Elucidation of the Role of NKR-P1: CLR Recognition Systems in Intestinal & Renal Epithelial Cell Homeostasis and Immunity

Elias Abou Samra

A Thesis Submitted to the

Faculty of Graduate and Postdoctoral Studies

In Partial fulfillment of the Requirements for the

PhD degree in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa

© Elias Abou Samra, Ottawa, Canada 2017

// Summary

Natural killer (NK) cells represent a crucial component of the innate immune system and are primarily regulated by the interactions of their activation and inhibitory receptors with ligands available on target cells. The genetically linked Ly49 and NKR-P1 family of receptors constitute two of the major regulatory receptor systems used by NK cells and have been shown to bind different ligands. Whereas the Ly49 receptors survey MHC-I ligands on target cells, the NKR-Pl receptor family members bind to various members of the C-type lectin-related (Clr) family. Interestingly, NKR-P1 and Clr haplotypes possess a stable genomic polymorphism across multiple mouse strains, suggesting that this inhibitory receptor:ligand relationship has an important role in the maintenance of host cellular cognate specificities. The NKR-P1 and Clr receptor-ligand pairs identified in mice include the NKR-P1B:Clr-b and the NKR-P1G:Clr-f interacting pairs. Previous RT-PCR and *in situ RNA* hybridization data generated by our laboratory determined that kidney tubular epithelium as well as the small and large intestinal epithelial cells specifically and highly expresses the *Clr-f* transcripts. Contrarily, the *Clr-b* transcripts were only detected on hematopoietic cells of various lymphoid organs and kidneys. Moreover, foregoing studies revealed that the loss of Clr-b following viral or chemical induced stress mediates NK cell killing of the target cell, suggesting a tissue-specific immune-surveillance mechanism in parallel with the global MHC-I-dependent missing-self model. However, the role of the NKR-P1B:Clr-b recognition-system have never been examined in the intestine. Additionally, the role of Clr-f in the kidney and intestines, where they are highly expressed, has not been investigated. For these reasons, I aimed in my thesis to provide a better understanding of the functional aspect of the NKR-P1B:Clr-b and NKR-P1G:Clr-f recognition systems in mediating gut mucosal and renal homeostasis, respectively.

First, in order to determine the role of NKR-P1B and Clrb receptor:ligand pair as a "missing-self" immunosurveillance system in the gut, I started by identifying the expression pattern of both the receptor and ligand on various intestinal cells. My results demonstrate that NK cells do not represent the major NKR-P1B-expressing cells in the gut lamina propria. Instead, ILC3 subsets constituted the predominant cell population expressing the receptor, whereas γδT cells composed a small fraction of NKR-P1B$^+$ lymphocytes. In addition, the NKR-P1B expression on myeloid cells was exclusive to colon macrophages and DC subsets. Interestingly, the highest percentage of NKR-P1B$^+$ immune cells was found in the gut, which suggests the dominant role of NKR-P1B in regulating immune functions at the level of intestinal mucosa. As expected, the expression of the NKR-P1B ligand, Clr-b, appeared on all innate immune cell types in the gut. Next, using oral infection models of *Salmonela typhimurium* and *Citrobacter rodentium*, I showed that NKR-P1B-deficient NK cells, ILC3 and γδ T cells are hyporesponsive compared to their WT counterparts. In particular, gut NKR-P1B-deficient NK cells and γδT cells secreted low levels of IFNγ cytokine while infected with *S.typhimurium*. Importantly, the decreased IFNγ secretion by NK and γδT cells was associated with an increased dissemination of the bacterium into the knockout spleens at day 5 post-infection. Likewise, I detected a significant decrease in IL-22 cytokine production by NKR-P1B-deficient ILC3 compared to their WT counterparts at both steady state and following *C.rodentium* infection.

Next, I address the potential role of Clr-f in the kidney. Renal tubular epithelial cells have been shown to express high levels of *Clr-f* transcripts. Epithelial cells constitute the major cellular component of kidney tubules and are well known to mediate metabolic waste excretion, reabsorption of essential molecules as well as other physiological functions, such as ions exchange and water retention. To determine the role of Clr-f in renal epithelial cells, I generated

a Clr-f-deficient mouse with the help of two of my previous lab colleagues. Importantly, chemical analysis on urine and serum samples from knockout and WT littermates indicated that Clr-f-deficient kidneys display a decreased filtration capacity. In particular, higher creatinine levels were detected in the Clr-f deficient serum. In addition, Clr-f-deficient mice appeared to have a lower fractional excretion of sodium (FENa) in their urine filtrates in comparison to WT excreted urine. Blood pressure measurements on the same mice at 12 and 24 weeks of age revealed a hypotensive phenotype in the Clr-f-deficient mice. Furthermore, pathological assessment of Clr-f-deficient kidneys exhibited moderate and aggravated lesions of the tubular epithelium along with marked glomerular mesangiolysis. Lastly, flow cytometry analysis on isolated lymphocytes from Clr-f-deficient and WT mice demonstrated comparable immune infiltrates between the two mouse genotypes.

Altogether, our data shows that the absence of Clr-f results in the development of glomerular and tubular lesions in an immune-independent manner leading to an abnormal kidney function. Additionally, the disruption of NKR-P1B:Clr-b recognition system results in abnormal innate immune cell number and function in the mouse intestine. These novel findings sheds light on the important role of Clr-f in maintaining healthy kidney morphology and function, as well as the crucial role for NKR-P1B:Clr-b interactions in mediating intestinal homeostasis at steady and infected states.

Acknowledgment

I would like to thank Dr. Andrew Makrigiannis for his guidance and support throughout my PhD degree. I would also like to thank my lab colleagues Dr. Munir Rahim, Dr. Ahmad Mahmoud, Andrew Wight and Megan Tu for their friendship and helpful advice.

I also give my honest thanks and credit to Dr. Paul MacPherson and Dr. Todd Fairhead, for being amazing thesis advisors. It was a great pleasure to work with such kind and knowledgeable professors in the scientific community.

Statement of Contributions

- Lab research associate, Dr. Munir Rahim
Thesis editing

- Honor's student Zachary Hickey,
Helped with the NKR-P1B screening profile study, Fig 2.1-2.6

- PhD candidate Ahmad Mahmoud
Helped with *S.typhimurium* and *C.rodentium* oral infections

- Postdoctoral fellow Dr. Haggag Zein & MD/PhD student Clayton Hall
Generated the targeting construct for the Clr-f ^{KO}

- PhD student Andrew Wight
Helped with the urine collection of Clr-f ^{WT} and Clr-f ^{KO} mice

- PhD candidate Lara Gharibeh, from the Nemer Lab, University of Ottawa
Helped with the blood pressure measurements of Clr-f ^{WT} and Clr-f ^{KO} mice

- Research technician Megan Fortier, from the Nemer Lab, University of Ottawa
Helped with the blood collection of Clr-f ^{WT} and Clr-f ^{KO} mice

- Dr. Subash Sad, University of Ottawa
Provided the *S.typhimurium* bacterium

- Dr. Brian K. Coombes, McMaster University
Provided the *C.rodentium* bacterium

- Dr. James Carlyle, University of Toronto
" Provided the NKR-P1B and Clr-b flow antibodies"

My sincere appreciation goes to my wife Renée Jarawan & my family Georges, Jouliana, Myriam and Maya Abou-Samra, whom I shared the ups and downs of being a PhD student. I owe my gratitude to my best friends and colleagues Lara Gharibeh and George Kanaan. You guys made it easier to be away from home. Finally, I would like to dedicate this thesis to my uncle and Godfather **Adel Abou-Samra** who lost his life before seeing me graduate.

List of Figures

Figure I: Schematic view of the differentiation of various innate lymphoid cells ILCs p.12

Figure II: Illustration of the basic structural and functional unit of the kidney p.25

Figure III: A sketch of a healthy kidney glomerulus p.28

Figure 1: NKR-P1B expression on the cells of the lymphoid and myeloid lineage in multiple gut compartments and secondary lymphoid organs p.54

Figure 2: Ly49 expression on spleen and gut immune cells p.59

Figure 3: Tissue-dependent distribution of NKR-P1B-expression immune cells in B6 mice p.62

Figure 4: Increased frequency and numbers of several innate immune cell types in the gut of NKR-P1B-deficient mice p.65

Figure 5: Increased small intestinal length but lower average body-weight in the NKR-P1B deficient mice p.69

Figure 6: Clr-b expression is broadly expressed on gut-resident leukocytes p.72

Figure 7: Defective NK and γδ T cells response to Salmonella typhimurium infection in the gut of NKR-P1B-deficient mice p.76

Figure 8: NKR-P1B^{WT} and NKR-P1B^{KO} mice showed similar survival kinetics following *S.typhimurium* infection *in vivo* p.80

Figure 9: Defective ILC3 responses to *Citrobacter rodentium* infection in the gut of NKR-P1B-deficient mice p.83

Figure 10: Generation of Clr-f-deficient mice p.87

Figure 11: Increased body-weight in Clr-f-deficient mice but comparable intestinal lengths and organ-weight in Clr-f-expressing tissues p.90

Figure 12: Disrupted kidney tubules in Clr-f- deficient mice p.94

Figure 13: Typical variants of mesangiolysis in glomeruli of Clr-f-deficient mice p.96

Figure 14: Equivalent frequency and numbers of several adaptive and innate immune cell types in different organs of Clr-f-deficient and WT mice p.98

Figure 15: Disrupted urine and serum creatinine levels suggest kidney tubular dysfunction in Clr-f-deficient mice p.103

Figure 16: Lower blood pressure measurements in Clr-f-deficient mice compared to their WT littermates p.105

Figure 17: Comparable sodium and potassium levels but decreased FENa in Clr-f-deficient mice p.107

Figure 18: Clr-f-deficient and WT mice show similar urine and serum electrolyte levels p.109

List of Tables

Table I. List of primers p.51

Table of Content

1. General introduction **p.1**

1.1 Innate immunity p.1

1.2 Natural Killer Cell (NK)

1.3 The role of NKR-P1:Clr interactions in NK cell biology

1.3.1 The role of NKR-P1:Clr interactions in the intestinal barrier function

1.4 Gut mucosal immunity p.6

1.4.1 NK cells in gut immunity

1.5 Innate lymphoid cell (ILC) biology p.9

1.5.1 Tissue distribution of ILCs in humans and mice

1.5.2 ILC subsets in human and murine gut immunity

1.5.2.1 ILC1

1.5.2.2 ILC2

1.5.2.3 ILC3

1.5.2.3.1 Role of ILC3 in maintaining Citrobacter rodentium infection

1.6 γδT cells development, distribution and function in human and murine gut immunity p.20

1.7 Kidney structure, function and vascularity p.24

1.7.1 Glomerulus

1.7.2 Renal tubular system

1.8 Renal Immunology p.29

1.8.1 Role of renal epithelial cells in the innate immune apparatus of the kidney

1.8.2 Role of γδT cells in kidney diseases

2. Rational and Objectives **p.35**

3. Materials and Methods **p.40**

3.1 Mice

3.2 Generation of Clr-f-deficient mice

3.3 Tissue sampling

3.4 Cell isolation from tissues

3.5 Flow cytometry staining

3.6 Bacterial preparation

3.7 Oral gavage, bacterial count and tissue collection

3.8 Histology and pathology scoring

3.9 In-vitro culturing and intracellular cytokine staining

3.10 In-situ hybridization

3.11 RT-PCR

3.12 Urine and plasma chemical waste and electrolyte measurement

3.13 Blood pressure measurements on 12 and 24-week-old mice

4. Results **p.52**

4.1 NKR-P1B expression on the cells of the lymphoid and myeloid lineage in multiple gut compartments and secondary lymphoid organs

4.2 Unlike the NKR-P1B receptor, Ly49 receptors are not highly expressed on immune cell subsets in the gut

4.3 Tissue-dependent distribution of NKR-P1B-expression immune cells in B6 mice

4.4 Increased frequency and numbers of several innate immune cell types in the gut of NKR-P1B-deficient mice

4.5 Increased small intestinal length but lower average body-weight in the NKR-P1B-deficient mice

4.6 Clr-b expression is broadly expressed on gut-resident leukocytes.

4.7 Defective NK and γδ T cells response to *Salmonella typhimurium* infection in the gut of NKR-P1B-deficient mice

4.8 NKR-P1B^{WT} and NKR-P1B^{KO} mice showed similar survival kinetics following *S.typhimurium* infection *in-vivo*.

4.9: Defective ILC3 responses to *Citrobacter rodentium* infection in the gut of NKR-P1B-deficient mice.

4.10 Generating the Clr-f-deficient mice

4.11 Kidney tubular and glomerular lesions in Clr-f-deficient mice

4.12 Urine and serum chemical analysis revealed disrupted kidney function in hypotensive Clr-f-deficient mice.

5. Discussion p.111

6. Summary and Future Perspectives p.128

7. List of References p.132

List of Abbreviations

ADR, Adrinamycin

AhR, aryl hydrocarbon receptor

BM, bone marrow

BP, blood pressure

C.rodentium, citrobacter rodentium

Ca, calcium

CD, cluster of differentiation

CD, crohn's disease

CFU, colonies forming units

CKD, chronic kidney diseases

Cl, chloride

Clr, C-type lectin related

CMV, cytomegalovirus

DAMPs, damaged-associated molecular patterns

DCs, dendritic cells

DETCs, dendritic epidermal T cells

ECs, epithelial cells

EDTA, ethylenediaminetetraacetic acid

Eomes, eomesodermin

FACS, fluorescence activated cell sorting

FBS, fetal bovine serum

FeNa, fractional sodium excretion

Gata-3, Gata-binding protein 3

GBM, glomerular basement membrane

GFR, glomerular filtration rate

GI, gastro-intestinal

GM-CSF, granulocyte-macrophage colony-stimulating factor

H&E, hematoxylin and eosin

HIF, hypoxia-inducible factor

HLA, Human leukocyte antigen

IBD, inflammatory bowel disease

Id2, inhibitor of DNA binding-2

iELs, intra-epithelial lymphocytes

IFN, interferon

Ig, immunoglobulin

IgAN, primary IgA nephropathy

IL, interleukin

ILC, innate lymphoid cells

ITIM, immunoreceptor tyrosine-based inhibitory motif

KACL, keratinocyte-associated C-type lectin

KGF-1, keratinocyte growth factor-1

KIR, Killer immunoglobulin-like receptor

LLT1, lectin-like transcript 1

LP, lamina propria

LTi, lymphoid tissue-inducer

MFI, mean fluorescence intensity

MHC-I, major histocompatibility complex class I

MNPs, mononuclear phagocytes

mRNA, messenger ribonucleic acid

Na/K, sodium potassium ratio

NCR, natural cytotoxicity receptor

NK, natural killer

NLRs, nucleotide-binding oligomerization domain-like receptors

P, phosphorus

PBS, phosphate buffered saline

PMA, phorbol 12-myristate 13-acetate

PRRs, pattern recognition receptors

RAG, recombination activation genes

RLRs, retinoic acid-inducible gene-I-like receptors

RNA, ribonucleic acid

ROR, retinoic acid receptor-related orphan receptor

ROS, reactive oxygen species

RT-PCR, reverse transcription polymerase chain reaction

S.typhimurium, salmonella typhimurium

Th, t-helper cells

TLRs, toll like receptors

TNF, tumour necrosis factor

TSLP, thymic stromal lymphopoietin.

UC, Ulcerative colitis

UPCR, urine protein to urine creatinine ratio

VEGF, vascular endothelial growth factor

WT, wild-type

1. General introduction

1.1 Innate immunity

The innate immune system plays a crucial role in the early phase of the immune response to pathogens, cancer, and tissue transplants. Innate immunity is composed of a diverse hematopoietic cell types including the natural killer (NK) cells, macrophages, dendritic cells and granulocytes, as well as non-hempatopoietic cells. Cells of the innate immune system collaborate with each other not only to contain and eliminate transformations and infections, but also to serve as a sentinel to alert and prime the adaptive arm of the immune system, to promote immunity when such pathogenic states exist. Such crosstalk between the innate and acquired immune systems is central to the mounting of a sterilizing immune response. The ascending scale of an immune response depends first on the ability of the innate immune system to perform general "self-nonself" discrimination and detect potential "danger" signals, then exhibit the release of a cytokine network and co-stimulatory molecules resulting in effector mechanisms such as phagocytosis and cytotoxicity. Finally, the acquired immune cells such as B and T cells, which function with refined specificity will eliminate any remaining traces of pathogens, generating the last stage of an immune response.

1.2 Natural Killer Cell (NK)

NK (natural killer) cells represent an arm of the innate immune system; these cells differentiate between healthy and abnormal cells by using a wide collection of cell surface receptors that control their activation, proliferation and effector functions. This distinguishes NK cells from other innate immune cells such as macrophages and granulocytes, which rely on conserved

pattern-recognition receptors. NK cell recognition involves the initial binding to potential target cells such as: virus-infected cells, tumor cells and MHC-disparate tissue transplants (Trinchieri et al. 1989, Biron et al. 1999). The integration of signals by NK cells transmitted by the interactions of their activation and inhibitory receptors with ligands available on target cells, will determine whether the NK cell detaches and moves on or stays and responds. Class I major histocompatibility complex (MHC-I) acts as ligands for inhibitory Ly49 receptor family members on mouse NK cells (Lanier, 2005). MHC-I is often down-regulated on virally-infected and tumor cells to avoid recognition and killing by cytotoxic T cells, in turn making them target for NK cells (Hewitt et al. 2003, Garcia-Lora et al. 2003). This is termed the MHC-I-dependent 'missing-self' response. Interestingly, erythrocytes lack the surface expression of MHC-I; however, these cells are spared from killing by NK cells (Lanier, 2005). One possible explanation could be that these cells have insufficient activating ligands expressed on their cell surface (Diefenbach et al. 2001, Arase et al. 2002). Alternatively, one could also suggest that other ligands on these cells, such as Clr-b, which is recognized by mouse inhibitory NKR-P1B/D receptor, could dampen NK cell activation (Kumar et al. 2005, Vivier et al. 2008). Once activated, NK cells release granzymes and perforin, which is responsible for the induction of programmed cell death of the target cells.

1.3 The role of NKR-P1:Clr interactions in NK cell biology

NK cell receptors are mainly structured as (Ig)-like folds of type I/type II transmembrane domains, along with an extracellular C-type lectin like domain (Yokoyama et al. 1993). Among the molecules belonging to the latter superfamily on mouse NK cells are the Ly49 and NKR-P1 family of receptors that have been shown to be genetically linked, but bind different ligands.

Whereas the Ly49 survey MHC-I ligands on the target cells, the NKR-Pl receptor family members bind to various members of the C-type lectin-related (Clr) family. The *Clr* gene family was first identified in the natural killer gene complex (NKC) in 2001 by sequencing of bacterial artificial chromosome (BAC) clones. In agreement with other C-type lectin genes, cytoplasmic, transmembrane and extracellular domains of Clr molecules are encoded by exons 1, 2, and 3-5, respectively. The Clr family of genes maps to the centromeric portion of the mouse NKC, between *Nkrp1a* and *Cd69* on chromosome 6 and is actually found intermingled among the *Nkrp1* genes. The murine *Nkrp1* gene family encodes for five proteins, including three stimulatory isoforms (NKR-P1A, C, F) and two inhibitory (NKR-P1B/D, G). Likewise, multiple members make up the Clr family namely: Clr-a, -b, -c, -d, -e, -f, -g, -h, -i, -j. The identified receptor-ligand pairs in mice include: NKR-P1B/D: Clr-b (Carlyle et al. 2004); NKR-P1F: Clr-c, -d, and -g; and NKR-P1G: Clr-f, -d, and -g (lizuka et al. 2003, Chen et al. 2011). It appears that the *Nkrp1-Clr* cluster is co-inherited *en bloc,* allowing a stable evolution of functional receptor-ligand pairs in order to maintain cellular host cognate specificities. As a result, the *Nkrp1* and *Clr* haplotypes demonstrate stable genomic polymorphism with focused diversity compared to the highly polymorphic and polygenic Ly49 receptors (Carlyle et al. 2006, Chen et al. 2011). In humans, similar but not homologous, the NKC-encoded receptor NKRP-1A (CD161) shows inhibitory function upon binding the Clr-related ligand LLT1 (Aldemir et al. 2005). Other human NKC-encoded receptor ligand pairs include AICL (*CLEC2B*) binding to the activating NK receptor, NKp80 (Bartel et al.2013, Klimosch et al. 2013) and KACL (*CLEC2A*) binding to NKp65 (Bauer et al. 2015). Interestingly, the Clr molecules are absent for immunoregulatory tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail or charged residues in their transmembrane domain, which suggest the unique and alternative functions of Clr family

members compared to the functions described for other NKC encoded proteins. Previous RT-PCR and microarray data detected the expression of several Clr family transcripts in different mouse tissues. However, recent work done by our laboratory using RT-PCR and *in situ RNA* hybridization, revealed the specific cell types expressing each of the Clr family members (Zhang et al. 2012). In particular, epithelial cells of the small and large intestine specifically and highly expressed *Clr-f*. Notably, *Clr-f* transcripts were also detected in kidney tubular epithelial cells whereas its most closely related Clr *"Clr-a"* was exclusively restricted to the gut. Contrarily, the *Clr-b* transcripts were broadly detected in the spleen, liver, thymus, lymph nodes and kidneys. However, unlike *Clr-f*, kidney *Clr-b* expression was not identified on renal epithelial cells. Instead, it was restricted to the glomerular and interstitial areas. The broadly expressed *Clr-b* transcripts on most hematopoietic cells are similar to expression patterns of MHC-I molecules. Being a ligand for the inhibitory NKR-P1B receptor on NK cells, Clr-b was proposed to play an important role in a novel form of recognition in order to prevent NK cell attack of normal cells. Expression of Clr-b on transfected cells protects them from NK-mediated lysis (Carlyle et al. 2004, Iizuka et al. 2003, Rahim et al. 2015). Clr-b expression was found to be down-regulated on a number of tumour cell lines (Carlyle et al. 2004). Furthermore, genotoxic stress induces a rapid down-regulation of Clr-b, in contrast the MHC-I expression in the stressed cells remained normal (Fine et al. 2010). A study done on rat cytomegalovirus-infected cells also showed a rapid loss in the expression of rat Clr-b (Voight et al. 2007). Collectively, the previous findings represent new insights into the missing-self hypothesis and strongly raise the role of an MHC-I-independent recognition system in controlling NK cell functions.

1.3.1 The role of NKR-P1:Clr interactions in the intestinal barrier function

The intestinal epithelium covers a huge area of 400 m^2 in humans and is considered as the most populated barrier surface (Peterson and Artis, 2014). The intestinal epithelial cells (IECs) are responsible for the intake of highly diverse nutrients while they are constantly challenged by potential pathogens including commensals and pathogenic microbiota. For that purpose, IECs must not only maintain the intestinal homeostasis, but also defend against invading pathogens. This is normally achieved by secreting highly glycosylated mucins, immunoglobulins and antimicrobial peptides (Galo et al. 2012, Neish et al. 2002, Hooper et al. 2010, Maloy et al. 2011) and through priming and regulating the diverse immune cell repertoire lining the intestinal epithelium, such as the intra-epithelial lymphocytes (IELs), innate lymphoid cells (ILCs), T cells, dendritic cells (DCs) and macrophages (Peterson et al. 2014). The dynamic coordination between the gut microbiome and the immune cell network requires complex immunoregulatory mechanisms. Several NK receptor ligands of the immunoglobulin superfamily have been described to contribute to this complex regulatory mechanism. Ligands of the natural killer group 2 member D (NKG2D), MICA, has been shown to be upregulated on IECs during chronic induced inflammation. Blockage of the NKG2D signaling through MICA, was shown to prevent $CD4^+$ T cell-mediated colitis in mice (Ito et al. 2008) as well as Crohn's disease in humans (Allez et al. 2007). Furthermore, previous work done by our lab demonstrated that IECs constitutively express Clr-f, the ligand for the inhibitory NKR-P1G receptor, suggesting that Clr-f may act as a health marker of epithelial cells in these tissues and prevents lymphocytic attack under normal conditions. Indeed, recent work done by Leibelt et al. revealed that an increased Clr-f expression after challenging the mice with poly(I:C), may constrain the reactivity of

intestinal intra-epithelial NKR-P1G positive γδT cell subsets towards the epithelial barrier that is constantly provoked by microbial stimuli (Leibelt et al. 2014).

1.4 Gut mucosal immunity

Antigens may enter GI tract through several routes. These include M-cells in the follicle-associated epithelium where local dendritic cells (DCs) can directly present antigens to T-cells within the Peyer's Patches (PP) (Mason et al. 2008). In the PP, DCs delivers cellular signals, which primes T-cells and activates B-cells immunoglobulin class switching from IgM to IgA that aids in inducing anti-inflammatory responses (MacPherson et al. 2004). Antigen loaded DCs can also traffic to other areas of the gut associated lymphoid tissue (GALT), these includes the mesenteric lymph nodes (MLN) for T-cell recognition and differentiation (Mason et al. 2008). The primed and differentiated lymphocytes in PP and MLNs traffic via the thorasic duct into the lamina propria (LP), which carries the major effector functions in the GALT and is populated with primed T and B cells, Intra-epithelial lymphocytes (IELs), DCs and macrophages. In addition, lamina propria DCs can also extend out into the intestinal lumen for direct antigens sampling (Mason et al. 2008). Alternatively, antigens can access the MLN's by crossing the epithelium of the villus lamina propria or they can be taken up by lamina propria macrophages and DCs that express MHC class I/II molecules, enabling them to prime local innate and adaptive immune cells (MacPherson et al. 2004, Mason et al. 2008).

1.4.1 NK cells in gut immunity

NK cell functions are extensively studied in spleen, liver and blood. However, data indicate that NK cells are not only present in lymphoid tissues or in circulation but also in peripheral tissues

where they can perform direct and indirect immunoregulatory roles (Shi et al. 2011). At the level of the gut mucosa, resident NK cells have varying phenotypes based on their status of maturation and function. However, the steps for the post-bone marrow NK cell development in the gut mucosal sites including migration, phenotype changes, education and maturation, in addition to the mechanisms for NK cell adjustment to the gut environment, are largely unknown (Leibelt et al. 2015). Current understanding of NK cell development and migration suggest that NK cells emerge from the bone marrow (BM) as a mix of mature and immature cells and migrate to mucosal tissue sites where they develop and acquire organ specific phenotypes (Leibelt et al. 2015). Once migrated, tissue specific microenvironments will influence NK cell development and education via cytokine milieu such as IL-15, TGF-β, IL-10 and the resident microbiome (Leibelt et al. 2015, Cheroutre et al. 2011). The maturation of murine NK cells is divided into 4 stages based on the expression of CD27 and CD11b markers that correspond with their cytolytic activity and inflammatory cytokines production (Carlyle et al. 2004, Chen et al. 2011). IEL and LP intestinal NK cells of naïve mice appear to be immature as they exhibit $CD27^{lo}$ $CD11b^{lo}$ surface expression. In humans, $CD56^{bright}$ NK cells mature into $CD56^{dim}$ NK cells by up-regulating the CD16 Fc-receptor and acquiring surface inhibitory killer cell immnuglobulin-like receptors (KIRs) as well as the ability to release perforin and granzyme (Huntington et al. 2009). The $CD56^{dim}$ subset constitutes 90% of total blood, bone marrow and spleen NK cells (Bjorkstom et al. 2010, Bjorkstom et al. 2013) whereas the gut, tonsils and uterus are enriched for $CD56^{bright}$ NK cells (Bjorkstom et al. 2013, Fehniger et al. 2003). Recently, human small and large intestinal NK cells are identified as $NKp46^{+}$ $NKp44^{-}$ $RoR\gamma t^{-}$ lymphocytes (Reynders et al. 2011, Takayama et al. 2010) and they display a high expression of NKG2A and low to absent levels of CD16 and KIRs (Chinen et al. 2007). Interestingly, human NK cells have been shown

to exhibit phenotypic alterations in patients with inflammatory bowel diseases (IBD) (Takayama et al. 2010, Yadav et al. 2011). The IFNγ NKp46^{+} NK cells were found to increase significantly in inflamed mucosa of patients with Crohn's disease (CD). On the other hand, genetic association studies have found that KIR2DL1 and KIR2DL3 gene frequencies are significantly lower in patients with ulcerative colitis (UC) in comparison to healthy controls (Jones et al. 2006, Yadav et al. 2011), suggesting that these receptors can be directly linked to IBD susceptibility. In mice, gut NK cells were shown to participate in immune responses against intracellular pathogens. The IFNγ-producing NK cells have been shown to prevent systemic spread of *listeria monocytogenes* during the early days of infection (Reynders et al. 2011, Bregenholt et al. 2001). Other investigation of murine *Salmonella Typhimurium* infection demonstrated that depletion of NK cells resulted in a greater colonization of the murine gut with Salmonella and inhibited the mice capacity in controlling the bacterial burden in comparison to WT mice (Ashkar et al. 2009). Subsequent studies determined that in addition to IFNγ, gut NK cells also produce the CCL3 chemokine during infection, leading to the recruitment of inflammatory monocytes into the site of infection (Schulthess et al. 2012). In humans, the role of NK cells in gut mucosal infections has been examined in HIV patients, where a greater number of NK cells in the intra-epithelium correlated with a better control of the viral load in the gut of these patients (Sips et al. 2012). These previous studies illustrate multiple functions of gut NK cells in directly controlling infections as well as coordinating and regulating the intestinal mucosal immune responses. Notably, bacterial versus parasitic infections highlighted large variations in gut NK cell responses. Demonstrating by then the complexity of gut NK immunoregulatory mechanisms and the need to understand how to take advantage of this cell type to develop a better mucosal defense.

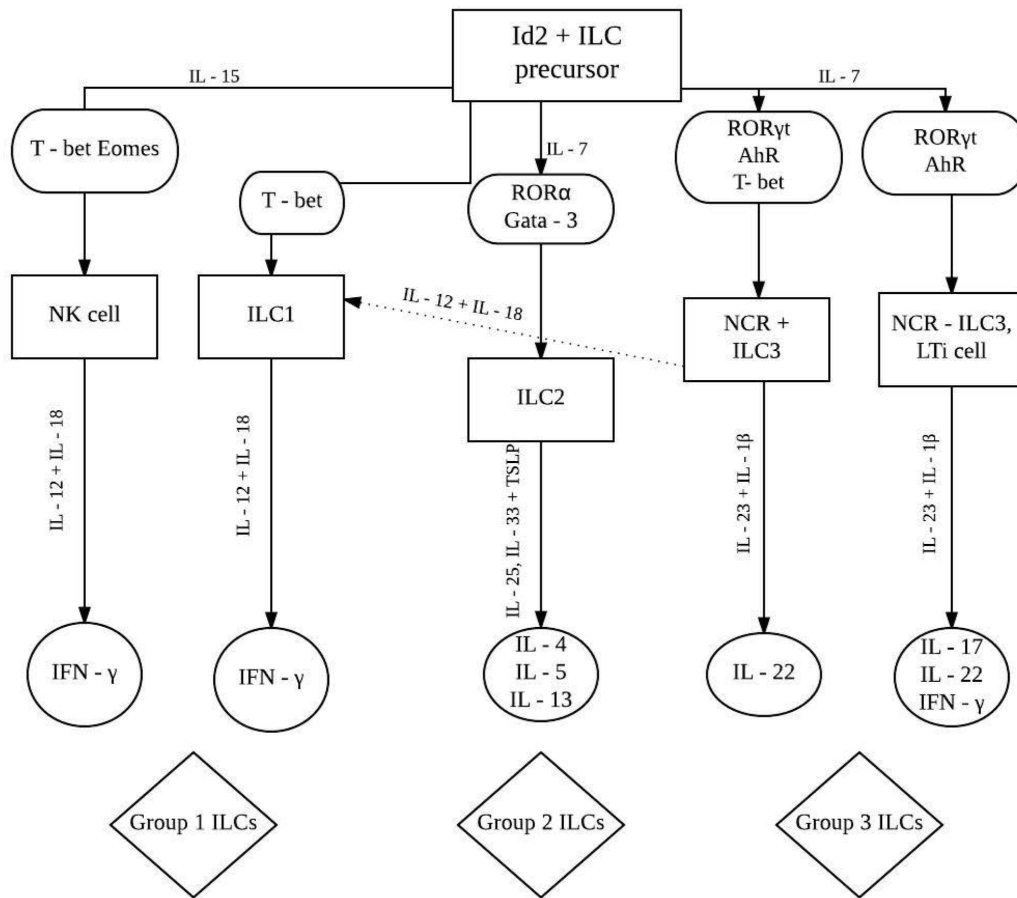
1.5 Innate lymphoid cell (ILC) biology

Innate lymphoid cells (ILCs) constitute a newly identified family of mononuclear hematopoietic cells with integrate innate and adaptive immune responses. ILCs are functional in the early stages of the immune response against microbes (Cella et al. 2009), in tissue repair (Scandella et al. 2008; Monticelli et al. 2011), containment of commensals (Sonnenberg et al. 2012), and in maintaining the epithelial integrity at barrier surfaces (Sonnenberg et al. 2011). ILCs are characterized as cell lineage marker negative (Lin^-) cells expressing the interleukin (IL)-2 and IL-7 receptor α subunits (Spits and Di Santo 2011). Unlike their adaptive counterparts (T and B cells), ILCs do not express rearranged antigen specific receptors (Spits & Cupedo 2012). Recent studies have identified three distinct ILC subsets on the basis of their differential requirements for transcription factors during development (Artis & Spits 2015). These include: group 1 ILCs (ILC1), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) which also includes the lymphoid tissue inducer (LTi) cells. All ILCs emerge from a common lymphoid progenitor that differentiates into numerous precursors that are committed to distinct cell lineage (Artis & Spits 2015). The integrin $\alpha 4\beta 7^+$ cell precursors also called α-lymphoid cell precursor (α-LP) cells expressing the chemokine receptor CXCR6 can develop into ILC3s and NK cells but not T cells or B cells (Cherrier et al. 2012; Klose et al. 2014). These cells will also develop into two distinct cell precursors expressing the Id2 transcriptional repressor. One of the $Id2^+$ precursors, which also express CD127 and the $\alpha 4\beta 7^+$ integrin, will develop into ILC1s, ILC2s and ILC3s, but not NK cells (Sedda et al. 2014), (Figure I). Id2 appears to be required at later stages of NK cells development, whereas early developmental stages are dependent on the transcription factor eomesodermin (Eomes) (Gordon et al. 2012; Spits et al. 2013). Further studies are required to improve our understanding of developmental checkpoints in the progression of different ILC

populations. Based on their phenotypical and functional characteristics, ILCs secrete discrete patterns of cytokine that mirror the cytokine-secretory profile of T helper cell subsets (Spits and Di Santo 2011). For example, in response to IL-12 and IL-18 (Bernink et al. 2013) ILC1s produce interferon gamma (IFNγ) and tumor necrosis factor (TNF) and have been shown to mediate immunity against parasites and intracellular bacteria (Klose et al. 2014). Conversely, ILC2s are responsive to IL-25, IL-33 (Neil et al. 2010; Saenz et al. 2010) and thymic stromal lymphopoietin (TSLP) (Mjosberg et al. 2012) and produce type 2 (T_H2) cell associated cytokines, in particular IL-5 and IL-13, as well as epidermal growth factor ligand amphiregulin, and have been shown to mediate anti-heminth immunity, tissue repair and allergic inflammation (Mjosberg et al. 2011; Monticelli et al. 2011; Wilhelm et al. 2011). Lastly, ILC3s including the lymphoid tissue inducers (LTi) respond to IL-23 (Cupedo et al. 2009; Sanos et al. 2009) and IL-1β (Hughes et al. 2010; Cella et al. 2010) by secreting Il-17A/F, IL-22, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF, promoting antibacterial immune responses, and mediating chronic inflammation (Cella et al. 2009; Cupedo et al. 2009; Satoh-Takayama et al. 2008, Sonnenberg et al. 2011). LTis were initially identified by Mebius et al. in 1997 who first described their important role during embryonic lymph node development. Post-nattily, gut LTis where shown to be essential for the formation of isolated lymphoid follicles (ILF) clusters and cryptopatches. The shared developmental transcription factors of ILCs with corresponding T cell subsets emphasizes on the functional similarities between the two cell lineages. For example, T-bet is indispensable for IFNγ production from ILC1s (Szabo et al. 2000), meanwhile it is considered to be the master transcription factor of T_H1 cells. On the other hand, RoRγt and the aryl hydrocarbon receptor (AHR) are essential for the development, survival and function of ILC3 subsets (Sun et al. 2000; Eberl et al. 2004; Kiss et al. 2011), as they are for T_H17 cells. On

the contrary, the transcriptional factor RoRα has been shown to play an important role in ILC2 development and function, but not on T_H2 cells (Halim et al. 2012). This fact implicates that some transcriptional regulators are specifically involved in ILCs development and function but not on the corresponding T cell subsets. As with the T helper subsets, current evidence revealed functional plasticity between ILC subsets in response to multiple cytokine signals. For example: *in-vitro* cultured murine $RoR\gamma t^+$ ILC3s stimulated with IL-12 and IL-18 pro-inflammatory cytokine mediators exhibit an increased IFNγ production whereas they lost their capacity to secrete IL-17 and IL-22 (Vonarbourg et al. 2010; Klose et al. 2013). Previous observations have also revealed that upon stimulation with IL-12, human $RoR\gamma t^+$ $NKp44^-$ ILC3 can differentiate *in-vitro* into $CD127^+$ ILC1 cells (Bernink et al. 2013). Due to their diverse function in mediating immunity against infection, microbiota interactions, and tissue repair; ILCs need to be closely regulated as unbalanced activation of these cells can induce severe inflammation and damage in the intestine (Buonocore et al. 2010), lung (Chang et al. 2011; Halim et al. 2012), skin (Kim et al. 2013; Roediger et al. 2013) and liver (McHedlidze et al. 2013).

Figure I: Schematic view of the differentiation of various innate lymphoid cells ILCs



1.5.1 Tissue distribution of ILCs in humans and mice

In the peripheral blood of healthy individuals, the CD127^{+} ILC phenotype constitutes 0.01 to 0.1% of total lymphocytes. The majority of the latter ILCs correspond to ILC2 (Mjosberg et al. 2011), whereas NKp44^{+} ILC3 and CD127^{-} ILC1 are almost absent (Fuchs et al. 2013). Interestingly, the absence of cytokine transcripts in healthy peripheral blood CD127^{+}ILC subsets indicates that they are not activated. The distribution of ILC subsets in humans is tissue dependent. For example, ILC2 and NKp44^{-} ILC3 are prevalently present in the healthy skin (Dyring-Andersen et al. 2014; Teunissen et al. 2014), whereas NKp44^{+} ILC3 is the prominent ILC subset in the intestine. Human NKp44^{-} and NKp44^{+} ILC3s have been detected in the spleen and were found to interact with stromal cells and B cells for survival signaling and antibody production (Magri et al. 2014). Murine ILCs have been spotted and characterized in adipose tissue (Moro et al. 2010; Molofsky et al. 2013), and were found to enhance the accumulation of eosinophils and stimulate the activation of macrophages, which are associated with metabolic homeostasis. Murine ILCs have been also found to reside in the liver and mediate liver fibrosis when stimulated with IL-33 (McHedlidze et al. 2013). So far, there is no data available about the presence of human ILCs in the liver and adipose tissue. Although ILCs have been detected in different mice organs, these cells reside primarily in the gut mucosa and are absent from the blood circulation. In contrast, NK cells are present in the circulation and from there are recruited to the site of inflammation to provide rapid IFNγ release and help their innate cell counterparts in fighting against pathogenic infections. In the following paragraphs, I will review the available knowledge on the effector functions of human and mice ILCs in controlling intestinal infections and mediating gut inflammation.

