The role of Cysteinyl leukotriene type 1 receptor (CysLTR1) during *Listeria monocytogenes* infection in mice

MSc (Med) in Clinical Science and Immunology

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Sibongiseni Kwakho Luntukazi Poswayo

Supervisors: Dr. Suraj P. Parihar

Co-Supervisors: Dr. Mumin Ozturk and Prof. Frank Brombacher

February 2020

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Abstract

South Africa recently experienced a Listeriosis outbreak, which was responsible for over 180 deaths, caused by an intracellular, rod-shaped bacilli called *Listeria monocytogenes* (*LM*). *LM* can infect both phagocytic and non-phagocytic cell types and induces its uptake by expressing internalin A and B, then secretes listeriolysin O (LLO), a virulence factor forming pores on the phagosome membrane to escape into the cytosol. Macrophages can phagocytose invading pathogens and induce innate inflammatory responses. Production of cytokines and eicosanoids by antigen presenting cells activates the adaptive immunity. Eicosanoids (epoxyeicosatreinoic acids, prostanoids and leukotrienes) are generated from metabolites of 20-carbon chained poly-unsaturated fatty acids and arachidonic acid. Leukotrienes (LTs) are generated from 5-lipoxygenase-metabolism of arachidonic acid to LTB_4 and cysteinyl LTs (cysLTs). CysLTs are pro-inflammatory lipids that have pathobiological functions in asthma. CysLTs function through three G-protein coupled receptors (CysLTR1, CysLTR2 and GPR99). The CysLTR1 and its ligands function has been well elucidated in asthmatic and allergic responses however, its role in bacterial infections is unknown. The aim of our study was to elucidate the role of CysLTR1 on disease progression in mice and macrophages infected with *LM*. In this study, we showed that CysLTR1 mRNA expression is upregulated by *LM* infection in WT macrophages and mice. Mice deficient of CysLTR1 had no defects at homeostasis. During time kinetic experiments with *LM*, CysLTR1 knockout mice displayed increased neutrophil recruitment and decreased lymphocyte cells at 3dpi, however, bacterial burdens were comparable to wild-type mice. In addition, macrophages deficient of CysLTR1 have no effect on the intracellular growth of *LM*. In conclusion, CysLTR1 signalling plays a role in lymphoid cell activation and neutrophilic recruitment during early *LM* infection, however, further studies are required to better understand the role of CysLTR1 during inflammatory responses.

Abbreviations

5-HPETE	5-hydroperoxy-eicosatetraenoic acid
γ-GL	Gamma-glutamyl leukotrienase
ActA	Actin polymerization factor A
AD	Alzheimer's disease
APCs	Antigen presenting cells
AA	Arachidonic acid
AERD	Aspirin-exacerbated respiratory disease
BMDMs	Bone marrow derived macrophages
Ca^{2+}	Calcium
Cas9	CRISPR associated protein 9
cAMP	Cyclic AMP or 3',5'-cyclic adenosine monophosphate
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine receptor
CD-	Cluster of Differentiation
CFUs	Colony forming units
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
CXCL	Chemokine (C-X-C motif) ligand
CysLTs	Cysteinyl leukotrienes
CysLTR-	Cysteinyl leukotriene receptor
CRISPA	Cluster regulatory interspace short palindromic repeats
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
cDC	Conventional DC
DMEM	Dulbecco's modified eagle medium

DNA	Deoxyribonucleic acid
Dpi	Days post infection
E-cadherin	Epithelial cadherin
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
H_2O	Dihydrogen monoxide or water
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HKLM	Heat-killed *Listeria monocytogenes*
IKK	Iκ Kinase
iNOS	Inducible nitric oxide synthase
IFN-	Interferon
IL-	Interleukin
Inl-	Internalin
KLRG1	Killer cell lectin like receptor G1
KO	Knockout
LLO	Listeriolysin O
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LM	*Listeria monocytogenes*
LO/LOX	Lipoxygenase
LTs	Leukotrienes
LTC_4S	Leukotriene C4 synthase
LTRA	leukotriene receptor antagonist
Macs	Macrophages

MAMPs	Microbial associated molecular patterns
MCP-	monocyte chemoattractant protein
MHC	Major histocompatibility complex class
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic acid
Mtb	Mycobacterium tuberculosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear factor κβ
NK cells	Natural Killer cells
NO	Nitric oxide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD1	programmed cell death protein 1
PG	Prostaglandin
PLA2	Phospholipase A2
PLC	Phospholipase C
Plc	Phospholipase
PPRs	Pathogen recognition receptors
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TB	Tuberculosis
TGF-β	Transforming growth factor beta
Th1	T helper 1
TK	Thymidine kinase

TNF	Tumor necrosis factor
TSA	Tryptic soy agar
TSB	Tryptic soy broth
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
WT	Wildtype

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CHAPTER 1: INTRODUCTION

1. Listeria monocytogenes

Listeria monocytogenes (*L. monocytogenes* or *LM*) was initially described as *Bacterium monocytogenes* by E.G.D. Murray in 1940, then later changed to the genus name *Listeria* in 1962 (1, 2). There are 17 Listeria genus species that have been recognized so far, namely; *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimer*, *L. marthii*, *L. innocua*, *L. grayi*, *L. fleschmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, *L. cornellensis*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia* and *L. booriae* (3). *L. monocytogenes* and *L. ivanovii* are the two only species that are known to be pathogenic, with *L. monocytogenes* being an important human foodborne pathogen (4, 5).

L. monocytogenes is a facultative intracellular Gram-positive bacterium that is the causative agent of Listeriosis (6). *LM* mostly causes infection in immunocompromised individuals, elderly people, pregnant women, and neonates (7–9). *LM* can be isolated from soil, plants and water, and can cause disease in both animals and humans (10, 11), and causes neonatal infection, sepsis and meningitis in immunocompromised individuals (12). In 2016, the centre for disease control (CDC) reported that listeriosis infects about 1600 immuno-incompetent individuals, which include pregnant women and neonates in the United State, with an estimate of 260 deaths each year (7). Between 2017 and 2018, South Africa had an outbreak of Listeriosis, with 674 reported cases and 183 patient deaths due to the disease of which 42% were neonates infected during pregnancy or delivery (13).

1.1. *L. monocytogenes* pathogenesis

L. monocytogenes is introduced into the human host through ingestion of contaminated foods. When the bacterium is ingested, it crosses the intestinal barrier through the blood and the lymphatic system to the liver and spleen. In the liver, the bacteria replicates in the hepatocytic macrophages and then spreads via blood to cross barriers such as brain and the placenta (14). Therefore, *LM* can infect macrophages and various other cell types and this ability is mostly facilitated by its virulence factors discussed below.

1.2. Host cellular invasion by *L. monocytogenes*

Invasion of the host cellular system by *LM* is initiated by two surface proteins, internalin A (InlA) and B (InlB). InlA interacts with epithelial cadherin (E-cadherin) expressed on epithelial cells, to induce *LM* uptake (15). While InlB induces *LM* uptake by various other

cells such as liver hepatocytes, tissue fibroblasts and epithelial cells by interacting with host tyrosine kinase, Met receptor (16–18). Therefore, InlA and InlB allow the internalization of *LM* by phagocytic and non-phagocytic cells, and it becomes entrapped in a vacuole.

Once internalized in a vacuole, to promote its survival *LM* secretes a pore-forming toxin, Listeriolysin O (LLO) and two phospholipases (PlcA and PlcB) depending on the cell type, to disrupt the phagosome membrane and promote its escape into the host cytoplasm (19). LLO forms pores around the phagosome, while PlcA and PlcB cause damage that results in the lysis of the phagosome membrane (20). In the cytoplasm, *LM* replicates and becomes motile by exploiting host actin through the actin polymerization factor (ActA), which enables the bacteria to polymerize host actin to propel into neighbouring cells. During this cell-to-cell spread, *LM* trapped in a double membrane vacuole when propelling from the cytoplasm through membrane protrusions into nearby cells (19–24). This mode of dissemination renders this bacterium virtually invisible to immune system. The secondary double-membrane vacuole can also be lysed by LLO, PlcA and PlcB, leading to the re-initiation of this infection cycle (19, 25). Through the expression of these virulence factors, *LM* is able to cross the intestinal barrier, and spreads through the bloodstream or lymph to the mesenteric lymph nodes, spleen and the liver and can cross the blood-brain barrier and fetoplacental barrier (24, 26).

1.3. Host cellular immunity to *L. monocytogenes* infection

During any pathogenic infection, the host must induce an appropriate immune response to clear the infection. The interaction between the innate and adaptive immunity is therefore is essential for the control of pathogenic infections. During *LM* infection, innate immunity is responsible for detecting and controlling the infection, and adaptive immunity clears and hence protection for future infections (27). The innate immune cells detect microbial and danger associated molecular patterns (MAMPs and DAMPs) through patter recognition receptors (PRRs) expressed on their cell surface and in endosomes (28). These innate immune cells include macrophages, neutrophils, dendritic cells (DCs), granulocytes and monocytes, and they are the first line of defence during intracellular bacterial infections. Although natural killer (NK) cells are lymphoid cells, they also play a role in innate immunity during tumour, viral and bacterial invasion.

LM expresses ligands that can be recognized by the toll-like receptor (TLR) 2 that recognizes bacterial peptidoglycan, lipotechoic acid and lipoproteins, TLR5 that recognizes *LM* flagellin, TLR9 which recognizes the CpG motif of the bacterium DNA, and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which recognizes muramyl dipeptide. Recognition of these MAMPs all result in the activation of the nuclear factor κB (NF-κB) which increases pro-inflammatory cytokine secretion (29–32). It has been shown that mice lacking CCR2 (a chemokine receptor for MCP-1 and MCP-3), IFN- γ and TNF receptor are susceptible to *LM* infection (33–35). These chemokines and cytokines are essential for the optimal innate immunity against *LM* infections, as they increase macrophage and neutrophil recruitment and enhance microbicidal functions to control bacterial burden before the onset of the adaptive immunity.

The macrophages and DCs play a crucial role during *LM* infection, they can engulf the invading pathogens, resulting in the activation of the innate immunity. *LM* infected DCs result in the recruitment of granulocytes, NK cells and monocytes to the site of infection in response to IL-12 secretion (36). Recruitment of NK cells enhances the production of IFN-γ required for further activation and maturation of DCs and macrophages (9, 36, 37). However, it has been also demonstrated that NK cell stimulation can be detrimental for the host during *LM* infection (38). *LM* secrets a protein and virulence factor, p60, which can indirectly stimulate NK cells (39). Activation and maturation of DCs is essential during infection, as it enhances surface co-stimulation receptors (CD80, CD86, CD70) and cytokines (IL-12p70, IL-18, IFN-α/β) (40). These DCs migrate to the infection-associated secondary lymphatics, where they interact with the naïve T cells in the draining lymph nodes (41). During *LM* infection DCs have been shown to be the source of IL-12 leading to resistance to infection through production of IFN-γ (42–44). Subsequently, IL-12 drives the rapid recruitment of NK cells, granulocytes and monocytes during *LM* infection (36).

Macrophages internalize the bacterium and it becomes entrapped in a vacuole or phagosome. As the phagosome matures, macrophages create a toxic environment for the bacterium to inhibit its escape into the cytosol. However, for an effective control of *LM* by macrophages, they must be activated. Activation of the macrophages infected with *LM* results in the generation of nitric oxide (NO) through hydrolysis of L-arginine by inducible nitric oxide synthase (iNOS) (45). Production of reactive oxygen and nitrogen species (ROS and RNS) by macrophages inhibits the escape of *LM* from the vacuole into the

cytosol (46). *LM*-infected macrophages secrete TNF, IL-12 and some chemokines (such as MIP2) which allow stimulation and recruitment of other immune cells. For instance, IL-12 regulates the development of T cells that secrete IFN-γ, TNF, and IL-2. TNF and IL-12 secretion by macrophages, increases the major histocompatibility complex class II (MHCII) and co-stimulatory molecule expression, enabling antigen presentation to T cells. This also leads to T cell and NK cell activation. IFN- γ secretion, together with TNF, result in complete activation of macrophages and *LM* killing through NO production (47–49). Macrophages also secrete IL-1, IL-6 and chemokines responsible for neutrophil recruitment and activation (50).

Production of CXCL1 and CXCL2 during *LM* infection recruits neutrophils to the site of infection (51). Neutrophils phagocytize the *LM* to limit its spread, and these are ingested by macrophages which kill the bacteria. Their ability to kill *LM* is contributed to by their ability to produce reactive oxygen species and are important for bacterial killing (52). Another mechanism that neutrophils employ to inhibit *LM* spread is neutralizing its virulence factor LLO. It has been shown that during phagocytosis by neutrophils, the matrix metalloproteinase-8 (MMP), an endoprotease that degrades LLO thereby prevents the bacteria from perforating the infected neutrophil, therefore, entrapping it within the phagosome (53).

T lymphocytes form a large component of the adaptive immunity and is essential for the clearance of *LM* and to generate memory for future infections. *LM* infected antigen presenting cells (APCs) present a peptide fragment of the antigen to T cells through major histocompatibility complex I (MHC-I) and II (MHC-II) (48). *LM* antigen presented by MHC-I by DCs/macrophages to CD8 T cells to mounts a protective cytotoxic T lymphocyte (CTL) response against *LM* infection (54). CD8 T cells induce immunity to *LM* by either lysing the infected cells via perforin and granzymes, or by secreting IFN-γ that in turn activates macrophages for an effective killing of the bacterium (55). It has been demonstrated that mice primed with killed *LM* generate long-term CD8 T cell mediated immune response to *LM* infection (56). Upon re-infection, CD8 memory T cells induce a rapid elimination of infected cells by invading pathogens to provide protective immunity (57). The memory CD8 T cells are recruited to the site of infection, where they secrete IFN-γ for macrophage activation and rapidly secrete granzyme and perforin directed to infected cells (58). In response to *LM* infection, IL-12 and type 1 interferons have been shown to induce CD8 T cell memory development (59). APCs can, however, present *LM*

antigens to CD4 T cells via the MHC-II resulting in IFN-γ secretion to further activate macrophages (60). CD4 T cells help memory CD8 T cell expansion after re-infection with the same pathogen (61) and arerequired for the long-term maintenance of memory $CD8^+$ T cells and function by maintaining their ability to respond to IL-7 and IL-15 (62). More recently, it has been shown that LLO specific CD4 T cells transition into long-term memory populations that resemble their parent effector T cells. These effector and memory T cells predominantly have a Th1 profile that result in production of IFN-γ, TNF, and IL-2 and minimal production of IL-17A. Deletion of CD4 T cells during *LM* infection results in increased bacterial burden, proving that memory CD4 T cells might play a crucial role in intestinal intracellular bacteria (63). Therefore, both CD4 and CD8 memory T lymphocytes play a role in the clearance and control of *LM* infection during secondary infection.

Antibodies have been shown to play a minor role during *LM* infection. Antibodies reduce early dissemination of *LM* to vital organs by trapping the bacteria and their antigens in secondary lymphoid organs where specific immune responses are initiated. B cells and antibodies can also facilitate the generation of the protective T cells responses (64). They have also been shown to play a role in generation of memory CD8 T cells during *LM* infection (65).

2. What are Eicosanoids?

Eicosanoids are a lipid mediator family of enzymatically generated metabolites of 20-carbon polyunsaturated fatty acids (PUFAs) (66, 67). Eicosanoid biosynthesis begins with the release of arachidonic acid (AA), esterified in the sn-2 domain of membrane phospholipids, by the action of phospholipase A_2 (PLA_2), particularly group IVA cytosolic $cPLA_2$ (68). Eicosanoids are synthesized primarily by three enzymatic oxygenation pathways, each involving a distinct family of enzymes; cyclooxygenases (COXs), epoxygenases, and lipoxygenases (LOs) (66). The enzymatic activities of prostaglandin G/H synthases or COXs, lipoxygenases, and epoxygenases respectively result in the formation of prostaglandins (PGs) and thromboxanes, leukotrienes, and epoxyeicosatrienoic acids (Figure 1).

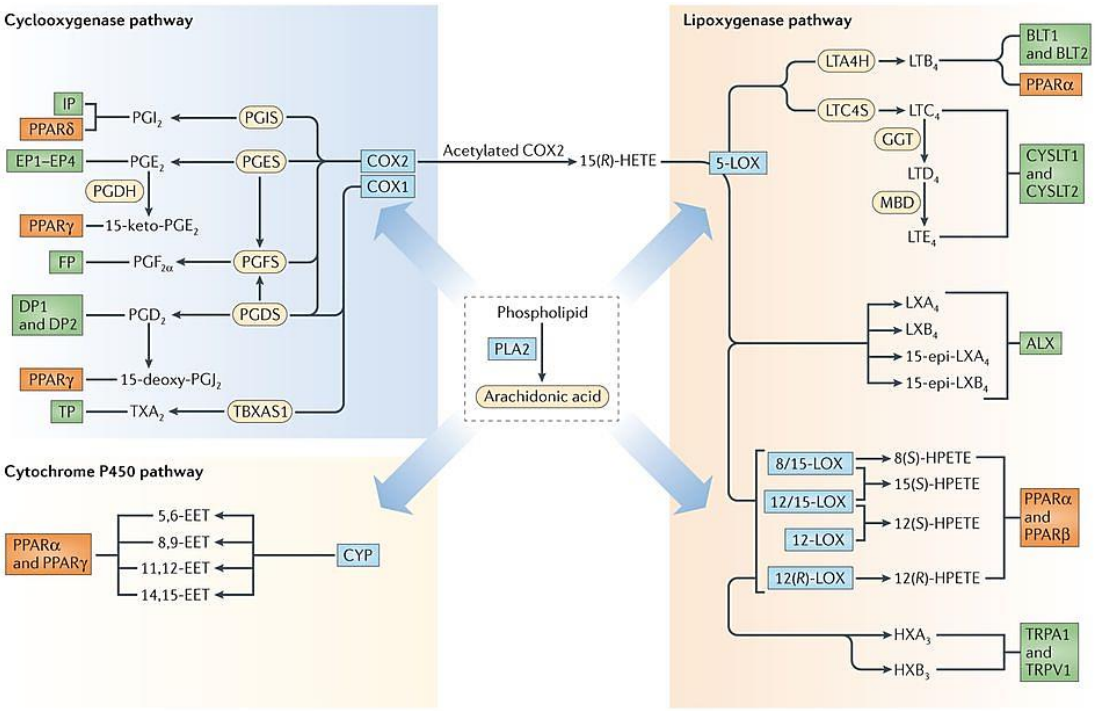

Figure 1: Eicosanoid synthesis and signalling receptors. Eicosanoid biosynthesis results from the esterification of the phospholipase A2 (PLA2), leading to the activation of the arachidonic acid (AA) pathway. The three enzymatic pathways involved in the synthesis of eicosanoids include cyclooxygenases (COX1/2), epoxygenases (also known as cytochrome P450, CYP) and lipoxygenases (LOX) (69).

Eicosanoids play an important role in the function of immune cells. Receptors for eicosanoids are widely expressed throughout the immune system and function at multiple levels in both innate and adaptive immunity. The immunoregulatory properties of eicosanoids and their receptors result from their ability to modulate the production of cytokines and the expression of cytokine receptors, cell surface molecules in both an autocrine and paracrine manner. They therefore provide a link between innate and adaptive immunity (70).

Prostaglandins and thromboxanes are collectively termed the prostanoids, which are widely generated in response to diverse stimuli and act in a paracrine or autocrine manner, while playing important roles in normal physiology and in diseases (68). Epoxyeicosatrienoic acids are autocrine and paracrine effectors in the cardiovascular system and kidneys. They regulate the transportation of ion and gene expression, while initiating vasodilation and have anti-inflammatory and pro-fibrinolytic effects (71). Leukotrienes induce acute inflammatory responses such as increased vascular permeability and recruitment of granulocytes, while prostaglandins also have pro-inflammatory effects by increasing vascular permeability but also exert immune-suppressive effects (72).

3. Synthesis of Leukotrienes

Leukotrienes (LTs) are a group of eicosanoids generated from the 5-lipoxygenase-(5-LO-) metabolism of AA to form leukotriene B_4 (LTB_4) and the cysteinyl LTs (CysLTs), LTC_4, LTD_4 and LTE_4 (73). LTs are lipid mediators that have potent proinflammatory activities (74, 75). LTs are predominately synthesized by inflammatory cells such as leukocytes, macrophages, and mast cells. The cellular activation by immune complexes, bacterial peptides, and other stimuli elicit a sequence of events that induce cPLA2 and 5-LO translocation to the nuclear envelope (76). The activation of both the cPLA2 and 5-LO enzymes involves increased intracellular calcium levels and enhances the activation of protein kinase C (PKC). The AA is converted to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and subsequently to an unstable intermediate, LTA_4, by the enzyme 5-LO (Figure 2) (77). 5-LO is required to be translocated from the cytosolic or nucleoplasmic compartment to the perinuclear envelope, where it acts in concert with 5-LO activating protein (FLAP), which is required for 5-LO to function enzymatically in intact cells (78).

LTA_4 is preferentially hydrolysed to the dihydroxy leukotrienes, LTB_4, by LTA_4 hydrolase in the neutrophils (79). However, in eosinophils, basophils, mast cells and macrophages, through conjugation of LTA_4 to a reduced glutathione by LTC_4 synthase (LTC_4S) forms LTC_4. LTC_4S is the terminal enzyme involved in the cysteinyl leukotriene (Cys-LT) synthesis (80–82). The formed LTC_4 is exposed to the cell surface through a specific energy dependent step. It is converted extra-cellularly to LTD_4 by a γ-glutamyl transpeptidase (γ-GT) or by a more functionally specific enzyme, γ-glutamyl leukotrienase (γ-GL) (83). LTD_4 is then converted to LTE_4 by a dipeptidase (84). LTE_4 is excreted in the urine without undergoing any chemical modification (85).

All the leukotrienes synthesised by the enzyme LTC_4S (LTC_4, LTD_4 and LTE_4), are collectively termed cysteinyl leukotrienes (CysLTs) since they are peptide-conjugated lipids. Leukotrienes were initially identified by their contractile properties on intestinal and bronchial smooth muscle (86, 87). However, they are now better recognised as potent inflammatory mediators that mediate a diverse array of biological responses (88).

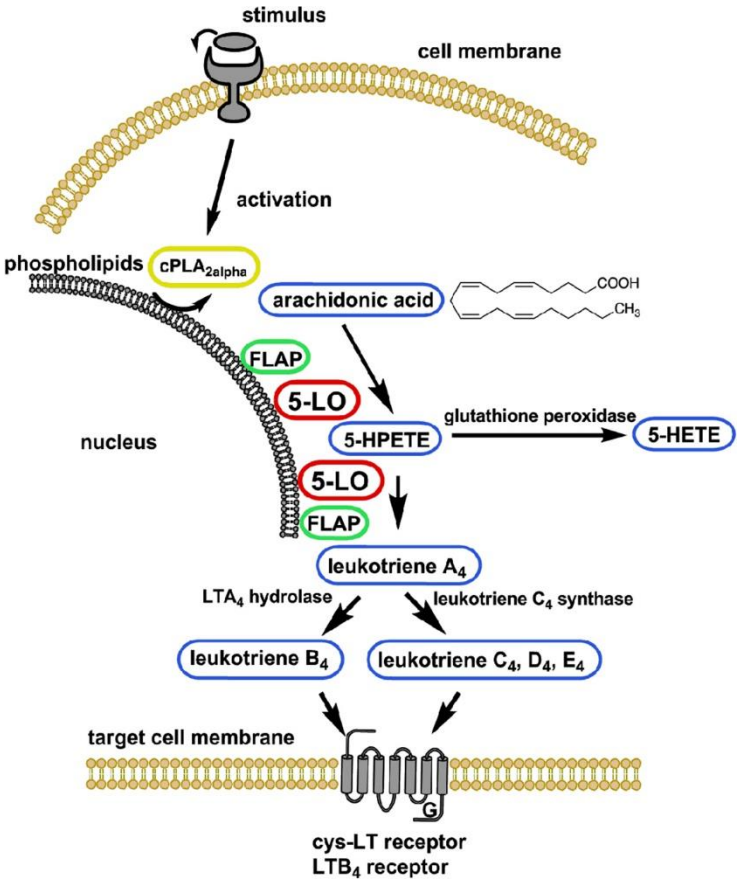

Figure 2: Biosynthesis of leukotrienes. Stimuli presented by the cellular membrane result in the deoxygenation of the arachidonic acid released by the cytosolic phospholipase A2alpha ($cPLA_{2alpha}$) from phospholipids, resulting in the formation of 5-HPETE and leukotriene A_4 (LTA_4). LTA_4 is further hydrolysed to leukotriene B_4 (LTB_4) by LTA_4 hydrolase or synthesized by leukotriene C_4 synthase to cysteinyl leukotrienes C_4, D_4, and E_4 (LTC_4, LTD_4 and LTE_4) (89).

3.1. Cysteinyl leukotriene receptors and their functions

Once the CysLTs have been synthesized, they bind to specific receptors to exert their biological functions. CysLTs exhibit several biological functions through two specific G protein-coupled receptor (GPCR) subtypes termed cysteinyl leukotriene type 1 receptor (CysLTR1) and CysLT type 2 receptor (CysLTR2) (78). CysLTR1 binds LTD_4 with a higher affinity than LTC4, whereas CysLTR2 binds both LTC_4 and LTD_4 with equal affinity (88). Another CysLT receptor, GPR99 has been recently identified to preferentially bind LTE_4 (90).

CysLTR1 and CysLTR2 signalling is mediated through intracellular mobilization of calcium (91). CysLTR2 is about 38% identical to CysLTR1 and are both expressed in the same tissues such as smooth muscle and substantially in myeloid cells. There is an partial

overlap of the two receptors, which suggest that they may have complementary and distinct functions (83). It has been shown that IL-4 significantly induces the expression of CysLTR1 and CysLTR2 at a protein and RNA level in monocytes and eosinophils. However, CysLTR2 expression is also induced in T cells and B cells when stimulated with IFN-γ (92). It has also been suggested that IL-4 and IL-13 can modulate CysLTR1 expression in human monocytes and macrophages, and their responsiveness to LTD_4 (93). There is very limited data describing the function of GPR99 or CysLTR3, however has been recently shown that during aeroallergen inhalation it regulates airway brush cells expansion and function through binding to the ligand, LTE_4 (94).

Besides their function on immune regulation, CysLT receptors have been suggested to have a functional role in signalling pathways. Once CysLTs bind to their specific CysLT receptors, they activate a cascade of downstream signalling pathways. Activation of CysLTR1 induces PI3K-Akt signalling, which leads to the nuclear translocation of B-catenin (β-cat) and the activation of target genes that include cyclin D1, COX-2 and c-Myc (Figure 3) (95). Akt/PKB activation leads to the activation of the IκB kinase (IKK) complex resulting to the subsequent degradation of IκB protein leading the translocation and activation of nuclear factor κB (NF-kB) (96). The signalling of CysLTR1 and CysLTR2 can activate the phospholipase C (PLC) and Ras-Raf-MEK-ERK pathways (95, 97, 98). This cascade of events results in the translocation of Erk 1/2 leading to the activation of genes that play a role in proliferation, migration and survival. Ligation of the CysLT receptors can alternatively activate the PKC pathway and the transcription factor cAMP response element-binding protein (CREB) (95).

Though the CysLT receptors may play a role in the activation and regulation of distinct signalling pathways, there is also a cross-regulation that occur between these receptors. Binding of LTC4 or LTD4 to CysLTR2 negatively regulates the signalling of CysLTR1 through the receptor heterodimerization. A newly identified CysLT receptor, GPR17, has also been reported as a ligand-independent negative regulator of CysLTR1 (98, 99).

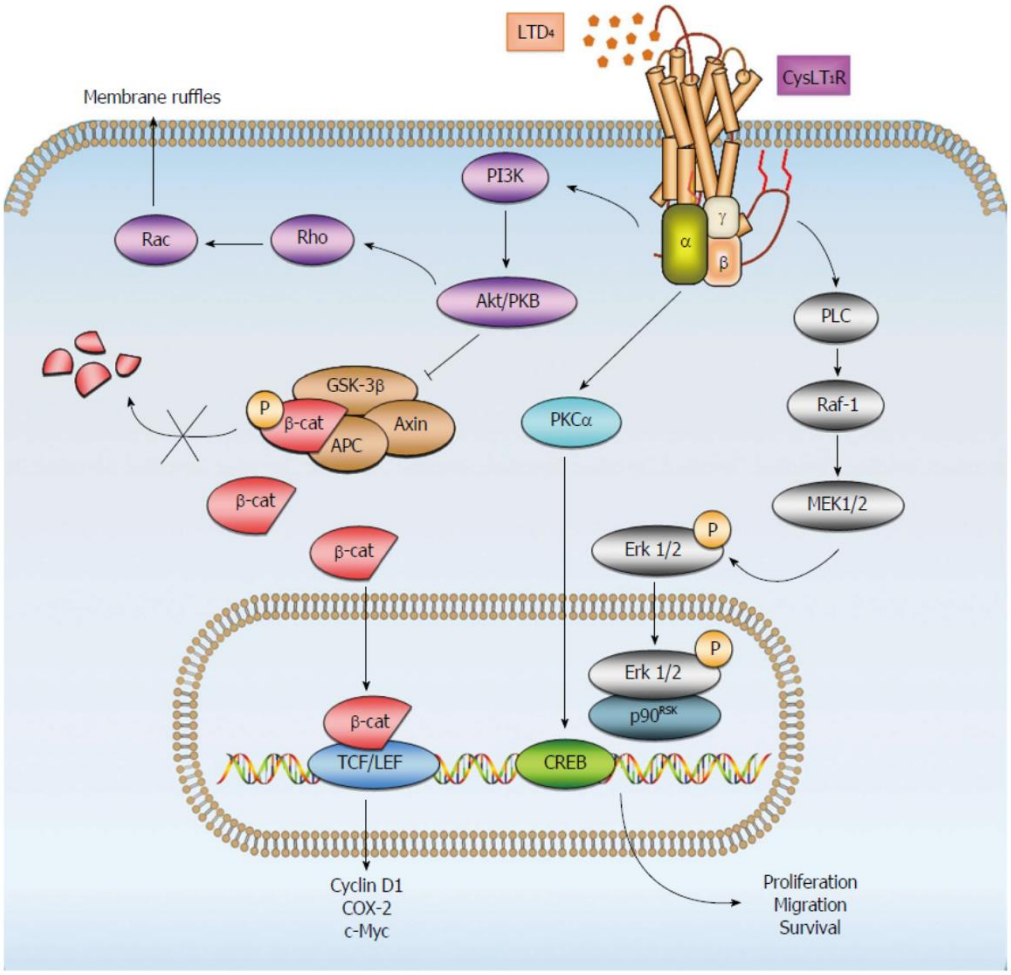

Figure 3: CysLTR1 cellular signalling pathways. Activation of cysLTR1 through binding of LTD_4 leads to the activation of PI3K resulting in β-cat membrane translocation and cyclin D1, COX-2 and c-Myc activation. Binding of LTD_4 to CysLTR1 may also induce membrane ruffles through P13K signalling. CysLTR1-LTD4 signalling activates the PLC pathway with a subsequent induction of MEK1/2, and alternately activates PKC and the transcriptional factor CREB resulting to cell proliferation, migration and survival (95).

4. Eicosanoids role in inflammation and related diseases

Eicosanoids do not only have a role in inflammatory diseases, but leukotrienes, particularly cysLTs, have been shown to play a significant role during brain injuries and in cognitive functions. Farias et al.(100), co-cultured blood neutrophils with rat neurons, glia cells and stimulated them with calcium and they detected high levels of LTC_4, LTD_4 and LTE_4. This suggested that glia cells and neurons participate in a transcellular biosynthesis of leukotrienes. They hypothesized that the LTC_4 formation may be unveiled in the genesis and progression of inflammatory responses as a result of brain injury (100). In 2009, using fluid percussion injury to model traumatic brain injuries, they showed that cysLTs are upregulated within an hour after the injury. The increased production of cysLTs was

contributed by neutrophils through a transcellular biosynthesis mechanism. They further demonstrated that treating the rats with MK-886 (FLAP antagonist), resulted in decreased cysLT synthesis, which correlated with a reduction in brain lesions (101). The same research group conducted another study demonstrating that leukotriene synthesis plays a role in the pathophysiology of traumatic brain injury (102). In a healthy brain, leukotrienes cannot be detected, however in a mechanism that involves neutrophils and brain cells, leukotrienes are rapidly synthesized in the brain after a traumatic injury (101, 103). By blocking leukotriene synthesis using MK-885, the researchers demonstrated that rats treated with FLAP inhibitor before or after brain injury had decreased leukotriene production, brain edema, attenuated brain-blood barrier disruption and deficits. MK-886 treatment also improved post-injury spatial learning and the memory of the rats (102). These results indicate that leukotrienes are not only released during brain injury, but they also affect cognitive functions, and targeting their production reverses their effect on brain and cognitive deficits.

Lai et al. 2014, conducted a study to investigate the effect of montelukast targeting CysLTR1 on spatial learning and memory in bilateral intracerebroventricular infusions of $A\beta_{1-42}$-injected mice. $A\beta_{1-42}$ is used as experimental model for Alzheimer's disease (AD) for testing learning and memory. They found that mice injected with Aβ1-42 had learning and memory deficits which were accompanied by increased inflammatory and apoptotic responses, supported by the NF-κβ p65, TNF-α and IL-1β increase, caspase-3 activation and decreased Bcl-2 in the hippocampus and cortex. The Aβ1-42 injection also increased CysLTR1 expression in both these regions of the brain. The effects of Aβ1-42 were reversed when mice were treated with montelukast. Their results suggested that the reversed Aβ1-42-induced cognitive defects by montelukast is partially contributed by inhibition of neuronal inflammation and apoptosis which is mediated by CysLTR1 signalling (104).

4.1. CysLTR1 in asthma and other inflammatory diseases

Eicosanoids (leukotrienes and prostaglandins) have been well elucidated in asthmatic inflammation. They have also been shown to play a role in aspirin-exacerbated respiratory disease (AERD). In a study by Mastalerz *et al.* 2014 (105), to investigate whether inhaled lysyl-aspirin on sputum supernatant induces eicosanoid production during bronchial challenge test. They compared asthmatic patients with hypersensitivity to nonsteroidal anti-inflammatory drugs with asthmatic patients that are aspirin tolerant. AERD patients had

significantly higher LTD_4, LTE_4, PGD_2. After challenging the patients with lysyl-aspirin, there was an increase in LTD_4 and LTE_4, while PGE_2 and LTB_4 decreased in AERD subjects (105).

A study conducted by Zhu *et al.* (106), aimed to identify whether the leukotriene receptor antagonist (LTRA) targets are present in the bronchial mucosa and if they are upregulated in asthma patients. LTRA is a type of medication or antagonist which works as leukotriene-related enzyme inhibitor or leukotriene receptor antagonist, opposing the function of the leukotriene mediators. These LTRAs include CysLTR1 antagonists (montelukast, zafirlukast, and pranlukast) and 5-LO antagonists (zileuton and Hypericum perforatum), and are used in asthma treatment (107, 108). They demonstrated bronchial mucosal eosinophils, neutrophils, mast cells, macrophages, B lymphocytes and plasma cells expressed CysLTR1, but T lymphocytes did not. They also showed that the inflammatory cells in patients with stable and exacerbated asthma was greater than in non-smoking and nonatopic control subjects. This study demonstrated that the LTRA targets are present in the bronchial mucosa and are upregulated during asthma and exacerbated asthma (106).

