THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

Investigating the Role of C-type Lectin Receptor Dectin-2 in Schistosome Immunopathology

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A thesis
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ABSTRACT

Parasitic flatworm *Schistosoma mansoni* infects avian and mammalian hosts and results in schistosomiasis. Adult worms lay eggs in the bloodstream, and most eggs are excreted through host fecal matter. A fraction of the eggs fails to pass through and wedge in liver and intestinal tissue. The presence of dying eggs and egg antigens results in a CD4 T cell-mediated inflammatory response. In mild pathology, the cytokine environment is Th2-directed, while in severe pathology the Th1 and Th17 cytokine environments dominate. We and others have shown that IL-1β expression leads to severe pathology. Major C-type lectin receptors CD209a, Dectin-2, and Mincle are crucial for egg-mediated IL-1β production in dendritic cells (DCs) and macrophages. Although inflammasomes are known to be important in egg-mediated IL-1β production, it has remained unclear whether the C-type lectin receptors induce IL-1β production via inflammasome activation. Our findings establish Dectin-2 as a central inducer of inflammasome and immunopathology in murine schistosomiasis. Understanding pathways leading to inflammation can ultimately lead to the identification of novel targets for therapeutic intervention.

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Chapter 1

Background and Introduction

Schistosomiasis, a disease caused by the parasitic flatworm *Schistosoma mansoni* (*S. mansoni*), occurs due to host contact with fresh water containing cercariae (larval form) which penetrate avian or mammalian skin and allow adult worms to enter the vasculature, migrate to the portal system, and lay eggs[1]. Cercariae exit snail intermediate hosts upon sunlight contact with the freshwater environment. This parasite affects more than 250 million people globally, making it the second most prevalent tropical parasitic disease with 200,000 annual deaths[2]. Praziquantel is the only licensed drug for treatment, but it only targets adult worms and some schistosome strains have demonstrated resistance[3]. No vaccine exists with a high reinfection rate, and some hybrids of schistosome strains have been discovered in Europe[4].

Inflammatory Responses during *S. mansoni* Infection

S. mansoni infection is characterized by proinflammatory responses against egg antigens trapped in various organs such as the liver and intestines that lead to granuloma development, portal hypertension, splenomegaly, hepatic fibrosis and fatal gastrointestinal hemorrhage[5]. The majority of schistosome infections follow mild pathology and a Th2 dominant cytokine environment after an initial Th1 response, but 5-10% of cases exhibit severe pathology with a Th1 and Th17-directed cytokine environment associated with an increased fatality rate[6,7,8]. Our lab uses the two mouse models for mild and severe pathology, C57BL/6 (BL/6) and CBA respectively, which mirror what we see in human patients[9,10]. BL/6 mice have a Th2-dominated cytokine environment that regulates the Th1/Th17 pro-inflammatory

response, allowing for a stronger anti-inflammatory response[8,9,10,11]. Th2 cytokines include interleukin (IL)-4, IL-5, IL-10, and IL-13 which are associated with resolving inflammation and repair, promoting immunoglobulin E (IgE) and eosinophilia activity[12]. While IL-10-producing Th17 cells serve a regulatory role, IL-1β and IL-23 induce pathogenic Th17 cells[13]. CBA mice have a Th17-dominated cytokine environment, facilitating a strong pro-inflammatory response associated with inflammation and tissue damage[9,10,11]. The molecular mechanisms responsible for perpetual inflammation during schistosome infection are not understood. However, we and others have shown that schistosome eggs induce proinflammatory cytokine IL-1β production in dendritic cells (DCs) in a phagocytosis-independent manner, suggesting that egg antigens activate cell surface receptors[10,14,15].

