The role of Interleukin -1 signaling in the immune defense and in the development of the T helper cell lineage

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CONTENTS

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LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

LIST OF TABLE

ABSTRACT

IL-1 is a pro-inflammatory cytokine which play an important role in the activation and regulation of host defence and immune responses to inflammation or injury. IL-1 is able to bind and activate IL1-RI and IL1-RII, which are found on many cells types. The role of the IL-1 signalling in the deployment of Th cell subsets, especially Th17 cells is well known. However, the specific cells which are responsible for the expression of IL-1 signalling in the immune defense and in the development of the Th cell lineage in response to infection, is still largely unclear. Therefore in this thesis, IL1-RI conditional knockout mice specifically in hematopoietic cells (IL1-RI *vaviCre*+) were generated. Using IL1-RI *vaviCre*+ mice in comparison with IL1-RI global knockout mice (IL1-RI$^{-/-}$) would determine whether the expression IL-1 signalling from hematopoietic cells is responsible for the immune defense and in the development of the Th1, Th2 and Th17 cells against gastrointestinal helminth *Trichuris muris* (*T.muris)* infections.

The generation of IL1-RI *vaviCre*+ mice have been investigated at the genomic and proteomic level in order to confirm that the *Il1-rI* gene is inactivated in hematopoietic cells. The characterisation of IL1-RI *vaviCre* + mice at the genomic level confirmed that the *Il1-rI* gene was obliterated successfully. At protein level the characterisation of IL1- RI *vaviCre* + mice confirmed that IL1-RI was dysfunctional in hematopoietic cells. Additionally, the development of the immune cells was investigated in IL1-RI *vaviCre* + and IL1-RI$^{-/-}$ mice. Our findings demonstrated that the lymphocyte development was not affected by the deletion of the IL1- RI gene. This data indicated that IL1- RI *vaviCre* + and IL1-RI$^{-/-}$ mice are vital *in vivo* models.

In high dose infection, both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice were able to clear the infections due to their ability to generate a Th2 response. Both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice infected with low dose of *T.muris* were susceptible to infections and showed high levels of Th1 cytokines. Thus, we hypothesised that IL1-RI signalling in hematopoietic cells was not required for worm expulsion and the generation of Th2 and Th1 response.

Interestingly, low dose *T.muris* infection showed a clear reduction in the Th17 cytokines IL22 and IL17 in both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice, suggesting that IL-1 signalling expressed from hematopoietic cells is responsible for the development of Th17 cells and secretion of IL17 and IL22. IL1- RI *vaviCre* + and IL1-RI $^{-/-}$ mice infected with low dose of *T.muris* also showed an increase in inflammation in the colon and decreased of goblet cell hyperplasia. It is well known that IL22 plays an important role in preventing tissue damage and repair. Thus, in this study IL22 global knockout mice (IL22 $^{-/-}$) were used to determine if the change in crypt lengths and goblet cell hyperplasia in IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ was due to an absence of IL22. Our finding showed that IL22 $^{-/-}$ mice infected with low dose of *T.muris* had increased crypt length and a reduction in goblet cells. The similar phenotype in crypt length and goblet cell hyperplasia between IL22 $^{-/-}$, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice suggested that a lack of IL22 in IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice is responsible for the change in mice phenotype. It also provides more evidence for the role of IL-1 signaling in hematopoietic cells in the generation of Th17 cells and in the production of its cytokine IL22.

IL1-RII is an inhibitor of IL1-RI, thus, in this study IL1-RII global knockout mice (IL1-RII $^{-/-}$) mice was used in comparison with IL1-RI $^{-/-}$ mice to verify the role of IL-1 signaling in the development of Th17 cells. Our finding showed an overexpression of IL17 and IL22 in IL1-RII $^{-/-}$ compared with IL1-RI $^{-/-}$ mice and a higher level of IL17 in IL1-RII $^{-/-}$ mice compared with IL1-RII $^{flox/flox}$ mice. This data confirmed that IL-1 signaling is important for the development of Th17 cells and the production of its cytokine IL17 and IL22.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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ABBREVIATIONS

APC	Antigen presenting cells
BAC	The bacterial artificial chromosome
BM	Bone marrow
C.rodentium	*Citrobacter rodentium*
CANP	Calcium-activated neutral protease
CD121a	Cluster of Differentiation 121a
CIA	Collagen induced arthritis
CNS	Central Nervous system
Con A	Concanavalin A
DCs	Dendritic cells
DSS	Dextran sodium sulphate
ECSI	Evolutionarily conserved signaling intermediate in Toll pathwas
ELISA	Enzyme linked immunesorbent assay
ERK1/2	Extracellular signal-regulated kinase 1/2
ES	Embryonic stem
FLP	Flippase
FRT	Flip-recombinase targets
H&E	Hematoxylin-eosin
HepG2	Hepatoma cell line
HET	Heterozygous
HOM	Homozygous mice
HR	Homologous recombination
HRP	horseradish-peroxidase
HS	Hypersensitive
iCre	Improved Cre
IFNγ	Interferon gamma
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IKK	IκB kinase
IL	Interleukin
IL-1	Interleukin-1
IL1-R AcP	IL-1R accessory protein
IL1-RII	IL-1 receptor type II
IL-1Ra	Receptor antagonist
IL-1Ra $^{-/-}$	IL-1Ra knockout mice
IL1-RI	IL-1 receptor type-I
IL1-RII $^{-/-}$	IL1- RII knockout mice
IL-4R	IL-4 receptor
ILC3	Innate lymphoid cells 3
IRAK	Serine/threonine IL-1 Receptor Associated Kinase
iTh17	Innate Th17 cells
iTreg	Induced T-regulatory
KLH	Keyhole limpet hemocyanin
KO mice	Knockout mice
L.major	*Leishmania major*
LB	Luria-Bertani broth
LCR	Locus control region
loxP	locus of crossover (x) in P1
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cells

LTi-like cells	Lymphoid tissue inducer cells -like cells
MAP	Mitogen-activated protein
MCP-1	Monocyte Chemoattractant Protein-1
MLN	Mesenteric lymph node
Muc2	Mucin2
N.D	Not detected
neo gene	The neomycin resistance gene
NeoR	Neomycin
NFκB	Nuclear transcription factor κB
NK	Natural killer
NKT	Natural killer T cells
NOD-like receptors	The nucleotide-binding oligomerization apoptosis inhibitor proteins-like receptors
OVA	Anti-ovalbumin
p.i	Post infections
PCR	Polymerase chain reaction
pre-B cells	Pregenitor B cells
pro-B cell	Progenitor B cells
PuroR	Puromycin
SCF	Stem cell factor
sIgM	IgM surface
STAT	Signalling transducer and activator of transcription
T. muris	*Trichuris muris*
T.trichiura	*Trichuris trichiura*
T2D	Type 2 diabetes
TAK-1	Transforming growth factor β-activated kinase-1
TCR	T-cells receptors
TDS	Trichuris dysentery syndrome
Tfh	Follicular helper T-cell
Th	T-helper
Th1	T-helper cells type 1
Th17	T-helper cells type 17
Th2	T-helper cell type 2
Th9	T-helper 9
TIR	Toll/interleukin-IL-1
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNFα	Tumor necrosis factors α
Tollip	Toll-interacting protein
Tr1	Regulatory type 1 cells
TRAF6	Tumour necrosis receptor-associated factor-6
T-reg	Regulatory T-cells
WB	Western Blots
WT	Wild type
γδ T	Gamma delta T-cells
APC	Antigen presenting cells
BAC	The bacterial artificial chromosome
BM	Bone marrow
C.rodentium	*Citrobacter rodentium*
CANP	Calcium-activated neutral protease

CHAPTER ONE: INTRODUCTION

This introduction will begin with an overview of pro-inflammatory cytokines including the interleukin-1 (IL-1) family of cytokines, IL-1 receptors, IL-1 signalling, the role of IL-1 in disease and the role of IL-1 in T-cell polarisation. It will culminate with a review of the generation of conditional knockout mice, followed by an overview of the cell type specific knockout mice used in this thesis. Finally, this introduction will finish with an overview of the infection model *Trichuris muris (T.muris)* which is used *in vivo* to test the role of IL-1signalling in the immune defence and for the development in the T helper cells lineage.

1.1 PRO-INFLAMMATORY CYTOKINES

1.1.1 IL-1 family of cytokines

IL-1 cytokines are reported as highly pro-inflammatory molecules, able to activate and regulate host defence and immune responses (Auron *et al*., 1984). The IL-1 family is a group of 11 cytokines, including IL-1α IL-1β, IL-1 receptor antagonist (IL-1Ra), IL‑18, IL‑33 and IL‑1F5–IL‑1F10, Figure 1-1 demonstrates the IL-1 family members (Dinarello, 2009, Sims and Smith, 2010). All IL-1 family members are potentially soluble proteins produced by different cell types (Boutin et al., 2003). IL-1 cytokines are primarily produced by macrophages, monocytes and dendritic cells (DCs). However, epithelial cells, lymphoid cells, vascular smooth muscle cells and hepatocytes also produce IL-1 (Kamari et al., 2007, Shimizu *et a*l., 2015). IL-1α and IL-1β are the most highly characterised IL-1 family members (Luheshi *et al*., 2009), both bind and then activate the same receptor, namely IL-1 receptor type-I (IL1-RI) and IL-1 receptor type-II (IL1-RII), which belong to the IL-1 family (Dinarello, 1996, Kamari et al., 2007, Cullinan et al., 1998). It was reported IL-1 induce the inflammation by promote cell adhesion molecules, cytokines, and chemokines in many types of cells such as epithelial cells, endothelial cells, synovial cells, and macrophages (Shimizu *et al*., 2015). IL-1Ra can also bind to IL1-RI and IL1-R II but it does not cause a biological response (Cullinan *et al*., 1998, Smith *et al*., 2003).

IL-1R accessory protein (IL1-R AcP) is an important member of the IL-1 family. It shares a 25% amino acid identity with IL1-RI and IL1-RII proteins (Greenfeder *et al*., 1995). IL1-R AcP was reported as a co-receptor for IL1-RI (Wesche et al., 1997b, Thomas et al., 2012). IL1-R AcP is required for high affinity binding between IL1-RI and IL-1 (Dinarello, 1996, Cullinan *et al*., 1998, Sims and Smith, 2010). Studies with IL1-RAcP-knockout (KO) mice and cells indicated that the presence of IL-1RAcP is required for IL-1 bioactivity (Cullinan *et al*., 1998).

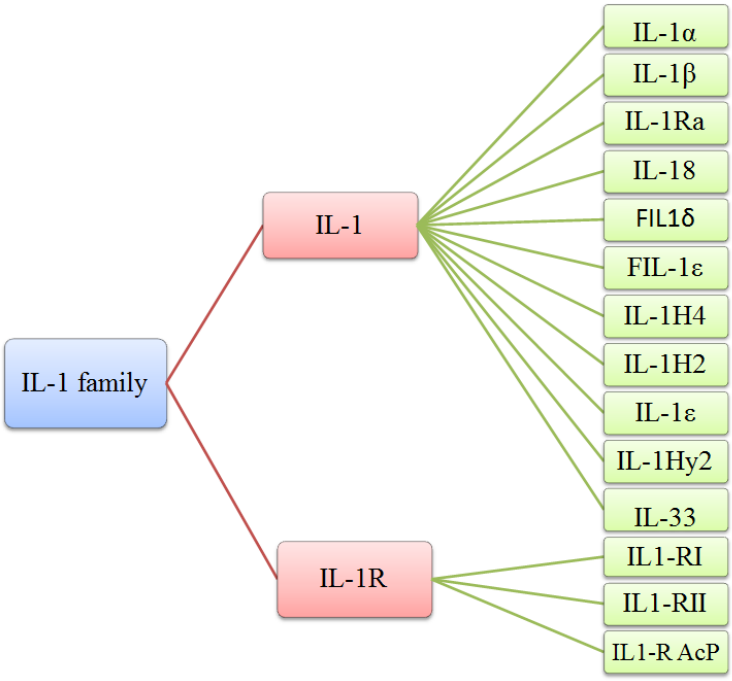


Figure 1-1: Overview of the IL-1 family members: The IL-1 family has two main groups IL-1 cytokine and IL-1 cytokine receptor. IL-1 cytokine has 11 members IL-1α IL-1β, IL-IL-1Ra, IL‑18, IL‑33, FIL-1δ, FIL-1ε, IL-1H4, IL-1H2, IL-1ε and IL-1Hy2. The IL-1 cytokine receptor has three members IL1-RI, IL1-RII and IL1-R AcP, as adapted from (Dinarello, 2009).

IL-1/IL1-RI signalling is regulated by many mechanisms. The activity of IL-1 can be regulated by two different inhibitors, IL-1Ra and IL1-RII (Smith *et al*., 2003). The function of IL-1Ra is an inhibitor of IL-1α and IL-1β (Burger et al., 1995). The competitive binding of IL-1Ra to the IL1-RI prevents IL1-RAcP from binding to IL1-RI. The absence of IL1-RAcP affects the binding affinity of the IL-1 ligands that prevents signals transduction (Cullinan *et al.*, 1998, Burger *et al.*, 1995, Dinarello, 2009). The soluble form of IL1-R II (IL-1sRII) enhances the inhibiting activity of the IL-1Ra (Burger

et al., 1995). A second inhibitor of the IL-1 system is IL1-RII. It binds strongly with IL-1β and poorly with IL-1α. IL1-R II lacks the entire intracellular domain, and is known to act as a decoy receptor. (McMahan *et al*., 1991, Giri *et al.*, 1994). The soluble form of IL1-sR AcP enhances the ability of IL-1sRII to inhibit IL1-RI action by elevating its affinity to bind to IL-1α and IL-1β (Smith *et al*., 2003).

1.1.2 IL-1β and IL-1α

IL-1α and IL-1β are reported as important mediators in acute and chronic inflammation. The activation and production of theses cytokines are regulated at several levels (Van Tassell *et al*., 2014). The biological effect of IL-1α and IL-1β is achieved by binding with IL1-RI but not IL1-RII because of the short cytoplasmic domain of IL1-RII (Sims *et al*., 1993). IL-1α and IL-1β cytokines are synthesise as precursor proteins pro- IL-1α and pro-IL-1β (31-34 kDa). Pro- IL-1α and pro- IL-1β are then converted to the mature form upon secretion (17-18 kDa) (Rubartelli et al., 1990, Mosley *et al*., 1987, Risbud and Shapiro, 2014). Interestingly, intracellular pro- IL-1α, not pro- IL-1β, can bind and activate IL1-RI (Mosley *et al*., 1987, Van Tassell *et al*., 2014).

Earlier studies that used radiolabelled pro-IL-1α to understand the internalisation and intracellular transport of IL-1 found that radiolabelled pro-IL-1α bound to receptors without degradation, internalised and trans-located to the nucleus (Curtis *et al.*, 1990). Interestingly, mature IL-1α is unable to bind to DNA while the pro - IL-1α /IL1-RI complex can bind directly to DNA (Weitzmann and Savage, 1992). Pro-IL-1α contains sequences similar to the nuclear localisation signal (KVLKKRR) (Wessendorf *et al.*, 1993). The function of intracellular pro - IL-1α is to regulate normal cellular differentiation (Dinarello, 2009). Mature IL-1α is produced when the pro-IL-1α is released by injured cells and then cleaved by a calcium-activated neutral protease (CANP), also known as calpain (Kobayashi *et al*., 1990). IL-1 α and TNFα play a crucial role in T-cells' differentiation by the induction of CD25 expression on early immature thymocytes, followed by maturation to CD4+CD8+ differentiation (Zuniga-Pflucker *et al*., 1995). It is

also required for T-cell activation in response to contact allergens (Nakae *et al.,* 2001b). Moreover, IL-1α is involved in diet-induced weight gain and atherosclerosis (Kamari *et al*., 2007) .

As mentioned earlier, IL-1β is synthesised as a pro-IL-1β peptide which is inactive. Pro-IL-1β is cleaved by cysteine proteases known as caspase-1 and converted to the mature IL-1β (Thornberry *et al*., 1992, Van Tassell *et al*., 2014). IL-1β can also be cleaved and activated by other proteases produced in an inflammatory environment such as trypsin, elastase, and chymotrypsin.(Hazuda *et al*., 1990). Caspase-1 also needs cleavage to be an active protein (Wilson *et al.*, 1994). Caspase-1 is synthesised as inactive pro-caspase-1. The nucleotide-binding oligomerization apoptosis inhibitor proteins-like receptors (NOD-like receptors) is a cytosolic protein which plays an important role in the regulation and activation of caspase-1 through a multi-protein complex known as inflammasome (Van Tassell et al., 2014). Caspase-8 and Caspase-9 are also required in the activation process of caspase-1 (Mariathasan and Monack, 2007). The regulation of caspase-1 is reported as another level of IL-1 regulation (Wilson *et al*., 1994). In caspase-1 deficient mice the production of IL-1α is decreased (Kuida *et al*., 1995), suggesting that the production of IL-1α is control by caspase-1.

IL-1β is known as a prototypical pro-inflammatory cytokine, as it is produced in response to disease or injury. IL-1β has extensive effects on gene expression including elevating cytokines, tissue remodelling enzymes and acute phase proteins (Dinarello, 1996, Li *et al*., 2008). Protein products produced by microbes induce the expression IL-1α and IL-1β by monocytes, macrophages, dendritic cells, B-cells and NK-cells (Dinarello, 1996). Moreover, IL-1β also plays an important role in the adaptive immune response by inducting the development of activated lymphocytes (Gery *et al*., 1972), as T-cell antibody production is dependent on IL-1β not IL-1α (Nakae *et al*., 2001b). IL- 1β is essential for the efficient clearance of bacterial infections (Miller *et al*., 2007). Moreover, IL-1β is a

potent regulator for cell death (apoptosis). IL-1Ra can be used to inhibit hypoxia-induced apoptosis (Friedlander *et al*., 1996).

1.1.3 IL-1 receptors

The cytokine receptor IL1-R is able to bind to IL-1α, IL-1β and IL-1Ra. IL1-R was cloned from human T-cells and fibroblasts and it was found that both cells possess a relative molecular mass of 80 kDa (Dower *et al*., 1986). IL1-R was cloned from T-cells in C57BL/6 mice and it was found that the length of extracellular binding portion of IL1-R was 319 amino acids containing three Ig-like extracellular domains (Sims *et al*., 1988). Moreover, Gay and Keith found that the cytoplasmic domain of IL1-R consists of 215 amino acids and has a strong homology with Toll receptors (Gay and Keith, 1991). IL1-R has a high affinity binding in response to low concentrations of IL-1α or IL-1β on human rheumatoid synovial cells cultures (Chin *et al*., 1988). Interestingly, a study has shown that B-cells and B-lymphoma had IL-1R with a lower molecular weight (60kDa) than T-cells (Horuk *et al*., 1987). This observation explains that IL1-R expressed by T-cells and B-cells are produced from different genes. It was also found that there are structural differences between T-cells and B-cells in the extracellular domains of the IL-1 receptors (Bomsztyk *et al*., 1989). Therefore, with the two different receptors McMahan *et al* suggested that IL-1RI was the 80kDa molecule, and IL1-RII was the 60kDa molecule. (McMahan et al., 1991, Sims *et al*., 1993).

The *Il1-rI* gene encodes a cytokine receptor that belongs to the interleukin-1 receptor family. It is also known as CD121a (Cluster of Differentiation 121a) and IL-1 receptor alpha chain. This gene locus is located on chromosome 1 and consists of 40281925-40373022 bp (MGI-Mouse Genome Informatics MGI, 2014), from where the IL-I receptor gene was cloned by gene expression screening (Sims *et al*., 1988). IL-1α and IL-1β bind with high affinity to the IL-1RI /IL-1RAcP complex is a binding requiring a two-step reaction. Firstly, IL-1 binds to IL-1RI causing a conformational change in IL1-RI. Secondly, the change in the IL1-RI allows IL1-RAcP to bind to the IL-1/IL1-RI complex

leading to signal transduction (Dower et al., 1989, Greenfeder *et al*., 1995). IL1-RAcP provides high stability signals that increase IL-1 binding affinity. Once signalling has been performed the complex is internalised and transferred to the nucleus by the golgi apparatus (Falk *et al*., 1989).

IL1-RII's extracellular portion also consists of three immunoglobulin domains. IL1-RII contains a short intracellular cytoplasmic tail that is 29 amino acids long. This indicates that two IL-1 receptors interact with different signal transduction pathways. IL1-RII is expressed by a numbers of cells including B and T-lymphocytes (McMahan *et al*., 1991). IL1-RII is cleaved from the cells and both the membrane and soluble form act as a decoy receptor (McMahan *et al*., 1991, Giri *et al.*, 1994, Lang *et al*., 1998). IL1-RII can bind to IL1-R AcP to down regulate IL-1 responsiveness (Lang *et al*., 1998).

1.1.4 IL-1 signalling

IL-1 signalling is activated in response to infection, tissue injury or stress (Jensen et al., 2000). Mitogen-activated protein (MAP) kinase p38, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase and nuclear transcription factor κB (NFκB) are the main pathways activated by IL-1 (Auron, 1998, Brikos *et al*., 2007).

Many cytokines are activated after secretion. However, some require processing at several levels. Figure 1-2 demonstrates the mechanism of IL-1α and IL-1β's expression and activation.

IL-1α and IL-1β processing, release and activation within responsive cells, starts with the translation of pro-IL-1α and pro-IL-1β in the cytoplasm of IL-1 expressing cells (Stevenson *et al*., 1992, Rubartelli et al., 1990). Next, pro-IL-1α/β is cleaved by calpain and caspase-1 to stimulate release of mature IL-1α/β (Kobayashi *et al*., 1990, Thornberry et al., 1992). Calpain is calcium- dependent Protease, which requires ATP for its function (Watanabe and Kobayashi, 1994). Both mature IL-1α and IL-1β then bind to and activate the single transmembrane domain type IL1-RI on responsive cells (Dinarello, 1996, Vigers *et al*., 1997). IL1-RAcP binds to the IL1-RI IL-1α or IL-β complex (Wesche *et al*., 1997b,

Dinarello, 1996). The only known signalling receptor for IL-1α and IL-1β is IL1-RI; it requires heterodimeristion with the protein IL1-RAcP for high affinity binding and signal transduction (Cullinan *et al*., 1998, Dinarello, 1996, Sims and Smith, 2010). The intra-cytoplasmic domains of IL1-RI and IL1-RAcP belong to the Toll like receptor (TLR) family reported as Toll/interleukin-IL-1 (TIR) (O'Neill and Bowie, 2007, Thomas et al., 2012). The creation of the IL-1/IL1-RI /IL1-RAcP complex enables recruitment of the adapter molecule MyD88, which also contains TIR domain, to the TIR domains of IL1-RI and IL1-RAcP (Medzhitov *et al*., 1998, Wesche *et al*., 1997a). Binding of IL-1 with the IL1-RII decoy receptor leads to loss of the TIR domain, which is required for signal transduction (Thomas *et al*., 2012).

Subsequently, the serine/threonine IL-1 Receptor Associated Kinase IRAK-1 IRAK-2 and the IRAK-4 are recruited and activated by MyD88 (Janssens and Beyaert, 2003). In a resting state, IRAK-1 is associated with a Toll-interacting protein (Tollip). The phosphorylation of Tollip and IRAK-4 is required for IRAK-1 activation (Burns *et al*., 2000). These multiple kinases cause the recruitment and activation of tumour necrosis receptor-associated factor-6 (TRAF6) (Janssens and Beyaert, 2003). The IRAK-1/TRAF6 complex interacts with transforming growth factor β-activated kinase-1 (TAK-1) within the cytoplasm, which then phosphorylates the IκB kinase (IKK) complex (Ninomiya-Tsuji *et al*., 1999). The degradation of the phosphorylated IKK is leading to translocate NFκB to the nucleus and activate gene expression. Alternatively, the interaction between TRAF6 and the TRAF associated protein named evolutionarily conserved signalling intermediate in Toll pathways (ECSI), causes activation of c-Jun through the Map kinase/JNK signalling cascade (Kopp *et al*., 1999).

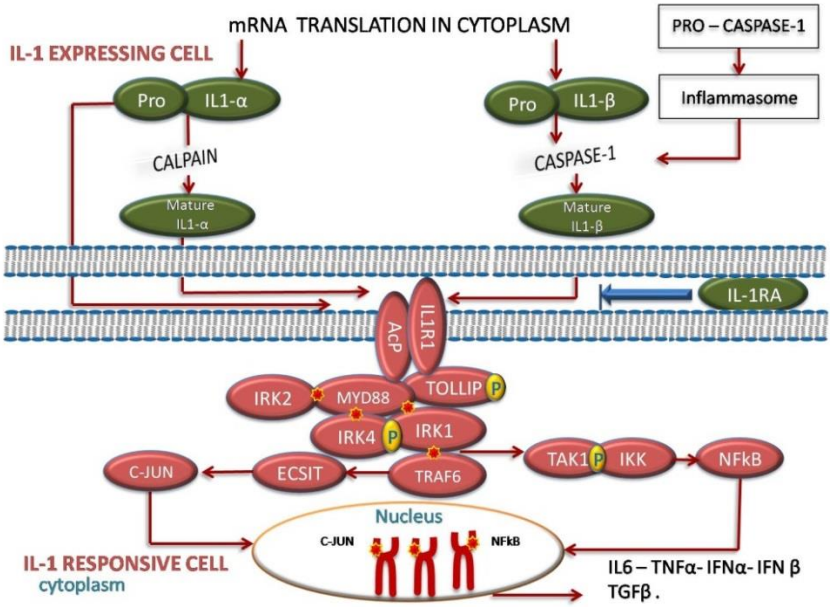


Figure 1-2: The mechanism of IL-1α and IL-1β expression and activation: Pro-IL-1α and pro-IL-1β are translated in the cytoplasm of IL-1 expressing cells. Then, pro-IL-1α/β is cleaved by calpain and caspase-1 to stimulate release of mature IL-1α/β. Both mature IL-1α/β are released from cells and bound to transmembrane IL1-RI on IL-1-responsive cells. This leads to the enrolment of IL-1RAcP (AcP) to IL1-RI. The recruitment and activation of a multi-protein complex to the cytoplasmic domain of the receptor dimer causes the activation of NFkB, mitogen-activated protein kinases, regulating gene expression and RNA stability.

1.1.5 The role of IL-1 in disease

Several studies indicate that IL-1 plays a crucial role in innate and adaptive immune responses as a result of similarities in the cytoplasmic domains of IL1-RI and Toll-like receptors (TLRs). Both receptors are involved in inflammatory and host-defence responses of injury and infection (O'Neill, 2008, Dinarello, 2009, Thomas *et al*., 2012). Whilst, IL-1 protects the organism due to its ability to enhance the host response to pathogens, its overproduction can cause pathological symptoms (Schreuder *et al.*, 1995). Several studies were therefore performed to understand IL-1 responding cells in a variety of inflammatory diseases.

Inhibition IL-β signalling has been reported as a standard of therapy for a large number of inflammatory diseases including autoimmune diseases such as rheumatoid arthritis, type 2 diabetes (T2D) and heart failure. Hence blocking IL-β leads to reduced disease severity.

The neutralisation of IL-β can be achieved by IL-1Ra, which binds to IL1-RI and prevents the activity of IL-β. Moreover, IL1-RII has a higher affinity binding to IL-β than IL1-RI. Therefore, the soluble or membrane associated IL1-RII can bind to the IL-β and prevent its activity but that requires the soluble form of IL1-RAcP (Dinarello, 2011). IL-1 was reported as an important mediator of chronic inflammatory joint diseases, including rheumatoid arthritis (Geiger *et al*., 1993, Bendele *et al*., 1999). These studies indicate that IL-1 is important for collagen induced arthritis and the addition of anti-IL-1β antibody or IL-1Ra, reduced arthritis incidence (Geiger *et al*., 1993, Bendele *et al*., 1999). Another study demonstrates that patients treated with IL-1Ra show a reduction in rheumatoid arthritis activity (Bresnihan *et al*., 1998). T2D is shown as immune mediated disease causing reduction in insulin production due to the destruction of pancreatic β-cells. It was shown that a high expression of IL-1 causes T2D that reduce insulin production and increase β-cell apoptosis (Banerjee and Saxena, 2012). In the last few years there have been several studies performed to investigate the role of IL-1 in T2D. It was shown that high production of IL-1β in human pancreatic cells caused a reduction in insulin secretion, cell proliferation and β-cell apoptosis (Banerjee and Saxena, 2012). Therefore, reduction of IL-1β can be used to improve insulin production through β-cells (Dinarello *et al*., 2010). Moreover, another study showed that IL-1 has an important role in the prevention of heart diseases such as atherosclerosis, atherothrombosis, heart failure and pericarditis (Van Tassell *et al*., 2014). In atherothrombotic disease the activation of IL-1 leads to enhancing the formation of atheromatous lesions and promoting vascular inflammation (Bujak and Frangogiannis, 2009). Thus, IL-1Ra the inhibitor of IL-1 can be used in several cardiovascular diseases (Bujak and Frangogiannis, 2009).

Furthermore, the biological effect of IL-1 includes the activation of inflammatory response and stimulates the production acute-phase proteins, in acute and chronic central nervous system (CNS) response to injuries. IL-1 has been reported as an endogenous pyrogen because a high expression of IL-1 increases body temperature and fever. Moreover, in the

murine models, the high expression of IL-1 causes brain trauma. This can be prevented by using the IL-1 inhibitor, IL-1Ra. It was shown that in the IL-1α/ IL-1β KO mice the brain injury was reduced 80%, while in the IL-1Ra KO mice the brain injury increased three-fold and led to an elevation in mortality (Pinteaux *et al*., 2009). The production of IL-1 in the CNS due to inflammation or injuries not only affects the brain; it also causes an effect in the systemic functions, which are important for defending against CNS diseases such as body temperature, function of neuroendocrine and peripheral immune system (Allan *et al*., 2005). IL-1 involves many CNS diseases such as brain trauma, epilepsy, Parkinson's, Alzheimer's diseases and stroke. In stroke patients, after stroke the concentration of IL-1β increases in the plasma compared with that of healthy people. A study showed that IL-1Ra, IL-6 and acute-phase proteins were increased in the plasma of stroke patient within 2-4 days; a higher concentration was shown in the advanced stage of the disease. Thus, the measurement of pro-inflammatory (IL-1α and IL-1β) and anti-inflammatory (IL-1Ra) molecules seen in after-stroke patients is important to determine the severity of the disease (Allan *et al*., 2005). The treatment of stroke patients with IL-1Ra shows an increase in blood neutrophil counts but no change was observed in IL-6 and C-reactive protein concentration and the patient had a better clinical outcome (Allan *et al*., 2005).

1.1.6 The role of IL-1 for T helper cell subsets.

CD4 T-helper (Th) cells are a lineage of lymphocytes that play an important role in the immune system. The cell population consists of at least three subpopulations namely Th1, Th2 and Th17 cells (Bettelli *et al*., 2007). Other new CD4 subsets have been identified, such as induced T-regulatory cells (iTreg), follicular helper T-cell (Tfh), the regulatory type 1 cells (Tr1) and the potentially distinct T-helper 9 (Th9). The differentiation of each Th subset depends on the complex network of cytokine signalling and transcription factors followed by genetic modifications. (Luckheeram *et al*., 2012).

Naive CD4 T-cells are activated after interaction with T-cells receptors (TCR) with antigen presenting cells (APC) in a particular cytokine milieu. The activated naive CD4 T

then differentiate into one of Th lineages Th1, Th2, Th17 or regulatory T-cells (Treg) that are based on the cytokines secreted during the interaction. For example IL-12, IL-4, IL-6 and IL-2 are important for the development of Th1, Th2, Th17 and Treg respectively. The differentiation of Th lineages involves up regulation of different master regulator transcription factors which are required to promote Th differentiation: T-bet for Th1; GATA3 for TH2 and RORγt for Th17 (Zhu *et al*., 2010, Russ *et al*., 2013). The active signalling transducer and activator of transcription (STAT) play an important role in the induction of transcription factors as well as the cytokine production by T-cells. STAT 6 can be activated by interferon gamma (IFNγ) and is important for T-bet stimulation. STAT 5 is activated by IL-4 and is required for induction of GATA3. STAT 3 is activated by TGF-β, IL-6, IL-21, and IL-23 and is responsible for the simulation of RORγt. The stimulated T-bet, GATA3 and RORγt lead to induction of Th1, Th2 and Th17 specific cytokine gene, respectively. Th lineages can be distinguished by their cytokine production. Th1, Th2 and Th17 cells are characterised by their ability to produce IFNγ, IL-4 and IL-17 respectively (Russ *et al*., 2013). In the last stage of differentiation each T-cell expresses one of the IL-1 family receptors IL-18R in Th1, IL-33R in Th2 and IL-1R in IL-17. Active STAT and IL-1 cytokine IL-18, IL-33 and IL-1β are able to induce cytokine production from Th1, Th2 and Th17 respectively (Guo *et al*., 2009, Zhu *et al*., 2010).

Th1 cells produce mainly IFNγ, IL-12 and are suitable against intracellular pathogens. Th2 cells respond by producing IL-4, IL-5 and IL-13, suitable for extracellular threats. Th17 cells generate cytokines such as IL-17, IL-22 and mediate immunity to extracellular bacteria and fungi; it also plays roles in autoimmunity and inflammation (Luckheeram *et al*., 2012, Fasnacht *et al*., 2009).

The interaction of IL-1 with the adaptive immune response and specifically, T-cell responses has been widely investigated. IL-1 (IL-1α or IL-1β) was reported to primarily stimulate T-cell functions (Dinarello, 2009). Earlier studies produced data that contradicts this; IL-1 has been shown to increase the proliferation of Th2 polarised cells and not Th1

cells. It also showed that Th2 are the only cells that express high affinity receptors for IL-1 (Lichtman *et al*., 1988). In 1998 Satoskar *et al* used *in vivo* models to understand the role of IL-1 in the regulation of Th1 and Th2. WT mice and IL1-RI null mice were both infected with parasite *Leishmania major (L.major)* or immunised with keyhole limpet hemocyanin (KLH). It was found that both infections drive a potent Th1response in WT mice, whereas IL1-RI null mice respond to both stimuli with increased Th2 response. This data demonstrates a role for IL-1 in the negative regulation of Th2 responses (Satoskar *et al*., 1998). Work with primary human T-cell cultures demonstrates that production of IL-4 was reduced by exposure to IL-1 (Sandborg *et al*., 1995). Another study showed that IL-1β up-regulated Th2 responses by increasing IL-4, a key Th2 cytokine production in airway hyperresponsiveness and airway inflammation (Johnson *et al*., 2005). Interestingly, there was a study indicating that IL-1α is important for enhancing Th1 development; this was shown when BALB/c mice were infected with *L.major.* It was found that a high production of IL-12 in response to the *L.major* infection occurred only when IL-1α was injected locally at the site of the infection (Von Stebut *et al*., 2003).

It was demonstrated that IL-17 cytokines are produced largely by Th17 cells. Th17 cells are the major source of IL-17 in many types of cells of the adaptive immune system. However, there are several types of innate immune cells such as gamma delta T cells (γδ T), innate Th17 cells (iTh17), natural killer cells (NK) , natural killer T cells (NKT) and macrophages which are able to produce IL-17 (Jin and Dong, 2013). It has also been shown that neutrophils are also capable to produce IL17 in response to IL15 (Mills, 2008).

IL17 cytokine family consists of six members which have been identified according to their similarity; IL17A IL17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F. The highly homology members of IL17 family are IL-17A and IL-17F are well investigated (Jin and Dong, 2013). The biological function of IL17 is mediate though the surface receptors on target cells (Jin and Dong, 2013). IL-17A binds to a complex of receptors consisting of IL-17RA and IL-17RC, while, IL-17F binds with IL-17RC and with

IL-17RA but with lower affinity than IL-17A (Ishigame *et al*., 2009, Kuestner *et al*., 2007). Epithelial cells, endothelial cells and fibroblasts have been reported as main responder cells to IL-17A and IL-17F (Shen and Gaffen, 2008). It was reported that IL-17A and IL-17F bind to the same receptor complex IL17RA and IL-17RC, this suggesting that IL-17A and IL-17F have similar functions (Ishigame *et al*., 2009). The main function of IL-17A and IL-17F in pathogenic infection, is promoting the production of antimicrobial peptides, cytokines (IL-6, G-CSF, GM-CSF), and chemokines (CXCL1, CXCL2, CXCL5) resulting in activation and recruitment of neutrophils and macrophages (Ishigame *et al*., 2009, Mills, 2008).

IL-1 especially IL-1β plays an essential role in the development of Th17 and secretion of its cytokine IL17 and IL22. A study with the IL-1RI deficient mice and cells indicated that IL-17 was reduced in response to antigen challenge (Sutton *et al*., 2006, Harris *et al*., 2008). The production of IL-23 which drives IL-17 production is dependent on IL-1β (Harris *et al*., 2008). Therefore, the production of IL-17 requires a combination of IL-23 and IL-1α or IL-1β. Another study used IL-1RI deficient mice showed that in γδ T cells IL-1β is required for IL23 function which is essential for the production of IL-17 and IL22 (Sutton *et al*., 2009). A study has shown that IL-6 in combination IL-1 and IL23 are able to induce all of Th17 cytokine IL17, IL17F and IL22 from naïve T-cells (Kimura *et al*., 2007, Chung *et al*., 2009). A study has reported that microbiota in intestinal macrophages induced IL-1β, but not IL-6 which leads to the induction of Th17 to produce IL17 and IL22 (Shaw *et al.*, 2012). A further study has shown that IL-1 signaling in T-cells is essential for Th17's early differentiation *in vitro* and *in vivo.* Regulated expression of the transcription factors, regulatory factors IRF4 and RORγT are important for Th17 polarization with TGF-β, IL-6 and IL-23. It was also demonstrated that the expression of IL-1RI is important for Th17 mediated autoimmunity and for early Th17 differentiation *in vivo* (Chung *et al*., 2009).

IL22 is a newly discovered cytokine produced mainly by Th17 cells (Chung *et al*., 2006). However, many types of lymphocytes are also able to produce IL-22, such as NK cells , Lymphoid tissue inducer cells (LTi) cells , Lymphoid tissue inducer cells -like cells (LTi-like cells) and γδ T cells (Zenewicz and Flavell, 2011). Some of these cells required IL-1 to produce IL22 such as Th17, LTi-like and γδ T and some required other cytokine such as IL12 and 1L18 to produced IL22 like NK cells (Chen *et al.*, 2013, Sutton *et al*., 2009). Dextran sodium sulphate (DSS) was used to generate colitis in WT mice; the source of IL-22 in DSS-induced colitis has been investigated. It was shown that when lamina propria cells from WT mice treated with DSS were stimulated with LPS, IL22 was significantly increased by Toll-like receptor (TLR) signalling. They also show that IL22 was produced through colonic $CD11c^{+}$ cells in response to the stimulation of TLR (Pickert *et al*., 2009). Another study has shown that Flagellins are expressed by bacteria, particularly pathogenic bacteria, in the gut and the lung, leading to the activation of TLR5 signalling in epithelial cells which stimulates the production of IL-17 and IL-22. It also demonstrated that the source of Th17 which produces of cytokines IL17 and IL22 was a new population of $CD3^{-}$ $CD127^{+}$ immune cells similar to LTi cells (Van Maele *et al*., 2010).

IL22 is the only cytokine produced by immune cells and acts only to non-haematopoietic such as epithelial cells, keratinocytes and fibroblasts cells (Wolk *et al*., 2004, Wolk and Sabat, 2006). IL22 is a members of IL10 cytokine family and acts via a heterodimeric receptor involving IL-22R and IL-10Rb (Wolk and Sabat, 2006). IL-22R is mainly present in epithelial cells, this provides IL22 with the unique ability of signalling specially to tissues (Zenewicz and Flavell, 2011). IL22 plays an important role in the response of tissue during inflammation through the activation of STAT3-signaling cascade. STAT3 is a transcription factor activated by many cytokines and growth factors. The activated STAT3 translocates to the nucleus and regulates genes involved in proliferation, migration, survival and apoptosis, depending on the cell type (Pickert *et al*., 2009, Zenewicz and Flavell, 2011) . It was reported that IL22 is able to promote anti-apoptotic pathways such

as STAT3, Akt and mitogen-activated protein kinase pathways and anti-microbial molecules such as b-defensin, Reg3c, lipocalin-2. These are important to prevent tissue damage and help with tissue repair (Zenewicz and Flavell, 2011). Very similar to the production process of IL-17, the production of IL22 from Th17 cells required IL23 and IL-1 (Marijnissen *et al*., 2011).

The function of IL-1 signaling for the immune system is still unclear. In this project the role of IL-1 was investigated using three different transgenic mice, global knockout mice IL-1RI, IL-1RII and conditional IL1-RI knockout in hematopoietic cells IL-1RI*vaviCre* mice. Gastrointestinal helminth *T.muris* was used as an infection model in the study. The infected IL-1RII, IL1-RI and IL1-RI*vaviCre* mice were analysed to investigate the role of IL-1 signaling for the immune defense and in the development of the Th lineages Th1, Th2 and Th17.

1.2 TRANSGENIC MICE

1.2.1 Generation of global knockout mice

Laboratory mice have an important role in the study of the human disease mechanisms (Austin *et al*., 2004). The murine models are an ideal organism in the study of human biology and disease due to a high level of homology between mice and human genomes (Gregory *et al*., 2002). The introduction of specified gene sequences into mammalian embryos is a useful technique in studying problems in gene regulation and cell differentiation. The first introduction of foreign DNA into the mouse was described in 1980 by Gordon *et al* (Gordon *et al*., 1980). The following study was generated by Brinster and Palmiter in 1984; in this study, mouse metallothionein I promoter-regulator was fused with a rat or human growth hormone structural genes and was introduced into mice. Offspring containing the new gene construction grew quickly, and they observed a large difference in growth rate when compared with wild mice, indicating that the gene was expressed successfully (Brinster and Palmiter, 1984).

In 1978 Thomas and Capecchi described a new method, which allowed inactivation of a specific locus within the mouse genome. In this method the chosen gene was inactivated by replacing the target gene fragment with a mutant version of this segment using homologous recombination in embryonic stem (ES) cells. Homologous recombination is a rare event in mammalian cells. Thus, the addition of the selective gene neomycin resistance in the targeted locus of the ES cell is required. The expression of neomycin resistance gene (neo gene) allows for the selection of the isolated mutant ES cells, as well as inactivating the predetermined gene (Thomas and Capecchi, 1987, Rajewsky *et al*., 1996). In order to generate germ-line chimaeras the microinjection of mutant ES cells into mouse blastocysts was performed (Bradley *et al*., 1984). The interbreeding of heterozygous mice produced homozygous mice with the desired mutation. This classical technique for gene targeting leads to the generation of global knockout mice. The global knockout mutation may be lethal to mice if the gene is involved in development. Moreover, global knockout mice may show complex phenotypes if the gene is expressed in different types of cells. Global knockout mice may show no phenotype due to compensation for the loss of gene function with another gene related (Rajewsky *et al*., 1996, de Boer *et al*., 2003). Moreover, the expression of an active selection marker gene in the targeted locus in the global knockout mice may affect the mutant phenotype (Rajewsky *et al*., 1996). Therefore, the generation of conditional knockout mice in which the specified gene is inactivated in a specified cell type or tissue is required to avoid these problems.

1.2.2 Generation of conditional knockout mice

The most common system that has been used for over 15 years for conditional gene targeting is the Cre-loxP system. This is widely used in biomedical and immunological research (Rajewsky *et al*., 1996, Schmidt-Supprian and Rajewsky, 2007). The first study that was performed in order to generate conditional gene targeting using the Cre-loxP system was in 1994 by Gu *et al*. In this study it was possible to delete the promoter and the first exon of the DNA polymerase beta gene in murine T-cells using bacteriophage directed

site-specific Cre recombinase. However this lead to a lethal phenotype (Gu *et al*., 1994) . Using Cre-loxP system recombination in mouse cells *in vitro* and *in vivo* has become a favoured approach because it offers a wide range of applications in gene targeting (Rajewsky *et al*., 1996). Cre-loxP system recombination consists of two main elements: the Cre recombinase enzyme and the loxP site from bacteriophage (locus of crossover (x) in P1). LoxP sites in a specific DNA sequence can be recognised by the genetic recombination enzyme Cre. The DNA sequence loxP site consist of a 34-bp sequence, this sequence divides into two 13-bp reversed repeats flanked by an 8-bp sequence (figure 3) (Kwan, 2002). The Cre recombinase enzyme excised the flanked DNA segment by two loxP sites. This leads to modification in the DNA sequence such as insertion, inversion, deletion, or translocation according to the location and orientation of the recognition site (figure 1- 3) (Abremski and Hoess, 1984, Rajewsky *et al*., 1996, Kwan, 2002).

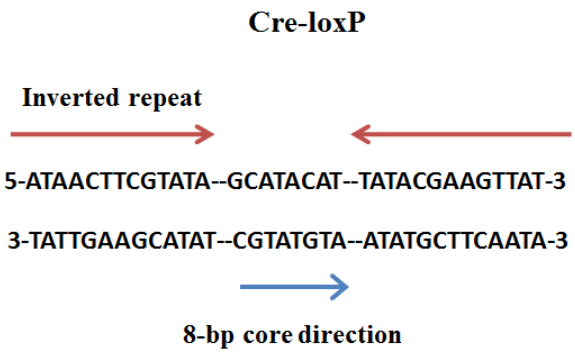


Figure 1-3: The structure of Cer-loxP: The DNA sequence loxP site consists of 34-bp sequence, this sequence is divided into two 13-bp reversed repeats flanked by an 8-bp sequence which determines the orientation of the recognition site. Adapted from(Kwan, 2002)

One of the most common strategies performed in the generation of a conditional knockout mouse is to delete the gene of interest using a targeting vector which contains 3-loxP. The targeting vector used to generate the loxP contains the selectable marker gene neo resistance gene. In this technique two of the loxP sequences flank the neo resistance gene and the third one is used to delete the exon after Cre recombination (Rajewsky *et al*., 1996, Kwan, 2002). Conditional knockout mice can be achieved by crossing mice containing the floxed target gene with the deleted selectable marker with mice expressing Cre in a

specific cell type (Gu *et al*., 1994, Rajewsky *et al*., 1996). Hence, the new offspring contain both the loxP flanked gene and Cre transgene. Moreover, the gene will only be excised in the tissue expressing Cre recombinase. There are a large number of mouse lines able to express Cre recombinase in different types of cells (Nagy *et al*., 2009). Thus, multiple conditional knockout mice can be generated through crossing floxed mice with the appropriate Cre lines. Figure 1-4 demonstrated the generation of conditional knockout mice using a 3-loxP site strategy.

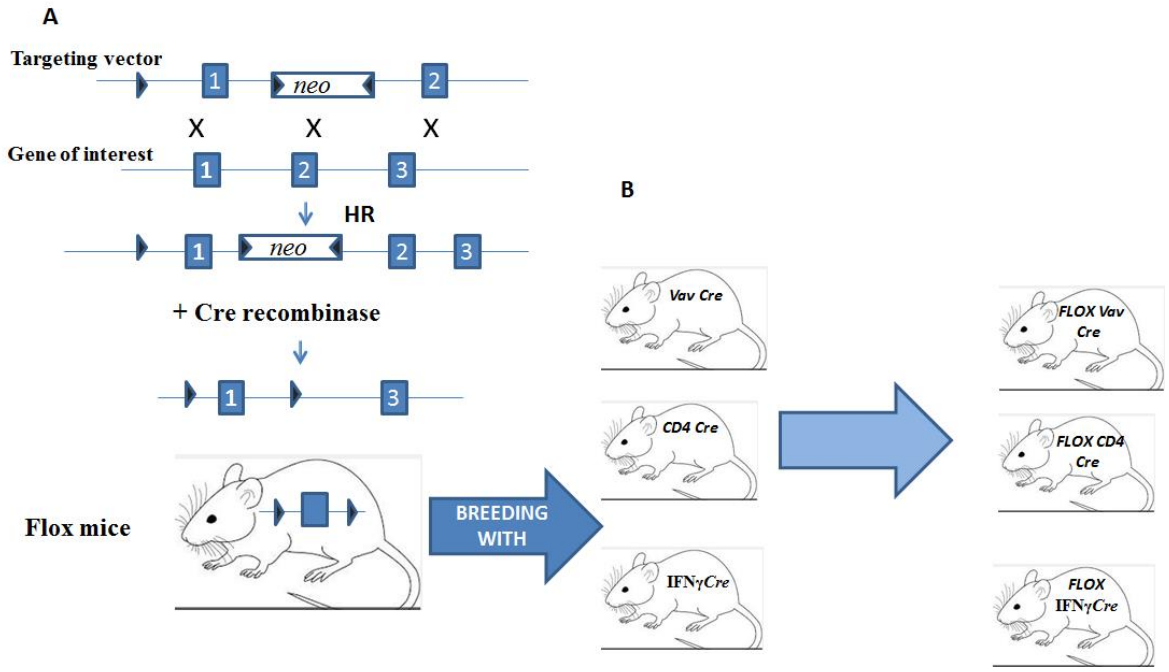


Figure 1-4: Generation conditional knockout mice using 3-loxP site strategy: (A) the gene targeting vectors contain 3 loxP sites, two of them flanking the selective gene neo gene and the third one used for exon deletion after the Cre recombination. The homologous recombination (HR) between the gene of interest in the genome of ES cells and targeting vectors is leading to genomic locus being modified. In these processes Cre recombinase deletes the flanking neo gene. The mouse line has been generated containing loxP site from modified ES cells. (B) Variety of Cre transgenic mice with different specialities can be used to generate mice with a conditional gene targeting by breeding them with flox mice. Adapted from (Rajewsky *et al*., 1996)

Studies performed on knockout interferon α/β showed that 100% deletion of loxP-flanked gene segment had been achieved in the liver and 98% in the lymphocytes upon infection. Few deletions were observed in other tissue. Thus, there is a variation in the response to Cre-loxP recombination in different cell type. However, efficiency of the Cre-loxP system can reach 100% in some cell types (Rajewsky *et al*., 1996).

1.2.3 *VavCre* conditional knockout mice

As mentioned previously the Cre-loxP systems can be used to create genomic modified mice, leading to the generation of conditional mouse mutants in specific tissues or cells (de Boer *et al*., 2003). Several mouse lines were reported to express Cre recombinase transgenes in specific hematopoietic cell subsets such as *LysMCre* macrophages and granulocytes, *CD19-Cre* B-lymphocytes and *CD4-Cre* T-lymphocytes (Clausen *et al*., 1999, Rickert *et al*., 1997, Aghajani *et al*., 2012). *VavCre* transgenic mice have been generated to inactivate genes in all hematopoietic cells and endothelial cells (Georgiades *et al*., 2002). It is known that the *vav* gene is expressed and regulates all hematopoietic and endothelial cells in adult and foetal mice (Georgiades *et al*., 2002). In these mice Cre recombinase expression was carried out using *vav* regulatory elements. Moreover, the element promoter of the *vav* gene was shown to be the driver of expression of the transgene, efficiently and exclusively, in all nucleated hematopoietic and endothelial cells (Georgiades *et al*., 2002) A study reported that *vav* gene regulatory sequences contain DNase I hypersensitive (HS) site, which is important for β –galactosidase (Bcl-2) activity. Furthermore, *vav* DNA sequences have been used to direct the expression of hematopoietic cells of Bcl-2 and two reporter transgenes (Ogilvy *et al*., 1998). In order to generate *vavCre* conditional knockout mice, *vav* regulatory sequence was ligated to the bacteriophage P1 Cre-recombinase cDNA to create the plasmid p*vavCre.* Then, two different transgenic mice *vavCre1* and *vavCre8* were generated using the p*vavCre* plasmid. Both mice were bred with R26R mice, which contain the lacZ reporter gene, to examine the level of *vavCre*-mediated recombination. LacZ reporter gene was expressed only when Cre recombination expression occurred. In the offspring, the Cre recombinase expression occurred in most hematopoietic and endothelial cells produced from the mice, confirming the mice were *VavCre/R26R* double heterozygous. This was confirmed using flow cytometry and histochemistry for Bcl-2, which plays an important role in the expression of hematopoietic endothelial cells (Georgiades *et al*., 2002).

In 2003 de Boer *et al* showed two differences in *vavCre* transgenic mice, they called them *vaviCre* and *hCD2iCre*. *VaviCre* mice expressing the codon improved *Cre (iCre)* under the control of *vav* promoter. *HCD2iCre* mice expressed *iCre* under the control of hCD2 promoter (de Boer *et al*., 2003). It was reported that the expression of *vav* cre vector (HS21/45 *vav*-hCD4) were able to drive the expression of a hCD4 transgene throughout hematopoietic cells and affects other cells such as endothelial cells (Georgiades *et al*., 2002, de Boer *et al*., 2003). Thus, in this study hCD4 gene was replaced with the *iCre* cDNA in the *vav* expression vector in order to limit conditional targeting to the hematopoietic system (de Boer *et al*., 2003). Moreover, *iCre* cDNA was also inserted into the hCD2 VA vector which has been shown to drive high expression of transgene specifically in T and B cells. The activity of the Cre at single-cell level was determined in *vaviCre* and *hCD2iCre* lines by crossing them with R26R-EYFP Cre reporter mouse. The R26R-EYFP reporter mouse has an EYFP transgene inserted into the ROSA26 locus by homologous recombination. EYFP is expressed only when a floxed transcriptional cannot sequence which inhibits transcription from the ROSA26. It was shown that *vav* promoter elements were capable to direct Cre-mediated recombination in all hematopoietic cells. While, hCD2 promoter and locus control region (LCR) were able to direct the expression of Cre recombinase to T and B cells only (de Boer *et al*., 2003). Thus, in this study *vaviCre* mouse was used to obliterate *Il1-rI* gene in all hematopoietic cells.

1.3 INFECTION MOUSE MODEL

1.3.1 *Trichuris trichiura*

Trichuris trichiura (T.trichiura) is one of the most prevalent human parasites in the population of human hosts. Studies show that *T.trichiura* is widely distributed worldwide, especially in Southeast Asia, Central Africa and southern India, with around 700-800 million people infected (de Silva *et al.*, 2003). Infection with *T.trichiura* is rarely fatal. However high levels of the infectious disease are related to *T.trichiura* (Stephenson *et al*.,

2000). Infection with *T.trichiura* usually coincides with other nematode infections such as *lumbricoides* (Anderson, 1986). A study reported that children are more likely to be infected with *T.trichiura* than adults, with infection reaching 95% in several parts of the world. Trichuris dysentery syndrome (TDS) occurs due to a heavy *T.trichiura* infection, resulting in rectal prolapse, anaemia, chronic dysentery and clubbing of the fingers (Stephenson *et al*., 2000).

There is no treatment or vaccine that can be used to provide immunity to helminth parasites. The available anti-helminthics treatment depends on post-infection treatment. Anthelmintics drug such as mebendazole and albendazole have a toxic effect to the cytoskeleton in the parasite (Abbas and Newsholme, 2009). A study has shown that anthelmintics drugs have a weak effect when they have been used for *T.trichiura.* Following treatment with a single oral dose of albendazole, the cure rate for infection with *T.trichiura* was 28% (Keiser and Utzinger, 2008). Therefore, further investigations need to be done to understand the role of the immune system in *T.trichiura* infections to provide a suitable vaccine and improved antihelminthic therapy.

1.3.2 *Trichuris muris*

Trichuris muris (*T.muris*) is a gastrointestinal helminth occurring in mice naturally. It has been used as a model to investigate human gut parasite *Trichuris trichiura (T.trichiura)* in order to understand the immune response to parasitic infection (Roach *et al*., 1988). Using *T.muris* provides useful information on the immune response to *T.trichiura* (Cliffe *et al*., 2005, Liu *et al*., 2006).

T. muris is a helminthic whipworm that infects the colon and the caecum of mice. In the large intestines of mice, adult females released the unembryonated eggs and then pass out in the mice faeces. Within 4-10 weeks, the unembryonated eggs become embryonated, mature and infective. The infective eggs stage is larvae L1, and the infective effect can remain successfully for a long time (Deschoolmeester and Else, 2002). *T.muris* life cycle starts by the invasion of *T.muris* infective eggs through the faecal-oral route of the animal

host. At 90 minutes post invasion, the eggs hatch in the cecum and release larvae, which in turn migrate up the epithelium of the colon (Deschoolmeester and Else, 2002). In this stage bacteria, for example *Eschericha coli*, *require* an optimum temperature (37°C) and pH 7 to provide the most favourable conditions for enhanced egg hatching (Hayes *et al*., 2010). The use of antibiotics can lead to a reduced worm number by inhibiting hatching (Hayes *et al*., 2010). The presence of large numbers of bacteria in the caecum explains why the highest worm burden is found in this part of the colon. The larvae then immediately penetrate the mucosal tissue of the large intestine and move to the crypt. They also penetrate the base of the caecal crypts by secreting enzymes (Klementowicz *et al*., 2012, Cliffe and Grencis, 2004). The migrations of larvae through epithelial cells leads to the formation of syncytial tunnel (Tilney *et al*., 2005). The larvae moult undergoes a number of stages at around days 9-11, 20-21 and day 28 post infections (p.i.), before becoming adult by day 35 p.i. (Klementowicz *et al*., 2012, Cliffe and Grencis, 2004). Figures 1-5 demonstrated *T.muris* life cycles.