Bouduad *et al*. 2018, conducted a study to investigate whether IL-33 is able to regulate cysLT receptor expression (109). It has been demonstrated that IL-33 plays a role in the initiation of immune responses during an asthmatic reaction (110–113). As mentioned, cysLTs have been well studied in asthmatic responses and it has also been shown that CysLTR1 is highly upregulated during asthma in leukocytes (106) however, the interaction between IL-33 and CysLTR1 was not fully understood. Boudaud *et al*., showed that IL-33 in human peripheral blood lymphocytes (PBLs) upregulated the expression of CysLTR1 at protein but not at mRNA level. The increased CysLTR1 protein expression was consistent with how these cells responded to LTD_4 through calcium mobilization and CD4 T cell migration. CysLTR1 expression was also increased in naïve and memory CD4 T cells in response to IL-33, opposing what was shown by Zhu *et al*., 2005 (106). This response was independent of APCs (109), and could be a therapeutic target for treatment of asthma.

CysLTR1 expression is not only induced by its binding ligands, various studies have demonstrated that CysLTR1 expression can be regulated by certain cytokines besides IL-33. For instance, CysLTR1 is upregulated by IL-13 and IL-4 in human monocytes and macrophages (114), IL-5 in eosinophils differentiated from HL-60 cells (115), TGF-β and IL-13 in bronchial smooth muscles (116) and IFN-γ in human airway monocytes and peripheral cells (117, 118). In 2015, Thivierge *et al*. (119), demonstrated that T cell

expression of CyLTR1 is elevated by allergen in a mechanism that involves secretion of IL-4, in turn, this would promote the response of CD4 T cells to cysLTs and Th2 immune activation (119). However, besides IFN-γ being proven to enhance the expression of CysLTR1 in some immune cells, it has not been demonstrated whether it can upregulate CysLTR1 in Th1 cells. Although CysLTR1 and its ligands induce a robust Th2 immune response, it would be essential to therefore, demonstrate whether they play a role in Th1 cell activation.

4.2. Role of eicosanoids in microbial infections

The function of PGE_2 has been studied in a handful of microbial infections. During acute mucosal infection with *Toxoplasma gondii* in mice, it has been demonstrated that production of PGE2 is associated with inflammatory monocytes. Neutrophil activation was inhibited by these monocytes in a PGE2-dependent manner. Inhibition of PGE2 by indomethacin or celecoxib increased the activation of neutrophils and led to host mortality post *T. gondii* infection. These results therefore, suggest that PGE2 interaction with inflammatory monocytes is required for the modulation of the neutrophil mediated pathology during pathogen-induced inflammation (120).

It has also been reported that treatment of alveolar macrophages with PGE2 during *Klebsiella pneumoniae* inhibited their killing ability, and this effect was reversed by addition of adenylyl cyclase antagonist, which mimicked the actions of EP2 and EP4 receptor inhibitors. Through EP2/EP4 receptors, PGE_2 suppresses the microbicidal action of alveolar macrophages, which is associated by reactive oxygen intermediates (ROI) generation, activation of cAMP, protein kinase A, and blocking of NADPH oxidase phosphorylation and translocation to the phagosome membrane (121). This indicates that PGE_2 suppresses the killing effect of macrophages by inhibiting the phagocytic activity in *Klebsiella pneumoniae* infections. During *K. pneumoniae* infection in LT deficient mice were more susceptible to infection compared to wildtype mice however, there were no defects in the recruitment of neutrophils to the lung. However; it was observed that bacterial phagocytosis and clearance were impaired in alveolar macrophages of the deficient mice. This defect was reversed by addition of LTB_4 *in vitro*, suggesting that LTs have a significant role during bacterial pneumonia in mice model (122).

Cyclooxygenase 2 (COX2), the enzyme responsible for PGE_2 synthesis, has also shown to play a role during bacterial infection. Moreno *et al*., showed that during *Mycobacteria*

tuberculosis (*Mtb*) time course infection in mice, PGE_2 expression is upregulated in a time-dependent manner. They also observed that when prostaglandin production is suppressed during TB infection, there were differences in the pathology and immune response at early and late phase of infection. Blocking COX2 during early *Mtb* infection increased TNF-α and IFN-γ expression while it decreased IL-1α and iNOS expression. However, during the chronic phase of infection, IL-1α, TNF-α, IFN-γ and iNOS expression were increased, whilst the IL-4 and IL-10 expression was suppressed (123). These results suggest that inhibition of prostaglandin production by COX, particularly PGE_2, has a significant role in the protection and disease progression during TB infection in mice.

Leukotrienes have also been shown to play a role on other bacterial infections. For instance, infection of murine mast cells with *Escherichia coli* (*E. coli*), increased significant amounts of LTB_4 and LTC_4. Moreover, using mast cell deficient mice, it was observed that leukotrienes are responsible for the recruitment of neutrophils to the site of infection and subsequently resulting in bacterial clearance (124).

More recently, it has been observed that *Brucella abortus* (*B. abortus*) upregulates the expression of 5-LO during infection in mice, upregulating the production of LTB_4 and lipoxin A4 (also a product of lipoxygenase). When comparing wildtype to 5-LO deficient mice, liver and spleen bacterial burden were decreased with reduced liver pathology. This was accompanied by increased production of IL-12, IFN-γ and iNOS during time course infection (125). This demonstrated that 5-LO plays a role in host susceptibility during *B. abortus* infection by suppressing the host protective Th1 immune response.

5. Role of Eicosanoids during *Listeria monocytogenes* infection

Hutchison *et al*., 1987, investigated whether the suppression of *LM* phagocytosis by macrophages was due to the low-molecular weight component (prostaglandin) in spleen culture supernatants. This study demonstrated that PGE_2 treated peritoneal macrophages able to supress phagocytosis of *LM* and addition of PGE_2 inhibitor, indomethacin, compelety abrogated the inhibitory effect on the phagocytosis. These results suggested that prostaglandins, especially PGE_2, are modulators of *LM* phagocytosis by macrophages (126).

In 2008, Noor *et al*., conducted a study where they investigated the regulation of AA release and production of eicosanoid by resident macrophages during bacterial infection. Their study elaborated that *LM* infection activated cPLA2α in resident peritoneal macrophages,

which resulted in the release of AA and production of eicosanoids, particularly LTC_4 and PGE_2. Furthermore, the activation of cPLA2α involved the virulence factor, LLO, and the host toll-like receptor 2 (TLR2). This study also demonstrated that cPLA2α activation has an important role in the suppression of TNF production by *LM*-infected macrophages, suggesting that eicosanoids play a role in regulating immune responses during *Listeria* infection (127).

More recently, Pitts *et al*., 2019, demonstrated that PGE_2 inhibit neutrophil-mediated clearance of *LM* (128). PGE_2 has been shown to inhibit reactive oxygen species (ROS), IL-12 and TNF production (120, 121, 129). Pitts *et al*., hypothesized that increased PGE_2 production in BALB/cByJ mice microenvironment will inhibit liver ability to kill *LM*, which will contribute to susceptibility of the mice. PMN cells harvested from BALB/cByJ or C57BL/6J mice had decreased ability to kill *LM* after pre-treatment with PGE_2 in vitro. Treatment with PGE_2 slowed PMN migration to the chemoattractant to LTB4, decreased PMN uptake of *LM*, and respiratory rupture by PMN was inhibited compared to cells that were not treated. Their results demonstrated that secretion of PGE_2 can inhibit the effector functions of neutrophils, resulting in reduced bacterial (*LM*) killing (128).

Eicosanoids, particularly prostaglandins, have been shown to regulate immune responses to *LM* infection. This could be contributed by the modulation of the phagocytic effects of macrophages during bacterial infection, suppression of chemokine production and regulation of TNF, IL-6 and IL-10 levels (130–132). However, the effect of cysteinyl leukotrienes on *LM* infection has not been elucidated. The role of CysLTR1 has been well elucidated in the etiology of airway inflammation and asthma (133). However, the function of the receptor and its associated ligands in bacterial infection is still unknown. Therefore, we aim to elucidate the role of CysLTR1 in disease progression in mice and macrophages infected with *Listeria monocytogenes*.

6. Rationale of the study

As mentioned, *Listeria monocytogenes (LM)* is a facultative intracellular Gram-positive bacterium that is the causative agent of Listeriosis (6). Macrophages and dendritic cells can phagocytose an invading microorganism and induce an innate inflammatory response. However, the DCs play a central role in the transition between the innate and the adaptive immunity (70). The production of eicosanoids is an early response to microbial infection that can regulate the innate immune response (134). *LM* stimulates the release of the

arachidonic acid and eicosanoid production by peritoneal macrophages and the activation of group IVA cytosolic phospholipase A_2. Increased production of endogenous mediators such as cytokines and eicosanoids can activate APCs, particularly DCs, which in turn activate the adaptive immune response (70).

As mention above, South Africa recently experienced a Listeriosis outbreak which resulted in 674 reported cases, with 42% neonates cases (13). In 2015, South Africa was ranked amongst the highest fertility rates in African countries (135), with an estimate of infant mortality rate in 2017 being 32.8 per 1000 live births (136). Furthermore, Africa continent has the highest prevalence HIV, with approximately 12,6% of South African living with HIV and women are mostly infected (136). It is well known that Listeriosis mostly affects immunocompromised individuals, therefore, HIV infected patients are at higher risk of *LM* infection.

Diagnosis of Listeriosis is difficult and can result in delayed treatment. This is because, Listeriosis can only be diagnosed by culturing *LM* bacterium from the patient's blood, cerebrospinal fluid or amniotic fluid, on a medium that is selective for this bacterium with no serological tests available for diagnosis of Listeria. Treatment of Listeriosis includes intravenous (IV) antibiotic administration to prevent, stop or slow down progression of severe disease. The two antibiotics used to treat this disease are IV ampicillin and Bactrim, however, it has been advised that patient treatment should be individualized for optimal outcomes (13).

The South African Listeriosis outbreak increased infant mortality, the chances of death among pregnant women, spontaneous abortions and neonate deaths in South Africa. The increased morbidity and mortality could be mostly contributed by the lack of rapid diagnostic tools for *LM* and delayed treatment. Therefore, a need for development for new interventions to diagnose, treat *Listeria monocytogenes* infections and to better understand the disease pathogenesis. Hence, this study is aimed at investigating host responses to *LM* infection, specifically a pro-inflammatory lipid modulator signalling pathway, namely cysteinyl leukotriene signalling. We aim to delineate the role of cysteinyl leukotrienes in pathogenesis of listeriosis by using cysteinyl leukotriene receptor knockout mice as a tool.

We conducted a pilot mortality study where CysLTR1$^{-/-}$ (KO) and CysLTR1$^{+/+}$ (WT) mice were infected with *LM*. Here, the CysLTR1-deficient mice presented with increased survival compared to wildtype mice during infection (Figure 4A). Furthermore, primary

bone-marrow derived macrophages were infected with *LM*, the gene expression of CysLTR1 and LTC_4S (the enzymes responsible for cysteinyl leukotriene synthesis) were both upregulated early after infection at one- and three-hours however transient as it decreased at six hours post-infection (Figure 4B & 4C). This suggests that the Cysteinyl leukotriene pathway might play a role in disease progression and/or control during *LM* infection. We therefore hypothesize that CysLTR1 deletion in mice will result in a host protective phenotype during *LM* infection.

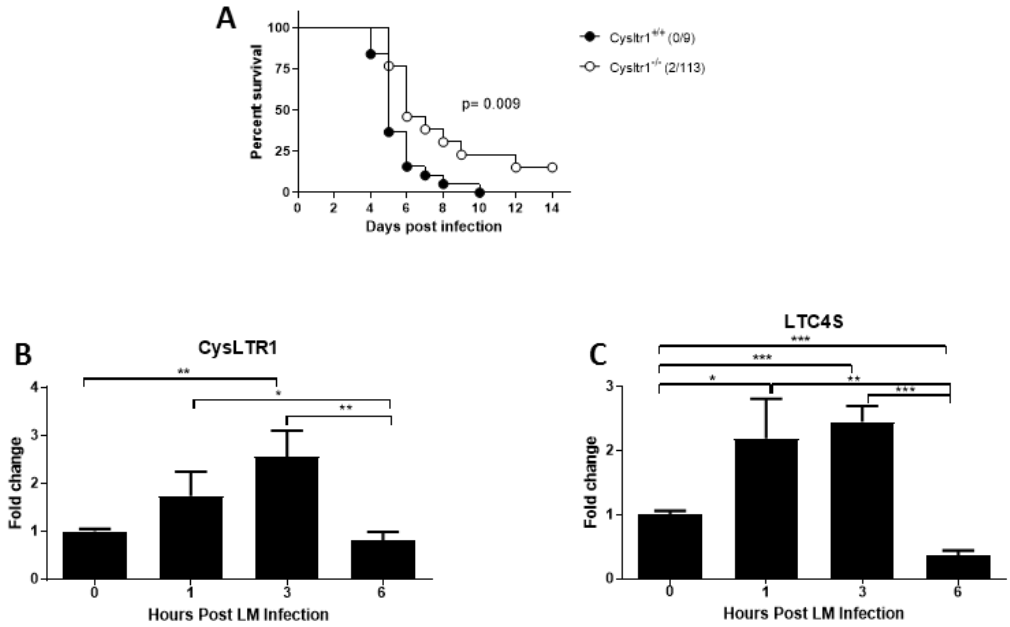

Figure 4: Mouse survival and CysLTR1 and LTC_4S expression following *L. monocytogenes* infection. A) $CysLTR1^{-/-}$ and $CysLTR1^{+/+}$ mice (C57BL/6) were infected with $1x10^6$ CFUs of *LM* and monitored over 15 days post-infection for survival, Statistically analysed by log-rank (Mantel-Cox) test **B-C)** C57BL/6 wildtype mouse bone marrow derived macrophages (BMDMs) were infected with MOI of 10 to determine the gene expression of CysLTR1 and LTC_4S using quantitative PCR. Error bar denotes mean ± SEM and analysed using the One-way ANOVA multiple comparison test ($p < 0.05$ *, $p < 0.01$**, $p < 0.001$***).

6.3.Aim and Objective

6.3.1. Aim of the study

We aim to understand the role of cysteinyl leukotriene signalling on disease progression during *Listeria monocytogenes* infection

6.3.2. Objectives

1. Measure the RNA expression levels of CysLTR1 in mice and macrophages by time course infection of wildtype mice and bone marrow-derived macrophages with *LM*

2. Characterization of CysLTR1-deficient mice and their littermate wild-type controls at naïve state
3. Determine the effect of CysLTR1 deletion on macrophage ability to control *LM* infection
4. Mortality and time kinetic studies to evaluate the effect CysLTR1 deletion during *LM* infection *in vivo*

CHAPTER 2: MATERIALS AND METHODS

1. Ethics statement

This study was performed under the strict guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). All mouse experiments conducted according to the protocol approved by the Animal Ethics Committee of the Faculty of Health Science, UCT (Protocol number: AEC 019/031). Mice were euthanised by halothane inhalation and death confirmed by cervical dislocation at the humane endpoint or terminating the experiment.

2. Mouse strains

CysLTR1 deficient mice ($Cysltr1^{-/-}$) were generated by breeding heterozygous ($Cysltr1^{-/+}$) female and male mice. CysLTR1 heterozygous ($Cysltr1^{-/+}$) mice were gifted by Dr Yoshihide Kanaoka of the Department of Medicine, Harvard Medical School (137). All mice were kept in ventilated cages under specific-pathogen-free condition in the biomedical animal facility of the UCT Faculty of Health Science. Mice were between 8-12 weeks of age and sex matched for each experiment, unless stated otherwise.

3. Mouse genotyping

DNA extraction from mice tails: DNA from mice tails was extracted by incubating tails overnight in lysis buffer at 56^{o}C overnight rotation and then centrifuged at 10000 rpm for 10 to 12 minutes. Supernatants were collected and added to isopropanol tubes, gently mixed by inverting the tubes. The tubes were then centrifuged for 6 minutes at 10000 rpm, the supernatants were removed without disturbing the DNA pellets. 70% cold ethanol and incubated for 10 minutes. To get rid of the excess ethanol, the tubes were centrifuged at 10000 rpm for 2 minutes and allowed to air dry. 500µl PCR water was added and the tubes were incubated for 20 minutes on a heating block to resuspend the DNA.

Genotyping by PCR: To determine the mice genotypes, DNA from mice tails was genotyped by PCR using the following reaction mix: the DNA sample was mixed with 10X buffer, 10mM dNTPs, 25mM $MgCl_2$, 5U/µL supertherm Taq, H_2O and 6.25µM wildtype or knockout primers. The PCR reaction was ran under the following conditions 94^{o}C denaturation for 2 minutes, 35 cycles of amplification (94^{o}C for 20 seconds, 58^{o}C for 30 seconds and 72^{o}C for 45 seconds), and extended annealing for 5 minutes at 72^{o}C. CysLTR1 deletion specific primers (**5'-ATCTTGTTCAATGGCCGATCCCAT-3'** and **5'-AAAACAATGACGTGCACTATAAAG-3'**) and the wildtype specific-primers (**5'-**

AAAACAATGACGTGCACTATAAAG-3' and **5'-AATCATGTATACTTGGAAGGCTGA-3'**) were used to confirm CysLTR1 deletion.

4. Bacterial culture

L. monocytogenes (virulent EGD strain) was cultured in Tryptic-Soy Broth (TSB) at 37ºC with overnight shaking at 170 rpm. Overnight culture was sub-cultured in fresh TSB media and grown to mid-log phase (OD reading of 0.6 to 0.8) at 37ºC. The bacterial culture was preserved in 20% glycerol and stored in -80ºC until use. The stock concentration bacteria were determined by plating random vails. The dose was determined by plating inoculum before and after performing infection.

5. Infection of mice with *L. monocytogenes* and determination of bacterial loads

Mortality studies: CysLTR1$^{-/-}$ and CysLTR1$^{+/+}$ mice on C57BL/6 background (10 per group, sex-matched and 8-12 weeks old) were infected with about 1x10^6 CFUs/200µL intraperitoneally and monitored for 15 days to compare the survival rate between the two groups. While CysLTR1$^{-/-}$ and CysLTR1$^{+/+}$ mice on BALB/c background were infected with about 2x10^5 CFUs/200µL intraperitoneally. When mice showed weight loss of 20% to their initial body weight and/or show signs of severe sickness (lack of grooming, hunched back, pale extremities) the mice were euthanized, and death confirmed by cervical dislocation.

Time-course studies: CysLTR1$^{-/-}$ and CysLTR1$^{+/+}$ mice on C57BL/6 background (5-6 mice per group) were infected with about 5x10^5 CFUs/200µL *L. monocytogenes* intraperitoneally and sacrificed 3 days and 7 days post infection (dpi). Bacterial burden in the liver and spleen of *LM* infected mice were determined at 3- and 7dpi. The organs from euthanized mice were aseptically removed, weighed and homogenized in 0.1% Tween-80 in saline. Neat, 10^1, 10^2 and 10^3-fold dilutions were plated on Tryptic-soy agar (TSA) plates and incubated at 37ºC for 24 hours to determine the bacterial burdens.

6. Histopathology of the liver, spleen and lung sections

Portions of the spleen, liver and lung from naïve mice were fixed in 10% neutral buffered formalin (NBF, 3.8-4% formaldehyde, 4 g/L NaH_2PO_4, 6.5 g/L Na_2HPO_4, pH 7.0) and cut into 3 sections (30-45 µm apart) and stained with haematoxylin and eosin (H&E) to determine any differences at naïve state. *L. monocytogenes*-infected liver and spleen tissue were fixed in 10 % NBF, cut into 3 sections (30-45 µm apart) and stained with H&E for

histopathology. Image acquisition and quantification was performed on Nikon 90i microscopy using NIS advanced software.

7. Cytokine responses in organs at naïve state and post *L. monocytogenes* infection

Homogenates of the lungs, liver and spleen of each mice were centrifuged at 3000 rpm for 5 minutes. The supernatants were collected and the levels of IL-17, IL-10, TNF (BioLegend); IFN-β, IFN-γ, IL-4, IL-6, IL-12p40, IL-12p70, IL-2, GM-CSF, IL-5, MCP-1/CCL2, TGF-β (BD Biosciences); IL-1α, IL-1β, CCL5/RANTES, CXCL-10/IP-10 (R&D Systems) were all measured by ELISA according to manufacturers' instructions and nitric oxide production using Griess reagent (138).

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8. Immune cell populations in tissues at naïve and *LM*-infected mice by flow cytometry

Single cell suspensions for the lung were obtained by digesting it in digestion buffer, DMEM containing 0.18 mg/ml Collagenase Type 1 (Sigma Aldrich) and 0.002 mg/ml DNAse I (Sigma Aldrich). The samples were rotated for 1 hour at 37^{o}C, cells passed through 100 and 70 µm sieve in tandem, then centrifuges cells at 1200 rpm for 10 minutes at 4^{o}C. The samples were then incubated with red blood cell (RBC) lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) to remove contaminating erythrocytes. The cells were resuspended in complete media (DMEM + 10% FCS+ penicillin/streptomycin) and counted by Trypan Blue exclusion assay to determine the total number of live cells.

Liver single cell suspensions were obtained by digesting in digestion buffer (DMEM + 5%FCS containing 0.11 mg/ml Collagenase type I and 0.11 mg/ml Collagenase type II and 0.001 mg/ml DNAse I) and incubated at 37^{o}C for 30 minutes. The cells were passed through 100 and 70 µm cell strainer in tandem and centrifuged at 1200 rpm for 5 min. The cells were later resuspended in 3 ml PBS + 3% FCS and 1.7mL isotonic Percoll was added on top, followed by gentle mixing by inversion, then centrifuged at 500 rpm for 10 minutes without brakes. The cells were then incubated in RBC lysis buffer for 5 minutes at room temperature, centrifuged at 1200 rpm for 5 minutes. The cells were resuspended in 1mL complete media and counted by Trypan Blue exclusion assay to determine the total number of live cells.

The single cell suspensions for the spleen and the thymus were prepared in complete media by meshing through 70 µm and 40 µm sieve in tandem and centrifuged at 1200 rpm for 5 minutes. The cells were incubated with RBC lysis buffer and centrifuged at 1200 rpm for another 5 minutes. The pellets were washed once with complete media and resuspend in 5mL media, then the cells were counted.

All the organ single cell suspensions (about $1x10^6$cells/well) were stained with either myeloid antibody mix (575V Viability dye, CD64 PeCy7, Ly6C PercP-Cy5.5, Cd11b V450, MHC II A700, CD103 PE, CD11c APC, SiglecF APC-Cy7, Ly6G FITC) or lymphoid antibody mix (575V Viability dye, CD4 V500, CD44 PE, NK1.1 APC-Cy7, CD3 A700, CXCR5 PeCy7, CD62L V450, TCRγδ PE-Texas Red, CD19 PercP-Cy5.5, CD8 APC, KLRG1 BV786, PD-1 FITC, Ki67 PE, all from BD Biosciences) for flow cytometry analysis of various immune cell populations and their activation status. The samples were acquired with BD LSRII and analysed using FlowJo™ Software version 10.6.

9. Intracellular staining of liver and spleen single cells

Liver and spleen single cells (3 and 7 dpi, $2x10^6$ cells/well) were seeded on a 96-well U-bottom plate 200µL/well. The cells were either stimulated with 20ng/mL PMA, 1µg/mL ionomycin and 2µM monensin or left unstimulated. Monensin was used to block the secretion of intracellular cytokines into the media. The cells were then incubated for 6 hours at 37^{o}C. The cells were transferred into a V-bottom plate and centrifuged at 500g for 5 minutes at 4^{o}C and washed the cells with 1xPBS. We stained the cells with extracellular antibodies (575V viability dye, CD3 PerCP, CD4 BV510 and CD8 APC), then permeabilized the cells for 30 minutes at 4^{o}C. We then stained the cells with intracellular antibodies (TNF PE, IFN-γ A700, perforin FITC and granzyme B V450, all from BD Biosciences) for 45 minutes at 4^{o}C in the dark. We performed flow cytometry acquisition using BD LSRII and analysed using FlowJo™ Software version 10.6.

10. Splenocyte re-stimulation for cytokines *ex vivo*

To re-stimulate splenocytes, we pre-coated a 48-well plate with 100µL anti-CD3 (20µg/mg) and incubated the plate at 37^{o}C for 30 minutes. After incubation, we pipetted out the anti-CD3 and added $1x10^6$ cells/well from spleen single cells of 3- and 7-day infected mice. We infected or stimulated the cells with heat-killed *LM* (HKLM) with a MOI of 1:10 and left cells in media. The plate was then incubated at 37^{o}C for 72 hours. After 72 hours, the plate was centrifuged at 1200 rpm for 5 minutes to separate any floating

cells and supernatants were collected into a new plate. We measured IFN-γ, IL-2, IL-4, IL-17, IL-10 and TGF-β secretion by re-stimulated splenocytes by cytokine ELISA.

11. Generation of Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were generated by flushing out bone marrow precursor cells from the mice tibia and femur bones. The cells were cultured at a concentration of about $13x10^6$cells/mL in 150 cm^2 sterile tissue culture grade CellStar (Greiner Bio-One) petri dishes with 50mL of macrophage differentiation media (DMEM containing 10% heat inactivated fetal calf serum, 5% heat inactivated horse serum, 30% L929 conditioned medium, 2mM L-glutamine, 1 mM Na-pyruvate, 100 µg/ml penicillin-streptomycin and 50µM β-mercaptoethanol) . The cells were incubated at 37ºC for 10 days under 5% CO_2 conditions with 50 ml macrophage differentiation media top-up on day 5.

12. Infection of macrophages and determination of bacterial growth

BMDM infection with *L. monocytogenes*: On day 10, macrophages were harvested and seeded $2x10^5$cells per well in 96-well plates for infection with *L. monocytogenes*. The cells were stimulated with 100nM LTD4 (Sigma Aldrich) or left unstimulated (unless stated otherwise) overnight at 37ºC under 5% CO_2 conditions.

Cells were then infected with *L. monocytogenes* with a multiplicity of infection (MOI) of 10 and incubated at 37ºC for 1, 3, 6 and 12 hours for determination of intracellular bacterial load. The supernatants were collected at each time point and stored at -20ºC for downstream ELISA applications.

Cells were lysed by adding 100µL 0.1% Triton X-100 in PBS and incubated at room temperature for 5 minutes to facilitate the release of intracellular bacteria. The lysates were serially diluted in 1x PBS and plated on TSA plates. The plates were incubated at 37ºC overnight and the colony forming units (CFUs) counted to determine bacterial burden.

13. Cytokine and Nitric oxide produced by macrophages post *L. monocytogenes* infection

Supernatants were thawed and cytokines such as TNF, IL-1α, IL-6, IL-12p40, and IL-10 were measured by ELISA and nitric oxide production using Griess reagent (138).

14. RNA Extraction

To extract RNA, tissue single cells or BMDMs were collected in 500µL Trizol. 1/5 volume of chloroform:isoamyl alcohol (49:1) mixture was added and vigorously mixed for 10 seconds and incubated for 2 minutes at room temperature. The samples were then centrifuged at 4^{o}C for 10 minutes at maximum speed (18000 rpm). The aqueous phase was removed and added into new tubes, half the amount of the original Trizol volume of chloroform:isoamyl was added and incubated for 2 minutes on ice. The samples were then centrifuged at 4^{o}C at maximum speed. The aqueous phase was transferred into new tubes and added 1/10 volume of sodium acetate, linear polyacrylamide (1µL/1mL Trizol) and isopropanol (half of the original Trizol volume). The samples were incubated at -20^{o}C overnight to precipitate the RNA. The next day, the samples were centrifuged at maximum speed for 20 minutes at 4^{o}C and supernatants were removed without disturbing the RNA pellets. The pellets were washed using ice cold 70% ethanol (as original Trizol volume), and vortexed to resuspend the pellets. The samples were centrifuged at 4^{o}C for 10 minutes at 7000g, removed the supernatants and washed with 500µL 70% ethanol and vortexed to mix. The resuspended RNA pellets were centrifuged again at 7000g for 10 minutes to remove excess salts. The pellets were air dried until the they were clear without over drying them. The pellets were resuspended in RNAse-free water and incubated at 60^{o}C for 10 minutes, with flicking every few minutes. The RNA concentration was measured using the ThermoFisher NanoDrop One UV Spectrophotometer.

15. Quantitative Real-time PCR

The cDNA from BMDMs and organ single cells were synthesized by using Transcriptor First Strand cDNA Synthesis Kit from Roche according to manufacturer's instructions. Quantitave RT-PCR was done to confirm the gene expression of Cysltr1, Cysltr2 and Ltc4s in the samples using specific set of primers represented in the table below.

Table 1: Set of primers used for the quantification of Cysltr1, Cysltr2 and Ltc4s mRNA expressions by qPCR.

Primer name	Forward (5'-3')	Reverse (5'-3')
Cysltr1	5'-TCTGTTGTGGGTTTCTTTGGC-3'	5'-GGAAGGCTGATTTCTCATGGT-3'

Cysltr 2	5'-TGTCACCAGTGTCAGGAGTG-3'	5'-ACTTTTGAGGACTCAGCTCCAA-3'
Ltc4s	5'-CAAGCCTACTTCTCCCTACAGGTG-3'	5'-GTTTACCTGGGCTCGGAAGAC-3'

16. Statistical analysis

All the data was analysed using GraphPad Prism version 8.0.2, statistically analysed using unpaired student t-test unless stated otherwise in figure legends. A P-value under 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

3.1. Expression of CysLT receptors and Ltc4s during *Listeria monocytogenes* infection

Cysteinyl leukotriene receptors (CysLTR1 and CysLTR2) are known to be involved in allergic responses (106–108, 133), however, it is unknown whether CysLTs play a role in bacterial infection. To determine whether bacterial infection could induce expression of CysLT receptors and the enzyme responsible for CysLT production, we infected mice with *L. monocytogenes* and measured mRNA expression of Cysltr1, Cysltr2 and leukotriene C_4 synthase (Ltc4s) in the liver and spleen. We investigated the mRNA expression of these genes in the liver and spleen since *L. monocytogenes* mostly disseminates in these organs (9, 48, 139). *L. monocytogenes* infection in mice resulted in an upregulation in Cysltr1, Cysltr2 and Ltc4s mRNA expression in the liver and spleen (Figure 1A and 1B), mostly at later stages of infection, suggesting that CysLT signalling might be affected during the later stages of *Listeria* infection in mice. We further investigated this effect at a cellular level to determine whether *LM* could induce the expression of these genes in bone marrow-derived macrophages (BMDMs). As *in vivo*, Cysltr1 and Ltc4s mRNA expression was upregulated at 3 hours post-infection in macrophages (Figure 1C), however in contrast to *in vivo*, Cysltr2 expression in BMDMs remained unaffected *in vitro*. At a macrophage level, cysLT signalling is affected at early stages of *LM* infection.

We were able to show that *LM* infection in mice and macrophages can induce Cysltr1, Cysltr2 and Ltc4s expression, we further asked whether this was dependent on live *LM* or its antigen was sufficient to activate CysLT signalling. We then infected BMDMs with heat-killed *LM* (HKLM) and measured mRNA expression of these genes. Interestingly, HKLM modestly increased Cysltr1 mRNA expression at 3 hours whereas downregulated at 12 hours post-infection, while it downregulated Cysltr2 and had no effect on Ltc4s mRNA expression (Figure 1D). These results suggest that viable *L. monocytogenes* is required to induce cysLT signalling in macrophages and mice.

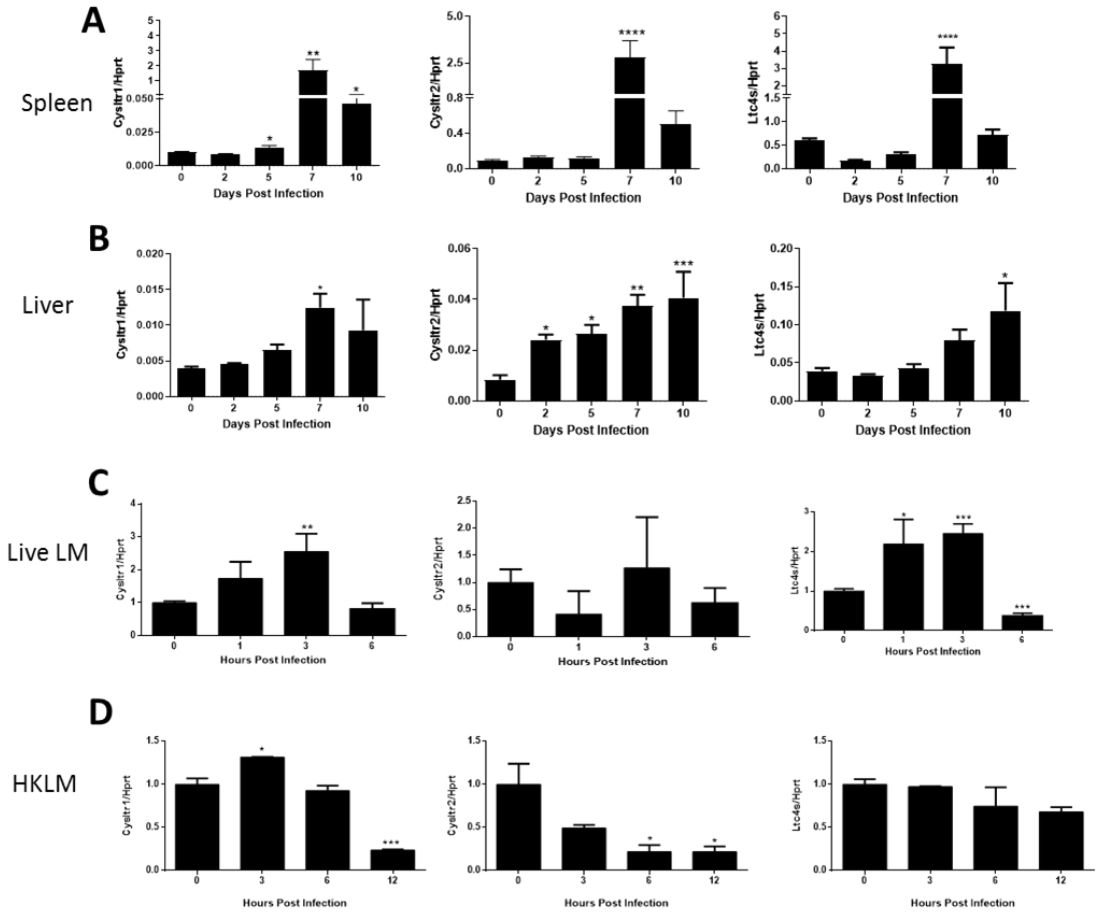

Figure 1: Expression of CysLT receptors and LTC$_4$S during *Listeria monocytogenes* infection in mice and macrophages. C57BL/6 wild-type mice were infected with 7.5x10^5 colony forming units (CFUs)/200µL *LM* intraperitoneally and sacrificed at the indicated time-points. **a)** spleen and the **b)** liver were collected from each mouse for each time point for RNA extraction and measured mRNA expression of Cysltr1, Cysltr2, and Ltc4s (*n = 3 mice per group*). Bone marrow-derived macrophages (BMDMs) from C57BL/6 wild-type mice were generated and infected with **c)** live *LM* or **d)** heat-killed *LM* (HKLM) with a multiplicity of infection (MOI) of 50 and collected RNA samples at different time-points (0, 1, 3, 6 and 12 hours post-infection) to measure Cysltr1, Cysltr2, and Ltc4s expression. Error bar denotes mean ± SEM and analysed using the One-way ANOVA multiple comparison test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.2. Generation of CysLTR1 knockout mice

CysLTR1 deficient mice were generated using a homologous recombination technique by replacing the 278 nucleotides of the CysLTR1 coding region with a neomycin resistance gene cassette (Figure 2A) (137). The mice were gifted by Dr. Yoshihide Kanaoka (Harvard University, USA) as CysLTR1 deficient on pure BALB/c and C57BL/6 backgrounds. We crossed the mice with our lab's BALB/c and C57BL/6 mice and confirmed the CysLTR1 deletion by PCR (Figure 2B). The wildtype specific primers were unable to amplify the knockout mice samples, while our CysLTR1 deletion specific primers were able to amplify

the mutant gene (333 bp) from deficient mice, confirming that CysLTR1 was deleted in our knockout mice.

