Murine leupaxin deletion results in spontaneous weight gain, tissue inflammation, and integrin signaling defects

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

IMMUNOLOGY

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Abstract

Immune cells are remarkable in their ability to gracefully travel throughout the body to maintain homeostasis and defend against pathogens. Key proteins during immune cell migration are the integrin proteins that bind to specific ligands for robust adherence of the leukocyte to the substrate. Integrin engagement allows for downstream signaling instructing the cell to reorganize its cytoskeleton and initiate morphological changes that occur during migration across the endothelial barrier. However, another less well studied function of integrins is its ability to signal for cell polarization during cytotoxic lymphocyte directed granule release. Primarily in natural killer (NK) cells, LFA-1 outside-in signaling orchestrates cellular polarization that moves the microtubule organizing center (MTOC) and lytic granules towards the intended target cell.

My research is to study the effects of leupaxin knockout on the individual NK cell and the leukocyte populations of the spleen, visceral adipose tissue, and liver of the mice. Leupaxin belongs to the paxillin family of proteins and is a cytoskeletal protein downstream of integrin signaling preferentially expressed in cells of hematopoietic origin. I found that leupaxin deletion reduced the granule polarization towards the MTOC during target cell killing by the NK cell but did not impede the NK cell cytotoxicity and degranulation. During cell crawling, leupaxin knockout NK cells have faster velocity on VCAM-1 and fibronectin. However, leupaxin deletion did not alter the RANTES chemokine directed transwell migration of NK cells. The integrin downstream signaling functionality of leupaxin may act as a fine-tuning mechanism to assist in granule polarization and reduce integrin protein turnover during cell crawling.

Overall, leupaxin knockout mice display altered metabolic activity as they have increased body weight compared to their wildtype (WT) counterparts. Sex differences can be observed in the leupaxin knockout mice as females have onset of increased weight gain at an earlier age along with significant weight differences in liver and visceral adipose tissue. Leupaxin knockout mice did not

have alterations to their splenic populations. Alterations in the CD44 by CD62L profile towards effector memory T cell subsets can be observed within the CD8 T cells of the visceral adipose tissue (VAT) and liver of both sexes. Innate immune cells display an increase in VAT macrophage population frequency and liver resident NK cell population frequency suggesting a possible onset of obesity related low-grade inflammatory condition. These results suggest that the leupaxin integrin signaling network is pivotal to the prevention of steady state activation of T cells.

Preface

This thesis is an original work by JiaQi Liu. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Maturation and activation of T-cells”, No. AUP00000305, approved August 13 2001, renewed April 21, 2020.

Acknowledgements

I would like to take this opportunity to thank everyone who supported me throughout the MSc program. I would not have been able to finish my degree without everyone's support. I am deeply grateful for my supervisor Dr. Hanne Ostergaard for accepting me as a student, giving me a second chance, and never-ending patience for the multiple difficulties when working with me. This is and will be one of the of the most important transition periods in my life. In addition, I like to thank my committee members Dr. Troy Baldwin and Dr. Jim Smiley for their suggestion and feedback on my project. I would also like to express my appreciation for Dr. Kevin Kane, everyone in the Ostergaard, Kane, and Baldwin labs for providing meaningful feedback and ideas during lab meetings. Lastly, I would like to express that I would not have completed this chapter of my life without the support and friendship of all my friends and family that lent me a helping hand during difficult times.

Table of Contents

List of Tables

List of Figures

List of Abreviations

-ve	Negative
ADAP	Adhesion and degranulation promoting adapter protein
ADCC	Antibody dependent cell cytotoxicity
ARP2/3	Actin-related proteins 2/3
APC	Antigen presenting cell
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X, apoptosis regulator
BCR	B cell receptor
Bid	BH3 interacting domain death agonist
BM	Bone marrow
CD	Cluster of differentiation
Cdc42	Cell division control protein 42 homolog
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTV	CellTrace Violet
DAG	Diacylglycerol
DC	Dendritic cell
dCs	defined calf serum
DISC	Death induced signalling complex
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
DPBS	Dulbecco's phosphate-buffered saline
EAT-2	SH2 Domain Containing 1B
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EOMES	Eomesodermin
ERK	Extracellular signalling regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FasL	Fas ligand
FBS	fetal bovine serum
fCs	Fetal calf serum

FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
Het	Heterozygous
HEV	High endothelial venule
hsCRP	High-sensitivity C-reactive protein
IFN	Interferon
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
ILK	Integrin linked kinase
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
IP3	Inositol trisphosphate
IQGAP1	IQ motif containing GTPase activating protein 1
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activating motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
JAB1	Jun Activation Domain-binding Protein 1
KO	Knockout
LAT	Linker for activation of T cells
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LPXN	Leupaxin
Mac-1	Macrophage-1 antigen
MACPF	Membrane Attack Complex/Perforin
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MICA/B	Major histocompatibility complex class I chain-related protein A/B
MEK	Mitogen-activated or extracellular signal-regulated protein kinase kinase
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cells
MTOC	Microtubule Organizing center
Mult1	Murine UL16-binding proteinlike transcript 1
MYH9	Non-muscle myosin heavy chain IIa

NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKP	Natural killer progenitor
NKT	Natural killer T
NLR	NOD like receptor
NMII	Non-muscle myosin II
PAMP	Pathogen associated molecular pattern
PAK	P21 activated kinase
PD-1	Programmed cell death protein 1
PI	PMA $^{+}$ ionomycin
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKCθ	Protein kinase Cθ
PLCγ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate
PP2A	Protein phosphatase 2A
PRR	Pattern recognition receptor
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PTP-PEST	Protein tyrosine phosphatase non-receptor type 12
PXN	Paxillin
Pyk2	Protein tyrosine kinase 2 beta
RANTES	Regulated on activation, normal T cell expressed and secreted
RasGRP1	Ras guanyl-releasing protein 1
Rae1/2	Ribonucleic acid export 1/2
Rap1	Ras-related protein 1
RhoA	Ras homolog family member A
RPMI	Roswell Park Memorial Institute
$S1P_5$	Sphingosine-1-phosphate 5
SH2	Src Homology 2
SHP1/2	Src homology region 2 domain-containing phosphatase-1/2
SLAM	Signalling lymphocyte activation molecule
SLO	Secondary lymphoid organ
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SMAC	Supermolecular activation cluster
SNARE	Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor
Sos	Sons of sevenless
SP	Single positive
TCR	T cell receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor

TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R1/-R2	TNF-related apoptosis-inducing ligand – receptor1/receptor2
TSP	Thymic seeding progenitor
Tyr	Tyrosine
ULBP	UL16-binding proteins
VAMP7	Vesicle-associated membrane protein 7
VAT	Visceral adipose tissue
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WASp	Wiskott-Aldrich syndrome protein
WT	Wild type
ZAP70	Zeta-chain-associated protein kinase of 70 KDa

Chapter 1: Introduction

1.1. The immune system

In nature, the defence against microbial pathogenic lifeforms by animals is dependent on the effective response of specific cells and proteins in the mammalian body called the immune system. In humans, these cells are not conglomerated into one distinctive organ, but rather distributed throughout the body at various regions to provide localized rapid response to infections. To reach the varying areas of the body, immune cells travel through the bloodstream or move through specialized vessels called the lymphatic system. During an immune response against a pathogen, the coordination between various types of cells help each other to distinguish self from non-self to remove pathogens without harming the body. The development of immune cells all begins in the bone marrow with the hematopoietic stem cell giving rise to two branches: the innate immune system and the adaptive immune system.

Innate immunity is an invariable, hereditary response that occurs without prior exposure to disease causing agents or foreign substances. Innate immunity is comprised of myeloid and lymphoid cells as well as circulating proteins that respond rapidly to pathogens in a non-antigen specific manner. The innate immune system proteins include C-reactive proteins, complement proteins, LPS binding proteins, and other proteins that help modulate the speed and intensity of the innate immune response. The main components of the innate immune system are the cells which are comprised of the myeloid lineage macrophages, monocytes, dendritic cells (DCs), eosinophils, basophils, mast cells, neutrophils, platelets, the lymphoid lineage natural killer (NK) cell, and several other lymphoid lineage populations [1]. Innate immune cells can detect unwanted conditions such as tissue damage, infection, cancerous growths through the activation of germline encoded sensory receptors called Pattern Recognition Receptors (PRRs) on innate immune cells with an example being the toll like receptors (TLRs) [2]. Using PRRs, myeloid cells can mount a rapid response within minutes of

encountering the conserved molecule expressed by pathogens (also know as pathogen associated molecular patterns [PAMP]). Transmembrane PRRs are the classical membrane receptors that activate immune cells through binding of cell extrinsic PAMPs leading to activation of immune cells. The second class of PRRs is the cytosolic class such as the NOD like receptors (NLR). NLRs sense pathogens that have bypassed the cell membrane and invaded the intracellular environment [2]. The third class of PRR is the secreted proteins that bind microbes and stimulate activation of host immunity such as the case of opsonins [3]. The resulting signaling of PRR activation leads to subsequent activities including transcription of immunity genes (ex. interferon [IFN] related genes), activation of phagocytosis in phagocytic cells, triggering of cytotoxic activity in NK cells, as well as transcriptional outcomes that amplify an immune response [2, 4].

Within the innate immune system, NK cells play the essential role in acting as the cytotoxic mechanism that can kill pathogen infected or defective cells [5, 6]. This rapid cytotoxic response by NK cells provides initial containment against any intracellular pathogen infections or cancerous growths [6]. A rapid response is possible as NK cells are part of the innate immune system and do not require prior priming before cytotoxicity. In contrast, the cells of the adaptive immune system require activation via antigen presenting cells (APCs). APCs are integral aspects of the innate immune system that bridge the innate immune system to the adaptive immune system. APCs, most notably dendritic cells but also includes B cells and macrophages, capture small moieties of pathogens called antigens and present the antigens to adaptive immune cells to prime them for activation [7, 8]. To clear an infection, the induction of the adaptive immune system, comprising of B and T cells, is required.

B and T cells develop from the lymphoid branch of the hematopoietic stem cell within the bone marrow. Unlike the germline encoded receptors of innate immunity, the receptors for B and T cell activation are produced through random genetic rearrangement which generate millions of possible

receptors for recognizing foreign antigens that illicit immune responses [9]. Naïve B cells enter secondary lymphoid organs and are primed for activation if APCs present strong affinity antigen to the B cell receptor (BCR) [10]. The activation of B cells require interaction between the B cell and T helper cells which allows for the B cell to enter multiple affinity selection and maturation stages occurring within the germinal center of SLOs. The end stage of development yields plasma cells which mediate the humoral immunity by secreting the BCR called antibodies that neutralize toxins and alert immune cells of foreign substance presence within the body [11]. T cells are the major players within the adaptive immune system that help control the severity of response while providing cytotoxic capabilities. T cells are split into various subtypes with the two most prominent subsets being the $CD4^+$ T cell and the $CD8^+$ T cell. $CD4^+$ T cells, also called T helper cells (Th cells), are the main modulators of the strength and type of an immune response. Th cells can be further subdivided into Th1, Th2, Th17, as well as other minor subtypes of helper T cells [12]. Th1 cells provide cytokines and signals such as IFNγ, TNFα, and IL-2 that initiate a cell mediated cytotoxic immune response against intracellular pathogens [12, 13]. Th2 cells secrete cytokines such as IL-4, IL-5, and IL-13 which help mediate the activation of humoral (also known as B cell antibody mediated) immune response against extracellular parasites, bacteria, allergens, and toxins [12, 13]. Like the NK cell, $CD8^+$ T cells are the effector cell for destroying pathogen infected cells or cancerous cells. However, unlike NK cells, $CD8^+$ T cells need prior antigen presentation by APCs before performing its cytotoxic function. Activation of the Th1 response utilizes $CD8^+$ T cells to become activated by APCs to transform into cytotoxic T lymphocytes (CTLs) which can kill off target cells that engage their T cell receptor (TCR) [12, 14].

1.2. NK cell biology

1.2.1. NK cell development

Recently, NK cells have been described as a subset of leukocytes within the innate lymphoid cells (ILC) group. All ILCs derive from a common precursor and give rise to multiple ILC subtypes including ILC1, ILC2, and ILC3s [15]. ILC2 and ILC3 subtypes secrete cytokines corresponding to Th2 and Th17 immune responses respectively. NK cells are one of two subtypes of ILC1s that produce Th1 cytokines such as IFNγ, further driving the inflammatory condition and cytotoxic activity during a response to intracellular pathogens [15]. Classical models of NK cell development support the NK cell developing from the common lymphoid progenitor (CLP) which gives rise to all lymphoid cells including the ILC subtypes [16].

The development of NK cells primarily occurs in the bone marrow with mature cells emigrating to various tissues of the body [16, 17]. All subsequent NK cell development information will be referring to murine NK cells. Starting from the parenchyma of the bone marrow, NK cell developmental intermediates egress towards sinusoids of the bone marrow and out to second lymphoid organs depending on the stage of maturation [16]. Cytokines produced by stromal cells residing in bone marrow provide important stimulating cytokines including IL-3, IL-7, c-kit, flt3 ligand, and IL-15 for promoting NK cell development [17, 18]. During development, the acquisition of the IL-15 receptor β chain (CD122) initiates the differentiation of CLPs into NK cells, by providing signals for functional maturation and survival. The development of NK cells following the IL-15 expressing NK progenitor introduces the expression of NK receptors in a sequential manner, starting with NK1.1, NKG2A, and NKp46 marking the stage of development called the immature NK cell stage [16-18]. The immature NK cell stage is subsequently followed by the expression of Ly49 receptors, CD49b, and CD11a progressing the development into the mature NK cell stage [16-18]. The transition from immature NK cells to mature NK cells can also be defined by the expression

of CD11b and CD27 following the maturation program of $CD11b^{lo}$ $CD27^{lo}$ → $CD11b^{lo}$ $CD27^{hi}$ → $CD11b^{hi}$ $CD27^{hi}$ → $CD11b^{hi}$ $CD27^{lo}$ [16-18]. $CD11b^{hi}$ $CD27^{hi}$ mature NK cells produce relatively more cytokines and display more robust cytotoxic potential post cytokine stimulation than $CD11b^{hi}$ $CD27^{lo}$ NK cells. $CD11b^{hi}$ $CD27^{lo}$ NK cells represent the end stages of NK development and arise from $CD11b^{hi}$ $CD27^{hi}$ NK cells [16].

1.2.2. NK cell trafficking

Movement of NK cells to various areas of the body is driven by small signalling molecules called chemokines that stimulate movement towards a high concentration gradient [19]. Chemokines are small molecular weight proteins under 10kDa that consist of several cysteine residues and disulphide bonds hence the CXC or CC naming nomenclature. They associate with chemokine receptors which are G protein coupled receptors that contain seven transmembrane domains and a cytoplasmic signaling domain. Depending on the destination of the leukocyte, these cells will express specific chemokine receptors that drive the movement of the cell to specific areas of the body.

Early trafficking of NK cells within the bone marrow is dependent on several chemokine receptors including CX3CR1 and CXCR4 [20, 21]. Immature NK cells and NKPs express $CXCR4^{hi}$ and $CX3CR1^{lo}$ which has been demonstrated to be important for NK cell trafficking within the bone marrow, especially the parenchyma [22]. CXCR4 expression gradually decreases during the NK cell maturation process while maturing NK cell transition from the parenchyma to the sinusoids is facilitated an increased CX3CR1 expression [21, 22]. The adherence of terminally mature NK cells to the sinusoids during transition from the parenchyma to the sinusoids is dependent on integrin receptors VLA-4 (CD49d/CD29) and LFA-1(CD11b/CD18) binding to their associated ligands VCAM-1 and ICAM-1 respectively [20]. These integrin receptors allow for tight binding to endothelial walls which moves the mature NK cells to the sinusoids where it encounters sphingosine-1-phosphate receptor 5 ($S1P_5$) [23]. $S1P_5$ is one of five sphingosine-1-phosphate G protein coupled

receptors and is selectively expressed by NK cells, facilitating NK cell egress out of the bone marrow to secondary lymphoid sites [22, 23].

Once NK cells enter circulation, NK cells travel to secondary lymphoid organs (SLOs) such as lymph nodes through high endothelial venules (HEVs), similar to the movement of other lymphocytes in circulation [24, 25]. HEVs are areas of the endothelium where the endothelial cells possess an unconventional "plump" morphology and express receptors specific for leukocyte attachment [25]. Adhesion protein receptors such as CD62L and LFA-1 (CD11b/CD18) on NK cells as well as other lymphocytes assist lymphocyte rolling and tight adhesion onto HEVs allowing for extravasation into secondary lymphoid organs [24-27]. How NK cells reach the various tissues post secondary lymphoid organ exit during steady state has been understudied.

1.2.3. NK cell target recognition

NK cells employ a wide array of activating and inhibitory receptors that act in unison to control the activation of cytotoxicity in NK cells. The best characterized activating NK cell receptor is NKG2D that recognizes ligands not normally present or present in low amounts on healthy cells [28]. NK cell activation via NKG2D is dependent on expression of induced-self proteins on stressed, malignant transformed, or pathogen infected cells. Murine NKG2D induced self ligands include Rae1, H60, and Mult1 while human NKG2D ligands include MICA/MICB and ULBP proteins [28]. Another large group of receptors that are vital for controlling NK cell activation/inhibition are the Ly49 receptors encoded by the *Klra* gene cluster. Although named Ly49 receptors, each different receptor may play opposing roles in NK cell cytotoxicity signalling with some inducing activation and others causing inhibition of activation. Some known inhibitory Ly49 receptors for NK activation include the Ly49A, LY49C, and Ly49G that recognize murine peptide bound H-2 gene family coded Major Histocompatibility (MHC) receptors [29]. This leads to the prevailing "missing self" theory of NK cells being activated by the decreased MHC receptor expression during viral interference in cell

programming resulting in less inhibitory signals sent to the NK cells (**Figure 1.1**) [30]. In the absence of MHC expression on target cell surfaces, the activating signals overpower the lowered inhibitory signals leading to NK cell cytotoxicity of the target cell. Activating and inhibitory Ly49 receptors both mostly detect MHC receptors and activating Ly49 such as Ly49D, Ly49P, Ly49W has been reported to associate with peptide bound H-2D^{d} an isoform of the H-2D MHC class I protein [197]. Activating Ly49 receptors can also recognize a greater variety of ligands with an example being the Ly49H receptor recognizing the cytomegalovirus protein CMV m157 [29].

Similarly, the NK cell NCR receptor family, which contains NKp30, NKp44, and NKp46, can trigger NK cell cytotoxicity when activated through monoclonal antibodies [31]. NKp46, commonly used as an NK cell identifying marker, is an activating receptor that binds to influenza viral hemagglutinin and may possibly bind to other unidentified ligands [31]. Another important activation receptor of the NK cell is the CD16/Fcγ receptor, which binds the Fc portion of antibodies and triggers antibody dependent cell cytotoxicity (ADCC) to targets bound by the IgG antibody [32]. The receptors described above only indicate a portion of the extensive repertoire of NK activating and inhibitory receptors with many identified receptors having no known ligands.

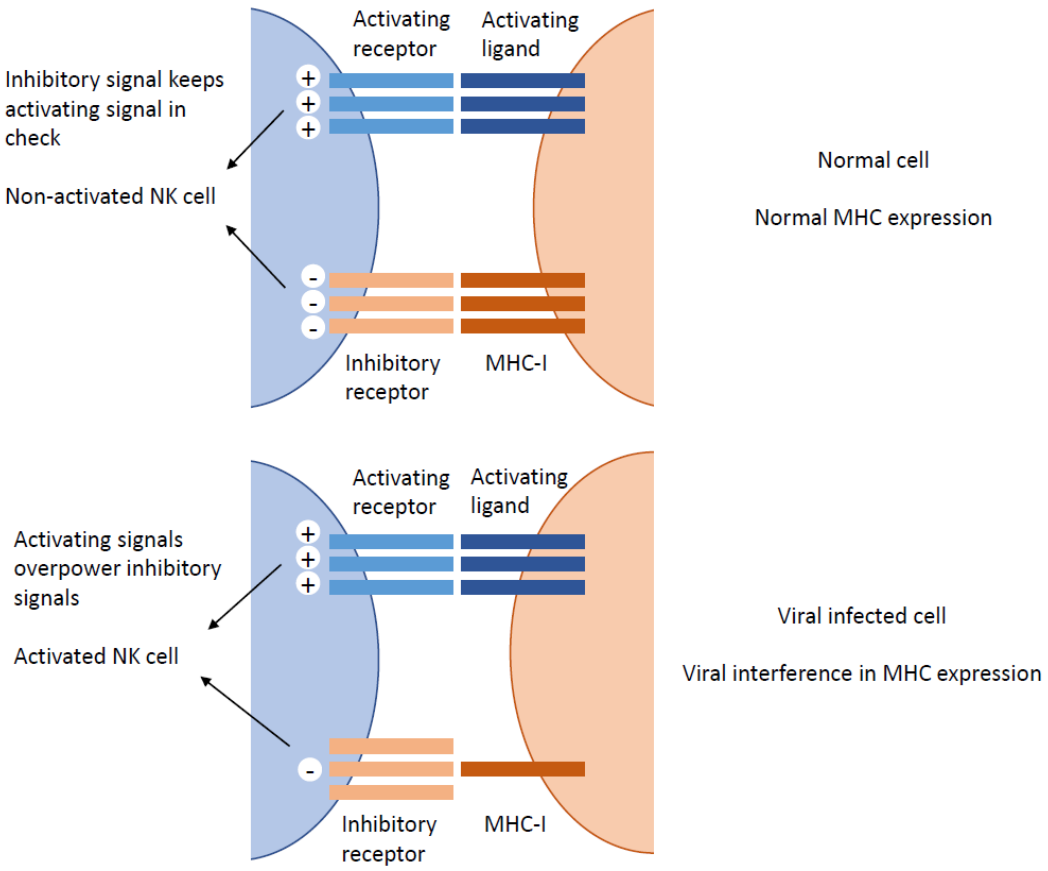


Figure 1.1. NK cell "missing self" activation theory. During normal conditions, NK cell activation is kept in check by the inhibitory ligands such as MHC-I binding to its associated inhibitory receptor on the NK cell. During intracellular pathogenic infections like viral infections, interference in gene expression of peptide presentation proteins with a major protein being MHC-I prevents its expression on the cell surface. The lack of MHC therefore provides less inhibitory signals for the NK cell causing the activating signal to overpower the inhibitory signals leading to NK cell activation.

1.2.4. Activation downstream signalling

As NK cells employ a wide selection of receptors to control the activation state of the cell, diverse pathways are utilized by the numerous receptors that ultimately lead to the activation of the NK cell cytotoxic program. In the case of activating receptors like CD16 that has bound antibody or NKp46, coupling to the immunoreceptor tyrosine-based activating motifs (ITAM) bearing proteins, such as Fcγ, will trigger a downstream signalling cascade leading to activation (**Figure 1.2**) [33]. Engagement of ITAM leads to the involvement of the Src family tyrosine kinases such as Lck that serves to transfer the signal to further protein tyrosine kinases of the Syk family including Syk and ZAP70. Fyn is a Src family tyrosine kinase that is phosphorylated by Lck and acts to propagate cell activation signals by phosphorylating Syk family members such as ZAP70 [34]. In addition to its role in activation, Fyn may play a role in fine-tuning/suppressive role in NK cell activation as Fyn knockout NK cells display a markedly increase in pro-inflammatory cytokine production post NKG2D activation [35]. Distinct from Fyn and Lck, the third Src family tyrosine kinase Lyn acts as a negative regulator in lymphocyte function [35, 36]. Genetic deficiencies of Lyn in mice show an increase in Fyn activity within lymphocytes and spontaneous development of autoimmunity, indicating that Lyn may play a role in the suppression of other Src-family kinase activity [37]. Zap70, also known as Zeta-chain-associated protein kinase 70, is a protein kinase that serves to kickstart the activation response of an activating receptor signal through phosphorylation of many proximal proteins.

Once the phosphorylation cascade starts, the next step involves the recruitment and phosphorylation of adaptor proteins including linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (SLP-76) [33, 34]. Both LAT and SLP-76 act as scaffolds in the signalling complex and propagate the activation signal through bringing proteins to proximity of one another. Scaffolding proteins bring together the Syk family kinases and the Vav family of guanine

nucleotide exchange factors (GEF) that exchange GDP on GTPases to GTP. GTPases are proteins that cleave bound GTP into GDP for energy during its enzymatic activity. In contrast to CD16, the activating receptor NKG2D does not propagate cell activation through ITAM bearing proteins, but rather it couples to DAP 10 or DAP12 molecules for signaling [33, 197]. DAP10 proximally activates phosphoinositide 3-kinase (PI3K) or adaptor proteins that subsequently phosphorylate the Vav family of GEFs [33]. A common target for PI3K is the phosphorylation of AKT a multitarget effector with roles in cell activation, growth, and metabolism. DAP12 signals for activation through ITAM recruitment [197].

Where activating receptors converge in signalling is the activation of Rac (a subfamily of the Ras superfamily of small GTPases) by the Vav GEFs which subsequently leads to the following chain of activities: activating of p21 activated kinase (PAK) → activation of mitogen-activated or extracellular signal-regulated protein kinase kinase (MEK) → activation of mitogen activated protein kinases (MAPK) → activation of extracellular signalling regulated kinase (ERK) [33]. This activation signalling cascade culminates in the polarization of the granules, cytokine secretion, and targeted release of cytotoxic granules at the bound target cell. In a similar manner, scaffolding proteins also recruits and phosphorylates the growth factor receptor-bound protein 2 (Grb2) adaptor and sons of sevenless (Sos) GEF complex contributing to MEK activation by the way of Ras and Raf [33]. Ras is a prototypical member of the Ras superfamily of small GTPases while the Raf family of kinases are signal transduction proteins in cell growth and proliferation pathways [38]. Another way of amplifying the NK cell activation signal is by the activation of phospholipase Cγ (PLCγ) by the Src family kinases which subsequently cleaves PIP_2 to IP_3 and diacylglycerol (DAG) [39]. IP_3 triggers Ca^{2+} release from the ER promoting phosphorylated NFAT transcription factor activity [40]. DAG recruits RasGRP1 a Ras superfamily protein as well as activates NFκB transcription factor

through protein kinase Cθ (PKCθ) [41, 42]. In both IP3 and DAG signaling, subsequent immune activating gene transcription is promoted.

Just as activating receptors engage ITAMs to propagate an activation signal, some of the inhibitor receptors of NK cells associate with immunoreceptor tyrosine-based inhibition motifs (ITIM) to halt activating signals [43, 44]. The majority of inhibitory Ly49 receptors are inhibitory and signal through ITIMs although some Ly49 receptors can be activating and utilize the DAP12 molecule for signaling [197]. Upon ITIM tyrosine phosphorylation, the tandem proteins of the SH2 family protein tyrosine phosphatases SHP-1 and SHP-2 are recruited to the receptor downstream site. These phosphatases serve to stop the signal through dephosphorylating key proteins in the signalling cascade such as SLP-76 and Vav [33, 44]. The balance between inhibition or activation of the NK cell profile is dependent on the magnitude of engagement of both activating and inhibitory receptors at the site of contact with NK target. A dynamic equilibrium is maintained on critical components of signalling such as Vav which tips towards phosphorylation and activation or de-phosphorylation and inhibition based on the relative strength of each signal received.

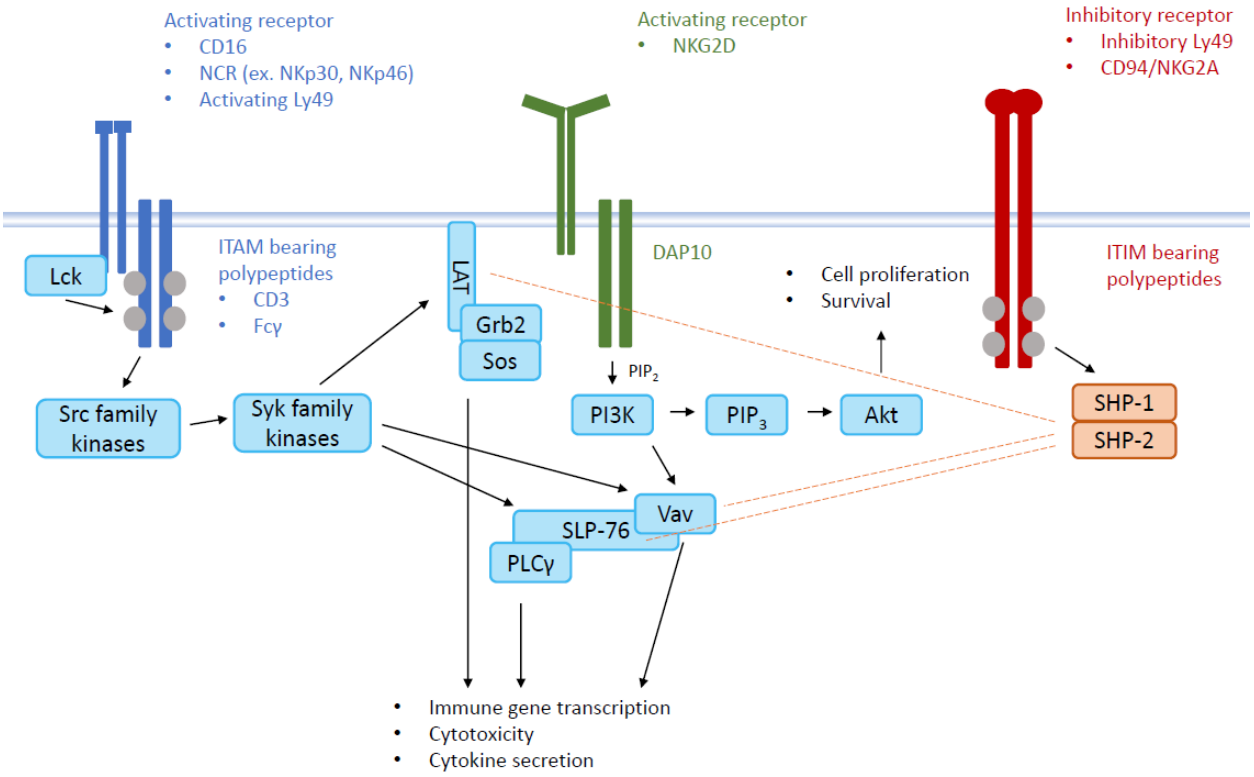


Figure 1.2. Signaling network downstream of NK cell activating and inhibitory receptors. Proteins in blue boxes represents activating pathways with arrows delineating the pathway progression. Proteins in red represents inhibitory pathways with dashed lines in red indicating areas of phosphatase activity and protein phosphorylation inhibition. Grey circles indicate ITIM or ITAM phosphorylation. The pathways shown only represent part of the entire activating receptor signaling network.

1.2.5. NK cell cytotoxicity

NK cells can recognise virally infected or cancerous cells through germline encoded receptors which send a signalling cascade resulting in the lysis of the target cell. NK cells can mediate cytotoxicity via two distinct pathways. They can kill target cells through directional release of cytotoxic granules containing perforin or granzyme B, or they can induce apoptosis in target cells through the death receptors Fas ligand (FasL) and TRAIL to engage Fas/CD95 or TRAIL-R1/-R2 [45].

NK cell degranulation is a process of targeted release of cytotoxic granules involving the directional movement and release against target cells. The release of cytotoxic granule contents occurs within a supramolecular structure called the immunological synapse (IS) [46]. Contents of the cytotoxic granule that help induce lysis include the granzyme family of proteins and perforin [45]. Perforin is a protein that polymerizes on target cell membranes to form a membrane attack complex [47, 48]. Perforin consists of three domains: The N-terminal membrane attack complex perforin like (MACPF)-dependent cytolysin pore-forming domain, a central epidermal growth factor (EGF) domain, and a C terminal membrane and $Ca2^{+}$ -binding C2 domain [48]. The polymerization of perforin is dependent on the pH and $Ca2^{+}$ concentration with the acidic pH present in cytotoxic granules preventing perforin from forming pores on the vesicle surface [45]. Perforin release during degranulation was initially thought to polymerize into pore like structures on the target cell membrane. However, there is virtually no evidence that perforin forms stable transmembrane domains [49]. Indeed, the formation of pores on a cell membrane that disrupts the osmotic balance and ion concentrations warrants a response to remove the defective membrane patch through endocytosis. Thus, it is likely that perforin disrupts the function and trafficking of the endosome after granzyme endocytosis into the target cell [45, 49]. Once within the endosome, it remains unclear whether the perforin polymerizes pores on the stable endosome membrane allowing for the granzymes to enter the cytosol or the perforin alters the endosome fate in an unknown manner that

results in granzyme release. Regardless of the mechanism of perforin action, the result is the release of granzymes into the cytosol triggering apoptosis through caspase activation [45].

Granzymes are a family of structurally related serine proteases that are secreted during the degranulation process and together induce apoptosis within the target cell [45].The function of individual granzyme family members are functionally redundant as genetic deficiencies within CTLs in any one granzyme protein still results in apoptosis or target cells *in vitro*, albeit more slowly in the case of Granzyme B deficiency [49]. Granzyme A and B are the most well studied enzymes with Granzyme B being a potent inducer for apoptosis. Granzyme B has many target cleavage sites but prefers to cleave after aspartate residues [45]. The target of Granzyme B *in vitro* includes caspase-3 and caspase-6, 7, 8, 9, 10 but caspase-3 cleavage is thought to be the most pivotal *in vivo* [45, 49]. Caspase-3 cleavage by Granzyme B results in caspase-3 activation and subsequently induces caspase dependent cell death via cleavage of various structural and regulatory proteins of the nucleus, cytoskeleton, and cytosolic proteins [45, 50]. In addition, Granzyme B mediates caspase independent cell death by cleaving the Bid protein [45, 50]. The cleaved, truncated tBid protein relocates to the mitochondria to interact with the pro-apoptotic proteins Bad and/or Bax resulting in the loss of mitochondrial membrane integrity. The lysed mitochondria further release pro-apoptotic proteins including cytochrome c, promoting cell apoptotic death.

Unlike granzyme mediated cell death, FasL and TRAIL signalling for cell death does not require entering of protein into the target cell cytosol. FasL is a transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily typically found within secretory cytotoxic granules [45, 52]. In NK cells and CTLs, the secretory granules that contain FasL are distinct from granules that contain granzymes and perforin [52]. FasL during granule secretion are quickly diffused along the membrane but constrained within the immunological synapse by LFA-1 [53]. Upon binding to Fas/CD95, the receptor/ligand complex multimerizes with other Fas/FasL complexes triggering the

formation of death induced signalling complex (DISC). DISC formation leads to caspase-8 and 10 mediated cell death signalling cascades [54]. TRAIL is also a transmembrane protein with homology with FasL and TNF. TRAIL has been shown to localize in vesicles containing CD107a [45]. The mechanism for TRAIL release from these vesicles remains a mystery with only the cytokines IL-2, IL-15, and IL-12 previously shown to stimulate the expression of TRAIL on NK cell surface [45]. However, the mechanism for the directed targeting of a target cell by NK cell TRAIL ligands during killing remains unknown.