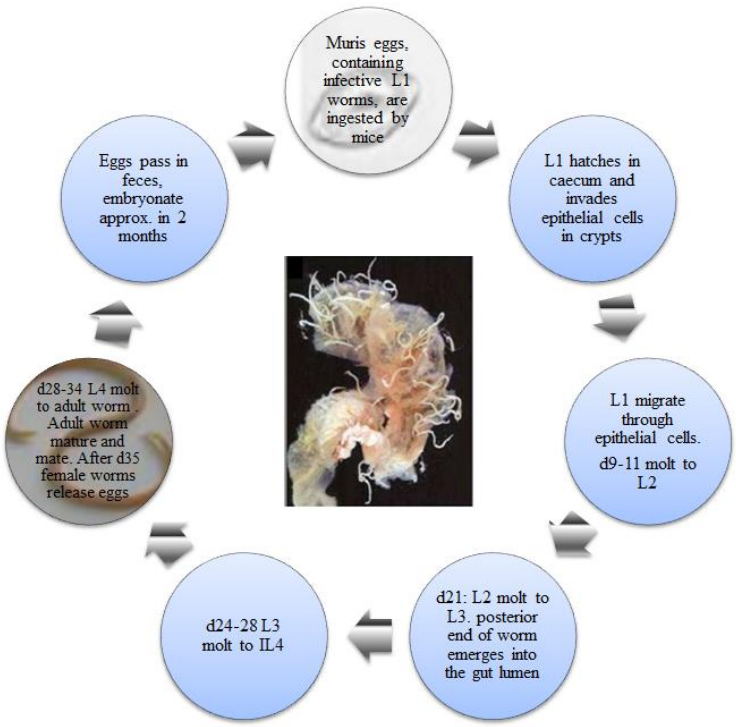


Figure 1-5: *T. muris* life cycle. Infective embryonated eggs, ingested by mice using gavage. Within 90 minutes eggs hatch in the caecum. The larvae then moult through many stages at around days 10, 20 and 28 post infections (p.i.) before becoming adult at day 35 p.i. The adult females release the eggs in the caecum and pass out the of mice in faeces.

The infection of mice with *T.muris* is a powerful tool to examine cytokine-mediated immunity. Moreover, worm expulsion is dependent on the mice strain. Most mice are able to expel the worms, while some mice strains such as AKR and B10.BR are unable to due to a susceptible immune response. NIH mice are the strain most resistant to *T.muris*, as it expels all worms by day 12 p.i. All BALB mice clear the infection between day 12 and day 21 p.i. C57BL6 and C57BL10 have a weak response to *T.muris* and worms can be found around day 27 p.i (Deschoolmeester and Else, 2002). By the study of these strains, much has been discovered about cell regulatory mechanisms which control the cell development causing chronic and acute infection. Acute infection was obtained when resistant mice were infected with *T.muris*. Resistant mice were able to expel the worms due to the development of the Th2 response. In susceptible mice infected with *T-muris*, chronic infection developed. Susceptible mice generated a Th1 response and failed to generate Th2. They were therefore unable to expel the worms (Else and Grencis, 1991, Else and Grencis, 1996).

1.3.3. *T.muris* encourage CD4 T-helper subset polarisation

The type CD4+ T-helper cell plays an important role in response to *T.muris* infection (Deschoolmeester and Else, 2002, Helmby and Grencis, 2003). The role of T-helper cells, in response to *T. muris* infections, was first investigated in 1983 by Lee *et al.* In this study a significant increase was observed in the T cell numbers of mesenteric lymph node (MLN), which transferred immunity in infected mice with *T.muris*. This indicated that T-cells play a crucial role in mediating inflammatory responses to this parasite (Lee *et al*., 1983). This data was confirmed by a study performed in 1991 by Ito Yoichi using nude mice. The study maintained *T.muris* infections and found that lymphoid cell transfer can be used to restore resistance to the parasite (Yoichi, 1991). Another study performed in 1995 by Koyama *et al.* showed that CD4+ T-cells are essential for the expulsion of *T.muris*. In this study mice were treated with monoclonal antibodies to either CD4 or CD8. It was found that CD4 T-cells, but not CD8 T-cells, were important for resistance to *T.muris*

(Koyama *et al*., 1995). In 1996 Else and Grencis performed a study that showed adoptive transfer of CD4 T-cells to the susceptible SCID mice conferred resistance to the *T.muris* leading to worm expulsion.

Moreover, CD4 T-cells were demonstrated to work against the larval stages. Thus, worm expulsion occurred before the worm reached the adult stage (Else and Grencis, 1996). Moreover, the presence of the CD4 T in the gut is important for *T.muris* expulsion (Deschoolmeester and Else, 2002, Cliffe and Grencis, 2004).

It is well known that the T-helper cell type 2 (Th2) responses are associated with host resistance and that the T-helper cells type 1 (Th1) responses are associated with host susceptibility to infection. Due to the Th2 responses, which involve expression of high levels of IL-4, IL-5, IL-9, IL-13 and a parasite-specific immunoglobulin G1 (IgG1) in resistance mice such as BALB/K, BALB/c are able to expel the worm. Conversely, susceptible mice strains such as AKR mount a Th1-type response with production of a high level of IFNγ, IL-18 and IL-12 and of parasite-specific immunoglobulin G2a (IgG2a) (Else and Grencis, 1991, Else and Grencis, 1996). The first study to describe the role of Th subset in *T.muris* infection was in 1991. It was found that IFNγ expression was high and IL5 expression was low in susceptible mice, with the converse expression in resistant mice (Else and Grencis, 1991).

It was shown that resistant mice, which mount a Th2 in response to *T.muris* infection, were able to generate a Th1 response and develop a chronic infection if the mice were infected with a low egg count of the *T.muris.* In this study Th2 cytokines; IL-4, IL-5, IL-9 and IgG1 were significantly decreased, while Th1 cytokines, IFNγ and IgG2 were significantly increased when the resistant BALB/K mice had been infected with a low dose of *T. muris* eggs (Bancroft *et al*., 1994). Thus, the change in the *T.muris* infection dose determines whether an acute or chronic infection occurs in the resistant mice such as C57BL/6, BALB/K and BALB/c. It was shown that a high dose infection with 100-200 eggs in C57BL/6 mice, developed an acute infection Th2 response, and the mice were able to

expel the worm. In a low dose infection with fewer that than 60 eggs, the mice developed a chronic infection Th1 response and the mice were unable to expel the worm (Bancroft *et al*., 2001).

1.3.3.1 T-helper cells type 1

The role of Th1 response in susceptible mice has been investigated. In normally susceptible mice the depletion of IFNγ caused the expulsion of *T.muris*, indicating that IFNγ played a crucial role in the establishment of chronic *T. muris* infections (Else *et al*., 1994, Grencis, 2001). The role of IFNγ in *T.muris* infections was compared between BALB/K resistant mice and B10.BR susceptible mice. In this study the MLN were restimulated *in vitro* with concanavalin A (Con A). This found that BALB/K mice showed low levels of IFNγ, while B10.BR showed high levels of IFNγ (Else *et al.*, 1992). The production of IFNγ during the infection of susceptible animals with T. *muris* is increased and this is important for the development of the chronic infection (Else *et al*., 1992). Infected IFNγ KO and IFNγ receptor KO mice with *T. muris* showed that absence of IFNγ or its receptors leads to an early production of Th2 response and quick worm expulsion compared to the wild type control (Grencis, 2001).

Moreover, through the use of IL-18 KO mice, it was found that IL-18 plays a crucial role in promoting Th1 response through down-regulation of the Th2 response via IL-13 and IL-4 reduction (Helmby *et al*., 2001). Additionally, IL-18 KO mice showed that the expression of the IFNγ in response to *T.muris* infections were similar in the IL-18 KO and wild type mice (Helmby *et al*., 2001). The importance of IL-18 in Th1 response was shown by the presence of IL-18 mRNA in the intestines of susceptible mice infected with *T.muris* (Helmby *et al*., 2001). Caspase-1 is required to convert pre-IL-18 to the bioactive form of IL-18. IL-18 protein correlated with its receptors and was quantifying in the lamina propria of the large intestine (Helmby *et al*., 2001).

Furthermore, IL-12 was reported as induction for the production of IFNγ, thus IL-12 is required for a strong Th1 response, determined by the treatment of resistant BALB/K mice with recombinant IL-12, resulting in generation of Th1 response (Bancroft *et al.*, 1997).

IL-27 is a member of the IL-12 family of cytokines and has similar biological activity to IL-12. It requires WSX-1 cytokine receptor for its function. IL-27 was reported to promote the Th1 response in mice susceptible to infection. WSX-1 KO mice showed that during the *T.muris* infection the interaction of IL-27/WSX-1 was increased in the susceptible AKR mice compared with the WSX-1 KO mice, which showed a resistance phenotype. This resistance occurred due to a decrease in the Th1 immune response and up-regulation in the Th2 immune response (Bancroft *et al*., 2004).

1.3.3.2 T-helper cells type 2

Th2 cytokines have been reported to play a critical role in the host's protection against *T. muris*. IL-4 is the key cytokine of the Th2 response. The blocking of IL-4 function using monoclonal antibody against IL-4 receptor (IL-4R) in resistant mice prevents the production of a Th2 response and generates a Th1 response. The IL-4 blocked mice showed a high level of IFNγ and IgG2 and a low level of IgG1. On the contrary, the treatment of susceptible mice with IL-4 complex (IL-4 and anti-IL-4 mAB) early in infection the leads to the generation of a Th2 response in resulting worm explosion (Else *et al*., 1994). Another study was performed to confirm this result using IL4 KO mice (C57BL/6 background). They found that these mice were susceptible to *T.muris* infection and that low levels of IL-5, IL-9, IL-13 and IgG1 and high levels of IFNγ and IgG2 were observed (Bancroft and Grencis, 1998). A study was reported that the sex and background of the IL-4 KO mice was important in the mice phenotype following the infection. It was shown that female IL-4 KO mice on BALB/c were resistant to infection, similar to the wild type control mice and produced high levels of IL-13 cytokine, which was important for the Th2 response (Bancroft *et al*., 2000).

In addition to IL-4, IL-13 has been reported as an important cytokine to generate Th2 response. IL-13 KO mice showed that by generating a Th2 response through the production of IL-4, IL-5 and IL-9, the mice were unable to expel the worm and showed high levels of IgG2 which led to the development of a chronic infection (Bancroft and Grencis, 1998). In order to verify the previous study, another experiment was generated using a soluble IL-13 receptor fusion protein to block the IL-13 action *in vivo.* In this study, female IL4 KO mouse, which showed resistance phenotype due to the high production of IL-13, were treated with a soluble IL-13 receptor fusion protein. This resulted in the failure of mice expel the worms (Bancroft *et al*., 2000).

Furthermore, another cytokine important for Th2 response to *T.muris* infection is IL-9 and a high level of IL-9 production was observed in resistant mice (Else *et al*., 1992). IL-9 is expressed from T-cells and non-T-cells in athymic mice that lack a thymus gland (Svetic *et al*., 1993). IL-9 was investigated by stimulating isolated MLNs from resistant and susceptible mice infected with *T.muris*. The resistant mice produced IL-9 early at day 4 pi, with no IL4 expression by this time point. The susceptible mice did not show an increased IL-9 production at any point, indicating that IL-9 is important in the generation of Th2 response. Moreover, susceptible mice treated with recombinant IL-9 resulted in reduced worm growth. A significant increase in the mast cells number was observed compared with untreated mice at day 34 pi. It was also shown that IL9 caused an elevation in the production of IgG1 (Faulkner *et al*., 1998). Moreover, the importance of IL9 was examined by injecting susceptible animals with IL-9 secreting cell. This resulted in a 50% reduction in the worm count in the injected mice (Faulkner *et al*., 1998).

One of the most important cytokines in the Th2 responses to antigen is IL-10 (Laouini *et al*., 2003). IL-10 has been reported as an important cytokine for the host's resistant survival during infection with *T.muris* (Schopf *et al*., 2002). In this study IL-10, IL-10/IL-4 or IL-10/IL-12 KO mice were examined. Both IL-10 and IL-10/IL-4 KO were susceptible to infection and produced high levels of the Th1 cytokine IFNγ and TNFα. While, IL-

10/IL-12 KO mice were resistant to the infection and produced a high level of Th2 cytokines IL-4, IL-5 and IL-13. This indicated that IL-12 is important for the infection susceptibility of IL-10 KO mice. Moreover, IL-10 and IL-10/IL-4 KO mice presented with intestinal inflammation, which was observed by a reduction in mucus production and mucosal ulceration. These mice did not survive more than 25 days pi. Treatment with antibiotics reduced the mortality and improved mucus production in the double KO IL-10/IL-4 but not IL-10 KO mice. This shows that outgrowth of opportunistic bacteria activated by *T.muris* larvae caused intestinal damage, which played a critical role in morbidity and mortality in the IL-10 KO mice (Schopf *et al*., 2002).

IL-25 has been reported as members of IL-17 cytokine. It is produced by active Th2 cells. IL-25 plays an important role in the generation of the Th2 response to the *T.muris* infection. A study using infected IL-25 KO mice has shown that these mice fail to produce Th2 responses and clear the infection. Moreover, treatment of the susceptible AKR mouse with exogenous IL-25 leads to the development of a Th2 response and resulting worm expulsion (Owyang *et al*., 2006).

1.3.3.3 T-helper cells type 17

In 2010 Levison et al. reported the up-regulation of Th17 in chronic *T.muris* infections, using *T.muris* infection in AKR mice (Levison *et al*., 2010). As mentioned before, IL-25 KO mice were unable to generate Th2 response and clear the infection. Thus, IL-25 KO was able to generate Th1 and develop chronic infection. IL25 KO showed high levels of IFN-γ and IL-17 indicating that Th17 is generated in chronic infections (Owyang *et al*., 2006). Moreover, IL-10 KO mice are highly susceptible to the *T-muris* infection. This showed that IL-10 KO mice produced a mixed Th1 and Th17 response to infection and showed strong inflammation in the cecum (Fasnacht *et al*., 2009).

A recent study has shown that IL-22 is a member of Th-17 family of cytokine, and is required for goblet cell activity, which plays a crucial role in worm expulsion. Although IL-22 KO mice were able to produced Th2 cytokines, the mice failed to expel the worms

(Turner *et al*., 2013). A very recent study in the Else lab showed that in the high dose infection of C57BL6 with *T.muris*, low levels of the IL-17A were expressed (Else, unpublished).

1.3.4 The mechanisms of *T.muris* worm explosion

1.3.4.1 Epithelial cell turnover

Crypt hyperplasia accompanied by epithelial cell proliferation has been associated with a chronic *T.muris* infection (Cliffe *et al*., 2007). IFNγ is an important cytokine in the induction of epithelial cell proliferation during infection. IFNγ is able to control the excessive crypt elongation during the chronic infection (Cliffe *et al*., 2005, Cliffe *et al*., 2007). In an acute infection, resistant mice have a mechanism that leads to fast worm expulsion, which is epithelial cell turnover acceleration. This mechanism is also known as epithelial escalator, where epithelial cells are removed by moving from the bottom to the top of the crypt. This fast epithelial cell turnover leads to the transfer of the embedded parasite in the epithelial layer to the top of the crypt and subsequent expulsion (Cliffe *et al*., 2005). Studies performed using IL-13 and IL-4 KO mice have shown that IL-13 not IL4 is important for acceleration in epithelial cell turnover (Bancroft et al., 2000, Cliffe *et al*., 2005).

In chronically infected susceptible AKR mice, *T.muris* promotes its own survival by inducing the production of IFNγ, which direct epithelial cell proliferation and induces the production of CXCL10 chemokine. This leads to a reduced cell escalator to the crypt. This encourages crypt cell hyperplasia and elevates the epithelial niche that the parasite inhabits (Cliffe *et al*., 2005). The difference between the susceptible and resistance mice in epithelial cell turnover is due to the difference in their immune response. Thus, the expression of Th1 and Th2 cytokine are associated with increased or decreased speed of epithelial turnover respectively (Klementowicz *et al*., 2012).

1.3.4.2 Goblet cells

Goblet cells have been reported as major secretors of mucus. The mucus barrier is an important part of the innate immune system, which protects the intestinal epithelium. Goblet cell hyperplasia is present during infection with *T.muris* in both susceptible and resistant mice (Artis *et al.*, 2004, Hasnain et al., 2010). Up-regulation of mucin (Muc2) the main component of mucus expression in the caecum is associated with worm expulsion and only observed in resistant mice. Studies in Muc2 KO mice have shown a significant delay in worm expulsion despite no change being observed in the immune response. Another study used Muc2 KO mice showed that IL-22/STAT3 signalling play an essential role in the maintenance of homeostasis in the mouse ileum by regulate the innate immunity defence genes such as Fut2, Reg3b, Reg3g, Relmb when the mucus barrier is lost (Sovran *et al*., 2015). This indicates that Muc2 is an important component for the immune response to *T-muris* infection in the gut and this function is independent of the immune system (Hasnain *et al*., 2010).

1. 4 IL -1 AND *T.MURIS*

The first study investigating the role of IL-1 in the development of Th2 response in the *T.muris* infection model was in 2004 by Helena Helmby and Richard K. Grencis. It was found that naïve T-cells from both IL-1α null and IL-1β null mice were unable *in vitro* to polarise into Th2 cells, as characterized by secretion of IL-4, IL-13 and IL-9. This data indicated that both IL-1α and IL-1β play essential roles in Th2 *in vitro* cell polarisation. To investigate whether this data also applies *in vivo,* IL-1α or IL-1β null mice were infected with a high dose of *T.muris* eggs, which induced a Th2 response. It was found that both IL-1α null and IL-1β null mice were unable to produce an effective Th2 response, with low levels of IL-4, IL-13 and IL-9 and therefore failed to expel the parasite (Helmby and Grencis, 2004). Another study performed by Neil E. Humphreys and Richard K. Grencis in 2009 demonstrated that IL-1α or IL-1β null mice were able to polarise Th2 but required IL-1 for cytokine secretion. It also showed that the signalling receptor for IL-1 (IL1-RI) and

IL1-RAcP were not necessary for the differentiation of Th2 or cytokine secretion (Humphreys and Grencis, 2009).

1.5 THE AIMS OF THIS THESIS

The role of the IL-1 signalling in the deployment of Th cells subset, especially Th17 is well known. However, the specific cells, which are responsible for IL-1 signalling in the generation of Th cells and in the immune deface is still largely unclear. In order to develop a greater understanding and clarify the role of IL-1 signalling *in vivo* we generated the conditional knockout mice, specific for inactivation of the *Il1-rI* gene in hematopoietic cells, IL1-RI *vaviCre,* and compared it with global knockout IL1-RI (IL1-RI$^{-/-}$). Moreover, IL1-RII is an inhibitor for IL1-RI, thus the comparison between IL1-RI $^{-/-}$ and IL1-RII $^{-/-}$ global knockout mice are a powerful method used to understand the role of IL-1 signalling in the immune system. In this study gastrointestinal helminth *T.muris* has been used as an infection model to study gut inflammation in the mouse mutant. Low and high doses of *T.muris* have been used to generate different immune responses Th1, Th17 and Th2 respectively. Th1, Th2 and Th17 cells have been evaluated according to their related cytokines. Moreover, different approaches such as worm burden, antibody response, cytokine responses and pathology of the mice have been analysed to study the role of IL-1signalling *in vivo*.

Aims of each chapter:

1- To generate conditional knockout mice IL1-RI *vaviCre* and confirmation of IL1-RI inactivation at the genome and protein level
2- To investigate the difference between IL1-RI *vaviCre+*, IL1-RI *vaviCre-* and IL1- RI $^{-/-}$ mice in lymphocyte development using flow cytometry.
3- To examine the role of the IL-1signalling in hematopoietic cells in T-cell functions and differentiation using low and high dose *T.muris* infections *in vivo* studies.
4- To demonstrate the relevance of IL-1 signalling in T-cell functions and differentiation in *vivo* studies using infected IL1-RII $^{-/-}$ mice that are challenged with low and high dose of *T.muris*.

CHAPTER TWO: MATERIAL AND METHOD

2.1 ANIMALS

Table 2.1 shows an overview of all the mice strains used in this thesis. IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice were generated by TaconicArtemis in Cologne, Germany. IL22 $^{-/-}$ mice were generated by Jean-Christophe Renauld at the Ludwig Institute of Cancer Research in Brussels, Belgium, and provided from Prof Richard Grencis's lab at the University of Manchester. IFNγ-R*vaviCre* mice were generated in Prof Werner Muller's labs at the University of Manchester in 2011.

In the Biological Services Unit at the University of Manchester all mice were maintained in individually ventilated cages. In accordance with the Animals (scientific procedures) Act 1986, the mice were euthanized by schedule 1 methods. All experiments performed in this project were approved by a Project Licence obtained from the Home Office (U.K.) and in accordance with University of Manchester guidelines

Mice strain	Functional abberation
C57BL/6J	Wildtype
IL1-RI ^{flox} / ^{flox}	IL1-RI flox
IL1-RII ^{flox} / ^{flox}	IL1-RII flox
IL1-RI $^{-/-}$	IL1-RI global knockout
IL1-RII $^{-/-}$	IL1-RI global knockout
IL1-RI *vaviCre*	IL1-RI Conditional knockout , *i*Cre expression in all hematopoietic cells
IFNγ-R*vaviCre*	IFNγR Conditional knockout, *i*Cre expression in all hematopoietic cells
IL22 $^{-/-}$	IL22 global knockout

Table 2.1 Mouse strains used for experiments and breeding. IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice were imported from TaconicArtemis in Cologne, Germany. IL22 $^{-/-}$ mice were generated by Jean-Christophe Renauld at the Ludwig Institute of Cancer Research in Brussels ,Belgium. IFNγ-R *vaviCre* mice were generated in Prof Werner Muller's lab at the University of Manchester in 2011. All mice were kept at the University of Manchester in accordance with the animals (scientific procedures) act of 1986.

2.1.1 Genotyping

The mouse ear biopsies were digested at 54°C with thermo shaker for one hour in 200µl of ear punches in PBND buffer (Appendix D) and 2µl a proteinase K (10mg/ml). After one hour the mouse ear punches were heated to 95 °C for 10 minutes and then cooled in ice for 10 minutes. After centrifugation at 13,000 rpm for 10min supernatant was transferred into a new tube and stored at 4°C for the genotyping Polymerase chain reaction (PCR).

2.1.1.1 Genotyping using PCR

PCR was used to genotype IL1-RI $^{-/-}$, IL1-RI ^{flox} / ^{flox}, IL1- RII $^{-/-}$, IL1-RII ^{flox} / ^{flox} and IL22 $^{-/-}$ mice and the different *vaviCre* line IL1-RI *vaviCre* and IFNγ-R *vaviCre* mice. All primers were ordered from Eurofins-MWG Operon (0.01µmol scale HPSF purification) and prepared to a concentration of 10pmol/µl. All primers are represented in the 5' to 3' orientation. One Taq Hot start DNA polymerase kit was used for all PCRs (BioLabs). The mastermix used in the PCR is shown in table 2.2. PCR products were run on 1% agarose gel electrophoresis and stained with SafeView (NBS Biologicals).

Volume	Component
5ul	One Taq standard reaction buffers
0.5 ul	10 mM dNTPs (Bioline)
0.5 ul	Forward primes 10pmol/ul
0.5 ul	Reverse primers 10pmol/ul
0.125 ul	One Taq Hot Start DNA Polymerase
17.375 ul	Nuclease free water
1.0 ul	Template DNA

Table 2.2 PCR Mastermix used One Taq Hot start DNA polymerase kit (BioLabs)

2.1.1.2 Detecting of IL1- RI ^{flox} / ^{flox} , IL1-RI $^{-/-}$, IL1-RII ^{flox} / ^{flox} IL1-RII $^{-/-}$, IL22 $^{-/-}$ IFNγ $^{flox/flox}$ and *vaviCre* allele.

Table 2.3 shows the primers' sequence, the size of the detected band, and the PCR programme used to detect IL1-RI $^{-/-}$, IL1-RI ^{flox} / ^{flox}, IL1-RII $^{-/-}$, IL1-RII ^{flox} / ^{flox} , IL22 $^{-/-}$, IFNγ $^{flox/flox}$ and *vaviCre* allele .

IL1-RI $^{flox/flox}$ allele

Primers	oilgos1: 2959_32	5- CTAGTCTGGTGGAACTTACATGC-3
	oilgos2: 2959_33	5- AACTGAAAGCTCAGTTGTATACAGC-3
Band size	Wt	267 bp
	Wt/flox	267bp , 432 bp
	flox/flox	432 bp
PCR programme	94°C	5 min (Denaturation)
	94°C	30 sce (Annealing temperature)
	62°C	30 sce (Elongation)
	68°C	1 min (Extension) because it is under 1000bp
	Go to 2	30 cycles
	72°C	10 min

IL1-RI $^{-/-}$ allele

Primers	oilgos1:2959_32	5- CTAGTCTGGTGGAACTTACATGC-3
	oligos4:2960_39	5- GATAAAGCAGAGCTGGAGACAGG-3
Band size	-/-	400 bp
PCR programme	95°C	5 min (Denaturation)
	95°C	30 sce (Annealing temperature)
	63°C	30 sce (Elongation)
	72°C	1.5 min (Extension) because it is under 1000bp
	Go to 2	35 cycles
	72°C	10 min

IL1-RII $^{flox/flox}$ allele

Primers	33	5- TGTCTCCATCAGACTGACTTTAGG -3
	34	5- ACCATGTCTGCCTGTTCACC -3
Size	Wt	228 bp
	Wt/flox	228 bp, 347 bp
	flox/flox	347
PCR programme	95°C 5'	5 min
	95°C	5 min (Denaturation)
	95°C	30 sce (Annealing temperature)
	63°C	30 sce (Elongation)
	72°C	1 min (Extension)
	Go to 2	35 cycles
	72°C	10 min

IL1-RII $^{-/-}$ allele

Primers	31	5- GTAGTGGGCAATCAGATGGAC -3
	34	5- ACCATGTCTGCCTGTTCACC -3
Band size	-/-	228 bp
PCR programme	95°C	5 min (Denaturation)
	95°C	30 sce (Annealing temperature)
	60°C	30 sce (Elongation)
	72°C	1 min (Extension)
	Go to 2	30 cycles
	72°C	10 min

IL22 $^{-/-}$ allele

Primers	NeoF	5-GAACAAGATGGATTGCACGCAGGTTC-3
	Neo R	5-CGATATTCGAAGCAGGCTA-3
	34	
Band size	-/-	585bp
PCR programme	95°C	5 min (Denaturation)
	95°C	30 sce (Annealing temperature)
	60°C	30 sce (Elongation)
	72°C	1 min (Extension)
	Go to 2	35 cycles
	72°C	10 min

IFNγ $^{flox/flox}$ allele

Primers	LoxP1	5’ – TGA GTT CCA AGC AAG ACA GA – 3’
	LoxPsite	5’ – AAG TTA TGG TCT GAG CTC GC – 3’
	LoxP2	5’ – CAG GGT AGA AAA GAT GTG CA – 3’
Band size	Wt	358bp
	Wt/flox	191bp, 358bp, 392bp
	flox/flox	191bp, 392bp
PCR programme	94C	5 min (Denaturation)
	94C	30 sec (Annealing temperature)
	62C	1 min (Elongation)
	68C	1 min (Extension)
	Go to 2	30 cycles
	68C	5 min

***Vav iCre* allele**

Primers	VaviCre For	5'-AGATGCCAGGACATCAGGAACCTG-3'
	VaviCre Rev	5'-ATCAGCCACACCAGACACAGAGATC-3'
Band size	VaviCre+	~200-300bp
PCR programme	94°C 5'	5 min Denaturation
	94°C	30 sce (Annealing temperature)
	56°C	30 sce (Elongation)
	68°C	1 min (Extension)
	Go to 2	30 cycles
	68	5 min
	10°C	10 min

Table 2.3 Paired primers, products band size and the PCR programme used in detecting IL1- RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, IL1-RII ^{flox} / ^{flox} ,IL1- RII $^{-/-}$, IL22$^{-/-}$ IFNγ $^{flox/flox}$ and *vaviCre* allele. All IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, IL1-RII ^{flox} / ^{flox} and IL1-RII primres were designed by TaconicArtemis in Cologne, Germany. IL22$^{-/-}$ primres was provided by Richard Grencis's lab.

2.1.1.3 Agarose gel electrophoresis

Agarose gels were prepared by heating 1% agarose (sigma) in 1x TBE (Appendix D) buffer. 6 µl of SafeView (NBS Biologicals) was added to the agarose gel. The gel was then transferred to the gel-casting tray with a comb. 5 µl of 1Kb DNA ladders (Bioline) was loaded in separate wells. 20 µl of sample/loading buffer mix (Bioline) was added to the other wells. Gels were run at 100V for 40 minutes in the presence of 1xTBE buffer. Bands were imaged using a BioView UV light (BIO DOC imaging system).

2.2 DNA SEQUENCING OF IL1-RI *vaviCre* – AND IL1-RI *vaviCre* +

2.2.1 DNA extraction from spleen cells:

Spleen cells were isolated as described below (see section 2.4.1). The spleen cells were centrifuged at 13000 rpm for 10 min. Then, the pellets were re-suspended in 300 µl 50 mM KOH and incubated in 98 °C for 45 min. After centrifugation at 13000 rpm for 10min, the supernatant was transferred to the new tube and 25µl of 1M Tris pH 8 was added to each tube and stored at 4°C for the PCR. Two set of primers were used to detect IL1-RI *vaviCre – and* IL1-RI *vaviCre* + allele. A PCR using oligos 1 and oligos 4 primers (1st primers) showed a band at approximately 1400 bp for IL1-RI *vaviCre* – (see 3.2.2.1.1). The size of the IL1-RI *vaviCre* – band was large and difficult to sequence. Therefore, another primer was designed WT F and WT R (2nd primers) using Primer 3 web page. The following table shows the sequences of the two set of primers, the size of the band detected , the PCR Master mix and the PCR programme used detected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + (Table 2.4).

IL1-RI *vaviCre* – and IL1-RI *vaviCre* +

1st primers	oilgos1: 2959_32	5- CTAGTCTGGTGGAACTTACATGC-3
	oligos4 :2960_39	5- GATAAAGCAGAGCTGGAGACAGG-3
Band size detected using 1st primers	IL1-RI *vaviCre* +	300bp
	IL1-RI *vaviCre* –	1400bp
2nd primers	WT F	5-GCCCCCTCACCTCAAATAGA-3
	WT R	5-GCCGAGCTATCCTCACACTC-3
Band size using 2nd primers	IL1-RI *vaviCre* –	650 bp
PCR programme	94°C	5 min (Denaturation)
	94°C	30 sce (Annealing temperature)
	63°C	30 sce (Elongation)
	68°C	1 min (Extension)
	Go to 2	30 cycles
	68°C	10 min

Table 2.4: Two set of primers, band size detected, and the PCR programme was used to detect IL1-RI *vaviCre – and* IL1-RI *vaviCre+*: The 1st set of primers was used to detect IL1-RI *vaviCre – and* IL1-RI *vaviCre+*. However, due to the large band size for

IL1-RI *vaviCre* – was detected when 1st primers was used, a 2nd primer was designed to amplify a smaller DNA fragment. The primers were ordered form Eurofins-MWG.

To extract DNA, gels were visualised under a UVI Tech UV transilluminator at 312nm. IL1-RI *vaviCre* – *was* detected at size ~ 400 bp and for IL1-RI *vaviCre*+ at size ~ 650bp bands were cut from the gel using a scalpel. The DNA was then extracted using the Qiagen Gel Extraction Kit protocol

2.2.2 Ligations

The extracted DNA was ligated into pGEMT – easy vector (PROMEGA) by adding 1µl of pGEMT–easy vector, 1µl of ligase buffer and 1µl of ligase enzyme to 7µl of the sample and incubated over night at 4 °C. The next day, the ligated DNA was transformed into DH 5 α *E.coli* bacteria (Invitrogen).

2.2.3 Transformation.

50µl of the DH 5 α was added to the ligated DNA and incubated for 30 minutes in ice. Then, it was heated at 42 °C for 1 minute. After that, 300 µl of SOC medium (Sigma) was added and incubated at 37°C for 1 hour. Next, the transformed bacteria was growing onto ampicillin agar plates (100µg/ml) over night at 37°C to allow the establishment of colonies

2.2.4 DNA extraction from bacteria.

From each plate one colony was incubated in 5 ml of Luria-Bertani broth (LB) containing 100µg/ml ampicillin and incubated at overnight in 37°C. The bacterial DNA was extracted the next day using Qiagen Miniprep kits.

2.2.5 DNA sequencing.

The PCR experiment was performed using and SP6 and T7 primers for each DNA extraction. The primers sequence, PCR Mastermix and the PCR programme was shown in table 2.5.

Primers	T7	5-TAATACGACTCACTA-3
	SP6	5- TATTTAGGACACT-3
PCR programme	95°C 5'	5 min (Denaturation)
	95°C	30 sce (Annealing temperature)
	63°C	30 sce (Elongation)
	72°C	1.5 min(Extension)
	Go to	35 cycles
	72°C	10 min

Table 2.5: The primer sequences and the PCR programme for detecting the extracted DNA from bacteria. The primers were ordered form Eurofins-MWG.

As shown in chapter 3 (3.2.2.1.2) the ligated DNA from IL1-RI *vaviCre* + spleen cells demonstrated a band at ~550 bp (150 from the vector + 400 from the ligated DNA). The ligated DNA from the IL1-RI *vaviCre* – spleen cells showed a band at ~ 800 bp (150 from the vector + 650 from the ligated DNA)**.** The samples plus either T7 or SP6 primers were sent for sequencing in the DNA sequencing facility at the University of Manchester.

2.3 EXPERIMENTAL MODELS

2.3.1. Infection with *Trichuris muris*

Infections with *T. muris* were performed at the University of Manchester. The mice were infected with a low dose (approx. 20 or 30 infective *T.muris* eggs) according to the infectivetiy of the embryonated egges for 21 days. The infectivetiy of the 20 embryonated egges were 75% and were 55% for the 30 embryonated egges. In high dose (approx. 200 *T.muris* eggs) were used to infected the mice for 21 days (provided by Prof. Else, University of Manchester). The eggs were counted in to the volume of 200µl and mice were infected orally by oral gavage. On day 21 post infection (p.i.), the mice were euthanized by CO_2 asphyxiation. Blood, mesenteric lymph nodes, snip of the proximal colon to the caecum and remaining colon and caecum were harvested from the mice.

Caecum and colon were kept in -20 °C for worm counts. The snip of the colon was fixed in 4% paraffin.

2.4 ISOLATION OF CELLS

2.4.1 Isolation of cells from the spleen

The spleen was removed from the mice and placed in washing buffer (Appendix D). The spleen was then homogenized being passed through a 100µm cell strainer (BD Biosciences). Centrifugation at 250x*g* for 5 minutes was then performed to pellet the cells which were treated with 3ml of erythrolysis lysing buffer (BD Pharm Lyse™, BD Biosciences) for 10 min at room temperature. The suspension was then washed by adding 10ml of washing buffer and centrifuged 250x*g* for 5 minutes. The cells were then re-suspended in complete RPMI medium, and counted using a cell counter (CASY system). In the flow cytometry experiment the cells were re-suspended in FACS buffer (Appendix D).

2.4.2 Isolation of cells from mesenteric lymph nodes (MLN)

The MLNs were harvested from mice and placed in a washing buffer (Appendix D). The lymph nodes were then homogenized by passing through a 100µm cell strainer (BD Biosciences). Centrifugation at 250x*g* for 5 minutes was then preformed to pellet the cells. The suspension was then washed with a washing buffer and re-suspended in complete RPMI (Appendix D) and counted using a cell counter (CASY system). In the flow cytometry experiment the cells were re-suspended in FACS buffer (Appendix D).

2.4.3 Isolation of cells from Bone marrow

The tibia and femur were removed from the mice and placed in a washing buffer (Appendix D). The tissues were then removed from the bones. The ends of the bones were trimmed, and the bone marrow was flushed out using a 5 mL syringe with washing buffer into a petri dish. The cells were then pushed through a 100µm cell strainer. After centrifugation at 250x*g* for 5 minutes, the cell pellet was re-suspended in an erythrocyte

lysis buffer and incubated for 10 minutes at room temperature. 10ml of washing buffer was added and then centrifuged 250x*g* for 5 minutes. Cells were then re-suspended in an FACS buffer and counted using a cell counter (CASY system).

2.5 COLLECTION OF CELLS AND SERUM FROM BLOOD

Blood was collected directly from the heart upon necropsy. The blood was incubated for 1 hour at 37°C. The blood was then centrifuged at 5,000x*g* for 8min twice to separate the serum. The serum was then stored at -80°C.

For the flowcytometry experiment blood was collected into a tube containing the anticoagulant heparin (Sigma). After centrifugation at 250x*g* for 5 minutes, the cells were treated with 1 ml of erythrocyte lysis buffer and incubated for 10 minutes at room temperature. Then, 4ml of washing buffer was added and centrifuged at 250x*g* for 5 minutes. Cells were then re-suspended in FACS buffer and counted using a cell counter (CASY system).

2.6 HISTOLOGY

The colon sections were removed placed in histology cassettes and fixed with 4% formalin for 24hrs. The colon sections were dehydrated through an alcohol series and cleared in xylene and paraffin. The sample were embedded in paraffin (Histocentre2, Shandon), 5μm sections were cut and collected onto gelatine coated glass slides and allowed to dry for 4 hours at 37°C . Before staining sections were de waxed and rehydrated by five minute incubations in citroclear (tCS biosciences), followed by five minute incubations in alcohol series from 100% to 50%. (Fisher Scientific), and then five minutes incubation in dH_2O.

2.6.1 Hematoxylin-eosin (H&E) staining

Sections were stained in Harris Haematoxylin (Raymond Lamb,Hampstead UK) for 3 minutes. They were then washed with tap water and differentiatied in acidified alcohol (1% HCl (Sigma-Aldrich) in 70% ethanol) for 10 seconds. Subsequently, sections were stained

with Eosin (Merck) for 4 minutes. Finally, the sections were washed in tap water and dehydrated through alcohol series, from 50% to 100%., followed by two changes of citroclear. The slides were mounted and coverslipped using DEPEX mounting media (BDH Laboratory Supplies). The slides were then scanned by the Panoramic viewer system and the 20 crypt lengths per section were measured using ImageJ software Figure 2-1

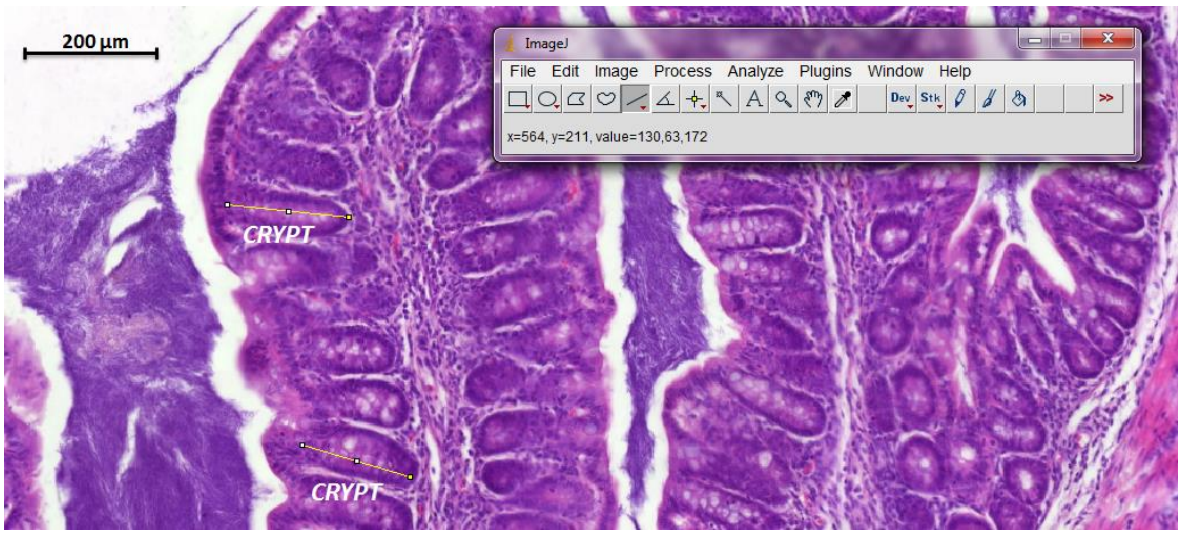


Figure 2-1 Mugerment of the crypt lengths: Mice were infected with a low or high dose of *T.muris***.** At day 21 p.i, colons were isolated from the mice and embedded in wax. 5 micron sections were cut and stained with hematoxylin and eosin. The slides were scanned by Panoramic Viewer system and the crypt lengths were measured using ImageJ software. Image J setting:

2.6.2 Goblet cell staining

The mucins in goblet cells were stained with 1% alcian blue (Sigma-Aldrich) in 3% acetic acid (Sigma-Aldrich, pH 2.5) for 5 minutes. Then, sections were washed in distilled water and treated with 1% periodic acid for 5 minutes (Sigma-Aldrich). They were washed in distilled water, then tap water for 5 mins and rinsed in distilled water. Sections were then treated with Schiff's reagent (Sigma) for 15mins. Slides were washed again in distilled water, tap water for 5 minutes and rinsed in distilled water before being counterstained with Mayer's haematoxylin (Sigma-Aldrich). Slides were washed in tap water, dehydrated , mounted and coverslipped using DEPEX mounting media (BDH Laboratory Supplies).Goblet cells were stained blue (acid mucins), magenta (neutral mucins) or purple (acid / neutral mixed mucins) with grey/blue nuclei. The slides were scanned by the

Panoramic Viewer system and the goblet cells were enumerated in 20 randomly selected crypts.

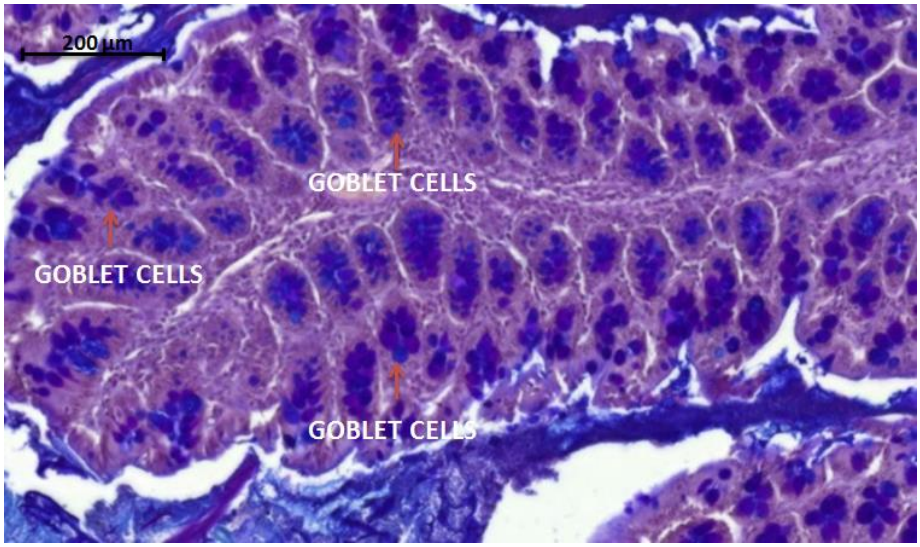


Figure 2-2 Intestinal goblet cells count: Mice were infected with low Aand high dose of *T. muris*. At day 21 colons were isolated from the mice and embedded in wax. 5 micron sections were cut and stained with goblet cells stain (periodic acid-Schiffs). The slides were scanned by Panoramic viewer system and the goblet cells were enumerated in 20 randomly selected crypts.

2.6.3 IL22 immunohistochemistry

Sections were de waxed and rehydrated as described above. Sections were heated in distialed water (dH_2O) in a microwave for ten minutes to retrieve the antigen. To block endogenous peroxide activity, sections were incubated in 0.3% (v/v) H_2O_2 for twenty minutes at 25^{o}C. Sections were then washed for five minutes with a washing buffer (1 x PBS/0.05% (w/v) BSA) on a shaking platform at room temperature. Sections were also treated with a blocking reagent (Perkin Elmer life sciences) for 30 minutes at room temperature in order to block non-specific binding. Sections were washed for five minutes twice with a washing buffer on a shaking platform at room temperature. Sections were incubated with 100µl of primary antibody anti-IL-22, 1/100; (Abcam) or with 2-3 drops of the control polyclonal IgG (Abcam) for one hour at room temperature and then washed for

five minutes twice with a washing buffer on a shaking platform. Sections were then incubated with 100µl of biotinylated goat anti-rabbit antibody (1/1000) (Dako cytomation) for 30 minutes at room temperature. Sections were washed for five minutes with a washing buffer on a shaking platform at room temperature. Sections were incubated with 100µl of streptavidin horseradish peroxidase (HRP) (1/1000) (Roche) for 30 minutes at room temperature Sections were again washed twice for five minutes with a washing buffer on a shaking platform at room temperature. Sections were incubated with 100µl of 3, 3'-diaminobenzidine (DAB) (DAP Refrigerator, Vector) for 4 minutes at room temperature. Sections were washed for five minutes twice with a washing buffer on a shaking platform at room temperature. Sections were counterstained with haematoxylin for one minute at room temperature, and then washed in tap water. Sections were dehydrated through alcohol series, from 50% to 100%., followed by two changes of citroclaer (tCS biosciences). Sections were mounted and coverslipped using DEPEX mounting media.

2.7 WORM BURDEN

After day 21 pi *T.muris* worms were counted in caecum and colon proximal to caecum. Caecum and colon proximal to caecum were removed from the mice and frozen at -20C until processed. Once defrosted, the caecum and colon were dissected gently and the faecal matter was washed out 3 times with PBS. At day 21 the worms are embedded deep in the muscular wall. Thus, worms were scraped out of the caecum into the Petri dishes using forceps. The worms were counted in the faeces matter as well as caecum and colon tissue using a microscope at x10 magnification.

2.8 RESTIMULATION OF SPLEEN CELLS

Spleen cells were isolated as described earlier (see section 2.4.1). $5x10^6$ cells/ml were plated in 96 well culture plate and stimulated with either 100ng/ml lipopolysaccharide (LPS) (sigma) or 20 ng/ml IL-1β (R&D) at 37°C, 5% CO2 for 24 hrs. After centrifugation

at 250x*g* for 5 minutes supernatants were collected and stored at –20°C for ELISA. The cell pellets were re-suspended in a urea lysis buffer for western blot (WB) experimentation.

2.9 RESTIMULATION OF MESENTERIC LYMPH NODE CELLS

Mesenteric lymph nodes were isolated as described earlier (see section 2.4.2). $5x10^6$ cells/ml were plated in 96 well culture plate and stimulated for 4 hours with 50 μg/ml 4 hour parasite E/S antigen (provided by Prof. Else, University of Manchester) at 37°C, 5% CO2 for 48 hrs. After centrifugation at 250x*g* for 5 minutes, supernatants were collected and stored at –20°C for ELISA assay.

2.10 WESTERN BLOTTING

$5x10^6$ spleen cells were stimulated with either 100ng/ml of LPS or 20 ng/ml of IL-1β at 37°C, 5% CO2 for 24 hours. Unstimulated spleen cells from mice were used as control cells. Cells were washed with cold PBS three times, then re-suspended in 500 ml western blot urea lysis buffer (Appendix D) and puls with 5 μl of Protease Inhibitor Cocktail (Sigma), and incubated on ice for 5 minutes. After centrifugation at 5000x*g* for 5 minutes, supernatants were collected and stored in –20°C. A nanodrop instrument was used to determine the concentration of the protein in each sample. The protein concentration was adjusted in all samples by the addition of lysis buffer. WB was performed using 4-12% BIS-Tris Gel (Invitrogen) and its running buffer MED SDS (Invitrogen). 24μl sample and 6μl sample /loading buffer mix (NuPAGE LDS Sample Buffer (4X), life and technology) was prepared and heated at 80°C for 5 minutes. 10 μl of electrophoresis marker (Sigma) was loaded into the first well followed by 20 μl from each sample. The gel was run at 100V until the tracking dye reached the bottom of the gel. After that, the gel was removed from the gel cassettes and placed in the nitrocellulose membrane (iBlot Transfer Stack, nitrocellulose**,** Invitrogen). Blotting was performed in 10 minutes using an iBlot dry blotting system Blots (Invitrogen). Blots were incubated with a blocking solution (5% w/v milk in TBS Tween (Appendix D)) for 90mins at room temperature. The blots were then

washed once for 1 minute with TBS Tween and incubated with a primary antibody anti-mouse IL1 Receptor 1, (1:1000 in in TBS Tween) and anti-mouse β-actin (1:1000 in TBS Tween) overnight at 4°C with rocking. Next day, the blots were washed 4 times with TBS Tween for 20 minutes. The blot were then incubated with the secondary antibody (horseradish-peroxidase (HRP) - conjugated goat anti-mouse or anti-rabbit (Cell signalling); 1:10,000 in TBS Tween) for 60 minutes. After one hour the blots were washed 4 times with TBS Tween for 15 minutes. The signals were developed by using the Western Blot Chemiluminescent Substrate (ECL western blotting substrate, Thermo)

2.11 *TRICHURIS MURIS* SPECIFIC IgG1 AND IgG2 ELISA

T. muris specific antibody responses were investigated in the serum of infected mice using ELISAs. ELISA plates were coated with 50µl/well of 5µg/ml *T. muris* antigen with 24 hours E/S in 0.05M in carbonate bicarbonate buffer (Appendix D) overnight at 4°C (provided by Prof. Else, University of Manchester) . Plates were then aspirated and washed 5 times with PBS Tween and incubated with 100µl/well of 3% BSA in PBS Tween (PBS-0.05 %Tween20, Sigma-Aldrich) to blocked the non-specific binding for 1 hr at 37°C. Plates washed with PBS Tween 5 times. The serums were diluted 1:20 – 1:2560 in PBS Tween and then added 50µl/well from each dilution to the plate and incubated for 1hr at 37°C. After 1 hour plates were aspirated and washed 5 times with PBS Tween and incubated with 50 µl/well of biotinylated rat anti-mouse IgG1 (BD Biosciences, 1:500) or IgG2a/c (BD Biosciences, 1:1000) for 1hr at room temperature. The plates were then aspirated and washed 5 times with PBS Tween and 75µl/well of streptavidin - β - peroxidase (SA-POD, Roche, 1:1000) was added to the plates and incubated for 1hr at room temperature. Finally, plates were aspirated and washed 5 times with PBS Tween and developed with 100 µl/well of 3,3', 5,5' tetramethylbenzidine (Ultra TMB ELISA superstrate, Thermo) . The reaction was stopped by adding 100 µl/well of 2N sulphuric acids (R&D). Plates were then read using a Dynex MRX11 plate reader (Dynex Technologies) at 405nm with a reference of 490nm.

2.12 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA was used to analyse cytokine and chemokine concentration in the re-stimulation spleen and lymph nodes (see 2.8 and 2.9). The concentration of TNFα, IL-6 and MCP-1 were determined in re-stimulated spleen using ELISA kits specific for TNFα, IL-6 and MCP-1 (BD Biosciences). The concentration of IFNγ, IL4, IL22 and IL17 was measured using ELISA kits specific for IFNγ, IL4, IL22 and IL17 (BD Biosciences). The Biosciences ELISA kits contained of the required buffers as well as the flat bottomed 96 well plates. Briefly, 96 well plates were coated with 100 µl/well of the capture antibody and incubated overnight at 4°C. The following day, plates were washed 5 times with PBS Tween (Appendix D) .The plates were then blocked with 300 µl/well of assay buffer for 1 hour then aspirated and washed 5 times with PBS Tween. Eight serial dilutions of the cytokine or chemokine recombinant protein was prepared using assay buffer. Then, 100 µl/well of the sample along with the recombinant cytokine dilution were incubated for 2 hours. After 2 hours, the plate were aspirated and washed 5 times with PBS Tween and incubated with a detection antibody for 1 hour. The plates were then aspirated and washed 5 times with PBS Tween before incubated with 100 µl/well of Avidin-HRP for 30 minutes. Again, plates were aspirated and washed 5 times with PBS Tween and incubated with 100 µl/well of TMB Solution for 15 minutes. The reaction was stopped with 50ul of 2N sulphuric acids stop solution (R&D), added for 5-10 minutes. The plate was read at 450 nm using a Dynex MRX11 plate reader (Dynex Technologies).

2.13 FLOW CYTOMETRY

2.13.1 Nine Fluorescence Parameter Flow Cytometry

Nine Fluorescence Parameter Flow Cytometry was performed as in the Frischmann and Müller study (Frischmann and Muller, 2006). Spleen cells, blood and lymph nodes cells were isolated as described in sections 2.4.1, 2.4.2, and 2.5. $5x10^6$ cells/ml cells were washed 3 times with an FACS buffer (Appendix D) and then stained with 1:200 mouse

serioblock FcR (ABD seroTec) in 4°C for 15 minutes to block all extracellular antibodies. The cells were then stained the primary antibody, which was directly coupled with a fluorochrome-labeled in FACS buffer on ice for 45 minutes. Table 2.6 shows antibodies used along with dilution. Unstimulated spleen cells from mouse were used as a control cells

Antibody	Clone	Supplier	Concentration
CD4 PE-Cy5	RM4-5	BD Pharmingen	1:500
CD8 PE-Cy5	53-6.7	BD Pharmingen	1:400
CD19 APC	1D3	BD Pharmingen	1:2000
IgM PE	eB121-15F9	eBioscience	1:600
IgD pacific blue	11-26c	eBioscience	1:400
F4/80 PE	BM8	eBioscience	1:200
CD49b pacific blue	DX5	eBioscience	1:30
Gr-1 PE	RB6-8C5	eBioscience	1:30
Gr-1 pacific blue	RB6-8C5	Bioscience	1/750
Viability stain APC	----------	Bioscience	1/1000

Table 2.6 Nine Fluorescence Parameter Flow Cytometry antibody.

The cells were then washed twice with FACS buffer, and then re-suspended in FACS buffer and analysed on LSR-Fortessa machine (BD Biosciences) using Cell Quest software (BD Biosciences). Samples were acquired by a live gate, without compensation and at least 10,000 events were recorded. The data were analysed using FlowJo (see gating strategy in Figure 4-1)

2.13.2 B cells FACS analysis

$5x10^6$ cells/ml spleen and bone marrow cells were isolated (see 2.4.1, 2.4.3) and washed 3 times with FACS buffer (Appendix D). The cells were then treated with 1:200 serioblock FcR (ABD seroTec) at 4°C for 15 minutes. After that, the cells were stained with the

primary antibody directly coupled fluorochrome-labeled in FACS buffer on ice for 45 minutes. Antibodies and dilutions used are shown in table 2.7. Unstimulated spleen and bone marrow cells were used as a control cells.

Antibody	Clone	Supplier	Concentration
B220 APC	RA3-6B2	eBioscience	1/640
IgM PE	eB121-15F9	eBioscience	1:300
IgD pacific blue	11-26c	eBioscience	1/800
c-Kit PE-Cy5	CD117	eBioscience	1/80
Viability stain APC-CY7	----------	eBioscience	1/1000

Table 2.7 B cells FACS antibody.

The cells were washed twice and re-suspended in FACS buffer. LSR-Fortessa machine (BD Biosciences) using Cell Quest software (BD Biosciences) was used to analyse the cells. Samples were acquired by a live gate without compensation, and at least 10,000 events were recorded. The data were analysed using FlowJo (see gating strategy in Figure 4-4)

2.14 STATISTICAL ANALYSIS

Statistical significance of differences between three groups or more was determined using one-way ANOVA followed by a Tukey multiple comparison post hoc test**.** Statistical significance of differences between two groups was determined using Student's t- test**.** All statistical analyses were performed using GraphPad Prism 6. Significant differences are illustrated by * =P≤0.05, ** =P≤0.01 *** =P≤0.001, **** =P≤0.0001.

CHAPTER THREE: CHARACTERISATION OF IL1-RI *vaviCre* CONDITIONAL KNOCKOUT MICE

3.1 INTRODUCTION

The role of IL-1 signalling in inflammation and host-defence responses to injury and infection is well documented. It plays an important role to assist in host protection against infection. (Dinarello, 2009, O'Neill, 2008, Thomas *et al*., 2012). Several types of cells produce IL-1; however the cells that are responsible for the functioning of IL-1 in the immune system during injury or infection are largely unknown. It has been demonstated that IL-1 signalling plays an important role in the differentiation of Th17 cells from naive T cells. Th17 cells have been reported to be an important T cell subset in autoimmunity and clearance of mucosal infection through the production of pro-inflammatory cytokines such as IL-17, IL-17F and IL-22 (Sims and Smith, 2010, Chung *et al*., 2009).

Conditional knockout mice in which the specified gene is inactivated in a specified cell type or tissue is a suitable model for this study. The bacteriophage-derived Cre-loxP recombination system is a powerful tool in order to generate conditional knockout mice, since it offers a wide range of applications for targeting genes (Rajewsky *et al*., 1996). There exist over 500 transgenic mouse lines that are able to express Cre recombinase within different cells types. Thus, the generation of conditional knockout mice can be achieved by crossing the mice containing the flox targeted gene with the appropriate Cre mice (Nagy *et al*., 2009). The generation of mice with the specific haematopoietic cell IL1-RI knockout (IL1-RI *vaviCre)* will enable an investigation to be conducted into establishing the cells responsible for the downstream signalling of IL-1.

The generation of IL1-RI *vaviCre* conditional knockout mice will be described, which will include information about mouse breeding and genotyping. This will be followed by an investigation of the IL1-RI delta allele at the genome and protein level in order to demonstrate that the *Il1-rI* gene is inactivated in hematopoietic cells.

3.2 RESULTS

3.2.1 The generation of IL1-RI *VaviCre* conditional knockout mice

Mice with the flox/flox IL-1 receptor type-I (IL1-RI $^{fl}/^{fl}$) gene will be used to generate mice with the specific haematopoietic cell (*vaviCre*) IL1-RI knockout. The IL1-RI$^{fl}/^{fl}$ transgenic mice were generated by TaconicArtemis in 2011 in Cologne, Germany and then bred as homozygous mice after being shipped to the University of Manchester.

