The role of Cysteinyl leukotriene type 1 receptor (CysLTR1) during *Listeria monocytogenes* and *Mycobacterium tuberculosis* infections in mice

Submitted by

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Declaration

I, Sibongiseni KL. Poswayo, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

I dedicate this thesis to my babies, Ayabukwa, Lubumba and Alunamida

May this be a constant reminder that with hard work and determination you can achieve anything you put your mind on.

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Abbreviations

5-LO	5-lipoxygenase
AA	Arachidonic acid
ActA	Actin polymerization factor A
ALOX5	Arachidonate 5-lipoxygenase
ALOX5AP	ALOX5 activating protein
APCs	Antigen-presenting cells
Arg1	Arginase 1
Ca^+	Calcium
CCL	Chemokine (C-C motif) ligand
CDC	Centre of Disease and Control and Prevention
cDC	Conventional Dendritic cells
cDNA	Complementary Deoxyribonucleic Acid
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
CYBB	Cytochrome b-245 heavy beta chain
CYP	Cytochrome P450
CysLT	Cysteinyl leukotriene
CysLTR	Cysteinyl leukotriene receptor
$cPLA_{2\alpha}$	Cytosolic phospholipase A_2
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EETs	Epoxyeicosatrienoic acids
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
HAND	HIV-associated neurocognitive disorders

HDT	Host Directed Therapy
HIV	Human immunodeficiency virus
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL-	Interleukin
IMMs	Inflammatory monocyte-derived macrophages
Inl-	Interlin
iNOS	Inducible nitric oxide synthase
IPS-1	Interferon-beta promoter stimulator 1
K. pneumoniae	*Klebsiella pneumoniae*
LLO	Listeriolysin O
Lm	*Listeria monocytogenes*
LXA_4	Lipoxin A_4
LOX	Lipoxygenase
LT	Leukotriene
LTA_4H	Leukotriene A_4 Hydrolase
LTC_4S	Leukotriene C_4 synthase
LTRA	Leukotriene receptor antagonist
MAIT cells	Mucosal-associated invariant T cells
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MtArgJ	Ornithine acetyltransferase
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation factor 88
MZMs	Marginal zone macrophages
NADPH	Nicotinamide dinucleotide phosphate
NF-κB	Nuclear factor kappa-B
NK cells	Natural killer cells
NLR	NOD-like receptor

NOD	Nucleotide-binding oligomerization domain
NOS2	Nitric oxide synthase 2
NOX2	Nicotinamide dinucleotide phosphate (NADPH) oxidase 2
PAMPs	Pathogen-associated molecular patterns
Plc	Phospholipase
PRK	Pranlukast
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative Polymerase Chain reaction
RIC	Reduced intensity conditioning
ROS	Reactive Oxygen Species
RPMs	Red pulp macrophages
SARS-Cov2	Severe acute respiratory syndrome coronavirus 2
STAT	Signal transducer and activator of transcription
TAK1	Transforming growth factor beta-activated kinase 1
TB	Tuberculosis
TGFβ	Transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
Tregs	regulatory T cells
WHO	World Health Organization

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Abstract

During infection, antigen-presenting cells release cytokines and eicosanoids (including leukotrienes, epoxyeicosatrienoic acids, and prostanoids) to activate adaptive immunity. Leukotrienes (LTs), an eicosanoids subset, are produced from arachidonic acid via 5-lipoxygenase metabolism, resulting in LTB4 and cysteinyl LTs (cysLTs; LTC4, LTD4 and LTE4). CysLTs, which are pro-inflammatory lipid mediators, play a role in asthma and interact with three G-protein-coupled receptors (CysLTR1, CysLTR2, and GPR99). While the role of CysLTR1 and its ligand has been studied in asthmatic and allergic responses, its function during bacterial infections remains unclear. Our study aims to elucidate the role of CysLTR1 in disease progression using C57BL/6 and Balb/c mice infected either with *Listeria monocytogenes* (*Lm*) or with *Mycobacterium tuberculosis* (*Mtb*). Using CysLTR1 knockout (*Cysltr1*$^{-/-}$) mice, we conducted time course and survival studies on both C57BL/6 and Balb/C genetic backgrounds. Our findings revealed that the function of CysLTR1 in bacterial infections is influenced by the host's genetic background and gender. Interestingly, CysLTR1 deletion did not impact survival or tissue pathology in C57BL/6 mice during *Lm* and *Mtb* infections. However, in *Lm* infection, CysLTR1 deletion led to increased recruitment of neutrophils to the liver and spleen, while in female *Cysltr1*$^{-/-}$ mice during *Mtb* infection, lung neutrophil recruitment was elevated. In Balb/C mice, CysLTR1 deletion during *Lm* infection affected survival in a sex-dependent manner, providing protection to females by reducing neutrophil recruitment. Conversely, Balb/C male mice were more susceptible to *Lm* infection. Notably, during *Mtb* infection, CysLTR1 deletion in Balb/C mice resulted in improved disease outcomes due to reduced lung neutrophils and pathology. In summary, CysLTR1 signalling significantly influences neutrophil recruitment and activation during intracellular bacterial infections, with its impact varying based on gender and genetic background.

Chapter 1: Literature review

1.1. Eicosanoids

Eicosanoids are a family of signalling molecules generated through the enzymatic or nonenzymatic oxidation of arachidonic acid or other polyunsaturated fatty acids (PUFAs) with 20-carbon units (1,2). They are a subcategory of oxylipins; a diverse carbon unit of fatty acids that have important roles as cellular signalling molecules (3). Eicosanoids have multiple subfamilies that include prostaglandins, thromboxanes, leukotrienes, lipoxins, eoxins and epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs). These families are further subcategorized into three different pathways based on the enzymes that initiate their metabolism. Prostaglandins and thromboxanes are synthesized through the metabolism of the AA by the enzymatic activities of the cyclooxygenases (COXs), COX-1 and COX-2 (4–7). Leukotrienes, lipoxins, eoxins, and hypoxins form part of the lipoxygenase (LOX) pathway (8–10). The EETs and HETEs are synthesized by the cytochrome p450 epoxygenase and form part of the cytochrome P450 (CYP) pathway (11–13) and the products of this pathway can be further metabolized by COXs. For the biosynthesis of each eicosanoid, free fatty acids (particularly the AA) must be available. The AA is only generated by phospholipase A_2 (PLA_2) from the membrane glycerophospholipid following cellular stimulation which results in increased calcium (Ca^{2+}) and/or phosphorylation (14–16), see **Figure 1.1** below.

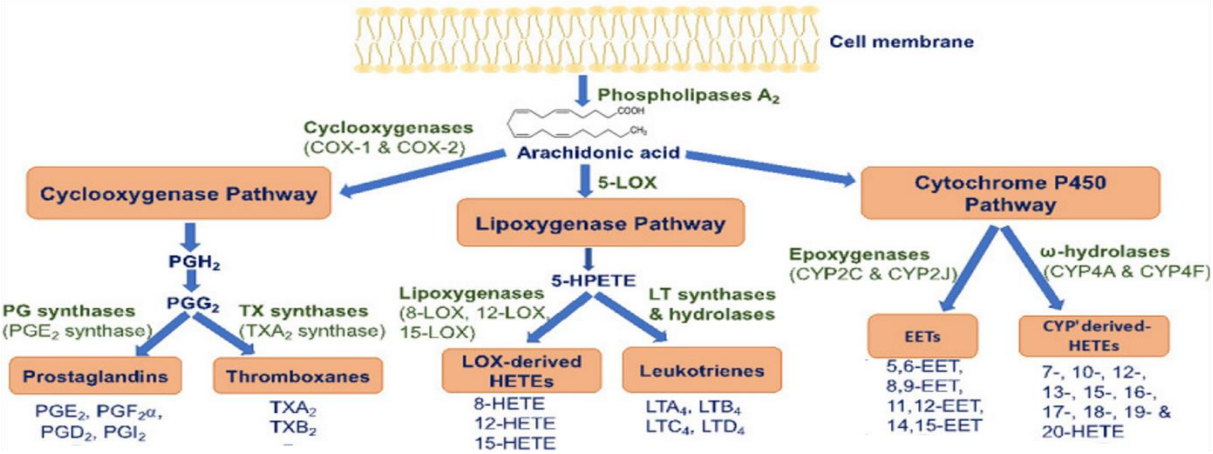

Figure 1.1: Eicosanoid biosynthesis from the arachidonic acid. Synthesis of eicosanoids begins with the esterification of the phospholipase A_2 (PLA_2) from the cell membrane for the activation of the AA. Enzymatic activities of three distinct enzyme families are involved in the production of the eicosanoids leading to their distinct classification, cyclooxygenases (COXs), lipoxygenases (LOXs) and epoxygenases (also known as cytochrome P450, CYP). Image adapted from reference (17).

1.1.1. Eicosanoid receptors

Eicosanoids exert their biological functions through their receptors. Eicosanoid receptors are mostly G-protein coupled receptors (GPCRs). Prostanoids act through eight GPCRs that include four E prostanoid receptors (Eps), EP1, EP2, EP3 and EP4 which bind PGE2 (18); two D prostanoid receptors (DP), DP1 and DP2 that bind PGD2 (19); while prostaglandin I_2 (PGI_2) binds to IP (20); and TxA2 binds to TP (21). The DP1 prostaglandin receptor is found in various cell types, including airway epithelial cells, vascular endothelium, airway smooth muscles, and platelets. On the other hand, DP2, also known as a chemoattractant receptor homologous molecule, expressed on Th2 cell (CRTH2), is specifically induced in inflammatory cells associated with asthma pathogenesis. These inflammatory cells include Th2 cells, eosinophils, and basophils. DP2 activation by PGD2 induces cellular chemotaxis and activation resulting in IL-4, IL-5, and IL-13 production (19). The EETs have effector functions on endothelial cells and smooth muscle cells. G-protein coupled receptor 40 (GRP40) was demonstrated to be a low-affinity receptor for EETs in the vasculature (22). The 12-HETE binds to the GPR31 receptor and induces tumor proliferation and metastasis (23), while GPR75 has been shown to

bind 20-HETE (24). Leukotriene B4 (LTB4) exerts its chemotactic and granulocyte activation effects through two receptors, leukotriene B4 receptors 1 and 2 (BLT1 and BLT2) (25). The cysteinyl leukotrienes (cysLTs, LTC4, LTD4 and LTE4), were initially thought to function through two receptors, CysLTR1 and CysLTR2 (26), however, a third receptor (GPR99/CysLTR3) was recently discovered (27).

1.1.2. Eicosanoids biological roles

Eicosanoids have been implicated in various biological functions. For instance, they regulate physiological responses, including tissue homeostasis, pain, host defences and inflammation (28), and have roles in the pathogenesis of such as cardiovascular diseases, diabetes, hypertension, and Covid-19 (29–32). Eicosanoids also contribute to reducing inflammation by facilitating the resolution of inflammatory processes, restricting immune cell infiltration, and initiating tissue repair mechanisms (33). Eicosanoid receptors are expressed throughout the immune system, they modulate cytokine production and expression of cell surface molecules involved in autocrine and paracrine processes. These results in a crosstalk between the innate and adaptive immune responses (34).

Specifically, prostanoids (collective term for prostaglandins and thromboxanes) are synthesized in response to diverse stimuli and function through paracrine and autocrine signalling pathways. They play significant roles in normal physiological processes and diseases (2). Prostaglandins have both pro-inflammatory and immune-suppressive effects (35). Epoxyeicosatrienoic acids (EETs) also have autocrine and paracrine effects on the kidneys and cardiovascular system. EETs modulate ion cellular transportation and gene regulation while triggering vasodilation. They also exhibit anti-inflammatory and pro-fibrinolytic effects (11). Leukotrienes, on the other hand, demonstrate acute inflammatory properties such as increased vascular infiltration and granulocyte recruitment (35).

1.2. The Leukotriene Family

The leukotriene family consists of the leukotrienes and cysteinyl leukotrienes (CysLTs). Leukotrienes are a family of inflammatory mediators that belong to the family of eicosanoids. Immune and nonimmune stimuli, such as antigens, immune complexes, complements, cytokines, pollutants etc., result in the metabolism of the arachidonic acid (AA) by 5-LO to form leukotrienes and eventually cysteinyl leukotrienes (CysLTs). In short, leukotriene synthesis begins with the translocation of cytosolic phospholipase A2 (cPLA2) and 5-LO to the nuclear envelope, where the AA is metabolised to form 5-hydroperoxyl eicosatetraenoic acid (5-HPETE). 5-HPETE is then converted to an unstable leukotriene intermediate, leukotriene A4 (LTA4), by the enzymatic activity of 5-LO. The dihydroxyl leukotriene, LTB_4, is formed via the hydrolysis of LTA_4 by LTA4-hydrolase. LTA_4 can also be conjugated to a reduced glutathione by leukotriene C_4 synthase (LTC_4S) to form LTC_4 (36). LTC_4, after undergoing hydrolysis, transforms into LTD_4 through the action of gamma-glutamyl leukotrienase. Subsequently, LTD_4 is synthesized further to produce LTE_4 with the help of a dipeptidase. Collectively, these three lipids (LTC_4, LTD_4, and LTE_4) are referred to as CysLTs due to their shared amino acid, cysteine (37). The production of cysLTs appears to be restricted to leukocytes, eosinophils, basophils, and macrophages. However, under the inflammatory stimulus, transcellular activity can result in cysLTs formation in endothelial cells (38). Because of this mechanism, cells that are unable to produce (such as vascular endothelial cells, platelets, and blood peripheral monocytes) or use (leukocytes) LTA4, can produce cysLTs from surrounding cells (39).

1.2.1. Cysteinyl leukotrienes and their receptors

The lipid mediators, cysLTs, are known for their biological functions and pathogenesis in asthma which is usually signified by excessive pulmonary inflammation. These mediators exert their functions through three G-couple protein receptors, cysteinyl leukotriene type 1 receptor

(CysLTR1, CysLTR2, and GPR99/CysLTR3). CysLTR1 is has highier affinity for bonding LTD4 than the other two cysLTs, LTC_4 and LTE_4, while CysLTR2 binds both LTC4 and LTD4 with equal affinity. CysLTR3 was recently discovered and is mainly stimulated by LTE4 (40,41). These receptors are widely expressed across a variety of cells and organs. For instance, CysLTR1 is expressed in the peripheral blood leukocytes such as eosinophils, monocytes, neutrophils, basophils, dendritic cells, B cells and T cells (42). CsyLTR2 is widely expressed in various organs like the heart, adrenal, lung, spleen, endothelium, and blood leukocytes (43). CysLTR3 on the other hand is mostly expressed in the tissue of the respiratory tract and kidneys (44). The CysLT receptors are located on the inflammatory cells' outer plasma membrane and interact with the cytoplasm G proteins resulting in Ca^{2+}increase and cAMP reduction (45).

CysLTR1 is typically associated with the traditional physiological responses triggered by CysLTs, which include bronchoconstriction, enhanced vascular permeability, activation of leukocytes, transcription factor activation, and the release of inflammatory mediators and cytokines (46). Elevation of intracellular Ca^{2+} is known to induce CysLTR1 signalling. Activated CysLTR1 signals through either or both Gq- and Gi-proteins depending on the type of cell (47). Montelukast, Pranlukast, and Zafirlukast are some of the CysLTR1 antagonists used as anti-asthmatic drugs (48). In mice, CysLTR2 deficiency and overexpression showed that it has no effect on bronchoconstriction regulation, but plays a role in lung inflammation, vascular permeability, and tissue fibrosis (49). It has also been demonstrated that CysLTR2 regulates the production of IL-33 in alveolar epithelial cells (50). Not much research has been done on CysLTR3 however, it has been shown that it increases IL-25 production in airway cells modulating cellular functions, resulting in vascular leakage during responses to inflammation (27,51), indicating that CysLTR3 plays a role in allergic and respiratory diseases.

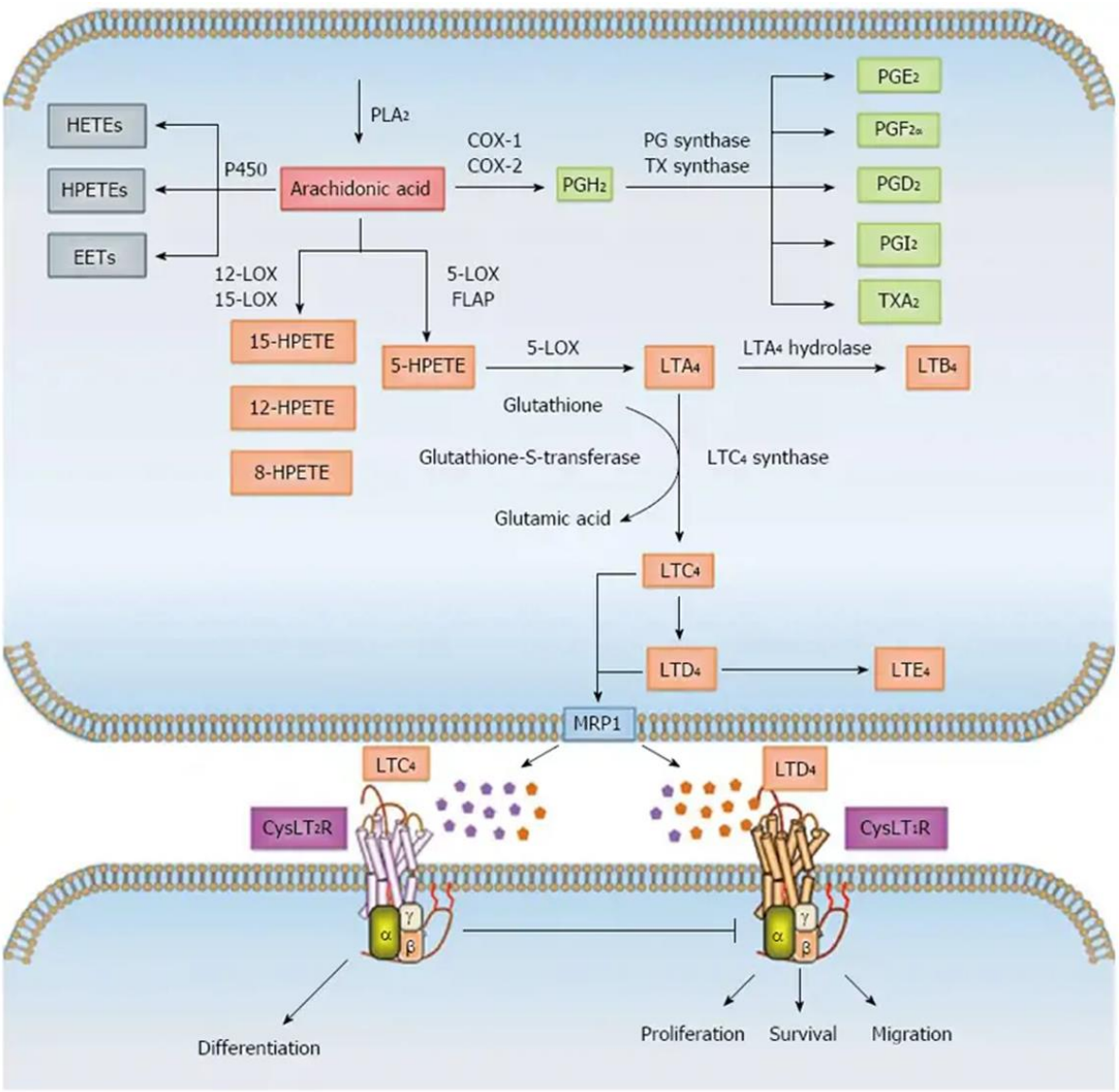

Figure 1.2: Graphic representation of the arachidonic acid metabolism to cysteinyl leukotrienes and their receptors. Cytosolic arachidonic acid (AA) is either metabolized through three enzymatic pathways: the cytochrome P450, cyclooxygenase (COX) or 5-lipooxygenase (5-LOX). The metabolism of the AA through the LOX pathway involves 5-LOX and 5-lipooxygenase activating protein (FLAP) resulting 5-HPETE. This is then subsequently processed to produces leukotrienes (LTs), from LTA4 to LTB_4 which is further metabolized to produce cysLTs (LTC_4, LTD_4 and LTE_4) through the enzymatic activity of leukotriene C_4 synthase (LTC_4S). CysLTs are transported through the multidrug resistance-associated protein (MRP) family efflux transporters where they can exert their biological functions through G protein-coupled receptors (CysLTR1 and CysLTR2). LTD_4 binding to CysLTR1 leads a sequence of signaling events resulting in cellular proliferation, survival and migration, while LTC_4 binding to CysLTR2 leads to cellular differentiation. Image adapted from (52).

1.2.2. Leukotrienes in asthmatic and allergic responses

As mentioned above, leukotrienes and cysteinyl leukotrienes play a vital role in the pathogenesis of asthma and allergic responses. In asthma, LTB_4 and the cysLTs have been shown to have a crucial role in the modulation of inflammation and experimentally cysLTs can mimic the pathological effects associated with asthma. Patients with asthma are sensitive to cysLT inhalation as these lipids tend to constrict the airways, promote mucus production, and blood vessel leakiness in the lungs. These are usually accompanied by increased recruitment of inflammatory cells, eosinophils, to the mucus lining in lung airways (53–56). Treatment with the 5-LO antagonist, zileuton, has been shown to improve symptoms in patients with asthma and who are aspirin intolerant (57,58).

In allergic responses, children with allergic rhinitis exposed to ragweed were observed to have increased production of LTC_4 in their nasal mucosal cells, such as must cells (59). While patients with seasonal allergic rhinitis produced high levels of LTB_4 and LTE_4 in their exhale breath condensation during and post-allergic season (60). Seasonal allergic rhinitis patients treated with the active oral 5-LO inhibitor, A-64077, showed attenuated allergen-induced nasal congestion, accompanied by reduced LTB_4 and 5-HETE secretion but not PGD_2 (61). Treatment of both asthmatic and allergic rhinitis with montelukast effectively improved patient outcomes (62). Further emphasising the role of cysLTs signalling, particularly via CysLTR1, in the pathogenesis of allergic responses.

1.2.3. Leukotriene role in inflammatory diseases

Leukotrienes, particularly cysLTs, are known for their inflammatory mediator effects. They have been implicated in various inflammation-related diseases in addition to their role in allergic responses and airway inflammatory diseases.

Neurodegenerative-related disease. Cysteinyl leukotrienes are not detectable in healthy brains, however, in post-traumatic injuries through a mechanism that involves neutrophils and brain cells, there is a rapid rise in cysLT production (63–65). One study demonstrated that Ca^{2+}-stimulated neutrophils cultured with rat neurons or glial cells produce high levels of CysLTs, suggesting these cells play a role in the transcellular synthesis of these lipids (66). Based on these findings, Farias et al (63), further demonstrated that following a traumatic brain injury cysLT production is immediately upregulated. Neutrophils attributed this increase to a transcellular biosynthesis mechanism. Treatment with MK-886, an antagonist for FLAP used to block LT and cysLT production, resulted in reduced cysLT synthesis and brain lesions (63). FLAP inhibition pre- and post-traumatic brain injury reduces leukotriene production, and brain swelling while improving blood-brain barrier disruption and cognitive deficits due to the injury. In rats, MK-886 treatment improved their spatial learning and memory (64). Therefore, cysLT and LT secretion are not only caused by brain injuries, but their increased production affection affects cognitive effects, therefore attenuating their production improves brain health and functions.

LTD_4 inflammatory effector functions have been examined in amyloid-β (Aβ) generation (an Alzheimer's disease (AD) model using neurons in vitro). Treatment of primary neurons with LTD_4 induced the generation of Aβ in a CysLTR1-mediated NF-κB signalling manner. And inhibition of CysLTR1 using pranlukast and inhibition of the NF-κB pathway reversed these effects (67). Inhibition of the 5-LO pathway was also shown to reduce the formation of Aβ in the transgenic AD-like amyloidosis mouse model, Tg2576 (68). In an AD mouse model using Aβ1-42 injections, mice had impaired learning and memory difficulties which were accompanied by increased CysLTR1 expression, and inflammatory and apoptotic responses. These effects were reduced post-treatment with CysLTR1 antagonists, montelukast (69). Leukotrienes have also been implicated in the pathology of neuropathic pain post-peripheral

nerve injury. Rats that had spared nerve injury (SNI) showed increased expression of LT synthesis and their receptors in the spinal cord and microglia. The inhibition of 5-LO or BLT1 and CysLTR1 receptors reduced the mechanical allodynia development, indicating that LTs have an intraspinal role in neuropathic pain (70).

Rheumatoid arthritis. Leukotrienes also have been shown to play an important role in acute and chronic inflammation such as rheumatoid arthritis (RA). LTB_4 was shown to be essential in the development of collagen-induced arthritis (mouse model for human RA) in mice. Inhibiting the LTB_4 receptor reduced the humoral immune responses to the inflammation caused by collage (71). FLAP-deficient mice also showed reduced arthritis severity scores when compared to their littermate controls, demonstrating an important role of leukotrienes in inflammation caused by this disease (72).

Chronic lung inflammation and scaring. Leukotrienes are involved in the pathogenesis of pulmonary fibrosis and targeting the 5-LO pathway has been proposed as a potential treatment for this disease (73,74). In mice with fibrosis (induced using bleomycin), cysLTs were shown to be important in chronic pulmonary inflammation. Inhibition or disruption of cysLT synthesis provides increased protection to alveolar septal thickening caused by macrophages, fibroblasts, and deposition of collagen. However, when CysLTR1 was inhibited, there was increased cysLT secretion in the bronchoalveolar lavage fluid and this was accompanied by increased septal thickening. These findings suggest that the chronic inflammation associated with fibrosis might be mediated via CysLTR2 signalling, while CysLTR1 plays a dual role in inflammation control (75).

Cancer. Leukotrienes have also been investigated in various types of cancer studies, such as prostate, pancreatic, intestinal, esophageal, and colorectal cancers (76–84). In prostate cancer, cancer cells had increased secretion of LTB_4. This did not correlate with inflammation,

suggesting that LTB_4 plays a role in prostate carcinogenesis and not hyperplasia (76). CysLTR1 has also been demonstrated to play a role in the pathogenesis of pancreatic cancer. CysLTR1 expression was found to be increased in pancreatic cancerous cells, and this expression was reduced in a concentration- and time-dependent manner that involved early apoptotic events when CysLTR1 was inhibited using montelukast (82). Blocking leukotriene synthesis by inhibiting the 5-LO pathway interferes with pancreatic cancer proliferation while inducing apoptosis. It was also demonstrated that pancreatic cancer tissues have upregulated 5-LO and LTB4-receptor (78) LTA4 hydrolase, the enzyme responsible for LTB_4 synthesis, is highly expressed in esophageal adenocarcinoma and its expression appears during early esophageal adeno-carcinogenesis events (79). Caco2 and HT29 cells, colon cancer cell lines, treated with 5-LO inhibitor and BTL1 antagonists had significantly reduced viability and proliferation due to apoptosis. Inhibition of BLT1 downregulated the LTB_4-mediated activation of the extracellular signal-regulated kinase (ERK) in these cancer cell lines. This suggests that blocking the LTB_4-signalling pathway triggers apoptosis through the attenuation of ERK activation in colon cancer cells (85). In a clinical pathologic trial conducted with colon cancer patients, CysLTR1 was significantly increased, and this correlated with poor survival outcomes (81). Interestingly, CysLTR2 doesn't seem to influence cell proliferation as CysLTR1 does. However, activation of CysLTR2 in colon cancer cells induced terminal cellular differentiation similar to that observed in the effect of the fermented fibre product, butyrate. A decrease in CysLTR2 was associated with poor disease outcomes in colon cancer cells (84). This therefore suggests that there should be a balance in the expression of cysLT receptors, as CysLTR1 enhances cancer cell growth and CysLTR3 inhibits it. Taken together, there is strong evidence that LTs and CysLTs play a major role in cancer carcinogenesis and their synthesis, or receptors have been potential candidates for the treatment of this disease and various other inflammatory diseases.

1.2.4. Leukotriene role in microbial infections

The inflammatory mediator effects of LTs and cysLTs are not restricted only to asthma, allergic responses, and inflammatory diseases, but have also been observed during microbial infections. This could be due to their ability to regulate both innate and adaptive immunity, and to modulate the inflammation caused by pathogens. The role of LTs has been implicated in various viral infections, including influenza virus, coronavirus, and respiratory syncytial virus infections (RSV). The mechanism viruses induce an infection can be dependent on the secretion of inflammatory mediators. RSV infection in children induces elevated LTC_4 production in the nasopharyngeal fluid than influenza A and parainfluenza viral infections. However, in all three viral infections, the induction of LTC_4 in nasal pharyngeals is accompanied by wheezing (86). Virus-inducing wheezing (RSV infection) has also been associated with increased IFN-γ in nasopharyngeal secretion that's correlated with increased cysLT production (87). During influenza virus infection, alveolar macrophages inhibit the secretion of LTD_4 and its autocrine activities to type 1 cells for better protection (88). LTB_4 signalling via the BLT1 receptor is important for the antimicrobial activity of neutrophils during viral infection. Gaudreault *et al*. (89), observed that treatment with LTB_4 reduced lung viral load accompanied by increased neutrophil antimicrobial peptides. Deletion of BTL_1 or neutrophil depletion reversed these effects in mice (89). LTB_4 activates TAK1 to induce IPS-1 and RIC axis of the NOD2 pathways, and this is required for the LTB_4-mediated anti-influenza effects (90). LTB_4 signalling induces type 1 interferon-α/β (IFN-α/β) and signal transducer and activator of transcription 1 (STAT1), this results in IFN-α secretion by interstitial macrophages and inhibits proliferation of inflammatory monocyte-derived macrophages (IMMs) during influenza A viral infection. Treatment with LTB_4 as the viral load peaks decreases IMM activation mediates organ destruction and increases mice survival (91).

LTs have been implicated in inflammatory diseases, and there have been multiple reports implicating their role in SARS-CoV2 infection. Montelukast has been proposed as a potential therapy for COVID-19, because of its anti-inflammatory effects (92–95). The targeted lipidomic analysis of bronchoalveolar lavage (BAL) of patients with severe COVID-19 disease, revealed increased fatty acids and inflammatory mediators (eicosanoids) that included thromboxanes, prostaglandins, and leukotrienes. Of the increased LTs, these included LTB_4, LTE_4, and eoxin E_4 (96) and this validated the potential role of eicosanoids, particularly LTs, in the pathobiology of COVID-19. In a cross-sectional COVID-19 study, LTB_4 levels were associated with reduced SARS-Cov2 burden in patients, while PGE_2 was linked with reduced symptoms. Neither of these lipids were linked to disease severity, however, LTB_4 may be involved in the control of viral loads (97). In one study, inhibition of LTB_4 did not affect SARS-Cov2 viral load or the symptoms associated with the disease in COVID-19 outpatients (98). LTB_4 antagonist might have not affected the viral infection as this study was performed in outpatients with mid-moderate symptoms. As mentioned, montelukast has been suggested as a possible therapy for COVID-19, Camera *et al.* (99), demonstrated that early administration of this drug can limit the acute and chronic lung tissue damage in patients inhibiting hyperinflammatory responses (99).

The human immunodeficiency virus (HIV) infection can induce neurological complications such as HIV-associated neurocognitive disorders (HAND) (100). This viral infection causes chronic inflammation in the brain due to activated immune cells and continuous neuroinflammation causes neuronal damage and degeneration as a function of time (101). Increased LTB_4 and LTD_4 secretion in a monocyte-astroglia coculture post-HIV-1 infection was shown to contribute to neuro-inflammation and -toxicity implicating the role of LTs in the neuropathogenesis of HIV (102). LTB_4 production was found to be elevated in the cerebrospinal fluids of patients with HIV (103). Secretion of LTs in neuropathological

conditions can result in the recruitment of potentially infected cells (like monocytes and lymphocytes) to the central nervous system (CSN). However, LTB_4 has been reported to have anti-HIV effects. LTB_4 promoted the secretion of antimicrobial proteins by neutrophils which inhibit HIV-1 infectiveness (104,105). In monocyte-derived microglia-like cells (MDMis) LTB_4 and LTD_4 treatment reduced HIV-1 viral replication, by altering the pH-independent entry and the viral life cycle early post-fusion events. These LTB_4 and LTD_4 effects in MDMis were partly mediated by the protein kinase C (PKC) signal transduction pathway (106). More recently, in vitro, macrophage infection with HIV and/or HIV gp120 stimulation resulted in reduced secretion of cysLTs downstream of p38 MAPK and contributed to neurotoxicity. Inhibition of p38 MAPK deprived the macrophages of LTC_4S, while the inhibition of CysLTR1 provided cerebrocortical neurons against toxins in both HIV-infected and gp120-stimulated macrophages. In vivo, deficiency of *Ltc4s* or *cysltr1* reversed the neuronal damage and impairment of spatial memory in HIVgp120tg mice (107). As previously mentioned, the LT role is associated with a lot of pulmonary-related diseases. HIV infection has also been linked with overrepresentation of pulmonary arterial hypertension (PAH). Individuals living with HIV and PAH (HIV-PAH) were shown to have high levels of HIV-X4 variants, increased ALOX5 expression, and increased secretion of LTs in their BALs (108). Taken together, these show the role of eicosanoids, particularly LTs, in HIV and HIV-associated co-morbidities.