1.5.2 ILC subsets in human and murine gut immunity

1.5.2.1 ILC1

The human gut contains CD127^{high} ILC1, characterized by the expression of IFNγ and T-bet transcripts and by the lack of NK cell markers such as Nkp44, CD16, CD56, granzymes and perforin (Fuchs et al. 2013). Since there is no exact equivalent of the human CD127^{high} ILC1 cells in mice, *in-vivo* function of these cells has not yet been precisely established. Nonetheless, murine T-bet^{+} IFNγ producing ILCs were found to contribute resistance against Salmonella enterica (Fuchs et al. 2013) and Toxoplasma gondii (Klose et al. 2013), indicating that human CD127^{high} ILC1 might also play a role in mediating immunity against pathogenic gut bacteria. Recent observations suggest that CD127^{high} ILC1 appearance in the gut is triggered by bacterial colonization in contrast to ILC3 and ILC2, which are early detected in the human fetal gut (Bernink et al. 2013). These findings support the hypothesis of human CD127^{high} ILC1s in mediating gut immunity during a pathogenic state. Interestingly, inflamed intestinal tissues from biopsies of Crohn's disease patients revealed the accumulation of IFNγ-producing CD127^{high} ILC1s, whereas the frequency of NKp44^{+} ILC3 was notably reduced, possibly through IL-12- and IL15-dependent differentiation of ILC3 into the ILC1 phenotype (Fuchs et al. 2013; Bernink et al. 2013). This observation is further supported by another study where they showed the absence of ILC1s in the gut lamina propria of alymphoid mice reconstituted with human immune system under normal conditions, yet these cells appeared following induction of colitis by dextran sodium sulphate treatment (Bernink et al. 2013). Another IFNγ secretory ILC1 subset has been also identified in humans. These ILC1 are characterized by the expression of CD103 and NK cell marker in contrast to their CD127^{high} counterparts. CD103^{+}ILC1 are predominantly present within the mucosal epithelium. The murine counterparts of human CD103^{+}ILC1 do not

express CD103, however they are distinguished by the expression of the intraepithelial CD160 marker. Murine intraepithelial ILC1 are potent producers of IFNγ in response to IL-12 and IL-15 *in-vitro* stimulation, as well as in response to anti-CD40 administration in an *in-vivo* model of induced colitis (Shui et al. 2012; Fuchs et al. 2013). Anti-NK1.1 depletion studies in mice indicate that ILC1 may contribute to severe intestinal inflammation; however multiple intra-epithelial cells express the NK1.1 marker including NK, and γδT cells (Fuchs et al. 2013). Thus, it is inappropriate to attribute the observed phenotype to ILC1. Until very recently, it was still controversial to arbitrate whether the RoRγt-independent ILC1s really exist in the gut, or do they originate from RoRγt^{+}ILC3s that up-regulate T-bet and down-regulate RoRγt in response to inflammatory signals, which leaves mucosal NK cells as the only identified ILC1 member in the murine gut.

1.5.2.2 ILC2

Recently, the ILC family has been expanded with the discovery of type-2 innate lymphoid cells (ILC2), originally described as nuocytes (Neil et al. 2010), natural helper cell (NHC) (Moro et al. 2010) and innate helper 2 cells (Ih2) (Price et al. 2010). ILC2 were first reported in Rag$^{-/-}$ mice as a non-B, non-T cell that has the capacity of secreting IL-5, IL-9 and IL-13 in response to IL-25, IL-33 and TSLP (Walker et al. 2013). ILC2 originate from a common lymphoid precursor (CLP) in the bone marrow and reside primarily in the intestines, airways and in fat associated lymphoid clusters. Recent studies in mice have found that ILC2 provide a critical source of IL-13, responsible in mediating innate responses against allergies and helminth infections (Fuchs et al. 2013). In case of intestinal parasites clearance such as *Nippostrongylus brasiliensis,* the production of IL-13 by ILC2 has shown to be essential in driving goblet cell mucus secretion and intestinal smooth muscle cell contraction, which are necessary physiological processes to effect

worm expulsion (Walker et al. 2013). Although phenotypical and functional ILC2 studies have been mostly conducted in mice models, similar populations have been identified in the human lung and intestines. These cells are defined in humans as lin^{-}IL-7Rα^{+}NKp44^{-}CD25^{+}CRTH2^{+} CD161^{+} and were found in higher percentages in the nasal polyps of patients with chronic rhinosinusitis (Walker et al. 2013; Mjosberg et al. 2012).

1.5.2.3 ILC3

In humans, ILC3 subsets have been detected in different mucosa associated lymphoid tissues such as the appendix, tonsils, Peyer's patches, colon and jejunum lamina propria and fetal mesenteric lymph nodes. In 2009, the first report appeared demonstrating the presence of human IL22-producing ILC3 in the healthy gut (Cella et al. 2009, Artis & Spits 2015). This ILC3 subset expresses the NKp44 NK cell receptor (Cella et al. 2009; Takayama et al. 2010) and CCR6 (Cella et al. 2009; Cella et. al. 2010), which were originally called NK22 cells. The production of IL-22 by ILC3 is responsible in promoting proliferation, and production of IL-10, antimicrobial peptide, and mucus by the intestinal epithelial cells (Cella et al. 2009). Human gut NKp44^{+}ILC3 also expresses transcripts for leukemia inhibitory factor (LIF), which is an IL-26 inducer (Cella et. al 2009), a cytokine that induces the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-8 by intestinal epithelial cells (Dambacher et al. 2009). Moreover, gut resident NKp44^{+} ILC3 produce the B cell activating factor (BAFF), supporting the activation and expansion of mature B cells (Cella et al. 2009; Cella et al. 2010), and the CCL20 chemokine that directs the migration of T and B-lymphocytes into the intestinal site (Cella et al. 2010). On the other hand, mice ILC3 subsets, in particular the LTi MHC class II (MHC-II) expressing cells have been shown to present commensal antigens to CD4^{+}T cells. Because LTi lack co-stimulatory molecules, the interaction between LTi and T cells did not result in the activation of

the latter cells. Instead, it induced tolerant T cell responses against derived commensal antigens (Hepworth et al. 2013). Human ILC3 were also found to express MHC-class II molecules, suggesting that MHC-II^{+} ILC3 might induce tolerant T cell immune responses in the human gut (Hepworth et al. 2013). A study conducted by Qiu et al. demonstrated that murine ILC3 could indirectly regulate Th17 cells via the aryl hydrocarbon receptor (AHR), which is a ligand dependent transcription factor. Notably, Ahr$^{-/-}$ ILC3 has a diminished IL-22 production, leading to increased intestinal susceptibility to filamentous bacteria, known to stimulate Th17 cells to expand. Further studies are needed to determine whether similar immune regulatory mechanism also exist in the human gut. The primary focus of previous studies was to determine the ability of ILC3 to modulate the function of different immune cell subsets. However, none of them examined the reciprocal modulation of ILC3 subsets. Mizuno et al. detected that *in-vitro* co-culture of intestinal RoRγt^{+}ILCs with CD14^{+} macrophages isolated from inflamed surgically resected specimens of patients with Chron's disease results in an enhanced IL-22 production by ILCs (Mizuno et al. 2014). Furthermore, in a mouse model of *Citrobacter rodentium* induced colitis, *in-vivo* depletion of mononuclear phagocytes (MNPs) resulted in severe inflammation after infection. However, IL-22 production by ILC3 in close proximity to MNPs rescued the observed phenotype. Together, these two findings reveal a critical role of macrophages in integrating inflammatory and microbial signals to regulate ILC3 function in the gut. *In-vitro*, human NKp44^{+} ILC3 are responsive to IL-23, IL-1β, IL-2 and IL-7 stimulation by producing IL-22 (Hughes et al. 2010; Cella et al. 2010; Crellin et al. 2010), which is further enhanced in the presence of TLR2 (Crellin et al. 2010). This discovery raised the question whether microbiota TLR agonists can also regulate the development of NKp44^{+} ILC3 in addition to the modulation of their function. Yet, human studies of fetal gut NKp44^{+} ILC3 revealed that their development

is independent of commensals (Hoorweg et al. 2012). Subsequent murine studies, demonstrated that the frequency of IL-22 producing ILC3 is similar in conventional versus germ-free mice (Sonnenberg et al. 2012). Although commensal microflora is apparently not necessary for ILC3 development, however it can influence ILC3 functions by causing the production of IL-25 by epithelial cells, which inhibits IL-22 secretion by ILC3 (Fuchs et al. 2013). Collectively, all previous studies illustrate the important role of murine intestinal ILC3 subsets in mediating gut immunity, either by direct control of epithelial cell function or indirectly through various intestinal cells cross-talk.

1.5.2.3.1 Role of ILC3 in maintaining *Citrobacter rodentium* infection

In addition to their critical role in maintaining intestinal homeostasis, ILC3s are also considered as potent innate immune effector cells against invading bacterial pathogens, such as *Citrobacter rodentium,* an extracellular gram-negative bacterium that causes acute colitis in mice and models human enterohemorrhagic and enteropathogenic *Escherichia coli* infections (Zheng et al. 2008; Cella et al. 2009). *C. rodentium* carry distinct genetic elements in its chromosomes, called pathogenicity islands (PAIs), referred to as locus of enterocyte effacement (LEE) (Diefenbach et al. 2013)**,** which allows the bacteria to attach to colonic epithelial cells resulting in effacement of brush border microvilli and in colonic mucosal hyperplasia (Diefenbach et al. 2013)**.** Although adaptive immunity is required for the eradication of *C. rodentium*; recent studies have showed that *C.rodentium* infected Rag$^{-/-}$ mice, which lack both T and B cells but retaining ILC3s, developed an IL-22-mediated response and were resistant for the first 30 days of infection (Satoh-Takayama et al. 2008; Zheng et al. 2008; Sonnenberg et al. 2011). Initially, CD11c^{+} dendritic cells were primarily identified as the source of IL-22 (Diefenbach et al. 2013). However, recent studies have revealed that ILC3 subsets are the main source of IL-22 during

C.rodentium infection, and depletion of ILCs led to a rapid death following infection (Diefenbach et al. 2013). In response to the expression of IL-23 by mononuclear pahgocytes, ILC3 secretes the IL-22 cytokine that acts exclusively on epithelial cells and mesenchymal stroma due to the restricted expression of the IL-22 receptor on non-hematopoietic cells. (Zheng et al. 2008; Diefenbach et al. 2013). In turn, IL-22 promotes STAT3-dependent expression of antimicrobial proteins such as RegIII and maintenance of intestinal epithelial barrier function (Zheng et al. 2008; Sonnenberg et al. 2011; Vaishnava et al. 2011). Previous studies have demonstrated that IL-22$^{-/-}$ mice displayed excessive epithelial disruption and were rapidly succumbed to infection (Satoh-Takayama et al. 2008, Sonnenberg et al. 2011). In summary, It's evident that ILC3 mediated IL-22 response is required for the early resistance to *C.rodentium;* however it is still unclear how IL-22 controls gene expression within epithelial cells and how this relates to immunity against *C.rodentium* infection.

1.6 γδT cells development, distribution and function in human and murine gut immunity

Both αβ and γδT cells arise from the same precursors in the thymus. However various ontological reports established that the development of γδT cell subsets precedes αβT cells in the thymus as observed in different species including mice and human (Haas et al. 1993). Interestingly, both human and murine expression of γδT cell receptor appears in an ordered series of overlapping waves, regulated by multiple enhancers, which are influenced by specific cytokines produced by the thymic epithelial cells (Appasamy et al. 1993, He et al. 1995), and the rearrangement of specific Vγ genes (Krangel et al. 1990, Wilson et al. 1994). Following thymic maturation, γδT cells expressing particular γδTCRs selectively home to various tissues. In mice, $V\gamma5^{+}\delta1$ T cells also called dendritic epidermal T cells (DETCs) are the first to emerge from the fetal thymus to predominate the intra-epithelial compartment of the skin (Girardi et al. 2006). Following emigration from the thymus, DETCs has been determined to play a role in mediating epidermal homeostasis and wound repair through their constitutive expression of insulin-growth factor 1 (IGF-1) and keratinocyte growth factor-1 (KGF-1) (Sharp et al. 2005). Interestingly, the generation of Vγ5Vδ1 cells is dependent on the presentation of IL-15 by non-lymphoid cells in the thymus (Schluns et al. 2004, Zhao et al. 2005), indicating the influence of cytokine signalling pathways in mediating the generation of the γδT repertoire and suggesting fundamental differences in the thymic selection between αβ and γδT cells (Komori et al. 2006). Whereas $V\gamma5^{+}$ T cells constitute most of the intra-epithelial lymphocytes (IELs) in the murine skin, $V\gamma1^{+}$ γδT cells represent one of the major subsets in circulation as well as in the secondary lymphoid organs (Pereira et al. 1995). Previous studies have indicated an important role of the $V\gamma1^{+}$

thymocyte population in multiple mouse models of diseases (Born et al. 2010). Further murine studies have also identified other γδT cell subsets of limited TCR diversity restricted to other epithelial tissues. These include $V\gamma6^{+}V\delta1$ T cells that consist the vast majority of IELs in the tongue and reproductive tract where they seem to respond to inflammation and exhibit immune-regulatory functions (Born et al. 2010, Itohara et al. 1989), and the Vγ7Vδ4/δ5 T cells that represent most of the intestinal IELs (Asarnow et al. 1989). Other studies revealed that gut homing γδT IEL precursors are dependent on the interaction between their surface chemokine receptor CCR9 and CCL25 secreted in the thymus (Komori et al. 2006, Wurbel et al. 2001). This interaction promotes the expression of integrin on the surface of γδT IEL precursors, which mediates their adhesion to the intestinal epithelium through binding to E-cadherin (Ericsson et al. 2004), revealing once again the divergent development pathways between αβT and γδT cells and the important role of soluble factors in the development and distribution of γδT cell subsets. Other then being generated from the thymus, cryptopatch progenitor cells that exist in the lamina propria of the intestine has been also identified as another source of gut γδT cell population in athymic mice (Emoto et al. 2004, Nonaka et al. 2005). However, cell-fate mapping studies revealed that γδT IEL progenitor do not mature within the cryptopatch locus of mice with intact thymus (Komori et al. 2006). Similar to their skin counterparts, the function of gut γδT IELs has been described to modulate epithelial homeostasis through the release of KGF-1in an IL-7 dependent manner (Yang et al. 2004, yang et al. 2005). Additionally, in an inflammatory model of chemically induced colitis, the transfer of γδT IELs into $TCR\delta^{-/-}$ recipient mice induced the production of IL-15 by adjacent epithelial cells and decreased the production of inflammatory recruitment factors such as IFN-γ and TNFα from intra-epithelial lymphocytes (Inagaki et al. 2004). All of these effects restored epithelial homeostasis and revealed the vital regulatory

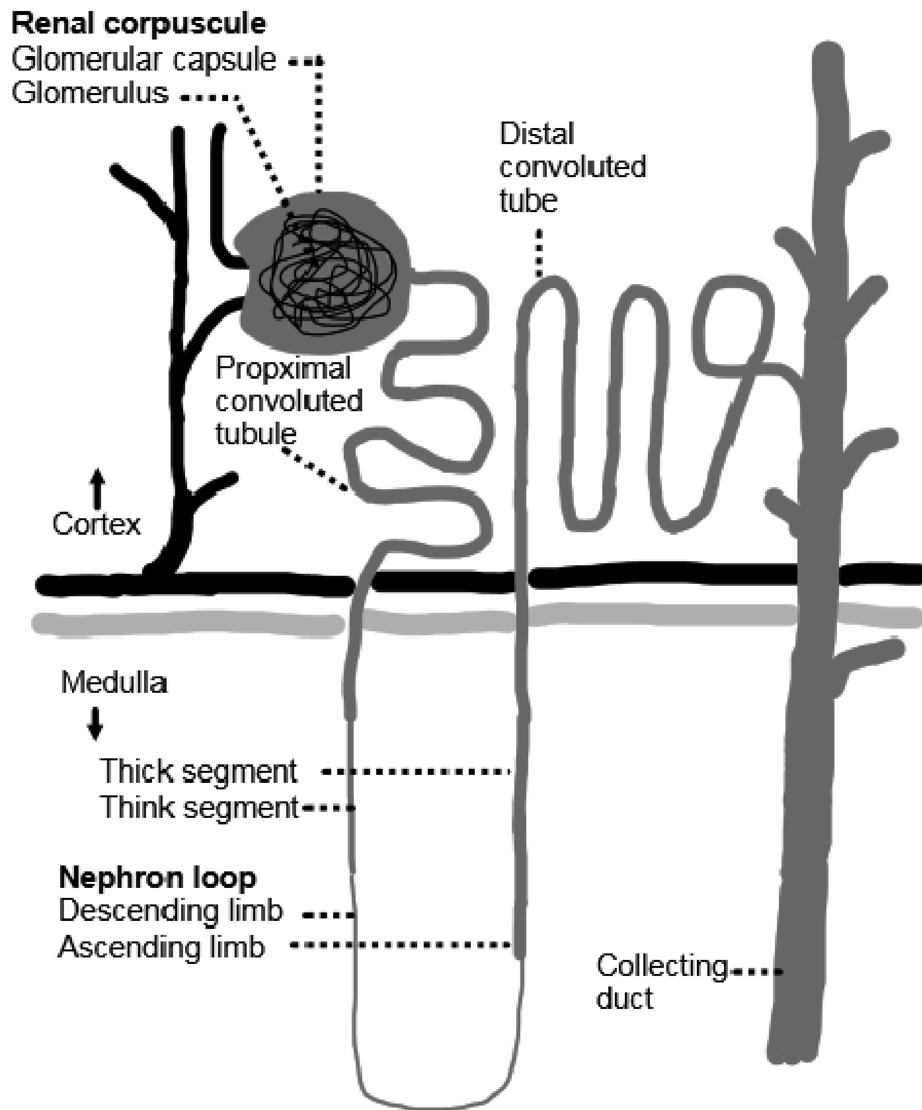
activities of γδT cells during intestinal inflammation. In humans, γδT cells expressing the Vδ1 chain paired with various Vγ chains are the first to emerge from the fetal thymus and vastly populate the intestinal epithelium (Girardi et al. 2006). Importantly, these cells have been reported to lyse stressed/transformed epithelial cells via NKG2D:MICA receptor:ligand interaction, as well as via the presentation of self-peptide by CD1 or stress induced MHC-1b molecules (Girardi et al. 2006). However, other mechanisms of γδ IEL activation have been also suggested. For example, in cytomegalovirus (CMV) infected patients, γδ IEL were capable of lysing CMV-infected epithelial cells in a TCR dependent manner, independently of MHC-I like molecules or NKG2D (Halary et al. 2005). Moreover, infection studies reported that Vδ1^{+}T cells respond to the LPS layer of gram-negative bacteria through specific TCR engagement. Importantly, this interaction was dependent upon the contact of γδT cells with resident dendritic cells (Das et al. 2004). On the other hand, human Vγ9Vδ2 T cells constitute 80% of the γδT cells in normal adult blood and have been demonstrated to promote cytotoxicity against multiple cancer cell lines as they were also found to infiltrate the lung and kidney epithelial tumors (Fisch et al. 1997). In contrast to the restricted intestinal intra-epithelial Vδ1 T cells, Vγ9Vδ2 T cells are capable of rearranging their TCR genes to produce functional diversity against intracellular and extracellular bacteria (Dieli et al. 2001), develop a memory phenotype and may also function as antigen-presenting cells (Brandes et al. 2005). Despite the fact that Vγ9Vδ2 T cells possess adaptive immune properties, they are still referred as part of the innate immune system where various restricted TCR rearrangements are considered as pattern recognition receptors (Girardi et al. 2006). In summary, the leading role of γδT cells in managing tissue homeostasis is unquestionable. However little is known about the antigens they recognize, the mechanisms of activation and the details lying behind their thymic positive selection, homing and expansion at

tissue specific sites (Girardi et al. 2006). Further understanding of development and function, as well as a detailed comparison of murine and human γδT cell subsets is needed to provide insights into the role of these cells in mediating tissue homeostasis, protection against infections and tumor surveillance.

1.7 Kidney structure, function and vascularity

The main function of the kidney is the production and the control of the urine composition to appropriately match the body's internal environment and requirements. This process is done by selective removal of toxic and excess substances from the blood plasma and subsequent controlled reabsorption of ions, salts, carbohydrates, small molecular weight proteins and water (Kurts et al. 2013). Structurally, the kidney is formed of an outer cortex that lies between individual inner medulla units. In turn, the medulla units form a series of conical structures called medullary pyramids that points towards the urine collecting system also called calyceal system. The human kidney bears 10-18 medullary pyramids, each one associated with a cortex shell, forming the functional and structural lobes of the kidney (MRCPath Lowe 2005). The human kidneys receive 25% of the cardiac output. Consequently, most common kidney diseases result from the blood vascular component abnormality. For this reason, it is important to realize that the kidney differs from other organs in their vascular supply. Normally, the vascular system provides the parenchymal tissues with oxygen and nutrients and carries metabolic waste materials away from the organ. In the kidney, however, the functional unit of the parenchyma called the nephron (Figure II), which is composed of two main components, the glomerulus and the cortical and medullary tubular system, serves the blood supply through the presence of two capillary systems (MRCPath Lowe 2005). The glomerulus tuft, which forms the first capillary system, receives blood from an afferent arteriole and acts as the site of initial blood filtration together with a second capillary system that arises from the efferent arteriole. Next, the efferent arteriole is divided into a complex capillary system that runs intimately in the interstitial spaces of the renal tubules, and thus ideally placed to take up any substances reabsorbed from the glomerular filtrate by tubular epithelial cells (MRCPath Lowe 2005).

Figure II: Illustration of the basic structural and functional unit of the kidney "nephron"



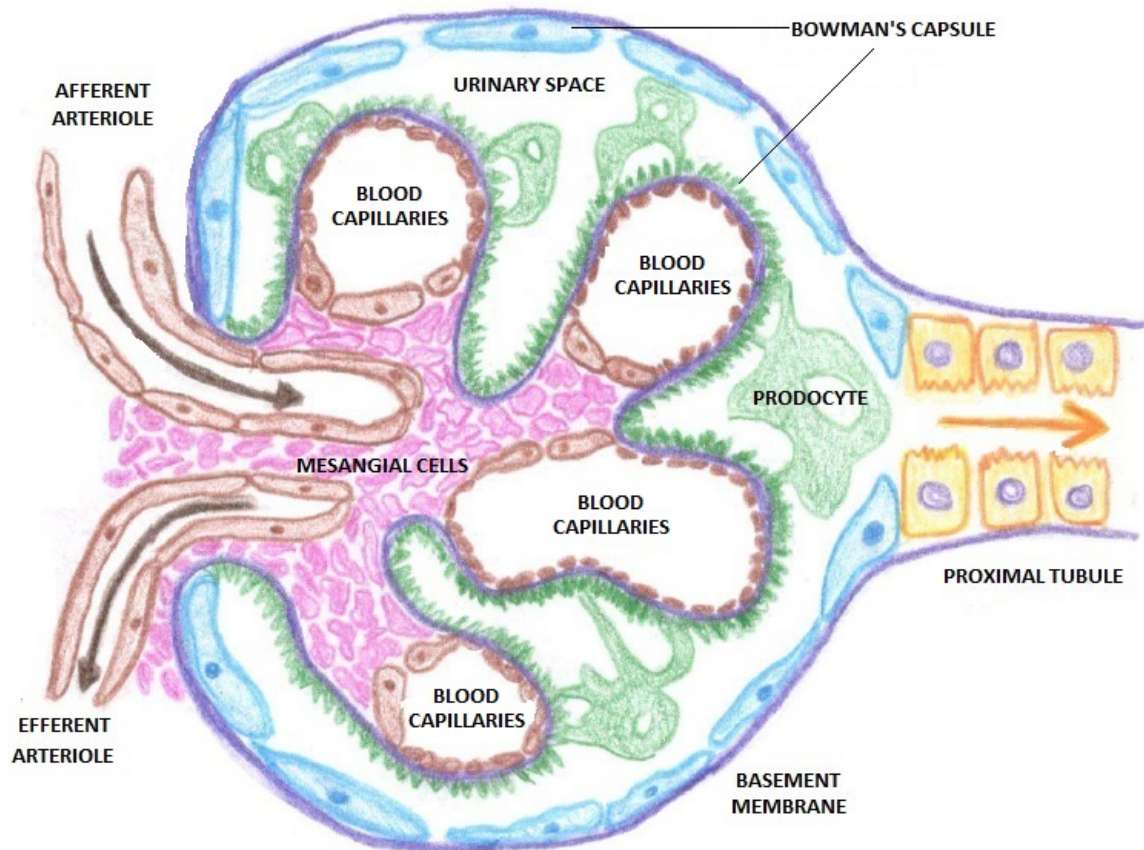
1.7.1 Glomerulus

The glomerulus is the first functional component of the nephron and is the initial site of partial filtration of the blood arriving from the afferent arteriole (Figure III). While entering the glomerulus the afferent arteriole splits up into several branches of capillaries network that is supported by its own strip of mesangium (MRCPath Lowe 2005). The exact mechanism of action of the mesangium is not well known. However, mesangium abnormalities in human glomerular diseases suggest that it is a vital functional component of the glomerulus. For example, in diabetes mellitus, the mesangial cells produce excessive amounts of a cellular matrix to form spherical nodules known as Kimmelstiel–Wilson nodules (MRCPath Lowe 2005). Additionally, in some cases of immune complexes deposition within the mesangium there is a proliferation of mesangial cells, which leads to the development of mesangial glomerulonephritis (Kurts et al. 2013). In the glomerulus, the filtration of blood occurs at the level of the glomerular filtration barrier, which comprises glomerular capillary endothelium, glomerular basement membrane (GBM) and epithelial podocytes attached to the outer surface of the GBM. Furthermore, the high polyanionic charge on the outer surface of GBM and podocytes works as a physiochemical barrier, preventing the passage of cationic molecules (Kurts et al. 2013, MRCPath Lowe 2005). Thereafter, the blood passes from the glomerular capillaries into the urinary space, which is enclosed by Bowman's capsule, before passing down the tubular system. The filtered blood leaves the glomerulus via the efferent arteriole and flows onward to provide an oxygenated blood supply to the highly metabolic active tubules, where controlled reabsorption and further excretions occurs (Kurts et al. 2013).

1.7.2 Renal tubular system

All molecules below 68kDa or the molecular size of albumin pass the glomerular filtration barrier and enter the tubules, which first comprises the proximal tubules that are basically a continuation of the Bowman's capsule and are vital components for extensive reabsorption of the glomerular filtrate (Kurts et al. 2013, MRCPath Lowe 2005). At the beginning, these tubules pursue a convoluted course with apical microvilli to provide a bigger surface area of absorption, as they are also rich with mithochondria for active transport of various components against gradients. The main function of proximal convoluted tubules involves the reabsorption of water, glucose and amino acids by using Sodium/Potassium ATPase pump, whereas large molecules are reabsorbed by endocytosis (MRCPath Lowe 2005). The proximal tubules then straighten and descend towards the medulla forming the thick and thin limb of the loop of Henle. This then loops back on it-self and re-enters the cortex forming the thick ascending limb of the loop of Henle. Throughout the loop, a hypertonic gradient is created, allowing the concentration of urine in the collecting duct system. In the cortex, close to the glomeruli, the distal tubule becomes convoluted and empties into a collecting duct. All over the distal tubules, sodium and bicarbonate ions are reabsorbed from urine in exchange for potassium and hydrogen (MRCPath Lowe 2005). These functions are dependent on the presence of aldosterone, which is a hormone secreted by the adrenal cortex (Kurts et al. 2013). All non-reabsorbed compounds passing from the tubular system into the collecting duct will form the urine. Lastly, the urine concentration will be further regulated under the influence of hypertonicity gradient and antidiuretic hormone all over the collecting duct (Kurts et al. 2013).

Figure III: A sketch of a healthy kidney glomerulus



1.8 Renal Immunology

Chronic kidney disease (CKD) affects 10% of the western population and is considered a serious cost-intensive and social condition especially in the elderly population as well as in younger age groups who progress to kidney failure and require kidney dialysis or transplantation (Kurts et al. 2013). Although recent discoveries have revealed multiple mechanisms lying behind several immune-pathological kidney disorders, the cross talk between the immune system and the kidney is still not widely studied inside the immunological community. Under various induced stress, the kidneys produce multiple hormones that can directly or indirectly affect the immune system. These include erythropoietin in response to hypoxia, renin, which induces angiotensin and aldosterone to regulate blood pressure and electrolyte balance, and vitamin D, which regulate bone homeostasis and phagocytic cell functions (Kurts et al. 2013). Inside the functional unit of the kidney (nephron), the glomerulus selectively filters molecules lower then 68kDa, whereas molecules above 68kDa are retained in blood. In addition, the glomerular barrier bears a high polyanionic charge on the outer surface of glomerular basement barrier and podocytes. Altogether, the size-selective and charge-dependent properties of the glomerular filtration promote the deposition of immune complexes in the glomerulus, which drives immune responses against kidney derived-autoantigens and causes autoimmune kidney diseases (Kurts et al. 2013). Besides the glomerular immune complex deposition, several immune mechanisms can also contribute to the progression of CKD. These include complement dysregulation or cell mediated inflammation through the interaction with kidney resident immune cells (Kurts et al. 2013). Despite the fact that CKD might not be immune initiated, unbalanced immune responses has been demonstrated to exacerbate the progression of the disease, hence their obvious implications for therapy.

1.8.1 Role of renal epithelial cells in the innate immune apparatus of the kidney

The tubulointerstitium, which is the space between the glomeruli and tubuli contains nearly all of the intra-renal immune system and is the initial site for the progression of nephritis (Kurts et al. 2013). Renal orchestrated innate immune responses do not only consist of professional immune cells but also of non-immune cells, such as epithelial cells (Hato et al. 2015). Other than acting as a physical barrier, renal tubular epithelial cells constitute a major functional component in the kidney and have been shown to be crucial in mediating innate immunity through the production of chemokines and cytokines, and through their ability to recognize and process pathogenic or auto-antigens using a sophisticated repertoire of pattern recognition receptors (PRRs) (Hato et al. 2015). As in the case of most body organs, epithelial cell numbers far exceed any other cell type, especially that of immune cells in the kidney, hence they can play a substantial role in mediating immune responses and maintaining homeostasis. In the kidney, PRRs including the Toll like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs), are widely expressed on myeloid cells but have been also found in renal epithelial cells (Hato et al. 2015). The expression of these receptors on both myeloid and epithelial cells direct their functions as sentinels against pathogen-associated molecular pattern (PAMPs) and/or damaged self-endogenous soluble molecules also called DAMPs, which include the high mobility box 1 (HMB1), heat shock proteins and extracellular ATP. The ligation between PRRs and their corresponding ligands, results in driving the activation of the adaptive immune system by inducing inflammation, phagocytosis and maturation of antigen presenting cells. Importantly, a prolonged inflammation in addition to a deregulated tissue repair mechanism will involve collateral tissue damage, leading to the progression of chronic kidney diseases. Indeed, previous studies reported that mice lacking intrinsic renal TLR4 or TLR2 had lesser tubular damage

following renal ischemia-reperfusion in comparison to WT mice (Wu et al. 2007, Leemans et al. 2005). These discoveries specifically indicate the role of TLRs in mediating tubular damage. Besides inducing inflammation, tubular epithelial cells have been described to express MHC-II and costimulatory proteins after various stimuli (Wuthrich et al. 1990, Niemann et al. 2002, Wahl et al. 2002), demonstrating their ability to present antigens to T cells and illustrating their shared functions with professional immune cells. Since renal tubular epithelial cells are found in specific confined structures inside the kidney, they can never accomplish higher levels of immune activities unless auxiliary immune cells supports them to do so (Hato et al. 2015). For this reason, further studies will be needed in order to elucidate the mechanisms of communication between renal tubular epithelial cells and immune cells. It is noteworthy that although I have taken renal tubular epithelial cell immunity from a centric view this cannot hinder the role of other non-immune cells that mediates certain types of renal injuries. For example, the expression of PRRs on the surface of glomerular podocytes and endothelium has been extensively proven to mediate glomerular injury (Banas et al. 2008, Chen et al 2011). The generated inflammatory signals from the glomeruli can spread into the tubules through the peritubular capillaries initiating an oxidative stress, which lead to the progression of chronic kidney diseases.

1.8.2 Role of γδT cells in kidney diseases

γδT cells consist only 0.5 to 0.6% of total circulating lymphocytes in normal individuals (Dechanet et al. 1999). However, foregoing studies have revealed significant increase of peripheral γδT cells during several infections such as malaria, Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV), and autoimmune diseases including rheumatoid arthritis, celiac disease, and multiple sclerosis (Dechanet et al. 1999). Interestingly, γδT cells have been shown to recognize non-classical MHC class I molecule as well as induced stressed molecules expressed on the surface of host-damaged cells during chronic inflammatory responses (Wu et al. 2004). In contrast to αβT cells, γδT cells also recognize unprocessed protein antigens independently of peptide/MHC complexes. Initial studies attributed several regulatory properties for γδT cells in mediating tissue-homeostasis and initiating tissue repair mechanisms (Dechanet et al. 1999, Wu et al. 2004). Yet, virus-induce myocarditis studies revealed that different γδT TCR subsets are associated with either regulation (Vγ1) (Huber et al. 2000) or pro-inflammatory (Vγ4) outcomes (Huber et al. 2002), suggesting that the functional property of a particular TCR γδT cell subset is dependent on the ligand-TCR pairing. Today, γδT cells are known to have the capabilities to recognize a wide array of stress-induced signals and to mount early local effector immune responses, a function referred to as "lymphoid stress surveillance" (Couzi et al. 2015). Accordingly, γδT cells acquire their functions within 1-3 days after stimulation, while naïve αβT cells activation requires at least 5-7 days to respond to a specific stimulus (Couzi et al. 2015). In the context of CMV seropositive kidney transplants patients, flow cytometry data determined a large expansion of γδT cells in the peripheral blood post-renal transplantation. Surprisingly, the expansion kinetic and phenotype of the acquired γδT cells was similar to that of $CD8^+$T cells in the periphery (Dechanet et al. 1999, Couzi et al. 2015 Couzi et al. 2009). These data demonstrate

that γδT cells represent a first line of defense against CMV infection in immune-compromised patients. However, the latter findings contradict with the proposed concept of early lymphoid stress surveillance. To reconcile with the adapted concept, recent studies have proposed the presence of two γδT cell populations. First, tissue resident γδT cells that expand and acquire rapid immune responses and second, lymphoid homing γδT cells that expanded in a conventional adaptive manner after being primed in the circulation (Vantourout et al. 2013). Importantly, alternative discoveries have demonstrated that tissue-resident γδT cells are also able to recirculate to the blood via afferent lymph and lymph nodes (Shekhar et al. 2012). The perception of these discoveries is well exemplified in the case of the most common form of glomerulonephritis in the developed world (Falk et al. 1995). IgA nephropathy (IgAN) is characterized by the accumulation of IgA antibody in the glomerular mesangium, which often leads to the progression of chronic kidney disease. Several evidences have suggested the role of γδT cells in mediating the progression of IgAN (Falk et al. 1995, Buck et al. 2002). Notably, abnormality within the most abundant γδT subpopulation in the intestinal mucosa has been associated with the mechanisms underlying the pathogenesis of IgAN (Buck et al. 2002). In particular, Vγ3 gene expression was significantly decreased in gut biopsies of patients with IgAN in comparison to those in the control individuals. Further studies revealed that the observed hole in the intestinal γδT cell repertoire was pursued with a renal expansion of γδT cells (Falk et al. 1995, Buck et al. 2002), suggesting a fundamental role of γδT cells in contributing to the progression of IgAN. Various reports have also associated the increased number of renal γδT cells with Adriamycin-induced nephropathy, which represent a rat model of progressive glomerulosclerosis similar to that observed in humans (Wu et al. 2004 & Ando et al. 2001). The expanding γδT cells in the kidneys of ADR-induced nephropathy used a restricted set of Vγ6Vδ1

T cells receptor (TCR) genes (Wu et al. 2004 Ando et al. 2001). Interestingly, an equivalent increase of the same renal γδT cell subset has been observed in the kidney during listeria monocytogenes infection (Wu et al. 2004, Ikebe et al. 2001), suggesting that this restricted set of renal γδT cell receptor genes may represent a pathway for innate recognition in different diseases. The role of γδT cells has been further investigated in the context of Heymann nephritis (HN), which represent an experimental rat model of autoimmune-mediated glomerulonephritis (GN) that mimics membranous GN in human (Salant et al. 1989). The prominent feature of active HN is defined by tubulointerstitial inflammation characterized by tubular injury and increased renal mononuclear cell infiltrates (Wu et al. 2004, Heymann et al. 1962). Work done by Rosenkranz et al. using γδT cell deficient mice (Rosenkranz et al. 2000) clearly demonstrated the permissive role of γδT cells in mediating renal diseases progression due to their ability in recruiting neutrophils, and activating local macrophages (Turner et al. 2012). Indeed, murine studies of crescentric glomerulonephritis revealed that pro-inflammatory cytokine production such as IL-17 by renal γδT cells is dependent on the production of IL-23 by kidney dendritic cells (Turner et al. 2012). Furthermore, the absence of IL-17 producing γδT cells limited neutrophil recruitment into the kidney site and ameliorated the renal injury (Turner et al. 2012). Altogether, these findings emphasize on the indirect role of γδT cells in contributing to kidney pathogenesis.

2. Rational and Objectives

As their name implies, NK cells rapidly seek out and destroy, without prior sensitization, host cells that have become infected by a variety of microbes (viruses, bacteria, fungi) or that have become transformed and cancerous. NK cells are able to distinguish between normal healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors that control their activation and aid them in the discrimination of self from non-self antigens (Raulet et al. 2001, Yokoyama et al. 2003). The presence of inhibitory receptors on NK cells defines their role in the "missing–self" hypothesis (Karre et al. 1986). NK cells recognize targets that have down-regulated or lost surface expression of classical and non-classical MHC class I molecules recognized by the inhibitory NK cell receptors (Karre et al. 1986; Vance et al. 1998). The "at-least-one" hypothesis states that in order to prevent autoimmunity, as NK cell develop they must express at least one inhibitory receptor for self-antigens in order to mature into effector cells; this prevents autoimmunity (Raulet et al. 2001). In addition, different NK cell subsets express different combinations of inhibitory receptors so that there is always at least one subset that can be activated upon detecting a target cell that has lost a given single self-antigen. NK cells integrate the stimulatory and inhibitory signals coming from the potential target cell to ultimately make the decision to ignore or kill the target cell. Two families of NK cell receptors have been shown to be highly important in the regulation of the conventional NK cells; these include the Ly49 family of lectin-like transmembrane proteins, which recognize class I MHC molecules and the related Nkrp1 family of receptors. The *Nkrp1* and *Clr* genes encode a receptor:ligand pair used by natural killer (NK) cells as a "missing-self" immunosurveillance system. *Nkrp1* and *Clr* genes are conserved across multiple mouse strains as well as different species, suggesting that this inhibitory receptor:ligand relationship has an important role in

immune regulation. Recent work has shown that NK cells lacking the NKR-P1B receptor fail to recognize Clr-b-deficient target cells and therefore have an impaired Clr-b-dependent missing-self recognition (Rahim et al. 2015). However, due to the lack of the inhibitory signals, NKR-P1B-deficient NK cells showed increased protection against B-cell lymphomas (Rahim et al. 2015). In humans, the NKR-P1B receptor homolog is known as NKR-P1A (CD161), which shows inhibitory function upon binding its ligand LLT1 (Aldemir et al. 2005; Rosen et al. 2005).

The mammalian gastrointestinal (GI) tract is the site of food digestion and nutrient uptake, but is also home to vast population of commensal and pathogenic microorganisms, collectively referred to as the gut microbiota (Kamada et al. 2013). The presence of both commensal and pathogenic bacteria in the gut lumen creates a daunting immunological task for the host mucosal immune system: be responsive when faced with harmful invasive pathogens yet also be tolerant when subjected to benign foreign antigens (Mason et al. 2008). Tilting this equilibrium in either direction can lead to harmful processes for the host. Mucosal lesions associated with inflammatory bowel diseases, such as ulcerative colitis (UC) and Crohn's disease (CD), were shown to be generated by a hyperactive immune response to commensal bacteria (Manichanh et al. 2012; Leach et al. 1996; Pene et al. 2008), which demonstrate the need for sufficient immunological tolerance. However, satisfactory immune responses are still paramount to host survival, as reduced or impaired immune responsiveness leaves the gut susceptible to invading pathogens (Yuan et al. 2004; Artis et al. 2008). Consequently, the mammalian immune system has evolved an intricate immune network specialized to handle such a delicate state of equilibrium. Particularly, the innate immune system performs general "self-nonself" discrimination and detects "danger" signals, then mounts messenger cascades and effector mechanisms that act as sentinels to alert and prime the adaptive immune system (Kumar et al.

2011; Schenten et al. 2011) in order to eliminate the pathogen and re-establish a healthy state (Kumar et al. 2011). In the context of a GI tract, multiple cellular components of the innate immune system are potentially involved in mediating tissue homeostasis. These include, the lymphoid and myeloid cells, such as the natural killer (NK) cells, innate lymphoid cells (ILC), γδT cells, macrophages and dendritic cells (DC) (Eberl et al., 2004, Inagaki et al. 2004, Yang et al. 2004, Spits et al. 2011, Hwang et al. 2013, Serafini et al. 2014, Allan et al. 2015). Since, NKR-P1 and the Ly49 family of receptors are highly important in the regulation of conventional spleen & liver NK cell functions. In addition, both ILCs and γδT cells have been shown to express NK cell receptors and are heavily implicated in intestinal immune functions. I hypothesized that the NKR-P1B-Clrb interaction mediates gut tissue-specific immune-regulatory functions, in contrast to the global Ly49:MHC-I immunosurveillance mechanism. To understand the potential role of NK cell receptors in modulating intestinal immune homeostasis, I first aimed to establish the NKR-P1B:Clr-b expression pattern on various intestinal immune cells. Next, I wanted to determine what missing-self system dominates NK as well as other innate lymphoid cells in various intestinal compartments. Finally, I studied the impact of NKR-P1B-deficiency on gut-resident immune cells function at steady state and during bacterial infections.

