INVESTIGATING THE ROLE OF ACTIVATION-INDUCED CYTIDINE DEAMINASE IN *CHLAMYDIA* PATHOGENESIS

by

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Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

Dedicated to my loving family for their endless support, and especially my Mom & Dad for their unconditional love.

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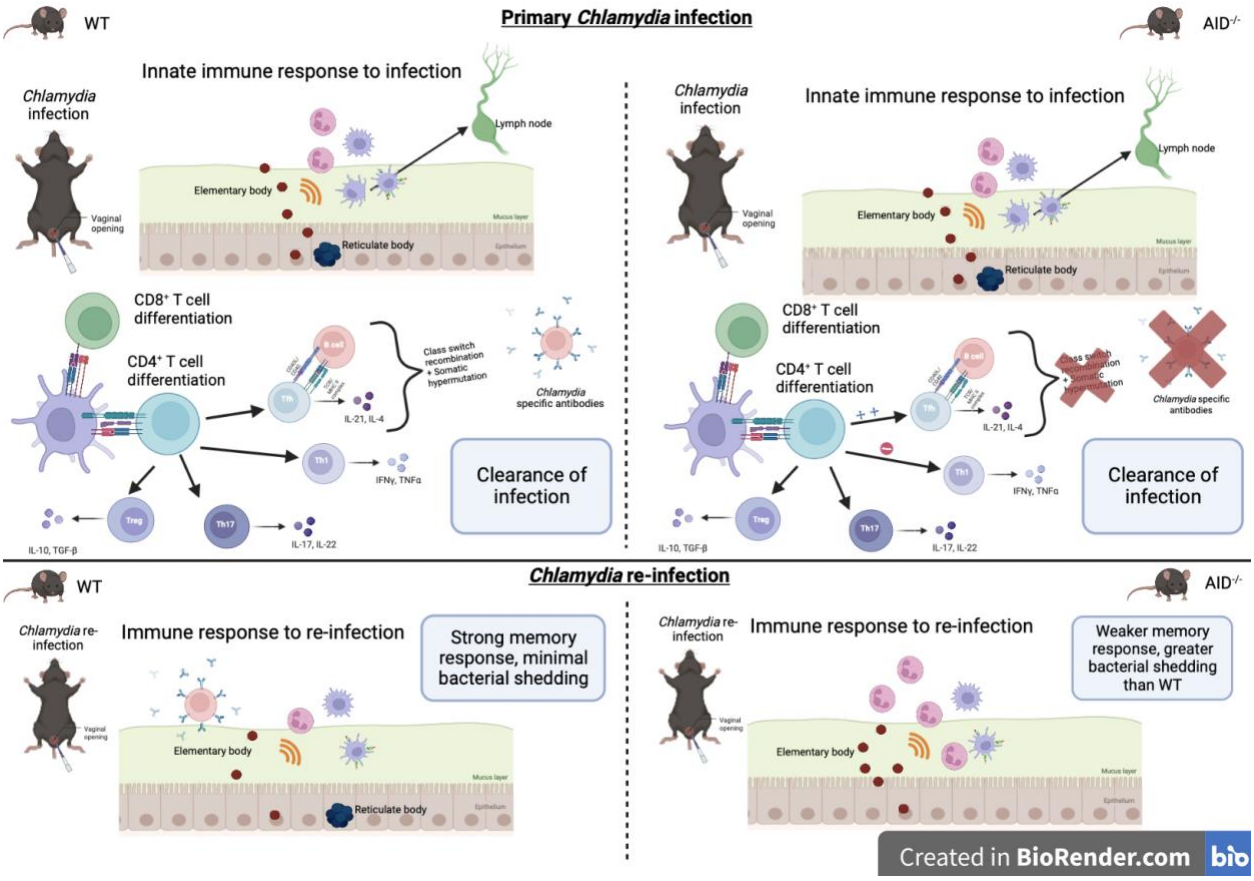
ABSTRACT

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infections around the world. The role of antibodies in Chlamydia pathogenesis remains inconclusive. Activation-induced cytidine deaminase (AID) is an enzyme critically required for B cell receptor diversification and the formation of high-affinity antibodies. The objective of this study is to determine the specific role of AID in murine models of primary and secondary Chlamydia genital infections using wild type (WT) and AID knockout (KO) mice. We showed that the bacterial burden, local immune cell infiltration and oviduct tissue pathology were comparable in two strains of mice following primary Chlamydia infection. In agreement with the known function of AID in secondary antibody production, AIDKO mice had minimal IgG1 and IgG2c production, but produced significantly greater IgM compared to WT mice. In addition, two strains of mice displayed divergent adaptive cellular immune profile following primary Chlamydia infection. While WT mice developed strong protective T helper 1 (Th1) immunity, AIDKO mice had elevated accumulation of suppressive regulatory T cells (Tregs) and follicular T helper cells (Tfh), the type of T cells collaborating with B cells for high-affinity antibody production. Accordingly, AIDKO mice had a delayed Chlamydia clearance, increased oviduct dilation and increased tissue inflammation compared to WT mice upon secondary challenge. Our data highlights a novel role of AID in host defense against Chlamydia infection via controlling both humoral and cellular immune responses. Further research is needed to dissect the mechanism whereby AID-deficiency in B cells affects the differentiation of T helper cells.

ABSTRACT


WT
Primary Chlamydia infection
AID-/-
Chlamydia infection
Innate immune response to infection
Lymph node
Elementary body
Reticulate body
Vaginal opening
Mucus layer
Epithelium
CD8+ T cell differentiation
CD4+ T cell differentiation
B cell
Th
IL-21, IL-4
Class switch recombination + Somatic hypermutation
Chlamydia specific antibodies
Th1
IFNγ, TNFα
Treg
Th17
IL-10, TGF-β
IL-17, IL-22
Clearance of infection
Chlamydia re-infection
Immune response to re-infection
Strong memory response, minimal bacterial shedding
Weaker memory response, greater bacterial shedding than WT
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LIST OF ABBREVIATIONS AND SYMBOLS USED

ACK	ammonium-chloride-potassium
AID	activation-induced cytidine deaminase
AIDKO	activation-induced cytidine deaminase knock-out
ANOVA	analysis of variance
BS	bovine serum
Cm	*Chlamydia muridarum*
CPAF	*Chlamydia* protease activating factor
CSR	class switch recombination
Ct	*Chlamydia trachomatis*
DAMP	damage-associated molecular patterns
DC	dendritic cell
DNA	deoxyribonucleic acid
EB	elementary body
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FOXP3	forkhead box P3
gDNA	genomic deoxyribonucleic acid
GT	genital tract
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HSP	heat shock protein
IFN	interferon
IFU	inclusion forming unit
Ig	immunoglobulin
IL	interleukin
mg	milligrams
ml	millilitres
mM	millimolar

MOMP	major outer membrane protein
MTOC	microtubule organization center
MZB	marginal zone B cell
NAAT	nucleic acid amplification test
NETs	neutrophil extracellular traps
ng	nanogram
NK	natural killer
OD	optical density
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDPN	podoplanin
PMN	polymorphonuclear neutrophil
PID	pelvic inflammatory diseases
pH	potential of hydrogen
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RB	reticulate body
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
SEM	standard error of mean
SPG	sucrose-phosphate-glutamic buffer
T3SS	type 3 secretion system
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TMB	tetramethylbenzidine substrate solution
TNF	tumour necrosis factor
Treg	regulatory T cell
WT	wild-type

5X	repeated, low dose infection schedule
°C	degrees celsius
µg	microgram
µl	microliter
µm	micrometer

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CHAPTER 1 INTRODUCTION

1.1 Overview of Chlamydia

The Chlamydiaceae family is among the most well-known and studied groups within the Chlamydiae phylum (Cheong et al., 2019; Elwell et al., 2016). The Chlamydiaceae family describes a group of obligate, intracellular pathogens with the ability to colonize a wide variety of hosts and exist in diverse environmental conditions (Bachmann et al., 2014; Cheong et al., 2019).Within this family, 11 species exist that can cause pathogenic disease in humans or animals (Bachmann et al., 2014). The *C. muridarum* species is a mouse pathogen that is often used for animal models of *Chlamydia* infections to mirror the course of infection that takes place in humans. *C. trachomatis, C. psitacci* and *C. pneumoniae* are the species that are pathogenic to humans and are responsible for numerous diseases that create a large healthcare burden around the world.

C. trachomatis infection remains one of the most common sexually transmitted infections around the world (Brunham & Rey-Ladino, 2005; Cheong et al., 2019; Fu et al., 2022) and accumulates health care costs over 500 million USD within the United States (Naglak et al., 2017) . Transmission can occur through oral, vaginal, and anal sex but can also be transmitted vertically from a mother to a newborn child during a natural vaginal delivery (Price, Ades, Soldan, et al., 2016; Zikic et al., 2018). *C. trachomatis* exists in 5 unique serovars that can cause a plethora of different diseases. The serovars A-C can infect conjunctival epithelial cells, causing trachoma, which without treatment, can lead to blindness (Faris et al., 2019) and is the leading cause of blindness from an infectious disease worldwide (Mohseni et al., 2022). The more commonly known serovars D-K are variants that are capable of infecting genital tract epithelial cells, and lead to urogenital infections, such as cervicitis, urethritis, or pelvic inflammatory disease in women, and epididymitis, prostatitis, proctitis or reactive arthritis in men (Mohseni et al., 2022). The serovars L1-L3 are a more rare form of the sexually transmitted infection that infect the lymphatic system of the genital tract, called lymphogranuloma venereum (LGV) and although not as common as infections perpetrated by the D-K serovars, reports of this disease are increasing in populations of men who have sex with men (Rawla et al., 2022).

1.2 Epidemiological data of *Chlamydia* infection in humans

It is estimated that 1 in 20 young women between the ages of 14-24 who are sexually active will be infected with *C. trachomatis* (Tjahyadi et al., 2022). Moreover, two thirds of new *C. trachomatis* infections occur in youths aged 15-24 (Tjahyadi et al., 2022). The region with the largest number of infections is America, closely followed by Africa (Huai et al., 2020). Cases of *C. trachomatis* infection are often asymptomatic, with up to 70% of infections in women and 50% of infections in men not experiencing symptoms after infection (Canada, 2021; Elwell et al., 2016).Without symptoms of infection, individuals are often not diagnosed, which leads to frequent reinfections, chronic cases of infection, and subsequent ascension of infection into the upper female reproductive tract due to the lack of treatment. Ascension of *C. trachomatis* infection into the upper female reproductive tract can lead to pelvic inflammatory disease, tubal factor infertility, ectopic pregnancy, and pelvic pain (Tjahyadi et al., 2022).

Currently, diagnosis with *C. trachomatis* infections is conducted using nucleic acid amplification tests (NAATs) done on vaginal or cervical swabs from women or urethral swabs and urine samples from men (Dukers-Muijrers et al., 2022; Price, Ades, Soldan, et al., 2016). Previously, methods such as culture and enzyme immunoassays were used for diagnostic purposes, particularly in early epidemiological studies where culturing played a critical role in diagnosing the disease (National Chlamydia Screening Program (NCSP), 2021). It is important to keep in mind that cultures have a lower sensitivity than the current NAATs and may have some influence on earlier epidemiological data. Reported cases of *Ct* infection have been rising in Canada between 2009 and 2018 (Canada, 2021). The number of cases reported in 2009 was 87,283 and this number climbed to 117,008 cases in the year 2018 (Canada, 2021). Additionally, both the United States and Canada reported an increase in *Ct* cases during the COVID-19 pandemic which could be partially attributed to the lack of preventive testing and treatment services that were available for sexually transmitted infections during the global pandemic (WHO, 2022).

1.3 *C. trachomatis* life cycle

C. trachomatis has a unique, biphasic developmental cycle where it can exist in two different forms. They can be elementary bodies (EBs), which are infectious, but metabolically inactive

extracellular form, or they can be reticulate bodies (RBs), which is the non-infectious, metabolically active and intracellular form (AbdelRahman & Belland, 2005; Gitsels et al., 2019). The initial goal of the EBs is to pass the plasma membrane of the host cell and establish an infection. To do so, the EBs bind to heparin sulfate proteoglycans at the surface of epithelial cells using ligands on the EB membrane OmcA, OmcB and major outer membrane protein (MOMP) for *C. trachomatis* (Gitsels et al., 2019). Once the EB adheres to the epithelial cell, it interacts with other cell surface receptors which include, but are not limited to: mannose receptor, mannose-6-phostphate receptor, epidermal growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor , ephrin receptor A2, protein disulfide isomerase, and ß1 integrin (Witkin et al., 2017). Upon binding to the host cell, EBs activate a functional type 3 secretion system (Gitsels et al., 2019). The type 3 secretion system is a bacterial structure which inject the effectors Tarp, CT166, CT694, and their chaperones into the host cell (Gitsels et al., 2019). This triggers chlamydia-induced actin remodeling, allowing the pathogen to enter into the host cell (Bastidas et al., 2013).

After entry into the host cell, the EBs reside inside of inclusion bodies that allow the EBs to rearrange into RBs (AbdelRahman & Belland, 2005; Witkin et al., 2017). The inclusion membrane is altered by the insertion of bacterial proteins to shunt it away from endosomal pathway and subsequent lysosomal fusion (Scidmore et al., 1996). Instead, it is diverted to the microtubule organizing center located near the peri-Golgi region(Scidmore et al., 1996). Transportation to the MTOC allows the EBs to interact with nutrient-rich compartments of the cell for intracellular survival. *C. trachomatis* species do not possess the appropriate metabolic machinery to synthesize its own energy and nutrients and thus rely on the host cell for essential amino acids like tryptophan (Witkin et al., 2017).

RBs utilize resources from the host cell to replicate via binary fission until the number of RBs inside inclusion bodies reaches a critical volume (Elwell et al., 2016). Subsequently, RBs transform back into the EB form before release from the host cell either through lysis or extrusion (Bastidas et al., 2013). Host cell lysis proceeds in two steps, with the first being the cysteine protease-dependent lysis of the inclusion body (Hybiske & Stephens, 2007). The second step involves rupturing of the host cell plasma membrane (Hybiske & Stephens, 2007). However,

host cell extrusion proves to be more complicated. Extrusion of the EBs from the host cell is dependent on actin polymerization with myosin II and RhoA regulating the exit and pinching off of the inclusion body (Hybiske & Stephens, 2007; Zuck & Hybiske, 2019).

Under undesirable and/or stressful conditions such as exposure to antibiotics or tryptophan starvation as a result of IFN-γ production by host immune cells, *Chlamydia* may alter gene transcription and metabolism to a persistent state where the RBs cease division but remain viable and culturable organisms (Witkin et al., 2017). This survival mechanism employed by *Chlamydia* allows it to endure the unfavourable conditions in the host until conditions become more favourable where they can resume normal replicative behaviours (Witkin et al., 2017). This state of chlamydial persistence is particularly harmful if it remains for extended periods of time and develops into a chronic chlamydial infection which wreak more havoc within the host reproductive system (Witkin et al., 2017).

1.4 Chlamydia disease sequelae

Genital *C. trachomatis* infections are the leading causes of pelvic inflammatory disease (PID), tubal factor infertility, and ectopic pregnancy (Witkin et al., 2017). This is in part due to the large proportion of asymptomatic cases where individuals are unaware that they are infected and therefore do not seek treatment (Mohseni et al., 2022; Rodrigues et al., 2022). Failing to seek treatment increases the risk of transmission and gives the pathogen an opportunity to ascend in the infected female genital tract (Rodrigues et al., 2022). Ascension of *C. trachomatis* in the female reproductive tract provides further possibilities for chronic infections and increased tissue damage that can contribute to numerous complications (den Hartog et al., 2005). Moreover, the risk of severe tubal pathology is increased in recurrent *Chlamydia* infections (den Heijer et al., 2019). Studies have also linked *Ct* infections with a heightened risk for developing cervical cancer (Bhuvanendran Pillai et al., 2022; Josefson, 2001; Paavonen et al., 2003). *C. trachomatis* can contribute to a higher risk of miscarriage, preterm birth, and stillbirth in previously infected women (Rours et al., 2011). These experiences, compounded with the stigma surrounding sexually transmitted infections, highlight the many risks *Ct* infections can have on a woman's physical, emotional, and mental wellness.

1.5 Host responses to Chlamydia infection

1.5.1 Innate immune response

The innate immune response is a broad defense strategy that exists to protect the body from pathogens at both the molecular and cellular levels. It is the first line of defense against infectious agents and provides both physical and chemical barriers to fight off infection while simultaneously priming specific adaptive immune responses to each unique pathogen.

The innate immune system must first distinguish between foreign entities and the body's own cells (Amarante-Mendes et al., 2018). This is carried out by pattern recognition receptors (PRRs) which are able to recognize distinct molecules found on pathogens (Amarante-Mendes et al., 2018; Redgrove & McLaughlin, 2014). These unique molecules are referred to as pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components and lipids (Janeway & Medzhitov, 2002). PRRs are also capable of recognizing damage-associated molecular patterns (DAMPs), such as stress-induced self-molecules (Janeway & Medzhitov, 2002). A subtype of PRR that is especially important is called the Toll-like receptors (TLRs) (Kawasaki & Kawai, 2014; Massari et al., 2013; Yadav et al., 2021). There are ten types of TLRs in humans and twelve types in mice that can be divided into two groups (Kawasaki & Kawai, 2014; Kumar et al., 2011). TLR1, TLR2, TLR4, TLR5,TLR6, and TLR11 are expressed on the surface of the cell and bind PAMPs related to microbial membrane components (Kawasaki & Kawai, 2014; Kumar et al., 2011). TLR3, TLR7, TLR8, and TLR9 are expressed intracellularly and detect microbial nucleic acids (Kawasaki & Kawai, 2014; Kumar et al., 2011; Yadav et al., 2021). TLRs trigger activation of MyD88-dependent and TRIF-dependent signalling cascades (Piccinini & Midwood, 2010; Redgrove & McLaughlin, 2014), leading to production of antimicrobial peptides, inflammatory chemokines, cytokines and lipid mediators that collectively organize the early immune response (Amarante-Mendes et al., 2018; Redgrove & McLaughlin, 2014). This includes the infiltration and expansion of neutrophils, macrophages, natural killer (NK) cells, and dendritic cells (Chen et al., 2019; Redgrove & McLaughlin, 2014; Vasilevsky et al., 2014). These mechanisms allow the host immune system to provide innate protection at early stages of *Chlamydia* infections and prime the adaptive immune responses (Redgrove & McLaughlin, 2014; Vasilevsky et al., 2014).

1.5.2 Mucosal barrier immune response

Upon initial infection, the first line of defense against *Ct* is our mucosal barrier that is made up of epithelial cells. Infected epithelial cells emit various signalling molecules and soluble mediators to combat the pathogen (Bastidas et al., 2013; Chen et al., 2019; Mohseni et al., 2022). Soluble mediators of the innate immune system include the complement system, PRRs, antimicrobial peptides, cytokines, and inflammatory molecules (e.g. mucin-1) (Jonsson et al., 2019; Scurtu & Simionescu, 2021). *Chlamydia* infection has been shown to induce infected epithelial cells to express various signalling molecules including CXC-chemokine ligand 1 (CXCL1), CXCL8 / IL-8, CXCL16, granulocyte/monocyte colony-stimulating factor (GM-CSF), IL-1α, IL-6, tumour-necrosis factor (TNF), CC-chemokine ligand 5 (CCL5), and CXCL10 (Brunham & Rey-Ladino, 2005; Chen et al., 2019). Collectively, the mucosal barrier immune response will initiate inflammation, trigger chemotaxis of certain immune cells, and activate local immune cells for an organized bactericidal response against *Chlamydia* (Xiang et al., 2021).

1.5.3 Neutrophil response

Neutrophils are the first population of innate immune cells to infiltrate the local site of infection and are the most abundant form of white blood cell (Redgrove & McLaughlin, 2014). A slew of chemoattractants signal neutrophil recruitment to the site of infection. They can be divided into four subfamilies, chemotactic lipids (i.e. leukotriene B_4), chemokines (such as CXCL1-CXCL3 and CXCL5-CXCL8), complement anaphylatoxins (C3a and C5a) and formyl peptides (e.g., N-formyl-Met-Leu-Phe), all of which function by activating specialized G protein-coupled receptors to guide neutrophil migration (Metzemaekers et al., 2020).

The conventional role of neutrophils in host defense against infection is to exert microbicidal properties by destroying invading pathogens (DeLeo & Allen, 2020; Kobayashi et al., 2018; Nauseef, 2007; Rosales, 2018). Neutrophils store an assortment of effector molecules in different types of granules and secretory vesicles (Mantovani et al., 2011). Primary granules hold the most toxic mediators such as elastase, myeloperoxidase, cathepsins, and defensins (Faurschou & Borregaard, 2003; Gierlikowska et al., 2021; Mantovani et al., 2011). Secondary granules are known as specific granules and contain a variety of substances including lactoferrin and matrix

metalloprotease 9 (MMP9) (Borregaard & Cowland, 1997; Gierlikowska et al., 2021; Lacy, 2006). The contents of tertiary granules overlap with those of secondary granules but tertiary granules are distinct due to their intrinsic buoyant densities when they are isolated by density gradient centrifugation (Kjeldsen et al., 1994; Udby & Borregaard, 2007). Secretory vesicles hold membrane-associated receptors necessary for early phases of neutrophil-mediated inflammatory responses (Faurschou & Borregaard, 2003). Granule mobilization and release is highly regulated due to their containment of tissue-destructive proteases(P. Lacy, 2006).

Neutrophils employ a variety of effector functions including phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) (**Figure 1.1**). Phagocytosis is critical to the microbicidal function of neutrophils (H. Chen et al., 2019; W. L. Lee et al., 2003; Rosales, 2018). Recognition of invading pathogens is mediated by receptors present on the neutrophils, such as TLRs, Fc receptors (FcγR1, FcγRII and FcγRIII) and opsonized complement molecules (Karsten et al., 2019; Wellington et al., 2003). Phagocytosis is most efficient in the presence of opsonins, such as neutralizing antibodies, to directly mediate what is called opsonophagocytosis (Gierlikowska et al., 2021). Antibody-coated pathogen can directly bind to the FcγRI, FcγRII, and FcγRIII receptors on neutrophils (Gierlikowska et al., 2021; Wellington et al., 2003). This efficient binding process enhances uptake and internalization of the pathogen by neutrophils. Once the pathogen is engulfed by neutrophils, it resides in a bacteria-containing vacuole known as the phagosome (Kobayashi et al., 2018). Within the phagosome, neutrophils can kill pathogens using either oxygen-dependent or oxygen-independent mechanisms (Kobayashi et al., 2018; Nauseef, 2007). The oxygen-dependent mechanism involves the production of a superoxide enzyme complex, NADPH oxidase that can be converted to other reactive oxygen species effective at killing bacteria (Kobayashi et al., 2018; Nauseef, 2007). Oxygen-independent methods of neutrophilic microbicidal activity involve antimicrobial peptides and enzymes stored in azurophilic and specific granules (DeLeo & Allen, 2020; Kobayashi et al., 2018). Neutrophil phagocytosis activates the migration of granules to fuse with the phagosome (Kobayashi et al., 2018). This fusion enriches the phagosome with diverse antimicrobial peptides including alpha-defensins, cathepsins, proteinse-3, elastase, azurocidin, and lysozymes (Kobayashi et al., 2018). Both oxygen-dependent and oxygen-independent mechanisms work collectively to destroy invading pathogens (DeLeo & Allen, 2020; Kobayashi et al., 2018).

Degranulation is another form of neutrophil-mediated pathogen killing that involves the sequential release of an array of different granules (Gierlikowska et al., 2021; Kolaczkowska & Kubes, 2013). Neutrophils can release antimicrobial proteins and enzymes stored in granules into the phagosome to kill engulfed bacteria (Kobayashi et al., 2018; Sengeløv et al., 1995), or into the extracellular space to kill extracellular bacteria, and contribute to leukocyte recruitment (Eichelberger & Goldman, 2020; Lacy, 2006). However, excessive neutrophil degranulation has been linked to a variety of inflammatory disorders and is known for perpetrating severe pathology and host tissue damage in a context-dependent manner (Eichelberger & Goldman, 2020; Lacy, 2006).

NET formation begins upon activation by several mediators, including IL-8, LPS, and IFN-α with complement 5a (Mantovani et al., 2011). Once NET formation begins, the neutrophils will loosen the chromatin DNA to allow formation of complexes with various granular and cytoplasmic proteins (Brinkmann et al., 2004; Kobayashi et al., 2018). NETs are composed of nuclear materials including DNA and histones, and are populated with proteins from primary, secondary, and tertiary granules (Mantovani et al., 2011). These complexes make up fibers that can aggregate and create a space with high concentrations of antimicrobial granule proteins at the local site of infection (Brinkmann et al., 2004; Kobayashi et al., 2018; Manda et al., 2014).Studies have shown that NETs can trap microorganisms such as *Escherichia coli, Staphylococcus aureuas, Shigella flexneris, Salmonella enterica, Candida albicans, and Leishmania amzonesis* (Brinkmann et al., 2004; Mantovani et al., 2011; Urban et al., 2009). By trapping pathogens, NETs can promote interaction between granule-derived proteins with trapped microorganisms, and affect their subsequent disposal (Mantovani et al., 2011). NET formation occurs within minutes, and has been suggested to be mediated by a process termed NETosis(Mantovani et al., 2011). NETosis is a form of cell death that deviates from classical apoptosis, only occurring during the formation of NETs (Mantovani et al., 2011). NETosis is characterized by chromatin decondensation and nuclear membrane disintegration to make way for NET formation (Mantovani et al., 2011). Although NETs provide an effective way for the neutrophils to immobilize and kill the invading pathogen, many microorganisms have evolved strategies to evade NETs (Kaplan & Radic, 2012; Manda et al., 2014; Mantovani et al., 2011).

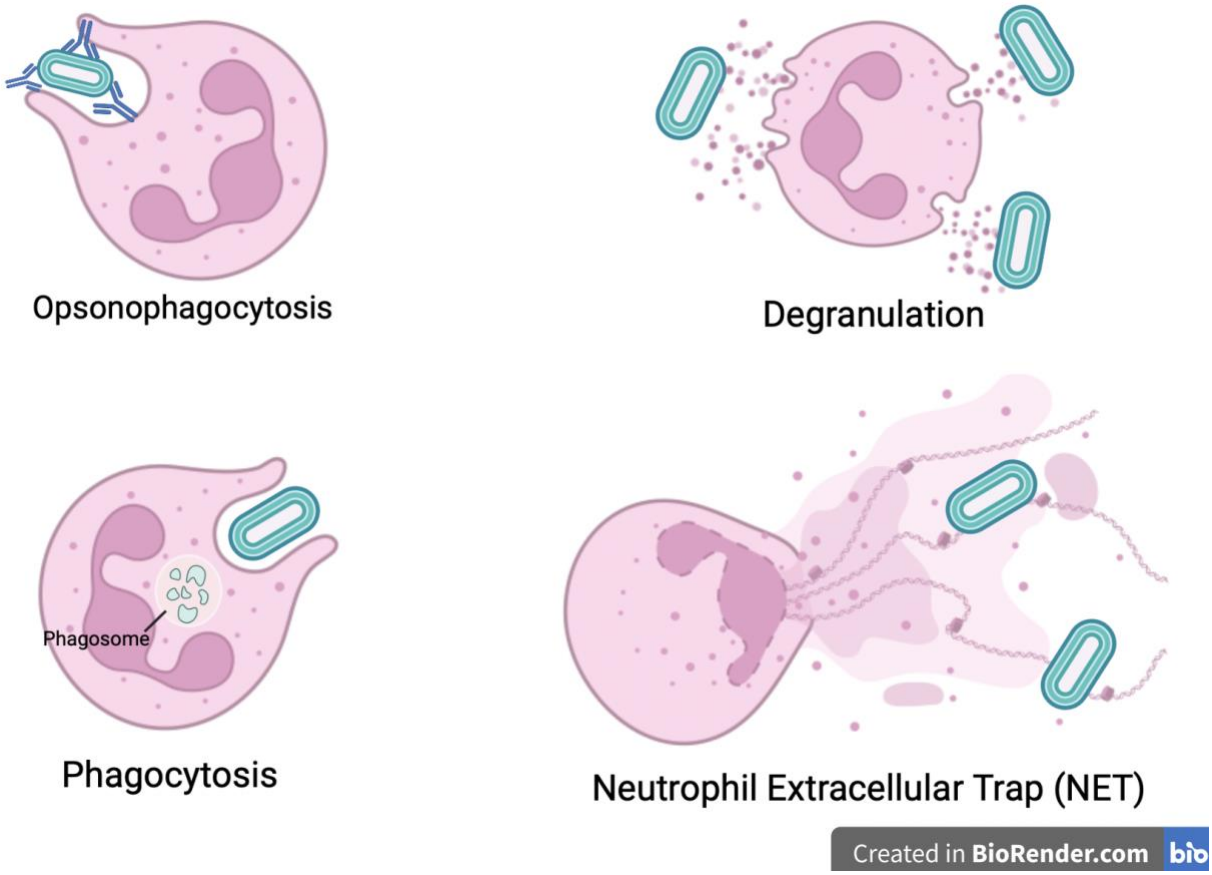


Figure 1.1: Neutrophil Bactericidal Functions
A diagram illustrating the various effector functions that neutrophils use to destroy pathogens.