1.2.5.1. MTOC reorientation and granule polarization during the NK cell killing cycle

Right after the discovery of NK cell cytotoxicity, it was shown that NK cells can serially kill multiple targets [45]. During an infection, most cytotoxicity is mediated by a small percentage of the total population of NK cells within the body killing in a serial manner [55]. Every killing cycle initiates with the recognition of ligands by activating receptors that overpower any signals sent by inhibitory receptors. Integrin receptors quickly form tight attachments towards the target cell followed by the formation of the immunological synapse (IS). Cell polarization begins with the movement of the MTOC towards the IS mediated by activating receptor and β2 integrin signalling as shown in **Figure 1.3** [56]. The MTOC reorientation is required for efficient killing and is thought to prevent bystander cell cytotoxicity but also implies that only one lytic IS can be formed, and NK cells can only kill one cell at a time.

Almost all studies regarding the reorientation process of the MTOC during cytotoxicity has been done in T cells which share a similar process to NK cells in MTOC reorientation. Thus, looking at T cells as a surrogate for NK cell MTOC reorientation, the directional movement of microtubules is brought about by the actions of motor proteins including dynein [57]. While the reorientation of the microtubules is better studied in T cells, the exact step-by-step mechanism remains mostly a mystery. Proximal signalling of TCR leads to the recruitment of the adaptor protein adhesion and

degranulation promoting adaptor protein (ADAP) to the site of the immunological synapse [58]. ADAP recruitment to the IS subsequently binds dynein which localizes the motor protein to the IS. Due to the localization of dynein to the IS, an attractive theory is that dynein acts as a motor that pulls the MTOC during reorientation. However, knockdown experiments of dynein have only minimally slowed down the reorientation of the MTOC [60], indicating other proteins may also facilitate MTOC movement in a functionally redundant manner to dynein. In addition to dynein, non-muscle myosin II (NM II) has been shown to assist in the reorientation of the MTOC with dynein and NM II double knockout cells having essentially no MTOC movement [60]. While dynein is localized at the MTOC, NM II is shown to localize in the opposite direction away from the IS and generates fibrous F-actin clusters behind the MTOC as it moves towards the IS [60]. Looking at the localization of both motor proteins, it is hypothesized that dynein pulls while NM II pushes the MTOC during reorientation with both proteins simultaneously facilitating its movement [59]. Other molecules within the activation signalling complex including Zap-70 and LAT also play a role in the contribution to MTOC reorientation although their exact role remains unclear [59]. Catalytic defects of Zap-70 or LAT deletion in CTLs both produce decreases in MTOC reorientation during T cell activation. Another scaffolding protein IQGAP1 has been demonstrated in NK cells to assists in the reorientation of the MTOC [64]. IQGAP1 directly interacts with Cdc42 which is hypothesized to facilitate the attachment of the microtubules to the F-actin at the IS site [65]. As IQGAP1 can interact with both actin cytoskeleton and microtubule associated proteins it is proposed that IQGAP1 serves as the linker between the two cytoskeletal structures [66]. The clearing of F-actin during the IS formation actin cytoskeleton rearrangement would then generate mechanical force to pull on the microtubules and move the MTOC towards the IS [66]. The movement of the MTOC is likely a concerted effort between the actions of the dynein and NM II motor proteins and mechanical force generated through actin pulling on the microtubules.

Following MTOC movement, the NK cell granules travel along with microtubules towards the MTOC resulting in the polarization of cytotoxic granules towards the IS and target cell (**Figure 1.3**). This step is called granule polarization. In NK cells the polarization process is dictated by integrin signalling as sole activation of β2 integrin LFA-1 can trigger polarization of lytic granules to the site of contact with ICAM-1 but does not trigger degranulation [70]. Like the MTOC reorientation process, little has been done in the advancement of knowledge on how lytic granules are signalled to polarize to the MTOC. Most proteins that signal downstream of the NK cell activating signal including Vav, Cdc42, Zap-70, and others have been shown to regulate granule polarization in NK cells in some capacity [71]. While other signalling molecules have unknown pathways in their mechanism of function in controlling NK cell polarization, signaling focused on the integrin-linked kinase (ILK)–Pyk2–paxillin core has been suggested to promote NK cell polarization during cytotoxicity [56]. In T cells, the signalling lymphocyte activation molecule (SLAM) receptors have been shown to promote cell polarization including the movement of lytic granules to the IS [72]. A cytosolic adaptor of the SLAM receptors, EAT-2 assists with the control of MTOC reorientation and granule polarization by linking the SLAM receptor to phospholipase Cγ, calcium mobilization, and Erk kinase [73]. Other adaptor proteins including LAT which are constitutively associated with 2B4 in glycolipid-enriched microdomains of the cell membrane have been shown to promote SLAM mediated cytotoxicity [74]. Movement of lytic granules along the microtubule network is dependent on motor proteins such as dynein [75]. The final stage of NK cell granule polarization is partially mediated by motor proteins including the non-muscle myosin heavy chain IIa (MYH9) that moves the granules along the F-actin network of the NK cell immunological synapse [76]. Removal of MYH9 significantly reduces granule polarization, granule velocity, and cytotoxicity NK cells but not MTOC reorientation [76]. Granules are then moved through the openings within the F-actin network and fused with the plasma membrane for content release [61].

The paxillin family of proteins which are adaptor proteins downstream of β2 integrin signalling have also been implicated in assisting granule polarization within NK cells [56, 89]. Paxillin knockdown has been shown to hinder the granule polarization process [89]. Leupaxin is another member of the paxillin family of proteins that have been shown to assist MTOC reorientation and granule polarization in human NK cell lines [56]. Generally, the paxillin family of proteins serve to connect integrin signalling to the actin cytoskeleton but how it contributes to granule polarization and motor protein activity remains a mystery [56, 68]. There has been evidence of paxillin associating with microtubule proteins directly [67], acting in a similar role to IQGAP1 with both paxillin and leupaxin denoted as upstream of the IQGAP1 protein in a β2 integrin microtubule signalling network proposed by the Long group [56, 69].

Fusion of lytic granules to the cell membrane is mediated by the soluble NSF attachment receptor (SNARE) proteins. These proteins reside on both the target plasma membrane called tSNAREs and vesicle membrane called vSNAREs. The major vSNARE of lytic granules is VAMP7/TI-VAMP with the protein colocalizing with T cell lytic granules throughout granule development [77]. With VAMP7 binding to membrane associated tSNAREs, the vesicle membrane of cytotxic granules fuse with the plasma membrane leading to the contents being released into the IS synaptic cleft. Once the release of granules is complete and target cell undergoes apoptosis, the NK cell detaches from its target cell to search for new targets for killing. The detachment process of cytotoxic lymphocytes likely relies on the deadhesion of integrin receptors within the IS. Where inside-out signaling is required for the activation of integrin receptors, it has been proposed that inside-out signaling is also required to maintain the conformational change that increases integrin receptor ligand affinity [198]. Thus as cytotoxicity commences and the target cell undergoes apoptosis, the endocytosis of activating receptors from the cell surface removes the inside-out signal required for maintaining

integrin activation and the integrin reverts back to the low affinity state allowing for the cytotoxic cell to detach from the dying target.

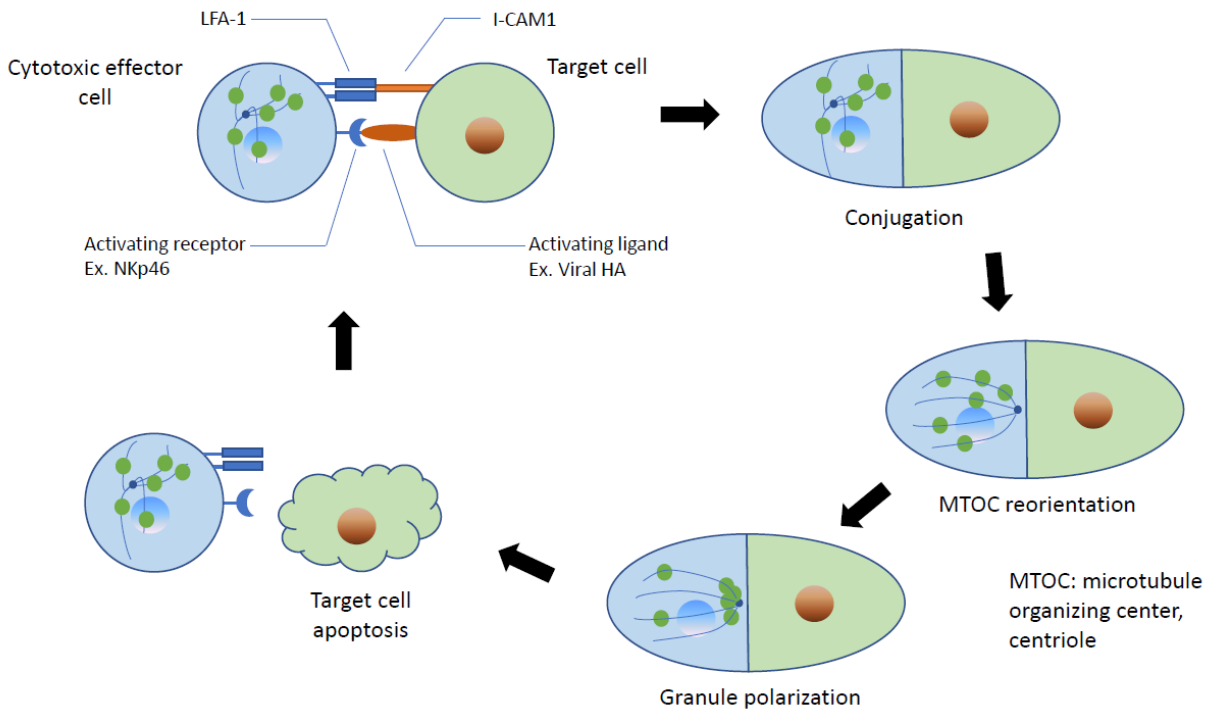


Figure 1.3. NK cell killing cycles. NK cell recognizes a target cell through activation receptors and its associated ligand along with LFA-1 binding to ICAM-1 for conjugation to the target cell. Once conjugation occurs, the formation of the immunological synapse issues signals for the reorientation of the MTOC towards the immunological synapse. Granules move along the microtubules towards the repositioned MTOC where they are subsequently released through the immunological synapse. The granule contents initiate target cell apoptosis and the NK cell releases the target and searches for a new target.

1.2.5.2. Immunological synapse structure

The immunological synapse is a highly organized structure formed during the engagement of an effector cell to target cell during cytotoxicity. The bullseye ring like structure of the IS is comprised of three distinct compartments: the central supramolecular activation cluster (cSMAC), the peripheral supramolecular activation cluster (pSMAC) and the distal supramolecular activation cluster (dSMAC) [78]. Again, the signals for cell activation and translocation of molecules has been extensively studied in T cells, insights in T cell SMAC structure may be informative on NK cell SMAC structure. TCR signals typically begins in the dSMAC of CTLs and migrate towards the cSMAC [79]. Upon reaching the cSMAC, the TCR signals come to a stop. The standing model proposes that once cytotoxicity occurs, the TCR is endocytosed into the cell for degradation by the ubiquitination machinery located at the cSMAC [80]. The translocation and docking of the MTOC to the cSMAC also dictates the terminal movement of lytic granules to move towards the MTOC and cSMAC culminating in the release of granular contents through the IS. In T cells it is well documented that granule release occurs through the cSMAC [81] while the exact location NK cell granule content release is unclear. Studies have indicated that within prototypical roughly formed NK cell SMAC center, 1-4uM diameter channels exist within the F-actin network that allow for lytic granule transit to the plasma membrane [46]

The second ring of the IS is the pSMAC. The pSMAC surrounds the cSMAC and is populated by integrin receptors such as LFA-1 to mediate adhesion to the target cell [82]. The tight binding on various adhesion receptors in the pSMAC ring has been suggested to act as a seal preventing the cytotoxic contents of the IS from leaking to neighboring cells [83]. Activating receptor binding such as the TCR in CTLs and CD16 in NK cells stimulates the opening of LFA-1 to an open conformation through a process called “inside out” signaling [84]. LFA-1 within the pSMAC is in its high affinity

conformation allowing for the stable interaction between the effector and target cells in addition to signaling cytoskeleton reorganization in the effector cell.

The outer third ring of the immunological synapse is called the dSMAC. Initial activating signal leads to the formation of a ring-shaped branched actin network in the cSMAC followed by retrograde flow of actin towards the dSMAC [82]. The dSMAC is abundant in F-actin which is critical for forming the barrier and edges of the IS. Specifically, the polymerized F-actin acts as a barrier to lytic granules fusion with the membrane serving to funnel the granules to clearings within the F-actin network located at the cSMAC. Once granules move through clearings at the F-actin network, fusion and release of granular contents can occur.

1.3. Integrin receptors

Integrins are a family of transmembrane receptors implicated in cell adhesion and motility. Each integrin receptor contains one α and one β subunit with there being 18 defined α subunits and 8 defined β subunits forming 24 known dimeric integrins [132]. Integrin protein structure contains 3 alternating conformations including bent, extended, and open conformations [133]. The integrin protein structure changes depending on the activation signals received by the cell. The bent conformation is the non-activated conformation and provides low affinity interactions that is associated with leukocyte rolling on endothelium during leukocyte recruitment to peripheral tissue [134]. When a cell receives activation signals, intracellular signal transduction changes the conformation of integrin receptors from bent to extended which have higher affinity to their respective ligands. Leukocytes interacts with various substrates, including endothelial lining and other cells through the strong affinity interactions mediated by integrin receptors. The β2 and β7 families of integrins are exclusively expressed on leukocytes while the β1 family is expressed on a wide variety of cells including leukocytes. Notable integrin receptors on leukocytes include LFA-1 (αLβ2) and VLA-4 (α4β1). The activation of integrin receptors during leukocyte recruitment to sites

of inflammation provides tight adhesion to endothelial lining allowing for cell arrest and extravasation.

Integrin α and β subunits are both transmembrane glycoproteins that include a large extracellular domain, a single membrane spanning domain, and an apart from β4, a cytoplasmic domain [135]. The cytoplasmic tail is what determines the conformation of the large extracellular domain through a process of "inside-out" signalling. The binding of ligands by the extracellular domain also facilitates regular "outside-in" signalling through the cytoplasmic tail and its associated proteins. During the resting/bent conformation of integrin proteins, the two subunits remain near each other. The bent conformation is still able to bind to ligands albeit with much lower affinity compared to its extended or open conformations [135]. Intracellular signalling upon receiving activation signals move the subunits of the integrin receptor apart from each other which alters the conformation of the integrin protein into its extended conformation [133]. The extended conformation exposes its ligand binding globular headpiece to potential ligands. The full strong affinity open conformation interaction is achieved when the integrin binds to its associated ligand.

1.3.1. Inside-out signaling

For leukocyte integrin receptors to achieve its full affinity and to its ligands and for clustering to occur, a series of intracellular signaling events need to occur in a process called inside-out signalling. Non-stimulated leukocytes generally have surface integrin receptors in the bent conformation that restrict the cell from forming tight adhesions to random targets. In the event of an activating signal received by a chemokine receptor or lymphocyte activating receptors such as the TCR or NKG2D, the intracellular signaling events open the integrin into the extended conformation and cluster towards a specific direction, allowing for the activated cell to tightly adhere to its intended target [136]. Various signaling proteins downstream of activating signals also play a role in the alteration of integrin conformation. Activating receptor signals recruiting LAT to the signaling complex initiates

the pathway for integrin inside out signaling. LAT in addition to SLP-76 both being large scaffolding proteins transduces the activating signals to the integrin signalosome through PKC and ADAP.

TCR signal transduction leads to the Rap GTPases being recruited to the cytoplasmic domains of integrin receptors [136]. Rap1 and Rap2 are proteins that are directly activated downstream of TCR/BCR or chemokine signaling and assist in LFA-1 and α4 integrin dependent adhesion. Rap1 recruits RapL which forms a complex with Mst1 that interacts with the α_L chain of LFA-1 to enhance integrin extended conformational change. Paxillin is another protein that acts downstream of TCR signalling while functioning as an interactor of the α subunit of the integrin receptor [136, 137]. Mutations in the amino acid sequence that block the interaction between paxillin and the α subunit tail promotes T cell spreading and tight adhesion during TCR activation. Paxillin acts as a negative regulator of integrin mediated tight adhesion in its phosphorylated form but acts as a positive regulator for integrin clustering formation in its unphosphorylated form [163].

Proteins that bind to the β subunit of the integrin receptor include talin and the kindlin family of proteins. Main features of proteins that bind to the β subunit is the inclusion of the FERM domain that interacts with β1, β2, and β3 integrin cytoplasmic tails [136]. Talin facilitates the extension of the integrin extracellular domain through its association with the β subunit cytoplasmic tail causing spatial changes of the integrin subunits. Deletion of talin in T cells leads to deficiencies in TCR mediated integrin activation [138]. Talin, in its resting state, forms a closed conformation with its FERM domain binding to its c-terminal rod domain, rendering it unable to interact with the β subunit cytoplasmic tail [139]. Upon inside out signal transduction, the talin protein becomes activated and the c-terminal rod domain disassociates from the FERM domain allowing talin to freely associate with the β subunit of the integrin receptor. Kindlins are another family of integrin associating FERM domain containing proteins that bind to the integrin β subunit cytoplasmic tail [136]. The kindlin family consists of Kindlin-1, 2, and 3 with kindlin-3 being exclusively expressed in leukocytes.

Kindlins appear to be co-activators that act in conjunction with talin to promote integrin activation. In kindlin-3 knockout mice, leukocytes show defects in the ability to arrest on endothelium during leukocyte recruitment to peripheral tissue [140]. It has also been suggested that kindlin association with integrins may contribute to their clustering to increase avidity of adhesion [141]. Recent developments have implicated leupaxin as a protein acting downstream of kindlin-3 that assists in the regulation of the stability and lifetime of integrin-cluster rich cellular protrusions called podosomes [87]. Paxillin phosphorylation in podosomes reduce their stability and promotes the dissolution of the actin rich protrusions while kindlin-3 recruits leupaxin, which recruits the phosphatase PTP-PEST, assisting in dephosphorylating paxillin and extending the lifetime of podosomes.

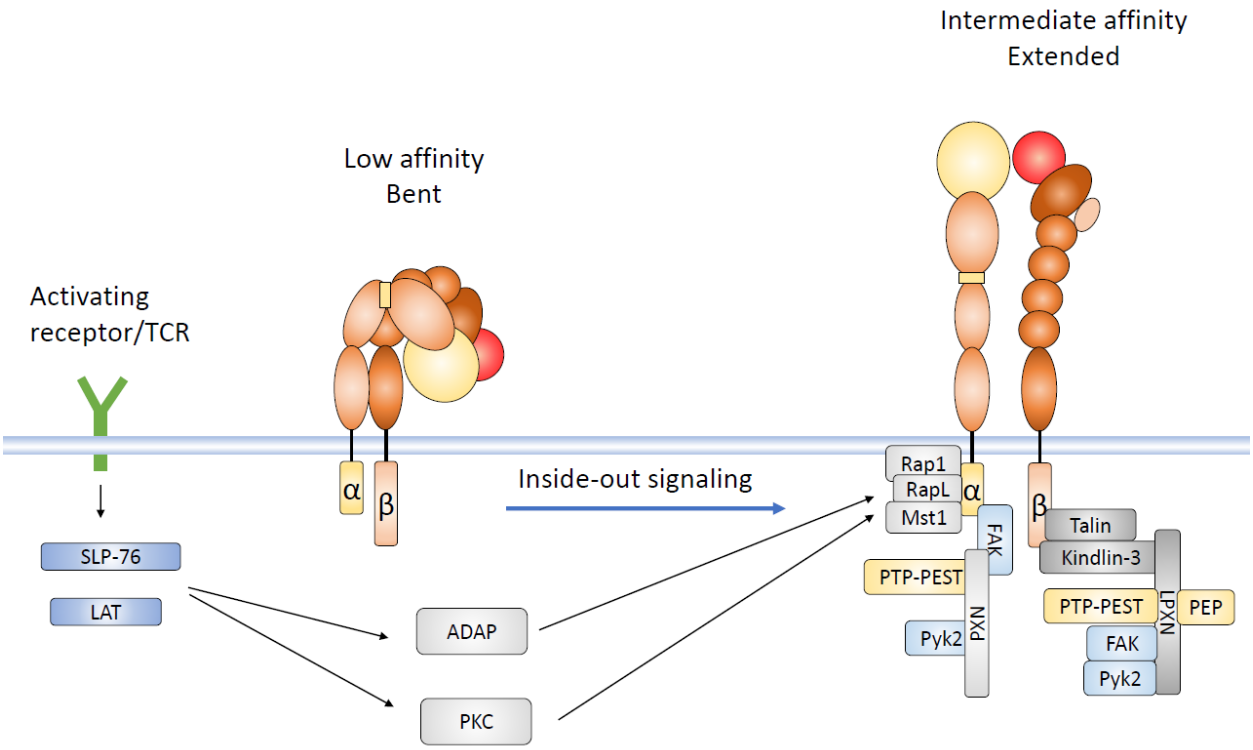


Figure 1.4. **A general overview of cytotoxic lymphocyte inside out signaling.** The induction of an activating receptor sends a signal downstream to adaptor proteins. Various proteins associated with these adaptor proteins including ADAP and PKC transmit the signal to the integrin receptor resulting in the recruitment of Rap1 to the alpha subunit cytoplasmic tail of the integrin receptor. Rap1 recruits RapL which forms a complex with Mst1 to facilitate the opening up of the integrin receptor to its extended conformation. Other proteins that associate with the alpha subunit includes paxillin and leupaxin that are adaptor proteins allowing for the localization of a wide variety of other enzymes to the integrin complex.

1.3.2. Outside-in signaling

The signaling events that occur post ligand-induced clustering of integrins is called outside-in signaling which are more canonical to regular receptor signaling pathways. In general, most outside-in signalling events have to do with the regulation of the actin cytoskeleton and microtubules for firm adhesion cell spreading and migration. However, there are some specifically integrin mediated events in some leukocytes that include the MTOC reorientation and granule polarization processes [56]. Furthermore, outside-in signalling in integrins provide co-signals that assist in leukocyte functions along with other activating signals such as IL-2 secretion and proliferation signaling by T cell TCR and β2 integrins. Initial events of integrin outside-in signaling is reminiscent of other activating receptors with the activation of the Src and Syk family kinases [136]. Kinase activity downstream of integrin activation constructs the integrin signalosome although the exact processes and proteins involved remains relatively unknown. In the process of phosphorylation, scaffolding proteins including SLP-76, ADAP, paxillin, and leupaxin are recruited to the cytoplasmic tail complex of the integrin receptors [136]. SLP-76 along with other adaptor proteins help form the signalosome of the integrin cytoplasmic tail. Focal adhesion kinase (FAK) and its homolog protein tyrosine kinase 2 beta (Pyk2) are both proteins that act downstream of Src family kinases and are recruited to the paxillin family of adaptor proteins [97, 142]. Both FAK and Pyk2 have been shown to play overlapping roles in promoting chemokine-induced macrophage motility and knockout of either proteins reduce the crawling velocity [143, 144]. FAK and Pyk2 are phosphorylated and activated by Src or Syk family kinases and serve to promote integrin cluster disassembly.

Following integrin clustering and integrin binding, numerous biochemical events and protein enzymatic activities culminate in the reorganization of the actin cytoskeleton. Most signaling pathways culminate in the activation of Rho GTPases. A key protein in the outside-in signaling pathway is the Vav family of Rho GTPases. The Vav proteins in turn activate proteins that modulate

actin fiber formation including Cdc42, Rac1, and RhoA [136]. All three are small GTPases that assist in the reorganization of the actin cytoskeleton [136, 145]. Another function of the Vav family of GTPases is the regulation of PLCγ and its subsequent Ca^{2+} signaling. Out of all the proteins identified downstream of Rho GTPase signaling, the Wiskott-Aldrich syndrome protein (WASp) is imperative to the integrin-based actin signaling. WASp is activated by numerous stimuli including the small GTPase Cdc42 when its bound to a GTP [136, 146]. Activated WASp unfolds into a conformation that allows it to bind to Arp2/3 which nucleates the polymerization of actin. The integrin pathway culminating in WASp activation alters the actin cytoskeleton to tailor the cell shape to the situational need.

In addition to adhesion events and actin cytoskeletal reorganization, integrin proteins on cytotoxic lymphocytes also aid in the various effector functions including killing and cytokine secretion. Proteins such as cytohesin-1 and JAB1 are activated post LFA-1 engagement [151]. Cytohesin-1 is phosphorylated during LFA-1 engagement but not TCR engagement. Cytohesin-1 deletion reduces IL-2 secretion from T cells post activation. JAB1 is a protein specifically associated with the LFA-1 signaling complex and dissociates from the complex during LFA-1 engagement to its ligand and activates the c-Jun complex formation. C-jun which binds to c-fos creating the AP-1transcription factor that regulates cell growth and cytokine secretion during lymphocyte activation. In another series of events during NK cell activation, integrin binding solely initiates the processes of MTOC reorientation and granule polarization. Unlike T cells which require the binding of the TCR to induce these processes, NK cells do not require any activating receptor engagement [70]. A signaling network comprising of ILK-Pyk2-Paxillin-leupaxin- RhoGEF7-Cdc42 and other proteins have been previously described downstream of β2 integrins to signal cell polarization [56]. Through the signaling of numerous effector and adaptor proteins, integrin signaling instructs NK cells to polarize

its cytoskeleton and cytotoxic granules although granule release is not controlled by integrin signaling.

1.3.3. Trafficking and cell-cell adhesions of lymphocytes

Lymphocyte usage of integrin mediated adhesion is mainly prevalent in two modes of action: extravasation across endothelium and attachment to other cells whether it be for antigen presentation or cytotoxicity. Lymphocyte extravasation across endothelium is a highly organized multi-step process that involves rolling, firm adhesion, crawling, transcellular migration, and migration through the basement membrane. In the event of tight adhesion to the endothelium, LFA-1 is the major contributor to lymphocyte adhesion during lymphocyte recruitment under inflammatory conditions for cells such as T and NK cells. VLA-4 is another contributor to the extravasation process of lymphocytes as deletion of LFA-1 still allows for lymphocytes to be recruited via VLA-4 in some tissue but not others [148]. Therefore, the involvement of integrin receptors is dependent on the expression of ligands on the endothelium within a specific tissue. The main ligand for LFA-1 is ICAM-1 and for VLA-4 is VCAM-1 although each integrin receptor has multiple ligands and each ligand can associate with different receptors. After adhesion, T and NK cells crawling along the endothelium searching for areas for transmigration. Due to the robust nature of the LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions, the cells can crawl against sheer flow and follow a chemotaxis gradient towards the site of inflammation [148, 149]. The crawling cells then send out LFA-1 and WASp rich podosomes to scan the surface for areas to move into the tissue across the endothelial barrier [150].

The second major involvement of integrin proteins in lymphocyte function is during attachment to cells during antigen presentation and target cell directed cytotoxicity. During cytotoxic lymphocyte binding to target cells, the formation of the SMAC contains the pSMAC ring which is enriched in β2 integrins like LFA-1 that co-stimulate lymphocyte cytotoxicity [82]. The binding of integrins during

target cell killing by effector cells is to mainly secure the effector onto the target cell mediated by the tight adhesion of integrins. In addition, a multitude of outside-in signaling events provide effector cells with polarization, cytokine production, and growth signals as described above.

1.4. Obesity and the immune system

Obesity is the accumulation of fat within the body to the extent where adverse health effects occur. These adverse health events are often due to the overt activation of the immune response. Weight gain is associated with a general low-grade systemic inflammation that leads to the systemic increase Th1 immune response leukocytes and Th1 associated cytokines. For those diagnosed with obesity, this systemic inflammation ultimately manifests in diseases such as type 2 diabetes and cancer. Numerous cell types play a role in the obesity induced inflammatory conditions with some major contributors being macrophages, ILCs, B cells, $CD4^+$ T cells, T_{reg}, and $CD8^+$ T cells [152].

Macrophages have two classifications of either M1 or M2 macrophages. M1 macrophages are the "classically activated" macrophages with prominence of M1 macrophage populations in inflamed adipose tissue. Generally, M1 macrophages are associated with a highly active Th1 response. These cells are activated by pro-inflammatory mediators such as LPS and IFNγ and secrete pro-inflammatory cytokines IL-6, IL-1β, inducible nitric oxide synthase (iNOS), and TNF-α [152]. M2 macrophages on the other hand are "alternatively activated" macrophages and promote tissue repair, angiogenesis, and anti-inflammatory conditions. These macrophages are induced by the Th2 cytokines IL-4 and IL-13 and are dominant in the adipose tissue of lean mice. M2 macrophages propagate Th2 anti-inflammatory responses by secreting IL-10, IL-1 decoy receptor, and arginase, thereby blocking IL-1β and iNOS activity [152]. The classical distinguishing marker between these two types of macrophages in adipose tissue is the surface expression of CD11c which is present on M1 macrophages and not on M2. During the transition from lean to obese phenotype, the M1/M2 axis of the macrophage population is characterized by a shift in polarization towards M1. During

obesity driven inflammation, several chemoattractants serve to recruit more circulating monocytes into the adipose tissue including MCP-1 (a well-characterized macrophage chemoattractant), leukotriene B4 (which is secreted by adipocytes and also stimulates chemotaxis of neutrophils), CX3CL1, as well a many others. Just as more obesity promotes monocyte recruitment, it also promotes macrophage retention. However, it is not only the recruitment and cytokine secretion of macrophages that drive the inflammation, macrophages also scavenge the tissue picking up antigens from possible invading pathogens to present to T cells which helps mount a Th1 adaptive immune response.

Both B and T cell types of the adaptive immune system are players in driving obesity induced inflammation. B cells, particularly those that have switched into the IgG class, promote adipose tissue inflammation and insulin resistance (IR0029. Insulin resistance being the reduction in responsiveness of cells within the body to the hormone insulin resulting in decreased blood glucose uptake by cells, increased blood glucose levels, and could potentially cause type 2 diabetes. B cells secrete pro-inflammatory cytokines including IL-8, IL-6, and TNF-α [155]. Along with cytokine production, B cell antigen presentation capabilities activate T cell responses. Both $CD4^+$ and $CD8^+$ T cells play pivotal roles during obesity driven inflammation. Th1 $CD4^+$ T cells directs the Th1 pro-inflammatory response by secreting Th1 cytokines such as IFNγ and recruiting other immune cells. A sharp increase in the Th1 $CD4^+$ T cell population numbers can be observed in mice fed high fat diet while Th2 and T_{reg} numbers remain static or decrease [157, 190]. A systemic deletion of the IFN-γ gene improves obesity-induced IR and lowers macrophage infiltration in AT. Increases in Th1 $CD4^+$ T cell population in the peripheral tissues of the body are therefore indicative of a mounting pro-inflammatory response and occurs during obesity. Conversely, Th2 $CD4^+$ T cells are anti-inflammatory and protect against obesity induced inflammation and diseases. Major cytokines that Th2 $CD4^+$ T cells secrete are IL-4, L-5, and IL-13. The frequency of Th2 $CD4^+$ T cells in adipose

tissue inversely correlates with insulin resistance and high-sensitivity C-reactive protein (hsCRP, a marker of systemic inflammation) while Th1 frequency directly correlates to hsCRP [158]. $CD8^+$ T cells are primarily killers of pathogen infected or abnormal cells but also functions as secretors of Th1 cytokines. Systemic depletion of $CD8^+$ T cells in mice effectively lower M1 macrophage populations, ameliorates pre-established tissue inflammation, and reduces insulin resistance [157, 188]. Reintroduction of $CD8^+$ T cells via adoptive transfer reinstated M1 macrophage populations, increased adipose tissue pro-inflammatory cytokine presence, and promoted insulin resistance. Thereby, $CD8^+$ T cells within tissues propagate pro-inflammatory immune responses during obesity.

Recent developments in the field of obesity related research had implicated the innate lymphoid cell population as critical mediators of both inflammatory and anti-inflammatory responses as mentioned above. Two important cells of the ILC subtype that are involved with obesity driven inflammation are the ILC1 and ILC2 cells. The function of these lymphocytes mirror $CD4^+$ T cells in their cytokine section role and are important regulators of an immune response [154]. ILC1s mirror the Th1 $CD4^+$ T cell subtype as both secrete Th1 cytokine to drive pro-inflammatory responses. In the case of NK cells, the corresponding cell of adaptive immunity would be $CD8^+$ T cells which also produce Th1 cytokines. These cells have been implicated in contributing to the development of non-alcoholic fatty liver disease [153]. ILC2s, which mirror the functions of the Th2 $CD4^+$ T cell, are a major source of Th2 associated cytokines including IL-5 and IL-13. The Th2 cytokine production promotes the polarization of the M1/M2 axis towards the M2 alternatively activated macrophages [152]. Furthermore, ILC2 promote the browning of adipocytes, turning from white to beige fat. Beige fat presence is more correlated with a lean body.

Curiously, defects in integrin receptors have been described as a cause of obesity within mice. It has been previously described that systemic deletion of the Mac-1 β2 integrin or ICAM-1 integrin ligand spontaneously increases the weight of the genetic knockout C57bl/6 mice [105]. However, others

have reported that a knock-in of a mutated β2 integrin receptor show normal weight gain but significantly increased insulin resistance [159]. β2 integrin receptors mostly function in the recruitment of leukocytes to areas of inflammation. The exact roles integrin β2 receptors play in the establishment of obesity associated inflammation and insulin resistance remains mostly a mystery. On the other side of the equation, β7 integrin knockout mice consistently display signs of protection from obesity-linked insulin resistance. Unlike β2 integrins, a mechanism of action has been proposed for β7 integrins where β7 integrins assist with localization of leukocytes to the gut epithelium [189]. The localization of intestinal epithelium leukocytes (IELs) to the gut epithelium reduces the Glucagon-like peptide-1 (GLP1) within blood plasma with has been linked to glucose tolerance. It remains to be discovered how β2 integrin deficiency can alter the motility/localization of leukocytes as pertaining to obesity.

1.5. Paxillin family of proteins

1.5.1. Paxillin family members

The paxillin family of proteins are multidomain cytoskeletal adaptor proteins that provide protein binding sites for signal transmission downstream of integrin receptors. The paxillin family of proteins include paxillin, leupaxin, and Hic-5 all of which are recruited to the integrin cytoplasmic tail during integrin activation and clustering. The protein structure of the paxillin family member consists of N-terminal half LD-motifs and C-terminal half LIM domains as shown in **Figure 1.6** [160]. LD motifs are leucine- and aspartate-rich motifs that have the consensus sequence LDXLLXXL and facilitate protein binding. LIM domains stand for Lin11, Isl-1, Mec-3 which are double zinc finger motifs that mediate protein-protein interactions between the paxillin family proteins and their binding partners.

Paxillin (PXN) is the first and most well characterized of the paxillin family of proteins. PXN is ubiquitously expressed in all cells and its deletion is embryonically lethal [161, 162]. This also

suggests that PXN function cannot be compensated by the other protein family members. PXN is a protein that exists in four alternatively spliced isoforms. The isoforms include α, β, γ, and δ with α being the most widely expressed and considered the canonical isoform of PXN [161]. Modification to the PXN protein including tyrosine phosphorylation and serine phosphorylation at multiple sites tightly regulate protein binding and spatial distribution of the protein within a cell [163]. An example of paxillin regulation by phosphorylation is the requirement of dephosphorylated PXN in the assembly of focal adhesions which are integrin-rich contact points between cells and the extracellular matrix [163]. The phosphorylation of Tyr31 and Tyr118 on PXN by FAK promotes focal adhesion disassembly. Serine phosphorylation of PXN at Ser178 by JNK regulates cell adhesion and migration [164].