The kidney's primary function is the production and the control of the urine composition in order to appropriately match the body's internal environment and requirements. This process is done by selective removal of toxic metabolic waste and excess fluids from the blood plasma and subsequent controlled reabsorption of electrolytes and minerals into the bloodstream. The human kidneys receive 25% of the cardiac output (MRCPath Lowe 2005, Kurts et al. 2013). Consequently, most common kidney diseases result from the blood vascular component abnormality. Renal epithelial cells (ECs) constitute the major cellular component of kidney

tubules and play an important role in recovering essential metabolites from the glomerular filtrates. In turn, tubular ECs mediate various physiological functions, including ion exchange, sugar absorption and water retention, which makes them among the most metabolic active and the most sensitive cells in response to lack of adequate oxygenation. Other than their key role in controlling the reabsorption and excretion of various molecules, tubular epithelial cells have also been shown to mediate functional cross-talk with adjacent capillary endothelial cells (Tasnim et al. 2012) and tubulointerstitial fibroblasts (Tan et al. 2016), which demonstrate their attributable endocrine and immunological properties. Therefore, kidney tubular epithelial cells play a key role in mediating a wide spectrum of physiological and immune functions (MRCPath Lowe 2005, Kurts et al. 2013, Hato et al. 2015). In particular, renal tubular epithelial cells have been shown to be crucial in mediating innate immunity through the production of chemokines and cytokines, and through their ability to recognize and process pathogenic or auto-antigens using a sophisticated repertoire of pattern recognition receptors (PRRs) (Hato et al. 2015). As in the case of most body organs, epithelial cell numbers far exceed any other cell type, especially that of immune cells in the kidney, hence they can play a substantial role in mediating immune responses and maintaining homeostasis. Since renal tubular epithelial cells are found in specific confined structures inside the kidney, they can never accomplish higher levels of immune activities unless auxiliary immune cells supports them to do so (Hato et al. 2015). For this reason, further studies will be needed in order to elucidate the mechanisms of communication between renal tubular epithelial cells and nearby immune cells. Recent work done by our laboratory using *in situ RNA* hybridization, revealed that the *Clr-f* transcript expression is confined to kidney tubular and intestinal epithelial cells (Zhang et al. 2012). Clr-f is a member of C-type lectin-related (Clr) proteins linked to the inhibitory NKR-P1G receptor in the mouse NK

gene complex (NKC) (Chen et al. 2011, Zhang et al. 2012, Kirkham et al. 2014). Being a ligand for the inhibitory NKR-P1G receptor, Clr-f may play a role in the control of multiple immune and/or non-immune functions in the kidney. Indeed, in his attempt to elucidate the role of Clr-f in mediating intestinal homeostasis, Leibelt et al demonstrated that intestinal epithelial cells upregulate their surface protein expression for Clr-f upon poly(I:C) challenge. He also identified the expression of the NKR-P1G receptor on a subpopulation of intraepithelial lymphocytes that exhibit a $CD103^{+}\gamma\delta T^{bright} NKG2A^{-}$ phenotype (Leibelt et al. 2014). These findings suggest that Clr-f acts as a stress ligand that inhibits the function of $NKR\text{-}P1G^{+}$ $\gamma\delta T$ cells towards the epithelial barrier in order to prevent autoimmunity. In order to examine the role of Clr-f in the context of modulating kidney functions, I first generated a Clr-f knockout mouse. Then I assessed the kidney pathology in Clr-f deficient mice in comparison to their controls. Finally, I used clinical assays to measure the kidney function in both knockout and wild-type mice.

3. Materials and Methods

3.1 Mice

C57BL/6 (B6) mice used in this study were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). NKR-P1B-deficient (*Nkrp1b*$^{-/-}$) mice on a B6 background were generated by our laboratory as previously described (Rahim et al. 2015). Clr-b-deficient mice on a B6 background were a gift to our laboratory and were generated as previously described (Kartsogiannis et al. 2008). All mice used in this study were between 7-10 weeks of age. Both female and male mice were used. All mice were bred and maintained at the Animal Care and Veterinary Services (ACVS) at Roger Guindon Hall (University of Ottawa, Ontario). All manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa animal ethics committee. Only mice littermates were used for functional studies.

3.2 Generation of Clr-f-deficient mice

The targeting construct containing a Clr-f genomic sequence with a floxed phosphoglycerate kinase (PGK)–neomycin cassette replacing exons 3, 4 and part of exon 5 of Clr-f was created by two of our previous lab members. The complete targeting construct was confirmed by sequencing at the Ottawa Hospitals Research Institute (OHRI) before electroporation into C57BL/6 Bruce-4 embryonic stem (ES) cells, followed by selection in G418. Meanwhile, the 5' and 3' Clr-f probes were tested in a restriction fragment length polymorphism (RFLP) analysis using thymic genomic DNA from a WT B6 mouse, which was digested with BamHI and PstI, respectively. Digested DNA fragments were resolved on 1% agarose gel and transferred to nylon membrane blots. *Clr-f* cDNA probes were labeled with radioactive ^{32}P-dCTP (Perkin Elmer) using the NEBlot kit (New England Biolabs, USA). Blots were probed with ^{32}P-labelled *Clr-f* cDNA probes in hybridization solution (10% (w/v) dextran sulfate; 1 M NaCl; 1% SDS).

Hybridization was carried out overnight at 65°C and post-hybridization washes were performed with a solution containing 2X SSC and 1% SDS pre-warmed to 65°C. The blots were exposed to photographic films to reveal the hybridization signals. The membranes were stripped with 0.2. SSC/ 0.2% SDS for 30 min at 85°C between different hybridizations. Neomycin-resistant clones were screened by Southern blot analysis, and a targeting efficiency of ~15% was observed. Selected ES clones were microinjected into blastocysts at the Clinical Research Institute of Montreal microinjection service (Montreal, Quebec) to generate the chimeric Clr-f^{neo} founder mice. The mice were screened by Southern blot analysis and were then bred with B6 females to produce Clr-$f^{wt/neo}$ heterozygous mice. Heterozygous mice were interbred to obtain Clr-$f^{neo/neo}$ mice. To remove the neomycin cassette, Clr-$f^{neo/neo}$ mice were bred with CMV-cre transgenic mice on a B6 background (The Jackson Laboratory). The resulting Clr-$f^{wt/lox}$ mice were interbred to produce Clr-$f^{lox/lox}$ mice. Mice were genotyped using specific primers (Table 1). PCR amplification was performed using the Accustart II Taq DNA polymerase (Quanta Biosciences). PCR mixture was prepared following the manufacturer's instructions. Phenol/Chloroform extracted ear DNA was used as templates. The following PCR cycling parameters were used: 30 amplification cycles of 94^0C for 40s, 60^0C for 45s, 72^0C for 60s. Wild-type (WT) and Clr-f-deficient littermates were used in all experiments unless otherwise indicated. All experiments carried on mice were approved by the institutional animal care and use committee (IACUC) of the University of Ottawa.

3.3 Tissue sampling

Kidney, liver, spleen, jejunum, colon and cecum were harvested from 12-weeks-old male mice. The kidney, liver and spleen were weighed and normalized to their corresponding mouse body weight. Kidneys were then cut transversally into two parts and fixed in 4% formaldehyde for

histology preparation. On the other hand, intestinal segments were measured immediately using a standard 30 cm ruler. Tibia measurements were also quantified using a digital caliber for normalization purposes.

3.4 Cell isolation from tissues

From the intra-epithelium

Following tissue collection, the fat was removed from each of the jejunum, colon and cecum. The intestinal samples were then washed several times with 1x cold PBS solution to remove fecal content. Using fine scissors the intestines were opened longitudinally and cut into 0.5cm pieces. The intestinal pieces were then incubated into two rounds of dissociation solution containing Ca/Mg-free Hank's Balanced Salt Solution (HBSS) solution (Lonza) supplemented with 10 mM HEPES (AMRESCO) and 5 mM EDTA (Invitrogen), and placed at 37^0C on a 100 RPM shaker for 20 min, followed by vigorous shaking for 30 seconds. The supernatant from each round of dissociation were collected and combined into a 50mL falcon tube on ice for intra-epithelial lymphocyte extraction. The cell suspension was then filtered through 70μm cell strainer (Fisher Scientific). The filtrate was centrifuged at 300 g for 10 min and the cell pellet was resuspended in DMEM (Lonza) supplemented with penicillin streptomycin (100 μg/mL) (VWR) and 10% FBS (Lonza). The latter cells were layered on a discontinuous 40-70% percoll gradient (GE Healthcare) and centrifuged at 600 g for 30 min at 4^0C with minimal acceleration and deceleration. Intra-epithelial lymphocytes were collected from the interphase of the two-percoll gradients. Cells were then pelleted, washed in PBS and used for flow cytometry analysis.

From the lamina propria

For the isolation of lymphocytes from the lamina propria, the remaining intestinal fragments from above, were removed and their connective tissue was dissociated using a digestion solution containing (Ca/Mg^{-}free HBSS, 0.75 mg/mL collagenase IV (Worthington) and 0.5 mg/mL DNAse I (Roche), and incubated at 37^{0}C and 100 RPM for 20 min. Following each of the two round of digestion, the supernatants of the intestinal fragments were collected and kept on ice for further lymphocyte enrichment, whereas tissue left overs were disposed. The cells in the supernatants were pelleted by centrifugation at 860 g at 4^{0}C for 10 min. To isolate all leukocytes, the cells were then re-suspended in 10 mL of 40% Percoll (GE Healthcare) and centrifuged at 900 g for 20 min at 20^{0}C. Leukocytes were then collected from the pellet. Alternatively, to enrich the cell sample for lymphocytes, the cells from the supernatant were re-suspended in 40% percoll and overlayed onto 80% Percoll. The samples were then centrifuged at 900 g for 20 min at 20^{0}C without applying break and lymphocytes were collected from the interface between the two Percoll gradients. In either case, the cells were then washed with 1x PBS and resuspended in 1x PBS to create a single cell suspension.

Mesenteric lymph nodes cell isolation

Mesenteric lymph nodes were removed from the intestinal mesentery and crushed through a 100µm cell strainer using a 5 mL syringe plunger and resuspended in10 mL 1x PBS. The filtrate was pelleted at 500 g at 20^{0}C for 5 min. Cells were then washed twice and resuspended in 1x PBS to create a single cell suspension.

Splenocytes isolation

Spleens were crushed using glass coverslips and splenocytes were resuspended in 1x PBS. Cells were pelleted by centrifugation at 500 g for 5 min at 20^{0}C. Pellets were then resuspended in

ACK lysis for 5 min to lyse red blood cells. Cells were then washed and resuspended in 1x PBS to create a single cell suspension.

Hepatic cell isolation

Livers were crushed through a 100μm cell strainer using a 10 mL syringe plunger and 30 mL of 1x PBS. Cells in the filtrate were then pelleted by centrifugation at 500 g at 20^0C for 5 min. Cell pellet was washed three times with 1x PBS. Cells were then resuspended in 37.5 % percoll and centrifuged at 680 g at 20^0C for 12 min. Leukocytes were collected from the pellet and were resuspended in ACK lysis for 5 min to lyse red blood cells. Cells were then washed and resuspended in 1x PBS to create a single cell suspension.

3.5 Flow cytometry staining

Following cell extraction from all of the above-mentioned organs, single cell suspensions were counted using trypan blue stain method (AMRESCO). To prepare leukocytes for flow cytometry analysis, $2x10^6$ cells were washed with FACS buffer (1x PBS, 0.5% BSA and 0.02% NaN_3) and centrifuged at 500 g for 5 min at 4ºC. Red blood cells were lysed using ACK buffer. Cells were then blocked with Fc block (anti-CD16/CD32, 2.4G2) (eBioscience) at 4ºC for 20 min. Following the blocking step, primary antibodies were appropriately diluted in FACS buffer and added to the cell suspension in a final volume of 50μl. The primary antibodies included fluorochrome-conjugated anti-TCRβ, anti-Ly49D (4E5), anti-MHC-II (BD Biosciences), anti-CD19, anti-CD11c, anti-CD11b, anti-Ly49 G (4D11), anti-Ly49 C/I/F/H (1411B) antibodies (eBioscience), anti-CD45 (eBioscience, clone 30-F11), anti-B220 (eBioscience, clone RA3-6B2), anti-TCRβ (eBioscience, clone H57-597), anti-CD8a (eBioscience, clone 53-6.7), anti-CD4 (eBioscience, clone GK1.5), anti-NKp46 (eBioscience, clone 29A1.4), anti-NK1.1 (eBioscience, clone PK136), anti-TCRγδ (eBiosience, clone UC7-13D5) and Fixable Viability

Dye (eBioscience, ref: 65-0865-14), biotin labeled anti-Clr-b (4A6) and anti-NKR-P1B (2D12) antibodies were gifts to our laboratory and have been previously described (Carlyle et al. 2004; Iizuka et al. 2003). Cells were then washed with FACS buffer and incubated with fluorochrome-conjugated streptavidin (eBioscience) for another 20 min at 4ºC. To stain for intracellular transcription factors, cells were washed in FACS buffer, re-suspended in IC fixation buffer (eBioscience) and incubated in dark at room temperature for 20 min. Afterwards, cells were washed twice using 1x permeabilization buffer (eBioscience). Intracellular antibodies, including anti-GATA-3 (BD Biosciences) and anti-RORγt (eBioscience), were appropriately diluted in permeabilization buffer and added to the cell mixture in dark for 20 min at room temperature. Finally, cells were washed twice and re-suspended in FACs buffer for flow cytometry analysis. Flow cytometry acquisition was performed on a CyAN-ADP using Summit Version 4.2 software (Beckman Coulter, Mississauga, ON, Canada) and/or using the Fortessa flow cytometer (BD biosciences) using DIVA acquisition software. Data were analyzed using Kaluza flow analysis software V (Beckman Coulter).

3.6 Bacterial preparation

Single bacterial colonies of *DBS100 Citrobacter rodentium* and *WT Salmonela typhimurium* were picked up from selective agar plates and cultured overnight in 3 mL sterile brain heart infusion (BHI) medium (BD Biosciences) and LB broth (BD Biosciences), respectively. Next, the 3 mL overnight cultures were transferred into a 250 mL flask containing 77mL of LB or BHI media, respectively. The flasks were placed on a shaking incubator (200rpm) at 37^0C and bacteria were grown until an optical density (OD) of 0.9 (λ=600nm) was reached. Afterwards, the culturing flasks were placed directly on ice. 1mL aliquots of each bacterium were prepared and kept at -80^0C for later infection studies. For determination of bacterial CFU, 10-fold serial

dilutions of the -80^{0}C bacterial aliquots were streaked onto BHI or LB-agar plates, followed by 2-day incubation at 37^{0}C under aerobic conditions. For long-term storage, bacteria were resuspended in 20% glycerol at a final volume of 100mL and stored at -80°C.

3.7 Oral gavage, bacterial count and tissue collection

In order to provoke a pathogenic mechanism of intestinal salmonellosis, mice were first treated with streptomycin for 24 hours prior to *S.typhimurium* infection (Barthel et al. 2003). Primarily, food and water were removed three hours before antibiotic administration. Next, 20mg of streptomycin (Sigma-Aldrich, S9137) in 200µL of 1x PBS was orally injected using a disposable animal feeding needle (Cadence science Ref 9931). The food and water were returned two hours post-treatment. On the following day, the bacteria was taken out from the -80^{0}C fridge, and rinsed once with sterile cold PBS. Mice were inoculated by gavage with 10^8 CFU of bacteria diluted in 200µL of 0.9% saline (NaCl) solution. Control groups were only inoculated with saline solution. Similar infection method was used for *C.rodentium*, without the antibiotic treatment. For bacterial cell count, spleens and feces were collected 5 days post *S.typhimurium* infection. Spleens were crushed using 100µm filters and splenocytes were re-suspended in 10mL of 1x cold PBS. Fecal pellets were placed in 500 µl of 4°C cold PBS and suspended homogeneously on ice by vortexing and pipetting. Homogenates were serially diluted and plated on streptomycin-supplemented LB agar plates and incubated at 37^{0}C overnight. Colonies were enumerated and normalized to the weight of the tissues. For histology, infected and non-infected colons were removed and washed several times with 1x cold PBS then fixed in 4% paraformaldehyde for 48 hours at 4^{0}C. Next, colons were transferred to 70% ethanol, embedded in paraffin and cut into 4µm sections.

3.8 Histology and pathology scoring

Colon sections (4μm) were stained with hematoxylin and eosin (H&E) (by the histology core facility at Roger Guindon Hall, University of Ottawa, Ontario) and were examined blindly by a gastrointestinal pathologist at the Ottawa hospital research institute (OHRI) to assess histological changes. Gut sections were then assessed for lamina propria and intra-epithelial lymphocytes infiltrates per 100 enterocytes at 40x magnification. Besides, kidney sections (3μm) were PAS stained (by the histology core facility at Roger Guindon Hall, University of Ottawa, Ontario) and were examined blindly by the pathologist at the Kidney Research Center (Ottawa Hospital). Kidney tissue sections were assessed for glomerular damage, tubular epithelial cell disruption and tubulointerstitial lymphocyte infiltrates. A semi-quantitative score was used to evaluate the degree of glomerular damage. A minimum of 50 glomeruli in each group was examined and the severity of the lesion was graded from 0 to +3: a +1 lesion represented an involvement of 30% of the glomerulus, +2 of 30–60%, while a +3 lesion indicated > 60% of the glomerulus was involved.

3.9 *In-vitro* culturing and intracellular cytokine staining

Following the trypan blue cell death exclusion method, colon lamina propria leukocytes were cultured in 96-well plates at a density of 10^6 cells/well in a total volume of 200μL of RPMI 1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamycin, and 10% heat-inactivated FBS and in the presence of brefeldin A (5μg/mL; eBioscience) for 5 hours at 37^0C in a humidified CO_2 incubator. For stimulating IFNγ release from NK and γδT cells, IL-12/IL-18 (25ng/mL) was added into the cell culture media. IL-1/IL-23 and IL-2/IL-1/IL-23 (10ng/mL) were added separately to the cell culture for stimulating IL-22 cytokine release from ILCs. Cultured cells were washed with a FACS buffer and incubated for 20 min at 4^0C in the dark with

anti-CD16 monoclonal antibody to block nonspecific antibody binding. Subsequently, cells were stained with anti-CD3 (BD Biosciences), anti-TCRγδ, anti-NKp46, anti-RORγt (eBioscience) for 30 min at 4^0C in the dark, as described above, to gate on lamina propria lymphocyte subpopulations. Intracellular IFNγ and IL-22 staining were performed as described above. Data were acquired using BDLSRFortessa (BD Biosciences) and analyzed using Kaluza Version 1.2 software (Beckman Coulter).

3.10 *In-situ* hybridization

In-situ hybridization analysis of *Clr-b* gene in the small intestinal tissue was performed as described previously (Gregorieff et al. 2010). Plasmids containing both of the antisense and sense *Clr-b* RNA probes were previously generated in our lab. Digoxigenin-labeled antisense and sense RNA probes were transcribed from linearized plasmids containing the *Clr-b* gene with T7 polymerases using DIG RNA labeling mix (Roche). RNA probes were purified with commercially available RNA purification columns (RNeasy Mini Kit, Qiagen). Purified probes were tested for integrity on a standard 1% agarose/ethidium bromide gel, quantitated using a Nanodrop DNA/RNA quantification system (Thermo Scientific) and aliquots were stored at -80°C. The intestinal tissue was collected from 8 weeks old B6 mice and fixed in 4% paraformaldehyde at 4°C with gentle shaking for up to 24 hours. Small intestinal sections were then sent to the Histology Core Facility (Roger Guindon Hall, University of Ottawa, Ontario) for paraffin embedding and 4µm sections were prepared. Intestinal tissue sections on glass slides were first deparaffinated, treated with 0.2 N HCl for 15 min and 30 µg/ml proteinase K at 37°C for 20 min, post-fixed in 4% paraformaldehyde for 10 min and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine solution twice for 5 min each. Slides were then pre-hybridized with hybridization solution (50% (v/v) formamide/5xSSC (pH 4.5); 2% (w/v)

blocking powder (Roche); 0.05% (w/v) CHAPS; 5 mM EDTA; 50 μg/ml heparin; 1 μg/ml yeast RNA) in a 58°C oven for 1.5 hours, and then incubated with hybridization solution containing 500ng/ml digoxigenin-labeled probe overnight at 58°C. Post-hybridization washes were performed with 2xSSC (pH 7.5) followed by washing with 50% formamide/2xSSC (pH 4.5) at 55°C three times for 20 min each. Slides were stained with sheep anti-digoxigenin alkaline phosphatase-conjugated antibody at 1/1000 dilution in blocking solution (Roche) at 4°C overnight. After several washes in Tris/NaCl buffer, slides were equilibrated in NTM buffer (0.1 M Tris-Cl pH 9.5, 0.1 M NaCl, 0.05 M MgCl2). Color development was performed by adding nitro blue tetrazolium /5-bromo, 4-chloro, 3-indoylphosphate (NBT/BCIP, Roche) substrates to the slides and slides were kept in the dark at room temperature until optimal staining was obtained. Slides were then counterstained with 0.025% methylgreen and visualized using a light microscope.

3.11 RT-PCR

Different tissues of interest (jejunum, colon, kidney, liver, heart) were excised, rinsed with PBS, frozen with liquid nitrogen and transferred to -80°C for storage before proceeding to RNA isolation. RNA was isolated from frozen tissues using RiboZol RNA extraction reagent (AMRESCO) following the manufacturer's instructions. Approximately 1 μg of RNA was transcribed into cDNA using verso cDNA kit (Thermo Scientific). PCR amplification was performed on 1 μl of cDNA product using specific primers (Table 1). PCR conditions used were: 94°C 30s, 58°C 30s, 72°C 60s, and 35 cycles. PCR products were visualized on 1% agarose gel stained with ethidium bromide.

3.12 Urine and plasma chemical waste and electrolyte measurement

Approximately 150 µL of urine was collected daily from 12-weeks-old male mice at the same time of the day for 5 consecutive days. The urine was centrifuged at 10000 rpm to get rid of debris and then stored at -80 °C. We also collected blood from the facial vein of the same mice in heparinized tubes and plasma was isolated by centrifugation at 3,000*g* for 5 min and stored at -80 °C. Urine and plasma samples were sent to Idexx Laboratories (Markham, Ontario) to measure the protein, creatinine and electrolytes concentrations.

3.13 Blood pressure measurements on 12 and 24-week-old mice

Systolic and diastolic blood pressures (BPs) were measured by tail-cuff plethysmography on 12 and 24 weeks old male Clr-f ^{KO} and WT mice at steady state using a BP2000 Visitech model. BP was measured daily in the same room and at the same hour of the day for 5 consecutive days. BP values of the last 3 consecutive measurements were used to calculate the mean BP value since the first 2 days were considered essential for mice adaptation.

Table I. List or primers

Primers name	**Forward sequence**	**Reverse sequence**
LoxP3	TCCTGGAAGGCTCTGGATTG	GACCTCGGAATGCCACTTGTC
WTFor3	CAGTGAGGTCAGATGTGGCAG	GACCTCGGAATGCCACTTGTC
ForNeo3	GACAATAGCAGGCATGCTG	GACCTCGGAATGCCACTTGTC
Clr-f (I)	TTGAAACGAGTTCCATGGGC	GGTCATAGAGCATCTGATTG
Clr-f (II)	ATGAATGCTGCAAAGGTTG	TCACACATGCCTTGGTAC
Clr-b	ACTCAGCTCCTCAGCTCTGA	GGCTAAAAAGCGTCTCTTGG
Clr-a	CAAAGGTTGAAGAGGCTTCC	TCACGCATGCTTTGGCACAT
GAPDH	ACTCACGGCAAATTCAACGGC	ATCACAAACATGGGGGCATCG

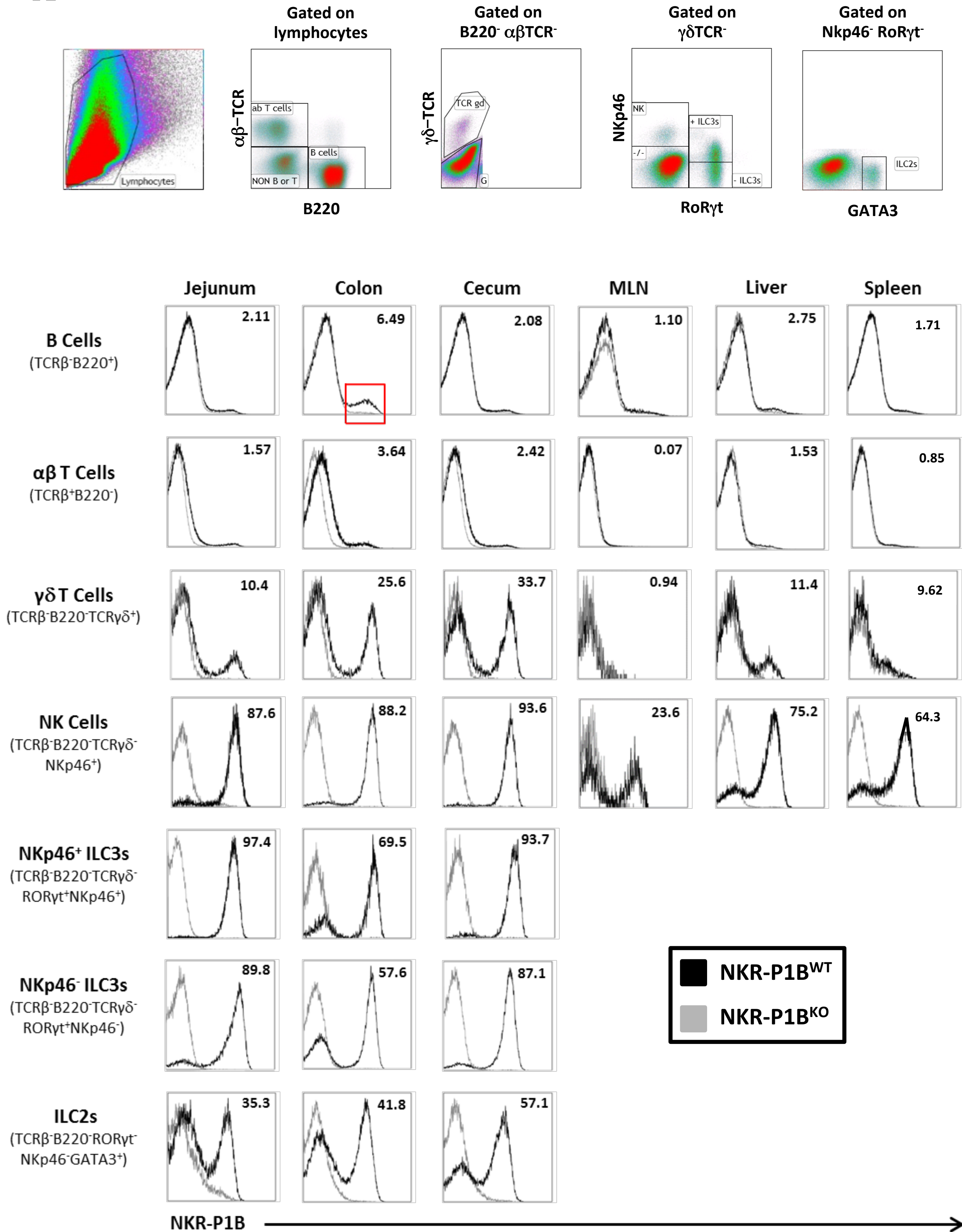
4. Results

4.1 NKR-P1B expression on the cells of the lymphoid and myeloid lineage in multiple gut compartments and secondary lymphoid organs

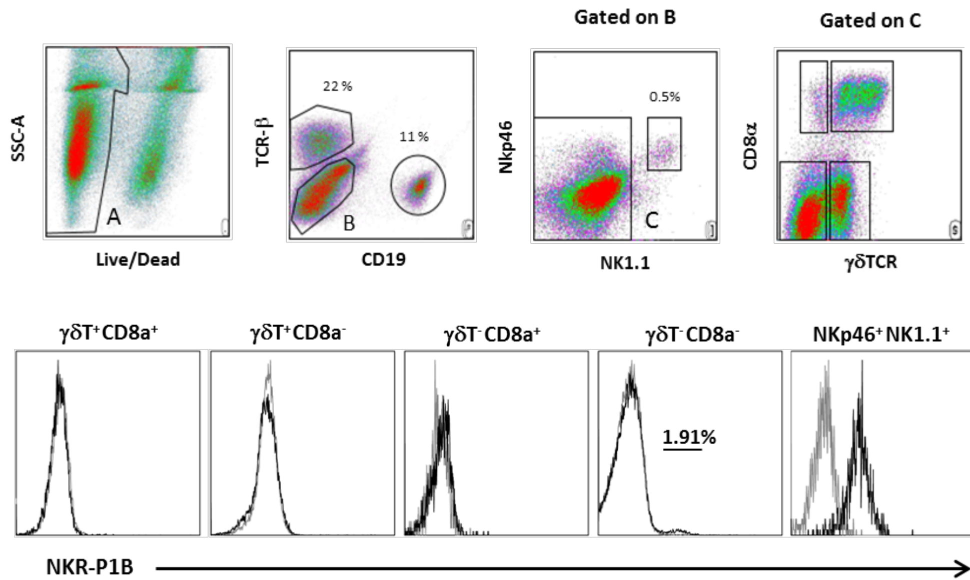
Since no previous study has ever screened for the protein expression of the NKR-P1B receptor in the gut, we first determined the expression pattern of NKR-P1B within the different intestinal compartments. Total lymphoid and myeloid cells were isolated from the lamina propria of the jejunum, colon, cecum and small intestinal intra-epithelium in addition to mesenteric lymph nodes (MLN), liver and spleen. Next, isolated cells were stained for NKR-P1B and various cell surface markers, and acquired on a flow cytometer to survey for the receptor expression on immune cells using the gating strategy shown in figure 1. We found that NKR-P1B is expressed on the cell surface of all lamina propria γδT cells (TCRβ^- B220$^-$ TCR$\gamma\delta^+$) NK cells (TCRβ^- NKp46$^+$RORγt$^-$), NKp46$^+$ ILC3s (TCRβ^-NKp46$^+$RORγt$^+$), NKp46$^-$ ILC3s (TCRβ^-NKp46$^-$ RORγt$^+$) and ILC2 (TCRβ^-NKp46$^-$RORγt$^-$GATA3$^+$) but not on TCRαβ T-cells (TCRβ^+) or B-cells (B220$^+$) (Fig.1A). Only a small percentage of NKR-P1B expressing cells were detected among the B220$^+$ cells in the colon (red square, Fig.1A). Circulating and tissue resident plasmacytoid dendritic cells (pDCs) are known to express the B220 marker (Omatsu et al. 2005). Thus, we isolated total leukocytes from the lamina propria of the jejunum, colon and cecum and stained for NKR-P1B on local DCs and macrophage subsets. High expression of NKR-P1B was detected on the surface of colon DCs (CD11c$^+$CD11b$^-$ and CD11c$^+$CD11b$^+$ subsets) in contrast to their counterparts in other gut lamina propria compartments, as well as the liver and spleen DC subsets (Fig.1C). We also showed a small proportion of colon macrophages expressing the NKR-P1B receptor on their surface (Fig.1C). Although, lamina propria γδT cells positively stained for the NKR-P1B receptor in different gut lamina propria compartments (Fig.1A), intra-

epithelial γδT cell subsets did not express NKR-P1B receptor (Fig.1B). The NKR-P1B expression on NK cells was used as a positive control for receptor expression (Fig.1A, B and C). As a negative control, we performed the same NKR-P1B staining on the cell isolated from NKR-P1B^{KO} mice. Comparison of gut lamina propria NKR-P1B^{+} NK and ILC3 subsets with their liver and spleen counterparts (Fig.1A) revealed that a higher percentage of these cells expressed NKR-P1B in the gut. In addition, we observed a higher percentage of NKR-P1B^{+} lymphocytes in the small intestine (12.57±8.98%) in comparison to spleen (2.94±0.83%) (Fig. 1D and E).

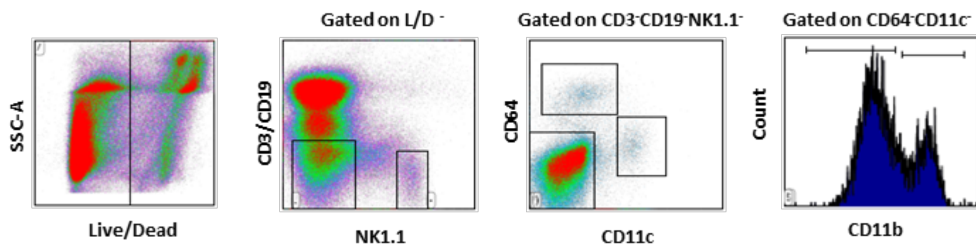
Figure 1: NKR-P1B expression on the cells of the lymphoid and myeloid lineage in multiple gut compartments and secondary lymphoid organs. (A) Flow cytometry analysis of NKR-P1B expression on all resident lymphocytes in the jejunum, colon, cecum and mesenteric lymph nodes and secondary lymphoid organs, including liver and spleen of WT B6 and NKR-P1B-deficient (KO) mice. Percentage of cells expressing NKR-P1B is indicated. The red square highlights the expression of the NKR-P1B receptor on a subset of colon $B220^+$ cells. **(B)** Flow cytometry analysis showing the expression of the NKR-P1B receptor on small intestinal intra-epithelial lymphocytes. **(C)** Flow cytometry analysis representing the NKR-P1B expression on specific dendritic cell and macrophage subsets in different gut compartments and secondary lymphoid organs of wild-type and $NKR\text{-}P1B^{KO}$ mice. NKR-P1B expression on NK cells served as a positive control; whereas NKR-P1B staining in $NKR\text{-}P1B^{KO}$ mice was used a negative control for each of the indicated cell subsets. Data is representative of multiple independent experiments (n=9). **(D)** Flow cytometry analysis showing the percentage of $NKR\text{-}P1B^+$ lymphocytes in the small intestine (SI) and spleen. One representative sample is shown from multiple separate experiments. **(E)** Bar graph showing the percentage of $NKR\text{-}P1B^+$ lymphocytes in the SI and the spleen (SI: n=6, spleen: n=5). Statistical analysis was performed using a two-tailed unpaired t-test; *, $P < 0.05$.

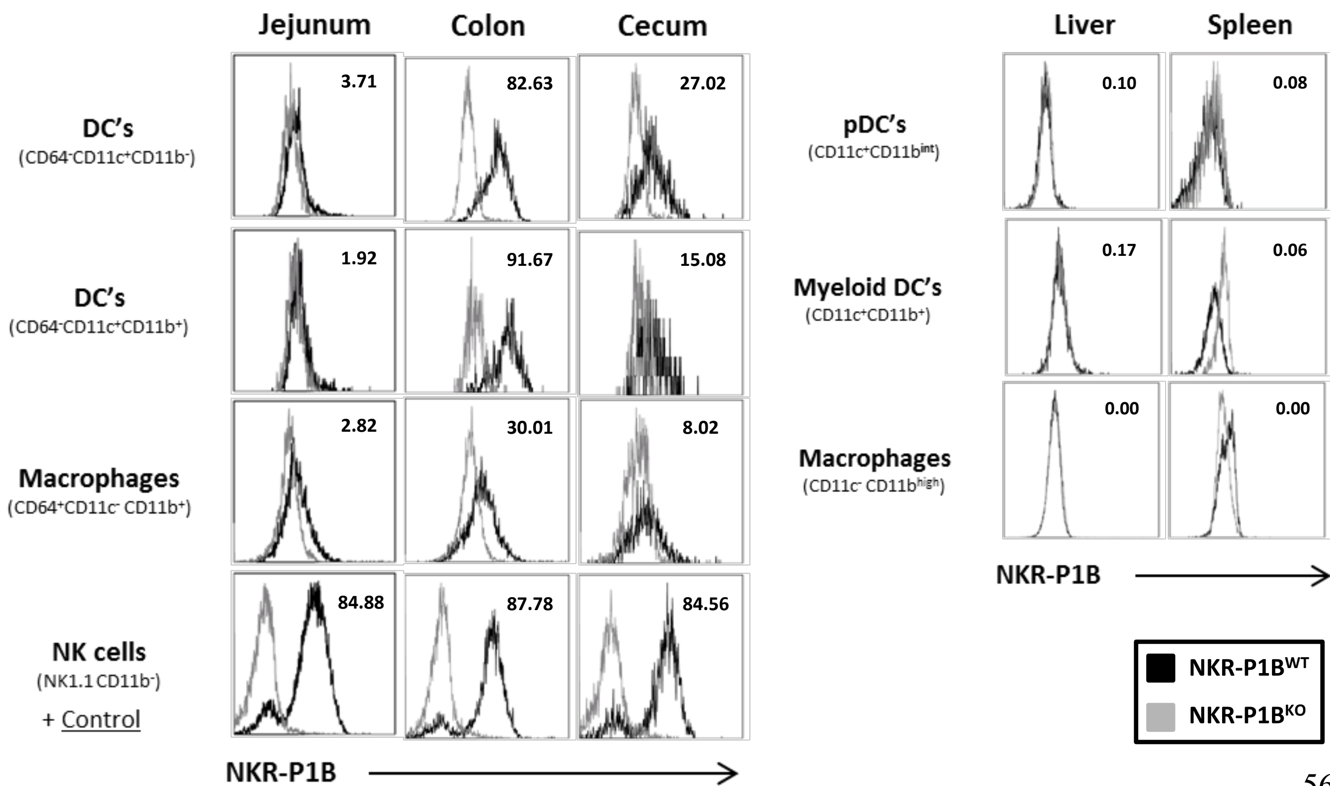

A
Gated on lymphocytes
Gated on B220⁻ αβTCR⁻
Gated on γδTCR⁻
Gated on Nkp46⁻ RoRγt⁻
Lymphocytes
αβ-TCR
ab T cells
B cells
NON B or T
B220
γδ-TCR
TCR gd
G
NKp46
NK
+ ILC3s
-/-
- ILC3s
RoRγt
ILC2s
GATA3
Jejunum
Colon
Cecum
MLN
Liver
Spleen
B Cells
(TCRβ⁻B220⁺)
2.11
6.49
2.08
1.10
2.75
1.71
αβ T Cells
(TCRβ⁺B220⁻)
1.57
3.64
2.42
0.07
1.53
0.85
γδ T Cells
(TCRβ⁻B220⁻TCRγδ⁺)
10.4
25.6
33.7
0.94
11.4
9.62
NK Cells
(TCRβ⁻B220⁻TCRγδ⁻ NKp46⁺)
87.6
88.2
93.6
23.6
75.2
64.3
NKp46⁺ ILC3s
(TCRβ⁻B220⁻TCRγδ⁻ RORγt⁺NKp46⁺)
97.4
69.5
93.7
NKp46⁻ ILC3s
(TCRβ⁻B220⁻TCRγδ⁻ RORγt⁺NKp46⁻)
89.8
57.6
87.1
ILC2s
(TCRβ⁻B220⁻RORγt⁻ NKp46⁻GATA3⁺)
35.3
41.8
57.1
NKR-P1B^WT
NKR-P1B^KO
NKR-P1B

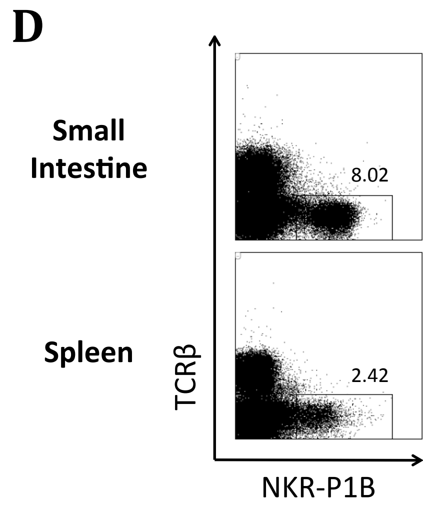
B

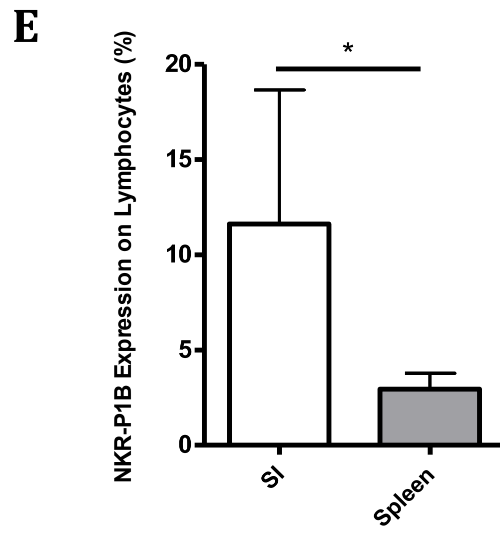


C






D
Small
Intestine
Spleen
TCRβ
NKR-P1B
8.02
2.42

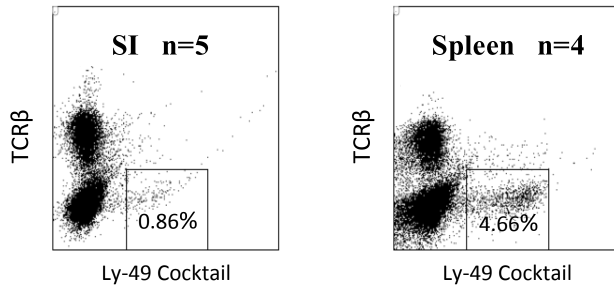

E
NKR-P1B Expression on Lymphocytes (%)
20
15
10
5
0
*
SI
Spleen

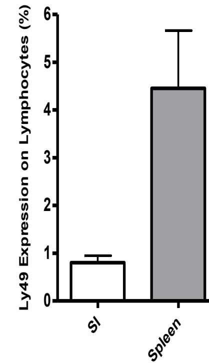
4.2 Unlike the NKR-P1B receptor, Ly49 receptors are not highly expressed on immune cell subsets in the gut

After showing that NKR-P1B is prevalent in the gut lamina propria and expressed on several innate immune cell subsets, including NK cells and ILCs, we wanted to check whether other lectin-like receptors are also expressed in the gut. Since the Ly49 family of receptors is well known to play an important role in directing NK cell function, we surveyed for the presence of Ly49 receptors on lamina propria intestinal lymphocytes. First, we looked at the overall expression of Ly49 receptor on total gut and spleen lymphocytes. We found that splenic lymphocytes (4.46±1.21%) have a higher frequency of Ly49 expression in comparison to the gut lamina propria (0.80±0.15%) (Fig.2A). Next, we checked Ly49 expression on intestinal NK cells and ILC subsets. Our data demonstrate that the percentage of gut-resident NK cells expressing the Ly49 receptor (19.92±2.20%) is significantly lower in comparison to their splenic counterparts (77.78±1.77%) (Fig. 2.2B, $P < 0.001$). On the other hand, ILC2 and ILC3s showed a complete absence of the Ly49 receptors on their surface (Fig.2B, $P < 0.001$).