Blue ovals represent the pathogen and pink cells represent neutrophils.

In the context of *Chlamydia* infection, neutrophils have been shown to limit the spread of *Ct* infection, with evidence of human neutrophils inactivating *Ct* in vitro (Redgrove & McLaughlin, 2014; Register et al., 1986; Tığlı, 2023). Neutrophil depleted mice have been shown to have up to 10-times greater bacterial burden than their neutrophil competent counterparts (Barteneva et al., 1996), highlighting a protective role for neutrophils in host defense against early *Ct* infection. However, it has become clear that *Chlamydia* infection can cause neutrophil paralysis, and reprogram neutrophils from short lived major immune effector cells to host cells suitable for *Chlamydia* replication and development (Chen et al., 2019; Frazer et al., 2011; Rajeeve et al., 2018; van Zandbergen et al., 2004).

Chlamydia can prolong neutrophil lifespans, and survive inside infected neutrophils (Sarkar et al., 2015; van Zandbergen et al., 2004; Wong et al., 2019). The *Chlamydia* infection activates PI3K pathway inside of neutrophils which stabilizes the anti-apoptotic protein Mcl-1 (Sarkar et al., 2015; Wong et al., 2019). In a separate study, *Chlamydia* infection is also associated with lower levels of reduced caspase-3 activity in infected neutrophils contributing to the antiapoptotic effect (van Zandbergen et al., 2004). *Chlamydia* is able to evade the host innate immune response by paralyzing neutrophils (Chen et al., 2019; Rajeeve et al., 2018). A chlamydial protease called chlamydial protease-like activating factor (CPAF) is identified as the bacterial effector responsible for preventing the activation of uninfected neutrophils (**Figure 1.2**)(Chen et al., 2010; Fenner, 2018; Rajeeve et al., 2018). CPAF can interfere with chemical signalling that would otherwise activate neutrophils (Christian et al., 2010; Rajeeve et al., 2018). CPAF can cleave the formyl peptide receptor 2 (FPR2) and release it from the surface of neutrophils (Rajeeve et al., 2018). This dysregulates neutrophil responses and prevents the formation of NETs, ultimately allowing the bacteria to survive and replicate in the neutrophils for extended periods of time (Rajeeve et al., 2018; van Zandbergen et al., 2004).

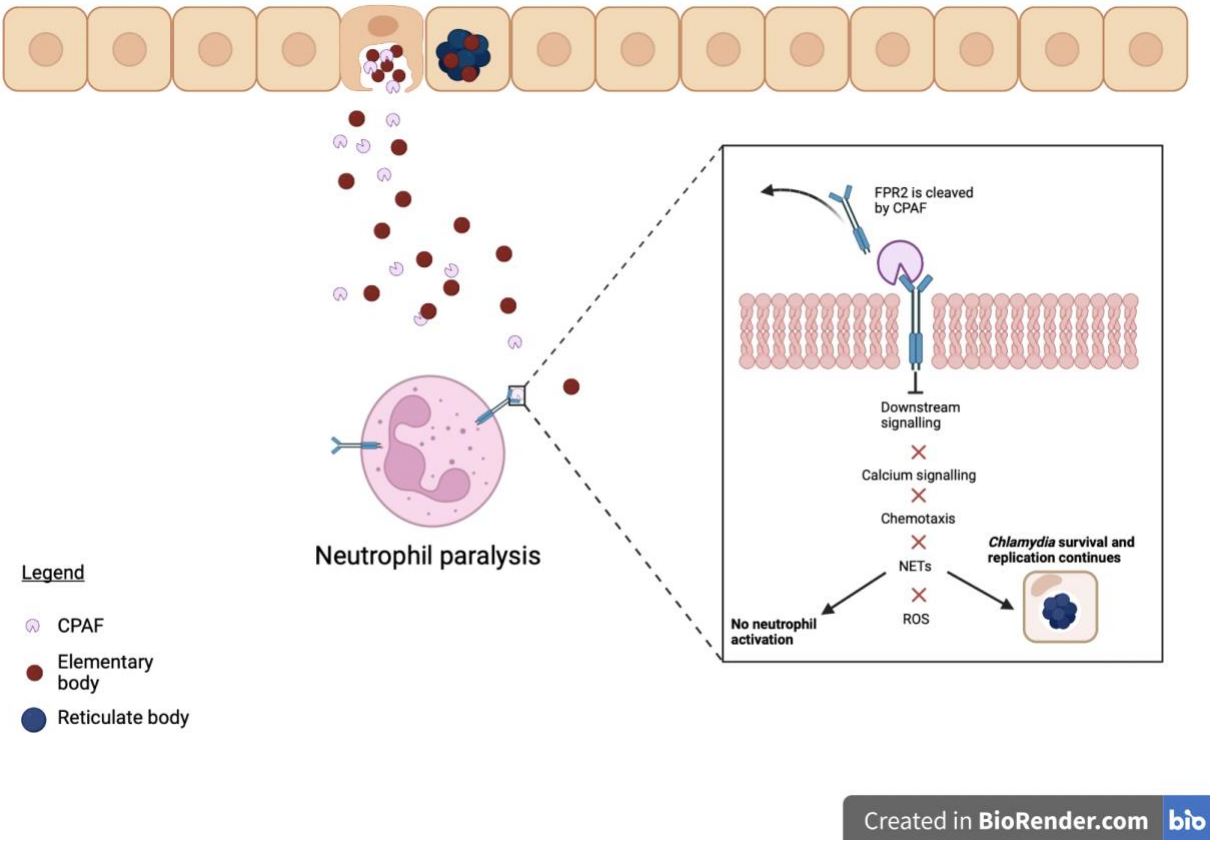


Figure 1.2: Chlamydia paralysis of host neutrophils

This diagram illustrates how Chlamydia uses the protease CPAF to paralyze neutrophils and enable pathogen survival and replication.

Due to the inflammatory nature of neutrophils, they have been suspected to play a large role in tempering tissue pathology as a result of chlamydial infection (Frazer et al., 2011; McQueen et al., 2021; Yang et al., 2021). Although important for modulating host adaptive immune responses, neutrophils may also promote host tissue damage, particularly during chronic infection. In acute inflammatory situations, neutrophilic responses are more beneficial rather than harmful – helping to limit the spread of infection with minor tissue damage (Barteneva et al., 1996; Naglak et al., 2017). In the context of chronic inflammation and continuous neutrophil activity, they are inadvertently pathogenic (Frazer et al., 2011; Lehr et al., 2018). When *Chlamydia* infects neutrophils and delays apoptosis, tissue-damaging molecules and cytokine production is increased, leading to more severe tissue damage (Frazer et al., 2011; Xiang et al., 2021).

1.5.4 Macrophage response

Macrophages differentiate from monocytes and are capable of both phagocytosis and antigen presentation. The conventional role of macrophages is to ingest and break down dead cells, tumour cells, debris, and foreign particles (Hirayama et al., 2017). They can secrete pro-inflammatory and antimicrobial mediators as part of the innate immune response (Hirayama et al., 2017).

In the context of chlamydial infection, macrophages migrate to the site of infection and remove bacteria via phagocytosis before destroying it via autophagy (Redgrove & McLaughlin, 2014). In mouse models of *Ct*, it was revealed that $CD11b^{+}$ macrophages were infiltrating the genital mucosa (Lausen et al., 2019). Macrophages exist in different subsets as well. There are classically activated macrophages which are well known for their anti-microbial effector mechanisms and phagocytic activity during acute infection, and then there are alternatively activated macrophages that are more important for tissue repair at sites of wound healing with less bactericidal abilities than their classical counterparts (Narasimhan et al., 2019; Tietzel et al., 2019). IFN-γ can activate classical macrophages so that they have increased antigen presenting capacity and secretion of pro-inflammatory cytokines to trigger an adaptive immune response (Narasimhan et al., 2019; Tietzel et al., 2019). These IFN-γ activated macrophages express high levels of CD64 which plays an important role in macrophage antibody-dependent cellular

cytotoxicity (Akinrinmade et al., 2017), anti-microbial effectors like inducible nitric oxide synthase or indoleamine-2,3,-dioxygenase and cytokines like IL-12p70 which helps to prevent immune injury by IFN-γ (Verma et al., 2014). Alternatively activated macrophages are activated via IL-4, IL-13 or other cytokines tend to express high levels of CD206 and anti-inflammatory cytokines such as IL-10 and TGF-β (Narasimhan et al., 2019; Tietzel et al., 2019). Although all types of macrophages are important to the immune system's fight against *Chlamydia* infection, *Chlamydia* has evolved mechanisms to evade the host immune response. However, the ability for *Chlamydia* to infect macrophages and disseminate infection depends on both the strain of *Chlamydia* and source of macrophage production (Herweg & Rudel, 2016). There has been evidence that *Ct* can use alternatively activated macrophages to their advantage. Tietzel et al. (2019) found that alternatively activated macrophages are good hosts for *Ct* because the bacteria can complete its developmental cycle within the infected macrophage and stimulate IL-10 production to further attenuate host immune responses.

Certain macrophage populations at the local site of infection appear to upregulate the biomarker Podoplanin (PDPN). PDPN is a transmembrane receptor reported to be expressed on inflammatory immune cells, particularly during cases of chronic inflammation (Chan et al., 2023; De Filippis et al., 2017). PDPN expressing macrophages are present in the murine genital tract post primary infection and macrophages expressing PDPN are more likely to migrate towards *Chlamydia* infected cells (Chan et al., 2023). In a similar vein, PDPN knockout macrophages function appropriately in response to *Heliobacter pylori* infection but have impaired migration due to dysfunctional filopodia formation (Cheok et al., 2021). Collectively, this data demonstrates a crucial role for PDPN in directing appropriate migration of macrophages to active infection sites within the body.

1.6 Adaptive immune response

The adaptive immune response is composed of two main mechanisms, humoral immunity and cell mediated immunity. Both mechanisms are carried out by lymphocytes and are important to the clearance of pathogens following the activation of our innate immune system.

Dendritic cells (DCs) are responsible for the initiation of adaptive immune system. After engulfing pathogens from the infected tissue sites, they migrate into the draining lymph nodes where they present the processed antigens to naïve CD4 and CD8 T cells (Liu, 2016). Pathogen-loaded DCs undergo a maturation process by upregulating MHC class I / II molecules and various costimulatory molecules, as well as production of different cytokines for immune polarization (Liu, 2016). *Ct* infection has been shown to trigger DCs to produce IL-12, the T cell recruiting chemokines CXCL10, and upregulate CCR7 which is critical for DC migration to local lymph nodes (Brunham & Rey-Ladino, 2005). Studies have provided evidence that the adoptive transfer of DCs that have been exposed to *Cm* protects mice against a subsequent infection (Su et al., 1998), demonstrating their importance in protection against chlamydial infections.

1.6.1 Cellular Immunity

Cell mediated immunity involves activated T cells to elicit their effector functions. In this branch of the adaptive immune response, mature T cells can directly interact with antigens either by killing an infected host cell or producing cytokines and chemokines to instruct phagocytic immune cells like macrophages to destroy the microbes they have eaten (Alberts et al., 2002; Helble & Starnbach, 2021). There are two cellular phenotypes that coordinate cell-mediated immunity, $CD4^+$ and $CD8^+$ T cells (Helble & Starnbach, 2021; Seder & Ahmed, 2003).

Naïve $CD4^+$ T cells can differentiate into multiple subtypes to accommodate the immune response to a variety of pathogens (Seder & Ahmed, 2003; Tığlı, 2023). $CD4^+$ T helper cells can secrete a diverse range of cytokines, chemokines and signalling molecules that enhance the responses of other adaptive immune cells and promote the development of memory T cell responses (Helble & Starnbach, 2021; Seder & Ahmed, 2003).

The $CD4^+$ T cell response is made up of five main subsets of the $CD4^+$ T helper cell: Th1, Th2, Th17, Regulatory T cells (Treg), and follicular T helper cells (Tfh) (**Figure 1.3**). Th1 cells are mainly involved in the type 1 immune responses that is important for clearing intracellular pathogens and are known for their expression of the cytokine IFN-γ and the master transcription

factor T-bet (Zhu & Zhu, 2020). Th2 cells express IL-4, IL-5, IL-13 and the master transcription factor GATA3 and stimulates the type 2 immune response that targets parasites such as helminths and critical for tissue repair (Zhu & Zhu, 2020). Th17 cells mainly express IL-17 and IL-22 and the master transcription factor RORγt and contribute to the type 17 immune response that targets extracellular pathogens like bacteria (Zhu & Zhu, 2020). Tregs express the master transcription factor FoxP3 and are an immunosuppressive subset of the $CD4^+$ T cell that maintains homeostasis and protects against immunopathology (Kondělková et al., 2010). Tfh cells are a special subset of $CD4^+$ T helper cell, they produce IL-21 and express Bcl6 and support B cells in the development of specific antibodies, and are necessary for germinal center formation, affinity maturation, and the development of memory B cells and plasma cells (Crotty, 2014).

When it comes to clearing chlamydial infections, $CD4^+$ T cells have proven to be indispensable. Nude mice, which lack a thymus and T cells, are unable to control chlamydial infections, but with the adoptive transfer of $CD4^+$ T cells primed against Chlamydia, they are able to control the infection (Brunham & Rey-Ladino, 2005). The importance of $CD4^+$ T cells in clearing chlamydial infections is highlighted by the fact that primed donor $CD4^+$ T cells, but not primed donor $CD8^+$ T cells provided naïve mice with significant immunity against *Cm* (Su & Caldwell, 1995).

Tfh cells are a subset of $CD4^+$ T cells that support B cells in developing high affinity antibodies and memory B cells (Crotty, 2014). Tfh cell differentiation occurs in several steps and is not defined by exposure to any one cytokine like Th1 cell differentiation which can be completely induced by IL-12 exposure in both *in vitro* and *in vivo* scenarios (Crotty, 2014). During dendritic cell priming of naïve $CD4^+$ T cells, the determinants regulating Tfh cell differentiation are IL-6, inducible costimulatory (ICOS), IL-2 and the strength of the T cell receptor signal (Tubo et al., 2013). Production of the transcription factor BCl-6 by the naïve $CD4^+$ T cell commits the cell to the route of becoming a Tfh cell (Basso et al., 2012). If the chemokine receptor CXCR5 is expressed during early stages of differentiation, it induces early Tfh cells to begin migrating towards the B cell follicle prior to further Tfh cell differentiation (Choi et al., 2013). Once early Tfh cells have reached the follicle, co-stimulation is taken over by activated B cells which

induce Tfh cells to upregulate their expression of CXCR5, ICOS and PD1 so that they are able to migrate into the B cell zone to aid in the formation of germinal centres and finally become fully mature Tfh cells within the germinal center (Crotty, 2014).

In a study done on the intracellular bacteria *Salmonella Typhi* , researchers found that IgM producing B cells along with expansion of a population of IFN-γ producing Tfh cells were induced upon antigen exposure (Perez-Shibayama et al., 2014). Given the importance of IFN-γ for resolution of chlamydial infections, Tfh cells in the draining lymph node may play a role in supporting the production of protective IFN-γ during active infections (Labuda & McSorley, 2018). However, this particular subset of $CD4^+$ T cells has not been widely studied in the context of *Chlamydia* infection and thus further research must be done to investigate the role that Tfh cells may play in eradicating chlamydial infections and re-infections.

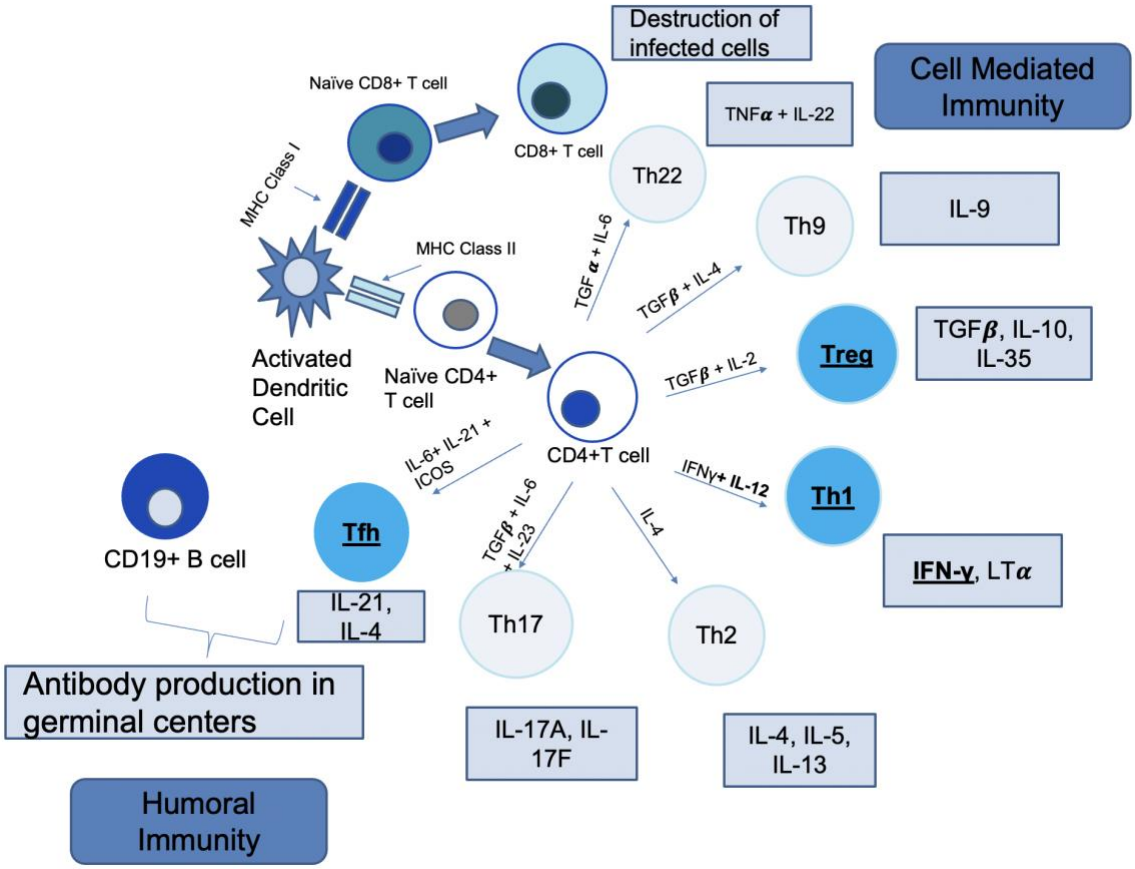


Figure 1.3: T cell subsets

This figure depicts the many subsets of T cells that exist, and the corresponding cytokines they are able to produce. Most notably, Th1, Tfh, and Treg cells are pertinent in studying the functional role of AID in *Chlamydia* pathogenesis.

1.6.1.1 Role of Th1/Th2/Th17 in Chlamydia infection

Specifically, it is the Th1 response and production of IFN-γ by $CD4^+$ T cells that appears to be critical to resolving *Chlamydia* infections. This conclusion was reached through many studies which demonstrate that mice deficient in MHC Class II molecules, IL-12, IFN-γ, IFN-γ receptor, or $CD4^+$ T cells are unable to control chlamydial infection (Brunham & Rey-Ladino, 2005; Helble &Starnbach, 2021; Lin et al., 2019). Th1 effector mechanisms in *Chlamydia* infection are multifaceted (Murray & McKay, 2021). Th1 immunity can control intracellular *Chlamydia* infection through IFN-γ induced production of the enzyme indeolamine-2,3-dioxygenase (IDO) (Brunham & Rey-Ladino, 2005). Activation of IDO by IFN-γ results in the depletion of tryptophan, and a lack of this amino acid results in the death of *Chlamydia* through tryptophan starvation (Brunham & Rey-Ladino, 2005; Murray & McKay, 2021). Th1 production of IFN-γ also upregulates production of inducible nitric oxide synthase (iNOS) which makes nitric oxide (NO) (Murray & McKay, 2021). NO is a well-known cytotoxic, anti-bacterial factor capable of damaging bacterial DNA (Murray & McKay, 2021; Ramsey et al., 2001).

Th1 and Th2 subsets of $CD4^+$ T cells regulate different immune responses specific to certain kinds of pathogens. The dynamic between Th1 and Th2 responses can provide insight into pathological and protective immune responses to *Chlamydia* infections.

While it is very clearly understood that Th1 and cell mediated immune responses are indispensable to clearing chlamydial infections, Th2 responses have been associated with pathological host responses to *Chlamydia*. This is largely due to the opposing effects of Th2 linked cytokines and transcription factors that hinder the differentiation of robust Th1 responses (Bretscher, 2019; Wynn, 2004). Women who express higher levels of Th2 cytokines are more likely to experience *Chlamydia*-induced infertility (Agrawal et al., 2009). Conversely, women who did not experience *Chlamydia* -induced infertility expressed higher levels of Th1 cytokines such as IFN- γ and IL-12 (Agrawal et al., 2009). These results suggest that Th1 polarized responses may play an important role in protecting the host from long term disease sequelae. Moreover, Th2 cytokines such as IL-13 play a role in promoting tissue remodelling and the development of fibrosis (Miguel et al., 2013; Wynn, 2004). This suggests that Th2 associated cytokines may be directly linked to pathology development during *Chlamydia* infection. This has

led to the traditional understanding that within the context of *Chlamydia* immunity, Th1 polarized responses are protective while Th2 polarized responses are harmful and directly interfere with the critical Th1 response.

Th17 responses are also induced during a *Chlamydia* infection (Bai et al., 2009; Scurlock et al., 2011). Th17 cells support bacterial clearance by stimulating chemokine production, recruiting neutrophils/macrophages, and supporting maintenance of the mucosal barrier (Blaschitz & Raffatellu, 2010; Luckheeram et al., 2012; Moore-Connors et al., 2013). Data from Moore et al. (2013) shows that Th17 and its effector cytokine IL-17 have important roles in the host immune response to *Chlamydia* infection. Although Th17 response appears to promote Th1 immunity, high levels of IL-17 have been associated with increased tissue damage (Bai et al., 2009; Moore-Connors et al., 2013). Thus, although Th17 responses are necessary for their protective functions, it is critical that this response is appropriately regulated to avoid excessive tissue damage.

1.6.1.2 Role of Regulatory T cell responses (Treg) in Chlamydia infection

A robust immune response to invading pathogens is important, but immunosuppression must exist to maintain immune tolerance and ward off collateral tissue damage and potential autoimmunity. This is where Tregs come in to play! Tregs are responsible for immunosuppression to maintain tissue homeostasis and self-tolerance (Kondělková et al., 2010). Tregs originate in the thymus and make up roughly 5-10% of the peripheral $CD4^+$ T cells in both mice and humans (Matos et al., 2021).

Tregs express the intracellular transcription factor forkhead box P3 (FoxP3) and are responsible for suppressing a variety of effector cell types (Matos et al., 2021). Mutations in the FoxP3 gene can lead to severe autoimmune diseases in humans and lymphoproliferative disorder in mouse models (Bennett et al., 2001; Brunkow et al., 2001). Because FoxP3 expression is so critical to appropriate Treg activity, it is a useful marker to identify levels of Tregs in mouse studies. Previously, Dr. Jessica Moore-Connors in the Wang lab demonstrated a novel role for $CD4^+CD25^+FoxP3^+$ Tregs in promoting inflammation in a mouse model of *Cm* infection via promoting Th17 response (Moore-Connors et al., 2013). There has also been evidence of harmful outcomes following Treg-induced immunosuppression during *Ct* ocular trachoma trials (Mabey

et al., 2014). Future work in this field of literature may aid in characterizing the impact of Tregs in the context of chlamydial infections.

1.6.1.3 Role of $CD8^+$ T cells in Chlamydia infection

$CD8^+$ T cell are able to recognize antigens presented on MHC class I molecules (Helble & Starnbach, 2021). They are able to upregulate cytokines and release perforin and granzymes to kill target cells (Helble & Starnbach, 2021). $CD8^+$ T cells can recognize *Ct* proteins and can perform targeted cell killing against chlamydial antigens (Helble & Starnbach, 2021; Fling et al., 2001), but have also been shown to be dispensable for clearance of Chlamydia in mice. This was proven when $CD8^{-/-}$ mice were able to clear infections at a similar rate to their WT counterparts (Brunham & Rey-Ladino, 2005; Helble & Starnbach, 2021; Johansson et al., 1997). Nonetheless, $CD8^+$ T cells produce important cytokines such as IFN-γ and form effector memory cells, supporting the defense against *Chlamydia* (Helble & Starnbach, 2021; Fling et al., 2001; Seder & Ahmed, 2003).

1.6.2 Humoral Immunity

Humoral immunity is otherwise known as the antibody response. In this branch of the adaptive immune response, B cells are activated by Tfh cells and produce pathogen-specific secondary antibodies, which are proteins termed immunoglobulins (Alberts et al., 2002). In addition, B cells can help activate T cells through antigen presentation and cytokine production(Alberts et al., 2002; Cano & Lopera, 2013). Various subtypes of B cells are actively involved in the humoral immune response. Their development begins in the bone marrow where immature B cells migrate towards secondary lymphoid organs (SLOs) for further differentiation during an ongoing immune response (Cano & Lopera, 2013). At the SLOs, by the guidance of B cell activation factor (BAFF) or A proliferation-inducing ligand (APRIL), transitional B cells differentiate into either marginal zone B cells (MZB) or migrate towards the germinal center to become follicular B cells (FB) (Cano & Lopera, 2013). Further differentiation can occur depending on specific antigen stimulation or cytokine microenvironment to create plasma cells or memory B cells (Nagasawa, 2006).

1.6.2.1 B and T cell interactions

There is a high interdependence between T cells and B cells during the initiation of the humoral response as well as for future immunological memory (Petersone et al., 2018; Tay et al., 2020). There are several key pathways that help to regulate these interactions (**Figure 1.4**). The most prominent pathway is the interaction between MHCII molecules on the B cells and T cell receptor (TCRs) (Sundberg et al., 2007). MHCII molecules display peptides to the TCR on $CD4^+$ T which determines if this peptide is a foreign or self-peptide (Sundberg et al., 2007). If the TCR recognizes a foreign peptide, it can trigger a response including the release of inflammatory cytokines and/or signaling for antibody secretion (Hennecke & Wiley, 2001). Another key pathway exists with CD80/86 and CD28. CD28 is a co-stimulatory surface marker found on the T cell that interacts with CD80/86 found on the surface of the B cell (Ville et al., 2015). This pathway helps to stabilize the immune response, heightens the intensity of TCR activation, and acts as a second signal for T cell activation (Fuse et al., 2006). This pathway is negatively regulated by CTLA-4 which is also able to bind CD80/86, with higher affinity (Fuse et al., 2006). Notably, CD40, found on the B cell and CD40L of the T cell, are critical to germinal center formation (Petersone et al., 2018). If CD40L is blocked when there is an active GC reaction, the response is halted (Takahashi et al., 1998). This demonstrates how continuous crosstalk and interactions between the CD40-CD40L are necessary to sustain GC reactions (Takahashi et al., 1998).

The interdependence of B cells and T cells is highlighted beautifully within the germinal centre where Tfh cells are sustained by antigenic stimulation from the B cells (Baumjohann et al., 2013). A GC forms in peripheral lymphoid tissues during T-cell dependent antibody responses to pathogens (Klein & Dalla-Favera, 2008). GC B cells exist in the light-zone and dark-zone of the GC and undergo somatic hypermutation (SHM) and class switch recombination (CSR) with the support of Tfh cells to produce a diverse range of high affinity antibodies (Kräutler et al., 2017). Without B cells, immature Tfh cells would be unable to transition to the mature Tfh phenotype within the GC (Kerfoot et al., 2011). Additionally, there is a positive correlation between the numbers of Tfh cells and GC B cells present in a GC at any given time, providing further evidence of their close relationship (Baumjohann et al., 2013).