Il1-rI gene containes 11 exons. TaconicArtemis examined all *Il1-rI* gene exons in order to identify the exon which could be delated and prevent *Il1-rI gene* function. They found that the delation of exon 5 prevented the function of *Il1-rI* gene.

The generation of the IL1-RI delta allele was achieved by flanking exon 5 with two loxP sites. The two positive selection markers neomycin (NeoR) and puromycin (PuroR) were flanked by two different flippase (FLP) recombination target sites of FRT (flip-recombinase targets) and F3 respectively. These were then inserted into exon 4 and 5 (Figure 3-1). The homologous recombinant clones were separated using NeoR and PuroR using a double positive selection in order to increase the co-integration efficiency of the two loxP sites. The bacterial artificial chromosome (BAC) clones from the C57BL/6J RPCI-23 BAC library were used to generate a targeting vector, which were then transferred into the C57BL/6N Tac ES cell line, resulting in the genomic locus being modified. Both selection markers were removed after Flp recombination. The flanked exon 5 with two loxP sites was deleted by Cre recombination, which led to loss of the function of the *Il1-rI* gene by obliterating part of the extracellular region (Ig-like-C2 type 2). It is also caused frame shift from exon 4 to all exons downstream (Figure3-1) (Appendix A, TaconicArtemis, Cologne, Germany, 2011).

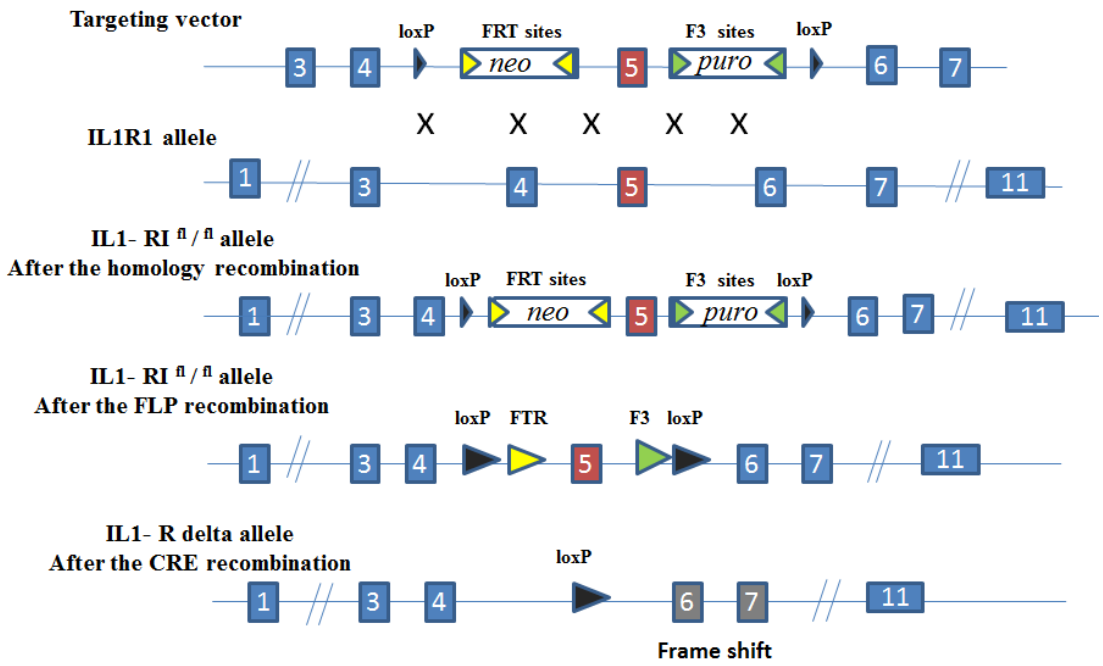
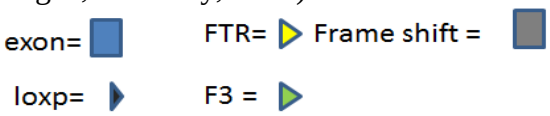


Figure 3-1: The generation of the IL1-RI delta allele: The BAC gene targeting vector contained two loxP sites flanked exon 5. The FLP recombination target sites FRT and F3 flanked the two positive selected markers of NeoR and PuroR respectively. The homologous recombination between the *Il1-rI* gene in the ES cells, and gene targeting vectors, leads to the genomic locus being modified (which obtains the IL1-RI $^{fl}/^{fl}$ allele). The FLP recombination leads to the removal of the positive selected markers NeoR and PuroR. The mice that have been modified with the IL1-RI delta allele have been generated by deleting exon 5, which is flanked by loxP sites with the expression of Cre recombinase. The deletion of exon 5 caused deletion of part of the extracellular region (Ig-like-C2 type 2) and frame shift from exon 4 to all exons downstream (Adapted from, Appendix A, TaconicArtemis, Cologne, Germany, 2011).



3.2.1.1 Mice breeding in order to generate IL1-RI *vaviCre*

The IL1-RI $^{fl/fl}$ mice were crossed with the IFNγ-R $^{fl/fl}$ *vaviCre*+ mice. The latter were generated in 2011 by Dr Ruth Forman, who is a member of Prof Muller's lab at the University of Manchester, UK. .IFNγ-R $^{fl/fl}$ *vaviCre*+ mice were used in the generation of IL1-RI $^{fl/fl}$ *vaviCre*+ mice as controls which only expressed *vaviCre*+.(ie .IFNγ-R$^{wt/wt}$).The *vaviCre*+mice originally used in the generation of IFNγ-R $^{fl/fl}$ *vaviCre*+ were provided from Dimitris Kioussis lab (de Boer et al., 2003)

As a result from the first breeding there were two different strains established, namely IL1-RI $^{fl/wt}$ / IFNγ-R $^{fl/wt}$ *vaviCre*+ and IL1-RI $^{fl/wt}$ / IFNγ-R $^{fl/wt}$ *vaviCre* – .The new strains were crossed with each other and produced further strains (Figure 3-2). IL1-RI $^{fl/fl}$ IFNγ -R $^{wt/wt}$ *vaviCre*+ and IL1- RI $^{fl/fl}$ / IFNγ-R $^{wt/wt}$ *vaviCre* – were chosen and crossed once more to obtain IL1-RI $^{fl/fl}$ IFNγ -R $^{wt/wt}$ *vaviCre*+. This breeding process provided both the experimental mice of IL1- RI $^{fl/fl/}$ IFNγ-R $^{wt/wt}$ *vaviCre*+ and the control mice of IL1-RI $^{fl/fl}$/ IFNγ-R $^{wt/wt}$ *vaviCre* – . Figure 3.2 demonstrates the breeding steps that were used to generate the conditional knockout mice IL1-RI *vaviCre.*

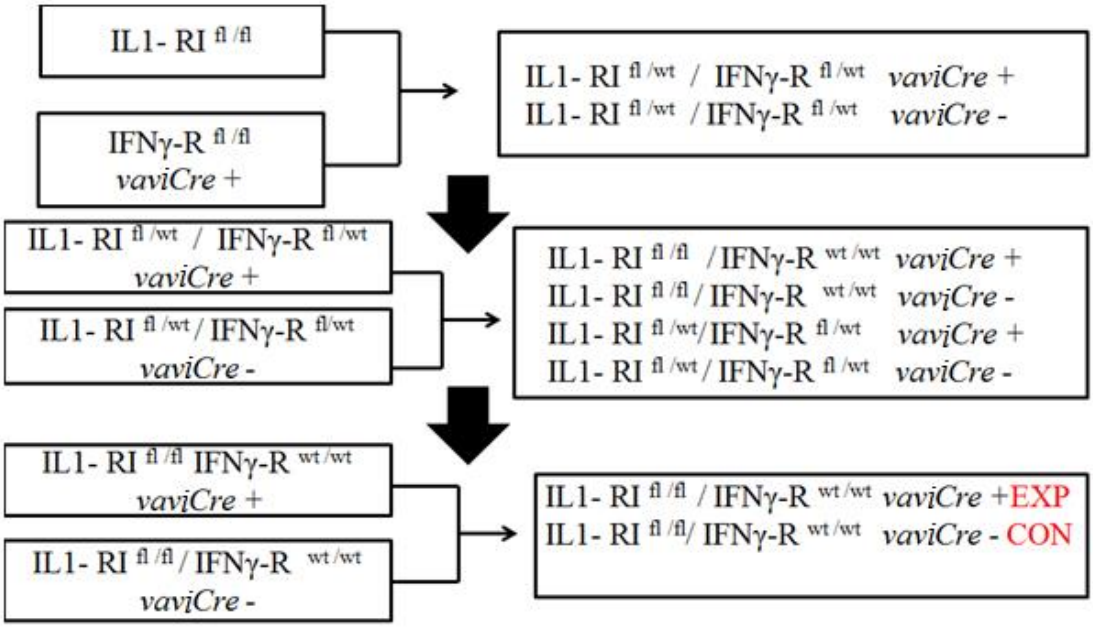


Figure 3-2 : Breeding steps that were used to generate the conditional knockout mice IL1- RI *vaviCre*. The IL1- RI $^{fl/fl}$ mice were first crossed with IFNγ-R $^{fl/fl}$ *vaviCre* + in order to induce the deletion of the floxed sequence in the IL1-RI $^{fl/fl}$ mice. Subsequently, the IL1- RI $^{fl/wt}$ / IFNγ-R $^{fl/wt}$ *vaviCre* + and IL1- RI $^{fl/wt}$ / IFNγ-R $^{fl/wt}$ *vaviCre* – were crossed, which resulted in 4 different mice groups. Finally, IL1- RI $^{fl/fl}$ IFNγ -R $^{wt/wt}$ *vaviCre* + and IL1- RI $^{fl/fl}$ / IFNγ-R $^{wt/wt}$ *vaviCre* – were chosen, and crossed once more to determine IL1- RI $^{fl/fl}$ IFNγ -R $^{wt/wt}$ *vaviCre* +. This provided the experimental and control mice.

3.2.1.2 Genotyping

The DNA extracted from mouse ear punches was used for PCR to genotype the IL1-RI *vaviCre* and IFNγ-R allele. TaconicArtemis designed two sets of primers in order to genotype the IL1-RI allele**.** The first set of primers :

- oligos 1 (2959-32: CTAGTCTGGTGGAACTTACATGC)**;** and
- oligos 2 (2959-33AACTGAAAGCTCAGTTGTATACA GC)

flanked the first loxP site and the second set of primers :

- oligos 3 (2960-40 : GGGGATGGAGGTAGAGGTATGG); and
- oligos 4 (2960-39: GATAAACGAGAGCTGGAGACAGG)

flanked the second loxP site (Figure 3-3)**.** Both primers can be used to detect the IL1-RI allele. Oligos 1 and oligos 2 were used routinely in genotyping IL1-RI mice. The fragment amplified with oligos 1 and 2 was used to detect heterozygous, homozygous and wild type alleles.

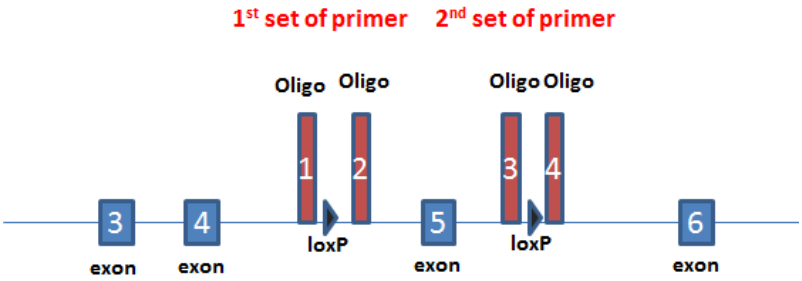


Figure 3-3: Two sets of premiers were used to genotype IL1-RI by PCR: Both sets of primers were provided by TaconicArtemis, which allowed for the detecting of heterozygous, homozygous and wild type alleles (adapted from Appendix C, TaconicArtemis, Cologne, Germany, 2011)

In the IL1-RI genotype, the wild type mice C57BL/6 showed one band at 267 bp, the heterozygous mice (wt/fl) showed two bands at 276 bp and 432 bp and the homozygous mice (fl/fl) showed only one band at 432 bp (Figure 3-4 A).

A *iCre* primers sequence Vav For: (AGATGCCAGGACATCAGGAACCTG) and Vav Rev (ATCAGCCACACCAGACACAGAGATC) were designed by previous members of Prof W. Müller's lab (Braunschweig, Germany). In the *vaviCre* genotype *vaviCre* +, one band showed at approximately ~ 200-300 bp, but there was no band for *vaviCre* – (Figure 3-4 B).

IFNγ-R primers loxP1: (TGAGTTCCGCAAGACAGA), loxP site (AAGTTATGGTCTGAGCTCGC) and LoxP2 (CAGGGTAGAAAAGATGTGCA) were provided in 2011 by Dr Ruth Forman a member of Prof Muller's lab at the University of Manchester. The IFNγ-R genotyping showed a band at 358 bp in the wild type mice bp; three bands at 191 bp; 358bp and 392bp in the heterozygous mice (wt/fl); two bands at 191 bp; and 392bp in the homozygous mice (fl/fl) (Figure 3-4 C).

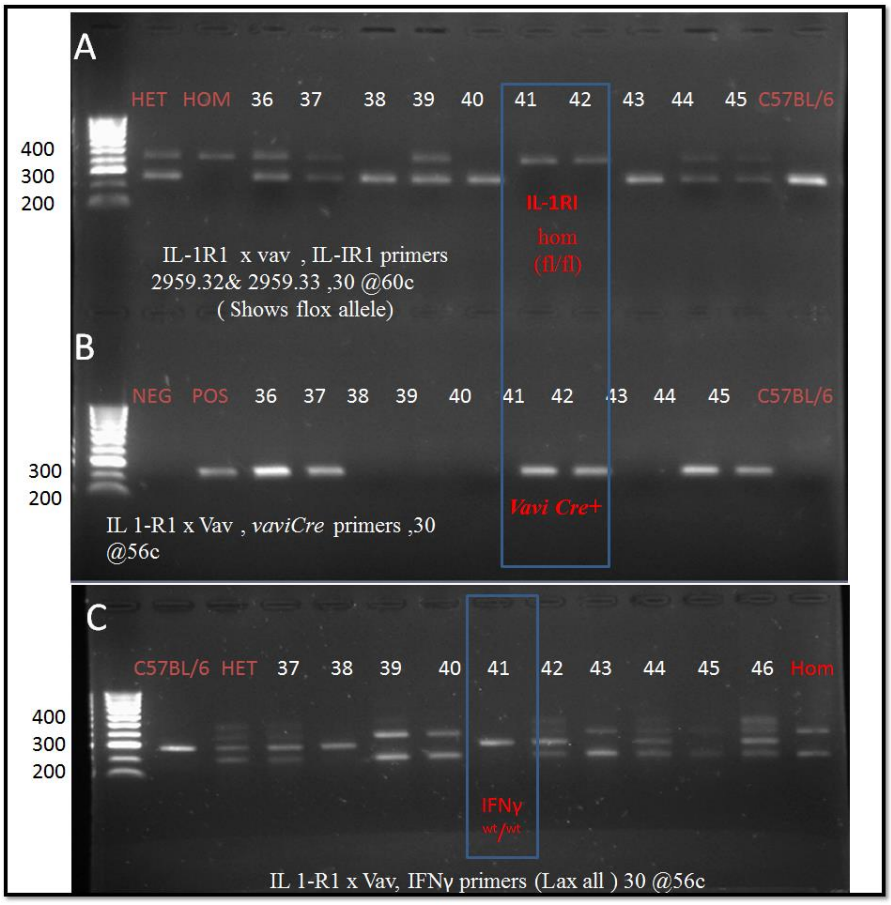


Figure 3-4: Genotyping PCRs for detecting (A) IL1-RI (B) *vaviCre* and IFNγ-R alleles: Mouse genomic DNA was extracted from ear punches, and PCRs were performed with the appropriate primers and visualised on a 1% agarose gel. (A) The IL1-RI wild type (WT) mice C57BL/6 showed one band at 267 bp; the heterozygous (HET) mice (wt/fl) showed two bands at 276 bp and 432 bp; and the homozygous mice (HOM) (fl/fl) showed one band at 432 bp. (B) The *vaviCre* + showed one band at approximately 200-300 bp, but there was no band for *vaviCre* – (C) The IFNγ-R WT mice C57BL/6 showed one band at 358 bp. The HET mice (wt/fl) showed three bands at 191 bp, 358 bp and 392 bp respectively; and the HOM mice (fl/fl) showed two bands at 191 bp and 392bp. IL1- RI $^{fl/fl}$ / IFNγ-R $^{wt/wt}$ *vaviCre* – or + were the target genotypes for the breeding process.

At the end of the breeding process, the experimental mice IL1-RI $^{fl/fl/}$ IFNγ-R $^{wt/wt}$ *vaviCre* + and the control mice IL1- RI $^{fl/fl}$/ IFNγ-R $^{wt/wt}$ *vaviCre* – were obtained (Figure 3-5).

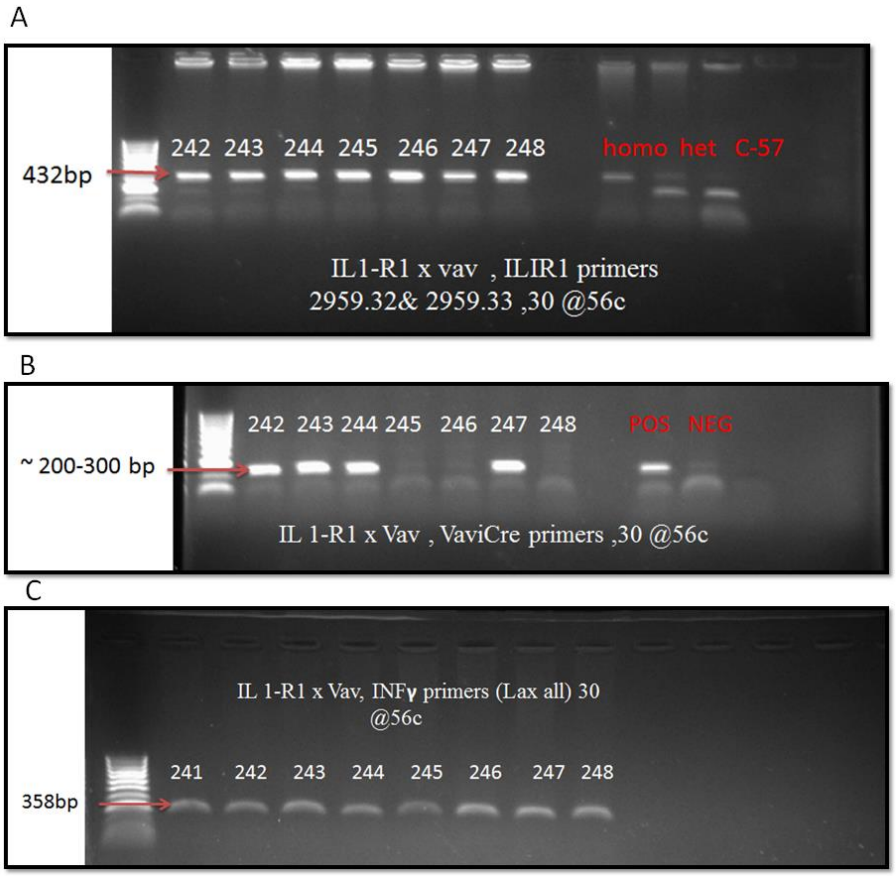


Figure 3-5: Genotyping PCRs for detecting (A) IL1-RI allele, (B) *vaviCre* allele and (C) IFNγR allele at the completion of the mice breeding: Mouse genomic DNA was extracted from ear punches, and PCRs were performed with the appropriate primers and visualised on a 1% agarose gel. (A) The PCR genotyping for the IL1-RI allele demonstrated that all mice were HOM (fl/fl) and the size of the band was 432 bp. (B) The PCR genotyping for *vaviCre* demonstrared that some mice were *vaviCre* +(242,243,244.247) at band size ~ 200-300 bp, and some were *vaviCre* – (245, 246, 248), and others did not demonstrate a band (C). The PCR genotyping for the IFNγR allele demonstrated that all mice were wild-type (wt/wt) and showed one band at 358 bp.

3.2.2 Investigation of the IL1-RI delta allele

Several experiments at the genome and proteine level were carried out in order to determine whether the *Il1-rI* gene and the IL1-R1 protein were inactivated specifically in hematopoietic cells. Spleen cells were used in these experiments.

3.2.2.1 Genome analysis of the IL1-RI delta allele

3.2.2.1.1Genotyping PCRs for detecting the IL1-RI delta allele

In order to examine the eradication of the IL1-RI exon 5, which was flanked by two loxP in IL1-RI *VaviCre* conditional knockout mice, a PCR was carried out using oligos1 and oligos 4. A PCR using both oligos 1 and oligos 4 primers were used to amplify the DNA segment determined by the two loxP sites containing exon 5 (Figure 3-6).

Figure 3-6: A PCR was used to amplify the DNA segment containing exon 5. The DNA fragment was amplified by PCR using both oligos 1 and oligos 4 to examine the presence of exon 5 in the *Il1-rI* gene within the IL1-RI *vaviCre* conditional knockout mice.

It was found that the wild-type mice C57BL/6 and the IL1-RI *vaviCre* – demonstrated a band at approximately 1000 bp. IL1-RI *vaviCre* + showed a band at ~400 bp, as demonstrated in Figure 3-7. This indicated that the segment flanked by the loxp site containing exon 5 was deleted successfully in the IL1- RI mouse strain.

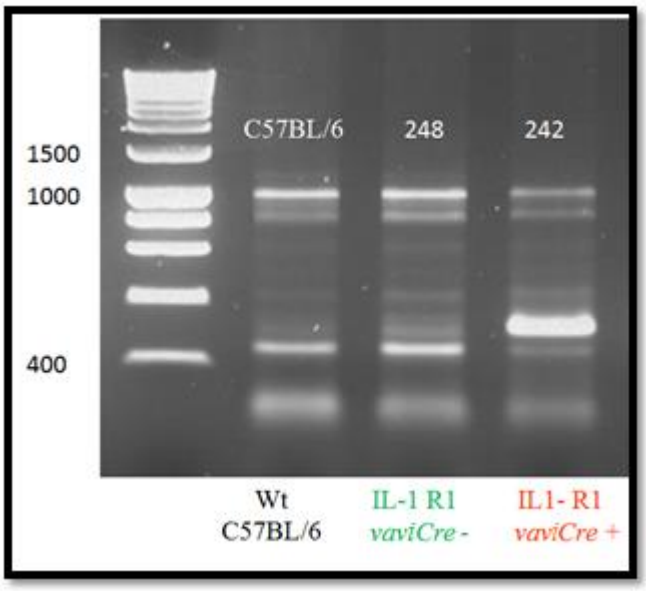


Figure 3-7: Genotyping PCRs for detecting the IL1-RI delta allele: The wild-type mice and the IL1-RI *vaviCre* – demonstrated a band at approximately 1000 bp. Whereas the IL-1-RI *vaviCre* + showed a band at approximately 400 bp.

The size of the IL1-RI *vaviCre* – band was large and difficult to sequence. Therefore, another primer was designed (WT F: GCCCCCTCACCTCAAATAGA and WT R: GCCGAGCTATCCTCACACTC) close to exon 5 in order to detect the IL1-RI *vaviCre* –. This demonstrated a band at approximately 650 bp (Figure 3-8).

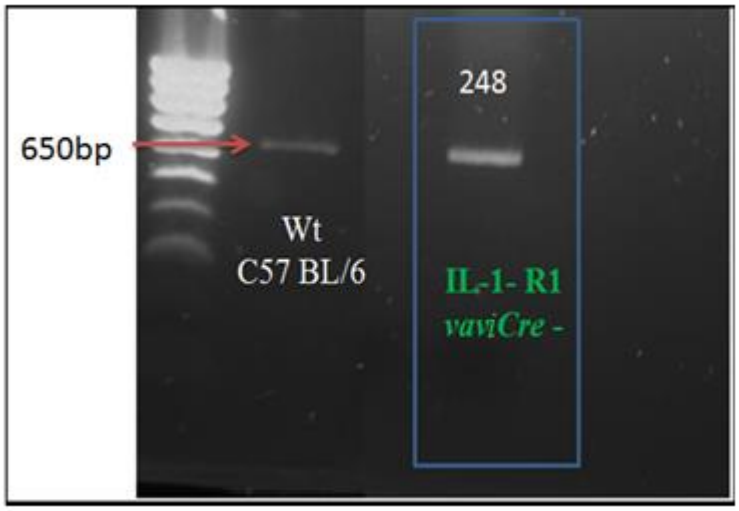


Figure 3-8: The IL1-RI *vaviCre* – detection by PCR*:* In order to sequence IL1-RI *vaviCre* –, a second primer was designed to amplify a smaller DNA fragment. Using the new primers, namely wild type mice and IL1-RI *vaviCre* –, a band was showed at approximately 650 bp.

3.2.2.1.2 DNA Sequencing for the IL1-RI delta allele

To analyses the DNA sequence for the IL1-RI delta allele**,** DNA fragments for IL1-RI *vaviCre* + (approximately 400 bp) and IL1-RI *vaviCre* – (approximately 650 bp) were cut from the agarose gel. The DNA was extracted from the gel using a QIA quick gel extraction kit from QIAGEN. The extracted DNA was ligated into pGEMT from PROMEGA. The ligated DNA was then transformed in *E.coli* bacteria DH5α and grown on ampicillin agars plates overnight at 37°C. From each plate, one clone was incubated with Luria-Bertani broth (LB) medium with 100µg/ml ampicillin overnight at 37°C. The bacterial DNA was extracted using a QIA prep spin Miniprep kit from QIAGEN. A PCR using primers T7 and SP6 was performed in order to confirem the successful ligation of the DNA into the vector. It was found that the ligated DNA from IL1-RI *vaviCre* + spleen cells demonstrated a band at ~550 bp (150 from the vector + 400 from the ligated DNA). The ligated DNA from the IL1-RI *vaviCre* – spleen cells showed a band at ~ 800 bp (150 from the vector + 650 from the ligated DNA) (Figure 3-9).

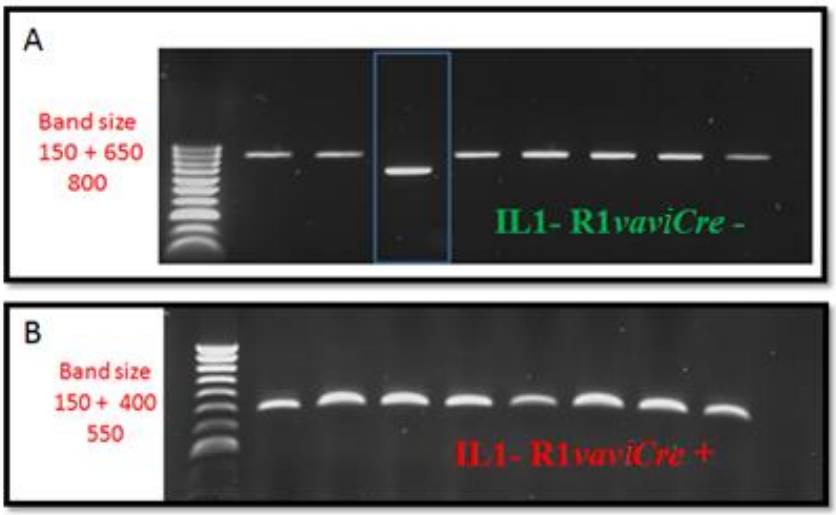


Figure 3-9: PCR for detecting ligated DNA from the IL1-RI *vaviCre* – and the IL1-RI *vaviCre*+: The successful ligation of the DNA into the vector showed (A) 800 bp in the IL1-RI *vaviCre*– (150 from the vector + 650 from the ligated DNA) and (B) 550 bp in IL1-RI *vaviCre* + (150 from the vector + 400 from the ligated DNA).

The samples plus either T7 or SP6 primer were sent for sequencing in the DNA sequencing facility at the University of Manchester. It was found that by observing the IL1-RI sequence from the UCSC Genome Browser website, the Exon 5 sequence was observed in the IL1-RI *vaviCre*– spleen cells. However, exon 5 was not present in IL1-RI *vaviCre* + spleen cells (Figure 3-10). Moreover, the DNA fragment flanked with the two loxPs was delated in the IL1-RI delta allele (Figure 3-10). This data demonstrated evidence to confirm the deletion of the DNA segment flanked with the two loxPs in the IL1-RI delta allele.

IL1-RI $^{flox/flox}$

agatgtagtttcaagaaaagtgctagaacatcctttgagagaatagattagaaatttaagccccttaagagagaacttttttataaca
gagatagtgttttgtgtttcattaaaaacatatgttctggggctgcagggatggctcagggattaatagcatgcatggctcttgcac
agaactagaatttgttttccagcatgcacagaaaaggcggctcacaactgcctataattacagatccacctgccgccccttctg
gcctccattggtactgtcctcgtgtgcacaaacctctaccgcacacatacacacactcataattaaagataaaataaaaataaatc
ctaaaagactaatgaaaagctaaatgcttcatacgtaacatggtagcgtctgtagccacttaatcagtaggtggaacacagcgta
ttttattacctttagcttacctagtctggtggaacttacatgcgaacatcatcttttagagatgaaagaatatgaaatgaatagtatcc
gtgcaggttcgagataattaaccctcactaaagccgcgggtcgactcgagcctaggc**LoxPataacttcgtataa**tgtatgc
tatacgaagttataagcttgaagttcctattccgaagttcctattctctagaaagtataggaacttcattctaccgggtaggggagg
cgcttttcccaaggcagtctggctattctctagaaagtataggaacttcctcgagccctatagtgattaattaaggaaggggctgt
ggtgttgagaaatgtcagcattattgcttcaatcacatcttctgtgaaataaatttgtcttcacccgtctttgtggcatacgcctctgg
gcttcaaattttgaaatgtaattatccccccctccat**gccccctcacctcaaataga**gataaaacatgctgtatacaactgagctt
tcagttactttctctgtcatgaatttgtgaaaaacagaagtgaaaaacttgtgttcctattccactgcacaggggagctcctaactgt
cccatgcctctttttatttattcttgtttagaactgtaaaccattccactgcacaggggagctcctaactgtcccatgcctctttttattt
attcttgtttag**aactgtaaacctctgcttcttgacaacgtgagcttcttcggagtaaaagataaactgttggtgaggaatg**
tggctgaagagcacagaggggactatatatgccgtatgtcctatacgttccgggggaagcaatatccggtcacacgag
taatacaatttatcacaataggtgagtcacagctctgacccctggtgttggcatgtggactggccttagagaccaccgttctg
atatcattgctctgttgtctaaaaaggaactaagtctaggaactggtcaatgactcacaaggtctattggtcacacaaagtaaaat
agggaagagacagtttgttttgactcatgtgtccagagagaatgcagttggttatggcgagggaggcatggaagctg**gagtgt**
gaggatagctcggcagtcgggaagaagggtgataggatttcatgggcacactggaagcgagaagttcctatactatttgaag
aataggaacttcggaataggaacttcatttaaatggttacccctaggccggccgctagcgatatcggatcc**loxPataacttcg**
tataatgtatgctatacgaagttatgccagcgaaagagagatagagctagacaaatgattgatagatgatagagatagatagtc
agaaatgggttcaactataaaatcttaaaacctcactccccccccccccacacacacacagtgacatcctgtctccagctctgc
tttatctaccaaaggttccacaacctttccagatagtagtaccagctggggaccaagtgttcaaactcatgagcctgtgggggga
tgttcatgttcatgttcaaaccacaatgcaaagtcttatggtcttaatttcatttttgttagtaagtagtacatggatattttcattactg
ctcctggtgcaatgctaattagacaaatgcattatgaattatactttccaatcaatcttgttaaatatcttcgatatcactgaaggtagt
tcctccaaggagctcccggactctctttagtgggtacatt

IL1-RI *vaviCre* –

cttctgtgaaataaatttgtcttcacccgtctttgtggcatacgcctctgggcttcaaattttgaaatgtaattatcccccccctccat**gccc**
cctcacctcaaatagagataaaacatgctgtatacaactgagctttcagttactttctctgtcatgaatttgtgaaaaacagaagtgaa
aaacttgtgttcctattccactgcacaggggagctcctaactgtcccatgcctctttttatttattcttgtttagaactgtaaaccattccac
tgcacaggggagctcctaactgtcccatgcctctttttatttattcttgtttag**AACTGTAAACCTCTGCTTCTTG**
ACAACGTGAGCTTCTTCGGAGTAAAAGATAAACTGTTGGTGAGGAATGTG
GCTGAAGAGCACAGAGGGGACTATATATGCCGTATGTCCTATACGTTCCG
GGGGAAGCAATATCCGGTCACACGAGTAATACAATTTATCACAATAGgtgagt
cacagctctgacccctggtgttggcatgtggactggccttagagaccaccgttctgatatcattgctctgttgtctaaaaaggaactaa
gtctaggaactggtcaatgactcacaaggtctattggtcacacaaagtaaaatagggaagagacagtttgttttgactcatgtgtcca

gagagaatgcagttggttatggcgagggaggcatggaagctg**gagtgtgaggatagctcggc**agtcgggaagaagggtgata
ggatttcatgggcacactggaaggagagagagaagtgg

IL1-RI *vaviCre* +

NNNNNNNNNNNCGCATGCTCCNGCCGCCATGGCGGCCGCGGGAATTCGATTC
TAGTCTGGTGGAACTTACATGCGAACATCATCTTTTAGAGATGAAAGAAT
ATGAAATGAATAGTATCCGTGCAGGTTCGAGATAATTAACCCTCACTAAAGCC
GCGGGTCGACTCGAGCCTAGGC**ATAACTTCGTATA**ATGTATGCTATACGAAGT
TATGCCAGCGAAAGAGAGATAGAGCTAGACAAATGATTGATAGATGATAGAG
ATAGATAGTCAGAAATGGGTTCAACTATAAAATCTTAAAACCTCACTCCCCCC
CCCCCCCACACACACACAGTGACATCCTGTCTCCAGCTCTGCTTTATCAA
TCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAA
CGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGT
AATCATGG

Figure 3-10: IL1-RI *vaviCre* – and IL1-RI *vaviCre* + DNA sequences: The genomic sequence of IL1-RI ^{fl} / ^{fl} was adapted from the UCSC Genome Browser website. The sequence in dark and light Blue in IL1-RI ^{fl} / ^{fl} showed the two sets of primers used to amplify the DNA fragments from IL1-RI *vaviCre* + and IL1-RI *vaviCre* – respectively. The position of the loxp sites (Brown) site was identified according to the sequence of the ligated DNA into pGEMT – easy vector generated by TaconicArtemis (Appendix A). The sequence in purple demonstrates exon 5, which was only present in the IL1-RI *vaviCre* – sequence. It was not present in the IL1-RI *vaviCre* + sequence. The two sequences in Pink and Green demonstrate the similarity between the IL1-RI ^{fl}/^{fl} and IL1-RI *vaviCre* +. The comparison between IL1-RI ^{fl} / ^{fl} and IL1-RI *vaviCre* + demonstrated that the DNA segment flanked with the two loxP was deleted.

Primers 1 Loxp site Sequence before loxp site
Primers 2 Exon 5 Sequence after loxp site

3.2.2.1.3 Lack the binding site of IL-1 in the IL1- RI delta allele

In order to investigate the structure prediction of the IL1-RI delta allele, both IL1-RI *vaviCre*– and IL1-RI *vaviCre* + DNA sequences were translated into amino acid sequences using the ExPASy bioinformatics resource portal website. The translated amino acid sequences were identified by comparing them with amino acid IL1-RI sequence taken from the UCSC Genome Browser website. Despite IL1-RI *vaviCre* – demonstrating a similar amino acid sequence to IL1-RI from the database, IL1-RI *vaviCre* + did not. This indicated that the deletion of the *Il1-rI* gene in IL1-RI *vaviCre* + resulted in amino acid modification (Figure 3-11).

IL1- RI amino acid sequence

MENMKVLLGLICLMVPLLSLEIDVCTEYPNQIVLFLSVNEIDIRKCPLTPNKMHGDTIIWYKNDSKTP
ISADRDSRIHQQNEHLWFVPAKVEDSGYYYCIVRNSTYCLKTKVTVTVLENDPGLCYSTQATFPQRL
HIAGDGSLVCPYVSYFKDENNELPEVQWYKNCKPLLLDNVSFFGVKDKLLVRNVAEEHRGDYICR
MSYTFRGKQYPVTRVIQFITIDENKRDRPVILSPRNETIEADPGSMIQLICNVTGQFSDLVYWKWNG
SEIEWNDPFLAEDYQFVEHPSTKRKYTLITTLNISEVKSQFYRYPFICVVKNTNIFESAHVQLIYPVPDF
KNYLIGGFIILTATIVCCVCIYKVFKVDIVLWYRDSCSGFLPSKASDGKTYDAYILYPKTLGEGSFSDLDT
FVFKLLPEVLEGQFGYKLFIYGRDDYVGEDTIEVTNENVKKSRRLIIILVRDMGGFSWLGQSSEEQIAI
YNALIQEGIKIVLLELEKIQDYEKMPDSIQFIKQKHGVICWSGDFQERPQSAKTRFWKNLRYQMPA
QRRSPLSKHRLLTLDPRDTKEKLPAATHLPLG

IL1- RI *vaviCre* – Amino acid sequence

MENMKVLLGLICLMVPLLSLEIDVCTEYPNQIVLFLSVNEIDIRKCPLTPNKMHGDTIIWYKNDSKTPISAD R D S R I H
Q Q N E H L W F V P A K V E D S G Y Y Y C I V R N S T Y C L K T K V T V T V L E N D P G L C Y S T Q A T F P
Q R L H I A G D G S L V C P Y V S Y F K D E N N E L P E V Q W Y K N C K P L L L D N V S F F G V K D K L L V
R N V A E E H R G D Y I C R Met S Y T F R G K Q Y P V T R V I Q F I T I D E N K R D R P V I L S P R N E T I E A
D P G S Met I Q L I C N V T G Q F S D L V Y W K W N G S E I E W N D P F L A E D Y Q F V E H P S T K R K Y
T L I T T L N I S E V K S Q F Y R Y P F I C V V K N T N I F E S A H V Q L I Y P V P D F K N Y L I G G F I I L T A T
I V C C V C I Y K V F K V D I V L W Y R D S C S G F L P S K A S D G K T Y D A Y I L Y P K T L G E G S F S D L D
T F V F K L L P E V L E G Q F G Y K L F I Y G R D D Y V G E D T I E V T N E N V K K S R R L I I I L V R D Met G
G F S W L G Q S S E E Q I A I Y N A L I Q E G I K I V L L E L E K I Q D Y E K Met P D S I Q F I K Q K H G V I C
W S G D F Q E R P Q S A K T R F W K N L R Y Q Met P A Q R R S P L S K H R L L T L D P V R D T K E K L P A
A T H L P L G Stop

IL1- RI *vaviCre* + Amino acid sequence

MENMKVLLGLICLMVPLLSLEIDVCTEYPNQIVLFLSVNEIDIRKCPLTPNKMHGDTIIWYKNDSKTPISADRDS
RIHQQNEHLWFVPAKVEDSGYYYCIVRNSTYCLKTKVTVTVLENDPGLCYSTQATFPQRLHIAGDGSLVCPYVS
YFKDENNELPEVQWYKMKTRGTDLLS

Figure 3-11: Comparison of the amino acid sequence between IL1-RI *vaviCre* – and IL1-RI *vaviCre* +: The DNA sequences for IL1-RI *vaviCre* – and IL1-RI *vaviCre* + were translated into amino acid sequences by the ExPASy bioinformatics resource portal website. The amino acid sequence for IL1-RI was taken from the UCSC Genome Browser website and was used in order to identify the translated sequences. The amino acid sequence for IL1-RI *vaviCre* – was similar to the amino acid sequence for IL1-RI from the UCSC website both containe the specifct membrane site (Red). The IL1-RI *vaviCre* + observed a modified amino acid sequence that compared with the amino acid sequences for IL1-RI *vaviCre* – and IL1-RI from the database.

The established amino acids sequences for IL1-RI *vaviCre* – and IL1-RI *vaviCre* + were used to create a ribbon diagram using the SWISS-MODEL repository website. According to the ribbon diagram of the IL-1β, which was bound to both IL1-RI and IL-1RAcP (Thomas et al., 2012), it was found that the conditional knockout mice IL-1-RI *vaviCre* + lacked the binding site of IL-1α and /or IL-1β (Figure 3-12). This data indicated that IL-1α and /or IL-1β bind normally to IL1- RI in the IL1-RI *vaviCre* – mice. However, the IL1-RI

was unable to bind to IL-1β in IL1-RI *vaviCre* + mice. The binding site for IL-1α is still unkown.

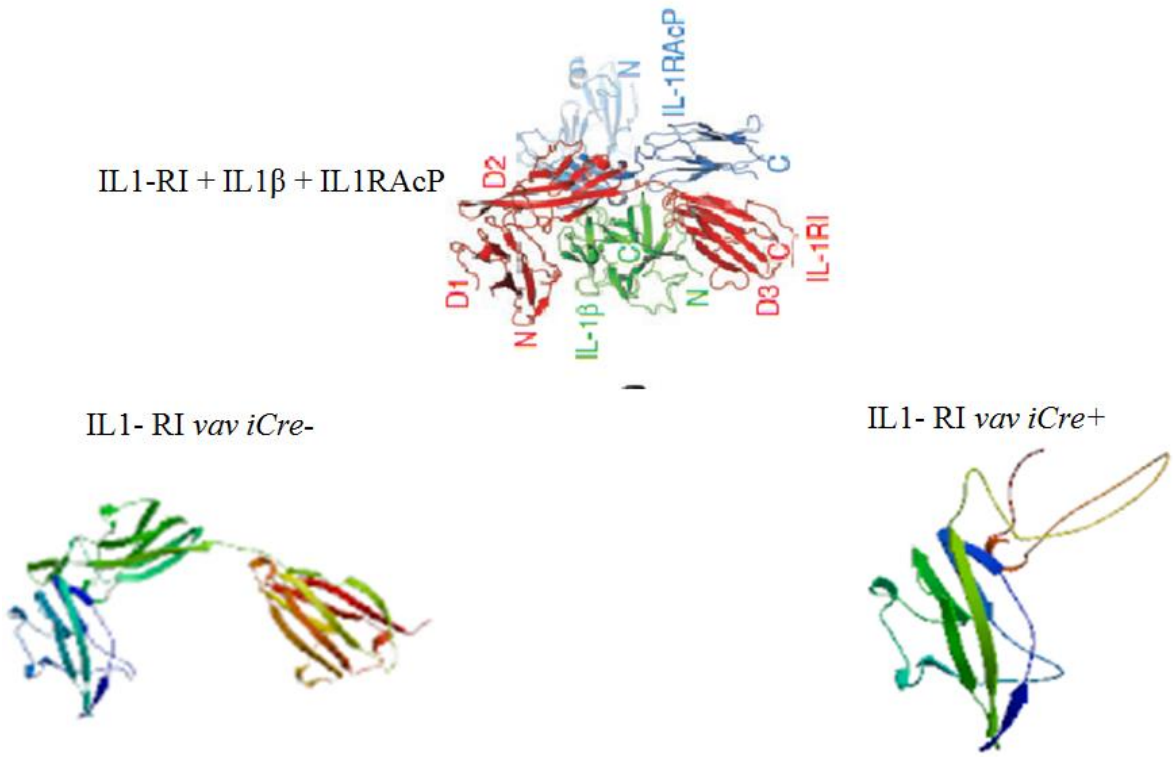


Figure 3-12: A comparison of the ribbon diagram of IL-1β bound to the obetween IL1-RI *vav* – and IL1-RI *vaviCre* +: According to the structures of IL1-RI + IL1β + IL1RAcP published by (Thomas et al., 2012), IL1-RI *vaviCre* + does not contain the binding site of IL-1β and the membrane exon. IL-1α binding site is not known. A SWISS-MODEL repository was used to create the structures.

3.2.2.2 Analysis of the IL1-RI delta allele on the protein level

3.2.2.2.1 The confirmation of abrogation of the IL1-RI protein expression in IL1-RI delta allele conditional knockout mice

To confirm the efficiency of IL1-RI deletion, Western Blots (WB) experiment was conducted in order to test differences in IL1-RI expression between the IL1-RI *vaviCre* – and IL1-RI *vaviCre* +, in response to IL-1β and LPS. In this experiment, the global knockout IL1-RI$^{-/-}$ mice spleen cells were used as a control to investigate whether the deletion of IL1- RI occurred only in hematopoietic cells.

The spleen was isolated from each mouse and 5x10^{6} spleen cells were stimulated with for 24 hours with either 100ng/ml of lipopolysaccharide (LPS) or 20 ng/ml of IL-1β. Unstimulated spleen cells from each transgenic mice were used as a control. The cells

were then lysed using the urea lysis buffer. A western blot experiment was performed using a gradient gel (4-12 %) that was blotted onto a nitrocellulose membrane. The membrane was then analysed for the presence of IL1-RI and the β-actin protein. It was found that IL1-RI *vaviCre* – cells showed an expression of IL1-RI that was evident through the presence of the IL1-RI band. This was observed in both unstimulated cells and cells stimulated with LPS or ILβ. In contrast, IL1-RI *vaviCre* + demonstrated an expression of IL1-RI only when the cells were stimulated with LPS. The IL1-RI protein was not expressed in response to ILβ in the IL1-RI *vaviCre* + cells. The IL1-RI$^{-/-}$ spleen cells did not express IL1-RI in response to the LPS or IL1β (Figure 3-13). This data demonstrates that the successful deletion of IL1-RI in the hematopoietic cells was achieved in IL1-RI *vaviCre* + mice.

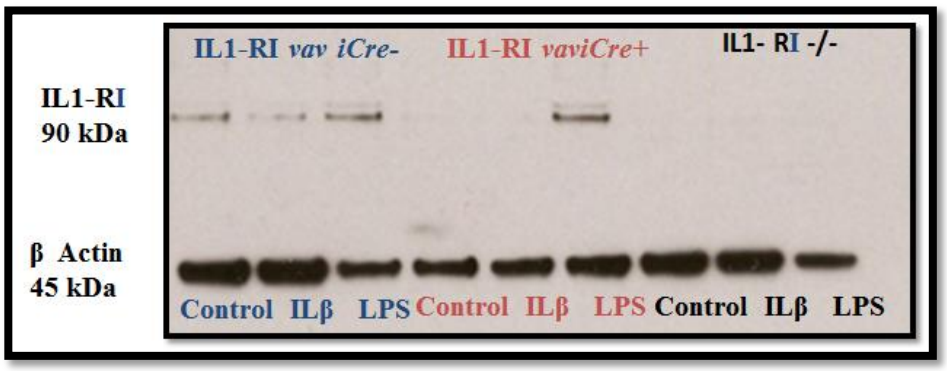


Figure 3-13. The Western Blot Analysis of IL1-R1 in IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI$^{-/-}$ spleen cells: Spleen cells from IL1-RI *vaviCre* –, IL1-RI, *vaviCre* + and IL1-RI$^{-/-}$ were stimulated for 24 hours with either 100 ng/ml LPS or 20 ng/ml IL-1β. Unstimulated spleen cells were used as a control for each mouse. A WB was performed to investigate the expression of IL1-RI. The WB detected a 90 kDa band which corresponded to the IL1-RI in IL1-RI *vaviCre* –. In IL1-RI *vaviCre* +, the WB detected a 90 kDa band only when the cells were stimulated with LPS. IL1-RI was not detected in IL1- RI$^{-/-}$. This experiment was performed twice and demonstrated similar results both times.

3.2.2.2.2 The IL1-RI delta allele showed a reduction in the concentration of IL6, TNFα and MCP-1 *in vitro*

Several studies have shown that both IL-1 α and IL-1β play an important role in the production of IL-6, Tumor necrosis factors α (TNFα) and the chemokine monocyte Chemoattractant Protein-1 (MCP-1) (Tsakiri et al., 2008b, Ajuebor et al., 1998, Ledesma et

al., 2004, Allan et al., 2005). The aim of this experiment was to identify defects in the production of IL6, TNF α and MCP-1 due to the deletion of IL1-RI in hematopoietic cells. In this experiment, spleen cells were harvested from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice, and stimulated with 100 ng/ml of LPS or 20 ng/ml of IL-1β. Supernatants were collected after 24 hours culture for ELISA assay, which was used to determine the concentration of IL-6, TNFα and MCP-1.

It was found that when stimulated with IL-1β, the concentration of TNFα in IL1-RI *vaviCre* – spleen cells was significantly higher than the concentration of the TNFα in IL1-RI *vaviCre* + spleen cells. When stimulated with LPS, both IL1-RI *vaviCre*– and IL1-RI *vaviCre* + spleen cells demonstrated elevated TNFα levels compared to unstimulated cells (Figure 3-14 A). The concentration of IL-6 increased significantly in the IL1- RI *vaviCre* – spleen cells when the cells were treated with IL-1β, compared with unstimulated IL1-RI *vaviCre* – spleen cells. The IL1-RI *vaviCre* – spleen cells stimulated with IL-1β showed a significant increase in the concentration of IL-6 compared with the IL1-RI *vaviCre* + spleen cells treated with IL-1β. Both the IL1-RI *vaviCre* – and IL1- RI *vaviCre* + spleen cells treated with LPS expressed significantly more IL-6 than untreated cells (Figure 3-14 B). The stimulation of IL1-RI *vaviCre* – spleen cells with IL-1β showed a significant increase in the concentration of MCP-1 compared to unstimulated IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells stimulated with IL-1β. The concentration of MCP-1 in the IL1- RI *vaviCre* – and IL1- RI *vaviCre* + spleen cells stimulated with LPS demonstrated no significant difference compared to unstimulated cells (Figure 3-14 C). This data demonstrates that the IL1-RI *vaviCre* – mice have normal IL1 signalling, whereas the responsiveness is abrogated in the IL1-RI *vaviCre* +.

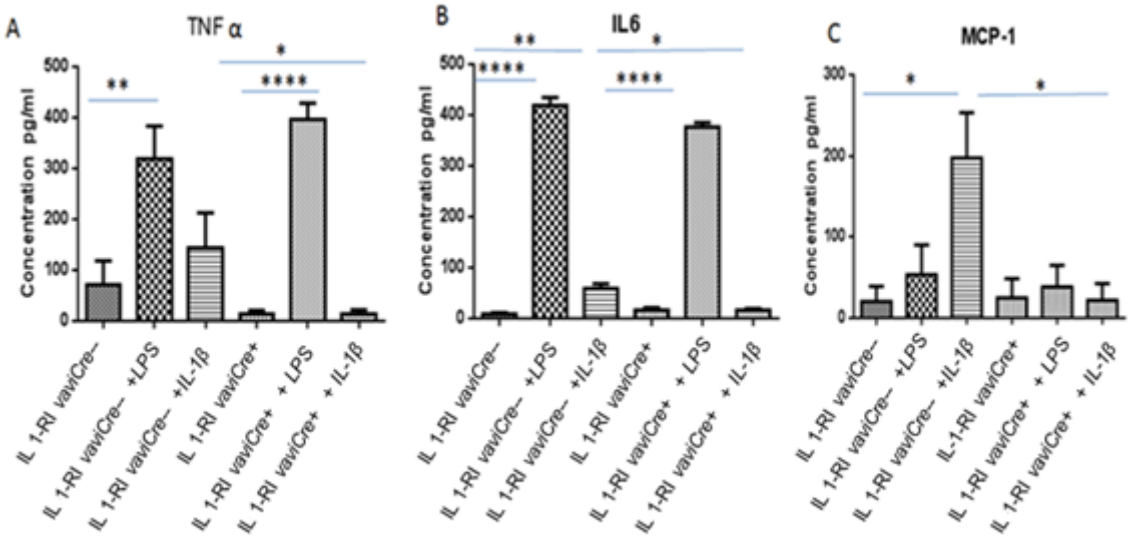


Figure 3-14: Graphical illustration of the concentration of TNF*α*, IL6 and MCP-1 in IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells:
Spleen cells from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were stimulated for 24 hours with 100 ng/ml of LPS or 20 ng/ml of IL-1β. A- IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells were treated with LPS and elevated the concentration of TNFα. A significant difference was found in the concentration of TNFα between IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells upon being stimulated with IL1β.B-. The concentration of IL-6 increased significantly in IL1-RI *vaviCre* – spleen cells when they were treated with either LPS or IL1β. The concentration of IL-6 increased significantly in IL1-RI *vaviCre* + mice when the spleen cells were stimulated with LPS only. The concentration of IL-6 was found to be significantly higher in IL1-RI *vaviCre* – mice than in IL1-RI *vaviCre*+ mice when the spleen cells were stimulated with IL-1β. No significant difference was observed in the IL-1-RI *vaviCre* – or in the IL1-RI *vaviCre* + spleen cells when stimulated with LPS. C- When spleen cells stimulated with IL1-β, IL1-RI *vaviCre* – mice demonstrated a significant increase in the concentration of MCP-1 within IL1-RI *vaviCre* – spleen cells, compared with unstimulated spleen cells from IL1-RI *vaviCre* – and stimulated spleen cells from IL1-RI *vaviCre*+ mice with IL-1β. The data is presented as a mean percentage (+/- SEM) per group, where N=6. To analyse the data, a one-way ANOVA was conducted, followed by a Tukey multiple comparison post hoc test. (* =P≤0.05, ** =P≤0.01 *** =P≤0.001, **** =P≤0.0001).

3.3 DISCUSSION

3.3.1 The Cre-loxP system used for the generation of conditional gene inactivation

The first aim of the study was to generate the conditional *Il1-rI* gene knockout mice with specific inactivation within hematopoietic cells (IL1-RI *vaviCre* mice). IL1-RI *vaviCre* was used to investigate whether hematopoietic cells were responsible for IL-1 signalling during injury or disease. In the last 15 years, the Cre-loxP system has been widely used to generate conditional knockout mice in a specific cells or tissues. The generation of deleting specific genes in the mouse requires breeding of two different types of mice. The first type expresses Cre recombinase and the second type has the specific gene of interest that is flanked by two loxP sites (Castrop, 2010). As discussed in chapter 1, *vaviCre* transgenic mice have been created to inactivate the *vav* gene in the majority of hematopoietic cells (Georgiades *et al*., 2002, de Boer *et al*., 2003). It has been reported that the *vav gene* is expressed and regulates hematopoietic cells in mice (Ogilvy *et al*., 1998, de Boer *et al*., 2003). It was demonstrated that the generated *vaviCre* transgenic mice enhance recombination in the majority of hematopoietic cells (de Boer et al., 2003).

The IL1-RI *vaviCre* mice described in this thesis have been generated by crossing IL1-RI $^{fl/fl}$ mice with IFNγ-R $^{fl/fl}$ *vaviCre* + mice in order to promote the Cre recombination in IL1-RI $^{fl/fl}$. Several breeding steps have been conducted to remove the two loxP sites and the Cre recombinase in the IFNγ-R gene. At the end of the breeding process, IL1-RI $^{fl/fl}$ *vaviCre* demonstrated the efficient recombination in the *Il1-rI* gene only.

The main drawback of using the Cre-loxP system for generating conditional deficient mice is the variability in the efficiency of recombination. This is due to a large number of tissue-specific promoters unable to express the Cre recombinase at levels that are sufficient enough in order to generate the efficient deletion and accessibility of the loxP sites in

target cells (Castrop, 2010). Therefore, the analysis that produced the conditional knockout mice confirmed that gene inactivation by Cre-loxP is important.

3.3.2 Analysis of the IL1-RI delta allele in the genome level

Subsequent to the successful generation of the IL1-RI delta allele in the IL1-RI *vaviCre* mice, a comparison between IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice (the control) was conducted at the genome level in order to validate the *Il1-rI* gene deletion. In this study, the deletion of the DNA segment flanked with two loxP sites containing exon 5 was confirmed by PCR. This was used to detect the IL1-RI delta allele, and then a comparison was conducted on the DNA sequences for IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice. The amplified PCR for extracted DNA from the IL1-RI *vaviCre* + mice demonstrated a smaller band compared to the DNA amplified from the IL1- RI *vaviCre* – mice. Moreover, the sequence of bands IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice from the PCR, were obtained and analysed based on the mouse sequence of IL1- RI. These were obtained from the UCSC Genome Browser. The comparison of DNA sequences for IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice demonstrated the absence of the DNA sequence flanked with the two loxP sites that were extracted from the IL1-RI *vaviCre* + mice.

A clear amino acid modification in the IL1-RI delta allele was also found bioinformatically. This was due to the deletion of exon 5 in the *Il1-rI* gene. The translated DNA sequence for IL1-RI *vaviCre* + and IL1-RI *vaviCre* – in the amino acid sequence were examined based on mouse amino acid sequences. These were provided by the UCSC Genome Browser.

In this study, the comparison between the ribbon diagram, which shows the structure of the protein IL1-RI (Thomas *et al*., 2012), and the ribbon diagrams that were generated using amino acid sequences for IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice , demonstrated that in the IL1-RI *vaviCre* + mice IL1-RI lacked IL-1β binding site.

Genomic data indicated that in the IL1-RI delta allele, the DNA fragment containing exon 5, flanked by two loxP sites was successfully obliterated. The deletion of the *Il1-rI* gene resulted in a modification within the amino acids. This led to the absence of IL-1α and/or IL-1β in the binding site IL1-RI.

3.3.3 The analysis of the IL1-RI delta allele at the protein level

Previous studies have demonstrated that during infection or disease the IL1-RI is predominately expressed within immune and endothelial cells. The binding of IL-1α and/or IL-1β to IL1-RI has been shown to activate the signalling downstream of IL1-RI, resulting in the activation of the NFkB pathway and the increase in production of the immune cytokines of IL-6 and TNFα (Dinarello, 1996).