C-Type Lectin Receptors and Schistosome Immunopathology

C-type lectin receptors (CLRs), a type of pattern recognition receptor (PRR) that recognizes carbohydrates such as those released from schistosome eggs, help to regulate gene expression in response to specific pathogens by inducing gene expression directly, or by modifying TLR signaling[8,16]. Macrophage-inducible C-type lectin (Mincle) and DC-associated C-type lectin 2 (Dectin-2) (CLEC6A) non-covalently associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules such as Fc receptor γ-chain (FcRγ) to induce downstream signaling[8,10,14,16,17]. This signaling primarily initiates or modifies NF-κB activity. Ligand binding facilitates adaptor protein ITAM phosphorylation and subsequent tyrosine kinase (Syk) recruitment and downstream signaling that engenders one of the previously described cytokine environments[8,16,17].

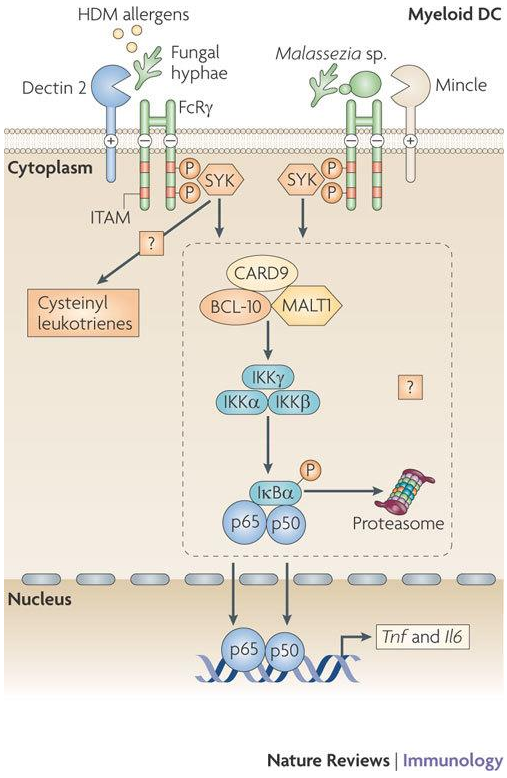


Figure 1. Ligands bind Dectin-2 and Mincle Associated with FcRγ and induce ITAM phosphorylation and Syk recruitment which leads to changes in gene expression and cytokine signaling8. Figure from Pearce et al. Nature Reviews Immunology 2(7):499-511.

The differences in dendritic CLR expression level may help to explain the deviation between these two pathologies. CBA mice DCs display markedly increased CD209a (SIGNR5, a homolog of human DC-SIGN) on their surface which senses schistosome egg glycans (carbohydrates)[17]. CD209a inhibition or mice with both copies of the CD209a gene knocked out (CD209a -/-) demonstrate this receptor's instrumental importance in proinflammatory Th17 cell development and immunopathology[14]. Moreover, schistosome-infected CD209a -/- mice show pathology similar to low pathology BL/6 mice. Related CLRs Dectin-2 and Mincle initiate schistosome egg-induced IL-1β and IL-23 while CD209a augments the production of IL-1β and IL-23 through sustained Raf-1 activation[14]. Our lab and others have shown that Dectin-2 and Mincle initiate an FcRγ-dependent signaling cascade involving Syk in murine models[14,15]. These CLRs synergize with CD209a to promote the development of Th17 cell-mediated

immunopathology and result in severe parasitic disease[14]. Additionally, murine Dectin-2 -/- bone marrow-derived dendritic cells (BMDCs) exhibit significantly decreased IL-1β production in BMDCs[14].

