 13B).

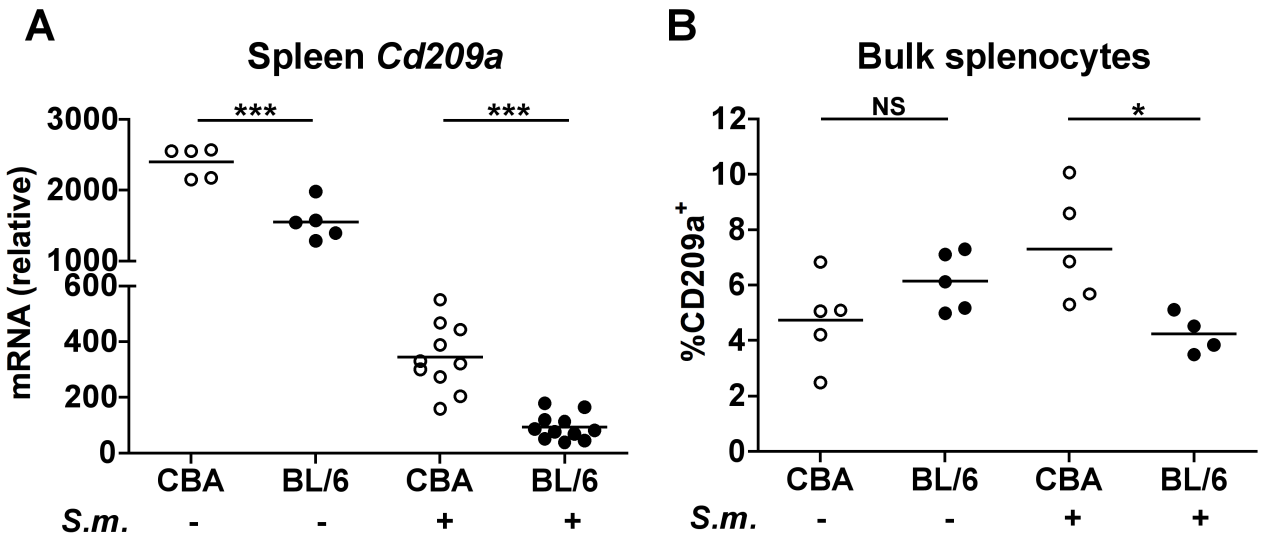

Figure 13. *CD209a* expression is elevated in normal and infected CBA vs. BL/6 spleen. (A) Normal or 7 week-infected CBA and BL/6 mice were obtained. RNA was purified from whole spleen tissue of individual normal or 7 week-infected mice and *CD209a* expression was assessed by qRT-PCR (mRNA relative to GAPDH). Data are from one representative experiment of two. (B) Splenocytes were isolated from normal and 7 week-infected CBA and BL/6 mice. Surface expression of CD209a was assessed by flow cytometric analysis. Data are from one representative experiment of three or four. For all figures, *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

CD209a expression by APC subpopulations is elevated in normal and schistosome-infected CBA mice.

The CD11c$^+$ subpopulation

To this date, several studies have demonstrated that human DC-SIGN is primarily expressed by DCs (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b; van Vliet et al., 2008; Geijtenbeek & Gringhuis, 2009). However, little is known about APC subpopulations that express CD209a in mice or whether expression varies between mouse strains. In particular, CD209a expression by APC subpopulations during schistosome infection is not documented. CD11c positive splenocytes from normal and infected CBA and BL/6 mice, largely representative of DCs, were assessed for CD209a expression by flow cytometric analysis. The percentage of CD209a-expressing CD11c$^+$ cells within normal and infected splenocytes (CD209a$^+$CD11c$^+$/bulk spleen and subpopulation-specific-gated CD209a$^+$CD11c$^+$/total CD11c$^+$) was higher in CBA mice. Moreover, CD209a-expressing CD11c$^+$ cells increased after 7 weeks of infection in CBA spleen, but remained unchanged in BL/6 (Fig. 14A,B).

CD209a surface expression was additionally imaged on normal CD11c$^+$ splenocytes from CBA and BL/6 mice using Aminis ImageStream flow cytometric imaging technology. CD209a was visible on the surface of both CBA and BL/6 CD11c$^+$ cells (Fig. 15A); however, higher percentages were present in CBA spleen confirming previous results (data not shown). Surface expression of CD209a was clearly visible on CD11c negative splenocytes indicating that cells other than DCs also express CD209a (Fig. 15B). Of note, clustering of CD209a was observed in the absence of surface

fixation demonstrating a membrane localization pattern reminiscent of DC-SIGN (Neumann et al., 2008).

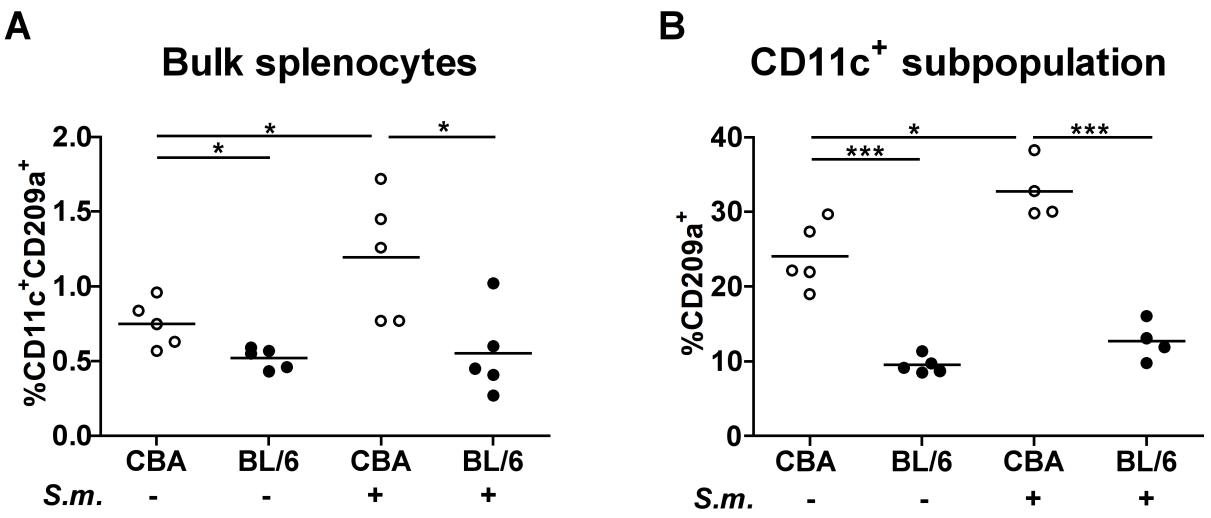

Figure 14. CD209a surface expression is elevated on CD11c$^+$ splenocytes from CBA vs. BL/6 mice and increases during infection. Splenocytes were isolated from normal and 7 week-infected CBA and BL/6 mice. (A) Surface expression of CD209a and CD11c was assessed by flow cytometric analysis. (B) The percentage of the CD11c$^+$ splenocyte subpopulation that expresses CD209a was assessed by separately gating for CD11c$^+$ cells. Data are from one representative experiment of three or four.

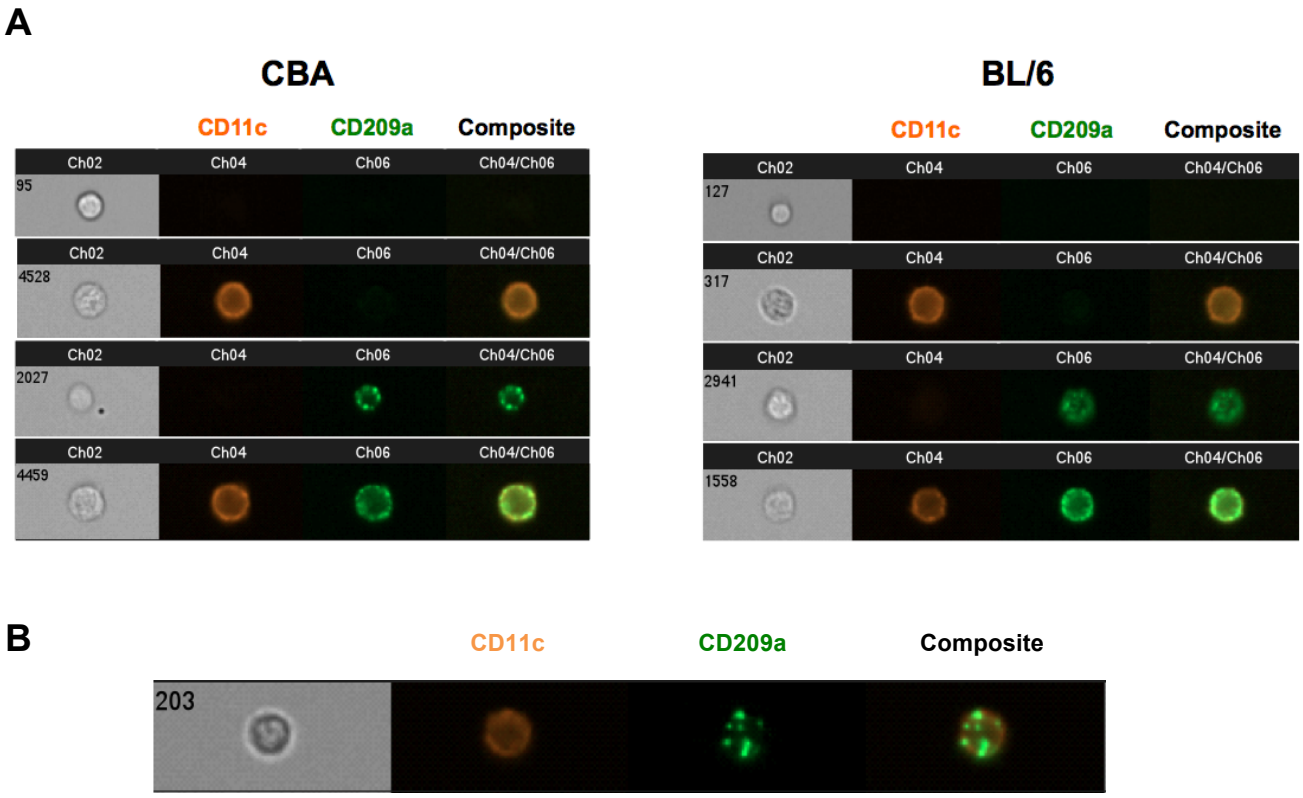

Figure 15. Imaging of CD209a on CBA and BL/6 DCs. Splenocytes were isolated from normal CBA and BL/6 mice. Red blood cell lysis was carried out and cells were labeled for CD11c and CD209a followed by 1% paraformaldehyde fixation. (A) Bright-field and fluorescent images of individual cells were captured by showing surface expression of CD11c (orange) and CD209a (green) by Amnis ImageStream flow cytometry. Composite images showing co-expression of CD11c and CD209a are shown. (B) CBA cells imaged in the absence of paraformaldehyde fixation showed clustering of CD209a. Data are from one representative experiment of three.

The $CD19^+$ subpopulation

Numerous studies have demonstrated that B cells can function as APCs and have the capacity to internalize and process antigen for presentation by MHC Class II (Kleijmeer et al., 1997; West et al., 1994; Lankar, 2002). We thus evaluated CD209a surface expression on splenic $CD19^+$ cells, largely representative of B cells, by flow cytometric analysis. While no difference was observed in normal bulk spleen, there was a greater percentage of double positive CD209a-expressing $CD19^+$ cells in infected bulk spleen ($CD209a^+CD19^+$/bulk spleen) from CBA compared to BL/6 mice (Fig 16A). Moreover, subpopulation-specific gating revealed that a higher percentage of $CD19^+$ cells ($CD209a^+CD19^+$/total $CD19^+$) expressed CD209a in both normal and infected CBA compared to BL/6 spleen (Fig. 16B). The overall percentage of $CD209a^+CD19^+$ splenocytes in infected BL/6 mice was consistently low likely indicating an infiltration and expansion of dissimilar cell populations.

The GR-1^+ subpopulation

While granulocytes are mainly recognized for their innate anti-microbial defense mechanisms, many studies have demonstrated that they can acquire a DC-like professional APC phenotype characterized by the up-regulation of MHC II as well as co-stimulation markers CD80, CD86, and CD83 (Iking-Konert et al., 2001; Iking-Konert et al., 2001; Culshaw et al., 2008). Murine MHC II-expressing neutrophils have also been reported to induce Th1 and Th17 responses (Abi Abdallah et al., 2011). We investigated the expression of CD209a on granulocytes using the pan-granulocyte marker Gr-1. There was no difference in CD209a expression by Gr-1^+ splenocytes from

normal CBA vs. BL/6 mice (Fig 17A). However, after 7 weeks of infection the percentage of Gr-1$^+$ CD209a$^+$ cells (CD209a$^+$Gr-1$^+$/bulk spleen) was elevated in bulk CBA compared to BL/6 spleen (Fig. 17A). Additionally, a significant increase in the percentage CD209a-expressing granulocytes was evident after subpopulation-specific gating (CD209a$^+$Gr-1$^+$/total Gr-1$^+$) (Fig. 17B). Taken together, these results indicate that an increase in granulocyte CD209a expression occurs during the development of severe, but not mild, murine schistosomiasis.

The F4/80$^+$ subpopulation

Macrophages are classic APCs known for their major roles in host defense, immunoregulation, and wound healing that continually probe the surrounding environment for foreign antigen through PRRs (Mosser & Edwards, 2008; Takeuchi & Akira, 2010; Murray & Wynn, 2011). Assessment of CD209a expression on F4/80$^+$ cells, broadly representative of macrophages, demonstrated that CD209a is expressed by F4/80$^+$ cells in both CBA and BL/6 mice (Fig. 18A,B). A slight increase in the percentage of F4/80$^+$CD209a$^+$ cells was observed in bulk spleen from infected CBA compared to BL/6 mice, (Fig. 18A) but no differences in expression were visible after subpopulation-specific gating.

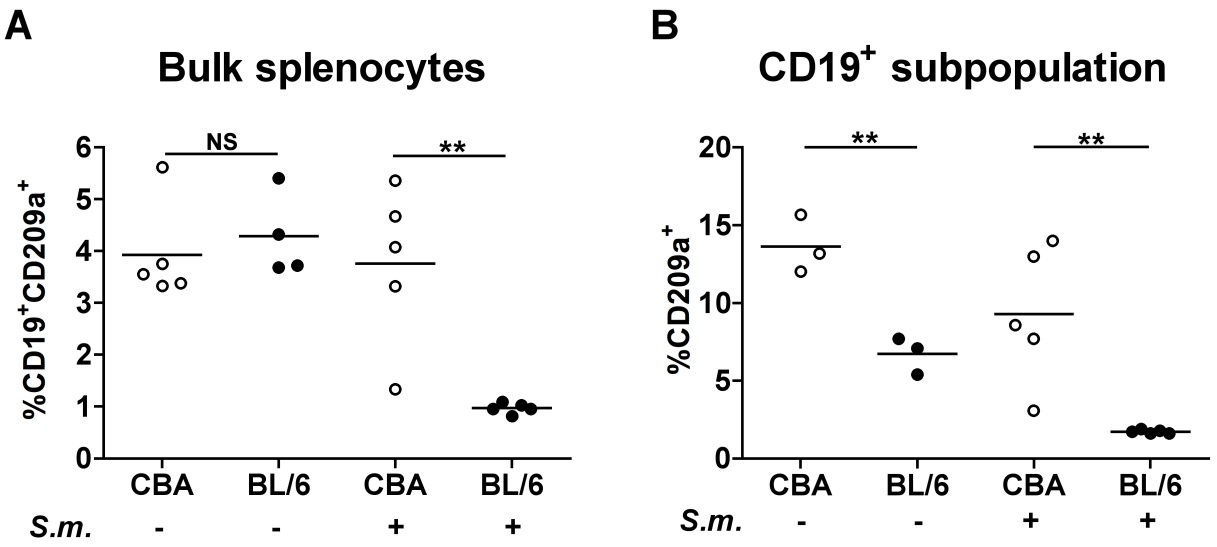

Figure 16. CD209a surface expression is elevated on CD19$^+$ splenocytes from CBA vs. BL/6 mice. Splenocytes were isolated from normal and 7 week-infected CBA and BL/6 mice. (A) Surface expression of CD209a and CD19 was assessed by flow cytometric analysis. (B) The percentage of the CD19$^+$ splenocyte subpopulation that expresses CD209a was assessed by separately gating for CD19$^+$ cells. Data are from one representative experiment of three or four.

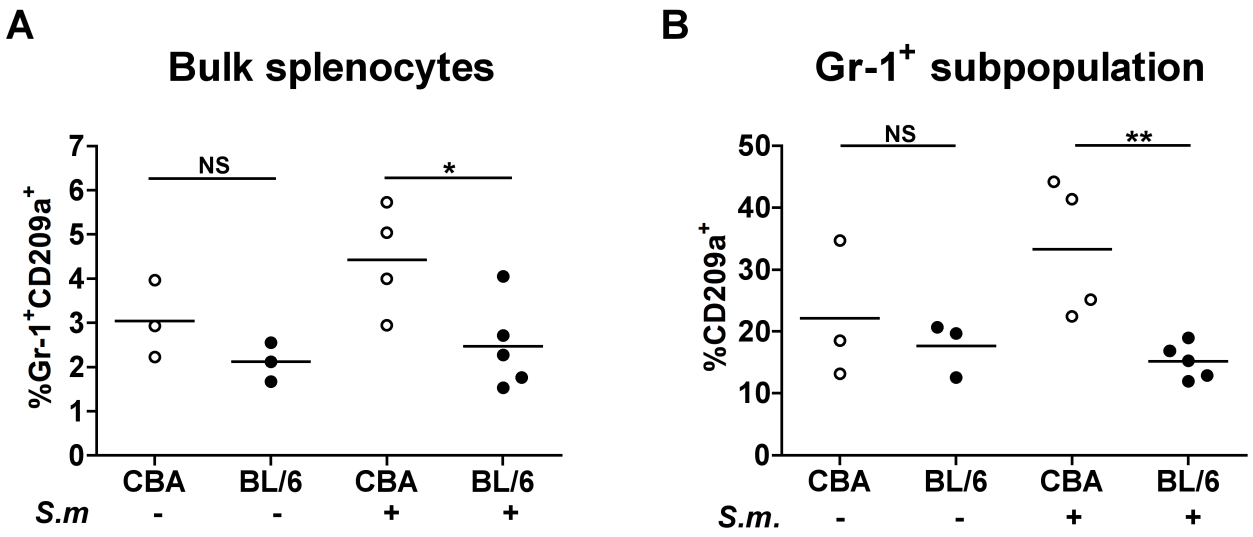

Figure 17. CD209a surface expression is elevated on Gr-1$^+$ splenocytes from infected CBA vs. BL/6 mice. Splenocytes were isolated from normal and 7 week-infected CBA and BL/6 mice. (A) Surface expression of CD209a and Gr-1 was assessed by flow cytometric analysis. (B) The percentage of the Gr-1$^+$ splenocyte subpopulation that expresses CD209a was assessed by separately gating for Gr-1$^+$ cells. Data are from one representative experiment of three or four.

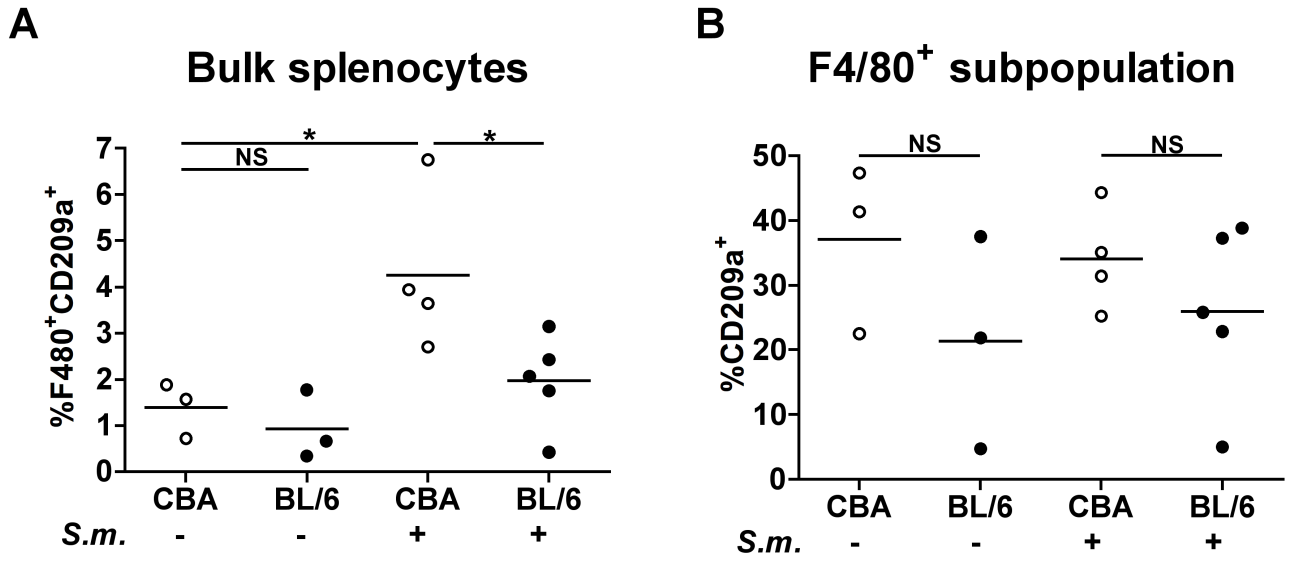

Figure 18. CD209a surface expression is elevated on F4/80+ splenocytes from infected CBA vs. BL/6 mice. Splenocytes were isolated from normal and 7 week-infected CBA and BL/6 mice. (A) Surface expression of CD209a and F4/80 was assessed by flow cytometric analysis. (B) The percentage of the F4/80$^+$ splenocyte subpopulation that expresses CD209a was assessed by separately gating for F4/80$^+$ cells. Data are from one representative experiment of three or four.

CD209a expression is elevated in the spleen of a wild-derived mouse strain prone to severe schistosomiasis

Previous investigation of *S. mansoni* infection in MOLF mice, a wild-derived strain of widely diverse genetic background compared to common inbred mice, demonstrated a strong predisposition to severe Th17-mediated pathology (Smith et al., 2011). We hypothesized that MOLF mice may express high levels of CD209a, in a comparable fashion to CBA mice. Whole spleen tissue was obtained from MOLF, CBA, and BL/6 mice and *Cd209a* transcript was quantified by qRT-PCR. Similar to CBA, *Cd209a* transcript was elevated in MOLF spleen (Fig. 19A). Additionally, splenocytes were obtained from normal MOLF, CBA, and BL/6 mice and DC CD209a expression was assessed by flow cytometric analysis. A higher percentage of splenic DCs expressed CD209a in MOLF and CBA mice relative to BL/6 (Fig. 19B). These data revealed elevated CD209a expression in a wild-derived mouse strain prone to severe pathology.