Leupaxin (LPXN) is a protein within the paxillin family of proteins thought to have similar binding partners to PXN and is poorly studied. The expression LPXN occurs in a wide variety of cells within the body but most notably, LPXN is the most highly expressed within the cells of hematopoietic origin. LPXN is recruited downstream of integrin signalling during cell adhesion and migration and its recruitment is mediated by the LIM3 domains [170]. Pyk2 associates with both PXN and LPXN while LPXN recruitment to focal adhesions suppresses PXN phosphorylation [97, 98, 165]. This led to the hypothesis that LPXN acts as a negative regulator of PXN whether it be for competing for protein binding or dephosphorylating PXN.

Hic-5 shows restricted distribution with the highest expressing cells being smooth muscle cells [166]. Like LPXN, Hic-5 is not very well characterized so little is known about its roles within a cell. Hic-5 knockout mice have been generated which are viable, so the protein does not play a major role in embryo development [167].

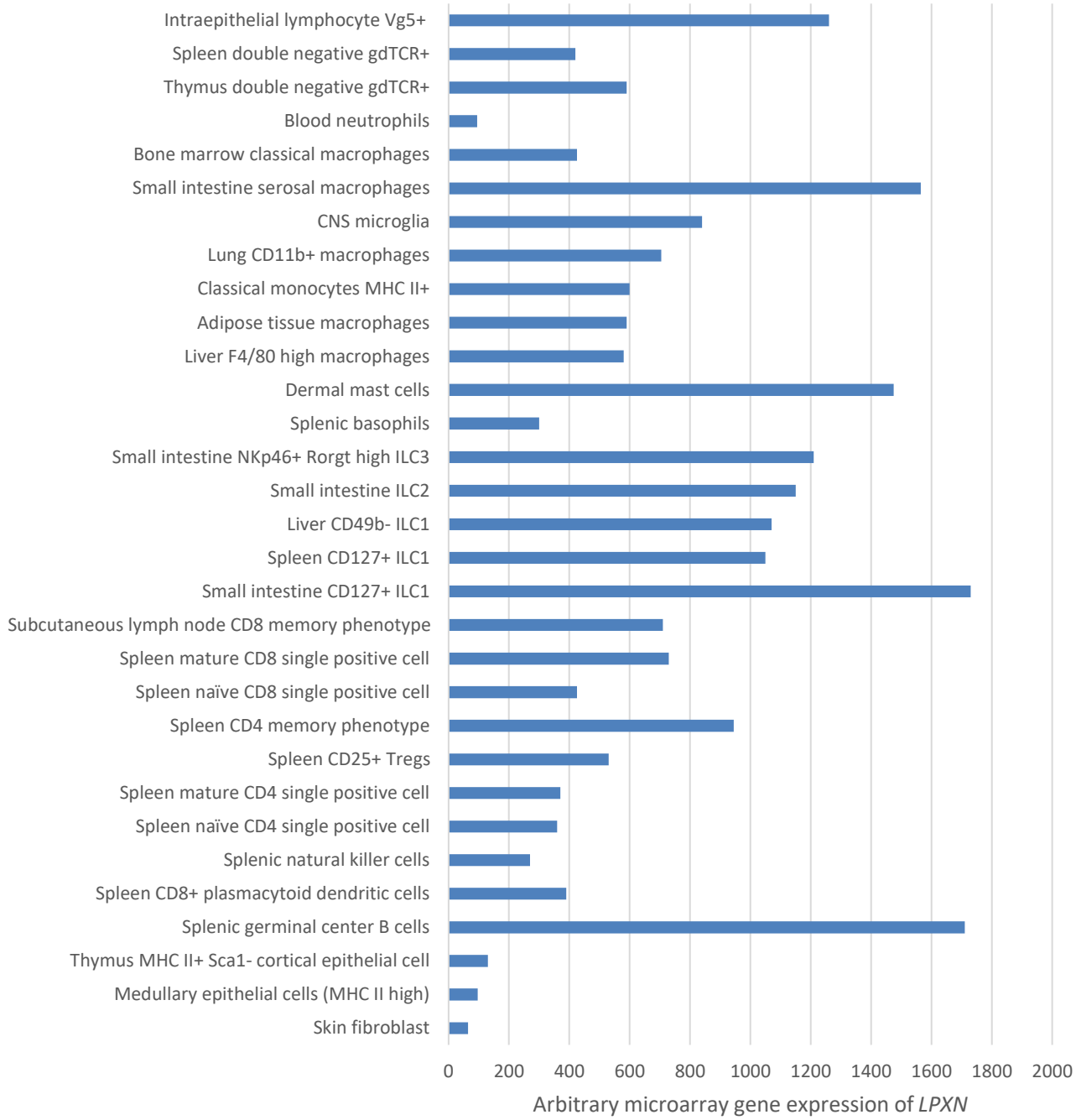


Figure 1.5. LPXN microarray gene expression chart. The RNA-seq data was collected from the website of Immunological Genome Project. The figure is created by Microsoft Excel.

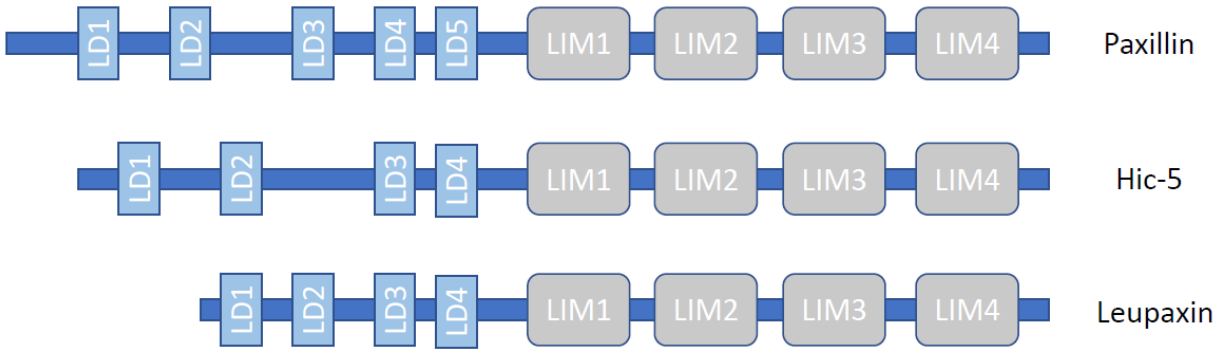


Figure 1.6. The paxillin family of proteins. The three members of the paxillin family of proteins including paxillin, leupaxin, and Hic-5. All three members contain LD motifs and LIM domains for facilitating protein-protein interactions. The LD motifs are leucine-rich motifs (LDXLLXXL). The LIM domains contain double-zinc finger motifs and are highly conserved among family members.

1.5.2. Leupaxin binding partners and functions

Curiously, all three members of the paxillin family of proteins have been implicated in the progression of cancer. Aberrant expression, mutation, and hyper phosphorylation of all and any of the three proteins can be found in prostate cancer, breast cancer, lung cancer, etc. PXN mutations and increased expression has been shown to be connected to increased lung cancer invasiveness [68, 168]. Similarly, LPXN is overexpressed in prostate cancer cells and its expression level correlates with the progression of the tumor [169]. Hic-5 plays a role in promoting breast cancer progression and is an indicator of metastatic behavior [167]. In all cases, the association of the paxillin family of proteins with cancer invasiveness, and metastatic behaviour gives insight to their role in regulating the downstream events of cell movement and integrin signaling.

Figure 1.5 shows the expression pattern of leupaxin occurring mainly within hematopoietic cells suggesting that leupaxin plays a major role in leukocyte integrin signaling. Moreover, expression seems to be more prominent in activated/tissue resident leukocytes as opposed to naïve/circulating leukocytes as seen in **Figure 1.5** which correlates with integrin activation during effector cell activation. Like PXN, previous data from our lab has shown that LPXN is also recruited to the signalling complex downstream of LFA-1 leukocyte integrin activation [172]. While both PXN and LPXN are structurally similar, the two proteins may act in opposition to one another. Knockdown of PXN decreases breast cancer cell adhesion while knockdown of LPXN produced the opposite effect of stimulating adhesion [170]. Thus, leupaxin association with integrin signaling may serve to antagonize the functions of paxillin.

As an adaptor protein, LPXN interacts with numerous proteins within the integrin signaling network depending on the phosphorylation modifications. LPXN is phosphorylated at multiple positions including residue 22, 62, 72, ~110, as well as other positions to facilitate its ability to be recruited to integrin signaling complexes and to bind to varying proteins [170]. LPXN is not only

tyrosine phosphorylated but shown previously by Dr. ShuGang Yao from our lab to be serine phosphorylated at the 54 position [172]. Protein interactions with LPXN is portrayed in **Figure 1.4**. FAK is a protein that is synonymous with integrin associated tyrosine kinase activity and binds to LPXN at its LD3 motif [165]. FAK association with PXN in turn phosphorylates PXN [163], whereas it is unknown whether FAK association with LPXN induces phosphorylation of LPXN or that binding to LPXN acts as a negative competitor to association with PXN. The homolog of FAK, Pyk2, has been well studied as an important binding partner of LPXN. Pyk2 serves as a kinase during integrin signaling and plays a role in the disassembly of trailing edges of cells during integrin mediated migration [171]. Whether LPXN recruitment of Pyk2 assists in its kinase function or negatively regulates it by bringing it away from PXN remains unknown. The protein kindlin-3 is a protein within in the kindlin family of proteins that is specifically expressed in leukocytes [99, 101]. Both LPXN and kindlin-3 are proteins that are present in high amounts in cells of hematopoietic origin and recently it was shown that these proteins interact within podosome signalling [87]. PXN phosphorylation promotes disassembly of integrin rich binding spots such as focal adhesions [68, 160]. In contrast, the kindlin-3 – leupaxin interaction has been shown to increase the stability of podosomes and prevent its early disassembly [87]. It is likely that PXN and LPXN are general integrin associated proteins that acts downstream of all integrin signaling. While in proximity within the integrin signaling complex, LPXN and its associated proteins may serve to dephosphorylate PXN so that the phospho-PXN cannot act to disassemble the integrin mediated adhesion complexes [87]. As LPXN interacts with the protein PTP-PEST, a phosphatase of the PEST family including PTP-PEST, PEP (encoded by the gene *ptpn22*), and PTP-HSCF [102, 178], this interaction could explain LPXN's ability to regulate PXN dephosphorylation through the recruitment of phosphatases. Western blots with HA antibodies of anti-FLAG immunoprecipitation from HEK293t cell co-transfected with FLAG-PEP and HA-LXPN show an interaction between HA-

LPXN and FLAG-PEP [199]. Therefore, both PTP-PEST and PEP from the PEST family of phosphatases interact with LPXN.

The role of LPXN within leukocytes remains relatively unknown as most studies of LPXN utilizes cancer cell lines or adherent cell lines. It was previously shown that LPXN acts as a negative regulator for BCR signaling [118]. Furthermore, the Long group has done extensive studies on integrin associated proteins and elucidated LPXN as an integrin downstream protein [56]. Knockdown of LPXN in human NK cell lines decreased MTOC reorientation and granule polarization. However, as most of these studies have been done in cell lines, it remains to be seen if these results are observable within primary cells.

1.5.3. Hypothesis and objectives of study

The overall hypothesis is that LPXN acts downstream of integrin signaling and acts as a negative regulator to integrin mediated motility but also serves to assist in leukocyte cytotoxic functions.

Specifically, the following questions will be addressed in my thesis:

1. Does the deletion of LPXN in primary NK cells affect target cell cytotoxicity and its associated cell mechanisms?
2. Does the deletion of LPXN alter the integrin mediated motility? Do chemotactic signals affect this change?
3. How does the deletion of LPXN alter the varying tissue resident leukocyte populations?

Chapter 2: Materials and method

2.1. Mice

The C57BL/6 mice were purchased from the Jackson Laboratory. All $LPXN^{fl/fl}$ and $LPXN^{KO/KO}$ mice generation was performed by Dr. Samuel Cheung and described in detail by Dr. ShuGang Yao [172]. The leupaxin floxed mice (C57BL/6N-A^{tm1Brd} $Lpxn^{tm1a(EUCOMM)Hmgu}$/WtsiPh) were purchased from the Sanger Institute in the UK. The leupaxin knockout (*LPXN* $^{KO/KO}$) mice were generated by breeding leupaxin floxed mice with CMV-Cre mice. $LPXN^{flox/WT}$ mice were bred with CMV-Cre mice and $LPXN^{WT/KO}$-CMV-Cre heterozygous offspring were screened for via PCR analysis. $LPXN^{WT/KO}$-CMV-Cre mice were bred with $LPXN^{WT/KO}$-CMV-Cre mice and $LPXN^{KO/KO}$ mice were screened for via PCR analysis. The C57BL/6 mice were housed in conventional housing facility (Health Sciences Laboratory Animal Service, University of Alberta). All animal studies were approved by the University Animal Policy and Welfare Committee at the University of Alberta and adhered to the guidelines put forward by the Canadian Council on Animal Care (protocol number AUP305).

2.2. Cells

2.2.1. Tumor cell lines

YAC-1 provided by Dr. K.P. Kane (University of Alberta) were maintained in RPMI 1640, with 5% heat-inactivated FCS, 20 mM Hepes, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin as described before [174]. The target cell L1210 lymphoma cell line is a gift from Dr. K.P. Kane (University of Alberta) and described before [173]. L1210 were maintained in RPMI 1640 supplemented with 8% dCS and penicillin/streptomycin and kept at 37°C with 5% CO_2.

2.2.2. NK cell generation

Ex vivo NK cells were isolated from spleen using the Stem Cell^{TM} mouse NK cell isolation kit via negative selection. In brief, spleens were isolated from 4-6 week old C57BL/6 mice, homogenized

and resuspended in D-PBS containing 2% FCS at a concentration of 100 million cells/mL. Rat serum and isolation cocktail were added to the cell solution, followed by incubation at room temperature for 10 minutes. The streptavidin rapidspheres were added to the cells and incubated at room temperature for 5 minutes. The cell solution D-PBS containing 2% FCS was topped to a final volume of 2.5 ml and placed into the magnet for 2.5 minutes. The NK cell containing solution was retrieved and cells were washed twice with RPMI containing 2% dCs.

IL-2 activated primary NK cells were obtained through spleen NK cell enrichment as previously described [175]. Mouse spleens were homogenized and $3x10^7$ splenocytes were placed into cell culture treated petri dishes. Petri dishes with splenocytes were incubated for 1h and subsequently non-adherent cells were collected to remove adherent monocytes from cell mixture prior to enrichment. $2x10^7$ cells from the cell mixture was then suspended in 10mL of complete media (10% FCS, 0.1 nM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and 53 nM 2-mercaptoethanol.) with 800U/mL recombinant human IL-2 (rIL-2) expressed in and isolated from Escherichia coli. The suspension was placed in a cell culture treated petri dish and incubated at 37°C with 5% CO_2 for 3 days. Post-incubation, non-adherent cells were removed, and remaining cells were resuspended with complete media and 800U/mL rIL-2. Cell were incubated for another 3 days and subsequently collected for use. Cell cultures analysed via flow cytometry were 90-95% $NK1.1^+$.

2.3. Antibodies

The goat anti-mouse granzyme B polyclonal IgG antibody was purchased from Santa Cruz Biotechnologies (Dallas, USA). The donkey anti-goat IgG secondary antibody conjugated with Alexa Fluor-488 and anti-mouse γ-tubulin monoclonal antibody conjugated with Alexa Fluor-647 was purchased from Abcam (San Fransisco, USA). The αCD3e –efluor450, αCD8 – FITC, αNKp46 –

PerCP eflour 710, αCD3e – PerCP efluor710, and αCD11a – APC antibodies were purchased from Thermo Fisher Scientific (Canada). The following antibodies: αCD107a – PE, αLy6G – BV711 HO, αCD4 – BUV737, αCD8 – BV605, αCD25 – FITC, αCD19 – BUV395, αNK1.1 – PE cy7, αCD11b – PE, αCD86 – FITC, αCD45.2 – PE, , αCD49a – BV605, αCD44 – PE cy5, and αF4/80 – BUV395 were purchased from BD Biosciences (Mississauga, ON). The following antibodies: CD1d(α-GalCer) tetramer – PE, αCD45.2 – APC eflour780, αCD45.2 – APC, CD19 – efluor450, αCD11c – PE cy7, αCD25 – APC, αCD69 – Biotin, streptavidin – BV711, αγδTCR – BV650, αCD62L – APC eflour780 were borrowed with the generosity from Dr. Troy Baldwin's lab (University of Alberta). The αCD49b – APCe780 antibody was borrowed with the generosity from Dr. Kevin Kane's lab (University of Alberta).

2.4. Reagents

Phorbol 12-myristate 13-acetate (PMA), Histopaque-1077, EDTA, and dimethyl sulfoxide (DMSO), and calcein-AM were purchased from Sigma-Aldrich (Mississauga, ON). The fetal bovine serum (FBS), defined calf serum (dCS), fetal calf serum (fCs), fetalclone I serum, and L-glutamine were purchased from GE Healthcare (Piscataway, USA). Ionomycin was purchased from Calbiochem (San Diego, USA). Fibronectin was purchased from EMD Millipore (Billerica, MA). The ICAM-1/Fc chimera was purchased from R&D Systems (Minneapolis, USA). The murine recombinant VCAM-1 and RANTES/CCL5 were purchased from Peprotech (New Jersey, USA). The fluorescence dye CellTracker green was purchased from Life Technologies (Burlington, ON). The fluorescent dye CellTrace Violet, eight well-chambered coverglass and coverslip were purchased from Fisher Scientific (Ottawa, ON). The 96 well V bottom microtiter plates, glass bottom microscopy dishes, cell culture petri dishes, and 0.5μm pore size transwell plated were purchased from Corning (New York, USA). The NK cell purification kit was purchased from StemCell (Vancouver, BC).

2.5. Calcein-AM cytotoxicity assay

Target cells were resuspended in complete media at a final concentration of $1x10^6$ cells/ml and incubated with 15 µM calcein-AM for 30 min at 37°C with occasional shaking. After two washes in complete medium cells were adjusted to $5x10^4$ cells/100µL. The test was performed in V bottom 96-well microtiter plates. 100uL of the calcein-AM target cell suspension was used in each replicate. IL-2 activate NK effector cells and calcein-AM labeled YAC-1 target cell or L1210 negative control were placed in each well at 20:1, 10:1, 5:1, and 1:1 effector:target ratios in triplicate. 6 wells were used for the 0:1 spontaneous release and maximum release (only target cells in medium plus 2% Triton X-100) assessment. After incubation at 37°C in 5% CO_2 for 4h, 75µL of each supernatant was harvested and transferred into new fluorescent reader plates. Samples were measured with a spectral plate reader (excitation filter: 485nm, band-pass filter: 530nm). Data were expressed as arbitrary fluorescent units (AFU). Percent lysis was calculated with the formula:

$$\%\ lysis = \frac{(\text{experimental well AFU}) - (\text{spontaneous release AFU})}{(\text{total release AFU}) - (\text{spontaneous release AFU})} \times 100\%$$

2.6. Degranulation assay

YAC-1 target cells were resuspended in 1mL RPMI $^+$ 2% fCs at a final concentration of $1x10^6$ cells/ml and incubated with CellTracker green fluorescent dye. IL-2 activated NK cells were incubated with CellTrace Violet (CTV) fluorescent dye. The concentration of both dyes was set to 1:1000 (5µM) and were incubated with cells for 30mins at 37°C with occasional shaking. NK cells were resuspended at $1x10^6$ cells/mL and YAC-1 cells were resuspended at $5x10^6$ cells/mL Four conditions were set up in 96well V bottom plates with negative (-ve) control, no target cell, 5:1 YAC-1:NK cell ratio, and NK cell with PMA $^+$ ionomycin (PI). The -ve control contained $1x10^5$ CTV labeled NK cells with no CD107a – PE antibodies. The no target cell condition contains $1x10^5$ CTV labeled NK cells. The 5:1 condition contains $1x10^5$ NK cells and $5x10^5$ YAC-1 target cells. The

PI condition contains $1x10^5$ NK cells with 2µM ionomycin and 100µg/mL PMA. All conditions were done in triplicates and incubated for 30mins at 37°C in 5% CO_2. Post incubation, brefeldin-A and CD107a – PE antibody was added to each condition at 1:1000 and 1:100 concentrations respectively and incubated again at 37°C in 5% CO_2 for 4.5 hours. Cells were collected, washed, and resuspended with ice cold DPBS + 0.5mM EDTA and incubated on ice for 30 minutes. Cells were collected and analyzed via flow cytometry. Degranulation was calculated based on CD107a – PE fluorescence.

2.7. Motility assay

The 8-well chambered coverglass was coated with 10ug/ml of ICAM-1, VCAM-1, or fibronectin for 48h at 4°C. The coated coverglass was washed 3 times with D-PBS before use. The IL-2 activated NK cells were collected, washed once with D-PBS. The dead cells were removed by density centrifugation. Histopaque-1077 was gently added to the bottom of the cell solution, followed by centrifugation at 800 rcf for 15 minutes at room temperature. When centrifugation was finished, the cell layer between histopaque and medium was gently collected and transferred to complete medium. The live cells were washed once with RPMI $^+$ 2% fCs and once with RPMI. 1×10^5 cells suspended in RPMI $^+$ 2%fCs at 5×10^5 cells/mL were transferred to each well and incubated for 30 minutes at 37°C incubator before imaging. The chambered coverglass was placed onto a brightfield microscopy stage and the temperature was maintained at 37°C. The cell migration was tracked for 30 minutes with the time interval of 10 seconds. Video analysis was done using ImageJ and cell motility analysis was done using the Chemotaxis plugin of ImageJ.

2.8. Transwell assay

Transwell insert membranes were coated with of ICAM-1, VCAM-1, or fibronectin for 48h at 4°C. The transwell inserts were washed 3 times with D-PBS before use. The dead cells were removed from the mixture through Histoplaque-1077 density gradient centrifugation as described above. 500µL of RPMI $^+$ 2% fCs or RPMI $^+$ 2% fCs $^+$ 100ng/mL RANTES (CCL5) were added to the 24

well cell culture plate, used as the bottom chamber of the transwell inserts. The live cells were washed once with RPMI $^{+}$ 2% fCs and 1×10^5 cells were transferred into the transwell insert top chamber topped up to a total volume of 200µL. The transwell inserts were added to the cell culture plates and were incubated for 2h at 37°C in 5% CO_2. The transwell inserts were removed from the cell culture plates post-incubation and the bottom chamber cells were collected resuspended in 300µL FACS buffer. All 300µL of cell containing solution was fed through the flow cytometer and the total number of cells was determined through forward and side scatter gating.

2.9. Fixed cell imaging of granule polarization

Fluorescent fixed cell imaging was used to determine the granule polarization of the NK cell granules. Live IL-2 activated NK cells were isolated through histoplaque-1077 density gradient centrifugation. YAC-1 target cells were labeled with CTV in the procedure described above for NK cells. 1×10^5 live NK cells were incubated with 2×10^5 YAC-1 target cells in FACS tubes for 10 minutes at 37°C in 5% CO_2. Cells were gently resuspended with a 200uL pipette tip cut open at the 10µL mark for wider pore size to prevent conjugates being pulled apart by sheer flow of the original small pore size. 1mL of ice-cold methanol was added to each FACS tube for fixation and permeabilization and incubated for 15mins at -20°C. FACS tubes were then topped up to 4mL with D-PBS to reduce methanol concentration and centrifuged. Methanol solution was decanted, and cell conjugates were then washed three times with D-PBS. Conjugates were resuspended in 100µL of DPBS $^{+}$2% dCs for blocking and incubated for 30 minutes at room temperature. Post-blocking, cell conjugates were resuspended in 100µL D-PBS $^{+}$ 2% dCs with 1:300 goat anti-mouse granzyme B antibody added. Conjugates were incubated for 30mins at room temperature and washed three times with D-PBS $^{+}$ 2% dCs. The conjugates were resuspended in 100µL D-PBS $^{+}$ 2% dCs added with 1:400 donkey anti-goat conjugated with Alexa Flour 488 and 1:400 of anti-γ tubulin antibody conjugated with Alexa Flour 647. Cell conjugates were incubated at room temperature for 30mins

then washed once with D-PBS $^{+}$ 2% fCs and three times with D-PBS. Cells were resuspended in 100µL of D-PBS and transferred to a glass bottom dish. Cells were allowed to settle to the bottom of the dish and imaging was performed using the Olympus IX-81 spinning disk confocal microscope. Images were merged and processed using ImageJ.

2.10. Mouse weight measurements

LPXN$^{fl/fl}$ mice which will be referred to as wildtype (WT), heterozygous (Het), and LPXN knockout (KO) offspring from five selected heterozygous parental groups were co-caged in the conventional housing facility (Health Sciences Laboratory Animal Service, University of Alberta). Three mice of WT, Het, and LPXN KO of both male and female were weighed on a weekly basis starting at 63 days old. Mice were weighed until 182 days old.

2.11. Mouse liver mononuclear cell preparation

Liver of mice 8-16 weeks old were retrieved, washed thoroughly with D-PBS, then manually digested through a 75µm mesh strainer using a syringe plunger into 10mL of RPMI $^{+}$ 2% fCs. Liver cells were then washed two times with RPMI $^{+}$ 2% fCs. Liver cells were resuspended in 40% Percoll and the solution was carefully overlaid on top of 70% Percoll as previously described [114]. The Percoll gradient was then centrifuged at 1260rcf for 30 minutes. After centrifugation, the middle mononuclear cell layer was collected from the Percoll gradient.

2.12. Mouse visceral adipose tissue leukocyte preparation

Leukocytes from visceral adipose tissue (VAT) mice 8-16 weeks old were isolated as previously described [176]. VAT was collected and thoroughly washed using D-PBS. The VAT was cut up into small pieces and placed into 20mL of DMEM $^{+}$ 2mg/mL collagenase IV and incubated for 3 hours at 37°C with shaking. After incubation, the solution was centrifuged with the DMEM supernatant aspirated. Cells were washed twice with DMEM and resuspended in 1mL ACK lysis buffer then

incubated for 4 minutes at room temperature. Cells were washed three times with RPMI $^{+}$ 2% dCs post incubation.

2.13. Flow cytometry

Cells were pre-treated with Live/Dead Aqua cell viability dye to distinguish between live and dead cells. For blocking, cells were incubated with RPMI $^{+}$ 2% dCs at 4°C for 30 minutes. For staining, cells were incubated with selected antibodies in RPMI $^{+}$ 2% dCs at 4°C for 30 minutes. Cells were washed three times with DPBS and resuspended in FACS flow buffer (1x PBS, 2-5 % (v/v) BSA, 2mM EDTA, and 2mM NaN_3) prior to analysis. Analysis was done using the BD LSRFortessa™.

2.14. Statistical analysis

Statistical analysis was performed with the software GraphPad Prism6. The statistical analysis used for specific experiment is shown in the legend below each figure.

Chapter 3: Leupaxin promotes granule polarization in NK cells while acting as a negative regulator during integrin mediated cell crawling

3.1. Introduction

NK cells are integral components of the innate immune system known for its role in killing malignant or pathogen infected cells. Unlike T cells which require prior activation by APCs before cytotoxicity against other cells can occur, NK cells can kill target cells right after development without the need for assistance from APCs. NK cell target recognition and activation are controlled by a pool of both activating and inhibitory germline encoded receptors on the cell surface that dictates whether a NK cell triggers its cytotoxic mechanism on a bound target cell. The most prevalent theory for NK cell activation is the missing self theory with interplay between activating and inhibitory receptors carefully balancing an equilibrium as explained in the introduction chapter [30, 46]. NK cells once activated by a target cell undergo the formation of the immunological synapse in the cleft between the NK cell and target cell which granules are released through.

Before degranulation can occur in NK cells, integrin signalling at the immunological synapse induce the polarization of lytic granules towards the immunological synapse [56, 70]. As the paxillin family of proteins function downstream of integrin receptors [86], I propose that leupaxin plays a role in the NK cell processes mediated via integrin signalling including granule polarization and motility. While the exact signal pathway of integrin signal transduction to cell polarization remains shrouded in mystery, the Long group has described an ILK-Pyk2-leupaxin-Cdc42 based network functioning in conjunction with paxillin to influence the reorientation of the MTOC [56]. Thus, it is suspected that leupaxin acts to fine tune the cellular responses of integrin “outside in” signalling.

Recently, leupaxin has also been shown to play a role in the downstream signaling of kindlin-3 in establishing stability of actin rich protrusions called podosomes [87]. Due to the tight knit association

of LPXN, podosomes, and integrin signaling, LPXN may play a role in promoting stability of integrin rich adhesion complexes and prevent early turnover. In concordance to this theory, overexpression of LPXN in breast cancer cell lines increased their adhesion during stimulation [170]. Deletion of LPXN should in theory increase actin structure disassembly and increase protein turnover thereby increasing cell motility velocity. However, most studies on the functionality of leupaxin has been done in adherent cells which have vastly different functions in comparison to leukocytes. Leukocytes express relatively high levels of leupaxin when compared to other cells of the body but its role in leukocytes remaining mostly unclear. The only concrete evidence of leupaxin activity in leukocytes shows that a decrease in leupaxin decreases MTOC reorientation and granule polarization in a human NK cell line [56]. However, no further exploration in NK cell integrin mediated function such as motility.

In this chapter, I aimed to investigate the role of leupaxin in NK cell integrin associated functions including granule polarization, degranulation, cytotoxicity, and motility. I used leupaxin KO mice generated via breeding leupaxin floxed mice with the CMV-Cre mice. Unlike previous studies with studying leupaxin function in human NK cell lines, I use primary murine NK cells for all experiments. Like previous studies that implicate leupaxin in integrin signalling, I found that leupaxin deletion leads to decreased granule polarization in primary murine NK cells. As integrin plays a major role in cell movement, I have also found that leupaxin deletion significantly increases crawling velocity on integrin ligands. My results confirm previous findings of leupaxin affecting granule polarization while introducing a novel role of leupaxin in NK cell non-stimulus driven integrin mediated motility.

3.2. Results

3.2.1. Leupaxin KO NK cells have decreased cytotoxic granule polarization post conjugation with YAC-1 target cell.

In NK cells the polarization of granules can be induced solely by the induction of LFA-1 engagement of ICAM-1 and subsequent downstream signalling [70]. The Long group has shown a novel role of leupaxin in the ILK-Pyk2-leupaxin-Cdc42 signalling pathway for inducing cell polarity during integrin receptor engagement [56]. As the previous study was performed in human NK cell lines that had perpetual granule polarization to the MTOC regardless of activating signal, I sought to define the contribution of leupaxin to granule polarization in primary murine NK cells. To address this, I conjugated IL-2 activated NK cells with YAC-1 cells to investigate the polarization of the granules in the killing process. The YAC-1 target cells were labeled with Cell Trace Violet and the total distance of the granzyme B containing granules to the MTOC, as visualized by staining for γ-tubulin, was measured. The leupaxin deficient IL-2 activated NK cells displayed a decreased degree of granule polarization 10 minutes into incubation with YAC-1 target cells shown in **Figure 3.1**. The LPXN knockout NK cells have a lower percentage of granules within a 2μm distance to the MTOC equating to fewer polarized granules. The granzyme B containing lytic granules also have an increased average distance from the granules to the MTOC (**Figure 3.1**). Thus, consistent with the role of leupaxin playing downstream of integrin signalling in NK cells [56], leupaxin also regulates the efficiency of granule polarization. In the LPXN knockout NK cells, over 60% the granules are still tightly polarized at the MTOC so the end result of granule polarization may not be affected by the deficiency. It is possible that LPXN KO may have decreased the rate of polarization of granules but other paxillin family adaptor proteins such as paxillin, which has been shown to regulate granule polarization, may compensate for the deletion of leupaxin [89].

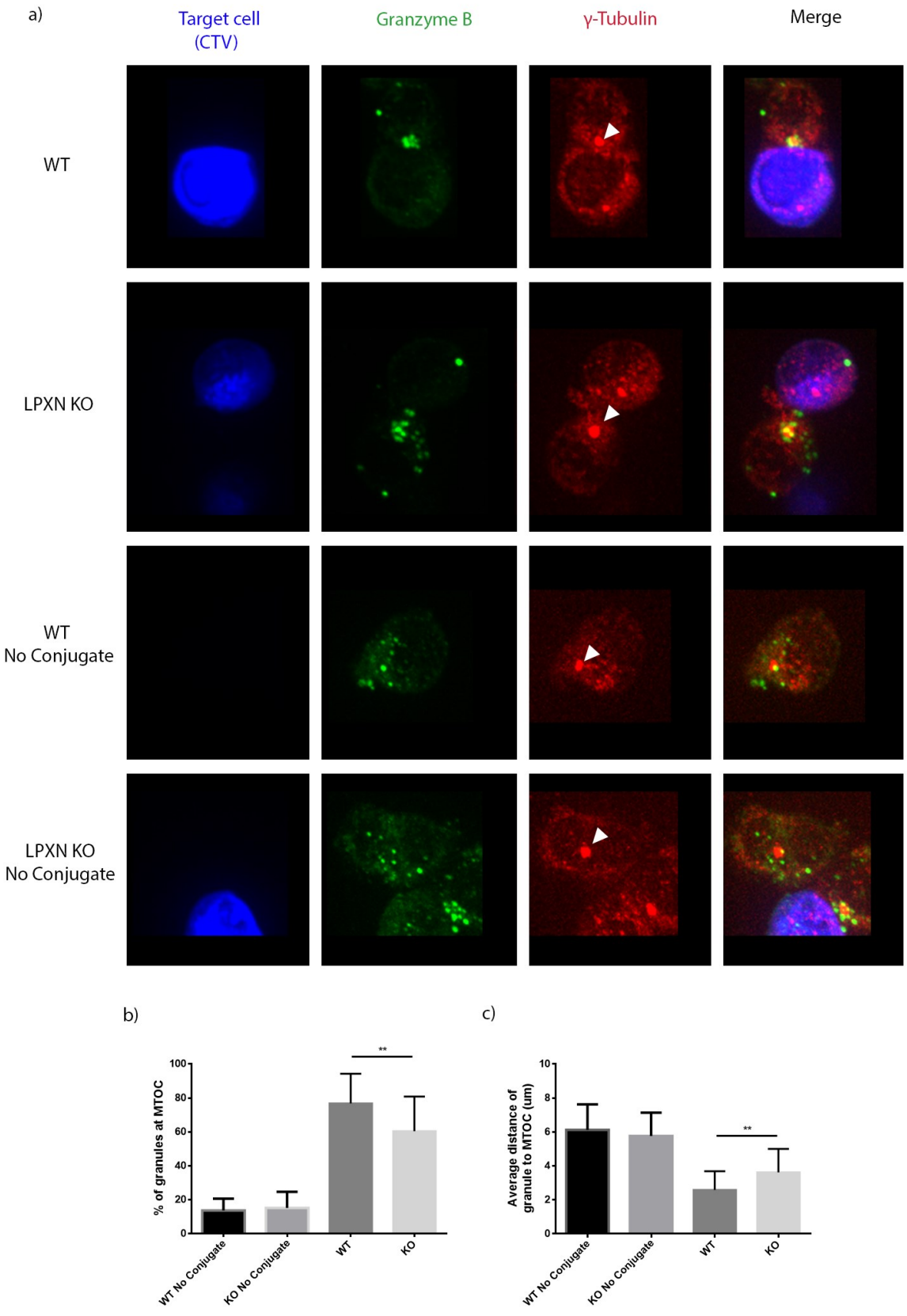

a)
Target cell (CTV)
Granzyme B
γ-Tubulin
Merge
WT
LPXN KO
WT No Conjugate
LPXN KO No Conjugate
b)
% of granules at MTOC
100
80
60
40
20
0
**
WT No Conjugate
KO No Conjugate
WT
KO
c)
Average distance of granule to MTOC (um)
10
8
6
4
2
0
**
WT No Conjugate
KO No Conjugate
WT
KO

Figure 3.1. Leupaxin KO NK cells have decreased cytotoxic granule polarization post conjugation with YAC-1 target cell. (A) IL-2 activated NK cell and Cell Trace Violet labeled YAC-1 target cell were mixed in a FACS tube at 1:1 effector target ratio, centrifuged at 100g for 2 minutes in a 4°C centrifuge, followed by a 10-minute incubation period at 37°C. 1mL of ice-cold ethanol was added to each sample immediately after the incubation period and incubated at -20°C for 15 minutes to allow for fixation and permeabilization. Samples were stained with anti-Granzyme B goat anti-mouse primary antibody, donkey anti-goat-AF488 secondary, and anti-γtubulin-AF647. The cells were imaged by spinning disk confocal microscopy and a projection of the merged optical sections is shown. White triangles within the γ-tubulin images indicate the MTOC. (B) Quantification of the percentage of granules that are at the MTOC. Granules within 2μm of the MTOC were considered polarized. (C) Quantification of the average distance of the granules to the MTOC. At least 30 cells were captured for each group from three independent conjugation experiments. All experiments were performed three times. The unpaired student's *t*-test was used for statistical analysis. The error bar represents standard error of the mean. ** represents $p < 0.01$.