This study conducted two experiments to determine the responsiveness of IL1-RI in both IL-1β and LPS in order to validate the inactivation of IL1-RI. LPS has been reported to be a strong immune stimulus by interacting with CD4 membrane receptors, which induce the production of cytokines TNFα, IL-1 and IL-6 (Meng and Lowell, 1997). The analysis of WB data demonstrated that the expression of IL1-RI in IL1- RI *vaviCre* – was observed in response to both IL-1β and LPS. In contrast, there was no observed expression of IL1-RI in response to IL-1β and LPS in spleen cells isolated from IL1-RI$^{-/-}$ mice. In IL1-RI *vaviCre* + spleen cells, IL1-RI was expressed in response to LPS, but not in IL-1β. It appears to be plausible that the expression of IL1-RI, found in response to LPS in IL1-RI *vaviCre* + spleen cells, is due to the presence of the non-hematopoietic cells such as epithelial and stromal cells, which are still able to express IL1-RI. It has been demonstrated that epithelial cells release IL-1β in response to antigen *in vivo*, and in inflammatory cells *in vitro* (Hastie *et al*., 1996). LPS has been reported as strong inducer of IL-1β production *in vivo* and *in vitro* (Scheibel *et al*., 2010, Joshi *et al*., 2006). The incubation of blood from wild type mice with LPS leads to the release of IL-1β (Fletcher *et al*., 1995). Previous studies using IL1-RI$^{-/-}$ mice have demonstrated that IL-1β activates the expression of IL1-RI in epithelial and stromal cells (Chen *et al*., 2012, Wohleb *et al*., 2014). Therefore, it is

suggested that the expression of IL1-RI, found in response to LPS in the IL1-RI delta allele, is may due to the production of IL-1β from LPS stimulated epithelial cells that cause an expression on IL1-RI. However, further WB experiments need to be conducted in order to investigate whether the expression of IL1-RI, found in response to LPS in IL1-RI *vaviCre* + cells, is due to the present of epithelial cells. The findings of WB experiment indicate that the specific deletion of IL1-RI in hematopoietic cells was successfully achieved.

ELISA was used to examine whether the deletion of the *Il1-rI* gene caused defect in the production of TNFα, IL-6 and MCP-1 in response to IL-1β and LPS. In the current study, the concentration of IL-6 and MCP-1 increased significantly in the IL-1β stimulated with IL1-RI *vaviCre* – spleen cells, compared to unstimulated IL1-RI *vaviCre* – spleen cells and IL-1β stimulated IL1-RI *vaviCre* + spleen cells. This result is supported by Tsakiri (2008), who demonstrated that primary neurones cells cultured with IL-1β have induced protein synthesis and the expression of IL-6 mRNA. Tsakiri *et al* (2008) also showed that both IL-1β and IL-1α successfully induced the chemokine MCP-1 (Tsakiri *et al*., 2008a).

The findings of the current study further indicated that there was no significant difference in the concentration TNFα when the IL1-RI *vaviCre* – spleen cells were stimulated with IL-1β compared to being unstimulated. This finding can be explained by a previous study which demonstrated that IL-1α not IL-1β was able to induce TNFα synthesis in glial cells (Andre *et al*., 2005). On the contrary, there was a significant difference found in the concentration of TNFα between IL1-RI *vaviCre* –and IL1-RI *vaviCre* + spleen cells upon being stimulated with IL-1β. A plausible explanation for this difference in concentration is due to the presence of IL-1α, which can bind and activate IL1-RI in the IL1-RI *vaviCre* – spleen cells. Further experiments are required to fully investigate and corroborate the observed difference. In further experiments IL-1α will be to use stimulate IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells. The data in the current study demonstrated that both IL1-RI *vaviCre* – and IL1-RI *vaviCre* + spleen cells exhibited a high concentration of

TNFα and IL-6 when stimulated with LPS. IL1-RI and TLR share similar functionality due to the similarity of the cytoplasmic domains between IL1-RI and TLR, which can offer one possible explanation for the this finding (O'Neill, 2008, Dinarello, 2009, Thomas et al., 2012). TLR4 is a member of the TLR family that is responsible for the response to LPS, which was demonstrated using C3H/HeJ mice. These demonstrated a mutation in the TLR4 gene (Poltorak *et al*., 1998). The stimulation of the TLR by LPS leads to the activation of a number of signalling pathways such as NFκB and MAPK proteins. These lead to an expression of cytokines such as IL-6 and TNFα (Smolinska *et al*., 2011, Li *et al*., 2012). The analysis of the IL-6 and TNFα cytokines and MCP-1 chemokine expression confirmed that IL1-RI is dysfunctional in the IL1-RI delta allele.

3.4 CONCLUSION

The results presented in this chapter demonstrated that a new line of mice have been successfully generated, namely the IL1-RI *vaviCre* conditional knockout mice. The genome analysis of the IL1- delta allele confirmed the deletion in IL1- RI. This causes the modification of the IL1-RI amino acid, which results in a lack of a binding site for both IL-1α and/or IL-1β. The proteomic analyses of the delta alleles found no expression of IL1-RI in the hematopoietic cells of IL1-RI *vaviCre* knockout mice. It also showed that the specific deletion of IL1-RI prevented signalling in IL1-RI, as well as the production of cytokine and chemokine. The new line of mice can be used to investigate whether the signalling of IL-1 in hematopoietic cells is required for immune defense. The mice can also be used to understand the development of the T-helper cell lineages Th1, Th2 and Th17 in response to parasitic infection.

CHAPTER FOUR: LYMPHOCYTE DEVELOPMENT IN IL1-RI *vaviCre* CONDITIONAL KNOCKOUT MICE

4.1 INTRODUCTION

IL-1 is a polypeptide cytokine that is involved in several biological processes, including endothelial cell stimulation, lymphocyte activation and mesenchymal tissue remodelling. It has been shown that IL-1 induces immunomodulatory molecules such as IL-2, IL-4, IL-6 and TNFα in several cell types such as lymphocytes, fibroblasts and endothelial cells. The IL-1 cell surface binding receptor IL1-RI binds both IL-1α and IL-1β in all cells, including lymphocytes and endothelials cells (Dinarello *et al*., 1989). In the human thymic epithelium, IL-1 acts as a growth factor for thymocytes. However, the use of IL-1α, IL-1β, or IL-1RI knockout mice found no role for IL-1 in thymic growth and functioning (Dinarello, 2009).

In T cells, IL-1α, or IL-1β combined with an antigen, have been reported as co-stimulators of T cell functioning (Dinarello, 2009). It has been found that IL-1β played an important role in the up-regulating of the expression of CD4 T cells in response to the antigen (Ben-Sasson et al., 2009). It was also found that IL-1 had a strong effect for promoting the of antigen-specific differentiation CD8 T cell, migration expansion and for memory (Ben-Sasson *et al*., 2013).

In B cells, IL-1 has been reported as a factor of growth and differentiation due to its ability to induce the production of IL-6 (Pike and Nossal, 1985, Dinarello, 2009). Furthermore, it has been shown that IL-1β (rather than IL-1α) is required for the production of B cells antibodies against type 1 T-independent antigen. This was found by using IL-1β $^{-/-}$ and IL-1α $^{-/-}$ mice (Nakae *et al*., 2001a).

Previous studies have demonstrated that IL-1β, which is expressed primarily by monocytes and macrophages, plays a role in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis (Pope and Tschopp, 2007). It has also been shown that IL-1β plays an important role in the cellular innate host defence mechanism. This involves phagocytosis and the killing of bacteria and fungi by neutrophils, monocytes, and

macrophages. IL-1β, TNFα, IFNγ and IL-18 cytokine are secreted upon being identified by the microorganism. These cytokines are important for activating neutrophils, monocytes, and macrophages in order to phagocytose the pathogen and to produce toxic oxygen and nitrogen radicals (Netea *et al*., 2010).

The deletion of IL1-RI may affect the development of lymphocytes, which may be cause an alteration in the mice phenotype. Therefore, the aim of the current chapter is to investigate the difference between IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice in the development of the lymphocyte. This is an important experiment , because if no difference is found in the lymphocyte development between IL1-RI *vaviCre* + mice, IL1-RI $^{-/-}$ mice and the control IL1-RI *vaviCre* – mice, the transgenic mice models IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ will provide a vital *in vivo* model for studying the role of IL-1 signalling . If the inflammatory cell composition of IL1-RI *vaviCre* + and IL1- RI $^{-/-}$ mice is found to have changed in comparison to the IL1-RI *vaviCre* – mice, this would suggest that the deletion of the *Il1-rI* gene is able to change the inflammatory pathway of IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. Hence, this would provide an inaccurate model.

4.2 RESULTS

4.2.1 The identification of differentiated immune cells

In order to investigate the development of lymphocyte in the IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice, the composition of leukocytes in the spleen, blood and mesenteric lymph nodes (MLN) were analysed. Using the nine fluorochrome labels and a specific gating strategy, different immune cells were identified as B cells, non-B cells, T cells, monocytes, neutrophils and NK cells (Figure 4-1). This method was established by Frischmann and Muller and was used to analyse blood leukocytes in different mice strains (Frischmann and Muller, 2006).

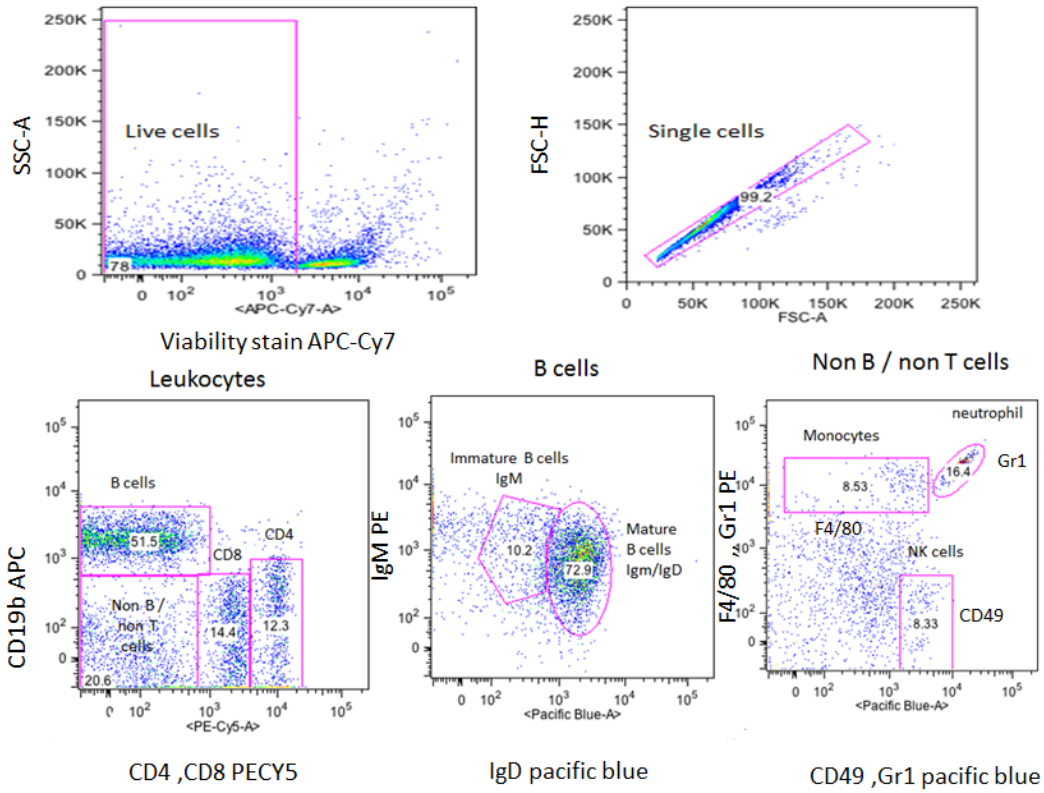


Figure 4-1: Nine fluorochrome labels and a specific gating strategy: The spleen, blood and MLN cells were isolated from the IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. Firstly, leukocytes were separated and differentiated into T cells (CD4^{+} and CD8^{+}) and were gated using PE-Cy5, B cells and non-B/non-T cells, and was gated using CD19-APC. Secondly, B cells were gated and differentiated into immature IgM using PE-IgM^{+}, and mature B IgM^{+}IgD^{+} cells by using (PE-IgM^{+} and Pacific Blue IgD^{+}). Lastly, non-B/non-T cells were separated into NK cells by using Pacific Blue CD49b^{+}, macrophages/monocytes using PE-F4/80^{+}, and into neutrophils by using Pacific Blue Gr1^{+} PE-Gr-1^{+}. Cells were acquired on the LSR-Fortessa machine (BD Biosciences) and the data was analysed using FlowJo.

In the spleen cells no significant difference was observed in the percentage of lymphocytes between IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1- RI $^{-/-}$ mice (Figure 4-2).

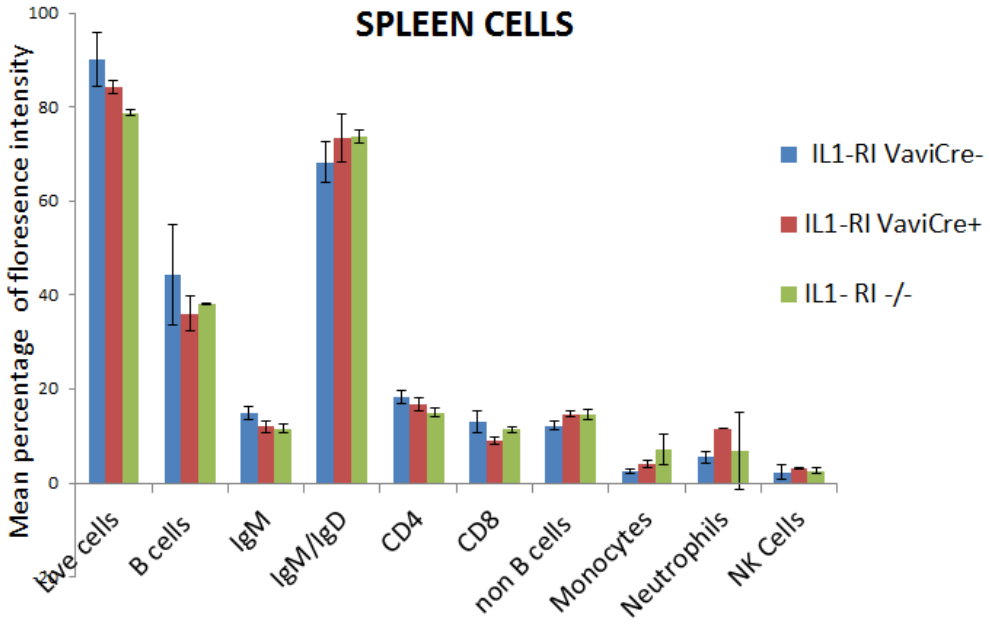


Figure 4-2: The analysis of lymphocytes in the spleen: Spleen cells were isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice and stained using the nine stain protocol. Cells were acquired on the LSR-Fortessa machine (BD Biosciences) and the data was analysed using FlowJo. There was no significant difference between the percentages of IL1-RI *vaviCre*–, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ spleen lymphocyte. The data is presented as mean percentages (+/- SEM) N=3. To analyse the data, a one-way ANOVA for each lymphocyte was conducted, followed by a Tukey multiple comparison post hoc test.

In the blood cells, a significant difference was found in the percentage of immature B cells ($IgM^{+}IgD^{-}$) between IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. Moreover, the percentage of mature B cells ($IgM^{+/}IgD^{+}$) increased significantly in the blood cells isolated from IL1-RI *vaviCre*+ and IL1-RI $^{-/-}$ mice in comparison to IL1-RI *vaviCre* – mice (Figure 4-3 A). In the MLN cells, the percentage of mature B cells ($IgM^{+/}IgD^{+}$) decreased significantly in IL1-RI *vaviCre* + mice in comparison to both IL1-RI *vaviCre* – and IL1-RI$^{-/-}$ mice. MLN cells that were isolated from IL1-RI *vaviCre* + mice demonstrated a significantly higher percentage of immature B cells ($IgM^{+}IgD^{-}$) than MLN cells isolated from the IL1-RI $^{-/-}$ mice (Figure 4-3 B).

This data indicated that no alterations were found in the development of T cells, macrophages monocytes, neutrophils and NK cells between IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. However, an alteration in the development of B cells was found in the blood and MLN. Therefore, B cell differentiation was further investigated.

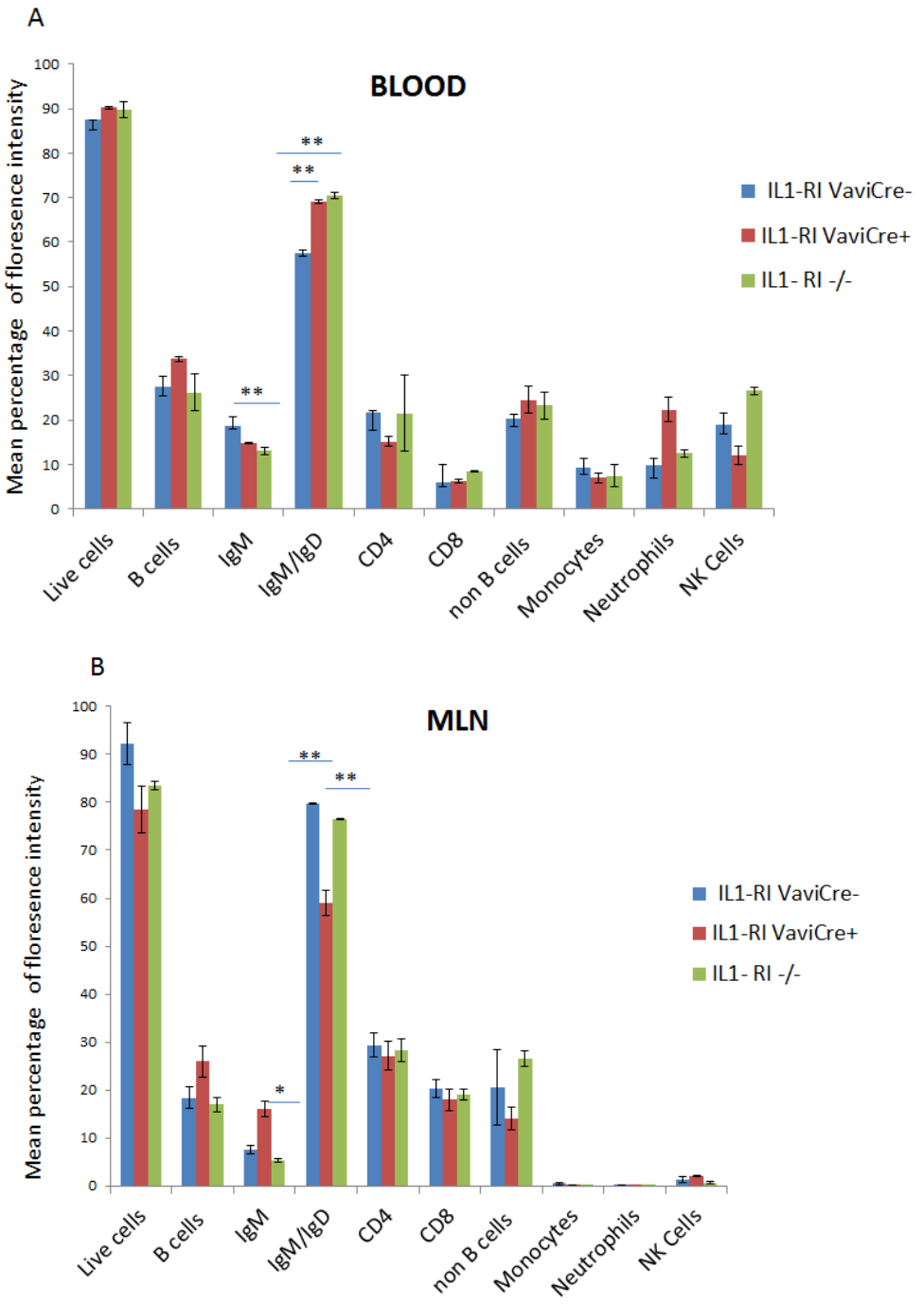


Figure 4-3: The analysis of blood and MLN lymphocyte cells: Blood and MLN cells were isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice and stained using the nine stain protocol. Cells were acquired on the LSR-Fortessa machine (BD Biosciences) and the data was analysed using FlowJo. (A) There were a significant difference between IL1- RI *vaviCre* + and IL1-RI $^{-/-}$ in the percentage of IgM^{+} cells found in the blood. In addition, the percentage of $IgM^{+/}IgD^{+}$ increased significantly in IL1- RI *vaviCre* – in comparison to IL1-RI *vaviCre*+ and IL1-RI $^{-/-}$. (B) The percentage of $IgM^{+/}IgD^{+}$ cells in the MLN decreased significantly in IL1-RI *vaviCre*+ mice in comparison to both IL1-R1 *vaviCre* - and IL1-RI $^{-/-}$ mice. Moreover, there was a significant difference in the percentage of IgM^{+} cells in the MLN of IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. The data is presented as mean percentages (+/- SEM) N=3. To analyse the data, a one-way ANOVA for each lymphocyte was conducted, followed by a Tukey multiple comparison post hoc test (* =P≤0.05, ** =P≤0.01).

4.2.2 B-cell differentiation in bone marrow and spleen

The main aim of the experment was to investigate the change in the development of B cells in both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice . Further investigation was required in order to determine whether the differences that were observed in the nine stain flow cytomety experiment were due to the deletion of IL1-RI, or due to a normal variation between the animal and organ.

In 1993 Ehlich *et al* described a flow cytomety protocol which investiagted the development of B cells in bone marrow (BM). This was described in two stages: (1) in progenitor B cells (pro-B cell); and (2) in pregenitor B cells (pre-B cells) that by used B cells surface marker (Ehlich *et al*., 1993).

In this protocol, the CD45R (B220) was used to identify B cells (CD45R/B220^{+}) in BM. In addition, CD43 was used to differentiate B cells (CD45R/B220^{+}) into pre-B cells (CD45R/B220^{+} CD43^{+}) and pro-B cell (CD45R/B220^{+} CD43^{-}) (Ehlich *et al*., 1993). Thus, in this study B cells were identified by using CD45R (B220) in the spleen and BM. It has been reported that IgM expressed on the surface of B cells identified in the early stage of the development of BM cells, in this stage B cells are immature. When the B cells exit the BM to the peripheral lymphoid organs such as lymph nodes, the spleen or intestinal mucosa tissues, B cells begin to express IgD and become mature (Geisberger *et al*., 2006). Therefore, in spleen cells, both IgM and IgD were used to differentiate B cells into immature B cells (IgM^{+}IgD$^{-)}$ and mature B cells (IgM^{+}IgD^{+}). It was demonstrated that CD117 (C-kit) is a type III receptor tyrosine kinase expressed in the earliest hematopoietic stem cell and plays an important role in cell signal transduction. It is usually found in C-kit phosphorylated and activated by the binding to the stem cell factor (SCF) (Miettinen and Lasota, 2005). The early development of B cells is dependent on BM stromal cells. In early pro-B cells, activated C-kit by the SCF causes an induction of the proliferation of pro-B cells (Janeway CA Jr, 2001). Therefore, in BM cells, the C-kit was used to differentiate

immature B cells (IgM^+IgD^-) into pre-B cells ($C\text{-}kit^-$) and pro B cells ($C\text{-}kit^+$). In this study a specific gating strategy was used to examine the development of B cells in the spleen and in the BM cells isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice (Figure 4-4).

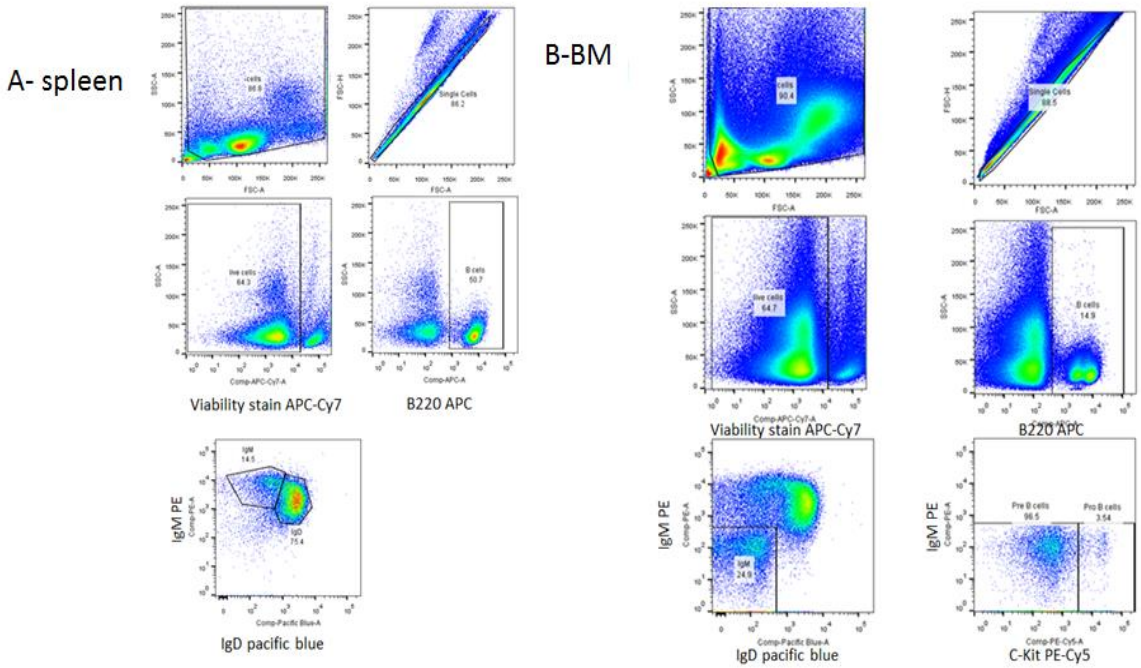


Figure 4-4 : B cells label gating strategy used for (A) the Spleen and (B) in the BM: Cells were isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. In both the spleen and BM cells, the B cells were identified and gated using APC-$B220^+$. Subsequently, B cells were gated and differentiated into immature B cells IgM^+IgD^- by using PE-IgM^+ and mature B cells IgM^+IgD^+ cells using PE-IgM^+ and pacific blue IgD^+. The IgM^+ cells were separated and differentiated only in BM into pre-B cells ($C\text{-}kit^-$) and pro -B cells ($C\text{-}kit^+$) using C-kit PE-CY5. Cells were acquired on the LSR- Fortessa machine (BD Biosciences) and the data was analysed using FlowJo.

In spleen cells, there was no significant difference found in the percentage of total B cells, immature B cells IgM^+IgD^- or mature B cells IgM^+IgD^+ between the IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. (Figure 4-5 A). Furthermore, in the BM there was no significant alteration found in the percentage of total B cells, immature B cells IgM^+IgD^- and mature B cells IgM^+IgD^+ between the IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice (Figure 4-5 B). This data indicates that B cells develop normally in the conditional knockout mice IL1-RI *vaviCre* + and in the complete knockout mice IL1-$RI^{-/-}$.

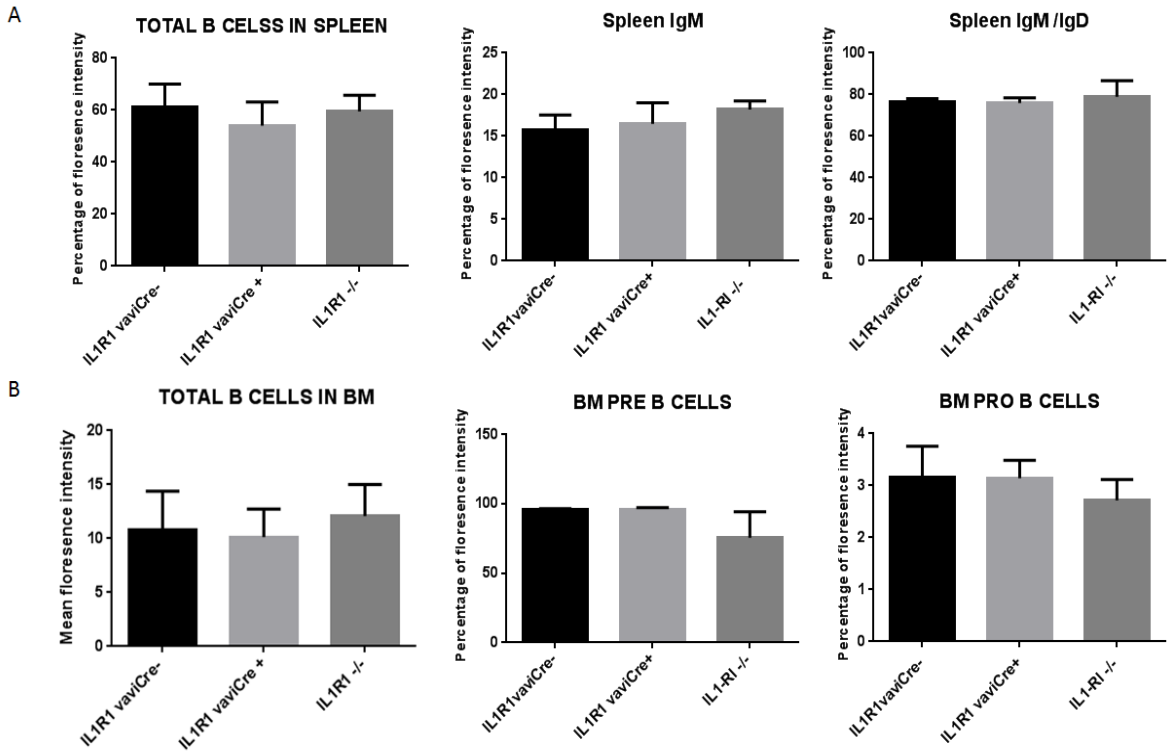


Figure 4-5: The analysis of B cells lymphocyte in the spleen and BM: Spleen and BM cells were isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice and stained using the B development stain protocol described in Ehlich *et al*. Cells were acquired on the LSR-Fortessa machine (BD Biosciences) and the data was analysed using FlowJo. (A) In spleen cells, no significant difference was found in the percentage of total B cells, immature $IgM^{+}IgD^{-}$ and mature $IgM^{+}IgD^{+}$ B cells between the IL1-RI *vaviCre* –, IL1- RI *vaviCre* + and IL1-RI $^{-/-}$ mice. (B) No significant difference was found in the percentage of total B cells, or in both immature $IgM^{+}IgD^{-}$ and $IgM^{+}IgD^{+}$ B cells in the BM isolated from the IL1-RI *vaviCre* –, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. The data above is presented as mean percentages (+/- SEM) N=3. To analyse the data, a one-way ANOVA for each lymphocyte was conducted, followed by a Tukey multiple comparison post hoc test

4.3 DISCUSSION

This chapter focussed on the lymphocyte development in the mice. Applying this monitoring method, in combination with the appropriate analysis, the most important immune effector cells were investigated. These were B cells (immature $IgM^{+}IgD^{-}$ and mature $IgM^{+/}IgD^{+}$ B cells), T cells (CD4 and CD8), macrophages/monocytes, neutrophils and NK cells in the blood (Frischmann and Muller, 2006).

As previously discussed, the IL-1 (especially the IL-1β) plays an important role in the activation and functioning of CD4 and CD8 T cells in response to an antigen (Ben-Sasson *et al*., 2009, Ben-Sasson *et al*., 2013). IL-1 acts as a growth and differentiation factor for B cells, and is considered to be important for the B cell functioning (Pike and Nossal, 1985, Dinarello, 2009, Nakae *et al*., 2001a). IL-1β that is released primarily by monocytes and macrophages plays an essential role in cellular innate host defence, which relies on active monocytes and macrophage (Netea *et al*., 2010). The deletion of IL1-RI may affect the development of lymphocytes, which may cause an alteration in the mice phenotype. Hence, this would provide an inaccurate model for the current study. Therefore, lymphocyte development in the conditional knockout mice IL1-RI *vaviCre+* and the global knockout mice $IL1\text{-}RI^{-/-}$ were investigated.

There was no significant alteration found in the percentage of T cells (CD4 and CD8), macrophages/monocytes, neutrophils and NK cells between the IL1-RI *vaviCre* – and the knockout mice IL1-RI *vaviCre* + and the $IL1\text{-}RI^{-/-}$ in the spleen, blood or MLN. However, a significant change was found in the percentage of B cells in the blood and in the MLN. Therefore, an additional flow cytomety experiment was conducted in order to establish whether the change was due to the deletion of IL1-RI.

The B cells flow cytomety experiment showed that there was no significant change in the development of B cells in the spleen or BM. Thus, it is suggested that the difference in the percentage of B cells found in the blood and in the MLN may be due to an additional

phenotype of these mice. It is not clear whether this is due to deletion of the *Il1-rI* gene or not as yet.

It is indicated that both the nine stain and B cells development flow cytometry experiments should be repeated with a larger number of mice in order to gain sufficient evidence. The data presented in this experiment indicated that lymphocytes develops normally in both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$. Furthermore, IL1-RI is not required for lymphocyte development.

4.4 CONCLUSION

The results presented in this chapter have demonstrated that lymphocyte development was found to be normal and was unaffected by the deletion of the *Il1-rI* gene in both the conditional knockout IL1-RI *vaviCre* + and global knockout mice IL1-RI $^{-/-}$. This indicates that both the IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice are vital *in vivo* models, and can be used for studying the role of the IL-1 signalling in immune defense, and also in the development of T-helper cell lineages Th1, Th2 and Th17 in response to parasitic infection.

CHAPTER FIVE: CHARACTERISATION OF *TRICHURIS MURIS* INFECTED IL1-RI *vaviCre* CONDITIONAL KNOCKOUT MICE

5.1 INTRODUCTION

T.muris is a helminthic whipworm that infects the colon and the caecum of mice and is capable of generating acute or chronic infection depending on the dose of infective eggs given to the mice. It was shown that C57BL/6 mice are resistant to high dose infections (200 eggs) and that these mice develop acute infections, a Th2 response, and are then able to expel the worm. Whilst in a low dose infection (< 60 eggs), the mice developed a chronic infection, a Th1 response and then the mice were unable to expel the worm (Bancroft *et al*., 2001, Hurst *et al*., 2013). Additionally, several studies have shown that Th17 cells response is up-regulation in chronic *T.muris* infection (Levison *et al*., 2010, Fasnacht *et al*., 2009).

The role of IL-1 in response to the *T.muris* infections was first investigated by Helena Helmby and Richard K. Grencis in 2004. By using IL-1α and IL-1β knockout mice, they found that IL-1 was required for the development of Th2 immune response and its cytokine secretion in response to high dose infection with *T.muris.* (Helmby and Grencis, 2004). Moreover, it was shown that the signalling components, IL1-RI and IL1-R AcP, are not required for the development of Th2 and cytokine secretion as well as *T. muris* expulsion (Humphreys and Grencis, 2009).

The cells which are responsible for the functioning of IL-1 in the immune system in response to pathogenic infection are largely unclear. Therefore, to address whether the signalling of IL-1 in hematopoietic cells is required for immune defence and in the generation of Th1, Th2 and Th17 cells, genetically engineered IL1-RI *vaviCre* conditional knockout mice were used. These mice were challenged with varying doses of the infective *T.muris* eggs. Following infection, the T-helper cell response was investigated using a variety of standard approaches such as worm burden, IgG1 and IgG2 antibody response, cytokine responses and histology. If IL-1 signalling expressed from hematopoietic cells is required for T-helper cell development, then it is hypostasised that mice lacking functional IL-1 receptors will not mount a T-helper cell response after.

5.2 RESULTS

5.2.1 Low and high dose infection for conditional knockout mice IL1-RI *vaviCre* with *T.muris*

To investigate the role of IL-1 signalling in hematopoietic cells in the immune defence and the in generation of Th1, Th2 and Th17 cells, IL1-RI *vaviCre* – and IL1-RI *vaviCre* + were infected with a low dose (approx. 20 infective *T.muris* eggs) and a high dose (approx. 200 *T.muris* eggs), for 21 days to challenge Th1, Th2 and Th17 phenotype respectively. Several approaches were used for the analysis: worm burden, IgG1 and IgG2 antibody response, cytokine responses and histology of the mice were used to investigate changes in the immune response in the absence of IL-1.

5.2.1.1 IL1-RI signalling in hematopoietic cells was not required for worm expulsion in high dose infection, while, the absence of IL1-RI signalled in hematopoietic cells impaired worm expulsion in low dose infections.

After 21 days p.i. infection with low or high doses of *T.muris*, the colon and the caecum were isolated from the IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice and a worm count was performed. It was found that the high dose infection IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were resistant to and able to clear the infection (Figure 5-1). Interestingly, it was found that IL1-RI *vaviCre* + mice, were more susceptible than IL1-RI *vaviCre* – mice when they were infected with low dose of *T.muris*. IL1-RI *vaviCre* + mice showed a significant increase in the worm count compared with IL1-RI *vaviCre* – mice (Figure 5-1).

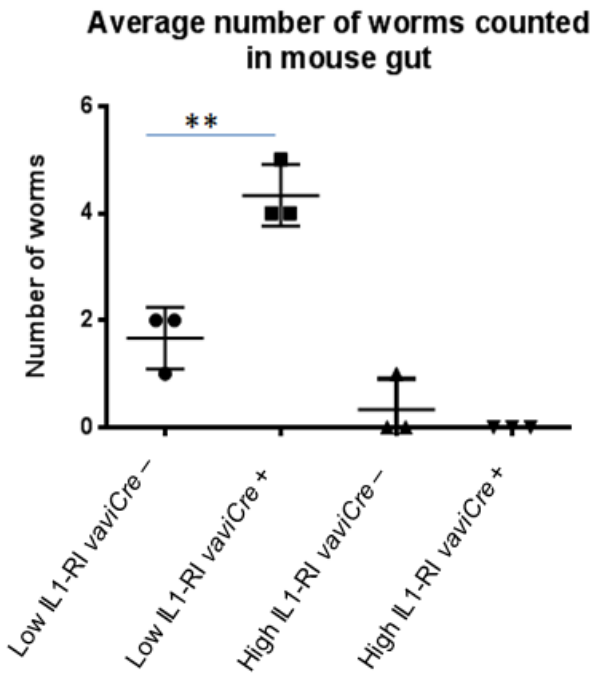


Figure 5-1: Worm burden of IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T.muris.* At 21 day p.i. with low or high dose of *T.muris* the worm burden was counted from isolated IL1-RI *vaviCre* – and IL1-RI *vaviCre* + colon and the caecum. Both IL1-RI *vaviCre* – and IL1-RI *vaviCre*+ infected with high dose *T.muris* eggs expelled the worms during the infection. In low dose infection IL1-RI *vaviCre*+ mice had a significantly higher number of worms than IL1-RI *vaviCre* – mice. The data is presented as a mean percentage (+/- SEM) per group N=3. One way ANOVA followed by a Tukey multiple comparison post hoc tests was used to analyse the data (** =P≤0.01).

5.2.1.2 IL-1 increased Th2 response in absence IL1-RI, While IL-1 signalling in hematopoietic cells is required for strong Th1 response

To investigate specific antibody IgG1 and antibody IgG2 responses to *T.muris* infection in IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice, blood was collected from low or high dose infected mice at 21 days p.i. and then centrifuged to separate the serum. ELISAs were performed to measure the IgG1 and IgG2 response in the serum. We found that IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were able to mount a mix of IgG1 and IgG2 responses when they were infected with low or high dose of *T.muris.* High dose infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice showed significant increase in IgG1 compared with naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice respectively. IgG1 increased significantly in IL1-RI *vaviCre* + mice compared with IL1-RI *vaviCre* – mice (Figure 5-2 A). Low dose infected IL1-RI *vaviCre* – mice mounted a significantly greater

IgG2 response compared with uninfected IL1-RI *vaviCre* – and infected IL1-RI *vaviCre* + mice. Low dose infected IL1-RI *vaviCre* + mice, had significantly elevated IgG2 levels compared with uninfected IL1-RI *vaviCre* + mice (Figure 5-2B). The change in the Th1 and Th2 responses between infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice and uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice, indicates that the low and high dose infections were performed successfully.

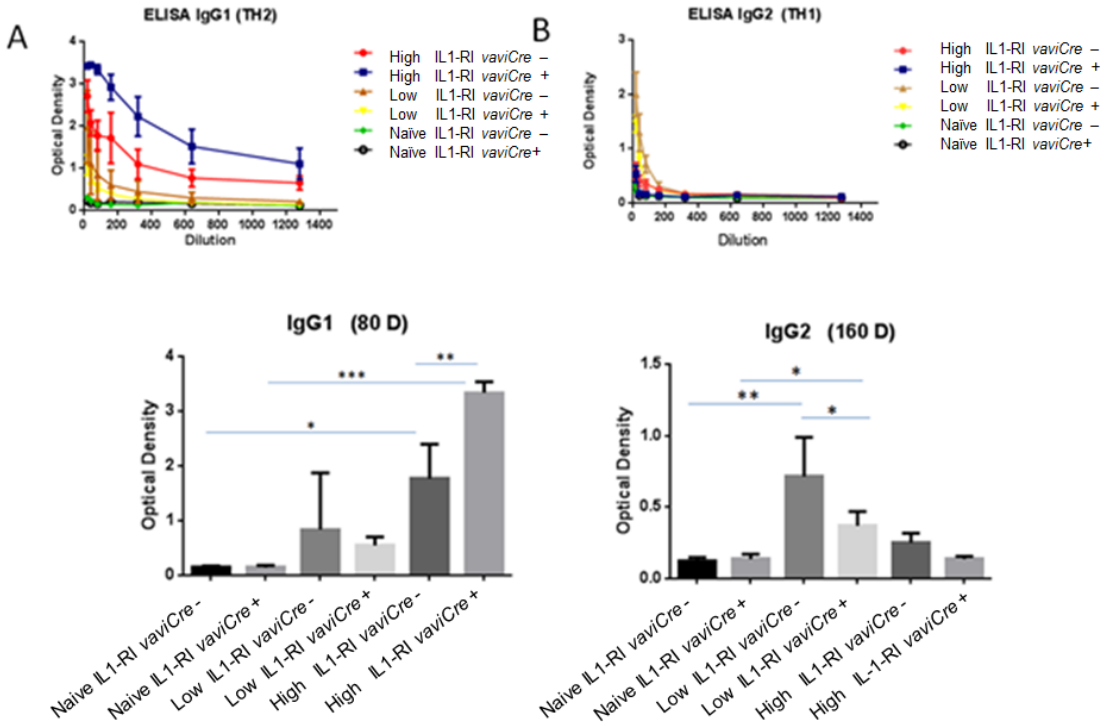


Figure 5-2: Specific *T.muris* IgG1 and IgG2 antibody in the serum of IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T. muris.* At day 21 p.i. with low or high doses of *T.muris*, ELISAs measured specific *T.muris* IgG1and IgG2 antibody in the serum of infected mice. (A) There was a significant increase in IgG1 response in high dose infected IL1-RI *vaviCre*+ and IL1-RI *vaviCre* – mice compared with naïve IL1-RI *vaviCre*+ and IL1-RI *vaviCre* – mice respectively. In high dose infected mice IgG1 increased significantly in IL1-RI *vaviCre*+ compared with IL1-RI *vaviCre* – mice (B) IL1-RI *vaviCre*+ and IL1-RI *vaviCre* – mice, infected with low dose *T.muris* had a significant increase in IgG2 levels compared to naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre*+ mice respectively. Moreover, in low dose infected mice IgG2 levels increased significantly in IL1-RI *vaviCre* – compared IL1-RI *vaviCre* + mice. 1:80 dlution (180D) and 1:160 dilution (1:160D) were chosen to analyse both IgG1 and IgG2 respectively. Data is presented as a mean percentage (+/- SEM) per group N=3. One- way ANOVA followed by a Tukey multiple comparison post hoc tests (* =P≤0.05, ** =P≤0.01 *** =P≤0.001).

5.2.1.3 Lack of IL-1 signaling in hematopoietic cells caused a reduction of the Th17 response as well as IL22 production in the mice infected with a low dose of *T.muris.*

Th1, Th2 and Th17's development was investigated according to their related cytokines IFNγ, IL4 and IL22 respectively. IFNγ, IL4 and IL22 cytokines were measured using ELISA. At day 21 p.i., with a low or high dose of ***T.muris***, the MLN were isolated from infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* **+** mice. Cells were then re-stimulated *in vitro* with a final concentration of 50 mg/mL parasite E/S antigen for 48 h.

It was found that the concentration of IFNγ and IL4, increased in the MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with high dose, compared with MLN cells isolated from naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice **(**Figure 5-3 A-B**).** MLN cells isolated from low dose infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice produced a low concentration of IL4 and a high concentration of IFNγ. However, the concentration of IFNγ in the MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with a low dose was higher than MLN cells isolated from IL1- RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with a high dose **(**Figure 5-3 A-B**).** Additionally, no significant difference was observed in the concentration of IFNγ and IL4 between MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T.muris* **(**Figure 5-3 A-B**).** In contract, in low dose infected mice the concentration of IL22 decreased significantly in MLN cells isolated from IL1-RI *vaviCre* **+** compared with MLN cells isolated from IL1-RI *vaviCre* – mice. Moreover, in high dose infection MLN cells isolated from IL1-RI *vaviCre* – mice had a higher concentration of IL22 levels than MLN cells isolated from IL1-RI *vaviCre* + but this difference was not significant **(**Figure 5-3 C**)**.

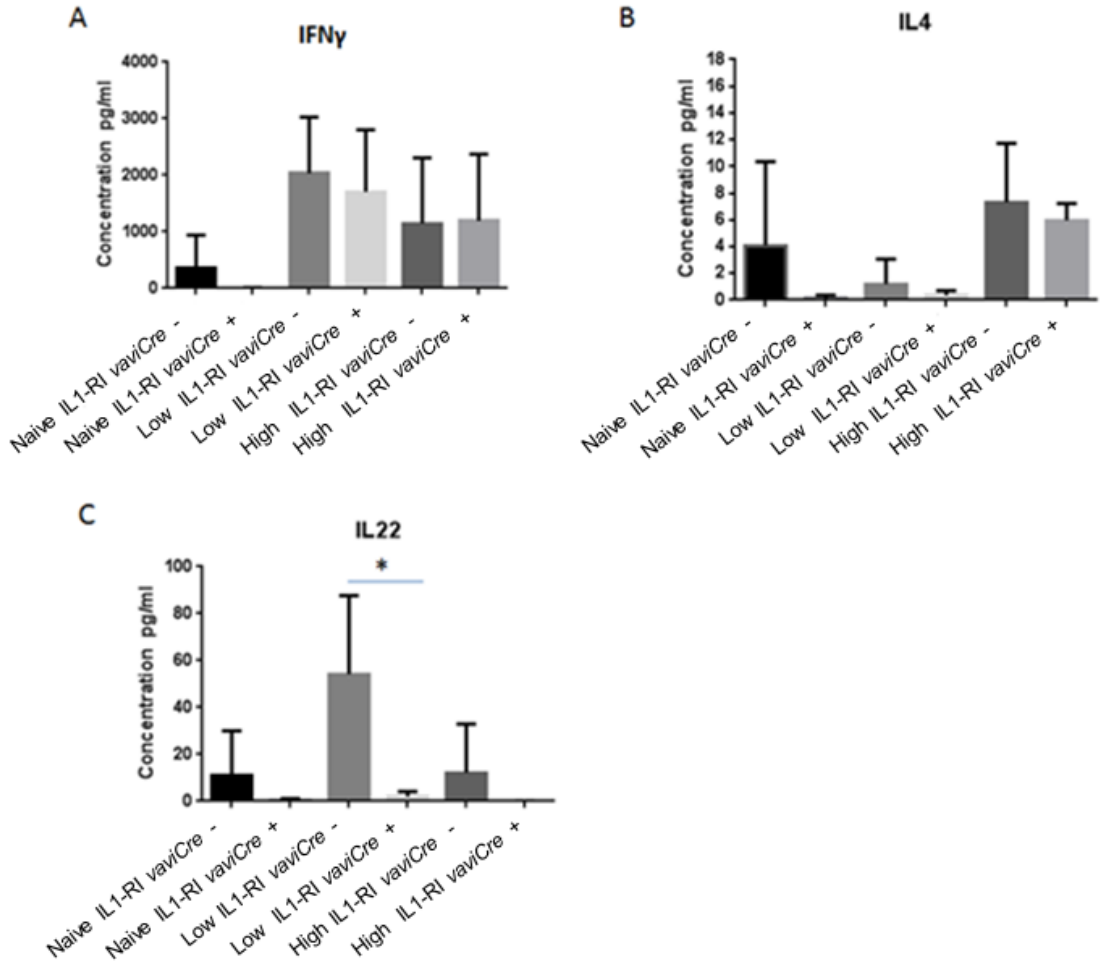


Figure 5-3: Graphical illustration of the concentration of IFNγ, IL4 and IL22 expressed by MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T.muris* : MLN cells were removed from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice at day 21 p.i with low or high dose of *T.muris*. MLN cells were then re-stimulated with a final concentration of 50 mg/mL parasite E/S antigen for 48 h. (A-B) The concentration of IFNγ and IL4 increased in the IL-RI *vaviCre* – and IL1-RI *vaviCre* + MLN cells isolated from mice infected with a high dose *T.muris* compared with MLN cells isolated from naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre* +. While, in MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with a low dose *T.muris* infection, the concentration of IL4 was very low and the concentration of IFNγ was very high. The concentration of IFNγ in the MLN cells isolated from RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low dose was higher than MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with a high dose. In addition, no significant difference was showed in the concentration of IFNγ and IL4 between MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose *T.muris*. (C). In low dose infections MLN cells isolated from IL1-RI *vaviCre* + mice was significant decreased in the concentration of the IL22 level, when compared with MLN cells isolated from IL1- RI *vaviCre* – mice. In high dose infection MLN cells isolated from IL1-RI *vaviCre* – mice had higher concentration of IL22 levels than MLN cells from isolated from IL1-RI *vaviCre* +, this difference was not significant. Data is presented as a mean percentage (+/- SEM) per group N=3. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to analyse the data. (* =P≤0.05).

5.2.1.4 Histological analysis of IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T.muris*

Crypt lengths on histology sections were used to measure the physical markers of colon inflammation (colitis). Histology was used to investigate goblet cells hyperplasia, which is responsible for secreting mucus into the gut and protecting the intestinal epithelium from invasion by foreign bodies such as *T.muris*. At day 21 p.i. with low or high dose T.*muris*, part of the colon from infected mice was removed and embedded in wax. Five micron sections were cut and stained with hematoxylin and eosin or goblet cells stain (periodic acid-Schiffs).

5.2.1.4.1 Increased crypt hyperplasia in IL1-RI *vaviCre* + mice infected with low dose of *T.muris* due to a lack of IL-1 signalling in hematopoietic cells

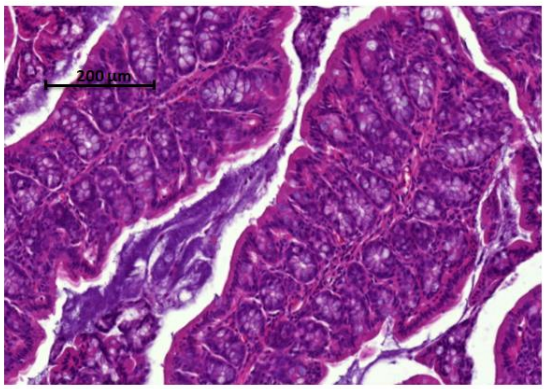
The measurement of crypt length shows that no significant different was observed in crypt length between IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with high dose of T.*muris* (Figure 5-4 B). In contrast, in low dose infections IL1-RI *vaviCre* + mice showed a significant increase in crypt hyperplasia compared with low dose infected IL1-RI *vaviCre* – and uninfected IL1-RI *vaviCre* + mice (Figure 5-4 A,C).

5.2.1.4.2 Decrease of goblet cell hyperplasia in IL1-RI *vaviCre* + infected with low or high doses of *T.muris* due to an absence of IL-1 signalling in hematopoietic cells

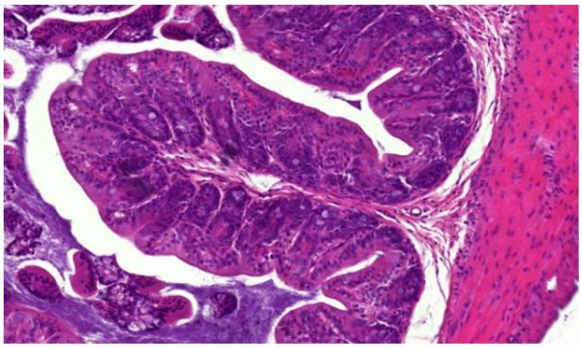
In the analysis of goblet cells we found that both low and high dose infections, had a significant decrease in the goblet cells numbers in IL1-RI *vaviCre* + mice compared with IL1-RI *vaviCre* – mice (Figure 5-5B,C)**.** No goblet cell hyperplasia was observed in IL1-RI *vaviCre* + mice infected with low or high doses of *T.muris*. Moreover, IL1-RI *vaviCre* – mice infected with high dose of *T.muris* had a significant increase in goblet cell numbers compared with the naïve IL1-RI *vaviCre* – mice (Figure 5-5A,B).