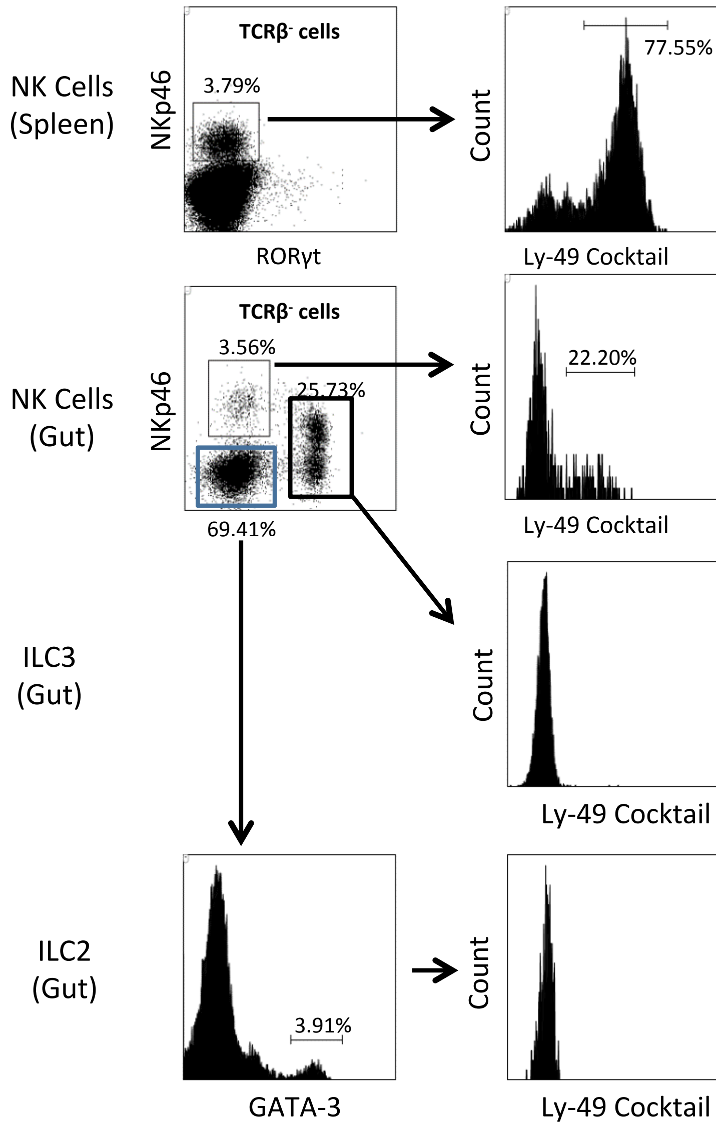
Figure 2: Ly49 expression on spleen and gut immune cells. **(A)** Flow cytometry analysis showing the percent of Ly49^{+} lymphocytes in the small intestinal lamina propria (gut) and the spleen. Data from one representative sample is shown (Gut n=5, Spleen: n=4). **(B)** Flow cytometry analysis showing Ly49 expression on NK cells, ILC3s and ILC2 from the small intestine and NK cells from the spleen of WT B6 mice. Stains for each cell type were repeated in multiple different experiments (Gut NK, ILC3s and ILC2: n=5, Spleen NK: n=4). The Ly49 cocktail is composed of antibodies against Ly49 C, I, F, H, D and G. Percentage of Ly49^{+} cells is shown. Statistical analysis was performed using a two-tailed unpaired t-test; ***, $P < 0.001$.

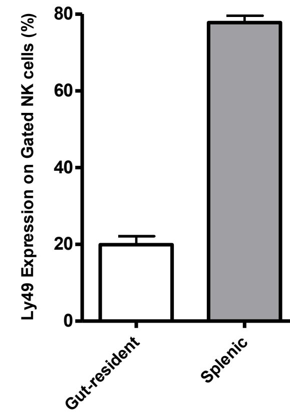
A





B





Ly49 Cocktail includes:

-1411B – (C,I, F, H)

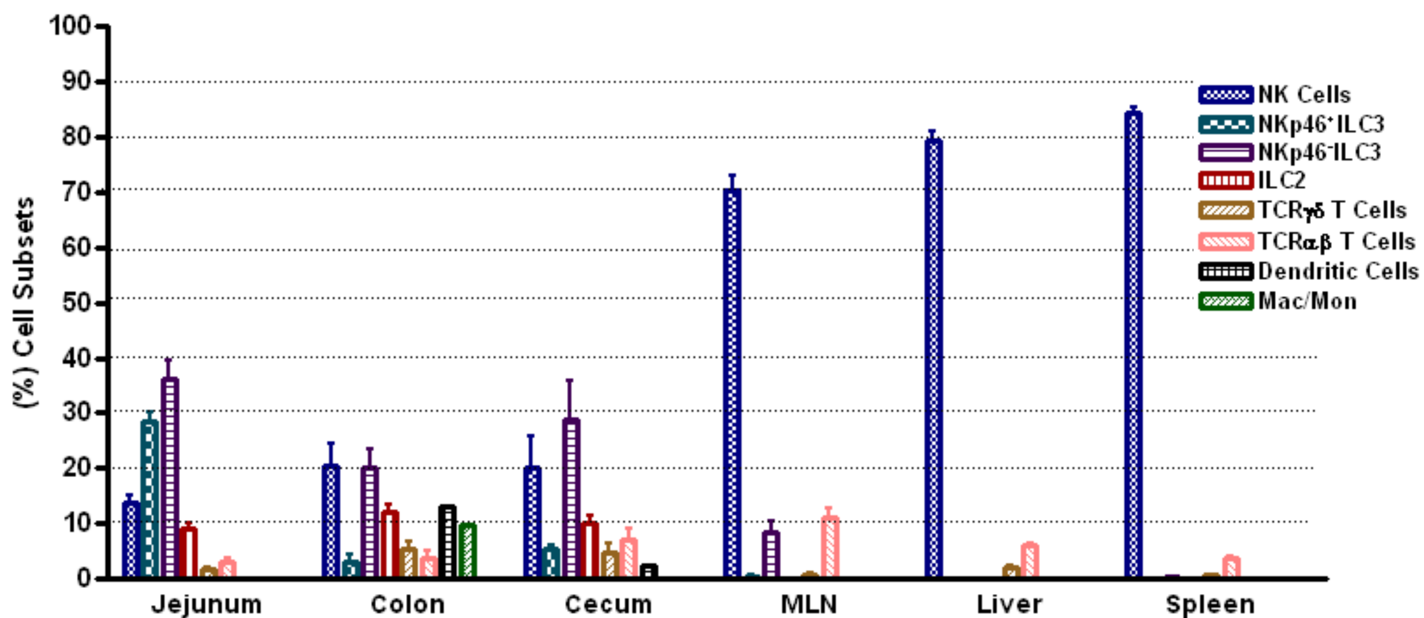
-4D11 – (G)

-4E5 – (D)

4.3 Tissue-dependent distribution of NKR-P1B-expression immune cells in B6 mice

As was shown in figure 1, we were able to identify various cell types expressing the NKR-P1B receptor including NK cells, ILC subsets and γδT cells in the gut. Moreover, expression of NKR-P1B in the gut is more dominant in comparison to Ly49 (Fig. 2) and to NKR-P1B expression in other organs, such as the spleen (Fig. 1D and 1E). In order to determine the tissue-specific composition of cells comprising the NKR-P1B$^+$ population in different mice tissues, we first gated on all NKR-P1B$^+$ leukocytes isolated from the jejunum lamina propria (LP), colon LP, cecum LP, mesenteric lymph nodes (MLN), liver and spleen of WT B6 mice. Next, we determined the cell type composition of NKR-P1B$^+$ population in these tissues using antibody staining and flow cytometry analysis (Fig.3). Interestingly, while comparing the composition of NKR-P1B$^+$ cells in the different organs, we found that NKp46$^-$ (42.73±2.75%) and NKp46$^+$ (24.79±4.91%) ILC3s make up the largest percentage of NKR-P1B$^+$ lymphocytes in the jejunum lamina propria (Fig.3). On the other hand, NK cells and Nkp46$^-$ILC3 make up the largest percentage of NKR-P1B$^+$ cells in both of the colon and cecum LP (Fig.3). In addition, the NKR-P1B$^+$ DCs (12.03±0.73%) and macrophages (9.41±0.67%) were unique to the colon LP (Fig.3). Moreover, ILC2 cell subset consisted 10-12 percent of total NKR-P1B$^+$ cells in different LP gut parts, whereas NK cells composed the majority of cells expressing the receptor in the mesenteric lymph nodes (69.88±3.51%), liver (78.66±2.42%) and spleen (83.29±1.59%).

Figure 3: Tissue-dependent distribution of NKR-P1B-expression immune cells in B6 mice. Flow cytometry analysis showing all identified NKR-P1B$^+$ lymphocyte subsets and their percentage composition of the NKR-P1B-expressing immune cell subsets in jejunum, colon, cecum, mesenteric lymph nodes, liver and spleen. Pooled data from at least three independent experiments (n=6) is shown.


100
90
80
70
60
50
40
30
20
10
0
(%) Cell Subsets
NK Cells
NKp46⁺ILC3
NKp46⁻ILC3
ILC2
TCRγδ T Cells
TCRαβ T Cells
Dendritic Cells
Mac/Mon
Jejunum
Colon
Cecum
MLN
Liver
Spleen

4.4 Increased frequency and numbers of several innate immune cell types in the gut of NKR-P1B-deficient mice

Our data revealed expression of NKR-P1B on multiple lymphoid cell types, including NK cells, ILC3s and γδT cells as well as myeloid cells, such as colon DCs and macrophages (Fig.1). In addition, ILC3 subsets and NK cells were found to comprise the largest percentages of NKR-P1B$^+$ lymphocytes in several segments of the gut lamina propria (Fig.3). Thus, it was important to determine whether the absence of the receptor could affect the percentages and numbers of these cell types in the gut LP among others. Single-cell suspensions of jejunum, colon and cecum LP, in addition to mesenteric lymph node, liver and spleen were stained with the designated surface markers and analyzed by flow cytometry using the gating strategy shown in Fig.1). Our data shows an increase in the percentages and numbers of γδT cells, LTi (Nkp46$^-$ ILC3) and ILC2 in the jejunum lamina propria of NKR-P1B-deficient mice in comparison to WT B6 mice (Fig.4A and B). We also detected a slight increase in the percentage but not the number of Nkp46$^+$ILC3 (ILC3) in the colon lamina propria of NKR-P1B^{KO} mice (Fig.4C and D). Similarly, cecum lamina propria ILC3 subsets appeared to increase their frequency but not their numbers in the knockout mice WT (Fig.4E and F). On the other hand, no statistical differences in the numbers of DC subsets or macrophages were detected in distinct parts of the gut lamina propria (Fig.4G, H and I). It is noteworthy to mention that most of the analyzed innate immune cell types in different parts of the gut LP displayed a pattern of increased cell frequency and numbers in the NKR-P1B-deficient mice in comparison to WT mice. On the contrary, mesenteric lymph node, liver and spleen innate immune cell types had similar percentages and numbers between the two mice genotypes (Fig.4J, K and L). Lastly, since T and B cells do not express the NKR-P1B receptor; we observed no change in their cell frequency or number as was expected (Fig.4A-L).

Figure 4: Increased frequency and numbers of several innate immune cell types in the gut of NKR-P1B-deficient mice. Flow cytometry analysis showing the average percentages and numbers of lymphoid cells **(A-F)** and myeloid cells **(G-I)** in distinct parts (jejunum n=8, colon n=6 and cecum n=7) of the gut lamina propria of WT and NKR-P1B^{KO} mice. (**J-L)** Flow cytometry analysis showing the average percentages and numbers of multiple lymphoid cells in the liver (n=6), mesenteric lymph nodes (n=8) and spleen (n=9) of WT and NKR-P1B^{KO} mice. Cells isolated from each of the indicated organs were counted prior to FACS staining. Cell numbers were determined by multiplying the percentage of each cell subset with their corresponding total cell count.

A

Jejunum

Cell Percentage
65 60 55 50 45 40 35 30 25 20 15 10 5 0
T Cells B Cells

Jejunum

Cell Percentage
6 5 4 3 2 1 0
* * *
γδ T NK ILC3 LTi ILC2

B

Jejunum

Cell Number
4.5×10^6 4.0×10^6 3.5×10^6 3.0×10^6 2.5×10^6 2.0×10^6 1.5×10^6 1.0×10^6 5.0×10^5 0
T Cells B Cells

Jejunum

Cell Number
4.5×10^5 4.0×10^5 3.5×10^5 3.0×10^5 2.5×10^5 2.0×10^5 1.5×10^5 1.0×10^5 5.0×10^4 0
* ** *
γδ T NK ILC3 LTi ILC2

C

Colon

Cell Percentage
50 40 30 20 10 0
T Cells B Cells

Colon

Cell Percentage
7 6 5 4 3 2 1 0
*
γδ T NK ILC3 LTi ILC2

D

Colon

Cell Number
1.1×10^6 1.0×10^6 9.0×10^5 8.0×10^5 7.0×10^5 6.0×10^5 5.0×10^5 4.0×10^5 3.0×10^5 2.0×10^5 1.0×10^5 0
T Cells B Cells

Colon

Cell Number
4.5×10^5 4.0×10^5 3.5×10^5 3.0×10^5 2.5×10^5 2.0×10^5 1.5×10^5 1.0×10^5 5.0×10^4 0
γδ T NK ILC3 LTi ILC2

E

Cecum

Cell Percentage
65 60 55 50 45 40 35 30 25 20 15 10 5 0
T Cells B Cells

Cecum

Cell Percentage
5 4 3 2 1 0
* *
γδ T NK ILC3 LTi ILC2

F

Cecum

Cell Number
4.0×10^6 3.0×10^6 2.0×10^6 1.0×10^6 0
T Cells B Cells

Cecum

Cell Number
2.3×10^5 2.0×10^5 1.8×10^5 1.5×10^5 1.3×10^5 1.0×10^5 7.5×10^4 5.0×10^4 2.5×10^4 0
γδ T NK ILC3 LTi ILC2

G

Jejunum

Cell Number
2.0×10^4 1.5×10^4 1.0×10^4 5.0×10^3 0
CD11b^- DC CD11b^+ DC CD64^+ Mac

H

Colon

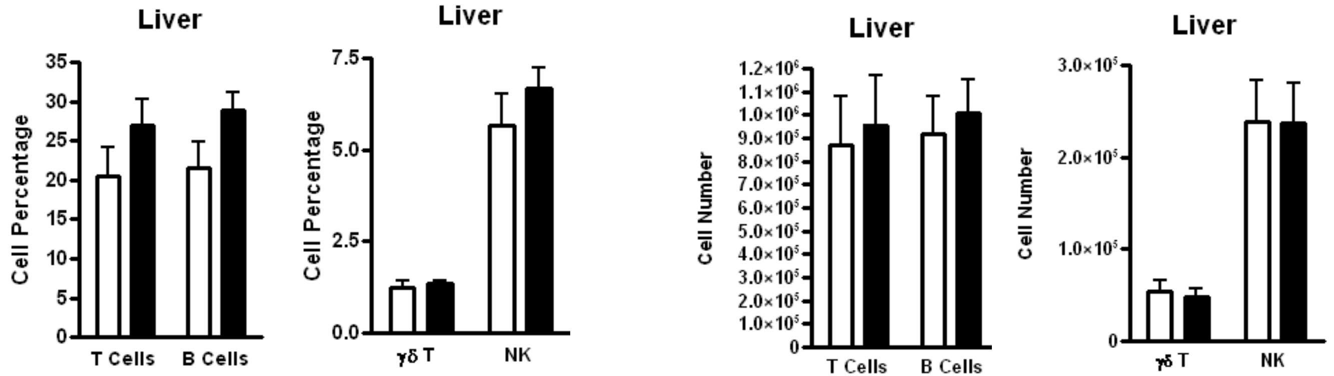
Cell Number
2.3×10^4 2.0×10^4 1.8×10^4 1.5×10^4 1.3×10^4 1.0×10^4 7.5×10^3 5.0×10^3 2.5×10^3 0
CD11b^- DC CD11b^+ DC CD64^+ Mac

I

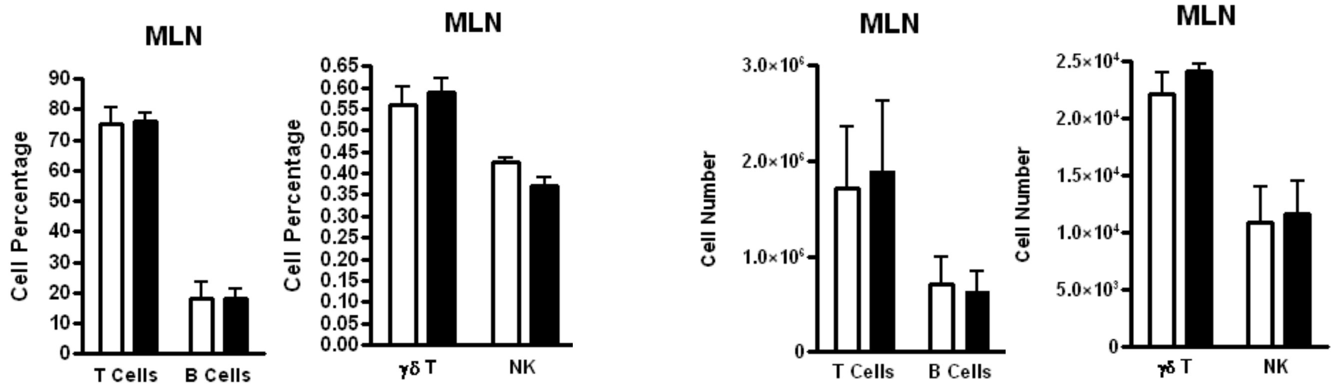
Cecum

Cell Number
3500 3000 2500 2000 1500 1000 500 0
CD11b^- DC CD11b^+ DC CD64^+ Mac

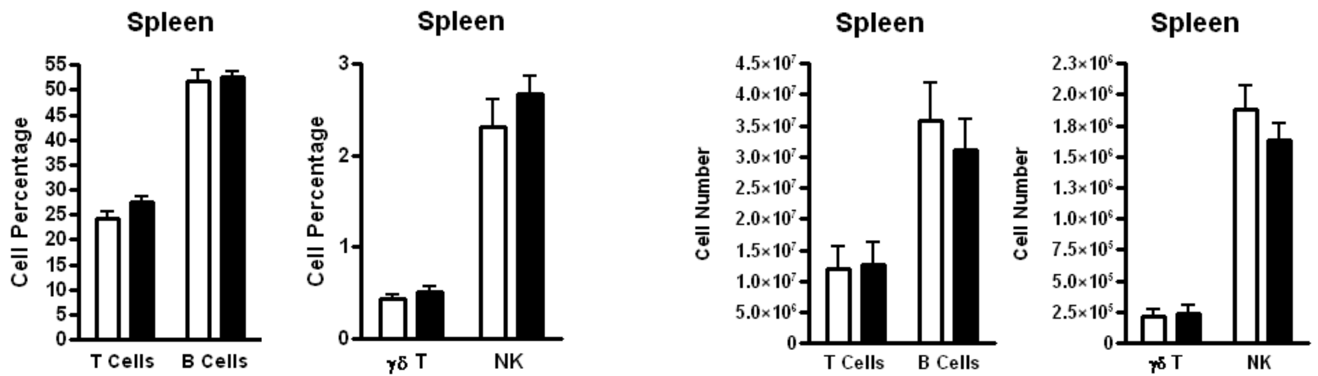
J



K



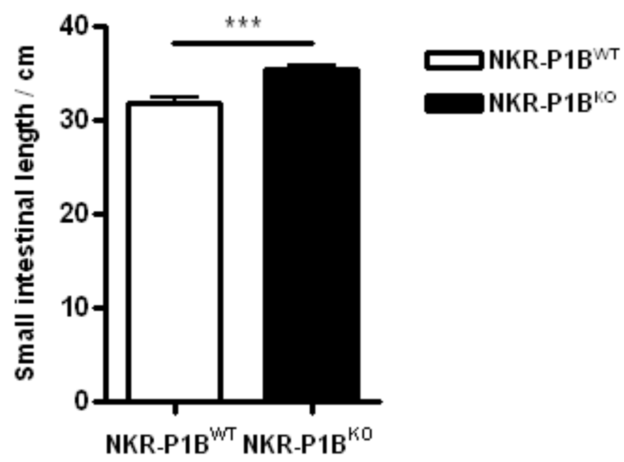
L

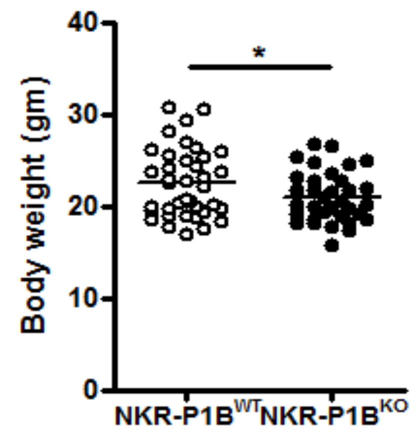


4.5 Increased small intestinal length but lower average body-weight in the NKR-P1B-deficient mice

To assess whether the lack of the NKR-P1B receptor can alter the development of the intestinal tract, we measured the small intestinal lengths and body weights of NKR-P1B-deficient mice compared to sex and age matched WT B6 control. The size of each mouse was taken into account by normalizing the length of the small intestine to the tibia length. While comparing the small intestinal length between the two genotypes, we found that the mean normalized length of the NKR-P1B^{KO} small intestine (36.57±0.92 cm) were measurably longer than those of their WT littermates (30.66±1.44 cm) (Fig.5A). Interestingly, the body weight measurements of the two mouse genotypes demonstrated that even though the NKR-P1B^{KO} mice had a longer small intestine, their average weight was significantly lower in comparison to the WT B6 mice (Fig.5B).

Figure 5: Increased small intestinal length but lower average body-weight in the NKR-P1B-deficient mice. (A) Intestinal lengths of the small intestine normalized with tibia length of NKR-P1B-deficient (NKR-P1B^{KO}) and wild-type (WT) B6 mice (WT: n=12, NKR-P1B^{KO}: n=14). **(B)** Body weights of NKR-P1B^{KO} and WT mice. Each data point represents the body weight of one mouse. Statistical analysis was performed using a two-tailed unpaired t-test; *, $P < 0.05$, ***, $P < 0.001$.

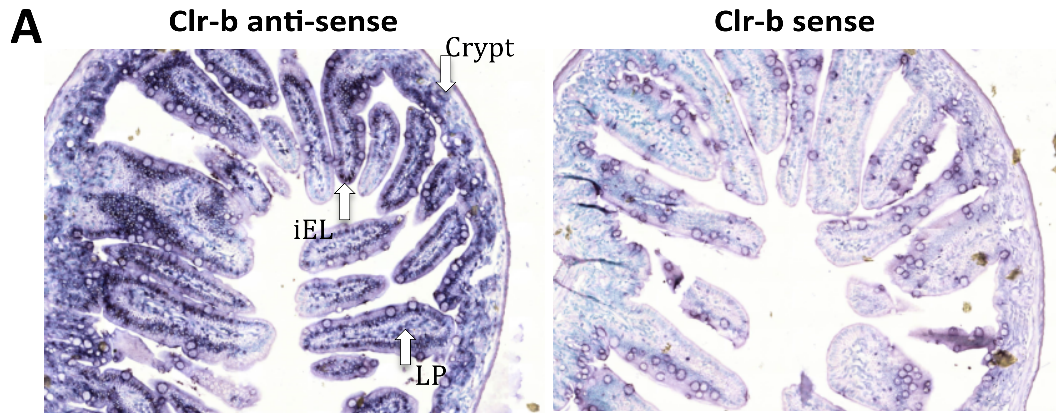

A
Normalized Small Intestinal Length
with Tibia Length
***
Small intestinal length / cm
40
30
20
10
0
NKR-P1B^WT
NKR-P1B^KO
NKR-P1B^WT
NKR-P1B^KO

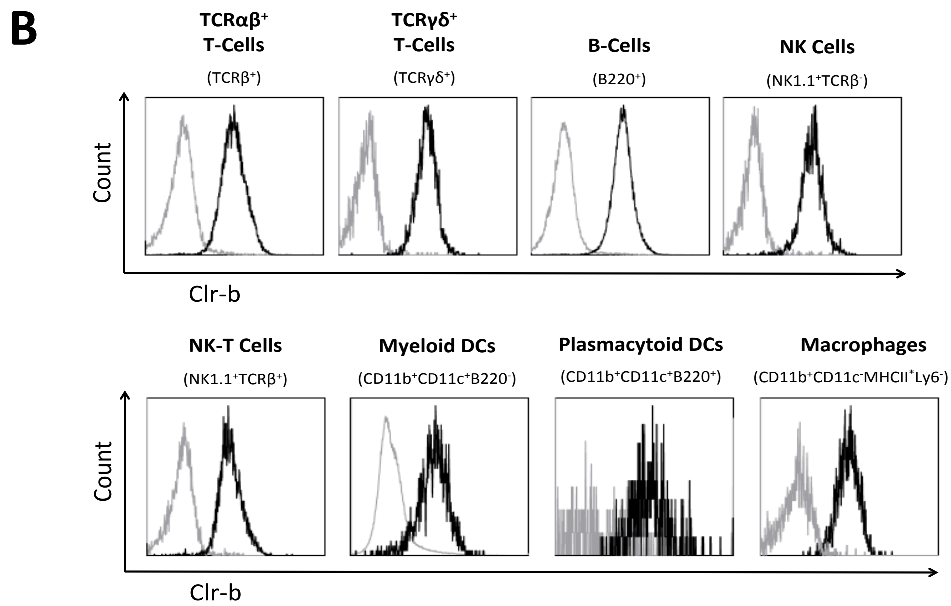

B
Body Weight
*
Body weight (gm)
40
30
20
10
0
NKR-P1B^WT
NKR-P1B^KO

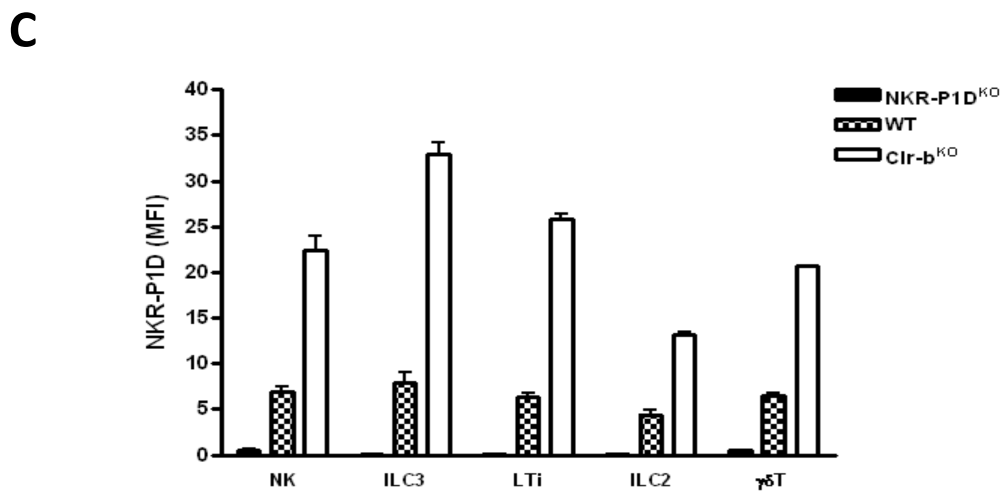
4.6 Clr-b expression is broadly expressed on gut-resident leukocytes.

Following characterization of the NKR-P1B receptor in the gut lamina propria, we checked for the mRNA and protein expression pattern of its respective ligand, Clr-b. Since the Clr-b antibody is not compatible for immunohistochemical staining, we generated a Clr-b mRNA probe in order to perform an *In-situ* hybridization. These data should indicate the sites of interaction between the NKR-P1B^{+} cells with their ligand in the gut. Clr-b appears to be expressed in the intra-epithelium, lamina propria and the crypt of the small intestine (Fig.6A). In order to identify the type of immune cells expressing the Clr-b ligand, we stained total leukocytes isolated from the small intestinal lamina propria of WT mice and Clr-b-deficient (Clr-b$^{-/-}$) mice with anti-Clr-b antibody for flow cytometry analysis. Clr-b expression was detected on TCRαβ T-cells, TCRγδ T-cells, B cells, NK cells, NK-T cells, myeloid dendritic cells (myeloid DCs), plasmaytoid dendritic cells (plasmacytoid DCs) and macrophages (Fig.6B). Interestingly, we detected higher expression of the NKR-P1B receptor on the small intestine NK cells, ILC3, LTi, ILC2 and γδT cells based on the comparison of NKR-P1B mean fluorescence intensity (MFI) between Clr-b$^{-/-}$ and WT mice (Fig.6C).

Figure 6: Clr-b expression is broadly expressed on gut-resident leukocytes. (A) *In-Situ* hybridization staining on 4μm small intestinal section using Clr-b digoxigenin-labeled anti-sense and sense RNA probes, transcribed from linearized plasmids containing the *Clr-b* gene with T7 polymerases. The sense Clr-b probe acts as a negative control. The *In-Situ* staining shown here is a representative of at least three independent repeats. **(B)** Flow cytometry analysis showing the Clr-b expression on small intestinal lamina propria resident leukocytes. Markers used to identify each cell subset are indicated. Stains for both the WT (black line) and the Clr-b-deficient (grey line) mice were repeated in multiple independent experiments (n=6). **(C)** Average NKR-P1B MFI on resident small intestinal lamina propria NK cells, NKp46^{+} ILC3s, NKp46^{-} ILC3s (LTi), ILC2 and γδT cells of NKR-P1B-deficient (NKR-P1B^{KO}), WT and Clr-b-deficient (Clr-b^{KO}) mice measured by flow cytometry (n=4 for each mouse genotype).


A
Clr-b anti-sense
Clr-b sense
Crypt
iEL
LP


B
TCRαβ+ T-Cells
(TCRβ+)
TCRγδ+ T-Cells
(TCRγδ+)
B-Cells
(B220+)
NK Cells
(NK1.1+TCRβ-)
Count
Clr-b
NK-T Cells
(NK1.1+TCRβ+)
Myeloid DCs
(CD11b+CD11c+B220-)
Plasmacytoid DCs
(CD11b+CD11c+B220+)
Macrophages
(CD11b+CD11c-MHCII+Ly6-)
Count
Clr-b

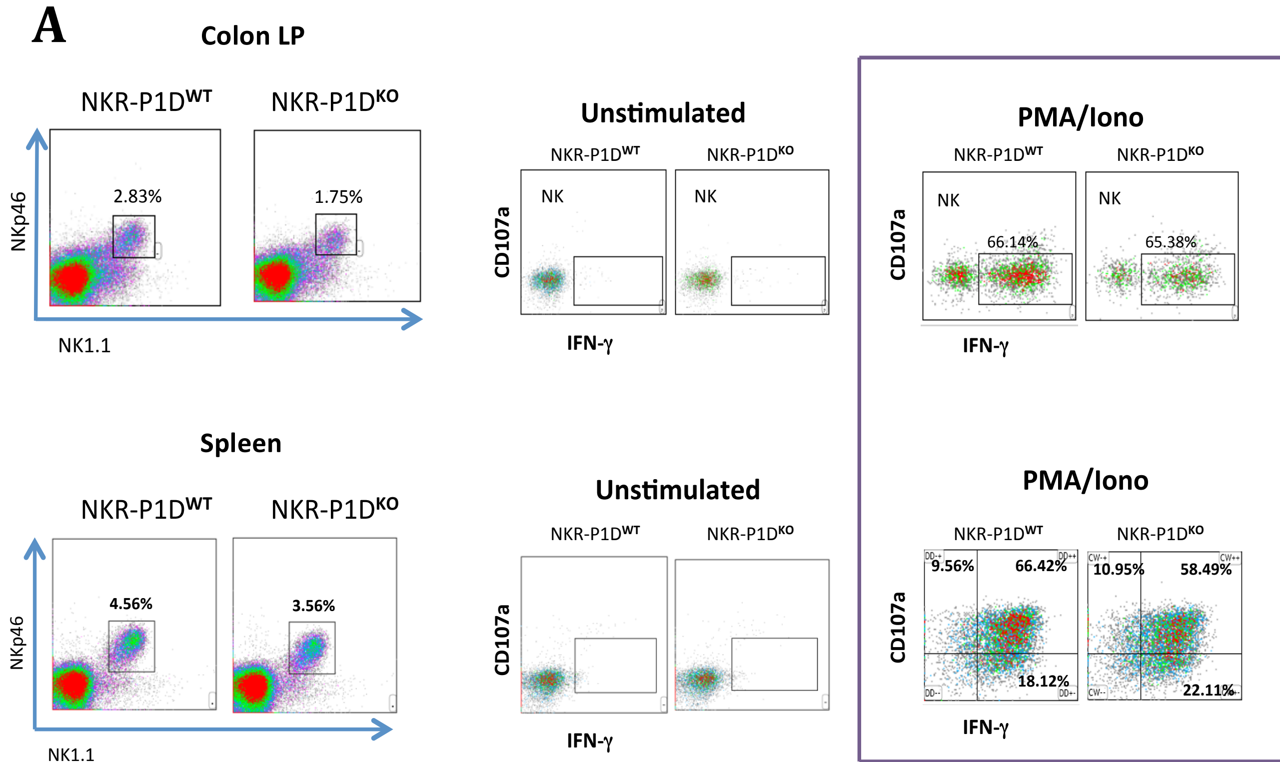

C
NKR-P1D (MFI)
40
35
30
25
20
15
10
5
0
NKR-P1D KO
WT
Clr-b KO
NK
ILC3
LTi
ILC2
γδT

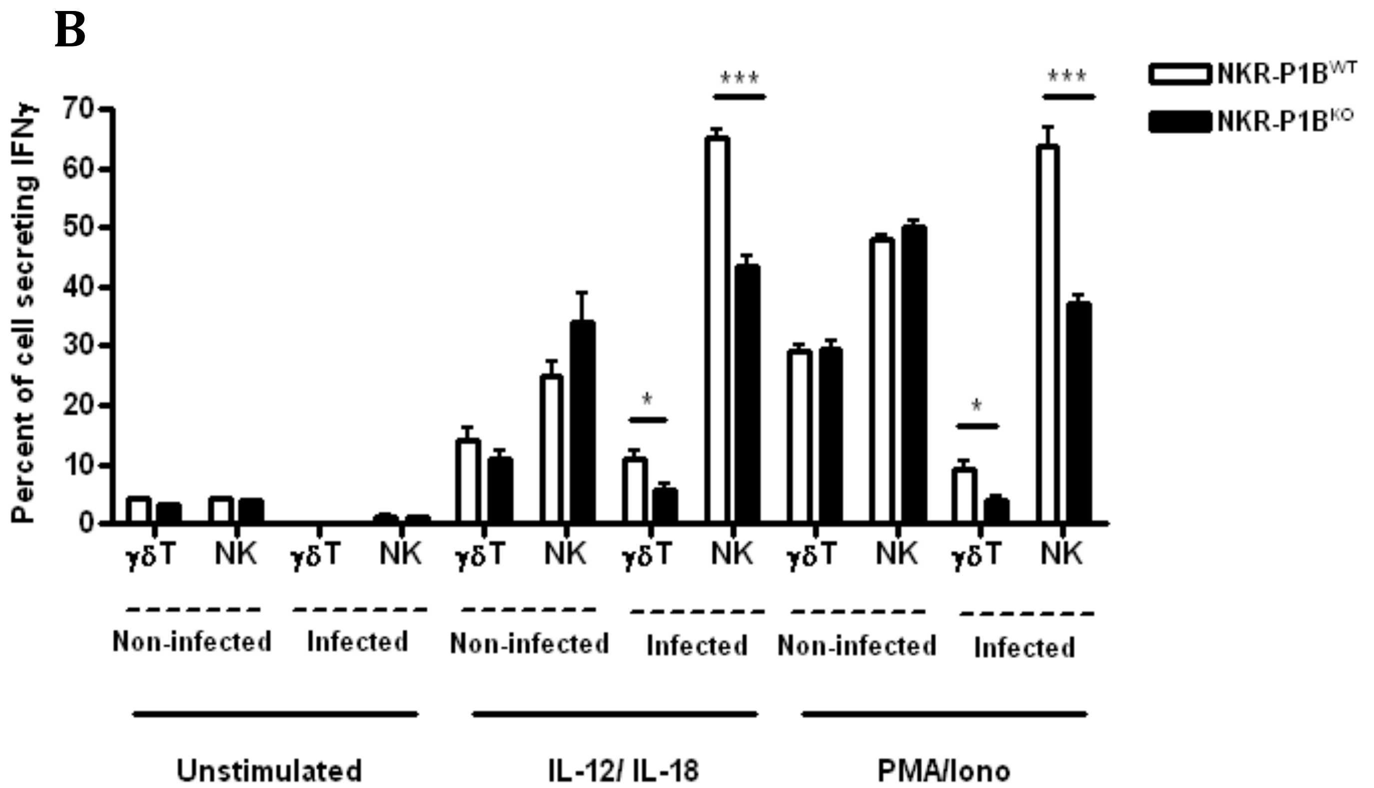
4.7 Defective NK and γδ T cells response to *Salmonella typhimurium* infection in the gut of NKR-P1B-deficient mice

Before studying the role of NKR-P1B receptor on gut NK cells during oral salmonella infection, we first decided to compare the gut NK cell function to that of their spleen counterparts. For that purpose, we used the IFNγ and CD107a intracellular flow stain on colon LP and spleen NK cells isolated from both WT and NKR-P1B^{KO} mice. The NKR-P1B^{KO} mice were used in this experiment in order to determine whether the observed functional difference is dependent on NK cells tissue localization, regardless of the presence or absence of the NKR-P1B receptor on their surface. Generally, NK cells mediated cytoxicity occurs through the release of cytoplasmic granules, such as granzymes and perforin, from cytoplasmic membrane-enveloped lysosomes. The lipid bilayer surrounding the cytotoxic granules contains lysosomal-associated membrane glycoproteins including CD107a (Zaritskaya et al. 2010). Our data demonstrate that colon lamina propria NK cells in both the WT and NKR-P1B-deficient mice do not express the CD107a in contrast to their splenic counterparts when stimulated with PMA/Ionomycin (Fig.7A, purple square). Hence, colon NK cells are unable to mediate cell cytoxicity through granzymes and perforin release. However, both spleen and colon NK cells were able to produce IFNγ cytokine, following PMA/Ionomycin stimulation (Fig.7A, purple square). Therefore, we decided to compare IFNγ production by the colon NK and γδ T cell in the WT and NKR-P1B-deficient mice after oral *S.typhimurium* infection. Five days following infection, NK and γδT cells were isolated from the colon lamina propria and were stimulated with IL-12/IL-18 or PMA/Iono. Following stimulation, both NK and γδT cell subsets isolated from the colon of infected NKR-P1B^{KO} mice had a lower percentage of IFNγ^{+} cells in comparison to their WT littermates (Fig.7 B). Previous studies have revealed the role of IFNγ cytokine in recruiting monocytes and

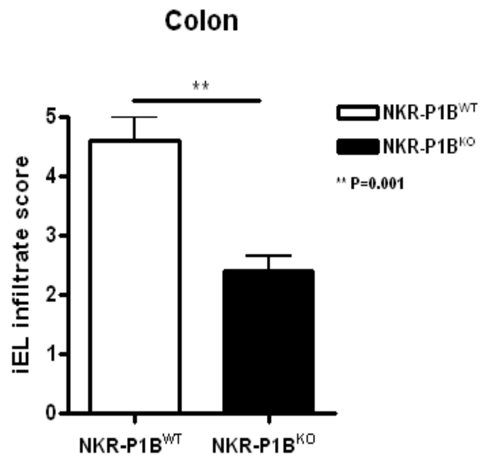
lymphocytes to the site of infection (Lee et al. 2009). Thus, we investigated whether the difference in IFNγ production between the WT and NKR-P1B-deficient NK and γδT cells, can influence the number of intra-epithelial lymphocytes in the context of oral *S.typhimurium* infection. Interestingly, a higher level of intra-epithelial lymphocyte infiltration was detected in the colon of WT mice compared to the NKR-P1B-deficient mice infected with *S. thyphymurium* (Fig.7C). To determine whether the IFNγ release from NK and γδT cells can affect the bacterial multiplication at the site of infection as well as its systemic dissemination, feces and spleens homogenates were prepared at day five post-infection and serial dilutions were plated on streptomycin-supplemented LB agar plates. After bacterial growth, colonies were enumerated and normalized to the weight of the tissue used for bacterial enumeration. Even though WT mice had a higher percentage of IFNγ^{+} NK and γδT cells in the colon LP during infection (Fig.7B), no statistically significant difference in bacterial load between the feces from NKR-P1B^{KO} and their WT littermates was detected (Fig.7D). In contrast, the bacterial loads in spleens (Fig.7E) of NKR-P1B^{KO} mice were significantly higher compared to their WT littermates. Thus, the absence of NKR-P1B receptor from the surface of gut NK and γδT cells results in lower IFNγ production and increased systemic bacterial dissemination following oral *S.typhimurium* infection *in-vivo*.