In parasitic infections, the role of LTs was first demonstrated using *Trypanosoma cruzi* that LTB_4 and LTC_4 increase the phagocytosis and killing of the parasites by peritoneal macrophages (109,110). In murine macrophages, LTB_4 stimulation induced nitric oxide production and TNFα secretion resulting in enhanced microbicidal effects of macrophages infected with *T. cruzi* (111). In monocyte-derived macrophages infected with *Toxoplasma gondii* LTB4 stimulation increased intracellular killing of the parasite in an IFN-γ dependent manner (112). In *Plasmodium berghei* (cause of cerebral malaria in mice), at the peak of

cerebral infection mice had increased LTB_4 levels that correlated with mice susceptibility to the infection. This suggests that LTs, particularly LTB_4, might have a detrimental effect on cerebral malaria (113). More recently, Mosala *et al.* (114) demonstrated that during *Schistosoma mansoni* infection, deletion of CysLTR1 results in a reduction in liver granulocytes, damage and IL-4 production. They also demonstrated that treatment of mice with montelukast (CysLTR1 antagonist) in combination with praziquantel results in reduced cellular infiltration to the liver and reduced egg burden during chronic infection. Together, their data demonstrate that combinational therapy (CysLTR1 inhibition and praziquantel) can be used as a prophylactic to treat chronic schistosomiasis infection (114). During *Histoplasma capsulatum* (fungi) infection, mice deficient in 5-LO were more susceptible to the infection and this was associated with increased fungi in the lungs together with TNF-α, IL-1, IL-6, and KC secretion (115). Therefore, the inability of these mice to secrete LTs affected their ability to modulate inflammatory responses, suggesting that LTs are required for the modulation of pulmonary histoplasmosis.

Other studies have also demonstrated the role of LTs in enhancing monocyte/macrophages' phagocytosis and microbicidal activities during bacterial infections. In *Klebsiella pneumonia* infection demonstrated that mice that do not produce LTs, 5-LO deficient mice, have increased lung bacteria and susceptibility to infection. Exogenous addition of LTB_4 in macrophages derived from 5-LO deficient mice showed improved phagocytic and bactericidal effects (116), indicating a critical role of LTs in the defence against bacterial pneumonia in murine models. It has also been demonstrated that the addition of not only LTB_4 but LTC_4, LTD_4 and 5-HETE postinfection in *K, pneumoniae* infected 5-LO deficient alveolar macrophages, significantly reduced intracellular bacterial survival in a dose-dependent manner. LTB_4 inhibition using BLT1 antagonist increased intracellular growth of *K. pneumoniae* while inhibiting CysLTR1 alone, or both CysLTR1 and CysLTR2 did not affect the survival of this bacteria. This indicated

that, although the addition of LTB4 and cysLTs enhances the bactericidal effects of alveolar macrophages, LTB_4 is the main metabolite primarily responsible for this function. Lastly, the increased leukotriene-mediated alveolar macrophage microbicidal effects require NADPHox activation, which leads to the generation of superoxide anion and H_2O_2 required for the killing of internalized bacteria (117). 5-LO deficient mice infected with *Streptococcus pneumoniae* also demonstrated increased lung bacteria with increased dissemination to the spleen, further demonstrating the importance of LTs in bacterial clearance and control. Treatment of these mice with LTB_4 effectively reversed these effects however, route and timing of administration matters. Aerosolized LTB_4 was more effective in improving bacterial killing when administered postinfection in 5-LO deficient mice, pretreatment with LTB_4 also significantly reduced lung *S. pneumoniae* in wildtype mice. This increased ability to clear the bacteria in response to LTB_4 treatment was associated with enhanced monocyte recruitment and the expression of p47phox by leukocytes (118).

In *Escherichia coli* infection of mast cells, the infection induced the release of LTs *in vitro*. Mice deficient in mast cells that were treated with LT-synthesis inhibitor, A-63162, had impaired recruitment of neutrophils and increased bacterial growth in peritoneal cavities. This demonstrates that mast cell derived LTs may be partly responsible for neutrophil recruitment and subsequent bacterial elimination during *E. coli* induced peritonitis (119). Another study also demonstrated that 5-LO deficient mice infected with *Achromobacter xylosoxidans* had impaired bacterial control, increased lung inflammation, and were more susceptible to the infection than their counterparts. Treatment with LTB_4 was also able to improve mice survival, bacterial control, and lung inflammation. LTB_4 promotes the gene and protein expression of α-defensin-1 during infection, which increases neutrophil antibacterial properties (120). Altogether, these studies demonstrated that leukotrienes, particularly LTB_4, not only mediate inflammation but also play a significant role in microbicidal cellular functions.

1.3. *Listeria monocytogenes* and *Mycobacterium tuberculosis*

Listeria monocytogenes (*Lm*, causative agent of listeriosis) and *Mycobacterium tuberculosis* (*Mtb*, the causative agent of tuberculosis) are both facultative intracellular bacteria and induce Th1 immunity. Early diagnosis and treatment implementation determine disease outcome for both listeriosis and tuberculosis (121,122). Timely diagnosis for both disease is very important, however, it is usually challenging, as microscopic examination at the initial phase of infection has limited sensitivity, bacterial cultures and nucleic acid amplification techniques are not always available resource limited settings.

1.3.1. Listeriosis and its pathogenesis

Listeriosis is one of the most severe foodborne diseases. It is caused by the facultative Gram-positive bacterium, *Listeria monocytogenes*. *Lm* was discovered in 1924 by EGD Murray and named *Bacterium monocytogenes*, however, its genus name was later changed to Listeria in 1940 (123,124). Of the 21 known Listeria (125) species (*L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. marthii*, *L. invanovii*, *L. seeligeri*, *L. rocourtiae*, *L. newyorkensis*, *L. cornellensis, L. grandensis*, *L. weihenstephanensis*, *L. riparia*, *L. booriae*, *L. grayi grayi*, *L. thailandensis*, *L. valentina*, *L. aquatica*, *L. fleischmannii*, *L. goaensis*, *L. floridensis*, *L. costaricensis*), *L. monocytogenes* and *L. ivanovii* are the two species known to be pathogenic in mammals (126–128). Listeriosis is a relatively rare disease, depending on the country and region of the world, 0.1 to 10 cases per million are reported each year. Even though reported cases are low, the high rate associated with death still make this disease a significant public concern (129). The Centre of Disease and Control (CDC) reported *Lm* infected about 1600 immunocompromised individuals, including pregnant women and neonates in the United States in 2016 (130). While, South Africa had a listeriosis outbreak between 2017 and 2018, with 674 reported cases and 183 deaths. About 42% of the deaths due to listeriosis were neonates infected during pregnancy or at birth (131).

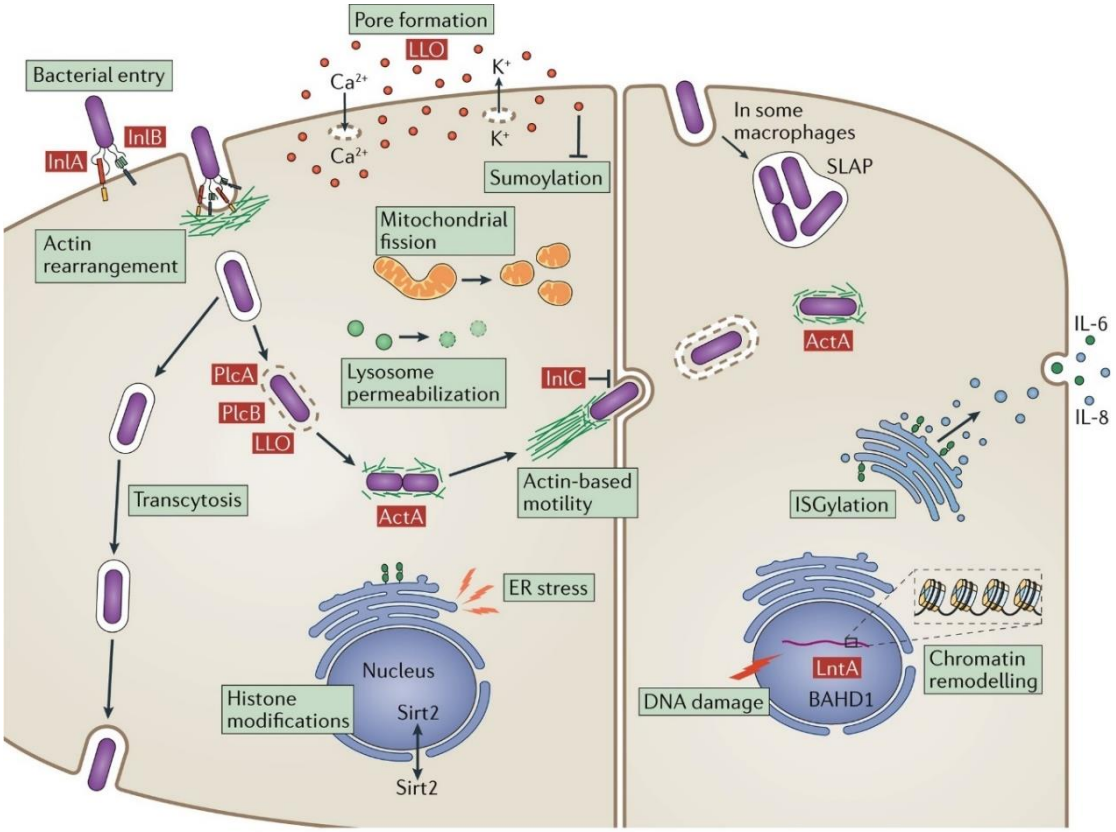

Figure 1.2: *Listeria monocytogenes* infection overview with non-phagocytic cells. Upon ingestion, *Lm* induces its uptake by non-phagocytic cells, it can replicate within the phagosome and uses various virulence factors, including LLO, internalins, phospholipases, and actin polymerization, to escape the phagosome and spread to neighbouring cells. LLO cytotoxicity effects also cause alteration in histone modifications, desumoylation and mitochondrial fission, it also causes stress to the endoplasmic reticulum, and permeabilization of the lysosome. Image adapted from reference (132).

Lm infection occurs through ingesting contaminated foods and can cross the intestinal barrier leading to its spread into the bloodstream to lymph nodes and dissemination to its intended organs, the liver and spleen. *Lm* exploits the host cell receptors and signalling to influence its entry and uses host actin to escape and evade killing (**Figure 1.2**). Once ingested, *Lm* can be internalized by phagocytic and non-phagocytic cells. However, in non-phagocytic cells, its uptake is receptor-mediated by two members of the 25 proteins, internalins, expressed by the bacteria, internalin A (InlA) and InlB. InlA binds to two host eukaryotic cell membrane receptors, E-cadherin (133–136) and InlB binds to Met, the receptor for hepatocyte growth factor/scatter factor (HGF/SF) (137–140). The bacteria then get internalized in a vacuole. *Lm*

then uses listeriolysin O (LLO), phospholipase A (PlcA) and PlcB to cause the vacuole to rapture enabling it to escape. LLO causes alteration in histone modification, desumoylation, mitochondrial fission, endoplasmic reticulum (ER) stress, and lysosomal permeabilization due to the pores caused by LLO extracellular activity (132). PlcA and PlcB induce phagosome membrane lysis, resulting in *Lm* escape into the cytoplasm (141). In the cytoplasm, the bacterium replicates and uses host host actin polymerization factor (ActA), which enables it to propel to the neighbouring cells (142,143). As the bacterium migrates to the next cell, it becomes encapsulated in two membrane vacuoles which can be lysed by the LLO, PlcA and PlcB toxic activities reinitiating the infection cycle (132,141,144,145). Through all these processes and secretion of virulence factors, *Lm* can cross both the blood-brain and fetoplacental barriers. *Lm* can replicate and thrive in various cell types, therefore changing various hast processes and organelles. For instance, infection causes chromatin packing changes resulting in altered downstream gene expression and can also lead to DNA damage (132). The host can contain and even clear the infections through the secretion of antimicrobial effectors and cytokines, discussed below.

1.3.2. Tuberculosis and its pathogenesis

Mycobacterium tuberculosis (*Mtb*), first discovered in 1882 by Robert Koch, is a Gram-positive bacterium that causes tuberculosis (TB). At that time, TB was reported to be responsible for one death out of every seven people living in the United States and Europe. For several years since its discovery, TB has been ranked as one of the deadliest infectious diseases globally. In 2019, the World Health Organization (WHO) reported about 10 million TB cases and about 1.2 million associated deaths (146). In 2020, TB became the second leading cause of death due to the Covid-19 outbreak. In the 2023 Global Tuberculosis Report, it was reported that there were 7.5 million new TB diagnoses, with an estimate of about 10.6 million TB cases and 1.3 million deaths (including people living with HIV) recorded in 2022 (147).

M. tuberculosis is an airborne bacterium that can be transmitted from one TB-infected individual to the next person. Once inside the respiratory tract and lungs, the primary infection begins with bacilli getting to the alveolar spaces it is engulfed by residential alveolar macrophages which act as a first line of defence (148–150). Failure to clear the bacteria results in the invasion of the lung interstitial tissue. Here, the bacteria infect the alveolar epithelium or alveolar macrophages that migrate to the lung parenchyma (149,151). Dendritic cells and other inflammatory cells can transport the bacterium to the lymph nodes where the T cells are then primed, leading to the recruitment of more immune cells, including B- and T cells (152). Activation and recruitment of these cells result in the formation of a granuloma (153,154). This stage of infection is classified as latent TB infection (LTBI), as the infection is contained and suppressed within the granuloma. The granulomas are formed to contain and inhibit the spread of the bacteria and can remain this way for many years (155). However, granulomas can also serve as bacterial population refuge where they can evade further recognition and subsequent killing by the host immune system (156). The disintegration of cells due to necrosis results in cavity formation leading to bacterial dissemination to other parts of the lungs (155). Once the bacteria are released into the airways, it results in active TB infection, and the disease can be transmitted to a new host (151) (see **Figure 1.3** below).

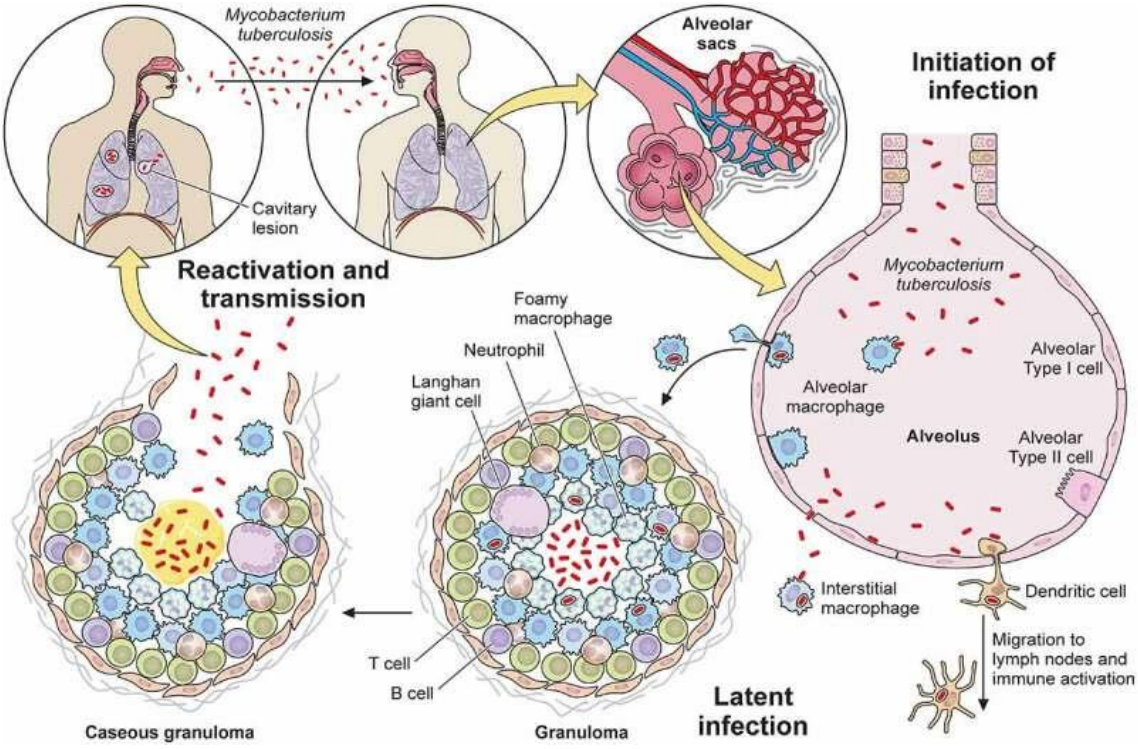

Figure 1.3: Tuberculosis infection cycle overview. Inhalation of the bacterium through the host's respiratory tract to the lungs resulting in the activation of the innate immunity with the alveolar space. The bacterium is phagocytosed by macrophages and dendritic cells, which result in the recruitment of more immune cells and activation of the adaptive immune system. The innate and adaptive immune responses can clear the bacteria or contain it in a solid granuloma. If contained in a granuloma, the bacteria can replicate, overwhelming the caseous granuloma resulting in its reactivation and transmission. Image adapted from reference (151).

Mtb has evolved various mechanisms to evade host immunity and promote its survival within the phagosome and eventually escape to the cytoplasm. *Mtb* employs various nontoxic virulence factors, which are divided into non-protein and protein virulence factors. The *Mtb* non-protein virulence factors include lipids, glycolipids, glycans, nucleic acids and metabolites, and are important surface molecules involved in the pathogen-host interaction, recognition, survival within various cells and virulence. The major non-protein virulence factors include phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM), lipoarabinomannan (LAM), and phthiocerol dimycocerosates (PDIMs). LM, LAM and PDIM are said to be involved in bacterial recognition by toll-like receptors (TLRs), resulting in immune response activation by APCs (157). LM activates macrophages through TLR2 and

TLR4, and upon di-acylatilation it is involved in the regulation and inhibition of the production of nitric oxide and secretion of cytokines (158). PMID enables mycobacterium's ability to evade the TLR-mediated detection of this pathogen, by delaying innate immune cell recruitment and the activation of the adaptive immunity to the site of infection (159,160).

Of the protein virulence factors, the PE/PPE family and the early secretory antigenic target (ESAT-6) secretion (ESX) system proteins have been shown to modulate cell death to either promote bacterial intracellular survival or promote bacterial dissemination. For instance, the PE proteins (PE17, PPE11 and PPE88) inhibit the secretion of IL-6, IL-12 and TNF, while also inducing necrosis promoting bacterial survival (161–163). PPE60, PE-PGRS19 and PE6 were shown to induce pyroptosis and suppress autophagy, resulting in bacterial dissemination (164–166). ESAT-6 on the other hand is a proapoptotic protein that increases expression of caspsase-3, -5, -7, and -8 (167,168). It also promotes the activation of inflammasome-induced pyroptosis, induction of neutrophil necrosis, formation of NETs, plasma membrane integrity destruction, and the induction of necroptosis (169–171). ESAT-6 also inhibits the maturation of the phagosome (172,173) and prevents phagosome-lysosome fusion (174), promoting bacterial persistence. Other protein families shown to have a role in intracellular survival and dissemination of *Mtb* (**Figure 1.4**) include the serine-threonine protein kinases [protein kinase (Pkn) PknE and PknF] which are proapoptotic and promote inhibition of the inflammasome (175–177); PknG inhibits phagolysosome fusion (178); the phosphatase [secreted acid phosphatase (SapM)] which promotes phagosome arrest and phagolysosome fusion restriction (179,180); and the protein tyrosine phosphatase (PtpA) which inhibits acidification in the phagosome (181–183). PtpA also promotes ferroptosis resulting in bacterial dissemination (184).

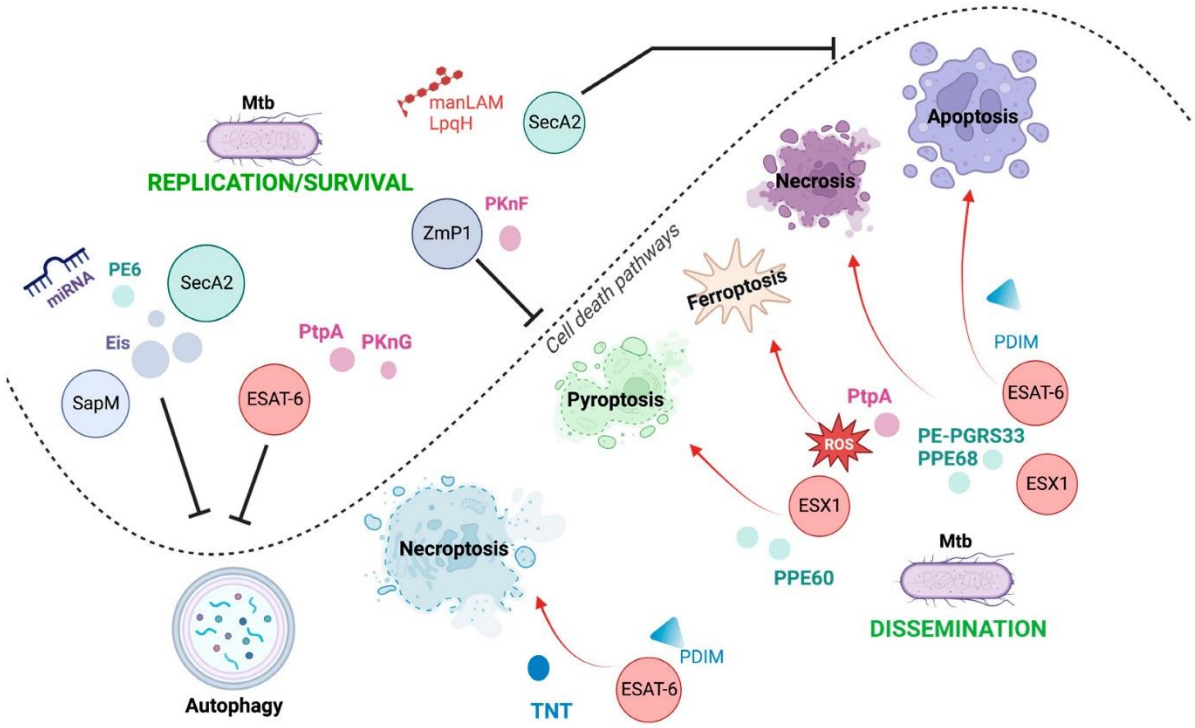

Figure 1.4. Schematic representation of the cell death pathways targeted by various *Mtb* virulence factors to promote its survival and dissemination. For survival and replication, *Mtb* uses various virulence factors to inhibit (black arrows) apoptosis, pyroptosis, and autophagy. For its dissemination, it induces (red arrows) necroptosis, pyroptosis, ferroptosis, necrosis and apoptosis using another range of virulence factors. Image adapted from (157).

1.3.3. Immunity to *Lm* and *Mtb* infections

Immunity to intracellular infection involves the innate and adaptive immune responses resulting in host protection. Innate immunity involves the identification of the pathogen. The host pattern recognition receptors (PRRs) identify a pathogen through their evolutionary conserved repetitive pathogen-associated molecular patterns (PAMPs) and initiate the activation of innate immunity. During *Lm* infections, PRRs like TLRs and nuclear-binding oligomerization domain (NOD)-like receptors (NLRs) have been implicated in the activation of innate immunity. For instance, the cell surface membrane bound TLR2 recognizes the lipoproteins of *Lm*. Mice deficient in TLR2 are more susceptible to *Lm* infection (185). This suggests TLR2 could be crucial in the activation of the innate immune response as the deletion

of TLR2 leads to improper activation of macrophages. MYD88, an adaptor surface molecule expressed downstream of various TLRs, is also important in the innate immune response to *Lm* infection (186). Cytosolic NOD1 and NOD2 recognize *Lm* peptidoglycan fragments resulting in the expression of antibacterial peptides and proinflammatory genes (187–189). It has been demonstrated that NOD1-deficient mice are also more susceptible to *Lm* infection (189).

TLR2, TLR4, TLR8, TLR9 and MyD88 play a more vital role in the activation of immunity against *Mtb* (190–196). Mice deficient in both TLR2 and TLR9 were more susceptible to *Mtb* infection and had impaired proinflammatory cytokine secretion (194). Inhibition of TLR2 and TLR4 inhibited the Mtb-induced NF-KB activation in vitro. Blocking of TLR4 demonstrated that *Mtb*-induced TNF-α was dependent on TLR signalling, while nitric oxide secretion was independent of TLRs or mediated by TLRs in a manner independent of MyD88 (195). In Indonesian and Russian populations, TLR8 was found to be increased during TB disease, suggesting its role in disease susceptibility (196). NOD2 has also been implicated in *Mtb* recognition by APCs, and it has been demonstrated that NOD2-deficient mice are more susceptible to *Mtb* infection than their counterparts (197–199). Dectin-1 and TLR4 recognition of *Mtb* plays a role in innate immune activation, independent of TLR2, resulting in the induction of Th1 and Th17 immune responses (200). These receptors together with their downstream signalling molecules determine the overall disease severity, susceptibility/protection, and the eventual outcome.

Recognition of pathogens by APCs, such as monocytes, macrophages, dendritic cells, and neutrophils, through the PRRs leads to a cascade of events that lead to the formation of a phagosome. As the phagosome matures from early to late phagosome, there are changes in the internal acidity, it gains GTPases, proteases, and acid hydrolases, leading to the formation of the highly acidic phagolysosome (201). Other antibacterial properties of the phagolysosome include the generation of toxic reactive oxygen species (ROS), reactive nitrogen intermediates

(RNIs), and antimicrobial peptides that include defensins, cathelicidins, lysozymes, lipases, and proteases (201,202). Phagolysosomal degradation of the bacteria resulting in presentation of antigens to CD4 T cells through the MCH class II molecules to initiate adaptive immunity.

The adaptive immune system is characterized by cell-mediated and humoral immunity, respectively, controlled by T cells and B cells. These lymphocytes express clonally variable antigen receptors, their cell-mediated immunity is antigen-specific and has immunological memory. T cell activation is initiated once bacterial peptides are presented by APCs through the αβ T cell receptor (TCR) and MHC-complex interaction. CD4 T cells recognize peptides presented through MHC-II, while CD8 T cells are activated through MHC-I. CD4 T cells are divided into subsets that include T-helper 1 (Th1), Th2, Th17, follicular T helper, and regulatory T (Tregs) cells. During *Mtb* infection, CD4 Th1 cells have a protective role which is accounted for by the secretion of pro-inflammatory cytokines IFN-γ, and TNF-α. The secretion of either of these cytokines results in the activation and recruitment of monocytes and granulocytes (203,204). In *Lm* infection, CD4 T cells are also associated with IFN-γ production aiding in the activation of macrophages (205). Depletion of CD4 T cells results in improper granuloma formation during *Lm* infection (206), signifying an important role of CD4 T cells in *Lm* control.

Th17 cell responses have been implicated in both *Mtb* and *Lm* infections. During *Lm* infection, the IL-17 produced by γδ T cells resulted in increased neutrophil recruitment to the liver and subsequent bacterial clearance, in a manner regulated by IL-23 (207). However, during *Mtb* infection, the IL-23/Th17 pathway might not be crucial in the control of infection as IL-17A and IL-23 knockout mice demonstrated insignificant susceptibility to *Mtb* infection (208,209), however, plays a role in the modulation of mycobacteria-induced inflammation (210). CD8 T cells eliminate pathogen-infected cells through contact-dependent lysis and cytokine secretion, and some studies have demonstrated that CD8 T cells are required for the effective killing of *Mtb* and *Lm* infection through an IFN-γ-dependent manner (211,212). In *Lm* infections, CD8

T cells were shown to respond to cytokines, such as IL-12 and IL-18, rapidly secreting IFN-γ after *Lm* infection (213–215).

Nonconventional T cells, include γδ T cells, NK cells, NKT cells, invariant NKT cells, and mucosal-associated invariant T (MAIT) cells, play a role in the immunity to intracellular pathogens and can be important connectors of the innate and adaptive immunity. It has been demonstrated that γδ T cells play a vital role in mediating host defence against bacterial infection and serves as a link between the innate and adaptive immune systems (216). During *Lm* infection, γδ T cells are a major source of IL-17A required for the innate immune response and activation of CD8 T cell response against *Lm* infection (217). Deletion of γδ T cells during chronic *Lm* infection was associated with liver necrosis, increased inflammation and disruption of macrophage functions mediated by TNF-α secreting CD8 T cells, accompanied by a reduction in IL-10 (218). In *Mtb* infection, γδ T cell disruption leads to increased regulation of the FAS and FAS ligand (molecules that regulate cell death), and this increase is associated with disease progression (219). These reports therefore suggest that γδ T cells play an important role in the regulation of both the inflammatory responses to intracellular bacteria and the overall elimination of the pathogen. NK cells in *Lm* infection have proved their importance of controlling bacterial growth. In nude rats without T cells, NK cells can recognize MCH antigens and are resistant to *Lm* infection, and there was reduced bacterial growth in the spleen which was eliminated at 3 days postinfection (220). T cell-deficient RAG knockout mice infected with *Mtb*, the infection stimulated an NK cell-dependent secretion of IFN-γ, which was also associated with resistance to *Mtb*, and regulation of granulocyte function (221). MAIT cells have a protective role during *Mtb* infection, as it has been reported that their levels in the peripheral blood and lungs of patients with TB are significantly reduced (222). They have been implicated in the priming of CD4 T cells at early stages of infection in a mechanism that involves IL-17A resulting in bacterial load reduction (223).

Proinflammatory cytokines (IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18) play a prevalent role in the control of intracellular bacterial infections and the regulation of both the innate and adaptive immune responses. Cytokine secretion influences the role of immune cells during infection and can influence disease outcomes. Some of the major important proinflammatory cytokines that play a role in controlling *Lm* and M*tb* infections include IFN-γ and TNF-α. IFN-γ mediates the transition from innate to adaptive immunity by enhancing the initiation of Th1 response through induction of IL-12 and IL-18 production (224). IFN-γ also regulates the cell-mediated immune response and activation of TH1 cells by upregulating the expression of MHC complex (225). IFN-γ deficient mice are highly susceptible to *Mtb* infection and have increased tissue pathology and necrosis (204), demonstrating its role in bacterial killing. Patients treated with IFN-γ had improved disease outcomes associated with a reduction in inflammatory cytokine secretion, more responsive CD4 T cells and reduced *Mtb* in the sputum (226). During *Lm* and *Mtb* infections, CD8 T cells also secrete IFN-y. In *Mtb* infection, CD8 T cells secreted some IFN-γ, but are more cytotoxic, with increased granzyme B and perforin secretion (227). In *Lm* infection, it has been demonstrated that naïve CD8 T cells rapidly respond to infection by secreting IFN-γ, and the response is mediated by IL-12 and IL-18 (213). Effector and memory CD8 T cells also secrete IFN-γ mediated by IL-12 and IL-18 actions in response to *Lm* infection (228).

As mentioned, TNF-α is another major cytokine that plays a role in bacterial control. This is due to the ability of TNF-α which activate macrophages and induce similar antimicrobial functions towards the pathogen, which include the production of inducible nitric oxide (iNOS), ROS, RNI, and autophagy. During *Lm* infection, memory CD4 T cells producing TNF-α provided more protection than those secreting IFN-γ (229). and During *Mtb* infection, TNF-α is involved in the granuloma formation and containment of the bacteria (230). TNF-α receptor-deficient mice are more susceptible to *Lm* infection and have increased bacterial dissemination

in the liver, spleen and brain, and organ pathology (231). While in *Mtb* infection, deletion of TNF-α in macrophages and neutrophils resulted in transient susceptibility to infection. T cells deficient in TNF-α provided early bacterial control while mice became more susceptible to chronic infection (203). This suggests T cell derived TNF-α is more important for host protection.