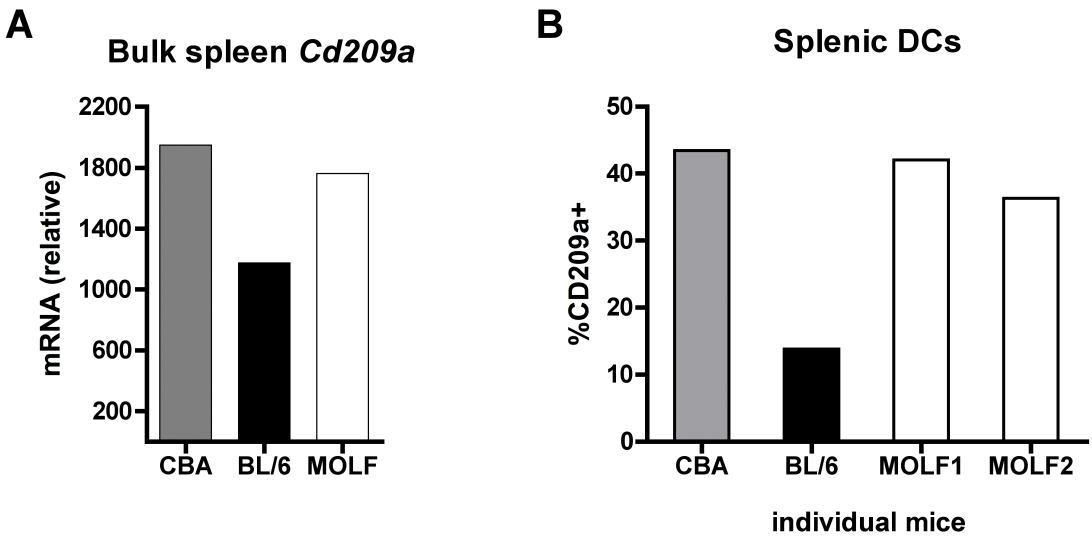

Figure 19. CD209a expression is elevated in MOLF spleen. (A) RNA was purified from whole spleen tissue of CBA, BL/6, and MOLF mice and *CD209a* expression was assessed by qRT-PCR (mRNA relative to GAPDH). (B) Splenocytes were isolated from normal CBA, BL/6, and MOLF mice. Surface expression of CD209a was assessed by flow cytometric analysis. Data is one representative experiment of two.

CD209a expression is higher in liver granuloma cells from infected CBA vs. BL/6 mice

The liver is the principal site of egg-induced granulomatous pathology in *S. mansoni* infection. Hepatic egg deposition provokes a robust inflammatory response resulting in densely packed granulomas composed of infiltrating DCs, neutrophils, macrophages, eosinophils, and T cells (Pearce & MacDonald, 2002; Larkin et al., 2012). We investigated *Cd209a* expression in livers from normal and infected CBA and BL/6 mice. There was no relative difference in *Cd209a* expression in normal CBA and BL/6 livers when assessed by qRT-PCR, suggesting low expression by resident lymphoid cells; however, we detected a significant increase in *CD209a* in CBA liver after 7 weeks of infection and the formation of granulomas (Fig. 20A). No change in *CD209a* expression was visible in BL/6 liver after infection (Fig. 20A). Moreover, elevated CD209a surface expression was additionally confirmed in bulk CBA liver granuloma cells (GCs) by flow cytometric analysis (Fig. 20B).

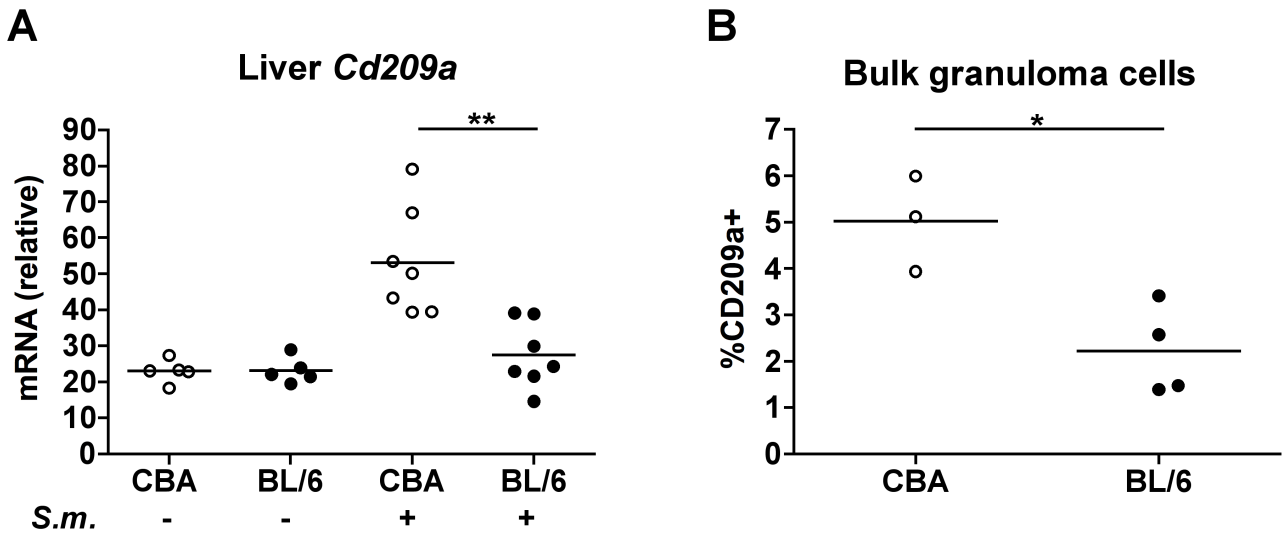

Figure 20. *CD209a* expression is elevated during *S. mansoni* infection in CBA vs. BL/6 liver. (A) Normal or 7 week-infected CBA and BL/6 mice were obtained. RNA was purified from whole liver tissue of individual mice and *CD209a* expression was assessed by qRT- PCR (mRNA relative to GAPDH). Data are from one representative experiment of two. (B) Liver GCs were isolated from livers by collagenase digestion followed by Lympholyte® cell separation. Surface expression of CD209a was assessed by flow cytometric analysis. Data are from one representative experiment of three. For all figures, *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

CD209a expression by granuloma cell subpopulations is higher in infected CBA vs. BL/6 mice

In light of the increase in liver and bulk GC CD209a in CBA mice, we further investigated surface expression of CD209a by GC subpopulations including DCs, granulocytes, and macrophages. Within GCs, the percentage of CD209a-expressing $CD11c^+$ DCs ($CD209a^+CD11c^+$/bulk GCs and subpopulation-specific-gated $CD209a^+CD11c^+$/total $CD11c^+$) was elevated in infected CBA mice compared to BL/6 (Fig. 21A,D). Similarly, CD209a expression by Gr-1^+ granulocytes and F4/80^+ macrophages was also higher bulk CBA GCs (Fig. 21B,C). Subpopulation-specific gating additionally revealed an increase in the percentage of CD209a-expressing Gr-1^+ cells ($CD209a^+$Gr-1^+/total Gr-1^+), and F4/80^+ cells ($CD209a^+$F4/80^+/total F4/80^+) within CBA, but not BL/6 GCs (Fig. 21E,F). Together, these data show that CD209a expression by CBA DCs, granulocytes, and macrophages, is significantly elevated at the foremost site of pathology in comparison to BL/6.

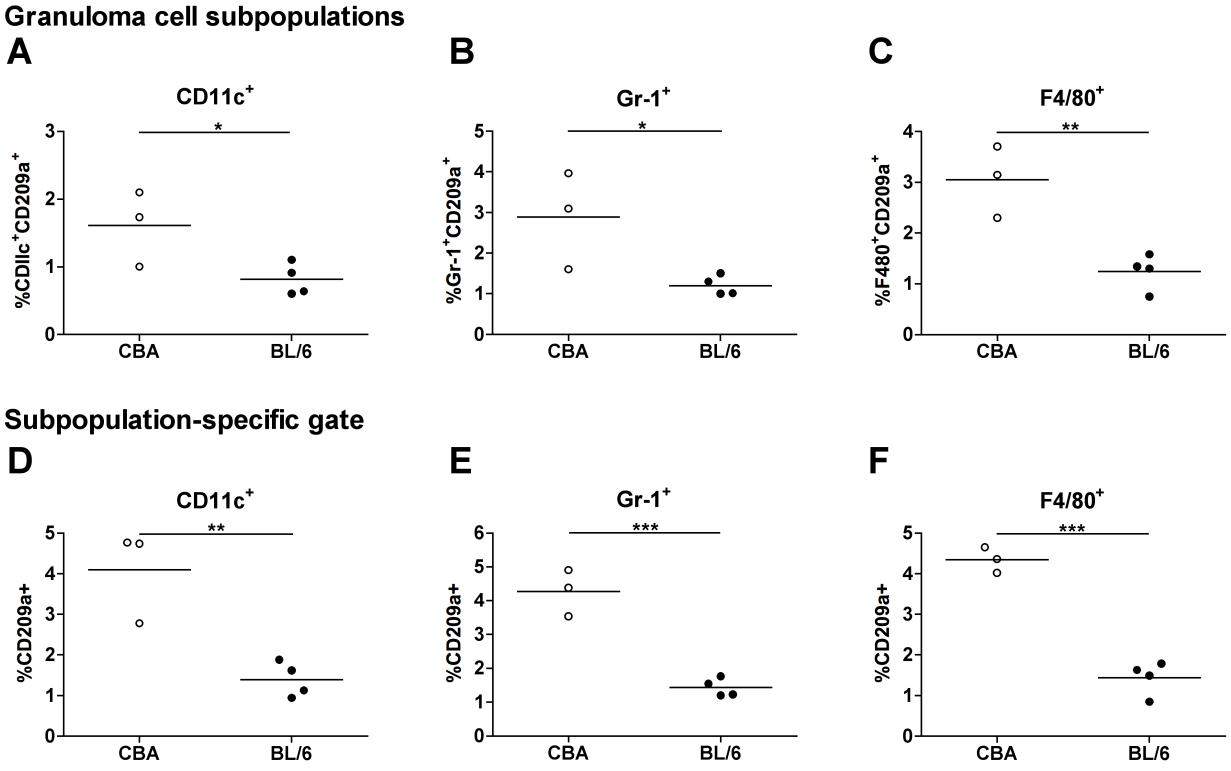

Figure 21. CD209a expression is higher in CBA liver granuloma cell subpopulations compared to BL/6. (A) CBA and BL/6 mice were infected with *S. mansoni* for 7 weeks. Liver GCs were isolated from livers by collagenase digestion followed by Lympholyte® cell separation. Surface expression of CD209a was assessed by flow cytometric analysis and GCs were additionally stained for cell subpopulation markers CD11c, Gr-1, and F4/80 (A-C). The percentage of each individual granuloma subpopulation that expresses CD209a was assessed by separately gating for $CD11c^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells (D-F). Data are from one representative experiment of three.

CD209a$^+$ cells are abundant in infected CBA spleen and liver sections

To this date, CD209a expression in schistosome-infected mouse tissues has never been explored by microscopy. To assess CD209a$^+$ cell localization and frequency in CBA and BL/6 tissues, infected spleen and liver cryostat sections were stained for CD209a and visualized with a confocal microscope (Fig. 22 and 23). In infected CBA spleen, CD209a$^+$ cells were abundant in CD11c-rich interfollicular T cell areas compared to BL/6 spleen (Fig. 22A-D); CD209a$^+$ cells were also present within B220$^+$ B cell-rich follicles in CBA, but not BL/6 spleen (Fig. 22A-B). Only CD11c-high, but not CD11c-intermediate or -low, cells are visible due to technical microscope limitations; as such, absence of co-localization between CD11c and CD209a suggests that CD209a may be more highly expressed by CD11c-low or -intermediate subpopulations. Staining also suggests that splenic APC subpopulations other than DCs also express CD209a as was previously demonstrated by flow cytometry. Within infected liver sections, marked infiltration of CD209a$^+$ cells was apparent mainly in the egg-induced granulomas (Fig. 23A,B). Most notably, in CBA livers the granulomas were larger and densely infiltrated with CD209a$^+$ cells (Fig. 23A), whereas in BL/6 livers the smaller granulomas contained fewer CD209a$^+$ cells (Fig. 23B). Differences in fluorescence intensity were documented quantitatively as shown in Fig. 23C,D. Considerable cell co-localization of CD209a and CD11c was also evident in CBA granulomas (Fig. 24). Together, these images demonstrate a higher frequency of CD209a$^+$ cells in lymphoid and lesional tissue of CBA mice.

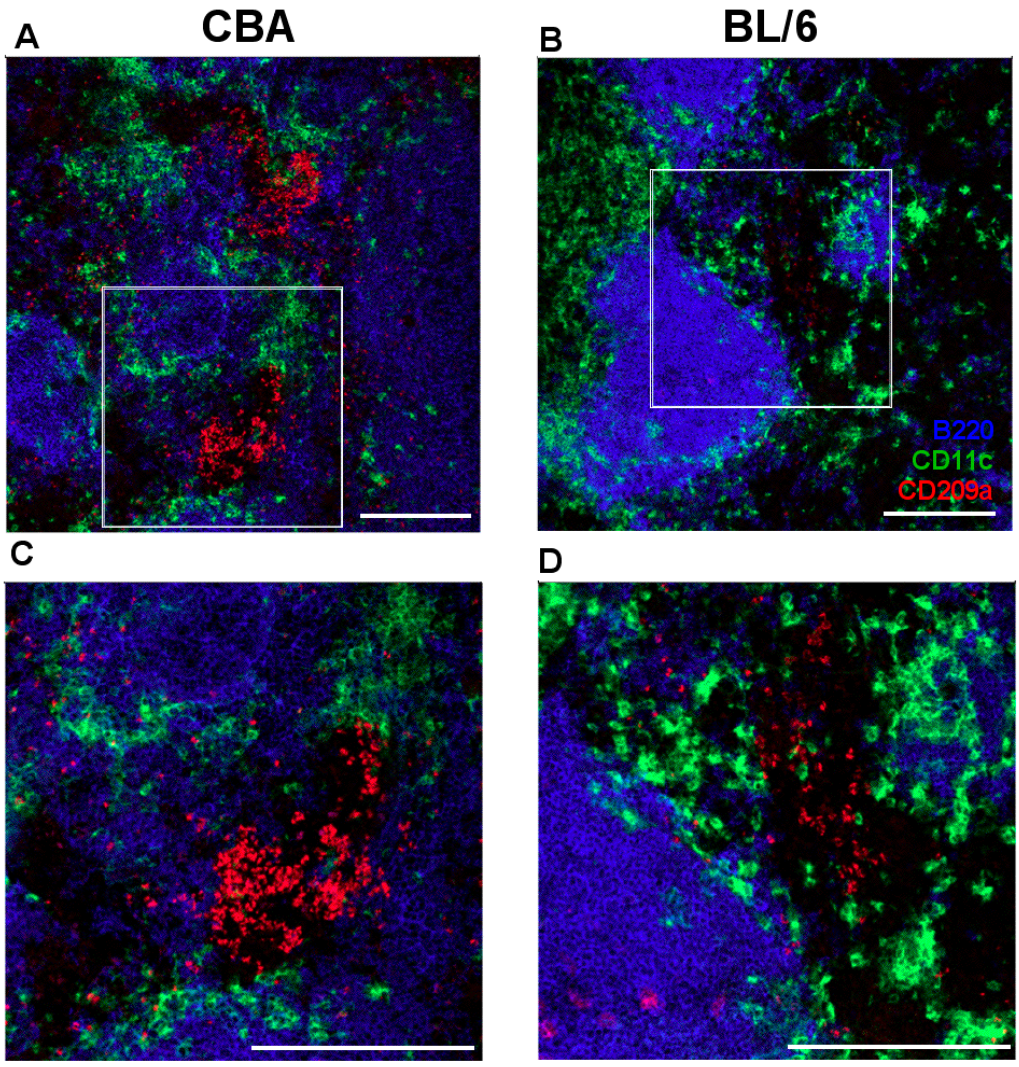

Figure 22. CD209a^{+} cells are abundant in CBA spleen compared to BL/6. CBA and BL/6 mice were infected for 7 weeks. OCT-embedded frozen spleen (A-D) cryostat sections were stained for CD209a (red) and imaged by confocal microscopy. CBA (A,C) and BL/6 (B,D) spleen sections were counterstained for CD11c (green) and B220 (blue). Scale bars represent 200 µm for both 100x and 200x magnifications. Images are representative of 5 mice examined per strain.

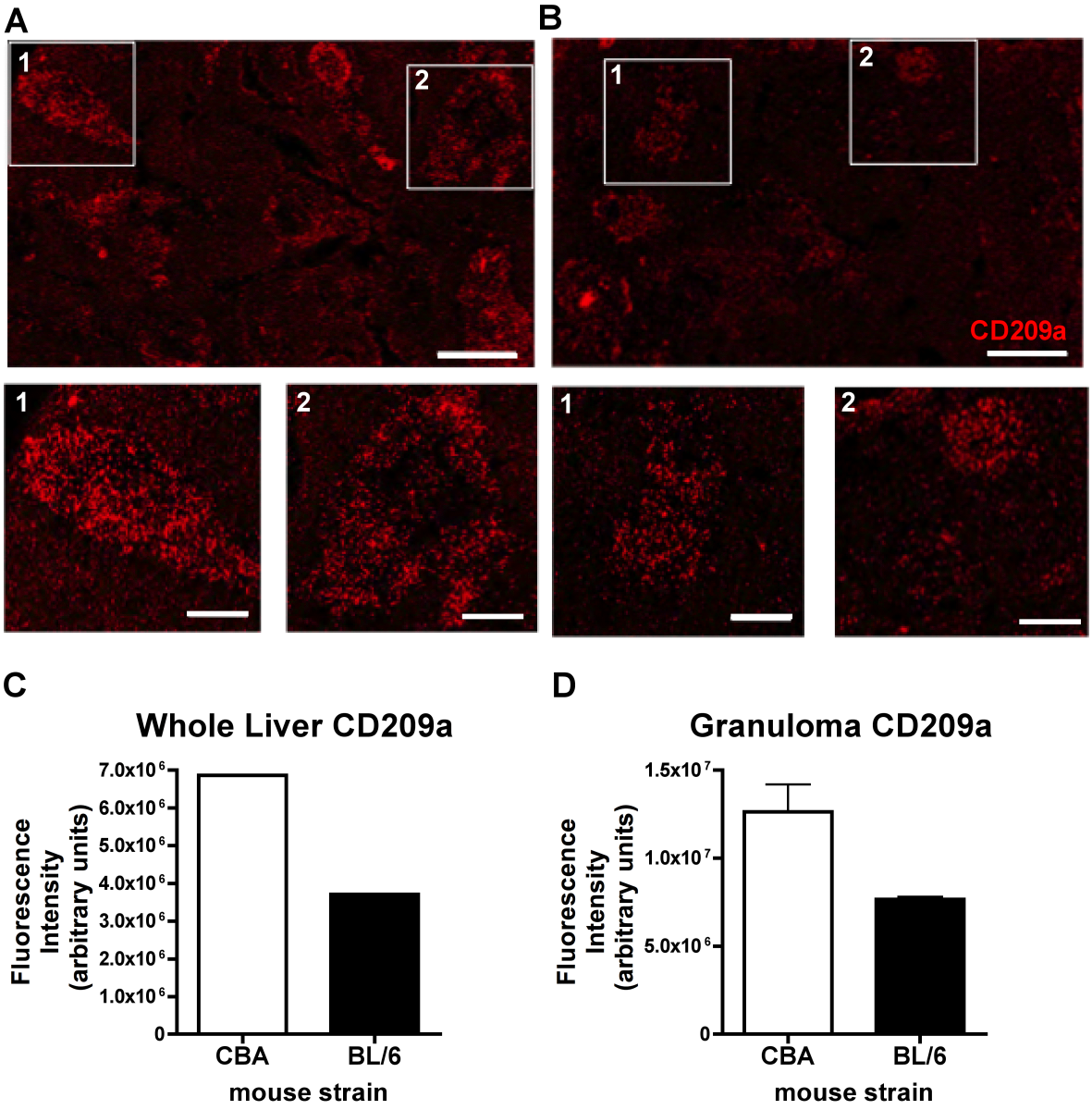

Figure 23. CBA liver granulomas are densely infiltrated with CD209a$^+$ cells compared to BL/6. CBA and BL/6 mice were infected for 7 weeks. OCT-embedded frozen CBA (A) and BL/6 (B) liver cryostat sections were stained for CD209a (red) and imaged by confocal microscopy. Scale bars represent 500 µm for panoramic view and 200 µm for 100x granuloma magnifications. (C-D) Average CD209a fluorescence intensity in whole liver (A,B) and granulomas (A1,2, B1,2) was quantified by Volocity 6.0 Software (PerkinElmer). Images are representative of 3 mice examined per strain.

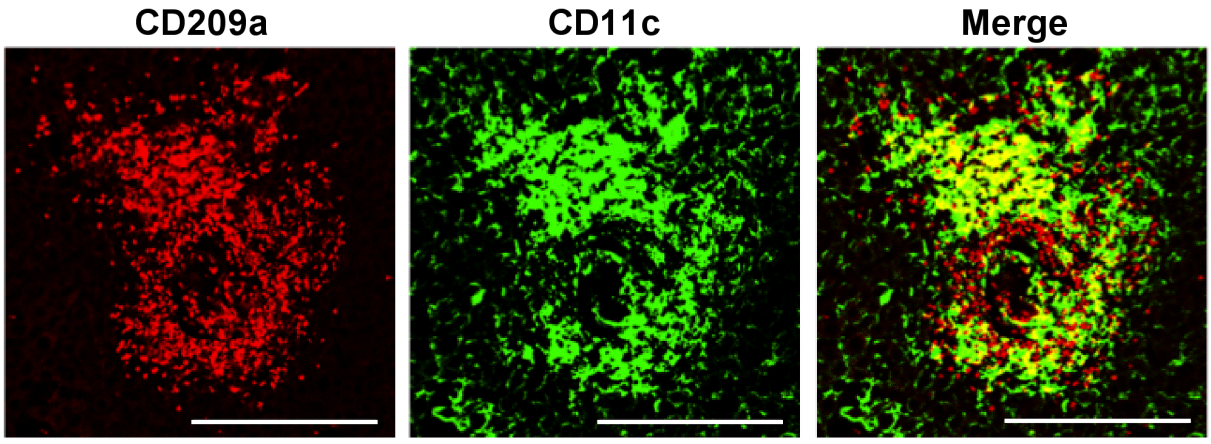

Figure 24. Co-localization of CD209a and CD11c in CBA granuloma. CBA mice were infected for 7 weeks. OCT-embedded frozen liver cryostat sections were stained for CD209a (red) and CD11c (green) and imaged by confocal microscopy. Merge demonstrates co-localization of CD209a and CD11c in a granuloma. Scale bars represent 200µm. Images are representative of 3 mice.

CD209a expression is elevated on DC subpopulations in infected CBA, but not BL/6 MLN.

CD209a has been previously documented on mouse monocyte-derived DCs with potent antigen presenting capacity (Cheong et al., 2010); however, little is known regarding CD209a expression by diverse DC subpopulations, particularly in schistosome infection. DC subpopulations are categorized according to a vast profile of surface molecules that include, but are not limited to, CD11c, CD11b, MHC Class II, CD8, CD4, B220, CD40, CD80, CD45 and CD103 (Merad et al., 2013). To determine the DC subpopulations that express CD209a in *S. mansoni* infection, mesenteric lymph node (MLN) cells from infected CBA and BL/6 mice were assessed for CD209a by flow cytometric analysis (Fig. 25A). As anticipated, CD209a expression was higher on total $CD11c^+$ cells from CBA compared to BL/6 MLN (Fig. 25B).

Classical DCs (cDCs) are a broad subpopulation of DC present in lymphoid and non-lymphoid tissues that have a superior ability to sense, process, and present antigen to naïve T cells (Merad et al., 2013). Such DCs are generally classified by $CD11c^+$ and MHC Class II expression. Within $CD11c^+$MHC Class II^+ cDCs, the percentage of CD209a surface-expression was elevated in CBA MLN compared to BL/6 MLN (Fig. 25C). There was no difference in the percentage of $CD209a^+$ $CD11c^+$MHC Class II- cDCs (Fig. 25D). This data indicates that CD209a expression is elevated on a potent antigen presenting subpopulation of DCs in the CBA MLN.