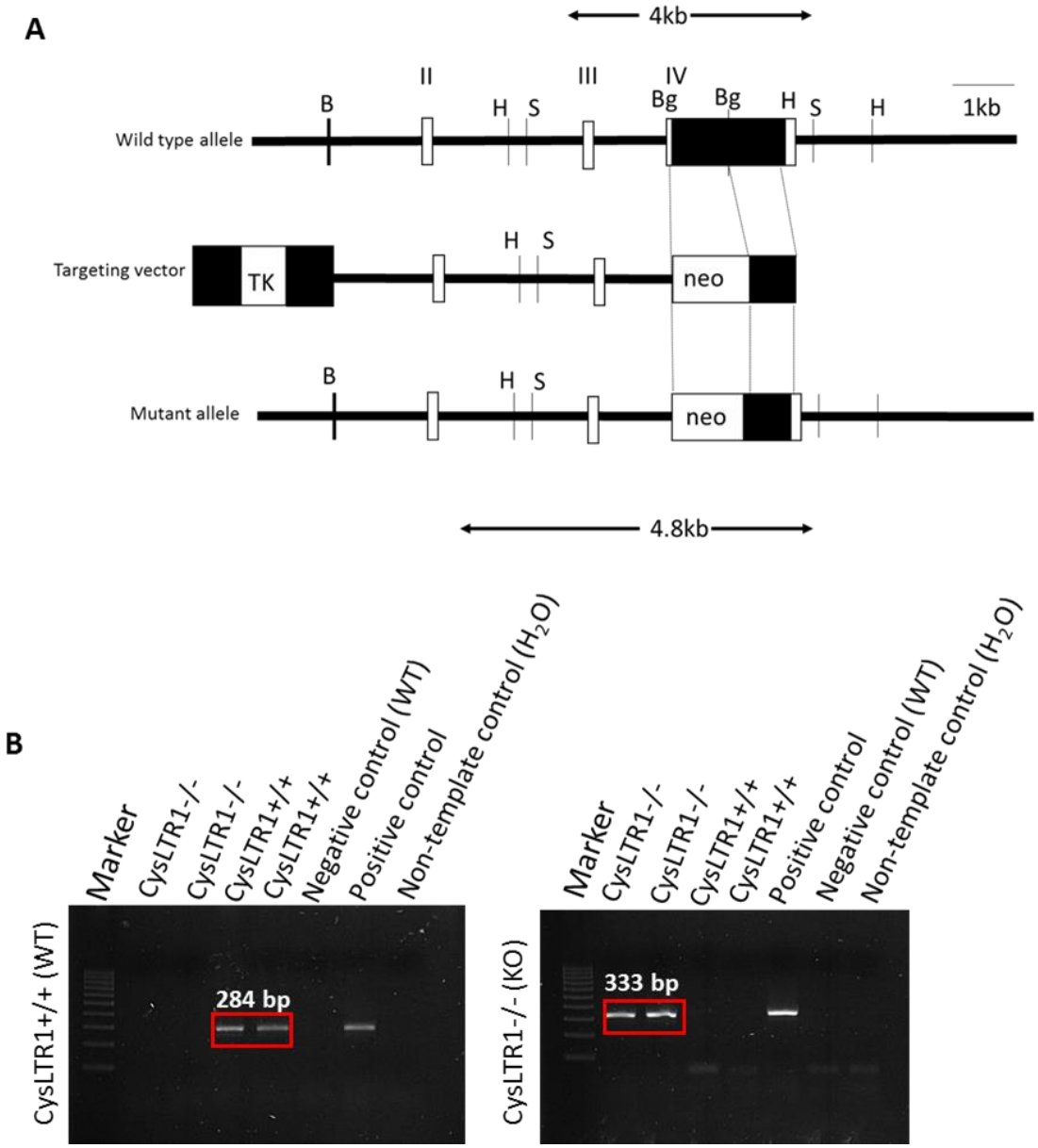

Figure 2: Generation of CysLTR1 deficient mice and confirmation of deletion. A) The generation of CysLTR1 deficient allele by homologous recombination, the upper structure represents the genomic arrangement of the mouse CysLTR1 gene, the middle is the targeting vector, and the lower structure represents the organization of the putative recombinant CysLTR1 allele. **B)** Genotyping of CysLTR1$^{-/-}$ mice.

3.3. Characterization of CysLTR1 deficient mice

Characterization of CysLTR1 Knockout mice and phenotypic analysis: CysLTR1 mice were generated by Kanaoka's group (137), however, full characterization of the mice has not been reported yet. Here, we report whether the deletion of CysLTR1 has any effects on homeostasis at naïve state. To examine this, we characterized 8-12-week-old C57BL/6 CysLTR1$^{-/-}$ and CysLTR1$^{+/+}$ (littermate controls) mice. At naïve state, there were no difference in body weights, organ weights or cell numbers between the two groups (Figure 3A-3C). We then validated the CysLTR1 deletion on mice organs by qPCR, and there was no mRNA expression of CysLTR1 in the thymus, spleen, liver, and lung of knockout mice. We then wanted to investigate whether CysLTR1 deletion influenced mRNA expression of the other cysLT receptor and enzyme responsible for the synthesis of cysLTs in these animals. As expected, there were no significant differences in the mRNA expression of Cysltr2 and Ltc4s between the knockout mice and their littermate controls (Figure 3D-3F). Cysltr1 and Cysltr2 genes are localized on different chromosomes, chromosomes X and 14 on the mouse genome respectively (137), therefore the deletion of one gene should not affect the expression of the other unless there are unknown feedback mechanisms. CysLTR1 knockout mice on BALB/c background, were characterized by Paballo Mosala, PhD candidate in our lab (manuscript in preparation).

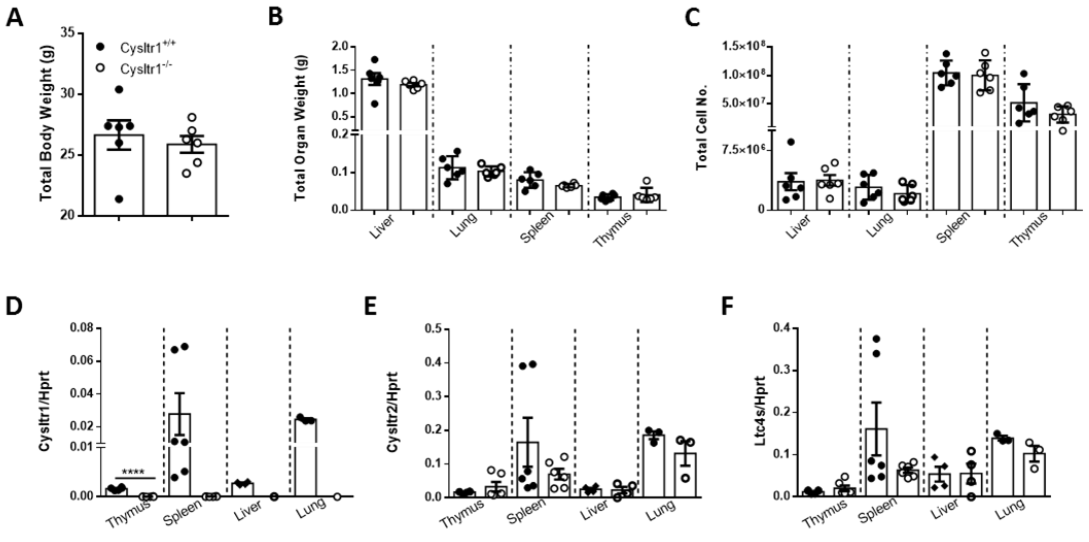

Figure 3: Characterization of CysLTR1 deficient mice. CysLTR1 deficient mice were sacrificed and characterized for any differences at naïve state by (*n =5-6 per group*), **A)** total body weight, **B)** organ weight. **C)** The organs were collected for single-cell suspension, total cell numbers were counted for each tissue. From the single-cell suspensions, we collected 2x10^6 cells for RNA extraction. The mRNA expression of **D)** Cysltr1 (to confirm deletion), **E)** Cysltr2 and **F)** Ltc4s was measured by qPCR. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with Welch's correction, ****p<0.0001.

Thymic generation of lymphocytes is not affected by CysLTR1 deletion: We confirmed that Cysltr1 was successfully deleted in our knockout mice, and the deletion of Cysltr1 had no effect on Cysltr2 and Ltc4s expression. We then wanted to determine whether Cysltr1 deletion had any effect at homeostasis. The thymus is the organ where the development and maturation of lymphocytes occurs and therefore, we wanted to see whether deletion of CysLTR1 had any effect in the development of these cells at naïve state by flow cytometry (gating strategy in Supplementary Figure S1). There were no differences in the thymus T cell percentages and cell numbers (Figure 4A-B).

Deletion of CysLTR1 has no effect on lung pathology and immune cells at naïve state: We then wanted to determine what effect CysLTR1 deletion has on the lung pathology and at the cellular level. We performed lung histopathology on CysLTR1 KO (knockout) mice and their littermate controls at naïve state and showed that the deletion of CysLTR1 does not have an effect on lung pathology (Figure 5A) nor the free lung alveolar spaces (Figure 5B). Consistent with Mao *et al*. 2018, who generated CysLTR1 knockout mice using CRISPR/Cas9 and also demonstrated that the deletion of this gene had no significant effect on lung pathology (140). The lung lymphocyte populations (B, NK, CD8 T, and CD4 T cells) had no significant differences in both cell percentages and cell numbers between the knockouts and their littermate controls (Figure 5C-D). Furthermore, within CD4 and CD8 T cell subsets ($CD62L^+$ $CD44^-$: naïve; $CD62L^+$ $CD44^+$: central memory; $CD62L^-$ $CD44^+$: effector/effector memory or activated cells; and $CD62L^-$ $CD44^-$: double negative cells) there were no significant differences (Figure 5E-F). We also looked at T cell exhaustion and homing markers (PD1 and KLRG1, respectively), KO lung CD4 T cells had significantly reduced $PD1^+$ $KLRG1^+$ and $PD1^+$ $KLRG1^-$ but increased $PD1^-$ $KLRG1^-$ CD4 T cells (Figure S7A). There was a significant reduction in KO $PD1^+$ $KLRG1^+$ CD8 T cells at naïve state (Figure S7B) indicating that KO CD4 and CD8 T cells in the lung have less proliferative capacity (141). The myeloid cell populations (neutrophils, interstitial macrophages (macs), eosinophils, alveolar macs, monocyte-derived dendritic cells (DCs), CD11b DCs, CD103 DCs, and monocytes) also had no significant differences at naïve state (Figure 5G-H).

We then measured cytokines in the lung homogenates at naïve state to determine whether the deletion influenced the ability of cells to release cytokines at naïve state in knockout mice. There were no significant differences in most of the pro-inflammatory cytokine (IL-12p40, IL-12p70, IL-6, IL-2, TNF, IL-1α and IL-1β) we measured (Figure 5I), however,

IFN-γ and IL-17 were reduced in our knockout mice. This reduction was not observed in the repeat experiments, indicating that it might likely be due to an artefact on the experiment. The lung anti-inflammatory (IL-4, IL-5, and IL-13); regulatory (IL-10 and TGF-β) cytokines, chemokine (RANTES or CCL5) and growth factor (GM-CSF) had no significant differences (Figure 5J-L). In the study conducted by Mao *et al*. 2018, they also demonstrated that nitric oxide production was affected by the deletion of CysLTR1 in the lung tissue and they also further showed this by measuring the protein expression of the nitric oxide mediator, DDAH1 by western blot. Both NO and DDAH1 were both reduced in their CysLTR1 knockout mice (140). However, nitric oxide levels in the lung in our CysLTR1 KO mice were not affected (Figure 5M). These results demonstrate that at naïve state, CysLTR1 has no major effect on lung pathology, recruitment of the immune cell populations, cytokines and nitric oxide secretion.

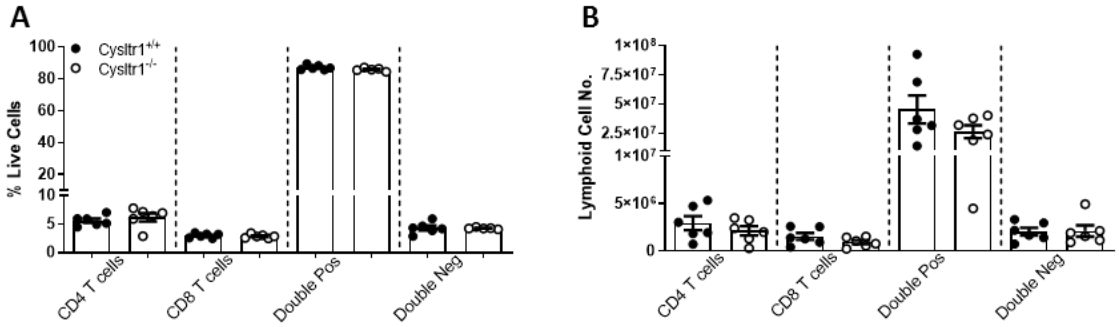

Figure 4: CysLTR1 deletion has no effect on thymic lymphoid cell populations. Thymus T cell generation was determined by $CD4^{high}$ $CD8^{low}$, $CD4^{low}$ $CD8^{high}$, $CD4^{high}$ $CD8^{high}$ and $CD4^{low}$ $CD8^{low}$ using flow cytometry from total live cells. Data is representative of three experiments (*n=5-6 mice/group*). Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction.

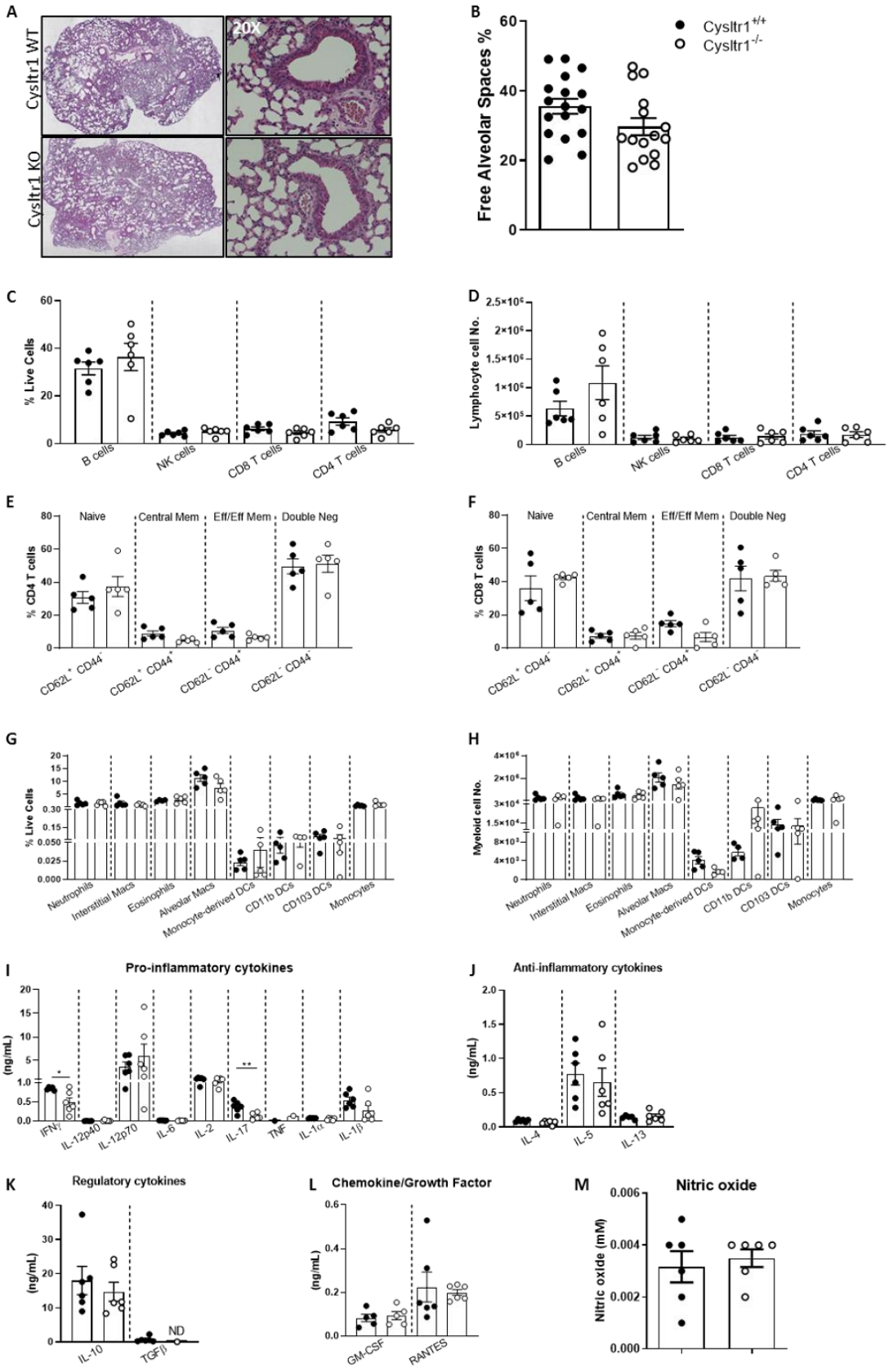

Figure 5: CysLTR1 deletion has no effect on lung homeostasis. CysLTR1 knockout and their littermate control mice were sacrificed at naïve state (*n=5-6 mice per group*) and the lung tissue was harvested for histology, flow cytometry, and ELISA. **A)** H&E staining of the lung for CysLTR1 KO and WT mice. **B)** Lung free alveolar air space quantification. **C-D)** Lung lymphoid cell

percentages of total live cells and cell numbers measured by flow cytometry. **E-F)** CD4 T cell and CD8 T cell subsets measured by flow cytometry. **G-H)** Myeloid cell percentages of total live cells and cell numbers. **I)** pro-inflammatory, **J)** Th2, **K)** regulatory cytokines, **L)** growth factor and chemokine by ELISA and **M)** nitric oxide from lung homogenates. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, *p<0.05, **p<0.01.

Liver pathology and homeostasis is not affected by CysLTR1 deficiency: The role of CysLTR1 has been mostly demonstrated in lung pathology and airway etiology because of its role in allergic and asthmatic responses. Because we are interested in the role of CysLTR1 during *LM* infection, we needed to determine whether the deletion of this receptor had any effect in pathology and the immune population at naïve state in the organs that are important during *LM* infection. We then looked at the effect of CysLTR1 deletion in the liver. Like in the lung, haematoxylin and eosin (H&E) staining showed no differences in the liver pathology of the KO and WT mice (Figure 6A). Flow cytometry analysis of the liver lymphoid cell percentages and numbers exhibited no differences between the KO and WT mice (Figure 6B-C). The CD4 and CD8 T cell subsets and follicular Th cells also had no differences at naïve state (Figure 6D-E). There were no differences between the percentages of WT and KO PD1 and KLRG1 expression by CD4 and CD8 T cells in the liver (Figure S7C-D). The myeloid cell population also had no significant differences (Figure 6F-G). However, like in the lung, we observed a reduction of IL-17 in the KO liver while all the other cytokines had no differences (Figure 6H). There were no differences in anti-inflammatory cytokine (IL-4, IL-5, and IL-13) and regulatory cytokines (IL-10 and TGF-β) (Figure 6I-J). However, there was a reduction in GM-CSF levels in KO liver homogenates compared to their littermate controls (Figure 6K). The nitric oxide production was not affected by CysLTR1 deletion (Figure 6L). These results demonstrate that the deletion of CysLTR1 overall has no major effect in liver homeostasis.

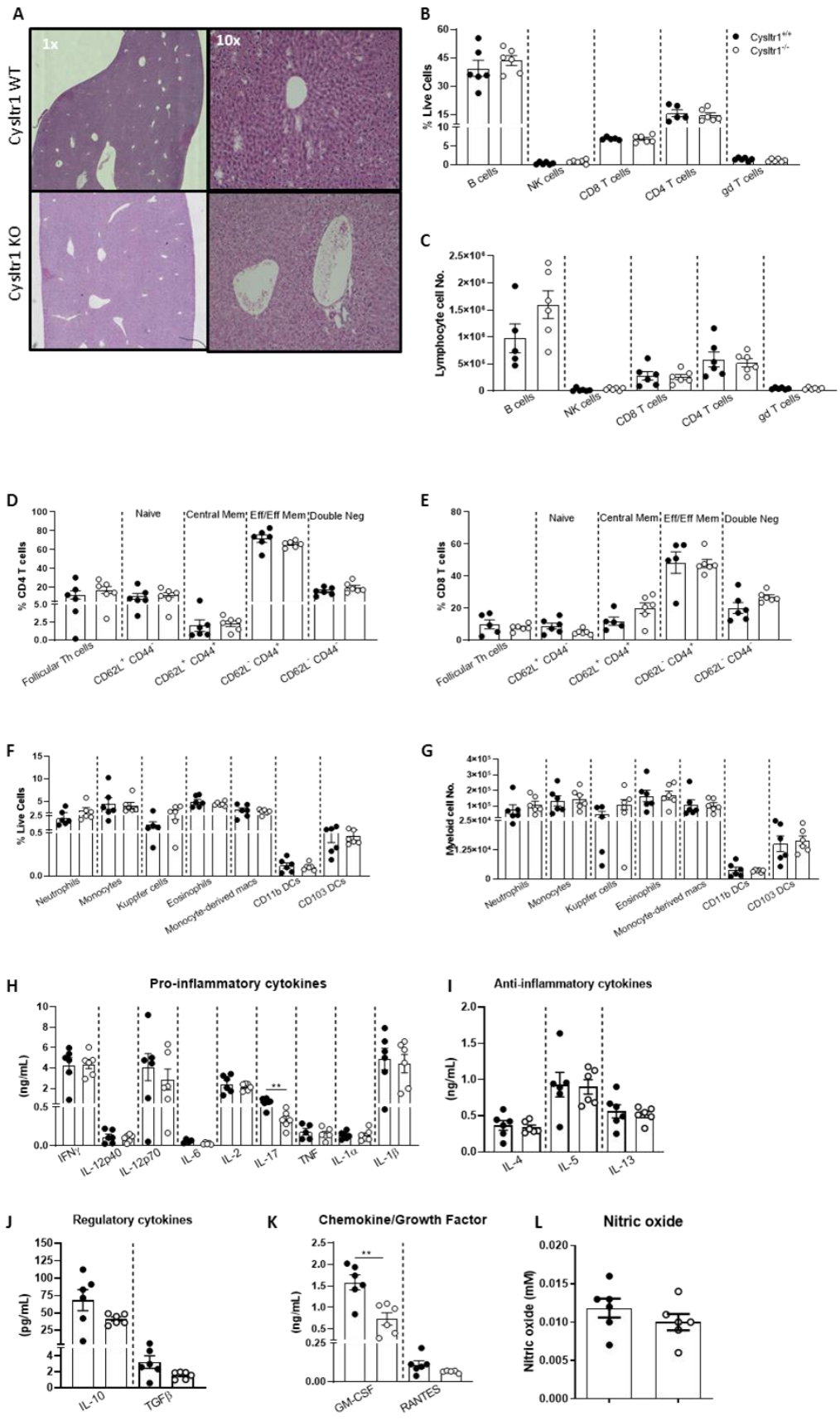

Figure 6: CysLTR1 deletion has no effect on liver pathology and immune cellular responses at the naïve state. CysLTR1 knockout and their littermate control mice were sacrificed at naïve state (*n=5-6 mice per group*) and the liver was harvested for histology, flow cytometry, and ELISA.

A) H&E staining of the liver for CysLTR1 KO and WT mice. **B-C)** Liver lymphoid cell percentages of total live cells and cell numbers measured by flow cytometry. **D-E)** CD4 T cell and CD8 T cell subsets measured by flow cytometry. **F-G)** Myeloid cell percentages of total live cells and cell numbers. **H)** pro-inflammatory, **I)** Th2, **J)** regulatory cytokines, **K)** growth factor (GMCSF) and chemokine (RANTES) by ELISA and **L)** nitric oxide from liver homogenates. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, **$p<0.01$.

CysLTR1 deficiency in mice has no effect on spleen pathology and immune cells at naïve state: Similar to lung and liver, we then determined the effect of CysLTR1 deletion on the mouse spleen and immune cell populations at naïve state. The H&E staining showed no differences between the CysLTR1 KO and WT mice spleen (Figure 7A). There were no differences in the lymphoid cell percentages and numbers between the two groups (Figure 7B-C). Similarly, CD4 and CD8 T cell subsets showed no differences in the percentages (Figure 7D-E). As expected, the naïve CD4 T cells ($CD62L^+$ $CD44^-$) were higher in both groups at a steady state. Exhaustion markers expressed by CD4 and CD8 T cells were comparable between KO and WT spleen cells, with the exception of $PD1^-$ $KLRG1^+$ CD8 T cells that were significantly increased in KO mice (Figure S7E-F) which indicates increased CD8 T cells in the spleen vasculature (142). The myeloid cell population demonstrated no differences both in cell percentages and cell numbers between the two groups (Figure 7F-G). The marginal zone macrophages were the least frequent myeloid cells in both groups. These results show that CysLTR1 deletion has no effect on mice spleen pathology and immune populations at naïve state.

Systemic cytokine and antibody production are not affected by CysLTR1 deletion: We then determined systemic cytokines and antibodies secreted in the blood at naïve state to evaluate whether the deletion of CysLTR1 might influence their secretion. There were no differences in cytokine secretion between the knockouts and their littermate controls (Figure 8A). However, we found a modest increase in total IgG and a reduction in IgG3 in the knockout mice (Figure 8B). These differences were not maintained when the experiment was repeated, therefore, CysLTR1 deletion has no effect on antibody and cytokine secretion at naïve state. Together, these results demonstrate that CysLTR1 deletion has no effect on organ pathology, immune cell populations, cytokine, and antibody secretion.

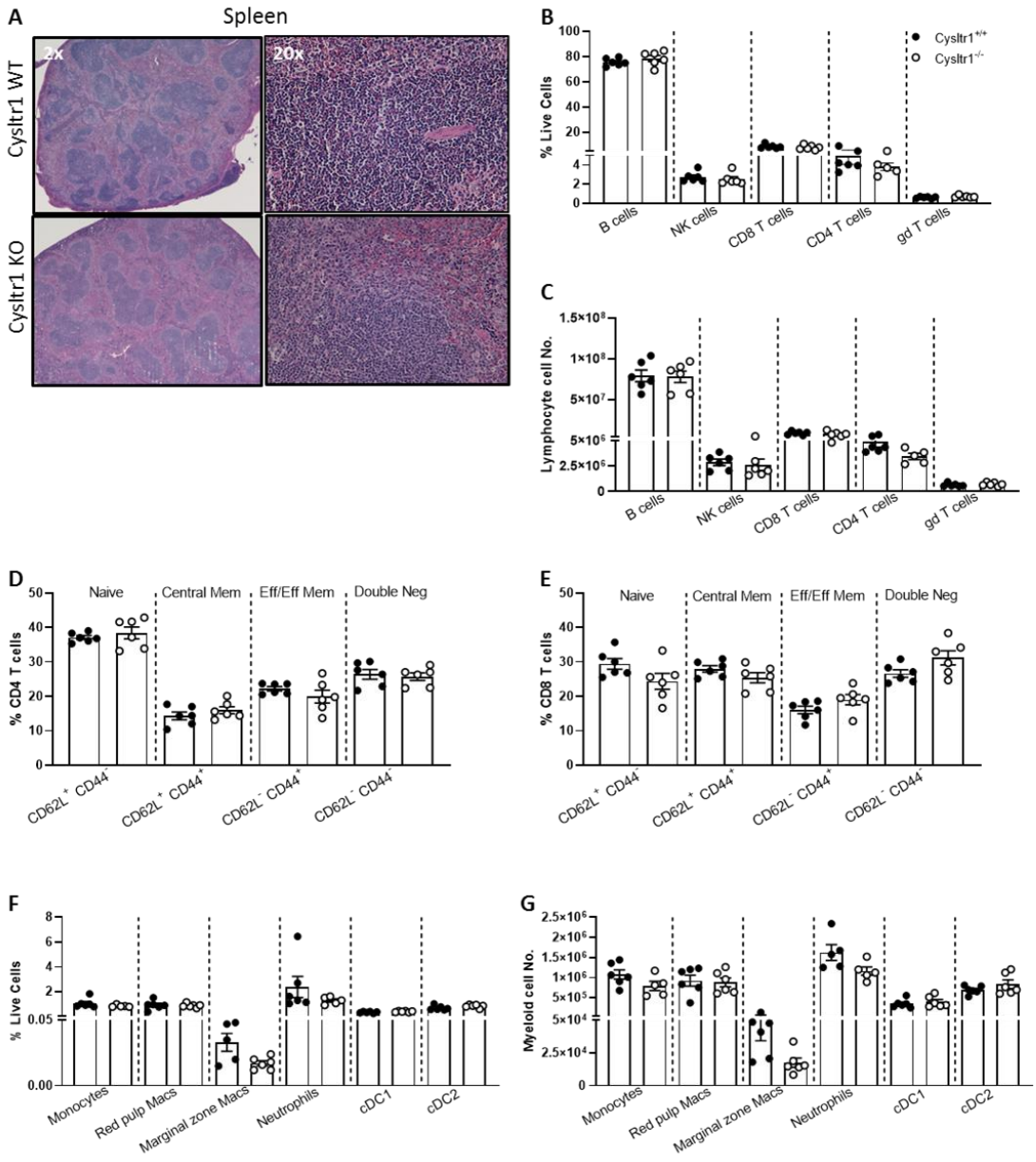

Figure 7: CysLTR1 deletion has no effect on spleen pathology and immune cell populations at naïve state. CysLTR1 knockout mice and their littermate control mice were sacrificed at naïve state (*n=5-6 mice per group*) and the spleen was harvested for histology and flow cytometry analysis. **A)** H&E staining of the spleen for CysLTR1 KO and WT mice. **B-C)** Spleen lymphoid cell percentages of total live splenocytes and cell numbers measured by flow cytometry. **D-E)** CD4 T cell and CD8 T cell subsets measured by flow cytometry. **F-G)** Myeloid cell percentages of total live splenocytes and cell numbers. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with Welch's correction.

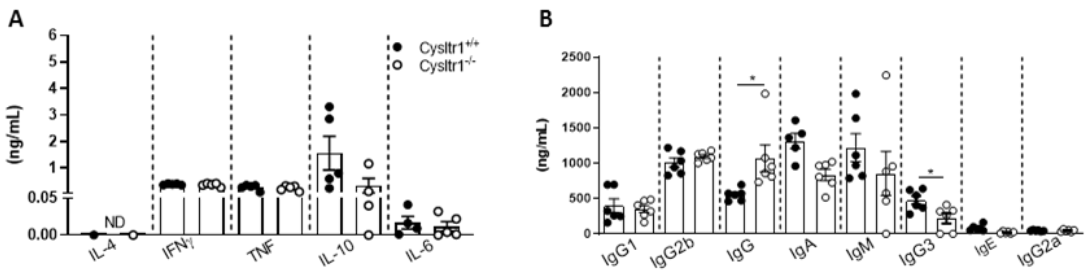

Figure 8: CysLTR1 deletion has no effect on serum cytokine and antibody production at naïve state. CysLTR1 knockout mice and their littermate control mice were sacrificed at naïve state (*n=5-6 mice per group*) and cardiac puncture was performed to collect blood for antibody and cytokine measurement. **A)** Measured cytokine (IL-4, IFN-γ, TNF, IL-10 and IL-6) and **B)** antibody (IgG1, IgG2b, IgG, IgA, IgM, IgG3, IgE and IgG2a) levels in the serum by ELISA. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, *p<0.05.

3.4. CysLTR1-deficient macrophages revealed similar bacterial growth during *L. monocytogenes* infection

We showed that CysLTR1 deletion at naïve state has no effect on the mouse homeostasis, suggesting no baseline defect in these animals. Our findings also demonstrated an upregulation of CysLTR1 in wildtype mice and macrophages, suggesting that this receptor might have an *LM* specific role. Therefore, we sought to investigate whether deletion of this receptor has any role in the control of *LM* infection *in vitro*. We generated BMDMs from C57BL/6 and BALB/c CysLTR1$^{+/+}$ (wildtype/WT) and CysLTR1$^{-/-}$ (knockout/KO) mice and then infected with *LM*. Macrophages can phagocyte and ingest invading pathogens, present antigens to T cells, and induce the production of cytokines that are required for the activation of other immune cells (143). We, therefore, wanted to determine the ability of the macrophages to phagocyte the bacteria by measuring ingested bacteria from BMDM lysates at different time points.

We found that there were no differences in the bacterial burden in KO and WT BMDMs in both backgrounds (Figure 9A-B). In addition, the ability of the macrophages to phagocyte *LM* was not affected by the deletion of CysLTR1 in both backgrounds determined at 1-hour post-infection. We then wanted to determine whether the secretion of cytokines and nitric oxide (NO), required for the effective killing of intracellular bacteria, was impaired in CysLTR1 KO macrophages. We measured NO production, cytokine and chemokine secretion at 6- and 12-hours after infection. Reactive nitrogen intermediates (RNI) such as NO synthase (NOS) have been shown to play a role in inhibiting the escape and growth of *LM* in macrophages (46, 144, 145). There were no differences in NO secretion by

CysLTR1$^{-/-}$ and CysLTR1$^{+/+}$ macrophages in both BALB/c and C57BL/6 (Figure 9C and 9E). We found that C56BL/6 knockout macrophages tend to increase in IL-10 secretion (Figure 9D), while BALB/c KO macrophages had a decreasing trend in the secretion of IL-10 (Figure 9F) post *LM* infection. At 12 hours post-infection, TNF secretion had a decreasing trend in both C57BL/6 KO BMDMs (Figure 9G) and BALB/c BMDMs (Figure 9I). The IL-6 secretion in C57BL/6 KO BMDMs at 12 hours post-infection had an increasing trend (Figure 9H), and in contrast it showed a decreasing trend in KO BALB/c macrophages (Figure 9J). MIP2 secretion in KO C57BL/6 (Figure 9K) and KO BALB/c (Figure 9M) macrophages was decreased. Macrophages from C57BL/6 (Figure 9L) and BALB/c (Figure 9N) had no major difference on RANTES/CCL5 secretion between the KO and wildtype macrophages post *LM* infection. In summary, CysLTR1 deletion had no effect on intracellular growth of *LM*, cytokine and chemokine as well as nitric oxide production. The differential cytokine secretion observed in macrophages could be due to genetic background of the mice.