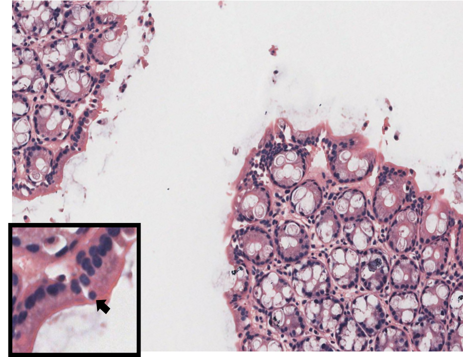
Figure 7: Defective NK and γδ T cells response to *Salmonella typhimurium* infection in the gut of NKR-P1B-deficient mice. (A) Flow cytometry analysis of IFNγ and CD107a expression in the colon LP and spleen NK cells (purple square). Same number of enterocytes and splenoctyes were stimulated with PMA and Ionomycin, and compared with non-stimulated group. Data are representative of at least three independent experiments. **(B)** NKR-P1B^{KO} and WT mice were orally infected with 10^8 CFU *S.typhimurium*. Five days later, isolated colon LP NK and γδT cells were stimulated with IL-12/IL-18 and PMA/Iono. IFNγ production was measured by intracellular staining and flow cytometry. Data are expressed as the means +/- standard deviation (SD) of at least three independent experiments (each done in triplicate). Statistical analysis was performed using a two-tailed unpaired t-test; *, P< 0.05**;** ***, P < 0.001 **(C)** Histological assessment showing iEL infiltrates at day 5 after *S.typhimurium* infection (iELs count per 100 surface enterocytes at 40x magnification). A representative photograph for hematoxylin and eosin (H&E) stained colon section from WT B6 mouse is shown (n=5). The arrow on the photograph indicates an intra-epithelial lymphocyte **(D and E)** CFU counts in both feces and spleens were evaluated at day 5 post-infection (p.i). Data are representative of at least 3 independent experiments (n=6). Statistical analysis was performed using a two-tailed unpaired t-test; ***, P < 0.001.


A
Colon LP
NKR-P1D^{WT}
NKR-P1D^{KO}
2.83%
1.75%
NKp46
NK1.1
Unstimulated
NK
CD107a
IFN-γ
PMA/Iono
66.14%
65.38%
Spleen
4.56%
3.56%
9.56%
66.42%
18.12%
10.95%
58.49%
22.11%

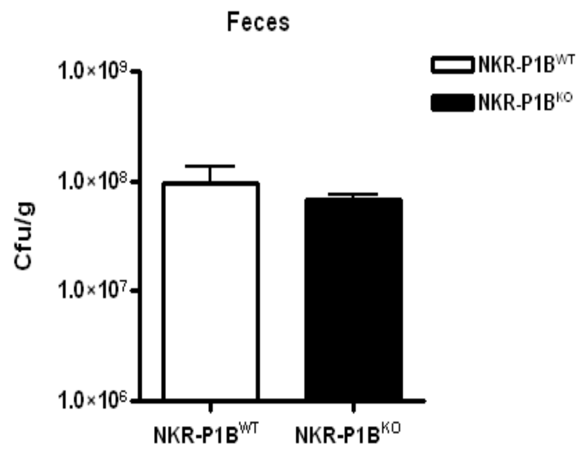

B
Percent of cell secreting IFNγ
NKR-P1B^{WT}
NKR-P1B^{KO}
γδT
NK
Non-infected
Infected
Unstimulated
IL-12/ IL-18
PMA/Iono

C

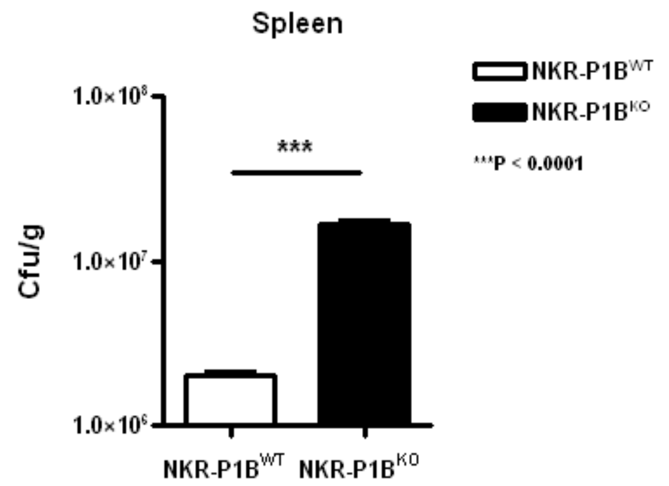




D



E

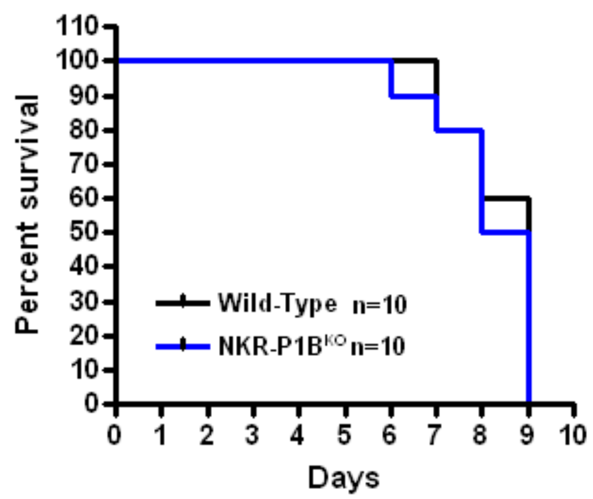


4.8 NKR-P1B^{WT} and NKR-P1B^{KO} mice showed similar survival kinetics following *S.typhimurium* infection *in-vivo*.

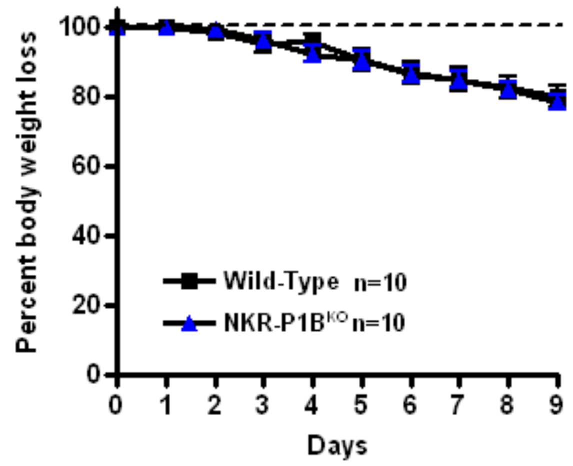
To investigate the influence of gut NKR-P1B receptor on the course of invasive Salmonella infection in vivo, NKR-P1B^{KO} and WT mice were pre-treated with streptomycin for 24 hours to deplete the naturally existing microbiota, which protects and hinders the ability of Salmonella to colonize the intestinal lumen (Barthel et al. 2003). Next, the mice were inoculated with 10^8 CFU of WT *S.typhimurium* strain. Our data demonstrate that both groups of mice started to randomly die by days 6-8, whereas fifty to sixty percent of knockout and WT mice respectively, died by day 9 of infection. None of the mice remained alive at the end of the observation period (Fig. 8A). We also monitored the body weight of the infected mice throughout the infection; our results indicate that both NKR-P1B^{KO} and WT mice responded similarly to the infection (Fig.8B).

Figure 8: **NKR-P1B^{WT} and NKR-P1B^{KO} mice showed similar survival kinetics following *S.typhimurium* infection *in-vivo*. (A)** Groups of age and sex-matched WT and NKR-P1B^{KO} littermates were infected with 10^8 CFU of *S.typhimurium*, and then monitored for survival for a period of 10 days. **(B)** The same groups of WT and NKR-P1B^{KO} mice were daily monitored for their body weight following *S.typhimurium* infection, until day 10 post-infection. Data are pooled from two independent experiments (n = 10 in each group).

A



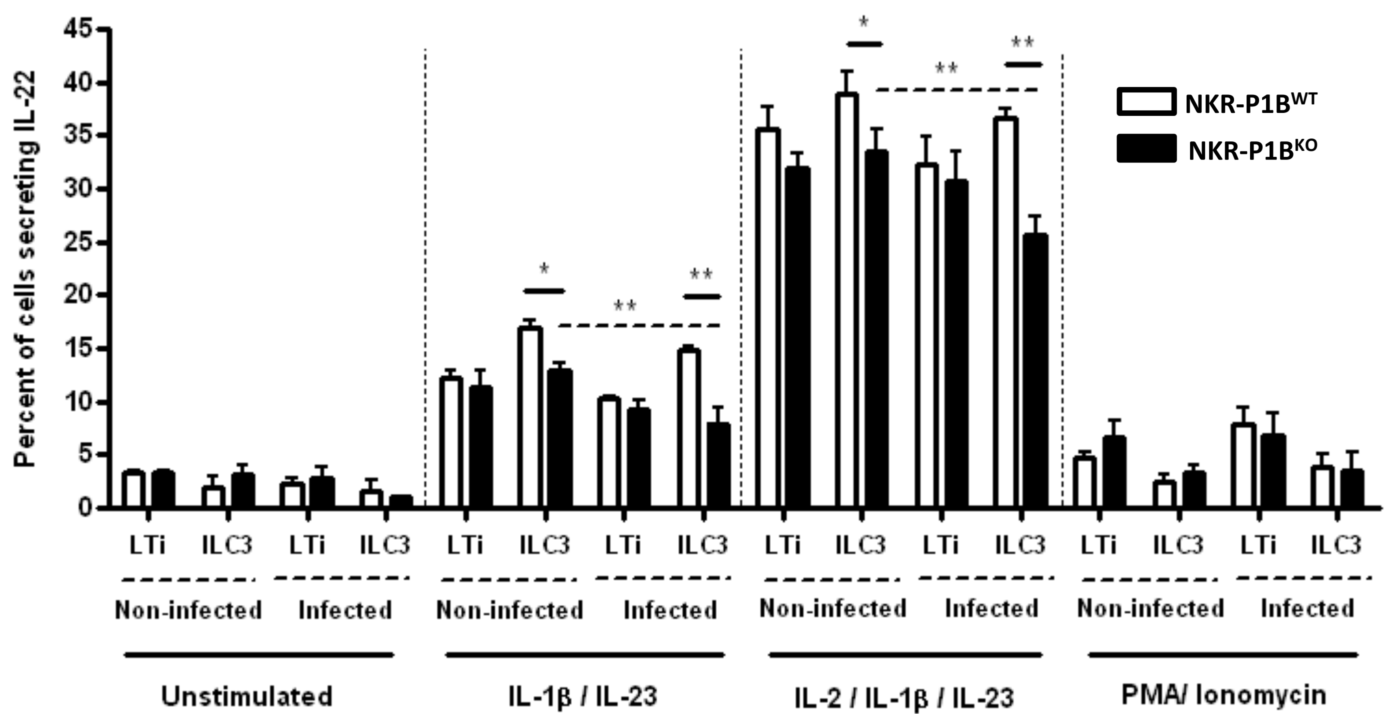
B



4.9: Defective ILC3 responses to *Citrobacter rodentium* infection in the gut of NKR-P1B-deficient mice.

Previous studies have revealed that ILC3 subsets are the main source of IL-22 during *C.rodentium* infection, and their depletion can lead to death in Rag^{KO} mice following infection (Diefenbach et al. 2013). We first decided to determine whether the absence of the NKR-P1B receptor on gut ILCs from NKR-P1B-deficient mice would affect their IL-22 cytokine secretion following oral *C.rodentium* infection. Five days following infection, total leukocytes were isolated from the colon lamina propria of WT and NKR-$P1B^{KO}$ mice and were stimulated for 5 hours with IL-1β/IL-23, IL-2/IL-1β/IL-23 and PMA/Iono. The intracellular IL-22 production by ILC3 and LTi was measured by flow cytometry analysis. NKR-$P1B^{KO}$ ILC3 were found to be hyporesponsive in comparison to their WT counterparts in both the un-infected and *C.rodentium*-infected mice, following IL-1β/IL-23 stimulation (Fig.9). Similar results were observed in response to IL-2/IL-1β/IL-23 cytokine stimulation. However, the addition of IL-2 significantly enhanced the IL-22 production from ILCs compared to the IL-1β/IL-23 stimulation alone (Fig. 2.9). Additionally, there were fewer IL-22-producing ILC3 in the infected compared to the uninfected NKR-$P1B^{KO}$ mice. Thus, similar to what we previously observed for NKR-$P1B^{KO}$ γδT and NK cells (Fig.7B), under stress conditions, such as *S.typhimurium or C.rodentium* infections, NKR-$P1B^{KO}$ innate immune cell subsets are hyporesponsive compared to their WT counterparts. . All together, the data suggest that NKR-P1B receptor is required for the optimal functioning of the innate immune cell subsets in the gut.

Figure 9: Defective ILC3 responses to *Citrobacter rodentium* infection in the gut of NKR-P1B-deficient mice. Isolated enterocytes from NKR-P1B^{WT} and NKR-P1B^{KO} colon lamina propria were stimulated with IL-1β/IL-23, IL-2/IL-1β/IL-23 and PMA/Iono, and the frequencies of IL-22 producing ILC3 and LTi were determined by gating on CD19^{-}CD3^{-}Nkp46^{+}RoRγt^{+} and CD19^{-}CD3^{-}Nkp46^{-}RoRγt^{+} cells, respectively, using flow cytometry. The data is representative of one of multiple separate experiments (n=6). Statistical analysis was performed using a two-tailed unpaired t-test; *, P < 0.05, **, P < 0.01.

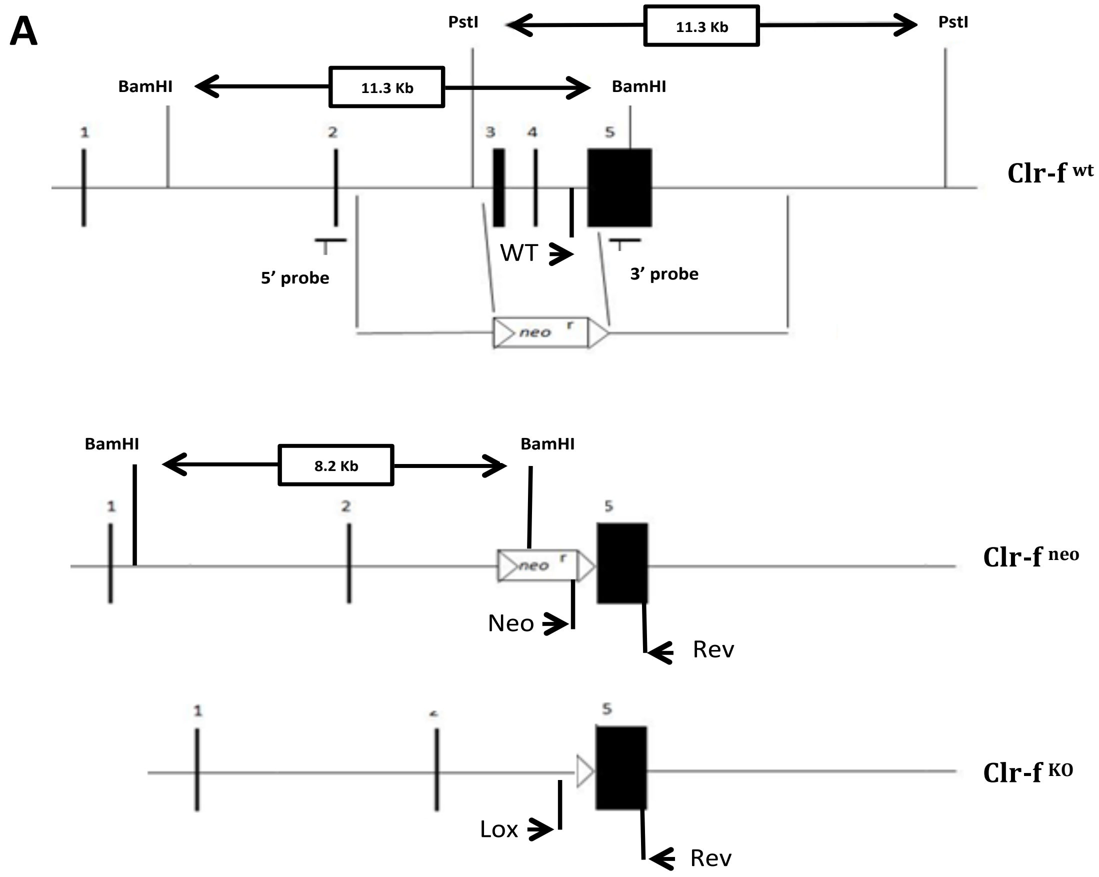

Percent of cells secreting IL-22
45
40
35
30
25
20
15
10
5
0
*
**
**
*
**
**
NKR-P1B^WT
NKR-P1B^KO
LTi
ILC3
LTi
ILC3
LTi
ILC3
LTi
ILC3
LTi
ILC3
LTi
ILC3
LTi
ILC3
LTi
ILC3
Non-infected
Infected
Non-infected
Infected
Non-infected
Infected
Non-infected
Infected
Unstimulated
IL-1β / IL-23
IL-2 / IL-1β / IL-23
PMA/ Ionomycin

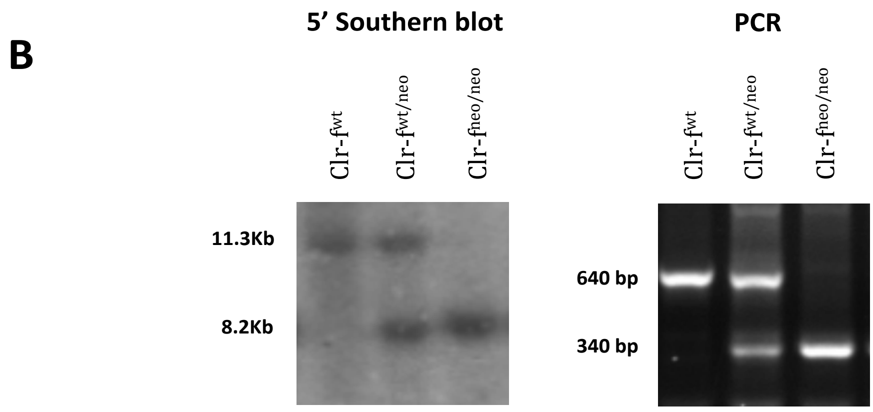
4.10 Generating the Clr-f-deficient mice

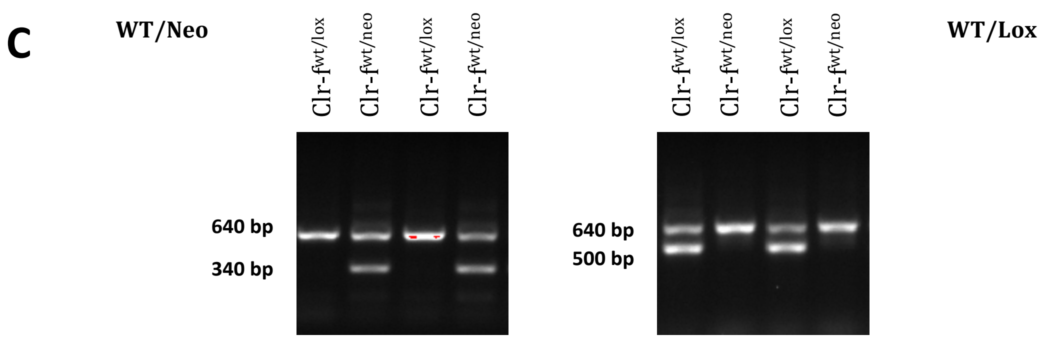
In order to study the role of the Clr-f ligand on renal epithelial cell function, we generated Clr-f-deficient mice on a B6 background. A targeting construct containing a floxed neomycin cassette was inserted into the *Clr-f* gene by homologous recombination, replacing exons 3, 4 and part of exon 5 in B6-ES cells (Fig.10A). Correctly targeted ES cell clones were selected by Southern blot analysis using multiple DNA probes, including a 5', 3' and a neomycin probe. Notably, our targeting strategy aimed to delete multiple exons at once in order to eliminate the occurrence of any possible RNA splicing events, which may lead to the production of partially or fully functional Clr-f ligand. Selected ES cells were microinjected into blastocysts and male founder mice bearing the *Clr-f* ^{neo} allele were bred to WT B6 females. The generated *Clr-f* $^{wt/neo}$ pups were genotyped by Southern blot and PCR analysis using a 5' DNA probe and PCR primers unique for the *Clr-f* targeted region, respectively (Fig.10A-B). Next, *Clr-f* $^{wt/neo}$ heterozygote offsprings were interbred to produce *Clr-f* $^{neo/neo}$ mice (Fig.10B). The resulting homozygote *Clr-f* $^{neo/neo}$ mice were then selected and bred with CMV-cre transgenic (Tg) mice to remove the neomycin cassette through Cre-mediated recombination. Finally, using specifically designed *Clr-f* PCR primers, we genotyped for *Clr-f* $^{wt/lox}$ mice (Fig.10C) and interbred them to obtain *Clr-f* $^{lox/lox}$ mice, termed as Clr-f ^{KO}. Since our attempt to generate a Clr-f antibody was unsuccessful, we confirmed the *Clr-f* deletion by semi-quantitative RT-PCR. Previous data generated by our lab have determined the expression of *Clr-f* transcripts in renal and intestinal epithelial cells as well as the liver but not in the heart (Zhang et al. 2012). Thus, we generated cDNA from Clr-f-expressing and non-expressing organs from both the Clr-f ^{KO} and WT mice. Our results show a complete loss of *Clr-f* transcripts in the jejunum, colon, cecum and kidneys of Clr-f-deficient mice without affecting the *Clr-b* transcripts levels (Fig.10D). However, we detected lower Clr-a

transcripts in the Clr-f ^{KO} mice when compared with their WT littermates (Fig.10D). The latter result might not be as surprising considering the fact that genes for Clr-a and Clr-f are closely linked in the NKC gene complex (Zhang et al. 2012). Importantly, our transcript analysis determined that Clr-a expression is restricted to the intestinal tissue, which probably won't affect the kidney functions in Clr-f-deficient mice. Clr-f ^{KO} mice appear healthy and fertile. We observed that Clr-f ^{KO} mice maintained an increased body weight in comparison to their WT littermates (Fig.11A); whereas the two mouse genotypes had similar intestinal lengths and organ weights in the Clr-f expressing tissues, while normalized to their corresponding tibia length and body weights respectively (Fig. 11B, C).

Figure 10: Generation of Clr-f-deficient mice. (A) *Clr-f* deletion strategy. Exons 3, 4 and part of exon 5 were replaced with a floxed neomycin (neo) cassette by homologous recombination in ES cells of B6 background. Correctly targeted ES cell clones were selected by Southern blot analysis and used to generate *Clr-f* ^{neo} mice. These mice were bred with CMV-cre transgenic mice to produce *Clr-f* ^{lox} mice. Filled boxes denote exons (numbered), and arrowheads represent PCR primers. The location of 5′ and 3′ Southern probes is underlined, and BamHI and *Pst*I restriction enzyme sites are shown. **(B)** Southern blot of BamHI-digested genomic DNA from mice of the indicated genotypes using the 5′ probe (left panel). PCR analysis confirming the Southern blot result (right panel) **(C)** PCR analysis of tail DNA from *Clr-f* ^{neo} and *Clr-f* ^{lox} mice. **(D)** Semi-quantitative RT-PCR analysis of Clr-f transcript expression in various organs, using primers specific for exons 1 and 5 to detect the full coding sequence of Clr-f. GAPDH transcript levels were used as an internal loading control for each of the indicated organs. Heart cDNA was used as a negative control for *Clr-f* transcripts expression. Two sets of primers were used to amplify Clr-f transcripts: **(I)** the reverse PCR primer is located inside the targeted region of exon 5, and, **(II)** the reverse PCR primer is located outside the targeted region of exon 5.


A
PstI
11.3 Kb
PstI
BamHI
11.3 Kb
BamHI
1
2
3
4
5
Clr-f wt
WT
5' probe
3' probe
neo r
BamHI
8.2 Kb
BamHI
1
2
5
neo r
Clr-f neo
Neo
Rev
1
5
Clr-f KO
Lox
Rev


B
5' Southern blot
PCR
Clr-fwt
Clr-fwt/neo
Clr-fneo/neo
11.3Kb
8.2Kb
Clr-fwt
Clr-fwt/neo
Clr-fneo/neo
640 bp
340 bp


C
WT/Neo
Clr-fwt/lox
Clr-fwt/neo
Clr-fwt/lox
Clr-fwt/neo
640 bp
340 bp
Clr-fwt/lox
Clr-fwt/neo
Clr-fwt/lox
Clr-fwt/neo
WT/Lox
640 bp
500 bp

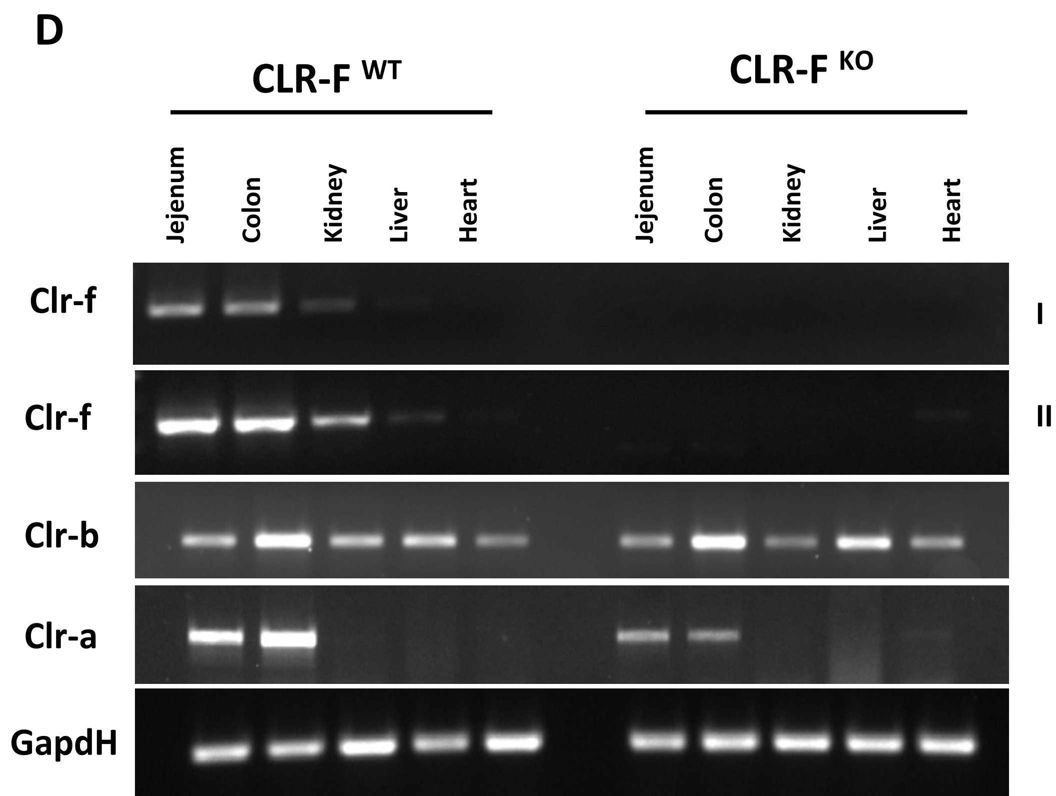
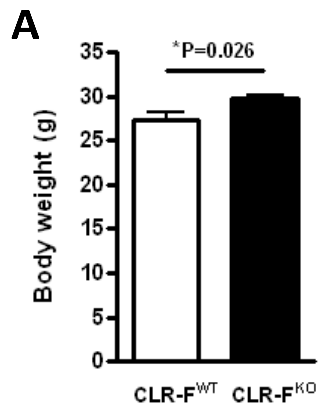
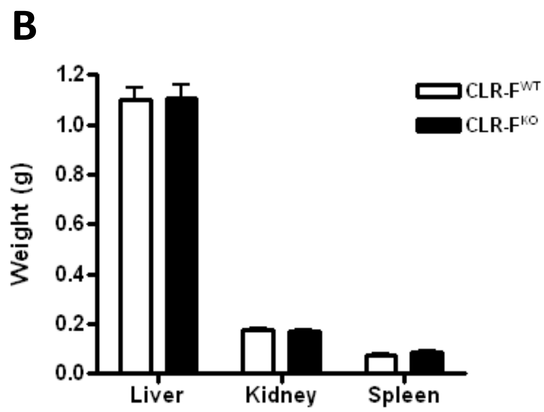
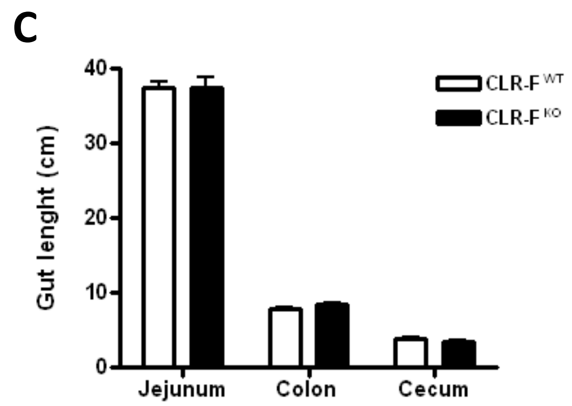

D
CLR-F WT
CLR-F KO
Jejenum
Colon
Kidney
Liver
Heart
Jejenum
Colon
Kidney
Liver
Heart
Clr-f
I
Clr-f
II
Clr-b
Clr-a
GapdH

Figure 11: Increased body-weight in Clr-f-deficient mice but comparable intestinal lengths and organ-weight in Clr-f-expressing tissues (A) Body weights of Clr-f-knockout (Clr-f ^{KO}) and wild-type (WT) B6 mice (Clr-f ^{WT} n=10, Clr-f ^{KO} n=12). Statistical analysis was performed using a two-tailed unpaired t-test; *, $P < 0.05$. **(B)** Liver, kidney and spleen weight measurements normalized to body weights of the same group of Clr-f ^{KO} and WT mice. Spleens were used as a negative control for the absence of the Clr-f ligand. **(C)** Using the same group of mice, we measured the intestinal lengths of the small intestine, colon and cecum normalized with their corresponding tibia length.


A
*P=0.026
Body weight (g)
35
30
25
20
15
10
5
0
CLR-F^WT
CLR-F^KO


B
Weight (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CLR-F^WT
CLR-F^KO
Liver
Kidney
Spleen


C
Gut lenght (cm)
40
30
20
10
0
CLR-F WT
CLR-F KO
Jejunum
Colon
Cecum

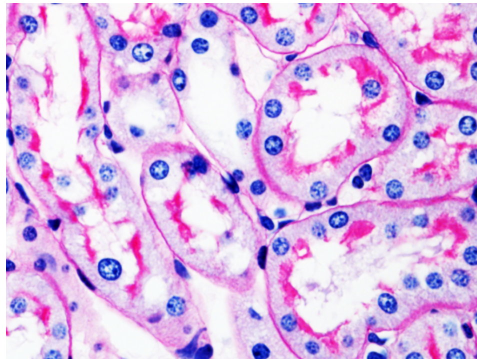
4.11 Kidney tubular and glomerular lesions in Clr-f-deficient mice

Since we know that the Clr-f ligand is expressed on renal tubules, it was obvious to determine whether Clr-f-deficient kidney tubules would display symptoms of morphological disorders or traces of immune-mediated inflammatory stress. For these purposes, we prepared 3 μm thick PAS-stained kidney sections collected from four 12 weeks old Clr-f ^{WT} and Clr-f ^{KO} mice, which were then examined by a pathologist at the kidney research center in the University of Ottawa. The data indicates that Clr-f-deficient kidneys exhibit varied proximal and distal tubular lesions compared to their WT control. These variations ranged from tubular epithelial cell flattening to nuclear displacement and the loss of brush borders (Fig.12B and C), accumulation of luminal necrotic debris (Fig.12C) and their complete disappearance in some of the examined areas (Fig.12D). Because we detected multiple variations of the observed pathology, we only report the scope of tubular damage without quantifying it. Although previous microarray and *In Situ* hybridization data indicated the absence of Clr-f transcripts from the renal glomeruli, Clr-f ^{KO} kidneys intriguingly demonstrated phenotypic changes in their glomerular mesangium, probably as a consequence of the tubular damage (Fig.13). Notably, the glomeruli of Clr-f ^{KO} kidneys suffered from capillary aneurisms (Fig.13A) that progressed to pronounced disruption of glomeruli and constant presence of necrotizing cellular debris in Bowman's spaces (Fig.13 B and D), and even to their complete disappearance (Fig.13C). Our pathology scoring results indicate that Clr-f-deficient mice have a significant higher glomerular damage in comparison to their WT littermates (Fig.13E). Interestingly, the prominent glomerular lesions didn't correlate with a significant increase in inflammatory cell infiltrates into the mesangium. Only isolated focal mononuclear inflammatory cells where detected in some of the damaged areas (Fig.13D). However, this is not considered enough to cause any renal inflammation, suggesting that Clr-f-

deficient tubular epithelial cells promote glomerular lesions in an immune-independent manner. Indeed, our flow cytometry analysis of immune cell populations in 12 weeks old Clr-f ^{WT} and Clr-f ^{KO} kidneys revealed comparable numbers and percentages of adaptive and innate immune cell types between the two mouse genotypes (Fig.14 A and B). Similar results were also detected in multiple tissue-resident lymphoid cells in the mesenteric lymph nodes, spleen and liver (Fig.14C, D and E), suggesting normal immune development in Clr-f ^{KO} mice compared to B6 WT mice.

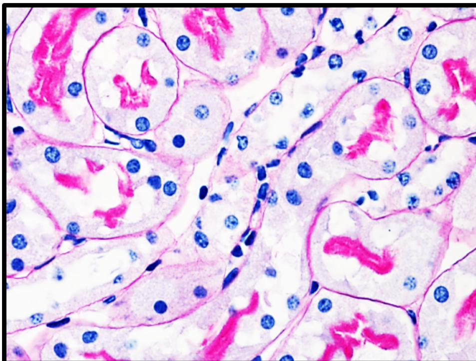
Figure 12: Disrupted kidney tubules in Clr-f- deficient mice. Representative images of tubular lesions with mosaic appearance in proximal and distal tubules. **(A)** Minimal epithelial disturbance. **(B)** Note flattened tubular epithelium with numerous cytoplasmic vacuoles (arrow) and nuclear apical displacement. **(C, D)** Moderate and aggravated lesions with disappearance of tubular epithelium. Areas of cell loss where tubular basement membrane covered by a thin layer of cytoplasm (arrowheads). Many detached cells can be seen in tubular lumina (long arrows) along with accumulated necrotic debris (asterisks) and loss of epithelial cell brush borders. PAS-staining of kidney (3μm) sections from 12 weeks old Clr-f ^{WT} and Clr-f ^{KO} (n=4) at 630X magnification.

Wild-Type

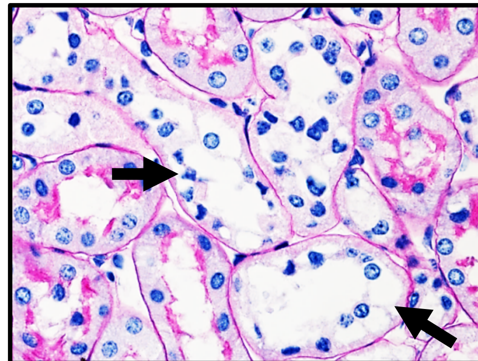


CLR-F knockout

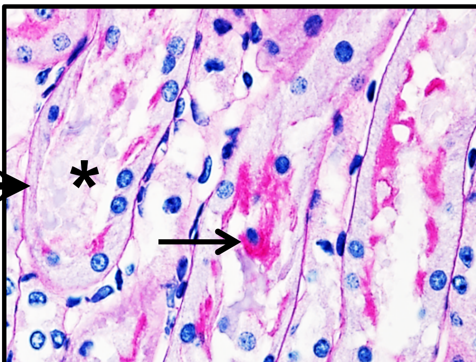
A



B



C



D

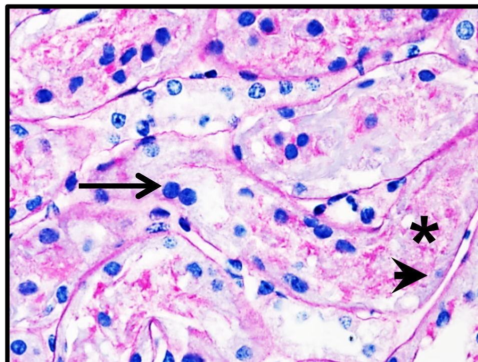
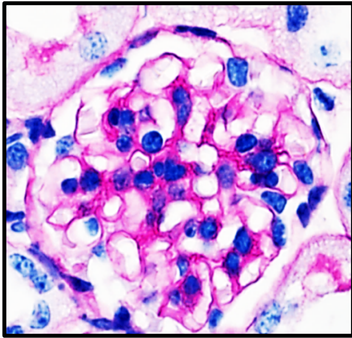
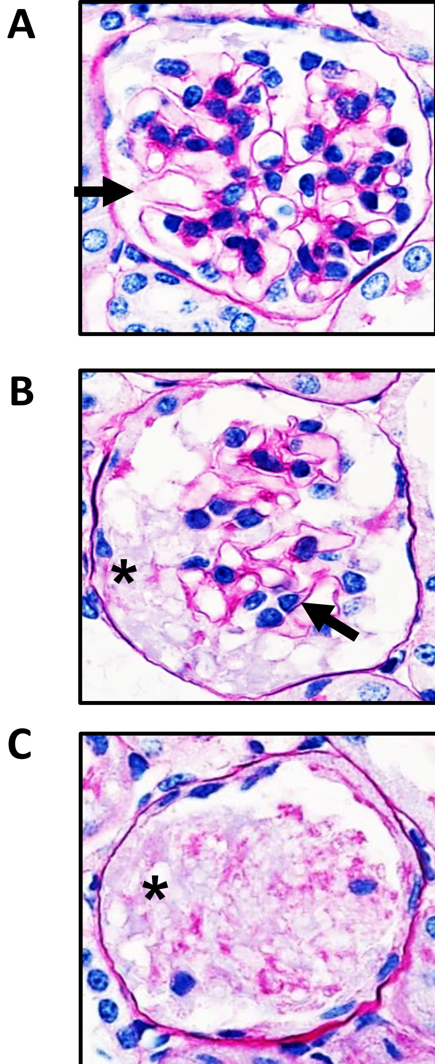
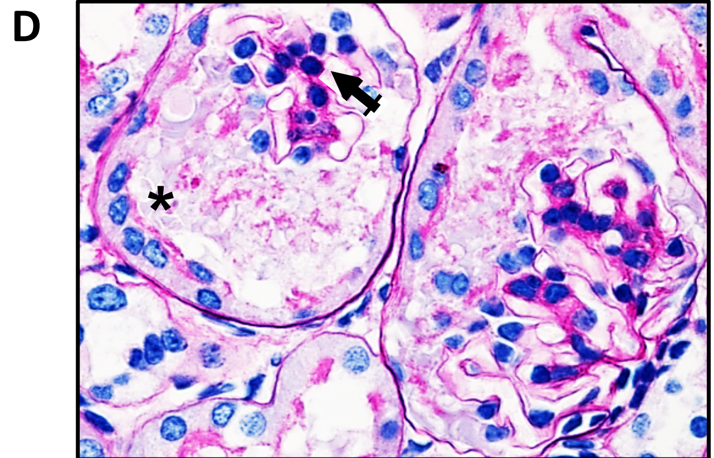


Figure 13: Typical variants of mesangiolysis in glomeruli of Clr-f-deficient mice. Primary phase **(A)** with capillary aneurisms (arrows) progresses to fragmentation and pronounced disruption of glomeruli **(B, D)**, and even to their complete disappearance **(C)**. Asterisks indicate constant presence of necrotizing cellular debris and plasma in Bowman's spaces. Notably, the intensive glomerular lesions occur without a significant increase in inflammatory cell infiltrates neither from the mesangium nor from parietal epithelium. Arrow in **(D)** indicates the presence of isolated focal mononuclear inflammatory cells. PAS-staining of kidney (3μm) sections from 12 weeks old Clr-f ^{WT} and Clr-f ^{KO} (n=4) at 630x magnification. **(E)** Pathology scoring of the observed glomerular lesions. Scores are based on the level of disappearance of the glomerulus: 1, <30% (A); 2, 30-60% (B); 3, >60% (C).