Lymphoid tissue-resident cDCs can be further categorized by the expression of CD8. $CD8^+$ DCs generally represent 20-40% of LN DCs and reside in a phenotypically

immature state within the LN prior to activation (Merad et al. 2013). Such $CD8^+$ DCs are characterized by the expression of a distinct profile of TLRs and CLRs including CD205, Clec9A, and langerin, which enhances the recognition of particular families of antigens (Merad et al., 2013). A higher percentage of $CD11c^+CD8^+$ DCs expressed CD209a in CBA compared to BL/6 MLN (Fig. 25E). This finding suggests that schistosome-infected CBA mice develop large population of $CD8^+$ cDCs with potential to recognize schistosome Ag through lectins such as CD209a.

Activated cDCs up-regulate various surface markers such as CD80 and CD86 (Merad et al., 2013). CD80, also called B7.1, is an important co-stimulatory molecule on DCs that interacts with CD28 during T cell activation. To investigate CD209a expression on activated DCs in infected mice, $CD11c^+$ cells were stained for CD80 and CD209a. The percentage of CD209a-expressing $CD11c^+CD80^+$ DCs was significantly elevated in CBA compared to BL/6 MLN (Fig. 25G); however, no difference in CD209a expression by CD80- DCs was visible between strains (Fig. 25H). This result demonstrates that a higher percentage of activated MLN DCs express CD209a in infected CBA mice, which are predisposed severe Th17-dependent pathology in murine schistosomiasis.

Finally, CD209a expression was assessed on $CD11b^+$ cDCs. This CD8- subpopulation represents a large portion of lymphoid tissue and non-lymphoid tissue resident cDCs that are often derived from monocyte precursors. More specifically, $CD11b^+CD103^+$ DCs are a migratory cDC subpopulation that enters lymphoid tissue following encounter with Ag (Merad et al., 2013). Although CD209a was expressed on migratory $CD11b^+CD103^+$ DCs and $CD11b^+$CD103- DCs, no difference was seen between strains (Fig. 25I,J).

Taken together, these data demonstrate that the frequency of CD209a-expressing cDC subpopulations with enhanced capacity to recognize and process Ag as well as prime naïve T cells is significantly higher in infected CBA, compared to BL6 MLN. Such CBA CD209a^{+} cDC subpopulations likely contribute to the development of schistosome-Ag induced Th17 cell responses that fail to develop in BL/6 mice.

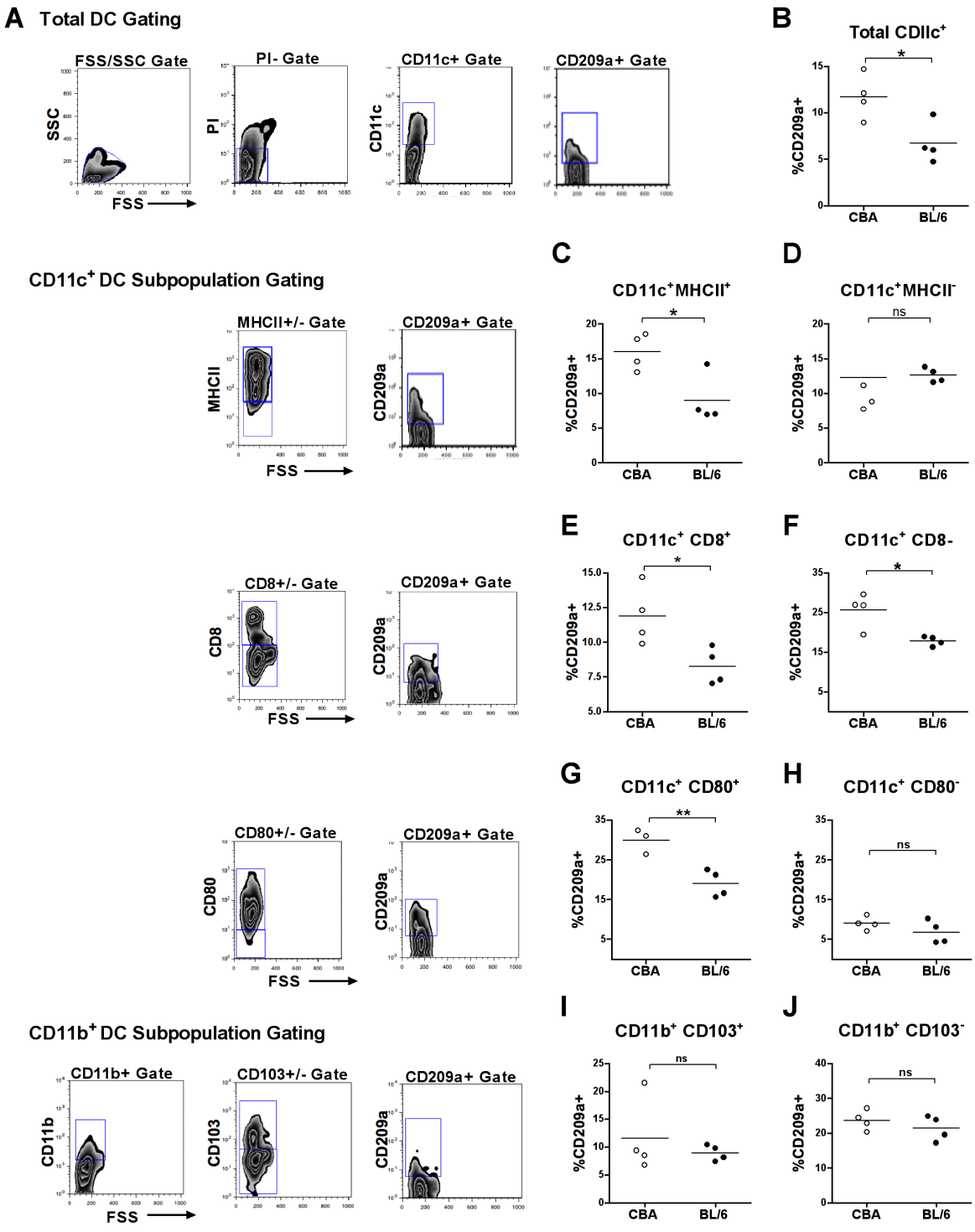

Figure 25. CD209a is elevated on DC subpopulations from infected CBA vs. BL/6 MLN. MLN cells were isolated from 7 week-infected CBA and BL/6 mice. Cells were

gated for viability by propidium iodide exclusion and surface expression of CD209a was assessed by flow cytometric analysis. MLN cells were additionally stained for DC population markers CD11c and CD11b (A-B). The percentage of individual DC subpopulations that expressed CD209a was assessed by separately gating for MHC II+ (C-D), CD8+ (E-F), CD80+ (G-H), and CD103+ (I-J) cells as shown in the strategy in panel A. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = not significant.

Egg-simulated CD11c+ cells, but not CD19$^+$, Gr-1$^+$, or F4/80$^+$ cells, elicit Th17 responses in vitro

While CD11c$^+$ cells are required for the elicitation of Th2 development in schistosome-infected BL/6 mice (Phythian-Adams et al., 2010), the specific APC population that stimulates Th17 cell differentiation in severe schistosomiasis is not known. To determine which CD209a-expressing APC subpopulation induces schistosome-specific Th17 cell responses, splenocytes from normal CBA mice were sorted and co-cultured with naïve CD4+ T cells together with anti-CD3/CD28-coated beads in the presence or absence of eggs (Fig. 26). CD11c$^+$, CD19$^+$, Gr-1$^+$, and F4/80$^+$ cell populations previously shown to express CD209a and largely representative of DCs, B cells, granulocytes, and macrophages were examined. CBA CD11c$^+$ cells induced robust IL-17 production by T cells in response to eggs (Fig. 27A); in contrast, minimal amounts of egg-specific IL-17 was produced by co-cultures containing CD19$^+$, Gr-1$^+$, or F4/80$^+$ cells (Fig. 27B,C,D). These results indicate that CD11c$^+$ cells, but not CD19$^+$, Gr-1$^+$, or F4/80$^+$ cells, are the APC subpopulation that most efficiently induces egg-specific IL-17 secretion by CD4$^+$ T cells.

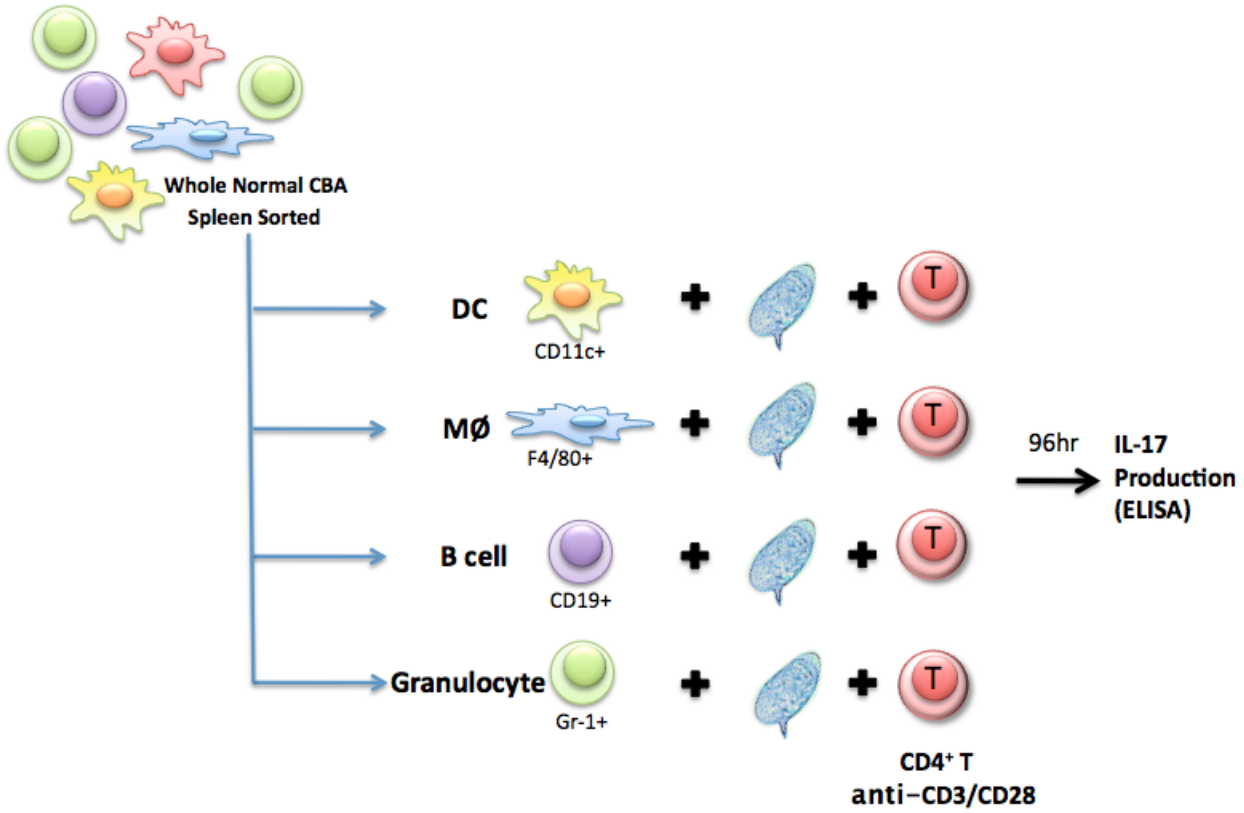

Figure 26. Schematic representation of egg-stimulated co-culture configurations involving sorted APC populations, naïve CD4$^+$ T cells, and eggs. Splenocytes were obtained from normal CBA mice and stained for CD11c, F4/80, CD19, or Gr-1 APC population markers for separation by Fluorescence Activated Cell Sorting (FACS). Individual cell populations were co-cultured with naïve CD4$^+$ T cells together with anti-CD3/CD28-coated beads in the presence or absence of eggs. IL-17 production was assessed by ELISA after 96 hr.

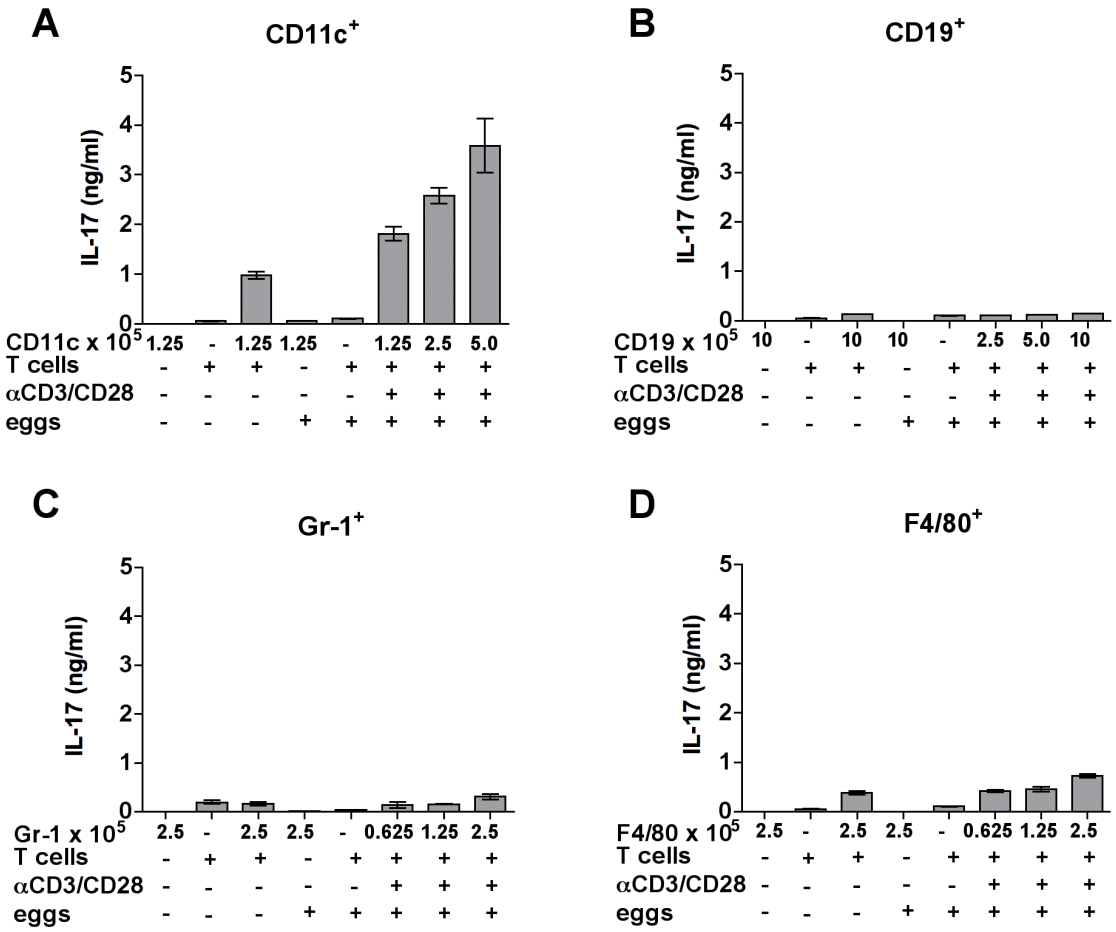

Figure 27. Egg-stimulated CD11c$^+$ cells, but not CD19$^+$, Gr-1$^+$, or F4/80$^+$ cells, elicit Th17 responses. Splenocytes from normal CBA mice were stained for CD11c, CD19, Gr-1 or F4/80 subpopulation markers and separated by fluorescence-activated cell sorting (FACS). The indicated concentrations of (A) CD11c$^+$, (B) CD19$^+$, (C) Gr-1$^+$, and (D) F4/80$^+$ cells were individually co-cultured with 1 x 10^6 CD4$^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs. IL-17 production in 96 hr culture supernatants was assessed by ELISA. Additional single-cell controls are shown. Data are from one representative experiment of two.

CBA, but not BL/6, DC CD209a expression increases in response to schistosome egg stimulation

Little is known of the regulation and kinetics of CD209a surface expression on APCs, particularly after exposure to schistosome Ag. In light of our previous data demonstrating that CD209a expression increases on the surface of splenic $CD11c^+$ cells after *S. mansoni* infection, and given the association of CD11c expression with DCs, we further investigated the kinetics of CD209a surface expression by BMDCs in the presence of live eggs. CBA and BL/6 BMDC were cultured in the presence or absence of eggs and CD209a expression was assessed after 0, 6, 18, 32, and 48 hr of stimulation by flow cytometric analysis. CD209a expression on CBA DCs increased over time in the presence of eggs (Fig. 28). In contrast, low CD209a expression on BL/6 DCs remained unchanged. These data suggest that CD209a surface expression is unregulated in DCs capable of inducing schistosome-specific Th17 development.

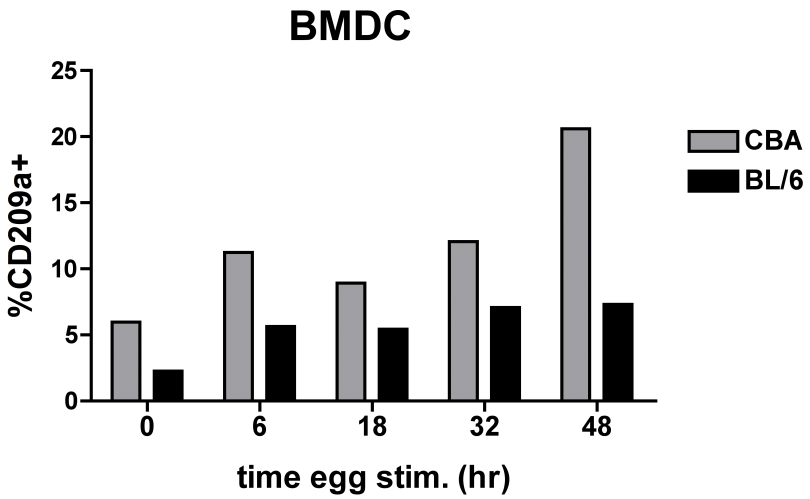

Figure 28. CBA, but not BL/6, DC CD209a expression increases in response to egg stimulation. CBA and BL/6 BMDCs were differentiated in medium containing GM-CSF over 7 days and cultured in the presence of live schistosome eggs. After 0, 6, 18, 32, and 48 hr of stimulation, cells were fixed with 1% paraformaldehyde and surface expression of CD209a was assessed by flow cytometric analysis. Data are one representative experiment of two.

CHAPTER 5

Characterization of CD209a Function in the Development of Schistosome-specific Pathogenic Th17 Cell Responses

5.1 Rationale

DC-SIGN is a versatile human CLR shown to bind natural schistosome egg-expressed glycans such as Le^X (van Die et al., 2003) and DC-SIGN-expressing human DCs elicit Th cell differentiation in response to mannose- and fucose-containing pathogen glycans (Gringhuis et al., 2009). Presently, the schistosome Ag-specific binding capacity and inflammatory function of the murine CLR, CD209a, are not documented; nonetheless, reports suggest that CD209a may be a marker of highly inflammatory APCs. Cheong et al. 2010, demonstrated that $CD209a^+$ monocyte-derived DCs (Mo-DC) possess powerful Ag-presenting capability (Cheong et al., 2010). In addition, a study on bacterial sepsis revealed that leukocyte cell-derived chemotaxin- 2 (LECT2) interacts with CD209a on murine macrophages to promote endocytosis, bacterial killing, and proinflammatory cytokine production (Lu et al., 2013). Other murine DC-SIGN homologues, including CD209b and CD209d, were confirmed to bind the schistosome glycan Le^X, but an *in vivo* function has not been identified for these receptors, particularly in the pathogenesis of schistosomiasis (Van Liempt et al., 2004; Powlesland et al., 2006; Saunders et al., 2009).

Our present investigation of CD209a in CBA and BL/6 mice revealed a significant enhancement in CD209a protein and mRNA expression by CBA DCs in comparison to

BL/6 DCs. The data also demonstrated that CD209a-expressing cDC subpopulations of powerful antigen presenting capacity develop in the MLN of CBA mice, but not BL/6 mice, over the 7-week coarse of infection. Furthermore, CD209a was most highly expressed on $CD11c^+$ DCs, the critical CBA APC that induces pathogenic Th17 cell development in response to schistosome Ag. Marked CBA $CD11c^+$ cell expression of CD209a was evident during infection with *S. mansoni* in spleen, MLN, as well as liver granulomas.

A CLR that mediates schistosome-specific Th17 cell responses during severe schistosomiasis has not been identified at this time; however, CLR family members, particularly within the Dectin subfamily, are well known to elicit robust pro-inflammatory responses and T cell IL-17 production (LeibundGut-Landmann et al., 2007; Takeuchi & Akira, 2010; Gringhuis et al., 2012). In light of the schistosome-binding capacity of human DC-SIGN and the marked elevation of CD209a on CBA DCs, which induce schistosome-specific T cell IL-17 production, we were curious to examine the function of CD209a in the development of Th17 cell responses to known DC-SIGN ligands. We hypothesized that CD209a expression may play a role in the CBA DC cytokine response to schistosome Ag as well as the subsequent induction of Th17 cells.

5.2 Materials and Methods

Parasite Isolation

Eggs were isolated from livers of 7- to 8-week infected mice under sterile conditions by a series of blending and straining techniques, as described in Shainheit et al 2008.

Cells

BMDCs. Bone marrow was flushed from femurs and tibias of normal CBA and BL/6 mice. Red blood cells (RBCs) were lysed with Tris ammonium chloride buffer and cells were cultured in complete-RPMI 1640 medium (Lonza) containing 10% FBS (Aleken Biologicals) and recombinant GM-CSF at 15ng/ml (Peprotech AF-315-03) or GM-CSF-containing supernatant from the J558L transfectant B cell hybridoma. The medium was changed on day 3 and 5 and cells harvested on day 7. CD11c $^{+}$ DC purity was >85% by flow cytometric analysis.

CD4^{+} T cells. Single-cell suspensions were prepared from the spleens of normal CBA, CBA Sm-p40-specific TCR Tg, and BL/6 mice, RBCs were lysed, and CD4^{+} T cells were purified by negative selection using CD4^{+} T cell isolation Kit II for mouse (Miltenyi Biotec). CD4^{+} T cell purity was >95% by flow cytometric analysis.

Co-cultures

Purified naïve CD4+ T cells from normal spleen and syngeneic BMDCs were cultured (8T:1DC) in complete-RPMI 1640 medium together with anti-CD3/CD28-coated beads ($2x10^{5}$ beads/ml, Dynabeads® Invitrogen) and freshly isolated live schistosome eggs for 96 hr.

Carbohydrate Stimulation

CBA and BL/6 BMDCs were derived with GM-CSF-containing supernatant from the J558L transfectant B cell hybridoma in cRPMI for 7 days. Cells were washed and plated at 1 x 10^6 cells per well in the presence or absence of mannose, fucose, Mannan from *Saccharomyces cerevisiae*, or schistosome eggs and Il-12p40 was assessed by ELISA after 24 hours.

Mannose Inhibition

CBA BMDCs were derived with GM-CSF-containing supernatant from the J558L transfectant B cell hybridoma in cRPMI for 7 days. Cells were washed and plated at 1.25 x 10^5 cells per well in the presence or absence of mannose. Four hours later, 1 x 10^6 naïve CBA $CD4^+$ T cells together with anti-CD3/CD28-coated beads were added to co-cultures in the presence or absence of live schistosome eggs. IL-17 production was assessed by ELISA after 96 hours.