A

Naïve



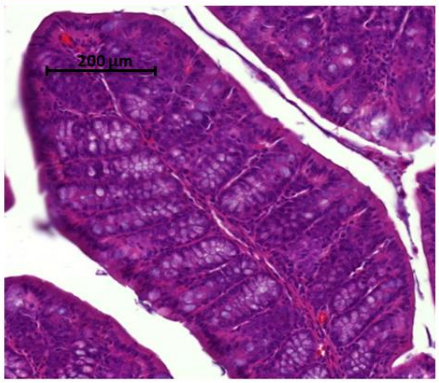
IL1- RI *vaviCre* -



IL1- RI *vaviCre* +

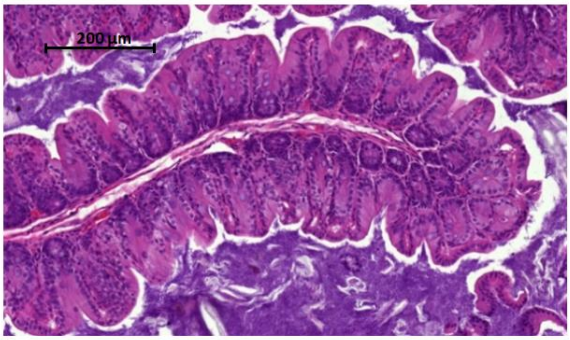
B

High Dose

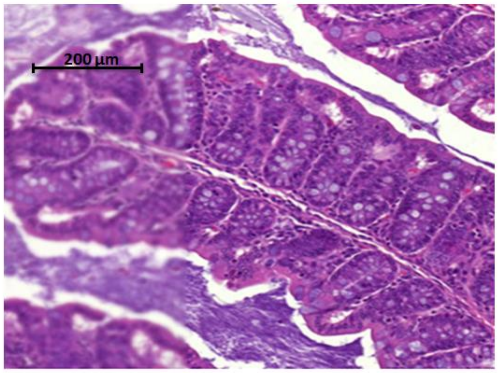


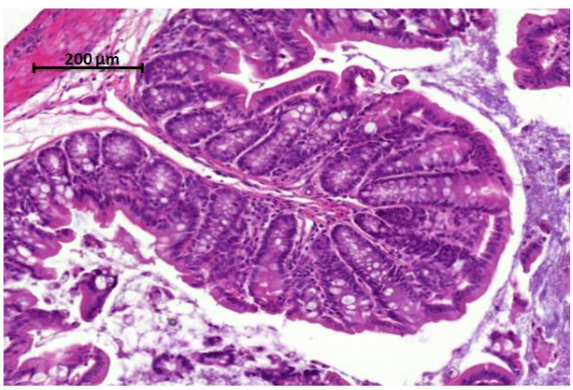
C

Low Dose



IL1- RI *vaviCre* -





IL1- RI *vaviCre* +

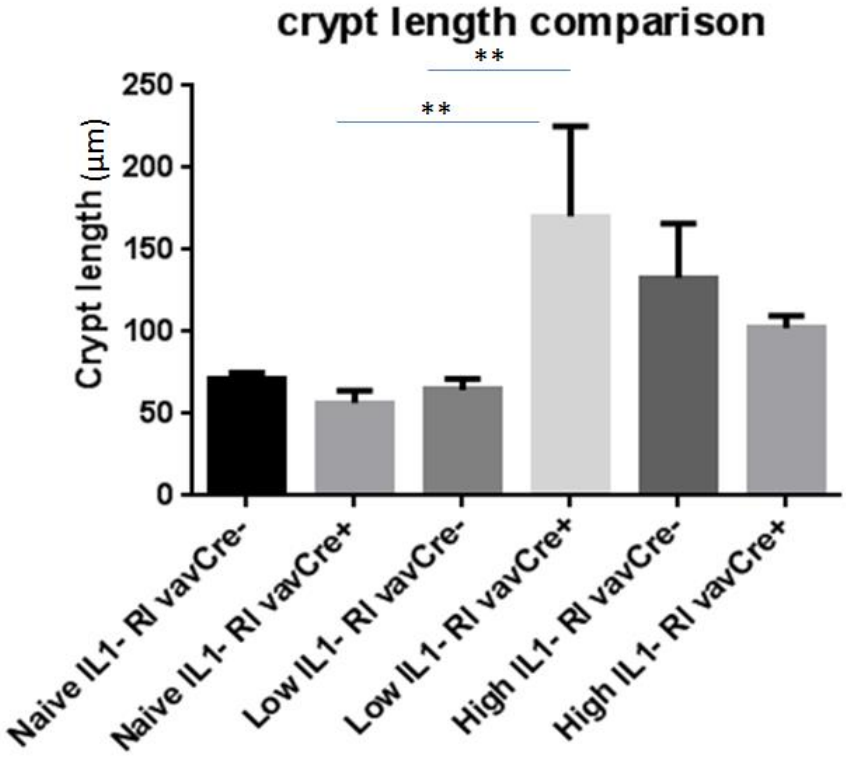
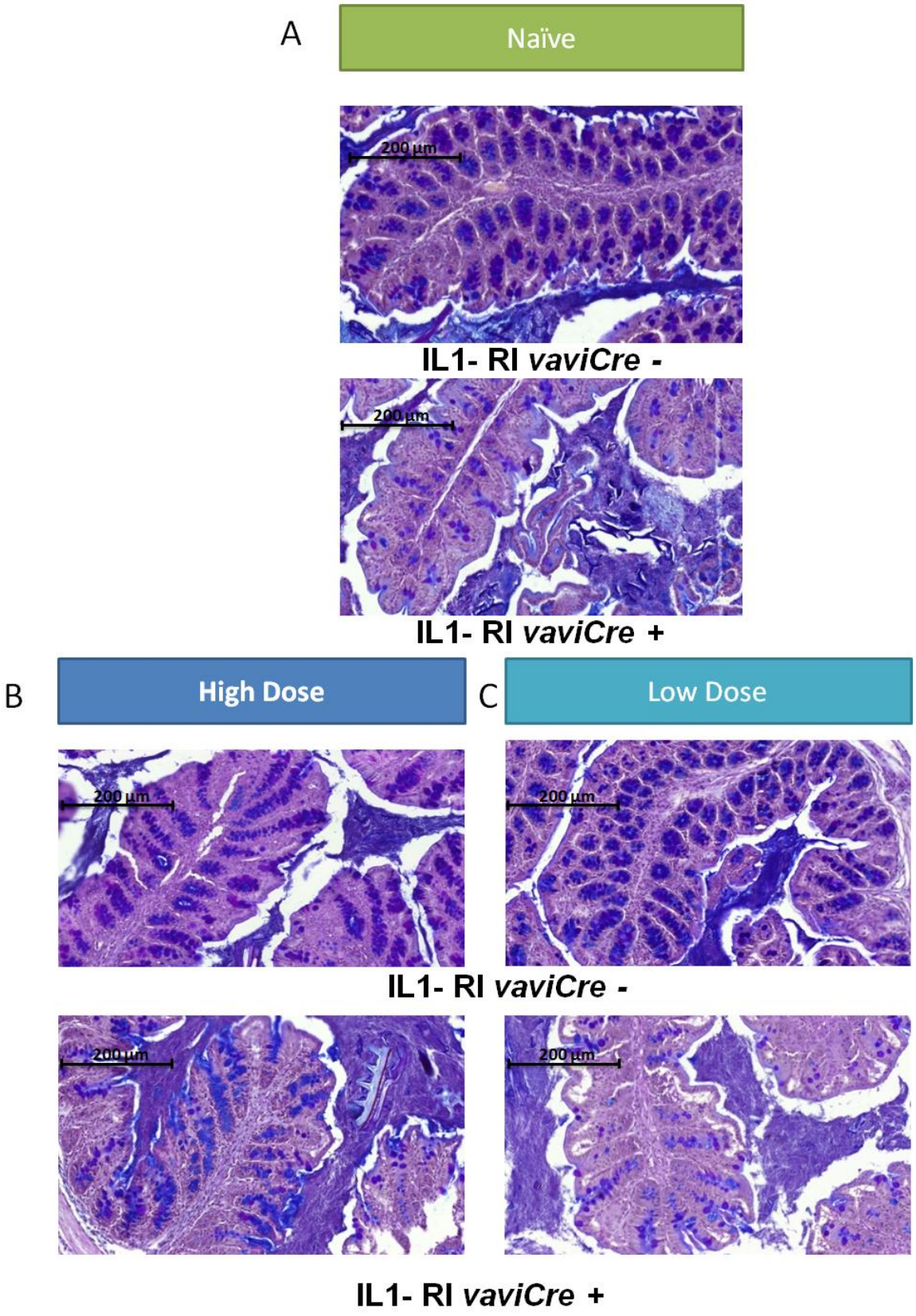


Figure 5-4: Colonic crypt lengths in IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice on day 21 p.i. with high or low dose of *T.muris*. Mice were infected with low or high dose of *T.muris*: At day 21 p.i, the colon was isolated from the mice and embedded in wax. 5 micron sections were cut and stained with hematoxylin and eosin. The slides were scanned by Panoramic viewer system and the crypt lengths were measured using ImageJ software. All pictures magnification are 10x. D: (B) *No* significant difference was observed in the crypt lengths between IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with high dose of *T.muris.*While, (A,C) in low dose infected IL1-RI *vaviCre* + mice had a significant increase in the crypt length compared with infected IL1-RI *vaviCre* – and naïve IL1-RI *vaviCre* + mice. The data is presented as a mean percentage (+/- SEM) per group N=3. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to analyse the data. (** =P≤0.01)


A
Naïve
200 µm
IL1- RI vaviCre -
200 µm
IL1- RI vaviCre +
B
High Dose
C
Low Dose
200 µm
200 µm
IL1- RI vaviCre -
200 µm
200 µm
IL1- RI vaviCre +

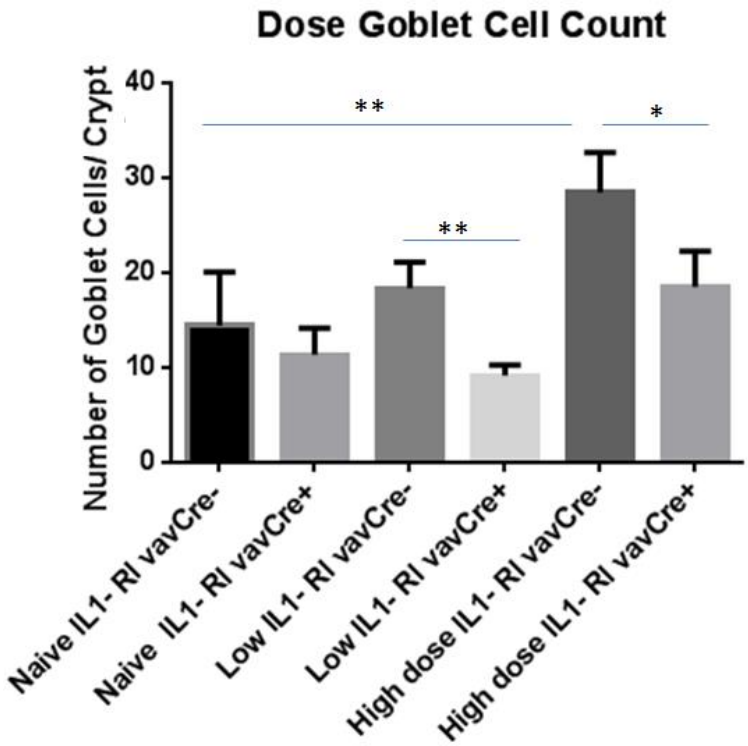


Figure 5-5: Intestinal goblet cells count in the IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice on day 21 p.i. with high or low dose of *T. muris.* Mice were infected with low or high dose of *T.muris*. At day 21 p.i the colon was isolated from the mice and embedded in wax. 5 micron sections were cut and stained with goblet cells stain (periodic acid-Schiffs). *The* slides were scanned by Panoramic viewer system and the goblet cells were enumerated in 20 randomly selected crypt. All pictures magnification are 10x.(B-C) There was a significant decreased in the goblet cell count between IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice infected with low or high dose of *T.muris.* (A-B) Moreover, the number of the goblet cells increased significantly in IL1-RI *vaviCre* – infected with high dose compared with naïve IL1-RI *vaviCre* – mice. The data is presented as a mean percentage (+/- SEM) per group N=3. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to analyse the data. (* =P≤0.05, ** =P≤0.01 *** =P≤0.001).

5.2.2 Low dose *T.muris* infected IL1-RI *vaviCre* +, IL1-RI $^{-/-}$ and IL22$^{-/-}$ mice.

In the previous experiment it was found that the obliteration *IL1- RI* gene in hematopoietic cells, which prevents IL-1 signalling, leads to a change in IL1-RI *vaviCre*+ phenotype in response to low dose infections with ***T. muris***. One of the important changes observed was a clear reduction on the Th17 response and its cytokine IL22. Low dose *T.muris* infections were repeated and global IL22 knockout mice (IL22 $^{-/-}$) mice were used to investigate if the reduction of Th17 response and it cytokine IL22, is responsible for the change in the IL1-RI *vaviCre* + phenotype. In this experiment global IL1-RI knockout mice (IL1-RI $^{-/-}$) were used and compared to the conditional knockout IL1-RI *vaviCre*+ mice to confirm the result and to investigate if the inhibition of IL-1 signaling in hematopoietic cells, leads to a change in the knockout mice phenotype in response to the *T.muris* infection. It was reported that C57BL/6 mice are slow responders to *T.muris* infection and that the generation of chronic infection can be achieved by infecting the mice with low dose (< 60) of infective eggs (Bancroft *et al*., 2001, Hurst *et al.*, 2013). Thus, in this experiment the mice were infected with 30 infective eggs of ***T.muris***.

5.2.2.1 Genotyping PCR for IL1-RI $^{-/-}$, IL1-RI ^{flox} / ^{flox} and IL22 $^{-/-}$

DNA extracted from mouse ear punches was used to genotype the homozygous IL1-RI ^{flox}/ ^{flox} mice and the delta mice IL1-RI $^{-/-}$ and IL22 $^{-/-}$ by PCR. IL1-RI $^{-/-}$ and IL1-RI ^{flox}/ ^{flox} mice were provided from Emmanuel Pinteaux lab. The primers used to genotyping IL1-RI ^{flox} / ^{flox} are oligos 3 (2960-39: CTAGTCTGGTGGAACTTACATGC) and oligos 4 (2960-40: AACTGAAAGCTCAGTTGTATACAGC) and IL1- RI ^{flox} / ^{flox} shows a band at 432 bp (Figure 5-6 A). Moreover, IL1-RI $^{-/-}$ genotyping shows a band at 400 bp using the oligos 1 (2959-32: CTAGTCTGGTGGAACTTACATGC) and oligos 4 (32960-39 :GATAAACGAGAGGAGACAGG) primers (Figure 5-6 B). Furthermore, IL22 $^{-/-}$ mice were generated by Jean-Christophe Reanauld at the Ludwig Institute of Cancer Research in Brussels, Belgium. IL22 $^{-/-}$ mice were provided from Richard Grencis lab, and its genotyping showed bands at 585 bp using

NeoF primer (GAACAAGATGGATTGCACGCAGGTTC) and

Neo R primer: (CGATATTCGAAGCAGGCTA) (Figure 5-6 C).

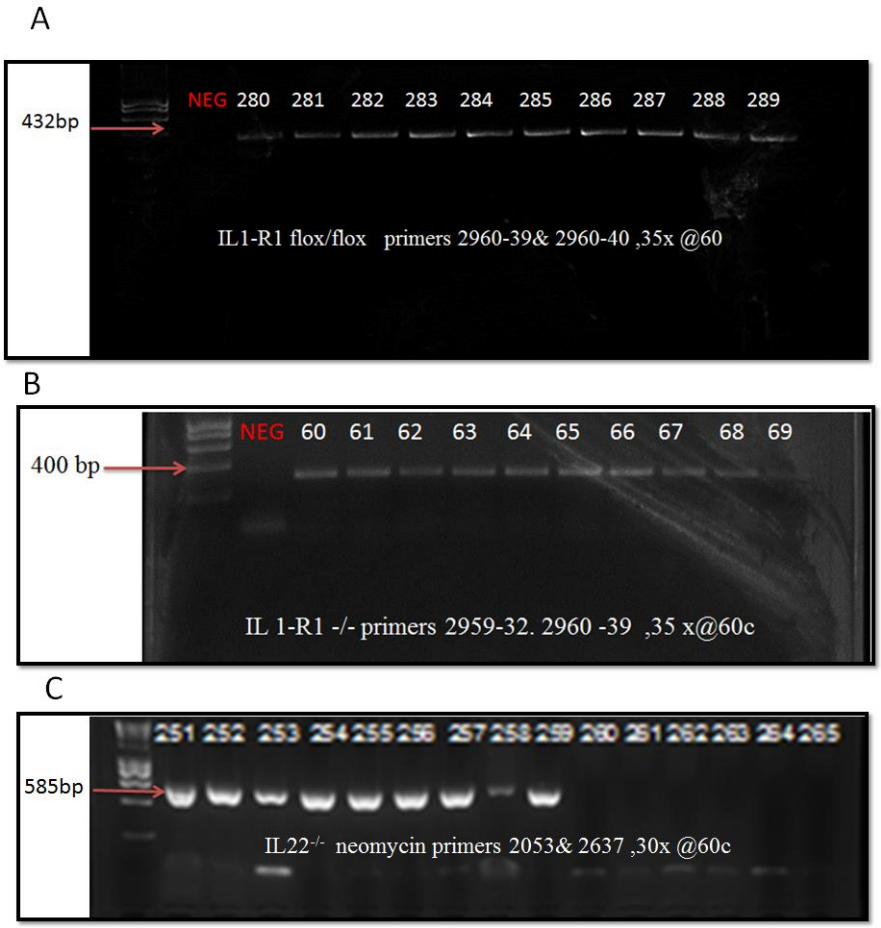


Figure 5-6: Genotyping PCRs for detecting (A) IL1-RI ^{flox} / ^{flox} (B) IL1-RI $^{-/-}$ and IL22$^{-/-}$: Mouse genomic DNA was extracted from ear punches . PCRs were performed with the appropriate primers and visualized on a 1% agarose gel. (A) The homozygous mice IL1-RI ^{flox} / ^{flox} showed a band at 432 bp. (B). The IL1- RI delta mice IL1- RI $^{-/-}$ showed a band at 400 bp. (C) The IL22 delta mice IL22 $^{-/-}$ showed a band at 585 bp.

5.2.2.2 IL1-RI *vaviCre* – mice were able to expel the worm when infected with a low dose of *T.muris* unlike IL1-RI $^{flox}/^{flox}$

At 21 day p.i, with a low dose of *T.muris,* the worms were counted from colon and caecum isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* **+,** IL1-RI $^{flox}/^{flox}$, IL1-RI $^{-/-}$, WT and IL22 $^{-/-}$. As expected, IL1-RI $^{flox}/^{flox}$, IL1-RI $^{-/-}$, WT and IL22$^{-/-}$ were susceptible to the infection. No significant difference was observed between IL1-RI $^{flox}/^{flox}$ and IL1-RI $^{-/-}$ mice and between WT and IL22 $^{-/-}$ mice respectively (Figure 5-7).Once again low dose infection IL1-RI *vaviCre* – mice were able to expel more worms than IL1-RI *vaviCre* + mice. IL1-RI *vaviCre* – mice had a significantly decreased in worm count compared with IL1-RI *vaviCre* + mice. Interestingly, the worms count in the IL1-RI $^{flox}/^{flox}$ mice was significantly higher than IL1-RI *vaviCre* – mice (Figure 5-7). IL1-RI $^{flox}/^{flox}$ was used as a control since they were the parental strain for the generation of IL1-RI $^{-/-}$ mice.

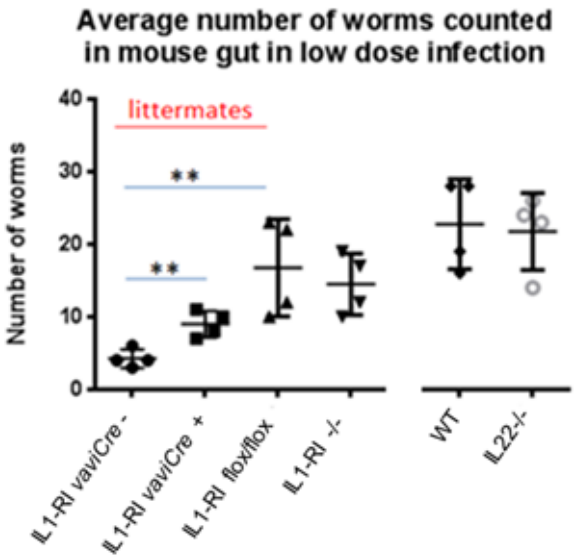


Figure 5-7: Worm burden of IL1-RI *vaviCre* –, IL1-RI *vaviCre* +, IL1-RI $^{flox}/^{flox}$, IL1-RI $^{-/-}$, WT and IL22$^{-/-}$ mice infected with a low dose of *T.muris.* At 21 day p.i. with a low dose of *T.muris* the worm burden was counted in the guts of the mice. No significant differences were observed in the worm count between IL1-RI $^{flox}/^{flox}$ and IL1-RI $^{-/-}$ mice and between WT and IL22 $^{-/-}$ mice. In contrast, in the littermates IL1- RI *vaviCre*+ and IL1-RI *vaviCre* – mice, the worm burden increased significantly in IL1- RI *vaviCre*+ compared with IL1-RI *vaviCre* – mice. Moreover, IL1-RI$^{flox}/^{flox}$ had a significant increase in the worm burden compared with IL1-RI *vaviCre* – mice. The data is presented as a mean percentage (+/- SEM) per group N=4. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI $^{flox}/^{flox}$ and IL1- RI $^{-/-}$ mice . T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (* =P≤0.05, ** =P≤0.01).

5.2.2.3 Increased dose of the low dose infection elevated the Th2 response in IL1-RI *vaviCre* mice.

ELISAs were performed to analyse *T.muris* specific antibody responses IgG1 and IgG2 in the serum of low dose infected mice at 21 days p.i. We found that IgG2 response increased significantly in infected IL1-RI *vaviCre* – mice compared with infected IL1-RI *vaviCre* + mice. Furthermore, there was a lower IgG2 response in IL1-RI $^{-/-}$ and IL22 $^{-/-}$ than IL1-RI ^{flox} / ^{flox} and WT mice respectively, but this difference was not significant (Figure 5-8).

Interestingly, low dose infection with 30 eggs cause an increased in Th2 response in IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice, but not in IL1-RI ^{flox} / ^{flox} and IL1-RI $^{-/-}$ mice. We found that infected IL1-RI *vaviCre* + mice showed a significant increase in IgG1 response compared with IL1-RI *vaviCre* – mice (Figure 5-9). IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice had a significant increase in IgG1 compared with uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice respectively. There was also a significant increase in IgG1 in IL1-RI *vaviCre* + compared with IL-RI ^{flox} / ^{flox} and IL1- RI $^{-/-}$ mice (Figure 5-9). Furthermore, no significant difference in IgG1 levels was observed between IL1-RI ^{flox} / ^{flox} and IL1- RI $^{-/-}$ and between WT and IL22 $^{-/-}$ mice (Figure 5-9).

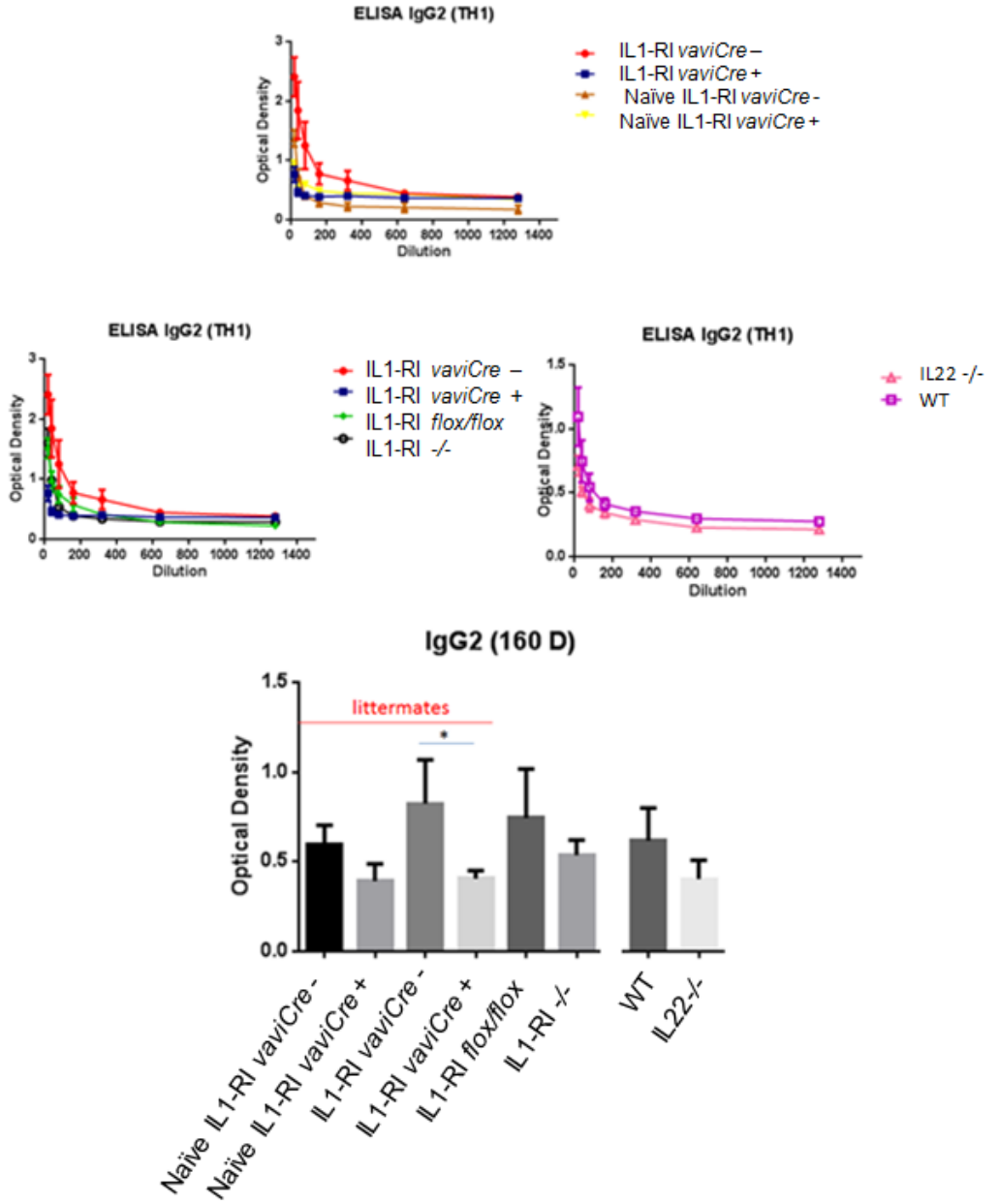


Figure 5-8: Specific *T. muris* antibody IgG2 levels in IL1-RI *vaviCre* –, IL1-RI *vaviCre* +, IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, WT and IL22$^{-/-}$ mice infected with low dose of *T. muris.* At 21 day p.i. with a low dose infection of *T.muris*, specific antibody to *T.muris* IgG1 and IgG2 was measured in the serum of infected mice by ELISAs. There was a significant increase in IgG2 response in infected IL1-RI *vaviCre* mice – compared with infected IL1-RI *vaviCre* + mice. IL1- RI $^{-/-}$ and IL22 $^{-/-}$ mice had lower IgG2 levels than IL1- RI ^{flox} / ^{flox} and WT mice respectively, but this difference was not significant. IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were littermates. 1:160 dilution (160D) was chosen for analysis. Data is presented as a mean percentage (+/- SEM) per group N= 4. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI ^{flox}/ ^{flox} and IL1- RI $^{-/-}$ mice . T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (* =P≤0.05)

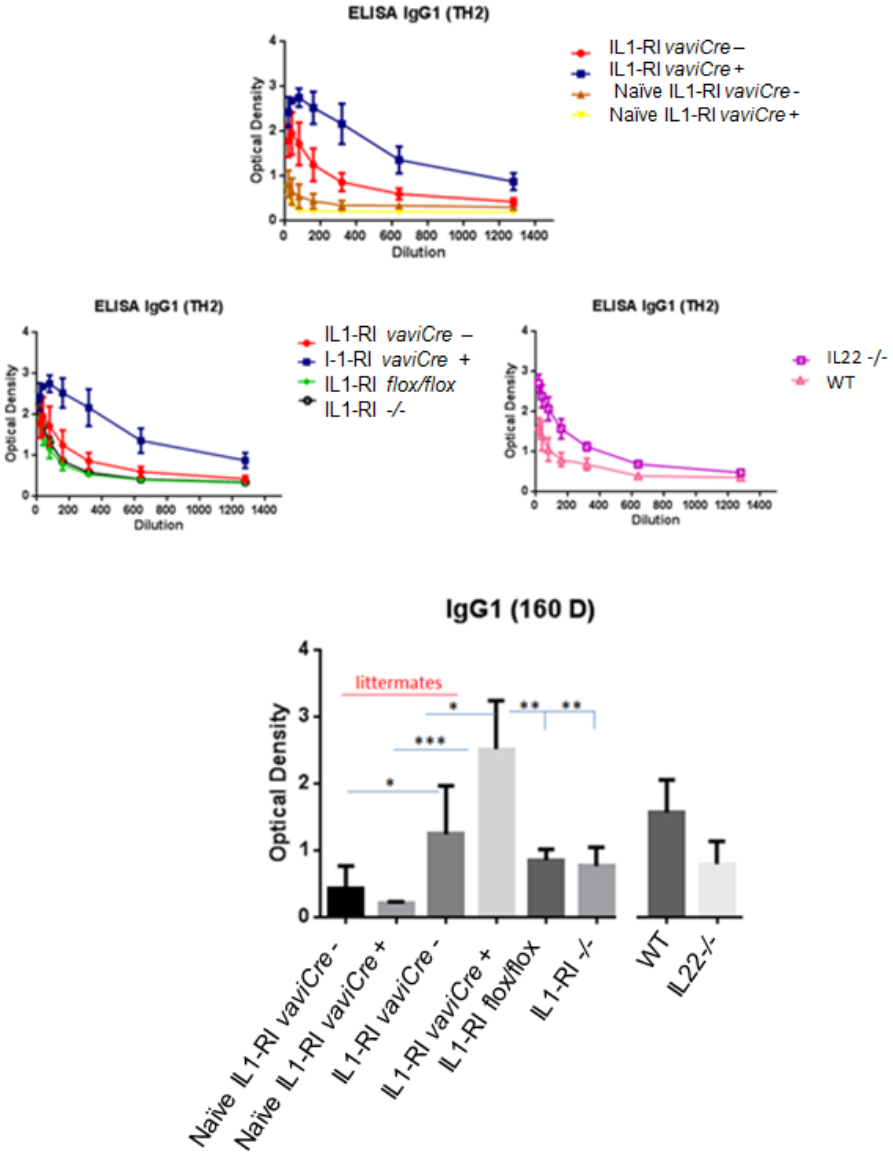


Figure 5-9: Specific *T.muris* antibody IgG1 levels in IL1-RI *vaviCre* –, IL1-RI *vaviCre* +, IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, WT and IL22 $^{-/-}$ mice infected with low dose of *T. muris.* At 21 day p.i. with low dose infection of *T muris*, specific antibody to *T.muris* IgG1and IgG2 was measured in the serum of infected mice by ELISAs. There was a significant increase in IgG1 response in infected IL1-RI *vaviCre*+ mice compared with naïve IL1- RI *vaviCre*+ mice and infected IL1-RI *vaviCre* – , IL1-RI ^{flox} / ^{flox} and IL1-RI $^{-/-}$ mice. In addition, in infected IL1-RI *vaviCre* – mice IgG1 levels increased significantly compared with naïve IL1-RI *vaviCre* – mice. Moreover, no significant differences in the levels of IgG1 were observed between infected IL1-RI ^{flox}/^{flox} and IL1-RI $^{-/-}$ mice and between infected WT and IL22 $^{-/-}$ mice. IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice are littermate. 1:160 dilution (160D) was chosen to analyse IgG1. Data is presented as a mean percentage (+/- SEM) per group N= 4. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI ^{flox}/ ^{flox} and IL1- RI $^{-/-}$ mice . T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (* =P≤0.05, ** =P≤0.01 *** =P≤0.001).

5.2.2.4 Reduction of IL22 and IL17 concentration in the mice that lack IL-1 signalling

MLN were isolated from mice infected with a low dose of *T.muris* at day 21 p.i**.** The cells were then re-stimulated in *vitro* with a final concentration of 50 mg/mL parasite E/S antigen for 48 h. MLN cells were used to evaluate the level of Th1, Th2 and Th17 according to their cytokines IL4, IFNγ and IL22 respectively by ELISA. In this experiment we measured the concentration of IL17, a member of Th17, in addition to IL22.

It was found that, the concentration of IFNγ increased significantly in the MLN isolated from infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice compared with MLN isolated from uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice respectively. MLN isolated from infected IL1-RI $^{flox}/^{flox}$, IL1- RI $^{-/-}$ WT and IL22 $^{-/-}$ mice produced a high concentration of IFNγ**.** However, no significant difference was observed in the concentration of IFNγ between MLN isolated from infected RI ^{flox} / ^{flox} and IL1-RI $^{-/-}$ mice and between MLN isolated from infected WT and IL22 $^{-/-}$ mice (Figure 5-10 A). Moreover, no change was observed in the concentration of IL4 between MLN isolated from infected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice and MLN isolated from uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. Furthermore**,** MLN isolated from IL1- RI $^{flox}/^{flox}$, IL1- RI $^{-/-}$, WT and IL22 $^{-/-}$ mice expressed a very low concentration of IL4 when infected with a low dose of *T.muris* (Figure 5-10 B). Additionally, the concentration of IL22 decreased significantly in MLN cells isolated from infected IL22 $^{-/-}$ mice compared with MLN cells isolated from infected WT mice. In MLN cells isolated from infected IL1-RI *vaviCre* **–** mice, IL22 expression increased significantly compared with MLN cells isolated from naïve IL1-RI *vaviCre* – mice**.** No change was observed in the concentration of the IL22 in MLN cells isolated from infected IL1-RI *vaviCre* + mice compared with MLN cells isolated from naïve IL1-RI *vaviCre* + mice. Moreover**,** there was a significant decrease in the concentration of IL22 in MLN cells isolated from infected IL1-RI *vaviCre* + mice and IL1-RI $^{-/-}$ mice compared with MLN cells isolated from infected IL1-RI *vaviCre* – and IL1-RI

$^{flox}/^{flox}$ mice respectively. Interestingly, the concentration of IL22 in MLN cells isolated from infected IL1-RI $^{flox}/^{flox}$ mice was significantly higher than MLN cells isolated from infected IL1-RI *vaviCre* – mice (Figure 5-10 C)**.**

The IL17 ELISA showed a significant decreased in the concentration of IL17 in MLN cells isolated from infected IL1-RI *vaviCre* + mice and IL1-RI $^{-/-}$ mice compared with MLN cells isolated from infected IL1-RI *vaviCre* – and IL1-RI $^{flox}/^{flox}$ mice respectively. Moreover, the concentration of IL17 increased significantly in infected IL22 $^{-/-}$ compared with infected WT mice.

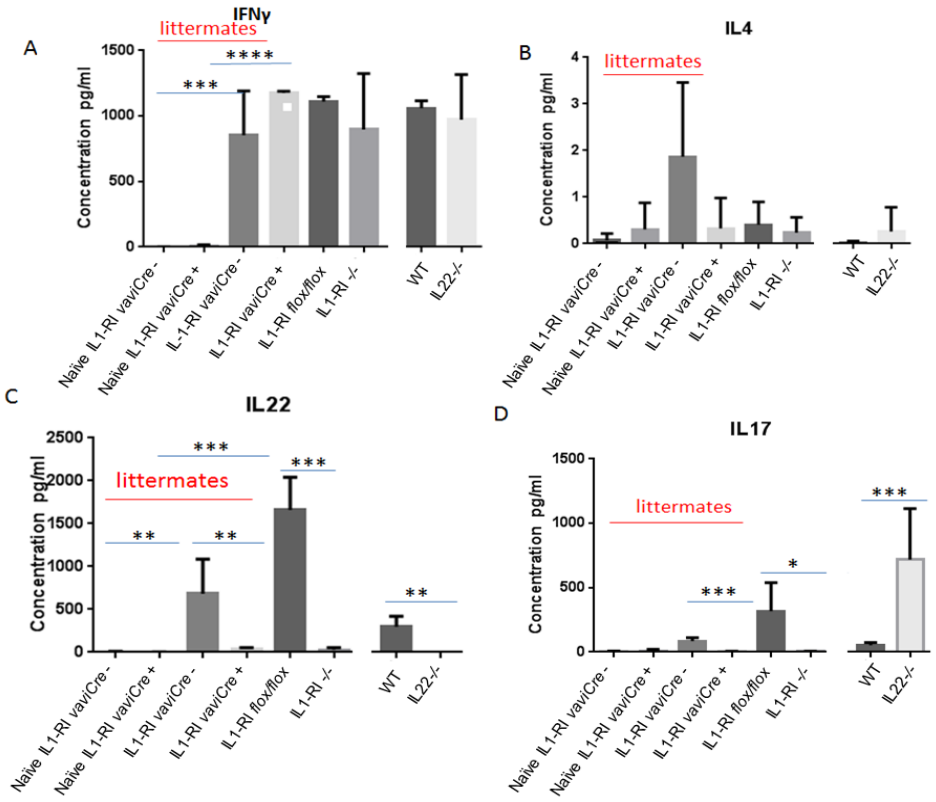


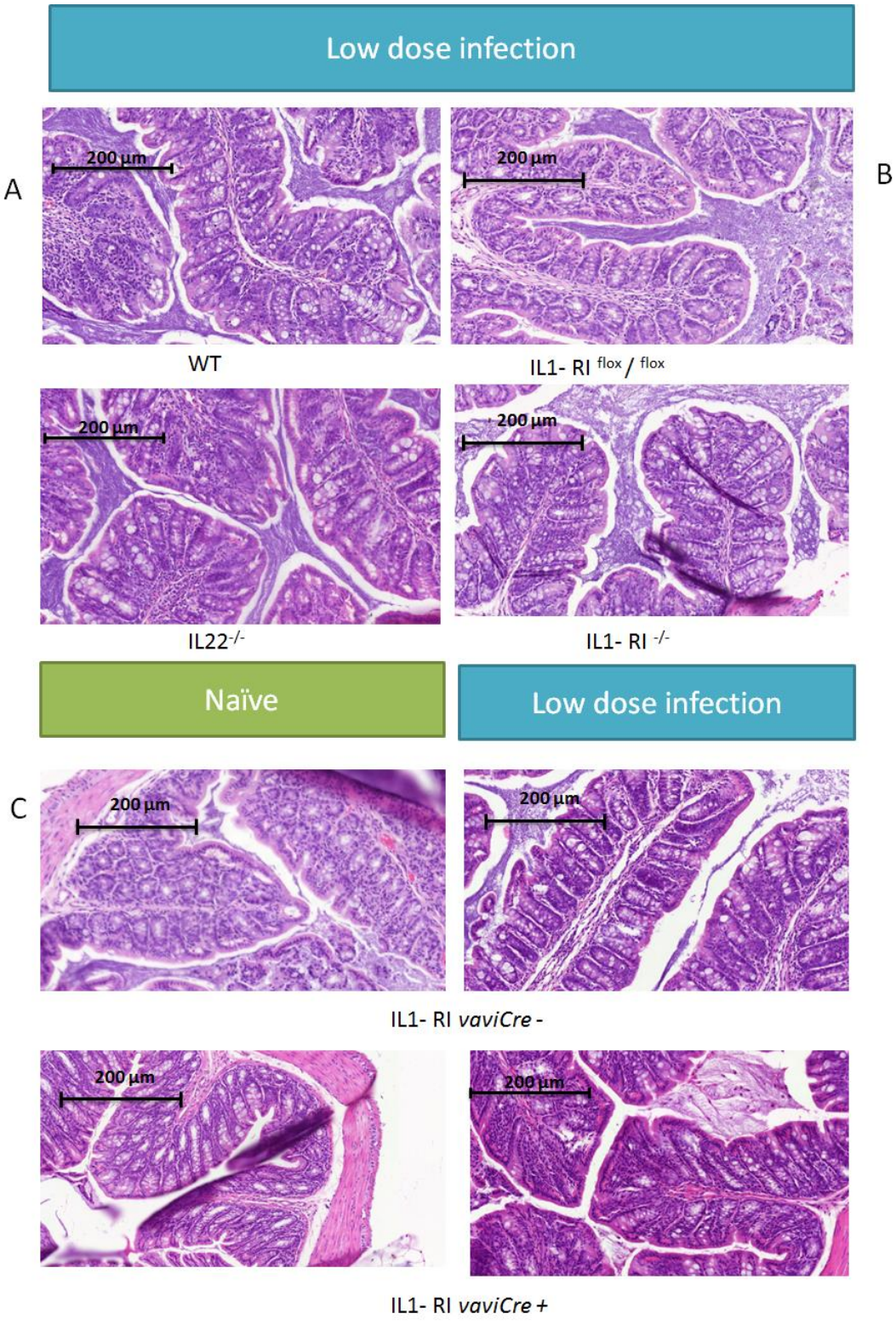
Figure 5-10: Graphical illustration of IFNγ, IL4, IL22 and IL17 concentration expressed by MLN cells isolated from IL1-RI *vaviCre* –, IL1-RI *vaviCre* +, IL1- RI ^{flox} / ^{flox} , IL1-RI $^{-/-}$ WT and IL22$^{-/-}$ mice infected low dose of *T. muris*: MLN cells were removed from the infected mice at day 21 p.i with a low dose of *T.muris*. . MLN cells were then re-stimulated with a final concentration of 50 mg/mL parasite E/S antigen and incubated for 48 h. (A) low dose infection with *T.muris* caused a significant increase in the the concentration of IFNγ in MLN cells isolated from IL1-RI *vaviCre* – and IL1-RI *vaviCre* + compared with MLN cells isolated from naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. MLN cells isolated from infected IL1-RI ^{flox}/^{flox}, IL1-RI $^{-/-}$ WT and IL22 $^{-/-}$ mice produced high concentrations of IFNγ. (B) No significant difference was observed in the concentration of IL4 between MLN cells isolated from infected and uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. In addition, a low concentration of IL4 was observed in MLN cells isolated from infected IL1-RI^{flox}/^{flox}, IL1-RI $^{-/-}$ WT and IL22 $^{-/-}$ mice. (C) The concentration of IL22 increased significantly in the MLN cells isolated from infected IL1-RI *vaviCre* – compared with MLN cells isolated from naïve IL1-RI *vaviCre* – mice. MLN cells isolated from infected IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ had a significant decreased in the concentration of IL22 compared with MLN cells isolated from infeted IL1-RI *vaviCre* – and IL1-RI ^{flox}/^{flox}.mice. The concentration of IL22 in MLN cells isolated from infected IL1- RI ^{flox}/ ^{flox} was significantly more than MLN cells isolated from infected IL1-RI *vaviCre* – mice. MLN cells isolated from infected WT had a significant elevation of IL22 levels compared with MLN cells isolated from infected IL22$^{-/-}$ mice (D) MLN cells isolated from infected IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice showed a significant decreased concentration of IL17 compared with MLN cells isolated from infected IL1-RI *vaviCre* – and IL1-RI ^{flox}/ ^{flox}.mice Furthermore, MLN cells isolated from infected IL22 $^{-/-}$ mice had a significant higher concentration of IL17 than MLN cells isolated from infected WT mice . IL1-RI *vaviCre* – and IL1-RI *vaviCre* + are littermate. Data is presented as a mean percentage (+/- SEM) per group N=4. The data is presented as a mean percentage (+/- SEM) per group N=4. One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI ^{flox}/ ^{flox} and IL1- RI $^{-/-}$ mice . T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (* =P≤0.05, ** =P≤0.01, *** =P≤0.001, **** =P≤0.0001).

5.2.2.5 Histology analysis of IL1-RI *vaviCre* –, IL-1-RI *vaviCre* +, IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$ WT and IL22$^{-/-}$ mice infected with a low dose of *T.muris*

As previously mentioned histology was used to investigate the change in the crypt length which indicates the level of colitis and also used to study the change in goblet cells hyperplasia which is responsible for secrete mucus into the gut during the infection. In this experiment in addition to the analysis of crypt length and goblet cell, histology was used to investigate the change in the expression of IL22 cytokine in the infected mice. Therefore, at day 21 p.i. with *T.muris*, part of colon from the infected mice was isolated and embedded in wax. Then, 5 micron sections were cut and stained with hematoxylin and eosin, goblet cells stain (periodic acid-Schiffs) or with anti-IL22 antibody.

5.2.2.5.1 Lack of IL-1 signalling reduces expression of IL-22 which is required for tissue repair

The measurement of crypt length showed that crypt hyperplasia increased significantly in infected IL22 $^{-/-}$ mice compared with WT mice (Figure 5-11 A). Moreover, infected IL1-RI *vaviCre* + mice had a significant increase in crypt length compared with infected IL1-RI *vaviCre* – and naïve IL1-RI *vaviCre* + mice. While, no significant change was observed in crypt length between infected IL1-RI *vaviCre* – mice and naïve IL1-RI *vaviCre* – mice (Figure 5-11 C) IL1-RI $^{-/-}$ mice showed a similar phenotype to IL1-RI *vaviCre* + mice. The crypt hyperplasia in IL1-RI $^{-/-}$ mice was significantly higher than IL1-RI $^{flox}/^{flox}$ mice (Figure 5-11 B).


Low dose infection
A
200 µm
B
200 µm
WT
IL1- RI flox / flox
200 µm
200 µm
IL22-/-
IL1- RI -/-
Naïve
Low dose infection
C
200 µm
200 µm
IL1- RI vaviCre -
200 µm
200 µm
IL1- RI vaviCre +

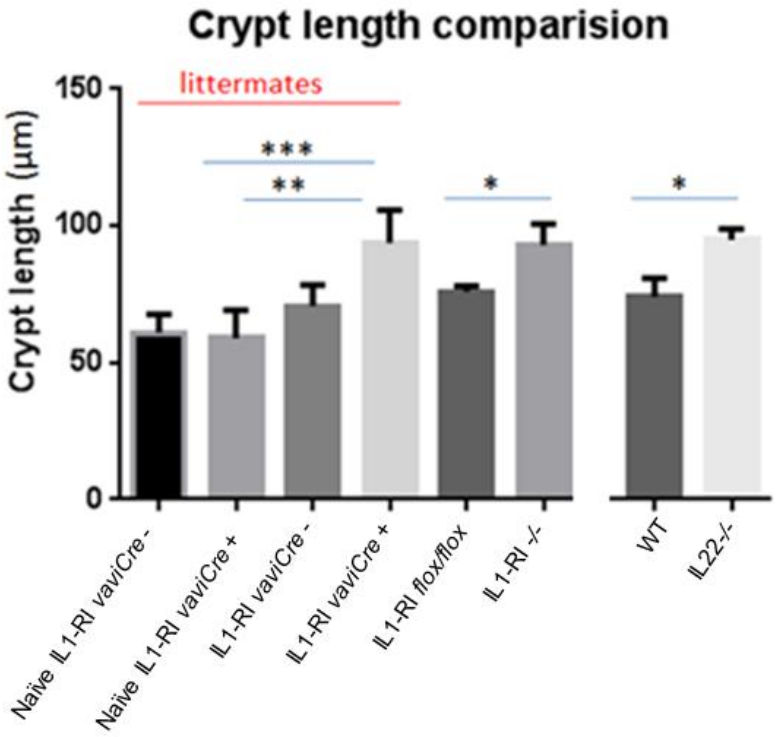


Figure 5-11: Colonic crypt lengths in IL1-RI *vaviCre* –, IL1-RI *vaviCre* + IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, WT and IL22 $^{-/-}$ mice infected with a low dose of *T.muris*:
Mice were infected with a low dose of *T.muris***.** At day 21 p.i, colons were isolated from the mice and embedded in wax. 5 micron sections were cut and stained with hematoxylin and eosin. The slides were scanned by Panoramic viewer system and the crypt lengths were *measured using ImageJ software.* All pictures magnification are 10 x. (A) Infected IL22 $^{-/-}$ mice showed a significant increase in crypt length compared with WT.(B) Infected IL1-RI *vaviCre* + mice had a significant increase in crypt length compared with infected IL1-RI *vaviCre* – and naïve IL1-RI *vaviCre* + mice. (C) Crypt length in infected IL1-RI $^{-/-}$ mice were significantly increased compared with IL1-RI ^{flox}/^{flox} mice. IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice are littermates**.** Data is presented as a mean percentage (+/- SEM) per group N=4. The data is presented as a mean percentage (+/- SEM) per group N=4. (B-C) One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI ^{flox}/ ^{flox} and IL1- RI $^{-/-}$ mice . (A) T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (* =P≤0.05, ** =P≤0.01, ***=P≤0.001).

5.2.2.5.2 Absence of IL-1 signalling reduces expression of IL22 which is required for the activation of goblet cells

The analysis of goblet cells has shown goblet cell hyperplasia was reduced significantly in infected IL22 $^{-/-}$ mice compared with infected WT mice (Figure 5-12 A). The same phenotype was observed in infected IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. Infected IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice showed a significant decrease in goblet cell hyperplasia compared with infected IL1-RI *vaviCre* – and IL1-RI $^{flox/flox}$ mice respectively. Moreover, there was significant increase in goblet cell number between infected IL1-RI *vaviCre* – and uninfected IL1-RI *vaviCre* – mice, while no significant change was showed between IL1-RI *vaviCre* + and uninfected IL1-RI *vaviCre* + mice (Figure 5-12 B-C).

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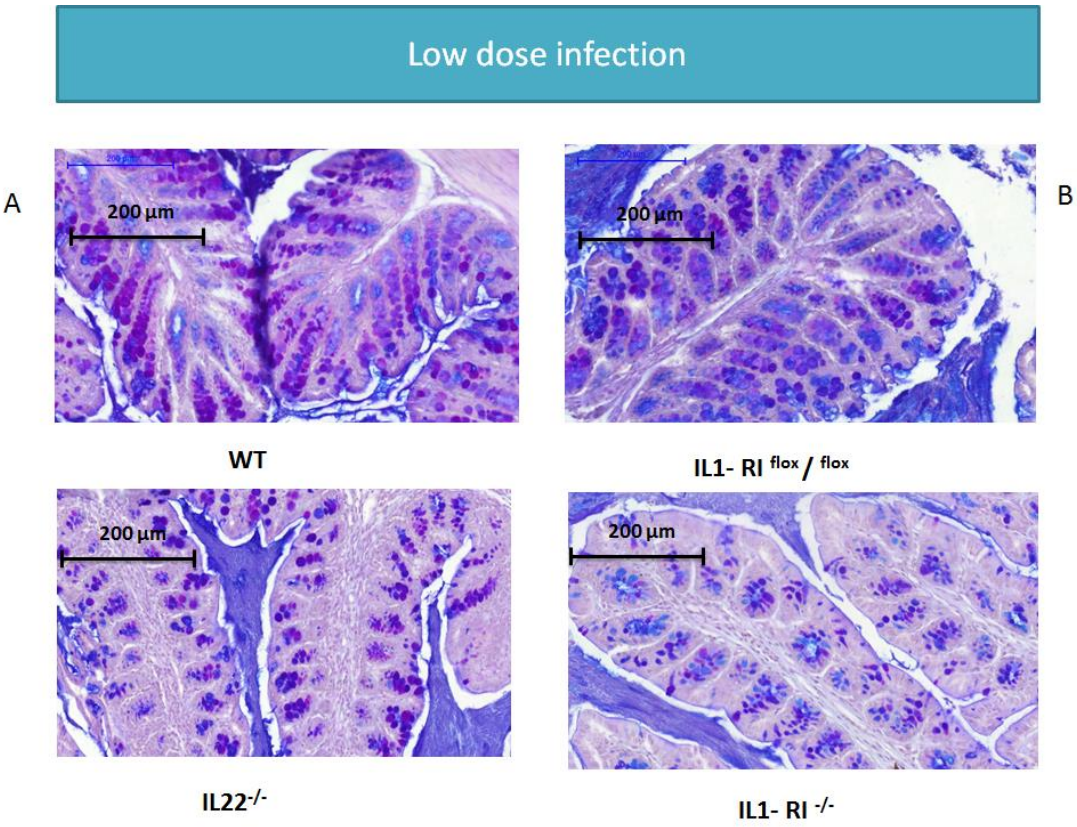

Low dose infection
A
200 µm
B
200 µm
WT
IL1- RI flox / flox
200 µm
200 µm
IL22-/-
IL1- RI -/-
Naïve
Low dose infection
C
200 µm
200 µm
IL1- RI vaviCre -
200 µm
200 µm
IL1- RI vaviCre +

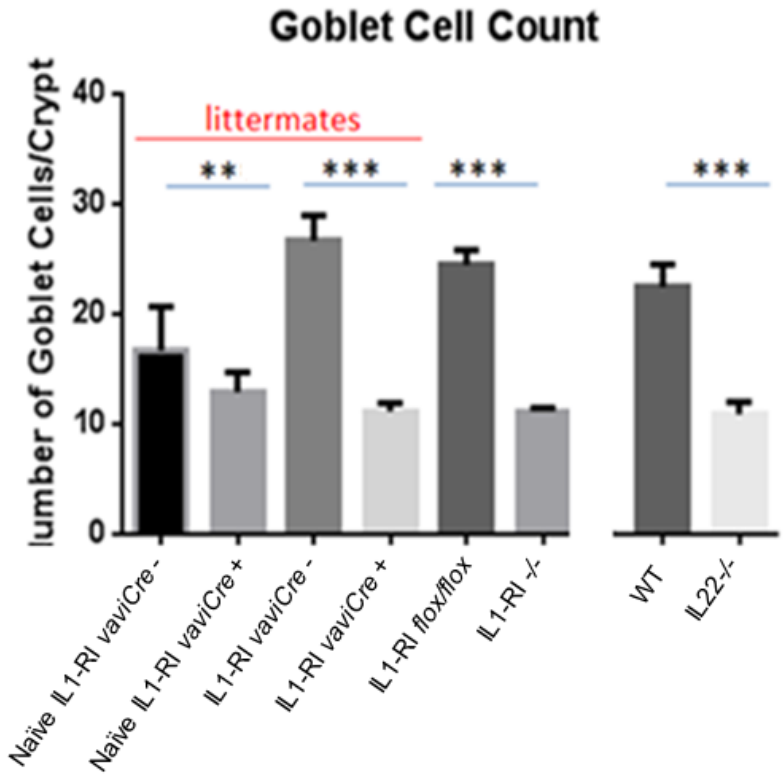


Figure 5-12: Intestinal goblet cell count of IL1-RI *vaviCre* –, IL1-RI *vaviCre* + IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, WT and IL22 $^{-/-}$ mice infected with a low dose of *T.muris.* Mice were infected with low dose of *T. muris*. At day 21 colons were isolated from the mice and embedded in wax. 5 micron sections were cut and stained with goblet cells stain (periodic acid-Schiffs). The slides were scanned by Panoramic viewer system and the goblet cells were enumerated in 20 randomly selected crypts. All picture magnifications are 10 X. A – B) Goblet cell hyperplasia was reduced significantly in IL1-RI $^{-/-}$ and IL22 $^{-/-}$ mice compared with IL1-RI ^{flox}/^{flox} and WT mice respectively. (C) Infected IL1-RI *vaviCre* + had significantly lower goblet cell numbers than IL1-RI *vaviCre* –. Moreover, there was a significant increase in the goblet cell numbers in the infected IL1-RI *vaviCre* – mice compared with naïve IL1-RI *vaviCre* – mice. IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice are littermates. The data is presented as a mean percentage (+/- SEM) per group N=4. (B-C) One- way ANOVA followed by a Tukey multiple comparison post hoc tests was used to determine the significant difference between IL1-RI *vaviCre* – , IL1-RI *vaviCre* +, IL1-RI ^{flox}/ ^{flox} and IL1- RI $^{-/-}$ mice . (A) T-test were used to determine the significant difference between WT and IL22$^{-/-}$ mice (*** =P≤0.001).

5.2.2.5.3 IL-1 signalling is necessary for the production of IL22

Previous experiments showed that an absence of IL-1 signalling leads to a reduction of IL22. In order to confirm this result, immunohistochemistry detecting IL-22 in the colon for was performed. It was found that IL22 $^{-/-}$ mice lack expression of IL22 completely compared with WT mice **(**Figure 5-13 A). IL1-RI $^{flox/flox}$ mice showed higher expression of IL22 than IL1-RI $^{-/-}$ mice **(**Figure 5-13 B). Moreover, the comparison between IL1-RI *vaviCre* **–** and IL1-RI *vaviCre* **+** mice has shown that IL1- RI *vaviCre* – mice had a high expression of IL22. While, IL1-RI *vaviCre* + showed a variation in the expression of IL22 between the crypts, some crypts had IL22 expression and some of the crypts did not (Figure 5-13 C)**.**

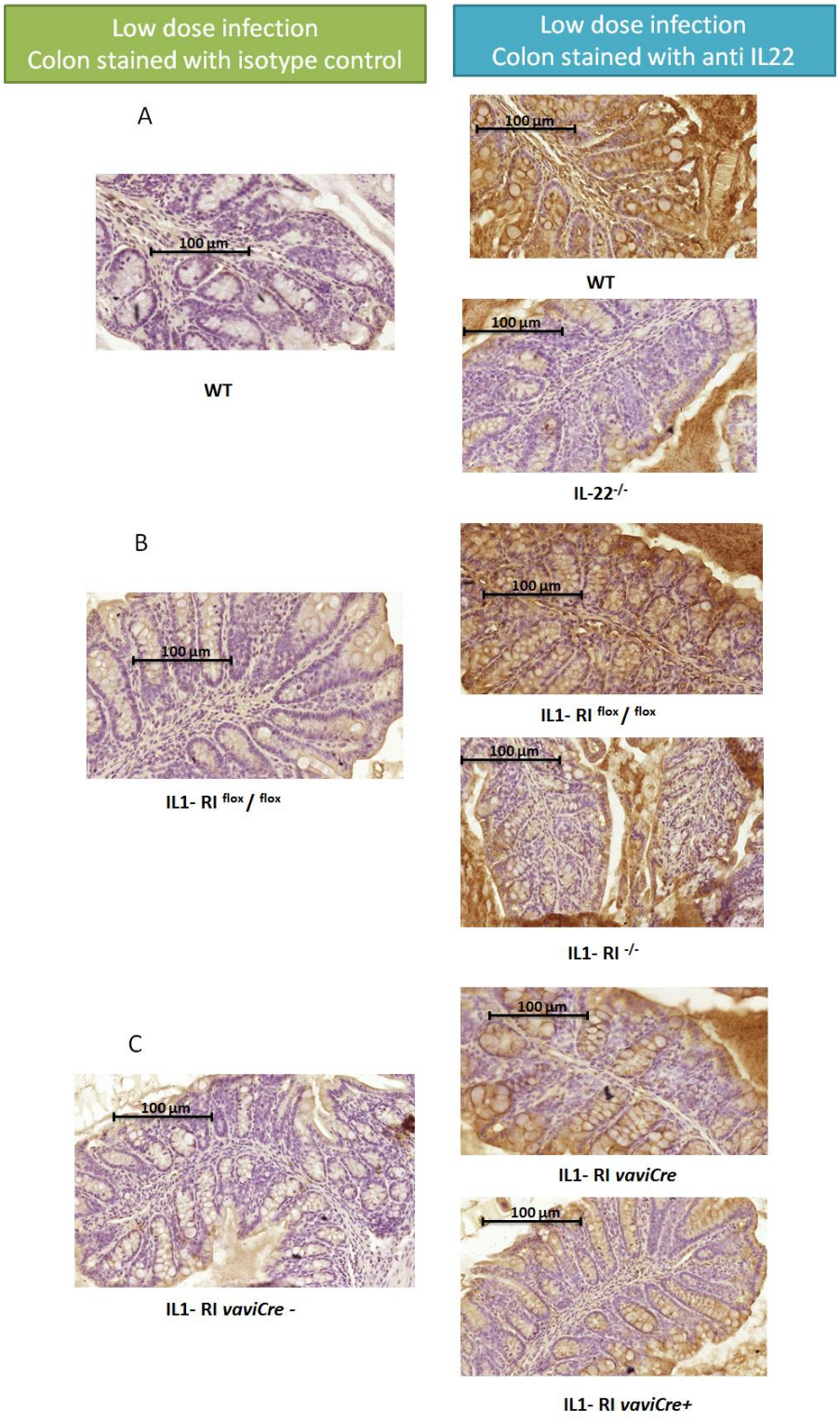


Figure 5-13: Immunohistochemistry for IL-22 of IL1-RI *vaviCre* –, IL1-RI *vaviCre* + IL1-RI ^{flox} / ^{flox}, IL1-RI $^{-/-}$, WT and IL22 $^{-/-}$ infected with a low dose of *T.muris:* The colonic tissue was embedded in wax. 5 micron sections were cut and stained with anti-IL-22 antibody (brown) or isotype control by immunohistochemistry and counterstained with haematoxylin (blue). (A) IL22 $^{-/-}$ mice had no IL-22 expression compared with WT (B) IL1-RI $^{-/-}$ and showed lower IL22 expression than IL1-RI ^{flox}/ ^{flox} (C) In IL1-RI *vaviCre* + mice, some crypts had IL-22 expression and some did not, while, all crypts in IL1-RI *vaviCre* – mice showed an expression of IL-22. The slides were scanned by Panoramic viewer system. All pictures magnification are 20X N=4.