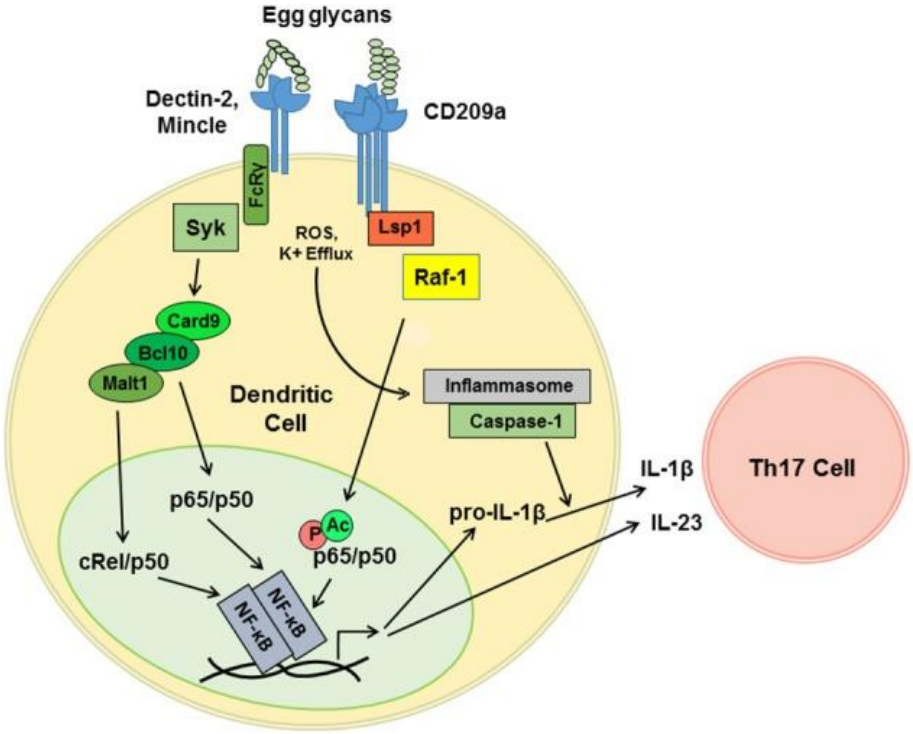


Figure 2. CD209a and Dectin-2/Mincle C-type lectin receptors on murine antigen presenting cells (APCs) synergize to promote Th17 cell-mediated response to schistosome egg glycans through distinct signaling pathways14. Figure from Kalantari et al. Cell reports 22(5):1288-1300.

Inflammasome Pathway in the context of *S. mansoni* Infection

Many studies have shown that schistosome egg-mediated IL-1β production is dependent on various inflammasomes such as NLRP3, AIM2, and NLRP6[18,19,20]. While egg-mediated IL-1β production via inflammasome activation is well established in the field, we do not know which cell surface receptors get activated and subsequently trigger inflammation via inflammasome activation. Inflammasomes are multiprotein cytosolic complexes comprised of a sensor protein and apoptosis-associated speck protein containing a CARD (ASC) adaptor protein which recruits pro-caspase-1 and induces inflammation. These

complexes form in response to stress or pathogen-derived signaling and oligomerization of pro-caspase-1 protein induces caspase-1-mediated pro-inflammatory responses including the secretion of IL-1β and IL-18[21,22,23]. Sensor protein nucleotide-binding domain and leucine-rich repeat-containing receptor (NOD-like receptor) (NLR) contains a signaling domain that allows for caspase-1 recruitment, a leucine-rich repeat (LRR) domain (regulates ligand binding), and a nucleotide-binding domain (NBD). AIM2-like receptor (ALR) can nucleate inflammasome assembly[24].

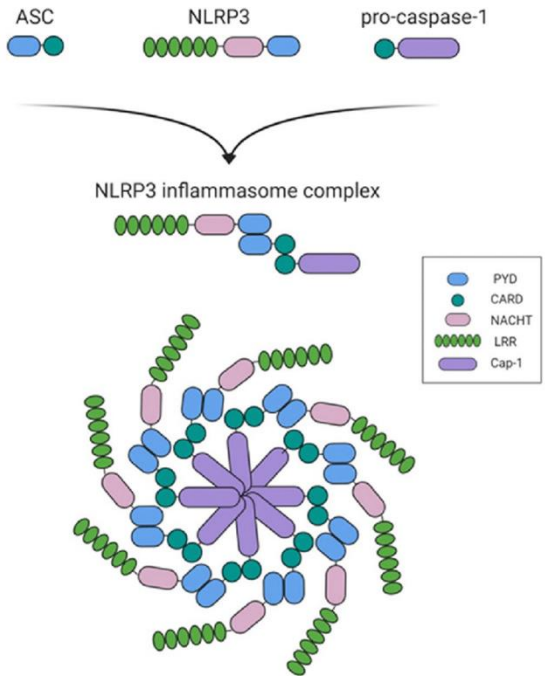


Figure 3. . NLRP3 inflammasome components and structure25. Figure from Seok et al. Archives of pharmacal research 44(1):16–35.