RNAi

Knockdown. CBA BMDCs derived with rGM-CSF were infected with lentivirus containing CD209a- or GFP-targeted shRNA (RNAi Platform of the Broad Institute, Cambridge, MA). Puromycin was used to select for infected cells and BMDCs were harvested for co-culture experiments on day 10.

Over-expression. The open reading frame (ORF) sequence for CD209a [28,50] was inserted into a lentiviral plasmid and packaged into viral particles in HEK293T cells with

Xtreme gene 9 DNA Transfection Reagent (Roche). Concentrated virus was used to infect BMDCs as described above.

CD209a analysis

Flow cytometry and cell sorting. Spleen cells from individual normal and infected CBA and BL/6 spleens were isolated and RBCs were lysed. Cells were blocked with rat IgG and stained with fluorescently labeled antibodies specific for CD11c (BD Pharmingen 553802) and biotin-conjugated anti-CD209a (BD Pharmingen 558073). Subsequently, cells were stained with Alexa Fluor® 647-conjugated streptavidin (Invitrogen S-21374). Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with Summit Software. BMDCs were gated for viably based on forward scatter and side scatter parameters as well as propidium iodide exclusion. Normal CBA splenocytes were sorted at the Tufts Pathology Flow Cytometry Core Facility with using the Abs listed above.

qRT-PCR. A TaqMan probe for *Cd209a* (Applied Biosystems Mm00460067) was used in combination with TaqMan® Gene Expression Master Mix.

Cytokine analysis

ELISA. Supernatants from 96 hr co-cultures were assessed for IL-1β, IL-23, and IL-17A using R&D Systems® ELISA kits.

qRT-PCR. RNA from co-cultures was obtained with Trizol® or TurboCapture 96 mRNA Kit (Qiagen) and cDNA synthesized with High Capacity Reverse Transcription Kit

(Invitrogen) or Sensiscript RT kit (Qiagen). TaqMan® probes for *Il17a* (Applied Biosystems Mm00439618) and *Gapdh* (Applied Biosystems 4352339E) were used in combination with TaqMan® Gene Expression Master Mix (Applied Biosystems).

Statistical analysis

ANOVA and student's t-tests were used to statistically analyze differences between groups. p-values of < 0.05 were considered significant, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

5.3 Results

CBA and BL/6 DCs elicit differential cytokine responses to DC-SIGN ligands

Binding of human DC-SIGN to mannose- and fucose-containing glycans such as the high-mannose moieties of HIV-1 gp120, *Mycobacterium tuberculosis,* various yeast strains as well as schistosome Le^X and pseudo-Le^Y glycoconjugates is well documented (Geijtenbeek et al., 2000a; van Die et al., 2003; Meyer et al., 2005; Gringhuis et al., 2009; Takahara et al., 2012). Furthermore, CD209a has been shown to retain structural and binding properties of DC-SIGN and DC-SIGNR (Park et al., 2001; Powlesland et al., 2006). We hypothesized that elevated CD209a expression by CBA DCs, but not BL/6 DCs, may confer the capacity to recognize DC-SIGN glycan ligands other than those present in schistosome Ag. To test this hypothesis, CBA and BL/6 BMDCs were cultured in the presence or absence of DC-SIGN ligands including schistosome Ag (eggs), Mannan from *S. cerevisiae*, fucose, or mannose for 24 hr and IL-12/23p40 was assessed in supernatant by ELISA. CBA BMDCs displayed robust and comparable dose-dependent responses to eggs and Mannan, whereas negligible cytokine production was seen in BL/6 BMDC supernatants (Fig. 29A, B). Stimulation with monomeric mannose or fucose did not elicit cytokine responses from CBA or BL/6 BMDCs (Fig. 29C,D). These data demonstrate that CBA and BL/6 BMDCs elicit differential cytokine responses to DC-SIGN ligands, which is likely due to the differential CLR expression.

CBA DCs elicit Th17 cell development in response to Mannan

In light of the strong CBA DC IL-12/23p40 response to Mannan, we tested the ability of CBA DCs to induce Mannan-specific Th17 development. When co-cultured together with naïve CD4+ T cells, anti-CD3/CD28-coated beads, and Mannan, CBA DCs induced T cell IL-17 production in response to Mannan (Fig. 30). These data demonstrate that CBA DC – T cell co-cultures elicit similar Th17 cell responses to the DC-SIGN ligand Mannan and schistosome Ag.

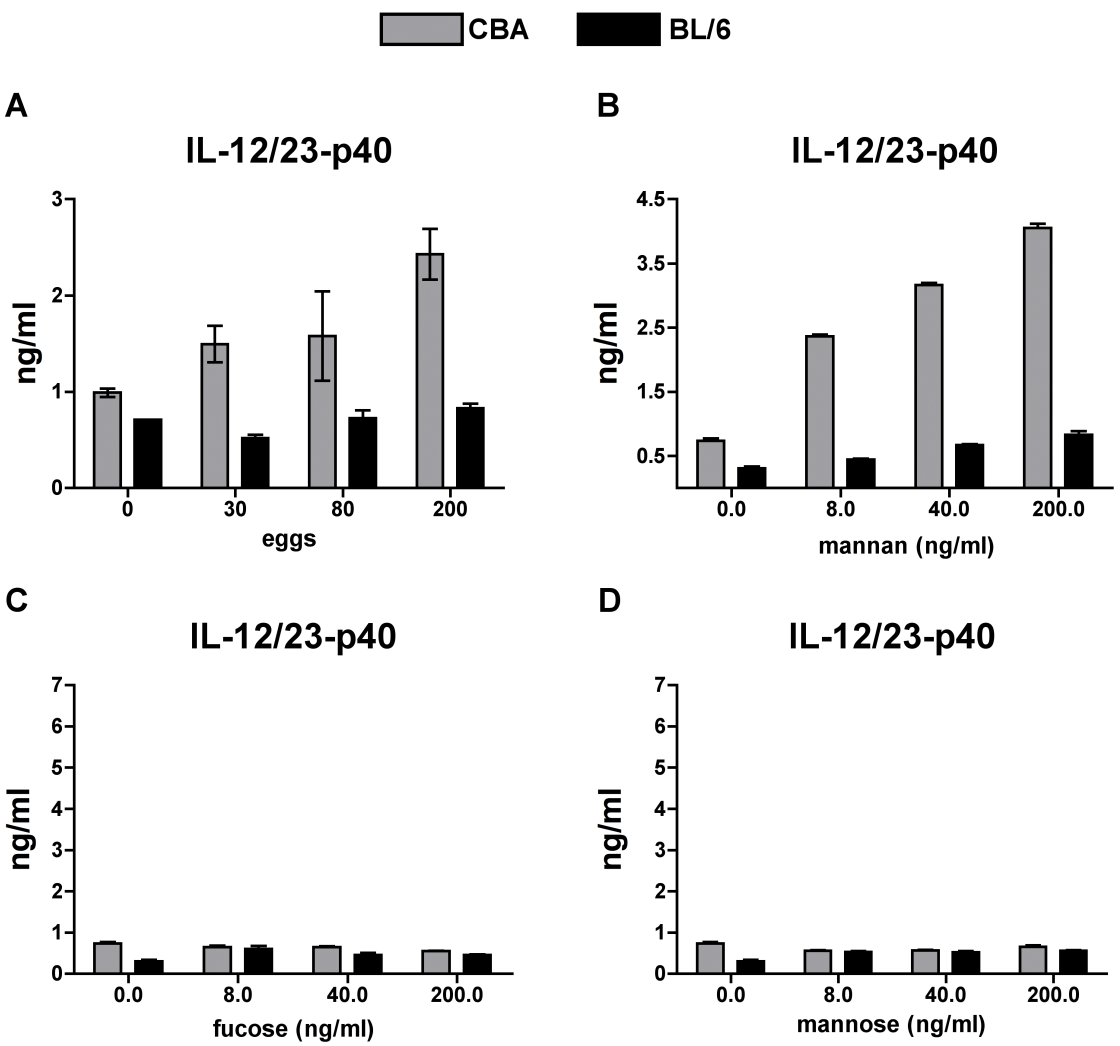

Figure 29. CBA, but not BL/6, DCs elicit IL-12/23p40 response to glycan Ags. CBA and BL/6 BMDCs were differentiated in medium containing GM-CSF over 7 days and cultured at $1x10^6$ cells per well in the presence of live eggs, Mannan, fucose, or mannose at the indicated concentrations. IL-12/23p40 production in supernatants was assessed after 24 hours by ELISA. Data are one representative experiment of three.

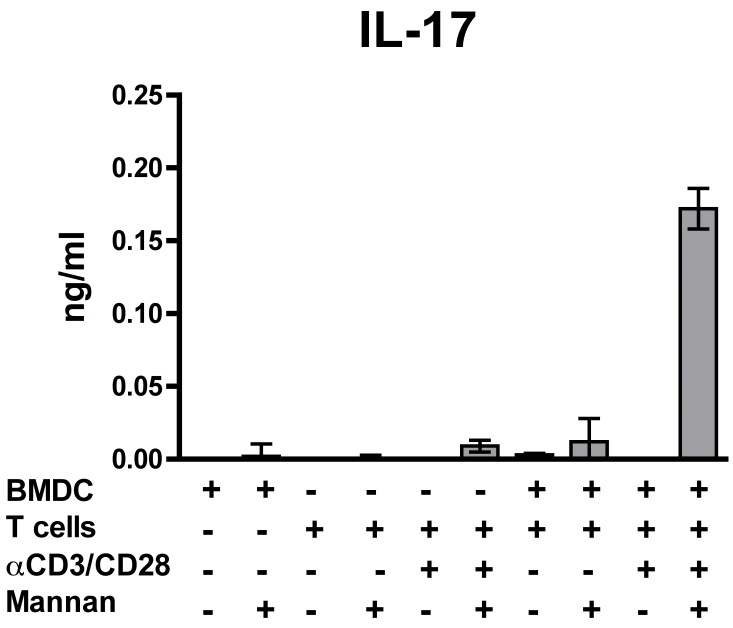

Figure 30. CBA DCs elicit T cell IL-17 in response to DC-SIGN ligand Mannan. CBA BMDCs were differentiated in medium containing GM-CSF over 7 days and cultured with naïve $CD4^+$ T cells together with anti-CD8/CD28-coated beads ± Mannan at 5µg/ml. IL-17 production in DC culture supernatants was assessed by ELISA after 96 hours. Additional single-cell controls are shown. Data are from one representative experiment of two.

Egg stimulated IL-17 production by CBA DCs is lectin dependent

Thus far, our findings have identified a strain-dependent difference in DC CLR expression that correlates with schistosome-specific pro-inflammatory cytokine responses and Th17 pathogenesis in murine schistosomiasis. To further investigate the function of DC-expressed CLRs, such as CD209a, in schistosome Ag-specific cytokine production, we attempted to block CLRs by competitive carbohydrate binding and assess the outcome on egg-specific Th17 cell development. Blocking of DC-SIGN by mannose-based carbohydrate antagonists, antibodies, as well as the DC-SIGN ligands Mannan and HIV-1 gp120 has previously been documented (Zeituni et al., 2009; Varga et al., 2013; Tomašić et al., 2014). As mannose did not stimulate CBA DCs previously (Fig. 29D), CBA BMDCs were blocked with mannose prior to co-culture with naïve CD4+ T cells and eggs (Fig. 31). CBA DC – T cell co-cultures that were pre-blocked with mannose had a dramatic dose dependent decrease in egg-specific T cell IL-17 production (Fig. 32). While mannose blocking is not specific to CD209a, these data suggest that elicitation of T cell IL-17 in response to eggs is largely lectin-activity.

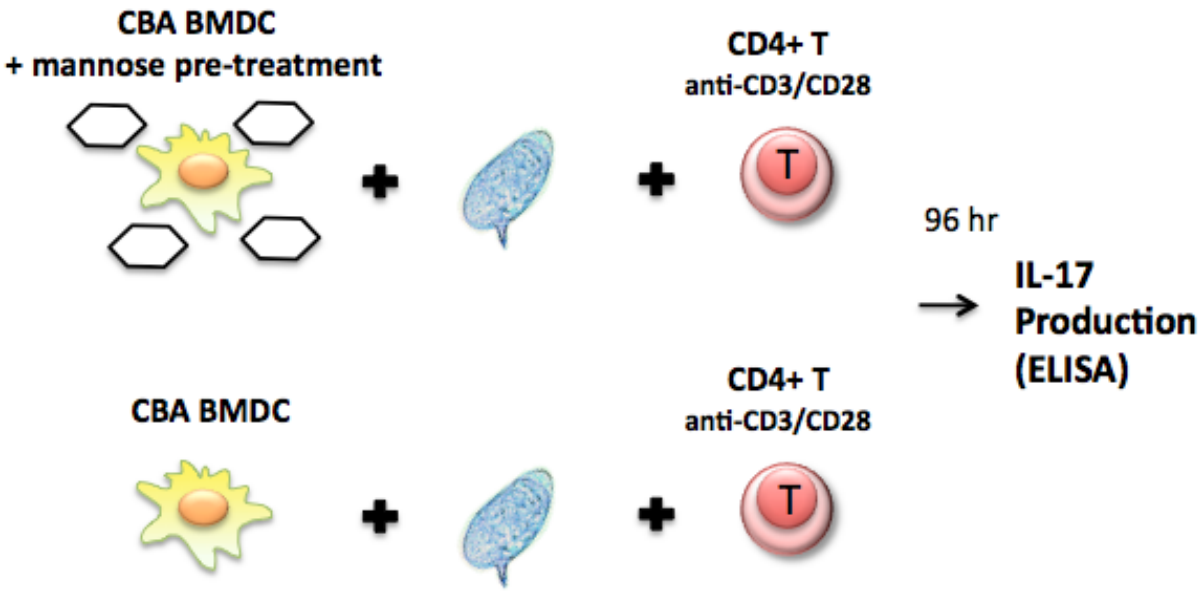

Figure 31. Schematic representation of mannose-blocking assay. CBA BMDCs were differentiated in medium containing GM-CSF over 7 days and pre-treated with mannose for four hours to block surface-expressed CLR carbohydrate binding domains. Mannose-blocking was followed by the addition of naïve $CD4^+$ T cells together with anti-CD8/CD28-coated beads ± live schistosome eggs. IL-17 production in supernatant was assessed after 96 hours by ELISA.

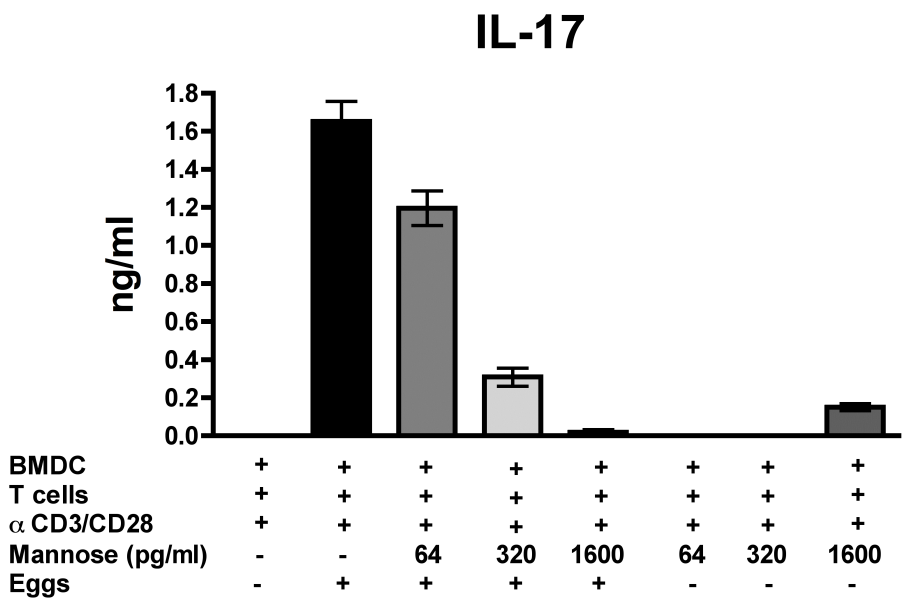

Figure 32. Egg-specific IL-17 production by CBA DCs is decreased in the presence of mannose. CBA BMDCs were differentiated in medium containing GM-CSF over 7 days and pre-incubated with mannose for 4 hr at the indicated concentrations. Following mannose incubation, BMDCs were co-cultured with naïve $CD4^+$ T cells together with anti-CD8/CD28-coated beads ± live schistosome eggs. IL-17 production in 96 hr culture supernatants was assessed by ELISA. Data are from one representative experiment of six.

CD209a expression on DCs is necessary for schistosome egg-induced Th17 cell responses

A murine CLR capable of inducing schistosome-specific Th17 cell development has not been documented. Given the potent Th17-inducing capacity of CBA $CD11c^+$ cells, we specifically examined the role of CD209a on DCs in eliciting Th17 cell responses to eggs. *Cd209a*-targeted short hairpin RNA (shRNA) was delivered into CBA BMDCs via lentiviral infection, resulting in an 86% knockdown as assessed by flow cytometric analysis and qRT-PCR (Fig. 33, Fig. 34A,B,C).

BMDCs in which CD209a expression was knocked down (shCD209a), or control BMDCs (Normal, shCTRL), were subsequently co-cultured with naïve $CD4^+$ T cells and anti-CD3/CD28- coated beads in the presence or absence of live eggs and Th17 cytokine production was assessed by ELISA and qRT-PCR (Fig. 33). In response to eggs, co-cultures containing shCD209a DCs produced significantly less IL-1β and IL-23 compared to normal or shCTRL DCs (Fig. 35A,B). Accordingly, there was a substantial decrease in egg-stimulated T cell IL-17 secretion and mRNA expression in co-cultures containing shCD209a DCs compared to co-cultures containing control DCs (Fig. 36A,B). Significantly, egg-stimulated shCD209a DCs also elicited lower IL-17 production by TCR transgenic (Tg) $CD4^+$ T cells specific for the immunodominant peptide of the Sm-p40 major schistosome egg Ag (Fig. 37)(Finger et al., 2005; Shainheit et al., 2011).

Further assessment of transcription factors required for lineage commitment of $CD4^+$ T cells revealed that egg-stimulated co-cultures comprised of T cells and shCD209a DCs exhibited significantly lower expression of the Th17-associated

transcription factor *Rorc.* In contrast, the Th1- and Th2-associated transcription factors, *Tbx21* and *Gata3*, were unchanged regardless of the DC population present in the cultures (Fig. 38A,B,C). Collectively, these data indicate that CD209a expression on CBA DCs is necessary for egg-stimulated IL-23 and IL-1β production, which induces Th17 cell differentiation.

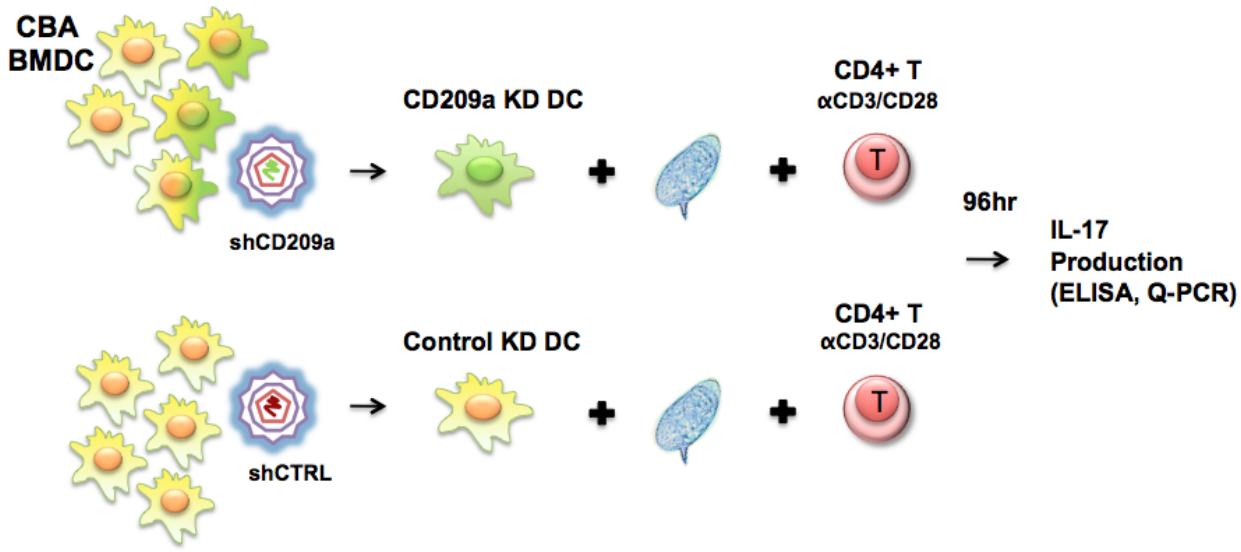

Figure 33. Schematic representation of lentiviral *Cd209a* knockdown in CBA DCs and subsequent co-culture with CD4+ T cells and eggs. Lentivirus encoding *Cd209a*-targeted shRNA was used to knockdown *Cd209a* expression in CBA BMDC. shCD209a DCs were co-cultured with naïve CD4+ T cells together with anti-CD3/CD28-coated beads ± schistosome eggs. Lentivirus encoding a GFP-targeted shRNA was used for a mock-knockdown in control BMDCs (shCTRL). IL-17 as well as Th17-associated cytokines and transcription factors were assessed after 96 hr by ELISA and qRT-PCR.

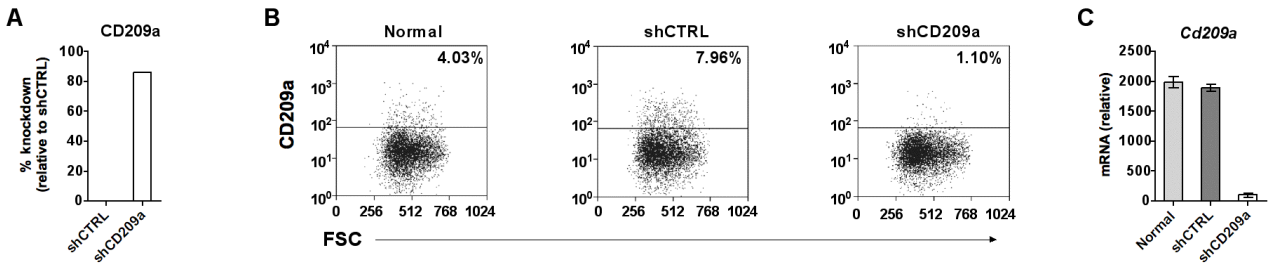

Figure 34. Lentiviral CD209a knockdown validation in CBA DCs. CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection and CD209a knockdown efficiency was assessed by flow cytometric analysis relative to CBA BMDCs receiving a GFP-targeted control shRNA (shCTRL) or normal BMDCs (A,B). *CD209a* expression knockdown was also assessed by qRT-PCR (mRNA relative to GAPDH) (C).