Within the GC, B cells differentiate into either plasma or memory B cells. Plasma cells are responsible for creating and secreting secondary specific antibodies (Allen & Sharma, 2023). Plasma cell differentiation is induced in a specific subset of high affinity GC B cells (Kräutler et al., 2017). These GC B cells receive support from the Tfh cells to increase activation of NF-κB within the B cell (Lau & Brink, 2020). The upregulation of NF-κB leads to increased expression of IRF4 which is the transcription factor necessary to induce plasma cell differentiation (Lau & Brink, 2020). The plasma cells exit the dark zone of the GC with increased life span and are sent to the bone marrow (Meyer-Hermann et al., 2012). Memory B cells also require support from the Tfh cells but typically have lower affinity than plasma cells for the antigen (Lau & Brink, 2020). The function of memory B cells are to remember primary encounters with antigens and to initiate effective protection against secondary infections (Crotty et al., 2003).

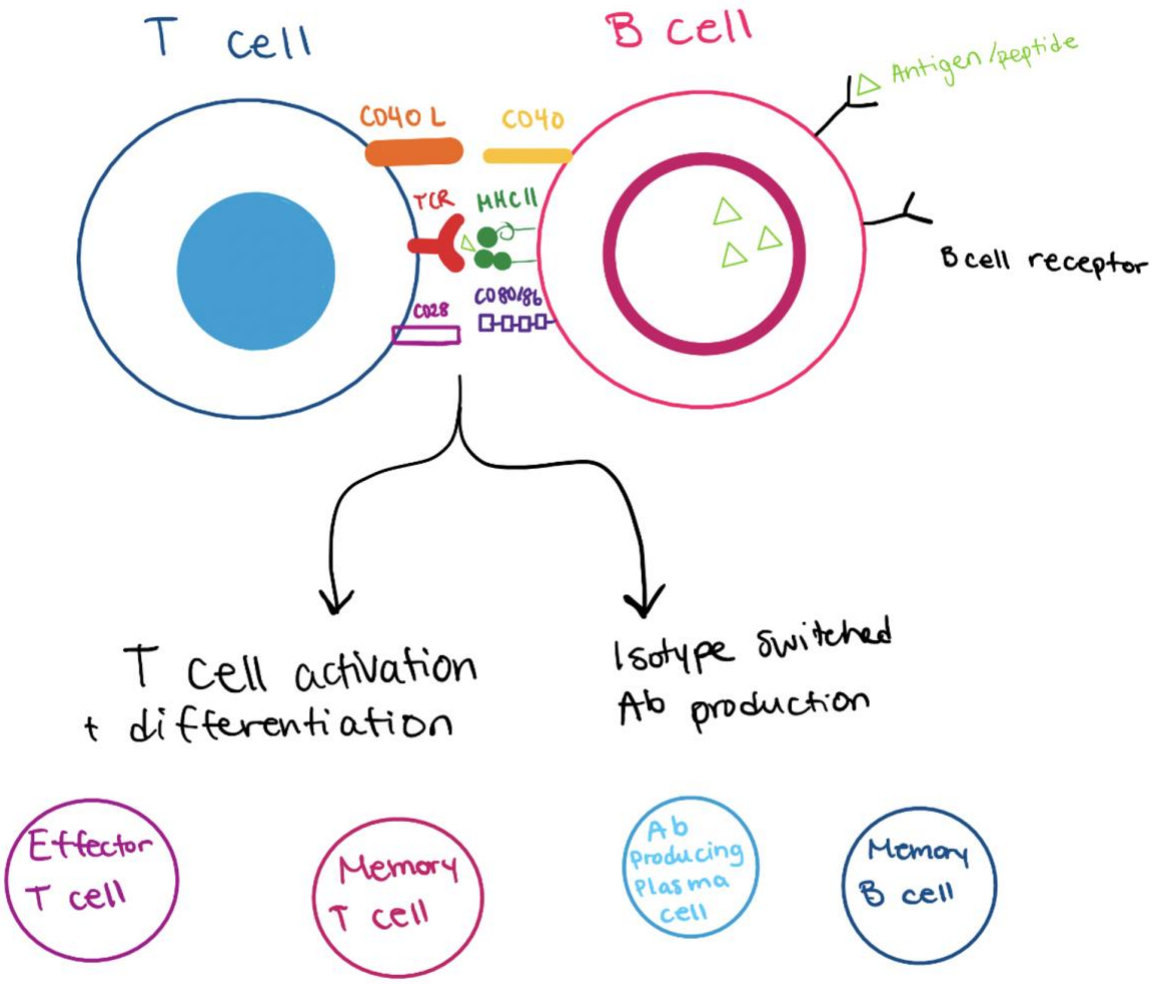


Figure 1.4 : B and T cell interactions

A figure depicting several important interactions between B and T cells. Once B cell receptors have recognized an antigen, activated B cells can then process and present antigens using MHC Class II that is recognized by TCRs. CD40 ligand and CD28 are then able to ligate with CD40 and CD80/86 respectively on the B cells.These interactions along with cytokine production will allow the B and T cells to differentiate into effector and memory subsets. B cells become antibody producing plasma and memory B cells, while T cells mature into effector or memory T cells.

1.6.2.2 Antibody involvement in Chlamydia clearance and controversy

Antibodies play an important role in the immune system to protect the host. They have several functions, which include the neutralization of the pathogen, phagocytosis, antibody-dependent cellular toxicity, and complement-mediated lysis of pathogens and/or infected cells (Forthal, 2014). Antibodies also play an important role in vaccines. Introducing foreign pathogens in a vaccine allows the body to encounter the antigen safely and develop specific antibodies and memory cells targeted to this antigen (Ginglen & Doyle, 2023). Subsequent encounters with the pathogen are met with a robust and rapid immune response because the immune system has already been primed (Ginglen & Doyle, 2023). Without a vaccine, the symptoms and side effects of a primary exposure to certain pathogens may prove to be fatal before the host immune system is able to mount an appropriate immune response (Ginglen & Doyle, 2023).

The importance of antibodies in mediating immunity against *Chlamydia* was first recognized in human studies where *Chlamydia*-specific antibodies have been correlated with lower rates of infection (Darville et al., 2019; Jawetz et al., 1965; Redgrove & McLaughlin, 2014). Since then, *Chlamydia* specific antibodies targeted to the major outer membrane protein (MOMP) have been shown to neutralize infections *in vitro* (Olsen et al., 2015; Peeling et al., 1984). Studies have proven that antibody-mediated neutralization of *Chlamydia* and antibody-dependent cellular cytotoxicity are important mechanisms for B cells in the fight against chlamydial infection (Olsen et al., 2015, 2017; Redgrove & McLaughlin, 2014). B cells also support formation of antibody-antigen complexes that amplify antigen presentation to the CD4+ T cell and encourage phagocytosis of infected cells (Igietseme et al., 2004; Li & McSorley, 2013).

Previously, many studies have used B cell deficient mice (μMT) to examine the role of antibodies in *Chlamydia* and have produced different results (Johansson et al., 1997; Morrison & Morrison, 2005; Su et al., 1997)). Su et al. (1997) provided evidence that μMT mice were able to control chlamydial infections in a comparable manner to their WT counterparts, but took longer to clear a secondary infection. Conversely, in the same year, Johansson et al. (1997) argued that although μMT mice lacked *Chlamydia* specific antibodies, they have almost complete immune protection similar to WT mice against reinfection. Similarly, Ramsey et al. (1988) found no difference in resistance to reinfection between μMT mice and WT mice, whereas Yang et al.

(1998) concluded that μMt mice are not immune to secondary challenge. The Morrison group (2005) used μMT mice to provide evidence that antibodies are important for immunity against genital tract reinfection by *Cm*. They noted that this antibody mediated protection heavily relied on $CD4^+$ T cell priming during primary infection (Morrison & Morrison, 2005).

Differing results between studies may be attributed to the multifactorial effector functions of B cells. B cells are a large component of our secondary lymphoid organs and play an important role in maintaining the lymphoid architecture (Li & McSorley, 2015; Petersone et al., 2018). Thus, the responses seen in μMT mice with defective $CD4^+$ T cell responses could be related to the disruption of lymphoid structures along with poorer antigen presentation to the T cells (Li & McSorley, 2015). B cells also play an important role in the maintenance of macrophages in the draining lymph nodes (Moseman et al., 2012). A defect in the maintenance of important phagocytic cells in infected μMT mice, combined with the lack of cytokine response in μMT mice, may explain why some studies see increased dissemination of *Chlamydia* in μMT mice (Li & McSorley, 2013; Moseman et al., 2012). When knocking out B cells, there is loss of the antibody response, but also a lack of cytokine response, and loss of antigen-presentation to support $CD4^+$ T cell priming. However, the exact relevance of B cells and antibody-mediated protection in *Chlamydia* pathogenesis must be further investigated.

1.7 Activation-induced Cytidine Deaminase

AID is encoded by the *AICDA* gene and its expression is induced by activated B cells, and CD154:CD40 signaling (Petersen-Mahrt et al., 2002) and activation of NF-κB pathways (Çakan & Gunaydin, 2022). Its expression is further supported by IL-4 and TGF-β signaling by $CD4^+$ T cells and TLR-activating antigen molecules (Çakan & Gunaydin, 2022; Xu et al., 2007). Negative regulation of AID, once infections have been cleared, is regulated by the potential negative regulators IRF4, BLIMP1,ID3, and ID2 (Mechtcheriakova et al., 2012). However, this process of regulation is not yet fully understood and requires further study.

AID was first identified by the Honjo group in 1999 through cDNA cloning (Muramatsu et al., 1999) where they deemed it as a new member of the RNA-editing deaminase family and speculated that it was involved with germinal center B cells in SHM and CSR (Muramatsu et al.,

2000). It has since been revealed that AID is a DNA cytidine deaminase that initiates SHM and CSR by deaminating deoxycytidines(dC) to deoxyuracils (dU), triggering DNA repair mechanisms. In SHM, AID deaminated dCs to dUs in antibody variable region genes during active immune responses (Stavnezer, 2011). The dUs can be removed by base-excision repair enzymes and the mismatch repair pathway(Dominguez & Shaknovich, 2014) (**Figure 1.5**). These repair pathways are prone to error, resulting in the generation of antibodies with high affinity antigen binding sites. These new high affinity antibodies that have diverse receptors and specificities with slight variations, compete for the limited resources and space in the germinal centre, and there is a positive selection for B cells producing antibodies with the highest affinity for the antigen (Durandy et al., 2006).

In CSR, AID activity introduces double stranded breaks (DSBs) in the DNA sequence within the switch regions that are located upstream of the immunoglobulin heavy chain constant region genes by deaminating dC residues at the top and bottom of the switch regions (Stavnezer, 2011) (**Figure 1.5**). This triggers the DNA repair mechanism whereby classical and alternative non-homologous end joining attempt to repair the DSB and allow the B cells to switch from expressing IgM to IgG or other secondary specific antibodies with the same antigen specificity (Dominguez & Shaknovich, 2014).

CSR modifies the ability for B cells to produce different isotypes, but these isotypes have the same antigen affinity because CSR does not affect the variable region. In contrast, SHM modifies the antigen specificity due to the changes in the variable region but do not have an effect on the isotype being expressed because it does not involve mutating the switch regions.

Outside of CSR and SHM, there has been evidence that AID expression in young transitional B cells can decrease self-antigen recognition (Çakan & Gunaydin, 2022). The central tolerance checkpoint for autoreactive B cells occurs within the bone marrow prior to peripheral tolerance where receptors are edited and deleted (Kuraoka et al., 2018). In AID deficiency models, the immature B cells are highly resistant to tolerization and B cell receptor induced apoptosis which can lead to impaired B cell tolerance and increased serum autoantibodies in AID deficient mice

(Kuraoka et al., 2011), supporting the idea that B cells play an important role in regulating central B cell tolerance.

Due to the highly mutagenic potential of AID, its expression is very tightly regulated in an effort to protect the body from potential aberrant expression of AID and its ability to target non-Ig genes. For example, there has been evidence that malfunction of repair machinery in response to AID-mediated mutations may contribute to the development of tumorigenesis (Dominguez & Shaknovich, 2014) and that wrongful expression of AID can lead to the development of B cell leukemias and lymphomas (Park, 2012). Although AID is critical to the formation of diverse, high affinity specific antibodies, there remains the possibility that when triggered in response to infections, deviant expression of AID may occur and lead to genome-wide targeting by AID, resulting in genomic instability if chromosomes are translocated and point mutations introduced where they should not be, both in B cells and non-B cells, ultimately resulting in the formation of cancer (Park, 2012).

Thus, it is imperative that AID is properly regulated, and this can be done through epigenetic regulation. DNA hypermethylation at the promoter of AICDA suppresses the expression of AID in B cells (Fujimura et al., 2008) whereas the AICDA gene is demethylated and enriched in H3k4me3 and H3k9ac/K14ac once B cells are activated (Crouch et al., 2007). The methylation of cytidines can protect DNA from the mutagenic effects of AID but it is not a universal safeguard against AID activity, especially if cytidines are located next to CpG motifs (Larijani et al., 2005).

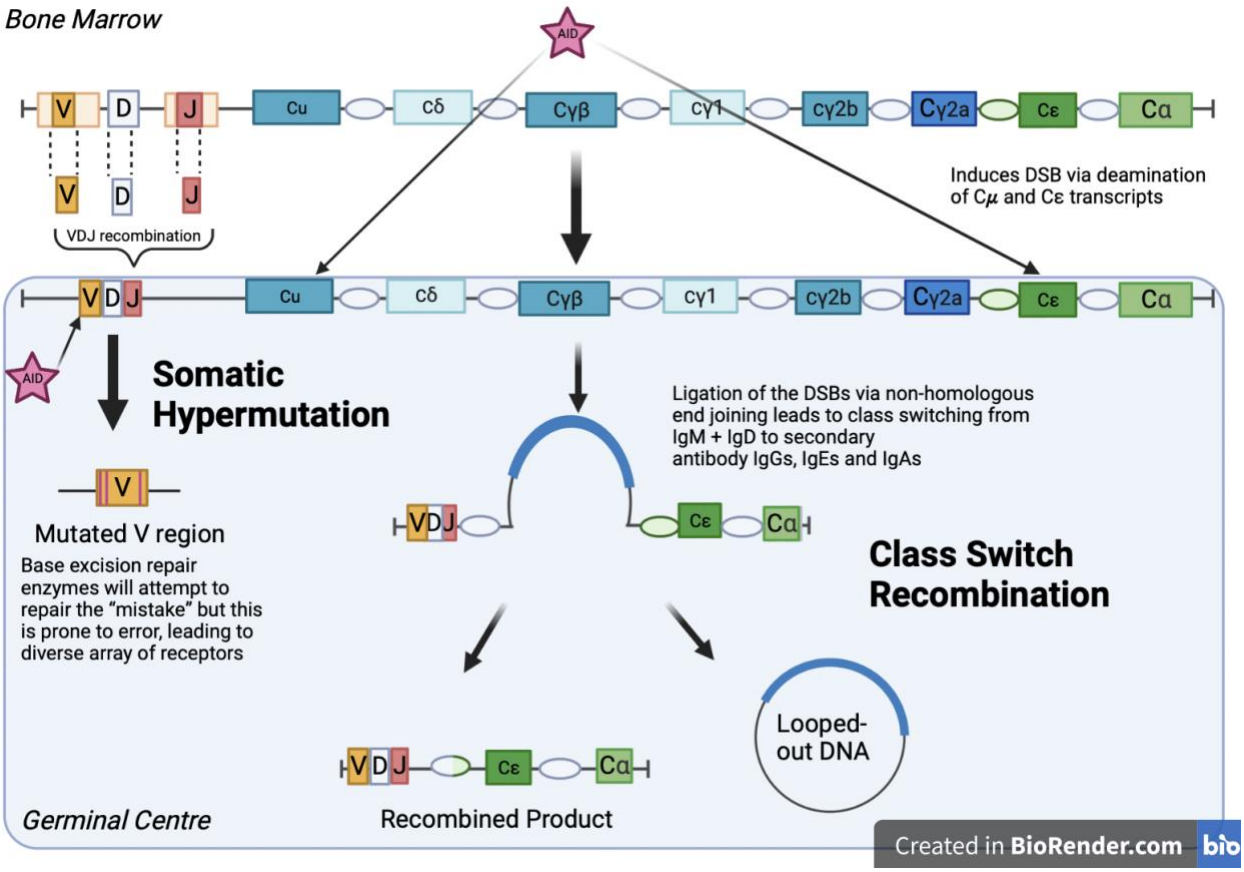


Figure 1.5 : Diagram of the conventional role of AID

The rectangles represent exons while the ovals represent switch regions. Recombination of the VDJ region occurs in the bone marrow, while CSR and SHM occur in the germinal centre. SHM introduces mutations into the recombined V exon to create B cells with diverse antigen specificity. CSR brings the constant region (Ce) closer to the V exon by deleting the regions upstream of the target constant region and switches the B cells to switch isotypes.

Adapted from: https://www.nature.com/articles/35080033/figures/1?proof=t%2Btarget%3D

1.8 AID deficiency in humans

AID deficiency is an extremely rare primary immunodeficiency for humans with Durandy et al. (2013) only being able to locate 45 patients scattered around the world to conduct their investigations on autosomal recessive AID-deficiency. They characterized AID deficiency in humans as a drastic defect in CSR which includes normal or increased levels of IgM and no detectable IgG or IgA secondary antibodies in the serum, as well as a complete lack of SHM in all but two patients. Furthermore, these patients have regular levels of lymphocytes but a decrease in $CD4^+$ T cells, significantly decreased levels of Tregs and lymphadenopathy which showed enlarged germinal centres. Due to the abnormal function of the immune system, AID deficient humans have increased susceptibility to infections and are prone to the development of autoimmune diseases (Quartier et al., 2004), increased levels of serum autoreactive antibodies, and inflammatory disorders (Durandy et al., 2013). Similarly, other studies have found that AID mutations in patients with hyper-IgM syndrome leads to a lack of CSR, SHM, in addition to lymph node hyperplasia with high levels of apoptosis (Revy et al., 2000).

The pathogenesis of autoimmunity seen in cases of AID-deficiency has not yet been fully elucidated but there are theories that seek to explain this phenomenon. A lack of AID may result in intrinsic B cell defects that stem from issues in both central and peripheral B-cell tolerance (Meyers et al., 2011) and defects in the control of B cell proliferation pinpointed by the enlarged germinal center phenotype. The decrease in Treg populations is also likely to allow for the survival of autoreactive T cells which would further exacerbate autoimmunity phenotypes (Durandy et al., 2013). Due to the extensive cross-talk between T and B cells within the immune system, these hypotheses are not exclusive but may work together to create severe defects in the immune system response that encourages development of autoimmunity and inflammatory conditions.

1.9 AID deficient mice

AID deficient mice, also known as AID knock out (AIDKO) mice, present a scenario where the innate immune response and B cell cytokine production remain intact, and there is a selective loss of specific antibodies with intact formation of germinal centers (Muramatsu et al., 2000). AID deficient mice present with significantly greater serum autoantibody, decreased capacity to

eliminate autoreactive B cells, lack CSR and SHM, and demonstrate a hyper IgM phenotype (Chen et al., 2010; Kuraoka et al., 2011; Muramatsu et al., 2000). These phenotypes indicate that AID may play important roles in negative regulation of systemic autoimmunity along with their known role in antibody diversification. Furthermore, AID deficient mice with murine lupus developed severe splenomegaly and suffered from extreme glomerulonephritis when compared to AID-competent controls, further supporting the concept that AID contributes to healthy negative regulation of the immune response (Chen et al., 2010).

Due to the highly mutagenic and potential tumorigenic nature of AID activity, many researchers have used AID deficient mice in cancer studies (Nonaka et al., 2016; Schubert et al., 2021) as well as acute infection studies, particularly those looking at the relationship between AID and inflammation (Hale, 2020). AID deficient mice are a great model for demonstrating the involvement of exact effector functions of B cells due to the ability to remove specific antibody response but maintain other effector functions from B cells such as cytokine signaling and antigen presentation (Malaviarachchi et al., 2020).

1.10 Animal models of Chlamydia infection

Both *in vitro* and *in vivo* models for chlamydial infection have been established and used in research over time. *In vitro* modeling can take the form of 2D cell-culture models involving immortalized cells like HeLa cells and McCoy cells, as well as HEC-1B, CaCo2, Hep2, and monocyte-macrophages (Filardo et al., 2022). These models have been used to isolate *Ct* from collected specimens and to study the biology and both molecular and cellular pathways of *Chlamydia* infection. More recently, there have been advancements in 3D cell-culture models that can help mimic the microenvironment that *Ct* exists in within host tissue, providing more insight into host-pathogen interactions through direct cell-to-cell contact (Nogueira et al., 2017). Among the many variations of 3D cell-culture models, human organoid models have been popular for studying *Ct*, with studies employing endometrial organoids generated from murine primary cells, ectocervical organoids from primary human cells as well as primary human fallopian tube organoids to study a chronic infection model of *Ct* and the effect on tubal pathology (Filardo et al., 2022).

In vivo studies generally involve several popular animal models to study female genital tract infections with *Ct*. Species of nonhuman primates such as the marmoset, grivet, baboon, and pig-tailed macaque have been previously used to study *Ct* genital tract infection (De Clercq et al., 2013). Most often, the pig-tailed macaque is preferred due to the high degree of similarity in the anatomy and physiology of the female reproductive tract between this species and humans. Pig-tailed macaques also have a 28-30 day menstrual cycle with vaginal microflora that is comparable to women (Patton et al., 1996). Guinea pigs have also been used along with the strain of *Chlamydia caviae* which causes guinea pig inclusion conjunctivitis (Murray, 1964). An advantage of this animal model is the ability for the infection to be transmitted sexually and perinatally (Filardo et al., 2022). Pig models have also been selected as a potential alternative animal model due to their highly ubiquitous expression of genes expressed between the porcine female reproductive tissues and human genital tissue (Tuggle et al., 2003).

Although many animal models exist to study genital *Ct* infections, by far the most used animals are mice. Mice are easy to handle, available in large numbers and there are many well-characterized inbred or knockout strains of mice available (Vanrompay et al., 2006). *Chlamydia muridarum* (*Cm)* establishes an infection in female mice that very closely resembles the protective immune responses and pathology seen in women infected by *Ct* (de la Maza et al., 1994) and infection by both *Ct* serovars and *Cm* are possible in mice (Barron et al., 1981). Furthermore, many murine models use the practice of synchronizing mouse estrous cycles prior to infection to decrease the confounding factors at play involving different hormone levels between specimens (De Clercq et al., 2013).

Cm infection of the murine female reproductive tract establishes a 4-8 week long genital infection that can be resolved spontaneously without treatment (Dockterman & Coers, 2021). Primary infection of *Cm* in murine models can elicit a robust immune response and genital pathology in mice, similar to what is seen in humans. By days 14 and 21 post infection, significant inflammatory infiltrate, mostly dominated by neutrophil populations in the oviduct can be detected in *Cm* infected mice, but resolves as early as 28 days post infection (Shah et al., 2005). After 28 days post infection, along with later timepoints, symptoms such as uterine horn dilation, tissue fibrosis and occlusion of the oviduct lumen can lead to oviduct dilation, otherwise

known as hydrosalpinx (Shah et al., 2005). The scarring and tissue fibrosis from genital *Cm* infection in mice has been correlated with infertility after primary *Cm* infections in mouse models. In contrast, it often takes a secondary *Ct* infection to establish significant pathology in human female reproductive tracts (Brunham & Paavonen, 2020) but the specific immune mechanisms influencing the development of pathology in humans has not been clarified and requires further research.

1.11 Rationale

Ct is still responsible for a large disease and socioeconomic burden around the world. Although antibiotics can cure symptomatic infections, a large majority of chlamydial infection go undetected which leads to cases of chronic infection and frequent reinfection. In these cases, women are prone to developing severe tissue fibrosis and tissue damage that can result in infertility and other ailments such as pelvic inflammatory disorder (Tjahyadi et al., 2022). Furthermore, antibiotics are currently the only treatment available for this disease, but the need for *Chlamydia* vaccines to decrease the healthcare burden of this bacterial infection has become more apparent as numbers continue to rise and a gap in knowledge into the pathophysiology caused by the host immune response remains (Phillips et al., 2019). In theory, vaccinating against chlamydial infections would be a great way to decrease the spread of infection. However, introducing chlamydial antigens primes the host immune system to respond with antibody mediated mechanisms of destroying the pathogen if the host is exposed to chlamydial antigens naturally. Unfortunately, the specific roles of antibodies in chlamydial infection have not yet fully been elucidated and this information needs to be further researched prior to the creation of a successful vaccine. If not, vaccinations may result in greater development of pathology in cases of infection and cause more harm than benefit.

The current understanding about the role of antibodies in chlamydia pathogenesis is that they may not play a critical role in clearing primary infection, but appear to have more of a role in protecting against reinfections (Su et al., 1997). Recent studies have come to this conclusion using μMT knockout mice. μMT knockout mice are able to clear *Chlamydia* from the female reproductive tract similarly to wild type controls (Malaviarachchi et al., 2020) but that wild type mice that are able to produce antibodies are more resistant to infection after secondary challenge

than the B cell deficient mice without *Chlamydia* specific antibodies (Su et al., 1997). Meyers et al. (2011) have also demonstrated that AID is necessary for both central and peripheral B cell tolerance and that a deficiency of AID leads to poor B cell tolerance and development of autoreactive B cells. Current knowledge in the field indicates that AID is also crucial to immune tolerance in addition to its role in the production of high affinity antibodies.

To this date, there is no evidence in the literature that the specific role of AID in *Chlamydia* pathogenesis has been investigated. Thus, it is reasonable to determine the functional role of AID in *Chlamydia* pathogenesis to provide insight into the role of AID as well as the role those specific antibodies play in pathology development and clearance of infection.

1.12 Hypothesis

Current knowledge in the field indicates that AID is crucial to immune tolerance in addition to its role in the production of high affinity antibodies. I hypothesize that AID deficiency promotes tissue pathology development following a secondary challenge through an antibody-independent mechanism.

1.13 Objectives

There are three main objectives to this project. The first is to determine the impact of the AID gene in controlling bacterial shedding by comparing differences in bacterial shedding between the AIDKO and WT strains of mice. The second objective is to determine the impact of AID gene in controlling Chlamydia induced tissue pathology. This includes examining gross pathology and oviduct dilation post infection in both strains of mice. Finally, the third objective is to determine the impact of the AID gene in B cell response and T cell response to chlamydial infections.

CHAPTER 2 MATERIALS & METHODS

2.0 Chlamydia strain and Purification of *Chlamydia muridarum*

Chlamydia muridarum (*Cm*) was originally obtained from Xi Yang (University of Manitoba, Winnipeg, Manitoba) and propagated in McCoy cells (American Type Culture Collection) according to procedures described previously (Brown et al., 2012). Briefly, McCoy cells at 90-100% confluency in 150mm culture dishes were infected with Cm crude stock for 48 to 72 hours. On purification day, the culture supernatant containing infectious EB was collected into 50 mL conical tubes (Sarstedt), which were first centrifuged at 500 xg for 10 minutes at 4ºC in a Beckman Coulter Allegra X-12R centrifuge to remove cellular debris. The EB-containing supernatant was then transferred into ultracentrifuge tubes, which were balanced and centrifuged at 22,500 x G for 1 hour at 4 ºC in a Beckman Coulter Optima L-100 XP Ultracentrifuge. After this centrifugation, the supernatant was discarded and the EB-containing pellet was resuspended in 2 mL of Sucrose-phosphate-glutamic buffer (SPG), aliquoted into small volume (20 µL), and stored at -80 ºC until setting up experiments. The EB was also purified from the monolayer using discontinuous density gradient centrifugation. A two-layer cushion was formed in ultra-centrifuge tubes using 30% Isovue-370^{TM} (Bracco Diagnostics) and 50% sucrose. Both the Isovue-370^{TM} and sucrose were diluted using 30 mM Tris-HCl. The cushion was created by adding 10 mL of the 50% sucrose solution to each tube and then slowly adding the 10 mL of the 30% Isovue-370^{TM} on top of the sucrose solution. The SPG containing monolayer-derived *Cm* was then added on top of each cushion and the tubes were balanced and centrifuged at 22,500 x G for 1 hour at 4 ºC with a slow acceleration and the break off. Following this centrifugation, the pellet was washed with SPG buffer twice then resuspended in 4 mL of SPG buffer. Once the pellet was resuspended, the monolayer-derived *Cm* was aliquoted into 30 and 100 µL tubes and stored at -80 ºC for future use. *Cm* titre was quantified as the number of inclusion-forming units (IFUs) per mL using quantitative Polymerase Chain Reaction (qPCR) as described in the section of **2.7.3**.