Wild-type


CLR-F knockout
A
B
C
*
*


D
*

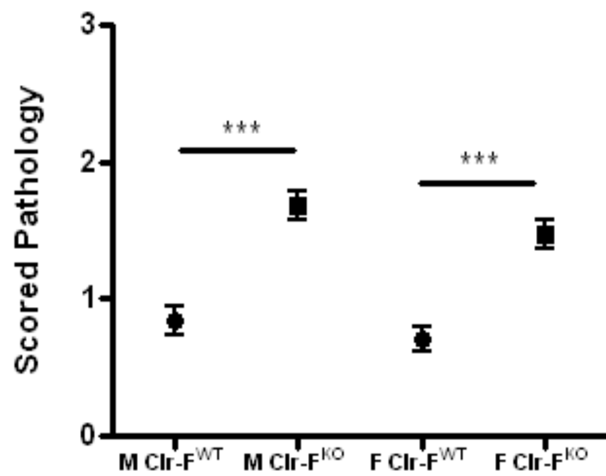
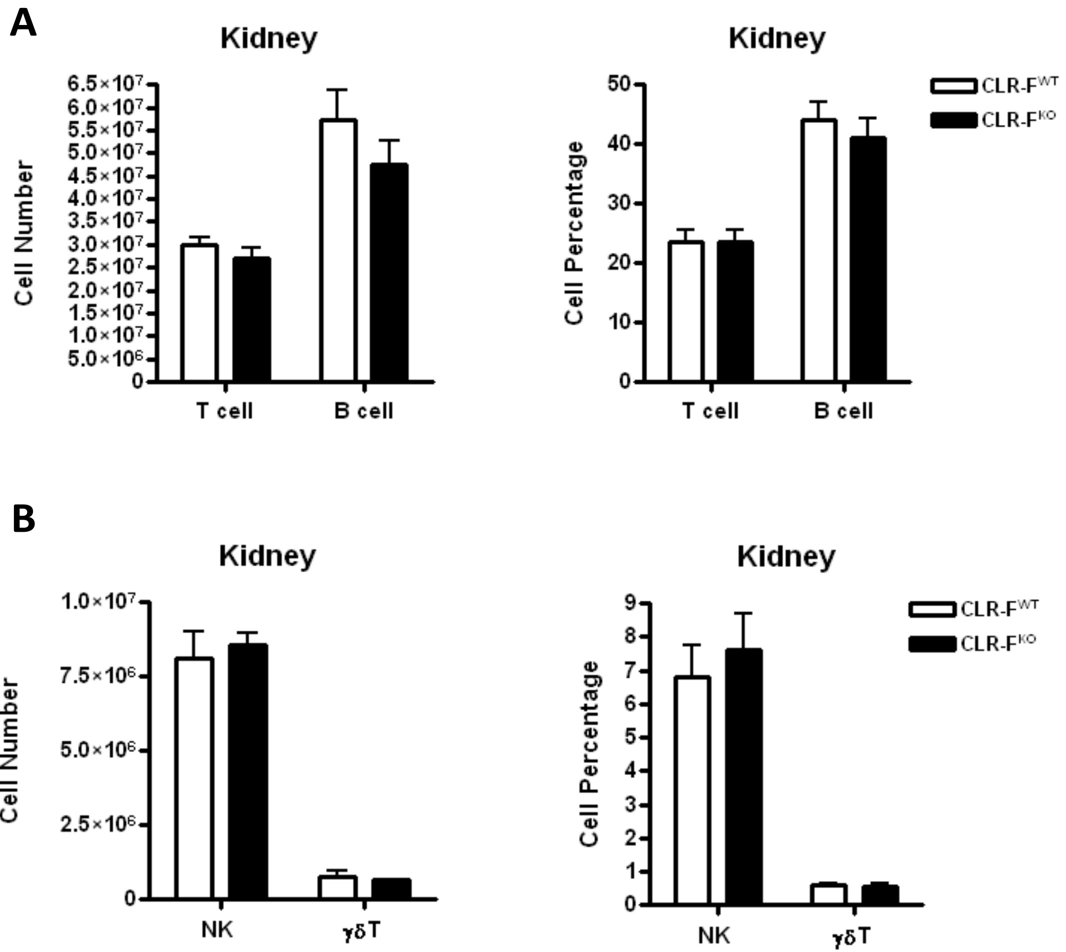
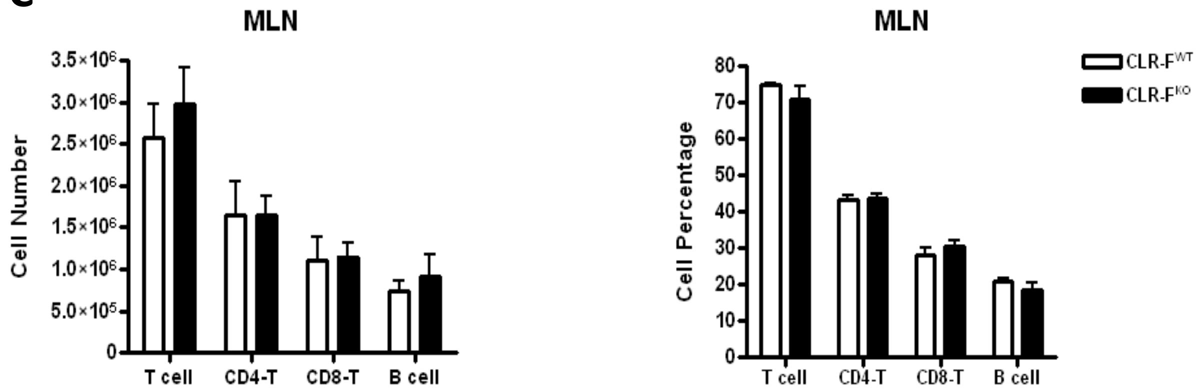

E
Glomeruli pathology scoring
Scored Pathology
0
1
2
3
***
***
M Clr-F^WT
M Clr-F^KO
F Clr-F^WT
F Clr-F^KO

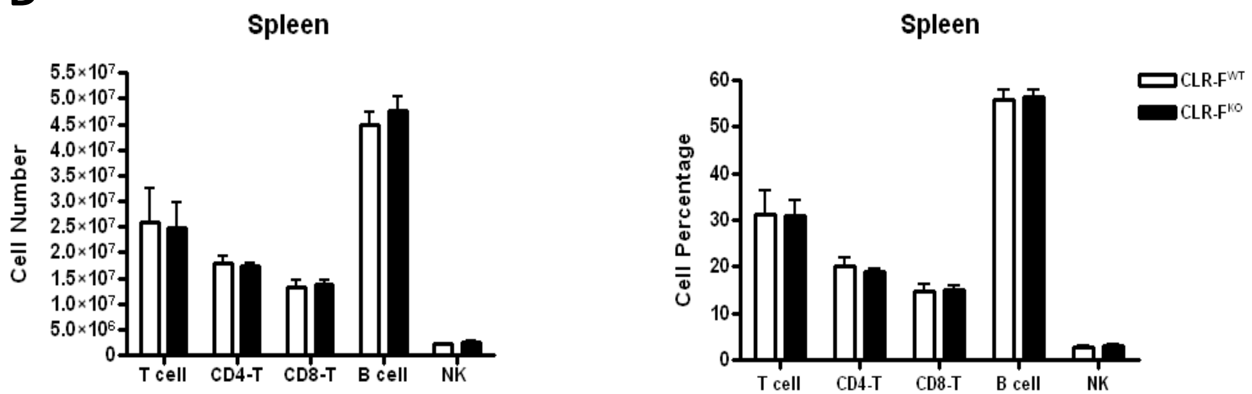
Figure 14: Equivalent frequency and numbers of several adaptive and innate immune cell types in different organs of Clr-f-deficient and WT mice. (A-B) Flow cytometry analysis showing the average numbers and percentages of adaptive and innate lymphoid cells in the kidney. **(C-E)** Flow cytometry analysis showing the average numbers and percentages of multiple tissue-resident lymphoid cells in the mesenteric lymph nodes (n=6), spleen (n=8) and liver (n=6) of WT and Clr-f ^{KO} mice. Cells isolated from each of the indicated organs were counted prior to FACS staining. Cell numbers were determined by multiplying the percentage of each cell subset with their corresponding total cell count.


A
Kidney
Cell Number
6.5×10^7
6.0×10^7
5.5×10^7
5.0×10^7
4.5×10^7
4.0×10^7
3.5×10^7
3.0×10^7
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
T cell
B cell
Kidney
Cell Percentage
50
40
30
20
10
0
T cell
B cell
CLR-F^WT
CLR-F^KO
B
Kidney
Cell Number
1.0×10^7
7.5×10^6
5.0×10^6
2.5×10^6
0
NK
γδT
Kidney
Cell Percentage
9
8
7
6
5
4
3
2
1
0
NK
γδT
CLR-F^WT
CLR-F^KO

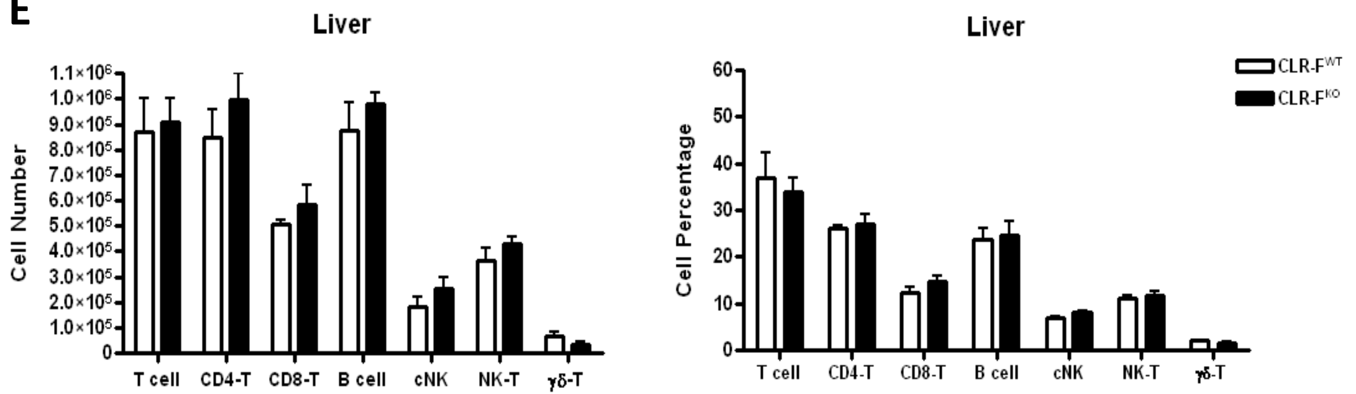
C



D



E

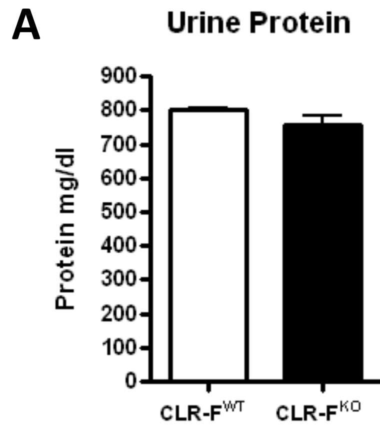


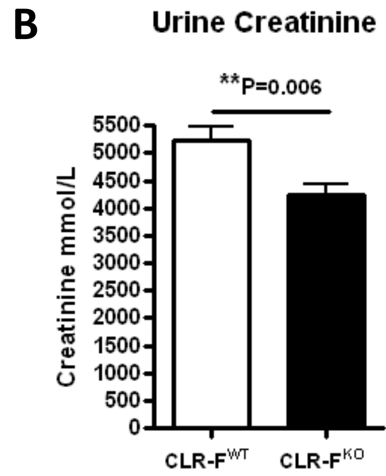
4.12 Urine and serum chemical analysis revealed disrupted kidney function in hypotensive Clr-f-deficient mice.

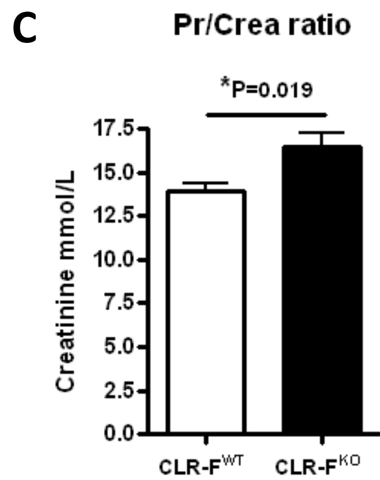
Generally, the kidney's primary role is to excrete chemical wastes (eg: creatinine) out of the bloodstream into the urine, while reabsorbing essential molecules back into the circulation (eg: albumin, glucose) (MRCPath Lowe 2005). Kidneys are also known to regulate blood pressure through the release of renin, which is an enzyme mainly synthesized by smooth muscle cells in the walls of the afferent arterioles. In particular, renin stimulates the secretion of aldosterone by the adrenal glands causing the renal tubules to retain sodium, water and causing arterial vasoconstriction, all of which help in maintaining normal body fluids and raising the blood pressure (MRCPath Lowe 2005). Since Clr-f is expressed on epithelial cells of kidney tubules, we checked whether its absence would alter the main kidney functions in Clr-f ^{KO} mice. Our chemical analysis on 12 weeks old males Clr-f ^{KO} and WT mice revealed normal levels of protein (Fig.15A) but lower creatinine levels in the urine of Clr-f deficient mice (Fig.15B) in parallel with a marked increase of blood creatinine compared to their WT littermates (Fig.15D). These results verify the observed kidney pathology (Fig. 14 and 15) and demonstrate an impaired filtration capacity of Clr-f-deficient kidneys. Urine protein to creatinine (UP/CR) ratio is significantly higher in Clr-f ^{KO} mice in comparison to their WT controls. The increased concentration of blood creatinine in comparison to their WT littermates indicates abnormal kidney filtration function in the Clr-f ^{KO} mice. Normally, nephrologists order the UP/CR test to take a fast snapshot on the renal status while monitoring a person with known kidney damage and not to indicate whether the patient is suffering from proteinuria (Paradeep 2016, Levey et al. 2003). In contrast, creatinine is regularly produced in the blood due to the breakdown of creatinine phosphate in muscles, which makes the measurement of serum creatinine levels a

useful method to designate the glomerular filtration rate (GFR) (Levey et al. 2003, Shimada et al. 2016). Other than the filtration of chemical wastes and the reabsorption of essential proteins, the kidney also controls salts and water balance, which is a key factor in the regulation of blood pressure (MRCPath Lowe 2005, Smith et al. 2006). Notably, Clr-f is expressed on tubular epithelial cells where it can affect electrolyte homeostasis. For these reasons, it was crucial to test whether Clr-f-deficiency can cause blood pressure dysregulation or influence the ions exchange. Our results determined that 12 weeks and 24 weeks old Clr-f ^{KO} mice had a lower systolic and diastolic blood pressure compared to their WT littermates (Fig. 16A and B). However, there were no differences in the urinary excretion of Na, K, Cl, P and Ca as well as plasma electrolyte concentrations between the two mouse genotypes (Fig. 17 and 18). Previous epidemiological reports have suggested the sodium/potassium (Na/K) ratio as a robust tool in the evaluation of blood pressure outcomes (Iwahori et al. 2016). Likewise, the calculation of fractional excretion of sodium (FENa) in terms of urine and plasma Na concentrations is considered a superior metric for urinary excreted Na, rather than the interpretation of urinary Na alone, as Na concentration varies with water reabsorption (Iwahori et al. 2016). We show that Clr-f ^{KO} mice display normal Na/K ratio but lower FENa, which indicates a decreased percentage of Na excretion in Clr-f ^{KO} mice compared to the WT control group. Altogether, our results suggest a minimal effect of Clr-f on renal electrolyte homeostasis while revealing its critical role in maintaining normal kidney filtration and regulated blood pressure.

Figure 15: Disrupted urine and serum creatinine levels suggest kidney tubular dysfunction in Clr-f-deficient mice. (A, B and D) Protein and creatinine measurements in urine and blood samples from 12 weeks old male Clr-f ^{KO} and WT mice. **(C)** Urine protein to creatinine (UP/CR) ratio is used to take into account the concentration of excreted urine at the time of collection.


A
Urine Protein
Protein mg/dl
900
800
700
600
500
400
300
200
100
0
CLR-F^WT
CLR-F^KO


B
Urine Creatinine
**P=0.006
Creatinine mmol/L
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CLR-F^WT
CLR-F^KO


C
Pr/Crea ratio
*P=0.019
Creatinine mmol/L
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
CLR-F^WT
CLR-F^KO

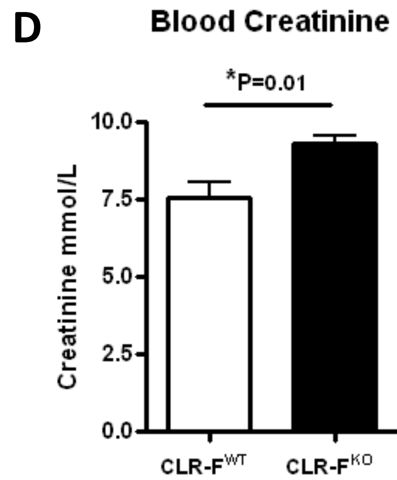
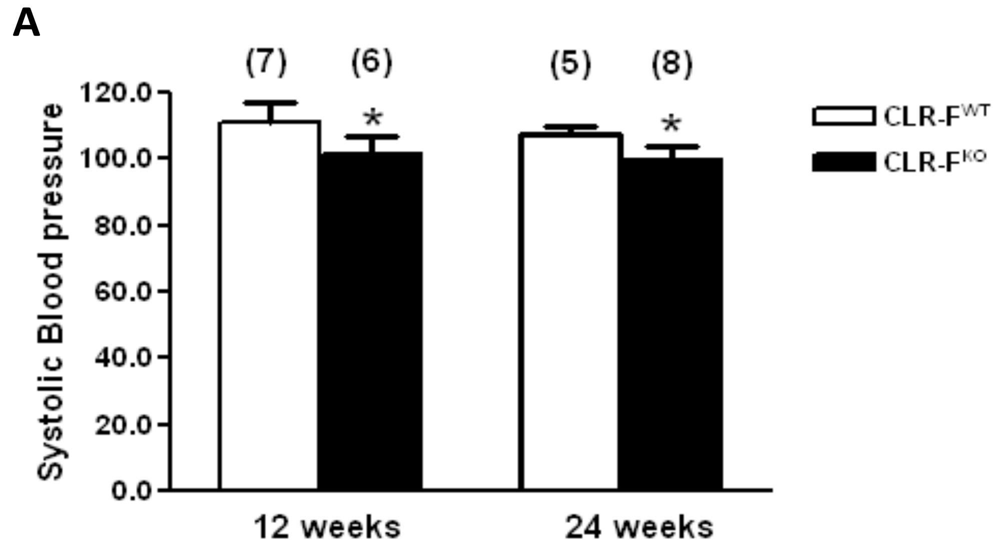

D
Blood Creatinine
*P=0.01
Creatinine mmol/L
10.0
7.5
5.0
2.5
0.0
CLR-F^WT
CLR-F^KO

Figure 16: Lower blood pressure measurements in Clr-f-deficient mice compared to their WT littermates. Systolic and diastolic blood pressures (BP) were measured at steady state by tail-cuff plethysmography in 12 and 24 weeks old male Clr-f ^{KO} and WT mice. The plotted BP values represent the mean of three-day measurements following two days of adaption period.

A



B

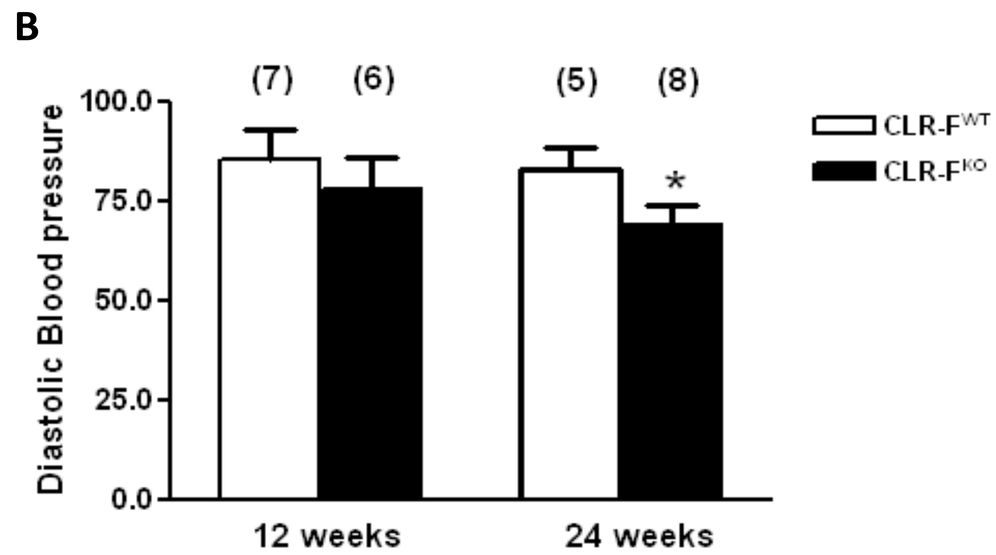
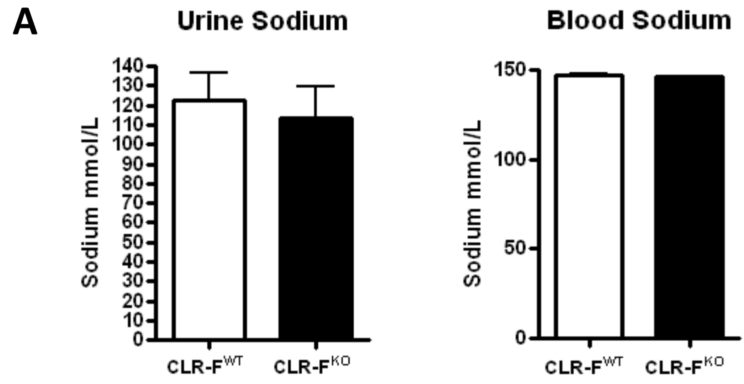
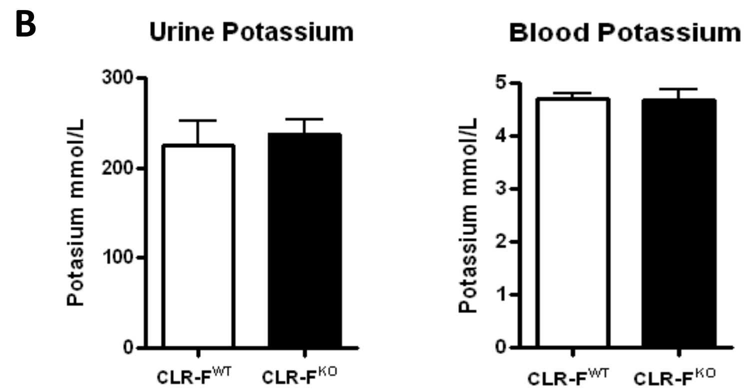
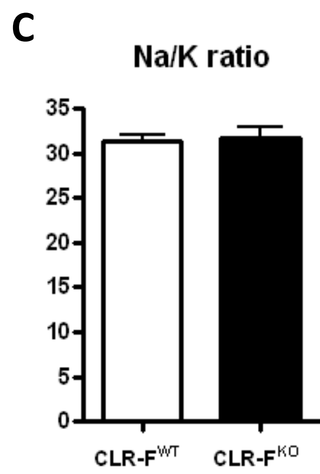


Figure 17: Comparable sodium and potassium levels but decreased FENa in Clr-f-deficient mice. (A-B) Sodium (Na) and Potassium (K) measurements on urine and blood samples from 12 weeks old male Clr-f ^{KO} and WT mice. **(C)** Urine Na/K ratio **(D)** Urinary fractional excretion of sodium (FENa) values in 12 weeks old male Clr-f ^{KO} and WT mice.


A
Urine Sodium
Sodium mmol/L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
CLR-F^WT
CLR-F^KO
Blood Sodium
Sodium mmol/L
150
100
50
0
CLR-F^WT
CLR-F^KO


B
Urine Potassium
Potasium mmol/L
300
200
100
0
CLR-F^WT
CLR-F^KO
Blood Potassium
Potassium mmol/L
5
4
3
2
1
0
CLR-F^WT
CLR-F^KO


C
Na/K ratio
35
30
25
20
15
10
5
0
CLR-F^WT
CLR-F^KO

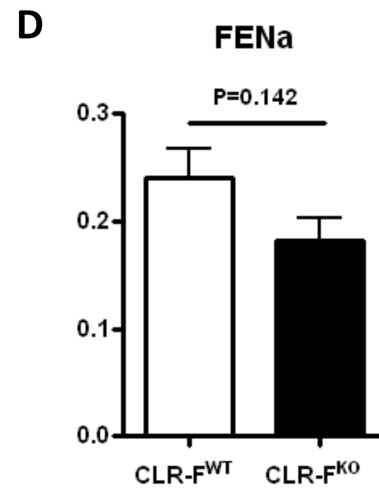
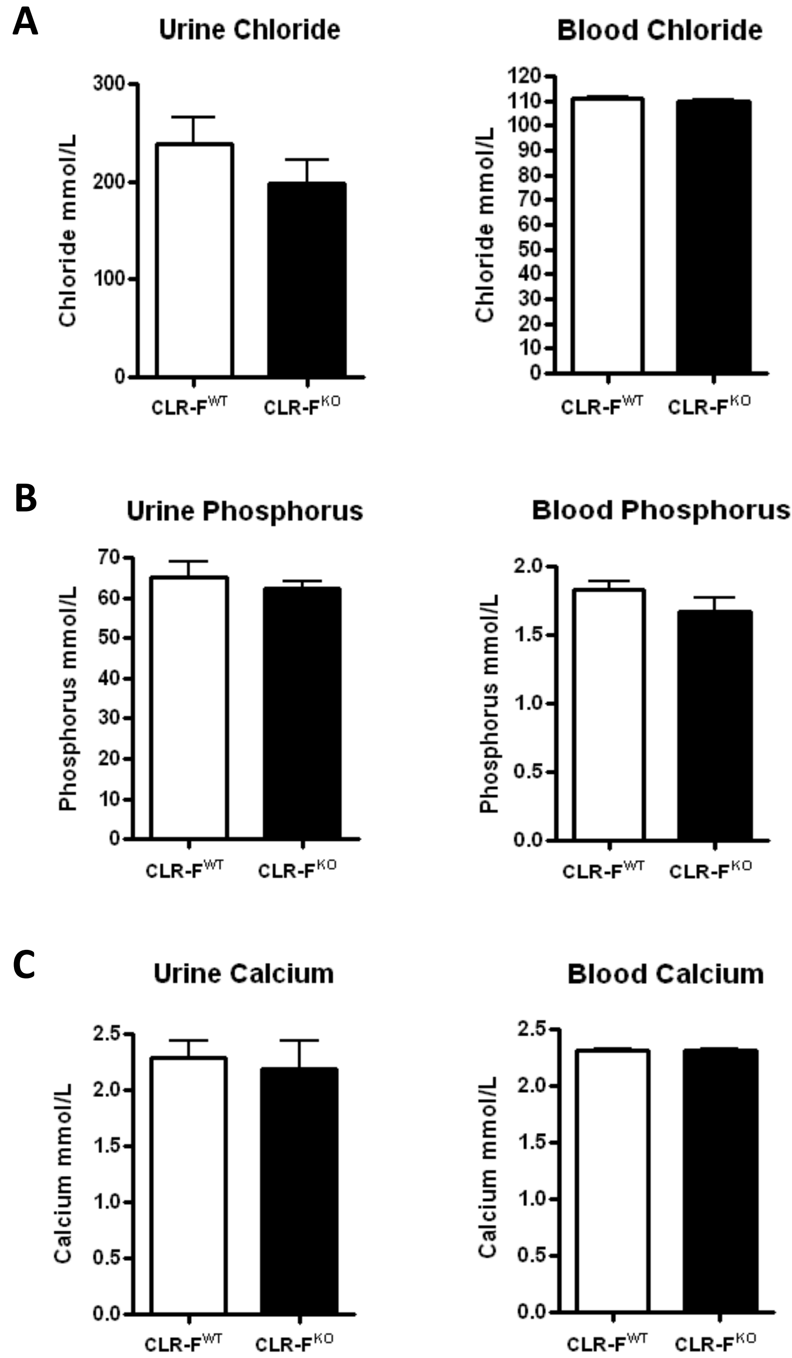

D
FENa
P=0.142
0.3
0.2
0.1
0.0
CLR-F^WT
CLR-F^KO

Figure 18: Clr-f-deficient and WT mice show similar urine and serum electrolyte levels. Bar graphs representing **(A)** urine and blood Chloride (Cl), **(B)** Phosphorus (P) and **(C)** Calcium (Ca) levels in 12 weeks old male Clr-f ^{KO} and WT mice.


A
Urine Chloride
Chloride mmol/L
300
200
100
0
CLR-F^WT
CLR-F^KO
Blood Chloride
Chloride mmol/L
120
110
100
90
80
70
60
50
40
30
20
10
0
B
Urine Phosphorus
Phosphorus mmol/L
70
60
50
40
30
20
10
0
Blood Phosphorus
2.0
1.5
1.0
0.5
0.0
C
Urine Calcium
Calcium mmol/L
2.5
Blood Calcium

5. Discussion

Surface expressed NK cell inhibitory and activating receptors have been shown to regulate NK cell-mediated surveillance of target cells (Iizuka et al. 2003, Rahim et al. 2015). Members of the NKR-P1 and the Ly49 family of receptors have been shown to be highly important in the regulation of conventional spleen and liver NK cell functions. NK cell receptors have also been expressed on the surface of non-NK cells, including ILCs and γδT cells, suggesting similar immune-regulatory mechanisms among these cells (Allan et al. 2015). ILCs and γδT cells play important roles in mediating intestinal homeostasis (Girardi et al. 2006, Hwang et al. 2013, Serafini et al. 2014, Leibelt et al. 2015). Thus, it was crucial to identify the expression of Ly49 and NKR-P1 receptors on the surface of intestinal ILCs and γδT cells and to acquire a better understanding on their potential role in intestinal immunity.

The expression of NKR-P1B and Ly49 NK cell receptors on various gut-resident lymphocytes

Using flow cytometry analysis on isolated lymphocytes from the mice intestines, we specifically showed the cellular expression pattern of the NKR-P1B receptor in different parts of the intestine. Gut-resident NK cells, ILC3 subsets and γδT cells all expressed the NKR-P1B receptor. Interestingly, the distribution of the receptor between various innate immune cells was organ specific. Our data indicate that the NKR-P1B expression is prevalent among the ILC3 lymphocytes within the lamina propria of the jejunum, colon and cecum whereas NK cells constitute most of the NKR-P1B^{+} population in mesenteric lymph nodes and secondary lymphoid organs, including liver and spleen. Unlike the high expression of the NKR-P1B receptor, Ly49 receptors were absent on ILC3s while only a small proportion of gut NK cells expressed the

Ly49 receptors. Moreover, our data revealed a significantly higher expression of the NKR-P1B receptor among intestinal leukocytes in comparison to isolated splenocytes. Previous study by Luci et al. (2009) showed that murine intestinal lamina propria NK cells display low levels of Ly49 in comparison to their spleen and liver counterparts. Inngierdingen et al. (2012) reported the presence of a prominent NKR-P1B^{bright} NK cell population in rat's gut-associated lymphoid tissue. Altogether, these results indicate that NKR-P1B is potentially the dominant receptor in regulating the functions of intestinal NK cells and ILC3s compared to Ly49, supporting the hypothesis that predicts the role of NKR-P1B:Clr-b interaction in mediating tissue-specific immune-regulatory functions (Chen et al. 2011, Rahim et al. 2015), in contrast to the suggested global Ly49:MHC-I immunosurveillance mechanism. Previous murine and human studies have determined the expression of the natural cytotoxicity NK cell receptor, Nkp46 in mice and Nkp44 in humans, on the surface of ILC3s (Spits et al. 2013). In our study, we revealed the selective predominance of the NKR-P1B NK cells receptor, but not of Ly49s on intestinal ILC3s, which is probably due to the genetic differences between the two receptors families. The genes coding for the Ly49 receptors are highly diverse in terms of gene content and gene organization (Rahim et al. 2015). Additionally, the MHC-I ligands for the Ly49 receptors are highly polymorphic molecules, which are encoded by genes present on a separate chromosome, thus allowing for independent segregation. This genetic diversity allows Ly49 receptors to bind a wide variety of molecules in an environment as heterogeneous as the intestine. In contrast to the highly diverse *Ly49* gene cluster, the genes encoding the NKR-P1 receptors and the Clr ligands are intermingled within the same genomic region, allowing for co-segregation of the genes and protection of the receptor-ligand interaction (Zhang et al. 2012, Rahim et al. 2014). Because of the tolerogenic nature of the intestinal environment, it is logical to suggest that gut-resident

immune cells have developed a selective evolutionary mechanism in order to express the highly conserved NKR-P1 receptors instead of the exceedingly polymorphic Ly49.

Identifying the expression of the NKR-P1B ligand Clr-b on gut-resident Leukocytes

After determining the intestinal cellular components that express the NKR-P1B receptor, it was crucial to determine Clr-b ligand expression in order to identify the potential interacting partners. First we performed *In-situ* hybridization on paraffin-embedded intestinal tissue sections from C57BL/6 wild-type mice using a generated Clr-b mRNA probe. Our *In-situ* stain detected the presence of Clr-b transcripts at different areas of the intestinal mucosa, including the small intestinal lamina propria, crypt and intra-epithelium. Previous microarray analysis revealed expression of Clr-b transcripts on all hematopoietic cells of various mouse organs, including the small intestine (Zhang et al. 2012). Using flow cytometry analysis we confirmed the broad expression of Clr-b on all lymphoid and myeloid immune cells within the small intestinal lamina propria. Hence, NKR-P1B:Clr-b recognition appears to be the regulation of multiple intestinal immune cell subsets. Interestingly, small intestinal innate lymphoid cells demonstrated an increased NKR-P1B mean fluorescence intensity (MFI) in Clr-b-deficient mice in comparison to their wild type counterparts. Our results agree with the previous findings, which also showed higher NKR-P1B expression on Clr-b-deficient spleen and liver NK cells (Chen et al. 2015). This could be a compensatory mechanism whereby the cells upregulate receptor expression in the absence of its ligand. Alternatively, absence of Clr-b binding may result in higher surface availability of NKR-P1B receptor for antibody binding and detection.

NKR-P1B-deficienct mice have abnormal intestinal development

To monitor whether the absence of the NKR-P1B receptor could affect the development of the intestinal tract in mice, we measured the intestinal lengths of NKR-P1B-deficient and WT mice and normalized them to the tibia length from the same mouse. The data show that NKR-P1B-deficient mice possess longer small intestines; despite having a significant lower body weight compared to their WT littermates. A potential mechanism for the observed variations in intestinal developmental between the two mice genotypes could be due to altered LTi cell functions. LTi cells are essential for the formation of lymph nodes during embryogenesis and are also present in the post-natal gut, where they are important in the structural regulation of cryptopatches and intestinal lymphoid structures (Eberl et al., 2004). Phenotypically, LTis do not express NKp46 in mice, but are differentiated from NKp46^{-} ILC3s as they express CD4. However, due to experimental limitations, these two classes of group 3 innate lymphoid cells were not differentiated during this study. Our results indicate that the cell number and percentage of Nkp46^{-} ILC3, which also include the LTi cells, are significantly higher in the NKR-P1B-deficient mice in comparison to WT mice. We also show that 80% of these cells express the NKR-P1B receptor. Therefore, it is reasonable to suggest that the NKR-P1B:Clr-b axis may play a role in modulating the functions of LTi, and its absence may lead to an abnormal development of the intestinal tract. Further studies are needed to clearly distinguish LTi cells and determine their function in the NKR-P1B-deficient mice.

Defective NK and γδ T cells response to *Salmonella typhimurium* infection in the gut of NKR-P1B-deficient mice

Gut-resident NK cells, γδT cells and ILC3s have been shown to protect the intestinal tract against dangerous pathogenic bacteria (Das et al. 2004, Inagaki et al. 2004, Satoh-Takayama et al. 2008, Ashkar et al. 2009). Although the implications of these cell types in gut immunity are becoming clear, the mechanisms regulating these cell types are poorly understood. We have now shown that NKR-P1B is expressed on intestinal ILC3, NK and γδT cells. Therefore, it was reasonable to suggest that NKR-P1B might play an important role in regulating not only intestinal NK cells, but also γδT cells and ILC3s. Few studies have addressed the role of NK cells during bacterial infections. *S.typhimurium* infection is one of the most common causes of foodborne illnesses worldwide. Murine models of *S.typhimurium* infection demonstrated an increased intestinal IFN-γ production in parallel with increased numbers of intestinal NK cells (Barthel et al. 2003, Harrington et al. 2007, Ashkar et al. 2009, Lapaque et al. 2009). Interestingly, IFNγ production by NK cells was dependent on its direct interaction with the IL-2/IL-15-secreting infected macrophages (Lapaque et al. 2009). Other types of lymphocytes have been also involved in fighting against *S.typhimurium* infection. Indeed, flow cytometry analysis on human peripheral blood demonstrated an increased proportion of NKR-P1A^{+} γδ and αβ T cells in salmonella infected individuals (Hoshina et al. 2012), suggesting an important role of NKR-P1A receptor, the human homologue of NKR-P1B, in the defense against salmonella infection. However, this study did not address the function of the receptor or the role of NKR-P1A^{+} cells in intestinal immunity. We used the NKR-P1B-deficient mice to determine the direct impact of NKR-P1B function on gut-resident NK and γδT cells. Our data demonstrated that the lack of NKR-P1B receptor on the surface of both NK and γδT cells renders them hyporesponsive

to stimuli compared to their WT counterparts. Normally, we would expect that the absence of the NKR-P1B inhibitory receptor on the surface of NK cells would augment the responsiveness to activating signals. However, the ablation of the NKR-P1B receptor rendered gut NK cells hyporesponsive to stimulation. Notably, the presence of inhibitory receptors is essential for licensing NK cells to respond to activation signals, a process known as NK cell education. Indeed, recent reports suggest that the acquisition of inhibitory Ly49 for self-MHC-I is a key step in the 'licensing' of developing NK cells to avoid a hyporesponsive state (Tu et al. 2014, Kadri et al. 2015). Further studies reported that the more of inhibitory receptors NK cells express on their surface the more educated and responsive they are to activating stimuli (Belanger et al. 2012, Rahim et al. 2015). Our data support the role of the NKR-P1B receptor in contributing to gut NK cell education and suggest that NKR-P1B-mediated immunosurveillance may be tissue-specific, rather than global as per the Ly49/KIR system in recognition of MHC-I molecules. On the other hand, there are no previous reports that describe the role of inhibitory receptors on γδT cells education, Hence, it would be difficult to conclude that the observed hyporesponsiveness of NKR-P1B deficient γδT cells is solely dependent on the absence of the receptor. Instead, it is more logical to propose that the low secretion of IFNγ by NKR-P1B-deficient NK cells during Salmonella infection could result in a more tolerant gut microenvironment, thus, preventing the activation of nearby γδT cells. Moreover, the remarkable increase in the percentage of IFNγ-producing NK cells in WT mice following Salmonella infection was not observed in the γδT cell population, which suggests that NK cells, rather than γδT cells, might play a more important role in controlling intestinal infections. Certainly, antibody-mediated depletion and adoptive transfer experiments by Kupz et al. (2013) demonstrated that $Thy1^{+}$ immature NK cells, are mainly responsible for intestinal immunity against Salmonella through the early production of IFNγ.