3.2.2. Leupaxin does not affect degranulation nor cytotoxicity of IL-2 activated NK cells when killing YAC-1 target cells.

Previous studies done on leupaxin in human NK cell lines showed decreased granule polarization upon siRNA mediated knockdown but did not delve into the cytotoxicity and its associated cellular mechanisms [56]. To investigate the cytotoxic functions of leupaxin knockout NK cells I first isolated NK cells from mouse spleen through negative selection or enrichment of IL-2 activated NK cells as described in Materials and Methods. Natural killer cells, like cytotoxic T cells, require signalling by the β2-integrin LFA-1 (CD11a/CD18), for adhesion to target cells and proper cytotoxicity. Although not directly mediated by integrin associated functions, degranulation is still based on the efficacy of the effector cell to form conjugates with the target cell. To examine if leupaxin deletion affected the degranulation of NK cells, NK cells were incubated with YAC-1 target cells and degranulation was measured by the fluorescence emitted by bound CD107a – PE antibody. The CD107a based degranulation suggested no differences in degranulation between WT and LPXN KO IL-2 activated NK cells (**Figure 3.2**).

Next, I measured the cytotoxicity of IL-2 activated NK cells when targeting YAC-1 target cells. The NK effector cells, and YAC-1 target cells were incubated at various effector ratios (**Figure 3.3**) and cytotoxicity was measured through the calcein-AM cytotoxicity assay. Leupaxin deficient *ex vivo* splenic NK cells from negative selection displayed the same YAC-1 killing capabilities as the WT *ex vivo* splenic NK cells (**Figure 3.3a**). *Ex vivo* splenic NK cell isolation was only performed once as it was thought that IL-2 activation of primary NK cells may enhance their cytotoxicity and provide more significant results. However, IL-2 activated NK cells deficient in leupaxin also did not display any differences in ability to kill YAC-1 target cell when compared to the WT (**Figure 3.3b**). Therefore, deficiency of LPXN did not alter the cytotoxic capabilities of primary NK cells against YAC-1 target cells. As LPXN KO NK cells still display proper cytotoxicity, it makes sense that

degranulation would also remain unaltered as it implies that proper conjugate formation is in place. Thus, the degranulation process of the NK cells is not affected by leupaxin deletion.

In addition to cytotoxicity, bystander cell cytotoxicity was measured through the calcein-AM cytotoxicity assay as well. The previous study by the Long group showed reduced granule polarization in leupaxin knockdown NK cells [56] while another study by Hsu et. al. showed bystander cell killing by human NK cell lines when the LFA-1/ICAM-1 interaction is blocked [88]. As leupaxin acts within the β2 integrin signalling complex, LPXN KO produced a lesser but similar phenotype to full LFA-1 deletion with granules not fully polarized towards the IS. When considering that granule convergence at the MTOC is ablated, the NK cell degranulation process induces cytotoxicity to bystander non-target cells, it is hypothesized that deletion of leupaxin in NK cells may possibly lead to reduced granule polarization and allow for bystander cell cytotoxicity of non-target cells within close proximity to the NK cell when activated by a target cell. To test this, I analysed bystander cell killing using the calcein-AM cytotoxicity assay. By adding the effector cell, target cell and bystander cell in the same well, during NK cell cytotoxic activity the bystander cells may be caught in the theoretical non-directional release of granule contents. Any increase in bystander cell cytotoxicity would be shown with an increase in calcein-AM release when the L1210 bystander cell is labeled the YAC-1 target cell is unlabeled. However, the results in **Figure 3.3c** and **Figure 3.3d** show no differences in bystander cell cytotoxicity of both *ex vivo* or IL-2 activated NK cells. One explanation is that in LPXN KO NK cells, the granules may still be converging at the MTOC just at a slower rate but still fully polarize over a longer period. This granule convergence can be clearly seen in **Figure 3.1**. Thus, one would see a decrease in polarization when measuring polarization at a specific timepoint but no differences in overall polarization of the granules or cytotoxicity. Future experiments using live cell imaging should be performed to test this hypothesis.

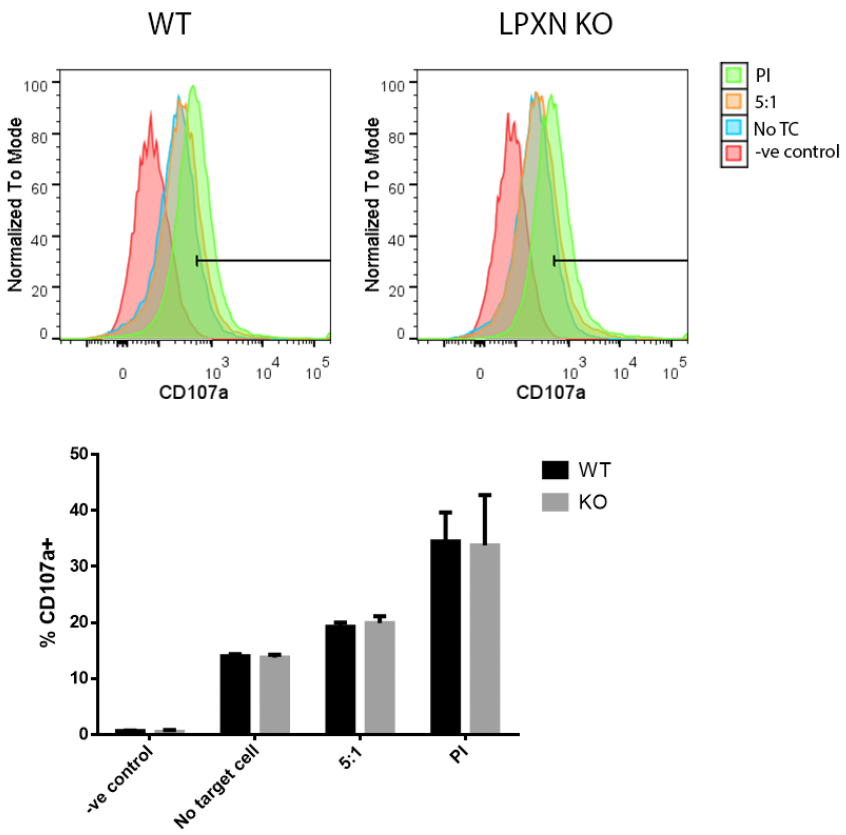


Figure 3.2 Leupaxin knockout does not affect degranulation capabilities of IL-2 activated NK cells. (Top) IL-2 activated NK cells were collected, labeled with Cell Trace Violet, and $1x10^5$ NK cells were placed into FACS tubes per sample. Sample were separated into 4 groups: negative control (-ve control), no YAC-1 target cell (no TC), 5:1 YAC-1 to NK cell ratio (5:1), and PMA 100ng/mL $^+$ ionomycin 1µg/mL (PI). $5x10^5$ Cell Tracker Green labeled YAC-1 target cells were added to indicated samples. Samples were gently mixed, centrifuged 100g for 2 minutes in a 4°C centrifuge, followed by a 30-minute incubation period. 1x BFA and 2µL of CD107a – PE was added to each sample post 30-minute incubation followed by a 4.5-hour incubation period. Cells were collected and analysed via flow cytometry post incubation. (Bottom) Graph indicating the degranulation of IL-2 activated NK cells with various stimuli. The unpaired student's *t*-test was used for statistical analysis. All results were non-significant. The error bar represents standard error of the mean.

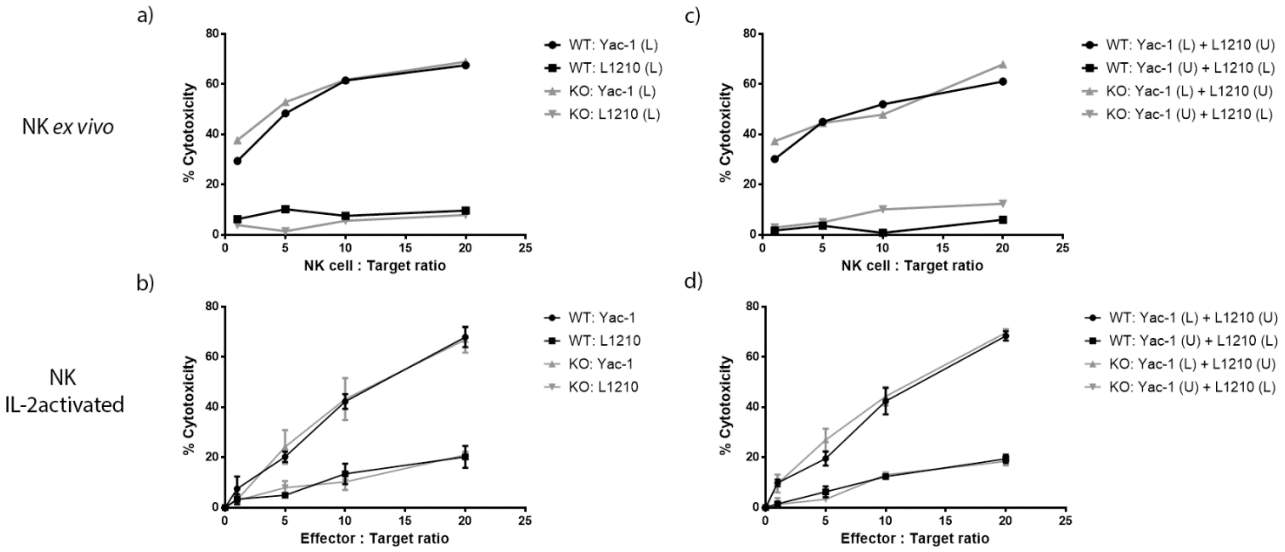


Figure 3.3 Leupaxin does not affect cytotoxicity of *ex vivo* and IL-2 activated NK cells when killing YAC-1 target cells. YAC-1 target cells were collected and labeled with Calcein-AM. NK cells were mixed with either YAC-1 target cells or L1210 negative control cells at various effector:target ratios in 96 well V-bottom plates. Cells were centrifuged at 100g for 2 minutes in a 4°C centrifuge, followed by incubation at 37°C for 5 hours. 100µL sample medium of each well is transferred to a black 96 well plate and read using plate reader for analysis of absorbance at 710nm. (A) *Ex vivo* NK cells where purified from splenocytes through negative selection. NK cells were mixed with either YAC-1 or L1210 labeled with Calcein-AM. Cytotoxicity assay performed with same procedure as in the methods section. (B) IL-2 activated NK cells were generated from mice spleen through incubation in complete media and 1000units/mL IL-2. Cytotoxicity assay performed as described in methods. (C) *Ex vivo* NK cells were mixed with both YAC-1 and L1210 with either YAC-1 or L1210 cells labeled with Calcein-AM. Bystander cytotoxicity was performed using the same cytotoxicity aassay procedure as mentioned in methods. (D) Bystander cytotoxicity assay performed with IL-2 activated NK cells with same procedure as described in C. Both experiments in A and C were only performed once with three repeats done for the IL-2 activated NK cells in B and

D. The unpaired student's *t*-test was used for statistical analysis. All results were non-significant. The error bar represents standard error of the mean.

3.2.3. Leupaxin knockout NK cells crawl faster than wildtype NK cells on fibronectin and V-CAM1 during non-directional crawling.

Integrin receptors play a critical part in the regulation of cell motility on extracellular matrix and endothelium lining [90, 91]. In vitro, placing lymphocytes onto proteins of the extracellular matrix will induce crawling movement [92]. Integrin receptors play a key role in regulating lymphocyte motility and leupaxin deletion may alter the outside-in signals cells receive from integrin signalling therefore altering the response to ligand binding. To explore the possibilities of leupaxin deletion altering cell motility, I used Fibronectin, ICAM-1, and VCAM-1 as substrates for various integrin receptors on the cell surface. Fibronectin is a common substrate for numerous integrin receptors on the cell surface and I use it as a general screen for integrin function deficiency [93]. ICAM-1 is best known for binding the LFA-1 integrin receptor and critical for chemokine mediated cell arrest on the endothelium [94]. VCAM-1 is a cell adhesion molecule that engages the VLA-4 receptor (α4β1) and complement receptor 4 (CD11c/CD18) which is a β2 integrin receptor [95]. When observing the motility of IL-2 activated NK cells during non-directional crawling on fibronectin, the knockout cells have a significant increase in velocity (**Figure 3.4a, b**). Similarly, the increase in velocity was observed with LPXN KO NK cells crawling on VCAM-1 (**Figure 3.4a, b**). This increase in velocity implies that leupaxin acts as a negative regulator to NK cell motility during non-chemotaxis directed crawling on integrin ligands. No difference was observed with NK cell crawling on ICAM-1 between WT and LPXN KO. The lack of difference on ICAM-1 is an anomaly as if LPXN acts downstream of all integrin receptors, LFA-1 mediated velocity should also be increased in LPXN KO. However, as NK cells crawling on ICAM-1 are already moving at double the speed of other substrates, the cell may be approaching its physical limit and any further increase in velocity is not possible for the NK cell. These results further confirmed that leupaxin acts downstream of integrin receptors acting as part of an inhibitory signalling pathway to integrin function. The directionality of the cell movement

patterns, which refers to the ability of the cell to move in a straight line, was measured to ensure the movement of leupaxin deficient NK cells are not abnormal when compared to the WT NK cells. No difference was observed between the WT and LPXN KO NK cells with regarding to motility on all three different substrates (**Figure 3.4c**). This confirms that the form and mechanism of NK cell crawling is normal and that no specific abnormal forms of movement can be detected in LPXN KO NK cells. Rather than just β2 integrins, which are highly expressed on leukocytes [96], the effects of leupaxin deficiency is most prominent when the β1 integrins are engaged in the case of fibronectin. Leupaxin may be playing a fine-tuning role in downstream signalling of all integrin receptors rather than just β2 integrins to negatively regulate integrin activation and cell motility in a non-stimulus driven environment.

The engagement of integrin receptors to their complementary molecules in the presence of an activation signal leads to tight adhesion and cell arrest on the endothelium [94]. In sites of inflammation, chemokine attractors lead activated cells to travel across the endothelium barrier using the LFA-1/ICAM-1 interaction as well as other activated high affinity integrin proteins [94]. In order to model directional cell movement through a permeable barrier such as the endothelium through high affinity integrin receptor engagement, I used an integrin ligand coated transwell assay. The transwell insert separates the wells of a 24 well plate into top and bottom chambers through a permeable membrane containing 5μm diameter pores. The top chamber of the assay was coated with fibronectin, ICAM-1, VCAM-1, and no coating with only cell culture treatment. NK cells placed in the top chamber, and RANTES chemokine attractant in the bottom chamber to induce NK cell migration into the bottom chamber. No differences were observed in the number of cells that had migrated to the bottom chamber in all 4 different conditions (**Figure 3.5**). The presence of chemokine providing an activation signal changes the conformation of the integrin receptors which may have led to bypassing any subtle effects leupaxin deletion may have been causing. Just as LPXN

KO NK cell cytotoxic function remains identical to WT during activation by YAC-1 target cells, leupaxin deletion does not affect the activation mediated migration through a permeable membrane.

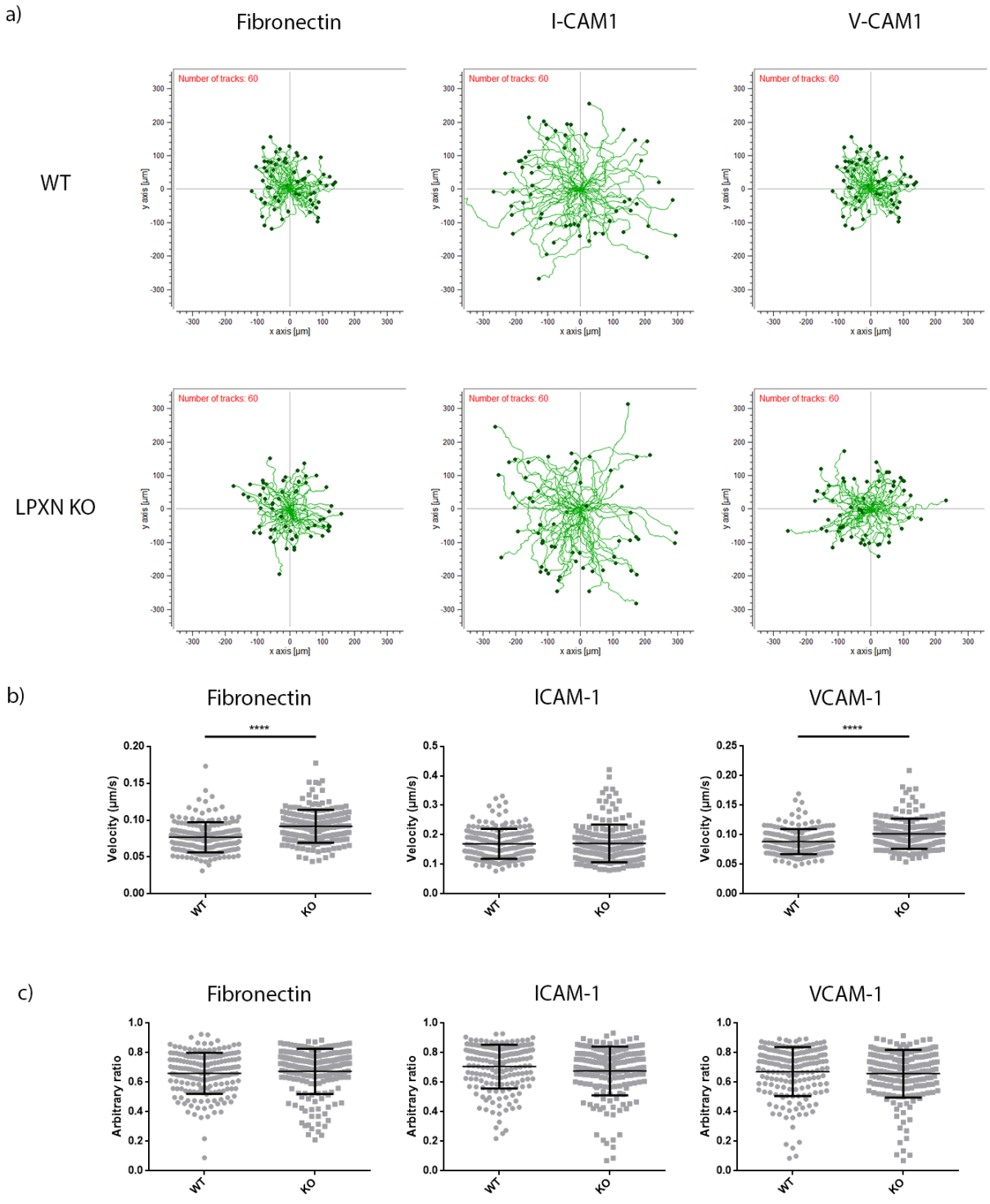


Figure 3.4 Leupaxin knockout NK cells crawl faster than wildtype NK cells on fibronectin and V-CAM1. (a) IL-2 activated NK cells were placed onto a coverglass coated with either fibronectin, I-CAM1, and V-CAM1. The migrating cells were tracked for 30 mins using a brightfield microscope

with time intervals of 10s between images. The migration patterns of the NK cells were analysed using ImageJ. At least 60 cells were analysed for each experiment and all experiments were performed three times. (b) Quantification of the velocity of the NK cells on varying substrates. (C) Quantification of the directionality of the NK cell crawling. Directionality is analysed through an arbitrary ratio of straightness of the cell's path with 1 being straight and 0 being non-straight. The unpaired student's *t*-test was used for statistical analysis. The error bar represents standard error of the mean. **** represents $p < 0.0001$.

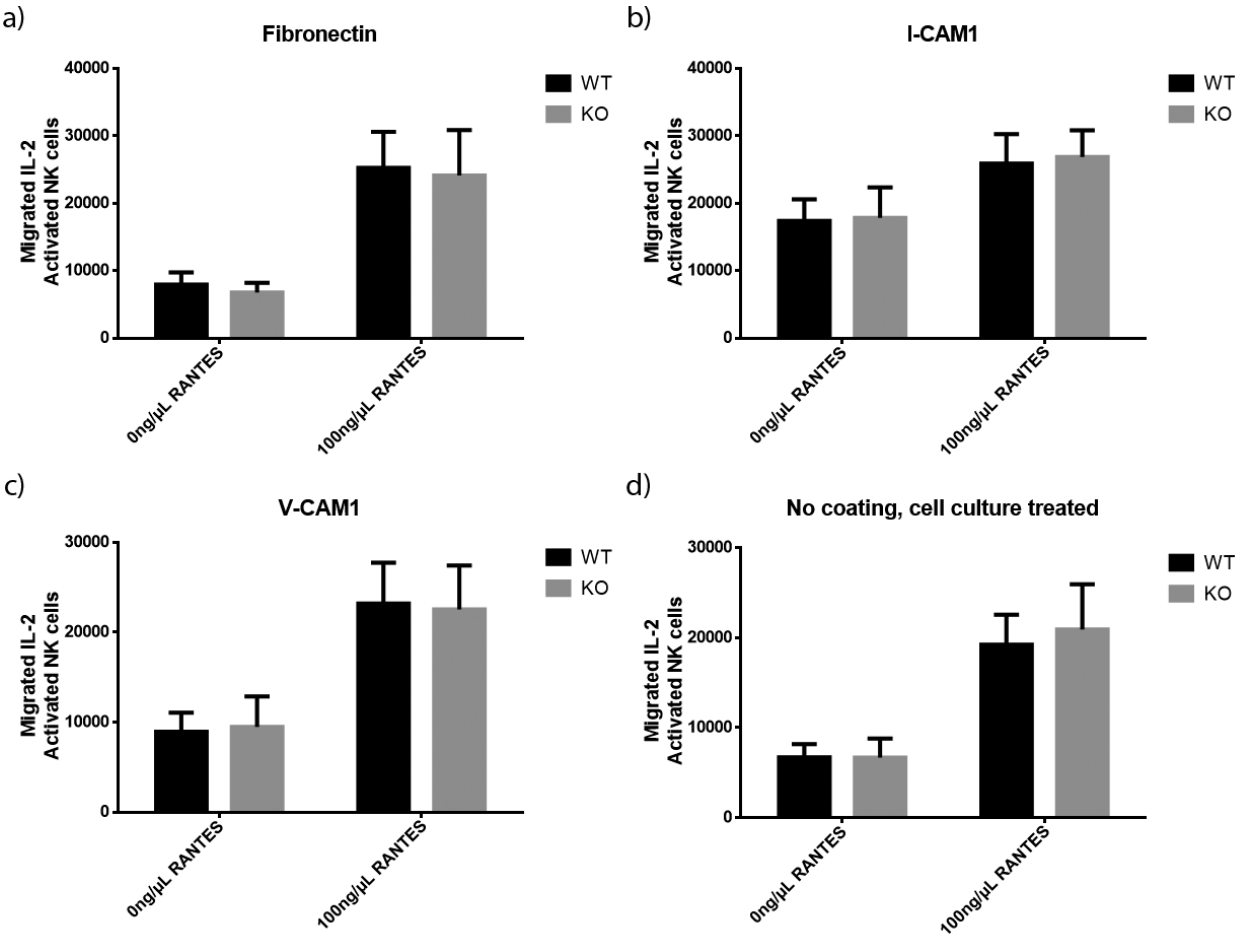


Figure 3.5. Leupaxin deficienct NK cells does not affect NK cell chemokine directed transwell migration. 5µM diameter pore transwell membranes were coated with fibronectin (A), I-CAM1 (B), V-CAM1 (C), or DPBS (no coating) (D). $1x10^5$ IL-2 activated NK cells were placed into the upper chamber of a transwell in a 200µL volume RPMI $^+$ 2% fCs. 500µL of RPMI $^+$ 2% fCs is added to the bottom chamber of each sample with 100ng/mL of RANTES being added as indicated. Samples were incubated at 37°C for 1-hour. Bottom compartment cells were collected post incubation and suspended in 300µL FACS buffer. All 300µL of cell containing solution was taken in analysed by the flow cytometer and the total number of cells was determined through forward and side scatter gating. All experiments were performed three times. The unpaired student's *t*-test was used for

statistical analysis. All results were non-significant. The error bar represents standard error of the mean.

3.3. Discussion

In this chapter I tested the various cytotoxic and integrin mediated functions of leupaxin deficient IL-2 activated NK cells. I have demonstrated that the leupaxin deficient NK cells display no differences in cytotoxic ability when compared to NK cells WT (**Figure 3.3**). In association with cytotoxicity, cytotoxic granule release by the IL-2 activated NK cells were not affected by the leupaxin deletion (**Figure 3.2**). When I observed the cytotoxic process with confocal imaging of the polarization process of the granules, the leupaxin deficient NK cells displayed a decrease in polarization and increase in average distance of the granules to the MTOC (**Figure 3.1**). Some studies have demonstrated that granule polarization is ablated when the LFA-1/ICAM-1 interaction is disrupted and no LFA-1 signalling occurs leading to bystander cell cytotoxicity [88]. I hypothesized that defective granule polarization in leupaxin deficient NK cells may cause bystander cell killing during NK cell cytotoxicity. In actuality, the confocal image in **Figure 3.1** clearly demonstrates that the leupaxin deficient NK cells still maintain proper granule polarization for most of the cytotoxic granules which explains the lack of bystander cell cytotoxicity in **Figure 3.3b**. Rather than a perpetual increase in granule distance to the MTOC as shown by the Long group in the human KHYG-1 NK cell line [56], another possibility is that the LPXN KO NK cell granules can polarize properly towards the MOTC albeit at a slower rate than the WT NK cells. The decrease in granule movement kinetics in the murine LPXN KO NK cells may have been the cause of the decrease in granule polarization when observing the granule polarization at a fixed timepoint. As others have discovered, paxillin is a key regulator in granule polarization post LFA-1 activation and disruption of paxillin phosphorylation results in decreased polarization of granules in primary human NK cells [89]. Leupaxin may play a supplementary role to paxillin in the integrin signalling complex in assisting the speed of which cytotoxic granule polarization occurs. Paxillin and leupaxin share similar cytosolic binding proteins such as Pyk2 indicating that leupaxin and paxillin may play some

overlapping roles in integrin signalling [97, 98]. Paxillin along with other proteins may act in unison with each other to have the most optimal granule polarization signal whereas the contribution of leupaxin is only a minor part of the whole. Leupaxin deficiency may cause the granule polarization signal to be less than optimal. However, due most aspects of the integrin signalling network including paxillin signalling remaining intact, cytotoxic granules in leupaxin deficient NK cells can still polarize properly to the MTOC over a longer period of time. As lytic granules are still released through the IS, bystander cytotoxicity does not occur. Further experiments with live cell imaging should be performed to visualize any possible differences in kinetics during granule polarization of NK cells.

I have demonstrated the role of leupaxin being a negative regulator of IL-2 activated NK cells during non-chemotaxis directed integrin mediated crawling on fibronectin and VCAM-1 (**Figure 3.4**). Crawling assay results suggests that in under non-chemokine driven conditions, leupaxin deficient NK cells have increased crawling velocities on fibronectin and VCAM-1 but not ICAM-1. This implies that leupaxin acts downstream of integrin signalling when it comes to actin cytoskeleton reorganization for motility. Recent publications have shone light on the interactions between kindlin-3 and leupaxin in regulating the podosome lifetime and stability in murine preosteoclasts [87]. Kindlin-3 is a well studied protein involved in the signalling cluster downstream of integrin receptors and acts as an adaptor protein regulating key aspects including integrin conformation, immune cell firm adhesion under sheer flow, and platelet aggregation [99, 100]. A disruption in kindlin-3 association with the beta subunit of the β2 integrin causes a decrease in the T cell adherence to ICAM-1 during low ligand concentration conditions [102]. Therefore, kindlin-3 is theorized to control stability of integrin clusters during cell movement and adhesion with leupaxin as part of its signalling network. Deletion of leupaxin in NK cells may remove a regulating factor on how kindlin-3 signals turnover rate of integrin mediated adhesion. Leupaxin may acts as a scaffolding protein

within the integrin – kindlin-3 network that maintains the stability of integrin clusters, preventing a high rate of turnover. A possible reduction in integrin/ligand binding stability due to a defective kindlin-3/leupaxin signalling network may explain the increase in crawling velocity on fibronectin and VCAM-1. Indeed, LPXN promotes dephosphorylation of PXN which in turn promotes focal adhesion stability [165]. Deletion of LPXN would provide an avenue for enhanced concentrations phosphorylated PXN to act on integrin clusters, promoting its disassembly, increasing turnover, and increasing velocity during cell crawling on integrin ligands.

As I do not see a difference in crawling in ICAM-1 I cannot make the same conclusion about the β2 integrin LFA-1. However, a reason for the lack of difference may be due to the speed of which NK cells travel on an ICAM-1 coated surface. The speed of NK cells crawling on ICAM-1 is almost double the speed on the two other substrates which may approach the physical limit on how fast the cell can crawl. If the cell is already moving at maximum velocity in the WT, then the removal of leupaxin will not be able to enhance the crawling velocity. Future experimental design to analyse the velocity of IL-2 activated NK cells on ICAM-1 may warrant titrating the ICAM-1 concentration such that the NK cells will not be moving speeds close to their physical limits. The exact method leupaxin employs to decrease cell motility remains unknown but a previous study has indicated that leupaxin associates with PTP-PEST in prostate cancer cells [102]. PTP-PEST, a protein tyrosine phosphatase, has been shown to be a potent negative regulator of lymphocyte activation when overexpressed and promotes motility and epithelial to mesenchymal like transition in colon cancer cells when knocked down [103, 104]. The PEST family of proteins along with potential other unknown phosphatases may regulate cell migration through the dephosphorylation of PXN thereby promoting leukocyte adhesion and spreading and slowing down crawling velocity [165].

As I observed IL-2 activated NK cell motility under non-stimulus conditions, I decided to use a transwell assay to observe cell migration when directed by a chemokine stimulus. The transwell

assay provided a permeable membrane that tries to mimic the movement of cells across the permeable endothelium. According to F**igure 3.5**, leupaxin does not play a role in the RANTES mediated directional migration of NK cells. As seen from previous data from our lab, during strong activating signals such as the N4 peptide on T cell activation, any effects leupaxin deficiency plays in cell activation is overpowered by the strong activation signals [172]. The N4 peptide is SIINFEKL amino acid sequence derived from chicken ovalbumin and is the cognate antigen to the TCR on OT-1 T cells. Data from our lab also suggests a decrease in MOTC reorientation when using variants of the N4 peptide namely T4 (SIITFEKL) peptide which has lower affinity and triggers activation of OT-1 T cells at a decrease in intensity compared to N4 [172]. Thus, it may be possible that the strong integrin activation signals provided by RANTES may bypass any subtle effects leupaxin may play in integrin mediated migration. Further looking into the movement of NK cells in an *in vivo* model would provide more explanation on how leupaxin affects cell migration across endothelial barriers.

Overall, this chapter demonstrates that LPXN acts downstream of integrin signaling but is not an absolute dictator of integrin function. Rather, LPXN seems to act as a fine-tuning mechanism for integrin mediated cellular functions such as granule polarization and motility. Likely, LPXN plays a role in assisting PXN in granule polarization or providing slight inhibition to phosphor-PXN mediated protein turnover during cell crawling. However, the data shows that these integrin mediated cellular functions still remains intact even in the absence of LPXN. Thus, LPXN is a fine-tuning protein that provides minor regulation to the integrin protein to prevent extremes in its signaling pathways.

Chapter 4: Leupaxin contributes to stabilizing weight gain and T cell activation profiles in the visceral adipose tissue and liver of mice.

4.1. Introduction

Inflammation is a mechanism of the body engineered to maintain tissue and organ homeostasis. The composition of immune cells within various tissues and the release of mediators determine the careful balance of inflammatory and anti-inflammatory responses. In the case of obesity, weight gain has been linked as a causative agent for the induction of a pro-inflammatory immune response [109]. Consequently, the inflammation may play contributory roles in insulin resistance, defective insulin secretion, and disruptions in other aspects of energy homeostasis. Unlike acute inflammation against pathogens, the obesity related inflammation involves the activation of aspects of innate and adaptive immunity that continues for prolonged periods of time, up to a lifetime. Moreover, acute inflammation is localized to specific areas of the body whereas the chronic obesity induced inflammation occurs in a systemic fashion [109].

Weight gain is a process closely involved with the hypertrophy of adipocytes in peripheral areas of the body including the visceral adipose tissue. The adipose tissue hosts a diverse population of leukocytes that promotes or suppresses adipocyte deposition depending on the cell and/or activation state [108]. Within the innate immune cells, macrophages above all other cells accumulated within the adipose tissue. The M1/M2 dichotomy of macrophage populations is a simplified way of describing the inflammatory state of the tissue. M1-like macrophages are classically activated and is associated obesity and inflammatory phenotype while M2-like macrophages promote homeostasis and repair and is often associated with lean or anti-inflammatory phenotype. Just as macrophage dichotomy indicates the present state of the adipose tissue, ILCs populations are closely linked to the propagation of either homeostatic, anti-inflammatory or the potentially detrimental pro-inflammatory body state [108]. ILC1s including specialized cytokine secretors and NK cells promote the

generation of a proinflammatory environment due to Th1 cytokine secretions such as IFNγ [111]. ILC2s, on the other hand, produce Th2 cytokines including IL-5 and IL-13 that promote an anti-inflammatory repair state of the tissue thereby suppressing pathogenic weight gain [108]. Innate immunity therefore plays a major role in the balance of obese versus non-obese body state.