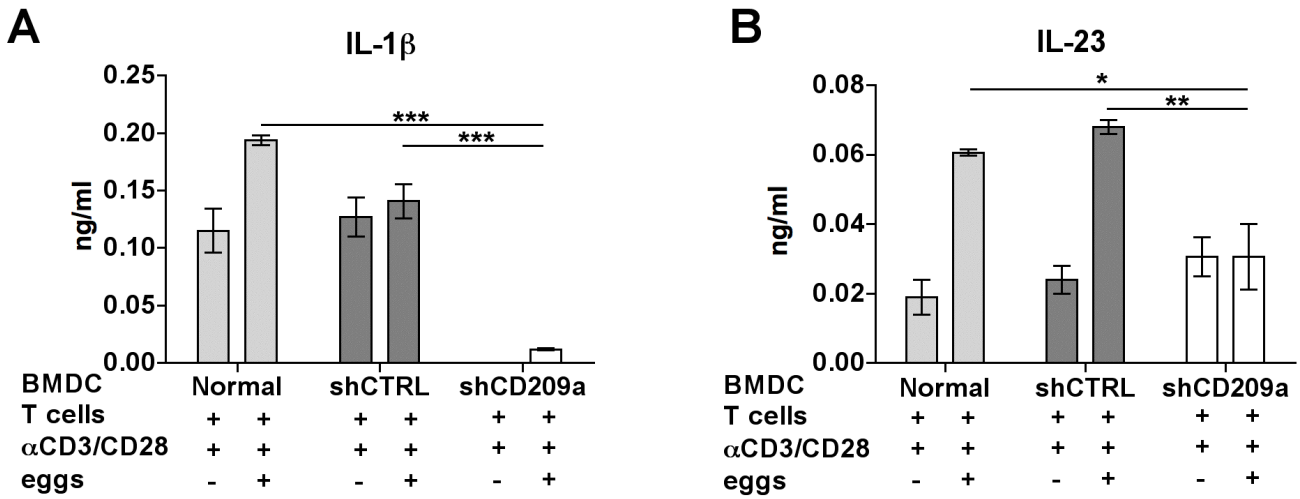

Figure 35. CD209a expression is necessary for schistosome egg-induced IL-1β and IL-23 production. CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection. Normal CBA, shCTRL, or shCD209a DCs were co-cultured with naïve CBA $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. (A-B) IL-1β and IL-23 in supernatants were assessed by ELISA. Bars represent the mean ± S.D. of three biological replicates of one representative experiment of five. For all figures, *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

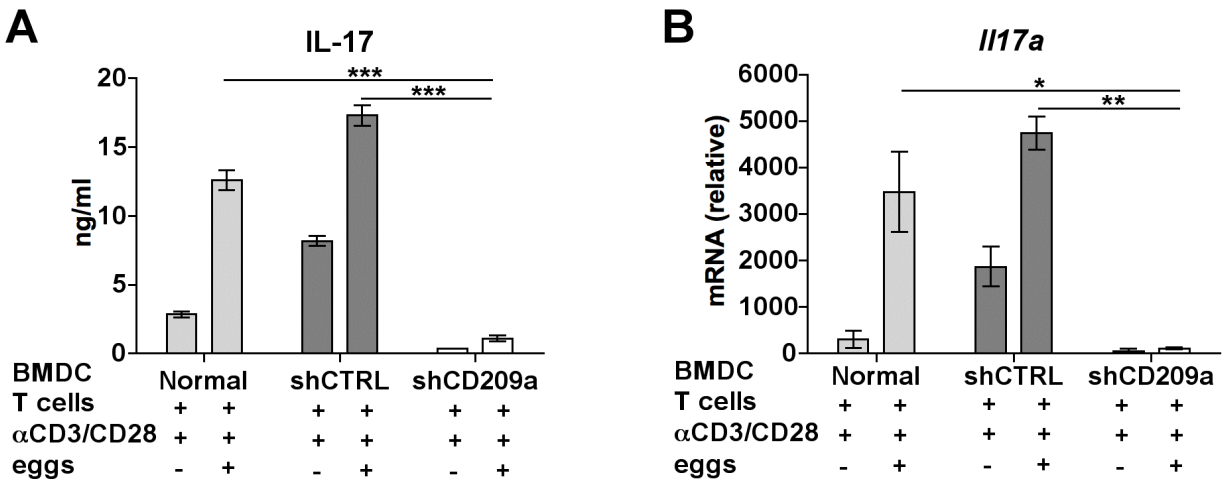

Figure 36. CD209a expression by DCs is necessary for the induction of schistosome egg-induced IL-17. CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection. Normal CBA, shCTRL, or shCD209a DCs were co-cultured with naïve CBA $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. (A) IL-17 in supernatants was assessed by ELISA. (B) *Il17a* was assessed by qRT-PCR. Bars represent the mean ± S.D. of three biological replicates of one representative experiment of five.

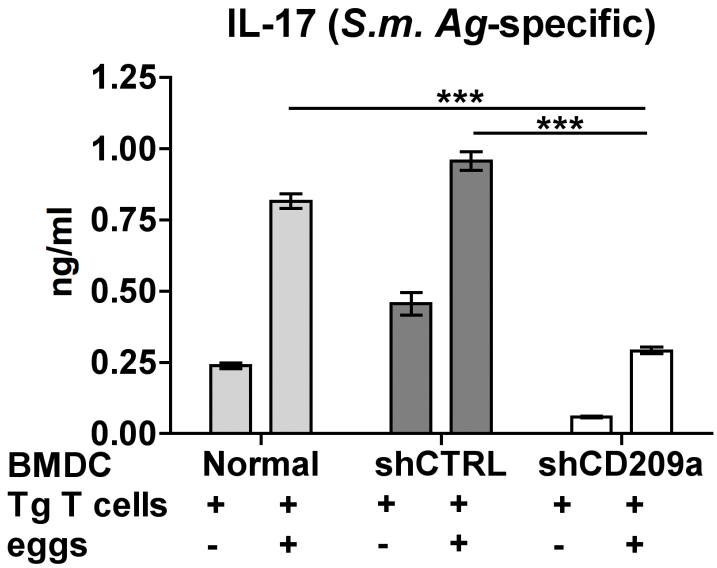

Figure 37. CD209a expression on DCs is necessary for the production of egg-stimulated IL-17 by *S. mansoni* Smp40 Ag- specific Transgenic CD4+ T cells. CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection. Normal CBA, shCTRL, or shCD209a DCs were co-cultured with *S. mansoni* major egg Ag Sm-p40-specific Tg T cells (Tg T) ± schistosome eggs. IL-17 was assessed by ELISA. Bars represent the mean ± S.D. of three biological replicates of one representative experiment of five.

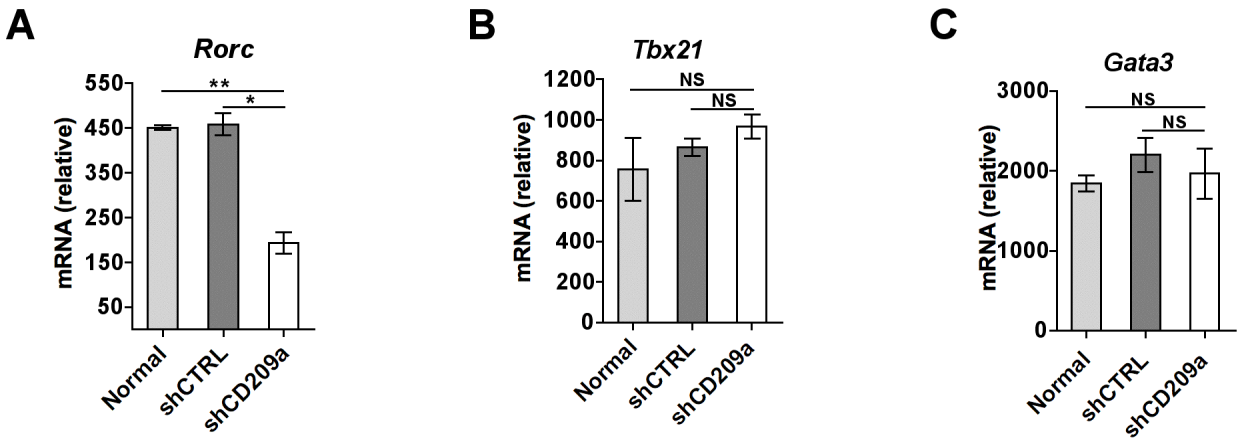

Figure 38. Th17-associated transcription factor *Rorc* is decreased in egg-stimulated shCD209a DC-T co-cultures. CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection. Normal CBA, shCTRL, or shCD209a DCs were co-cultured with naïve CBA $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. Transcription factors *Rorc, Tbx21,* and *Gata3* were assessed by qRT-PCR in egg-stimulated co-cultures. Bars represent the mean ± S.D. of three biological replicates of one representative experiment of five.

CD209a over-expression confers on BL/6 DCs the capacity to induce Th17 cell responses

CD209a expression is significantly lower in BL/6 than in CBA DCs (Fig. 12,14,21,25,28), and BL/6 DCs fail to induce Th17 cell responses to eggs *in vitro* (Rutitzky et al., 2005, 2008; Shainheit et al., 2008, 2011). We thus investigated whether CD209a over-expression might confer on BL/6 DCs the capacity to induce egg-specific Th17 cell responses. A lentiviral vector was utilized to over-express CD209a in BL/6 BMDCs, which was successfully accomplished as determined by flow cytometric analysis and qRT-PCR (Fig. 39, Fig. 40A,B,C).

BL/6 CD209a-expressing BMDCs were co-cultured with naïve BL/6 $CD4^+$ T cells and anti-CD3/CD28-coated beads in the presence or absence of live eggs and Th17-associated cytokine production was assessed by ELISA and qRT-PCR. Egg-stimulated CD209a-expressing BMDCs produced higher levels of IL-1β and IL-23 in comparison to control BMDCs (Fig. 41A,B). IL-17 secretion and mRNA expression was also enhanced in CD209a BMDC co-cultures compared to co-cultures containing control BMDCs (Fig. 42A,B). Additionally, *Rorc* expression was elevated in CD209a BMDC co-cultures compared to control BMDCs (Fig. 43A), but there were no significant changes in *Tbx21* or *Gata3* expression (Fig. 43B,C). Overall, these results confirm that CD209a expression enables egg-stimulated DCs to induce IL-1β and IL-23 secretion leading to Th17 cell development.

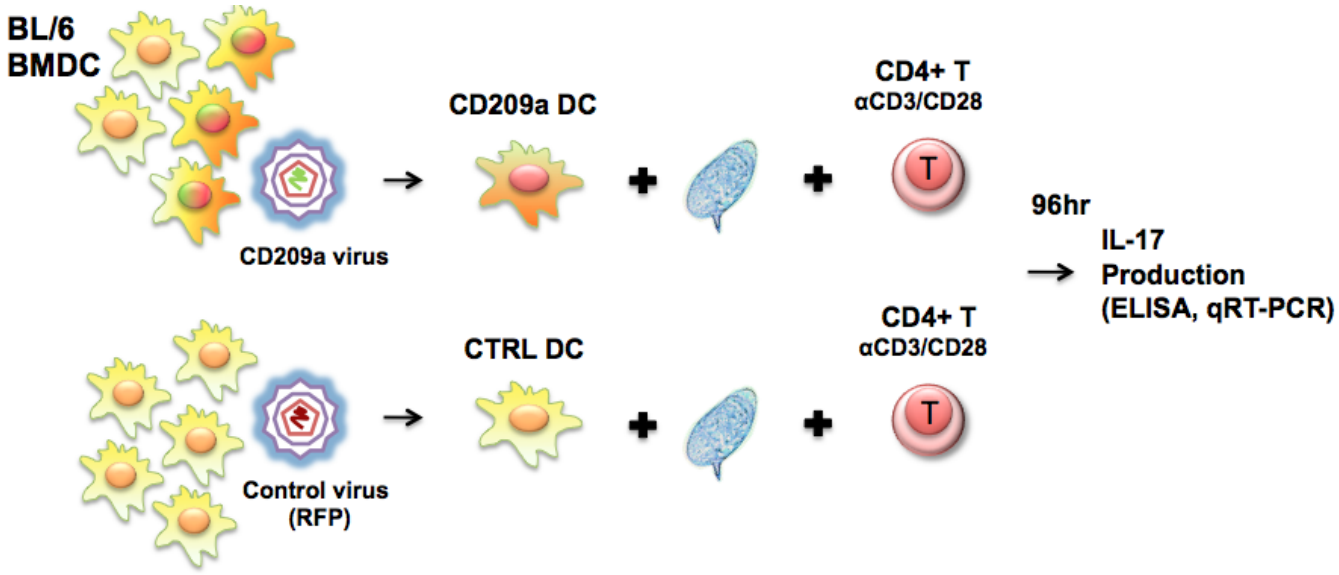

Figure 39. Schematic representation of lentiviral *Cd209a* over-expression in BL/6 BMDC and subsequent co-culture with naïve CD4$^+$ T cells and eggs. Lentivirus encoding *Cd209a* was used to over-express *Cd209a* in BL/6 BMDC. CD209a-expressing DCs were co-cultured with naïve CD4$^+$ T cells together with anti-CD3/CD28-coated beads ± schistosome eggs. Lentivirus encoding RFP was used for over-expression in control BMDCs (CTRL). IL-17 as well as Th17-associated cytokines and transcription factors were assessed after 96 hr by ELISA and qRT-PCR.

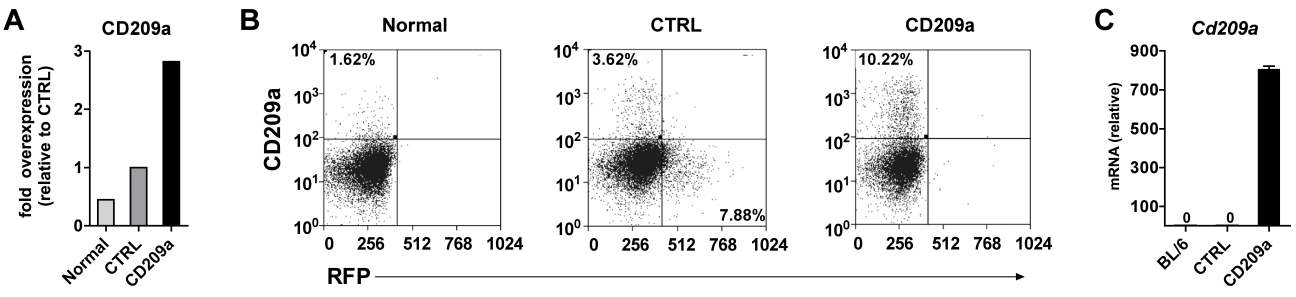

Figure 40. Lentiviral *Cd209a* over-expression validation in BL/6 BMDC. (A-B) BL/6 BMDC were differentiated in rGM-CSF-containing medium over 10 days. CD209a over-expression was achieved using a lentiviral vector and confirmed by flow cytometric analysis relative to BL/6 control BMDC that over-expressed RFP (CTRL). (C) *CD209a* over-expression was also assessed by qRT-PCR (mRNA relative to GAPDH).

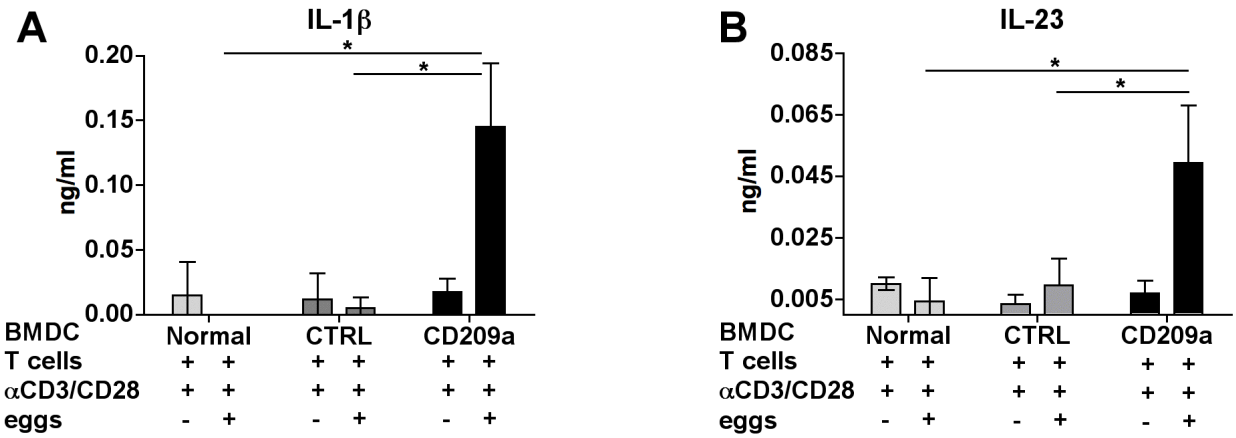

Figure 41. CD209a expression is critical for schistosome-induced IL-1β and IL-23 production. BL/6 BMDC were differentiated in rGM-CSF-containing medium over 10 days. CD209a over-expression was achieved using a lentiviral vector. Normal BL/6, CTRL, or CD209a DC were co-cultured with naïve BL/6 $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. IL-1β and IL-23 in supernatants was assessed by ELISA. Bars represent the mean ± S.D of three biological replicates of one experiment representative of three. For all figures, *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

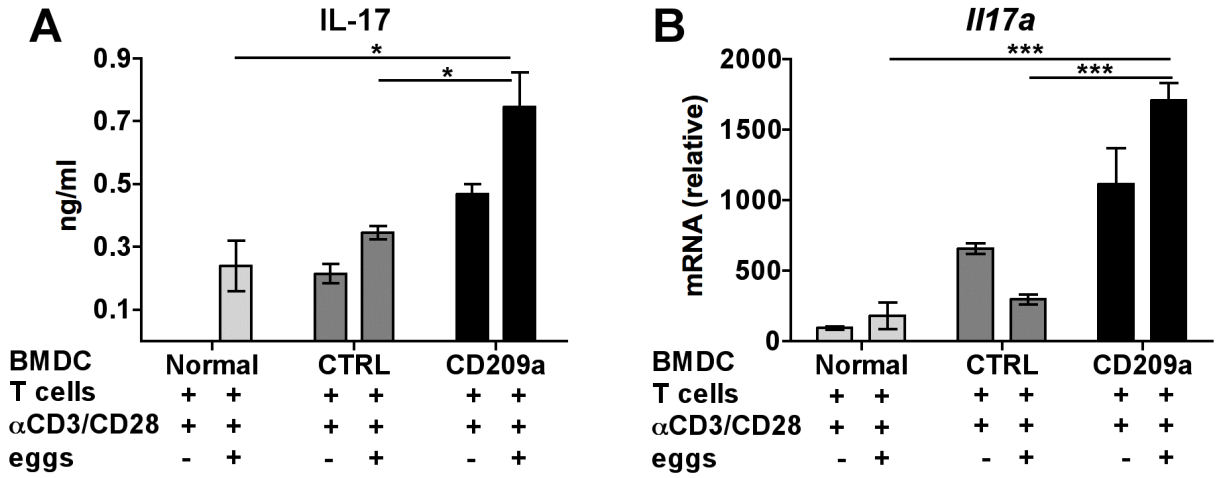

Figure 42. CD209a over-expression confers on BL/6 DC the capacity to induce Th17 cell responses. BL/6 BMDC were differentiated in rGM-CSF-containing medium over 10 days. CD209a over-expression was achieved using a lentiviral vector. Normal BL/6, CTRL, or CD209a DC were co-cultured with naïve BL/6 $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. IL-17 in supernatants was assessed by ELISA. *Il17a* was assessed by qRT-PCR. Bars represent the mean ± S.D of three biological replicates of one experiment representative of three.

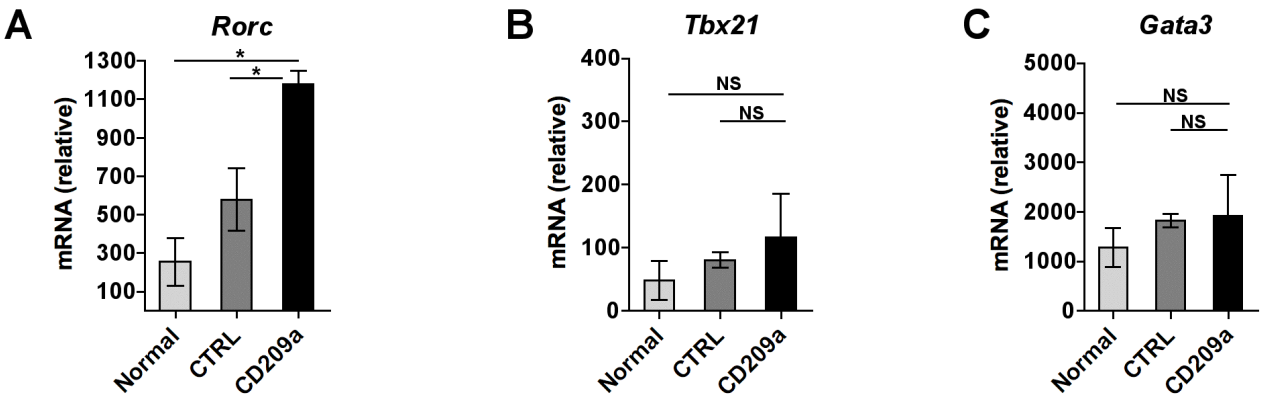

Figure 43. Th17-associated transcription factor *Rorc* is elevated in egg-stimulated BL/6 CD209a DC-T co-cultures. BL/6 BMDC were differentiated in rGM-CSF-containing medium over 10 days. CD209a over-expression was achieved using a lentiviral vector. Normal BL/6, CTRL, or CD209a DCs were co-cultured with naïve BL/6 $CD4^+$ T cells together with anti-CD3/CD28-coated beads and schistosome eggs for 96 hr. Transcription factors *Rorc*, *Tbx21,* and G*ata3* were assessed by qRT-PCR in egg-stimulated co-cultures. Bars represent the mean ± S.D of three biological replicates of one experiment representative of three.

CHAPTER 6

Characterization of CD209a Associated Signaling Responses to Schistosome Antigen

6.1 Rationale

Pro-inflammatory signaling pathways that materialize in DCs upon PRR ligation have been well documented for several human and murine PRR families (Akira et al., 2006; Takeuchi & Akira, 2010); however, little is known of the signaling responses associated with murine CD209 homologues. While mitogen-activated protein (MAP) kinase activation has been reported following cross-linking of CD209b (SIGNR1) (Numazaki et al., 2009) as well as CD209d (SIGNR3) (Tanne et al., 2009), the molecular mechanisms critical for Th17 cell-associated DC cytokine production downstream of murine CD209 family members, particularly in response to schistosome products, are largely undescribed. Nonetheless, a signalosome of signaling proteins was characterized downstream of human DC-SIGN ligation by fucose- and mannose-containing pathogen glycans. Gringhuis et al. revealed that DC-SIGN ligation by ManLam stimulates MAP kinase activation and modification of NFκB leading to subsequent IL-12p40, IL-10, IL-12p35, and IL-6 production (Gringhuis et al., 2007; Gringhuis et al. 2009). Further studies by Caparrós et al. confirmed a similar mechanism involving extracellular signal-related kinases 1 and 2 (ERK1/2) and phosphoinositide-3 kinase (PI3K) activation following DC-SIGN engagement by ligand (Caparros, 2006).

The present study in Chapter 5 demonstrated that CD209a expression by DCs is critical for the production of IL-23 and IL-1β, which leads to subsequent development of Th17 cells in response to schistosome antigen. Although human DC-SIGN expression by DCs was not previously associated with Th17 cell responses, ligation of DC-SIGN does indeed lead to the production of IL-12p40, a subunit of the Th17-associated DC cytokine IL-23 (Gringhuis et al., 2007; Gringhuis et al., 2009). Additionally, stimulation of other CLRs, such as the Dectin family, leads to IL-23 production and subsequent Th17 cell development during fungal infection (LeibundGut-Landmann et al., 2007). Thus, we investigated further into the CD209 family-associated MAP-kinase signaling mechanisms in CBA vs. BL/6 DCs. We hypothesized that MAP kinase signaling responses which occur downstream of human DC-SIGN in response to fucose-containing Ag, may also materialize in CBA DCs upon schistosome egg stimulation. Furthermore, these signaling mechanisms may play a role in CBA DC production of IL-1β and IL-23 in response to schistosome Ag.