Various PRRs facilitate NLRP3 activation typically through sensing pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS). Hematopoietic cells such as macrophages express NLRP3. Their transcription and activity depend on two different signals. Toll-like receptor (TLR) activation and various NF-κB-activated ligands act as the first signal and induce pro-IL-1β transcription, the precursor to IL-1β[18,21, 22]. Many components can act as appropriate TLR agonists to induce this signaling pathway such as lipopolysaccharide (LPS) or Pam3CSK4, a synthetic

triacylated lipopeptide (LP). The second signal is stress- or pathogen-associated. Importantly, we and others have shown that live schistosome eggs contain both signals for IL-1β production[14,15].

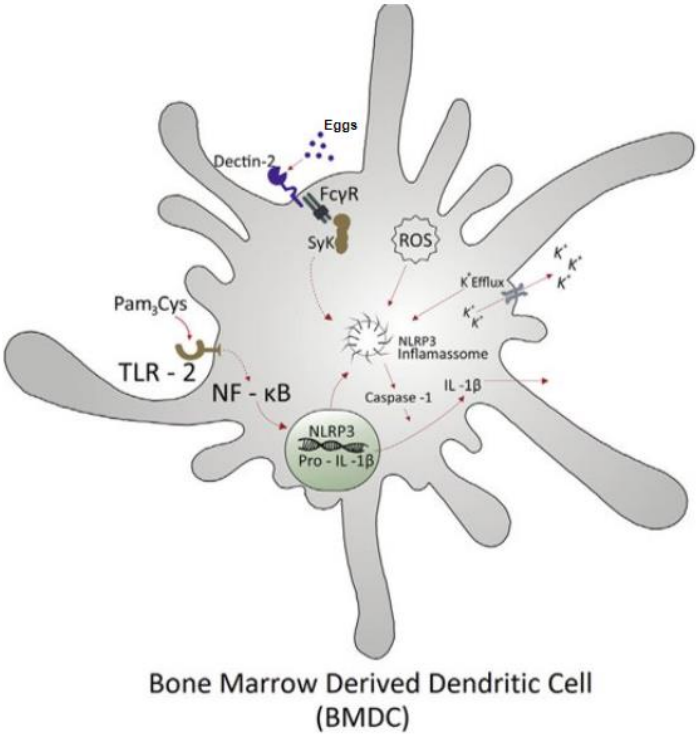


Figure 4. Pam3CSK4 (signal 1) and eggs (as signals 1 and 2) activate dendritic cell (DC) surface receptors and induce NLRP3 inflammasome assembly and activation producing caspase-1 to cleave pro-IL-1β into IL-1β and stimulate pro-inflammatory response[18]. Figure adapted from Sanches et al. Microbes and infection 22(10):534–539.**

While our lab uses live eggs in our experiments, others use soluble egg antigen (SEA) which binds the Dectin-2/FcRγ complex and activates Syk which engenders reactive oxygen species (ROS) and potassium efflux[14,15,18]. Since we know that major CLRs CD209a, Dectin-2, and Mincle are crucial for egg-mediated IL-1β production in DCs[14,] we hypothesized that these receptors induce inflammasome activation and IL-1β production upon schistosome egg stimulation.