The adaptive immune system also contributes to the inflammatory balance of the peripheral tissues especially when it comes to weight gain. Resident lymphocytes within an adipose tissue including $CD4^+$ and $CD8^+$ T cells are critical participants in obesity-induced inflammation [108]. While $CD8^+$ T cells are clearly involved with increased inflammation within adipose tissue [113], the role of $CD4^+$ T cells is much less clear cut. Depending on the balance of Th1 or Th2-like responses of the $CD4^+$ T cell population within the adipose tissue, these lymphocytes can reflect proinflammatory pathogenic outcomes (Th1) or homeostatic maintenance (Th2-like) [108]. In a similar manner to adipose deposition in adipose tissue, the liver also breaks into non-alcoholic fatty liver phenotype when inflammation runs rampant. The immunology of inflammation driven weight gain of the liver has resemblances to other areas of the body with a Th1 driven response. A unique feature of the liver is the presence of specialized liver resident NK cells that behave like ILC1s mainly focusing on secreting Th1 cytokines [114]. During liver fatty weight gain, NK cells are converted to a more ILC1-like phenotype to promote cytokine secretion [111]. Th1 response propagating cells including $CD8^+$ T cells, NK cells, and ILC1s are critical to the mounting pro-inflammatrory immune response required for non-alcoholic fatty liver disease [116]. The generation of Th1 response without pathogenic influences is the hallmark of the body undergoing shifts towards possible obesity phenotype.

Integrin proteins have been shown to play a role in obesity suppression although the exact method of their function in this manner remains undetermined. Previous publications show that the removal of β2 integrin proteins or their corresponding ligands have generated heavier mice under both high fat

diet and normal chow diet conditions [105]. Measurements of insulin levels in ICAM-1 KO mice indicates elevate insulin levels during high fate diet feeding suggesting insulin resistance may be occurring [191]. As leupaxin acts downstream of integrin signalling, it remains a possibility that weight gain of LPXN KO mice may be altered. The role of the paxillin family of proteins in influencing the weight gain of mice has remained unstudied due to paxillin knockouts being embryonically lethal. Indeed, in our LPXN KO mice I observed an increase in weight gain in the LPXN KO mice during the spleen isolation procedures in chapter 3. Also, CD4 and CD8 T cells of the liver and adipose tissue exhibit a more activated state. I also found that liver resident NK cell population is increased in LPXN KO mice. My results present a novel finding of the leupaxin protein promoting T cell activation within the visceral adipose tissue and liver in addition to promoting weight gain of LPXN KO mice.

4.2. Results

4.2.1. LPXN KO *mice have increased weights compared to their WT counterparts.*

Previous studies have shown that genetic removal of β2 integrin signalling, whether that be through the removal of the receptor or its primary ligand, leads to increased weight gain compared to WT male mice during both normal chow and high fat diets [105]. Previous data from our lab shows that leupaxin has been shown to play a role in the signalling complex of integrins and is phosphorylated during LFA-1 activation through plate bound αLFA-1 antibodies [172]. If leupaxin deletion leads to integrin signalling deficiency and defective integrin signalling is linked to increased weight gain, I decided to take a measurement of mice weight over time to observe any differences in weight gain of the leupaxin knockout mice. Weights were measured weekly for both male and female WT, heterozygous, and leupaxin knockout co-caged mice. In both the male and female mice, leupaxin deficient mice have increased weight gains in comparison to the WT (**Figure 4.1a**). The weight difference for female leupaxin deficient mice occurs at a much earlier age starting around 105 days while male leupaxin deficient mice show differences in weight starting around 140 days. When weighing liver (**Figure 4.1b**) and visceral adipose tissue pads (**Figure 4.1c**) I see an increase in the liver and VAT pad in the female mice but not male mice at 8-16 weeks. This sex difference in weight observed may be an indication of the differing immune responses between female and male mice. As female mice generally have higher disposition for inflammatory conditions than male mice, the removal of leupaxin may cause more of an increase in the steady state inflammatory conditions within various tissues of the body [106]. A important difference between the male and female mice with respect to weight and organ weight is that the LPXN KO male mice did not show any overall weight increases until ~20 weeks old, well beyond the measurement age for the VAT and liver of 8-16 weeks old. In contrast the female mice displayed an earlier increase in overall weight at ~7 weeks old occurring before the organ weight measurement age of 8-16 weeks old. Therefore, this explains

the increase seen in females but not males. The increased inflammation is likely linked with the increased adipose deposition and weight gain within female mice which becomes more pronounced at a much earlier age. An unknown aspect that was not screen for was the intestinal epithelial leukocytes which harbors an unique leukocyte population that controls various aspects of weight gain and the immunomodulation within the gut epithelial sites [192].

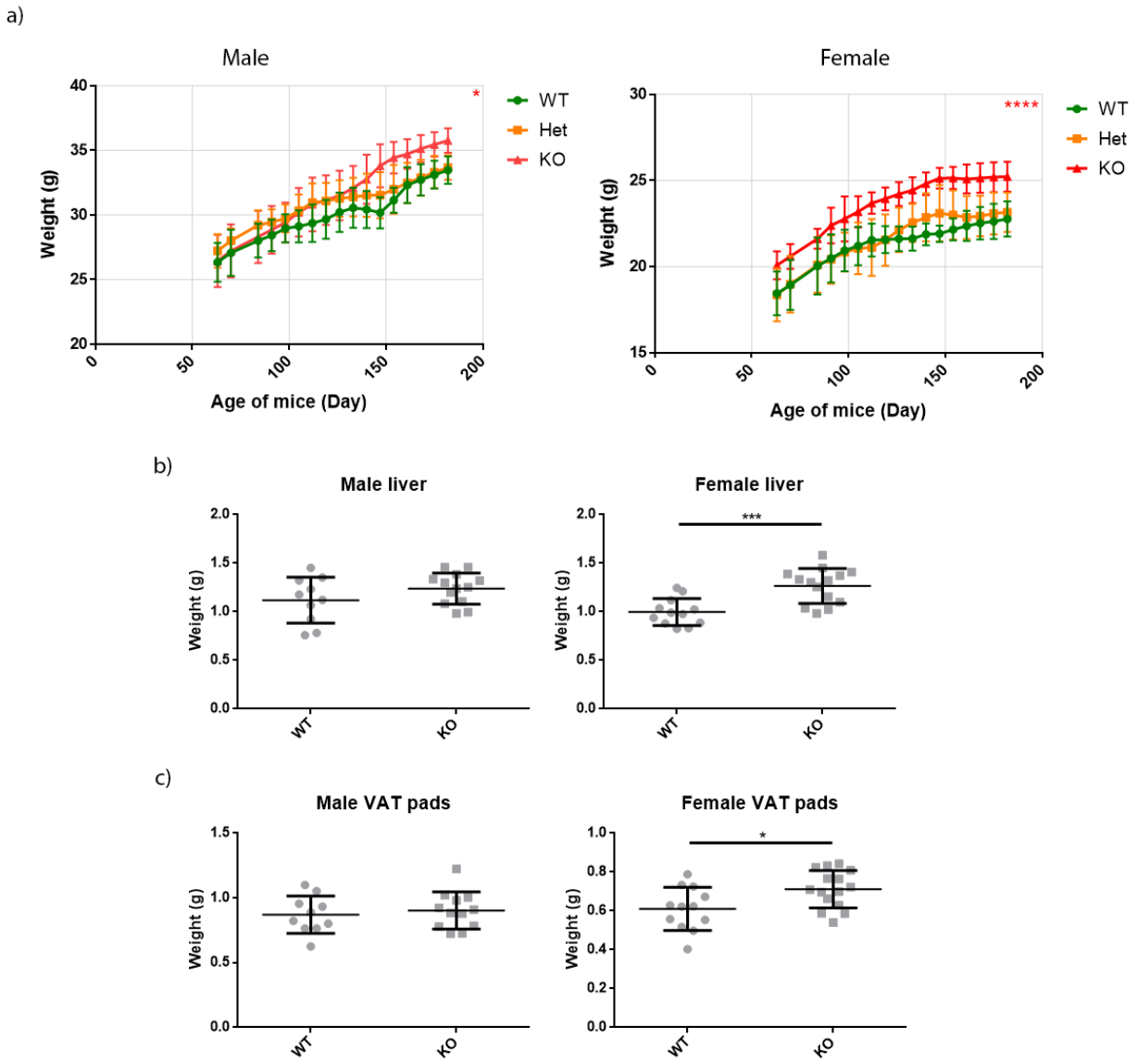


Figure 4.1. **LPXN KO mice have increased weights compared to their wildtype counterparts**. (a) Mice from heterozygous parents were co-caged and weighed over a 120-day time period with intervals of 7 days. Both male (left) and female (right) mice have 3 mice per group. Weighing started at 63 days and ended at 182 days old. All mice have birthdates that are 1 week apart. (b) Liver from mice euthanized at 8-16 weeks old were weighed. (c) Weights of visceral adipose tissue pads from mice euthanized at 8-16 weeks old were obtained. * represents $p < 0.05$, *** represents $p < 0.001$, and **** represents $p < 0.0001$. One-way ANOVA was used for statistical analysis of mice weight

gain and unpaired student's T test was used for statistical analysis of liver and VAT weight. The error bar represents standard error of the mean.

4.2.2. Leupaxin deletion does not affect frequency of specific leukocyte populations in spleen.

When observing the expression levels of leupaxin, cells of hematopoietic origin express elevated levels of the protein when compared to cells not of hematopoietic origin (**Figure 1.5**). While leukocytes do not explicitly form long term adhesion bonds with extracellular matrix, it does require tight transient adhesion to targets such as endothelium or other cells. During leukocyte development, cell contact and localization via integrin receptors also provides signals for proliferation and survival [107]. Leupaxin deletion may therefore affect leukocyte development owing to its role downstream of the integrin receptor signaling. As I wanted to observe if the deletion of leupaxin affects any of the leukocyte populations in the body, I first addressed specific cell populations in the spleen, which is a lymphoid organ functioning as the storage area for a large diverse population of leukocytes while performs roles in removing old red blood cells, providing a maturation site for B cells, and providing a site for potential T cell activation. The markers used and the cell types identified within the spleen are defined in **Table 4.1**. The lack of leupaxin within splenic white blood cells did not alter the CD4 T cell and CD8 T cell populations in female mice (**Figure 4.2a, b**) nor in male mice (**Figure 4.4a, b**). Next, I measured the CD44 by CD62L activation profile of T cells. CD44 is a glycoprotein found on activated cell surfaces and is expressed on all activated conventional T cells. CD62L or L-selectin is a marker expressed in naïve cells and downregulated during activation but is expressed again on central memory T cells whereas effector memory T cells lack the expression of CD62L. The activation profiles of the CD4 and CD8 T cells within the spleen did not display any alteration in both female (**Figure 4.2c-f**) and male (**Figure 4.4c-f**) LPXN KO mice when compared to WT mice. The percentage of $CD4^+$ T cells expressing CD25 in splenic leukocytes remains unchanged in female LPXN KO mice as well (**Figure 4.2g, h**). In the male mice we see a trend in decreasing $CD4^+$ $CD25^+$ T regulatory cells (**Figure 4.4g, h**) which might indicate possible decrease overall downregulation of leukocyte activation. Another possibility might be that the T regulatory cells are entering peripheral

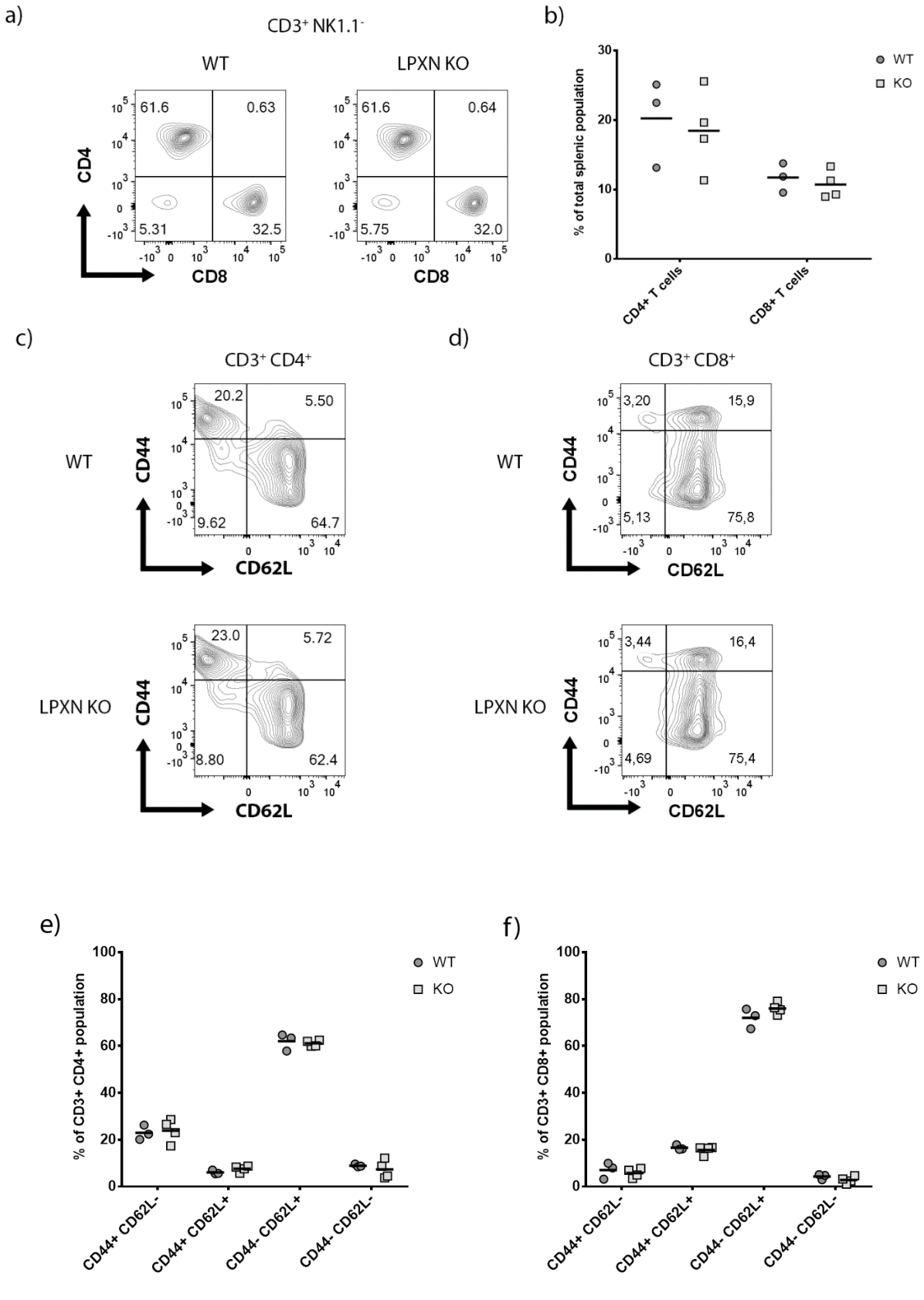
tissue at a higher frequency causing a decrease in T_{reg} cells within the spleen. Total splenocyte numbers do not differ between WT and LPXN KO mice (**Figure 4.2i** and **Figure 4.4i)**.

Leupaxin deletion did not affect the splenic γδ T cell and DN T cell populations within the mice as no difference was observed between the WT and LPXN KO (**Figure 4.3a, b** and **Figure 4.5a, b**). The $CD19^+$ B cell population did not have any differences in population between WT and LPXN KO (**Figure 4.3c, d** and **Figure 4.5c, d**). Following the pattern of other splenic leukocyte populations, the $F4/80^+$ splenic monocytes and macrophages did not display any differences in population between WT and LPXN KO (**Figure 4.3e, f** and **Figure 4.5e, f**). Neutrophils in the spleen of both WT and LPXN KO remained similar in population frequency (**Figure 4.3e, f** and **Figure 4.5e, f**). Lastly, NK cells did not have any significant differences in the splenic population frequency between the WT and LPXN KO (**Figure 4.3e, f** and **Figure 4.5e, f**).

When observing the leupaxin gene expression from **Figure 1.5**, it becomes apparent that cells that express high levels of leupaxin have a more activated phenotype and tissue resident localization while splenic populations have relatively low expression of leupaxin. This may suggest that splenic leukocyte populations, which are mostly un-activated or have not received any stimulus, may not be significantly affected by the deletion of leupaxin. Development of varying leukocyte populations is inferred to be unaffected by leupaxin deletion as well. Overall, leupaxin deficiency does not alter the murine splenocyte populations that I examined.

Cell type	Identifying flow cytometry antibody markers
$CD4^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$
$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^+$
Naïve $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^-$, $CD62L^+$
Central memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^+$
Effector memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^-$
T_{reg} cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$, $CD25^+$
$\gamma\delta$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^-$, CD1d tetramer $^-$, $\gamma\delta TCR^+$
Double Negative (DN) T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^-$, CD1d tetramer $^-$, $\gamma\delta TCR^-$
B cells	$CD3^-$, $NK1.1^-$, $CD19^+$
Neutrophils	$CD3^-$, $Ly6G^+$, $F4/80^-$
Monocytes and macrophages (spleen)	$CD3^-$, $Ly6G^-$, $F4/80^+$
NK cells	$CD3^-$, $NK1.1^+$

Table 4.1. The corresponding flow cytometry antibody markers used to identify the varying cell types within the mouse splenocyte population.


a)
CD3+ NK1.1-
WT
LPXN KO
61.6
0.63
5.31
32.5
61.6
0.64
5.75
32.0
CD4
CD8
b)
% of total splenic population
WT
KO
CD4+ T cells
CD8+ T cells
c)
CD3+ CD4+
WT
20.2
5.50
9.62
64.7
LPXN KO
23.0
5.72
8.80
62.4
CD44
CD62L
d)
CD3+ CD8+
WT
3,20
15,9
5,13
75,8
LPXN KO
3,44
16,4
4,69
75,4
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
f)
% of CD3+ CD8+ population

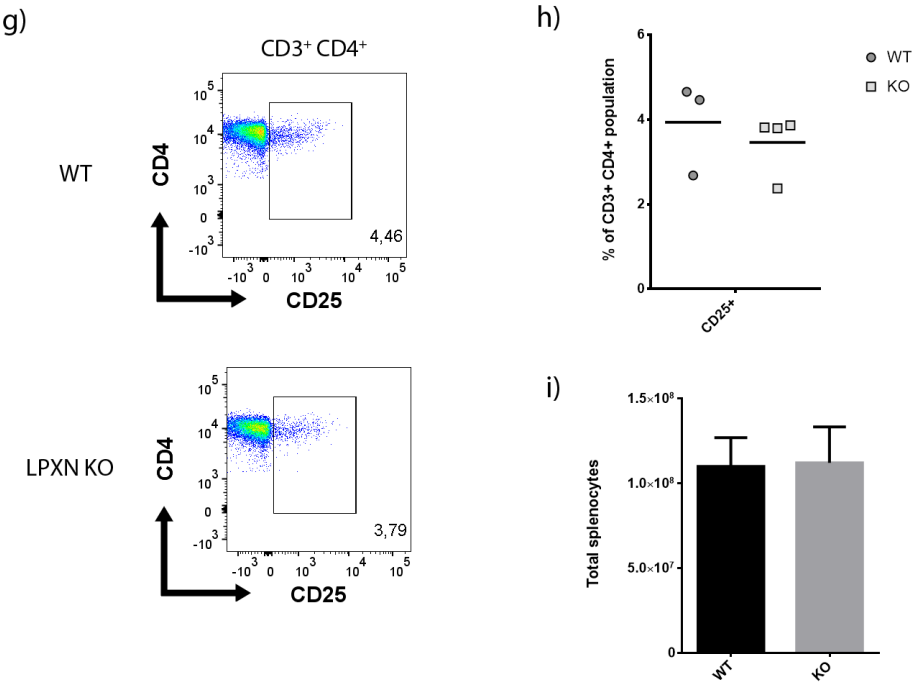


Figure 4.2. Leupaxin deficiency does not affect the T cell populations and activation states in the female mice spleen. Spleen from 8-16 week old female WT and LPXN KO mice were homogenized. All cells are gated for live and CD45.2^{+} CD3^{+}. (a) Cells were analysed by flow cytometry and flow plots of CD3^{+} CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of CD4^{+} and CD8^{+} T cells in the mice spleen. CD44 by CD62L flow plots of CD4^{+} (c) or CD8^{+} (d) T cells of WT or LPXN KO splenocytes. Quantification of the CD44 by CD62L activation states of the CD4^{+} (e) or CD8^{+} (f) T cells. (g) Flow plot of CD4^{+} CD25^{+} T cells. (h) Quantification of CD25^{+} T cell percentage of CD3^{+} CD4^{+} splenocyte population. (i) Quantification of the total population numbers of splenocytes. All results were not significantly different as determined by the unpaired student's *t*-test statistical analysis.

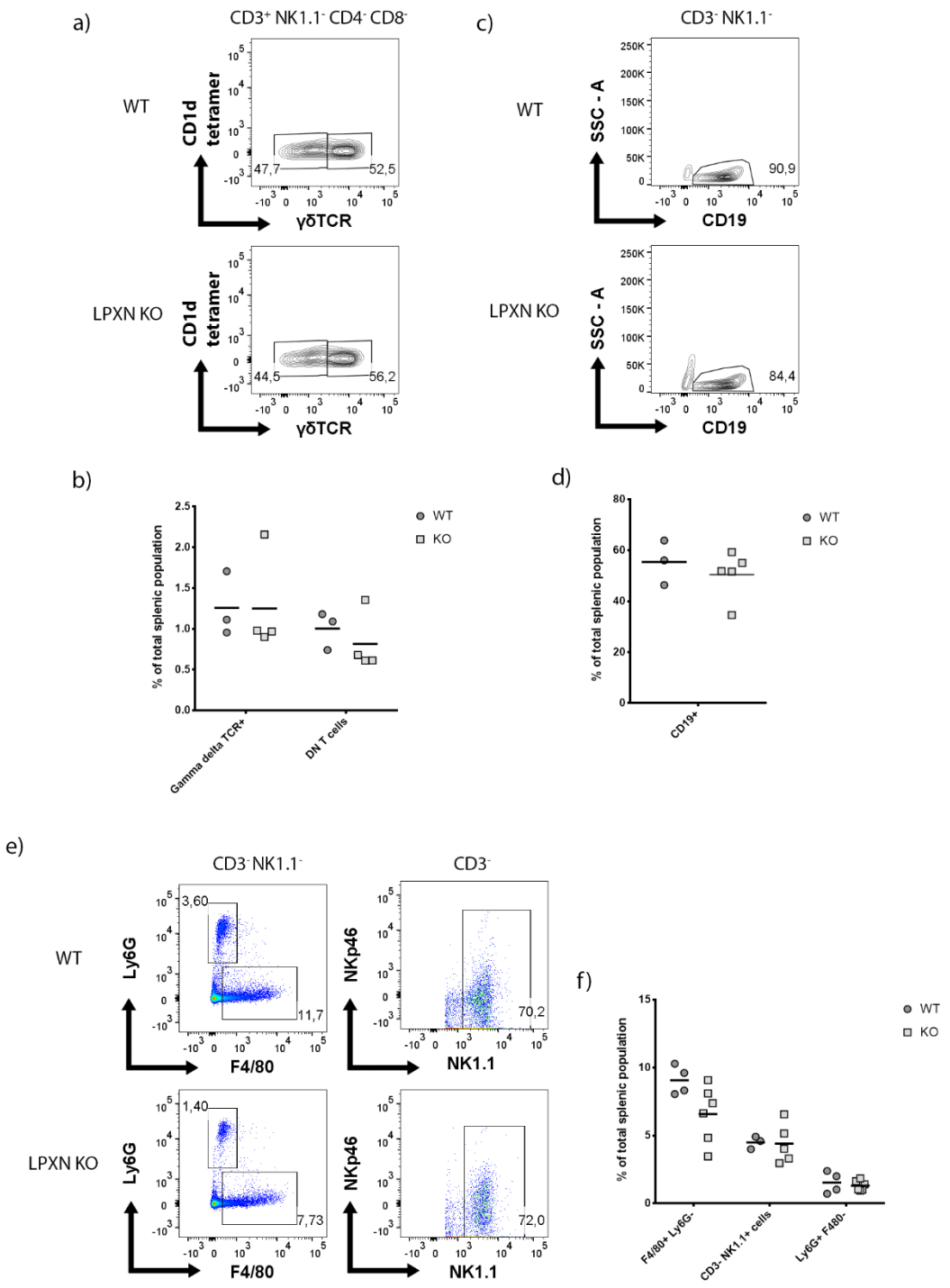
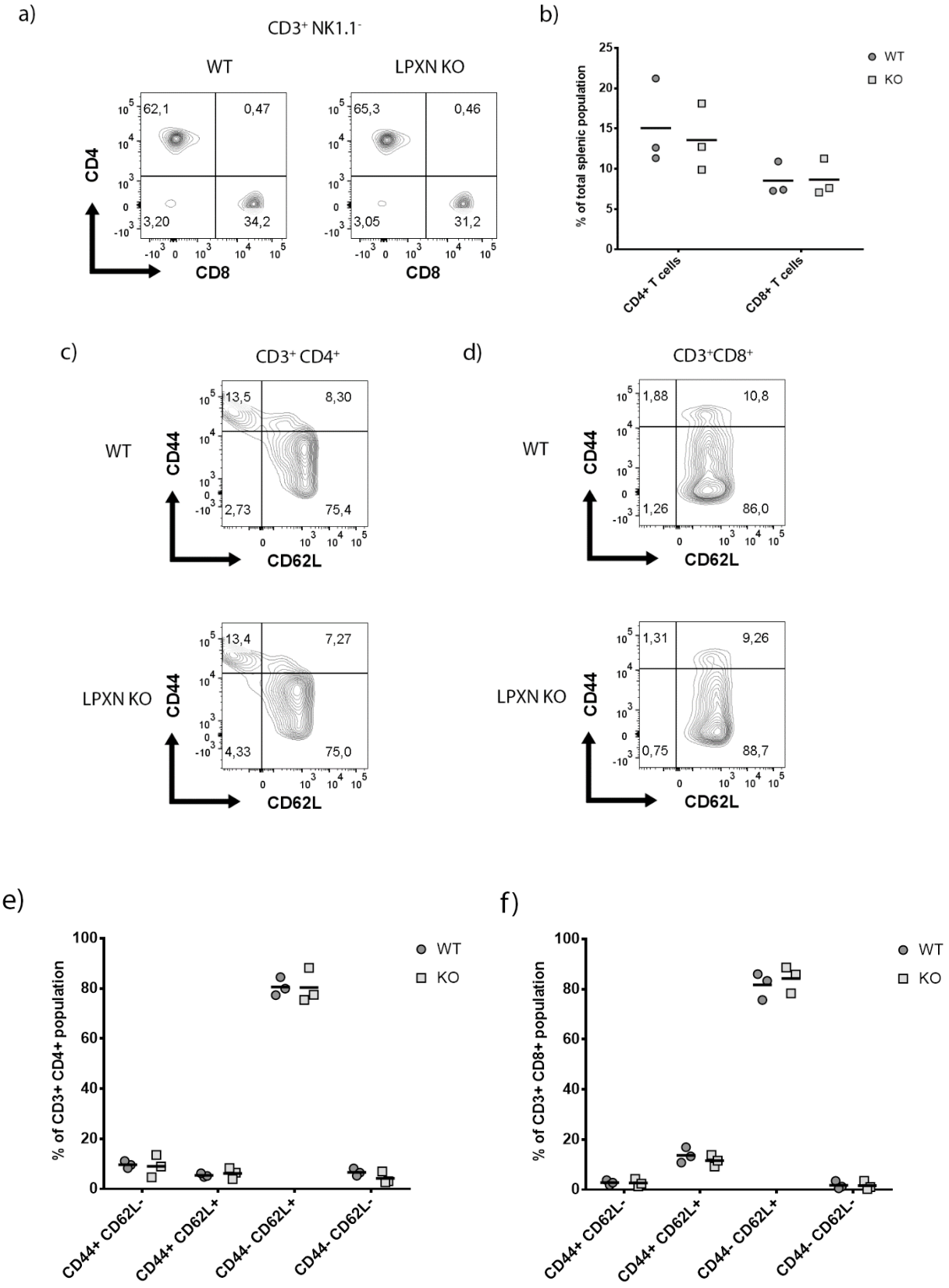


Figure 4.3. Leupaxin knockout does not affect the γδ T cell, double negative T cell, CD19^{+}, neutrophil, macrophage and NK cell populations in the spleen from female mice. Spleens from female WT and LPXN KO mice were homogenized. All cells are gated for live and CD45.2^{+}. (a)

Cells were analysed by flow cytometry and flow plots of cells stained for $CD3^+$, $NK1.1^-$ $CD4^-$, $CD8^-$, CD1d tetramer, and γδTCR are shown. (b) Quantification of the percentages of γδ T cells and double negative T cells in the mice spleen. (c) Flow plots of cells stained for $CD3^-$ $CD19^+$. (d) Quantification of $CD3^-$ $CD19^+$ cell percentages in splenocyte population. (e) Flow plots of cells stained for Neutrophils, monocytes, and macrophages (left). Flow plots of cells stained for NK cells (right). (f) Quantification of $Ly6G^+$ $F4/80^-$ neutrophils, $Ly6G^-$ $F4/80^+$ monocytes and macrophages, and $CD3^+$ $NK1.1^+$ NK cell percentages out of splenocyte population. All results were not significantly different as determined by the unpaired student's *t*-test statistical analysis.


a)
CD3+ NK1.1-
WT
LPXN KO
62,1
0,47
3,20
34,2
65,3
0,46
3,05
31,2
CD4
CD8
b)
% of total splenic population
WT
KO
CD4+ T cells
CD8+ T cells
c)
CD3+ CD4+
WT
13,5
8,30
2,73
75,4
LPXN KO
13,4
7,27
4,33
75,0
CD44
CD62L
d)
CD3+CD8+
WT
1,88
10,8
1,26
86,0
LPXN KO
1,31
9,26
0,75
88,7
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
f)
% of CD3+ CD8+ population

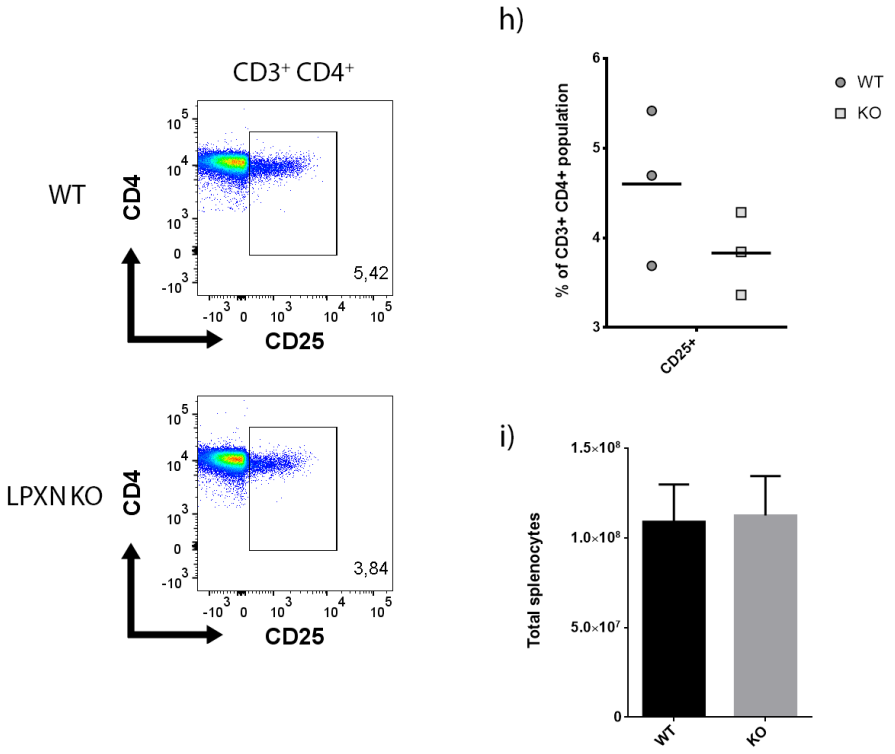


Figure 4.4. Leupaxin knockout does not affect the T cell populations and activation states in spleens from male mice. Spleen from male WT and LPXN KO mice were homogenized. All cells are gated for live and CD45.2$^+$ CD3$^+$. (a) Cells were analysed by flow cytometry and flow plots of CD3$^+$ CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of CD4$^+$ and CD8$^+$ T cells in the mice spleen. CD44 by CD62L flow plots of CD4$^+$ (c) or CD8$^+$ (d) T cells of WT or LPXN KO splenocytes. Quantification of the CD44 by CD62L activation states of the CD4$^+$ (e) or CD8$^+$ (f) T cells. (g) Flow plot of CD4$^+$ CD25$^+$ T cells. (h) Quantification of CD25$^+$ T cell percentage of CD3$^+$ CD4$^+$ splenocyte population. (i) Quantification of the total population numbers of splenocytes. All results were not significantly different as determined by the unpaired student's *t*-test statistical analysis.