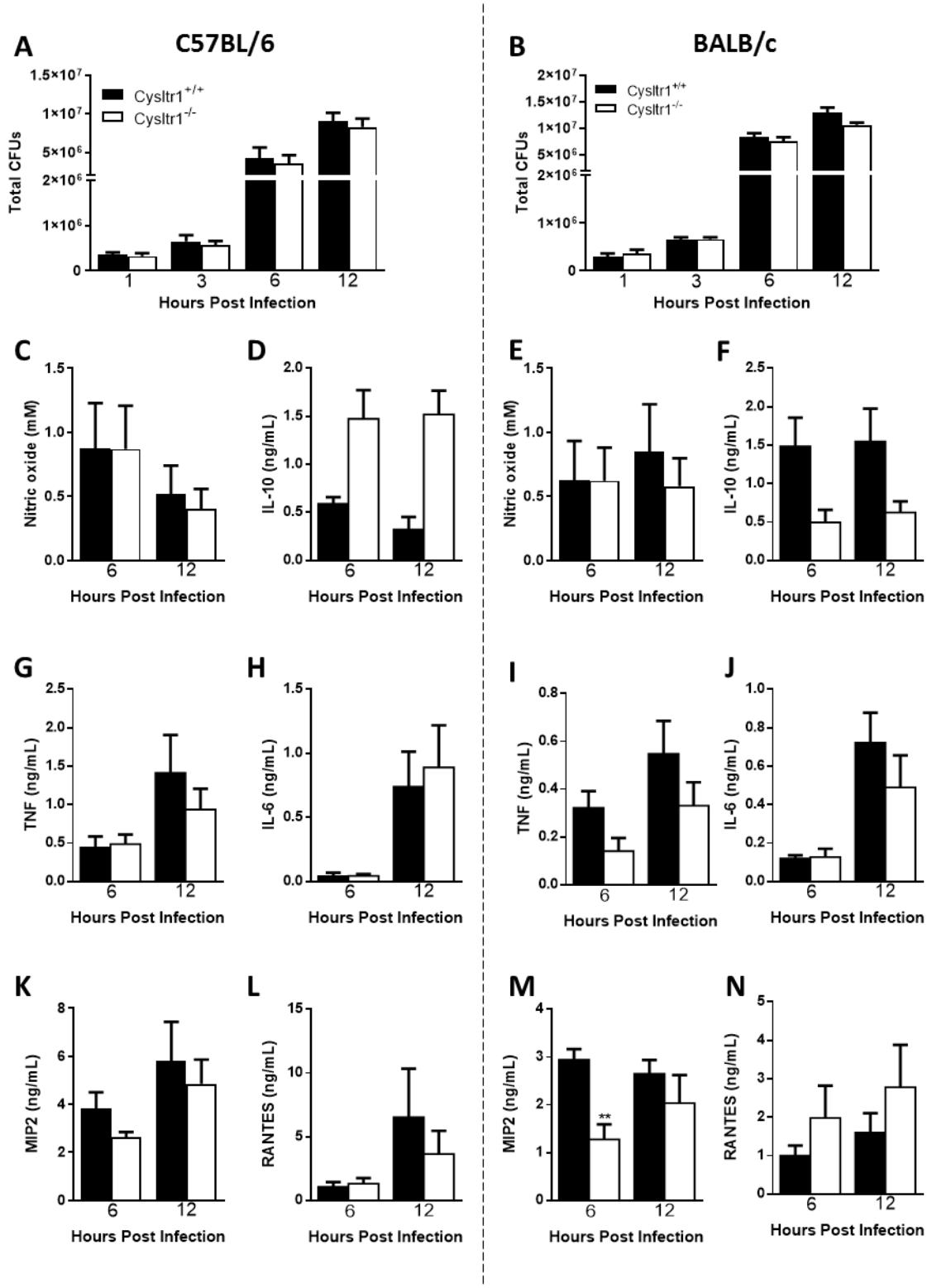

Figure 9: CysLTR1 knockout macrophage control of *LM* infections and secreted cytokines. C57BL/6 and BALB/c BMDMs from CysLTR1 wildtype and knockout mice were generated for 10 days and infected with 2x10^5 CFUs *LM* for 1 hour, 3, 6 and 12 hours. **A-B)** Bacterial burden was measured in macrophages by plating CFUs. **C&E)** Nitric oxide in supernatants. **D&F)** IL-10, **G&I)** TNF, **H&J)** IL-6, **K&M)** MIP2 and **L&N)** RANTES were measured from cell supernatants using ELISA. Data is a representative of quadruplicates and two pooled experiments. Error bar denotes mean ± SEM and analysed using the unpaired student t-test, *p<0.05.

3.5. CysLTR1 is dispensable during *L. monocytogenes* infection in mice

CysLTR1 deficiency result in increased liver neutrophils and monocytes during *LM* infection: Thus far, we have established that CysLTR1 deletion has no effect on mice at naïve state, and it does not have an effect on intracellular growth bacteria in macrophages *in vitro*. We then investigated whether the deletion of this receptor affects host protection during *LM* infection *in vivo*. We infected CysLTR1 KO and WT mice with *LM* and sacrificed at three- and seven-days post-infection (3- and 7-dpi). At each time point, we measured the bacterial burden in liver and spleen. In the liver, we found no differences in bacterial loads at both 3- and 7-dpi (Figure 10A). At 3dpi, the KO mice had increased liver cell numbers, but not at 7-dpi (Figure 10B) and liver weights (Figure 10C) were comparable to their littermate controls. Furthermore, histopathology analysis of the liver showed that there were no differences in lesion areas between the wildtype and knockout mice (Figure 10D and 10E) at both time points. We then measured immune cell recruitment by flow cytometry in the liver (gating strategy in Figure S2 and S4). Myeloid cells are a crucial part of the immune system as they initiate the innate immune responses during host invasion by pathogens and play an important role in the activation of adaptive immunity. Three days post-infection, neutrophil cell percentages in KO mice were significantly increased during *LM* infection while there were no significant differences in other myeloid cell populations (Figure 10F). In CysLTR1 KO livers the absolute numbers of monocytes and neutrophils were significantly increased compared to littermate controls 3 days post-*LM* infection (Figure 10H). At 7 days of post-*LM* infection, there were no differences in myeloid cell percentages except for an increase in monocyte-derived macrophage percentages in KO mice (Figure 10G). However, there were no observed differences in the myeloid cell numbers (Figure 10I). These results show that CysLTR1 deficiency results in increased neutrophil and monocyte recruitment to the liver early after *LM* infection.

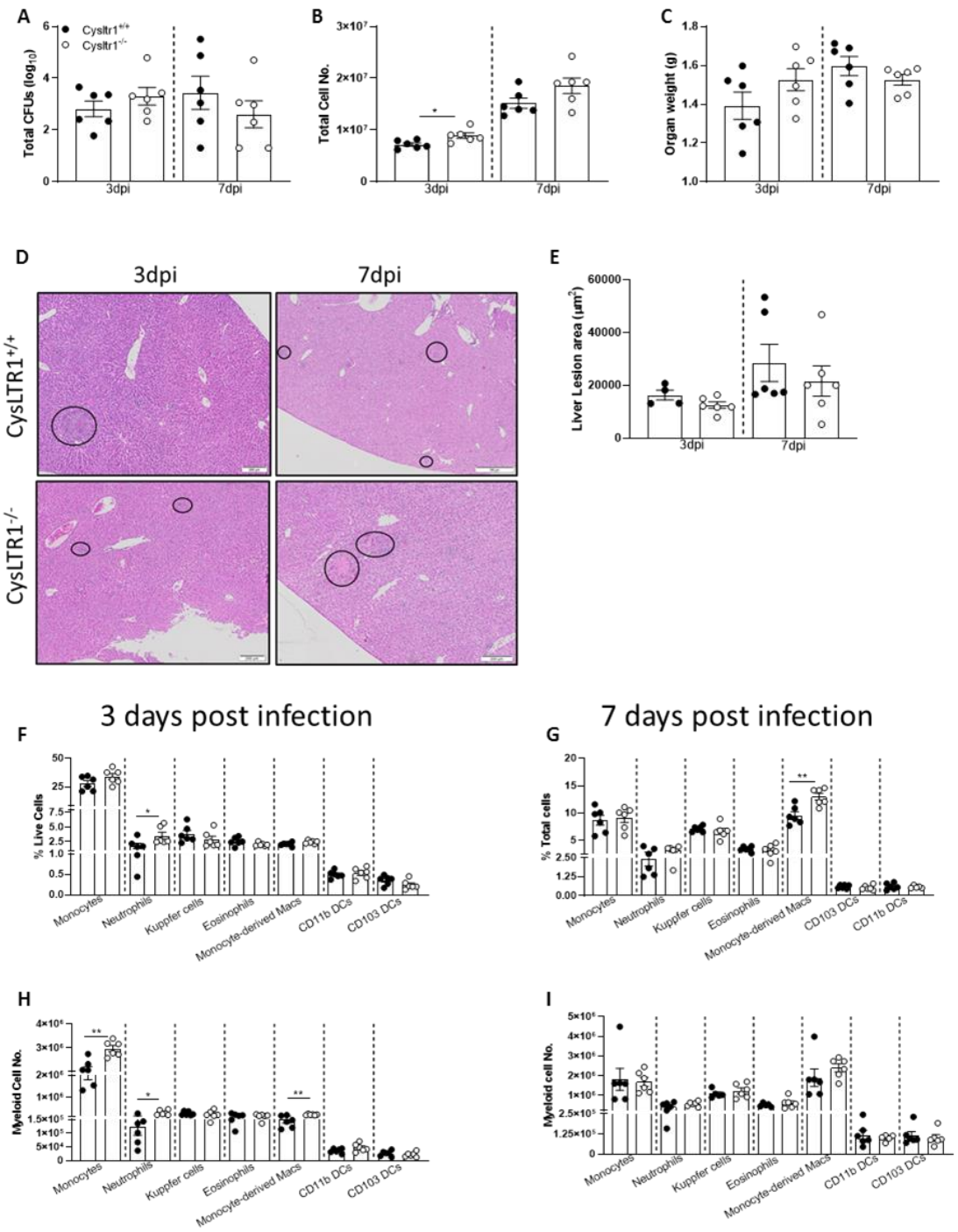

Figure 10: CysLTR1 deficiency in mice increased liver neutrophils early after *L. monocytogenes* infection. CysLTR1 KO mice and their littermate controls were infected with about 1.1x10^5 *LM* CFUs/200µL per mouse intraperitoneally. The mice were sacrificed at 3- and 7-dpi (*n = 6 mice per group*) and the liver were collected to measure **A)** bacterial burden by plating liver homogenates on tryptic soy agar (TSA) plates for CFUs, **B)** cell numbers by making single-cell suspensions, **C)** organ weights and **D)** H&E staining for immunohistology analysis. **E)** liver lesions were measured using the Olympus-VS-ASW-L100 imaging system, lesions are indicated by the circles. The single cells were then stained for myeloid cell population analysis by flow cytometry. **F-G)** Liver myeloid cell percentages of live cells 3- and 7-dpi. **H-I)** Liver myeloid cell numbers 3- and 7-dpi. Data is representative of two experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with Welch's correction, (*p<0.05, **p<0.01).

CysLTR1 deficient mice have reduced B cell, NK cell and CD4 T cell percentages at early stages of infection with *LM*: We then analysed the lymphoid cell populations since they are important for the normal function of the immune system. In the liver lymphoid cell percentages at the 3 days post-*LM* infection, we observed a significant reduction in B cells, NK cells and CD4 T cells in KO mice while there were no differences in other cell populations (Figure 11A), however, cell numbers had no significant differences except for γδ T cells (Figure 11C). At 7dpi, we observed no differences in the lymphoid cell percentages (Figure 11B), however, CD8 T cell numbers were significantly increased in KO mice (Figure 11D). We then looked at CD4 and CD8 T cell subsets post-infection, at 3dpi there were no differences in CD4 T cell subsets (naïve, central memory, and effector/effector T cells) and at 7dpi there were no differences in naive and effector/effector CD4 T cells, while there was a significant decrease in central memory CD4 T cells (Figure 11E and 11F). The CD8 T cell subsets both at 3- and 7dpi had no differences except increased effector/effector memory cells at 3 dpi (Figure 11G and 11H). We also looked at exhaustion and homing of CD4 and CD8 T cells at 3- and 7-days post-*LM* infection using exhaustion and parenchymal markers PD1 and KLRG1, and found no differences in CD4 exhausted T cells at 3dpi (Figure S8A), and at 7dpi there were a significant reduction in vascular, less differentiated $PD1^{-}$ $KLRG1^{+}$ KO cells (Figure S8B). There was a significant increase in parenchymal $PD1^{+}$ $KLRG1^{+}$ cells and reduction in $PD1^{-}$ $KLRG1^{-}$ KO CD8 T cells 3dpi (Figure S8C), while there were no differences in CD8 T cell exhaustion at 7dpi (Supplementary Figure S8D). Furthermore, we determined T cell proliferation by measuring Ki67 expression. At 3- and 7-dpi, CD4 and CD8 T cell proliferation showed similar proliferation between the two groups of animals (Figure 11I and 11J). This suggest that CystLTR1 deletion has no effect on T cell proliferation

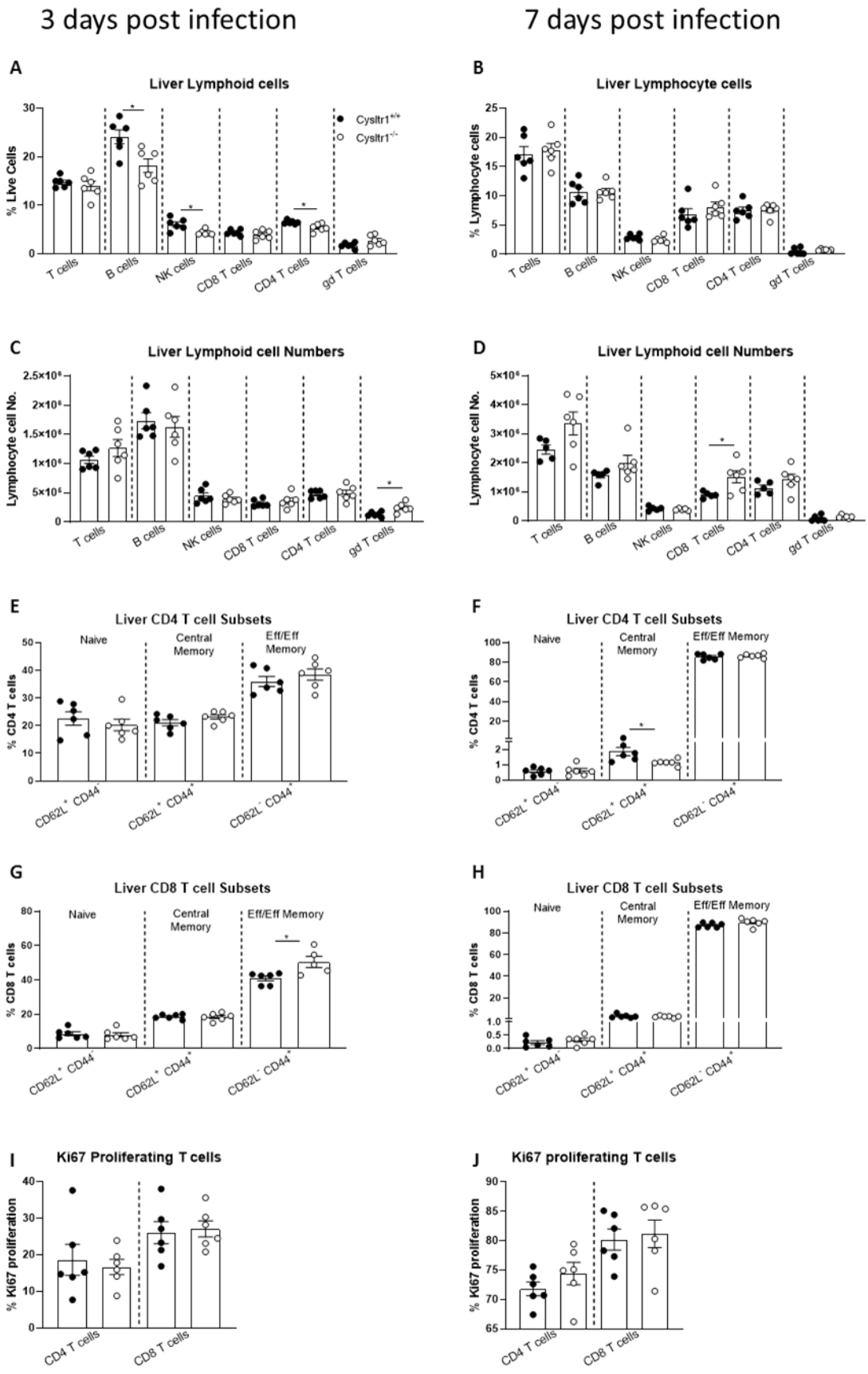

Figure 11: Effect of CysLTR1 deletion in liver lymphocyte population post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with about $1.1x10^5$

LM CFUs/200µL per mouse intraperitoneally and sacrificed the mice at 3- and 7-dpi (*n = 6 per group*). Liver single cells were stained for the lymphoid cell population for analysis by flow cytometry. **A-B)** Liver lymphoid cell percentages of total live cells, **C-D)** lymphoid cell numbers, **E-F)** CD4 T cell subsets, **G-H)** CD8 T cell subsets and **I-J)** CD4 and CD8 T cell proliferation measured by Ki67 at 3- and 7-dpi. Data is representative of two independent experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with Welch's correction, (*$p<0.05$).

CysLTR1 deficiency increased T cell specific host responses after *L. monocytogenes* infection in mice: To explore cell-specific cytokine responses, we re-stimulated liver single-cell suspensions with phorbol myristate acetate (PMA) and ionomycin (PMA/ionomycin) or left untreated for 6 hours to measure cytokine (IFN-γ and TNF), pore-forming protease (perforin) and serine protease (granzyme B) secretion by CD4 and CD8 T cells 3- and 7-days post-*LM* infection by flow cytometry (using the gating strategy in Figure S6). PMA/ionomycin is used for activation of the transcription factors NF-KB and NFAT which lead to cytokine secretion by T cells (146). We chose secretion of these cytokines and proteins by T cells because they have been shown to play a protective role during *L. monocytogenes* infection (147–154). There was no effect on IFN-γ production by $CD4^+$ T cells regardless of stimulation (Figure 12A), whilst IFN-γ was significantly reduced in $CD8^+$ T cell production in mice lacking Cystlr1 (Figure 12B). There was a significantly increased production of perforin and granzyme B in CD8 T cells in unstimulated KO liver cells, but not with PMA/ionomycin stimulation (Figure 12C and 12D). Granzyme B and perforin have been shown to be produced mostly by CD8+ T cells. At 7dpi, IFN-γ production by $CD4^+$ T cells was significantly increased after PMA/ionomycin stimulation in KO mice (Figure 13A). We also measured TNF production in $CD4^+$ T cells at 7dpi, and there was a significant increase in TNF secretion in unstimulated cells, and an increasing trend in PMA/ionomycin cells (Figure 13B). Collectively, these results suggest that liver CD4 and CD8 T cells from CysLTR1 KO mice have a general trend of increased cytotoxic and IFN-γ and TNF secretion except for decreased IFN-γ secretion at 3dpi.

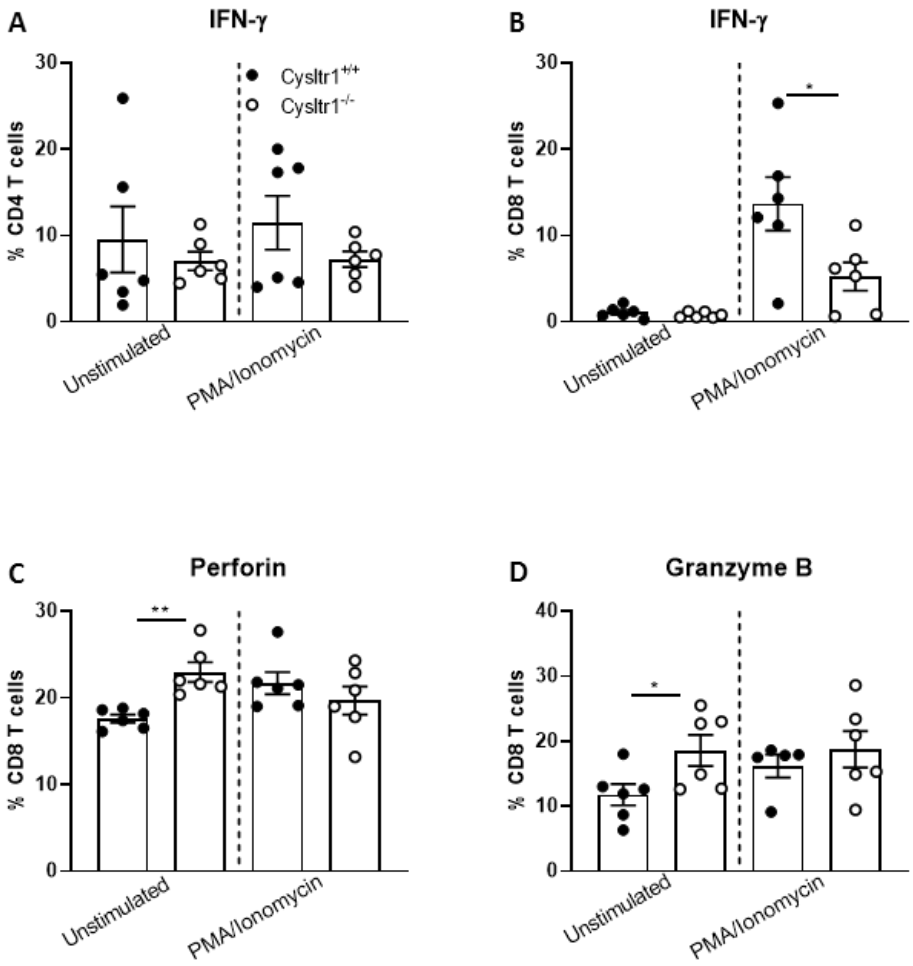

Figure 12: Liver CD8 T cells from CysTLR1 deficient mice have increased perforin and granzyme B production early after *LM* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3dpi (*n = 6 per group*). Liver single cells were stimulated for cytokine production using PMA/ionomycin (stimulated) or left unstimulated. Flow cytometry was used to determine percentages of **A-B)** IFN- γ produced by CD4 and CD8 T cells, **C)** perforin and **D)** granzyme B by CD8 T cells 3dpi. Error bars denote mean ± SEM and analysed using the unpaired, student t-test with Welch's correction, (*$p<0.05$, **$p<0.01$).

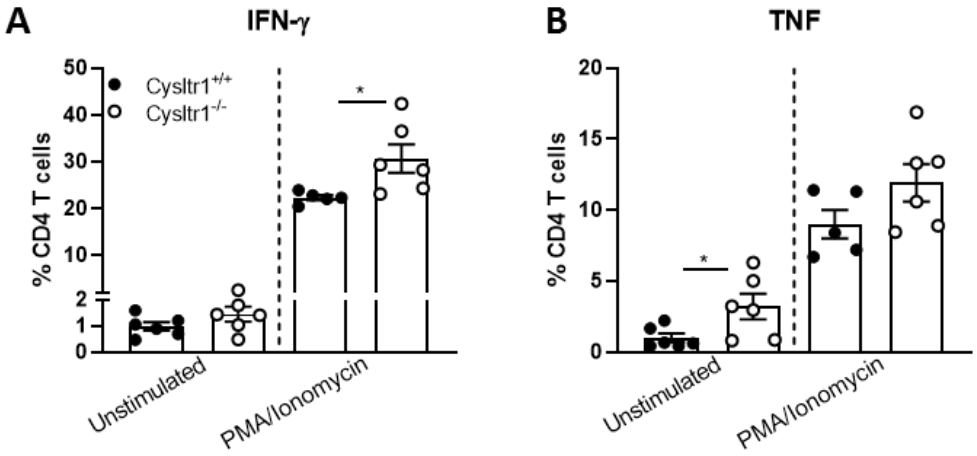

Figure 13: Liver CD4 T cells from CysLTR1 deficient mice have increased production of IFN-γ and TNF after *L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and sacrificed at 7dpi (*n = 6 per group*). Liver single cells were stimulated for cytokine production using PMA/ionomycin (stimulated) or left unstimulated. Flow cytometry was used to determine percentages of **A)** IFN- γ and **B)** TNF produced by CD4 T cells at 7dpi. Error bars denote mean ± SEM and analysed using the unpaired student t-test with Welch's correction, (*$p<0.05$).

CysLTR1 deficiency results in decreased pro-inflammatory cytokines at early *LM* infection: We then measured nitric oxide, cytokines (pro-inflammatory, regulatory and Th2 cytokines), chemokines and growth factors at 3- and 7-days post-*LM* infection in liver homogenates. There were no significant differences in NO production in the liver at both time-points (Figure 14A). The IL-1α production was significantly increased in liver homogenates at 7dpi (Figure 14B), whilst IL-1β remained unaffected (Figure 14C) in the KO mice. There was a significant increase of IFN-β at 7dpi in liver homogenates (Figure 14D) of KO mice. IFN-γ, IL-17, and IL-12p70 production were significantly reduced in KO mice at 3dpi while there were no differences at 7dpi (Figure 14E, 14F, and 14I). There were no differences in other proinflammatory cytokines; IL-6 and IL-12p40 between the KO and their littermate controls (Figure 14G and 14H). We also assessed the regulatory (TGF-β and IL-10) and Th2 (IL-4) cytokines, and there were no significant differences in TGF-β, IL-10 and IL-4 production between the two groups (Figure 14J-14L). We then measured chemokines and growth factors and found a significant reduction in CXCL2, CXCL10, and GM-CSF production at 3dpi (Figure 15A, 15F, and 15H) whereas CCL3 and CXCL1 production were increased at 7dpi (Figure 15C and 15D) by KO mice. However, at later stages of the infection, these differences were not observed instead, KO mice have increased CCL3 and CXCL1 levels. Overall, these results suggest that at the early stages

of infection there are slight decrease in the levels of Th1 (IL12p70, GM-CSF), Th17 (IL17) cytokines and chemo-attractants (CXCL2 and CXCL10) in Cysltr1 KO mice.

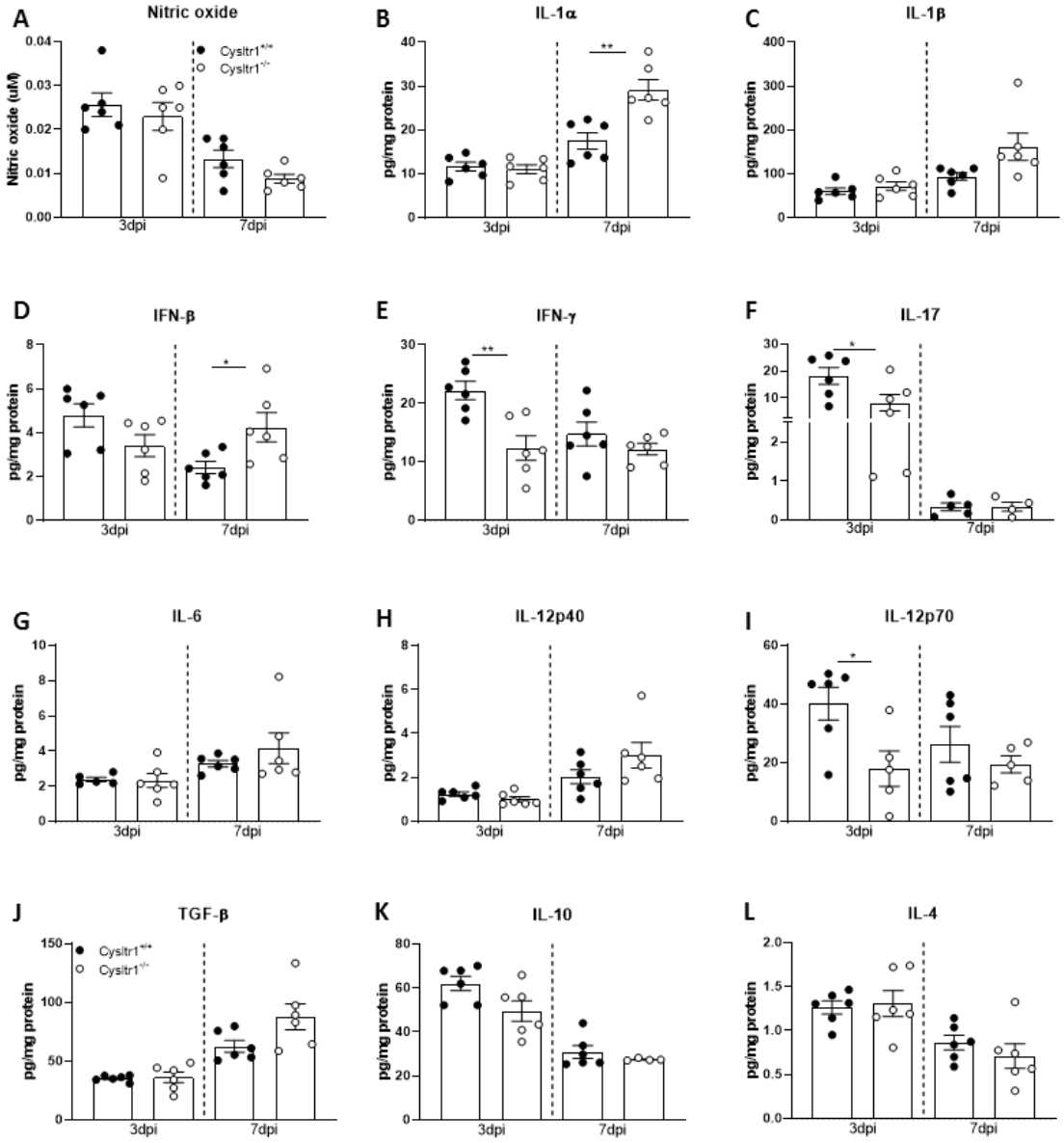

Figure 14: Nitric oxide, pro-inflammatory, regulatory and Th2 cytokine levels in the liver of CysLTR1 knockout mice after *L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and sacrificed the mice at 7dpi (*n = 6 per group*). Collected the liver for homogenates and measured **A)** nitric oxide, pro-inflammatory; **B)** IL-1α, **C)** IL-1β, **D)** IFN-β, **E)** IFN-γ, **F)** IL-17, **G)** IL-6, **H)** IL-12p40, **I)** IL-12p70, regulatory; **J)** TGF-β, **K)** IL-10 and Th2 cytokines; **L)** IL-4 and by ELISA. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*p<0.05, **p<0.01).

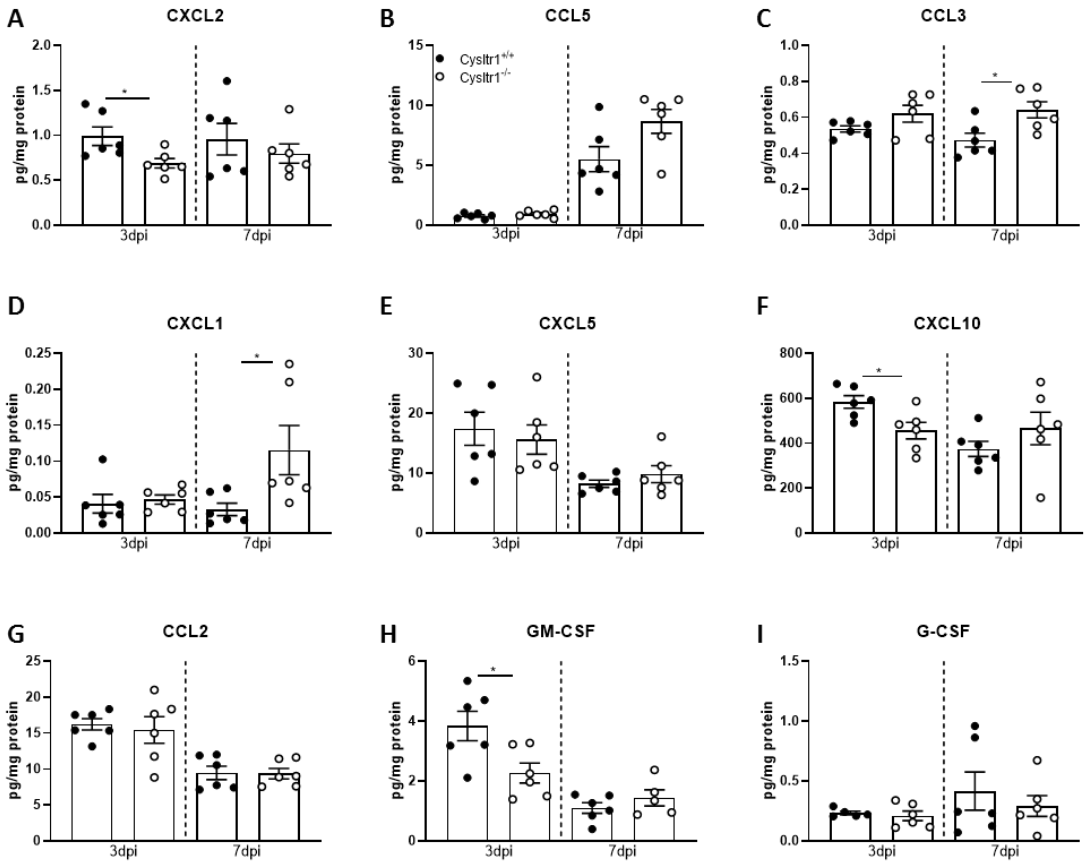

Figure 15: Chemokine and growth factor levels in the liver of CysLTR1 knockout mice after *L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and sacrificed the mice at 3- and 7-dpi (*n = 6 per group*). Liver homogenates were collected and measured for **A)** CXCL2, **B)** CCL5/RANTES, **C)** CCL3, **D)** CXCL1, **E)** CXCL5, **F)** CXCL10, **G)** CCL2, **H)** GM-CSF and **I)** G-CSF concentration by ELISA. Data is representative of three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*$p<0.05$).