1.3.4. Eicosanoids in *Lm* and *Mtb* infections

Eicosanoids, as mentioned above, are known for their inflammatory mediator properties, and an increase in cytokines and eicosanoid production is crucial for the activation of adaptive immune responses. For this reason, eicosanoids have been shown to play an important role in microbial infections. During *Lm* infection, Noor *et al.* (232) have demonstrated that *Lm* activates $cPLA_{2\alpha}$ resulting in the production of the eicosanoids PGE_2, PGI_2 and LTC_4 in peritoneal macrophages in a mechanism that involves LLO and host TLR2 interactions. $cPLA_{2\alpha}$-deficient macrophages and mice showed that the internalization of *Lm* was significantly reduced. The activation of $cPLA_{2\alpha}$ results in TNF-α suppression, as $cPLA_{2\alpha}$ deficient macrophages had increased TNFα secretion after *Lm* infection. Furthermore, they also showed that COX-1 and COX-2 inhibition using indomethacin increased TNF-α secretion in $cPLA_{2\alpha}$ wildtype macrophages, demonstrating that prostaglandins play a role in the suppression of TNF-α production during *Lm* infection (232). Interestingly, prostaglandins, particularly PGE_2, have been identified as a phagocytosis modulator during *Lm* infection in peritoneal macrophages (233). Inhibition of COX-2 or disruption of PGE_2 synthesis results in impaired priming of CD8 T cells during *Lm* infection and exogenous addition of PGE_2 reversed these effects (234). Another study demonstrated that the optimal production of PGE_2 by macrophages and dendritic cells requires *Lm* to escape into the cytosol, as infection with *Lm* lacking LLO induces less PGE_2 secretion (235). Neutrophils play a major role in the innate immunity against *Lm* infection and subsequent clearance of the bacteria. One study has demonstrated that pre-

treatment of neutrophils with PGE_2 inhibits their ability to kill *Lm* since PGE_2 reduces chemotaxis of neutrophils and inhibits their phagocytosis ability and production of ROS during *Lm* infection (236). Together these studies demonstrate an important role of PGE_2 in the phagocytic properties of APCs and priming of proper CD8 T cells responses against *Lm* infection.

In *Mtb* infection, cyclooxygenases, particularly PGE_2, have anti-mycobacterial properties, therefore resulting in better *Mtb* control. During *Mtb* infection, apoptotic human macrophages were shown to induce expression of $cPLA_{2\alpha}$ independently of the secretion of TNF-α, resulting in caspase 3 activation, apoptosis, and subsequent killing of *Mtb* (237). Like in *Lm* infection, disruption of PGE_2 syntheses by deleting prostaglandin synthase (PGES) in mice and macrophages results in increased bacterial burden post-*Mtb* infection (238). Divangahi *et al*. (239) demonstrated that in macrophages infected with virulent *Mtb*, PGE_2 is required for membrane repair, inhibition of necrosis and *Mtb* killing (239). In another study by Mayer-Barbar *et al.* (240), showed that BAL fluid of mice infected with *Mtb* had increased bacterial load that was associated with an eicosanoid imbalance, decreased PGE_2 and COX-2 and increased LXA_4 and LTB_4. Interestingly, they found that in $Il1r1^{-/-}$ mice, *Mtb* infection induces a reduction of PGE_2 and $PGF_{2\alpha}$ while increasing LXA_4 and LTB_4 in the BAL fluid. The reduction in PGE_2 in IL-1R1 deficient mice was associated with poor disease outcomes, however, treatment with PGE_2 rescued survival and enhanced the bacterial clearance. These results suggest that IL-1 signalling might provide host resistance to *Mtb* infection by triggering PGE_2 synthesis, which in turn helps modulate bacterial growth and containment (240)**1.3.5. Leukotrienes in *Mtb* infection**

The 5-LO pathway, particularly lipoxins, has been associated with increased susceptibility to *Mtb* infection. Lipoxin A_4 (LXA_4) has been associated with increased bacterial growth and hindered bacterial control. LXA_4 sera levels were elevated in wildtype mice, and this was

accompanied by increased IL-12, IFN-γ and NOS2 mRNA levels in the lungs when compared to 5-LO deficient mice. LTB_4 sera levels, on the other hand, were only elevated 10 days postinfection and subsequently decreased as infection progressed. 5-LO-deficient mice were less susceptible to the infection and had reduced bacterial loads, and treatment with LXA_4 analogue reversed these effects. These results suggest that LXA_4 negatively regulated the Th1 antimicrobial properties favouring bacterial survival (241). Zebrafish LTB_4 hydrolase mutant studies demonstrated that reduction in LTB_4 secretion results in increased anti-inflammatory LXA_4 and this permits mycobacterial growth by inhibiting the production of TNF-α. And in humans with heterozygous *LTA4H* gene have better disease outcomes than those with homozygous *LTA4H* (242). Optimal levels of LTB_4 are required for better control of the mycobacterial growth. It has been demonstrated that reduced LTB_4 results in increased anti-inflammatory lipoxin secretion, while high levels cause high inflammation, and both these extremes lead to inadequate bacterial control (243).

Neutrophils play an integral role in the phagocytosis and clearing of pathogens. *M. bovis* infected neutrophils had increased secreted levels of LTB_4 and blocking LTB_4 syntheses by inhibition of the 5-LO pathway using MK-886 results in reduced antibacterial effects of neutrophils (244). This suggests that LTs, particularly LTB_4, are essential for the antimicrobial properties of neutrophils. LTB_4 expression was significantly increased in the lungs of mice infected with *Mtb*. *In vivo,* daily administration of MK-886 increased mice lung bacterial loads and mice susceptibility to the infection. This susceptibility was associated with reduced pro-inflammatory cytokines (TNF-α, IFN-γ, IL-12, and IL-6) secretion and NO_2 production (245). A study by Franco *et al.* (246) demonstrated that BCG-immunized mice challenged with *Mtb* then treated with MK-886 still have increased bacterial burden, and impaired recruitment of leukocytes to the lungs. This further proves the importance of leukotrienes in protection against *Mtb* infection. The authors also demonstrated that the immunized mice were still more

protected than the unimmunized mice, suggesting that LTs are not required for the protective phenotype induced by vaccination (246).

All the above discussed studies evaluated the role of LTB_4 by either using 5-LO deficient mice or inhibiting the 5-LO pathway however, this pathway involves other LTs besides LTB4, and we cannot be certain that the observed results are specific to LTB_4 signalling. Banerjee *et al.* (247) recently addressed these concerns. They demonstrated that in individuals with active pulmonary TB, LTB_4 and LTB_4R expressions were increased when compared to healthy individuals. Upon completion of anti-TB treatment, the expression of this LT and its receptor are significantly reduced, suggesting that LTB_4-signalling corresponds to host bacterial load, and because of the pro-inflammatory properties of LTB_4, its signalling might be enabling the bacterial survival and spread. STAT1/2 and NADPH complex were identified as potential downstream signal mediators of LTB_4 in TB patients. After validating that *Mtb* infection induces LTB_4 expression in THP-1 macrophages, the authors were able to prove that LTB_4 signalling regulates both STAT1 and STAT2 activities and the activation of the NADPH oxidase during *Mtb* infection in macrophages by specifically inhibiting LTB_4R using U75302. They further demonstrated that inhibition of this receptor in macrophages and mice during *Mtb* infection results in the reduction of the bacterial burden (247). This demonstrates that LTB_4 plays a crucial role in disease progression, via inhibition of ROS production through NADPH oxidase and regulation of STAT1/STAT2 signalling. Inhibition of LTB_4 or its signalling receptor could be a potential host-directed therapy target for TB treatment as an adjunctive to the current regimen.

1.4. Problem Statement

Infectious diseases continue to be the world's leading cause of mortality, with developing countries more vulnerable. *Mycobacterium tuberculosis* (*Mtb*) is one of the leading causes of death from infectious diseases worldwide, with South Africa being one of the countries with

the highest tuberculosis (TB) burden. In 2017, there were about 56 000 reported deaths amongst individuals living with HIV that were co-infected with TB, with 322 000 reported TB incidents and 12 000 multi-drug-resistant incidents in South Africa (248). The increased development of drug-resistant TB could be attributed to many factors, but the prescribed length of treatment, treatment cost and the lack of rapid laboratory diagnostic tools are major contributing factors.

South Africa is not only ranked amongst the top ten countries with the highest burden of tuberculosis, but it also has the highest percentage of individuals living with HIV, and those co-infected with TB/HIV. These individuals are more vulnerable to opportunistic infections, such as Listeriosis and others. Moreover, it is difficult to diagnose listeriosis, which results in delayed treatment. Therefore, there is an unmet medical need to develop new intervention strategies to treat and prevent the spread of TB and listeriosis infections.

Thus far, eicosanoids, especially prostaglandins, have been observed to modulate immune responses during *Lm* and *Mtb* infections. This modulation may involve influencing macrophage phagocytic activity during bacterial infection, suppressing chemokine production, and regulating levels of TNF, IL-6, and IL-10 (249–251). However, the impact of cysteinyl leukotrienes on *Lm* and *Mtb* infection remains unclear.

There is currently no evidence supporting the role of cysLTs and their binding receptors in the pathogenesis and immune regulation of *Lm* infection. In *Mtb*, a couple of researchers have provided some evidence that CysLTR1 could have a significant effect on disease outcomes. Evidently, Mishra *et al*. (252) demonstrated Pranlukast (PRK), an approved FDA CysLTR1 inhibitor, was able to inhibit *Mtb* growth by targeting an enzyme responsible for the biosynthesis of arginine, ornithine acetyltransferase (MtArgJ) of the pathogen. They demonstrated that the bactericidal effects of PRK were due to its ability to reduce the bacteria's arginine levels, therefore, attenuating its growth and survival. In *Mtb*-infected macrophages,

the antibacterial effects of PRK treatment were also associated with reduced 5-LO, Cox-2, FLAP, and CysLTR1 expression. Mice treated with PRK or a combination of PRK and rifampicin had improved lung pathology and reduced bacterial burden, indicating that PRK has the potential to be used as a combinational drug with the current TB regimen (252). Another study demonstrated that PRK decreases MtArgJ's downstream metabolites, including N-acetylglutamate, arginosuccinate, L-arginine, L-ergothioneine, and L-phenylalanine (253).

Mishra *et al.* (252), did demonstrate the effect of PRK in *Mtb* control, however, they also demonstrated that PRK is not a specific inhibitor of CysLTR1. Although PRK does inhibit the expression of CysLTR1, it also inhibits the 5-LO and COX-2 pathways. Therefore, it is unclear whether the reduction in macrophage and mice bacterial loads is due to the direct effects of the drug on the bacterial MtArJ or inhibition of the LTs and prostaglandins biosynthesis. The purpose of the current study is to investigate whether CsyLTR1 signalling pathway plays any significant role in *Lm* and *Mtb* control and to evaluate if it can be a target for host-directed therapy for both diseases caused by these bacteria. Based on the discussed evidence regarding eicosanoids, specifically LTs, we hypothesize that CysLTR1 deletion will provide host protection during *Lm* and *Mtb* infection in mice.

1.4.1. Aim and Objectives

This project aimed to understand the role of cysteinyl leukotriene signalling in disease progression during bacterial infections, specifically *L. monocytogenes* and *M. tuberculosis*. This aim will be achieved through the following objectives:

- Characterizing the germline CysLTR1 knockout mice at naïve state.
- Time kinetics experiments to evaluate the role of CysLTR1 in immune responses and pathology during *Mtb* and *Lm* infections.

- Survival studies to determine whether CysLTR1 influences the outcome of listeriosis and tuberculosis in mice.
- Investigate the underlying mechanism driving the outcome of listeriosis and tuberculosis in mice.

By addressing these objectives, this project will provide valuable insights into the complex interplay between cysteinyl leukotriene signalling and bacterial infections, potentially paving way for the development of novel therapeutic strategies.

Chapter 2: Manuscript in preparation

The background- and sex-specific role of Cysteinyl leukotriene type 1 receptor (CysLTR1) in *Listeria monocytogenes* infection in mice

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2.1. Abstract

Leukotriene B4 (LTB4) and cysteinyl leukotrienes (cysLTs; LTC4, LTD4, and LTE4) are a class of eicosanoids produced by the 5-lipoxygenase-metabolism of arachidonic acid (AA). CysLTs, pro-inflammatory lipid mediators, play physiological roles in allergic and asthmatic reactions. CysLTR1, CysLTR2, and GPR99/CysLTR3 are the G protein-coupled receptors through which cysLTs carry out their biological activities. The CysLTR1 function has been thoroughly investigated in asthmatic and allergy-related illnesses, however, CysLTR1 role in bacterial infections have not been fully understood. Using CysLTR1-deficient mice, the current work sought to assess the function of CysLTR1 during *Listeria monocytogenes* (*Lm*) infection in male and female C57BL6 and Balb/C mice. *Lm* infection increased CysLTR1 expression in the liver and spleen tissues of wildtype C57BL/6 and Balb/C mice. The deletion of CysLTR1 had no effect on mice homeostasis. During *Lm* infection, CysLTR1-deficiency had no effect on bacterial burden. We demonstrate for the first time, that CysLTR1 signalling influences neutrophil recruitment to the liver and spleen of infected mice differently depending on the host background, with C57BL/6 mice having increased neutrophils, whilst reduced in Balb/C mice at later stages of infection. The deletion of CysLTR1 in C57BL/6 mice does not affect their survival during *Lm* infection, however Balb/C female mice (and not male mice) are more protected to *Lm* infection in the absence of CysLTR1. In conclusion, CysLTR1 signalling influences neutrophil recruitment in a background-dependent manner, while host survival is sex-specific in Balb/C mice.

2.2. Introduction

Cellular activation by immune complexes, bacterial peptides and other stimuli results in the arachidonic acid metabolism by 5-lipoxygenase (5-LO) to generate eicosanoids; with leukotriene B4 (LTB4) and cysteinyl leukotrienes (LTC4, LTD4, and LTE4) forming the leukotriene subset of the eicosanoids. Leukotrienes are lipid mediators that induce proinflammatory responses and are primarily synthesized by inflammatory cells such as eosinophils, basophils, mast cells, dendritic cells, and macrophages (254–256). Cysteinyl leukotrienes (CysLTs) are synthesized by the enzymatic activity of leukotriene C4 synthase (LTC4S) and are peptide conjugated. They exhibit their biological functions through specific G protein-coupled receptors, CysLT type 1 receptors (CysLTR1), CysLTR2 and CysLTR3/GPR99 (27,257). CysLTR1 binds LTD_4 with a higher affinity than LTC4, whereas CysLTR2 binds both LTC_4 and LTD_4 with equal affinity (258). GPR99, on the other hand, has been shown to preferentially bind LTE_4 (259).

CysLTs are also known as proinflammatory bioactive lipids that have a pathobiological function in asthma and allergic responses (260). In brief, CysLTs are primarily involved in eosinophil and mast cell induced bronchoconstriction in asthma. There have been antagonists developed to treat asthma by inhibiting production of CysLTs or their receptors known as leukotriene receptor antagonists (LTRAs). The inhibitors either work as leukotriene-related enzyme inhibitor or leukotriene receptor antagonist, opposing the function of the leukotriene mediators. These include CysLTR1 antagonists (montelukast, zafirlukast, and pranlukast) and 5-LO antagonists (zileuton and Hypericum perforatum) (261,262). For instance, CysLTR1 antagonists have been shown to reduce symptoms and improve the quality of life for allergic rhinitis patients by affecting systemic inflammation associated with the disease and reducing allergen-induced early- and late-airway responses (263,264).

While CysLTs and their receptors have been intensively studied in allergic and asthmatic responses, there have been a handful of studies done to understand the leukotrienes' role in bacterial infections. One of those studies demonstrated that mouse mast cells during *Escherichia coli* infection secrete significant amounts of LTB4 and LTC4, and mast cell deficient mice failed to secrete these leukotrienes resulting in impaired neutrophil recruitment and bacterial clearance (265). Leukotriene deficient mice were shown to be more susceptible to *Klebsiella pneumoniae* infection, however the lack of leukotrienes did not affect neutrophil recruitment to the site of infection but impaired alveolar macrophage ability to phagocytose and clear the bacteria (116). Leukotrienes have been shown to play a protective role in mice infected with *Mycobacterium tuberculosis*. Mice treated with MK 886, a 5-LO pathway inhibitor, had reduced LTB4, increased lung bacterial burden, reduced nitric oxide and proinflammatory cytokine production (266).

Although the leukotrienes have been implicated in disease modulation during bacterial infections, the role of CysLTs and their specific receptors has not been well elucidated. In the present study, we evaluated the role of CysLTR1 using gene knockout mice during *Listeria monocytogenes* (*Lm*) infection. *L. monocytogenes* infection model has been well established to evaluate and define the paradigms of cell-mediated immunity. In the early 1960s, Mackaness used *Lm* to demonstrate the importance of cellular immunity activation in bacterial infection control, as primary infection with *Lm* resulted in host susceptibility while upon preexposure the mice became more resistant (267). *Lm* has since been used as a model to study the host innate and adaptive immunity. Here, we demonstrated for the first time that *Lm* infection induces CysLT receptors and enzyme expression in both C57BL/6 and Balb/C mice backgrounds. The deletion of CysLTR1 influences mice survival differently depending on mice backgrounds and sex. C57BL/6 mice survival was not affected by the deletion of CysLTR1 during *Lm* infection, while Balb/C female were more protected from the infection than their

male counterparts. We observed that *Lm* infection resulted in either increased or reduced neutrophils to the spleen and liver in CysLTR1 deficient C57BL/6 and Balb/C mice, respectively. CysLTR1 signalling influences immunity to *Lm* infection in a host dependent manner influenced by sex in Balb/C mice.

2.3. Materials and Methods

2.3.1. Ethics

All animal studies adhered to the rigorous guidelines outlined in the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). Additionally, mouse experiments followed the approved protocol by the Animal Research Ethics Committee (AREC: 022/039) at the Faculty of Health Science, University of Cape Town.

2.3.2. Mouse strains

CysLTR1-deficient mice (*Cysltr1*$^{-/-}$) were created by breeding heterozygous (*Cysltr1*$^{-/+}$) animals from Balb/C and C57BL/6 backgrounds. Dr. Frank Austen from Harvard Medical School generously provided CysLTR1 heterozygous mice on both backgrounds (268). These mice were housed in ventilated cages under specific-pathogen-free conditions at the research animal facility of the UCT Faculty of Health Science. For most experiments, mice aged 8-12 weeks were used, with sex matching unless otherwise specified.

2.3.3. Genotyping

Genomic DNA extracted from mice ear clips underwent PCR genotyping. The reaction mix which included 10X buffer, 10mM dNTPs, 25mM MgCl2, 5U/µL SuperTherm Taq, water, and 6.25µM wildtype or knockout primers. PCR conditions involved denaturation at 94°C for 2 minutes, followed by 35 amplification cycles (94°C for 20 seconds, 58°C for 30 seconds, and 72°C for 45 seconds), with an extended annealing step at 72°C for 5 minutes. Specific primers targeting CysLTR1 deletion (5’- ATC TTG TTC AAT GGC CGA TCC CAT -3’ and 5’- AAA ACA ATG ACG TGC ACT ATA AAG -3’) and wildtype (5’- AAA ACA ATG ACGT GCA CTA TAA AG -3’ and 5’- AAT CAT GTA TAC TTG GAA GGC TGA -3’) were used for confirmation.

2.2.4. *Listeria monocytogenes* culture and mice infections

Bacterial culture: *L. monocytogenes* (strain EGD) was grown in Tryptic-Soy Broth (TSB) at 37°C with overnight shaking at 170 rpm. The overnight culture was then transferred to fresh TSB and grown until reaching mid-log phase (OD 0.6-0.8) at 37°C. The bacterial culture was preserved in 20% glycerol and stored at -80°C until needed. Prior to experiments, the concentration of bacteria in stock was determined by plating samples from random vials on Tryptic-Soy agar (TSA) plates.

Mortality studies: Balb/C mice are known to be more susceptible to *Lm* infection than C57BL/6 mice (269). Therefore, *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice, both on C57BL/6 background and aged 8-12 weeks, were intraperitoneally infected with approximately 1x10^6 CFUs/200µL (an established laboratory lethal dose 50 (LD50) for C57BL/6 mice) of bacteria. They were monitored over 15 days to compare survival rates between the two groups. Conversely, *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice on BALB/c background were infected intraperitoneally with around 2x10^5 CFUs/200µL, which was established as the LD50 for Balb/C mice. Mice experiencing either a 20% weight loss from their initial body weight or showing severe sickness signs (lack of grooming, hunched back, pale extremities) were humanely euthanized to prevent suffering, and death was confirmed by cervical dislocation.

Time-course studies: C57BL/6 *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice were intraperitoneally infected with approximately 5x10^5 CFUs/200µL of *Listeria monocytogenes* and were euthanized at 3- and 7- days post-infection (dpi). Similarly, Balb/C *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice were intraperitoneally infected with about 2x10^4 CFUs/200µL of *Lm* and were euthanized at 3- and 7-dpi. The bacterial load in the liver and spleen of these *Lm*-infected mice was assessed at both 3- and 7-dpi using previously established methods (270).

2.3.5. Liver and spleen single cell suspensions

Liver single cell suspensions were prepared by enzymatic digestion in a buffer consisting of DMEM supplemented with 5% FCS, containing 0.11 mg/ml Collagenase type I, 0.11 mg/ml Collagenase type II, and 0.001 mg/ml DNase I, followed by incubation at 37°C for 30 minutes. The digested cells were filtered through 100µm and 70µm cell strainers sequentially and then centrifuged at 500g for 5 minutes. The resulting cell pellet was resuspended in 3 ml of PBS supplemented with 3% FCS. Next, 1.7 mL of isotonic Percoll was layered on top, gently mixed, and centrifuged at 500 rpm for 10 minutes without brakes. The cells were incubated briefly in RBC lysis buffer at room temperature, followed by centrifugation at 500g for 5 minutes. The cells were resuspended in 1 mL of complete media and counted using the Trypan Blue exclusion method to determine the number of viable cells.

For spleen processing, the organs were mechanically homogenized in complete media and filtered through 70µm and 40µm cell strainers sequentially. The resulting suspension was centrifuged at 500g for 5 minutes, and the pellet was treated with RBC lysis buffer followed by another centrifugation at 500g for 5 minutes. The cells were washed once with complete media, resuspended in 5 mL of media, and counted using the same method as described for liver cells.

2.3.6. Immune cell populations

Organ single cell suspensions (~$1x10^6$ cells per well) were labelled with either a myeloid antibody cocktail (including Fixable Viability stain 575V, CD64 PeCy7, Ly6C PercP-Cy5.5, Cd11b V450, MHC II A700, CD103 PE, CD11c APC, Siglec-F APC-Cy7, Ly6G FITC) or a lymphoid antibody cocktail (575V Viability dye, CD4 V500, CD44 PE, NK1.1 APC-Cy7, CD3 A700, CXCR5 PeCy7, CD62L V450, CD19 PercP-Cy5.5, CD8 APC, KLRG1 BV786, PD-1 FITC, Ki67 PE; all antibodies sourced from BD Bioscience). Flow cytometry analysis was

performed to study diverse immune cell populations, employing the gating strategies detailed in *Appendices A*, *B*, and *C*. The samples were acquired with BD LSRII and analysed using FlowJo™ Software version 10.6.

2.3.7. Cytokine profiles in tissue homogenates

Liver and spleen homogenates from mice were centrifuged at 1000g for 5 minutes to obtain supernatants. The levels of IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, and IFN-γ (BD Biosciences) in these supernatants were quantified using ELISA, following the manufacturers' instructions as previously outlined (270).

2.3.8. Histopathology

Each animal's liver lobe was preserved in 10% neutral buffered formalin (NBF), which contains approximately 3.8-4% formaldehyde, 4 g/L NaH2PO4, and 6.5 g/L Na2HPO4 at pH 7.0. The fixed organs were sectioned into three slices spaced 30-45 μm apart and stained with haematoxylin and eosin (H&E) to assess histopathological changes post Listeria monocytogenes (Lm) infection. Slides were digitally scanned at 20X magnification using the Virtual Slide VS120 microscope from Olympus, Japan, and analysis was conducted using Qu Path version 0.3.2 for quantification purposes

2.3.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated from liver and spleen tissues of infected mice using the RNeasy® Mini kit (Qiagen), following the manufacturer's protocol. The concentration of RNA was determined using the ThermoFisher NanoDrop One UV Spectrophotometer. Subsequently, cDNA synthesis and quantitative PCR (qPCR) were conducted according to methods previously detailed by Jones *et al.* (271), using the primers used are on *Appendix E*.

2.3.10. Statistical analysis

All data presented were analysed using GraphPad Prism 10.0 software, employing the Student's t-test. Statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

2.3. Results

2.3.1. CysLTR1 deletion in C57BL/6 has no effect on mice homeostasis.

With CysLTR1-deficient mice, our initial investigation aimed to determine the impact of CysLTR1 signalling on host homeostasis. The CysLTR1 knockout (*Cysltr1*$^{-/-}$) mice were created using homologous recombination, wherein a neomycin resistance gene cassette replaced 278 nucleotides within exon 4 of the Cysltr1 coding region (**Figure 2.1A**) (272), and gifted as pure *Cysltr1*$^{-/-}$ on Balb/C and C57BL/6 backgrounds. We successfully confirmed the deletion of CysLTR1 in these mice by genotyping the *Cysltr1*$^{+/+}$ (wildtype) and *Cysltr1*$^{-/-}$ mice (**Figure 2.1B).** Due to the insertion of a neomycin cassette into the mutant allele, the *Cysltr1*$^{-/-}$ mice exhibit a gene size of 333 bp, differing from the wildtype CysLTR1 gene size of 284 bp.

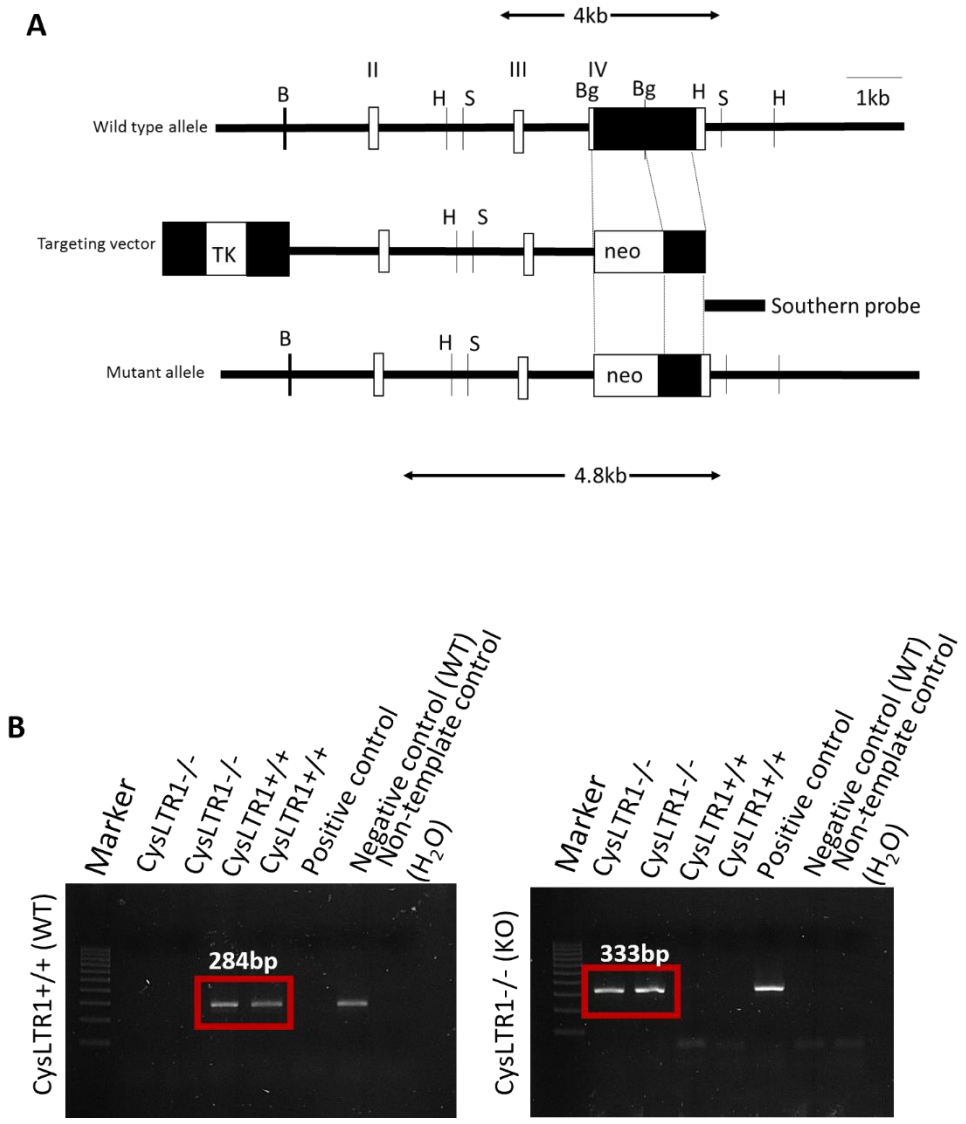

Figure 2.1: Generation of CysLTR1 deficient mice and confirmation of deletion. A) Homologous recombination generation of CysLTR1 deficient mice, the upper structure represents the genomic arrangement of the mouse CysLTR1 gene, the middle is the targeting vector, and the lower structure represents the organization of the putative recombinant CysLTR1 allele. *Bam*HI (*B*), *Bgl*II (*Bg*), *Hin d*III (*H*), and *Sca*I (*S*) are the restriction enzyme sites. **B)** Genotyping of *Cysltr1*$^{-/-}$ mice.

Both the Balb/C and C57BL/6 background *Cysltr1*$^{-/-}$ mice were characterized at steady state by comparing them to their *Cysltr1*$^{+/+}$ littermate control mice. C57BL/6 *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice were comparable at naïve state with no differences in weight (**Figure 2.2A**), organ weight (**Figure 2.2B**), organ cell numbers (**Figure 2.2C**), and organ specific immune cells (**Figure 2.2G-L**). Furthermore, we validated that CysLTR1 deletion in tissues (**Figure 2.2D**) by measuring its mRNA expression and confirmed successful deletion in all organs at steady state.

The deletion of CysLTR1 has no effect on the expression of CysLTR2 or LTC4S (**Figure 2.2E -F**). CysLTR1 and CysLTR2 are 31% homologous and are on different chromosomes. CysLTR1 is expressed on the X chromosome in both mice and humans, position Xq13-Xq21 in humans (262,272,273), therefore deletion of CysLTR1 cannot affect expression of CysLTR2. LTC4S on the other hand, is the enzyme responsible for the synthesis of CysLTs, its expression is not regulated or influenced by these receptors. We also observed no major differences in cytokine production in the lung and liver of naïve mice (**Figure 2.2M-N**). Balb/C *Cysltr1*$^{-/-}$ mice were characterized by other members in our laboratory (114) and no major differences were observed at the naïve state. Thus, the deletion of CysLTR1 had no impact on immune system homeostasis irrespective of the genetic background.