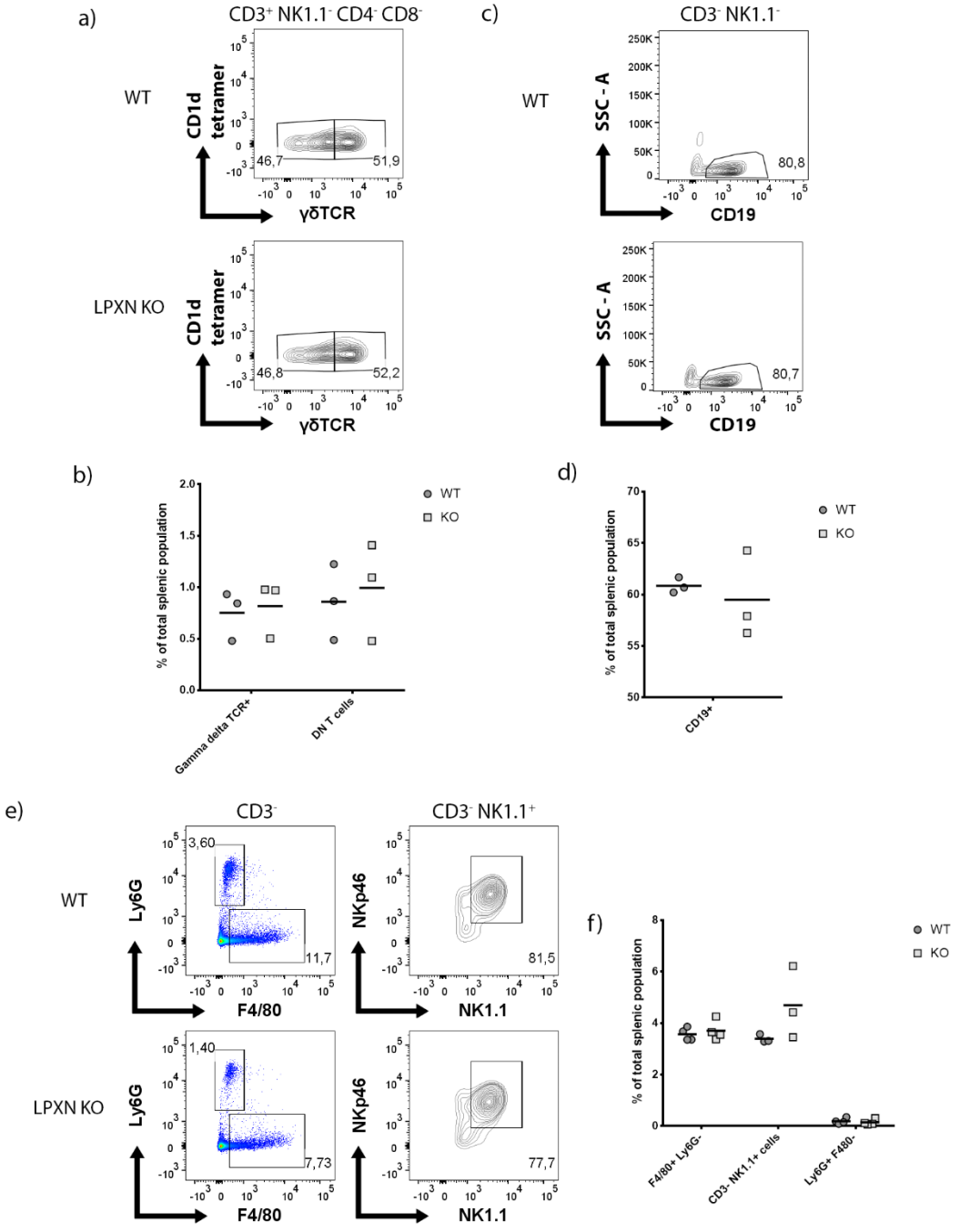


Figure 4.5. Leupaxin knockout does not affect the γδ T cell, double negative T cell, CD19^{+}, neutrophil, macrophage, and NK cell populations in the male mice spleen. Spleen from male WT and LPXN KO mice were homogenized. All cells are gated for live and CD45.2^{+}. (a) Cells were analysed by flow cytometry and flow plots of cells stained for CD3^{+}, NK1.1^{-} CD4^{-}, CD8^{-}, CD1d

tetramer, and γδTCR are shown. (b) Quantification of the percentages of γδ T cells and double negative T cells in the mice spleen. (c) Flow plots of cells stained for $CD3^-$ $CD19^+$. (d) Quantification of $CD3^-$ $CD19^+$ cell percentages in splenocyte population. (e) Flow plots of cells stained for Neutrophils, monocytes, and macrophages (left). Flow plots of cells stained for NK cells (right). (f) Quantification of $Ly6G^+$ $F4/80^-$ neutrophils, $Ly6G^-$ $F4/80^+$ monocytes and macrophages, and $CD3^+$ $NK1.1^+$ NK cell percentages out of splenocyte population. All results were not significantly different as determined by the unpaired student's *t*-test statistical analysis.

4.2.3. Leupaxin deletion increases the steady state T cell activation in the visceral adipose tissue.

One of the hallmarks of obesity is the induction of a chronic low-grade inflammation across various organs of the body [108]. Inflammatory conditions are introduced as a consequence of increased weight gain and contributes to the numerous obesity related diseases including insulin resistance. The adipose tissue harbours a diverse population of immune cells, making it an immune organ that links metabolism and immunity [109]. In the case of LPXN KO mice, I first saw an increase in weight gain of the mice and investigated if increased T cell activation is occurring in the adipose tissue as is common with obesity. As all groups of mice are fed normal chow diet, any weight increases are solely due to the varying conditions created by the altered cell populations within the peripheral tissues of the body. Based on the result of LPXN KO mice displaying higher body weight, I decided to investigate the visceral adipose tissue leukocyte population to examine if differences occur between the WT and LPXN KO. All examined cell types and their corresponding flow cytometry antibody markers are shown in **Table 4.2**.

Leupaxin deficiency does not significantly alter the percentage of $CD4^+$ and $CD8^+$ T cells within the $CD3^+$ NK1.1- population in the VAT of female (**Figure 4.6a**) and male (**Figure 4.8a**) mice. However when I examined the CD44 by CD62L activation profiles of the LPXN KO VAT $CD4^+$ T cells in female mice I saw a significant increase in the $CD44^+$ $CD62L^+$ population which corresponds to the central memory T cells (**Figure 4.6b, e**). The male LPXN KO mouse VAT $CD4^+$ T cell activation profile is the same as the WT (**Figure 4.8b, e**). The CD8 activation profile of both female (**Figure 4.6c, f**) and male (**Figure 4.8c, f**) LPXN KO mice show an increase in $CD44^+$ $CD62L^-$ $CD8^+$ T cells indicating an increase in the percentage of effector memory T cell in the total $CD8^+$ population. The $CD8^+$ T cell CD44 by CD62L activation profile of female mice show significant decreases in $CD44^-$ $CD62L^+$ T cell percentages while the male activation profile trends towards having lower $CD44^+$ $CD62L^+$ and $CD44^-$ $CD62L^+$ population frequencies although the results are not significant. This

suggests that naïve/non-activated T cell percentage of total $CD8^+$ T cell population is decreasing in the VAT of LPXN KO mice. The percentage of $CD4^+$ T cells that are $CD25^+$ corresponding to T_{reg} cells did not show any significant differences between WT and LPXN KO mice (**Figure 4.6g, h** and **Figure 4.8g, h**).

Looking at the other leukocyte populations, the iNKT population defined by CD1d tetramer binding and are suppressive of tissue inflammation [187], are not significant alter by the LPXN deficiency of both sexes (**Figure 4.7a, b** and **Figure 4.9a, b**). γδ T cell which is a regulatory population that helps control adipose homeostasis and thermogenesis [183], remain unchanged within the LPXN KO in comparison to the WT in both sexes (**Figure 4.7a, b** and **Figure 4.9a, b**). The LPXN KO neutrophil $Ly6G^+$ population, which are a small percentage of the total leukocyte population but contributes to macrophage influx during obesity associates inflammation [184], remains the same as the WT in both sexes (**Figure 4.7a, b** and **Figure 4.9a, b**). Similar to previous results, the $CD19^+$ B cell population which promotes or suppresses inflammation depending on local environmental factors [185], remains the same between the WT and LPXN KO mice (**Figure 4.7c, d** and **Figure 4.9c, d**). NK cells within the mice VAT generally plays a similar role to $CD8^+$ T cells by promoting tissue inflammation [154]. The LPXN KO mice $NK1.1^+$ $NKp46^+$ NK cell population frequency trends to be higher in the female mice (**Figure 4.7e, f**) while having no differences in the male mice (**Figure 4.9e, f**). The significant differences start to occur when observing the macrophage population which influx into the adipose tissue and increase in population numbers during tissue inflammation [152]. We see a trend towards albeit not significant increase in population frequency of $CD11b^+$ $F4/80^+$ macrophages in female LPXN KO mice (**Figure 4.7e, f**) and a significant increase in the macrophage $CD11b^+$ $F4/80^+$ population frequency of male leupaxin knockout mice (**Figure 4.9e, f**). The macrophage population can be subdivided into M1 which are pro-inflammatory, and M2 which are regulatory and anti-inflammatory [152]. The LPXN KO mice M1 macrophage population defined by $CD11c^+$ $CD86^+$

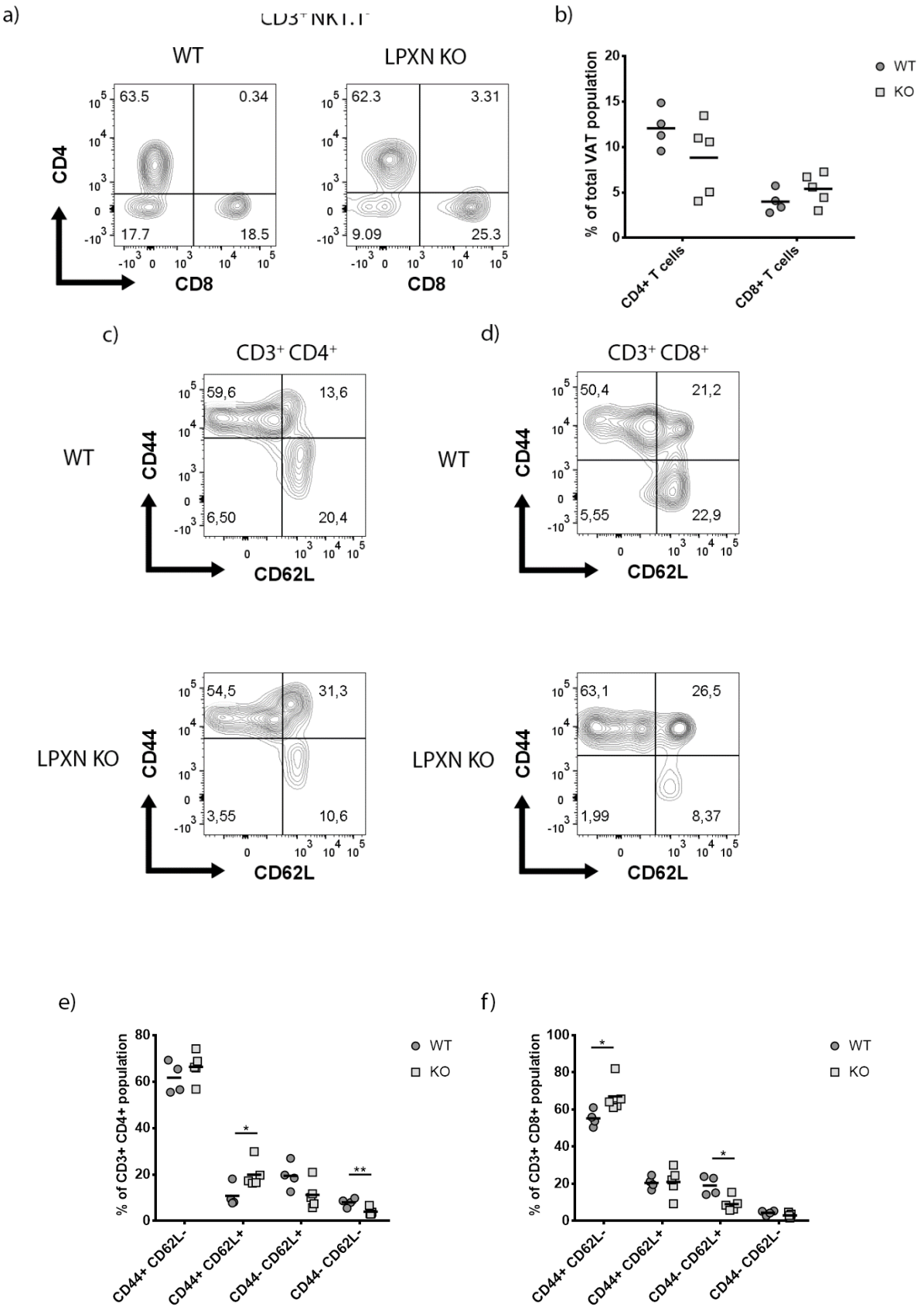
does no display any significant differences in comparison to the WT in both sexes (**Figure 4.7e, g** and **Figure 4.9e, g**). Knowing that LPXN acts downstream of integrin signaling, I sought to observe the β2 integrin alpha subunits expression on the total leukocyte population. β2 integrin alpha subunits CD11a, CD11b, and CD11c expression on adipose leukocyte cell surface was not changed by the removal of leupaxin (**Figure 4.7h** and **Figure 4.9h**).

The deletion of leupaxin introduces an increase in T cell activation in the visceral adipose tissue during steady state normal chow diet feeding. $CD8^+$ T cells in both male and female mice display a marked increase in population frequency which is generally associated with Th1 inflammation and insulin resistance [158]. The male T cell activation seems to be less intense than the female mice as no increases in $CD4^+$ T cell activation was observed in the male whereas a significant increase is seen in the female $CD44^+$ $CD62L^+$ $CD4^+$ T cells. Several innate leukocyte populations including macrophages (especially M1 macrophages) and NK cells are contributors to the weight gain driven tissue inflammation through the secretion of pro-inflammatory cytokines including TNFα and IFNγ [186]. The female mice also trend towards an increase in NK cell population while the male mice did not have any significant differences. This VAT NK cell difference in female mice corresponds to the earlier weight gain seen in the female mice indicating a more pronounced Th1 associated immune response is occurring in the female mice. However, it remains unknown if the innate cells initiate the T cell activation or if T cell activation produces cytokines that drives changes to the innate immune cell populations. As obesity has been closely linked to generating a low-grade inflammation across the body, inflammation may play a key role in the establishment of obesity and weight gain. The inflammatory environment generated by the increased activation of leukocytes may be a factor in contributing to the increased deposition of adipose tissue in leupaxin deficient mice. As I observed some of the major differences between the WT and KO to be T cell populations, verifying whether T cells are responsible in this shift in leukocyte activation may provide a basis on how these changes

are initiated. To examine the role of T cells, depletion of either CD4 or CD8 T cells and observing if the weight gain and macrophage frequency increase is reverted might provide evidence of the immune response being perpetrated by T cells. It remains to be explored if the shift in activation profiles are caused by resident T cells or an influx newly of activated T cells. I would like to further investigate whether tissue resident cells are being activated to produce the phenotype or more activated cells are being recruited from circulation by using a *in vivo* adoptive transfer of T cells tracking experiment.

Cell type	Identifying flow cytometry antibody markers
$CD4^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$
$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^+$
Naïve $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^-$, $CD62L^+$
Central memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^+$
Effector memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^-$
T_{reg} cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$, $CD25^+$
iNKT cells	$CD3^+$, $NK1.1^+$, CD1d tetramer $^+$
$\gamma\delta$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^-$, CD1d tetramer $^-$, $\gamma\delta TCR^+$
Double Negative (DN) T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^-$, CD1d tetramer $^-$, $\gamma\delta TCR^-$
B cells	$CD3^-$, $NK1.1^-$, $CD19^+$
Neutrophils	$CD3^-$, $Ly6G^+$, $F4/80^-$
Macrophages (Liver and Adipose)	$CD3^-$, $Ly6G^-$, $CD11b^+$, $F4/80^+$
M1 Macrophages	$CD3^-$, $Ly6G^-$, $CD11b^+$, $F4/80^+$, $CD11c^+$, $CD86^+$
NK cells	$CD3^-$, $NK1.1^+$, $NKp46^+$

Table 4.2. The corresponding flow cytometry antibody markers used to identify the varying cell types within the mouse VAT leukocyte population.


a)
CD3+ NK1.1-
WT
LPXN KO
63.5
0.34
17.7
18.5
62.3
3.31
9.09
25.3
CD4
CD8
b)
% of total VAT population
WT
KO
CD4+ T cells
CD8+ T cells
c)
CD3+ CD4+
WT
59,6
13,6
6,50
20,4
LPXN KO
54,5
31,3
3,55
10,6
CD44
CD62L
d)
CD3+ CD8+
WT
50,4
21,2
5,55
22,9
LPXN KO
63,1
26,5
1,99
8,37
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
f)
% of CD3+ CD8+ population

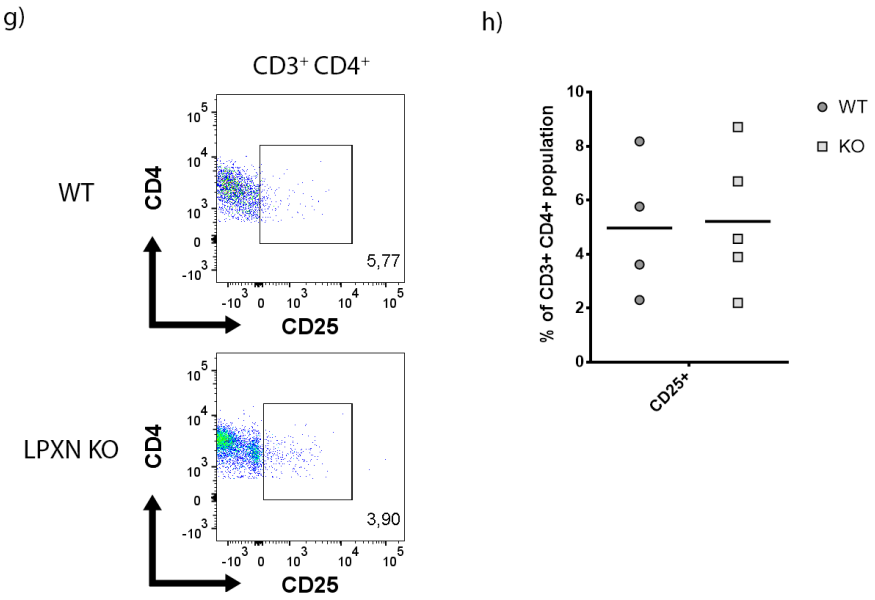
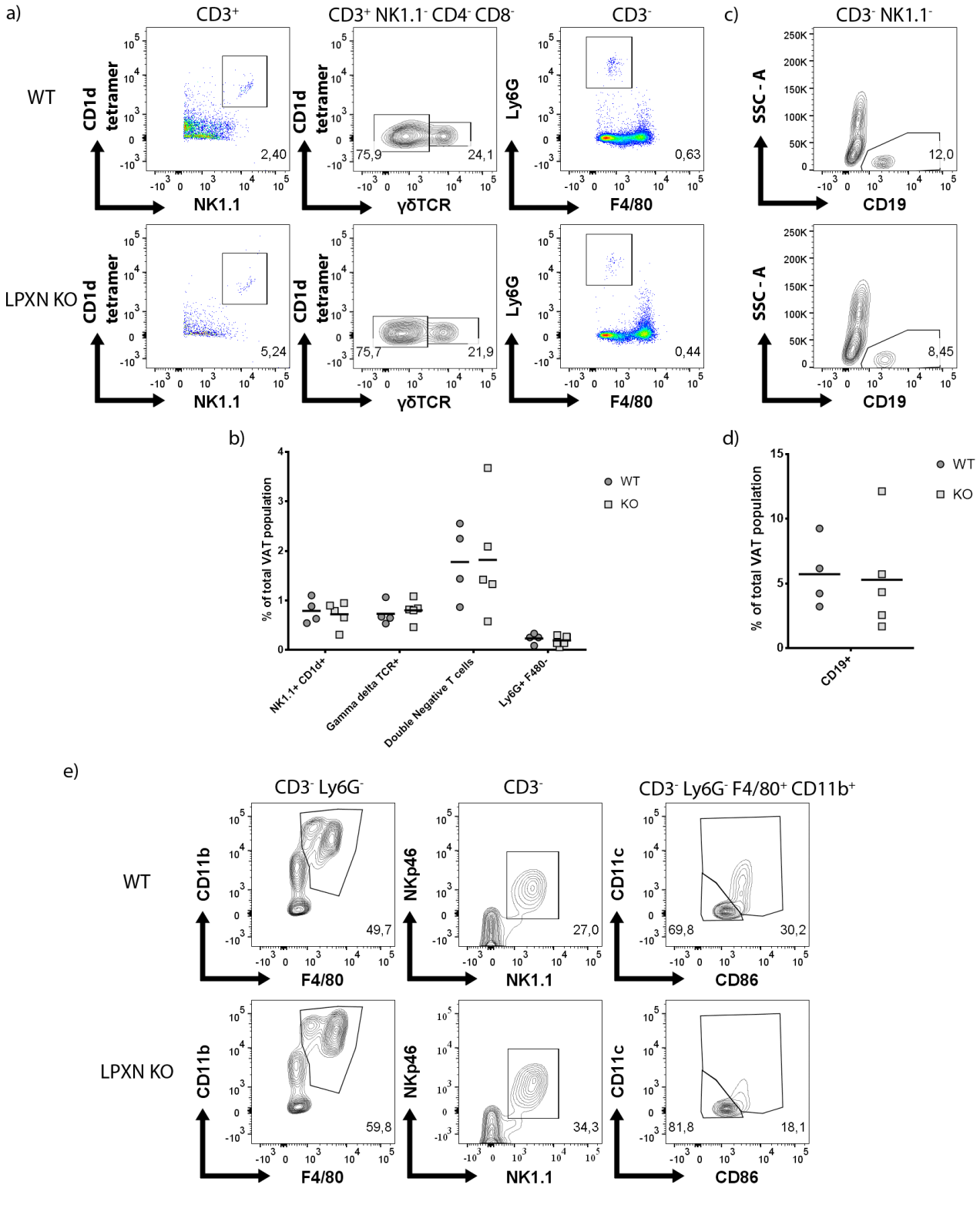


Figure 4.6. Leupaxin knockout increases CD4$^+$ central memory T cell and CD8 effector memory T cell presence in female VAT. Visceral adipose tissue from female WT and LPXN KO mice were digested via collagenase IV in DMEM for 2-hours and subsequently separated via centrifugation at 4°C for 10-minutes. All cells were gates on live CD45.2$^+$. (a) Cells were analysed by flow cytometry and flow plots of CD3$^+$ CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of CD4$^+$ and CD8$^+$ T cells in the mice VAT. CD44 by CD62L flow plots of CD4$^+$ (c) or CD8$^+$ (d) T cells of WT or LPXN KO VAT leukocytes. Quantification of the CD44 by CD62L activation states of the CD4$^+$ (e) or CD8$^+$ (f) T cells. (g) Flow plot of CD4$^+$ CD25$^+$ T cells. (h) Quantification of CD25$^+$ T cell percentage of CD3$^+$ CD4$^+$ VAT leukocyte population. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.


a)
CD3+
CD3+ NK1.1- CD4- CD8-
CD3-
c)
CD3- NK1.1-
WT
LPXN KO
CD1d tetramer
NK1.1
γδTCR
Ly6G
F4/80
SSC - A
CD19
2,40
75,9
24,1
0,63
12,0
5,24
75,7
21,9
0,44
8,45
b)
% of total VAT population
WT
KO
NK1.1+ CD1d+
Gamma delta TCR+
Double Negative T cells
Ly6G+ F480-
d)
CD19+
e)
CD3- Ly6G-
CD3-
CD3- Ly6G- F4/80+ CD11b+
CD11b
NKp46
CD11c
CD86
49,7
27,0
69,8
30,2
59,8
34,3
81,8
18,1

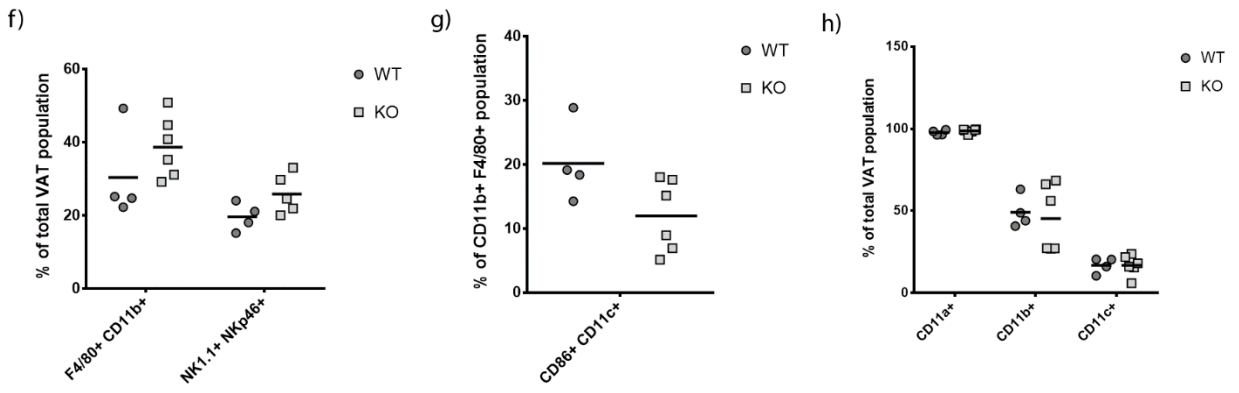
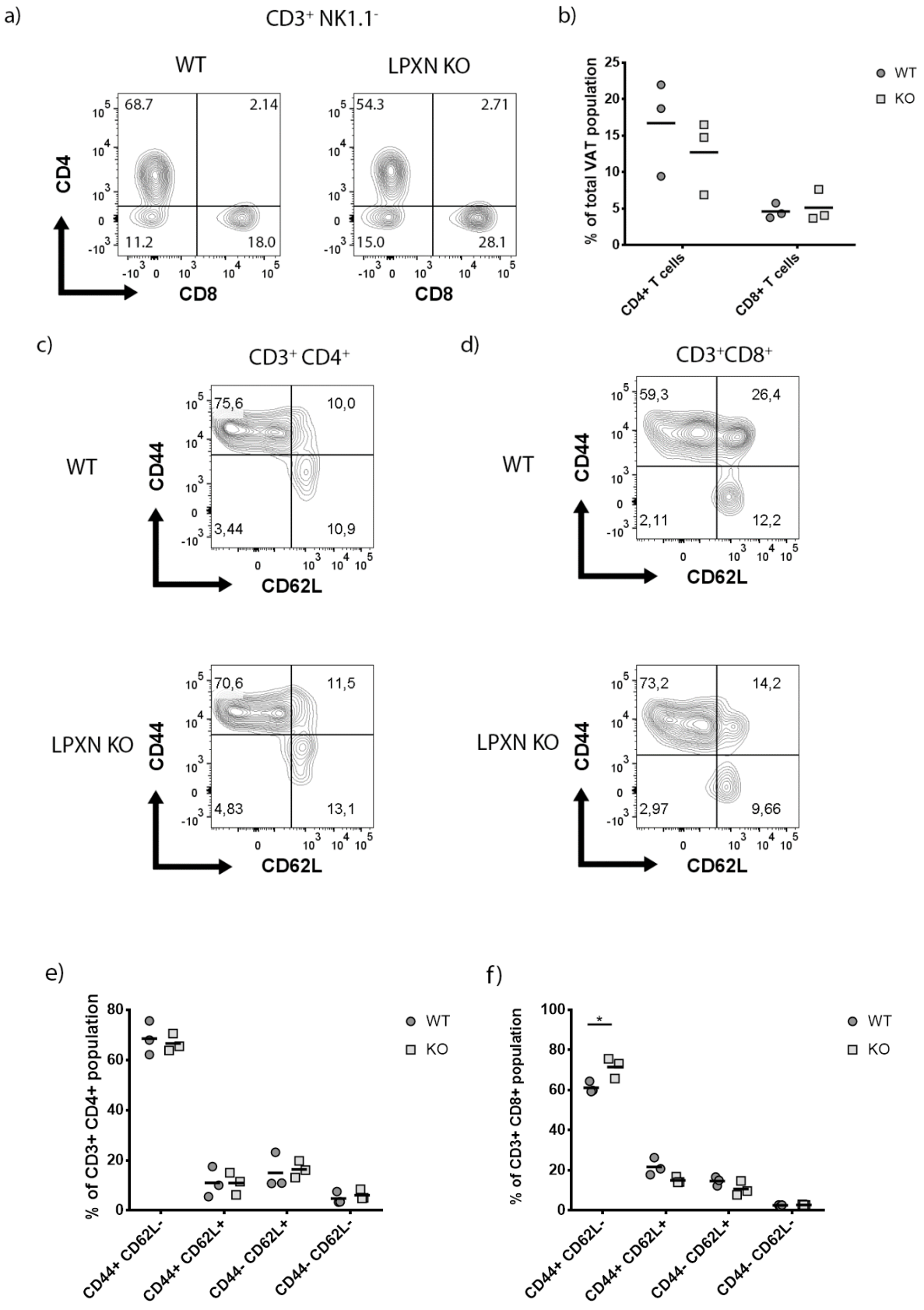


Figure 4.7. Leupaxin knockout does not affect the iNKT, γδ T cell, DN T cell, Ly6G$^+$ F4/80$^-$, CD19$^+$, Macrophage, M1 macrophage, NK, CD11a$^+$, CD11b$^+$, CD11c$^+$ population frequencies in the female VAT. Visceral adipose tissue from female WT and LPXN KO mice were digested via collagenase IV in DMEM for 2-hours and subsequently separated via centrifugation at 4°C for 10-minutes. All cells were gates on live CD45.2$^+$. (a). Flow plots of the analysis for iNKT cells, γδ T cells, and Ly6G$^+$ F4/80$^-$ neutrophils are shown. (b) Quantification of the percentages of the iNKT cell, γδ T cell, DN T cell and Ly6G$^+$ F4/80$^-$ neutrophils in the total population of VAT. (c) Flow plots of CD19$^+$ B cells cells are shown. (d) Quantification of the percentage of cells in total VAT leukocyte population that are CD19$^+$. (e) Flow plots of macrophages, NK cell, and M1 macrophages are shown. (f) Quantification of the percentage of macrophages and NK cells within the total VAT leukocyte population. (g) Quantification of the M1 macrophages percentage of the total macrophage population. (h) Quantification of the percentage of cells in total VAT CD45.2$^+$ population that are CD11a$^+$, CD11b$^+$, and CD11c$^+$. All results were not significantly different as determined by the unpaired student's *t*-test statistical analysis.


a)
CD3+ NK1.1-
WT
LPXN KO
CD4
CD8
68.7
2.14
11.2
18.0
54.3
2.71
15.0
28.1
b)
% of total VAT population
CD4+ T cells
CD8+ T cells
WT
KO
c)
CD3+ CD4+
WT
LPXN KO
CD44
CD62L
75,6
10,0
3,44
10,9
70,6
11,5
4,83
13,1
d)
CD3+CD8+
59,3
26,4
2,11
12,2
73,2
14,2
2,97
9,66
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
f)
% of CD3+ CD8+ population
*

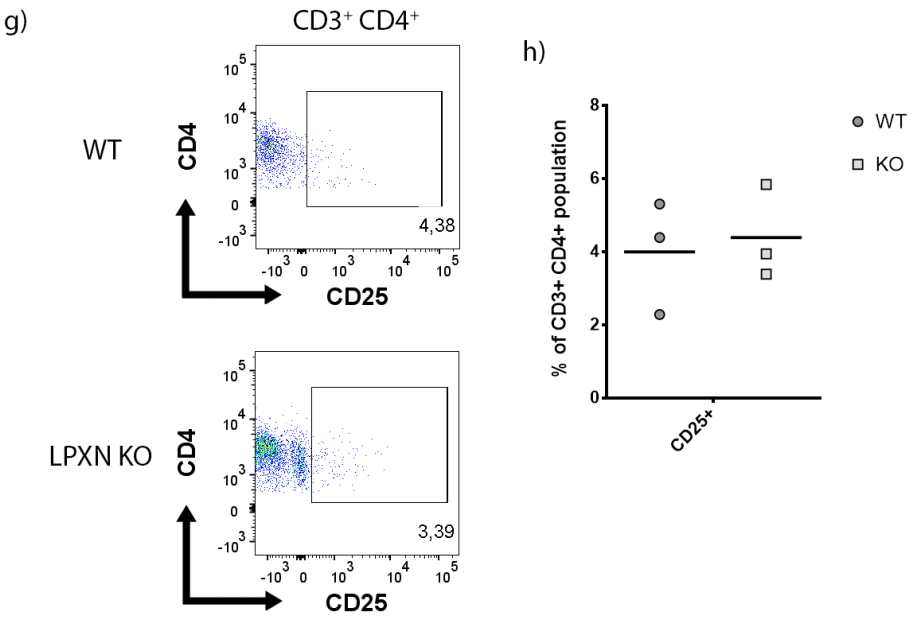
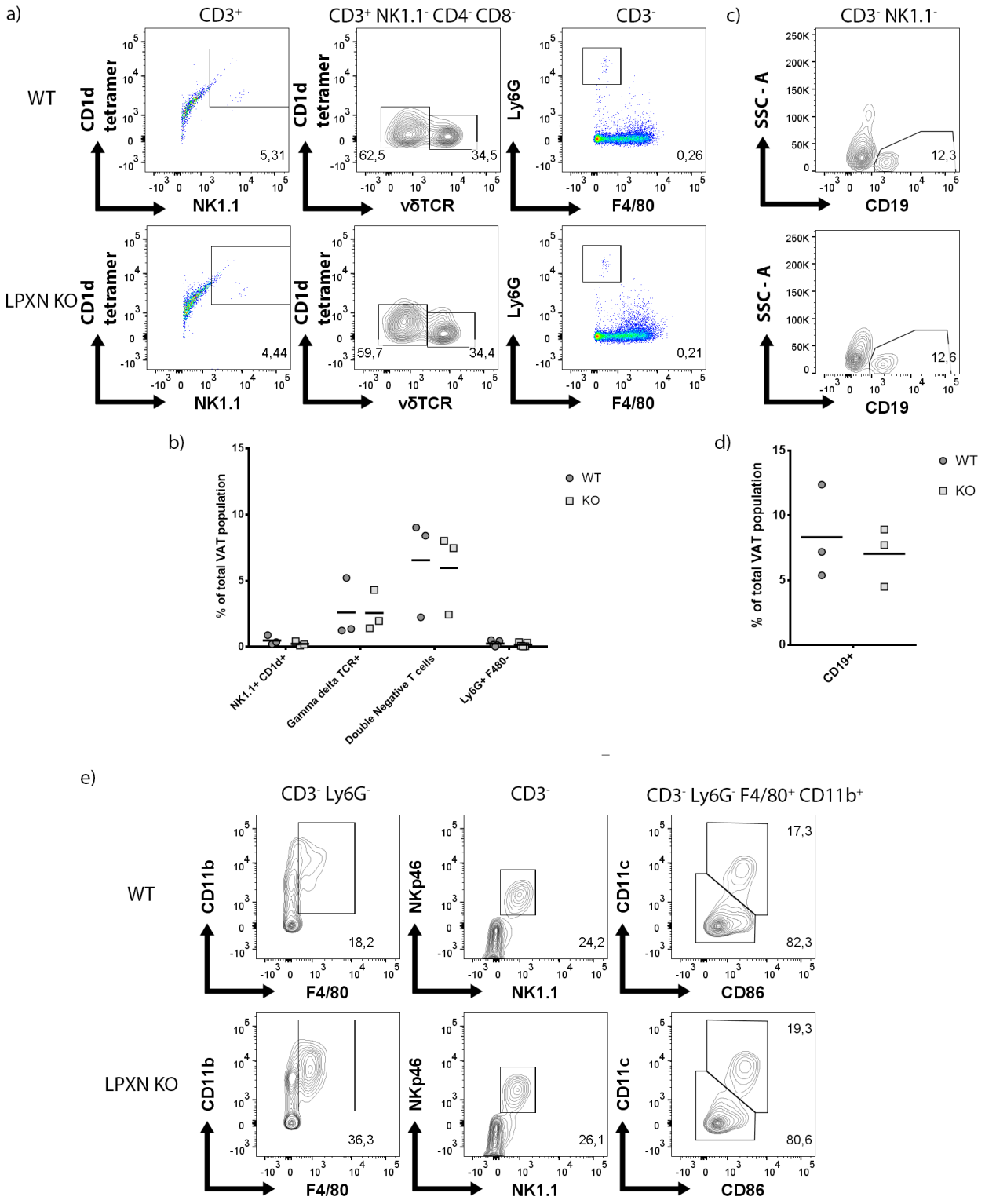


Figure 4.8. Leupaxin knockout increases CD8$^+$ effector T cell presence in male VAT. Visceral adipose tissue from male WT and LPXN KO mice were digested via collagenase IV in DMEM for 2-hours and subsequently separated via centrifugation at 4°C for 10-minutes. All cells were gates on live CD45.2$^+$ (a) Cells were analysed by flow cytometry and flow plots of CD3$^+$ CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of CD4$^+$ and CD8$^+$ T cells in the mice VAT. CD44 by CD62L flow plots of CD4$^+$ (c) or CD8$^+$ (d) T cells of WT or LPXN KO VAT leukocytes. Quantification of the CD44 by CD62L activation states of the CD4$^+$ (e) or CD8$^+$ (f) T cells. (g) Flow plot of CD4$^+$ CD25$^+$ T cells. (h) Quantification of CD25$^+$ T cell percentage of CD3$^+$ CD4$^+$ VAT leukocyte population. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.


a)
CD3+
CD3+ NK1.1- CD4- CD8-
CD3-
WT
LPXN KO
CD1d tetramer
NK1.1
vδTCR
Ly6G
F4/80
5,31
62,5
34,5
0,26
4,44
59,7
34,4
0,21
c)
CD3- NK1.1-
SSC - A
CD19
12,3
12,6
b)
% of total VAT population
WT
KO
NK1.1+ CD1d+
Gamma delta TCR+
Double Negative T cells
Ly6G+ F480-
d)
CD19+
e)
CD3- Ly6G-
CD3-
CD3- Ly6G- F4/80+ CD11b+
CD11b
NKp46
CD11c
CD86
18,2
24,2
17,3
82,3
36,3
26,1
19,3
80,6

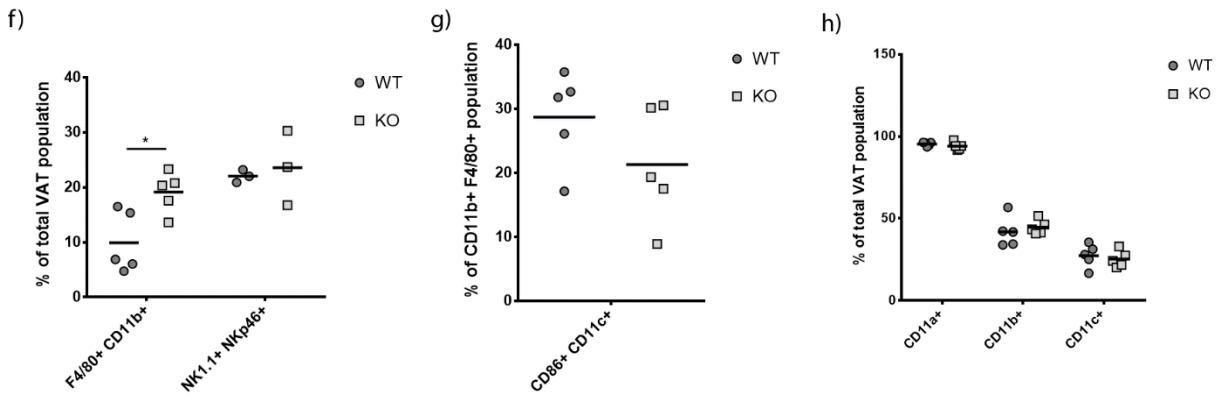


Figure 4.9. Leupaxin knockout increases the percentage of macrophages in the total population of the male VAT. Visceral adipose tissue from male WT and LPXN KO mice were digested via collagenase IV in DMEM for 2-hours and subsequently separated via centrifugation at 4°C for 10-minutes. All cells were gates on live $CD45.2^+$ (a). Flow plots of the analysis for iNKT cells, γδ T cells, and $Ly6G^+$ $F4/80^-$ neutrophils are shown. (b) Quantification of the percentages of the iNKT cell, γδ T cell, DN T cell and $Ly6G^+$ $F4/80^-$ neutrophils in the total population of VAT. (c) Flow plots of $CD19^+$ B cells cells are shown. (d) Quantification of the percentage of cells in total VAT leukocyte population that are $CD19^+$. (e) Flow plots of macrophages, NK cell, and M1 macrophages are shown. (f) Quantification of the percentage of macrophages and NK cells within the total VAT leukocyte population. (g) Quantification of the M1 macrophages percentage of the total macrophage population. (h) Quantification of the percentage of cells in total VAT $CD45.2^+$ population that are $CD11a^+$, $CD11b^+$, and $CD11c^+$. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.