6.2 Materials and Methods

Parasite isolation

Eggs were isolated from livers of 7- to 8-week infected mice under sterile conditions by a series of blending and straining techniques, as described in Shainheit et al 2008.

CD209a analysis

Flow cytometry and cell sorting. DC2.4 cells (Shen et al., 1997) kindly provided by

Dr. Cheolho Cheong were blocked with rat IgG and stained with a biotin-conjugated anti-CD209a antibody (BD Pharmingen 558073). Subsequently, cells were stained with Alexa Fluor® 647-conjugated streptavidin (Invitrogen S-21374). Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with FlowJo Software.

qRT-PCR. A TaqMan probe for *Cd209a* (Applied Biosystems Mm00460067) was used in combination with TaqMan® Gene Expression Master Mix.

Signaling protein analysis

Intracellular Staining. BMDCs were washed, fixed with 3% PFA, blocked with rat IgG, and stained with a fluorescently labeled antibody (Ab) specific for CD11c. Subsequently, cells were washed, permeabilized with methanol, and stained with a primary Ab specific for phospho-ERK (Cell Signaling 4695) followed by a fluorescently labeled secondary anti- rabbit IgG Ab (Invitrogen A11034). Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with Summit Software.

Western Blot. BMDCs were washed, lysed, and prepared with Laemmli's SDS-sample buffer (Boston Bioproducts). Samples were run on an SDS-PAGE gel and transferred to an Immobilon-P PVDF (Millipore) membrane, which was then blocked in 5% BSA. The activation of MAP-kinases was detected with Abs specific for phospho-SRC Tyr416 (Cell Signaling 2101), phospho-RAF-1 Ser 338 (Cell Signaling 9427), phospho-ERK1/2 Thr202/Tyr204 (Cell Signaling 4695P), phospho-JNK Thr183/Tyr185 (Cell Signaling

9251S), and phospho-p38 Thr180/Tyr182 (Cell Signaling 9215S). Total kinase expression was detected with Abs specific for SRC (Cell signaling 32G6), RAF-1 (Cell Signaling 9422P), ERK1/2 (Cell Signaling 4695), JNK (Cell Signaling 9252), p38 (Cell Signaling 9212), and GAPDH (Cell Signaling 2118S).

Cytokine analysis

ELISA. Supernatants from 96 hr co-cultures were assessed for IL-17A using R&D Systems® ELISA kits.

qRT-PCR. RNA from co-cultures was obtained with Trizol® or TurboCapture 96 mRNA Kit (Qiagen) and cDNA synthesized with High Capacity Reverse Transcription Kit (Invitrogen) or Sensiscript RT kit (Qiagen). TaqMan® probes for *Il17a, Il23a, Il1b, CD209a* (Applied Biosystems Mm00439618), and *Gapdh* (Applied Biosystems 4352339E) were used in combination with TaqMan® Gene Expression Master Mix (Applied Biosystems).

DC2.4 cells.

DC2.4 bone marrow-derived BL/6 DC cell line was obtained from Dr. Cheolho Cheong (IRCM, Montréal, Québec, Canada) (Shen et al., 1997). DC2.4 cells were maintained in cRPMI medium containing 10% FBS, penicillin/streptomycin, and 2-mercaptoethanol. DC2.4 cell lines stably expressing CD209a or RFP (CTRL) were generated using lentiviral vectors encoding CD209a or RFP and subsequent puromycin selection. Expression of CD209a was confirmed by flow cytometric analysis and qRT-PCR.

ERK Inhibition

CD209a-expressing or CTRL (RFP) DC2.4 cells were plated at $1x10^6$ cells per well and pre-incubated with a 10uM concentration of ERK inhibitor U0126 plus DMSO or DMSO alone for 1 hr prior to egg stimulation. Cells were stimulated with live schistosome eggs for 5 minutes or 24 hrs and samples were collected for assessment by Western blot, qRT-PCR, or ELISA.

EGTA Calcium Chelation

Normal, CD209a-, or RFP-expressing DC2.4 cells were plated at $1x10^6$ cells per well and pre-incubated for 1 hr with 5µg/ml EGTA for calcium-specific chelation of C-type lectin receptors. Cells were stimulated for 5 minutes with live schistosome eggs and samples were collected for assessment by Western blot.

6.3 Results

CD209a expression is associated with schistosome egg-stimulated MAP-kinase activation in DCs

While signaling pathways associated with CD209a are unknown to this date, recent studies have demonstrated that the MAP kinases Rapidly Accelerated Fibrosarcoma-1 (RAF-1) and ERK1/2 are activated in DCs following ligation of human DC-SIGN (Caparros, 2006; Gringhuis et al., 2007, 2009; Johnson et al., 2012). We therefore investigated MAP kinase activation in CBA and BL/6 BMDCs (Fig. 44). Following stimulation with live eggs, there was a steady increase in phospho-ERK1/2 in CBA BMDCs as determined by intracellular staining, which was not apparent in BL/6 BMDCs (Fig. 45A,B). This striking increase in phospho-ERK1/2 was still clearly evident at 24 hours post egg stimulation in CBA BMDCs by Western blot, at which time little or no phospho-ERK was detectable in BL/6 BMDCs (Fig. 46C). An increase in the phosphorylation of the upstream MAP kinase RAF-1 as well as SRC kinase was also detected in CBA compared to BL/6 BMDCs (Fig. 46A,B). By contrast, there was no significant activation of the MAP kinases p38 or JNK (Fig. 46D,E). These results are consistent with the notion that egg-stimulated CD209a expression on CBA DCs is associated with SRC, RAF-1, and ERK1/2 MAP kinase activation.

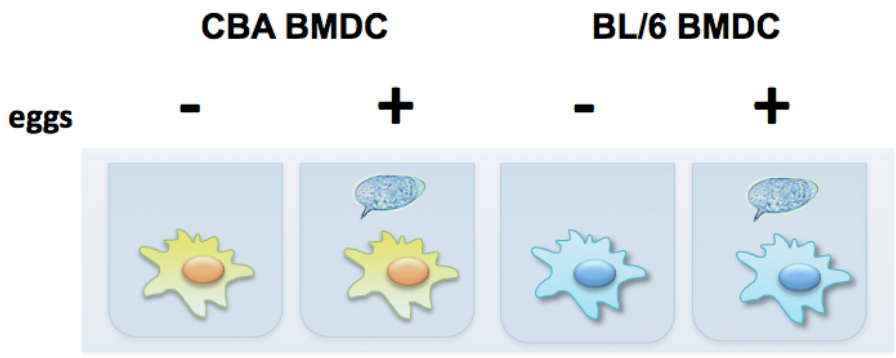

Figure 44. Schematic representation of BMDC – egg co-cultures for MAP-kinase activation assay. CBA and BL/6 BMDCs were cultured in the presence or absence of live eggs for 0 min, 20 min, 40 min, 80 min, 240 min, or 24 hr in the presence or absence of eggs. Phospho-ERK1/2 was assessed by intracellular staining or western blot. SRC, RAF-1, p38, and JNK phosphorylation was assessed by Western blot after 24 hr.

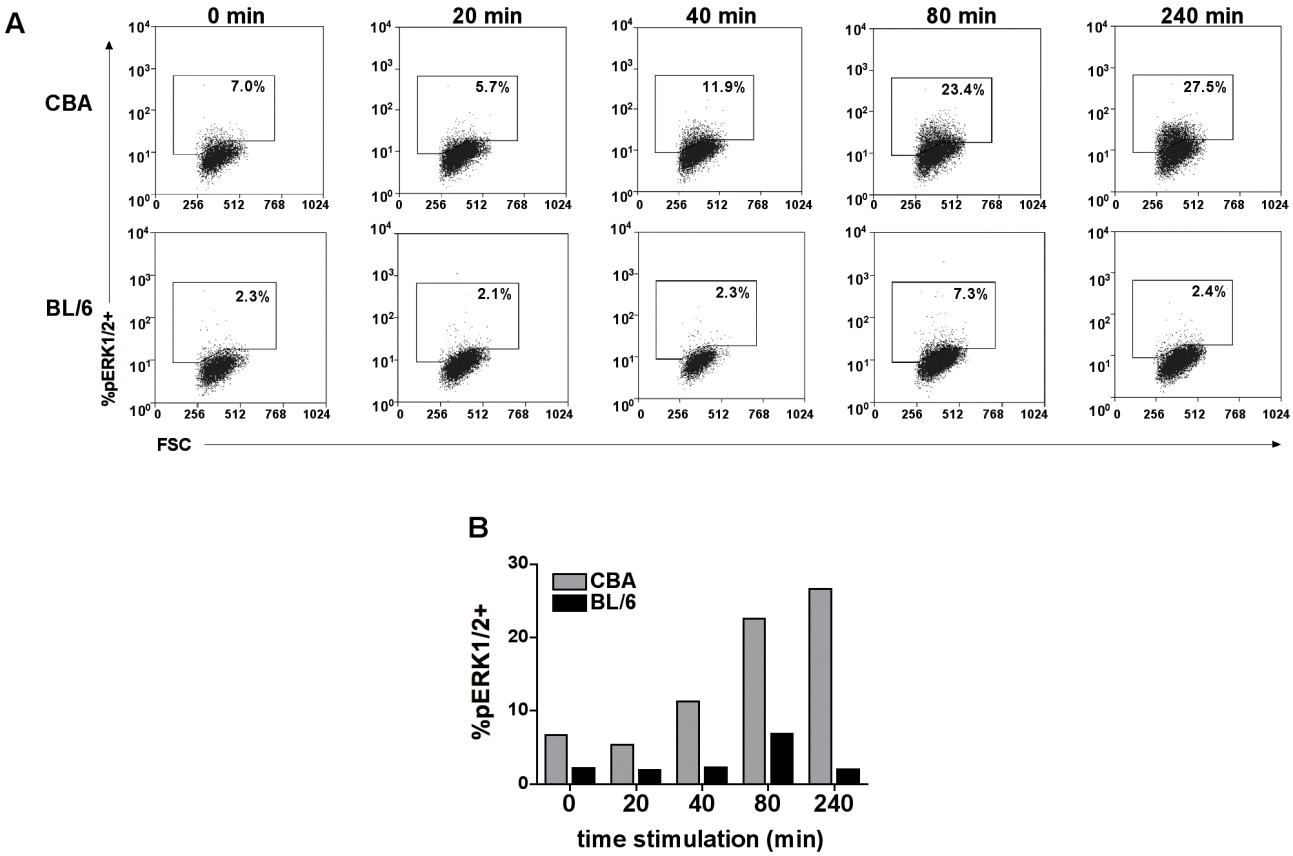

Figure 45. CBA DCs elicit rapid and sustained ERK1/2 activation in response to eggs. (A-B) CBA and BL/6 BMDCs were differentiated in GM-CSF-containing medium over 7 days and cultured with schistosome eggs for the indicated time periods. Cells were fixed, permeablized and analyzed by intracellular staining for phospho-ERK1/2. Flow cytometry plots were gated for viability and $CD11c^+$ cells, which were > 80% for both strains. Data are from one representative experiment of three.

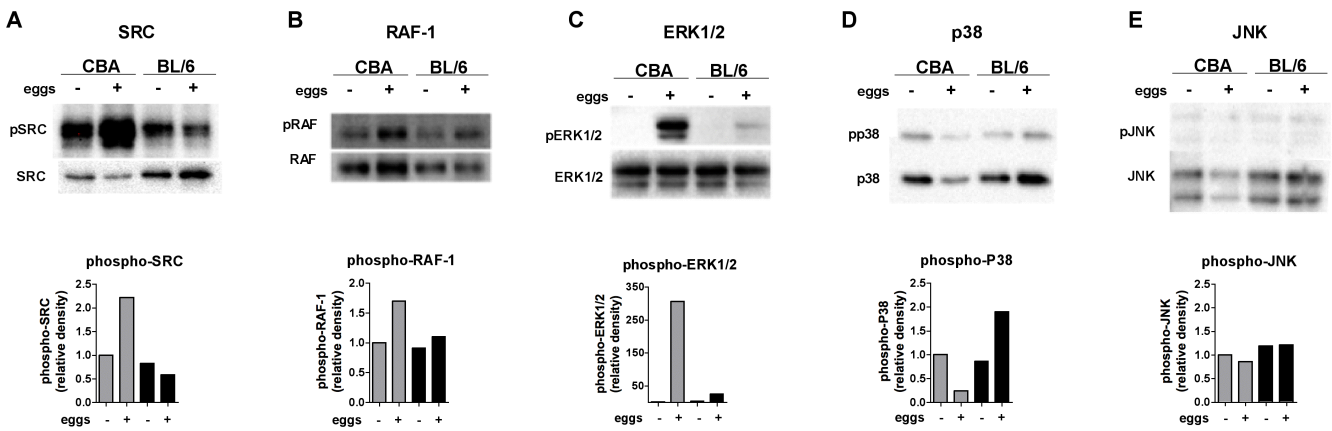

Figure 46. Egg-stimulated CBA DCs elicit elevated MAP-kinase activation compared to BL/6 DCs. (A-B) CBA and BL/6 BMDC were differentiated in GM-CSF-containing medium over 7 days and cultured ± schistosome eggs for 24 hr and phosphorylated vs. total SRC, RAF-1, ERK1/2, JNK, and p38 expression were assessed by Western blot. Data are from one representative experiment of four.

CBA DCs elicit ERK1/2 responses to the DC-SIGN ligand Mannan

High-mannose moieties have been shown to activate MAP-kinase responses downstream of DC-SIGN ligation (Gringhuis et al., 2009). To compare CBA and BL/6 DC capacity to elicit MAP-kinase activation in response to Mannan, CBA and BL/6 BMDCs were stimulated with Mannan for 20 minutes and phospho-ERK1/2 was assessed by western blot (Fig 47). Mannan-stimulated ERK1/2 activation was greater in CBA compared to BL/6 BMDCs; in contrast, there was no strain-dependent difference in the magnitude of ERK/12 activation in response to LPS (Fig. 47). These data demonstrate that CBA DCs have an enhanced capacity to respond to DC-SIGN ligands in comparison to BL/6.

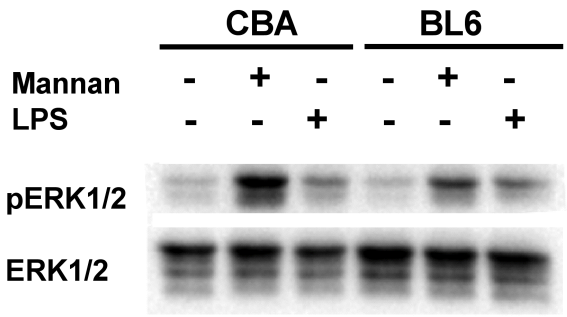

Figure 47. CBA DCs elicit an enhanced MAP-kinase response to Mannan in comparison to BL/6 DCs. CBA and BL/6 BMDC were differentiated in GM-CSF-containing medium over 7 days and cultured ± 5ug/ml Mannan or 100ng/ml LPS for 20 minutes. Cells were assessed for phosphorylated vs. total ERK1/2 expression by Western blot. Data are one representative experiment of two.

CD209a-expressing DC cell line elicits egg-specific Th17 cell responses and ERK1/2 activation

Signaling mechanisms downstream of DC-SIGN homologues that promote schistosome Ag-specific Th17 cell development have not been characterized. To determine if egg-specific ERK1/2 activation by DCs is specifically dependent on CD209a expression, the DC2.4 BL/6 transformed bone-marrow-derived DC line, previously shown to function like cDCs and present antigen via MHC I and MHC II (Shen et al., 1997)(Fig. 48A), was utilized to explore signaling mechanisms in greater depth. A lentiviral vector was used to over-express CD209a in DC2.4 cells and subsequent puromycin selection successfully generated stable CD209a-expressing DC2.4 cells (Fig 48B). CD209a expression was validated by flow cytometric analysis and qRT-PCR (Fig. 49A,B). RFP-expressing DC2.4 cells were used as a control (CTRL). CD209a-expressing DC2.4 cells were confirmed to be functionally comparable to primary CD209a-expressing BMDC in co-culture analyses demonstrating enhanced Th17-cytokine production in response to schistosome eggs in comparison to CTRL DC2.4 cells (Fig. 50). As such, CD209a-expressing DC2.4 cells elicited increased egg-specific IL-17 and *Il1b* in co-culture with T cells from infected BL/6 mice in comparison to CTRL DC2.4 cells (Fig. 50A,B). Additionally, mature IL-1β was apparent in egg-stimulated single cell CD209a-expressing DC2.4 cell cultures after 24 hours by Western blot (Fig. 50C).

To investigate the requirement of CD209a for schistosome Ag-specific ERK1/2 activation, CD209a-expressing and CTRL DC2.4 cells were assessed for their capacity

to stimulate egg-specific phospho-ERK1/2. After 5 minutes of egg stimulation, egg-specific phospho-ERK1/2 activation was apparent in CD209a-expressing DC2.4 cells, but not CTRL DC2.4 cells (Fig. 51A). Elevated ERK1/2 activation was still apparent after 24 hours (Fig. 51B).

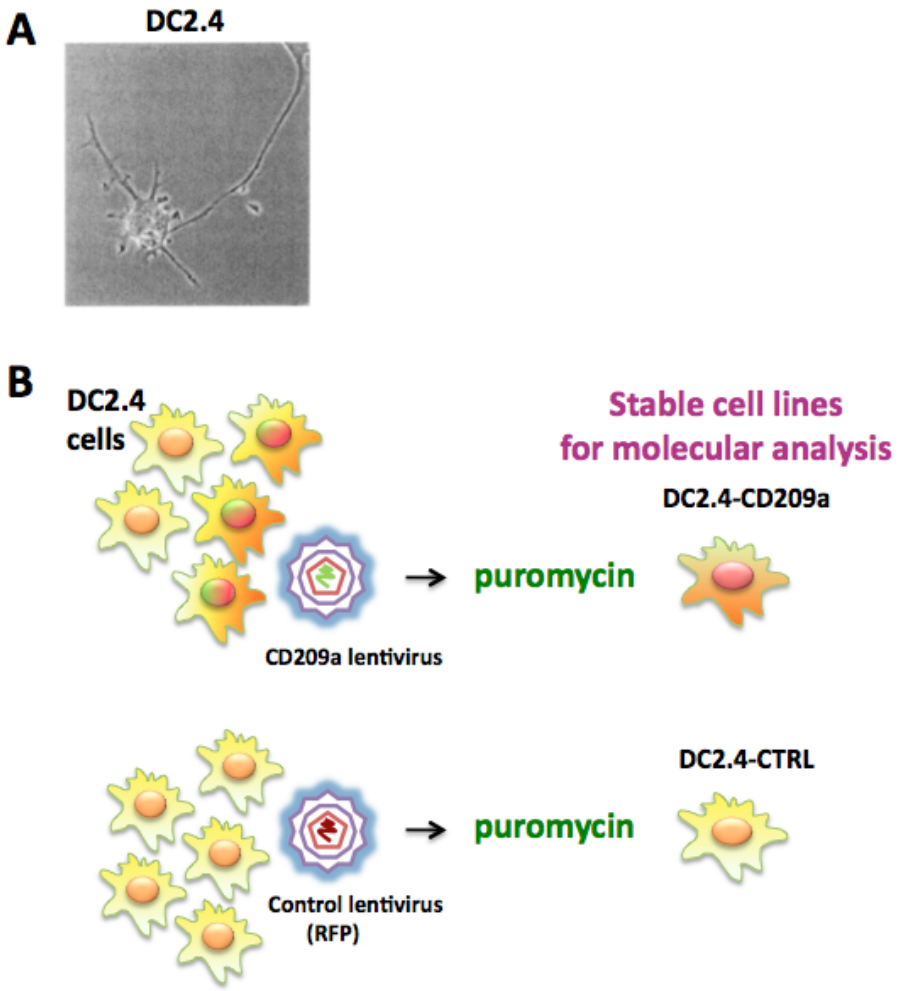

Figure 48. The generation of a CD209a-expressing dendritic cell line. DC2.4 cells are a transformed BL/6 bone marrow-derived cell line (image from Shen et al., 1997). Stable DC2.4 cell lines expressing CD209a or RFP (CTRL) were generated using lentiviral vectors encoding CD209a or RFP. Following lentiviral infection, CD209a- or RFP-expressing cells were positively selected for in puromycin-containing medium. Cells were expanded and maintained in cRPMI medium for molecular analysis.

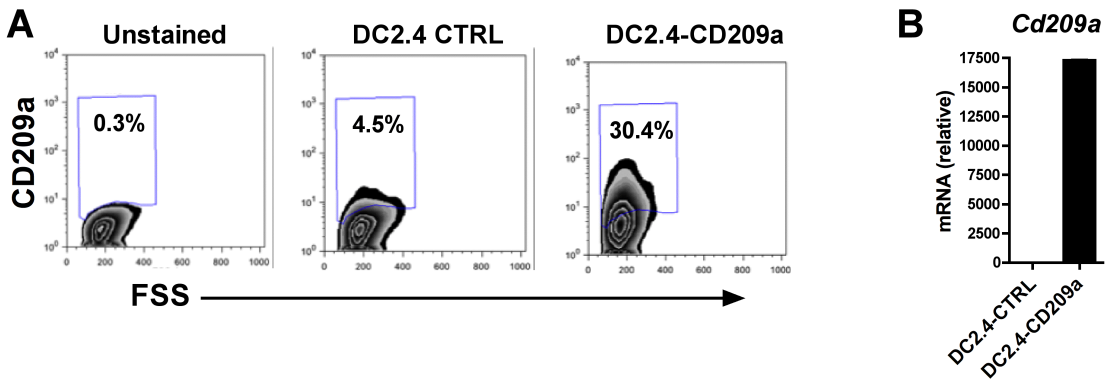

Figure 49. CD209a-expressing vs. CTRL DC2.4 cells. The DC2.4 BL/6 bone marrow-derived DC line was stably grown in cRPMI. A lentiviral vector was utilized to express CD209a or RFP (CTRL) and puromycin selection was carried out to generate stable expression. CD209a expression was confirmed by flow cytometric analysis and qRT-PCR.

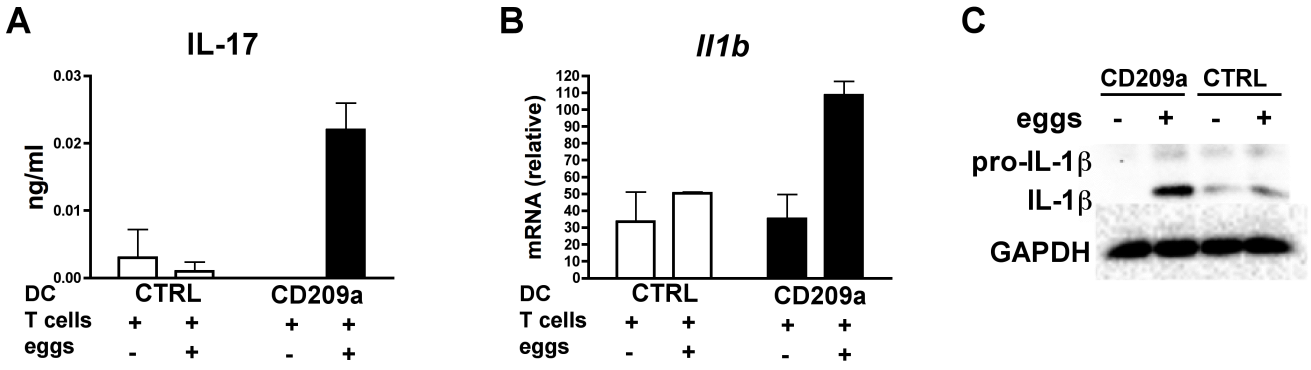

Figure 50. CD209a expression confers on DC2.4 cells the capacity to induce schistosome egg-specific Th17 cell responses. CD209a-expressing or CTRL DC2.4 cells were co-cultured with CD4+ T cells from infected BL/6 mice ± eggs for 96 hours. (A-B) IL-17 in supernatants was assessed by ELISA and *Il1b* transcript was assessed by qRT-PCR. (C) CD209a-expressing or CTRL DC2.4 cells were cultured ± eggs for 24 hours and pro- vs. mature IL-1β were assessed by Western blot. Data are one representative experiment of two.