CysLTR1 deletion results in an increased recruitment of neutrophils in the spleen after *LM* infection: The spleen plays an important role during host immune responses to blood-borne bacterial infections (155), due to its unique structural organization. The spleen can connect innate and adaptive immunity resulting in effective clearance of blood pathogens (156). Therefore, we then evaluated the effect of CysLTR1 deletion on the ability of the spleen to control *LM* infection in mice. CysLTR1 deficient mice were infected with *LM* and sacrificed at 3- and 7-dpi to determine the bacterial burden and immune responses by flow cytometry. At both time points, the KO and WT mice had comparable bacterial burdens in the spleen (Figure 16A). We made single-cell suspensions and found that at 7dpi the KO mice had significantly reduced cell numbers (Figure 16B) with no

apparent differences in spleen weights at both time points (Figure 16C). Even though there seemed to be no differences in the control of *LM* infection between the two mice groups, there seemed to be a reduction in cellular recruitment into the spleen of CysLTR1 deficient mice at 7dpi, a time point regarded as chronic stage of infection during *LM* infection. This revealed more tissue distraction in KO mice compared to their littermate controls indicated by the white pulp (black arrow) and red pulp (red arrow) atrophy (Figure 16D). White pulp atrophy could characterize the loss of lymphocytes in T cell and B cell areas of the spleen, and red pulp atrophy is due to decreased hematopoietic cells (157, 158). We then assessed immune cell populations in response to *LM* infection by flow cytometry analysis. At 3dpi, we saw an increasing trend in myeloid cell percentages (monocytes, red pulp macrophages, marginal zone macrophages, conventional DC1, and DC2) of CysLTR1 KO mice compared to their littermate WT controls. Moreover, we observed a significant increase in neutrophil percentages (Figure 17E), which were maintained in cell numbers in KO mice (Figure 16G), suggesting CysLT signalling might play an important role in the recruitment of neutrophils. At 7dpi, there were no differences in spleen myeloid cell percentages of the two groups, except for a significant increase in marginal zone macrophages and neutrophil cell percentages of KO mice (Figure 16F). Amongst myeloid cell numbers, there was a decreasing trend in KO cell populations, with a significant reduction in cDC1 (Figure 16H), this reduction in KO myeloid cell numbers could be attributed to the decreased cell numbers harvested from the spleen. This suggest that increased neutrophil percentages both at 3dpi and 7dpi can highlight the role of CysLT signalling in inflammation in both tissue and *L. monocytogenes*-specific contexts.

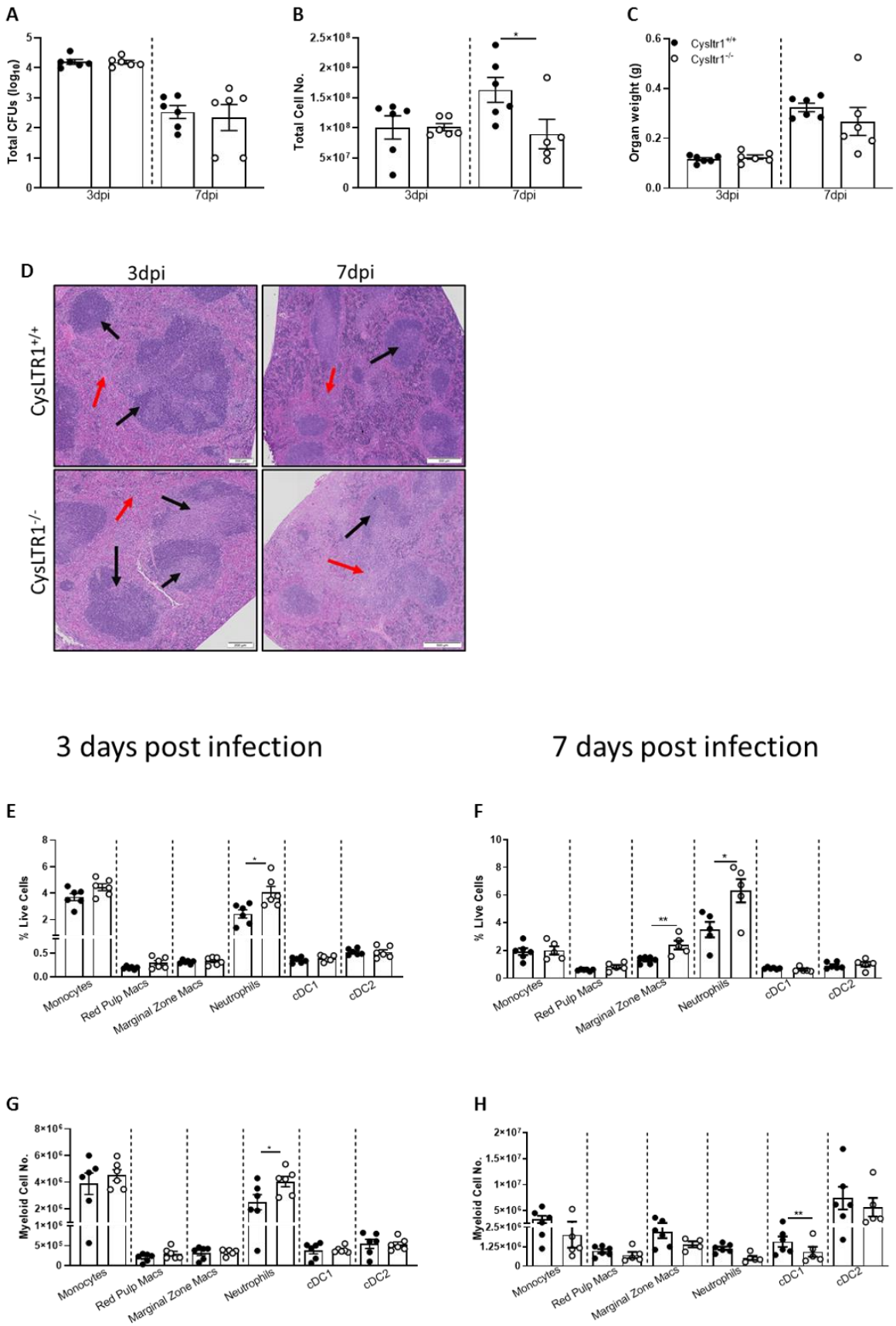

Figure 16: CysLTR1 deficiency in mice increased splenic neutrophils after *L. monocytogenes* infection. CysLTR1 KO mice and their littermate controls were infected with about 1.1x10^5 *LM* CFUs/200µL per mouse intraperitoneally. The mice were sacrificed at 3- and 7-dpi (*n = 6 mice per*

group) and spleen were collected to measure **A)** bacterial burden by plating spleen homogenates on TSA plates for CFUs, **B)** cell numbers by making single-cell suspensions and **C)** organ weights. **D)** Spleen histopathology was done by staining with H&E and the images were captured using the Olympus-VS-ASW-L100 imaging system. The single cells were then stained for myeloid cell population analysis by flow cytometry. **E-F)** Spleen myeloid cell percentages of total live splenocytes at 3- and 7-dpi. **G-H)** Spleen myeloid cell numbers 3- and 7-dpi. Data is representative of two experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*$p<0.05$, **$p<0.01$).

CysLTR1 deficient spleen lymphocytes are significantly decreased early after *LM* infection: We then assessed at lymphocyte population in the spleen at 3- and 7-days post-*LM* infection. At 3dpi, there was a significant reduction in the KO lymphoid cell percentages (T cells, B cells, NK cells, CD8 T cells, and CD4 T cells) except for γδ T cells which were comparable to littermate controls (Figure 17A). The lymphoid cell numbers, however, had no differences between the KO and WT (Figure 17C). At 7dpi, the lymphoid cell percentages were comparable between the two groups, but the KO mice had significantly reduced NK cells (Figure 17B). The lymphoid cell numbers were significantly reduced in the KO mice compared to their controls (Figure 17D). We then looked at T cell subsets at 3dpi and there was no difference in CD4 follicular T helper cells, while there was a significant reduction in KO CD4 naïve T cells, but increased central memory T cells and effector-effector T cells (Figure 17E), while there were no differences in CD4 T cell subsets at 7dpi (Figure 17F). There was an increasing trend in KO CD8 follicular T helper cells, while there was a significant reduction in naïve, and central memory, but increased effector-effector CD8 T cells 3dpi (Figure 17G). At 7dpi however, there was only a significant reduction in KO CD8 central memory T cells while the other subsets had no differences between the two groups (Figure 17H). We measured T cell exhaustion or activation by looking at PD1 and KLRG1 expression by CD4 and CD8 T cells at 3- and 7-dpi. At 3dpi, there was a significant increase in both KO CD4 and CD8 T cells expression of $PD1^+$ $KLRG1^+$ markers which indicates increased activated vascular cells, while there was a reduction in $PD1^-$ $KLRG1^-$ cells (Figure S9A and S9C). However, at 7dpi, there were no differences between CD4 and CD8 T cell exhaustion markers between the KO and WT mice (Figure S9B and S9D). We measured T cell proliferation, at 3dpi KO CD8 T cell proliferation was significantly increased (Figure 17I), while there was a significant increase in KO CD4 and CD8 T cell proliferation at 7dpi (Figure 17H). These results show that Cysltr1 KO mice have decreased NK cells in the spleen, increased effector and proliferative T cell phenotype at the early stages of the infection.

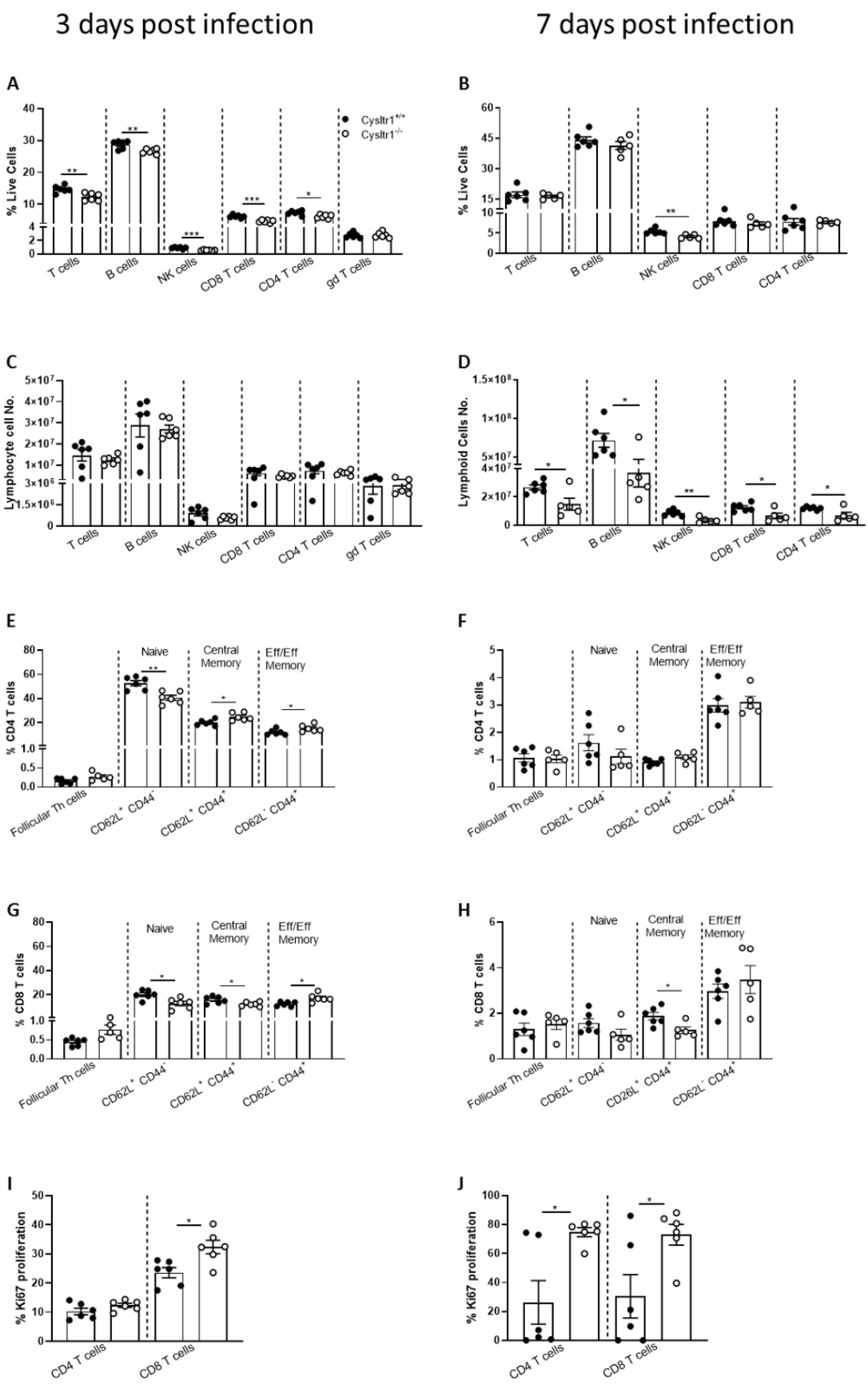

Figure 17: Effect of CysLTR1 deletion in mice spleen lymphocyte population post *L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with

about $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3- and 7-dpi (*n = 6 per group*). Spleen single cells were stained for lymphoid cell population analysis by flow cytometry. **A-B)** Spleen lymphoid cell percentages of total live splenocytes, **C-D)** lymphoid cell numbers, **E-F)** CD4 T cell subsets, **G-H)** CD8 T cell subsets and **I-J)** CD4 and CD8 T cell proliferation measured by Ki67 at 3- and 7-dpi. Data is representative of two independent experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*p<0.05, **p <0.01, ***p<0.001).

After *LM* infection, CysLTR1 deficient mice showed an early decrease in IFN-γ and a late increase in TNF production by CD4 T cells: We then stimulated splenocytes with PMA/ionomycin or left unstimulated to measure IFN-γ, Granzyme B, perforin and TNF secretion by flow cytometry. At 3dpi, the percentage of KO CD4 T cells producing IFN-γ were significantly reduced in unstimulated cells (Figure 18A). However, KO CD8 T cells producing IFN-γ were unaffected (Figure 18B). There was a significant decrease in KO CD8 T cells producing perforin (Figure 18C). CysLTR1 KO CD8 T cells production of granzyme B had no effect when compared to the WT littermate controls (Figure 18C and 18D). At 7dpi, there was a significant increase in TNF production by KO CD4 T cells post PMA/ionomycin stimulation (Figure 19B) and a similar trend in IFN-γ production (Figure 19A). Interestingly, even though CD4 and CD8 T cells have higher activation markers at 3dpi, they were secreting lower Th1 and cytotoxic cytokines.

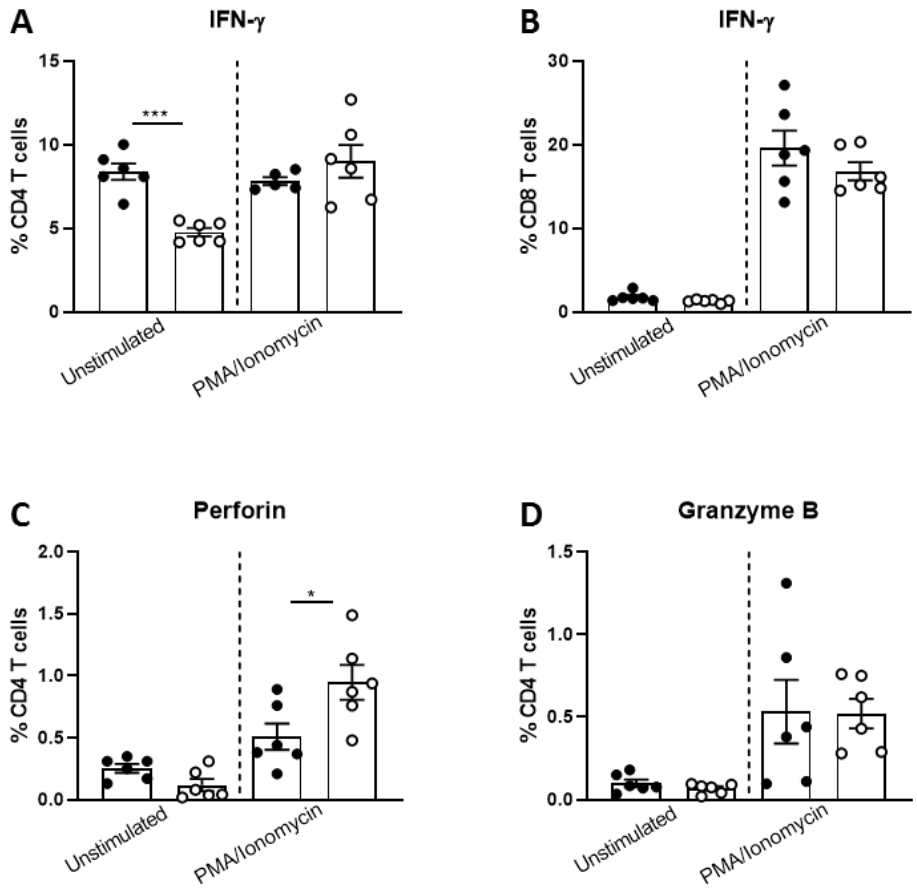

Figure 18: CysLTR1 deficient CD8 T cells have decreased production of IFN-γ and perforin at three days post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3dpi (*n = 6 per group*). Splenocytes were stimulated for cytokine production using PMA/ionomycin (stimulated) or left unstimulated. Flow cytometry was used to determine percentages of **A-B)** IFN-γ produced by CD4 and CD8 T cells, **C)** perforin and **D)** granzyme B by CD8 T cells 3dpi. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*p<0.05, **p<0.01, ***p<0.001).

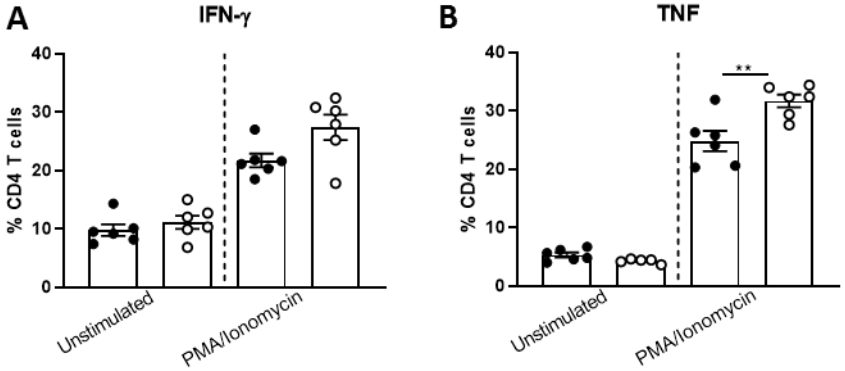

Figure 19: CysLTR1 deficient mice CD4 T cells have increased production of IFN-γ and TNF at seven days post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice

were sacrificed at 7dpi (*n = 6 per group*). Splenocytes were stimulated for cytokine production using PMA/ionomycin (stimulated) or left unstimulated. Flow cytometry was used to determine percentages of **A)** IFN-γ and **B)** TNF produced by CD4 T cells at 7dpi. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (**p<0.01).

CysLTR1 deficiency had no effect on spleen cytokine and chemokine profile after *LM* infection: We determined spleen cytokine responses in the tissue homogenates. Nitric oxide production was unaffected at both 3- and 7-dpi (Figure 20A). We measured pro-inflammatory cytokines by ELISA, and there was a significant increase in IL-1α at 3dpi in the KO compared to WT (Figure 20B). At both 3dpi and 7dpi, IL-17, IL-12p70, and IL-23 secretion were undetectable (data not shown). The other pro-inflammatory cytokines, however, were comparable in KO and WT at both time points. In addition, there were no significant differences in the production of TGF-β and IL-10 secretion in both KO and WT mice (Figure 20H and 20I), however, a significant decrease in IL-4 secretion at 7dpi in KO homogenates (Figure 20J). We then measured chemokine (CXCL2, CCL5, CCL3, CXCL1, CXCL5, and CXCL2) and growth factor (GM-CSF) secretion by KO and WT mice in spleen homogenates, and there were no significant differences in all measured chemokines and growth factors (Figure 21A-21G). Altogether, this suggests that the spleen cytokine/chemokine profile was similar between WT and Cysltr1 KO samples.

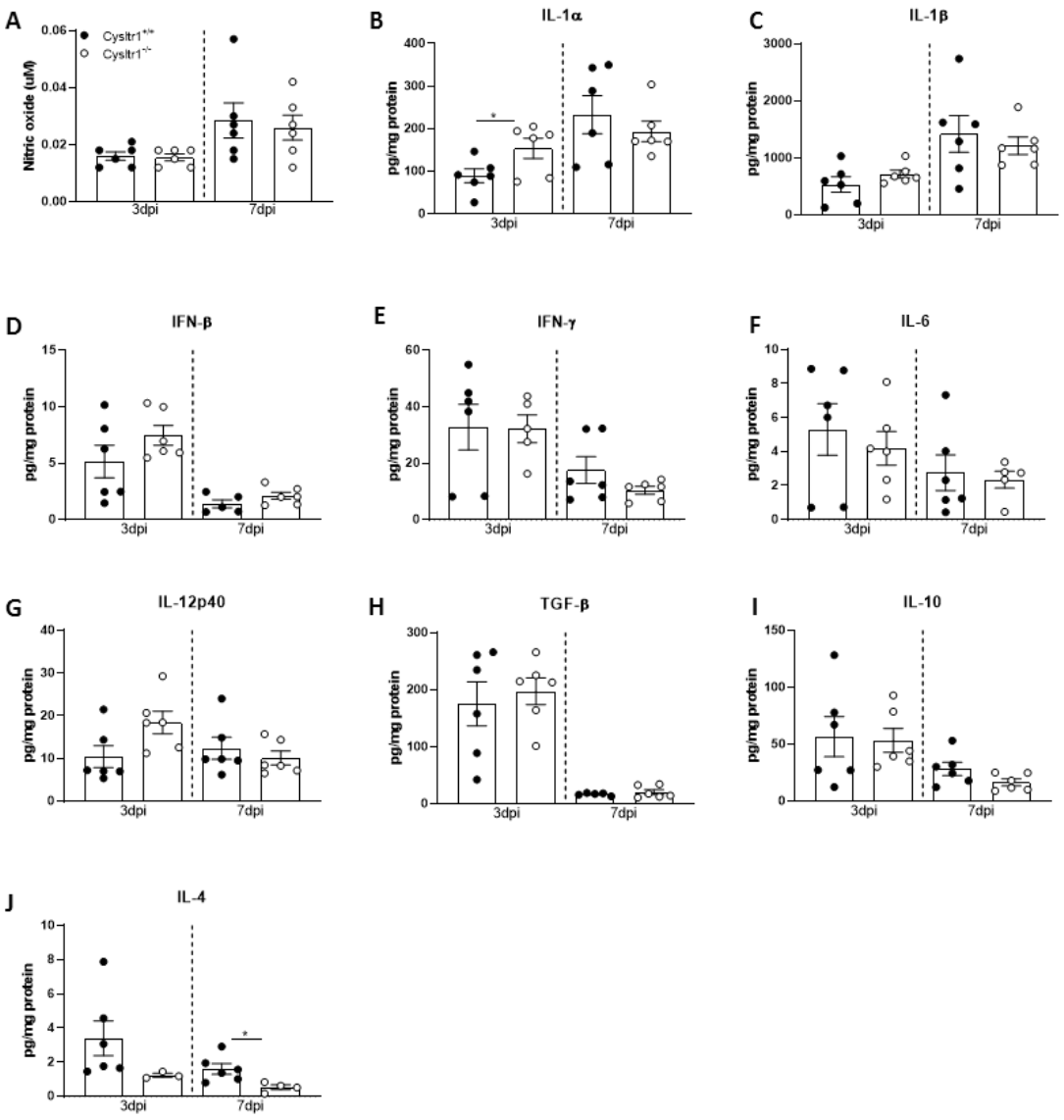

Figure 20: Nitric oxide, pro-inflammatory, regulatory and Th2 cytokine production in CysLTR1 knockout spleen homogenates post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3 and 7dpi (*n = 6 per group*). The spleen was collected for homogenates and measured **A)** nitric oxide, pro-inflammatory; **B)** IL-1α, **C)** IL-1β, **D)** IFN-β, **E)** IFN-γ, **F)** IL-6**,** and **G)** IL-12p40, regulatory; **H)** TGF-β, **_I)** IL-10 and Th2 cytokines **J)** IL-4 by ELISA. Data is representative of two experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction, (*p<0.05, **p<0.01).

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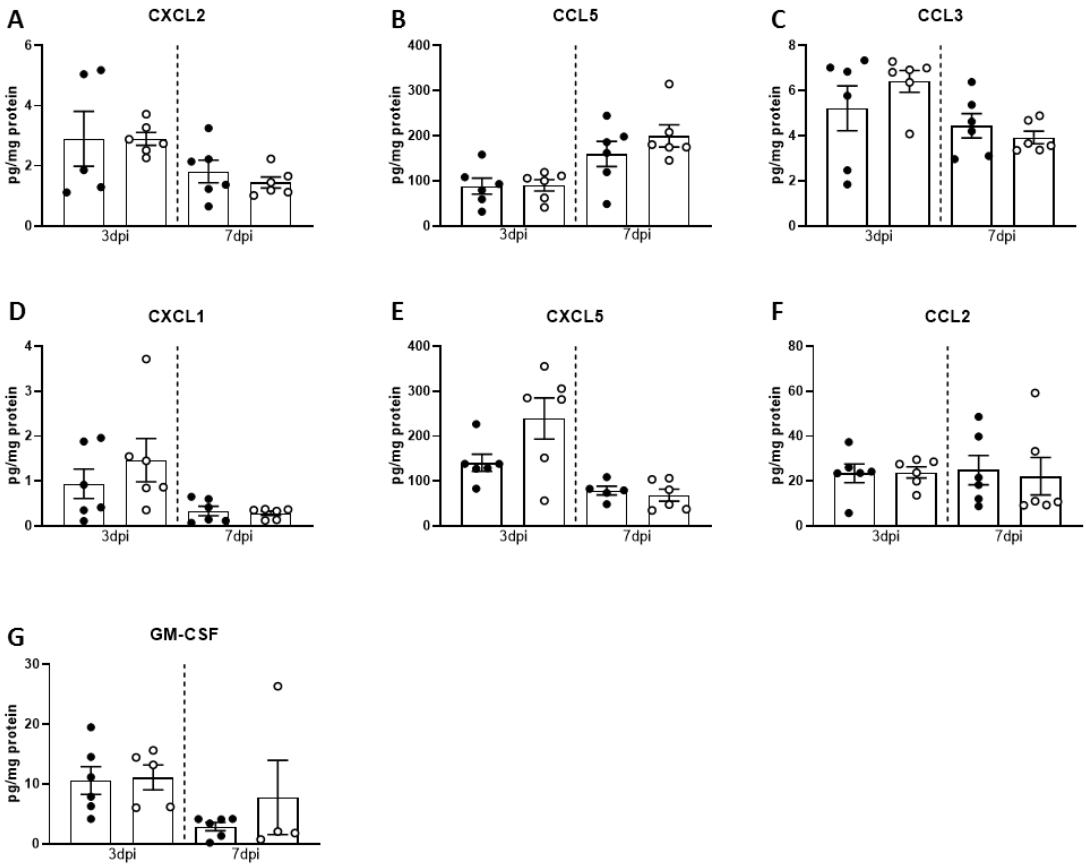

Figure 21: Chemokine and growth factor production in CysLTR1 knockout spleen homogenates post *L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3- and 7-dpi (*n = 6 per group*). The spleen was collected for homogenates and measured **A)** CXCL2, **B)** CCL5/RANTES, **C)** CCL3, **D)** CXCL1, **E)** CXCL5, **F)** CCL2 and **G)** GM-CSF by ELISA. Data is representative of two to three experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction.

CysLTR1 deficient splenocytes have increased IFN-γ production upon re-stimulation *ex vivo*: Following total cytokine concentrations measurement in the spleen, we also stimulated splenocytes with anti-CD3 antibody, heat-killed *LM* (HKLM) or left them untreated and measured cytokine secretion potential of T cells. Treating cells with HKLM was done to determine the antigen-specific T cells responses. At 3dpi, there were no differences in IFN-γ secretion (Figure 22A), while there was a significant increase in IFN-γ production by KO splenocytes post-HKLM re-stimulation (Figure 22B). IL-2 secretion from stimulated splenocytes was similar at 3dpi (Figure 22C), whereas HKLM stimulation of KO splenocytes resulted in a significant reduction at 7dpi (Figure 22D). However, IL-4, IL-17, IL-10 and TGF-β secretion by splenocytes was comparable between the KO and WT cells both at 3- and 7-days post-*LM* infection (Figure 22E-22L). Decreased IL-2

secretion is actually in line with decreased T cell numbers in the spleen at 7dpi which can explain decreased maintenance of these cells.

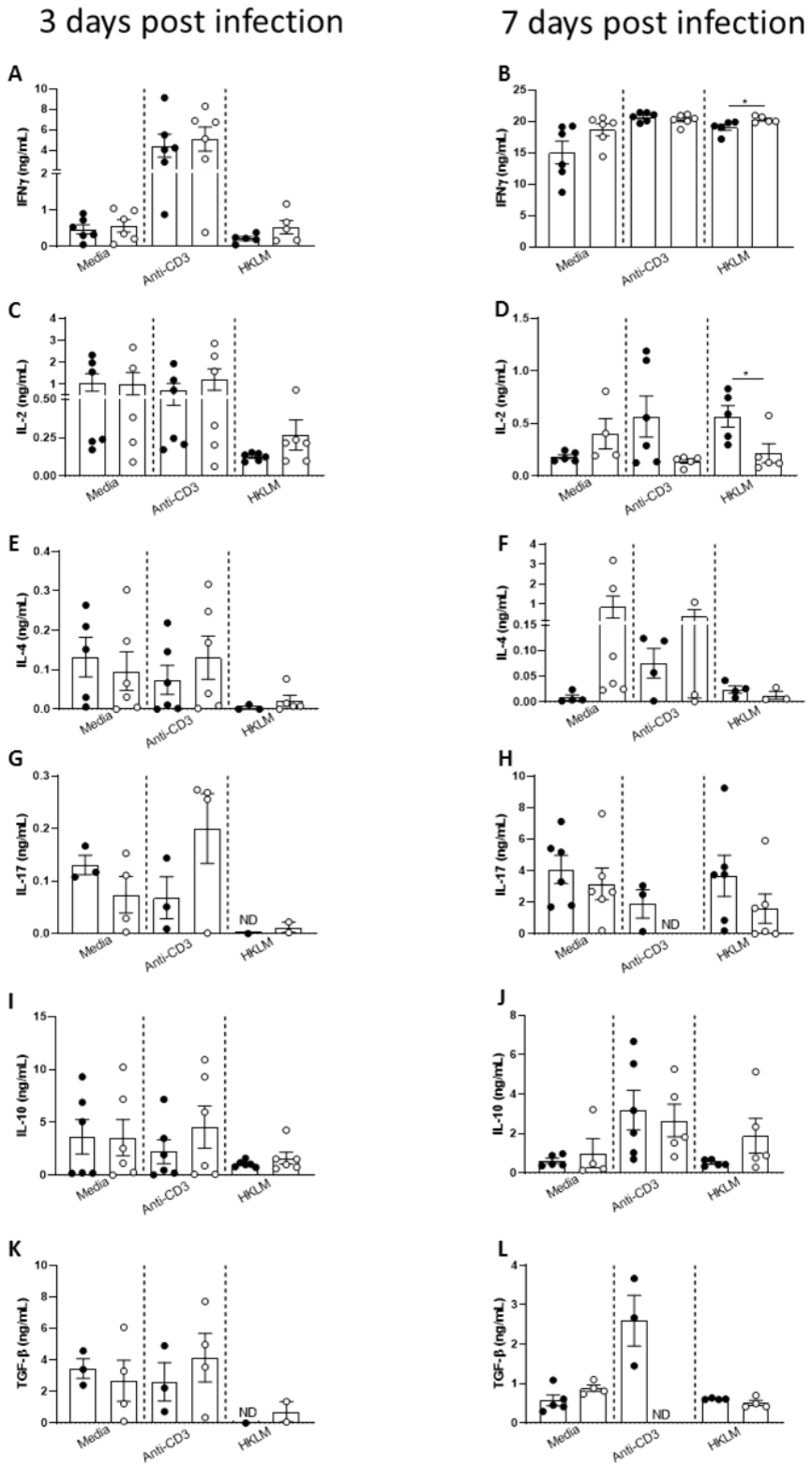

Figure 22: Re-stimulation of KO splenocytes with heat-killed *LM* increased IFN-γ production seven days post-infection. CysLTR1 deficient mice and their littermate controls were infected with $1.1x10^5$ L*M* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3- and

7-dpi (*n = 6 per group*). Splenocytes were stimulated for cytokine production using anti-CD3, heat-killed *LM* (HKLM) or left unstimulated for 3 days at 37^{o}C. **A-B)** IFN-γ, **C-D)** IL-2, **E-F)** IL-4, **G-H)** IL-17, **I-J)** IL-10 and **K-L)** TGF-β levels were measured in supernatants by ELISA. Data is representative of two experiments. Error bars denote mean ± SEM and analysed using the unpaired, student t-test with/without Welch's correction, (*p<0.05).

CysLTR1 deficiency had no effect on systemic cytokine secretion after *LM* infection: We then measured systemic cytokines at 3 and 7 days in serum by ELISA post-*LM* infection. There were no significant differences in cytokine levels measured at both time points (Figure 23A and 23B). At 3dpi, there was an increasing trend in IFN-γ, IL-17 secretion in KO compared to the WT controls. This increasing trend was reversed in all cytokines measured at 7dpi. This suggests that as in the spleen, serum cytokine levels do not differ significantly; however, we observed some differences in cytokine/chemokine levels in the liver, again highlighting tissue-specific effects of CysLT signalling.

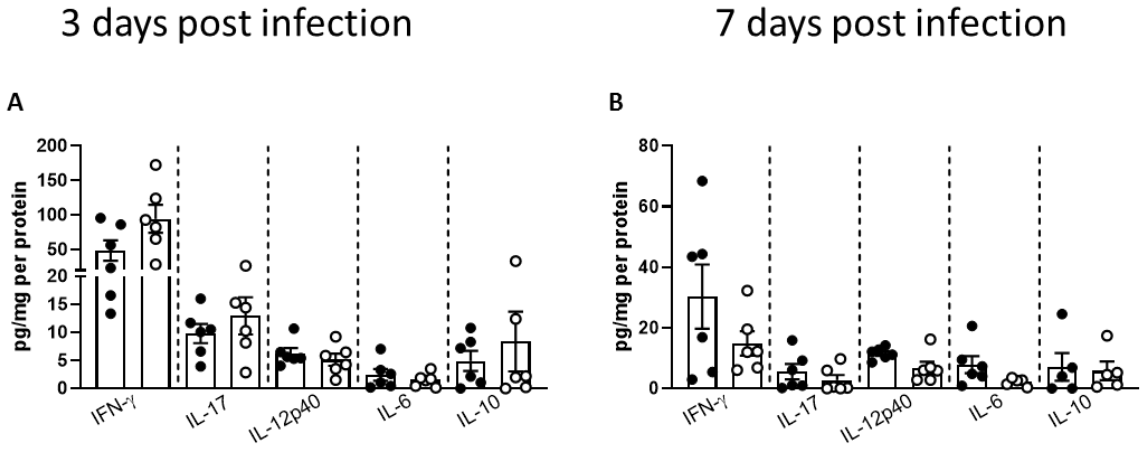

Figure 23: CysLTR1 deletion has no effect on serum cytokines during L. monocytogenes infection in mice. CysLTR1 deficient mice and their littermate controls were infected with 1.1x10^{5} *LM* CFUs/200µL per mouse intraperitoneally and the mice were sacrificed at 3- and 7-dpi (*n = 6 per group*). The cardiac puncture was performed to collect blood for cytokine levels in response to *LM* infection. **A-B)** Measured cytokine (IFN-γ, IL-17, IL-12p40, IL-6, and IL-10) secretion in the blood by ELISA 3- and 7-dpi. Data is representative of two experiments. Error bars denote mean ± SEM and analysed using the unpaired student t-test with/without Welch's correction.