4.2.4. Leupaxin deficiency increases activated T cell population frequency in the liver leukocyte population

The liver plays a key function in the breakdown of metabolites during the feeding and digestion process. In a similar manner to adipose tissue, Th1 immune cells play a major role in the establishment of non-alcoholic fatty liver and obesity [115]. Th1 cytokine secreting $CD4^+$ T cells and $CD8^+$ cytotoxic T cells are both contributing factors to obesity in the T cell compartment of the immune system. Thus, looking at leupaxin deficient mice and their weight gain, I examined the T cells of the liver as an indication of the inflammatory condition. All identified cells and their associating flow cytometry markers are shown on **Table 4.3**.The CD4 T cells and CD8 T cell populations both have no difference in percentage of total population of both female (**Figure 4.10a, b**) and male (**Figure 4.12a, b**) leupaxin knockout mouse liver. However, the activation profile of both CD4 and CD8 T cells follow the same pattern of having less CD44- $CD62L^+$ and more $CD44^+$ CD62L- T cells in female leupaxin knockout mice (**Figure 4.10c-f**). Male leupaxin knockout mice display only have significantly higher $CD4^+$ $CD44^+$ $CD62L^-$ while CD8 T cells follow the trend of having higher $CD44^+$ CD62L- populations but not being statistically significant (**Figure 4.12c-f**). $CD4^+$ $CD25^+$ T regulatory cells show no difference between the WT and leupaxin knockout mice (**Figure 4.10g, h** and **Figure 4.12g, h**). The T cell analysis indicates an activated T cell profile indicating that an immune profile with a more active phenotype is occurring in the LPXN KO mouse liver in comparison to the WT.

Next, I observed the other non-conventional T cell and innate leukocytes in the liver. The reasoning behind observing these leukocyte populations is the same as the reasoning for observing them in the VAT. In both the liver and VAT these cells play regulatory or pro-inflammatory roles that help maintain homeostasis or shifts the steady state towards an activated pro-inflammatory phenotype. γδ T cell population frequency does not differ between WT and LPXN KO (**Figure 4.11a, b** and **Figure**

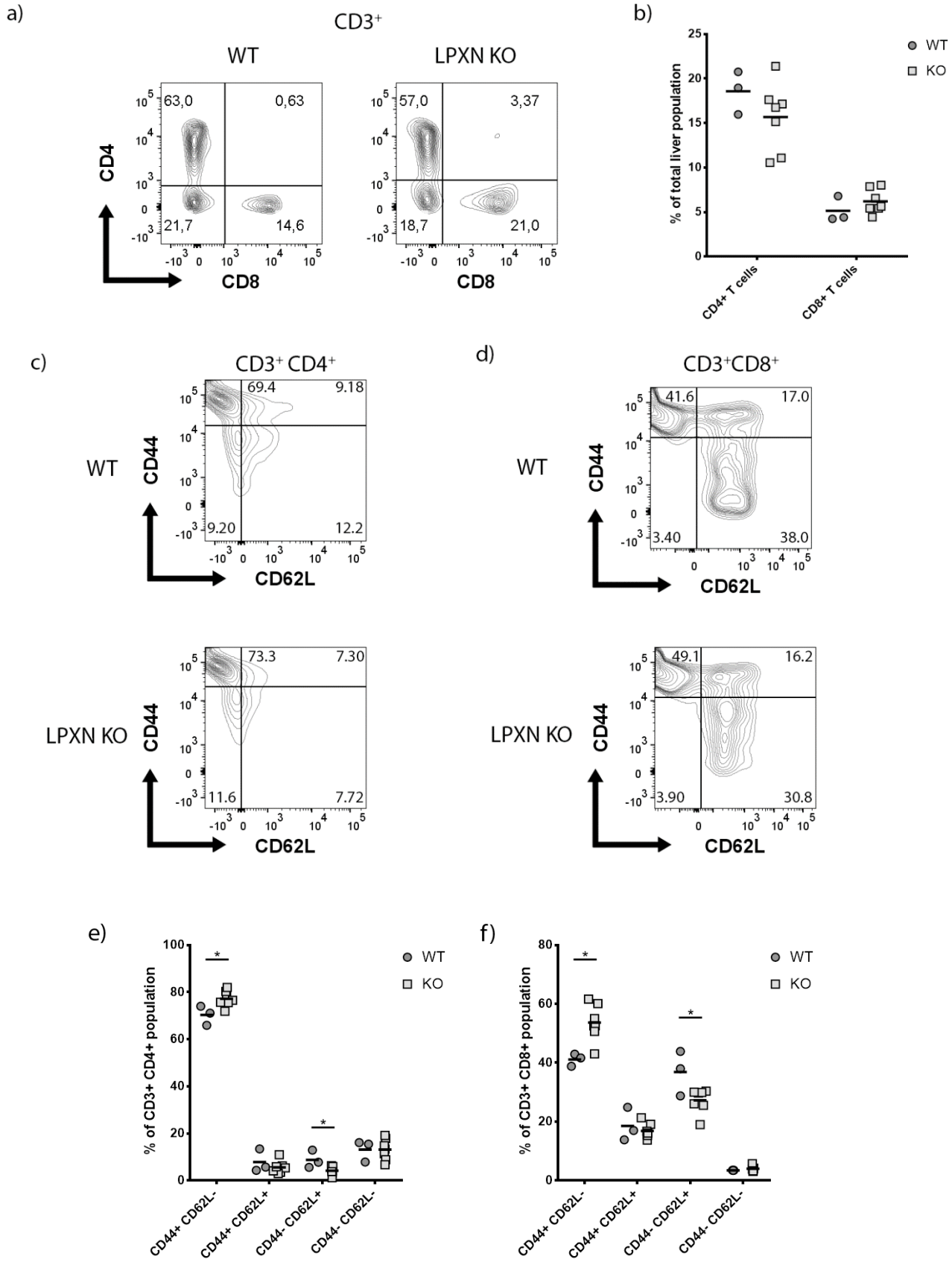
4.13a, b). Neutrophil population frequency also remains unaltered (**Figure 4.11a, b** and **Figure 4.13a, b**). NKT cells, defined by $CD3^+$ $NK1.1^+$, also do not display any significant differences between the WT and LPXN KO (**Figure 4.11a, c** and **Figure 4.13a, c**). $CD19^+$ B cells remain the same between the WT and KO (**Figure 4.11a, d** and **Figure 4.13a, d**). Liver $CD11b^+$ $F4/80^+$ macrophage populations also display no significant differences between WT and LPXN KO (**Figure 4.11e, f** and **Figure 4.13e, f**). The liver contains a unique resident population of NK cells that have close resemblance to ILC1s [114]. These liver resident NK cells would be prominently involved in any Th1 pro-inflammatory responses during fatty liver phenotype manifestation [116]. Examination of $NK1.1^+$ $NKp46^+$ $CD49a^+$ CD49b- liver resident NK cells show that female leupaxin knockout mice have a higher percentage of the total population comprised of the liver resident NK cell (**Figure 4.11e, g**) while the male mice trends to having an increase although the result is not significant (**Figure 4.13e, g**). I also looked at the NK cell population frequency which all did not display any significant differences in the LPXN KO mice but also trends to having an increase in the LPXN KO (**Figure 4.11e, g** and **Figure 4.13e, g**). Likely the lymphocytes that have a greater role in cytokine secretion and regulation of the inflammatory environment such an ILC1 like liver resident NK cells are more sensitive to the low-grade inflammation. NK cells which is mainly for cytotoxicity with less capabilities for cytokine secretion might be therefore less responsive to the low-grade inflammation compared to the ILC1 like liver resident NK cells. M1 macrophage population defined by $CD11c^+$ $CD86^+$ also did not significantly differ between the WT and LPXN KO (**Figure 4.11e, h** and **Figure 4.13e, h**). Like the visceral adipose tissue, leupaxin does not affect the population frequency of cells expressing β2 integrin alpha subunits on the cell surface of liver leukocytes (**Figure 4.11i** and **Figure 4.13i**).

Overall an increase in the activation of T cells towards a more activated profile can be observed in the liver. Innate Th1 cytokine producing cells either have a significant increase or trend towards

having an increase in the percentage of the total population. An overview of the liver leukocyte population describes an increase in the Th1 cytokine producers including increases in population frequency of ILC1in females and $CD8^+$ T cells in both female and male mice livers. One possible explanation for this immune cell activation is the removal of leupaxin may prevent possible phosphatases from being recruited to the signalling site and over phosphorylation of proteins such as paxillin or other kinases such as Src family kinases that play a critical role in the activation of innate immune cells and T cells [102, 103]. $CD8^+$ T cell activation leads to cell proliferation bringing about the increases in population frequency and Th1 cytokine secretion.

Cell type	Identifying flow cytometry antibody markers
$CD4^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$
$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^+$
Naïve $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^-$, $CD62L^+$
Central memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^+$
Effector memory $CD4^+$/$CD8^+$ T cells	$CD3^+$, $NK1.1^-$, $CD4^+$ or $CD8^+$, $CD44^+$, $CD62L^-$
T_{reg} cells	$CD3^+$, $NK1.1^-$, $CD4^+$, $CD8^-$, $CD25^+$
NKT cells	$CD3^+$, $NK1.1^+$
$\gamma\delta$ T cells	$CD3^+$, $NK1.1^-$, $CD4^-$, $CD8^-$, $\gamma\delta TCR^+$
B cells	$CD3^-$, $NK1.1^-$, $CD19^+$
Neutrophils	$CD3^-$, $Ly6G^+$, $F4/80^-$
Macrophages (Liver and Adipose)	$CD3^-$, $Ly6G^-$, $CD11b^+$, $F4/80^+$
M1 Macrophages	$CD3^-$, $Ly6G^-$, $CD11b^+$, $F4/80^+$, $CD11c^+$
NK cells	$CD3^-$, $NK1.1^+$, $NKp46^+$, $CD49a^-$, $CD49b^+$
Liver resident NK cells (ILC1s)	$CD3^-$, $NK1.1^+$, $NKp46^+$, $CD49a^+$, $CD49b^-$

Table 4.3. The corresponding flow cytometry antibody markers used to identify the varying cell types within the mouse liver leukocyte population.


a)
CD3+
WT
LPXN KO
63,0
0,63
21,7
14,6
57,0
3,37
18,7
21,0
CD4
CD8
b)
% of total liver population
WT
KO
CD4+ T cells
CD8+ T cells
c)
CD3+ CD4+
WT
LPXN KO
69.4
9.18
9.20
12.2
73.3
7.30
11.6
7.72
CD44
CD62L
d)
CD3+CD8+
41.6
17.0
3.40
38.0
49.1
16.2
3.90
30.8
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
f)
% of CD3+ CD8+ population

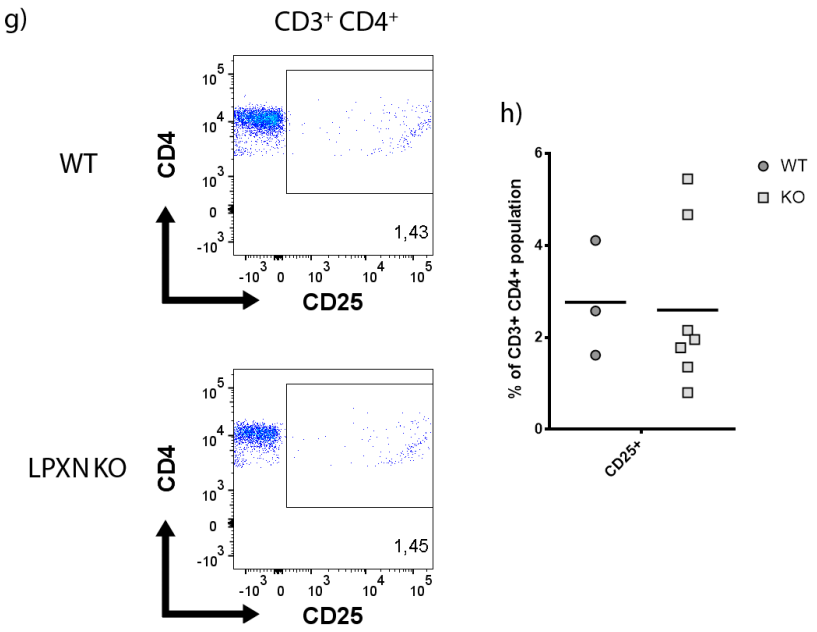
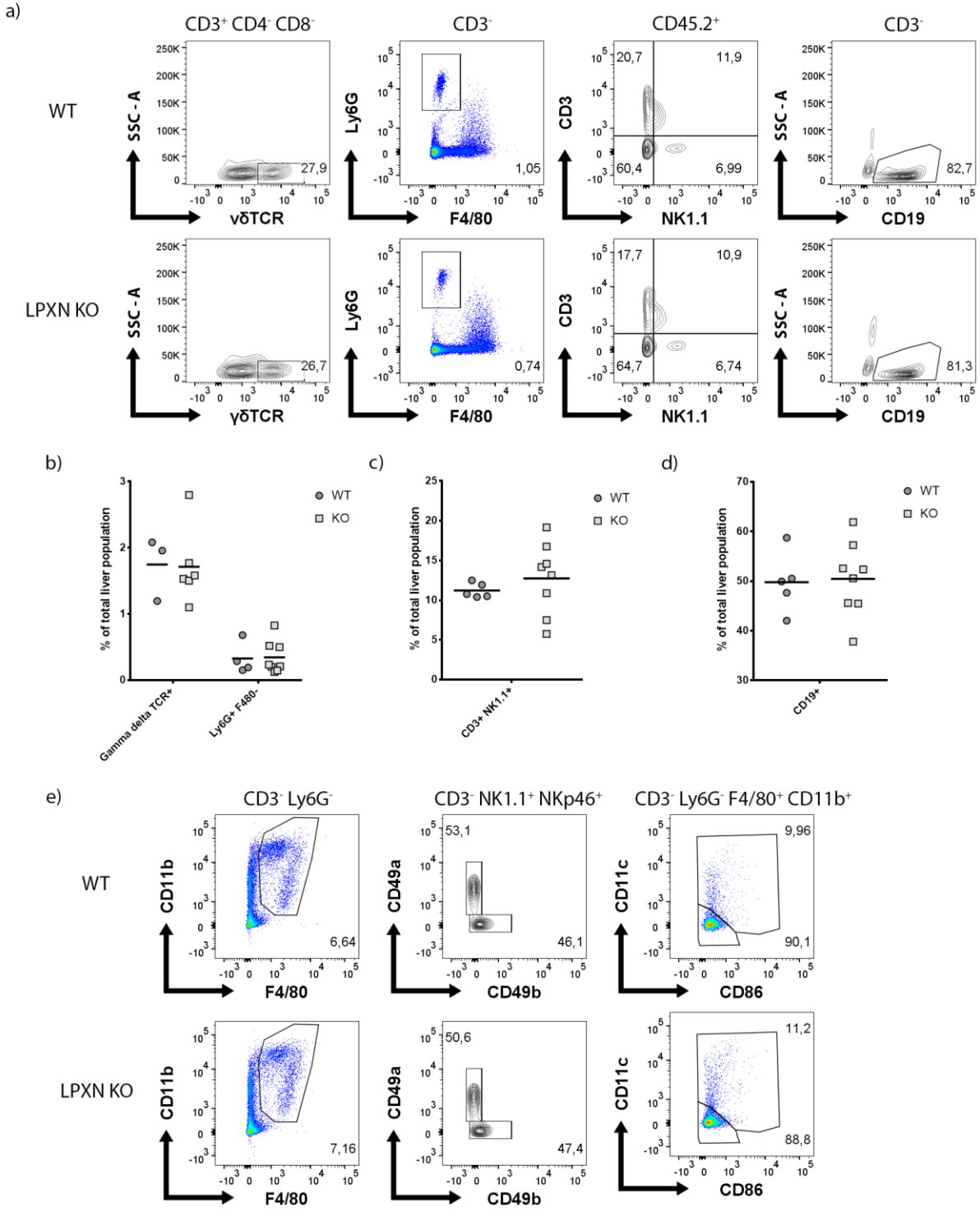


Figure 4.10. Leupaxin knockout increases CD4$^+$ and CD8$^+$ effector memory T cell presence while decreasing naïve T cell population frequency in the female mouse liver. Liver from female WT and LPXN KO mice were digested via stainless steel mesh. Subsequent liver mononuclear cells were isolated via 40%/70% Percoll gradient. All cells were gates on live CD45.2$^+$. (a) Cells were analysed by flow cytometry and flow plots of CD3$^+$ CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of CD4$^+$ and CD8$^+$ T cells in the mice liver. CD44 by CD62L flow plots of CD4$^+$ (c) or CD8$^+$ (d) T cells of WT or LPXN KO liver leukocytes. Quantification of the CD44 by CD62L activation states of the CD4$^+$ (e) or CD8$^+$ (f) T cells. (g) Flow plot of CD4$^+$ CD25$^+$ T cells. (h) Quantification of CD25$^+$ T cell percentage of CD3$^+$ CD4$^+$ liver leukocyte population. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.


a)
CD3+ CD4- CD8-
CD3-
CD45.2+
CD3-
WT
LPXN KO
SSC - A
vδTCR
γδTCR
Ly6G
F4/80
CD3
NK1.1
CD19
27,9
1,05
20,7
11,9
60,4
6,99
82,7
26,7
0,74
17,7
10,9
64,7
6,74
81,3
b)
c)
d)
% of total liver population
WT
KO
Gamma delta TCR+
Ly6G+ F480-
CD3+ NK1.1+
CD19+
e)
CD3- Ly6G-
CD3- NK1.1+ NKp46+
CD3- Ly6G- F4/80+ CD11b+
CD11b
CD49a
CD11c
CD49b
CD86
6,64
53,1
46,1
9,96
90,1
7,16
50,6
47,4
11,2
88,8

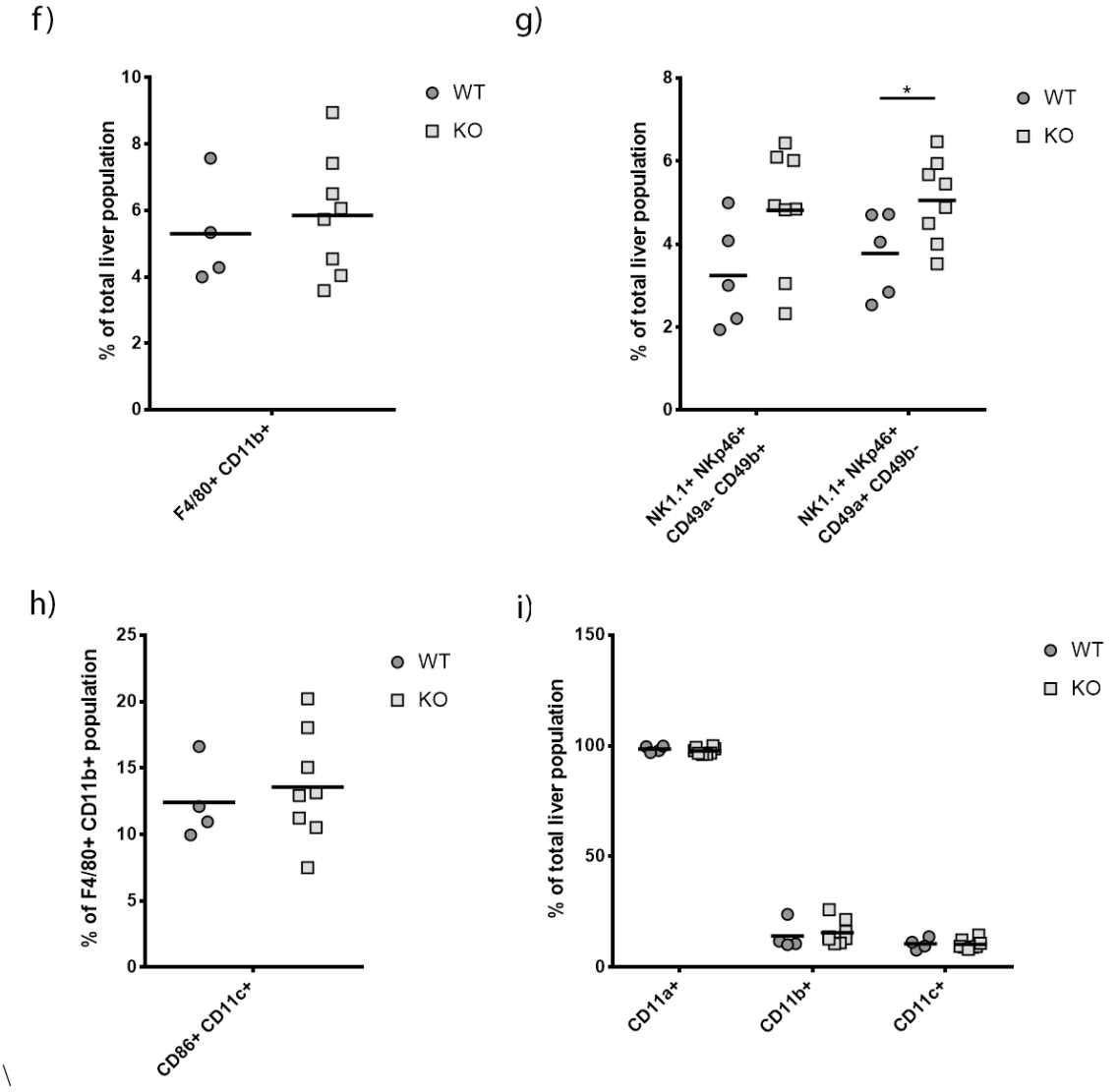
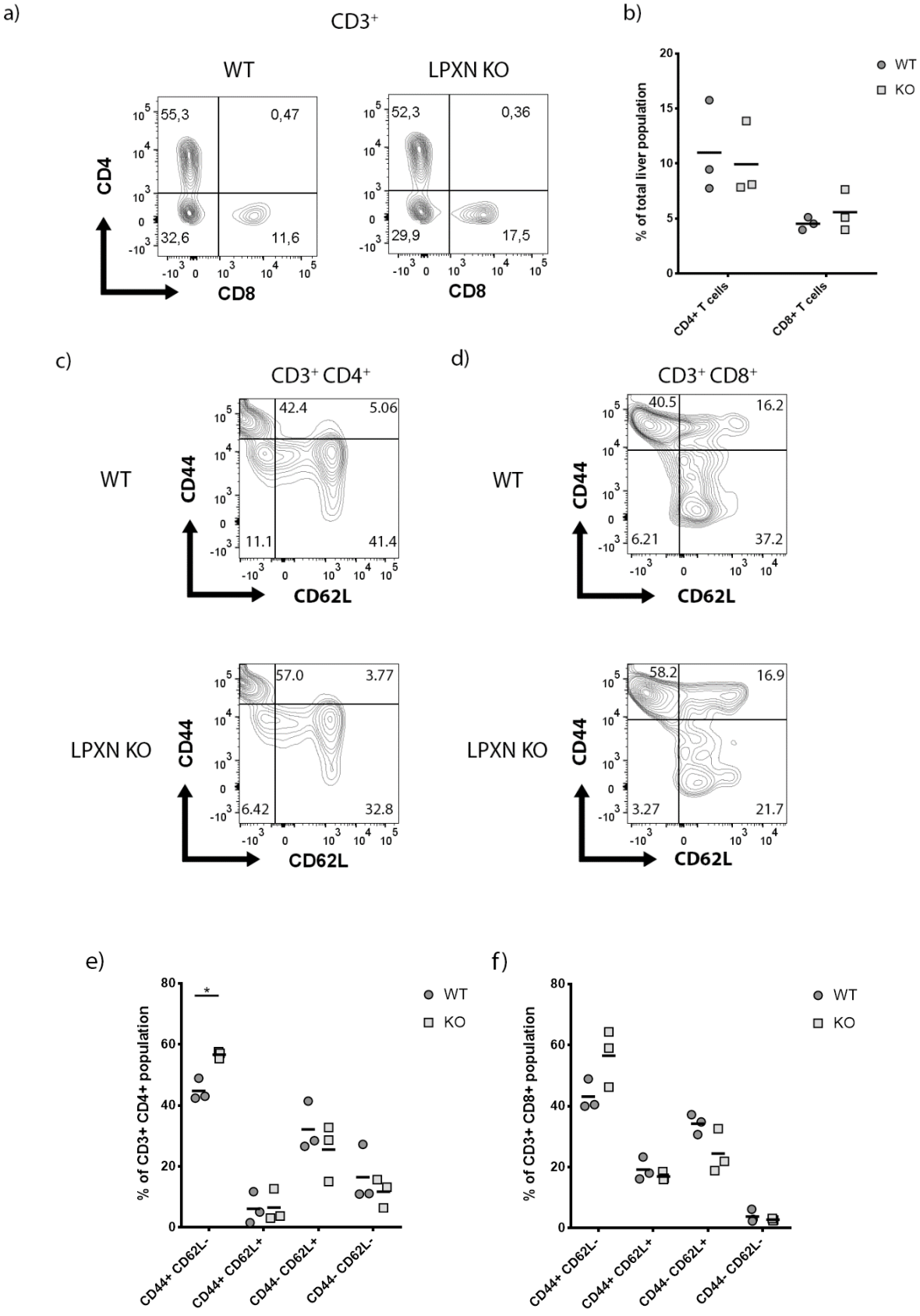


Figure 4.11. Leupaxin knockout increases the percentage of CD49a$^+$ CD49b- liver resident NK cells within the total leukocyte population in the female mouse liver. Liver from female WT and LPXN KO mice were digested via stainless steel mesh. Subsequent liver mononuclear cells were isolated from liver solution via 40%/70% Percoll gradient. All cells were gates on live CD45.2$^+$. (a) Flow plots for γδ T cells, F4/80$^-$ Ly6G$^+$ cells, NKT cells, and CD19$^+$ B cells are shown. (b) Quantification of the percentages of the, γδ T cell and Ly6G$^+$ F4/80$^-$ neutrophils within the total liver leukocyte population. (c) Quantification of the percentages of the NKT cells within the total liver leukocyte population (d) Quantification of the percentage of cells that are CD19$^+$ within the total

liver leukocyte population. (e) Flow plots for macrophages, NK cells, liver resident NK cells, and M1 macrophages are shown. (f) Quantification of the percentage of macrophages within the total liver leukocyte population. (g) Quantification of the percentage of NK cells and liver resident NK cells within the total population. (h) Quantification of the percentage of M1 macrophages within the total macrophage population. (i) Quantification of the percentage of cells that are $CD11a^+$, $CD11b^+$, and $CD11c^+$ within the total liver leukocyte population. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.


a)
CD3+
WT
LPXN KO
CD4
CD8
55,3
0,47
32,6
11,6
52,3
0,36
29,9
17,5
b)
WT
KO
% of total liver population
CD4+ T cells
CD8+ T cells
c)
CD3+ CD4+
WT
LPXN KO
CD44
CD62L
42.4
5.06
11.1
41.4
57.0
3.77
6.42
32.8
d)
CD3+ CD8+
40.5
16.2
6.21
37.2
58.2
16.9
3.27
21.7
e)
% of CD3+ CD4+ population
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
CD44- CD62L-
*
f)
% of CD3+ CD8+ population

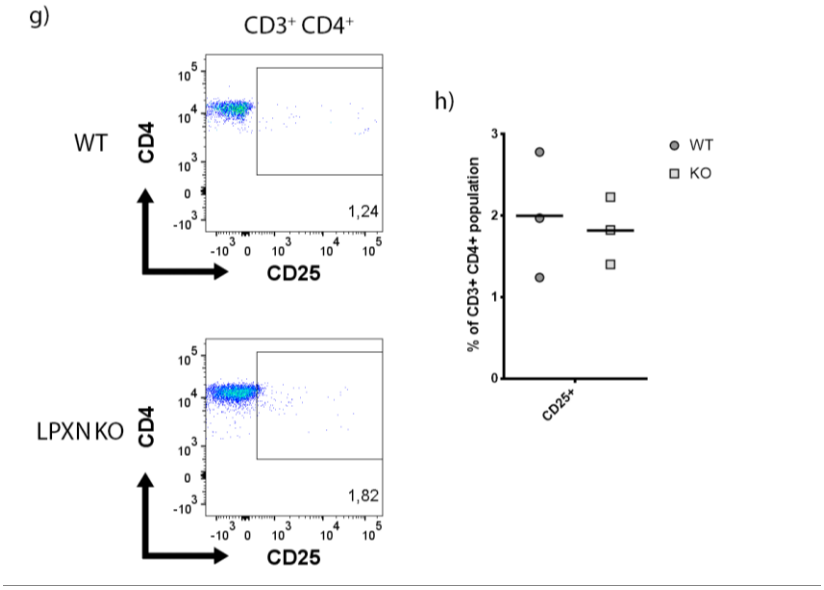
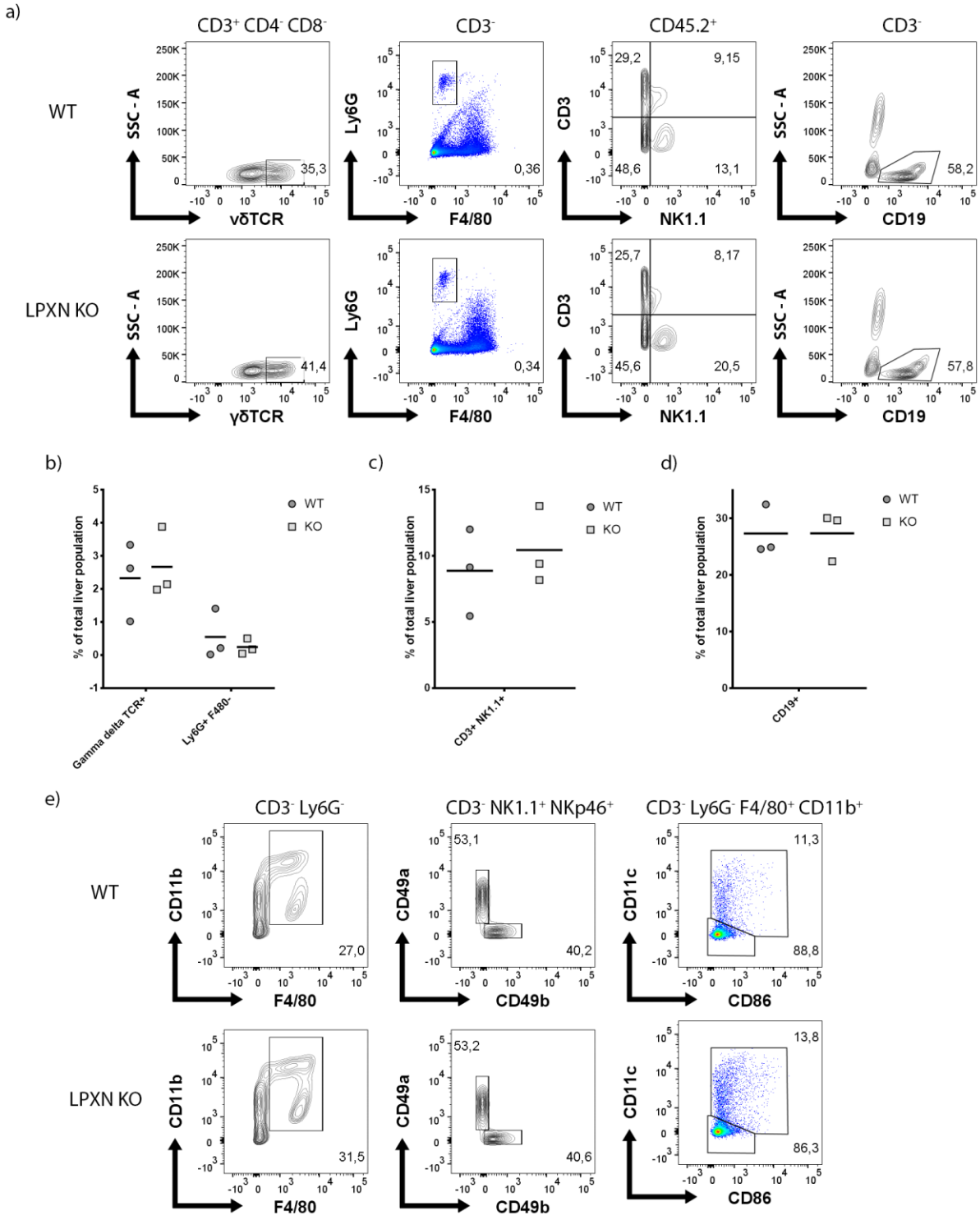