Chapter 2

Materials and Methods

Optimization Experiments

To optimize the amount of Dectin-2 blocking antibody (MCA2415, BioRad) used to block this receptor on murine immortalized macrophages (MIMs) (BL/6 background) without causing cell death, these cells were cultured in complete culture medium (DMEM, 10% FBS, 0.1% PennStrep). $1x10^6$ cells were seeded into each well of a 6 well plate with complete culture medium for 24 hours. Cells were then stimulated with different concentrations (15 µg/mL, 10 µg/mL, 5 µg/mL, 2 µg/mL 1 µg/mL, 0 µg/mL (negative control)) of Dectin-2 blocking antibody for 1 hour. Cell viability was assessed by combining 10 µL of cell suspension with 10 µL of trypan blue dye and using cell counter. The same procedure was repeated in the context of schistosome eggs with two concentrations (5 µg/mL, 7.5 µg/mL) of Dectin-2 blocking antibody chosen based on viability from the first evaluation compared to negative control.. For each plate, wells were either stimulated with 1,000 schistosome eggs for 24 hours after 1 hour of Dectin-2 blocking antibody treatment (if applicable) or acted as negative control. Cell viability was assessed the same way as described previously.

Experimental Design

To conduct *in vitro* investigations of the effects of Dectin-2 inhibition on inflammasome activation, MIM were cultured in a complete culture medium (DMEM, 10% FBS, 0.1% PennStrep). $1x10^6$ cells were seeded in each well for 24 hours. Treatment groups were control, Dectin-2 blocking antibody, eggs, and Dectin-2 blocking antibody + eggs. Dectin-2 blocking antibody wells were stimulated for 1 hour prior to egg stimulation. Egg wells were stimulated 1000 *S. mansoni* eggs for 24 hours, while

the remaining two wells received no extra stimulation. Cells were then spun down at 4ºC and 1,000-1,200 rpm for 5 minutes and new media was added to each well following supernatant removal. Cells were removed from the plate and 10 uL of cell suspension was combined with 10 uL of trypan blue dye to assess cell viability in the cell counter. Cells were then spun down at 4ºC and 1,000-1,200 rpm for 5 minutes again to re-adhere to the plate. The media was removed and 100 µL of radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific) with 1:10 dithiothreitol (DTT) (Thermo Fisher Scientific) and 1:100 Halt Protease Inhibitor (Thermo Fisher Scientific) was added to each well with cells and incubated on ice for 30 minutes. Lysate was collected and stored. Levels of ASC (adaptor protein for all inflammasomes) and GAPDH (total protein) expression in cell lysates were analyzed using western blot. GAPDH was used as a loading control to consider the relative expression of ASC. The experiment was conducted twice on two different sets of samples.

Western Blotting

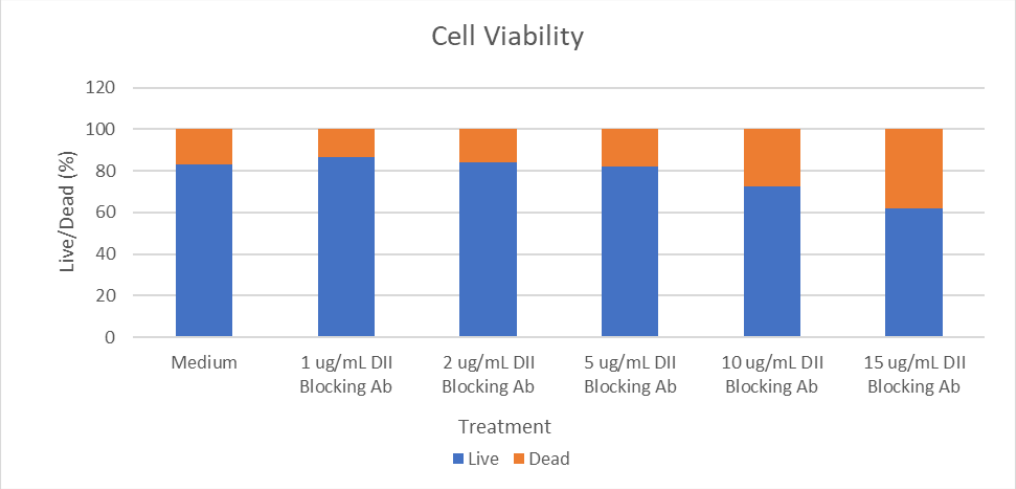
Previously described cell lysate samples were loaded and ran on native PAGE 4-20% Mini-Protean pre-cast gels and transferred onto nitrocellulose membrane according to manufacturer instructions (both BioRad). PBST solution (1000 mL PBS with 0.5 mL Tween20) with 5% BSA was used to block the membrane. The membrane was analyzed for antibodies specific to ASC (67824S, Cell Signaling Technology) and GAPDH (2118S, Cell Signaling Technology).