To make heat killed Cm (HK*Cm),* aliquots of purified *Cm* were incubated at 70 ºC for 30 minutes. This HK*Cm* was used as chlamydial antigens for *in vitro* recall stimulation and in antibody ELISAs.

2.1 Mice and *In vivo* procedures

C57BL/6 wild type mice as well as C57BL/6 mice CD45.1 congenic mice were bred in-house and used as the 'wild-type'(WT) strain for this project. Activation-induced cytidine deaminase knock out (AIDKO) mice were originally obtained from Dr. Tasuku Honjo and then bred in-house for the study. Mice between the ages of 6 to 10 weeks of age were used for this work. The animals were housed in pathogen-free conditions within the Carleton Animal Care Facility (CACF) at Dalhousie University. All protocols for this project were approved by the animal care committee at Dalhousie University, in line with the guidelines provided by the Canadian Council of Animal Care.

2.1.1 Genotyping

AIDKO mice used for breeding were genotyped to confirm the absence of the AID gene. All genotyping work was performed by Dr. Julia Liu at the IWK Health Centre. Spleens were excised from naïve AIDKO and WT mice. RNA was isolated from the spleens using RNeasy Mini Kit (Qiagen, Canada). Following RNA extraction, cDNA was synthesized with the QuantiTect RT kit (Qiagen, Canada). AID gene specific primers 118: 5'-GGC-TGA-GGT-TAG-GGT-TCC-ATC-TCA-G-3'; and 119:5'-GAG-GGA-GTC-AAG-AAA-GTC-ACG-CTG-GA-3', were used (Integrated DNA Technologies, Iowa, US). The primers were combined with the cDNA to be run in an RT-PCR. The RT-PCR cycle had a 5-minute initial activation step at a temperature of 95° C, before 42 cycles were run at 72° C with the final extension step lasting 10 minutes. After the RT-PCR, the products were run on a 1% agarose gel with electrophoresis to reveal genotyping results (**Figure 2.1**).

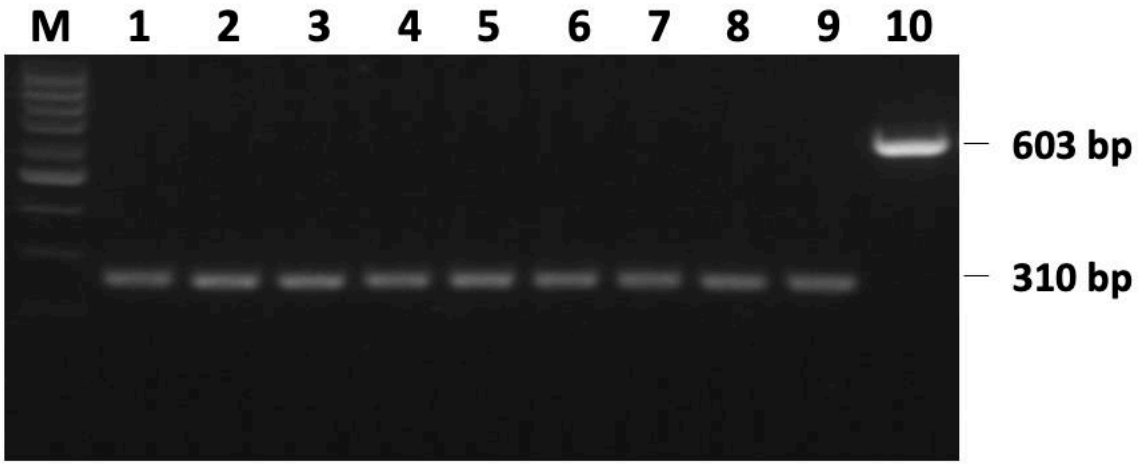


Figure 2.1: AIDKO breeders lack the AID gene

The figure depicts the genotyping results for the AIDKO mouse breeders compared to a WT control mouse. The AIDKO gene length is 310 base pairs while the WT gene is 603 base pairs. Columns 1-3 show the results for the male and two female mice of breeding cage #1. Columns 4-5 show the results for male and two female mice of breeding cages #2. Columns 7-9 show the results for the male and two female mice of breeding cage #3. Column 10 shows the genotyping results for the WT mouse used as a control (Picture courtesy of Dr. Julia Liu).

2.1.2 Intravaginal Cm infections

Mice estrous cycles were synchronized using 2.5 mg subcutaneous injections of Depo-Provera (Pfizer, New York) diluted with phosphate buffered saline (PBS) at 10 and 3 days prior to initial *Cm* infection (**Figure 2.2**). Isofluorane was used to anesthetize animals prior to injections. On days 0, 2, 4, 6 and 8, mice were infected intravaginally with 1.35 x 10^4 IFU *Cm* resuspended in 10µL of SPG, monitored every 2-3 days and sacrificed on days 17, 30, 52 and 132 post infection. For the challenge experiment, pre-infected mice and a group of naïve mice were infected intravaginally with 3 x 10^4 IFU *Cm* on day 130 and monitored daily for a week (**Figure 2.3**).

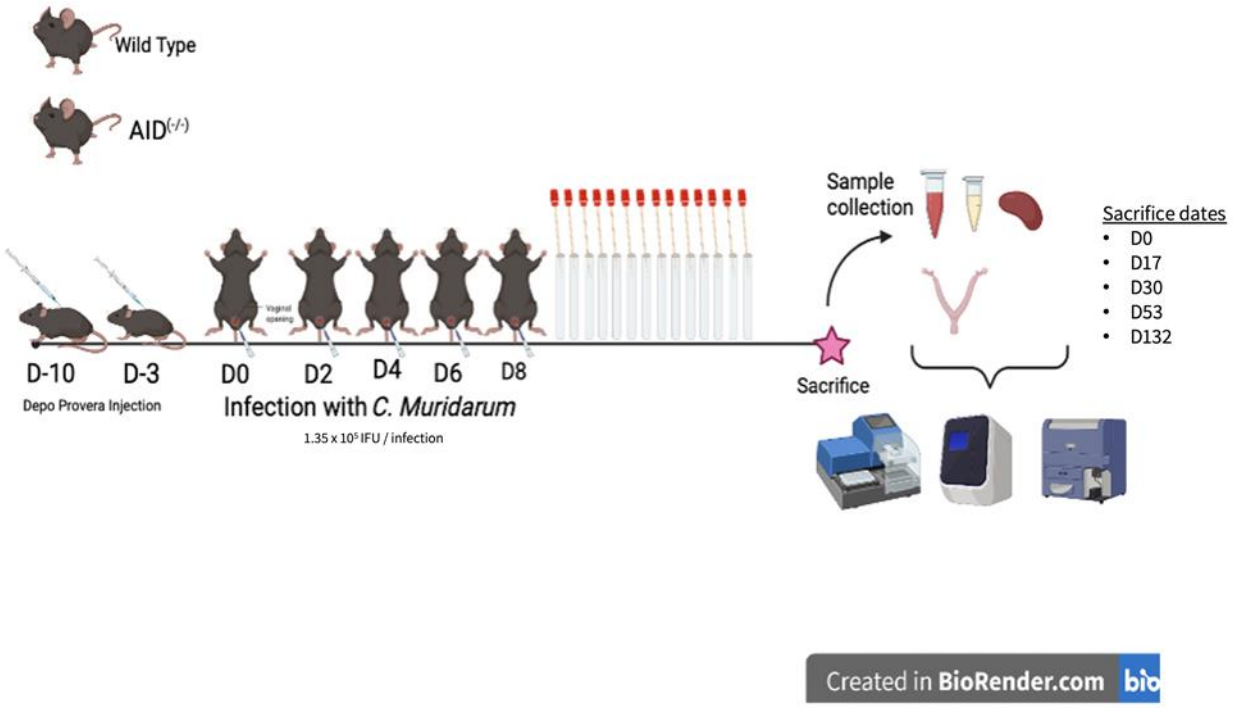


Figure 2.2: Diagram of the general in vivo study design

This model involves the use of AIDKO mice and WT mice. Mice are given Depo-Provera injections 10 days and 3 days out from initial infection in order to regulate their hormone levels and remove hormonal differences as a confounding factor. Next, the mice are given 5 low dose infections (1.35 x 105 IFU / infection) every other day over the course of 10 days. SPG is used as a mock-infection for control groups.

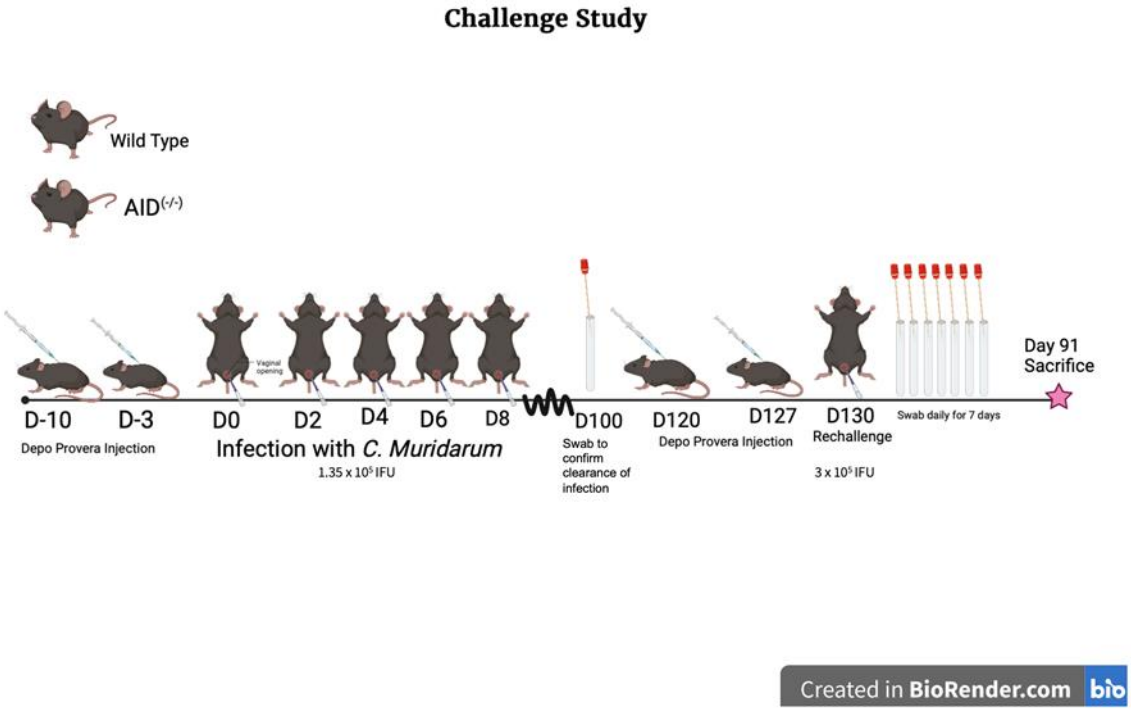


Figure 2.3: Diagram of the re-challenge in vivo study design

This model involves the use of AIDKO mice and WT mice. Mice are given Depo-Provera injections 10 days and 3 days out from initial infection in order to regulate their hormone levels and remove hormonal differences as a confounding factor. Next, the mice are given 5 low dose infections (1.35 x 105 IFU / infection) every other day over the course of 10 days. SPG is used as a mock-infection for control groups. The mice are given time to naturally clear the infection prior to 100 days post infection where a vaginal swab is taken for qPCR to check for complete clearance of infection. Depo-Provera injections are given 10 and 3 days out from the re-challenge date. On day 130, the mice are re-infected with a single higher dose of Cm (3 x 105 IFU / infection) and swabbed for 7 consecutive days before the sacrifice.

2.1.3 Vaginal swabbing

Intravaginal swabbing of the mouse vaginal canal was performed over the course of infection to assess the levels of bacterial burden. Calcium alginate nasopharyngeal swabs (Puritan, Maine, USA) were inserted into the vaginal canal. The swab was then rotated clockwise for 15 seconds and then counter clockwise for 15 seconds. Once the swabs were collected, they were placed into a 1.5 mL Eppendorf tube containing 350 µL of SPG and a sterile glass bead. The handle of the swab was cut to fit inside the closed tube and specimens were stored on ice until they were ready for further processing. The tubes were subsequently vortexed for 30 seconds each and then placed into a tube revolver (ManSci Inc. Lab Revolution) to be centrifuged for 1 hour at 4ºC. Post centrifugation, 100 µL of the sample was directly aliquoted into 500µL of DNAzol (Molecular Research Center Inc., OH, USA) and stored at 4ºC until the genomic (g)DNA isolation was performed. The remainder of the sample was aliquoted into a sterile 500 µL Eppendorf and stored at -80ºC.

2.1.4 Sacrifice

Mice were sacrificed at different end points (Days 0, 17, 30, 52 and 132 post primary infection and day 7 post challenge) to gather a full immune profile throughout an active infection. An incision was made on the ventral side of the mouse and the spleen and genital tracts were harvested and placed into RPMI (Cytiva, USA) containing 5% Bovine Serum and placed on ice until further processing. Photos of the excised genital tracts were taken at sacrifice against a green coloured paper inside of a plastic paper protector for consistency between experiments. A ruler was used in the photo to help gauge the size of the genital tracts and cysts.

Blood samples were collected on the sacrifice day for serum collection. A 23-gauge BD PrecisionGlide™ needle was used to perforate the skin of the cheek and give access to the submandibular vein for blood collection. 250 µL of blood was collected into a 1.5 mL Eppendorf for serum isolation. The blood was allowed to clot at room temperature for 30 minutes prior to being centrifuged at 4 ºC in a Thermo Scientific Sorvall Legend Micro 21R benchtop centrifuge for 30 minutes at 21,000 x g. Using a micropipette, the serum above the clot was collected and stored at -80ºC until antibody ELISA assay.

2.1.5 Single cell splenocyte isolation

To create a single-cell suspension of splenocytes, whole spleens were crushed between 2 sterile glass slides into a petri dish of 10 mL of RPMI mixed with 5% BS. Each slide was rinsed with 5 mL of the RPMI + 5% BS mixture and the total volume (20 mL) was transferred to a 50 mL conical tube. The petri dish was rinsed with 10 mL of RPMI + 5% BS and this was added to the 50 mL conical tube. The tubes were centrifuged in a Beckman Coulter Allegra X-12R centrifuge at 525 xg in 4 ºC for 10 minutes. The supernatant was discarded, and the pellet was resuspended gently with a vortex. After resuspension, 5 mL of ammonium-chloride-potassium (ACK) lysis buffer was added to the tube to lyse red blood cells (RBC). This mixture was incubated for 5 minutes at room temperature (RT), then 20 mL of RPMI + 5% BS was added to stop the reaction. The splenocytes were subsequently filtered through 40 µm cell strainers (Sarstedt, Germany) into new conical tubes. 5 µL of cells were taken from the tube and diluted with 45 µL of Trypan Blue (Gibco, Oakville, ON) for counting using a hemocytometer (Hasser Scientific, Horsham, PA) under a Leica DML microscope. While cells were being counted the tubes were centrifuged in a Beckman Coulter Allegra X-12R centrifuge at 525 xg in 4 ºC for 10 minutes prior to the cells being resuspended in RPMI + 5% BS at a concentration of 5x 10^6 cells/mL. The cells are then used for flow cytometry staining or seeded into tissue culture dishes for *in vitro* recall stimulation.

2.1.6 Spleen *in vitro* recall

To observe cytokine recall responses, splenocytes were re-suspended in complete (c)RPMI medium with 1 x 10^6 cells in 200 µL per well in a 96 well U-bottom tissue culture plate (Sarstedt). Each sample was plated in sextuplicate for each stimulation condition of which there were 4; culture medium alone, dilutions of heat-killed *Cm* at 1/50 (4.8 x 10^6 IFU/ mL), 1/100 (2.4 x 10^6 IFU/ mL), 1/200 (1.2 x 10^6 IFU/ mL), or 0.05 µL/well of anti-mouse CD3 (Bio X Cell, Lebanon, NH). All cultures were incubated at 37 ºC for 72 hours. Following the 72-hour incubation, each plate was centrifuged for 20 minutes at 600 xg at 4 ºC in an Allegra X-12R centrifuge. Following the centrifugation, the supernatant was collected and stored at -20 ºC for future Cytokine ELISAs.

2.1.7 Single cell isolation of murine genital tract

To create a single-cell suspension of genital tract cells, the genital tract was bathed in 2.5 mL Hanks Balanced Salt Solution (HBSS) (Wisent Bio products) and minced finely with surgical scissors in their collection tubes. The tubes were centrifuged at 525 xg at 4 ºC for 10 minutes in a Beckman Coulter Allegra X-12R centrifuge. The supernatant was collected and stored at -80 ºC, the minced tissue was resuspended in 2 mL of 1X PBS and centrifuged down at 525 xg at 4 ºC for 10 minutes in a Beckman Coulter Allegra X-12R centrifuge to wash away fat and debris. The 1X PBS supernatant was discarded and the tissue was resuspended in a mixture of 2.5 mL of HBSS and 25 units of collagenase I, collagenase II, and collagenase IV each (Sigma Aldrich). The samples were inverted 5 times to mix the solution and then placed in a 37 ºC water bath for 35 minutes, with the tubes being inverted every 5-10 minutes to ensure even digestion of the tissue. After incubation, the samples were removed from the water bath and filtered through a 40 µm cell strainer (Sarstedt) into a 50 mL conical tube and centrifuged down at 525 xg at 4 ºC for 5 minutes in a Beckman Coulter Allegra X-12R centrifuge. The remaining tissue was scraped off the filter into the original tube and the supernatant from the conical tube was transferred back to the remaining tissue for further digestion for 40 minutes in the water bath. The cell pellet in the 50 mL conical tube was resuspended in 2 mL of HBSS. After completion of the second incubation, the filter centrifugation was repeated with an additional rinse of the filter using 2 mL of HBSS. After centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 2 mL of RPMI + 5% BS and counted using a hemocytometer with trypan blue staining.

2.2 Genomic DNA isolation and Chlamydia quantification using quantitative Polymerase Chain Reaction (qPCR)

The 100 µL of sample obtained from processed vaginal swabs or 10 µL of purified chlamydia in 90 µL of distilled H_2O was added to 500 µL of DNAzol (Molecular Research Center Inc.). Following a minimum of 10 minutes incubation in DNAzol at room temperature, 250 µL of 100% ethanol was added to the solution and tubes were inverted gently to avoid breaking genomic DNA (gDNA). The 100% ethanol and DNAzol mixture was incubated for 5 minutes at RT to precipitate gDNA. Following this incubation, the samples were centrifuged in a Thermofisher Scientific Sorvall Legend Micro 21R benchtop centrifuge for 5 minutes at a

temperature of 4 ºC at 14,800 xg. The supernatant was carefully discarded from each sample following the centrifugation and tubes remained inverted and were blotted on a clean paper towel and then 750 µL of 70% ethanol was added to the gDNA pellet. The samples were inverted several times to mix the pellet and then centrifuged as previously described. This wash step was repeated, and then while discarding the supernatant after the second wash, a Kim Wipe was carefully used to dab away any remaining supernatant. 100µL of 8 mM sodium hydroxide was added to resuspend the gDNA pellet and 3.8 µL of 1M HEPES Buffer (Molecular Resaerch Center Inc.) was added to adjust the pH to 7.2 prior to storage at -20 ºC until used in qPCR reactions.

The titer of *Cm* in gDNA preparations was quantified using qPCR. Standards and samples were diluted in a separate V-bottom plate. A standard stock of *Cm* gDNA with a titre of 4.75 x10^6 IFU was diluted 10x using UltraPure™ DNase and RNase free distilled water (Thermo Fisher) and then serially diluted 5x to create a standard curve that started at 95,000 IFU of *Cm* down to 7.6 IFU. Each vaginal swab sample was diluted 10x by UltraPure™ DNase and RNase free distilled water. PCR reactions were setup in 10µl volume reactions in 96 well qPCR plates (RNAase-, DNAase-, DNA-, and PCR inhibitor free, Bio-Rad). Each reaction contained 2.5 µL of diluted sample, 5 µL of PerfeCTaSYBR Green Fastmix (Quantabio), 2 µL of UltraPure™ DNase and RNase free distilled water, 0.25 µL of 10 µM forward primer (5'-CGC CTG GG AGT ACA ACTC GC-3'), and 0.25 µL of 10 µM reverse primer (5'- CCA ACA CCT CAC GGC ACG AG-3'). In each PCR run, gDNA pooled from naïve mice were included as a background control.

Once the qPCR plate was loaded, a cover was applied to the plate, then it was centrifuged in an Allegra X-12R centrifuge for a centrifugation at 600×g for 5 minutes at 4 ºC to remove excess bubbles and ensure all liquid was drawn to the bottom of the qPCR plate. A Bio-Rad CFX96 Touch Real-Time PCR Detection System machine was used to run the plate. The qPCR cycle used was: 95 ºC for 2 minutes, 40 cycles at 95 ºC for 10 seconds, followed by 60 ºC for 20 seconds. Once 40 cycles were completed, a gradual decrease began from 10 seconds at 95 ºC, 30 seconds at 65 ºC, 5 seconds at 95 ºC and then a final 10 minutes at 10 ºC. The accompanying Bio-Rad CFX Maestro software was used for establishing a standard curve and further data

analysis. The accompanying software to the Bio-Rad CFX96 Touch Real-Time PCR Detection System machine, CFX Maestro was used to process the data retrieved from the experiment.

2.3 Histopathology: Oviduct dilation

For long term end points (D132 and D137), whole and/or partial genital tracts were harvested, placed in histological cassettes and fixed in 10% formalin. The genital tract samples were then paraffin embedded, sectioned, as well as stained with hematoxylin and eosin (H&E) through the IWK Pathology department. Then, the slides viewed using a Leica DM2500 microscope and digital images were taken at 5x magnification of the oviducts. The largest oviduct and/or cyst in each sample was chosen for further analysis. These images were then analyzed for cross sectional oviduct dilation measured in microns using the NIH Image J software (National Institutes of Health).

2.4 Flow Cytometry

Surface and intracellular markers were stained using fluorescent antibodies to examine the immune response post infection. Samples were fixed after staining and stored away from light at 4 ºC and acquired within 48 hours for surface staining and within 24 hours for intracellular staining. To create compensation controls, OneComp eBeads^{TM} (Thermo Fisher) were stained with single stains of each individual antibody. Acquisition for panels were performed on either the BD FACS Celesta (BD Biosciences) or the BD LSR Fortessa SORP (BD Biosciences). FCS Express 7 (De Novo Software, Pasadena, CA USA) software was used to analyze the raw flow cytometry data.

2.4.1 Flow cytometry for surface staining

Two multi-parameter flow cytometry panels (see **Table 2.1 and 2.2**) were constructed and used to profile immune responses in the single cell suspensions isolated from the genital tracts and spleens, respectively. Cells were plated in a volume of 1 x 10^6 cells / well in a 96 well V-bottom plate. The cells were centrifuged in an Allegra X-12R centrifuge at 525 xg for 5 minutes at 4 ºC and the RPMI + 5% BS supernatant was dumped. Cells were resuspended in 1X PBS and the centrifugation step was repeated to wash excess protein off the cells and prepare them for viability staining. The 1X PBS wash was dumped and the cells were resuspended in 50µL of

fixable viability dye eFluor^{TM}506 (Thermo Fisher), diluted 1:3000 in 1X PBS. The plate was incubated away from light at 4 ºC for 30 minutes. Following the incubation, cells were washed with 200 µL of FACS buffer, the centrifugation step was repeated and the supernatant was dumped. The samples were blocked using 50 µL of FACS buffer with 10% normal rat serum (Thermo Fisher) and they were incubated away from light at 4 ºC for 20 minutes. After blocking, the 1X PBS wash and centrifugation step were repeated. 50 µL of the FACS antibody staining cocktail (**Table 2.1 and Table 2.2**) was added to the samples, and individual fluorescence minus one controls (FMOs) were added to their respective wells. The samples were incubated away from light at 4 ºC for 20 minutes. Following staining, the 1X PBS wash and centrifugation step were repeated two times. For surface stains, the cells were then fixed with 200 µL of FACS Fixing buffer and stored at 4 ºC before being acquired by BD LSRFortessa cell analyzer within 24 hours.

2.4.2 Flow cytometry for intracellular staining

In some experiments, the frequency of Treg cells was determined in the spleen samples. A separate multi-parameter flow cytometry panel was used (**Table 2.3**). Following the surface staining procedure as described above, each sample received 200 µL of IC Fixation Buffer (Thermo Fisher) then incubated at RT for 1 hour. After the 1 hour incubation, the cells were centrifuged in an Allegra X-12R centrifuge at 600 xg for 5 minutes at 23ºC. The supernatant was dumped and the cells were washed with 200 µL of FoxP3 Permeabilization buffer (Thermo Fisher) and the centrifugation step was repeated. The wash and centrifugation step was repeated once more. After the second wash, the supernatant was dumped and the cells were re-suspended in 50 µL of FACS Buffer + 10% rat serum and incubated away from light for 15 minutes at RT. After the 15-minute incubation, the cells were centrifuged at 600 xg for 5 minutes at 23ºC. The supernatant was dumped and cells were resuspended in 50 µL of diluted FoxP3 PE antibody (**Table 2.3**). The cells were protected from light and incubated with the antibody for 30 minutes at RT. After the incubation with the intracellular antibody, the cells were washed with 200 µL of permeabilization buffer and the centrifugation step was repeated. The supernatant was dumped and the rinse and centrifugation steps were repeated once more. Samples were then resuspended in 200 µL FACS Buffer and transferred to 5 mL flow cytometry tubes for acquisition by BD FACSCelesta within 24 hours.

2.5 Enzyme linked immunosorbent assay (ELISA)

2.5.1 Indirect Antibody ELISA

Indirect ELISAs were used to investigate levels of antibody present in serum samples obtained from mice. High-protein-binding, 96 well microplates (Grenier Bio-One) were coated with 50 µL of ELISA coating solution (**Table 2.4**) and either 500 IFU of HK*Cm* per sample or 5 µg of an isolated *Cm* antigen TC0912C,-0582,-0047,-0326. TC0582 and TC0912C are *Cm* proteins that are considered to be pathology-associated antigens (Zeng et al., 2012), while TC0047 and TC0326 are designated as non-pathology associated antigens (Zeng et al., 2012). Pathology designated antigens were preferentially recognized by mice that developed hydrosalpinx post infection with *Cm*. The coated plates were placed at 4 ºC overnight.

The following morning, the plates were washed 3 times with 200 µL of ELISA wash buffer (**Table 2.4**) in a BioTek plate washer. The wash was dumped over a sink and blotted onto paper towel. The plates were then blocked with 200 µL of ELISA Blocking buffer (**Table 2.4**) and incubated for 2 hours at RT. Serum samples were serially diluted using ELISA Blocking buffer with a starting concentration of 1:50 in a separate 96-well V bottom plate (Sarstedt). After the 2-hour incubation, the 3-cycle plate wash and blotting steps were repeated. Diluted serum samples or a negative control (ELISA wash buffer) was loaded into each well and plates were placed at 4 ºC overnight.

On the third day, the plates were washed 5 times and the blotting step was repeated. Secondary antibodies of goat anti-mouse IgG1, IgG2c, and IgM conjugated with horseradish peroxidase (HRP) (Novus Biologicals, USA and Invitrogen, USA) diluted 1:5,000, 1:25,000 and 1:40,000 respectively. 50 µL of the diluted antibody was added to each well of their separate plates and incubated at RT For 2 hours, away from light. After the incubation, the plates were washed for 7 cycles and the blotting step was repeated. 50 µL of 1X tetramethylbenzidine (TMB) (Thermo Fisher) was added to each well and the plates were incubated away from light for 8 minutes before 50 µL of H_2SO_4 stop solution was added to stop the reaction. Plates were then placed in the Biotech Synergy HT plate reader to measure the OD readings of each well within 5 minutes after addition of the H_2SO_4 stop solution and the data was collected on the Gen5 3.11 software.

2.5.2 Sandwich Cytokine ELISA

To measure *in vitro* recall of cytokines in splenocyte supernatant, a sandwich ELISA was used. Capture antibodies against IL-17, IL-13, IFNγ, IL-10 and IL-4 (Thermo Fisher) were diluted using 1X assay diluent (Thermo Fisher) as per directed on the manufacturer's website. Each well received 50 µL of their plates respective diluted capture antibody and was incubated overnight at 4ºC.