Figure 4.12. Leupaxin knockout increases $CD4^+$ and $CD8^+$ effector memory T cell presence while decreasing naïve T cell population frequency in the male mouse liver. Liver from female WT and LPXN KO mice were digested via stainless steel mesh. Subsequent liver mononuclear cells were isolated via 40%/70% Percoll gradient. All cells were gated on live $CD45.2^+$. a) Cells were analysed by flow cytometry and flow plots of $CD3^+$ CD4 by CD8 of WT and LPXN KO is shown. (b) Quantification of the percentages of $CD4^+$ and $CD8^+$ T cells in the mice liver. CD44 by CD62L flow plots of $CD4^+$ (c) or $CD8^+$ (d) T cells of WT or LPXN KO liver leukocytes. Quantification of the CD44 by CD62L activation states of the $CD4^+$ (e) or $CD8^+$ (f) T cells. (g) Flow plot of $CD4^+$ $CD25^+$ T cells. (h) Quantification of $CD25^+$ T cell percentage of $CD3^+$ $CD4^+$ liver leukocyte population. The unpaired student's *t*-test was used for statistical analysis. * represents $p<0.05$.


a)
CD3+ CD4- CD8-
CD3-
CD45.2+
CD3-
WT
LPXN KO
SSC - A
vδTCR
γδTCR
Ly6G
F4/80
CD3
NK1.1
CD19
35,3
0,36
29,2
9,15
48,6
13,1
58,2
41,4
0,34
25,7
8,17
45,6
20,5
57,8
b)
c)
d)
% of total liver population
WT
KO
Gamma delta TCR+
Ly6G+ F480-
CD3+ NK1.1+
CD19+
e)
CD3- Ly6G-
CD3- NK1.1+ NKp46+
CD3- Ly6G- F4/80+ CD11b+
CD11b
CD49a
CD11c
CD49b
CD86
27,0
53,1
40,2
11,3
88,8
31,5
53,2
40,6
13,8
86,3

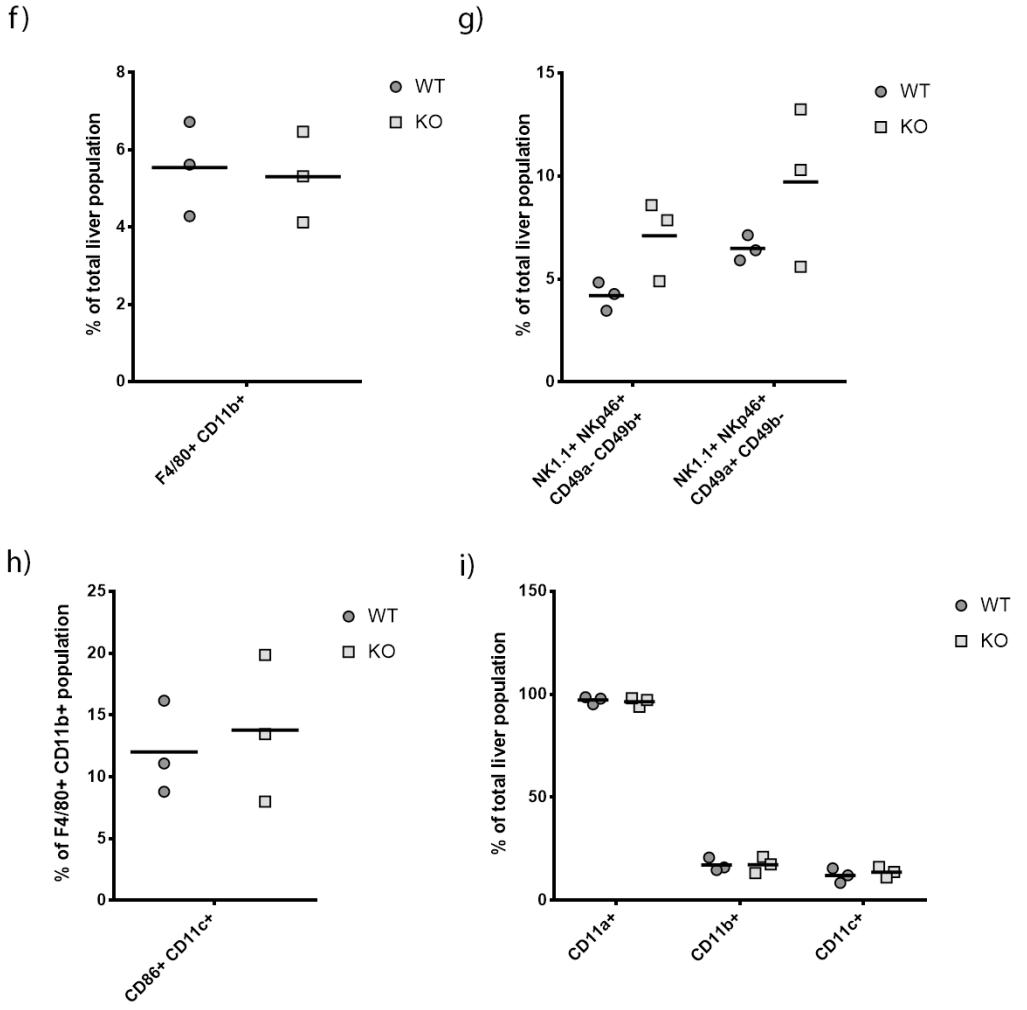


Figure 4.13. Leupaxin knockout does not significantly alter the NKT, γδ T cell, Ly6G^{+} F4/80-, CD19^{+}, Macrophage, M1 macrophage, NK, Liver resident NK cell, CD11a^{+}, CD11b^{+}, and CD11c^{+} population frequencies in the male mouse liver. Liver from male WT and LPXN KO mice were digested via stainless steel mesh. Subsequent liver mononuclear cells were isolated from liver solution via 40%/70% Percoll gradient. All cells were gates on live CD45.2^{+}. (a) Flow plots for γδ T cells, F4/80^{-} Ly6G^{+} cells, NKT cells, and CD19^{+} B cells are shown. (b) Quantification of the percentages of the, γδ T cell and Ly6G^{+} F4/80^{-} neutrophils within the total liver leukocyte population. (c) Quantification of the percentages of the NKT cells within the total liver leukocyte population (d) Quantification of the percentage of cells that are CD19^{+} within the total liver

leukocyte population. (e) Flow plots for macrophages, NK cells, liver resident NK cells, and M1 macrophages are shown. (f) Quantification of the percentage of macrophages within the total liver leukocyte population. (g) Quantification of the percentage of NK cells and liver resident NK cells within the total population. (h) Quantification of the percentage of M1 macrophages within the total macrophage population. (i) Quantification of the percentage of cells that are $CD11a^+$, $CD11b^+$, and $CD11c^+$ within the total liver leukocyte population. The unpaired student's *t*-test was used for statistical analysis.

4.3. Discussion

In this chapter, I have shown that the leupaxin knockout mice have an increase in weight in both male and female mice. Previously, the Wagner group classified the β2 integrin receptors as a "new class of obesity genes" where the deletion of Mac-1 (CD11a/CD18) or the LFA-1 ligand ICAM-1 spontaneously increased the weight for C57BL/6J mice on normal chow diet [105]. While the removal of β2 integrin genes in mice leads to a condition similar to leukocyte adhesion deficiency type 1 in humans, the removal of leupaxin does not recreate this ailment in mice. However, the spontaneous increases in weight gain can be observed in leupaxin knockout mice. In both the case of leupaxin deletion and integrin receptor deletion, I assume a signalling pathway dependent on leupaxin that prevents the spontaneous induction of low-grade inflammation by leukocytes. As mice age, the deposition of adipose tissue in peripheral sites of the body increases. Looking at older mice, unpublished data from our lab indicates that at old age of ~360 days old, the WT mice catches up in weight to the leupaxin knockout mice, with both mice weighing the same and becoming obese. Thus, leupaxin deficient mice exhibit weight gain at an earlier age in comparison to WT mice but result in the same obese phenotype at later stages of life. Female leupaxin knockout mice exhibit the increase in weight gain at a much earlier age than male mice (**Figure 4.1**). While B6 mice diet induced weight-gain is heightened in male mice, differences due to LPXN KO is much more pronounced in female mice. This might imply factors within the female mice that are absent in the male mice, which may include a higher propensity of developing inflammatory conditions, are creating the physiological differences seen in weight and leukocyte activation. Sexual dimorphism between male and female inflammatory condition development is commonly studied. In mice, females have a disposition for immune activation, for example, the development of inflammatory diseases such as allergic airway inflammation are more prevalent in female mice compared to male mice [106]. An innate propensity for better protection against deadly pathogens in female mice compared to male

mice further confirms the heightened immune response females produce. This higher immune activation might provide an possibly explanation on how a slight alteration to elevate T cell activation in female leupaxin knockout mice may be associated with the earlier weight as more inflammation may occur in the female mice body in comparison to the male mice leading to higher leptin resistance. Further identifying which catalytic proteins leupaxin recruits to integrin binding sites including the other two members of the PEST family of phosphatases may demystify how integrin signalling deficiency results in increased weight gain in mice.

In recent times, the interest in leupaxin as a signalling molecule within the β2 integrin receptor LFA-1 signalling complex has grown due to its discovery in interacting with kindlin-3 in regulating podosome stability [87]. In this chapter, rather than looking at the defect leupaxin removal induces on the cellular scale, I observed the effects leupaxin removal plays in the visceral adipose tissue and liver on a population level. In both the male and female mice, the spleen leukocyte populations of the WT and leupaxin deficient mice are identical (**Figure 4.2-5**). When looking at the mRNA expression of leupaxin, mainly cells of activated phenotypes express high levels of leupaxin in comparison to the naïve/cells found in the spleen which have relatively low expression of leupaxin (**Figure 1.5**). The lack of strong expression of leupaxin in splenic leukocytes due to their non-activated nature may be a cause for the lack of difference found in the spleen of the leupaxin deficient mice. In addition, we can infer that the development of leukocytes is not affected by the whole-body removal of leupaxin.

When I observed the leukocytes in both VAT and liver of WT and LPXN KO mice, a significant increased in activated T cells could be found in the LPXN KO mice. Obesity and inflammation are tightly linked through numerous factors including the actions of various hormones and cytokines. Leptin is a hormone primarily secreted from the adipocytes and is a critical hormone in controlling energy balance and diet intake [195]. It may be possible that in LPXN KO mice, the increased

leukocyte activation drives an inflammatory process that leads to leptin resistance causing increased hunger and food intake. Leptin resistance has been linked with the production of proinflammatory cytokines such as TNFα [196]. It may be that the deletion of LPXN in B6 mice provides a role in the generation of proinflammatory cytokines that sends signals across various tissues of the body for leptin resistance.

An interesting find was the increase of central memory $CD44^+$ $CD62L^+$ phenotype in the CD4 T cells of female mice VAT leukocytes (**Figure 4.6**). This phenotype was not found in the male mice VAT $CD4^+$ T cells. The $CD4^+$ T cell activation difference might give insight to part of the reason why females exhibit an increase in weight earlier than male mice. While Belkaid and colleagues have reported the visceral adipose tissue being a reservoir for memory CD4 T cells, the cells have the effector memory $CD44^+$ CD62L- phenotype although the mice used in their study was C57BL/6NTac and they do not state any specific genders [117]. Thus, this increase in central memory T cells may be a unique feature in the reverse role of inflammation inducing obesity to manifest in the case of leupaxin deletion.

Looking into the integrin signaling function of LPXN may give insight on possibilities of how this $CD62L^+$ population can occur. In the case of a listeria monocytogenes mice infection model, Bose et. al have observed increased $CD8^+$ $CD44^+$ $CD62L^+$ memory cell development of CD11a deficient mice [193]. $CD4^+$ T cell population was not mentioned in the listeria monocytogenes infection model study. In a similar manner, another study introduced a moesin deletion mice model which generated increased $CD62L^+$ T cell populations across the SLOs of the mice [194]. Moesin is a member of the ERM family of adaptor proteins that link integrin mediated signaling from the cytoplasmic membrane to the actin cytoskeleton. Both LPXN and moesin being integrin linked adaptor proteins influencing the cytoskeletal rearrangement strikes a similarity between their functions and may provide an understanding of how LPXN is able to cause this increase in $CD62L^+$ T cells although no

definite conclusions can be drawn. However, the VAT $CD4^+$ T cells are the only population with the increase in CD62L (**Figure 4.6**) while the $CD8^+$ T cells in the female VAT (**Figure 4.6**) and $CD4^+$ (**Figure 4.10**) and $CD8^+$ T cells (**Figure 4.10**) within the female liver display no signs of increase in $CD44^+$ $CD62L^+$ T cells. A possible explanation of the difference observed may be due to the LPXN deletion being a systemic deletion. As LPXN is expressed within every cell of the body and every cell expressed integrin proteins, it remains possible the removal of LPXN may affect distinct tissues differently. Regardless, the adipose tissue memory T cells show increased population frequency in leupaxin knockout mice as seen in **Figure 4.6-9**, which may act to provide the inflammation associated with the deposition of adipose tissue and may even act as an unintended protective barrier against infections and possibly cancerous growths.

Obesity has been linked to a chronic low-grade inflammation occurring throughout the body and an increase in the memory T cell population in the adipose tissue [113, 117]. This increase in weight gain of leupaxin knockout mice may be a cause for the increase in $CD44^+$ CD62L- CD4 and CD8 T cell percentage in the liver and VAT. In general, an increase in effector memory T cells indicate a Th1 response and increase in inflammatory signals in accordance with mice obesity phenotype. During obesity induced inflammation, Th1 cell populations are magnified while Th2 and regulatory populations remain stagnant as seen in the $CD25^+$ $CD4^+$ T cell analysis in **Figure 4.6g,h**, **Figure 4.8g,h**, **Figure 4.10g,h**, and **Figure 4.12g,h** . Like the adipose tissue, the liver displays an increase in Th1 cytokine producers such as liver resident NK cells (**Figure 4.11e, g** and **Figure 4.13e, g**). Significant differences in the flow plots between male and female livers were observed with male livers exhibiting much more percentages in the CD44- $CD62L^+$ population. This may be another factor in the mouse sexual dimorphism with a larger increase in population frequency of $CD44^+$ $CD62L^-$ effector memory type T cell in female mice contributing to the earlier increase in fatty tissue deposition and weight gain within the female mouse liver.

A previous study indicated that leupaxin plays a role in the negative regulation of B cell mediated signalling [118]. However, previous data from our lab by Dr. ShuGang Yao indicated that the TCR activation mediated cytotoxic mechanisms including $CD8^+$ T cell degranulation and cytotoxicity is not affected in leupaxin knockout OT-1 T cells [172]. Nevertheless, this does not rule out the prospect of leupaxin altering the signalling threshold of TCR mediated activation. As leupaxin activity is linked with integrin signalling, it may be possible that leupaxin recruits the phosphatases to the immunological synapse during integrin engagement when T cells encounter APCs and other cells.

Having been shown to interact with leupaxin, the PEST family of phosphatases are a likely candidate for phosphatases that carry out the inhibitory role of leupaxin [102]. The phosphatases would presumably dephosphorylate some of the proteins within the TCR signaling complex, thus dampening the signal. Without the presence of leupaxin, the T cell activation signals are either stronger or are prolonged which may possibly lead to the increased T cell activation as seen in the VAT and liver. Thus, an overactivation of immune cells during steady state may shift the balance of immune homeostasis to a Th1 response which in turn produce an influx of Th1 cytokines such as IFNγ. Increased dissemination of Th1 inflammatory signals leads to the systemic low-grade inflammation, possibly contributing to the increase in weight gain during feeding for the leupaxin knockout mice.

Further looking into the response and survival of leupaxin knockout mice to cancerous growths or pathogenic infections may elucidate if the removal of leupaxin influences the active immune response. Due to the leupaxin knockout mice already supporting an activated T cell profile during normal conditions, it may be possible that leupaxin knockout mice respond and clear infections or cancerous growths with a much higher success rate. The inflammatory condition may prime the immune system to mount a stronger response to pathogens or cancerous growths. In addition, it may

be prudent to examine if any specific populations are making a major contribution to the inflammatory condition of LPXN KO mice. To perform this, I would use monoclonal antibodies against a specific cell type to remove certain cells such as $CD8^+$ T cells from the immune milieu.

Chapter 5: General discussion

5.1. Conclusion

5.1.1. LPXN acts downstream of integrin outside-in signaling during NK cell cytotoxicity and crawling

Leupaxin has been previously demonstrated to regulate the ability of cytotoxic granules to polarize to the MTOC [56]. However, as this study was performed in human NK cell lines, I endeavoured to explore the functions of leupaxin in primary NK cells. Within the LPXN KO IL-2 activated NK cells, I showed that the granules have greater distance to the MTOC and less granules polarized to the MTOC after 10 minutes of contact with YAC-1 target cells (**Figure 3.1**). This suggests that LPXN is involved with the outside-in signaling cascade downstream of β2 integrin receptors. This result is synonymous with the results obtained by the Long group which implicated LPXN as part of a signaling network to the microtubule proteins. Its likely that the association of LPXN and Pyk2 plays a major role in this signaling network [67, 102]. However, as I see most of the granules still converge at the MTOC in **Figure 3.1**, it explains the lack of bystander cell cytotoxicity or alterations in normal cytotoxicity seen in **Figure 3.3**. Rather LPXN KO may introduce a decrease in the kinetics of granule polarization but still maintain the ability of granules to polarize properly.

As paxillin has a ubiquitous role in all integrin signaling networks, I would argue that LPXN acts in general downstream of all integrin as a protein recruited to the β subunit cytoplasmic tail. However, the role of LPXN in integrin mediated granule polarization seems to be a separate signaling network from the ability of leupaxin to regulate crawling velocity. Previously published results have demonstrated that LPXN interacts with kindlin-3 which is recruited to the β subunit cytoplasmic tail in leukocytes during integrin engagement to its ligand [99, 100]. LPXN, kindlin-3, and β2 integrins are also proteins specially or preferentially expressed in leukocytes which promotes the ideas of these proteins acting within the leukocyte integrin signaling network. Kindlin-3 along with Talin facilitates

the conformational change of integrin proteins as well as integrin protein clustering. The deletion of LPXN by CRISPR/Cas9 in RAW cells alters the stability of integrin rich protrusions [87]. In a way, this mirrors the phenotype of LPXN knockdown in adherent cells reducing cell adhesion [170]. LPXN is acting as a positive regulator of integrin cluster stability and preventing protein turnover in both cases. In general, a decrease in adhesive property of the cell would suggest increase in crawling velocity as the quicker disassembly of integrin clusters would have less avidity to its ligands to keep the cell stuck to one spot. Indeed, in the results seen in **Figure 3.4**, I observed an increase in the crawling velocity of LPXN KO cells on VCAM-1 and fibronectin, supporting the idea of LPXN KO lowering the adhesiveness of NK cells on integrin ligands. As kindlin-3 plays roles in both integrin conformational change and integrin clustering [99, 100, 101], it remains unknown which aspect of integrin activation LPXN plays a role in.

How LPXN is able to perform its function of prolonging integrin stability and acting as a negative regulator is likely due to association with various phosphatases. The PEST family of protein tyrosine phosphatases including PTP-PEST, PTPN22, and PTP-HSCF are the most prominent phosphatase candidate of facilitating LPXN mediated integrin stability. PTP-PEST has been shown to bind directly to LPXN in prostate cancer cells [102]. The recruitment of PTP-PEST to LPXN likely also provides an avenue for PTP-PEST to dephosphorylate various proteins including Pyk2 within the integrin signaling complex. LPXN KO removes a recruitment method of the PEST family of phosphatases which may result in over-phosphorylation of varying proteins promoting integrin cluster disassembly. PXN is a prime candidate of the phosphatase activity as non-phosphorylated PXN promotes focal adhesion assembly and phosphorylated PXN promotes focal adhesion disassembly [164]. Therefore, as LPXN KO removes a method of phosphatase recruitment, the decrease of phosphatase activity within close proximity of the integrin signaling complex may cause an increase in phosphorylated proteins such as PXN promoting the disassembly of integrin clusters

[165]. LPXN is not the only protein to associate with PTP-PEST as PXN has also been to bind to PTP-PEST [177]. Therefore, LPXN most likely plays a fine-tuning role in the signaling complex to help augment PXN functionalities. The subtle fine-tuning effects of LPXN also seem to be overpowered by a strong stimulus in vitro as seen in the usage of RANTES in the transwell migration assay in **Figure 3.5**.

5.1.2. LPXN deletion increases the activation profile of tissue resident leukocytes

The appearance of weight gain is often associated with a chronic low-grade inflammation that occurs throughout the body [157]. I have demonstrated that the LPXN KO mice gain weight at a faster rate than the WT C57bl/6 mice as seen in **Figure 4.1**. Furthermore, LPXN KO introduces a difference in the weight gain patterns of male and female mice with female mice displaying the difference in weight at an earlier age. A possible reason for how this increase propensity for female LPXN KO mice to gain weight compared to male LPXN KO mice may be due to increased inflammation and leptin resistance [195, 196]. This weight gain is also associated with an increase in the frequency of activated T cells in both the $CD4^+$ and $CD8^+$ T cells of the female VAT and liver as seen in **Figure 4.6** and **Figure 4.10** respectively. In a similar manner, the male mice also show increased frequencies of $CD8^+$ T cells in the VAT (**Figure 4.8**) and $CD4^+$ and $CD8^+$ in the liver (**Figure 4.12**). General increases in activated lymphocyte populations within the adipose tissue indicate an inflammatory condition which is likely occurring in the LPXN KO mouse tissue. As I did not examine any inflammation markers in my experiments, I cannot conclusively declare that inflammation is occurring within the various tissues of the body. Future experiments on determining pro-inflammatory cytokine concentration within the interstitial fluid and circulation may provide evidence of an immune response happening. Obesity induced inflammation is characterized by increases in numbers of $CD8^+$ T cell and Th1 associated cell populations while Th2 and regulatory cell population numbers remain stagnant or decrease [157]. Indeed, the increase I saw in $CD8^+$ T cell

in LPXN KO mice while having $CD25^+$ $CD4^+$ T cell frequencies similar to the WT demonstrates a possible inflammatory condition occurring. LPXN in the mouse body therefore may be acting as a suppressor of steady state lymphocyte activation where its removal introduces host systemic inflammation.

Curiously, LPXN KO OT-1 mice that contain a transgenic TCR designed to specifically recognize the OVA peptide SIINFEKL from chicken ovalbumin do not spontaneously gain weight. However, these mice do display an increased incidence of sickly-looking mice and death even when housed in a germ-free facility (unpublished observation). It is likely that the LPXN KO induced weight gain requires polyclonal TCRs which can maintain a stable low-grade inflammation without the establishment of lethal autoimmunity as seen in the monoclonal TCR OT-1 mice (unpublished observations). Whatever the trigger is for T cell activation in OT-1 mice, once the trigger occurs most identical TCR T cells will undergo activation and produce massive inflammatory effects that becomes lethal. With polyclonal TCRs within LPXN KO mice, only a small number of T cells subset will trigger activation with most T cells remaining dormant which may establish a low amount of inflammation but not to the lethal levels observed in OT-1 T cells.

The mechanism of which LPXN suppresses lymphocyte activation is unknown. As others have discovered, LPXN acts as a negative regulator of BCR signaling in B cells [118]. The phosphatase recruiting function of LPXN likely plays a role in its inhibitory mechanism. Both PTP-PEST and PTPN22 have been implicated in the suppression of lymphocyte activation [103, 178]. However, the role of PTP-PEST in lymphocyte suppression is debated with overexpression inhibiting lymphocyte activation while mice with T cell specific conditional deficiency of PTP-PEST have lower T cell responses [103, 179]. While PTP-PEST activity is questioned, PTPN22, which is solely expressed in cells of hematopoietic origin, has confirmed antagonizing action against lymphocyte activation. PTPN22 forms a complex with Csk, a potent inhibitor of T cell activation, to dephosphorylate the Src

family kinases, preventing their kinase activity [179]. As LPXN acts downstream of β2 integrin proteins which cluster around the pSMAC during lymphocyte activation through activating receptors, it likely recruits PEST family of proteins to the pSMAC ring. This recruitment of PTP-PEST family of proteins close to the site of the activation signalosome might provide an explanation on how LPXN is able to suppress lymphocyte activation through dephosphorylation of TCR downstream signaling proteins.

Recently, integrin and its associated proteins have been increasingly implicated in augmenting the immune cytotoxic response. Notably, laylin which is a transmembrane glycoprotein associating with LFA-1 and talin has been discovered to have increased expression in tumor infiltrating $CD8^+$ T cells [188]. Unlike LPXN which does not enhance cytotoxicity through its deletion, laylin expression on tumor infiltrating CTLs increases their cytotoxicity. Laylin deletion also introduces reduced tumor resistance unlike the increased tumor resistance found in LPXN deficient mice from preliminary experiments performed in our lab. Due to laylin's association with talin and the contrasting phenotypes observed, its likely that laylin promotes integrin associated functions in an opposite manner of LPXN. As new discoveries unfold, it is clear that integrins although not critical for the cytotoxicity mechanism, is vital in controlling the aim and direction of the immune response.

5.2. Model of action

Putting together the collective information on LPXN, I propose that LPXN acts downstream of integrin signaling in several different pathways as shown in **Figure 5**. First, during cell crawling on endothelium, LPXN associated with the β subunit cytoplasmic tail through binding to kindlin-3. LPXN then recruits members of the PEST family of phosphatases to the integrin signaling complex. The PEST family phosphatases then dephosphorylate integrin signaling proteins including PXN. The dephosphorylated form of PXN promoted integrin cluster assembly and stability while the phosphorylation by FAK promotes integrin cluster disassembly and protein turnover [156, 163]. LPXN, by recruiting the PEST family of phosphatases, dephosphorylates PXN to reduce protein turnover and promote adhesion. The second signaling pathway involving LPXN is during NK cell engagement to a target cell. Integrin association with ligands on the target cell trigger a signaling cascade starting from ILK [56]. The ILK–LPXN–Pyk2–Cdc42 signaling network supplements the ILK – PXN – RhoGEF7 – Cdc42 signaling network with both leading to activation of microtubule associated proteins leading to lytic granule polarization. The third theoretical pathway is the involvement of LPXN in lymphocyte activation signal dampening. The activation of the TCR in T cells assembles the SMAC with the activating receptor at the cSMAC and integrin receptors clustering to the pSMAC ring, surrounding the TCR. The integrin receptors again recruit LPXN through kindlin-3 to the beta subunit cytoplasmic tail where it then recruits the PEST family phosphatases to dephosphorylate TCR downstream proteins and dampen the activation signal.

The most notable PEST family member in this case is PTPN22 which has potent activation suppressing capabilities but its association with LPXN has yet to be confirmed. Theoretically, the PEST family phosphatases bind to LPXN and dephosphorylates TCR signaling proteins including the Src family kinases, thus halting the T cell activation signal transduction at a critical stage. This would reduce the amount of spontaneous T cell activation in during normal conditions or steady state.

Lessened T cell activation would equate to less cytokines such as IFNγ produced and less inflammatory Th1 phenotype. The reduced inflammation would prevent the spontaneous weight gain.

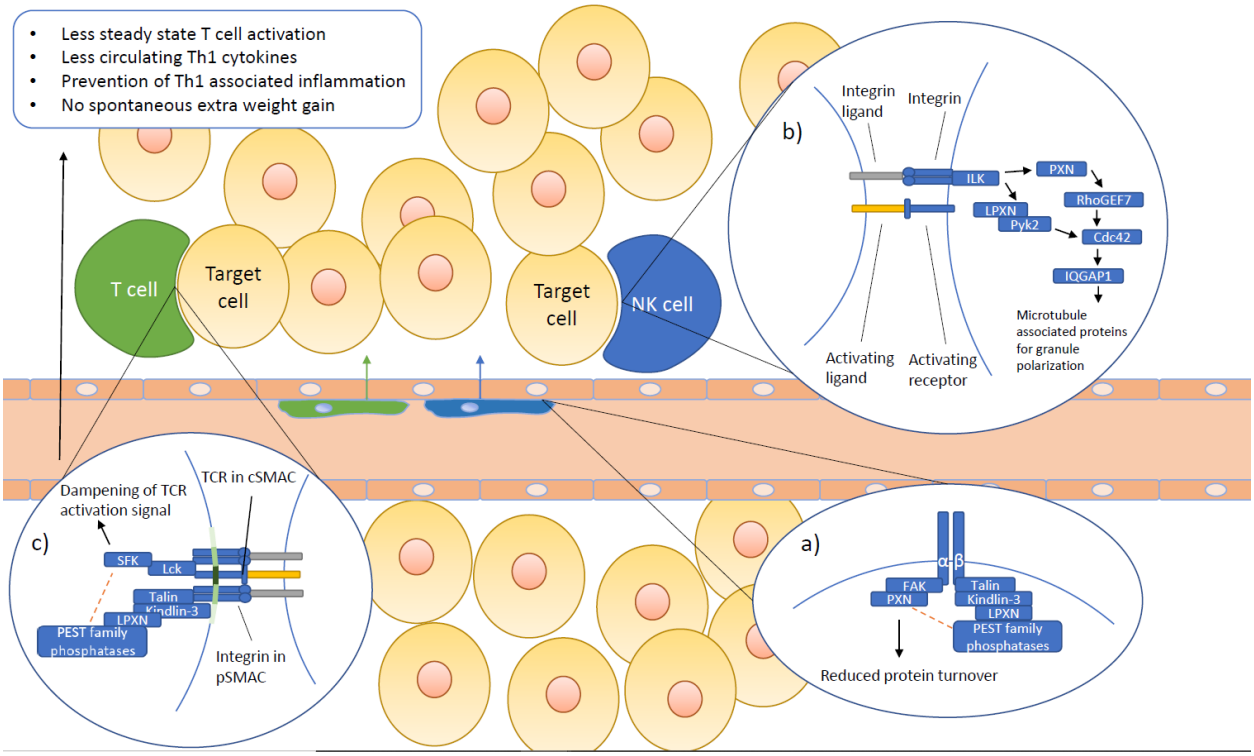


Figure 5. The model for three pathways of LPXN action downstream of integrin signaling. Leupaxin (LPXN) is a protein that acts downstream of integrin signaling in a variety of pathways. a) During crawling on the endothelium, integrins associate with its ligand and activates outside-in signaling. The proteins Talin and Kindlin-3 are recruited to the integrin β subunit cytoplasmic tail which then allows LPXN to be recruited to the β subunit cytoplasmic tail. LPXN then recruits the PEST family phosphatases to dephosphorylate various proteins of the integrin signaling complex. One protein dephosphorylated by LPXN involvement is paxillin (PXN). The dephosphorylated form of PXN promoted integrin cluster assembly and stability while the phosphorylation by FAK promotes integrin cluster disassembly and protein turnover. Thus, LPXN and associated phosphatases dephosphorylates PXN to reduce protein turnover and promote adhesion. b) When NK cells attach to their target cell, the engagement of integrins promotes the polarization of granules towards the target cell through a variety of signaling molecules. LPXN is involved in the ILK–LPXN–Pyk2–Cdc42 signaling network. LPXN involvement supplements the ILK – PXN –

RhoGEF7 – Cdc42 signaling network with both signaling cascades leading to activation of microtubule associated proteins promoting lytic granule polarization. c) Integrin receptors during TCR engagement cluster in the pSMAC which surrounds the TCR in the cSMAC. Theoretically, integrin signaling again recruits LPXN through kindlin-3 which in turn recruits the PEST family of phosphatases. The PEST family phosphatases, most notably PTPN22, dephosphorylates Src family kinases thereby dampening the TCR activation signal. However, this idea remains hypothetical as PTPN22 has yet been shown to associate with LPXN. The dampening of the TCR signal would prevent overactivation of a T cell population, inflammatory conditions, and weight gain.

5.3. Future directions

I showed that LPXN KO NK cells crawl at a slower speed than WT NK cells which would indicate an inhibitory activity. As I mentioned in **Figure 5**, the PEST family of phosphatases are the prime candidates for LPXN phosphatase activity leading to its inhibitory role. Most notably, PTPN22 is a potent inhibitor of lymphocyte activation so its possible association with LPXN is an area of intrigue. Thus, future experiments regarding LPXN should include pulldown assays of LPXN to see if any of the PEST family of phosphatases associate with LPXN.

The PEST family of phosphatases possibly target various proteins downstream of integrin signaling. One of the main candidates of PEST phosphatase action is PXN. Thus, testing if the phosphorylation status of PXN is altered in any way in LPXN KO lymphocytes would be a good conformation to the hypothesis. As phosphorylated PXN promotes the protein turnover and disassembly of integrin clusters, LPXN KO might increase the phospho-PXN in the cell rendering it less adhesive. In line with the idea of LPXN KO generating a cell with less adhesiveness, the ability of LPXN KO lymphocytes to crawl under sheer flow like the crawling process on endothelium should be determined.

While the LPXN KO NK cells display reduced granule polarization, most of the granules appear to be able to fully polarize to the MTOC. It may be possible that the decrease in granule polarization is due to a slower kinetic for the movement of granules to the MTOC. LPXN may act as a supplemental role to the granule polarization network, hastening the process. Thus, to check if the granule polarization has reduced kinetics in LPXN KO, live cell imaging of the cytotoxicity process should be performed.

T cell activation profile in the LPXN KO mice peripheral tissue show an increase in activated effector T cell frequency. The individual T cells may also have higher propensity for activation as the

removal of LPXN theoretically removes phosphatases for dampening the immune response. Thus, to observe T cell activation, the phosphorylation status of the Src family kinases should be monitored. Another possible protein candidate for monitoring T cell activation is to observe the phosphorylation status of ERK as most pathways culminate in the phosphorylation of ERK. These observations will provide insight on if LPXN removal does indeed prevent dephosphorylation of TCR downstream proteins leading to overactivation. Ova specific T cells should be activated with the weaker T4 or G4 peptide rather than the extremely strong N4 peptide as overly strong activation signals might bypass any subtle effects LPXN may play in the T cell activation process.

With LPXN KO mice displaying a greater degree of inflammation is its tissues, more activated $CD8^+$ T cells also can be found in the peripheral tissues. The presence of activated T cells would drive a Th1 association immune response which may heighten any immune response to any foreign infections. Thus, to test if the LPXN removal alter the immune response against pathogens or malignant growths in any way, viral infection studies and tumor injection studies should be performed. This would elucidate if the LPXN KO immune cells have better responses to infections. Also examining the location of immune cells within tumor microenvironments might provide insight to the migration capabilities of the leukocytes during infection.

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