Chapter 3

Results

Dectin-2 Blocking Antibody Optimization in Murine Immortalize Macrophages

A)



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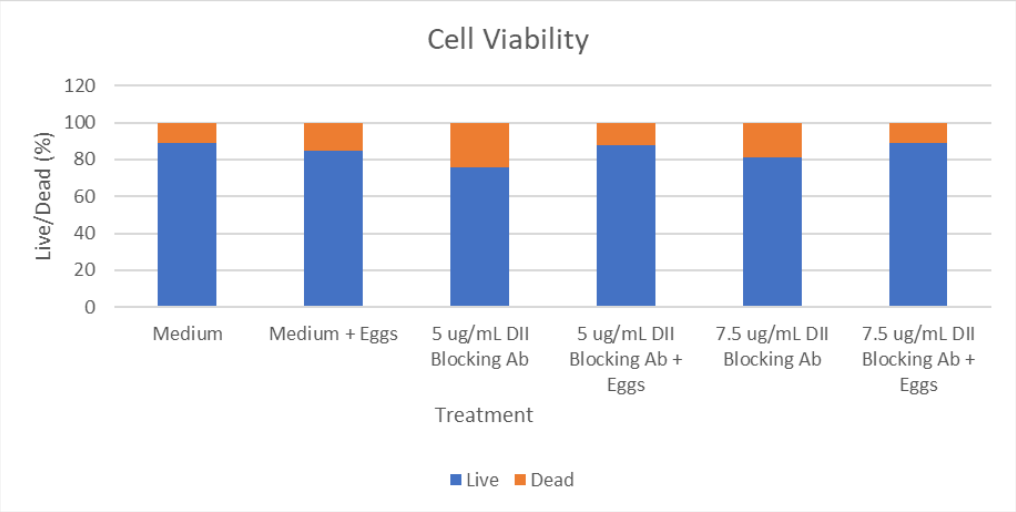


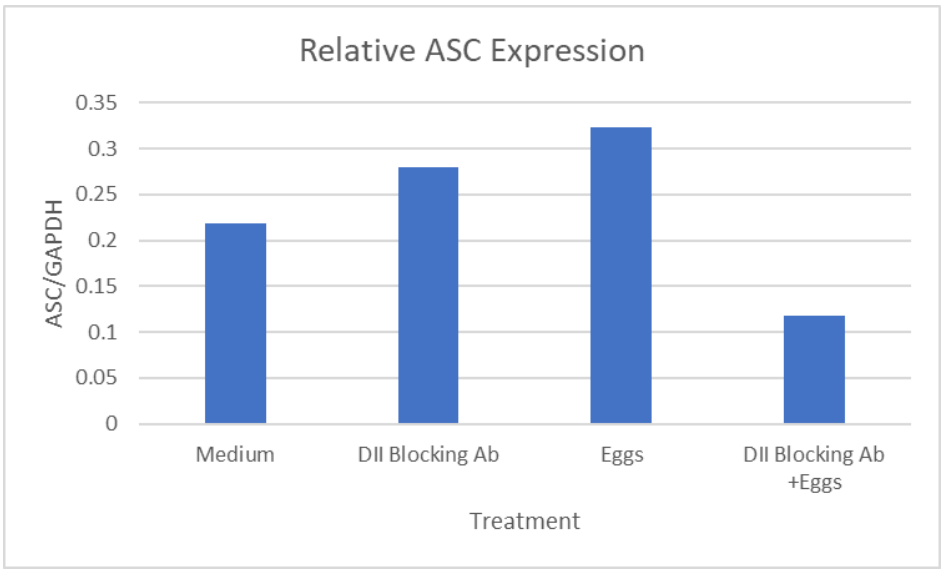
Figure 5. Anti-mouse Dectin-2 antibody concentration optimization as evaluated by murine immortalized macrophage (MIM) cell viability (live/dead counts) (A-B). (A) MIMs treated with various concentrations of Dectin-2 blocking antibody for 1 hour prior to live/dead staining. (B) MIMs treated with 2 different concentrations of Dectin-2 blocking antibody selected based on initial optimization assay for 1 hour followed by 1,000 schistosome eggs (if applicable) for 24 hours prior to live/dead staining