3.6. Survival benefit in CysLTR1 deficient mice is dependent on genetic background of animals during *L. monocytogenes* infection.

We were able to show that CysLTR1 is highly expressed in the mice liver and spleen during *LM* infection, and KO mice have Moreover, sub-lethal dose of LM infection to evaluate

the role of CysLTR1 as a function of time revealed no major differences in bacterial loads, however, increased neutrophilic recruitment and reduced lymphoid cells in the spleen. We then evaluated the effect of CysLTR1 deletion has on the survival of mice during *LM* infection by using LD_{50} dose. We infected C57BL/6 CysLTR1 KO mice and their littermate controls with about $1x10^6$ *LM* CFUs/200µL intraperitoneally and monitored over a period of 15 days. CysLTR1 KO mice showed a modest increase in susceptibility, however, not significant to the *LM* infection when compared to littermate controls (Figure 24A). Consistent with our other data with BALB/C mice, we also performed mortality experiment with BALB/C CysLTR1 KO mice. Remarkably, we found that deletion of CysLTR1 in BALB/C mice displayed susceptibility during *LM* infection (Figure 24B). We speculate this could be due to the increased neutrophilic recruitment and tissue pathology caused by neutrophilia and likely predisposed susceptibility of BALB/C background.

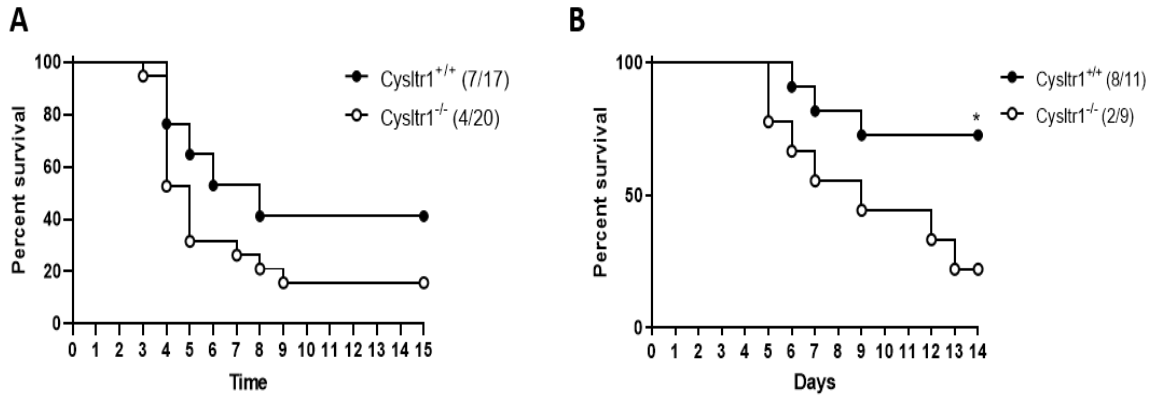

Figure 24: Effect of CysLTR1 deletion on mice survival during *L. monocytogenes* infection. C57BL/6 $CysLTR1^{+/+}$ and $CysLTR1^{-/-}$ mice (*n= 9-10 mice per group*) were infected with $1x10^6$ CFUs *LM* per 200µL and monitored for survival over 15 days (two pooled experiments). And BALB/c $CysLTR1^{+/+}$ and $CysLTR1^{-/-}$ mice (n= 9-11 mice/group) were infected with $3.8x10^5$ CFUs *LM* per 200µL and monitored for survival over 14 days. **A)** C57BL/6 and **B)** BALB/c mice survival curve post infection with *LM*. Statistically analysed by log-rank (Mantel-Cox) test, (*$p<0.05$).

CHAPTER 4: DISCUSSION

4.1. CysLT receptors and Ltc4s expression in response to *LM* infection

Cysteinyl leukotrienes have been well studied in asthmatic and allergic responses, with mostly pathophysiological functions of CysLTs in asthma being regulated through CysLTR1. And as a result, several antagonists (montelukast, pranlukast, and zafirlukast) for CysLTR1 have been developed and are being used in clinical practice (108). CysLTR1 and CysLTR2 induce similar signalling events, which include calcium (Ca^{2+}) influx and inositol phosphate accumulation in the cells (159–161). However, they mediate different responses *in vivo* and have different cellular and tissue distribution (162–164). CysLTR1 has been shown to be mainly expressed in the peripheral blood leukocytes, including eosinophils, monocytes, neutrophils, basophils, DCs, B cells, T cells, mast cells, interstitial lung macrophages and bronchial smooth muscle cells, and in the lung, spleen, pancreas, small intestines and other tissues (90, 162, 163, 165, 166). CysLTR2, on the other hand, has been shown to be highly expressed in tissues, such as the heart, adrenal tissue, lung, spleen, endothelium tissue, and in the peripheral blood leukocytes (163). Even though these receptors are widely expressed in different cells and tissues, it is unknown whether their expression can be induced by a bacterial infection and their potential role during bacterial infection. We showed that *LM* infection induce both CysLTR1 and CysLTR2 expression together with LTC_4S expression both *in vivo* and *in vitro*. *In vivo*, the expression of these receptors and LTC_4S are highly upregulated at a later stage of infection, while at macrophage level CysLTR1 and LTC_4S are upregulated early after infection. Interestingly, macrophages stimulated with HKLM, showed CysLTR1, CysLTR2 and LTC_4S mRNA expression was unaffected, suggesting that live *LM* is required to induce their expression. CysLTs are calcium 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 (167). It has been demonstrated that *LM* transiently induces calcium influx through the production of LLO. It is believed that *LM* induces extracellular Ca^{2+} mobilization by LLO, and activates the downstream Ca^{2+}-dependent signalling which is required for cell invasion (168). This could, therefore, suggest that *LM* might manipulate the CysLT pathway for Ca^{2+} flux for an efficient cellular invasion.

4.2. Characterization of CysLTR1 deficient mice at naïve state

CysLTR1 knockout mice were generated by Yoshihide Kanaoka's group (137), but the comprehensive immunological characterization of the mice has not been reported. In order to understand the role of this receptor and its signalling during intracellular bacterial infection, its deletion at naïve state should have no significant effect on mice. We first confirmed deletion by genotyping the KO and WT mice and able to show a successful deletion of the Cysltr1 gene. The KO mice have a 333bp gene size because of the neomycin resistance gene insertion into the mutant allele, instead of the actual Cysltr1 gene size (284bp). We further validated CysLTR1 deletion on tissues by measuring its mRNA expression and additionally, its deletion had no effect on Cysltr2 and Ltc4s mRNA expression. The reason why CysLTR2 expression is not affected by Cysltr1 deletion in mice, even though they are 31% homologous (169), shows that there are no feedback mechanisms involved in the expressions of CysLT receptor genes. Cysltr2 gene is located on chromosome 13, position 13q14 in humans (170), while it is located in chromosome 14 in mice (137). Cysltr1 is located on the X chromosomes both mice and humans, position Xq13-Xq21 in humans (108, 137, 171). LTC_4S, the enzyme responsible for the synthesis of cysteinyl leukotrienes, is not affected by Cysltr1 deletion, therefore this receptor has no effect on the enzyme's expression or regulation. Full characterization of CysLTR1 KO mice revealed that CysLTR1 deletion does not alter mice immunity or organ pathology at naïve state. It was interesting that lung, liver, and spleen B cells in both WT and KO mice were higher compared to other lymphoid populations. B cells are believed to play a pivotal role in immunity because of their ability to produce antibodies, however their role is dependent of environmental stimulations such as growth factors, cytokines and intercellular interactions (172). As mentioned above, CysLTR1 can be expressed by various cells and tissues including bronchial smooth muscle cells, phagocytic cells and B lymphocytes. In B cells, CysLTR1 is said to be upregulated post-stimulation with CD40L and these cells have increased responsiveness to LTD_4 in terms of antibody production (173). However, at steady state, CysLTR1 does not influence B cell recruitment or development but might play a role in antibody production during immune responses. It has been demonstrated that hematopoietic progenitor cells can be stimulated by a variety of cytokines into mature blood cells which include B cells, basophils, monocytes to macrophages, neutrophils, eosinophils and T cells through specific receptors and CysLTR1 is expressed on the surface of these immune cells at various stages of development (166).

This, therefore, suggests that CysLTR1 signalling may play a role in both lymphoid cell and myeloid cell generation and recruitment however, we show that at naïve state CysLTR1 has no major effect on the generation of these populations. Suggesting, CysLTR1 signalling is potentially involved in immune regulation in response to inflammatory ques and pathogenic infection. We measured cytokine secretion in organ homogenates and serum, and there were no significant differences between the KO and WT cytokine secretion at naïve state. Taken together, our results demonstrated that CysLTR1 deletion in mice has no effect on organ pathology and proportion of immune populations. This had a small effect on cytokine and antibody secretion at naïve state.

4.3. Effect of CysLTR1 deletion in macrophages for the control of *LM* infection *in vitro*

Macrophages are important innate immune cells and they also play a key role in the initiation of adaptive immunity. Activation of macrophages increases phagocytosis and killing properties, membrane receptor expression, and secretion of cytokines to induce an appropriate pro-inflammatory immune response (174, 175). During *LM* infection, IFN-γ potentiates peritoneal macrophages to prevent its escape to the cytosol, inhibiting its growth. Moreover, IFN-γ, LPS, IL-6, and anti-IL-10 stimulated bone marrow-derived macrophages also inhibit *LM* escape and cytoplasmic growth (176, 177). In this study, we demonstrated that macrophages deficient of CysLTR1 showed comparable control of *LM* infection to wildtype macrophages. Despite no differences in bacterial burden in KO and WT BMDMs from C57BL/6 and BALB/c background, they varied in their cytokine profiles. Nitric oxide production was comparable at insufficient levels in both backgrounds in KO and WT macrophages, which explains the inability of macrophages to control *LM* infection. NO is a toxic L-arginine radical synthesized by inducible nitric oxide synthase in activated macrophages. IFN-γ and LPS are known to be potent NO synthase inducers in macrophages (178). During *in vitro* infections with pathogens such as *Leishmania major*, *Toxoplasma gondii* and *Mycobacterium bovis*, NO exhibits antimicrobial properties (179–181). It has also been shown that it may be an important molecule during *LM* killing by macrophages stimulated by IFN-γ (182), however, there are studies contradicting this. During *LM* infection increased expression of nitric oxide synthase 2 (NOS2) and production of NO has been related to increased *LM* cell-to-cell spread (183). It has also been proposed that NO delays macrophage phagosome maturation that contain the bacteria, slowing the macrophage killing effect (184). During early allergic reactions, CysLTs and

NO are one of the mediators released following antigen-antibody interactions (185). It has also been shown that during an allergic response, nasal obstruction induced by LTD_4 is inhibited by pranlukast and N^{ω}-nitro-L-arginine methyl ester (L-NAME), NO synthase inhibitor (186), suggesting that NO secretion by activated CysLTR1 might have an essential role allergic inflammation. The comparable NO secretion in C57BL/6 BMDMs was accompanied by an increasing trend in IL-10 secretion. In contrast, BALB/c KO BMDMs showed a decreasing trend in IL-10 secretion. IL-10 is an inhibitor of macrophage pro-inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) production (187, 188). It has been shown to decrease nitric oxide levels and iNOS expression in LPS stimulated macrophages (189). Moreover, one study has demonstrated that IL-10 inhibits cysLT-induced activation of monocytes and dendritic cells (190). Our results show that: 1) Bacterial burden between the KO and WT macrophages generated from both backgrounds is comparable, accompanied by similar production of NO, which explains bacterial survival within these cells. 2) Based on the available literature, there is a possibility that NO is insufficient for effective killing of *LM*, as it has been shown that both ROI and RNI play a role in entrapping *LM* within the phagosome. Therefore, even though there is evidence that NO promotes the cell-to-cell spread of *LM*, activated macrophages have been shown to retain the bacterium within the phagosome through localization of RNI or ROI to the vacuole (46). 3) We have shown that *LM* infection induces expression of CysLT receptors and their enzyme. Even though CysLTR1 activation induces NO secretion in Th2 settings of allergic responses, activation of this receptor in Th1 settings with different environmental cues might have a different role. Here, we demonstrate that NO secretion was not affected in CysLTR1 knockout macrophages in *LM* infection. 4) The differences we observed in cytokine profiles between the two backgrounds suggest that macrophages generated from C57BL/6 are slightly different from BALB/c mice which have different immune regulation patterns. For instance, C57BL/6 KO macrophages have an increasing trend in IL-10 secretion, which inhibits pro-inflammatory cytokine secretion required for effective killing of *LM*. This increased IL-10 production resulted in a decreasing trend in TNF secretion is which required for effective killing of *LM*, and MIP2, which has been shown to provide resistance to *LM* infection (191). However, in BALB/c KO macrophages, the anti-inflammatory IL-10 secretion has a decreasing trend which is accompanied by decreasing trend in IL-6, TNF and MIP2, also validating their inability to kill *LM*. Most studies, however, have reported that for an effective secretion of pro-inflammatory cytokines and effective *LM* killing by

macrophages, macrophages must be pre-conditioned by either IFN-γ or LPS. Therefore, further *in vitro* experiments are required to assess *LM* growth in IFN-γ/LPS-stimulated macrophages.

4.4. Effect of CysLTR1 deletion during *LM* infection in mice

Neutrophils play the first line of defence during *LM* infection by direct killing of infected cells or the bacterium itself, however, there are conflicting reports on the role of neutrophils during the control of *LM* infection. Neutrophils in the liver have been demonstrated to kill bacteria from apoptotic hepatocytes or by directly lysing infected hepatocytes (192, 193). They are also known to secrete chemokines that recruit more immune cells to the site of infection (194). However, Shi *et al*., 2011, demonstrated that mice depleted of neutrophils are less susceptible to *LM* infection than those depleted of monocytes, suggesting that neutrophils are dispensable during *LM* infection (195). Our study further adds that, even a significant increase in neutrophils in CysLTR1 KO mice at 3 and 7 dpi, had no differences in organ bacterial burdens. This, therefore, validates that neutrophils may have no to minimal effect on bacterial killing, and potentially play a role in the spread of the bacterium and tissue pathology instead.

More recently, neutrophils were shown to regulate macrophage inflammatory signalling by inducing a rapid and sustained suppression of NF-κB activation (196). Interestingly, at 7dpi in the liver of KO mice, there was a significant increase in monocyte-derived macrophages, while neutrophils percentages were comparable between the KO and WT at this time-point. This suggests that increased neutrophils might be inhibiting the recruitment of these macrophages, hence the inability to control bacterial burden, as these cells have been shown to be unable to kill *LM* infection (197). It has been reported that CysLT receptor expression in neutrophils is lesser compared to other cells, but upon 5-LOX activation neutrophils are able to secrete LTA_4 to cells that can synthesize LTB_4 and CysLTs. LTB_4 activates neutrophils causing the release of proteases, myeloperoxidases and reactive oxygen species, which can cause tissue destruction (198). However, when neutrophils are stimulated with LTC_4 and LTD_4, they have moderate activation of Ca^{2+} mobilization and nitric oxide production when compared to LTB_4 stimulation (199). Deletion of CysLTR1 has no effect on leukotriene (LT) and cysteinyl leukotrienes (CysLTs) synthesis, however, it surely affects CysLT activities or signalling. Here, we demonstrated that the deletion of CysLTR1 promoted neutrophil recruitment during *LM* infection. The increase in the recruitment of neutrophils in CysLTR1 deficient mice during *LM* infection could be due to

LTB_4 action, though, further investigation is required. However; increased frequencies and possible augmented activation of neutrophils can also explain the increased tissue destruction in KO mice spleen.

We also demonstrated that at early *LM* infection, liver and spleen lymphoid cells were significantly reduced in KO mice. In the liver, this reduction was significant in B cells, NK cells, and CD4 T cells, while in the spleen all the lymphoid cells, except for γδ T cells, were significantly reduced. The lymphoid cells are important for adaptive immunity against *LM* infection. During *LM* infection, B cells are said to be susceptible to the infection (200) and are killed through apoptosis depending on the infection dose of *LM* (201, 202). During innate immunity to *LM* infection, NK cells are said to produce IFN-γ which is required to increase myeloid cell antimicrobial activities against invading intracellular bacteria (36, 37). Therefore, NK cells induce host immunity to bacterial infections. However, Clark *et al*., 2016, demonstrated that activated NK cells increase host susceptibility to *LM* infection which is independent of their IFN-γ production. They correlated the increased susceptibility to *LM* infection with enhanced production of IL-10 by NK cells, which was promoted by the *LM* virulence factor p60 (38). CD4 T cells also participate in host protection during *LM* infection, they differentiate into Th1 cells inducing the production of Th1-derived cytokines, such as IFN-γ (203–205). CD8 T cells induces immunity to *LM* by either lysing the infected cells by direct exposure of intracellular bacteria to perforin and granzymes, or by secreting IFN-γ that in turn activates macrophages for effective killing of the bacteria (55). As aforementioned, CysLTs are potent inducers of Ca^{2+} influx and it has been demonstrated that when LTD_4 binds to CysLTR1 there is an induction of Ca^{2+} fluxes, which result in chemotaxis of αβ and γδ T cells to the inflamed tissue (206). Therefore, deletion of CysLTR1 could be reducing Ca^{2+} influx and recruitment of lymphocytes to the site of infection, hence reduction in the lymphoid cell population in the liver and spleen of KO mice during *LM* infection. During inflammatory responses like in rhinosinusitis allergic reaction, leukocytes that express CD45 on their cell surfaces, which include T cells, B cell and monocytes, CysLTs play a role in their activation and recruitment. It has been demonstrated that CysLTR1 expression in these cells is highly upregulated during allergic inflammation and suggested that CysLTR1-expressing cells maybe selectively recruited to the site of inflammation or may replicate and survive through the influence of CysLTs (207). Therefore, increased lymphoid cells in the liver and spleen of WT mice during *LM* infection could be due to

increased CysLTR1 expression in response to the infection and its interaction with its binding ligands which result in recruitment of lymphoid cells to the site of infection. Even though the increased recruitment of CD4 T cells in the liver of WT mice; in terms of activation, proliferation and IFN-γ production, they are comparable to those of KO mice. This further explains the comparable bacterial control observed between the KO and WT mice. Interestingly, though CD8 T cells between the two groups were similar but KO mice had slightly increased CD8 effector/effector memory T cells, decreased IFN-γ production, and increased perforin and granzyme B secretion. However, the granzyme B and perforin secretion were not efficient enough to kill the bacteria on their own as, IFN-γ production by T cells is required for activation of macrophages and subsequent secretion of granzyme B and perforin (58). Despite increased granzyme B and perforins, KO mice had significantly increased CD8 KLRG1$^+$ PD1$^+$ T cells, indicating these cells are exhausted and therefore not able to contain bacterial growth. Nevertheless, this validates that CysLTR1 and its ligands might play a role in terminal differentiation of lymphocytes during inflammatory responses. It is possible that during secondary infection with *LM* these cells will be able to mount a protective immune response. In the spleen of KO mice, we observed a decrease in CD4 naïve T cells, increased CD4 central memory, effector/effector memory T cells, and KLRG1$^+$ PD1$^+$ T cells. This demonstrates that, even though there was a significant reduction in CD4 T cells, *LM* infection still results in their activation and CysLTR1 deletion might be affecting their survival and effector phenotypes. This also affected the production of IFN-γ by KO CD4 T cells, and perforin by KO CD8 T cells, subsequently their ability to clear *LM* infection. CD8 T cells of KO mice, however, had a significant reduction of naïve and central memory cells, increased effector/effector memory T cells and proliferation. Despite reduced in percentages, they were active and proliferating but were not effective at clearing the bacteria. Moreover, these cells are unable to produce sufficient IFN-γ, perforin and granzyme B which may be a result of exhaustion (KLRG1$^+$ PD1$^+$ phenotype).

In liver, we showed a significant reduction in IFN-γ, IL-17 and IL-12p70, and a decreasing trend of IFN-β. All these cytokines are required for the effective clearance of *LM* infection and their reduction further validates the inability of CysLTR1 deficient mice to control *LM* infection. Chemokines play a crucial role during immune defence because of their ability to attract immune cells to the site of infection or inflamed tissue (208). CysLTR1 KO mice has significantly reduced production of CXCL2, CXCL10 and growth factor, GM-CSF

early after infection. CXCL2 is secreted by mast cells and macrophages as a chemoattractant for early recruitment of neutrophils (209). CXCL10 has been associated with a variety of diseases as it is involved in chemotaxis, apoptosis, cellular growth and angiogenesis (210). CXCL10 receptor, CXCR3, is predominantly expressed in T cells, NK cells, dendritic cells, macrophages and B cells therefore, CXCL10 plays a role in recruitment of these cells (210–212). The reduction in production of CXCL10 in the liver correlates with the reduction of lymphoid cells in these KO mice. This therefore suggests that CysLTR1 deletion might be affecting the ability of macrophages and monocytes to secrete this chemokine to attract lymphoid cells to the site of infection. The reduced IFN-γ production also influenced the reduction of CXCL10, because it has been shown that leukocytes produce of CXCL10 in response to IFN-γ (213). The inability of CysLTR1 KO mice to recruit lymphoid cells to the site of infection is due to reduced pro-inflammatory cytokine production and chemokine secretion required for chemotaxis of lymphoid cells. However, we aim to investigate further the reason why mice have increased neutrophil recruitment while at the same time decreased lymphocyte chemotaxis with an increased exhausted phenotype.

4.5. Effect of CysLTR1 deletion on mice survival during *LM* infection

Lastly, we conducted mortality studies to determine the effect of CysLTR1 deletion on mice survival. This revealed that the KO mice appeared to be more susceptible to *LM* infection than their littermate controls. Our time-course experiments partially validate the susceptible phenotype observed in survival studies. For instance, KO mice have increased neutrophils which could cause tissue destruction, reduced recruitment of T cells and decreased pro-inflammatory cytokines thereby hampering the optimal adaptive immune responses. Moreover, a mortality study for CysLTR1 KO mice on BALB/c background, showed that CysLTR1-deficient mice were indeed susceptible to *LM* infection. These results suggest that intact CysTLR1 signalling contribute to host protection against listeriosis. Further studies will focus on the analysis of tissue pathology and systemic cytokine responses in mice.

4.6. Conclusion and future studies

In this study, we demonstrated for the first time that *LM* infection induces expression of CysLTR1, CysLTR2, and LTC_4S both *in vivo* and *in vitro*. We also demonstrated that the deletion of CysLTR1 has no effect on homeostasis in mice. CysLTR1 deletion had no effect

on *LM* growth in macrophages, with differential cytokine responses from both C57BL/6 and BALB/c background. However, we intend to conduct more studies to investigate the effect of CysLTR1 signalling at the cellular level when these macrophages are pre-treated with IFN-γ or other stimulants (LPS, TNF and PGE_2) before *LM* infection which can better mimic macrophage responses *in vivo*. We suspect that there will be differences in the ability of WT and KO macrophages to control *LM* infection and in their cytokine and nitric oxide production. We also reported that CysLTR1 deletion *in vivo* has no effect on bacterial control, however, KO mice had increased neutrophils and reduced lymphoid cells. As mentioned before, CysLTs play a role in the recruitment of lymphocytes to the site of infection or inflamed tissue (206), we, therefore, suggest that deletion of CysLTR1 might have affected the attraction of these lymphoid cells and also the secretion of chemokines required for activation and recruitment of inflammatory cells. In conclusion, CysLTR1 signalling plays a role in lymphoid cell activation and recruitment during early immune responses to *LM* infection and regulates the recruitment of neutrophils.

Further studies: WE will activate of KO and WT macrophages with IFN-γ and/or LPS to demonstrate whether the inability of CysLTR1 deficient macrophages to control *LM* infection is due to its deletion and in turn insufficient inactivation on macrophages. We also will infect WT macrophage with mutant *LM* strains that lack the expression of the virulence factors, such as LLO or ActA, to determine which virulence factor is required for the upregulation of CysLT receptors and LTC_4S expression. Since we observed a stronger phenotype on BALB/c mice during mortality studies, we plan on conducting time course studies in this background to further understand the immune mechanisms. Furthermore, we will perform secondary infections on C57BL/6 mice, which will give us a better understanding of whether CysLTR1 play a role in memory responses.

REFERENCES

1. Murray EGD, Webb RA, Swann MBR. 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). J Pathol Bacteriol 29:407–439.
2. Pirie JHH. 1940. *Listeria*: Change of name for a genus of bacteria [7]. Nature.
3. Orsi RH and, Wiedmann M. 2016. Characteristics and distribution of *Listeria spp.*, including Listeria species newly described since 2009. Appl Microbiol Biotechnol 5273–5287.
4. Scallan E, Hoekstra RM, Angulo FJ, Tauxe R V., Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg Infect Dis 17:7–15.
5. Monnier L. 2010. Human Listeriosis Caused by *Listeria ivanovii*. Emerg Infect Dis 16:136–138.
6. Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, Prasad SP, Vijila HM. 2007. *Listeria*--review of epidemiology and pathogenesis. J Microbiol Immunol Infect 40:4–13.
7. Centers for Disease Control and Prevention. 2016. *Listeria* (Listeriosis) | Listeria | CDC.
8. Lamont RF, Sobel J, Mazaki-Tovi S, Kusanovic JP, Vaisbuch E, Kim SK, Uldbjerg N, Romero R. 2011. Listeriosis in human pregnancy: a systematic review. J Perinat Med 39:227–236.
9. Pamer EG. 2004. Immune responses to *Listeria monocytogenes*. Nat Rev Immunol 4:812–823.
10. Roberts AJ, Wiedmann M. 2003. Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. Cell Mol Life Sci 60:904–918.
11. Camejo A, Carvalho F, Reis O, Leitão E, Sousa S, Cabanes D. 2011. The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence 2:379–394.
12. Schlech WF, Acheson D. 2000. Foodborne Listeriosis. Clin Infect Dis 31:770–775.
13. 2018. WHO | Listeriosis – South Africa. WHO.

14. Lecuit M. 2007. Human listeriosis and animal models. Microbes Infect 9:1216–1225.

15. Gaillard, J.L., Berche, P., Frehel, C., Gouln, E., and Cossart P. 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell 65:1127–1141.

16. Lecuit M, Ohayon H, Braun L, Mengaud J, Cossart P. 1997. Internalin of *Listeria monocytogenes* with an Intact Leucine-Rich Repeat Region Is Sufficient To Promote Internalization. Am Soc Microbiol 65:5309–5319.

17. Yang, Shen, Naujokas, Morag Park and KI. 2000. InlB-Dependent Internalization of Listeria Is Mediated by the Met Receptor Tyrosine Kinase. Cell 103:501–510.

18. Gao X, Lorinczi M, Hill KS, Brooks NC, Dokainish H, Ireton K, Elferink LA. 2008. Met Receptor Tyrosine Kinase Degradation Is Altered in Response to the Leucine-rich Repeat of the *Listeria* Invasion Protein Internalin B. J Biol Chem 284:774–783.

19. Pizarro-Cerdá J, Kühbacher A, Cossart P. 2012. Entry of *Listeria monocytogenes* in mammalian epithelial cells: An updated view. Cold Spring Harb Perspect Med 2.

20. Camargo AC, Woodward JJ, Nero LA. 2016. The Continuous Challenge of Characterizing the Foodborne Pathogen *Listeria monocytogenes*. Foodborne Pathog Dis 13:405–416.

21. Domann E, Wehland J, Rohde M, Pistor S, Hartl M, Goebel W, Leimeister-Wächter M, Wuenscher M, Chakraborty T. 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J 11:1981–90.

22. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P. 1992. *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. Cell 68:521–531.

23. Cossart P. 2011. Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. Proc Natl Acad Sci 108:19484–19491.

24. Radoshevich L, Cossart P. 2018. Listeria monocytogenes: Towards a complete picture of its physiology and pathogenesis. Nat Rev Microbiol 16:32–46.

25. Travier L, Lecuit M. 2014. *Listeria monocytogenes* ActA: A new function for a

"classic" virulence factor. Curr Opin Microbiol 17:53–60.

26. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, González-Zorn B, Wehland J, Kreft J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev 14:584–640.

27. Unanue ER. 1997. Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. Immunol Rev 158:11–25.

28. Mogensen TH. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 22:240–273.

29. Seki E, Tsutsui H, Tsuji NM, Hayashi N, Adachi K, Nakano H, Futatsugi-Yumikura S, Takeuchi O, Hoshino K, Akira S, Fujimoto J, Nakanishi K. 2002. Critical Roles of Myeloid Differentiation Factor 88-Dependent Proinflammatory Cytokine Release in Early Phase Clearance of *Listeria monocytogenes* in Mice . J Immunol 169:3863–3868.

30. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410:1099–1103.

31. Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Núñez G. 2008. The Cytosolic Sensors Nod1 and Nod2 Are Critical for Bacterial Recognition and Host Defense after Exposure to Toll-like Receptor Ligands. Immunity 28:246–257.

32. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nuñez G, Flavell RA. 2003. Nod2-Dependent Regulation of Innate and Adaptive Immunity in the Intestinal Tract. Biochem Biophys Res Commun 5:18.

33. Jia T, Serbina N V., Brandl K, Zhong MX, Leiner IM, Charo IF, Pamer EG. 2008. Additive Roles for MCP-1 and MCP-3 in CCR2-Mediated Recruitment of Inflammatory Monocytes during *Listeria monocytogenes* Infection . J Immunol 180:6846–6853.

34. Harty JT, Bevant MJ. 1995. Specific immunity to *Listeria monocytogenes* in the absence of IFNγ. Immunity 3:109–117.

35. Pfeffer K, Matsuyama T, Kündig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Krönke M, Mak TW. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L.*

monocytogenes infection. Cell 73:457–467.

36. Kang SJ, Liang HE, Reizis B, Locksley RM. 2008. Regulation of Hierarchical Clustering and Activation of Innate Immune Cells by Dendritic Cells. Immunity 29:819–833.

37. Humann J, Bjordahl R, Andreasen K, Lenz LL. 2007. Expression of the p60 Autolysin Enhances NK Cell Activation and Is Required for *Listeria monocytogenes* Expansion in IFN-γ-Responsive Mice . J Immunol 178:2407–2414.

38. Clark SE, Filak HC, Guthrie BS, Schmidt RL, Jamieson A, Merkel P, Knight V, Cole CM, Raulet DH, Lenz LL. 2016. Bacterial Manipulation of NK Cell Regulatory Activity Increases Susceptibility to *Listeria monocytogenes* Infection. PLoS Pathog 12:e1005708.

39. Schmidt RL, Filak HC, Lemon JD, Potter TA, Lenz LL. 2011. A LysM and SH3-Domain Containing Region of the *Listeria monocytogenes* p60 Protein Stimulates Accessory Cells to Promote Activation of Host NK Cells. PLoS Pathog 7:e1002368.

40. Steinman RM, Hemmi H. 2006. Dendritic cells: Translating innate to adaptive immunity. Curr Top Microbiol Immunol 311:17–58.

41. Sallusto F, Lanzavecchia A. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 177:134–140.

42. Zhan Y, Xu Y, Seah S, Brady JL, Carrington EM, Cheers C, Croker BA, Wu L, Villadangos JA, Lew AM. 2010. Resident and Monocyte-Derived Dendritic Cells Become Dominant IL-12 Producers under Different Conditions and Signaling Pathways. J Immunol 185:2125–2133.

43. Mitchell LM, Brzoza-Lewis KL, Henry CJ, Grayson JM, Westcott MM, Hiltbold EM. 2011. Distinct responses of splenic dendritic cell subsets to infection with *Listeria monocytogenes*: Maturation phenotype, level of infection, and T cell priming capacity ex vivo. Cell Immunol 268:79–86.

44. Kapadia D, Sadikovic A, Vanloubbeeck Y, Brockstedt D, Fong L. 2011. Interplay between CD8α+ Dendritic Cells and Monocytes in Response to *Listeria monocytogenes* Infection Attenuates T Cell Responses. PLoS One 6:e19376.

45. Nathan C. 1997. Perspectives Series: Nitric Oxide and Nitric Oxide Synthases.

Inducible Nitric Oxide Synthase: What Difference Does It Make? J Clin Invest 100:2417–2423.

46. Myers JT, Tsang AW, Swanson JA. 2003. Localized reactive oxygen and nitrogen intermediates inhibit escape of *Listeria monocytogenes* from vacuoles in activated macrophages. J Immunol 171:5447–53.

47. Torres D, Barrier M, Bihl F, Quesniaux VJF, Maillet I, Akira S, Ryffel B, Erard F. 2004. Toll-like receptor 2 is required for optimal control of *Listeria monocytogenes* infection. Infect Immun 72:2131–9.

48. Edelson BT, Unanue ER. 2000. Immunity to *Listeria* infection. Curr Opin Immunol 12:425–431.

49. Beckerman KP, Rogers HW, Corbett JA, Schreiber RD, McDaniel ML, Unanue ER. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to *Listeria monocytogenes*. J Immunol 150.

50. Cousens LP, Wing EJ. 2000. Innate defenses in the liver during *Listeria* infection. Immunol Rev. Blackwell Munksgaard.

51. Ebe Y, Hasegawa G, Takatsuka H, Umezu H, Mitsuyama M, Arakawa M, Mukaida N, Naito M. 1999. The role of Kupffer cells and regulation of neutrophil migration into the liver by macrophage inflammatory protein-2 in primary listeriosis in mice. Pathol Int 49:519–532.

52. Witter AR, Okunnu BM, Berg RE. 2016. The Essential Role of Neutrophils during Infection with the Intracellular Bacterial Pathogen *Listeria monocytogenes* . J Immunol 197:1557–1565.

53. Eusondia A, Vadia S, Nackerman CC, Oghumu S, Satoskar AR, McLeish KR, Uriarte SM, Stephanie S. 2014. The Pore-forming Toxin Listeriolysin O is Degraded by Neutrophil Metalloproteinase-8 and Fails to Medaite *Listeria monocytogenes* Intracellular Survival in Neutrophils. J Immunol 192:234–244.

54. Lara-Tejero M, Pamer EG. 2004. T cell responses to *Listeria monocytogenes*. Curr Opin Microbiol 7:45–50.

55. Harty JT, Badovinac VP. 2002. Influence of effector molecules on the CD8+ T cell response to infection. Curr Opin Immunol 14:360–365.

56. Lauvau G, Vijh S, Kong P, Horng T, Kerksiek K, Serbina N, Tuma RA, Pamer EG. 2001. Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. Science (80-) 294:1735–1739.