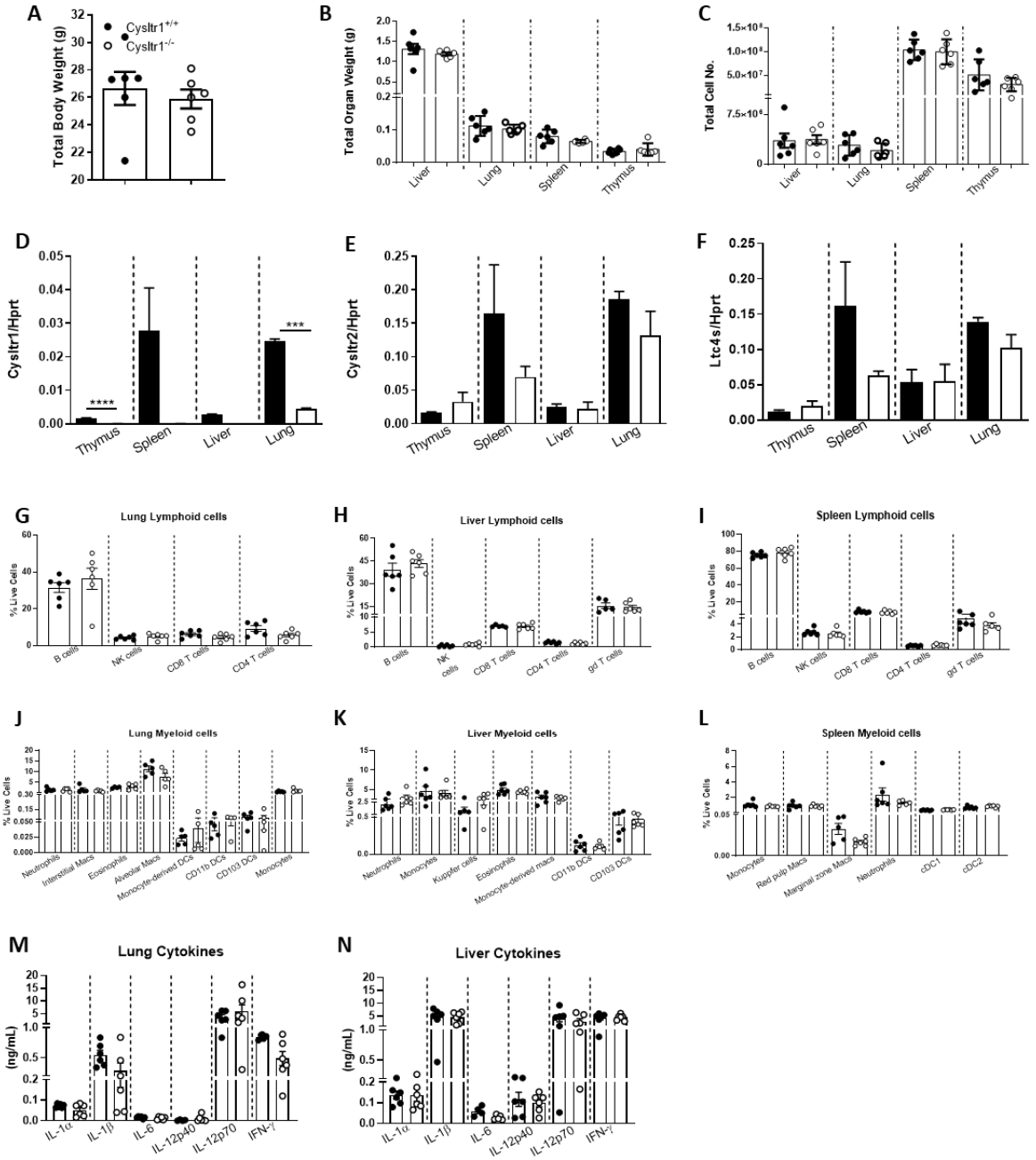

Figure 2.2: CysLTR1 deletion has no effect on immune homeostasis at naïve state and does not alter CysLTR2 and LTC4S expression. C57BL/6 CysLTR1 deficient mice and their littermate controls were sacrificed and characterized for any differences at naïve state by comparing **A)** total body weight, **B)** organ weight, **C)** cell numbers from the organs. We used $2x10^6$ cells for RNA extraction. The mRNA expression of **D)** *Cysltr1* (to confirm deletion), **E)** *Cysltr2* and **F)** *Ltc4s* was measured by qPCR. **G)** Lung, **H)** Liver, and **I)** spleen lymphoid cell populations (B cells, NK cells, CD8 and CD4 T cells). Myeloid cell populations in the **J)** lung, **K)** liver, and **L)** spleen analysed by flow cytometry. **M)** Lung and **N)** Liver cytokines measured in homogenates at naïve state by ELISA. Data is representative of three experiments. Error bars denote mean ± SEM of n = 6 per group and analysed using the unpaired student t-test with Welch's correction, ****p<0.0001.

2.3.2. *Listeria monocytogenes* infection induces CysLTR1 expression.

Cysteinyl leukotrienes and their receptors are known to be involved in asthmatic and allergic responses. Here, we determine whether cysteinyl leukotrienes play a role in bacterial infection. Firstly, we determined whether infection with *Lm* induces the expression of CysLT receptors; CysLTR1 and CysLTR2, and the cysLT enzyme; leukotriene C4 synthase (LTC4S). We measured mRNA expression of these genes in the liver and spleen of *Lm*-infected C57BL/6 and Balb/c mice by qPCR. Indeed, *Lm* infection in mice resulted in an upregulation in *Cysltr1*, *Cysltr2* and *Ltc4s* mRNA expression in the C57BL/6 spleen (**Figure 2.3A**) and liver (**Figure 2.3B**), mostly at later stages of infection. *Cysltr1*, *Cysltr2* and *Ltc4s* expression in Balb/C spleen were significantly reduced early at 2-dpi and upregulated at chronic 7-dpi (**Figure 2.4A**). In the Balb/C liver, *Cysltr1* was downregulated at 2-dpi and upregulated at 10-dpi, though the expression of *Cysltr2* and *Ltc4s* had no significant differences (**Figure 2.4B**). Taken together, these results indicate that *Lm* infection alters CysLT expression and may influence its pathogenesis.

2.3.3. CysLTR1 deletion had no effect on bacterial growth irrespective of sex and genetic background during *Listeria monocytogenes* infection

We established that the deletion of CysLTR1 has no effect on host homeostasis at naïve state. We next assessed how deleting this receptor affects *Lm* infection outcomes in mice. *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice were infected with *Lm* and sacrificed at 3- and 7-dpi, to measure organ bacterial load in the spleen and liver. At both time-points, organ bacterial loads were comparable in C57BL/6 and Balb/C female (**Figure 2.3C and 2.4C)** and male mice **(Figure 2.3D and 2.4D**). This suggests that deletion of CysLTR1 has no effect on host tissue bacterial control. In addition, C57BL/6 organ weights were also unaffected by deletion of the receptor during infection (**Figure 2.3E-F**). While Balb/C females also had comparable organ weights, Balb/C *Cysltr1*$^{-/-}$ male showed reduced spleen weights at 7-dpi **(Figure 2.4E-F**). C57BL/6

female *Cysltr1*$^{-/-}$ spleen had significantly decreased cell numbers at 3-dpi which were then comparable to those of control mice at 7dpi (**Figure 2.3G**). C75BL/6 *Cysltr1*$^{-/-}$ male liver had a minor increase in cell numbers at 3-dpi, while their spleen had reduced cell numbers at 7-dpi (**Figure 2.3H**). Balb/C female *Cysltr1*$^{-/-}$ spleen cell numbers were significantly reduced at 3-dpi, while both the liver and spleen cell numbers were the same as those of controls at 7-dpi (**Figure 2.4G**). Additionally, Balb/C male *Cysltr1*$^{-/-}$ liver cells were increased while spleen cells were reduced at 3-dpi, but these numbers were comparable at 7-dpi (**Figure 2.4H**). Overall, the deletion of CysLTR1 does not influence the host bacterial control but does influence the total cell numbers in the tissue. These effects are mostly consistent and were observed in spleen at 3dpi in both backgrounds and sexes.

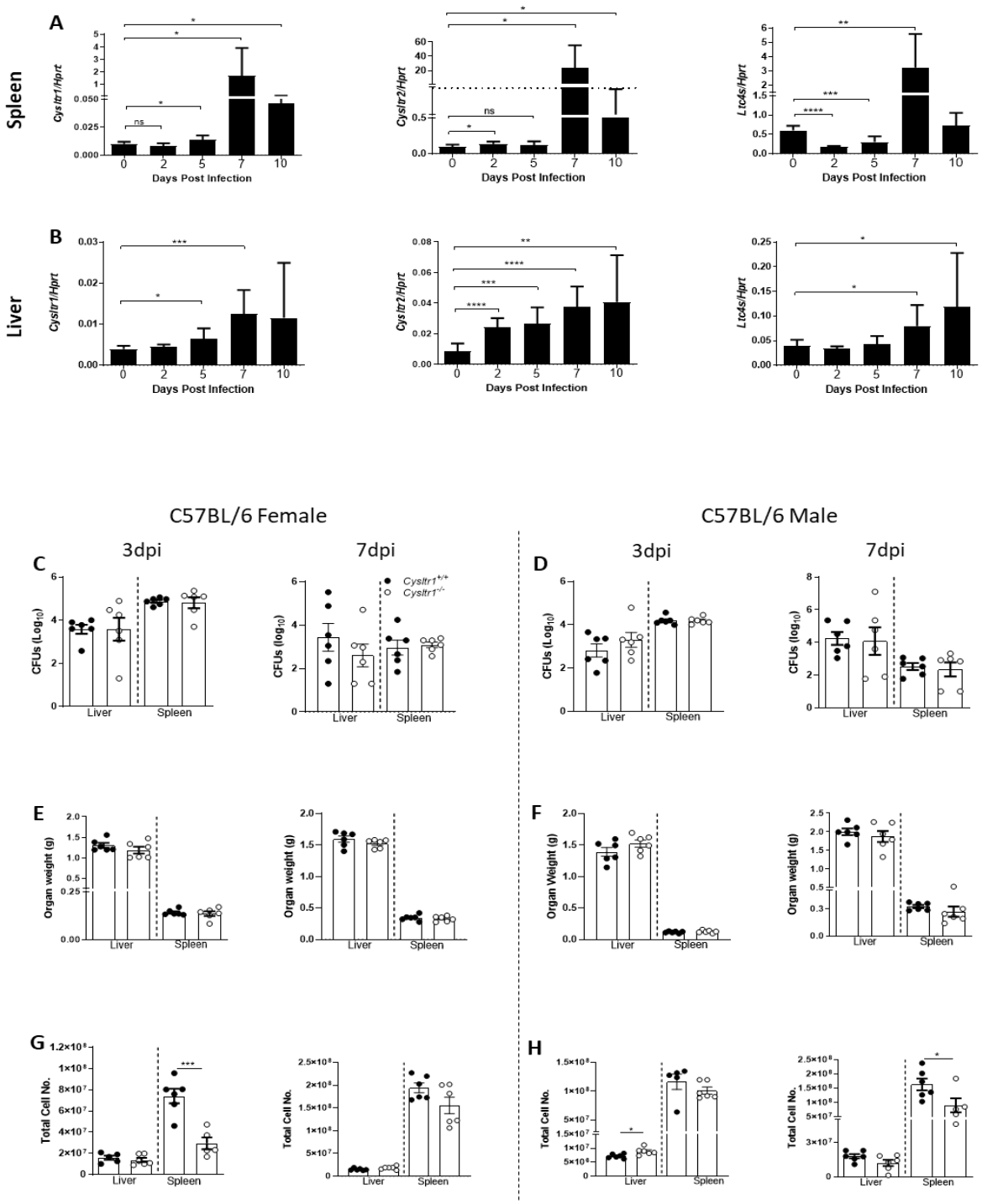

Figure 2.3: ***Listeria monocytogenes*** **infection increases CysLTR1 expression, and deletion of CysLTR1 in C57BL/6 mice has no effect on bacterial control**. C57BL/6 wildtype mice were infected with 7.5x10^5 CFUs/200µL *L. monocytogenes* intraperitoneally and sacrificed at various time-points post-infection. **A)** The spleen and **B)** liver were collected from each mouse for each time point for RNA extraction and mRNA expression of *Cysltr1*, *Cysltr2* and *Ltc4s* measured by qPCR. C57BL6 *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ male and female mice were infected with 5x10^5 CFUs of *L. monocytogenes* intraperitoneally and sacrificed 3- and 7-days post infection (dpi). Mice spleen and liver were collected to determine **C-D)** bacterial burden, **E-F)** organ weights, and **G-H)** cell numbers. Experiments were performed once. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

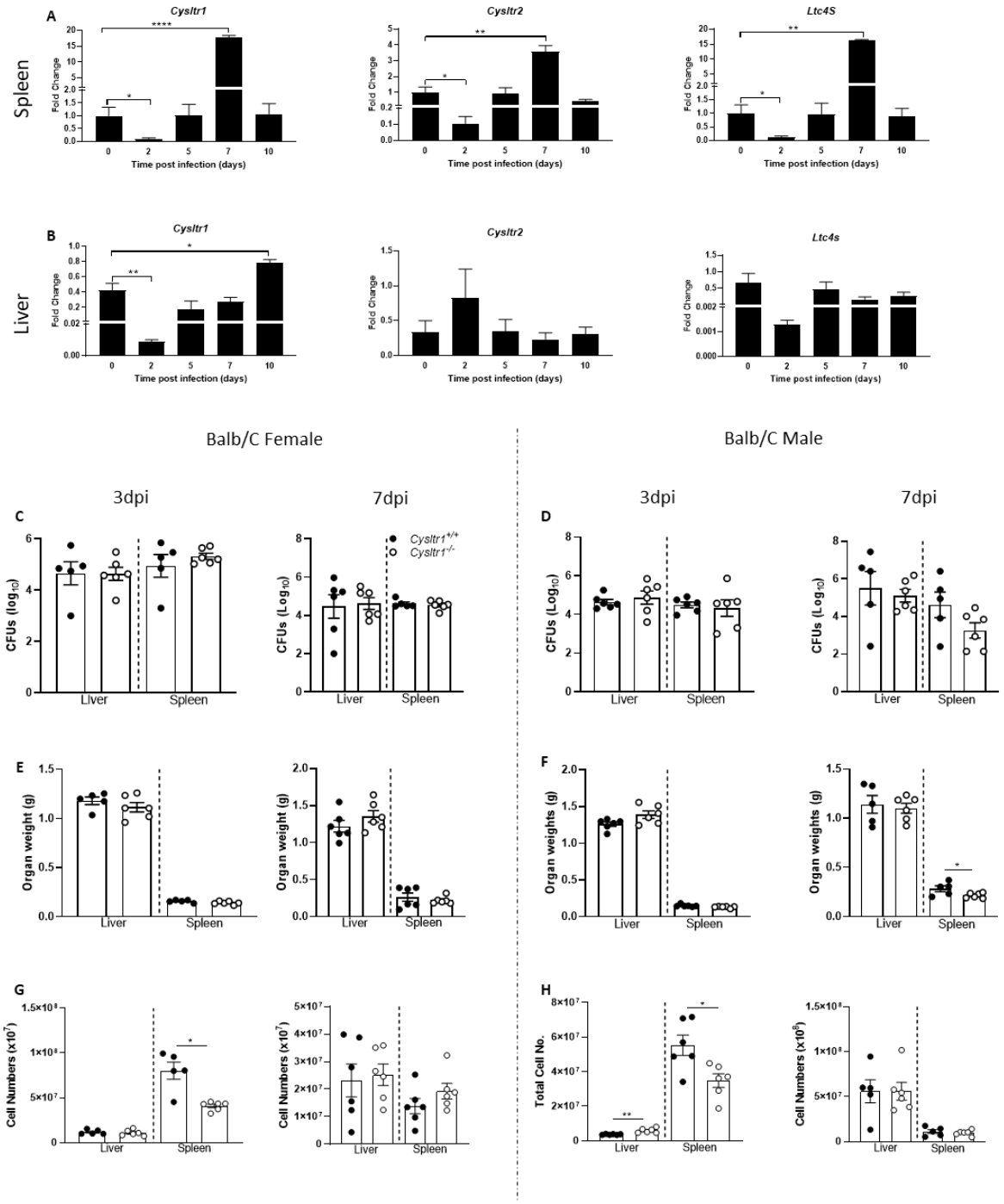

Figure 2.4: *Listeria monocytogenes* infection transiently downregulates CysLTR1 expression followed by an increase at later timepoints in Balb/C wildtype mice. Balb/C wildtype mice were infected with 2.5x10^4 CFUs/200µL *L. monocytogenes* intraperitoneally and sacrificed at various time-points post-infection. **A)** The spleen and **B)** liver were collected from each mouse for each time point for RNA extraction and measured mRNA expression of CysLTR1, CysLTR2 and Ltc4s by qPCR. Balb/c *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ male and female mice were infected with 2x10^4 CFUs/200µL *L. monocytogenes* intraperitoneally and sacrificed 3- and 7-dpi. Mice spleen and liver were collected to determine **C-D)** bacterial burden, **E-F)** organ weights, and **G-H)** cell numbers. Data is representative of two independent experiments. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.3.4. CysLTR1 deletion influences spleen neutrophil recruitment and cytokine secretion during *Listeria monocytogenes* infection.

Given the observed differences in the total cell numbers in the tissues, we assessed spleen and liver immune cells by flow cytometry. At 3-dpi, C57BL/6 female *Cysltr1*$^{-/-}$ mice had significantly reduced spleen CD8 T cells, while at 7dpi had reduced B cells and increased CD4 and CD8 T cell frequencies (**Figure 2.5A**). Male C57BL/6 *Cysltr1*$^{-/-}$ spleens had significantly reduced B cells, CD4, CD8 T cells and NK cell frequencies at 3-dpi, while these populations, except for reduced NK cells, were comparable at 7-dpi (**Figure 2.5B**). At 3-dpi, Balb/C female Cysltr1$^{-/-}$ splenocytes had increased NK cells, while males had increased B cell percentages. At 7-dpi, both Balb/C female and male *Cysltr1*$^{-/-}$ splenocytes had significantly increased CD4 T cells (**Figure 2.6A-B**). Interestingly amongst myeloid cells, C57BL/6 *Cysltr1*$^{-/-}$ female (**Figure 2.5C**) and male (**Figure 2.5D**) had significantly increased neutrophils at both time points, while there weren't differences in other myeloid cells, except increased cDC2 in females (**Figure 2.5C**) at 3-dpi and increased marginal zone macrophages in males (**Figure 2.5D**) at 7-dpi. We did not observe major differences in Balb/C myeloid cell populations, however, female *Cysltr1*$^{-/-}$ splenocytes had increased neutrophils and marginal zone macrophages (MZMs) at 3-dpi, while at 7-dpi neutrophils were significantly reduced and there was an increased in red pulp macrophages (RPMs, **Figure 2.6C**). At 3-dpi, Balb/C male mice had comparable splenocyte myeloid cells, while at 7-dpi *Cysltr1*$^{-/-}$ male spleens had reduced neutrophils, accompanied by increased RPMs and conventional dendritic cells 1 (cDC1, **Figure 2.6D**). We then measured spleen cytokine secretion to determine immune responses to infection. At both 3- and 7-dpi, C57BL/6 female mice spleen has comparable cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IFN-γ) secretion during *Lm* infection (**Figure 2.5E**). In C57BL/6 males, most cytokines were comparable between *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice except increased IL-1α and IL-6 at 3- and 7-dpi, respectively (**Figure 2.5F**). At 3-dpi,

Balb/C female *Cysltr1*$^{-/-}$ spleen had reduced IL-1β, IL-6 and IL-10 secretion, while at 7-dpi *Cysltr1*$^{-/-}$ had reduced IL-6 when compared to the littermate controls (**Figure 2.6E**). At both timepoints, male spleens had comparable cytokine profile (**Figure 2.6F**). In summary, CysLTR1 signalling in the spleen influences neutrophil and lymphoid cell populations differently between the C57BL/6 and Balb/C genetic backgrounds in mice.

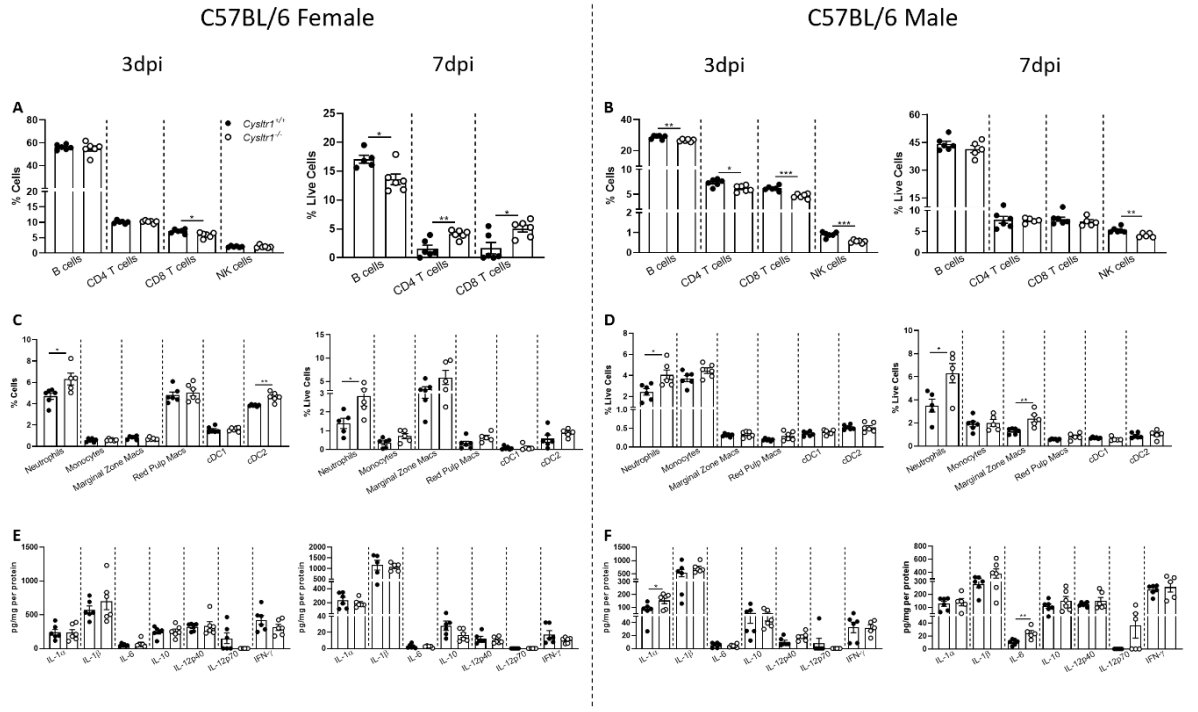

Figure 2.5: The deletion of CysLTR1 in C57BL/6 mice increase neutrophil frequencies in spleen but has no effect on cytokines in *Listeria monocytogenes* infection. C57BL/6 mice were sacrificed, spleens were collected, and single cell suspensions were prepared and stained for flow cytometry analysis at 3- and 7-dpi. **A-B)** Spleen lymphoid cells (B cells, CD4 T cells, CD8 T cells and NK cells), and **C-D)** myeloid cells (neutrophils, monocytes, marginal zone macrophages, red pulp macrophages, conventional dendritic cells 1 (cDC1), and cDC2) were measured. At 3dpi, immune cells were not gated from live cells. Mice spleen was homogenized and measured **E-F)** cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IFN-γ) secretion measured by ELISA. Experiments were only performed once. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

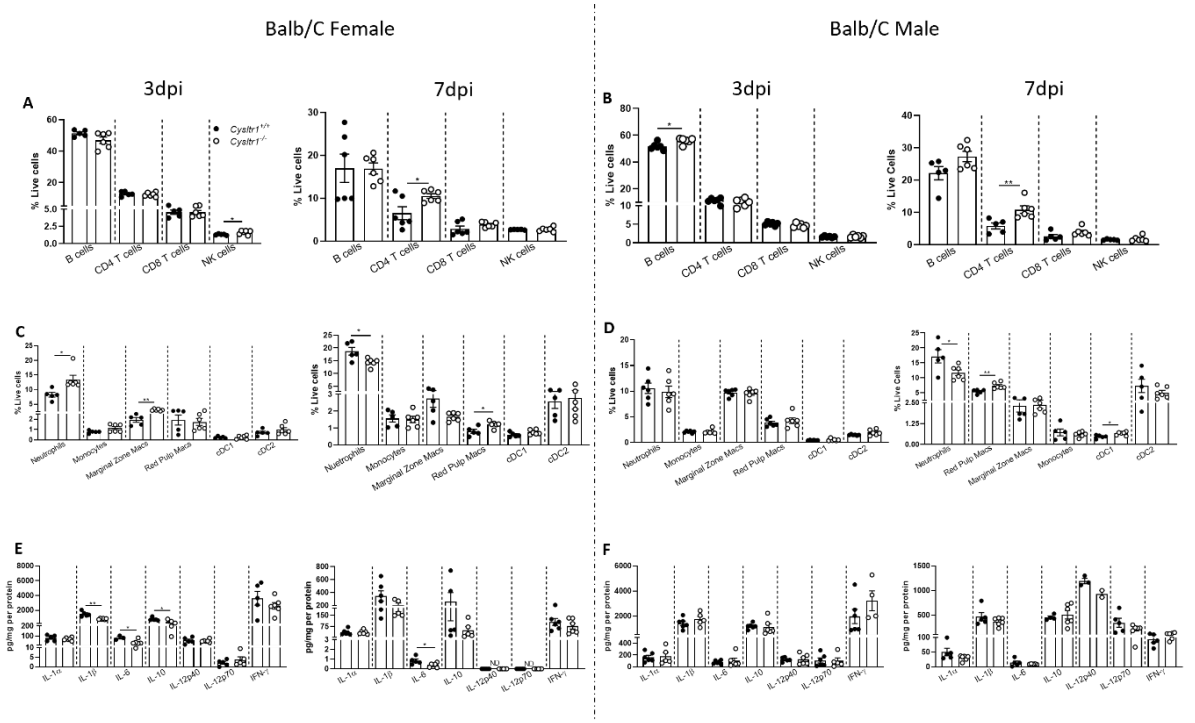

Figure 2.6: CysLTR1 deficiency in BALB/c mice results in reduced spleen neutrophils during chronic stage of *Listeria monocytogenes* infection. Balb/c mice were sacrificed, and spleens were collected, and single cell suspensions were prepared and stained for flow cytometry analysis at 3- and 7-dpi. **A-B)** Spleen lymphoid cells (B cells, CD4 T cells, CD8 T cells and NK cells), and **C-D)** myeloid cells (neutrophils, monocytes, marginal zone macrophages, red pulp macrophages, conventional dendritic cells 1 (cDC1), and cDC2) were measured. Mice spleen was homogenized and measured **E-F)** cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IFN-γ) secretion by ELISA. Data is representative of two-four independent experiments. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test, (*p<0.05, **p<0.01).

2.3.5. Increased liver neutrophils in CysLTR1-deficient C57BL/6 male mice have no effect on pathology.

The liver, as the spleen, is a vital organ that is affected during *Lm* infection, and liver-specific immune response is crucial for disease outcome (274). We, therefore, assessed liver immune cells by flow cytometry. C57BL/6 females had comparable liver lymphoid cells at both time points (**Figure 2.7A**), while male *Cysltr1*$^{-/-}$ had significantly reduced CD4 T, NK, and B cell percentages at 3-dpi, and at 7-dpi they had reduced CD4 and CD8 T cells (**Figure 2.7B**). Balb/C female *Cysltr1*$^{-/-}$ liver had increased B cells and NK cells at 3-dpi, while only NK cell frequencies were reduced at 7-dpi (**Figure 2.8A**). Male Balb/C *Cysltr1*$^{-/-}$ liver also had increased CD4 and CD8 T cells at 3-dpi, and these were significantly increased at 7-dpi

accompanied by increased B cell percentages (**Figure 2.8B**). Together this data suggests that CysLTR1 signalling differentially regulates T cells during *Lm* infection in male mice based on their background.

When evaluating myeloid cell populations, we observed that female C57BL/6 *Cysltr1*$^{-/-}$ liver had reduced monocytes at 3-dpi and increased monocyte-derived macrophages at 7-dpi (**Figure 2.7C**). Male C57BL/6 *Cysltr1*$^{-/-}$ liver had increased neutrophils at 3- and 7-dpi while increased monocyte-derived macrophage frequencies were observed at 7-dpi (**Figure 2.7D**). Balb/C female mice had comparable liver myeloid cells at 3-dpi, with an increasing trend of neutrophils in *Cysltr1*$^{-/-}$ mice (**Figure 2.8C**). At 7-dpi, *Cysltr1*$^{-/-}$ neutrophils were significantly reduced, while monocytes were increased when compared to littermate controls (**Figure 2.8C**). At 3-dpi, Balb/C male neutrophils were comparable between the groups, while *Cysltr1*$^{-/-}$ mice had increased liver eosinophils and monocytes. Additionally, at 7-dpi, Balb/C *Cysltr1*$^{-/-}$ male mice had reduced (not significant) neutrophils, and increased Kupffer cells, CD11b DCs, and CD103 DCs (**Figure 2.8D**). We then measured cytokine production in liver homogenates, and C57BL/6 female mice had comparable secretion at 3-dpi, while there was a significant increase in IL-1α and IL-1β secretion in *Cysltr1*$^{-/-}$ liver at 7-dpi (**Figure 2.7 E**). At 3-dpi, C57BL/6 male *Cysltr1*$^{-/-}$ liver homogenates had reduced amounts of IFN-γ, while there were no differences observed at 7-dpi in cytokine production (**Figure 2.7 F**). Balb/C female and male liver cytokine secretion was comparable between the knockout and controls at 3-dpi, while *Cysltr1*$^{-/-}$ female mice had reduced liver IL-6 levels and *Cysltr1*$^{-/-}$ males had reduced IL-12p70 at 7-dpi (**Figure 2.8E-F**). Liver histopathology analysis showed minimal pathology differences in both female and male C57BL/6 mice as depicted by the lesions and quantified lesion sizes (**Figure 2.7G-H**). Balb/C females, however, had comparable pathology at 3-dpi, and significantly reduced *Cysltr1*$^{-/-}$ liver lesions at 7-dpi (**Figure 2.8G**). Interestingly, Balb/C *Cysltr1*$^{-/-}$ male mice had reduced pathology at 3-dpi, but increased microabscess/lesion formation at 7-dpi (**Figure**

2.8H). Taken together, these results indicate that deletion of CysLTR1 in Balb/C females increases neutrophils at early timepoints but reduces at later timepoints accompanied by decrease in pathology. In male Balb/C mice, deletion of CysLTR1 influences liver pathology during *Lm* infection despite the minimal effects on myeloid cells and secretion of cytokines. While in C57BL/6 male mice, CysLTR1 deletion increases neutrophil recruitment in a cytokine independent manner and has no effect on organ pathology.

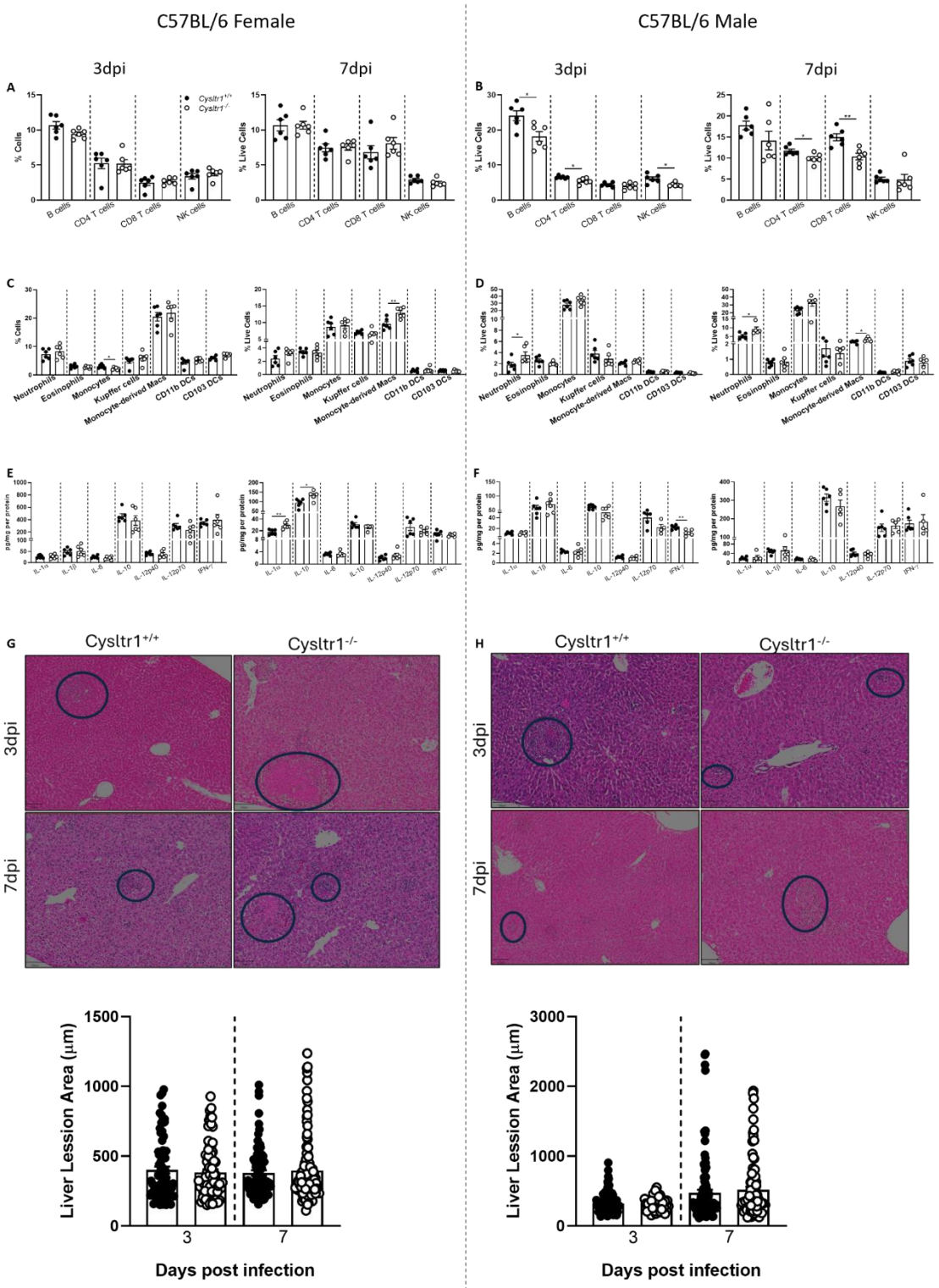

Figure 2.7: Deletion of CysLTR1 increases C57BL/6 male mice liver neutrophils during *Listeria monocytogenes* infection. C57BL/6 mice were sacrificed, and livers were collected, prepared single cell suspensions, and then stained for flow cytometry analysis at 3- and 7-dpi. **A-B)** Liver lymphoid cells (B cells, CD4 T cells, CD8 T cells and NK cells), and **C-D)** myeloid cells (neutrophils, eosinophils, monocytes, Kupffer cells, monocyte-derived macrophages, CD11b DCs and CD103 DCs) were measured. At 3dpi, immune cells were not gated from live cells. Mouse liver was homogenized and measured **E-F)** cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IFN-γ) secretion measured by ELISA. **G-H)** H&E staining for immunohistology analysis. Liver lesion quantification was performed using Qu Path version 0.3.2. Experiments were only performed once. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test, (*p<0.05, **p<0.01).