5.3 DISCUSSION

The main aim of this study was to investigate the role of IL-1 signalling in hematopoietic cells in the immune response and in the development of Th1, Th2, and Th17. Helminthic whipworm *T.muris* is an ideal tool which can be used in varying doses to challenge the mice to generate Th1, Th2, and Th17. Studies using *T.muris* showed that the type of the immune response developed in the infected mice will determine whether the mice will be resistant or susceptible to *T.muris* (Hurst and Else, 2013b). C57BL/6 mice are slow responders to ***T.****muris*, thus giving a high dose infection of *T.muris* results in an acute infection and the mice are able to develop a Th2 response and expel the worms by approximately day 21 p.i. In contrast, the low dose infection of C57BL/6 with ***T.****muris* results in a chronic infection and the mice are able to develop a Th1 response and unable to clear the infection (Bancroft *et al*., 2001, Hurst *et al*., 2013, Hurst and Else, 2013a). Several studies showed that a chronic infection of *T.muris* results in an up-regulation of Th17 cells response (Levison *et al*., 2010, Fasnacht *et al*., 2009). Therefore, both low and high doses of *T.muris* were used in this study to promote the generation of Th1, Th2, and Th17 in the IL1-RI *vaviCre* conditional knockout mice. In the current study several phenotypic changes were observed in IL1-RI *vaviCre* + mice when infected with a low dose of *T.muris.* The most interesting finding was that IL1-RI *vaviCre* + mice showed a clear reduction in the Th17 response and its cytokine IL22. IL1-RI $^{-/-}$ and IL22 $^{-/-}$ mice were infected with a low dose of *T.muris* and compared with IL1-RI *vaviCre* + mice. IL1-RI $^{-/-}$ mice was used to confirm the result and to investigate if the inhibition of IL-1 signaling in hematopoietic cells is responsible for the change in IL1-RI *vaviCre* + mice's phenotype. IL22 $^{-/-}$ mice were used to investigate if the reduction of Th17 response and it cytokine IL22 leads to the change in the IL1-RI *vaviCre* + mice's phenotype.

5.3.1 Role of IL-1 in *T.muris* worm expulsion.

Successful worm expulsion in high dose infected resistant mice is associated with a strong Th2 immune response with high expression of IL4, IL-5, IL-9 and IgG1, while susceptibility in low dose infection is due to a stronger Th1 response with high levels of IFNγ, IL-18 and IL-12 and IgG2 (Else and Grencis, 1991, Bancroft *et al.*, 2001). It was shown that in high dose infections both IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were able to clear the infection and showed significantly increased IgG1 and a high concentration of IL4 that compared with uninfected IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. This data indicates that IL1-RI signalling in hematopoietic cells was not required for the worm explosion in the mice infected with a high dose infectionof *T.muris*. This data confirms the study by Humphreys and Grencis, which showed that IL-1 not IL1-RI and IL-1R AcP is important for the expulsion of *T.muris* (Humphreys and Grencis, 2009). They showed that null IL-1RI mice (IL1-RI$^{-/-}$) and null IL-1R AcP (IL-1R AcP$^{-/-}$) were able to generate Th2 cells and release it cytokines resulting in *T.muris* expulsion. While, null IL-1α $^{-/-}$ (IL-1α $^{-/-}$) and null IL-1β$^{-/-}$ (IL-1β$^{-/-}$) mice were susceptible and a showed high Th1 response, they were unable to expel the worm. It was shown that the MLN from infected IL1-RI $^{-/-}$ and IL-1RAcP$^{-/-}$ mice, which were re-simulated with E/S, produced more Th2 cytokines in response to exogenous IL-1 (Humphreys and Grencis, 2009). In addition, the treatment of IL-1α $^{-/-}$ and IL-1β$^{-/-}$ MLN cells with exogenous IL-1α or IL-1β restores the Th2 response and its cytokine secretion (Helmby and Grencis, 2004, Humphreys and Grencis, 2009). This data indicated that IL-1 plays a critical role in the development of Th2-mediated immunity to *T.muris*.

One unanticipated finding was the observation that IL1-RI *vaviCre* + mice infected with a low dose of *T.muris* were more susceptible for the *T.muris* infection than IL1-RI *vaviCre* – mice. Both mouse models showed a high IgG2 response and concentration of IFNγ compared with naïve IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. This data indicated that

the absence of IL1-RI signalling in hematopoietic cells impaired the worm expulsion in IL1-RI *vaviCre* mice infected with a low dose of *T.muris*.

Moreover, IL-1RI $^{-/-}$, IL1-RI $^{flox}/^{flox}$, IL22 $^{-/-}$ and WT mice were susceptible to a low dose *T.muris* infection and no significant difference was observed in worm burden between IL1-RI $^{-/-}$ and IL22 $^{-/-}$ compared with IL1-RI $^{flox}/^{flox}$ and WT mice. They showed a high IgG2 response and concentration of IFNγ. However, there was a significant decreased in the worm burden between IL1-RI *vaviCre* – and IL1-RI $^{flox}/^{flox}$ mice. One possible explanation for the change in IL1-RI *vaviCre* – in worm burden which have to be similar to IL1- RI ^{flox} / ^{flox} is because the background of the IFNγ-R $^{flox/flox}$ *vaviCre* + mice, which were used to generate IL1-RI *vaviCre* mice, is 129 which is highly resistance to *T.muris*. Thus, in further investigation, it might be possible to sequence IL1-RI *vaviCre* to see if there remains some of 129 mice sequence which may have caused this phenotype.

5.3.2 Role of IL-1 in Th1 and Th2 response.

It was reported that IgG2 and IgG1 are markers for Th1 and Th2 lymphocytes respectively (Mountford *et al*., 1994). It was shown that there was a significant increase in IgG1 response in IL1-RI *vaviCre* + mice compared with IL1-RI *vaviCre* – and uninfected IL1-RI *vaviCre* + mice. This result was observed when IL1-RI *vaviCre* + and IL1-RI *vaviCre* – mice were infected with a high dose of *T.muris* and when the mice were infected with 30 instead of 20 eggs in a low dose infection. This result match study has shown that when WT cells are primed with OT-II mice cells and immunized with anti-ovalbumin (OVA) and LPS in IL1-RI $^{-/-}$ recipients, IL-1 causes an increase concentration of serum IgG1 (Ben-Sasson *et al*., 2009). The increase of IgG1 in IL1-RI *vaviCre* – mice and IL1-RI *vaviCre* + mice when infected with an increased dose of low dose *T.muris* infection, provides more evidence for a difference in the IL1-RI *vaviCre* genetic background.

In addition, our findings have shown an increase in IgG2 when in low dose infected IL1-RI *vaviCre* + in compared with uninfected IL1-RI *vaviCre* + .It aslo exhibited an increase in IgG2 when IL1-RI *vaviCre* –, IL1-RI ^{flox} / ^{flox} and WT mice infected with low dose of

T.muris compared with IL1-RI *vaviCre* +, IL1-RI $^{-/-}$ and IL22 $^{-/-}$ mice. Several studies have shown that IL-1 is important for collagen induced arthritis and the addition of anti-IL-1β antibody or IL-1Ra reduced the arthritis incidence (Geiger *et al*., 1993, Bendele *et al.*, 1999). There was a study which investigated the role of B-cells immunity in collagen induced arthritis (CIA) using IL22 $^{-/-}$ mice. This study demonstrated that IgG2 levels were reduced significantly in IL22 $^{-/-}$ mice at early stage of disease (Odilia Corneth1, 2011). Thus, absences of IL-22 which produces dependent on IL-1 signaling in low dose infected IL1-RI *vaviCre* – mice and IL1-RI $^{-/-}$ mice may causes a reduction of IgG2 levels. This data indicated that IL-1 is capable of inducing Th1 and Th2 responses in the absence of IL1-RI in hematopoietic cells. However, IL-1 signalling plays an essential role in the generation of strong Th1 response.

5.3.3 Role of IL-1 in the development of Th1, Th2 and Th17 cells

The evaluation of the development of Th1, Th2 and Th17 was investigated according to their cytokine production; IFNγ for Th1, IL4 for Th2 and IL17 and IL22 for Th17. In the current study, no change was observed in the concentration of IFNγ and IL4 between MLN cells isolated from infected IL1-RI *vaviCre* – mice and MLN cells isolated from infected IL1-RI *vaviCre* + mice. Furthermore no change was observed in the concentration of IFNγ and IL4 between MLN cells isolated from infected IL1- RI $^{-/-}$ mice and MLN cells isolated from IL1-RI ^{flox} / ^{flox} mice when infected with low or high dose of *T.muris*. This result is consistent with studies, which have shown that IL1-RI and IL1-RAcP are not required for Th1, Th2 responses (Humphreys and Grencis, 2009). They have shown that both MLN cells from IL1-RI $^{-/-}$ and IL1-RAcP$^{-/-}$ mice were able to polarise into Th1 with a high concentration of IFNγ and into Th2 with a high concentration of IL-13 and IL-9 (Humphreys and Grencis, 2009). This data indicated that the deletion of IL1-RI did not affect the development and the cytokine secretion of Th1 and Th2 cells. In contract, in low or high dose *T.muris* infection, MLN cells isolated from infected IL1-RI *vaviCre* + mice had lower concentration of IL-22 than MLN cells isolated from infected IL1-RI *vaviCre* –

mice. The change in the concentration of IL-22 between MLN cells isolated from infected IL1-RI *vaviCre* – mice and MLN cells isolated from infected IL1-RI *vaviCre* + mice was observed significantly in low dose but not in high dose *T.muris* infection due to the ability of a chronic *T.muris* infection to trigger Th17 cells (Levison *et al*., 2010, Fasnacht *et al*., 2009). The same phenotype was observed in MLN cells isolated from infected IL-1R1$^{-/-}$ mice which shows a significant decrease in the concentration of IL-22 compared with MLN cells isolated from infected IL1-RI ^{flox} / ^{flox} mice when they were infected with a low dose of *T.muris*. In low dose infection the concentration of IL17 is reduced significantly in MLN cells isolated from infected IL1-RI *vaviCre* + mice and IL-1R1$^{-/-}$ mice compared with MLN cells isolated from infected IL1-RI *vaviCre* – and IL1-RI $^{flox}/^{flox}$ mice. The concentration of IL22 in MLN cells isolated from infected IL1-RI $^{flox/flox}$ mice was significantly higher than MLN cells isolated from infected IL1-RI *vaviCre* – mice. This result is consistent with several studies showing that IL-1 is important for the development of Th17 and its cytokine production IL17 and IL22. It was reported that IL-1 plays an important role in the generation of Th17 cells, a study with IL-1RI deficient mice indicated that IL-17 levels were decreased in response to Keyhole limpet hemocyanin (KLH) in the presence of LPS that in compered with wild-type C57BL/6 mice (Sutton *et al*., 2006). The development and the expansion of Th17 cells are driven by the cytokines IL-6, TGF-β, IL-21, IL-1, and IL-23 (Sutton *et al*., 2009) . IL-6, TGF-β and IL21 are able to induce the differentiation of naive murine T cells into Th17 cells (Sutton *et al*., 2009). IL23, which play an important role in the production of IL17, requires IL-1β or IL-1α for its function. A study with IL-1RI deficient mice has demonstrated that IL-23 cannot induce the production of IL-17 in the absence of IL-1 (Sutton *et al*., 2006). Another study used IL1-RI deficient mice shown that the innate immune cells gamma delta T cells (γδ T cells) which provide an early source of IFNγ in mucosal tissues and express IL-23R and the transcription factor RORγT couple to produce IL-17, IL-21, and IL-22 in response to IL-1 β and IL-23 (Sutton *et al*., 2009) .

Studies have shown that IL-1 signaling in T-cells is essential for Th17 early differentiation *in vitro* and *in vivo.* Regulation of the expression of the transcription factors, regulatory factors IRF4 and RORγT are important for Th17 polarization with TGF-β, IL-6 and IL-23. It also demonstrated that the expression of IL1-RI is important for Th17-mediated autoimmunity and for early Th17 differentiation *in vivo* (Chung *et al*., 2009). IL22 is a novel cytokine expressed by IL-17 producing T-cells (Chung *et al.*, 2006). It was reported that IL22 expressed from Th17 cells after stimulation with IL23 and IL-1 *in vitro* (Marijnissen *et al*., 2011). A study shows that IL-6 in combination with IL-1 and IL23 is able to induce all Th17 cytokine IL17, IL17F and IL22 from naïve T-cells (Chung *et al*., 2009). A study shows that IL-1β, but not IL-6, is encouraged by the presence of the microbiota in intestinal macrophages and is essential for the induction of TH17 cells. Microbiota induced IL-1β acts on IL-1R1-expressing T cells and that leads to the generation of Th17 cells and production of IL17 and IL22 (Shaw *et al*., 2012).

This data indicated that the obliteration of *IL1- RI* gene, which inhibits IL-1 signalling, leads to a reduction of IL22 and IL17. This demonstrated that IL-1 signalling is required for the deployment of Th17 and its cytokine secretion.

A similar phenptype in the the expresstion of IL22 and IL17 between IL1- RI $^{-/-}$ and IL1-RI *vaviCre* + mice in compared with IL1- RI $^{flox/flox}$ and IL1-RI *vaviCre* - mice respectively , indicated that the expression of IL-1 in hematopoietic cells is responsible for the cytokine secretion of Th17 cells (IL22 and IL17). The change was observed between IL-RI ^{flox} / ^{flox} and IL1-RI *vaviCre* – mice in the IL22 expression suggesting a difference in genetic background between the two mice strains.

5.3.4 Role of IL-1 in colitis, goblet cells hyperplasia and the production of IL22 in colonic tissues

Histology was used in this study to investigate the level of the colon inflammation (colitis) and the goblet cells hyperplasia which is responsible for the secretion of mucus, protecting

the intestinal epithelium from invasion by foreign bodies such as *T.muris*. It was reported that goblet cells produce molecules containing a range of mucins and antimicrobial proteins which play an important role in the innate immune defence mechanisms in the gut, against both bacterial and helminth infections (Turner *et al*., 2013).

The measurement of crypt length in IL1-RI *vaviCre* + mice infected with a low dose showed an increase of crypt hyperplasia compared with naïve IL1-RI *vaviCre* + mice. While, no significant difference was observed between infected IL1-RI *vaviCre* – mice and naïve IL1-RI *vaviCre* – mice. One possible explanation is that infected IL1-RI *vaviCre* + mice had significantly more worms compared with IL1-RI *vaviCre* – mice. The worms in IL1-RI *vaviCre* + mice may promote their own survival by inducing the production of IFNγ which direct epithelial cell proliferation and induces the production of CXCL10 chemokine. This leads to a reduction in the cell escalator to the crypt as well as worm expulsion (Cliffe *et al*., 2005). In a high dose infection no significant difference was shown in crypt hyperplasia between IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice. One possible explanation for this result is that, IL1-RI *vaviCre* – and IL1-RI *vaviCre* + mice were able to clear the infection when infected with a high dose by a mechanism called crypt cell escalator. It was reported that, in acute infection of resistancet mice, crypt cell's exhibit hyperplasia in response to *T. muris* infection due to the 'crypt cell escalator' which is responsible for fast worm expulsion (Cliffe *et al*., 2005, Klementowicz *et al*., 2012).

Additionally, low dose infections of IL22 $^{-/-}$ mice have shown a significant increase in crypt hyperplasia compared with WT mice. This data indicates that IL-22 plays an important role in reducing the inflammation response. It was reported that IL22 is able to promote anti-apoptotic pathways such as STAT3, Akt, mitogen-activated protein kinase pathways and expression of anti-microbial molecules such as b-defensin, Reg3c, lipocalin-2, which is important into preventing tissue damage and tissue repair (Zenewicz and Flavell, 2011). Our findings were consistent with a study, which showed that IL22 knockout mice treated with DSS to generate colitis, had delayed intestinal wound healing

compared with WT mice (Pickert *et al*., 2009). The same phenotype was observed in IL1-RI *vaviCre* + mice and IL1-RI $^{-/-}$ mice which shows a significant increase in the crypt hyperplasia compared with IL1-RI *vaviCre* – mice and IL1-RI $^{flox/flox}$ mice. This data demonstrates that the absence of IL-1 signaling, affects the production of IL22, which is important for tissue repair. A similar phenotype in crypt hyperplasia was observed between IL1-RI *vaviCre* + and IL1-RI$^{-/-}$ mice, indicating that IL-1 signaling in hematopoietic cells may be responsible for IL22 production.

Several studies have shown that goblet cell hyperplasia is present during infection with *T. muris* in both susceptible and resistance mice (Artis *et al*., 2004, Hasnain *et al.*, 2010). It was found that in low dose infection, goblet cell hyperplasia decreased significantly in IL22$^{-/-}$ mice compared with WT mice. This data indicates that IL22 is important for goblet cells hyperplasia. This result was supported by a study that shows that IL22 has an essential role in goblet cells activation, by promoting the induction of many goblet cells markers such as Muc1 and Muc3 (Turner *et al.*, 2013). Low and high dose infected IL1-RI *vaviCre* + mice show a significant reduction in goblet cell hyperplasia compared with IL-1-RI *vaviCre* – mice. The same phenotype was observed in low dose infected IL1-RI $^{-/-}$ mice which shows a significantly lower goblet cell counts than IL-RI $^{flox/flox}$ mice. Therefore, we demonstrated that the absence of IL-1 signaling affects the production of IL22, which is important for goblet cell hyperplasia. A similar phenotype in goblet cells has been observed between IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice, indicating that IL-1 signaling in hematopoietic cells may be responsible for IL22 production which activates goblet cells.

It was shown that the main source of intestinal mucin is Muc2 and its absence cause colitis in the mice (Burger-van Paassen *et al*., 2011). A study used Muc2$^{-/-}$ knockout mice showed that Muc2$^{-/-}$ were unable to produce mucus and did not show positive Muc2 goblet cells. It demonstrated that Muc2$^{-/-}$ mice had an increased villus length and number of Ki67^{+} epithelial cells in compared with WT mice which indicate an induction in epithelial cells

proliferation. In the absence of Muc2, IL-22/STAT3 signalling play an important role in the maintenance of homeostasis in the mouse ileum by regulating the innate immunity defence genes such as Fut2, Reg3b, Reg3g, Relmb (Sovran *et al.*, 2015). In further investigation, it may be useful to use IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice to examine the change in Muc2 in the absence of IL-1 signaling which is required for IL22 production.

The study demonstrates the role of IL22 in goblet cells shows that when IL22$^{-/-}$ mice are infected with a high dose of *T.muris*, goblet cell hyperplasia was decreased which leads to impaired worm expulsion, despite the presence of high Th2 cytokine levels (IL-4, IL-5 and IL-13) (Turner *et al*., 2013). One explanation for the worm expulsion was shown in IL1-RI *vaviCre* + mice which are unable to produce IL22 by Th17 cells due to the deletion of *Il1-rI* gene is IL1-RI *vaviCre* + mice may be able to produce IL22 independent of IL-1 signalling. It was shown that the combination of IL12 and IL18 secreted by activated macrophages leads to the induction of NK cells to produceIL-22 (Zenewicz and Flavell, 2011). Moreover, it was reported that, lamina propria cells from WT mice treated with DSS to generate colitis were able to produce IL22 through colonic CD11c^{+} cells in response to the stimulation of TLR4 with LPS (Pickert *et al*., 2009). Another study shows that, the new population CD3^{-} CD127^{+} immune cells which are similar to LTi cells, are able to produced IL22 through the activation of TLR5 by Flagellins expressed by particularly pathogenic bacteria in the gut and the lung (Van Maele *et al*., 2010).

It was reported that activation of transcription factor STAT3 play an important role in the development of Th17 cells (Yang *et al*., 2007). There was a study investigating the role of STAT3 activation in CD4+ T cells during host defence in mice in response to *Citrobacter rodentium* (*C. rodentium*) which cause colitis through enterohemorrhagic or enteropathogenic bacteria *Escherichia coli*. They found that lamina propria cells isolated from *C. rodentium* infected mice which are deficient in STAT3 in CD4+ cells (Stat3^{-CD4}) completely lose IL22 production by CD4+ lamina propria cells only in the later stage of the infection (Backert *et al*., 2014).They demonstrated that Stat3^{-CD4} mice were susceptible

to the infection due to the absence of IL22 which causes a defect in intestinal epithelial barrier and facilitate bacterial invasion and systemic distribution of bacteria. It also showed that in early stage of the infection with *C. rodentium*, innate lymphoid cells (ILC3s) from $Stat3^{-CD4}$ were able to produce IL22 independent of STAT3 (Backert et al., 2014). These studies indicate that the sources of IL22 are CD4+ T cells which produce IL22 which is dependent on IL-1 signalling. However there are many cells able to produce IL22 independent of IL-1 signalling such as NK, ILC3s, colonic $CD11c^{+}$ and $CD3^{-}$ $CD127^{+}$ cells during gut inflammation.

There are some differences between IL1-RI *vaviCre* + mice and IL22 $^{-/-}$ mice. The delation of the *Il1-rI gene* in IL1-RI *vaviCre*+ inhibits the production of IL17 through Th17 cells, whereas $IL22^{-/-}$ mice are still able to produce IL17. The role of IL17 in high dose *T.muris* infections is still unclear. Interestingly, a very recent study in the Grencis lab showed that when $IL22^{-/-}$ mice were infected with a high dose of *T.muris*, they were sometimes susceptible and sometime resistant (Grencis, unpublished). Thus, we suggested that sometimes $IL22^{-/-}$ mice were able to activate sufficient quantities of goblet cells independent of IL-22, resulting in worm expulsion. It was reported that goblet cells exist in the gastrointestinal (GI) tract, which has commensal microbiota. GI epithelium microbes are able to regulate the production of the mucin secreted from goblet cells by activating different signaling pathways such as NF- κB signaling pathway and secretory elements (Kim and Khan, 2013). This indicated that the both IL1-RI *vaviCre* + and IL22 $^{-/-}$ mice are able to activate goblet cells independently of IL22. Further experiments need to be undertaken to understand the role of IL22 and IL17 in high dose *T.muris* infections.

IL22 is the only cytokine, which is produced by immune cells and acts only onto non-haematopoietic such as epithelial cell, keratinocytes and fibroblasts cells (Wolk *et al*., 2004, Wolk and Sabat, 2006). It is a members of IL-10 cytokine family, signalling through a heterodimeric receptor involving IL-22R and IL-10Rb, and activates anti-apoptotic pathways such as STAT3 (Wolk and Sabat, 2006). IL-22R is mainly present in epithelial

cells. The best target of IL22 is colonic epithelial cells, keratinocytes and hepatocytes (Zenewicz and Flavell, 2011) . Thus, in this study the immunohistochemistry in colonic tissue for anti-IL22 was used to confirm the previous experiments which showed that absence of IL-1 signalling leads to a reduction of IL22. In this experiment no expression of IL22 was observed in IL22 $^{-/-}$ mice compared with WT mice. Moreover, IL1-RI $^{-/-}$ showed a lower expression of IL22 than IL1-RI $^{flox/flox}$ mice. The comparison between IL1-RI *vaviCre* **–** and IL1-RI *vaviCre* + mice showed that IL1-RI *vaviCre* – mice had a high expression of IL22, whereas, IL1-RI *vaviCre* + show that some crypts had IL22 expression and some of them had not. This data indicated that IL-1 plays an important role in the production of IL22**.** It also confirms that there are some cells able to produce IL22 independent of IL-1 signalling.

5.4 CONCLUSION

The results presented in this chapter show that IL-1 not IL1-RI is required for *T.muris* worm expulsion. It is also shown that IL-1 plays an important role in the development of Th2-mediated immunity to *T. muris*. IL-1 is essential for the generation of the Th1 and Th2 response and is independent of IL1-RI. The deletion of IL1-RI did not affect the development, and the cytokine secretion of Th1 and Th2 cells. While, IL-1 signalling is important for the development of Th17 cells and the secretion of its cytokines IL17 and IL22. Moreover, IL-1 expressed from hematopoietic cells is responsible for IL22 production. IL22 plays an important role in the activation of goblet cells and in tissue repair. It also showed that many cells able to produce IL22 independent of IL-1 signalling.

CHAPTER SIX: CHARACTERISATION OF *TRICHURIS MURIS* INFECTED IL-1RII KNOCKOUT MICE

6.1 INTRODUCTION

IL-1α and IL-1β are both pro-inflammatory cytokines expressed by a large number of cells such as macrophages, monocytes epithelial cells, lymphoid cells, vascular smooth muscle cells and hepatocytes (Kamari *et al*., 2007). These cytokines are able to bind and activate two difference receptors: IL1-RI and IL1-RII (Dinarello, 1996, Kamari *et al.*, 2007, Cullinan *et al.*, 1998). The biological activity of IL-1 is regulated by inhibitors IL-1Ra and IL1-RII (Smith *et al*., 2003). IL-1Ra can bind to both IL1-RI and IL1-RII, but is unable to induce signal transduction. It has been demonstrated that the soluble form of IL1-R II (IL-1sRII) enhances the inhibiting activity of the IL-1Ra (Burger *et al*., 1995), and binds strongly with IL-1β but poorly with IL-1α. IL1-RII lacks the entire intracellular Toll/interleukin-IL-1 (TIR) domain, and is known to act as a decoy receptor (McMahan *et al*., 1991, Giri *et al*., 1994). The soluble form of IL1-RAcP (IL1-sRAcP) enhances the ability for soluble form of IL1-RII (IL-1sRII) to inhibit the action of IL1-RI through increasing its affinity to bind with IL-1α and IL-1β (Smith *et al*., 2003). It was reported that the binding of IL1-R II with pro- IL-1α prevents the cleavage and activity of IL-1α (Zheng *et al*., 2013).

As IL1-RII has been demonstrated to be an inhibitor of IL1-RI, this chapter used IL1-RII $^{-/-}$ global knockout mice in order to demonstrate the relevance of IL-1 signalling in T cell functions and differentiation *in vivo*.

Il1-rII gene containes 9 exons. TaconicArtemis investigated all *Il1-rII* gene exons in order to identify the exon which could be delated and prevent *Il1-rIIgene* function. They found that the deletion of exon 3 could prevent the function of *Il1-rII* gene .The IL1-RII delta allele generation was achieved by flanking exon 3 with two loxP sites. Two different flippase (FLP) recombination target sites (FRT (flip-recombinase targets) and F3) flanked the two positive selection markers neomycin (NeoR) and puromycin (PuroR). These were then inserted into exon 2 and 3 (Figure 6-1). The homologous recombinant clones were separated using NeoR and PuroR, through a method of double positive selection in order to

increase the co-integration efficiency of the two-loxP sites. The bacterial artificial chromosome clone (BAC) from the C57BL/6J RPCI-23 BAC library was used to generate a targeting vector, which was then transferred into the C57BL/6N Tac ES cell line, resulting in the modification of the genomic locus. The selection markers were removed after the recombination of Flp. The exon 3 was flanked by two loxP sites that can be deleted by Cre recombination, which led to the loss of the function of the *Il1-rII* gene due to the deletion of the Ig-like C2 type 1 domain. The deletion of exon 3 also caused a frame shift from exon 2 to exons 4 and 5 (Figure 6-1) (TaconicArtemis, Cologne, Germany).

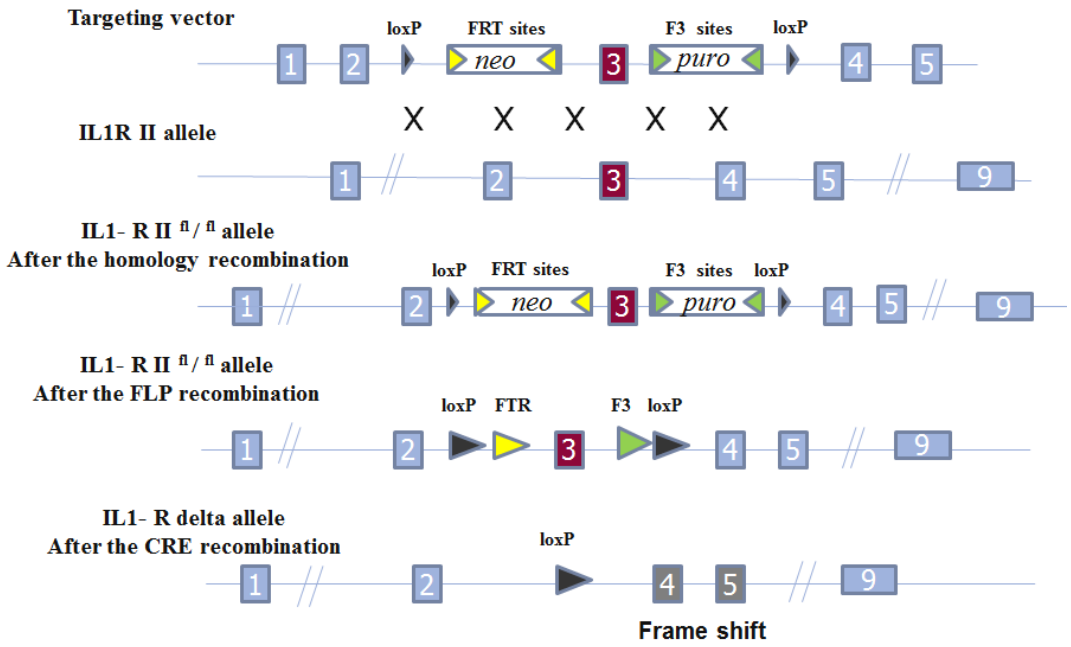
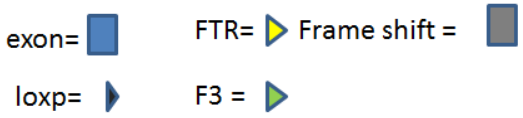


Figure 6-1: The generation of the IL1-RII delta allele: The BAC gene targeting vector contained two loxP sites flanked exon 3. The FLP recombination target sites FRT and F3 flanked the two positive selected markers of NeoR and PuroR respectively. The homologous recombination between the *Il1-rII* gene in the ES cells, and gene targeting vectors, leads to the genomic locus being modified (which obtains the IL1-RII $^{fl}/_{fl}$ allele). The FLP recombination leads to the removal of the positive selected markers NeoR and PuroR. The mice that were modified with the IL1- RII delta allele were generated by deleting exon 3, which was flanked by loxp sites with the expression of Cre recombinase. The deletion of exon 3 caused the dilating of the Ig-like C2 type 1 domain and a further frame shift from exon 2 to all downstream exons (Adapted from, TaconicArtemis, Cologne, Germany).



In chapter five it was demonstrated that IL1-RI signalling played an important role in the development of Th17 and its cytokine production. Therefore, the comparison between the IL1-RI $^{-/-}$ and IL1-RII $^{-/-}$ mice is a powerful method in order to confirm the role of IL-1 signaling in the development of Th17 and its cytokine production. It is also an important method for understanding the role of IL-1signalling in the immune system. In this study, the IL1-RII $^{-/-}$ and control IL1-RII ^{flox} / ^{flox} mice were challenged with varying doses of the infective *T.muris* eggs in order to stimulate a Th1, Th2 and Th17 response. Several standard approaches, such as worm burden, IgG1 and IgG2 antibody response, cytokine responses and histology were used to investigate the T helper cell response in relation to infection. If the IL-1 signalling expression is necessary for the development of T-helper cells, then it is hypothesised that mice lacking functional IL1-RII will show an overexpression of of T helper cells response.

6.2 RESULTS

6.2.1 Genotyping PCR for IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice

DNA extracted from mouse ear punches was used to genotype the homozygous IL1-RII ^{flox} / ^{flox} mice and the delta IL1-RII $^{-/-}$ mice through PCR. IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice were provided by the Emmanuel Pinteaux lab. IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice were obtained from TaconicArtemis in Cologne, Germany. The primers that were used to genotype IL1- RI ^{flox} / ^{flox} were (33: GTAGTGGGCAATCAGATGGAC) and

(34: ATCTACATCAGAATGCTCACAAGC). The IL1-RII ^{flox} / ^{flox} mice showed a band at 347 bp and the IL1-RI ^{wt} / ^{wt} showed band at 228 bp and the (Figure 6-2 A).

Moreover, IL1-RI $^{-/-}$ genotyping showed a band at 300 bp using primers of (31: GTAGTGGGCAATCAGATGGAC) and

(34: ACCATGTCTGCCTGTTCACC) (Figure 6- 2 B).

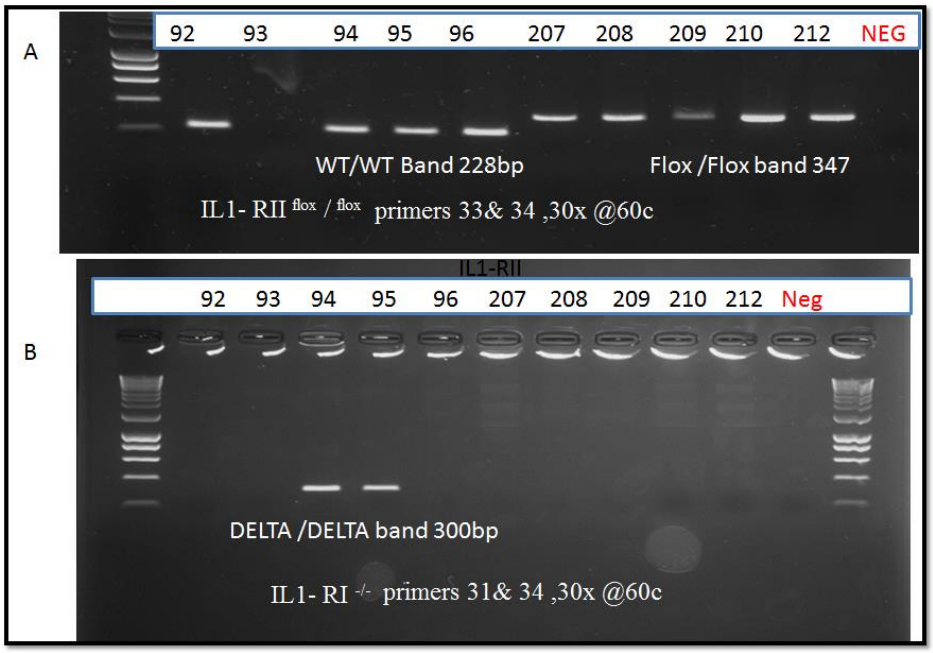


Figure 6-2: Genotyping PCRs for detecting (A) IL1-RII ^{flox} / ^{flox} and (B) IL1-RII $^{-/-}$: Mouse genomic DNA was extracted from ear punches. PCRs were performed with the appropriate primers and visualised on a 1% agarose gel. (A) The homozygous mice IL1-RII ^{flox} / ^{flox} (207,208,209,210,2012) showed a band at 347 bp and the IL1-RII ^{wt} / ^{wt} mice (92,94,95,96) showed a band at 228 bp (B) The delta IL1- RII $^{-/-}$ mice (94,95) showed a band at 300 bp.

6.2.2 Low and high dosage of *T.muris* infection for both IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice

In order to investigate the relevance of IL-1 signalling in T cell functions and differentiation *in vivo*, both IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ were infected with a low dose (approx. 20 infective *T.muris* eggs) and a high dose (approx. 200 *T.muris* eggs). This was conducted over a period of 21-days in order to induced phenotypes Th1, Th2 and Th17 respectively. Subsequent to infection, the change of immune response in absence of IL1-RII was investigated by using several approaches: worm burden, IgG1 and IgG2 antibody response, cytokine responses and histology.

6.2.2.1 Obliteration of the *Il1-rII* gene did not affect worm burden in mice infected with either a low or high infection dose of *T.muris*

After 21 days of being infected with either a low and high dose of *T. muris*, the colon and the caecum was isolated within both IL1- RII ^{flox} / ^{flox} and IL1-RII$^{-/-}$ mice and a worm count

was conducted. In the high dose infection, it was found that both mice were resistant to the infection and able to expel the worms (Figure 6-3 A). In low dose infection, the mice were susceptible to the infection and unable to expel the worms. There was no significant difference observed between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ when infected with the low dose of *T. muris* (Figure 6-3 B).

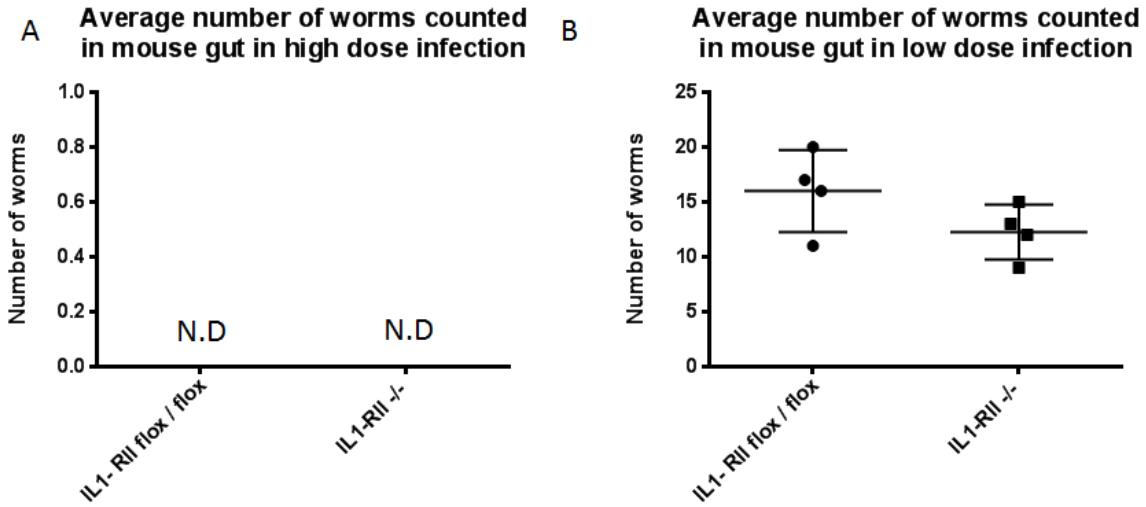


Figure 6-3: Worm burden of IL1-RII $^{flox / flox}$ and IL1-RII $^{-/-}$ mice upon being infected with either (A) a high or (B) a low dose of *T.muris.* At 21 days p.i, with either a low or high dose of *T.muris*, the worm burden was counted from isolated IL1- RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ colon and the caecum. (A) Both sets of mice that were infected with a high dose of *T. muris* eggs expelled the worms during the infection (B) In the low dose infection, the mice were susceptible and no significant difference was observed between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice (N=4). A t-test was used to analyse the data (N.D = not detected).

6.2.2.2 There were no observable difference in the responses of Th1 and Th2 between IL1-RII $^{flox}/^{flox}$ and IL1-RII $^{-/-}$ mice upon being infected with a low or high dose of *T.muris*

It has been reported that IgG2 and IgG1 are markers for Th1 and Th2 lymphocytes respectively (Mountford *et al*., 1994). To investigate the specific response of IgG1 and IgG2 antibodies in relation to the infection of *T.muris* in both IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice, blood was collected from mice infected with a low and a high dose at 21 days p.i. Blood sample then centrifuged to separate the serum. ELISAs were performed to measure the IgG1 and IgG2 response in the serum. It was found that the mice (both IL1-RII $^{flox}/^{flox}$ and IL1-RII $^{-/-}$) infected with either a low or high dose of *T.muris*, expressed a high quantity of IgG1 and IgG2 respectively. However, there was no significant difference observed in the quantity of IgG1 and IgG2 between IL1-RII $^{flox\ flox}$ and IL1-RII $^{-/-}$ when they were infected with the *T.muris* eggs. (Figure 6-4 A-B). In the high dose infection, IgG1 increased significantly within infected mice compared to uninfected mice (Figure 6-4 A).

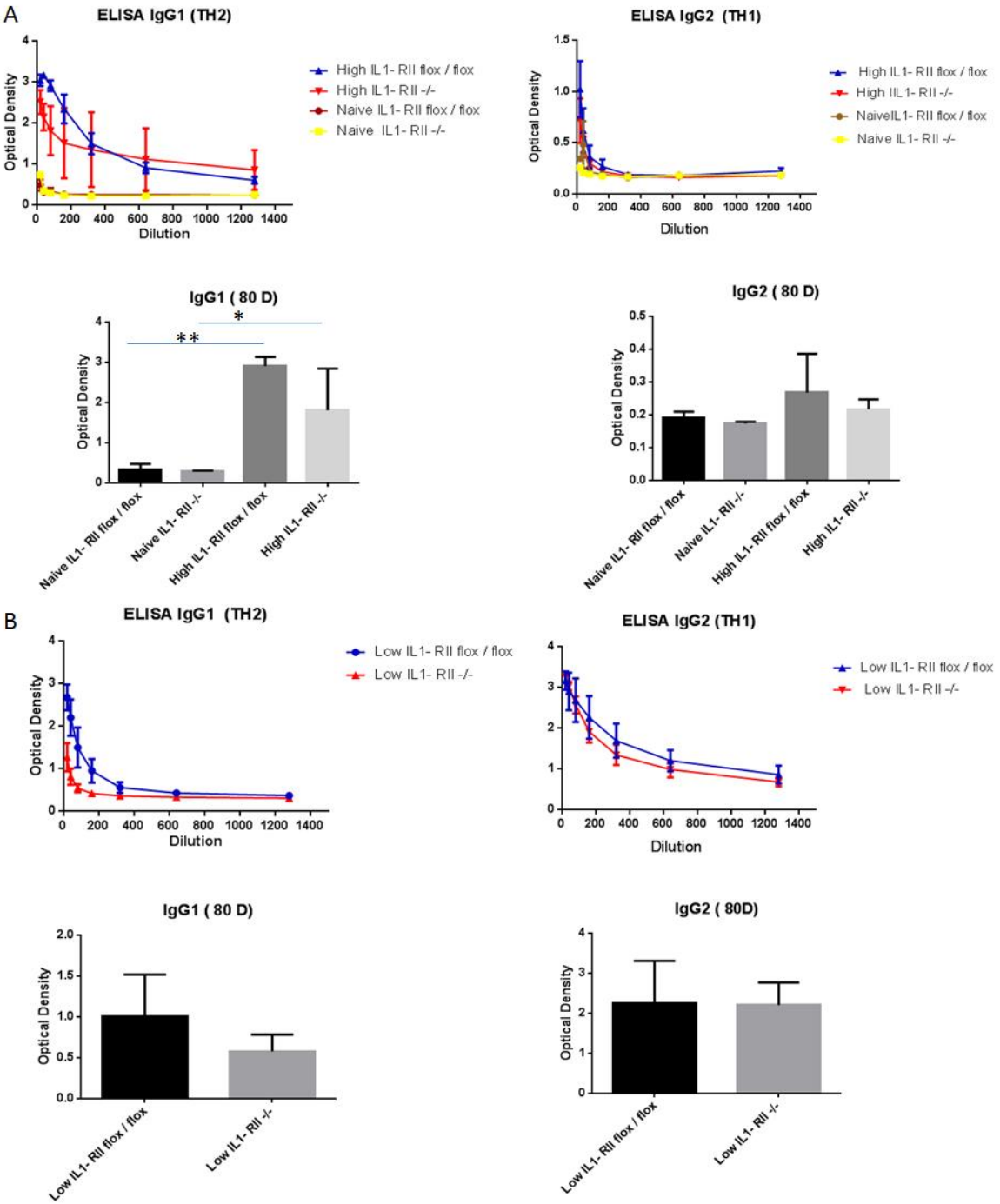


Figure 6-4: Specific *T.muris* IgG1 and IgG2 antibodies found in the serum of IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with either (A) a high or (B) a low dose of *T. muris.* At p.i day 21 (both with low and high dose of *T.muris*), specific *T.muris* IgG1 and IgG2 antibodies were measured in the serum of ELISA infected mice. (A) There was a significant increase in response of IgG1 found in IL1-RII ^{flox}/^{flox} and IL1-RII $^{-/-}$ mice infected with a high dose compared with naïve IL1-RII ^{flox}/ ^{flox} and IL1-RII $^{-/-}$ respectively. (A-B) IL1-RII ^{flox}/^{flox} and IL1-RII $^{-/-}$ expressed a high quantity of IgG1 and IgG2 when infected with a high or low dose of *T.muris.* There was no significant difference observed in IgG1 and IgG2 between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ when infected with either a high or low dose of *T.muris*. A 1:80 dilution (80D) was chosen to analyse both IgG1 and IgG2. Data is presented as a mean percentage (+/- SEM) per group N=4. (A) A one-way ANOVA was conducted, which was followed by a Tukey multiple comparison post-hoc test (B). T-tests were used to analyse the data (* =P≤0.05, ** =P≤0.01).

6.2.2.3 The IL1-RII$^{-/-}$ mice infected with a low dose of *T.muris* showed an increase in IL17 and a reduction in IL22 compared to IL1-RII ^{flox}/ ^{flox} mice

The development of Th1, Th2 and Th17 was investigated according to their related respective cytokines IFNγ, IL4 and IL22. At p.i day 21 the MLN were isolated from the IL1-RII ^{flox}/ ^{flox} and IL1-RII$^{-/-}$ mice infected with a low or high dose of *T.muris*. 50 mg/mL parasite E/S antigen was used to stimulate MLN cells for 48 hours. An ELISA was preformed to measure the concentration of the cytokines IFNγ, IL4 and IL22. IL17, a member of Th17, was investigated within the infected mice in response to a low dose of ***T.*** *muris*.

It was found that in response to a high dose of the *T.muris* infection, the concentration of IFNγ, IL4 and IL22 increased significantly in MLN cells isolated from IL1-RII ^{flox}/ ^{flox} and IL1-RII $^{-/-}$ mice, compared to MLN cells isolated from uninfected mice respectively **(**Figure 6-5 A-B-C). Moreover, no significant difference was observed between IL1-RII ^{flox}/ ^{flox} and IL1-RII $^{-/-}$ mice in the concentration of IFNγ and IL4 expressed by MLN cells **(**Figure 6-5 A-B).There was a reduction in the concentration of IL22 in MLN cells isolated from IL1-RII $^{-/-}$ mice compared to MLN cells isolated from IL1- RII ^{flox}/ ^{flox} mice. However, this difference was not significant **(**Figure 6-5C**).**

MLN cells isolated from mice (both IL1-RII ^{flox}/ ^{flox} and IL1-RII $^{-/-}$) infected with a low dose of *T.muris*, demonstrated no significant change in the concentration of either IFNγ or IL4 **(**Figure 6-6 A-B). The concentration of IL22, expressed by MLN cells that were isolated from IL1-RII $^{-/-}$ mice, decreased significantly in comparison to MLN cells isolated from IL1-RII ^{flox}/ ^{flox} mice **(**Figure 6-6 C). In contrast, MLN cells isolated from IL1-RII $^{-/-}$ mice had a significantly higher concentration of IL17 than MLN cells from isolated from IL1-RII ^{flox} / ^{flox} mice **(**Figure 6-6 D).

The concentration of IL17 and IL22 within MLN cells that were isolated from IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} infected mice (with a low dose) were compared to MLN cells isolated

from IL1-RI $^{-/-}$ and IL1-RI ^{flox} / ^{flox} mice also infected with a low dose. This was discussed in chapter five. It was found that the concentration of IL22 reduced significantly in MLN cells isolated from IL1-RI $^{-/-}$ and IL1-RII $^{-/-}$ mice compared to MLN cells isolated from IL1-RI ^{flox} / ^{flox} and IL1- RII ^{flox} / ^{flox} mice respectively **(**Figure 6-7 A). MLN cells isolated from IL1-RII $^{-/-}$ mice expressed significantly more IL22 than MLN cells isolated from IL1-RI $^{-/-}$ mice. (Figure 6-7 A). MLN cells isolated from IL1-RI $^{-/-}$ mice saw a significant reduction in the concentration of IL17 compared to MLN cells isolated from IL1- RI ^{flox} / ^{flox} mice. The concentration of IL17 expressed by MLN cells isolated from IL1-RII $^{-/-}$ mice increased significantly when compared to MLN cells isolated from IL1- RII ^{flox} / ^{flox} mice **(**Figure 6-7 B). The concentration of IL17 increased significantly within MLN cells isolated from IL1-RII $^{-/-}$ mice compared with MLN cells isolated from IL1-RI $^{-/-}$ mice (Figure 6-7 B).

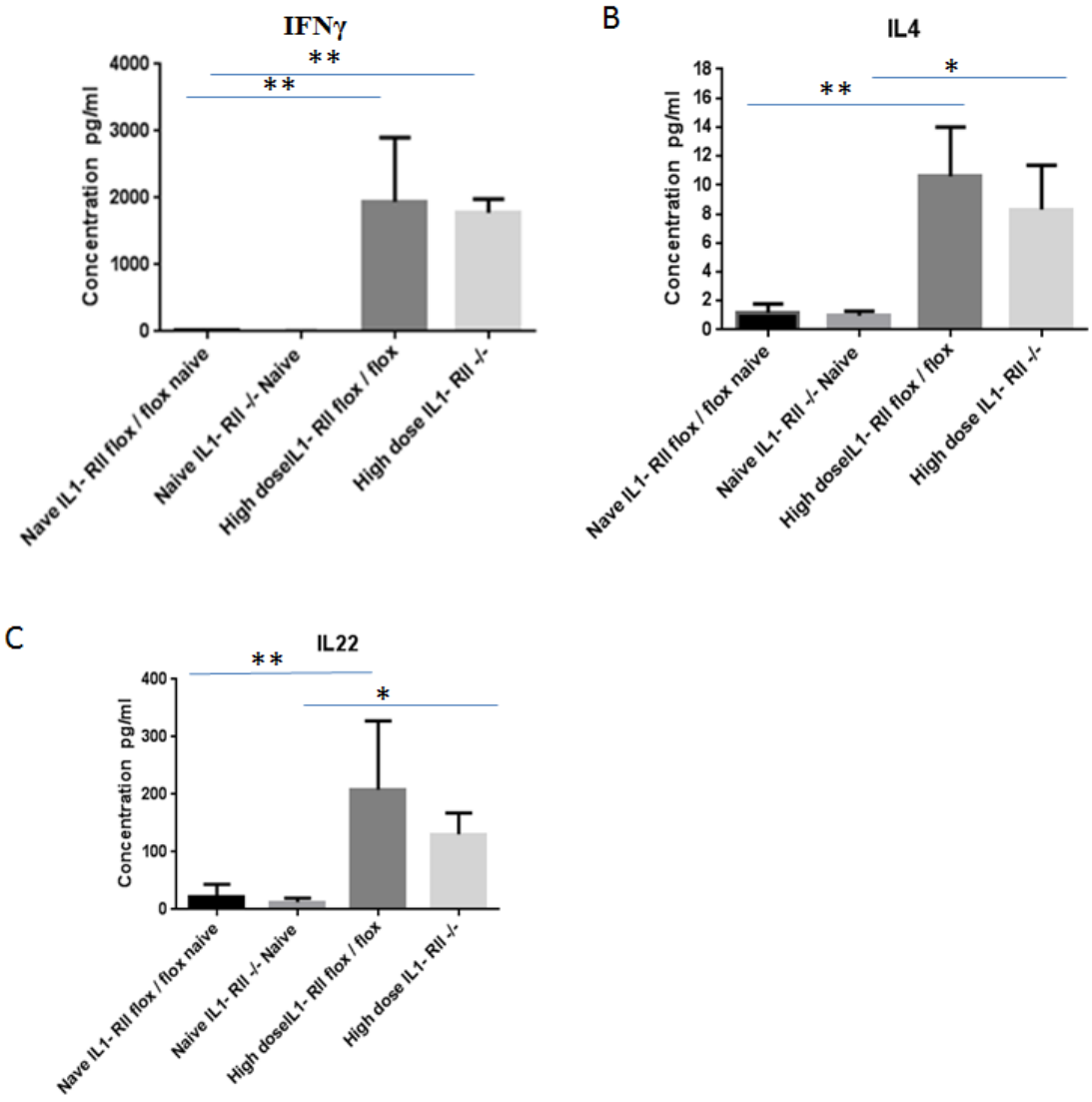


Figure 6-5: This graph illustrates the concentration of IFNγ, IL4 and IL22 expressed by MLN cells isolated from IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice that were infected with a high dose of *T.muris*: MLN cells were removed from IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice with a high dose of *T.muris* at day 21 p.i. MLN cells were then re-stimulated with a final concentration of 50-mg/mL parasite E/S antigen for 48 hours. (A-B-C) The concentration of IFNγ, IL4 and IL22 increased significantly in both IL-1RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice MLN cells isolated from mice infected with a high dose of *T.muris*, compared to MLN cells isolated from both naïve IL-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice. (A-B) No significant difference was observed in the concentration of IFNγ and IL4 between MLN cells isolated from IL1-RII ^{flox} / ^{flox} mice and IL1-RII$^{-/-}$ mice. (C) There was decrease in the concentration of IL22 in MLN cells isolated from IL1-RII $^{-/-}$ mice compared to MLN cells isolated from IL1-RII ^{flox} / ^{flox} mice, but this difference was not significant. Data is presented as a mean percentage (+/- SEM) per group N=4. A one- way ANOVA was conducted, followed by Tukey multiple comparison post hoc tests to analyse the data (* =P≤0.05, ** =P≤0.01).

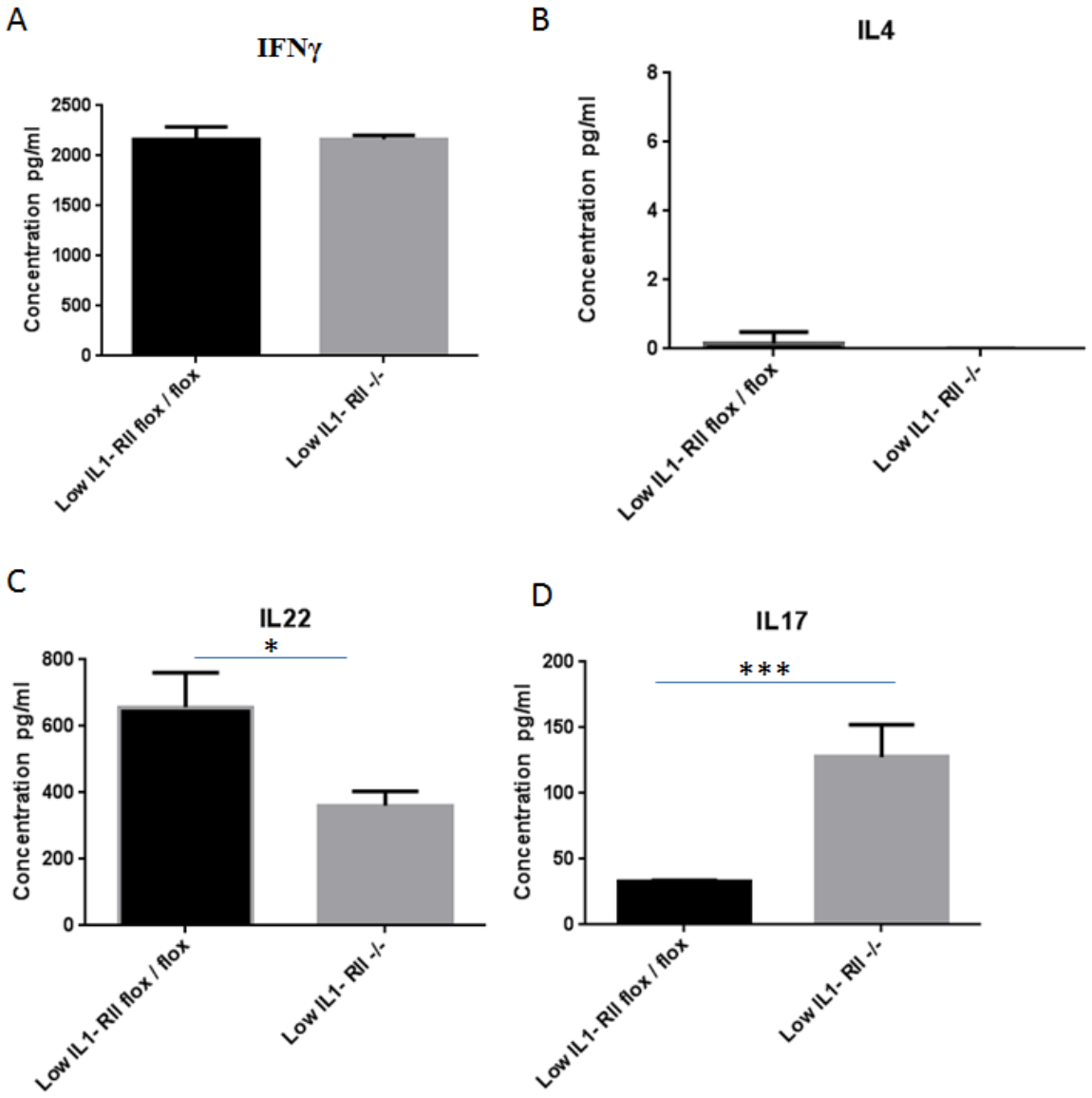


Figure 6-6: This graph illustrates the concentration of IFNγ, IL4, IL22 and IL17 expressed from MLN cells that were isolated from mice IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ infected with low dose of *T.muris*: MLN cells were removed from both IL-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice at day 21 p.i with low dose of *T.muris*. MLN cells were then re-stimulated with a final concentration of 50-mg/mL parasite E/S antigen for 48 hours. (A-B) Upon being infected with a low dose of *T. muris* no significant difference was observed in the MLN cells isolated from either IL-RII ^{flox} / ^{flox} or IL1-RII $^{-/-}$ mice in the concentration of IFNγ and IL4 (C) The concentration of IL22 expressed by MLN cells were isolated from IL1-RII $^{-/-}$ mice decreased significantly when compared to MLN cells isolated from IL-1RII ^{flox} / ^{flox}. (D) There was a significant increase in the concentration of IL17 expressed by MLN cells that were isolated from IL1-RII $^{-/-}$ mice, compared to MLN cells isolated from IL1-RII ^{flox} / ^{flox}. Data is presented as a mean percentage (+/- SEM) per group N=4. A t-test was used to analyse the data (* =P≤0.05, *** =P≤0.001).