The following morning, the plates were washed in a BioTek plate washer for 3 cycles using 200 µL of ELISA wash buffer (**Table 2.4**). The wash was discarded over a sink and blotted on paper towel before 200 µL of 1X assay diluent (Thermo Fisher) was added to each well to block the plates. Plates were blocked at RT for 2 hours. During the incubation period, the samples were diluted using 1X assay diluent; 1:2 for IL-10, IL-13, IL-4, 1:10 for IL-17 and 1:20 for IFNγ. Lyophilized standards with known concentrations determined by the manufacturer (Thermo Fisher) were solubilized using 1X assay diluent (Thermo Fisher) and serially diluted 1:2, 8 times to make 8 standard dilution points. After the two hour incubation, the 3-cycle wash step and blotting step was repeated. 100 µL of diluted sample, standards or ELISA wash buffer, used as a negative control, was loaded into their respective wells. This plate was incubated at RT for 1-2 hours. After this incubation, the plates were washed for 5 cycles and the blotting step was repeated. Biotin-conjugated, secondary detection antibodies for each listed cytokine above (Thermo Fisher) was diluted using 1X assay diluent (Thermo Fisher) as per manufacturer instructions. 50 µL of this diluted detection antibody was added to plates according to their cytokine. The plates were incubated at RT for two hours before the 5-cycle wash and blotting step was repeated. 50 µL of 1X streptavidin-horseradish peroxidase (SAV-HRP) (Thermo Fisher) was loaded into each well and the plates were incubated for 20 minutes. After the 20-minute incubation, plates were washed for 7 cycles and the blotting step was repeated prior to the addition of 50 µL of 1X TMB to each well. Plates were incubated away from light until colour appeared in the 7^{th} well of the diluted standards before the reaction was stopped using 50 µL of H_2SO_4 stop solution. Plates were then placed in the Biotech Synergy HT plate reader to measure the OD readings of each well within 5 minutes after addition of the H_2SO_4 stop solution and the data was collected on the Gen5 3.11 software.

2.6 Statistical analysis

To analyze and visualize data, Prism 9 software (GraphPad, La Jolla, CA) was used. For investigating a single variable between two strains, unpaired t-tests were used. Analysis of data sets that involved two independent variables and compared two groups was carried out using a 2-way ANOVA with a Šídák's multiple comparisons test. All data is presented as the mean ± standard error of mean (SEM). Significance is as indicated by the *P* value with < 0.05 as *, <0.01 as **, and a *P* value of <0.001 as ***.

Table 2.1: List of fluorochrome conjugated anti-mouse antibodies used for phenotyping immune subsets present in genital tracts.

Antibody Target	Fluorochrome Conjugate	Catalogue Number	Manufacturer	Volume used per test (50 µL)
CD11b	APC	17-0112-82	Thermo Fisher	0.125 µL
CD31	Brilliant Violet 711 ™	740690	BD Biosciences	0.125 µL
CD19	PE-Cyanine7	25-0193-82	Thermo Fisher	0.042 µL
CD4	AF700	100430	BioLegend	0.1 µL
CD8a	PE	12-0081-85	Thermo Fisher	0.33 µL
CD45	Brilliant Violet 421 ™	103134	BioLegend	0.062 µL
F4/80	Brilliant Violet 650 ™	123149	BioLegend	0.167 µL
Ly6G	FITC	11-9668-82	Thermo Fisher	0.25 µL
MHC II	Brilliant Violet 786 ™	107645	BioLegend	0.033 µL
Podoplanin (PDPN)	PE/Dazzle™ CF 594	127420	BioLegend	0.25 µL
Viability Stain	eFluor™ 506	65-0866-18	Thermo Fisher	1 µL

Table 2.2: List of fluorochrome conjugated anti-mouse antibodies used for phenotyping Tfh cells and subsets of B cells in the spleens

Antibody Target	Fluorochrome Conjugate	Catalogue Number	Manufacturer	Volume used per test (50 µL)
CD138	Brilliant Violet 421 ™	142508	BioLegend	2.5 µL
CD3e	Brilliant Violet 605 ™	100351	BioLegend	0.167 µL
PDL2	PerCP-eFluor™ 710	46-9972-82	Invitrogen	1.25µL
CD95	Brilliant Violet 786™	107645	BioLegend	0.167 µL
CD4	FITC	11004285	eBioscience	0.25 µL
GL7	PE	12-5902-82	Invitrogen	0.3 µL
CD38	AF700	56-0381-82	Invitrogen	0.16 µL
CXCR5	APC/Fire™ 750	145534	BioLegend	2.5 µL
CD19	PE-Cyanine7	25-0193-82	Thermo Fisher	0.042 µL
Viability Stain	eFluor™ 506	65-0866-18	Thermo Fisher	1 µL

Table 2.3: List of fluorochrome conjugated anti-mouse antibodies used for phenotyping Treg cells in the spleens

Antibody Target	Fluorochrome Conjugate	Catalogue Number	Manufacturer	Volume used per test (50 µL)
CD19	Brilliant Violet 421 ™	115538	BioLegend	1 µL
FoxP3	PE	12577382	Invitrogen	0.5 µL
CD3	Brilliant Violet 650 ™	100229	BioLegend	0.625 µL
CD4	APC	17-0042-82	Invitrogen	0.5 µL
Viability Stain	eFluor™ 506	65-0866-18	Thermo Fisher	1 µL

Table 2.4: Buffers, reagents and media used for *in vivo* and *in vitro* experiments

Name	Ingredients	Supplier	Amount
RPMI + 5% Bovine Serum	Bovine Serum	Sigma Aldrich	25 mL
	RPMI-1640 Medium 1X	SH30027.01 - Cytiva	500 mL
Complete RPMI Medium (cRPMI)	Fetal Bovine serum	Sigma Aldrich	100 mL
	HEPES buffer	Wisent Bio	1 mL
	Penicillin/streptomycin (Pen Strep)	Thermo Fisher	1 mL
	L-glutamine	Thermo Fisher	1 mL
Ammonium-chloride-potassium buffer (ACK Lysis Buffer)	NH4Cl	BioShop	16.6 g
	KHCO3	BioShop	2 g
	EDTA	Bioshop	0.04 g
ELISA Coating Buffer (pH 9.6 +/- 0.2)	Na2CO3	BioShop	1.59 g
	NaHCO3	BioShop	2.93 g
	Distilled H2O		975 mL
ELISA Wash buffer	1 X PBS	Made in Lab	4L
	Tween 20	CAS #56-40-6 BioShop	2 mL
ELISA Blocking Buffer	ELISA Wash Buffer		100 mL
	Bovine Serum Albumin	BioShop	2 g
Cytokine ELISA Diluent	5X ELISA/ELISPOT Diluent	88-7137-88 Invitrogen	5 mL
	Distilled H2O		40 mL
H2SO4 Stop Solution	Sulfuric Acid		2 mL
	Distilled H2O		18 mL
Fluorescence-activated cell sorting buffer (FACS)	1 X Phosphate buffered saline	Made in lab	995 mL
	Inactive bovine serum	Sigma Aldrich	5 mL

Name	Ingredients	Supplier	Amount
Phosphate buffered saline (PBS)	NaCl	BioShop	8 g
Phosphate buffered saline (PBS)	KCl	BioShop	0.2 g
Sucrose-phosphate-glutamic buffer (SPG) pH 7.2	Na2HPO4	BioShop	1.15 g
	KH2PO4	BioShop	0.2 g
	Sucrose	BioShop	75.3 g
Sucrose-phosphate-glutamic buffer (SPG) pH 7.2 FACS Fixing Buffer	KH2PO4	BioShop	0.42 g
	K2HPO4	BioShop	1.25 g
	Monosodium glutamate	BioShop	0.92 g
	1X PBS		360 uL
FACS Fixing Buffer	10% Formalin	Sigma Aldrich	40 uL

CHAPTER 3 RESULTS

Primary Infection

3.1 AID deficiency has no impact on bacterial shedding during primary infection

Firstly, to assess whether AID deficiency has an impact on the bacterial shedding post primary infection by Cm, WT and AIDKO mice were infected with five repeated infections of *Cm* (1.35 x 10^4 IFU per infection) every other day over the course of ten days. The infected mice were swabbed every 2-3 days and gDNA isolation was performed on the collected swabs to quantify bacterial shedding in mice using qPCR. We found the levels of bacterial burden and overall kinetics of bacterial shedding were comparable in AIDKO and WT mice, with both strains shedding little to no bacteria past day 34 (**Figure 3.1**). The results from this experiment provide evidence that AID deficiency has little to no impact on bacterial shedding during primary infection.

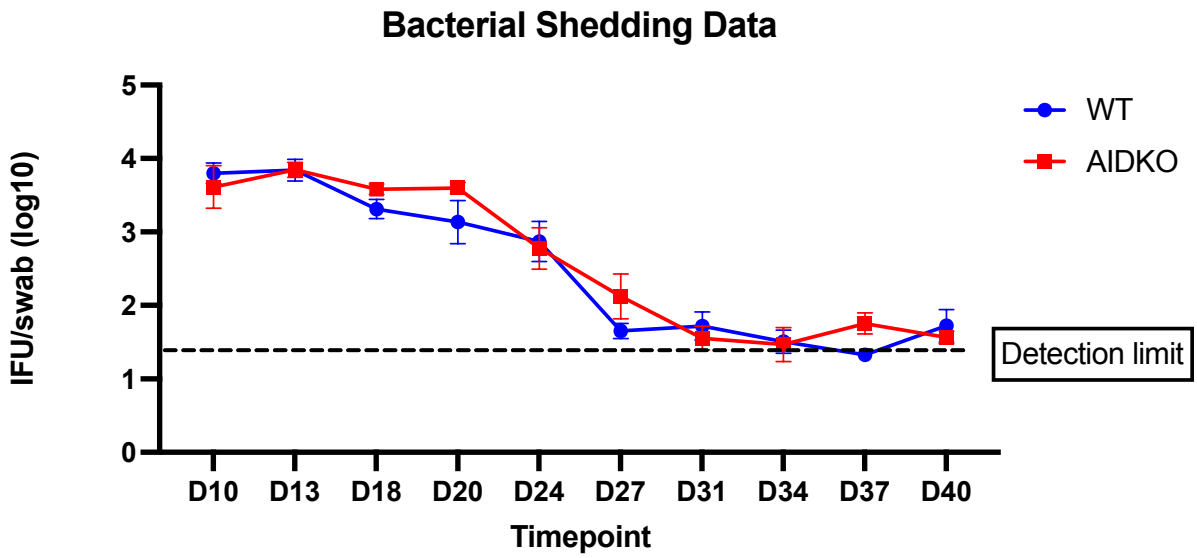


Figure 3.1: Bacterial shedding post primary infection is comparable between strains

AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were swabbed every 2-3 days with a calcium alginate tipped swab. Genomic DNA (gDNA) was isolated and the bacterial level was determined by qPCR. The data is presented as mean ± SEM(n=6-11). No statistical difference was observed between strains, analysis was conducted using a two-way ANOVA and a Šídák's multiple comparisons test. Detection limit was determined by sample collection from naïve uninfected mice.

3.2 AID deficiency leads to hyper IgM syndrome and lack of antigen specific antibody production

Previous reports of AID deficiencies established a hyper IgM syndrome and lack of secondary specific antibody production (Bao et al., 2022; Muramatsu et al., 1999). With this knowledge, we sought to measure the level of IgM and IgG production in both WT and AIDKO mice. Serum samples taken from mice at D0 and D30 post infection were assayed using the conventional *Cm* specific antibody ELISA assay. Multiple *Cm* antigens including TC0047, TC0582, TC0326 and TC0921 and whole HKCM were used to coat the plates. Notably, TC0582 and TC0912C antigens have been characterized as pathogenic antigens while TC0047 and TC0326 are considered as non-pathogenic ones (Zeng et al., 2012).

We found that AIDKO mice produced significantly greater IgM specific for almost all tested antigens compared to WT counterparts who had little IgM response. Specifically, TC0582 ($P \leq 0.01$), TC0326 ($P \leq 0.01$), TC407 ($P \leq 0.05$) and whole HKCM ($P \leq 0.01$) as well as visibly more IgM against TC0912c were observed in AIDKO mice (**Figure 3.2**). In contrast, there is no IgG response in AIDKO mice (**Figure 3.3a**). The WT mice produced significantly greater IgG1 against whole HKCM ($P \leq 0.05$) and visibly greater IgG1 specific for TC0582, TC0326, TC047, and TC0912c than the AIDKO mice (**Figure 3.3a**). Similarly, WT mice produced significantly more IgG2c specific for HKCM ($P \leq 0.001$) and TC0912c ($P \leq 0.05$), and visibly more IgG2c specific for TC0582, TC0326 and TC047 when compared to their AIDKO counterparts (**Figure 3.3b**).

Collectively, the antibody ELISA results indicate that AID deficiency leads to a hyper IgM syndrome in response to chlamydial infection and a lack of IgG1 and IgG2c *Cm* specific antibody production after primary infection. The data is highly consistent with the expected phenotype of AIDKO mice.

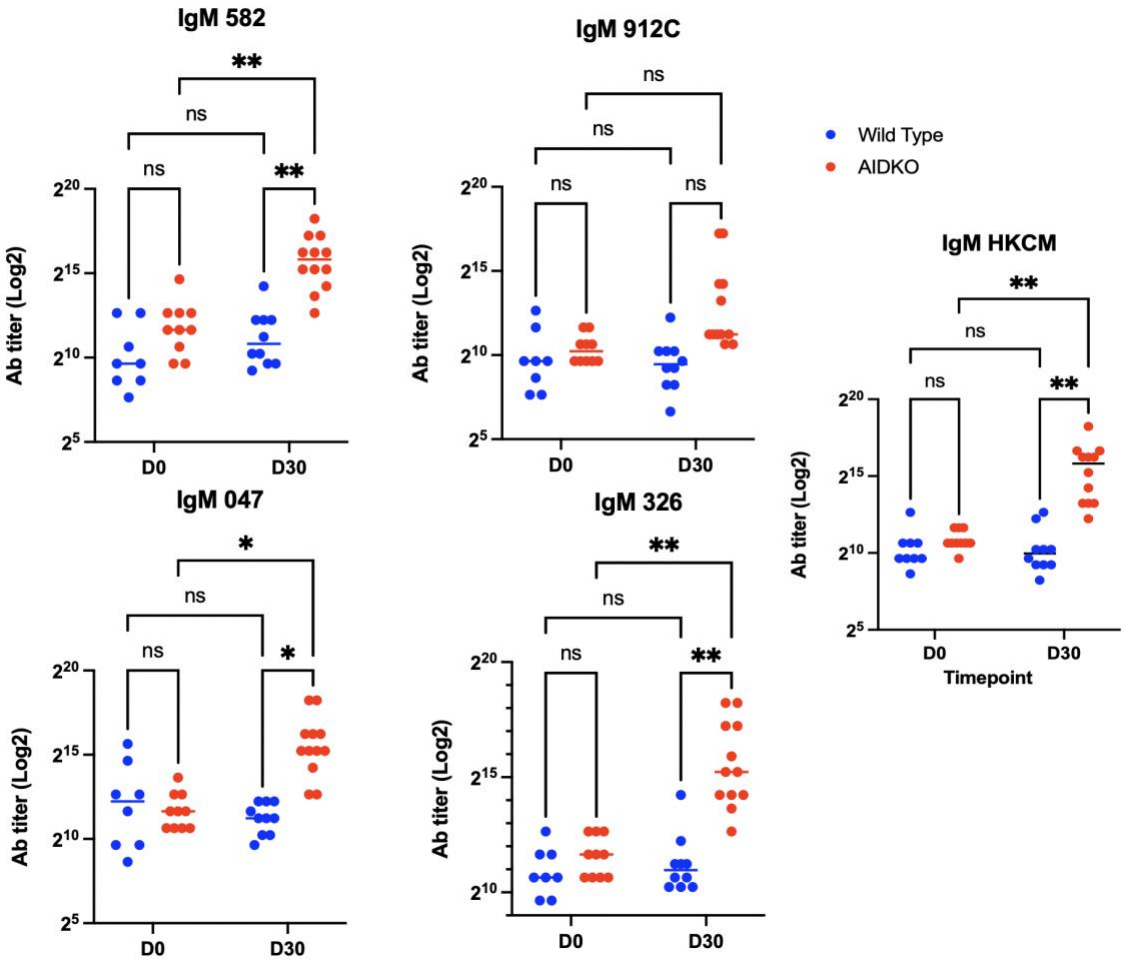
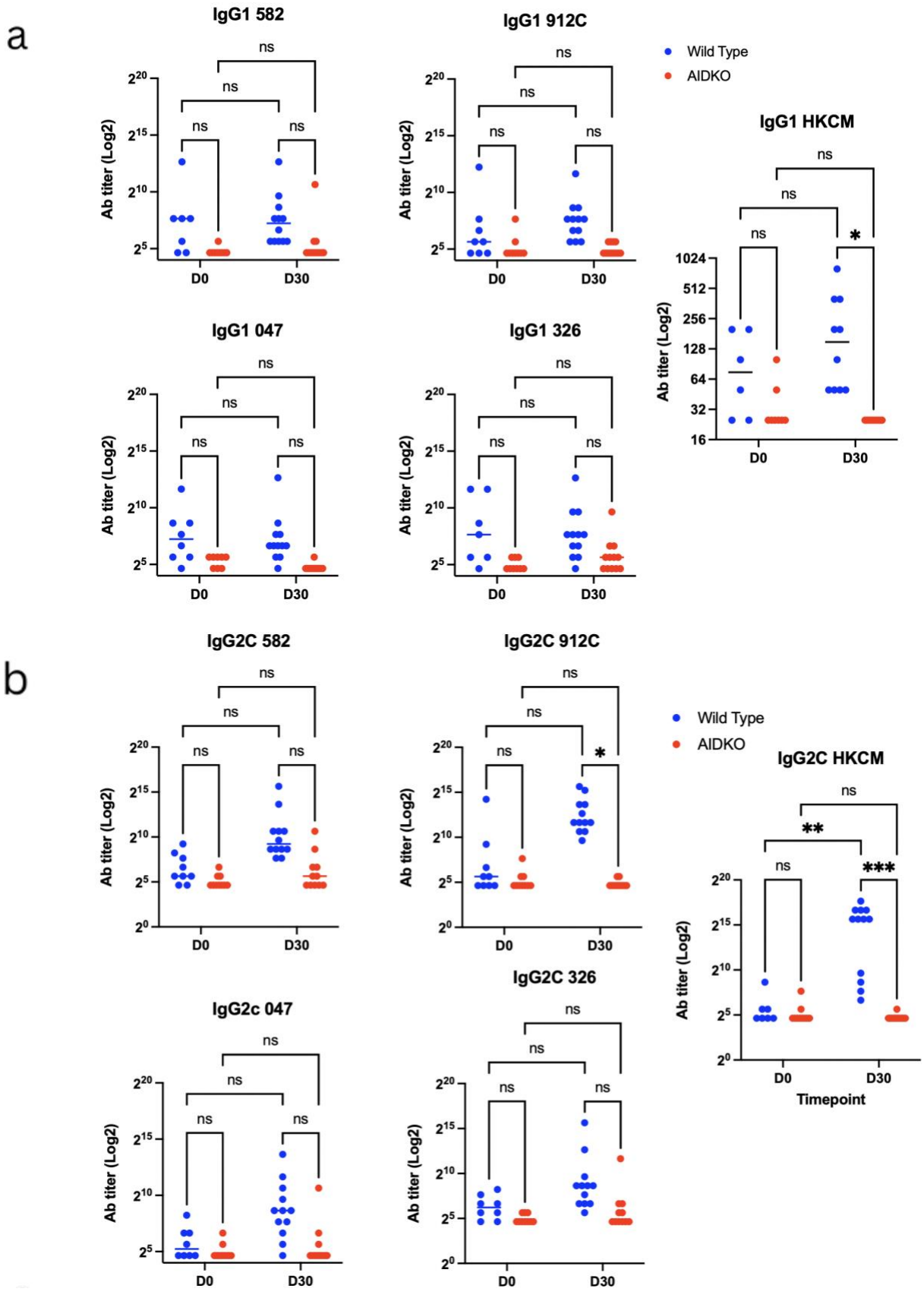


Figure 3.2: AIDKO mice produce significantly more IgM in response to primary infection

AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were sacrificed on day 30 and serum was collected and serially diluted for ELISA in order to measure antibody titres of IgM against whole HKCM, TC0912C, TC0047, TC0326 and TC0582. Data is presented with the mean ±SEM (n=6-12); *P≤ 0.05, ** P≤ 0.01 with a two-way ANOVA and a Šídák's multiple comparisons test.


a
IgG1 582
IgG1 912C
IgG1 047
IgG1 326
IgG1 HKCM
Wild Type
AIDKO
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IgG2C 582
IgG2C 912C
IgG2C 326
IgG2c 047
IgG2C HKCM
**
***
Timepoint

Figure 3.3: WT mice produce significantly more IgG2c in response to primary infection AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were sacrificed on day 30 and serum was collected and serially diluted for ELISA in order to measure antibody titres of IgG1 (a) and IgG2c (b) against whole HKCM, TC-0912C, TC-0047, TC-0326 and TC-0582. Data is presented with the mean ± SEM (n=6-12); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001 with a two-way ANOVA and a Šídák's multiple comparisons test.

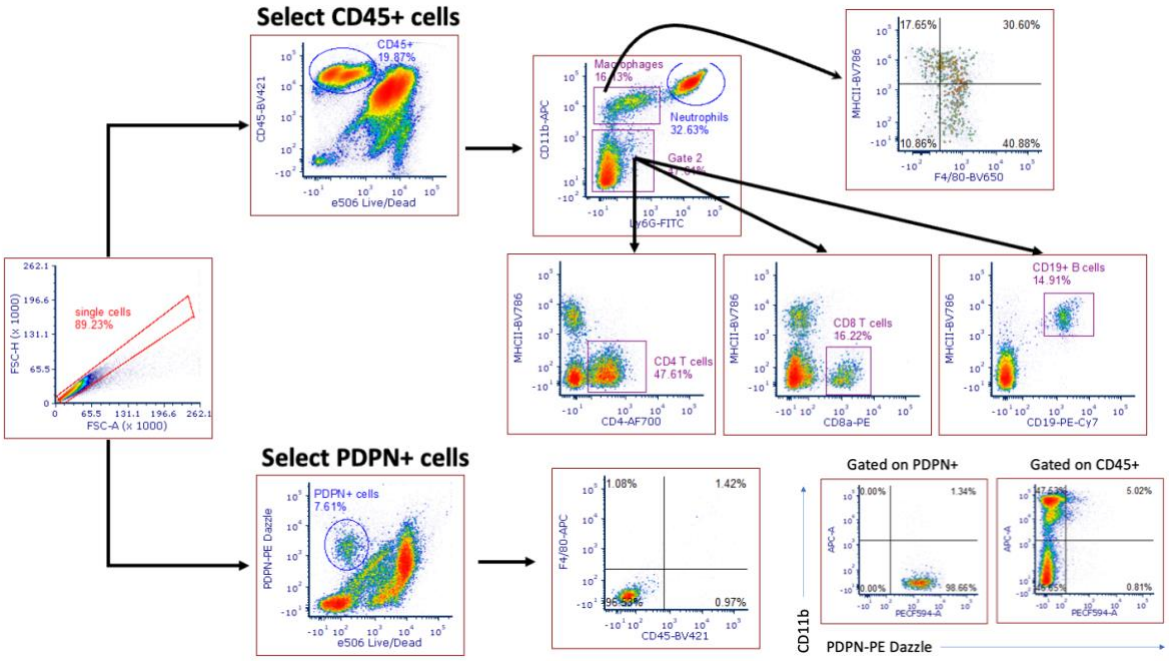
3.3 AID deficiency does not greatly impact the cellular response at the local site of infection

In order to understand the functional role of AID in host responses to repeated *Cm* infections, a multi-parameter flow cytometry panel was used to phenotype the main immune cell subsets in the single cell suspensions of genital tracts isolated from WT and AIDKO mice on days 0, 17, 30 and 53 post *Cm* infections. The frequency of various immune cells among the total CD45+ leukocyte population was determined following a conventional gating strategy to select live single cells (**Figure 3.4a**). PDPN is primarily present on endothelial cells of lymphatics but can be upregulated in many cell types including fibroblast cells and macrophages during states of chronic inflammation such as a persistent chlamydial infection (De Filippis et al., 2017). So, we included a fluorescent antibody recognizing mouse PDPN to identify tissue structure cells.

We found the total average total number of cells isolated from WT and AIDKO mice were comparable at all time points. However, it was clear that the infection led to significant inflammatory responses in both strains of mice as there was marked increases in cell recovery at day 17 ($P \leq 0.01$ in WT mice and $P \leq 0.0001$ in AIDKO mice) compared to naïve counterparts (**Figure 3.4b**). The cell recovery returned to comparable levels to naïve mice by day 30 post primary infection. Of interest, there appeared to be a rebound at D53 post infection.