Our data also show that despite being a good producer of IFNγ, gut-resident NK cells are unable to degranulate and hence lack the cytotoxicity functions. It has been shown that the IFNγ production by mucosal NK cells limits systemic bacterial dissemination and sustains the accumulation of inflammatory leukocytes into sites of infection (Kupz et al. 2013, Dolowschiak et al. 2016). As expected, our pathology scoring on day 5 infected colons, demonstrates increased number of intra-epithelial lymphocytes (iELs) in WT mice compared to NKR-P1B-deficient mice. However, both mice genotypes had similar bacterial count in their feces, suggesting that the recruitment of iELs doesn't play a role in controlling luminal bacterial load. Interestingly, we detected a significantly larger bacterial counts in the spleen of NKR-P1B-deficient mice compared to their WT littermates on day 5 post-infection. Despite the large bacterial counts detected in the spleens of NKR-P1B-deficient mice at day 5 of infection, both WT and knockout mice showed similar survival kinetics at the infection dose used in this study. These results indicate the early role of the NKR-P1B receptor in sustaining minimal bacterial dissemination during the first days of infection.

Defective ILC3 responses to *Citrobacter rodentium* infection in the gut of NKR-P1B-deficient mice

The human intestinal epithelium covers a 400 square meters of surface area (Peterson and Artis, 2014) and averages 1.41 square meters in mice (Casteleyn et al. 2010). It maintains homeostasis and limits infection by providing an important physical barrier between the gut luminal content and the reactive immune cells of the underlying lamina propria (Peterson and Artis, 2014). The epithelium communicates with the lamina propria immune cells through cytokine messengers. Specifically, intestinal epithelial cells express the IL-22 receptor and responds to IL-22 secreted by immune cells within the epithelial barrier (Cella et al. 2009, Sonnenberg et al. 2011). ILC3s are immune-regulatory cells present primarily in the GI tract of mice and humans. In mice, NKp46^{+} ILC3s are important producers of IL-22 in the gut. Interestingly, IL-22 cytokine has been shown to be required for health and proliferation of epithelial cells, ensuring the fortification of the intestinal barrier (Cella et al. 2009, Sonnenberg et al. 2011, Peterson and Artis, 2014). Notably, the absence of Nkp46^{+} ILC3s has been associated with increased susceptibility to *Citrobacter rodentium* infection, a murine pathogen that models human entero-pathogenic *Escherichia coli* infections (Satoh-Takayama et al. 2008). On the other hand, Nkp46^{-} ILC3 has been found to release high amounts of IL-17 and IFNγ. IL-17 is a pro-inflammatory cytokine necessary for the activation and recruitment of neutrophils, allowing the clearance of invading microbes (Buonocore et al. 2010, Cho et al. 2016). However, the depletion of NKp46^{-} ILC3 (LTi) has been shown to ameliorate colitis in B and T-cell deficient mice infected with *Helicobacter hepaticus* (Buonocore et al. 2010), suggesting a role in mediating intestinal homeostasis, similar to Nkp46^{+} ILC3 (ILC3). ILC3 subsets constituted the highest percentage of the NKR-P1B expressing cells in the gut. Thus, it was crucial to determine the function and the

implication of the NKR-P1B receptor on the surface of ILC3 and LTi cells in regulating mucosal immunity under steady and pathogenic state. Our results showed that NKR-P1B-deficient ILC3 is hyporesponsive compared to the WT ILC3 following various stimuli. Moreover, ILC3 isolated from NKR-P1B-deficient colons infected with *C. rodentium* secreted lower levels of IL-22 compared to the uninfected NKR-P1B-deficient ILC3, suggesting an important role of the NKR-P1B in regulating ILC3 function during *C. rodentium* infection. Both NKR-P1B-deficient and WT LTi secreted similar amounts of IL-22 following stimulation. These results suggest that the regulatory mechanisms responsible for the IL-22 production by LTi are independent of the NKR-P1B receptor. Notably, the addition of IL-2 into the stimulation media significantly increased IL-22 production from both ILC subsets without altering the observed functional differences between the WT and NKR-P1B-deficient mice. This might be due to the stimulation of the IL-2 receptor on the surface of colon DCs, which may in turn activate ILC3. Indeed, similar activation mechanisms have been previously described for DC-mediated T cell activation (Wuest et al. 2011). On the other hand, PMA/Iono stimulation was unable to induce the IL-22 production by ILC3 subsets (Hoorweg et al. 2012). Previous reports have shown that the engagement of NKR-P1B with Clr-b inhibits conventional spleen and liver NK cells (Iizuka et al. 2003, Rahim et al. 2015). However, it is not surprising that NKR-P1B appears to have an activating function in ILC3s because other inhibitory receptors, for example Ly49Q on plasmacytoid dendritic cells (Tai et al. 2008), have previously been shown to have similar activating role in innate immune cells. Alternatively, this could be an indirect effect of cytokine and chemokines, which have been shown to modulate IL-22 production by ILC3s. Satoh-Takayama et al. has demonstrated the critical role of CXCL16 and IL-23 co-expression by intestinal DCs following *C. rodentium* infection. He showed that the absence of CXCR6 chemokine receptor on the surface of ILC3

impaired their IL-22 secretion and disabled the mucosal immunity against *C. rodentium*. Interestingly, CXCR6 chemokine receptor deficiency selectively decreased the number of Nkp46^{+} ILC3 in the intestinal lamina propria but not of LTi (CD4^{+} ILC3) (Satoh-Takayama et al. 2014). Also during *C. rodentium* infection, Seo et al. (2015) indicated the crucial role of CD11c^{+}CD11b^{+} intestinal lamina propria macrophages in mounting the activation of IL-22-producing ILC3 in an IL-1β dependent manner. Since we have shown that NKR-P1B is selectively expressed on colon DCs and macrophages, it could be proposed that NKR-P1B-deficient colon DCs and macrophages have an altered chemokine and cytokine production profile, consequently affecting ILC3 activation and IL-22 production. Overall, a large number of murine studies have reported the critical role of IL-22 production by ILC3s in mediating protection against *C. rodentium,* which is mostly produced in an IL-23, IL-1β and CXCL16-dependent manner (Satoh-Takayama et al. 2014, Seo et al. 2015). However, other type of lymphocytes, including Th17 cells, are able to secrete large quantities of IL-22 in the presence of IL-6, IL-23 and IL-1β (Zenewicz et al. 2011), which might mask the specific effect of ILC3. Therefore, further studies with NKR-P1B deficiency on a Rag-deficient background, which lacks all T and B cells, are required to reveal the specific role of NKR-P1B expressing ILC3s during pathogenic intestinal infection.

Clr-f in mediating immune and physiological kidney function

The interaction between the immune system and the kidney has not received much attention among the immunological community. However, various innate and adaptive immune mechanisms have been shown to contribute to the progression of chronic kidney diseases (CKD), even in the absence of an immune-mediated cause (Imig et al. 2013). We showed that Clr-f-deficient kidneys exhibit tubular and glomerular lesions in comparison to kidneys from the WT mice. This observation suggests that interrupting the interaction between the Clr-f ligand with its receptor, NKR-P1G, could result in the development of pathological lesions in the kidney. Unfortunately, we have not been able to identify the NKR-P1G-expressing immune cells in the kidney, which would be expected to interact with Clr-f expressed on renal tubules. This is partly due to our inability to acquire an antibody specific to NKR-P1G. In a study to elucidate the role of Clr-f in mediating intestinal homeostasis, Leibelt et al used antibodies specific to NKR-P1G and Clr-f to show that NKR-P1G receptor is expressed on a $CD103^{+}$ $\gamma\delta T^{bright}$ $NKG2A^{-}$ subpopulation of the intestinal intraepithelial lymphocytes, and that the intestinal epithelial cells upregulate their surface expression for Clr-f upon poly(I:C) challenge (Leibelt et al. 2014). These findings suggest that Clr-f acts as a stress ligand that inhibits the function of $NKR\text{-}P1G^{+}$ γδT cells towards the epithelial barrier in order to prevent autoimmunity.

Various clinical studies have emphasized on an indirect role of γδT cells in contributing to kidney pathogenesis, such as in mediating the progression of IgA nephropathy (IgAN) (Falk et al. 1995, Buck et al. 2002). As its name indicates, IgAN is characterized by the accumulation of IgA antibody in the glomerular mesangium and often leads to the progression of chronic kidney disease. Interestingly, detected intestinal γδT dysfunction followed by the expansion of renal γδT cells has been associated with the mechanisms underlying the pathogenesis of IgA nephropathy

(IgAN) (Falk et al. 1995, Buck et al. 2002). Similarly, an increased number of renal γδT cells have been also detected in a rat model of progressive glomerulosclerosis, also called Adriamycin-induced nephropathy (Wu et al. 2004 & Ando et al. 2001). On the contrary, we detected comparable numbers and percentages of renal γδT cells between the Clr-f-deficient mice and their WT littermates, which eliminate the possibility that the observed kidney phenotype in Clr-f-deficient mice mimics those previously described to be associated with immune dysfunction. Foregoing studies have further investigated the role of γδT cells in the context of Heymann nephritis (HN) (Salant et al. 1989). The prominent feature of active HN is defined by tubulointerstitial inflammation characterized by tubular injury and increased renal mononuclear cell infiltrates (Wu et al. 2004, Heymann et al. 1962). Although, our histology assessment revealed the presence of tubular lesions, only focal and inconsistent glomerular mononuclear cell infiltrates were detected in Clr-f-deficient kidneys. Work done by Rosenkranz et al. using γδT cell deficient mice (Rosenkranz et al. 2000) clearly demonstrated the permissive role of γδT cells in mediating renal diseases progression due to their ability in recruiting neutrophils, and activating local macrophages (Turner et al. 2012). In other words, immune-mediated kidney diseases normally reveal infiltration of inflammatory cells whether in the interstitial space or at the glomerular level. Our data has not revealed an increased or dysregulation of renal γδT cells, which commonly lead to increased mononuclear cell infiltrates, in Clr-f-deficient kidney. It is noteworthy that our analysis is based on the assumption that renal γδT cells express the NKR-P1G receptor, in a similar fashion to the previously described mucosal intra-epithelial γδT cells by Leibelt et al. (2014). However, multiple murine studies have identified diverse γδT cell subsets of limited TCR diversity that selectively home to various epithelial tissues. These include $V\gamma5^{+}\delta1$ T that predominate the intra-epithelial compartment of

the mice skin (Girardi et al. 2006) and has been determined to play a role in mediating epidermal homeostasis and wound repair (Sharp et al. 2005), the Vγ6^{+} δ1 T cells that consist the vast majority of IELs in the tongue and reproductive tract where they seem to respond to inflammation and exhibit immune-regulatory functions (Born et al. 2010, Itohara et al. 1989), and the Vγ7Vδ4/δ5 T cells that represent most of the intestinal IELs (Asarnow et al. 1989). Importantly, virus-induce myocarditis studies revealed that different γδT cell subsets are associated with either regulation (Huber et al. 2000) or pro-inflammatory outcomes (Huber et al. 2002). Altogether, these findings suggest that our assumption of kidney resident γδT cells expressing the NKR-P1G receptor might not be correct, since different γδ TCR exhibit distinct functional properties. Further transcript analysis will be necessary to determine whether renal γδT cells express the NKR-P1G receptor or whether other immune cell types in the kidney express this receptor.

Renal tubular ECs mediate various physiological functions, including ion exchange, water retention and possess high reabsorptive capabilities. Other then that, the capillary network surrounding the tubular ECs, is located downstream the glomerular capillaries, which renders them among the most sensitive cells in response to renal hypoxia (Tanaka et al. 2015). Our blood pressure measurements determined that Clr-f-deficient mice are hypotensive compared to their WT littermates. In addition, the Clr-f-deficient mice displayed high concentrations of creatinine in their blood, which commonly indicates a decreased glomerular filtration rate (GFR) and tubular dysfunction **(**Levey et al. 2003, Shimada et al. 2016, Paradeep 2016**).** Previous clinical observations have correlated the development of renal failure with preceding episodes of relative hypotension (Vincent et al. 2009, Lehman et al. 2010, Onuigbo et al. 2015). One would suggest that the link between hypotension and kidney diseases is a cause-and-effect relationship.

However, various mechanisms can be responsible for the apparent link. For example, the lack of oxygenation at the tubulointerstial level due to the decreased blood pressure inside the tubular capillaries may develop renal hypoxia (Tanaka et al. 2015). Recent studies have reported that the partial pressure in the renal cortex and medulla in various animals is between 20-60 and 10-30 mmHg, respectively, with some studies reporting even lower values (Tanaka et al. 2014, Tanaka et al. 2015). The innate existence of a physiological hypoxic state together with the high metabolic nature of tubular epithelial cells has compelled nephrologists to consider renal tissue hypoxia as a viable model for the progression of chronic kidney disease (Tanaka et al. 2015, Fu et al. 2016). It is possible that renal hypoxia could be the cause of the observed kidney phenotype in the Clr-f-deficient mice. However, we have not been able to define the origin of hypotension in the Clr-f-deficient mice. Notably, the blood pressure (BP) is regulated by multiple interacting physiological systems. These include the renal, cardiovascular and endocrine systems (Coffman et al. 2011, Messaoudi et al. 2015). Since the absence of Clr-f on renal tubular epithelial cells resulted in their damage, it is reasonable to suggest that this may result in a decrease in BP through disrupting the control of sodium and water reabsorption by tubular epithelial cells. It would be normal to expect an increased water and sodium reabsorption by Clr-f-deficient kidneys as a typical compensatory mechanism for the detected hypotension (Coffman et al. 2011, Messaoudi et al. 2015). Indeed, Clr-f-deficient mice exhibited a significant increase in their body-weight compared to their WT littermates, which could indicate body fluid retention. Generally, the fluids within the body have a regulated osmotic pressure depending on the amount of proteins, sugars and salts dissolved in the blood (Messaoudi et al. 2015). Importantly, electrolytes including organic salts, such as NaCl, possess a great osmotic power in comparison to other molecules because they can dissociate into multiple ions. For this reason, when tubular

epithelial cells reabsorb abnormal amount of salts into the blood, it is always coupled with fluid shift to maintain osmotic balance (MRCPath Lowe 2005, Body Fluids 2013). Interestingly, the Clr-f-deficient mice exhibited lower percentage of urinary sodium excretion, also refereed as FeNa, in comparison to their WT control group. Surprisingly, the observed decrease in urinary Na did not correlate with an increased Na reabsorption into the blood of the knockout mice. Moreover, the two mouse genotypes showed similar concentrations of various electrolytes in both of their urine and blood samples. In summary, Clr-f does not appear to regulate BP through direct handling and excretion of electrolytes by tubular epithelial cells. Alternatively, dysfunctional responses of the closely associated peritubular endothelial cells may also play a role in modulating tubular epithelial injury and regulation of BP (Tasnim et al. 2012, Messaoudi et al. 2015). In particular, this can be achieved by increasing vasodilatory responses such as nitric oxide (NO) production (Messaoudi et al. 2015). Further studies, including measurement of endothelial nitric oxide synthase (NOS3), are required to address these possibilities (Messaoudi et al. 2015). Endothelial cells are normally protected by autocrine and paracrine signals that include the release of vascular endothelial growth factor (VEGF). Several studies have demonstrated that macrophages (Harmey et al. 1998, Duffy et al. 2004) and the epithelial cells of proximal and distal tubules (Tasnim et al. 2012, Tanaka et al. 2015) have the capacity to produce VEGF. In addition, endothelial cells of the peritubullar capillaries and the glomerular capillary loop express the VEGF receptors (Tanaka et al. 2015), hence its crucial role in maintaining the health of renal capillaries. Interestingly, the VEGF expression was shown to be among the list of 100 genes that respond to hypoxia-inducible factor (HIF), which is predominantly produced by tubular epithelial cells in the hypoxic kidney (Tanaka et al. 2015). Moreover, the VEGF production by macrophages is regulated by the augmentation of HIF responses (Tanaka et al.

2015). We have found that the closely related NKR-P1B receptor is broadly expressed on the intestinal innate lymphoid cells, as well as the colon macrophage and DC subsets. Similarly, NKR-P1G may have a broader expression profile on immune cells than previously described (Leibelt et al. 2014). NKR-P1G expression on kidney resident DCs or macrophages would be expected to play a role in regulating their function through interaction with Clr-f expressed in the renal tubules. Therefore, Clr-f-deficiency on kidney tubular epithelial cells might directly affect their VEGF production leading to dysfunctional epithelial-endothelial cross-talk. Finally, since Clr-f transcripts are absent from kidney glomeruli (Zhang et al. 2012) the observed glomerular damage in the Clr-f-deficient mice could be secondary to the damaged Clr-f-expressing kidney tubules, which might directly or indirectly affect the glomerular capillaries through renal-mediated hypoxia.

In conclusion, the exact function and immune-mechanisms of action of Clr-f in the kidney needs further investigations. Our data suggest that lack of Clr-f from tubular epithelial cells has minimal effect on renal electrolyte homeostasis while likely reflecting imbalanced water reabsorption and revealing its critical role in maintaining normal kidney filtration, which can all contribute to hypotension and the progression of renal hypoxia.

6. Summary and Future Perspectives

In our attempt to study the function of the NKR-P1B receptor in mediating intestinal homeostasis, we showed its broad expression on the gut-resident ILC3, NK and γδT cell, as well as DC and macrophages in the colon of WT mice. This broad expression pattern is different than what was previously thought and observed in the spleen, where NKR-P1B is expressed specifically on NK cells (Carlyle et al. 2004). In addition, NKR-P1B expression is more predominant in ILC3 than the NK cells in the gut. Moreover, NKR-P1B but not Ly49 inhibitory receptors appear to be predominantly expressed on the gut-resident NK cells. NKR-P1B has been shown to be an inhibitory receptor, and its engagement by Clr-b inhibits NK cell function in mice (Carlyle et al. 2004, Zhang et al. 2012). In contrast, our results demonstrated that both NKR-P1B-deficient NK and γδT cells in the gut were hyporesponsive and secreted low levels of IFNγ compared to their WT counterparts upon *S. thyphimurium* infection. Similarly, NKR-P1B-deficient ILC3s displayed a deficiency in IL-22 cytokine secretion upon *C. rodentium* infection in the gut. It is possible that the absence of the NKR-P1B receptor may have an effect on the normal development of the innate intestinal immune cells, thereby rendering them hypo-functional. Further studies using blocking antibodies against the NKR-P1B receptor in WT mice are required to fully understand its role in the intestinal innate immune cell function. Moreover, NKR-P1B deficiency also resulted in the dissemination of Salmonella infection to the spleen, which is not observed in the WT mice. Despite the hyporesponsive nature of NK, γδT and ILC3 cells, NKR-P1B-deficient mice did not show higher susceptibility to infection by Salmonella and Citrobacter, compared to the WT mice. A competent adaptive immune response may compensate for the hyporesponsive innate immune response in these mice. It is therefore necessary to analyze innate immune cell function using these bacterial infection models on Rag-deficient

mice, in which T and B cells are absent. Taken together, the NKR-P1B receptor expression on various innate immune intestinal cells is a significant finding and sparks interest into further investigating on how the NKR-P1B:Clr-b recognition system works to regulate mucosal immunity. The human NKR-P1A is an inhibitory receptor on NK cells and has been considered as the functional homolog of the mouse NKR-P1B (Kirkham et al. 2014). Previous clinical findings have reported that human NK cells exhibit phenotypic alterations in patients with inflammatory bowel diseases (IBD) (Takayama et al. 2010, Yadav et al. 2011). In addition, human ILCs appear to have diverse function in mediating immunity against infection (eg: *Citrobacter rodentium*) and normal microbiota, and abnormal ILC3 numbers have been observed in human models of autoimmune diseases, such as Crohn's disease (CD) and ulcerative colitis (UC) (Buonocore et al. 2010). Also, the human NKR-P1A$^+$ γδT cells have been reported to increase in numbers during *Salmonella typhimurium* infection, and were suggested to play an important role in the early defense mechanism against bacterial infection (Hoshina et al. 2012). In summary, our NKR-P1B-deficient mice may prove to be a useful model to study human NKR-P1A receptor functions, in order to fully understand the mechanisms underlying regulation of gut-resident innate lymphoid cell functions, and thus intestinal immunity and health.

We showed earlier in the results section that Clr-f-deficient kidneys have a damaged tubular system and a disrupted filtration capacity compared to their WT control group. Our observations revealed that the discovered phenotype is independent of lymphocytic innate-immune infiltrates, since we detected comparable numbers and percentages of renal γδT and NK cells between the two mouse genotypes. However, we have also showed that the closely related NKR-P1B receptor is broadly expressed on intestinal innate lymphoid cells, as well as macrophages in the colon and DC subsets. Similarly, NKR-P1G may also have a broader expression profile on

various immune cell types. It is crucial to keep in mind that renal DCs and macrophages are in close proximity with the tubular system (Ferenbach et al. 2008, Weisheit et al. 2015) and constitute the majority of resident immune cells, whereas fewer lymphocytes are restricted to the renal tissue (Weisheit et al. 2015). In particular, $CD11c^+$ dendritic cells appear to be restricted to the cortical tubulointerstitium of healthy kidney and mostly absent from the glomeruli. On the other hand, renal macrophages are found in the glomeruli, but are more abundant in the medulla (Ferenbach et al. 2008, Hochheiser et al. 2013, Weisheit et al. 2015). The location of dendritic cells within the tubulointerstitium represents the optimal site for tubular self-antigens sampling as well as for accessing intravascular antigens and small molecular weight antigens that escaped the initial glomerular filtration (Ferenbach et al. 2008). Importantly, several studies have associated the intra-renal accumulation of DCs and macrophages with the progression of chronic kidney disease (Ferenbach et al. 2008, Hochheiser et al. 2013, Weisheit et al. 2015). Since we don't have the antibody against the NKR-P1G receptor, it will be necessary at first to perform a transcript analysis in order to identify the interacting immune cell partners with the Clr-f expressing tubular epithelial cells. In case renal DCs and Mac do not appear to express the receptor, another straightforward approach that will help study their role in mediating the observed kidney phenotype would include the depletion of renal DCs and macrophages using clodronate liposomes (Weisheit et al. 2015) or better, using chemokine receptor blockage (Weisheit et al. 2015, Tacke et al. 2007, Vielhauer et al. 2010). The inhibition of chemokine receptors, such as CX3CR1 is a highly desirable therapeutic technique against DCs and macrophage-mediated renal inflammation, because it has few side effects on the myeloid subset of other organs (Weisheit et al. 2015). However, it is noteworthy that under inflammatory conditions, the recruitment of monocytes into the renal tissue is not solely restricted to CX3CR1.

Other chemokine receptors, such as CCR1, CCR2, and CCR5 have been implicated to compensate for the loss of CX3CR1 (Tacke et al. 2007, Vielhauer et al. 2010). Since we don't know the immune cell types that actually express the NKR-P1G receptor, it will first be necessary to analyze renal innate immune cell function using Rag-deficient Clr-f ^{KO} mice, in order to determine the role of adaptive immune arm in mediating the observed phenotype. Similarly, TCRδ-deficient mice can also be used to test for the role of γδT cells in mediating renal homeostasis. Finally, to test for the role of NK cells in mediating the observed tubular and glomerular damage, NK1.1 depletion studies can also be performed on various Clr-f ^{KO} mice background. Altogether, the latter experimental suggestions assume that the renal immune system decision of whether to induce immunity or not is based on the agreement and the integration of signals between several immune cell types in order to reduce the likelihood of autoimmunity.

7. List of References

Aldemir, H., Prod'homme, V., Dumaurier, M.-J., Retiere, C., Poupon, G., Cazareth, J., … Braud, V. M. (2005). Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor. *Journal of Immunology (Baltimore, Md.: 1950)*, *175*(12), 7791–7795.

Allan, D. S. J., Kirkham, C. L., Aguilar, O. A., Qu, L. C., Chen, P., Fine, J. H., … Carlyle, J. R. (2015). An in vitro model of innate lymphoid cell function and differentiation. *Mucosal Immunology*, *8*(2), 340–351. http://doi.org/10.1038/mi.2014.71

Allez, M., Tieng, V., Nakazawa, A., Treton, X., Pacault, V., Dulphy, N., … Toubert, A. (2007). CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions. *Gastroenterology*, *132*(7), 2346–2358. http://doi.org/10.1053/j.gastro.2007.03.025

Ando, T., Wu, H., Watson, D., Hirano, T., Hirakata, H., Fujishima, M., & Knight, J. F. (2001). Infiltration of canonical Vgamma4/Vdelta1 gammadelta T cells in an adriamycin-induced progressive renal failure model. *Journal of Immunology (Baltimore, Md.: 1950)*, *167*(7), 3740–3745.

Appasamy, P. M., Kenniston, T. W., Weng, Y., Holt, E. C., Kost, J., & Chambers, W. H. (1993). Interleukin 7-induced expression of specific T cell receptor gamma variable region genes in murine fetal liver cultures. *The Journal of Experimental Medicine*, *178*(6), 2201–2206.

Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., & Lanier, L. L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science (New York, N.Y.)*, *296*(5571), 1323–1326. http://doi.org/10.1126/science.1070884

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews Immunology*, *8*(6), 411–420. http://doi.org/10.1038/nri2316

Artis, D., & Spits, H. (2015). The biology of innate lymphoid cells. *Nature*, *517*(7534), 293–301. http://doi.org/10.1038/nature14189

Asarnow, D. M., Goodman, T., LeFrancois, L., & Allison, J. P. (1989). Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. *Nature*, *341*(6237), 60–62. http://doi.org/10.1038/341060a0

Ashkar, A. A., Reid, S., Verdu, E. F., Zhang, K., & Coombes, B. K. (2009). Interleukin-15 and NK1.1+ cells provide innate protection against acute Salmonella enterica serovar Typhimurium infection in the gut and in systemic tissues. *Infection and Immunity*, *77*(1), 214–222. http://doi.org/10.1128/IAI.01066-08

Banas, M. C., Banas, B., Hudkins, K. L., Wietecha, T. A., Iyoda, M., Bock, E., … Alpers, C. E. (2008). TLR4 links podocytes with the innate immune system to mediate glomerular injury. *Journal of the American Society of Nephrology: JASN*, *19*(4), 704–713. http://doi.org/10.1681/ASN.2007040395

Bartel, Y., Bauer, B., & Steinle, A. (2013). Modulation of NK cell function by genetically coupled C-type lectin-like receptor/ligand pairs encoded in the human natural killer gene complex. *Frontiers in Immunology*, *4*, 362. http://doi.org/10.3389/fimmu.2013.00362

Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., … Hardt, W.-D. (2003). Pretreatment of Mice with Streptomycin Provides a Salmonella enterica

Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host. *Infection and Immunity*, *71*(5), 2839–2858. http://doi.org/10.1128/IAI.71.5.2839-2858.2003

Bauer, B., Spreu, J., Rohe, C., Vogler, I., & Steinle, A. (2015). Key residues at the membrane-distal surface of KACL, but not glycosylation, determine the functional interaction of the keratinocyte-specific C-type lectin-like receptor KACL with its high-affinity receptor NKp65. *Immunology*, *145*(1), 114–123. http://doi.org/10.1111/imm.12432

Bélanger, S., Tu, M. M., Rahim, M. M. A., Mahmoud, A. B., Patel, R., Tai, L.-H., … Makrigiannis, A. P. (2012). Impaired natural killer cell self-education and “missing-self” responses in Ly49-deficient mice. *Blood*, *120*(3), 592–602. http://doi.org/10.1182/blood-2012-02-408732

Bernink, J. H., Peters, C. P., Munneke, M., te Velde, A. A., Meijer, S. L., Weijer, K., … Spits, H. (2013). Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nature Immunology*, *14*(3), 221–229. http://doi.org/10.1038/ni.2534

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P., & Salazar-Mather, T. P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual Review of Immunology*, *17*, 189–220. http://doi.org/10.1146/annurev.immunol.17.1.189

Björkström, N. K., Kekäläinen, E., & Mjösberg, J. (2013). Tissue-specific effector functions of innate lymphoid cells. *Immunology*, *139*(4), 416–427. http://doi.org/10.1111/imm.12098

Björkström, N. K., Riese, P., Heuts, F., Andersson, S., Fauriat, C., Ivarsson, M. A., … Malmberg, K.-J. (2010). Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood*, *116*(19), 3853–3864.

Body Fluids. Retrieved from http://anatomyandphysiologyi.com/body-fluids (November 2013)

Born, W. K., Yin, Z., Hahn, Y.-S., Sun, D., & O'Brien, R. L. (2010). Analysis of gamma delta T cell functions in the mouse. *Journal of Immunology (Baltimore, Md.: 1950)*, *184*(8), 4055–4061. http://doi.org/10.4049/jimmunol.0903679

Brandes, M., Willimann, K., & Moser, B. (2005). Professional antigen-presentation function by human gammadelta T Cells. *Science (New York, N.Y.)*, *309*(5732), 264–268. http://doi.org/10.1126/science.1110267

Bregenholt, S., Berche, P., Brombacher, F., & Di Santo, J. P. (2001). Conventional alpha beta T cells are sufficient for innate and adaptive immunity against enteric Listeria monocytogenes. *Journal of Immunology (Baltimore, Md.: 1950)*, *166*(3), 1871–1876.

Buck, K. S., Foster, E. M., Watson, D., Barratt, J., Pawluczyk, I. Z. A., Knight, J. F., … Allen, A. C. (2002). Expression of T cell receptor variable region families by bone marrow gammadelta T cells in patients with IgA nephropathy. *Clinical and Experimental Immunology*, *127*(3), 527–532.

Buonocore, S., Ahern, P. P., Uhlig, H. H., Ivanov, I. I., Littman, D. R., Maloy, K. J., & Powrie, F. (2010). Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature*, *464*(7293), 1371–1375. http://doi.org/10.1038/nature08949

Cai, G., Anumanthan, A., Brown, J. A., Greenfield, E. A., Zhu, B., & Freeman, G. J. (2008). CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nature Immunology*, *9*(2), 176–185. http://doi.org/10.1038/ni1554

Carlyle, J. R., Jamieson, A. M., Gasser, S., Clingan, C. S., Arase, H., & Raulet, D. H. (2004). Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proceedings of*

the National Academy of Sciences of the United States of America, *101*(10), 3527–3532. http://doi.org/10.1073/pnas.0308304101

Carlyle, J. R., Mesci, A., Fine, J. H., Chen, P., Bélanger, S., Tai, L.-H., & Makrigiannis, A. P. (2008). Evolution of the Ly49 and Nkrp1 recognition systems. *Seminars in Immunology*, *20*(6), 321–330. http://doi.org/10.1016/j.smim.2008.05.004

Carlyle, J. R., Mesci, A., Ljutic, B., Belanger, S., Tai, L.-H., Rousselle, E., … Makrigiannis, A. P. (2006). Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *Journal of Immunology (Baltimore, Md.: 1950)*, *176*(12), 7511–7524.

Casteleyn, C., Rekecki, A., Van der Aa, A., Simoens, P., & Van den Broeck, W. (2010). Surface area assessment of the murine intestinal tract as a prerequisite for oral dose translation from mouse to man. *Laboratory Animals*, *44*(3), 176–183. http://doi.org/10.1258/la.2009.009112

Cella, M., Fuchs, A., Vermi, W., Facchetti, F., Otero, K., Lennerz, J. K. M., … Colonna, M. (2009). A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*, *457*(7230), 722–725. http://doi.org/10.1038/nature07537

Cella, M., Otero, K., & Colonna, M. (2010). Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(24), 10961–10966. http://doi.org/10.1073/pnas.1005641107

Chang, Y.-J., Kim, H. Y., Albacker, L. A., Baumgarth, N., McKenzie, A. N. J., Smith, D. E., … Umetsu, D. T. (2011). Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nature Immunology*, *12*(7), 631–638. http://doi.org/10.1038/ni.2045

Chen, J., John, R., Richardson, J. A., Shelton, J. M., Zhou, X. J., Wang, Y., … Lu, C. Y. (2011). Toll-like receptor 4 regulates early endothelial activation during ischemic acute kidney injury. *Kidney International*, *79*(3), 288–299. http://doi.org/10.1038/ki.2010.381

Chen, P., Bélanger, S., Aguilar, O. A., Zhang, Q., St-Laurent, A., Rahim, M. M. A., … Carlyle, J. R. (2011). Analysis of the mouse 129-strain Nkrp1-Clr gene cluster reveals conservation of genomic organization and functional receptor-ligand interactions despite significant allelic polymorphism. *Immunogenetics*, *63*(10), 627–640. http://doi.org/10.1007/s00251-011-0542-8

Cheroutre, H., Lambolez, F., & Mucida, D. (2011). The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Reviews. Immunology*, *11*(7), 445–456. http://doi.org/10.1038/nri3007

Cherrier, M., Sawa, S., & Eberl, G. (2012). Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *The Journal of Experimental Medicine*, *209*(4), 729–740. http://doi.org/10.1084/jem.20111594

Chinen, H., Matsuoka, K., Sato, T., Kamada, N., Okamoto, S., Hisamatsu, T., … Hibi, T. (2007). Lamina propria c-kit+ immune precursors reside in human adult intestine and differentiate into natural killer cells. *Gastroenterology*, *133*(2), 559–573. http://doi.org/10.1053/j.gastro.2007.05.017

Cho, C. G., Gong, S. H., Kim, H.-B., Song, J.-J., Park, J. H., Lim, Y.-S., & Park, S.-W. (2016). Role of group 3 innate lymphoid cells during experimental otitis media in a rat model. *International Journal of Pediatric Otorhinolaryngology*, *88*, 146–152. http://doi.org/10.1016/j.ijporl.2016.07.001

Coffman, T. M. (2011). Under pressure: the search for the essential mechanisms of hypertension. *Nature Medicine*, *17*(11), 1402–1409. http://doi.org/10.1038/nm.2541

Couzi, L., Pitard, V., Moreau, J.-F., Merville, P., & Déchanet-Merville, J. (2015). Direct and Indirect Effects of Cytomegalovirus-Induced γδ T Cells after Kidney Transplantation. *Frontiers in Immunology*, *6*, 3. http://doi.org/10.3389/fimmu.2015.00003

Couzi, L., Pitard, V., Netzer, S., Garrigue, I., Lafon, M.-E., Moreau, J.-F., … Déchanet-Merville, J. (2009). Common features of gammadelta T cells and CD8(+) alphabeta T cells responding to human cytomegalovirus infection in kidney transplant recipients. *The Journal of Infectious Diseases*, *200*(9), 1415–1424. http://doi.org/10.1086/644509

Crellin, N. K., Trifari, S., Kaplan, C. D., Satoh-Takayama, N., Di Santo, J. P., & Spits, H. (2010). Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2. *Immunity*, *33*(5), 752–764. http://doi.org/10.1016/j.immuni.2010.10.012

Cupedo, T., Crellin, N. K., Papazian, N., Rombouts, E. J., Weijer, K., Grogan, J. L., … Spits, H. (2009). Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nature Immunology*, *10*(1), 66–74. http://doi.org/10.1038/ni.1668

Dambacher, J., Beigel, F., Zitzmann, K., De Toni, E. N., Göke, B., Diepolder, H. M., … Brand, S. (2009). The role of the novel Th17 cytokine IL-26 in intestinal inflammation. *Gut*, *58*(9), 1207–1217. http://doi.org/10.1136/gut.2007.130112

Das, H., Sugita, M., & Brenner, M. B. (2004). Mechanisms of Vdelta1 gammadelta T cell activation by microbial components. *Journal of Immunology (Baltimore, Md.: 1950)*, *172*(11), 6578–6586.

Déchanet, J., Merville, P., Bergé, F., Bone-Mane, G., Taupin, J. L., Michel, P., … Moreau, J. F. (1999). Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients. *The Journal of Infectious Diseases*, *179*(1), 1–8. http://doi.org/10.1086/314568

Diefenbach, A. (2013). Innate lymphoid cells in the defense against infections. *European Journal of Microbiology & Immunology*, *3*(3), 143–151. http://doi.org/10.1556/EuJMI.3.2013.3.1

Diefenbach, A., Jensen, E. R., Jamieson, A. M., & Raulet, D. H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*, *413*(6852), 165–171. http://doi.org/10.1038/35093109

Dolowschiak, T., Mueller, A. A., Pisan, L. J., Feigelman, R., Felmy, B., Sellin, M. E., … Hardt, W.-D. (2016). IFN-γ Hinders Recovery from Mucosal Inflammation during Antibiotic Therapy for Salmonella Gut Infection. *Cell Host & Microbe*, *20*(2), 238–249. http://doi.org/10.1016/j.chom.2016.06.008

Duffy, A. M., Bouchier-Hayes, D. J., & Harmey, J. H. (2004). Vascular Endothelial Growth Factor (VEGF) and Its Role in Non-Endothelial Cells: Autocrine Signalling by VEGF. In J. H. Harmey, *VEGF and Cancer* (pp. 133–144). Boston, MA: Springer US. Retrieved from http://link.springer.com/10.1007/978-1-4419-9148-5_13

Dyring-Andersen, B., Geisler, C., Agerbeck, C., Lauritsen, J. P. H., Gúdjonsdottir, S. D., Skov, L., & Bonefeld, C. M. (2014). Increased number and frequency of group 3 innate lymphoid cells in nonlesional psoriatic skin. *The British Journal of Dermatology*, *170*(3), 609–616. http://doi.org/10.1111/bjd.12658

Eberl, G., Marmon, S., Sunshine, M.-J., Rennert, P. D., Choi, Y., & Littman, D. R. (2004). An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nature Immunology*, *5*(1), 64–73. http://doi.org/10.1038/ni1022

Emoto, Y., Emoto, M., Miyamoto, M., Yoshizawa, I., & Kaufmann, S. H. E. (2004). Functionally active CD8alphabeta+ TCRgammadelta intestinal intraepithelial lymphocytes in athymic nu/nu mice. *International Immunology*, *16*(1), 111–117.

Ericsson, A., Svensson, M., Arya, A., & Agace, W. W. (2004). CCL25/CCR9 promotes the induction and function of CD103 on intestinal intraepithelial lymphocytes. *European Journal of Immunology*, *34*(10), 2720–2729. http://doi.org/10.1002/eji.200425125

Falk, M. C., Ng, G., Zhang, G. Y., Fanning, G. C., Roy, L. P., Bannister, K. M., … Knight, J. F. (1995). Infiltration of the kidney by alpha beta and gamma delta T cells: effect on progression in IgA nephropathy. *Kidney International*, *47*(1), 177–185.

Fehniger, T. A., Cooper, M. A., Nuovo, G. J., Cella, M., Facchetti, F., Colonna, M., & Caligiuri, M. A. (2003). CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood*, *101*(8), 3052–3057. http://doi.org/10.1182/blood-2002-09-2876

Ferenbach, D., & Hughes, J. (2008). Macrophages and dendritic cells: what is the difference? *Kidney International*, *74*(1), 5–7. http://doi.org/10.1038/ki.2008.189

Fine, J. H., Chen, P., Mesci, A., Allan, D. S. J., Gasser, S., Raulet, D. H., & Carlyle, J. R. (2010). Chemotherapy-induced genotoxic stress promotes sensitivity to natural killer cell cytotoxicity by enabling missing-self recognition. *Cancer Research*, *70*(18), 7102–7113. http://doi.org/10.1158/0008-5472.CAN-10-1316

Fisch, P., Meuer, E., Pende, D., Rothenfusser, S., Viale, O., Kock, S., … van der Bruggen, P. (1997). Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. *European Journal of Immunology*, *27*(12), 3368–3379. http://doi.org/10.1002/eji.1830271236

Fu, Q., Colgan, S. P., & Shelley, C. S. (2016). Hypoxia: The Force that Drives Chronic Kidney Disease. *Clinical Medicine & Research*, *14*(1), 15–39. http://doi.org/10.3121/cmr.2015.1282

Fu, Q., Colgan, S. P., & Shelley, C. S. (2016). Hypoxia: The Force that Drives Chronic Kidney Disease. *Clinical Medicine & Research*, *14*(1), 15–39. http://doi.org/10.3121/cmr.2015.1282

Fuchs, A., & Colonna, M. (2013). Innate lymphoid cells in homeostasis, infection, chronic inflammation and tumors of the gastrointestinal tract. *Current Opinion in Gastroenterology*, *29*(6), 581–587. http://doi.org/10.1097/MOG.0b013e328365d339

Fuchs, A., Vermi, W., Lee, J. S., Lonardi, S., Gilfillan, S., Newberry, R. D., … Colonna, M. (2013). Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. *Immunity*, *38*(4), 769–781. http://doi.org/10.1016/j.immuni.2013.02.010

Gallo, R. L., & Hooper, L. V. (2012). Epithelial antimicrobial defence of the skin and intestine. *Nature Reviews. Immunology*, *12*(7), 503–516. http://doi.org/10.1038/nri3228

Garcia-Lora, A., Algarra, I., & Garrido, F. (2003). MHC class I antigens, immune surveillance, and tumor immune escape. *Journal of Cellular Physiology*, *195*(3), 346–355. http://doi.org/10.1002/jcp.10290

Girardi, M. (2006). Immunosurveillance and immunoregulation by gammadelta T cells. *The Journal of Investigative Dermatology*, *126*(1), 25–31. http://doi.org/10.1038/sj.jid.5700003

Gordon, S. M., Chaix, J., Rupp, L. J., Wu, J., Madera, S., Sun, J. C., … Reiner, S. L. (2012). The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity*, *36*(1), 55–67. http://doi.org/10.1016/j.immuni.2011.11.016

Gregorieff, A., & Clevers, H. (2010). In situ hybridization to identify gut stem cells. *Current Protocols in Stem Cell Biology*, *Chapter 2*, Unit 2F.1. http://doi.org/10.1002/9780470151808.sc02f01s12

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., & Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(22), 12445–12450.