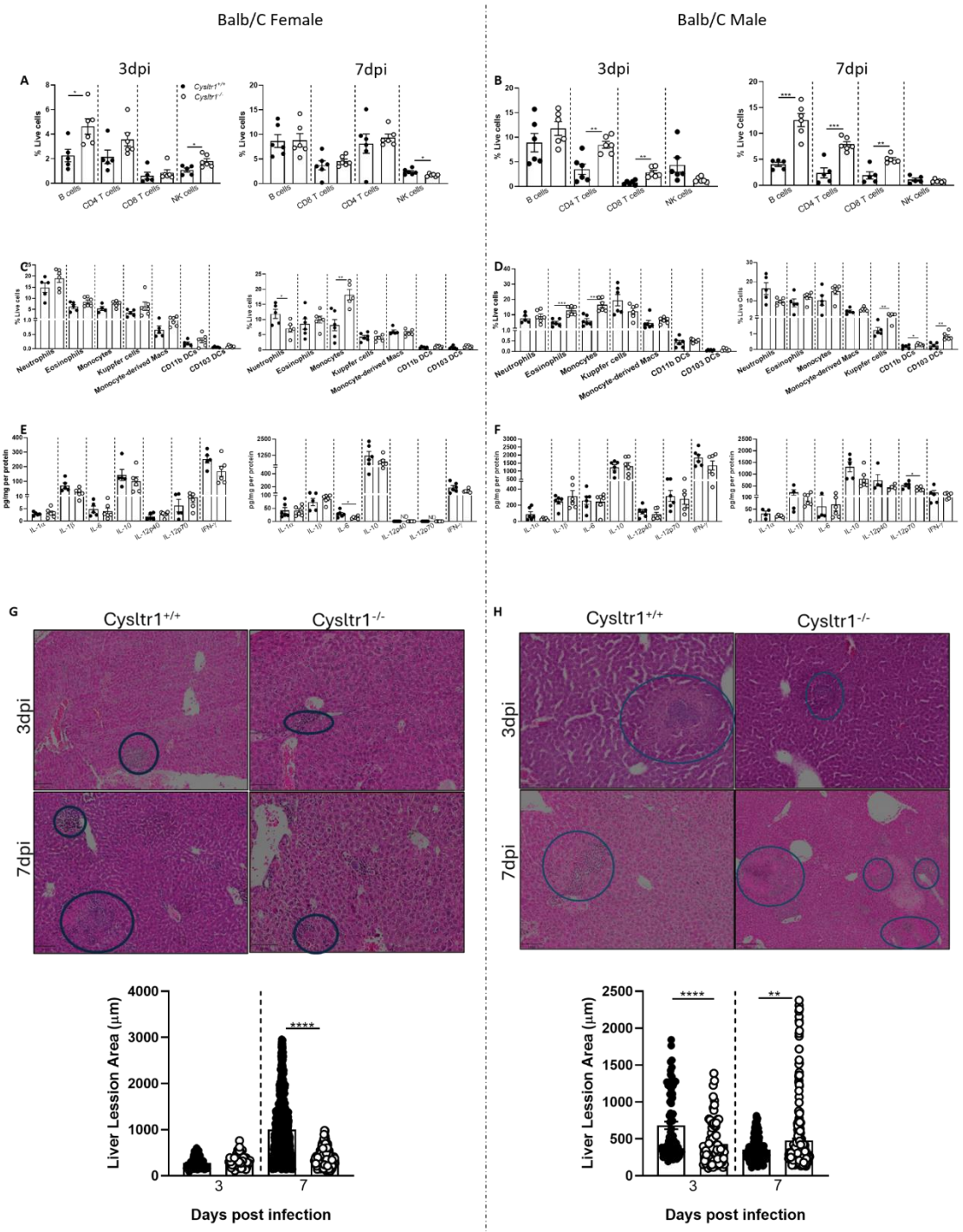

Figure 2.8: CysLTR1 deficiency in BALB/c mice results in reduced liver neutrophils with no effect on cytokines in chronic *Listeria monocytogenes* infection. Balb/C mice were sacrificed, and livers were collected, prepared single cell suspensions, and then stained for flow cytometry analysis at 3- and 7-dpi. **A-B)** Liver lymphoid cells (B cells, CD4 T cells, CD8 T cells and NK cells), and **C-D)** myeloid cells (neutrophils, eosinophils, monocytes, Kupffer cells, monocyte-derived macrophages, CD11b DCs and CD103 DCs) were measured. Mouse liver was homogenized and measured **E-F)** cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IFN-γ) secretion measured by ELISA. **G-H)** H&E staining for immunohistology analysis. Liver lesion quantification was performed using Qu Path version 0.3.2. Data is representative of two-four independent experiments. Error bars show mean ± SEM of n= 5-6 mice/group and analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.3.6. The survival of CysLTR1-deficient mice is dependent on genetic background and sex in *Listeria monocytogenes* infection.

We have shown that deletion of CysLTR1 in C57BL/6 and Balb/C mice had no effect on bacterial control but plays a role in neutrophil recruitment in a background dependant manner, and pathology in a sex-dependent manner in Balb/C mice. We then assessed whether the deletion of this receptor affects mice survival during *Lm* infection. We infected mice with *Lm* lethal dose of 50 percent (LD50, $1x10^6$CFUs/200µL for C57BL/6 mice and $2x10^5$CFUs/200µL for Balb/C mice) and monitored their survival over 15 days. C57BL/6 female (**Figure 2.9A**) and male (**Figure 2.9B**) *Cysltr1*$^{-/-}$ and *Cysltr1*$^{+/+}$ mice had comparable survival. Balb/C female *Cysltr1*$^{-/-}$ mice, on the other hand, were significantly protected (**Figure 2.9C**), while *Cysltr1*$^{-/-}$ male mice were more susceptible to *Lm* infection than their counterparts (**Figure 2.9D**). The deletion of CysLTR1 has no effect on the survival of C57BL/6 mice, however, in Balb/c mice, deficiency of this receptor has divergent outcomes (protective in females and detrimental in males) of the survival of animals.

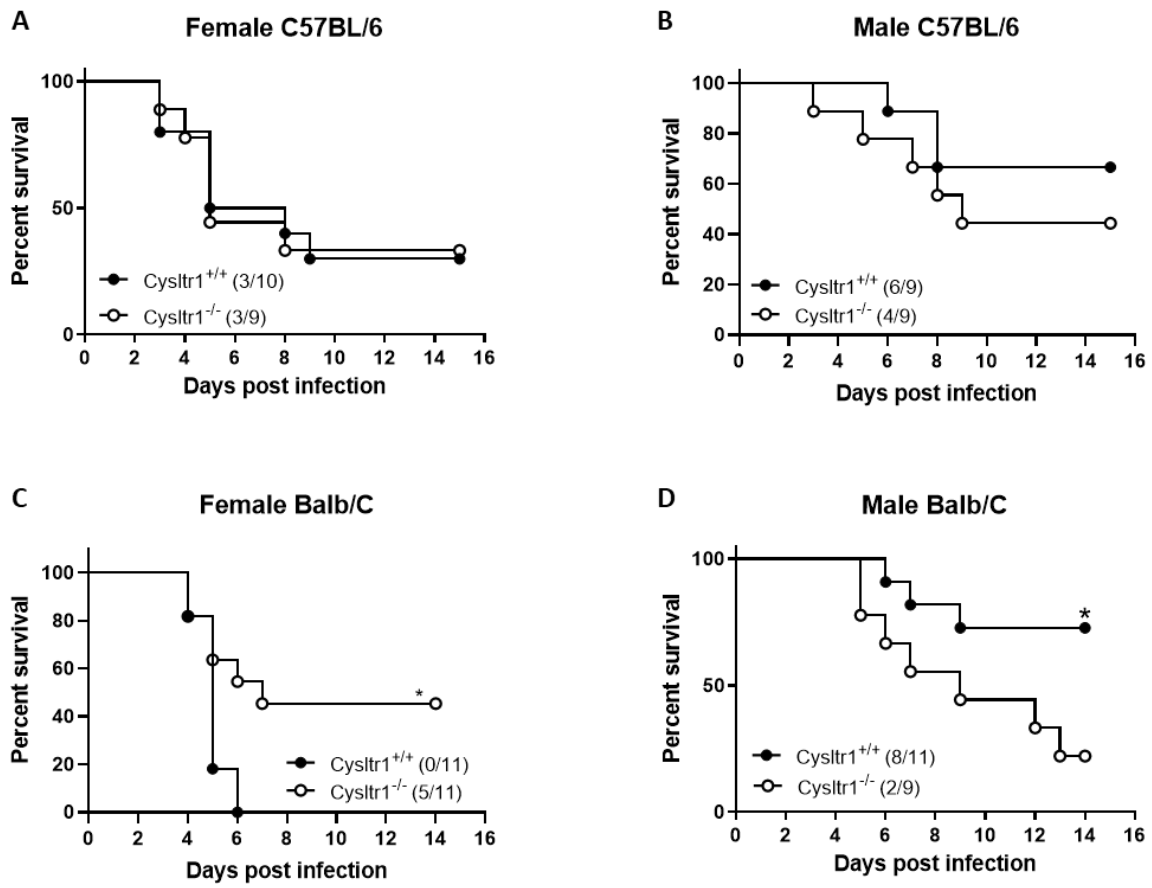

Figure 2.9: CysLTR1 is dispensable in C57BL/6 mice but divergent survival outcomes in Balb/C mice during *Listeria monocytogenes* infection. C57BL/6 *Cysltr1*$^{+/+}$ and *Cysltr1*$^{-/-}$ mice (n= 9-10 mice/group) were infected with 1x10^6 CFUs *Lm*/200µL, Balb/C *Cysltr1*$^{+/+}$ and *Cysltr1*$^{-/-}$ mice (n= 9-11 mice/group) were infected with 3x10^5 CFUs *Lm*/200µL and animals were monitored for survival over 15 days. **A)** C57BL/6 female, **B)** C57BL/6 male, **C)** Balb/C female, and **D)** Balb/C male mice survival curve after infection with *Lm*. Each mortality experiment was done once. Survival analysis was performed by log-rank (Mantel-Cox) test, (*p<0.05).

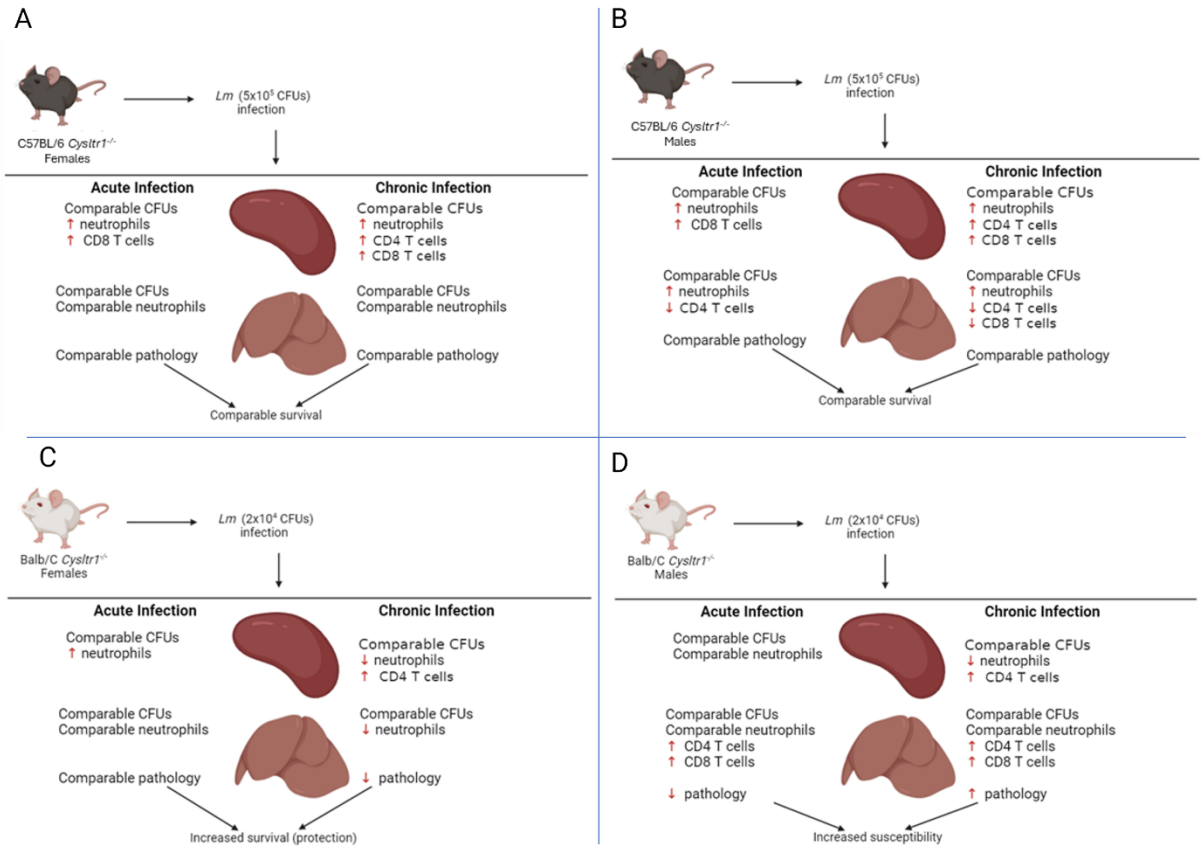

Figure 2.10: Schematic summary of major results during *Listeria monocytogenes* infection. A) CysLTR1 deletion in C57BL/6 female mice has no effect on bacterial control, results in increased spleen neutrophils, CD4 and CD8 T cells and has no effect on mice survival. **B)** CysLTR1 deletion in C57BL/6 male mice has no effect on spleen and liver bacterial burden, results in increased neutrophils and comparable liver pathology resulting in comparable survival. **C)** In female Balb/C mice, CysLTR1 deletion had no effect of bacterial growth, resulted in reduced spleen and liver neutrophils and reduced liver pathology during chronic infection leading in mice protection. **D)** CysLTR1 deletion in Balb/C male mice has no effect on bacterial control, resulted in increased neutrophils and increased liver pathology during late infection leading to increased mice susceptibility to *Lm* infection.

2.4. Discussion

The role of cysteinyl leukotrienes (CysLTs) has been well elucidated in asthma and allergic responses, with pathophysiological functions of cysLTs being modulated by CysLTR1 in asthma. This resulted in the development of several antagonists for CysLTR1 used to clinically treat asthma related diseases (262). Though both CysLTR1 and CysLTR2 induce similar signalling events including calcium (Ca^{2+}) flux and inositol phosphate secretion (275–277), they modulate different responses and are differentially distributed in the tissue and cellular level (278–280). In a study by Carion *et al.* (281) to study the role of CysLTR1 and CysLTR2 in corneal infection, they demonstrated that *Pneudomonas aeruginosa* infection differentially induced their mRNA expression in Balb/C and C57BL/6 mice (281), suggesting mice background influences the expression of these receptors. Indeed, our results showed that *Lm* infection also induced CysLTR1, CysLTR2 and LTC_4S expression in the liver and spleen of Balb/C and C57BL/6 mice (**Figure 2.3A-2.3B and 2.4A-2.4B**). Consistent with the findings by Carion *et at.* (281), there are genetic background specific differences in the expression of CysLT receptors during bacterial infections. In C57BL/6 mice, CysLT pathway gene expression increases with infection, whilst there is a transient early downregulation followed by increased expression at later stage of infection in Balb/C mice.

There have been a few studies documenting the leukotrienes' role in bacterial infections, particularly LTB4, but not the CysLTs (117,118,120,265,282–284). Here, we demonstrate that CysLTR1 signalling is influenced by both sex and genetic background. CysLTR1 deletion had no effect on bacterial burden regardless of genetic background or sex. Interestingly, the deletion of CysLTR1 affected organ lymphoid populations differently for each background and sex with each timepoint during *Lm* infection. The lymphoid cells are important for adaptive immunity against *Lm* infection. For instance, B cells contribute to susceptibility to *Lm* infection (285) *in vitro* and are killed via apoptosis in a dose-dependent manner (286,287), which *Lm* exploits to

successfully establish infection. Mice deficient of B cells were shown to be susceptible to *Lm* infection, since B cells and the antibodies are required for the induction T cell response (288). B cells are required for sustaining the generation of memory CD8 T cells during the infection (289). NK cells are required for the proper activation of the innate immunity to *Lm* infection, via production of IFN-γ for antimicrobial activities of myeloid cells (290,291). NK cells, therefore, induce host immunity to bacterial infections. In contrast, activated NK cells can increase host susceptibility to *Lm* infection, through increased IL-10 secretion, independent of IFN-γ (292).

CD4 T cells participate in host protection during *Lm* infection by differentiating naïve T cells into Th1 cells which produce cytokines, such as IFN-γ (293–295). However, there have been reports that CD4 T cells are less protective during *Lm* infection when compared to CD8 T cells (296). It has also been demonstrated that depletion of CD4 T cells in *Lm* infection increases antigen-specific CD8 T cells, which confers more protection (297). CD8 T cells induce immunity to *Lm* by either lysis of the infected cells by perforin and granzymes, or by secreting IFN-γ that in turn activates macrophages for an effective killing of the bacterium (298). Mittrucker *et al*.(297) demonstrated that mice immunized with inactive *Lm* had increased *Lm*-specific CD8 T cells and protected against subsequent infection with *Lm* infection. We found female C57BL/6 *Cysltr1*$^{-/-}$ mice had reduced spleen CD8 T cells, while male *Cysltr1*$^{-/-}$ spleen lymphoid cells were reduced early after infection, however, despite reduction in these lymphoid cells, these changes did not translate towards the susceptibility of these mice (**Figure 2.5A-B and 2.7B**).

CysLTs are potent inducers of calcium (Ca^{2+}) influx, and it has been shown that when LTD_4 binds to CysLTR1 there is an induced Ca^{2+} fluxes, which results in chemotaxis of αβ and γδ T cells to the inflamed tissue (299). Therefore, deletion of CysLTR1 itself or through reducing Ca^{2+} influxes could result in reduced recruitment of lymphocytes in the liver and spleen of

C57BL/6 males and females. CysLTs are significantly elevated, involved in the activation and recruitment of leukocytes that express CD45 such as T cells, B cells, and monocytes, during inflammatory reactions to rhinosinusitis allergic reaction, indicating that these cells are specifically drawn to the site of inflammation or may replicate and survive through the influence of CysLTs (300). Therefore, increased lymphoid cells in the liver and spleen for C57BL/6 *Cysltr1*$^{+/+}$ male mice could be due to increased CysLTR1 expression in response to *Lm* infection and its interaction with its binding ligands which results in the recruitment of lymphoid cells. Consistently, we found expression of *Cysltr1* in both liver and spleen increased in mice during *Lm* infection. In the liver, Balb/C *Cysltr1*$^{-/-}$ had increased CD4 and CD8 T cells at early stages, which together with NK cells were also increased at chronic infection (**Figure 2.8A-B**), contradicts the assumption that recruitment to the site of infection requires CysLTR1 as observed in male C57BL/6 mice. This difference in lymphoid cellular recruitment could be influenced by the genetic variation of these mice backgrounds and their resistance or susceptibility to the *Lm* infection. For instance, Balb/C and C57BL/6 mice have MHCI and MHCII restricted epitopes that recognise certain *Lm* peptide amino acids of its virulence factors, LLO and p60. In Balb/C mice, the MHCI specifically recognize LLO_{91-99} and $p60_{217-225}$ and this results to a strong CD8 T cell response to the *Lm* infection (301). In C57BL/6, the MHCII epitope interacts with the $LLO_{190-201}$ peptide resulting in strong CD4 T cell and CD8 T cell responses (302). This therefore suggests that deletion of CysLTR1 in Balb/C mice increases T cell responses, possibly by inducing/inhibiting one of the MHC epitopes and vice versa in C57BL/6 mice.

The most interesting observations were the differences in neutrophil frequencies in mice deficient in CysLTR1. We observed different trends in neutrophil frequences both in the liver and spleen of Balb/C and 57BL/6 mice, and these were also somewhat influenced by the gender of the mice. C57BL/6 *Cysltr1*$^{-/-}$ male mice had consistently increased neutrophils at both early

(innate stage of immunity) and late (adaptive stage of immunity) in both the spleen and liver, while the *Cysltr1*$^{-/-}$ females only had increased neutrophils in the spleen (**Figure 2.5C-D and 2.7C-D**). In Balb/C female *Cysltr1*$^{-/-}$ mice showed increased neutrophils at early stage followed by a significant reduction at later stage of infection, while the male *Cysltr1*$^{-/-}$ mice had a significant reduction at chronic stages, but not significant in the liver (**Figure 2.6C-D and 2.8C-D**). It has been well documented that the initiation of the innate immunity by neutrophils, NK cells and activated macrophages are critical in control of *Lm* infection. Neutrophils kill bacteria infected apoptotic hepatocytes or by lysing the infected cells directly (303,304). Neutrophils secrete chemokines that are required for the recruitment of more immune cells to the site of infection (305) and consequently to initiate adaptive immunity. Neutrophils are crucial for clearing *Lm* bacteria and preventing their spread to the central nervous system compartment (306). However, the depletion of neutrophils in mice during *Lm* infection leads to less susceptibility than in mice that are depleted of monocytes, suggesting that neutrophils are dispensable (307). Neutrophils have also been shown to regulate macrophage inflammatory signalling by inducing a rapid and sustained suppression of NF-κB activation (308). As mentioned, CysLTs are Ca^{2+} flux inducers, and calcium plays a crucial role in a variety of eukaryotic cellular signalling processes, which include exocytosis, contraction, metabolism, gene transcription, fertilization, and proliferation, and regulates actin microfilament (309). For instance, intracellular Ca^{2+} upregulation triggers neutrophil phagosome activities, which include NADPH oxidase activation and granule fusion with phagosomes. LTB_4, which neutrophils produce, interacts with the LTB_4 receptor to propel neutrophil migration (310). LTB_4 receptors and other G protein-coupled receptors facilitate neutrophil aggregation at the site of injury or infection (311). This therefore suggests that LTs, CysLTs and their receptors play a role not only in neutrophil recruitment but also in their overall function and Ca^{2+} regulation. Our data indicates that genetic background influences lymphoid cellular

recruitment and neutrophil recruitment. In the absence of CysLTR1, C57BL/6 mice had consistently increased neutrophils post *LM* infection except for C57BL/6 female *Cysltr1*$^{-/-}$ liver neutrophils, while Balb/C had a reduction of neutrophils at later stages of infection. However, the different neutrophil recruitment profiles did not have any effect on their antimicrobial properties in both backgrounds. Interestingly though, deletion of CysLTR1 had no effect on C57BL/6 mice liver pathology (**Figure 2.7G-H**), which is no surprise for the female mice. However, C57BL/6 *Cysltr1*$^{-/-}$ male mice had increased neutrophil recruitment to the liver, and which did not reflect on augmented pathology. This suggests that even though CysLTR1 signalling influences neutrophil migration, it has no effect on bacterial control and tissue pathology. In Balb/C mice however, female mice had a comparable number of microabscesses at earlier stages, but as the infection progressed *Cysltr1*$^{+/+}$ mice had increased lesion numbers when compared to *Cysltr1*$^{-/-}$ mice. *Cysltr1*$^{-/-}$ male mice had reduced lesion numbers at earlier timepoint, however, these increased with disease progression (**Figure 2.8G-H**). These results indicate background specific role of CysLTR1 signalling, and in Balb/C background this role might also be influenced by sex differences. In Balb/C female *Cysltr1*$^{-/-}$ liver pathology is comparable to that of the littermate controls, and this correlated with the comparable liver neutrophils at early stages of infection, while the reduced liver pathology correlates with reduced neutrophils in chronic infection. This suggests that liver pathology in these mice might be induced by the recruitment of neutrophils.

Moreover, survival analysis showed that CysLTR1 deletion on C57BL/6 female mice had no effect regardless of sex (**Figure 2.9A-B**). However, C57BL/6 male *Cysltr1*$^{-/-}$ started to succumb earlier and were slightly more susceptible, which may be due to the reduced lymphocytes, required for the adaptive immunity, and subsequent clearing of the bacteria, and consistently increased neutrophils could be detrimental to the host through tissue destruction. In Balb/C female *Cysltr1*$^{-/-}$ mice, despite, the reduced neutrophils and in line with the reduced liver

pathology at chronic infection led to less susceptibility to the infection (**Figure 2.9C**). Balb/C male *Cysltr1*$^{-/-}$ mice were more susceptible (**Figure 2.9D**) likely due to increased pathology at the inflammation resolution stage. It is no surprise to observe sex differences, as there is growing evidential data indicating sex dimorphisms in the biology and pharmacology of eicosanoids (312). Leukotriene biosynthesis is modulated by testosterone, making LTs more prominent in females. For example, female but not male Apc$^{min/+}$ mice (mouse model used to study adenomatous polyposis and colorectal tumors) lacking CysLTR1 had reduced tumors and decreased inflammation in the small intestines (313), suggesting that CysLTR1 is detrimental for the control of tumors and inflammation in females. And here, we demonstrate that in Balb/C females, its absence provides protection to *Lm* infection possibly by reducing liver pathology and neutrophil recruitment.

Overall, we demonstrated that *Lm* infection induced CysLTR1, CysLTR2 and LTC4S expressions in the tissues of mice. The deletion of CysLTR1 in mice does not affect their immune homeostasis at steady state. CysLTR1 deletion has no effect on bacterial control, however, influences cellular recruitment differently between the two backgrounds. We not only demonstrate that sex differences might influence the phenotype observed by the deletion of CysLTR1, but we also report that the role of CysLTR1 signalling in intracellular bacterial infection is also influenced by host genetic background. In conclusion, CysLTR1 modulated lymphoid cell and neutrophil recruitment during *Lm* infection, and its deletion affects disease progression in mice differently based on both gender and genetic background.

2.5. Acknowledgements

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Chapter 3: Manuscript in preparation

The background-specific role of cysteinyl leukotriene type 1 receptor (CysLTR1) during *Mycobacterium tuberculosis* infection in mice

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3.1. Abstract

Tuberculosis (TB) is one of the major public health concerns as it is one of the leading causes of death due to infectious diseases. Host-directed therapies (HDTs) provide a promising avenue for combating this disease. Inflammatory mediators, specifically eicosanoids, have been proposed as potential targets for conjunctive HDT to treat TB. Cysteinyl leukotrienes (CysLTs) are part of the eicosanoid leukotriene family with proinflammatory mediator effects that have been intensively studied in asthma and allergic responses. The role of cysLTs and their receptors has not been fully evaluated during *Mtb* infection. In this study, we investigated the role of cysLT receptor 1 (CysLTR1) during *Mtb* infection in mice with two distinct genetic backgrounds. Using CysLTR1 deficient mice, we demonstrated that the deletion of this receptor does not affect *Mtb* control however, it influences mice survival differently based on their genetic background. In Balb/C mice, deletion of this receptor provided protection against *Mtb* infection by reducing neutrophil recruitment and lung pathology. In C57BL/6 mice, deletion of CysLTR1 does not affect mice survival, however, increased neutrophils in female during *Mtb* infection. In conclusion, CysLTR1 signalling affects host susceptibility and neutrophil activity in a background-specific manner.

3.2. Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), is one of the leading causes of death from infectious diseases globally. According to the World Health Organisation, in 2022 there were 10.6 million TB cases and 1.3 million deaths due to this disease (147). Chest X-rays (314) and bacterial cultures provide less false negatives than other diagnostic tests available for TB detection (314,315). However, for low- to middle-income countries, X-rays can be expensive. Bacterial cultures, on the other hand, take several weeks to obtain the results, but this is still the best test as it can also detect whether the patient has sensitive TB or drug-resistant TB. Once diagnosed, TB can be treated, however, the lengthy treatment period could lead to non-adherence resulting in the development of drug- and multi-drug-resistant TB (316). Therefore, new diagnostic tools, more efficient and shorter treatment regimens are required for the treatment and eradication of this disease.

In recent years, host-directed therapy (HDT) has been identified as a promising approach in the treatment of *Mtb*. HDT repurposes already available FDA-approved drugs to kill *Mtb* and improve tissue destruction by reducing inflammation by targeting the host immune system (317). For example, eicosanoids, a family of enzymatically generated metabolites of arachidonic acid (AA), are a promising target for HDT for the treatment of *Mtb* (318,319). The eicosanoid family is made up of multiple subfamilies that are subdivided into three distinct families based on the enzymes responsible for their generation from the AA. These enzymes include cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP). Of interest to the current study are the eicosanoids generated by LOXs, particularly the leukotriene family. Leukotrienes are a family of lipid mediators that include leukotrienes (LTB4) and cysteinyl leukotrienes (cysLTs; LTC_4, LTD_4 and LTE_4) synthesized through the metabolism of the AA by 5-lipooxygenase (5-LO) (8,9,320).

During *Mtb* infection, 5-LO deficiency in mice was associated with better disease outcomes whilst LTB_4 increases the susceptibility to the infection, suggesting that LTB_4 plays a role in *Mtb* immunopathogenesis (318). Various other studies have linked LTB_4 inhibition with poor disease outcomes during *Mtb* infection (244–246). However, these studies used 5-LO-deficient mice or directly inhibited this pathway using 5-LO antagonists, resulting in disruption of LTB_4 and cysLTs signalling. CysLTs signal through three G protein-coupled receptors (CysLTR1, CysLTR2, and CysLTR3) (26,27). CysLTs are proinflammatory lipid mediators that have pathobiological functions in asthma and of the three cysLT receptors, CysLTR1 has been well elucidated in the etiology of airway inflammation and asthma (53–56,60,263). Subsequently, multiple antagonists for CysLTR1 have been developed and are being clinically used for the treatment of asthma and allergic reactions (62).

CysLTR1 antagonist, pranlukast (PRK), has been shown to directly inhibit *Mtb* growth by specifically inhibiting the arginine biosynthesis of the bacterium. The treatment of mice with this inhibitor also improved disease outcomes due to a reduction in bacterial burden and improved tissue pathology *in vivo*. However, PRK also inhibited the 5-LO and COX pathways (252), suggesting this is not specific to CysLTR1. Treatment with PRK does not provide evidence for the specific role of CysLTR1 signalling during *Mtb* infection since it has anti-*Mtb* effects and inhibits pathways upstream of CysLTR1.

In the present study, we elucidated the specific role of CysLTR1 during *Mtb* infection by using CysLTR1 deficient (*Cysltr1*$^{-/-}$) mice. We demonstrated that the deletion of CysLTR1 in mice affected survival differently depending on their genetic background during *Mtb* infection, with Balb/C mice being more protected from the infection. While in C57BL/6 mice, deletion of the receptor did not affect their survival. We showed that CysLTR1 signalling affects CD4 T cell activation in a similar manner in both Balb/C and C57BL/6 mice, while neutrophil recruitment and activation is genetic background- and partly sex-specific in Balb/C mice. The observed

protection in Balb/C mice deficient in CysLTR1 was attributed to reduced lung pathology and decreased neutrophil infiltration. Together, our results demonstrated that CysLTR1 is a potential target for HDT against *Mtb*. However, the host background and gender may significantly influence its efficacy.

3.3. Methods and Materials

3.3.1. Ethics

All animal experimental procedures conducted in this study were in strict accordance with the South African National Standard (SANS 10386:2008) and the Animal Research Ethics Committee (Protocol number: AREC 022/024) of the Faculty of Health Sciences, University of Cape Town.