Within the single cell suspensions, we were able to identify $CD45^+$ leukocytes and $PDPN^+CD45^-$ tissue structure cells (**Figure 3.4a**). There was significant induction of $CD45^+$ lymphocytes after primary infection at D17 in both strains ($P \leq 0.01$ in WT and $P \leq 0.001$ in AIDKO). However, there were no significant differences between WT and AIDKO samples in either percentage or absolute cell number at any timepoint (**Figure 3.4c**). Similarly, the number and percentages of live $PDPN^+$ cells in the genital tract were significantly upregulated post primary infection ($P \leq 0.01$ in WT and $P \leq 0.0001$ in AIDKO), however there were no significant differences seen between the strains at any timepoint (**Figure 3.4d)**. Notably, over 90% of $PDPN^+$ cells were $CD45^-CD11b^-$ tissue structure cells. We noticed that the expression of PDPN on $CD11b^+$ macrophages was much lower compared to tissue structure cells (**Figure 3.4a**). Collectively, these results demonstrate that AID deficiency does not impact the inflammatory responses, expansion of tissue structure cells and recruitment of innate immune cells to the local site of infection during primary infection.

a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Neutrophils
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CD11b-APC
Ly6G-FITC
17.65%
30.60%
10.86%
40.88%
MHCII-BV786
F4/80-BV650
single cells
89.23%
FSC-H (x 1000)
FSC-A (x 1000)
CD4 T cells
47.61%
CD4-AF700
CD8 T cells
16.22%
CD8a-PE
CD19+ B cells
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CD19-PE-Cy7
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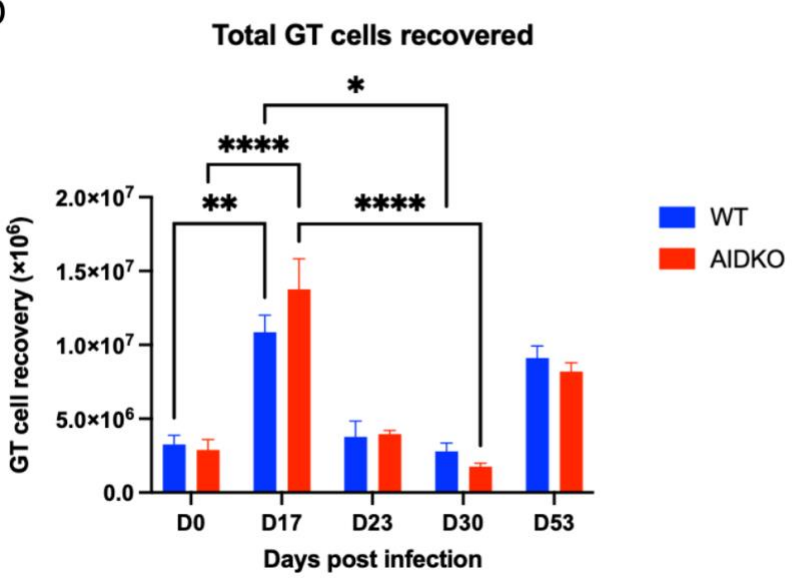
PE-CF594-A
CD11b
PDPN-PE Dazzle

b


Total GT cells recovered
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2.0×10^7
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1.0×10^7
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GT cell recovery (×10^6)
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D17
D23
D30
D53
Days post infection
WT
AIDKO

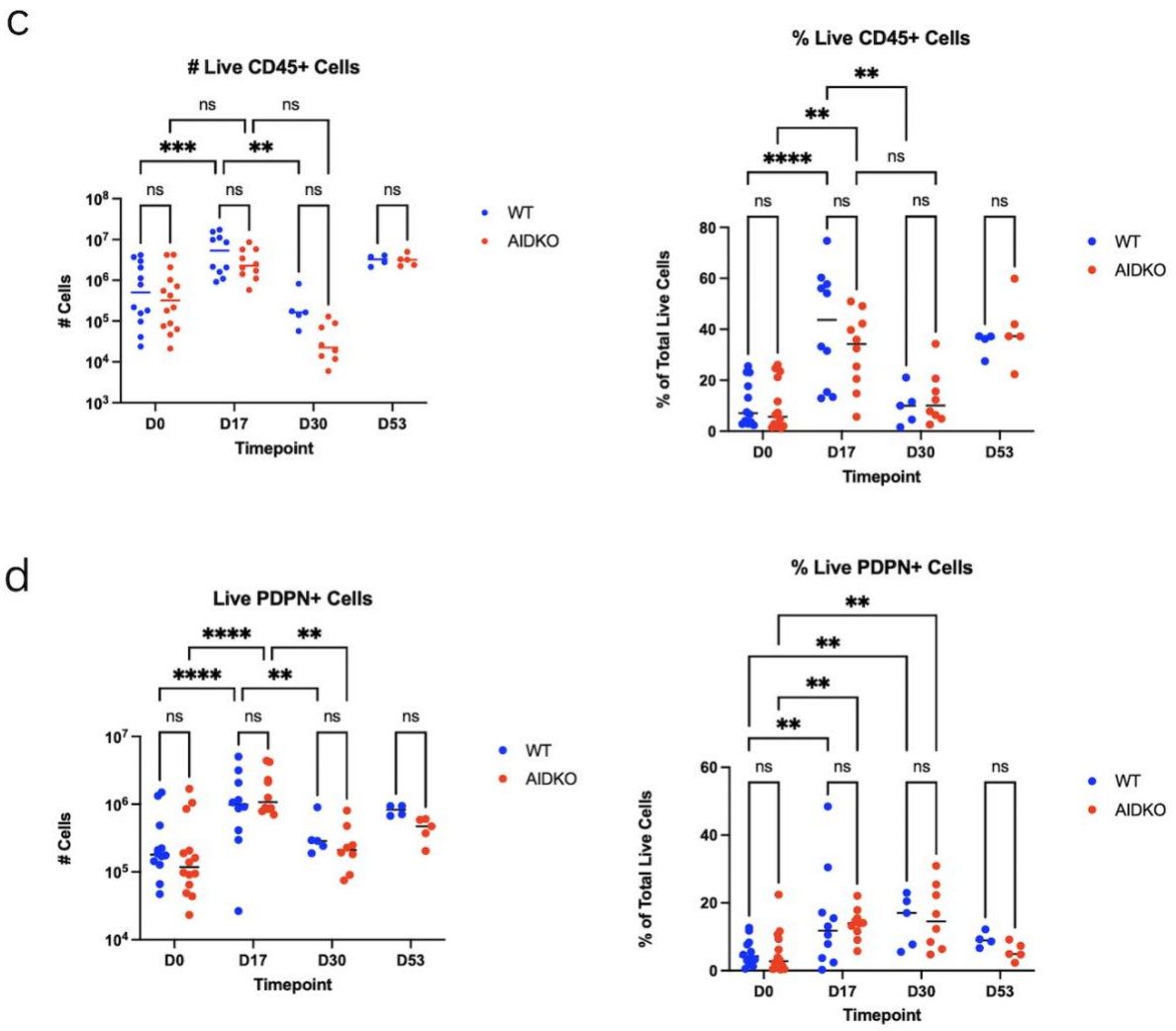


Figure 3.4: Gating strategy and separation of genital tract immune cell populations

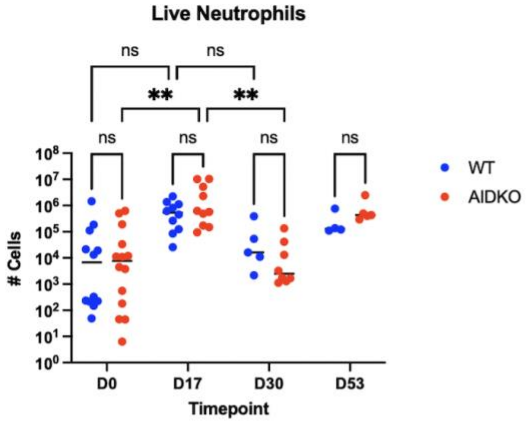
AIDKO and WT mice were intravaginally infected with 5 low doses of *Cm* and sacrificed at days 0, 17, 30, and 53. Flow cytometry analysis was conducted to phenotype genital tract cells. The gating strategy is illustrated in panel (a). The total number of cells isolated from genital tracts at all timepoints (b). The frequency and absolute number of $CD45^+$ leukocytes (c) and $PDPN^+$ tissue structure cells (d). Data are presented with the mean ± SEM (n=4-14 mice per strain per time point); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, ****P≤0.0001, with a two-way ANOVA and a Šídák's multiple comparisons test. Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

Neutrophils were defined as $CD11b^{high}Ly6G^{+}$ cells (**See Figure 3.4a**). There was a significant increase in neutrophil accumulation at D17 post infection ($P \leq 0.01$), but both the numbers and percentages of neutrophils were comparable between strains (**Figure 3.5a**). This indicates that AID deficiency has no impact on the neutrophil response kinetics after primary infection.

F4/80-expressing tissue macrophages were mapped to the $CD11b^{low}$ population, which expressed various amounts of MHC class II (**Figure 3.4a**). Although there was a significant increase in $CD11b^{low}F4/80^{+}MHCII^{high/low}$ macrophage populations post primary infection in both strains ($P \leq 0.01$ in WT and $P \leq 0.001$ in AIDKO), the numbers and percentages of macrophages present at the local site of infection were comparable between AIDKO and WT mice (**Figure 3.5b**). Notably, an increase in relative abundance of tissue macrophages in both strains occurred at D30 post infection, which was delayed compared to the kinetics of neutrophils.

We further analyzed the activation status of $PDPN^{+}$ cell population by checking the expression of MHCII level on $PDPN^{+}F4/80^{-}$ parenchymal cells (**Figure 3.5c),** and $PDPN^{+}F4/80^{+}$ macrophages (**Figure 3.5d**). Remarkably, *Cm* infection caused significant upregulation of MHCII on $PDPN^{+}F4/80^{-}$ parenchymal cells, and significant expansion of $PDPN^{+}/F4/80^{+}MHCII^{+}$ macrophages in infected WT and AIDKO mice. However, numbers and percentages of both $PDPN^{+}$ subpopulations remained comparable between strains. Collectively, our data indicate that *Cm* infection triggered substantial tissue inflammation involving both tissue macrophages and tissue structure cells, yet AID deficiency has no or little impact on local tissue inflammation as well as macrophage kinetics and macrophage functional status after primary *Cm* infection.

a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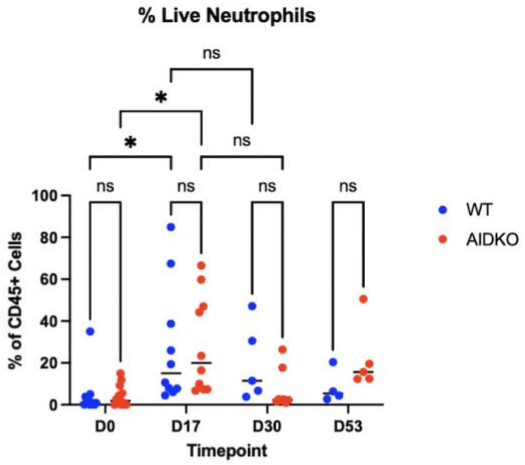
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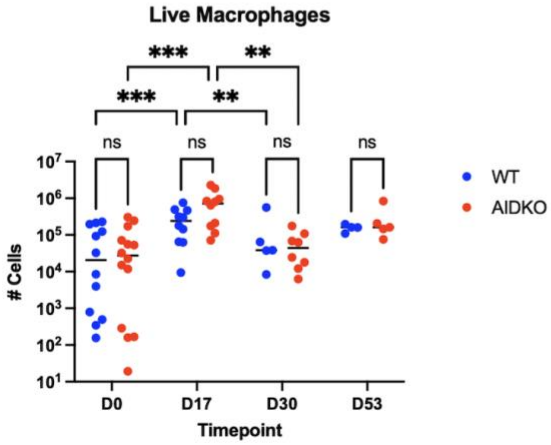
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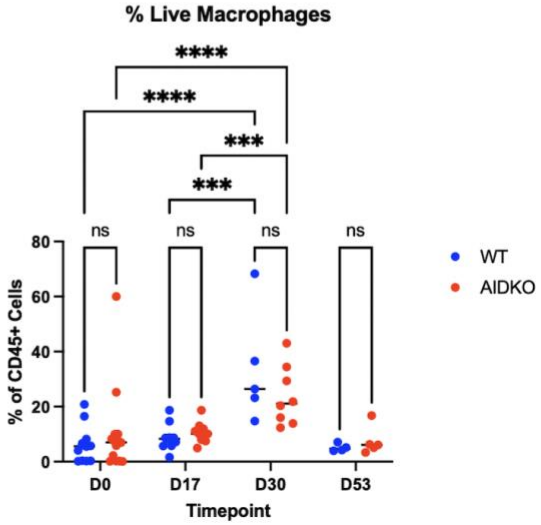
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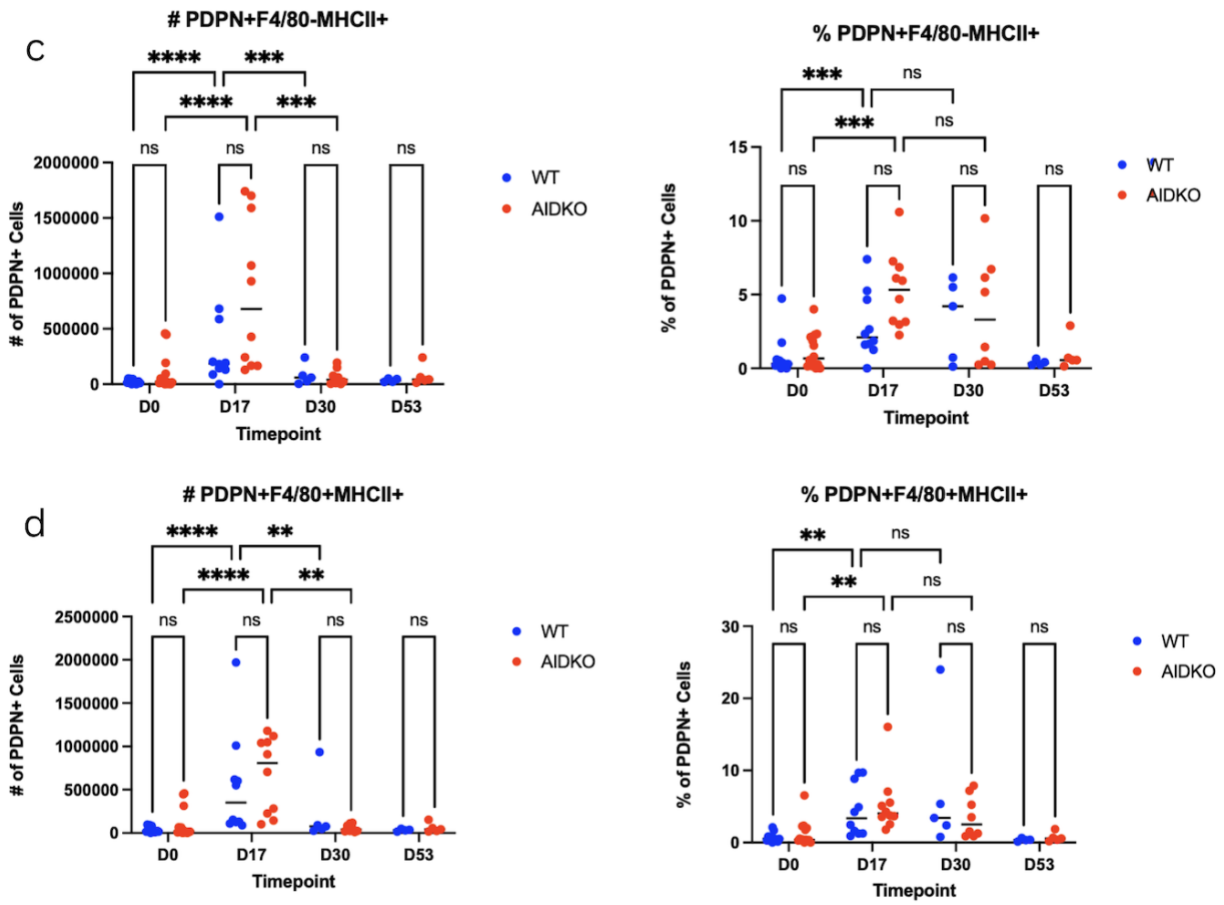


Figure 3.5: Genital tract innate immune cell subsets

The FACS samples were collected as described in Figure 3.4. The frequency and absolute number of neutrophils (a), macrophages (b), $PDPN^{+}F4/80^{-}$ parenchymal cells (c), and $PDPN^{+}F4/80^{+}$ macrophages (d) are presented with the mean ± SEM (n=4-14 mice per strain per time point); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, ****P≤0.0001, with a two-way ANOVA and a Šídák's multiple comparisons test. Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

Within the $CD45^+$ lymphocyte populations, we further examined specific subsets of immune cells from that belong to the adaptive immune system. $CD4^+$ and $CD8^+$ T cells were defined as $CD45^+CD4^+CD11b^-$ cells and $CD45^+CD8\alpha^+CD11b^-$, respectively (**Figure 3.4a**). $CD4^+$ T cells of both strains increased significantly post primary infection at D17 ($P \leq 0.001$) but no significant differences in the numbers or percentages of $CD4^+$ T cells present at the local site of infection were discovered between strains at any timepoint (**Figure 3.6a**). Similar data were obtained with $CD8^+$ T cells (**Figure 3.6b**). Once again, our data indicate that AID deficiency has little influence on the local T cell responses to primary infection.

Naturally, we were interested in investigating whether AID deficiency has an impact on the B cell response to primary infection due to the well-known involvement of AID and B cell interactions. $CD19^+$ B cells were identified as $CD45^+CD19^+MHCII^+$ cells (**Figure 3.6c**). Similar to the T cell population expansion in the genital tract, there is a significant expansion of the B cell populations post primary infection ($P \leq 0.001$) (**Figure 3.6c**). Notably, we see a significant difference in the number of $CD19^+$ B cells present at the local site of infection between the WT and AIDKO mice at D17 post primary infection ($P \leq 0.05$). These numbers return to comparable levels between both strains at later timepoints (**Figure 3.6c**). This shows that AID deficiency has an influence on the early local B cell response to primary infection. This leads to the conclusion that AID deficiency leads to significantly greater B cell recruitment to the local site of infection at early timepoints post primary infection.

In summary, the local T cell responses to primary Cm infection are unaffected by the AID deficiency, but the local B cell responses are significantly impacted.

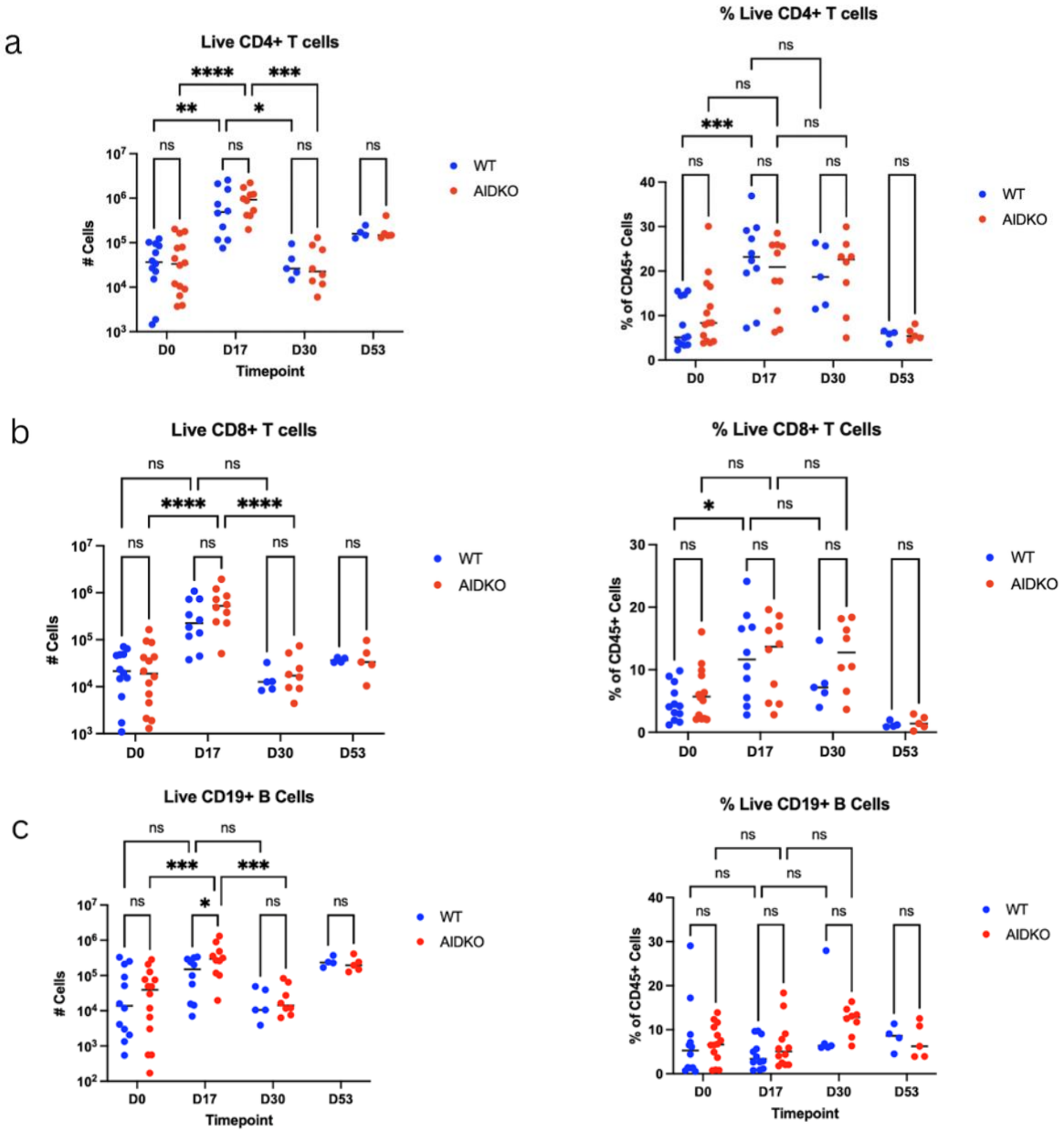


Figure 3.6: Genital tract adaptive immune cell subsets

The FACS samples were collected as described in Figure 3.4. The frequency and absolute number of $CD4^+$ T cells (a), $CD8^+$ T cells (b), $CD19^+$ B cells (c) are presented with the mean ± SEM (n=4-14 mice per strain per time point); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, ****P≤0.0001, with a two-way ANOVA and a Šídák's multiple comparisons test. . Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

3.4 AID deficiency has significant impact on systemic lymphoid compartments

In parallel with the murine genital tract immune phenotyping, we also examined the impact of AID deficiency in the immune response at the systemic level. Another multi-parameter flow cytometry panel (**Table 2.2**) was used to phenotype the main T and B cell subsets relevant to antibody production and the germinal center reactions in the spleen. The frequency of various immune subsets including total CD4 T cells and B cells, Tfh cells, GC B cells, plasma cells as well as memory B cells, was determined following a conventional gating strategy to select live single cells (**Figure 3.7a**). The total number of splenocytes isolated from WT and AIDKO mice were comparable at all time points (**Figure 3.7b**), suggesting AID deficiency does not impact the overall homeostasis of splenocytes following primary infection.

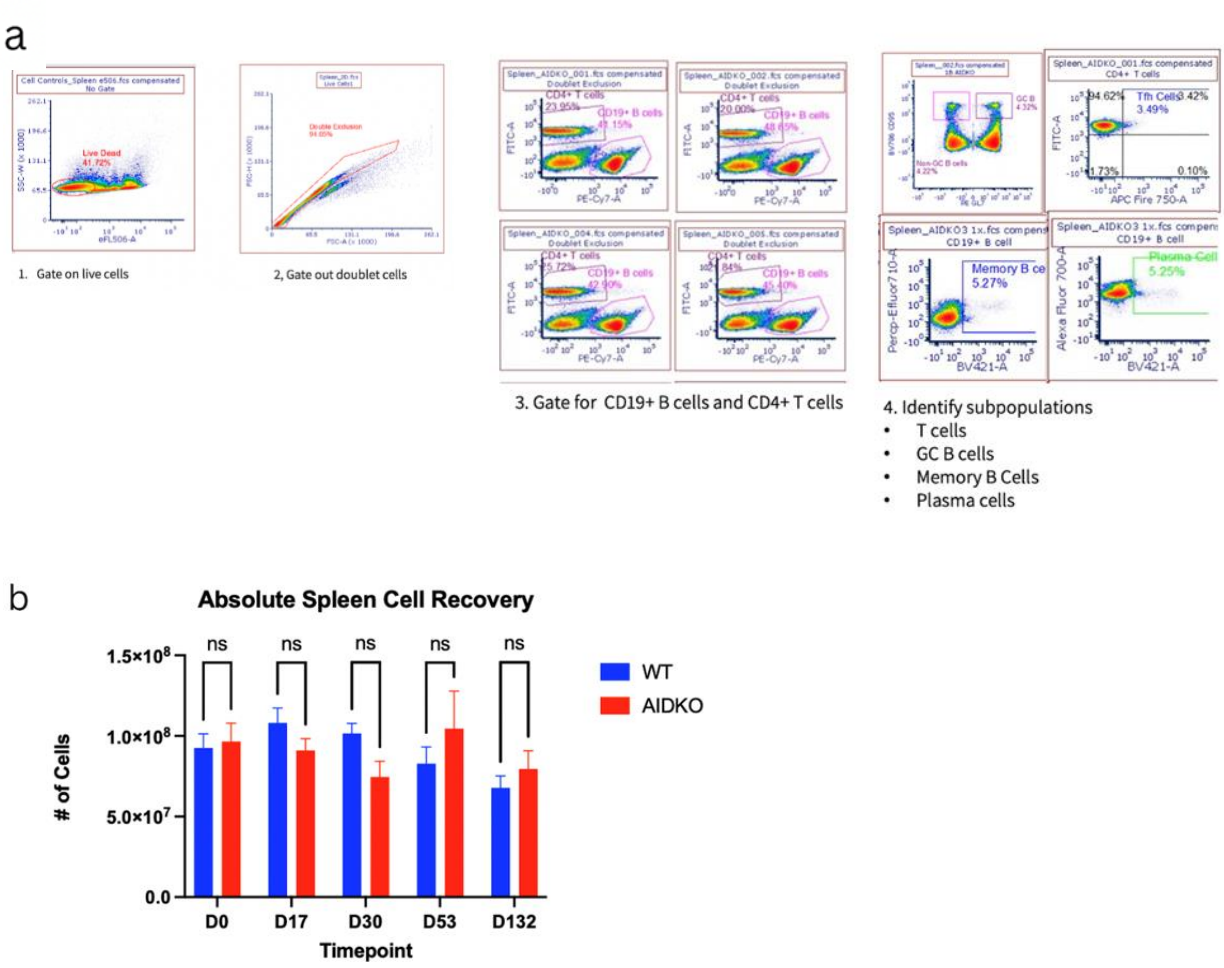
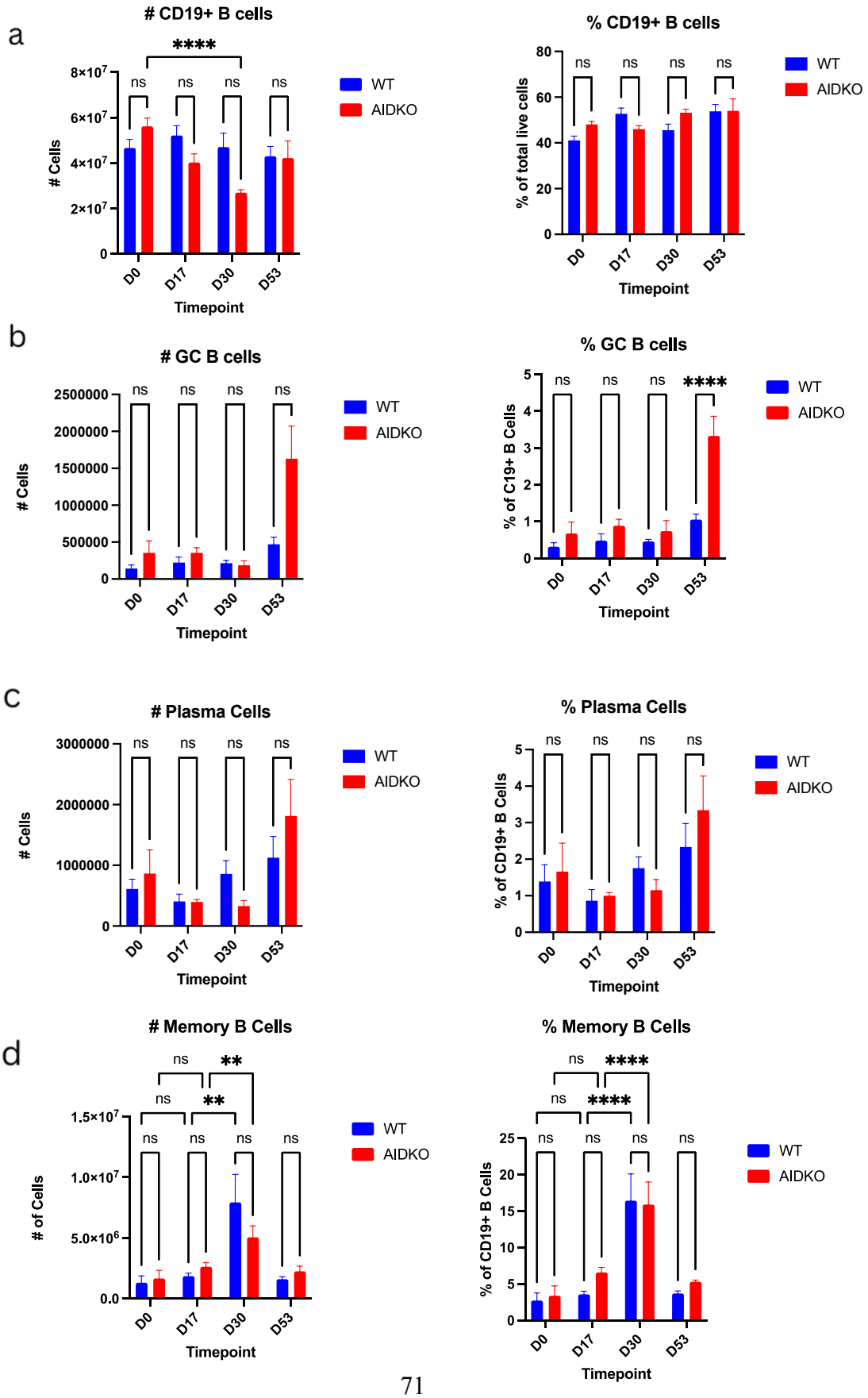


Figure 3.7: Gating strategy and absolute cell recovery of splenocytes

AIDKO and WT mice were intravaginally infected with 5 low doses of Cm and sacrificed at days 0, 17, 30, and 53. Flow cytometry analysis was conducted to phenotype splenocytes. The gating strategy is illustrated in panel (a), and the total number of cells isolated from spleens at all timepoints (b). Data is presented with the mean ± SEM (n=4-14 mice per strain per time point); with a two-way ANOVA and a Šídák's multiple comparisons test. Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

Given the known function of AID in B cells, we first investigated the systemic B cell response. The first population examined were the $CD19^+$ B cells present in the spleen. $CD19^+$ B were defined as $CD19^+CD4^-$ cells (**Figure 3.7a**). There are no significant differences found between strains at any timepoint in regard to the percentage of $CD19^+$ B cells (**Figure 3.8a**). However, the number of $CD19^+$ B cells in the AIDKO mice, but not in WT mice, gradually decreased between D0 to D30 post primary infection to a statistically significant level ($P \leq 0.0001$) (**Figure 3.8a**).