Haas, W., Pereira, P., & Tonegawa, S. (1993). Gamma/delta cells. *Annual Review of Immunology*, *11*, 637–685. http://doi.org/10.1146/annurev.iy.11.040193.003225

Halary, F., Pitard, V., Dlubek, D., Krzysiek, R., de la Salle, H., Merville, P., … Déchanet-Merville, J. (2005). Shared reactivity of V{delta}2(neg) {gamma}{delta} T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. *The Journal of Experimental Medicine*, *201*(10), 1567–1578. http://doi.org/10.1084/jem.20041851

Halim, T. Y. F., Krauss, R. H., Sun, A. C., & Takei, F. (2012). Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity*, *36*(3), 451–463. http://doi.org/10.1016/j.immuni.2011.12.020

Halim, T. Y. F., MacLaren, A., Romanish, M. T., Gold, M. J., McNagny, K. M., & Takei, F. (2012). Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell

development and allergic inflammation. *Immunity*, *37*(3), 463–474. http://doi.org/10.1016/j.immuni.2012.06.012

Hao, L., Klein, J., & Nei, M. (2006). Heterogeneous but conserved natural killer receptor gene complexes in four major orders of mammals. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(9), 3192–3197. http://doi.org/10.1073/pnas.0511280103

Harmey, J. H., Dimitriadis, E., Kay, E., Redmond, H. P., & Bouchier-Hayes, D. (1998). Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor beta-1. *Annals of Surgical Oncology*, *5*(3), 271–278.

Harrington, L., Srikanth, C. V., Antony, R., Shi, H. N., & Cherayil, B. J. (2007). A role for natural killer cells in intestinal inflammation caused by infection with Salmonella enterica serovar Typhimurium. *FEMS Immunology and Medical Microbiology*, *51*(2), 372–380. http://doi.org/10.1111/j.1574-695X.2007.00313.x

Hato, T., & Dagher, P. C. (2015). How the Innate Immune System Senses Trouble and Causes Trouble. *Clinical Journal of the American Society of Nephrology: CJASN*, *10*(8), 1459–1469. http://doi.org/10.2215/CJN.04680514

He, W., Zhang, Y., Deng, Y., & Kabelitz, D. (1995). Induction of TCR-gamma delta expression on triple-negative (CD3-4-8-) human thymocytes. Comparative analysis of the effects of IL-4 and IL-7. *Journal of Immunology (Baltimore, Md.: 1950)*, *154*(8), 3726–3731.

Hepworth, M. R., Monticelli, L. A., Fung, T. C., Ziegler, C. G. K., Grunberg, S., Sinha, R., … Sonnenberg, G. F. (2013). Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature*, *498*(7452), 113–117. http://doi.org/10.1038/nature12240

Hewitt, E. W. (2003). The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology*, *110*(2), 163–169.

Heymann, W., Hunter, J. L., & Hackel, D. B. (1962). Experimental auto-immune nephrosis in rats: III. *Journal of Immunology (Baltimore, Md.: 1950)*, *88*, 135–141.

Hochheiser, K., Heuser, C., Krause, T. A., Teteris, S., Ilias, A., Weisheit, C., … Kurts, C. (2013). Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. *The Journal of Clinical Investigation*, *123*(10), 4242–4254. http://doi.org/10.1172/JCI70143

Hooper, L. V., & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature Reviews. Immunology*, *10*(3), 159–169. http://doi.org/10.1038/nri2710

Hoorweg, K., Peters, C. P., Cornelissen, F., Aparicio-Domingo, P., Papazian, N., Kazemier, G., … Cupedo, T. (2012). Functional Differences between Human NKp44(-) and NKp44(+) RORC(+) Innate Lymphoid Cells. *Frontiers in Immunology*, *3*, 72. http://doi.org/10.3389/fimmu.2012.00072

Hoshina, T., Kusuhara, K., Saito, M., Mizuno, Y., & Hara, T. (2012). NKRP1A+ γδ and αβ T cells are preferentially induced in patients with Salmonella infection. *Human Immunology*, *73*(6), 623–628. http://doi.org/10.1016/j.humimm.2012.04.006

Huber, S. A., Graveline, D., Newell, M. K., Born, W. K., & O’Brien, R. L. (2000). V gamma 1+ T cells suppress and V gamma 4+ T cells promote susceptibility to coxsackievirus B3-induced myocarditis in mice. *Journal of Immunology (Baltimore, Md.: 1950)*, *165*(8), 4174–4181.

Huber, S. A., Sartini, D., & Exley, M. (2002). Vgamma4(+) T cells promote autoimmune CD8(+) cytolytic T-lymphocyte activation in coxsackievirus B3-induced myocarditis in mice: role for CD4(+) Th1 cells. *Journal of Virology*, *76*(21), 10785–10790.

Hughes, T., Becknell, B., Freud, A. G., McClory, S., Briercheck, E., Yu, J., … Caligiuri, M. A. (2010). Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity*, *32*(6), 803–814. http://doi.org/10.1016/j.immuni.2010.06.007

Huntington, N. D., Legrand, N., Alves, N. L., Jaron, B., Weijer, K., Plet, A., … Di Santo, J. P. (2009). IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *The Journal of Experimental Medicine*, *206*(1), 25–34. http://doi.org/10.1084/jem.20082013

Hwang, Y. Y., & McKenzie, A. N. J. (2013). Innate lymphoid cells in immunity and disease. *Advances in Experimental Medicine and Biology*, *785*, 9–26. http://doi.org/10.1007/978-1-4614-6217-0_2

Iizuka, K., Naidenko, O. V., Plougastel, B. F. M., Fremont, D. H., & Yokoyama, W. M. (2003). Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. *Nature Immunology*, *4*(8), 801–807. http://doi.org/10.1038/ni954

Ikebe, H., Yamada, H., Nomoto, M., Takimoto, H., Nakamura, T., Sonoda, K. H., & Nomoto, K. (2001). Persistent infection with Listeria monocytogenes in the kidney induces anti-inflammatory invariant fetal-type gammadelta T cells. *Immunology*, *102*(1), 94–102.

Imig, J. D., & Ryan, M. J. (2013). Immune and inflammatory role in renal disease. *Comprehensive Physiology*, *3*(2), 957–976. http://doi.org/10.1002/cphy.c120028

Inagaki-Ohara, K., Chinen, T., Matsuzaki, G., Sasaki, A., Sakamoto, Y., Hiromatsu, K., … Yoshimura, A. (2004). Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *Journal of Immunology (Baltimore, Md.: 1950)*, *173*(2), 1390–1398.

Innate Immunity of the Gut: Mucosal Defense in Health and Di... : Journal of Pediatric Gastroenterology and Nutrition. (n.d.). Retrieved August 8, 2016, from http://journals.lww.com/jpgn/Fulltext/2004/05000/Innate_Immunity_of_the_Gut__Mucosal_Defense_in.1.aspx

Inngjerdingen, M., Kveberg, L., & Vaage, J. T. (2012). A novel NKR-P1B(bright) NK cell subset expresses an activated CD25(+)CX(3)CR1(+)CD62L(-)CD11b(-)CD27(-) phenotype and is prevalent in blood, liver, and gut-associated lymphoid organs of rats. *Journal of Immunology (Baltimore, Md.: 1950)*, *188*(6), 2499–2508. http://doi.org/10.4049/jimmunol.1003939

Ito, Y., Kanai, T., Totsuka, T., Okamoto, R., Tsuchiya, K., Nemoto, Y., … Watanabe, M. (2008). Blockade of NKG2D signaling prevents the development of murine CD4+ T cell-mediated colitis. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *294*(1), G199-207. http://doi.org/10.1152/ajpgi.00286.2007

Itohara, S., Nakanishi, N., Kanagawa, O., Kubo, R., & Tonegawa, S. (1989). Monoclonal antibodies specific to native murine T-cell receptor gamma delta: analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(13), 5094–5098.

Iwahori, T., Ueshima, H., Torii, S., Saito, Y., Fujiyoshi, A., Ohkubo, T., & Miura, K. (2016). Four to seven random casual urine specimens are sufficient to estimate 24-h urinary sodium/potassium

ratio in individuals with high blood pressure. *Journal of Human Hypertension*, *30*(5), 328–334. http://doi.org/10.1038/jhh.2015.84

Jones, D. C., Edgar, R. S., Ahmad, T., Cummings, J. R. F., Jewell, D. P., Trowsdale, J., & Young, N. T. (2006). Killer Ig-like receptor (KIR) genotype and HLA ligand combinations in ulcerative colitis susceptibility. *Genes and Immunity*, *7*(7), 576–582. http://doi.org/10.1038/sj.gene.6364333

Kadri, N., Thanh, T. L., & Höglund, P. (2015). Selection, tuning, and adaptation in mouse NK cell education. *Immunological Reviews*, *267*(1), 167–177. http://doi.org/10.1111/imr.12330

Kamada, N., Seo, S.-U., Chen, G. Y., & Núñez, G. (2013). Role of the gut microbiota in immunity and inflammatory disease. *Nature Reviews. Immunology*, *13*(5), 321–335. http://doi.org/10.1038/nri3430

Kärre, K., Ljunggren, H. G., Piontek, G., & Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*, *319*(6055), 675–678. http://doi.org/10.1038/319675a0

Kartsogiannis, V., Sims, N. A., Quinn, J. M. W., Ly, C., Cipetic, M., Poulton, I. J., … Gillespie, M. T. (2008). Osteoclast inhibitory lectin, an immune cell product that is required for normal bone physiology in vivo. *The Journal of Biological Chemistry*, *283*(45), 30850–30860. http://doi.org/10.1074/jbc.M801761200

Kim, B. S., Siracusa, M. C., Saenz, S. A., Noti, M., Monticelli, L. A., Sonnenberg, G. F., … Artis, D. (2013). TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Science Translational Medicine*, *5*(170), 170ra16. http://doi.org/10.1126/scitranslmed.3005374

Kirkham, C. L., & Carlyle, J. R. (2014). Complexity and Diversity of the NKR-P1:Clr (Klrb1:Clec2) Recognition Systems. *Frontiers in Immunology*, *5*, 214. http://doi.org/10.3389/fimmu.2014.00214

Kiss, E. A., Vonarbourg, C., Kopfmann, S., Hobeika, E., Finke, D., Esser, C., & Diefenbach, A. (2011). Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science (New York, N.Y.)*, *334*(6062), 1561–1565. http://doi.org/10.1126/science.1214914

Klimosch, S. N., Bartel, Y., Wiemann, S., & Steinle, A. (2013). Genetically coupled receptor-ligand pair NKp80-AICL enables autonomous control of human NK cell responses. *Blood*, *122*(14), 2380–2389. http://doi.org/10.1182/blood-2013-01-479790

Klose, C. S. N., Flach, M., Möhle, L., Rogell, L., Hoyler, T., Ebert, K., … Diefenbach, A. (2014). Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*, *157*(2), 340–356. http://doi.org/10.1016/j.cell.2014.03.030

Klose, C. S. N., Kiss, E. A., Schwierzeck, V., Ebert, K., Hoyler, T., d'Hargues, Y., … Diefenbach, A. (2013). A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature*, *494*(7436), 261–265. http://doi.org/10.1038/nature11813

Komori, H. K., Meehan, T. F., & Havran, W. L. (2006). Epithelial and mucosal gamma delta T cells. *Current Opinion in Immunology*, *18*(5), 534–538. http://doi.org/10.1016/j.coi.2006.06.001

Krangel, M. S., Yssel, H., Brocklehurst, C., & Spits, H. (1990). A distinct wave of human T cell receptor gamma/delta lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *The Journal of Experimental Medicine*, *172*(3), 847–859.

Kumar, H., Kawai, T., & Akira, S. (2011). Pathogen recognition by the innate immune system. *International Reviews of Immunology*, *30*(1), 16–34. http://doi.org/10.3109/08830185.2010.529976

Kumar, V., & McNerney, M. E. (2005). A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nature Reviews. Immunology*, *5*(5), 363–374. http://doi.org/10.1038/nri1603

Kupz, A., Scott, T. A., Belz, G. T., Andrews, D. M., Greyer, M., Lew, A. M., … Strugnell, R. A. (2013). Contribution of Thy1+ NK cells to protective IFN-γ production during Salmonella typhimurium infections. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(6), 2252–2257. http://doi.org/10.1073/pnas.1222047110

Kurts, C., Panzer, U., Anders, H.-J., & Rees, A. J. (2013). The immune system and kidney disease: basic concepts and clinical implications. *Nature Reviews. Immunology*, *13*(10), 738–753. http://doi.org/10.1038/nri3523

Lanier, L. L. (2005). NK cell recognition. *Annual Review of Immunology*, *23*, 225–274. http://doi.org/10.1146/annurev.immunol.23.021704.115526

Lapaque, N., Walzer, T., Méresse, S., Vivier, E., & Trowsdale, J. (2009). Interactions between human NK cells and macrophages in response to Salmonella infection. *Journal of Immunology (Baltimore, Md.: 1950)*, *182*(7), 4339–4348. http://doi.org/10.4049/jimmunol.0803329

Leach, M. W., Bean, A. G., Mauze, S., Coffman, R. L., & Powrie, F. (1996). Inflammatory bowel disease in C.B-17 scid mice reconstituted with the CD45RBhigh subset of CD4+ T cells. *The American Journal of Pathology*, *148*(5), 1503–1515.

Lee, P. Y., Li, Y., Kumagai, Y., Xu, Y., Weinstein, J. S., Kellner, E. S., … Reeves, W. H. (2009). Type I Interferon Modulates Monocyte Recruitment and Maturation in Chronic Inflammation. *The American Journal of Pathology*, *175*(5), 2023–2033. http://doi.org/10.2353/ajpath.2009.090328

Leemans, J. C., Stokman, G., Claessen, N., Rouschop, K. M., Teske, G. J. D., Kirschning, C. J., … Florquin, S. (2005). Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *The Journal of Clinical Investigation*, *115*(10), 2894–2903. http://doi.org/10.1172/JCI22832

Lehman, L.-W., Saeed, M., Moody, G., & Mark, R. (2010). Hypotension as a Risk Factor for Acute Kidney Injury in ICU Patients. *Computing in Cardiology*, *37*, 1095–1098.

Leibelt, S., Friede, M. E., Rohe, C., Gütle, D., Rutkowski, E., Weigert, A., … Steinle, A. (2015). Dedicated immunosensing of the mouse intestinal epithelium facilitated by a pair of genetically coupled lectin-like receptors. *Mucosal Immunology*, *8*(2), 232–242. http://doi.org/10.1038/mi.2014.60

Levey, A. S., Coresh, J., Balk, E., Kausz, A. T., Levin, A., Steffes, M. W., … National Kidney Foundation. (2003). National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Annals of Internal Medicine*, *139*(2), 137–147.

Luci, C., Reynders, A., Ivanov, I. I., Cognet, C., Chiche, L., Chasson, L., … Tomasello, E. (2009). Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nature Immunology*, *10*(1), 75–82. http://doi.org/10.1038/ni.1681

Macpherson, A. J., & Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science (New York, N.Y.)*, *303*(5664), 1662–1665. http://doi.org/10.1126/science.1091334

Magri, G., Miyajima, M., Bascones, S., Mortha, A., Puga, I., Cassis, L., … Cerutti, A. (2014). Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells. *Nature Immunology*, *15*(4), 354–364. http://doi.org/10.1038/ni.2830

Mahmoud, A. B., Tu, M. M., Wight, A., Zein, H. S., Rahim, M. M. A., Lee, S.-H., … Makrigiannis, A. P. (2016). Influenza Virus Targets Class I MHC-Educated NK Cells for Immunoevasion. *PLoS Pathogens*, *12*(2), e1005446. http://doi.org/10.1371/journal.ppat.1005446

Maloy, K. J., & Powrie, F. (2011). Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*, *474*(7351), 298–306. http://doi.org/10.1038/nature10208

Manichanh, C., Borruel, N., Casellas, F., & Guarner, F. (2012). The gut microbiota in IBD. *Nature Reviews. Gastroenterology & Hepatology*, *9*(10), 599–608. http://doi.org/10.1038/nrgastro.2012.152

Mason, K. L., Huffnagle, G. B., Noverr, M. C., & Kao, J. Y. (2008). Overview of gut immunology. *Advances in Experimental Medicine and Biology*, *635*, 1–14. http://doi.org/10.1007/978-0-387-09550-9_1

McHedlidze, T., Waldner, M., Zopf, S., Walker, J., Rankin, A. L., Schuchmann, M., … Wirtz, S. (2013). Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity*, *39*(2), 357–371. http://doi.org/10.1016/j.immuni.2013.07.018

Mebius, R. E., Rennert, P., & Weissman, I. L. (1997). Developing lymph nodes collect CD4+CD3-LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity*, *7*(4), 493–504.

Messaoudi, S., He, Y., Gutsol, A., Wight, A., Hébert, R. L., Vilmundarson, R. O., … Nemer, M. (2015). Endothelial Gata5 transcription factor regulates blood pressure. *Nature Communications*, *6*, 8835. http://doi.org/10.1038/ncomms9835

Mizuno, S., Mikami, Y., Kamada, N., Handa, T., Hayashi, A., Sato, T., … Kanai, T. (2014). Cross-talk between RORγt+ innate lymphoid cells and intestinal macrophages induces mucosal IL-22 production in Crohn's disease. *Inflammatory Bowel Diseases*, *20*(8), 1426–1434. http://doi.org/10.1097/MIB.0000000000000105

Mjösberg, J., Bernink, J., Golebski, K., Karrich, J. J., Peters, C. P., Blom, B., … Spits, H. (2012). The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity*, *37*(4), 649–659. http://doi.org/10.1016/j.immuni.2012.08.015

Mjösberg, J. M., Trifari, S., Crellin, N. K., Peters, C. P., van Drunen, C. M., Piet, B., … Spits, H. (2011). Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nature Immunology*, *12*(11), 1055–1062. http://doi.org/10.1038/ni.2104

Molofsky, A. B., Nussbaum, J. C., Liang, H.-E., Van Dyken, S. J., Cheng, L. E., Mohapatra, A., … Locksley, R. M. (2013). Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *The Journal of Experimental Medicine*, *210*(3), 535–549. http://doi.org/10.1084/jem.20121964

Monticelli, L. A., Sonnenberg, G. F., Abt, M. C., Alenghat, T., Ziegler, C. G. K., Doering, T. A., … Artis, D. (2011). Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature Immunology*, *12*(11), 1045–1054. http://doi.org/10.1031/ni.2131

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., … Koyasu, S. (2010). Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*, *463*(7280), 540–544. http://doi.org/10.1038/nature08636

(MRCPath.), A. S., & Lowe, J. S. (2005). *Human Histology*. Elsevier/Mosby.

Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K. A., … McKenzie, A. N. J. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*, *464*(7293), 1367–1370. http://doi.org/10.1038/nature08900

Neish, A. S. (2002). The gut microflora and intestinal epithelial cells: a continuing dialogue. *Microbes and Infection / Institut Pasteur*, *4*(3), 309–317.

Niemann-Masanek, U., Mueller, A., Yard, B. A., Waldherr, R., & van der Woude, F. J. (2002). B7-1 (CD80) and B7-2 (CD 86) expression in human tubular epithelial cells in vivo and in vitro. *Nephron*, *92*(3), 542–556. http://doi.org/64084

Nonaka, S., Naito, T., Chen, H., Yamamoto, M., Moro, K., Kiyono, H., … Ishikawa, H. (2005). Intestinal gamma delta T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles. *Journal of Immunology (Baltimore, Md.: 1950)*, *174*(4), 1906–1912.

Omatsu, Y., Iyoda, T., Kimura, Y., Maki, A., Ishimori, M., Toyama-Sorimachi, N., & Inaba, K. (2005). Development of murine plasmacytoid dendritic cells defined by increased expression of an inhibitory NK receptor, Ly49Q. *Journal of Immunology (Baltimore, Md.: 1950)*, *174*(11), 6657–6662.

Onuigbo, M. A. C., & Agbasi, N. (2015). Intraoperative hypotension - a neglected causative factor in hospital-acquired acute kidney injury; a Mayo Clinic Health System experience revisited. *Journal of Renal Injury Prevention*, *4*(3), 61–67. http://doi.org/10.12861/jrip.2015.13

Paradeep, A. (2016, July 25). Chronic Kidney Disease Workup: Approach Considerations, Renal Function Formulas, Renal Ultrasonography. Retrieved September 21, 2016, from http://emedicine.medscape.com/article/238798-workup

Pène, J., Chevalier, S., Preisser, L., Vénéreau, E., Guilleux, M.-H., Ghannam, S., … Gascan, H. (2008). Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *Journal of Immunology (Baltimore, Md.: 1950)*, *180*(11), 7423–7430.

Pereira, P., Gerber, D., Huang, S. Y., & Tonegawa, S. (1995). Ontogenic development and tissue distribution of V gamma 1-expressing gamma/delta T lymphocytes in normal mice. *The Journal of Experimental Medicine*, *182*(6), 1921–1930.

Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews. Immunology*, *14*(3), 141–153. http://doi.org/10.1038/nri3608

Plougastel, B., Dubbelde, C., & Yokoyama, W. M. (2001). Cloning of Clr, a new family of lectin-like genes localized between mouse Nkrp1a and Cd69. *Immunogenetics*, *53*(3), 209–214.

Pott, J., & Hornef, M. (2012). Innate immune signalling at the intestinal epithelium in homeostasis and disease. *EMBO Reports*, *13*(8), 684–698. http://doi.org/10.1038/embor.2012.96

Price, A. E., Liang, H.-E., Sullivan, B. M., Reinhardt, R. L., Eisley, C. J., Erle, D. J., & Locksley, R. M. (2010). Systemically dispersed innate IL-13-expressing cells in type 2 immunity.

Proceedings of the National Academy of Sciences of the United States of America, *107*(25), 11489–11494. http://doi.org/10.1073/pnas.1003988107

Qiu, J., Guo, X., Chen, Z.-M. E., He, L., Sonnenberg, G. F., Artis, D., … Zhou, L. (2013). Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity*, *39*(2), 386–399. http://doi.org/10.1016/j.immuni.2013.08.002

Rahim, M. M. A., Chen, P., Mottashed, A. N., Mahmoud, A. B., Thomas, M. J., Zhu, Q., … Makrigiannis, A. P. (2015). The mouse NKR-P1B:Clr-b recognition system is a negative regulator of innate immune responses. *Blood*, *125*(14), 2217–2227. http://doi.org/10.1182/blood-2014-02-556142

Rahim, M. M. A., & Makrigiannis, A. P. (2015). Ly49 receptors: evolution, genetic diversity, and impact on immunity. *Immunological Reviews*, *267*(1), 137–147. http://doi.org/10.1111/imr.12318

Raulet, D. H., Vance, R. E., & McMahon, C. W. (2001). Regulation of the natural killer cell receptor repertoire. *Annual Review of Immunology*, *19*, 291–330. http://doi.org/10.1146/annurev.immunol.19.1.291

Reynders, A., Yessaad, N., Vu Manh, T.-P., Dalod, M., Fenis, A., Aubry, C., … Tomasello, E. (2011). Identity, regulation and in vivo function of gut NKp46+RORγt+ and NKp46+RORγt- lymphoid cells. *The EMBO Journal*, *30*(14), 2934–2947. http://doi.org/10.1038/emboj.2011.201

Roediger, B., Kyle, R., Yip, K. H., Sumaria, N., Guy, T. V., Kim, B. S., … Weninger, W. (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nature Immunology*, *14*(6), 564–573. http://doi.org/10.1038/ni.2584

Rosen, D. B., Bettadapura, J., Alsharifi, M., Mathew, P. A., Warren, H. S., & Lanier, L. L. (2005). Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. *Journal of Immunology (Baltimore, Md.: 1950)*, *175*(12), 7796–7799.

Rosenkranz, A. R., Knight, S., Sethi, S., Alexander, S. I., Cotran, R. S., & Mayadas, T. N. (2000). Regulatory interactions of alphabeta and gammadelta T cells in glomerulonephritis. *Kidney International*, *58*(3), 1055–1066. http://doi.org/10.1046/j.1523-1755.2000.00263.x

Saenz, S. A., Siracusa, M. C., Perrigoue, J. G., Spencer, S. P., Urban, J. F., Tocker, J. E., … Artis, D. (2010). IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*, *464*(7293), 1362–1366. http://doi.org/10.1038/nature08901

Salant, D. J., Quigg, R. J., & Cybulsky, A. V. (1989). Heymann nephritis: mechanisms of renal injury. *Kidney International*, *35*(4), 976–984.

Sanos, S. L., Bui, V. L., Mortha, A., Oberle, K., Heners, C., Johner, C., & Diefenbach, A. (2009). RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nature Immunology*, *10*(1), 83–91. http://doi.org/10.1038/ni.1684

Satoh-Takayama, N., Serafini, N., Verrier, T., Rekiki, A., Renauld, J.-C., Frankel, G., & Di Santo, J. P. (2014). The chemokine receptor CXCR6 controls the functional topography of interleukin-22 producing intestinal innate lymphoid cells. *Immunity*, *41*(5), 776–788. http://doi.org/10.1016/j.immuni.2014.10.007

Satoh-Takayama, N., Vosshenrich, C. A. J., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., … Di Santo, J. P. (2008). Microbial flora drives interleukin 22 production in intestinal NKp46+

cells that provide innate mucosal immune defense. *Immunity*, *29*(6), 958–970. http://doi.org/10.1016/j.immuni.2008.11.001

Scandella, E., Bolinger, B., Lattmann, E., Miller, S., Favre, S., Littman, D. R., … Ludewig, B. (2008). Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nature Immunology*, *9*(6), 667–675. http://doi.org/10.1038/ni.1605

Schenten, D., & Medzhitov, R. (2011). The control of adaptive immune responses by the innate immune system. *Advances in Immunology*, *109*, 87–124. http://doi.org/10.1016/B978-0-12-387664-5.00003-0

Schluns, K. S., Nowak, E. C., Cabrera-Hernandez, A., Puddington, L., Lefrançois, L., & Aguila, H. L. (2004). Distinct cell types control lymphoid subset development by means of IL-15 and IL-15 receptor alpha expression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(15), 5616–5621. http://doi.org/10.1073/pnas.0307442101

Schulthess, J., Meresse, B., Ramiro-Puig, E., Montcuquet, N., Darche, S., Bègue, B., … Cerf-Bensussan, N. (2012). Interleukin-15-dependent NKp46+ innate lymphoid cells control intestinal inflammation by recruiting inflammatory monocytes. *Immunity*, *37*(1), 108–121. http://doi.org/10.1016/j.immuni.2012.05.013

Sedda, S., Marafini, I., Figliuzzi, M. M., Pallone, F., & Monteleone, G. (2014). An overview of the role of innate lymphoid cells in gut infections and inflammation. *Mediators of Inflammation*, *2014*, 235460. http://doi.org/10.1155/2014/235460

Seo, S.-U., Kuffa, P., Kitamoto, S., Nagao-Kitamoto, H., Rousseau, J., Kim, Y.-G., … Kamada, N. (2015). Intestinal macrophages arising from CCR2(+) monocytes control pathogen infection by

activating innate lymphoid cells. *Nature Communications*, *6*, 8010. http://doi.org/10.1038/ncomms9010

Serafini, N., Klein Wolterink, R. G. J., Satoh-Takayama, N., Xu, W., Vosshenrich, C. A. J., Hendriks, R. W., & Di Santo, J. P. (2014). Gata3 drives development of RORγt+ group 3 innate lymphoid cells. *The Journal of Experimental Medicine*, *211*(2), 199–208. http://doi.org/10.1084/jem.20131038

Shekhar, S., Milling, S., & Yang, X. (2012). Migration of γδ T cells in steady-state conditions. *Veterinary Immunology and Immunopathology*, *147*(1–2), 1–5. http://doi.org/10.1016/j.vetimm.2012.03.016

Shi, F.-D., Ljunggren, H.-G., La Cava, A., & Van Kaer, L. (2011). Organ-specific features of natural killer cells. *Nature Reviews. Immunology*, *11*(10), 658–671. http://doi.org/10.1038/nri3065

Shimada, M., Dass, B., Ejaz, A. A., & Acknowledgements. (n.d.). Assessment of elevated creatinine. Retrieved September 21, 2016, from http://bestpractice.bmj.com/best-practice/monograph/935.html

Shui, J.-W., Larange, A., Kim, G., Vela, J. L., Zahner, S., Cheroutre, H., & Kronenberg, M. (2012). HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. *Nature*, *488*(7410), 222–225. http://doi.org/10.1038/nature11242

Sips, M., Sciaranghella, G., Diefenbach, T., Dugast, A.-S., Berger, C. T., Liu, Q., … Alter, G. (2012). Altered distribution of mucosal NK cells during HIV infection. *Mucosal Immunology*, *5*(1), 30–40. http://doi.org/10.1038/mi.2011.40

Smith, P. L., Buffington, D. A., & Humes, H. D. (2006). Kidney epithelial cells. *Methods in Enzymology*, *419*, 194–207. http://doi.org/10.1016/S0076-6879(06)19009-6

Sonnenberg, G. F., Fouser, L. A., & Artis, D. (2011). Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nature Immunology*, *12*(5), 383–390. http://doi.org/10.1038/ni.2025

Sonnenberg, G. F., Monticelli, L. A., Alenghat, T., Fung, T. C., Hutnick, N. A., Kunisawa, J., … Artis, D. (2012). Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science (New York, N.Y.)*, *336*(6086), 1321–1325. http://doi.org/10.1126/science.1222551

Sonnenberg, G. F., Monticelli, L. A., Elloso, M. M., Fouser, L. A., & Artis, D. (2011). CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity*, *34*(1), 122–134. http://doi.org/10.1016/j.immuni.2010.12.009

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., … Vivier, E. (2013). Innate lymphoid cells--a proposal for uniform nomenclature. *Nature Reviews. Immunology*, *13*(2), 145–149. http://doi.org/10.1038/nri3365

Spits, H., & Cupedo, T. (2012). Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annual Review of Immunology*, *30*, 647–675. http://doi.org/10.1146/annurev-immunol-020711-075053

Spits, H., & Di Santo, J. P. (2011). The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nature Immunology*, *12*(1), 21–27. http://doi.org/10.1038/ni.1962

Sun, Z., Unutmaz, D., Zou, Y. R., Sunshine, M. J., Pierani, A., Brenner-Morton, S., … Littman, D. R. (2000). Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science (New York, N.Y.)*, *288*(5475), 2369–2373.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., & Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*, *100*(6), 655–669.

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., … Randolph, G. J. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *The Journal of Clinical Investigation*, *117*(1), 185–194. http://doi.org/10.1172/JCI28549

Tai, L.-H., Goulet, M.-L., Belanger, S., Toyama-Sorimachi, N., Fodil-Cornu, N., Vidal, S. M., … Makrigiannis, A. P. (2008). Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *The Journal of Experimental Medicine*, *205*(13), 3187–3199. http://doi.org/10.1084/jem.20080718

Takayama, T., Kamada, N., Chinen, H., Okamoto, S., Kitazume, M. T., Chang, J., … Hibi, T. (2010). Imbalance of NKp44(+)NKp46(-) and NKp44(-)NKp46(+) natural killer cells in the intestinal mucosa of patients with Crohn's disease. *Gastroenterology*, *139*(3), 882–892, 892–3. http://doi.org/10.1053/j.gastro.2010.05.040

Tan, R. J., Zhou, D., & Liu, Y. (2016). Signaling Crosstalk between Tubular Epithelial Cells and Interstitial Fibroblasts after Kidney Injury. *Kidney Diseases*. http://doi.org/10.1159/000446336

Tanaka, S., Tanaka, T., & Nangaku, M. (2014). Hypoxia as a key player in the AKI-to-CKD transition. *American Journal of Physiology. Renal Physiology*, *307*(11), F1187-1195. http://doi.org/10.1152/ajprenal.00425.2014

Tanaka, S., Tanaka, T., & Nangaku, M. (2015). Hypoxia and Dysregulated Angiogenesis in Kidney Disease. *Kidney Diseases (Basel, Switzerland)*, *1*(1), 80–89. http://doi.org/10.1159/000381515

Tasnim, F., & Zink, D. (2012). Cross talk between primary human renal tubular cells and endothelial cells in cocultures. *American Journal of Physiology. Renal Physiology*, *302*(8), F1055-1062. http://doi.org/10.1152/ajprenal.00621.2011

Teunissen, M. B. M., Munneke, J. M., Bernink, J. H., Spuls, P. I., Res, P. C. M., Te Velde, A., … Mjösberg, J. (2014). Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. *The Journal of Investigative Dermatology*, *134*(9), 2351–2360. http://doi.org/10.1038/jid.2014.146

Trinchieri, G. (1989). Biology of natural killer cells. *Advances in Immunology*, *47*, 187–376.

Tu, M. M., Mahmoud, A. B., Wight, A., Mottashed, A., Bélanger, S., Rahim, M. M. A., … Makrigiannis, A. P. (2014). Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells. *Cancer Research*, *74*(14), 3684–3694. http://doi.org/10.1158/0008-5472.CAN-13-3021

Turner, J.-E., Krebs, C., Tittel, A. P., Paust, H.-J., Meyer-Schwesinger, C., Bennstein, S. B., … Panzer, U. (2012). IL-17A production by renal γδ T cells promotes kidney injury in crescentic GN. *Journal of the American Society of Nephrology: JASN*, *23*(9), 1486–1495. http://doi.org/10.1681/ASN.2012010040

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., … Hooper, L. V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science (New York, N.Y.)*, *334*(6053), 255–258. http://doi.org/10.1126/science.1209791

Vance, R. E., Kraft, J. R., Altman, J. D., Jensen, P. E., & Raulet, D. H. (1998). Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *The Journal of Experimental Medicine*, *188*(10), 1841–1848.

Vantourout, P., & Hayday, A. (2013). Six-of-the-best: unique contributions of γδ T cells to immunology. *Nature Reviews. Immunology*, *13*(2), 88–100. http://doi.org/10.1038/nri3384

Vielhauer, V., Kulkarni, O., Reichel, C. A., & Anders, H.-J. (2010). Targeting the recruitment of monocytes and macrophages in renal disease. *Seminars in Nephrology*, *30*(3), 318–333. http://doi.org/10.1016/j.semnephrol.2010.03.006

Vincent, J.-L., & Castanares Zapatero, D. (2009). The role of hypotension in the development of acute renal failure. *Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association - European Renal Association*, *24*(2), 337–338. http://doi.org/10.1093/ndt/gfn679

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology*, *9*(5), 503–510. http://doi.org/10.1038/ni1582

Voigt, S., Mesci, A., Ettinger, J., Fine, J. H., Chen, P., Chou, W., & Carlyle, J. R. (2007). Cytomegalovirus evasion of innate immunity by subversion of the NKR-P1B:Clr-b missing-self axis. *Immunity*, *26*(5), 617–627. http://doi.org/10.1016/j.immuni.2007.03.013

Vonarbourg, C., Mortha, A., Bui, V. L., Hernandez, P. P., Kiss, E. A., Hoyler, T., … Diefenbach, A. (2010). Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt(+) innate lymphocytes. *Immunity*, *33*(5), 736–751. http://doi.org/10.1016/j.immuni.2010.10.017

Wahl, P., Schoop, R., Bilic, G., Neuweiler, J., Le Hir, M., Yoshinaga, S. K., & Wüthrich, R. P. (2002). Renal tubular epithelial expression of the costimulatory molecule B7RP-1 (inducible costimulator ligand). *Journal of the American Society of Nephrology: JASN*, *13*(6), 1517–1526.

Walker, J. A., & McKenzie, A. N. J. (2013). Development and function of group 2 innate lymphoid cells. *Current Opinion in Immunology*, *25*(2), 148–155. http://doi.org/10.1016/j.coi.2013.02.010

Weisheit, C. K., Engel, D. R., & Kurts, C. (2015). Dendritic Cells and Macrophages: Sentinels in the Kidney. *Clinical Journal of the American Society of Nephrology: CJASN*, *10*(10), 1841–1851. http://doi.org/10.2215/CJN.07100714

Wilhelm, C., Hirota, K., Stieglitz, B., Van Snick, J., Tolaini, M., Lahl, K., … Stockinger, B. (2011). An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nature Immunology*, *12*(11), 1071–1077. http://doi.org/10.1038/ni.2133

Wilson, A., Held, W., & MacDonald, H. R. (1994). Two waves of recombinase gene expression in developing thymocytes. *The Journal of Experimental Medicine*, *179*(4), 1355–1360.

Wu, H., Chen, G., Wyburn, K. R., Yin, J., Bertolino, P., Eris, J. M., … Chadban, S. J. (2007). TLR4 activation mediates kidney ischemia/reperfusion injury. *The Journal of Clinical Investigation*, *117*(10), 2847–2859. http://doi.org/10.1172/JCI31008

Wu, H., Knight, J. F., & Alexander, S. I. (2004). Regulatory gamma delta T cells in Heymann nephritis express an invariant Vgamma6/Vdelta1 with a canonical CDR3 sequence. *European Journal of Immunology*, *34*(8), 2322–2330. http://doi.org/10.1002/eji.200324780

Wuest, S. C., Edwan, J. H., Martin, J. F., Han, S., Perry, J. S. A., Cartagena, C. M., … Bielekova, B. (2011). A role for interleukin-2 trans-presentation in dendritic cell-mediated T cell activation in

humans, as revealed by daclizumab therapy. *Nature Medicine*, *17*(5), 604–609. http://doi.org/10.1038/nm.2365

Wurbel, M. A., Malissen, M., Guy-Grand, D., Meffre, E., Nussenzweig, M. C., Richelme, M., … Malissen, B. (2001). Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood*, *98*(9), 2626–2632.

Wuthrich, R. P., Glimcher, L. H., Yui, M. A., Jevnikar, A. M., Dumas, S. E., & Kelley, V. E. (1990). MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. *Kidney International*, *37*(2), 783–792.

Yadav, P. K., Chen, C., & Liu, Z. (2011). Potential role of NK cells in the pathogenesis of inflammatory bowel disease. *Journal of Biomedicine & Biotechnology*, *2011*, 348530. http://doi.org/10.1155/2011/348530

Yang, H., Antony, P. A., Wildhaber, B. E., & Teitelbaum, D. H. (2004). Intestinal intraepithelial lymphocyte gamma delta-T cell-derived keratinocyte growth factor modulates epithelial growth in the mouse. *Journal of Immunology (Baltimore, Md.: 1950)*, *172*(7), 4151–4158.

Yokoyama, W. M., & Plougastel, B. F. M. (2003). Immune functions encoded by the natural killer gene complex. *Nature Reviews. Immunology*, *3*(4), 304–316. http://doi.org/10.1038/nri1055

Yokoyama, W. M., & Seaman, W. E. (1993). The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annual Review of Immunology*, *11*, 613–635. http://doi.org/10.1146/annurev.iy.11.040193.003145

Zaritskaya, L., Shurin, M. R., Sayers, T. J., & Malyguine, A. M. (2010). New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Review of Vaccines*, *9*(6), 601–616. http://doi.org/10.1586/erv.10.49

Zenewicz, L. A., & Flavell, R. A. (2011). Recent advances in IL-22 biology. *International Immunology*, *23*(3), 159–163. http://doi.org/10.1093/intimm/dxr001

Zhang, Q., Rahim, M. M. A., Allan, D. S. J., Tu, M. M., Belanger, S., Abou-Samra, E., … Makrigiannis, A. P. (2012). Mouse Nkrp1-Clr gene cluster sequence and expression analyses reveal conservation of tissue-specific MHC-independent immunosurveillance. *PloS One*, *7*(12), e50561. http://doi.org/10.1371/journal.pone.0050561

Zhao, H., Nguyen, H., & Kang, J. (2005). Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nature Immunology*, *6*(12), 1263–1271. http://doi.org/10.1038/ni1267

Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., … Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, *14*(3), 282–289. http://doi.org/10.1038/nm1720