57. Khan SH, Badovinac VP. 2015. *Listeria monocytogenes*: a model pathogen to study antigen-specific memory CD8 T cell responses. Semin Immunopathol. Springer Verlag.

58. Zenewicz LA, Shen H. 2007. Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview. Microbes Infect 9:1208–1215.

59. Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF. 2009. Programming for CD8 T Cell Memory Development Requires IL-12 or Type I IFN. J Immunol 182:2786–2794.

60. Daugelat S, Ladel CH, Schoel B, Kaufmann SH. 1994. Antigen-specific T-cell responses during primary and secondary *Listeria monocytogenes* infection. Infect Immun 62:1881–8.

61. Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Bensinger SJ, Ehst BD, Griffith TS, Green DR, Schoenberger SP. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature 434:88–93.

62. Williams MA, Holmes BJ, Sun JC, Bevan MJ. 2006. Developing and maintaining protective CD8+ memory T cells. Immunol Rev 211:146–153.

63. Romagnoli PA, Fu HH, Qiu Z, Khairallah C, Pham QM, Puddington L, Khanna KM, Lefrançois L, Sheridan BS. 2017. Differentiation of distinct long-lived memory CD4 T cells in intestinal tissues after oral *Listeria monocytogenes* infection. Mucosal Immunol 10:520–530.

64. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, Zinkernagel RM. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science (80-) 286:2156–2159.

65. Shen H, Whitmire JK, Fan X, Shedlock DJ, Kaech SM, Ahmed R. 2003. A Specific Role for B Cells in the Generation of CD8 T Cell Memory by Recombinant Listeria monocytogenes . J Immunol 170:1443–1451.

66. Khanapure S, Garvey D, Janero D, Gordon Letts L. 2007. Eicosanoids in

Inflammation: Biosynthesis, Pharmacology, and Therapeutic Frontiers. Curr Top Med Chem 7:311–340.

67. Stillwell W, Stillwell W. 2016. Bioactive Lipids, p. 453–478. *In* An Introduction to Biological Membranes. Elsevier.

68. Loick H, Theissen J. 1994. Die Eicosanoide als Mediatoren beim ARDS. AINS - Anästhesiologie · Intensivmed · Notfallmedizin · Schmerztherapie 29:3–9.

69. Dennis EA, Norris PC. 2016. Eicosanoid Storm in Infectrion and Inflammation. Nat Rev Immunol 15:511–523.

70. Harizi H, Gualde N. 2005. The impact of eicosanoids on the crosstalk between innate and adaptive immunity: the key roles of dendritic cells. Tissue Antigens 65:507–514.

71. Spector AA, Fang X, Snyder GD, Weintraub NL. 2004. Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function. Prog Lipid Res 43:55–90.

72. Rocca B, FitzGerald GA. 2002. Cyclooxygenases and prostaglandins: shaping up the immune response. Int Immunopharmacol 2:603–630.

73. Peters-Golden M, Brock TG. 2003. 5-Lipoxygenase and FLAP. Prostaglandins Leukot Essent Fat Acids 69:99–109.

74. Rogerio AP, Anibal FF. 2012. Role of leukotrienes on protozoan and helminth infections. Mediators Inflamm 2012.

75. Machado ER, Ueta MT, Lourenço E V, Anibal FF, Sorgi CA, Soares EG, Roque-Barreira MC, Medeiros AI, Faccioli LH. 2005. Leukotrienes play a role in the control of parasite burden in murine strongyloidiasis. J Immunol 175:3892–3899.

76. Funk CD. 2001. Prostaglandins and leukotrienes:advances in eicosanoid biology. Science (80-) 294:1871–1875.

77. Rouzer CA, Matsumoto T, Samuelsson B. 1986. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. Proc Natl Acad Sci U S A 83:857–61.

78. Ghosh A, Chen F, Thakur A, Hong H. 2016. Cysteinyl Leukotrienes and Their Receptors: Emerging Therapeutic Targets in Central Nervous System Disorders. CNS Neurosci Ther 22:943–951.

79. Evans JF, Dupuis P, Ford-Hutchinson AW. 1985. Purification and characterisation of leukotriene A4 hydrolase from rat neutrophils. Biochim Biophys Acta - Gen Subj 840:43–50.

80. Nicholson DW, Ali A, Vaillancourt JP, Calaycay JR, Mumford RA, Zamboni RJ, Ford-Hutchinson AW. 1993. Purification to homogeneity and the N-terminal sequence of human leukotriene C4 synthase: a homodimeric glutathione S-transferase composed of 18-kDa subunits. Proc Natl Acad Sci U S A 90:2015–9.

81. Lam BK, Penrose JF, Freeman GJ, Austen KF. 1994. Expression cloning of a cDNA for human leukotriene C4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A4. Proc Natl Acad Sci U S A 91:7663–7.

82. Welsch DJ, Creely DP, Hauser SD, Mathis KJ, Krivi GG, Isakson PC. 1994. Molecular cloning and expression of human leukotriene-C4 synthase. Proc Natl Acad Sci U S A 91:9745–9.

83. Kanaoka Y, Boyce JA. 2014. Cysteinyl Leukotrienes and Their Receptors; Emerging Concepts. Allergy Asthma Immunol Res 6:288–295.

84. Lee CW, Lewis RA, Corey EJ, Austen KF. 1983. Conversion of leukotriene D4 to leukotriene E4 by a dipeptidase released from the specific granule of human polymorphonuclear leucocytes. Immunology 48:27–35.

85. Kanaoka Y, Boyce JA. 2004. Cysteinyl Leukotrienes and Their Receptors: Cellular Distribution and Function in Immune and Inflammatory Responses. J Immunol 173:1503–1510.

86. Dahlén S-E, Hedqvist P, Hammarström S, Samuelsson B. 1980. Leukotrienes are potent constrictors of human bronchi. Nature 288:484–486.

87. Alabaster VA, Bakhle YS. 1976. Release od smooth muscle-contracting substances from isolated perfused lungs. Eur J Pharmacol 35:349–360.

88. Kanaoka Y, Boyce JA. 2004. Cysteinyl Leukotrienes and Their Receptors: Cellular Distribution and Function in Immune and Inflammatory Responses. J Immunol 173:1503–1510.

89. Steinhilber D, Fischer AS, Metzner J, Steinbrink SD, Roos J, Ruthardt M, Maier TJ. 2010. 5-Lipoxygenase: Underappreciated Role of a Pro-Inflammatory Enzyme in Tumorigenesis. Front Pharmacol 1:143.

90. Kanaoka Y, Maekawa A, Austen KF. 2013. Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. J Biol Chem 288:10967–10972.

91. Peters-Golden M, Gleason MM, Togias A. 2006. Cysteinyl leukotrienes: multi-functional mediators in allergic rhinitis. Clin Exp Allergy 36:689–703.

92. Early SB, Barekzi E, Negri J, Hise K, Borish L, Steinke JW. 2007. Concordant Modulation of Cysteinyl Leukotriene Receptor Expression by IL-4 and IFN-γ on Peripheral Immune Cells. Am J Respir Cell Mol Biol 36:715–720.

93. Thivierge M, Stankova J, Rola-Pleszczynski M. 2018. IL-13 and IL-4 Up-Regulate Cysteinyl Leukotriene 1 Receptor Expression in Human Monocytes and Macrophages. J Immunol 167:2855–2860.

94. Bankova LG, Dwyer DF, Yoshimoto E, Ualiyeva S, McGinty JW, Raff H, von Moltke J, Kanaoka Y, Frank Austen K, Barrett NA. 2018. The cysteinyl leukotriene 3 receptor regulates expansion of IL-25–producing airway brush cells leading to type 2 inflammation. Sci Immunol 3:eaat9453.

95. Savari S, Vinnakota K, Zhang Y, Sjölander A. 2014. Cysteinyl leukotrienes and their receptors: bridging inflammation and colorectal cancer. World J Gastroenterol 20:968–77.

96. Madrid L V, Mayo MW, Reuther JY, Baldwin Jr AS. 2001. Akt Stimulates the Transactivation Potential of the RelA/p65 subunit of NF-κB Through Utilization of the IκB Kinase and Activation of the Mitogen Activated Protein Kinase p38. J Biol Chem 25:18934–18940.

97. Thompson C, Cloutier A, Bossé Y, Poisson C, Larivée P, McDonald PP, Stankova J, Rola-Pleszczynski M. 2008. Signaling by the cysteinyl-leukotriene receptor 2. Involvement in chemokine gene transcription. J Biol Chem 283:1974–84.

98. Hennen S, Wang H, Peters L, Merten N, Simon K, Spinrath A, Blättermann S, Akkari R, Schrage R, Schröder R, Schulz D, Vermeiren C, Zimmermann K, Kehraus S, Drewke C, Pfeifer A, König GM, Mohr K, Gillard M, Müller CE, Lu QR, Gomeza J, Kostenis E. 2013. Decoding signaling and function of the orphan G protein-coupled receptor GPR17 with a small-molecule agonist. Sci Signal 6:ra93.

99. Maekawa A, Balestrieri B, Austen KF, Kanaoka Y. 2009. GPR17 is a negative

regulator of the cysteinyl leukotriene 1 receptor response to leukotriene D4. Proc Natl Acad Sci U S A 106:11685–11690.

100. Farias SE, Zarini S, Precht T, Murphy RC, Heidenreich KA. 2007. Transcellular biosynthesis of cysteinyl leukotrienes in rat neuronal and glial cells. J Neurochem 103:1310–1318.

101. Farias S, Frey LC, Murphy RC, Heidenreich KA. 2009. Injury-Related Production of Cysteinyl Leukotrienes Contributes to Brain Damage following Experimental Traumatic Brain Injury. J Neurotrauma 29:1977–1986.

102. Corser-Jensen CE, Goodell DJ, Freund RK, Serbedzija P, Murphy RC, Farias SE, Dell'Acqua ML, Frey LC, Serkova N, Heidenreich KA. 2014. Blocking leukotriene synthesis attenuates the pathophysiology of traumatic brain injury and associated cognitive deficits. Exp Neurol 256:7–16.

103. Voigt C, Donat CK, Hartig W, Förschler A, Skardelly M, Stichtenoth D, Arendt T, Meixensberger J, Schuhmann MU. 2012. Effect of leukotriene inhibitors on evolution of experimental brain contusions. Neuropathol Appl Neurobiol 38:354–366.

104. Lai J, Hu M, Wang H, Hu M, Long Y, Miao MX, Li JC, Wang XB, Kong LY, Hong H. 2014. Montelukast targeting the cysteinyl leukotriene receptor 1 ameliorates Aβ 1-42 -induced memory impairment and neuroinflammatory and apoptotic responses in mice. Neuropharmacology 79:707–714.

105. Mastalerz L, Celejewska-Wójcik N, Wójcik K, Gielicz A, Januszek R, Cholewa A, Stręk P, Sanak M. 2014. Induced sputum eicosanoids during aspirin bronchial challenge of asthmatic patients with aspirin hypersensitivity. Allergy 69:1550–1559.

106. Zhu J, Qiu Y-S, Figueroa DJ, Bandi V, Galczenski H, Hamada K, Guntupalli KK, Evans JF, Jeffery PK. 2005. Localization and Upregulation of Cysteinyl Leukotriene-1 Receptor in Asthmatic Bronchial Mucosa. Am J Respir Cell Mol Biol 33:531–540.

107. Scott JP, Peters-Golden M. 2013. Antileukotriene Agents for the Treatment of Lung Disease. Am J Respir Crit Care Med 188:538–544.

108. Singh RK, Tandon R, Dastidar SG, Ray A. 2013. A review on leukotrienes and their receptors with reference to asthma. J Asthma 50:922–931.

109. Boudaud M, Turcotte S, Stankova J, Rola-Pleszczynski M. 2018. IL-33 Upregulates Cysteinyl Leukotriene Receptor Type 1 Expression in Human Peripheral Blood CD4+ T Lymphocytes. J Immunol 201:2787–2798.

110. Préfontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, Martin JG, Hamid Q. 2010. Increased IL-33 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol 125:752–754.

111. Préfontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, Lemière C, Martin JG, Hamid Q. 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. J Immunol 183:5094–103.

112. Qi F, Wang D, Liu J, Zeng S, Xu L, Hu H, Liu B. 2015. Respiratory macrophages and dendritic cells mediate respiratory syncytial virus-induced IL-33 production in TLR3- or TLR7-dependent manner. Int Immunopharmacol 29:408–415.

113. Chu DK, Llop-Guevara A, Walker TD, Flader K, Goncharova S, Boudreau JE, Moore CL, In TS, Waserman S, Coyle AJ, Kolbeck R, Humbles AA, Jordana M. 2013. IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. J Allergy Clin Immunol 131:187-200.e8.

114. Thivierge M, Stankova J, Rola-Pleszczynski M. 2014. IL-13 and IL-4 Up-Regulate Cysteinyl Leukotriene 1 Receptor Expression in Human Monocytes and Macrophages. J Immunol 167:2855–2860.

115. Thivierge M, Doty M, Johnson J, Stanková J, Rola-Pleszczynski M, Mackay IS, Kay AB, Hamid QA. 2000. IL-5 up-regulates cysteinyl leukotriene 1 receptor expression in HL-60 cells differentiated into eosinophils. J Immunol 165:5221–6.

116. Espinosa K, Bossé Y, Stankova J, Rola-Pleszczynski M. 2003. CysLT1 receptor upregulation by TGF-β and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD4. J Allergy Clin Immunol 111:1032–1040.

117. Amrani Y, Moore PE, Hoffman R, Shore SA, Panettieri RA. 2001. Interferon- γ Modulates Cysteinyl Leukotriene Receptor-1 Expression and Function in Human Airway Myocytes. Am J Respir Crit Care Med 164:2098–2101.

118. Early SB, Barekzi E, Negri J, Hise K, Borish L, Steinke JW. 2007. Concordant modulation of cysteinyl leukotriene receptor expression by IL-4 and IFN-γ on

peripheral immune cells. Am J Respir Cell Mol Biol 36:715–720.

119. Thivierge M, Turcotte S, Rola-Pleszczynski M, Stankova J. 2015. Enhanced cysteinyl-leukotriene type 1 receptor expression in T cells from house dust mite-allergic individuals following stimulation with Der p. J Immunol Res 2015:384780.

120. Grainger JR, Wohlfert EA, Fuss IJ, Bouladoux N, Askenase MH, Legrand F, Koo LY, Brenchley JM, Fraser IDC, Belkaid Y. 2013. Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. Nat Med 19:713–721.

121. Serezani CH, Chung J, Ballinger MN, Moore BB, Aronoff DM, Peters-Golden M. 2007. Prostaglandin E $_2$ Suppresses Bacterial Killing in Alveolar Macrophages by Inhibiting NADPH Oxidase. Am J Respir Cell Mol Biol 37:562–570.

122. Bailie MB, Standiford TJ, Laichalk LL, Coffey MJ, Strieter R, Peters-Golden M. 1996. Leukotriene-deficient mice manifest enhanced lethality from *Klebsiella pneumonia* in association with decreased alveolar macrophage phagocytic and bactericidal activities. J Immunol 157:5221–4.

123. Rangel Moreno J, Estrada García I, De La Luz García Hernández M, Aguilar Leon D, Marquez R, Hernández Pando R. 2002. The role of prostaglandin E2 in the immunopathogenesis of experimental pulmonary tuberculosis. Immunology 106:257–66.

124. Malaviya R, Abraham SN. 2000. Role of Mast Cell Leukotrienes in Neutrophil Recruitment and Bacterial clearance in Infectious Peritonitis. J Leukoc Biol 67:841–846.

125. Fahel JS, Souza MB de, Gomes MTR, Corsetti PP, Carvalho NB, Marinho FA V., Almeida LA de, Caliari M V., Machado FS, Oliveira SC. 2015. 5-Lipoxygenase Negatively Regulates Th1 Response during *Brucella abortus* Infection in Mice. Infect Immun 83:1210–1216.

126. Hutchison DL, Myers RL. 1987. Prostaglandin-mediated suppression of macrophage phagocytosis of *Listeria monocytogenes*. Cell Immunol 110:68–76.

127. Noor S, Goldfine H, Tucker DE, Suram S, Lenz LL, Akira S, Uematsu S, Girotti M, Bonventre J V, Breuel K, Williams DL, Leslie CC. 2008. Activation of cytosolic phospholipase A2alpha in resident peritoneal macrophages by *Listeria*

monocytogenes involves listeriolysin O and TLR2. J Biol Chem 283:4744–55.

128. Pitts MG, D'Orazio SEF. 2019. Prostaglandin E2 Inhibits the Ability of Neutrophils to Kill *Listeria monocytogenes*. J Immunol 202:3474–3482.

129. Van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. 1995. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J Exp Med 181:775–9.

130. Shinomiya S, Naraba H, Ueno A, Utsunomiya I, Maruyama T, Ohuchida S, Ushikubi F, Yuki K, Narumiya S, Sugimoto Y, Ichikawa A, Oh-ishi S. 2001. Regulation of TNFα and interleukin-10 production by prostaglandins I2 and E2: studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. Biochem Pharmacol 61:1153–1160.

131. Akaogi J, Yamada H, Kuroda Y, Nacionales DC, Reeves WH, Satoh M. 2004. Prostaglandin E $_2$ receptors EP2 and EP4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF-α and IL-6 production. J Leukoc Biol 76:227–236.

132. Chen B-C, Liao C-C, Hsu M-J, Liao Y-T, Lin C-C, Sheu J-R, Lin C-H. 2006. Peptidoglycan-induced IL-6 production in RAW 264.7 macrophages is mediated by cyclooxygenase-2, PGE2/PGE4 receptors, protein kinase A, I kappa B kinase, and NF-kappa B. J Immunol 177:681–93.

133. Singh RK, Gupta S, Dastidar S, Ray A. 2010. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. Pharmacology 85:336–49.

134. Noor S, Goldfine H, Tucker DE, Suram S, Lenz LL, Akira S, Uematsu S, Girotti M, Bonventre J V, Breuel K, Williams DL, Leslie CC. 2008. Activation of cytosolic phospholipase A2alpha in resident peritoneal macrophages by *Listeria monocytogenes* involves listeriolysin O and TLR2. J Biol Chem 283:4744–55.

135. United Nations. 2015. World Fertility Patterns 2015 Data Booklet.

136. ISI House. 2017. Mid-year population estimates 2017. South Africa.

137. Maekawa A, Frank Austen K, Kanaoka Y. 2002. Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. J Biol Chem 277:20820–20824.

138. Granger DL, Taintor RR, Boockvar KS, Hibbs JB. 1996. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. Methods Enzymol 268:142–151.

139. Zhang T, Abel S, Abel Zur Wiesch P, Sasabe J, Davis BM, Higgins DE, Waldor MK. 2017. Deciphering the landscape of host barriers to *Listeria monocytogenes* infection. Proc Natl Acad Sci 114:6334–6337.

140. Mao J, Hu X, Pang P, Zhou B, Zhang Y, Li D, Shan H. 2018. Establishment of a CRISPR/Cas9-Mediated Cysltr1 Knockout Mouse Model and iTRAQ-Based Proteomic Analysis. PROTEOMICS - Clin Appl 12:1700087.

141. Moguche AO, Shafiani S, Clemons C, Larson RP, Dinh C, Higdon LE, Cambier CJ, Sissons JR, Gallegos AM, Fink PJ, Urdahl KB. 2015. ICOS and Bcl6-dependent pathways maintain a CD4 T cell population with memory-like properties during tuberculosis. J Exp Med 212:715–728.

142. Sakai S, Kauffman KD, Schenkel JM, McBerry CC, Mayer-Barber KD, Masopust D, Barber DL. 2014. Cutting Edge: Control of *Mycobacterium tuberculosis* Infection by a Subset of Lung Parenchyma–Homing CD4 T Cells . J Immunol 192:2965–2969.

143. Shaughnessy LM, Swanson JA. 2007. The role of the activated macrophage in clearing *Listeria monocytogenes* infection. Front Biosci 12:2683–92.

144. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie Q, Sokol K, Hutchinson N, Chen H, Mudget JS. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81:641–650.

145. Shiloh MU, MacMicking JD, Nicholson S, Brause JE, Potter S, Marino M, Fang F, Dinauer M, Nathan C. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity 10:29–38.

146. Ai W, Li H, Song N, Li L, Chen H. 2013. Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. Int J Environ Res Public Health 10:3834–3842.

147. Badovinac VP, Harty JT. 2000. Adaptive immunity and enhanced CD8+ T cell response to *Listeria monocytogenes* in the absence of perforin and IFN-gamma. J

Immunol 164:6444–52.

148. Pitts MG, Myers-Morales T, D'Orazio SEF. 2016. Type I IFN Does Not Promote Susceptibility to Foodborne *Listeria monocytogene*s. J Immunol 196:3109–16.

149. McCormack R, Bahnan W, Shrestha N, Boucher J, Barreto M, Barrera CM, Dauer EA, Freitag NE, Khan WN, Podack ER, Schesser K. 2016. Perforin-2 Protects Host Cells and Mice by Restricting the Vacuole to Cytosol Transitioning of a Bacterial Pathogen. Infect Immun 84:1083–1091.

150. Mouchacca P, Chasson L, Frick M, Foray C, Schmitt-Verhulst A-M, Boyer C. 2015. Visualization of granzyme B-expressing CD8 T cells during primary and secondary immune responses to *Listeria monocytogenes*. Immunology 145:24–33.

151. Carrero JA, Vivanco-Cid H, Unanue ER. 2008. Granzymes drive a rapid listeriolysin O-induced T cell apoptosis. J Immunol 181:1365–74.

152. Serbina N V, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. 2003. TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. Immunity 19:59–70.

153. Badovinac VP, Harty JT. 2000. Intracellular staining for TNF and IFN-γ detects different frequencies of antigen-specific CD8+ T cells. J Immunol Methods 238:107–117.

154. Grivennikov SI, Tumanov A V., Liepinsh DJ, Kruglov AA, Marakusha BI, Shakhov AN, Murakami T, Drutskaya LN, Förster I, Clausen BE, Tessarollo L, Ryffel B, Kuprash D V., Nedospasov SA. 2005. Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils: Protective and Deleterious Effects. Immunity 22:93–104.

155. Aoshi T, Carrero JA, Konjufca V, Koide Y, Unanue ER, Miller MJ. 2009. The cellular niche of listeria monocytogenes infection changes rapidly in the spleen. Eur J Immunol 39:417–425.

156. Mebius RE, Kraal G. 2005. Structure and function of the spleen. Nat Rev Immunol 5:606–616.

157. Elmore SA. 2006. Enhanced Histopathology of the Spleen. Toxicol Pathol 34:648–655.

158. Suttie AW. 2006. Histopathology of the Spleen. Toxicol Pathol 34:466–503.

159. Thompson MD, Capra V, Takasaki J, Maresca G, Rovati GE, Slutsky AS, Lilly C, Zamel N, McIntyre Burnham W, Cole DE, Siminovitch KA. 2007. A functional G300S variant of the cysteinyl leukotriene 1 receptor is associated with atopy in a Tristan da Cunha isolate. Pharmacogenet Genomics 17:539–549.

160. Yaddaden L, Véronneau S, Thompson MD, Rola-Pleszczynski M, Stankova J. 2016. Cellular signalling of cysteinyl leukotriene type 1 receptor variants CysLT1-G300S and CysLT1-I206S. Prostaglandins Leukot Essent Fat Acids 105:1–8.

161. Brochu-Bourque A, Véronneau S, Rola-Pleszczynski M, Stankova J. 2011. Differential signaling defects associated with the M201V polymorphism in the cysteinyl leukotriene type 2 receptor. J Pharmacol Exp Ther 336:431–439.

162. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams DL, Ford-Hutchinson AW, Caskey CT, Evans JF. 1999. Characterization of the human cysteinyl leukotriene CysLT1 receptor. Nature 399:789–793.

163. Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, Stocco R, Bellefeuille JN, Abramovitz M, Cheng R, Williams DL, Zeng Z, Liu Q, Ma L, Clements MK, Coulombe N, Liu Y, Austin CP, George SR, O'Neill GP, Metters KM, Lynch KR, Evans JF. 2000. Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 275:30531–30536.

164. Beller TC, Maekawa A, Friend DS, Austen KF, Kanaoka Y. 2004. Targeted gene disruption reveals the role of the cysteinyl leukotriene 2 receptor in increased vascular permeability and in bleomycin-induced pulmonary fibrosis in mice. J Biol Chem 279:46129–46134.

165. Sarau HM, Ames RS, Chambers J, Ellis C, Elshourbagy N, Foley JJ, Schmidt DB, Muccitelli RM, Jenkins O, Murdock PR, Herrity NC, Halsey W, Sathe G, Muir AI, Nuthulaganti P, Dytko GM, Buckley PT, Wilson S, Bergsma DJ, Hay DWP. 1999. Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. Mol Pharmacol 56:657–663.

166. Figueroa DJ, Breyer RM, Defoe SK, Kargman S, Daugherty BL, Waldburger K, Liu

Q, Clements M, Zeng Z, O'neill GP, Jones TR, Lynch KR, Austin CP, Evans JF. 2001. Expression of the Cysteinyl Leukotriene 1 Receptor in Normal Human Lung and Peripheral Blood Leukocytes. Am J Respir Crit Care Med 163:226–233.

167. Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21.

168. Dramsi S, Cossart P. 2003. Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line. Infect Immun 71:3614–3618.

169. Liu M, Yokomizo T. 2014. The role of leukotrienes in allergic diseases. Allergol Int 64:17–26.

170. Thompson MD, Takasaki J, Capra V, Rovati GE, Siminovitch KA, Burnham WMI, Hudson TJ, Bossé Y, Cole DEC. 2006. G-protein-coupled receptors and asthma endophenotypes: The cysteinyl leukotriene system in perspective. Mol Diagnosis Ther 10:353–366.

171. Zhang J, Migita O, Koga M, Shibasaki M, Arinami T, Noguchi E. 2006. Determination of structure and transcriptional regulation of CYSLTR1 and an association study with asthma and rhinitis. Pediatr Allergy Immunol 17:242–249.

172. Law CL, Clark EA. 1994. Cell-cell interactions that regulate the development of B-lineage cells. Curr Opin Immunol 6:238–247.

173. Lamoureux J, Stankova J, Rola-Pleszczynski M. 2006. Leukotriene D 4 enhances immunoglobulin production in CD40-activated human B lymphocytes. J Allergy Clin Immunol 117:924–930.

174. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969.

175. Raetz M, Hwang SH, Wilhelm CL, Kirkland D, Benson A, Sturge CR, Mirpuri J, Vaishnava S, Hou B, Defranco AL, Gilpin CJ, Hooper L V., Yarovinsky F. 2013. Parasite-induced T H 1 cells and intestinal dysbiosis cooperate in IFN-γ-dependent elimination of Paneth cells. Nat Immunol 14:136–142.

176. Portnoy DA, Schreiber RD, Connelly P, Tilneys LG. 1989. γ Interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J Exp Med 170:2141–2146.

177. Myers JT, Tsang AW, Swanson JA. 2003. Localized Reactive Oxygen and Nitrogen Intermediates Inhibit Escape of *Listeria monocytogenes* from Vacuoles in Activated Macrophages . J Immunol 171:5447–5453.

178. Nussler AK, Billiar TR. 1993. Inflammation, immunoregulation, and inducible nitric oxide synthase. J Leukoc Biol 54:171–178.

179. Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S. 1990. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. J Immunol 144:4794–7.

180. Adams LB, Hibbs JB, Taintor RR, Krahenbuhl JL. 1990. Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine. J Immunol 144:2725–9.

181. Flesch IE, Kaufmann SH. 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect Immun 59:3213–8.

182. Beckerman KP, Rogers HW, Corbett JA, Schreiber RD, McDaniel ML, Unanue ER. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to *Listeria monocytogenes*. J Immunol 150:888–95.

183. Cole C, Thomas S, Filak H, Henson PM, Lenz LL. 2012. Nitric Oxide Increases Susceptibility of Toll-like Receptor-Activated Macrophages to Spreading Listeria monocytogenes. Immunity 36:807–820.

184. Farrell A. 2012. NO means yes to Listeria infection. Nat Med 18:868–868.

185. Chiba Y, Oshita M, Sakai H, Misawa M. 2007. Involvements of cysteinyl leukotrienes and nitric oxide in antigen-induced venodilatation of nasal mucosa in sensitized rats in vivo. J Smooth Muscle Res 43:139–144.

186. Mizutani N, Nabe T, Imai A, Sakurai H, Takenaka H, Kohno S. 2001. Markedly Increased Nasal Blockage by Intranasal Leukotriene D4 in an Experimental Allergic Rhinitis Model: Contribution of Dilated Mucosal Blood Vessels. Jpn J Pharmacol 86:170–182.

187. Bogdan C, Vodovotz Y, Nathan C. 1991. Macrophage deactivation by interleukin 10. J Exp Med 174:1549–55.

188. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041–8.

189. Huang CJ, Stevens BR, Nielsen RB, Slovin PN, Fang X, Nelson DR, Skimming JW. 2002. Interleukin-10 inhibition of nitric oxide biosynthesis involves suppression of CAT-2 transcription. Nitric Oxide - Biol Chem 6:79–84.

190. Woszczek G, Chen L-Y, Nagineni S, Shelhamer JH. 2008. IL-10 Inhibits Cysteinyl Leukotriene-Induced Activation of Human Monocytes and Monocyte-Derived Dendritic Cells. J Immunol 180:7597–7603.

191. Czuprynski CJ, Brown JF, Steinberg H, Carroll D. 1998. Mice lacking the murine interleukin-8 receptor homologue demonstrate paradoxical responses to acute and chronic experimental infection with *Listeria monocytogenes*. Microb Pathog 24:17–23.

192. Conlan JW, Dunn PL, North RJ. 1993. Leukocyte-mediated lysis of infected hepatocytes during listeriosis occurs in mice depleted of NK cells or CD4+ CD8+ Thy1.2+ T cells. Infect Immun 61:2703–2707.

193. Rogers HW, Callery MP, Deck B, Unanue ER. 1996. *Listeria monocytogenes* induces apoptosis of infected hepatocytes. J Immunol 156:679–84.

194. Bennouna S, Bliss SK, Curiel TJ, Denkers EY. 2003. Cross-Talk in the Innate Immune System: Neutrophils Instruct Recruitment and Activation of Dendritic Cells during Microbial Infection. J Immunol 171:6052–6058.

195. Chao Shi, Tobias M. Hohl, Ingrid Leiner, Michele J. Equinda, Xiaozhou Fan, Eric G. Pamer. 2011. Ly6G+ Neutrophils Are Dispensable for Defense against Systemic *Listeria monocytogenes* Infection. J Immunol 187:5293–5298.

196. Marwick JA, Mills R, Kay O, Michail K, Stephen J, Rossi AG, Dransfield I, Hirani N. 2018. Neutrophils induce macrophage anti-inflammatory reprogramming by suppressing NF-κB activation article. Cell Death Dis 9:655.

197. Czuprynski CJ, Campbell PA, Henson PM. 1983. Killing of *Listeria monocytogenes* by human neutrophils and monocytes, but not by monocyte-derived macrophages. RES J Reticuloendothel Soc 34:29–44.

198. Crooks SW, Stockley RA. 1998. Leukotriene B4. Int J Biochem Cell Biol 30:173–178.

199. Theron AJ, Steel HC, Tintinger GR, Gravett CM, Anderson R, Feldman C. 2014. Cysteinyl leukotriene receptor-1 antagonists as modulators of innate immune cell function. J Immunol Res 2014:16.

200. Menon A, Shroyer ML, Wampler JL, Chawan CB, Bhunia AK. 2003. In vitro study of *Listeria monocytogenes* infection to murine primary and human transformed B cells. Comp Immunol Microbiol Infect Dis 26:157–174.

201. Bhunia AK, Steele PJ, Westbrook DG, Bly LA, Maloney TP, Johnson MG. 1994. A six-hour in vitro virulence assay for *Listeria monocytogenes* using myeloma and hybridoma cells from murine and human sources. Microb Pathog 16:99–110.

202. Bhumia AK and XF. 1999. Examination of Cytopathic Effect and Apoptosis in Listeria monocytogenes-Infected Hybridoma B-Lymphocyte (Ped-2E9) Line In Vitro -Journal of Microbiology and Biotechnology | Korea Science 398–403.

203. Kaufmann SHE. 1993. Immunity to Intracellular Bacteria. Annu Rev Immunol 11:129–163.

204. Mittrücker H-W, Köhler A, Kaufmann SHE. 2000. Substantialin vivo proliferation of CD4+ and CD8+ T lymphocytes during secondaryListeria monocytogenes infection. Eur J Immunol 30:1053–1059.

205. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. 1993. Development of T H 1 CD4 + T cells through IL-12 produced by Listeria-induced macrophages. Science (80-) 260:547–549.

206. Prinz I, Gregoire C, Mollenkopf H, Aguado E, Wang Y, Malissen M, Kaufmann SHE, Malissen B. 2005. The Type 1 Cysteinyl Leukotriene Receptor Triggers Calcium Influx and Chemotaxis in Mouse αβ- and γδ Effector T Cells. J Immunol 175:713–719.

207. Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH. 2002. Leukotriene-Receptor Expression on Nasal Mucosal Inflammatory Cells in Aspirin-Sensitive Rhinosinusitis. N Engl J Med 347:1493–1499.

208. Hughes CE, Nibbs RJB. 2018. A guide to chemokines and their receptors. FEBS J 285:2944–2971.

209. De Filippo K, Dudeck A, Hasenberg M, Nye E, Van Rooijen N, Hartmann K, Gunzer M, Roers A, Hogg N. 2013. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood 121:4930–4937.

210. Liu M, Guo S, Stiles JK. 2011. The emerging role of CXCL10 in cancer. Oncol Lett 2:583–589.

211. Loetscher M, Loetscher P, Brass N, Meese E, Moser B. 1998. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. Eur J Immunol 28:3696–3705.

212. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 101:746–754.

213. Luster AD, Ravetch J V. 1987. Biochemical characterization of a γ interferon-inducible cytokine (ip-10). J Exp Med 166:1084–1097.