Having demonstrated that AID alters the systemic B cell response, we next examined specific subsets of $CD19^+$ B cells. Because AID plays a prominent role in specific secondary antibody diversification, we wanted to investigate the B cell subsets that pertain to producing high affinity antibodies such as germinal center B cells (GC B cells). GC B cells were defined as $CD19^+$ $CD95^+GL7^+$(**Figure 3.7a**). At early timepoints post primary infection, GC B cells levels were comparable between strains and did not appear to change drastically in response to primary infection (**Figure 3.8b**). However, by D53 post primary infection, the AIDKO mice had significantly greater percentages of GC B cells present in the spleen ($P \leq 0.0001$) and visibly higher numbers as well compared to WT counterparts (**Figure 3.8c**). A similar trend was observed in the $CD19^+$ $CD138^+PDL2^-$ plasma cells in the AIDKO mice. In comparison, memory B cells with the phenotype of $CD19^+PDL2^+CD138^-$ showed a significant increase in percentages and numbers present in both strains at D30 ($P \leq 0.0001$) but a decrease back to baseline levels by D53 (**Figure 3.8d**). Therefore, AID deficiency greatly alters systemic B cell responses to *Cm* infection, particularly at later time points, which is in agreement with the known function of AID in B cell functions.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CD19+ B cells
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60
40
20
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b
GC B cells
2500000
2000000
1500000
1000000
500000
% GC B cells
5
4
3
2
1
% of C19+ B Cells
c
Plasma Cells
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% Plasma Cells
% of CD19+ B Cells
d
Memory B Cells
**
1.5×10^7
1.0×10^7
5.0×10^6
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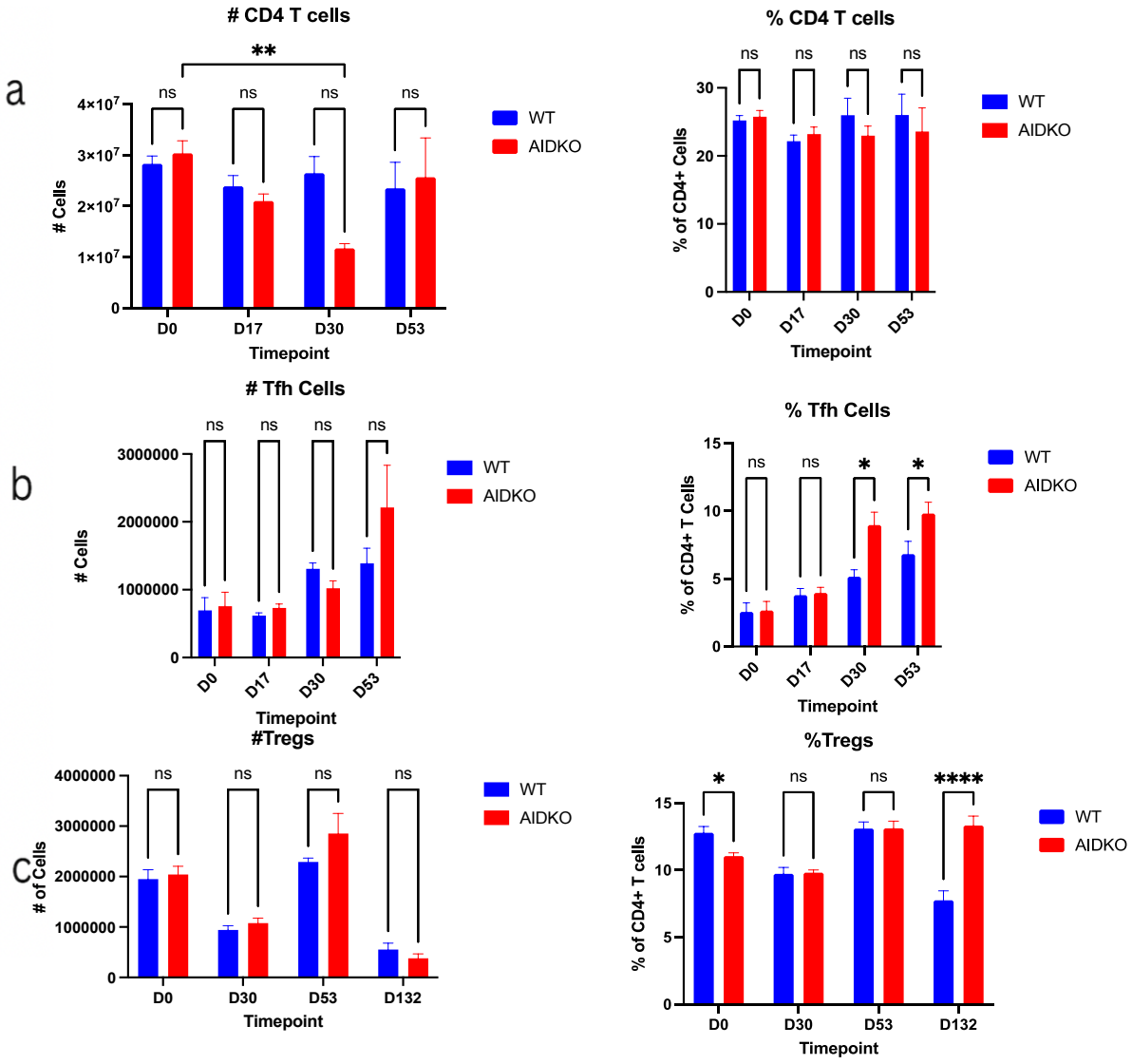
# of Cells
% Memory B Cells
25
20
15
10

Figure 3.8: AID deficiency leads to distinct systemic B cell responses to *Chlamydia* infection AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were sacrificed at several timepoints post primary infection and spleens were excised on the sacrifice day. Different B cell populations were identified using surface staining, and flow cytometry acquisition. These populations included $CD19^+$ B cells (a), GC B cells (b), plasma cells (c), and memory B cells (d). Data is presented with the mean ± SEM (n=5-15 mice per group); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, ****P≤0.0001, with a two-way ANOVA and a Šídák's multiple comparisons test. Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

Given the nature of T and B interaction and altered B cell responses in AIDKO mice, we next examined the $CD4^+$ T cells defined as $CD4^+CD19^-$ population in our panel (**Figure 3.7a**). Although there were no significant differences in percentages of numbers of $CD4^+$ T cell present at the spleen post primary infection between strains, we observed a marked reduction in the number of $CD4^+$ T cells in the spleen of AIDKO mice, but not in WT mice, at D30 post primary infection (**Figure 3.9a**). Based on the expression of CXCR5, we were able to further identify Tfh subset, which were defined as $CD4^+CXCR5^+$(**Figure 3.7a**). Of interest, the percentages of Tfh cells in both strains of mice were not altered at the early phase of infection, however this trend began to change at later time points (**Figure 3.9b**). By D30 and D53 post primary infection, AIDKO mice produced significantly greater percentages of Tfh populations than their WT counterparts ($P \leq 0.05$) (**Figure 3.9b**). This indicates that AID deficiency resulted in an heightened Tfh response to primary infection.

This observation prompted us to examine whether AID deficiency has an impact on immunosuppression and the Treg response at later timepoints post primary infection. We constructed additional panel (**Table 2.3**) to specifically examine the $CD4^+FoxP3^+$ regulatory T cells in few experiments. Although WT mice appeared to have more Tregs present in the spleen in naïve mice (D0) ($P \leq 0.05$), the percentages evened out by D53 post primary infection (**Figure 3.9c**). It was not until D132 post primary infection where we observed significantly greater percentages of Tregs present in the spleen in the AIDKO mice relative to the WT mice $P \leq 0.0001$) (**Figure 3.9c**). These results suggest that AID deficiency may not play a large role in impacting the regulation of the T cell response at earlier time points but that there are long lasting implications of primary *Chlaymdia* infection in AID deficient hosts.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CD4 T cells
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Timepoint
WT
AIDKO
% CD4 T cells
ns
ns
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30
20
10
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% of CD4+ Cells
D0
D17
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Timepoint
WT
AIDKO
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Tfh Cells
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0
% of CD4+ T cells
D0
D30
D53
D132
Timepoint
WT
AIDKO

Figure 3.9: AID deficiency leads to significant differences in the systemic T cell response to *Chlamydia* infection

AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were sacrificed at several timepoints post primary infection and spleens were excised on the sacrifice day. Different T cell populations were identified using surface staining and intracellular staining with antibodies and flow cytometry acquisition. These populations included CD4+ T cells (a), Tfh cells (b), and Tregs(c). Data is presented with the mean ± SEM (n=5-15 mice per strain per time point); *P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, ****P≤0.0001, with a two-way ANOVA and a Šídák's multiple comparisons test. Flow cytometry data was collected over several experiments. D0 results were pooled from 3 separate experiments, D17 results came from a single experiment, D30 results were pooled from 2 separate experiments, D53 results were pooled from 2 separate experiments.

After establishing that AID deficiency has an impact on the systemic T cell response, particularly with altered Tfh and Tregs cell numbers post primary infection, we sought to assess whether this would extend to other $CD4^+$ T helper lineages, such as Th1 response. To investigate this, splenocytes from AIDKO and WT mice were processed into single cell suspensions on D0, D30 and D53 post primary infection and cultured for 72 hours with or without HK*Cm* stimulation. The culture supernatants were collected and assayed via sandwich ELISA to quantify the production of IFN-γ, IL-4/IL-13, IL-10, and IL-17. The IFN-γ was used as a measurement of the Th1 response. IL-4/IL-13 were used to measure the Th2 response. IL-10 was used to measure the Treg response, and IL-17 was used to measure the Th17 response.

The IL-4/IL-13, and IL-10 were below the detectable level, indicating that these responses were minimal. However, the WT mice demonstrated a visibly greater IFN-γ response in the D30 samples but there was no statistical difference observed at this timepoint. However, with the D53 timepoint samples, the WT mice produce significantly greater levels of IFN-γ than the AIDKO mice ($P \leq 0.05$) (**Figure 3.10a**). Detection of IL-17 production in D30 samples was minimal, but WT mice have visibly greater production of IL-17 in the D53 samples (**Figure 3.10b**).

Comparatively, WT mice had a greater ratio of IFN-γ response to IL-17 response in the D30 samples but this trend is not reflected in the D53 samples (**Figure 3.10c**). Collectively, this indicates that the WT mice have a more robust Th1 response to chlamydial antigens and supported the idea that the AIDKO mice with their altered T cell responses, particularly Tregs, which inhibit appropriate Th1 responses upon a secondary encounter with chlamydial antigens.

In addition to the observed changes in systemic responses, this data demonstrates that, in addition to regulate B cell function, AID plays a role in regulating the $CD4^+$ T cell response, likely promoting Tfh cell differentiation and inhibiting Th1 responses that impact the immune response to subsequent encounters with *Cm*.

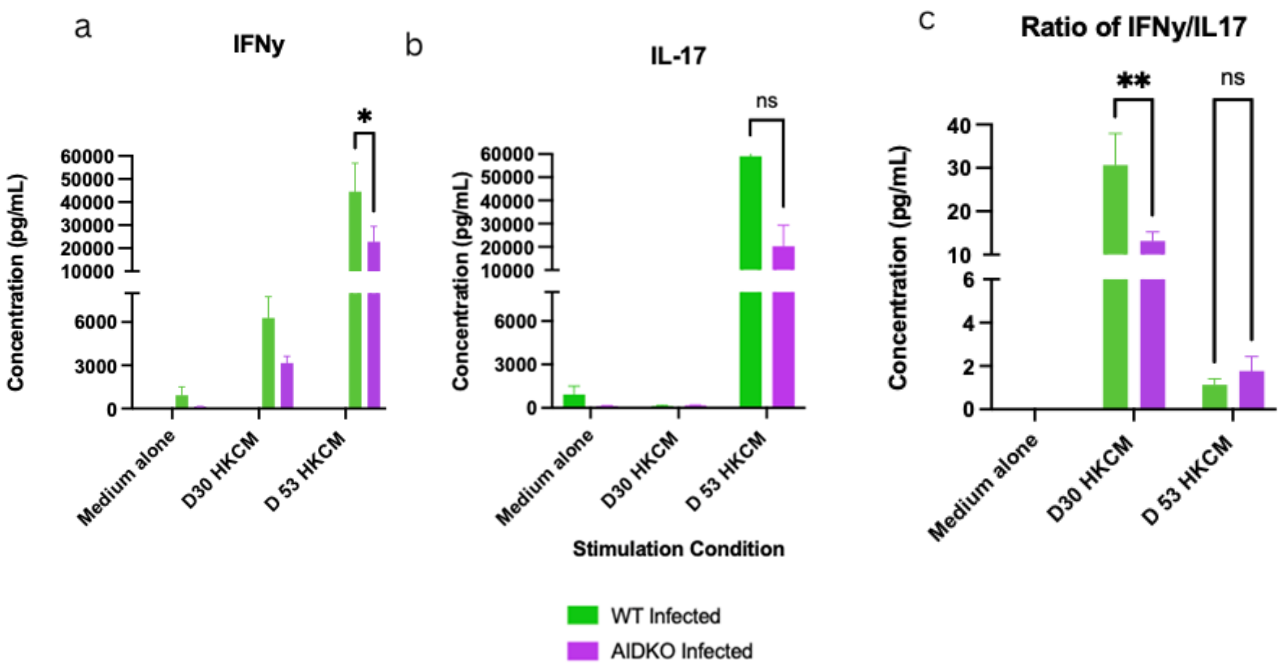


Figure 3.10: AIDKO deficiency alters IFN-γ production *in vitro* after HK*Cm* stimulation of primed splenocytes

AIDKO and WT mice were intravaginally infected with five low doses of *Cm*. Splenocytes isolated at D30 and D53 post primary infection were stimulated with medium or HK*Cm* for 72 hours. Supernatants were collected from culture at 72 hours and assayed in a sandwich ELISA to measure (a) IFN-γ and IL-17 (b) production in response to chlamydial antigens. The ratio of IFN-γ to IL-17 production is also examined (c). Data is presented as mean ± SEM (n=3-5 mice per group); *P≤0.05,** P≤0.01 using a two-way ANOVA and a Šídák's multiple comparisons test.

3.5 AID deficiency does not impact oviduct dilation post primary infection

Given the similarities between strains observed in the levels of cell dynamics in the genital tract as a result of chlamydial infection, we sought to investigate whether this trend was reflected in the tissue pathology post primary infection. The genital tracts of naïve and infected AIDKO and WT mice were excised at D0, D96 and D132 post primary infection. Genital tracts were fixed in 10% formalin and sent to the IWK pathology department to be processed into histopathology slides. Both AIDKO and WT naïve oviducts were used as a control as they demonstrate a lack of oviduct dilation and the oviducts retain a healthy, ovular shape (**Figure 3.11**). Both AIDKO and WT infected oviducts at D96 and D132 post primary infection demonstrate a degree of dilation and a loss of healthy oviduct shape (**Figure 3.11**). Both strains showed significant increases in oviduct dilation when compared to naïve samples but there are no significant difference in the amount of dilation between the strains (**Figure 3.12**). Despite significant differences in systemic T cell and B cell responses in two strains of mice, AID deficiency has no impact on the oviduct dilation and tissue pathology development post primary infection.

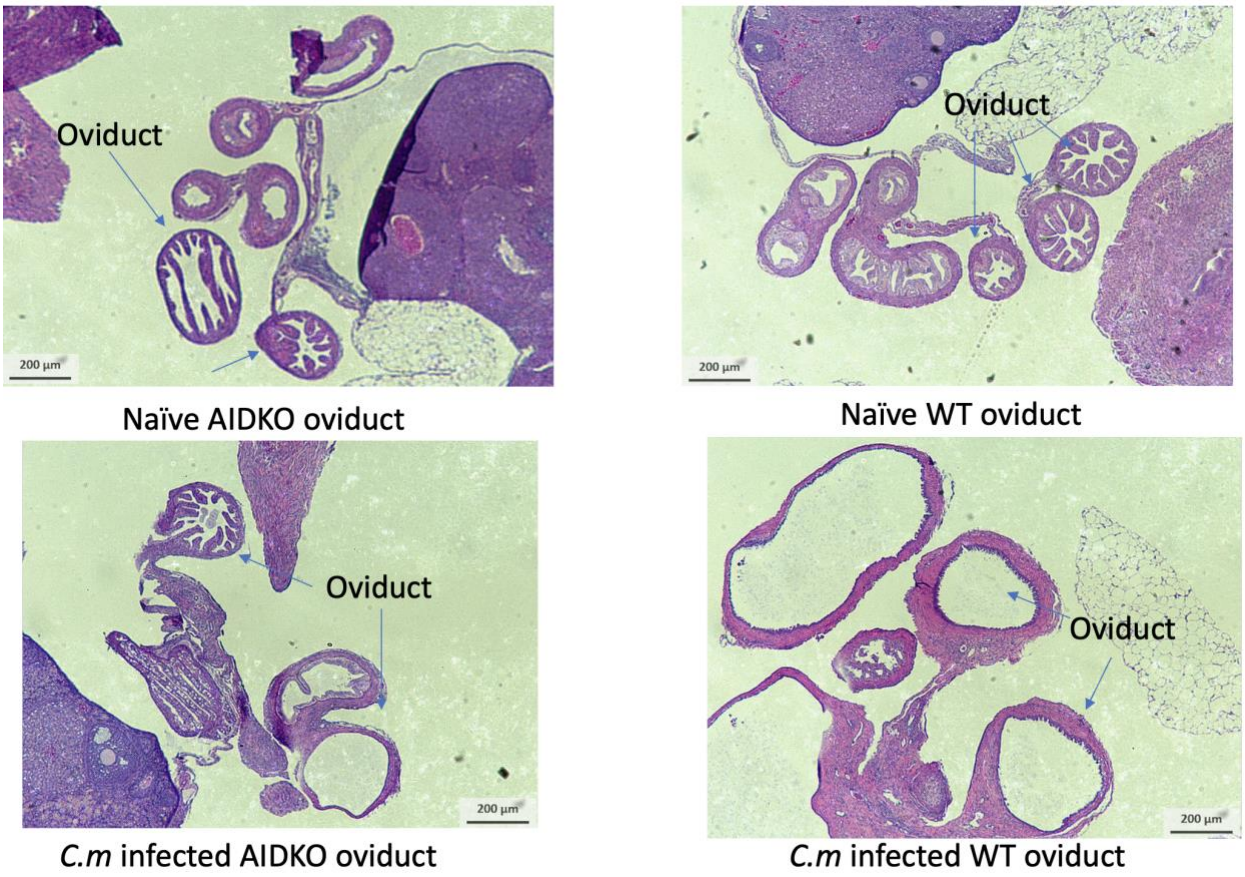


Figure 3.11: Histology slides of WT and AIDKO mouse naïve and infected oviducts

A view of the histology slides showing the internal structure of a naïve (top), and infected (bottom) murine oviduct in AIDKO and WT strains. Cross sections of oviducts that were measured using ImageJ are highlighted by blue arrows in the photograph.

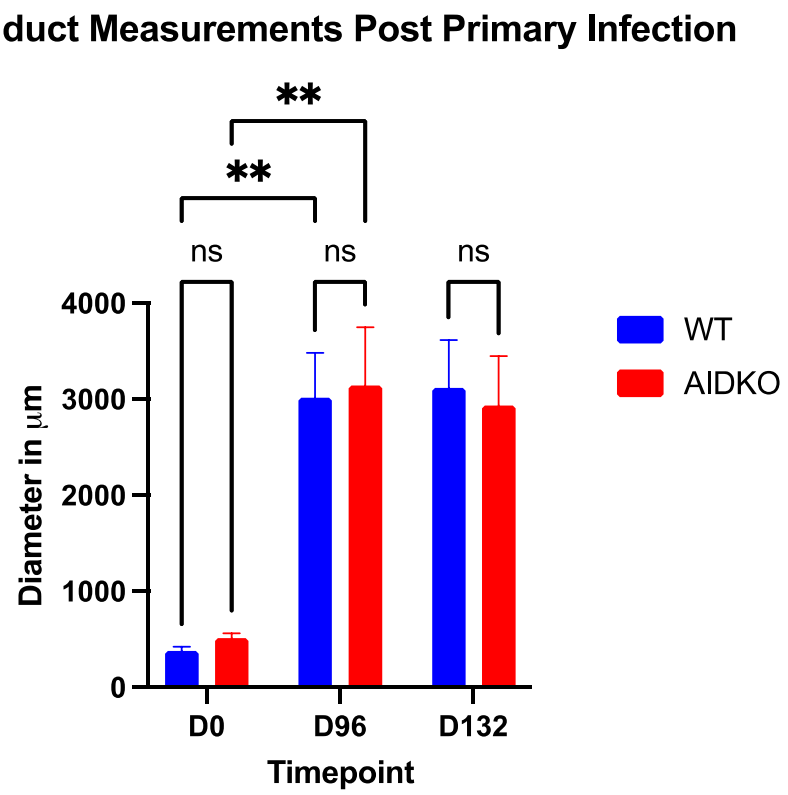


Figure 3.12: Oviduct dilation post primary infection

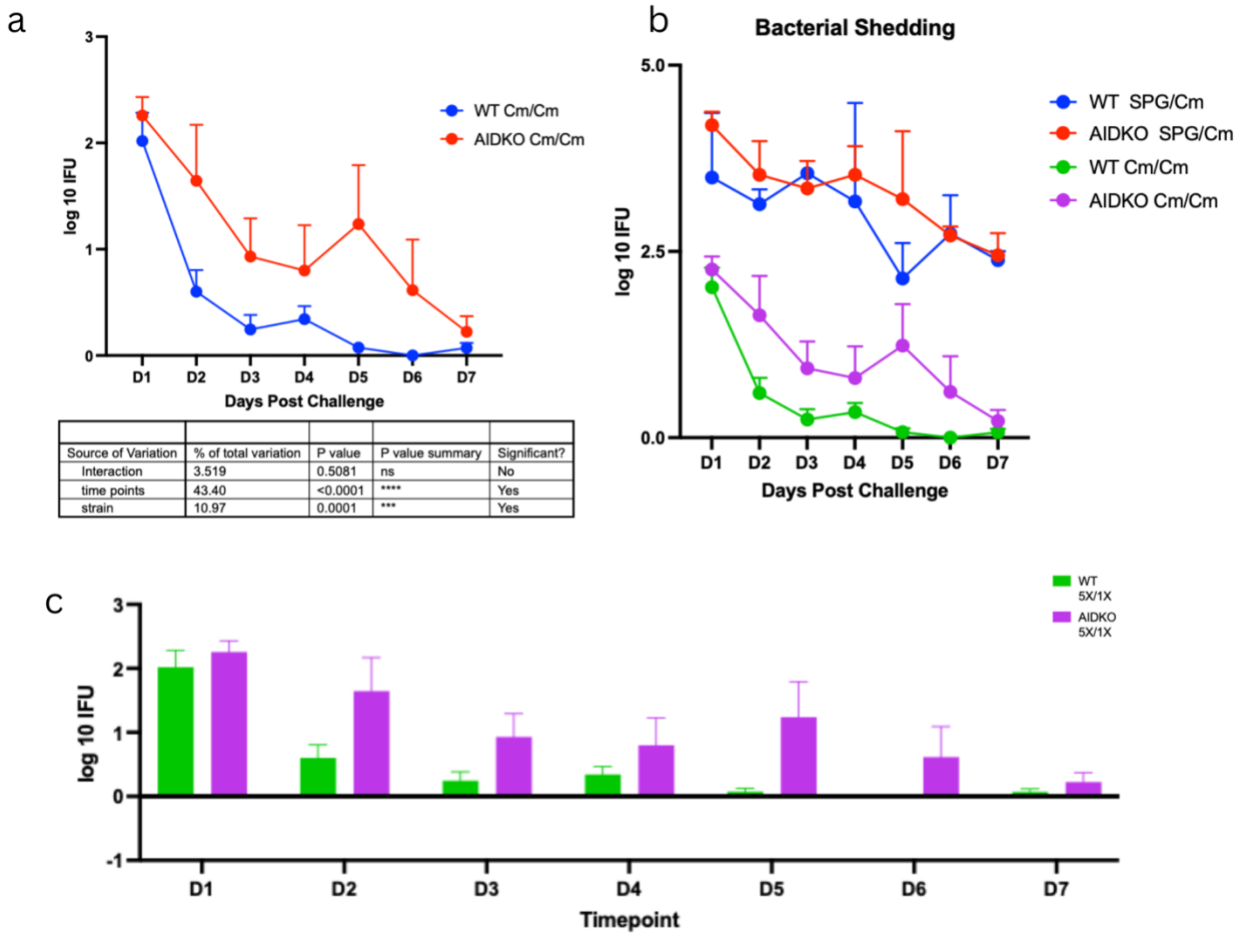
AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were sacrificed at several timepoints post primary infection and GTs were excised on the day of sacrifice. GTs were preserved in 10% formalin for histology before being analyzed through ImageJ for oviduct dilation to examine the severity of tissue damage at the local site of infection. Data is presented with the mean ± SEM (n=5-7 mice per group); *P≤ 0.05, ** P≤ 0.01 with a two-way ANOVA and a Šídák's multiple comparisons test.

Secondary Infection

3.6 Secondary antibodies against Chlamydia muridarum provide significant protection against reinfection

Given the general protective nature of Th1 immunity and antibodies against subsequent reinfections, we sought to determine whether AID deficiency, and a lack of protective antibodies and weakened Th1 immunity would impact the course of *Cm* challenge. Thus, we conducted a challenge experiment which followed the same course of infection as the general *in vivo* studies with five low dose infections but allowed for complete clearance of primary infection and then a subsequent re-infection at a later timepoint to elucidate the differences in immune responses to reinfection with and without AID and secondary antibody responses (**Figure 2.2**). Both AIDKO and WT mice were subjected to the typical hormone regulation prior to the five low dose infections and are referred to as the primed group. Control cages of the unprimed mouse group were also given Depo-Provera injections and SPG mock infections following the same timeline. After 130 days and confirmed clearance of the primary infection, both the primed and unprimed mice were given the same dosage of *Cm*.

The mice were swabbed every day following the secondary infection to fully capture the bacterial shedding trends between the strains in response to a re-infection. What we found is that the AIDKO mice have significantly higher bacterial burden than their WT counterparts upon re-challenge ($P \leq 0.0001$) (**Figure 3.13a**), although both strains demonstrated decreased bacterial burden when compared to unprimed control mice (**Figure 3.13b**). Moreover, after the qPCR data collected from the swabs is normalized, the WT mice essentially stopped shedding bacteria after day 4 post re-infection while the AIDKO mice continued to shed bacteria up to day 7 post re-infection (**Figure 3.13c**). This provides evidence that AID deficiency has an impact on the immune response to reinfection that makes AIDKO mice more susceptible to reinfection.



Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.519	0.5081	ns	No
time points	43.40	<0.0001	****	Yes
strain	10.97	0.0001	***	Yes

Figure 3.13: Bacterial shedding data post after re-infection

qPCR results for vaginal swabs for 7 days after re-infection of the pre-infected group. Vertical axis is the log10 IFU and horizontal axis is timepoint of swab collection. gDNA was isolated from swab samples and then diluted for qPCR. The results show that (a) AIDKO mice have significantly greater bacterial shedding than WT mice after re-infection and that (b) there were no differences between strains in bacterial shedding for the mock-infected group upon challenge but there was a marked decrease in bacterial shedding seen in the pre-infected group with the AIDKO mice shedding significantly more bacteria than the pre-infected WT mice. (c) Normalized challenge data from the pre-infected group showed that the WT mice stopped shedding bacteria after 4 days post re-infection while the AIDKO mice continued to shed bacteria for the entire 7-day swab schedule.

3.7 AID deficiency significantly impacts tissue pathology triggered by reinfection

Since the bacterial burden and oviduct dilation data from the primary infections correlate, we sought to determine if there is a difference in the pathology after a reinfection. Genital tracts were collected 7 days post re-infection and fixed in 10% formalin and sent to the IWK pathology department to be processed into histopathology slides. The AIDKO have significantly greater oviduct dilation than the WT mice in response to re-infection ($P \leq 0.05$) (**Figure 3.14**). This correlates with the increased bacterial burden post re-infection seen in the AIDKO strain of mice similar to how the bacterial burden and oviduct dilation data correlate from the primary infection results. This allows us to conclude that AID deficiency significantly impacts tissue pathology development triggered by a subsequent re-infection.