3.3.2. Mouse strains

CysLTR1 deficient mice (*Cysltr1*$^{-/-}$) were generated by breeding heterozygous (*Cysltr1*$^{-/+}$) animals on Balb/C and C57BL/6 backgrounds. *Cysltr1*$^{-/+}$ mice on both backgrounds were gifted by Dr Frank Austen of the Department of Medicine, Harvard Medical School (268). The *Cysltr1*$^{-/+}$ mice were backcrossed to generate *Cysltr1*$^{-/-}$ and littermate control, *Cysltr1*$^{+/+}$ mice at the University of Cape Town (UCT) Animal Research Facility, Faculty of Health Sciences. All experiments were conducted on mice that were aged 8-12 weeks and sex matched.

3.3.3. Infection with *Mtb*

Mice were anaesthetized and then intranasally infected with *Mtb* HN878 through administration of 25µL/nostril. One day after infection, mice were euthanized, lungs collected, homogenized, and plated for colony forming units (CFUs) to determine the infection dose.

3.3.4. Organ bacterial loads

At the indicated time points, mice were euthanized to collect the right lower lungs and spleens to homogenize. The homogenates were serial diluted (10-fold dilutions) and plated on Middlebrook 7H11 agar plates that were supplemented with OADC and glycerol. Plates were then incubated at 37ºC for 2-3 weeks and CFUs counted to determine organ bacterial burden.

3.3.5. Lung single-cell suspensions

At each time point, the left lung lobes of each mouse were collected, chopped with a scalpel and incubated in digestion buffer (DMEM, DNase 1 and collagenase type 1) for an hour at 37°C. To obtain a single-cell suspension, the digested lungs are passed through 100um and 70um cell strainers in tandem followed by the red cell lysis buffer (150mM NaCl, 10mM $KHCO_3$ and 0.1mM Na_2-EDTA). The cells are then washed with complete media (DMEM + 10% FCS) and resuspended at $10x10^6$/ml.

3.3.6. Immune cell populations

To determine immune cell populations, lung single cells ($1x10^6$ cells) were stained for lymphoid (Viability dye Bv605, CD4 Bv421, CD8 Bv510, CD44 PE, NK1.1 APC-Cy7, CD3 A700, CD62L APC, CD19 PercP-Cy5.5, all from BD Bioscience) and myeloid (Viability dye Bv605, Ly6G FITC, Ly6C PerCP-Cy5.5, SiglecF APC-Cy7, CD11b Bv421, CD11C APC, CD103 PE, CD64 PE-Cy7, MHCII A700, MerTK Bv786, all from BD Bioscience) specific surface markers for flow cytometry analysis. Stained fixed cells were acquired using the BD LSR Fortessa (BD Biosciences Immunocytochemistry Systems) flow cytometer and the data was analysed using FlowJo v10.8.1_CL software. The gating strategy used to identify various immune cells is in *Appendix A* and *D*.

3.3.7. Cytokine and chemokine profile in homogenates

Organ cytokines and chemokines were measured using the standard sandwich ELISA protocol. Streptavidin-HRP conjugated antibodies were detected using the TMB Microwell Peroxidase substrate (KPL internation), while streptavidin-AP conjugated antibodies were detected using p-nitrophenyl phosphate disodium salt hexahydrate (Sigma). Measured cytokines and chemokines included IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-23, IFN-y, TGF-β, CCL3, CCL5, CXCL1 and CXCL2 (coating, standard and detection antibodies purchased from BD

Biosciences, BioLegend and R&D Scientific). The optical density for each ELISA plate was measured using the VersaMax™ microplate spectrophotometer (Molecular Devices, Sunnyvale, California).

3.3.8. Histopathology

The second right lung lobes of *Mtb*-infected mice were collected in neutral buffered formalin (3.8-4% formaldehyde, 4 g/L NaH_2PO_4, 6.5 g/L Na_2HPO_4, pH 7.0) for fixation. The fixed lobes were then embedded in wax, cut into 3 sections of 30-35µm apart and stained with haematoxylin and eosin (H&E) to determine any histopathological differences. Slides were scanned at 40x magnification using the Virtual Slide VS120 microscope (Olympus, Japan) and quantification was performed using Qu Path version 0.3.2.

3.3.9. Neutrophil sorting

Single cells from infected lungs ($10x10^6$ cells) were either stained with viability dye Bv605 and an antibody mix of CD11b Bv421 and Ly6G FITC to isolate neutrophils. The stained cells were then sorted using the BD Flow Cytometer sorter for $CD11b^+$ $Ly6G^+$ (neutrophils) cells from the live cell population. Neutrophils were also isolated using the Anti-Ly6G MicroBeads UltraPure, mouse (Miltenyi Biotec) following the manufacturer's instructions. The purity of the neutrophils was determined by flow cytometry. Isolated neutrophils were spun at 400xg for 5 minutes and pellets were resuspended in Buffer RLT for RNA isolation.

3.3.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA collected from sorted neutrophils was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To obtain cDNA, normalized RNA was then reverse transcribed using the Transcriptor First Strand cDNA synthesis kit from Roche following the manufacturer's instructions. qPCR for various gene transcripts was performed as

previously described by Jones et al (18). The primers used for each targeted gene transcript are listed in *Appendix E*.

3.3.11. Statistical analysis

All data presented were analysed using GraphPad Prism 10.0 software, employing the Student's t-test. Statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3.4. Results

3.4.1. Mycobacterial burden is not affected by the deletion of CysLTR1 in mice during *Mycobacterium tuberculosis* infection.

To investigate the role of CysLTR1 in Mtb infection, C57BL/6 *Cysltr1*$^{-/-}$ and Balb/C *Cysltr1*$^{-/-}$ mice were infected with HN878 *Mtb* and sacrificed at 3- and 12-wpi for Balb/C mice and C57BL/6 female mice, and 6- and 12-wpi for C57BL/6 males. At the indicated time points, bacterial burden in lung and spleen was comparable between the knockout and control male or female mice (**Figure 3.1A-B and 3.2A-B**). C57BL/6 female *Cysltr1*$^{-/-}$ mice had significantly increased lung and spleen weights at 3-wpi, while there were no significant differences between the groups at 12-wpi (**Figure 3.1C**). C57BL/6 male *Cysltr1*$^{-/-}$ lungs and spleens had comparable weights at both time points (**Figure 3.1D**). Balb/C female *Cysltr1*$^{-/-}$ mice lungs were significantly smaller than control mice at 3-wpi, while, together with the spleens, they were comparable at 12-wpi (**Figure 3.2C**). Balb/C male *Cysltr1*$^{-/-}$ mice had reduced spleen weights at 3-wpi, however, the lungs and spleens were comparable to controls at 12-wpi (**Figure 3.2D**). At both 3- and 12-wpi, C57BL/6 female *Cysltr1*$^{-/-}$ lungs had increased cell numbers (**Figure 3.1E**), while male mice had comparable lung cell numbers (**Figure 3.1F**). Balb/C female *Cysltr1*$^{-/-}$ lung cell numbers were reduced at 3-wpi but not at 12-wpi and male lungs at 3- and 12-wpi had similar cell numbers (**Figure 3.2E-F**). Histopathology analysis showed that *Mtb* infection in C57BL/6 female mice had a comparable effect on lung pathology at both time points, although the *Cysltr1*$^{-/-}$ lungs had slightly increased, but not significant, alveolar air space than the controls at 12-wpi (**Figure 3.1G**). C57BL/6 *Cysltr1*$^{-/-}$ male lungs had increased alveolar air space at 6-wpi but were comparable at 12-wpi (**Figure 3.1H**). Balb/C *Cysltr1*$^{-/-}$ female mice had reduced lung alveolar spaces at 3-wpi and had no effect at 12-wpi (**Figure 3.2G**). Male Balb/C *Cysltr1*$^{-/-}$ mice, on the other hand, had consistently increased free alveolar spaces in the lungs at both 3- and 12-wpi (**Figure 3.2H**). Taken together, CysLTR1 deletion

does not affect bacterial loads, however, this receptor influences lung cell recruitment in female mice and lung pathology outcomes in Balb/C mice.

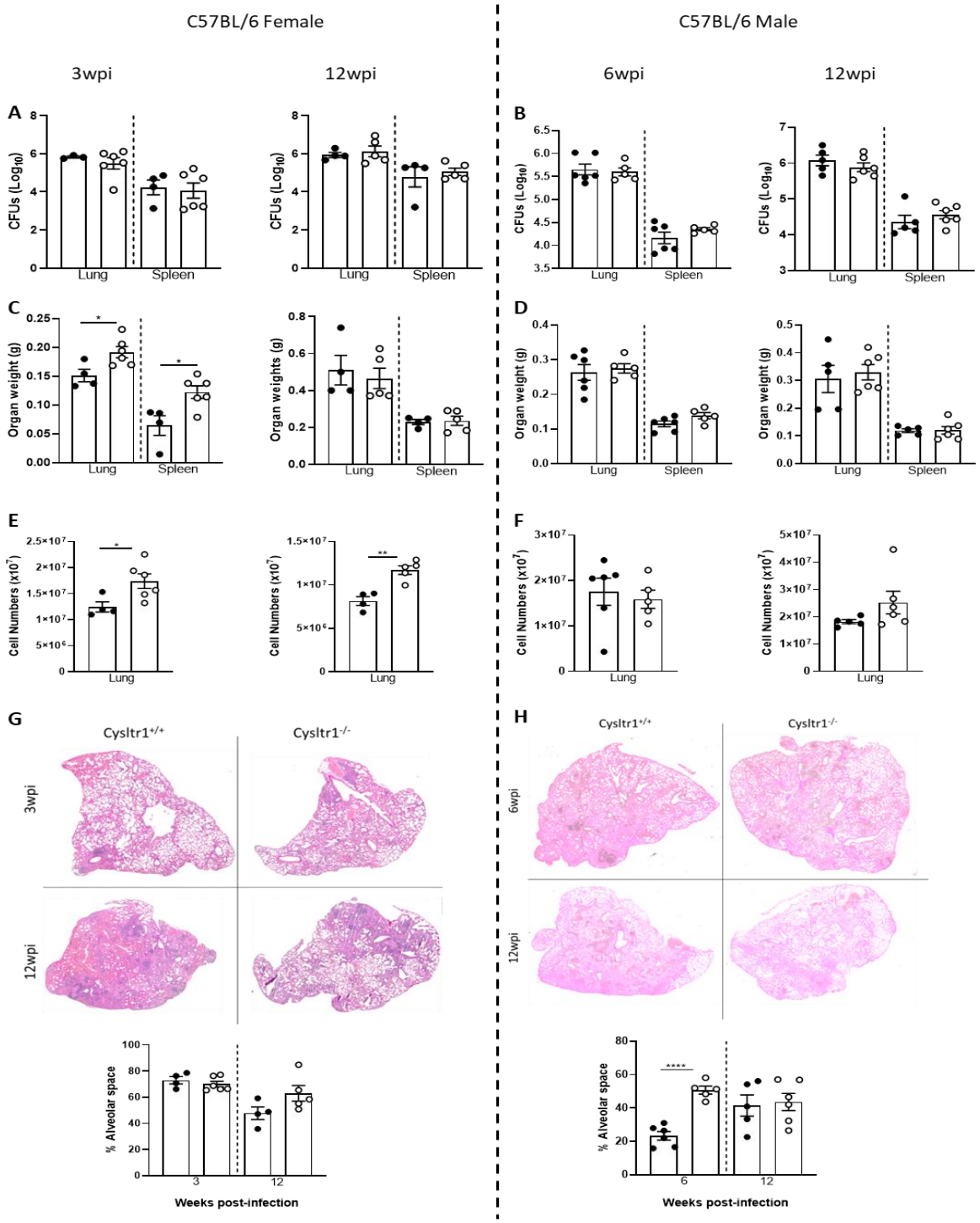

Figure 3.1: CysLTR1 deletion in C57BL/6 mice does not affect bacterial burden and pathology in *Mycobacterium tuberculosis* infection. C57BL/6 littermate controls (*Cysltr1*$^{+/+}$) and knockout (*Cysltr1*$^{-/-}$) mice were intranasally infected with about 100CFUs HN878 Mtb strain and sacrificed at 3- and 12- weeks post-infection (wpi) for females, and at 6- and 12-wpi for males. At each time point, **A-B)** the bacterial load in the lungs and spleen, **C-D)** organ weights, and **E-F)** lung cell numbers were measured. **G-H)** Lung H&E staining for immunohistology analysis. Lung free alveolar airspace percentage quantification was performed using Qu Path version 0.3.2. Female experiments were performed twice, the 6wpi male was performed once and the 12wpi data represents two independent experiments. Error bars denote mean ± SEM of n= 4-6 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, ****p<0.00001).

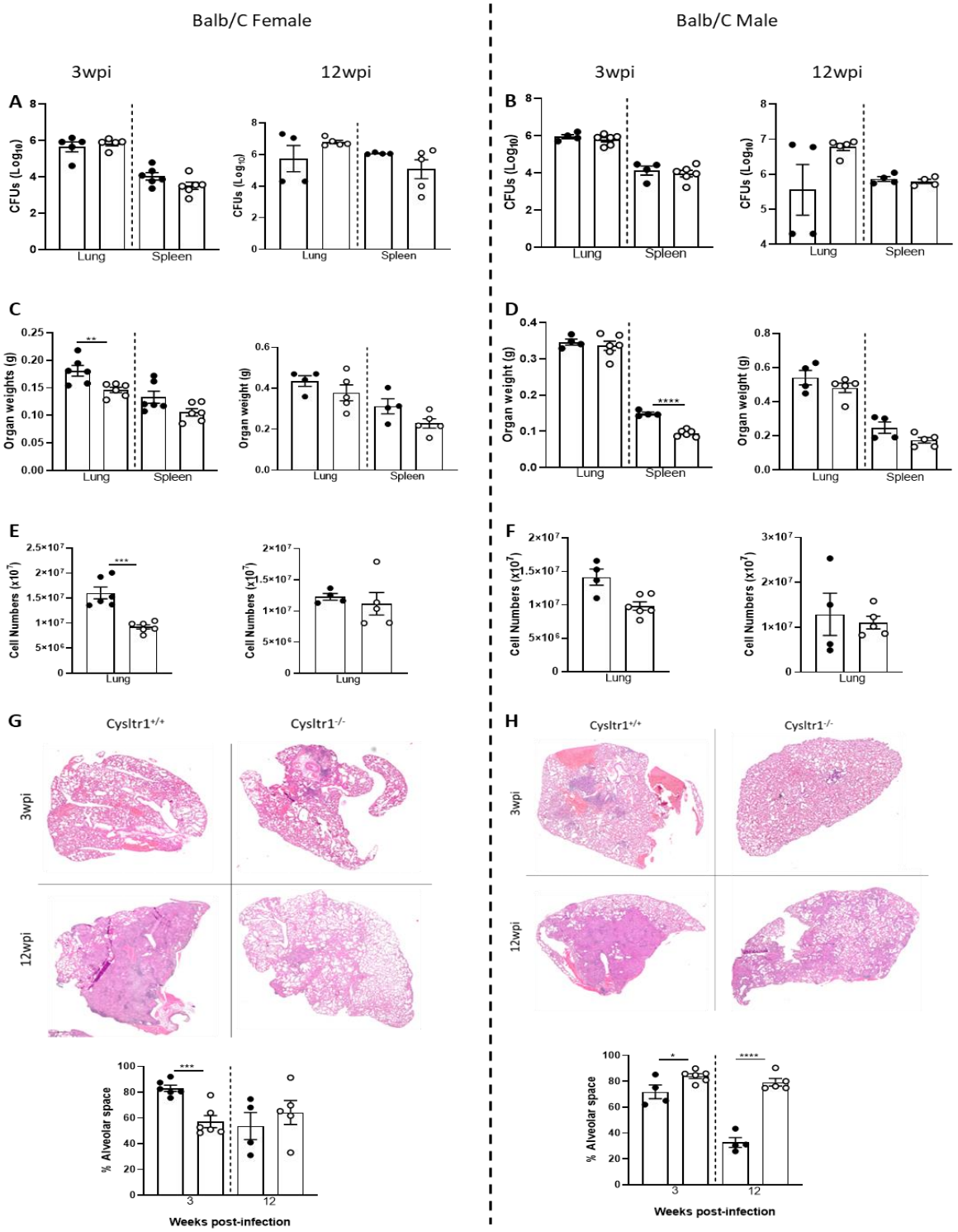

Figure 3.2: CysLTR1 deficiency improved lung pathology in Balb/C mice. Balb/C *Cysltr1*$^{+/+}$ and *Cysltr1*$^{-/-}$ mice (n= 4-6 mice/group) were infected with about 100CFUs HN878 intranasally for 3 and 12 weeks. At 3- and 12-wpi mice were sacrificed and measured their **A-B**) organ bacterial burden, **C-D**) organ weights, and **E-F)** lung cell numbers. **G-H)** Lung H&E staining for immunohistology analysis. Lung free alveolar airspace percentage quantification was performed using Qu Path version 0.3.2. Female data is a representation of two independent experiments and the male experiments were performed once. Error bars denote mean ± SEM of n= 4-6 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.00001).

3.4.2. CysLTR1 deletion influences neutrophil recruitment to the site of infection.

We investigated how the deletion of CysLTR1 influences immune responses during *Mtb* infection. We measured lung immune populations by flow cytometry using the gating strategies in *Appendix A* and *D* to identify the different immune cell types. Both C57BL/6 female and male *Cysltr1*$^{-/-}$ mice had comparable lymphoid cell populations, except for significantly reduced NK cells in male *Cysltr1*$^{-/-}$ at 12-wpi (**Figure 3.3A-B**). While there were no differences in CD4 and CD8 T cells, we measured their activation by expression of CD62L and CD44. At 3-wpi, female C57BL/6 *Cysltr1*$^{-/-}$ lungs had significantly reduced naïve cells and increased effector/effector (eff/eff) memory CD4 T cells, while at 12-wpi *Cysltr1*$^{-/-}$ CD4 naïve cells were significantly reduced, and the memory cells were comparable to controls (**Figure 3.3C**). C57BL/6 male mice, on the other hand, had similar CD4 T cell subsets at 6-wpi, however, at 12-wpi male *Cysltr1*$^{-/-}$ lungs had increased naïve CD4 T cells (**Figure 3.3D**). *Cysltr1*$^{-/-}$ female lung CD8 T cells had increased eff/eff memory cells at 3-wpi, but no differences were observed in other subsets at 3- and 12-wpi (**Figure 3.3E).** Male *Cysltr1*$^{-/-}$ lungs, had reduced CD8 naïve T cells at 6-wpi, increased naïve CD8 and reduced eff/eff memory CD8 T cells at 12-wpi (**Figure 3.3F**). On the other hand, Balb/C *Cysltr1*$^{-/-}$ lungs had comparable lymphoid cells in *Mtb* infection at both time points (**Figure 3.4A-B**). Balb/C female *Cysltr1*$^{-/-}$ CD4 and CD8 T cells had significantly reduced eff/eff memory cells at 3-wpi, while *Cysltr1*$^{-/-}$ CD4 naïve T cells were increased at 12-wpi (**Figure 3.4C and 3.4E**). Balb/C male *Cysltr1*$^{-/-}$ mice had increased central memory and reduced eff/eff memory CD4 T cells at 3-wpi, and increased CD4 naïve T cells at 12-wpi, while CD8 T cell subsets were comparable at both time points (**Figure 3.4D and 3.4F**). Interestingly, in lung myeloid cell populations, most of these cells were comparable between C57BL/6 *Cysltr1*$^{-/-}$ and control mice, for both female and male mice at each time point (**Figure 3.3G-H**), except C57BL/6 female *Cysltr1*$^{-/-}$ mice had increased neutrophils at both 3- and 12-wpi (**Figure 3.3G**). At both time points, Balb/C *Cysltr1*$^{-/-}$ female mice had comparable

myeloid cell populations except reduced CD103 DCs at 3-wpi and reduced neutrophils at 12-wpi (**Figure 3.4G**). Balb/C male *Cysltr1*$^{-/-}$ mice also had comparable myeloid cells, except for reduced eosinophils and increased monocyte-derived DCs at 3- and 12-wpi respectively. Male *Cysltr1*$^{-/-}$ mice, however, had slightly increased neutrophils at 3-wpi and not significant at 12-wpi (**Figure 3.4H**). The deletion of CysLTR1 does not have much effect on lung lymphoid cells, however, influences cellular infiltration and neutrophil recruitment during *Mtb* infection.

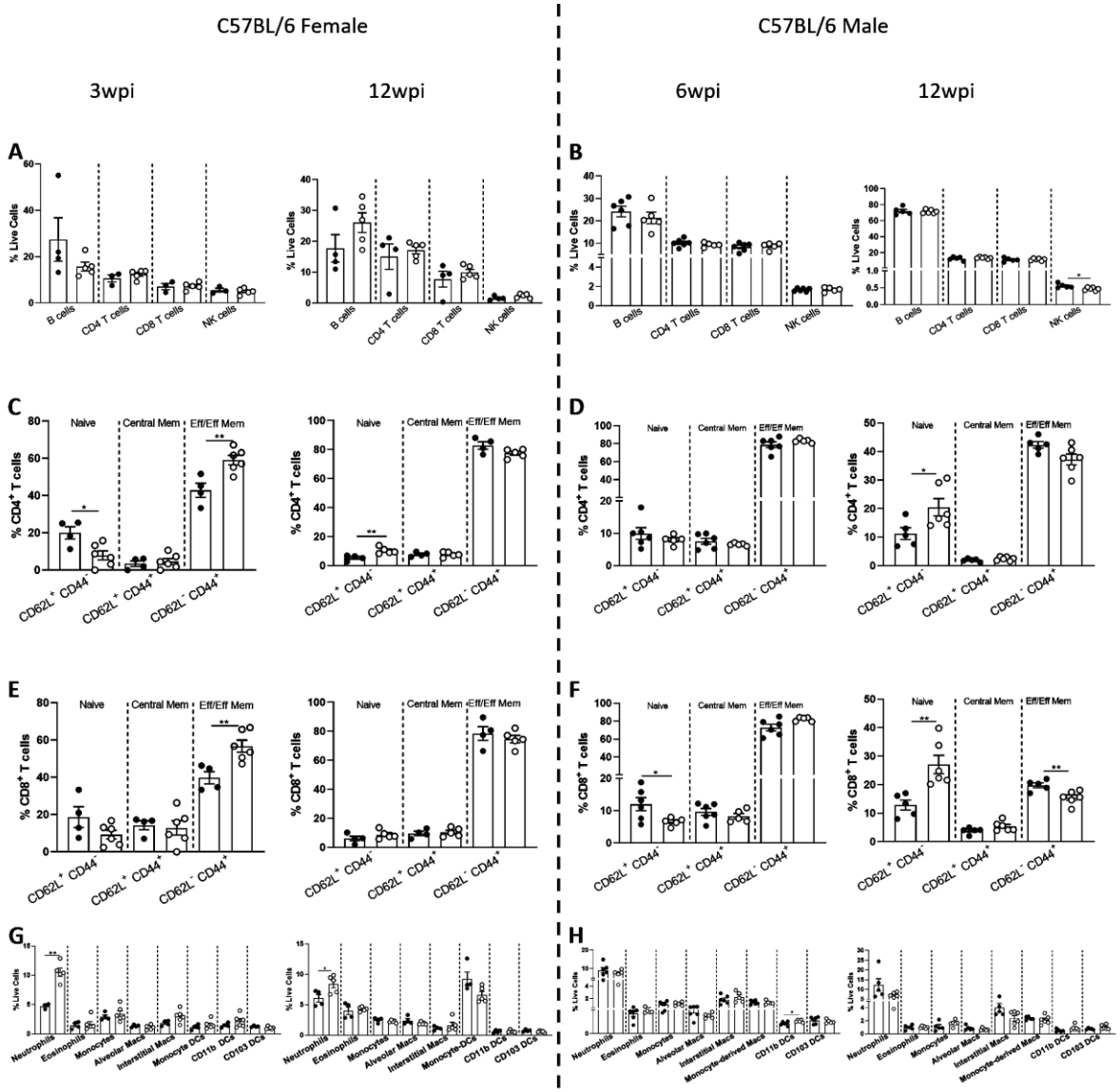

Figure 3.3: CysLTR1 deletion increased neutrophil recruitment in C57BL/6 female mice. C57BL/6 mice were intranasally infected with 100CFUs HN878 Mtb and sacrificed at 3- and 12-wpi for females and at 6- and 12-wpi for males. At each time point lung single cells were stained and analysed by flow cytometry to determine **A-B)** lung lymphoid cell populations, **C-D)** CD4 T cell subsets, **E-F)** CD8 T cell subsets, and **G-H)** myeloid cell populations. Female experiments were performed twice, the 6wpi male experiment was performed once and the 12wpi data represents two independent experiments. Error bars denote mean ± SEM of n= 4-6 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, **p<0.01).

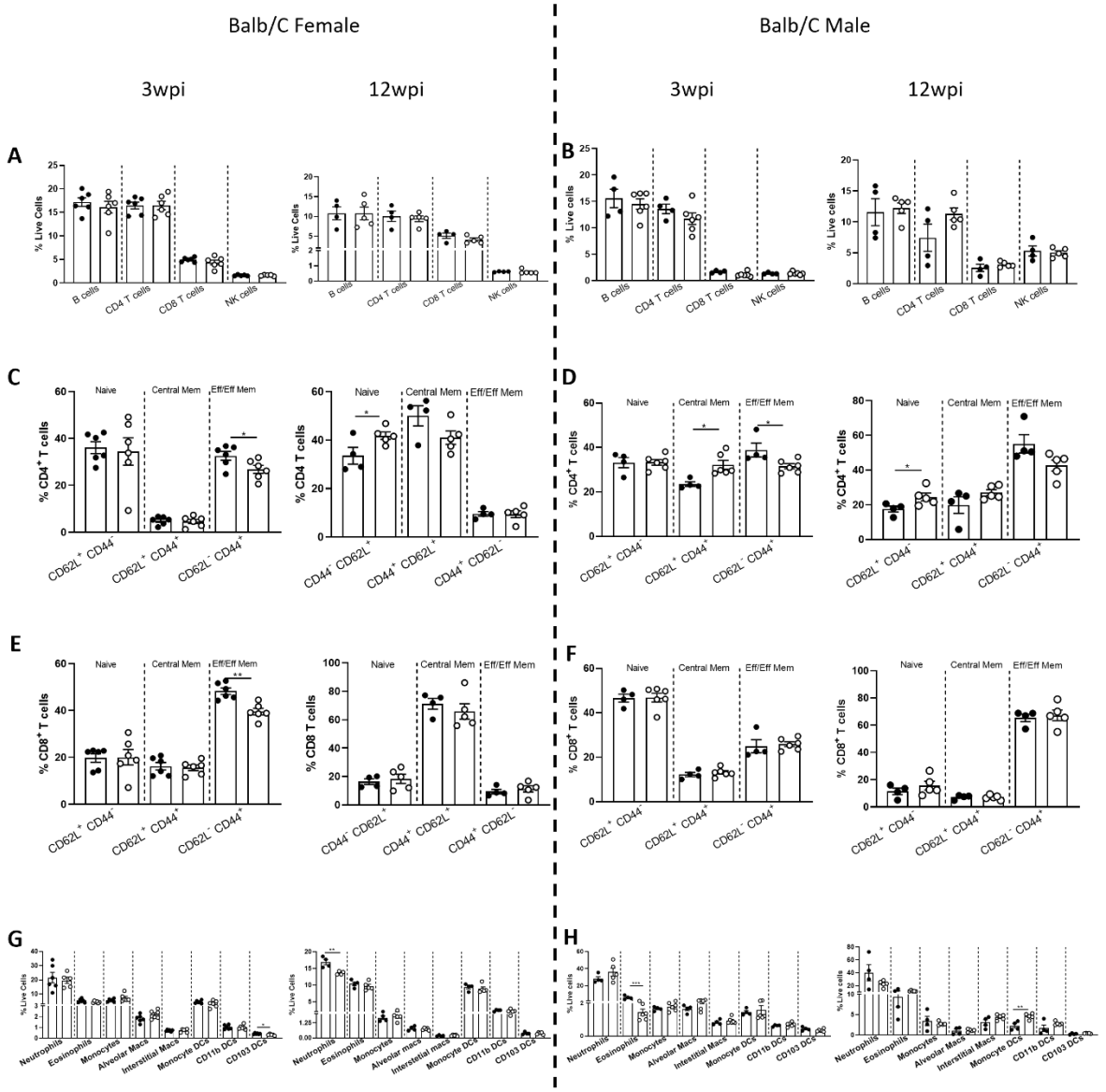

Figure 3.4: CysLTR1 deletion reduces lung neutrophils at later stages of *Mtb* infection in Balb/C mice. Balb/C mice were intranasally infected with 100CFUs HN878 Mtb and sacrificed at 3- and 12-wpi and lung **A-B)** lymphoid cells, **C-D)** CD4 T cell subsets, **E-F)** CD8 T cell subsets, and **G-H)** myeloid cell populations were analysed by flow cytometry from single-cell suspensions. Female data is a representation of two independent experiments and the male experiments were performed once. Error bars denote mean ± SEM of n= 4-6 mice/group and the data was analysed using the unpaired student T-test, (*p<0.05, **p<0.01, ***p<0.001).

3.4.3. CysLTR1 deletion resulted in differential cytokine responses in a sex- and genetic background-dependent manner in *Mtb* infection.

We then determined cytokines and chemokines in the lung homogenates by ELISA. C57BL/6 female *Cysltr1*$^{-/-}$ mice had reduced IL-1α, IL-6, IL-10 and IL-12p70 at 3-wpi, and increased TGF-β secretion at 12-wpi (**Figure 3.5A**). There were no differences in C57BL/6 male *Cysltr1*$^{-}$

$^{-/-}$ mice at 6-wpi (**Figure 3.5B**), and both C57BL/6 female and male mice had comparable chemokine secretion at any time point (**Figure 3.5C-D**). Deletion of this receptor appears to influence cytokine secretion early after *Mtb* infection in C57BL/6 female mice. Given this and that C57BL/6 male lungs had comparable cytokine and chemokine secretion at 6-wpi (early chronic infection), it is feasible that there will be no major differences at 12-wpi. Balb/C females *Cysltr1*$^{-/-}$ mice showed comparable levels of cytokines and chemokines at both time points (**Figure 3.5E and 3.5G**). Balb/C male *Cysltr1*$^{-/-}$ mice showed increased IL-1α, IL-6, IL-10, IL-12p70, IL-23, TGF-β and CXCL2 secretion at 3-wpi and had no differences at 12-wpi (**Figure 3.5F and 3.5H**). These results indicate that the deletion of CysLTR1 in Balb/C male mice increased pro-inflammatory cytokine production in response to *Mtb* infection. Deletion of CysLTR1 influences cytokine secretion early after infection in a sex- and gender-specific manner in TB.