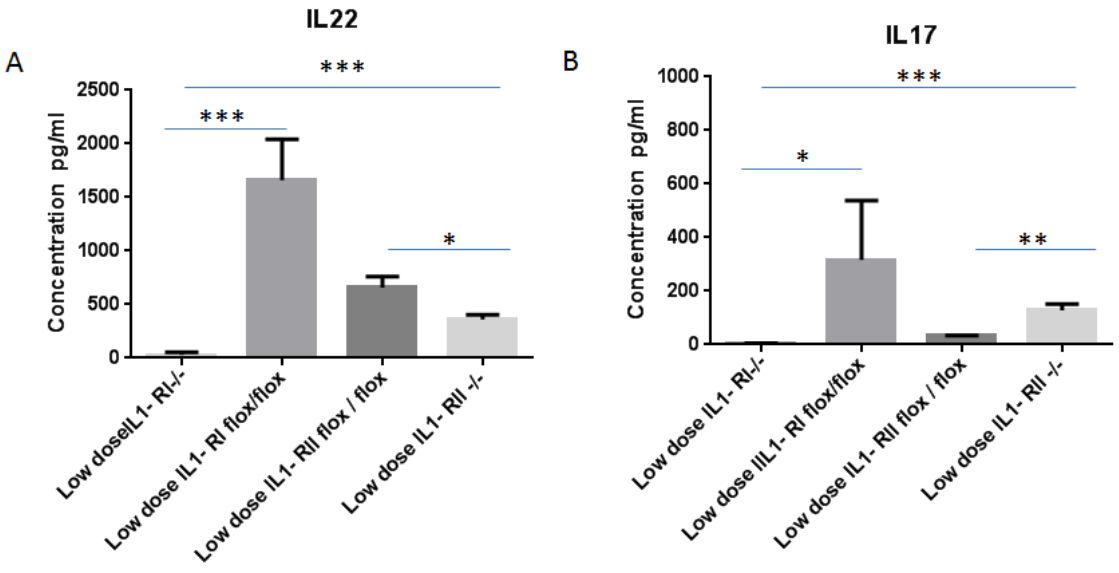


Figure 6-7: A comparison of the concentration of IL-22 and IL-17 expressed by MLN cells isolated from both IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice, and IL1-RI $^{-/-}$ IL1- RI ^{flox} / ^{flox} mice (presented in chapter 5) when infected with low dose of *T.muris*: MLN cells were harvested from IL1-RII ^{flox} / ^{flox,}, IL1-RII $^{-/-}$, IL1-RI $^{-/-}$ and IL1-RI ^{flox} / ^{flox} mice at day 21 p.i with low dose of *T.muris*. MLN cells were then re-stimulated with a final concentration of 50-mg/mL parasite E/S antigen for 48 hours. (A) There was a significant decrease in the concentration of IL22 in MLN cells when isolated from both IL1-RI $^{-/-}$ and IL1-RII $^{-/-}$ mice in comparison to MLN cells isolated from IL1-RI ^{flox} / ^{flox} and IL1-RII ^{flox} / ^{flox} mice. The concentration of IL22 in MLN cells isolated from IL1-RII $^{-/-}$ mice increased significantly when compared to MLN cells isolated from IL1-RI $^{-/-}$ mice. (B) The concentration of IL17 in MLN cells isolated from IL1-RI $^{-/-}$ mice decreased significantly when compared to MLN cells isolated from IL1-RI ^{flox} / ^{flox} mice. The concentration of IL17 increased significantly in MLN cells that were isolated from IL1-RII $^{-/-}$ mice in comparison to MLN cells isolated from IL1-RII ^{flox} / ^{flox} mice. There was a significant increase in the concentration of IL17 within MLN cells isolated from IL1-RII $^{-/-}$ mice in comparison to MLN cells isolated from IL1-RI $^{-/-}$ mice. Data is presented as a mean percentage (+/- SEM) per group N=4. Multiple t- tests were used to analyse the data (* =P≤0.05, ** =P≤0.01, *** =P≤0.001).

6.2.2.4 Histological analysis of IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with both low and high dose of *T.muris*

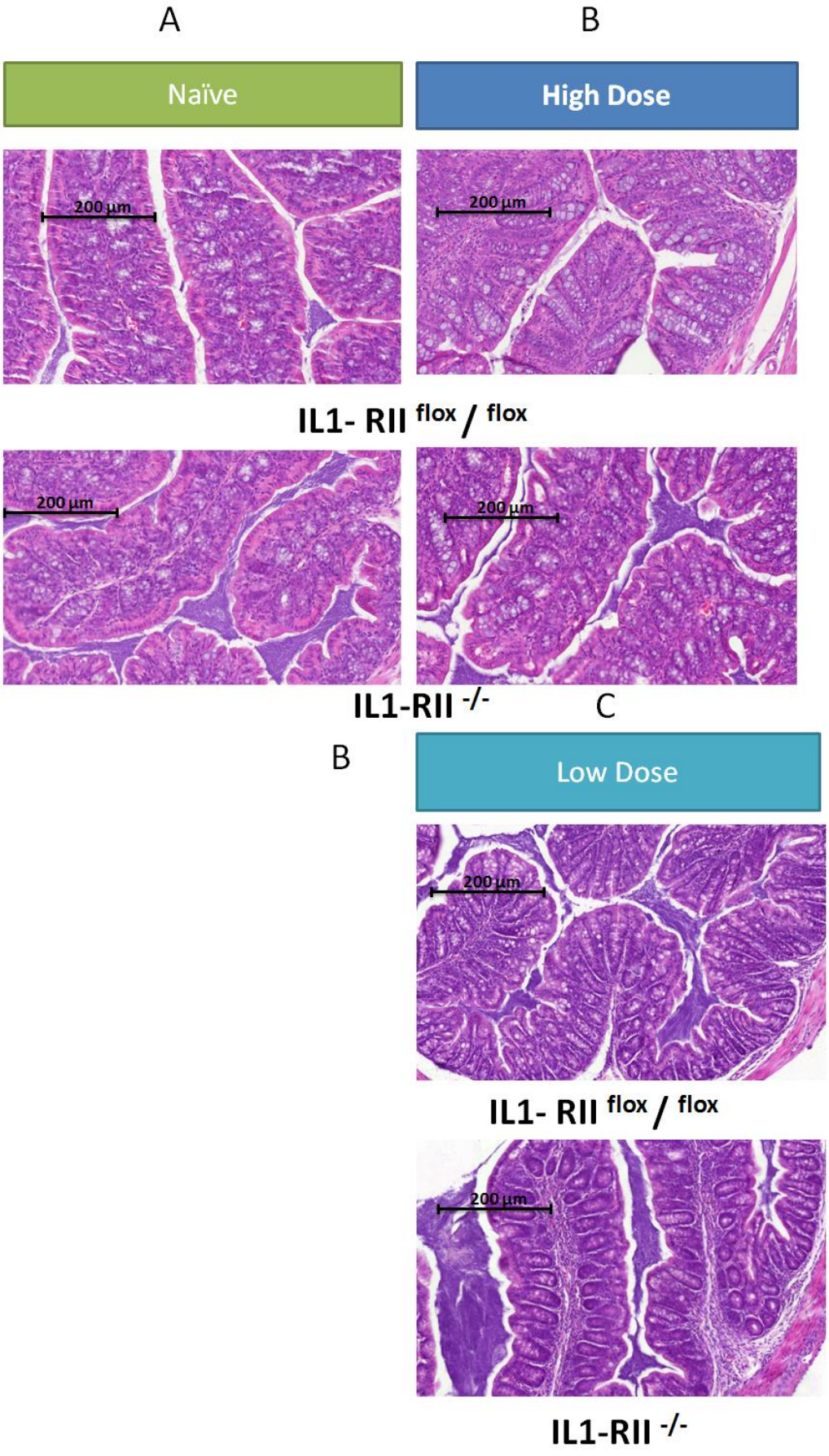
A histology analysis was used to investigate the level of colon inflammation (colitis) according to crypt lengths and the intestinal goblet cells hyperplasia in IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice. It was used to examine the expression of IL22 found in the gut of IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice. The colon from both IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ infected mice was isolated at day 21 p.i and embedded in wax. 5 micron-sections were cut and stained with hematoxylin,eosin, goblet cells stain (periodic acid-Schiffs), or with the anti-IL22 antibody.

6.2.2.4.1 The absence of IL1-RII did not affect the level of colitis in the mice infected with either a low or high dose of *T.muris*

The measurement of crypt length demonstrated that no significant difference was observed in crypt hyperplasia between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with a high dose of *T.muris* (Figure 6-6 A). There was also no significant change observed in crypt length between the IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with a high dose and uninfected IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice respectively (Figure 6-8 A,B). IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice showed an increase in crypt length when they were infected with a low dose of *T.muris***.** However, there was no significant difference in crypt hyperplasia between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice (Figure 6-8 C)

6.2.2.4.2 The deletion of *Il1-rII* gene resulted in *a* reduction in goblet cell hyperplasia in the mice infected with low or high dose of *T.muris.*

In the analysis of goblet cells a significant decrease was found in the goblet cell hyperplasia in IL1-RII $^{-/-}$ compared to IL1-RII ^{flox} / ^{flox} , when the they were infected with either a low or high dose of *T.muris* (Figure 6-9 A,C). Furthermore, IL1-RII ^{flox} / ^{flox} mice infected with a high dose of *T.muris* had a significantly higher goblet cell count than naïve IL1-RII ^{flox} / ^{flox} mice (Figure 6-9 A-B).


A
B
Naïve
High Dose
200 µm
200 µm
IL1- RII flox / flox
200 µm
200 µm
IL1-RII -/-
C
B
Low Dose
200 µm
IL1- RII flox / flox
200 µm
IL1-RII -/-

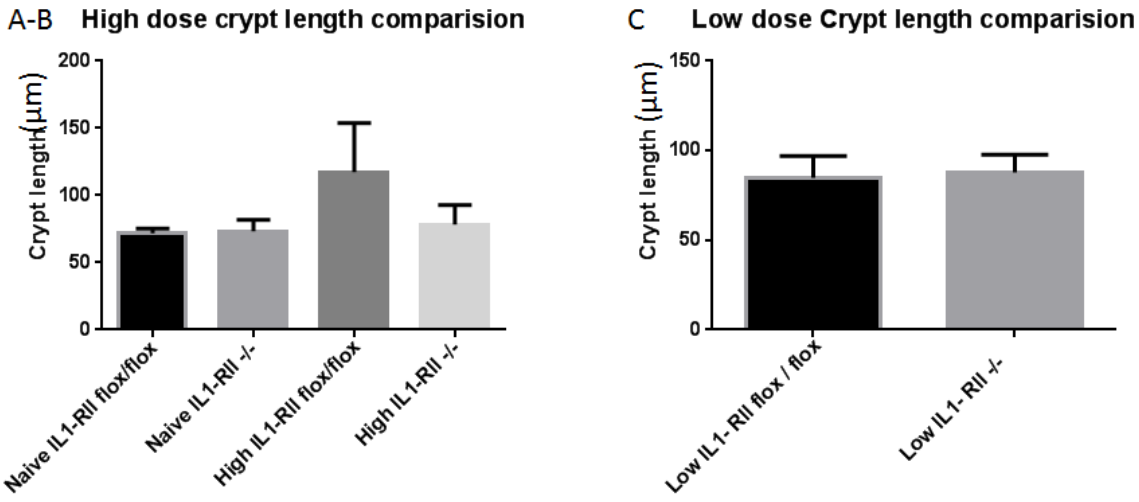
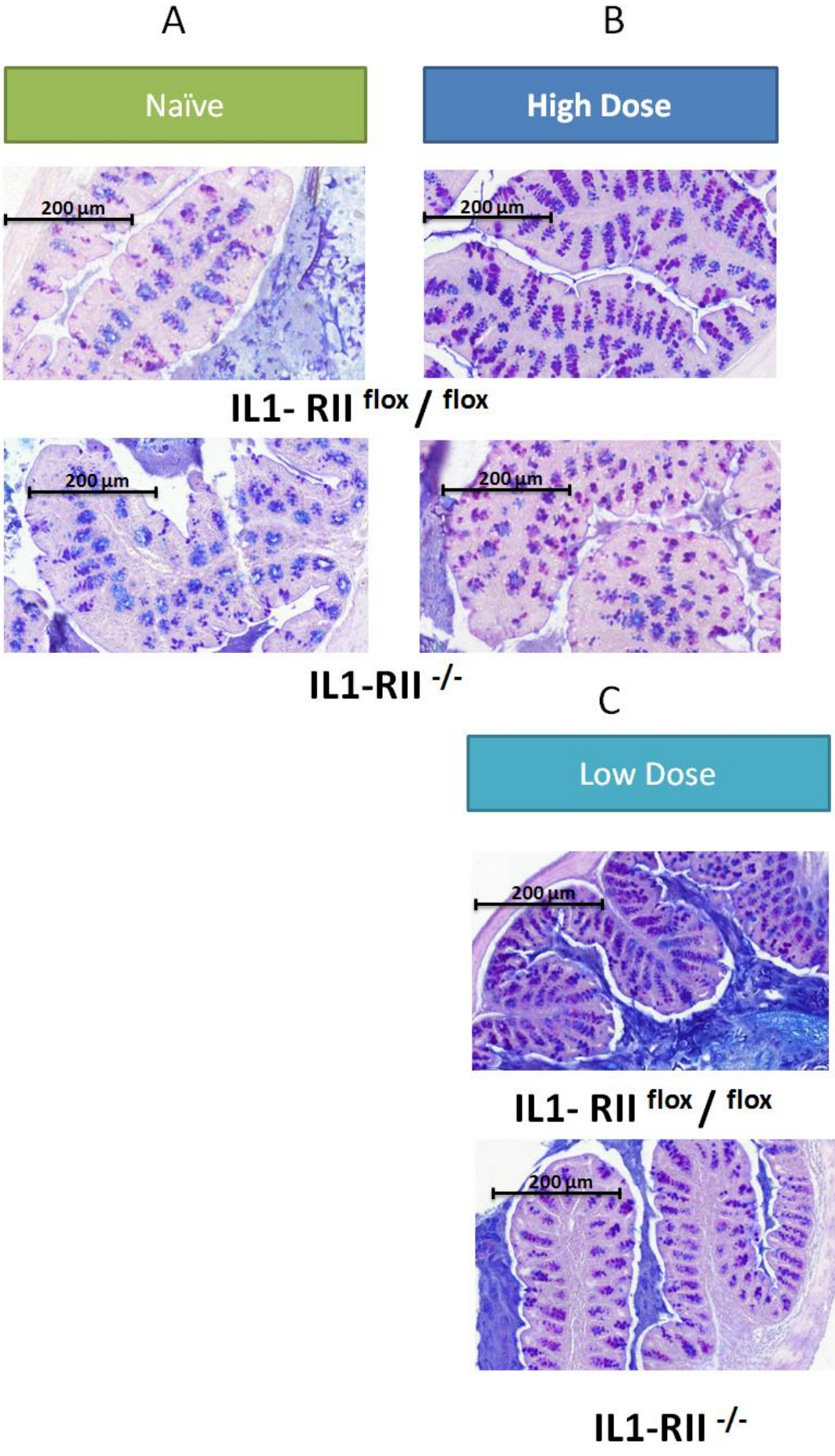


Figure 6-8: The colonic crypt lengths in IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice on day 21 p.i when infected with either (A) a high or (D) a low dose of *T.muris* Mice were infected with a low or high dose of *T.muris*. At day 21 p.i, the colon was isolated from the mice and embedded in wax. 5 micro-sections were cut and stained with hematoxylin and eosin. The slides were scanned by the panoramic viewer system and the crypt lengths were measured using ImageJ software. The pictures are all magnified by 10x. (A,C) No significant difference was observed in the crypt lengths between IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice that were infected with high or low dose of *T.muris*. (A-B) IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with a high dose did not show a change in crypt length in comparison to naïve IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice. (D) IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice infected with a low dose caused an increase of crypt length. The data is presented as a mean percentage (+/- SEM) per group N=4.(A-B) A one- way ANOVA was followed by a Tukey multiple comparison post hoc tests, and (C) a t-test was used to analyse the data.


A
B
Naïve
High Dose
200 µm
200 µm
IL1- RII flox / flox
200 µm
200 µm
IL1-RII -/-
C
Low Dose
200 µm
IL1- RII flox / flox
200 µm
IL1-RII -/-

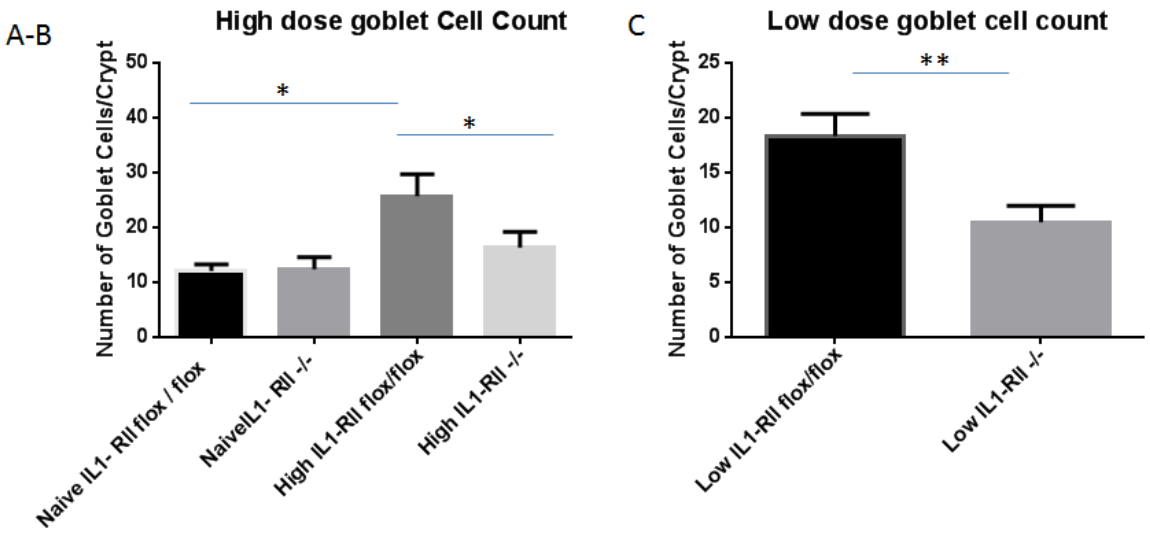


Figure 6-9: The intestinal goblet cell count found in IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice on day 21 p.i infected with (A) a high and (C) a low dose of *T.muris.* Mice were infected with a low and high dose of *T.muris*. At day 21 p.i the colon was isolated from the mice and embedded in wax. 5 micro-sections were cut and stained with a goblet cell stain (periodic acid-Schiffs). The slides were scanned by the panoramic viewer system and the goblet cells were enumerated in 20 randomly selected crypts. All pictures are magnified by 10x. (A-C) The IL1-RII $^{-/-}$ mice infected with either a low or high dose of *T.muris* had a significant reduction in the goblet cell numbers compared to IL1-RII ^{flox} / ^{flox} mice. (A-B) The number of the goblet cells increased significantly in IL1-RII ^{flox} / ^{flox} mice infected with a high dose compared to naïve IL1-RII ^{flox} / ^{flox} mice. The data is presented as a mean percentage (+/- SEM) per group N=4. (A-B) A one-way ANOVA was followed by a Tukey multiple comparison post hoc test, and (C) demonstrates the t-test that was conducted to analyse the data. (* =P≤0.05, ** =P≤0.01).

6.2.2.4.3 The reduction of the IL22 expression seen within infected IL1-RII $^{-/-}$ mice compared to infected IL1-RII $^{flox/flox}$ mice with a low or high dose of *T.muris*

The expression of IL22 was investigated in the colon using immunohistochemistry for anti-IL22. It was found that the IL1-RII $^{-/-}$ mice showed a reduction in the IL22 expression compared to IL1-RII ^{flox} / ^{flox} mice when they were infected with either a low or high dose of *T.muris* (Figure 6-10 A-B).

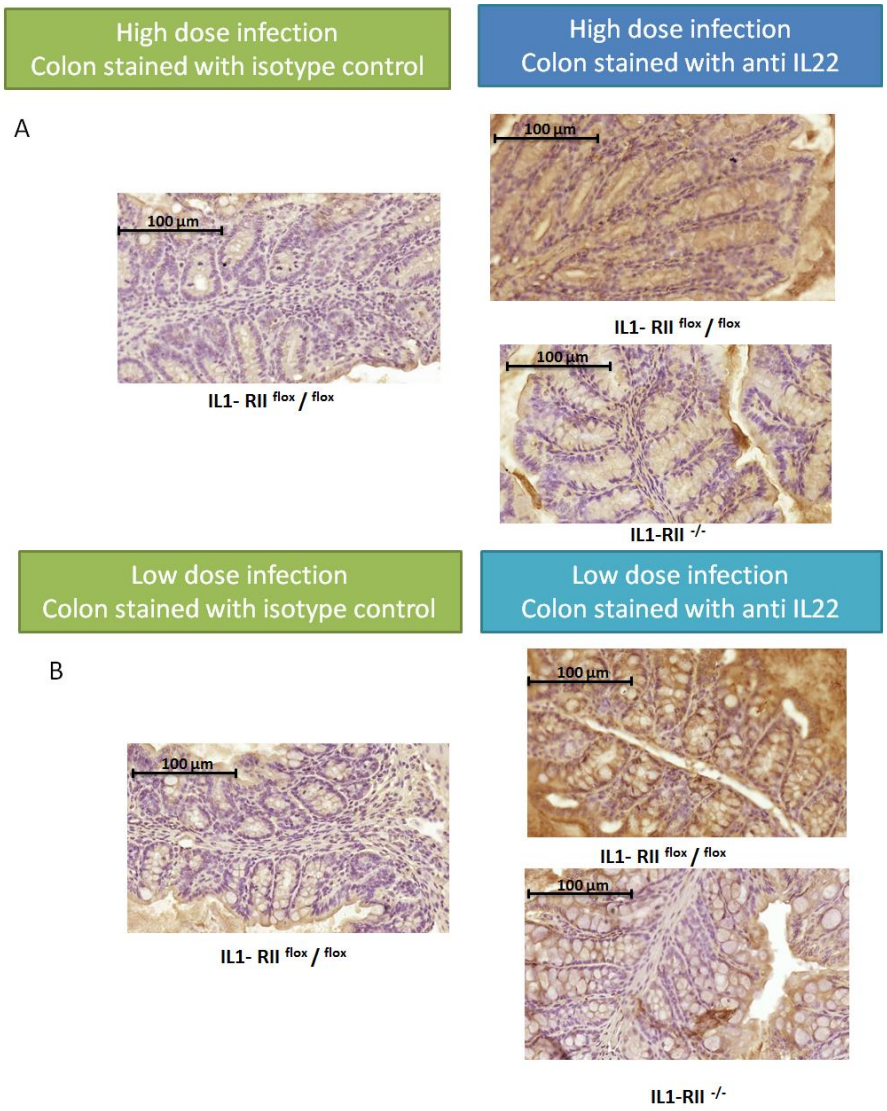


Figure 6-10: The immunohistochemistry of IL1-RII ^{flox} / ^{flox} and IL1-RII $^{-/-}$ mice on day 21 p.i with (A) a high or (B) a low dose of *T.muris:* The colonic tissue was embedded within the wax. 5 micro-sections were cut and stained with either the anti-IL-22 antibody (brown) or isotype control by immunohistochemistry and then counterstained with haematoxylin (blue). The IL1-RII $^{-/-}$ mice had a reduction in the IL22 expression compared to IL1-RI ^{flox} / ^{flox} mice when they were infected with either a low or high dose of *T.muris*. The slides were scanned by the panoramic viewer system. All pictures are magnified by 20X N=4.

6.3 DISCUSSION

It has been reported that IL-1Ra and IL1-RII are an inhibitors receptors, both regulate the biological activity of IL-1 (Smith *et al.*, 2003). Therefore, the aim of this chapter was to investigate the role of IL-1 signalling seen in the immune response and in the development of Th1, Th2 and Th17 cells by using IL1-RII global knockout mice (IL1-RII $^{-/-}$). Chapter 5 demonstrated that IL1-RI signalling plays an essential role in the development of Th17 cells and within their cytokine production. The current study has verified the role of IL-1 signaling within the development of Th17 cells and in their cytokine production by comparing IL1-RI $^{-/-}$ and IL1-RII $^{-/-}$ mice. In this study, a low and high dose of *T.muris* infection was used in order to challenge the IL1-RII $^{-/-}$ and control IL1-RII ^{flox} / ^{flox} mice to develop Th1, Th2 and Th17 phenotype. The development of Th1, Th2 and Th17 cells were evaluated according to their cytokine production; IFNγ for Th1, IL4 for Th2 and IL17 and IL22 for Th17.

It was demonstrated that upon being infected with a high dose of *T.muris*, both IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice expelled the worms and mounted a significantly higher level of IgG1 than uninfected mice. MLN cells isolated from both IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice when they were infected with a high dose of *T.muris* expressed a significantly increased concentration of IFNγ, IL4 and IL22 compared to uninfected mice. It was reported that C57BL/6 mice responded slowly to the high *T.muris* infection dose and were able to clear the infection within 21 days. This was found to associate with a strong Th2 immune response in a high expression of IL4, IL-5, IL-9 and IgG1 (Bancroft *et al*., 2001, Hurst *et al*., 2013, Hurst and Else, 2013a).

Several studies have demonstrated that C57BL/6 mice infected with low dose of *T.muris* are unable to expel the worms and demonstrate a stronger Th1 response with high levels of IFNγ, IL-18 and IL-12 and IgG2 (Bancroft *et al*., 2001, Hurst *et al*., 2013, Hurst and Else, 2013a) The current study demonstrated that the IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice infected with low dose of *T.muris* were susceptible to the infection and showed a high

expression of IgG2. MLN cells isolated from low dose infected IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice demonstrated a high concentration of IFNγ and a low concentration of IL4.

This study has demonstrated that a high dose of *T.muris* infection showed no significant change in the level of IgG1 observed between IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice, Additionally, no significant change was found in the concentration of IFNγ, IL4 and IL22 between MLN cells isolated from IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice infected with a high dose of *T.muris*. IL1-RII $^{-/-}$ and IL1- RII ^{flox} / ^{flox} mice infected with a low dose also showed no change in the expression of IgG2 or worm burden between the two types of mice. The comparison between MLN cells isolated from the mice showed no significant change in the concentration of IFNγ and IL4 when infected with low dose of *T.muris.* The possible explanation for this finding is that the biological effect of both IL-1α and IL-1β is achieved through the binding with IL1-RI but not with IL1-RII due to the short cytoplasmic domain (TIR domain) of IL1-RII which important for the association with MyD88 (Sims *et al*., 1993, Shimizu *et al*., 2015). In this previous study murine pre-B-cell lymphoma was used, which required IL-1 signalling to express the IgM surface (sIgM). This was found through blocking IL1-RI in murine pre-B-cell lymphoma 70Z/3 with the Monoclonal antibody M147. This blocks the binding of IL-1 to murine IL1-RI but not for IL1-RII, which completely prevents the response of IL-1 and the expression of sIgM when the cells are stimulated with IL-1 (Sims *et al*., 1993). This study also used the human hepatoma cell line HepG2 that produced a noticeable amount of both receptors. They investigated the induction of IL8 thorough IL-1β and found that the expression of IL8 was inhibited by the anti-IL1-RI and not anti-IL1-RII (Sims *et al*., 1993). This indicated that IL1-RI was responsible for the biological effects of IL-1α and IL-1β.

In chapter 5 it was demonstrated that the concentration of the Th17 cytokines IL17 and IL22 significantly decreased in the absence of IL1-RI. The current chapter demonstrates that upon being infected with a low dose of *T.muris*, the concentration of IL17 significantly increased in the MLN isolated from IL1-RII $^{-/-}$ mice compared to IL1-RII ^{flox} / ^{flox} mice.

The comparison of IL17 and IL22 between the low dose infected IL1-RI $^{-/-}$ mice and IL1-RII $^{-/-}$ mice demonstrated that both cytokines increased significantly in the MLN cells isolated from IL1-RII $^{-/-}$ compared to IL1-RI $^{-/-}$ mice. The possible explanation for this finding could potentially be because IL1-RII has been reported to be an inhibitor of IL1-RI (Smith *et al.*, 2003), which is important for the generation of Th17 cells. As previously discussed, several studies have demonstrated that IL-1 signalling plays a critical role in Th17 cell differentiation and in the secretion of IL17 and IL22 cytokines (Chung *et al.*, 2009, Sutton *et al.*, 2009, Sutton *et al.*, 2006). It was revealed that the soluble form of IL1-RAcP (IL1-sRAcP) enhanced the ability for IL-1RII (IL-1sRII) to inhibit action of IL1-RI by increasing its affinity to bind to both IL-1α and IL-1β (Smith et al., 2003). It was also reported that the soluble form of IL-1sRII enhanced the inhibition of IL-1Ra, whereas, the soluble form of IL-1RI (IL-1sRI) prevented it. Interestingly, these two inhibitors were able to eradicate each other's effects (Burger *et al.*, 1995). It is well known that the competitive binding of IL-1Ra to IL1-RI prevents IL1-RAcP from binding to IL1-RI. The absence of IL1-RAcP affects the binding affinity of the IL-1 ligands, which has been shown to prevent signal transduction (Cullinan *et al.*, 1998, Burger *et al.*, 1995, Dinarello, 2009). It was reported that with the highest affinity, IL-1sRII binds to IL-1β but not to IL-1α, and leads to an inhibition of the activity of IL-1β (Giri *et al.*, 1994). The binding of IL1-R II with pro- IL-1α prevents the cleavage and activity of IL-1α (Zheng *et al.*, 2013). Thus, it is suggested that absence of IL1-RII allows IL-1sRI to prevent the inhibition of IL-1Ra. It also effectively increases the concentration of both IL-1α and IL-1β, which are able to signal through IL1-RI, leading to an increase in the production of IL17.

Several studies have used global IL-1Ra knockout mice (IL-1Ra $^{-/-}$) in order to investigate the role of IL-1 in the development of Th17. The role of IL-1 in the development of Th17 was investigated by using IL-1Ra $^{-/-}$ mice, which developed autoimmune arthritis. This study found that the lymph nodes of IL-1Ra $^{-/-}$ mice had a high concentration of IL21. They showed that in naïve CD4 T cells, the expression of IL-1Ra was induced by both IL21 and

IL-1, and were able to inhibit TGF-β-induced Foxp3, resulting in Th17 cell differentiation in absence of IL6 (Ikeda *et al*., 2014).

Interestingly, the current findings demonstrated that when infected with either a low or high dose of *T.muris*, there was a reduction in the concentration of IL22 found in the MLN cells isolated from IL1-RII $^{-/-}$ mice compared to IL1- RII ^{flox} / ^{flox} mice. Due to the ability of a chronic *T.muris* infection to stimulate Th17 cells, the change in concentration of IL-22 was significant for a low dose but not for a high dose between MLN cells isolated from infected IL1-RII ^{flox} / ^{flox} mice and MLN cells isolated from infected IL1-RII $^{-/-}$ mice (Levison *et al*., 2010, Fasnacht *et al*., 2009). IL-1Ra $^{-/-}$ mice were found to have a progressive erosive arthritis. IL-1Ra $^{-/-}$ mice demonstrated a high level of IL17 in tissue that was either mildly or severely inflamed, while a high level of IL22 was observed only in severely inflamed tissue (Marijnissen *et al*., 2011). Thus, it is suggested that increasing the infection period may increase the level of the inflammation, and therefore could potentially increase the concentration of IL22 within IL1-RII $^{-/-}$ mice. Another possible explanation for these findings, as previously mentioned, could potentially be because IL1-RII is able to bind and inhibit the activity of IL-1β (Giri *et al*., 1994). It is also able to bind to pro- IL-1α, which leads to the inhibition of cleavage and the activity of IL-1α (Zheng *et al*., 2013). It is proposed that the absence of IL1-RII may cause a defect in the balance between IL-1α and IL-1β, which may potentially lead to this phenotype.

A histology analysis was used to investigate the change in colitis and in goblet cells hyperplasia between IL1-RII $^{-/-}$ and IL1- RII ^{flox} / ^{flox} mice. The crypt length demonstrated no significant change in crypt hyperplasia between IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice infected with a high dose. There was also no significant difference between the mice in their crypt hyperplasia when they were infected with a high dose of *T. muris.*

One possible explanation for this result is because the high dose infected mice were able to expel the worm through the crypt cell escalator. It was reported that within acute infected

resistant mice, the crypt cell exhibited hyperplasia in response to the *T.muris* infection due to the 'crypt cell escalator' that is responsible for fast worm expulsion (Cliffe *et al*., 2005, Klementowicz *et al*., 2012).

IL1-RII $^{-/-}$ and IL1-RII ^{flox} / ^{flox} mice infected with a low dose demonstrated no change in the crypt hyperplasia. The main function of IL22 is to promote anti-apoptotic pathways such as STAT3, Akt, mitogen-activated protein kinase pathways, anti-microbial molecules such as b-defensin, Reg3c, and lipocalin-2, which are important for preventing tissue damage and for also helping to repair tissue (Zenewicz and Flavell, 2011). As previously mentioned, MLN isolated from IL1-RII $^{-/-}$ mice had a higher IL22 expression compared to IL1-RI $^{-/-}$ mice when infected with a low dose of *T.muris*. This data indicates that IL1-RII $^{-/-}$ mice were able to produce sufficient quantities of IL22 via the IL1-RI signalling pathway that is required for tissue repair.

The analysis of goblet cells in mice infected with either a high or low dose of *T.muris* showed that IL1-RII $^{-/-}$ mice had reduced goblet cells hyperplasia compared to IL1-RII ^{flox} / ^{flox} mice. As discussed in chapter 5, IL22 plays an essential role in goblet cell activation, by promoting the induction of goblet cells markers such as Muc1 and Muc3 (Turner *et al*., 2013). This data provides further evidence for the reduction of IL22 level in IL1-RII $^{-/-}$ mice.

The current study used immunohistochemistry in colonic tissue for anti-IL22 to confirm the reduction of IL22 in IL1-RII $^{-/-}$ mice. This experiment demonstrated that the expression of IL22 decreased in IL1-RII $^{-/-}$ mice compared to IL1-RII ^{flox} / ^{flox} mice when infected with low or high dose of *T.muris.* This data confirms the decrease in concentration of IL22 in IL1-RII $^{-/-}$. Further experiments are required in order to understand the role of IL22, as well as to understand the level of IL-1α and IL-1β in the absence of IL1-RII $^{-/-}$ for infections of both low and high doses of *T.muris*. Null IL-1α and IL-1β mice can be used to investigate

the cytokines that are responsible for the development of Th17 cells and cytokines IL22 and IL17.

6.4 CONCLUSION

The results presented in this chapter demonstrate that the absence of IL-1RII did not affect the development or cytokine secretion of Th1 and Th2 cells. This indicates that IL-1RI is responsible for the biological effects of IL-1α and IL-1β. As IL-1RII is an inhibitor of IL-1RI, the deletion of the *Il1-rII* gene causes an overexpression of IL17 and IL22 in the mice infected with a low dose of *T.muris.* This finding was observed when IL1-RII$^{-/-}$ was compared with IL1-RI$^{-/-}$ mice As previously mentioned the soluble form of IL-1sRII enhances the inhibition of IL-1Ra, whereas the soluble form of IL-1sRI prevented it. Thus, we hypothesized that the absence of IL1-RII allowed IL-1RI to prevent the inhibition activity of IL1-Ra and that caused an increse in IL17 and IL22 Figure (6-11).This demonstrates that IL-1 signalling is important for the development of Th17 cells and for the secretion of cytokines IL17 and IL22. However , a reduction in the level of IL22 was found in IL1-RII $^{-/-}$ mice compared to IL1-RII ^{flox} / ^{flox} mice, which caused a reduction in goblet cells hyperplasia within IL1-RII $^{-/-}$. This change may be due to the low level of inflammation or in the defect of the balance between IL-1α and IL-1β.

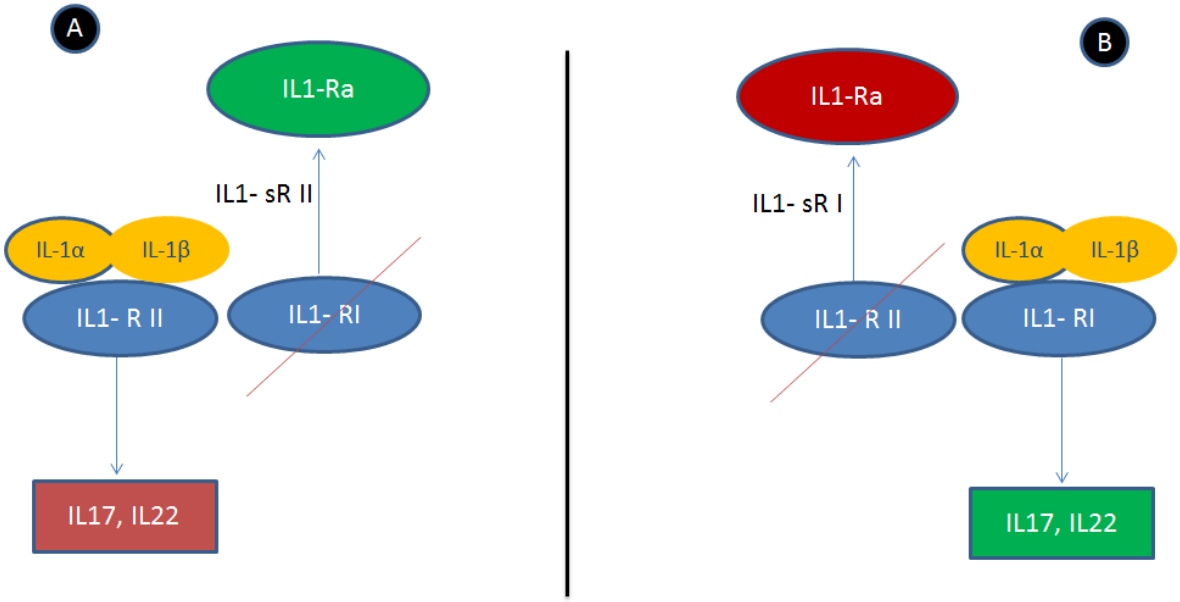


Figure 6-11: Hypothesis for phenotypes observed in IL1-RI$^{-/-}$ and IL1-RII$^{-/-}$ regarding the expression of IL17 and IL22. (A) In absence of IL-1RI, IL-1α and /or IL-1β bind to IL-1RII and that leads to the prevention of both signal transduction and the production of IL17 and IL22. Moreover, the soluble form of IL-1sRII enhances the inhibition of IL-1Ra. (B) In contrast, IL-1α and /or IL-1β are able to bind to IL-1RI in absence of IL-1RII. The presence of IL-1sRI prevents the inhibitory activity of IL1-Ra. This leads to an increase in the production of IL22 and IL17.

CHAPTER SEVEN: GENERAL DISCUSSION

7.1 SUMMARY

IL-1 is a pro-inflammatory cytokine consisting of two molecules; IL-1α and IL-1β, which play an important role in the activation and regulation of host defence and immune responses to inflammation or injury (Sims *et al*., 1993, Auron, 1998). Both IL-1α and IL-1β are able to bind and activate IL1-RI and IL1-RII, which are found on many cells types such as T-cells, fibroblasts, and endothelial cell (Dinarello, 1996, Kamari *et al*., 2007, Cullinan *et al*., 1998). There are many cells capable to produce IL-1 such as epithelial cells, lymphoid cells, vascular smooth muscle cells and hepatocytes. However, macrophages and monocytes are the primarily source for IL-1 (Kamari *et al*., 2007).

Th17 cells appear to be essential for autoimmunity and clearance of mucosal infection through the production of pro-inflammatory cytokines such as IL-17A, IL-17F and IL-22 (Chung *et al*., 2009). Several studies showed that IL-1 signalling plays an essential role in the differentiation of Th17 cells and production of its cytokine IL17 and IL22 (Chung *et al*., 2009, Sutton *et al*., 2006, Mills, 2008). The role of IL-1 signalling in the development of Th1, Th2 and Th17 has been investigated using global knockout IL1-RI (IL1-RI$^{-/-}$) mice. It was shown that the immunization of IL1-RI$^{-/-}$ mice with Keyhole limpet hemocyanin (KLH) and LPS induced the production of Th1 and Th2 cytokine IFN-γ and IL-10 respectively. The expression of the Th17 cytokine, IL17, was reduced in IL1-RI$^{-/-}$ mice, compared with wild-type C57BL/6 mice (Sutton *et al*., 2006). This data suggested that IL-1 signalling is required for the production of the Th17 cytokine, IL17. There are many cells able to produce IL17 and/or IL22. Some of these cells required IL-1 signalling to produce IL22 and/or IL17 like Th17 and some of them not (discussed in section 7.2.1).

The specific cells which are responsible for the expression of IL-1 signalling in the immune defense and in the development of the T helper cell lineage in response to pathogenic infection, is still largely unclear. Therefore in this thesis, IL1-RI conditional knockout mice specifically in hematopoietic cells (IL1-RI *vaviCre*) mice were used. IL1-

RI *vaviCre* mice are able to express IL1-RI in cells type such as epithelial cells and fibroblasts. Using IL1-RI *vaviCre* mice would determine whether the expression IL-1 signalling from hematopoietic cells is responsible for the immune response against parasitic infection. IL1-RI *vaviCre* mice were compared with IL1-RI$^{-/-}$ mice in order to develop a greater understanding and clarify the role of IL-1 signalling *in vivo*. Mice with cell type specific knockouts can be used to further identify the cell type involved in IL-1 signalling.

It was known that IL1-RII is decoy receptor for IL-1 because it lacks an TIR signalling domain. As mentioned earlier, IL-1 is able to bind and activate two receptors IL1-RI and IL1-RII. However, the biological effect of IL-1 is achieved only by binding with IL-1RI not IL1-RII. The binding of IL-1 with IL1-RII cannot transduce the signal, because IL1-R II lacks the TIR domain, which required for the association with MyD88 (Sims et al., 1993, Shimizu *et al*., 2015). The inhibitor receptors IL-1Ra and IL1-R II have been shown to regulate the biological activity of IL-1 (Smith *et al*., 2003, Shimizu *et al*., 2015). Thus, in the current study, global IL1-RII knockout mice (IL1-RII$^{-/-}$) was also used and compared with IL1-RI $^{-/-}$ mice in order to better understand the role of IL-1 signalling in the immune system.

Gastrointestinal helminth *Trichuris muris* (*T.muris*) has been utilized in this thesis as an infection model to investigate gut inflammation in the mouse mutant. *Trichuris muris* is a well-known model of intestinal nematode infection, the expulsion of the parasite is dependent on the development of a Th2 response. Varies doses of *T-muris* infection have been used to challenge the mice to generate different immune responses; Th1, Th2 and Th17. It is well known that the low dose infection of C57BL/6 with ***T.****muris* results in a chronic infection and the mice are able to generate a Th1 and Th17 response. While, the high dose infection with ***T.*** *muris* leads to an acute infection and the mice developed Th2 response. (Bancroft *et al*., 2001, Hurst *et al*., 2013, Hurst and Else, 2013a, Levison *et al*., Fasnacht *et al*., 2009).

7.1.1 The successful generation of a conditional knockout mice IL1-RI *vaviCre*

In this thesis, the generation and characterisation of IL1-RI *vaviCre* mice has been described (chapter three). The generation of IL1-RI flox/flox mice was performed by TaconicArtemis in 2011 in Cologne, Germany. The deletion of the floxed sequence in the IL1-RI $^{fl/fl}$ mice was induced by crossed IL1-RI ^{flox} / ^{flox} mice with the IFNγ-R $^{flox/flox}$ *vaviCre* + mice, which promoted the Cre recombination in IL1-RI $^{flox/flox}$. The conditional knockout IL1-RI $^{flox/flox}$ *vaviCre* + (IL1-RI *vaviCre* +) mice and its control IL1-RI $^{flox/flox}$ *vaviCre* – (IL1-RI *vaviCre* –) mice were generated successfully at the end of the breeding process.

IL1-RI delta allele has been investigated at the genomic and proteomic level in order to confirm that the *Il1-rI* gene is inactivated in hematopoietic cells. The characterisation of IL1-RI *vaviCre* + mice at the genomic level confirmed that the *Il1-rI* gene was obliterated successfully. Our finding demonstrated an absence of the DNA sequence flanked with the two loxP sites in IL1-RI *vaviCre* + mice. It also showed that the deletion of the DNA sequence flanked with the two loxP sites in IL1-RI *vaviCre* + mice causing a clear amino acid modification. The ribbon diagram of IL1-RI in IL1-RI *vaviCre* + mice demonstrated that IL1-RI *vaviCre* + mice lacked a IL-1α and /or IL-1β binding site compared with the ribbon diagram of IL1-RI performed by (Thomas *et al*., 2012).

The characterisation of IL1-RI *vaviCre* + mice at protein level confirmed that IL1-RI was dysfunctional in hematopoietic cells. The *in vitro* analysis of IL1-RI *vaviCre* + demonstrated that IL1-RI *vaviCre* + spleen cells were capable of expressing IL1-RI when stimulated with LPS but not IL-1β. Therefore, we hypothesised that the expression of IL1-RI, observed in response to LPS in the IL1-RI *vaviCre* + spleen cells, may be due to the production of IL-1β from LPS stimulated epithelial cells which are still capable of expressing IL1-RI.

It has been reported that IL-1β is a potent inducer of IL-6 and MCP-1 synthesis in the primary neurone cells (Tsakiri *et al*., 2008a). Conversely, IL-1α is effective at inducing

TNFα synthesis in epidermal cells (Andre *et al*., 2005). Thus, in this study the production of IL6, TNFα and the chemokine MCP-1 was investigated in the absence of IL1-RI in hematopoietic cells in response to IL-1β and LPS. Our finding demonstrated that after 24 hour of being stimulation with IL-1β the concentration of IL6, TNFα and MCP-1 decreased significantly in IL1-RI *vaviCre* + spleen cells compared with the control IL1-RI *vaviCre* – spleen cells. As IL-1α but not IL-1β is able to induce TNFα, we suggested that the presence of IL-1α in the IL1- RI *vaviCre* – spleen cells were able to bind and activate IL1- RI. Moreover, in this study both IL1-RI *vaviCre* + and IL1-RI *vaviCre* – spleen cells had a high expression of IL6 and TNFα when they were stimulated with LPS. This may due the ability of LPS to activate many signalling pathways such as NFκB and MAPK proteins though TLR4, which leads to the expression of cytokines such as IL-6 and TNFα (Smolinska *et al*., 2011, Li *et al*., 2012).

This data indicted the new IL1-RI *vaviCre* conditional knockout mice have been successfully generated. This new mouse line can be used to examine whether the signalling of IL-1 in hematopoietic cells is required for immune defense and the generation of Th cells subset.

7.1.2 IL1-RI *vaviCre* and IL1-RI$^{-/-}$ mice are vital and suitable *in vivo* model for studying the role of IL-1 signalling

IL-1 plays an important role in the activation and functioning of many lymphocytes such as CD4 and CD8 T cells, B cells, monocytes and macrophage (Ben-Sasson *et al*., 2013, Ben-Sasson *et al*., 2009, Nakae *et al*., 2001a, Netea *et al*., 2010). The deletion of IL1-RI may cause a defect in the development of lymphocytes, which may cause an alteration in the mouse phenotype. Hence, this would provide an inappropriate model for the current study. Therefore, in this study the development of the immune cells were identified as B cells, non-B cells, T cells, monocytes, neutrophils and NK cells in spleen were investigated in spleen, blood and mesenteric lymph nodes (MLN) of the mice (described in chapter four).

Due to the alteration was obvesrved in the development of B cells in blood and MLN, B cell differentiation was investigated (describe in chapter four).

Our finding demonstrated that the lymphocyte development was found in both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice, were not affected by the deletion of the Il1-rI gene. This data indicated that both transgenic mice IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ are vital *in vivo* models and suitable to use in this study.

7.1.3 IL-1 signalling expressed from hematopoietic cells is responsible for the development Th17 and secretion of its cytokine IL17 and IL22

In chapter five, the role of IL-1 signalling in hematopoietic cells during the development of Th1 and Th2, Th17 cells in response to low and high dose of ***T. muris*** has been investigated using the new mice line IL1- RI *vaviCre* compared with IL1-RI $^{-/-}$ mice. Our findings showed that both IL1- RI *vaviCre* + and IL1-RI $^{-/-}$ mice infected with high dose of ***T. muris*** were able to clear the infections due to their ability to generate a Th2 response with high expression of IL4 and IgG1. Thus, we suggested that IL1-RI signalling in hematopoietic cells was not required for the worm expulsion and the generation of Th2 response. In low dose infection both IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice were susceptible to infections and showed high level of IFNγ and IgG2 compared with uninfected mice. However, there was a reduction in the level of IgG2 in IL-1-RI *vaviCre* +, IL1-RI $^{-/-}$ and IL22 $^{-/-}$ mice compared with IL-1-RI *vaviCre* –, IL1-RI ^{flox} / ^{flox} and WT mice when the mice where infected with low dose of *T.muris*. We hypothesised that IL-1 is able to promote Th1 and Th2 responses in the absence of IL1-RI in hematopoietic cells. However, IL-1 signalling appears to be important for the generation of a strong Th1 response.

In this study, low dose infection with *T.muris* showed a clear reduction in the concentration of the Th17 cytokine IL22 and IL17 in MLN isolated from IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice. The similar phenotype in the concentration of IL17 and IL22 was observed between IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice suggesting that IL-1 signalling response expressed

from hematopoietic cells is responsible for the development of Th17 cells and secretion of IL17 and IL22.

Additionally, an increase of the colon inflammation (colitis) and decreased of the goblet cells hyperplasia in low dose infected IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice compared with low dose infected IL1-RI *vaviCre* – and IL1-RI $^{flox/flox}$ mice was observed in this study. IL22 has been reported as an important cytokine in promoting anti-apoptotic pathways such as STAT3, Akt, mitogen-activated protein kinase pathways and anti-microbial molecules such as b-defensin, Reg3c, lipocalin-2, which is important in preventing tissue damage and tissue repair (Zenewicz and Flavell, 2011). It also plays an important role in the activation of goblet cells through the induction of many goblet cells markers such as Muc1 and Muc3 (Turner *et al*., 2013). Thus, in this study IL22 global knockout mice (IL22 $^{-/-}$) were used to determine if the change in IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice phenotype in crypt lengths and goblet cells hyperplasia is due to an absence of IL22. Our finding showed that IL22 $^{-/-}$ mice infected with low dose of ***T. muris*** had increased crypt length and a reduction in goblet cells count compared with WT mice. The similar phenotype in crypt length and goblet cell hyperplasia between IL22 $^{-/-}$, IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice suggesting that a lack of IL22 in IL1-RI *vaviCre* + and IL1-RI $^{-/-}$ mice is responsible for the change in mice phenotype. It also provides more evidence for the role of IL-1 signaling in hematopoietic cells in the generation of Th17 cells and in the production of its cytokine IL22.

7.1.4 Confirm of the role of IL-1 signalling in the development of Th17 cell using IL1-RII $^{-/-}$ deficient mice

IL1-RII is an inhibitor of IL1-RI, thus, in this study IL1-RII $^{-/-}$ mice was used in comparison with IL1-RI $^{-/-}$ mice (presented in chapter five) to verify the role of IL-1 signaling in the development of Th17 cells. IL1-RII $^{-/-}$ mice and it control IL1-RII $^{flox/flox}$ mice were also infected with low and high dose of ***T. muris***. Our finding showed that, IL1-RII $^{-/-}$ and IL1-RII $^{flox/flox}$ mice infected with high dose of ***T. muris*** were resistant to

infection, both mice were able to expel the worm and showed high level of IFNγ, IL4, IL22 and IgG1. Moreover, IL1-RII$^{-/-}$ and IL1-RII $^{flox/flox}$ mice were susceptible to infection and showed high level of IFNγ and IgG2 when they were infected with low dose of ***T. muris.*** This data suggested that absence of IL1-RII did not affect the worm expulsion and the development of Th1 and Th2 cells.

In this study, the concentration of IL17 increased significantly in MLN cells isolated from IL1-RII $^{-/-}$ mice compared with IL1-RII $^{flox/flox}$ mice when infected with low dose of ***T. muris***. Moreover, the comparison of MLNs isolated from IL1-RII $^{-/-}$ mice and IL1-RI $^{-/-}$ mice demonstrated that IL22 and IL17 increased significantly in the MLN cells isolated from IL1-RII $^{-/-}$ compared to IL1-RI $^{-/-}$ mice. This data indicated that, absence of the IL1-RII, an inhibitor of IL1-RI (Smith *et al*., 2003), leads to an overproduction of the Th17 cytokine IL17 and IL22. This confirms that IL-1 signalling is essential for the development of Th17 cells and the production of IL17 and IL22.

Additionally, no significant change was observed in crypt hyperplasia between IL1-RII $^{-/-}$ and IL1- RII ^{flox} / ^{flox} mice infected with a high or low dose of *T. muris*. This is due to the ability of IL1-RII $^{-/-}$ mice to expel the worms when infected with high dose of *T. muris* and its capability to produce sufficient amount of IL22 by IL1-RI signalling pathway required for tissue repair.

Interestingly, this study showed a reduction in the concentration of IL22 in MLN cells isolated from IL1-RII $^{-/-}$ mice compared with IL1-RII ^{flox} / ^{flox} mice infected with low or high dose *T.muris*. This change was confirmed by the analysis of goblet cell hyperplasia of in IL1-RII $^{-/-}$ and IL1- RII ^{flox} / ^{flox} mice. It showed that the goblet cell count decreased significantly in IL1-RII $^{-/-}$ compared with IL1- RII ^{flox} / ^{flox} mice. The change in the level of IL22 was also verified by using immunohistochemistry in colonic tissue for anti-IL22 which showed a decrease in IL22 expression in IL1-RII $^{-/-}$ mice compared with IL1- RII ^{flox} / ^{flox} mice when infected with low or high dose of *T.muris*. IL1-RII has been reported to

activate the inhibition activity of IL-1Ra (Burger *et al*., 1995). There was a study, which showed the IL-1Ra$^{-/-}$ express IL22 only in severely inflamed tissue (Marijnissen *et al*., 2011). Thus, we suggest that increasing the infection period may increase the level of the inflammation, and therefore may raise the concentration of IL22 in IL1-RII $^{-/-}$ mice.7.2

7.2 DISCUSSION AND FUTURE WORK

7.2.1 What are the cellular sources of IL-17 and IL22, and which are dependent on IL-1 signalling?

Th17 cells have been reported as a major source IL22 and IL17 (Jin and Dong, 2013, Chung *et al*., 2006). However, there are various types of cells capable to produce IL22 and IL17. It was reported that innate immune cells such as gamma delta T cells (γδ T cells), innate Th17 (iTh17), natural killer (NK) and natural killer T cells (NKT), macrophages and neutrophils are able to produce IL17 (Jin and Dong, 2013, Mills, 2008). Moreover, the production of IL22 have been reported from many cells type such as NK cells, Lymphoid tissue inducer cells (LTi) cells, Lymphoid tissue inducer cells -like cells (LTi-like cells), γδ T cells and innate lymphoid cells (ILC3) in addition to Th17 cells (Zenewicz and Flavell, 2011). Some of these cells require IL-1 to produce IL22 and/or IL17 like Th17 cells. For example, it has been shown that the expression of IL-1 is necessary for LTi-like and γδ T cells to produce IL22 and/or IL17. NK cells and neutrophils are capable of producing IL22 and/or IL17 independent of IL-1 signalling (Chen *et al*., 2013, Sutton *et al*., 2009, Backert *et al*., 2014, Mills, 2008).

This study showed the important role of IL-1 signalling in hematopoietic cells in the development of Th17 cells and in the secretion IL22 and IL17 that by using IL1-RI *vaviCre* mice. We suggested that in IL1-RI *vaviCre* mice the deletion of *Il1-rI* gene through the expression of *vaviCre* recombinase may occur in most cells responsible for the development of Th17 cells and the production of IL17 and IL22. Thus, further experiments need to be undertaken to investigate which specific hematopoietic cell subsets are involved

in IL-1 signalling and how that affects the development of Th17 cells and the secretion of IL17 and IL22. Several mouse lines were reported to express Cre recombinase transgenes in specific hematopoietic cell subsets such as macrophages and granulocytes (*LysMCre*), , T-lymphocytes (*CD4Cre*) and T and B cells (*hCD2vaviCre)* (Clausen *et al*., 1999, Aghajani *et al*., 2012, de Boer *et al*., 2003).These transgenic mice can be used to obliterate *Il1-rI* gene specifically in macrophages and granulocytes or T-lymphocytes.

It was reported that IL-1 signalling plays an essential role in the early differentiation of Th17 cells *in vitro* and *in vivo* (Chung *et al*., 2009). It has been shown to be involved in the regulation of Th17 transcription factors IRF4 and RORγT (Chung *et al.*, 2009). Several studies reported that the combination of TGF-β and IL-6 with IL-1 and IL23 are able to induce the transcription factor STAT3, which stimulates RORγT which is required for the differentiation of Th17 cells and production of IL17, IL17F and IL22 (Russ *et al*., 2013, Mills, 2008). RORγT is also required for the secretion of IL22 from ILC3 cells and IL17 from NKT cells and γδ T cells. (Mills, 2008, Guo *et al*., 2014).