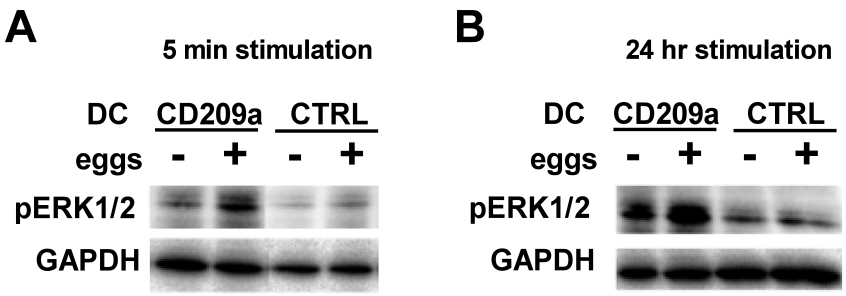

Figure 51. CD209a expression confers on DC2.4 cells the capacity to induce schistosome egg-specific ERK1/2 activation. (A-B) CD209a-expressing or CTRL DC2.4 cells were cultured ± eggs for 5 minutes or 24 hours. Phospho-ERK1/2 and GAPDH expression were assessed by Western blot. Data are one representative experiment of two.

DC CD209a expression is necessary for pERK dependent IL-1β and IL-23 production

Given previous documentation of DC-SIGN-specific MAP-kinase signaling upstream of cytokine production (Gringhuis et al., 2009), we evaluated whether ERK1/2 activation was necessary for the production of DC IL-1β and IL-23 secretion in response to schistosome egg stimulation (Fig. 52). ERK signaling was effectively inhibited using U0126, an inhibitor of MAPK/ERK kinase (MEK). DC2.4 cells were pre-incubated with U0126 prior to egg stimulation, which abrogated phosphorylation of ERK as shown in Figure 53A,B after 5 minutes and 24 hours. ERK1/2 phosphorylation in egg-stimulated CD209a-expressing DC2.4 cells was also greatly decreased in the presence of EGTA (Fig. 53A), a calcium specific chelator, which is consistent with the notion that egg-specific ERK1/2 activation is CLR-dependent. Significantly, CD209a-expressing cells that were pre-incubated with U0126 failed to produce mature IL-1β protein after egg stimulation (Fig. 53B). This observation is supported by a dramatic decrease in egg-specific *Il1b* and *Il23a* transcript in U0126-treated CD209a-expressing DC2.4 cells (Fig. 53C,D). Taken together, these data demonstrate that CD209a-dependent ERK1/2 activation is necessary for DC production of the Th17-associated cytokines IL-1β and IL-23.

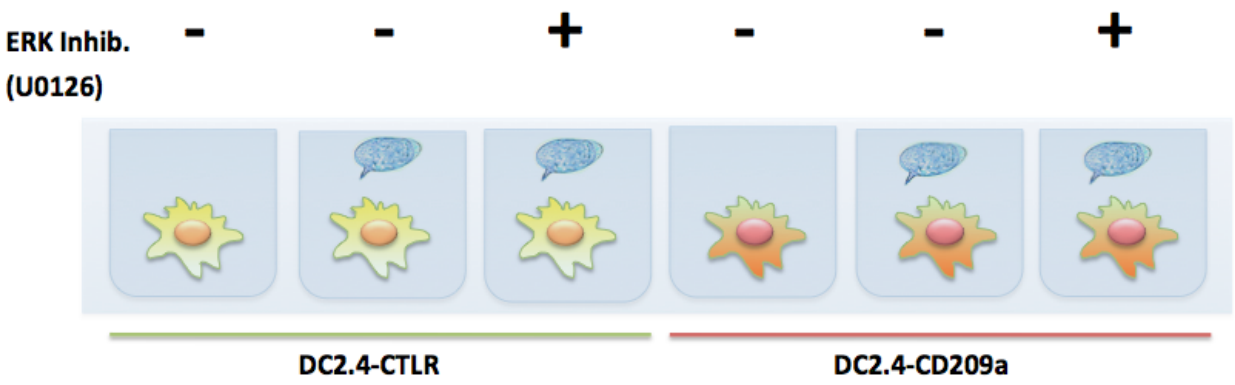

Figure 52. Schematic representation of ERK1/2 inhibition in DC2.4 cells. Stable BL/6 DC2.4 cell lines expressing CD209a or RFP (CTRL) were pretreated with the ERK MAP-kinase activation inhibitor U0126 (inhibitor of MEK) for 1 hour. Following inhibition pretreatment, DC2.4 cells were incubated ± schistosome eggs for 5 minutes or 24 hours. ERK phosphorylation and Th17-associated cytokines IL-1β and IL-23 were assessed by Western blot and/or qRT-PCR.

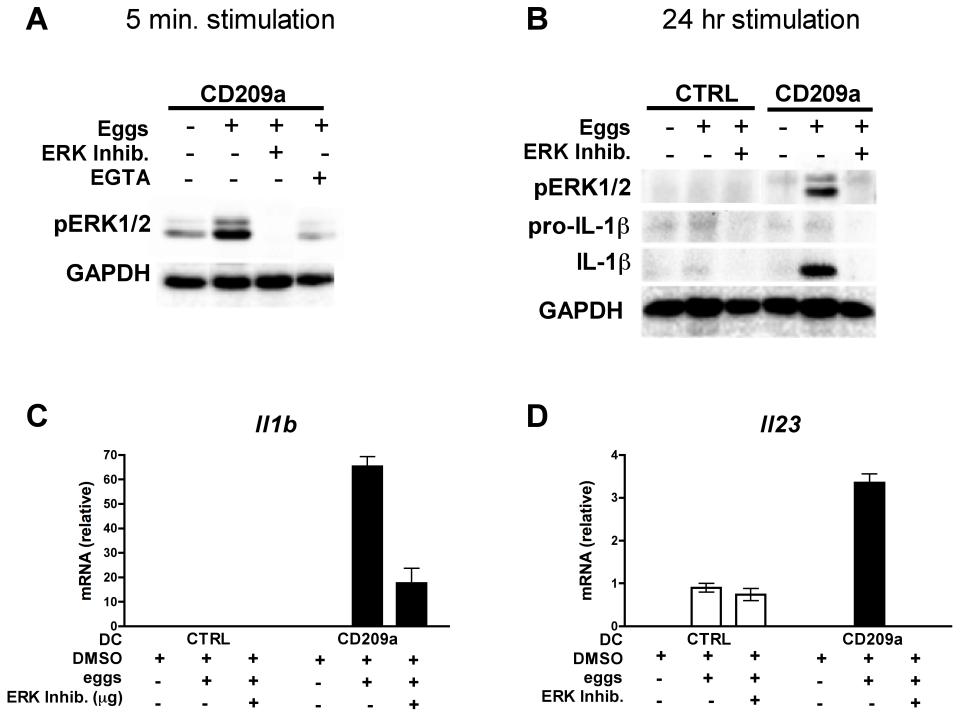

Figure 53. CD209a-dependent DC IL-1β and IL-23 production requires ERK1/2 activation. (A) CD209a-expressing DC2.4 cells were incubated with ERK1/2 activation inhibitor U0126 (inhibitor of MEK kinase) or EGTA calcium-specific chelator for 1 hour. DCs were stimulated with eggs for 5 minutes and phospho-ERK1/2 was assessed by Western blot. (B) U0126-treated vs. untreated CD209a-expressing DCs were additionally stimulated with eggs for 24 hours. Phospho-ERK1/2 and pro- vs. mature IL-1β protein were assessed by Western blot. CTRL DC2.4 cells were included as a negative control. (C-D) *Il1b* and *Il23a* transcript in U0126-treated vs. untreated CD209a-expressing and CTRL DC2.4 cell 24 hour cultures was additionally quantified by qRT-PCR.

SECTION III: DISCUSSION

Discussion

Overall summary of results

Innate immune cells are essential elements at the forefront of host defense. Assorted APC populations incessantly scan the surrounding biological environment for signs of infection and abnormality (Gordon, 2002; Akira et al., 2006; Takeuchi & Akira, 2010). DCs are powerful APCs that detect foreign Ag through a vast array of PRRs and induce the differentiation of naïve $CD4^+$ T cells into effector T cell populations. Diverse cellular, environmental, and genetic factors may influence DCs to bias Th cell differentiation towards Th1, Th2, Th17, or Treg cell lineages (Figdor et al., 2002; Villadangos & Schnorrer, 2007; Merad et al., 2013). In murine *S. mansoni* infection, the development of Th17 cell responses is detrimental to the host, rather than protective, and leads to severe pathology. Potent proinflammatory properties of IL-17 stimulate deleterious hepatic inflammation in response to highly immunogenic parasite Ags (Rutitzky et al., 2005; Rutitzky et al., 2008; Shainheit et al., 2008; Shainheit et al., 2011; Larkin et al., 2012). In the present investigation, we explored the role of innate immunity in the orchestration of Th17 cell responses during murine schistosomiasis. In consideration of the strong evidence that DCs are critical mediators of schistosome-specific Th differentiation (Shainheit et al., 2008, Phythian-Adams et al., 2010), we examined the genetic profile of DCs from mice that develop divergent Th responses in schistosome infection.

Comprehensive genetic profiling analyses exposed major differences in the expression of PRRs between CBA and BL/6 DCs. We found a strikingly higher (18X) expression of the CLR CD209a in CBA compared to BL/6 DCs. Aside from remarkable baseline differences in the expression, CD209a was significantly elevated at the site of severe hepatic pathology as well as in the spleen during pronounced splenomegaly in infected CBA mice, but not BL/6. While various APC subpopulations expressed CD209a, surface expression was particularly enhanced on DCs from CBA spleen, MLN, as well as liver granuloma cells. Furthermore, CD209a expression on CBA DCs, but not on B cells, granulocytes, or macrophages, was necessary for the induction of pathogenic $CD4^+$ Th17 cell responses to eggs *in vitro*, an observation that supports previous reports demonstrating that DCs induce schistosome-specific Th cell responses (Shainheit et al., 2008; Phythian-Adams et al., 2010). Also, CD209a was elevated on cDC subpopulations with strong Ag presentation capability, defined by markers including MHC Class II, CD8, CD80, and CD86, in infected CBA MLN (Villadangos & Schnorrer, 2007; Merad et al., 2013). Finally, CD209a surface expression was elevated on DCs from MOLF wild-derived mice which were previously demonstrated to develop severe schistosomiasis and elevated Th17 cell responses (Smith et al., 2011).

Subsequent functional analysis of CD209a expression on DCs revealed a role for CD209a in schistosome-specific Th17 cell development. Gene silencing of CD209a in CBA DCs, and over-expression of CD209a in BL/6 DCs, demonstrated that CD209a expression is necessary for DC production of IL-1β and IL-23 in response to live eggs as well as subsequent $Ror\gamma t^+$ Th17 cell development. Investigation of the signaling responses associated with DC CD209a expression revealed signaling patterns

reminiscent of human DC-SIGN signaling (Caparros, 2006; Gringhuis et al., 2009), including the activation of SRC, RAF-1, and ERK1/2 MAP kinases, but not p38 or JNK. In agreement with this observation, CBA DCs, but not BL/6 DCs, elicited comparable IL-12/23p40 and phospho-ERK1/2 responses to both the DC-SIGN ligand Mannan (Geijtenbeek et al., 2000a; Hong et al., 2007) and schistosome eggs, whereas there was no difference in the response to LPS. Finally, CD209a expression on CBA DCs and DC2.4 cells was required for ERK1/2-dependent IL-1β and IL-23 production in response to eggs. These data reveal a novel ERK-dependent CLR-mediated mechanism that leads to the development of Th17 cell responses to schistosome Ag (Fig. 54).

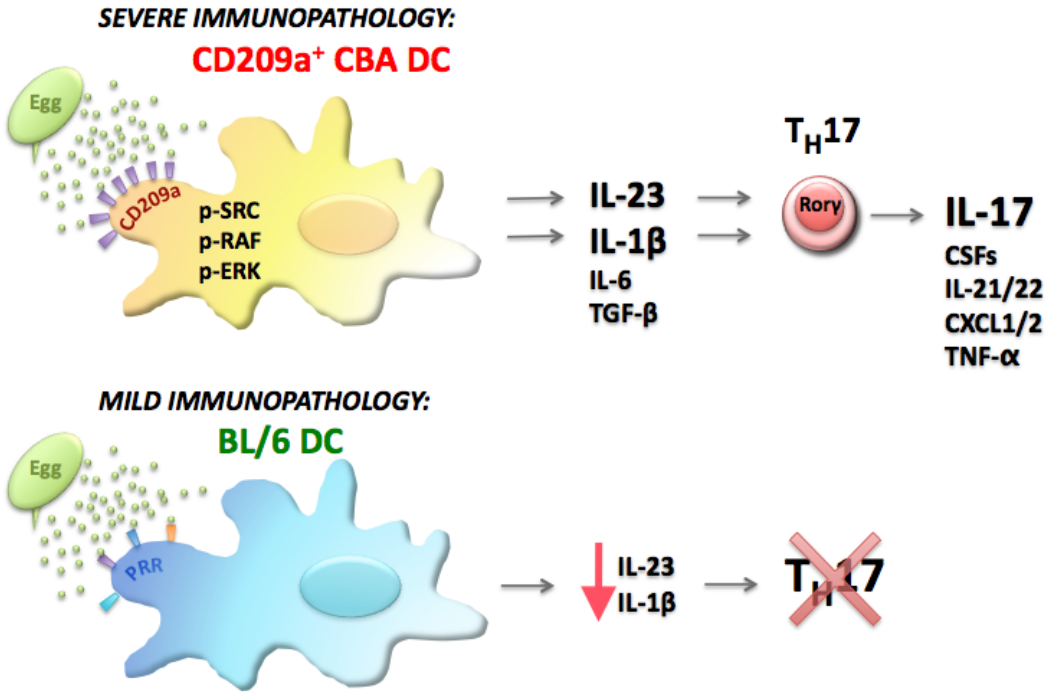

Figure 54. Role of DC CD209a expression in egg-induced pathogenic Th17 cell responses. In severe schistosomiasis, elevated expression of CD209a on CBA DCs is required for MAP-kinase-dependent IL-23 and IL-1β DC cytokine production leading to subsequent Th17 cell development and the secretion of the highly pro-inflammatory cytokine IL-17. In mild schistosomiasis, CD209a expression is negligible on BL/6 DCs, which express few PRRs and fail to elicit schistosome-specific MAP-kinase activation or Th17-associated cytokine responses.

The CD209 gene family

CD209 gene family members have been documented in humans and non-human primates as well as an array of other species in the animal kingdom including rats, mice, horses, cattle, bats, birds, squirrels, and dogs. Evolutionary studies suggest that multiple *CD209* genes originated following duplication events in early anthropoid ancestors 40 million years ago (Ortiz et al., 2008). In humans, the *CD209* genes code for DC-SIGN and the related protein L-SIGN (DC-SIGNR, *Cd209l*), which both cluster on chromosome 19. In primates, both DC-SIGN and L-SIGN are expressed by phagocytic cells and play a pivotal role in host defense responses. L-SIGN is more abundantly expressed in the lymph node and liver sinus endothelia, and thus named "L"-SIGN. Other non-human primates also possess a third *CD209* family member, CD209L2 (*Cd209l2)*, a *CD209* homologue believed to be deleted in humans due to selective pressure (Geijtenbeek et al., 2000a; Bashirova et al., 2003; Ortiz et al., 2008; Tanne et al., 2009). While little is known of the function of *CD209* genes in most other species, sequence variation provides insight into the evolutionary history of this CLR gene family.

Biochemical properties of the CD209 family

CD209 family members are Type II transmembrane proteins with several conserved features that have been retained across species; namely, an extracellular carbohydrate binding domain (CBD), a neck region composed of repeating sequences,

a calcium binding domain, and various intracellular tail motifs that are believed to be important for receptor internalization and signaling (Figdor et al., 2002; Powlesland et al., 2006; Ortiz et al., 2008). Of particular interest are the conserved 23-amino acid sequence repeats of CD209 family neck regions. In primates, *CD209* genes encode for 5 to 9 neck sequence repeats. Human DC-SIGN, first described in Curtis et al., 1992, as a HIV-1 gp120-binding lectin, has 7.5 sequence repeats, which were demonstrated to be critical for receptor tetramerization by hydrodynamic analyses (Curtis et al., 1992; Ortiz et al., 2008; Serrano-Gómez et al., 2008). Sequence analysis suggests that the DC-SIGN neck region adopts an α–helical confirmation and forms coiled-coils upon oligomerization (Menon et al., 2009). Various studies have suggested that the length and sequence of the DC-SIGN and DC-SIGNR neck regions is important for efficient Ag binding, particularly in the context of HIV-1 (Menon et al., 2009; Zhu D. et al., 2010; da Silva et al., 2011; Boily-Larouche et al., 2012).

To this date, eight murine CD209 homologues, CD209a – g plus one pseudogene, have been characterized, all of which cluster closely together on chromosome 8 proximal to the genes for CD23 and LSECtin (Park et al., 2001; Powlesland et al., 2006) (Fig. 55). Interestingly, outside of primate *CD209* genes, murine *CD209b* and *CD209c* are the only other CD209 genes in the animal kingdom known to contain more than one 23-amino acid repeat in the neck region. Other CD209 genes, including murine *CD209a*, code for one conserved 23-amino acid neck sequence (Park et al., 2001; Powlesland et al., 2006; Ortiz et al., 2008). Both the varied number of neck sequence repeats, as well as the difference in *CD209* gene homologues in primates and mice, may be indicative of selective pressure that resulted

from the necessity to recognize PAMPs, cell surface antigens, or commensal motifs. Thus, the function of CD209 homologues in diverse species may highly contrast the current functional paradigm of human DC-SIGN or DC-SIGNR (Koppel et al., 2005; Ortiz et al., 2008).

Sequence analysis of human and murine CD209 carbohydrate binding domains (CBDs) indeed suggests that these gene families followed disparate evolutionary paths in diverse species (Powlesland et al., 2006; Ortiz et al., 2008). Interestingly, the CBD sequences of human DC-SIGN and DC-SIGNR have greater homology to each other than to any of the murine CD209 homologues. Based on monosaccharide binding assays, the CBDs of murine CD209b, CD209d, and CD209g are thought to be the most similar to the human DC-SIGN CBD, which has a higher relative affinity for fucose compared to mannose (Powlesland et al., 2006). Nonetheless, DC-SIGN was shown to recognize pathogen glycans composed of various different carbohydrate constituents including the fucose-based Le^X and mannose-based ManLam (van Die et al., 2003; Meyer et al., 2005; van Liempt et al., 2006a; Gringhuis et al., 2009). Numerous efforts to block DC-SIGN CBD-binding to HIV-1 gp120 with mannose- and fucose-based glycomimetic molecules have been documented (Timpano et al., 2008; Varga et al., 2013; Tomašić et al., 2014). More specifically, binding of oligosaccharides by DC-SIGN is coordinated by the side chains of various amino acids in the CBD including Val^{351}, Ser^{360}, Glu^{358}, and the aromatic ring of Phe^{313}. Such interactions are stabilized by calcium ions that bind both within the primary CBD and at a proximal Ca^{2+} ion-binding domain. Depending on the ligand, biochemical glycan-CBD interactions may differ slightly (Feinberg et al., 2001; Meyer et al., 2005).

In terms of functional mammalian glycan-binding capacity, the CBD of murine CD209d, which was demonstrated to have the highest relative affinity for fucose- and GalNAc-containing glycan Ags, most strongly resembled the preferential glycan-binding properties of DC-SIGN in a mammalian glycan binding array analysis (Powlesland et al., 2006; van Liempt et al., 2006a). In contrast, Powlesland et al. demonstrated that CD209a and CD209f have a higher relative affinity for mannose- compared to fucose-containing mammalian glycans, thus suggesting binding capacities that are more functionally comparable to DC-SIGNR (Koppel et al., 2005; Powlesland et al., 2006). Regardless of these observations, little is known of the parasite Ag-binding capacity of murine CD209 homologues. While CD209b was shown to recognize soluble schistosome egg Ags *in vitro*, it fails to play a role in the development of pathology during *S. mansoni* infection *in vivo* (Saunders et al., 2009). DC-SIGN expressed on DCs has been shown to bind to natural *S. mansoni* glycans Le^X and pseudo-Le^Y (van Die et al., 2003; Meyer et al., 2005). Investigation of the affinity of the CD209a CBD for natural schistosome glycans will be necessary to fully understand the *S. mansoni* egg glycan binding-capacity of this CLR.

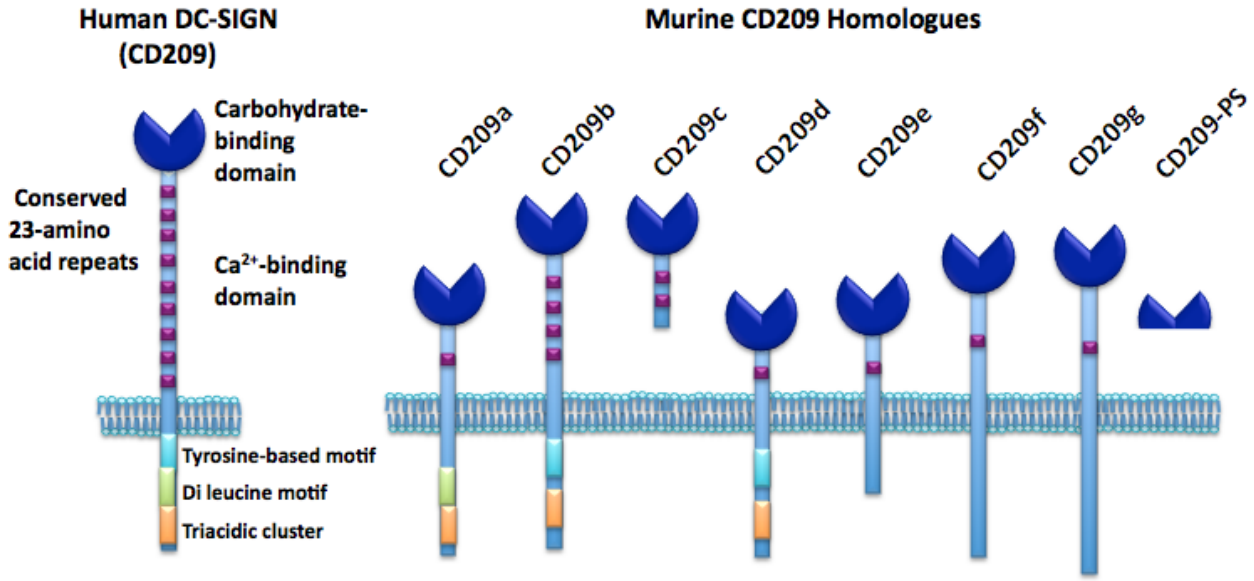

Figure 55. The murine CD209 homologues. Human DC-SIGN is a Type I CLR containing one extracellular CBD, a calcium binding domain, and a long neck region with conserved 23-amino acid repeating sequences. The DC-SIGN cytoplasmic tail contains a tyrosine-based motif of undescribed function, a di leucine motif, and a triacidic cluster all thought to be important for signaling and/or receptor-antigen internalization (Figdor et al., 2002; T. B. Geijtenbeek 2000a; Koppel et al., 2005). Eight murine homologues of human DC-SIGN have been identified including CD209a – g and one pseudogene (PS). Murine CD209 homologues each contain one extracellular CBD with the exception of CD209-PS, which contains only a truncated CBD. CD209b and CD209c contain four and two conserved 23-amino acid sequence repeats in the neck region, respectively. One sequence repeat is present in all other murine homologues excluding CD209-PS. Neck region length is believed to constitute to receptor oligomerization (Ortiz et al., 2008; Serrano-Gómez et al., 2008). Conserved cytoplasmic tail motifs present in human DC-SIGN have been identified in some of the murine homologues including CD209a, CD209b, and CD209d (Park et al., 2001; Koppel et al., 2005; Powlesland et al., 2006). Diagram adapted from Powlesland et al., 2006.