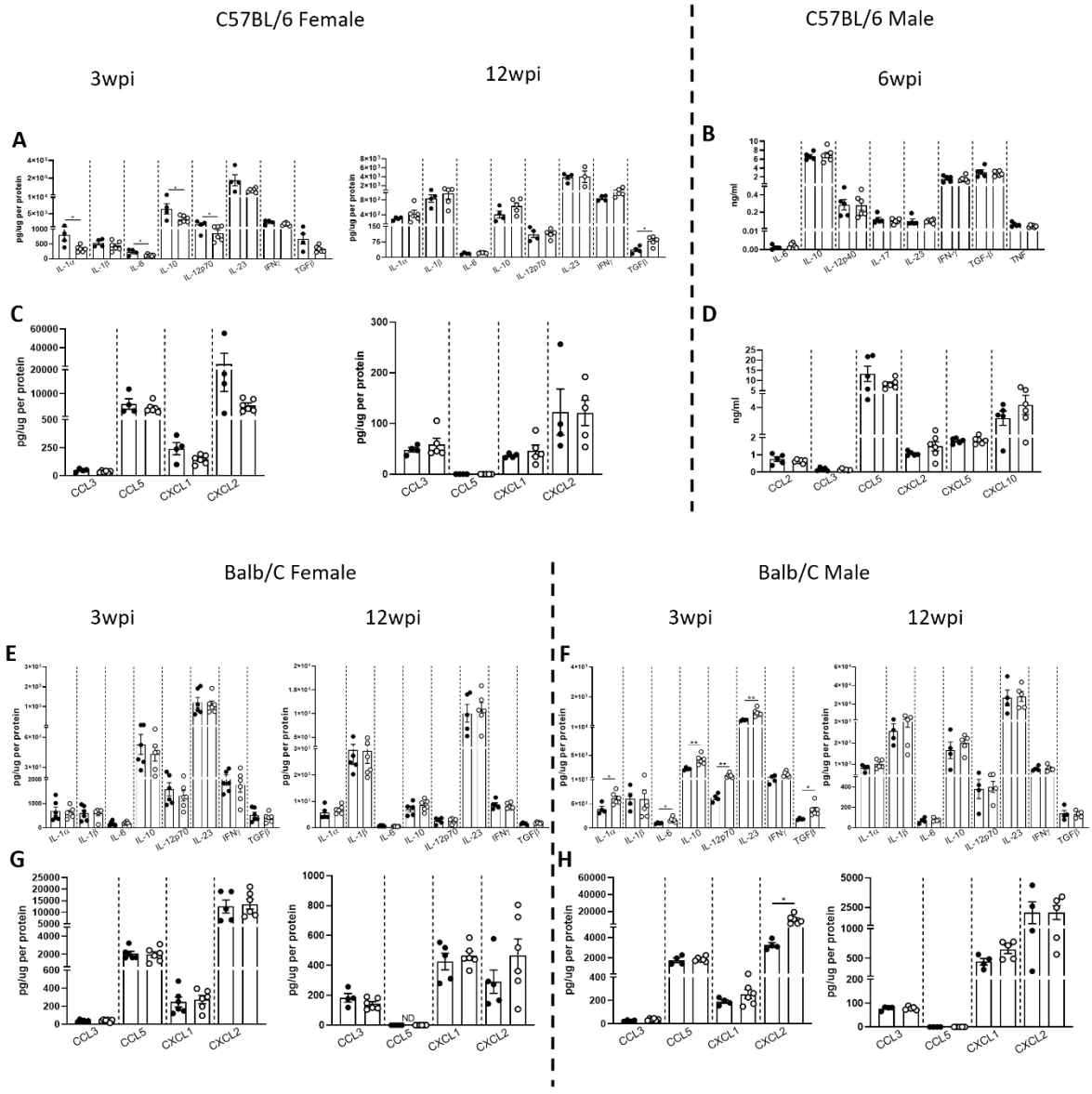

Figure 3.5: CysLTR1 deletion influence early proinflammatory cytokines in the lungs differently depending on mice background and gender. C57BL/6 and Balb/C *Cysltr1*$^{+/+}$ and *Cysltr1*$^{-/-}$ mice were intranasally infected with 100CFUs HN878 *Mtb* strain and sacrificed at 3-, 6- (C57BL/6 male mice) and 12-wpi. **A-B and E-F)** Cytokines, and **C-D and G-H)** chemokines were measured in lung homogenates by ELISA. Experiments on female mice were performed twice, the 6wpi C57BL/6 male experiment and Balb/C male experiments were performed once. Error bars denote mean ± SEM of n= 4-6 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, **p<0.01).

3.4.4. Neutrophils showed reduced myeloperoxidase (*Mpo*) expression in CysLTR1-deficient Balb/C mice.

We have demonstrated that the deletion of CysLTR1 influence neutrophil recruitment and lung pathology based on gender and genetic background. Given the observed differences in neutrophils, we sorted neutrophils from the lungs of *Mtb*-infected mice at 3- and 12-wpi to understand their potential role in disease outcome. We sorted lung neutrophils by using anti-Ly6G microbeads and confirmed purity by flow cytometry (**Figure 3.6A**). In male C57BL/6 mice, CysLTR1 deletion had no effect on neutrophil recruitment post-*Mtb* infection, suggesting that neither receptor nor neutrophils significantly impact disease outcome. Consequently, C57BL/6 male lung neutrophils were not isolated for further analysis. We then performed quantitative real-time PCR to measure the mRNA expression of various genes. Firstly, we measured the expression of myeloperoxidase (MPO), a member of the heme peroxidase-cyclooxygenase superfamily that plays a role in neutrophil trafficking and activation (321). Neutrophil *Mpo* expression was comparable at 3-wpi, however, significantly reduced at 12-wpi in Balb/C *Cysltr1*$^{-/-}$ females (**Figure 3.6B**). In contrast to female, Balb/C *Cysltr1*$^{-/-}$ males, *Mpo* expression was significantly reduced in neutrophils at both time points (**Figure 3.6B**). In C57BL/6 female *Cysltr1*$^{-/-}$ mice the *Mpo* expression was reduced at 3-wpi and had no effect at 12-wpi (**Figure 3.6B**). Both nitric oxide synthase-2 (NOS2) and arginase-1 (Arg1) are known to play a crucial role in *Mtb* pathogenesis (322). Balb/C *Cysltr1*$^{-/-}$ female neutrophils displayed comparable *Nos2* and *Arg1* expression at 3-wpi, while significantly reduced *Nos2* and *Arg1* expression at 12-wpi (**Figure 3.6C-D**). Balb/C *Cysltr1*$^{-/-}$ male neutrophils showed no difference in *Nos2* and *Arg1* expression at 3-wpi whilst significantly increased Nos2 and Arg1 reduced at 12-wpi (**Figure 3.6C-D**). In C57BL/6 *Cysltr1*$^{-/-}$ female neutrophils had increased Nos2 expression at 3-wpi and decreased at 12-wpi with no change in Arg1 expression (**Figure 3.6C-D**). Nicotinamide dinucleotide phosphate (NADPH) oxidase 2 (NOX2), also known as

cytochrome b-245 heavy beta chain (CYBB), is responsible for neutrophil extracellular traps (NETs) formation and reactive oxygens species formation, contribute to better control of *Mtb* infection by neutrophils (323). In Balb/C female *Cysltr1*$^{-/-}$ neutrophils, the - *Cybb* expression was comparable at both time points. However, Balb/C *Cysltr1*$^{-/-}$ male neutrophils showed upregulated *Cybb2* expression at 3-wpi, but not at 12-wpi. C57BL/6 female *Cysltr1*$^{-/-}$ mice showed that *Cybb* expression had no effect at 3-wpi, whilst reduced *Cybb* expression in neutrophils at 12-wpi (**Figure 3.6E**).

We also measured the expression of chemokines (Cxcl2, CCL5 and CCL2) required for the recruitment of neutrophils. Neutrophil *Cxcl2* expression in Balb/C mice and C57BL/6 females were comparable at both time points (**Figure 3.7A**). Balb/C *Cysltr1*$^{-/-}$ female neutrophils had reduced *Ccl5* expression at 3-wpi whereas males had no difference at both time points. C57BL/6 *Cysltr1*$^{-/-}$ female had reduced expression of Ccl5 at 12-wpi (**Figure 3.7B**). Balb/C *Cysltr1*$^{-/-}$ female neutrophils showed reduced expression of *Ccl2* at both time points, while the expression remained unchanged in Balb/C male. In C57BL/6 *Cysltr1*$^{-/-}$ neutrophils had no change at 3-wpi, however, significantly lower expression of *Ccl2* at 12-wpi (**Figure 3.7C**). Tumour necrosis factor alpha (TNF-α) and interleukin 1 alpha (IL-1α) are pro-inflammatory cytokines that are essential in the control of *Mtb* (324). Neutrophil *Tnf-α* expression was comparable in Balb/C *Cysltr1*$^{-/-}$ females (**Figure 3.7D**). In Balb/C *Cysltr1*$^{-/-}$ male neutrophils had significantly increased *Tnf-α* induction at 12-wpi (**Figure 3.7D**). The expression of *Il1α* was not affected in either females or males in Balb/C mice (**Figure 3.7E**). In C57BL/6 female *Cysltr1*$^{-/-}$ neutrophils had increased expression of *Il1α* at 3-wpi, but not at 12-wpi (**Figure 3.7E).** Taken together, the reduced neutrophils in Balb/C mice correlate with reduced *Mpo* expression and subsequently decreased lung pathology in the absence of CysLTR1 in *Mtb* infection.

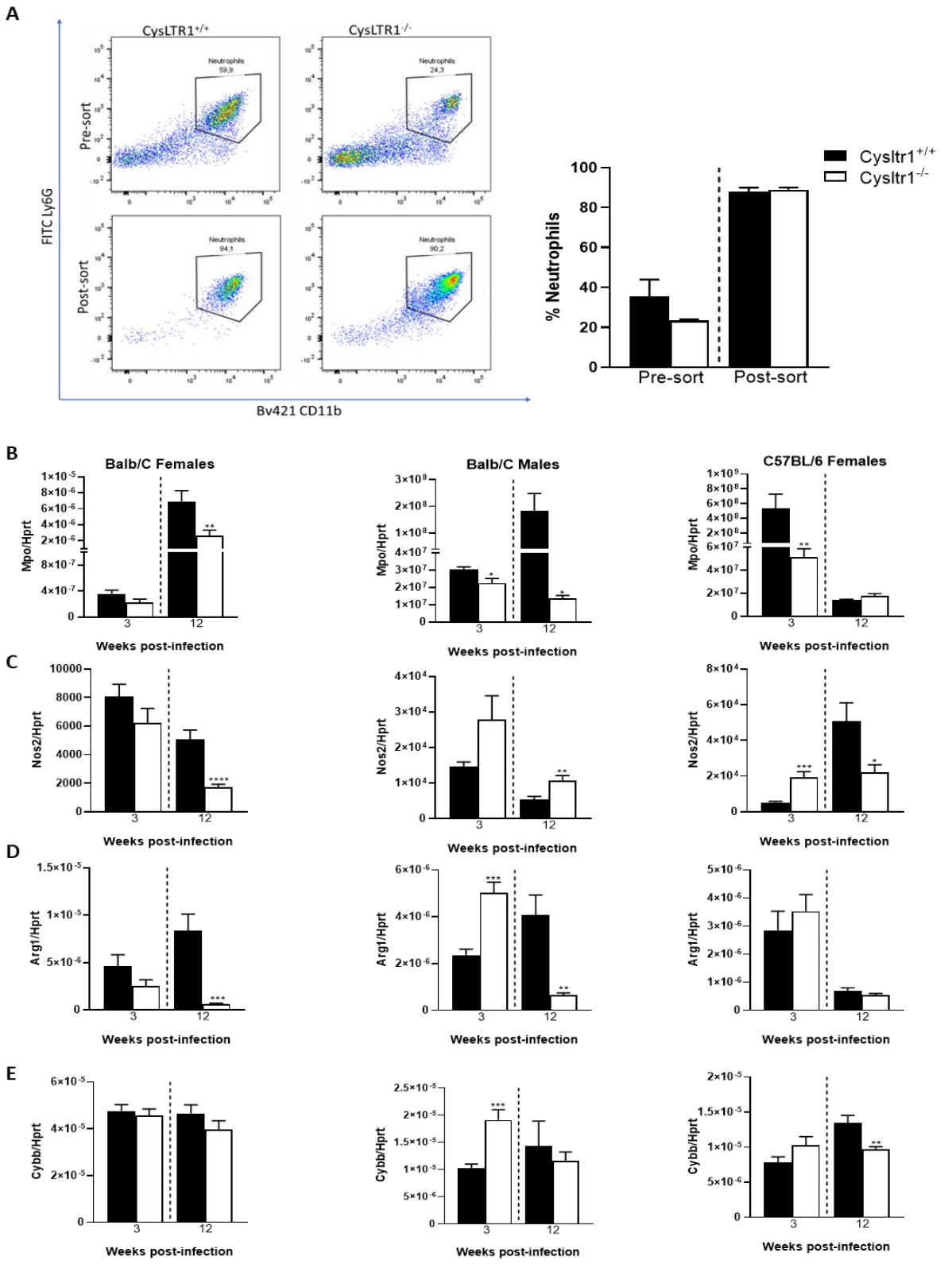

Figure 3.6: CysLTR1-deficient neutrophils reduced myeloperoxidase expression during *Mycobacterium tuberculosis* infection. Balb/C mice and C57BL/6 female mice were intranasally infected with 100CFUs HN878 Mtb and sacrificed at 3- and 12-wpi. Lung neutrophils were sorted by FACS sort or using anti-Ly6G microbeads and **A)** purity was confirmed by flow cytometry. **B)** *Mpo*, **C)** *Nos2*, **D)** *Arg1*, and **E)** *Cybb* mRNA expression in neutrophils was measured by qPCR. Error bars denote mean ± SEM of n= 3-4 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.00001).

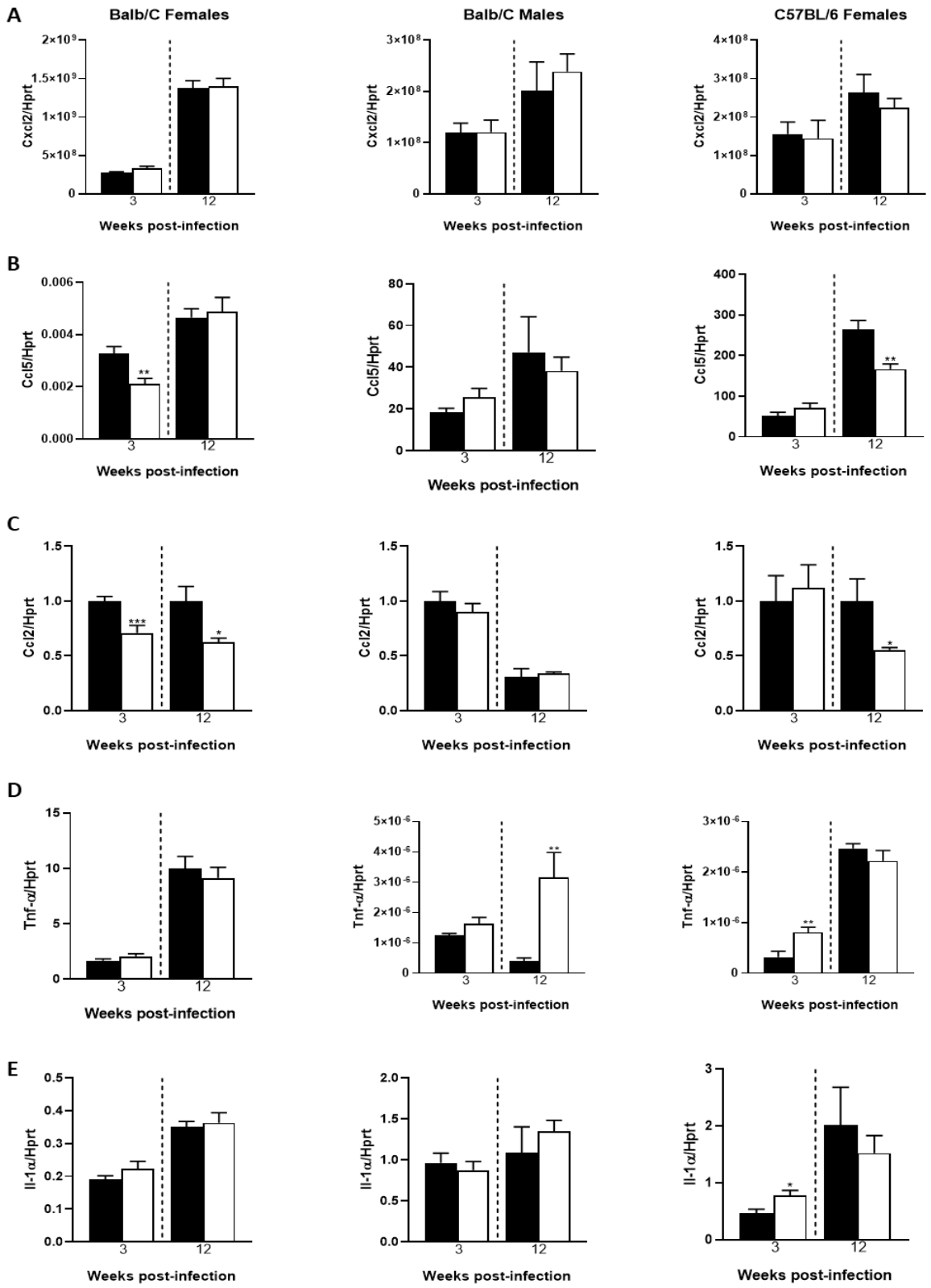

Figure 3.7: Balb/C female neutrophils deficient in CysLTR1 had reduced CCL2 expression during *Mycobacterium tuberculosis* infection. Balb/C mice and C57BL/6 female mice were intranasally infected with 100CFUs HN878 Mtb and sacrificed at 3- and 12-wpi. Lung neutrophils were sorted and **A)** *Cxcl2*, **B)** Ccl5, **C)** *Ccl2*, **D)** *Tnf-α* and **E)** *Il-1α* mRNA expression was measured by qPCR. Error bars denote mean ± SEM of n= 3-4 mice/group and the data was analysed using the unpaired student t-test, (*p<0.05, **p<0.01, ***p<0.001).

3.4.5. Deletion of CysLTR1 is protective during *Mycobacterium tuberculosis* infection in Balb/C mice.

We have demonstrated that deletion of CysLTR1 in mice has no effect on bacterial burden, however, affects cellular and neutrophil recruitment to the lungs, and improves lung pathology in Balb/C mice. We sought to understand whether the deletion of this receptor will influence the survival of mice during a high-dose (~200 CFUs/mouse) *Mtb* infection. We found that the deletion of CysLTR1 in C57BL/6 mice had no effect on the survival irrespective of the gender (**Figure 3.8A-3.8B**). In contrast to C57BL/6 mice, Balb/C *Cysltr1*$^{-/-}$ mice showed a significant increase in survival when compared to controls which succumbed to the infection earlier than knockout animals (**Figure 3.8C-3.8D**). Taken together, these results indicated that CysLTR1 deletion influence the survival of mice is genetic background-specific during *Mtb* infection.

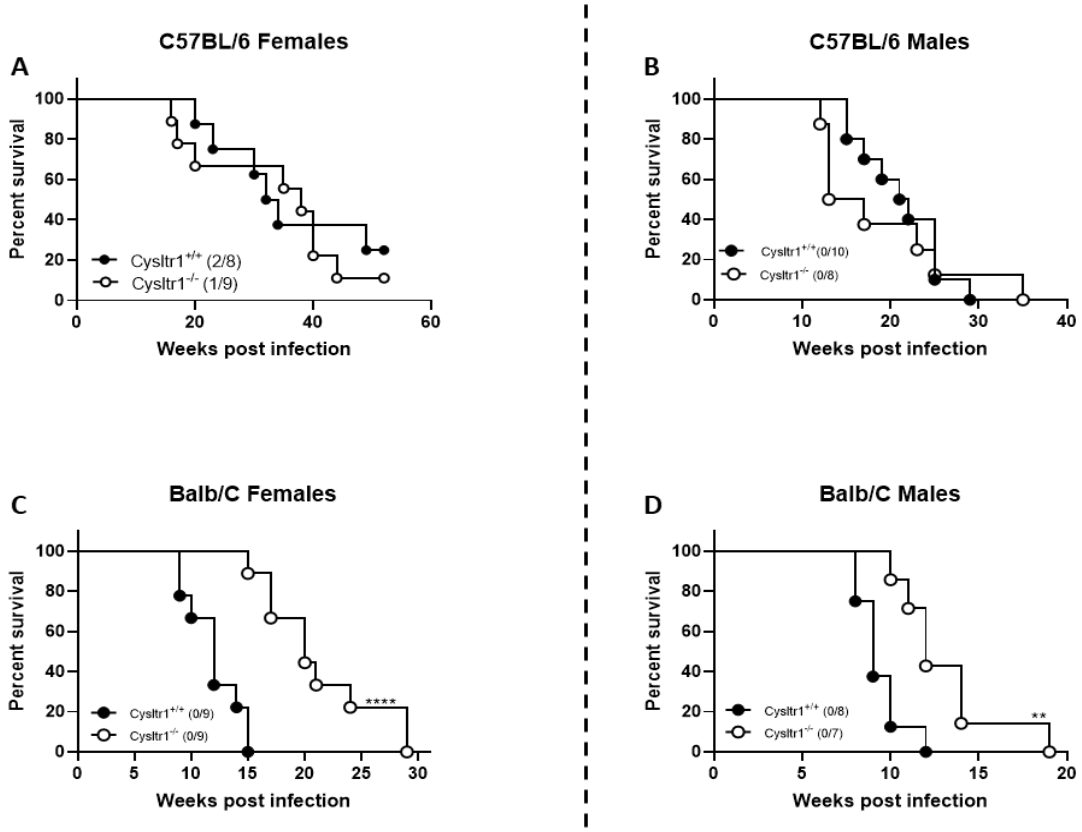

Figure 3.8: Enhanced survival of CysLTR1-deficient Balb/C mice during *Mycobacterium tuberculosis* infection. C57BL/6 and Balb/C *Cysltr1*$^{+/+}$ and *Cysltr1*$^{-/-}$ mice were intranasally infected with high dose (~ 200CFUs) HN878 Mtb for mortality experiments. Mice were monitored and euthanized at humane endpoints. A) C57BL/6 female, B) C57BL/6 male, C) Balb/C female, and D) Balb/C male survival curve. Statistically analysed by log-rank (Mantel-Cox) test, (**$p<0.01$, ****$p<0.00001$).

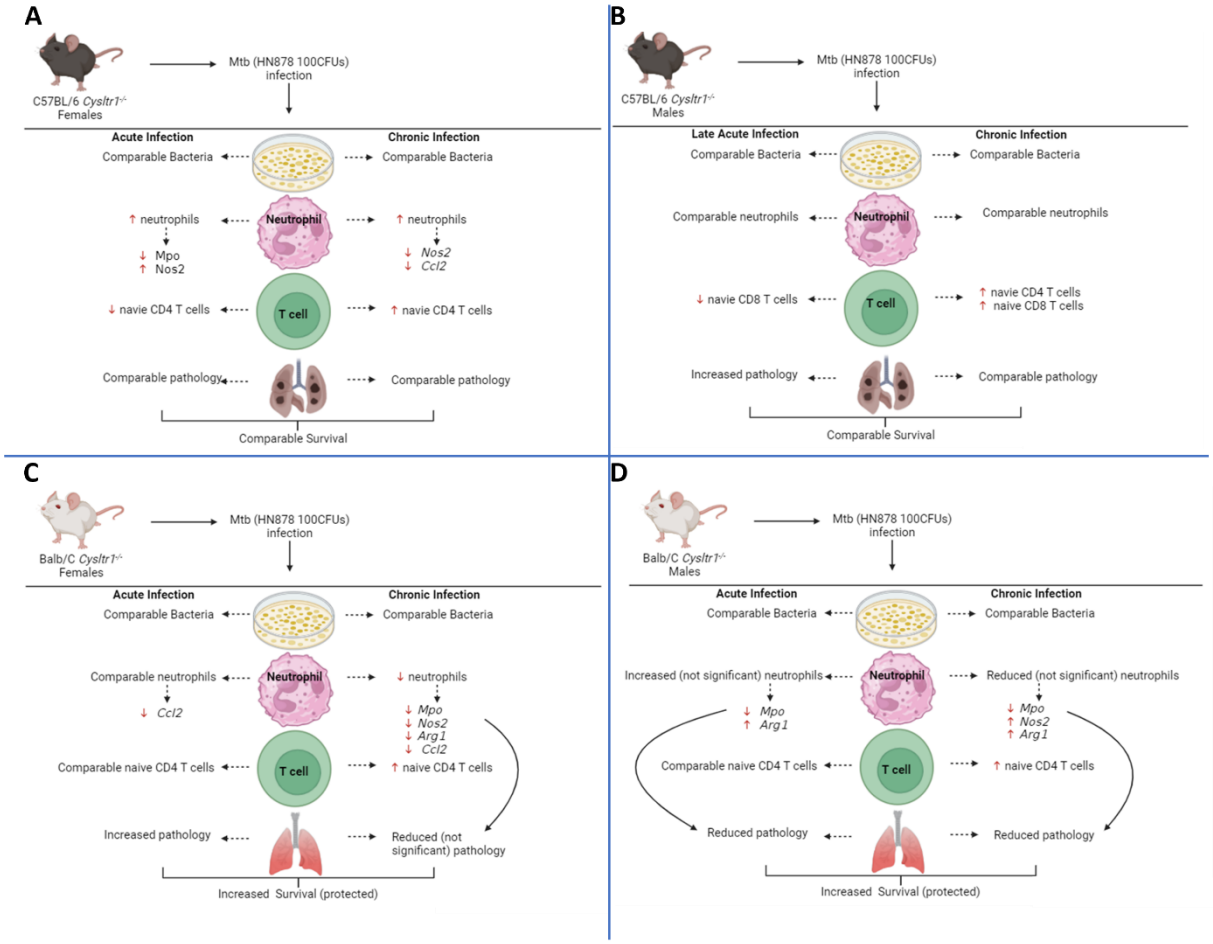

Figure 3.9: Schematic summary of major results during *Mycobacterium tuberculosis* infection. A) Deletion of CysLTR1 in C57BL/6 female mice has no effect on bacterial growth, results in increased neutrophils and naïve CD4 T cells but comparable lung pathology and no effect on mice survival. **B)** In C57BL/6 male mice, CysLTR1 deletion had no effect on bacterial burden, neutrophils, lung pathology and has no effect on mice survival. **C)** CysLTR1 deletion in Balb/C female mice had no effect on bacterial burden, resulted in reduced neutrophils with reduced *Mpo*, *Nos2*, *Arg1* and *Ccl2* expression leading to improved lung pathology and increasing mice survival during Mtb infection. **D)** In Balb/C male mice, CysLTR1 deletion resulted in neutrophils with reduced *Mpo* expression (less activation) and reduced lung pathology during chronic infection leading to increased mice survival.

3.5. Discussion

Host-directed therapies (HDTs) offer a new promising avenue to combat TB disease. Eicosanoids have been proposed as possible targets for HDT as they regulate both the innate and adaptive immune responses to reduce bacterial infection and improve pathology. For instance, Marakalala *et al.* (325) demonstrated that eicosanoids (ALOX5, ALOX5 activating protein [ALOX5AP], and LTA4H) were enhanced in the necrotic centres and cells that bordered the caseum, implying an important role of eicosanoids in human TB granuloma formation. Another study by Mishra *et al.* (326) reported that macrophages treated with CysLTR1 antagonist, pranlukast (PRK), are more efficient at eliminating intracellular bacterial growth by reducing pathogen pro-survival pathways including 5-LO signalling, eicosanoid synthesis and CysLTR1 signalling. Given PRK is a CysLTR1 antagonist, these results demonstrate that it is nonspecific since it affects the pathways upstream to CysLTR1. Using CysLTR1 knockout mice, we examined the role of CysLTR1 in most widely used Balb/C and C57BL/6 genetic background and gender of animals during *Mtb* infection *in vivo*.

Given the gender- and background-dependent differences reported for the role of eicosanoids and CysLTR1 (281,312,313), we conducted study including the gender and mouse background. In both C57BL/6 and Balb/C mice, deletion of CysLTR1 does not affect the ability of mice to control *Mtb* growth (**Figure 3.1A-B and 3.2A-B**). This contradicts the finding with PRK, which resulted in reduced bacterial growth (326). This difference could be attributed to the antagonist's ability to not only inhibits CysLTR1 but also 5-LO and the biosynthesis of eicosanoids that mediate the control of the bacterium. In our model, the deletion of CysLTR1 does not affect the upstream signalling pathways of eicosanoids. Therefore, the deletion and inhibition of CysLTR1 does not affect bacterial control, and CysLTR1 signalling alone has no antimicrobial properties. Interestingly, our study revealed that CysLTR1 deletion influences lung histopathology and this is dependent on the mice background. For instance, C57BL/6

female *Csyltr1*$^{-/-}$ mice had no effect whereas male increased free alveolar spaces indicating improvement of tissue pathology (**Figure 3.1G-H**). In Balb/C mice, on the other hand, the deletion of CysLTR1 resulted in increased pathology early, however, decreased (though not significant) pathology at later time point. Male Balb/C *Cysltr1*$^{-/-}$ mice increased free alveolar spaces at both time points (**Figure 3.2G-H**). Increased lung free alveolar space indicate improvement in inflammation/pathology reflecting better disease outcomes. In Balb/C mice, deletion of this gene results in less pathology during chronic *Mtb* infection, indicating a background-specific effect of CysLTR1 signalling.

CD4 T cells play a vital role in the initiation of adaptive immunity and activation of immune cells required for the proper clearance of the bacteria and its control. In *Mtb* infection, activated CD4 T cells secrete INF-γ and TNF-α which are required not only for the bacterial killing but for the activation and recruitment of monocytes/granulocytes to the site of infection (203,204). The only study that has linked CysLT receptor expression with T cell activation and maturation in children with hyperplasic tonsils showed that there is an increase in the expression of CysLTR1 and CysLTR2 in $CD3^{+}$ mitotic large lymphocytes (327). These lymphocytes are undergoing differentiation and maturation, suggesting CysLTs play a role in T cell activation and differentiation. In *Mtb* infection, the deletion of CysLTR1 does not affect lymphoid cell recruitment, however, interestingly, both C57BL/6 and Balb/C *Cysltr1*$^{-/-}$ mice had significantly increased naïve CD4 T cells at chronic stage (**Figure 3.3C-D and 3.4C-D**), which upon antigen recognition differentiate into specific CD4 T cell subtypes that is dependent on the cytokines milieu in the microenvironment (328).

Neutrophils are essential to the host defence against infections and play a key role in the innate immunity. They are one of the most abundant host immune cells and are the first to arrive at the site of infection (329,330). In TB patients, neutrophils are the predominantly infected phagocytes (331) and are referred to as double-edged swords, as they are crucial for the

protection against early but detrimental in late stages of infection (332). During chronic infection, neutrophils are associated with increased lung damage and severity of the disease. This leads to uncontrolled inflammation and dysfunctional neutrophils, resulting in more recruitment and activation of pro-inflammatory neutrophils (333). They play a role in the dissemination of the bacteria resulting in systemic infection (331,333,334). Here, we demonstrated that in *Mtb* infection, the deletion of CysLTR1 results in reduced neutrophils in the lungs of Balb/C mice at later stages of infection (**Figure 3.4G-H**) and correlated with improved lung pathology. Interestingly, despite the consistently increased neutrophils in female *Cysltr1*$^{-/-}$ C67BL/6 during the early and late stages of *Mtb* infection (**Figure 3.3G**), the lung pathology remained unaffected (**Figure 3.1G**). This suggesting that neutrophils alone are not responsible for the lung pathology and that their impact is likely dependent on genetic background.

The crosstalk between neutrophils and CD4 T cells has been linked to better disease outcomes. CD4 T cells are known to regulate neutrophil activation and function through the recruitment of these granulocytes to the site of infection (335), while CD8 T cells delay neutrophil apoptosis (336,337). Due to their dynamic interactions with neutrophils, CD4 T cells are vital during *Mtb* infection. For instance, Th1 and Th17 cells mediate the protection and pathology of TB through their interactions with neutrophils. In acute *Mtb* infections, Th17 cellular interactions with neutrophils lead to protection against the disease, while in chronic settings this becomes detrimental (338). Besides being regulated by T cells, neutrophils can act as APCs and present antigens to antigen-specific memory CD4 T cells through the MHCII complex and costimulatory molecules (339). The cytokines generated by these neutrophils are required for the induction of CD4 T cell responses (340–343). Interestingly, a study by Minns *et al.* (344), demonstrated that unstimulated neutrophils inhibited the proliferation and activation of T cells. T cells at early stages of activation were more prone to inhibition by neutrophils, while naïve

T cells are not responsive to neutrophils at all (344). This could explain the observed increased naïve CD4 T cells and neutrophils in C57BL/6 female *Cysltr1*$^{-/-}$ mice in chronic stages (**Figure 3.3C and 3.3G**). During chronic *Mtb* infection in Balb/C mice, however, the deletion of CysLTR1 possibly inhibits CD4 T cell activation (hence increased naïve CD4 T cells, which concomitantly showed decreasing trend in activated $T_{eff/mem}$ cells), reducing the secretion of stimulatory cytokines to decrease recruitment and activation of immune cells, hence the reduction in neutrophils.

It is no surprise that CysLTR1 signalling influences neutrophil recruitment as the LT pathway, particularly LTB4, has been implicated in the chemotaxis of neutrophils (345–347). We show that during *Mtb* infection, deletion of CysLTR1 affects neutrophils differently depending on the host genetic background and gender. It is unclear why these differences have no effect on the ability of neutrophil ability to control/kill the bacilli, or how in C57BL/6 female *Cysltr1*$^{-/-}$ mice the increased neutrophils have no effect on the lung pathology. Therefore, we isolated neutrophils from infected mice and assessed various gene expressions by qPCR. Myeloperoxidase (MPO) is one of the main enzymes secreted by neutrophils upon activation (348). MPO activates neutrophils in an autocrine and paracrine manner. It binds to CD11b/CD18 integrins leading to neutrophil degradation, inducing the production of integrins and NADPH oxidase activation (349). Its secretion within the neutrophil containing pathogen rapidly increases the cell's microbicidal activity, however, when released to the outside of the cells it induces tissue damage, enhancing disease pathogenesis (348). Therefore, MPO is not only a marker for neutrophil activation but is also an indicator of tissue pathology. Our Balb/C *Cysltr1*$^{-/-}$ mice showed reduced lung pathology and neutrophils at later stages of infection also had decreased *Mpo* gene expression (**Figure 3.6B**), further validating that the recruited neutrophils are less activated, resulting in decreased pathology and better disease outcome. However, in C57BL/6 *Cysltr1*$^{-/-}$ female mice, despite the increase in neutrophils the *Mpo*

expression was reduced early after infection and rendered neutrophils less active. The differences in *Mpo* expression could be due to both gender and genetic background of the mice. Moreover, our observation is consistent with the study which reported higher MPO activity in BALB/C mice compared to C57BL/6 mice, which may be associated with varying level of chemoattractant CCL2 production in these animals (350).