There was a study investigating the role of STAT3 signalling in the production of IL22 from ILC3s and Th cells in response to *Citrobacter rodentium (C.rodentium)* infection. In this study *Rorc Cre* mice which also known as *RORγTCre* mice were crossed with Stat3-floxed mice in order to delete *STAT3* gene in RORγt-expressing cells. They found that, STAT3 signalling is important for the secretion of IL-22 and IL-17 from both ILC3s and Th cells. It also showed that STAT3 signalling is critical for the development of RORγt in Th cells not in ILC3s. This suggested that the regulation of IL22 production by STAT3 in ILC3s is not via RORγt (Guo *et al*., 2014).

IL-1 with other cytokines is necessary for the activation of STAT3, which stimulates RORγt, which is required for the production of IL17 and IL22. Therefore, *RORγTCre* mice is a suitable model and can be used to inactivate IL1-RI in RORγt-expressing cells such as

Th cells, NKT cells, ILC3s cells and γδ T cells in order to examine the role of IL-1 signalling in these cells in the production of IL17 and IL22 .

7.2.2 Does IL-1α and/or IL-β influence the development of Th17 cells and the production of IL17 and IL22?

IL-1α or IL-β are the best characterized member of the IL1 family (Luheshi *et al.*, 2009). Both cytokines are capable of binding and activating IL1-RI and IL1-RII (Dinarello, 1996, Kamari *et al.*, 2007, Cullinan *et al*., 1998). IL-1α and IL-1β are produced as inactive precursors pro-IL-1α and pro-IL-1β, incapable of binding to their receptor. IL-1β is activated by caspase-1, which required formation of inflammasome. IL-1α is activated by calpain (Zheng *et al*., 2013). IL‑1α and IL‑1β signal through the same receptor complex, thus both cytokines have identical biological activities. However, IL‑1α and IL‑1β differ in several points. IL‑1β is produced mainly by monocytes and macrophages, while IL‑1α is highly expressed by keratinocytes and endothelial cells. IL‑1β is produced and circulates systemically, whereas IL‑1α is acts locally in the producing cells due to its associated to the cell plasma membrane (Sims and Smith, 2010). There was study investigate the expression of *IL-1* gene in mice infected with *Yersinia enterocolitica 08*. They found that mice infected with *Yersinia enterocolitica 08* showed a delay in the induction of IL-1α mRNA compared to IL-1β mRNA. This data indicated that IL‑1α and IL‑1β are regulated differentially at the transcriptional level (Rausch *et al*., 1994).

The role of IL-1 in the induction of IL17 production from T cells was investigated using IL1-RI $^{-/-}$ mice. The *in vitro* analysis of T cells from IL1-RI $^{-/-}$ mice showed that IL23 alone did not promote the production of IL17. It also showed that IL23 promoted IL17 production from T cells which was improved by IL-1α or IL-1β (Sutton *et al*., 2006). Another study which used IL1-RI $^{-/-}$ mice showed that γδ T cells which express IL-23R and RORγT couple to produce IL-17, IL-21, and IL-22 in response to IL-1 β and IL-23 *in vitro* (Sutton *et al*., 2009). This data indicated that both cytokines; IL‑1α and IL‑1β are

important for the production of IL17 and IL22. Further work is required to develop a greater understanding and clarify the role IL-1α and IL-1β in the production of IL17 and IL22 *in vivo*. The generation of IL-1α $^{flox/\ flox}$ and IL-1β $^{flox/\ flox}$ is required in order to create global knockout mice of IL-1α (IL-1α $^{-/-}$) and IL-1 β (IL-1 β $^{-/-}$). IL-1 α $^{-/-}$ and IL-1 β $^{-/-}$ are appropriate models which can be used in the investigation of the role of IL-1α and IL-1β in the production of IL17 and IL22 *in vivo*.

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APPENDIX

APPENDIX A

Milestone Documentation

Milestone 1 Report for

University of Manchester

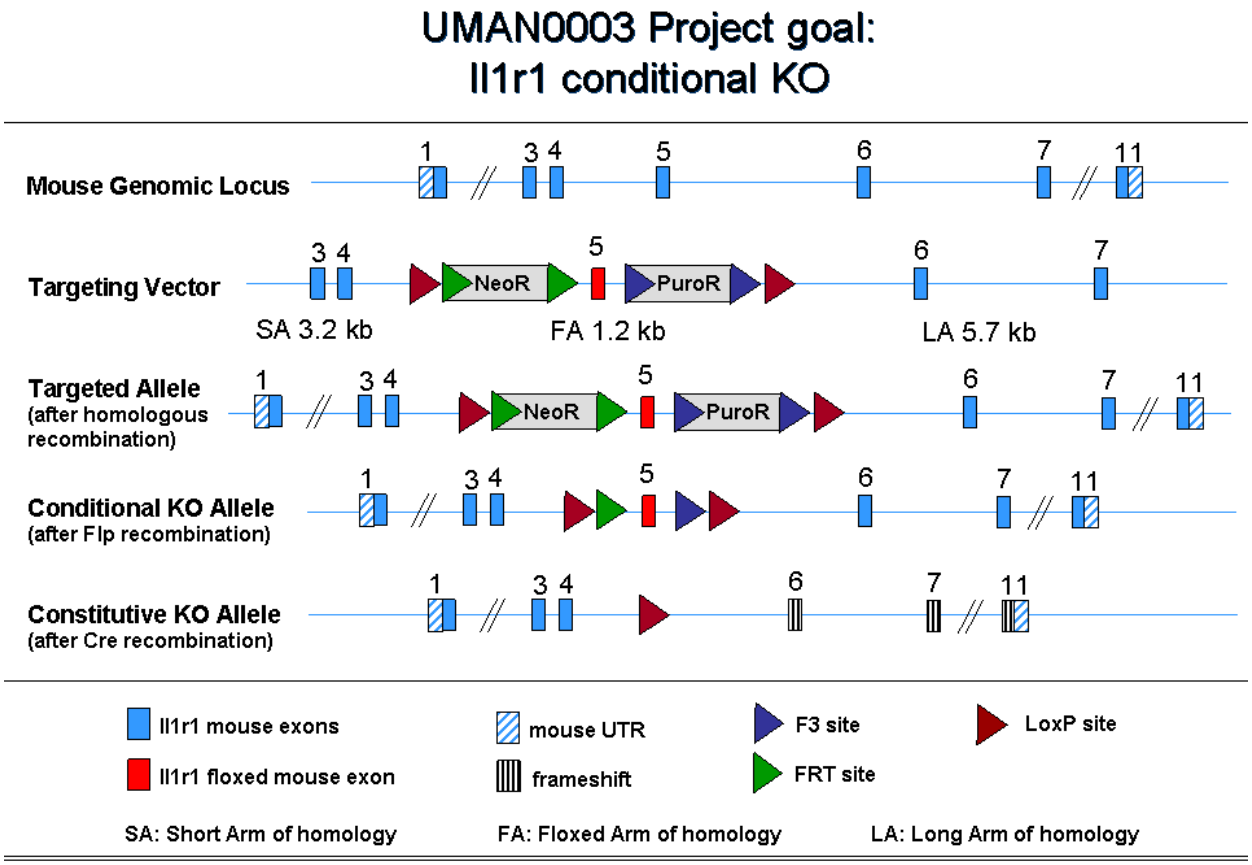
Conditional KO

Il1r1 (UMAN0003)

Summary

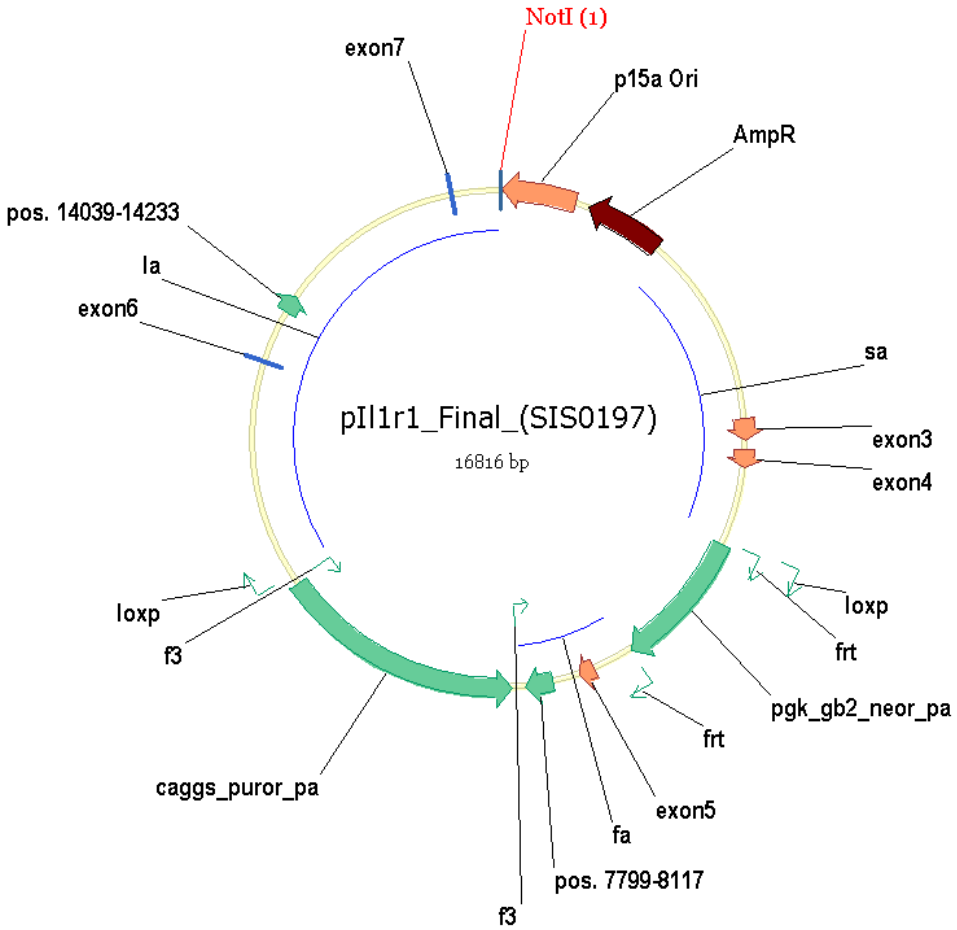
Customer	University of Manchester
Project	Il1r1
Project Type	Conditional KO
Description	Generation of the targeting vector
Official Symbol for Target Gene	Il1r1
Official Name for Target Gene	interleukin 1 receptor, type I
Gene Identifier	Ensembl gene ID: ENSMUSG00000027241 ; NCBI gene ID (Entrez ID): 16177
Chromosomal Localization	1
Official Start Date	2010-01-11
Official Finish Date	2010-03-24
Issue Date	2010-04-06
Comment	documentation checked GR
Full Title / Revenue ID	UMAN0003 Il1r1 KO / 109370

Overview of the strategy



Targeting strategy allows generation of conditional and constitutive Knock-out Il1r1 alleles. Targeting strategy is based on transcript ENSMUST00000027241.
Il1r1 exon 1 contains the translation initiation site.
Exon 5 has been flanked by LoxP sites (size of floxed arm 1.2 kb).
Selection markers have been flanked by FRT (NeoR) and F3 (PuroR) sites and inserted into intron 4 and intron 5, respectively.
Homologous recombinant clones will be isolated using double positive selection (Neomycin and Puromycin resistance) in order to increase the efficiency of co-integration of both loxP sites.
Targeting vector has been generated using BAC clones from the C57BL/6J RPCI-23 BAC library and will be transfected into TaconicArtemis C57BL/6N Tac ES cell line.
Conditional KO allele after Flp-mediated removal of selection markers.
Constitutive KO allele after Cre-mediated recombination. Deletion of exon 5 should result in loss of function of the Il1r1 gene by deleting part of the extracellular region (Ig-like-C2 type 2) and by generating a frame shift from exon 4 to all downstream exons (premature Stop codon in exon 6). In addition the resulting transcript may be a target for Non-sense Mediated RNA Decay and may therefore not be expressed at significant level.
Remaining recombination sites will be located in non-conserved regions of the genome.
Comment: In case the truncated mRNA encoded by the constitutive KO allele would be transcribed and translated, a protein containing the first 175 amino acids of the Il1r1 protein might be expressed. If stable this truncated protein would contain part of the extracellular region (Ig-like-C2 type 1). It is unclear whether this truncated protein would have any physiologically relevant function.

Final vector



Vector Sequence

BASE COUNT 4356 a 3967 c 4251 g 4242 t 0 n
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61 tcatgtggca ggagaaaaaa ggctgcaccg gtgcgtcagc agaatatgtg atacaggata
121 tattccgctt cctcgctcac tgactcgcta cgctcggtcg ttcgactgcg gcgagcggaa
181 atggcttacg aacggggcgg agatttcctg gaagatgcca ggaagatact taacagggaa
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Methods

Vector Construction Liquid ET-cloning: Mouse genomic fragments (obtained from the C57BL/6J RPCIB-731 BAC library) and selected features (such as recombination sites and selection markers, as indicated on the vector map) were assembled together using ET-cloning and restriction enzymes. The confirmed sequence of the final targeting vector is shown. Further information is available on request.

APPENDIX B

Milestone Documentation

Milestone 2 Report for

University of Manchester

Conditional KO

Il1r1

(UMAN0003)

Summary

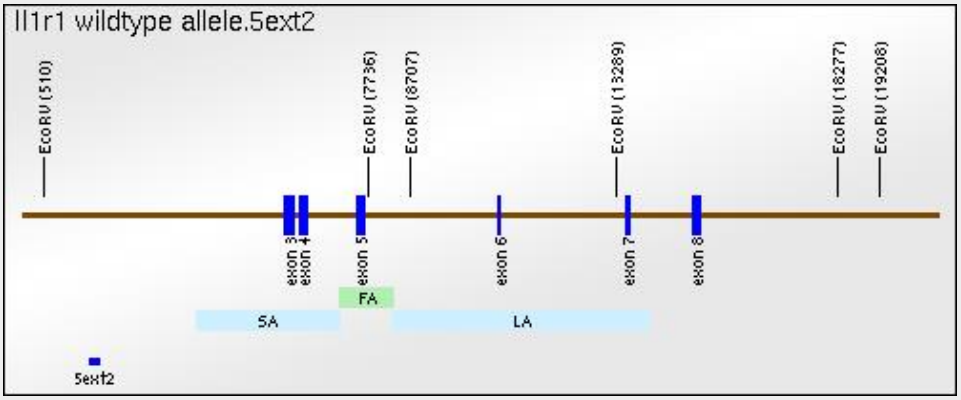
Customer	University of Manchester
Project	Il1r1
Project Type	Conditional KO
Nomenclature	C57BL/6-*Il1r1*^{tm2205Arte}
Description	Generation of heterozygous targeted C57BL/6 NTac ES cells
Official Symbol for Target Gene	Il1r1
Official Name for Target Gene	interleukin 1 receptor, type I
Gene Identifier	Ensembl gene ID: ENSMUSG00000027241 ; NCBI gene ID (Entrez ID): 16177
Chromosomal Localization	1
Official Start Date	2010-04-01
Official Finish Date	2010-06-07
Issue Date	2010-06-11
Comment	Documentation checked GR
Full Title / Revenue ID	UMAN0003 Il1r1 KO / 109370

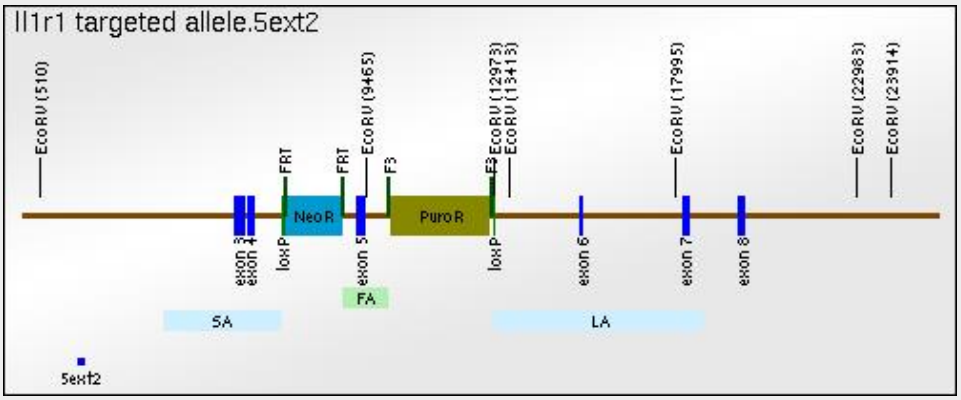
Summary table: Transfection of ES cells

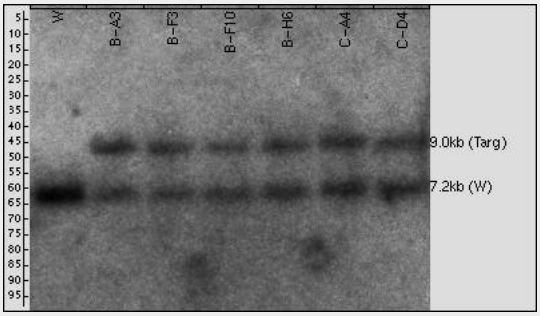
Transfection 3839	
Transfection date	2010-04-01
Transfection method	Electroporation
Vector	pIl1r1_Final_(SIS197)
ES cell line	Art B6/3.5 (genetic background: C57BL/6NTac)
Quality control of the ES cell clone or ES cell line used for transfection	The karyotype analysis done at Chrombios GmbH (Germany) by multicolor fluorescence in situ hybridisation with probes for all murine chromosomes (mFISH) shows that the cell line meets our quality standard for parental ES cell lines used for targeting experiments. PCR testing of 27 murine pathogens (incl. Mycoplasma) at Taconic US shows no contamination. Cells were tested successfully for their germline competence.
Enrichment Method	G418 selection, Puromycin selection
Method for Screening	PCR analysis
# of clones analysed	282
# of targeted clones	9
Targeting frequency	3,2%
IDs of expanded clones	B-A3, B-F3, B-F10, B-H6, C-A4, C-D4
Method for Validation	Southern Blot analysis
Quality control: Mycoplasma	negative, tested by PCR analysis
IDs of validated clones	B-A3, B-F3, B-F10, B-H6, C-A4, C-D4

Southern Blot Analysis

Homologous recombination at the 5' side



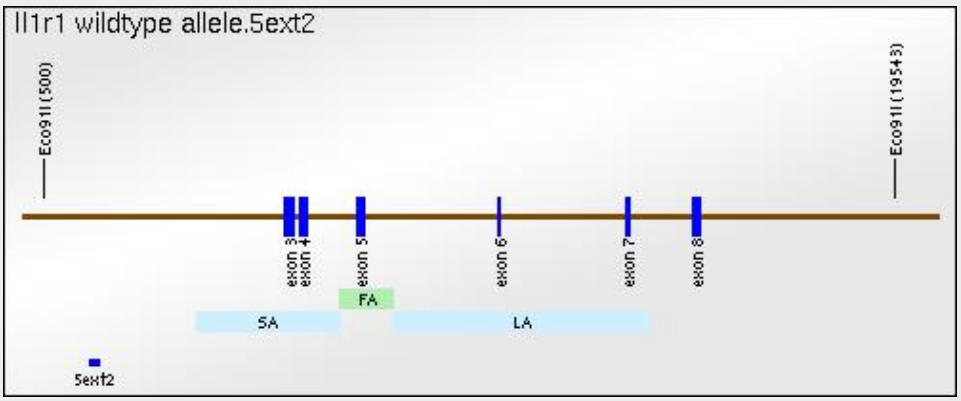


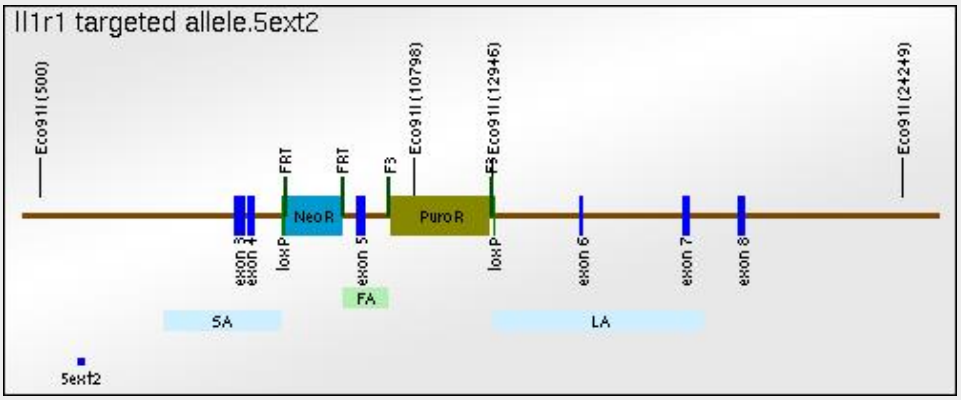


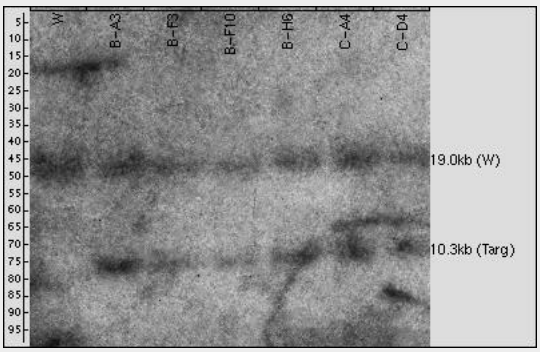
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6, C-A4, C-D4. Method: Digestion with: EcoRV, Probe: 5ext2

Results: Detects correct homologous recombination (Targ) at the 5' side in all clones.

Homologous recombination at the 5' side





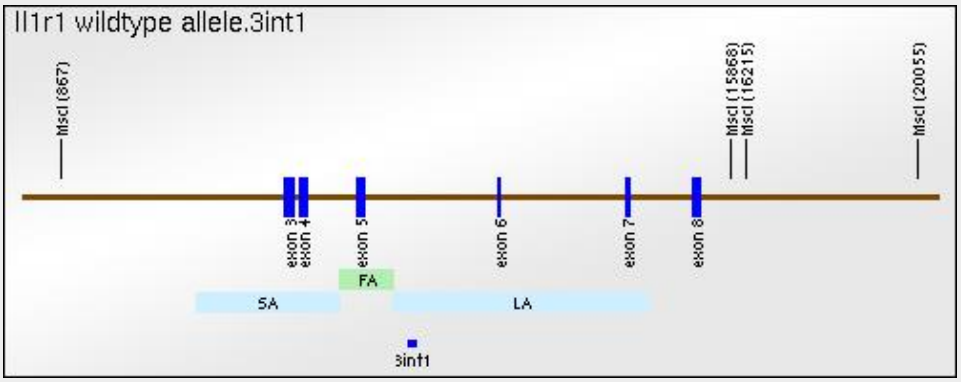


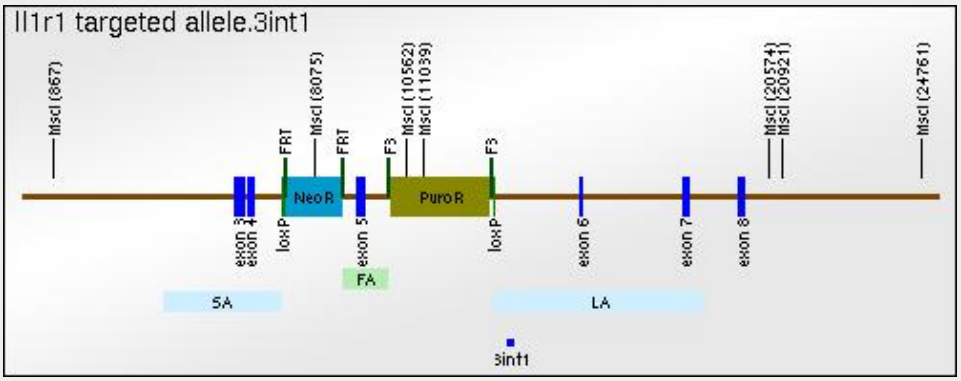
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

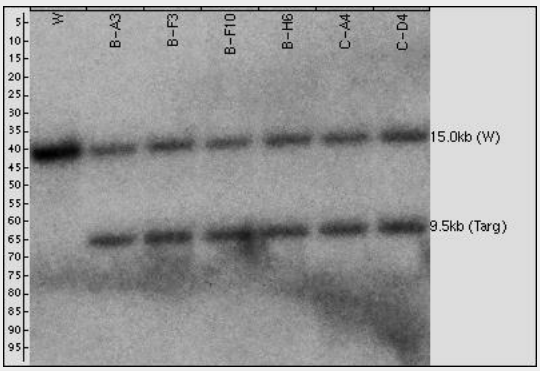
C-A4, C-D4. Method: Digestion with: Eco91I, Probe: 5ext2

Results: Detects correct homologous recombination (Targ) at the 5' side in all clones.

Homologous recombination at the 3' side and single integration





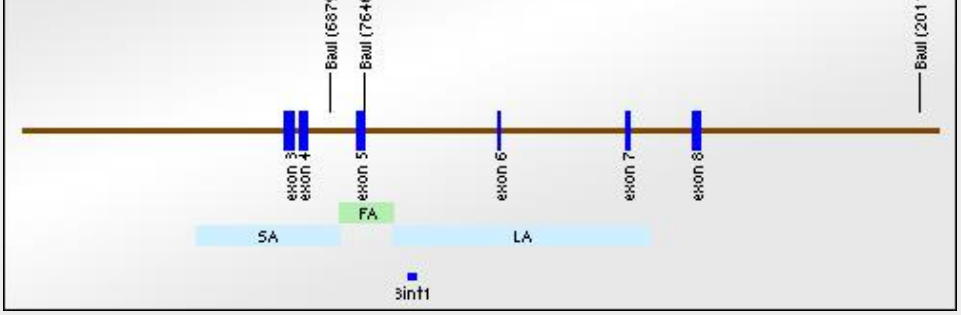


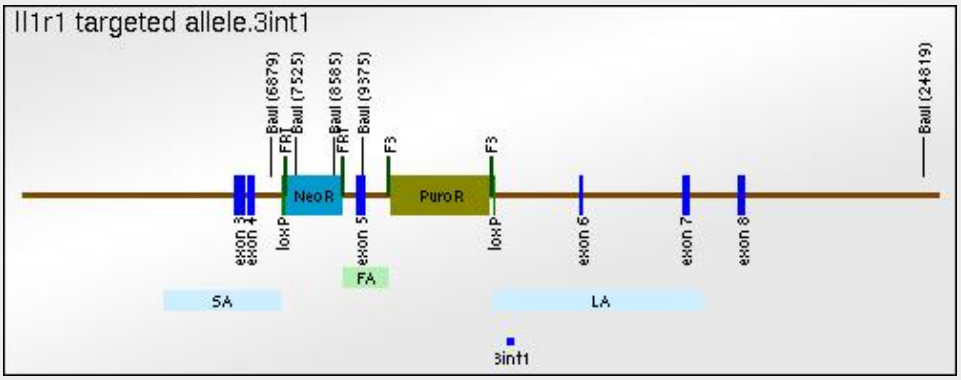
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

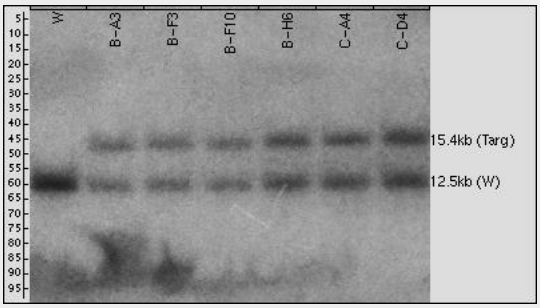
C-A4, C-D4. Method: Digestion with: MscI, Probe: 3int1

Results: Detects correct homologous recombination (Targ) at the 3' side and single integration in all clones.

Homologous recombination at the 3' side and single integration





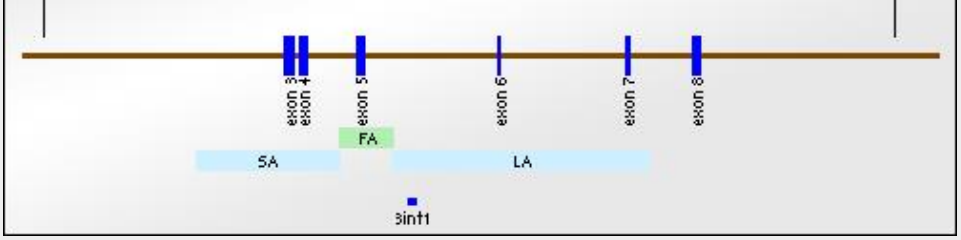


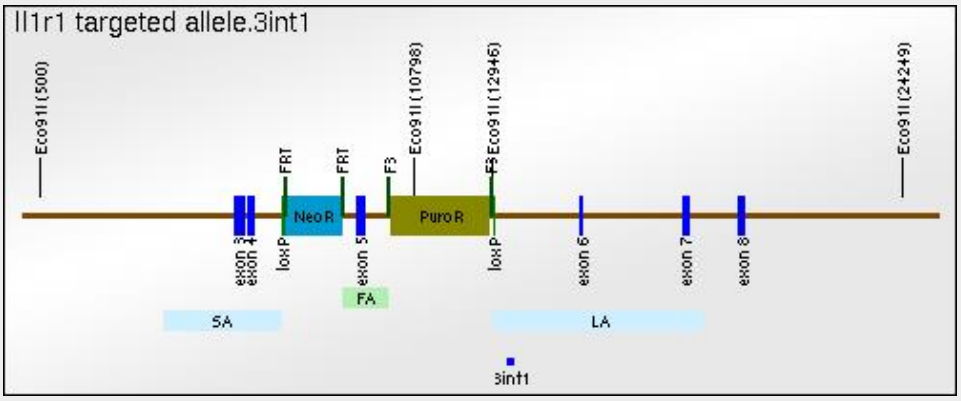
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

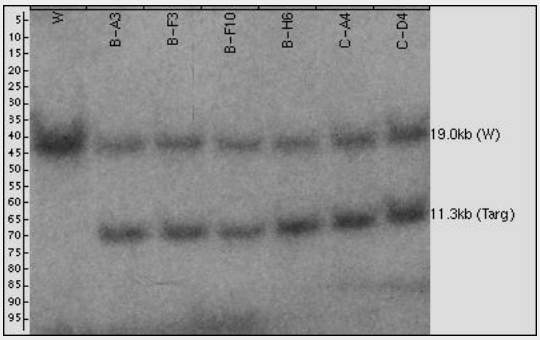
C-A4, C-D4. Method: Digestion with: BauI, Probe: 3int1

Results: Detects correct homologous recombination (Targ) at the 3' side and single integration in all clones.

Homologous recombination at the 3' side and single integration





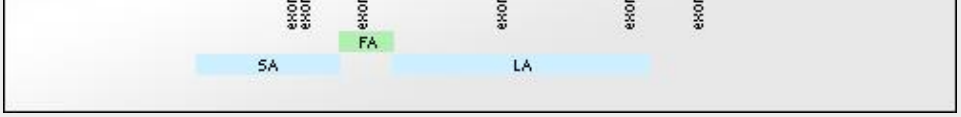


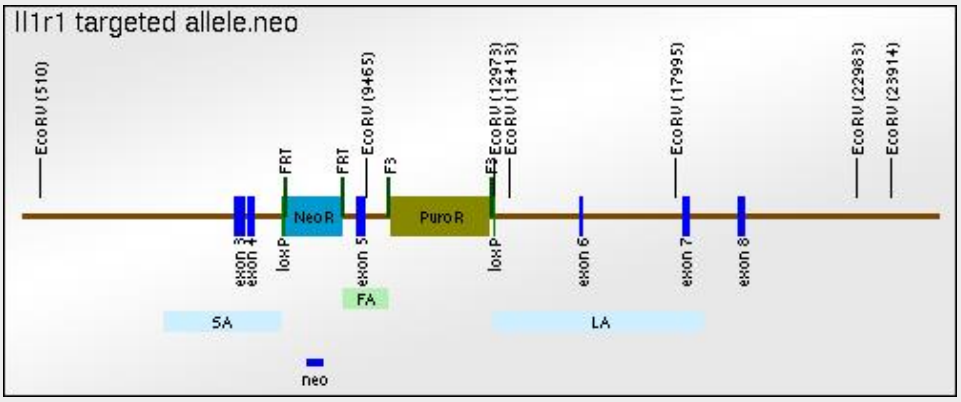
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

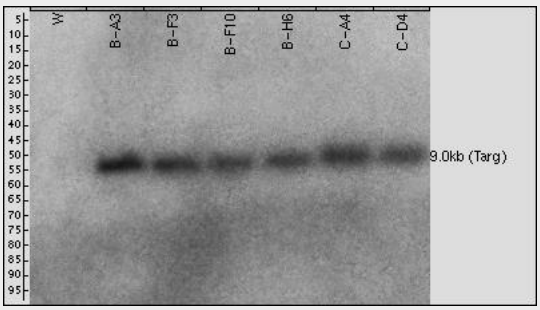
C-A4, C-D4. Method: Digestion with: Eco91I, Probe: 3int1

Results: Detects correct homologous recombination (Targ) at the 3' side and single integration in all clones.

Homologous recombination at the 5' side and single integration





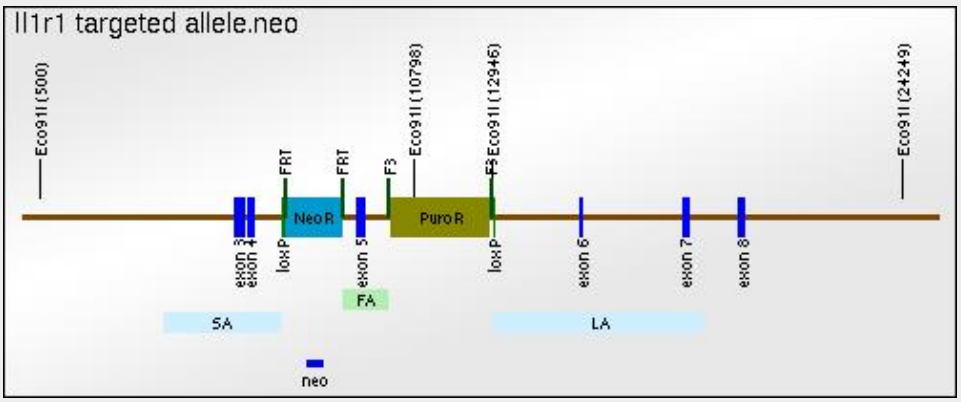


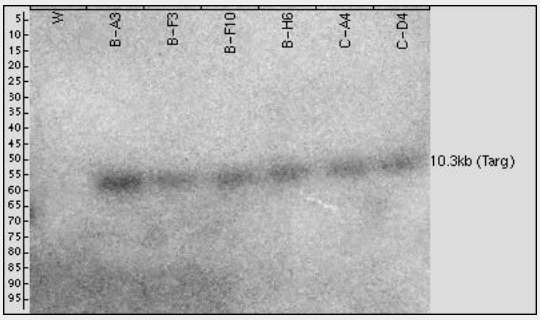
Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

C-A4, C-D4. Method: Digestion with: EcoRV, Probe: neo

Results: Detects correct homologous recombination (Targ) at the 5' side and single integration in all clones.

Homologous recombination at the 5' side and single integration





Material: Genomic DNA from W, B-A3, B-F3, B-F10, B-H6,

C-A4, C-D4. Method: Digestion with: Eco91I, Probe: neo

Results: Detects correct homologous recombination (Targ) at the 5' side and single integration in all clones.

Transfection method

Transfection of mouse ES cells to introduce a targeting vector via homologous recombination The quality-tested ES cell line (see table: Transfection of ES cells) was grown on a mitotically inactivated feeder layer comprised of mouse embryonic fibroblasts in ES cell culture medium containing Leukemia inhibitory factor and Fetal Bovine Serum.

The cells were electroporated with the linearized DNA targeting vector(s) according to Taconic Artemis' Standard Operation Procedures. The selection mechanisms used are listed in the table 'Transfection of ES cells - Enrichment Method'.

Resistant ES cell colonies (ES clones) with a distinct morphology were isolated on day 8 after transfection and analysed by Southern Blotting and/or PCR in a primary screen. Homologous recombinant ES cell clones were expanded and frozen in liquid nitrogen after extensive molecular validation as described above.

Probe Information for Southern Blot Analysis

Primer sequences for the PCR amplification of the Il1r1 5ext2 probe: sense:
CCAGAGCATTCAGGTCTCTCTTAGG
antisense: CAGGTCTGGAGGTTCTCAAATATCC

Primer sequences for the PCR amplification of the Il1r1 3int1 probe: sense:
GCATTATGAATTATACTTTCCAATC
antisense: TGTCCCACCACTGTACTATATCTCC

APPENDIX C

Milestone Documentation

Milestone 3 Report for

University of Manchester

Conditional KO

Il1r1 (UMAN0003)

Creation of conditional Knock-Out mice by breeding with B6 Flp_deleter TG

Summary

Customer	University of Manchester Project
Il1r1 KO	
Project Type	Conditional KO
Nomenclature	C57BL/6-*Il1r1*^{tm2205Arte}
Description	Creation of conditional Knock-Out mice by breeding with B6 Flp_deleter TG
Official Start Date	2010-06-14 Official Finish Date
Date	2011-07-21 Issue Date 2011-07-22
Comment	Genotyping aspects checked Final
Milestone Full Title / Revenue ID KO / 109370	UMAN0003 Il1r1

Production of Chimeric Mice

ESC Clone	Il1r1 3839 B-A3	Il1r1 3839 B-F3	Il1r1 3839 B-F10
Karyotype 40, XY	n/a	n/a	n/a
Transferred blastocysts	48	50	108
Transfers	3	3	6
Litters	3	3	3
Pups born	18	9	14
Weaned Chimeras	13	9	7
> 50% chimeric male pups	6	2	7

G0 Mice Details

Chimeras for clone Il1r1 3839 B-A3					
Id	DoB	Sex	% Chimerism	Status	Used in ChB
139430	2010-07-01	f	50 - 75	sacrificed	
139431	2010-07-01	f	50 - 75	sacrificed	
139432	2010-07-01	f	25 - 50	sacrificed	
139433	2010-07-01	f	25 - 50	sacrificed	
139434	2010-07-01	f	25 - 50	sacrificed	
139422	2010-07-01	m	50 - 75	sacrificed	11441
139423	2010-07-01	m	50 - 75	sacrificed	11695
139424	2010-07-01	m	50 - 75	sacrificed	11696
139425	2010-07-01	m	50 - 75	sacrifice	
139426	2010-07-01	m	50 - 75	sacrificed	
139427	2010-07-01	m	50 - 75	sacrifice	
139428	2010-07-01	m	25 - 50	sacrifice	
139429	2010-07-01	m	25 - 50	sacrifice	

Chimeras for clone Il1r1 3839 B-F3					
Id	DoB	Sex	% Chimerism	Status	Used in ChB
144279	2011-01-03	m	50 - 75	sacrifice	12362
144280	2011-01-03	m	50 - 75	sacrificed	12363
144281	2011-01-03	m	25 - 50	sacrificed	
144282	2011-01-03	m	25 - 50	sacrificed	
144283	2011-01-03	m	25 - 50	sacrificed	
144284	2011-01-03	m	25 - 50	sacrificed	
144285	2011-01-03	m	25 - 50	sacrificed	
144286	2011-01-03	f	25 - 50	sacrificed	
144287	2011-01-03	f	25 - 50	sacrificed	

Chimeras for clone Il1r1 3839 B-F10					
Id	DoB	Sex	% Chimerism	Status	Used in ChB
146733	2011-04-09	m	100	sacrifice	
146734	2011-04-09	m	50 - 75	sacrifice	
146735	2011-04-09	m	50 - 75	sacrifice	
146736	2011-04-09	m	50 - 75	sacrifice	
146737	2011-04-09	m	50 - 75	sacrifice	
146738	2011-04-09	m	50 - 75	sacrifice	
146739	2011-04-09	m	50 - 75	sacrifice	

Breeding Details

Breeding details for clone: Il1r1 3839 B-F3 x FLP deleter								
Breeding #	Type	Setup	Stop	DOB Pups	# born	# germline	# weaned	# typed
12362	ChB	2011-03-31		2011-05-10 2011-06-02 2011-06-25 2011-07-19	6 6 4 6	0 4 4	0 4 4	 4 4
12363	ChB	2011-03-31	2011-06-29	2011-04-23 2011-05-28 2011-06-22 2011-07-19	3 5 8 10	0 0 0	0 0 0	

Breeding details for clone: Il1r1 3839 B-F3 x C57BL/6 NTac								
Breeding #	Type	Setup	Stop	DOB Pups	# born	# germline	# weaned	# typed
12749	B	2011-07-15				n/a		

Breeding details for clone: Il1r1 3839 B-A3 x FLP deleter								
Breeding #	Type	Setup	Stop	DOB Pups	# born	# germline	# weaned	# typed
11441	ChB	2010-08-24	2010-12-13	2010-09-28 2010-10-25 2010-11-20 2010-12-12	6 9 3 6	0 0 0 0	0 0 0 0	
11695	ChB	2010-10-19	2011-05-10	2010-11-07 2010-12-03 2010-12-29 2011-01-21 2011-02-15 2011-03-09 2011-04-01 2011-04-24 2011-05-21	9 8 11 9 8 10 7 7 2	0 0 0 1 1 0 0 0 0	0 0 0 1 1 0 0 0 0	 1 1
11696	ChB	2010-10-19	2010-12-27					

G1/G2 Genotyping Results

II1r1: cond/+ Tg(CAG-Flpe): tg/+						
#	Mouse Id	Breeding Id	DOB	Sex	Status	Used in B
1	147576	12362	2011-06-02	m	Breeding	12749
2	147577	12362	2011-06-02	m		
3	148422	12362	2011-06-25	m	Ship	
4	148423	12362	2011-06-25	m	Ship	
5	148424	12362	2011-06-25	f	Ship	
6	148425	12362	2011-06-25	f	Ship	

A combination of the following genotypes is available:

II1r1: W Tg(CAG-Flpe):						
#	Mouse Id	Breeding Id	DOB	Sex	Status	Used in B
1	147578	12362	2011-06-02	f	sacrificed	
2	147579	12362	2011-06-02	f	sacrificed	

II1r1: W Tg(CAG-Flpe): tg/+						
#	Mouse Id	Breeding Id	DOB	Sex	Status	Used in B
1	144511	11695	2011-01-21	f	sacrificed	
2	145300	11695	2011-02-15	f	sacrificed	

Genotyping results pending						
#	Mouse Id	Breeding Id	DOB	Sex	Status	
1	147578	12362	2011-06-02	f	sacrificed	
2	147579	12362	2011-06-02	f	sacrificed	

Legend	
W	Wildtype
tg/+	random transgenes heterozygote
cond/+	Heterozygous flanked

Genotyping Analysis / PCR Standard Operation Procedure

PCR SOP ID: 2959

Genotyping PCR performed according to SOP 2959 detects heterozygous/homozygous conditional and wildtype alleles.

Primers

2959_32: CTAGTCTGGTGGAACTTACATGC

2959_33: AACTGAAAGCTCAGTTGTATACAGC

Reaction

5µl PCR Buffer 10x (Invitrogen)

2µl MgCl2 (50mM)

1µl dNTPs (10mM)

1µl Primer 2959_32 (5µM)

1µl Primer 2959_33 (5µM)

0,2µl Taq (5U/µl, Invitrogen)

37,8µl H2O

2µl DNA

Program

Standard

95°C 5'

95°C 30"

60°C 30"

72°C 1'

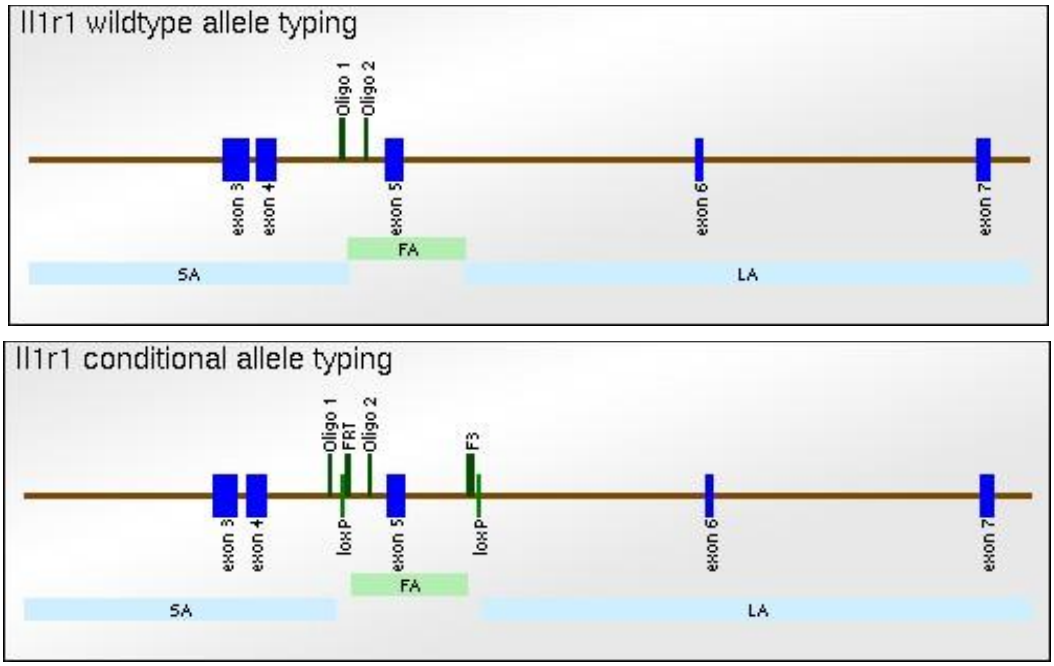
35 cycles

72°C 10'

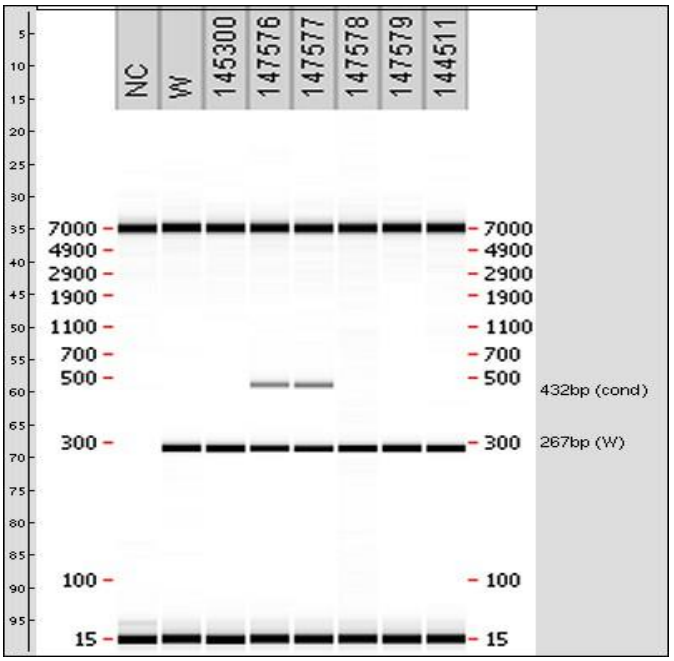
Expected Fragments [bp]

267(W),432(cond),267(W)+432(cond)

Genotyping Analysis According to PCR SOP 2959



The fragment amplified with oligos 1 (2959_32: CTAGTCTGGTGGAACTTACATGC) + 2 (2959_33: AACTGAAAGCTCAGTTGTATACAGC) detects heterozygous/homozygous conditional and wildtype alleles.



PCR SOP ID: 2960

Genotyping PCR performed according to SOP 2960 detects heterozygous/homozygous conditional and wildtype alleles.

Primers

2960_40: GGGGATGGAGGTAGAGGTATGG

2960_39: GATAAAGCAGAGCTGGAGACAGG

Reaction

5µl PCR Buffer 10x (Invitrogen)

2µl MgCl2 (50mM)

1µl dNTPs (10mM)

1µl Primer 2960_40 (5µM)

1µl Primer 2960_39 (5µM)

0,2µl Taq (5U/µl, Invitrogen)

37,8µl H2O

2µl DNA

Program

Standard

95°C 5'

95°C 30"

60°C 30"

72°C 1'

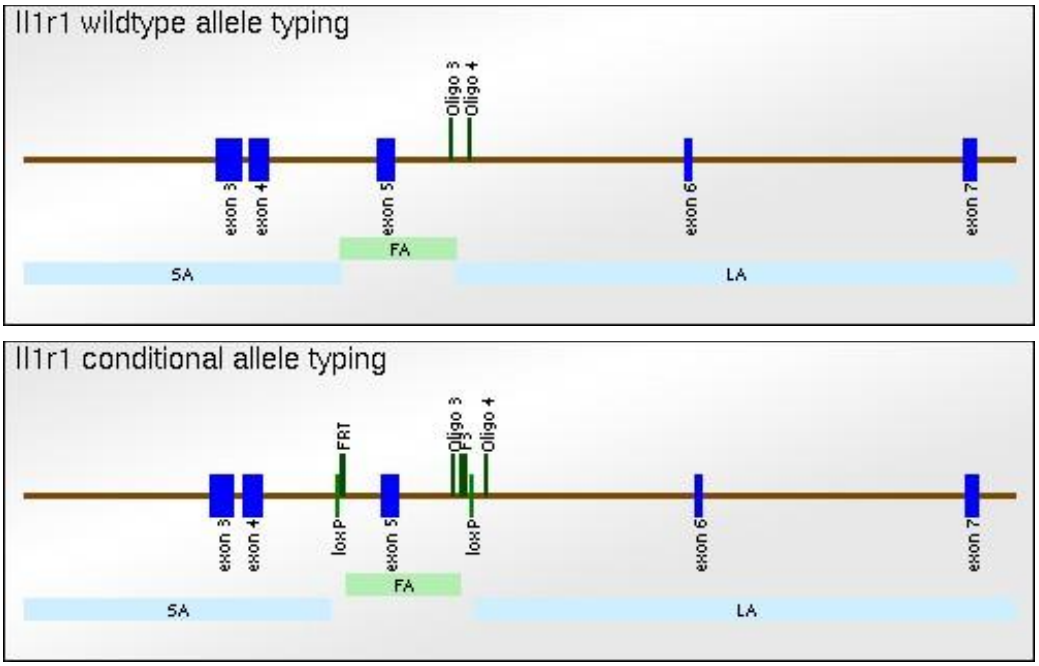
35 cycles

72°C 10'

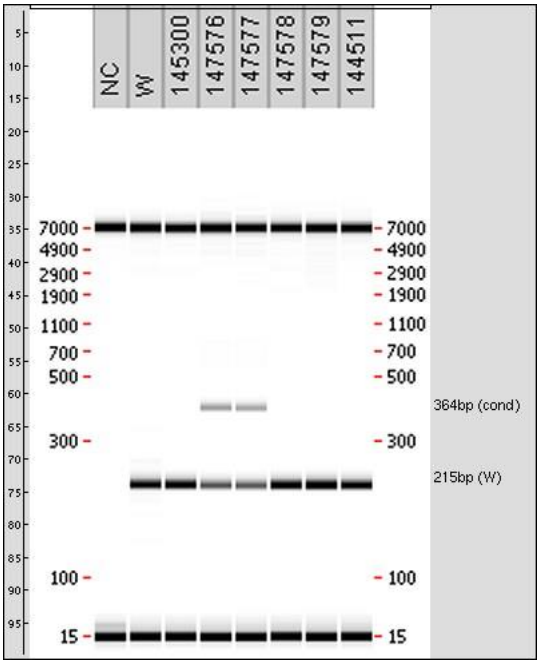
Expected Fragments [bp]

215(W),364(cond),215(W)+364(cond)

Genotyping Analysis According to PCR SOP 2960



The fragment amplified with oligos 3 (296040: GGGGATGGAGGTAGAGGTATGG) + 4 (2960_39: GATAAAGCAGAGCTGGAGACAGG) detects heterozygous/homozygous conditional and wildtype alleles.



PCR SOP ID: 1307

(A.k.a. ART Generic GEN FLPe)

Please note: PCR-protocol has been modified at 2010-03-15.

Genotyping PCR performed according to SOP 1307 detects the Flp transgene and the 1307+Control creates an additional

control fragment at 585bp (PCR-ID 1260).

Primers

1307_1: Flpe_as_GGCAGAAGCACGCTTATCG

1307_2: Flpe_s_GACAAGCGTTAGTAGGCACAT

Reaction

5µl PCR Buffer 10x (Invitrogen)

2µl MgCl2 (50mM)

1µl dNTPs (10mM)

1µl Primer 1307_1 (5µM)

1µl Primer 1307_2 (5µM)

0,2µl Taq (5U/µl, Invitrogen)

37,8µl H2O

2µl DNA

Program

Standard

95°C 5'

95°C 30"

60°C 30"

72°C 1'

35 cycles

72°C 10'

Expected Fragments [bp] 343(targ)

PCR SOP ID: 1307 + Control
(A.k.a. ART Generic GEN FLPe)
Please note: PCR-protocol has been modified at 2010-03-15.
Genotyping PCR performed according to SOP 1307 detects the Flp transgene and the 1307+Control creates an additional
control fragment at 585bp (PCR-ID 1260).
Primers

1307_1: Flpe_as_GGCAGAAGCACGCTTATCG

1307_2: Flpe_s_GACAAGCGTTAGTAGGCACAT

1260_1: GAGACTCTGGCTACTCATCC

1260_2: CCTTCAGCAAGAGCTGGGGAC

Reaction
5µl PCR Buffer 10x (Invitrogen)
2µl MgCl2 (50mM)
1µl dNTPs (10mM)

1µl Primer 1307_1 (5µM)

1µl Primer 1307_2 (5µM)

1µl Primer 1260_1 (5µM)

1µl Primer 1260_2 (5µM)

0,2µl Taq (5U/µl, Invitrogen)

35,8µl H2O

2µl DNA

Program

Standard

95°C 5'

95°C 30"

60°C 30"

72°C 1'

35 cycles

72°C 10'

Expected Fragments [bp] 343(targ)

Expected Control Fragments [bp] 585(c)

Methods

Diploid injection

After administration of hormones, superovulated BALB/c females were mated with BALB/c males. Blastocysts were isolated from the uterus at dpc 3.5. For microinjection, blastocysts were placed in a drop of DMEM with 15% FCS under mineral oil. A flat tip, piezo actuated microinjection-pipette with an internal diameter of 12 - 15 micrometer was used to inject 10-15 targeted C57BL/6NTac ES cells into each blastocyst. After recovery, 8 injected blastocysts were transferred to each uterine horn of 2.5 days post coitum, pseudopregnant NMRI females. Chimerism was measured in chimeras (G0) by coat colour contribution of ES cells to the BALB/c host (black/white). Highly chimeric mice were bred to strain C57BL/6 females. Depending on the project requirements, the C57BL/6 mating partners are non-mutant (W) or mutant for the presence of a recombinase gene (Flp-Deleter or Cre-deleter or CreER inducible deleter or combination of

Flp-deleter/CreER). Germline transmission was identified by the presence of black, strain C57BL/6, offspring (G1).

Genotyping Analysis

Sample analysis has been performed by using a Caliper LabChip GX device. Results are displayed in the Virtual Gel format, additional Graph and Data Summary Table formats are available on request. Marker bands present in each lane (15 bp and 7000 bp in size) are used for automated analysis and annotation of genotyping results (NC = negative control, PC = positive control, W = Wildtype).

Formal Nomenclature:	**C57BL/6-*Tg(CAG-Flpe)2 Arte***
Synonyms:	ART0011 FLP_deleter TG, Flp deleter, CAGGS-Flp
Background Strain:	C57BL/6
Generation	G19
Strain Derivation	The mutant was derived by random integration of the transgene CAGGS-Flpe in C57BL/6 one-cell embryos.
Strain Description:	General ubiquitously active Flp deleter. In vitro-evolved FLP recombinase (FLPe) under the control of the chicken-□-actin promoter and an hCMV immediate early enhancer. The use of the chimeric CMV enhancer/□-actin promoter leads to a ubiquitous expression profile in eukaryotes. The FLPe recombinase carries a minimal, nuclear localization signal from the SV40 larger T antigen. Additional elements of the expression cassette are an intron with splice donor and acceptor sites upstream of the FLPe gene and a synthetic polyadenylation signal downstream of the FLPe.
Mating System:	Homozygous x Homozygous
Breeding and Husbandry	This strain is maintained by homozygous sibling matings. Expected coat color from breeding: black.
Side effects	No side effects
References	- Buchholz F., Angrand P.O. and Stewart A.F., Nature Biotechnology 16, 657-662 (1998) - Schaft J., et al., Genesis 31, 6-10 (2001)
Additional Documentation	- PCR SOP 1307

APPENDIX D

Ear Lysis Buffer (PBND Buffer) : 50mM KCl, 10mM Tris, 2.5mM $MgCl_2$, 0.1mg/ml gelatine, 0.4% w/v NP40, 0.4% w/v Tween 20

TBE buffer 1 M Tris base, Boric acid 1 M and 0.02 M EDTA

Complete RPMI: RPMI-1640 media containing 10% v/v fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin

Washing buffer : PBS-2 containing 2% v/v fetal calf serum and 100 μg/ml streptomycin.

FACS buffer: PBS-2, 2% FCS, 0.05% sodium azite

Urea lysis buffer **7**M Urea, 4%SDS, 90 mM TRIS-HCL, adjusted to pH 6.8, 0.1% Bromophenol blue

TBS x10: 250mM Tris, 1.25M NaCl adjusted to pH 8.0 with HCl

1xTBS-Tween :100ml 10x TBS, 890ml dH_2O, 1% v/v Tween-20

Carbonate bicarbonate buffer: 15mM Na_2CO_3, 35mM $NaHCO_3$ adjusted to pH 9.6

Blocking solution : 1M TRIS HCl adjusted to pH 7.5 , 0.15M NaCl , 0.5g Blocking powder (Perkin Elmer life sciences).