SUPPLEMENTARY FIGURES

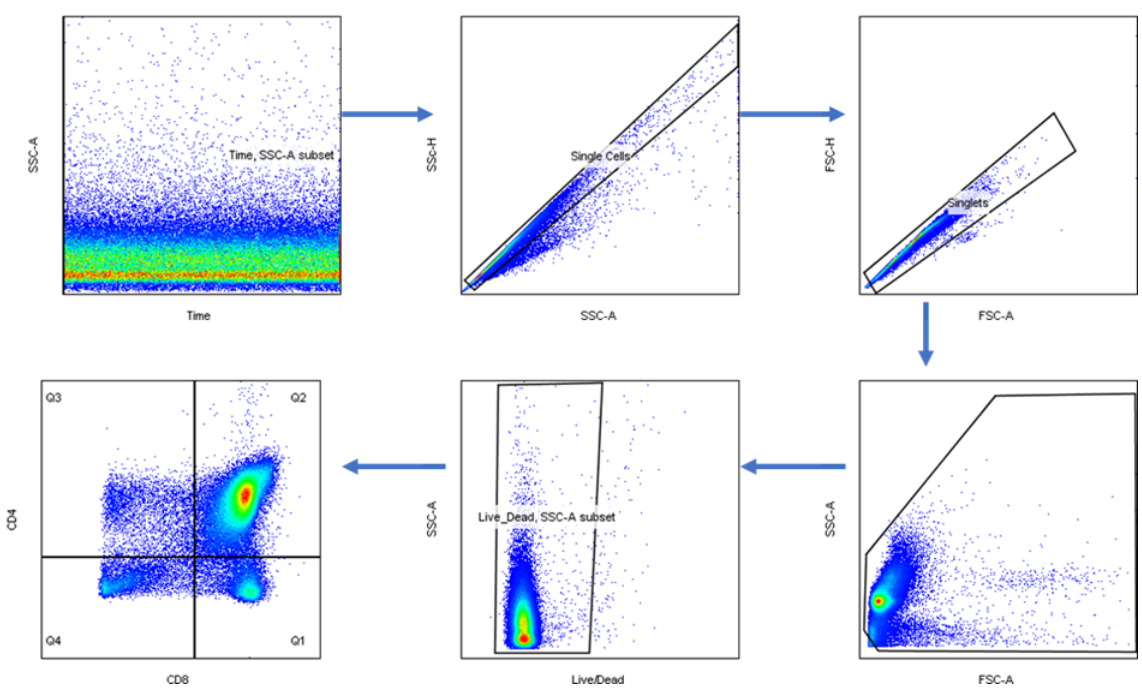

Figure S1: Gating strategy to identify thymus T cells. From the time gate, we gated for single cells, then singlets, then we removed the debris. We then gated for live cells from SSC-A and live/dead. From the live cells we gated for the $CD4^+$ CD8-, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^+$ and $CD4^-$ $CD8^-$ T cells using a CD4 and CD8 markers. The analysis was done using FlowJo Version 10.6.

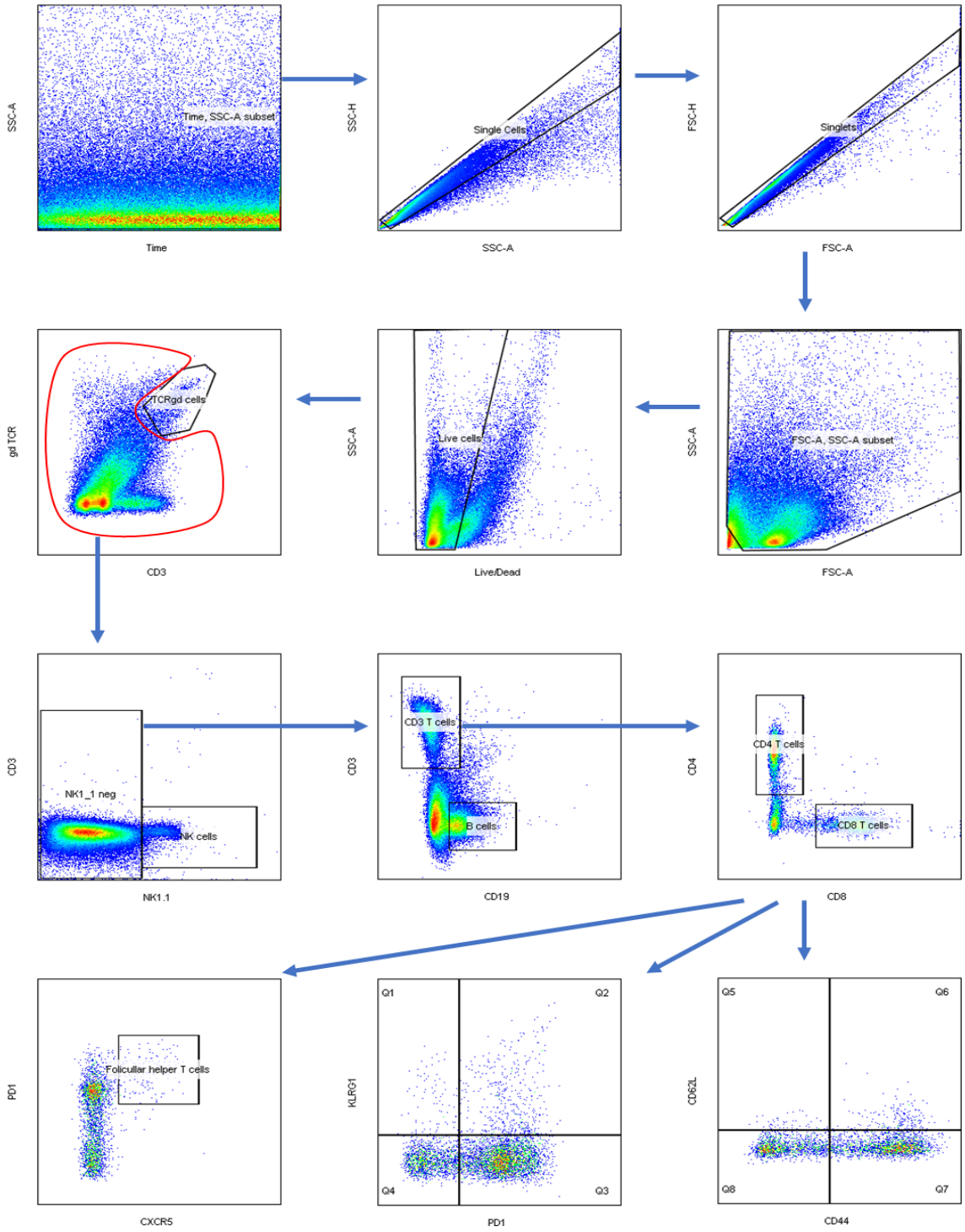

Figure S2: Lung, liver and spleen gating strategy for lymphoid cell populations. From the time gate, we gated for single cells, to singlets, then gated out the debris, then gated for live cells from SSC-A and live/dead. From the live cells, we gated for γδ T cells (γδTCR$^+$ CD3$^+$) using γδTCR and CD3 markers. From the rest of the cells, gated for NK cells (CD3$^-$ NK1.1$^+$). From NK1.1$^-$ cells we gated for CD3 T cells (CD3$^+$ CD19$^-$) and B cells (CD3$^-$ CD19$^+$). From CD3$^+$ cells we gated for CD4 T cells (CD4$^+$ CD8$^-$) and CD8 T cells (CD4$^-$ CD8$^-$). From both CD4$^+$ and CD8$^+$ T cells we gated for follicular T helper cells (CXCR5$^+$ PD1$^+$), parenchymal/vascular and exhausted/terminally differentiated cells using KLRG1 and PD1, naïve T cells (Q5: CD62L$^+$ CD44-), central memory T cells (Q6: CD62L$^+$ CD44$^+$) and effector/effector memory T cells (Q7: CD62L$^-$ CD44$^+$). The analysis was done using FlowJo version 10.6.

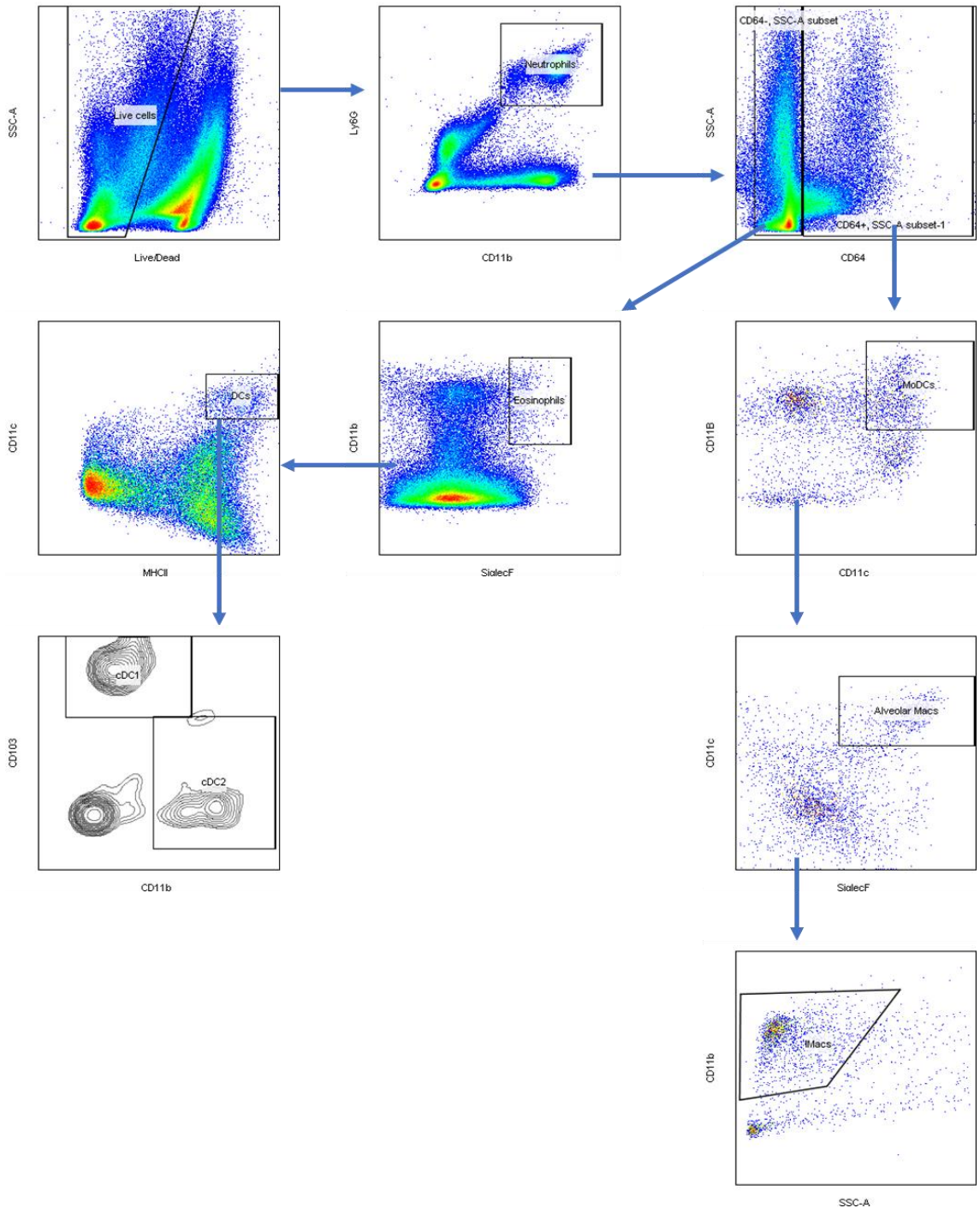

Figure S3: Lung myeloid cell populations gating strategy. From the time gate, we gated for single cells, to singlets, then gated out the debris (not shown), then gated for live cells from SSC-A and live/dead. From the live cells, we gated for neutrophils ($Ly6G^+$ $CD11b^+$). From the negative population we gated for $CD64^+$ cells and $CD64^-$ cells. From the $CD64^-$ cells we gated for eosinophils ($CD11b^+$ $SiglecF^+$). From the negative population, we gated for DCs ($CD11c^+$ $MHCII^+$). From DCs we gated for cDC1 ($CD103^+$ $CD11b^-$) and cDC2 ($CD103^-$ $CD11b^+$). And from the $CD64^+$ cells we gated for monocyte derived DCs (MoDC, $CD11b^+$ $CD11^+$). From the negative cells we gated for alveolar macrophages ($CD11c^+$ $SiglecF^+$) and from the negative population we gated for interstitial macrophages (SSC-A $CD11b^+$) using SSC-A and CD11b. The analysis was done using FlowJo volume 10.6.

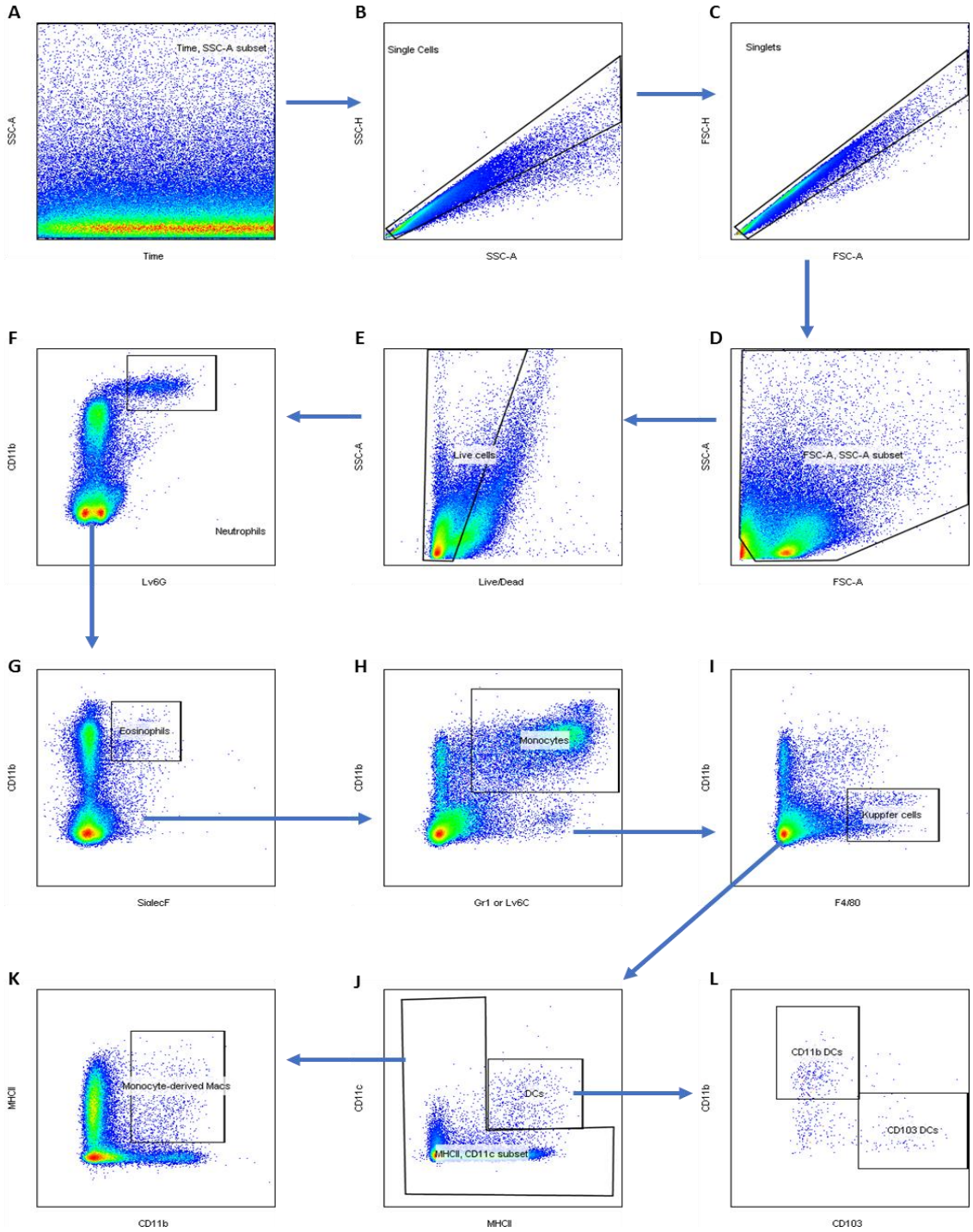

Figure S4: Liver myeloid cell populations gating strategy. From the time gate, we gated for single cells, to singlets, then gated out the debris, then gated for live cells from SSC-A and live/dead. From the live cells, we gated for neutrophils ($Ly6G^+$ $CD11b^+$). From the neutrophil negative population, we gated for eosinophils ($CD11b^+$ $SiglecF^+$). From the eosinophil negative population, we gated for monocytes ($Ly6C^+/Gr1^+$ $CD11b^+$). From the monocyte negative population, we gated for Kupffer cells ($CD11b^{mid}$ $F4/80^+$). From the negative population, we gated for DCs ($CD11c^+$ $MCHII^+$). From the DCs, we gated for cDC1 ($CD103^+$ $CD11b^-$) and cDC2 ($CD103^-$ $CD11b^+$). From the $CD11c^+MHCII^+$ subset, we gated for monocyte derived macrophages ($CD11b^+$ $MHCII^+$). The analysis was done using FlowJo version 10.6.

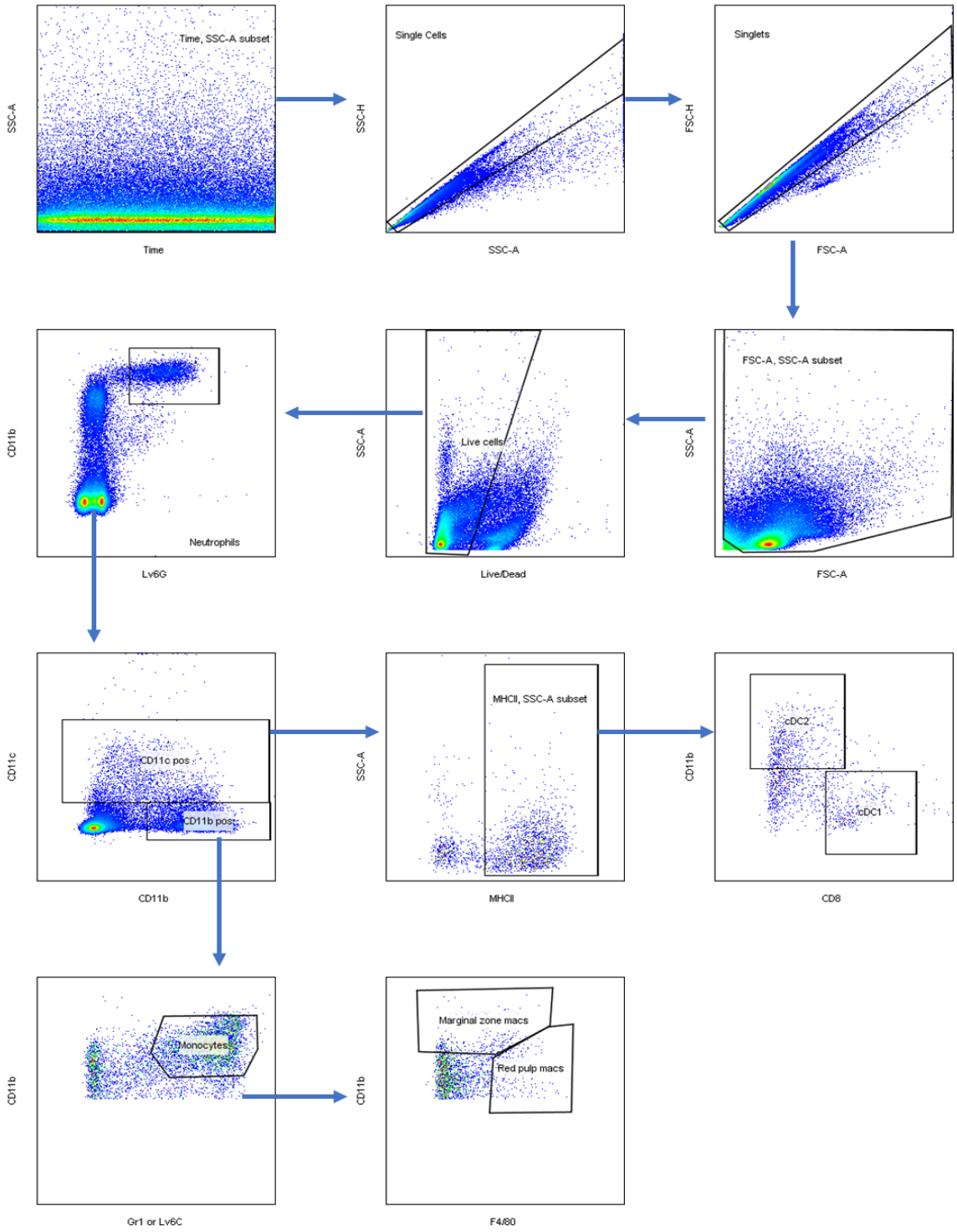

Figure S5: Spleen myeloid cell populations gating strategy. From the time gate, we gated for single cells, to singlets, then gated out the debris (not shown), then gated for live cells from SSC-A and live/dead. From the live cells, we gated for neutrophils ($Ly6G^+$ $CD11b^+$). From the negative population, we gated for CD11b positive and CD11c positive cells. From the CD11b positive cells we gated for MHCII SSC-A subset, from which we gated for cDC1 ($CD11b^+$ $CD8^-$) and cDC2 ($CD11b^-$ $CD8^+$). From CD11c positive cells, we gated for monocytes () and from the negative population, we gated for marginal zone macrophages ($CD11b^+$ $F4/80^{low}$) and red pulp macrophages ($CD11b^{low}$ $F4/80^+$). The analysis was done using FlowJo volume 10.6.

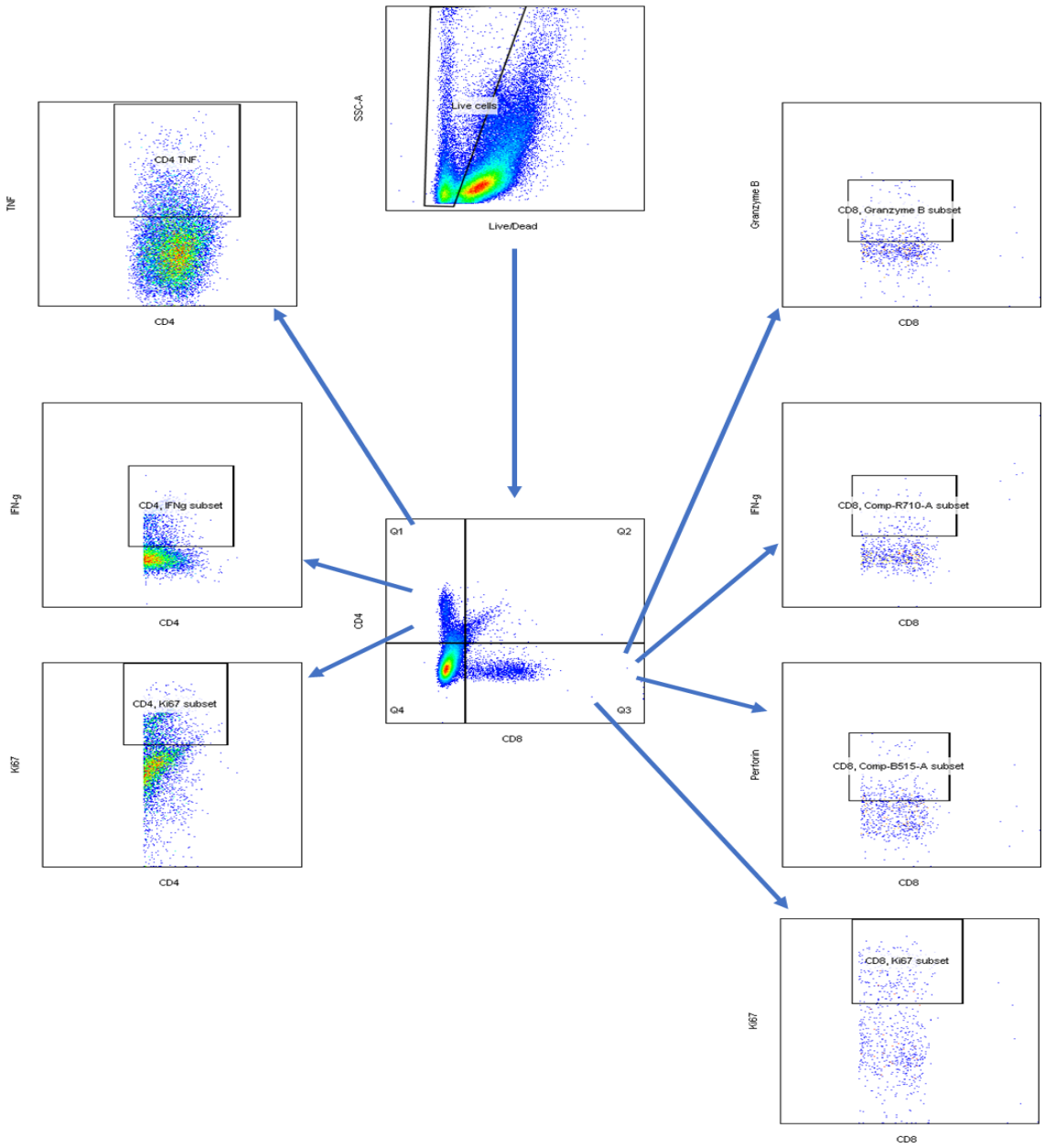

Figure S6: Liver and spleen T cell intracellular staining gating strategy. From the time gate, we gated for single cells, then singlets, then we removed the debris. We then gated for live cells from SSC-A and live/dead. From the live cell gating, we gated for the CD4 and CD8 T cells. From CD4 T cells we gated for CD4 T cell cytokines, TNF ($CD4^+$ TNF^+), IFNy ($CD4^+$ $IFNy^+$) and proliferating CD4 T cells ($CD4^+$ $Ki67^+$). From $CD8^+$ cells we gated for cytokines, granzyme B ($CD8^+$ granzyme B+), perforin ($CD8^+$ $perforin^+$), IFNy ($CD8^+$ $IFNy^+$), and proliferating CD8 T cells ($CD8^+$ $Ki67^+$). The analysis was done using FlowJo version 10.6.

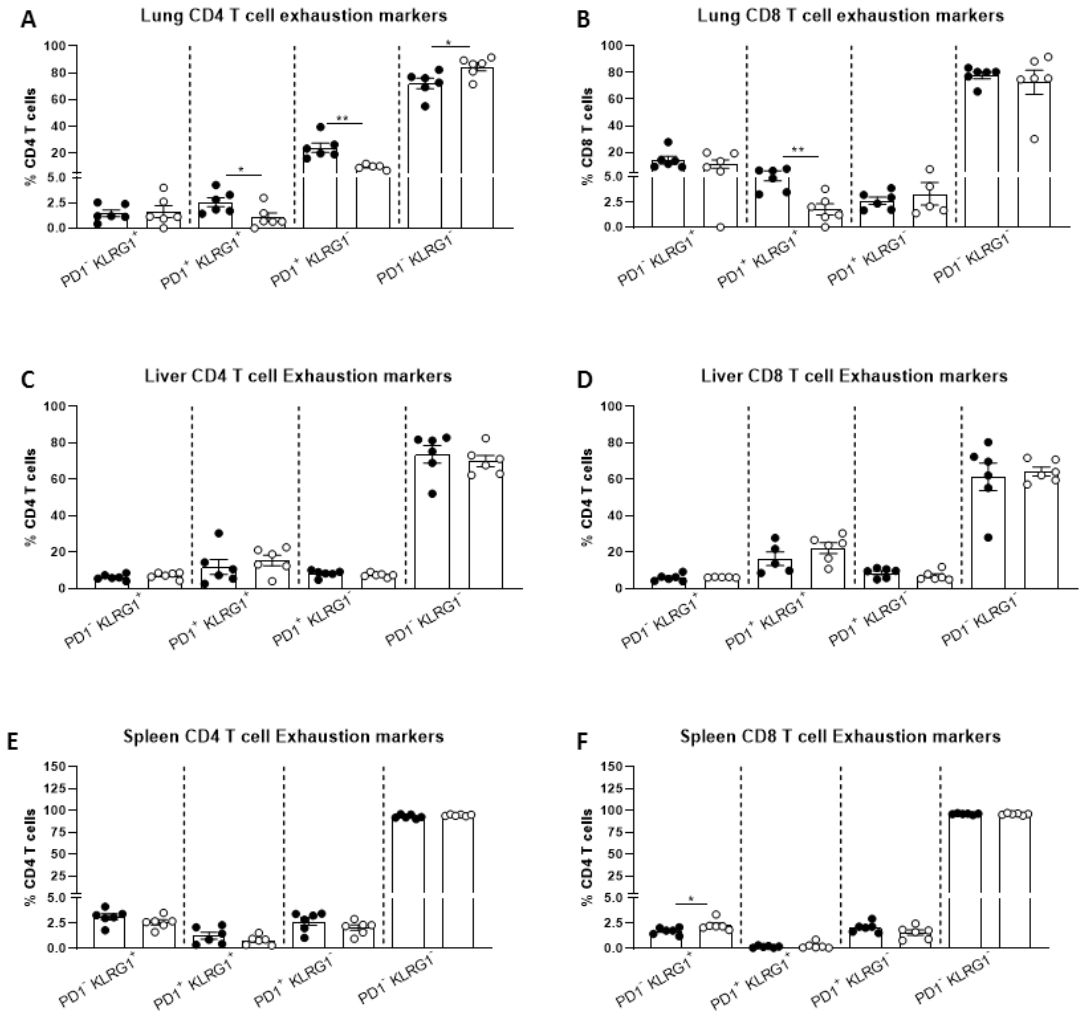

Figure S7: Lung, liver and spleen CD4 and CD8 T cell exhaustion markers at naïve state. CysLTR1 deficient mice and their littermate controls were sacrificed. A-B) Lung, C-D) liver and E-F) spleen single cells were stained for CD4 and CD8 T cell exhaustion markers, PD1 and KLRG1. Data is representative of three independent experiments. Error bars denote mean ± SEM and was statistically analysed using the unpaired student T test with/without Welch's correction, (*p<0.05, **p<0.01).

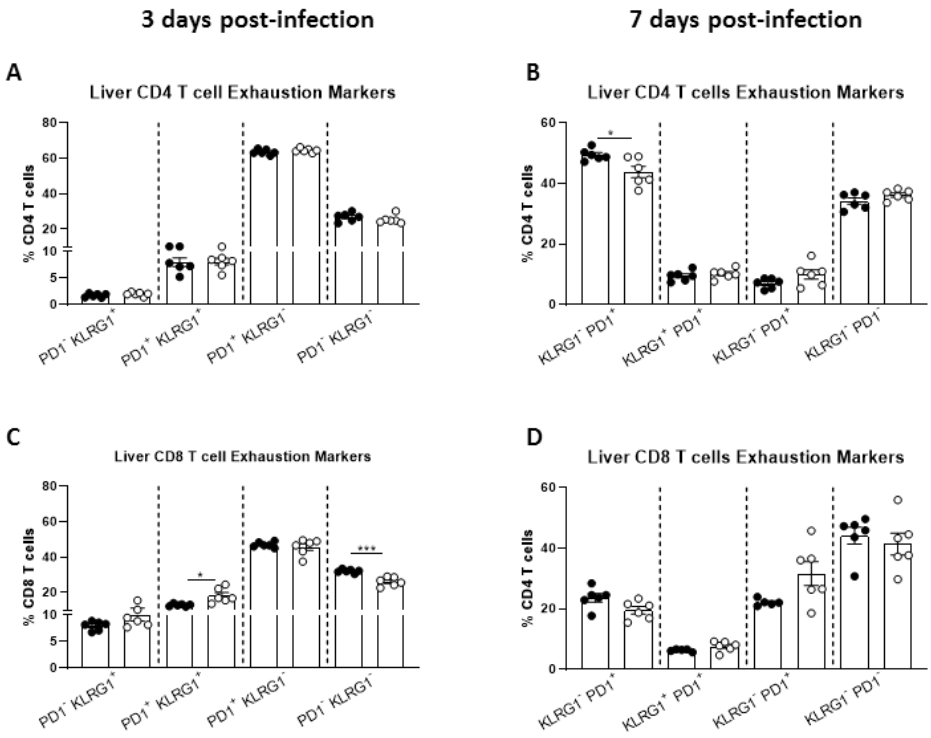

Figure S8: Liver CD4 and CD8 T cell exhaustion and homing markers post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with about $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and sacrificed the mice at 3- and 7-dpi (*n = 6 per group*). *A-D)* Liver single cells were stained for CD4 and CD8 T cell exhaustion and homing markers, PD1 and KLRG1 at 3- and 7-dpi. Data is representative of two independent experiments. Error bars denote mean ± SEM and were statistically analysed using the unpaired student T-test with Welch's correction, (*$p<0.05$, ***$p<0.001$).

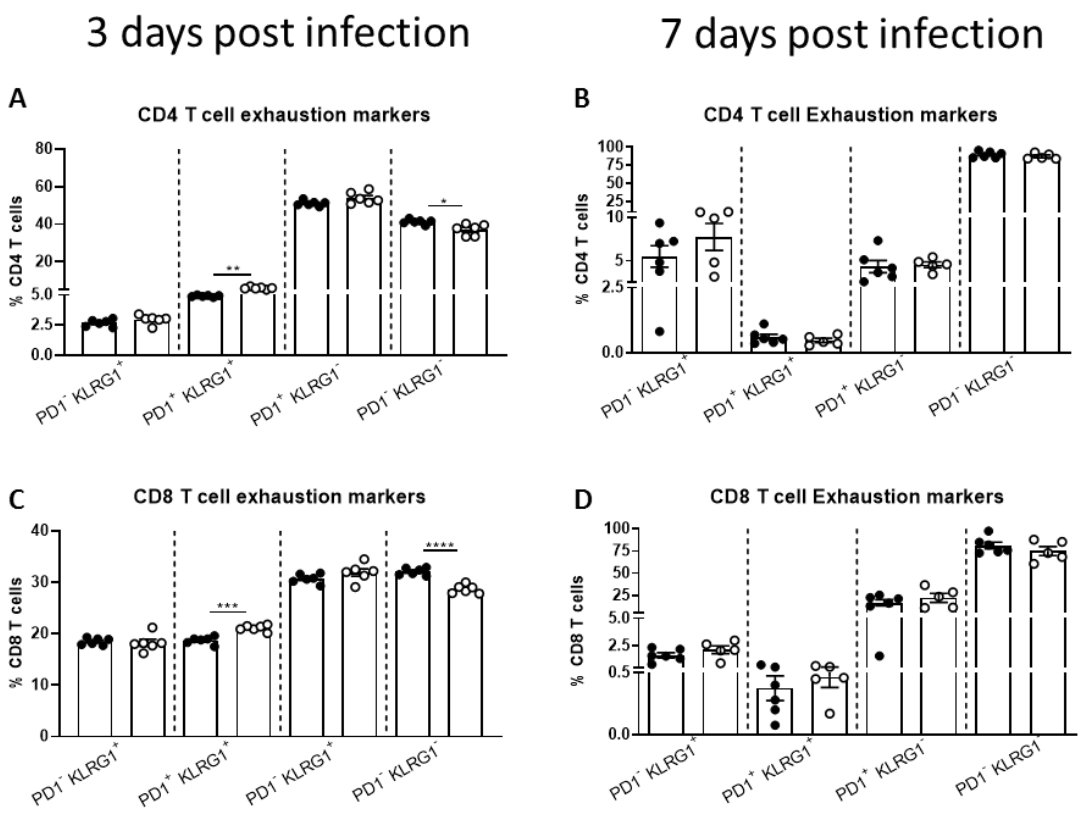

Figure S9: Spleen CD4 and CD8 T cell exhaustion markers post-*L. monocytogenes* infection. CysLTR1 deficient mice and their littermate controls were infected with about $1.1x10^5$ *LM* CFUs/200µL per mouse intraperitoneally and sacrificed the mice at 3- and 7-dpi (*n = 6 per group*). **A-D)** Liver single cells were stained for CD4 and CD8 T cell exhaustion markers, PD1 and KLRG1 at 3- and 7-dpi. Data is representative of two independent experiments. Error bars denote mean ± SEM and were statistically analysed using the unpaired student T-test with/without Welch's correction, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).