Optimization of Dectin-2 blocking antibody was conducted. MIMs were treated with medium (negative control), 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, or 15 µg/mL of Dectin-2 blocking antibody for 1 hour. The first three treatments (1 µg/mL, 2 µg/mL, and 5 µg/mL) of the blocking antibody demonstrated consistent live/dead counts as compared to control. However, 15 µg/mL, and to a lesser extent, 10 µg/mL exhibited an increase in cell death as compared with control (Figure 4A). It is unlikely that concentrations below 5 µg/mL will sufficiently block Dectin-2, so these concentrations were not considered further. Moreover, elevated cell death may skew results to be less reflective of Dectin-2-induced inflammasome activation as opposed to increased inflammatory signaling associated with cell death. The decrease in viable cells seen with 10 µg/mL of Dectin-2 blocking antibody treatment, but not with 5 µg/mL suggests that the optimal concentration may exist between these two values.

Therefore, MIMs were left untreated or treated with either 5 µg/mL, or 7.5 µg/mL of Dectin-2 blocking antibody for 1 hour followed by 24-hour incubation with or without 1,000 schistosome eggs. Live/dead counts across all considered groups were relatively similar. Therefore, 7.5 µg/mL of Dectin-2 blocking antibody was chosen as the concentration utilized in inflammasome experiments as it was the maximum amount of Dectin-2 blocking antibody that could be used without exacerbating cell death.

Blocking Dectin-2 Signaling Decreases ASC Expression in Murine Immortalized Macrophages

A)



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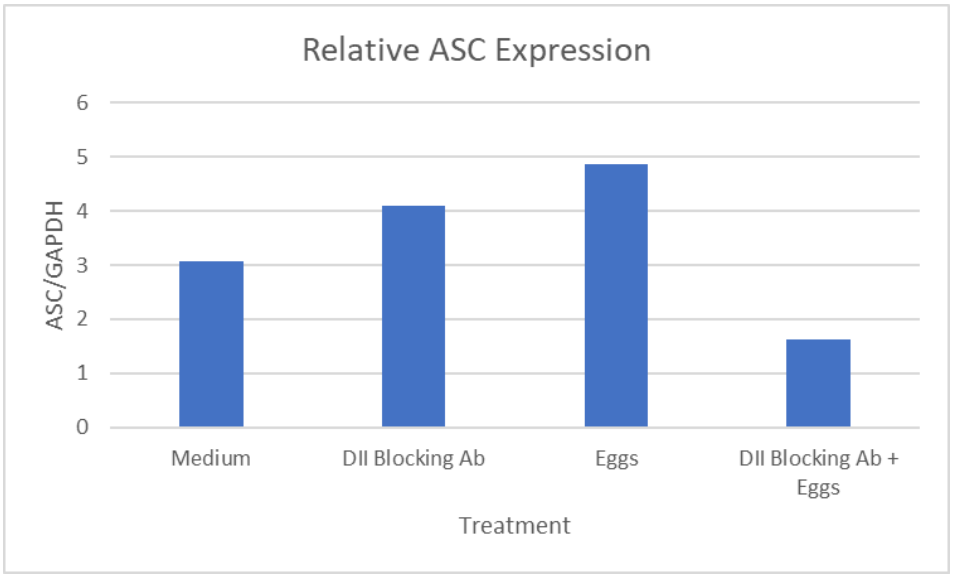

Figure 6. Blocking Dectin-2 receptor on murine immortalized macrophages (MIMs) reduces downstream ASC expression (A, B). Western blot analysis results expressed as ASC/GAPDH to quantify relative ASC expression for $1x10^6$ MIM cell lysates after treatment with 7.5 µg/mL of Dectin-2 blocking antibody followed by 1,000 schistosome eggs for 24 hours.