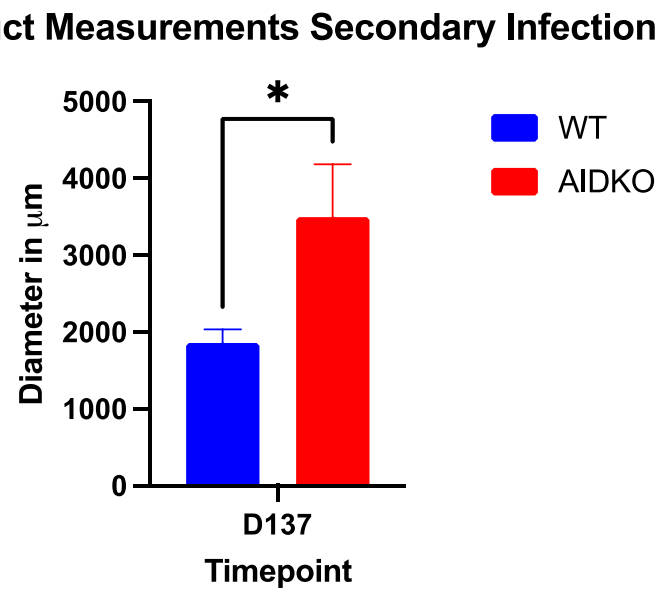


Figure 3.14: Oviduct dilation after re-infection

AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were given time to clear the primary infection before a re-infection was given. Mice were sacrificed 7 days after re-infection and GTs were excised on the day of sacrifice. GTs were preserved in 10% formalin for histology before being analyzed through ImageJ for oviduct dilation to examine the severity of tissue damage at the local site of infection. Data is presented with the mean ± SEM (n=6-7 mice per group); *P≤ 0.05 with a two-way ANOVA and a Šídák's multiple comparisons test.

3.8 AID deficiency significantly impacts the neutrophilic response at the local site of infection during a re-infection

To assess the immune response at the local site of infection during a re-infection, genital tracts were collected 7 days post re-infection and processed into single cell suspensions prior to flow cytometry staining and subsequent acquisition on the BD LSR Fortessa SORP. We wanted to investigate neutrophil infiltration in response to a re-infection to see if these results could correlate with the increased bacterial burden and oviduct dilation seen in the AIDKO mice. Neutrophils were defined as $CD11b^{high}Ly6G^{+}$ cells (**Figure 3.4a**). The results show that the WT mice have a significant reduction in the numbers and percentages of neutrophils present at the local site of infection during a re-infection ($P \leq 0.01$) (**Figure 3.15**). This decrease is not seen in the AIDKO mice, indicating that the re-infection triggers a greater inflammatory response in the AIDKO mice than in the WT mice. This data correlates with the increased bacterial shedding seen in the AIDKO mice post reinfection (**Figure 3.13a**) and bolsters the idea that AIDKO mice are more susceptible to reinfection.

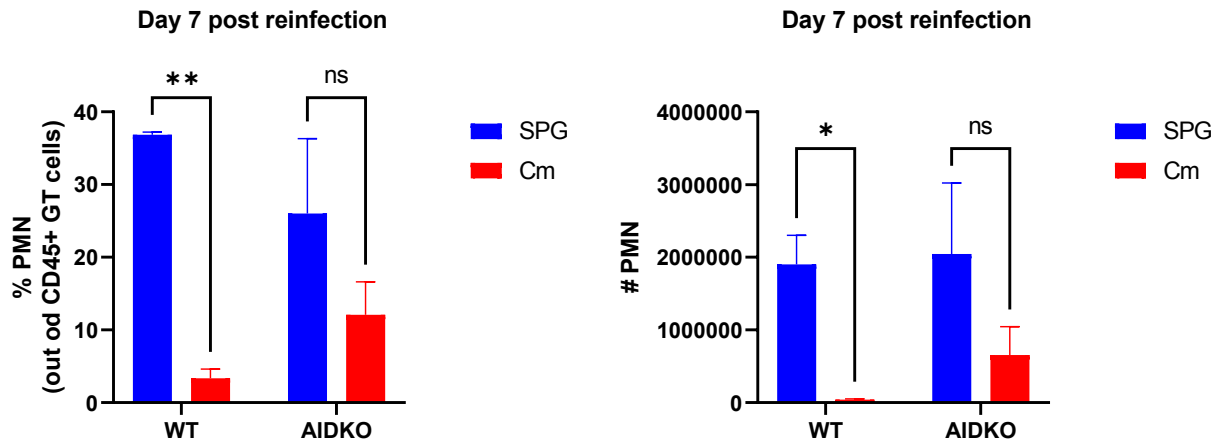


Figure 15: WT mice demonstrate a marked reduction in PMN infiltration GT in response re-infection

AIDKO and WT mice were intravaginally infected with 5 low doses of *C. muridarum* with the first day of infection being day 0. Mice were given time to clear the primary infection before a secondary challenge was given. Mice were sacrificed 7 days after re-infection and GTs were excised on the day of sacrifice. GTs were processed into single cell suspensions prior to surface staining with antibodies and flow cytometry acquisition to examine PMN infiltration to the local site of infection after re-infection. Data is presented with the mean ± SEM (n=3-6 mice per group); *P≤ 0.05, ** P≤ 0.01, with a two-way ANOVA and a Šídák's multiple comparisons test.

CHAPTER 4 DISCUSSION

4.1 Discussion of key findings

Despite decades of research, researchers have not reached a unanimous conclusion on the role of antibodies in *Chlamydia* pathogenesis. The use of μMT mice has been very popular to investigate the influence of antibodies in host immune responses and pathology development but leaves room for many confounding factors such as the loss of cytokine production from B cells. To isolate the role of antibodies without affecting the other facets of the B cell response, in this study, we turn to AIDKO mice. Prior to this study, the specific role of AID in *Chlamydia* pathogenesis has not been investigated. We use AIDKO mice and WT mice in our mouse model to examine the differences in pathogenesis and immune responses that would take place after *Cm* infection between strains. We hypothesize that AID deficiency would promote tissue pathology development after re-infection through antibody-independent mechanisms. Using this model, we sought to pinpoint the intricacies of host immune response to chlamydial infection without the presence of AID, CSR, SHM and thus a lack of secondary antibody production.

4.1.1 Repeated, low dose infection model instead of conventional single, high dose infection model more closely mimics natural human infections

In literature, the conventional model for *Chlamydia* infection is a single, high dose infection (Li & McSorley, 2015; Morrison & Morrison, 2005; Naglak et al., 2017; Rank et al., 2003). However, this model does not appropriately mimic the human experience with *Chlamydia* infection that is often complicated by asymptomatic infection that results in chronic infections and frequent reinfections. A previous student in the Wang lab, Monica Surette examined the differential immune responses that resulted from repeated, low dose *Cm* infections in comparison to a single high dose *Cm* infection. The results from her Master's thesis indicated that the repeated, low dose infection model parallel the reports of the human experience with *Chlamydia* infection in regard to enhanced tissue pathology and a reduction in protective immunity from subsequent infections (Surette, 2022). In hopes of producing results that can provide more in-depth insight into the host immune response to chlamydial infection in humans, we employed the usage of the repeated, low dose infection model for all instances of primary infection.

4.1.2 Use of AIDKO mice instead of μMT mice provides greater insight on the specific influence of antibodies in Chlamydia pathogenesis

In addition to using a repeated, low dose infection model rather than a conventional, single, high dose inoculation, we opted to use AIDKO mice rather than μMT mice. We chose to use AIDKO mice because previously, authors have used μMT mice and found many conflicting results. Namely, Su et al. (1997) concluded that μMT mice controlled a primary chlamydial infection similarly to WT mice but were not as efficient in clearing secondary infections, while Johansson et al. (1997), in the same year, using the same strain of mice, found that μMT mice have similar immune protection to WT mice in response to reinfection. These differing results can be explained if we examine the multi-faceted roles that B cells fulfill during an immune response. Not only do B cells produce antibodies, but they are capable of producing cytokines, are involved in $CD4^+$ T cell activation, via co-stimulation and antigen presentation, and regulating other immune cells for immune homeostasis (LeBien & Tedder, 2008). The use of μMT mice fails to consider the influence of losing these multi-functional responsibilities that B cells fulfill, and how this may affect the immune response to both primary infection and subsequent reinfections. Although μMT mice can provide correlative evidence of the role of antibodies in chlamydial pathogenesis, we are losing the cytokine response and other roles that B cell play during an active immune response, so the results cannot be used as direct evidence of antibody effect.

Furthermore, AID does not affect early immune response such as the cytokine response from B cells, but rather only B cell receptor diversification, maturation and hypermutation which are the later stages of antibody response. In fact, our model maintains IgM production but excludes subsequent isotypes. Therefore, we are losing the secondary antibodies made against *Cm* but we maintain the general IgM antibody response to more closely parallel the regular immune response and isolate the influence of secondary specific antibodies

4.1.3 AIDKO mice have comparable bacterial shedding trends and oviduct dilation post primary infection

The results from our study revealed that *Cm* infection induces a very similar primary infection in regard to bacterial burden and pathology between WT and AIDKO strains. Generally the

understanding is that AID is expressed in lymphoid tissues, and is responsible for regulating CSR and SHM for the diversification of secondary antibodies (Mechtcheriakova et al., 2012). If we examine this information through the lens of the results from this study, it is understandable that AID deficiency would have little impact on the bacterial shedding trends and pathology development post primary infection. Antibodies are created to adapt the immune response during a primary infection but also to improve recognition of the pathogen in subsequent encounters (Alberts et al., 2002). Thus, during a primary infection, lack of specific antibodies against *Chlamydia* due to AID deficiency is unlikely to influence clearance of infection. To further support this, it has been reported that specific anti-chlamydial antibody responses act mainly to enhance protective T-cell responses but that mice incapable of producing secondary specific antibodies are still able to resolve primary infection (Hafner et al., 2008).

With comparable levels of inflammation seen at the local site of infection post primary infection, it is understandable that oviduct dilation as a result of pathology development would also be comparable between strains. Moreover, neutrophils are one of the first immune cells to respond to the local site of infection and they have been deemed responsible for increasing immunopathology post chlamydial infection (Lijek et al., 2018). We observed an influx of neutrophils post primary infection in both strains, but the numbers and percentages remained comparable, providing further justification for the similarities in oviduct pathology development between strains post primary infection.

4.1.4 AIDKO have altered humoral responses to primary infection

A characteristic of both AID deficient mice and humans is a hyper IgM syndrome and lack of secondary antibodies such as IgG and IgA (Muramatsu et al., 1999). Our results confirmed that this phenotype persists during *Chlamydia* infection in murine models. We observed that the AIDKO mice produce significantly more IgM but the WT mice produce significantly more IgG1 and IgG2c antibodies in response to chlamydial antigens. It is important to note that although the AIDKO mice produce greater amounts of IgM, the IgM results seen in the two strains may be showing different types of IgM. The WT IgM has the ability to undergo SHM while the AIDKO IgM cannot undergo SHM. This reveals the possibility for differences in pentameric versus hexameric IgM as a result of mutations in the J chain which can affect its behaviour (Gong &

Ruprecht, 2020). The hexameric IgM is more effective at activating complement which may result in faster destruction of bacteria than pentameric IgM (Gong & Ruprecht, 2020). Future studies may consider examining the exact polymers of IgM produced by either strain during *Chlamydia* infections for further clarification.

Additionally, despite the well-known concept that AID deficient mice and humans are unable to perform class switching in B cells because AID plays a critical role in CSR, we see low levels of serum IgG1 and IgG2c in both naïve and infected AIDKO mouse serum. However, the levels of secondary antibody observed in the AIDKO mice is comparable to that of the naive WT mice and does not significantly increase in the serum post infection, thus, this may be attributed to baseline level readings being high or there being a similar phenomenon of low serum levels of secondary antibody in the mouse serum which is seen in hyper IgM syndrome in humans with AID deficiency. We have confirmed that the AIDKO strain we are using lack the AID gene through genotyping and excluded the possibility of the wrong strain of mice being used in this experiment.

4.1.5 AIDKO mice have relatively similar immune responses to primary infection at the local site of infection

Both WT and AIDKO mice should have comparable levels of containing bacterial infection post primary *Cm* infection due to the comparable populations of neutrophils, tissue macrophages, and stromal macrophages that infiltrate the local site of infection. With an intact innate immune response, both strains are able to quickly detect and alert the body of an active *Chlamydia* infection through antimicrobial peptides and inflammatory cytokine production (Helble & Starnbach, 2021). An interesting observation was that the neutrophil and stromal macrophage frequencies were increased by D17, whereas tissue macrophage frequencies were not increased until D30. Neutrophils are among the first immune cells to infiltrate the local site of infection so it is unsurprising that their population expands faster than macrophage populations (Barteneva et al., 1996; Rosales, 2018). Tissue macrophages are often found circulating in the bloodstream, therefore, it may take them longer to arrive at the local site of infection in the genital tract whereas the stromal macrophages are already present and ready to activate in response to infection (Epelman et al., 2014).

We know that cell mediated immunity, specifically the Th1 response, plays a central role in fighting *Chlamydia* infections. Our results demonstrate that the $CD4^+$ T cell response at the local site of infection appears to be unaffected by the AID deficiency, allowing for appropriate clearance of primary infections that parallel WT mice. The $CD8^+$ T cell response has been delegated with a more supportive role during active *Chlamydia* infections, often upregulating effector cytokines such as IFNγ, but have largely been deemed as dispensable for clearance of *Cm* infection (Johansson et al., 1997). Nonetheless, $CD8^+$ T cell recruitment remains intact at the local site of infection, further perpetrating a typical immune response to chlamydial infection in both strains of mice. Future studies may consider measuring the activity of $CD8^+$ T cells in addition to their presence.

At the local site of infection, the only cell population with significant differences between strains was the number of $CD19^+$ B cells at D17. Although we did not examine expansion of autoimmune cells in this study, it is well known that both AID-deficient humans and AIDKO mice often suffer from autoimmune conditions (L. Chen et al., 2010). This may be in part due to the evidence that shows AID is critical for establishing proper central and peripheral B-cell tolerance (Meyers et al., 2011). Subjects with AID deficiencies have defective central and peripheral B-cell tolerance checkpoints, which can lead to an accumulation of excessive numbers of autoreactive B cells that impart the signature hyper IgM syndrome seen in AIDKO mice and AID-deficient patients(Meyers et al., 2011), but may also explain the increased numbers of B cells observed in the AIDKO mice. Additionally, chlamydial infection may induce greater expansion of IgM producing B cells in AIDKO mice than the WT mice that could explain these results. However, future studies could examine specific subtypes of B cells present at D17 in the genital tract to further clarify these results.

4.1.6 AIDKO mice have significantly different immune responses at the systemic level to primary infection

Although there were not many differences observed in the immune response at the local site of infection, we were curious as to whether AID deficiency would affect the systemic immune response to *Chlamydia* infections. Studies on WT and AIDKO B6-lymphoproliferative (lpr) mice

show that although both lpr strains have splenomegaly, the AIDKO lpr mice have significantly larger spleens than their WT lpr counterparts (Chen et al., 2010), but there were no differences observed between the WT B6 mice and AIDKO mice also included in the study. Our results did not reflect splenomegaly seen in the lpr mice before or after *Cm* infection. In fact, the absolute cell recovery from the excised spleens does not increase in the spleen, indicating that there is no response. These results are in concordance with the data from Chen et al. 2010 with the WT B6 and AIDKO mice that were not lpr.

No significant differences were observed between strains in regard to the numbers and percentages of $CD4^+$ T cells found in the spleen at any timepoint but there was a significant decrease in the number of $CD4^+$ T cells between the D0 to D30 timepoints present in the AIDKO mice. However, this number returned to baseline levels by D53. This was a particularly intriguing observation because a similar phenomenon was seen in the number of $CD19^+$ B cells in the spleen within the same time frame with no significant differences between strains at any given timepoint post primary infection either. A potential explanation to this observed phenomenon could mirror what is seen in AID deficient humans where a decrease in $CD4^+$ T cells is observed and has been attributed to T cell exhaustion from repeated infections (Durandy et al., 2013). It is possible that, due to the persistent nature of *Cm* infections and robust B cell activity, the T cells are constantly being activated and thus are subject to subsequent apoptosis. Similarly, the normal B cell response may be perturbed due to the AID deficiency and lead to dysfunctional B cell responses. B cell exhaustion has yet to be defined but cases of this have been reported in post antigen-activated human B cells (Ahmed et al., 2019). It would be worthwhile to consider examining regulatory B cells and their behaviour in chronic *Cm* infections to see if dysfunctional regulation of B cell populations could lead to overactivation and subsequent exhaustion and decline in B cell numbers in the spleens of AIDKO mice overtime after primary *Cm* infections.

Due to the perturbations observed in $CD4^+$ T cell populations over time in the spleen, we were interested in examining subpopulations of $CD4^+$ T cells such as Tfh cells which are critical in the GC. What we found was that by D30 and bridging into D53 post primary infection, the AIDKO mice have significantly higher percentages of Tfh cells than their WT counterparts. This

observation indicates that AID deficiency plays a role in regulating the $CD4^+$ T response by promoting Tfh cell differentiation. We wonder then, if this will have an effect on the critical Th1 response to *Cm* infection. The sandwich cytokine ELISA results show that the WT mice have visibly greater IFNγ responses in the D30 samples and significantly greater IFNγ production in the D53 samples when compared to AIDKO splenocytes of the same respective timepoint. Thus, our results demonstrate that the promotion of Tfh cell differentiation as a result of AID deficiency inhibits a robust Th1 response to secondary encounters with *Cm* antigens.

Interestingly, the WT mice have significantly greater percentages of Tregs than AIDKO mice at baseline and no significant differences were observed between strains at either D30 or D53 timepoints. However, by D132 the AIDKO mice have significantly higher percentages of Tregs than their WT counterparts. Human studies have shown decreased numbers of Tregs in AID deficient patients that was attributed to the involvement of switched memory B cells in generating and maintaining Tregs in the human immune system (Durandy et al., 2013; Meyers et al., 2011). Thus, the baseline results of WT mice having greater percentages of Tregs than AIDKO mice agrees with the findings in human studies but the later timepoint results where the AIDKO mice have greater percentages of Tregs does not agree with the findings from human studies. This may be attributed to differences between murine and human immune system mechanisms, as well as a gap in the literature that examines the Treg levels in AID deficient humans subject at later timepoints similar to the D132 timepoint post primary *Cm* infection.

No significant differences were found between strains at any timepoint within the $CD19^+$ B cell, plasma cell or memory B cell populations post primary infection. However, at D53 post primary infection, the AIDKO mice have significantly greater percentages of GC B cells in the spleen than their WT counterparts. This is in concordance with previous studies that proved AIDKO mice to have larger GCs and thus, an abnormal accumulation of GC B cells in the spleen (Zaheen et al., 2009). It is worthwhile to note that the larger GC structure seen during AID deficiency is reflected in human studies as well (Revy et al., 2000). Moreover, GC B cells of AIDKO mice have been shown to have reduced levels of apoptosis (Zaheen et al., 2009) which may explain the persistently higher percentages of GC B cell observed at D53 post primary infection. Normal regulation of the immune response led to normal levels of apoptosis in the WT mice while this

apoptosis did not occur in the AIDKO mice, resulting in the significantly greater populations of GC B cells observed at D53. Future experiments could consider measuring only dividing B cells where a larger difference in B cell populations could be seen.

4.1.7 AID deficiency dysregulates Th1 and Th17 responses, which likely affects response to future reinfections

The cytokine ELISA results show no measurable levels of IL-4, IL-13, or IL-10. The lack of IL-10 production was unexpected but can be attributed to dysfunctional IL-10 standards skewing the readings of the plates. This can easily be remedied through the purchase of new reagents and repeating the experiment to gather more accurate data. The lack of IL-4/IL-13 production indicates that little to no Th2 response was seen. This conclusion is in agreement with other studies in the field that found virtually no Th2 cells in mice intravaginally infected with *Cm* (Labuda & McSorley, 2018; Li & McSorley, 2013). Th2 responses in *Chlamydia* infection are largely considered nonessential. This is supported by evidence that genetic deletion of IL-4 or IL-4Rα has no impact on *Cm* bacterial burden in comparison to WT mice (Li & McSorley, 2013). Furthermore, transfer of *Cm* specific Th2 clones to nude mice provide no protection while transfers of Th1 clones provides robust protection against *Cm* infections (Hawkins et al., 2002).

Th1 responses are significantly greater in the D53 WT mice samples when compared to their AIDKO counterparts. This indicates that the WT mice can induce a more robust Th1 response during *in vitro* stimulation with chlamydial antigens than AIDKO mice. Moreover, the Th17 responses in WT mice are significantly greater than those of AIDKO mice by D53 post primary infection but this trend was not reflected in the D30 samples. This disconnect may be attributed to delayed Th17 responses to *Chlamydia* infections previously observed (Moore-Connors et al., 2013). But overall, it is clear to us that the WT mice can induce a more robust and effective immune response during *in vitro* stimulation with chlamydial antigens when compared to AIDKO mice.

4.2 Challenge study rationale

The results we gathered from the *in vivo* experiments post primary infection confirmed our understanding of the role of AID that has been described in literature, and that the influence of

antibody is unimportant in clearing primary infection. However, these results did not provide us insight as to how AID deficiency would impact the immune response to a re-infection. Thus, we decided to use the challenge study to investigate this. The challenge study follows the same general model but provides the opportunity for complete clearance of infection prior to a re-infection.

4.2.1 AIDKO mice have greater bacterial shedding after re-infection and greater oviduct dilation

The first parameter we wanted to examine after the re-challenge was whether there was a difference between strains in bacterial shedding. The results showed that the AIDKO mice have significantly higher bacterial burden than the WT mice post re-challenge. This proved to us that the WT mice were better protected against a secondary challenge than the AIDKO mice. These results are in concordance with the findings of Su et al. (1997) and the Morrison group (2005) who concluded that antibodies are important immunity against genital tract reinfections. However, the Morrison group stated that the antibody mediated protection against reinfection relies heavily on proper $CD4^+$ T cell priming during the primary infection. Collectively, with our results from primary infection that indicated AID plays a role in regulating $CD4^+$ T cell differentiation, we are more inclined to believe that these results depend more heavily on the cellular response rather than antibody mediated protection. Specifically, we know that the AIDKO mice preferentially differentiate more Tfh cells and have an inhibited Th1 response. This may lead to improper priming of $CD4^+$ T cells during primary infection and make the AIDKO mice more vulnerable to subsequent reinfections in which they have greater bacterial burden.

Due to the difference observed in levels of bacterial shedding between strains, we sought to determine if this has an effect on pathology. The results showed that the AIDKO mice have significantly greater oviduct dilation than WT mice after a reinfection. This suggests that antibody production is not the perpetrator for severe pathology development which contradicts the theory whereby *Ct* associated antibodies are correlated with pathology development and tubal factor infertility (Dadamessi et al., 2005; Toye et al., 1993).

4.2.2 AIDKO mice have stronger immune responses to reinfection

If the antibodies were not the culprit behind increased GT pathology after subsequent reinfections, we wonder if this could be a result of more aggressive immune responses that were necessary in the AIDKO mice who were poorly protected against reinfection. An overreactive inflammatory response is likely to result in more severe tissue damage and may be linked to the tissue damaging cytokines produced during the active immune response. The first population of cells we wanted to examine were polymorphonuclear neutrophils (PMNs) at the local site of infection. The results show that the WT mice demonstrate a significant reduction in PMN infiltration in the GT after a reinfection, but the AIDKO mice do not have the same level of reduction. This conveys the idea that the immune response was greater in the AIDKO mice likely due to the greater levels of bacterial burden.

Neutrophils have been proven to at least be partially responsible for inducing pathology development in murine models of *Cm* GT disease (Lee et al., 2010). Lacy et al. (2011) conducted a study on guinea pigs with ocular infections induced by *C. caviae.* They depleted the guinea pigs of neutrophils using rabbit antineutrophil antiserum and evaluated ocular pathology and immune responses. The results showed that guinea pigs that have been depleted of neutrophils have dramatically decreased ocular pathology in both clinical and histological aspects (H. M. Lacy et al., 2011). In a similar vein, Lee et al. (2010) depleted mice of neutrophils using a neutrophil-depleting monoclonal antibody shortly after *Cm* urogenital infection. They found that the neutropenic mice have relatively similar courses of infection as their neutrophil competent control counterparts, but have developed significantly less tissue pathology when examined using histopathology (Lee et al., 2010). This discovery was accompanied by a decrease in the amount of matrix metalloproteinase-9 (MMP-9) produced by the neutropenic mice, and they concluded that the inflammation and production of MMP-9 play a role in inducing fibrosis and contributing to infertility in mouse models of chlamydial upper genital tract infections. When this information is studied considering our data, it is plausible that the increased pathology seen in AIDKO mice post reinfection is a result of the stronger immune response to reinfection. Increased PMN infiltration provides an opportunity for increased exposure of epithelial cells to tissue damaging cytokines and subsequent severe pathology development.

4.3 Implications for vaccine development

It is estimated that roughly 130 million new infections of sexually transmitted *Ct* infections occur annually worldwide, making it the most common sexually transmitted bacterial pathogen in the world (Newman et al., 2015; Price, Ades, Welton, et al., 2016). The most efficient way to control the spread of sexually transmitted *Chlamydia* infections would be the development of a vaccine (Brunham, 2013). However, despite work in *Chlamydia* vaccinology beginning in the 1960s, progress has been slow (de la Maza et al., 2021). Critical challenges in vaccine development involve finding vaccine delivery and adjuvant systems that are able to induce robust immune responses, but not trigger pathological immune responses (Brunham, 2013). Only recently has a phase 1 clinical trial for a *Ct* vaccine against genital infections been completed (de la Maza et al., 2021).

The findings from this study contribute to the field of knowledge surrounding host immune responses to *Chlamydia* infections by supporting the idea that antibodies are not largely responsible for severe pathology development after subsequent infections, and that inducing a proper Th1 response during primary infection supports better protection against subsequent infections.

4.4 Limitations and future directions

The focus of this study was to determine the contribution of AID in *Chlamydia* pathogenesis, which we were able to do in both primary infection and reinfection scenarios. However, there were certain limitations to this study. One limitation would be that we discovered a contribution of AID to $CD4^+$ T cell regulation which presents an additional factor to consider when trying to elucidate the role of secondary antibodies during the immune response to *Chlamydia*. Future studies need to be done to determine the mechanistic reasoning behind the AIDKO phenotype immune response to *Chlamydia* infections. For such a study, both WT And AIDKO mice could be immunized against *Cm*. Then, prior to a re-challenge, both strains would be depleted of $CD4^+$ and $CD8^+$ T cells. In this situation, the memory T cell response is lost in both strains, and we can isolate the role of antibodies in protecting the host from subsequent reinfections.

It is important to consider the possible confounding effects that the unique microbiomes may have in influencing host responses. The vaginal flora may differ between individual mice and between species, potentially resulting in differential immune responses to *Chlamydia* infection. This can be addressed by testing for differences in the natural microbiome of the mice using swabbing methods. If large differences exist, it may be beneficial to house both strains of mice in the same cages.

Additionally, we can look to murine models as an efficient and relatively accurate way to examine the pathogenic immune mechanisms perpetrated by chlamydial infections that may be extrapolated to further investigate the immune mechanisms that mediate human chlamydial disease. It is important to note however, that although murine models of chlamydial infection can mimic many aspects of human infection, mice are often raised in pathogen-free facilities with specific living conditions that is not realistic to humans and the mice are able to spontaneously clear *Cm* infection whereas humans are often asymptomatic which leads to chronic cases of infection and frequent reinfections.

4.5 Conclusions

We can conclude that AID deficiency does not lead to many differences in the immune response to primary infection at the local site of infection. Both AIDKO and WT mice have similar trends in bacterial shedding and pathology development following primary infections.
However, there are significant systemic changes in the host immune response. Additionally, it appears that AID deficiency has a much larger impact on host immune response to subsequent reinfections and impacts the critical memory responses necessary for immunity.

To summarize the most important findings from this study, we reveal an unexpected role for AID in regulating $CD4^+$ T cell differentiation. AID deficiency skews the immune system to preferentially differentiate $CD4^+$ T cells into Tfh cell populations and inhibits robust Th1 responses. *Chlamydia* specific $CD4^+$ IFN-γ response have been associated with protective immunity against reinfection (Bakshi et al., 2018). The inhibition of a proper Th1 response interferes with appropriate $CD4^+$ T cell priming during primary infection and makes the AIDKO mice more susceptible to subsequent reinfections. This results in greater bacterial burden

triggering a more vigorous immune response which ultimately causes more pathology development in the AIDKO mice after reinfection.

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