MPO directly regulate nitric oxide (NO) functions during acute inflammation (351). As expected, we demonstrated that reduction in *Mpo* expression correlated with reduced *Nos2* expression in neutrophils, however, this appears gender dependent in *Cysltr1*$^{-/-}$ Balb/c mice. This observation was also the genetic background specific since reduction in *Mpo* was accompanied by increased *Nos2* expression in *Cysltr1*$^{-/-}$ C57BL/6 females (**Figure 3.6C**). Mice deficient in Arginase 1 (*Arg1*) were shown to have decreased lung bacterial burden, and this was associated with increased NO secretion (352), suggesting that NO is more important than *Arg1* in the control of *Mtb*. Another study demonstrated that mice lacking both *Nos2* and *Arg1* have increased bacterial burden and lung pathology. *Arg1* expression was associated with necrosis and increased bacterial growth in the lungs (322). Therefore, the reduced *Arg1* expression (**Figure 3.6D**) observed in Balb/C *Cysltr1*$^{-/-}$ mice further validates the better lung pathology of these mice during *Mtb* infection. C57BL/6 female mice had a comparable expression of *Arg1*, hence the comparable lung pathology. These results, therefore, suggest that in Balb/C wildtype mice arginase secretion could be the driving force of the lung pathology in *Mtb* infection, and its expression might be CysLTR1 signalling dependent. Lastly, monocyte chemoattractant protein 1 (MCP-1), also known as CCL2, is produced by myeloid cells and plays a role in bacterial clearance through the recruitment of neutrophils to the site of infection (353). In patients with pulmonary TB, CCL2 is linked to disease severity possibly because of increased cellular infiltration or inflammation associated with the increased bacterial dissemination (354). In latently infected individuals, CCL2 was linked with the regulation of

the integrity of the granuloma (355). MCP-1 has also been identified as a downstream effector of CysLTR1 signalling (356). We found that the deletion of CysLTR1 in female Balb/C and C57BL/6 mice leads to a reduction in *Ccl2* expression in neutrophils, as CCL2 is downstream CysLTR1, and deletion of this receptor impacts its expression and consequently influences the neutrophil recruitment in *Mtb* infection. Interestingly, in males, its expression remains unchanged, revealing gender-dependent role of this chemoattractant. This reduction in *Ccl2* expression in Balb/C females may lead to decreased neutrophil recruitment resulting in better disease outcomes. In female C57BL/6 mice, decreased *Ccl2* might contribute for better lung pathology. Lastly, we revealed a background-specific role of CysLTR1 signalling on survival during *Mtb* infection. C57BL/6 mice, irrespective of gender, have similar survival rates, indicated that CysLTR1 signalling is dispensable in these mice. However, in Balb/C mice, CysLTR1 deficiency is beneficial to the host, resulting in significantly improved survival outcomes. Therefore, CysLTR1 is crucial for the pathogenesis of *Mtb* in Balb/C mice.

Overall, the role of CysLTR1 is background-specific during *Mtb* infection. Deletion of this receptor did not affect C57BL/6 mice while increasing protection in Balb/C mice in *Mtb* infection. CysLTR1 signalling though, does affect cellular recruitment and activation based on background and gender. C57BL/6 female mice had increased lung neutrophils during infection in the absence of CysLTR1, while did not affect male neutrophils. Though *Cyslt1*$^{-/-}$ female mice had increased neutrophils but found to be less active (reduced *Mpo*), hence they had comparable lung pathology. In Balb/C mice, the protection observed by CysLTR1 deletion is driven by reduced lung pathology accounted for by reduced neutrophils. In female Balb/C mice, CysLTR1 deletion provided protection by inhibiting neutrophil activation and recruitment in chronic infection by downregulating the expression of *Mpo*, *Nos2*, *Arg1* and *Ccl2* collectively resulted in improved lung pathology. In male Balb/C mice, *Cysltr1*$^{-/-}$ neutrophils, despite reduced *Mpo* expression (which controls NO production), had increased

Nos2 and *Arg1* expression at chronic infection. *Nos2* expression may be increased to regulate/balance *Arg1* effects to decrease lung damage, as evident by reduced pathology. In conclusion, CysLTR1 signalling has no effect on host bacterial control, however, it regulates neutrophil activity resulting in tissue destruction and unfavourable disease outcomes in a background-specific manner during *Mtb* infection.

3.6. Acknowledgements

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Chapter 4: Concluding Remarks

In this study, we assessed and evaluated the role of CysLTR1 signalling in mice during *Lm* and *Mtb* infection. Various studies have demonstrated that different mice backgrounds respond differently to *Lm* (357–359), and it has been well documented that eicosanoids have sex-dependent role (281,312,313). We therefore evaluated the effect of deleting CysLTR1 in mice of both C57B/6 and Balb/C genetic backgrounds while comparing gender during *Lm* and *Mtb* infections. Firstly, we demonstrated that deletion of CysLTR1 has no effect on host homeostasis at steady state in C57BL/6. The characterization of Balb/C mice was performed by Mosala *et at* (114) from our laboratory and reported no major differences at naïve state.

During *Lm* or *Mtb* infection, CysLTR1 deletion had no effect on bacterial burdens in the tissues. Interestingly, deletion of CysLTR1 in mice induced neutrophil recruitment during both *Lm* and *Mtb* infection, however this is genetic background- and sex-dependent. In C57BL/6 female mice, neutrophils were increased in spleens *Cysltr1*$^{-/-}$ mice during *Lm* infection but not in the liver. In male mice, deletion of CysLTR1 significantly increased neutrophils in both the liver and spleen during *Lm* infection. During *Mtb* infection, female C57BL/6 *Cysltr1*$^{-/-}$ mice had increased lung neutrophils, while no differences were observed in lungs from male mice. This demonstrated a sex-specific role of CysLTR1 signalling that might also be dependent on the type of bacterial and/or site of infection. In Balb/C female *Cysltr1*$^{-/-}$ mice, we observed significant reduction in neutrophils at later stages of infection during *Lm* (at 7-dpi) and *Mtb* (at 12-wpi). In male Balb/C *Cysltr1*$^{-/-}$ mice, the spleen also had reduced neutrophils during *Lm* infection, while the liver had slightly reduced neutrophils at 7-dpi, and similar results were observed in male lung during *Mtb* infection at 12-wpi. These results suggest that in Balb/C mice during *Lm* and *Mtb* infection, CysLTR1 deletion results in reduced recruitment of neutrophils to the site of infection at later stages of infection.

In C57BL/6 mice deletion of CysLTR1 had no effect on organ pathology regardless of increased neutrophils in the liver of male mice during *Lm* infection and in the lung of female mice during *Mtb* infection. During *Mtb* infection, we speculate that the comparable expression of *Mpo* in C57BL/6 female mice could explain the lung pathology in these mice. Despite the deletion of CysLTR1 increasing neutrophil recruitment, it has minimal effect on their activation and possibly release of tissue damaging toxins during infection. The deletion of CysLTR1 in female Balb/C mice resulted in reduced organ pathology at later stages of *Lm* and *Mtb* infection. *Cysltr1*$^{-/-}$ Balb/C male mice had increased liver pathology during *Lm* infection, but those infected with *Mtb* had reduced lung pathology at later infection timepoints. In *Mtb* infection, the reduction in tissue pathology was associated with reduced *Mpo* expression. In female *Cysltr1*$^{-/-}$ mice, this was further accompanied by reduction in *Nos2*, *Arg1* and *Ccl2* expression in neutrophils. Therefore, CysLTR1 signalling play a role in neutrophil recruitment and activation resulting in tissue damage.

C57BL/6 *Cyltr1*$^{-/-}$ mice had comparable survival during both *Lm* and *Mtb* infection regardless of gender. This together with comparable organ bacterial loads and tissue inflammation, confirms that CysLTR1 signalling plays minimal role during the immunopathogenesis of *Lm* and *Mtb* in C57BL/6 mice. In Balb/C mice, deletion of CysLTR1 in females increases their protection during *Lm* and *Mtb* infection. Balb/C *Cysltr1*$^{-/-}$ male mice showed increased survival during *Mtb* infection. However, CysLTR1 deletion in Balb/C male mice infected with *Lm* increased their susceptibility to the infection, in line with the increased pathology observed in the liver. The increased survival of *Cysltr1*$^{-/-}$ mice during *Lm* and *Mtb* infection is accompanied by reduced neutrophils and organ pathology at later stages of infection. In both *Lm* and *Mtb* infections, neutrophils play both a protective and detrimental role in host protection (306–308,333). They are required for early control of the infection, however at later stages of infection they can result in tissue destruction and uncontrollable inflammation resulting in poor

disease outcomes. Here, we demonstrated that in Balb/C mice, deletion of CysLTR1 regulated neutrophil recruitment and activation likely resulting in better survival.

At present, antagonists targeting CysLTR1 are used for treating asthma and allergic responses. Given that FDA-approved drugs already exist for this receptor, repurposing them for listeriosis and tuberculosis treatment is viable option to test as monotherapy or adjunctive therapy for these infectious diseases. However, further experiments are necessary prior to assess its efficacy in humans for translation studies.

Numerous studies have shown gender differences in eicosanoid/leukotriene production (279, 310, 311). Additionally, there is evidence suggesting that asthmatic women and girls respond better to montelukast treatment compared to male counterparts (357–359), likely due to hormonal differences. In a study by Lima et al., which examined leukotriene pathway polymorphisms in asthma patients from various ethnic groups treated with montelukast, it was found that differences in polymorphisms of genes in the leukotriene pathway (ALOX5, LTC4S, and LT4H) can be associated with exacerbation of asthmatic reactions. These genetic variations contribute to differences in how patients respond to montelukast treatment (360). Therefore, based on our study's results, clinical treatment of TB and listeriosis targeting CysLTR1 should consider the predisposition to eicosanoids between males and females and take into account the host genetic background. Further studies are needed to tailor appropriate treatment regimens for individual patients. In conclusion, we have showed that CysLTR1 signalling is influenced by the host gender and genetic background during intracellular bacterial infections. CysLTR1 plays a role in neutrophil recruitment, organ pathology and influences mice survival in a background- and gender-specific manner during *Lm* infection, while this is only background-specific during *Mtb* infection. Therefore, CysLTR1 has a potential to be a target for development of HDTs to reduce and resolve tissue destructions during these intracellular bacterial infections, with consideration of the host’s gender and genetic background.

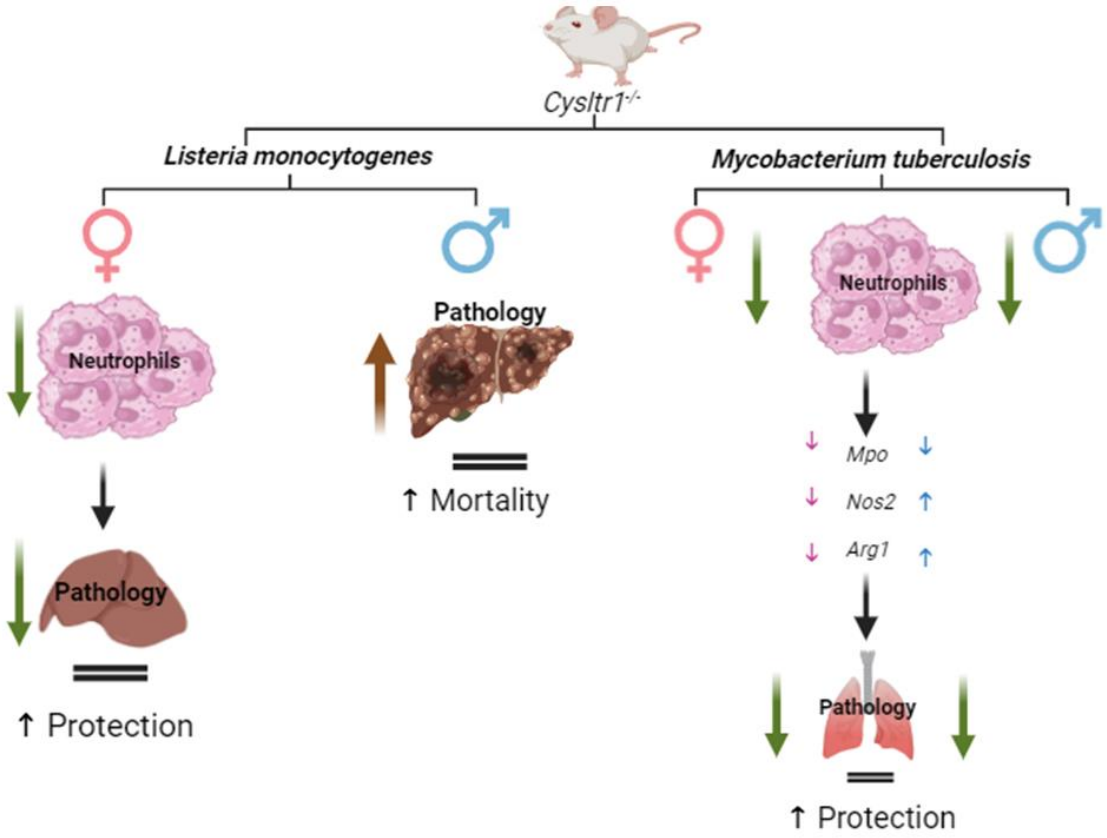

Figure 4.1: Graphical Abstract. CysLTR1 deletion in Balb/C mice reduces neutrophils during chronic bacterial infections, leading to reduced pathology resulting in better survival. In Balb/C male mice however, CysLTR1 deletion increased liver pathology resulting in increased susceptibility to *Lm* infection.

Future studies: Since we observed a stronger phenotype in Balb/C mice during both *Lm* and *Mtb* infections, we intend to evaluate whether we will observe a similar effect when we treat Balb/C mice with montelukast (a CysLTR1 antagonists). Balb/C wildtype mice infected with *Mtb* will be treated with montelukast in conjunction with anti-TB drugs to evaluate if inhibition of CysTR1 can be used as a conjunctive therapy to combat TB.

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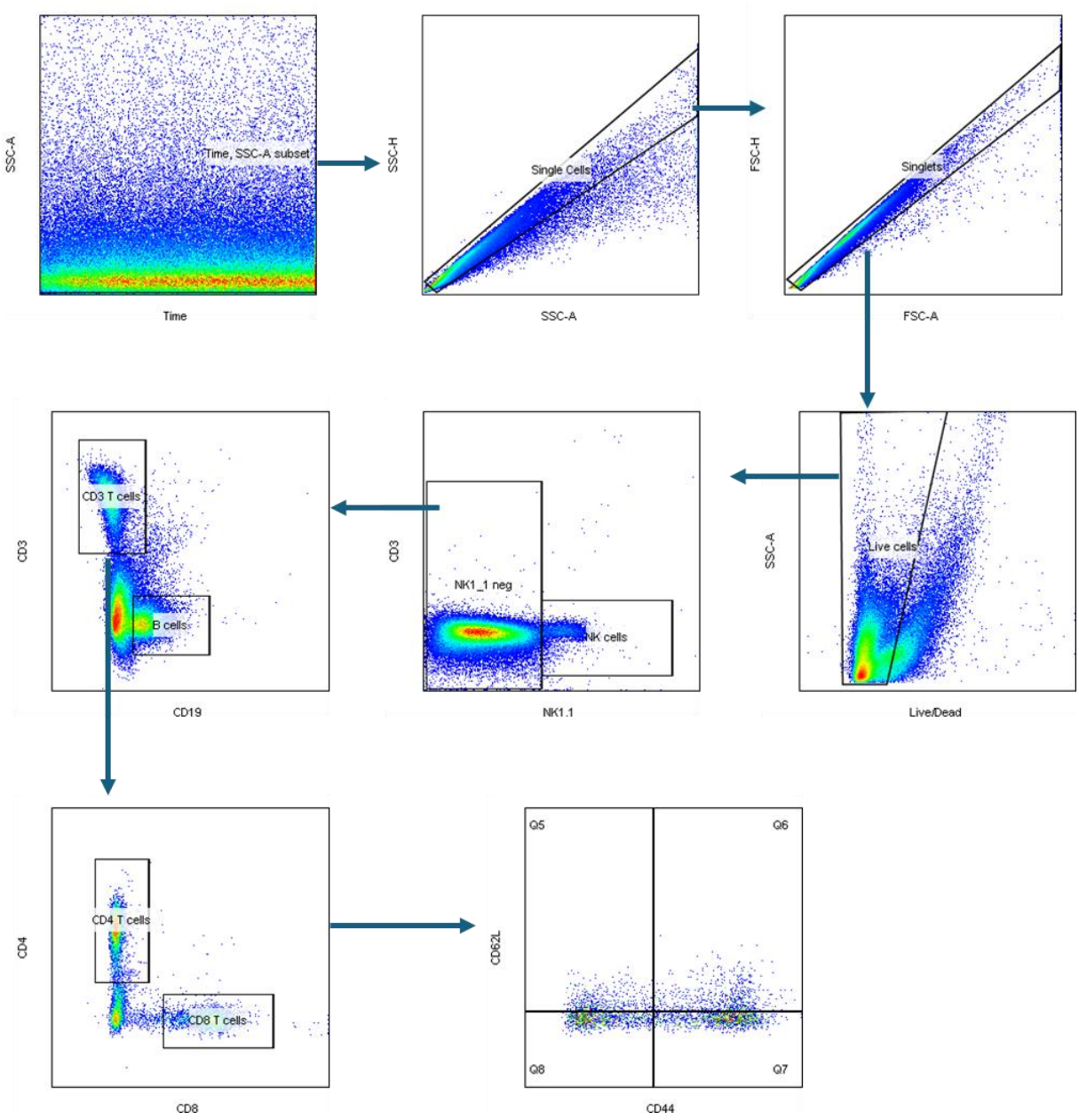
Appendices

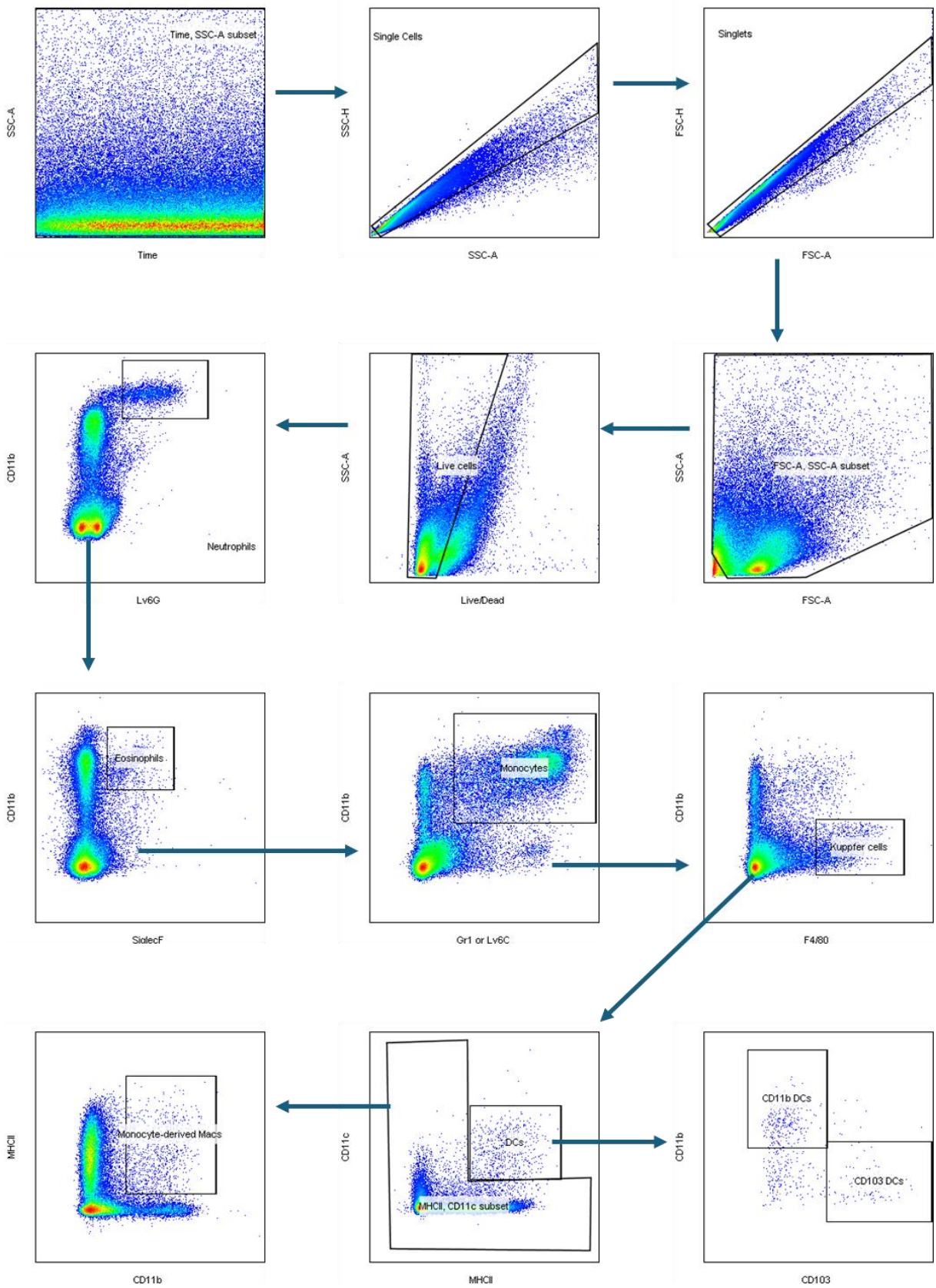
Appendix A

Liver, Spleen, and Lung Lymphoid Gating Srategy



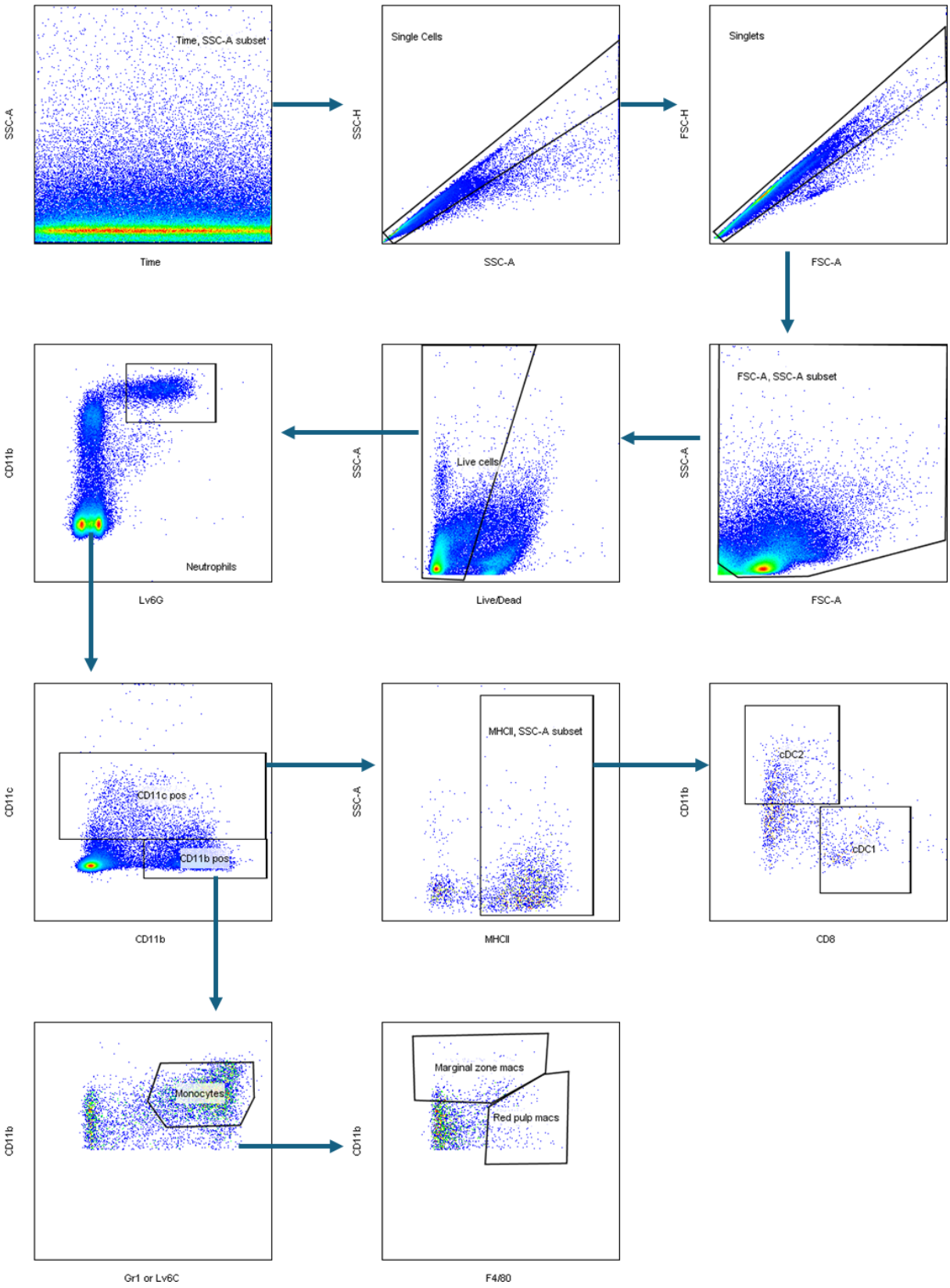
Appendix B

Liver Myeloid cell Gating Strategy



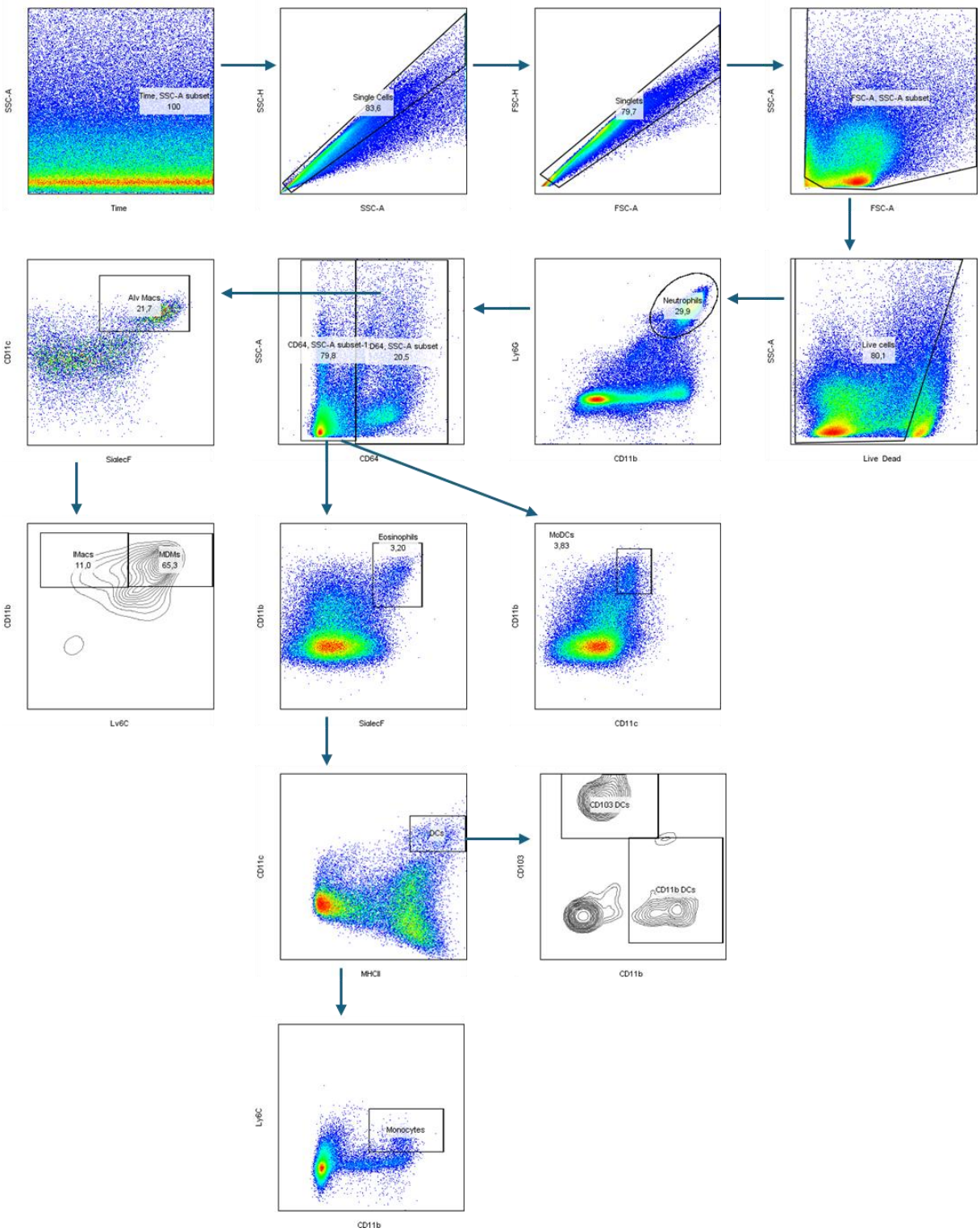
Appendix C

Spleen Myeloid cell Gating Strategy



Appendix D

Lung Myeloid cell Gating Strategy



Appendix E

Table 1: Target Gene Primer Sequences

Gene Target	Primer Sequence
Hprt - Forward	5’ – GTT GGA TAT GCC CTT GAC – 3’
Hprt - Reverse	5’ – AGG ACT AGA ACA CCT GCT – 3’
CysLTR1 - Forward	5'-TCT GTT GTG GGT TTC TTT GGC-3'
CysLTR1 - Reverse	5'-GGA AGG CTG ATT TCT CAT GGT-3'
CysLTR2 - Forward	5'-TGT CAC CAG TGT CAG GAG TG-3'
CysLTR2 - Reverse	5'-ACT TTT GAC GCA TCA GCT CCA A-3'
Ltc4s - Forward	5'-CAA GCC TAC TTC TCC CTA CAG GTG-3'
Ltc4s - Reverse	5'-GTT TAC CTG GGC TCG GAA GAC-3'
Arg1 - Forward	5'-CAG AAG AAT GGA AGA GTC AG-3'
Arg1 - Reverse	5'-CAG ATA TGC AGG GAG TCA CC-3'
Ccl2 - Forward	5'-CTC TCT CTT CCT CCA CCA CCA T-3'
Ccl2 - Reverse	5'-TGG GGC GTT AAC TGC ATC TG-3'
Ccl5 - Forward	5'-ATG GAT CTC CCA CAG CCT CT-3'
Ccl5 - Reverse	5'-TCC TTC GAG TGA CAA ACA CGA C-3'
Cybb - Forward	5'-GTG GTT GGG GCT GAA TGT CT-3'
Cybb - Reverse	5'-AGT GCT GAC CCA AGG AGT TT-3'
Cxcl2 - Forward	5'-CTC TCA AGG GCG GTC AAA AAG-3'
Cxcl2 - Reverse	5'-CAG CGA GGC ACA TCA GGT A-3'
Il-1α - Forward	5’ – CGC TTG AGT CGG CAA AGA AAT C – 3’
Il-1α - Reverse	5’ – ATA CTG TCA CCC GGC TCT CC – 3’
Mpo - Forward	5'-GGA CTG GAT TTG CCT GCT CT-3'
Mpo - Reverse	5'-ACC GAT CAC CAT CAC GTA GC-3'
Nos2 - Forward	5’ – AAC TGC AAG AGA ACG GAG AAC G – 3’
Nos2 - Reverse	5’ – AAC ATT CTG TGC TGT CCC AGT – 3’
Tnfα - Forward	5'-TCT CAT CAG TTC TAT GGA CC-3'
Tnfα - Reverse	5'-GGG ACT AGA CAA GGT ACA AC-3'