The role of Dectin-2 in stimulating inflammasome assembly and activation was investigated. MIMs were treated with 7.5 µg/mL Dectin-2 blocking antibody for 1 hour followed by eggs for 24 hours. ASC is an adaptor protein for most inflammasomes and therefore indicative of their activation. GAPDH was used as a loading control to consider the relative expression of ASC. Protein levels of ASC in cell lysates were measured using western blotting and divided by total protein as measured by GAPDH in the same way to quantify relative ASC expression. Cells stimulated with Dectin-2 blocking antibody prior to stimulation with schistosome eggs showed decreased relative ASC expression when compared to schistosome eggs only in both trials (Fig 4. A- B.).

Discussion

We and others have shown previously that schistosome egg-mediated IL-1β production leads to severe pathology. Moreover, our lab demonstrated CLRs CD209a, Dectin-2 and Mincle to be crucial for schistosome egg-mediated IL-1β production. However, the mechanisms facilitating this response remain uncharacterized. Previous studies have demonstrated the connection between inflammasome and IL-1β in response to schistosome egg antigen *in vivo* and *in vitro*[15]. Our lab has shown recently *in vitro* that schistosome eggs are upstream of inflammasome activation leading to IL-1β production in murine ASC -/- BMDCs. Specifically, when inflammasome assembly was impaired by eliminating ASC expression, IL-1β production was also shown to be inhibited when cells were stimulated with eggs and nigericin (positive control)[26].

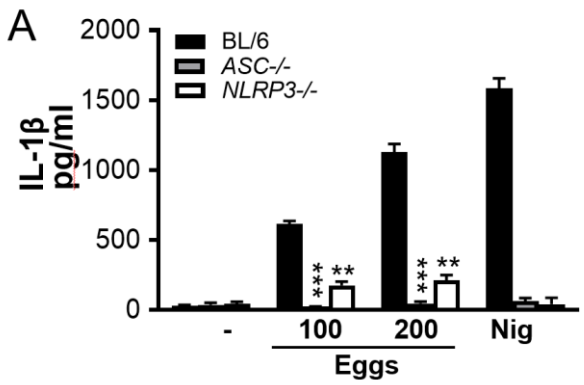

Figure 7. Bone marrow-derived dendritic cells (BMDCs) from BL/6, ASC-/- and NLRP3-/- mice were cultured for 24 hours with the indicated number of live (or no) eggs, or lipopolysaccharide (LPS) plus Nigericin (Nig). IL-1β in supernatants was measured by enzyme-linked immunosorbent assay (ELISA)[26].

This thesis investigated the role of CLR Dectin-2 in inflammasome activation by treating MIMs with Dectin-2 blocking antibody and assessing ASC protein expression in response to schistosome egg stimulation using western blot. The results demonstrated that treatment with Dectin-2 blocking antibody prior to stimulation with schistosome eggs reduces ASC expression, which suggests that Dectin-2 is upstream of inflammasome activation during *S. mansoni* infection. To further corroborate this relationship, Dectin-2 -/- mice should be infected with 80-85 *S. mansoni* cercariae through intraperitoneal injection (i.p.) as described in previous protocols or tail immersions as a more pathologically relevant model. We expect to see decreased inflammation and pathology in the Dectin-2 -/- model as compared with control. However, while the relationship between Dectin-2 receptor and subsequent inflammatory pathways can be clarified with knockout models, the potential of blocking antibodies as a therapeutic intervention is more clinically relevant. Therefore, it would be useful to repeat the experiment described in this thesis by treating BMDCs with Dectin-2 blocking antibody and examine downstream ASC expression through western blot. Furthermore, potential reduction in IL-1β in the supernatant should be evaluated using Enzyme-Linked Immunosorbent Assay (ELISA) to confirm this proinflammatory pathway.

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**Source image in Figure 4 modified by Maria Colon to state “eggs” over initial label “SEA” to adjust figure applicability to lab techniques