CD209 family function in host defense

In humans, CD209 is a molecule of widespread function within the realm of carbohydrate recognition. As reflected in the nomenclature, the natural ligand of DC-SIGN is intercellular adhesion molecule-3 (ICAM-3), one of several transmembrane glycoproteins and intercellular adhesion molecules that facilitate DC – T cell interactions (Geijtenbeek et al., 2000b). Indeed, DC-SIGN was named for its function in the facilitation of primary immune responses. DC-SIGN was demonstrated to bind with relatively high affinity to ICAM-3 on resting T cells, alongside other lower-affinity adhesion molecules such as LFA-1. Such interactions stabilize transient DC – T cell interactions at the immune synapse, promoting efficient TCR engagement (Geijtenbeek et al., 2000b). Currently, there is no evidence suggesting that murine CD209 homologues also bind adhesion molecules to promote cell-cell interactions during DC – T cell responses.

Besides its role in cell adhesion, human DC-SIGN is a promiscuous CLR capable of binding a variety of mannose- and fucose-containing bacterial and viral glycans while also mediating endocytosis and cytokine production (Meyer et al., 2005; van Liempt et al., 2007; Hong et al., 2007; Johnson et al., 2012). Before the name “DC-SIGN” was coined, it was originally discovered for its role as a membrane-associated HIV-1 glycoprotein gp120-binding lectin (Curtis et al., 1992). DC-SIGN is indeed the main CLR expressed by DCs that facilitates trans-infection of HIV into human $CD4^+$ lymphocytes (Geijtenbeek et al., 2000a; da Silva et al., 2011). Interestingly, polymorphisms in the *CD209* gene have been shown to effect the efficacy of HIV-1 virus transfer to $CD4^+$ T

cells in adults as well as from mother to child during vertical transmission (Selvaraj et al., 2009; da Silva et al., 2011; Boily-Larouche et al., 2012; da Silva et al., 2012). As DC-SIGN is a PRR for various bacterial and viral pathogens, human polymorphisms have additionally been linked to the susceptibility and development of disease during infection with dengue virus, *M. tuberculosis*, and invasive pulmonary aspergillosis (Selvaraj et al., 2009; K. Chang et al., 2012; Sainz et al., 2012; Alagarasu et al., 2013).

The murine DC-SIGN homologue, CD209a, was the first CD209 homologue originally discovered and termed “mouse DC-SIGN” (Park et al., 2001). Peptide sequence analysis has shown that CD209a retains many structural properties of human DC-SIGN including carbohydrate- and calcium-binding domains in the extracellular peptide sequence, a conserved neck region sequence, and tri-acidic cluster and dileucine motifs in the cytoplasmic tail (Park et al., 2001; Figdor et al., 2002; Powlesland et al., 2006). Such cytoplasmic tail motifs promote receptor internalization and may function in receptor-Ag uptake (Figdor et al., 2002). In contrast to DC-SIGN, there is no evidence to suggest that CD209a is capable of dimerization, which could potentially impact the capacity of this CLR to stably bind glycan Ag. We have shown that CD209a is highly expressed on the surface of $CD11c^+$ DCs and exhibits a pattern of surface localization, clustering, and internalization that is reminiscent of DC-SIGN (Neumann et al., 2008). On human DCs, DC-SIGN clusters and signals from lipid rafts within the plasma membrane (Caparros, 2006; Neumann et al., 2008). Additionally, DC-SIGN has been shown to internalize and target SEA to lysosomal compartments containing MHC class II molecules (van Liempt et al., 2007). Future analysis of the potential cellular components that contain CD209a, including the plasma membrane, endosomes, and

lipid rafts, will provide a greater understanding of the functional potential of this murine CLR, particularly in relation to schistosome Ag internalization and signaling capacity.

A current topic of great interest in the field of immunology is the diverse assortment of DC subpopulations and their function in disease, particularly in relation to Ag presentation and Th differentiation. Here we demonstrated that CD209a is highly expressed by DC subsets of powerful APC capacity in infected CBA compared to BL/6 mice, particularly on cDC subpopulations that are characterized by the expression of MHC Class II, CD80, and CD8 (Villadangos & Schnorrer, 2007; Merad et al., 2013). While little was known of specific CD209a function prior to this study, particularly in regards to schistosome Ag, a few reports have suggested that CD209a is a marker of a highly inflammatory APC subset (Cheong et al., 2010; Lu et al., 2013). Along these same lines, $CD209a^+$ monocyte-derived DC (Mo-DC) were shown to possess powerful Ag-presenting capability (Cheong et al., 2010). A more recent study on bacterial sepsis was the first to reveal that leukocyte cell-derived chemotaxin-2 interacts with CD209a on murine macrophages to promote endocytosis, bacterial killing, and cytokine production (Lu et al., 2013). Our data also suggest that $CD209a^+$ DCs are highly inflammatory DCs. Further in depth analysis of cell surface markers as well as DC subset-specific transcription factor expression will elucidate a more accurate characterization of $CD209a^+$ DCs. Additionally, functional analysis of individual $CD209a^+$ cDC subsets will pinpoint the particular cDC populations that most efficiently facilitate Th17 cell responses *in vitro* and *in vivo*. Without question, it is possible that CD209a marks a novel cDC subset.

CD209 family signaling properties

Signaling events associated with CD209a have not been characterized prior to this investigation; however, various human studies have documented MAP kinase activation downstream of DC-SIGN that leads to cytokine production in response to a variety of glycan Ags (Caparros, 2006; Gringhuis et al., 2007; Gringhuis et al., 2009; Johnson et al., 2012). MAP kinases are evolutionarily conserved signaling molecules essential for mediating rapid communication of extracellular signals to the nucleus during diverse cellular processes (Kolch, 2005; Wellbrock et al., 2004). Such rapid signaling cascades are crucial during PRR-mediated responses to pathogen and enable immediate defense mechanisms and cytokine production. In human DCs, Gringhuis et al. showed that in the presence of TLR4 stimulation, DC-SIGN ligation by ManLam stimulates the recruitment of a signalosome of scaffolding and signaling proteins that leads to MAP kinase activation, modification of NFκB, and IL-12p40, IL-10, IL-12p35, and IL-6 production. In contrast, DC-SIGN stimulation by the fucose-based glycan Le^X from *Helicobacter pylori* stimulated an alternate signaling pathway, excluding RAF-1 activation and leading to the production of IL-10 (Gringhuis et al., 2007; Gringhuis et al., 2009). Although such human studies have not specifically investigated the role of DC-SIGN signaling cascades in the development of Th17 cell responses, the p40 subunit of IL-12 is shared by the Th17-inducing DC cytokine IL-23 (Gee et al., 2009). Future in depth analysis of Th cell development following DC-SIGN stimulation, particularly in the absence of co-stimulation by other PRRs, may reveal a broader Th cell polarization capacity for DC-SIGN.

Various other reports have linked human DC-SIGN to the activation of MAP-kinase signaling. Specifically, Caparrós et al., 2006, revealed that ERK1/2 and PI3K, but not p38, are activated following engagement of human DC-SIGN leading to Th2-assocaited cytokine production. This study also demonstrated that DC-SIGN from lipid rafts co-precipitates with tyrosine kinases Lyn and Syk in human DCs (Caparros, 2006). A similar report documenting interactions between DC-SIGN and syncytial virus glycoprotein G also demonstrated ERK/1/2 stimulation post DC-SIGN ligation (Johnson et al., 2012). While MAP kinase activation has been reported following cross-linking of CD209b (SIGNR1) (Numazaki et al., 2009) as well as CD209d (SIGNR3) (Tanne et al., 2009), the signaling mechanisms critical for Th17 cell associated cytokine production downstream of murine DC-SIGN homologues, particularly in response to schistosome products, are largely unknown.

Our findings demonstrate that CD209a expression on DCs is necessary for egg-stimulated Th17 cell responses. Importantly, we also demonstrated that CD209a-dependent DC production of IL-1β and IL-23 requires ERK1/2 activation. To this date, few studies have described the role of ERK1/2 in Th17 cell development. Signaling through ERK1/2 was previously shown to promote IL-23p19 and IL-1β production leading to Th17 cell differentiation in a study of primary human fibroblasts (Zhu et al., 2012). A recent study also demonstrated that Th17 cell development, but not Treg cell development, fails to occur in the presence of ERK1/2 inhibitor (Liu et al., 2013). In particular, the pathogenicity of Th17-polarized cells in a model of colitis was significantly decreased following ERK1/2 inhibition (Liu et al., 2013).

More relevant to immunity in schistosome infection, ERK1/2 activation, in the

absence of significant JNK or p38 activation, has also been reported in response to lacto-*N*- fucopentaose III (LNFPIII) and ES62 from *Acanthocheilonema viteae*; however, these studies focus on the anti-inflammatory properties of helminth-related molecules and the induction of Th2 cell responses (Thomas et al., 2003; Kane et al., 2004; Goodridge et al., 2005; Carvalho et al., 2009; Harnett et al., 2010). Indeed, numerous studies have established the immunomodulatory Th2-promoting properties of helminth products that may signal through receptors such as human DC-SIGN and the MR (van Riet et al., 2007; Steinfelder et al., 2009; Carvalho et al., 2009; Everts et al., 2009; Harnett et al., 2010; Klaver et al., 2013). Prior to this study, the only CLRs known to facilitate Th17 cell responses were Dectin-1 and Dectin-2 which signal via Syk kinase and CARD9 in response to fungal Ags resulting in IL-23 production (Robinson et al., 2006; LeibundGut-Landmann et al., 2007; Gringhuis et al., 2012).

In the current study, SRC kinase was also associated with the development of pathogenic egg-stimulated cytokine responses. SRC Family Kinases (SFKs) are a family of SRC Homology Domain (SH)-containing signaling proteins that play crucial roles in the regulation of cell proliferation, metabolism, and survival (Brown & Cooper, 1996; Thomas & Brugge, 1997; Boggon & Eck, 2004); hence, dysregulation of SFKs has been linked to cancer (Creedon & Brunton, 2012; Liu et al., 2013; Gargalionis et al., 2014). Moreover, SFKs are important mediators of innate inflammatory responses in APCs, particularly in relation to PRR signaling mechanisms (Boggon & Eck, 2004; LeibundGut-Landmann et al., 2007; Byeon et al., 2012; Kuka et al., 2010). In DCs, SFK activation was shown to be necessary for TLR3- and TLR8-mediated IL-12 production and subsequent Th1 differentiation (Kuka et al., 2010). Significantly, Dectin-mediated

cytokine production is dependant on the activation of SFK, Syk, during infection with fungi and bacteria (LeibundGut-Landmann et al., 2007; Robinson et al., 2009; Gringhuis et al., 2012; Lin et al., 2013). In addition to Dectin family CLRs, CLEC-2, also signals through a Syk-dependent mechanism that involves a cytoplasmic YXXL-containing tyrosine-based motif (Fuller et al., 2007; Hughes et al., 2010; Séverin et al., 2011). Importantly, SRC family kinases have been associated with CD209 family signaling processes. Human DC-SIGN co-precipitates with the SFKs Lyn and Syk in lipid rafts following receptor ligation (Caparros, 2006). Additionally, crossing-linking of murine CD209b expressed on RAW264.7 macrophages induced SFK activation including Lyn, Hck, and Frg, which were similarly localized to CD209b-containing lipid rafts (Numazaki et al., 2009). A thorough biochemical analysis of CD209a cytoplasmic regions, as well as a broad investigation of SFKs in CD209a-expressing DCs, including Syk, Lyn, Fyn, Lck, and Frg activation, will elucidate the role of SFKs in CD209a-dependent inflammation to schistosome Ag.

In sum, severe pathology in murine schistosomiasis is dependent on $CD4^+$ Th17 cell responses, as seen in infected CBA mice. In the present study we demonstrate that pathogenic Th17 cell cytokine responses to live *S. mansoni* eggs are largely dependent on the expression of CD209a by DCs. In response to eggs, DCs elicit CD209a-dependent ERK1/2 activation, which leads to the production of IL-23 and IL-1β. Future work investigating the outcome of pathology in *S. mansoni*-infected CD209a deficient CBA mice will clarify the function of CD209a in murine schistosomiasis *in vivo.*

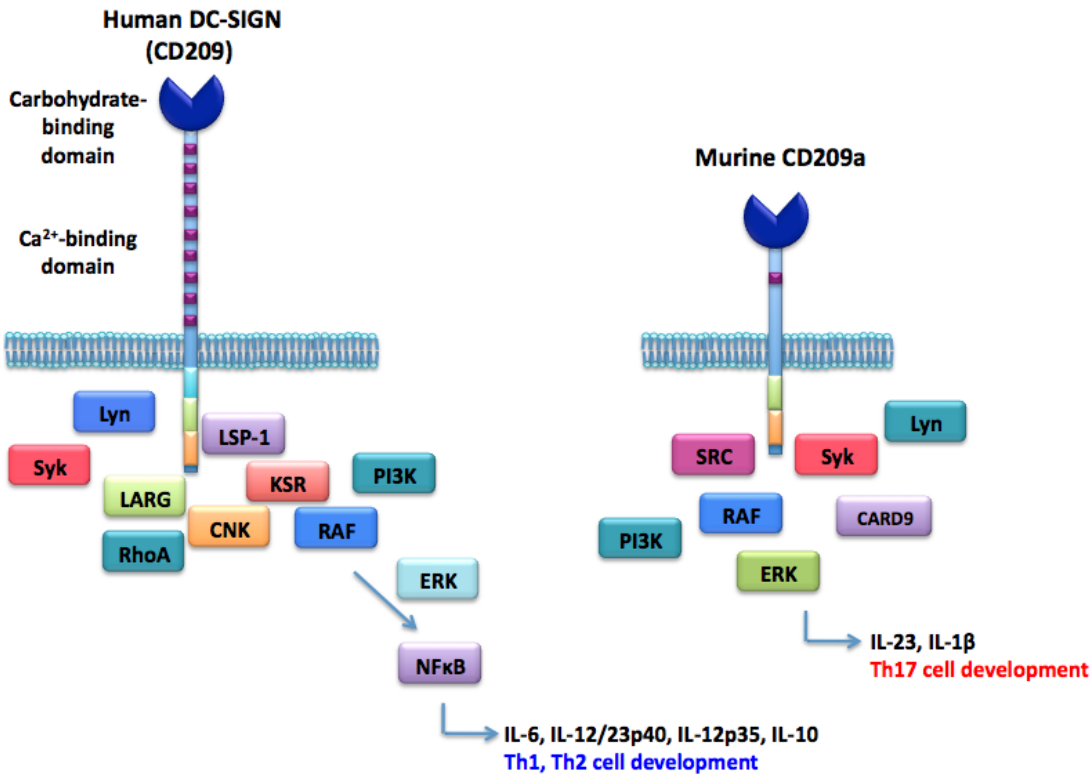

Figure 56. Signaling of human CD209 and murine CD209a. Signaling pathways in human DC-SIGN have been described following ligation by mannose- and fucose-containing Ag. A DC-SIGN signalosome including the F-actin binding protein LSP-1, scaffolding proteins CNK and KSR, the RhoA GTPase, and Rho guanine nucleotide-exchange factor, LARG, has been documented (Gringhuis et al., 2007, 2009). MAP-kinase activation following DC-SIGN engagement includes activation of RAF-1 leading to downstream ERK1/2-phosphorylation and the modification of NFκB, which is associated with the production of IL-6 IL-12/23p40, IL-12p35, and IL-10 production promoting Th1/Th2 responses (Gringhuis et al., 2007, 2009). DC-SIGN is also associated with PI3K activation and precipitates with Lyn and Syk in lipid rafts (Caparros, 2006). Presently, murine CD209a expression on CBA DCs was associated with SRC, RAF-1, and ERK1/2 activation following egg stimulation. ERK1/2 was critical for CD209a-dependent DC production of IL-23 and IL-1β. CLR-associated SFKs Lyn and Syk as well as CARD9 are potential CD209a signaling molecules that may mediate Th17 cell development (Caparros, 2006; LeibundGut-Landmann et al., 2007; Marakalala et al., 2011).

Significance and future directions of CD209

The present study is the first to discover a proinflammatory CLR-mediated pathway required for the development of pathogenic Th17 cells that mediate severe disease in murine schistosomiasis. Our findings reveal that DCs are the original source of a pathogenic response dictated by the CLR, CD209a, that results in T cell IL-17 production. Characterization of the CD209a pathway discloses a novel PRR mechanism facilitating IL-17 production in response to schistosome egg Ag. Currently, an equivalent human CLR that fuels Th17-mediated disease in schistosomiasis has not been described. This discovery prompts the investigation of human CLR function in the development of severe human schistosomiasis, as well as other Th17-mediated immune diseases. In particular, immediate investigation of human DC-SIGN as well as DC-SIGNR in severe schistosomiasis and the development of schistosome-specific Th17 cell responses is warranted. Considering the promiscuous binding behavior of human CD209 proteins, which recognize numerous molecules (Geijtenbeek et al., 2000a; van Die et al., 2003; Koppel et al., 2005; van Liempt et al., 2006b; Johnson et al., 2012), the discovery of a proinflammatory function for DC-SIGN could be relevant to various infectious and/or inflammatory diseases. Thus, our findings are a significant contribution to the current paradigm of CLR-mediated immunity in severe schistosomiasis; this is the first report implicating a CD209 homologue in Th17-mediated disease.

Importantly, DC-SIGN is a documented PRR for schistosome Ag (Meyer et al., 2005; van Die et al., 2003); as such, the role of DC-SIGN in human schistosomiasis is

already a topic of considerable importance in regions of Africa endemic for *S. mansoni* and *S. heamatobium*. Analysis of DC-SIGN surface expression by APC subpopulations in the peripheral blood of infected individuals is being investigated by collaborators in Senegal. Following our report on CD209a, investigation of other human CLRs in human schistosomiasis, besides DC-SIGN, will likely be pursued as well. Indeed, of all PRRs, CLRs are the family recognized for their capacity to induce Th17 cell responses; namely, the Dectins and CD209a (Robinson et al., 2009; Marakalala et al., 2011; Gringhuis et al., 2012; Ponichtera et al., 2014). Moreover, our present findings also warrant the investigation of DCs in severe human schistosomiasis, as well as DC MAP-kinase activation in the development of Th17-mediated disease. Overall, discovery of the CD209a proinflammatory pathway uncovers new candidate molecules for research and drug development.

An overarching problem in human schistosomiasis is the lack of beneficial treatment for severe liver disease in 10% of infected individuals. PZQ eliminates the helminth, but does not readily alleviate severe hepatic granulomatous inflammation (Hotez et al., 2010). Our classification of a DC-mediated inflammatory pathway discloses relevant targets for the development of novel therapeutic approaches that could aid in alleviation of schistosome egg Ag-stimulated granulomatous inflammation. In our system, CD209a-expressing DCs were the original source of pathogenic cytokine responses; thus elimination of this pathogenic DC subset during severe hepatic schistosomiasis could block Th17-mediated disease progression. A CD209a-specific antibody-drug conjugate of cytotoxic nature could be utilized to specifically target and kill CD209a^{+} DCs, thus eradicating the source of Th17 cell induction. Such therapy in

combination with PZQ could be beneficial for preventing advanced liver fibrosis and morbidity.

Along these lines, cytokines and signaling molecules in the CD209a pathway are potential targets for therapeutic intervention of Th17-mediated disease in schistosomiasis. Besides the obvious approach to neutralize IL-23, IL-1β, and IL-17, the inhibition of ERK1/2 activation is another prospect. In our system, ERK1/2 was required for CD209a-dependent DC pro-inflammatory cytokine production. An ERK1/2 inhibitor that is either targeted to liver tissue or specifically to $CD209a^+$ DCs is a possible therapeutic approach to block the development of proinflammatory cytokine production at the site of schistosome eggs. Further investigation of the CD209a pathway will reveal additional potential drug targets. Of interest is SRC kinase, which was associated with CD209a expression on DCs. SFKs mediate Th17 cell development following Dectin stimulation (Robinson et al., 2009; Marakalala et al., 2011; Lin et al., 2013); thus, future investigation will determine the function of SFKs in the CD209a pathway. Additional assessment of molecules associated with the CD209a cytoplasmic tail is necessary to fully understand the pathway.

In continuation of the present study, of great interest is the *in vivo* role of CD209a. To determine the contribution of CD209a in outcome of disease in schistosomiasis, CBA mice deficient for CD209a will be assessed for hepatic granulomatous pathology. Th1, Th2, and Th17 cytokine production by MLNCs, splenocytes, and liver GCs from infected CD209a-deficient mice will be analyzed. A CD11c-specific conditional CD209a knockout (KO) mouse will be utilized to assess the requirement of CD209a on DCs in the development of severe Th17-mediated disease.

Finally, to further explore the pathogenic potential of $CD209a^{+}$ DCs, CBA CD209a-expressing DCs, or CBA CD209a KO DCs, will be adoptively transferred into infected $H\text{-}2^{K}$ congenic BL/6 mice for assessment of liver pathology. A transgenic DC-SIGN-expressing mouse is also of great interest.

Lastly, to explore the schistosome glycan-binding properties of CD209a, a recombinant CD209a protein containing the extracellular CBD, Ca^{2+} binding domain, and neck region will be tested in a glycan array encompassing 100 natural schistosome egg glycans. High-affinity ligands will be functionally tested for stimulatory capacity in DC and DC – T cell co-cultures. Identification of functional schistosome ligands for CD209a will contribute to our understanding of CD209a binding properties and serve as a useful tool for in investigation CD209a-specific inflammatory pathways.

In conclusion, schistosomiasis is a serious, potentially fatal parasitic disease that affects 200,000,000 individuals around the globe, leading to 200,000 deaths annually (Fallon, 2000; Pearce & MacDonald, 2002; Larkin et al., 2012). The present study identified a CLR-mediated pathway necessary for induction of pathogenic Th17 cells associated with severe disease in humans and murine schistosomiasis (Larkin et al., 2012; Mbow et al., 2013). The discovery of CD209a is a major contribution to our understanding of CLR-mediated disease in severe schistosomiasis and discloses a novel mechanism for the development of Th17-mediated disease.

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